Doctoral Thesis

Regio- and stereoselective hydroxylations with Sphingomonas sp. HXN-200

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Regio- and Stereoselective Hydroxylations with *Sphingomonas* sp. HXN-200

A dissertation submitted to the
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for the degree of
Doctor of Sciences

presented by

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Abstract

*Sphingomonas* sp. HXN-200 was found to be highly effective and regioselective for the hydroxylations of *N*-substituted azetidines and piperidines, giving the corresponding 3-hydroxy azetidines and 4-hydroxypiperidines in 91-98% yield, respectively. High yield (70-83%) preparations have also been achieved by using frozen/thawed cells as biocatalyst. Hydroxylations of *N*-tert-butoxycarbonyl azetidine and *N*-benzyl piperidine were carried out in bioreactor on 1 L scale affording the corresponding hydroxylated products in 2.140 g and 2.072 g, respectively, providing convenient and practical syntheses of 3-hydroxyazetidines and 4-hydroxypiperidines. For the first time, lyophilized cells were used successfully in hydroxylation without the addition of cofactor.

The strain HXN-200 was also found to be able to hydroxylate *N*-benzyl pyrrolidin-2-one and *N*-tert-butoxycarbonyl pyrrolidin-2-one with high regioselectivity and excellent enantioselectivity, affording (S)-4-hydroxy-pyrrolidin-2-ones in 99.9% and 92% ee, respectively. Simple crystallization increased the ee of (S)-4-hydroxy-\(N\)-tert-butoxycarbonyl pyrrolidin-2-one from 92% to 99.9%. High activity, excellent regioselectivity, and moderate enantioselectivity were also observed in the hydroxylation of *N*-benzyl piperidin-2-one and *N*-tert-butoxycarbonyl piperidin-2-one with *Sphingomonas* sp. HXN-200, which afforded the corresponding (R)-4-hydroxy-piperidin-2-ones in 31% ee and 68% ee, respectively. High yield preparations of the (R)-4-hydroxy-piperidin-2-ones are demonstrated both in shaking flask and bioreactor.

*Sphingomonas* sp. HXN-200 can also be employed to catalyze the trans-dihydroxylation of *N*-substituted 1,2,5,6-tetrahydropyridines and 3-pyrrolines giving the corresponding 3,4-dihydroxypiperidines and 3,4-dihydroxypyrrolidines, respectively, with high activity and enantioselectivity. The trans-dihydroxylation reaction is sequentially catalyzed by a monooxygenase and an epoxide hydrolase in the strain via an epoxide intermediate. Preparative trans-dihydroxylation of *N*-Phenoxy carbonyl 1,2,5,6-tetrahydro-pyridine and *N*-benzyloxy carbonyl 3-pyrrolines with frozen/thawed cells of *Sphingomonas* sp. HXN-200 afford (+)-(3R,4R)-3,4-dihydroxy-piperidine and (+)-(3R,4R)-3,4-dihydroxy-pyrrolidine in 96% ee both with 60% and 80% yield, respectively. These results represent first example of enantioselective trans-dihydroxylation with non-terpene substrates and with bacterial catalyst, thus significantly extending this methodology in practical synthesis of valuable and useful trans-diols.
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Hydrolysis of N-benzyloxycarbonyl-3,4-epoxy-pyrrolidine and cyclohexene oxide with the epoxide hydrolase of Sphingomonas sp. HXN-200 gave the corresponding vicinal trans-diols in 95% ee and 87% ee, respectively, representing the first example of enantioselective hydrolysis of a meso-epoxide with a bacterial epoxide hydrolase. Enantioselective hydrolysis of racemic epoxide N-phenoxy carbonyl-3,4-epoxypiperidine with Sphingomonas sp. HXN-200 afforded 34% of (-)-N-phenoxy carbonyl-3,4-epoxy piperidine in >99% ee. Further hydrolysis of (-)-N-phenoxy carbonyl-3,4-epoxypiperidine with the same strain afforded (-)-(3S, 4S)-N-phenoxy carbonyl-3,4-dihydroxy-piperidine in 96% ee and 92% yield. Thus, (-)-N-phenoxy carbonyl-3,4-dihydroxy-piperidine can be accessed by double hydrolysis whereas the (+)-enantiomer can be obtained by dihydroxylation with Sphingomonas sp. HXN-200.

Sphingomonas sp. HXN-200 was found to contain an NADH-dependent soluble alkane monooxygenase that is different from the well-known membrane-bound three-component alkane hydroxylase system (AlkB system) in Pseudomonas putida GPo1. The soluble alkane monooxygenase was purified and characterized as a P450 enzyme, namely P450pyr. The P450pyr monooxygenase was purified by a) 40-60% ammonium sulphate cut of cell-free extracts, b) hydrophobic interaction chromatography, and c) anion exchange chromatography. Subsequently, the N-terminal sequence of the purified P450pyr enzyme was determined and the molecular weight was established as 47.3KDa by MS. The Component B was purified by a) anion exchange chromatography, and b) gel filtration chromatography. The molecular weight of component B is determined as 11.2KDa by MS. The purified P450pyr is not active by itself. However, combine with other components restores the activity of the monooxygenase. The 47-kDa P450pyr shows a clear absorption peak at 449nm in the CO difference spectrum.

The homology model of P450pyr was established based on the sequence identity and the existing X-ray structure of P450terp. The sequence alignment of P450pyr was obtained with ClustalX and used to generate the 3D homology model via automated protein homology modeling server – SWISS-MODEL. The heme-binding signature of the P450pyr was then found to be FGFGHI HRCVG (AA359-368). The homology model of P450pyr was used to characterize substrate/enzyme complexes. Docking of the substrates on to the structural model of P450pyr with AutoDock program generated the preferred conformations of substrates inside the catalytic pocket of the enzyme. The results allowed the identification of the
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preferred substrate bound conformation and its related crucial residues at enzyme active site. The calculated preferences of the hydroxylation site and its stereoselectivity for substrates were in good consistence with the experimental outcomes.


Sphingomonas sp. HXN-200 kann auch für die katalytische trans-Dihydroxylierung von N-substituierten 1,2,5,6-Tetrahydropyridinen und 3-Pyrrolinen zu 3,4-Dihydroxypiperidinen und 3,4-Dihydroxypyrrolidinen, ebenfalls mit hoher Aktivität und Selektivität, verwendet werden. Die trans-Dihydroxylierungsreaktion wird erst durch eine Mono-oxygenase und anschliessend durch eine Epoxidhydrolase über ein Epoxidzwischenprodukt in HXN-200 katalysiert. Die preparative trans-Dihydroxylierung von N-Phenoxy carbonyl 1,2,5,6-tetrahydro-pyridin und N-Benzyl oxycarbonyl 3-pyrrolin durch gefrorene/laufgetaute Sphingomonas sp. HXN-200 Zellen führte zu (+)-(3R,4R)-3,4-Dihydroxypiperidin und (+)-(3R,4R)-3,4-Dihydroxypyrrolidine mit 96% ee und Ausbeuten mit 60% bzw. 80%. Zum ersten Mal wurde mit diesen Ergebnisse eine enantioselektive trans-Dihydroxylierung mit einem bakteriellen Katalysator mit nicht-terpenen Substraten demonstriert. Daher stellt dies
Zusammenfassung

eine wichtige Erweiterung der Methoden der angewandten Synthese nützlicher Trans-Diole dar.

Die Hydrolyse von N-benzyloxycarbonyl-3,4-epoxy-pyrroolidin und Cyclohexenoxiden mit der Epoxidhydrolase von Sphingomonas sp. HXN-200 ergaben die entsprechenden vicinalen trans-Diole mit 95% ee bzw. 87% ee. Dies stellt erstmals ein Beispiel für die enantioselektive Hydrolyse eines meso-Epoxide mithilfe einer bakteriellen Epoxidhydrolase. Die enantioselektive Hydrolyse racemischer Epoxide wie N-phenoxy carbonyl-3,4-epoxy piperidin mit Sphingomonas sp. HXN-200 ergab 34% (-)-N-phenoxy carbonyl-3,4-epoxy piperidin in >99% ee. Die Hydrolysis von (-)-N-phenoxy carbonyl-3,4-epoxy piperidin mit dem gleichen Kulturstamm ergab (-)-(3S, 4S)-N-phenoxy carbonyl-3,4-dihydroxy-piperidine mit 96% ee und 92% Ausbeute. Also ist (-)-N-phenoxy carbonyl-3,4-dihydroxy-piperidin über doppelte Hydrolyse zugänglich, während das (+)-Enantiomer über die Dihydroxylierung mit Sphingomonas sp. HXN-200 erreicht werden kann.

Es wurde festgestellt, dass Sphingomonas sp. HXN-200 eine lösliche, NADH-abhängige Alkan-monooxygenase (AM) enthält, welche sich von dem bekannten Pseudomonas putida GPo1 Alkanhydroxylasesystem unterscheidet, das sich durch ein membrangebundenes Dreikomponentensystem auszeichnet. Diese lösliche AM wurde gereinigt und als P450pyr charakterisiert, das den P450 Enzymen zuzuordnen ist. P450pyr wurde zunächst bei a) 40-60% Ammoniumsulphatkonzentrationen aus Zellfreieextrakten gewonnen und mit b) hydrophober Interaktionschromatographie und c) Anionenaustauschchromatographie aufgereinigt. Danach wurde die N-terminale Sequenz des gereinigten P450pyr Enzysms bestimmt und das Molekulargewicht mit 47.3 kDa mit Hilfe der Massenspektroskopie (MS) ermittelt. Die B-Komponente wurde mit a) Anionenaustauschchromatographie und b) Grössenausschluss chromatographie gereinigt. Das Molekulargewicht der B-Komponente wurde mit MS mit 11.2 kDa bestimmt. Reines P450pyr ist alleine nicht aktiv, jedoch kann man durch Kombination mit anderen Komponenten seine ursprüngliche Monoxygenase-aktivität wiederherstellen. Das 47.3 kDa grosse P450pyr zeigt einen definierten Absorptionspeak bei 449 nm im CO-Differenzspektrum.

Das Homologiemodel von P450pyr wurde aufgrund der Sequenzidentität und der vorhandenen Kristallstuktur von P450terp konstruiert. Der Mehrfachsequenzvergleich von P450pyr wurde mit ClustalX ermittelt und dazu benutzt ein dreidimensionales
**Zusammenfassung**

List of Publications from this thesis:


List of patent applications from this thesis


Chapter 1

Introduction

Abstract

This chapter serves as a general overview for the research described in the following chapters. First of all, the selective hydroxylation of non-activated carbon atoms, both chemically and biologically, is reviewed. Secondly, the selective chemical and enzymatic oxidation of C-C double bonds, including asymmetric dihydroxylation, was summarized. Thirdly, the biocatalyst screening and the substrate engineering for the selective hydroxylation of pyrrolidine and its derivatives are briefly introduced and the results are discussed as a background for the thesis. Finally, aims and contents of the thesis are listed.
1.1 Selective hydroxylation of non-activated carbon atoms

Saturated hydrocarbons, for example, alkanes from natural gas and crude oil, are the most abundant chemical raw materials of all naturally occurring organic molecules. Non-activated carbon atoms are rather inert chemical structure that cannot be used directly for chemical synthesis. Although they are readily oxidized completely (burning to CO$_2$ and H$_2$O), they are still among the most difficult chemicals to be oxyfunctionalized in a controlled manner with mild reaction conditions. For example, $n$-hexane is able to resist attack with boiling nitric acid, sulfuric acid, chromic acid and potassium permanganate. Although alkane oxidation is a thermo-dynamically favored reaction, it is very difficult to do it in a selective fashion. Thus far, oxidation of non-activated carbon atoms remains a challenge in conventional organic chemistry. Despite the great success in organic synthesis in the last century, most of the organic transformations still require and initiate at active functional groups, such as hydroxy-, keto-, and activated H (like $\text{–NH}$), in the starting materials. However, the selective oxidation of alkanes is one of the most important technologies and a key objective in chemical industry.$^1$ The requirements for the selective oxidation of non-activated carbon atoms have stimulated the research for novel oxidative reactions and more efficient oxidation procedures.

Chemically, hydrocarbon oxidations performed with inorganic oxidizing agents like chromium and manganese oxides, halogen, nitric acids, and molecular oxygen, are rather inefficient and barely selective.$^{2,3}$ As an example, Nylon-6 is manufactured on a scale of $10^6$ tons per year with Dupont’s cyclohexane oxidation process, in which cyclohexane is oxidized to a mixture of cyclohexanol and cyclohexanone. The conversion of the cyclohexane is kept under 5% in order to avoid over-oxidation. The unreacted cyclohexane needs to be extracted and recycled. Therefore, to increase the efficiency and selectivity of hydrocarbon oxidation has been the goal of both academic and industrial research efforts. Although the oxidation of alkanes leading to alcohols and carbonyl compounds has considerable potential from both ecological and economical point of views, current oxidation technologies have several drawbacks: the reaction conditions are often harsh, the reagent mixture is corrosive, and the reaction is often unselective. Therefore, it’s necessary to develop alternatives for the replacement of these environmentally unfavorable traditional oxidation processes with new oxidation methodologies.
Selective chemical catalysts may enable green chemical processes with enormous economical and environmental benefits. The development of selective catalysts for the oxidation of non-activated carbon atoms has attracted considerable attentions of synthetic chemists in the last two decades. While limited examples of the alkane oxyfunctionalization processes have been applied industrially, a much larger number of these oxidative reactions are being investigated in academic and industrial laboratories. The implementation of novel catalytic processes for the conversion of hydrocarbons to the oxygenated compounds requires the discovery, development, and further understanding of catalysts that exhibit high activity, selectivity, and stability.

**Biocatalysis are going to be a promising tool for solving these problems.**

Over the past decades, biocatalysis has received increasing attention as an attractive approach in numerous processes and its utilization is now increasing dramatically\(^4\). Advantages of using microorganisms over more common chemical reagents are that they are inexpensive sources to generate chirality, energetically favorable, and they can be regenerated. The demand for optically active compounds, particularly in the strongly regulated pharmaceutical industry, has increased the demand for biocatalytic processes towards fine chemical applications. Since these biocatalysts can afford products with high regio- and enantioselectivity, enzyme-catalyzed reaction steps have already been integrated in the development of synthetic routes for the industrial production of optically active molecules\(^5\).

Biological oxidation processes are very interesting because non-activated carbon atoms can be oxidized directly by the use of microorganisms. Oxygenases are important biocatalysts for the selective oxidation of non-activated carbon atoms. The initial activation of non-activated carbon atoms by oxygenases is the fundamental strategy used in microbial catabolism of aliphatic hydrocarbons, a fascinating and valuable chemical transformation, where the aliphatic substrate is transformed to the corresponding alcohol by an oxygenase in the first step. Further enzymatic oxidation leads to the formation of acid *via* an aldehyde intermediate, and the carbon acid is then completely degraded *via* beta-oxidation. These oxygenases can be used in biotransformations of achiral substrates to yield enantio-enriched, or even enantiopure, oxidized products.


1.1.1 Chemical hydroxylation of non-activated carbon atoms

The chemical oxidation reaction is an electron transfer process from substrate to a specified reagent – oxidant. A variety of chemical reactions, such as hydroxylation, epoxidation, and dihydroxylation, are classified as oxidation reactions. The name of each reaction indicates the corresponding oxyfunctionalization of the substrates. Many selective chemical oxidation methods are available and found their applications in industry. However, the conventional reaction conditions, such as temperature, are often harsh. The reaction selectivity and turnover numbers are various with different substrates. The oxidants are often corrosive, flammable, reactive, and even explosive. The reactions may be dangerous, because of fire, explosion and uncontrollable exothermic reactions. Because most of the emergencies are caused by radical-chain decomposition of the oxidant, the storage of them often needs special containers and considerable attentions.

Attempts at the selective oxidation of alkanes encounter two important problems. First, the regio- and stereoselectivity of alkane oxidations are problematic. Second, considering chemo-selectivity, the initial products of alkane oxidation are often more reactive with the oxidant than the alkane itself. For example, in the methane oxidation process, the C-H bond in methanol is 11 kcal/mol weaker than it in methane (93 and 104 kcal/mol, respectively), which means that compared with methane, methanol is a much better substrate for the oxidation reaction. The resulting oxidation of methanol will decrease the desire product yield significantly.

The development of new methodologies for the selective functionalization of the C-H bond is one of the most impressive challenges and exciting areas of chemistry, which have been attacked with a combination of technologies from organic synthesis, molecular recognition, catalysis, and metal coordination chemistry. Although there are considerable research efforts to achieve a certain level of selectivity, the selective oxidation of saturated C-H bond has proven to be difficult to accomplish with synthetic catalysts. Most of the heterogeneous, as well as homogeneous, inorganic catalysts are limited in catalyzing the shape-selective (terminal) oxidation of saturated simple alkanes. Despite the large number of investigations in this area, practical selective oxidations of saturated hydrocarbons are still rather rare. Among the various strategies available for chiral molecule production, asymmetric catalysis attracts
considerable attentions. The generation of the chirality is mainly due to the contribution of asymmetry property of the catalyst ligands. For the selective oxidation approach, the titanium-catalyzed epoxidation of allylic alcohols\textsuperscript{7}, and the osmium-catalyzed dihydroxylation of alkenes\textsuperscript{8} are important achievements. Analogies derived from nature, where oxygenases are well known for the selective oxidation of organic compounds, have also been useful: biomimetic approaches provided an impressive start for the highly selective oxidation of organic substrates with small well-designed enzyme active site mimics. Examples are the impressive achievements in the development of synthetic oxygenase active site mimics in the research groups of Groves\textsuperscript{9}, Jacobsen\textsuperscript{10}, and Katsuki\textsuperscript{11}. The following sections focus on the research efforts achieved in the selective hydroxylation of C-H bond with the employment of inorganic molecular sieves and chiral catalysts derived from synthetic ligands.

(1) Selective hydroxylation of non-activated carbon atoms

There are several publications concerning the various ways that selective oxyfunctionalization of alkanes has been attempted\textsuperscript{12}. Most of these deals with selective hydroxylation of functionalized alkanes. There is also some progress on the selective hydroxylation of saturated nonfunctionalized hydrocarbons. In alkane oxidation, both radical and electrophilic reagents, such as superacid, prefer to attack tertiary over secondary and primary C-H bonds (C-H bond reactivity: allylic C-H > benzylic C-H > 3º >> 2º >> 1º). However, the regioselectivity of alkane oxidations is often poor, for instance, there is no significant preference for one 2º site over another 2º site; and for a desired terminal oxidation, the reaction preference is often in the opposite direction.

Oxidation with heterogeneous catalysis plays an important role in the chemical industry\textsuperscript{13}. Nearly all of the monomers used for the fiber and plastic production are obtained from these oxidation processes. Despite its importance in the chemical industry, the selectivity and efficiency of heterogeneous oxidation is rather lower. Since the selectivity is brought by the solid nature of the heterogeneous catalyst, it is very difficult to define the active sites\textsuperscript{14}, which appear indistinguishable from the rest of the solid. The development of new catalysts and reactions for selective heterogeneous oxidations has progressed slowly in recent years\textsuperscript{15}. New developments in selective oxidation include the direct oxidation benzene to phenol and the aerobic oxidation of methanol to formaldehyde. Among them, one of the highlights is the shape-selective terminal alkane oxidation.
Framework-substituted molecular sieves are the main heterogeneous microporous catalysts used for the shape-selective oxyfunctionalization of saturated alkanes. With the replacement of the original molecular sieves Al\textsuperscript{III} site by catalytic active transition metal ions (Co\textsuperscript{III}, Mn\textsuperscript{III}, Fe\textsuperscript{III}), the sieves are designed to allow free access of molecular oxygen and only the terminal methyl groups of alkanes. Shape-selective oxyfunctionalization can be achieved by optimizing the cage dimension and the catalytic transition metal ion. In a representative terminal \textit{n}-octane oxidation reaction\textsuperscript{16}, despite the promising regioselectivity, the efficiency is still poor. Although the C1 selectivity can be increased up to 50\%, the catalytic reaction only affords 7\% yield after 24h with a turnover number of 3-5 h\textsuperscript{-1}. Considering the low efficiency of these reactions, a new reaction engineering approach for selective oxidation\textsuperscript{17} has recently been explored.

Selective oxyfunctionalization of alicyclic hydrocarbons are rather problematic. Barton\textsuperscript{18} and co-workers demonstrated the selective oxidation of secondary over tertiary hydrogen in adamantane (the so-called Gif system\textsuperscript{19}). With iron salt, zinc, and molecular oxygen in a pyridine/acetic acid suspension, non-activated methylene groups can be oxidized to ketones. Although the system generally does not attack either primary or tertiary carbons, it represents an unusual radical mechanism which is under continuous investigation and remains controversial\textsuperscript{20}. For the cyclohexane oxidation, where all of the C-H bonds are the same, the points to be considered are the reaction efficiency and the substrate over-oxidation. The prospects of selective oxidation of alicyclic hydrocarbon containing non-equivalent C-H bonds with metal complex and molecular sieve catalysts still appear bleak.

Heterogeneous catalysis generally exhibits easier separation procedures for the catalyst from the reaction mixture and higher potential for continuous processing, whereas \textbf{homogeneous oxidation} usually shows more efficient heat transfer, milder reaction conditions, and higher selectivities. Historically, homogeneous selective oxidations are used for many large-scale processes, such as the \textit{p}-xylene oxidation to terephthalic acid, propylene epoxidation to propylene oxide, and ethylene oxidation to acetaldehyde. Based on catalytic mechanisms, they are mainly classified into three categories: organometallic and redox chemistry based oxidations; organic radical mechanism based oxidations; and selective oxygen atom transfer mechanism based oxidations\textsuperscript{21}. The organic radical mechanism based inorganic catalytic oxidation is rather unselective, and can only be used in some special cases,
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such as the \( p \)-xylene oxidation to terephthalic acid. The last mechanism is mainly involved in epoxidation processes, and is discussed below.

It has been shown that organometallic alkane activation can reverse the regioselectivity pattern (1'\( > \)2'\( > \)3'), whereas the organic radical involved mechanism prefer to attack tertiary over secondary and primary C-H bonds (3'\( > \)2'\( > \)1'). The strong C-H bond can be selectively activated in the presence of a weaker C-H bond; for example, aromatic C-H bond can be activated before benzylic C-H bonds in toluene\(^{22}\).

Shilov\(^{23}\) reported one of the earliest examples of C-H activation. It was found that deuterium can be incorporated into alkanes with \( K_2PtCl_4 \) and acetic acid in \( D_2O \) solution, and the formation of RCl and ROH were detected in the presence of \( H_2PtCl_6 \). The \( PtCl_4(H_2O_2)_2 \) complex has been proposed as the active catalytic species, and the observed selectivity pattern is 1'\( > \)2'\( > \)3'.

Recently, several new types of Shilov reactions have been developed, and the scope and limitations of these reactions have been examined\(^{24}\). The methyl group of \( p \)-toluenesulfonic acid is selectively oxidized 1.5 times faster than the corresponding hydroxymethyl group of the product\(^{25}\). No oxidation of the aldehyde product was observed. Sen reported the important 'chelate effect' in the selective oxidation of functionalized organic compounds such as aliphatic alcohols and carboxylic acids\(^{26}\). The observed reactivity was shown to be as follows: \( \alpha \text{-CH} << \beta \text{-CH} < \gamma \text{-CH} < \delta \text{-CH} \) for alcohols and \( \alpha \text{-CH} << \beta \text{-CH} < \gamma \text{-CH} \geq \delta \text{-CH} \) for acids. The phenomenon suggests a reaction mechanism involving initial substrate coordination to the platinum center followed by substrate activation.

\[
\begin{align*}
\text{Pt}^{\text{III}} + \text{RCH}_3 & \rightleftharpoons \text{Pt}^{\text{IV}} \text{CH}_2\text{R} \quad H_2O \quad \text{Pt}^{\text{III}} + \text{RCH}_2\text{OH}
\end{align*}
\]

Proposed catalytic mechanism of the Shilov reaction

Although the conversions of the Shilov reaction can reach 40-70%, the reagent used in the catalysis is rather expensive, the decomposition of the catalyst to platinum(0) during the reversible reoxidation to the active catalytic form appears problematic, and the selectivity of
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these reactions is not high enough. All of these drawbacks limit the practical large-scale application of this reaction.

Kim and co-workers\textsuperscript{27} reported the stereoselective hydroxylation of alkanes by $m$-chloro perbenzoic acid ($m$-CPBA) in the presence of simple metal salt catalysts, such as Mn(II)-, Fe(II)-, and Co(II)-perchlorates. The catalytic activity and enantioselectivity of these metal salt catalysts have been compared. It was shown that the enantioselectivity are the same or slightly superior to that of the metalloporphyrins – M(TPFPP)(ClO$_4$). Good regio- and enantioselectivity can be obtained, hydroxylation of \textit{cis}-1,2-dimethylcyclohexane afford the corresponding \textit{cis}- and \textit{trans}-alcohol in a 81:1 ratio with 82\% regioselectivity and 81\% conversion, and a total turnover of 5-20 can be reached. Since metal perchlorates are potentially explosive, the reaction is limited to small scales and should be handled with great care.

\textbf{(2) Biomimetic catalysis towards non-activated C-H bond hydroxylation}

Selective organic reactions are normally achieved by manipulation of the intrinsic reactivity of functional groups in the reactants. In non-catalyzed oxidation process, it is quite difficult to selectively oxidize an isolated methyl group in a substrate that also contains C-C double bonds or hydroxyl groups, which can be oxidized more easily. Therefore, oxidation of the methyl group can only be achieved by blocking the other more active functional groups or by activating the methyl group itself. In contrast, enzymatic selectivities are independent of the functional groups of the substrates, where the enzyme catalyst takes the advantage of the regio- and stereoselectivity of the formed enzyme/substrate complex. Selective oxidation of a methyl group occurs when the methyl group is the only reactive group within the distance that the enzymatic oxidizing group can reach in its enzyme/substrate complex and the reactive C-C double bonds or hydroxyl groups are beyond this range. Rather than reactivity control in traditional chemical synthesis, enzymatic selectivity is achieved by geometric control. This essential difference has attracted considerable interest among synthetic chemists. If the geometric control inherent in enzyme catalysis could be incorporated into organic synthetic methodology by appropriate catalyst designed to imitate enzyme catalytic active sites\textsuperscript{28}, “enzymatic” style transformations could be achieved through organic chemical catalysis, such processes are referred to as “biomimetic catalysis”.

--- 8 ---
Although conventional oxidations are rather inefficient and barely selective, nature has developed an excellent solution by using metalloproteins as catalysts in selective hydrocarbon oxidation. Iron-containing enzymes play a key role in these biologically essential transformations. For example, cytochrome P450 selectively oxidizes the long aliphatic side chain of cholesterol for the biosynthesis of the hormone progesterone; methane mono-oxygenase (MMO) convert methane to methanol as part of the metabolism of methanotrophs; copper containing oxidases are involved in catechol degradation. In all these reactions, a C-H bond is oxidized to an alcohol that is susceptible to further transformations.

There are already many reports in which biomimetic metalloporphyrins are used for selective alkane hydroxylations and alkene epoxidations. Except the promising asymmetric epoxidation achieved by metalloporphyrins and its derivatives, the majority of these studies are focused on understanding of catalytic mechanism of enzymes, including heme containing oxygenases and non-heme oxygenases. A variety of oxidants such as iodosylarenes, hydrogen peroxide, alkyl hydroperoxides, and hypochlorites, have been used in these transformations as oxygen donor.

**a) Selective catalytic mimics of cytochrome P450s**

The extensive investigations of the heme-containing P450s over the last two decades have given us a deep view of oxygen activation and selective hydrocarbon oxidation by an iron center. New advances in ligand design have been summarized. Moreover, a number of reviews have been published on the use of metalloporphyrins, such as Fe, Mn, Ru, and strong oxidants, such as NaOCl and ROOH, as enzyme model systems.

**Regio/Shape selectivity**

The first example of biomimetic catalysis with cytochrome P450s was reported by Groves. By using iodosylbenzene as the oxidant and the first synthetic analog of a P450 active site, [Fe(III)TPP(Cl)], as catalyst, selective hydroxylation of cyclohexane was achieved. Metalloporphyrins with sterically hindered groups have been used in several selective hydroxylation reactions. The selectivity of several metalloporphyrins were investigated, such as [Fe(TFSPP)(Cl)], [Mn(TTMPP)(OAc)], [Fe(T(2-MeP))(Cl)], [Fe(TTMPP)(Cl)], [Fe(TMP)(Cl)], and [Mn(TTPPP)(OAc)]. The unhindered or modestly hindered metalloporphyrins, such as metal complex with TPP or TTMPP ligands, exhibit poor
Regioselectivity of the primary C-H bond and other sites was shown in the hydroxylation of branched alkanes. The secondary alcohol is the major product (>90%) in the hydroxylation of 2,2-dimethylbutane, an interesting structure containing both 2º site and two different 1º sites, with unpocketed metalloporphyrins, either Mn(TPP)(OAc) or Mn(TTMPP)(OAc). However, the formation of the secondary alcohol (3,3-dimethylbutan-2-ol) become a minor product (<25%) with the deeply pocketed Mn(TTPPP)(OAc) catalyst. The hydroxylation prefers the most exposed methyl group (formation of 3,3-dimethylbutan-1-ol) to the hindered tert-butyl methyl groups (formation of 2,2-dimethyl-butanol).

As expected, the unpocketed porphyrins afford exclusively secondary alcohols in the hydroxylation of alkanes, which can be explained with the relative C-H bond strengths (3º >> 2º >> 1º). These enzyme-like, regio/shape selective hydroxylations with the pocketed metalloporphyrins enable the promising preference of the sterically most accessible methyl groups.

There are also some investigations reported in heterogeneous biomimetic catalysis. Two main classes of catalysts have been developed: micro-liquid systems that utilize micelles or lipid bilayers, and micro-porous solids with interior catalytic heme sites.

It was shown that porphyrins could be incorporated into micelles or bilayers structure. Shilov41 reported the incorporation of a long-tailed iron porphyrin complex into Triton X-100 micelles or phospholipid vesicles. Hoshino and Groves have succeeded in incorporating the metalloporphyrins into lipid bilayers vesicles42. Interestingly, one approach uses the orientation of Mn-porphyrins in a thermotropic nematic liquid crystal to control substrate access to the catalytic center43. However, the selectivities of these methods are modest, and the approaches are impractical.
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The use of porphyrin modified zeolites as shape selective catalysts have been reported. Balkus has reported an incorporation method that uses a perfluorinated Ru phthalocyanine as the seed for the surrounding growth of zeolite NaX. Oxidation of cyclohexane to cyclohexanone is catalyzed with slow turnover rate (0.03 sec\(^{-1}\)) without loss of activity after 20,000 turnovers with 70% yield. The advantage of these systems is the oxidative robustness, but their selectivities are often lower than those of homogeneous system. Nevertheless, the nonselective surface oxidations, difficulties in releasing the trapped products inside the zeolite porous, and the low efficiency limited the application of the approach.

Other porphyrin incorporation approaches include layered phosphonates and minerals intercalation, and silica surface attachment. Selectivities with these materials are low.

Enantioselectivity

Several chiral metalloporphyrins have been developed for the asymmetric hydroxylation. Groves reported several iron(III) complexes with chiral vaulted binaphthyl-porphyrins (TBNAPP). Hydroxylation of a variety of alkyl-substituted aromatics affords 40-72% ee with 19-72% yield. Enantioselectivities are higher with lower yields. The stereoselective hydroxylation of cyclohexane and its analogs by high-valent metalloporphyrin, [Fe(TF\(_4\)TMAR)]\(^{5+}\), in the presence of \(\text{H}_2\text{O}_2\) have been reported. The hydroxylation catalyzed by this Fe-porphyrin complex was found to be highly stereospecific, since the hydroxylation of cis- and trans-1,2-dimethylcyclohexane afforded tertiary alcohol products with >99% retention of the configuration. More interestingly, the catalyst is able to catalyze the hydroxylation of alkenes even at -40°C. Breslow described a manganese-porphyrin complex with four cyclodextrin auxiliaries attached. A steroid substrate was hydroxylated only at the C6 position with 167 turnovers before the catalyst was totally destroyed.

For a practical catalytic oxidation reaction, the catalyst should be oxidatively robust compare to the substrate. However, simple metalloporphyrins are easily decomposed under oxidizing conditions. Cook reported that the robustness of metalloporphyrins can be improved by the steric protection: Mn( TPP)(OAc) has a half-life of 5 min, Mn(TTMPP)(OAc) of 10 min, and Mn(TTPPP)(OAc) of 25 hours.

In summary, metalloporphyrins containing sophisticated chiral auxiliaries, steric hindrance groups, and strong electron-withdrawing groups could afford the oxygenated product with
high shape/regio\textsuperscript{54}, stereoselectivity\textsuperscript{55} and high turnover number. However, the difficulties in catalyst synthesis, their stability and recovery, and especially the high cost of these catalysts, have so far made their synthetic application impractical.

**b) Selective catalytic mimics of the diiron nonheme oxygenase**

Synthetic diiron oxygenase model mimicking the active site of enzymes, such as methane monooxygenase and toluene monooxygenase, have been investigated intensively\textsuperscript{56}. Several ligands for the preparation of chelate diiron complexes have been reported, such as tris-(pyrazol)borate, polypyridyl-(single bridging phenoxy), \textit{m}-xylenediamine bis(triacid)imide, 2,6-diaryl benzoic acid, and dibenzofuran 4,6-diacetic acid. The normally used oxidants are alkyl hydroperoxides and hydrogen peroxide. Successful usage of molecular oxygen as oxidant turned out to be quite challenge with the biomimetic nonheme catalysts, and no results have been published yet.

The majority of the researches in this area are focused on mechanical studies. There are only a few examples for catalytic reactions were reported. For instance, Trukhan\textsuperscript{57} reported the synthesis of [Fe\textsubscript{2}OL(Obz)](ClO\textsubscript{4})\textsubscript{2} complex, which can selectively catalyze the oxidation of methane to methanol with H\textsubscript{2}O\textsubscript{2} as oxidant. Some progress has also been made in the synthesis of new ligands for the preparation of dinuclear active site mimics\textsuperscript{58}. A special case is that of Ovanesyan\textsuperscript{59} who showed that the iron complex formed during the thermal treatment of the FeZSM-5 zeolite is capable of oxidizing methane to methanol at 100\% selectivity with nitrous oxide as oxygen source.

**(3) Asymmetric benzylic hydroxylation**

The asymmetric hydroxylation of the benzylic C-H bond was first achieved by Groves in 1989\textsuperscript{10b} with high regioselectivity at the benzylic position and moderate enantioselectivity by
employing a chiral iron-porphyrin complex as catalyst. As an example, the hydroxylation of ethyl benzene has been carried out under anaerobic conditions in dichloromethane at 0°C with the chiral binaphthyl iron-porphyrin complex. \((R)\)-1-phenyl ethanol was afforded in 40% yield and 41% ee.

Katsuki has developed the Mn-salen complex for the enantioselective C-H oxidation\(^6\). The oxidation of 1,1-dimethylindan with iodosylbenzene (PhIO) in chlorobenzene with the Mn-salen catalyst afforded the corresponding hydroxylated product with 29% conversion and 64% ee. With an improved concave type Mn-salen catalyst, the enantioselectivity can be increased up to 90% ee in 20 hr with 25% conversion.

\[
\begin{align*}
\text{Mn} & \quad \text{PhPh} \\
& \quad \text{PF}_6^+ \quad \text{OH}
\end{align*}
\]

\(4)\) Asymmetric allylic oxidation

The allylic carbon of an alkene can be regio- and enantioselectively acyloxyxylated in the presence of copper and peracid derivatives, the so-called Kharasch reaction\(^6\), which provides an enantioselective route to allylic alcohols. Recent developments have focused on copper complexes\(^6\). The Cu(I) complex, prepared \textit{in situ} from bisoxazoline and Cu(I) trifluoromethanesulfonate, has been used for the selective allylic oxidation of cyclohexenes by Pfaltz and coworkers\(^6\).\(^3\)

Cyclopentene can be converted to \((S)\)-2-cyclopentenyl benzoate in 84% ee at \(-20^\circ\text{C}\) with 61% yield. It has been shown that acetonitrile and acetone are the suitable solvents. These catalytic complexes can only undergo 10-15 turnovers without loss of enantioselectivity.

\[
\begin{align*}
\text{Cu(I) triflate} & \quad \text{PhCO}_2\text{-Bu, -20°C} \\
\text{O} & \quad \text{Ph}
\end{align*}
\]
Similar copper-complex catalysts have been developed in different research groups, such as Andrus catalyst\textsuperscript{64}, Muzart catalyst\textsuperscript{65}, Feringa catalyst\textsuperscript{66}, Andersson catalyst\textsuperscript{67}, and Singh catalyst\textsuperscript{68}. Other optically active copper(II)-tris(oxazoline) complexes have been developed by Katsuki\textsuperscript{69} as biomimetic catalysts which mimic the active site of non-heme oxygenases.

In summary, the iron-porphyrin complex, salen Mn(III) complexes, and Cu(I, II) complexes derived from chiral bis-oxazoline, tris-oxazoline, and bicyclic $\alpha$-amino acids ligands are the best catalysts known for the selective oxidation of C-H bonds, but limited to benzylic and allylic substrates. Although many reports have shown the potential of the selective chemical oxidation catalysts, the substrate range remains limited and the catalytic efficiency and selectivity are still low. Clearly, the development of these catalysts has long way to go.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Representative catalytic reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Structure 1" /></td>
<td><img src="image2.png" alt="Reaction 1" /></td>
</tr>
<tr>
<td>4Å mol. sieves</td>
<td>49% Yield, 81% ee</td>
</tr>
<tr>
<td><img src="image3.png" alt="Structure 2" /></td>
<td><img src="image4.png" alt="Reaction 2" /></td>
</tr>
<tr>
<td></td>
<td>58% Yield, 81% ee</td>
</tr>
</tbody>
</table>

(5) Asymmetric $\alpha$-hydroxylation of enolates and enol derivatives

Compounds containing $\alpha$-hydroxy carbonyl groups are important building blocks and auxiliaries for the synthesis of many biologically active molecules, such as antibiotics, pheromones, and sugars\textsuperscript{70}. In organic synthesis, enolates are generally prepared \textit{in situ} from
enolizable carbonyl substrates with an appropriate base and followed by treatment with oxidizing reagent to afford the corresponding \( \alpha \)-hydroxy carbonyl product. With chiral enolates or asymmetric oxidants, optically active products are accessible. Most common bases used in this reaction are the metal alkoxides and amides. Molecular oxygen can be employed as oxidant.

The reaction undergoes an unstable \( \alpha \)-hydroperoxy intermediate, which is subsequently reduced to the \( \alpha \)-hydroxy carbonyl product\(^{71}\). In early studies, \( \alpha \)-hydroxylation of an adjacent carbonyl group was achieved with control by a chiral auxiliary, which could differentiate the two faces of an enolate\(^{72}\). Followed by oxygen transfer, the corresponding \( \alpha \)-hydroxy ketones, esters, or acids can be produced after cleavage of the chiral auxiliaries. In the following representative reaction, \( \alpha \)-hydroxy acid was obtained from the corresponding chiral enolates derived from pyrrolidine-type imines with \( N \)-sulfonyloxaziridine oxidant\(^{73}\).

Davis\(^{74}\) developed reagent-controlled asymmetric oxidations of enolates to \( \alpha \)-hydroxy carbonyl compounds using camphorsulfonyl oxaziridine as the oxidant. This method afforded moderate to excellent ee (60-95%) for most carbonyl compounds such as acyclic keto esters, amides, and \( \alpha \)-oxo ester enolates.

It is worth noting that sodium enolates of ketones normally give high stereoselectivity with camphorsulfonyl oxaziridine as the oxidant. The highest stereoselectivity has been observed
in the oxidation of the sodium enolate of deoxybenzoin, in which benzoin can be obtained in over 95% ee\textsuperscript{75}.

Zhu and co-workers further extended this method to prochiral enol silyl ethers and prochiral enol esters\textsuperscript{76}. The corresponding product can be easily converted to $\alpha$-hydroxyl ketones with high enantioselectivity.
1.1.2 Enzymatic hydroxylation of non-activated carbon atoms

Together with a large number of available enzymes and the increasing need for enantiopure chemical intermediates, successful applications of enzymatic catalysis are growing rapidly. High throughput screening techniques now allow the rapid identification of new enzymes with desired properties. Enzyme engineering and substrate engineering methodologies have permitted the modification of existing enzyme activities, resulting in enzymatic reactions that are better suited to industrial needs. These new developments have steadily increasing impact on the development of new chemical processes. As a result, biocatalytic processes are now more often considered as alternatives to traditional chemical methods. Enzymes can compete with other catalysts, with high specificity and selectivity. These advantages can lead to successful biocatalytic processes especially when reactions cannot be catalyzed by other means.

Advantages of using enzymes as catalysts:

The most important advantage that biocatalysis bring to organic synthesis is the selectivity. Higher selectivity means better production of single enantiomer, fewer side products, and reduced environmental loads. Chemo-selectivity can be extremely useful in cases where a reaction is desired on one chemical functional group in the presence of a chemically more reactive moiety. It is defined as the ability of enzyme to act on one functional group in the presence of other reactive functional groups. Regioselectivity is also a significant advantage of enzyme catalysis. It is defined as the ability of the enzyme to act on one location in a molecule. Stereoselectivity is the most important benefits of enzyme catalysis. It is defined as the ability of enzyme to act on a single enantiomer, enantio precursor or diastereomer.

The ability to catalyze reactions under mild conditions is another important property of enzymes catalysts. Enzymes typically function at ambient temperature, atmospheric pressure, and neutral pH with minimal byproduct formation. Enzymes are proteins, and completely biodegradable. In addition, enzyme processes generate fewer waste disposal problems. When aqueous solutions are used, solvent consumption can be reduced. The mild operating conditions require lower energy input, resulting in lower costs and lower emissions of greenhouse gases. Therefore, the enzymatic catalysis processes are environmental friendly. Enzyme catalyzed reactions usually have high turnover numbers, with rate accelerations
ranging from $1 \times 10^6$ to $1 \times 10^{10}$. Considering that catalysis is normally carried out at ambient temperature and pressure, the catalytic power of enzyme is remarkable.

**Comparison of enzyme and chemical catalyst prices**

In spite of the advantages mentioned above, biocatalysis is still not considered first by many synthetic chemists. Quite often, biocatalysis is attempted only after other possible synthetic methods have failed. Among the reasons for the chemists to do so, one of the most important beliefs is that the enzymes are too expensive.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Approx. price $/Kg</th>
<th>Chemical catalyst</th>
<th>Approx. price $/Kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine liver esterase</td>
<td>15,000</td>
<td>BINAP</td>
<td>40,000</td>
</tr>
<tr>
<td>Penicillin amidase</td>
<td>10,000</td>
<td>Sharpless catalyst</td>
<td>10,000</td>
</tr>
<tr>
<td>Aspartase</td>
<td>10,000</td>
<td>Rh(PPh3)Cl</td>
<td>2,000</td>
</tr>
<tr>
<td>Lipase</td>
<td>5,000</td>
<td>Jacobsen catalyst</td>
<td>1,000</td>
</tr>
<tr>
<td>Glucoamylase</td>
<td>100</td>
<td>Raney nickel</td>
<td>30</td>
</tr>
</tbody>
</table>

As shown in the table, price of some enzymes is not more expensive than that of the chemical catalysts. Furthermore, the real cost of a catalytic process is not determined only by the catalyst, but by the overall catalytic processes. The cost contribution of an enzyme to the final product can be quite low. For example, $p$-fluoro-L-phenylalanine, which has a selling price of 500 $/Kg, is produced biocatalytically with the enzyme used for the production costing only 20-30 $ per kilogram product.

Considering these potential advantages, biocatalysis can often create the basis for a better process for the production of specialty and fine chemicals. In practice, biooxidations can be carried out *in vitro* with purified enzymes, as well as *in vivo* with whole cells. Considering the cofactor dependence of the oxygenases and the difficulty of *in situ* regeneration of the cofactors, biooxidation processes are often carried out with whole cells which is rather cheap.

With molecular oxygen as oxidant, the monooxygenase catalyzed the insertion of one oxygen atom into a non-activated C-H bond or C-C double bond in substrates, while the second oxygen is reduced by NAD(P)H with the formation of H$_2$O. Dioxygenase catalyzes the selective insertion of two oxygen atoms from molecular oxygen into the substrates.
Biooxidation was first reported in 1950s for microbial steroid hydroxylation\textsuperscript{78}. Since then, it was investigated intensively in the last few decades, including the hydroxylation of terpenes in the perfume industry and the selective oxidation of synthetic substrates, to produce (chiral) alcohols. In addition, biocatalytic oxidations of pharmaceuticals and xenobiotic compounds have been extensively studied.

Considering the selectivity of biooxidations, it is not yet possible to predict the regio- and stereoselectivity with a given substrates subjected to oxidation by a new enzyme. It is also difficult to predict the acceptance of a new substrate with a known enzyme, in which oxidative biotransformation of certain molecules have already been examined. Therefore, the screening of microorganisms is often the method of choice for performing a desired transformation. Developing a catalytic model may help the prediction of substrate range with a known enzyme. Holland\textsuperscript{79} has summarized these models for the hydroxylation with \textit{Beauveria bassiana} ATCC 7159, and some common features are concluded. The optimized distance between binding and hydroxylation positions in the active site of the enzyme is 4 to 7 Å. It has been proposed that the efficient substrate binding required both polar and nonpolar interactions with the enzyme, and the existence of multiple binding modes for a single substrate is common. Whether these factors are applicable to a wide range of oxygenase remains uncertain, but data for a range of cytochrome P450 enzymes give cause for optimism in this regard\textsuperscript{80}.

(1) \textbf{Enzymatic hydroxylation of non-activated hydrocarbons}

A variety of substrates have been successfully employed for selective hydroxylations with several classes of enzymes\textsuperscript{81}. Cytochrome P450 monooxygenases are the most prominent hydroxylation enzymes. They are widespread in nature, existing in all kind of organisms from bacteria to human beings, and used to hydroxylate terpenes, steroids, and other complex natural products\textsuperscript{81,82}. Other hydroxylation enzymes include methane monooxygenase\textsuperscript{83} and alkane hydroxylase\textsuperscript{84}.  

\textbf{Cytochrome P450 System}

The cytochrome P450 was initially characterized by Klingenberg\textsuperscript{85} and Garfinkel\textsuperscript{86} as a special cytochrome which absorbed UV light maximally at 450nm with the formation of the enzyme carbon monoxide complex. The cytochrome P450 (CYP) system is now known to constitute a superfamily of enzymes with a molecular weight of 45-55 kDa and iron porphyrin.
Most P450 enzymes catalyze the oxidative transformation of a large number of endogenous and exogenous substrates, such as alkane hydroxylation, aromatic hydroxylation, alkene epoxidation, oxidative dehalogenation, and N-oxidation\(^87\). Many P450 proteins function to convert hydrophobic substrates to more hydrophilic derivatives that can be easily eliminated from the body. A number of cytochrome P450 catalyzed biohydroxylations have been reported, and some P450 enzymes, including P450cam\(^88\) and P450BM3\(^89\), have been purified for mechanistic investigations. However, the complexity of the P450 enzymes and their general fragility during isolation have restricted the most practical biohydroxylations to whole cells.

As shown above, in a typical catalytic cycle of P450-catalyzed hydroxylation, the substrate binds to the active site on the enzyme as the first step which initialized the release of the water molecule and the second step where the iron atom is reduced to the ferrous state by an electron transferred from NAD(P)H via the reductase. In step 3, molecular oxygen binds the P450-substrate complex. A second electron is transferred from NAD(P)H to the complex via P450 reductase in step 4. In the last step, the complex rearranges with insertion of one atom of oxygen into the substrate to yield a product and molecular water.

The tertiary structures have been obtained for several soluble bacterial P450 proteins\(^90\), such as P450cam\(^91\), P450terp\(^90c\), P450eryf\(^90d\), and P450BM3\(^92\). Recently a membrane-bound eukaryotic P450 enzyme has been crystallized and the X-ray structure has been determined\(^93\).
It appears that only the sequences closely surrounding two key residues in P450s are strongly conserved: a highly conserved threonine in the I helix involved in oxygen binding; and the C-terminal cysteine residue which is part of the highly conserved consensus sequence F(G/S)XGX(H/R)XCGX(I/L/F)A, known as the heme-binding domain at the P450 active site. Although the sequence identity between any two P450s with known 3-D structures does not exceed 30%, the overall topology of these proteins is similar. The solved structures show conserved structural features such as a four-bundle helical core, and the secondary structure exhibits a helix rich right side and a β sheet containing left side, with the heme wedged between these domains.

The most dramatic variations between the P450 structures are found in regions responsible for substrate access and binding. The crystal structure of P450BM3 indicates clearly the opened access channel for the long chain fatty acid substrates, whereas in P450cam this channel is closed. The broad substrate specificity of individual P450s, the hydrophobic properties of the active sites and the preference of P450 enzyme for hydrophobic substrates suggest that the interactions of substrates with P450 enzymes are dominated by hydrophobic and steric factors rather than ionic interactions.
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Hydroxylations catalyzed by P450 enzymes play a key role in the detoxification metabolism in living cells. Such reactions are also of interest in the regio- and stereoselective hydroxylation of non-activated C-H bond for chemical synthesis. One of the most intensive investigated enzymes for synthetic purposes is the cytochrome P450BM3 from *Bacillus megaterium*.

Comparison of P450 structures
Chapter 1. Introduction

It has been reported that P450BM3 is able to catalyze the enantioselective hydroxylation of fatty acids at the \( \omega-1 \), \( \omega-2 \), and \( \omega-3 \) position with rather low regioselectivity, resulting in a mixture of these products\(^9^7\). Further studies have extended the application of this enzyme to vicinal diols and aromatic derivatives of fatty acids. The enantioselectivity of the products obtained in the first case are moderate (60-70% ee)\(^9^8\), whereas the hydroxylation of a variety of non-functionalized arylalkanes afford good to high enantiomeric excess\(^9^9\).

The cytochrome P450cam mutant Y96A was able to catalyze the hydroxylation of the functionalized cyclohexanes and the benzyloxycarbonyl- (Cbz) protected piperidines, which themselves are not substrates\(^1^0^0\). Modeling studies of the substrates-enzyme active site interaction suggest that there is no hydrogen bond formation between protein side chains and the carbonyl oxygen of the Cbz group\(^1^0^1\). In addition, the size and flexibility of the aromatic protecting group seems to be crucial for the regioselectivity of the hydroxylation. Moreover, the Cbz protection group shows rather good stability either to microbial carbamate hydrolases\(^1^0^2\) or to benzylic hydroxylation\(^1^0^3\), which suggests that degradation of the Cbz group in whole cells is generally low.

\[
\begin{align*}
\text{Ph} & \quad \frac{\text{P450camY96A}}{\text{Ph}} \\
\text{OH} & + \quad \text{Ph} \\
\text{OH} & + \quad \text{Ph} \\
\text{Ph} & \quad \text{OH} \\
\text{OH} & \quad \text{Ph} \\
\end{align*}
\]

Methane monooxygenases

Methane monooxygenases\(^1^0^4\) (MMOs) isolated from *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b are capable of hydroxylation of simple alkanes with NADH
Chapter 1. Introduction

as cofactor. The reactions have been intensively investigated with methanogenic microorganisms as well as purified enzymes in the last decades, and the X-ray structure of the enzyme was obtained\textsuperscript{105,106}. It was reported that the MMO from \textit{Methylococcus capsulatus} is able to hydroxylate a series of simple alkanes to alcohols, such as \textit{n}-pentane to (\textit{R})-2-pentanol. However, with this enzyme the highest efficiency is obtained with methane as substrate, while for substrates with increased chain length, the yields are significantly reduced\textsuperscript{106}.

\textbf{AlkB system}

\textit{Pseudomonas putida} GPo1 is able to grow on alkanes ranging from pentane to dodecane. The alkane hydroxylase responsible for the hydroxylation activity is an integral membrane-bound non-heme iron monooxygenase, namely AlkB. The total AlkB system consists of three components: the AlkB monooxygenase, rubredoxin and the rubredoxin reductase\textsuperscript{107}. It has been shown that the AlkB alkane hydroxylase system is capable of hydroxylating a wide range of linear, branched, and cyclic alkanes, except the one containing tertiary carbon. Substituted cyclic alkanes are normally hydroxylated in the \textit{trans}-4 ring positions in the substrates, but not at the alkyl-substituents themselves. Alkyl benzenes, such as ethylbenzene and its 3- or 4-substituted derivatives, are also good substrates of this enzyme\textsuperscript{108}.

\textbf{Other oxygenases}

2-Hydroxybiphenyl 3-monoxygenase (HbpA), which catalyses the regioselective hydroxylation of 2-hydroxy-biphenyl to 2,3-dihydroxy-biphenyl, has been found to be stable in organic/aqueous two-phase systems. Preparative hydroxylation has been performed successfully with isolated enzyme with \textit{in situ} NADH regeneration \textit{via} a formate dehydrogenase cycle\textsuperscript{109}. Dioxygenases, such as naphthalene dioxygenase, can also perform hydroxylation reactions. This biotransformation appears to be a function of the property of the dioxygenase active site iron-oxo complex\textsuperscript{110}.

\textbf{(2) Whole cell hydroxylation}

Although the enzymatic hydroxylation reaction is known to be the first step either in a detoxification process in living organisms, or in an oxidative degradation pathway in metabolism, relatively few hydroxylations are synthetically performed with isolated enzymes. Unlike hydrolases, most oxygenases are unstable in purified form and difficult to work with. In addition, oxygenases are cofactor dependent. For these reasons the use of oxygenases is far less common than that of hydrolytic enzymes. Considering the cost of effective \textit{in situ}
cofactor regeneration, the employment of whole cell catalysts is method of choice.

A number of hydroxylations catalyzed by bacterial or fungal strains have been reported and summarized\textsuperscript{111}. Recombinant strains containing the necessary hydroxylation enzyme, such as the expression of a microbial hydroxylase from \textit{Pseudomonas putida} into \textit{E. coli}, were also used for hydroxylation\textsuperscript{112}. Due to the wide range of substrate specificities and the unpredictable biohydroxylation results of different strains\textsuperscript{81,113}, the biohydroxylations discussed in the following section were based on the different types of substrates.

\textbf{a) Hydroxylation of aliphatic substrates}

Some of the simple aliphatic fatty acids can be hydroxylated at specific positions with microorganisms. A \textit{Pseudomonas} species is able to convert the oleic acid substrate to (\textit{R}, \textit{R})-7,10-dihydroxy-8-(\textit{E})-octadecenoic acid\textsuperscript{114}, whereas the fungus \textit{Gaeumannomyces graminis} hydroxylates a variety of unsaturated fatty acids at C-8 position\textsuperscript{115}. There are some examples for the hydroxylation of amino acids, among which the 4-hydroxylation of L-proline with \textit{Helicoceras oryzae}\textsuperscript{116} and the \textit{threo} hydroxylation of L-aspartic acid with \textit{Papularia arundinis}\textsuperscript{117}.

\textit{N}-heterocyclic compounds, which are the main topic of this thesis, have also been explored for the hydroxylation with microorganisms. The hydroxylation of \textit{N}-benzoyl piperidine derivatives with \textit{Beauveria bassiana} (ATCC 7159) has been reported in later 1960s with low yield (3-48\%) and side reaction\textsuperscript{118}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{hydroxylation.png}
\end{figure}

While the enzymatic hydroxylations of benzyloxy-carbonyl- (Cbz) and benzoyl- (bz) protected piperidine afford only the 4-hydroxylate products, the hydroxylation of the \textit{N}-
benzyloxycarbonyl 2-methyl-piperidine results in a mixture. Moreover, the hydroxylation of 
N-benzyol 2-methyl-piperidine gives not only a mixture of hydroxylated isomers, but also the 
overoxidized 4-keto-piperidine.

The biohydroxylation of N-substituted pyrrolidin-2-ones and piperidin-2-ones are rather 
difficult. It has been reported that hydroxylation of N-benzyl-, N-benzoyl- and N-phenyl 
acetyl-pyrrolidin-2-one with Beauveria bassiana (ATCC 7159) afford the corresponding N-
benzyl 5-hydroxy-pyrrolidin-2-one, N-benzyol 4-hydroxy-pyrrolidin-2-one and N-phenyl 
acetyl 4-hydroxy-pyrrolidin-2-one with 11%, 21%, and 5% yield, respectively, in very low 
e.e\(^1\). Moreover, several byproducts are formed in each case. Hydroxylation of N-phenyl-, N-
benzyl-, or N-benzyol-4-piperidin-2-ones with Beauveria bassiana ATCC 7159 gave the 
corresponding 4-hydroxy piperidin-2-ones in low concentration with a yield of 7\(^%\)\(^1\)^, 10\(^%\)\(^1\), 
and 27\(^%\)\(^1\), respectively.

The proposed model for the enzymatic hydroxylation of these amides suggests that the 
distance between the amide oxygen and the hydroxylation site are particularly important\(^8\)^\(^1\),\(^12\)^\(^2\).

The “docking/protecting” concept has been employed to modify a substrate to enable a 
biohydroxylation that originally did not occur, or to improve a given biohydroxylation with 
higher regio- and stereoselectivity. With this concept, a variety of compounds, such as 
ketones, carboxylic acids, and alcohols, have been hydroxylated with microbial strains that 
would have failed to be carried out otherwise. N-containing auxiliaries are also demonstrated 
to enable the hydroxylation of cyclopentanone derivatives\(^1\)^\(^2\).
b) Allylic hydroxylation

Allylic hydroxylations of a series of bicyclic compounds are carried out by *Rhizopus arrhizus* ATCC 11145 with high yield and enantioselectivity\(^\text{124}\). Several fungi\(^\text{125}\), such as *Mucor plumbeus* CBS 110-16\(^\text{126}\), have also been reported to perform allylic hydroxylations of bicyclic ketones. The *Rhodococcus opacus* PWD4 is able to hydroxylate D-limonene at the allylic position in the cyclic ring affording enantiopure (+)-trans-carveol\(^\text{127}\), while (-)-perillyl alcohol is hydroxylated by *Catharanthus roseus* at the allylic position in the side chain\(^\text{128}\).

Substrates such as toluene and ethylbenzene undergo benzylic hydroxylation with the fungus *M. isabellina* ATCC 42613\(^\text{129}\). Further investigations have extended the benzylic hydroxylation to a variety of substituted aromatic and hetero-aromatic substrates\(^\text{130}\).

Substituted propylbenzene can be hydroxylated at benzylic position with *Bacillus megaterium*, leading to the formation of (R)-alcohol\(^\text{131}\). *Aspergillus niger* and *Cunninghamella elegans* are able to perform benzylic hydroxylations of substituted 1,2,3,4-tetrahydroquinolines\(^\text{132}\), whereas *Helminthosporium* sp. NRRL 4671\(^\text{133}\) hydroxylate only terminal and sub-terminal positions of the alkyl groups of substituted aromatics.
hydroxylations of dialkylpyridines have been carried out with *Beauveria bassiana* and *Curvularia* species\textsuperscript{134}. By using *Pseudomonas* and *Rhodococcus* species, aryl methyl groups can be oxidized directly to carboxylic acids, presumably via hydroxylation\textsuperscript{135}. It has been shown that dioxygenases from *Pseudomonas* species, such as toluene and naphthalene dioxygenases\textsuperscript{136}, can also perform benzylic hydroxylation of indene and its derivates\textsuperscript{137}. For example, *Pseudomonas putida* UV4 can hydroxylate saturated benzocarboxycles\textsuperscript{138} and dihydro-benzofuran\textsuperscript{139} to give the corresponding benzylic alcohols.

d) Aromatic hydroxylation

Enzymatic hydroxylation of simple aromatic substrates has been studied for the preparation of phenolic products. Biphenyl aromatics can be hydroxylated at 4 positions in high yield by *Aspergillus parasiticus*, and the reaction can be scaled up to kilogram scale\textsuperscript{140}. Aniline can be converted to 2- and 4-aminophenol by several *Aspergillus* strains\textsuperscript{141}. Other strains capable of hydroxylation of simple aromatics include *Nocardia asteroides* IFO 3384\textsuperscript{142}, *Fusarium verticillioides*\textsuperscript{143}, and *Beauveria bassiana* ATCC 7159\textsuperscript{144}.

Aryl alkyl acids and their analogs can be hydroxylated to phenol derivatives by various microorganisms. Kuge reported the hydroxylation of 2-phenyl propionic acid in the *para* position with *Streptomyces rimosus* ATCC 10907\textsuperscript{145}. Hydroxylation of phenyl acetic acid with *Humicola* and *Chaetomium* species affords 2-hydroxy products\textsuperscript{146}, whereas the 3-hydroxy isomer can be produced by *Rhizoctonia solani*\textsuperscript{147}. Moreover, the 5-hydroxy phenyl acetic acids can be prepared with *Beauveria bassiana*\textsuperscript{148}.

Hydroxylation of heterocyclic aromatic compounds, such as substituted pyridines, quinolines, and pyrazines, can also be accomplished with various microorganisms. The strains involved in these biotransformations include *Alcaligenes faecalis*\textsuperscript{149}, *Arthrobacter oxydans*\textsuperscript{135a}, *Alcaligenes eutrophus*\textsuperscript{150}, *Pseudomonas acidovorans*\textsuperscript{150}, *Rhodococcus erythropolis*\textsuperscript{151}, and *Pseudomonas putida* UV4\textsuperscript{152}.

\[ 
\begin{array}{c}
\text{O} \\
\text{O} \\
\text{R} \\
\longrightarrow \\
\text{R} \\
\text{R} \\
\text{R} \\
\text{COOH} \\
\end{array} \\
\begin{array}{c}
\text{O} \\
\text{R} \\
\longrightarrow \\
\text{R} \\
\text{R} \\
\text{N} \\
\end{array} \\
\begin{array}{c}
\text{O} \\
\text{N} \\
\text{R} \\
\end{array} \\
\begin{array}{c}
\text{R} \\
\text{N} \\
\end{array} \\
\text{R} \\
\]

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1.2 Enantioselective oxidation of C-C double bonds

1.2.1 Chemical oxidation

1.2.1.1 Chemical asymmetric epoxidation

Optically active epoxides are important building blocks used in organic synthesis. Since the ring of epoxides can be enantioselectively opened to form bifunctional compounds\textsuperscript{153}, many chemical methods have been developed for the synthesis of enantiopure epoxides. The following section presents an overview of various existing strategies for chemical asymmetric epoxidations of allylic alcohols, $\alpha, \beta$-unsaturated ketones and non-functionalized simple alkenes.

(1) Selective epoxidation of allylic alcohols

In the 1970s, it was found that VO(acac)$_2$ can catalyse the selective epoxidation of allylic alcohols with alkyl hydroperoxide and chiral auxiliary ligands\textsuperscript{154}.

Several new chiral ligands for the asymmetric epoxidation of allylic alcohols catalysed by vanadium complexes have been developed in recent years\textsuperscript{155}. Although most of them exhibit rather low enantioselectivity, Yamamoto presented some promising examples\textsuperscript{156}.

Katsuki and Sharpless\textsuperscript{157} have described the most widely used asymmetric epoxidation reaction in 1980. Together with Ti(IV) alkoxide, enantiopure tartrate ester, and $t$-BuOOH, a wide variety of allylic alcohols can be epoxidized with better than 90% ee. Further
investigation indicated that the conversion of the epoxidation could be increased by addition of molecular sieves. A catalyst containing 5mol% titanium tetra-isopropoxide and 6mol% tartrate is recommended as the standard.

The stereochemistry of the Sharpless epoxidation\textsuperscript{158}

The reaction can also be used for the kinetic resolution of racemic sec-allylic alcohols. When a racemic sec-allylic alcohol is epoxidized, the enantiomer with the group oriented to the complex have a lower reaction rate\textsuperscript{159}. When one enantiomer reacts faster affording the erythro epoxy alcohol, the other is enriched in its chirality. Higher enantiomeric excess of the unreacted allylic alcohol can be achieved in most cases. Moreover, with an excellent enantio-selective ratio, for instance $K_R/K_S > 500$, both the remaining allylic alcohol and the epoxide can be obtained with high ee.

When meso diallyl alcohols are used, only one of the four possible epoxides is formed\textsuperscript{160}.
Chapter 1. Introduction

The kinetic resolution can also be extended to other sec-alcohol systems, which contain a similar structure to meso diallyl alcohols\(^1\). The resolution of \(\alpha\)-Furfuryl toluenesulfonamide can be achieved with a modified reaction condition, although the enantioselectivity is opposite to that observed for allylic alcohols.

The mechanism\(^{162}\), synthetic applications, the scope and limitations of the reaction have been reviewed\(^{163}\) many times. The Sharpless epoxidation method, although it seems universal, only results in high enantioselectivity with primary or secondary allylic alcohols. This reaction is also sensitive to the preexisting substrate chirality\(^{164}\). The \((R)\) or \((S)\) configuration of a starting secondary allylic alcohol can affect the \(cis/trans\) character of the epoxide formed. Furthermore, if the neighbor carbon of the hydroxyl group carries a sterically hindered group, a decrease or even a complete loss of enantioselectivity is observed\(^{165}\). In some cases, the formed epoxy-alcohol is unstable to the reaction conditions. The Lewis acidic titanium IV catalyst opens the epoxide, leading to a diol, which binds with the chiral complex and inactivates it\(^{166}\).

(2) Selective epoxidation of electron-deficient alkenes

Most of the effective oxidants for the epoxidation of electron-deficient alkenes are hydroperoxides\(^{167}\), such as hydrogen peroxide\(^{168}\) and \(\text{tert}-\text{butyl hydroperoxide (t-BuOOH, TBHP).}\) They have all been used to carry out the selective epoxidation of \(\alpha, \beta\)-unsaturated carbonyl compounds. The reaction is performed first by a nucleophilic addition of the hydroperoxide anion to the electron-deficient \(\beta\)-carbon, and then followed by epoxidation \textit{via} elimination of hydroxide or alkoxide anion. Since the reaction proceeds through a thermodynamically favored partially \(\text{C}_\text{\alpha-C}_\text{\beta}\) single-bonded intermediate, both \textit{cis}- and \textit{trans}-alkenes often form the same product\(^{169}\).
Wynberg\textsuperscript{170} reported the first enantioselective epoxidation of \( \alpha, \beta \)-unsaturated carbonyl compounds. The enantioselectivity is rather low with a 55\% ee as the best result. Colonna and Takahashi reported the epoxidation of naphthoquinones and chalcone with bovine serum albumin\textsuperscript{171} and cyclodextrin\textsuperscript{172}. The use of bovine serum albumin give an unpredictable moderate enantioselectivity (50-77\% ee), and with cyclodextrin, the enantioselectivity never exceeded 41\% ee.

Julia and co-workers have developed an improved method\textsuperscript{173}, where alkaline aqueous hydrogen peroxide and insoluble poly amino acids were used for the synthesis. Colonna and Carriere\textsuperscript{174} suggested that a hydrogen-bonded interaction of the substrate with poly amino acid catalysts introduced the enantioselectivity of this reaction. Furthermore, it was shown that a 1:1 gram ratio of catalyst to substrate gave the most efficient conversion. Amino acids Ala, Leu, and isoLeu were found to be the best catalyst; toluene and CCl\(_4\) were the most suitable solvents. It was found that most of the compounds behaved well under standard procedure affording 55-95\% yield with 80-86\% ee.

Ferreira and Bezuidenhoudt report the successful application of this method to electron-rich polyoxygenated chalcones\textsuperscript{175}. The epoxidations are carried out with poly-S-Ala and poly-R-Ala in a triphasic mixture with 40-84\% ee.

In spite of the previously developed selective epoxidation of chalcone-type substrates, Roberts introduced an impressive extension of this reaction\textsuperscript{176}. The substrate range can be extended to ketones with aliphatic substituents, C\(_2\)-symmetrical \( \alpha, \beta \)-unsaturated ketones, dienes, and bis-dienes. Employment of a biphasic system in this reaction was also reported\textsuperscript{177}.

Organometallic complexes have been employed in several asymmetric epoxidation approaches based on electron-deficient system. Various salen-Mn(III) complexes are shown to be effective catalysts for the asymmetric epoxidation of alkenes. Among them, the commercially available Jacobsen catalyst\textsuperscript{178} has been widely applied. For instance, the epoxidation of \( \textit{cis} \)-ethyl cinnamate affords a mixture of \( \textit{cis} \)- and \( \textit{trans} \)-epoxide (4:1) in 82-98\% yield with 93\% ee for both isomers.
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Good enantiomeric excess can be achieved for the asymmetric epoxidation of α, β-unsaturated ketones with N-methyl pseudoephedrine and diethylzinc\textsuperscript{179}. Shibasaki\textsuperscript{180} describes an efficient catalytic asymmetric epoxidation of enones using lanthanoid complexes with 83-94\% ee.

(3) Selective epoxidation of simple (non-functionalized) alkenes

Asymmetric epoxidation of normal alkenes have also been successfully achieved in the past decades\textsuperscript{181}. In general, type-I metal complex catalysts\textsuperscript{182}, such as VO(OR)\textsubscript{3} and MoO\textsubscript{2}(OR)\textsubscript{2}, catalyze the selective epoxidation by activation of an oxidant and subsequently transfer an oxygen from oxidant to alkenes\textsuperscript{154b}. The oxidation states of the metal ions do not change during the reaction, these reaction types are therefore more suitable for the epoxidation of alkenes containing coordinating functional groups, such as allylic alcohols. For the epoxidation of non-functionalized alkenes, the reaction is rather slow. On the other hand, type-II metal complex catalysts, like the cytochrome P450s found in living cells, catalyze the epoxidation by oxidation of a metal ion with oxidant to the high-valent metal oxide and subsequent migration of an oxygen to alkene by the reduction of the metal ion, which can be reoxidized \textit{in situ} to the high-valent metal oxide.

The investigation of the catalytic selective epoxidation of non-functionalized alkenes with metalloporphyrin complexes and its equivalents has attracted considerable attention. Besides iron porphyrins, many other metal complexes have also been developed to catalyze oxygen-transfer epoxidations with the corresponding high-valent metal oxides. Considering the
enantioselectivity, the recognition of the alkenes to force one of their enantiofaces to the active metal center, which is controlled by the enzyme cavity in biological oxidations, could be achieved through non-covalent interactions between alkenes and the chiral ligands. Various types of metal catalyst have been developed, including metalloporphyrin and metallosalen complexes.

a) Metalloporphyrin

Groves reported the first example of alkene epoxidation by an optically active chloroferritetraphenylporphyrin catalyst with low enantiomeric excess\textsuperscript{183}. Although the reaction shows moderate enantioselectivity with some styrene derivatives (51\% ee), the other substrates afford less than 20\% ee. A number of metalloporphyrins have been used for the epoxidation of non-functionalized alkenes\textsuperscript{184}. Iodosylbenzene, hydrogen peroxide, hypochlorite derivatives, and molecular oxygen have been employed as oxidants.

Inoue has reported an optically active Mn(III)-porphyrin complex that could catalyze the asymmetric epoxidation of styrene derivatives with 8-58\% ee in the presence of imidazole which is dedicated to block the unhindered face of the porphyrin ring by coordinating to the manganese ion\textsuperscript{185}.

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Substrate</th>
<th>Conversion (%)</th>
<th>ee (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>95</td>
<td>83 (S)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>90</td>
<td>82 (S)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>74</td>
<td>55 (S)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>78</td>
<td>72 (S)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>55 (1S, 2R)</td>
</tr>
</tbody>
</table>

Collman\textsuperscript{186} has reported a threitol-strapped Mn(III)-porphyrin complex that combined the advantages of the above two types of chiral metalloporphyrins. Good enantioselectivity can be achieved for the epoxidation of conjugated mono- and cis-disubstituted alkenes in the
presence of 1,5-dicyclohexylimidazole. Although various oxidants have been employed in the reaction, iodosylbenzene affords the best enantioselectivity.

b) Metallosalen

Metallosalen complex has a structure similar to that of metalloporphyrin. As expected from the structure similarity, chiral metallosalen complexes can also be oxidized to the high-valent oxo species and various oxygen transfer reactions can be performed including epoxidation.\(^{187}\)

The porphyrin ligand has a metal ion center and its peripheral carbons are all sp\(^2\). Considering the construction of an optically active metalloporphyrin catalyst, the chiral auxiliaries should be placed outside of the porphyrin ring. Therefore, it is rather difficult to synthesize an efficient metalloporphyrin catalyst. By taking advantage of the structural features of salen ligand, partially surrounding a metal center and containing two sp\(^3\) carbons in its ethylenediamine moiety, the chirality can be introduced to the two sp\(^3\) carbons and the C3, C3' position can be substituted with bulky or chiral groups.

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Substrate</th>
<th>Oxidant</th>
<th>Conversion (%)</th>
<th>ee (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PHIO</td>
<td>60</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>NaOCl</td>
<td></td>
<td>78</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>NaOCl</td>
<td></td>
<td>40</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>NaOCl</td>
<td></td>
<td>80</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>NaOCl</td>
<td></td>
<td>91</td>
<td>88</td>
<td></td>
</tr>
</tbody>
</table>
Because of the non-planar conformation of the salen complex, the alkene epoxidation happens from the downward side of the ligand. The orientation of the incoming alkenes is mainly induced by the repulsion between C3-substituent, \( sp^3 \)-C-substituent in the salen complex and the substitution group in the alkenes. However, when the substrate is a conjugated alkene, \( \pi-\pi \) antibonding repulsion between salen catalyst and the alkene also contributes to the substrate orientation, especially for the cis- and tri-substituted conjugated alkenes, resulting in excellent enantioselectivity. Among the various C3-substituents, the 2-phenyl-naphthyl group serves as the best space controller. Although the choice of the oxidant depends on the substrates, iodosylbenzene and sodium hypochlorite are generally used oxidants for terminal epoxidation.

The mono-, di- and tri-substituted alkenes can be epoxidized with salen-Mn(III) complexes with 80-98% ee. The regio- and diastereoselective epoxidation of conjugated polyenes is limited to some \( \alpha,\beta-\gamma,\delta \)-unsaturated esters. Asymmetric epoxidation of trans-alkenes remains an unsolved problem. The reaction may be applicable only with special substrates. Trans-epoxide can be obtained in the epoxidation of dienes and enynes.

Chiral salen complexes are relatively easily synthesized from substituted ethylenediamines and salicylaldehydes, and now more than 100 complexes have now been investigated. These catalysts are very effective for the epoxidation of conjugated cis-disubstituted and tri-substituted alkenes. The yields are often higher than 80% and the enantioselectivity often lead to enantiomeric excess above 90%. However, the salen complexes are not particularly robust, and the total turnover numbers are typically less than 40.

Although the conjugated cis-alkenes normally are good substrates for Jacobsen epoxidation reactions, it has been suggested that the epoxidation of conjugated alkenes proceeds through a
radical intermediate in which the C-C bond between the radical and its neighbor are rotatable, resulting in the formation of a diastereomeric epoxide. Therefore, the epoxidation of \textit{cis}-\beta-methylstyrene gave a mixture of \textit{cis-} and \textit{trans-}epoxides\textsuperscript{192}. Further developments indicate that this undesired rotation could be suppressed by low temperature. For instance, by decreasing the temperature to \(-78^\circ\text{C}\), the enantioselective epoxidation of styrene affords 86\% ee\textsuperscript{193}.

(4) Other biomimetic epoxidations

Deeply pocketed Mn(TTPPP)(OAc) shows enhanced selectivity for the epoxidation of the most exposed double bond or terminal epoxidation of dienes\textsuperscript{53}. Collman and co-workers\textsuperscript{54e,55} have synthesized a variety of Mn(III) picnic-basket porphyrins, which can be designed and optimized for specific substrates. Good selectivity was observed by using C6PBP and PXLPBP as ligands. In the epoxidation of the \textit{cis}-2-octene \textit{vs} cyclooctene, the selectivity reached more than 1000:1 with Mn(PXLPBP) as catalyst. The selectivity for terminal oxidation \textit{vs} internal epoxidation is modest. Moore and Suslick\textsuperscript{194} have synthesized a variety of sterically hindered poly(phenylester) and poly(phenylamide) dendrimer-metalloporphyrins. The regioselectivity observed for the \textit{meta}-substituted dendrimer-metalloporphyrins is good, but still less than that of the bispocketed TTPPP-porphyrins. In contrast, the \textit{ortho}-substituted species exhibits excellent regioselectivity in the epoxidation of 1-alkene and \textit{cis-}cyclooctene and of non-conjugated dienes. Molecular modeling shows that the substrate access to the metal center is limited to a 0.5nm gap between the dendrimer substituents; such steric hindrance apparently induces the regio/shape selectivity. Turnover numbers of \textit{ortho}-substituted dendrimer metallo-porphyrins, [Mn(T(2',6'-GIAP)P)Cl], are typically 1-2 sec\textsuperscript{-1}, which is slightly slower than that of the unhindered Mn(TPP)Cl (3-4 sec\textsuperscript{-1}).

A new chiral porphyrin with (S)-binaphthyl-L-alanine straps was synthesized. (\textit{R})-styrene oxide can be obtained in >90\% ee by catalytic epoxidation of styrene with F\textsubscript{3}PhIO as oxidant\textsuperscript{195}. Mansuy\textsuperscript{196} reported selective epoxidation of para-chlorostyrene with various chloroiron basket-handle porphyrins in 12-50\% ee with 35-50\% yield. Several \(\beta\)-methyl styrenes were epoxidized with manganese(III) TBNP chloride in 15-40\% ee\textsuperscript{197}. Substituted styrenes can also be epoxidized by chloroiron twin-coronet porphyrins with 11-89\% ee\textsuperscript{198}. Konishi\textsuperscript{199} and Halterman\textsuperscript{200} reported that the enantioselectivity of the alkene epoxidation obtained with strapped dihexyldeuteroporphyrin derivatives could be achieved from 8\% ee for 4-chlorostyreneoxide to 58\% ee for indene oxide. Epoxidation of arene-conjugated \textit{cis-}
alkenes were achieved in 41-76% ee. The epoxidation of 1,2-dihyronaphthalene can be obtained in 88% ee by a Mn complex with 1,2-diphenoxyethane strapped porphyrin\textsuperscript{201,55}. The chiral pocket of this class of porphyrins can be optimized for specific substrates with the modification of chiral threitol straps. The catalytic epoxidation could reach 100 turnovers and the enantioselectivity is different with oxidants varying from 10% to 80% ee. Breslow\textsuperscript{202} described a manganese-porphyrin complex with four cyclodextrin auxiliaries attached. Catalytic epoxidation of styrene was achieved at a rate of 40 turnovers·min\textsuperscript{-1} and afforded up to 5500 turnovers with 75% ee\textsuperscript{203}. 

(5) Aerobic asymmetric epoxidation

Although the oxidation of organic compounds is an exothermic process, dioxygen normally serves as a weak one-electron oxidant (E\textsubscript{0} = -0.3 eV) and the thermodynamic barrier for the formation of superoxide O\textsubscript{2}\textsuperscript{-} or HO\textsubscript{2}\textsuperscript{-}, which initiates the reaction, is high. Chemical oxidations with oxygen require its conversion to radical forms, which act as strong one-electron oxidants, or into a form capable of performing two-electron oxidations, such as hydrogen peroxide. However, molecular oxygen is an economical and environmentally friendly oxidant. It is used for most of the biological oxidation processes in living cells. Mukaiyama has reported the utilization of molecular oxygen for the terminal oxidation of alkanes with a Mn catalyst, including Mn-salen complexes, in the presence of an aldehyde as reductant. This aerobic epoxidation can be performed in an enantioselective manner with a chiral metal complex catalyst\textsuperscript{204}. It is worth noting that the enantioselectivity in the aerobic epoxidation is opposite to that observed in the epoxidation with iodosylbenzene and NaOCl.

1.2.1.2 Asymmetric cis-dihydroxylation

Catalytic selective dihydroxylation of C-C double bonds has become a widely used practical method to access chiral alcohols\textsuperscript{205}. The most attractive advantage of this reaction is the high regio- and stereoselectivity for a broad variety of substrates.

The OsO\textsubscript{4}-mediated dihydroxylation of alkenes has been established in the 1930s. Although the osmylation reaction developed by Criegee is not economical and ecological friendly, the reaction has been further investigated by using cheap cooxidants to reoxidize the Os(VI) glycolates, such as \textit{t}-BuOOH\textsuperscript{206}, N-methylmorpholine N-Oxide\textsuperscript{207}, and K\textsubscript{3}Fe(CN)\textsubscript{6}/K\textsubscript{2}CO\textsubscript{3}\textsuperscript{208}. It has been noted in Criegee’s pioneering work that tertiary amines could accelerate the osmylation reaction. With a chiral amine, asymmetric \textit{cis}-dihydroxylation of a prochiral
alkene can be achieved. Later on, more than 400 alkaloids are investigated, and the chinchona alkaloid derivatives, developed by Hentges and Sharpless\(^{209}\), have so far proven to be the best catalytic system for \textit{cis}-dihydroxylation.

\[
\text{OsO}_4 + \text{NR}_3 \rightarrow \text{Catalytic} \quad \text{Stoichiometric} \rightarrow \text{"Os"}
\]

The Osmylation of alkenes

Together with ligands such as phthalazine (PHAL), diphenylpyrimidine (PYR), and anthraquinone (AQN) analogs, derivatives of dihydroquinine (DHQ) and dihydroquinidine (DHQD) are now the choice of catalytic \textit{cis}-dihydroxylation of almost all types of alkenes. A broad range of alkenes can be oxidized in an easy and convenient way to give \textit{cis}-diols with \(\text{ee} > 90\%\). These catalysts are relatively cheap and, more important, both enantiomer of the diols are accessible. Recent advances are rather rare, mainly focused on novel ligand synthesis\(^{210}\), catalyst recovery\(^{211}\), and new methodologies development\(^{212}\) such as the employment of ionic liquids and catalyst immobilization.

\[
\begin{align*}
\text{DHQD} & \quad \text{top (i)-attack} \\
\text{DHQ} & \quad \text{bottom (i)-attack}
\end{align*}
\]

Enantioselectivity prediction model — ‘mnemonic device\(^{213}\)’ of AD reactions
Chapter 1. Introduction

Important ligands used in asymmetric cis-dihydroxylation reactions

Representative enantioselective AD reactions

<table>
<thead>
<tr>
<th>Substrates</th>
<th>(DHQD)_2PHAL</th>
<th>(DHQD)_2PYR</th>
<th>(DHQD)_2DPP</th>
<th>(DHQD)_2AQN</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>70 (S)</td>
<td>83 (S)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C_8H_{17}</td>
<td>84 (R)</td>
<td>89 (R)</td>
<td>89 (R)</td>
<td>92 (R)</td>
</tr>
<tr>
<td>Cl_3C</td>
<td>86 (S)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C_4H_9</td>
<td></td>
<td></td>
<td>92 (R)</td>
<td></td>
</tr>
<tr>
<td>C_5H_{11}</td>
<td></td>
<td></td>
<td></td>
<td>99 (R)</td>
</tr>
<tr>
<td>CO_2Et</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ph</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Comparison of asymmetric cis-dihydroxylation reaction with enzyme

<table>
<thead>
<tr>
<th>Catalyst / Enzyme</th>
<th>Turnover number (min^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chymotrypsin^a</td>
<td>6000</td>
</tr>
<tr>
<td>Asymmetric cis-dihydroxylation^b</td>
<td>3000</td>
</tr>
<tr>
<td>DNA polymerase^a</td>
<td>900</td>
</tr>
<tr>
<td>Tryptophan synthetase^a</td>
<td>120</td>
</tr>
</tbody>
</table>

In summary, the AD reaction follows a highly stereospecific $cis$-addition of two OH groups to C-C double bonds. The formation of the side-products, such as epoxides and $trans$-diols, are rather rare. The AD reaction has broad substrate range, and tolerating most of the organic functional groups. It typically proceeds with high chemo- and enantioselectivity, without the requirement of a directing or sterically demanding group. The enantioselectivity can be predicted, the exceptions are rather rare and only exist with the reactions yielding low ee. The reaction have moderate conditions, can be scaled up and the catalysts have high turnover numbers. The disadvantage of the reaction is that osmium compounds are highly toxic. Therefore, great attention should be given to both operation and disposal procedures.
1.2.2 Enzymatic oxidation

1.2.2.1 Cis-dihydroxylation

The enzymatic conversion of a C-C double bond to a vicinal diol is defined as microbial dihydroxylation. This process is broadly divided into two groups: the direct conversion of arenes or alkenes into cis-dihydrodiols with dioxygenase enzymes; and the enzymatic epoxidation of the C-C double bonds with a monooxygenase followed by hydrolysis to generate the trans-vicinal diols. \textit{Trans}-dihydroxylations via an epoxide intermediate are carried out in a similar manner to biohydroxylations, but arene \textit{cis}-dihydroxylations often require more strict biotransformation conditions and relatively few organisms are available to do so.

The initial step for the bacterial arene degradation is usually a \textit{cis}-dihydroxylation of the aromatic ring. Since the first investigation of the arene dioxygenase\textsuperscript{214} for the production of \textit{cis}-dihydroarene diols by Gibson in the late 1960s, the reaction has been intensively studied in relation to the microbial degradation of xenobiotic aromatic compounds and, so far, more than 300 such reactions\textsuperscript{215} have been reported. The \textit{cis}-dihydroxylation reactions are catalyzed
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by a family of non-heme iron dependent dioxygenases, such as toluene dioxygenase (TDO), naphthalene dioxygenase (NDO), biphenyl dioxygenase (BPDO), and benzoate dioxygenase (BZDO). Among them, naphthalene dioxygenase is the most well investigated enzyme. It has been shown that NDO contains three components: an iron-sulfur flavoprotein that acts as a reductase; an iron-sulfur ferredoxin which transfers electrons from NAD(P)H; and an oxygenase with a catalytic mononuclear iron active site\textsuperscript{216}. The catalytic domain of NDO, which has been characterized by NDO X-ray crystallography\textsuperscript{217}, contains several hydrophobic amino acids in a flattened and elongated cavity that is capable of accepting aromatic substrates, such as naphthalene.

Proposed stepwise (A) and concerted (B) mechanisms of arene dioxygenases\textsuperscript{218}

The practical application of this important biotransformation into synthetic chemistry has been rather slow. It is interesting to note that almost 20 years elapsed from Gibson’s report in 1968 to the first application of cyclohexadiene \textit{cis}-dial in synthesis by Ley in 1987\textsuperscript{219}. Later, it was recognized that some dioxygenase enzymes, such as TDO, exhibit broad substrate specificity and this has led to a steady growth in the number of reports of \textit{cis}-dihydrodiol metabolites. The increasing applications of the arene dioxygenase enzymes in enantioselective synthesis have been tremendous\textsuperscript{220} in the last decades. However, the reaction had no equivalent in organic chemistry until 1995, when Motherwell reported the first chemical equivalents - the racemic synthesis of conduritols and inositols by photochemical osmium tetroxide oxidation of benzene\textsuperscript{221}.

Dioxygenases responsible for the formation of \textit{cis}-dihydro diols from arene substrates are quite often found in bacterial organisms. Wild-type bacterial strains capable of using arenes as a carbon source generally contain \textit{cis}-dial dehydrogenase enzymes which rapidly catalyze the
dehydrogenation of the cis-diol products to afford the corresponding catechols which undergo further dioxygenase-catalysed oxidation to yield ring-opened products. For a practical enzymatic cis-dihydroxylation, the accumulation of cis-dihydrodiol metabolites in the wild-type strain is required. To do so, the subsequent enzyme in the arene degradation pathway, such as cis-dihydrodiol dehydrogenase, must be suppressed. An alternative approach is to express the arene dioxygenase in a host organism that does not contain the cis-dihydrodiol dehydrogenase genes. Furthermore, the attempts to overcome the low enzyme activity and the dioxygenase instability have led to the construction of recombinant strains, such as *E. coli* TG2 and *E. coli* JM109222.

The availability of recombinant strains lacking cis-dihydrodiol dehydrogenases has allowed the investigation of dioxygenase regioselectivity by the isolation of arene cis-dihydroxylation products. Similar to the TDO-catalyzed cis-dihydroxylation reactions, the majority of arene cis-dihydroxylations afford the 2,3-dihydroxy products. In some cases, the carboxy group present in benzoic acids could direct the cis-dihydroxylation to the 1,2-position yielding an ipso-cis-dihydrodiol. Relatively few examples for the formation of cis-dihydrodiol at the 3,4-position of a mono-substituted benzene substrate have been reported223.

It is difficult to give definitive information on the most appropriate dioxygenase for a particular biotransformation. In general, TDO is suitable for substituted benzene substrates and bicyclic arenes, NDO is largely used in the cis-dihydroxylation of bi- and tri-cyclic arenes, BPDO is more appropriate for larger polycyclic aromatics (3–5 membered) and BZDO is particularly useful for benzoic acid substrates215.

Together with mutant and recombinant strains and large fermenters, it has been possible to
provide \textit{cis}-dihydrol products commercially from the mid-1980s. However, in spite of the fact that the production of arene \textit{cis}-dihydrols by whole cell biotransformation has been possible more than ten years, to date, there are only a few examples, such as indigo\textsuperscript{224}, polyphenylene\textsuperscript{225}, and indinavir\textsuperscript{226}, where \textit{cis}-dihydrols have been used in industrial production processes.

One of the reasons for this slow growth of industrial applications is the scale-up of these biotransformation processes. The bioproduct, \textit{cis}-dihydrols, is highly water-soluble and, in many cases, inherently instable. These properties complicate the isolation process, which is often the slowest and least efficient part of the overall process. The product isolation often requires repeated liquid-liquid extraction with an organic solvent, careful control of the pH and temperature to minimize losses through thermal or acid-catalyzed decomposition, addition of inorganic salt to increase the ionic strength of the aqueous liquor, and evaporation under reduced pressure. Alternative approaches have been examined, such as the selective removal of the \textit{cis}-diols onto solid absorbents\textsuperscript{227}, and continuous extraction \textit{via} two-phase biotransformations\textsuperscript{228}. 
1.2.2.2 *Trans*-dihydroxylation

Optically active epoxides and their corresponding vicinal diols are important building blocks in organic chemistry. Aliphatic alkene epoxidation can result in the formation of the epoxide, which can then be transformed to the corresponding *trans*-vicinal diols by subsequent hydrolysis with epoxide hydrolase. The synthesis of optically active *trans*-diols has been investigated intensively in recent years\cite{229}, and both chemical and enzymatic methodologies have been developed. While asymmetric *cis*-dihydroxylation can be achieved with Sharpless catalyst\cite{230} or a dioxygenase\cite{231}, epoxidation of a C-C double bond followed by hydrolysis could give rise to the asymmetric “*trans*-dihydroxylation”, which are realized by using a biological system containing a monooxygenase and an epoxide hydrolase\cite{232}. There are successful examples such as the fungus-catalyzed *trans*-dihydroxylations of several acyclic terpenes\cite{233}, limonene\cite{234}, and *α*-terpinene\cite{234}. However, enantioselective *trans*-dihydroxylation of a non-isoprene C-C double bond has been synthetically unsuccessful: several eukaryotic systems (plants, animal and fungi) were known to metabolize aromatic compounds transforming the C-C double bond into the corresponding *trans*-dihydrodiol with rather low yield\cite{235}; bioconversion of 1-benzyl-3-methyl-1,2,5,6-tetrahydro pyridine with *Cunninghamella verticillata* VKPM F-430 gave a mixture of 3,4-*trans*-diol and two mono-hydroxylated products\cite{236}. Moreover, *trans*-dihydroxylation catalysts reported so far are limited to the eukaryotic systems.

Since the epoxidation and hydrolysis are both involved in the *trans*-dihydroxylation, the epoxidation of C-C double bonds and the hydrolysis of epoxide are reviewed in the following section.

(1) Epoxidation of C-C double bonds

The Sharpless epoxidation method was the first developed chemical approach, which enabled the asymmetric epoxidation of allylic alcohols, whereas Jacobsen catalysts are more suitable for the epoxidation of *trans*- and terminal olefins. The Sharpless dihydroxylation process allows direct dihydroxylation of olefins with relatively satisfactory yield and enantioselectivity. This method also affords an alternative method for synthesizing the corresponding *trans*-epoxide with *trans*-substituted olefins as substrates\cite{237}. However, the limitations in substrate specificities and the requirements of heavy metal-based catalysts, which are expensive and possible sources for industrial pollution, have made the industrial
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application of these methods economically and environmentally unfriendly. As alternatives to these chemical approaches, a number of environmentally gentle enzymatic methods for the synthesis of optically active epoxides and trans-diols have been developed, and the considerable efforts have been summarized."\(^{238}\)

Epoxidation of C-C double bonds in aromatic or aliphatic substrates can be catalyzed by oxygenases such as cytochrome P450s, \(\omega\)-monooxygenases, and methane monooxygenases.

**Heme-containing monooxygenases**

Cytochrome P450s are among the most important enzymes in this group. They have been found in all types of living cells, such as bacteria, yeast, fungi, plants, and insects. The P450 enzymes oxygenate hydrophobic xenobiotics as the first and essential detoxication step in living organisms, and lead to water-soluble metabolites which can be easily excreted from the cell. The catalytically active site in cytochrome P450 enzymes is a ferroporphyrin, which can activate molecular oxygen and incorporate one of its two oxygen atoms into an organic molecule. Depending on the chemical structure of the substrates, monooxygenases are able to catalyze the hydroxylation of non-activated carbon atoms as well as the epoxidation of alkene C-C double bonds.

Epoxidation of various alkenes by P450 enzymes has been studied. Ruettinger\(^{239}\) reports the epoxidation of fatty acids such as palmitoleic acid by a cytochrome P450 enzyme from *Bacillus megaterium*. It has been shown that both the epoxidation and the hydroxylation reaction can be catalyzed by the same P450 monooxygenase. Ortiz has demonstrated that P450cam, isolated from *Pseudomonas putida*, could also stereoselectively epoxidize styrene derivatives\(^{240}\) to afford (1\(S\), 2\(R\))-epoxides with 78% ee.

![Epoxidation by P450cam from P. putida](image)

Several cytochrome P450s have been cloned and overexpressed in various hosts in recent years\(^{241}\). However, synthetic applications of these overexpressed enzymes have not been described. Levinger\(^{242}\) reported the site directed mutagenesis of a heme-containing human myoglobin, in which the phenylalanine in the heme pocket of this protein was replaced by
tyrosine. Compared with the wild type enzyme, the tyrosine mutant exhibited 25 times higher activity for the oxidation of styrene with hydrogen peroxide as oxidant. Moreover, the enantiomeric excess of the formed (R)-styrene oxide is increased to 98% ee (60% yield), whereas the wild type enzyme gives the racemic epoxide.

The ability to epoxidize C-C double bonds in organic molecules are not restricted to the cytochrome P450s, and some non-heme monooxygenases are able to do so as well.

\textbf{\textit{\(\omega\)-Monooxygenases}}

Abbott\textsuperscript{243} reported that the \(\omega\)-monooxygenases from \textit{Pseudomonas oleovorans} was able to hydroxylate aliphatic molecules as well as stereoselectively epoxidize terminal alkenes. The epoxidation of 1,7-octadiene afforded (R)-7,8-epoxy-1-octene in 80% ee, which could be further epoxidized to the corresponding diepoxide\textsuperscript{244}. The enzyme has been isolated and the enzymatic mechanism has been investigated\textsuperscript{245}.

These research efforts have led to the industrial synthesis of the \(\beta\)-blockers Metoprolol and Atenolol. Epoxidation of the prochiral allyl ethers with \textit{P. oleovorans} affords the corresponding (S)-epoxides with excellent enantiomeric excess. Refinement of this approach has led to the construction of a recombinant strain where an \textit{alk} gene from \textit{P. oleovorans} has been cloned into a \textit{P. putida} host. The product accumulates in this strain and can not be further oxidized. Further investigation revealed that the \textit{P. oleovorans} monooxygenase was capable to epoxidize various types of alkenes, including allylphenyl ethers and allylbenzene with high stereoselectivity\textsuperscript{246}.

\[ R = CH_3OCH_2CH_2 \]

\textit{R}\textsubscript{O}CH\textsubscript{2}H

\[ R = CH_2OCH_2CH_3 \]

\textit{R}\textsubscript{O}H

\[ CH_2\text{NHiPr} \]

\textit{OH}

\textit{P. oleovorans} 98.4% ee

\textit{R} = CH\textsubscript{3}OCH\textsubscript{2}CH\textsubscript{2}

\textit{Metoprolol}

\text{Synthesis of Metoprolol with stereoselective microbial epoxidation}\textsuperscript{247}

\textbf{Methane Monooxygenases}

Methane monooxygenases (MMOs), which are able to oxidize methane to methanol, have been successfully isolated from several microorganisms, such as \textit{Methylosinus trichosporium}, \textit{Methylococcus capsulatus}, and \textit{Methylococcus organophilum}\textsuperscript{248}. MMOs are also able to
oxidize series of hydrocarbons, such as aliphatic alkanes, alkenes, and aromatic substrates\(^{249}\). It has been shown that the same enzymatic system was responsible for the hydroxylation and the epoxidation process. Hou reported the epoxidation of short-chain alkenes (C2-C4) by MMOs\(^{250}\). Ohno observed epoxidation of short-chain alkenes by MMOs with 14-28% ee\(^{251}\). Seki studied the epoxidation of halogenated allyl derivatives with \textit{M. trichosporium}. Again, the observed ee of the epoxides were low\(^{252}\).

**Xylene oxygenase**

It has been discovered that \textit{P. putida} strain can growth on toluene and xylenes as carbon sources. The Xylene oxygenase (XO) in this strain is responsible for the hydroxylation of the aromatic substrates\(^{246}\), and the XO gene was cloned into an \textit{Escherichia coli} host. It has been observed that this enzyme epoxidizes styrene to produce (\textit{S})-styrene oxide, which is obtained in 93% ee. Similarly, \textit{m}-chlorostyrene was epoxidized with 95% ee. However, epoxidation of \textit{p}-chlorostyrene affords only 37% ee whereas \textit{p}-methylstyrene was oxidized at the methyl group. Improvements of these biotransformations with two-liquid phase fermentations have also been developed to increase the productivity\(^{253}\).

**Fungi**

Archelas reported that the epoxidation of (\textit{S})-sulcatol derivatives with the fungus \textit{Aspergillus niger} affords the corresponding (2\textit{S}, 5\textit{S}) epoxide, the precursor of pheromone pityol, in 50% yield (100% ee)\(^{254}\).

\[
\begin{align*}
\text{OR} & \xrightarrow{\text{Aspergillus niger}} \text{OR} & (2\textit{S}, 5\textit{S}) \text{Pityol} \\
\text{H}_2\text{C} & \xrightarrow{\text{Penicillium sp.}} \text{H}_2\text{C} & \text{90\% ee}
\end{align*}
\]

However, it is worth noting that such an accumulation of epoxide with whole cell fungi is rather rare. In fact, the formed epoxide is generally metabolized further to the corresponding vicinal diol. Several fungal strains, such as \textit{Cunninghamella elegans} and \textit{Syncephalastrum racemosum}, can epoxidize aromatic substrates, but the formed epoxides are directly
hydrolyzed into the less toxic corresponding trans-diols. The enzymes responsible for these oxidations are reported as cytochrome P450s\(^{255}\). Another study showed that the epoxidation of the alkene precursor of the antibiotic fosfomycin could be accessed in low concentrations, such as 0.5 g/L, with 20 fungi in 90% ee\(^{256}\).

**Haloperoxidases**

The chloroperoxidase from *Caldaryomyces fumago* is able to catalyze the halide independent direct epoxidation of alkenes, such as styrene and its derivatives, with hydrogen peroxide as oxidant\(^{257}\). Further studies\(^{258}\) indicate that the best substrates are aliphatic cis-disubstituted olefins. The enantioselectivities are variable from 49% ee to 97% ee with moderate yields depending on different alkene substituents.

![Reaction Scheme](image)

The use of chloroperoxidase for the synthesis of a biologically active (R)-(−)-mevalonolactone was described by Lakner\(^{259}\). Enantioselective epoxidation of methallylpropionate followed by chemical synthesis afforded the product in 57% overall yield and 93% ee.

![Reaction Scheme](image)

In some cases, the application of these enzymes is limited by enzyme deactivation with substrate, such as aliphatic terminal alkenes\(^{260}\), and by the sensitivity of the enzyme with the hydrogen peroxide oxidant. Very recently, direct epoxidation reactions catalyzed by haloperoxidase have been summarized\(^ {261}\).

**Others**

The preparation of 1,2(R)-epoxy-2-methylalkane, precursor of prostaglandin \(\omega\)-chains has been achieved by *Nocardia corallina* strain in 76-90% ee\(^ {262}\).
Mahmoudian reported the isolation of 18 bacterial strains which were able to produce optically active epoxides with excellent enantioselectivity (up to 98%)\textsuperscript{263}. However, it has been shown that the high enantioselectivity is due to the second-step enantioselective hydrolysis of the racemic epoxide produced by initial epoxidation. This provides an example for the use of epoxide hydrolases for the preparation of enantiopure epoxides.

As mentioned above, enantioselective epoxidation of C-C double bonds is possible with various monooxygenases of different types and origins, either as purified enzymes or as wild type or recombinant strains. These enzymes are generally cofactor dependent and quite often only low epoxide concentrations can be reached in the fermentor because of product inhibition. In spite of some promising progress with two-phase systems\textsuperscript{253, 264}, no practical process have been achieved thus far.

(2) Epoxide hydrolysis

The uses of epoxide hydrolases (EH), which are able to enantioselectively hydrolyze epoxides, provide an alternative approach for the production of optically active epoxides as well as chiral trans-diols. These enzymes have been known for a long time, and exist in bacteria\textsuperscript{265}, fungi\textsuperscript{266}, yeasts\textsuperscript{267}, plants\textsuperscript{268}, insects\textsuperscript{269}, and mammalian cells\textsuperscript{270}. Until recently, epoxide hydrolases were mainly studied for their biological role and catalytic mechanism. Several epoxide hydrolases have been purified and overexpressed, and mechanistic studies have been reported\textsuperscript{271}. The discovery of epoxide hydrolases in bacterial \textit{Rhodococcus} sp. and the fungus \textit{Aspergillus niger} prompted the increasing interest in the biocatalytic applications of these enzymes\textsuperscript{272}.

**Catalytic mechanism**

The solved X-ray structure of \textit{A. radiobacter} AD1 epoxide hydrolase revealed the proposed $\alpha/\beta$-hydrolase fold topology of the enzyme and the presence of a catalytic triad in the active site. Two tyrosine residues (Tyr152 and Tyr215) present in the cap domain are positioned close to Asp107 in such a way that they can be involved in catalysis by activating the substrate or by donating a proton to the leaving group\textsuperscript{273}. Sequence alignment studies showed
that the two tyrosines are crucial amino acid residues and conserved in all known soluble epoxide hydrolases from microorganisms, mammals and plants.\textsuperscript{274}

The mechanism consists of two steps, the first step resulting in the formation of a covalent intermediate (A) and a subsequent hydrolysis step (B). The oxygen atom in the epoxide is bound in the active site between Tyr152 and Tyr215. The epoxide is opened by a nucleophilic attack from Asp107, resulting in the formation of the intermediate. In the second step, a water molecule, activated by proton abstraction by His275, attacks the ester group of the covalent intermediate and the diol is released.

**Bacterial EH**

The utilization of the bacterial epoxide hydrolases for practical syntheses started in the late 1960s. Niehaus\textsuperscript{275} described the enantioselective hydrolysis of cis- and trans-9,10-epoxystearic acids by a *Pseudomonas* sp. Strain. An epoxide hydrolase\textsuperscript{276}, which was isolated from the *P. putida* strain, was used in the synthesis of tartaric acid from the precursor epoxide. Michaels\textsuperscript{277} described the hydrolysis of epoxypalmitate by *Bacillus megaterium*. Later on, de Bont\textsuperscript{278}, Escoffier\textsuperscript{279}, Jacobs\textsuperscript{280}, and Nagasawa\textsuperscript{281} studied the enzymatic hydrolysis of short-chain epoxides and epoxy-steroids with *Nocardia* sp., *Mycobacterium aureum*, *Pseudomonas* sp., and *Corynebacterium* sp. strains. However, these enzymes either have not been explored deeply or afford only very low enantioselectivity.

Faber\textsuperscript{282} observed epoxide hydrolase activity from a *Rhodococcus* sp. strain, which was previously used for the enzymatic hydrolysis of nitriles. This cofactor-independent, soluble *Rhodococcus* EH was further characterized\textsuperscript{283}. Aliphatic straight-chain terminal epoxides as...
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well as glycidyl derivatives can be accepted as substrates with low to moderate enantio-selectivities\(^{284}\). However, this initiated a comprehensive screening for enantioselective bacterial epoxide hydrolases\(^{285}\). It appears that EH enzymes are generally associated with the genus *Rhodococcus*, *Nocardia*, *Mycobacterium* and *Arthrobacter*\(^{286}\). Functionalized epoxides, as well as epoxide hydrolase catalyzed kinetic resolution, have been used in the total synthesis of \((S)-\text{frontalin}\) and \((R)-\text{mevalonolactone}\)\(^{287}\).

With 2-methyl-1,2-epoxy-heptane as substrate, enzymatic hydrolysis with the *Nocardia* EH1 enzyme and a sequential acid catalyzed hydrolysis step afford the \((S)-\text{diol}\) in 98% yield and 98% ee\(^{288}\). The enzyme catalyzed ring opening of the epoxide occurred at the terminal carbon atom, with the subsequent chemical hydrolysis of the remaining epoxide at another non-terminal carbon atom, yielding the trans-diol in high ee and conversion.

\[
\begin{align*}
\text{Nocardia EH1} & \quad \text{H}^+ / \text{dioxane} / \text{H}_2\text{O} \\
\text{98% ee; 98% yield}
\end{align*}
\]

Moreover, Archer reported the chemo-enzymatic conversion of 1-methyl-1,2-epoxy-cyclohexane to the corresponding \((1S,2S)-\text{diol}\) in a one-pot process with a *Corynebacterium* epoxide hydrolase and acid-catalyzed hydrolysis\(^{289}\). These two examples illustrate a valuable phenomenon, which has also been observed with other substrates\(^{268,290}\), since it allows the preparation of a single enantiomer of the formed *vicinal*-diol with the opposite enantiomer of the remaining epoxide from a racemic epoxide substrate.

There are also investigations reported by industrial researchers, where the enantioselective epoxide hydrolase activity has been observed in more than 50 different microorganisms\(^{291}\). However, it is worth to mention that the enantioselective hydrolyses of *meso*-epoxides with bacterial epoxide hydrolases have not been reported, except the investigation discussed in this thesis.

**Fungal EH**

As the bacterial epoxide hydrolases often prefer aliphatic epoxides as substrates, the epoxide hydrolases from fungal origin exhibit the supplementary ability. Pioneering work has shown that various fungal epoxide hydrolases\(^{292}\) are capable of hydrolyzing a variety of
epoxides with promising enantioselectivities. Racemic geraniol N-phenyl carbamate has been efficiently hydrolyzed by the fungus *Aspergillus niger*, affording the remaining (6S)-epoxide in 42% yield with 94% ee\(^ {293} \).

\[ \text{A. niger} \]
\[ \text{racemic} \rightarrow \text{(6S) - epoxide} \]

Following this methodology, the preparation of both enantiomers of the biologically active Bower's compound can be achieved in higher than 96% ee\(^ {294} \). Similarly, a diastereoselective hydrolysis of the exocyclic limonene epoxides can be achieved using the same fungi for the preparation of either enantiopure stereoisomer\(^ {293} \).

Similar results have also been obtained in the enantioselective hydrolysis of styrene oxide with *A. niger*, affording the (S)-enantiomer in 96% ee\(^ {295} \). Opposite enantioselectivity has been accessed with the fungus *Beauveria bassiana*, leading to the (R)-enantiomer in 98% ee. However, both of the hydrolysis reactions afford the corresponding R-diol.

\[ \text{B. bassiana} \]
\[ \text{(S) ee = 96\%} \]
\[ \text{(R) ee = 62\%} \]

\[ \text{A. niger} \]
\[ \text{(S) ee = 96\%} \]
\[ \text{(R) ee = 62\%} \]

It has been shown that substituted styrene derivatives, such as para-substituted styrene oxide\(^ {296} \) and \( \beta \)-substituted derivatives\(^ {290b} \), could also be accepted by these fungi as substrates. In the latter case, the hydrolysis of phenylpropene oxide affords the enantiopure (1R, 2R)-diol in 42% yield. With the same range of epoxides a shift in regioselectivity was observed from electron-donating to electron-withdrawing substituents with *Syncephalastrum racemosum*\(^ {297} \). A kinetic study revealed the mechanism, in contrast to that of the *A. niger* enzyme, as a concerted process implying a general acid activation. Recently, it has been shown that *A.*
niger could also be used to obtain optically pure (S)-2,3- and 4-pyridyl-oxiranes. For practical applications, 330 mM \( p \)-nitrostyrene epoxide has been hydrolyzed within 6 h for the preparation of (S)-\( p \)-nitrostyrene epoxide (49% yield, 99% ee). Moreover, controlled acid hydrolysis of the reaction mixture led to an overall yield of 94% of (R)-diol (ee 80%). The recrystallized (R)-diol (99% ee) can be further used for the preparation of the biologically active enantiomer of Nifenalol.

Another application of the enantioselective fungal hydrolysis is the preparation of indene oxide, which can be used for the synthesis of the HIV protease inhibitor Indinavir. Racemic epoxyindene can be rapidly hydrolyzed by \( B. \) bassiana to afford the corresponding (1R, 2S)-epoxide (98% ee, 20% yield) and (1R, 2R)-trans-diol (69% ee, 48% yield). Similarly, epoxy-dihydronaphthalene can also be hydrolyzed to afford the (1R, 2R)-diol in 98% ee.

Yeast EH

Epoxide hydrolase activity has also been detected in various yeast strains. The yeast \( R. \) glutinis showed a wide substrate range with aliphatic and aromatic epoxides. Further screenings showed that enantioselective epoxide hydrolase activity is relatively common in various yeasts.

Mammalian EH

The investigation of enantioselective hydrolysis of epoxides using mammalian EHs began in the 1970s. Several mammalian epoxide hydrolases, such as microsomal EH, cholesterol EH, and leukotriene A4 EH, have been identified. A variety of substrates, including racemic or prochiral, aromatic or aliphatic compounds, can be efficiently hydrolyzed by these enzymes. Among them, the most extensively studied mammalian epoxide hydrolases are the membrane bound microsomal EH (mEH) and the soluble EH (sEH). The microsomal EH is generally involved in the detoxification of xenobiotic epoxides, which are produced by cytochrome P450 catalyzed oxidations, whereas the soluble EH is involved in both the detoxification of epoxides and the biosynthesis of endogenous compounds.

Belluci reported the enantioselective hydrolysis of cycloalkane oxides and epoxides of heterocycles. Successful hydrolyses of meso-cycloalkene oxides have been shown with both microsomal EH and soluble EH enzymes to afford the corresponding trans-diol with 40-90%
ee depending on different ring sizes.

![](image)

The enantioselectivity of the hydrolysis of substituted styrene oxides by sEH and mEH is usually low to moderate. An exception is the mEH-catalyzed hydrolysis of cis-β-substituted styrene oxides. It has been shown that β-substituted derivatives could be hydrolyzed by rabbit liver mEH, resulting in the formation of the remaining epoxide and the trans-diol with good enantioselectivity. The hydrolysis of cis-β-methyl styrene oxide and cis-β-ethyl styrene oxide afford nearly optically pure (1S, 2R)-epoxides and (1R, 2R)-diols. Similarly, the hydrolysis of cis-β-substituted aliphatic epoxides by mEH quite often leads to high enantioselectivity.

Although mammalian epoxide hydrolases exhibit high enantioselectivity with specific substrates, the low availability of the enzymes limits the production of optically active epoxides or diols in gram scale. The uses of these mammalian epoxide hydrolases for the large-scale production have not been described. In comparison, epoxide hydrolases from microbial sources such as fungi and bacteria can be easily obtained in larger amounts and, therefore, have a much higher potential to be used for the industrial production of optically active epoxides and the corresponding trans-diols.
1.3 Backgrounds and overview of the thesis

1.3.1 Backgrounds

Our capability to learn from Mother Nature is one of the main reasons why human beings are superior to other living forms. One of such capability is the use of microorganisms, such as brewing beers and baking bread. The past two centuries have already seen the discovery of microorganisms, the definition of what they are, and gradually understanding of the biosynthetic pathways they carry out, and finally, it has been realized that tremendous different kinds of compounds could be prepared via biosynthesis.

Over the past decades, biocatalysis has received increasing attention as an attractive method in numerous processes and its uses are still increasing dramatically. The demand for single-enantiomer compounds, particularly in the strongly regulated pharmaceutical industry, has increased the demand for biocatalytic process for fine chemical applications. The uses of biocatalysts in organic synthesis have considerable advantages. Firstly, one of the most important benefits is the high selectivity, such as chemo-, regio-, and enantioselectivity. Secondly, enzymes can also catalyze the reactions that are chemically unfeasible. A prominent example is the regio- and stereo-selective hydroxylation of the non-activated carbon atoms. Furthermore, another important feature is that enzyme can catalyze sequential reactions, such as the enantioselective trans-dihydroxylation of C-C double bonds. Finally, enzymatic processes normally use water as solvent, making them environmental friendly.

To make biocatalysis economically more competitive with existing technologies, molecular biology is often used to improve the productivity and the catalytic properties of the enzymes with cloning and overexpression of the genes in suitable hosts. Genetic engineering can adapt enzymes to unnatural reaction conditions or modify them in such a way that they can perform designed reactions that originally they didn’t\textsuperscript{312}. Substrate engineering is employed in altering the enantioselectivity and the catalytic activity of a known biotransformation, as well as modifying a substrate analog to enable the catalytic reaction with compounds that originally were not accepted by the enzyme\textsuperscript{123,313}. However, for a desired reaction with specified selectivity, the most important task is the discovery of appropriate biocatalyst.
Regio- and stereoselective hydroxylation of non-activated carbon atoms remains a challenge in organic chemistry. Nature solved this problem via monooxygenases, which use molecular oxygen as oxidant to insert one O atom at a specific carbon atom and reduce the second O atom with electrons from NAD(P)H. This relatively complex reaction often requires a metal center and additional proteins for electron transfer from electron-donating reduced cofactors.

A number of monooxygenases are reported, and some of them have been purified, cloned, and overexpressed. The X-ray structure of soluble methane monooxygenase (sMMO), soluble bacterial P450s (P450cam, P450BM3) have been determined, and the insights into the catalytic mechanisms have been obtained. However, the synthetic applications of these well-known monooxygenases are rather limited, mainly due to the narrow substrate range and the poor regio- and stereoselectivity. With the advantage of broad substrate range, nowadays, fungal catalysts are often used in laboratorial hydroxylations. However, they suffer from several drawbacks, such as low activity, poor regio- and stereoselectivity, side reactions, and difficult to handle. It is therefore necessary to discover and develop new biohydroxylation catalysts with high activity, high regio- and stereoselectivity, new substrate specificity, and broad substrate range. These catalysts should also be easy to handle, thus improving the acceptance of using them for routine hydroxylations in organic laboratory.
1.3.2 The discovery of *Sphingomonas* sp. HXN-200 for the hydroxylation of *N*-benzyl pyrrolidine

Previously, Li et al. reported the discovery of new biohydroxylation catalyst for the regio- and stereoselective hydroxylation of pyrrolidines\(^{318}\) to afford the corresponding (\(R\))- and (\(S\))-3-hydroxypyrrolidines which are useful pharmaceutical intermediates.

Microorganisms involved in alkane degradation pathways as a source for biocatalysts

Organisms able to use \(n\)-alkanes as sole source of carbon and energy can be used as a catalyst source for the screening the desired biocatalytic reaction.

The alkane degrading pathway
In order to find the appropriate catalyst, 70 alkane-degrading strains were screened on a microtiter plate for the 3-hydroxylation of N-benzyl pyrrolidinone. It was shown that 12 alkane-degrading strains were able to catalyze the hydroxylation of N-benzyl pyrrolidinone to N-benzyl 3-hydroxypyrrolidinone. While the well-known membrane-bound AlkB-containing *P. oleovorans* GP01 affords the (*R*)-product in 52% ee, *Sphingomonas* sp. HXN-200 exhibits much higher activity, excellent regioselectivity, and the opposite enantioselectivity, giving the (*S*)-product in 53% ee.

![Alkane degrading strains](image-url)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Strain</th>
<th>Activity (U/g CDW)</th>
<th>Enantiomeric excess (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HXN-1100</td>
<td>0.4</td>
<td>70 (<em>R</em>)</td>
</tr>
<tr>
<td>2</td>
<td>HXN-400</td>
<td>0.05</td>
<td>65 (<em>R</em>)</td>
</tr>
<tr>
<td>3</td>
<td><em>P. putida</em> P1</td>
<td>0.1</td>
<td>62 (<em>R</em>)</td>
</tr>
<tr>
<td>4</td>
<td><em>P. oleovorans</em> GP01</td>
<td>0.1</td>
<td>52 (<em>R</em>)</td>
</tr>
<tr>
<td>5</td>
<td>BC20</td>
<td>0.1</td>
<td>40 (<em>R</em>)</td>
</tr>
<tr>
<td>6</td>
<td>HXN-1500</td>
<td>0.3</td>
<td>25 (<em>R</em>)</td>
</tr>
<tr>
<td>7</td>
<td>HXN-500</td>
<td>1.1</td>
<td>10 (<em>R</em>)</td>
</tr>
<tr>
<td>8</td>
<td>HXN-200</td>
<td>0.6</td>
<td>53 (<em>S</em>)</td>
</tr>
<tr>
<td>9</td>
<td>HXN-100</td>
<td>0.3</td>
<td>10 (<em>S</em>)</td>
</tr>
<tr>
<td>10</td>
<td>HXN-1900</td>
<td>1.0</td>
<td>&lt; 10 (<em>S</em>)</td>
</tr>
<tr>
<td>11</td>
<td>HXN-1000</td>
<td>0.1</td>
<td>&lt; 10 (<em>S</em>)</td>
</tr>
<tr>
<td>12</td>
<td>HXN-600</td>
<td>0.3</td>
<td>0</td>
</tr>
</tbody>
</table>

It was also found that *Sphingomonas* sp. HXN-200 could accept different substituted pyrrolidines as substrates. Changing the docking/protecting group from benzyl to benzyloxy-carbonyl resulted in a 3-fold increase of the activity and improvement of the enantiomeric excess of the hydroxylated product from 53% (*S*) to 75% (*R*).
The strain HXN-200 can be grown easily to high cell density, and the frozen/thawed cells are easy to handle, and used for the gram-scale preparations. The ee of (S) and (R)-3-hydroxy pyrrolidines can be easily increased to 95% by crystallization.

1.3.3 Goal of the thesis

In this PhD thesis, we first aim to further develop the *Sphingomonas* sp. HXN-200 as a routine biohydroxylation catalyst, and to explore the synthetic potential for the production of other important hydroxylated compounds.

The primary selected biocatalytic reaction to be investigated during this project is the hydroxylation of non-activated hydrocarbons, since the success of this reaction will have a significant influence and will enrich the research on the oxidative functionalization of hydrocarbons as well as on pharmaceutical synthesis. The next reaction to be investigated is the enantioselective trans-dihydroxylation, which is unique and able to afford synthetically useful trans-diols.

In order to understand the influence of substrate engineering on the biocatalytic reactions, a series of substrates with various docking/protecting groups will be investigated and the difference will be studied.

To get a fundamental understanding of this novel biocatalyst, the monooxygenase responsible for the hydroxylation will be purified from the strain HXN-200 and characterized.

Finally, the substrate/enzyme interaction, which may leave significant scope for a structural understanding of the phenomenon observed in the biohydroxylation, will be studied by computational modeling.
1.3.4 Overview of the thesis

Chapter 2, 3, and 4 describe the regio- and stereoselective biohydroxylation of a series of N-substituted azetidines, piperidines, pyrrolidine-2-ones, and piperidine-2-ones. Both the reaction activity and the enantioselectivity can be altered with a variety of different heads and tails in substrates. Gram scale preparations of the formed optical active products, which are important pharmaceutical intermediates, have also been carried out. Moreover, the use of lyophilized cell powder for the enantioselective hydroxylation is described in Chapter 2.

Chapter 5 describes the discovery of the epoxide hydrolase activity in Sphingomonas sp. HXN-200, and the first example of enantioselective hydrolysis of meso-epoxide to afford the corresponding trans-diol. Chapter 6 demonstrates the enantioselective trans-dihydroxylation of C-C double bonds which has been achieved in high yield and ee with the strain HXN-200 by the sequential enzymatic epoxidation/hydrolysis reaction.

The enzyme responsible for the hydroxylations was purified and characterized as a soluble cytochrome P450 monooxygenase, namely P450pyr. The purification procedures and the partial characterization are summarized in Chapter 7. Moreover, a three-dimensional structural homology model is developed based on the amino acid sequence of the P450pyr enzyme and the X-ray structure of the P450terp. The substrate/enzyme interactions are studied in Chapter 8, and these provide good explanations for the regio- and enantioselectivity of the enzymatic biohydroxylation reactions.

Finally, the overall conclusions and the future perspectives of the research described in this thesis will be discussed in Chapter 9.
Reference


Chapter 1. Introduction


Chapter 1. Introduction


Chapter 1. Introduction


Chapter 1. Introduction


Chapter 1. Introduction


Chapter 1. Introduction


Chapter 1. Introduction


Chapter 1. Introduction


Chapter 1. Introduction

Chapter 1. Introduction


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Chapter 1. Introduction


Chapter 2

Practical Syntheses of N-Substituted 3-Hydroxyazetidines and 4-Hydroxypiperidines by Hydroxylation with Sphingomonas sp. HXN-200

Abstract

Hydroxylation of N-substituted azetidines 11-12 and piperidines 15-19 with *Sphingomonas* sp. HXN-200 gave 91-98% of the corresponding 3-hydroxyazetidines 13-14 and 4-hydroxy-piperidines 20-24, respectively, with high activity and excellent regioselectivity. High yield preparations (2 g product/L) were achieved with frozen/thawed cells as biocatalyst. For the first time, rehydrated lyophilized cells were successfully used for the biohydroxylation.
Introduction

3-Hydroxyazetidines and 4-hydroxypiperidines are useful pharmaceutical intermediates. For example, 3-hydroxyazetidines are used in the synthesis of oral carbapenem antibiotics L-036 \(1\) and L-084 \(2\), antiepileptic Dezinamide \(3\), and antihypertensive Azelnidipine \(4\). 4-hydroxypiperidines are used for the preparation of allergic rhinitis drugs Ebastine \(5\) and Betotastine besilate \(6\), antibacterial Nadifloxacin \(7\), antiallergy/antiasthmatic Linazolast \(8\), and agents for antiplatelet therapy Lamifiban \(9\) and Sibrafiban \(10\) (Scheme 1). In practice it is often advantageous, if not required, to use 3-hydroxyazetidine and 4-hydroxypiperidine in their \(N\)-protected form.

![Chemical structures of various compounds](image)

There are synthesis routes to 3-hydroxyazetidine and its \(N\)-substituted derivatives, but each of them has drawbacks: amination of epichlorohydrin is limited to primary hindered amines\(^3\); amination of 1,3-dichloro-2-propyl ether gives low yield\(^4\); halogenation of 2,3-
epoxyamines has only one example for tert-butyl amine\textsuperscript{3g}; the route via 1-azabicyclo [1.1.0]
butane requires a special reagent\textsuperscript{3h}; and reduction of azetidinone requires difficult to obtain
starting materials\textsuperscript{3i}. Syntheses of 4-hydroxypiperidine and \(N\)-substituted 4-hydroxypiperidine
are also not straightforward: preparations involving reduction of \(N\)-substituted 4-
piperidones\textsuperscript{4a-e}, hydrogenation of 4-hydroxypyrindine or \(N\)-substituted 1\(H\)-pyridin-4-one\textsuperscript{4f-h}, or
hydrogenation and cyclization of 3-hydroxy-glutaronitride\textsuperscript{4i} give low overall yields in multi-
steps; syntheses via Mannich-type cyclization of formaldehyde with benzyl-but-3-enyl-
amine\textsuperscript{4j} or with \(N\)-benzylammonium trifluoroacetate and allyl-trimethyl-silane\textsuperscript{4k} are not
practical; hydroboration of \(N\)-trimethylsilanyl- or \(N\)-benzyloxylcarbonyl-1,2,5,6-tetrahydro-
pyridine\textsuperscript{4l-n} gives a mixture of 3- and 4-hydroxy piperidines.

Regioselective hydroxylation of azetidine and piperidine represents one of the simplest
ways for preparing the hydroxylated derivatives. While selective hydroxylation on non-
activated carbon atom remains still a challenge in synthetic chemistry\textsuperscript{5}, biohydroxylation can
be a useful tool for this type of transformation\textsuperscript{6-7}. However, no successful biohydroxylation of
azetidine or \(N\)-substituted azetidines has been reported thus far. Hydroxylations of \(N\-
substituted piperidines with \textit{Beauveria sulfurescens} ATCC 7159\textsuperscript{7i,8} or \textit{Aspergillus niger} VKM
F-1119\textsuperscript{9} are known, but the low activity, yield, and product concentration (less than 0.1 g/L)
limit their synthetic applications.

Results and discussion

\textit{Sphingomonas} sp. HXN-200 was grown on \(n\)-octane vapor in 30 L E2 medium\textsuperscript{10} at 30°C
and 1500 rpm for 90 h to a cell density of 8.5 g/l. The cells were harvested and the cell pellets
(2.5 Kg wet cells consisting of 10\% dry cells) were stored at -80°C. The frozen/thawed cells
were used for hydroxylation of \(N\)-substituted azetidines 11-12 and piperidines 15-19.
Substrates 11 and 12\textsuperscript{11} were synthesized by reaction of azetidine with phenyl chloroformate
and di-tert-butyl-dicarbonate in 67\% and 83\% yield, respectively. Piperidines 15\textsuperscript{12}, 16\textsuperscript{8a}, and
18\textsuperscript{13} were prepared according to established procedures, and 17\textsuperscript{14} was prepared in an
improved yield of 82\%.

Hydroxylations were performed with frozen/thawed cells of \textit{Sphingomonas} sp. HXN-200
on a 10-mL scale in the exploratory stage, and the bioconversions were followed by HPLC
analyses. As shown in table 1, hydroxylation of azetidine 11-12 gave the desired 3-hydroxy-
azetidine 13-14 with high activities (15-17 U/g cdw, U = µmol/min, cdw = cell dry weight) and high conversions. No byproducts were detected, indicating the excellent regioselectivity and clean biotransformation. Moreover, higher product concentrations could be achieved as follows: hydroxylation of 10 mM solutions of 11 and 12 gave 82% and 89% of 3-hydroxyazetidine 13 and 14, respectively. This demonstrates the first successful biohydroxylation of azetidines.

Table 1. Hydroxylation of N-substituted azetidines 11-12 with frozen/thawed cells (4.0 g cdw/l) of Sphingomonas sp. HXN-200.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>Activity (µmol/min)</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 (5.0)</td>
<td>13</td>
<td>15</td>
<td>82</td>
</tr>
<tr>
<td>11 (7.0)</td>
<td>13</td>
<td>16</td>
<td>89</td>
</tr>
<tr>
<td>11 (10.0)</td>
<td>13</td>
<td>17</td>
<td>98</td>
</tr>
<tr>
<td>12 (5.0)</td>
<td>14</td>
<td>17</td>
<td>82</td>
</tr>
<tr>
<td>12 (10.0)</td>
<td>14</td>
<td>17</td>
<td>89</td>
</tr>
</tbody>
</table>

活性是在前30分钟内测定的。b转化率是通过HPLC分析确定的；误差限：2%的陈述值。

Similarly, hydroxylation of piperidines 15-19 afforded the desired 4-hydroxypiperidines 20-24. As shown in table 2, high activity was obtained in hydroxylation of 15-18 (12-29 U/g cdw), while moderate activity (4.5 U/g cdw) was observed for the hydroxylation of 19, probably due to the steric hindrance of the N-benzoyl group in the substrate. No other regioisomers were detected during hydroxylation, indicating excellent regioselectivity. Hydroxylation of 15 was a clean reaction, while hydroxylation of 16-19 gave a small amount of the corresponding 4-ketones. Nevertheless, over 91% of 4-hydroxypiperidines 20-24 were formed in hydroxylation of 15 (5 mM), 16 (2 mM), 17 (8 mM), 18 (6 mM) and 19 (5 mM), respectively.
Preparative hydroxylations were carried out on 60-mL to 2-L scales with frozen/thawed cells, shown in table 3. Hydroxylation of azetidine 12 (15.8 mM) on 1-L scale gave 2.140 g (79%) of 3-hydroxyazetidine 14. Hydroxylation of 11 on 60-ml scale gave 13 in 81% yield. Similarly, hydroxylation of piperidine 15 (5.0 mM) at a cell concentration of 4.0 g cdw/l gave 4-hydroxypiperidine 20 in 83% yield. The product concentration was easily increased to 2.072 g/L by use of a higher cell density (10.2 g cdw/l) and higher substrate concentration (15 mM). Compounds 21, 22, 23, and 24 were also prepared in good yields by hydroxylation of 16-19, respectively. These results are clearly superior to those obtained with other hydroxylation systems.

Table 2. Hydroxylation of N-substituted piperidines 15-19 with frozen/thawed cells (4.0 g cdw/l) of Sphingomonas sp. HXN-200.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Prod.</th>
<th>Activity$^a$ (U/g cdw)</th>
<th>Conv.$^b$(%) 0.5 h</th>
<th>Conv.$^b$(%) 1 h</th>
<th>Conv.$^b$(%) 2 h</th>
<th>Conv.$^b$(%) 3 h</th>
<th>Conv.$^b$(%) 5 h$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 (5.0)</td>
<td>20</td>
<td>49</td>
<td>77</td>
<td>94</td>
<td>98</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 (2.0)</td>
<td>21</td>
<td>12</td>
<td>71</td>
<td>83</td>
<td>87</td>
<td>87</td>
<td>91 (1.4)</td>
</tr>
<tr>
<td>16 (3.0)</td>
<td>21</td>
<td>13</td>
<td>49</td>
<td>61</td>
<td>65</td>
<td>68</td>
<td>69</td>
</tr>
<tr>
<td>17 (7.0)</td>
<td>22</td>
<td>19</td>
<td>31</td>
<td>53</td>
<td>84</td>
<td>91</td>
<td>94 (5.1)</td>
</tr>
<tr>
<td>17 (8.0)</td>
<td>22</td>
<td>18</td>
<td>26</td>
<td>51</td>
<td>76</td>
<td>88</td>
<td>94 (4.1)</td>
</tr>
<tr>
<td>18 (5.0)</td>
<td>23</td>
<td>29</td>
<td>69</td>
<td>91</td>
<td>94</td>
<td>94</td>
<td>94 (6.3)</td>
</tr>
<tr>
<td>18 (6.0)</td>
<td>23</td>
<td>27</td>
<td>54</td>
<td>79</td>
<td>86</td>
<td>93</td>
<td>93 (6.6)</td>
</tr>
<tr>
<td>19 (2.0)</td>
<td>24</td>
<td>4.5</td>
<td>27</td>
<td>50</td>
<td>78</td>
<td>91</td>
<td>97 (1.8)</td>
</tr>
<tr>
<td>19 (3.0)</td>
<td>24</td>
<td>4.0</td>
<td>16</td>
<td>32</td>
<td>57</td>
<td>74</td>
<td>90 (1.7)</td>
</tr>
</tbody>
</table>

$^a$Activity was determined over the first 30 min. $^b$Conversion was determined by HPLC analysis; error limit: 2% of the stated values. $^c$Number in bracket is the conversion to the corresponding 4-ketones at 5 h, no bracket indicates no ketones formed.
To facilitate the application of this interesting biohydroxylation system in organic synthesis, we developed a lyophilized cell powder preparation of *Sphingomonas* sp. HXN-200 as a practical catalyst for use in organic synthesis. Hydroxylation of piperidine 15 (5 mM) with the rehydrated catalyst powder at a density of 4.0 g cdw/l afforded 80% of 4-hydroxypiperidine 20. Comparing with a similar hydroxylation with frozen/thawed cells, shown in Figure 1, 85% of the activity was achieved with the lyophilized powder. It has been shown that hydroxylation with *Sphingomonas* sp. HXN-200 is NADH dependent. The fact that rehydrated lyophilized cells are able to carry out such a NADH dependent hydroxylation indicates that these cells are capable of retaining and regenerating NADH at rates equal to or exceeding the rate of hydroxylation. Although it is known that lyophilized microbial cells retain activities for hydrolytic reactions after rehydration, our result is the first example of the use of lyophilized cells for a cofactor dependent hydroxylation.

### Table 3. Preparation of *N*-substituted 3-hydroxyazetidines 13-14 and 4-hydroxypiperidines 20-24 by hydroxylation with frozen/thawed cells of *Sphingomonas* sp. HXN-200.

<table>
<thead>
<tr>
<th>substrate</th>
<th>scale (mM)</th>
<th>cell density (g cdw/l)</th>
<th>time (h)</th>
<th>conv. (%)</th>
<th>yield (%)</th>
<th>yield (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>4.0</td>
<td>4.0</td>
<td>1.5</td>
<td>98</td>
<td>81.0</td>
<td>37.5</td>
</tr>
<tr>
<td>12</td>
<td>15.8</td>
<td>10.2</td>
<td>5.0</td>
<td>83</td>
<td>79.0</td>
<td>2140</td>
</tr>
<tr>
<td>15</td>
<td>5.0</td>
<td>4.0</td>
<td>4.0</td>
<td>98</td>
<td>82.9</td>
<td>1501</td>
</tr>
<tr>
<td>15</td>
<td>15.0</td>
<td>10.2</td>
<td>5.2</td>
<td>98</td>
<td>76.2</td>
<td>2072</td>
</tr>
<tr>
<td>16</td>
<td>2.0</td>
<td>4.0</td>
<td>3.0</td>
<td>96</td>
<td>70.2</td>
<td>33.0</td>
</tr>
<tr>
<td>17</td>
<td>7.0</td>
<td>4.0</td>
<td>4.0</td>
<td>91</td>
<td>83.2</td>
<td>43.6</td>
</tr>
<tr>
<td>18</td>
<td>5.0</td>
<td>4.0</td>
<td>2.0</td>
<td>96</td>
<td>69.5</td>
<td>69.3</td>
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<tr>
<td>19</td>
<td>2.0</td>
<td>4.0</td>
<td>5.0</td>
<td>83</td>
<td>71.5</td>
<td>29.3</td>
</tr>
</tbody>
</table>

*Conversion was determined by HPLC analysis; error limit: 2% of the stated values. *Yield of the isolated pure product. *Substrate was added at different time points.*

**Conclusion**

Hydroxylations of *N*-substituted azetidines and piperidines with *Sphingomonas* sp. HXN-200 are highly effective and regioselective, giving the corresponding 3-hydroxy azetidines and 4-hydroxypiperidines in 91-98%, respectively. High yield (70-83%) preparation of 13-14 and 20-24 have been achieved by use of frozen/thawed cells as biocatalyst. Hydroxylations of *N*-tert-butoxycarbonyl-azetidine 12 and *N*-benzyl piperidine 15 were carried out on 1 L scale affording 12 in 2.140 g and 15 in 2.072 g, respectively, providing with convenient and practical syntheses of 3-hydroxyazetidines and 4-hydroxypiperidines. For the first time,
lyophilized cells have been used successfully in hydroxylation without addition of cofactor. These catalysts can now be used in routine hydroxylations.

Figure 1. Biohydroxylation of \( N \)-benzylpiperidine 15 (5.0 mM) to 20 with rehydrated lyophilized cells and frozen/thawed cells of \( Sphingomonas \) sp. HXN-200 (4.0 g cdw/l) in 20 ml 50 mM K-phosphate buffer (pH=8.0) containing glucose (2%).
Experimental Section

**General Methods.** $^1$H- and $^{13}$C-NMR spectra were determined at 300 K in CDCl$_3$, chemical shifts in ppm relative to TMS, coupling constants $J$ in Hz. Mass spectra were obtained by Atmospheric Pressure Chemical Ionization at 40 eV with Hewlett Packard 1100 LC-MS. Melting points are uncorrected. Analytic high-performance liquid chromatography (HPLC) was carried out with a Hewlett Packard 1050 instrument. IR spectra were measured in CHCl$_3$. Optical rotations were determined using a PERKIN ELMER 241 polarimeter. CD spectra were measured in acetonitrile at 20°C on a JASCO J-710 spectropolarimeter. Bioconversion was analyzed by HPLC. The purity of the products was established by GC analyses with a Chrompack CP–Sil-5CB column (25 m x 0.32mm, temperature program: 60°C for 2 min, increase to 280°C at a rate of 25°C /min, then 280°C for 1 min).

**Growth of Sphingomonas sp. HXN-200.** Cells of *Sphingomonas* sp. HXN-200 were produced by growth on $n$-octane in 30 L E2 medium.$^7b$ The growth curve is given in Figure 2.

![Figure 2. Growth of Sphingomonas sp. HXN-200 on n-octane in 30 L E2 medium in a bioreactor](image)

**Materials.** Piperidine (99%), azetidine hydrochloride (97%), $\sigma$-valerolactam (97%), benzylbromide (98%), benzylchloroformate (95%), phenylchloroformate (97%), and di-tert-butyl-dicarbonate (99.5%) were purchased from Fluka. Compound 19 (98%) was purchased
from Lancaster. Compounds 15, 16, and 18 were prepared according to the published procedures.

**Chemical preparation of substrates.**

**N-Phenoxycarbonyl-azetidine 11.** A solution of phenyl chloroformate (1.35 ml, 10.7 mmol) in THF (2 ml) was added to a stirred mixture of azetidine hydrochloride (1.00 g, 10.7 mmol) and NaHCO₃ (2.06 g, 24.6 mmol) in THF-water (1:1, 10 ml) at 0°C, and the mixture was stirred at rt for 2 h. The product was extracted into CHCl₃ and washed with aq. Na₂CO₃ and then with water. The organic phase was dried over Na₂SO₄, filtered, and solvent evaporated. Column chromatography on a silica gel column afforded 66.7% (1.264 g) of 11. Rₐ 0.15 (ethyl acetate/hexane 2:8); mp 41.1-42.3°C; Purity: 96.8% (GC, t_R = 8.10 min); ¹H NMR (300 MHz, 243K) δ 7.41-7.34 (m, 2 H), 7.24-7.19 (m, 1 H), 7.14-7.10 (m, 2 H), 4.22 (t, 2 H, J = 7.8), 4.12 (t, 2 H, J = 7.8), 2.32 (quin, 2 H, J = 7.8); ¹³C NMR (75 MHz) δ 154.19 (s), 150.58 (s), 129.30 (d), 125.29 (d), 121.66 (d), 50.04 (t), 48.94 (t), 15.48 (t); MS m/z 178 (100%, M+1); IR (CHCl₃) ν 1716, 1595 cm⁻¹.

**N-tert-Butoxycarbonyl-azetidine 12.** To a mixture of azetidine hydrochloride (1.87 g, 20 mmol) and NaHCO₃ (6.72 g, 0.08 mol) in water (50 ml) was added di-tert-butyl dicarbonate (5.45 g, 25 mmol) and the mixture was stirred for 60 h. The product was extracted into ethyl acetate, the organic phase was dried over Na₂SO₄, and the solvent evaporated. Purification by column chromatography on silica gel afforded 83.4% (2.62 g) of 12 as colorless oil. Purity: 99.5% (GC, t_R = 5.39 min); Rₐ 0.46 (ethyl acetate/hexane 5:5); ¹H NMR (400 MHz) δ 3.86-3.79 (m, 4 H), 2.12-2.03 (m, 2 H), 1.35 (s, 9 H); ¹³C NMR (100 MHz) δ 157.29 (s), 79.96 (s), 50.37 (t), 29.34 (q), 16.28 (t); MS m/z 114 (50%, M-C₃H₇), 102 (100%, M-C₅H₅N); IR (CHCl₃) ν 1685 cm⁻¹.

**N-Phenoxycarbonyl piperidine 17.** A solution of phenyl chloroformate (14.5 ml, 115 mmol) in THF (20 ml) was added dropwise at 0°C to a stirred mixture of piperidine (11.5 ml, 115 mmol) and NaHCO₃ (12.5 g, 149 mmol) in THF-water (1:1, 100 ml). The mixture was then stirred at rt for 2 h, and CHCl₃ (200 ml) and 5% aqueous Na₂CO₃ (50 ml) were added. The organic layer was separated, washed with water (50 ml), dried over Na₂SO₄, filtered, and concentrated in vacuum. The crude product was purified by crystallization in hexane to give 19.3 g (81.8%) of 17. Purity: 99.3% (GC, t_R = 9.05 min); ¹H NMR (300 Hz) δ 7.37-7.08
General procedure for small-scale hydroxylation.

The frozen cells of *Sphingomonas* sp. HXN-200 were suspended to a cell density of 4.0 g/L in 10 ml of 50 mM K-phosphate buffer containing glucose (2% w/v) at pH = 8.0 in a 100 ml Erlenmeyer flask. Substrates were added either directly or as a solution in 10% MeOH to a final concentration of 2-10 mM. The mixture was shaken at 200 rpm at 30°C for 1-5 h and the reaction was followed by HPLC analysis of samples that were prepared by taking aliquots (0.1-0.2 ml) from a bioconversion mixture at predetermined time points and mixing with an equal volume of MeOH followed by centrifugation to remove the cells.

HPLC analyses:

Column: Hypersil BDS-C18 (5 µm, 125 mm x 4 mm); eluent: a mixture of A (10 mM K-phosphate buffer, pH 7.0) and B (acetonitrile); flow: 1.0 ml/min.; detection: UV at 210, 225, and 254 nm; retention time: 2.1 min for 13, 5.4 min for 11 (A/B 70:30); 2.7 min for 14, 9.2 min for 12 (A/B 75:25); 3.0 min for 20, 5.2 min for 15 (A/B 85:15); 2.0 min for 21, 8.8 min for 16 (A/B 55:44); 1.7 min for 22, 6.6 min for 17 (A/B 55:45); 1.5 min for 23, 5.6 min for 18 (A/B 50:50); 1.5 min for 24, 5.4 min for 19 (A/B 70:30); the conversion was quantified by comparing the integrated peak areas at 210 nm of the samples with the substrate and product standards.

General procedure for preparative hydroxylation with frozen/thawed cells of *Sphingo-

omonas* sp. HXN-200 in a shaking flask.

Substrate was added to 40-100 ml of cell suspension (4.0 g/L) of *Sphingomonas* sp. HXN-200 in 50 mM K-phosphate buffer (pH 8.0) containing glucose (2%) in a 500 ml shaking flask. The mixture was shaken at 200 rpm and 30°C for 1.5-5 h and the bioconversion was followed by analytical HPLC. The cells were removed from the bioconversion mixture by centrifugation and the pH of the supernatant was adjusted to 11–12 by addition of KOH followed by extraction with ethyl acetate. The organic phase was separated, dried over Na₂SO₄, and the solvent was removed by evaporation. The product was purified by column chromatography on silica gel. The results are given in table 2.
Chapter-2 Practical syntheses of 3-hydroxy azetidines and 4-hydroxy piperidines

**N-Phenoxy carbonyl 3-hydroxy azetidine 13.** $R_f$ 0.13 (n-hexane/ethyl acetate 1:1); mp 100.8-102.6°C; Purity: > 99.9% (HPLC); $^1$H NMR (400 MHz) $\delta$ 7.38-7.08 (m, 5 H), 4.57 (m, br, 1 H), 4.30 (s, br, 2 H), 3.97 (s, br, 2 H), 3.14 (s, br, 1 H); $^{13}$C NMR (100 MHz) $\delta$ 155.63 (s), 151.96 (s), 130.41 (d), 126.52 (d), 122.65 (d), 62.56 (d), 60.85 (t), 59.95 (t); MS m/z 194 (100%, M+1), 113 (12%); IR (CHCl$_3$) $\nu$ 3401, 1720, 1595 cm$^{-1}$.

**N-Benzyloxycarbonyl 4-hydroxypiperidine 21.** $R_f$ 0.12 (n-hexane/ethyl acetate = 1:1); colorless liquid; Purity: 99.4% (GC, $t_R = 10.69$ min); $^1$H NMR (200 MHz) $\delta$ 7.37-7.12 (m, 5 H), 5.12 (s, 2 H), 3.97-3.79 (m, 3 H), 3.21-3.08 (ddd, 2 H, $J = 13.6, 9.4, and 3.5$), 1.91-1.82 (m, 2 H), 1.65 (s, 1 H), 1.57-1.39 (ddt, 2 H, $J = 13.0, 9.0, and 4.1$); $^{13}$C NMR (50 MHz) $\delta$ 155.28 (s), 136.80 (s), 128.50 (d), 128.00 (d), 127.86 (d), 67.41 (d), 67.13 (d), 41.35 (t), 34.06 (t); MS m/z 236 (100%, M+1), 222 (17%), 192 (76%); IR (CHCl$_3$) $\nu$ 3452, 1689.1 cm$^{-1}$.

**N-Phenoxy carbonyl 4-hydroxypiperidine 22.** $R_f$ 0.11 (n-hexane/ethyl acetate 1:1); mp 116.5-118.3°C; Purity: 99.5% (GC, $t_R = 10.26$ min); $^1$H NMR (200 MHz) $\delta$ 7.40-7.06 (m, 5 H), 4.10-3.85 (m, 3 H), 3.28 (s, br, 2 H), 1.98-1.85 (m, 2 H), 1.81 (s, 1 H), 1.66-1.48 (ddt, 2 H, $J = 13.0, 8.8, and 4.1$); $^{13}$C NMR (50 MHz) $\delta$ 153.75 (s), 151.42 (s), 129.26 (d), 125.25 (d), 121.73 (d), 67.08 (d), 41.62 (t), 33.97 (t); MS m/z 222 (100%, M+1), 206 (24%); IR (CHCl$_3$) $\nu$ 3452, 1710, 1594 cm$^{-1}$.

**N-tert-Butyloxycarbonyl 4-hydroxypiperidine 23.** $R_f$ 0.20 (n-hexane/ethyl acetate 1:1); mp 64.6-66.5°C; Purity: 99.4% (GC, $t_R = 7.64$ min); $^1$H NMR (300 MHz) $\delta$ 3.87-3.78 (m, 3 H), 3.07-2.98 (ddd, 2 H, $J = 13.5, 9.7, and 3.4$), 2.03 (s, br, 1 H), 1.89-1.66 (m, 2 H), 1.52-1.38 (m, 11 H); $^{13}$C NMR (75 MHz) $\delta$ 154.86 (s), 79.57 (s), 67.69 (d), 41.26 (t), 34.17 (t), 28.44 (q); MS m/z 202 (7%, M+1), 146 (24%), 102 (100%); IR (CHCl$_3$) $\nu$ 3440, 1681 cm$^{-1}$.

**N-Benzoyl 4-hydroxypiperidine 24.** $R_f$ 0.14 (ethyl acetate); mp 89.6-90.5°C; Purity: 98.4% (GC, $t_R = 10.22$ min); $^1$H NMR (500 MHz 253K) $\delta$ 7.46-7.38 (m, 5 H), 4.22 (m, 1 H), 3.92 (m, 1 H), 3.66 (dt, 1 H, $J = 13.8, 4.5$), 3.32 (t, 1 H, $J = 9.6$), 3.16 (ddd, 1 H, $J = 13.7, 9.3,$ and 3.3), 2.90 (s, 1 H), 1.95 (m, 1 H), 1.80 (m, 1 H), 1.60 (ddd, 1 H, $J = 13.0, 9.0,$ and 4.0 Hz), 1.46 ppm (ddd, 1 H, $J = 12.7, 9.0,$ and 3.9); $^{13}$C NMR (125 MHz) $\delta$ 170.37 (s), 135.49 (s), 129.64 (d), 128.46 (d), 126.57 (d), 66.78 (d), 44.91 (t), 39.34 (t), 34.24 (t), 33.57 (t); MS m/z 206 (100%, M+1); IR (CHCl$_3$) $\nu$ 3429, 1621, 1579 cm$^{-1}$.
General procedure of preparative biohydroxylation with frozen/thawed cells of Sphingomonas sp. HXN-200 on 1-2 L scale.

Substrate was added to 1-2 L cell suspension of Sphingomonas sp. HXN-200 in 50 mM of K-phosphate buffer (pH 8.0) containing glucose (2%, w/v) in a 3 L bioreactor, the mixture was stirred at 1500 rpm and at 30°C under the introduction of air at 1 L/min. The biotransformation was followed by analytical HPLC. Work-up according to the same procedure described above and purification by column chromatography on silica gel afforded the pure products. The results are summarized in table 3.

**N-Benzyl-4-hydroxypiperidine 20.** \( R_f 0.20 \) (ethyl acetate/MeOH 8:2); mp 62.8-63.8°C\(^2\); Purity: 98.5 % (GC, \( t_R = 8.52 \) min); \(^1\)H NMR (300 MHz) \( \delta 7.31 \) (s, 2 H), 7.30 (s, 2 H), 7.29-7.21 (m, 1 H), 3.68 (m, 1 H), 3.50 (s, 2 H), 2.75 (dt, 2 H, \( J = 11.6, 4.0 \) Hz), 2.14 (dt, 2 H, \( J = 12.2, 2.8 \) Hz), 1.91-1.82 (m, 2 H), 1.78 (s, br., 1 H), 1.64-1.52 (m, 2 H). \(^{13}\)C NMR (75 MHz) \( \delta 138.41 \) (s), 129.08 (d), 128.15 (d), 126.95 (d), 68.07 (d), 62.92 (t), 50.99 (t), 34.49 (t); MS m/z 192 (100%, M+1); IR (CHCl3) \( \nu 3446, 1602 \) cm\(^{-1}\).

**Preparation of N-tert-Butoxycarbonyl-3-hydroxyazetidine 14.**

1 L suspension of the frozen/thawed cells (102 g with 10.2 g cdw) in 50 mM of K-phosphate buffer (pH 8.0) containing glucose (2%, w/v) which contributes to the intracellular regeneration of cofactors was stirred at 1500 rpm and at 30°C under the introduction of air at 1 L/min in a 3 L bioreactor. Substrate 12 was added at different time points: 10.1 mmol at the beginning, 2.0 mmol at 30 min, 2.0 mmol at 147 min, and 1.7 mmol at 180 min. The biotransformation was followed by analytical HPLC and stopped at 5 h by centrifugation. The pH of the supernatant was adjusted to 11–12 by addition of KOH followed by extraction with ethyl acetate. The organic phase was separated, dried over Na₂SO₄, and the solvent was removed by evaporation. The product was purified by column chromatography on silica gel (\( R_f 0.27, n\)-hexane/ethyl acetate 1:1) to give 14 in 79% yield (2.140 g).

**N-tert-Butoxycarbonyl-3-hydroxyazetidine 14.** \( R_f 0.27 \) (n-hexane/ethyl acetate 1:1); mp 44.8-46.8°C; Purity: 99.7 % (GC, \( t_R = 6.95 \) min); \(^1\)H NMR (400 MHz) \( \delta 4.53 \) (m, 1 H); 4.11-4.07 (dd, 2 H, \( J = 10.4, 7.2 \) Hz), 3.78-3.74 (dd, 2 H, \( J = 8.8, 4.4 \) Hz), 3.60 (s, 1 H); 1.38 (s, 9.
Biohydroxylation of N-benzyl piperidine 1 with lyophilised cells and frozen/thawed cells of Sphingomonas sp. HXN-200.

Frozen/thawed cells of Sphingomonas sp. HXN-200 were lyophilized at low temperature for 3 days to dry powder. These lyophilized cells were suspended into 20 ml of 50 mM K-phosphate buffer containing glucose (2% w/v) at pH = 8.0 to a cell density of 4.0 g/L in a 100 ml Erlenmeyer flask. Piperidine 1 was added to the mixture to a concentration of 5mM. The mixture was shaken at 200 rpm at 30°C for 4 h and the reaction was followed by HPLC analysis of samples that were prepared according to the procedure described above.

For comparison, hydroxylation of piperidine 1 (5 mM) was carried out in a 20 ml suspension of the frozen/thawed cells in 50 mM K-phosphate buffer (4.0 g/L) containing glucose (2% w/v) at pH = 8.0 and the mixture was shaken at 200 rpm at 30°C for 4 h. The reaction was followed by HPLC analysis and the results are given in Figure 1.
Reference


Chapter-2  Practical syntheses of 3-hydroxy azetidines and 4-hydroxy piperidines


Chapter-2 Practical syntheses of 3-hydroxy azetidines and 4-hydroxy piperidines


Chapter 3

Preparation of (S)-N-substituted 4-hydroxy-pyrrolidin-2-ones by regio- and stereoselective hydroxylation with *Sphingomonas* sp. HXN-200

Abstract

Enantiopure (S)-N-substituted 4-hydroxy-pyrrolidin-2-ones have been prepared for the first time by regio- and stereoselective hydroxylation of the corresponding pyrrolidin-2-ones by use of a biocatalyst. Hydroxylation of 6 and 8 with Sphingomonas sp. HXN-200 afforded 68% of (S)-7 in > 99.9% e.e. and 46% of (S)-9 in 92% e.e., respectively. Simple crystallization increased the e.e. of (S)-9 to 99.9% in 82% yield.
Chapter-3  Preparation of (S)-N-substituted 4-hydroxy-pyrrolidin-2-ones

Introduction

Optically active 4-hydroxy-pyrrolidin-2-one and its N-substituted derivatives are useful intermediates for the preparation of several pharmaceuticals. The (S)-enantiomers, for example, can be used in the synthesis of an oral carbapenem antibiotic CS-834\(^1\) 1 and nootropic drug (S)-Oxiracetame\(^2\) 2; the (R)-enantiomers can be used in the preparation of an antidepressant agent (R)-Rolipram\(^3\) 3, an anticonvulsant (R)-\(\gamma\)-amino-\(\beta\)-hydroxybutyric (GABOB)\(^4\)-5 4, and antihyperlipoproteinemic L-Carnitine (vitamin B\(_7\))\(^5\) 5.

Several methods for synthesis of optically active 4-hydroxy-pyrrolidin-2-one and its N-substituted derivatives have been developed, but each has one or more drawbacks: 1) syntheses via direct cyclization\(^4c,6\) or cyclization with ammonia\(^7\) or with alkyl- or aralkyl amine\(^8\) need optically active precursors which can not be prepared easily; 2) preparation involving reduction of (S)-N-benzyl-4-hydroxy-pyrrolidin-2,5-dione\(^9\) is multi-step and needs special reagents; 3) synthesis from (2S, 4R)-4-hydroxyproline\(^10\) needs expensive starting material; 4) synthesis via photochemical rearrangement of special oxaziridines\(^5\) occurs with low yield; 5) resolution of racemic 4-hydroxy-pyrrolidin-2-ones with stereoselective esterase\(^11\) is a low yield process and needs the preparation of the racemates.

Regio- and stereoselective hydroxylation of pyrrolidin-2-ones is the simplest route for preparing optically active 4-hydroxy-pyrrolidin-2-one and its N-substituted derivatives.
However, regio- and stereoselective hydroxylation on non-activated carbon atom remains a challenge in synthetic chemistry\textsuperscript{12}. On the other hand, biohydroxylation can be a useful tool for this type of transformation\textsuperscript{13-14}. However, selective biohydroxylation of pyrrolidin-2-ones has proven to be very difficult. Hydroxylation of \textit{N}-benzoyl- and \textit{N}-phenylacetyl-pyrrolidin-2-one with \textit{Beauveria sulfurescens} (ATCC 7159), a well-known fungus for hydroxylation, gave only 21\% of \textit{N}-benzoyl-4-hydroxy-pyrrolidin-2-one and 5\% of \textit{N}-phenylacetyl-4-hydroxy-pyrrolidin-2-one, respectively, in very low e.e.\textsuperscript{6b}. Moreover, several by-products were formed in each case.

In our previous study on biohydroxylation of pyrrolidines\textsuperscript{14a}, we found that \textit{Sphingomonas} sp. HXN-200\textsuperscript{15} is an excellent biocatalyst for regio- and stereo-selective hydroxylation of \textit{N}-substituted pyrrolidines giving the corresponding optically active 3-hydroxy-pyrrolidines. Here, we report a simple and practical synthesis of \((S)-N\)-substituted 4-hydroxy-pyrrolidin-2-ones by hydroxylation of the corresponding pyrrolidin-2-ones with \textit{Sphingomonas} sp. HXN-200 as biocatalyst.

\textbf{Table 1.} Hydroxylation of 6 to 7 with resting cells (4.0 g/L) of \textit{Sphingomonas} sp. HXN-200

\begin{table}[h]
\begin{center}
\begin{tabular}{cccccccc}
\hline
 & Glucose & Activity\textsuperscript{a} & 0.5h & 1h & 2h & 3h & 5h \\
6 (mM) & (\%) & (U/g CDW) & & & & & \\
2.0 & 0 & 2.6 & 15 & 19 & 22 & 22 & 23 \\
2.0 & 2 & 4.4 & 26 & 41 & 62 & 69 & 70 \\
3.0 & 2 & 4.6 & 18 & 29 & 49 & 58 & 65 \\
4.0 & 2 & 4.1 & 12 & 19 & 36 & 47 & 57 \\
5.0 & 0 & 3.0 & 7.0 & 8.0 & 9.0 & 10 & 10 \\
5.0 & 2 & 4.3 & 10 & 14 & 24 & 36 & 47 \\
\hline
\end{tabular}
\end{center}
\textsuperscript{a} Activity was determined over the first 30min.
\end{table}
Exploratory hydroxylation of 6 and 8 was performed with resting cells of *Sphingomonas* sp. HXN-200 on 10 ml scale and followed by analytical HPLC. Hydroxylation of 6 and 8 afforded the desired 4-hydroxy products 7 and 9, respectively. Comparison of the retention time and the UV absorption area at 210 nm with the standards of 6-9 suggested the conversion to the products.

As shown in Table 1, hydroxylation of 2 mM of *N*-benzyl-pyrrolidin-2-one 6 with resting cells (4.0 g/L) of *Sphingomonas* sp. HXN-200 in the presence of glucose (2%, w/v) for 5 h gave 70% of the desired *N*-benzyl-4-hydroxy-pyrrolidin-2-one 7 as main product. The addition of glucose increased the conversion significantly, this is because the biohydroxylation is cofactor dependent and the addition of glucose contributed to the intracellular regeneration of cofactors. This effect was also observed in hydroxylation of 5 mM of 6; the conversion to 7 at 5 h was increased from 10% to 47% by addition of 2% of glucose. Hydroxylation of 3 mM and 4 mM of 6 for 5h gave 65% and 57% of 7, respectively, with activity of 4.6 and 4.1 U/g CDW (U = μmol/min, CDW = cell dry weight) in the first 30 min.

Higher activity was observed for hydroxylation of *N*-tert-butoxycarbonyl-pyrrolidin-2-one 8 with resting cells (4.0 g/L) of *Sphingomonas* sp. HXN-200. As shown in Table 2, hydroxylation of 14 mM of 8 gave an activity of 11 U/g CDW and 63% conversion to 9 at 5 h. Interestingly, both conversion and activity are not very much dependent on the starting concentration of substrate, which is advantageous for practical bioconversions. 57-80% of 9 were formed in hydroxylation of 2-16 mM of 8 for 5 h. No byproduct was formed in biohydroxylation of 8, demonstrating the excellent regioselectivity of the biocatalyst.

**Table 2.** Hydroxylation of 8 to 9 with resting cells (4.0 g/L) of *Sphingomonas* sp. HXN-200
Table 3. Preparation of 7 and 9 by hydroxylation of 6 and 8 with resting cells of Sphingomonas sp. HXN-200, respectively.

<table>
<thead>
<tr>
<th>substrate (mM)</th>
<th>cells (g/L)</th>
<th>prod.</th>
<th>conv.(%)</th>
<th>yield (%)</th>
<th>e.e.(S)(%)</th>
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</thead>
<tbody>
<tr>
<td>6 3.0</td>
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<td>7</td>
<td>66</td>
<td>55</td>
<td>&gt;99.9</td>
</tr>
<tr>
<td>6 6.0</td>
<td>8.0</td>
<td>7</td>
<td>59</td>
<td>42</td>
<td>&gt;99.9</td>
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<td>75</td>
<td>68</td>
<td>&gt;99.9</td>
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<td>10</td>
<td>9</td>
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<td>42</td>
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</tr>
</tbody>
</table>

Biohydroxylation of 8 (14 mM) with 4.0 g/L of resting cells of Sphingomonas sp. HXN-200 afforded 9 in 39% yield (1.10 g/L). Similarly, increase of cell density to 8.0 g/L improved the yield to 1.29 g/L (46%). Further improvement was achieved by use of more substrate and
more cells: hydroxylation of 20 mM of 8 with 10 g/L of resting cells of *Sphingomonas* sp. HXN-200 gave the pure product 9 in 42% yield (1.69 g/L). Based on our experience, the yield of 7 and 9 can be further improved by performing the hydroxylation in a bioreactor.

For determination of e.e. of the biohydroxylation products 7 and 9, standard (R)- and (S)-7 and 9 were synthesized from the corresponding (R)- and (S)-4-tert-butyldimethylsilyloxy-pyrrolidin-2-one 10, which was prepared according to the known procedure\textsuperscript{17}. As shown in Scheme 1, benzylation of (R)- and (S)-10 afforded the corresponding (R)- and (S)-11 in 19% and 23% yield, respectively. Deprotection of 11 gave (R)- and (S)-7 in 41% and 34% yield, respectively. Similarly, treatment of (R)- and (S)-10 with Boc\textsubscript{2}O, DMAP, and Et\textsubscript{3}N afforded 89% and 92% of (R)- and (S)-12, respectively. Deprotection of 12 gave the corresponding (R)- and (S)-9 in 8.1% and 16% yield, respectively. Here, the yields were not optimized. The structures of (R)- and (S)-7, 9, 11, and 12 were identified by \textsuperscript{1}H-NMR, \textsuperscript{13}C-NMR and MS spectra.

![Scheme 1](image)

| a. NaH, THF, 0°C, BnCl, 19% of (R)-11; 23% of (S)-11. b. Bu\textsubscript{4}NF, THF, 41% of (R)-7; 34% of (S)-7. c. (Boc)\textsubscript{2}O, DMAP, Et\textsubscript{3}N, 89% of (R)-12; 92% of (S)-12. d. Bu\textsubscript{4}NF, THF, 0°C, acetic acid, 8.1% of (R)-9; 16% of (S)-9. |

The e.e. of 7 and 9 were determined by HPLC with a chiral column. As shown in Fig. 1, hydroxylation of *N*-benzyl-pyrrolidin-2-one 6 afforded (S)-*N*-benzyl-4-hydroxy-pyrrolidin-2-one 7 in >99.9% e.e.. Hydroxylation of *N*-tert-butoxycarbonyl-pyrrolidin-2-one 8 gave (S)-*N*-
tert-butoxycarbonyl-4-hydroxy-pyrrolidin-2-one 9 in 92% e.e., shown in Fig. 2. The enantiomeric excess of biohydroxylation product 9 varies from 90 to 92% (S) in three preparations given in Table 3. The e.e. of 9 can be increased from 92% to 99.9% (S) in 82% yield by simple crystallization from n-hexane/ethyl acetate (2:1).

Figure 1. HPLC separation with a chiral column: (a) mixture of (S) and (R)-7. (b) biohydroxylation product 7.

Figure 2. HPLC separation with a chiral column: (a) mixture of (S) and (R)-9. (b) biohydroxylation product 9.

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In summary, a simple and practical method was developed for the synthesis of (S)-N-substituted 4-hydroxy-pyrrolidin-2-ones by hydroxylation of the corresponding pyrrolidin-2-ones with *Sphingomonas* sp. HXN-200.
Experimental section

General Methods

$^{1}$H- and $^{13}$C-NMR spectra were recorded at 400 MHz ($^{1}$H-NMR) and 100 MHz ($^{13}$C-NMR) on a Varian 400 instrument at 20°C in CDCl$_3$. MS analyses were carried out on HPLC-MS Hewlett Packard 1100. IR spectra were recorded at PERKIN-ELMER 1600 Series FTIR.

HPLC analysis

Hypersil BDS-C18 column (125mm×4mm); UV detection at 210 nm; acetonitrile/10mM K-phosphate buffer (pH=7.0) 20/80 as eluent; flow at 1 ml/min.; retention time: 2.7 min for 7 and 8.1 min for 6; 2.7 min for 9 and 6.7 min for 8.

Chiral HPLC analysis

(a) The e.e. of 7 was determined by analytical HPLC. Column: Chiralpak AS; UV detection at 210 nm; eluent: $n$-hexane/isopropanol 4:1; flow: 1.0 ml/min.; $T_R$ (S) = 20.3 min; $T_R$ (R) = 30.5 min. (b) The e.e. of 9 was determined by analytical HPLC. Column: Chiralcel OB-H; UV detection at 210 nm; eluent: $n$-hexane/isopropanol 7:1; flow: 0.5 ml/min.; $T_R$ (R) = 17.9 min; $T_R$ (S) = 22.6 min.

General procedure for small-scale hydroxylation

Substrate 6 or 8 (2-16 mM) was added to 10 ml of cell suspension (4.0 g/L) of Sphingomonas sp. HXN-200 in 50mM potassium phosphate buffer (pH = 8.0) containing glucose (0-2%, w/v) in a 100ml shaking flask. The mixture was shaken at 200 RPM and 30°C for 5 h. Samples (100 μl) were taken out at different times, mixed with methanol (100 μl), and the cells were removed by centrifugation. The supernatant was analyzed by HPLC.

Preparation of (S)-N-benzyl-4-hydroxy-pyrrolidin-2-one 7

To 50 ml of cell suspension (4.0 g/L) of Sphingomonas sp. HXN-200 in 50mM potassium phosphate buffer (pH = 8.0) containing glucose (2%, w/v) in a 500ml shaking flask was added 6 (26.3 mg, 0.15 mmol). The mixture was stirred at 200 RPM and 30°C. The reaction was followed by analytical HPLC and was stopped at 5 h with 66% conversion. The cells were removed by centrifugation and the product was extracted into ethyl acetate. The organic phase was dried over Na$_2$SO$_4$, filtered, and evaporated. Purification by column chromatography on
Chapter 3 Preparation of (S)-N-substituted 4-hydroxy-pyrrolidin-2-ones

silica gel (Rf of 7 = 0.13 and Rf of 6 = 0.50, ethyl acetate/methanol 9:1) afforded 15.8 mg (55%) of 6 as white powder.

Preparation of (S)-N-tert-butoxycarbonyl-4-hydroxy-pyrrolidin-2-one 9

N-tert-butoxycarbonyl-2-pyrrolidinone 8 (129.5mg, 0.70mmol) was added to 50 ml of cell suspension (8.0 g/L) of Sphingomonas sp. HXN-200 in 50 mM K-phosphate buffer (pH=8.0) containing glucose (2%) in a 500 ml shaking flask. The mixture was shaken at 200 rpm and 30°C and the bioconversion was followed by HPLC analysis of samples that were taken out at different times (6 x 0.10 ml). The reaction was stopped at 5 h with 66% conversion to 9. The cells were removed by centrifugation and the product was extracted into ethyl acetate. The organic phase was dried over Na2SO4, filtered, and evaporated. Purification by column chromatography on silica gel (Rf = 0.28, ethyl acetate) afforded 46% (63.3 mg) of the pure product. e.e. of 9: 92% (S).

Increase of e.e. of (S)-9 by crystallization

The biohydroxylation product (S)-9 (92% e.e., 26.3mg) was dissolved in 1.5ml of n-hexane/ethyl acetate (2/1) and crystallized at 0°C for 24 h. 21.6mg (82%) of the crystals of (S)-9 were obtained in 99.9% e.e.

Data of bio-products 7 and 9

(S)-N-benzyl-4-hydroxy-pyrrolidin-2-one 7: mp 107.3-108.0°C. [α]25D = -34.1° (c = 1.00, CHCl3). 1H-NMR (300MHz, CDCl3): δ 7.35-7.19 (m, 5 H, aromatic H), 4.52-4.38 (m, 3 H, NCH2Ph, H-C(4)), 3.48 (dd, 1 H, J = 10.9 and 5.6 Hz, H_A-C(5)), 3.26 (s, br., 1 H, OH), 3.18 (dd, 1 H, J = 10.8 and 2.0 Hz, H_B-C(5)), 2.70 (dd, 1 H, J = 17.4 and 6.6 Hz, H_A-C(3)), 2.43 ppm (dd, 1 H, J = 17.3 and 2.5 Hz, H_B-C(3)); 13C-NMR (75MHz, CDCl3): δ 172.94 (s, CO); 135.97 (s), 128.70 (d), 127.60 (d, aromatic C); 64.27 (d, C-4); 55.71 (t, CH3Ph); 46.32 (t, C-5); 41.14 ppm (t, C-3); MS: m/z 192.1(M+1, 100), 174.1(9). IR (cm⁻¹): 3401, 3007, 2928, 1682, 1483, 1435, 1082.

(S)-N-tert-butoxycarbonyl-4-hydroxy-pyrrolidin-2-one 9: 1H-NMR (300MHz, CDCl3): δ 4.47 (s, 1 H, H-C(4)), 3.88 (dd, 1 H, J = 11.9 and 5.1 Hz, H_A-C(5)), 3.77 (d, 1 H, J = 11.8 Hz, H_B-C(5)), 3.15 (s, 1 H, OH), 2.77 (dd, 1 H, J = 17.7 and 6.1 Hz, H_A-C(3)), 2.43 (d, 1 H, J = 17.7 Hz, H_B-C(3)), 1.52 ppm (s, 9 H, 3 CH3); 13C-NMR (75MHz, CDCl3): δ 172.8 (s, COO); 150.05 (s, CO); 83.19 (s, C(CH3)3); 63.03 (d, C-4); 55.31 (t, C-5); 42.71 (t, C-3); 28.03 ppm
Chapter-3  Preparation of (S)-N-substituted 4-hydroxy-pyrrolidin-2-ones

(S)-N-tert-butoxycarbonyl 4-hydroxy-pyrrolidin-2-one 9 after recrystallization

mp 133.5-134.6°C. [α]25 D = +2.1° (c = 1.86, CHCl3).

Data of chemically synthesized compound 7, 9, 11, and 12

(S)-N-benzyl-4-hydroxy-pyrrolidin-2-one 7: 1H-NMR (400MHz, CDCl3): δ 7.35-7.20(m, 5 H, aromatic H), 4.51-4.45(m, 3 H, NCH2Ph, H-C(4)), 3.49(dd, 1 H, J = 10.8Hz, 5.6Hz, Hα-C(5)), 3.19(dd, 1 H, J = 10.8Hz, 1.6Hz, Hβ-C(5)), 2.74(dd, 1 H, J = 17.6Hz, 6.8Hz, Hα-C(3)), 2.43ppm(dd, 1 H, J = 17.6Hz, 2.4Hz, Hβ-C(3)); 13C-NMR (100MHz, CDCl3): δ 174.08(s, CO); 137.09(s), 129.84(d), 129.13(d), 128.75(d) (aromatic C); 65.59(d, C-4); 56.67(t, CH2Ph); 47.38(t, C-5); 42.27ppm(t, C-3); MS: m/z 192.1(M+1, 100), 174.1(9); IR (cm -1): 3400, 3007, 2928, 1681, 1483, 1435, 1262, 1082.

(R)-N-benzyl-4-hydroxy-pyrrolidin-2-one 7: 1H-NMR (400MHz, CDCl3): δ 7.35-7.22(m, 5 H, aromatic H), 4.52-4.42(m, 3 H, NCH2Ph, H-C(4)), 3.49(dd, 1 H, J = 11.2Hz, 6.0Hz, Hα-C(5)), 3.19(dd, 1 H, J = 11.2Hz, 1.2Hz, Hβ-C(5)), 2.73(dd, 1 H, J = 17.6Hz, 6.8Hz, Hα-C(3)), 2.45ppm(dd, 1 H, J = 17.6Hz, 2.4Hz, Hβ-C(3)); 13C-NMR (100MHz, CDCl3): δ 174.04(s, CO); 137.05(s), 129.86(d), 129.13(d), 128.76(d) (aromatic C); 65.50(d, C-4); 56.78(t, CH2Ph); 47.43(t, C-5); 42.29ppm(t, C-3); MS: m/z 192.1(M+1, 100), 174.1(9); IR (cm -1): 3400, 3007, 2928, 1681, 1483, 1435, 1262, 1082.

(S)-N-tert-butoxycarbonyl-4-hydroxy-pyrrolidin-2-one 9: 1H-NMR (400MHz, CDCl3): 4.43(s, 1 H, H-C(4)), 3.86(dd, 1 H, J = 12.0Hz, 6.0Hz, Hα-C(5)), 3.77(d, 1 H, J = 11.6Hz, Hβ-C(5)), 2.76(dd, 1 H, J = 17.6Hz, 6.0Hz, Hα-C(3)), 2.49(d, 1 H, J = 17.6Hz, Hβ-C(3)), 1.48ppm(s, 9 H, 3CH3); 13C-NMR (100MHz, CDCl3): δ 173.72(s, COO); 151.05(s, CO); 84.25(s, C(CH3)3); 64.13(d, C-4); 56.30(t, C-5); 43.75(t, C-3); 29.03ppm(q, CH3); MS: m/z 202 (M+1, 2), 146.0 (100), 128.0 (13), 113.0 (12), 102.1 (81); IR (cm -1): 3399, 2983, 1782, 1747, 1715, 1370, 1308, 1152, 1078, 1022, 848.

(R)-N-tert-butoxycarbonyl-4-hydroxy-pyrrolidin-2-one 9: 1H-NMR (400MHz, CDCl3): 4.47(d, J = 3.6Hz, 1 H, H-C(4)), 3.86(dd, 1 H, J = 11.6Hz, 4.4Hz, Hα-C(5)), 3.75(d, 1 H, J =
12.0Hz, $H_B$-$C(5)$), 2.75($dd$, 1 H, $J = 17.6$Hz, 6.0Hz, $H_A$-$C(3)$), 2.50($d$, 1 H, $J = 17.6$Hz, $H_B$-$C(3)$), 1.50ppm($s$, 9 H, 3CH$_3$); $^{13}$C-NMR (100MHz, CDCl$_3$): $\delta$ 173.59($s$, COO); 151.06($s$, CO); 84.26($s$, C(CH$_3$)$_3$); 64.17($d$, C-4); 56.28($t$, C-5); 43.76($t$, C-3); 29.05ppm($q$, CH$_3$); MS: m/z 202 (M+1, 2), 146.0 (100), 128.0 (13), 113.0 (12), 102.1 (81); IR (cm$^{-1}$): 3399, 2983, 1782, 1747, 1715, 1370, 1308, 1152, 1078, 848.

(R)-N-benzyl-4-tert-butyldimethylsilyloxy-pyrrolidin-2-one 11: $^1$H-NMR (400MHz, CDCl$_3$): $\delta$ 7.37-7.22($m$, 5 H, aromatic H), 4.62-4.34($m$, 3 H, NCH$_2$Ph, H-$C(4)$), 3.45($ddd$, 1 H, $J = 10.0$Hz, 5.6Hz, 2.0Hz, $H_A$-$C(5)$), 3.12($dd$, 1 H, $J = 10.4$Hz, 3.2Hz, $H_B$-$C(5)$), 2.66($ddd$, 1 H, $J = 16.8$Hz, 6.8Hz, 2.0Hz, $H_A$-$C(3)$), 2.40($dd$, 1 H, $J = 16.8$Hz, 2.8Hz, $H_B$-$C(3)$); 0.85($s$, 9 H, C(CH$_3$)$_3$); 0.046($d$, 3 H, $J = 3.2$Hz, SiCH$_3$); 0.005ppm($s$, 3 H, $J = 2.4$Hz, SiCH$_3$); $^{13}$C-NMR (100MHz, CDCl$_3$): $\delta$ 173.90($s$, CO); 137.23($s$), 129.73($d$), 128.93($d$), 128.58($d$) (aromatic C); 66.27($d$, C-4); 56.96($t$, CH$_2$Ph); 47.07($t$, C-5); 42.56($t$, C-3); 26.72($q$, CH$_3$-CSi); 18.98($s$, SiC); -3.75($q$, SiCH$_3$); -3.86ppm($q$, SiCH$_3$).

(R)-N-tert-butoxycarbonyl-4-tert-butyldimethylsilyloxy-pyrrolidin-2-one 12: $^1$H-NMR (400MHz, CDCl$_3$): 4.36($m$, 1 H, H-$C(4)$), 3.84($dd$, 1 H, $J = 12.0$Hz, 4.8Hz, $H_A$-$C(5)$), 3.60($dd$, 1 H, $J = 11.6$Hz, 2.8Hz, $H_B$-$C(5)$), 2.69($dd$, 1 H, $J = 17.6$Hz, 6.0Hz, $H_A$-$C(3)$), 2.50($dd$, 1 H, $J = 17.6$Hz, 3.2Hz, $H_B$-$C(3)$), 1.51($s$, 9 H, 3CH$_3$); 0.86($s$, 9 H, C(CH$_3$)$_3$); 0.057($s$, 3 H, SiCH$_3$); 0.051ppm($s$, 3 H, SiCH$_3$); $^{13}$C-NMR (100MHz, CDCl$_3$): $\delta$ 173.23($s$, COO); 151.10($s$, CO); 84.05($s$, C(CH$_3$)$_3$); 64.90($d$, C-4); 56.50($t$, C-5); 44.20($t$, C-3); 29.00($q$, CH$_3$-CO); 26.70($q$, CH$_3$-CSi); 19.01($s$, SiC); -3.75($q$, SiCH$_3$); -3.82ppm($q$, SiCH$_3$).
Reference


(15) (a) Sphingomonas sp. HXN-200 was isolated from a waste air filter by Plaggemeier, Th.; Schmid, A.; Engesser, K. at University of Stuttgart. (b) For growth conditions see Ref. 14a.

(16) N-Benzyl-3-hydroxy-pyrrolidin-2-one was formed as byproduct. Ratio of 7/byproduct is about 5/1.

Chapter 4
Regio- and stereoselective hydroxylation of \( N \)-substituted piperidin-2-ones with \textit{Sphingomonas} sp. HXN-200

Abstract

High activity, excellent regioselectivity, and moderate enantioselectivity were achieved in the hydroxylation of $N$-benzyl-piperidin-2-one and $N$-tert-butoxycarbonyl-piperidin-2-one with *Sphingomonas* sp. HXN-200. High yield preparations of the 4-hydroxy-piperidin-2-ones were demonstrated in a bioreactor and in a shaking flask by use of the frozen/thawed cells as biocatalyst. The absolute configurations for the hydroxylated bio-products were established by chemical correlation.
Introduction

While regio- and stereoselective hydroxylation of non-activated carbon atoms remains a challenge in organic chemistry\(^1\), Nature has found a general solution for this reaction \textit{via} oxygenases. Several biological systems have been successfully applied in the hydroxylation of steroids and several other alicyclic compounds\(^2\)\textsuperscript{-11}. Thus far, soluble cytochrome P450 monooxygenases such as P450 cam\(^12\) and P450 BM-3\(^13\), soluble methane monooxygenase (sMMO)\(^14\), and membrane-bound alkane hydroxylase (AlkB) of \textit{P. putida} GPO\(^15\) have been well investigated. In practice, the filamentous fungus \textit{Beauveria bassiana} ATCC 7159 has been widely used in organic laboratories for hydroxylation of a broad range of substrates\(^3\), \textsuperscript{5,9,10c,11}, although the activity and product concentration are rather low. We have recently discovered that \textit{Sphingomonas} sp. HXN-200, an alkane-degrading bacterium containing a soluble monooxygenase, is a highly active and regio- and stereoselective catalyst for hydroxylations\(^16\). The easy to handle frozen/thawed cells of \textit{Sphingomonas} sp. HXN-200 have been successfully used in the high yield preparation of several pharmaceutical intermediates such as \textit{N}-substituted (\textit{S})- and (\textit{R})-3-hydroxyprrolidines, (\textit{S})-4-hydroxy pyrrolidin-2-ones, 3-hydroxyazetidines, and 4-hydroxy piperidines\(^16\). Now, we want to further explore the synthetic application of this catalytic system in the hydroxylation of other alicyclic compounds.

Optically active 4-hydroxy-piperidin-2-one is a useful synthon for preparing antibiotics such as Blasticidin S\(^17\)\textsuperscript{a-b} and penem derivatives\(^17\textsuperscript{c}\), \textit{\beta}-amino acid equivalent 4-aminopiperidin-2-ones\(^17\textsuperscript{d}\), and peptidomimetics with \textit{Homo-Freidinger} lactam substructure\(^17\textsuperscript{e-f}\). Chemical preparation of 4-hydroxy-piperidin-2-one, including the oxidation of \textit{N}-substituted 4-piperidinopiperidine\(^18\textsuperscript{a}\), 1,2,3,6-tetrahydropyridine\(^18\textsuperscript{a}\), or 4-hydroxypiperidine\(^18\textsuperscript{b}\), resulted in a mixture. Preparation \textit{via} biohydroxylation is also difficult: hydroxylation of \textit{N}-phenyl-, \textit{N}-benzyl-, or \textit{N}-benzoyl-4-piperidin-2-ones with \textit{Beauveria bassiana} ATCC 7159 gave the corresponding 4-hydroxypiperidin-2-ones in low concentration with a yield of 7\%\(^6\), 10\%\(^11\textsuperscript{e}\), and 27\%\(^11\textsuperscript{e}\), respectively. Here we report our results on the hydroxylation of \textit{N}-substituted piperidin-2-ones with \textit{Sphingomonas} sp. HXN-200, preparative biotrans-formations, and the stereochemistry of the hydroxylation products.
Results and discussion

For hydroxylation studies, cells of Sphingomonas sp. HXN-200 were grown on n-octane vapor in 30 L E2 medium, as previously described\textsuperscript{16a-b}. The cells were harvested and the cell pellets were stored at -80°C. The frozen/thawed cells were used for biohydroxylation.

2.1. Small-scale biohydroxylation of 1-2

Based on the previous results on hydroxylation with Sphingomonas sp. HXN-200\textsuperscript{16}, the hydrophobic N-benzyl- and N-tert-butoxycarbonyl piperidin-2-one 1-2 were chosen as substrates. Compounds 1-2 were prepared according to established procedures\textsuperscript{19-20}. Small-scale hydroxylations were performed with frozen/thawed cells of Sphingomonas sp. HXN-200 in 10 ml 50 mM K-phosphate buffer (pH = 7.0-8.0) at a cell density of 4.0 g cdw/L. Glucose (2%, w/v) was added to improve the conversion via regeneration of the cofactor, and the mixture was shaken at 200 rpm and 30°C. Aliquots (0.1-0.2 ml) were taken out from the bioconversion mixture at predetermined time points, diluted in methanol, and the cells were removed by centrifugation. The samples were analyzed by reverse phase HPLC to follow the reaction in the aqueous phase. No extraction was needed, which is advantageous in comparison with GC analysis. Biohydroxylation of 1-2 afforded the desired product 3-4. The conversion was quantitated by comparing the integrated peak areas at 210 nm of the samples with the substrate and product standards. The results are given in table 1.

High activity (12-15 U/g cdw) was observed in hydroxylation of N-benzyl-piperidin-2-one 1. Biotransformation of a 10 mM solution of 1 gave 90% of N-benzyl-4-hydroxy-piperidine-2-one 3 at pH = 8.0. Changing the “docking/protecting” group to a tert-butoxycarbonyl group resulted in a slightly lower hydroxylation activity (7.7-9.0 U/g cdw). Nevertheless, hydroxylation of a 10 mM solution of 2 produced 69-75% of N-tert-butoxycarbonyl-4-hydroxy-piperidine-2-one 4. While higher activity and conversion were achieved at pH = 8.0 for the reaction of 1 to 3, no significant pH effect was observed for the transformation of 2 to 4. In both cases no other hydroxylation products were formed, indicating the excellent regioselectivity of the alkane monooxygenase of Sphingomonas sp. HXN-200. Moreover, no overoxidation products were detected, suggesting that the bioconversion is rather clean. Obviously, Sphingomonas sp. HXN-200 is significantly better than Beauveria sulfurescens ATCC 7159 in hydroxylation of N-substituted piperidin-2-one\textsuperscript{6,11e}. 
Table 1. Hydroxylation of N-substituted piperidin-2-ones 1-2 with frozen/thawed cells of *Sphingomonas* sp. HXN-200 (4.0 g cdw/L).

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate (mM)</th>
<th>Prod. pH</th>
<th>Activity a (U/g cdw)</th>
<th>0.5 h</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
<th>5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 (8.0)</td>
<td>3</td>
<td>7.5</td>
<td>12</td>
<td>18</td>
<td>36</td>
<td>55</td>
<td>68</td>
<td>77</td>
</tr>
<tr>
<td>2</td>
<td>1 (10.0)</td>
<td>3</td>
<td>7.5</td>
<td>12</td>
<td>14</td>
<td>27</td>
<td>40</td>
<td>47</td>
<td>51</td>
</tr>
<tr>
<td>3</td>
<td>1 (10.0)</td>
<td>3</td>
<td>8.0</td>
<td>15</td>
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<td>58</td>
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<td>83</td>
</tr>
<tr>
<td>4</td>
<td>2 (10.0)</td>
<td>4</td>
<td>7.0</td>
<td>9.0</td>
<td>11</td>
<td>25</td>
<td>45</td>
<td>59</td>
<td>68</td>
</tr>
<tr>
<td>5</td>
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<td>4</td>
<td>7.5</td>
<td>8.6</td>
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<td>24</td>
<td>47</td>
<td>64</td>
<td>74</td>
</tr>
<tr>
<td>6</td>
<td>2 (10.0)</td>
<td>4</td>
<td>8.0</td>
<td>7.7</td>
<td>9.2</td>
<td>21</td>
<td>40</td>
<td>55</td>
<td>66</td>
</tr>
</tbody>
</table>

*Activity was determined over the first 30 min. Conversion was determined by HPLC analysis; error limit: 2% of the stated values. Number in bracket is the ee determined by HPLC analysis with a chiral column.*

2.2. Determination of the ee for bioproduct 3-4

For determination of ee, a racemic standard of 3 was prepared chemically: oxidation of the bioproduct 3 with Jones reagent gave the corresponding ketone 5 in 34% yield; reduction of 5 with...
with NaBH₄ afforded the racemic 3 in 95% (Scheme 1). The two enantiomers were separated by HPLC on a chiralpak AS column with retention times of 8.7 and 10.4 min. Similarly, a pure sample of the bioproduct 4 was separated by HPLC on a chiralcel OB-H column with retention times of 28.3 and 30.8 min for the two enantiomers.

As shown in table 1, the ee of the bioproducts 3 and 4 are about 30-36% and 65-71%, respectively. No significant influence of pH on the ee was observed. While the ee of bioproduct 4 remained nearly unchanged during the biotransformation, there was a slightly decrease of ee of 3 (Figure 1). This is probably due to a trace alcohol dehydrogenase activity in the whole cells. While excellent enantioselectivity was observed in hydroxylations of N-benzyl- and N-tert-butoxycarbonyl pyrrolidine-2-one¹⁶c, hydroxylation of 1-2 gave only moderate to good enantioselectivity.

![Figure 1. Biotransformation of 1 (10mM) to 3 (— ee of 3; — conversion of 3) and of 2 (10mM) to 4 (— ee of 4; — conversion of 4) with frozen/thawed cells of Sphingomonas sp. HXN-200 (4 g cdw/L) at pH = 8.0.](image)

2.3. Preparative biohydroxylation

As an example of preparation on a gram scale, bioconversion of N-benzyl-piperidin-2-one 1 was carried out in a 3 L bioreactor. Biotransformation of substrate 1 (1.52 g, 8.04 mmol) in 1 L suspension of the frozen/thawed cells in 50 mM of K-phosphate buffer (pH = 8.0) containing glucose (2%, w/v) at a cell density of 10 g cdw/L for 5 h gave 75% conversion to
3. Work-up and chromatographic purification afforded 1.168 g (71%) of the pure 3 as white powder with an [α]_D^{25} of -6.5 (c=1.10, CHCl₃) and an ee of 31% (Figure 2a).

![HPLC analysis of ee for bioproduct (-)-3](image1)

(a)

![HPLC analysis of ee for bioproduct (+)-4](image2)

(b)

**Figure 2.** a) HPLC analysis of ee for bioproduct (-)-3. b) HPLC analysis of ee for bioproduct (+)-4.

Preparation of 4 was demonstrated by biotransformation with a shaking flask. Biohydroxylation of 2 (199 mg) in 100 ml 50mM K-phosphate buffer (pH = 8.0) containing glucose (2%, w/v) at a cell density of 4.0 g cdw/L gave 71% of conversion to 4 at 5 h. Extraction of the product with n-butanol/ethyl acetate(1:1) and purification by flash
chromatography afforded 96.2 mg (45%) of 4 in 68 % ee (Figure 2b) with an [α]$_D^{25}$ of +6.7 (c=1.50, CHCl$_3$). The low isolated yield is probably caused by the incomplete extraction of the product 4 which is rather hydrophilic. Continuous extraction would increase the yield.

The existence of an OH group in the bioproducts 3-4 was evidenced by the absorption at 3382 and 3446 cm$^{-1}$ in the IR spectra and the signal at 2.64 and 2.60-2.30 ppm in the $^1$H NMR spectra, respectively. This was further confirmed by the MS signal at 206 and 215 (16 higher than the corresponding substrate), the resonance for H-C-OH at 4.64 and 4.21 ppm in the $^1$H NMR spectra, and the signal for C-OH at 65.23 and 65.17 ppm in the $^{13}$C NMR spectra, respectively. The existence of the signals of the NCH$_2$ and NCOCH$_2$ for both 3 and 4 in the $^1$H NMR spectra exclude the possibility of OH groups at positions 6 or 3. The couplings of H-C-OH with NCOCH$_2$ and not with NCH$_2$ in the $^1$H- and (H, H)-COSY NMR spectra suggest clearly that the OH group in 3-4 is at the 4-position.

2.4. Stereochemistry of the bioproducts 3-4

The CD spectrum of the bioproducts 3 and 4 was determined, and compared with (R)- and (S)-N-benzyl-4-hydroxy-pyrrolidin-2-one 6 and (R)- and (S)-N-tert-butoxycarbonyl-4-hydroxy-pyrrolidin-2-one 7 (Figure 3).

![Scheme 2](image)
Figure 3. a) CD spectra of bioproduct (-)-3 and (R)- and (S)-N-benzyl-4-hydroxy-pyrrolidin-2-one 6 (in acetonitrile). b) CD spectra of bioproduct (+)-4 and (R)- and (S)-N-tert-butoxy-carbonyl-4-hydroxy-pyrrolidin-2-one 7 (in acetonitrile).

As shown in Scheme 3, deprotection of bio-(+)-4 in 68% ee afforded 84% of (-)-8 with an $[\alpha]_{25}^D$ of -1.8 ($c = 2.24$, MeOH) and 99% purity (HPLC). Since the $[\alpha]_{25}^D$ of (+)-(S)-8 in 96% ee is +2.4 ($c = 2.0$, MeOH)$^{21}$, the absolute configuration of bio-(+)-4 can be deduced as (+)-(R)-4. The prepared (-)-(R)-8 was then transformed into (R)-3 by protection, benzylaition, and deprotection. The resulting (R)-3 has an ee of 68% and the same MS and retention time in HPLC and GC as those of the bio-(−)-3. Comparison of the chiral HPLC chromatograms of the synthetic (R)-3 and bio-(−)-3, shown in Figure 3, established the absolute configuration of bio-(−)-3 as (−)-(R)-3.
Chapter-4  Regio- and stereoselective hydroxylation of piperidin-2-ones

Figure 4. (a) HPLC analysis of synthetic (R)-3 and bio-(-)-3 with a chiralpak AS column (250 x 4.6 mm) and n-hexane/isopropanol (75/25) as eluent at a flow rate of 0.5 ml/min. (b) HPLC analysis of synthetic (R)-3 and bio-(-)-3 with a Hypersil BDS-C18 column (5 µm, 125 mm x 4 mm) and 10 mM ammonium acetate buffer (pH 7.0) /acetonitrile (80/20) as eluent at a flow rate of 0.5 ml/min.

Conclusion

Hydroxylations of N-substituted pyrrolidine-2-ones 1-2 with Sphingomonas sp. HXN-200 are highly active and regioselective. Preparative transformations of 1-2 with the frozen/thawed cells afforded the corresponding 4-hydroxy pyrrolidine-2-ones 3-4 in high concentration and good yield. Same enantioselectivity was observed for hydroxylation of 1 and 2, giving the corresponding product 3-4 in 31% ee (R) and 68% ee (R), respectively.
Experimental section

General Methods. \(^{1}\)H- and \(^{13}\)C-NMR spectra were determined at 300 K in CDCl\(_3\), chemical shifts in ppm relative to TMS, coupling constants \(J\) in Hz. Mass spectra were obtained by Atmospheric Pressure Chemical Ionization at 40 eV with a Hewlett Packard 1100 LC-MS apparatus. Melting points are uncorrected. Analytic high-performance liquid chromatography (HPLC) was carried out with a Hewlett Packard 1050 instrument. IR spectra were measured in CHCl\(_3\). Optical rotations were determined using a PERKIN ELMER 241 polarimeter. CD spectra were measured in acetonitrile at 20°C on a JASCO J-710 spectropolarimeter. Bioconversion was analyzed by HPLC. The purity of the products was established by GC analyses with a Chrompack CP–Sil-5CB column (25 m x 0.32mm, temperature program: 60°C for 2 min, increase to 280°C at a rate of 25°C /min, then 280°C for 1 min).

Materials. Compounds 1\(^{19}\), and 2\(^{20}\) were prepared according to published procedures. Cells of Sphingomonas sp. HXN-200 were produced by growth on \(n\)-octane in 30 L E2 medium\(^{22}\) as previously reported\(^{16a-b}\).

\(N\)-Benzy1-piperidin-2,4-dione 5. A solution of chromium (VI) oxide (30 mg, 0.30 mmol) in sulfuric acid (30 µl) and water (90 µl) was added dropwise at 0°C to a stirred solution of \(N\)-benzy1-4-hydroxypiperidin-2-one 3 (61 mg, 0.30 mmol) in acetone (5 ml). The mixture was stirred at rt for 5 min and the reaction was stopped by addition of isopropanol (2 ml). After evaporation, the residue was treated with water (2 ml) and the product was extracted into CHCl\(_3\) (100 ml) and ethyl acetate (50 ml). Drying over Na\(_2\)SO\(_4\), filtration, and evaporation afforded the crude product that was purified by column chromatography on silica gel giving 20.7 mg (34 %) of 5\(^{23}\). Purity: 95.7% (GC); \(R_f\) 0.32 (ethyl acetate), \(^{1}\)H NMR (400 Hz) \(\delta\) 7.36-7.25 (5H, m), 4.68 (2H, s), 3.48 (2H, \(J = 6.0\)), 3.42 (2H, s), 2.53 (2H, \(J = 6.0\)); \(^{13}\)C NMR (100 MHz) \(\delta\) 204.60 (s), 167.42 (s), 137.32 (s), 129.97 (d), 129.20 (d), 129.00 (d), 51.10 (t), 49.99 (t), 43.38 (t), 39.75 (t); MS (m/z): 204 (100%, M+1).

Racemic \(N\)-benzy1 4-hydroxypiperidin-2-one 3. A mixture of \(N\)-benzy1-piperidin-2,4-dione 5 (6.5 mg, 0.032 mmol) and NaBH\(_4\) (2.4 mg, 0.064 mmol) in CH\(_2\)Cl\(_2\)/acetic acid (9:1, 2 ml) was stirred at rt for 10 h. The product was extracted into CHCl\(_3\) (50 ml). Drying over
Na₂SO₄, filtration, and evaporation afforded the crude product that was purified by column chromatography on silica gel giving 6.2 mg (95 %) of 3. Purity: 95.9% (GC); $R_f$ 0.34 (ethyl acetate/MeOH 9:1).

**General procedures for small-scale hydroxylation.** The frozen cells of *Sphingomonas* sp. HXN-200 were thawed and suspended to a cell density of 4.0 g cdw/L in 10 ml of 50 mM K-phosphate buffer containing glucose (2% w/v) at pH =7.0 - 8.0 in a 100 ml Erlenmeyer flask. Substrates were added directly to a final concentration of 2-10 mM. The mixture was shaken at 200 rpm at 30°C for 5 h. Aliquots (0.1-0.2 ml) were taken from a bioconversion mixture at predetermined time points and mixed with an equal volume of MeOH followed by centrifugation to remove the cells. The samples were analyzed by HPLC to quantify the conversion. At the same time, aliquots (0.5 ml) were taken from a bioconversion mixture, the cells were removed, and the product was extracted with ethyl acetate (0.5 ml). These samples were analysed by HPLC with a chiral column to determine the ee. All results are summarized in table 1.

**HPLC analyses.** Column: Hypersil BDS-C18 (5 μm, 125 mm x 4 mm); eluent: a mixture of A (10 mM K-phosphate buffer, pH 7.0) and B (acetonitrile); flow: 1.0 ml/min.; detection: UV at 210, 225, and 254 nm; $t_R$ of 3: 1.8 min, $t_R$ of 1: 4.4 min (A/B 70:30); $t_R$ of 4: 1.8 min, $t_R$ of 2: 4.3 min (A/B 70:30).

**Determination of ee by HPLC.** Chiral column (250 mm x 4.6 mm), UV detection at 210 and 254 nm, eluent A: n-hexane, and eluent B: isopropanol. For 3: chiralpak AS column; flow rate 1.0 ml/min, A/B (75/25); $t_R$: 8.7 and 10.4 min. For 4: chiralcel OB-H column; flow rate 0.5 ml/min, A/B (95/5); $t_R$: 28.3 and 30.8 min.

**Preparation of N-benzyl-4-hydroxypiperidin-2-one 3 by biohydroxylation in a bioreactor.** N-Benzyl-piperidin-2-one 1 (1.52 g, 8.04 mmol) was added to a 1 L suspension (10 g cdw/g) of frozen/thawed cells of *Sphingomonas* sp. HXN-200 in 50 mM of K-phosphate buffer (pH = 8.0) containing glucose (2%, w/v) in a 3 L bioreactor, the mixture was stirred at 1500 rpm and at 30°C under the introduction of air at 1 L/min. The biotransformation was followed by analytical HPLC and stopped at 5 h. After removing the cells, the supernatant pH was adjusted to 11–12 by addition of KOH followed by extraction with ethyl acetate. The organic phase was separated, dried over Na₂SO₄, and the solvent was removed by
evaporation. The crude product was purified by flash chromatography on silica gel (methanol/ethyl acetate 5:95, \( R_f \) 0.05) giving the pure \( \mathbf{3} \) (1.168 g, 71% yield) as a white powder. ee: 31% (\( S \)); purity: 98.4% (GC), 99.2% (HPLC); \([\alpha]_D^{25} = -6.5 \) (c 1.10, CHCl\(_3\)); mp 98.1–99.7°C\(^{11}\); \(^1\)H NMR (400 MHz) \( \delta \) 7.33–7.22 (5H, m), 4.64 (1H, d, \( J = 14.4 \)), 4.55 (1H, d, \( J = 14.4 \)), 4.18 (1H, m), 3.41 (1H, m), 3.14 (1H, quin, \( J = 6.0 \)), 2.73 (1H, dd, \( J = 16.0, 4.4 \)), 2.64 (1H, s, br), 2.50 (1H, dd, \( J = 16.0, 4.4 \)), 1.95 (1H, m), 1.84 (1H, m); \(^{13}\)C NMR (100 MHz) \( \delta \) 169.87 (s), 137.85 (s), 129.68 (d), 128.93 (d), 128.45 (d), 65.23 (d), 51.03 (t), 44.15 (t), 42.01 (t), 31.33 (t); MS (m/z): 206 (100%, M+1), 188 (8%); IR (CHCl\(_3\)): 3382, 1632 cm\(^{-1}\).

Preparation of \( N\)-tert-butoxycarbonyl-4-hydroxy-piperidin-2-one \( \mathbf{4} \) by biohydroxylation in a shaking flask. \( N\)-tert-butoxycarbonyl-piperidin-2-one \( \mathbf{2} \) (199 mg, 1.00 mol) was added to a 100 ml suspension (4.0 g cdw/L) of frozen/thawed cells of Sphingomonas sp. HXN-200 in 50 mM K-phosphate buffer (pH 8.0) containing glucose (2%) in a 500 ml shaking flask. The mixture was shaken at 200 rpm and 30°C for 5 h and the bioconversion was followed by analytical HPLC. The cells were removed from the bioconversion mixture by centrifugation and the pH of the supernatant was adjusted to 11–12 by addition of KOH followed by extraction with ethyl acetate/n-butanol(1:1). The organic phase was collected, water was removed by azeotropic distillation with MeOH, and the solvent was dried over \( \text{Na}_2\text{SO}_4 \) and removed by evaporation. The product was purified by column chromatography on silica gel (ethyl acetate, \( R_f \) 0.21), yielding 96.2 mg (45%) of \( \mathbf{4} \) as a white powder.

\( N\)-tert-butoxycarbonyl-4-hydroxy piperidin-2-one \( \mathbf{4} \): ee: 68% (\( R \)); purity: 97.7% (GC), 99.9% (HPLC); \([\alpha]_D^{25} = +6.7 \) (c 1.50, CHCl\(_3\)); mp 61.9-63.6°C; \(^1\)H NMR (400 MHz) \( \delta \) 4.21 (1H, s), 3.83 (1H, dd, \( J = 12.0, 4.4 \)), 3.57 (1H, dd, \( J = 12.0, 4.4 \)), 2.74 (1H, d, \( J = 15.6 \)), 2.53 (1H, d, \( J = 15.6 \)), 2.60-2.30 (1H, s, br), 2.02 (1H, d, \( J = 3.2 \)), 1.88 (1H, m), 1.50 (9H, s); \(^{13}\)C NMR (100 MHz) \( \delta \) 170.89 (s), 153.39 (s), 84.25 (s), 65.17 (d), 44.75 (t), 43.12 (t), 31.81 (t), 29.03 (q); MS (m/z): 215 (14%, M), 116 (100%), 100 (39%); IR (CHCl\(_3\)): 3446, 1766, 1715 cm\(^{-1}\).
References


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Chapter-4  Regio- and stereoselective hydroxylation of piperidin-2-ones


(21) The preparation and absolute configuration of (+)-(S)-8 were described in the following reference: Vink, M. K. S.; Schortinghuis, Ch. A.; Luten, J.; van Maarseveen, J. H.; Schoemaker, H. E.; Hiemstra, H.; Rutjes, F. P. J. T. *J. Org. Chem.* **2002**, *67*, 7869-7871. We thank Prof. Floris Rutjes at University of Amsterdam for providing us with the [α]_{25}^{D} value of (+)-(S)-8.


Chapter 5

Regio- and enantioselective hydrolysis of alicyclic epoxides with a bacterial epoxide hydrolase from *Sphingomonas* sp. HXN-200

Hydrolysis of $N$-benzyloxycarbonyl-3,4-epoxy-pyrrolidine and cyclohexene oxide with the epoxide hydrolase of *Sphingomonas* sp. HXN-200, respectively, gave the corresponding vicinal trans-diols in high ee and yield, representing the first example of enantioselective hydrolysis of a *meso*-epoxide with a bacterial epoxide hydrolase.
Introduction

Epoxide hydrolase (EH), an enzyme that catalyses the enantioselective addition of water to an epoxide forming the corresponding vicinal diol, has been extensively investigated,¹ and several microbial EHs have been applied to prepare enantiopure epoxide via kinetic resolution.² Theoretically, EH could also catalyse the enantioselective hydrolysis of a meso-epoxide to give the corresponding trans-diol in high ee and 100% yield, providing a simple and green synthesis in addition to the other known chemical methods.³⁻⁴ Mammalian epoxide hydrolase (mEH) is known to hydrolyse meso-epoxides such as cycloalkene oxides⁵⁻⁶ and acyclic 1,2-disubstituted epoxides⁶⁻⁷ with high enantioselectivity. However, synthetic applications are limited due to the poor availability of mEH. Microbial epoxide hydrolases can easily be produced in large amounts, but examples of hydrolysis of meso-epoxides with such EHs are rare: only a membrane-associated yeast EH from Rhodotorula glutinis was found to enantioselectively hydrolyse cyclohexene- and cyclopentene oxide;⁸ low enantioselectivity was observed in hydrolysis of cyclohexene oxide with a fungal EH;⁹ and thus far no bacterial EH was found to accept a meso-epoxide as substrate.¹⁰ Here we report the first example of highly enantioselective hydrolysis of a meso-epoxide catalysed by a bacterial EH from Sphingomonas sp. HXN-200 and the high yield preparation of the corresponding vicinal trans-diols.

Sphingomonas sp. HXN-200 is an alkane-degrading strain and known to regio- and stereoselectively catalyse the hydroxylation of a series of aliphatic heterocycles.¹¹ Cells of Sphingomonas sp. HXN-200 were produced by growth on n-octane in 2 L E2 medium,¹¹ the harvested cell pellets were stored at -80°C, and the frozen/thawed cells were used for hydrolysis. 3,4-Epoxypyrrolidine 1 was chosen as substrate since the trans-diol product is a useful synthetic intermediate for the preparation of antibiotics,¹² Sialyl Lewis X mimetics¹³ and aza-sugars.¹⁴ Substrate 1 was prepared by epoxidation of N-benzyloxy-carbonyl-3-pyrroline with mCPBA,¹⁴ and small-scale hydrolysis of 1 (10-20 mM) were performed with frozen/thawed cells (10 g/L) of Sphingomonas sp. HXN-200 in 10 ml 50 mM K-phosphate buffer (pH = 8.0) containing glucose (2%) at 30°C. Aliquots (0.1-0.2 ml) were taken from the bioconversion mixture at predetermined time points, diluted in MeOH, and the cells were removed by centrifugation. The samples were analyzed by reverse phase HPLC to follow the reaction directly in the aqueous phase. The conversion was quantitated by comparing the integrated peak areas at 210 nm of the samples with the product standard which was prepared
by hydrolysis of 1 with TFA. In all the cases, the desired diol 2 was generated without formation of any byproduct. The conversion was 98-99% for the hydrolysis of 10-15 mM of substrate (table 1). The activity reached 17-18 U/g cdw. The ee of diol 2 was determined by HPLC analysis with a chiral column. It remained unchanged during the biotransformation at 95%. To our knowledge, this is the first example of highly enantioselective hydrolysis of N-containing meso-epoxide catalysed by either a chemical or a biological catalyst.

Preparative hydrolysis was performed with 15 mM of 1 in 100 ml cell suspension (10 g/L) of *Sphingomonas* sp. HXN-200 (Figure 1). 97% conversion was reached after 5 h, and 289.1 mg (81.3%) of 2 was isolated with 98.9% purity, 95% ee and an $\alpha_2^{25} +7.6$ (c=1.80, CHCl$_3$). The bio-product 2 is identical with the authentic sample synthesized chemically, with respect to MS, $^1$H- and $^{13}$C-NMR, UV, and IR spectra.

![Diagram](image)

**Table 1 Hydrolysis of N-benzyloxy carbonyl-3,4-epoxy-pyrrolidine 1 with frozen/thawed cells of Sphingomonas sp. HXN-200**

<table>
<thead>
<tr>
<th>Concentration of 1 (mM)</th>
<th>Activity $^a$ (U/g cdw)</th>
<th>Conversion (%) $^b$ 0.5 h 1 h 2 h 3 h 4 h 5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0</td>
<td>17</td>
<td>52 72 88 98 99 99</td>
</tr>
<tr>
<td>15.0</td>
<td>18</td>
<td>37 53 70 86 96 98</td>
</tr>
<tr>
<td>20.0</td>
<td>18</td>
<td>27 43 56 70 79 85</td>
</tr>
</tbody>
</table>

$^a$Biotransformation was performed in 10 ml cell suspension (10 g cdw/L) in 50 mM K-phosphate buffer (pH=8.0) containing glucose (2%), and the activity was determined over the first 30 min. $^b$Conversion was determined by HPLC analysis; error limit: 2% of the stated values.
Figure 1 Preparation of 2 by hydrolysis of 1 (15 mM) with frozen/thawed cells (10 g/L) of Sphingomonas sp. HXN-200

The absolute configuration of bioproduct (+)-2 was established by chemical correlation: deprotection of bioproduct (+)-2 by hydrogenation with 20% Pd(OH)$_2$/C gave 94% of 3,4-dihydroxyproline with an $[\alpha]_D^{25}$ of -18.6 (c = 0.80, in MeOH). Since (3$S$, 4$S$)-3,4-dihydroxyproline has an $[\alpha]_D^{26}$ of +20.7 (c = 0.30, in MeOH),$^{15}$ the configuration of bioproduct (+)-2 can be deduced to be (3$R$, 4$R$).

To further explore the hydrolysis potential, cyclohexene oxide 3 was selected as the second substrate, since it represents a carbocyclic meso-epoxide and the product 4 is an useful synthon. The enzymatic reaction was performed at 25°C instead of 30°C to reduce the non-enzymatic hydrolysis rate. Hydrolysis of 3 (10-20 mM) was examined with frozen/thawed cells (13 g/L) of HXN-200 in 50mM Tris-HCl (pH = 7.5) and followed by GC analysis of samples that were prepared by taking aliquots (0.2 ml) at predetermined time points, removing the cells, and extracting with ethyl acetate (1:2). The conversion was quantitated by comparison of the integrated peak areas of the samples and the product standard and correction with the extraction efficiency. Here again, only the desired trans-diol 4 was formed, and the conversion reached >95% at 7 h (table 2). Hydrolysis of 1 (20 mM) under the same conditions with cells which were boiled for 20 min revealed that the non-enzymatic hydrolysis was only about 1.2% at 7 h. The ee of the product 4 was determined as 86-87% by GC analysis with a chiral column, and the configuration was established as (1$R$,2$R$) by comparison of the retention time with those of (1$R$,2$R$)- and (1$S$,2$S$)-4. This stereochemistry
outcome is similar to the hydrolysis with mEH\textsuperscript{5-6} and the membrane-associated yeast EH from \textit{R. glutinis}.\textsuperscript{8}

\begin{equation}
\text{HO}
\begin{array}{c}
\text{O} \\
\text{3}
\end{array}
\xrightarrow{\text{HXN-200, 87\% ee}}
\begin{array}{c}
\text{OH} \\
\text{OH} \\
\text{4}
\end{array}
\end{equation}

Table 2: Hydrolysis of cyclohexene oxide 3 with frozen/thawed cells and soluble cell-free extracts of \textit{Sphingomonas} sp. HXN-200

<table>
<thead>
<tr>
<th>3 (mM)</th>
<th>Cells (g cdw/L)</th>
<th>CFE\textsuperscript{a} (g prot./L)</th>
<th>Scale (ml)</th>
<th>Activity\textsuperscript{b} (U/g)</th>
<th>Time (h)</th>
<th>Conv.\textsuperscript{c} (%)</th>
<th>ee\textsuperscript{d} of 4 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>13</td>
<td>20</td>
<td>1.8</td>
<td>7</td>
<td>99</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>13</td>
<td>12</td>
<td>3.1</td>
<td>7</td>
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<td>5</td>
<td>1.4</td>
<td>14</td>
<td>91</td>
<td>85</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Cell free extract. \textsuperscript{b}Average activity for the whole reaction period. U/g cdw and U/g protein for whole cell and cell-free transformation, respectively. \textsuperscript{c}Conversion was determined by GC analysis; error limit: 2\% of the stated values. \textsuperscript{d}Ee was determined by GC analysis with a chiral column; error limit: 2\% of the stated values.

Different from the \textit{R. glutinis} EH, the HXN-200 EH was found to be a soluble enzyme. The preparation of the soluble cell-free extracts of HXN-200 involved suspending the frozen/thawed cells in 50 mM Tris-HCl (pH 7.5) to a cell density of 31 g/L, passing them through the French press to open the cells and release intracellular enzymes, and removing the cell wall fragments, membranes, and membrane-associated proteins by ultracentrifugation at 244,000 g at 4°C for 45 min. Hydrolysis of 3 (10-20 mM) with these soluble cell-free extracts (20 g protein/L) gave the corresponding diol (1\textit{R},2\textit{R})-4 in 85-86\% ee and >90\% conversion.

Preparative hydrolysis was performed with 10 mM of 3 in 150 ml cell suspension (10 g/L) of \textit{Sphingomonas} sp. HXN-200 at 25°C. Higher than 99\% conversion was reached after 22 h, and 159.3 mg (91.4\%) of 4 was isolated in 99.6\% purity and 87\% ee.
In summary, the soluble epoxide hydrolase of *Sphingomonas* sp. HXN-200 has been found to catalyse the hydrolysis of *N*-benzyloxy carbonyl-3,4-epoxy-pyrrolidine and cyclohexene oxide with high enantioselectivity. The high yield preparation of the corresponding vicinal *trans*-diols in high ee has been demonstrated by simple enzymatic hydrolysis. This provides us with the first efficient bacterial EH for hydrolysis of a meso-epoxide.
Experimental Section

Preparation of (3R,4R)-N-Benzoyloxycarbonyl-3,4-dihydroxy-pyrrolidine

(3R,4R)-N-Benzoyloxycarbonyl-3,4-dihydroxy-pyrrolidine 2 was prepared by hydrolysis of N-benzoyloxycarbonyl pyrrolidine 1 (329 mg, 1.5 mmol) in a 100 ml cell suspension (10.0 g/L) of Sphingomonas sp. HXN-200 in 50 mM K-phosphate buffer (pH 8.0) containing glucose (2%) in a 500 ml shaking flask at 200 rpm and 30°C for 5 h. The reaction was stopped by removing the cells via centrifugation, and the product was extracted into ethyl acetate. The organic phase was separated, dried over Na₂SO₄, and the solvent was removed by evaporation. Purification by column chromatography on silica gel (Rf 0.27, ethyl acetate) afforded 289.1 mg (81.3%) of 2.

HPLC analysis of 1 and 2. Column: Hypersil BDS-C18 (5 μm, 125 mm x 4 mm); eluent: 10 mM K-phosphate buffer (pH=7.0)/acetonitrile (70/30); flow: 1.0 ml/min; detection: UV at 210, 225, and 254 nm; t_R of 2: 2.0 min, t_R of 1: 4.2 min. The ee of 2 was determined by HPLC analysis with a chiral column (Chiralpak AS, 250 mm x 4.6 mm). eluent: n-hexane/isopropanol (97/3); flow rate: 1.0 ml/min; UV detection at 210 nm; t_R: 114.1 and 130.4 min.

Data of (3R,4R)-N-Benzoyloxycarbonyl-3,4-dihydroxy-pyrrolidine 2: 95% ee; 98.9% purity; [α]D²⁵ +7.6 (c=1.80, CHCl₃); ¹H NMR (CDCl₃, 400 MHz) δ 7.34-7.26 (m, 5 H, Ph-H), 5.06 (s, 2 H, PhCH₂); 4.07 (s, br, 2 H, CHOH x2); 3.86 (s, br, 1 H, OH), 3.63 (d, 2 H, J = 10.4 Hz, CH(H)N x2), 3.35 (t, 2 H, J = 10.4 Hz, CH(H)N x2), 2.63 (s, br, 1 H, OH); ¹³C NMR (CDCl₃, 100 MHz) δ 156.71 (s, C=O), 137.43 (s), 129.58 (d), 129.18 (d), 128.89 (d) (aromatic C), 76.27 (d), 75.63 (d) (CHOH x2), 68.28 (t, PhCH₂), 52.85 (t), 52.51 (t) (CHN, x2); APCI-MS(40eV) m/z 238 (30%, M+1), 194 (100%); IR (CHCl₃): 3406 (br, OH), 1693 (C=O) cm⁻¹.

GC analysis of 3 and 4. Column: Optima-5-0.25 μm (25 m x 0.32 mm); temperature program: 60° for 1 min, then to 140° at 15°/min, and finally to 260° at 49°/min); t_R of 3: 2.46 min, t_R of 4: 4.54 min. The ee of 4 was determined by GC analysis with a chiral column (lipodex-A, 25 m x 0.25 mm). temperature program: 60° for 1 min, then to 120° at 2°/min, 120° for 1 min, and finally to 160° at 40°/min); t_R of (1S,2S)-4: 26.13 min, t_R of (1R,2R)-4: 26.45 min.
### Reference


Chapter-5  Regio- and enantioselective hydrolysis of alicyclic epoxides


Chapter 6

Enantioselective trans-dihydroxylation of non-activated C-C double bonds of aliphatic heterocycles with Sphingomonas sp. HXN-200

Abstract

1. R = CO₂Ph
2. R = CO₂Ph
3. R = CO₂Ph (96% ee, 60% yield)
4. R = CO₂Ph
5. R = CO₂Ph
6. R = CO₂CH₂Ph (96% ee, 80% yield)

The bacterial strain *Sphingomonas* sp. HXN-200 was employed to catalyze the *trans*-dihydroxylation of *N*-substituted 1,2,5,6-tetrahydropyridines 1 and 3-pyrrolines 4 giving the corresponding 3,4-dihydroxypiperidines 3 and 3,4-dihydroxypyrrolidines 6, respectively, with high enantioselectivity and activity. The *trans*-dihydroxylation was sequentially catalyzed by a monooxygenase and an epoxide hydrolase in the strain with epoxide as intermediate. While both epoxidation and hydrolysis steps contributed to the overall enantioselectivity in *trans*-dihydroxylation of 1, the enantioselectivity in *trans*-dihydroxylation of the symmetric substrate 4 was generated only in the hydrolysis of meso-epoxide 5. The absolute configurations for the bioproducts (+)-3 and (+)-6 were established as (3*R, 4*R) by chemical correlations. Preparative *trans*-dihydroxylation of 1a and 4b with frozen/thawed cells of *Sphingomonas* sp. HXN-200 afforded the corresponding (+)(3*R, 4*R)-3,4-dihydroxypiperidine 3a and (+)(3*R, 4*R)-3,4-dihydroxy pyrrolidine 6b in 96% ee both and in 60% and 80% yield,
respectively. These results represent first examples of enantioselective trans-dihydroxylation with non-terpene substrates and with bacterial catalyst, thus significantly extending this methodology in practical synthesis of valuable and useful trans-diols. Enantioselective hydrolysis of racemic epoxide 2a with *Sphingomonas* sp. HXN-200 gave 34% of (-)-2a in >99% ee which is a versatile chiral building block. Further hydrolysis of (-)-2a with the same strain afforded (-)(3S, 4S)-3a in 96% ee and 92% yield. Thus, both enantiomers of 3a can be prepared by biotransformation with *Sphingomonas* sp. HXN-200.
Introduction

Enantiomerically pure vicinal diols are very useful intermediates in the synthesis of a number of important pharmaceuticals and biologically active molecules, and asymmetric dihydroxylation of C-C double bond provides a simple access to these diols. While asymmetric cis-dihydroxylation can be achieved with Sharpless catalyst\(^1\) or a dioxygenase,\(^2\) epoxidation of a C-C double bond followed by hydrolysis could give rise to the asymmetric “trans-dihydroxylation”. The latter can be realized by use of a biological system containing a monooxygenase and an epoxide hydrolase.\(^3\) Successful examples are the fungus-catalyzed trans-dihydroxylations of several acyclic terpenes,\(^4-6\) limonene,\(^7\) and \(\alpha\)-terpinene.\(^7\) However, enantioselective trans-dihydroxylation of a non-isoprene C-C double bond has been synthetically unsuccessful: several eukaryotic systems (plants, animal and fungi) were known to metabolize aromatic compounds transforming the C-C double bond into the corresponding trans-dihydrodiol with low yield;\(^8\) bioconversion of 1-benzyl-3-methyl-1,2,5,6-tetrahydro pyridine with Cunninghamella verticillata VKPM F-430 gave a mixture of 3,4-trans-diol and two mono-hydroxylated products.\(^9\) Moreover, trans-dihydroxylation catalysts reported so far are limited to the eukaryotic systems. We are interested in expanding the scope of enzymatic trans-dihydroxylation in practical syntheses by developing a highly active and easy to handle bacterial catalyst with new substrate specificity. Previously, we found that Sphingomonas sp. HXN-200, an alkane-degrading bacterium, contains a soluble alkane monooxygenase that catalyzes the regio- and stereoselective hydroxylation of non-activated carbon atom of various aliphatic heterocycles.\(^10\) Recently we also found a soluble epoxide hydrolase in this strain that catalyzes the enantioselective hydrolysis of alicyclic meso-epoxides.\(^11\) Considering that the alkane monooxygenase might catalyze the epoxidation of a C-C double bond, the strain HXN-200 might be a promising catalyst for trans-dihydroxylation of non-isoprene C-C double bond of alicyclic compounds.

\(N\)-Substituted 1,2,5,6-tetrahydropyridine and 3-pyrroline are interesting substrates, since trans-dihydroxylation of these compounds could give the corresponding optically active trans-3,4-diols. While (3\(R\), 4\(R\))-3,4-dihydroxypiperidine is a potent inhibitor of \(\beta\)-glucuronidase\(^{12a}\) and an intermediate for the preparation of xylanase inhibitor isofagomine,\(^{12b}\) (3\(R\), 4\(R\))-3,4-dihydroxypyrrolidine is a useful intermediate for the preparation of biologically active Sialyl Lewis X mimetics,\(^{13a}\) new carbapenem\(^{13b}\) and cephalosporin\(^{13c}\) antibiotics, and novel carbohydrate mimics.\(^{13d}\) The chemical synthesis of (3\(R\), 4\(R\))-3,4-dihydroxypiperidine
requires 10 steps from either D-arabinose$^{12a, 14}$ or D-tartaric acid$^{12b, 15}$ with low overall yield, whereas the preparation of (3R, 4R)-3,4-dihydroxypyrrolidine involves a difficult reduction step from D-tartaric acid.$^{16, 13b}$ Here, we report the highly enantioselective trans-dihydroxylation of $N$-substituted 1,2,5,6-tetrahydropyridine and 3-pyrrolines with *Sphingomonas* sp. HXN-200 as biocatalyst and the high yield preparation of (3R, 4R)-3,4-dihydroxypiperidine and (3R, 4R)-3,4-dihydroxypyrrolidine. In addition, we report the practical synthesis of enantiomerically pure 3,4-epoxypiperidine and (3S, 4S)-3,4-dihydroxypiperidine by enantioselective hydrolysis with the same strain.

**Result and Discussion**

*Enantioselective trans-dihydroxylation with Sphingomonas* sp. HXN-200.

![Scheme 1](image)

**Scheme 1.** *Trans*-dihydroxylation of $N$-substituted 1,2,5,6-tetrahydropyridines 1a,b and 3-pyrrolines 4a,b with *Sphingomonas* sp. HXN-200.

*Sphingomonas* sp. HXN-200$^{10c}$ was grown on n-octane in E2 medium as described,$^{10b}$ the cells were harvested, and the cell pellets were stored at -80°C. The easy to handle frozen/thawed cells were subsequently used for *trans*-dihydroxylation studies. Since 1,2,5,6-tetrahydropyridine and 3-pyrrole themselves were not substrates for the monooxygenase of *Sphingomonas* sp. HXN-200, docking/protecting groups$^{17,10}$ were introduced. $N$-Phenoxycarbonyl- and $N$-benzyloxy carbonyl groups were shown to be excellent docking/protecting groups for the hydroxylation of pyrrolidine,$^{10b}$ thus being selected as the
Substrate 1a was synthesized in 70% yield by treatment of 1,2,5,6-tetrahydropyridine with phenyl chloroformate, and 1b and 4a were prepared by similar procedures. To facilitate the analysis of the bioconversion, epoxides 2a,b and 5a,b and diols 3a,b and 6a,b were also prepared chemically: epoxidation of 1a and 4a with m-CBPA gave 79% and 67% of 2a and 5a, respectively; hydrolysis of 2a and 5a with TFA afforded the corresponding trans-diol 3a and 6a in 52% and 82% yield, respectively; similar reactions gave the epoxides 2b and 5b and diols 3b and 6b. The structures of all synthesized compounds were confirmed by the MS, IR, 1H and 13C NMR spectra.

Small-scale biotransformations were performed with frozen/thawed cells (cell concentration: 10 g cdw/L, cdw = cell dry weight; 1 g cdw corresponds to about 4 g frozen cell weight) of Sphingomonas sp. HXN-200 in 10 ml 50 mM K-phosphate buffer (pH = 8.0). Glucose (2%, w/v) was added to increase the yield via regeneration of the cofactor for the epoxidation, and the mixture was shaken at 200 rpm and 30°C. Aliquots (0.1-0.2 ml) were taken from the bioconversion mixture at predetermined time points, diluted in methanol, and the cells were removed by centrifugation. The samples were analyzed by reverse phase HPLC to follow directly the reaction in aqueous phase. The conversion was quantitated by comparing the integrated peak areas at 210 nm of the substrate and product in an analytic sample with the corresponding standards.

During the biotransformation of 1a,b and 4a,b, the epoxides 2a,b and 5a,b were formed and then hydrolyzed to the corresponding diols 3a,b and 6a,b (Figure 1). The formed epoxides and diols were extracted from the reaction mixture and purified, and their structures were identified by 1H and 13C NMR analyses. These results suggested that the alkane monooxygenase of HXN-200 is able to catalyze the epoxidation of non-activated C-C double bond. No hydrolysis of the chemically prepared epoxide 2a,b and 5a,b were observed with the cells that were boiled for 20 min, therefore, the hydrolysis during dihydroxylation must be catalyzed by the epoxide hydrolase from the strain.

As shown in table 1, the average epoxidation activity during the first 30 min is quite high for 1a (10 mM) and 1b (10 mM): 16 and 18 U/g cdw (U = µmol/min), respectively. While the
Table 1: Enantioselective trans-dihydroxylation of N-substituted 1,2,5,6-tetrahydropyridines 1a-b and 3-pyrrolines 4a-b with frozen/thawed cells (10 g cdw/L) of *Sphingomonas* sp. HXN-200.

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aBiotransformation was performed in 10 ml cell suspension in 50 mM K-phosphate buffer (pH=8.0) containing glucose (2%), and activity is the epoxidation activity based on the total product formation over the first 30 min. bEe was given in brackets. cConversion and ee were determined by HPLC analysis; error limit: 2% of the stated values; “-” for ee means the opposite enantiomer is in excess.
diol 3a was formed in 94% at 5 h, only 65% of the diol 3b was observed at the same time. Nevertheless, biotransformation of 1b at 8 mM for 5 h afforded 82% of 3b. The epoxidation activity for 4a (5-8 mM) was also quite high (9-12 U/g cdw), but the diol 6a was formed in only 45-48% at 5 h. In contrast, biotransformation of 4b (2-3 mM) showed lower epoxidation activity (4.4-5.0 U/g cdw), but gave 64-97% of the diol 6b. In fact, the diol formation depends on both epoxidation and hydrolysis. As shown in Figure 1, the concentration of the epoxides 2a,b and 5a,b reached maxima at 0.5-1.0 h. The epoxides 2a,b and 5b were rapidly hydrolyzed afterward, while the epoxide 5a was hydrolyzed rather slowly resulting in low conversion to 6a. It was also clearly shown in Figure 1 that the epoxidation rate became much slower after 0.5 h in all the cases, possibly due to the inhibition of the monooxygenase by the formed epoxides.
Figure 1: Trans-dihydroxylation of 1a,b and 4a,b with frozen/thawed cells (10 g cdw/L) of Sphingomonas sp. HXN-200. a) 1a (10 mM); b) 1b (8 mM); c) 4a (5 mM); d) 4b (3 mM).

The alkane monoxygenase of HXN-200 showed higher epoxidation activity for the six-member ring substrate 1a,b than for the five-member ring substrates 4a,b. Epoxidation of 1,2,5,6-tetrahydropyridine 1a and 3-pyrroline 4a with a N-phenoxy carbonyl docking/protecting group is slightly less active than the hydroxylation of the corresponding saturated substrate piperidine and pyrrolidine,10b,10d respectively. Epoxidation of N-benzyl oxycarbonyl-1,2,5,6-tetrahydropyridines 1b is more active than the hydroxylation of the corresponding piperidine,10d whereas epoxidation of 3-pyrroline 4b is less active than the hydroxylation of N-benzyl oxycarbonyl-pyrrolidine.10b
To investigate the enantioselectivity for the trans-dihydroxylation, analytic samples were prepared by taking 0.5 ml aliquots from biotransformation mixture and extracting with equal volume of ethyl acetate. HPLC analysis with a chiral column (Chiralcel OB-H, OD-H, or Chiralpak AS) gave the ee values of the epoxide 2a and diols 3a,b and 6a,b. During dihydroxylation of 1a (10 mM), the ee of the diol (+)-3a was 98% at 30 min and then decreased to 77% at 5 h (Figure 1a). The ee of the epoxide (+)-2a was 48% at 30 min and changed to >-99% at 5 h (Table 1). These results suggested the following: a) the enantioselective epoxidation of 1a initially gave (+)-2a in about 73% ee; b) (+)-2a was preferably hydrolyzed affording (+)-3a in very high ee at the beginning; c) (-)-2a was also hydrolyzed at a slower rate resulting in decrease of the ee of (+)-3a with time; d) pure (-)-2a remained at 5 h as a result of resolution. Similar results for the trans-dihydroxylation of 15 mM solution of 1a were obtained (Table 1). Reaction for 4 h gave 82% of (+)-3a in 93% ee.

Changing the docking/protecting group of 1 from the phenoxycarbonyl to the benzyloxy-carbonyl group resulted in lower enantioselectivity for the trans-dihydroxylation. As shown in Figure 1b, the ee of the diol 3b was 51% at 30 min and remained nearly unchanged during the reaction. Although attempt to determine the ee of the epoxide 2b by HPLC analysis with a chiral column (Chiralcel OB-H, OD-H, OJ or Chiralpak AS) failed, the constant low ee value of 3b during the reaction suggested that the enantioselectivity for hydrolysis of 2b is very poor. It can be thus assumed that epoxidation of 1b gave the epoxide 2b in about 50% ee. The fact that HXN-200 catalyzes the enantioselective epoxidation of 1a,b is remarkable, since enantioselective epoxidation of the aliphatic heterocyclic C-C double bond was proven to be difficult with microbial enzyme: only Rhodococcus rhodochrous 9703 (ATCC19067) was reported to oxidize 1b giving the corresponding epoxide as the major product with no information about the ee.\textsuperscript{21}

In the case of trans-dihydroxylation of the symmetric substrate 4a,b, the enantioselectivity was generated only in the hydrolysis step, since epoxidation gave meso-epoxide 5a,b. While biotransformation of 4a with a phenoxycarbonyl docking/protecting group gave diol 6a in 78% ee, much higher enantioselectivity was observed in dihydroxylation of 3b with a benzyloxy-carbonyl docking/protecting group: the diol 6b was formed in 95% ee.

To our knowledge, these are the first examples of enantioselective trans-dihydroxylation with a bacterial catalyst. These are also the first enantioselective dihydroxylations of 1,2,5,6-
tetrahydropyridines and 3-pyrrolines, since Osmium-catalyzed dihydroxylations of 1,2,5,6-tetrahydro pyridines and 3-prrolines resulted in racemic and meso-cis-diols, respectively.\textsuperscript{22-23}

**Enantioselective hydrolysis of racemic epoxides 2a,b and meso-epoxides 5a,b.**

To investigate the second reaction step of the trans-dihydroxylation, enzymatic hydrolysis was performed with racemic epoxides 2a,b and meso-epoxides 5a,b as substrates, respectively. The biotransformation was carried out with frozen/thawed cells in 10 ml 50 mM K-phosphate buffer (pH = 8.0) and followed by HPLC analysis as described above. In each case, the expected trans-diol was formed without any byproduct. Hydrolysis of (±)-2a was examined with three different concentrations (Table 2), and the enzymatic activity reached 15-18 U/g cdw which is nearly the same as the epoxidation activity for 1a. As expected, (+)-2a was hydrolyzed faster than (-)-2a, giving (-)-2a in high ee at 5 h. The enantioselectivity factor $E$ was calculated as 12.8 (for 20 mM at 5 h) from the equation $E = \ln(1 - c)(1 - ee_s)/\ln(1 - c)(1 + ee_s)$.\textsuperscript{24}

![Figure 2: Hydrolysis of (±)-2a,b and 5a,b (all 10 mM) to the corresponding 3a,b and 6a,b with frozen/thawed cells (10 g cdw/L) of Sphingomonas sp. HXN-200.](image)

As shown in Figure 2, 2b was hydrolyzed as fast as 2a. Although the ee of 2b could not be determined, the ee of the formed diol 3b was found to be very low. The enantioselectivity factor was calculated as 2.7 (for 10 mM at 4 h) according to the equation $E = \ln[1 - c(1 + ee_p)]/\ln[1 - c(1 - ee_p)]$.\textsuperscript{24} Hydrolysis of the meso-epoxide 5b is the fastest one among all hydrolysates, and the trans-diol 6b was obtained in 95% ee and >99% conversion.\textsuperscript{11} For this asymmetric reaction, $E$ was calculated as 39 according to the equation $E = (1 + ee_p)/(1 -}$
Hydrolysis of 5a showed the lowest activity (6 - 9 U/g cdw), which explains why a significant amount of 5a remained in the trans-dihydroxylation of 4a (Figure 1c). The diol 6a was formed in 67% ee, corresponding to an $E$ of 5.1. Obviously, the epoxide hydrolase of HXN-200 prefers substrates with special size: N-phenoxy carbonyl epoxy piperidine 2a and N-benzyloxycarbonyl epoxypyrrolidine 5b have similar sizes, and both of them are excellent substrates for the hydrolysis with high activity and enantioselectivity; hydrolysis of 2b with one carbon more or 5a with one carbon less is significantly less enantioselective. Comparison of the retention times in chiral HPLC chromatograms suggested the same enantiomer in excess for diols 3a-b and 6a-b obtained from dihydroxylation of 1a,b and 4a,b and from hydrolysis of 2a,b and 5a,b, respectively.

Table 3: Preparation of (+)-3a, (+)-6b, (-)-2a, and (-)-3a by biotransformation with frozen/thawed cells (10 g cdw/L) of Sphingomonas sp. HXN-200.

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*aConversion and ee were determined by HPLC analysis; error limit: 2% of the stated values.

*bYield of the isolated pure product. *cEpoxidation activity was determined form the total product formation over the first 30 min. *dHydrolysis activity was determined over the first 30 min.

Preparative trans-dihydroxylation of 1a and 4b and absolute stereochemistry.

To demonstrate the synthetic applicability of the enantioselective trans-dihydroxylation, preparative biotransformations were performed with frozen/thawed cells as biocatalyst in a shaking flask (Scheme 2). Bioconversion of 1a (10 mM) on a 100 ml scale at a cell density of 10 g cdw/L gave 91% of (+)-3a at 3 h. Extraction of the product with n-butanol/ethyl acetate (1:1) and purification by flash chromatography on silica gel afforded 77% of (+)-3a in 90% ee (Table 3). Reaction of 1a at an increased concentration (15 mM) for 3 h gave (+)-3a in 96% ee with 83% conversion, and the pure product was isolated in 60% yield (106.9 mg) with an $[\alpha]_D^{25}$ of + 4.24 ($c = 1.44$ in CHCl$_3$). Similarly, biotransformation of 4b (2.0 mM) on a 100 ml
scale for 3 h gave 95% of (+)-6b. Workup and chromatographic purification afforded 80% (37.8 mg) of the pure product in 96% ee with an $[\alpha]_D^{25}$ of +7.56 ($c = 1.80$ in CHCl$_3$).

Scheme 2. Preparation of (+)-3a and (+)-6b by enantioselective trans-dihydroxylation of 1a and 4b with Sphingomonas sp. HXN-200 and stereochemistry correlation of bioproducts 3a,b and 6a,b.

To establish the absolute configuration, bioproduct (+)-3a was transformed into 3,4-dihydroxy piperidine hydrochloride 7 in 72% yield by treatment with 6N HCl (Scheme 2). The obtained compound 7 has an $[\alpha]_D^{25}$ of -14.0 ($c = 0.50$, in MeOH), while the chemically
Table 2: Hydrolysis of N-substituted 3,4-epoxy-piperidines 2a-b and 3,4-epoxy-pyrrolidines 5a-b with frozen/thawed cells (10 g cdw/L) of *Sphingomonas* sp. HXN-200.

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<td>27 (95)</td>
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</table>

*dBiotransformation was performed in 10 ml cell suspension in 50 mM K-phosphate buffer (pH=8.0) containing glucose (2%), and the activity was determined over the first 30 min. *EE was given in brackets. *Conversion and ee were determined by HPLC analysis; error limit: 2% of the stated values. *Data from reference 11.*
synthesized (3R, 4R)-7\textsuperscript{25} has an [\alpha]_D\textsuperscript{25} of -15.0 (c = 0.24, in MeOH) that was determined in this study. Therefore, the absolute configuration of bioproduct (+)-3a can be deduced as (3R, 4R). Treatment of (-)-7 with benzyl chloroformate gave (+)-(3R, 4R)-3b in 71% yield. Comparison of the retention times of (+)-(3R, 4R)-3b and bioproduct 3b in chiral HPLC chromatograms suggested the (+)-(3R, 4R) for the major enantiomer of bioproduct 3b. Deprotection of bioproduct (+)-6b by hydrogenation gave 94% of (-)(3R, 4R)-8 with an [\alpha]_D\textsuperscript{25} of -18.6 (c = 0.80, in MeOH), thus establishing the absolute configuration of bioproduct (+)-6b as (3R, 4R).\textsuperscript{11,26} Reaction of (-)-8 with phenyl chloroformate afforded (+)-(3R, 4R)-6a in 65% yield, and comparison of the retention times in chiral HPLC analysis established the absolute configuration as (+)-(3R, 4R) for the major enantiomer of bioproduct 6a.

Preparation of (-)-2a and (-)-3a by hydrolysis with *Sphingomonas* sp. HXN-200.

The epoxide hydrolase of HXN-200 was shown to be enantioselective in hydrolysis of racemic 2a, thus enabling the preparation of enantiomerically pure epoxide 2a via resolution (Figure 3). Hydrolysis of racemic 2a (15 mM) for 3 h gave (-)-2a in >99.9% ee. Work-up and purification afforded 34% of (-)-2a with an [\alpha]_D\textsuperscript{25} of -18.05 (c = 1.23 in CHCl\textsubscript{3}) (Table 3).

![Figure 3: Hydrolysis of (±)-2a (15 mM) to 3a with frozen/thawed cells (10 g cdw/L) of *Sphingomonas* sp. HXN-200.](image)

This synthesis is important, since enantiopure epoxide 2a is very difficult to prepare by either chemical or enzymatic methods and it can be a versatile chiral building block. Following the highly regioselective ring opening of racemic 3,4-epoxypiperidine with HBr,\textsuperscript{27}
(-)-2a could be transformed into enantiopure trans-4-bromo-3-hydroxy-piperidine which can be further transformed into other enantiopure compounds such as cis-4-amino-3-hydroxy- and cis-3,4-dihydroxy-piperidine. Moreover, (-)-2a is a useful intermediate for the preparation of single enantiomer of antidepressant Ifoxetine sulfate28 and prokinetic agent Cisapride hydrate.29-30

![Scheme 3](image)

**Scheme 3.** Preparation of (-)-2a and (-)-3a by hydrolysis with *Sphingomonas* sp. HXN-200.

While (+)-3a was prepared from 1a by enantioselective trans-dihydroxylation, (-)-3a was also successfully prepared by hydrolysis of (-)-2a (5 mM) with HXN-200 (Scheme 3). The hydrolysis activity for (-)-2a is 6.6 U/g cdw, which is half of that for the racemic 2a. Nevertheless, (-)-2a was totally converted to the corresponding trans-diol 3a after 6 h of biotransformation (Table 3). Workup and chromatographic purification afforded (-)-3a in 92% yield and 96% ee with an $[\alpha]_D^{25}$ of -4.05 ($c = 0.65$ in CHCl$_3$). The high ee of the product indicates that the ring opening of (-)-2a is highly regioselective.

**Conclusion**

*Sphingomonas* sp. HXN-200 containing a monooxygenase and an epoxide hydrolase catalyzes the trans-dihydroxylation of *N*-phenoxy carbonyl-1,2,5,6-tetrahydropyridine 1a and *N*-benzyl oxycarbonyl-3-pyrroline 4b, respectively, with high activity and high enantioselectivity. While enantioselectivity for the trans-dihydroxylation of 1a is a combination of those for the epoxidation of 1a and hydrolysis of 2a, the enantioselectivity for the trans-
dihydroxylation of symmetric substrate 4b is due only to the hydrolysis of meso-epoxide 5b. The absolute configuration was established as (3R, 4R) for both (+)-3a and (+)-6b by chemical correlations. Preparative trans-dihydroxylations of 1a and 4b with frozen/thawed cells of Sphingomonas sp. HXN-200 as biocatalyst afforded the corresponding (+)-3,4-dihydroxypiperidine 3a and (+)-3,4-dihydroxypyrrolidine 6b in 60% and 80% yield, respectively, and both in 96% ee. These highly yielding and highly enantioselective trans-dihydroxylations provide the simplest syntheses of the trans-diols which are useful pharmaceutical intermediates. These are the first enantioselective trans-dihydroxylations of a non-isoprene C-C double bond of an aliphatic substrate and the first bacterium-catalyzed enantioselective trans-dihydroxylations, thus significantly extending this useful methodology in organic synthesis.

Enantioselective hydrolysis of the racemic epoxide 2a with the epoxide hydrolase of HXN-200 afforded (-)-2a, a versatile chiral building block, in >99% ee and 34% yield. Further hydrolysis of (-)-2a with HXN-200 showed excellent regioselectivity and gave (-)-(3S, 4S)-3a in 96% ee and 92% yield. Thus, both enantiomers of 3a can be prepared in high ee by biotransformation with Sphingomonas sp. HXN-200.
Experimental Section

General Methods. \(^1\)H- and \(^{13}\)C-NMR spectra were determined at 300 K in CDCl\(_3\) with chemical shifts in ppm relative to TMS and coupling constants \(J\) in Hz. Mass spectra were obtained by atmospheric pressure chemical ionization at 40 eV. IR spectra were measured in CHCl\(_3\). Melting points are uncorrected. Optical rotations were determined on a polarimeter. Bioconversion was analyzed by HPLC or LC-MS, and the ee of the products was determined by HPLC on a chiral column. The purity of the products was established by GC analyses with a Chrompack CP–Sil-5CB column (25 m x 0.32mm, temperature program: 60°C for 2 min, increase to 280°C at a rate of 25°C /min, then 280°C for 1 min).

Materials. Compound 4b was commercially available. Compounds 1b,\(^{31}\) 2b,\(^{31}\) 4a,\(^{32}\) 5b,\(^{33}\) and 6b\(^{33}\) were prepared according to known procedures. Cells of Sphingomonas sp. HXN-200 were produced by growth on \(n\)-octane in E2 medium according to the published procedure.\(^{34}\)

\(N\)-Phenoxycarbonyl-1,2,5,6-tetrahydropyridine 1a. A solution of phenyl chloroformate (1.50 ml, 12.0 mmol) in THF (5 ml) was added dropwise to a stirred mixture of 1, 2, 5, 6-tetrahydropyridine (0.996 g, 12.0 mmol) and NaHCO\(_3\) (1.30 g, 15.6 mmol) in THF-water (1:1, 12 ml) at 0°C, and the mixture was stirred at rt for 3 h. CHCl\(_3\) (20 ml) and 5\% aqueous Na\(_2\)CO\(_3\) (10 ml) were added, the organic phase was separated, and the aqueous phase was extracted with CHCl\(_3\) (50 ml). The combined organic phase was dried over Na\(_2\)SO\(_4\), filtered, and the solvent was removed by evaporation. Column chromatography on silica gel gave 1.6953 g (69.6 \%) of 1a\(^{18}\) as solid. mp 55.9-56.7°C; \(R_f = 0.38, (n\text{-hexane/ethyl acetate 8/2}); purity: 99.0 \% (GC, \(t_R = 8.20 \text{ min})\); \(^1\)H NMR (CDCl\(_3\), 400 MHz) \(\delta 7.36 (t, 2 H, J = 7.4), 7.20 (t, 1 H, J = 7.2), 7.12 (d, 2 H, J = 7.2), 5.90 (s, 1 H), 5.73 (s, 1 H), 4.15 (s, 1 H), 4.04 (s, 1 H), 3.74 (s, 1 H), 3.65 (s, 1 H), 2.25 (s, 2 H); \(^{13}\)C NMR (CDCl\(_3\), 100 MHz) \(\delta 155.2, 152.5, 130.3, 126.3, 122.9, 126.7, 126.2, 125.4, 124.8, 44.9, 44.2, 41.6, 26.4, 25.9; MS m/z 204 (100\%, M\(+1)); IR (CHCl\(_3\)) \(\nu 1713, 1425, 1238, 1204 \text{ cm}^{-1}.

\(N\)-Phenoxycarbonyl-3,4-epoxypiperidine 2a. m-CPBA (1.43 g, 8.34 mmol) was added to a solution of 1a (740.6 mg, 3.64 mmol) in CH\(_2\)Cl\(_2\) (20 ml) and the mixture was stirred at rt for 24 h. 1N NaOH (15 ml) was added and the mixture was extracted with CH\(_2\)Cl\(_2\) (3 x 20 ml). The organic phase was separated, dried over Na\(_2\)SO\(_4\), and the solvent was removed. Column
chromatography on a silica gel gave 631.2 mg (79.0%) of 2a with 99.7% purity (GC, \( t_R = 9.02 \) min). The spectroscopic data are the same as those for (-)-2a reported below.

**N-Phenoxycarbonyl-3,4-dihydroxypiperidine 3a.** TFA (0.30 ml) was added to a solution of 2a (30 mg, 0.14 mmol) in CHCl₃ (2 ml) and the mixture was stirred at reflux for 6 h. pH was adjusted to 8.0 by addition of 10% Na₂CO₃ and the mixture was extracted with CHCl₃ (3 x 20 ml). The organic phase was dried over Na₂SO₄ and the solvent was removed. Column chromatography on silica gel afforded 16.7 mg (51.5%) of 3a with purity of 99.3% (GC, \( t_R = 10.30 \) min). The spectroscopic data are the same as those for (+)-3a reported below.

**N-Benzylloxy carbonyl-3,4-dihydroxypiperidine 3b.** Similar to the preparation of 3a, hydrolysis of 2b (30 mg, 0.12 mmol) gave 19.9 mg (61.6%) of 3b with 98.7% purity (GC, \( t_R = 9.53 \) min). The spectroscopic data agree with those reported in ref. 12b.

**N-Phenoxycarbonyl-3,4-epoxy-pyrrolidine 5a.** Similar to the preparation of 2a, reaction of m-CPBA (0.52 g, 3.0 mmol) with 4a (284 mg, 1.50 mmol) gave 205.0 mg (66.7%) of 5a as solid. mp 72.1-73.0°C; \( R_f \) 0.42 (n-hexane/ethyl acetate = 1/1); purity: 99.0% (GC, \( t_R = 9.05 \) min); \(^1\)H NMR (CDCl₃, 400 MHz) \( \delta \) 7.35 (t, 2 H, \( J = 7.4 \)), 7.20 (t, 1 H, \( J = 7.2 \)), 7.11 (d, 2 H, \( J = 7.2 \)), 4.04 (d, 1 H, \( J = 13.2 \)), 3.95 (d, 1 H, \( J = 13.2 \)), 3.77 (s, 2 H), 3.58 (d, 1 H, \( J = 13.2 \)), 3.48 (d, 1 H, \( J = 13.2 \)); \(^{13}\)C NMR (CDCl₃, 100 MHz) \( \delta \) 154.7, 152.1, 130.4, 126.5, 122.8, 56.6, 56.1, 48.7; MS m/z 206 (100%, M+1); IR (CHCl₃) ν 1721, 1420, 1392, 1204 cm\(^{-1}\).

**N-Phenoxycarbonyl-3,4-dihydroxy-pyrrolidine 6a:** similar to the preparation of 3a, reaction of 5a (30.0 mg, 0.15 mmol) with TFA (0.30 ml) afforded 26.9 mg (82.4%) of 6a with 97.6% purity (GC, \( t_R = 9.30 \) min). The spectroscopic data are the same as those for (+)-6a reported below.

**General procedure for bioconversion on a small-scale.**

Frozen cells of *Sphingomonas* sp. HXN-200 were thawed and suspended to a cell density of 10 g cdw/L in 10 ml of 50 mM K-phosphate buffer containing glucose (2% w/v) at pH = 8.0 in a 100 ml Erlenmeyer flask. Substrates were added either directly (1a, 2b, 4a, 5a) or as a MeOH solution (1b, 2a, 4b, 5b) to a final concentration of 2-20 mM. The mixture was shaken at 200 rpm at 30°C for 3-5 h and the reaction was followed by HPLC analysis of samples that
were prepared by taking aliquots (0.1-0.2 ml) from a bioconversion mixture at predetermined
time points and mixing with an equal volume of MeOH followed by removal of the cells by
centrifugation. The ee of the products was determined by HPLC analysis on a chiral column,
and the samples was prepared by taking aliquots (0.5 ml) from a biotransformation mixture,
mixing with equal volume of ethyl acetate, removing the cells and separating the organic
phase after centrifugation, drying over sodium sulfate, and filtering. The results are listed in
table 1-2.

**HPLC analysis of conversion**: column: Hypersil BDS-C18 (5 µm, 125 mm x 4 mm); eluent:
a mixture of A (10 mM K-phosphate buffer, pH 7.0) and B (acetonitrile); flow rate: 1.0
ml/min; detection: UV at 210, 225, and 254 nm; t_R of 3a: 1.6 min, t_R of 2a: 3.4 min, t_R of 1a:
9.1 min (A/B 65:35); t_R of 3b: 1.8 min, t_R of 2b: 4.1 min, t_R of 1b: 10.8 min (A/B 70:30); t_R of
6a: 1.8 min, t_R of 5a: 3.3 min, t_R of 4a: 7.5 min (A/B 70:30); t_R of 6b: 2.0 min, t_R of 5b: 4.2
min, t_R of 4b: 9.2 min (A/B 70:30).

**HPLC analysis of ee**: chiral column (250 mm x 4.6 mm); UV detection at 210 nm; a
mixture of A (n-hexane) and B (isopropanol). For 2a: Chiralcel OB-H column, flow rate: 0.5
ml/min, A/B (60/40), t_R: 49.4 and 55.4 min. For 3a: Chiralcel OB-H column, flow rate: 0.5
ml/min, A/B (95/5), t_R: 92.7 and 110.4 min. For 3b: Chiralpak AS column, flow rate: 1.0
ml/min, A/B (93/7), t_R: 25.9 and 29.0 min. For 6a: Chiralcel OD-H column, flow rate: 0.5
ml/min, A/B (85/15), t_R: 18.1 and 25.5 min. For 6b: Chiralpak AS column, flow rate: 1.0
ml/min, A/B (97/3), t_R: 114.1 and 130.4 min.

**General procedure for biotransformation on a preparative scale.**
Substrate was added to 20-100 ml suspension of frozen/thawed cells of *Sphingomonas sp.*
HXN-200 (10.0 g cdw/L) in 50 mM K-phosphate buffer (pH 8.0) containing glucose (2%) in
a 100-500 ml shaking flask. The mixture was shaken at 200 rpm and 30°C for 3-6 h. The cells
were removed by centrifugation and the supernatant was extracted with *n*-butanol/ethyl
acetate (1:1) (for 3a, 6b) or ethyl acetate (for 2a). The organic phase was separated, dried over
Na_2SO_4, and the solvent was removed by evaporation. The product was purified by column
chromatography on silica gel and the results are summarized in table 3.
(+)-(3R,4R)-N-Phenoxy carbonyl-3,4-dihydroxy-piperidine 3a. Trans-dihydroxylation of 1a (152.3 mg, 0.75 mmol) on a 50 ml scale gave 106.9 mg (60.0%) of (+)-3a as solid. mp 136.8-137.5°C; ee: 96% (3R, 4R); [α]D²⁵ +4.24 (c 1.44, CHCl₃); Rf 0.32 (ethyl acetate); purity: 99.3 % (GC, tR = 10.30 min); ¹H NMR (CDCl₃, 400 MHz) δ 7.36 (t, 2 H, J = 7.8), 7.20 (t, 1 H, J = 7.4), 7.08 (d, 2 H, J = 8.4), 4.34-4.15 (m, 2 H), 3.54-3.45 (m, 2 H), 3.12-2.77 (m, 3 H), 2.04-1.96 (m, 1 H), 1.90 (s, 1 H), 1.59-1.56 (m, 1 H); ¹³C NMR (CDCl₃, 100 MHz) δ 155.2, 152.2, 130.4, 126.6, 122.8, 74.5, 73.9, 72.9, 72.5, 49.2, 49.0, 43.8, 43.5, 32.4; MS m/z 238 (9%, M+1), 118 (100%); IR (CHCl₃) ν 3614, 3425, 1713, 1428, 1204 cm⁻¹.

(+)-(3R,4R)-N-Benzylcarbonyl-3,4-dihydroxy-pyrrolidine 6b. Trans-dihydroxylation of 4b (40.6 mg, 0.20 mmol) on a 100 ml scale gave 37.8 mg (79.8%) of (+)-6b as a syrup. ee: 96% (3R, 4R); [α]D²⁵ +7.56 (c 1.80, CHCl₃); Rf 0.27 (ethyl acetate); purity: 98.9 % (GC, tR = 10.61 min). The spectroscopic data agree with those for (3R, 4R)-6b reported in ref. 13a.

(-)-N-Phenoxy carbonyl-3,4-epoxy-piperidine 2a. Hydrolysis of racemic 2a (164.3 mg, 0.75 mmol) on a 50 ml scale afforded 54.9 mg (33.5%) of (-)-2a as a syrup. ee: >99.9%; [α]D²⁵ -18.05 (c 1.23, CHCl₃); R, 0.24 (hexane/ethyl acetate = 2/1); purity: 99.7 % (GC, tR = 9.02 min); ¹H NMR (CDCl₃, 400 MHz) δ 7.36 (t, 2 H, J = 7.8), 7.20 (t, 1 H, J = 7.2), 7.10 (dd, 2 H, J = 7.2, 6.8), 4.08-3.97 (m, 2 H), 3.80 (d, 0.5 H, J = 17.2), 3.67 (dt, 0.5 H, J = 13.2, 5.2), 3.55 (dt, 0.5 H, J = 13.2, 5.2), 3.39-3.27 (m, 2.5 H), 2.16 (dt, 1 H, J = 14.8, 4.4), 2.08-1.98 (m, 1 H); ¹³C NMR (CDCl₃, 100 MHz) δ 155.1, 154.9, 152.3, 130.4, 126.5, 122.8, 51.8, 51.6, 51.4, 51.1, 44.0, 43.8, 39.2, 38.7, 25.8, 25.2; MS m/z 220 (90%, M+1), 118 (100%); IR (CHCl₃) ν 1716, 1428, 1205 cm⁻¹.

(-)-(3S,4S)-N-Phenoxy carbonyl-3,4-dihydroxy-piperidine 3a. Hydrolysis of (-)-2a (21.9 mg, 0.10 mmol) on a 20 ml scale gave 21.7 mg (91.6%) (-)-3a. Purity: 97.9 % (GC, tR = 10.30 min). ee: 96% (3S, 4S); [α]D²⁵ -4.05 (c 0.65, CHCl₃). ¹H NMR (CDCl₃, 300 MHz) δ 7.36 (t, 2 H, J = 7.8), 7.20 (t, 1 H, J = 7.5), 7.08 (d, 2 H, J = 7.8), 4.38-4.15 (m, 2 H), 3.56-3.47 (m, 2 H), 3.18-2.75 (m, 2 H), 2.52 (s, 2 H), 2.05-1.95 (m, 1 H), 1.60-1.55 (m, 1 H); ¹³C NMR (CDCl₃, 75 MHz) δ 153.6, 151.0, 129.2, 125.4, 121.6, 73.4, 72.9, 71.9, 71.5, 48.2, 48.1, 42.7, 42.4, 31.4. MS m/z 238 (100%, M+1); IR (CHCl₃) ν 3621, 3457, 1713, 1428, 1209 cm⁻¹.
Absolute stereochemistry of bioproducts. Transformation of bioproduct (+)-3a to (-)-(3R, 4R)-3,4-dihydroxypiperidine hydrochloride 7: A suspension of bioproduct (+)-3a (50.0 mg, 0.21 mmol) in MeOH (1 ml) and 6 N HCl (5 ml) was stirred at reflux for 2 h. pH was adjusted to 10 by addition of 12 N NaOH and the solvent was evaporated to dryness. The residue was treated with MeOH and purified by column chromatography on silica gel (Rf 0.18, CHCl3/MeOH/25%aq NH3H2O = 8:3:1) to afford 20.2 mg (81.8%) of 3,4-dihydroxy-piperidine. Part of this product (10.0 mg, 0.085 mmol) was dissolved in MeOH (2 mL), HCl (32%, 50µl) was added, and the resulting mixture was evaporated to dryness giving 11.5 mg (87.7 %) of (-)-7 as solid. [α]D25 –14.0 (c 0.50, MeOH). The spectroscopic data agree with those for (3R, 4R)-7 reported in ref. 12a. [α]D25 of a synthetic sample of (3R,4R)-725 was determined as –15.0 (c 0.24, MeOH).

Transformation of (−)-7 to (+)-(3R,4R)-N-benzyloxy carbonyl-3,4-dihydroxy-piperidine 3b. Phenyl chloroformate (0.050 ml, 0.397 mmol) in THF (2 ml) was added dropwise to a mixture of (−)-7 (10 mg, 0.085 mmol) and NaHCO3 (160 mg, 1.905 mmol) in THF/H2O (1:1, 4 ml) at rt, and the mixture was stirred for 20 h, and ethyl acetate (30 ml) was added. The organic phase was separated, dried over Na2SO4, filtered, and the solvent was removed by evaporation. Column chromatography on silica gel gave 15.2 mg (71.2%; 0.061 mmol) of (3R,4R)-3b. [α]D25 +3.51 (c 0.76, CHCl3); Rf 0.38 (ethyl acetate); purity: 97.9% (GC, tR = 9.53 min). The spectroscopic data agree with those reported in ref. 12b. The major enantiomer of bioproduct 3b has the same retention time as the chemically prepared (+)-(3R,4R)-3b.

Transformation of bioproduct (+)-6b to (−)-(3R, 4R)-3,4-dihydroxy pyrrolidine 8. A mixture of bioproduct (+)-6b (100 mg, 0.42 mmol) and 20% Pd(OH)2/C (55 mg) in MeOH (3.0 ml) was stirred under hydrogen for 24 h and filtered through celite. Column chromatography on silica gel afforded 8 in 93.9% (40.8 mg) as waxy solid. Rf 0.17, (CH2Cl2/MeOH/25%aq NH3H2O 8:3:1); [α]D25 –18.6 (c 0.80, MeOH). 1H NMR (D2O, 400 MHz) δ 4.03-4.01 (m, 2 H), 3.08 (dd, 1 H, J = 12.8, 4.4); 2.73 (d, 1 H, J = 12.8); 13C NMR (D2O, 100 MHz) δ 79.6, 54.6; MS (m/z): 104 (100%, M+1). [α]D26 of (3S,4S)-8 was reported to be + 20.7 (c 0.3, MeOH).26

Transformation of (-)-8 to (+)-(3R,4R)-N-phenoxy carbonyl-3,4-dihydroxy-pyrrolidine 6a. Similar to the transformation of (-)-7 to (+)-3b, reaction of (-)-8 with phenyl
chloroformate (0.050 ml, 0.397 mmol) in THF (2 ml) gave 14.0 mg (64.9%, 0.063 mmol) of (3R,4R)-6a as solid. mp 125.4-126.1°C;  [α]D25 +17.71 (c=0.70, MeOH); Rf 0.22 (ethyl acetate); purity: 97.8 % (GC, tR = 9.30 min); 1H NMR (CDCl3, 300 MHz) δ 7.36 (t, 2 H, J = 7.4), 7.20 (t, 1 H, J = 7.2), 7.12 (d, 2 H, J = 7.2), 4.22 (m, 1 H), 4.18 (m, 1 H), 3.86 (dd, 1 H, J = 12.1, 4.4), 3.76 (dd, 1 H, J = 12.1, 4.4), 3.58 (d, 1 H, J = 12.1), 3.50 (d, 1 H, J = 12.1), 2.13 (s, 2 H); 13C NMR (CD3OD, 75 MHz) δ 155.3, 152.5, 130.2, 126.4, 122.7, 76.2, 75.4, 53.3; MS (m/z): 224 (100%, M+1); IR (CHCl3) ν 3620, 3444, 1714, 1408 cm⁻¹. The major enantiomer of bioproduct 6a has the same retention time as the chemically prepared (+)-(3R,4R)-6a.

Acknowledgment: We thank Prof. Y. Ichikawa at Johns Hopkins University for providing an authentic sample of (3R, 4R)-7 and Prof. K. Engesser at University of Stuttgart for supplying us with the HXN-200 strain.
Reference


Chapter-6  Enantioselective trans-dihydroxylation of non-activated C-C double bonds


[25] An authentic sample of (3R, 4R)-7 prepared according to reference [12a] was given by Prof. Ichikawa, Y. at Johns Hopkins University.


Chapter 7

Purification and Partial Characterization of Cytochrome P450_{pyr} of *Sphingomonas* sp. HXN-200
Abstract

*Sphingomonas* sp. HXN-200 was found to contain an NADH-dependent soluble bacterial P450 enzyme that catalyses the regio- and stereoselective hydroxylation of non-activated carbon atoms. The soluble P450pyr was purified from its native bacterial host by the following procedures: a) 40-60% ammonium sulphate cut of cell-free extracts, b) hydrophobic interaction chromatography, and c) anion exchange chromatography. The purified P450pyr showed a single band on SDS-PAGE. Its’ molecular weight was established as 47.3KDa by MS, and the N-terminal amino acid sequences were determined by Edman degradation. The CO-reduced difference spectra of P450pyr had a maximum at 449nm, which represents the property of a typical P450 hemeprotein. The Component B is purified by a) anion exchange chromatography, and b) gel filtration chromatography. The molecular weight of component B is determined as 11.2KDa by MS. Enzyme activity was determined by using LC-MS with N-phenoxycarbonyl-piperidine as substrate. The purified P450pyr is not active by itself. However, combine with other components restore the activity of the monooxygenase as 118 U/g Protein.
Introduction

Regio- and stereoselective hydroxylation of non-activated carbon atoms remains one of the most impressive challenges in organic chemistry. However, it can be carried out with biocatalysis using molecular oxygen as oxidant\(^1\). These biotransformations are biologically catalyzed by monooxygenases. Among them, cytochrome P450s are the most prominent monooxygenases, and wide-spread in all kinds of organisms from bacteria to human beings. The cytochrome P450 (CYP) system is now known to constitute a superfamily of enzymes with a molecular weight of 45-55 kDa and iron porphyrin. Most P450 enzymes catalyze the oxidative transformation of a large number of endogenous and exogenous substrates by the insertion of an oxygen atom into a C-H bond or a C-C double bond\(^2\). Many P450 enzymes function to convert hydrophobic substrates to more hydrophilic derivatives that can be easily eliminated from the body. It has been recognized for their prominent role in the oxidative metabolism of pharmaceuticals and xenobiotic compounds in humans as well as biodegradation of environmental hazardous chemicals. A number of cytochrome P450s have been reported in recent years, including 207 bacterial forms separated in 36 families\(^3\). Some of them have been characterized during studies of the metabolism of biologically active macrolide ring molecules, such as the antibiotic erythromycin\(^4\), the anthelmintic avermectin\(^5\), and the anticancer epothilone\(^6\). Some bacterial P450 enzymes are involved in oxidative biotransformations of natural compounds such as phytotoxin thaxtomin\(^7\) and \(\alpha\)-terpineol\(^8\) or synthesized chemicals such as thio-carbamate herbicide\(^9\), ethyl tert-butyl ether pollutants\(^10\) and long-chain fatty acids\(^11\). Recently, P450cam, P450terp and P450BM3 were shown to catalyze the oxidation of alternative substrates, such as the oxidation of substituted thioanisoles to sulfoxide and the epoxidation of substituted styrenes\(^12\), with rather poor selectivity. The well-characterized camphor hydroxylase P450cam from *Pseudomonas putida* has been engineered to convert other substrates, including halogenated aromatic compounds such as alkylchlorobenzene\(^13\). Some P450s, such as P450cam\(^14\) and P450BM3\(^15\), have been purified, cloned, and overexpressed, their X-ray structures of some P450s are determined, and the insights into the catalytic mechanisms have been obtained. However, the synthetic applications of these P450 enzymes are rather limited, mainly due to the narrow substrate range, and the poor regio- and enantioselectivity.

Recently, we discovered that *Sphingomonas* sp. HXN-200, an alkane-degrading bacterium, is able to catalyze the highly active, regio- and stereoselective hydroxylation of remarkably
broad range of substrates, such as \( n \)-alkanes, cyclic alkanes, \( N \)-heterocycles, spiro-

oxazolidines, and benzoxazole derivatives\(^{16} \). It has been successfully used in the preparation

of several pharmaceutical intermediates such as (\( S \))- and (\( R \))- \( N \)-substituted 3-hydroxy-

pyrrolidines, (\( S \))-4-hydroxy-pyrrolidin-2-ones, (\( R \))-4-hydroxy-piperidin-2-ones, 3-hydroxy-

azetidines, 4-hydroxy-piperidines\(^{17} \). Here, we report the purification and characterization of

the soluble bacterial P450pyr hydroxylase from \textit{Sphingomonas} sp. HXN-200.

\textbf{Experimental sections}

\textbf{Materials}

Yeast extract and Bacto-tryptone were obtained from Difco. \( N \)-Phenoxycarbonyl piperidine

was synthesized as described previously\(^{17b} \). Sodium dithionate was purchased from Merck. All other chemicals used were purchased from Fluka. Phenyl Sepharose High Performance (hydrophobic interaction column material) and Source-15Q (strong anion exchange column material) was obtained from Pharmacia. Superformance\( ^{\circ} \) size exclusive column was purchased from Merck. Standard proteins for SDS (sodium dodecyl sulfate) gel electro-

phoresis were purchased from BioRad.

\textbf{Culture of Sphingomonas sp. HXN-200}

\textit{Sphingomonas} sp. HXN-200 was cultured aerobically in a 2.5-liter bioreactor containing

2.0 liters of the E2 medium, pH 7.1, at 30\( ^\circ \)C under agitation at 1500 rpm. Octane vapor was

supplied as the sole carbon and energy source. The pH of the growing culture was

automatically adjusted at pH 7.1 using 25\% ammonia solution. Cells were harvested by

centrifugation at a cell density of 4 g/L in exponential phase. The cell pallets were then stored

at -80\( ^\circ \)C until use.

\textbf{Enzyme activity assay}

The reaction mixture of P450pyr, other enzyme components which are obtained from

different chromatographic steps, 1 mM NADH and \( N \)-phenoxy carbonyl-piperidine (PCPI),

0.5ml 50 mM Tris-HCl buffer (pH 7.5), in a 1 ml falcon tube was incubated on a rotary shaker at

30\( ^\circ \)C for a predetermined time. The formed 4-hydroxy \( N \)-phenoxy carbonyl piperidine was quantified by using MS peak area of HPLC-MS chromatography in a Hewlett Packard LC-

MS 1100 instrument with a Macherey-Nagel Nucleosil 100-5 C18 pre-column with UV and MS detector. Flow: 0.5ml/min; Solvent A: 10mM ammonia acetate buffer containing 10\% of acetonitrile; Solvent B: acetonitrile. Gradient (Solvent A/B): 92/8(2min), 75/25(3min),
92/8(1min). MS (SIM mode): MSD 1, 206, EIC = 205.5-206.5; MSD 2, 222, EIC = 221.5-223.5.

**Determination of the protein concentration**

The protein concentration was determined as described by Bradford with bovine serum albumin as the standard. During purification of the P450pyr, protein concentration was determined by measuring the absorption at 595 nm.

![Standard curve of protein concentration](image)

**Cell disruption**

Frozen cell paste of *Sphingomonas* sp. HXN-200 were thawed and resuspended in 30 ml of 50 mM Tris-HCl, pH 7.5, containing 0.1 mM pefabloc, 1 mM DL-dithiothreitol, and 10% glycerol, with a cell density of 30 g/L. Lysis was carried out by pass cell suspension through a SLM AMINCO French pressure cell (3 times). The lysate was centrifuged (2.45×10^6 g) for 45 min to remove the cellular debris. 99% activity was found in supernatant. Protein concentration of the cell free extract was determined as 12 mg/ml. Compared with whole cell biotransformation with 2% glucose, 86.3% activity was obtained with cell free extracts.

**Purification control**

All purification procedures except hydrophobic interaction chromatography were carried out at 4°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to monitor the purification procedure, and determine the molecular. SDS-PAGE was performed in 12 or 15% polyacrylamide gel, and the protein bands were visualized by staining with Coomassie brilliant blue R-250.
The P450pyr monooxygenase is purified by a) 40-60% ammonium sulphate cut of cell-free extracts, b) hydrophobic interaction chromatography, and c) anion exchange chromatography. The Component B is purified by a) anion exchange chromatography, and b) gel filtration chromatography.

### Results and Discussion

#### Enzyme activity assay

The reaction mixture of P450pyr, other enzyme components, NADH and N-phenoxycarbonyl piperidine (PCPI) in 0.5ml 50 mM Tris-HCl buffer (pH 7.5), was incubated on a rotary shaker at 30°C for a predetermined time. The formed 4-hydroxy N-phenoxycarbonyl piperidine was quantified by HPLC-MS.

![Figure 1. LC-MS analysis of the enzyme activity](image)

**NADH dependence**: After incubating 2mM PCPI in 1ml cell free extracts for 30min, 31% conversion (without NADH) and 83% conversion (with 2mM NADH) was observed respectively. Obviously, the P450pyr is NADH dependent (Table 1).

<table>
<thead>
<tr>
<th>Conversion (%), Activity (U/g protein)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell debris with 2mM NADH</td>
<td>0.77%, 0.04*</td>
</tr>
<tr>
<td>Cell free extracts</td>
<td>18.3%, 1.02</td>
</tr>
<tr>
<td>Cell free extracts with 2mM NADH</td>
<td>72.7%, 4.04</td>
</tr>
<tr>
<td>Cell free extracts after storage in -20°C for 1 week</td>
<td>33.0%, 1.83</td>
</tr>
</tbody>
</table>

* The activity of the cell debris was determined as U/g CDW.
NaCl inhibition: As shown in Table 2, the activity of the P450pyr decreased with the increasing of NaCl concentration in Tris/HCl buffer. With 0.3M NaCl in the buffer, only 30% original activity was observed. The activity was tested with 1mM NADH and 0.6mM PCPI, shaking at 30°C, 30min. The P450pyr fraction employed in this experiment is taken from the 40-60% ammonia sulfate cut.

Table 2. NaCl inhibition

<table>
<thead>
<tr>
<th>NaCl concentration (M)</th>
<th>0.0</th>
<th>0.1</th>
<th>0.2</th>
<th>0.3</th>
<th>0.4</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity (U/g protein)</td>
<td>1.40</td>
<td>1.08</td>
<td>0.55</td>
<td>0.48</td>
<td>0.34</td>
<td>0.16</td>
</tr>
<tr>
<td>Conversion (%)</td>
<td>44.1%</td>
<td>34.0%</td>
<td>17.4%</td>
<td>15.0%</td>
<td>10.7%</td>
<td>5.2%</td>
</tr>
</tbody>
</table>

Relationship of the activity and the assay time: It was found that the enzyme activity is decreasing with extension of the assay time. Therefore, the time for enzyme activity assay should be less than 4 min in order to keep the linear catalytic ratio.

Comparison of the cells grown in LB medium with and without n-octane

It has been shown that the cells of HXN-200 grow in LB medium are not active for the hydroxylation of non-activated carbon atoms, but the ones induced by octane are active. As shown in Figure 3, compared with the cells of HXN-200 grown with LB medium, a new protein band with the molecule weight around 50KDa was observed with the cells grown on n-octane. This indicates the possible position of the monooxygenase in SDS-PAGE.
Chapter-7  Purification and Partial Characterization of Cytochrome P450pyr

Purification of P450pyr

(1) Ammonium sulfate precipitation

The soluble cell free extract was subjected to ammonium sulfate precipitation. Ammonium sulfate with predetermined quantity was then added to the cell free extracts. After 20 min incubation on ice, the sample was centrifuged at 14,000 RPM for 4 min at 4 °C. The precipitation was resuspended in 50mM Tris/HCl (pH = 7.5) with the same volume. As shown in Table 3, 95% activity was found in 40-60% precipitation.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>0%</th>
<th>30%</th>
<th>40%</th>
<th>50%</th>
<th>60%</th>
<th>70%</th>
<th>80%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precipitation Activity</td>
<td>0</td>
<td>0</td>
<td>0.04</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Supernatant Activity</td>
<td>2.78</td>
<td>5.22</td>
<td>5.19</td>
<td>1.94</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 3. Activity (U/g protein) of Ammonia sulfate precipitation

**Figure 3.** SDS-PAGE of ammonia sulfate precipitation

M: Marker; C8: Cell free extracts of HXN-200 grown on LB medium induced by octane; LB: Cell free extracts of HXN-200 grown on LB medium; Cf: Cell free extracts of HXN-200 grown on E2 medium induced by octane; 40P: 40% ammonia sulfate precipitation; 40-60: 40-60% ammonia sulfate precipitation; 60S: Supernatant of 60% ammonia sulfate precipitation.

Pre-test with the strong anion exchange column Source 15Q

The anion exchange chromatography with Source 15Q column shows rather high resolution, where the P450pyr system is separated as two different fractions (15Q_A and 15Q_B in Figure 4). Each of the two fractions is not active by themselves, but their
combinations restore the activity for the hydroxylation. The first fraction contains the P450pyr 50KDa protein band which could be the monooxygenase, and the second fraction contains the other components. The first fraction 15Q_A is therefore used for the activity assay in the purification of the component B, while the second fraction 15Q_B is employed in the determination of the active monooxygenase fractions.

15Q_A: P450pyr containing fraction from Source 15Q column with loading the cell free extracts of HXN-200; 15Q_B: Component B containing fraction from Source 15Q column with loading the cell free extracts of HXN-200; 33, 34, 35: Active fractions from hydrophobic interaction chromatography.

Figure 4. SDS-Page of active fractions from 15Q and HIC column

(2) Hydrophobic interaction chromatography

A 40-60% ammonium sulfate cut was loaded on to a Phenyl Sepharose hydrophobic interaction column equilibrated with 20 mM Tris/HCl buffer (pH 7.5) containing 1 mM DL-dithiothreitol, 10% glycerol and 1M-ammonium sulphate at 10°C. P450pyr containing fractions were then eluted with a linear gradient of ammonium sulphate from 1000 mM to 0 mM within 100 ml washing volume and another 50ml continuously elution with a flow rate of 1 ml/min. Fractions containing P450pyr were eluted out in 110-125 ml (33-35 in Figure 4) and determined with the enzyme assay described above.
Chapter-7  Purification and Partial Characterization of Cytochrome P450pyr

(3) Anion exchange chromatography

Fractions containing P450pyr were concentrated in a Millipore ultrafree centrifugal filter containing a BIOMAX 5k NMWL membrane. The concentrated fractions were then loaded on a SOURCE 15Q anion exchange columns equilibrated with 20 mM Tris/HCl buffer (pH 7.5) containing 1 mM DL-dithiothreitol and 10% glycerol. The protein was eluted with a linear NaCl gradient (0-500 mM NaCl) and collected in 2 ml fractions. The cytochrome P450pyr was eluted out in 27-35ml (15Q2-3 in Figure 7). Purified protein was stored at -20°C for later use.

Figure 5. Spectrum of FPLC with a Phenyl Sepharose hydrophobic interaction column

Figure 6. Spectrum of FPLC with a Source 15Q anion exchange column
Figure 7. SDS-Page of active fractions from 15Q column (15Q2, 3, 4, and 5 represent the active fractions from current anion exchange chromatography.)

### Purification table of P450pyr

<table>
<thead>
<tr>
<th>Protein Conc. [mg/ml]</th>
<th>Sample volume [ml]</th>
<th>Total protein [mg]</th>
<th>Specific Activity [U/g pro]</th>
<th>Total Activity [mU]</th>
<th>Yield [%]</th>
<th>Fold purification [x-fold]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>13.4</td>
<td>4.0</td>
<td>53.6</td>
<td>11.1</td>
<td>595.0</td>
<td>100</td>
</tr>
<tr>
<td>AS cut</td>
<td>4.3</td>
<td>4.0</td>
<td>17.2</td>
<td>32.3</td>
<td>555.6</td>
<td>93</td>
</tr>
<tr>
<td>HIC</td>
<td>0.52</td>
<td>20.0</td>
<td>10.4</td>
<td>45.6</td>
<td>474.2</td>
<td>80</td>
</tr>
<tr>
<td>IEC 15Q</td>
<td>0.07</td>
<td>2.0</td>
<td>0.14</td>
<td>118</td>
<td>16.5</td>
<td>2.8</td>
</tr>
</tbody>
</table>

**Determination of the N-terminal amino acid sequence**

The NH$_2$-terminal amino acid sequence of P450pyr enzyme was determined by automated Edman degradation method as MEHTG QSAAA TMPLD SIDVS I.

**Determination of molecular mass**

The molecular mass of the native P450pyr was determined by a MALDI-TOF MS as 47.3 KDa. The molecular mass of P450pyr was also calibrated under native condition with the reference proteins on a Merck Superformance size exclusive columns equilibrated with 20 mM Tris/HCl buffer (pH 7.5) containing 1 mM DL-dithiothreitol, 10% glycerol and 200 mM NaCl.
Purification of P450pyr Component B

(1) Anion exchange chromatography

The cell free extractions were directly loaded to a SOURCE 15Q anion exchange column equilibrated with 20 mM Tris/HCl buffer (pH 7.5) containing 1 mM DL-dithiothreitol, 10% glycerol, the protein was eluted with a NaCl gradient (1-1000 mM NaCl) and collected in 2 ml fractions. Component B was then eluted at 44-50 ml (22-23 in Figure 9) and then stored at -20°C.

Figure 8. Spectrum of FPLC with a Source 15Q anion exchange column
Cf: Cell free extracts of HXN-200 grown on E2 medium induced by octane; 15, 16, 17, 18: P450pyr containing fraction from Source 15Q column with loading the cell free extracts of HXN-200; 22, 23: Component B containing fraction from Source 15Q column with loading the cell free extracts of HXN-200.

Figure 9. SDS-Page of active fractions from 15Q column

(2) Size exclusive chromatography

After concentration with Millipore ultrafree centrifugal filter, the enriched component B was loaded onto a Merck Superformance size exclusive column equilibrated with 20 mM Tris/HCl buffer (pH 7.5) containing 1 mM DL-dithiothreitol, 10% glycerol and 200 mM NaCl. Component B was then eluted as a symmetric peak at 62-70 ml (B in Figure 11) and stored at -20°C.

Figure 10. Spectrum of FPLC with a Merck Superformance size exclusive column
Chapter 7  Purification and Partial Characterization of Cytochrome P450pyr

Figure 11. SDS-Page of active fractions from Gel Filtration column (B represents the active fractions from current gel filtration chromatography)

### Purification table of component B

<table>
<thead>
<tr>
<th></th>
<th>Protein Conc. [mg/ml]</th>
<th>Sample volume [ml]</th>
<th>Total protein [mg]</th>
<th>Specific Activity [U/g pro]</th>
<th>Total Activity [mU]</th>
<th>Yield [%]</th>
<th>Fold purification [x-fold]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>13.4</td>
<td>4.0</td>
<td>53.6</td>
<td>11.1</td>
<td>595.0</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>IEC 15Q</td>
<td>2.0</td>
<td>3.8</td>
<td>7.6</td>
<td>76.0</td>
<td>577.6</td>
<td>97</td>
<td>6.8</td>
</tr>
<tr>
<td>Gel Filtration</td>
<td>0.076</td>
<td>4.0</td>
<td>0.304</td>
<td>1613.7</td>
<td>490.6</td>
<td>82</td>
<td>145.4</td>
</tr>
</tbody>
</table>

### Determination of the N-terminal amino acid sequence

The NH\textsubscript{2}-terminal amino acid sequence of Component B was determined by automated Edman degradation method as MPTVTYV.

### MS of sAMO component B

Voyager Spec #1=>RSM2000 BP = 11233.8, 19637

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— 181 —
As shown above, the molecular mass of component B was determined by a MALDI-TOF MS as 11.2KDa.

**SDS-PAGE of the purified P450pyr and component B**

![SDS-PAGE Image]

**Spectral characteristics of cytochrome P450pyr**

![Spectral Characterization Image]

Figure 12. The CO difference spectra of P450pyr

UV-visible spectroscopy from the difference spectrum between samples of reduced P450pyr saturated with carbon monoxide and of the reduced P450pyr was examined. The
P450pyr was reduced by adding several grains of sodium dithionite (ca. 2 mg) into the enzyme solution, and the glass cuvette was then exposed to carbon monoxide (CO) for 2 min by gently bubbling the CO gas through the cuvette. The CO difference spectra (Figure 12), which were determined with a Varian CARY-1E UV-Visible spectrophotometer, shows a clear absorption at 449 nm, which revealed the typical characteristics of a bacterial P450. Native P450pyr showed an absorption peak at 422 nm in the absence of substrate.

In summary, P450pyr of *Sphingomonas* sp. HXN-200 was inducible with *n*-octane as the sole carbon source. The cell lysis procedure with a French pressure cell has a relatively good recovery of the activity, where 99% activity was found in the supernatant after ultracentrifuge. It was shown that the ammonium sulphate precipitation step (40%-60% cut) was necessary to remove large amount of other proteins. In the purification of P450pyr monooxygenase, the subsequent anion exchange chromatography with Source 15Q column after hydrophobic interaction chromatography did not give good recoveries: it appeared that the total activity of the enzyme lost a lot. However, the hydrophobic interaction chromatography still works as a powerful step. Combining hydrophobic interaction chromatography with anion exchange chromatography gives efficient purification of P450pyr. Combining anion exchange chromatography with size exclusion chromatography gives good separations of Component B as well. The purified P450pyr monooxygenase is not active by itself. However, combine with specific fractions obtained during the purification procedure restore the activity of the enzyme as 118 U/g Protein. The molecular mass of P450pyr enzyme was determined as 47.3 KDa by MS, and its UV absorption spectrum showed typical P450 hemeprotein properties.
Chapter-7 Purification and Partial Characterization of Cytochrome P450pyr

Reference


(3) http://cpd.ibmh.msk.su


Chapter 8

Substrate/Enzyme Interaction of P450pyr

— an *In Silico* study
Abstract

The variable active-site structure of P450 enzymes is the major modulators of substrate and product specificity. Even closely related P450s do not share all substrates and do not always lead to the same preferred hydroxylation selectivities. It is difficult to understand the differences in their specificities, since only a few three-dimensional P450 structures have been determined. Based on the X-ray structure of bacteria cytochrome P450terp, the homology model of P450pyr was established. The sequence alignment of P450pyr with the template was obtained with ClustalX and was used to generate the 3D homology model via automated protein homology modeling server – SWISS-MODEL. The homology model of P450pyr was then used to characterize substrate/enzyme complexes by docking the known substrates to the homology model with AutoDock program. The results allowed the identification of the preferred substrate bound conformation and its related crucial residues at enzyme active site. The calculated preferred hydroxylation site and the stereoselectivity were obtained for each substrate and were found to be consistent with experimental observation.
Introduction

Regio- and stereoselective hydroxylation of non-activated carbon atoms catalyzed by P450 enzymes play a key role in the biosynthetic pathways as well as in chemical synthesis. Cytochrome P450s is ubiquitous monooxygenase that can catalyze the insertion of oxygen into C-H bond or a C-C double bond. P450s are usually divided into soluble, like most bacterial P450s, and membrane bound monooxygenase. To date, X-ray structure of several bacterial P450s have been determined, such as P450cam, P450terp, P450BM3 and P450eryF. While P450cam catalyzes the first step of camphor metabolism to 5-exo-hydroxy-camphor, cytochrome P450terp catalyzes the key step of biodegradation of terpineol. Self-sufficient P450BM-3 was found to hydroxylate long-chain fatty acids at the ω-1, ω-2, and ω-3 position. P450eryF catalyzes the stereospecific 6(S)-hydroxylation of deoxyerythronolide B in the biosynthesis of erythromycin. Recent work has shown that P450cam, P450terp and P450BM-3 can also oxidize unnatural substrates, such as substituted thioanisoles and substituted styrenes, with relatively poor selectivity. The solved structure of these bacterial P450s shows conserved structure features such as a four-bundle helical core consisting of helices D, E, I, and L. The secondary structure exhibits a helix rich right side and a β sheets containing left side, with the wedged heme between these domains. Study of those soluble P450s has provided important information for the understanding of the catalytic mechanism and substrate enzyme interactions of P450 enzymes. The different substrate specificity of individual P450s, the hydrophobic property of the active sites, and the preference of hydrophobic substrates suggested that the interaction of substrates with P450 enzymes is dominated by hydrophobic and steric factors other than ionic interactions. A better understanding of the substrate/enzyme interaction could help the prediction of new substrates, regio- and enantioselectivity, and facilitate the substrate engineering as well as the protein engineering for the designed specificities.

Recently, we found that P450pyr from Sphingomonas sp. HXN-200 is able to catalyze the hydroxylation of a broad range of substrates, such as n-alkanes, cyclic alkanes, N-heterocycles, spiro-oxazolidines, and benzoxazole derivatives, with highly activity, high regioselectivity, and good to excellent enantioselectivity. The P450pyr has been purified and partially characterized, and the genes of P450pyr has been identified, sequenced, cloned, and overexpressed. However, the structural basis of substrate recognition is still unclear, since three-dimensional structure of P450pyr is still not available. Homology modeling approach
is therefore attractive for the investigation of P450pyr/substrates interaction. The basic premise of P450pyr homology modeling is that the bacterial P450s share a basic threedimensional structure\textsuperscript{11}. Although the sequence identity among P450cam, P450BM3, and P450terp is less than 30%, the structural similarity of all significant secondary structural elements is conserved across the available bacterial P450 structures. Homology modeling of the conserved core of P450s is therefore considered as a reliable approach even with lower identity between the targets P450 enzyme and the template\textsuperscript{12}. Here we report the homology modeling of P450pyr with the known structure of the bacterial P450terp as template, the substrate/enzyme interaction study using AutoDock program\textsuperscript{13}, and the prediction of regio- and stereoselectivity with known substrates.

Materials and Methods

*Sequence Alignment and Comparative Modeling* – For template searching, the P450pyr sequence was aligned with all the sequences at the National Center for Biotechnology Information (NCBI) with the BLAST program\textsuperscript{14}. The following P450 sequences and crystal structures came from Protein Data Bank\textsuperscript{15}: P450cam (1k2o), P450terp (1cpt), P450eryF (1jin), P450BM3 (1jpr).

*3D Model of Substrates and the Enzyme* – The 3D structures of P450pyr substrates were created and optimized with the ChemDraw and Chem3D Ultra modules of ChemOffice 2002. Partial charges for the substrates were then assigned by computing the Gasteiger charges with AutoDockTools. The rotatable bonds were defined using the AutoTors module of AutoDock 3.0. Since the conformational changes of the active site of the macromolecule with and without a ligand are rather small, the enzyme is assumed to be rigid, which could be more reasonable for the helix enriched P450 enzymes. Polar hydrogen atoms in P450pyr were added to enzyme by AutoDockTools program and assigned with Lennard-Jones 12-10 hydrogen bonding parameters. Partial charges were added by calculating the Kollman united atom charges. Atomic solvation parameters were assigned using AddSol program.

*Flexible Docking of Substrates into the Active Site of the P450pyr Model* – A Lamarckian genetic algorithm (LGA) with adaptive local search method is employed for the space search of the ligand\textsuperscript{16}. A grid-based method is used for energy evaluations, where the interaction energies, including van der Waals and electrostatic energies, in each grid point surrounding the enzyme are precalculated with the defined atom-atom types using AutoGrid 3.0 with 60 ×
60 × 60 points spaced at 0.375 Å distance. The grid was centered on the heme-Fe iron at the active site. AMBER force field\textsuperscript{17} was applied for the computation of nonbonded interaction energies. The Fe\textsuperscript{2+} iron parameters were the same as those described in the AutoDock website\textsuperscript{18}. All docking jobs were run on a Dell Precision 330 computer with Pentium IV 1.5 GHz CPU and 256MB memory running Mandrake 9.0.

**LGA parameters** – The size of the initial random populations were 50 individuals, the maximum number of energy evaluations was $2.5 \times 10^5$, the maximum number of generations was $2.7 \times 10^4$, the number of top individuals that survived for the next generation was 1, the mutation probability was 0.02, the crossover probability was 0.80, and the number of generations for picking the worst individuals was 10. The maximum iterations per local search was set to 300, the probability of performing a local search for an individual was set as 0.06, the maximum number of consecutive successes or failures before doubling or halving the step size was 4, and the parameter for the termination of the local search was 0.01 as the lower bound on the step size. 30 docking were performed in each docking run; the clustering tolerance of the root mean square positional deviation was 1.0 Å. The main goal of this study was to identify the bound conformation and its related crucial residues; therefore, a local search of the conformational space inside the active site cave was necessary. Thus, to minimize the meaningless search, the initial position of the ligand was placed in the active site by modifying the corresponding parameters in the ligand.enzyme.dpf file.
Results and Discussion

Sequence Alignment – The P450pyr was previously purified, and 27 N-terminal amino acids sequences were obtained. The gene of P450pyr monooxygenase was then identified and sequenced as 416 amino acid residues. Pairwise sequence alignments with BLAST revealed that the level of sequence identity between P450pyr and other P450s ranges from 62% with P450CB15, 28% with P450terp, 25% with P450eryF, 22% with P450cam, and no identity with P450BM3. Since the X-ray structure of P450CB15 is not available, the P450terp was used as template for the homology modeling study. The multiple-sequence alignment of P450s was obtained with ClustalX (Figure 1). This initial sequence alignment of P450pyr and P450terp was used to generate the three-dimensional homology model via the automated protein homology-modeling server – SWISS-MODEL. The heme-binding signature of P450pyr enzyme was found to be FGFGI HRCVG (AA359 - 368).

![Figure 1. The alignment of P450pyr sequence with its homologous sequences.](image)
Comparison of the homology model of P450pyr with the X-ray structure of P450cam, P450eryF, and P450terp reveals that all of these P450s are built with similar conserved “core structures” and the binding pocket of the P450pyr is larger than anyone else (Figure 2), giving rise to a broader substrate range for this enzyme.

![Comparison of P450 structures](image)

**Figure 2. Structures comparison of P450pyr with other known P450s**

Different from P450cam, in which no access channel to the catalytic pocket can be observed from the surface of the protein, two access channels to the catalytic heme site presents clearly in the homology model of P450pyr (Figure 3).
Figure 3. Identification of two substrate access channels (up: front view; down: top view)
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The overall surface of the P450pyr enzyme was calculated by Swiss-PdbViewer (Figure 4). The color represents the atom type: blue represents the nitrogen atom; red represents the oxygen atom; yellow represents the sulphur atom; and white represents the carbon atom. The enlarged heme pocket shows that the ferric iron locates in the corner of the heme pocket, which explains the high regioselectivity exhibited in the hydroxylation of the N-heterocyclic substrates with this enzyme. In the top viewed active site pocket, the right and down region, including Leu252, Ile102 and Phe403, exhibits a hydrophobic area. The Phe403, which located in the middle of this region, provides a hydrophobic barrier for the hydrophilic directing groups in substrates, such as the carbonyl group in the pyrrolidin-2-one substrates. A rather hydrophilic portion is shown in the left and top region of the heme active site, including Gly255, which may contribute to inducing the orientation of the hydrophilic group in the substrate. Moreover, the rather large cavity of the P450pyr active site affords the possibility for the binding of the substrate with bulky directing/protecting groups, which explains the broad substrate range of this enzyme.

Figure 4. The overall surface of P450pyr model and the close look of the heme pocket
The Rebound Catalytic Mechanism – Followed the accepted rebound mechanism, the mechanism of the P450pyr catalyzed hydroxylation reaction was suggested (Scheme 1).

![Scheme 1. The rebound mechanism of the P450pyr catalyzed hydroxylation](image)

The substrate is first bound to the catalytic pockets, which triggers the electron transfer from ferredoxin-type protein to high-spin ferric species 1, resulting in the formation of 2. Dioxide is then incorporated into the vacant heme site to generate the oxy intermediate 3. With the addition of the second electron, the ferric iron-peroxo species 4 is generated. The ferric iron-hydroperoxo species 5 is then formed by protonation of 4. Uptake of a second proton leads to the formation of iron-oxo species 6 which oxidize the substrate via a so-called ‘rebound’ step (TS2). Theoretical calculation revealed that the energy barrier for the rebound process is as lower as 1 kcal/mol. Compared with the 24 kcal/mol energy barrier for the C-H bond activation process (TS1), which is the rate-determining step, the rebound process is recognized as a barrierless step, resulting in experimentally high conserved stereoselectivity. Based on this mechanism, the preference of the modeled catalytic regio- and stereoselectivity are determined by the substrate binding to the catalytic pockets.

Substrate Docking – To study the substrate binding to the enzyme catalytic center, known substrates were docked to the enzyme by AutoDock program. The rotatable bonds in the substrates were not limited in the computing except the substrate N-benzoyl pyrrolidine where the limited rotation of the amide bond was observed in the NMR spectrum. The total docked
energy is calculated as the sum of the total nonbonded intermolecular interaction energy between each ligand atom and the P450pyr enzyme and the nonbonded intramolecular energy of the ligand. The substrate/enzyme complex model with the minimum docking energy was selected as the preferred substrate binding conformation. The clusters that docked outside the active site pocket of the P450 enzyme are ranked with computation but not considered for result evaluation.

Camphor and the P450cam are selected in order to validate the accuracy of the docking procedure. The docking of camphor to P450cam was performed with the above mention AutoDock parameter. The result of minimal energy conformation is in good consistence between the docked orientations with the X-ray binding conformation, as shown in Figure 5. The yellow molecule presents the camphor generated by the docking program.

Figure 5. Comparison of the docking result with the X-ray structure of P450cam with its natural substrate – camphor. (Left: front view; Right: back view)

Comparison of the Binding Conformation of N-benzyl pyrrolidine (BnPy) with and without Proton – In order to evaluate the influence of the nonpolar substrate proton in the docking study, both of the N-benzyl pyrrolidine substrates with and without proton were calculated. The two final docking conformations with minimal docking energy are nearly identical, demonstrating that the nonpolar protons in the substrates do not influence the docking results. Thus, in the following studies, nonpolar protons are omitted during the docking processes in order to facilitate the computation.
Figure 6. Docking of N-benzyl pyrrolidine to the P450pyr with and without proton

Substrate Binding Regio and Stereoselectivity of the hydroxylation – Nine representative substrates of P450pyr enzyme are chosen for the docking study. The biohydroxylation of these nine compounds catalyzed by the P450pyr are regio- and stereoselective, which enabled the characterization of this docking methodology with both regio- and stereoselectivity data.

It is showed that the amino acid Gly255, Leu252 and Ile102 serve as important residues for the substrates binding in the P450pyr active site. Nearly in all cases, the hydrophobic substitution of the substrates, such as the aromatic phenyl ring and the tert-butyl group, shows the interaction with the hydrophobic amino acid residue Leu252. In case of the pyrrolidine, this binding determined the orientation of the heterocyclic ring to the heme. In case of the pyrrolidin-2-one and piperidin-2-one substrates, the hydrophobic barrier generated by Phe403 strongly influences the binding flexibility of the carbonyl containing N-heterocyclic ring, and the hydrophilic residue - Gly255 induces the binding conformation of the carbonyl containing substrates in the heme pocket additionally. The space of the active site cavity is large enough
to allow the binding d/p group from smaller benzyl to the larger benzyloxy carbonyl group, including the bulky tert-butoxycarbonyl group.

Based on the ‘rebound’ catalytic mechanism, the shortest distance between the substrate carbon and the heme ferric iron\textsuperscript{25} was characterized as the preferred hydroxylation site for the evaluation of the docking results. Its corresponding preferences towards regio- and stereoselectivity of the P450pyr catalyzed biohydroxylation were summarized and the calculated value was presented in the subsequential pictures. The computed docking results, including preferred hydroxylation site and the stereoselectivity, have found to be consistent with experimental observations for all substrates except the N-tert-butoxycarbonyl pyrrolidine, where the calculated enantio preference gives the opposite enantioselectivity (Table 1).

The rather lower docking energy of the tert-butoxycarbonyl group containing substrates indicates a weak interaction of the bulky tert-butoxycarbonyl group with this region, which explains the lower enantioselectivity in the hydroxylation of N-tert-butoxycarbonyl protected pyrrolidine and pyrrolidin-2-one. One exception is the piperidin-2-one substrates where the energy-minimized conformation of the six-member head is not flat any more but rather chair shaped, this strongly influenced the enantioselectivity of the biohydroxylation, resulting in a dramatic decrease of the enantioselectivity in the hydroxylation of N-benzyl piperidin-2-one.
<table>
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<tr>
<th>Substrate</th>
<th>Experimental Regioselectivity</th>
<th>Preferred Enzyme-selectivity</th>
<th>Docking Energy (Kcal/mol)</th>
<th>Preferred Enzyme-selectivity</th>
<th>Docking Energy (Kcal/mol)</th>
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<td>-7.70</td>
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Table 1. Results of flexible docking of the investigated p450/Pyr substrates to the active site of the p450/Pyr homology model.
1) Binding conformation of $N$-benzyl pyrrolidine (BnPy):
2) Binding conformation of $N$-benzoyl pyrrolidine (BzPy):
3) Binding conformation of N-phenoxycarbonyl pyrrolidine (PCPy):
4) Binding conformation of $N$-benzyloxycarbonyl pyrrolidine (BCPy):
5) Binding conformation of \( N\text{-}tert\text{-}butoxycarbonyl \) pyrrolidine (BocPy):
6) Binding conformation of \(N\text{-tert-butoxycarbonyl pyrrolidin-2-one (BocPYO):}\)
7) Binding conformation of $N$-benzyl pyrrolidin-2-one (BnPYO):
8) Binding conformation of \( N\text{-}\text{tert-} \text{butoxycarbonyl piperidin-2-one (BocPO):} \)
9) Binding conformation of N-benzyl piperidin-2-one (BnPO):
10) Comparison of the binding conformation of pyrrolidines:
11) Comparison of the binding conformation of pyrrolidin-2-one:
12) Comparison of the binding conformation of piperidin-2-one:
Conclusions

A homology model of P450pyr was generated with the X-ray structure of P450terp as templates. The catalytic pocket is larger than P450terp, P450cam and easily accessible, indicating a broad substrate range. The heme is located in the corner of the active site pockets, suggesting an excellent regio- and enantioselectivity for the biohydroxylation. The ligand-binding conformations were established by a docking approach using AutoDock Program. The low-energy conformational models suggest that the Leu252, Ile102 and Phe403 are the important active site residues for the substrate binding. The docking analysis of the nine substrates shows that the most important factors in the selectivity for the P450pyr catalyzed hydroxylation are hydrophobic d/p group in the substrates, the type of the substrate ring, and the hydrophobicity and the geometry of the heme site. The predicted selectivities from the docking results are in good agreement with the experimental data and could provide useful information on P450pyr catalyzed hydroxylations. Key residues for hydroxylation have been identified and the docking approach with the homology model of P450pyr can be a useful tool to improve the enantioselectivity either by substrate engineering or protein engineering.
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Reference


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(9) Manuel bouza, unpublished data.


(15) http://www.rcsb.org/pdb/


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(18) http://www.scripps.edu/pub/olson-web/doc/autodock/faq.html#HEME


(20) http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html

(21) Due to the technical problem of the SwissModel server, the attempt of using multi-template in the construction of the P450pyr homology model failed.


Chapter 9

General Conclusion
Sphingomonas sp. HXN-200, an \(n\)-alkane-degrading bacterium, was found to catalyze the hydroxylation of a variety of \(N\)-heterocyclic compounds with high activity, high yield, excellent regioselectivity, and good to excellent enantioselectivity, representing by far the best monooxygenase among all known systems for these reactions. The whole-cell hydroxylations have been used for the preparation of several pharmaceutical intermediates.

Hydroxylations of \(N\)-substituted azetidines and piperidines with Sphingomonas sp. HXN-200 are highly effective and regioselective, giving the corresponding 3-hydroxy azetidines and 4-hydroxypiperidines in 91-98\%, respectively. High yield (70-83\%) preparations have been achieved by use of frozen/thawed cells as biocatalyst. Hydroxylations of \(N\)-tert-butoxycarbonyl azetidine and \(N\)-benzyl piperidine were carried out on 1 L scale affording hydroxylated products in 2.140 g and 2.072 g, respectively, providing a convenient and practical syntheses of 3-hydroxyazetidines and 4-hydroxypiperidines. It was also shown that lyophilized cells could be employed in hydroxylation without addition of cofactor.

For the first time, enantiopure (\(S\))-\(N\)-substituted 4-hydroxy-pyrrolidin-2-one was prepared by regio- and stereoselective hydroxylating pyrrolidin-2-ones with Sphingomonas sp. HXN-200. Hydroxylation of \(N\)-benzyl pyrrolidin-2-one and \(N\)-tert-butoxy carbonyl pyrrolidin-2-one afforded 68\% of (\(S\))-\(N\)-benzyl 4-hydroxy-pyrrolidin-2-one in > 99.9\% e.e. and 46\% of (\(S\))-\(N\)-tert-butoxycarbonyl 4-hydroxy-pyrrolidin-2-one in 92\% e.e., respectively. The e.e. of the latter compound can be increased to 99.9\% in 82\% yield with simple crystallization process.

High activity, excellent regio-selectivity, and moderate enantioselectivity were achieved in the hydroxylation of \(N\)-benzyl piperidin-2-one and \(N\)-tert-butoxycarbonyl piperidin-2-one with Sphingomonas sp. HXN-200. \(N\)-substituted piperidine-2-ones were hydroxylated by Sphingomonas sp. HXN-200, affording (\(R\))-\(N\)-benzyl 4-hydroxy-piperidin-2-one in 31\% ee and (\(R\))-\(N\)-tert-butoxy carbonyl 4-hydroxy-piperidin-2-one in 68\% ee respectively. The absolute configuration was established by chemical correlation. High yield preparations of the 4-hydroxy-piperidin-2-ones were demonstrated in a bioreactor and in a shaking flask by use of the frozen/thawed cells of HXN-200 as biocatalyst.
Sphingomonas sp. HXN-200 was found to contain a soluble epoxide hydrolase. Hydrolysis of N-benzyloxy carbonyl-3,4-epoxy-pyrrolidine and cyclohexene oxide with the epoxide hydrolase of Sphingomonas sp. HXN-200, gave the corresponding vicinal trans-diols in high ee and yield, representing the first example of enantioselective hydrolysis of a meso-epoxide with a bacterial epoxide hydrolase.

The strain of HXN-200 was able to catalyze the trans-dihydroxylation of N-substituted 1,2,5,6-tetrahydropyridines and 3-pyrrolines with high activity and enantioselectivity, affording the corresponding 3,4-dihydroxypiperidines and 3,4-dihydroxypyrrolidines, respectively. The absolute configurations of the products (+)-N-substituted 3,4-dihydroxypiperidines and (+)-N-substituted 3,4-dihydroxypyrrolidines were established as (3R, 4R) by chemical correlations. Preparative trans-dihydroxylation of N-phenoxy carbonyl 1,2,5,6-tetrahydropyridine and N-benzyloxy carbonyl 3-pyrroline with frozen/thawed cells of Sphingomonas sp. HXN-200 afforded the corresponding (+)-(3R, 4R)-3,4-dihydroxypiperidine and (+)(3R, 4R)-3,4-dihydroxy pyrrolidine in 96% ee both and in 60% and 80% yield, respectively. These results represent first examples of enantioselective trans-dihydroxylation with non-terpene substrates and with bacterial catalyst, thus extending this methodology in practical synthesis of valuable trans-diols.

The trans-dihydroxylation was sequentially catalyzed by a monooxygenase and an epoxide hydrolase in the strain with epoxide as intermediate. While both epoxidation and hydrolysis steps contributed to the overall enantioselectivity in trans-dihydroxylation of N-substituted 1,2,5,6-tetrahydropyridines, the enantioselectivity in trans-dihydroxylation of the symmetric substrate N-substituted 3-pyrrolines were generated only in the hydrolysis of meso-epoxide.

Enantioselective hydrolysis of racemic N-phenoxy carbonyl-3,4-epoxypiperidine gave 34% of (-)-N-phenoxy carbonyl-3,4-epoxypiperidine in >99% ee. Further hydrolysis of (-)-N-phenoxy carbonyl-3,4-epoxypiperidine with the same strain afforded (-)-(3S, 4S)-N-phenoxy carbonyl 3,4-dihydroxypiperidine in 96% ee and 92% yield. Thus, both enantiomers of N-phenoxy carbonyl 3,4-dihydroxypiperidine can be prepared by biotransformation with Sphingomonas sp. HXN-200.

Sphingomonas sp. HXN-200 was found to contain an NADH-dependent soluble P450 monooxygenase. The novel cytochrome P450pyr and the component B were purified from its
native bacterial host. P450pyr has a molecular weight of 47.3KDа, and N-terminal amino acid sequences were determined. The CO-reduced difference spectra of P450pyr had a maximum at 449nm. The molecular weight of component B is determined as 11.2KDа by MS. The purified P450pyr is not active by itself. However, combine with other components restores the activity.

The homology model of P450pyr was established based on the sequence identity and the known X-ray structure of bacteria P450terp. The homology model of P450pyr was then used to characterize substrate/enzyme complexes by docking the known substrates to the homology model with AutoDock program. The results allowed the identification of the substrate bound conformations and its related crucial residues at enzyme active site. The calculated preferred hydroxylation site and its stereoselectivity were found to be consistent with experimental observation.
Appendix
1) $^1$H-NMR (400MHz, CDCl$_3$) and $^{13}$C-NMR (100MHz, CDCl$_3$) spectra of bioproduct $N$-phenoxycarbonyl 3-hydroxyazetidine 13:
2) $^1\text{H-NMR}$ (400MHz, CDCl$_3$) and $^{13}\text{C-NMR}$ (100MHz, CDCl$_3$) spectra of bioproduct $N$-tert-butoxycarbonyl 3-hydroxyazetidine 14:
3) $^1$H-NMR (300MHz, CDCl$_3$) and $^{13}$C-NMR (75MHz, CDCl$_3$) spectra of bioproduct $N$-benzyl 4-hydroxypiperidine 20:
4) $^1$H-NMR (200MHz, CDCl$_3$) and $^{13}$C-NMR (50MHz, CDCl$_3$) spectra of bioproduct N-benzyloxycarbonyl 4-hydroxypiperdine 21:
5) $^1$H-NMR (200MHz, CDCl$_3$) and $^{13}$C-NMR (50MHz, CDCl$_3$) spectra of bioproduct $N$-phenoxycarbonyl 4-hydroxypiperidine 22:
6) $^1$H-NMR (300MHz, CDCl$_3$) and $^{13}$C-NMR (75MHz, CDCl$_3$) spectra of bioproduct $N$-tert-butoxycarbonyl 4-hydroxypiperidine 23:
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9) $^1$H-NMR (400MHz, CDCl$_3$) and $^{13}$C-NMR (100MHz, CDCl$_3$) spectra of chemically synthesized $N$-tert-butoxycarbonyl azetidine 12:
10) $^1$H-NMR (300MHz, CDCl$_3$) and $^{13}$C-NMR (75MHz, CDCl$_3$) spectra of chemically synthesized N-Phenoxy carbonyl piperidine 17:
Appendix for Chapter-3

1) $^1$H-NMR(300MHz, CDCl$_3$) spectrum of biocatalytically synthesized $N$-benzyl-4-hydroxy-pyrrolidin-2-one 7:
2) $^{13}$C-NMR(75MHz, CDCl$_3$) spectrum of biocatalytically synthesized $N$-benzyl-4-hydroxy-pyrrolidin-2-one 7:
3) $^1$H-NMR(300MHz, CDCl$_3$) spectrum of biocatalytically synthesized $N$-tert-butoxycarbonyl 4-hydroxy-pyrrolidin-2-one 9:
4) $^{13}$C-NMR(75MHz, CDCl$_3$) spectrum of biocatalytically synthesized $N$-tert-butoxycarbonyl 4-hydroxy-pyrrolidin-2-one 9:
Appendix for Chapter-4

1) $^1$H-NMR (400MHz, CDCl$_3$), $^{13}$C-NMR (100MHz, CDCl$_3$), and (H, H)-COSY spectra of bioproduct N-benzyl 4-hydroxypiperidin-2-one $3$: 
2) $^1$H-NMR (400MHz, CDCl$_3$), $^{13}$C-NMR (100MHz, CDCl$_3$), and (H, H)-COSY spectra of bioproduct $N$-tert-butoxycarbonyl 4-hydroxypiperidin-2-one 4:
3) $^1$H-NMR (400MHz, CDCl$_3$), $^{13}$C-NMR (100MHz, CDCl$_3$) spectra of chemically synthesized $N$-benzyl piperidin-2,4-dione 5:
1) $^1$H and $^{13}$C NMR spectra of bioproduct (-)-2a
2) $^1$H and $^{13}$C NMR spectra of bioproduct (+)-3a
3) $^1$H and $^{13}$C NMR spectra of bioproduct (-)-3a
4) $^1$H and $^{13}$C NMR spectra of bioproduct (+)-6b
5) $^1$H and $^{13}$C NMR spectra of chemically synthesized 1a
6) $^{1}H$ and $^{13}C$ NMR spectra of chemically synthesized 2a
7) $^1$H and $^{13}$C NMR spectra of chemically synthesized 3a
8) $^1$H and $^{13}$C NMR spectra of chemically synthesized 5a
9) $^1$H NMR spectra of chemically synthesized 6a
10) $^1$H and $^{13}$C NMR spectra of chemically synthesized (+)-3b
11) $^1$H and $^{13}$C NMR spectra of chemically synthesized (+)-6a
12) $^1$H and $^{13}$C NMR spectra of chemically synthesized (-)-7
13) $^1$H and $^{13}$C NMR spectra of chemically synthesized (-)-8
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