Propositions belonging to the thesis
“Enzymatic Reactions of Alcohols: Oxidation and Kinetic Resolution”
Yu-Xin Li

1. The terms “nonracemic”, “enantiomerically pure”, and “optically pure” are not equivalent although they are widely used as such.

2. The oxidation of water by the o xo ammonium cation derived from TEMPO is highly unlikely.

3. The mechanism proposed by Kawai et al., involving disproportionation of a phenoxy radical, for the laccase-catalysed oxidation of a lignin model compound, is unlikely.
   see also p. 15 of this thesis.

4. The conclusion that laccase does not accept 2,6-disubstituted benzyl alcohols as substrates is premature.

5. The preparation of (R)-2-aryloxypropionate herbicides from the tosylate of (S)-lactates is economically more attractive than from (S)-2-chloropropionate

6. The synthesis of carnitine published by Pellegata et al. starting from β-pinene, is unattractive both from an economical and environmental viewpoint. It is inconsistent with most of the 12 principles of Green Chemistry.

7. The living cell, where numerous conversions are performed in ‘one-pot’ without isolation of intermediates, is the best example of cascade catalysis.

8. The lengthy, bureaucratic procedures followed by the Dutch immigration authorities for granting work permit/entrance visa, are not consistent with the policy of Dutch universities to attract more foreign students and post-docs.

9. In a Chinese classroom the students listen to the teacher. In a Dutch classroom the students communicate with the teacher.

10. Invention is the mother of necessity.

These propositions are considered defensible and as such have been approved by the supervisor, Prof. R.A. Sheldon.
Stellingen bij het proefschrift
“Enzymatic Reactions of Alcohols: Oxidation and Kinetic Resolution”
Yu-Xin Li

1. De termen niet-racemisch, enantiomeerzuiver en optisch zuiver zijn niet equivalent ofschoon ze dikwijls zo worden gebruikt.

2. De oxidatie van water door het o xoammonium-kation van TEMPO is hoogst onwaarschijnlijk.

3. Het door Kawai et al. voorgestelde mechanisme voor de oxidatie van een lignine-modelverbinding, dat een disproportionering van een fenoxyradicaal inhoudt, is onwaarschijnlijk.

4. De conclusie dat 2,6-digesubstitueerde benzylalcoholen niet door laccase als substraat worden geaccepteerd, is voorbarig.

5. De bereiding van (R)-2-aryloxypropionaat-herbiciden uitgaande van het tosylaat van (S)-lactaat is economisch aantrekkelijker dan uitgaande van (S)-2-chloorpropionaat.

6. De synthese van carnitine, gepubliceerd door Pellegata et al. uitgaande van β-pineen, is zowel uit economische als milieutechnische overwegingen een uiterst onaantrekkelijke route. De route is in strijd met de meeste van de twaalf principes van de Groene Chemie.

7. De levende cel, waarin multistep-syntheses worden uitgevoerd in ‘één pot’ zonder scheiding van intermediaren, is het beste voorbeeld van cascade-katalyse.

8. De lange, bureaucratische procedures die door de Nederlandse Immigratiedienst worden gehanteerd inzake het verkrijgen van een werkvergunning/visum zijn niet consistent met de policy van Nederlandse universiteiten om meer buitenlandse promovendi en post-docs in dienst te nemen.

9. In de Chinese collegezaal luisteren de studenten naar de docent. In een Nederlandse collegezaal communiceren de studenten met de docent.

10. Uitvinding is de moeder van behoefte.

Deze stellingen worden verdedigbaar geacht en zijn als zodanig goedgekeurd door de promotor, Prof. R.A. Sheldon.
Enzymatic Reactions of Alcohols: Oxidation and Kinetic Resolution

Yu-Xin Li
Front cover: The traditional Chinese game – go. Designed by Yutian Li (Kevin)
Enzymatic Reactions of Alcohols:
Oxidation and Kinetic Resolution

Proefschrift

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus prof. dr. ir. J.T. Fokkema,
voorzitter van het College voor Promoties,
in het openbaar te verdedigen

op dinsdag 24 februari 2004 om 15.30 uur

don

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General Introduction

There is currently much interest in the use of biocatalysis in organic synthesis. Compared to conventional chemical synthesis, biocatalysis has many potential advantages: mild and environmentally friendly reaction conditions, high catalytic efficiency, and high stereo-, regio-, and chemoselectivity [Klibanov, 2001; Walsh, 2001]. Therefore, biocatalysis has been widely used by industry [Sheldon, 1993; Wandrey et al., 2000]. More than 100 biotransformation processes are currently carried out on an industrial scale, ranging from commodity chemicals to pharmaceutical and agrochemical precursors [Straathof et al., 2002; Thayer, 2001; Zaks, 2001; Schulze and Wubbolts, 1999].

The increasing interest in biocatalysis is driven by the need for safer, more targeted products (e.g. only the active enantiomer of a chiral drug to be produced) and more environmentally benign processes (e.g. less waste, less energy consumption). Recent progress in molecular biology, protein engineering and the use of high-throughput screening techniques [Wahler and Reymond, 2001; Bornscheuer and Pohl, 2001] are facilitating the commercial application of biotransformations.

The enzymes are classified into six classes according to the Enzyme Commission (EC) (Table 1). Table 2 shows the various enzyme types that are commonly used in organic synthesis [Koeller and Wong 2001].

<table>
<thead>
<tr>
<th>Enzyme class</th>
<th>Reaction type</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC 1. Oxidoreductases</td>
<td>Oxidation/reduction</td>
</tr>
<tr>
<td>EC 2. Transferases</td>
<td>Transfer of functional groups (acyl, sugar, phosphate, C1 units) from donor to acceptor</td>
</tr>
<tr>
<td>EC 3. Hydrolases</td>
<td>Hydrolysis (esters, amides, nitriles, epoxides, etc)</td>
</tr>
<tr>
<td>EC 4. Lyases</td>
<td>Cleavage of C-C, C-N and C-O bonds and the reverse reaction</td>
</tr>
<tr>
<td>EC 5. Isomerases</td>
<td>Racemisation, epimerization</td>
</tr>
<tr>
<td>EC 6. Ligases</td>
<td>Formation of C-C, C-O, C-SS, C-N bonds with consumption of ATP</td>
</tr>
</tbody>
</table>
**General introduction**

Table 2 Enzymes used in organic synthesis

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esterase, lipase</td>
<td>Ester hydrolysis and formation</td>
</tr>
<tr>
<td>Amidase (protease, acylase)</td>
<td>Amide hydrolysis and formation</td>
</tr>
<tr>
<td>Dehydrogenase</td>
<td>Oxidoreduction of alcohols and ketones</td>
</tr>
<tr>
<td>Oxidase</td>
<td>Oxidation</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>Oxidation, epoxidation, halohydration</td>
</tr>
<tr>
<td>Kinase</td>
<td>Phosphorylation (ATP dependent)</td>
</tr>
<tr>
<td>Aldolase, transketolase</td>
<td>Aldol reaction (C-C bond)</td>
</tr>
<tr>
<td>Glycosidase, glycosyltransesterase</td>
<td>Glycosidic bond formation</td>
</tr>
<tr>
<td>Phosphorylase, phosphatase</td>
<td>Formation and hydrolysis of phosphate</td>
</tr>
<tr>
<td>Sulfotransferase</td>
<td>Formation of sulfate ester</td>
</tr>
<tr>
<td>Transaminase</td>
<td>Amino acid synthesis (C-N bond)</td>
</tr>
<tr>
<td>Isomerase, lyase, hydratase</td>
<td>Isomerization, addition, elimination</td>
</tr>
</tbody>
</table>

Oxidoreductases and hydrolases are the two most prominent classes of enzymes. Oxidoreductases (EC 1) catalyse oxidations and reductions. They are able to catalyse the oxidation of hydrocarbons (alkanes, alkenes and aromatics) and a wide variety of functional groups. This class includes the dehydrogenases (hydrogen transfer), oxidases (electron transfer to molecular oxygen), oxygenases (oxygen transfer from molecular oxygen) and peroxidases (electron transfer to peroxide).

Hydrolases (EC 3) catalyse the (enantioselective) hydrolysis of various bonds, including esters, amides, nitriles, and epoxides. For example, esterases or lipases catalyse the hydrolysis of racemic esters to give optically active acids and/or alcohols. In general, esterases from microorganisms or animal sources are employed in the preparation of chiral acids (Scheme 1, reaction 1), while lipases are more often used for preparing chiral alcohols (reactions 2 and 3) [Roberts and Poignant, 2002]. Examples of various hydrolases used in synthetic biotransformations are shown in Table 3 [Sheldon, 2000].
General introduction

\[
\begin{align*}
R\cdot CO_2Me & \xrightarrow{\text{esterase}} H_2O & R\cdot CO_2H + MeOH & (1) \\
R\cdot OCOMe & \xrightarrow{\text{lipase}} H_2O & R\cdot OH + MeCO_2H & (2) \\
R^1CO_2R^2\ast & \xrightarrow{\text{lipase}} H_2O & R^1CO_2H + R^2\ast OH & (3)
\end{align*}
\]

* chiral center

Scheme 1 Esterase and lipase catalysed hydrolyses

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>EC Number</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Esterases, Lipases</td>
<td>3.1.1</td>
<td>RCO_2R'</td>
</tr>
<tr>
<td>Proteases (e.g. subtilisin, chymotrypsin)</td>
<td>3.4</td>
<td>RCONHR'</td>
</tr>
<tr>
<td>Amidases (e.g. penicillin acylase)</td>
<td>3.5.1</td>
<td>RCONHR'</td>
</tr>
<tr>
<td>Nitrilases</td>
<td>3.5.5</td>
<td>RCN</td>
</tr>
<tr>
<td>Glycosidases (e.g. β-galactosidase)</td>
<td>3.2</td>
<td>Glycosides</td>
</tr>
<tr>
<td>Epoxide hydrolase</td>
<td>3.3.2.3</td>
<td>Epoxides</td>
</tr>
<tr>
<td>Dehalogenases</td>
<td>3.8</td>
<td>RCI</td>
</tr>
</tbody>
</table>

Table 3 Hydrolases and their substrate scope

Aims and outline of this thesis

The general goal of the research described in this thesis has been to explore the enzymatic oxidation and kinetic resolution of alcohols, and thereby develop practical processes for the preparation of carbonyl compounds and enantiomerically pure alcohols and/or their esters from the corresponding alcohols, on the basis of high chemo- or enantio-selectivity, low environmental impact and low cost, and wide applicability. Consequently, this thesis consists of two parts: (i) the application of laccase (EC 1.10.3.2), a multi-copper containing polyphenol oxidase, in the oxidation of alcohols (Part I, chapters 1 to 4), and (ii) the use of lipase B from *Candida antarctica* (EC 3.1.1.3) in the preparation of enantiomerically pure secondary alcohols and/or their esters [Part II, chapters 5 to 8].

Previous work in our group [Dijksman, 2001] using Ru-TEMPO and Cu-TEMPO combinations for the aerobic oxidation of alcohols unraveled the unique role played by TEMPO as a hydrogen acceptor, which can be recycled by molecular oxygen [see
Chapter 1]. Inspired by this work, TEMPO was used in combination with a copper-containing enzyme to investigate if it would have a beneficial effect. Laccase was chosen because it is a cheap and readily available oxidase, with relatively broad substrate specificity. Indeed, laccase-TEMPO proved to be an effective combination, but the reaction mechanism seems to be different to that of the above-mentioned Cu/TEMPO system. The detailed studies on the process, including optimization of reaction conditions and mechanistic and kinetic investigations, are reported in chapters 2-4.

In the second part the synthesis of optically active secondary alcohols, including enantiomerically pure cyanohydrins, is reported, using CAL-B-catalysed kinetic resolution in the absence or presence of racemisation catalyst. A general introduction is described in Chapter 5. We have established a straightforward route to the preparation of cyanohydrin acetates, with high enantiomeric purities, by kinetic resolution. Our investigations on the dynamic kinetic resolution of cyanohydrins catalysed by CAL-B and ion exchange resin are presented in chapters 6 and 7. In Chapter 8 several new ruthenium catalysts are shown to be effective for the racemisation of secondary alcohols and compatible with CAL-B-catalysed kinetic resolution.

References

Sheldon, R.A. (2000) Personal communication, with permission
Part 1

Chapter 1

An Introduction to TEMPO and Laccase-Catalysed Oxidations of Alcohols

Abstract: Catalytic oxidation of alcohols can be achieved by abiological or enzymatic catalysis. TEMPO-catalysed oxidation of alcohols, with oxygen donors such as hypochlorite, is widely used in organic synthesis. The reactions can be performed under mild conditions with high selectivities and activities. TEMPO and its analogues can also mediate enzymatic reactions, such as the laccase-catalysed aerobic oxidation of alcohols. The application of laccases, a class of polyphenol oxidases, in the catalytic oxidations of alcohols and other non-phenolic compounds is relatively new. The primary substrates of laccases are phenols and arylamines. Alcohols, owing to their higher oxidation potentials, cannot be oxidised by laccases alone. However, with the help of mediators, e.g. TEMPO, laccase is able to catalyse the aerobic oxidation of non-phenolic compounds such as alcohols. The laccase-mediator system is, therefore, of interest in paper and pulp bleaching, textile dyes decolourisation, wastewater and soil treatment, and organic synthesis.
1. Introduction

The oxidation of primary and secondary alcohols to the corresponding aldehydes and ketones, respectively, plays an important role in organic synthesis [Sheldon and Kochi, 1981; Hudlicky, 1990]. Traditionally, such transformations have been performed with stoichiometric quantities of oxidants, such as chromium (VI) salts. These systems work well in small-scale reactions. On an industrial scale, however, they generate copious amounts of heavy metal-containing waste, which are harmful to the environment and require extra costs for their disposal. Therefore, the search for selective, more environmentally benign oxidation processes is of significant importance [Sheldon et al. 2002].

Chemo- or biocatalytic oxidations of alcohols, employing dioxygen or hydrogen peroxide as the terminal oxidant, constitute greener alternative methodologies. Roughly 400 alcohol oxidases have been identified, which act on the CH-OH group of donors [http://www.chem.qmul.ac.uk/iubmb/enzyme]. They catalyse the oxidative dehydrogenation of primary and secondary alcohols with dioxygen to form the corresponding aldehydes and ketones, respectively, with concomitant reduction of dioxygen to H₂O₂. One of the enzymes in this group is galactose oxidase (GO, EC 1.1.3.9), a copper-containing fungal metalloenzyme. GO catalyses the oxidation of primary alcohols or the 6-hydroxy group in galactose-containing carbohydrates to the corresponding aldehydes [Borman et al. 1999; Jazdzewski et al. 2000]. This enzyme exhibits a broad substrate specificity [Whittaker and Whittaker, 2001]. The alcohol oxidase (AO) from the methylotrophic yeast, Pichia pastoris, also showed a wide substrate specificity. Many unbranched primary alcohols, including propargyl alcohol, 2-chloroethanol, 2-cyanoethanol were effectively oxidised to the corresponding aldehydes [Dienys et al. 2003]. Vanillyl-alcohol oxidase (VAO, EC 1.1.3.38) can convert a wide range of 4-hydroxybenzyl alcohols and 4-hydroxybenzylamines into the corresponding aldehydes [van den Heuvel et al. 2001a; 2001b].

Among the various chemo-catalytic oxidations of alcohols, one of the most extensively studied and industrially relevant methods involves the use of TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) and its analogues, a class of stable N-oxyl radicals, in conjunction with hypochlorite as the terminal oxidant. Unfortunately, this method
suffers from serious drawbacks: the production of substantial amounts of inorganic salts and chlorinated byproducts caused by the use of hypochlorite. Consequently, there is a definite need for systems which employ dioxygen (or H₂O₂) as the terminal oxidant.

2. TEMPO and its analogues as oxidation catalysts

2.1. Properties and preparations of nitroxy1 radicals

TEMPO and its analogues are a class of stable nitroxy1 radicals with the general structure 1. Nitroxy1 radicals which have no hydrogen atoms at the α-carbons exhibit high stability. In contrast, when they contain a hydrogen on the α-carbon, they undergo facile disproportionation to form a nitrone and an N-hydroxylamine [Bowman et al. 1971].

\[ \text{R} \]

\[ \text{N} \]

\[ \text{O} \]

Stable nitroxy1 radicals have proven to be useful in a variety of applications, for instance, as spin probes in biological systems [Janzen, 1971; Keana, 1978] and as radical inhibitors [Riemenschneider, 1994; Han et al. 1999] or as catalysts in oxidation processes. The use of nitroxy1 radicals as oxidation catalysts has been reviewed [Hunter et al. 1984; Bobbitt and Flores, 1988; de Nooy et al. 1996], with emphasis on the oxidation of alcohols.

Nitroxy1 radicals can, in principle, be obtained by the oxidation of N-hydroxylamines, or secondary amines, or reduction of nitroso compounds [Rozantsev and Sholle, 1971a; 1971b]. The most convenient and commonly used method is the oxidation of secondary amines with hydrogen peroxide in the presence of a catalytic amount of sodium tungstate (Scheme 1) [Rozantsev, 1970]. 4-Oxo-2,2,6,6-tetramethylpiperidine (triacetoneamine), prepared by condensation of three equivalents of acetone with ammonia, is the most readily available starting material [Sosovsky and Konieczny, 1976].

Recently, the synthesis and application of a number of optically active nitroxy1
An introduction to TEMPO and laccase-catalysed oxidations of alcohols

radicals have been reviewed [Naik and Braslau, 1998].

Scheme 1 Synthesis of TEMPO and derivatives

2.2. Oxidation of alcohols mediated by TEMPO and its analogues

Nitroxyl radicals are oxidised, through one-electron transfer, to form the corresponding oxoammonium salts. The latter are strong oxidants capable of oxidising a number of functional groups [Bobbitt and Flores, 1988; de Nooy et al. 1996]. Oxoammonium salts oxidise primary and secondary alcohols to the corresponding carbonyl derivatives. The reactions are so fast that complete conversion can be obtained even in a few minutes [Anelli et al. 1987]. The oxoammonium salts are reduced to the hydroxylamines (Scheme 2). For simplicity, TEMPO is used as a representative nitroxyl radical in the following text unless specified.

Scheme 2 Oxidation of alcohols by oxoammonium salt
An obvious advantage of the stoichiometric use of oxoammonium salts is that there is no need for additional oxidant, which might interfere with the reaction. However, the drawback is that at least one equivalent of oxoammonium salt is needed [Bobbitt, 1998]. If additional acid scavenger is added, an excess of oxoammonium salt is necessary due to the loss of oxidant via syn proportionation (Scheme 3).

![Scheme 3 Syn proportionation between oxoammonium and hydroxylamine](image)

Scheme 3 Syn proportionation between oxoammonium and hydroxylamine

Most research on applying TEMPO in the oxidation of alcohols was focused on its use in catalytic quantities in combination with oxygen donors which act as the so-called primary or terminal oxidants. The most widely used protocol was introduced by Anelli et al. [1987] and uses bleach as the terminal oxidant and KBr as the co-catalyst. Other oxidants include m-chloroperoxybenzoic acid [Rychnovsky and Vaidyanathan, 1999], periodic acid [Kim and Nehru, 2002], N-chlorosuccinimide [Einhorn et al. 1996], and sodium bromite [Inokuchi et al. 1990]. When TEMPO was used with trichloroisocyanuric acid as the primary oxidant, aliphatic, benzylic and allylic alcohols and amino alcohols were rapidly and chemoselectively oxidised to carbonyl compounds [De Luca et al. 2001]. More recently, iodine was used as the terminal oxidant in TEMPO-mediated alcohol oxidations [Miller and Hoermer, 2003]. In these systems, TEMPO is used in catalytic amounts, typically 1 mol%. TEMPO-mediated oxidation of alcohols is characterized by mild reaction conditions, high chemoselectivity and high reaction rate. It exhibits a high preference for primary alcohols above secondary alcohols. In diols [Miyazawa and Endo, 1985; Siedlecka et al. 1990] or in mono- or polysaccharides [Chang and Robyt, 1996; Bragd et al. 2001], the primary hydroxyl groups are selectively oxidised. In some cases, however, TEMPO mediated oxidation of primary alcohols is accompanied by overoxidation to carboxylic acids [Anelli et al. 1987; Bragd et al. 2002].

The commonly accepted mechanism of TEMPO-mediated oxidation of alcohols by a terminal oxidant, e.g. hypochlorite, or by electrooxidation, is shown in Scheme 4. TEMPO is first oxidised by a terminal oxidant or at an electrode, via one-electron
An introduction to TEMPO and laccase-catalysed oxidations of alcohols

transfer, to form an oxoammonium ion. Then the alcohol is oxidised by the oxoammonium ion to give the corresponding aldehyde or ketone and the hydroxylamine (TEMPOH).

Scheme 4 TEMPO-mediated hypochlorite/bromide oxidation of alcohol

It is reported [de Nooy et al. 1996; Cecchetto et al. 2001] that under strongly acidic conditions (< pH 2), the oxoammonium cation is formed by acid-promoted disproportionation of TEMPO, and TEMPO is regenerated from the hydroxylamine by a terminal oxidant. Semmelhack et al. [1986] proposed the formation of an adduct 2, which undergoes a cyclic elimination to form the carbonyl compound. Ma and Bobbitt [1991], on the other hand, suggested elimination via an external base, as shown in 3.

On the basis of the steric effects on selectivity, de Nooy et al. [1995] suggested that adduct 2 was favoured under alkaline conditions whereas under acidic conditions the adduct 3 was favoured.

Recently, a mild and highly selective oxidation of primary and secondary alcohols to the corresponding aldehydes and ketones, respectively, with (acetoxyiodo)benzene in the presence of a catalytic amount of TEMPO was reported [De Mico et al. 1997]. The reactions were complete in a few hours at room temperature, without any noticeable
overoxidation to carboxylate. However, a drawback was the use of CH₂Cl₂ as the solvent and the need for stoichiometric amounts (1.2 eqn) of PhI(OAc)₂. Sakuratan and Togo [2003] have improved this system by using TEMPO together with poly(4-diacetoxyiodo-styrene). This reaction was performed in acetone, yielding aldehydes and ketones from the corresponding primary and secondary alcohols, respectively, without any overoxidation (Scheme 5).

![Scheme 5 Oxidation of alcohols by TEMPO-poly[4-(diacetoxyiodo)styrene]](image)

Another variation on this theme was reported by Bolm and co-workers [2000] who used TEMPO together with oxone as the primary oxidant. The mild reaction conditions of this system could even be applied in the presence of sensitive silyl protective groups. In the oxidation of methyl α-D-glucopyranoside, oxone (potassium monopersulfate) showed a reaction rate 25 times higher than that of peracetic acid. On the other hand, the former produced a larger amount of by-product [Bragd et al. 2002]. More recently, Bobbitt and co-workers [Merbouh et al. 2002] reported an efficient 4-acetamido-TEMPO-mediated oxidation of aldoses to aldaric acids, using elemental chlorine or bromine as the terminal oxidant. The use of elemental halogens as the terminal oxidants has several advantages compared with the use of bleach, such as the omission of the preparation of bleach from chlorine and increase in the volumetric efficiency. The amount of 4-acetamido-TEMPO was only 0.1 mol%.

In order to improve the stability and activity of TEMPO under various conditions, and for easy separation of products and recycling of catalytic systems, TEMPO may be immobilized by attaching to organic polymers [Miyazawa and Endo, 1988; MacCorquodale et al. 1990] or silicas [Tsubokawa et al. 1995; Bolm and Fey, 1999]. Fey and co-workers [2001] used silica-supported TEMPO in the hypochlorite oxidation of benzylic, aliphatic and cyclic alcohols, and the catalyst could be recycled for 10 runs. MCM-41 type mesoporous silica grafted [Verhoef et al. 1999; Brunel et al. 2001] and sol-gel entrapped TEMPO [Ciriminna et al. 2000] are also active, selective, and recyclable heterogeneous catalytic systems for the oxidation of alcohols with NaOCl as
An introduction to TEMPO and laccase-catalysed oxidations of alcohols

terminal oxidant. Dijksman et al. [2000] reported the use of a polymer immobilized TEMPO (PIPO), which was prepared from the commercial amine Chimassorb 944 (Scheme 6), for the oxidation of both aliphatic and benzylic alcohols by bleach. The conversion and selectivity were both above 99%. It was furthermore shown that PIPO gave faster rates of oxidation than the other immobilized forms of TEMPO referred to above.

![Scheme 6 Synthesis of PIPO](image)

TEMPO-catalysed electrochemical oxidation of alcohols has also been reported [Semmelhack et al. 1983; Deronzier et al. 1987; Xia and Li, 1997; Kishioka et al. 1998; Schnatbaum and Schafer, 1999]. The drawback of this method is the stoichiometric requirement for a base, which acts as the deprotonating agent for the alcohols [Kashiwagi, et al. 1996].

Contrary to the o xoammonium mechanism presented above, in Ru-TEMPO and Cu-TEMPO based systems another mechanism is operative. Dijksman et al. [2001] proposed the mechanism for the TEMPO/RuCl₂(PPh₃)₃ catalysed aerobic oxidation of alcohols which is shown in Scheme 7. In this mechanism, the alcohol undergoes dehydrogenation by the ruthenium catalyst, yielding the corresponding carbonyl compound and a ruthenium hydride. The TEMPO abstracts a hydrogen atom from the ruthenium hydride to form the ruthenium complex 4, in which a second molecule of TEMPO has undergone one-electron reduction to the nitrooxide anion. Replacement of the latter by alkoxide, followed by β-hydride elimination, affords the carbonyl compound and regenerates the ruthenium hydride catalyst. TEMPO is regenerated by spontaneous reaction of TEMPOH with molecular oxygen.
Scheme 7 Mechanism of TEMPO-ruthenium catalysed aerobic oxidation of alcohols

The insights obtained from the Ru-TEMPO system led to a re-interpretation of the Cu-TEMPO catalysed oxidation of alcohols reported by Semmelhack et al. [1984]. It turned out that in the latter system Cu-centered rather than oxoammonium based oxidative dehydrogenation of the alcohol takes place (Scheme 8) [Dijksman et al. 2003]. This mechanism was consistent with results obtained from kinetic isotope effects and Hammet correlation studies with a series of benzylic alcohols. The system can be regarded as a mimic for galactose oxidase, where a similar two-electron oxidation at a monocopper center takes place.

Scheme 8 Mechanism of CuCl/TEMPO catalysed aerobic oxidation of alcohols

Based on the mechanism in Scheme 8, we assumed that laccase, being a Cu(II)-containing enzyme (see later), in combination with TEMPO would be capable of oxidative kinetic resolution of secondary alcohols, if it involved a direct Cu-alcohol interaction. However, our preliminary results showed that although the oxidations of
both primary and secondary alcohols were surprisingly effective, no enantioselectivity was observed with secondary alcohols. As we shall show later, it turned out that laccase-TEMPO does not follow the mechanism depicted in Scheme 8.

During the course of our studies on laccase-TEMPO, Fabbrini et al. [2001] published their results on the use of laccase-TEMPO in the aerobic oxidation of benzylic alcohols. Earlier patents described the use of the combination TEMPO and laccase for carbohydrate oxidation [Viikari et al. 1999; Jetten et al. 2000]. However, in these studies, TEMPO and laccase were used in amounts as high as 30 mol% and 150 units per mmol substrate, respectively. Furthermore, many kinetic and mechanistic features of this system remain unclear. Our and their promising preliminary results stimulated us to perform further mechanistic and kinetic studies on the laccase-TEMPO combination with the aim of improving the practical utility of this system (see later).

3. Laccase-mediator system: an efficient tool for the oxidative transformation of non-phenolic compounds

Laccases are glycosylated multi-copper containing oxidases (\(p\)-dihydroxybenzene:dioxygen oxidoreductase, EC 1.10.3.2). They are widely distributed in higher plants (e.g., Rhus species Chinese or Japanese lacquer trees, peaches and tea leaves) [Keilin and Mann, 1939], fungi (e.g., Basidiomycetes Polyporus (Coriolus), Pleurotus and Pholiota, Ascomycetes Neuropora, Podospora and Aspergillus, Deuteromycetes) [Fahraeus et al. 1958; Benfield et al. 1964; Bollag and Leonowicz, 1984] and bacteria [Alexandre and Zhulin, 2000]. Laccase was first discovered in the white sap (latex) of trees in 1883 [Yoshida, 1883] and was named and characterized as a copper-containing oxidase ten years later [Bertrand, 1895]. In nature, plant laccases are implicated in the wound response of woody tissues [Bao et al. 1993], whereas fungal laccases are involved in lignin degradation [Ander and Erikkson, 1976].

Laccase catalyses the reaction of a phenolic substrate, such as lignin, by one-electron transfer, to give an aryloxy radical, accompanied by reduction of oxygen to water in a four-electron transfer process [Malmstrom, 1969]. The aryloxy radical is consequently converted \textit{via} a second one-electron oxidation to a variety of products, as illustrated for the lignin model compound (Scheme 9) [Kawai et al. 1988].
Scheme 9 Possible mechanism of degradation of phenolic β-1 model compounds by laccase of *C. versicolor*. (A) Cα oxidation, (B) Cα -Cβ cleavage, (C) alkyl-phenyl cleavage

In general, a laccase molecule contains four copper ions (with some exceptions where examples of laccase containing 5 or 6 coppers have been reported) [Reinhammar, 1984]. The four copper ions are classified into three types according to their spectroscopic properties: one type 1 (T1), one type 2 (T2) and a pair of type 3 (T3) coppers. T1 copper is coordinated with two histidines and one cysteine. T2 copper is coordinated with two histidines and one water molecule. Two T3 coppers are each coordinated with three histidines and bridged with hydroxide (Figure 1) [Bertrand *et al.* 2002a; 2002b].

Laccases have been reviewed in a number of publications, with regard to their enzymology and electron transfer mechanism [Messerschmidt, 1993; 1997; Solomon *et al.* 1996; ten Have and Teunissen, 2001], and spectroscopic properties and reaction kinetics [Reinhammar, 1984]. It is known that T1 copper exhibits extensive electron transfer at 610 nm, giving its blue colour to the enzyme. The electrons are abstracted from the substrate by T1 copper and then transferred to the T2/T3 cluster by the pathway shown in Scheme 10. T2 copper is required for dioxygen reactivity and the reduction of oxygen into water occurs at the T2/T3 trinuclear site [Sundaram *et al.* 1997; Palmer *et al.* 1999].
An introduction to TEMPO and laccase-catalysed oxidations of alcohols

Figure 1 Copper sites in laccase from Trametes versicolor, (a) T2/T3 cluster, (b) T1 site, adapted from [Bertrand et al. 2002b]

Scheme 10 Electron transfer from substrate to laccase

Laccase catalyses the aerobic oxidation of many kinds of aromatic compounds containing electron donating substituents, including o- and p-diphenols, aminophenols, polyphenols, polyamines, lignin and some arylidiamines. It can catalyse decarboxylation and demethoxylation reactions [Dec et al. 2001], e.g., 4-hydroxymandelic acid and 2-(4-hydroxyphenyl)glycine were readily converted into 4-hydroxybenzaldehyde by laccase at room temperature [Agematu, 1993]. Laccase can, in the presence of so-called mediators, also catalyse the oxidation of a range of non-phenolic compounds [Kawai et al. 1999a; 1999b; Srebotnik and Hammel, 2000], such as aromatic methyl groups [Potthast et al. 1995] polycyclic aromatic hydrocarbons (PAHs) [Collins et al. 1996], benzylic primary and secondary alcohols [Bourbonnais and Paice, 1990; Potthast et al.}
1996; Majchercky et al. 1999] and the primary hydroxyl group of carbohydrates [Viikari et al. 1999a]. The biotechnological applications of laccases, such as in pulp bleaching, decolourisation, removal of organopollutants from waste water, and organic synthesis, have attracted increasing interest from both academic and industrial sides [Mayer and Staples, 2002].

In the rest of this chapter the focus will be on the laccase-mediator system (LMS) catalysed transformation of non-phenolic compounds.

3.1 Laccase-mediator systems

Attempts to use the laccase isolated from white-rot basidiomycetes in pulp delignification by analogy with the natural biodegradation of wood by these laccase-containing fungi failed to achieve extensive delignification and brightening effect [Call and Mucke, 1997]. This is because in vivo, the laccase in white-rot basidiomycetes degrades lignins in cooperation with certain secondary metabolites. These small molecular metabolites can migrate from the enzyme to the lignocellulose complex and attack the lignin [Leonowicz et al. 2001]. The role of these metabolites was referred to as mediation. Laccase-catalysed oxidation of non-phenolic compounds is generally believed to be largely dependent on the redox potential of the copper(II) of the enzyme which can differ significantly depending on the source of the laccase. Since the highest redox potential of laccase is no more than 800 mV, laccases alone do not have sufficient reactivity towards unactivated substrates, which would require an oxidant with a higher redox potential [Li et al. 1999]. This problem can be circumvented by using laccases in combination with mediators [Fabbrini et al. 2002], thus broadening their applicability.

The first natural mediator of laccase, 3-hydroxyanthranilic acid (3-HAA, 6), was discovered by Eggert and co-workers in Pycnoporus cinnabarinus catalysed ligninolysis [Eggert et al. 1995; 1996]. The laccase-producing fungus P. cinnabarinus secretes a metabolite, 3-HAA, which was readily converted by laccase into cinnarbinic acid (7) through highly reactive intermediates. In the presence of 3-HAA, laccase isolated from P. cinnabarinus catalysed the oxidation of non-phenolic compound 8, which was not oxidised by laccase alone. Similarly, 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol, which are secreted by white-rot fungi as secondary plant metabolites, effectively mediated the oxidation of polycyclic aromatic hydrocarbons (PAH), known
Environmental pollutants, by *T. versicolor* laccase [Johannes and Majcherczyk, 2000].

2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, 9), the first synthetic and one of the most intensively investigated mediators, was shown already in 1990 to mediate laccase-catalysed oxidation of the non-phenolic lignin model compounds 10 and 11, which were non-oxidisable by laccases alone [Bourbonnais and Paice, 1990]. Veratryl alcohol (10) and the β-O-4 lignin model compound (11) were converted into the corresponding α-carbonyl compounds, using laccase-ABTS. The conversion of veratryl alcohol was above 90 % after 6 days, and of β-O-4 lignin model compound 12 % after 3 days. This was an important step in the utilisation of utilisation of laccase-mediatior systems for the oxidation of non-phenolics.

N-hydroxy compounds are another category of important synthetic mediators for laccase-catalysed biotransformations [Call, 1994]. The compounds in this group include N-hydroxybenzotriazole (HBT, 12), violuric acid (VLA, 13), N-hydroxyacetonilide (NHA, 14) [Call and Mucke, 1997, Xu et al. 2000], and (substituted) N-hydroxyphthalimide (NHPI, 15) [Fritz-Langhals and Kunath, 1998].
Chapter 1

However, HBT and VLA were found to deactivate the laccase, VLA to a greater extent than HBT [Li et al. 1999]. This deactivating effect of VLA on laccase has also been observed in the LMS-catalysed decolourisation of textile dyes [Soares et al. 2002]. It is likely that free radicals derived from the mediators react with the aromatic amino acids in the laccase [Li et al. 1999]. In the presence of veratryl alcohol or non-phenolic lignin model compounds, however, this deactivation of laccase by the mediator radical was suppressed [Bourbonnais et al. 1997]. This might be due to the more rapid reactions between these substances and the mediator radicals.

2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO) and its analogues (16–18) have recently been successfully used for improving the laccase-catalysed oxidation of alcohols or primary hydroxyl groups in carbohydrates [Jetten et al. 2000; Viikari et al. 1999b; Fabbrini et al. 2001; also see chapters 2, 3 and 4].

Recently, considerable synergistic effects of polyoxometalates in combination with laccases have been observed in the aerobic oxidation of hydrazonaphthalene type dyes [Carneiro et al. 2000] and in pulp delignification [Balakshin et al. 2001b], although the mechanism is not yet clear.

The usually accepted mechanism of laccase-mediator systems is presented in Scheme 11. The oxidised mediator is responsible for the oxidation of the substrate.

\[ \text{O}_2 \xrightarrow{\text{Laccase}} \text{Mediator}_{\text{ox}} \xrightarrow{\text{R-CH}_2\text{OH}} \text{H}_2\text{O} \xrightarrow{\text{Laccase}_{\text{ox}}} \text{Mediator} \xrightarrow{\text{R-CHO}} \]

Scheme 11 Redox cycle of oxidation of alcohols by laccase-mediator system
An introduction to TEMPO and laccase-catalysed oxidations of alcohols

A mechanistic study showed that ABTS was first oxidised by laccase to form a cation radical, which was further converted into its dication. Both the cation radical and dication were able to oxidise benzyl alcohols by abstracting a H-atom from the substrates (Scheme 12) [Majcherczyk et al. 1999].

\[
\begin{align*}
\text{H}_2\text{O} & \quad \text{O}_2 \\
\text{laccase-oxi} & \quad \text{laccase-red} \\
\text{ABTS} & \quad \text{ABTS}^+ & \quad \text{ABTS}^{2+} \\
\text{CH}_{3}\text{OH} & \quad \text{CHO} & \quad \text{CH}_{3}\text{OH} \\
\text{OCH}_3 & \quad \text{OCH}_3 & \quad \text{OCH}_3
\end{align*}
\]

Scheme 12 Oxidation of veratryl alcohol by laccase-ABTS

On the other hand, Bourbonnais et al. [1998] suggested that ABTS\textsuperscript{2+} was responsible for oxidising benzylc alcohols, whereas the cation radical can only oxidise phenolic compounds. Similarly,Potthast et al. [1996] found that no reaction was observed between the cation radical of ABTS and benzyl alcohol in the absence of laccase.

The oxidation of N-hydroxy compounds by laccase is similar to the oxidation of phenols [Xu et al. 2000]. It involves a single electron transfer as the initial oxidation step to form a nitroxy radical and a proton. The reaction rate is dependent on the redox potential difference between the laccase and the mediator. The radical derived from the N-hydroxy mediator is relatively stable and has sufficient potency to oxidise lignin in pulp.

TEMPO-mediated oxidation of alcohols by laccases was proposed to involve an oxoammonium cation as the active oxidant analogous to TEMPO-mediated chemical oxidations (vide supra, also see chapters 2-4). Baiocco et al. [2003] proposed that the oxidised mediator in Scheme 11 follows either an electron transfer (ET) or a radical hydrogen atom transfer (HAT) route of oxidation of the substrates. The laccase-HBT, laccase-NHPI and laccase-VLA follow the HAT mechanism, whereas laccase-ABTS favors an ET route. In contrast, laccase-TEMPO system would proceed through an ionic oxoammonium based mechanism [Fabbrini et al. 2002]. However, this issue is not
resolved and in chapters 2-4, the laccase-TEMPO will be further discussed.

3.2 Bioremediation by LMS

As discussed above, a variety of compounds, which are readily oxidised by laccases to form active intermediates, can be used as mediators to enable oxidation of a number of non-phenolic compounds, which are usually not oxidised by laccases alone. Therefore, laccase-mediator systems (LMS) have found wide applications in the biodegradation of non-phenolic compounds and in organic synthesis. In the presence of mediators, laccase has been used for demethylation and delignification of kraft pulp resulting in a substantial decrease in the pulp kappa value [Bourbonnais et al. 1992; 1996; Archibald et al. 1997; Balakshin et al. 2001a]. Compared to conventional pulp bleaching techniques, laccase-catalysed biobleaching requires much lower temperatures and pressures, and no toxic chlorinated organic compounds are generated.

In the textile industry, coloured effluents represent a major environmental problem. Many laccase-containing white rot fungi or purified fungal laccases are able to decolourise various industrial dyes [Rodriguez et al. 1999; Abadulla et al. 2002]. The decolourisation ability depends largely on the substrate structures and/or the laccase sources, as well as the enzyme preparations and conditions [Nyanhongoa et al. 2002]. The mediator also affects the decolourisation efficiency [Reyes et al. 1999; Soares et al. 2001].

The removal of phenols and polycyclic aromatic hydrocarbons (PAHs), which are present in waste waters from a number of industries, has been achieved successfully with laccase-catalysed transformations in the presence of mediators [D’Annibale et al. 1999, Duran and Esposito, 2000; Majcherczyk et al. 1998; Johannes et al. 1996]. It was supposed that the LMS oxidation of PAHs proceeds by several single electron transfer steps [Johannes et al. 1998]. The main oxidation products of PAHs are quinones [Rama et al. 1998]. In the laccase-catalysed transformation of chlorinated phenols, some phenolic acids were found to inhibit and some to enhance the oxidation efficiency. For instance, sinapinic acid enhanced the degradation of 2,4,5- and 2,4,6-trichlorophenols, 2,4-dichlorophenol and 4-chlorophenol, whereas ferulic acid and p-coumaric acids inhibited the degradation of 2,4,6-trichlorophenol, 2,3- 2,4- and 2,6-dichlorophenols and 2-chlorophenol [Itoh et al. 2000]. This was probably due to the different reactivities of the intermediates produced from the mediators by laccase.
3.3 Application of laccase in organic synthesis

In the presence of mediators such as HBT and NHPI, laccases can catalyse the aerobic oxidation of aromatic methyl groups or benzylic alcohols into the corresponding benzaldehydes [Potthast et al. 1995, 1996]. The reactions take place under mild conditions without further oxidation of the aldehyde products (Scheme 13). The benzyl alcohol formed in the procedure of Scheme 13 was further oxidised into the corresponding benzaldehyde. Allylic alcohols were selectively oxidised to aldehydes without oxidation of the double bonds [Fritz-Langhals and Kunath, 1998]. Clearly, LMS oxidation of alcohols has potential advantages over stoichiometric oxidations with e.g. dichromium salts or hypochlorite. The reaction is successful only with activated aromatics containing (multiple) electron-donating substituents as in the example shown in Scheme 13.

Scheme 13 Oxidation of activated methyl benzene by laccase-mediator

In addition to ABTS and HBT, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) and its 4-substituted analogues have been used for mediating the laccase-catalysed aerobic oxidation of carbohydrates [Jetten et al. 2000; Viikari et al. 1999a, 1999b] and benzylic alcohols [Fabbri et al. 2001; also see chapters 2, 3 and 4] to yield the corresponding aldehydes. This will be the subject of studies in chapters 2-4 of this thesis.

Other applications of laccase in organic synthesis include the Coriolus hirsutus laccase catalysed oxidative condensation of 3-methyl-2-benzothiazolinone hydrazone with 3-dimethylanilinebenzoic acid to form a blue indamine dye [Baker et al. 1996], and the Trametes versicolor laccase catalysed polymerization of α-naphthol [Aktas et al. 2000].
Chapter 2

Laccase-TEMPO Catalysed Aerobic Oxidation of Alcohols - Influence of Reaction Conditions

Abstract: The oxidation of a number of primary and secondary alcohols by laccase-TEMPO was investigated in aqueous buffer and in aqueous-organic solvents under varying reaction conditions. The optimum temperature was found around room temperature. The optimum pH was found at 4.5 - 4.8 when the reaction was performed in aqueous buffer, whereas in water-toluene system the reaction was independent of the aqueous pH. Laccase activity decreases in organic solvents. The water-miscible solvent 2-butanol was shown to be the best solvent for laccase activity preservation. Water-toluene was also a good solvent for laccase-TEMPO oxidation. However, in the ionic liquids C₄mimBF₄ and C₄mimDCA, laccase-TEMPO showed low activity.
1. Introduction

The oxidation of alcohols is of significant importance in organic synthesis. Numerous methods have been developed (see Chapter 1), ranging from the traditionally stoichiometric oxidations to catalytic systems using transition metals [Sheldon and van Bekkum, 2001]. Recently, environmentally benign "green" methods have drawn much attention [Sheldon et al. 2002; ten Brink et al. 2000]. As one of the "green" methods, enzymatic oxidation of alcohols is an interesting and active subject. The major reasons include the general advantages of enzymatic catalysis that high chemo- and stereo-selectivity can be obtained under mild reaction conditions. According to IUPAC, around 400 alcohol oxidases (EC 1.1) have been found capable of oxidising the hydroxyl groups of alcohols [http://www.chem.qmul.ac.uk/iubmb/enzyme], including the well documented copper-containing enzyme galactose oxidase (EC 1.1.3.9).

The use of laccases (benzenediol:dioxygen oxidoreductases; EC 1.10.3.2) for the oxidation of alcohols is relatively new, and has attracted increasing interest only recently [Bourbonnais and Paice, 1990]. This is because laccases are not alcohol oxidases, but polyphenol oxidases. The primary substrates of laccases are normally phenolic or analogous aromatic compounds containing electron donating substituents, including o- and p-diphenols, aminophenols, polyphenols, polyamines, aromatic amines, and lignins. The major sources of laccases are wood-decaying white-rot fungi [Fahraeus et al. 1958].

With the help of some oxidisable compounds, usually referred to as mediators, laccases can oxidise non-phenolic compounds. There are two reasons for employing mediators: one is that the macromolecular enzyme is too big to enter the wood cells to degrade the lignin. Therefore, a small molecule, i.e. mediator, which is oxidised by laccase to form an active oxidant in vivo, is used to enter and attack the interior of the tight lignocellulose, thus delignifying the wood tissues [Leonowicz et al. 2001]. Another reason for employing a mediator in vitro is based on the redox potential principle. Non-phenolic compounds usually have higher redox potentials than laccases. In other words, laccases have insufficient potential to oxidise these compounds. A proper mediator is readily oxidised by laccase to form an intermediate, which is able to oxidise the non-phenolic compounds [Call and Mucke, 1997]. Therefore, laccase-mediator systems have been extensively studied, for instance, in biobleaching in the paper and
pulp industry, dye decolourisation, food industry, and organic synthesis [Mayer and Staples, 2002; see also Chapter 1].

Up to now, most studies have been focused on the azino compound 2,2' azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS, I) [Bourbonnais and Paice, 1990] and the triazole compound N-hydroxy-1,2,4-benzotriazole (HBT, II) [Srebotnik and Hammel, 2000]. Only a few reports have been published on using 2,2,6,6-tetramethylpiperidinyl-1-oxo (TEMPO, III) as a mediator of laccase [Viikari et al. 1999a, Jetten et al. 2000, Fabbrini et al. 2001].

As TEMPO is widely used in the chemical oxidation of alcohols [de Nooy et al. 1996; see also Chapter 1], the use of TEMPO with laccase is of great interest as a catalyst for the aerobic oxidation of alcohols. However, the laccase-TEMPO system is only effective for the primary alcohol moiety in carbohydrates [Jetten et al. 2000] or primary benzylic alcohols [Fabbrini et al. 2001]. Moreover, the amounts of TEMPO and laccase used were quite high compared to substrate (30 mol% and 150 U/mmol substrate, respectively). No detailed reports regarding the effect of the reaction conditions on the efficiency of laccase-TEMPO oxidation of alcohols were available.

Here we report the use of laccase-TEMPO for the oxidation of both primary and secondary alcohols. The effects of various reaction parameters have been investigated with the aim of providing a sound basis for optimisation of these reactions.
2. Results and discussion

The effects of aqueous pH, reaction temperature, and reaction medium were studied, using benzyl alcohol and 1-phenylethanol as the model primary and secondary alcohol respectively. Laccase from *Coriolus versicolour* (CvL) was used as the benchmark enzyme. Laccases from other sources were also used for comparison. Preliminary experiments showed no oxidation products formed in the absence of either laccase, TEMPO or oxygen, confirming the combined effect of laccase and TEMPO. All reactions were carried out at room temperature under a molecular oxygen atmosphere. 0.1 M Acetate buffer at pH 4.5 was used as the aqueous medium.

2.1 Comparison of different laccase preparations

First, laccases from different sources and different preparations were compared in the oxidation of benzyl alcohol in acetate buffer (Table 1) in the presence of TEMPO. The laccase from *Coriolus versicolour* (CvL) was shown to be more active than the other preparations. Therefore we chose CvL as the benchmark enzyme.

<table>
<thead>
<tr>
<th>Laccase</th>
<th>Conv (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Coriolus versicolour</em> laccase</td>
<td>74.4</td>
</tr>
<tr>
<td></td>
<td>81.9 b</td>
</tr>
<tr>
<td><em>Trametes villosa</em> laccase</td>
<td>36.1</td>
</tr>
<tr>
<td></td>
<td>45.6 b</td>
</tr>
<tr>
<td>NS 51002</td>
<td>41.1</td>
</tr>
<tr>
<td>NS 51003</td>
<td>1.6</td>
</tr>
<tr>
<td><em>Pycnoporus cinnabarinus</em> laccase</td>
<td>10.9</td>
</tr>
</tbody>
</table>

* [Sub]: 140 mM, Lac/Sub: 25 U/mmol, TEMPO/Sub: 15 mol%, Acetate buffer (pH4.5), 4 h, O₂; b Lac/Sub: 47 U/mmol, TEMPO/Sub: 15 mol%, Water-toluene (1:1), 24 h
2.2 Influence of aqueous pH

Laccase activity is pH dependent. The optimum pH for laccase from Trametes (Coriolus) is reported to be in the range of 3.0 - 7.5, depending on enzyme sources and the properties of substrates [Call and Mucke, 1997]. In our system, i.e. CvL/TEMPO-catalysed oxidation of 1-phenylethanol in acetate buffer (AB), the conversion of the alcohol at pH 4.8 was faster than at pH 3.5 and 7.0 (Fig 2). This is probably because at higher pH values laccase becomes inhibited due to deprotonation of groups in the active sites [Reinhammar, 1984]. It was similarly observed by Naqui and Varfolomeev [1980] that at neutral pH, Polyporus versicolor laccase activity decreased compared with the activity at pH 4.5.

Figure 2 Influence of aqueous pH on oxidation of 1-phenylethanol by CvL-TEMPO. [Sub]₀: 125 mM, Lac/Sub: 47 U/mmol, TEMPO/Sub: 15 mol%, O₂, r.t.

2.3 Influence of temperature

The dependence of laccase activity on temperature is related to both enzyme sources and the reaction systems. It is reported that the acceptable temperature for laccase from Pleurotus ostreatus ranges from 25°C to 60°C, with an optimum of ca. 50°C and rapid loss of activity above 60°C [http://www.tienzyme.com/laccase.htm]. In C. versicolor laccase catalysed biobleaching by mediation of 1-hydroxybenzotriazole (HBT), however, the optimum temperature is 40 °C [Balaskin et al. 2001a]. In our CvL-TEMPO system,
Laccase-TEMPO catalysed oxidation of alcohols: influence of reaction conditions

the highest catalytic efficiency (based on the conversion of substrate) was obtained at around room temperature (20-30 °C) (Fig 3) in either aqueous buffer, or an aqueous-toluene (W-T) biphasic system. At temperatures below 10 °C or above 40 °C, the activity of CvL-TEMPO decreased sharply. It is likely that at higher temperatures the enzyme is denatured and at lower temperatures the enzyme is not active.

![Graph showing relative activity vs temperature](image)

**Figure 3** Influence of temperature on CvL-TEMPO oxidation of benzyl alcohol. W-T: water-toluene (1:1); AB: acetate buffer (pH 4.5)

2.4 Influence of concentrations of substrate, TEMPO, and laccase

The effects of concentrations of substrate, laccase and TEMPO were investigated by using benzyl alcohol as the substrate. The conversions were obtained from GC analysis after 4 h of reaction. The amount of laccase is expressed as activity units per mmol of substrate (Lac/Sub, U/mmol). The amount of mediator (TEMPO) is expressed as the mole percent of mediator to substrate (Med/Sub, mol%)

The results are shown in Figures 4 to 6. The conversion of benzyl alcohol increases linearly with increasing concentration of substrate and the amount of mediator in the investigated ranges. Increasing the amount of laccase also increases the conversion, but the trend is not simply a linear relationship. The order in substrate is investigated in more detail in Chapter 4; for 1-phenylethanol the order was determined as 1.
Figure 4 Influence of initial concentration of benzyl alcohol on its conversion by CvL-TEMPO catalysis. Lac/Sub: 25 U/mmol, Med/Sub: 10 mol%, r.t. O₂

Figure 5 Influence of mediator amount on conversion of benzyl alcohol by CvL-TEMPO catalysis. [Sub]₀: 140 mM, Lac/Sub: 25 U/mmol, r.t. O₂

Figure 6 Influence of laccase amount on conversion of benzyl alcohol by CvL-TEMPO catalysis. [Sub]₀: 140 mM, Med/Sub: 10 mol%, r.t. O₂
2.5 Enzyme stability in the laccase-TEMPO system

The stability of laccase in the presence of TEMPO was checked using two methods (see the experimental). Method A is to reuse the aqueous solution after benzyl alcohol was oxidised by laccase-TEMPO. In the second cycle of the laccase solution without extra addition of TEMPO, the conversion of substrate decreased from 100 to 26%. In the third cycle when an extra TEMPO (10 mol%) was added, the conversion was 24% (Table 2). Since there was no oxidation reaction in the absence of laccase, we conclude that the laccase activity was only 24% of the initial value.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Conv (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 (^a)</td>
</tr>
<tr>
<td>1</td>
<td>26 (^b)</td>
</tr>
<tr>
<td>2</td>
<td>23.7 (^c)</td>
</tr>
</tbody>
</table>

[Sub]: 140 mM, 24 h; \(^a\) Lac/Sub: 25 U/mm, Med/Sub: 10 mol%; \(^b\) No addition of TEMPO; \(^c\) TEMPO 10 mol% was added

In method B, laccase was first incubated with TEMPO in aqueous buffer at room temperature in the presence of oxygen for a certain time prior to use in the oxidation of benzyl alcohol. The conversion was much lower than without pre-incubation (Fig 7). Hence, we conclude that reaction between laccase and TEMPO decreases the activity of this system. Presumably the oxoammonium cation, formed by laccase catalysed oxidation of TEMPO, oxidises amino acid residues that are essential for activity or glycosyl moieties, resulting in loss of activity.

Figure 7 Effect of pre-incubation of laccase with TEMPO on oxidation of benzyl alcohol.
[Sub]: 140 mM, Lac/Sub: 25 U/mm, Med/Sub: 10 mol%, 4 h, r.t. O₂
2.6 Laccase catalysed oxidations in aqueous-organic solvents

The use of enzymes in organic solvents is of importance in organic synthesis, because of the poor solubility of many organic compounds in water. Our initial motive to study the influence of organic solvents on laccases was prompted by the successful use of lipases in non-conventional solvents [Klibanov, 2001] and based on the fact that most of the substrates of laccases have poor solubility in water. Although the effect of organic solvents has been investigated with both native and immobilized laccases [Milstein et al. 1994; Rogalski et al. 1995; Mai et al. 1999; Rodakiewicz-Nowak 2000], there are no reports available regarding the use of laccase-TEMPO systems in organic solvents under practical conditions.

Therefore, we investigated the effect of organic solvents on laccase-TEMPO catalysed oxidation of alcohols. For a better understanding of the influence of organic solvents on laccase activity, the oxidation of ABTS in the presence of water-miscible organic solvents was studied.

2.6.1 Laccase activity in the oxidation of ABTS in water containing organic solvents

ABTS is readily oxidised by laccase to form the ABTS cation radical via electron transfer. On further oxidation the dication of ABTS is obtained (Scheme 1) [Majcherczyk et al. 1999]. Both the cation radical and the dication have high redox potentials, 0.680 V and 1.09 V (NHE), respectively. Therefore, ABTS has been widely investigated as an efficient mediator of laccase for pulp delignification [Bourbonnais et al. 1997] and oxidation of benzylic alcohols [Potthast et al. 1996]. The dark green cation radical of ABTS has a maximum absorbance at around 420 nm. This reaction has also been used as a laccase activity assay.
Laccase-TEMPO catalysed oxidation of alcohols: influence of reaction conditions

\[ \text{ABTS} \xrightarrow{+e} \text{ABTS}\textsuperscript{+} \xrightarrow{-e} \text{ABTS}\textsuperscript{2+} \]

Scheme 1 Formation of cation radical and dication from ABTS

It is known that the presence of organic solvents usually affects the stability, selectivity, and activity of enzymes through direct or indirect interactions with enzyme molecules. In general, the catalytic activity displayed by enzymes in organic solvents is lower than in water [Klibanov, 1997]. Rodakiewicz-Nowak et al. [2000] investigated the effects of solvents on P. radiata laccase (PRL) and P. oryzae laccase (POL) catalysed oxidation of 2,6-dimethoxyphenol and syringaldazine. The conclusion was that solvents behaved like inhibitors. The inhibitory effects decreased with increasing solvent hydrophobicity. Khmelnitsky et al. [1991] also showed that the denaturation ability of organic solvents depends more or less linearly on log P (partition coefficient between octanol and water) values of the solvents.

We have measured the laccase activity in the oxidation of ABTS in aqueous buffer containing water-miscible solvents. As shown in Fig 8, organic solvents indeed deactivate the laccase, probably by denaturing the protein. The highest activity was obtained in the absence of organic solvents. The activity of the laccase decreased with increasing amounts of organic solvent. Laccase was highly deactivated when organic solvent was used in 50 % (v/v) in each case. In 1,2-dimethoxyethane (DME), the laccase activity dropped very fast. Even when only 10 % of DME was used in aqueous buffer,
the laccase was almost completely deactivated. This indicates that DME has a strong inhibitory effect on laccase activity. This might be due to an interaction between DME and laccase to form a complex, which changes the active conformation of the enzyme molecule, thus deactivating the enzyme [Uhing et al. 1981; Bertrand et al. 2002a].

Figure 8 Relative reactivity of laccase in the presence of aqueous organic solvents

Among the solvents tested, t-butanol showed the highest activity preservation when the volume of the solvent in the solution is no more than 30 %. In 30 % aqueous t-butanol, laccase activity remained around 60 %, whereas in acetone or acetonitrile, the activity was only around 20 % of the initial value.

The apparent kinetic constants ($K_m$ and $V_{max}$) of CvL were determined in acetate buffer (AB) containing 10 % (v/v) of water-miscible organic solvents (Table 3). The correlation between Log $P$ and $V_{max}$ (Figure 9) also showed that the deactivation of laccase decreased with increasing hydrophobicity of organic solvent (except for DME).
Table 3 Kinetic parameters of CvL (on oxidation of ABTS) in the presence of organic solvents

<table>
<thead>
<tr>
<th>Solvent (10% v/v) in AB</th>
<th>K_m (mM)</th>
<th>V_max (μmol min^{-1} mg^{-1})</th>
<th>Log P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No organic solvent</td>
<td>0.0581</td>
<td>1.3770</td>
<td></td>
</tr>
<tr>
<td>t-Butanol</td>
<td>0.1085</td>
<td>0.8077</td>
<td>0.509</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>0.0642</td>
<td>0.5910</td>
<td>-0.34</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.0743</td>
<td>0.5465</td>
<td>-0.24</td>
</tr>
<tr>
<td>DMSO</td>
<td>0.1567</td>
<td>0.6331</td>
<td>-1.350</td>
</tr>
<tr>
<td>DME</td>
<td>0.0678</td>
<td>0.0592</td>
<td>-0.440</td>
</tr>
<tr>
<td>DMF</td>
<td>0.1964</td>
<td>0.4768</td>
<td>-1.01</td>
</tr>
<tr>
<td>THF</td>
<td>0.1521</td>
<td>0.6761</td>
<td>0.327</td>
</tr>
</tbody>
</table>

\(^a\) Scifinder 2002 version; \(^b\) Bollag, 1992

Figure 9 Correlation between the activity of laccase (V_max) and the hydrophobicity of organic solvent (Log P). A: DMSO, B: DMF, C: DME, D: Acetone, E: Acetonitrile, F: THF, G: t-Butanol

In the presence of ionic liquids (IL) C_4mimBF_4 and C_4mimDCA, we failed to detect any laccase activity, because ABTS was not oxidised by laccase under the used conditions even when only 10% (v/v) of the IL was present in the aqueous solution. This analytical method is not applicable to biphasic water-toluene (W-T) systems, because they are not homogeneous.
2.6.2 Laccase-TEMPO-catalysed aerobic oxidation of alcohols in water-organic solvents

Based on the results of laccase activity in the oxidation of ABTS in aqueous organic solvents, we investigated the catalytic effect of laccase-TEMPO on the aerobic oxidation of alcohols in aqueous acetate buffer containing water miscible solvents t-BuOH and acetonitrile. We also investigated the use of the ionic liquids [bmim][BF₄] and [bmim][DCA] and water-immiscible toluene and MTBE as co-solvents, although the activity of laccase in these systems could not be determined by UV spectroscopy. The results are shown in Figure 10.

![Conversion of 1-phenylethanol by laccase-TEMPO in different media](image)

**Figure 10** Conversion of 1-phenylethanol by laccase-TEMPO in different media. [Sub]₀: 140 mM, Lac/Sub: 25 U/mmol, TEMPO/Sub: 15 mol%, 24 h. AB: acetate buffer, PB: phosphate buffer

* [Sub]₀: 125 mM, Lac/Sub: 47 U/mmol

As was observed in the oxidation of ABTS, t-butanol was the best co-solvent for preserving the laccase-TEMPO activity, although acetonitrile, MTBE, and toluene also gave good conversion of alcohol. In the presence of ionic liquids, the laccase-TEMPO system exhibited only poor activities.
2.6.3 Laccase-TEMPO catalysed aerobic oxidation of alcohols in water-toluene (W-T) biphasic systems

From the viewpoint of "greenness", t-butanol is preferred to toluene. On the other hand, as toluene is water-immiscible and an excellent solvent for many organic compounds, product separation is simpler in biphasic W-T than in water-t-butanol system. Therefore, for further investigations of the effect of reaction conditions we performed the oxidation of 1-phenylethanol in biphasic W-T systems.

The conversion decreased when the volume of toluene was increased (Table 4). In dry toluene, the enzyme precipitated and no reaction was observed. Since the reaction is dependent on the pH of aqueous buffer (see Figure 2), we have also investigated the effect of aqueous pH in W-T systems. Variation of aqueous pH values did not affect the conversion (Table 4).

<table>
<thead>
<tr>
<th>Toluene (%v/v)</th>
<th>Aqueous pH 4.8</th>
<th>24 h</th>
<th>48 h</th>
<th>Aqueous pH 7.0</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>99.0</td>
<td>--</td>
<td>--</td>
<td>94.4</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>25</td>
<td>74.1</td>
<td>97.1</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>50</td>
<td>36.5</td>
<td>56.4</td>
<td>37.5</td>
<td>56.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>31.7</td>
<td>53.6</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>85</td>
<td>16.7</td>
<td>25.7</td>
<td>13.4</td>
<td>24.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>10.0</td>
<td>13.0</td>
<td>7.1</td>
<td>11.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[Sub]₀: 125 mM, Lac/Sub: 94 U/mmol, TEMPO/Sub: 15 mol%, r.t. O₂

Table 5 Conversion of 1-phenylethanol under various conditions

<table>
<thead>
<tr>
<th>Lac/Sub (U/mmol)</th>
<th>TEMPO/Sub (mol%)</th>
<th>30 mol%</th>
<th>15 mol%</th>
<th>5 mol%</th>
</tr>
</thead>
<tbody>
<tr>
<td>94</td>
<td></td>
<td>61.9</td>
<td>36.5</td>
<td>11.8</td>
</tr>
<tr>
<td>47</td>
<td></td>
<td></td>
<td>51.1</td>
<td>27.6</td>
</tr>
<tr>
<td>23.5</td>
<td></td>
<td></td>
<td>31.5</td>
<td>19.4</td>
</tr>
</tbody>
</table>

[Sub]₀: 125 mM, W-T (1:1), 24 h, r.t. O₂
On the other hand, increasing the amount of TEMPO or laccase in W-T increased the conversion as expected (Table 5). When TEMPO was increased from 5 mol% to 15 mol%, i.e. 3-fold, the conversion of 1-phenylethanol also increased 3-fold.

To explore the substrate scope, some other secondary alcohols were also tested for oxidation by laccase-TEMPO in W-T biphasic media (Table 6). It is clear that benzylic alcohols react faster than alicyclic alcohols. Aliphatic acyclic alcohols were not active.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>W-T (9:1)</th>
<th>W-T (1:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>23 h</td>
<td>23 h</td>
</tr>
<tr>
<td>1-Phenylethanol</td>
<td>68.1</td>
<td>36.3</td>
</tr>
<tr>
<td>1-Phenyl propanol</td>
<td>51.1</td>
<td>11.3</td>
</tr>
<tr>
<td>Cyclohexanol</td>
<td>31.9</td>
<td>12.8</td>
</tr>
<tr>
<td>2-Hexanol</td>
<td>5.4</td>
<td>--</td>
</tr>
<tr>
<td>2-Octanol</td>
<td>8.9</td>
<td>2.2</td>
</tr>
<tr>
<td>1-Phenylethanol</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1-Phenylethanol</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Borneol</td>
<td>--</td>
<td>10.8 (^d)</td>
</tr>
<tr>
<td>Menthol</td>
<td>--</td>
<td>6.3 (^d)</td>
</tr>
</tbody>
</table>

\(^a\) [Sub]₀: 125 mM, Lac/Sub: 47 U/mmol, TEMPO/Sub: 15 mol%; \(^b\) Surfactant PEG3400: 5 mM; \(^c\) Lac/Sub: 70.8 U/mmol; \(^d\) Lac/Sub: 94 U/mmol, TEMPO/Sub: 15 mol%, W-T (3:1)

2.7 Influence of the reaction medium

We found that when the reaction medium was changed from acetate buffer (AB) to phosphate buffer (PB) at the same pH value, the conversion increased (Table 7). This suggests that PB is a better reaction medium for the laccase-TEMPO system. In water-t-butanol (9:1), however, there was no difference between these two buffers. This might be due to the fact that the organic solvent plays a dominant role on the enzyme activity in water-organic solvent systems. Therefore, the effect of PB or AB on the reaction could not be distinguished.
Table 7 Oxidation of alcohols by laccase-TEMPO: The effect of the medium (Conv %)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>AB</th>
<th>PB</th>
<th>AB+ t-BuOH (9:1)</th>
<th>PB+ t-BuOH (9:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzyl alcohol</td>
<td>60.5</td>
<td>78.2</td>
<td>34.5</td>
<td>39.5</td>
</tr>
<tr>
<td></td>
<td>74.4</td>
<td>97.8</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>1-phenylethanol</td>
<td>--</td>
<td>--</td>
<td>86.1</td>
<td>80.3</td>
</tr>
</tbody>
</table>

[Sub]₀: 140 mM, Lac/Sub: 25 U/mmol, Med/Sub: 10 mol%, 4 h. AB: 0.1 M acetate buffer at pH 4.5, PB: 0.1 M phosphate buffer at pH 4.5; a Med/Sub: 15 mol%; b 24 h.

As it is known that the oxoammonium salt is not stable under acidic conditions [Thomas and Mohanty, 1982], we investigated the stability of TEMPO-ClO₄ in both phosphate buffer and acetate buffer. The formation rates of TEMPO radical in both buffers are similar (Figure 11), thus excluding that the medium had an effect on the stability of oxoammonium salt.

![Decomposition of TEMPO-ClO₄ in buffer](image)

Figure 11 Stability of oxoammonium salt (TEMPO-ClO₄) in acidic buffers at r.t.

On the other hand, we measured the $V_{max}$ and $K_m$ of CvL in PB in the oxidation of ABTS. From the kinetic parameters presented in Table 8, it can be concluded that CvL has higher activity in PB compared to AB. Possibly, in PB the protein structure changes to a more stable formation, thereby leading to an enhanced rate for one-electron transfer.
Table 8 Kinetic constants of CvL in different buffers (pH 4.5, 25 °C)

<table>
<thead>
<tr>
<th>Buffer</th>
<th>K_m (mM)</th>
<th>V_max (μmol min⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M Acetate buffer (AB)</td>
<td>0.0581</td>
<td>1.377</td>
</tr>
<tr>
<td>0.1 M Phosphate buffer (PB)</td>
<td>0.0435</td>
<td>1.886</td>
</tr>
</tbody>
</table>

3. Conclusions

Laccase-TEMPO can be used for oxidation of benzylic and cyclic secondary alcohols in aqueous buffer as well as in water-organic solvents. This broadens the scope of laccase-mediator systems. The optimum temperature is between 20 and 30 °C. In aqueous medium, the optimum pH is 4.5 – 4.8, whereas in water-toluene systems, the reaction is independent of the aqueous pH value. Organic solvents can inhibit the laccase activity. The effect of organic solvent on laccase activity depends on the structure and hydrophobicity of the solvent. t-Butanol is the best solvent concerning laccase activity preservation. Toluene, acetonitrile, and MTBE also are good solvents for laccase-TEMPO catalysed oxidations. Laccase-TEMPO activity was enhanced in phosphate buffer compared to acetate buffer.

4. Experimental

4.1 Enzymes and chemicals

Laccase from Coriolus versicolour (CvL) was purchased from Juelich Fine Chemicals, Germany, as a lyophilized powder. The content of the protein is 8.8 mg/g solid (data from Juelich Fine Chemicals). NS 51002 and NS 51003 were gifts from National Starch & Chemicals, USA. Trametes villosa laccase was donated by Novozyme, Denmark. Pycnoporus cinnabarinus laccase was a gift from Prof. Kragl of Rostock University, Germany. TEMPO was obtained from BASF, Germany. ABTS was purchased from Fluka. All the liquid alcohols were distilled before use. The other chemicals were purchased from Aldrich or Acros and used as received. 0.1 M acetate buffer at pH 4.5 was used for preparing solutions for activity assay and as oxidation reaction medium unless otherwise specified.
4.2 Analysis methods

Substrate conversion was determined by GC with column WAX 52 CB (on Varian 3400 CX) or Sil 5 CB (on Varian STAR 3400). FID detector and temperature program (70 °C for 9 min, then increased at a rate of 10 °C/min to 250 °C for 6 min) were used. Dodecane or hexadecane was used as internal standard. The products were characterized by GC-MS. UV measurements were run on a Varian Cary UV-Vis Spectrophotometer.

4.3 Laccase activity assay

The laccase activity was determined spectroscopically at 25 °C using ABTS as substrate. To a UV cuvette, a certain amount of ABTS (4.3 – 9.6 mg) was added to 2.0 ml of 0.1 M acetate buffer (pH 4.5) containing 2.0 to 7.0 μg of laccase. The absorbance change at 420 nm was recorded against time to give a linear line with a slope of k. The oxidation rate of ABTS using a certain amount of enzyme was expressed as:

\[ V = \frac{k \times 1000 \text{ (μmol)}}{E_{420} \times E \text{ (min*mg)}} \]

where \( V \) represents the oxidation rate, \( k \) is the slope of the above linear line, and \( E_{420} \) is the extinction coefficient of ABTS at 420 nm, which is valued 36,000 M\(^{-1}\) cm\(^{-1}\) [Childs and Bradski, 1975]. E represents the amount of laccase.

One activity unit (U) of the enzyme was defined as 1 μmol ABTS oxidised per minute under the stated assay conditions, that is the \( V \). The activity of all the laccase preparations used in this study was determined as shown in Table 9. Each sample was measured 4 to 5 times, and the average value was adopted.

<table>
<thead>
<tr>
<th>Laccase sample</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Coriolus versicolor</em></td>
<td>0.96 (U/mg)</td>
</tr>
<tr>
<td>NS 51002</td>
<td>1.6 (U/μl)</td>
</tr>
<tr>
<td>NS 51003</td>
<td>1.8 (U/μl)</td>
</tr>
<tr>
<td><em>Trametes villosa</em></td>
<td>0.7 (U/μl)</td>
</tr>
<tr>
<td><em>Pycnoporus cinnabarinus</em></td>
<td>4.7 (U/ml)</td>
</tr>
</tbody>
</table>
4.4 Measurement of $K_m$ and $V_{max}$ in the presence of 10 %v/v water-miscible organic solvent

0.1 M Acetate buffer (pH 4.5) was used to prepare the stock solutions of CvL (5.1 μg/ml) and ABTS (1.96 mM). To a 2 ml UV cuvette were added 1 ml of the laccase solution, 0.2 ml of organic solvent, 0.02 – 0.5 ml of the ABTS solution. Finally, 0.1 M acetate buffer (pH 4.5) was added to make the total volume of 2 ml. The absorbance change at 420 nm and the oxidation rate ($V$) of ABTS were recorded and calculated the same way as in section 4.3. Table 10 shows a typical procedure and results in aqueous acetonitrile solution (10 %v/v)).

<table>
<thead>
<tr>
<th>ABTS solution (ml)</th>
<th>[ABTS] (mM)</th>
<th>$V$ (μmol/(min*mg))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>0.0196</td>
<td>0.138</td>
</tr>
<tr>
<td>0.05</td>
<td>0.049</td>
<td>0.265</td>
</tr>
<tr>
<td>0.1</td>
<td>0.098</td>
<td>0.333</td>
</tr>
<tr>
<td>0.2</td>
<td>0.196</td>
<td>0.444</td>
</tr>
<tr>
<td>0.5</td>
<td>0.49</td>
<td>0.547</td>
</tr>
</tbody>
</table>

The Lineweaver-Burk plot of reciprocal of oxidation rate ($1/V$) against the reciprocal of ABTS concentration ($1/[ABTS]$) is shown in Figure 12. The ratio of $K_m/V_{max}$ corresponds to the slope (0.1087). The intercept relates to $1/V_{max}$ (1.6921). The $K_m$ and $V_{max}$ for CvL in various aqueous solvents are shown in Table 3.

![Lineweaver-Burk plot](image)

Figure 12 $1/V$ against $1/[ABTS]$ in aqueous acetonitrile solution (10 %v/v)
4.5 Typical procedure for laccase-TEMPO catalysed oxidations

A mixture of 1-phenylethanol (61 mg, 0.5 mmol), dodecane (34 mg, internal standard), laccase (24.5 mg, 23.5 U), and TEMPO (11.7 mg, 0.075 mmol) in acetate buffer (pH 4.5, 4 ml) was placed in a 10 ml glass vial. The vial was connected with an oxygen cylinder to keep the system under atmospheric pressure of oxygen. The mixture was stirred at room temperature for a certain time. After reaction, the mixture was washed with diethyl ether (2 × 4 ml). The organic solution was dried with anhydrous sodium sulfate, centrifuged, and analyzed with GC.

4.6 Stability test of laccase

Method A: After the first reaction (performed as in section 4.5) was finished, the reaction mixture was washed with a 2-fold volume of diethyl ether. After phase separation, the aqueous solution containing laccase was used directly for the next reaction without addition of extra laccase. In some experiments extra TEMPO was added.

Method B: Laccase (14.6 mg, 14 U) and TEMPO (8.7 mg, 0.056 mmol) were added into 0.1 M acetate buffer (pH 4.5). The mixture was stirred for a few minutes till the TEMPO dissolved, and then left at room temperature under oxygen atmosphere for a certain time. Then benzyl alcohol (60.5 mg, 0.56 mmol) and internal standard (hexadecane, 30 mg) were added to this solution and the reaction was run for 4 h, followed by work-up as in section 4.5.
Chapter 3

Reactivity of TEMPO and its Derivatives in Laccase Catalysed Oxidation of Alcohols

Abstract: A series of TEMPO-like nitroxy radicals were studied as mediators of laccase (from Coriolus versicolour) in the oxidation of both primary and secondary benzylic alcohols. TEMPO, 4-acetamido-TEMPO, and 4-hydroxy-TEMPO were the most active mediators for laccase. Competition experiments between benzyl alcohol and 1-phenylethanol showed that TEMPO displayed a higher activity for primary alcohols than 4-acetamido-TEMPO and 4-hydroxy-TEMPO, whereas the latter two were more effective with secondary alcohols.
Reactivity of TEMPO and its derivatives in laccase-catalysed oxidation of alcohol

1. Introduction

TEMPO (2,2,6,6-Tetramethylpiperidinyl-1-oxy) has widely been used for oxidation of functional groups [Bobbitt and Flores, 1988, de Nooy et al. 1996, Sheldon et al. 2002, also see Chapter 1]. It has also been used as a mediator of laccase in the aerobic oxidation of alcohols [Fabbriani et al. 2001] and carbohydrates [Viikari et al. 1999a; 1999b; Jetten et al. 2000]. The oxidation of a nitroxy radical by removal of the unpaired electron is a favourable process because it leads to a considerable strengthening of the nitrogen-oxygen bond [Rozantsev 1970, p.99]. Any oxidising agent possessing a sufficiently high oxidation potential ($E^0$) can oxidise the nitroxy radical to form an oxoammonium ion. TEMPO ($E^0 = 0.53$ V) can be oxidised by laccases ($E^0 = 0.48$ -0.78 V) [Reinhammar, 1984] to form an oxoammonium ion which readily oxidises alcohols.

The TEMPO-like radical in biocatalytic laccase systems is proposed to form an oxoammonium ion in situ, which acts as the actual oxidant to convert the alcohol into a carbonyl compound (Scheme 1) [Viikari et al. 1999a, Fabbriani et al. 2001]. The thus formed hydroxylamine reacts with another molecule of TEMPO oxoammonium to give 2 molecules of TEMPO. Laccase is then assumed to reoxidise the nitroxy. In turn the laccase is regenerated from its reduced state by oxygen, coupled with the formation of water.

Scheme 1 Postulated mechanism of laccase-TEMPO oxidation of alcohols

However, in the very few publications regarding laccase-TEMPO oxidations, the reaction is slow and the concentration of TEMPO and laccase are quite high compared to
the alcohol. From the viewpoint of cost-effectiveness, the amount of expensive TEMPO should be decreased, or replaced by a cheaper mediator.

To improve the process to a more practical level, we have investigated systematically the reactivity of a series of nitroxy radicals as mediators of laccase in the oxidation of both primary and secondary benzylic alcohols.

2. Results and discussion

In all experiments, unless otherwise specified, 0.1 M acetate buffer (AB) at pH 4.5 was used as the aqueous buffer. Reactions were carried out at room temperature under an oxygen atmosphere. TEMPO-like nitroxy radicals are referred to as mediators (M) and alcohols as substrates (S), although the alcohols were not the real substrates converted directly by laccase in the proposed mechanism (Scheme 1).

In this study, the stable nitroxy radicals in Figure 1 were tested as mediators of laccase. These radicals are easily synthesized and commercially available [Rosantsev and Sholle, 1971a; Sosnovsky and Konieczny, 1976].

![Figure 1 TEMPO and its derivatives used in this study](image)

The different nitroxy radicals were used in the oxidation of benzyl alcohol and the results are shown in Table 1.
Reactivity of TEMPO and its derivatives in laccase-catalysed oxidation of alcohol

Table 1 Aerobic oxidation of benzyl alcohol catalysed by laccase and nitroxyl radicals

<table>
<thead>
<tr>
<th>Nitroxyl radical</th>
<th>Conv (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEMPO</td>
<td>100</td>
</tr>
<tr>
<td>Aa-TEMPO</td>
<td>95.7</td>
</tr>
<tr>
<td>Oh-TEMPO</td>
<td>100</td>
</tr>
<tr>
<td>O-TEMPO</td>
<td>2.7</td>
</tr>
<tr>
<td>Am-TEMPO</td>
<td>6.5</td>
</tr>
<tr>
<td>Ac-TEMPO</td>
<td>20.4</td>
</tr>
<tr>
<td>Mo-TEMPO</td>
<td>26</td>
</tr>
<tr>
<td>Bz-TEMPO</td>
<td>&lt;1</td>
</tr>
<tr>
<td>TEMIM</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Conditions: [Sub]₀: 140 mM, Lac/Sub: 25 U/mmol, Med/Sub: 10 mol%, 24 h, r.t. O₂

Among the nitroxyl radicals tested above, O-TEMPO, Am-TEMPO, and TEMIM showed very low activity for mediating laccase catalysed oxidation. Ac-TEMPO was moderately active. The conversion of benzyl alcohol was complete after 24 h in the systems mediated by TEMPO, Oh-TEMPO, and Aa-TEMPO. It is surprising that Bz-TEMPO showed no activity.

This difference can not be explained simply on the basis of redox potential (E⁰), although O-TEMPO has a higher E⁰ than the other radicals. All the E⁰ values of the nitroxyl radicals (Table 2) are lower than or equivalent to the E⁰ of CvL. Therefore, the CvL has sufficient oxidation potential to oxidise the nitroxyl radicals to form the corresponding oxoammonium ions as was proposed in Scheme 1.

Table 2 Redox potential of some nitroxyl radicals and laccase

<table>
<thead>
<tr>
<th>Nitroxyl radical</th>
<th>E⁰ (V)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEMPO</td>
<td>0.53</td>
<td>[a]</td>
</tr>
<tr>
<td>Oh-TEMPO</td>
<td>0.59</td>
<td>[a]</td>
</tr>
<tr>
<td>Am-TEMPO</td>
<td>0.68</td>
<td>[a]</td>
</tr>
<tr>
<td>O-TEMPO</td>
<td>0.78</td>
<td>[b]</td>
</tr>
<tr>
<td>CvL</td>
<td>0.78</td>
<td>[c]</td>
</tr>
</tbody>
</table>


The oxoammonium salt of O-TEMPO was previously shown to be unstable in
weakly acidic and basic medium [Thomas and Mohanty, 1982]. Cyclic voltammetry revealed that the O-TEMPO oxoammonium ion decomposed irreversibly into non-radical species at pH above 3.5.

The low stability of O-TEMPO was also observed by Abakumov and Tikhonov [1969] and was due to reaction of the O-TEMPO with acids. When they treated the free radical O-TEMPO with a benzene solution of trichloroacetic acid, a mixture of the corresponding hydroxylamine and a ring-opened nitroso compound was formed (Scheme 2).

![Scheme 2 Interaction of O-TEMPO with acid](image)

In our system, the O-TEMPO is presumably oxidised by laccase to form its oxoammonium ion, and the latter undergoes ring cleavage to form a \(-\text{N}=\text{O}\) compound (Scheme 3), thus losing its oxidising ability. In acetate buffer, the oxoammonium cation can be coupled with the acetate anion leading to decomposition via the mechanism shown in Scheme 3.

![Scheme 3 Probable decomposition of O-TEMPO in acidic medium](image)

The higher reactivity of TEMPO and Oh-TEMPO is probably a result of the higher stability and stronger oxidative ability of their oxoammonium ions.

In the case of Am-TEMPO, the situation is more complicated. Under acidic conditions, the Am-TEMPO can be converted into the corresponding oxoammonium cation either by disproportionation (Scheme 4) or by laccase oxidation. The amino group
Reactivity of TEMPO and its derivatives in laccase-catalysed oxidation of alcohol

in either the radical structure or the oxoammonium structure can readily be oxidised by
the oxoammonium cation. In addition, it is reported that amines can react easily with
nitroxy radicals in an acid medium [Neiman et al. 1964; Medzhidov et al. 1966]. These
possible reactions of Am-TEMPO may cause its deactivation.

\[ \begin{align*}
\text{NH}_2 & \quad \text{NH}_2 \\
\text{N} & \quad \text{N} \\
\text{O} & \quad \text{N} \\
\text{O} & \quad \text{O}\end{align*} \]

Scheme 4 Proposed reaction of Am-TEMPO in acidic buffer

Nakatsuji et al. [1999] reported that 4-amino-TEMPO is more readily oxidised than
TEMPO and Oh-TEMPO. They found by cyclic voltammetry that TEMPO and
Oh-TEMPO showed only one oxidation potential corresponding to nitroxy radical,
while 4-amino-TEMPO showed two oxidation potentials, related to the oxidation of
amino group and nitroxy radical, respectively.

The instability of Am-TEMPO was confirmed by the UV absorption change of
4-Amino-TEMPO in acidic medium. When Am-TEMPO was incubated in AB at pH 4.5,
the absorbance maximum at 430 nm increased. This could be due to the formation of
group –N=O (unfortunately we could not isolate the product). On the other hand, there
was no absorbance change in TEMPO system under the same conditions (Figure 2).

Figure 2 Absorption of Am-TEMPO (a) and TEMPO (b) in acetate buffer at pH 4.5
When the amino group in Am-TEMPO is protected with an acetyl group, the resulting amide moiety is stable towards oxidation. Hence, Aa-TEMPO is an efficient mediator.

The comparison between TEMPO, Oh-TEMPO, and Aa-TEMPO (Figure 3) shows that Oh-TEMPO and Aa-TEMPO have similar reactivity in the oxidation of benzylic alcohol, whereas TEMPO is more reactive.

![Graph showing reactivity of nitroxyl radicals in the laccase catalysed aerobic oxidation of benzyl alcohol.](image)

Figure 3 Reactivity of nitroxyl radicals in the laccase catalysed aerobic oxidation of benzyl alcohol. [Sub]₀: 125 mM, Lac/Sub: 47 U/mmol, Med/Sub: 15 mol%, r.t. O₂

In contrast, with the secondary alcohol, 1-phenylethanol, Oh-TEMPO and Aa-TEMPO showed higher activity than TEMPO (Table 3). This difference was more pronounced in water-toluene (W-T = 1:1) than in aqueous buffer.

Table 3 Conversion of 1-phenylethanol by laccase-nitroxyl radicals catalysed aerobic oxidation

| Mediator     | Acetate buffer | W-T (1:1) a
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TEMPO</td>
<td>66.8</td>
<td>27.6 (44.0)</td>
</tr>
<tr>
<td>Oh-TEMPO</td>
<td>76.8</td>
<td>60.7 (80.8)</td>
</tr>
<tr>
<td>Aa-TEMPO</td>
<td>78.5</td>
<td>81.7</td>
</tr>
<tr>
<td>O-TEMPO</td>
<td>2.4</td>
<td>--</td>
</tr>
<tr>
<td>Am-TEMPO</td>
<td>2.4</td>
<td>--</td>
</tr>
<tr>
<td>TEMIM</td>
<td>--</td>
<td>3.9 (4.2)</td>
</tr>
<tr>
<td>Fremy's salt</td>
<td>--</td>
<td>0</td>
</tr>
</tbody>
</table>

Conditions: [Sub]₀: 125 mM, Lac/Sub: 47 U/mmol, Med/Sub: 15 mol%, r.t. O₂

a 5.5 h; b 24 h; c Data in parentheses were after 48 h
Reactivity of TEMPO and its derivatives in laccase-catalysed oxidation of alcohol

O-TEMPO, Am-TEMPO, and TEMIM again showed no activity in laccase catalysed oxidation, nor did Fremy’s salt.

To confirm the different reactivities of TEMPO, Aa-TEMPO, and Oh-TEMPO towards different substrates, the relative reactivity of benzyl alcohol against 1-phenylethanol (expressed as \( k_1/k_2 \)) was determined in intermolecular competition reactions and calculated from the following equation (Table 4).

\[
\frac{k_1}{k_2} = \frac{\ln(1 - \text{Conv}_1)}{\ln(1 - \text{Conv}_2)}
\]

where: \( k \) is the rate constant

Conv represents the conversion

subscript 1 represents benzyl alcohol

subscript 2 represents 1-phenylethanol

<table>
<thead>
<tr>
<th>Mediator</th>
<th>AB (pH 4.5) (5 h)</th>
<th>W-T (1:1) (23 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conv₁/Conv₂</td>
<td>( k_1/k_2 )</td>
</tr>
<tr>
<td>TEMPO</td>
<td>76.8/35.2</td>
<td>3.37</td>
</tr>
<tr>
<td>Oh-TEMPO</td>
<td>40.6/32.1</td>
<td>1.34</td>
</tr>
<tr>
<td>Aa-TEMPO</td>
<td>51.6/31.8</td>
<td>1.89</td>
</tr>
</tbody>
</table>

Conditions: Benzyl alcohol and 1-phenylethanol were contained in the same amount, each with a concentration of [Sub]₀ 62.5 mM, Lac/Sub: 47 U/mmol, Med/Sub: 15 mol%.

In all cases benzyl alcohol was converted faster than 1-phenylethanol in aqueous buffer. When toluene was added into the medium, this difference was even bigger. On the other hand, Oh-TEMPO and Aa-TEMPO showed higher affinity to 1-phenylethanol than TEMPO did, especially in W-T.

3. Conclusions

A number of stable TEMPO-like nitroxy radicals were tested as mediators of the laccase catalysed aerobic oxidation of alcohols in acidic buffer and in water-toluene media. Among them TEMPO, 4-hydroxy-TEMPO, and 4-acetamido-TEMPO are the most effective mediators. No oxidation reaction occurred in the absence of either laccase,
TEMPO, or oxygen. Laccase-catalysed oxidation of benzyl alcohol with TEMPO mediation is faster than with 4-hydroxy-TEMPO and 4-acetamido-TEMPO. However, for the oxidation of the secondary alcohol 1-phenylethanol, the reactivity in 4-hydroxy-TEMPO and 4-acetamido-TEMPO systems is higher than in TEMPO system. This difference is more distinct in water-toluene systems.

4. Experimental

4.1 Enzymes and chemicals

Laccase from *Coriolus versicolor* (CvL) was purchased from Juelich Fine Chemicals as a lyophilized powder. The content of the protein is 8.8 mg/g solid (data from the enzyme supplier). TEMPO was obtained from BASF, Germany. The other nitroxyl radicals were purchased from Acros. 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was purchased from Fluka. 1-Phenylethanol and benzyl alcohol were distilled before use. The other chemicals were purchased from Aldrich or Acros and used as received. 0.1 M acetate buffer at pH 4.5 was used for preparing solutions for the activity assay and as oxidation reaction medium unless otherwise specified.

4.2 Laccase activity assay

The laccase activity was determined spectroscopically at 25 °C using ABTS as substrate. To an UV cuvette, a certain amount of ABTS (4.3 – 9.6 mg) was added to 2.0 ml of 0.1 M acetate buffer (pH 4.5) containing 2.0 to 7.0 μg of laccase. The absorbance change at 420 nm was recorded for 5 min (ε₄₂₀ = 36,000 M⁻¹ cm⁻¹ [Childs and Bradely, 1975]). One unit (U) of the enzyme was defined as 1 μmol ABTS oxidised per min under the stated assay conditions.

4.3 Typical procedure for alcohol oxidation by laccase-nitroxyl radical

A mixture of benzyl alcohol (54 mg, 0.5 mmol), dodecane (34 mg, internal standard), laccase (24.5 mg, 47 U), and TEMPO (11.7 mg, 0.075 mmol) in acetate buffer (pH 4.5, 4 ml) was placed in a 10 ml glass *vial*. The *vial* was connected with an oxygen source to keep the system under atmospheric pressure of oxygen. The mixture was
stirred at room temperature for a certain time. After reaction, the mixture was washed with diethyl ether (2 × 4 ml). The organic solution was dried with anhydrous sodium sulfate, centrifuged, and analyzed with GC.

4.4 Analysis methods

Alcohol conversion was analyzed by GC with column WAX 52 CB (on Varian 3400 CX) or Sil 5 CB (on Varian STAR 3400). FID detector and temperature program (70 °C for 9 min, then increased at a rate of 10 °C/min to 250 °C for 6 min) were used on either column. Dodecane or hexadecane was used as internal standard. The products were characterized by GC-MS.
Chapter 4

Kinetic and Mechanistic Investigations on Laccase-Catalysed Oxidation of Alcohols in the Presence of TEMPO and its Analogues

Abstract: The kinetics and mechanism of laccase (from *Coriolus versicolour*, CvL) catalysed aerobic oxidation of alcohols, in the presence of TEMPO and its analogues as mediators were investigated. Reaction kinetics together with kinetic isotope effects are consistent with the mechanism involving oxoammonium intermediacy. Laccase is not stable, however, in the presence of oxoammonium salts, presumably due to degradation via oxidation of essential amino acid residues or the glycosyl moieties on the periphery of the enzyme.
1. Introduction

Laccase-mediated systems have been extensively studied in pulp and paper biobleaching, dye decolourisation, and organic synthesis. Among a number of mediators, TEMPO and its analogues have recently been shown to be effective in the laccase catalysed aerobic oxidation of alcohols [Viikari et al. 1999b; Jetten et al. 2000; Fabbrini et al. 2001; also see chapters 2 and 3].

The mechanism proposed for laccase-TEMPO catalysed aerobic oxidation is shown in Scheme 1 [Viikari et al. 1999a].

![Scheme 1 Proposed mechanism of laccase-TEMPO catalysed oxidation of alcohols](image)

In this process, the TEMPO (I, R = H) is first oxidised by laccase, via one-electron transfer to form the corresponding oxoammonium ion (II), which acts as the active oxidant of alcohol. Heterolytic oxidation of the alcohol by the latter affords the corresponding carbonyl compound together with the formation of hydroxylamine (III). I is regenerated via syn proportionation between III and II [de Nooy et al. 1996]. In this catalytic cycle, laccase is the oxidant of TEMPO (I) and oxygen regenerates the oxidised form of laccase.

The advantage of the laccase-TEMPO reaction is that molecular oxygen is the oxidant and the only by-product is water, and laccase is a readily available, natural catalyst [Fahraeus et al. 1958]. A disadvantage of this process are the large amounts of laccase (150 Unit /mmol substrate) and TEMPO (30 mol%) that are needed to obtain a reasonable rate of reaction [Fabbrini et al. 2001].
Chapter 4

The aim of this study was to obtain kinetic and mechanistic information regarding laccase-TEMPO catalysed aerobic oxidations of alcohols, in order to provide a basis for further improvement of the process. No kinetic studies of the laccase-TEMPO system have been previously reported.

2. Results and discussion

According to the above mechanism, the real substrate of laccase is TEMPO. The alcohol does not react with the enzyme but with an intermediate oxoammonium ion, formed by laccase oxidation of TEMPO. For the sake of simplicity we hereafter refer to the alcohol as the substrate (Sub) and TEMPO or its analogues as the mediator (Med). Laccase is abbreviated as Lac in some cases.

In our system, a constant diffusion of oxygen into the reaction mixture solution was obtained by performing reactions under oxygen atmosphere. The reactions can also be carried out under air. There was no difference between these two systems (Figure 1). Since oxygen binding to laccase has a low constant of about 20 µM, laccase usually works under enzyme saturation with oxygen [Rodakiewicz-Nowak et al. 2000]. In the case of bubbling oxygen through the solution before - and not during - the reaction, because of low solubility of oxygen in water, the conversion of alcohol stopped when all the oxygen was consumed.

![Graph showing conversion vs time](image)

Figure 1 Oxidation of furfuryl alcohol by laccase-Aa-TEMPO. [Sub]₀: 140 mM, Med/Sub: 10 mol%, Lac/Sub: 23.5 U/mmol. ✷ oxygen bubbled for 0.5 h before reaction, ■ oxygen, ▲ air
2.1 Kinetic studies

In laccase-TEMPO catalysed aerobic oxidation of benzyl alcohols, the reaction systems are usually biphasic mixtures. This is due to the poor solubilities of the substrate alcohols, the product aldehydes, or the mediator in water. On the other hand, laccase always stays in the water phase. Therefore, there may exist interfacial mass transfer limitations of either substrate and/or product or mediator compound. In addition, it is difficult to take samples accurately from a biphasic system during the reaction process. To solve these problems we chose water soluble furfuryl alcohol as the substrate and 4-acetamido-TEMPO (Aa-TEMPO) as the mediator. As they are all water soluble materials the system is monophasic.

Our preliminary experiments (see Chapter 2) showed that the optimum aqueous pH and temperature for laccase-TEMPO catalysed oxidations were in the range of pH 4.5 to 5.0 and 20 to 30 °C, respectively. Therefore, unless otherwise specified, all the reactions were performed at room temperature (~ 25 °C) in 0.1 M acetate buffer (AB) at pH 4.5, and under an oxygen atmosphere to keep constant diffusion of oxygen into the reaction mixture. The results are based on the conversion of furfuryl alcohol to furfuryl aldehyde as determined by GC analysis.

We had previously shown that there was no oxidation of 1-phenylethanol or benzyl alcohol in the absence of mediator, or laccase, or oxygen (Chapters 2 and 3). This was also observed with furfuryl alcohol, thus confirming the need for all three components. As is proposed in Scheme 1, the alcohol is oxidised by the o xoammonium ion derived from the mediator (represented as \( M_{ox} \)) and the rate of substrate conversion can be expressed as:

\[
V = -\frac{d[Sub]}{dt} = k[Sub][M_{ox}] 
\]  

(1)

Where \( k \) is the rate constant

\([M_{ox}]\) is the concentration of oxoammonium ion

\([Sub]\) is the concentration of substrate

Because the \( M_{ox} \) is regenerated in situ by laccase, its concentration should be constant. Therefore, the reaction is only dependent on the concentration of substrate.

\[
V = -\frac{d[Sub]}{dt} = k_{ox}[Sub] 
\]  

(2)

Indeed, this has been observed in the experiments at Med/Sub of 15 mol%. In the range of initial substrate concentration ([Sub]₀) from 25 mM to 200 mM, the plots of
logarithm of substrate concentration (Ln [Sub]) against time always show very linear behaviour, indicating a pseudo first order reaction (Figure 2a). At all the tested levels of [Sub]₀, the overall rate constants (kₐₑₛₚ) in Eq. (2) are within the same range, although the highest rate was observed at [Sub]₀ of 80-140 mM (Figure 2b).

Figure 2 Effect of furfuryl alcohol concentration [Sub]₀ on its oxidation by laccase-acetamido-TEMPO. (a) reaction kinetics under different substrate concentration [Sub]₀, * 200 mM, × 140 mM, ▲ 80 mM, ■ 50 mM, ♦ 25 mM. Conditions: Med/Sub: 15 mol%, Lac/Sub: 47 U/mmol, r.t. O₂; (b) observed rate constant vs substrate concentration

This is in accordance with the results obtained from oxidation of 1-phenylethanol by laccase-TEMPO [see Chapter 2], which also showed a first order reaction (Figure 3).

Figure 3 Conversion of 1-phenylethanol by laccase-TEMPO at pH 4.8. Conditions: [Sub]₀: 125 mM, Lac/Sub: 47 U/mmol, TEMPO/S: 15 mol%, r.t. O₂

The initial reaction rate Vᵢₙᵢ is linearly dependent on [Sub]₀ when [Sub]₀ is no higher
than 140 mM. At low concentration, the reaction is very slow. No further increase of $V_{\text{ini}}$ was observed at $[\text{Sub}]_0$ higher than 140 mM (Figure 4). This indicates saturation kinetics with regard to the alcohol.

![Figure 4](image-url) Effect of furfuryl alcohol concentration $[\text{Sub}]_0$ on the initial rate ($V_{\text{ini}}$) of its oxidation. *Conditions:* Med/Sub: 15 mol%, Lac/Sub: 47 U/mmol, r.t. O$_2$

However, when the amount of mediator (Med/Sub) is changed, the kinetics become more complicated (Figure 5). At the levels of Med/Sub less than 15 mol%, the reactions are not first-order anymore, nor second-order, with respect to substrate. This means that the $[M_{\text{ox}}]$ in Eq. 1 is not a constant, and should be taken into account in the reaction kinetics.

![Figure 5](image-url) Effect of mediator amount Med/Sub on the oxidation of furfuryl alcohol. ◆ 2 mol%, ■ 5 mol%, ▲ 10 mol%, × 15 mol%. *Conditions:* $[\text{Sub}]_0$: 140 mM, Lac/Sub: 47 U/mmol, r.t. O$_2$
On the other hand, the initial rate is less dependent on the amount of mediator (Figure 6). The $V_{\text{ini}}$ does not show much difference in the range of Med/Sub from 5 mol% to 15 mol% (see also below in part 2.2). This means that at the beginning of the reaction, only 5 mol% mediator is sufficient to give a high reaction rate. These results also indicate that the reaction rate is zero order with respect to oxoammonium at the initial stage of the reaction at levels of above 5 mol% mediator.

Figure 6 Effect of mediator amount Med/Sub on the initial rate ($V_{\text{ini}}$) of oxidation of furfuryl alcohol. Lac/Sub: 47 U/mmol, r.t. $O_2$  ◆ [Sub]₀ 140 mM, ■ [Sub]₀ 80 mM

The enzyme amount is expressed as the laccase activity units relative to substrate (Lac/Sub, U/mmol). Its effect on reaction rate is shown in Figure 7. It is clear that the reaction rate is linearly dependent on the amount of laccase used.

Figure 7 Effect of laccase activity on the initial rate ($V_{\text{ini}}$) of oxidation of furfuryl alcohol. [Sub]₀: 140 mM, Med/Sub: 10 mol%, r.t. $O_2$

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2.2 Mechanistic studies

According to the proposed mechanism of laccase-mediator system, the mediator is oxidised by laccase to form an active intermediate, which oxidises the substrate [Eggert et al. 1996; Call and Mucke, 1997]. This mechanism was also adopted for laccase-TEMPO oxidation of alcohols as shown in Scheme 1 and is supported by our experimental results. However, the reaction process seems more complex.

The primary kinetic isotope effects (k_H/k_D) in laccase-nitroxyl radical catalysed oxidation reactions were measured and compared to the k_H/k_D ratio for TEMPO⁺-ClO₄⁻ under the same conditions (Table 1). The k_H/k_D ratios are in the same range, consistent with the intermediacy of o xo ammonium ions in the laccase-TEMPO system. Small differences may be attributed to differences in reaction conditions (e.g. ClO₄⁻ as anion).

<table>
<thead>
<tr>
<th></th>
<th>AB (pH 4.5)</th>
<th>W-T (1:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laccase-TEMPO</td>
<td>2.05</td>
<td>2.32</td>
</tr>
<tr>
<td>Laccase-Oh-TEMPO</td>
<td>2.54</td>
<td>2.61</td>
</tr>
<tr>
<td>Laccase-Aa-TEMPO</td>
<td>2.51</td>
<td>3.19</td>
</tr>
<tr>
<td>TEMPO⁺-ClO₄⁻</td>
<td>3.58</td>
<td>2.97</td>
</tr>
</tbody>
</table>

Table 1 Kinetic isotope effect (k_H/k_D)

Substrate: \( p \)-methylbenzyl alcohol-\( 1d \): 125 mM, Med/Sub: 15 mol%, Lac/Sub: 47 U/mmol, TEMPO⁺-ClO₄⁻: 1 equiv., 24 h

We found that the o xo ammonium cation derived from TEMPO is unstable in acidic buffer. When the perchlorate salt (TEMPO⁺-ClO₄⁻) was incubated in AB at pH 4.5, the formation of the TEMPO radical from TEMPO⁺-ClO₄⁻ was 17 % and 32 % in 2 h and 22 h, respectively, at room temperature under air atmosphere. In other words, the decomposition of o xo ammonium ion competes with the oxidation of alcohol by the o xo ammonium ion.

These results can also explain the above deviation of reaction rate from first order (Figure 4) at lower TEMPO amounts. In the laccase-TEMPO system, TEMPO is first oxidised by laccase to form an o xo ammonium ion, which then converts the alcohol to the corresponding aldehyde or ketone. Initially, a constant amount of TEMPO is efficiently converted into the o xo ammonium cations, which subsequently oxidise the alcohol. At longer reaction times, however, the reaction slows down. This could be due to the fact that o xo ammonium ion undergoes side reactions, in addition to its conversion to TEMPO
(as shown in Chapter 2, Figure 11), leading to a decreased availability. Only when TEMPO is used in relatively large amounts can the supply of oxoammonium ion be sufficient to maintain smooth oxidation of the alcohol.

We also found that deactivation of laccase occurs in the presence of TEMPO and oxygen, resulting in a decrease of the substrate conversion. This was observed in the oxidation of benzyl alcohol with a preincubated solution of laccase-TEMPO (Table 2).

<table>
<thead>
<tr>
<th>Entry</th>
<th>Pre-incubation time (h)</th>
<th>Conv (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>60.5</td>
</tr>
<tr>
<td>2</td>
<td>19</td>
<td>27.7</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>19.0</td>
</tr>
<tr>
<td>4</td>
<td>19</td>
<td>34.7</td>
</tr>
<tr>
<td>5</td>
<td>19</td>
<td>36.9</td>
</tr>
<tr>
<td>6</td>
<td>19</td>
<td>37.6</td>
</tr>
<tr>
<td>7</td>
<td>19</td>
<td>53.2</td>
</tr>
<tr>
<td>8</td>
<td>19</td>
<td>42.1</td>
</tr>
<tr>
<td>9</td>
<td>19</td>
<td>46.6</td>
</tr>
</tbody>
</table>

*Conditions: [Sub]$_0$: 140 mM, Lac/Sub: 25 U/mmol, TEMPO/Sub: 10 mol%, 4 h.  
*Extra addition of TEMPO (10 mol%);  
*Extra addition of laccase (25 U/mmol);  
*Reaction time: 8 h;  
*(Lac + TEMPO) incubated under N$_2$ atm.;  
*Laccase alone incubated under N$_2$ atm.;  
*Laccase alone incubated under O$_2$ atm.

When laccase was preincubated with TEMPO, under oxygen atmosphere in acetate buffer (pH 4.5) at room temperature, for a certain time before adding the substrate, the conversion of alcohol decreased compared with no preincubation time (entries 1, 2, 3), even when an extra amount of TEMPO (entry 4) or extra laccase (entry 5) was added to the reaction system. As we already know from previous experiments (vide supra) that oxoammonium is not stable in water, the results in Table 2 also indicate that laccase is unstable in the presence of oxoammonium. The deactivation of laccase is presumably due to degradation via oxidation of essential amino acid residues or the glycosyl moieties of the enzyme. On the other hand, when the reaction time was prolonged (entry 6), the conversion was improved. This suggests that there is little further deactivation of the laccase once the alcohol substrate has been added, i.e., the oxoammonium cation reacts
with the alcohol rather than with the laccase.

To confirm the instability of laccase in the presence of o xo ammonium cation, the laccase-TEMPO was also incubated under nitrogen. We assume that under nitrogen atmosphere o xo ammonium cation can not be formed catalytically from TEMPO by laccase oxidation, thus the enzyme should not be deactivated. Indeed, in this case the activity of laccase-TEMPO was much higher than upon incubation under oxygen (entry 7). In the absence of TEMPO, there was no significant difference between the incubation of laccase under nitrogen (entry 8) and under oxygen (entry 9).

3. Conclusions

Laccase catalysed aerobic oxidation of alcohols mediated by TEMPO and its derivatives is a rather complex process. The role of TEMPO and its derivatives is to form an o xo ammonium intermediate, which oxidises the alcohol to aldehyde or ketone. Because of the instability of the o xo ammonium salt of TEMPO and its derivatives, and/or the deactivation of laccase by reaction with the o xo ammonium cation, a relatively large amount of TEMPO or laccase is necessary for smooth conversion of alcohols.

4. Experimental

4.1 Enzyme and chemicals

Laccase from Coriolus versicolour (CvL) was purchased from Juelich Fine Chemicals as a lyophilized powder. The content of the protein is 8.8 mg/g solid (data from the enzyme supplier). TEMPO was obtained from BASF, Germany. ABTS was purchased from Fluka. All the liquid alcohols were distilled before use. The other chemicals were purchased from Aldrich or Acros and used as received. 0.1 M acetate buffer at pH 4.5 was used for preparing solutions for activity assay and as oxidation reaction medium unless otherwise specified.

4.2 Analysis

GC analyses were performed on a Varian GC with column CP Wax 52CB (50m ×
0.53mm, o.d. 0.70mm, df 2.0). Temperature programme (70 °C for 9 min, then increased at a rate of 10 °C/min to 250 °C for 6 min) was used on either column. Conversions and yields were calculated on basis of remaining alcohols using dodecane or t-amyl alcohol as internal standards.

$^1$H NMR spectra were collected on a 300 MHz Varian Inova spectrometer.

IR analyses were done on Perkin-Elmer Spectrum One FT-IR Spectrometer.

UV measurements were run with a Varian Cary UV-Vis Spectrophotometer.

4.3 Oxidation reaction

General procedure:

The substrate and internal standard were added into 0.1 M acetate buffer, followed by addition of laccase (from *Coriolus versicolor*) and TEMPO. The reaction system was connected with an oxygen cylinder to keep the oxygen constant, and stirred at room temperature. Samples were taken at certain time intervals and quenched with diethyl ether and dried over anhydrous Na$_2$SO$_4$ before GC analyses.

Intramolecular kinetic isotope effect ($k_H$/$k_D$):

α-Monodeutero-p-methylbenzyl alcohol was used as the substrate in the above procedure. After complete conversion of alcohol to aldehyde (monitored with TLC), the reaction mixture was quenched with MTBE and dried over Na$_2$SO$_4$. Removal of the solvent under vacuum gave a mixture of TEMPO and p-methylbenzaldehyde. Both labeled and unlabeled aldehydes were isolated and purified by column chromatography using petroleum-CH$_2$Cl$_2$ (5:5). The $k_H$/$k_D$ was determined by $^1$H NMR for the intensity of α-proton.

Oxidation of benzyl alcohol with preincubated solution of laccase-TEMPO:

Laccase and TEMPO were added into 0.1 M acetate buffer (pH 4.5). The mixture was stirred for a few minutes till the TEMPO dissolved, and then left at room temperature under oxygen atmosphere for a certain time (Table 2). Then benzyl alcohol and internal standard hexadecane were added to this solution and the mixture was stirred for the desired time, followed by extraction with diethyl ether and work-up as mentioned above.
4.4 Preparation of TEMPO-ClO$_4$ and TEMPO-BF$_4$ and their use in oxidation of alcohols [Bobbitt, 1998]

To a suspension of TEMPO (3.95 g, 25.3 mmol) in water (20 ml) was added dropwise a solution of 70% HClO$_4$ (3.66 g, 25.6 mmol) in water (5 ml) under ice-cooled conditions in 15 min, followed by addition of 15% NaOCl solution (6.4 g, 12.8 mmol) with ice-cooling in 10 min. The slurry reaction mixture was stirred for further 1 h at below 4 °C, and then the yellow precipitate was filtered and washed with 5% NaHCO$_3$ solution, ice-water, and ether. The solid was dried under vacuum overnight to give the product TEMPO-ClO$_4$ 3.0 g (yield 46.4%). m.p 148-149 °C.

TEMPO-BF$_4$ was prepared by the same procedure as above, using 35 % HBF$_4$ (6.5 g, 25.3 mmol) instead of 70% HClO$_4$. Yield 9.0%, m.p. 160-161 °C.

The oxidation of alcohols by oxoammonium salt was carried out stoichiometrically with equimolar amounts of alcohol and oxoammonium salt at room temperature.
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Chapter 5

Preparation of Chiral Alcohols by Chemo-Enzymatic Kinetic Resolution and Asymmetric Synthesis

Abstract: Enantiomerically pure secondary alcohols can be prepared by asymmetric hydrogenation (AH) or asymmetric transfer hydrogenation (ATH) of achiral ketones, or by kinetic resolution of racemic alcohols. Kinetic resolutions are achieved via chemical or enzymatic catalysis. The enzymatic kinetic resolutions (EKR) and EKR with in situ racemisation of the unreacted enantiomer, i.e. dynamic kinetic resolution (DKR) are reviewed. Methods for the synthesis of enantiomerically pure cyanohydrins are reviewed as a class of functionalized and versatile secondary alcohols.
1. Introduction

Chiral secondary alcohols are widely used as intermediates and chiral auxiliaries for the synthesis of pharmaceuticals and other fine chemicals [Sheldon, 1993; Stinson, 1992]. Unlike chiral acids or amines, which can be resolved via diastereomeric crystallization of appropriate salts, alcohols do not form salts, thus cannot be obtained by this direct method.

Enantiomerically pure alcohols are usually obtained by asymmetric hydrogenation or transfer hydrogenation (ATH) of achiral ketones, or by (dynamic) kinetic resolution of racemic alcohols. Many transition-metal complexes, with variations at the metal center or the ligand moiety, have shown efficiency in the ATH. They are the subjects of many reviews, for example by Chen et al. [2002], Noyori and Ohkuma [2001], Everaere et al. [2001], Yamakawa et al. [2000], Petra et al. [2000], Alonso et al. [1999], and Noyori and Hashiguchi [1997].

Kinetic resolution of secondary alcohols involves chemical kinetic resolution (CKR) or enzymatic kinetic resolution (EKR). Transition metal complexes play an important role in CKR. The subject has been extensively reviewed [Somfai, 2000]. For example, palladium-catalysed aerobic oxidative kinetic resolution of secondary alcohols using (-)-sparteine as the chiral ligand is a potentially interesting method [Ferreira and Stoltz, 2001; Jensen et al. 2001; Mueller and Sigman, 2003].

It is worth noting that chiral nitroxy radicals [Naik and Braslau, 1998; Arterburn, 2001] have also been used for the resolution of racemic alcohols, either by chemical or by electro-oxidative enantioselective oxidations. Examples include chiral nitroxy radical 1 [Kashiwagi et al. 1999; Ma et al. 1993] and 2 [Kuroboshi et al. 2000], and nitroxy peptides [Formaggio et al. 2002]. In the presence of a chiral base, e.g. (-)-sparteine, achiral TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) mediated electro-oxidation of racemic alcohols can also afford (R)-alcohols with up to 99% ee [Rychnovsky et al. 1996; Kashiwagi et al. 1996]. The use of nitroxy radicals can avoid using metal compounds and in some cases toxic reagents. Moreover, nitroxy radical-mediated oxidations are usually carried out under mild conditions [see chapters 1-4].

In this chapter, enzymatic kinetic resolution (EKR), with emphasis on EKR with in situ racemisation, i.e. dynamic kinetic resolution (DKR), is reviewed. Methods for the
synthesis of enantiomerically pure cyanohydrins, a class of versatile, functionalized secondary alcohols, are also reviewed.

2. Enzymatic Kinetic Resolution (EKR)

Enzyme-catalysed enantioselective hydrolyses, esterifications or transesterifications are especially useful methods for the preparation of enantiomerically pure alcohols [Faber and Riva, 1992; Roberts, 1999]. For example, secondary alcohols were resolved by *Aspergillus niger* aminoacylase catalysed transesterification, giving the corresponding (R)-esters in 50% yield with enantioselectivity $E > 500$ [Bakker et al. 2000]. Optically active aryl trifluoromethyl carbinols (3) are important intermediates in the synthesis of polyfunctional bioactive molecules [Abouabdellah et al. 1998]. They have been prepared by lipase catalysed enantioselective acylation of the corresponding racemic alcohols with vinyl acetate or by enantioselective hydrolysis of the racemic esters [Kato et al. 2001].

Enzymatic transesterification of alcohols is reversible and usually requires a large excess of acyl donor to achieve a reasonable degree of conversion [Cambou and Klibanov, 1984]. In order to render the process irreversible, various activated esters such as vinyl acetate (VA) [Degueil-Castaing et al. 1987; Kaminska et al. 1996], isopropenyl acetate (IPA) [Wang and Wong, 1988; Ghanem and Schurig, 2001], trifluoroethyl esters [Hoff et al. 2000], 2,2,2-trichloroethyl esters [Kirchner et al. 1985], cyanomethyl esters
Preparation of chiral alcohols by chemo-enzymatic catalysis

[West et al. 1988], and acid anhydrides [Bianchi et al. 1988] have been used. Among them, the enol esters are the most popular acyl donors [Hanefeld, 2003]. A kilogram scale preparation of enantiomerically pure aralkyl alcohols via lipase-catalysed acylation of secondary alcohols with succinic anhydride has been achieved in organic solvent [Gutman et al. 1993]. The major advantage of this method is the ease of separating the ester from the unreacted alcohol.

The moderate enantioselectivity of the esterifications of racemic alcohols with vinyl acetate can be improved by using other vinyl esters which are more bulky in the acyl moiety [Ottosson and Hult, 2001; Nakamura et al. 1998], such as vinyl propanoate and vinyl butanoate [Peter et al. 1998] or vinyl 3-arylpropanoates [Kawasaki et al. 2001]. Microwave irradiation was also found to be effective in increasing both the reaction rates and enantioselectivities of lipase-catalysed acylation of alcohols [Lin and Lin, 1998].

A more reactive acyl donor, 1-ethoxyvinyl ester (4), has recently been reported for lipase catalysed kinetic resolution of alcohols (Scheme 1) [Kita et al. 2000]. The advantages of this ester include the formation of an unreactive co-product, ethyl acetate, unlike the acetaldehyde liberated from vinyl acetate (VA), which deactivates some lipases [Weber et al. 1997]. The reactivity of 1-ethoxyvinyl ester is 2-3 times higher than that of VA in the reactions using C. rugosa lipase.

\[
\begin{align*}
\text{R}^1\text{R}^2\text{OH} & \quad \overset{\text{lipase}}{\underset{\text{R}^3\text{OCOCH}_3}{\longrightarrow}} \\
\text{R}^1\text{R}^2\text{O}^\text{Et} & \quad \text{VA: R}^3 = \text{H} \\
\text{R}^1\text{R}^2\text{O}^\text{Me} & \quad \text{IPA: R}^3 = \text{Me} \\
\end{align*}
\]

Scheme 1 Lipase-catalysed kinetic resolution of alcohols

Lipase B from Candida antarctica (CAL-B, commercial name Novozym 435) shows a high preference for the acylation of (R)-enantiomers of chiral alcohols [Orrenius et al. 1996]. Among the lipases used for enantioselective esterification of secondary alcohols, CAL-B was shown to afford the highest enantioselectivity (E), compared with Lipozyme (lipase from Rhizomucor miehei) and lipases from Pseudomonas sp. and Candida rugosa [Brown et al. 2000; Wehtje et al. 1997].
3. Dynamic Kinetic Resolution (DKR)

The obvious disadvantages of kinetic resolution processes are the maximum yield of 50% and consequent laborious separation of the product from the remaining substrate [Strauss and Faber, 1999]. Therefore, dynamic kinetic resolution (DKR), which combines kinetic resolution with in situ racemisation of the unreactive enantiomer, has attracted increasing interest from both academic and industrial laboratories.

![Scheme 2 Kinetic resolution with in situ racemisation](image)

Lipase-catalysed acylations of chiral alcohols can be performed in organic solvents with high chemo-, regio- and enantioselectivity. Hence, enzymatic resolution with lipases, coupled with in situ racemisation, is a potentially interesting DKR approach to the synthesis of enantiomerically pure alcohols. As shown in Scheme 2, in order to obtain 100% yield of the desired product (S)-B, the racemisation step should be faster than the formation of (R)-B.

Recently, many reviews covering the concept of DKR have appeared, with emphasis on the combination of lipase-catalysed resolution coupled with transition metal-catalysed racemisation [Kim et al. 2002; Huerta et al. 2001; Azerad and Buisson, 2000; El Gihani and Williams, 1999]. In this chapter a brief review and some recent new examples are presented.

3.1 DKR with ruthenium-catalysed racemisation

Ruthenium complexes are, up to now, the most efficient and extensively studied catalysts for the racemisation of secondary alcohols. A pioneering example was reported by the Bäckvall group, using the dimeric ruthenium complex 5 [Larsson et al. 1997].
Racemisation of (R)-1-phenylethanol was complete in 45 h with only 2 mol% of 5. However, this catalyst was not compatible with the conventional acyl donors such as vinyl acetate and isopropenyl acetate, because the acetaldehyde or acetone that is generated from the acyl donors acts as a hydrogen acceptor and oxidises the substrate alcohol to ketone. This problem was solved by employing p-chlorophenyl acetate as the acyl donor. Although the reaction with p-chlorophenyl acetate is slower than with isopropenyl acetate [Choi et al. 2002], the formed p-chlorophenol does not react with the catalyst 5. This methodology has been successfully applied to the DKR of racemic benzylic or aliphatic secondary alcohols [Persson et al. 1999], β-azido alcohols [Pàmies and Bäckvall, 2001], diols [Kim et al. 2001], chloro alcohols [Pàmies and Bäckvall, 2002], and hydroxyacid derivatives [Huerta and Bäckvall, 2001; Huerta et al. 2000].

The DKR reaction of alcohols using 5 was carried out under argon atmosphere and one equivalent of acetophenone was added to balance the hydrogen-consuming process, which leads to the oxidation of the substrate into ketone. Subsequent experiments showed that when the reaction was performed in the presence of a catalytic amount of oxygen (2 mol%), the additional ketone is not necessary [Koh et al. 1999]. This was probably due to the effect of in situ formed acetophenone during the DKR.

Park and co-workers reported an indenyl-rhenium complex 6 to be an excellent racemisation catalyst for alcohols. This catalyst can effectively racemise secondary alcohols at room temperature in the presence of strong base KOH [Koh et al. 1998]. However, efforts to combine this catalyst with enzymatic resolution failed because the acyl donor was hydrolysed by KOH. When 3 equivalents of triethylamine were used, instead of KOH, 1-phenylethanol was selectively acylated with p-chlorophenyl acetate under DKR conditions in 85% yield and 96% ee [Koh et al. 1999].

A more recent ruthenium catalyst reported by Park and co-workers is the aminocyclopentadienyl ruthenium chloride 7, which showed high racemisation activity in the presence of a catalytic amount of KOBu⁺ [Choi et al. 2002]. This catalyst racemises
secondary alcohols rapidly at room temperature without the aid of hydrogen mediators. Moreover, it is compatible with both lipases and isopropenyl acetate.

The Sheldon group has reported that ruthenium complexes 8 and 9 are also effective racemisation catalysts for secondary alcohols with KOH or TEMPO (2,2,6,6-tetramethylpiperidinyl-1-oxy) as co-catalyst. In combination with CAL-B catalysed resolution, complex 9 showed high activity for in situ racemisation in toluene [Dijksman et al. 2002]. This system was the subject of study, described in Chapter 8, where various Ru-complexes were used in DKR of racemic alcohols.

In the DKR of allylic alcohols mediated by 10 and lipase from Pseudomonas cepacia (PCL), the (R)-esters were obtained in 90% yield and >99% ee [Lee et al. 2000]. Using Novozym 435 in combination with complex 10 (X = Cl) and (R,S)-α-methyl phenylglycinamide as the ligand, 1-phenylethanol was selectively acylated with isopropenyl butanoate to give the corresponding (R)-ester in 97% yield and >99% ee [Verzijl et al. 2001].
3.2 DKR with other racemisation methods

The first report on the application of transition metal catalysts for the racemisation of secondary alcohols in enzymatic resolution processes was published by the group of Williams [Dinh et al. 1996]. The same group used a palladium complex, PdCl₂(MeCN)₂, together with a hydrolase in the DKR of cyclic allylic acetates, giving the corresponding alcohols in moderate yields and ee's [Allen and Williams, 1996]. Recently, Kim and co-workers [Choi et al. 1999] reported that in lipase PCL or CAL-B catalysed resolution of acyclic allylic acetates mediated by Pd(PPh₃)₄-phosphine complex, the alcohols were formed in >80% yield and >98% ee.

Racemisation of cyanohydrins with bases has been combined with enzymatic kinetic resolution, using PCL [Inagaki et al. 1992] or CAL-B [Li et al. 2002]. The alkaline anion exchange resin, Amberlite IRA904, efficiently catalyses the racemisation of mandelonitrile and does not inhibit CAL-B catalysed acylation. This is the subject of Chapter 7 in this thesis.

Spontaneous racemisation under the reaction conditions was observed in the enzymatic kinetic resolution of 6-hydroxypyranone [van den Heuvel et al. 1997] and 5-hydroxy-2H-furanone and 5-hydroxy-2H-pyrrolinone [van der Deen et al. 1996]. The racemisation of hemiacetal was presumed to proceed via reversible ring-opening of the pyranone (Scheme 4).

\[
\begin{align*}
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{OH} \\
\text{O} & \quad \text{OH} \\
\end{align*}
\]

Scheme 4 Racemisation of cyclic hemiacetal

In the presence of triethylamine, the racemic tricyclic acyloan (±)-endo-3-hydroxytricyclo[4.2.1.0²,⁵]non-7-en-4-one was dynamically resolved via the transient formation of the meso-enediol isomer (Scheme 5) under lipase-catalysed
transesterification conditions to give the single chiral acetate \((-\)-endo-3-acetoxytricyclo[4.2.1.0\(^2,5\)]non-7-en-4-one in 75% yield and 97% ee [Taniguchi and Ogasawara, 1997].

Scheme 5 Dynamic kinetic resolution involving a meso enediol intermediate

4. Chiral Cyanohydrins: Application and Preparation

Cyanohydrins, or \(\alpha\)-hydroxy nitriles, are important building blocks in synthetic chemistry. They are widely used in the preparation of a number of industrially important classes of fine chemicals [Kruse, 1992; Effenberger, 1994; Kanerva, 1996; Johnson and Griengl, 1997; North, 2003]. They can be converted into \(\alpha\)-hydroxycarboxylic acids, \(\alpha\)-hydroxyaldehydes, acyloins, vicinal diols, \(\beta\)-amino alcohols and \(\beta\)-hydroxy-\(\alpha\)-amino acids. In the agrochemical industry, for example, a range of commercially important and environmentally benign pyrethroid insecticides, such as cycloprothrin, fluvanilate, deltamethrin, and cypermethrin, are derivatives of the cyanohydrin of \(m\)-phenoxybenzaldehyde [Vijverberg, 1988; Ramos Tombo and Bellus, 1991; Krief, 1994]

Cyanohydrins are formed by the addition of hydrogen cyanide (HCN) to aldehydes or ketones as shown in Scheme 6. The direct addition process utilises highly toxic gaseous HCN. To avoid handling free HCN gas, a cyanide salt can be used for \textit{in situ} generation of HCN in acidic buffer was employed [Smitskamp-Wilms \textit{et al.} 1991].

\[
\begin{align*}
\text{RCOR}^1 + \text{HCN} &\rightleftharpoons \text{RCOHN}^\text{R}^1^\text{R}^2
\end{align*}
\]

Scheme 6 Formation of cyanohydrins from hydrogen cyanide and carbonyl compounds
Preparation of chiral alcohols by chemo-enzymatic catalysis

Alternatively acetone cyanohydrin can be used for the transhydrocyanation of ketones or aldehydes. This process involves two successive steps: acetone cyanohydrin decomposes to HCN and acetone under basic conditions, followed by addition of HCN to carbonyl compounds (Scheme 7) [Klempier et al. 1993].

\[
\text{HO-CN} \rightleftharpoons \text{O} + \text{HCN}
\]

\[
\text{R}^{1}\text{R}^{2} + \text{HCN} \rightleftharpoons \text{HO-CN}
\]

Scheme 7 Transhydrocyanation from acetone cyanohydrin to carbonyl compounds

Many cyanohydrins of importance as building blocks in organic synthesis are chiral and are used in enantiomerically pure form [Brussee and van der Gen, 2000]. Enantiomerically pure cyanohydrins can be prepared by asymmetric synthesis or by (dynamic) kinetic resolution.

4.1 Abiological catalytic asymmetric synthesis

By mimicking the highly stereospecific characteristics of natural enzymes, synthetic cyclic dipeptides 11 were found to be capable of catalysing the addition of HCN to aldehydes, giving optically active cyanohydrins [Oku and Inoue, 1981].

\[
\text{R}^{1}\text{O-NN-CO-}\text{R}^{2}
\]

A variety of cyclic dipeptides has been studied [Hulst et al. 1997; Gregory 1999]. Among them cyclo[((S)-Phe-(S)-His] 11 (R\(^{1}\) = benzyl, R\(^{3}\) = imidazoyl methyl, R\(^{2}\) = R\(^{4}\) = H) was the first and the most extensively studied catalyst for asymmetric synthesis of cyanohydrins from either aliphatic or aromatic aldehydes [Asada et al. 1985; Kobayashi et al. 1986]. It catalysed asymmetric addition of HCN to benzaldehyde, for example, giving (R)-mandelonitrile with ee up to 97% [Tanaka et al. 1990].

A number of metal-complexes have also been used as catalysts for the asymmetric synthesis of non-racemic cyanohydrins. The complex formed from titanium alkoxide and the Schiff’s base modified acyclic dipeptide esters 12 enantioselectively catalyses the
enantioselective hydrocyanation of benzaldehyde to give (R)-mandelonitrile with ee > 88% [Mori et al. 1991].

When $N$-(3,5-dibromosalicylidene)-(S)-valine piperidide was used as the ligand, an opposite (S)-cyanohydrin was obtained with up to 97% ee [Nitta et al. 1992]. The titanium-Schiff's base complex accepts a variety of aldehydes, giving the corresponding cyanohydrins with ee up to 96% [Hayashi et al. 1994].

Other chiral titanium complexes have been used, including 13 [Minamikawa et al. 1988], titanium-diisopropyl tartrate [Hayashi et al. 1992] and a complex formed from Ti(i-PrO)$_4$ and the chiral ligand 14.

Chiral salen-type compounds were recently found to be effective ligands for metal-catalysed asymmetric trimethylsilylcyanation of aldehydes. For example, the complex formed from Ti(i-PrO)$_4$ and ligand 15 afforded (R)-mandelonitrile with up to 87% ee [Pan et al. 1996]. Other aromatic and aliphatic cyanohydrins, however, were obtained only with 22-87% ee using this complex [Jiang et al. 1997].
Using 16 (R = R' = H) as the ligand, Ti(i-PrO)₄ catalysed the trimethylsilylcyanation of aldehydes to their trimethylsilylated (S)-cyanohydrins with 62-77% ee [Belokon et al. 1996]. The more sterically hindered ligand with R = R' = t-butyl gave the (S)-cyanohydrins in up to 92% ee [Belokon et al. 1997]. However, this catalyst system was not effective for the asymmetric trimethylsilylcyanation of ketones.

The trimethylsilylcyanation of ketones is catalysed by a bimetallic Ti-salen-type complex 17, affording trimethylsilylated cyanohydrins in up to 100% yield with 72% ee. The most interesting characteristic of this catalyst is that the reaction can be run at room temperature and atmospheric pressure, with a catalyst loading of only 0.5 mol% [Belokon et al. 1999].

In addition to titanium, other metals, in combination with salen-type ligands, have been used for the asymmetric synthesis of non-racemic cyanohydrins: zirconium alkoxides [Yamasaki et al. 2001], aluminum(III) [Hamashima et al. 1999, 2001], magnesium(II) [Corey and Wang, 1993], and lanthanum(III) [Qian et al. 1998; Aspinall et al. 1999]. The complex of vanadium(IV) and 16 (R = R' = t-butyl) [Belokon et al. 2000] was shown to be effective in loadings as low as 0.1 mol%, converting aromatic and
aliphatic aldehydes into the corresponding (S)-trimethylsilylated cyanohydrins with 68-95% ee. However, this complex was much less effective for the reaction of ketones.

The recoverable chiral monometallic aluminum catalyst 18 was recently shown to be highly effective for the trimethylsilylcyanation of aldehydes; the best result was >99% ee and 99% yield [Casas et al. 2002], with (substituted) benzaldehyde as the substrates. The loading of the catalyst was 10 mol%.

![Diagram of catalyst 18]

4.2 Oxynitrilase catalysed asymmetric synthesis

The main disadvantages of chemo-catalytic methods, such as low temperature (<-40 °C), low substrate concentration (0.2-0.6%) and vulnerable catalysts severely limit their industrial applications. In contrast, biocatalysis proceeds under mild conditions, and enzymes are easier to handle.

Enzymes that catalyse the formation and cleavage of cyanohydrins are known as oxynitrilases, or hydroxynitrile lyases (HNLs). In nature, these enzymes catalyse the cleavage of a glycogenic cyanohydrin to release hydrocyanic acid (HCN) as a response to cell damage. Additionally, the HCN liberated functions as a nitrogen source in the cell’s amino acid synthesis [Johnson et al. 2000]. The HNLs are widely distributed in around 3000 plants [Hickel et al. 1996], but HNLs from only 11 cyanogenic plants have been purified and characterized [Schmidt and Griengl, 1999]. HNL catalyses the reversible formation of cyanohydrins from the corresponding aldehydes or ketones and HCN or from acetone cyanohydrin in a transhydrocyanation process. In the presence of an excess of HCN or acetone cyanohydrin, the formation of cyanohydrins takes the priority (Scheme 7).

Although the first report on the preparation of cyanohydrins catalysed by HNLs was published nearly a hundred years ago [Rosenthaler, 1908], and the low optical activity was explained one decade later [Kriebel and Wieland, 1921], there was not much progress until the 1960’s when an enzyme isolated from bitter almonds (Prunus
Preparation of chiral alcohols by chemo-enzymatic catalysis

amygdalus), PaHNL, was investigated in the synthesis of (R)-cyanohydrins [Becker et al. 1966]. (R)-Mandelonitrile was obtained in an optical purity of 90% and a yield of 96%. This was the first breakthrough in the utilisation of HNLs for preparative synthesis of non-racemic cyanohydrins. A second important advance in the use of HNLs in synthetic applications was achieved in the mid-1990's by application of cloning and genetic modification techniques. Several expression systems were found to be suitable for the recombinant production of HNLs, including Escherichia coli, Saccharomyces cerevisiae, and Pichia pastoris [Hasslacher et al. 1996; Forster et al. 1996; Breithaupt et al. 1999]. This opened the door to commercial application of HNLs. *Hevea brasiliensis* is currently produced on scales of up to 50,000 L by overexpression [Griengl et al. 2000].

The HNLs that are currently used in the preparation of non-racemic cyanohydrins are shown in Table 1. PaHNL, MbHNL, and HbHNL tolerate considerable structural variation in substrates. They accept a range of aliphatic, aromatic and heteroaromatic aldehydes and ketones. In contrast, LuHNL only converts aliphatic aldehydes and ketones into (R)-cyanohydrins, whereas SbHNL is only stereoselective with aromatic aldehydes and ketones, affording (S)-cyanohydrins.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Natural substrate</th>
<th>Abbrev.</th>
<th>Speci.</th>
<th>EC number</th>
<th>Substrate</th>
<th>R¹</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Prunus amygdalus</em></td>
<td>(R)-mandelonitrile</td>
<td>PaHNL</td>
<td>R</td>
<td>4.1.2.1</td>
<td>Alkyl,</td>
<td>H,</td>
<td></td>
</tr>
<tr>
<td>(almonds)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>aryl, allyl, alkyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Linum usitatissimum</em></td>
<td>acetone</td>
<td>LuHNL</td>
<td>R</td>
<td></td>
<td>Alkyl</td>
<td>H,</td>
<td></td>
</tr>
<tr>
<td>(flax seedlings)</td>
<td>cyanoxydrin</td>
<td></td>
<td></td>
<td></td>
<td>alkyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sorghum bicolor</em></td>
<td>(S)-4-hydroxy-man</td>
<td>SbHNL</td>
<td>S</td>
<td>4.1.2.1</td>
<td>Aryl</td>
<td>H,</td>
<td></td>
</tr>
<tr>
<td>(millet seedlings)</td>
<td>delonitrile</td>
<td></td>
<td></td>
<td></td>
<td>alkyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Manihot esculenta</em></td>
<td>Acetone</td>
<td>MeHN</td>
<td>S</td>
<td>4.1.2.3</td>
<td>Alkyl,</td>
<td>H,</td>
<td></td>
</tr>
<tr>
<td>(manioc leaves)</td>
<td>cyanoxydrin</td>
<td></td>
<td></td>
<td></td>
<td>aryl, allyl, alkyl</td>
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<td></td>
</tr>
<tr>
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<td>(S)-butan-2-one cyanoxydrin</td>
<td>MeHN</td>
<td>S</td>
<td>4.1.2.3</td>
<td>Alkyl,</td>
<td>H,</td>
<td></td>
</tr>
<tr>
<td><em>Hevea brasiliensis</em></td>
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<td>HbHNL</td>
<td>S</td>
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<td>H,</td>
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<tr>
<td>(rubber tree leaves)</td>
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<td></td>
</tr>
</tbody>
</table>

Since PaHNL is readily available in large amounts from almond meal, it is the first choice for the preparation of (R)-cyanohydrins. It catalysed formation of (R)-cyanohydrins in moderate to good yield (54-90%) with moderate to high ee (76-98%)
[Effenberger et al. 1987; Johnson and Griengl, 1999]. PaHNL, adsorbed on crystalline cellulose, showed excellent enantioselectivity, in diisopropyl ether, for the addition of HCN to aromatic, heteroaromatic, aliphatic, and allylic aldehydes [Effenberger 2000].

As shown in Table 1, SbHNL, MeHNL and HbHNL are (S)-specific oxynitriilases. SbHNL from millet (Sorghum bicolor) was the first (S)-HNL used for cyanohydrins synthesis. SbHNL catalysed the addition of HCN to aromatic aldehydes, but was not effective with aliphatic aldehydes and ketones as substrates [Effenberger et al. 1990; Kiljunen and Kanerva, 1994].

Aromatic and some heteroaromatic (S)-cyanohydrins were also prepared in high ee by using the HbHNL in aqueous buffer, but some nitrogen containing heteroaromatic carbonyl compounds, e.g. pyridine-2-, -3-, -4-aldehyde, afforded racemic cyanohydrins due to blank non-enzymatic hydrocyanation. Phenoxy- and benzyloxy-substituted aliphatic aldehydes also gave racemic cyanohydrins [Schmidt et al. 1996]. In this case it turned out that the oxygen atom in the vicinity of the aldehyde moiety has influence on the enantioselectivity [Roda et al. 1999]. HbHNL has also been used for the synthesis of α,β-unsaturated (S)-cyanohydrins [Klempier et al. 1995]. However, both HbHNL and PaHNL did not show chiral discrimination between the enantiomers of α- and β-substituted alkyl or alkoxy aldehydes [Roda et al. 2002], thus are not suitable for the resolution of these substrates.

In order to avoid the use of purified enzymes, thereby reducing costs in terms of time and money, the apple, apricot, cherry and plum meals as well as almond meal were used as sources of hydroxynitrilase for the synthesis of aliphatic and aromatic cyanohydrins with high enantiopurity (usually >90% ee). Apple meal is more effective than almond meal with sterically hindered aldehydes (e.g. pivalaldehyde) as substrates [Zandbergen et al. 1991; Kiljunen and Kanerva, 1997a; 1997b].

In general, enzymatic activity is higher in water than in organic media. However, reaction in water generally leads to lower enantiomeric purity of the products because the competing non-enzymatic reaction is more favoured in aqueous media. Furthermore, product racemisation in the aqueous phase leads to decreased enantioselectivities. In order to suppress the non-enzymatic addition of HCN to aldehydes, low pH values (3-4.5) are needed [Schmidt et al. 1996], but low pH decreases the enzyme activity [Hanefeld et al. 1999]. For example, at pH 4.0 HbHNL retains only 20% of its optimum (approximately at pH 6.0) catalytic activity [Johnson and Griengl, 1999].
Preparation of chiral alcohols by chemo-enzymatic catalysis

Therefore, biphasic mixtures were employed, in which the non-enzymatic reaction was suppressed to a large extent and the enantiomeric purity of the cyanohydrins was considerably increased [Effenberger et al. 1987]. For instance, in buffer/MTBE system, both allylic and heteroaromatic cyanohydrins were obtained in more than 95% conversion with above 98% ee, whereas in aqueous buffer most of them were obtained in much lower ee, in some cases as racemic products. In a mixture of 10% (v/v) of diisopropyl ether and aqueous buffer, (R)-cyanohydrins were obtained with up to 97% ee at 100% conversion by using powdered almond meal [Huhtanen and Kanerva, 1992].

Griengl et al. [1998] found that carboxylic esters and long chain or branched dialkyl ethers such as MTBE and DIPE were the most suitable solvents for HbHNL catalysed reactions. However, for the production of cyanohydrins with PaHNL in biphasic systems, van der Gen and co-workers [Loos et al. 1995] found that methyl tert-butyl ether (MTBE) was the preferred solvent, conversions being higher than in ethyl acetate. They also found that the temperature and pH of the system have significant effects on the ee of the product, but not on the conversion. On the other hand, increasing the ratio of buffer to organic solvent increases the conversion but decreases the ee of the product.

Recently, Lin and co-workers investigated the utilisation of crude (R)-HNLs from several sources in micro-aqueous organic solvents [Lin et al. 1999; Chen et al. 2001]. The (R)-cyanohydrins were prepared in high enantioselectivity from aromatic and aliphatic aldehydes and methyl ketones in the temperature range 4 to 30 °C.

As a safer alternative to free gaseous HCN, acetone cyanohydrin has been used as the cyanide source in transhydrocyanations. For example, PaHNL catalysed transhydrocyanation of a variety of aromatic and aliphatic aldehydes gave (R)-cyanohydrins with up to 99% ee [Ognyanov et al. 1991]. Similarly, transhydrocyanation catalysed by a crude extract from S. bicolour gave (S)-mandelonitrile with 90% ee in 91% yield [Kiljun and Kanerva, 1994]. SbHNL was also employed in transhydrocyanations to prepare (S)-heteroaromatic cyanohydrins with 80-90% ee in moderate to good yield [Effenberger and Eichhorn, 1997]

4.3 Lipase-catalysed kinetic resolution

Lipase-catalysed kinetic resolution of cyanohydrins includes enantioselective esterification of free cyanohydrins and enantioselective hydrolysis and transesterification of cyanohydrin esters. Compared to catalytic asymmetric synthesis of cyanohydrins from
prochiral aldehydes or ketones, kinetic resolution suffers from the disadvantages of low yield (max. 50%) and more steps, particularly in the separation of the reaction products. On the other hand, the cyanohydrin (ester) products are more stable towards racemisation under the conditions of lipase-catalysed resolutions in organic solvents.

Lipases from several sources can be used for the kinetic resolution. Their activities and enantioselectivities depend on the enzyme sources, substrates, and reaction conditions. For the lipase-catalysed transesterification (Scheme 8), lipases from *Pseudomonas* sp. [Roos et al. 1998] and *Candida antarctica* [Hanefeld et al. 2000; also see Chapter 6] gave (S)-cyanohydrins and (R)-esters, whereas lipase from *Candida rugosa* gives (R)-cyanohydrins and (S)-esters [Effenberger et al. 1991].

![Scheme 8 Lipase catalysed kinetic resolution of cyanohydrin ester](image)

By using n-butanol for the PCL-catalysed transesterification of α-cyano-3-phenoxybenzyl acetate (CPBAc), in diisopropyl ether (DIPE), the (S)-cyanohydrin was obtained with an ee >96% after 46% conversion. The remaining ester (R)-CPBAc was racemised by treatment with triethylamine in DIPE or toluene at 65°C [Fishman and Zviely, 1998].

A novel strategy has been developed for the preparation of (S)-3-phenoxybenzaldehyde cyanohydrin 19 from racemic α-cyano-3-phenoxybenzyl butyrate without separation of the labile (R)-cyanohydrin from the reaction mixture [Fadnavis et al. 2001]. The (R)-cyanohydrin was decomposed by aqueous imidazole to the corresponding aldehyde, which was recovered by distillation and reused as the starting material, whereas the (S)-cyanohydrin butyrate was hydrolyzed by PCL affording the (S)-cyanohydrins (Scheme 9).
Preparation of chiral alcohols by chemo-enzymatic catalysis

Scheme 9 One-pot bi-enzymatic resolution of α-cyano-3-phenoxybenzyl butyrate

*Candida antarctica* lipase B (CAL-B) shows the opposite enantiomer discrimination to *C. rugosa* in the kinetic resolution of cyanohydrin esters. It was shown to be highly enantioselective (E = 100) in the deacylation of mandelonitrile acetate, yielding the (S)-mandelonitrile and the (R)-acetate [Hanefeld *et al.* 2001]. A more general method was developed for CAL-B catalysed enantioselective transesterification of cyanohydrin acetates, using 1-propanol as the co-substrate. This is presented in Chapter 6.

In order to avoid racemisation of the labile cyanohydrins formed in the kinetic resolutions and/or elimination of HCN, a lipase-catalysed protection of the cyanohydrins was performed in concert with the lipase-catalysed kinetic resolution. This process gave access to both enantiomers in pure form and in good yields [Veum *et al.* 2002].

As mentioned earlier, the maximum yield in a conventional kinetic resolution process cannot exceed 50%. The unwanted enantiomer needs to be separated from the reaction mixture and can either be racemised in a separate step, or be discarded. This adds to the cost of the process. To overcome this disadvantage, kinetic resolution with *in situ* racemisation, *i.e.* dynamic kinetic resolution (DKR), is of interest.

An elegant approach to dynamic kinetic resolution of cyanohydrins was developed by combining a lipase-catalysed esterification with an anion-exchange resin-catalysed racemisation of cyanohydrins in one pot (Scheme 9) [Inagaki *et al.* 1991; 1992]. However, this process took as long as one week to reach completion. A further kinetic investigation was carried out on this process using CAL-B for the resolution of mandelonitrile. This is the subject of Chapter 7 in this thesis.

Scheme 9 One-pot chemo-enzymatic synthesis of enantiopure mandelonitrile acetate
Chapter 6

CAL-B Catalysed Enantioselective Synthesis of Cyanohydrins
- A Facile Route to Versatile Building Blocks

Abstract: A straightforward process for the preparation of optically active cyanohydrins, important building blocks for the synthesis of drugs and agrochemicals, has been established. The lipase B from Candida antarctica (CAL-B) catalyses the kinetic resolution of the racemic cyanohydrin acetates under mild conditions. Optimization of the process led to a user-friendly synthesis of (S)- and (R)-cyanohydrins.
CAL-B catalysed enantioselective synthesis of cyanohydrins

1. Introduction

Due to their multifunctional character, cyanohydrins, especially the non-racemic forms, have been applied widely in the synthesis of a range of biologically active compounds. Usually, the derivatization of cyanohydrins (Figure 1) can be performed: (1) at the cyano group; (2) at the hydroxy group; (3) at the chiral carbon center; (4) at the R substituent.

\[
\begin{align*}
H & \quad \text{OH} \\
R & \quad \text{CN}
\end{align*}
\]

Figure 1 General structure of chiral cyanohydrins

The derivatives of chiral cyanohydrins are important intermediates for the synthesis of drugs and agrochemicals [Kruse, 1992; Effenberger, 1994]. Therefore, the enantioselective synthesis of cyanohydrins has attracted considerable attention. The asymmetric synthesis of enantiomerically pure cyanohydrins can be catalysed by small molecules, e.g. peptides [Oku and Inoue, 1981; Hulst et al. 1997; Gregory et al. 1999], or by hydroxynitrile lyases [Griengl et al. 1997; Marcus et al. 1998; Johnson and Griengl, 1999].

Lipases catalysed kinetic resolution of racemic cyanohydrins is also an effective way to obtain enantiomerically pure cyanohydrins [Inagaki et al. 1992; Effenberger et al. 1991; Lundell et al. 1998]. However, there is no general and straightforward lipase catalysed formation of chiral cyanohydrins. The lipase B from Candida antarctica (CAL-B, the commercially available immobilized preparation is known as “Novozym 435” or “chirazyme L-2, c.-f., C2, Lyo”) is known for its exceptional stability under harsh reaction conditions and it can be used in a large variety of organic solvents [Anderson et al. 1998]. Recently, its successful application in the enantioselective deacylation of racemic mandelonitrile acetate, yielding (R)-mandelonitrile acetate and (S)-mandelonitrile, was described [Hanefeld et al. 2001].

Here we report the results of the optimization of these reaction conditions and their utilisation for the enantioselective formation of a range of different cyanohydrins (Scheme 1). The commercial preparation of CAL-B, chirazyme L-2, was used as the enzyme.
Scheme 1 Chemo-enzymatic preparation of chiral cyanohydrins from aldehydes. BTEAC: benzyltrimethylammonium chloride; CAL-B: *Candida antarctica* lipase B

2. Results and discussion

The racemic cyanohydrin acetates (*rac-2* in Scheme 1) necessary for this study were prepared in a straightforward one step procedure [Fishman *et al.* 1998]. The aldehydes 1, sodium cyanide and acetic anhydride were stirred in water/dichloromethane at 2-10 °C in the presence of a phase transfer catalyst. This process gave a quantitative conversion of the aldehydes. Thus, the use of any free HCN was avoided. The remaining cyanide in the reaction mixture was destroyed with alkaline 20% aqueous FeSO₄.

In the enzymatic transesterification reaction using 1-propanol, the racemic cyanohydrin acetate (*rac-2*) was converted into propyl acetate and (*S*)-cyanohydrin (*S*)-3, while the (*R*)-acetate (*R*)-2 remained unmodified. The configurations of (*S*)-3 a-j were elucidated by comparing their retention times on chiral HPLC to that of the corresponding compounds obtained by transcyanation between acetone cyanohydrin and the aldehydes, using the (*S*)-selective hydroxynitrile lyase from *Hevea brasiliensis* (HbHNL) [Griengl *et al.* 1998; Hanefeld *et al.* 2001] as the catalyst.

The main emphasis of this study was on the enzymatic step, i.e. the CAL-B catalysed kinetic resolution of racemic cyanohydrin acetates (*rac-2*). The reaction conditions were investigated, using 2a as a model substrate [Vijverberg, 1988; Mitsuda *et al.* 1990; Sheldon, 1993], by varying the temperature, the ratio of 1-propanol to cyanohydrin acetate as well as the enzyme concentration. Then the optimized conditions were applied for the resolution of different cyanohydrin acetates.
2.1 Effect of temperature

In general, one would expect that reaction at very low temperatures would result in poor conversion, whereas higher temperatures have a beneficial effect on the reaction rate [Iglesias, et al. 1997; Pchelka, et al. 2000].

As expected, the reaction proceeded considerably faster when the temperature was increased from 25 °C to 60 °C (Figure 2). At 60 °C, the reaction reached conversion of 50% (theoretically complete conversion) in around 5 h, whereas at 40 °C, the complete conversion was achieved after 20 h only. At room temperature, the reaction was extremely slow. On the other hand, the enantiomeric excess (ee) of the product decreased when the temperature increased. This might be due to the fact that the equilibrium between the cyanohydrin and the corresponding aldehydes shifted to the aldehydes at higher temperature. The temperature was not increased above 60 °C, since CAL-B is known to be unstable at higher temperatures [Anderson et al. 1998], and also to avoid the rapid racemisation of cyanohydrin at higher temperatures (see Chapter 7).

![Figure 2 Temperature effect on CAL-B catalysed transesterification of 2a. Conditions: [2a] =0.1 M, 1-PrOH /2a =3 eq., CAL-B /2a =100 mg/mmol. ▲ 25 °C ◆ 40 °C ■ 60 °C](image)

2.2 Effect of ratio of 1-propanol to cyanohydrin acetate (2a)

In the CAL-B catalysed transesterification of 2a with 1-propanol, when the amount of 1-propanol was increased from 2 equiv. to 3 equiv. relative to the racemic substrate 2a, there was no difference with respect to the conversion. The ee of the product dropped slightly at a higher 1-propanol to 2a ratio (Figure 3).
2.3 Effect of enzyme concentration

In general, if the enzymatic reaction is not in the diffusion-control range, the more the enzyme used, the higher the activity can be obtained. Figure 4 clearly shows that when enzyme amount relative to the substrate (mg/mmol) was increased from 60 to 100 mg/mmol, the conversion of substrate 2a also increased. The time for complete conversion shortened from 20 h to 8 h. At both levels of CAL-B concentrations, the ee’s of the product do not show much difference. Hence, we concluded that a suitable ratio of CAL-B to rac- substrate is 100 mg/mmol substrate.

2.4 Kinetic resolution under optimized conditions

Based on the above results, the optimum conditions for the enzymatic resolution of
CAL-B catalysed enantioselective synthesis of cyanohydrins

m-phenoxy mandelonitrile acetate (2a) were as follows: [2a] 0.1 M, [PrOH] 0.2 M, CAL-B 100 mg/mmol substrate, temperature 60 °C. Under these conditions, the results of CAL-B catalysed transesterification of m-phenoxy mandelonitrile acetate with 1-propanol are shown in Table 1.

Table 1 Kinetic resolution of m-phenoxy mandelonitrile acetate (2a) using CAL-B

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Conv (%) of rac-2a</th>
<th>ee (%) of (S)-3a</th>
<th>ee (%) of (R)-2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.98</td>
<td>&gt;99</td>
<td>28.14</td>
</tr>
<tr>
<td>2</td>
<td>33.9</td>
<td>99.0</td>
<td>52.44</td>
</tr>
<tr>
<td>4</td>
<td>45.0</td>
<td>&gt;99</td>
<td>86.09</td>
</tr>
<tr>
<td>5</td>
<td>49.36</td>
<td>97.16</td>
<td>95.4</td>
</tr>
<tr>
<td>6</td>
<td>50.54</td>
<td>96.93</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

Conditions: [2a] 0.1 M, [PrOH] 0.2 M, CAL-B 100 mg/mmol 2a, 60 °C

These conditions were applied to a number of rac-cyanohydrin acetates. The enantioselectivity (E) for the aromatic substrates was always significantly above 30 and for a, c, d, g and h well above 100 (Table 2). However, for the aliphatic substrates (i and j) studied higher conversions were obtained but ee's were very low. Hence, we conclude that the CAL-B does not show enantioselectivity with respect to aliphatic cyanohydrin acetates.

Table 2 Results of the CAL-B catalysed resolution of rac-cyanohydrin acetates (2)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conv (%) of rac-2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ee (%) of (S)-3&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ee (%) of (R)-2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>E&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 h / 5 h</td>
<td>3 h / 5 h</td>
<td>3 h / 5 h</td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>46.5 / 49.5</td>
<td>&gt;99 / 97.2</td>
<td>86.1 / 95.4</td>
<td>260</td>
</tr>
<tr>
<td>2b</td>
<td>45.2 / 51.7</td>
<td>92.1 / 88.6</td>
<td>76.1 / 94.9</td>
<td>55</td>
</tr>
<tr>
<td>2c</td>
<td>45.5 / 49.4</td>
<td>&gt;99 / 98.5</td>
<td>82.7 / 96.8</td>
<td>360</td>
</tr>
<tr>
<td>2d</td>
<td>34.7 / 44.6</td>
<td>&gt;99 / 98.3</td>
<td>52.7 / 79.1</td>
<td>280</td>
</tr>
<tr>
<td>2e</td>
<td>51.0 / 53.1</td>
<td>92.0 / 87.6</td>
<td>95.8 / &gt;99</td>
<td>80</td>
</tr>
<tr>
<td>2f</td>
<td>44.3 / 52.9</td>
<td>89.0 / 82.3</td>
<td>70.8 / 92.4</td>
<td>35</td>
</tr>
<tr>
<td>2g</td>
<td>47.8 / 51.1</td>
<td>95.9 / 94.5</td>
<td>87.8 / &gt;99</td>
<td>140</td>
</tr>
<tr>
<td>2h</td>
<td>50.6 / 51.8</td>
<td>94.2 / 92.0</td>
<td>96.5 / &gt;99</td>
<td>125</td>
</tr>
<tr>
<td>2i</td>
<td>55.0 / 88.2</td>
<td>29.8 / 11.7</td>
<td>36.4 / 87.3</td>
<td>2</td>
</tr>
<tr>
<td>2j</td>
<td>65.4 / 85.4</td>
<td>30.2 / 14.0</td>
<td>57.2 / 81.8</td>
<td>3</td>
</tr>
</tbody>
</table>
**Conditions:**  [2] 0.1 M, [PrOH] 0.2 M, CAL-B 100 mg/mmol 2, 60 °C

\[ \text{Conv} = \frac{ee_{(R) - 2}}{ee_{(R) - 2} + ee_{(S) - 3}} \]

- The conversions were determined as \( \text{Conv} \) = $ee_{(R) - 2}$ / ($ee_{(R) - 2} + ee_{(S) - 3}$).
- The ee's were determined by chiral HPLC.
- E was calculated according to ref. [Rakels et al. 1993]

### 2.5 Reuse of CAL-B

Recycling experiments, under the optimum conditions with 2g as a substrate, showed that the activity of CAL-B remained unchanged while the enantioselectivity (E) decreased from 140 in the first use to 55 in the third cycle (Table 3). The decrease in selectivity could be due to the fact that some product, which was not washed off thoroughly, remained on the enzyme carrier and racemised.

<table>
<thead>
<tr>
<th>Use</th>
<th>Conv (%) of rac-2g</th>
<th>ee (%) of (S)-3g</th>
<th>ee (%) of (R)-2g</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>51.1</td>
<td>94.5</td>
<td>&gt;99</td>
<td>140</td>
</tr>
<tr>
<td>2</td>
<td>52.0</td>
<td>86.9</td>
<td>94.2</td>
<td>51</td>
</tr>
<tr>
<td>3</td>
<td>52.2</td>
<td>87.7</td>
<td>95.8</td>
<td>59</td>
</tr>
</tbody>
</table>

**Conditions:** [2g] 0.1 M, [PrOH] 0.2 M, CAL-B 100 mg/mmol 2g, 60 °C, 5 h

An obvious disadvantage of kinetic resolutions of racemates is that the maximum yield is limited to 50%. For a practical application of kinetic resolution it is, therefore, necessary to use the remaining enantiomer. The remaining cyanohydrin acetates can easily be separated from the cyanohydrins and racemised and then reused for kinetic resolution [Fishman et al. 1998; Roos et al. 1998]. Alternatively the (R)-enantiomer of the unconverted cyanohydrin acetates can be hydrolysed to produce the (R)-cyanohydrins. It is also possible to mesylate the cyanohydrins and subsequently invert the mesylate with NaOAc [Hirohara et al. 1998]. Another way is to invert the cyanohydrin via a Mitsunobu reaction, thus a high yield of the desired enantiomer, (R) or (S) is ensured [Vanttinen and Kanerva, 1995].

The most interesting approach to overcome the drawback of kinetic resolution would be the dynamic kinetic resolution (DKR), a process of kinetic resolution coupled with in situ racemisation of the unreacted enantiomer. Theoretically, this method can afford the
CAL-B catalysed enantioselective synthesis of cyanohydrins

desired enantiomerically pure product in 100% yield [Ward, 1995; El Gihani and Williams, 1999; also see Chapter 5]. With DKR, the separation of product from the remaining enantiomer of the substrate is also omitted.

3. Conclusions

In summary, a straightforward method for the preparation of optically active cyanohydrins and their acetates with excellent enantioselectivity has been developed. Its ease, and the general availability and stability of CAL-B, should help to further establish enantiomerically pure cyanohydrins as building blocks in organic chemistry.

4. Experimental

4.1 Materials and Methods

The aldehydes, sodium cyanide, acetic anhydride, and the phase transfer catalyst benzyltriethylammonium chloride (BTEAC) were purchased from Acros or Aldrich and used as received. The enzymatic reactions were run in dry toluene, which was obtained from Aldrich. Molecular sieves (4A) were obtained from Aldrich and used as supplied. CAL-B (Chirazyme L-2, c.-f., C2, Lyo) was a gift from Roche Diagnostics (Penzberg, Germany). Its activity was determined by titrating the butyric acid liberated from the hydrolysis of tributyrin with 100 mM sodium hydroxide. The enzyme was stored in a desiccator with silica gel as a drying agent at room temperature. The activity of the stored enzyme (3.7 U/mg) remained unchanged over a period of half a year. HPLC analyses were conducted with a Waters 510 pump, a Chiracel OD or OB-H column (0.46 x 25 cm), a Waters 486 UV detector at 215 nm and a mixture of hexane and 2-propanol (92:8) as eluent. The flow rate was 1.0 ml/min. 1,3,5-Trimethylbenzene was used as an internal standard.

4.2 Enzyme activity measurement

48.5 ml 10 mM potassium phosphate buffer (pH 7.0) was incubated in a thermostated vessel at 25 °C, equipped with a propeller stirrer (the stirring speed is set to such a value that a further enhancement of the stirrer speed does not increase the base consumption per min). After the addition of 1.47 ml tributyrin, the pH-stat system was started to keep
the pH at 7.0. When the pH stabilized, 5-10 U of the CAL-B was added. The consumption of 100 mM sodium hydroxide was monitored for 10 to 15 min. The specific activity was calculated from the base consumption in the linear part of the graph. 1 μmol of NaOH consumed per min corresponded to 1 unit (1 U) of CAL-B activity.

4.3 General procedure for the enzymatic resolution

To the solution of a rac-cyanohydrin acetate 2 (0.5 mmol) in dry toluene (5ml) was added CAL-B (50 mg, dried overnight in a dessicator) and 2 equivalents of 1-propanol. The reaction mixture was stirred at 60 °C under nitrogen atmosphere. Samples were taken at certain time intervals and diluted with n-hexane for HPLC analysis.

On a larger scale, 601 mg (10 mmol) 1-propanol was added to 876 mg (5 mmol) racemic 2a and 500 mg CAL-B in 50 ml toluene. After 3 h the enzyme was filtered off, the toluene was evaporated and the residue was chromatographed on silica gel (petroleum ether 9 : ethyl acetate 1) to yield 427 mg (R)-2a (97%, ee = 99%) and 329 mg (S)-3a (99%, ee = 95%).

4.4 Reuse of enzyme CAL-B

After each reaction, the enzyme was filtered off and washed with dry toluene (3 × 30 ml). After drying at room temperature under vacuum for around 3 h, the enzyme was used directly for the next reaction as described in 4.3.

4.5 General procedure for the preparation of rac-cyanohydrin acetates

To a mixture of sodium cyanide (20 mmol), benzyltriethylammonium chloride (BTEAC) (0.75 mmol), H2O (5 ml) and CH2Cl2 (5 ml) was added dropwise a solution of aldehyde (14.5 mmol), acetic anhydride (18.5 mmol) and CH2Cl2 (9 ml). During the addition the stirred reaction mixture was cooled to below 10 °C using an ice bath. After the addition was complete, the mixture was stirred under ice cooling for additional 2 h and then at room temperature overnight.

The phases were separated. The aqueous layer was washed with CH2Cl2 (2 x 5 ml). The organic layers were combined and the solvent was removed in vacuo to give the crude product. The aqueous phase containing cyanide was neutralised using alkaline aqueous FeSO4 solution.
CAL-B catalysed enantioselective synthesis of cyanohydrins

Purification of the crude aromatic products 2a-h was done on silica chromatography using a mixture of ethyl acetate and petroleum ether (1:5) as the eluent. The two aliphatic acetates were purified by distillation (50-55 °C /63 mbar for 2i and 115-118 °C /45 mbar for 2j). The yields/isolated yields were shown in Table 4.

Yield /isolated yield (%); \(^1\)H NMR (CDCl\(_3\))

2a: 96.5 /81.8; 7.00-7.37 (9H, Ar), 6.34 (1H), 2.14 (3H, CH\(_3\)-C=O)
2b: 80.8 /63.8; 7.03-7.35 (4H, Ar), 6.37 (1H), 2.38 (3H, CH\(_3\)-O), 2.16 (3H, CH\(_3\)-C=O)
2c: 76.2 /55.5; 7.45-6.91 (4H, Ar), 6.33 (1H), 3.79 (3H, CH\(_3\)-O), 2.10 (3H, CH\(_3\)-C=O)
2d: 99.0 /75.5; 7.30-7.32 (4H, Ar), 6.36 (1H), 2.38 (3H, CH\(_3\)-Ar), 2.14 (3H, CH\(_3\)-C=O)
2e: 92.8 /74.7; 7.22-7.41 (4H, Ar), 6.35 (1H), 2.37 (3H, CH\(_3\)-Ar), 2.13 (3H, CH\(_3\)-C=O)
2f: 93.2 /86.4; 7.41-7.51 (4H, Ar), 6.38 (1H), 2.17 (3H, CH\(_3\)-C=O)
2g: 90.5 /84.7; 7.44-7.45 (4H, Ar), 6.38 (1H), 2.17 (3H, CH\(_3\)-C=O)
2h: 87.1 /73.8; 7.43-7.53 (7H, Ar), 6.40 (1H), 2.15 (3H, CH\(_3\)-C=O)
2i: 96.8 /96.4; 5.16-5.18 (1H, C-CH-O), 2.15 (1H, C-CH-C; 3H, CH\(_3\)-C=O), 1.07-1.13 (3H, CH\(_3\)-C-C)
2j: 96.8 /96.0; 5.30-5.34 (1H, CH-O), 2.13 (3H, CH\(_3\)-C=O), 1.53-1.91 (2 x 2H, CH\(_2\)-CH\(_2\)), 0.97-1.02 (3H, CH\(_3\)-C-C)

4.6 Enantioselective synthesis of (S)-cyanohydrins catalysed by HbHNL

An aqueous solution of (S)-hydroxynitrile lyase from Hevea brasiliensis (HbHNL, 3530 U/ml, pH 4.7, 2 ml) was mixed with methyl \(t\)-butyl ether (10 ml), followed by addition of an aldehyde (5 mmol). To this solution was added acetone cyanohydrin (850 mg, 10 mmol). The biphasic reaction mixture was stirred vigorously at room temperature for 1 hour. The layers were separated and the aqueous phase was extracted with methyl \(t\)-butyl ether (3 \(\times\) 10 ml). Removal of the organic solvent under vacuum gave the final product (S)-cyanohydrin.
Chapter 7

Enantioselective Formation of Mandelonitrile Acetate – Investigation of a Dynamic Kinetic Resolution

Abstract: Investigations of the separate reactions of a dynamic kinetic resolution and the combined reactions revealed that the overall sequence is highly susceptible to the water-concentration in the reaction mixture. While the racemisation and the formation of mandelonitrile as well as its kinetic resolution proceeded rapidly, when performed independently, the dynamic kinetic resolution was severely hampered by the undesired hydrolysis of the acyl donor to give acetic acid. The utilisation of drying reagents and neutralising agents in order to suppress the formation of acetic acid or its consequences were investigated.
1. Introduction

Enantiomerically pure cyanohydrins and their esters are important building blocks in organic chemistry [Gregory, 1999; Johnson et al. 2000; Effenberger et al. 2000]. Their synthesis has, therefore, attracted considerable attention [Gröger, 2001; Brussee et al. 2000]. One particularly elegant approach is the dynamic kinetic resolution of cyanohydrins formed in situ from an aldehyde (1) and acetone cyanohydrin (2) as cyanide source. The utilisation of an enantioselective lipase for catalysing the esterification leads to a high yield and enantiomeric purity of the formed cyanohydrin ester (6) (Scheme 1). This method was first published in 1991 [Inagaki et al. 1991] and in more detail in 1992 [Inagaki et al. 1992]. These papers have been quoted frequently but only one report has since described the application of the methodology [Kanerva, 1994]. Since all the chemicals and the enzyme utilised in this synthesis of the cyanohydrin acetates (6) are readily available this is somewhat surprising. All the more so, since the method has even been included in a book on preparative biotransformations [Inagaki et al. 1999]. The main drawback of the published synthesis is the relatively long reaction time of 6-8 days. In order to obtain a better insight into the possible reasons for the long reaction time we investigated this multi component reaction and its separate steps. A few modifications were introduced. We used the highly selective Candida antarctica lipase B (CAL-B, chirazyme L-2, c.-f., C2, Lyo) rather than Pseudomonas cepacia lipase [Inagaki, et al. 1991] in our studies. The study was performed in dry toluene (instead of diisopropyl ether), a solvent that proved to be particularly suitable for the kinetic resolution of cyanohydrin acetates with CAL-B. Furthermore no zeolite molecular sieve was added for water adsorption [Fontes et al. 2002], since this might also act as a Lewis or Brønsted acid [Flanigen, 2001], thereby disturbing the hydrocyanation which is catalysed by alkaline Amberlite (OH form) [Inagaki et al. 1999]. Benzaldehyde (1) was used as a model substrate, since it is known to be a particularly good substrate for this reaction sequence [Hanefeld et al. 2001].
Three separate reactions constitute the overall reaction sequence from benzaldehyde (1) to the final product, (S)-mandelonitrile acetate (6). The elimination of HCN from acetone cyanohydrin (2) as well as the addition of HCN to benzaldehyde (1) are reversible, base catalysed, reactions. Under acidic conditions 2, 4 and 5 are stable and the reactions are extremely slow in either direction. The two reversible reactions are essential for the formation and racemisation of mandelonitrile (4 + 5), the substrate of the enzyme reaction. They can be driven to completion by the third, irreversible reaction.

This third step is the CAL-B catalysed acylation of 4 with iso-propenyl acetate (7) to form (S)-mandelonitrile acetate (6) and acetone (3). The enantiopurity of the product depends on the enantioselectivity ($E$) of the enzyme for the specific substrate under the specific conditions. In a kinetic resolution the $ee$ of the product decreases with the degree of conversion, since the availability of the favored substrate enantiomer (here 4) decreases much more rapidly than that of the less favored substrate enantiomer (here 5). In order to achieve the greatest possible enantiomeric purity in an enzymatic dynamic kinetic resolution, it is therefore important not only to utilise an enzyme with a high $E$ value, but also to ensure that the substrate remains (almost) racemic throughout its conversion. Thus, it is essential that the racemisation is faster than the acylation. Therefore, we first studied the reactions individually and then in combination. In the one-pot reaction, the molar ratio of 1 to acetone cyanohydrin (2) to iso-propenyl acetate (7) was fixed at 1:2:3.
2. Results and Discussion

The equilibrium constants and the equilibration times of the combined first two reactions were determined utilising 0.05 mol equivalents of alkaline Amberlite. The concentrations of 1 and 2 were quantified simultaneously by IR (Figure 1a). Even at room temperature the equilibrium of the combined reactions was reached within 5 h (Figure 1b).

![Figure 1a. IR-spectra of the measurements of the reaction of 1 and 2 in the presence of Amberlite (0.0625 equiv. OH') to form 3, 4 and 5. The concentration of 1 was measured at 2733 and 2815 cm⁻¹ and the concentration of 2 at 972 cm⁻¹. The equilibrium constant for the racemic reaction was determined as $K_{eq} = 6.53$. A: absorbance](image)

![Figure 1b. Reaction of 1 and 2 in the presence of Amberlite (0.0625 equiv. OH') to form 3, 4 and 5. The concentration of HCN is not shown. • 4+5; ■ 3; △ 1; × 2](image)

The racemisation of 5 under reaction conditions (40 or 60 °C) proceeded completely within an hour or less (Figure 2), which is in accordance with data reported earlier [Kanerva et al. 1994]. Moreover, the racemisation reaction was not influenced by the
presence of CAL-B. It was therefore anticipated that the elimination and addition reactions of HCN would not form the bottleneck of the overall procedure.

Figure 2. Racemisation of 5 at 40 (♦) and 60 °C (■) in the presence of Amberlite (0.05 equiv. OH⁻)

In the CAL-B catalysed acylation of the racemic mixture of 4 and 5 with 7, only 4 is converted. Several conditions for the esterification of the racemate were investigated (Figure 3). At 25, 40 and 60 °C in toluene, with different enzyme concentrations, CAL-B always showed excellent selectivity (E [Rakel et al. 1993] always larger than 400). In every case 6 was formed with an ee ≥ 99%. The reaction rate was proportional to the amount of enzyme.

Figure 3. Kinetic resolution of rac-mandelonitrile (4 + 5) with CAL-B and 7 at different temperatures and enzyme concentrations. ♦ 25 °C and 37 U/ml; ■ 40 °C and 37 U/ml; ▲ 60 °C and 37 U/ml; × 25 °C and 74 U/ml; * 40 °C and 74 U/ml; ● 60 °C and 74 U/ml.
**Investigation of a dynamic kinetic resolution on mandelonitrile acetate**

These results clearly indicate that the separate reactions are fast and proceed with high selectivity. As required for a successful dynamic kinetic resolution the racemisation is significantly faster than the acylation if either 370 or 740 units of CAL-B per mmol of substrate are utilised. In order to achieve overall reaction times that are significantly shorter than those described in the literature, the reaction temperature should be kept at least at 40 °C. No side products were detected in any case. Moreover, Amberlite did not catalyse the acylation step, ruling out any interference of the chemical catalyst with the enzyme catalysed reaction.

These results indicate that the combination of the racemisation of mandelonitrile (4 + 5) and its kinetic resolution should lead to the fast and highly selective formation of (S)-mandelonitrile acetate (6). This is indeed the case during the initial period of the reaction (Figure 4).

![Figure 4. Dynamic kinetic resolution of rac-mandelonitrile (4 + 5) in the presence of 7, CAL-B and Amberlite (0.05 equiv. OH) at 60 °C. ♦ 1; ■ 4+5; ▲ 6](image)

Comparison of Figure 3 and Figure 4 suggests that the observed slowing down of the formation of 6 can be attributed to the formation of 1 which is expected to be an inhibitor of the enzyme. In the course of the reaction, however, a change in rates is observed. Instead of a rapid racemisation and acylation of mandelonitrile, the concentration of 1 seems to almost stabilise on a plateau. Moreover, the ee of 5 increases, a clear indication of a kinetic resolution without racemisation of the initially racemic starting material. This strongly suggests that the Amberlite is deactivated during the course of the reaction. Similar results were obtained at 40 °C. The same observations were also made when performing the overall reaction of 1 (0.1 M) with 2 and 7 in the presence of CAL-B (37 U/ml) and Amberlite (0.05 equiv. OH'). The reaction was extremely slow and even after
47 h the yield of 6 was only 9.5%.

Recently, it has been demonstrated that the presence of even very small quantities of water can lead to a significant enzyme-catalysed hydrolysis of the acylating reagent [Weber et al. 1999]. The dry toluene utilised in the reactions described above contained 120 ppm of water. In order to investigate the consequences of this amount of water, the CAL-B catalysed hydrolysis of rac-mandelonitrile acetate and 7 in dry toluene (obtained from Aldrich) was examined separately. Both compounds were initially rapidly hydrolyzed. At 25 °C and in the presence of 120 ppm of water, 18% of rac-mandelonitrile acetate and 21% of 7 were hydrolyzed within 5 hours. The hydrolysis proceeded even after an amount of water equal to the original amount of water in the dry toluene had been consumed. This indicates that water adsorbed on the enzyme and its carrier must also be utilised in this hydrolysis. During the dynamic kinetic resolution of 4 + 5 some enzymatic hydrolysis of 6 and 7 can be expected as well, although 6 and 7 were stable in the presence of Amberlite alone. If this is indeed the case, the acetic acid that is formed will quickly and completely neutralise the alkaline ion exchanger, which is present in only 0.05 mol equivalents. The reaction mixture turns from alkaline to acidic. This stabilizes 2, 4 and 5. Such neutralisation regenerates water that had been consumed by hydrolysis, so the water concentration will remain constant, while the alkaline Amberlite will be depleted (Scheme 2).

\[
\text{7} \quad \text{O} \quad \text{O} \quad \text{Amberlite}^\ominus \text{OH}^\ominus \quad \text{H}_2\text{O} \quad \rightarrow \quad \text{O} \quad \text{Amberlite}^\ominus \text{Ac}^\ominus \quad \text{H}_2\text{O}
\]

Scheme 2 Deactivation of racemisation catalyst by the hydrolysis of the acyl donor

Upon deactivation of the racemisation catalyst, the dynamic kinetic resolution is turned into a conventional kinetic resolution. In addition, not all of 1 has at this stage been converted into 4 and 5 nor has all of 2 been converted into HCN and acetone (3). Therefore, the concentration of 4 + 5 is relatively low. Further drying of the solvent with molecular sieve prior to the incubation of rac-mandelonitrile acetate with CAL-B lowered the water content to 60 ppm. This did reduce the rate of the hydrolysis, but the problem remained. In order to overcome this problem and to ensure that the reaction medium remained alkaline 0.25 mol equivalent of base was added in the form of Amberlite. This did indeed lead to an improvement of the overall reaction. The
Investigation of a dynamic kinetic resolution on mandelonitrile acetate

conversion of 1 was significantly improved and the yield of 6 after 45 h was higher than before (15.9 instead of 9.5 %).

This clearly demonstrates that the Amberlite and CAL-B catalysed synthesis of cyanohydrin acetate 6 is very sensitive to competing hydrolysis and the acids released thereby. In order to overcome this problem and to improve the yield of 6 a large amount of Amberlite (1.0 mol OH⁻ equiv) was added. However, instead of improving the yield, only 2.1 % 6 were formed after 45 h. Moreover, this reaction mixture turned brownish, something not observed in the other reactions. This points to a base induced polymerisation of the HCN, which is well known to yield brown and even black products [Wadsten et al. 1959]. Adding more base, in order to neutralise the released acid does not circumvent the problem of hydrolysis. It seems to be necessary to suppress hydrolysis or its consequences more efficiently.

3. Conclusions

In summary, low concentrations of alkaline Amberlite efficiently catalyse the formation and racemisation of mandelonitrile (4 + 5) and do not inhibit the enzyme catalysed acylation. Similarly CAL-B is an excellent catalyst for the enantioselective formation of cyanohydrin acetates (6). However, the combination of these reactions into a dynamic kinetic resolution is severely hampered by the acid formed by the hydrolysis of 6 and 7. Utilisation of larger quantities of base does not solve this problem, possibly due to the polymerization of HCN.

4. Experimental

4.1 Materials and Methods

Benzaldehyde, acetone, acetone cyanohydrin and isopropenyl acetate were distilled prior to use and were stored under nitrogen. Technical grade rac-mandelonitrile from Acros was purified by silica gel column chromatography and stored under nitrogen. Dry toluene was obtained from Aldrich. Molecular sieves (4A) were obtained from Aldrich and used as supplied. CAL-B (Chirazyme L-2, c.-f., C2, Lyo) was a gift from Roche Diagnostics (Penzberg, Germany). Its activity was determined according to the procedure developed by Roche Diagnostics (see Chapter 6). The enzyme was stored in a desiccator.
Chapter 7

with silica gel as a drying agent at room temperature. The activity of the stored enzyme (3.7 U/mg) remained unchanged over a period of half a year. Amberlite IRA904 was purchased from Acros and conditioned according to literature (Inagaki et al. 1992a). HPLC analyses were conducted with a Waters 510 pump, a Chiracel OB-H column (0.46 x 25 cm), a Waters 486 UV detector at 215 nm and a mixture of hexane and iso-propanol (92:8) with 0.1% acetic acid as eluent. The flow rate was 1.0 ml/min. 1,3,5-Trimethylbenzene was used as an internal standard. Retention times: 1,3,5-trimethylbenzene 3.7 min, 7 5.7 min, 1 7.4 min, 5 12.4 min; 4 13.2 min; 6 15.1 min and (R)-mandelonitrile acetate 20.3 min. The water concentration in toluene was measured by Karl-Fischer-Titration with a Mettler DL35. Hydranal®-Titrant 2 (from Riedel-deHaen) was used as titrant. IR analysis was performed with a Perkin-Elmer FT-IR Spectrometer Spectrum 1000 in a KBr-cell with a path-length of 0.115 mm. Absorptions were measured at 2733 and 2815 cm⁻¹ (1) and 972 cm⁻¹ (2).

4.2 Kinetic Resolution of rac-Mandelonitrile (4 + 5)

In an 8 ml vessel rac-mandelonitrile (4 + 5) (66.5 mg, 0.5 mmol) and iso-propenyl acetate (7) (125 mg, 1.25 mmol) were dissolved in 5 ml dry toluene. A baseline sample was taken with a microsyringe. The solution was then transferred to a nitrogen gas flushed vessel with CAL-B (50 mg, 185 U or 100 mg, 370 U) in it. The reaction system was closed with a rubber septum and stirred at the desired temperature (25, 40 or 60 °C). Samples (15 µl) were taken with a microsyringe at the time intervals given in Figure 3 and diluted with 1 ml solvent (same as the mobile phase for HPLC) for chiral HPLC analysis. The enantiomeric excess of 6 was always > 99 %.

4.3 Dynamic Kinetic Resolution of rac-Mandelonitrile (4 + 5)

In an 8 ml vessel rac-mandelonitrile (4 + 5) (66.5 mg, 0.5 mmol) and iso-propenyl acetate (7) (125 mg, 1.25 mmol) were dissolved in 5 ml dry toluene. A baseline sample was taken with a microsyringe. The solution was then transferred to a nitrogen gas flushed vessel with CAL-B (50 mg, 185 U) and Amberlite IRA904 (OH⁻ form, 19.2 mg, 0.025 mmol OH⁻) in it. The reaction system was closed with a rubber septum and stirred at 60 °C. Samples (15 µl) were taken with a microsyringe at the time intervals given in Figure 4 and diluted with 1 ml solvent (same as the mobile phase for HPLC) for chiral HPLC analysis. The enantiomeric excess of 6 was always > 99 %. 

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4.4 Racemisation of (R)-mandelonitrile (5)

Amberlite IRA904 (OH⁻ form, 19.1 mg, 0.025 mmol OH⁻) was added to a solution of 5 (66.5 mg, 0.5 mmol) in dry toluene (5 ml). The reaction mixture was stirred at the desired temperature (40 or 60 °C). Samples (15 μl) were taken with a microsyringe at the time intervals given in Figure 2 and analyzed by chiral HPLC to measure the ee-values.

4.5 Chemical transcyanation between benzaldehyde (1) and acetone cyanohydrin (2) and vice versa

Amberlite IRA 904 (OH⁻ form, 38.5mg, 0.05 mmol OH⁻) was added to a solution of 1 (84.8 mg, 0.8 mmol) and 2 (70.0 mg, 0.82 mmol) in dry toluene (8 ml). The mixture was stirred in a closed system at room temperature (25 °C). Samples were taken (at the times given in figure 1b) with a syringe for infrared spectroscopic analysis (see Material and Methods). After 5 h the equilibrium situation was reached with 0.35 mmol 1, 0.16 mmol 2, 0.66 mmol 3 and 0.45 mmol rac-mandelonitrile.

The reverse reaction was carried out similarly using racemic mandelonitrile (109.3 mg, 0.82 mmol) and 3 (51.0 mg, 0.88 mmol) in dry toluene (8 ml). After 5 h the equilibrium situation was reached with 0.38 mmol 1, 0.14 mmol 2, 0.74 mmol 3 and 0.44 mmol rac-mandelonitrile.

4.6 Hydrolysis of rac-Mandelonitrile acetate or iso-propenyl acetate (7)

rac-Mandelonitrile acetate (or 7) (0.5 mmol) and CAL-B (50 mg, 185 U) were added to toluene (5 ml) that contained 120 ppm water or toluene (5 ml) that contained 60 ppm water (obtained by drying the dry toluene from Aldrich with molecular sieves). The reaction mixture was stirred at room temperature (25 °C). The conversion was measured by chiral HPLC. After 5 h in the presence of 120 ppm H₂O, 21.2 % 7 and 18.6 % rac-mandelonitrile acetate were hydrolyzed. After 5 h in the presence of 60 ppm H₂O, 12.9 % rac-mandelonitrile acetate was hydrolyzed.

4.7 One-pot synthesis of (S)-mandelonitrile acetate (6) from benzaldehyde (1), acetone cyanohydrin (2) and iso-propenyl acetate (7)

1 (84.8 mg, 0.8 mmol), 2 (136 mg, 1.6 mmol) and 7 (240 mg, 2.4 mmol) were added to dry toluene (8 ml). To this solution different OH⁻ equivalents of Amberlite IRA904
(OH\textsuperscript{-} form, 30.8 mg/0.05 equiv, 154 mg/0.25 equiv, or 616 mg/1.0 equiv) and CAL-B (80 mg, 296 U) were added. The reaction mixture was stirred at 40 °C and 15 μl samples were taken for the analysis by chiral HPLC. The enantiomeric excess of 6 was always > 99 %. The results are shown in Table 1.

Table 1. One-pot synthesis of 6 from 1, 2 and 7 in the presence of different amounts of Amberlite (OH\textsuperscript{-} form)

<table>
<thead>
<tr>
<th>Amberlite (OH\textsuperscript{-} equiv.)</th>
<th>Time (h)</th>
<th>Yield of 6 (%)</th>
<th>Conv. of 1 (%)</th>
<th>ee of 5 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>21</td>
<td>1.8</td>
<td>49.7</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>2.1</td>
<td>47.9</td>
<td>6.2</td>
</tr>
<tr>
<td>0.25</td>
<td>21</td>
<td>9.1</td>
<td>60.1</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>15.9</td>
<td>67.3</td>
<td>0.8</td>
</tr>
<tr>
<td>0.05</td>
<td>20</td>
<td>4.1</td>
<td>40.3</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>9.5</td>
<td>59.7</td>
<td>3.0</td>
</tr>
</tbody>
</table>
Investigation of a dynamic kinetic resolution on mandelonitrile acetate
Chapter 8

Ruthenium Catalysts for Racemisation and Dynamic Kinetic Resolution of Secondary Alcohols

Abstract: Four ruthenium-based catalytic systems were shown to catalyse the racemisation of chiral secondary alcohols. For example, 4,4,2-Trimethyl-2-pyridin-2-yl-1,3-oxazolidine was demonstrated, for the first time, to be an efficient ligand for ruthenium-catalysed racemisation of secondary alcohols. The combination of [TosN(CH$_2$)$_2$NH$_2$]RuCl(p-cymene)/TEMPO or [RuCl$_2$(p-cymene)]$_2$/TEMPO/(−)-sparteine catalysed the *in situ* racemisation of chiral secondary alcohols during enzymatic resolution in toluene.
1. Introduction


The kinetic resolution (KR) of racemic secondary alcohols by hydrolytic enzymes such as lipases and esterases is probably currently the most used route to obtain a wide range of enantiomerically enriched alcohols or their esters. KR, however, exhibits major disadvantages: the theoretical maximum yield cannot exceed 50% and laborious separation of the product from the remaining substrate is often needed. As a consequence, alternative methods for direct conversion of a racemic mixture to a single enantiomer are highly advantageous. One of the many approaches [Ward, 1995, Strauss and Faber, 1999] to reach this target is to employ a racemisation catalyst for the in situ conversion of less reactive enantiomers to more reactive enantiomers together with the enzyme. The KR combined with in situ racemisation is called dynamic kinetic resolution (DKR) [El Gihani and Williams, 1999; Chapter 5].

Among a number of transition metals, including palladium, iridium and rhodium, which are capable of racemising alcohols [Allen and Williams, 1996; Dinh et al, 1996], ruthenium complexes [Larsson et al, 1997; Persson et al, 1999; Koh et al, 1998; 1999] have been shown to be the most effective catalysts (1-3, Figure 1). However, the major drawback of these systems is the requirement of large amounts of additives, i.e. respectively 1 equivalent of acetophenone and 3 equivalents of triethylamine based on the substrate, to achieve their activity. In addition, the conventional acyl donors such as vinyl acetate and isopropenyl acetate are not compatible with the catalyst (1). Very recently, Choi et al [2002] have successfully used aminocyclopentadienyl ruthenium complex (4) for the DKR of secondary alcohols at room temperature.
2. Results and discussion

2.1 Racemisation with ruthenium-diamine complex

In previous experiments Dijksman [2001] showed that the systems RuCl$_2$(PPh$_3$)$_3$/TEMPO and ruthenium-diamine complex 5/KOH were efficient catalytic systems for the racemisation of chiral secondary benzylic alcohols in tert-amyl alcohol. However, attempts to combine these systems with lipase catalysed acylation failed. As a continuation of this work, the racemisation of (S)-1-phenylethanol by the ruthenium complex 5 was re-investigated under different conditions.
Table 1. Racemisation of (S)-1-phenylethanol with 5 as catalyst precursor

<table>
<thead>
<tr>
<th>Entry</th>
<th>co-catalyst (mol%)</th>
<th>solvent</th>
<th>e.e. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>---</td>
<td>tert-butanol</td>
<td>99</td>
</tr>
<tr>
<td>2</td>
<td>KOH (1)</td>
<td>tert-butanol</td>
<td>22 (0)</td>
</tr>
<tr>
<td>3</td>
<td>KOH (2)</td>
<td>tert-butanol</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>KOH (1)</td>
<td>tert-amyl alcohol</td>
<td>3 (0)</td>
</tr>
<tr>
<td>5</td>
<td>KOH (1)</td>
<td>toluene</td>
<td>84</td>
</tr>
<tr>
<td>6 b</td>
<td>KOH (3)</td>
<td>toluene</td>
<td>13 (10)</td>
</tr>
<tr>
<td>7</td>
<td>K₂CO₃ (1)</td>
<td>tert-butanol</td>
<td>96</td>
</tr>
<tr>
<td>8</td>
<td>DBU (1) c</td>
<td>tert-butanol</td>
<td>55</td>
</tr>
<tr>
<td>9</td>
<td>DABCO (1) d</td>
<td>tert-butanol</td>
<td>80</td>
</tr>
<tr>
<td>10</td>
<td>triethylamine (1)</td>
<td>tert-butanol</td>
<td>75</td>
</tr>
<tr>
<td>11</td>
<td>pyridine (1)</td>
<td>tert-butanol</td>
<td>98</td>
</tr>
<tr>
<td>12 b</td>
<td>TEMPO (4.5)</td>
<td>tert-butanol</td>
<td>79</td>
</tr>
<tr>
<td>13 b</td>
<td>TEMPO (4.5)</td>
<td>tert-amyl alcohol</td>
<td>75 (48)</td>
</tr>
<tr>
<td>14 b</td>
<td>TEMPO (4.5)</td>
<td>toluene</td>
<td>41 (15)</td>
</tr>
</tbody>
</table>

Conditions: 1 mmol (S)-1-phenylethanol, 1 mol% catalyst 5, 3 ml solvent, N₂ atmosphere, 70°C, 24h. a ee's in parentheses for reactions after 48h. b 1.5 mol% catalyst 5. c 1,8-diazabicyclo[5.4.0]undec-7-ene. d 1,4-diazabicyclo[2.2.2]octane.

* The results in this table were obtained in collaboration with Dr. Arné Dijksman (see thesis Dijksman 2001)
As the results in Table 1 show, compound 5 alone is inactive as a catalyst for the racemisation of (S)-1-phenyl ethanol (entry 1). Addition of a base (KOH) as co-catalyst (which itself is inactive as a catalyst) to 5 leads to a substantial increase in activity (entries 2, 3 and 4). The choice of base was found to be essential. For example, racemisation of (S)-1-phenylethanol with 5 in the presence of other bases resulted in much lower activities (entries 7–11).

The role of base was proposed to involve abstraction of a proton from the amino group of the ligand, resulting in the elimination of HCl to form the active catalyst 6, which racemises the alcohol via a hydrido-ruthenium intermediate 7 (Scheme 1) [Dijksman et al., 2002].

Scheme 1 Racemisation of secondary alcohol catalysed by ruthenium-diamine complex

Based on the known catalytic activity of RuCl₂(PPh₃)₃/TEMPO for alcohol racemisation (see above), we used the combination complex 5/TEMPO as the racemisation catalyst. Indeed, TEMPO could act as a co-catalyst although the activity was found to be lower than with KOH (entries 12-14).

Solvents were shown to play an important role in the RuCl₂(PPh₃)₃/TEMPO system [Dijksman, 2001]. t-Butanol was better than toluene and other solvents tested. This effect was also found when 5 was used as the racemisation catalyst (Table 1). When KOH was used as the co-catalyst, the best results were obtained in t-butanol or t-amyl alcohol. On the other hand, toluene proved to be the best solvent when TEMPO was used as the co-catalyst (entries 12-14). The use of toluene as solvent is more compatible with the enzyme and acyl donor (see Section 2.3) and, hence, more suitable for DKR of chiral secondary alcohols.
2.2 Racemisation with $[\text{RuCl}_2(p\text{-cymene})]_2$

Since $[\text{RuCl}_2(p\text{-cymene})]_2$ (3) displays high catalytic activity in the aerobic oxidation of benzylic and allylic alcohols [Lee and Chang, 2000], and the natural (-)-sparteine is a good ligand in oxidative kinetic resolution of alcohols with Pd [Ferreira and Stoltz, 2001], we studied the oxidative kinetic resolution of 1-phenylethanol with oxygen in the presence of 3, TEMPO and (-)-sparteine. Unfortunately no enantioselectivity was observed, although the conversion of 1-phenylethanol was 26% after 24 h. This prompted us to investigate the racemisation ability of this system. Indeed, the complex 3 has been reported to catalyse the racemisation of secondary alcohols in the presence of 1 equiv of triethylamine [Lee et al, 2000]. As we expected, racemisation of (S)-1-phenylethanol was observed by the catalytic system of 3/TEMPO/(-)-sparteine (Table 2, entries 1, 2). A blank experiment showed that 3 is essential for the racemisation (entry 3).

In the absence of (-)-sparteine the combination of 3/TEMPO showed very low racemisation activity (entry 4). The couple 3/(-)-sparteine showed only moderate activity in the absence of TEMPO (entry 5). Comparison between entries 4, 5 and 1 indicates a synergic effect of TEMPO and (-)-sparteine in combination with $[\text{RuCl}_2(p\text{-cymene})]_2$ for the racemisation of secondary alcohols.

Although the system 3/TEMPO/(-)-sparteine showed high racemisation activity, the drawback of this system is the use of relatively expensive TEMPO and a large amount of (-)-sparteine (1 equivalent). This prompted us to search for an alternative method.

Based on the observation that a variety of bidentate nitrogen ligands are effective ligands for ruthenium catalysts, we studied 2-pyridyl-oxazolidines 8 (8a $R=\text{CH}_3$, 8b $R=\text{H}$) in combination with $[\text{RuCl}_2(p\text{-cymene})]_2$ for the racemisation of 1-phenylethanol. The 1,3-oxazolidines 8a and 8b were readily prepared from 2-amino-2-methyl-propanol and 2-acetylpyridine or 2-pyridinecarboxyaldehyde [Fülöp et al, 1991] (see Experimental).
Table 2 Racemisation of (S)-1-phenylethanol with 3 and various co-catalysts

<table>
<thead>
<tr>
<th>Entry</th>
<th>Co-catalyst (mol%)</th>
<th>ee (%) after 23 h a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TEMPO (4) /(-)-Sparteine (12)</td>
<td>12 b</td>
</tr>
<tr>
<td>2</td>
<td>TEMPO (4) /(-)-Sparteine (100)</td>
<td>7 b (4)</td>
</tr>
<tr>
<td>3</td>
<td>TEMPO (4) /(-)-Sparteine (100)</td>
<td>99 c</td>
</tr>
<tr>
<td>4</td>
<td>TEMPO (10)</td>
<td>97 (44)</td>
</tr>
<tr>
<td>5</td>
<td>(-)-Sparteine (15)</td>
<td>47 b</td>
</tr>
<tr>
<td>6</td>
<td>K₂CO₃ (50) /8a (5)</td>
<td>10 (0)</td>
</tr>
<tr>
<td>7</td>
<td>K₂CO₃ (20) /8a (5)</td>
<td>15 (5)</td>
</tr>
<tr>
<td>8</td>
<td>K₂CO₃ (50) /8a (12) c</td>
<td>99</td>
</tr>
<tr>
<td>9</td>
<td>K₂CO₃ (50) /8b (10)</td>
<td>99</td>
</tr>
<tr>
<td>10</td>
<td>K₂CO₃ (50) /8b (11) d</td>
<td>95</td>
</tr>
<tr>
<td>11</td>
<td>K₂CO₃ (50) /8b (10) e</td>
<td>99</td>
</tr>
<tr>
<td>12</td>
<td>K₂CO₃ (50) /8b (11) f</td>
<td>94</td>
</tr>
</tbody>
</table>

Conditions: 0.5 mmol (S)-1-phenylethanol, 2 mol% catalyst 3, 3 ml toluene, N₂ atmosphere. a ee's in parentheses for reactions after 47 h; b 26 h; c No ruthenium catalyst; d In tert-amyl alcohol; e Extra acetophenone 15 mol% was added; f Molecular sieve 3A (from Aldrich)15 mg was added.

As we anticipated, the system 3/8a/K₂CO₃ indeed showed high racemisation activity (entries 6, 7). However, 3/8b/K₂CO₃ did not show any activity (entries 9-11). This suggests that the hydrogen atom at the 2 position of oxazolidine should be avoided. We surmised that this may, in some way, be connected with tautomerism of 8a and 8b.

It has been known for some time that 1,3-oxazolidines exist as mixtures of ring-chain tautomers (Scheme 2) [Fülöp et al, 1989; Alva-Astudillo et al, 1985]. The ring-chain tautomeric ratios depend on the characteristics of the ring, the substituents at the 2-position, and the solvent. In IR spectra, a broad and strong peak at 3417 cm⁻¹, which corresponds to a OH group, was observed in 8b but not in 8a (Figure 2). A sharp, moderately strong peak at 3297 cm⁻¹ related to secondary amine -NH- was observed in both 8a and 8b. These observations suggest that compound 8a mainly exists in the ring form, whereas 8b contains a large amount of the open-chain form. The ¹H NMR and ¹³C NMR spectra for pure 8a in CDCl₃, characterized by the signal intensities of the three protons of the methyl group at the 2-position, showed that 8a exists for 98% in the
Ruthenium catalysts for racemization and dynamic kinetic resolution of secondary alcohols

ring-form. Attempts to obtain pure $8b$ failed because a small peak close to $8b$ shown by GC spectrum always exists (Figure 3).

Scheme 2 Tautomeric equilibrium of oxazolidine 8

Figure 2 IR spectra of $8a$ and $8b$

m/z: 193 ($M^+$)

m/z: 179 ($M^+$)

Figure 3 GC/MS spectra of $8a$ and $8b$
The results in Table 2 taken together with the above observations suggest that in order to form an active ruthenium catalyst the oxazolidine should be in the ring form, in which the two nitrogen atoms coordinate to the ruthenium. In the open-chain form of 8b, coordination of the oxazolidine nitrogen atom to the ruthenium is hindered by strong intramolecular hydrogen-bonding (9).

![Chemical structure](image)

On the other hand, in the preparation of 8b, when 3A molecular sieve (MS) was added into the reaction mixture and stirred for 4 h, the product thus obtained was a solid and showed a high racemisation activity in combination with [RuCl₂(p-cymene)]₂ (Table 3, entries 1-3). The reason is not yet clear. We assumed that the product thus obtained was the immobilized 8b onto MS (hereafter referred to as 8b/MS). This immobilization might help to improve the coordination to the ruthenium. The mechanism of racemisation of secondary alcohols with ruthenium in the presence of 8a and 8b and the role of molecular sieve need to be further investigated.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Co-catalyst (mol%)</th>
<th>8b/MS (mg)</th>
<th>ee (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>K₂CO₃ (28)</td>
<td>4.4</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>K₂CO₃ (50)</td>
<td>4.3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>3</td>
<td>KO'Bu (8)</td>
<td>4.4</td>
<td>&lt;1</td>
</tr>
<tr>
<td>4</td>
<td>/</td>
<td>4.4</td>
<td>95</td>
</tr>
<tr>
<td>5</td>
<td>MS 3A (15 mg)</td>
<td>/</td>
<td>95</td>
</tr>
<tr>
<td>6</td>
<td>MS 3A (15 mg)</td>
<td>/</td>
<td>99</td>
</tr>
<tr>
<td>7</td>
<td>K₂CO₃ (28)</td>
<td>4.4</td>
<td>99</td>
</tr>
</tbody>
</table>

Conditions: 0.5 mmol (S)-1-phenylethanol, 2 mol% catalyst 3, 3 ml toluene, N₂ atmosphere. *ee was determined by HPLC after 23 h; *Molecular sieve 3A was stirred in the reaction mixture during preparation of 8b, see Experimental; *Blank experiments without 3
2.3. Dynamic kinetic resolution

Although the combination of 5/KOH is an active catalytic system for the racemisation of chiral secondary (benzylic) alcohols, initial attempts to combine lipase-catalysed acylation with this catalytic racemisation system in either tert-amyl alcohol or tert-butyl alcohol were not successful, i.e. only enantioselective acylation and no racemisation-activity was observed. It was subsequently shown that both the lipase and the acyl donor had a negative influence on the ruthenium-catalysed racemisation. The ruthenium catalyst 5 was deactivated by the enzyme and the acyl donor in alcoholic solvents as was shown in separate experiments (Table 4, entries 1–3). Because of this we re-examined the racemisation results and decided to repeat the experiments mentioned above in the less polar solvent toluene. Racemisation was indeed observed in the presence of either the lipase or the acyl donor using 5/KOH as the catalytic system in toluene as solvent (Table 4, entries 4–6).

As discussed above (Table 1), TEMPO can be used instead of KOH as the co-catalyst in combination with ruthenium complex 5. In this case, toluene as solvent gives better results than alcoholic solvents. With this combination, racemisation was observed in toluene in the presence of either the lipase or the acyl donor (Table 4, entries 7–9).

Table 4. Racemisation of (S)-1-phenylethanol by catalyst 5 in the presence of additives

<table>
<thead>
<tr>
<th>Entry</th>
<th>Co-catalyst</th>
<th>Solvent</th>
<th>Additive</th>
<th>ee (%) after 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>KOH</td>
<td>tert-Amyl alcohol</td>
<td>---</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>Novozym 435</td>
<td></td>
<td>99 (99)</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>CPA</td>
<td></td>
<td>99 (96)</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>Toluene</td>
<td>---</td>
<td>13 (10)</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>Novozym 435</td>
<td></td>
<td>4 (0)</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>CPA</td>
<td></td>
<td>89 (79)</td>
</tr>
<tr>
<td>7</td>
<td>TEMPO</td>
<td>Toluene</td>
<td>---</td>
<td>42 (15)</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>Novozym 435</td>
<td></td>
<td>63 (45)</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>CPA</td>
<td></td>
<td>72 (63)</td>
</tr>
</tbody>
</table>

Conditions: 1 mmol (S)-1-phenylethanol, 1.5 mol% catalyst 5, 3 ml tert-butanol, 50 mg Novozym 435, N₂ atmosphere, 70°C. a 3 mol% KOH or 4.5 mol% TEMPO. b CPA = 2 mmol p-chlorophenyl acetate. c ee's in parentheses for reactions after 48h.
Subsequently, the system 5/TEMPO was used in combination with enzymatic resolution (Table 5). As we anticipated, in situ racemisation occurred, resulting in a DKR (entries 1-3). In the systems of 5/KOH (entries 4, 5) or RuCl₂(PPh₃)₃/TEMPO (entry 8), both the conversion and the yield were lower than in the 5/TEMPO catalysed system. In addition, in the system of 5/KOH, the enzyme was almost completely deactivated in 24 h (entry 4).

The formation of 10-15% acetophenone was observed during the reaction. With the 5/TEMPO system the addition of extra acetophenone did not affect the dynamic kinetic resolution (entry 3 vs entries 6 and 7). TEMPO had a significant effect on both conversion and yield. When the ratio of TEMPO to 5 was raised from 1 to 3, the conversion and yield increased from 79% and 63% to 91% and 76%, respectively (entries 2, 3). In addition to toluene, some other solvents were also tested in the DKR (entries 10-15). Chlorobenzene (entry 14) showed similar results to toluene (entry 2). In the other solvents, the yields were lower than in toluene.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Cat (mol%)</th>
<th>KOH (mol%)</th>
<th>TEMPO (mol%)</th>
<th>Solvent</th>
<th>Conv %</th>
<th>Yield of (S)-ester %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5</td>
<td>4.5</td>
<td></td>
<td>Toluene</td>
<td>82 (72)</td>
<td>68 (64)</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>5</td>
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<td>79</td>
<td>63</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>15</td>
<td></td>
<td>Toluene</td>
<td>91</td>
<td>76</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
<td>3</td>
<td></td>
<td>Toluene</td>
<td>61 (55)</td>
<td>55 (54)</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>5</td>
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<td>Toluene</td>
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<td>5</td>
<td>15</td>
<td></td>
<td>Toluene</td>
<td>91</td>
<td>75</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>15</td>
<td></td>
<td>Toluene</td>
<td>90</td>
<td>78</td>
</tr>
<tr>
<td>8</td>
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<td>15</td>
<td></td>
<td>Toluene</td>
<td>61</td>
<td>56</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>5</td>
<td></td>
<td>Toluene</td>
<td>72</td>
<td>65</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>5</td>
<td></td>
<td>Acetone</td>
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<td>49</td>
</tr>
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<td>11</td>
<td>5</td>
<td>5</td>
<td></td>
<td>2-Pentanone</td>
<td>73</td>
<td>62</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
<td>5</td>
<td></td>
<td>n-Hexane</td>
<td>72</td>
<td>46</td>
</tr>
<tr>
<td>13</td>
<td>5</td>
<td>5</td>
<td></td>
<td>DME</td>
<td>78</td>
<td>53</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
<td>5</td>
<td></td>
<td>Ph-Cl</td>
<td>83</td>
<td>61</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
<td>5</td>
<td></td>
<td>iso-Octane</td>
<td>56</td>
<td>39</td>
</tr>
</tbody>
</table>

Conditions: 1 mmol 1-phenylethanol, 3 mmol p-chlorophenyl acetate, catalyst complex 5,
50 mg Novozym 435, 3 ml solvent, N₂ atmosphere, 70°C, 48h. ee of 1-phenylethyl acetate >99% in all cases. Data in parentheses for reactions after 24 h. 0.25 eq. acetophenone was added. 1.0 eq. acetophenone was added Catalyst: RuCl₂(PPh₃)₃. Catalyst: 1-Amino-2-indanol-[RuCl₂(p-cymene)]₂ complex. 1,2-Dimethoxyethane

The results of DKR experiments using [RuCl₂(p-cymene)]₂ (3) in the presence of various ligands, which showed high racemisation activities as discussed above, are shown in Table 6. In the presence of TEMPO /(-)-sparteine (Table 6, entry 2) or 8b/MS /K₂CO₃ (Table 6, entry 3), catalyst 3 catalysed in situ racemisation of 1-phenylethanol under the conditions of CAL-B-catalysed kinetic resolution. However, the combination of 3-8a-K₂CO₃ showed no racemisation activity under the same conditions, although a high racemisation activity was observed in the absence of lipase (Table 2, entries 6 and 7).

Table 6 Dynamic kinetic resolution of 1-phenylethanol catalysed by 3 and CAL-B

<table>
<thead>
<tr>
<th>Entry</th>
<th>8a /K₂CO₃ (mol%)</th>
<th>TEMPO /(-)-Sparteine (mol%)</th>
<th>8b/MS /K₂CO₃ (mg/mol%)</th>
<th>Conv (%)</th>
<th>Yield of (S)-ester (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11 /20</td>
<td>--</td>
<td>--</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>2</td>
<td>--</td>
<td>4 /14</td>
<td>--</td>
<td>85</td>
<td>77</td>
</tr>
<tr>
<td>3</td>
<td>--</td>
<td>--</td>
<td>7.4 /20</td>
<td>74</td>
<td>68</td>
</tr>
</tbody>
</table>

Conditions: 1 mmol 1-phenylethanol, 3 mmol p-chlorophenyl acetate, catalyst 3 (2 mol%), 50 mg Novozym 435, 3 ml toluene, N₂ atmosphere, 70°C, 48h. ee of (S)-1-phenethyl acetate >99% in all cases.

3. Conclusions

In summary, we have developed four new catalytic systems which are capable of catalysing the racemisation of chiral secondary alcohols. Three of these systems were shown to catalyse the in situ racemisation of 1-phenylethanol during enzymatic acylation. Among them [TosN(CH₂)₂NH₂]RuCl₂(p-cymene)/TEMPO was studied further in combination with CAL-B, with which enantiomerically pure (>99% ee) (S)-1-phenylethyl acetate was obtained in 76% yield at 91% conversion. The only side product observed was acetophenone, formed presumably by oxidation of the substrate by
TEMPO. 4,4,2-Trimethyl-2-pyridin-2-yl-1,3-oxazolidine and 4,4-dimethyl-2-pyridin-2-yl-1,3-oxazolidine were shown for the first time to be efficient ligands for ruthenium-catalysed racemisation of secondary alcohols. Further investigations are needed to optimize the reaction conditions and elucidate the mechanism.

4. Experimental

4.1 Materials and methods

Novozym 435 (C. antarctica lipase B, CAL-B) was a kind donation from Novo Nordisk, Denmark. HPLC with chiral columns OD or OB-H was used for determining the ee. The eluent was a mixture of n-hexane and 2-propanol (95:5). Conversion was analyzed by GC with a WAX 52 CB column (on Varian 3400 CX). FID detector and temperature program were used. Dodecane or hexadecane was used as internal standard. Products were characterized by GC-MS or NMR.

4.2 Reaction procedures

Preparation of 4-chlorophenyl acetate (CPA): To a solution of 4-chlorophenol (6.44 g, 50 mmol), Et$_3$N (15.1 ml, 150 mmol), and DMAP (125 mg, 1.0 mmol) in MTBE (80 ml) was added a solution of acetyl chloride (3.9 ml, 55 mmol) in MTBE (20 ml) dropwise. The reaction mixture was stirred at room temperature overnight (16-24 h), followed by washing with 1 M HCl (3 x 50 ml) and distilled water (3 x 50 ml). The yellow organic phase was dried over anhydrous Na$_2$SO$_4$. The solvent was evaporated under vacuum and the crude product (yield 95%) was purified with silica column chromatography (petroleum/ethyl acetate = 9/1) to give 4-chlorophenyl acetate (CPA) as a colourless to yellowish oil (6.38 g, 74.8%).

$^1$H NMR (400 MHz, CDCl$_3$, TMS): $\delta$ 7.32 (2H, aromatic), 7.01 (2H, aromatic), 2.25 (3H, CH$_3$)

$^{13}$C NMR (100 MHz, CDCl$_3$, TMS): $\delta$ 169.3, 149.1, 131.1, 129.4, 122.9, 20.9

Preparation of N-p-tosylethylenediamine (TSEDA): A mixture of p-toluenesulfonyl chloride (5.7 g, 30 mmol) in dichloromethane (50 ml) was added dropwise to an efficiently stirred solution of ethylenediamine (10 ml, 150 mmol) in dichloromethane (40 ml) at room temperature. After addition, the mixture was stirred at room temperature for
Ruthenium catalysts for racemization and dynamic kinetic resolution of secondary alcohols

an additional 1.5 h, followed by washing with distilled water (2 x 30 ml) and drying over anhydrous Na₂SO₄. The solvent was removed under vacuum to give a white powder (5.04 g, 77.8%, mp 116.5-118.5 °C).

¹H NMR (400 MHz, CDCl₃, TMS): δ 7.75 (2H, aromatic), 7.31 (2H, aromatic), 2.95 (2H, CH₂-N-S), 2.79 (2H, C-CH₂-N), 2.43 (3H, CH₃)

¹³C NMR (100 MHz, CDCl₃, TMS): δ 143.3, 136.9, 129.7, 127.0, 77.3, 76.7, 45.2, 40.8, 21.5

Preparation of [RuCl₂(p-cymene)]₂ (3): A solution of RuCl₂·3H₂O (4.01 g, 40%, 7.7 mmol) and α-terpinene (23 ml, 85-90%, 127 mmol) in ethanol (140 ml) was heated under reflux for 5 h under a nitrogen atmosphere, followed by cooling down to 4 °C. Unreacted RuCl₂·3H₂O was filtered off and washed with ether (3 x 25 ml). Petroleum ether (40-60 °C, 30 ml) was added to the combined filtrate, resulting in the formation of a red precipitate, which was filtered off and dried under vacuum to give a red powder (2.93 g, 62.2%).

¹H NMR (400 MHz, CDCl₃, TMS): δ 5.35 (2H, aromatic), 5.48 (2H, aromatic), 2.97-2.87 (1H, CH-C), 2.16 (3H, CH₃), 1.29 (6H, C-(CH₃)₂)

¹³C NMR (100 MHz, CDCl₃, TMS): δ 101.2, 96.7, 81.3, 80.5, 30.6, 22.1, 18.9

Preparation of (N-p-Tosylethlyenediamine)RuCl(p-cymene) (5): To a solution of [RuCl₂(p-cymene)]₂ (0.612 g, 1 mmol) and triethylamine (0.56 ml) in 2-propanol (60 ml) was added TSEDA (0.428 g, 2 mmol). The mixture was flushed with N₂, and then heated under reflux for 1 h under a nitrogen atmosphere. After cooling down to room temperature, 2-propanol and excess Et₃N were removed under vacuum and the red residue was dissolved in CH₂Cl₂ (50 ml), washed with H₂O (3 x 20 ml) and dried over NaSO₄. The solvent was removed under vacuum to give red crystals (0.805 g, 83.3%).

¹H NMR (400 MHz, CDCl₃, TMS): δ 7.75 (2H, tosyl aromatic), 7.15 (2H, tosyl aromatic), 5.65-5.50 (broad, 4H, cymene aromatic), 3.05 (broad, 2H, tosyl-N-CH₂), 2.80 (1H, cymene CH-C), 2.73 (broad, 2H, S-C-CH₂-N), 2.33 (3H, tosyl CH₃), 2.12 (3H, cymene CH₃), 1.22 (3H, cymene C-CH₂)

4,4,2-Trimethyl-2-pyridin-2-yl-1,3-oxazolidine (8a): To a solution of 2-amino-2-methyl-1-propanol (1.51 g, 17 mmol) and p-toluenesulfonic acid hydrate
(28.5 mg, 0.15 mmol) in dry toluene (50 ml) was added freshly distilled 2-acetylpyridine (1.82, 15 mmol). The mixture was refluxed overnight under nitrogen with azeotropically removal of water using a Dean-Stark set-up. After removal of the solvent under vacuum, the yellow residue was dissolved in CH$_2$Cl$_2$ (30 ml), washed with water and dried over Na$_2$SO$_4$. Removal of the solvent gave crude product (2.33 g, 80.3% yield). Purification over a silica gel column (ethyl acetate : petroleum ether = 8 : 2) gave pure liquid 8a.

$^1$H NMR (400 MHz, CDCl$_3$, TMS): δ 8.56 (1H, H$_d$), 7.69-7.63 (2H, H$_a$+H$_b$), 7.16 (1H, H$_c$), 3.71-3.38 (2H, H$_f$), 1.66 (3H, H$_g$), 1.32 (3H, H$_h$), 1.04 (3H, H$_i$), 2.73 (3H, H$_r$ of the chain form)

$^{13}$C NMR (100 MHz, CDCl$_3$, TMS): δ 164.2, 148.6, 136.5, 122.2, 119.4, 97.2, 76.8, 59.4, 29.7, 28.4, 26.4

**Preparation of 4,4-Dimethyl-2-pyrind-2-yl-1,3-oxazolidine (8b):** To a solution of 2-amino-2-methyl-1-propanol (185.0 mg, 2.07 mmol) in absolute ethanol (20 ml) was added freshly distilled 2-pyridinecarboxaldehyde (220.2 mg, 2.05 mmol). The mixture was stirred at room temperature overnight. The solvent and excess starting materials were removed under vacuum to give a crude yellow oily product (338.3 mg, 92.7% yield). Purification over a silica gel column (ethyl acetate : petroleum ether = 8 : 2) gave pure liquid 8b.

$^1$H NMR (400 MHz, CDCl$_3$, TMS): δ 8.60 (1H, H$_d$), 7.72 (1H, H$_b$), 7.43 (1H, H$_e$), 7.24 (1H, H$_c$), 5.56 (1H, H$_f$), 3.71-3.53 (2H, H$_g$), 1.39 (3H, H$_h$), 1.32 (3H, H$_i$)

![Structure of 8a and 8b](image_url)

In the above procedure of preparation of 8b, if 3A molecular sieve (from Aldrich) was added into the reaction mixture and stirred, after filtering off the molecular sieve and removal of solvent from the filtrate, a gray-white powder (mp >210 °C) was obtained. The powder was not soluble in most solvents, such as chloroform, toluene, THF, DMF, and DMSO. Therefore, the analysis data are not available. This solid, which was different from the 8b obtained as above, was used in the racemisation (Table 2, entries 12-14).
Ruthenium catalysts for racemization and dynamic kinetic resolution of secondary alcohols

A typical procedure for dynamic kinetic resolution of 1-phenylethanol: Catalyst (1.5% m) (TsRuCl 7.25 mg, 0.015 mmol) and co-catalyst (3% m) (KOH 1.68 mg, 0.03 mmol or TEMPO 4.5% m, 7.0 mg) were added to toluene (3 ml) and stirred at 70 °C for one hour under nitrogen atmosphere. After the solution was cooled down to room temperature, rac-1-phenylethanol (122 mg, 1 mmol), p-chlorophenyl acetate (511 mg, 3 mmol) and Novozyme 435 (50 mg) were added. The system was flushed with nitrogen gas for short time and then closed and stirred at 70 °C. Samples were taken at regular time intervals for measurement of the conversion with GC and ee with chiral HPLC.
References for Part II


125
References for part 2

References for part 2

Kriebel, V.K. and Wieland, W.A. (1921) J. Am. Chem. Soc. 43, 164-175
References for part 2

Rosenthaler, L. (1908) Biochem Z 14, 238-253
Summary

Characterised by its high catalytic activity, high stereo-, regio-, and chemoselectivity, and mild reaction conditions, biocatalysis is one of the most efficient methods to meet the "green" requirements in organic synthesis. This thesis is divided into two parts, in which respectively two enzymes play a major part. The oxidase enzyme laccase is applied for the oxidation of alcohols, while lipase enzyme is used for the kinetic resolution of alcohols.

Part I deals with the application of laccase-TEMPO systems in the oxidation of alcohols. In Chapter 1 a general introduction is given concerning TEMPO-catalysed oxidation of alcohols and laccase-mediator system (LMS) catalysed biotransformation of nonphenolic compounds. The primary substrates of laccases are phenolic compounds, which have low redox potentials and can be oxidised by laccase via electron transfer. In the presence of TEMPO however, laccases can oxidise non-phenolic compounds, such as benzylic alcohols.

Chapter 2 describes a study on the effects of various reaction conditions on the laccase-TEMPO-catalysed oxidation of alcohols. For laccase from Coriolus versicolor an optimum pH of 4.5-4.8 and an optimum temperature of 20-30 °C were found. Although organic solvents inhibited the activity of laccase, the laccase-TEMPO system was still active in the presence of up to 30% v/v water-miscible or water-immiscible solvents. It was also observed that laccase-TEMPO displayed a higher activity in phosphate buffer than in acetate buffer.

In Chapter 3 the effect of TEMPO and its derivatives on the laccase-catalysed aerobic oxidation of alcohols was studied. The efficiency of TEMPO and its derivatives as mediators was found to depend on their structure as well as the solvent. In order to further improve the laccase-TEMPO system to a more practical level, kinetic and mechanistic investigations were performed with laccase from Coriolus versicolor (CvL). These are described in Chapter 4. From these investigations it followed that the instability of oxoammonium salts in acidic buffers, and the deactivation of laccase in the presence of these same oxoammonium salts can be held responsible for the relatively high amounts of laccase and TEMPO needed in the oxidation of alcohols.

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In Part II the goal was to prepare chiral secondary alcohols using lipase-catalysed kinetic or dynamic kinetic resolution. In Chapter 5 a general introduction to enzymatic kinetic resolution of secondary alcohols is presented. Cyanohydrins form a separate class of alcohols and are regarded as highly versatile intermediates in organic synthesis, therefore their preparation in enantiomerically pure form is reviewed in more detail. A straightforward method for the preparation of optically active cyanohydrins and their acetates has been developed which resulted in excellent enantioselectivity. This method is described in Chapter 6. The results of investigations on dynamic kinetic resolution of cyanohydrins with CAL-B (immobilized lipase) and alkaline Amberlite IRA 904, are presented in Chapter 7. The racemisation of cyanohydrins by Amberlite was very fast, however both the acyl donor and the cyanohydrin acetate turned out to be labile to hydrolysis. Chapter 8 deals with the ruthenium complex-catalysed racemisation of secondary alcohols and its application in the dynamic kinetic resolution. Four systems were developed which proved to be active in racemising secondary alcohols efficiently. Three of these systems also displayed high activity in the CAL-B-catalysed kinetic resolution of secondary alcohols.
Samenvatting

Biokatalyse wordt gekenmerkt door een hoge katalytische activiteit, een hoge stereo-, regio- en chemoselectiviteit, en milde reactieomstandigheden. Daarom is biokatalyse bij uitstek één van de meest efficiënte methoden om te kunnen voldoen aan de “groene” eisen die tegenwoordig aan de organische synthese worden gesteld. Dit proefschrift is opgedeeld in twee delen waarin twee enzymen de hoofdrol spelen. Het oxidase-enzym laccase kan worden ingezet voor de oxidatie van alcoholen, terwijl een lipase-enzym wordt gebruikt voor de kinetische resolutie van alcoholen.

In deel 1 wordt de toepassing van laccase-TEMPO-systemen in de oxidatie van alcoholen beschreven. Hoofdstuk 1 is een algemene inleiding over TEMPO-gekatalyseerde oxidatie van alcoholen en de laccase-gekatalyseerde biotransformatie van niet-fenolische verbindingen in aanwezigheid van een zogenaamde mediator. De primaire substraten van laccase zijn fenolische verbindingen, die een lage redox-potentiaal hebben en geoxideerd kunnen worden door laccase via elektronenoverdracht. In aanwezigheid van TEMPO echter, kan laccase ook niet-fenolische verbindingen, zoals benzylische alcoholen, oxideren.

In hoofdstuk 2 wordt het effect van de verschillende reactiecondities op de laccase-TEMPO-gekatalyseerde oxidatie van alcoholen beschreven. Voor laccase van Coriolus versicolor werd een pH-optimum van 4.5-4.8 en een optimale temperatuur van 20-30°C gevonden. Ondanks het feit dat organische oplosmiddelen de activiteit van laccase verminderden, was het laccase-TEMPO-systeem nog steeds werkzaam in media die tot 30 volumeprocenten (zowel een met water mengbaar als niet-mengbaar) oplosmiddel bevatten. Experimenteel werd ook vastgesteld dat laccase-TEMPO een hogere activiteit vertoonde in een fosfaatbuffer dan in een acetaatbuffer.

In hoofdstuk 3 wordt het effect van de aanwezigheid van TEMPO en derivaten hiervan, op de laccase-gekatalyseerde oxidatie van alcoholen met luchtzuurstof bestudeerd. Het bleek dat de efficiëntie van deze zogenaamde mediatoren of co-katalysatoren zowel van hun structuur als van het gebruikte oplosmiddel afhing. Om het laccase-TEMPO-systeem verder te kunnen optimaliseren tot een potentieel industrieel proces, werden kinetische en mechanistische studies uitgevoerd met het laccase van
Coriolus versicolor (CvL). Deze worden beschreven in hoofdstuk 4. Hieruit kon worden geconcludeerd dat de instabiliteit van o xoammoniumionen in zure buffers en de deactivering van laccase door deze zelfde o xoammoniumionen, er de oorzaak van waren dat relatief grote hoeveelheden laccase en TEMPO nodig waren voor de oxidatie van alcoholen.

In deel II van het proefschrift worden de door lipase gekatalyseerde kinetische en dynamisch kinetische resolutie toegepast met het doel chirale secundaire alcoholen te bereiden. In hoofdstuk 5 wordt een algemene inleiding tot de kinetische resolutie van alcoholen gegeven, met speciale aandacht voor de bereiding van enantiomeren zuivere cyaanhydrinen. Cyaanhydrinen vormen een aparte klasse van alcoholen, ze worden gezien als veelzijdige intermediaren binnen de organische synthese. Er werd een eenvoudige en doeltreffende methode ontwikkeld voor de bereiding van optisch actieve cyaanhydrinen en de acetaten ervan, die resulteerde in een hoge enantioselectiviteit. Deze methode wordt beschreven in hoofdstuk 6. De resultaten van een onderzoek naar de dynamisch kinetische resolutie van cyaanhydrinen, door gebruik te maken van CAL-B (geimmobiliseerd lipase) en alkalisch Amberlyte IRA 904, worden beschreven in hoofdstuk 7. De racemisatie met behulp van Amberlyte verliep erg snel, echter zowel de acyldonor als het cyaanhydrineacetaat bleken gevoelig voor hydrolyse. Hoofdstuk 8 behandelt de racemisatie van alcoholen met rutheencomplexen als katalysatoren en de toepassing ervan in de dynamisch kinetische resolutie. Vier systemen werden ontwikkeld waarvan bleek dat ze in staat waren om secundaire alcoholen efficiënt te racemiseren. Drie van deze systemen vertoonden ook een hoge activiteit in de CAL-B-gekatalyseerde kinetische resolutie van secundaire alcoholen.
Acknowledgement

Changing living circumstances, especially from the very familiar Chinese culture to the European culture, is in some sense a challenge to my life. After almost four years' of working and studying in the Netherlands, I have learnt a lot from both academic and social sides. Ik hou van verse melk en kaas, maar niet van regen en veel wind. Here I would like to give my first thanks to Roger, my supervisor. Without your kindly invitation, I couldn't have come here the first year, and then of course not to say the following years. Your resourceful ideas and preciseness on research, as well as your friendliness are very impressive and helpful to me. Your perfect English helped me to finish this thesis without grammatical mistakes.

I very much appreciate the daily supervision from Ulf and Isabel. It was a pleasant time working with both of them. I would like to thank them both not only for helping me on my research, but also for improving my English, although English is not their native language. Their enthusiasm for research also encouraged me. I thank Thomas for giving me an opportunity to work in his group on enzymatic synthesis of cyanohydrins. That was a nice experience for me starting with enzymes.

Of course, Mieke vd K. is greatly acknowledged for her kind help and arrangements. She also helped me to learn about the Dutch culture and society. Ik kan nu gemakkelijk Nederlands spreken! I also thank Adrie (Kluyver Lab) and Fred for their valuable discussions and suggestions.

Rina, my student in Delft, I would like to thank you for your joining my research project and your work on laccase-TEMPO-catalysed oxidations as depicted in chapters 2 and 3. Whether you go to university or to work somewhere, Ik wens jou verder veel succes.

My acknowledgements go to Kristina, Joop and Anton for their work on NMR analysis, to Adrie for GC-MS analysis, and to Frank and Lars K. for saving my time by solving the problems of GC or HPLC.

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李玉新
Curriculum vitae

Yu-Xin Li was born on October 2nd 1963 in Hunan, P. R. China. After 4 years of study at the Hunan University, he received his B.Sc. diploma in Chemical Technology in 1984. He then moved to the University of Petroleum (Beijing), where he obtained the M.Sc. diploma in Chemical Engineering in 1987, under supervision of Prof. Guanghua Yang and Prof. Xia Shi. From 1987 to 1990, he worked at the Jianghan Petroleum Institute and from 1990 to 1999, at the Hunan Research Institute of Chemical Industry. During this period, he mainly worked on the process development of agrochemicals. He also spent two years as an administrative officer in a governmental department, and two years in the Hunan Chemical Information Center. In May 1999 he joined the research groups of Prof. Sheldon and Prof. Maschmeyer, Delft University of Technology.
List of publications

Li, Y.-X., Hanefeld, U. and Maschmeyer, T. (1999) Enzyme catalytic synthesis of optically active cyanohydrins. *Hunan Huagong* 29 (6), 1-6 (Eng)


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Sub]₀</td>
<td>Initial substrate concentration</td>
</tr>
<tr>
<td>3-HAA</td>
<td>3-Hydroxyanthranilic acid</td>
</tr>
<tr>
<td>4-CPA</td>
<td>4-Chlorophenyl acetate</td>
</tr>
<tr>
<td>Aa-TEMPO</td>
<td>4-Acetamido-2,2,6,6-tetramethylpiperidinyl-1-oxy</td>
</tr>
<tr>
<td>AB</td>
<td>Acetate buffer</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2’-Azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)</td>
</tr>
<tr>
<td>Ac-TEMPO</td>
<td>4-Amino-4-carboxylic-2,2,6,6-tetramethylpiperidinyl-1-oxy</td>
</tr>
<tr>
<td>AH</td>
<td>Asymmetric hydrogenation</td>
</tr>
<tr>
<td>Am-TEMPO</td>
<td>4-Amino-2,2,6,6-tetramethylpiperidinyl-1-oxy</td>
</tr>
<tr>
<td>ATH</td>
<td>Asymmetric transfer hydrogenation</td>
</tr>
<tr>
<td>BTEAC</td>
<td>Benzyltriethylammonium chloride</td>
</tr>
<tr>
<td>C₅₇mimBF₄</td>
<td>1-Butyl-3-methylimidazolium tetrafluoroborate</td>
</tr>
<tr>
<td>C₅₇mimDCA</td>
<td>1-Butyl-3-methylimidazolium dicyanoamine</td>
</tr>
<tr>
<td>CAL-B</td>
<td><em>Candida antarctica</em> lipase B</td>
</tr>
<tr>
<td>CKR</td>
<td>Chemical kinetic resolution</td>
</tr>
<tr>
<td>Conv</td>
<td>Conversion</td>
</tr>
<tr>
<td>CPBAc</td>
<td>α-Cyano-3-phenoxycylacetate</td>
</tr>
<tr>
<td>CvL</td>
<td><em>Coriolus versicolor</em> laccase</td>
</tr>
<tr>
<td>DIPE</td>
<td>Di-isopropyl ether</td>
</tr>
<tr>
<td>DKR</td>
<td>Dynamic kinetic resolution</td>
</tr>
<tr>
<td>DME</td>
<td>1,2-Dimethoxyethane</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-Dimethyl formamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>dppe</td>
<td>1,1’-Bis(diphenylphosphino)ferrocene</td>
</tr>
<tr>
<td>E</td>
<td>Enantioselectivity</td>
</tr>
<tr>
<td>ee</td>
<td>Enantiomeric excess</td>
</tr>
<tr>
<td>EKR</td>
<td>Enzymatic kinetic resolution</td>
</tr>
<tr>
<td>HbHNL</td>
<td><em>Hevea brasiliensis</em> hydroxynitrile lyase</td>
</tr>
<tr>
<td>HBT</td>
<td>N-hydroxy benzotriazole</td>
</tr>
<tr>
<td>HNL</td>
<td>Hydroxynitrile lyase</td>
</tr>
<tr>
<td>IPA</td>
<td>Isopropenyl acetate</td>
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<td>Kₐₑq</td>
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<tr>
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<td>Mediator</td>
</tr>
<tr>
<td>Med/Sub</td>
<td>Mediator mole percent on basis of substrate</td>
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<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
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<tr>
<td>MeHNL</td>
<td><em>Manihot esculenta</em> hydroxynitrile lyase</td>
</tr>
<tr>
<td><em>M</em>&lt;sub&gt;ox&lt;/sub&gt;</td>
<td>Oxoammonium ion of mediator</td>
</tr>
<tr>
<td>MTBE</td>
<td>Methyl <em>tert</em>-butyl ether</td>
</tr>
<tr>
<td>NAD(P)</td>
<td>Nicotinamide adenine dinucleotide (phosphate)</td>
</tr>
<tr>
<td>NHA</td>
<td>N-Hydroxy acetanilide</td>
</tr>
<tr>
<td>NHPI</td>
<td>N-Hydroxyphthalimide</td>
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<td>OMW</td>
<td>Olive mill wastewater</td>
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<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
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<td>PaHNL</td>
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<tr>
<td>PB</td>
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<td>SbHNL</td>
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<tr>
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<td>Substrate</td>
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<td>T</td>
<td>Toluene</td>
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<td>THF</td>
<td>Tetrahydrofuran</td>
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<td>Vinyl acetate</td>
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<td>VLA</td>
<td>Violuric acid</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum reaction rate</td>
</tr>
<tr>
<td>W</td>
<td>Water</td>
</tr>
<tr>
<td>W-T</td>
<td>Water-toluene</td>
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