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Regio-selective hydroxylation of *gem*-difluorinated octanes by alkane hydroxylase (AlkB)

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ABSTRACT

gem-Difluoromethylene substituted molecules constitute a distinct class of fluorinated compounds. In this study, special chemistry has been developed for their preparation based on the highly selective terminal hydroxylation of these *gem*-difluorinated octanes by AlkB (alkane hydroxylase) from *Pseudomonas putida* Gpo1 to form *gem*-difluorinated octan-1-ols. The hydroxylation reaction is performed by whole-cell catalysis. Identification of the distal- and proximal-hydroxylation products was made by ^{1}H, ^{13}C, and ^{19}F NMR; GC and GC/MSD; and/or by comparison with authentic standards in GC. To the best of our knowledge, we have obtained the first synthesis of 2,2-, 3,3- and 4,4-difluorooctan-1-ols, from simple and inexpensive starting materials.

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Alkane monooxygenase (AlkB), an integral membrane-bound diiron ω-hydroxylase, is responsible for introducing molecular oxygen regio-selectively into the unactivated terminal methyl group of *n*-octane to yield 1-octanol and water, as shown in Eq. 1.[1] Using this protein, the gram-negative bacterium *Pseudomonas putida* Gpo1 (formerly known as *Pseudomonas oleovorans*) is able to utilize linear medium-chain-length alkanes (C_3–C_{12}) as the sole feedstock.[2] The oxidation of alkyl cyclohexane and benzene derivatives by AlkB is also highly regio-selective, with tight control exerted at the C-4 position of the six-membered ring in alkyl cyclohexane and at the omega methyl position in alkyl benzenes.[3] This selection has been attributed to the steric factors and/or hydrophobic interactions between substrates and the AlkB.

$$C_8H_{18} + O_2 + NADH + H^+ \rightarrow C_8H_{17}OH + H_2O + NAD^+ \quad (1)$$

AlkB contains a nonheme diiron center as a catalytic site.[2c] It has been suggested that the hydroxylation process proceeds through a nonconcerted radical process similar to nonheme diiron soluble methane monooxygenase (sMMO) from methanotropic bacteria.[4] Given that AlkB generates a long-lived radical intermediate during catalysis according to diagnostic probes,[5] it is puzzling that AlkB activates the sp^3 C–H bond in the highly regio-specific manner that it does. Like many membrane proteins, it has been difficult to isolate the AlkB and maintain its activity outside of the bilayer. The crystal structure and the metal active site environment of AlkB have remained elusive.

Regio-selective hydroxylation of aliphatic compounds are among the most challenging reactions in synthetic chemistry due to the high C–H bond energy of the terminal methyl group (98 kcal/mol). This difficult problem has been gaining increasing attention in recent years because hydroxylated aliphatic precursors are used extensively in the chemical industry.[6] Fluorines are also of high importance in pharmaceuticals and agrochemicals because their presence in organic molecules can advantageously alter their chemical and biological profiles, including enhanced stability, lipophilicity, membrane permeability, and bioavailability.[7] In fact, 30−40% of agrochemicals and 20% of pharmaceuticals on the market are estimated to contain fluorine atoms.[7,8] Therefore, there is considerable interest in developing a systematic strategy to efficiently incorporate fluorine atoms into biologically active molecules.[7,9]

In this work, an *Escherichia coli*–alkane hydroxylase (AlkB) system has been developed for whole-cell catalysis. It was constructed from the nonheme diiron monooxygenase (AlkB) co-expressed together with two other soluble co-factors, rubredoxin and rubredoxin reductase in *E. coli* cells. These co-factors are required to provide the electrons required for the turnover of the enzyme when sufficient amounts of NADH are supplied to the *E. coli*.[3]

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Negative controls of *E. coli* without the AlkBGT expression displayed no evidence of bioconversion of these substrates. Aside from 1-octanol, there was no detectable octyl aldehyde or acid observed during the operations of the *E. coli*–AlkB whole-cell system using *n*-octane as the substrate. These results indicate that further oxidation exerted by an alcohol dehydrogenase could be neglected in this recombinant catalysis system.[2,3]

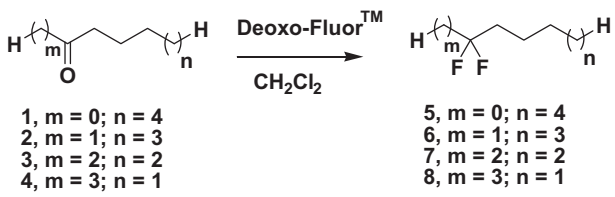
As an illustration of the utility of our *E. coli*–AlkB whole-cell system as a synthetic tool, the enzymatic chemical conversion of 1,1-, 2,2-, 3,3- and 4,4-difluorooctanes to their corresponding terminal alcohols was accomplished as the major products in excellent yields and with the desired selectivity at the ω-position. Thus, we have developed a method to systematically synthesize variable regioselective *gem*-difluorinated octan-1-ols. In addition, other minor products, which were otherwise difficult to prepare in the laboratory, were obtained from these chemoenzymatic conversions.

We have carried out *gem*-difluorination of the octane derivatives employing the fluorination reagent, Deoxo-Fluor™ [bis(2-methoxyethyl)aminosulfur trifluoride] (Matrix Scientific) in CH_2Cl_2.[10] 1-Octanal and 2-, 3-, 4-octanones (**1**–**4**), were used as the starting material to produce 1,1-, 2,2-, 3,3-, and 4,4-difluorooctanes (**5**–**8**) in 43–80% yields (Scheme 1). All the products were monitored by GC or GCMSD at 120 °C under isothermal conditions (t_R = 5.04, 4.87, 4.91, and 4.90 min for **5**–**8**, respectively). 1,1,1-Trifluorooctane **19** was synthesized by a modified sulfinatodehalogenation system.[11–14]

When the *E. coli*–AlkB whole-cell system (1.0 mL, $O.D._{600}$ = 30) was treated with **5**–**8**, gas chromatographic separation at 120 °C isothermal) revealed distal C–H activation at the terminal methyl carbon. The products **9**–**12** appeared at retention times 9.36, 8.39, 8.69, and 8.47 min, respectively. The higher retention times observed for the products reflected their increased polarity after the aliphatic hydroxylation. To determine the regio-selectivity of the hydroxylation, we have recorded both the ^{1}H NMR and ^{13}C NMR spectra of (*R*)-*O*-acetylmandelic acid derivatives of the corresponding alcohol obtained by esterification of fluorinated octanols with (*R*)-2-acetoxy-2-phenylethanoate in the presence of dicyclohexylcarbodiimide (DCC) and a catalytic amount of 4-dimethylaminopyridine (DMAP) (Scheme 2). These spectral data indicated that the major oxidation had occurred at the omega position (Supplementary data).

GC intensities of the various products were used to quantify conversion yields. Following a 3 h whole-cell catalysis, GC analysis yielded conversions of 43%, 68%, 40%, and 43% for **9**, **10**, **11** and **12**, respectively. Under similar catalytic conditions, the yield for the production of **20** from **19** was 27% (Scheme 3). The relatively lower yield of **20** versus **9**–**12** may just reflect the longer bond length and van der Waals radius of the C–F bond (1.35 and 1.47 Å) compared with the C–H bond (1.09 and 1.20 Å).[15] Perhaps, the size of the pocket is restricting the entry of the substrate into the protein. A similar behavior has been also observed in the recent study of P450 BM-3.[11] Interestingly, when *n*-octane and 1,7-octandiene were used as substrates in the AlkB whole-cell catalysis, the conversion yields were relatively poor, 1.2% and 1.1%, respectively, after a 3 h incubation.

When 3,3-difluorooctane **7** and 4,4-difluorooctane **8** were activated by AlkB, we observed minor products at t_R = 7.76 and 8.34 min, respectively, in the GC. These minor products corresponded to the proximal terminal alcohols. To identify these products, we synthesized 2,2-, 3,3- and 4,4-difluorooctan-1-ols to serve as authentic standards (Scheme 4).



Scheme 1. Preparation of 1,1-, 2,2-, 3,3- and 4,4-difluorooctanes.

In order to accomplish the highly efficient synthesis of 2,2-, 3,3- and 4,4-difluorooctan-1-ols (**33**, **17** and **18**), we have developed a synthetic strategy that uses 1,2-, 1,3- and 1,4-octanediols (**21**–**23**) as the starting materials. The synthetic procedure was straight-forward, involving only four synthetic steps in good yields (Scheme 4).

1,2-Octanediol **21** is commercially available (Acros). To achieve the synthesis of diol **22**, methyl 3-oxooctanoate was synthesized by coupling hexanoyl chloride with meldrum's acid.[16] After the reduction of methyl 3-oxooctanoate via $LiAlH_4$, we obtained the octane-1,3-diol **22**. The diol **23** was obtained by the reduction of γ-octanoic lactone by $LiAlH_4$. After protection of the primary alcohol by a benzyl group, **24**–**26** were readily oxidized to their corresponding ketones **27**–**29** (Scheme 4). *gem*-Difluorination was then accomplished by subjecting **27**–**29** to Deoxo-Fluor™. After deprotection by heterogeneous hydrogenation on Pd/C, we obtained the target 2,2-, 3,3-, and 4,4-difluorooctan-1-ol **33**, **17** and **18**, respectively, in 21%, 11% and 15% overall yield.

Compounds **17** and **18** provided authentic standards for the identification of the minor products derived from the oxidation of **7** and **8** in the AlkB whole-cell system (Scheme 3). The product ratio of 6,6-difluorooctan-1-ol to 3,3-difluorooctan-1-ol was 7.6:1.0 after a 3 h conversion of **7**, and their GC yields were 40% and 5.2%, respectively. In the case of **8**, the product ratio of 5,5-difluorooctan-1-ol to 4,4-difluorooctan-1-ol was 7.5:1.0, and their GC yields are 43% and 5.7%, respectively. We could not obtain a comparable GC signal for the proximal product **33** upon the treatment of substrate **6** by the whole-cell catalysis. A similar outcome was obtained for fluorinated substrate **5**. The structures of the proximal products were also established from the spectral data (^{1}H, ^{13}C, ^{19}F NMR, EI-MS and HR-ESI-MS in Supplementary data).

The substantially higher yields of oxidation of the *gem*-difluorooctanes relative to the *n*-octane by the AlkB whole-cell system reflect the higher k_{cat} and lower K_M expected for the fluoro-hydrocarbons in AlkB. Similar observations have previously been reported by this laboratory for the oxidation of fluorooctanes versus *n*-octane by cytochrome P450 BM-3.[11] The greater electrostatic and van der Waals interactions between the fluorinated substrates and the enzyme pocket introduced by the $-CF_2-$ and CF_3- enhance the binding affinity and presumably K_M. Structural and electronic factors lower the activation free energy and accelerate k_{cat}.[11,17] Other factors include the greater membrane permeability of the fluorinated substrates,[7,9b] which might come into play during prolonged catalysis, although theoretical calculations of water–octanol partition co-efficients suggest that the permeability of fluorinated octanes across the cell membranes are similar to that of *n*-octane (Table S2, Supplementary data).[7c] In any case, we can conclude that the hydrophobic pocket of AlkB is more readily accessed by the fluorinated octanes than the corresponding alcohols, because we do not observe any diol formation during *n*-octane oxidation by the *E. coli*–AlkB whole-cell system.

In conclusion, we have developed an efficient procedure for the preparation of *gem*-difluorinated octan-1-ols starting from *gem*-difluorooctanes. Enzymatic conversion of *gem*-difluorooctanes by AlkB in the *E. coli* whole-cell system yielded regio-selective hydroxylation at the primary carbon in 40–68% yield. The high throughputs of the hydroxylated products with the fluorine substituents suggest that this system offers a reasonable approach toward green conversions of the *gem*-difluorooctanes to the difluorinated octan-1-ols. The availability of these *gem*-difluorooctan-1-ols should facilitate the straightforward synthesis of many interesting *gem*-difluorooctan-1-ol-derived compounds via aliphatic chain extension and further functionalization, a strategy

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