Biotransformations of Turpentine Constituents: Oxygenation and Esterification

Marica Lindmark-Henriksson

Doctoral Thesis

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Publikationen kan beställas från:
Kungl Tekniska Högskolan
To my dear parents

To Emelie and Anton

with love
Lindmark-Henriksson Marica
Biotransformations of Turpentine Constituents:
Oxygenation and Esterification

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This thesis describes methods to obtain value–added compounds from TMP-turpentine obtained from the spruce, *Picea abies*. The methodology focuses on biotransformations using two approaches: an oxygenation approach (*i.e.* oxygenation of terpene hydrocarbons by cell cultures) and an esterification approach (*i.e.* lipase-catalysed transesterification of vinyl acetate with terpene alcohols, and a further fractionation of the TMP-turpentine).

The main constituents of the turpentine, $\alpha$-pinene, $\beta$-pinene and limonene, were subjected to a *P. abies* suspension culture. Allylic oxidation formed the major products for $\alpha$-pinene and $\beta$-pinene, which were further oxidised to their respective aldehyde or ketone. One of the minor products from $\alpha$-pinene, cis-verbenol, was not only transformed into verbenone but also isomerised to trans-verbenol. Limonene gave limonene-(1,2)-epoxide as the major product.

Fractionation of monoterpenes is accomplished through physical separation methods, chromatography and distillation, and lipase-catalysed transesterification of vinyl acetate with terpene alcohols. The esters of myrtenol and *trans*-pinocarveol were separated from the more slowly reacting alcohols such as borneol and carveol by use of a combination of the *Mucor miehei* lipase and *Candida antarctica* lipase A as catalysts. Furthermore, the non-reacting tertiary terpene alcohols were separated from the reacting alcohols in a single step by *Candida antarctica* lipase A.

Lipase-catalysed (*Candida antarctica* lipase B and *Pseudomonas cepacia* lipase) transesterification of vinyl acetate with sterically hindered secondary alcohols unexpectedly yielded hemiacetals or hemiacetal esters. The reaction conditions required to obtain these side products have been studied.
Keywords: *Picea abies, Pinaceae*, Essential oils composition; Terpene alcohol; Hemiacetal; Hemiacetal ester, TMP-turpentine; Monoterpene; α-Pinene; β-Pinene; Limonene; Verbenol; Pinocarveol; Borneol; Myrtenol; Suspension cell culture; Biotransformation; Lipase-catalysed; Oxidation; Allylic oxidation; Transesterification; Autoxidation; Separation.
This thesis is mainly based on the following papers, manuscripts and appendices. In the thesis, these are referred to as I-V and Appendix 1 or 2.

I. **Transformation of α-Pinene Using *Picea abies* Suspension Culture.**

II. **Transformation of Terpenes Using a *Picea abies* Suspension Culture.**

III. **Formation of Hemiacetal Esters in Lipase-Catalysed Reactions of Vinyl Esters with Hindered Secondary Alcohols.**

IV. **Hemiacetals and their esters as side-products in lipase-catalysed transesterifications of vinyl esters with sterically hindered alcohols.**

V. **Lipase-Catalysed Acylation as a Method of Separating Monoterpene Alcohols in *Picea abies* Turpentine.**

Reprints of papers, I and III were made with kind permission from the publishers: American Chemical Society and Elsevier.

Appendices:

1. **Chemical analyses of turpentine from the thermomechanical pulping (TMP) process.** Henriksson-Lindmark M.

2. **Biotransformations of monoterpenes.** Henriksson-Lindmark M.
### Abbreviations and equations

#### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Ac</td>
<td>acetyl</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>c</td>
<td>conversion</td>
</tr>
<tr>
<td>CAL</td>
<td><em>Candida antarctica</em> lipase</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CRL</td>
<td><em>Candida rugosa</em> lipase</td>
</tr>
<tr>
<td>DMAPP</td>
<td>dimethylallyl diphosphate</td>
</tr>
<tr>
<td>E</td>
<td>enantiomeric ratio (equation 1)</td>
</tr>
<tr>
<td>ee</td>
<td>enantiomeric excess (equation 2)</td>
</tr>
<tr>
<td>ent</td>
<td>enantiomer</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FPP</td>
<td>farnesyl diphosphate</td>
</tr>
<tr>
<td>GPP</td>
<td>geranyl diphosphate</td>
</tr>
<tr>
<td>His</td>
<td>histidine</td>
</tr>
<tr>
<td>HP</td>
<td>Hewlett Packard</td>
</tr>
<tr>
<td>IPP</td>
<td>isopentenyl diphosphate</td>
</tr>
<tr>
<td>LPP</td>
<td>linalyl diphosphate</td>
</tr>
<tr>
<td>MML</td>
<td><em>Mucor miehei</em> lipase</td>
</tr>
<tr>
<td>MPLC</td>
<td>medium pressure liquid chromatography</td>
</tr>
<tr>
<td>NAA</td>
<td>naphthalene acetic acid</td>
</tr>
<tr>
<td>NADP</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>PCL</td>
<td><em>Pseudomonas cepacia</em> lipase</td>
</tr>
<tr>
<td>SCA</td>
<td>Svenska Cellulosa Aktiebolaget</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>TBME</td>
<td>tert-butyl methyl ether</td>
</tr>
<tr>
<td>TLL</td>
<td><em>Thermomyces lanuginose</em> lipase</td>
</tr>
<tr>
<td>TMP</td>
<td>thermomechanical pulping</td>
</tr>
</tbody>
</table>
Equations

The enantiomeric ratio (E) is defined as the initial reaction rate ratio between the two enantiomers in a racemic mixture. E can for example be defined by the conversion (c) of a reaction and the enantiomeric excess of the substrate (eeₘ) as:

\[
E = \frac{\ln[(1-c) \cdot (1+eeₘ)]}{\ln[(1-c) \cdot (1-eeₘ)]}
\]  
Equation (1)

The percentage of enantiomeric purity, or enantiomeric excess (ee), of a chiral compound consisting of the enantiomeric R and S-forms, is for [S] > [R] defined as:

\[
\% \text{ ee} = \frac{[S] - [R]}{[S] + [R]} \cdot 100
\]  
Equation (2)

The enantiomeric excess (ee) could either be calculated for the substrate (eeₘ) or for the product (eeₚ).
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1. Introduction

1.1. Essential oils

The utilisation of essential oils, as for example offerings or perfumes, did very early become a tradition of mankind. As early as 3200 BC the Egyptians had developed technologies for producing cedar wood oils (Ohloff, 1994).

Essential oils originate from plants, fruits and vegetables and hence are products from a natural resource. The essential oils can either be tapped directly from the source as for cedar or pine essential oil, or obtained by extraction with a solvent. Essential oils can also be obtained by pressing, as exemplified by orange peel essential oil.

The main constituents of several essential oils are terpenes (see below, 2.2), the principal subject of this thesis.

1.2. Commercial interests in natural products

The classification ‘Natural’ has an economical impact on commercial products because of the interest of the consumer in natural flavours and fragrances. This classification should not be confused with the terminology used by chemists for “Natural Product Chemistry”, which is defined as the formation, structure and properties of secondary metabolites (Torssell, 1997).

The definition of ‘Natural’ is stated by the Code of Federal Regulations (1990) in USA and by the previous council of the European communities. The USA regulations define a ‘Natural’ flavour as the “essential oil, oleoresin, essence or extractive, distillate of any product of roasting, which contains the flavouring constituents derived from a bark, root, leaf or similar plant material” (Cheetham, 1997). The European guidelines define ‘Natural’ aroma compounds as “isolated by physical, enzymatic, or microbiological processes or traditional food preparation processes” (Berger, 1995). If transformation of a ‘Natural’ raw material is needed, the reaction must thus be carried out using enzymes or microorganisms in order to retain its ‘Natural’ classification. Isolation of the product from a ‘Natural’ source is similarly acceptable. If the product occurs in nature but is made using a non-natural process it is classified as ‘Nature-identical’ (Cheetham, 1997).

Because ‘Natural’ products are commercially more valuable than those of other origins, the flavour and fragrance industry is interested in producing them for economic profit. However, not all ‘Natural’ products have the desired
properties for commercial exploitation. For example, due to their generally uncharacteristic and undesirable odours, ‘Natural’ terpene hydrocarbons are of low interest for use in, for example, perfumes. Hence, these hydrocarbons are often physically removed from an essential oil to obtain a better quality. However, the terpene hydrocarbons are easily available at low cost and therefore they are ideal starting material for ‘Natural’ or ‘Nature-identical’ products (Ohloff, 1995). In contrast, the oxygenated terpenes have a more distinct and pleasant odour and hence, they are more attractive for the flavour and fragrance industry.

2. Turpentine and terpenes

2.1. Turpentine

In the thermomechanical pulping (TMP)-process (Tienvieri et al. 1999) pulp is produced mainly for manufacturing of wood-containing papers, such as newsprint and magazine papers. The conifer Norway spruce, *Picea abies*, is the main raw material for the TMP-process (see Figure 1 in Appendix 1). Hence, the SCA Ortviken paper mill in Sundsvall, Sweden, uses fresh non-barked spruce wood as the main raw material. Often, a small amount of saw chips from spruce is mixed with the wood.

The logs are barked and cut into chips. The chips are presteamed before they are introduced into the pressurised preheaters by plug screw feeders that reduce the water content. In the refining process the chips are heated to 140 °C at 4 bar and disintegrated to pulp. Then water is added until a proper pulp-concentration is reached. Thus, this process involves only mechanical treatment of the raw material. The production capacity of TMP-pulp at the Ortviken plant is about 780 000 tons / year (2002) and the annual production of thermomechanical pulp in Sweden is about 2 700 000 tons.

Turpentine is a mixture of volatile terpenoids originating from, for example, conifer wood and is always obtained when softwood is used as the raw material for the pulping process. When the heated wood chips pass through the refiner, the volatile extractives are distilled with the steam evolved. After cooling the steam from the refiner, the Ortviken plant yields a condensate (TMP-turpentine), which is collected and taken to a turpentine decanter. Because turpentine is
lighter than and poorly soluble in water, the turpentine will float on top of the water layer in the decanter. About 0.2 kg TMP-turpentine per ton pulp can be collected. Hence, it would be possible to obtain 540 tons of TMP-turpentine in Sweden every year. The TMP-turpentine is of a high quality compared to turpentine from other pulping processes such as the kraft pulp-turpentine, which due to the nature of the latter process contains odorous sulphur compounds.

Today, turpentine is used mainly as solvents and diluents for oil-based paints, varnishes, and pharmaceuticals as well as perfume additives. In the pulping industry, however, the turpentine produced is often used as fuel. Provided that the value of TMP-turpentine can be increased over the fuel price, the industry will be encouraged to exploit it. The goal of the work presented in this thesis, is to make it possible to produce value-added compounds or fractions from TMP-turpentine by using methods that can be classified as within the realm of ‘Natural’. I have chosen to work with biotransformations.

2.2. Terpenes

The common names for isoprenoids are terpenes (hydrocarbons) and terpenoids (oxygenated terpenes). The isoprenoids constitute the largest and most widely distributed class of secondary metabolites, i.e. compounds produced by plants and microbes, which are not essential to the growth of the organism. Terpenes are used for several tasks in nature, for example as pheromones and for chemical defence against microbes and insects (Torssell, 1997).

As early as 1887, von Wallach (1887) suggested that isoprene (2-methylbutadiene) is the building block of terpenes and he earned the Nobel Price in chemistry in 1910 for this concept. The isoprene units are ‘linked in a special way according to the isoprene rule (von Wallach, 1887, Ruzicka, 1953). Hence, the tail of one unit is linked to the head of the next one (Figure 1).

![Figure 1. The isoprene rule: Head to tail coupling of two isoprene units giving limonene.](image-url)
The terpenes are classified based on the number of isoprene-units linked (Table 1). More than 6 000 compounds with mono-, sesqui-, and diterpene structures have been detected and their structures determined (Connolly et al. 1991). The vast majority of these are hydrocarbons or oxygen containing terpenes such as alcohols. There are compounds with the isoprenoid skeleton, either lacking one, or with one extra carbon atom, named nor-terpenes and homo-terpenes respectively.

Table 1. Classification of terpenes based on Sjöström (1981).

<table>
<thead>
<tr>
<th>Prefix</th>
<th>Number of carbon atoms</th>
<th>Number of isoprene (C5H8) units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemi</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Mono</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>Sesqui</td>
<td>15</td>
<td>3</td>
</tr>
<tr>
<td>Di</td>
<td>20 (e.g. resin acids)</td>
<td>4</td>
</tr>
<tr>
<td>Sester</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>Tri</td>
<td>30 (e.g. steroids)</td>
<td>6</td>
</tr>
<tr>
<td>Tetra</td>
<td>40 (e.g. carotenoids)</td>
<td>8</td>
</tr>
<tr>
<td>Poly</td>
<td>&gt;40 (e.g. rubber)</td>
<td>&gt;8</td>
</tr>
</tbody>
</table>

The monoterpenes are C₁₀-terpenes. The monoterpenes can be further subdivided into acyclic, mono-, bi-, as well as various polycyclic compounds. These subclasses can be even further divided into groups according to their carbon skeleton. For example, the menthanes, belong to the monocyclic monoterpenes, whereas the pinanes and fenchanes belong to the bicyclic monoterpenes (see 2.4.1, Figure 7).
Several terpenes are chiral. The two enantiomers of a chiral compound often show different biological activities, including odour (Ohloff 1990, Berger 1995). The fragrance of the menthane group member \((4R)\)-(+)\-limonene (citrus fruit) differs from the turpentine like fragrance of \((4S)\)-(−)\-limonene (Figure 2).

**Figure 2.** The two enantiomers of limonene have different fragrances.
2.3. Biosynthesis of monoterpenes

The biosynthesis of terpenoids has been the subject of much research. The biosynthesis of monoterpenes, such as the rearrangement of GPP to linalyl diphosphate, cyclisation and dehydration of rearrangement products has been studied in, for example conifers (Croteau 1987, Bernard-Dagan 1988, Savage et al. 1995, Phillips and Croteau 1999, Sjödin et al., 2000). Enzymes catalysing the biosynthesis of monoterpenes have also been studied (Savage et al. 1994, Savage et al. 1995, Bohlmann and Croteau, 1999, Phillips et al. 1999, Fäldt et al., 2003).

It is still unclear where in the plant cell the biosynthesis of terpenoids takes place. One theory is that the monoterpenoids, diterpenoids and tetraterpenoids are synthesised in the plastids (chloro- or leucoplasts) of the cell, and that the sesquiterpenoids are synthesised in the cytosol of the cell (Rohmer, 1999, Rohmer et al. 2001). The diphosphates (i.e. IPP, GPP and FPP) are exchanged between the two pathways in the active transport between the cytoplasm and the plastid in the plant cell (Rohmer, 1999).

The biosynthesis of monoterpenes from primary metabolites can schematically be divided into four steps:

**Step 1.** Synthesis of the isoprene unit, isopentenyl diphosphate (IPP), which is the fundamental precursor of all isoprenoids (Figure 3). Two major biosynthetic pathways leading to IPP exist, i.e. the mevalonate pathway (Figure 3a, Dewick, 1999, McCaskill and Croteau, 1997) and the mevalonate independent pathway, "the Rohmer pathway" (Figure 3b, Rohmer, 1999, Rohmer et al., 2001). Both pathways lead to IPP and enzymes catalyse the individual steps. Many of these enzymes have been isolated and are well characterised (Bach, 1995, McGarvey, 1995, Rohmer, 2001, Hoeffler et al., 2002). For instance, the isomerisation of IPP into DMAPP is catalysed by IPP isomerase (Figure 3).
Figure 3. The first step in the biosynthesis of terpenoids is shown. Formation of isopentenyl diphosphate by: \textbf{a.} the mevalonate pathway (upper scheme). \textbf{b.} the mavalonate independent pathway \textit{i.e.} the “Rohmer pathway” (lower scheme). The diphosphate group is abbreviated as \textendash OPP.
Step 2. Formation of the allylic prenyl diphosphate, geranyl diphosphate (GPP) (Figure 4). GPP is the precursor of all monoterpenes.

![Figure 4](image-url) The second step in the biosynthesis of monoterpenes. PP= diphosphate.

Step 3. The elaboration of the geranyl diphosphate, *i.e.* GPP into monoterpenes (Figure 5). These reactions are catalysed by specific monoterpene synthases (Schwab *et al.*, 2002). Experimental evidence has shown that at least two enzymatic routes leading to each of the enantiomers of the monoterpenes exist (Wise and Croteau, 1999).

The formation of the pinanes begins with an isomerisation (Figure 5, step 1-2) and cyclisation (Figure 5, step 3) of GPP to the α-terpinyl cation via linalyl diphosphate (LPP) (Cane, 1999, Wise and Croteau, 1999). Although many efforts to observe LPP have been made, this postulated intermediate has not yet been found (Schwab *et al.*, 2000).
Figure 5. The third step in the biosynthesis of the monoterpene class, pinanes. Formation of \( \alpha \)-pinene and \( \beta \)-pinene from the monoterpene precursor geranyl diphosphate (GPP) (Cane, 1999, Wise and Croteau, 1999).

An intramolecular Markovnikov addition to the cyclohexenyl double bond of the \( \alpha \)-terpinyl cation forms the pinyl cation (Figure 5, step 4). Further, the pinyl cation is deprotonated, either from the adjacent methylene (step 5a) or methyl group (step 5b) to form \( \alpha \)-pinene or \( \beta \)-pinene respectively. Both enantiomers of \( \alpha \)-pinene occur naturally, whereas \( \beta \)-pinene occurs almost always as the nearly enantiomerically pure (–)-(1S)-enantiomer.
**Step 4.** Secondary chemical modifications of the parent isoprenoid product. These secondary enzymatic reactions include hydroxylation by the monooxygenases, cytochrome P-450 (see below 3.3.1), redox transformations and isomerisations (McCascill and Croteau, 1997). Oxygenated pinane monoterpenes, such as verbenol and pinocarveol are formed from \( \alpha \)- or \( \beta \)-pinene respectively (Figure 6, Wise and Croteau, 1999).

![Diagram of the transformation of \( \alpha \)-pinene and \( \beta \)-pinene to verbenol and pinocarveol respectively.](image)

**Figure 6.** The transformation of \( \alpha \)-pinene and \( \beta \)-pinene to verbenol and pinocarveol respectively is shown as an example of the last step in the biosynthesis of oxygenated monoterpenes. This step includes the secondary enzymatic reactions of the parent monoterpene to the oxygenated one.
2.4. Constituents of the TMP-turpentine

Softwood (e.g. spruce) oleoresin contains mono-, sesqui- and diterpenes while hardwood (e.g. birch) oleoresin mainly contains higher terpenes and their derivatives, such as sterols and other polypropenols. In spruce wood the oleoresin is deposited in resin ducts (canals), which can be found in all parts of the tree. Spruce oleoresin contains 50% resin acids and 20-30% monoterpenes (Sjöström, 1981). The remainder consists of other terpenoids and fatty acid esters.

Turpentine from the TMP-process at Ortviken was fractionated by distillation and medium pressure liquid chromatography (MPLC) and analysed by GC-MS (Appendix 1). The major constituents were identified using reference substances and retention indices (Davies, 1990, Appendix 1). The TMP-turpentine consisted of monoterpenes (74%, V/V), sesquiterpenes (13%, V/V) and diterpenes (1%, V/V). Ninety-two percent (V/V) of these terpenes were non polar and eight percent (V/V) were oxygenated, polar terpenes (Appendix 1).

Although 0.18% of the dry weight of wood from *P. abies* consists of monoterpenes and 0.04% consists of sesqui-terpenes and diterpene hydrocarbons (Assarsson and Åkerlund, 1966), only 0.014% turpentine is obtained from one ton of dry wood at the Ortviken TMP-paper mill. In order to investigate the possibility to increase the terpene yield from the TMP-process, wood chips of *P. abies* were steamed (Appendix 1). This resulted in an increased yield (0.12% of the dry weight) and a similar composition of terpenes compared to that achieved in the TMP-process of Ortviken mill (Appendix 1). This result demonstrated that it would be possible to produce a sixteen-fold increase in the turpentine yield in the plant.

Leakage of highly volatile compounds at different points in the process or incomplete removal during the steam distillation of the raw material could explain the loss of terpenes in the process used today. In addition, the loss of turpentine during wood storage is dramatic if storage time exceeds four weeks (Gullichsen and Lindeberg, 1999). Thus, processing of fresh wood will result in higher turpentine yields.

Preliminary results showed that the content of chemicals, such as terpene derivatives in the press water from the plug screw feeders in the process at Ortviken was too low to be commercially exploitable, hence no further work was undertaken to utilise it. (Lindmark *et al.* 1998).
2.4.1. Monoterpenes

The composition of monoterpane hydrocarbons in the xylem/phloem oleoresin of *P. abies* (Kimland and Norin, 1972, Persson et al., 1996) and in the oil obtained after steam distillation of spruce wood (Groth, 1958) has been investigated. This oil contains mainly (±)-α-pinene and (−)-β-pinene and minor quantities of other monoterpane hydrocarbons. This is in agreement with the results of our analysis of the composition of monoterpenes in the TMP-turpentine (Table 2). Thus, we have detected and identified about 30 major monoterpenes from the TMP-turpentine (Figure 7, 8, Appendix 1).

<table>
<thead>
<tr>
<th>Acyclic:</th>
<th>Bicyclic:</th>
<th>Tricyclic:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pinane skeleton</td>
<td>Camphane skeleton</td>
</tr>
<tr>
<td>Myrcene</td>
<td>α-Pinene</td>
<td>β-Pinene</td>
</tr>
</tbody>
</table>

**Figure 7.** Some of the monoterpane hydrocarbon constituents of the TMP-turpentine. The structures of the major constituents are framed.

α- and β-pinene are important intermediates in manufacturing of synthetic aroma compounds and are also used as flavouring and fragrance ingredients. Limonene is also an important monoterpane. It shows for example insecticidal properties. It is also used extensively in the perfume and flavour industries and in manufacturing of polymers and adhesives. It is also an important chiral starting material for organic synthesis (Connolly, 1991).
Table 2. The relative composition of monoterpene hydrocarbons in the TMP-turpentine compared with the monoterpene hydrocarbon content in kraft pulp-turpentine*.

<table>
<thead>
<tr>
<th>Monoterpene</th>
<th>TMP-turpentine (%)*</th>
<th>(+)/(-)**</th>
<th>Kraft pulp-turpentine (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tricyclene</td>
<td>0.7</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>Thujen</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>42.0</td>
<td>62/38</td>
<td>54.3</td>
</tr>
<tr>
<td>Sabinene</td>
<td>0.7</td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>α-Fenchene</td>
<td>0.2</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>β-Pinene</td>
<td>27.0</td>
<td>3/97</td>
<td>20.4</td>
</tr>
<tr>
<td>Camphene</td>
<td>1.7</td>
<td></td>
<td>1.1</td>
</tr>
<tr>
<td>3-Carene</td>
<td>5.4</td>
<td></td>
<td>10.0</td>
</tr>
<tr>
<td>α-Terpinene</td>
<td>0.2</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>Limonene</td>
<td>17.5</td>
<td>19/81</td>
<td>9.4</td>
</tr>
<tr>
<td>p-Cymene</td>
<td>0.7</td>
<td></td>
<td>0.4</td>
</tr>
<tr>
<td>γ-Terpinene</td>
<td>0.3</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>Terpinolene</td>
<td>1.2</td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td>Myrcene</td>
<td>2.2</td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>β-Phellandrene</td>
<td>0.3</td>
<td>7/93</td>
<td>0.2</td>
</tr>
</tbody>
</table>

*The relative composition of the monoterpene hydrocarbon constituents analysed by GC-MS. The differences in the response factors were neglected. **The ratios between the enantiomers were analysed by GC-MS using the peak purity software for control of overlapping peaks.

Kraft pulp-turpentine is often based on wood from pine instead of spruce. The ratio of monoterpene hydrocarbons is lower in kraft pulp-turpentine (47%), from the Östrand paper mill in Sundsvall, than in the TMP-turpentine (60%). Furhtermore, the composition of monoterpenes in the kraft pulp-turpentine is slightly different from that in the TMP-turpentine (Table 2). For example, the relative amount of 3-carene is higher in the kraft pulp-turpentine compared to that in the TMP-turpentine.

The composition of the monoterpenes in the TMP-turpentine remained relatively constant during a sampling period of one year, which was also true for the relative amount of the fraction of terpenes of a higher molecular weight (Appendix 1). Thus, the TMP-mill of Ortviken was found to be a source of monoterpenes of even quality.
The oleoresin from *P. abies* also contains minor amounts of volatile monoterpene alcohols, such as terpinene-4-ol, α-terpineol, myrtenol and borneol (Figure 8, Kimland and Norin, 1972). Our analyses show that these monoterpene alcohols are also the most abundant ones in the TMP-turpentine with α-terpineol as the dominating one (Table 3).

**Figure 8.** Some oxygenated monoterpenes in the TMP-turpentine. The structures of the major ones are framed.
Ketones and esters were also found in the oxygenated monoterpene fraction of the TMP-turpentine (Figure 8, Table 3).

Table 3. The relative amounts of some oxygenated monoterpenes in the TMP-turpentine from *P. abies*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Relative amount (%)</th>
<th>Concentration (mg/ml)</th>
<th>(+)/(-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borneol</td>
<td>0.4</td>
<td>0.8</td>
<td>62/38³</td>
</tr>
<tr>
<td>Bornyl acetate</td>
<td>0.5</td>
<td>0.7</td>
<td>-</td>
</tr>
<tr>
<td>Myrtenol</td>
<td>0.2</td>
<td>0.6</td>
<td>3/97⁴</td>
</tr>
<tr>
<td>Myrtenyl acetate</td>
<td>1.1</td>
<td>0.02</td>
<td>-</td>
</tr>
<tr>
<td><em>trans</em>-Pinocarveol</td>
<td>0.2</td>
<td>0.6</td>
<td>-</td>
</tr>
<tr>
<td>Terpinene-4-ol</td>
<td>0.8</td>
<td>1.1</td>
<td>-</td>
</tr>
<tr>
<td>α-Terpineol</td>
<td>2.7</td>
<td>7.1</td>
<td>19/81⁴</td>
</tr>
</tbody>
</table>

¹ The relative amount is the peak area of the compound divided by the sum of all peak areas from the TMP-turpentine (GC-MS). ² The ratios between the enantiomers were analysed by GC-MS using the peak purity software (HP) for control of overlapping peaks. ³ The ee of borneol was determined by integration of a single ion spectrum of m/z 110. ⁴ The ee of myrtenol and α-terpineol were determined by integration of the full scan (TIC) spectrum.

α-Terpineol, borneol and their acetates, are of great importance in perfumery. Myrtenol, 1,8-cineol, fenchol, linalool, carvone, and terpinene-4-ol are also of importance as fragrance constituents (Ohloff, 1997). Pinocarvone is known as a sex attractant for the pine beetle and *cis*-verbenol is a sex attractant component of *Ips typographus* (Schlyter *et al.*, 1987) and *Ips confusus* a pest on *Pinus ponderosa* (Connolly, 1991). *trans*- and *cis*-Verbenols have frequently been reported as insect semiochemicals and are therefore of use in the monitoring or mass trapping of bark beetles (Schlyter *et al.*, 1987, Lindgren and Borden, 1993, Miller, 2000).
2.4.2. Sesquiterpenes, diterpenes

Sesquiterpenes have also been found in the oleoresin of Norway spruce (Kimland and Norin 1972). Sesquiterpenes, both as hydrocarbons and oxygenated ones, constitute 13% (V/V) of the TMP-turpentine. Bicyclic sesquiterpenes with the cadinane skeleton are the main constituents of this fraction (Figure 9). Some diterpenes present in the TMP-turpentine have the cyclic cembrane skeleton (Figure 10).

**Figure 9.** Examples of sesquiterpenes with the cadinane skeleton that are found in the TMP-turpentine.

**Figure 10.** The diterpene cembrene.
2.4.3. Non-terpenoid constituents

Our studies show that TMP-turpentine also contains non-terpenoids such as alcohols, aldehydes, and aromatic derivatives, as well as straight chain and branched alkanes (Figure 11). Among the aldehydes, the malodours smelling hexanal and heptanal were present along with decadienal. These aldehydes could be the result of either autoxidation or enzymatic degradation of fatty acids (Schieberie and Grosch, 1981, Back, 2001, Nordermeer et al., 2000).

![Chemical structures](image)

**Figure 11.** Non-terpenoid constituents of the TMP-turpentine. 1) Hexanol, 2) hexanal, 3) heptanol, 4) heptanal, 5) 2,4-decadienal, 6) palmitic acid, 7) 1-allyl-4-methoxybene, 8) 1-isopropyl-2-methoxy-4-methylbene.

The spruce wood oleoresin also contained \( n \)-alkanes in homologous series ranging from \( C_{11} \) to \( C_{33} \), with \( C_{22} \) to \( C_{27} \) as main constituents (Assarsson and Åkerlund, 1966). Such compounds were also present in the TMP-turpentine. After distillation of the TMP-turpentine, the residue (12% V/V) was found to contain 17% palmitic acid (Figure 11), a small amount of some other fatty acids and diterpenes as resin acids and aliphatic branched and unbranched hydrocarbons of high molecular weight. (Appendix 1).
3. Transformation of terpenes by a *Picea abies* suspension culture

3.1. Introduction

Microbial transformations of terpenes, were carried out by Mayer and Neuberg (1915) as early as 1915. They transformed (+)-citronellal to (+)-citronellol by yeast. Biotransformation constitutes one approach to achieve the desirable classification of the products as ‘Natural’. Terpene flavour and fragrance compounds are synthesised by several microorganisms and higher plants and the conversion of low value monoterpenes to value-added ones has been investigated (Demyttenaere *et al.*, 2001, van der Werf *et al.*, 1997, Berger, 1995, Krasnobajew, 1984). However, these methods have been thwarted by the fact that the transformations lead to several products from a single substrate and in most cases, the yields are low.

The bacteria of the genus *Pseudomonas*, have the unusual ability to degrade and live on monoterpenes. They transform the monoterpenes before degrading them to carbon dioxide and water (Hungund *et al.*, 1970). Most other bacteria and fungi can only perform the transformation of terpenes, when grown in energy-rich media. This has been called co-metabolism.

3.2. Biotransformation products

Preliminary results showed that oxygenated monoterpenes were formed when we used TMP-turpentine as substrate for a *P. abies* suspension culture. Thus, α-pinene, β-pinene and limonene - major constituents of *P. abies* turpentine - were chosen as substrates for further investigations of the oxygenation ability of these cultures. Because of the explorative nature of this investigation, we have focused on the qualitative product pattern observed rather than on the yields of the products. Hence, in our earlier work (I) no corrections for differences in response factors (GC-MS) were performed. For useful applications it is of course necessary to have control over yields and mass balances between products and substrates.

Cultivation of *P. abies* cultures has been discussed by Mårtensson (2002) and has been reported by for example von Arnold and Hakman (1986). The *P. abies* suspension culture was grown in liquid nutrient medium according to Gupta and Durzan (1986) with some exceptions (Appendix 1). No optimisation of the
composition of nutrient medium and its constituents was performed. As expected for undifferentiated cells (Corbier and Ehret, 1986), no terpenes were produced by the P. abies suspension culture in the absence of a terpene substrate (I).

3.2.1. Biotransformation products of α-pinene

Biotransformations of α-pinene to verbenols and verbenone by a pure enzyme system of the monoxygenase P-450cam (Table 4, entry 2), by plant cells (Table 4, entry 3-5) and by the use of microorganisms (Table 4, entry 6-13) are reported.

Table 4. Biotransformations of α-pinene described in literature.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Microorganism/plant/enzyme</th>
<th>Allylic oxidation / and further oxidation</th>
<th>Main products</th>
<th>Other reactions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Picea abies</td>
<td>trans-verbenol, verbene, myrtanol</td>
<td>α-terpineol, trans-pinocarveol, trans-sobrerol</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>P. 450cam</td>
<td>cis-verbenol, verbene, myrtanol</td>
<td>α-pinene epoxide</td>
<td>Bell et al., 2003</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Rosa centifolia</td>
<td>trans, cis-verbenol</td>
<td></td>
<td>Corbier and Ehret, 1986</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Nicotiana tabacum</td>
<td>verbene, myrtanol</td>
<td>α-pinene epoxide, verbanone</td>
<td>Hirata et al., 1994</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Hyssop officinalis</td>
<td>trans-verbenol</td>
<td></td>
<td>Karp and Croteau, 1992</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Aspergillus sp. Penicillum sp.</td>
<td>trans-verbenol, verbene, myrtanol</td>
<td>trans-pinocarveol, sobrerol</td>
<td>Agraval et al., 1999</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Bacidomyctes</td>
<td>trans-verbenol, myrtanol, verbene, myrtanol</td>
<td>trans-pinocarveol, sobrerol, α-terpineol, carveol, carvone</td>
<td>Busmann and Berger, 1994a</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Pholiota squarrosa</td>
<td>trans-verbenol, verbene, myrtanol</td>
<td>trans-pinocarveol, p-menth-2-ene-1,8-diol</td>
<td>Busmann and Berger, 1994b</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Aspergillus niger</td>
<td>trans-verbenol</td>
<td>trans-sobrerol</td>
<td>Prema and Bhattacharyya, 1962</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Aspergillus niger</td>
<td>cis-verbenol, verbene, myrtanol</td>
<td>sobrerol</td>
<td>Rama Devi and Bhattarcharya, 1978</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Serrata marcescens</td>
<td>trans-verbenol, myrtanol, verbene, myrtanol</td>
<td>trans-sobrerol</td>
<td>Wright et al., 1986</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Armillariella mellea</td>
<td>trans-verbenol, verbene, myrtanol</td>
<td>sobrerol, carveol, carvacrol</td>
<td>Draczynska et al., 1985</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Armillariella mellea</td>
<td>verbene</td>
<td>trans-pinocarveol, α-terpineol, carvacrol</td>
<td>Draczynska et al., 1985</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Armillariella mellea</td>
<td>verbene</td>
<td>α-terpineol, sobrerol</td>
<td>Draczynska-Lusiak and Siewinski, 1989</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Pseudomonas PL</td>
<td>limonene, perillyl alcohol,</td>
<td></td>
<td>Shukla et al., 1968</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>P. putida</td>
<td>α-pinene epoxide</td>
<td></td>
<td>Gibbon and Pirth, 1971</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Pseudomonas sp.</td>
<td>myrtanol</td>
<td>borneol, perillyl acid</td>
<td>Shukla et al., 1968</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>P. fluorescence</td>
<td>myrtanol</td>
<td>α-pinene epoxide, trans-limonene-epoxide</td>
<td>Best et al., 1987</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>P. maltophilia</td>
<td>limonene, borneol, camphor, perillyl acid, 2-(4-methyl-3-cyclohexenylidene)-proponic acid</td>
<td>Narushima et al., 1982</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Pseudomonas sp.</td>
<td>myrtanol</td>
<td>α-terpineol, p-menth-2-ene-1,2-diol</td>
<td>Rhodes and Winskill, 1985</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Candida tropicalis</td>
<td>myrtanol</td>
<td></td>
<td>Chatterjee et al., 1999</td>
<td></td>
</tr>
</tbody>
</table>
In our study α-pinene was transformed by the *P. abies* suspension culture to mainly *trans*-verbenol and verbenone (Figure 12, I). *trans*-Verbenol was further transformed to verbenone (Figure 13). *trans*-Pinocarveol, *cis*-verbenol, myrtenol and α-terpineol were minor products and sobrerol was present in only some samples (I). Hence, biotransformation of α-pinene by the *P. abies* suspension culture was characterised mainly by oxidation at the allylic position. Other observed reactions were oxidative cleavage of the cyclobutane ring and oxidation of the terminal carbons in the C6 positions, producing monocyclic terpene alcohols.

![Figure 12](image)

**Figure 12.** Biotransformation products of (1R)-α-pinene with a *P. abies* suspension culture. The structures of the major products are framed.
Enantioselectivity has previously been found in biotransformations of α-pinene by plant cell suspension cultures of *Nicotiana tabacum* grown in the medium of Murashige and Skoog (1962) (Hirata *et al.*, 1994). However, our transformation of rac-α-pinene by a *P. abies* suspension culture showed only a very low preference for (1R)-α-pinene (I).

3.2.1.1. Isomerisation of cis-verbenol

cis-Verbenol has been transformed into verbenone using plant cells of *Nicotiana tabacum* and *Cannabis sativa* (Hamada, 1988, Takeya *et al.*, 1977) and the formation of trans-verbenol from cis-verbenol has been observed after biotransformation using *Solanum aviculare* plant cells (Vanek *et al.*, 1989). In the biotransformation of cis-verbenol by the *P. abies* suspension culture, the compound was isomerised to trans-verbenol (I).

![Figure 14](image)

**Figure 14.** Tentative mechanism of the biotransformation of (1S)-(4H)-cis-verbenol into (1S)-(4H)-trans-verbenol, which is further transformed to (1S)-verbenone.

This isomerisation was studied by biotransformation of a deuterium labelled (1S)-(4H)-cis-verbenol, which formed trans-verbenol with retention of the 4-
deuterium (I). Only very small amounts of verbenols were formed when
verbenone was used as a substrate. Thus, the isomerisation of cis-verbenol to
trans-verbenol did not proceed via ketone. Instead an enzyme bound allylic
cation was suggested to be involved in the reaction (Figure 14, I).

3.2.2. Biotransformation products of β-pinene
Several pathways and products have been reported for the biotransformation of
β-pinene (Table 5, Figure 15).

Table 5. Biotransformations of β-pinene described in literature.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Microorganism/plant</th>
<th>Main products</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Picea abies</td>
<td>trans-pinocarveol, myrtenol, α-terpineol</td>
<td>Dracznyska et al., 1985</td>
</tr>
<tr>
<td>2</td>
<td>Armillariella mellea</td>
<td>trans-pinocarveol, myrtenol, perillyl alcohol, hydroxyl-α-terpineol</td>
<td>Devi et al., 1978</td>
</tr>
<tr>
<td>3</td>
<td>Aspergillus niger NC1M 612</td>
<td>trans-pinocarveol</td>
<td>Bhattcharyya and Ganapathy, 1965</td>
</tr>
<tr>
<td>4</td>
<td>Aspergillus niger</td>
<td>pinocarveol, myrtenol</td>
<td>Bhattacharyya and Ganapathy, 1965</td>
</tr>
<tr>
<td>5</td>
<td>Pleurotus flabellatus</td>
<td>trans-pinocarveol, pinocamphone, eucalyptol, myrtenol, myrtenal, 1,4-cineol, 1,4-cineol, fenchone</td>
<td>Busmann and Berger, 1994a</td>
</tr>
<tr>
<td>6</td>
<td>Sewage bacterium (Seuberts medium)</td>
<td>α-terpineol, camphor borneol, isoborneol,</td>
<td>Dhavlikar et al., 1974</td>
</tr>
<tr>
<td>7</td>
<td>Sewage bacterium (Czapek-Dox medium)</td>
<td>trans-pinocarveol, pinocarvone?*</td>
<td>Dhavlikar et al., 1974</td>
</tr>
<tr>
<td>8</td>
<td>Hyssop officinalis</td>
<td>trans-pinocarveol</td>
<td>Karp and Croteau, 1992</td>
</tr>
<tr>
<td>9</td>
<td>Pseudomonas sp</td>
<td>myrtenol, borneol</td>
<td>Shukla et al., 1968</td>
</tr>
<tr>
<td>10</td>
<td>Ganoderma applanatum</td>
<td>eucalyptol, 1,4-cineol, myrtenol, myrtenal,</td>
<td>Busmann and Berger, 1994a</td>
</tr>
<tr>
<td>11</td>
<td>Bacidomycetes</td>
<td>α-terpineol, eucalyptol, perillyl alcohol, 1,4-cineol, carveol</td>
<td>Busmann and Berger, 1994a</td>
</tr>
</tbody>
</table>

Dhavlikar et al. (1974) did not determine the structure of the compound, however the massspectra (GC-MS) showed: M+ 150. Hence, it could have been pinocarvone.

The main biotransformation product from β-pinene by the P. abies suspension culture was trans-pinocarveol (Figure 15, 16, II). This is a common biotransformation product of β-pinene (Table 5, entry 1-8). The products myrtenol, α-terpineol, pinocarvone, pinocamphone, and some other minor products from β-pinene were also identified in the product mixture.
Figure 15. Tentatively biotransformation pathways of β-pinene using a P. abies suspension culture. The structure of the major biotransformation product are framed.

Figure 16. Biotransformation of (1S)-β-pinene using a P. abies suspension culture. The major product was trans-pinocarveol. *Response factor corrected integrated peak area (GC-MS) relative to the total corrected integrated area in percent.
The biotransformation of primary products from β-pinene was also investigated (Figure 17). Thus, (1R)-(−)-myrtenol, (1R)-(−)-trans-pinocarveol, (1S)-(−)-cis-pinocarvol, and (4S)-α-terpineol were used as substrates (II). Except for α-terpineol, all the substrates tested were transformed within one minute. The amounts of the products were low and the compositions did not change during the 15-days of observation period.

![Figure 17](image)

**Figure 17.** Biotransformation products of pinocarveols and myrtenol by means of a *P. abies* suspension culture. The composition of the product mixtures was calculated from integrated areas (GC-MS). The differences in response factors were neglected.

*trans*-Pinocarveol formed pinocarvone and myrtenol formed myrtenal and myrtanol. The reduction of the double bond in myrtenol leading to the saturated alcohol, myrtanol, was similar to the reduction observed during the formation of pinocamphone and isopinocamphone in the biotransformation of β-pinene. Biotransformation of *cis*-pinocarveol gave mainly pinocarvone and pinocamphone. Thus, no similar isomerisation to the one discussed above (3.2.1.1) involving a *cis*- to *trans*-verbenol epimerisation (I) was observed for
cis-pinocarveol. However, it may be possible that cis-pinocarveol isomerised to trans-verbenol, which thereafter rapidly formed pinocarvone.

3.2.3. Biotransformation products of limonene

Several biotransformation products of limonene have been reported in the literature (Figure 18, reviews Demyttenaere, 2001, Mikami, 1988). This interest for limonene as a starting material in biotransformations is likely due to the low cost of and wide availability of limonene in various essential oils such as oils of lemon, orange, caraway, dill and bergamot.

Several pathways for the biotransformation of limonene are reported (Figure 18).

Figure 18. Some biotransformation pathways for limonene found in the recent literature. (i) van der Werf et al. (2000), (ii) van der Werf et al. (1999), (iii) Vanek et al. (1999), (iv) McCaskill and Croteau (1997), (v) Onken and Berger (1999), (vi) Trytek and Fiedurek (2002), (vii) Karp et al. (1990), (viii) Noma et al. (1992), (ix) Tan et al. (1998).
Most of the microorganism and plant cell cultures seem to use more than one biotransformation pathway, which gives rise to a complex mixture of products. However, some exceptions are the biotransformation of rac-limonene by *Penicillium digitatum* that formed optically pure (4R)-(+)\-\textalpha\-terpineol in a good yield (up to 0.46 g/l) (Kieslich et al., 1986) and the transformation of (+)-limonene by *Corenyspora cassiicola*, which resulted in (1S, 2S, 4R)-p-menth-8-ene-1,2-diol as the only product in an even better yield (900g / 1300g) (Abraham et al., 1986). An interesting result is that some microorganism and plant cell cultures have been shown to transform one pure enantiomer of limonene into a mixture of (6S)- and (6R)-carveol (Berger, 1995, Vanek et al., 1999).

When (4R)-(+)\-limonene was subjected to transformation by the *P. abies* suspension culture, only a small amount of products was formed (Figure 19). Due to this low transformation rate compared to \textalpha\- and \beta\-pinene (Figure 20, II), limonene as a substrate was not further investigated.

**Figure 19.** Relative amounts of the biotransformation products of limonene in a *P. abies* suspension culture after 24 hours. The amounts are the relative integrated GC-MS areas of the peaks. The differences in response factors are neglected.
3.3. Monoterpenes as substrates for the *P. abies* culture

In comparison with β-pinene and limonene, α-pinene proved to be a rapidly reacting substrate for the *P. abies* suspension culture (Figure 20, II).

**Figure 20.** The biotransformation of a) α-pinene, b) β-pinene and c) limonene by a *P. abies* suspension culture forming their main products, *trans*-verbenol, *trans*-pinocarveol and limonene-(1,2)-epoxide, respectively. *Integrated peak area (GC-MS) relative to the total integrated area in percent. The differences in response factors are neglected.

The main transformation products of α-pinene, β-pinene and limonene formed by a *P. abies* suspension culture are shown in Figure 21.

Allylic oxidation seems to be the most favourable pathway, although several other products also occurred. The enantioselectivity of the reactions leading to the major products were low. However, a small increase in the enantioselectivity in the further transformation of the alcohols into their respective ketones or aldehydes was observed.

Enzymes or enzyme systems present in the *P. abies* suspension culture mediate the formation of the products. No identification of enzymes involved in these reactions was performed.

Enzymes catalysing biotransformations (van der Werf, 1997) and degradation (Trudgill, 1994) of monoterpenes are described in reviews. Cytochrome P-450 dependent enzymes, as described below (3.3.1), catalyses the biosynthesis of monoterpenes (McCaskill and Croteau, 1997).
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction</th>
<th>Major products</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Pinene</td>
<td>Allylic oxidation</td>
<td>trans-Verbenol, cis-Verbenol, Verbenone, Myrtenol</td>
</tr>
<tr>
<td></td>
<td>Oxidative cleavage and oxidation</td>
<td>α-Terpineol, (−)-trans-Sobrerol, (+)-trans-Sobrerol</td>
</tr>
<tr>
<td></td>
<td>Other reactions</td>
<td>trans-Pinocarveol</td>
</tr>
<tr>
<td>β-Pinene</td>
<td>Allylic oxidation</td>
<td>trans-Pinocarveol, Pinocarvone, Pinocamphone</td>
</tr>
<tr>
<td></td>
<td>Oxidative cleavage and oxidation at C7</td>
<td>α-Terpineol</td>
</tr>
<tr>
<td></td>
<td>Other reactions</td>
<td>Myrtenol</td>
</tr>
<tr>
<td>Limonene</td>
<td>Allylic oxidation</td>
<td>Carveol, Perillyl alcohol</td>
</tr>
<tr>
<td></td>
<td>Epoxidation</td>
<td>Limonene-1,2-epoxide</td>
</tr>
<tr>
<td></td>
<td>Oxidation with ring closure</td>
<td>1,8-Cineole</td>
</tr>
</tbody>
</table>

**Figure 21.** The main transformation products of α-pinene, β-pinene and limonene in the biotransformation by a *P. abies* suspension culture.
3.3.1. Monooxygenases

Oxido-reductases, which catalyse oxidations and reductions, are divided into subclasses on the basis of the type of substrate and the type of hydrogen or electron donor (acceptor) that is utilised. Monooxygenases (subgroup of oxido-reductases) catalyses the introduction of one atom of dioxygen into the substrate using cofactors furnishing oxidised organic products and a molecule of water (Figure 22). Monooxygenases could also catalyse the formation of epoxides.

Several oxido-reductases require the presence of cofactors in order to carry out their reaction. Cofactors can either be covalently bound to the active site of the enzyme forming a prosthetic group or acting as a co-substrate, interacting with the enzyme. The most commonly utilised cofactor for biosynthetic processes with oxido-reductases tends to be the nicotineamide adenine dinucleotides, NADH or NADPH (Bugg, 1999).

![Figure 22. Reactions catalysed by monooxygenases.](image)

The monooxygenases, catalysing hydroxylation of monoterpenes usually consist of three components, which take part in transporting electrons and catalysing the reaction (Figure 23-24, van der Werf, 1995):

1. A FAD flavoprotein reductase component, catalysing the electron release from NAD(P)H;
2. An iron-sulphur protein acting as an electron carrier between the flavoprotein and the cytochrome P-450 component;
3. A cytochrome P-450 containing component, which catalyses the oxidation of the substrate.
Cytochromes are protein cofactors that are essential as transporters of redox equivalents to the catalytically active sites of many oxidoreductases. Cytochrome P-450 is the terminal electron carrier of numerous monooxygenases of mammalian liver microsomes, adrenal mitochondria and bacterial systems. The cytochrome P-450 isolated from camphor-oxidising microorganisms has been studied most. Cytochrome P-450 dependent systems perform enzymatic hydroxylations in the biosynthesis of oxygenated isoprenoids (McCaskill and Croteau, 1997). One example is the allylic hydroxylation of (–)-β-pinene in hyssop (*Hyssopus officinalis*), forming pinocamphone and isopinocamphone via *trans*-pinocarveol (Karp and Croteau, 1992).
3.4. Biotransformation of monoterpane hydrocarbons

There are some important facts to have in mind when working with biotransformation of monoterpenes:

- Monoterpenes are toxic substrates, even to whole cells.
- Monoterpene hydrocarbons are poorly soluble in water.
- The volatility of monoterpenes causes problems during biotransformations, such as losses of both substrates and products.
- Biotransformations of terpenes often proceed along several metabolic pathways leading to a mixture of products.
- Most monoterpane biotransformations result in low yields.
- Monoterpenes are relatively unstable compounds. They can, for example, undergo spontaneous autoxidation.

The autoxidation of monoterpenes is discussed below (3.4.1).

Terpenoids in general have antimicrobial activities. This results in inhibition of culture growth or lysis of cells if too great an amount of a substrate is used. Inhibition of growth of cell cultures normally starts at terpene concentrations higher than 0.05% V/V (van der Werf et al., 1997). According to Brown et al. (1987) \( \alpha \)-pinene, for example, is fatal to the growth of the suspension culture of the plant Pelargonium fragrans, at a concentration of 0.5 g/l. The products resulting from the biotransformation can also be toxic. For example a bacteria of the Pseudomonas sp., obtained from soil, increases the cell lysis when exposed to borneol, which is a transformation product of \( \alpha \)-pinene (Shukla et al., 1968). \( \alpha \)-Pinene oxide, another biotransformation product of \( \alpha \)-pinene, inhibits the growth of Pseudomonas putida at a concentration above 0.2% (v/v) (van Keulen et al., 1998).

In order to avoid the problems associated with antimicrobial substrates, repeated or continuous and slow additions of the substrate or an organic-liquid two-phase system can be useful when performing biotransformations (van der Werf et al., 1997).

In order to avoid the toxic effects of monoterpenes, a low substrate concentration (0.16 - 0.32 g/l) was used in the biotransformations of monoterpenes by the suspension culture from P. abies (I-II).
The solubility of the monoterpene hydrocarbons in aqueous solutions is low. The contact area between the cell culture and the substrate could be increased either by using a two-phase system or by purging the substrate into the bottom of the suspension culture as a saturated vapour in an inert gas. Preliminary results of using heptane and the nutrition medium in a two-phase system in the biotransformation of α-pinene did not give any increased yields (unpublished results) and needs to be further optimised.

Due to the volatility of the monoterpenes the work-up procedure must be fast and efficient. The filtration procedure used for the reaction mixture from the *P. abies* biotransformation is one operation in which the monoterpenes can easily evaporate if the filtration does not proceed quickly enough.

However, the fact that many biotransformations result in low yields is mainly due to the mixture of products obtained from a single substrate. Choosing for example optimised growth conditions for the culture can solve this problem. Among others, Dhavlikar *et al.* (1974) have shown that the nutrition medium is an important factor in biotransformations of monoterpenes. Thus, the products can change depending on the medium used. When Seuberts medium is used in the biotransformation of β-pinene by a culture obtained from *Pseudomonas* sp., borneol is found as the main product. When, in contrast, Czapek-Dox medium is used as the nutrition medium, the main product is trans-pinocarveol (Dhavlikar *et al.*, 1974).

It is also known, that an optimised amount of the hormone, naphthalene acetic acid (NAA), in the nutrient medium (Murishige and Skoog, 1962, supplemented with hormones) of callus cultures of *Melissa officinalis* sp., leads to the formation of an essential oil, that contains mainly oxygenated monoterpenes instead of sesquiterpene hydrocarbons (Binder and Abou-Mandour 2000).

So far, we have not performed any work on the optimisation of the conditions for the *P. abies* cell growth.

### 3.4.1. Autoxidation

The term *autoxidation* is used for oxidation of an organic compound with molecular oxygen, usually via a free radical chain process. An initiator begins the free-radical chain process. The initiator could for instance be a metal, which will form a peroxiradical bond to the metal (Figure 25).
Figure 25. Molecular oxygen forms a peroxiradical with a metal. This peroxiradical will react with the olefin.

Autoxidation of olefins is mechanistically described as epoxidations and allylic oxidations (Sheldon and Kochi, 1981, Drago, 1991). The epoxidations start with an attack on the double bond, while allylic oxidations begin with abstraction of allylic hydrogen. These reactions involve free-radical intermediates (Figure 26). The allylic oxidation forms a hydroperoxide (Figure 26), which will, for example, decompose to an alcohol and oxygen.

**Attack on the double bond:**

**Abstraction of allylic hydrogen:**

Figure 26. Autoxidations of olefins could proceed either by attack on the double bond or by abstraction of the allylic hydrogen via free-radical intermediates.
3.4.1.1. Autoxidation products of α-pinene, β-pinene and limonene

Generally the autoxidation products of monoterpenes are the same as those formed by biotransformations (Moore et al., 1956, Bhattarchayya et al., 1960, Rothenberg et al., 1998). This makes it difficult to discriminate between autoxidation products and biotransformation products. For instance, the biotransformation products from α-pinene by the P. abies suspension culture - verbenone, verbenol and soberol - have also been found among the autoxidation products of α-pinene (Table 6, II).

A small amount of autoxidation products was formed when α-pinene or β-pinene was added to our nutrient medium in the absence of suspension culture (Table 6, I, II). However, this amount was much smaller than the amounts of products formed from the same substrate by the P. abies suspension culture. The suspension culture and the nutrient medium treatments produced different compositions of the products, with a higher extent of the respective ketone in the nutrient medium (Table 6). Neither trans-pinocarveol - the main product by the P. abies suspension culture - nor pinocamphone was produced from β-pinene by autoxidation in the nutrient medium. The amount of the produced pinocarvone was also lower than the amount produced by the suspension culture.

We did not detect any autoxidation products from limonene at the conditions used. However, our biotransformation products limonene-(1,2)-epoxide, carveol and carvone have been detected among the autoxidation products of limonene (Anandraman and Reineccius, 1986).
Table 6. The major oxidation products of (1R)-α-pinene (24 hours) or (1S)-β-pinene (8 days) after treatment with either a nutrient medium (autoxidation) or as biotransformation products after treatment with a *P. abies* suspension culture in a nutrient medium.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Products</th>
<th>mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoxidation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td><img src="image1" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><img src="image2" alt="Image" /></td>
<td>0.024</td>
</tr>
<tr>
<td>4</td>
<td><img src="image3" alt="Image" /></td>
<td>0.008</td>
</tr>
<tr>
<td>5</td>
<td><img src="image4" alt="Image" /></td>
<td>0.003</td>
</tr>
<tr>
<td>Biotransformation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><img src="image5" alt="Image" /></td>
<td>0.009</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6" alt="Image" /></td>
<td>0.064</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7" alt="Image" /></td>
<td>0.021</td>
</tr>
<tr>
<td>8</td>
<td><img src="image8" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>9</td>
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</tr>
<tr>
<td>10</td>
<td><img src="image10" alt="Image" /></td>
<td></td>
</tr>
<tr>
<td>Autoxidation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><img src="image5" alt="Image" /></td>
<td>0.018</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6" alt="Image" /></td>
<td>0.006</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7" alt="Image" /></td>
<td>0.006</td>
</tr>
<tr>
<td>8</td>
<td><img src="image8" alt="Image" /></td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td><img src="image9" alt="Image" /></td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td><img src="image10" alt="Image" /></td>
<td>-</td>
</tr>
<tr>
<td>Biotransformation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><img src="image5" alt="Image" /></td>
<td>0.066</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6" alt="Image" /></td>
<td>0.039</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7" alt="Image" /></td>
<td>0.008</td>
</tr>
<tr>
<td>8</td>
<td><img src="image8" alt="Image" /></td>
<td>0.320</td>
</tr>
<tr>
<td>9</td>
<td><img src="image9" alt="Image" /></td>
<td>0.303</td>
</tr>
</tbody>
</table>

1) α-Pinene; 2) β-pinene; 3) verbenone; 4) trans-verbenol; 5) cis- verbenol; 6) pinocarvone; 7) α-terpineol; 8) 1,8-cineole; 9) trans-pinocarveol; 10) pinocamphone.
4. Biotransformation by a horseradish culture and chloroperoxidase

4.1. Introduction

Peroxidases (EC 1.11.X.X) are a sub-group of oxido-reductases catalysing the oxidation of substrates using a peroxide, most often hydrogen peroxide as oxidant. Peroxidases catalyse the general reaction showed in Figure 27. To carry out their reactions peroxidases are either dependent or independent on a cofactor.

![Figure 27. A peroxidase-catalysed reaction of a substrate (R₂H).](image)

Peroxidases can however also act as monooxygenases and hence molecular oxygen is consumed as the oxygen donor. The oxygenation of monoterpenes by peroxidases may be useful to yield value-added compounds. No biotransformation of α-pinene or β-pinene by a pure enzyme preparation of peroxidase appears to be described in the literature. However, limonene is transformed into carvone as the major product by means of a mixture of a peroxidase, horseradish peroxidase and an oxygenase, glucose oxidase (Trytek and Fiedurek, 2002).

In contrast to P-450 enzymes (3.3.1), peroxidases do require neither electron transport proteins nor cofactors. A characteristic feature of most peroxidases is their low substrate specificity.

The reaction cycle of most peroxidases is described in Figure 28. They react through a ping-pong mechanism, in which two radical intermediates are produced in each catalytic cycle (Dunford, 1991). The first step is an oxidation of the resting enzyme (ferriperoxidase Fe^{3+}) by H₂O₂. The unstable intermediate compound I, consisting of ferryl iron (Fe^{4+}) and a porphyrin π cation radical, is formed. A reducing substrate converts compound I back to the resting enzyme via compound II. Alternative reaction mechanisms for peroxidases do exist (Dunford, 1990, Campa, 1990, Griffin, 1990). Example of such is the mechanism for chloroperoxidase-catalysed reactions (4.3).
Figure 28. The conventional cycle of peroxidase-catalysed reactions. RH=substrate: • indicates a free radical.

The reducing substrates are oxidised to free radical intermediates (Figure 28). These free radicals can thereafter react by several pathways and may for example dimerise, react with another substrate molecule, or attack various oxygen containing spieces. Finally alcohols, carbonyl compounds and epoxides are formed.

Peroxidases are divided into two superfamilies, the plant peroxidases and the animal peroxidases, each with different structural features. Plant peroxidases (POD) are widely distributed in plants and are important during plant growth and development and in stress responses. The plant peroxidases are further divided into three classes, I-III. Plants normally produce two classes of peroxidases, the intracellular Class I ascorbate peroxidases found in the cytosol and chloroplasts and the extracellular Class III peroxidases.

Horseradish peroxidase belong to the Class III peroxidases, which are heme containing (protoporphyrin IX), monomeric glycoproteins catalysing the oxidation of substrates with O_2 or H_2O_2, forming water as a coproduct.
4.2. Horseradish hairy root culture

Horseradish is known to contain large amounts of Class III peroxidases, which makes it interesting to employ terpenes as substrates for a horseradish culture (Appendix 2). This culture may tentatively also host other enzyme classes, such as monooxygenases.

Biotransformation of \( \alpha \)-pinene by the horseradish hairy root culture furnished a mixture of products (Figure 29) similar to that observed in the biotransformation of \( \alpha \)-pinene by the \textit{P. abies} suspension culture (as described in 3.2.1, I). Also the composition of the mixture changed with time similar to what was observed in the \textit{P. abies} suspension culture. Hence, during prolonged reactions the verbenols were transformed to verbenone either via trans-verbenol (as described in 3.2.1.1) or directly by oxidation to verbenone.

\[ \text{(1R)-\textit{\textbf{\textsf{1}}} \text{-Pinene}} \rightarrow \text{(1R)-\textit{\textbf{\textsf{1}}} \text{-trans-Verbenol}} + \text{(1R)-\textit{\textbf{\textsf{1}}} \text{-cis-Verbenol}} + \text{(1R)-\textit{\textbf{\textsf{1}}} \text{-Verbenone}} + \text{(1S)-\textit{\textbf{\textsf{1}}} \text{-Myrtenol}} + \text{trans-Sobrerol} \]

\textbf{Figure 29.} Biotransformation products of (1R)-\( \alpha \)-pinene by a horseradish hairy root culture. The major products are enclosed with borders.

However, no enzyme assays were performed to assess the enzyme activities in the horseradish culture. In addition, no experiments were made in order to determine the extent of autoxidation in the enzyme-free reaction medium.
4.3. Chloroperoxidase

The oxidative chlorination of certain substrates is catalysed by chloroperoxidase (CPO, EC 1.11.1.10). The catalytic mechanism of CPO differs somewhat from the conventional cycle of peroxidase-catalysed reactions (Figure 28). The enzyme compound III consisting of ferryl iron (Fe$^{6+}$) is formed after the conventional compound II, with the last step leading to the resting enzyme under speculation. Furthermore, there are structural similarities of the heme group between chloroperoxidase and P-450 cytochromes (Griffin, 1990).

Several organic compounds can act as substrates for the CPO, forming epoxides and diols. Styrene and α-methyl styrene are used as substrates for CPO, furnishing styrene epoxide and 2-phenyl-1,2-propane diol, respectively (Zaks and Dodds, 1995).

The structural similarities of the above mentioned substrates to limonene led to the investigation of the catalytic effect of chloroperoxidase on limonene. α-Pinene and β-pinene were also used as substrates. However, no products were found in either of the experiments. This was due to the precipitation of the enzyme already after one hour of contact with the substrates (Appendix II).
5. Transformation of terpenes by lipases

5.1. Introduction

Lipases (EC 3.1.1.3) belong to a class of enzymes called hydrolases and are members of a family of enzymes, which in biological systems, mainly hydrolyse carboxylic esters in the form of triacyl glycerol esters (fats). In vivo, enzymes mostly perform their catalytic processes in aqueous media. Yet, as early as 1900 Kastle and Loevenhart (1900) showed that lipases are able to catalyse esterification reactions in nonaqueous media. It was not until the work of Klibanov and co-workers that enzyme-catalysis in organic media achieved attention (Zacks and Klibanov, 1984, Cambou and Klibanov, 1984). In water, hydrolysis is the dominating reaction. By changing to an organic medium, the reverse reaction - esterification - is favoured instead. Thus, in organic media, lipases catalyse acyl transfer reactions. When an ester is used as acyl donor a transesterification occurs (Figure 30).

\[
\begin{align*}
&\text{R}_1\text{O} - \text{R}_2 + \text{R}_3\text{OH} \rightleftharpoons \\
&\text{R}_1\text{O} - \text{R}_3 + \text{R}_2\text{OH}
\end{align*}
\]

\textbf{Figure 30.} General reaction of a transesterification catalysed by lipases.

Lipases react according to a mechanistic model named the bi-bi Ping-Pong model (Kraut, 1977, Straathof \textit{et al.}, 1992). Lipases follow the reaction mechanism used by all serine hydrolases, involving a catalytic triad of amino acid residues consisting of serine, histidine and aspartate or glutamate (Figure 31, Fersht, 1999).
Medium engineering can increase the efficiency of the lipase-mediated reactions for a specific substrate by changing the temperature, the nature of acyl donor, the enzyme to substrate/acyl donor ratio, the concentration, or by the influence of additives. Some recent reviews discussing lipase-catalysed reactions in organic solvents and their synthetic and industrial applications include those by Kazlauskas and Bornscheuer (1998), Berglund and Hult (2000), Carrea and Riva (2000), and Reetz (2002).

Hydrolase-catalysed resolution by acylation of alcohols is a useful method in organic chemistry (Faber, 1997, van der Werf et al., 1997). Examples of factors controlling the enantioselectivity of the reaction are the reaction medium (organic solvent, water activity, substrate and acyl donor concentration), the substrate structure and the lipase (chemical modification, DNA shuffling and directed evolution) as described in a review by Berglund (2001).
For secondary alcohols Kazlauskas has suggested a rule for prediction of the expected enantioselectivity of the majority of lipases (Kazlauskas et al., 1991). The configuration of the fast reacting enantiomer is shown in Figure 32.

![Figure 32](image1.png)

**Figure 32.** Model for predicting the enantioselectivity of lipases for a secondary alcohol based on Kazlauskas et al. (1991). M and L indicate the medium- and the large-sized substituent, respectively in the fast reacting enantiomer.

Vinyl acetate is known to undergo efficient lipase-catalysed irreversible transesterifications with alcohols as substrates. The irreversibility in such reactions is due to the liberation of vinyl alcohol, which is rapidly and irreversibly tautomerised to acetaldehyde (Figure 33, Faber, 1997, Santiello, 1993). The secondary monoterpene alcohol, menthol, has been successfully resolved via enantioselective acylation with vinyl acetate catalysed by commercially available lipases (Koshiro et al., 1985, Wu et al., 1996).

We have investigated the possibility to use transesterification of vinyl acetate catalysed by several lipases as a method for the resolution and separation of terpene alcohols (V).

![Figure 33](image2.png)

**Figure 33.** Transesterification of an alcohol with vinyl acetate catalysed by a lipase. The liberated vinyl alcohol is tautomerised to acetaldehyde.
5.2. Lipase-catalysed transesterification

The lipases used in these experiments are listed in Table 7. They are all commercially available.

Table 7. Commercially available lipases used for the studies of resolution and separation of monoterpene alcohols.

<table>
<thead>
<tr>
<th>Lipase</th>
<th>Short</th>
<th>Supplier</th>
<th>Trade name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Burkholderia cepacia</em></td>
<td>PCL</td>
<td>Amano</td>
<td>lipase PS</td>
</tr>
<tr>
<td>other names:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas cepacia,</em></td>
<td></td>
<td>Roche</td>
<td>Chirazyme® L-6</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fungal:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Candida antartica A</em></td>
<td>CAL-A</td>
<td>Novo Nordisk</td>
<td>SP526</td>
</tr>
<tr>
<td><em>Candida antartica B</em></td>
<td>CAL-B</td>
<td>Novo Nordisk</td>
<td>Novozyme 435</td>
</tr>
<tr>
<td><em>Mucor miehei</em></td>
<td>MML</td>
<td>Novo Nordisk</td>
<td>SP524</td>
</tr>
<tr>
<td>other name:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rhizomucor miehei</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Thermomyces lanuginose</em></td>
<td>TLL</td>
<td>Novo Nordisk</td>
<td>SP523</td>
</tr>
<tr>
<td>other name:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Humicola lanuginosa</em></td>
<td></td>
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</tr>
</tbody>
</table>

In the first series of experiments, the major primary and secondary monoterpene alcohols in TMP-turpentine - borneol and myrtenol - were used as model substances, and were subjected to lipase-catalysed transesterification of vinyl acetate (Figure 34, V).

The primary alcohol, myrtenol reacted fast using PCL-L6 in TBME (c = 98%, 24 hours, room temperature) while the secondary alcohol, borneol, reacted slower. However, when CAL-A was used as catalyst, acceptable yields of the bornyl ester were obtained (c = 68%, 14 days, 30 °C). The enantioselectivities (E) of these reactions were low (E = 2) in all experiments. When PCL-PS, PCL-L6 or CAL-B was used as a catalyst for the transesterification of vinyl acetate with borneol, the hemiacetal ester was detected as an unexpected side-product (Figure 34, entry 1, see 5.2.1, III, IV).
In the second series of experiments the polar fraction from the TMP-turpentine was subjected to transesterification catalysed by five lipases: TLL, MML, CAL-A, PCL-PS or PCL-L6 (Figure 34, entry 2, V). The primary alcohols were esterified in a short time, whereas the secondary alcohols needed a longer conversion time. Still, they did all act as substrates. As expected, due to steric hindrance the tertiary terpene alcohols were unreactive (cf. Ospiran et al., 1996). TLL did not show any activity.

**Figure 34.** Schematic picture of the experiments performed on the lipase-catalysed transesterifications of vinyl acetate with terpene alcohols. 1) Borneol, 2) myrtenol, 3) trans-pinocarveol.
When MML or CAL-A was used as catalysts, the myrtenol in the TMP-alcohol mixture was fully converted to the corresponding ester within 24 h and a slower reaction rate was observed for PCL-L6 as a catalyst (Figure 35 a). In contrast, a very fast rate of conversion of myrtenol was observed when it was used as the single substrate for the PCL-L6 catalysed reaction (Figure 34, entry 1). Probably, compounds present in the terpene alcohol mixture could have acted as enzyme inhibitors or even as denaturating agents. Also, a slight change in the enzyme/substrate ratio might have been overlooked due to difficulties in determining the actual substrate concentration in the mixture of the terpene alcohols from the turpentine.

However, MML only converted about 30% of trans-pinocarveol, in the mixture of alcohols obtained from the TMP-turpentine, in 14 days (Figure 35 b). This result indicated that it would be possible to separate myrtenol from trans-pinocarveol by MML-catalysed transesterification of vinyl acetate (see below 6.4). The highest yield of bornyl acetate from the terpene alcohol mixture was obtained when the catalyst was lipase CAL-A.

**Figure 35.** Formation of terpene esters in the transesterification of vinyl acetate with the mixture of alcohols from the TMP-turpentine by means of lipases. a) Formation of myrtenyl acetate by MML, CAL-A and PCL-L6-catalysed transesterifications. b) Formation of myrtenyl acetate and trans-pinocarvyl acetate by MML-catalysed transesterification. *Integrated GC-MS area of peak in relation to the integrated area of an internal standard.*
5.2.1. Side-products

When the transesterification of vinyl acetate with borneol was catalysed by either of the two *Pseudomonas* sp. lipases (PCL-PS, PCL-L6) or by the lipase from *Candida antarctica* B (CAL-B), the yield of bornyl acetate was reduced due to the formation of the hemiacetal or the acetylated hemiacetal of borneol (Figure 36, III, IV). It is known that side-reactions can occur when using vinyl esters as acyl donors (Bianchi *et al.*, 1988, Xie, 1991), however the nature of these reactions has not been described.

![Figure 36](image)

**Figure 36.** Acetylated hemiacetal of borneol formed in the transesterification reaction of vinyl acetate and borneol using the lipases PCL-PS, PCL-L6 or CAL-B as catalysts.

In order to determine whether the formation of hemiacetal esters is a general reaction for slow-reacting, sterically hindered alcohols, the alcohols 2-17 (Figure 37) were used as substrates in transesterifications of vinyl acetate using the above-mentioned lipases as catalysts (III, IV). Formation of hemiacetal ester side-products also occurred, when the alcohols 2-11 (Figure 37) were used as substrates in transesterifications catalysed by *Pseudomonas cepacia* lipases and *Candida antarctica* lipase B. Formation of hemiacetal side-products occurred with substrates 1, 7-11 (Figure 37).
Figure 37. Transesterification of vinyl acetate with sterically hindered alcohols catalysed by *Pseudomonas cepacia* or *Candida antarctica* lipase B. 1) (+)-Borneol, 2) Isoborneol, 3) *Endo*-norborneol, 4) 3,3,8a-trimethyl-2,3,4,6,7,8,8a-octahydronaphthalen-1-ol, 5) 1,2,3,4-tetrahydronaphthalen-1-ol, 6) decahydronaphthalen-1-ol, 7) 2-Adamantol, 8) 1-Phenyl-1-butanol, 9) 3-Methyl-1-phenyl-1-butanol, 10) 3,3-Dimethyl-1-phenyl-1-butanol, 11) 3-Chloro-1-phenyl-1-propanol, 12) *Exo*-norborneol, 13) (+)-Isopinochampheol, 14) (–)-trans-pinocarveol, 15) 2-Methylhexanol, 16) α-Terpineol, 17) Terpinene-4-ol.

From alcohols 12-15 (Figure 37) only esters were produced and the substrates 16-17 were found to be unreactive due to sterical hindrance. The identities of the hemiacetal esters from the substrates 1-11 (Figure 37) were determined by GC-MS analysis. The identity of the hemiacetal esters of the substrates 1, 4-6 was also confirmed by NMR analysis (III, IV). Based on our experiments, no general correlation could be established between the extent of formation of a hemiacetal or hemiacetal ester and the structure of the substrate, the lipase used or the reaction conditions.
The hemiacetal ester was probably produced via esterification of the initially formed hemiacetal, from the acetaldehyde liberated in the acylation reaction (Figures 31, 33). Experiments excluding the enzyme did not result in any product while in experiments using a purified CAL-B, the hemiacetal ester was detected (IV). Hence, the lipase itself was the active catalyst in the formation of the hemiacetal ester side-products. Our results indicate that both steps yielding the hemiacetal ester are probably lipase-mediated (Figure 38, IV).

Whereas 2-adamantol and vinyl acetate, either with or without added acetaldehyde in the presence of CAL-B or PCL-L6, furnished the hemiacetal, similar experiments in the absence of enzyme gave no trace of a hemiacetal or its ester (Table 8). Furthermore, experiments with added acetaldehyde but excluding the vinyl acetate produced no side product. Hence, both lipase and vinyl acetate seem to be prerequisites to obtain both the hemiacetal and the hemiacetal ester of the substrate (Figure 38).

**Table 8.** Formation of a hemiacetal of 2-adamantol.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Acyl donor</th>
<th>Enzyme</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-adamantol</td>
<td>−</td>
<td>−</td>
<td>No product</td>
</tr>
<tr>
<td>−</td>
<td>−</td>
<td>PCL-L6/CAL-B</td>
<td>No product</td>
</tr>
<tr>
<td>2-adamantol</td>
<td>−</td>
<td>−</td>
<td>No product</td>
</tr>
<tr>
<td>−</td>
<td>PCL-L6/CAL-B</td>
<td></td>
<td>No product</td>
</tr>
</tbody>
</table>
Four diastereomers of the hemiacetal ester were formed from racemic borneol, *i.e.* two pair of enantiomers (Figures 39, 40). When one enantiomer of borneol was used as substrate, two hemiacetal esters were formed and one of them was formed in diastereomeric excess. We were unable to determine which one of the diastereomers, A or B that was the major one (Figure 39).

**Figure 39.** Formation of acetylated hemiacetal during lipase-catalysed transesterification of vinyl acetate with borneol. Enz-H = PCL-PS, PCL-L-6 or CAL-B.

**Figure 40.** The GC-chromatogram (column: β-dex 120) of the lipase-catalysed (PCL-PS) transesterification of vinyl acetate with racemic borneol in heptane after 14 days at roomtemperature. The two enantiomers of bornyl acetate did not separate. 1) A or B; 2) ent-A or ent-B; 3) ent-B or ent A; 4) B or A (Figure 39).
However, assuming that Kazlauskas rule (Kazlauskas et al., 1991, described in 5.1) is valid for the acylation of hemiacetals, the major products from (–)- and (+)-borneol will be the hemiacetal esters B and ent-A respectively (Figure 41).

Adding acetaldehyde to the reaction mixture with borneol as a substrate increased the diastereoselectivity (from dr = 2:1, to dr = 5:1) (Table 9, entry 1-2) (IV). The diaseteroselectivity could be even more improved by performing the same experiment on a larger scale (dr = 98:2) (Table 9, entry 3). Products of aldol condensation reactions were detected in experiments with propanal as additive (IV). Lipase (CAL-B)-mediated aldol reactions have been reported in the literature (Branneby et al., 2003).

**Table 9.** Transesterification of vinyl acetate with (–)-borneol.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate</th>
<th>Lipase/mg</th>
<th>Additives/mg</th>
<th>c (%)</th>
<th>Products (%</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(–)-Borneol</td>
<td></td>
<td></td>
<td></td>
<td>Ester</td>
<td>Ent-A?</td>
</tr>
<tr>
<td>1</td>
<td>a</td>
<td>PCL-L6/4.8</td>
<td>VinylAc/ 128</td>
<td>97-98</td>
<td>8-20</td>
<td>59-66</td>
</tr>
<tr>
<td>2</td>
<td>a</td>
<td>PCL-L6/4.8</td>
<td>VinylAc/ 128</td>
<td>74-86</td>
<td>0</td>
<td>83-85</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CH₃CHO/ 11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>b</td>
<td>PCL-L6/55.5</td>
<td>VinylAc/ 282</td>
<td>100</td>
<td>0.4</td>
<td>97.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CH₃CHO/ 196</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a. 0.65 ml solution of (–)-borneol 0.1 M in TBME. b. 123 mg (–)-borneol dissolved in 4 ml of TBME (the conversion was determined after 20 days).
The hemiacetal esters are sensitive to acids, which catalyse the cleavage to acid, acetaldehyde and alcohol. Thus, borneol was recovered from its corresponding acetylated hemiacetal upon chromatography on silica gel. Therefore, these side products are easily overlooked in enzymatic resolutions and their presence could result in lower \( ee \)-values than expected due to decomposition of hemiacetal esters during work-up. Liu et al. (2000) report lower \( ee \)-values than expected after hydrolysis of their initial product. They did not report any side-product formation in their CAL-B-catalysed resolution of 3-chloro-2-(2-thienyl)-1-propanol. Formation of hemiacetal esters offers one explanation for the unexpectedly low \( ee \) observed for the remaining substrate.

**Figure 42.** Resolution of 1) 3-chloro-2-(2-thienyl)-1-propanol by CAL-B-catalysed transesterification of vinylbutyrate gave a lower \( ee_p \) than expected from \( E=299 \), after hydrolyses of the product (Lie et al, 2000). Could the formation of a hemiacetal or hemiacetal ester be the explanation?
6. Separation of the terpenes from the TMP-turpentine

6.1. Introduction

Separation of the more valuable terpenes present in TMP-turpentine is one approach to value-added compounds. Such an approach will, of course, include physical methods such as chromatography or distillation. The processes for separation must be as simple as possible in order to facilitate future work on a larger scale. We have investigated whether enzymatic methods can be used to separate terpene alcohols from mixtures obtained by distillation and chromatography.

6.2. Chromatography

One fraction of oxygenated terpenes and one fraction of hydrocarbons were obtained from TMP-turpentine by the use of flash chromatography on silica gel (V). The terpene hydrocarbons were first filtered through the column and then eluted by pentane until almost no unpolar compounds were left on the column. Diethyl ether (15%) in pentane was used to elute the esters and the ketones from the column. The remaining terpene alcohols were eluted with diethyl ether.

6.3. Distillation

The crude TMP-turpentine was fractionated by distillation (Appendix 1). However, the monoterpane alcohols were mixed with some of the mono- and sesquiterpene hydrocarbons. To solve this problem, flash chromatography of the crude TMP-turpentine was performed prior to distillation (V). Distillation of the polar fraction gave a mixture of oxygenated compounds, which was now free from sesquiterpenes and other compounds of high molecular weight.
6.4. Lipase-catalysed transesterification as a separation method

Good yields of terpene acetates were obtained using lipase-catalysed transesterification of vinyl acetate with a polar terpene alcohol fraction, obtained by MPLC of the crude TMP-turpentine (Appendix 1, V). The content of acetates in was increased from 0.5% to 8.2% by CAL-A-catalysed transesterification, at room temperature. At 30 °C the acetate content was further increased to 13.6% in two weeks of treatment.

A second alcohol fraction was obtained by flash chromatography in combination with distillation (V). By starting with this latter alcohol fraction and by using a one step reaction with CAL-A as catalysts, an alcohol fraction containing α-terpineol as the major constituent was obtained in good yield (Figure 43, unpublished results).

![Diagram](image)

**Figure 43.** Separation of oxygenated terpenes from TMP-turpentine utilising chromatography, distillation and lipase-catalysed (CAL-A) transesterification of vinyl acetate with monoterpenic alcohols. 1) α-Pinene, 2) β-pinene, 3) limonene, 4) myrtenyl acetate, 5) trans-pinocarvyl acetate, 6) bornyl acetate, 7) α-terpineol.
If the lipases MML and CAL-A were used sequentially, each of the acetates of myrtenol, trans-pinocarveol, and borneol were obtained in good yields (Figure 44, V). These compounds are all attractive flavours and fragrances. The myrtenol acetate was however contaminated by the malodours esters of hexanol, heptanol, octanol, and nonanol (V).

**Figure 44.** Separation of oxygenated terpenes from TMP-turpentine using chromatography, distillation and lipase-catalysed (MML and CAL-A) transesterification of vinyl acetate with monoterpene alcohols. 4) Myrtenyl acetate, 5) trans-pinocarvyl acetate, 6) bornyl acetate, 7) α-terpineol. Values in % refer to the purity of the isolated compound.
7. Conclusions and future work

The aim of this thesis was to demonstrate methods of achieving value-added compounds from the TMP-turpentine. The methods described are successful in this respect and the usefulness of biotransformations to reach the goals set has been demonstrated. Hence, by use of a *P. abies* suspension culture (I, II) and the horseradish hairy root culture (Appendix 2), the main constituents of the TMP-turpentine (Appendix 1) were oxygenated in a satisfactory way, however, in low yields. Additionally, the lipase-catalysed transesterifications of vinyl acetate made it possible to separate the TMP-turpentine into acetates or fractions of desirable terpene alcohols (V). When performing enzyme-catalysed resolutions involving vinyl acetate, the formation of hemiacetal esters in the lipase-catalysed reactions of sterically hindered alcohols (III, IV) is a discovery worth keeping in mind.

Further experiments should be made with the aim to clarify the mechanism for the side-product formation during lipase-catalysed transesterification of vinyl acetate with sterically hindered secondary alcohols.

Other oxygenating enzymes should be screened for their ability to transform the monoterpenes hydrocarbon constituents. For instance, hydratases, and a mixture of glucose peroxidase and horseradish peroxidase have been found to transform limonene into α-terpineol and carvone, respectively (Tan *et al.*, 1998, Trytek and Fiedurek, 2002).

The yield of the transformation by *P. abies* suspension culture should be increased. The work-up procedure should also be further improved. The composition of the nutrition medium should be optimised in order to induce the cultures to perform biotransformations that result in a desired product pattern.

Also of interest is the possibility of the *P. abies* suspension culture in use for biotransformation of other substrates than terpenes, such as other cyclic unsaturated hydrocarbons. Is the isomerisation seen in the biotransformation of α-pinene (*cis*-verbenol into *trans*-verbenol) common for other types of non-terpene substrates? Such reactions may be useful in organic synthesis.

Further investigations using the horseradish hairy root culture should be performed. The *P. abies* suspension culture gave the same products as the horseradish culture. Work-up of the latter culture broth was much easier and therefore low product losses should be expected.
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/Marica
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References


