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P450-catalyzed regio- and stereoselective oxidative hydroxylation of disubstituted cyclohexanes: creation of three centers of chirality in a single CH-activation event

Adriana Ilie [a,b], Rubén Agudo [a,b], Gheorghe-Doru Roiban [a,b], Manfred T. Reetz [a,b,*]

[a] Department of Chemistry, Philipps-Universität Marburg, Hans-Meerwein Str., 35032 Marburg, Germany
[b] Max-Planck-Institut für Kohlenforschung, Kaiser-Wilhelm-Platz 1, 45470 Mülheim an der Ruhr, Germany

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This paper is dedicated to the memory of Harry H. Wasserman

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ABSTRACT

Wild-type P450-BM3 is able to catalyze in a highly regio- and diastereoselective manner the oxidative hydroxylation of non-activated disubstituted cyclohexane derivatives lacking any functional groups, including *cis*- and *trans*-1,2-dimethylcyclohexane, *cis*- and *trans*-1,4-dimethylcyclohexane, and *trans*-1,4-methylisopropylcyclohexane. In all cases except chiral *trans*-1,2-dimethylcyclohexane as substrate, the single hydroxylation event at a methylene group induces desymmetrization with simultaneous creation of three centers of chirality. Certain mutants increase selectivity, setting the stage for future directed evolution work.

1. Introduction

Enzymes are being increasingly used as catalysts in the production of fine chemicals, stereoselectivity often being the focus of interest.[1] Of particular importance are those transformations, which are difficult or impossible using state of the art synthetic transition metal catalysts or organocatalysts. The toolbox of organic chemists is greatly enriched by the development of complementary methods.[1,2] Until recently the traditional limitations of enzymes as catalysts in synthetic organic chemistry and biotechnology have prevented broad application, but fortunately the advent of directed evolution[3] as a viable protein engineering technique has changed the situation so that even retrosynthetic approaches based on enzymes are now emerging.[4] One of the current challenges in synthetic organic methodology development is regio- and stereoselective CH-activating oxidative hydroxylation of simple and complex organic compounds RH → ROH. Significant progress using synthetic catalysts or reagents has been achieved in this exciting area,[5] but a number of problems remain unsolved. A complementary approach is to use cytochrome P450 monooxygenases,[6] and indeed a number of industrial processes utilizing natural forms (wild-type, WT) are known.[7]

Whenever WT P450 monooxygenases fail to be regio- and stereoselective in a transformation of synthetic interest, which is required for applications, two strategies are possible to solve this problem: (1) Test mutants generated previously for other substrates and hope that a good catalyst will be found.[8] (2) Apply directed evolution,[3] which has a higher probability of success.[9] Directed evolution has been applied to P450 enzymes for more than decade, *but the control of both regio- and stereoselectivity remained elusive for a long time.* In early work, Arnold and co-workers reported the application of directed evolution to the P450-catalyzed oxidative hydroxylation of linear alkanes such as octane or nonane, which resulted in some improvement of regioselectivity, but poor to moderate enantioselectivity.[9c] Later Zhao et al. utilized saturation mutagenesis in order to improve and invert the enantioselectivity of P450(pyr) as a catalyst in the hydroxylation of *N*-benzylpyrrolidine at the 2-position, wild-type (WT) being slightly (*S*)-selective (43% ee) and the best mutants leading to 65% ee (*S*) and 83% ee (*R*).[9d] Later Li et al. performed directed evolution in order to boost (*S*)-selectivity to 98% ee, but enhanced (*R*)-selectivity was not reported.[9f] Recently we reported laboratory evolution of a P450 monooxygenase

* Corresponding author. Tel.: +49 (0)6421 28 25500; fax: +49 (0)6421 28 25620; e-mail address: reetz@mpi-muelheim.mpg.de (M.T. Reetz).

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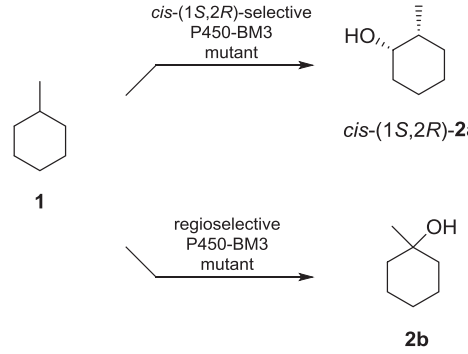
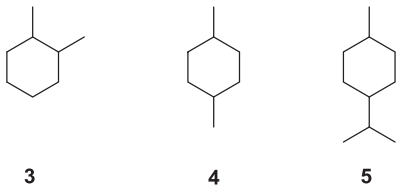
enabling both pronounced regio- and enantioselectivity in oxidative hydroxylation, both (*R*)- and (*S*)-selective mutants being evolved.[10] Using cyclohexene carboxylic acid methyl ester as substrate, highly (*R*)- and (*S*)- selective (>95% ee) mutants of P450-BM3[11] were evolved using iterative saturation mutagenesis (ISM),[2,12] essentially complete regioselectivity in favor of the 3-hydroxy products being observed.[10] In another recent study, >95% regio- and >95% diastereoselectivity in the oxidative hydroxylation of steroids was ensured by 2β- and 15β-selective mutants generated by directed evolution on the basis of ISM.[13] A number of other recent studies likewise addressed the challenge of regio- and stereoselectivity, rational design or directed evolution being used to generate appropriate mutants.[9] In many cases only one of the two possible enantiomers was accessed by protein engineering. Complete control of regio- and stereoselectivity as well as access to both stereoisomers on an optional basis remains a challenge for many types of substrates, as in the case of such simple molecules as *1-methylcyclohexene*.[9l]

P450 monoxygenases are Fe-heme dependent enzymes, the catalytically active high-spin intermediate [heme-Fe=O] (Compound I) inducing H-atom abstraction of the substrate RH with formation of the radical R• followed by rapid C–O bond formation and release of the product ROH.[6,14] This means that high regio- and stereoselectivity requires the compound to be held in a single specific pose with the respective C–H moiety pointing to the catalytically active species at an optimal angle of 130°.[14b,c] Substrates having functional groups may undergo H-bonding as one of the determining factors in positioning the compound in the large binding pockets of CYPs, which means that selective hydroxylation of substrates lacking such anchor points can be expected to be difficult, 1-methylcyclohexene being an example.[9l]

In order to address these challenges, directed evolution has been applied to small molecules, which are devoid of any functional groups (not even olefinic bonds), the hydroxylation of achiral methylcyclohexane **1** being a rare successful example.[15] Two different P450-BM3 mutants were evolved utilizing ISM, which led to regioselective hydroxylation with favored formation of *cis*-(1*S*,2*R*)-**2a** (71% regioselectivity; 94% diastereoselectivity; 92% enantioselectivity), and **2b** (71% regioselectivity), respectively.[15] In the case of desymmetrization **1** → *cis*-(1*S*,2*R*)-**2a**, two chiral centers are created in a single activation step, a phenomenon that is of considerable synthetic value.

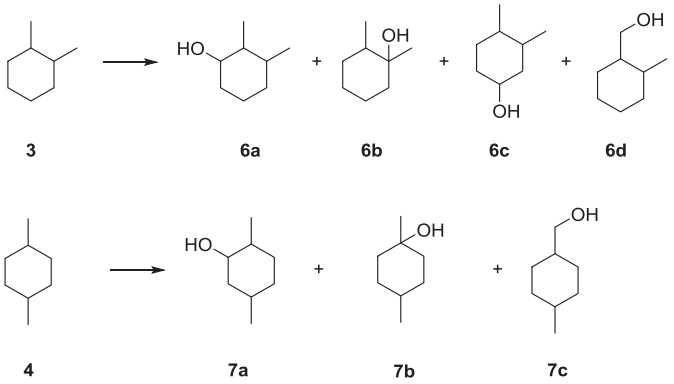


We were interested in the question whether a single oxidative hydroxylation can lead to the creation of *three* new centers of chirality and therefore considered disubstituted cyclohexanes as model compounds in this challenging endeavor. The selective oxidative functionalization of 1,2-dimethylcyclohexane (**3**) and 1,4-dimethylcyclohexane (**4**) using P450-BM3 as catalyst has not been attempted thus far. We also surmised that the regioselective P450-mediated oxidation of 1,4-methylisopropylcyclohexane (**5**) may constitute a novel access to biologically active compounds such as menthol or stereoisomers thereof. The present study constitutes an exploratory investigation of testing P450-BM3 as a catalyst in the oxidation of substrates **3**–**5**.



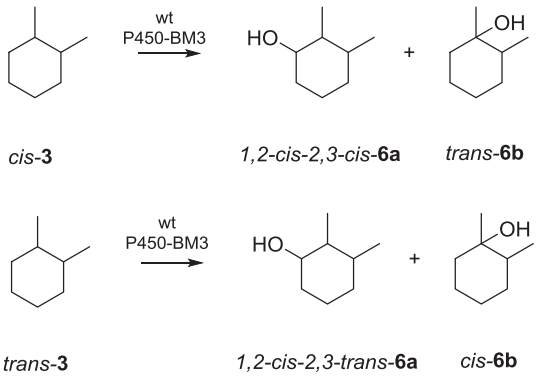
2. Results and discussion

In initial experiments, WT P450-BM3 was used as the biocatalyst in the oxidation of substrates **3** and **4**, which can lead to different regio-, distereo-, and enantiomers as indicated in Scheme 1. Hydroxylation at the methylene groups gives rise to molecules, which have three chiral centers. This ensures greater complexity and therefore added value to the products, provided some degree of stereoselectivity is achieved. This would be a highly desirable feature, but it also makes unambiguous stereochemical assignments more difficult.



Scheme 1. Possible oxidation products by WT P450-BM3 catalyzed oxidation of substrates **3** and **4**.

By GC–MS analyses, we have identified all products arising from the P450-BM3 catalyzed oxidation of 1,2-dimethylcyclohexane (**3**) and 1,4-dimethylcyclohexane (**4**) (Schemes 2 and 3) Subsequently, the products were fully characterized by comparison with authentic commercially available or separately prepared samples. Initially, we used mixtures of *cis*- and *trans*-**3**, but the GC chromatograms of the crude product mixtures proved to be too complex



Scheme 2. Oxidation products obtained by WT P450-BM3 catalyzed oxidation of substrates *cis*-**3** and *rac-trans*-**3**.