# LACCASE IN ORGANIC SYNTHESIS AND ITS APPLICATIONS

A Dissertation
Presented to
The Academic Faculty

by

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In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in the
School of Chemistry and Biochemistry

Georgia Institute of Technology
December 2008

# LACCASE IN ORGANIC SYNTHESIS AND ITS APPLICATIONS

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Date Approved: October 22, 2008

# ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Dr. Arthur J. Ragauskas, for his instruction, encouragement, advice, and support throughout my graduate education. I also would like to thank my thesis committee, Dr. David M. Collard, Dr. Uwe H. F. Bunz, Dr. John Cairney, and Dr. Preet M. Singh for their insightful comments and support throughout this project.

I would like to especially thank Dr. Leslie Gelbaum. His guidance and support in teaching me the techniques of NMR spectroscopy has helped me immensely in this research. I also would like to thank Dr. Robert Braga for conducting the IR experiments in the synthesis of *p*-naphthoquinone project.

I also appreciate the assistance of all of my co-workers in Dr. Ragauskas' lab that allowed me to complete my research tasks, and especially, I would like to thank the following colleagues:

Dr. Yunqiao Pu for his kind help in NMR spectroscopy; Lenong Allison for her strong support through my study; Dr. Kristina Knutson for her training in how to measure laccase activity; Dong Ho Kim for his assistance in bulk acid group testing and handsheet making; Shaobo Pan and Shoujian Hu for their kind help in taking SEM images; Dr. Nan Jiang for his helpful advice for my synthetic work; Lee Goetz and Poulomi Sannigrahi for their friendship and support during my study; Rajalaxmi Dash for her help in FT-IR experiments; and Abdullah Zettili, summer undergrad student, for helping me in the synthesis of *ortho*-naphthoquinone project.

I am pleased to acknowledge the IPST endowment fund and the Royal Thai Government Scholarship for providing me with financial support throughout my years of study. Ms. Tuwanda Strowbridge is appreciated for her guidance and help in the paper testing lab.

Lastly, I would like to thank my parents, family, and friends for always supporting and encouraging me in my education.

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# NOMENCLATURE

| | |
|---|---|
| 4-HBA | 4-Hydroxybenzoic acid |
| 7-ADCA | 7-Aminodesaacetoxycephalosporanic acid |
| ABTS | 2,2'-Azinobis-(3-ethylbenzylthiozoline-6-sulphate) |
| Ala | Alanine |
| Ar | Aromatic |
| Arg | Arginine |
| Asp | Aspatic acid |
| ATP | Adenosine triphosphate |
| DA | Diles-Alder |
| DABCO | 1,4-Diazabicyclo[2.2.2]octane |
| DCS | Dodecanesulfonate |
| dDP | 5,5-Di-*n*-dodecyl-2-hydroxy-1,3,2-dioxaphosphorinan-2-one |
| DMAP | 4-Dimethylaminopyridine |
| DMSO | Dimethylsulfoxide |
| DOPA | 3,4-Dihydroxyphenylalanine |
| DP | Degree of polymerization |
| DS | Dodecylsulfate |
| E | Enzyme |
| EC | Enzyme commission |
| EPR | Electron paramagnetic resonance |
| ES | Enzyme-substrate complex |
| ESCA | Electron spectroscopy for chemical analysis |
| ET | Electron transfer |

| | |
|---|---|
| EtOAc | Ethyl acetate |
| FMO | Frontier molecular orbital |
| FRPSG | Fluorous reverse-phase siclica gel |
| FT-IR | Fourier Transform Infrared |
| Gly | Glycine |
| HAA | Hydroxyanthanilic acid |
| HBT | N-hydroxybenzotriazole |
| HCl | Hydrochloric acid |
| His | Histidine |
| HMBC | Heteronuclear multiple bond coherence |
| HMQC | Heteronuclear multiple quantum coherence |
| HOMO | Highest occupied molecular orbital |
| $K_m$ | Michaelis-Menten constant |
| Lac | Laccase |
| LASCs | Lewis acid/surfactant combined catalysts |
| $Ln(OTf)_3$ | Lanthanide triflate |
| LUMO | Lowest unoccupied molecular orbital |
| MCD | Magnetic circular dichorism |
| ML | Middle lamella |
| M-M | Michaelis Menten |
| MS | Mass spectroscopy |
| NaCl | Sodiun chloride |
| NaOH | Sodium hydroxide |
| NMR | Nucleae magnetic resonance |
| o.d. | Oven dried |

| | |
|---|---|
| $OH^-$ | Hydroxide anion |
| PAA | 4-Hydroxyphenylacetic acid |
| PEG | Polyethyleneglycol |
| Phe | Phenylalanine |
| PMP | p-Methoxyphenyl |
| QDA | Quinone Diels-Alder |
| RT | Room temperature |
| RTILs | Room temperature ionic liquids |
| $Sc(OTf)_3$ | Scandium triflate |
| SDS | Sodium dodecyl sulfate |
| SEM | Scanning electron microscope |
| Ser | Serine |
| $SH^-$ | Hydrosulfide anion |
| T1 | Copper atom type 1 |
| T2 | Copper atom type 2 |
| T3 | Copper atom type 3 |
| TAPPI | Technical association of the pulp and paper industry |
| TEMPO | 2,2,6,6-tetramethyl-1-piperidinyloxyl |
| TLC | Thin layer chromatography |
| TMP | Thermomechanical pulp |
| UF | Urea/formaldehyde |
| UV | Ultraviolet |
| VA | Violuric acid |
| $V_{max}$ | Maximum reaction velocity |
| XAS | Cu K-edge X-ray spectroscopy |

# SUMMARY

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2), a multi-copper-containing oxidoreductase enzyme, is able to catalyze the oxidation of various low-molecular weight compounds, specifically, phenols and anilines, while concomitantly reducing molecular oxygen to water. Moreover, due to their high stability, selectivity for phenolic substructures, and mild reaction conditions, laccases are attractive for fine chemical synthesis. In this study, new green domino syntheses were developed by conducting reactions in an aqueous medium, an environmentally-friendly solvent, and using laccase as a biocatalyst.

The first study presents a work on the synthesis of naphthoquinones in the aqueous medium. Herein, laccase was used to oxidize *o*- and *p*-benzenediols to generate *o*- and *p*-benzoquinones *in situ*. These quinones then underwent Diels-Alder and oxidation reactions to generate napthoquinone products. This reaction system can yield naphthoquinones in up to 80% yield depending on the structure of the starting hydroquinone and diene.

The next part of this thesis reports the cascade synthesis of benzofuran derivatives from the reaction of catechols and 1,3-dicarbonyl compounds via oxidation-Michael addition in the presence of laccase and $Sc(OTf)_3$/SDS in an aqueous medium. Depending on the substrates, one-pot yields of benzofurans averaged 50-79%. In the absence of $Sc(OTf)_3$, these yields decreased to 45-65%. Hence, the use of Lewis acid was critical for efficient synthesis of the desired compounds. From an environmental concern, this system still produced a hazardous waste from the transition metal catalyst. Therefore, the

development of alternative methodologies to replace the lanthanide metal catalyst in this synthesis is a high priority to enhance the overall green chemistry aspect. As a consequence, lipase was used as a catalyst to replace $Sc(OTf)_3$ for the synthesis of benzofuran derivatives. The laccase/lipase co-catalytic system provides the benzofuran products in a good yield. In addition, this catalytic system was also able to catalyze the reaction of anilines and catechol.

Besides its application in organic synthesis, laccase also has an application in fiber modification. Therefore, in the last part of this thesis, laccase was applied to the modification of high-lignin softwood kraft pulp. This modification demonstrates the potential of laccase-facilitated grafting of amino acids to high lignin content pulps to improve their physical properties in paper products which resulted from the increase of carboxylic acid group of the fibers. A unique two-stage laccase grafting protocol was developed. Fibers were first treated with laccase, followed by grafting reactions with amino acids. The bulk acid group content was measured, and a variety of amino acids, including glycine, phenylalanine, serine, arginine, histidine, alanine, and aspartic acid, were examined. The effects of laccase dosage and amino acids on fiber modification were studied. In this study, histidine provided the best yield of acid groups on pulp fiber and was used in the preparation of handsheets for physical strength testing. Laccase-histidine-treated pulp showed an increase in the strength properties of the resulting paper.

# CHAPTER 1

# INTRODUCTION

## 1.1 Introduction

In recent years, the use of natural catalysts, enzymes, in the development of organic synthesis reactions has received a steadily increasing amount of attention due to their synthetic, economical, and, especially, environmental advantages [1,2]. The enzymes are able to promote reactions under very mild conditions of temperature, pH, and pressure. Moreover, to address the challenges of green chemistry, the possibility of using water to replace the hazardous classical organic solvents in enzyme-catalyzed reactions is another advantage. In addition to its environmental benefits, the use of water as a solvent is both inexpensive and safe. The main purpose of this dissertation is to create environmentally-friendly synthetic procedures by conducting the reactions in an aqueous medium in the presence of a biocatalyst.

The main biocatalyst used in this dissertation is laccase. Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2), a multi-copper-containing oxidoreductase enzyme, is able to catalyze the oxidation of various low-molecular weight compounds, including benzenediols, aminophenols, polyphenols, polyamines, and lignin-related molecules, while concomitantly reducing molecular oxygen to water [3-10]. Because of its high stability, selectivity for phenolic substructures, and mild reaction conditions, laccase is attractive for fine chemical synthesis [11-19]. Therefore, interest in the potential use of laccase in organic synthesis has recently increased. Laccase also finds a wide variety of industry applications, including food, pulp and paper, textile, cosmetics, and nanobiotechnology industries [20,21]. Recently, laccase applications have shifted toward fiber modification. Laccase has been reported to catalyze biografting of a variety

of substrates to technical lignins and lignin-rich cellulosic fibers [22-31]. Therefore, the utilizing of laccase in green synthetic chemistry and in fiber modification was the main focus of this research study.

In this dissertation, the synthesis of *p*-naphthoquinones and related structures via Diels-Alder reaction of *p*-quinone generated by laccase and dienes in an aqueous media was investigated. This study is described in Chapter 4. Chapter 5 further explores the laccase-triggered Diels-Alder reaction of 1,2-hydroquinone and dienes for the synthesis of *o*-naphthoquinones. Next, the cascade synthesis of benzofuran derivatives is investigated in Chapter 6. This synthesis was conducted from the reaction of catechols and 1,3-dicarbonyl compounds via oxidation-Michael addition in the presence of laccase and $Sc(OTf)_3$/SDS under air at room temperature in aqueous media. However, from an environmental perspective, this system still produces a hazardous waste from the transitional metal catalyst. Therefore, the development of alternative methodologies to replace the lanthanide metal catalyst in this synthesis is a high priority in order to enhance the overall green chemistry aspect of this one-pot synthetic reaction. As a consequence, the enzyme named lipase was used as an alternative catalyst in conjunction with laccase for the synthesis of benzofuran derivatives. In addition, this laccase/lipase co-catalytic system was further investigated to catalyze the Michael addition of anilines and catechols. The details of these studies are described in Chapter 7.

In addition, laccase also finds an application in fiber modification. In the last part of this research study, Chapter 8, laccase was applied to the modification of high-lignin softwood kraft pulp. This modification demonstrates the potential of laccase-facilitated grafting of amino acids to high lignin content pulps to improve their physical properties

in paper products by increasing the carboxylic acid group of the fibers. Finally, some overall conclusions and recommendations for future work complete the document.

## 1.2 Objectives

Recently, the increasing concern for the environment and for safe chemical procedures requires the development of new green synthetic methods. Therefore, the focus of this research is to develop new environmentally-friendly synthetic chemistry for the synthesis of a wide variety of compounds. To address the challenges of green chemistry, this study focuses on using a safer chemical, the enzyme laccase, in catalytic amount, using an environmentally-benign solvent, water, and conducting the reaction at ambient temperature. The major objectives of this research are summarized as follows:

- Determine the potential use of laccase in organic synthesis
- Develop new green chemistry synthesis by using a green reagent and a green solvent, which are laccase and water, respectively.

Besides green synthetic applications, this study also investigated the application of laccase in a new green procedure for modifying lignin-rich cellulosic fibers in an aqueous medium. The major objectives of this fiber modification research are summarized as follows:

- Evaluate the feasibility of a system utilizing laccase to graft amino acids with lignin-rich cellulosic fibers.
- Develop a new green procedure for fiber modification.

- Determine conditions where the laccase-facilitated grafting system is the most effective for modifying fibers.
- Evaluate the effect of laccase-facilitated grafting treatment on paper strength properties.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Green Chemistry

### 2.1.1 Definition of Green Chemistry

Green chemistry, also called sustainable chemistry, is a chemical philosophy encouraging the design of products and processes that reduce or eliminate the use and generation of hazardous substances. The U.S. Presidential Green Chemistry Challenge, March 1995, defines green chemistry as,

"the use of chemistry for source reduction or pollution prevention, the highest tier of the risk management hierarchy as described in the Pollution Act of 1990. More specifically, green chemistry is the design of chemical products and processes that are more environmentally benign"

### 2.1.2 Twelve Principles of Green Chemistry

Green chemistry is a highly effective approach to pollution prevention because it applies innovative scientific solutions to real-world environmental situations. The 12 Principles of Green Chemistry, originally published by Paul Anastas and John Warner in Green Chemistry: Theory and Practice [32]. These principles help to explain what the definition means in practice. The principles cover such concepts as:

- the design of processes to maximize the amount of raw material that ends up in the product;

- the use of safe, environment-benign substances, including solvents, whenever possible;
- the design of energy efficient processes;
- the best form of waste disposal: do not create it in the first place.

The 12 principles are [32]:

1. Prevent waste: Design chemical syntheses to prevent waste, leaving no waste to treat or clean up.
2. Design safer chemicals and products: Design chemical products to be fully effective, yet have little or no toxicity.
3. Design less hazardous chemical syntheses: Design syntheses to use and generate substances with little or no toxicity to humans and the environment.
4. Use renewable feedstock: Use raw materials and feedstock that are renewable rather than depleting. Renewable feedstock are often made from agricultural products or are the wastes of other processes; depleting feedstock are made from fossil fuels (petroleum, natural gas, or coal) or are mined.
5. Use catalysts, not stoichiometric reagents: Minimize waste by using catalytic reactions. Catalysts are used in small amounts and can carry out a single reaction many times. They are preferable to stoichiometric reagents, which are used in excess and work only once.
6. Avoid chemical derivatives: Avoid using blocking or protecting groups or any temporary modifications if possible. Derivatives use additional reagents and generate waste.

7. Maximize atom economy: Design syntheses so that the final product contains the maximum proportion of the starting materials. There should be few, if any, wasted atoms.
8. Use safer solvents and reaction conditions: Avoid using solvents, separation agents, or other auxiliary chemicals. If these chemicals are necessary, use innocuous chemicals. If a solvent is necessary, water is a good medium as well as certain eco-friendly solvents that do not contribute to smog formation or destroy the ozone.
9. Increase energy efficiency: Run chemical reactions at ambient temperature and pressure whenever possible.
10. Design chemicals and products to degrade after use: Design chemical products to break down to innocuous substances after use so that they do not accumulate in the environment.
11. Analyze in real time to prevent pollution: Include in-process real-time monitoring and control during syntheses to minimize or eliminate the formation of byproducts.
12. Minimize the potential for accidents: Design chemicals and their forms (solid, liquid, or gas) to minimize the potential for chemical accidents including explosions, fires, and releases to the environment.

## 2.2 Water as Solvent in Organic Synthesis

In order to move toward sustainable technologies, developing more benign synthetic procedures in chemical synthesis is important. This development can be achieved by several approaches, including reducing the amount of waste, the energy usage, and the use of volatile, toxic and flammable solvents. Therefore, many alternative solvents have been proposed to replace classical organic solvents. The most well-known of these alternate reaction media are listed below [33]:

- Use of water as solvents
- Reactions under solventless/solvent-free conditions
- Supercritical carbon dioxide (31.1 ºC, 73 atm)
- Supercritical water (374 ºC, 218 atm)
- Room-temperature ionic liquids

Herein, the use of water as a reaction media is the main focus of this thesis. The use of water as a medium for organic reaction is one of the finest solutions to the problem of solvent toxicity and disposal. Water is the cheapest, safest and most non-toxic solvent in the world. In addition, many surprising discoveries, such as an increase of reaction rates and reaction selectivity, have been made when using water as a reaction medium. The use of an aqueous medium affords both advantages and disadvantages, some of which are listed below [34]:

Advantages:

- Inflammable and anhydrous solvents are not needed
- Economical saving
- Abundant, cheap, not toxic and environmental friendly

- Protection-deprotection of functional groups such as OH, COOH may not be necessary
- Water-soluble compounds can be used directly without derivatization
- pH control
- Preferred solvent for enzyme catalyzed reactions
- Possibility of using additives such as mineral salts, surfactants, cyclodextrins
- Possibility of isolating products by decanting or filtration

Disadvantages:

- Not inert
- High boiling point
- Problems isolating highly water-soluble products
- Carbocarbon acid (pKa > 17) and water-sensitive reagents cannot be used

In the early 1980s, Breslow and Rideout were the first to show that Diels-Alder reactions were greatly accelerated in water [35]. This discovery triggered a more widespread interest toward the development of organic reaction in water. In the past 20-30 years, the potential benefits of using aqueous media have been recognized, and reactions including pericyclic, Michael additions, condensation, oxidation, reduction and organometallic reactions have been reported [36-41]. Among the organic reactions investigated in aqueous medium, the pericyclic reactions, especially Diels-Alder reaction, has been the most widely studied [34,38,42,43]. The following section highlights some Diels-Alder reactions that can be performed successfully in water.

### 2.2.1 Diels-Alder Reactions

The Diels-Alder reaction is a [4 + 2] cycloaddition in which a diene (4-π component) reacts with a dienophile (2- π component) to provide a six-membered ring. Bond-forming and bond-breaking processes are concerted in the six-membered transition state (Figure 1). Most dienophiles are of the form –C=C–Z or Z–C=C–Z', where Z and Z' are electron-withdrawing groups, such as CHO, COR, COOH, COCl, COAr, CN, $NO_2$, Ar, $CH_2OH$, $CH_2Cl$, $CH_2NH_2$, $CH_2CN$, $CH_2COOH$, halogen, $PO(OEt)_2$, or C=C [44]. Particularly common nucleophile are maleic anhydride and quinones. The Diels-Alder reactions with quinones will be discuss in detail in the next section. When one or more heteroatoms are present in the diene and/or dienophile framework, the cycloaddition is called a hetero-Diels-Alder reaction. The Diels-Alder reaction is of great value in synthetic organic chemistry because it creates the very useful cyclohexene ring.

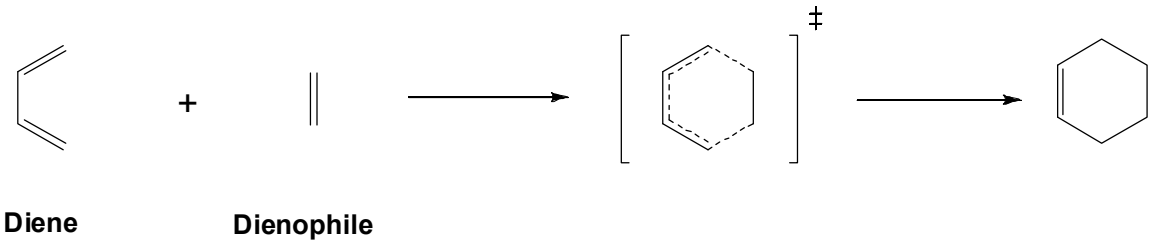



**Figure 1.** Diels-Alder reaction of 1,3-butadiene with ethylene.

The reactivity, regiochemistry, and stereochemistry of the Diels-Alder reaction can be explained by frontier molecular orbital theory (FMO). As applied to cycloaddition reactions the rule is that reactions are allowed only when all overlaps between the highest occupied molecular orbital (HOMO) of one component and the lowest unoccupied

molecular orbital (LUMO) of the other are in phase such that a positive lobe overlaps only with another positive lobe and a negative lobe only with another negative lobe. These orbitals are the closest in energy [44]. Figure 2 illustrates the molecular orbitals of alkenes and conjugated dienes, and the two dominant orbital interactions of symmetry allowed Diels-Alder cycloaddition.

The reactivity of a Diels-Alder reaction depends on the energy difference between HOMO and LUMO of the two components [43]. The lower the energy difference, the lower is the transition state energy of the reaction. The energy level of both HOMO and LUMO depends on the substituents. Electron-withdrawing groups lower their energy, while electron donating groups increase their energy. For normal electron-demand Diels-Alder reaction, the reaction is controlled by HOMO of diene and LUMO of dieneophile (Figure 2). Therefore, the reactions are accelerated by electron-donating substituents in the diene and by electron-withdrawing substituents in the dienophile. In contrast, the inverse electron-demand Diels-Alder reaction is controlled by LUMO of diene and HOMO of dienophile (Figure 2). Therefore, the reactions are accelerated by electron-withdrawing groups in the diene and by electron-donating groups in the dienophlie.

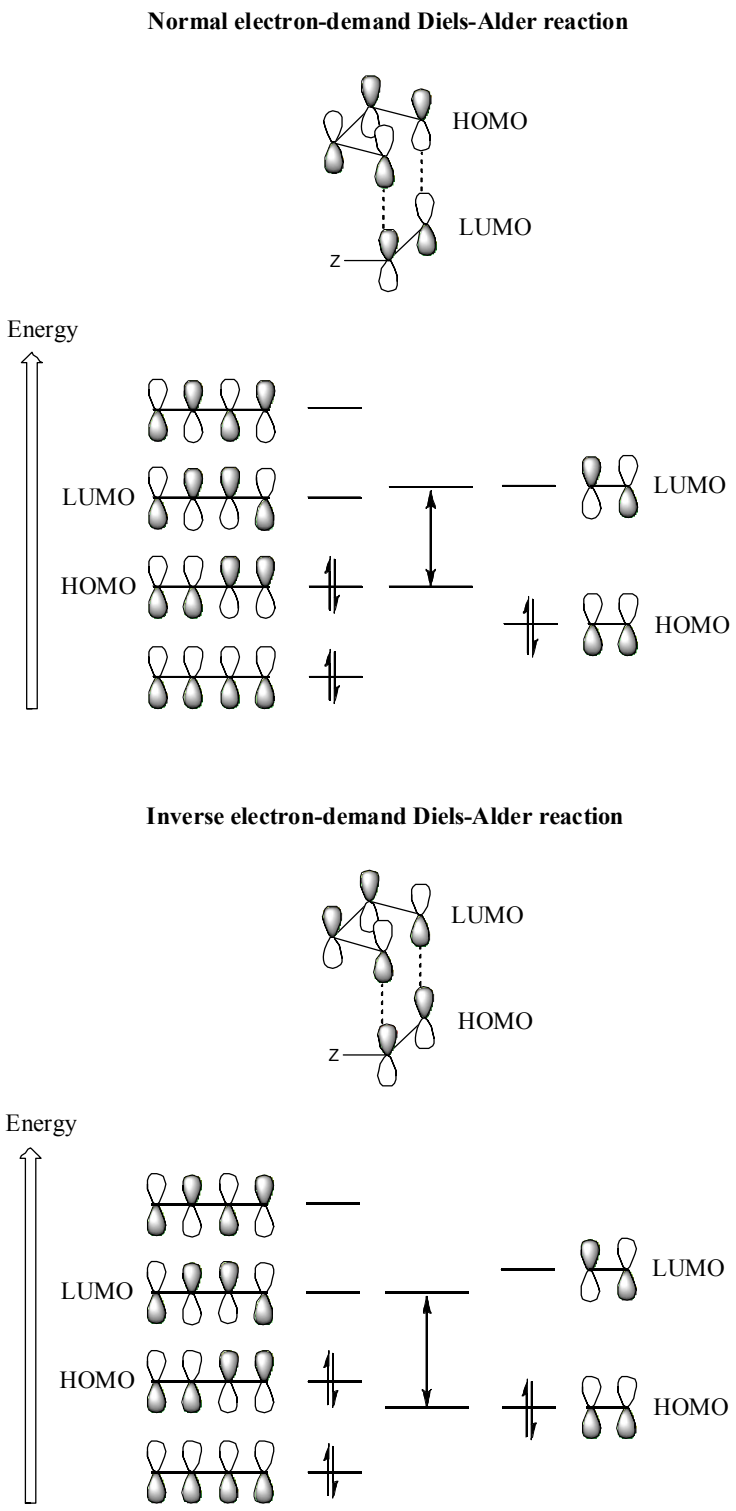



**Figure 2.** Schematic drawing of the molecular orbitals of alkenes and conjugated dienes and the orbital interaction for normal and inverse electron demand Diels-Alder reactions.

In addition, the regioselectivity of the Diels-Alder reaction can also be explained by FMO theory. The regiochemistry is controlled by the orbital coefficients of the atoms forming the σ-bonds. The σ-bonds form in such the way that the orbitals that have larger coeffients (larger lobes in Figure 3) overlap together. The regioselective is increased when the difference between the orbital coefficents of the two end atoms of diene and two atoms of dienophile increase [43].

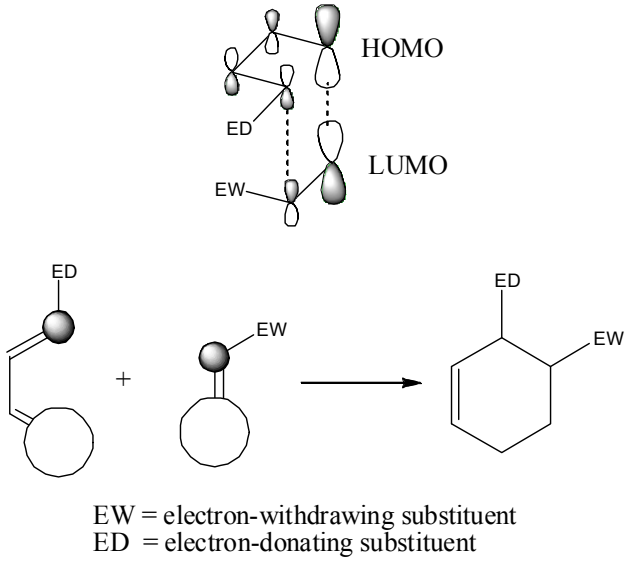



**Figure 3.** Example of the regioselectivity of normal electron-demand Diels-Alder reaction controlled by the orbital coefficients of the atoms forming the σ-bonds.[43]

The FMO theory can be used to explain the stereochemistry of the Diels-Alder reaction. The Diels-Alder reactions are suprafacial reactions and have two suprafacial approached named *endo* and *exo*. In *endo* approach, the bulkier sides of diene and dienophile lie one above the other. In *exo* approach, the bulkier side of one component is under the small side of the other. Therefore, the *exo* addition mode is expected to be preferred because of less steric repulsive interactions than in the *endo* approach.

However, it appears that the *endo* adduct is usually the major product. This *endo* preference can be explained by the FMO theory that the *endo* approach is kinetically favored because of the additional nonbonding interaction called "secondary orbital interaction" which stabilizes the *endo* transition state by lowering the trasition state energy (Figure 4)[43]. This secondary orbital interaction can not be formed in the exo approach.

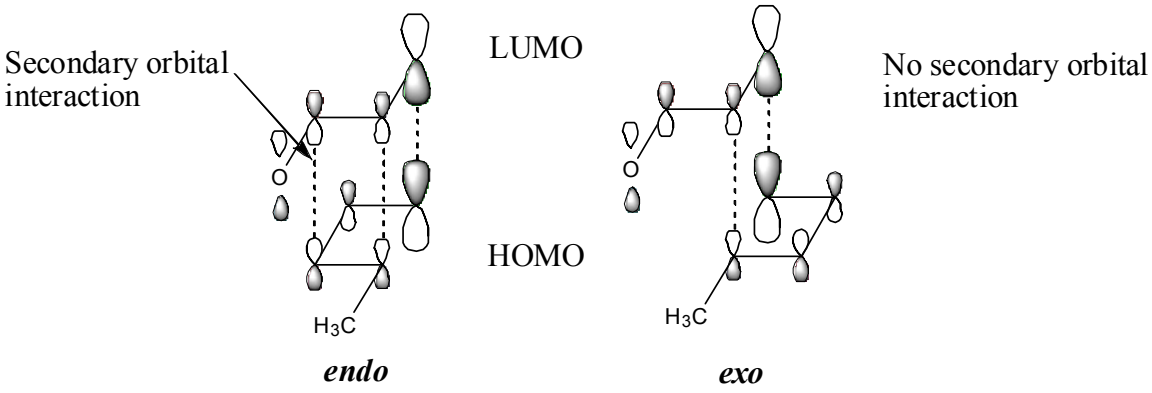



**Figure 4.** The *endo* and *exo* approach of the Diels-Alder reaction between piperylene and acrolein and the secondary orbital interaction in the *endo* transition state.[43]

The main part of this dissertation focuses on the chemistry of quinonoid compounds. Therfore, the next section will be discussed about the Diels-Alder reaction of quinonoid compounds. Then, the Diels-Alder reactions carried out in the water under conventional conditions of temperature and pressure will be illustrated next.

2.2.1.1 Quinone Diels-Alder Reaction

The quinone Diels-Alder (QDA) reaction (Figure 5) is a useful synthetic pathway and many studies showed that the QDA adducts can be used as suitable starting points for the synthesis of a wide variety of natural compounds, many of which are highly functionalized.

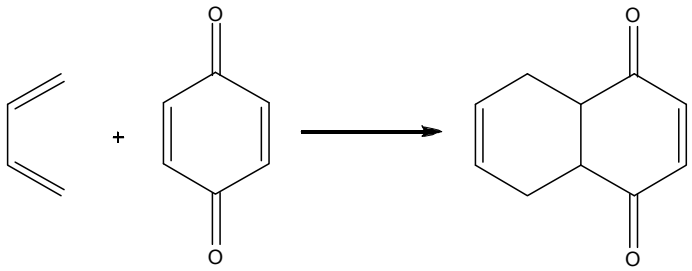


**Figure 5.** The quinone Diels-Alder (QDA) reaction.

An elegant example of the significance of QDA reactions in synthetic organic chemistry was shown by R. B. Woodward in 1952. Woodward et al. created the route to syntheis the steroids cortisone and cholesterol by using the QDA adduct of 2-methoxy-5-methyl-*p*-benzoquinone and butadiene as a precursor for this synthesis. The bicyclic adduct was formed *via* the intermediacy of *endo* transition state as illustrated in Figure 6.

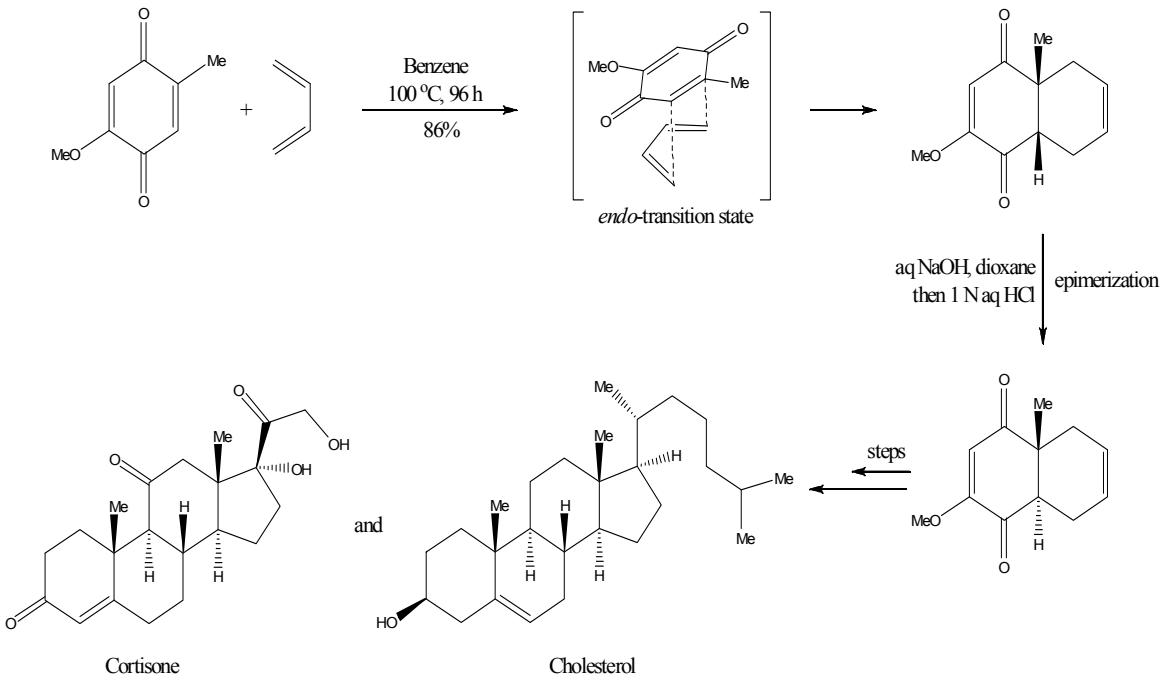



**Figure 6.** A quinone-based Diels-Alder reaction as the key step in the total synthesis of the steroid hormones cortisone and cholesterol.

Many studies have been reported the Diels-Alder reaction of quinonoid compounds and several of these studies were reviewed by K. T. Finley [45]. Examples of uncatalyzed and catalyzed quinone Diels-Alder reaction are summarized in Table 1.

**Table 1.** The examples of uncatalyzed and catalyzed quinone Diels-Alder reaction.

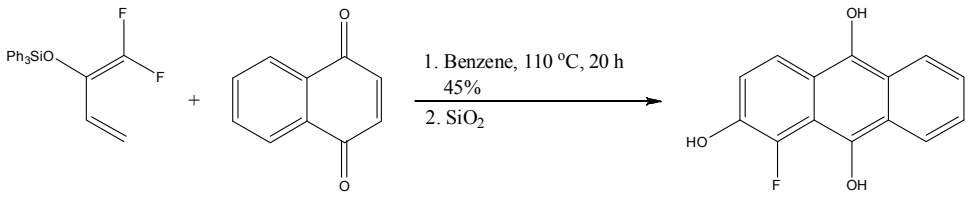
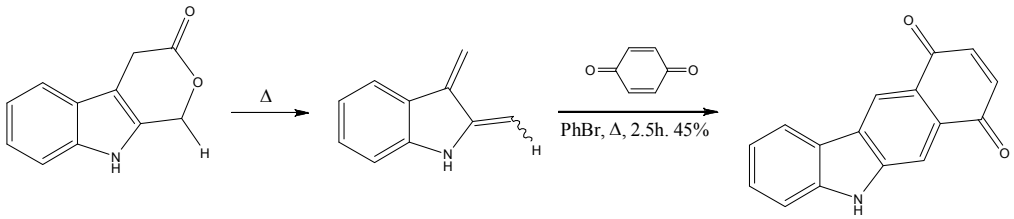
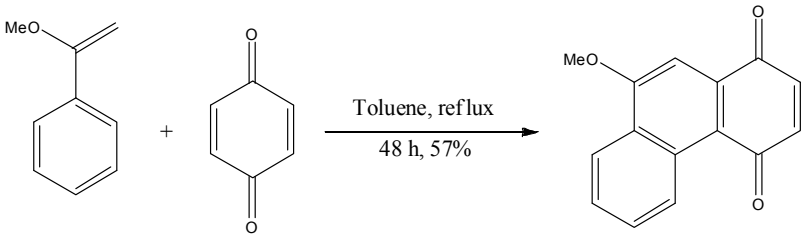
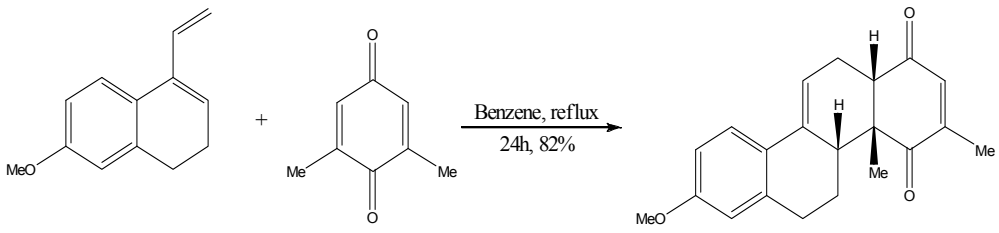
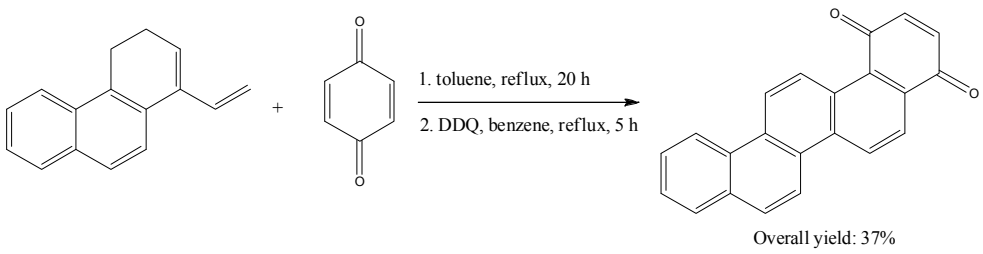
| Reaction | Reference |
|---|---|
|  | [46] |
|  | [47] |
|  | [48] |
|  | [49] |
|  | [50] |

**Table 1.** (Continued)

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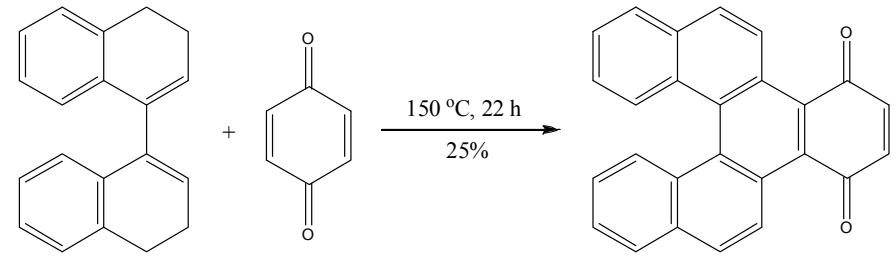
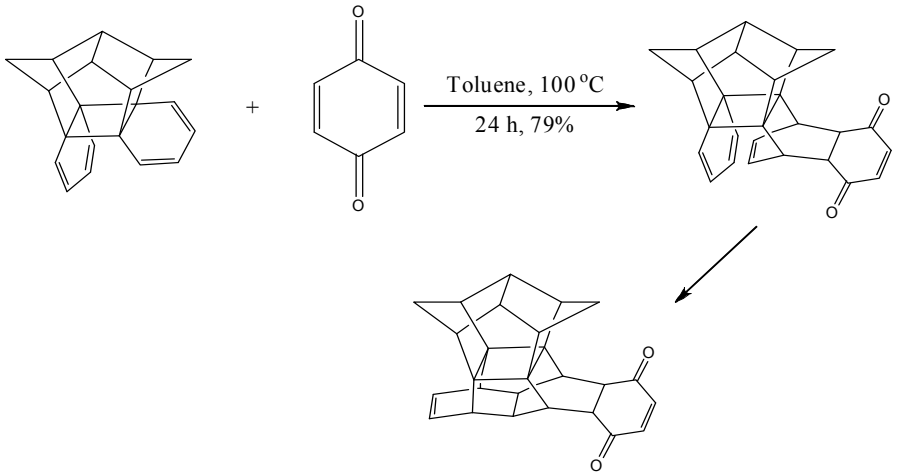
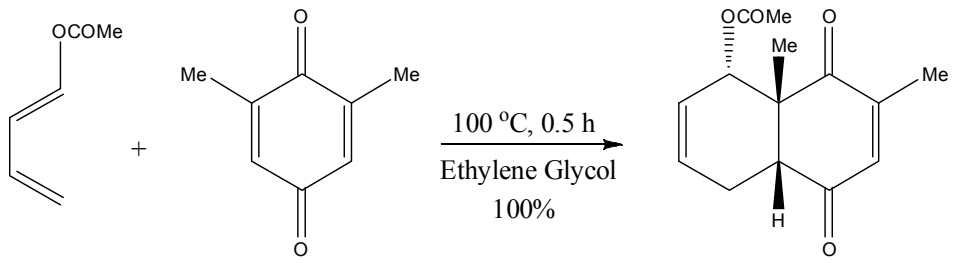
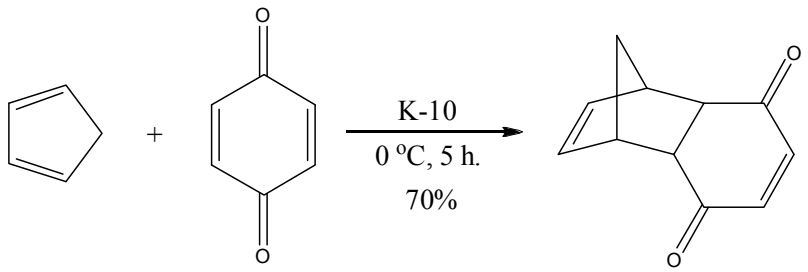
|   | [51] |
|   | [52] |
|   | [53] |
|   | [54] |

**Table 1.** (Continued)

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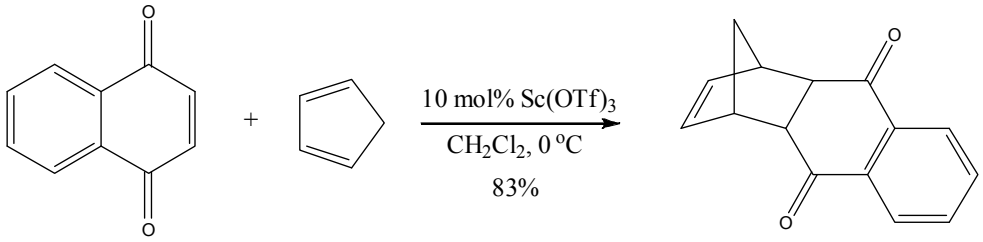
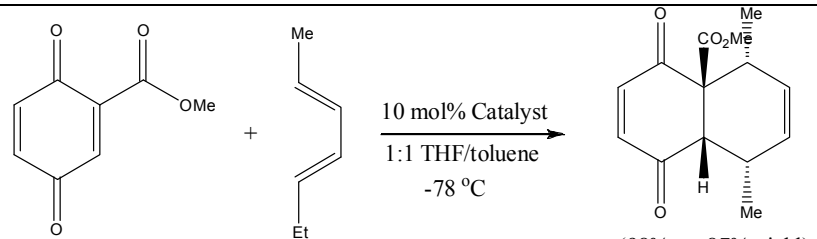
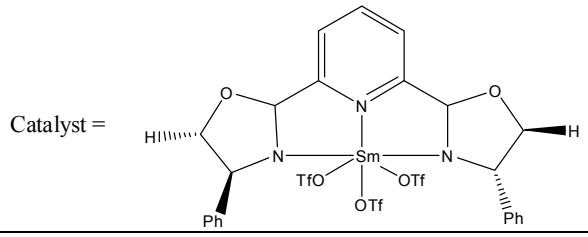
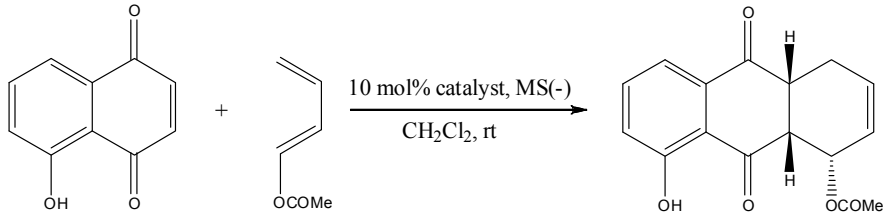
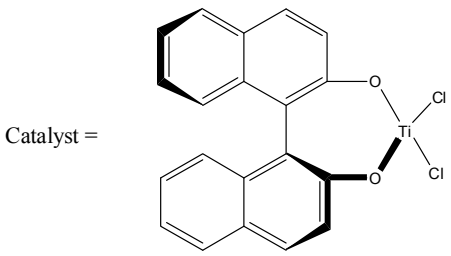
|---|---|
|  | [55] |
|  <br>  | [56] |
|  <br>  | [57] |

The important application of quinone Diels-Alder reaction is to generate the QDA adducts which can be used as the starting points in the total synthesis of various of natural

compounds [58-60]. For example, a QDA reaction was used to construct the tricyclic framework for the total synthesis of forskolin derivative [61]. The tricyclic carbon skeleton of the analogue of forskolin was generated *via* a Diels-Alder cycloaddition between a quinone and a vinyl cyclohexene as illustrated in Figure 7.

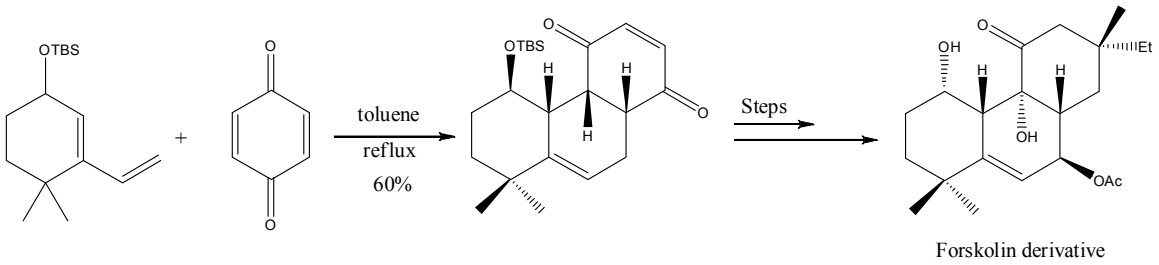



**Figure 7.** A Diels-Alder reaction of quinone and a vinyl cyclohexene as the key step in the total synthesis of forskolin derivative.[61]

Recently, the Nicolaou group reported the use of Mikami's catalyst ((S)-BINOL-$TiCl_2$) in the total synthesis of the unique terpenoid (-)-colombiasin A [62,63]. The first step of this synthesis involved a selective asymmetric Diels-Alder reaction of Danishefsky-type diene and quinone in the presence of the Mikami catalyst (30 mol%) as shown in Figure 8. After many steps, (-)-colombiasin A was received in 32% overall yield. White and Choi extended the versatility of this Mikami's catalyst in their total synthesis of (-)-ibogamine [64]. In this study, the Diels-Alder reaction of 1,4-benoquinone and 1,3-diene catalyzed by Mikami's catalyst was used as the key step in an asymmetric synthesis leading to the alkaloid (-)-ibogamine (Figure 9). The preparation of (-)-ibogamine was preceeded in 14 steps from 1,4-benzoquinone and the final product was received in 10% overall yield.

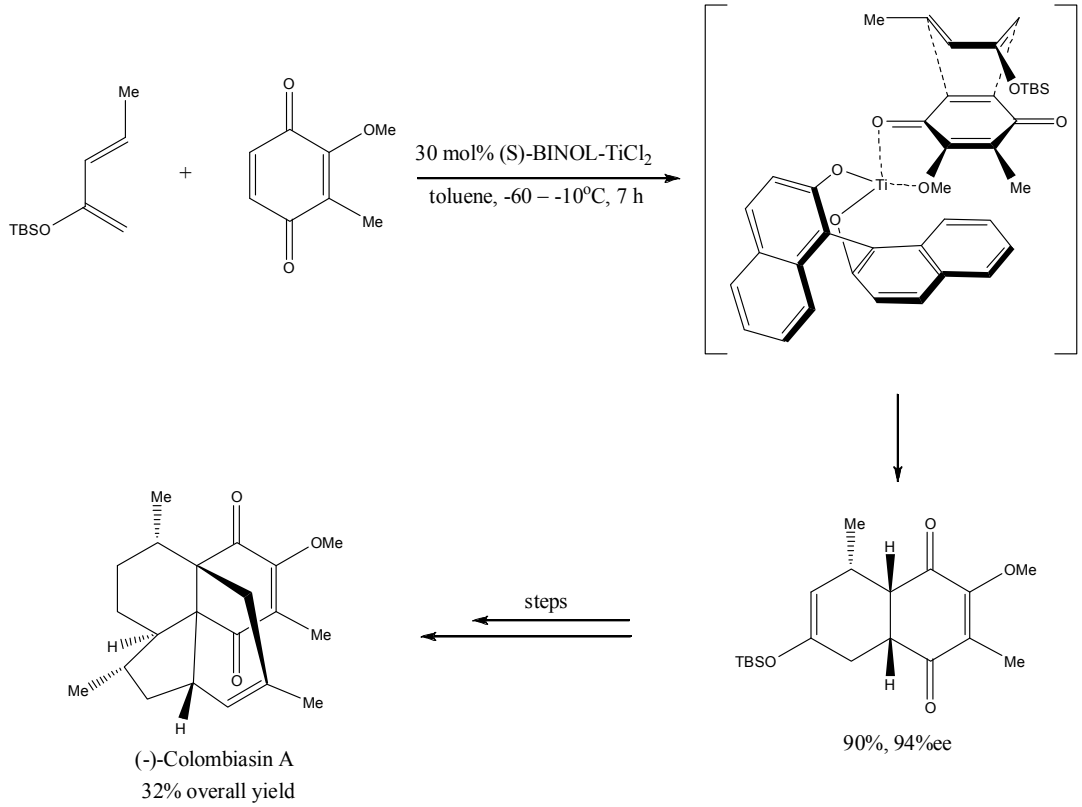



**Figure 8.** A Diels-Alder reaction of Danishefsky-type diene and quinone in the presence of the Mikami's catalyst for the total synthesis of (-)-colombiasin A.[62,63]

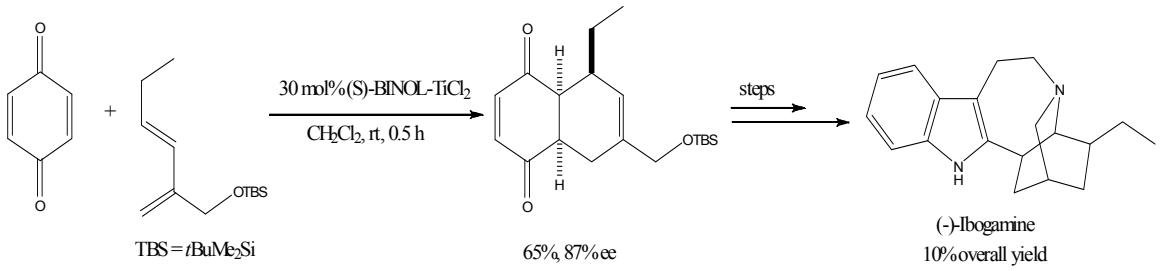



**Figure 9.** A Diels-Alder reaction of 1,3-diene and 1,4-benzoquinone in the presence of the Mikami's catalyst as a key step for the total synthesis of ibogamine.[64]

Most recently, Jocobsen et al. [65] reported the application of the Cr-catalyzed asymmetric quinone Diels-Alder Reaction for the total syntheses of (-)-colombiasin A and (-)-elisapterosin B. The QDA adduct was used as a precursor for these syntheses. The synthesis of (-)-colombiasin A was accomplished in 11.5% overall yield as summarized in Figure 10.

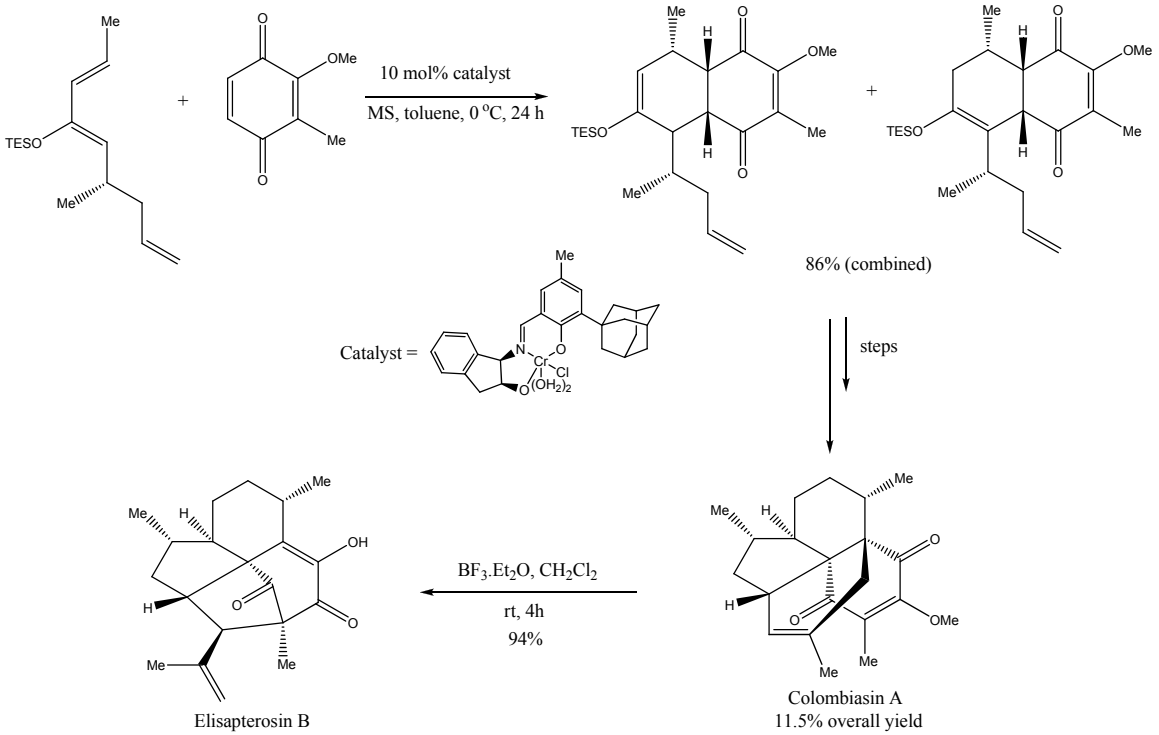
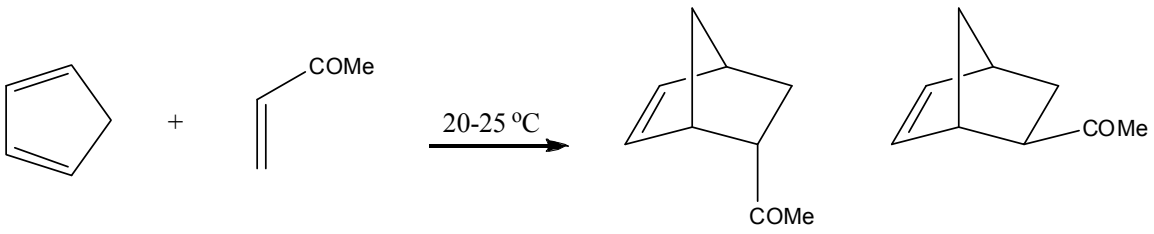



**Figure 10.** Cr-catalyzed asymmetric quinone Diels-Alder reaction as a key step for the total syntheses of (-)-colombiasin A and (-)-Elisapterosin B.[65]

2.2.1.2 Uncatalyzed Diels-Alder Reaction in Aqueous Medium

In 1931, Diels and Alder provided the first report of an uncatalyzed aqueous Diels-Alder reaction of furan and maleic anhydride [66,67]. However, the first kinetic study of acceleration of Diels-Alder reaction in water was studied by Rideout and

Breslow in 1980 [35]. In this study, they discovered that the reaction between cyclopentadiene and methyl vinyl ketone in water was 740 times faster than in the apolar hydrocarbon isooctane (Figure 11). By adding lithium chloride (salting-out agent) the reaction rate increased 2.5 times further. The authors suggested that this unusual acceleration in water was attributed to the polarity of the medium and hydrophobic interaction (hydrophobic packing of diene and dienophile). The presence of lithium chloride increased the reaction rate because the salt made the apolar reactants less soluble in water and in so doing it enhanced the hydrophobic interaction.



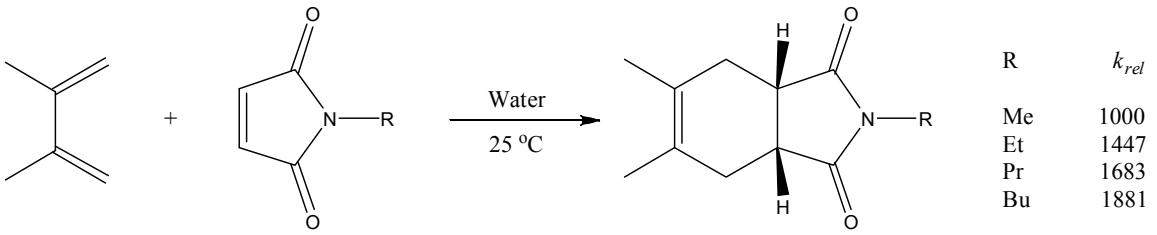

| | $k_{rel}$ |
|---|---|
| Isooctane | 1 |
| Methanol | 13 |
| Water | 740 |
| Water + LiCl | 1818 |

| | Endo/Exo |
|---|---|
| Cyclopentadiene | 3.9 |
| Ethanol | 8.5 |
| Water | 21.4 |

**Figure 11.** Diels-Alder reaction between cyclopentadiene and methyl vinyl ketone in water and organic solvents.[35]

Several experimental studies [68-71] and computer simulations [72] seem to indicate that the rate enhancement of the aqueous Diels-Alder reactions are due to the enforced hydrophobic interactions and hydrogen bonding interactions. The term "enforced" is used to stress the fact that the association of the nonpolar reagents is driven

by the reaction and only enhanced by water. For instance, Engberts and his co-workers [71] reported a kinetic study of a Diels-Alder reaction of 2,3-dimethyl-1,3-butadiene and with N-methyl-, N-ethyl-, N-propyl-, and N-butylmaleimide in different solvents. These reactions were accelerated in water relative to organic solvents as a result of enhanced hydrogen bonding and enforced hydrophobic interactions during the activation process. In addition, the acceleration increased as the hydrophobic character of the alkyl chain of the dienophile increased (Figure 12).

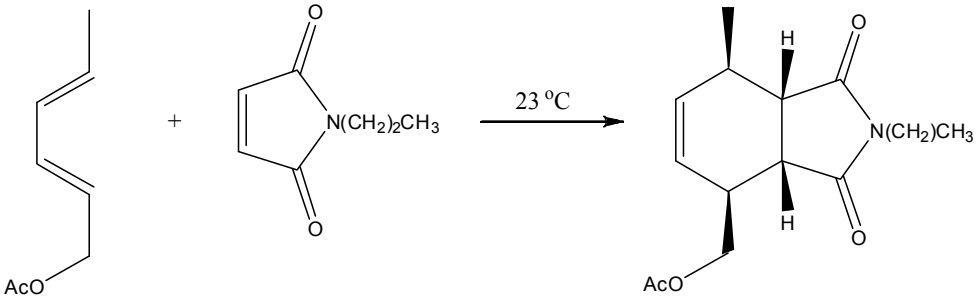



| R | $k_{rel}$ |
|---|---|
| Me | 1000 |
| Et | 1447 |
| Pr | 1683 |
| Bu | 1881 |

**Figure 12.** Relative reaction rate ($k_{water}$/ $k_{n\text{-}hexane}$) of Diels-Alder reaction between 2,3-dimethyl-1,3-butadiene and N-alkylmaleimides.[71]

Moreover, Sharpless and his colleagues [38] studied the cycloaddition of the water insoluble *trans,trans*-2,4-hexadienyl acetate and *N*-propylmaleimide under various conditions. The results of this study showed that the reaction in water suspension provided substantial rate acceleration over homogeneous solution and the reaction in a protic solvent such as methanol performed faster rate than in nonprotic solvent such as acetonitrile and toluene (Figure 13). These results show that hydrogen bonding and hydrophobic effects both are important for rate acceleration. Recently, Kumar and Tiwari [73] explored three simple Diels-Alder reactions involving cyclopentadiene with methyl

acrylate, ethyl acrylate and butyl acrylate both in water and room temperature ionic liquids (RTILs). They found that these Diels-Alder reaction in water are faster than in RTILs. The reduction of reaction rate in RTILs can be attributed to the absence of hydrophobic interactions and weaker hydrogen bonding in RTILs.

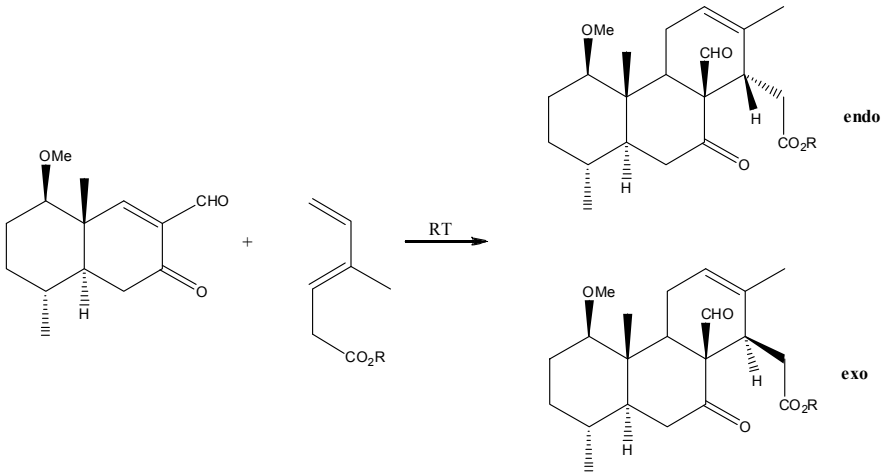



| Solvent | Time (h) | Yield (%) |
|---|---|---|
| Toluene | 144 | 79 |
| Acetonitrile | >144 | 43 |
| Methanol | 48 | 82 |
| Neat | 10 | 82 |
| Water | 8 | 81 |

**Figure 13.** Diels-Alder reaction between *trans,trans*-2,4-hexadienyl acetate and *N*-propylmaleimide under various conditions.[38]

Beside the rate enhancement, the enhancement of *endo*/*exo* selectivity of the aqueous Diles-Alder reaction was also observed. Breslow et al. [74] also noted that the *endo* addition of the reaction of cyclopentadiene with methyl vinyl ketone is more favored when the reaction is carried out in water than when it is performed in organic solvents (Figure 11). The *endo* preference in water were explained by the need to minimize the transition state surface area in aqueous medium, thus favoring the more compact *endo* transition state more than the extended *exo* transition state. Another

example is the study of Grieco and his co-workers [75]. They examined the Diels-Alder reaction between the α,β-unsaturated ketoaldehyde and ethyl 4-methyl-3,5-hexadienoate (R = Et) in water and in hydrocarbon solvents (Figure 14). They found that the reaction rate was doubled and both the reaction yield and the *endo* selectivity was enhanced when conducting the reaction in aqueous medium. The best result was observed when conducting the reaction of diene sodium carboxylate (R = Na). The reaction was completed in 5 hours and the *endo* adduct is 75% of the diastereoisomeric reaction mixture. In 1993, Paul et al. [76] applied this Diels-Alder reaction as a key step in the synthesis of chaparrinone and other quassinoids (naturally occurring substances with antileukemic activity). Recently, Utley et al. [77] reported the efficient formation of the *endo*-Diels-Alder adducts of the reaction between *ortho*-quinodimethanes, generated cathodically in aqueous electrolyte, and *N*-methylmaleimide.




| R | Solvent | time (h) | endo/exo | Yield (%) |
|---|---|---|---|---|
| Et | PhH | 288 | 0.85 | 52 |
| Et | $H_2O$ | 168 | 1.3 | 82 |
| Na | $H_2O$ | 5 | 3.0 | 100 |

**Figure 14.** Diels-Alder reaction between α,β-unsaturated ketoaldehyde and ethyl 4-methyl-3,5-hexadienoate.[75]

Several studies have been reported the hetero Diels-Alder cycloadditions in aqueous medium. For example, Kibayashi et al. explored the Diels-Alder reactions of the nitroso moiety of the N-acylnitroso, a powerful dienophile, with a diene in water. The N-acylnitroso compounds were generated in situ by periodate oxidation and then reacted with dienes to form the Diels-Alder adducts. This N-acylnitroso compounds can be trapped rapidly, especially in an intramolecular reaction such as the reaction of the in situ-generated N-acylnitroso compound in Figure 15 that immediately cyclized to *cis* and *trans*-1,2-oxainolactams [78]. Kibayashi et al. also used this acylnitroso approach in the syntheses of (-)-swainsonine and (-)-pumiliotoxin [79].

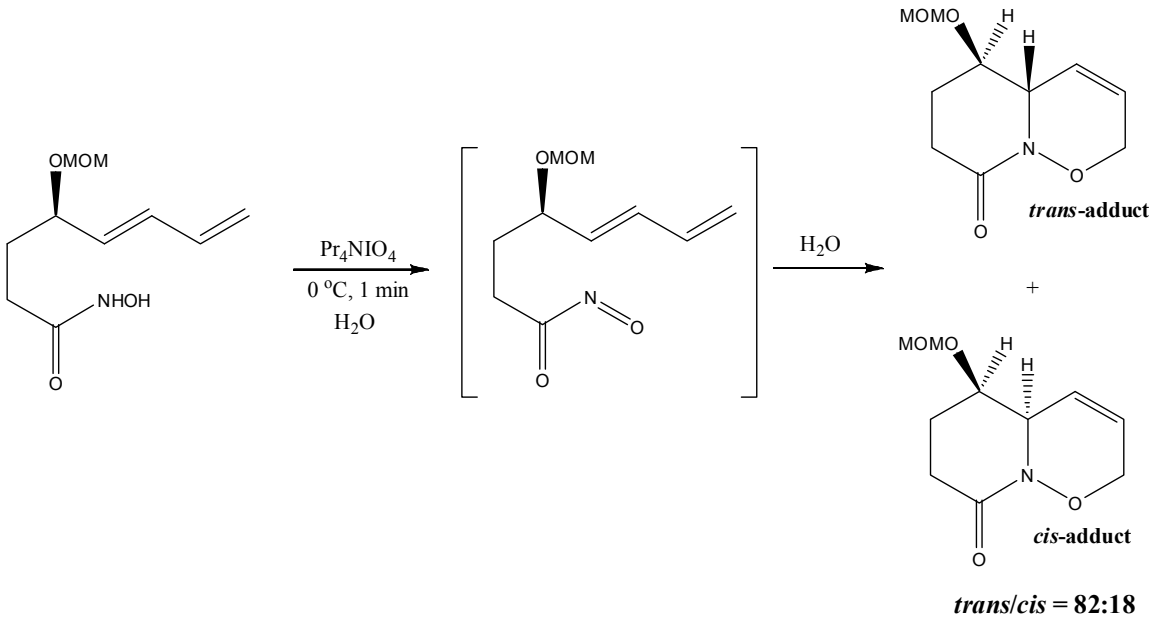
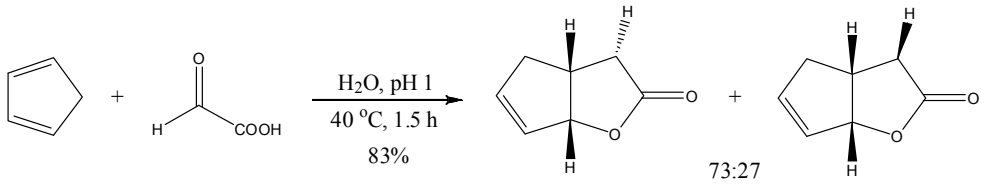
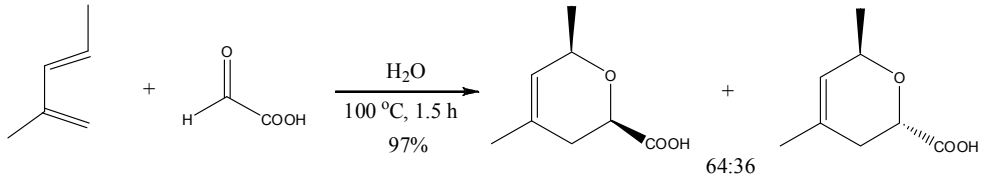
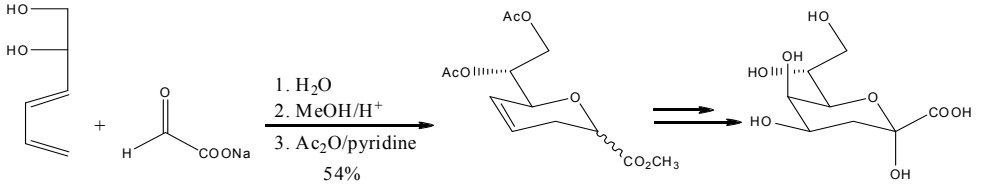
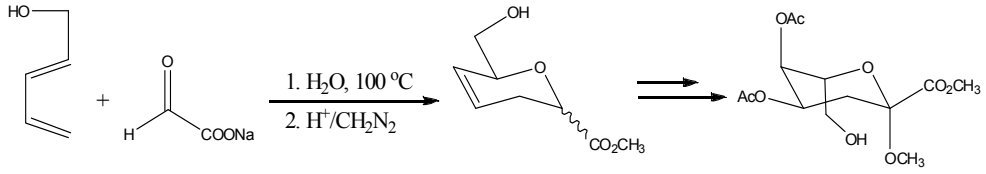
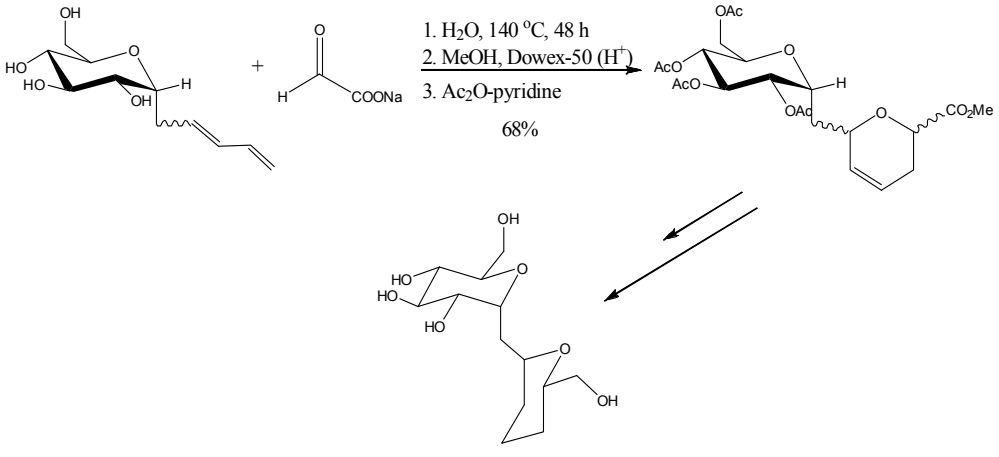



**Figure 15.** Intramolecular hetero Diles-Alder reaction of N-acylnitroso compound.[78]

Lubineau and coworkers [80,81] have shown that glyoxylic acid, pyruvaldehyde, and glyoxal were shown to react with cyclic or non-cyclic dienes *via* the aqueous hetero Diels-Alder reaction to give the corresponding cycloadducts and/or α-hydroxy γ-lactones in a good yield. Moreover, they also used this approach to prepare key starting compounds for the enantioselective synthesis of 3-deoxy-D-manno-2-octulosonic acid [82] and ketodeoxyheptulosonic acid derivatives [83]. Lubineau et al. have done the extensive work in the studied of the aqueous Diels-Alder reactions to prepare optically active oligosaccharides [84,85]. Some examples of Lubineau's work are summarized in Table 2. Another example for intramolecular hetero-Diels-Alder reaction in water was reported by Grieco and Kaufman [86]. They examined the intramolecular Diels-Alder reaction of iminium ions in polar media such as 5.0 M lithium perchlorate-diethyl ether and water. In hot water, the tricyclic amine product can be obtained as the exclusive diastereomer in 80% yield (Figure 16). They suggested that water appears to be the polar solvent of choice for this reaction system because the use of lithium perchlorate-diethyl ether as polar solvent led to some major problems. These problems occurred from the fact that weak acid (lithium perchlorate) in highly polar media become strong acids and protonation of the tethered dienes with concomitant diene isomerization is competitive with cycloaddition.

**Table 2.** Example of the hetero Diels-Alder reactions studied by Lubineau et al.

| Reactions | Reference |
|---|---|
|   | [80] |
|   | [81] |
|   | [82] |
|   | [83] |
|   | [85] |

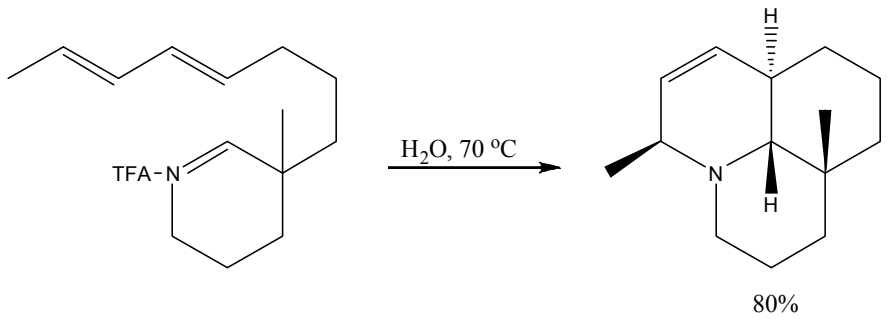



**Figure 16.** Intramolecular imino-Diels-Alder reactions.[86]

2.2.1.3 Lewis-Acid-Catalyzed Diels-Alder Reaction in Aqueous Medium

In recent years, a number of water-tolerant Lewis acids have been used to catalyze various Diels-Alder reactiond in aqueous medium [34]. In 1993, Kobayashi [55] reported the use of scandium trifate, $Sc(OTf)_3$ for the Diels-Alder reaction in aqueous medium. This catalyst was stable in water and easily recovered to reused. Many other Lewis acids have been reported to catalyze Diles-Alder reactions in water. Engberts [87,88] reported the use of aqua-complexing agents including $Co(NO_3)_2.6H_2O$, $Ni(NO_3)_2.6H_2O$, $Cu(NO_3)_2.3H_2O$, and $Zn(NO_3)_2.4H_2O$ as Lewis acid catalysts for Diels-Alder reaction in aqueous medium. The Diels-Alder reactions performing in aqueous medium in the presence of these metal catalysts were faster than the aqueous reactions without the catalysts, and $Cu^{2+}$ ion showed to be the best catalyst in this study. However, the catalysts worked efficiently only if they formed a chelate with the dienophile, and complexation with α-amino acids (see Figure 17) which induces asymmetry in the Diels-Alder reaction as in the copper-catalyed the reaction of 3-phenyl-1-(2-pyridyl)-2-propen-1-one with cyclopentadiene (Figure 18) [89]. This cycloaddition occurs *endo*-stereoselectively in 3 days with high yield and with acceptable enantioselectivity (*ee* = 74%). Therefore, this is

the first enantioselective Lewis acid-cataltzed Diels-Alder reaction in water. Recently, Engberts and Mubofu [90] reported a comparative study of specific acid catalysis (hydrochloric acid) and Lewis acid (i.e. copper (II) nitrate) catalysis of Diels–Alder reactions in aqueous medium. They found that the reaction rate is 40 times faster with copper catalysis than with hydrochloric acid catalysis at equimolar amounts of copper(II) nitrate and hydrochloric acid and under the same reaction conditions.

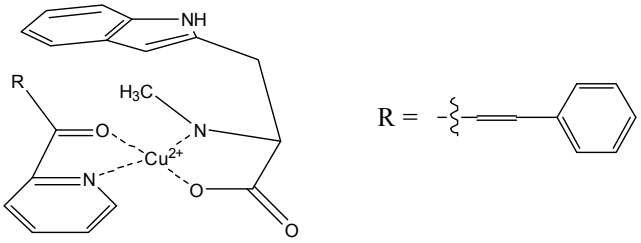
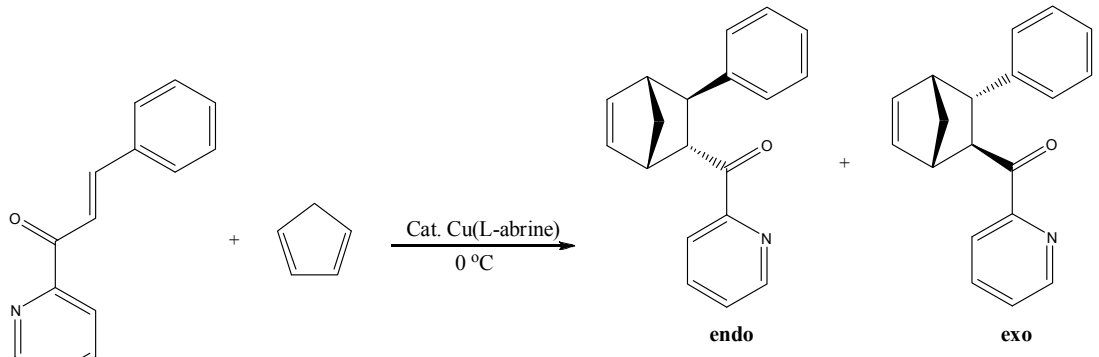



**Figure 17.** Complexation of Cu(L-abrine) catalyst and 3-phenyl-1-(2-pyridyl)-2-propen-1-one.[89]




| Solvent | ee (%) |
|---|---|
| $H_2O$ | 74 |
| EtOH | 39 |
| $CH_3CN$ | 17 |
| THF | 24 |
| $CHCl_3$ | 44 |

**Figure 18.** The enantioselectivity of copper (L-arabine) catalyzed Diels-Alder reactions of 3-phenyl-1-(2-pyridyl)-2-propen-1-one with cyclopentadiene.[89]

Many studies have now used water-tolerant Lewis acid, lanthanide triflates ($Ln(OTf)_3$) [91] together with $Bi(OTf)_3$ [92], $Sc(OTf)_3$ [93] and $In(OTf)_3$ [94,95] to catalyze the Diels-Alder reactions in water. For example, Wang et al. [96] studied the use of $Ln(OTf)_3$ to catalyze the aqueous aza-Diels-Alder reaction of an aldehyde and amine hydrochloride with diene. Figure 19 shows a representative reaction of this study. The product (*endo* + *exo*) was isolated in only 4% yield when no $Ln(OTf)_3$ was added. However, the yield of the product was increased to 64% when the lanthanide catalyst was added.

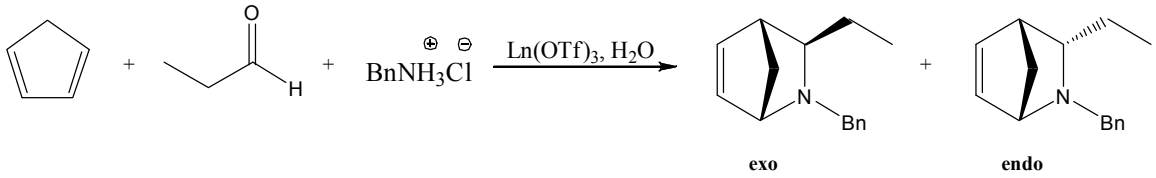



**Figure 19.** The aqueous aza-Diels-Alder reaction using lanthanide triflate.[96]

Lanthanide triflates were also shown to catalyze imino Diels-Alder reactions of imines with dienes or alkenes which were developed by Kobayashi and his co-workers [97]. Here, they reported a three-component coupling reactions between aldehydes, amines, and dienes or alkenes which were successfully carried out by using lanthanide triflate as a catalyst to afford pyridine and quinoline derivatives in high yields (Figure 20). Recently, Taguchi et al. [95] developed indium(III) triflate catalyzed intramolecular Diels-Alder reaction of ester-tethered 1,7,9-decatrienoates in aqueous media. This reaction gave the cycloadducts in good yield with perfect endo-selectivity and $In(OTf)_3$ is recyclable without troublesome purification.

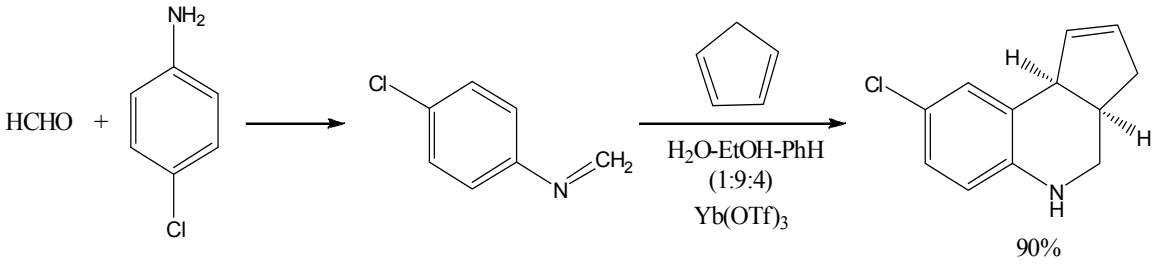
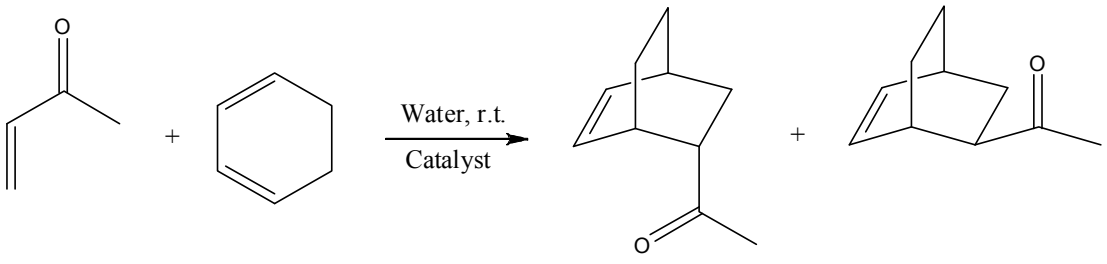



**Figure 20.** $Yb(OTf)_3$-catalyzed Diels-Alder reaction between N-benzylideneaniline as azadiene and cyclopentadiene.[97]

Lewis acid/surfactant combined catalysts (LASCs) such as $M(DS)_n$, $M(DCS)_n$, [98,99] and $Cu(dDP)_2$ [100] (M = lanthanides, Sc, Yb, Cu, Zn, Ag, Mn, Co; *n* = 1, 2, 3; DS = dodecylsulfate, DCS = dodecanesulfonate, dDP = 5,5-di-*n*-dodecyl-2-hydroxy-1,3,2-dioxaphosphorinan-2-one) have recently been prepared. However, reports on their catalytic ability in Diels-Alder reactions are discrepant.

Indium trichloride [101,102] and methylrhenium trioxide [103] are also water-tolerant Lewis acids, and have been reported to catalyze Diels-Alder cycloadditions in water. Some examples of these catalyst in the cycloaddition of methyl vinyl ketone and 1,3-cyclohexadiene are illustrated in Figure 21.




| | endo/exo | Yield (%) |
|---|---|---|
| $InCl_3$ | 90:10 | 87 |
| $MeReO_3$ | 99:1 | 91 |

**Figure 21.** The Diels-Alder reaction of methyl vinyl ketone and 1,3-cyclohexadiene catalyzed by indium trichloride or methylrhenium trioxide.[101,103]

Recently, Nishikido et al. [104] reported fluorous reverse-phase silica gel (FRPSG)-supported Lewis acids catalyzed Diels-Alder reactions in water, and the FRPSG-supported Lewis acids could be recycled by simple filtration after the reaction. Yu et al. [105] examined the use of water-soluble organotungsten Lewis acid, $[OP(2\text{-}py)_3W(CO)(NO)_2](BF_4)_2$ to catalyze Diels-Alder reactions under conventional heating or microwave heating conditions. The cycloaddition reactions were efficiently conducted in either water or in an ionic liquid, 1-butyl-3-methylimidazolium hexafluorophosphate. Most recently, Litz [106] reported Flextyl P$^{TM}$, a novel Ti(IV) performance catalyst, catalyzed the aqueous Diels-Alder reaction of 1,3-cyclohaxadiene with 1,4-benzoquinone. The catalyst improved conversion by 22% versus the uncatalyzed reaction.

## 2.3 Biocatalysis

### 2.3.1 Enzymes

Enzymes are natural catalysts that accelerate the rate of reactions. Like all catalysts, enzymes work by lowering the activation energy ($E_a$ or $\Delta G^{\ddagger}$) for a reaction, thus dramatically increasing the rate of the reaction. Enzymes are composed of one or more polypeptides organized in a specific three-dimensional structure through interactions between the functional groups on the amino acid constituents. These interactions include ionic bonding, covalent bonding, hydrogen bonding, and van der waal's forces. Some of the outstanding features of the enzymes include high substrate specificity, specificity in promoting only one biochemical reaction with their substrate ensuring synthesis of a specific biomolecular product without the concomitant production of by products, stereospecificity, and regeospecificity, which they express in catalysis.

#### 2.3.1.1 Nomenclature and Classification

An enzyme's name is often derived from its substrate or the chemical reaction it catalyzes, with the word ending in "ase". For identification purpose, the International Union of Biochemistry and Molecular Biology have developed a nomenclature for the enzymes. Every enzyme has a four-digit number in the general form EC A.B.C.D, where EC stands for 'Enzyme Commission'; the following properties are encoded:

A indicates to which of the six main divisions (classes) the enzyme belongs,

B stands for the subclass, indicating the substrate class or the type of transferred molecule,

C indicates the nature of the co-substrate,

D is the individual enzyme number.

Enzymes have been classified into six categories according to the type of reaction they catalyze. These six classes of enzymes are listed below:

- Class 1 – Oxidoreductases: catalyze oxidation/reduction reactions,
- Class 2 – Transferases: transfer a functional group such as methyl or phosphate group,
- Class 3 – Hydrolases: catalyze the hydrolysis of C-O, C-N, O-P and C-S bonds,
- Class 4 – Lyases: catalyze the addition or removal of some chemical groups of substrate by mechanism other than oxidation, reduction, or hydrolysis,
- Class 5 – Isomerases: catalyze isomerization changes within a single molecule,
- Class 6 – Ligases: catalyze the joining together of two compounds coupled with the hydrolysis of a diphosphate bond in ATP or a similar triphosphate.

#### 2.3.1.2 Enzyme Mechanism

Enzymes are three-dimensional proteins that possess an "active site". At the active site, specific amino acids interact with the substrate, and the tranfornation of substrate take places. In order to understand enzyme catalysis, some models have been proposed.

##### *2.3.1.2.1 'Lock-and-Key' Mechanism*

In 1894, Emil Fischer [107] developed the first proposal for a general mechanism of enzymatic action. He hypothesized that an enzyme and its substrate form a complex very much like a "lock and key"; therefore, each enzyme is very substrate specific and its structure is completely rigid. However, this model can not explain why many enzymes do

act on large substrates, while they are inactive on smaller counterparts. Moreover, this hypothesis can not explain why many enzymes can convert a variety of nonnatural compounds besides their natural substrates [108]. Thus, another model had to be developed.

*2.3.1.2.2 Induced-Fit Mechanism*

Daniel Koshland [109] suggested a modification to the lock and key model that the enzymes are not entirely rigid but rather represent delicate and soft structures. During the formation of the enzyme-substrate complex, the enzyme can change its conformation under the influence of the substrate structure so as to wrap itself around its guest (Figure 22). This phenomenon was denoted as the 'Induced Fit'. The induced fit theory states a) precise orientation of catalytic groups is required for enzyme action b) the substrate causes changes in the amino acids at the active site c) the changes in the catalytic structure caused by a substrate will bring the catalytic groups into proper alignment whereas a non-substrate will not achieve this.

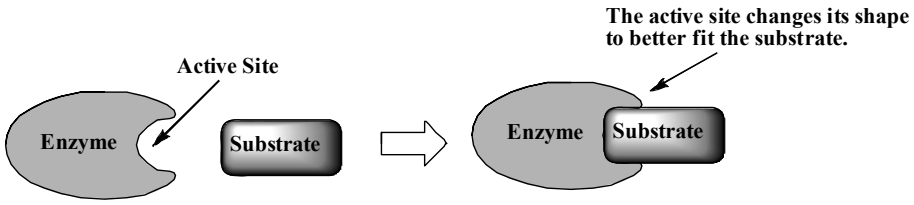



**Figure 22.** The induced fit mechanism for enzyme catalysis.

2.3.1.3 Enzyme Kinetics

The rate at which an enzyme converts substrate to products is referred to as its "activity". When a smaller amount of enzyme can convert a greater amount of substrate it is said to be more "active". The reaction kinetics have been characterized for many enzymes. Enzymatic activity is the productivity of the enzyme defined under strict standard conditions. Michaelis and Menten [110] used a simple unimolecular reaction to extract relationships used for predicting the kinetic properties of enzymes (Equation 1). The symbols that describe the reaction are E=Enzyme and S=Substrate. The reaction described by Michaelis and Menten proceeds in three phases. The *initial or stationary phase* is an important phase as it is at this point where substrate and enzyme come together for the intimate contact at the enzyme active site for the reaction. The second phase of the enzyme reaction is the *steady state* where the enzyme is assumed to be completely saturated with substrate and the rate of the reaction is dependant on the amount of enzyme (E) or enzyme-substrate complex (ES). According to Michaelis Menten (M-M) kinetics, the rate-limiting step is the conversion from ES to the product (P). The Michaelis Menten relationship is stated in Equation 1.

$$V = V_{max}\,[S]/[S]+K_m$$

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$$

**Equation 1.** The Michaelis-Menten Equation (V=reaction velocity; Vmax = maximum reaction velocity; [S] = substrate concentration; $K_m$ = michaelis-menten constant; E = enzyme; S = substrate, P = product).

$K_m$ is the M-M constant and $k_3$ is the turnover constant. These factors are important for gauging the efficiency of an enzyme-substrate system. $K_m$ is the concentration of substrate required for an enzyme to reach one-half of its maximum velocity or $V_{max}$. Essentially, $K_m$ is an indicator of the sensitivity or affinity of a particular enzyme for a certain substrate (Figure 23).

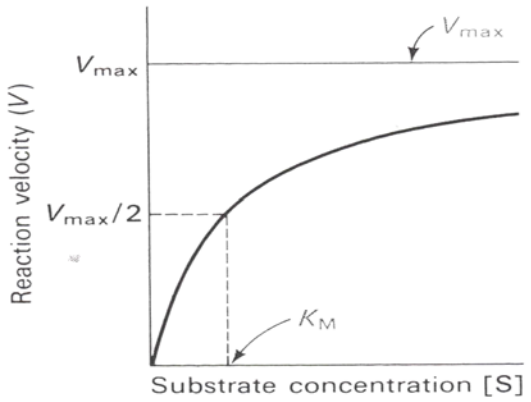



**Figure 23.** The graphical definition of the $K_m$ and $V_{max}$ Parameters in the Michaelis Menten Equation

The turnover number is the rate at which the enzyme-substrate complex is converted to the product, which indicates the ability of the enzyme to convert substrate into product. Since $k_3$ is the rate of formation of the product and $K_m$ is the affinity of the enzyme for the reactants, the value $k_3/K_m$ is usually a measure of the total enzyme productivity [111], therefore, achieving a maximum velocity at a low substrate concentration is ideal. Eventually the substrate concentration becomes limiting, and the reaction reaches its asymptotic limit [111] (Figure 23). Kinetic units can be elucidated by a relationship derived by Lineweaver and Burk (Equation 2).

$$1/V=1/V_{max}+K_m/V_{max}\ x1/[S]$$

**Equation 2.** Lineweaver and Burk equation for determining $K_m$ and $V_{max}$

Plotting the reciprocal of reaction rate vs. the reciprocal substrate concentration allows one to obtain $1/V_{max}$ at the y-intercept and $-1/K_m$ at the x-intercept.

2.3.1.4 Advantages and Disadvantages of Biocatalyst

*2.3.1.4.1 Advantages of Biocatalysts [108]*

- *Enzymes are very efficient catalysts*: Compare to the nonenzymatic reactions, the rates of enzyme-mediated processes are accelerated by a factor of $10^8$-$10^{10}$.
- *Enzymes are environmentally benign reagents*.
- *Enzymes act under mild conditions*: Enzymes act in a range of about pH 5-8, and in a temperature range of 20-40 ºC. This minimizes problems of undesired side reactions. However, there are some thermostable enzymes that can be performed at high temperature.
- *Enzymes are compatible with each other*: Several biocatalytic reactions can be carried out in a reaction cascade in one reactor because enzymes normally function under the same or similar conditions.
- *Enzymes are not bound to their natural role*: Enzymes can catalyze a variety of nonnatural substrates and often they are not required to work in water.
- *Enzymes can catalyze a broad spectrum of reactions*.
- *Enzymes display selectivity*: Three major types of selectivity are chemoselectivity, regioselectivity and diastereoselectivity, and enantioselectivity.
- *Valuable resource for green chemistry*

*2.3.1.4.2 Disadvantages of Biocatalysts [108]*

- *Enzymes are provided by nature in only one enantiomeric form*.
- *Enzymes require narrow operation parameters*: If a reaction proceeds too slow under given parameter of temperature and pH, there is only a narrow operational window for alteration. High temperature and extreme pH lead to deactivation of the enzymes.
- *Enzymes display their highest catalytic activity in water*.
- *Some Enzymes are bound to their natural cofactors such as NAD(P)H, and chemical energy (ATP)* : These cofactors are relatively unstable molecules and are prohibitively expensive to use in stoichiometric amounts.
- *Enzymes are prone to inhibition phenomena*: Many enzymatic reactions are prone to substrate- or product-inhibition, which causes the enzyme to cease to work at higher substrate and/or product concentrations, a factor which limits the efficiency of the process.
- *Enzymes may cause auto-immune responses including allergies*

### 2.3.2 Enzymes in Domino Reactions

Domino or cascade reactions involve two or more bond-forming transformations, which take place under the same reaction conditions, without adding additional reagents and catalysts, and in which the subsequent reactions result as a consequence of the functionality formed by bond formation or fragmentation in the previous step all occurring in one-pot [112]. The domino reaction is often proceeded via highly reactive intermediates.

In recent years, the availability of enzymes has increased. Therefore, the use of enzymes in the development of domino reaction has also increased in address the challenges of Green Chemistry. Many studies involve enzyme-initiated domino reactions have been reported [1,113-115]. Emzyme-initiated domino reactions follow a common reaction sequence. Firstly, the enzyme modifies a group ('trigger' group) in the starting material, generating a reactive intermediate that can undergo a subsequent domino reaction consisting of a (i) fragmentation, (ii) rearrangement, (iii) cyclization such as Diels-Alder reaction, or (iv) an intramolecular substitution affecting cyclization.

#### 2.3.2.1 Enzyme-Triggered Diels-Alder Reaction

The first successful combination of an enzymatic with a nonenzymatic transformation within a domino process was reported by Waldmann et al. in 1996 [116,117]. They reported the synthesis of highly functionalized bicycle[2.2.2]octenes by a tyrosinase-initiated hydroxylation-oxidation of phenols followed by a Diels-Alder (DA) reaction with electron rich dienophiles (see Figure 24). These studies, conducted in chloroform in the presence of oxygen, provided a unique three-step one-pot reaction of bicyclic DA products in high yields with the key intermediate being reactive *ortho*-quinones.

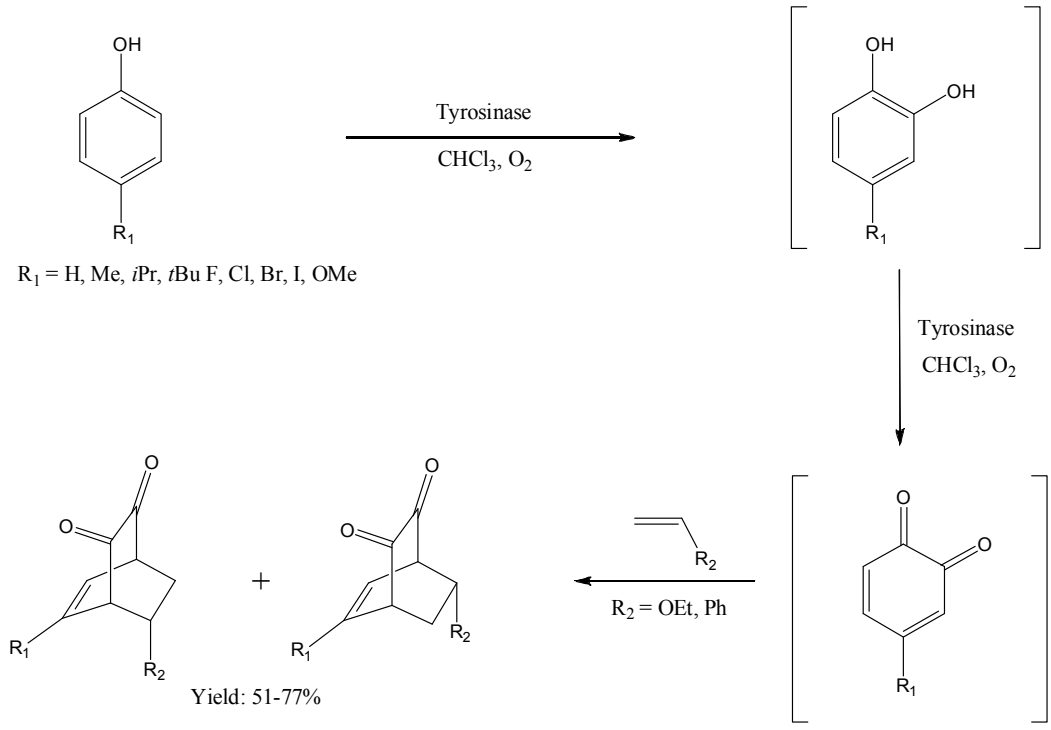



**Figure 24.** A cascade reaction involving o-quinones obtained by an enzyme-initiated hydroxylation-oxidation sequence combined with a Diels-Alder reaction.[116,117]

Kita and his co-worker [118,119] reported the first one-pot synthesis of optically active 7-oxabicyclo[2.2.1]heptenes catalyzed by lipase, the hydrolase enzyme that act on carboxylic ester bonds. As illustrated in Figure 25, the first step of this reaction was the kinetic resolution of racemic furfuryl alcohol derivatives *via* acyl transfer catalyzed by lipase. The next step was the intramolecular Diels-Alder reaction of the intermediate to provide 7-oxabicyclo[2.2.1]heptene derivatives. Most recently these authors reported the use of a lipase and a ruthenium catalyst to prepare polysubstituted decalines with high optical and chemical yields from racemic alcohols [120].

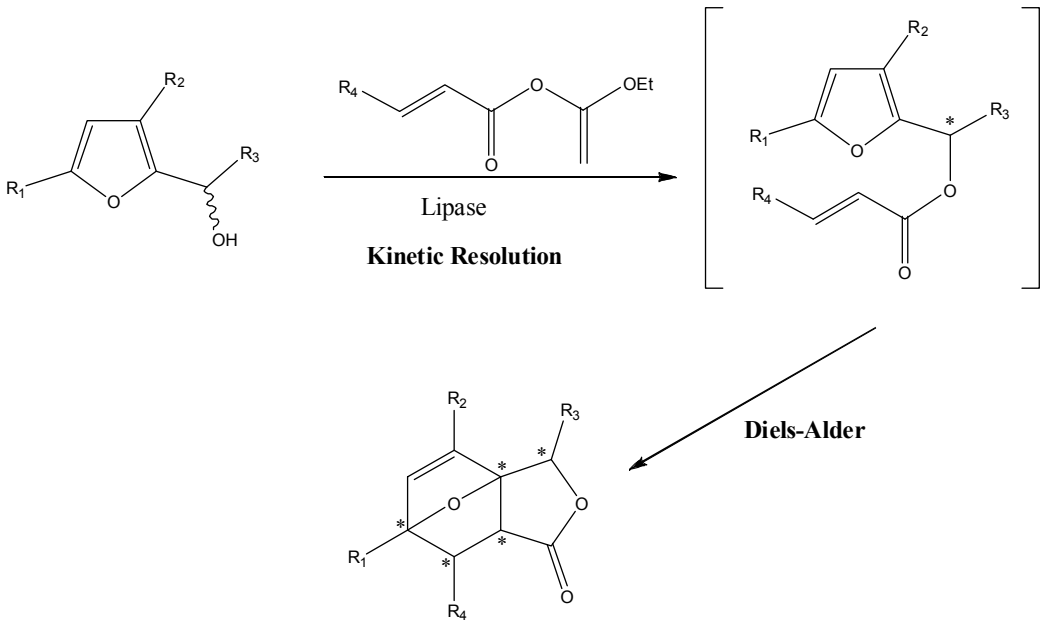



**Figure 25.** Lipase catalyzed-domino reaction in the one-pot synthesis of optically active 7-oxabicyclo[2.2.1]heptenes (* represents chiral center).[118,119]

2.3.2.2 Enzyme-Triggered Rearrangement

Skeleton rearrangements are a special class of reactions in organic synthesis because they often lead to products of exceptional structure. β-Glucosidase has been reported to initiate rearrangement of multifloroside by the Shen group [121]. Multifloroside was subjected to β-glucosidase in acetate buffer. The domino process started by enzymatic cleavage of a glycoside, and then a rearrangement subsequently took place to generate jasmolactone analogues as the final products in a rather low yield (10-20%) (Figure 26).

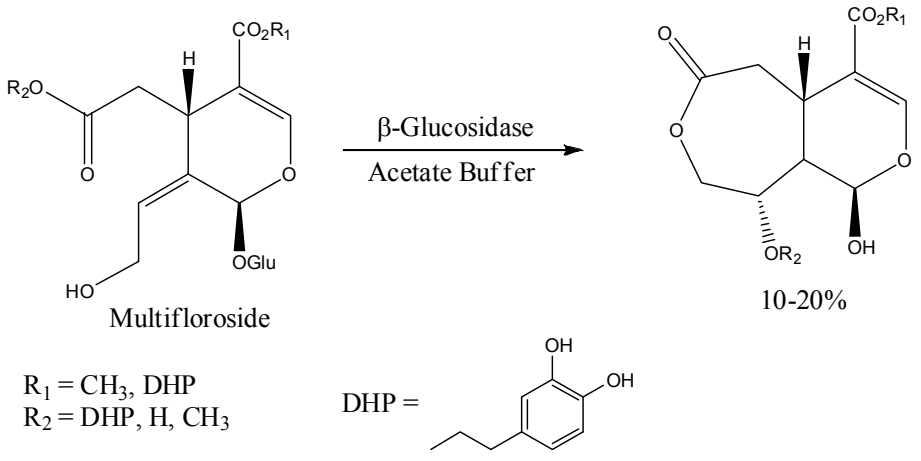
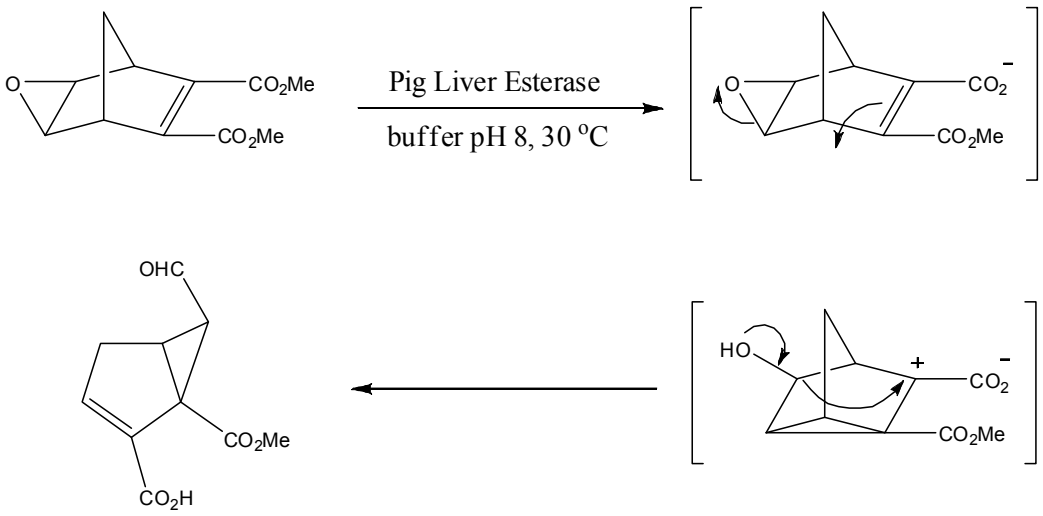



**Figure 26.** β-Glucosidase-triggered rearrangement of multifloroside in aqueous medium.[121]

An unusual enzyme-triggered asymmetric rearrangement was observed by Ohno and his co-workers when they attempted to hydrolyze the asymmetric tricyclic diester in an asymmetric fashion using porcine liver esterase [122]. First, a hemiester was form by hydrolysis and then immediately underwent a Meinwald rearrangement to furnish the final bicycle[3.1.0]hexane framework (Figure 27).




100%, ee = 48%

**Figure 27.** The synthesis of bicycle[3.1.0]hexane compound *via* enzyme-triggered Meinwald rearrangement.[122]

During the development of a new method for the synthesis of paclitaxel, an unexpected enzymatic dehydration-initiated rearrangement was discovered by Kim et al. [123]. The 7-triehtylsilyl derivative of 10-deacetylbaccatine III served as a precursor for this cascade reaction (Figure 28). In the presence of trichloroacetic anhydride as the acyl donor, this precursor was acylated by *Rhizopus delemar* lipase at the 13-hydroxy group, and underwent the dehydration-rearrangement to form the tricyclic diterpene intermediate. After a prolonged reaction time, the intermediate underwent a second dehydration to form the final product.

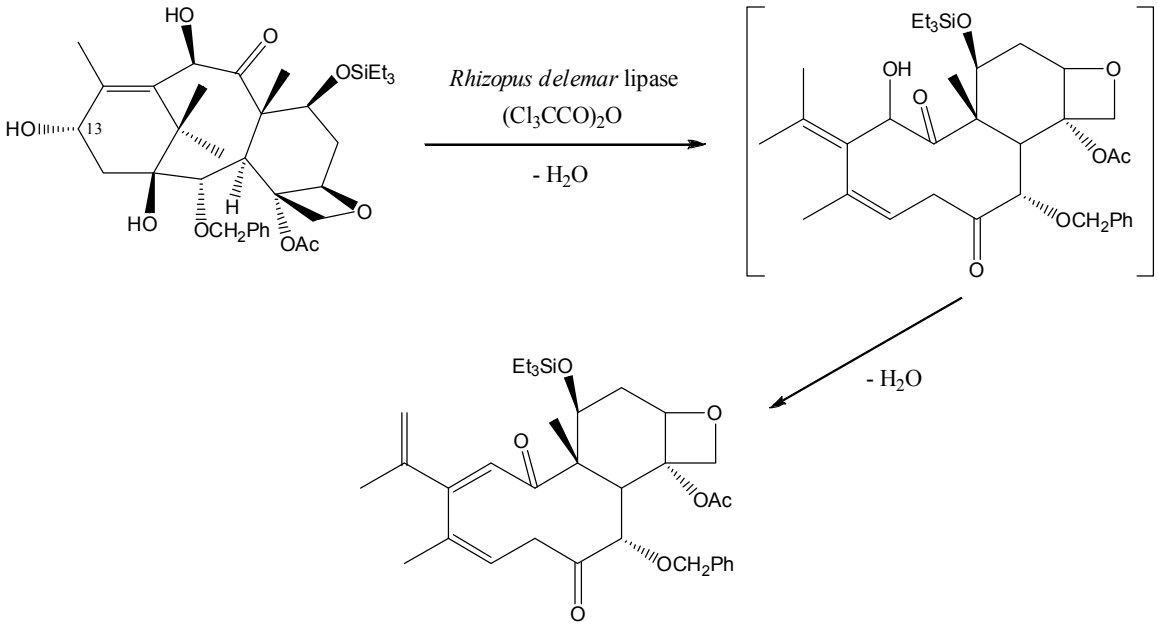



**Figure 28.** Enzymatic dehydration-initiated Rearrangement of paclitaxel precursors.[123]

2.3.2.3 Enzyme-Triggered Fragmentation

The Schaap group [124] presented the use of aryl esterase to catalyze the cleavage of a naphthyl acetate-substituted dioxetane in aqueous buffer at ambient temperature. The 1,2-dioxetane moiety of the naphtyl acetate was cleaved via hydrolysis by porcine liver esterase, thus generating the free intermediate naphtholate anion which subsequently underwent fragmentation reaction to form the naphthol methyl ester and admantone with the concurrent chemiluminescence (Figure 29).

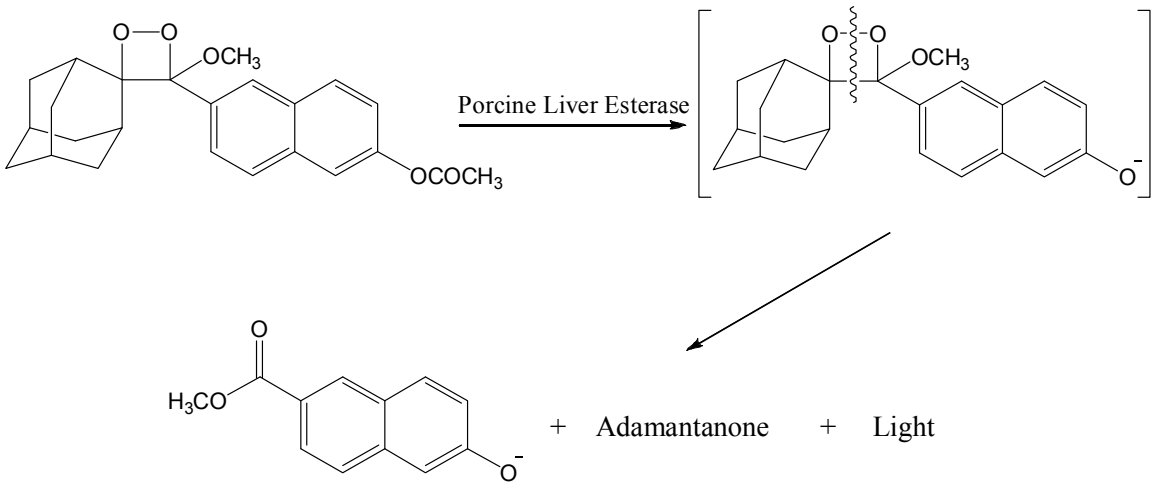



**Figure 29.** Ester hydolysis-initiated dioxetane fragmentation.[124]

During the synthesis of *N-Ras* lipopeptides, Waldmann et al. [125] developed a new protecting group for amino, hydroxyl, and carboxy moieties containing a *p*-acetoxybenzyloxycarbonyl group. In this study, lipase was first used to cleave the acetate group of the *p*-acetoxybenzyloxycarbonyl moiety to liberate the phenolate anion. Then, this intermediate anion underwent a fragmentation to generate a quinone methide with liberation of the desired products. This strategy was also applicable to solid-phase synthesis. The aromatic moiety that was to build the scaffold was linked on to a macroscopic polymeric carrier via a spacer-arm which acted as an enzymatically labile anchoring group [126]. This method is useful for combinatorial chemistry and parallel synthesis for the production of compound libraries attached to polymeric supports.

2.3.2.4 Enzyme-Triggered Intramolecular Substitution Affecting Cyclization

Enzyme-triggered intramolecular substitution affecting Cyclization reactions normally start with the enzymatic hydrolysis of an ester or epoxide to form the

hydroxylate or hydroxyl group which acts as a nucleophile to attack an electrophile *via* intramolecular $S_N2$ reaction in the second step. As in the work of Tamm et al., they conducted the asymmetric hydrolysis of *meso*-epoxy diester using porcine liver esterase in aqueous medium (Figure 30) [127]. In this cascade reaction, carboxylate anion was liberated by enzymatic hydrolysis of the more accessible (equatorial) carboxy ester. This carboxylate anion acted as nucleophile and attacked the epoxide moiety to generate the corresponding hydroxyl γ-lactone. Due to a conformation change of the intermediate during lactone formation, the remaining axial ester moiety was converted into the more accessible equatorial ester which could be additionally hydrolyzed by the esterase. This led to the formation of the final chiral product in 96% ee.

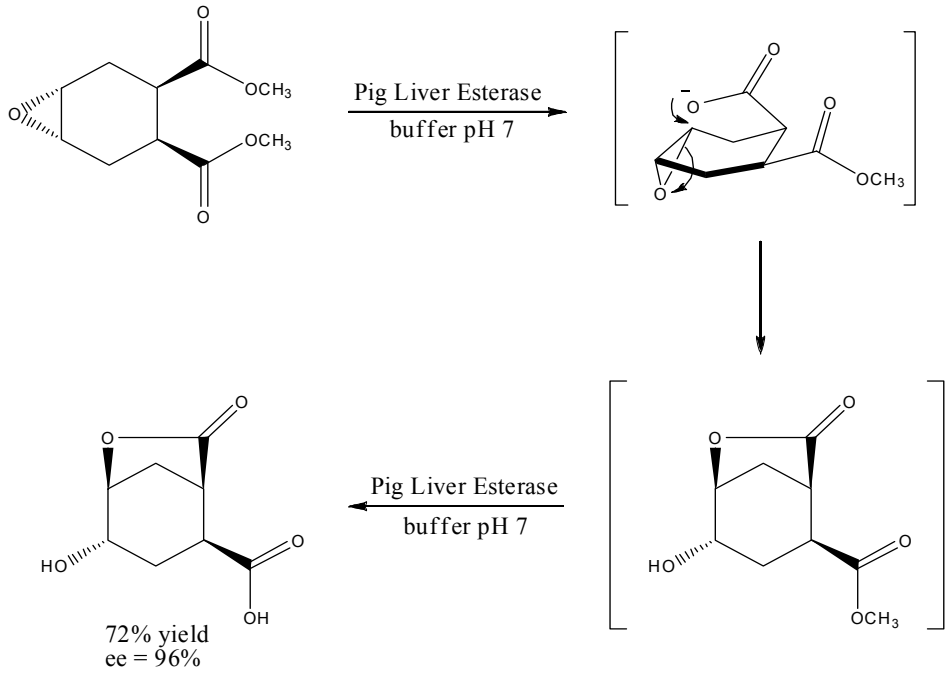



**Figure 30.** Enzymatic liberation of carboxylate anoin for the formation of γ-lactone.[127]

Another example of enzymatic hydrolysis of ester to liberate the carboxylate anion was reported by Williams et al [128]. In this study, the diepoxide underwent bis-cyclization by the pig liver esterase, with stereospecific opening of each epoxide ring in a 5-exo-tet manner to form the final product. The reaction mechanism is summarized in Figure 31.

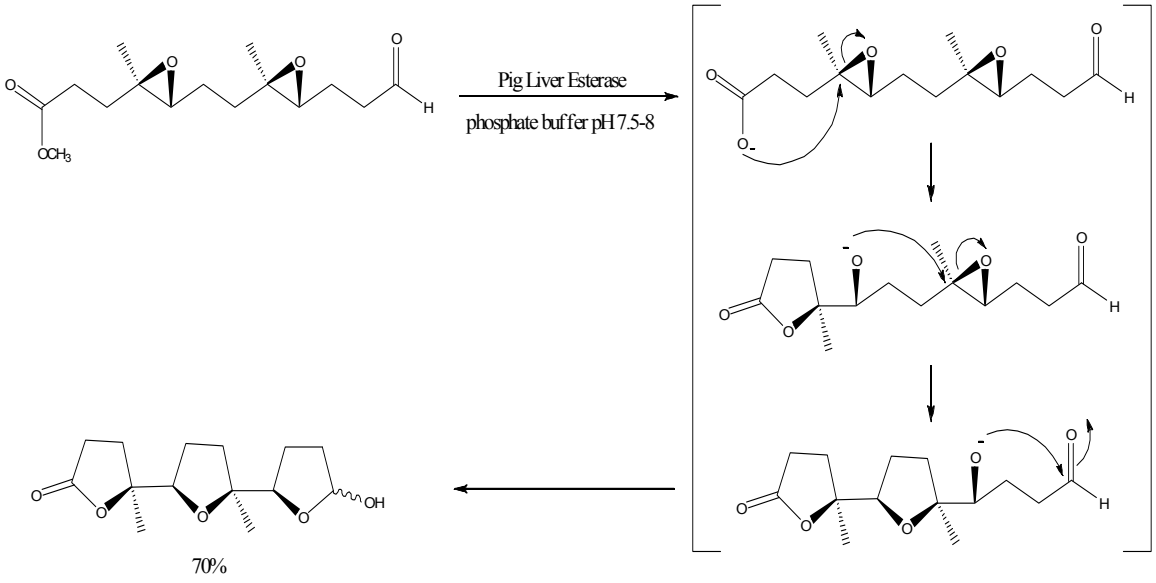



**Figure 31.** Enzyme-initiated a tree-step $S_N2$ cascade reaction of the diepoxide compound.[128]

An alcoholic group generated from the enzymatic hydrolysis of ester or epoxide can also act as nucleophile in a cascade reaction. For example, the ester moiety of a diasteromeric mixture of (±)-epoxy ester was hydrolyzed by a crude immobilized enzyme preparation (NOVO SP 409), or whole lyophilized cells of *Rhodococcus reythropolis* NCIMB 11540 to generate the corresponding intermediate alcohol (Figure 32). The alcohol immediately opened the epoxide in an $S_N2$ fashion to furnish the corresponding diastereomeric tetrahydrofuran derivatives [129].

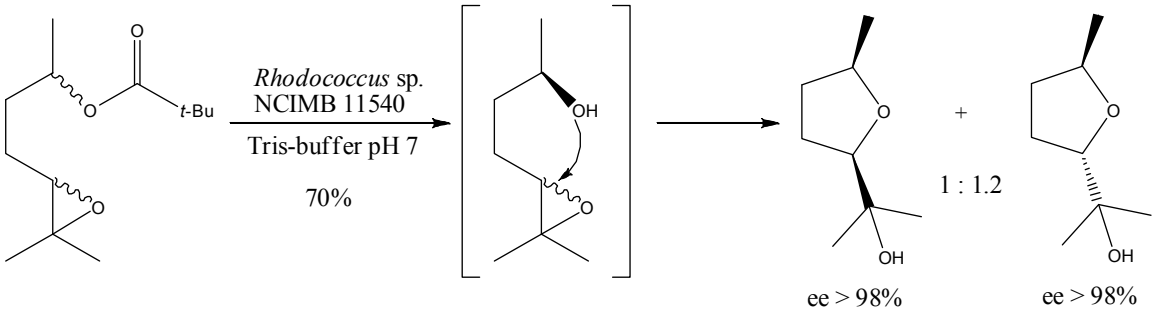



**Figure 32**. Cyclisation of a diasteromeric mixture of (±)-epoxy ester initiated by enzymatic generated hydroxyl group.[129]

In the following example, the diol nucleophile was generated by emzymatic hydrolysis of an epoxide to initiate a cascade reaction. For instance, the biohydrolysis of (±)-2,3-disubstituted *cis*-chloroalkyl-epoxides (Figure 33) [130]. First, bacterial epoxide hydrolases (*Mycobacterium paraffinicum* NCIMB 10420) hydrolyzed the racemic epoxide to form the corresponding diol which underwent spontaneous ring closure to yield the final cyclic product. This synthetic strategy has been used in asymmetric synthesis of many bioactive compounds [131-133].

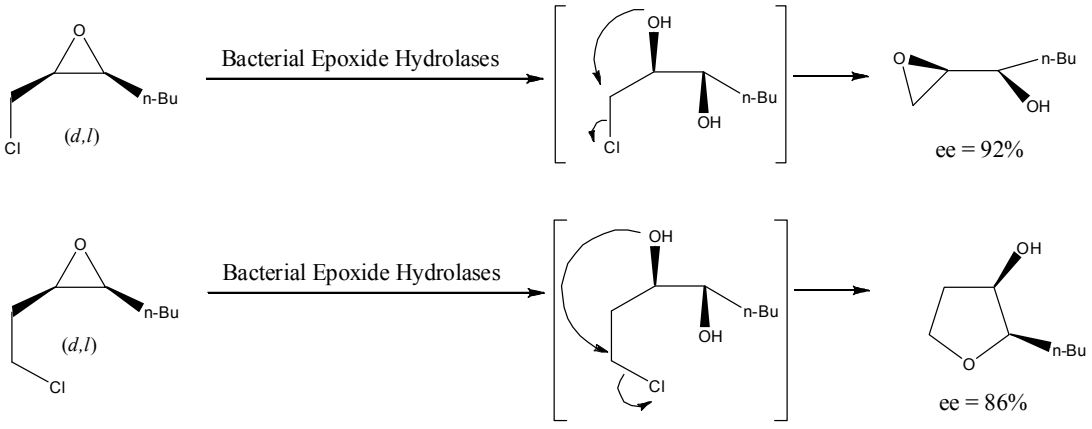



**Figure 33.** Epoxide hydrolases-initiated cyclisation of haloalkyl-oxiranes.[130]

The enzyme triggered cyclisation of bis-epoxides using bacterial epoxide hydrolase was investigated by Faber and his co-workers [134]. In this study, the tetrahydrofuran products were generated through two secondary pathways as illustrated in Figure 34. The products contain four stereogenic centers which constitute the central core of bioactive *Annonaceous* acetogenins.

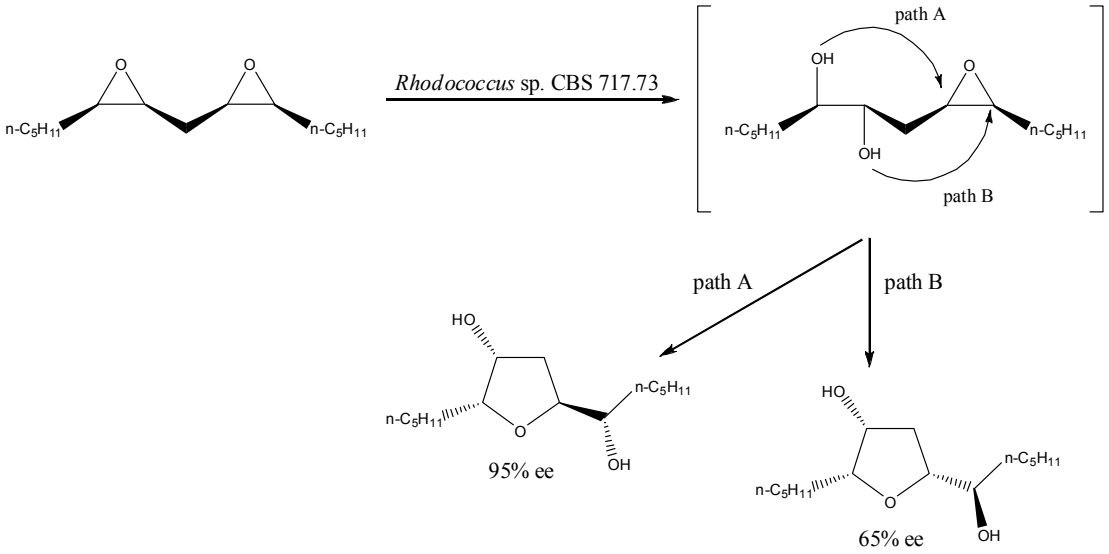



**Figure 34.** Enzyme-triggered transformation of *meso*-bis-epoxides.[134]

2.3.2.5 Enzyme-Triggered Other Type of Reactions

In 2005, the Kita group [135] developed a lipase-catalyzed domino kinetic resolution of α-hydroxynitrone intramolecular 1,3-dipolar cycloaddition reactions which successfully applied in the asymmetric total synthesis of (-)-rosmarinecine (Figure 35).

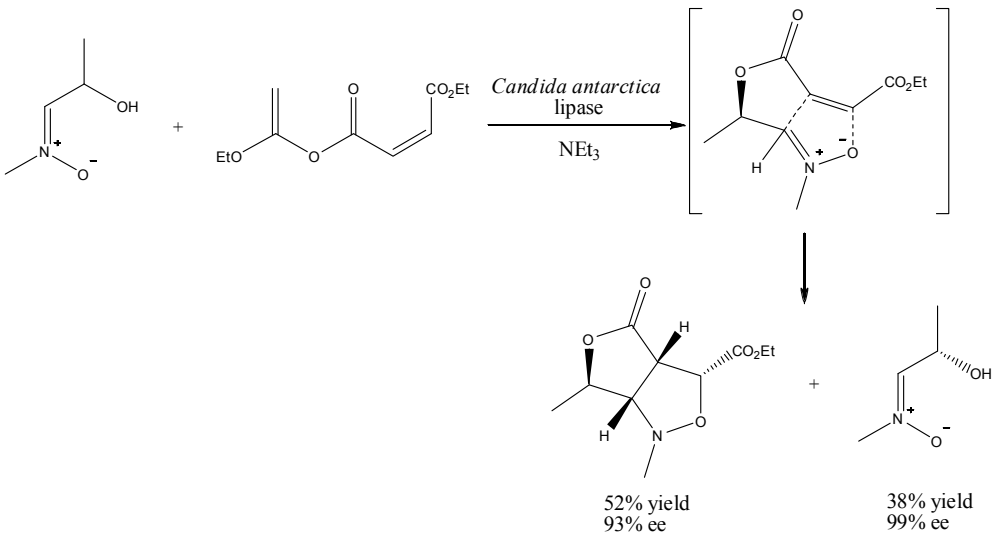



**Figure 35.** Enzyme-catalyzed intramolecular 1,3-dipolar cycloaddition reaction.[135]

Another recent development reported by Faber et al. [136] is a biocatalytic hydrogen-transfer reduction of halo ketones into enantiopure epoxides. The enzyme used in this study is either *Rhodococcus ruber* as lyophilized cell catalyst or an alcohol dehydrogenase prepared from *Pseudomonas fluorescens* DSM 50106 (PF-ADH).

2.3.2.6 Multienzymatic One Pot Reactions

The use of a multienzyme to catalyze organic reactions is an interesting approach in the application of domino reactions. There is no limit to the number of enzymes that can be used in a single reactor to produce a complex structure in a domino fashion. Since 1990, many studies have been reported on the use of multienzyme cocktails in the synthesis of many natural products including the synthesis of β-D-glucuronides [137], 2'-deoxy-N-acetyllactosamine [138], sialyl oligosaccharides [139], precorrin-5 [140],

sialylated antigen T-epitope [141], fluoroshikimic acids [142], cefazolin [143,144], and aromatic D-amino acid [145].

Sheldon and his co-workers [146] reported a two step, one pot enzymatic synthesis of cephalexin from D-phenylglycine nitrile in 2002. Two enzymes which are nitrile hydratase and penicillin G acylase were used in this approach. First, the D-phenylglycine was hydrated by nitrile hydratase to form the corresponding amide which subsequently underwent acylation reaction with 7-aminodesaacetoxycephalosporanic acid (7-ADCA) by penicillin G acylase to generate cephalexin (Figure 36).

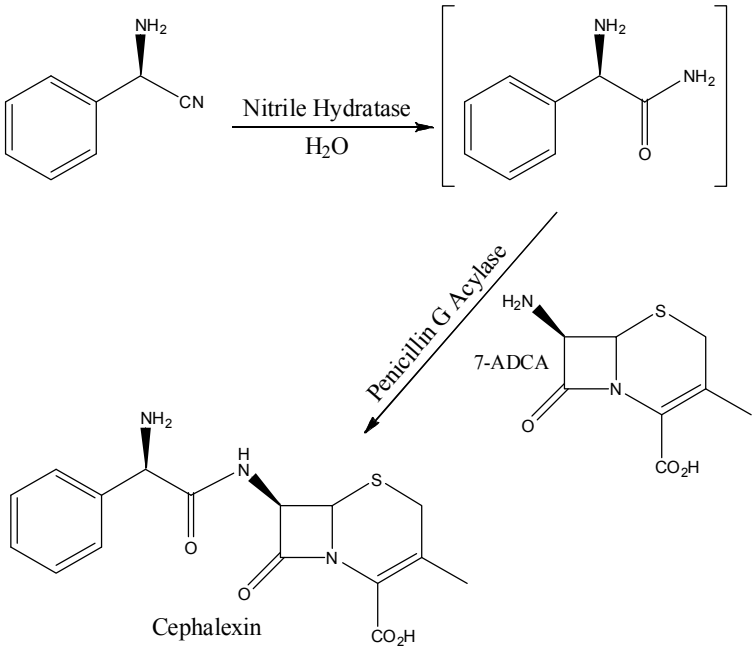



**Figure 36.** Two enzymetic reactions for the synthesis of cephalexin.[146]

Wong et al. [147] developed the four enzyme system for the synthesis of L-fructose. In this study, L-glyceraldehyde was produced in situ from glycerol in the

presence of galactose oxidase, catalase, rhamnulose-1-phosphate aldolase, and acid phosphatase (Figure 37).

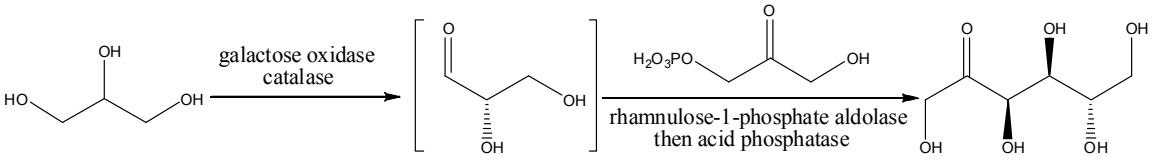



**Figure 37.** Four enzyme system for domino synthesis of L-fructose.[147]

Most recently, Kroutil et al. [148] reported the one pot, two step, two enzyme cascade reaction for the synthesis of enantiopure epoxide. In this study, enantiopure (R)- and (S)-epoxides were obtained by the reaction which combined either (R)- or (S)-selective alcohol dehydrogenase with a non-selective halohydrin dehalogenase. First, the pro-chiral α-chloro ketone was streoselectively reduced to the halohydrins as an intermediate by alcohol dehydrogenase, and then the intermediate was converted to epoxide by a non-enantioselective halohydrin dehalogenase (Figure 38).

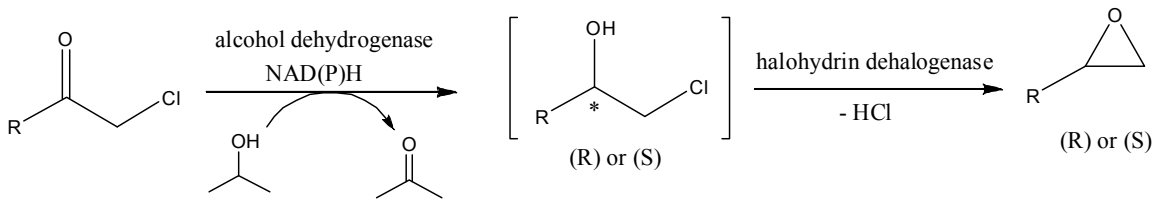



**Figure 38.** Two enzyme system for the synthesis of enantiopure epoxide.[148]

## 2.4 Laccase

### 2.4.1 Distribution in Nature

Laccase (EC 1.10.3.2, *p*-diphenol:oxygen oxidoreductase) is an enzyme belonging to the family of multicopper blue oxidase which typically found in plant and fungi. Laccase can catalyze the oxidation of a variety of compounds including ortho and para-diphenols, polyphenols, aminophenols, polyamines, lignins, aryldiamines, and a number of inorganic ions, while reducing molecular dioxygen to water [12,149-152].

Laccase was first discover by Yoshida in 1883 in the sap of lacquer tree *Rhus vernicifera* [153] and the enzyme has been characterized in great detail later in 2001 by Huttermann et al. [154]. However, the report of laccase in other plant species is more limited and partially characterized. These laccases include laccases form *Rhus succedanea* [155], *Acer pseudoplatanus* [156], *Pinus taeda* [157,158], *Populus euramericana* [159], *Liriodendron tulipifera* [160], *Nicotiana tobacco* [161], *Lolium perenne* [162], and *Zea mays* [163]. In plant, laccase participates in the formation of polymer lignin *via* radical-based mechanisms [156,164,165].

A few year later after the discovery of the plant laccase by Yoshida, fungal laccases were discovered by Bertrand in 1896 [166]. The majority of laccases characterized so far were isolated from fungi, and the reports of their presence in more and more fungal species have been published [167,168]. Up to now, more than 100 laccases have been purified from fungi, and laccase from the wood-rotting white-rot basidiomycetes were the most enzyme purified. The wood rotting fungi that produce laccase are *Trametes versicolor*, *T. hirsute* (*C. hirsutus*), *T. ochracea*, *T. villosa*, *T. gallica*, *Cerrena maxima*, *Coriolopsis polyzona, Lentinus tigrinus*, *Pleurotus eryngii*, etc.

Laccases have several roles in fungi including lignin degradation, morphogenesis, fungal plant-pathogen/host interaction, and stress defence [8,167,168].

There are also some reports about laccase activity in bacteria [169,170]. Moreover, proteins with features typical of laccases have recently been identified in insects [171].

### 2.4.2 Laccase Structure

Laccases are glycoproteins which often occur as isoenzymes that oligomerize to form multimeric complexes. The molecular weight of the monomer ranges from about 50 to 130 kD. The carbohydrate moiety of laccases consisting of mannose, N-acetylglucosamine, and galactose ranges from 10 to 45% of the protein mass in laccases. This carbohydrate moiety is believed to be responsible for the stability of the enzyme [3,152].

For the catalytic activity, the active site of laccases contains four copper atoms which are one type-1 (T1) copper and a tree-nuclear cluster (T2/T3) consisting of one type-2 (T2) and two type-3 (T3) coppers. T1 copper atom is located at the distance of about 12 Å from the T2/T3 site, and T2 copper atom is located at the distance of about 4 Å from T3 copper atoms [172-174]. The T1 copper has a trigonal coordination with two histidine and one cysteine, and the axial ligand of T1 is methionine in the bacterial (CotA) [173] and leucine or phenylalanine in fungal laccases. The T1 copper confers the typical blue color to multicopper proteins due to the strong absorption around 600 nm. This intense absorption caused by the covalent copper-cysteine bond. Moreover, type-1 copper is the site where substrate oxidation takes place because of its high redox potential

of ca. +790 mV. Type-2 copper is coordinated by two histidines and type-3 coppers are coordinated by six histidines. Type-2 copper shows only weak absorption in the visible region and reveals paramagnetic properties in electron paramagnetic resonance (EPR) studies. While type-3 coppers, a binuclear copper site with copper paired antiferromagnetically through a hydroxyl bridge, exhibit the absence of an EPR signal. The T3 site can be characterized by electron absorption at 330 nm (oxidized form) [155,175,176]. In addition, the trinuclear cluster (T2/T3 site) is where the reduction of molecular oxygen and release of water takes place. Figure 39 illustrated a scheme of active site of laccase CotA from *Bacillus subtilis*.

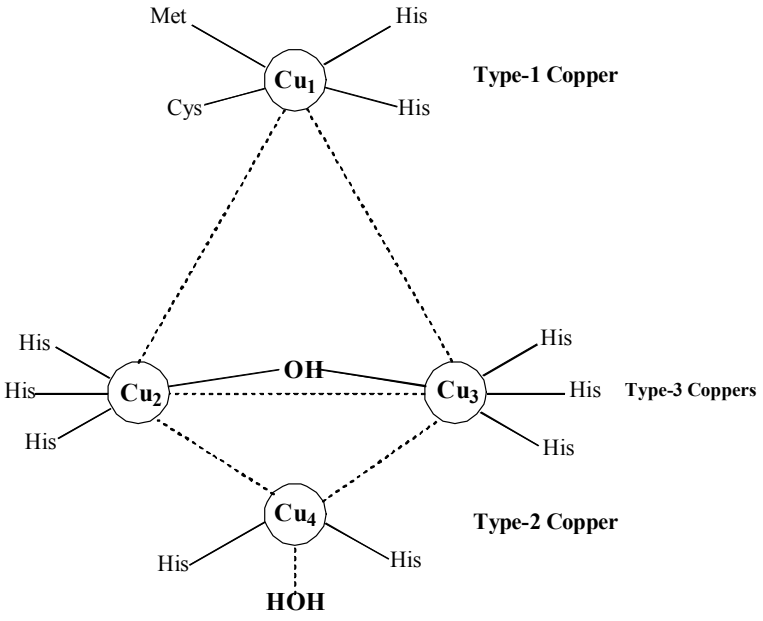



**Figure 39.** Active site of laccase CotA from *Bacillus subtilis* (adapted from Enguita et al. [173]).

Up to now, the three-dimensional structure [177] have been determined for five fungal laccases from *Coprinus cinereus* (with the T2 copper removed) [178], *Trametes*

*versicolor* [179,180], *Pycnoporus cinnabarinus* [181], *Melanocarpus albomyces* [182] and *Rigidoporus lignosus* [174]. Moreover, the three-dimensional structure of laccase CotA from endospores of *Bacillus subtilis* has also recently been published [173,183].

### 2.4.3 Catalytic Mechanism and Properties

Laccase catalysis is proposed to comprised three major steps [155,184,185]:

1. Type-1 copper is reduced by accepting electrons from the reducing substrate.
2. Electrons are transferred ~13 Å from type-1 copper to the trinuclear T2/T3 cluster.
3. Molecular oxygen is activated and reduced to water at the trinuclear T2/T3 cluster.

Figure 40 shows the catalytic cycle of laccase showing the mechanism of four-electron reduction of a dioxygen molecule to water at the enzyme copper sites [186]. Dioxygen molecule interacts with the completely reduced trinuclear cluster (T2/T3) via a $2e^-$ process ($k \approx 2 \times 10^6\ M^{-1}s^{-1}$) to produce the peroxide intermediate which contains the dioxygen anion [187]. One oxygen atom of the dioxygen anion bound with the T2 and T3 copper ions and the other oxygen atom coordinated with another copper ion of T3. Then, the peroxide intermediate undergoes a second $2e^-$ process ($k > 305\ s^{-1}$) [172], and the peroxide O-O bond is splitted to produce a native intermediate which is a fully oxidized form with the three copper centers in the trinuclear site mutually bridged by the product of full $O_2$ reduction with at least one Cu-Cu distance of 3.3 Å. This native intermediate form of lacccase was confirmed by the combination of Cu K-edge x-ray spectroscopy (XAS) and magnetic circular dichorism (MCD) studied by Solomon et al. [150].

Moreover, a combination of model studies and calculations has further demonstrated that the three copper centers in the trinuclear cluster are all bridged by a $\mu_3$-oxo ligand [188]. This structure has a single $\mu_3$-oxo ligand bridging all three coppers at the center of the cluster, with the second oxygen atom from $O_2$ either remaining bound or dissociated from the trinuclear site as shown in the native intermediate structure in Figure 40. This $\mu_3$-oxo bridged structure of the native intermediate provides a relatively stable structure that serves as the thermodynamic driving force for the $4e^-$ process of $O_2$ reduction, and also provides efficient electron transfer (ET) pathways from T1 site to all of the copper centers in the trinuclear cluster [188]. This efficient ET pathways lead to the fast reduction of the fully oxidized trinuclear cluster in the native intermediate to generate the fully reduced site in the reduce form for further turnover with $O_2$. The native intermediate can slowly convert to a completely oxidized form called "resting" laccase which has the T2 copper isolated from the couple-binuclear T3 centers. The decay of the native intermediate to the resting enzyme proceeds via successive proton-assisted steps as illustrated in Figure 41 [189]. The first proton binds at $\mu_3$-oxo center and then the second proton binds at T3 $OH^-$ bridge. Finally, the three copper centers in the trinuclear cluster are uncoupled to form the resting form of laccase. The slow decay of the native intermediate is due to the rearrangement of the $\mu_3$-oxo-bridge, the rate limiting step, from inside to outside of the cluster. The T1 site of this resting laccase can be reduced by a substrate. However, the electron-transfer rate onto the trinuclear cluster (T2/T3) is too low to be significant for catalysis [150,155].

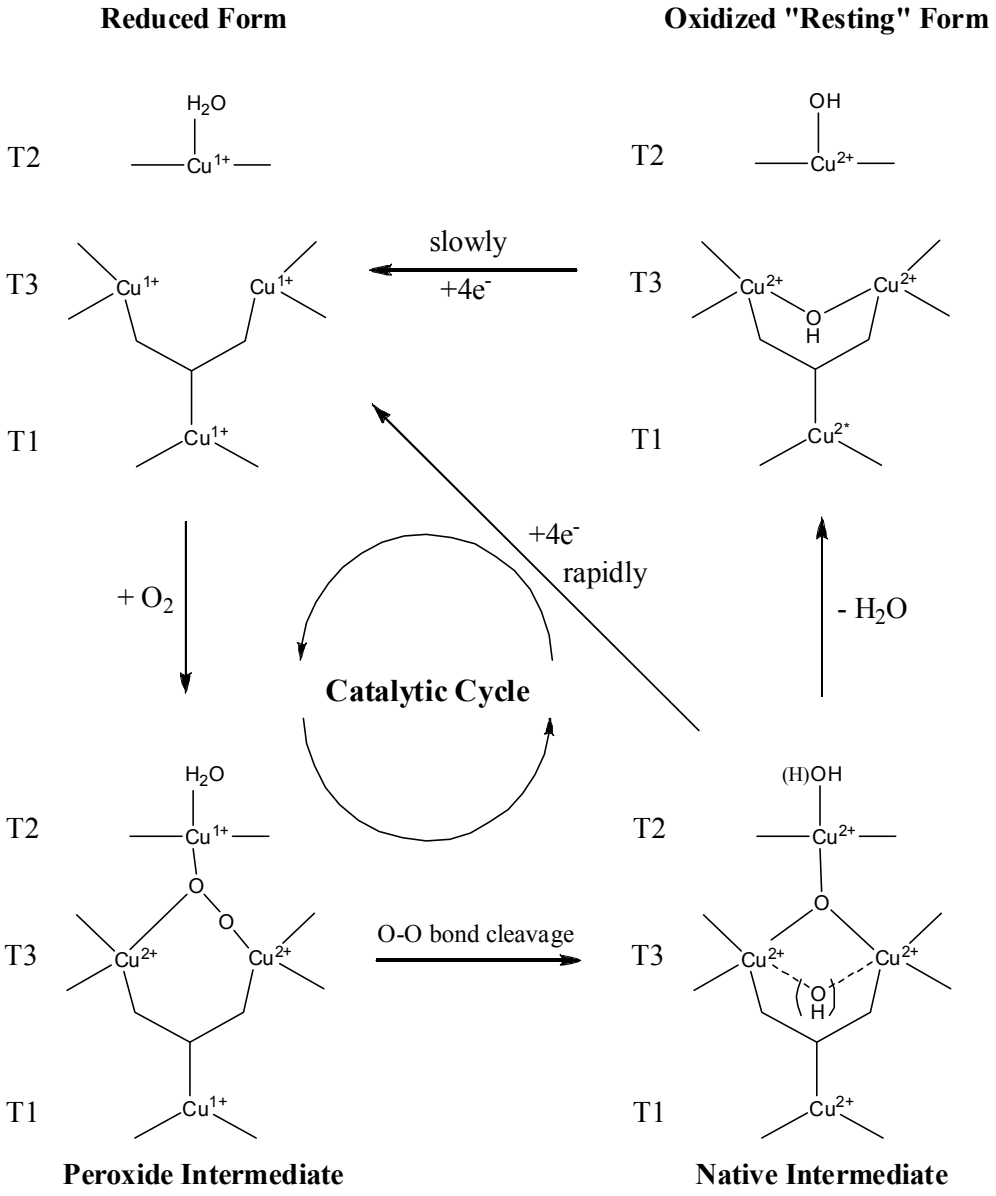



**Figure 40.** Catalytic cycle of laccase showing the mechanism of four-electron reduction of a dioxygen molecule to water at the enzyme copper sites (adapted form Shleev et al. and Solomon et al. [186,188]).

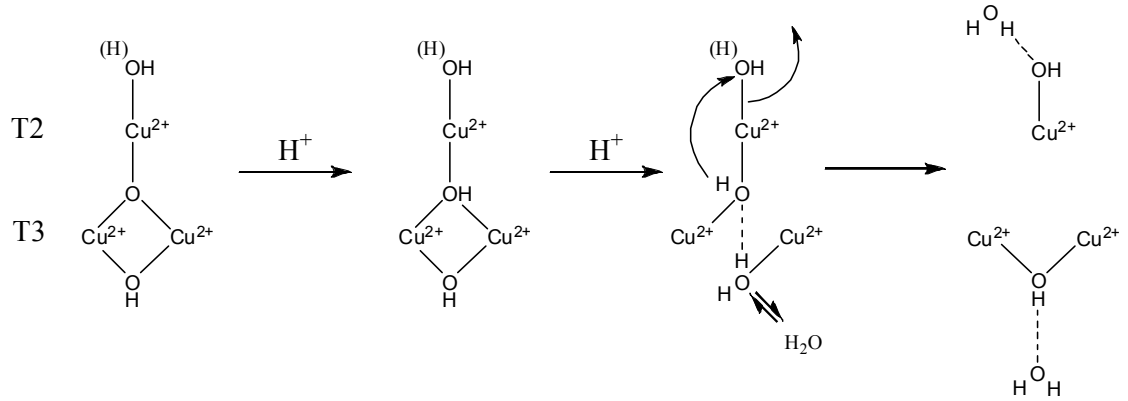



**Figure 41.** Proposed decay mechanism of the native intermediate to the resting laccase.[189]

Laccase can catalyze the oxidation of a variety of compounds including ortho and para-diphenols, polyphenols, aminophenols, polyamines, lignins, aryldiamines, and a number of inorganic ions [12,149-152]. Laccase will abstract an electron from substrates which produces a free radical, and reduce oxygen to water. The simplify scheme of laccase-catalyzed redox cycles for substrate oxidation and the example of the oxidation of hydroquinone by laccase are illustrated in Figure 42.

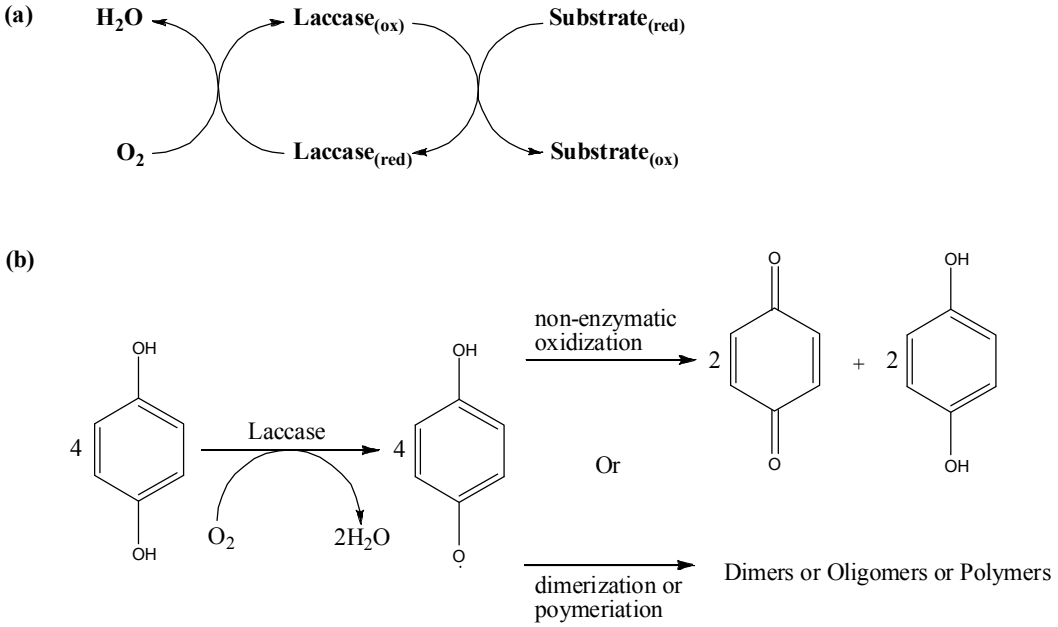



**Figure 42.** (a) Scheme of laccase-catalyzed redox cycles for substrate oxidation; (b) The example of the oxidation of hydroquinone by laccase.

Fungal laccases typically exhibit pH optima in the range from 3.5 to 5.0 when the substrates are hydrogen atom donor compounds, and the pH-dependence curve is bell-shaped [190-197]. The optimum pH for phenolic compounds can actually increase at higher pH to a limit. The limit for increasing the pH during substrate oxidation results from the balance between the redox potential difference between the substrate and the inhibition of the T2/T3 copper site by the binding of $OH^-$ ion [198,199]. The pH optimum of plant laccases for substrates that are donors of hydrogen atoms was different from that of fungal laccases. For example, laccase from Rhus vernicifera exhibited maximal activity in neutral and weak alkaline solution [198].

The optimal temperature of laccases usually do not differ from other extracellular ligninolytic enzymes with in the range from 50º to 70 ºC [168]. However, there are a few

enzymes with the optima below 35 ºC such as the laccase from G. lucidum with the highest activity at 25 ºC [200].

A wide spectrum of compounds has been described to inhibit laccase. These inhibitors include small inorganic anions such as azide, cyanide, fluoride and hydroxide. These ions bind with the T2/T3 site and this prevents the electron transfer from T1 site onto T2/T3 site and inhibits the enzymatic activity [198,201]. Other inhibitors such as metal ion ($Hg^{+}$), fatty acids, quaternary ammonium detergents, have been shown to either replace or chelate the copper centers, or de nature the protein [149].

### 2.4.4 Laccases in Organic Synthesis

Due to the catalytic and electrocatalytic properties of laccases, laccases have received much attention from researcher in last decades as well as have shown the potential of their wide application in several industrial and biotechnological processes [21,152]. Moreover, laccases also pose the possibility of their application in fine organic synthesis because of their ability to oxidize a variety of compounds [4]. The redox potential of laccase is in the range of 0.5 to 0.8 mV (vs. normal hydrogen electrode [NHE]) [198]. In the reactions where the substrate to be oxidized has a higher redox potential than laccase or the substrate is too large to penetrate into the enzyme active site, the presence of so-called ‘chemical mediator’ may be required to facilitate the reaction. First, the mediator reacts with the laccase to form a strongly oxidizing intermediate. Then, this oxidized mediator interacts with the bulky or high redox-potential substrate. The mediators that are widely used such as N-hydroxybenzotriazole (HBT), 2,2’-azinobis-(3-ethylbenzylthiozoline-6-sulphate) (ABTS), Violuric acid (VA), 3-

Hydroxyanthanilic acid (HAA), and 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO) (Figure 43) [9,202]. However, this section will focus only on the laccase-catalyzed reaction in the absence of mediators.

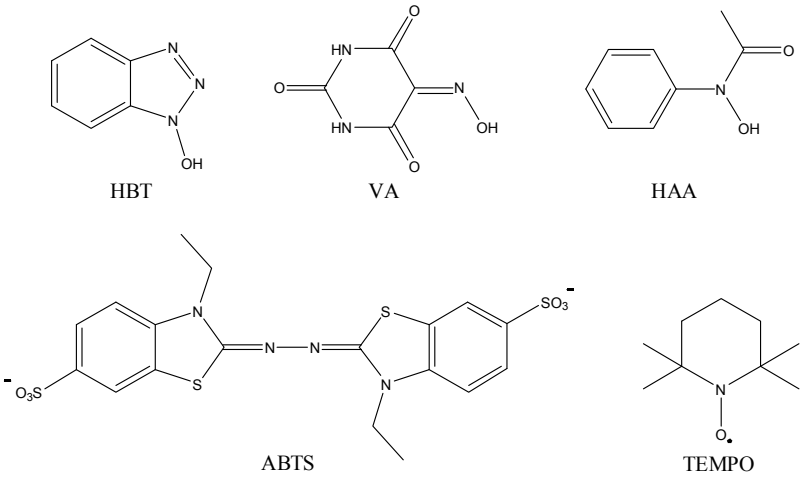



**Figure 43.** Chemical structure of laccase mediators.

2.4.4.1 Laccase-Catalyzed Oxidation Reaction

*2.4.4.1.1 Laccase-Catalyzed Transformation of Phenolic and Other Compounds*

Laccases have been reported to oxidize many phenolic compounds [198,203-207]. For example, Trejo-Hernandez and his co-workers [203] studied the use of laccase in the crude extract of the residual compost of *Agaricus bisporus* to oxidize phenolic compounds including guaiacol, 2,6-dimethoxyphenol, ventral alcohol, aniline, and phenol. All tested substrates formed insoluble products after being oxidized except for ventral alcohol that was transformed to a soluble aldehyde. The relative activity of the compost extract was 2,6-dimethoxyphenol > guaiacol > phenol > ventril alcohol >

aniline. Recently, the product of the oxidation of 2,6-dimethoxyphenol by *Rhus* laccase was determined for the first time by Wan et al. The reaction was conducted in water-organic solvent system. They found that only one product, 3,3',5,5'- tetramethoxy,1,1'-biphenyl-4,4'-diol (Figure 44), was produced [206].

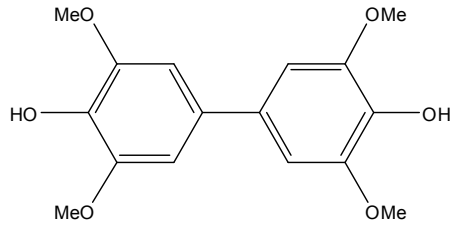



**Figure 44.** Stucture of 3,3',5,5'- tetramethoxy,1,1'-biphenyl-4,4'-diol produced by laccase catalyzed the oxidation of 2,6-dimethoxyphenol.[206]

Monolignols including isoeugenol, coniferyl alcohol, and ferulic acid have also been investigated for the laccase-catalyzed oxidation reactions. Chen and his co-workers [208] studied the oxidation of isoeugenol and coniferyl alcohol by laccase from *Rhus vernicifera* (tree) and *Pycnoporus coccineus* (fungus) in acetone-water (1:1, v/v). The rate of *Pycnoporus* laccase-catalyazed oxidation of isoeugenol and coniferyl alcohol is approximately 3 to 7 times faster than the rate of Rhus laccased-catalyzed oxidation. The rate of the oxidation depends on the nature of both monolignol and laccase (Figure 45).

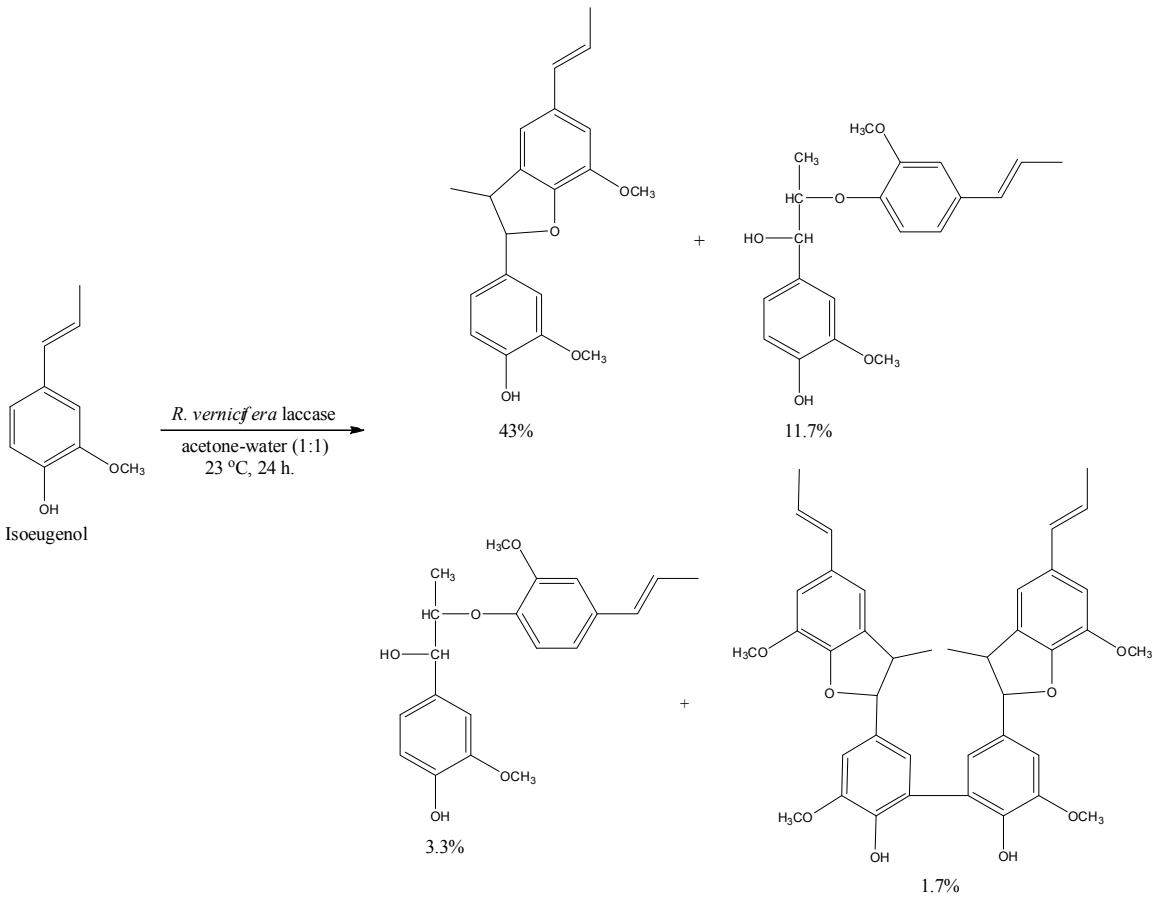



**Figure 45.** Dimer and tetramer products from the oxidation of isoeugenol alcohol by laccase.[208]

Nishida and Fukuzumi [209] examined the transformation of ferulic acid by white rot fungus, *Trametes versicolor*, in a medium containing glucose and ethanol under aerobic condition. The ferulic acid was transformed into coniferyl alcohol, coniferylaldehyde, dihydroconiferyl alcohol, vanillic acid, vanillyl alcohol, 2-methoxyhydroquinone and 2-methoxyquinone. Falconnier et al. [210] also reported the biotransformation of ferulic acid to vanillin by the white rot fungus *Pycnoporus cinnabarinus* I-937 (Figure 46).

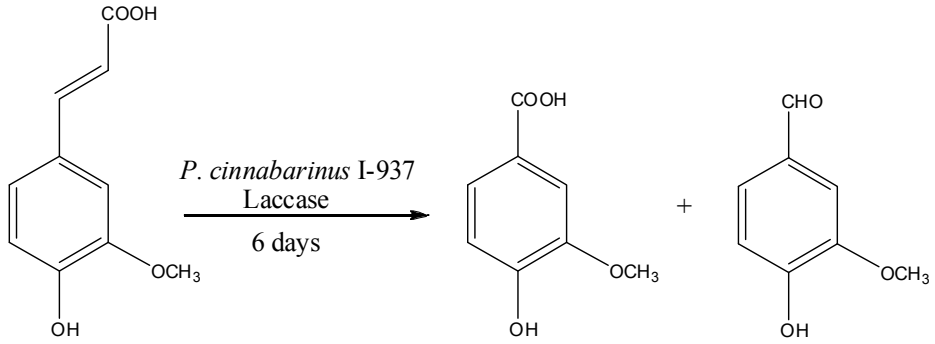



**Figure 46.** Biotransformation of ferulic acid by laccase.[210]

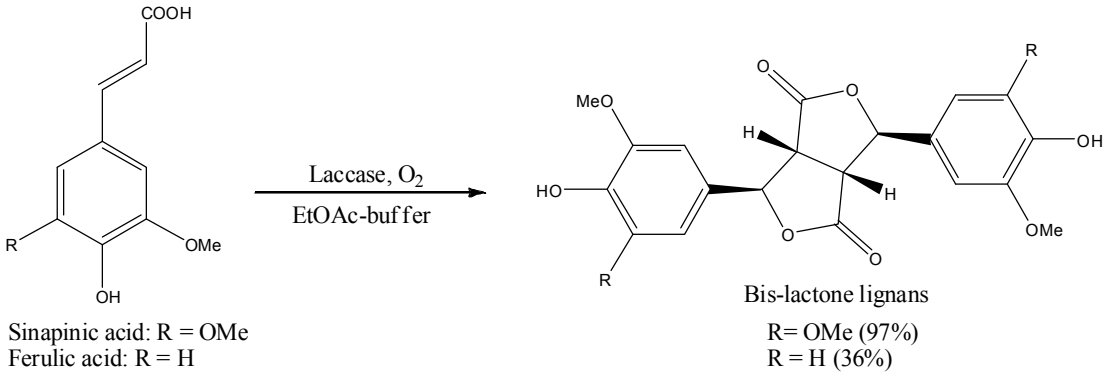



**Figure 47.** The synthesis of bis-lactone lignans.[211]

The oxidation of ferulic acid by laccase was recently used to synthesize phenolic colorants [212]. The oxidation was conducted in a biphasic hydro-organic system consisting of ethyl acetate and sodium phosphate buffer to generate the intermediate stable yellow products. This biphasic system facilitates the separation of the yellow product which were soluble only in the organic phase and prevent the polymerization of this intermediate to form brown polymer by reducing the activity of laccase in the presence of organic solvent. They suggested that this yellow color compound can be used

as food colorants. However, this yellow compound is still in progress to elucidate the structure. Moreover, the synthesis of bis-lactone lignan was reported to perform via the transformation of sinapinic acid and ferulic acid by laccase in biphasic system (Figure 47) [211] .

Azo dyes, the largest group of colorants used in industry are able to oxidize by laccase [213-215]. Renganathan and Chivukula [213] examined the oxidation of phenolic azo dyes catalyzed by laccase from *Pyricularia oryzae*. Laccase oxidized azo dyes to 4-sulfonylhydroperoxide, quinone compound, and other products (Figure 48). This study suggests that laccase oxidation can result in the detoxification of azo dyes. Most recently, Rehorek et. al. [214] reported a simultaneous combination of laccase and ultrasound treatment in acetate buffer (pH 4.5) at 40 ºC for the degradation of azo dyes such as acid oranges and direct blue dyes. The degradation process was monitor by UV-Vis spectrometry and HPLC analysis. Compare to laccase or ultrasound treatment, the stimultaneous treatment with laccase and ultrasound showed at least the same or higher degradation rates of the azo dyes. Besides the degradation of azo dyes, laccase was also reported to catalyze the formation of azo dyes by oxidative coupling between *o*-, *m*-, and *p*-methoxyphenols and 3-methyl-2-benzothiazolinene hydrazone [216].

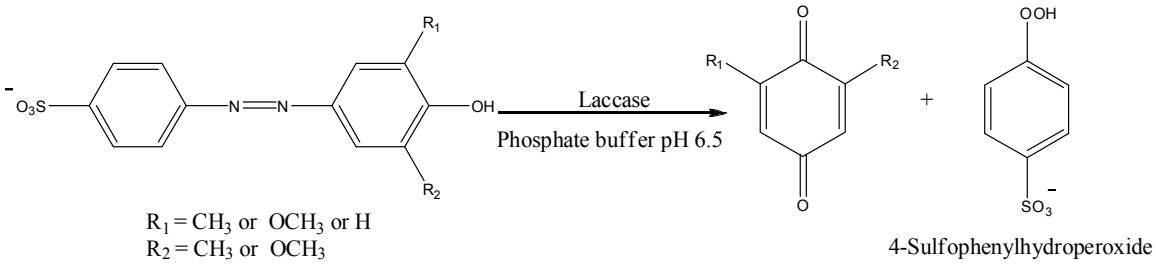



**Figure 48.** The oxidation of phenolic azo dyes by laccase.[213]

The transformation of other compounds such as steroid hormones [6,217,218], alkaloids [219], flavonols [220], procyanidin B-2 [221], and *N*-(2-alkylamino-4-phenylimidazol-1-yl)-acetamides [17] have been reported. The examples of these studies are summarized in Table 3.

**Table 3.** Some examples of laccase mediated transformation of natural compounds.

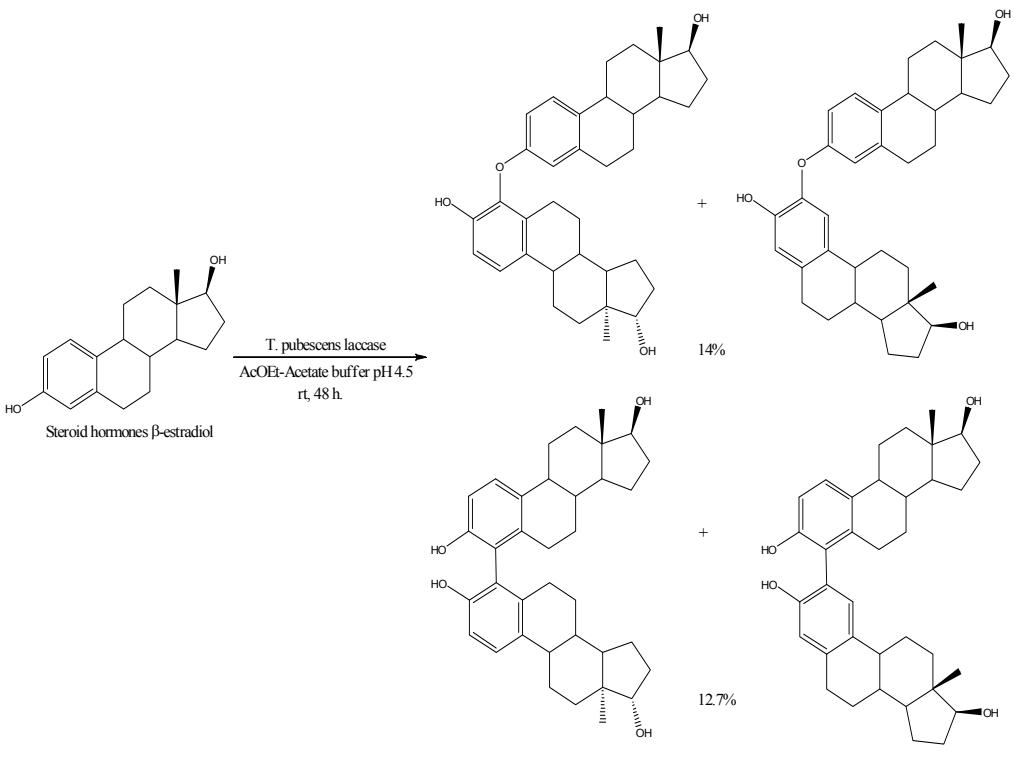
| Reaction | Reference |
|---|---|
|  | [6] |

**Table 3.** (Continued)

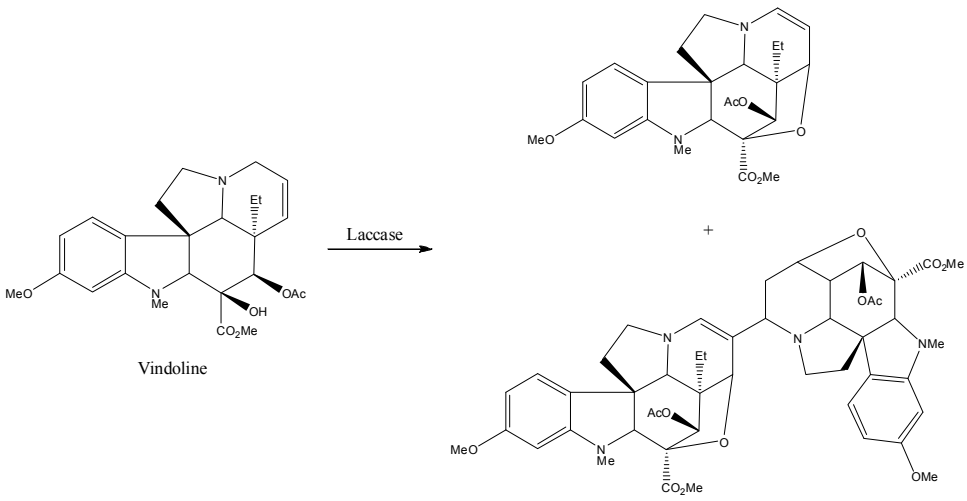
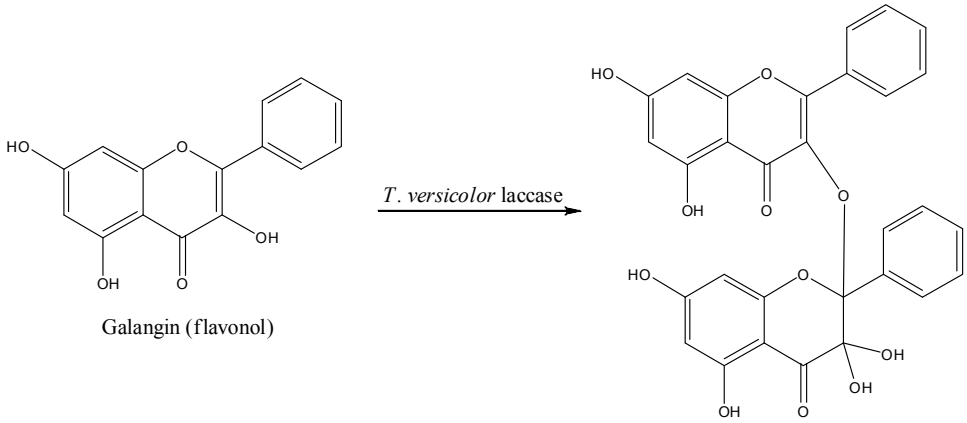
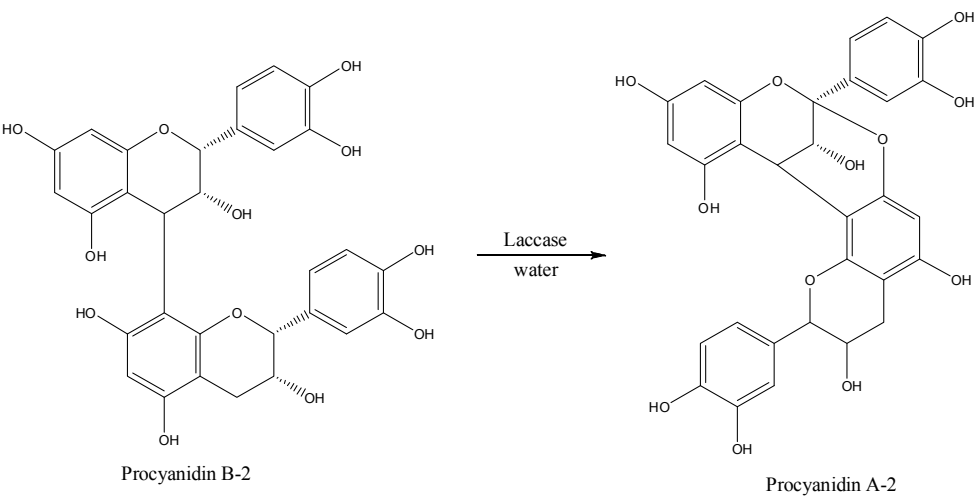
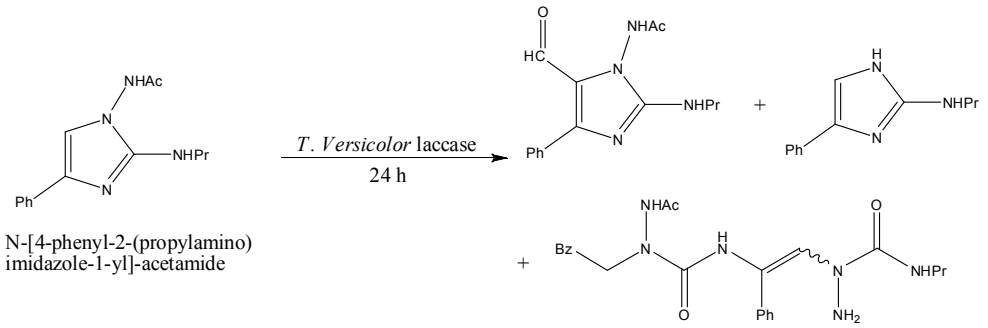
| | |
|---|---|
|  | [219] |
|  | [220] |
|  | [221] |

**Table 3.** (Continued)

| | |
|---|---|
|  <br>N-[4-phenyl-2-(propylamino) imidazole-1-yl]-acetamide | [17] |

*2.4.4.1.2 Lacccase-Catalyzed Oxidative Deprotection Reactions*

Moreover, the use of laccase in oxidative deprotection for peptide synthesis has been developed. A method to remove phenylhydrazide protecting group of both α- and γ-carboxyl group by laccase have been proposed by Semenov et. al. [222]. The deblocking method was performed under mild condition in aqueous medium and pH 7.0 in the presence of oxygen. Therefore, this deprotection method lead to non-oxidative modification without destruction of amino acid side chains. Recently, Rutjes and his co-workers [223] reported the oxidative deprotection of *p*-methoxyphenyl (PMP)-protected amines by laccase under mildly acidic condition (Figure 49). In addition, they found that the use of mediators lead to an extension of the substrate scope and increase reaction rate.

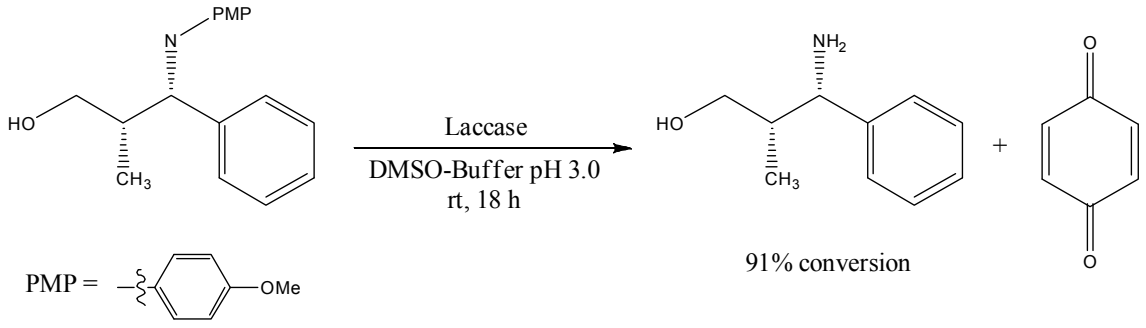
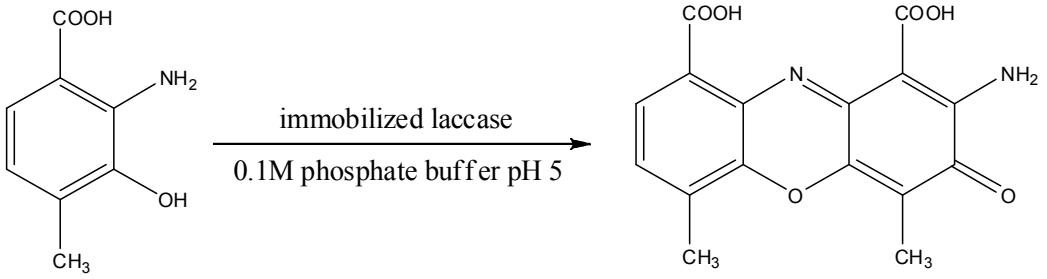



**Figure 49.** The oxidative deprotection of *p*-methoxyphenyl (PMP)-protected amines by laccase.[223]

*2.4.4.1.3 Laccase-Catalyzed Oxidative Coupling for the Synthesis of the Pharmaceutical Importance Compounds*

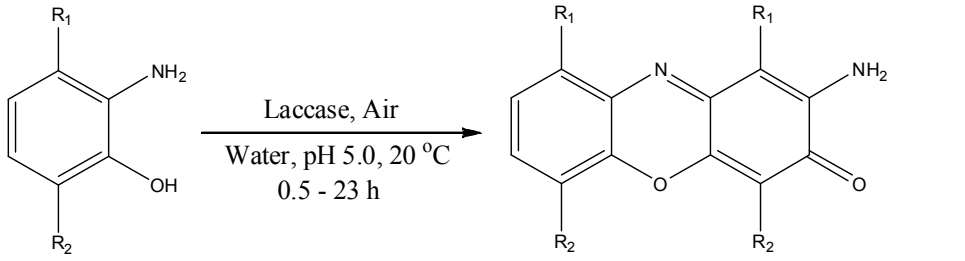
Laccase have been reported to use for the synthesis of the pharmaceutical importance compounds by oxidative coupling of the desired substrates to form the corresponding dimer products. Some of the phenoxazinone chormophores having antibiotic activity have successfully been synthesized via laccase-catalyzed oxidative coupling reactions [224-227]. The synthesis of these phenoxazinone chormophores involved the formation of aminophenoxy radicals by oxidation of *o*-aminophenols by laccase at the first step. These radicals then underwent coupling and cyclocondensation reaction to form the corresponding products. However, the reaction mechanism of this synthesis is still under investigation. For example, actinocin, chormophore of actinomycin antibiotics, was synthesized by laccase mediated oxidation of 4-methyl-3-hydroxyanthranilic acid (4-M-3-HAA) (Figure 50) [224]. Laccase used in this study was immobilized in polyacrylamide gel. The reaction proceeded successfully in aqueous medium and in 60% acetonitrile.




4-M-3-HAA Actinocin 74%

**Figure 50.** The synthesis of actinocin by laccase mediated oxidation of 4-methyl-3-hydroxyanthranilic acid.[224]

Recently, Giurg et. al. [225] reported the synthesis of 2-amino-3H-phenoxazin-3-one including actinocin, cinnabarinic acid, and questiomycin A by the catalytic oxidative cycloaddition of *o*-aminophenols. These reactions were conducted in the presence of laccase and oxygen in aqueous medium (Figure 51).




Questiomycin A: $R_1 = R_2 = H$
Cinnabarinic acid: $R_1 = COOH$, $R_2 = H$
Actinocin: $R_1 = COOH$, $R_2 = CH_3$

**Figure 51.** The synthesis of 2-amino-3H-phenoxazin-3-ones by the laccase catalyzed oxidative cycloaddition of *o*-aminophenols.[225]

The sulfonate analogue of cinnabarinic acid was recently synthesized by laccase mediated the oxidative dimerization of 3-hydroxyorthanilic acid (Figure 52) [226].

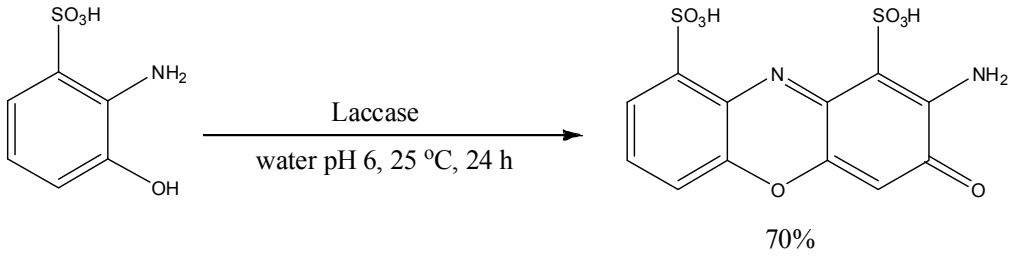



**Figure 52.** The synthesis of the sulfonate analogue of cinnabarinic acid by laccase mediated the oxidative dimerization of 3-hydroxyorthanilic acid.[226]

Forti and his co-workers [5] reported the transformation of *trans*-resveratrol (3,5,4'-trihydroxystilbene) by laccase from *Myceliophtora thermophyla* and from *Trametes pubescens* to generate the dehydrodimer product that has an antioxidant properties (Figure 53).

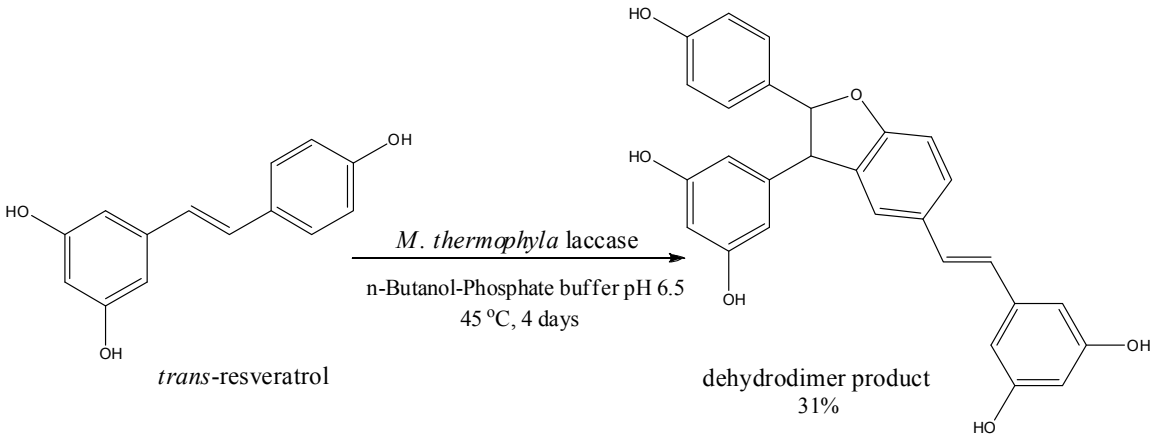



**Figure 53.** The transformation of *trans*-resveratrol (3,5,4'-trihydroxystilbene) by laccase.[5]

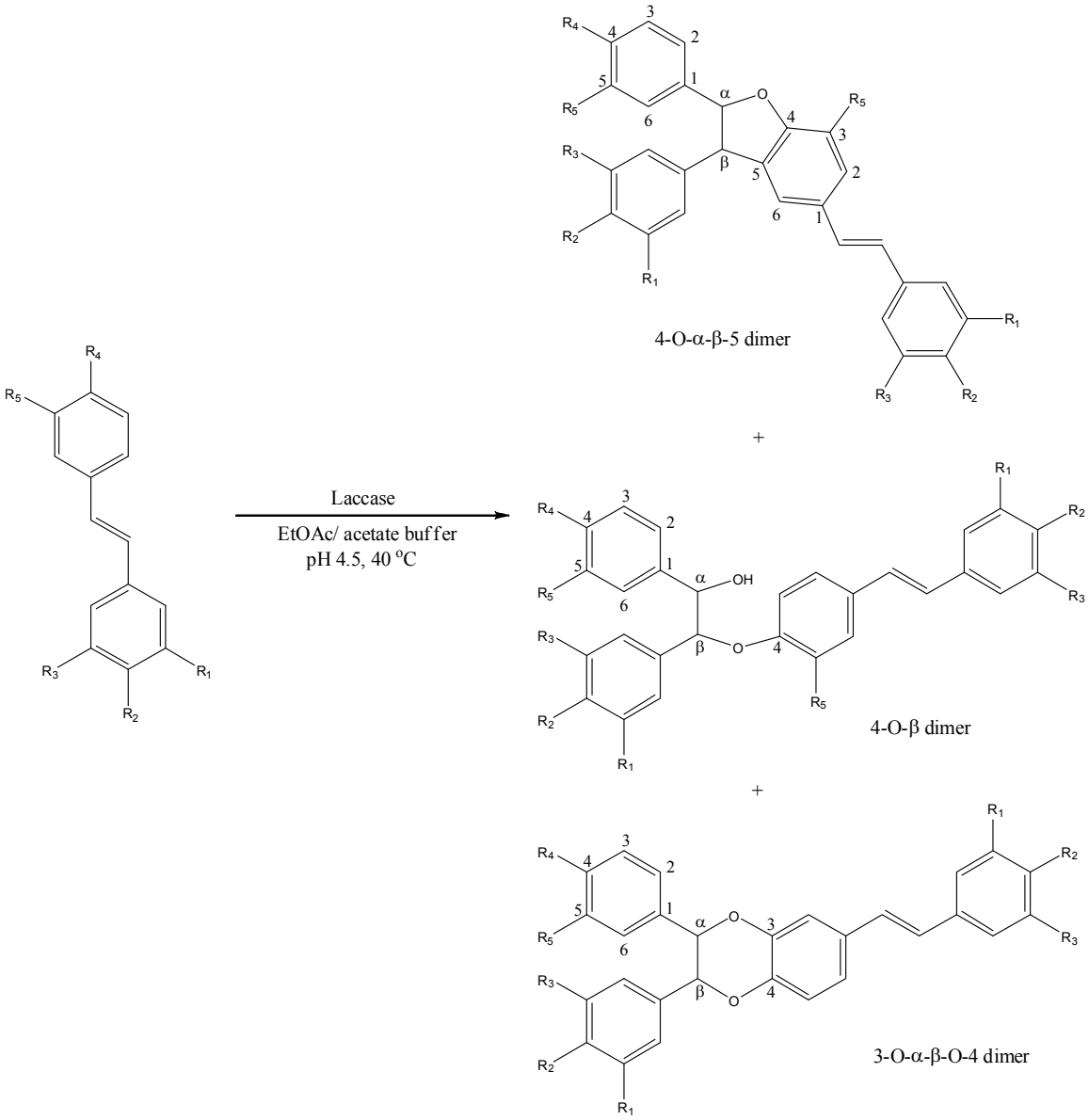



**Figure 54.** The oxidation of a seires of hydroxystilbenes by laccase.[228]

These authors recently reported the oxidation of a series of hydroxystilbenes, analogues of the phytoalexin resveratrol by laccase from *Trametes pubescens* in ethyl acetate/acetate buffer system [228]. In this study, three different dimeric product were identified with the main product usually being 4-O-α-β-5 dimers. These products were proposed to be generated via radical-radical coupling dimerization reactions (Figure 54).

Other biological active compounds have already been prepared. Antioxidant gelatin-catechin conjugates have already been synthesized by the laccase-catalyzed oxidation of catechin in the presence of gelatin in an aqueous medium [229]. Moreover, the dimerization of Penicillin X [230], totarol [231], flavonolignan silybin [232], and salicylic ester [13] by laccase have already been reported.

*2.4.4.1.4 Laccase-Catalyzed Oxidative Cross-coupling Reactions*

Laccases show to catalyze the oxidative cross-coupling reaction between different molecules. Oxidative coupling of hydroquinone and mithramicine [233] or (+)-catechin [16] have been examined. In the study of the cross coupling reaction between hydroquinone and (+)-catechin, *Rhus vernicifera* laccase catalyzed the formation of two new catechin-hydroquinone adducts (Figure 55). In this study, hydroquinone served as both a shuttle oxidant and a reactant during laccase oxidations.

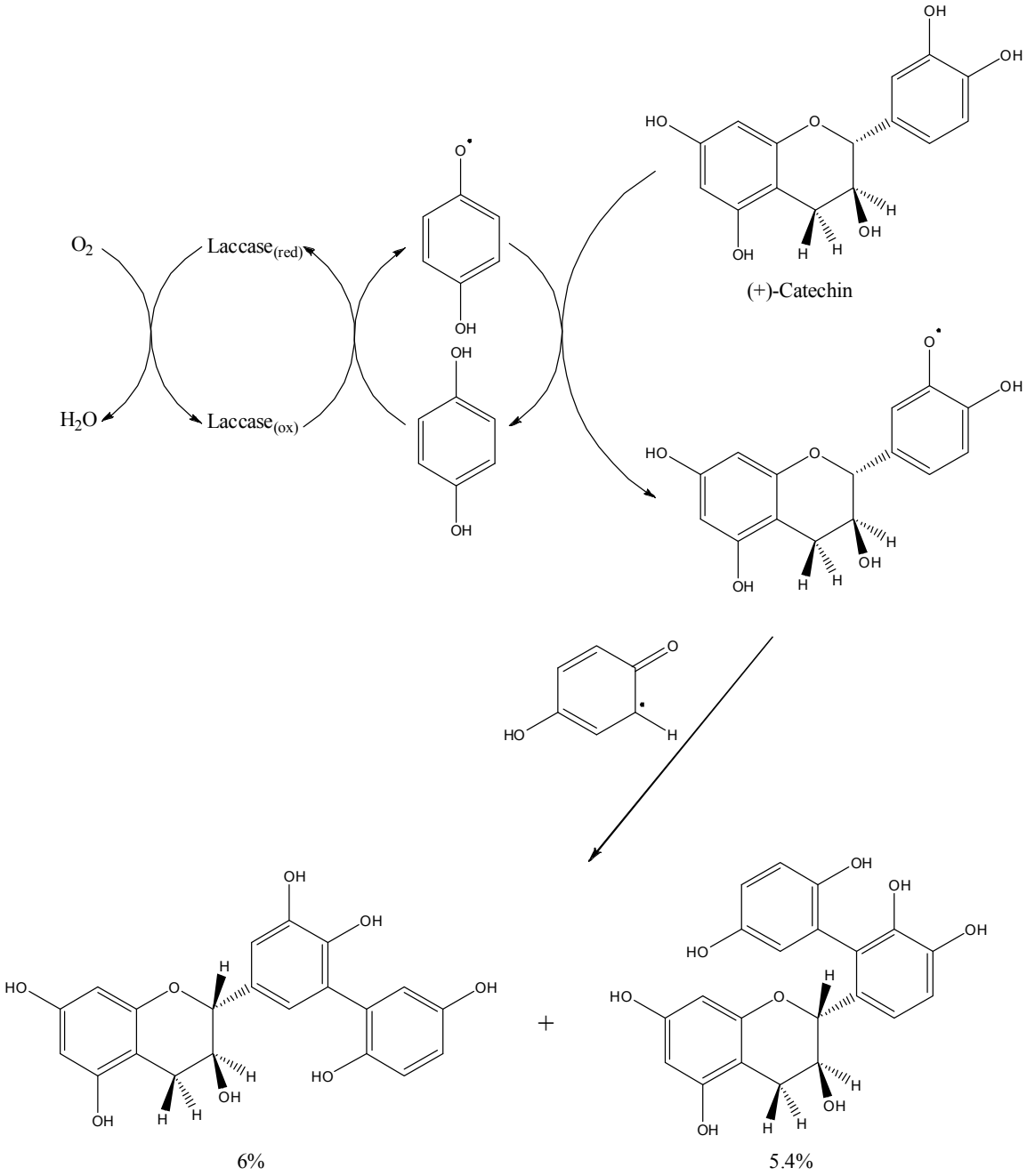



**Figure 55.** Laccase catalyzed the formation of catechin-hydroquinone adducts.[16]

Schauer et al. [15] reported the derivatization of the natural compound 3-(3,4-dihydroxyphenyl)-propionic acid (dihydrocaffeic acid) *via* N-coupling reaction with amines in the presence of laccase and oxygen in aqueous medium. The products of these reactions were formed by a R-$NH_2$ attack of a cation radical of dihydrocaffeic acid (Figure 56). Later, they also studied laccase catalyzed a heteromolecular coupling of dihydrocaffeic acid with 4-aminobenzoic acid in different reactor [234].

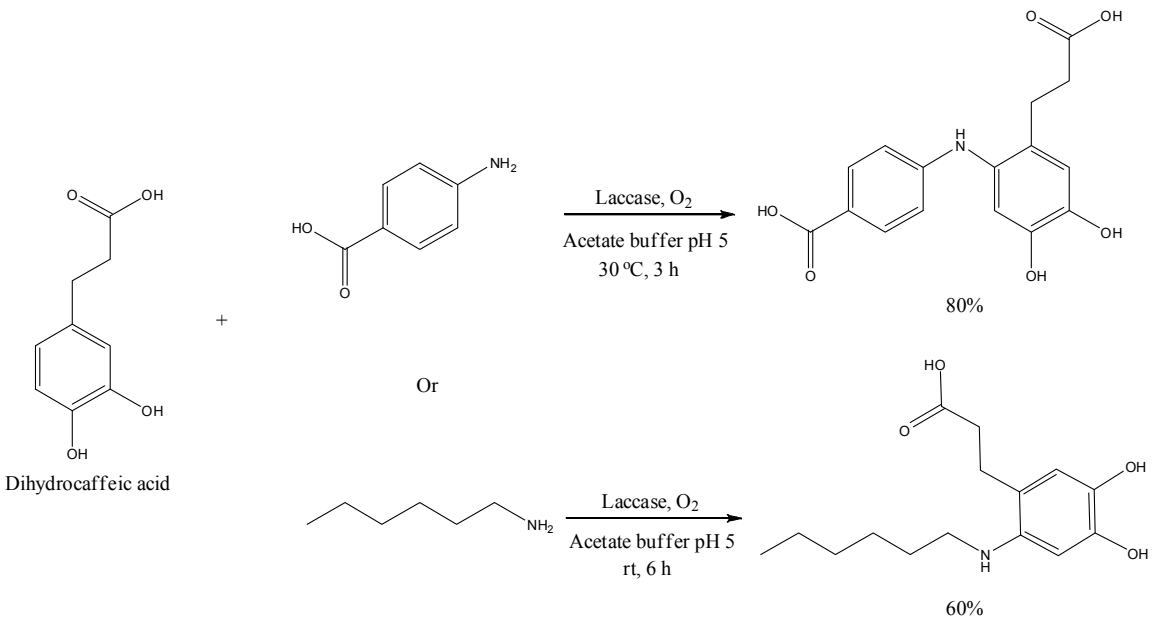



**Figure 56.** Laccase catalyzed N-coupling of dihydrocaffeic acid and amines.[15]

A recent example of laccase catalyzed cross-coupling reaction is the synthesis of Tinuvin, the benzotriazol base UV-absorber [235]. Laccase from Trametes hirsute was used to catalyze the coupling reaction of 3-(3-*tert*-butyl-4-htdroxyphenyl)propionic acid methylester to 1*H*-benzotriazole (Figure 57). This cross-coupling reaction occurred when 1*H*-benzotriazole was applied in four-fold molar excess.

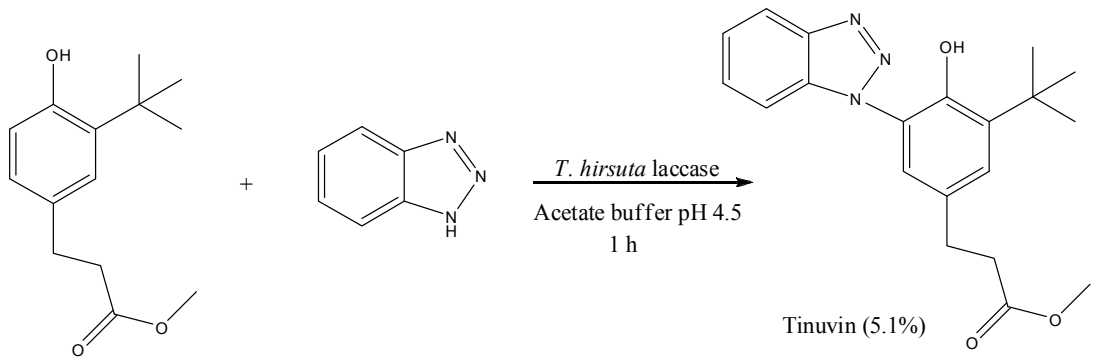



**Figure 57.** The synthesis of Tinuvin by a laccase-catalyzed reaction.[235]

Another recent example is the formation of protein-oligosaccharide conjugates [236]. The formation of hetero-cross-coupling between tyrosine side chain of α-casein and phenolic acid of hydrolyzed oat spelt xylan was catalyzed by laccase from *Trametes hirsula*. This study shows another use of laccase in the modification of the biopolymer.

2.4.4.2 Laccase-Mediated Formation of Intermediate Quinones in Organic Synthesis

In this section, all reactions proceeded *via* the quinonoid intermediates of laccase substrates. Laccase first oxidized the phenolic substrate to form phenolic radical which further underwent nonenzymatic oxidation to generate quinonoid intermediate. The quinonoid intermediate then reacted with other compounds to provide the corresponding product (Figure 58).

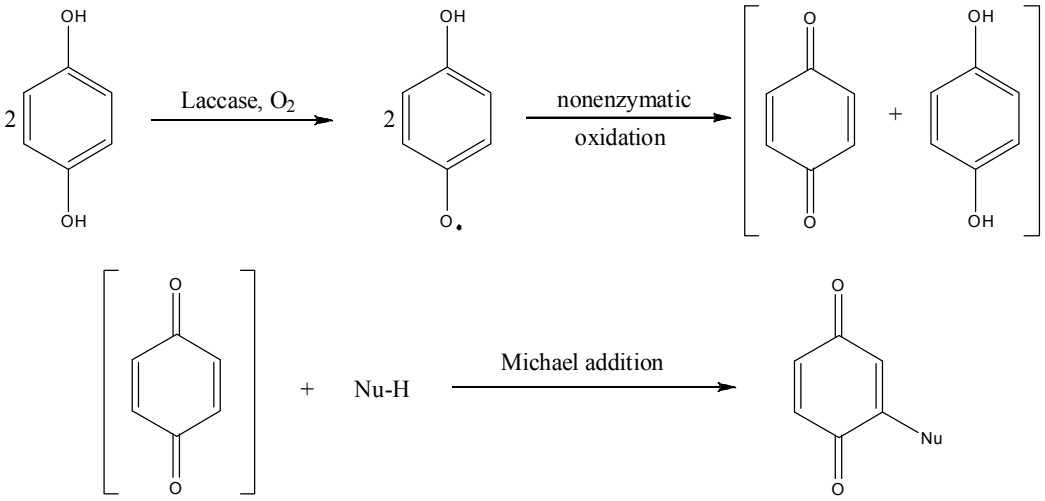



**Figure 58.** Mechanism of laccase mediated the formation of quinonoid intermediate for Michael addition reaction.

Many studies of laccase-catalyzed synthesis of aminoquinones have been reported [11,18,237]. Aminoquinones were synthesized by nuclear amination of p-hydroquinones with primary aromatic amines in the presence of fungal laccase. The mechanism of these reactions is likely to be proposed via Michael addition of primary amine to the quinoniod intermediate (Figure 59a). In addition, this strategy also used to derivatize unprotected amino acid L-tryptophane (Figure 59c) [238]. The laccase-catalyzed amination was also used in the synthesis of bioactive compounds such as β-lactam antibiotic cephalosporins (Figure 59d) [239] and novel penicellins (Figure 59e) [240]. Recently, Manda et al. [241] showed that the quinonoid intermediate of laccase substrate can react with solvent such as water, methanol, and other alcohols to form the C-O bond cross-coupling products (Figure 59b). Besides laccase-catalyzed amination of p-hydroquinone, Laccase-catalyzed amination of o-hydroquinone, such as laccase mediated Michael addition of $^{15}$N-sulfapyridine to protocatechuic acid, have also been reported [242].

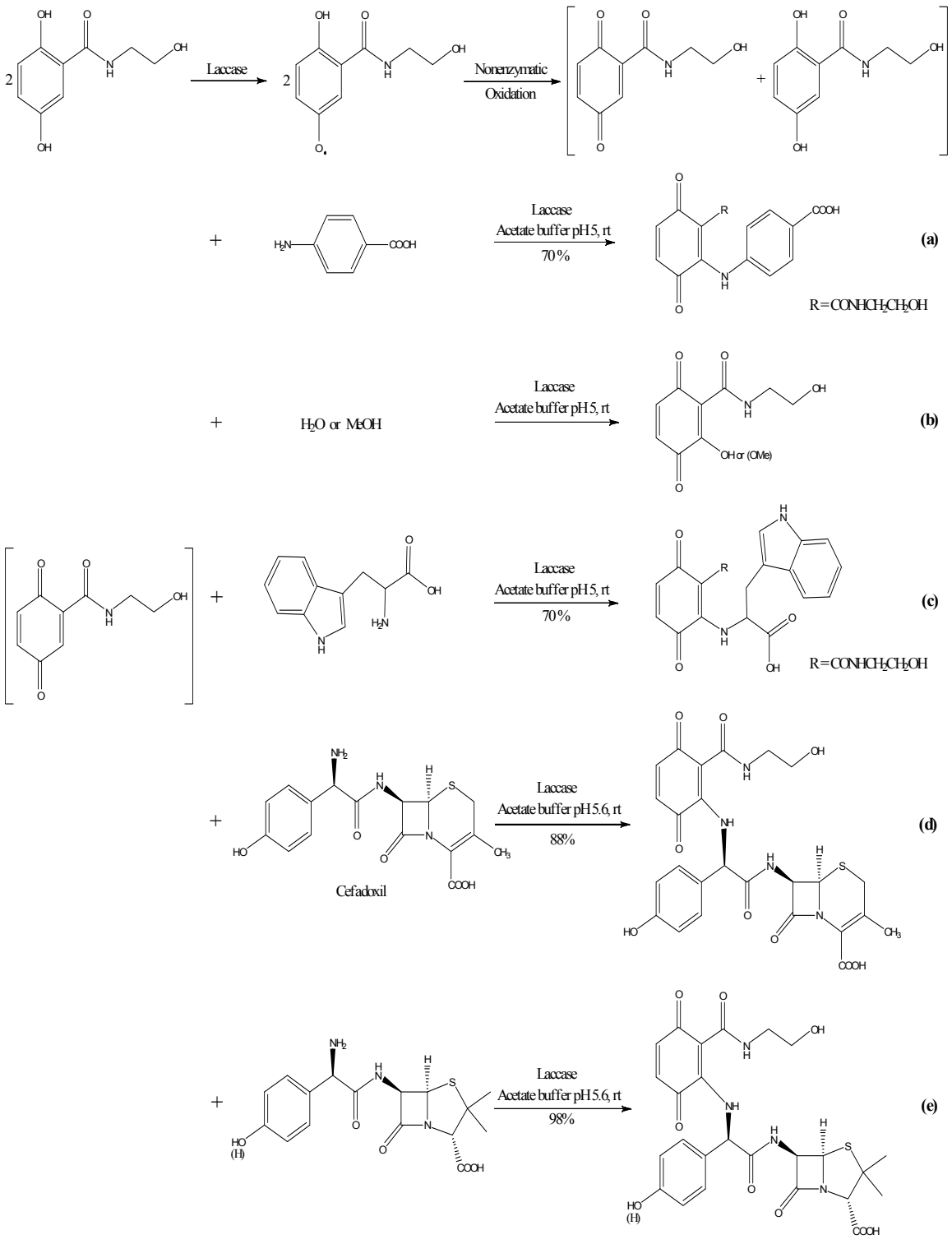
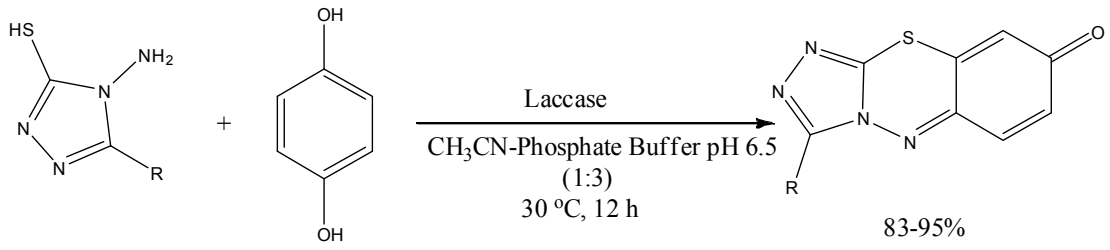



**Figure 59.** Laccase mediated amination reaction.[11,238-241]

Laccase-mediated formation of intermediate quinone can be used in the domino reaction. For example, Bhalerao et al. [243] reported laccase catalyzed one step synthesis of 3-substituted-1,2,4-triazolo(4,3-β)(4,1,2)benzothiadiazine-8-ones (Figure 60).




R = $CH_3$, $C_2H_5$, Ph, Ph-OMe, Ph-$CH_3$, Ph-Cl, Ph-Br

**Figure 60.** The synthesis of 3-substituted-1,2,4-triazolo(4,3-β)(4,1,2)benzothiadiazine-8-ones by laccase mediated reaction of 5-substituted-4-amino-3-mercapto-1,2,4-triazoles and hydroquinone.[243]

Recently, Leutbecher et al. [19] studied the synthesis of O-heterocycles via laccase-catalyzed domino reaction between 4-hydroxy-6-methyl-2H-pyran-2-ones with catechols. Moreover, Laccase initiated domino reaction of cyclohexane-1,3-diones with catechols for the synthesis of 3,4-dihydro-7,8-dihydroxy-2*H*-dibenzofuran-1-ones has been developed (Figure 61) [244]. The products yield ranging from 70% to 97%.

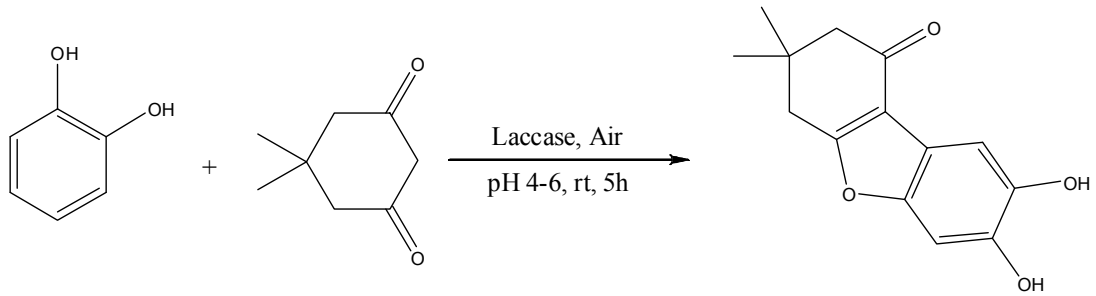



**Figure 61.** Laccase initiated domino reaction of cyclohexane-1,3-diones with catechols.[244]

2.4.4.3 Laccase-Catalyzed Polymerization Reaction

Laccases have shown to catalyze polymerization reaction of many compounds including acrylamide [245], 2-hydroxydibenzofuran [246], phenolic pollutants [247], 1-naphtol [248,249], catechol [250], 4-cholroguaicol [251], Bisphenol A [252], and aniline [253-255]. Some examples of these laccase catalyzed polymerization are shown in Table 4.

**Table 4.** Substrates, reaction conditions, and products from laccase catalyzed polymerization reactions.

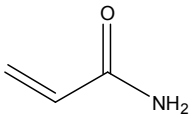
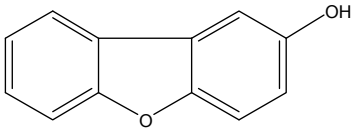
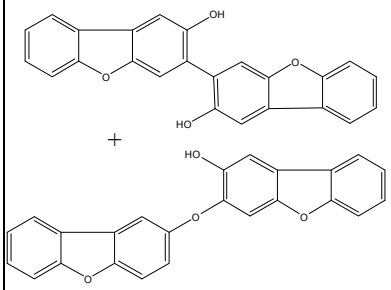
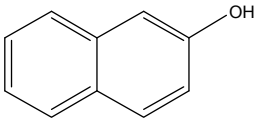
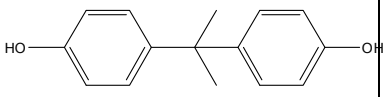
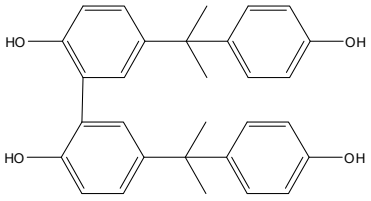
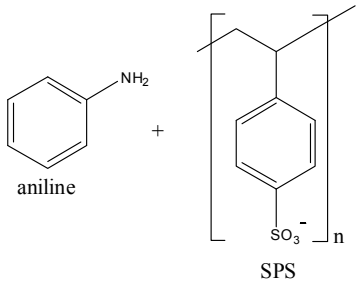
| Substrate | Reaction condition | Products | Reference |
|---|---|---|---|
| Acrylamide  | Laccase, water, 65 ºC, 4 h | Polyacrylamide (MW > $6 \times 10^5$) | [245] |
| 2-hydroxydibenzofuran  | Laccase, acetate buffer pH 5, 30 ºC, 3 h | Dimers such as  + Trimers and Oligomers | [246] |
| 1-naphthol  | Laccase, acetone-acetate buffer pH 5, 25 ºC | Orange colored poly(1-naphtol) Average MW = 4920 Da | [248] |
| Bisphenol A  | Laccase, phosphate buffer pH 6, rt, 4 days | Dimer  and Oligomers | [252] |

**Table 4.** (Continued)

| | | | |
|---|---|---|---|
| Aniline + Sulfonated polystyrene (SPS)<br>  <br> | Laccase, Citrate-phosphate buffer pH 3.5-4.4, 20 ºC | SPS-polyaniline complex | [255] |

In addition, many natural or artificial natural products have been synthesized by laccase-catalyzed polymerization reaction. Kobayashi and his co-workers developed a method for the preparation of artificial urushi [256-258]. Urushi is an insoluble polymeric film formed by the crosslinking of urushiol monomer whose structure is a catechol derivative with unsaturated hydrocarbon chain consisting of monoenes, dienes, and trienes at 3-, or 4-position of catechol. The artificial urushi in this study was prepared by laccase-catalyzed crosslinking of new urushiol analogues under mild conditions without the use of organic solvents (Figure 62).

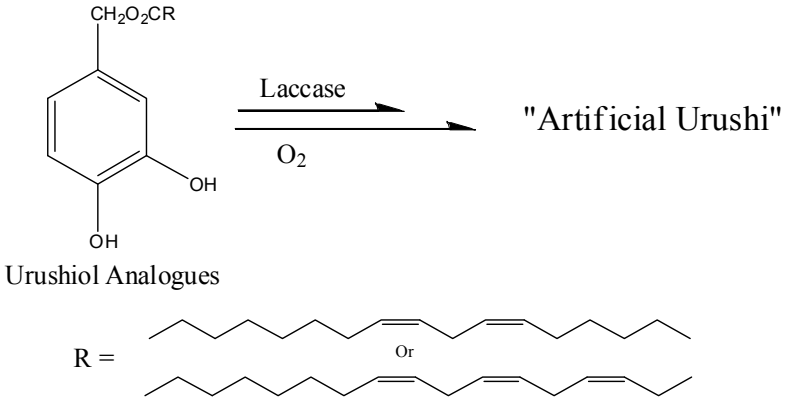



**Figure 62.** The synthesis of artificial urushi by laccase-catalyzed polymerization of urushiol analogues.[258]

Rutin is one of the most famous glycosides of flavanoid widely present in many plants and has been reported to have biological activities including antioxidant, antihypertensive, antiinflammatory, and antihemorrhagic activities. Therefore, Kobayashi et al. [14] synthesized poly(rutin) by laccase-catalyzed oxidative polymerization of rutin to amplify the antioxidant activity of rutin.

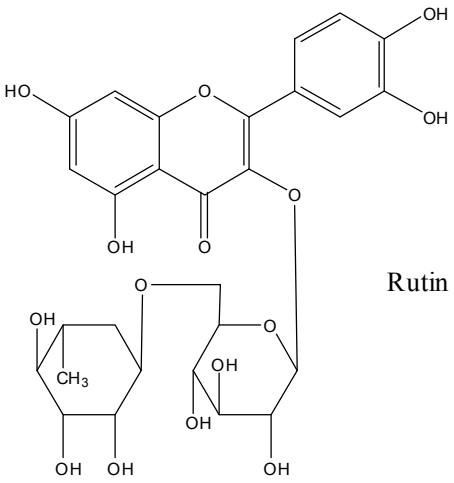
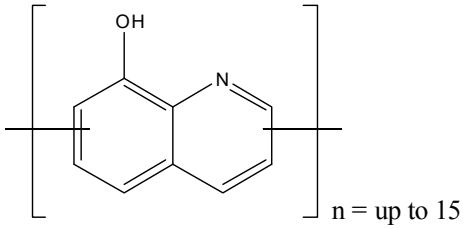



**Figure 63.** Structure of Rutin.

These authors also synthesized poly(catechin) [259], a new class of flavonoid polymers, via the polymerization of catechin by laccase in a mixture of acetone-acetate buffer solvent. Poly(catechin) exhibited greatly amplified superoxide scavenging activity and xanthine oxidase inhibitory activity compared with catechin. Moreover, Burton and Ncanana [260] recently reported laccase-catalyzed polymerization of 8-hydroxyquinoline to yield an antioxidant aromatic polymer (Figure 64). Eisenman et al. [261] reported the use of *Cryptococcus neoformans* laccase to catalyzed the synthesis of melanin from both D- and L-3,4-dihydroxyphenylalanine (DOPA).




Poly(8-hydroxyquinoline)

**Figure 64.** The structure of poly(8-hydroxyquinoline).[260]

### 2.4.5 Laccase in Fiber Modification

Enzyme facilitated lignocellulosic fiber modification is recently a growing field of research and interest [262]. Enzyme technology offers an environmentally friendly method for modifying the fibers. Moreover, enzymatic treatment conditions are often milder and less damaging to the fiber than chemical treatment. Laccase is one of the enzymes used for the surface modification of lignocellulosic fibers [20,263,264].

Fibers often have a set of their own properties. These properties, such as water-binding capability, flexibility, rigidity, hydrophilicity, hydrophobicity, and the ability to adhere to themselves and to other materials, depend on the structure and the composition of the major components of the fiber which are cellulose, hemicellulose, and lignin [263]. Altering these fiber properties is a tremendous opportunity to produce a new value-added material from this renewable resource.

The first part of this section will discuss the chemical composition and structure of the lignocellulosic fibers. Next, the recent development in fiber modification by laccase will be discussed.

### 2.4.5.1 Lignocellulosic Fibers

#### *2.4.5.1.1 Chemical Composition*

The three main natural polymers of lignocellulosic fibers are cellulose, hemicellulose, and lignin.

Cellulose is a straight-chain polysaccharide composed of D-glucose repeating units which are linked together by β-1,4-glycosidic linkages at the $C_1$ and $C_4$ positions as shown in Figure 65 [265]. The degree of polymerization (DP) of cellulose in native wood is around 10,000 but can decrease to less than 2000 after pulping [266]. The numerous hydroxyl groups on the chain backbone of cellulose macromolecules lead to the formation of both intermolecular and intramolecular hydrogen bonds. These hydrogen bonds stiffen the straight chain and promote aggregation, forming a crystalline structure [267]. Bundles of cellulose molecules are aggregated together in the form of microfibrils

with regions of high order (crystalline regions), and regions of low order (amorphous regions). Microfibrils build up and form fibrils which form cellulose fibers.

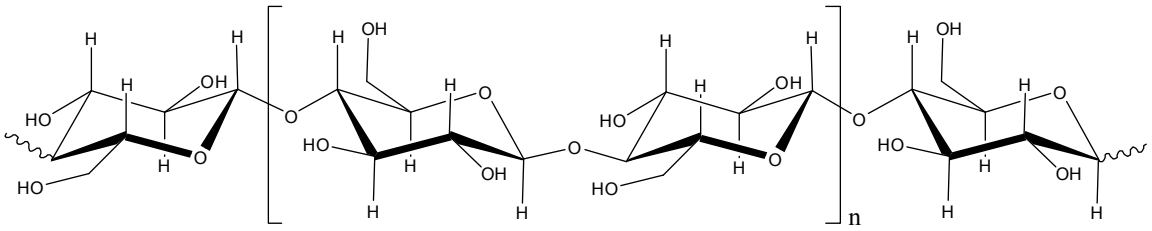


**Figure 65.** Chemical structure of cellulose.[265]

Cellulose has six crystalline polymorphs of which cellulose I and II are the most commonly found [265,268]. Cellulose I, the native form produced in plant and other organisms, is composed of parallel cellulose chains forming dense, highly hydrogen bonded sheets. Natural cellulose I exists as two crystal phases, named $I_\alpha$ and $I_\beta$. The relative amount of $I_\alpha$ and $I_\beta$ depends on their origins. For example, some algae and bacterial cellulose tend to be rich in $I_\alpha$ while cotton, wood, and ramie fiberstend to be rich in $I_\beta$ [269,270]. Recently, Langan et al. [271,272] studied the crystal structure and hydrogen-bonding system in cellulose $I_\alpha$ and $I_\beta$ from using synchrotron X-ray and neutron fiber diffraction. They found that cellulose $I_\alpha$ and $I_\beta$ can both be described as dense, highly hydrogen bonded sheets of parallel chains organized in sheet packed in a "parallel-up" fashion. These two allomorphs show no hint of intersheet O-H···O hydrogen bonding.The main difference between $I_\alpha$ and $I_\beta$ is the stacking of these sheets which is displaced in the chain direction. The second sheet of both allomorphs is shifted in the "up" direction by about c/4 relative to the first sheet. The third sheet in $I_\alpha$ is also shifted

up by about c/4 with respect to the second sheet, but in $I_{\beta,}$ it is shifted in "down" direction by about c/4 relative to the second sheet. Therefore, there is a relative difference of about c/2 in the position of the third sheet with respect to the second sheet in $I_\alpha$ and $I_\beta$. These authors also proposed that the most likely route for solid-state conversion of cellulose $I_\alpha \rightarrow I_\beta$ is the relative slippage by c/2 at the interface of the second and third sheets. They also indicated that weak C-H···O hydrogen bonding also contributes to cellulose crystal cohesion in both $I_\alpha$ and $I_\beta$. There are more C-H···O inter-sheet bonds in $I_\beta$ than in $I_\alpha$. This contributes to the stability of $I_\beta$ over $I_\alpha$.

Cellulose II consists of antiparallel cellulose chains that are arrange into less dense sheets and shows to have hydrogen bonding both within sheets and between sheets [273].

Hemicelluloses are branched heteropolysaccharides consisting of a number of different sugar building units including glucose, xylose, mannose, galactose, and arabinose (Figure 66). Hemicellulose is an amorphous polymer and this is attributed to the low degree of polymerization (DP = 50-300), and the branch structure. Hemicellulose is very hydrophilic, soluble in alkali, and easily hydrolyzed in acids [274]. The proportions and the composition of hemicellulose vary from one species to another. Hemicellulose content is typically 20-30% in softwood and 25-35% in hardwood [275]. Table 5 summarizes the DP and percentage of the major hemicelluloses in softwoods and hardwoods. Galactoglucomannans and arabinoglucuronoxylan are the two main hemicelluloses in softwood (Figure 67) while glucuronoxylan is the main hemicellulose in hardwood [276].

**Table 5.** The degree of polymerization and percentage of the major hemicelluloses in softwoods and hardwoods.[277]

| Hemicellulose type | | Percentage in wood (%) | Degree of polymerization (DP) |
|---|---|---|---|
| Softwoods | Galactoglucomannans | 11-25 | 100 |
| | Arabinoglucuronoxylan | 7-10 | 100 |
| Hardwoods | Glucuronoxylan | 15-30 | 200 |
| | Glucomannan | 2-5 | 200 |

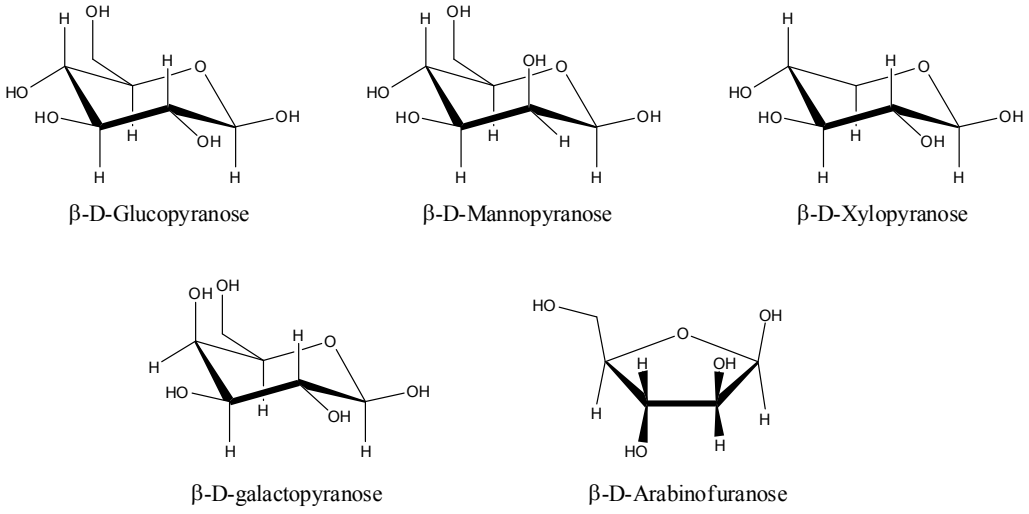



**Figure 66.** Sugar monomers in hemicellulose.

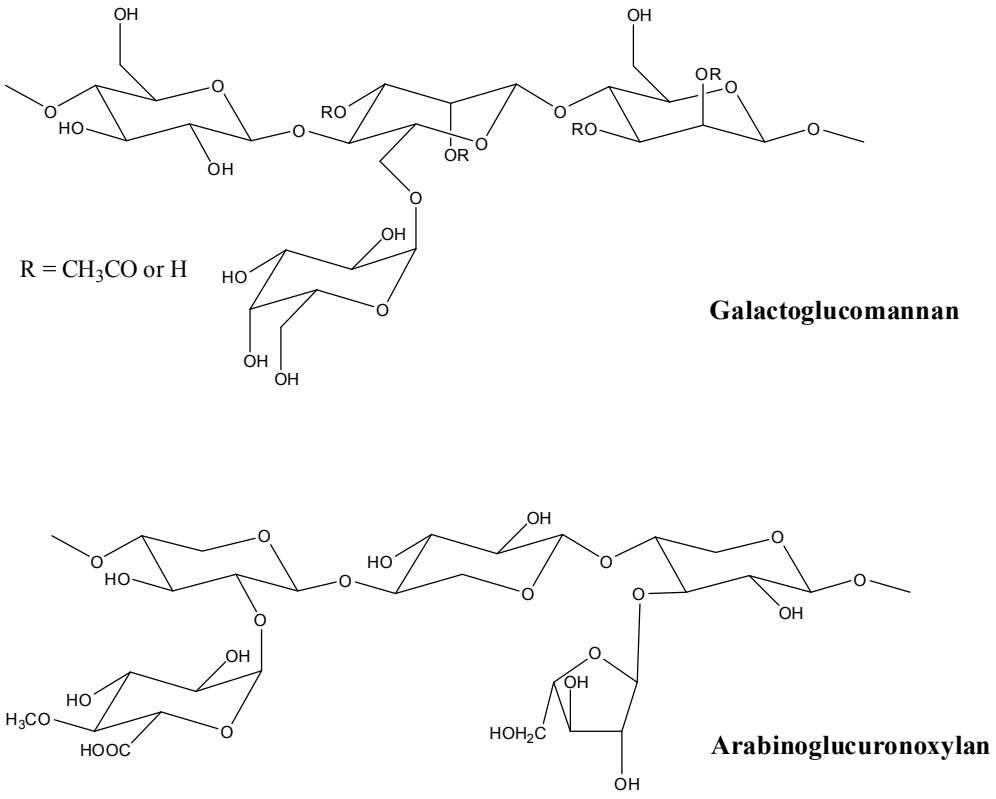
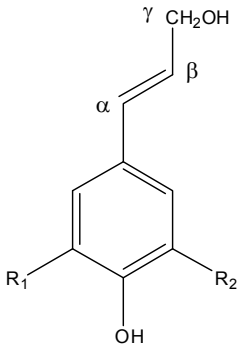



**Figure 67.** Structure of hemicelluloses in softwood.[276]

Lignin, the second most abundant natural polymer on earth, is a complex aromatic polymer most commonly derived from wood and an integral part of the cell walls of plants. Lignin is totally amorphous and hydrophobic in nature. It gives rigidity to the plants. Lignin macromolecule is a crosslinked three-dimentional phenolic polymer made up of hydroxyphenylpropane units [278]. Due to the difficulty in isolating lignin without modification, the original structure of native lignin is not yet known. However, numerous information from lignin degradation products and model compound studies provides the evidence that lignin formation originates from the polymerization of three different hydroxyphenylpropane units known as monolignols. These monolignols are sinapyl, coniferyl, and *p*-coumaryl alcohol as illustrated in Figure 68 [279].




Coniferyl alcohol (softwood/hardwood): $R_1 = OCH_3$, $R_2 = H$

*p*-Coumaryl alcohol (softwood/hardwood): $R_1 = R_2 = H$

Sinapyl alcohol (hardwood): $R_1 = R_2 = OCH_3$

**Figure 68.** The structure of monolignols.[279]

The polymerization of lignin is believed to proceed via the formation and subsequent coupling of phenoxy radicals [278,280]. Figure 69 illustrates five main resonance structures of the phenoxy radical which will undergo coupling reaction to form a wide variety of linkages. The phenylpropane units are linked by C-C and C-O bonds. Eight common interunit linkages in lignin are shown in Figure 70 [279]. Table 6 shows the percentage of linkages found in hardwood and softwood lignin. The β-O-4 ether linkage is the most abundant linkage in lignin, approximately 50% of total linkages in softwood lignin. In addition, functional groups, including hydroxyl, methoxyl, and carbonyl groups, have been identified in lignin.

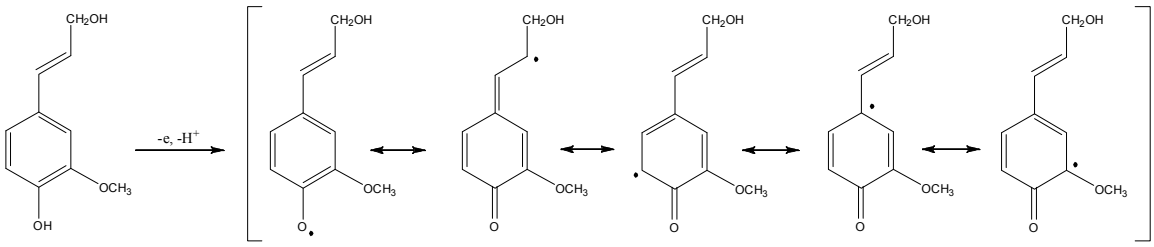



**Figure 69.** Resonance structures of lignin precursors.[278]

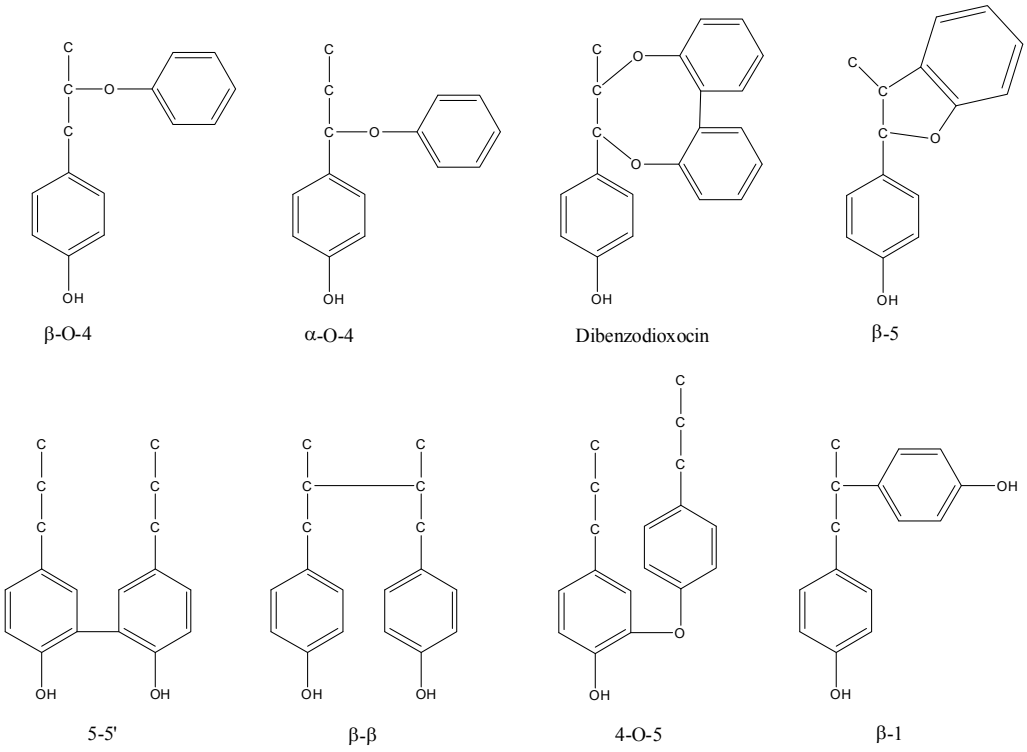



**Figure 70.** Structure of eight different lignin linkages.[281-283]

**Table 6.** The percentage of different lignin linkages in hardwood and softwood.[279,284]

| Type | Name | Percentage found in wood (%) | |
|---|---|---|---|
| | | Hardwood | Softwood |
| β-O-4 | β-aryl ether | 60 | 45 - 50 |
| α-O-4 | α-aryl ether | 7 | 6 - 8 |
| β-5 | Phenylcoumaran | 6 | 9 - 12 |
| 5-5’ | Biphenyl and Dibenzodioxocins | 7 | 18 - 25 |
| 4-O-5 | Diphenyl ether | 5 | 4 - 8 |
| β-1 | 1,2-diphenylpropane | 7 | 7 - 10 |
| β-β | β-β linked structures | 3 | 3 |

*2.4.5.1.2 The Effect of Kraft Pulping on Fiber Composition*

The major chemical pulping process in North America is the kraft process. The objective of any chemical pulping process is to remove enough lignin from cellulosic fibers to produce a pulp suitable for the manufacture of paper and other related products. In a conventional kraft cook, the wood chips are treated with an aqueous solution of sodium hydroxide (NaOH) and sodium sulfide ($Na_2S$), known as white liquor, in a large pressure vessel called a digester. The white liquor and the wood chips are then heated to a cooking temperature of about 170 ºC, typically reached after 1 – 1.5 hours. This allows the cooking liquor to impregnate the chips. The cook is then maintained at the cooking temperature for about 2 hours. Then, the contents are discharged into a blow tank to disintergrate the softened chips into fibers [285]. During the kraft pulping treatment, the hydroxide ($OH^-$) and hydrosulfide anion ($SH^-$), presenting in the pulping liquor, react with the lignin. This reaction causes the lignin polymer to fragment into smaller water/alkali-soluble fragment which are then dissolved as phenolate or carboxylate ions. Hemicellulose and some cellulose are also chemically attacked and dissolve to some

extent. Typically, approximately 90% of lignin, 50% of the hemicellulose and 10% of cellulose is removed in kraft pulping process [285].

The degradation of lignin during kraft pulping mainly proceeds through the cleavage of ether linkages, with a concomitant generation of free phenolic hydroxyl groups. The liberation of these phenolic hydroxyl group results in an increase of hydrophilicity of the lignin and the lignin fragments. As a consequence, the solubility of lignin in the pulping liquor is increased. However, the carbon-carbon linkages are more stable and tend to remain after the pulping process. At the end of kraft pulping, the remaining or residual lignin content is typically about 4-5% (by weight) [280,285].

Chakar and Ragauskas [280] recently reviewed the softwood kraft lignin process chemistry. Two main lignin reactions, which are degradation and condensation reactions, occur during kraft pulping. The major degradation reactions are the cleavage of α-aryl and β-aryl ether bonds [286]. α-Aryl lingkages are shown in Figure 71. The quinone methide intermediate is formed after the α-aryl bond cleavage. This quinone methide intermediate can react with $SH^-$ to generate a benzyl mercaptide structure. Then, the mercaptide anion attacks the β-carbon to yield a thiirane intermediate and eliminates the β-aryloxy group as illustrated in Figure 71. In addition, the terminal hydroxymethyl group of the quinone methide intermediate can be eliminated as formaldehyde to yield an alkali-stable enol ether (Figure 71) [287,288]. The cleavage of the β-aryl ether bond is summarized in Figure 72. This cleavage involves the attack of an ionized hydroxyl group present on the α- or γ-crabon.

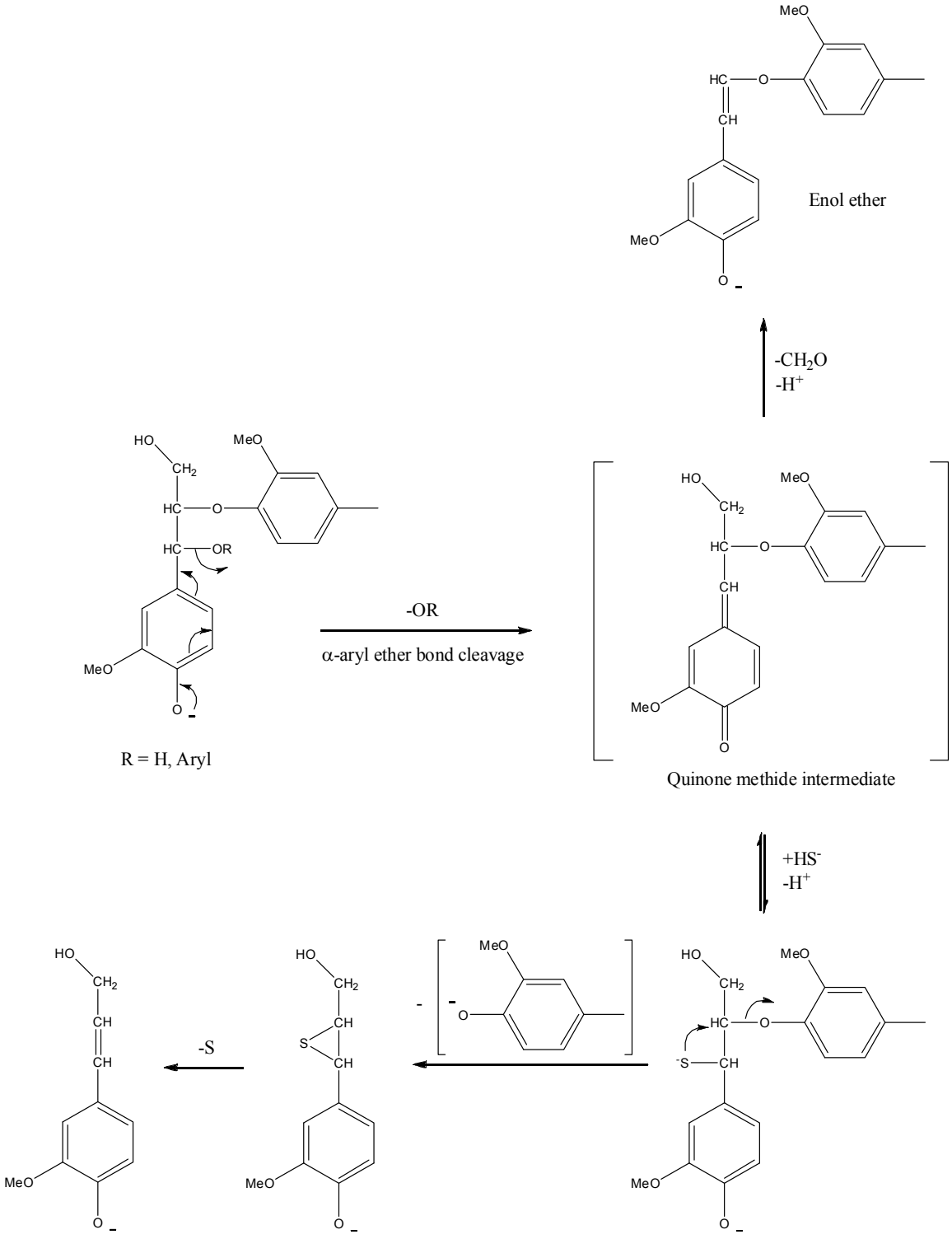



**Figure 71.** Alkaline cleavage of α-aryl ether bond, sulfidolytic cleavage of β-aryl ether bonds in phenolic arylpropane units, and conversion into enol-ether units of quinone methide intermediates.[280]

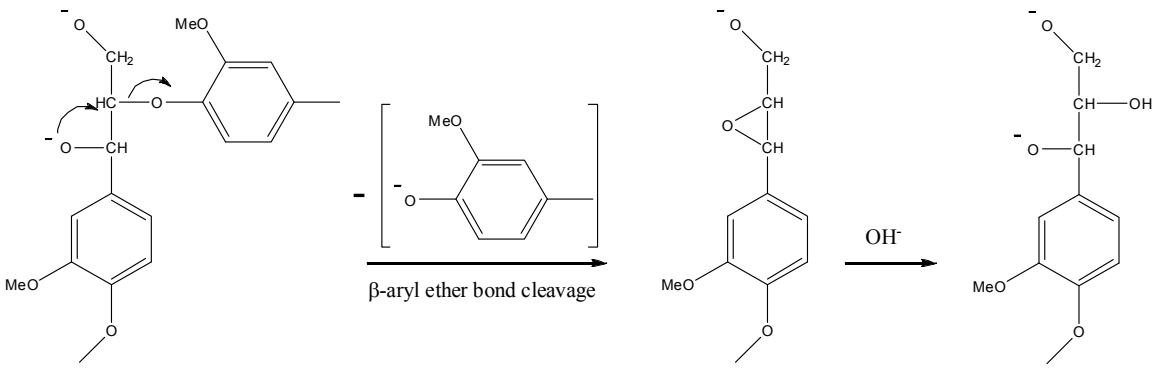



**Figure 72.** β-aryl ether bond cleavage in nonphenolic arylpropane unit.[280]

During the kraft pulping, the quinone methide intermediate acts as an acceptor which can react with necleophiles such as $SH^-$, $OH^-$, and lignin nucleophiles (e.g., carbanions from phenolic structures). Therefore, these nucleophiles compete for quinone methide intermediates. The condensation reaction proceeds via Michael addition between quinone methide intermediate and phenolated ion, followed by the abstraction of a proton and rearomatization to form the corresponding product. However, when the structures contain a good leaving group, such as an aroxyl group, at the β-carbon, the cleavage of β-aryl ether linkages will predominate over condensation reactions [280]. Figure 73 summarizes the proposed competitive addition of these necleophiles.

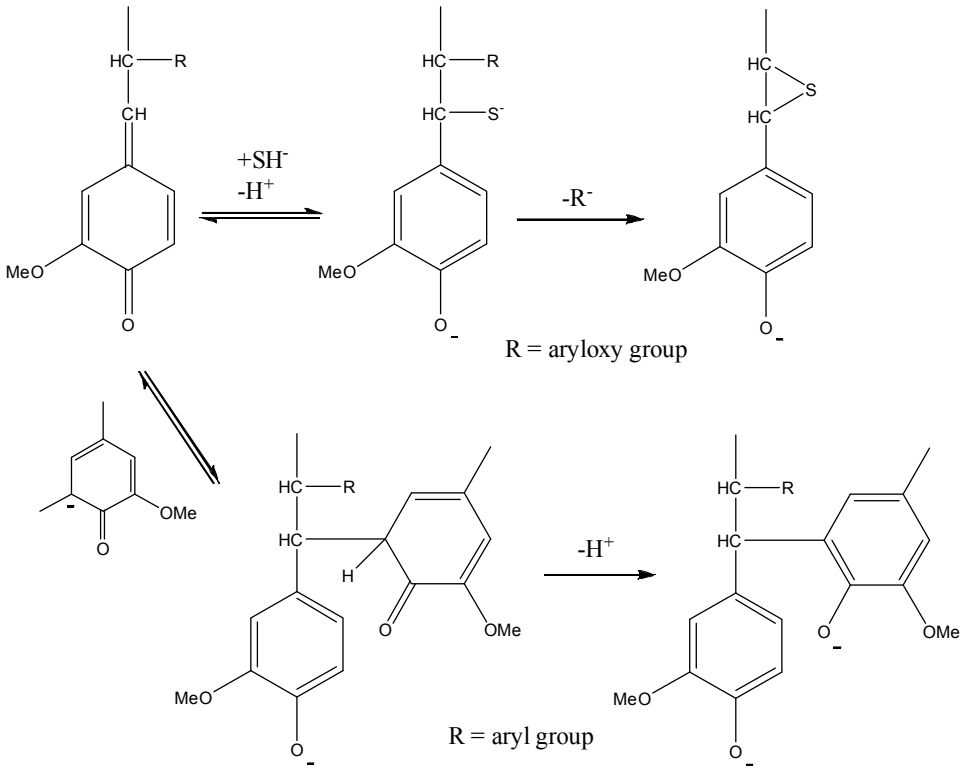



**Figure 73.** Competitive addition of external ($SH^-$) and internal (phenolate ion) nucleophiles to quinone methide intermediates.[280]

Moreover, the carboxylic acid group content of the residual lignin is affected by the kraft pulping process. Froass, Ragauskas, and Jiang [289] reported that the carboxylic acid group content of the lignin increases as delignification proceeds. The enhancement of carboxylic groups in residual lignin after kraft pulping is also reported by Jiang and Argyropoulos [290]. This enhancement is accompanied by a decrease in the amount of aliphatic hydroxyl groups.

Polysaccharides, including hemicellulose and cellulose, are also degraded during the kraft process. The hemicellulose content is reduced by approximately 40%. The dissolution of hemicellulose is caused by the combination of peeling and alkaline hydrolysis reactions. The peeling reaction can be ended via the stopping reaction which

converts the reducing end group to a stable carboxylic acid group [276]. Therefore, it can be assumed that virtually all carbohydrate end groups have been converted to carboxylic acids at the end of kraft pulping. Figure 74 summarized the peeling and stopping reactions of polysaccharides during kraft pulping.

10% of cellulose is removed during the kraft pulping process. This low loss of cellulose is due to the low accesssability of $OH^-$ into the crystalline region of the cellulose. In addition, about 90% of the extractives in wood are removed [285]. Table 7 shows yield values for individual wood composition after kraft pulping of Scots pine (*Pinus sylvestris*, softwood) and birch (*Betula verrucosa*, hardwood).

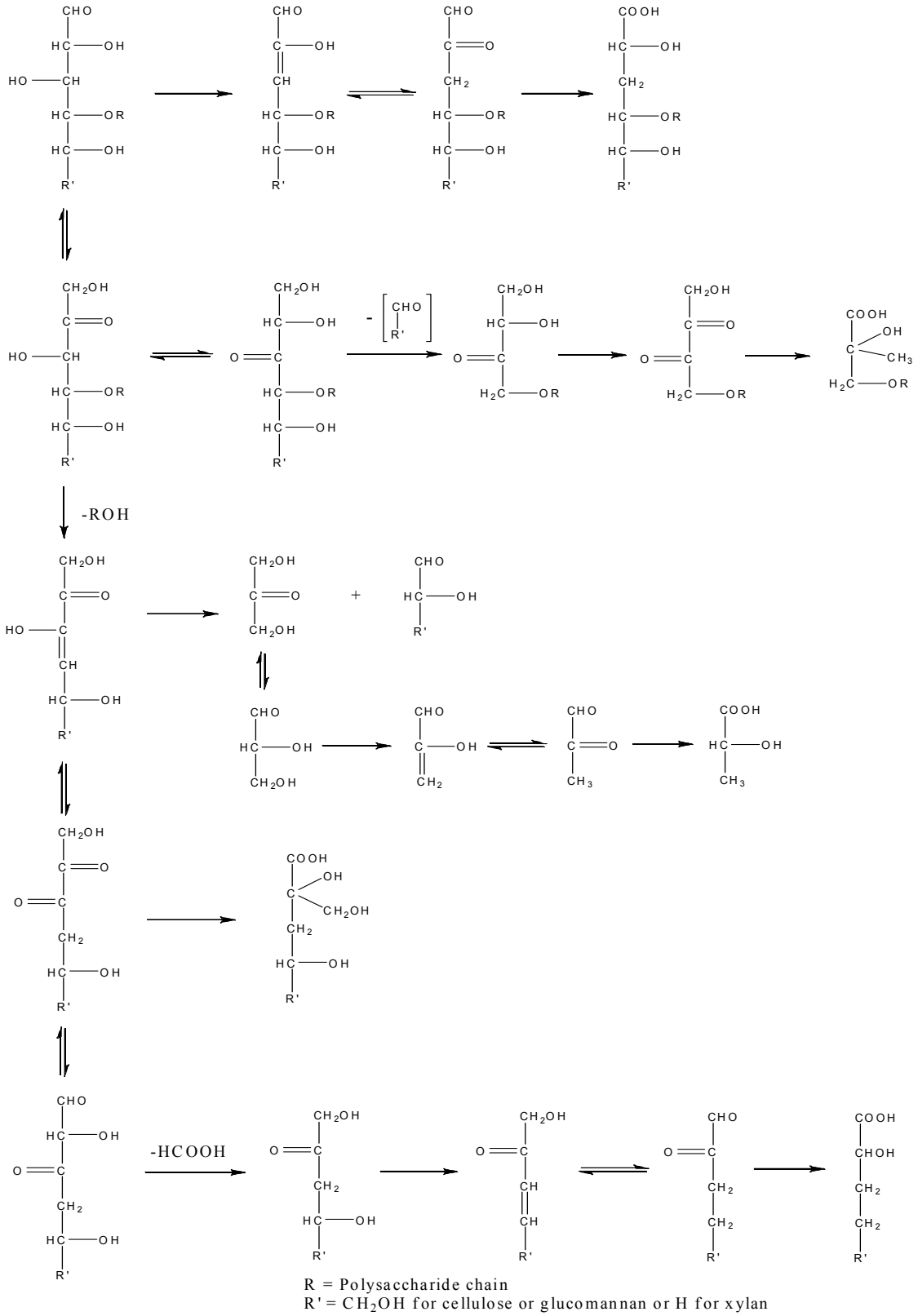



**Figure 74.** Scheme illustrates peeling and stopping reactions of polysaccharides during kraft pulping. [291]

**Table 7.** Yield values for individual pulp components after kraft pulping of Scots pine (a softwood) and birch (a hardwood).[292]

| Wood Component | Yield (%, on dry-wood basis) | | | |
|---|---|---|---|---|
| | Pine | | Birch | |
| | Original | After pulping | Original | After pulping |
| Cellulose | 39 | 35 | 40 | 34 |
| Glucomannan | 17 | 4 | 3 | 1 |
| Xylan | 8 | 5 | 30 | 16 |
| Other carbohydrates and various components | 5 | - | 4 | - |
| Sum of carbohydrates | 67 | 44 | 74 | 51 |
| Lignin | 27 | 3 | 20 | 2 |
| Pitch | 4 | 0.5 | 3 | 0.5 |
| Sum of components (yield) | 100 | 47 | 100 | 53 |

*2.4.5.1.3 Structure of Lignocelluosic Fibers*

Lignocellulosic fibers are composed of hollow cellulose fibrils held together by a lignin and hemicellulose matrix. The cell wall of a fiber has a complex, layered structure as illustrated in Figure 75. The hollow center of the fiber called lumen, and the sublayers of the cell wall consisting of a thin primary wall and a thicker secondary wall. The primary wall has a lower amount of cellulose and a higher amount of lignin compared to the secondary wall. Cellulose microfibrils from the primary wall are organized in a loose network almost perpendicular to the cell axis. The secondary wall is made up of three layers, S1, S2, and S3 [293]. The secondary wall's microfibrils have a parallel arrangement. Each layer of the secondary wall has a different microfibrillar angle, the angle between the fiber axis and the microfibrils. The microfibrillar angle in S1, S2, and S3 layers are 50-70º, 10-30º, and 60-90º, respectively [293]. The microfibrils, providing mechanical strength to the fiber, are made up of 30-100 cellulose molecules in extended

chain conformation. The thick S2 layer determines the mechanical properties of the fiber. The amorphous phase in the cell wall consists of hemicellulose, lignin, and in some cases pectin. The hemicellulose molecules are bonded with cellulose microfibrils by hydrogen bonding. This cellulose-hemicellulose network is believed to be the main structure component of the fiber cell. The compound that binds the two adjacent primary walls together is called the middle lamella. The middle lamella (ML) is primarily composed of lignin that holds the fibers together in the wood ultrastructure. The length of typical softwood fibers is approximately 2.5-7.0 mm and the width is approximately 25-50 μm. Typical hardwood fibers are approximately 0.8-1.6 mm long and 14-40 μm wide.

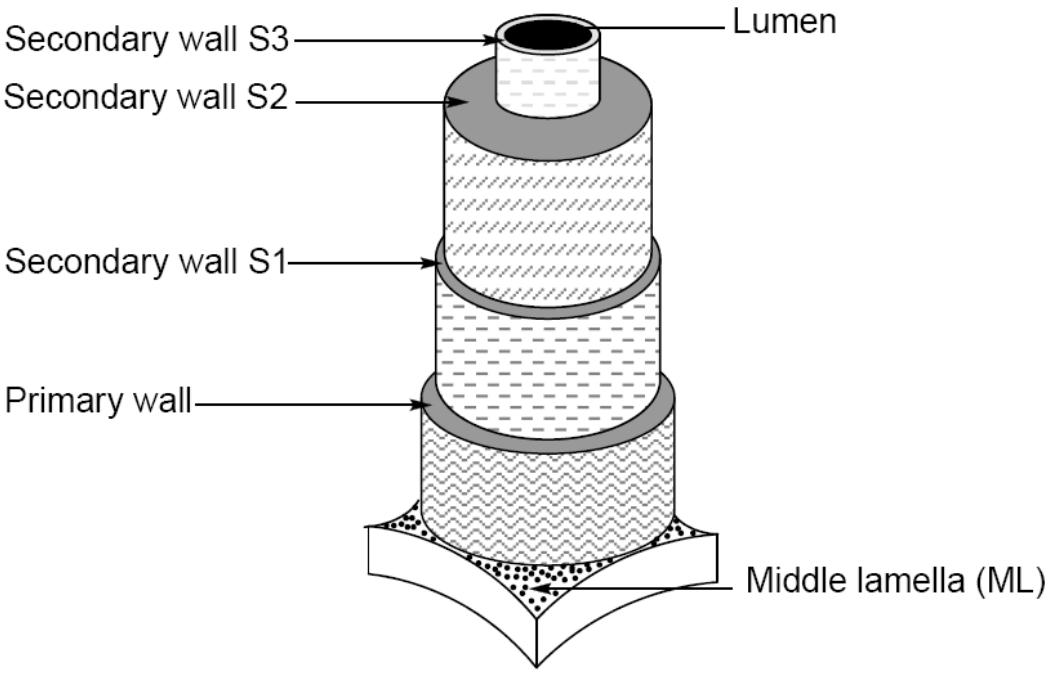



**Figure 75.** A softwood tracheid (fiber) cell wall structure (Adapted from Coté [294]).

2.4.5.2 Laccase Application in Fiber Modification

Recently, laccase research studies have shifted toward fiber modification. Lignocellulosic fibers compose of lignin, the macro phenoxylic structure which can be oxidized by laccase to form the phenoxylic radical in the fibers. These radicals appear to undergo polymerization with each other or undergo coupling reaction with other compounds. Therefore, they have been used to graft a variety of substrates onto the fiber which leads to the alteration of fiber surface. Moreover, depending on the grafting materials, the properties of the modified fibers can be designed to suit the end product.

Laccases have been applied for bonding of fiberborads, particle boards, paper boards, and kraft-liner board [295-298]. The auto adhesion of wood fiber and particles has been achieved using laccase for activation of the surface lignin. Laccase first oxidized lignin at the surface fibers to generate the lignin phenoxy radicals. These radicals then underwent the crosslinking reaction to form a crosslinked-network of lignin between fibers. Laccase-catalyzed polymerization of lignin through cross-linking of lignin phenoxy radicals led to the bonding and strength enhancement of lignocellulosic materials. Recently, the internal bonding of particle boards was improved by laccase-catalyzed funtionalization with 4-hydroxy-3-methoxybenzylurea [299]. In this study, 4-hydroxy-3-methoxybenzylurea was used as a functional compound to graft with spruce wood particle by laccase. The presence of the urea group in this funtionalized wood particle led to crosslinking between the funtionalized wood particles and resin in subsequent glueing processes (Figure 76), which improved the strength properties of the particle boards.

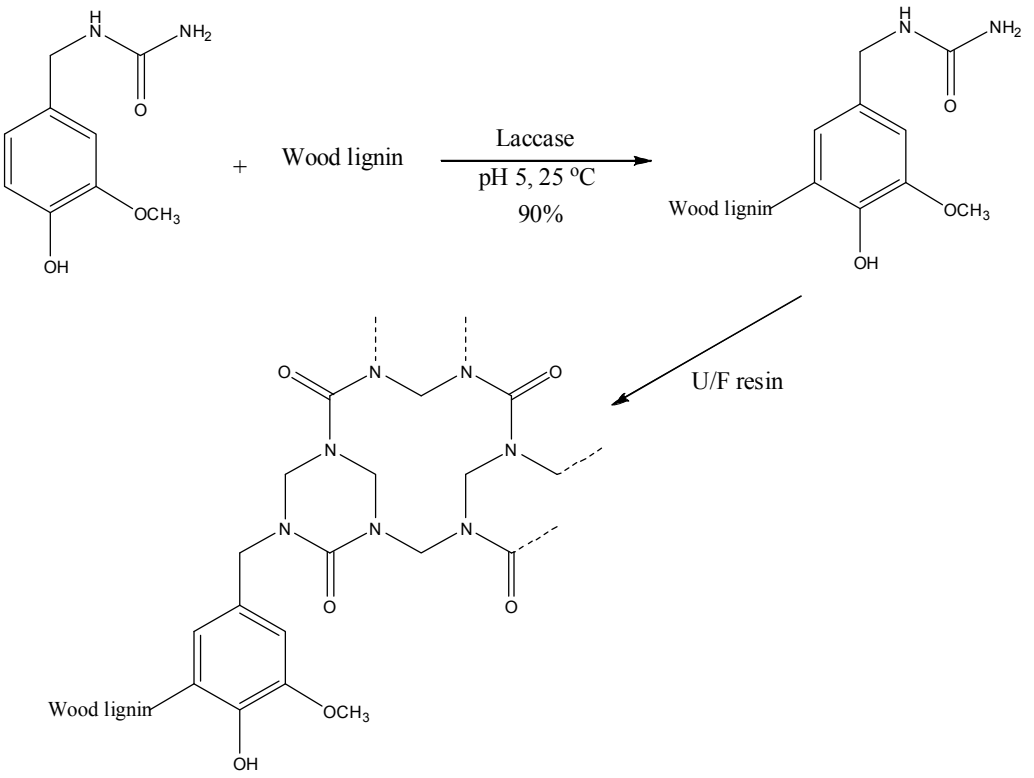



**Figure 76.** Laccase catalyzed grafting of lignin with 4-hydroxy-3-methoxybenzylurea, followed by chemical crosslinking to urea/formaldehyde (UF) resin in the subsequent glueing process.[299]

Besides catalyzing auto cross-linking between lignin, laccases have been used to catalyze the grafting reaction of various materials onto technical lignin. For example, guaiacol sulfonate has been grafted onto lignin by laccase resulting in an increase of the water solubility of lignin [22]. This reaction was initiated by an oxidation of lignin and guaiacol sulfonate by using laccase to generate phenoxy radicals of both components. These radicals then underwent the coupling reaction with each other to form guaiacol sulfonate-grafted lignin. Huttermann et al. reported that the lignin phenoxy radicals

formed by the laccase catalyzed oxidation reaction are so active that they can also react with nucleophlies such as cellulose and starch. Therefore, this study shows that carbohydrate can be covalently bonded with lignin via the laccase catalyzed reaction of lignin with cellulose [23]. Moreover, Mai et al. reported many studies involving the grafting of lignin with synthetic polymers derived from acrylic and acrylamide to create a new class of engineering plastics [24-27]. The presence of both laccase and peroxides such as dioxane peroxides were essential in the copolymerization of acrylamide and acrylic with lignin. In addition, the results from many experiments, such as solubility testing, elemental analysis, UV-Vis, FT-IR, and $^{13}$C-CPMAS spectroscopy, provided evidence of grafting. In case of acrylamide-lignin copolymer, when freeze-dried this copolymer appeared as homogeneous fibril-like particulates. The proposed mechanism of the enzymatical grafting is illustrated in Figure 77.

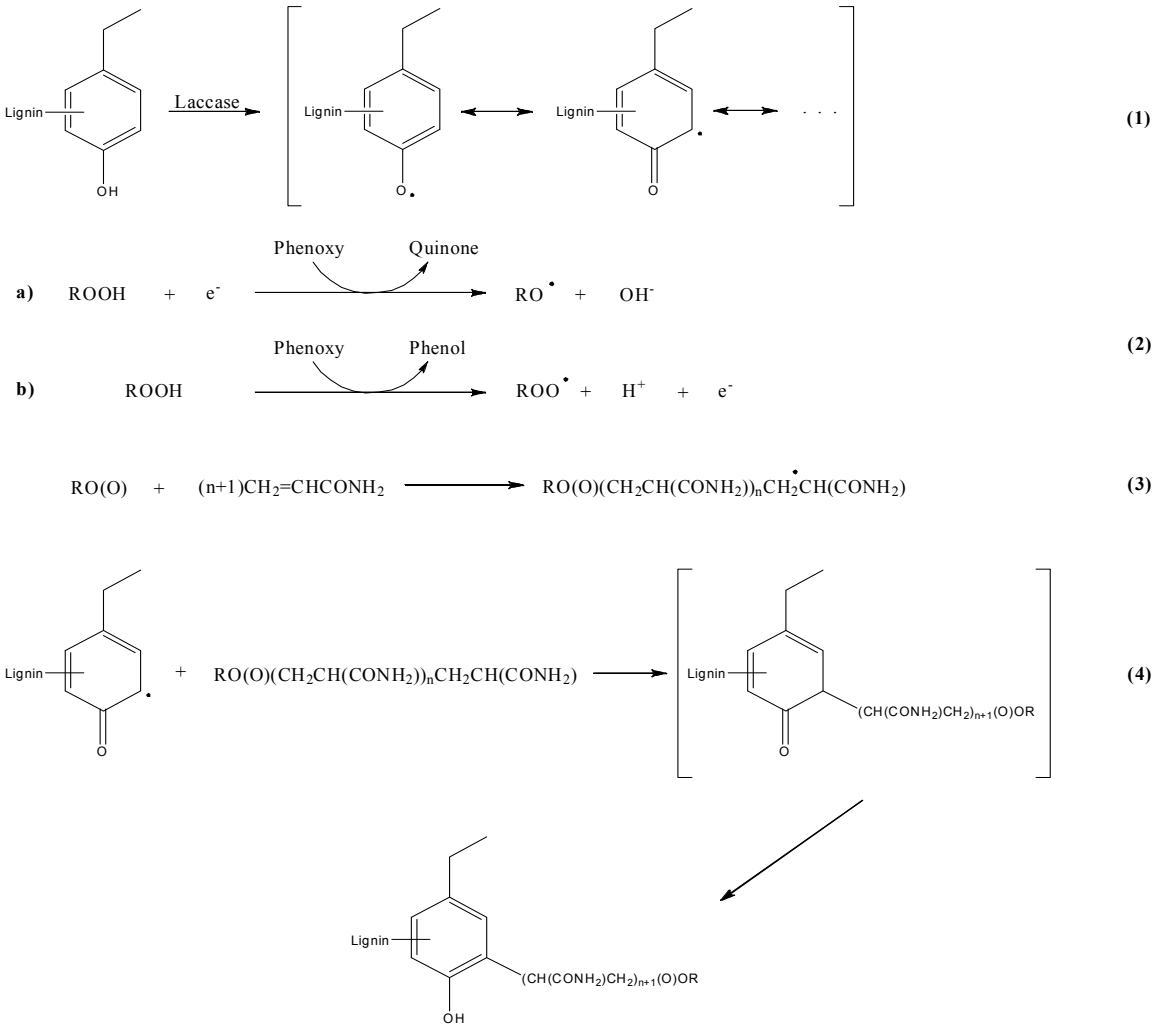
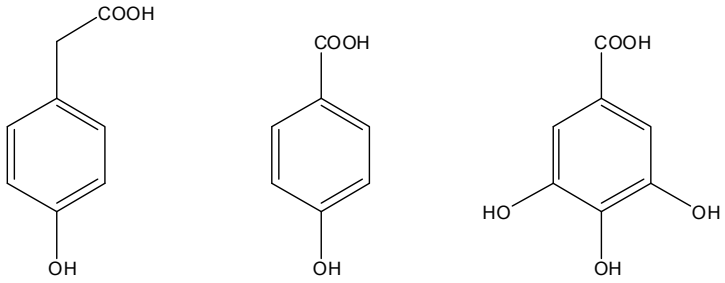



**Figure 77.** Proposed mechanism of chemoenymatically induced graft copolymerization between lignin and acrylamide. [25]

In addition, lignocellulosic fibers have been reported to be grafted with a variety of low molecular weight compounds. Chandra et al. modified high-lignin softwood kraft pulp by grafting with phenolic acids (Figure 78), including 4-hydroxyphenylacetic acid (PAA) [30], 4-hydroxybenzoic acid (4-HBA) [31], and gallic acid [29], in the presence of laccase. The grafting of these phenolic acids was performed in water (pH 4.5) at 45 ºC for 2-4 hours and resulted in an increase of carboxylic acid groups, water retention, tensile strength, and burst strength of the resulting paper. Table 8 summarizes some of the paper strength test results of the phenolic-grafted pulp experiments. The strength increases were due to the improvement of hydrogen bonding between fibers and the cross-linking between phenoxy radicals within the sheet.




PAA 4-HBA Gallic acid

**Figure 78.** Phenolic acids for the modification of high kappa pulp.

**Table 8.** Paper strength test result for high lignin kraft pulp treated with laccase and phenolic acids.

| | Treatment | | | |
|---|---|---|---|---|
| Physical properties of paper | Control | Laccase | Phenolic acid | Laccase + Phenolic acid |
| 4-Hydroxybenzoic acid (4-HBA)-treated experiment [31] | | | | |
| Apparent density ($g/cm^3$) | 0.43 | 0.44 | 0.42 | 0.47 |
| Burst index ($kPa.m^2/g$) | 2.38 | 2.39 | 2.42 | 2.95 |
| Tensile index (N.m/g) | 36.65 | 38.87 | 36.98 | 42.10 |
| 4-Hydroxyphenylacetic acid (PAA)-treated experiment [30] | | | | |
| Apparent density ($g/cm^3$) | 0.38 | 0.39 | 0.38 | 0.39 |
| Burst index ($kPa.m^2/g$) | 1.76 | 2.10 | 1.76 | 2.16 |
| Tensile index (N.m/g) | 31.40 | 33.46 | 30.56 | 34.54 |
| Gallic acid-treated experiment [29] | | | | |
| Apparent density ($g/cm^3$) | 0.41 | 0.42 | 0.42 | 0.43 |
| Burst index ($kPa.m^2/g$) | 2.46 | 2.40 | 2.41 | 2.68 |
| Tensile index (N.m/g) | 33.9 | 33.8 | 34.0 | 40.3 |
| Wet tensile index (N.m/g) | 1.38 | 1.74 | 1.21 | 2.26 |

Viikari et al. [28] reported the modification of the fiber surfaces of thermomechanical pulp (TMP) by laccase and tyramine via a two-stage functionalization method. This method consists of an enzymatic activation of fiber surfaces followed by the addition of radicalized compounds that react preferentially through radical coupling. The degree of bonding in this study was determined by electron spectroscopy for chemical analysis (ESCA) which showed an increase in nitrogen content which originated from nitrogen in tyramine. The results showed that the nitrogen content of laccase-tyramine treated unbleached and bleached TMP increased to 0.6% and 1.5%, respectively. In addition, the FTIR spectra of tyramine-grafted samples indicated the

formation of ether linkages at 1060 $cm^{-1}$. Therefore, the authors suggest that tyramine was bond by ether linkage to the pulp. The proposed structure of the modified fiber is illustrated in Figure 79. The mechanism was suggested to start with one electron oxidation of the phenolic hydroxyl groups of both lignin and tyramine to generate the corresponding radicals. These radicals then react via a radical coupling reaction to form the corresponding tyramine-bonded lignin (Figure 80).

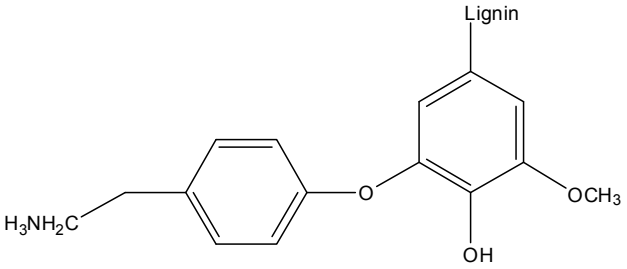



**Figure 79.** The proposed structure of the modified TMP with tyramine by laccase.[28]

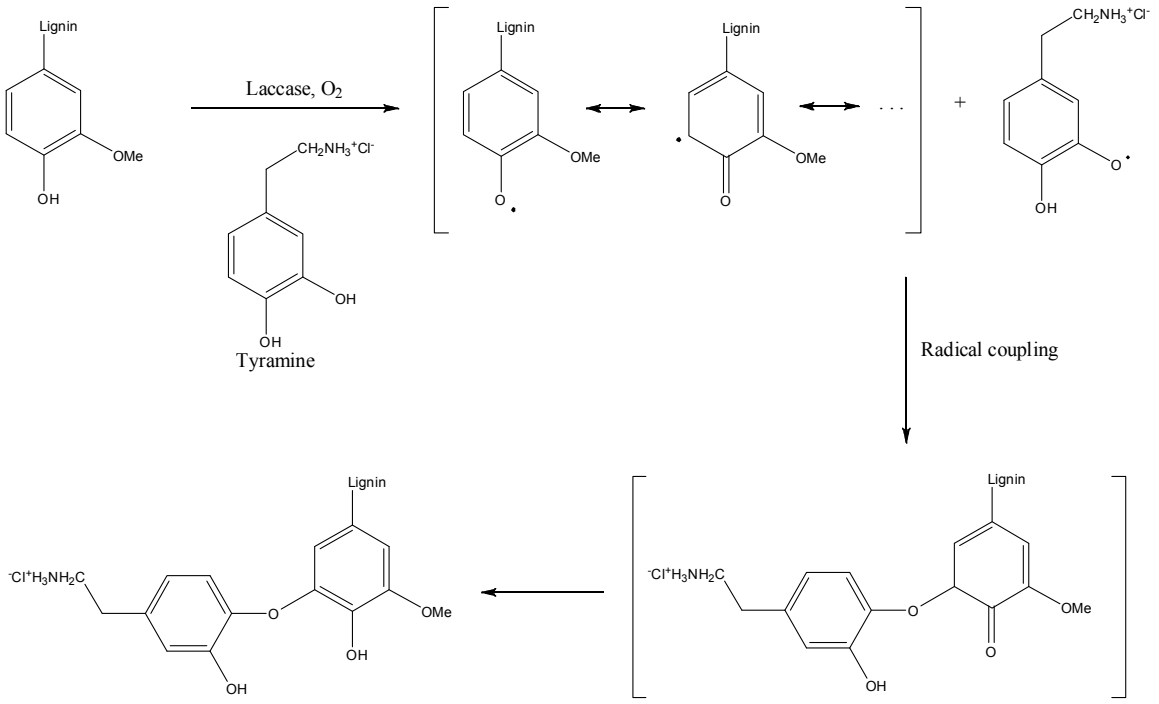



**Figure 80.** Proposed mechanism for grafting of tyramine to lignin by laccase.[28]

Recently, Elegir and his co-workers developed antimicrobial cellulose packaging through laccase-mediated grafting of antimicrobial active phenolic compounds, such as caffeic acid and isoeugenol, with unbleached kraft liner fibers [300]. Schroder et al. [301] reported the grafting of lignocellulosic surfaces with methoxyphenols and hydroquinone catalyzed by laccase to generated color and bacterial resistant lignocellulosic fibers. Moreover, Kim et al. [302] examined enzymatic polymerization on the surface of functionalized cellulose fibers. In Kim's study, laccase catalyzed the polymerization of catechol on the surface of aminized cellulose to from polycatechol-coated aminized cellulose (Figure 81).

Lignin itself has also been reported to be grafted onto lignocellulosic fibers. For example, an ultra-filtered lignin isolated from kraft black liquor was linked with kraft liner pulp and chemi-thermo-mechanical pulp by laccase from *Trametes pubescens.* This modification provided more than a twofold increase in wet strength of kraft liner pulp [303].

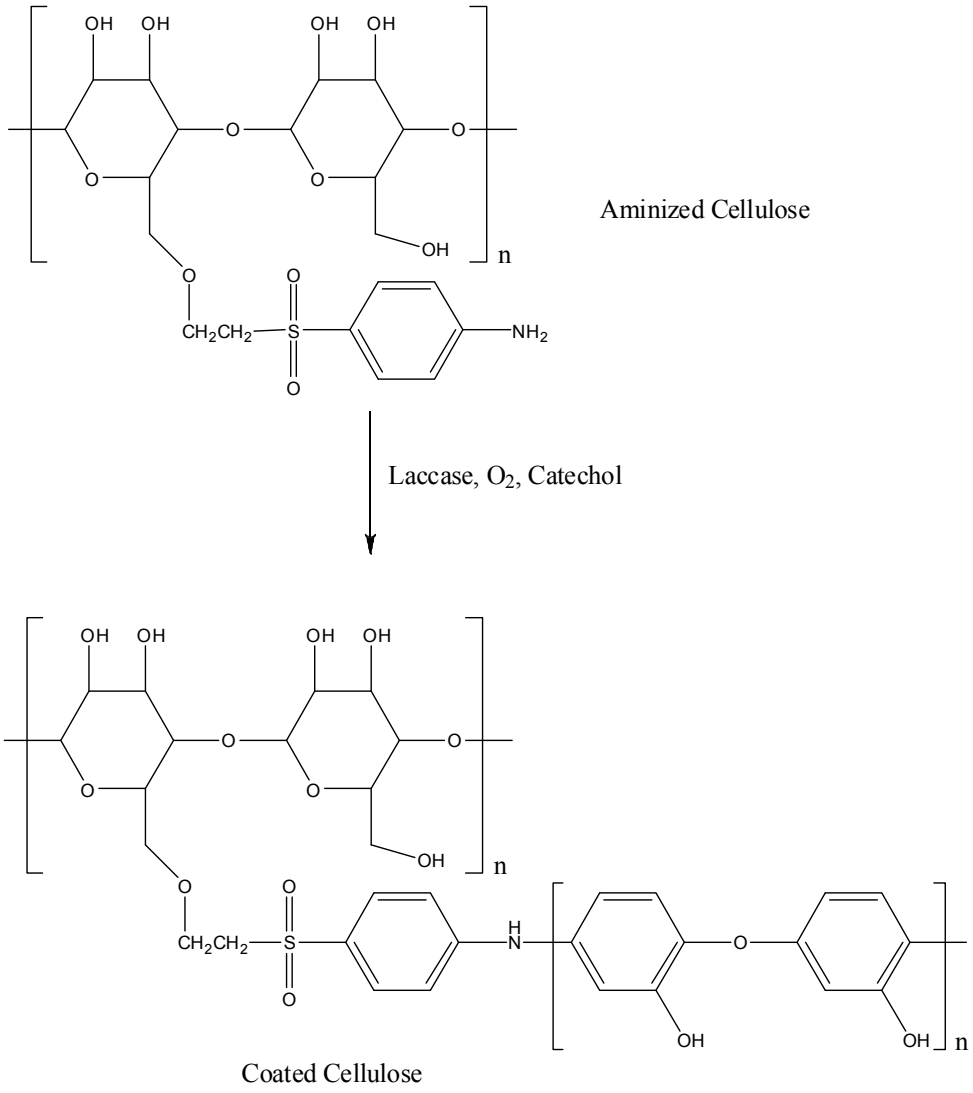



**Figure 81.** Laccase catalyzed Coupling reaction of aminized cellulose with catechol.[302]

### 2.4.6 Conclusions

Due to their high stability, selectivity for phenolic substructures, and mild reaction conditions used in laccase-catalyzed reactions, laccases are attractive for fine chemical synthesis and numerous synthetic processes have now been reported. A number of the laccase catalyzed reactions provide routes for the synthesis of biologically active compounds that have pharmaceutical significance. Moreover, the use of laccase as a biocatalyst in the synthetic methods is primarily used to develop more environmentally friendly processes when compared to the usual chemical-based synthetic processes that involve the use or disposal of harzardous chemicals. The laccase catalytic processes produce water as the sole-by product, and therefore could be ecologically friendlier. For example, the chemical synthesis of phenoxazine derivatives involves the condensation of the highly toxic, nitroso compounds, at elevated temperatures. Therefore, laccase was used instead of chemical reagent to catalyze the synthesis of phenoxazines in water at ambient temperature to provide greener synthetic method [225,226].

The laccase-catalyzed reactions are comparable to the chemical routes regarding to reaction rate, purity of the products, stability of the products in the reaction medium, and yields. For example, the formation of products from the nuclear amination reaction-catalyzed by laccase is comparable with reaction using sodium iodate as oxidant [237]. However, there are still some disadvantages of using laccase in the organic synthesis including the presence of buffer salts and protein in reaction medium makes the isolation process more difficult, the price of laccase is more expensive than chemical reagents, and the requirement of sufficient amount of oxygen for the catalytic system.

Apart from the use of laccase in organic synthesis, laccase-assisted modification of wood also has potential in the area of the forest products industry. The main benefits of laccase-catalyzed biografting of molecules to wood fibers are probably the absence of harmful solvents and chemicals and the mild reaction conditions. Due to the versatility, non-toxicity, and mild application conditions of laccase technology, laccase is likely to remain the subject of intensive investigations in many areas of biocatalyst applications.

## 2.5 Lipases

### 2.5.1 A General Account

Lipases (EC 3.1.1.3, triacylglycerol hydrolase) belong to the family of hydrolases that act on carboxylic ester bonds. Their physiological role is to catalyze the hydrolysis of triglycerides to diglycerides, monoglycerides, fatty acids, and glycerols. They can also catalyze the formation of acylglycerols from free fatty acids and glycerol (Figure 82) [304-306].

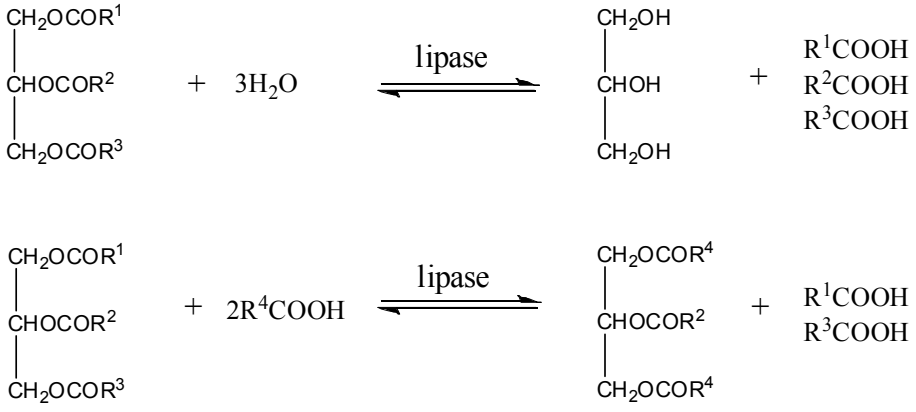



**Figure 82.** Lipase-catalyzed reactions of triacylglycerols.[307]

Lipases are widely found in animals, plants, and microorganisms [308,309]. Currently, several lipases are commercially available. The majority of commercial lipases are produced by fungi, yeast, and bacteria because of the ease of cultivating these microorganisms on a large scale. In general, lipases are extracellular-acidic glycoproteins. The molecular size of lipases is between 20 and 60 kDa [304]. Structural characteristic include an α/β-hydrolase fold and a nucleophilic elbow where the catalytic serine is located [307,310]. In addition, most lipases contain a 'lid' which is a helical oligopeptide that shields the active site. This lid will open to provide free access for the substrate when the enzyme interacts with a hydrophobic interface such as a lipid droplet. Therefore, lipase changes into an activated form by substrate activation at the lipid-water interface. This phenomenon is called interfacial activation and is unique structural characteristic of this class of enzymes [304,311].

Lipases can be classified into three major groups according to their ability to hydrolyze glycerides [304]. The first group is termed as 1,3-specific because they can hydrolyze only the terminal positions of triglycerides. Since their substrate range is not limited to triglycerides, this group can be regarded as lipases capable of hydrolyzing primary and to a small extent secondary esters. Lipases in this group include lipases of *Rhizopus* and *Rhizomucor.* The second lipase group can be termed as nonspecific because they can hydrolyze both primary and secondary esters. The last group consists of those few lipases that are positionally nonspecific but show fatty acid selectivity, cleaving only ester bonds wherein the fatty acid is of particular type. In addition, lipases may also exhibit chain length specificity.

In general, most animal lipases exhibit pH optima on the alkaline side, pH 8.0 - 9.0, while most microbial lipases show maximum stability in the neutral pH range [312]. Most lipases are optimally active at temperatures between 30 and 40 ºC [304]. Usually, animal and plants lipases are less thermostable than the microbial extracellular lipases [313].

The broad synthetic potential of lipases is largely because they possess broad substrate specificity and tolerate organic solvents. Substrates other than triglycerides include aliphatic, alicyclic, bicylic, and aromatic esters. Moreover, a wide range of thioesters and activated amines can also be substrates for lipases. Lipases can be employed for a variety of reactions such as esterification, interesterification, acidolysis, alcoholysis, and aminolysis (Figure 83) [304,307,311,314-317]. In addition, lipases do not require cofactors, and usually exhibit high chemoselectivity, regioselectivity, and enantioselectivity. These properties make lipases the most versatile biocatalyst. Besides the application of lipases in synthetic chemistry, the application of lipases are also found in the detergent, food, leather, textile, oil and fat, cosmetic, paper and pharmaceutical industries [305,318,319].

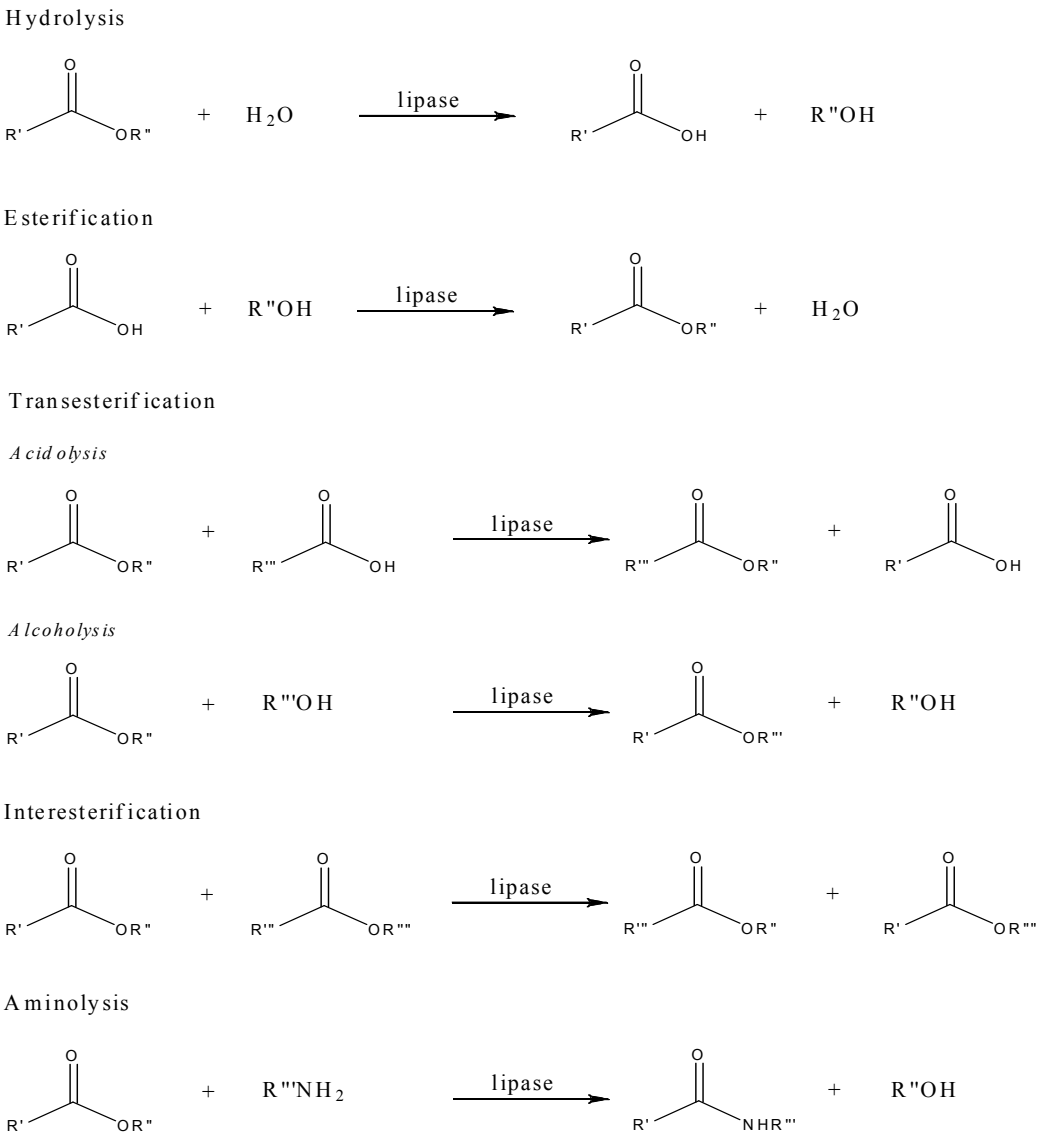
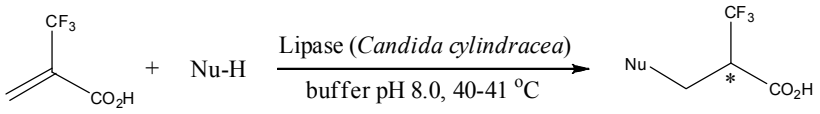



**Figure 83.** Examples of lipase-catalyzed reactions.[304]

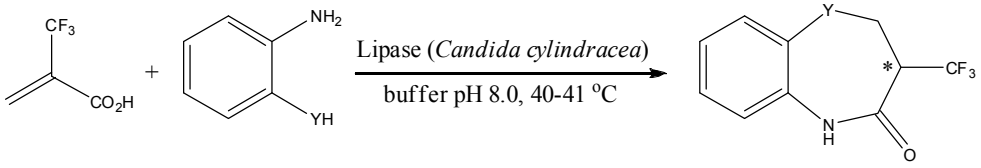
Besides catalyzing the reactions in Figure 83 above, lipases are reported to catalyze the Michael addition reaction, the 1,4-addition of a nucleophile to an α,β-unsaturated carbonyl compound. The proposed mechanism reported to involve the stabilization of the negative charge of the transition states in the oxyanion hole of the active site, and the His-Asp pair serves as a proton shutter. The following section will focus on the Michael reaction catalyzed by lipases.

### 2.5.2 Lipase-Catalyzed Michael Reaction

In 1986, Kitazume et al. [320] showed the possibility of hydrolases including lipase from *Candida cylindracea* to catalyze Michael addition reactions. In this study, optically active aliphatic and heterocyclic compounds possessing a trifluoromethyl group were synthesized via an enzymatic chiral Michael addition reaction of 2-(fluoromethyl)propenoic acid. The reactions were conducted in buffer solution pH 8.0 ($Na_2HPO_4$ and $KH_2PO_4$ solution) at 40 ºC and yielded the chiral products in the range of 40 to 90 % (Figure 84).




| Nu-H | Time (h) | Yield (%) | % e.e. |
|---|---|---|---|
| $H_2O$ | 34 | 48 | 70 |
| $Et_2NH$ | 92 | 47 | 71 |
| $PhNH_2$ | 40 | 76 | 39 |




| Y | Time (h) | Yield (%) | % e.e. |
|---|---|---|---|
| O | 21 | 83 | 41 |
| S | 20 | 86 | 47 |
| N | 24 | 56 | 38 |

**Figure 84.** Asymmetric Michael addition reaction of 2-(trifluoromethyl)propenoic acid catalyzed by lipase from *Candida cylindracea* (* represents chiral center).[320]

Torre et al. [321] has reported that lipase B from Candida antractica (CAL-B) can catalyze a Michael addition of a secondary cyclic and non-cyclic amine to acrylonitrile. The reactions were conducted in toluene at 30 ºC. In the presence of CAL-B, the rate of the reactions were up to 100-fold faster than the reaction in absence of the biocatalyst. The proposed mechanism of this process is summarized in Figure 85. The mechanism starts with the accommodation of acrylonitrile in the active site. Then, the conjugated addition of the necleophile leads to a zwiterionic intermediate stabilized by both the oxyanion hole and the His-Asp pair. This His-Asp pair catayzes proton transference from the incoming nucleophile to the α-carbon. Finally, a new acrylonitrile molecule shifts the final product, leading to a new catalytic cycle.

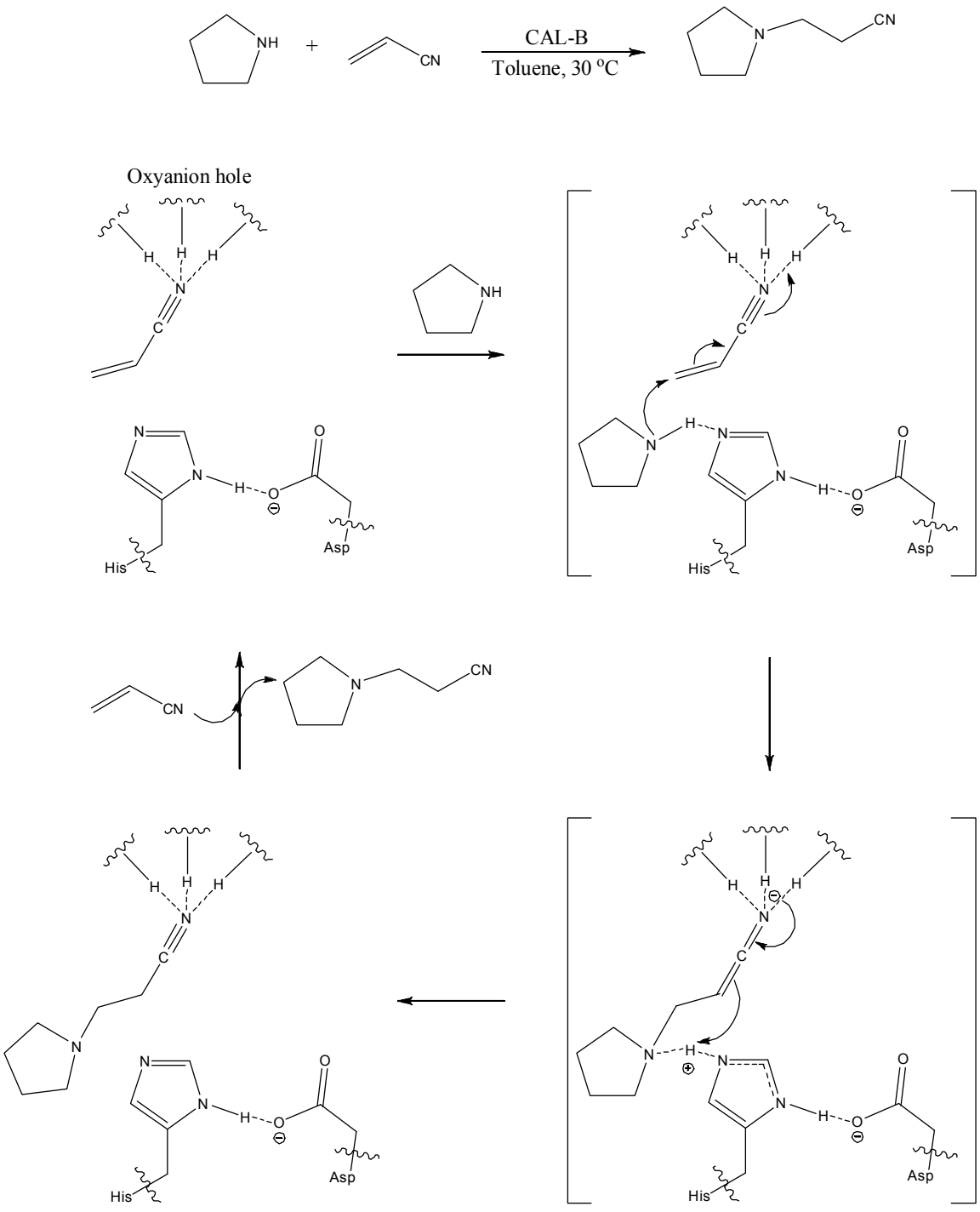
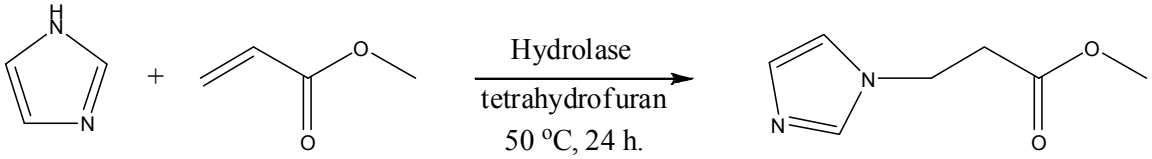



**Figure 85.** Proposed mechanism of lipase catalyzed Michael addition of pyrrolidine and acrylonitrile.[321]

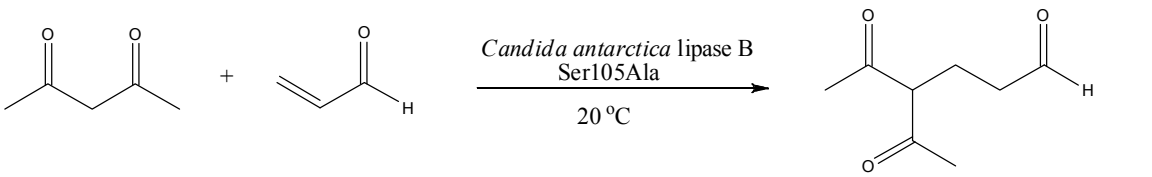
Recently, Dai and his co-workers [322] showed the ability of lipase M from *Mucor javanicus* in the synthesis of pyrimidine derivatives containing a branched sugar which may possess potential antitumor and antivirus activities. In this study, lipase M catalyzed the Michael addition reaction of pyrimidine with disaccharide acrylate in pyridine at 50 ºC for 72 hours to obtain the final products in yields from 56 to 75%. In addition, the study of hydrolase-catalyzed Michael addition of imidazole derivatives to acrylic monomers in organic medium has also been investigated [323]. A variety of hydrolases were used as catalysts in this study and the reactions were conducted in organic solvents at 50 ºC for 24 hours. All hydrolases were found to be able to catalyze this Michael addition reaction and lipase M showed to be the most efficient hydrolase with the percent conversion close to 100% after 24 hours. Figure 86 illustrates some results of this study.




| Hydrolase | Conversion (%) |
|---|---|
| None | Not detected |
| Alkaline protease from *Bacillus subtilis* | 93.0 |
| Proteinase from *Aspergillus oryzae* | 81.9 |
| Lipase B acrylic resin from *Candida Antarctica* | 88.7 |
| Lipase from *Candida cylindracea* | 86.0 |
| Amano Lipase M, from *Mucorjavanicus* | 96.0 |

**Figure 86.** Michael addition of imidazole and methyl acrylate catalyzed by a variety of hydrolases.[323]

Berglund et al. exhibited the possibility of ultilizing the mutant, *C. Antarctica* B Ser105Ala, to catalyze the Michael addition of thiol and amine nucleophiles to α,β-unsaturated carbonyl compounds in organic solvent [324]. The mutant enzyme was designed by the substitution of Ser 105 to Alanine in the active-site of *C. Antarctica* lipase B. This mutation led to a change in the catalytic mechanism of the enzyme. According to turnover numbers from kinetic studies, the Ser105Ala mutant of *C. Antarctica* lipase B was more efficient than the wild-type enzyme, *C. Antarctica* lipase B, for the catalysis of the Michael type reaction. Recently, they also studied the use of this Ser105Ala mutant of *C. Antarctica* lipase B in the catalysis of carbon-carbon bond formation between 1,3-dicarbonyls and α,β-unsaturated carbonyl compounds (Figure 87) [325]. The ability of wild-type and Ser105Ala mutant of *C. Antarctica* lipase B to catalyze this Michael reaction was investigated under solvent free conditions. The results showed that the reactions proceeded approximately 1.3 to 830 times faster with the mutant than with the wild-type enzyme. In addition, the uncatalyzed reaction, without enzyme, demonstrated a very low reaction rate. This indicates that the enzyme catalyzed the Michael addition reactions.




100% conversion (in less than 10 minute)

**Figure 87.** Michael addition of acetylacetone to acrolein catalyzed by a *C. Antarctica* lipase B Mutant.[325]

# CHAPTER 3

# EXPERIMENTAL MATERIALS AND PROCEDURES

## 3.1 Materials

### 3.1.1 Chemicals

All chemicals, except 2-methoxyhydroquinone, were obtained from Aldrich. 2-Methoxyhydroquinone was obtained from TCI America. All chemicals were used as received without further purification. Solvents, including ethyl acetate, hexane, petroleum ether, and acetone, were obtained from VWR and used as received without further purification. Water in all experiments was deionized water.

### 3.1.2 Enzymes

Laccase (EC 1.10.3.2) used in this study was donated by Novozymes (Franklinton, North Carolina). The laccase (NOVO NS51002) was isolated from the white-rot fungus *Trametes villosa* and expressed in an *Aspergillus* host. Lipases were purchased from Aldrich. Unit definition of each lipase is different depending on the method that Aldrich used to measure lipase activity. All enzymes were kept frozen until use.

#### 3.1.2.1 Enzyme Assay

Laccase activity was determined by oxidation of 2,2'-azinobis-(3-ethylbenzyl thiozoline-6-sulphonate) (ABTS) [326].The assay mixture contained 25 μM ABTS, 0.10 M sodium

acetate (pH 5.0), and a suitable amount of enzyme. The oxidation of ABTS was followed by an absorbance increase at 420 nm ($\varepsilon_{420}$ = 3.6 x 104 $M^{-1}cm^{-1}$) (see Figure 88 and Figure 89). Enzyme activity was expressed in units (U = μmol of ABTS oxidized per minutes).

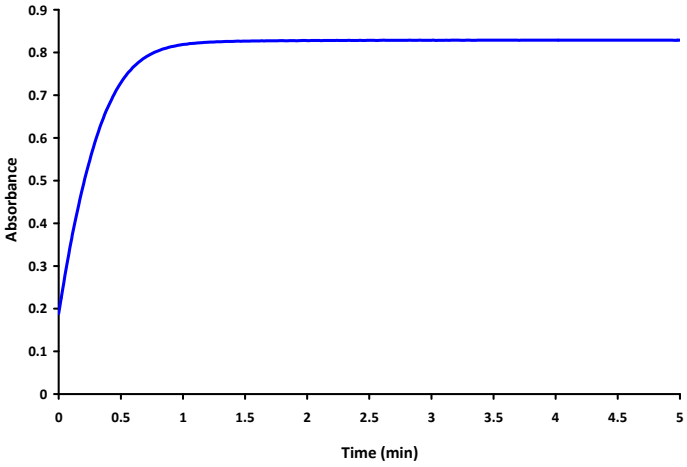



**Figure 88.** Graph illustrates the absorbance increase of laccase-oxidized ABTS at 420 nm.

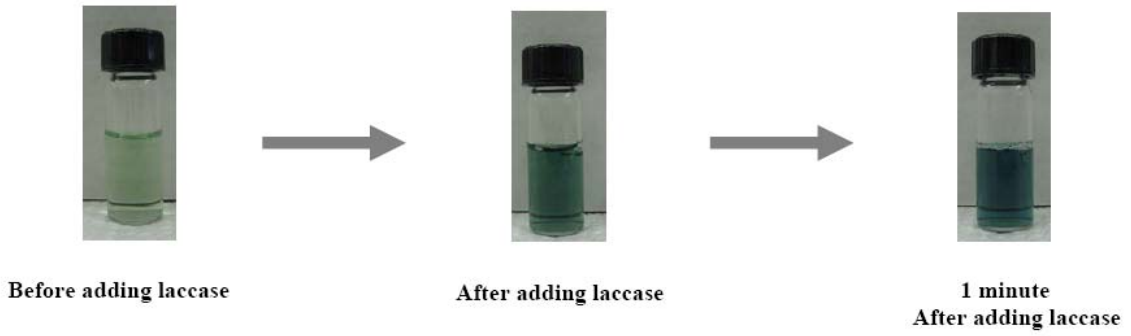



**Figure 89.** Picture illustrates the changing in color of ABTS (in water) after adding laccase. The color changes from bright green to dark green.

### 3.1.3 Pulp

A commercial linerboard softwood kraft pulp (17% of lignin content, kappa number is 113) was obtained from a southeastern U.S.A manufacturing facility. The lignin content of the kraft pulps was determined by $KMnO_4$ titration of the pulp following TAPPI method T-236 [327] and expressed as a "kappa number". This value is an indirect measurement of lignin content (% lignin content = 0.15 x kappa number). The pulp was exhaustively washed until the filtrate was pH neutral and colorless. Pulp was air dried and soxhlet extracted (see Figure 90) for 24 hours with acetone with subsequent washing with water prior to all treatments.

**Figure 90.** Photograph of the equipment set for soxhlet extraction.

## 3.2 Experimental Procedures for the Use of Laccase in Organic Synthesis

### 3.2.1 General Information

All reactions were monitored by TLC. TLC was performed on aluminum sheets precoated with silica gel 60 F254 (EMD Chemicals). Melting point was measured using electrothermal MEL-TEMP instrument.

Column chromatography was performed on Combiflash Companion instrument (Teledyne Isco company) (Figure 91). The Combiflash Companion is a flash chromatography system which provides a fully automated system from solvent injection to product collection. Columns used with this instrument are pre-packed columns (RediSep columns). RediSep normal-phase silica flash columns were used in this study. The column size is 12 g or 40 g, depending on sample size. The linear gradient elution was used to separate mixture of the products and the flow rate is 25 – 40 ml/min.

**Figure 91**. Picture of Combiflash Companion instrument (Teledyne Isco company) with 40 g RediSep normal-phase silica flash columns

### 3.2.2 Analytical Analysis Procedures

#### 3.2.2.1 $^{1}$H-NMR Characterization of the Products

$^{1}$H-NMR spectra were recorded on a Bruker Advance/DMX-400 instrument operating at 400 MHz. The qualitative $^{1}$H experiments were performed using using a 90$^{o}$ pulse and 3.0 s delay. The acquisitions were performed at room temperature with 24 – 120 scans and a 1 Hz line broadening.

#### 3.2.2.2 $^{13}$C-NMR Characterization of the Products

$^{13}$C NMR spectra were recorded on a Bruker Advance/DMX-400 instrument operating at 100 MHz. Acquisition was performed using a 90$^{o}$ pulse with a gate-decoupling pulse sequence and 2.0 s delay between repetitions. The acquisitions were performed at room temperature with 400 - 4000 scans and a 10 Hz line broadening.

#### 3.2.2.3 Fourier Transform Infrared (FTIR) Spectroscopy

Fourier Transform Infrared (FTIR) transmission spectra were collected for each of the samples in the solid state using a Magna-IR System 550 (Nicolet Instrument Corporation). Number of scans was 64 for each sample. Pellets were formed by pressing mixtures of 3 mg of dry sample and 500 mg of dry spectroscopy grade potassium bromide (KBr) at 15000 psi for 3 min. under vacuum.

#### 3.2.2.4 Mass Spectroscopy

Mass and high resolution mass spectra were carried out in The Georgia Institute of Technology Bioanalytical Mass Spectrometry Facility. The mass analysis was

performed in VG instruments 70SE. The VG-70SE is capable of high resolution (~ 50,000 at 10% valley) and accurate mass measurement (< 5 ppm) analyses. It is equipped with a dedicated GC and is capable of ionization via electron impact (EI) and chemical ionization (CI) for analysis of low molecular mass (< 700 Da), non-polar, volatile molecules.

3.2.2.5 UV/Vis for Enzyme Activity Measurement

Laccase activity was measured using a Perkin-Elmer Lambda 900 UV/vis spectrometer (Perkin Elmer, Waltham, MA, USA) equipped for measuring liquid samples. The Ultraviolet-visible (UV/vis) absorbtion spectra were scan at 420 nm for 5 minutes. The example spectrum is shown in Figure 88.

### 3.2.3 General Procedure of the Synthesis of 1,4-Naphthoquinones and Related Structures. (Chapter 4)

Oxygen was bubbled to a stirred solution of 30 ml of 0.10M acetate buffer (pH 4.5) and laccase (100 U) at 70 °C for 30 minutes. Next, *p*-hydroquinone (1.00 mmol) and diene (2.00 mmol) were added into the reaction mixture, and stirred under air, at 70 °C (Figure 92). In the first three hours of the reaction, 100 U of laccase was added each hour. After 24 hours of the reaction, the reaction mixture was extracted by EtOAc (3 x 30 ml). The organic phase was combined, dried over $MgSO_4$, and evaporated. The resulting crude products were purified by Combiflash Companion instrument using Redisep normal-phase silica column. Ethyl acetate and hexane (linear gradient: 0 – 30% EtOAc)

were used as an eluent to obtain the products. Products were characterized by $^{1}$H-NMR, $^{13}$C NMR and MS.

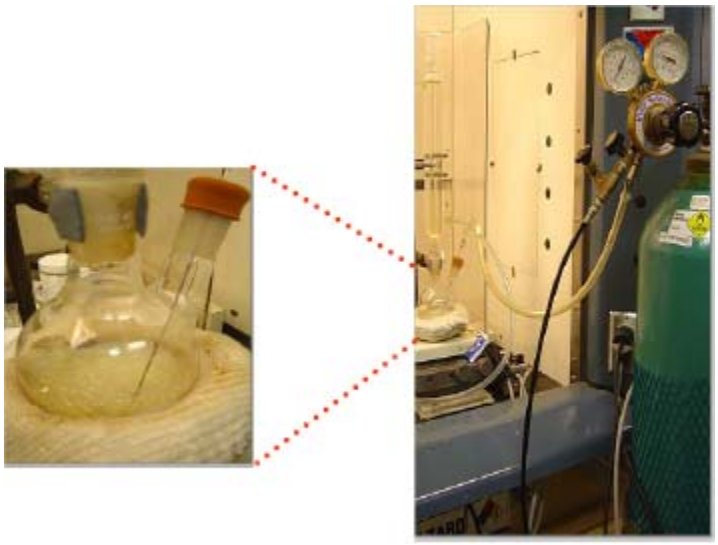


**Figure 92.** The reaction setting of the synthesis of 1,4-naphthoquinones and related structures *via* laccase-catalyzed Diels-Alder reaction.

### 3.2.4 General Procedure of the Synthesis of *o*-Naphthoquinones. (Chapter 5)

In a 250-mL round-bottom flask, 20 ml of cold 0.10M acetate buffer pH 4.5 and diene (10.00 mmol) were mixed together. The flask was then placed in an ice bath over a stirring plate. Next, 1.00 mmol of catechol dissolved in 20 mL of 0.10M acetate buffer, and laccase (100U) were added to the flask drop-wise. In the next three hours of the reaction, 100 U of laccase was added each per hour.The reaction was then stirred under room temperature. After 24 hours of the reaction, the reaction mixture was extracted by EtOAc (3 × 30 ml). The organic phase was combined, dried over $MgSO_4$, and evaporated. The resulting crude products were purified by Combiflash Companion

instrument using Redisep normal-phase silica column. Ethyl acetate and petroleum ether (linear gradient: 0 – 30% ethyl acetate) were used as an eluent to obtain the product. Products were characterized by $^{1}$H-NMR, $^{13}$C NMR and MS.

### 3.2.5 General Procedure of the Synthesis of Benzofuran Derivatives via Laccase-Oxidation-Michael Addition. (Chapter 6)

In a 250-mL round-bottom flask, 30 ml of 0.10 M phosphate buffer pH 7.0 and catechol (1.00 mmol) were mixed together. Next, 100 U of lacase was added to reaction mixture and then, 1,3-dicarbonyl compound (2.00 mmol), $Sc(OTf)_3$ (0.20 mmol, 0.0984 g), SDS (0.20 mmol, 0.0576g), and laccase (100 U) were added. The reaction was then stirred under air at room temperature for 1-4 hours. After the reaction was finished, the reaction mixture was then filtrated and washed with water to collect the precipitate product. If the product did not precipitate, the reaction mixture was extracted by EtOAc (3 × 30 ml). The organic phase was combined, dried over $MgSO_4$, and evaporated. The resulting crude products were purified by Combiflash Companion instrument using Redisep normal-phase silica column. Ethyl acetate and petroleum ether (linear gradient: 0 – 20% ethyl acetate) were used as an eluent to obtain the benzofuran product. Products were characterized by $^{1}$H-NMR, $^{13}$C NMR and MS.

### 3.2.6 General Procedure of the Synthesis of Benzofuran Derivatives Using Laccase-Lipase Co-Catalytic System. (Chapter 7)

In a 250-mL round-bottom flask, 30 ml of 0.10 M phosphate buffer pH 7.0 and catechol (1.00 mmol) were mixed together. Next, 100 U of laccase was added to reaction mixture and then, 1,3-dicarbonyl compound (2.00 mmol) and 924 U of lipase PS were added. The reaction was then stirred under air at room temperature for 4 hours. After the reaction was completed, the reaction mixture was extracted by EtOAc (3 × 30 ml). The organic phase was combined, dried over $MgSO_4$, and evaporated. The resulting crude products were purified by Combiflash Companion instrument using Redisep normal-phase silica column. EtOAc and petroleum ether (linear gradient: 0 – 20% ethyl acetate) were used as an eluent to obtain the benzofuran product. Products were characterized by $^{1}$H-NMR, $^{13}$C NMR and MS.

### 3.2.7 General Procedure for the Reaction of Catechols and Anilines Catalyzed by Laccase-Lipase Co-Catalytic System. (Chapter 7)

In a 250-mL round-bottom flask, 30 ml of 0.10 M phosphate buffer pH 7.0 and catechol (1.00 mmol) were mixed together. Next, 100 U of laccase was added to reaction mixture and then, aniline (2.00 mmol) and 924 U of lipase PS were added. The reaction was then stirred under air at room temperature for 3.5 hours. After the reaction was finished, the reaction mixture was filtered to collect the solid red color product**.** Products were characterized by $^{1}$H-NMR, $^{13}$C NMR and MS.

## 3.3 Experimental Procedures for the Use of Laccase in Fiber Modification

### 3.3.1 Pulp Treatment

Laccase (80 U/1g pulp) and an amino acid (3.2 mmol/1g pulp) were added with stirring to a 5% consistency [mass pulp/(mass pulp + water)] aqueous suspension of linerboard pulp buffered to pH 7 with 0.10 M sodium phosphate solution. The resulting slurry was stirred for 4 h at room temperature and then left stand 20 h. After treatment, the pulp sample was filtered, washed with deionized water until the filtrate was colorless and air-dried. Typically, pulp mass recovery was 95% (on oven dried weight basis).

### 3.3.2 Bulk Acid Group Measurement

Conductrometric titration for bulk acids was based on the work of Katz [328]. In brief, pulp (1.50 g o.d.) was stirred in 300.00 ml of 0.10 M HCl for 1 hour followed by rinsing in a fine fritted funnel with deionized water. The sample was then re-suspended in 250.00 ml of 1 mM NaCl solution, spiked with 1.50 ml of 0.10 M HCl and titrated against 0.05 M NaOH at 0.25 ml increments in an atmosphere of nitrogen, recording the conductivity at each increment. The titration data was plotted as conductivity vs. volume of NaOH to determine the milli-equivalent of acid groups per g of pulp (Figure 93). Trend lines were added in Excel in order to draw lines through each linear region on the graph. A line across the "flat" portion of the curve was plotted too. The intersections of the left trendline and the right trendline with the flat line were obtained, and their X-axis values are represented by *A* and *B* (Figure 93). The carboxylic acid content of pulp fibers

is obtained using Equation 3. The reported results were the average of two measurements which typically differed by less than 3%.

$$\text{RCOOH content} = \frac{(B - A) \times 5}{w} \text{ mmol/ 100 g o.d. pulp}$$

where w is the oven dried (o.d.) weight of the pulp sample in grams.

**Equation 3.** The equation used to calculate for the carboxylic content of pulp fibers.

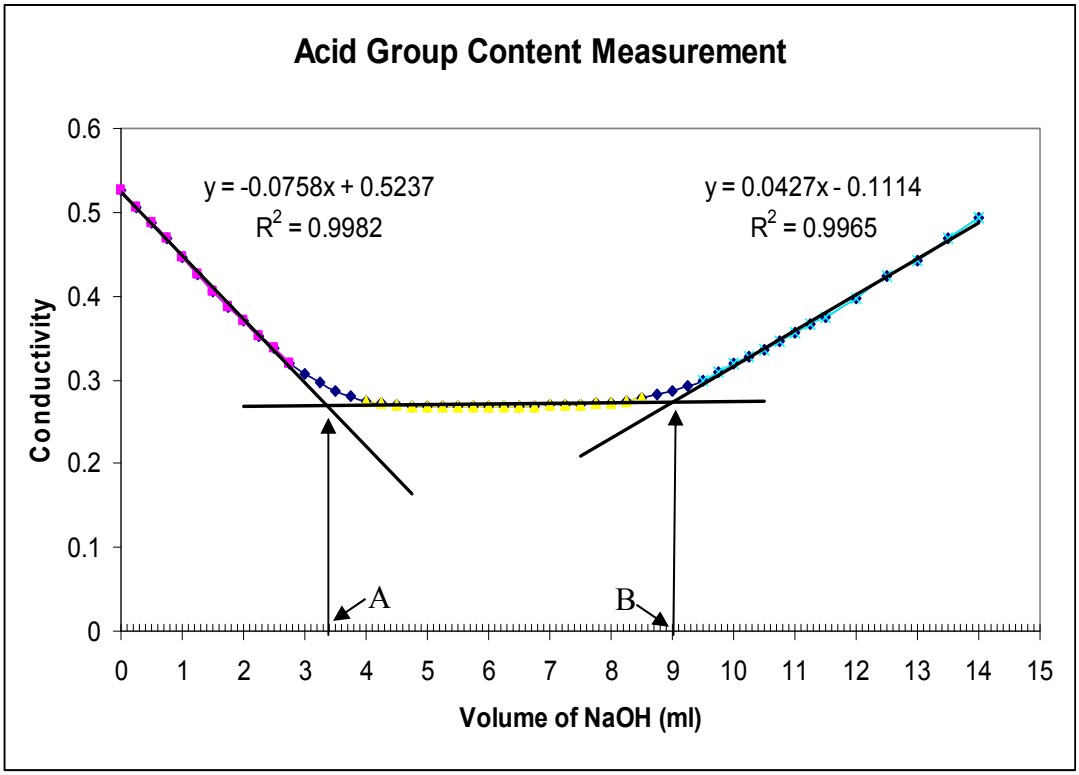



**Figure 93.** The titration data plotted as conductivity vs. volume of NaOH for the calculation of carboxyl group (RCOOH) content using conductivity method.

### 3.3.3 Pulp Refining and Handsheet Formation

Treated pulps and control were disintegrated for 30,000 revolutions (Figure 94) and then were refined in a PFI mill (Figure 95) for 3,000 revolutions according to TAPPI Standard T 248 [327]. Handsheets were formed according to TAPPI Standard T 205 [327] (Figure 96) and TAPPI conditioned (23 ˚C, 50% relative humidity) for at least 24 hours before physical testing.

**Figure 94.** Picture of instrument used for pulp disintegration.

**Figure 95.** The PFI mill for the laboratory refining of pulp.

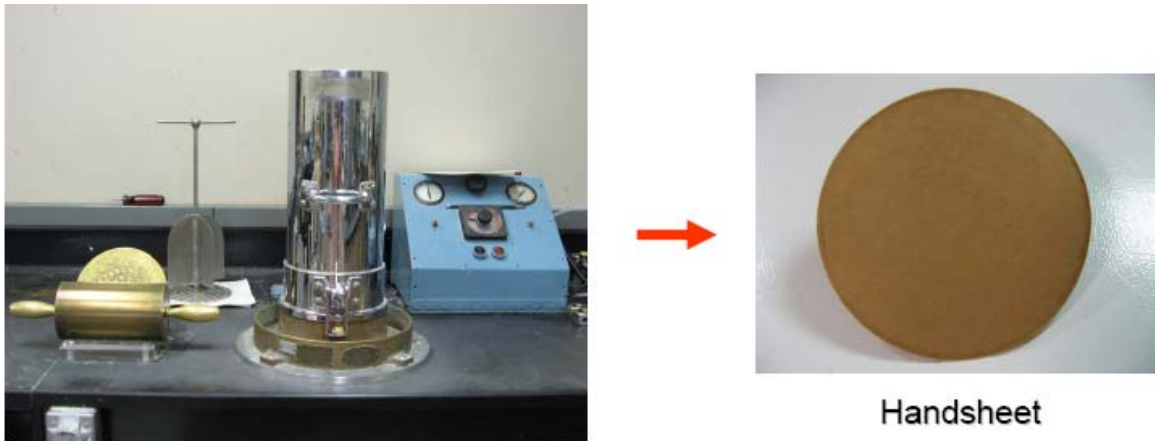



**Figure 96.** Handsheet making apparatus (left) and handsheet made from liner board softwood kraft pulp (right).

### 3.3.4 Paper Physical Tests

Apparent density, tensile strength, tearing resistance, and wet tensile strength were determine according to TAPPI methods T 210, T 494, T 414, and T 456 [327]. Apparent density was measured using a Lorenzten and Wettre caliper gauge. Tensile testing was carried out on an Lorentzen and Wettre Alwetron tensile tester, and wet tensile testing was measured on an Instron tester connected to a data analysis system running Test Works Software (Figure 97). Tear tests were performed on an Elmendorf tearing tester (Figure 98).

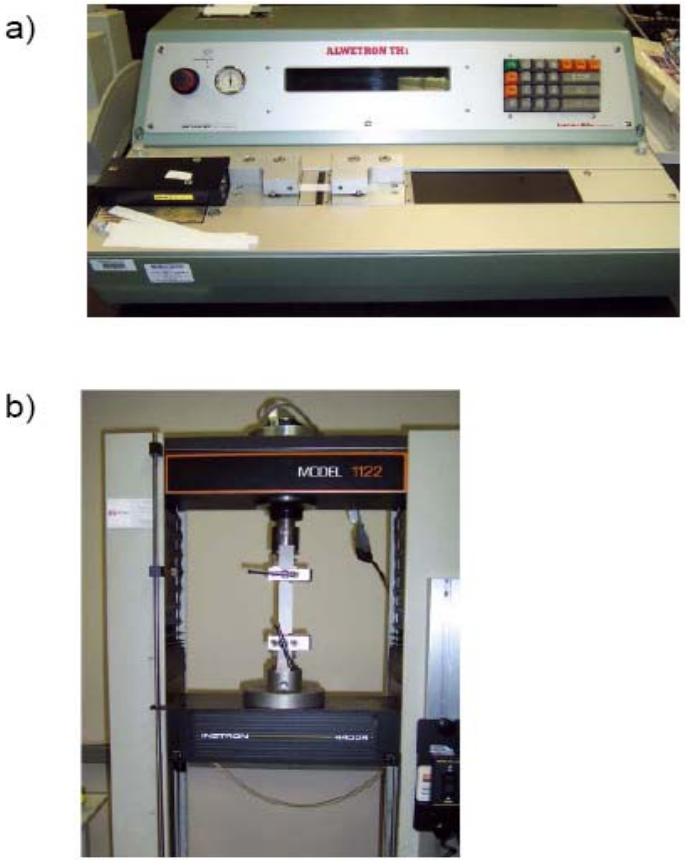



**Figure 97.** Tensile testers a) an Lorentzen and Wettre Alwetron tensile tester; b) an Instron tensile tester.

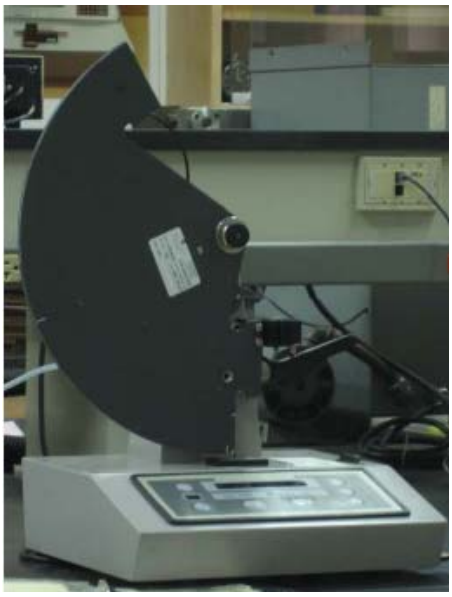


**Figure 98.** An Elmendorf tearing tester.

### 3.3.5 Nitrogen Analysis

Nitrogen analysis was performed on oven dry samples (24 hours, 105°C) by elemental microanalysis at Huffman Laboratories, Inc., Golden, CO. The results are reported on a dried sample basis.

Nitrogen is determined on a Thermo Flash analyzer. The technique is the classical Dumas method, with thermal conductivity detection. The method is described in ASTM D5373 (coal) and ASTM D5291 (petroleum products).

Weighed samples are combusted in oxygen at 950°C. The combustion products, including N and NOx, are swept with a helium carrier gas through combustion catalysts, scrubbers, and through a tube filled with reduced copper. The copper removes excess oxygen and reduces NOx to $N_2$. The $N_2$ is then separated from other gases on a chromatography column and measured with the thermal conductivity detection.

Precision is usually given as ± 0.3% absolute or 3% relative whichever is larger. The detection limit can be lowered by using larger samples. For organic materials 0.02% can be obtained. Lower detection levels can be obtained for samples consisting largely of inert materials such as soils.

### 3.3.6 Scanning Electron Microscope (SEM)

The SEM pictures of handsheets were taken using a Hitachi S-800 FE-SEM. The handsheet sample was stuck on the SEM sample holding stub by the conductive double side sticky carbon film and then was coated with alloy of Au/Pt prior to analysis.

# CHAPTER 4

# ONE-POT SYNTHESIS OF 1,4-NAPHTHOQUINONES AND RELATED STRUCTURES WITH LACCASE[i]

## 4.1 Introduction

The most abundant and available resource on the planet, one in which biochemical processes take place, is the aqueous medium, water. Recently, water has begun to be regarded as an environmentally friendly solvent in organic chemistry. In addition to its environmental benefits, the use of water as a solvent is both inexpensive and safe. In recent decades, the study of the organic reactions in aqueous solvent has accelerated and many, often, surprising discoveries have been made [36-38,41,329]. Breslow and Rideout [35] were the first to show the beneficial effects of water on the reactivity and selectivity of Diels-Alder reaction, quantitatively. This discovery stimulated further research in this area. Shortly after, several studies showed that many chemical reactions (such as pericyclic, condensation, oxidation, and reduction reactions) could be conducted efficiently in the aqueous medium [41,330-335].

Among the organic reactions investigated in the aqueous medium, the most widely studied reaction is the Diels-Alder reaction [34,43], a powerful tool frequently

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[i] This manuscript was published in [Green Chemistry, 2007, 9, 475-480]- Reproduced by permission of The Royal Society of Chemistry (RSC). It is entitled as "One-pot synthesis of 1,4-naphthoquinones and related structures with laccase". The other author is Dr. Arthur J. Ragauskas from the School of Chemistry and Biochemistry at the Georgia Institute of Technology.

employed to synthesize six-membered ring systems and one of the most useful reactions for introducing structural complexity in (total) synthesis [336-339]. The Diels-Alder reaction has many useful variations, one of which is its use in the synthesis of anthraquinones and naphthoquinones [340,341]. Naphthoquinones have attracted considerable attention in total synthesis because of their wide spectrum of biological activities, such as antitumor [342,343], wound healing [344], anti-inflammatory [344], and antimicrobial [345] and antiparasitic activities [346,347]. Another useful application of the Diels-Alder reaction is the quinone Diels-Alder (QDA) reaction (Figure 99) [56,61,65,348,349]. In this reaction, quinones are employed as dienophiles, which normally possess electron-withdrawing groups. This classed of quinones are usually unstable and difficult to isolate. To overcome these difficulties, many studies have focused on the Diels-Alder reaction of in situ-generated quinones [350-352]. Herein, we report the use of the enzyme, laccase, used in the in situ generation of quinones.

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are multi-copper-containing oxidoreductase enzymes widely distributed in plants and fungi. They are able to catalyze the oxidation of various low-molecular weight compounds, specifically, phenols and anilines; while concomitantly, reducing molecular oxygen to water [3-7,149,167]. Moreover, due to their high stability, selectivity for phenolic substructures, and mild reaction conditions used in laccase-catalyzed reactions, laccases are attractive for fine chemical synthesis. Therefore, interest in the potential use of these enzymes in organic synthesis has recently increased [11,13]. Indeed, a number of laccase-catalyzed reactions has been reported [11-19]. Recently, laccase was examined in the field of enzyme-initiated domino reaction chemistry. For example, utilizing their well known

propensity to oxidize phenolics, Lalk et al. [11] reported a laccase catalyzed a nuclear animation tandem reaction. These studies have demonstrated the synthetic research capabilities of this oxidative enzyme.

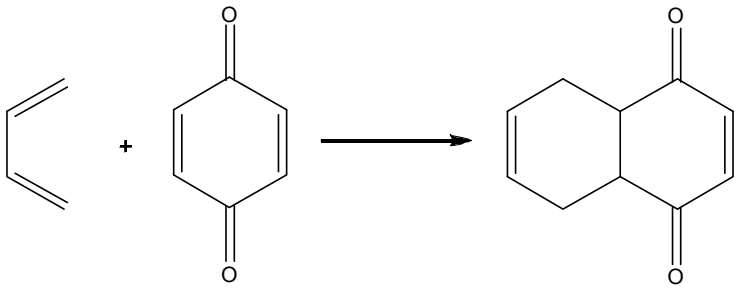


**Figure 99.** The Quinone Diels-Alder (QDA) reaction.

This study presents work on the synthesis of 1,4-naphthoquinones and related structures in the aqueous medium. In this procedure, para-quinone, generated in situ from the oxidation of para-hydroquinone by laccase, underwent the quinone Diels-Alder reaction with a diene, and then the Diels-Alder adduct was converted directly into dihydro 1,4-naphthoquinone. Upon extended treatment, this initial product was further oxidized to naphthoquinone as summarized in Figure 100. The effects of a laccase dose and temperature on these reactions, with the reaction of 2-methoxyhydroquinone (**1a**) and 2,3-dimethyl-1,3-butadiene (**2a**) as a model system, are reported here. This study also investigated the sensitivity of this reaction system to a variety of para-hydroquinones and dienes.

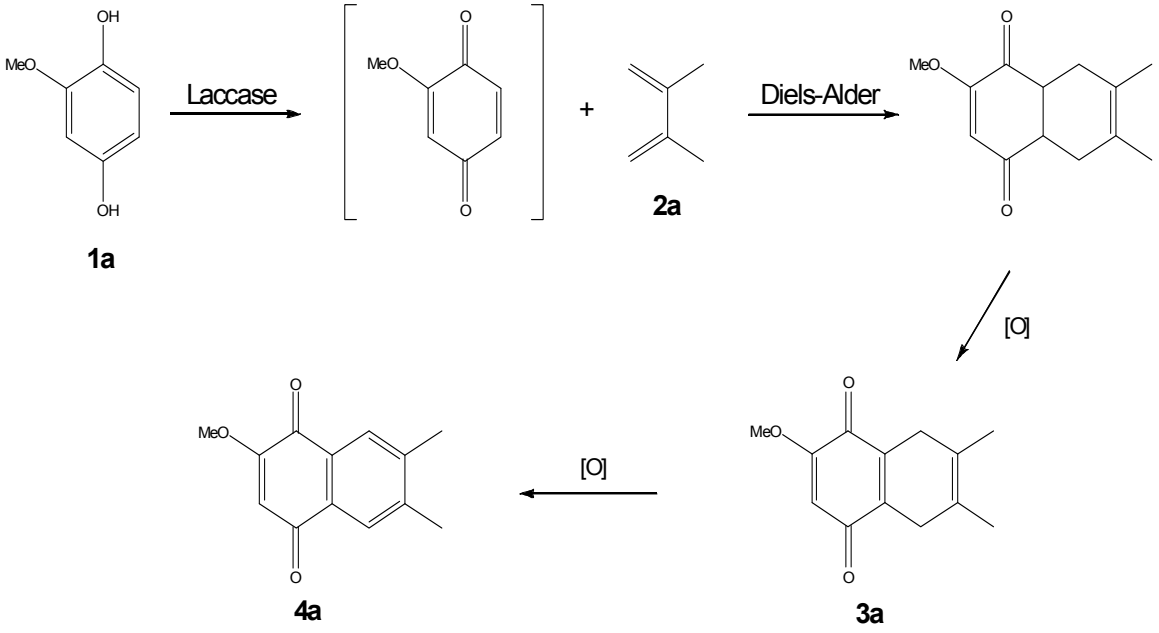



**Figure 100.** The proposed reaction pathway of laccase-catalyzed Diels-Alder reaction of 2-methoxyhydroquinone (**1a**) and 2,3-dimethyl-1,3-butadiene (**2a**).

## 4.2 Experimental Section

### 4.2.1 Materials

2-Methoxyhydroquinone was obtained from TCI America. Other hydroquinones, dienes, and reagents were obtained from Aldrich. All chemicals were used as received. Laccase (EC 1.10.3.2) from *Trametes Villosa* was donated by Novo Nordisk Biochem, North Carolina.

### 4.2.2 Enzyme Assay

Laccase activity measurement is described in Chapter 3 (Experimental Materials and Procedures).

### 4.2.3 General Procedure for the Study of the Effect of Laccase Dose and Temperature

Oxygen was bubbled to a stirred solution of 30 ml of 0.10M acetate buffer (pH 4.5) and laccase (¼ of the total amount of laccase used in this reaction) at a desired temperature for 30 minutes. Next, 2-methoxyhydroquinone (**1a**) (1.00 mmol) and 2,3-dimethyl-1,3-butadiene (**2a**) (2.00 mmol) were added to the reaction mixture, and stirred under air. In the first three hours of the reaction, ¼ of the total amount of laccase was added each hour. After the reaction reached the desired reaction time, the reaction mixture was extracted by EtOAc (3 x 30 ml). The organic phase was combined, dried over $MgSO_4$, and evaporated. Then the quantitative analyses of **3a** and **4a** were determined by $^1$H-NMR spectroscopy of the crude mixture using 10 μl of pentrafluorobenzaldehyde as an internal standard and using 0.5 ml of $CDCl_3$ as a NMR solvent. The example $^1$H-NMR spectrum is illustrated in Figure 101. Peak at 5.87 ppm (C-H) is used to calculate yield of compound **3a** and peak at 7.77 ppm (C-H aromatic) is used to calculate yield of compound **4a**.

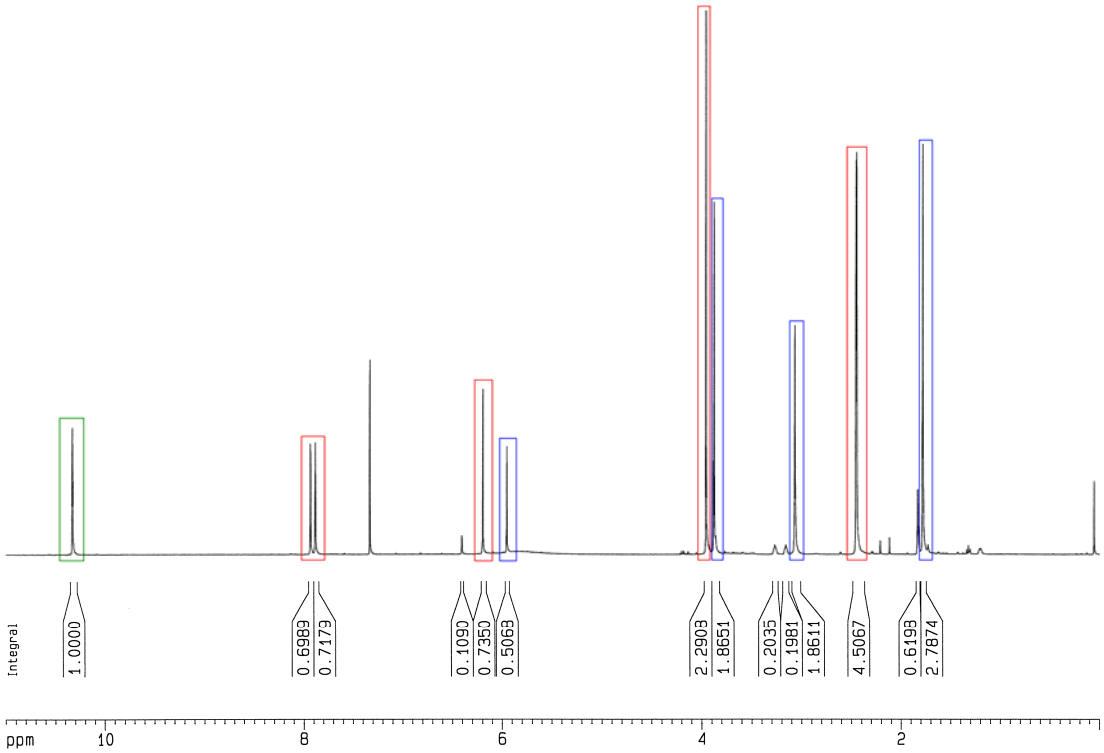



**Figure 101.** [1]H-MNR spectrum of crude mixture from the laccased-catalyzed reaction of of 2-methoxyhydroquinone (**1a**) and 2,3-dimethyl-1,3-butadiene (**2a**). Peaks of compound **3a** are illustrated in blue boxes. Peaks of compound **4a** are illustrated in red boxes. Peak of pentafluorobenzaldehyde is illustrated in green box.

### 4.2.4 General Procedure of the Synthesis of 1,4-Naphthoquinones and Related Structures.

The detail of the reaction procedure is described in Chapter 3 (Experimental Materials and Procedures).

### 4.2.5 Product Characterization

Products **3a**, **3b**, **3c**, **3d**, **4a**, **4b**, **4c**, **4d**, **4e**, **4g**, and **4h** have been previously reported and characterized. Compounds **3e**, **3g**, and **4f** have also been previously reported but without proper spectral characterization. Structures **3f** and **3h** are, to the best of our knowledge, new compounds. The NMR spectra of **3f** and **3h** were shown in Appendix A.1. All known products provided satisfactory analytical and spectroscopic data corresponding to the reported literature values.

5,8-Dihydro-2-methoxy-6,7-dimethyl-1,4-naphthoquinone (**3a**)

Yellow crystal; mp 134-136 °C (from EtOH) (lit. [353], 133-135 °C); $^{1}$H NMR (400 MHz; $CDCl_3$): δ 1.73 (s, 6H), 3.02 (s, 4H), 3.81 (s, 3H), 5.87 (s, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$): δ 18.1, 18.1, 30.3, 30.7, 56.1, 106.9, 121.7, 137.5, 140.2, 158.4, 181.7, 187.1; *m/z* (EI) 218 ($M^+$, 33%), 216 (100), 201 (38), 187 (40), 175 (35), 159 (9), 145 (10), 117 (32), 91 (12), 69 (15), 51 (6), 39 (4); *m/z* (EI) 218.09211 ($C_{13}H_{14}O_3$ requires 218.09429).

5,8-Dihydro-2-methoxy-5,7-dimethyl-1,4-naphthoquinone (**3b**)

Orange-yellow crystalline solid; mp 118-119 °C (from EtOH) (lit. [354], 118.5-120.5 °C); $^{1}$H NMR (400 MHz; $CDCl_3$): δ 1.16 (d, $J$ = 6.9 Hz), 1.80 (s, 3H), 2.93 (md, $J$ = 23.4 Hz, 1H), 3.15 (md, $J$ = 23.4 Hz, 1H), 3.29 (m, 1H), 3.81 (s, 3H), 5.47 (m, 1H), 5.88 (s, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$): δ 19.7, 20.9, 24.7, 32.8, 56.1, 106.8, 116.7, 136.3, 140.2, 142.5, 158.5, 181.4, 187.0; *m/z* (EI) 218 ($M^+$, 63%), 203 (100), 175 (73), 133 (12), 119 (54), 91 (24), 69 (20), 51 (5); *m/z* (EI) 218.09108 ($C_{13}H_{14}O_3$ requires 218.09429).

5,8-Dihydro-2-methoxy-5,8-dimethyl-1,4-naphthoquinone (**3c**)

Orange plates; mp 109-110 °C (from EtOH) (lit. [355], 110-112 °C); $^{1}$H NMR (400 MHz; $CDCl_3$): δ 1.16 (s, 3H), 1.18 (s, 3H), 3.36-3.37 (m, 2H), 3.81 (s, 3H), 5.75 (m, 2H), 5.86 (s, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$): δ 22.5, 23.0, 29.2, 29.4, 56.0, 107.1, 128.7, 142.1, 144.6, 158.2, 181.5, 186.6; *m/z* (EI) 218 ($M^+$, 52%), 203 (100), 175 (84), 133 (14), 119 (66), 91 (33), 69 (26), 39 (6); *m/z* (EI) 218.09397 ($C_{13}H_{14}O_3$ requires 218.09429).

5,8-Dihydro-2,6,7-trimethyl-1,4-naphthoquinone (**3d**)

Yellow needles; mp 88-89 °C (from EtOH) (lit. [356], 87-89 °C); $^{1}$H NMR (400 MHz; $CDCl_3$): δ 1.72 (s, 6H), 2.04(s, 3H), 2.99 (s, 4H), 6.54 (s, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$): δ 15.8, 18.2, 20.2, 30.5, 30.7, 121.8, 121.9, 133.0, 133.0, 139.5, 145.6, 187.3, 187.5; *m/z* (EI) 202 ($M^+$, 100%), 187 (36), 159 (67), 119 (27), 91 (18), 39 (9); *m/z* (EI) 202.10014 ($C_{13}H_{14}O_2$ requires 202.09938).

5,8-Dihydro-2,5,8-trimethyl-1,4-naphthoquinone (**3e**)

Yellow liquid; $^{1}$H NMR (400 MHz; $CDCl_3$): δ 1.25 (d, $J$ = 2.2 Hz, 3H), 1.26 (d, $J$ = 2.2 Hz, 3H), 2.10 (s, 3H), 3.43 (m, 2H), 5.83 (d, $J$ = 2.7, 2H), 6.61(s, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$): δ 15.7, 22.8, 22.8, 29.3, 29.5, 128.8, 128.9, 133.2, 144.0, 144.1, 145.4, 186.9, 187.3; *m/z* (EI) 202 ($M^+$, 100%), 187 (79), 159 (41), 119 (56), 91 (26), 39 (7); *m/z* (EI) 202.09985 ($C_{13}H_{14}O_2$ requires 202.09938).

1,4-Dihydro-6-methoxy-1,4-ethanonaphthalene-5,8-dione (**3f**)

Yellow needles; mp 123-124 °C (from EtOH); $^{1}$H NMR (400 MHz; $CDCl_3$): δ 1.35 (d, $J$ = 6.8 Hz, 2H), 1.49 (d, $J$ = 8 Hz, 2H), 3.80 (s, 3H), 4.34 (br s, 1H), 4.37 (br s,

1H), 5.76 (s, 1H), 6.39 ( br s, 2H); $^{13}$C NMR (100 MHz, $CDCl_3$): δ 24.4, 33.4, 33.7, 56.2, 105.9, 133.5, 133.7, 146.0, 149.2, 158.5, 178.1, 183.7; $\nu_{max}/cm^{-1}$ 3055, 2938, 2869, 1668, 1642, 1624, 1598, 1582, 1452, 1380, 1224, 1135, 1013, 868, 818; *m/z* 216 ($M^+$, 21%), 188 (100, M - $CH_2CH_2$), 173 (39), 158 (52), 130 (14), 102 (28), 89 (33), 69 (14), 51 (8); *m/z* (EI) 216.08073 ($C_{13}H_{12}O_3$ requires 216.07864).

1,4-Dihydro-6-methyl-1,4-ethanonaphthalene-5,8-dione (**3g**)

Yellow needles; mp 81-82 °C (from EtOH) (lit. [357], 83-84 °C); $^1$H NMR (400 MHz; $CDCl_3$): δ 1.34 (m, 2H), 1.47 (m, 2H), 2.03 (d, *J* = 1.6 Hz, 3H), 4.31 (br m, 1H), 4.35 (br m, 1H), 6.38 (dd, *J* = 2.7 Hz, 3.8 Hz, 2H), 6.44 (q, *J* = 1.6 Hz, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$): δ 15.7, 24.5, 33.5, 33.7, 132.1, 133.6, 133.7, 144.8, 148.1, 148.1, 183.9, 184.1; *m/z* (EI) 200 ($M^+$, 14%), 172 (100, M - $CH_2CH_2$), 144 (27), 116 (14), 104 (18), 76 (10), 39 (3); *m/z* (EI) 200.08399 ($C_{13}H_{12}O_2$ requires 200.08373).

1,4-Dihydro-6-bromo-1,4-ethanonaphthalene-5,8-dione (**3h**)

Orange crystals; mp 104-106 °C (from EtOH); $^1$H NMR (400 MHz; $CDCl_3$): δ 1.39 (d, *J* = 8.5 Hz, 2H), 1.53 (d, *J* = 8 Hz, 2H), 4.35 (br m, 1H), 4.44 (br m, 1H), 6.42 (t, *J* = 3.4 Hz, 2H), 7.15 (s, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$): δ 24.5, 24.6, 33.9, 34.8, 133.5, 133.7, 136.7, 137.0, 147.9, 148.9, 175.8, 181.1; ; $\nu_{max}/cm^{-1}$ 3043, 2998, 2935, 2869, 1660, 1645, 1627, 1571, 1445, 1331, 1302, 1263, 1233, 1051, 892, 777; *m/z* (EI) 266 (M + 2, 9%), 264 ($M^+$, 9%), 238 (82), 236 (80), 185 (10), 157 (100), 129 (41), 101 (21), 76 (11), 51 (7); *m/z* (EI) 263.97755 ($C_{12}H_9O_2Br$ requires 263.97859).

2-Methoxy-6,7-dimethyl-1,4-naphthoquinone (**4a**)

Yellow crystal; mp 165-167 °C (from EtOH) (lit. [358], 169-171 °C); $^{1}$H NMR (400 MHz; $CDCl_3$): δ 2.36 (s, 6H), 3.87 (s, 3H), 6.06 (s, 1H), 7.77 (s, 1H), 7.82 (s, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$): δ 19.9, 20.2, 56.3, 109.5, 127.1, 127.6, 128.9, 129.9, 142.9, 144.2, 160.2, 180.2, 185.1; *m/z* (EI) 216 ($M^+$, 100%), 201 (33), 187 (38), 186 (27), 158 (8), 145 (8), 130 (15), 117 (25), 103 (5), 91 (5), 69 (4), 51 (4), 39 (3); *m/z* (EI) 216.07925 ($C_{13}H_{12}O_3$ requires 216.07864).

2-Methoxy-5,7-dimethyl-1,4-naphthoquinone (**4b**)

Yellow powder; mp 149-151 °C (from EtOH) (lit. [354], 146-148 °C); $^{1}$H NMR (400 MHz; $CDCl_3$): δ 2.40 (s, 3H), 2.62 (s, 3H), 3.87 (s, 3H), 6.07 (s, 1H), 7.45 (s, 1H), 7.85 (s, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$): δ 17.0, 21.2, 56.2, 107.9, 124.1, 128.6, 131.6, 134.8, 140.3, 144.2, 160.8, 181.9, 184.7; *m/z* (EI) 216 ($M^+$, 100%), 201 (73), 186 (8), 128 (7), 117 (27), 103 (4), 91 (4), 77 (5), 63 (4), 51 (4), 39 (2); *m/z* (EI) 216.07918 ($C_{13}H_{12}O_3$ requires 216.07864).

2,6,7-Trimethoxy-1,4-naphthoquinone (**4c**)

Golden yellow solid; mp 232-234 °C (lit. [359], 234-235 °C); $^{1}$H NMR (400 MHz; $CDCl_3$): δ 3.90 (s, 3H), 4.04 (s, 6H), 6.07 (s, 1H), 7.51 (s, 1H), 7.54(s, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$): δ 56.3, 56.5, 107.7, 108.1, 109.0, 125.4, 126.9, 152.9, 153.8, 160.3, 179.4, 184.5; *m/z* (EI) 248 ($M^+$, 100%), 233 (10), 219 (37), 205 (6), 177 (17), 162 (10), 149 (28), 134 (6), 119 (6), 93 (3), 69 (6), 63 (3); *m/z* (EI) 248.06705 ($C_{13}H_{12}O_5$ requires 248.06847).

2-Methoxy-1,4-naphthoquinone (**4d**)

Yellow solid; mp 179-182 °C (lit. [360], 178-182 °C); $^{1}$H NMR (400 MHz; $CDCl_3$): δ 3.93 (s, 3H), 6.18 (s, 1H), 7.71-7.75 (m,2H), 8.06-8.13 (m, 2H); $^{13}$C NMR (100 MHz, $CDCl_3$): δ 56.4, 109.8, 126.1, 126.6, 130.9, 131.9, 133.3, 134.3, 160.4, 180.0, 184.7; *m/z* (EI) 188 ($M^+$, 100%), 173 (40), 158 (36), 102 (40), 89 (52), 76 (20), 69 (10), 50 (10), 39 (2); *m/z* (EI) 188.04625 ($C_{11}H_8O_3$ requires 188.04734).

2,6,7-Trimethyl-1,4-naphthoquinone (**4e**)

Yellow solid; mp 104-105 °C (lit. [361], 105-106 °C); $^{1}$H NMR (400 MHz; $CDCl_3$): δ 2.18 (s, 3H), 2.40(s, 6H),6.77 (s, 1H), 7.78 (s, 1H), 7.82 (s, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$): δ 16.4, 16.4, 20.1, 127.0, 127.5, 130.1, 130.2, 135.4, 143.3, 143.3, 147.8, 185.3, 185.7; *m/z* (EI) 200 ($M^+$, 100%), 185 (11), 172 (36), 157 (16), 144 (9), 132 ( 27), 115 (4), 104 (10), 77 (6), 63 (4), 51 (5), 39 (4); *m/z* (EI) 200.08187 ($C_{13}H_{12}O_2$ requires 200.08373).

2-Methyl-6,7-dimethoxy-1,4-naphthoquinone (**4f**)

Orange-yellow solid; mp 211-212 °C (lit. [362], 211-212.5 °C); $^{1}$H NMR (400 MHz; $CDCl_3$): δ 2.16 (s, 3H), 4.01 (s, 6H), 6.73 (s, 1H), 7.47 (s, 1H), 7.51 (s, 1H); $^{13}$C NMR (100 MHz, $CDCl_3$): δ 16.5, 56.5, 56.5, 107.5, 108.0, 111.4, 126.9, 127.1, 135.2, 147.7, 153.2, 184.6, 185.1; *m/z* (EI) 232 ($M^+$, 100%), 202 (31), 189 (19), 136 (12), 93 (7), 39 (8); m/z (EI) 232.08528 ($C_{13}H_{12}O_4$ requires 232.07356).

2-methyl-1,4-naphthoquinone (menadione) (**4g**)

Bright yellow solid; mp 104-105 °C (lit. [360], 103-104 °C); $^{1}$H NMR (400 MHz; $CDCl_3$): δ 2.17 (s, 3H), 6.83 (s, 1H), 7.71-7.73 (m, 2H), 8.03-8.09 (m, 2H); $^{13}$C NMR

(100 MHz, $CDCl_3$): δ 16.4, 126.0, 126.4, 132.0, 132.1, 133.5, 133.5, 135.5, 148.1, 184.9, 185.4; *m/z* (EI) 172 ($M^+$, 100%), 144 (23), 115 (24), 104 (34), 76 (22), 50 (9); *m/z* (EI) 172.05149 ($C_{11}H_8O_2$ requires 172.05243).

2-Bromo-6,7-dimethyl-1,4-naphthoquinone (**4h**)

Yellow solid; mp 156-158 °C (lit. [363], 156-159 °C); $^1H$ NMR (400 MHz; $CDCl_3$): δ 2.42 (s, 6H), 7.45 (s, 1H), 7.82 (s, 1H), 7.91(s,1H); $^{13}C$ NMR (100 MHz, $CDCl_3$): δ 20.2, 127.8, 128.8, 129.6, 139.9, 140.1, 144.1, 144.5, 177.9, 182.6; *m/z* (EI) 266 ($M^+$ + 2, 77%), 264 ($M^+$, 77%), 185 (100), 157 (53), 128 (25), 103 (7), 77 (9), 51 (9), 39 (3); *m/z* (EI) 263.97489 ($C_{12}H_9O_2Br$ requires 263.97859).

## 4.3 Results and Discussion

### 4.3.1 Preliminary Study of the Reaction System

Laccase-catalyzed reaction of 1,4-benzoquinones and dienes was initially investigated by using **1a** and **2a** as the model reagents and laccase as an oxidizing agent. Laccase first converted **1a** to 2-methoxy-1,4-benzoquinone, and then the quinone reacted with diene **2a** *via* the Diels-Alder reaction. The Diels-Alder adducts then underwent further oxidation to generate 5,8-dihydro-2-methoxy-6,7-dimethyl-1,4-naphthoquinone (**3a**) and 2-methoxy-6,7-dimethyl-1,4-naphthoquinone (**4a**).

In this preliminary study, the total amount of laccase used in the reaction was 1000 U/ 1g substrate, and the equivalence ratio of 2-methoxy hydroquinone and 2,3-dimethyl-1,3- butadiene was 1:2, to enhance the likelyhood that no *in situ*-generated 2-methoxy benzoquinone remained to further oxidize the Diels-Alder adducts. The reaction was conducted in 0.10M acetate buffer pH 4.5, in the presence of oxygen at 50 °C, for 24 hours (Figure 102). A pH of 4.5 was chosen for this reaction system because many studies have shown that this pH is the optimum pH for laccase activity in the formation of quinone, as in the work of Ishihara, and Leonowicz et al. and Ragauskas [364-366]. In this reaction system, vigorous stir was required to disperse **2a**, which is slightly dissolved in water, in an emulsion to increase the reaction rate between the *in situ*-generated quinone and **2a**. Moreover, the hydrophobic interactions between relatively apolar quinone and **2a** forced them into close proximity and favour the Diels-Alder reaction products.

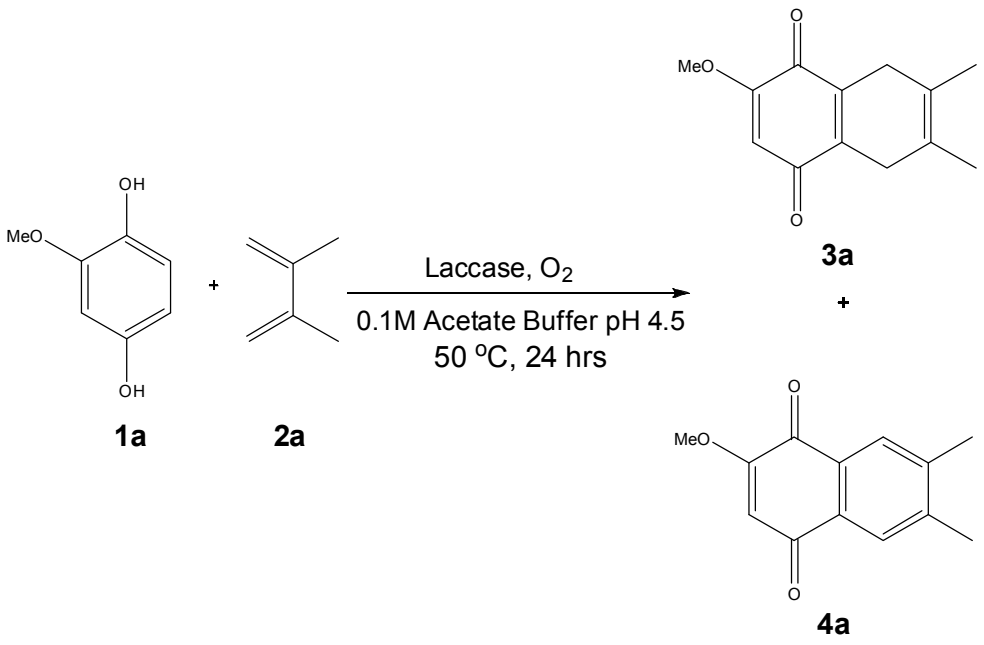



**Figure 102.** The preliminary reaction system for laccase-catalyzed aqueous Diels-Alder reaction of 2-methoxyhydroquinone (**1a**) and 2,3-dimethyl-1,3-butadiene (**2a**).

In the preliminary study, we examined the effect of oxygen on the formation of the products. We found that the quantity of oxygen affected the reaction. When an excessive amount of oxygen, such as bubbling oxygen throughout the reaction or pressurizing with oxygen at $9.9974 \times 10^5$ N/m$^2$ (145 psi), was used, the main product was 2-methoxy-1,4-benzoquinone (26%) and very small amounts of **3a** and **4a** were generated. In contrast, stirring the reaction under air generated **3a** (13%) and **4a** (45%). However, we also found that bubbling oxygen for 30 minutes into a laccase/buffer solution before adding all the reagents and gradually adding ¼ of the laccase (250 U/1g substrate) at the beginning of each of the first four hours of the 24-hour reaction improved the yield of **3a** and **4a** to 15% and 50%, respectively. After

this reaction procedure was examined, the control reaction adding no laccase was studied. The result showed that when no laccase was added to the system, no desired products were obtained. Therefore, the oxidizing agent, laccase, must be added to generate 2-methoxy-1,4-benzoquinone *in situ*. This quinone then underwent further reaction with diene to generate **3a** and **4a**.

### 4.3.2 The Effect of Laccase Dose

After the preliminary study, the next approach was to optimize the reaction conditions. The optimization was studied by investigating the effects of laccase dose and temperature. The laccase doses used in these experiments were 500, 1000, 2000, and 4000 U/ 1 g of **1a**. The reaction was conducted at 50 °C. The method for this study is described in the experimental section. The quantitative study of **3a** and **4a** was measured by $^{1}$H-NMR spectroscopy using tetrafluorobenzaldehyde as an internal standard. Figure 103 shows the results of the study.

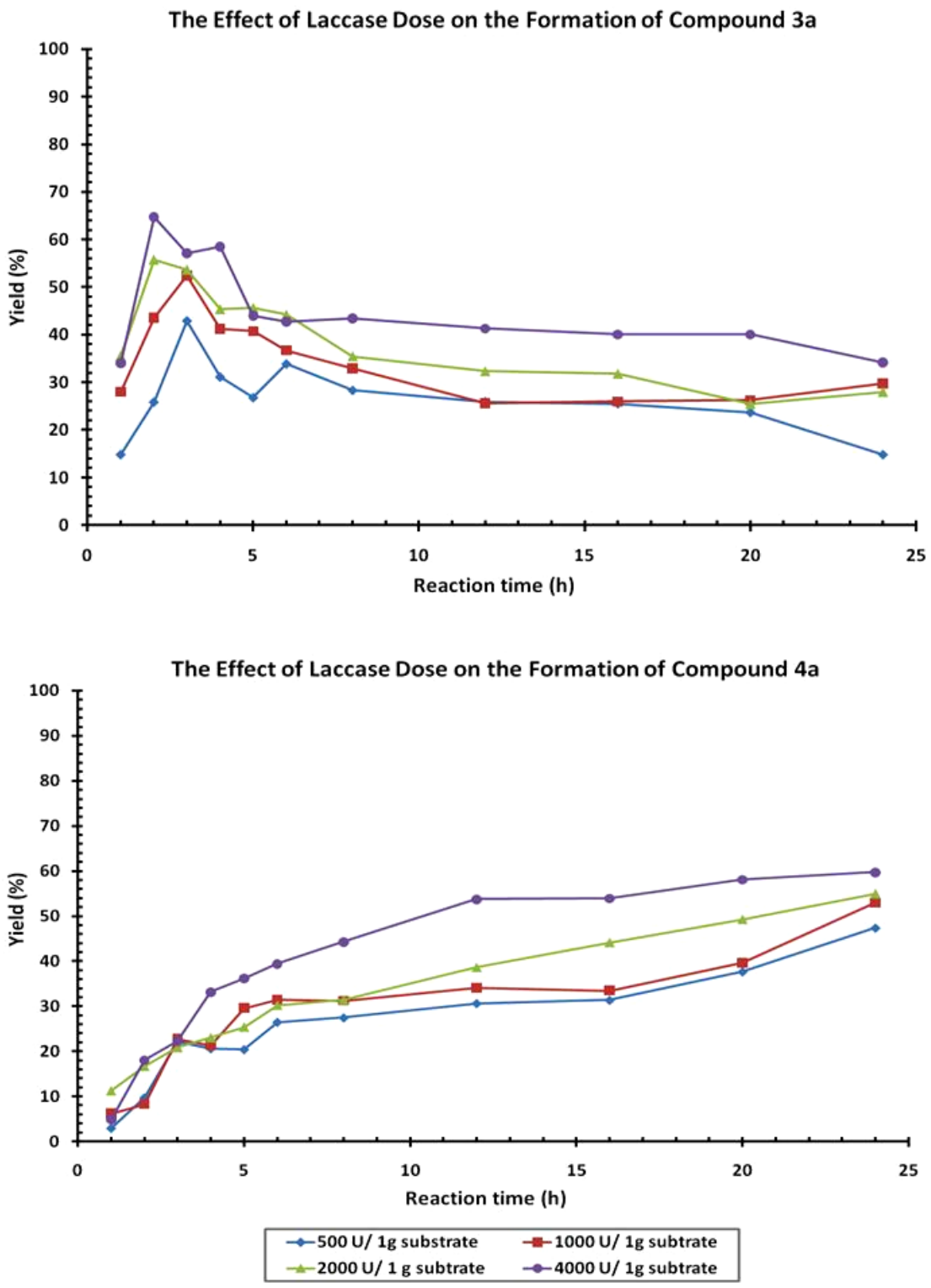



**Figure 103.** The effect of laccase dose on the formation of compound **3a** and **4a**. The percent yield of **3a** and **4a** was measured by $^{1}$H-NMR spectroscopy.

According to Figure 103, the amount of laccase in the reaction affected the formation of **3a** and **4a**. When the amount of laccase used in the reaction was increased from 500 to 4000 U/ 1g substrate, the percent yield of **3a** and **4a** at the end of the reaction (24 hours) also increased from 15% to 34% and from 47% to 60%, respectively. In addition, the formation of **3a** sharply increased in the first three hours, and then decreased gradually throughout the reaction. In contrast, the formation of **4a** increased slightly in the first two hours and then gradually increased after the third hour of the reaction. The explanation of this result is that the formation of **3a** was predominant at the beginning of the reaction, and upon further treatment, some of **3a** was gradually oxidized to generate **4a**, leading to the continual increase in the yield of **4a** at the longer reaction time. The proposed reaction pathway is summarized in Figure 100. The first step was the oxidation of **1a** by laccase to form 2-methoxy benzoquinone and then this quinone underwent the Diels-Alder reaction with **2a** to generate the Diels-Alder adduct. The Diels-Alder adduct was then oxidized by either laccase or quinone in the reaction solution to generate **3a** and upon further treatment, **3a** was oxidized to **4a**. To confirm the proposed reaction pathway, we stirred **3a** in 0.10M acetate buffer, pH 4.5 at 70 ˚C for 24 hours, with either laccase (4000 U/1g of **3a**) or with 2-methoxybenzoquinone (model quinone) (1equiv.). The results show that the percent conversion of **3a** to **4a** was 35% and 16% with laccase and 2-methoxybenzoquinone respectively. Therefore, these results show that both laccase and quinone in the reaction solution can oxidize **3a** to generate **4a**. However, laccase appears to be a better oxidizing agent than the quinone in this reaction system.

### 4.3.3 The Effect of Temperature

As demonstrated in the previous section, laccase dose has an influence on the formation of compounds **3a** and **4a**. The more laccase we used, the more products we obtained. Another factor that should affect the reaction is temperature. Thus, the experiment was conducted at different temperatures, including 25 °C, 50 °C, 70 °C, and 100 °C. The reaction procedure was the same as that used before except 4000 U/ 1g of **1a** was used. Figure 104 illustrates the effect of temperature on the reaction.

It is obvious that when **4a** was formed, its yield increased when the temperature of the reaction increased. For example, at the end of the reaction, the percent yield of **4a** was 17%, 60%, and 87% for the reaction at 25 °C, 50 °C, and 70 °C, respectively. In contrast, the formation of **3a** exhibited a different response to temperatures. For the reaction at 50 °C and 70 °C, the amount of **3a** sharply increased in the first two hours, and then decreased after the second hour. However, the decrease at 70 °C was faster than that at 50 °C because a higher temperature can more easily accelerate the conversion of **3a** to **4a**. For the reaction at 25 °C, the formation of **3a** gradually increased throughout the reaction. Moreover, we found that 2-methoxy-6,7-dimethyl-4a,5,8,8a-tetrahydro-1,4-naphthoquinone, the Diels-Alder adduct, was the main product of the reaction at 25 °C, instead of **3a** and **4a**. Therefore, this reaction best underwent the quinone Diels-Alder reaction to generate the Diels-Alder adduct at a low temperature, and upon further treatment, the Diels-Alder adduct was slowly converted to **3a**, and only a small amount of **4a** was obtained. For the reaction at 100 °C, no products were obtained because, at this high temperature, laccase was denatured.

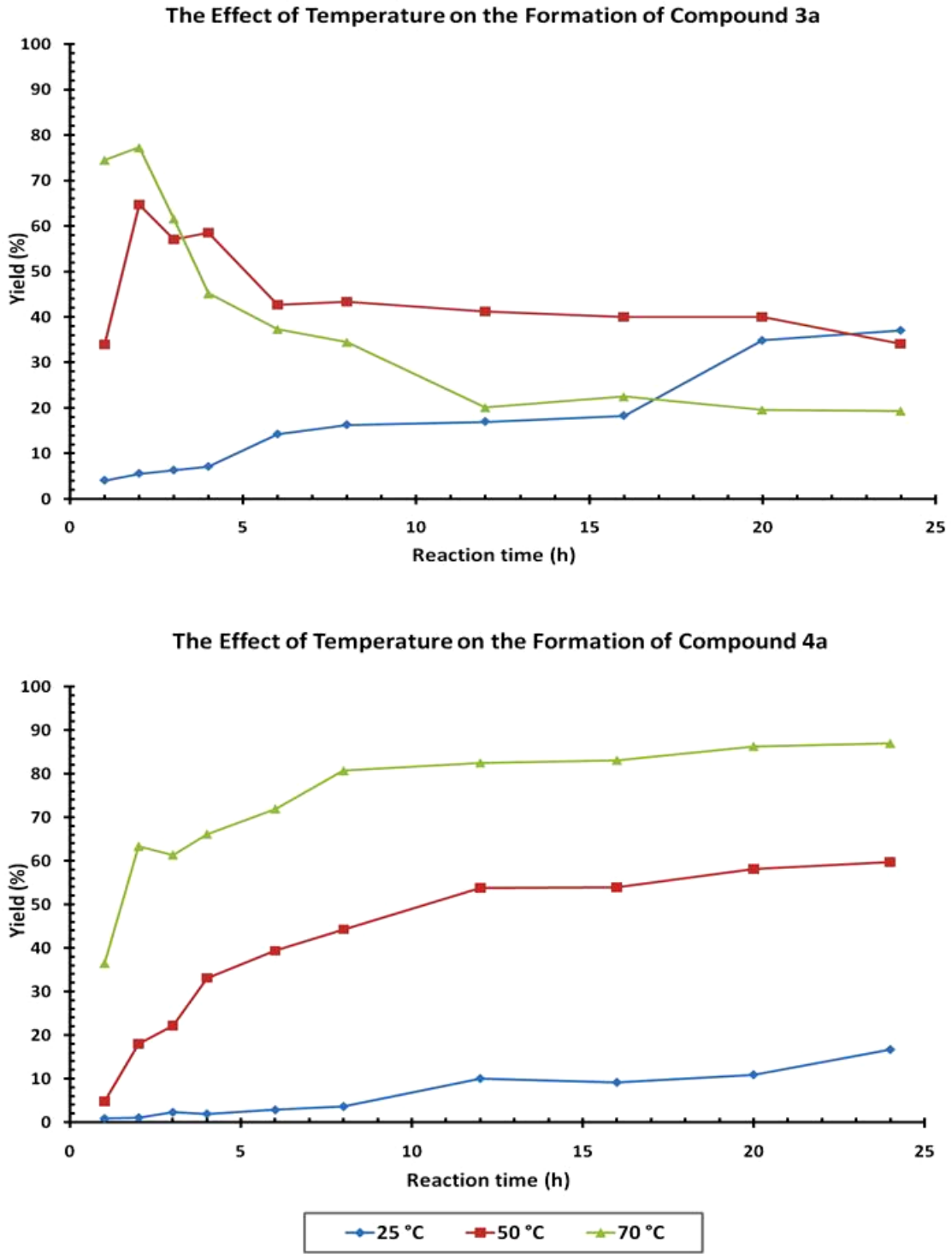



**Figure 104.** The effect of temperature on the formation of compound **3a** and **4a**. The percent yield of **3a** and **4a** was measured by $^{1}$H-NMR spectroscopy. (No products were obtained at 100 °C.)

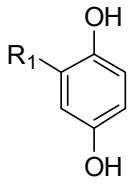
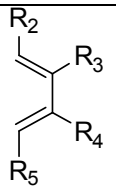
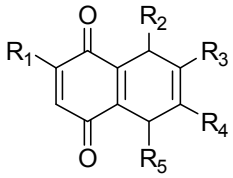
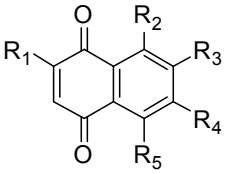
### 4.3.4 The Reaction of *p*-Hydroquinones and Dienes

From the optimization experiments, we chose to conduct the reaction with 4000 U of laccase/1g substrate at 70 °C for 24 hours to investigate the reaction of various *para*-hydroquinones and dienes as shown in Table 9.

In this experiment, three different *p*-hydroquinones, in which $R_1$ represented the OMe, Me, and Br groups, were used and conducted with a variety of dienes. The data in Table 9 shows that in most cases, 1,4-naphthoquinone products (**4**) were obtained as major products, and only small amounts or none of dihydro 1,4-naphthoquinone products (**3**) were obtained. However, when dienes have alkyl groups at $R_2$ and $R_5$ (e.g., **2c** and **2f**), only **3**-type products were formed. In addition, when $R_1$ is the OMe group, the yield of products was higher than when $R_1$ is the Me or Br groups. Although quinones with a Br substituent, a strong electron-withdrawing group, have been proven to be very reactive dienophiles for the Diels-Alder reaction, it produced a lower yield of the products than quinones with an OMe substituent, an electron-donating group. This result can be explained by the substrate affinity of laccase, which varies, depending on the substituents and their recipocal positions on the aromatic ring. Therefore, *p*-hydroquinones that have higher affinity to laccase are more easily oxidized, and generate higher amounts of the starting quinone that react with diene in the first step of the reaction. In this case, *p*-hydroquinones with the Br substituent have lower affinity to laccase than *p*-hydroquinones with OMe. This result agrees with that of a study that reported the high affinity of the phenolic compounds bearing the methoxyl group to laccase [367]. In addition, substituents also have effect on redox potential of hydroquinone starting material. Xu [198] showed that the

electron-withdrawing substituents reduce the electron density at the phenoxy group and increase redox potential of molecule, thus making it more difficult to be oxidized and less reactive in surrendering electron to the substrate pocket in laccase. In contrast, the presence of the electron-donating substituents results in the reduction in redox potential. Therefore, in this study, *p*-hydroquinone with the OMe group is more easily oxidized than that with the Br group.

**Table 9.** The reaction of *p*-hydroquinones and dienes[a].

| *p*-Hydroquinone | Diene | Yield of Products (%)[b] | |
|---|---|---|---|
|  **1** |  **2** |  **3** |  **4** |
| **1a**: $R_1$ = OMe | **2a**: $R_2 = R_5$ = H, $R_3 = R_4$ = Me | **3a** (10%) | **4a** (60%) |
| **1a** | **2b**: $R_2 = R_4$ = H, $R_3 = R_5$ = Me | **3b** (9%) | **4b** (55%) |
| **1a** | **2c**: $R_3 = R_4$ = H, $R_2 = R_5$ = Me | **3c** (46%) | - |
| **1a** | **2d**: $R_2 = R_5$ = H, $R_3 = R_4$ = OMe | - | **4c** (12%) |
| **1a** | **2e**: $R_3 = R_4 = R_5$ = H, $R_2$ = OMe | - | **4d** (79%), $R_2$ =H |
| **1b**: $R_1$ = Me | **2a** | **3d** (20%) | **4e** (22%) |
| **1b** | **2c** | **3e** (19%) | - |
| **1b** | **2d** | - | **4f** (4.28%)[c] |
| **1b** | **2e** | - | **4g** (40%), $R_2$ = H |
| **1c**: $R_1$ = Br | **2a** | - | **4h** (21%) |
| **1a** | **2f**:  | **3f** (64%) | - |
| **1b** | **2f** | **3g** (49%) | - |
| **1c** | **2f** | **3h** (51%) | - |

[a]Reaction conditions: The reaction of *p*-hydroquinone (1 equiv.) and diene (2 equiv.) was stirred with laccase (4000U/1g substrate) in 0.10M acetate buffer pH 4.5 at 70 ˚C for 24 hours. [b]Yield of isolated products was calculated base on the amount of 1,4-benzoquinone starting materials. [c]Found 26% of methylbenzoquinone as another product.

## 4.4 Conclusions

Here, we reported a new green chemistry synthesis of 1,4-naphthoquinones and related structures by using both a nonhazardous oxidizing agent, laccase, and the environmentally benign solvent, water. This study also shows another potential use of laccase as an oxidizing agent in organic synthesis. Moreover, the reaction system we used in this study produced the 1,4-naphthoquinone products in high yield. However, the reactivity of the reaction depends on the substrate specificity of laccase and the reactivity of both generated quinones and dienes. For instance, the presence of the electron-donating substituents, such as OMe group, results in the reduction in redox potential and makes *p*-hydroquinone more easily oxidized. Therefore, in this study, methoxy-hydroquinone provided higher yield of the product than methyl- or bromo-hydroquinone. In addition, both temperature and laccase dose effect on the formation the corresponding products. Therefore, the reaction condition have to be controlled to obtain the desired products.

# CHAPTER 5

# LACCASE-GENERATED QUINONES IN 1,2-NAPHTHOQUINONE SYNTHESIS VIA DIELS-ALDER REACTION[ii]

## 5.1 Introduction

The combination of enzymatic with nonenzymatic transformations for tandem reactions was first reported by Waldmann and co-workers in 1998 [117]. They reported the synthesis of highly functionalized bicycle[2.2.2]octenes by a tyrosinase-initiated hydroxylation-oxidation of phenols followed by a Diels-Alder (DA) reaction with electron rich dienophiles (see Figure 105). These studies, conducted in chloroform, provided a unique three-step one-pot reaction of bicyclic DA products in high yields with the key intermediate being reactive *ortho*-quinones. The applicability of enzyme catalyzed domino reactions in green chemistry has only recently been fully appreciated.

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[ii] This manuscript was published in Tetrahedron Letters, 2007, 48, 2983-2987. It is entitled as **"**Laccase-generated quinones in naphthoquinone synthesis via Diels-Alder reaction". The other authors are Abdullah Zettili from Department of Physical and Earth Science, School of Chemistry at Jacksonville State University and Dr. Arthur J. Ragauskas from the School of Chemistry and Biochemistry at the Georgia Institute of Technology. 

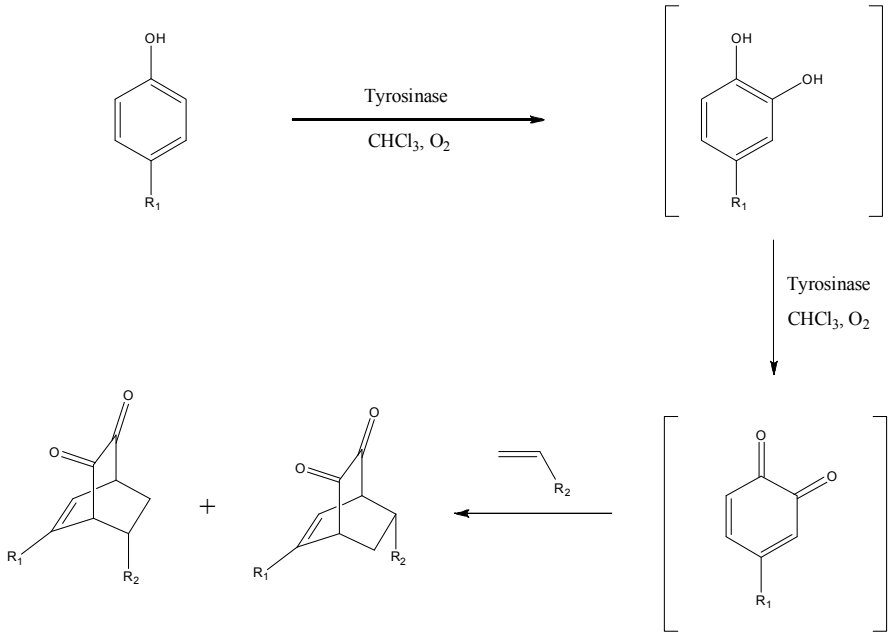



**Figure 105.** The example of enzyme-initiated reaction cascade reported by Waldmann and co-workers.[117]

In the previous Chapter, the successful synthesis of *p*-naphthoquinones via laccase-catalyzed Diels-Alder reaction between in situ-generated *p*-quinones and dienes in aqueous medium was described. To broaden substrate spectrum for this laccase-catalyed Diels-Alder reaction system, this Chapter further investigated the use of this reaction system for the synthesis of *o*-naphthoquinones. In this study, a series of substituted *o*-naphthoquinones were synthesized via an aqueous cascade reaction between acyclic dienes and *in-situ* generated o-quinones. The *ortho*-quinones were synthesized in situ by the oxidation of the corresponding *o*-benzohydroquinone by laccase. The initial Diels-Alder adduct was shown to undergo further oxidization by laccase and/or quinone to yield the desired *o*-naphthoquinones (see Figure 106).

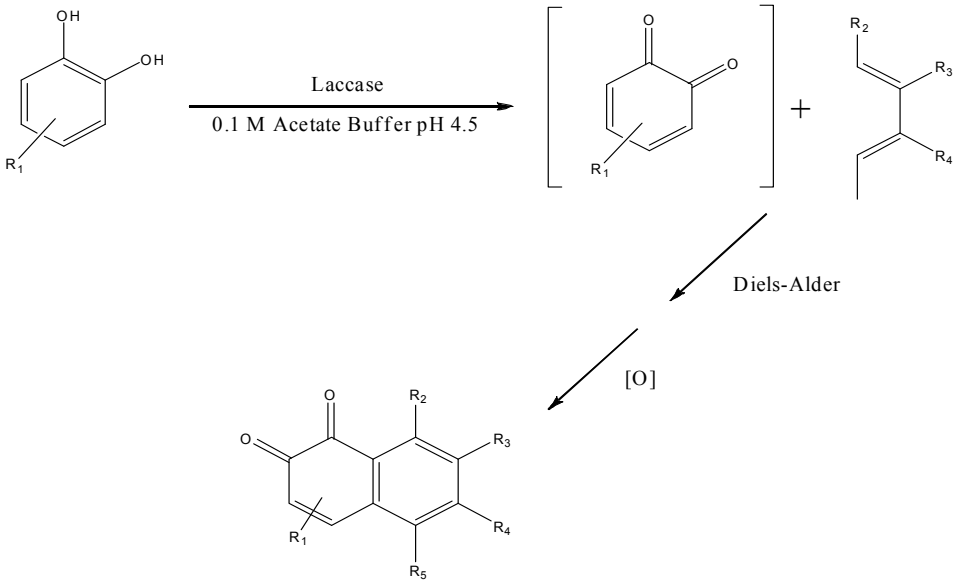



**Figure 106.** Laccase-initiated cascade synthesis of substitute *o*-naphthoquinones *via* aqueous Diels-Alder reaction.

Therefore, this Chapter summarizes our interests in the use of laccase for the synthesis of substituted *o*-napthoquinones. Naphthoquinones are naturally occurring compounds which have attracted interest in total synthesis because of their wide range of biological activity including antitumor [342,343,368], wound healing [344], anti-inflmmatory [344], and antimicrobial [345] and antiparasitic activities [346,347].

## 5.2 Experimental Section

### 5.2.1 Enzyme Assay

Laccase activity measurement is described in Chapter 3 (Experimental Materials and Procedures).

### 5.2.2 General Procedure of the Synthesis of *o*-Naphthoquinones

The detail of the reaction procedure is described in Chapter 3 (Experimental Materials and Procedures).

### 5.2.3 Typical Experimental Procedure for *p*-Naphthoquinone Synthesis

*p*-Hydroquinone (1.00 mmol), 1-acetoxy-1,3-butadiene (2.00 mmol), and laccase (100 U) were stirred in 40 ml of 0.10M acetate buffer pH 4.5 under air at 55 °C. In the next three hours of the reaction, 100 U of laccase was added each per hour. After 24 hours of the reaction, the reaction mixture was extracted by EtOAc (3 × 30 ml). The organic phase was combined, dried over $MgSO_4$, and evaporated. The resulting crude products were purified by Combiflash Companion instrument using Redisep normal-phase silica column. Ethyl acetate and petroleum ether (linear gradient: 0 – 20% ethyl acetate) were used as an eluent to obtain the products.

### 5.2.4 Product Characterization

Most compounds have been previously reported and characterized except the two compounds which are 4,7,8-trimethyl-1,2-naphthoquinone (**6e**) and 4-methyl-6,7-dimethoxy-1,2-naphthoquinone (**6f**). The NMR spectra of compound **6e** and **6f** are shown in Appendix A.2. All known products provided satisfactory analytical and spectroscopic data corresponding to the reported literature values.

6,7-Dimethyl-1,2-naphthoquinone (**6a**)

Orange-red needles: mp 147-148 °C (lit. [369],146-147 °C); $^{1}$H NMR ($CDCl_3$, 400 MHz) $\delta$ 2.32 (s, 3H, $CH_3$), 2.34 (s, 3H, $CH_3$), 6.33 (d, $J$ = 10 Hz, 1H, CH), 7.09 (s, 1H, Ar), 7.35 (d, $J$ = 10 Hz, 1H, CH), 7.84 (s, 1H, Ar); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 19.6, 20.2, 127.0, 129.5, 131.3, 131.6, 132.7, 140.1, 145.6, 146.0, 178.9, 181.3.

4,6,7-Trimethyl-1,2-naphthoquinone (**6b**)

Orange needles; mp. 119 °C (decomp.) (lit. [369],120 °C (decomp.); $^{1}$H NMR ($CDCl_3$, 400 MHz) $\delta$ 2.33 (s, 3H, $CH_3$), 2.36 (s, 3H, $CH_3$), 2.37 (s, 3H, $CH_3$), 6.31 (s, 1H, CH), 7.25 (s, 1H, Ar), 7.88 (s, 1H, Ar); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 19.5, 20.6, 20.6, 126.8, 127.9, 129.1, 131.3, 133.4, 139.9, 145.4, 154.1, 179.7, 180.9; MS (EI) *m/z* 200 ($M^+$, 100%), 172 (76), 157 (35), 128 (20), 91 (4), 77 (5), 51 (5); HRMS (EI) calcd for $C_{13}H_{12}O_2$ requires 200.08373, found 200.08264.

3-Methoxy-6,7-dimethyl-1,2-naphthoquinone (**6c**)

Maroon needles: mp 230-232 °C (lit. [369],231-233 °C); $^{1}$H NMR ($CDCl_3$, 400 MHz) $\delta$ 2.26 (s, 3H, $CH_3$), 2.3 (s, 3H, $CH_3$), 3.81 (s, 3H, $OCH_3$), 6.35 (s, 1H, CH), 6.96 (s, 1H, Ar), 7.75 (s, 1H, Ar); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 19.3, 20.3, 55.6, 110.5, 115.1,

120.9, 130.0, 131.8, 136.4, 137.2, 151.3,176.7, 178.2; MS (EI) *m/z* 216 ($M^+$, 80%), 188 (100), 173 (38), 159 (34), 145 (41), 117 (93), 91 (16), 57 (11), 51 (9); HRMS (EI) calcd for $C_{13}H_{12}O_3$ requires 216.07864, found 216.07910.

4-*t*-Butyl-6,7-dimethyl-1,2-naphthoquinone (**6d**)

Orange crystals [370]: $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ 1.46 (s, 9H, *t*-Bu), 2.27 (s, 3H, $CH_3$), 2.35 (s.3H, $CH_3$), 6.38 (s, 1H, CH), 7.67 (s, 1H, Ar), 7.87 (s, 1H, Ar); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 19.3, 20.9, 30.9, 36.8, 124.4, 130.0, 130.8, 132.1, 138.8,144.5, 164.6, 179.8, 182.3.

4,7,8-Trimethyl-1,2-naphthoquinone (**6e**)

Orange solid; mp. 118 °C (decomp.); $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ 2.36 (s, 3H, $CH_3$), 2.37 (s, 3H, $CH_3$) 2.59 (s, 3H, $CH_3$), 6.32 (s, 1H, CH), 7.29 (d, $J$ = 8 Hz, 1H, Ar), 7.41 (d, $J$ = 8 Hz, 1H, Ar); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 17.6, 20.9, 21.2, 124.5, 126.3, 129.8, 134.8, 135.6, 141.8, 144.1, 154.9, 181.6, 183.5; MS (EI) *m/z* 200 ($M^+$, 31%), 172 (100), 157 (22), 141(11), 129 (38), 115 (12), 102 (4), 77 (7), 63 (7), 51 (8), 44 (27); HRMS (EI) calcd for $C_{13}H_{12}O_2$ requires 200.0837, found: 200.0840.

4-Methyl-6,7-dimethoxy-1,2-naphthoquinone (**6f**)

Red needles: mp. 124 °C (decomp.); $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ 2.37 (s, 3H, $CH_3$), 3.97 (s, 3H, $OCH_3$), 4.03 (s, 3H, $OCH_3$), 6.25 (s, 1H, CH), 6.92 (s, 1H, Ar), 7.61 (s, 1H, Ar); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 20.7, 56.3, 108.7, 112.0, 125.2, 125.9, 130.8, 150.5, 153.2, 154.6, 178.3, 181.0; MS (EI) *m/z* 232 ($M^+$, 54%), 204 (100), 189 (37), 175 (4), 161 (9), 133 (9), 118 (8), 105 (12), 77 (5), 63 (7), 39 (6); HRMS (EI) calcd for $C_{13}H_{12}O_4$ requires 232.07356, found 232.07343.

4-Methyl-1,2-naphthoquinone (**6g**)

Orange needles: mp. 110 °C (decomp.) (lit. [371] ,109 °C (decomp.); $^{1}$H NMR ($CDCl_3$, 400 MHz) $\delta$ 2.40 (s, 3H, $CH_3$), 6.38 (s, 1H, CH), 7.53 (d and t, 2H, Ar), 7.70 (t, $J$ = 8 Hz, 1H, Ar), 8.09 (d, $J$ = 8 Hz, 1H, Ar); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 20.5, 78.69, 126.3, 127.3, 129.7, 130.6, 131.0, 135.4, 153.7, 179.5, 180.3; MS (EI) *m/z* 172 ($M^+$, 4%), 144 (100), 129 (4), 115 (71), 101 (4), 89 (7), 63 (7), 57 (6), 39 (5); HRMS (EI) calcd for $C_{11}H_8O_2$ requires 172.05243, found 172.05172.

1,4-Naphthoquinone (**7a**)

Yellow-brownish solid: mp. 127-128 °C (lit. [372] , 128 °C); $^{1}$H NMR ($CDCl_3$, 400 MHz) $\delta$ 6.98 (s, 2H, CH), 7.75 (m, 2H, Ar), 8.07 (m, 2H, Ar); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 126.4, 131.8, 133.9, 138.6, 185.0.

2-Methyl-1,4-naphthoquinone(menadione) (**7b**)

Bright yellow solid: mp. 104-105 °C (lit. [360] ,103-104 °C); $^{1}$H NMR ($CDCl_3$, 400 MHz) $\delta$ 2.17 (s, 3 H, $CH_3$), 6.83 (s, 1H, CH), 7.71-7.73 ( m, 2H, Ar), 8.03-8.09 (m, 2H, Ar); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 16.4, 126.0, 126.4, 132.0, 132.1, 133.5(x2), 135.5, 148.1, 184.9, 185.4; MS (EI) *m/z* 172 ($M^+$, 100%), 144 (23), 115 (24), 104 (34), 76 (22), 50 (9); HRMS (EI) calcd for $C_{11}H_8O_2$ requires 172.05243, found 172.05149.

2-Methoxy-1,4-naphthoquinone (**7c**)

Yellow needles: mp. 179-182 °C (lit. [373], 178-182 °C); $^{1}$H NMR ($CDCl_3$, 400 MHz) δ 3.93 (s, 3H, $OCH_3$), 6.18 (s, 1H, CH), 7.73 (dq, $J$ = 1 Hz and 7 Hz, 2H, Ar), 8.06 (dd, $J$ = 1 Hz and 7 Hz, 1H, Ar), 8.11 (dd, $J$ = 1 Hz and 7 Hz, 1H, Ar); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 56.4, 109.8, 126.1, 126.6, 130.9, 131.9, 133.3, 134.3, 160.4, 180.0, 184.7; MS

(EI) *m/z* 188 ($M^+$, 100%), 173 (40), 158 (36), 102 (40), 89 (52), 76 (20), 69 (10), 50 (10), 39 (2); HRMS (EI) calcd for $C_{11}H_8O_3$ requires 188.04734, found 188.04625.

2-Bromo-1,4-naphthoquinone (**7d**)

Yellow powder: mp.130-131 °C (lit. [374], 130-132 °C); $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ 7.52 (s, 1H, CH), 7.75-7.80 (m, 2H, Ar), 8.09 (m, 1H, Ar), 8.16 (m, 1H, Ar); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 126.8, 127.7, 130.8, 131.6, 134.0, 134.3, 140.0, 140.3, 177.8, 182.3.

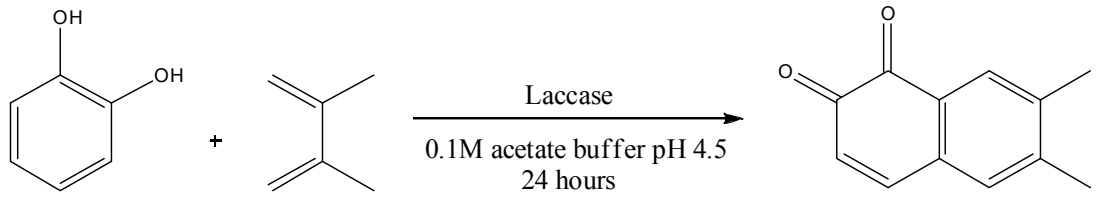
2-Chloro-1,4-naphthoquinone (**7e**)

Yellow solid: mp. 112-113 °C (lit. [375], 112-113 °C); $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ 7.23 (s, 1H, CH), 7.75-7.82 (m, 2H, Ar), 8.09 (m, 1H, Ar), 8.17 (m, 1H, Ar); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 126.7,127.5, 131.3, 131.7, 134.1, 134.5, 135.9, 146.3, 177.9, 182.7.

## 5.3 Results and Discussion

Initially, we focused our attention on the reaction of laccase with 1,2-catechols yielding the corresponding *o*-quinones which have an interesting reactivity profile in cycloaddition reactions [376], and have been used in *o*-naphthoquinone synthesis. In a preliminary study, the reaction of catechol (**5a**) and 2,3-dimethyl-1,3-butadiene (**2a**) in the presence of laccase was investigated. As summarized in Table 10, optimal yields of 6,7-dimethyl-1,2-naphthoquinone (**6a**) was achieved when the reaction was conducted with 1 equivalence of 1 and 10 equivalence of 2 in the presence of laccase in 0.1 M acetate buffer pH 4.5 at 3 ˚C for the first two hour of the reaction. The reaction mixture was then warmed to room temperature and stirred for another 22 hours.

**Table 10.** Preliminary study of the laccase-catalyzed reaction of catechol (**5a**) and 2,3-dimethyl-1,3-butadiene **(2a)** in aqueous medium



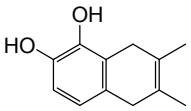

**5a** **2a** **6a**

| Entry | **5a** : **2a** (equiv.) | Temperature | Yield[a] of **6a** (%) |
|---|---|---|---|
| 1 | 1:10 | 3 °C (2 h), RT | 47 |
| 2 | 1:10 | RT. | 10 |
| 3 | 1:10 | 60 °C | No product formed |
| 4 | 1:5 | 3 °C (2 h), RT | 8 |
| 5 | 1:15 | 3 °C (2 h), RT | 32 |

[a]Isolated yield.

An excess of the diene was required to overcome the intrinsic instability of the *o*-benzoquinone as it will undergo competing decomposition, dimerization, and polymerization if insufficient diene is present for the Diels-Alder reaction [369]. In addition, the reaction temperature and medium were shown to have an effect on the reaction outcome. For example, if the reaction was preformed at room temperature or 60 ˚C the yield of **6a** was diminished to only 10 and 0 %, respectively. This result was attributed an increase in the rate of decomposition and polymerization of the in-situ generated o-quinone. Therefore, we retarded the rate of decomposition and polymerization by maintaining the initial reaction temperature to 3 ˚C for the first two hours and then allowing the reaction mixture warm to room temperature. This cascade reaction system provided 47 % of **6a**. Next, we examined whether the increase of reactant solublility by replacing acetate buffer solvent with organic or biphasic organic/water solvent can enhance the reaction. The results of these experiments are summarized in Table 11 . The reaction performed in aqueous acetate buffer at pH 4.5, generally known to be the optimum pH for laccase activity in the formation of quinone [364-366], provided the best result (see Table 11). The lower percent yield in other solvent systems was due to a decrease of laccase activity in organic and aqueous-organic mixed solvent [377,378]. Moreover, the Diels-Alder reaction has shown to exhibit higher reactivity and selectivity in aqueous medium than in organic solvent [35]. Interestingly, the use of a 1:1 acetate buffer/chloroform medium, provided the aromatized DA adduct (5,8-dihydro-6,7-dimethyl-1,2-naphthoquinone) instead of fully oxidized product (**6a**).

**Table 11.** Solvent effect on the laccase-catalyzed reaction of catechol (**5a**) and 2,3-dimethyl-1,3-butadiene (**2a**)[a]

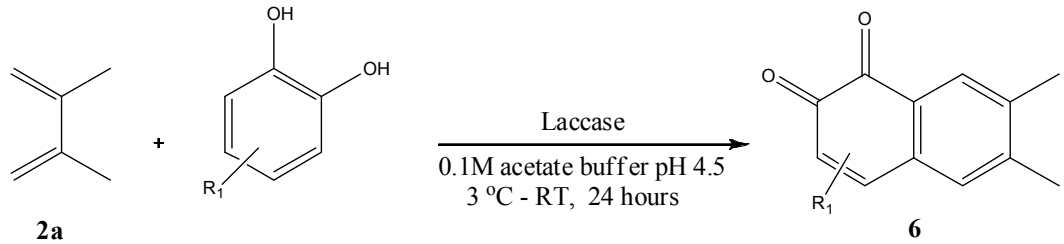
| Entry | Solvent | Yield[b] of **6a** (%) |
|---|---|---|
| 1 | 0.1 M Acetate buffer pH 4.5 | 47 |
| 2 | Water | 18 |
| 3 | 5% Aqueous PEG 2000 | 25 |
| 4 | *p*-Dioxane | 0 |
| 5 | 1:1 *p*-Dioxane/acetate buffer | 8 |
| 6 | 1:1 Ethylene Glycol/acetate buffer | 15 |
| 7 | 1:1 MeOH/acetate buffer | 18 |
| 8 | 1:1 Chloroform/acetate buffer | 0% of **6a**<br>27% of  |

[a]Reaction conditions: **5a** (1equiv) and **2a** (10equiv.) was stirred with laccase (4000U/1g substrate) in solvent at 3 °C for 2 hours and then stirred at room temperature for another 22 hours.
[b]Isolated yield.

After developing the optimum reaction conditions, the reaction of a variety of catechol substrates with diene **2a** were examined by using the procedure for the synthesis of *o*-naphthoquinones in the experimental section and these results are summarized in Table 12. The results show that the reaction depended on the reactivity of the in situ-generated *o*-quinones. The very high reactivity quinones, such as 3-methoxy-1,2-benzoquinone and 4-chloro-1,2-benzoquinone, which have rich electron donating group (OMe) and strong electron withdrawing group (Cl), respectively, did not provide good yields of the *o*-naphthoquinone product (entries 4 and 5). These quinones preferently

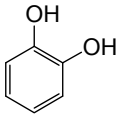
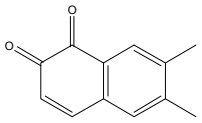
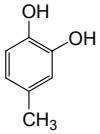
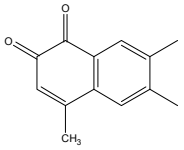
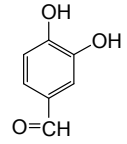
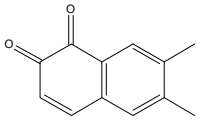
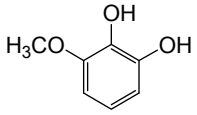
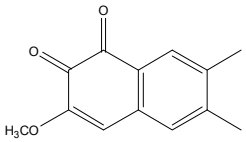
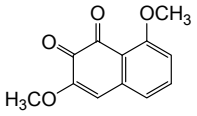
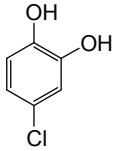
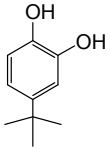
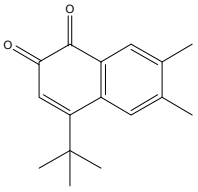
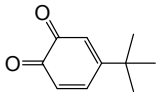
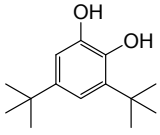
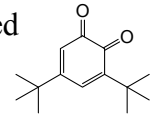
underwent dimerization and polymerization. For example, in-situ synthesis 3-methoxy-1,2-benzoquinone by laccase from the corresponding hydroquinone yielded 32% of the undesired product, which was generated by the decarbonylation and oxidation of the dimerization intermediate, and only 11% of naphthoquinone product. Besides the reactivity of the in situ-generated quinones, steric factor also affected the formation of the product. Quinones with bulky groups provided very low yield of the products such as 4-tert-butyl-1,2-benzoquinone yielded only 14% product for 4 day reaction, and 3,5-di-tert-butyl-1,2-benzoquinone gave no product but 97% of it remaining in the reaction solution (entries 6 and 7). From Table 12, the in-situ generated *o*-quinones with moderate reactivity clearly exhibited higher yields of the *o*-naphthoquinone adduct (entries 1-3), and 4-methyl-1,2-benzoquinone provided the highest yield (57%) in this reaction system (entry 2).

**Table 12.** The study of laccase-catalyzed reaction of **2a** with a variety of catechol substrates in aqueous medium




**2a** **6**

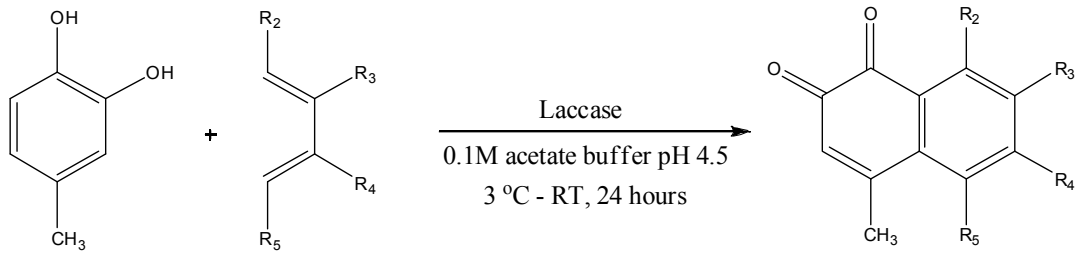
10 : 1

| Entry | Catechol | Product | Yield[a] (%) |
|---|---|---|---|
| 1 |  | **6a**  | 47 |
| 2 |  | **6b**  | 57 |
| 3 |  | **6a**  | 28 |
| 4 |  | **6c**  | 11 and 32% of  |
| 5 |  | - | no product formed |
| 6[b] |  | **6d**  | 14 and 15% of  |
| 7 |  | - | no product formed 97% of quinone  |

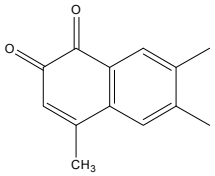
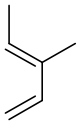
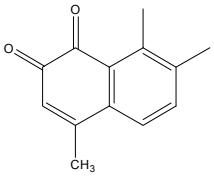
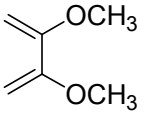
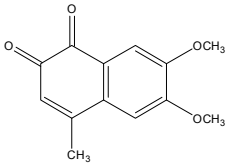
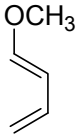
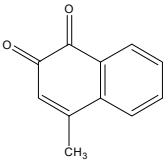
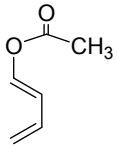
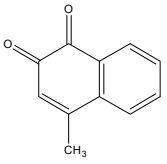
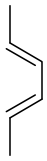
[a]Isolated yield; [b]96 hour reaction.

The versatility of this system for a variety of dienes was investigated by using 4-methylcatechol as starting material to generate 4-methyl-1,2-benzoquinone *in situ*. Table 13 demonstrates that many dienes can be used to react with 4-methyl-1,2-benzoquinone to generate *o*-naphthoquinone products in moderate to high yield. Optimal results were achieved when 1-methoxy-1,3-butadiene and 1-acetoxy-1,3-butadiene were used as diene reagent (entries 4 and 5). Both dienes provided very high yields of the product, and only 2 equivalence of 1-acetoxy-1,3-butadiene was needed. This high yielding reaction can be attributed to the elimination of the methoxy or acetoxy group that 'pushed' the reaction forward to the product. The proposed mechanism of the elimination of the methoxy or acetoxy is illustrated in Figure 107. During the Diels-Alder reaction step, the steric effect of the substituent make the reaction occurred only at the less substituent side.

**Table 13.** The study of laccase-catalyzed reaction of 4-methylcatechol with a variety of dienes in aqueous medium




1 : 10 **6**

| Entry | Diene | Product | Yield[a] (%) |
|---|---|---|---|
| 1 |  | **6b**  | 57 |
| 2 |  | **6e**  | 71 |
| 3 |  | **6f**  | 10 |
| 4 |  | **6g**  | 77 |
| 5[b] |  | **6g**  | 76 |
| 6 |  | - | no product formed |

[a]Isolated yield; [b]Only 2 equivalence of 1-acetoxy-1,3-butadiene was used.

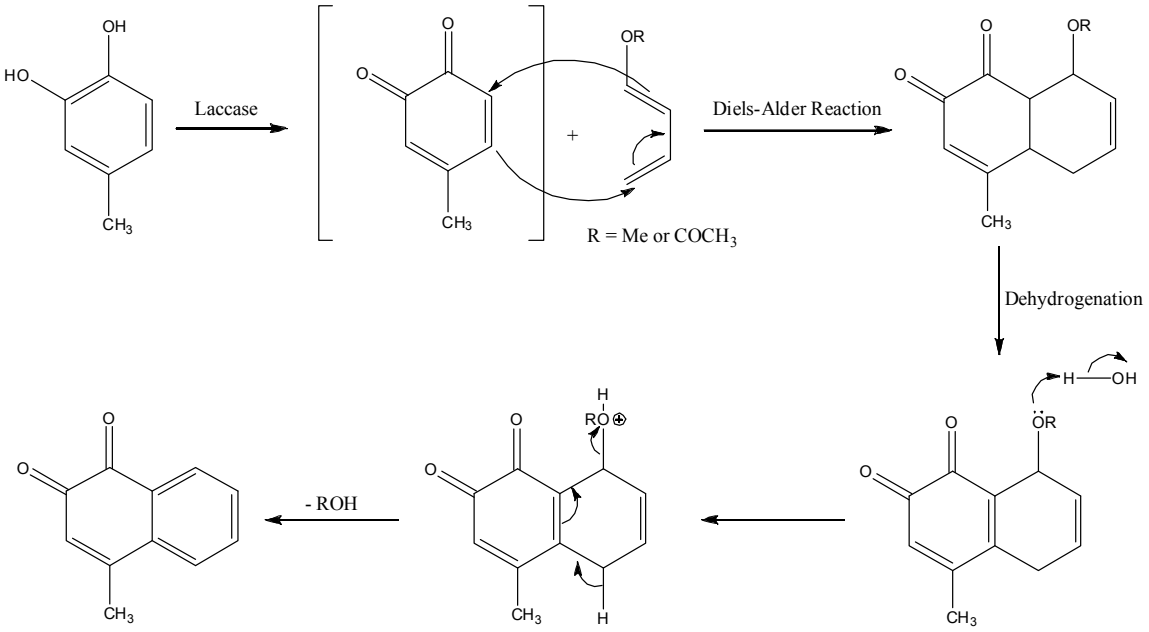
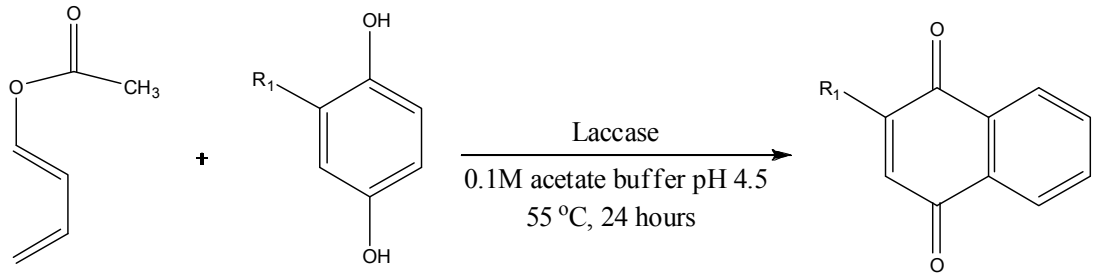



**Figure 107.** The proposed mechanism for the elimination of methoxy or acetoxy from the reaction of 4-methyl-1,2-benzoquinone and 1-methoxy-1,3-butadiene or 1-acetoxy-1,3-butadiene in the presence of laccase in aqueous medium.

In this study, we also conducted *p*-naphthoquinone synthesis by using a variety of 1,4-benzohydroquinones as a substrate for laccase to generate 1,4-benzoquinone *in situ*. As the result of o-quinone reaction above, the reactive 1-acetoxy-1,3-butadiene was chosen for this study. However, we found that the reaction of these less reactive *p*-benzoquinones gave very low yield of the desired product at low temperature. Therefore, the reaction was conducted at 55 °C for *p*-naphthoquinone synthesis, and 1 equivalence of 1,4-benzohydroquinone and 2 equivalence of diene were used (Table 14). The procedure for *p*-naphthoquinone synthesis is summarized in the experimental section. The results in Table 14 show that this reaction system can be used for a one-pot synthesis of *p*-naphthoquinones in excellent overall yield.

**Table 14.** The study of laccase-catalyzed reaction of 1-acetoxy-1,3-butadiene with a variety of 1,4-benzohydroquinone in aqueous medium at 55 $^{o}$C




2 : 1 **7**

| Entry | $R_1$ | Product | Yield[a] (%) |
|---|---|---|---|
| 1 | H | **7a** | 67 |
| 2 | $CH_3$ | **7b** | 75 |
| 3 | $OCH_3$ | **7c** | 81 |
| 4 | Br | **7d** | 67 |
| 5 | Cl | **7e** | 69 |

[a]Isolated yield.

## 5.4 Conclusions

In summary, an efficient green chemistry synthesis of *o*-naphthoquinone using laccase as an oxidant in aqueous medium was developed. In this reaction, laccase was used to oxidize *o*-diphenols to generate *o*-quinones *in situ* which further underwent Diels-Alder reaction and oxidation to form *o*-napthoquinone product. Due to the high reactivity of the *in situ*-generated *o*-quinones, the reactions have to conduct at low temperature (3 $^{o}$C to room temperature) to retard the side reactions, dimerization and polymerization. This reaction system can yield *o*-naphthoquinones up to 77% depending on the exact structure of the starting hydroquinone and diene. The reactions of 1-methoxy-1,3-butadiene and 1-acetoxy-1,3-butadiene provided very high yields of the product. This high yielding reaction can be attributed to the elimination of the methoxy or acetoxy group that 'pushed' the reaction forward to the product. In addition, this study also shows that the reaction of the reactive 1-acetoxy-1,3-butadiene and 1,4-hydroquinones catalyzed by laccase provided the yield of the corresponding *p*-naphthoquinones up to 80%.

# CHAPTER 6

# CASCADE SYNTHESIS OF BENZOFURAN DERIVATIVES VIA LACCASE OXIDATION–MICHAEL ADDITION[iii]

## 6.1 Introduction

The provious Chapters reported the green cascade syntheses of p- and o-naphthoquinone derivatives *via* Diels-Alder reaction catalyzed by laccase in aqueous medium [379,380]. These reactions provided the satified results for the sythesis of corresponding naphthoquinone products. To demonstrate another synthetic research capability of laccase, herein, this Chapter presents the first laccase-catalyzed carbon-carbon bond formation *via* oxidation-Michael addition for the cascade synthesis of benzofuran derivatives. Benzofurans have attracted much attention due to their broad spectrum of pharmacological activities [381-386] such as, anticancer, antimicrobial, antioxidant, and anti-HIV-1 activities. Therefore, the syntheses of benzofuran derivatives have been extensively investigated [387-391]. Most of these synthetic methods involve the formation of an annellated furan ring by the intramolecular cyclization of benzene

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[iii] This manuscript was published in Tetrahedron, 2007, 63, 10958-10962. It is entitled as **"**Cascade synthesis of benzofuran derivatives via laccase oxidation-Michael addition". The other authors are Dr. Leslie Gelbaum and Dr. Arthur J. Ragauskas from the School of Chemistry and Biochemistry at the Georgia Institute of Technology. This chapter is reproduced with the kind permission of from [Tetrahedron]. 

derivatives. These procedures involve either multi-steps, rigorous reaction conditions, or expensive reagents. Recently, Nematollahi et al. [392-395] and Bu et al. [396] reported the one-pot synthesis of polyhydroxylated benzofurans via the oxidation of catechols by an electrochemical method or sodium iodate, respectively, in the presence of 1,3-dicarbonyl compounds. Nevertheless, using biocatalysis in the preparation of polyhydroxylated benzofurans has never been reported. This study reports the first study at accomplishing this synthesis via a biocatalyst.

In this procedure, *ortho*-quinones, generated in situ from the oxidation of catechols by laccase, underwent the Michael addition reaction with 1,3-dicarbonyl compounds, and then, underwent intramolecular cyclization to benzofuran derivatives (see Figure 108). In addition, this study investigated the reaction system in the presence of either Lewis acid or Lewis base to improve reaction condition, and documented the recyclability of the catalytic system.

## 6.2 Experimental Section

### 6.2.1 General Information

All chemicals were obtained from Aldrich and used as received without further purification. Laccase (EC 1.10.3.2) from *Trametes Villosa* was donated by Novo Nordisk Biochem, North Carolina. $^{1}H$ and $^{13}C$ NMR spectra were recorded on a Bruker-400 spectrometer operating at 400 MHz for $^{1}H$ and 100 MHz for $^{13}C$. For HMBC correlations, the experiment was operated in a Bruker-DRX 500 spectrometer. Column chromatography was performed on Combiflash Companion instrument (Teledyne Isco company) using RediSep normal-phase flash columns. TLC was performed on aluminum sheets precoated with silica gel 60 F254 (EMD Chemicals). Mass spectra were carried out in The Georgia Institute of Technology Bioanalytical Mass Spectrometry Facility.

### 6.2.2 Enzyme Assay

Laccase activity measurement is described in Chapter 3 (Experimental Materials and Procedures).

### 6.2.3 General Procedure of the Synthesis of Benzofuran Derivatives via Laccase-Oxidation-Michael Addition.

The detail of the reaction procedure is described in Chapter 3 (Experimental Materials and Procedures).

### 6.2.4 Product Characterization

Products **9a** [394], **9b** [396], and **9c** [394] are known compounds, and their $^{1}$H-NMR and $^{13}$C NMR data are consistent with those in literature. Structure **9d** is, to the best of our knowledge, new compounds and its NMR spectra are shown in Appendix A.3.

3-Acetyl-5,6-dihydroxy-2-methylbenzofuran (**9a**)

White solid; mp. 238-239 ˚C (lit. [394], 236-238 ˚C); $^{1}$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 2.51(s, 3H, $CH_3$), 2.67 (s, 3H, $CH_3$), 6.92 (s, 1H, Ar-H), 7.35 (s, 1H, Ar-H), 9.03 (br s, 1H, OH), 9.10 (br s, 1H, OH); $^{13}$C NMR (DMSO-$d_6$, 100 MHz) $\delta$ 15.2, 30.7, 97.7, 106.3, 117.1, 117.2, 143.4, 144.1, 146.9, 160.6, 193.8; MS (EI) *m/z* 206($M^+$, 92%), 191 (100), 163 (36), 135 (14), 95 (6), 89 (4), 63 (4), 53 (3), 43 (19); HRMS (EI) 206.05838($C_{11}H_{10}O_4$ requires 206.05791).

Ethyl-5,6-dihydroxy-2-methyl-3-benzofuran carboxylate (**9b**)

White solid; mp. 180-182 ˚C (lit. [396,397], 180-182 ˚C); $^{1}$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 1.34 (t, $J$ = 7 Hz, 3H, $CH_3$), 2.62 ( s, 3H, $CH_3$), 4.27 (q, $J$ = 7 Hz, 2H, $CH_2$), 6.91(s, 1H, Ar-H), 7.22 (s, 1H, Ar-H), 9.03 ( br s, 1H, OH), 9.11 (br s, 1H, OH); $^{13}$C NMR (DMSO-$d_6$, 100 MHz) $\delta$ 14.1, 14.3, 59.8, 97.8, 105.9, 108.1, 116.9, 143.4, 144.2, 147.1, 161.1, 163.8; MS (EI) *m/z* 236($M^+$, 92%), 207 (100), 191 (33), 93 (4), 43 (6); HRMS (EI) 236.07061 ($C_{12}H_{12}O_5$ requires 236.06847).

3-Acetyl-5,6-dihydroxy-2,7-dimethylbenzofuran (**9c**)

White solid; mp. 218-220 ˚C (lit. [394], 217-219 ˚C); $^{1}$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 2.24 (s, 3H, $CH_3$), 2.49 (s, 3H, $CH_3$), 2.68 (s, 3H, $CH_3$), 7.22 (s, 1H, Ar-H), 8.41 (br s, 1H, OH), 9.28 (br s, 1H, OH); $^{13}$C NMR (DMSO-$d_6$, 100 MHz) $\delta$ 8.9, 15.3, 30.7, 103.2,

107.1, 116.2, 117.4, 141.8, 143.1, 146.5, 160.5, 193.9; MS (EI) *m/z* 220($M^+$, 85%), 205 (100), 177(21), 149 (4), 102 (5), 43 (13); HRMS (EI) 220.07490 ($C_{12}H_{12}O_4$ requires 220.07356).

Ethyl-5,6-dihydroxy-2,7-dimethyl-3-benzofuran carboxylate (**9d**).

White-yellow solid; mp. 183-185 ˚C; $^1$H NMR (DMSO-$d_6$, 400 MHz) $\delta$ 1.35 (t, $J$ = 7 Hz, 3H, $CH_3$), 2.25 (s, 3H, $CH_3$), 2.66 (s, 3H, $CH_3$), 4.29 (q, $J$ = 7 Hz, 2H, $CH_2$), 7.13 (s, 1H, Ar-H), 8.42 (s, 1H, OH), 9.31 (s, 1H, OH); $^{13}$C NMR (DMSO-$d_6$, 100 MHz) $\delta$ 8.9, 14.2, 14.2, 59.8, 102.8, 107.2, 108.3, 115.9, 141.9, 143.0, 146.7, 161.0, 163.9; MS (EI) *m/z* 250($M^+$, 98%), 221 (100), 176(11), 93 (4), 43 (7); HRMS (EI) 250.08453($C_{13}H_{14}O_5$ requires 250.08412).

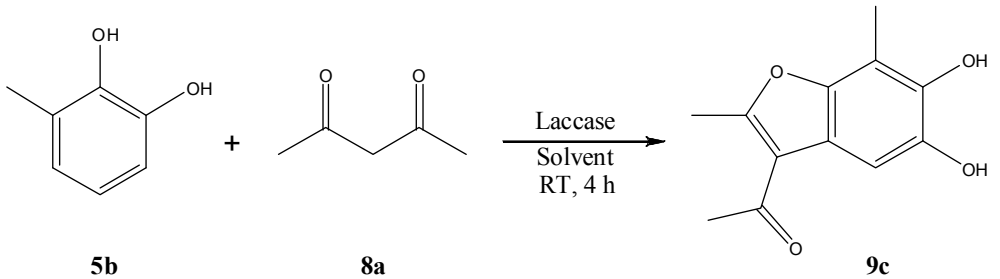
## 6.3 Results and Discussion

### 6.3.1 Preliminary Study and the Effect of pH on the Reaction System

In a preliminary study, the reaction of 3-methylcatechol (**5b**) and acetylacetone (**8a**) in the presence of laccase was investigated. The reaction was carried out under air at room temperature (23 ˚C) in the aqueous buffer solution for 4 hours. This reaction system was chosen to be a model reaction for this study because the product, 3-acetyl-5,6-dihydroxy-2,7-dimethylbenzofuran (**9c**), gradually precipitated during the reaction and was easy to recover by filtration after the reaction.

The effect of pH on this reaction system was initially studied. As summarized in Table 15, the optimal yields of **9c** were achieved when the reaction was conducted at pH 7.0. At pH 4.5, no product formed because this low pH was not basic enough to deprotonate alpha-proton from acetylacetone to facilitate the Michael addition reaction with the *in situ* generated *o*-quinone. At a higher pH value of 8.0, only a small yield of **9c** was received due to laccase activity which was dramatically decreased at this pH [199,366]. Therefore, only a small amount of starting catechol was oxidized and reacted subsequently with acetylacetone. Moreover, the ratio of **5b** and **8a** also affected the yield of **9c**. The result shows that the yield of **9c** increased when using **5b** and **8a** in 1:2 ratio (entry 2).

**Table 15.** The effect of pH on the laccase-catalyzed reaction of 3-methylcatechol (**5b**) and acetylacetone (**8a**)




**5b** **8a** **9c**

| Entry | Solvent/ pH | **5b**:**8a** (equiv) | Yield[a] of **9c** (%) |
|---|---|---|---|
| 1 | 0.1 M Phosphate buffer pH 7.0 | 1:1 | 46 |
| 2 | 0.1 M Phosphate buffer pH 7.0 | 1:2 | 64 |
| 3 | 0.1 M Acetate buffer pH 4.5 | 1:2 | 0 |
| 4 | 0.1 M Phosphate buffer pH 8.0 | 1:2 | 6 |

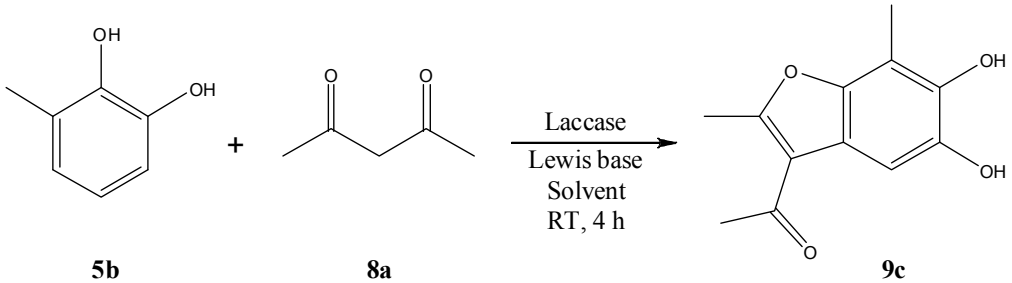
[a]Isolated yield.

### 6.3.2 The Effect of Lewis Bases on the Reaction System

After this preliminary study, the next phase was to improve the yield of the product by enhancing Michael-addition step. Traditionally, Michael reactions are catalyzed by strong bases such as alkali metal, alkoxides or hydroxides [398]. However, these strongly basic conditions can lead to a number of side- and subsequent reactions, and especially for this reaction system, the *in situ*-generated *o*-quinones are easily decomposed in the presence of hydroxides [376]. Recently, Xia et al. [399] reported the use of a Lewis base to catalyze Michael addition of azide ion to cyclic enones in water. Herein, adding Lewis base to the catalyzed Michael addition step was investigated. Table 16 reveals that the best yield of **9c** was obtained when using pyridine as Lewis base in phosphate buffer pH 7.0, and the ratio of **5b**:**8a**: pyridine was 1:2:1. While the use of

stronger Lewis acid such as 4-dimethylaminopyridine (DMAP) and 1,4-diazabicyclo[2.2.2]octane (DABCO) provided only a low yield of the product. Although the use of pyridine gave the best result for this reaction system, the yield of the product (54%, entry 3) was still much lower than the yield of the product (64%, Table 15, entry 2) accomplished without pyridine. According to these results, adding basic reagents into this reaction did not enhance the reaction efficiency, especially, when a strong base was used.

**Table 16.** The effect of Lewis bases on the laccase-catalyzed reaction of 3-methylcatechol (**5b**) and acetylacetone (**8a**)




**5b** **8a** **9c**

| Entry | Lewis bases | Solvent | **5b**: **8a**: Lewis base (equiv) | Yield[a] of **9c** (%) |
|---|---|---|---|---|
| 1 | Pyridine | Water | 1: 2: 0.5 | 33 |
| 2 | Pyridine | 0.1 M Phosphate buffer pH 7.0 | 1: 2: 0.5 | 40 |
| 3 | Pyridine | 0.1 M Phosphate buffer pH 7.0 | 1: 2: 1 | 54 |
| 4 | DMAP | 0.1 M Phosphate buffer pH 7.0 | 1: 2: 1 | 9 |
| 5 | DABCO | 0.1 M Phosphate buffer pH 7.0 | 1: 2: 1 | 13 |

[a]Isolated yield.

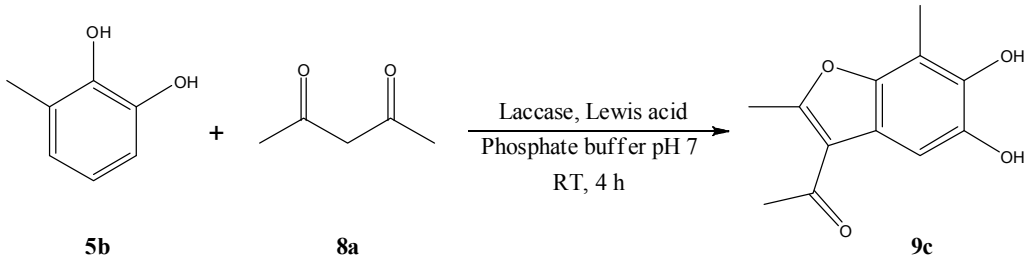
### 6.3.3 The Effect of Lewis Acids on the Reaction System

In order to circumvent the alkaline conditions above, we decided to investigate the reaction in the presence of a Lewis acid as an alternative method. Lewis acid-catalyzed Michael reactions have been developed, allowing the reaction to be carried out under milder conditions with high efficiency [400]. Our studies focus on the use of water as reaction medium to avoid the use of organic solvents which have become an environmental concern. Studies by Kobayashi et al. have showed that the rare earth metal triflates ($Sc(OTf)_3$, $Yb(OTf)_3$, etc.) can be used as Lewis acid catalyst in water-containing solvents [401,402]. Therefore, we examined a variety of Lewis acids including the water-compatible Lewis acid, $Sc(OTf)_3$ and $Yb(OTf)_3$, for the synthesis of **9c**. The reaction was carried out under the optimal condition in the preliminary study (Table 15, entry 2) but Lewis acids were varied. The results of this Lewis acid study is summarized in Table 17. The results show that the water-stable Lewis acid, $Sc(OTf)_3$ and $Yb(OTf)_3$ can enhance Michael addition step for this reaction system and provided a very good yield of **9c**. $Sc(OTf)_3$ showed better result than $Yb(OTf)_3$. However, we have to use 0.2 equiv of $Sc(OTf)_3$ to obtain the highest yield of **9c** (74%, entry 2) because using only 0.1 equiv of $Sc(OTf)_3$ did not have any effect on the reaction yield (63%, entry 1) when compared to the reaction without $Sc(OTf)_3$ (64%, Table 15, entry 2).

As we conducted the reaction in aqueous medium, the main drawback was the low solubility of organic substances. To overcome this problem, a small amount of surfactant, sodium dodecyl sulfate (SDS, 20 mol %) was used to improve the solubility, and the result shows a small increase of product yield from 74% to 76% (Table 17, entry

3). This result agrees with Kobayashi's work on the study of surfactant-aided Lewis acid catalysis in aqueous aldol reaction [403].

**Table 17.** The effect of Lewis acids on the laccase-catalyzed reaction of 3-methylcatechol (**5b**) and acetylacetone (**8a**)




**5b** **8a** **9c**

| Entry | Lewis acid | **5b**: **8a**: Lewis acid (equiv) | Yield[a] of **9c** (%) |
|---|---|---|---|
| 1 | $Sc(OTf)_3$ | 1: 2: 0.1 | 63 |
| 2 | $Sc(OTf)_3$ | 1: 2: 0.2 | 74 |
| 3 | $Sc(OTf)_3$/ SDS | 1: 2: 0.2 | 76 |
| 4 | $Yb(OTf)_3$ | 1: 2: 0.2 | 72 |
| 5 | $InCl_3.4H_2O$ | 1: 2: 0.2 | 71 |
| 6 | $CuCl_2$ | 1: 2: 0.2 | 49 |

[a]Isolated yield

### 6.3.4 The Synthesis of Benzofuran Derivatives

After successfully conducting the optimization experiments described above, we chose to conduct further synthesis of benzofuran derivatives by introducing 1 mmol of substituted catechols and 2 mmol of 1,3-dicarbonyl compounds in 0.1M phosphate buffer (pH 7.0), in the presence of laccase, 20 mol% of $Sc(OTf)_3$, and 20 mol% of SDS under air at room temperature. The proposed reaction pathway of this catalytic system is

illustrated in Figure 108, and the result of the reaction of various catechols and 1,3-dicarbonyl compounds are summarized in Table 18.

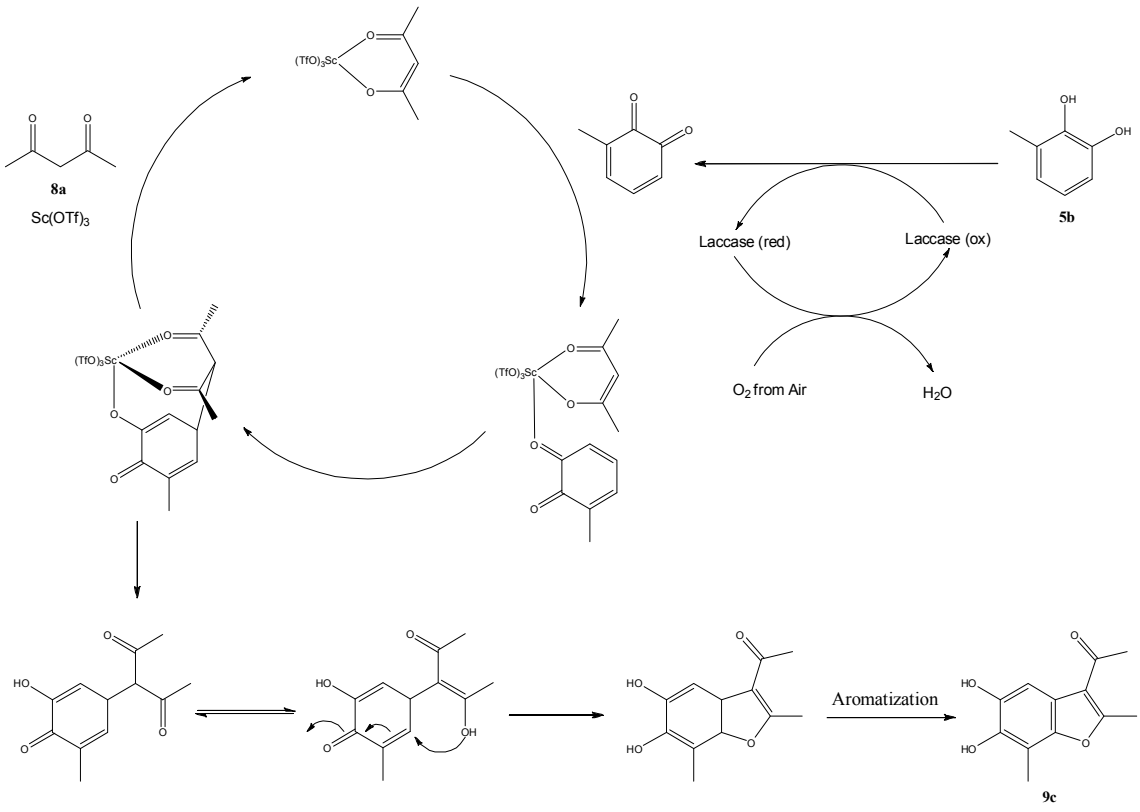
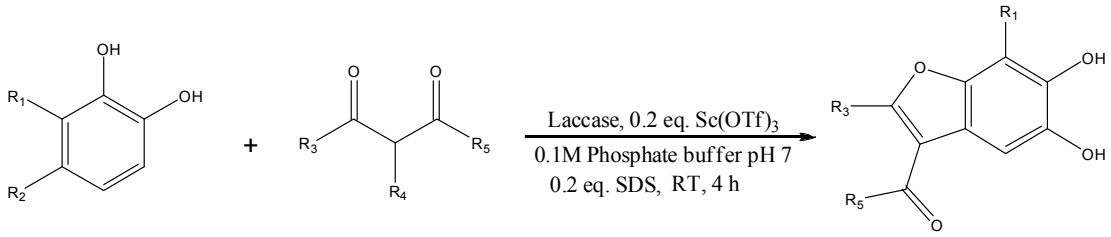
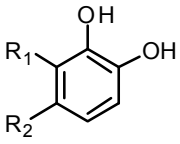
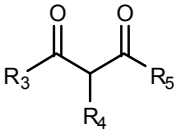
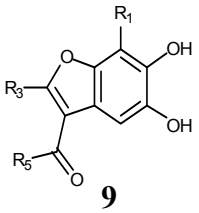



**Figure 108.** Proposed mechanism of laccase/$Sc(OTf)_3$ catalytic system for the synthesis of 3-acetyl-5,6-dihydroxy-2,7-dimethylbenzofuran (**9c**).

**Table 18.** The study of the laccase/$Sc(OTf)_3$-catalyzed reaction of catechols and 1,3-dicarbonyl compounds for benzofuran synthesis




| Entry | Catechol | 1,3-Dicarbonyl compound | Product (%yield)[a] |
|---|---|---|---|
| |  **5** |  **8** |  **9** |
| 1 | **5a**: $R_1 = R_2 = H$ | **8a**: $R_3 = R_5 = Me$, $R_4 = H$ | **9a** (68%) |
| 2 | **5a** | **8b**: $R_3 = R_5 = Me$, $R_4 = Cl$ | **9a** (66%)[b] |
| 3 | **5a** | **8c**: $R_3 = Me$, $R_4 = Cl$, $R_5 = OEt$ | **9b** (46%)[b] |
| 4 | **5b**: $R_1 = Me$, $R_2 = H$ | **8a** | **9c** (76%) |
| 5 | **5b** | **8b** | **9c** (79%)[b] |
| 6 | **5b** | **8c** | **9d** (48%)[b] |
| 7 | **5c**: $R_1 = OMe$, $R_2 = H$ | **8a** | - |
| 8 | **5d**: $R_1 = F$, $R_2 = H$ | **8a** | - |
| 9 | **5e**: $R_1 = H$, $R_2 = Cl$ | **8a** | **9a** (9%) |
| 10 | **5f**: $R_1 = H$, $R_2 = COOH$ | **8a** | **9a** (11%) |

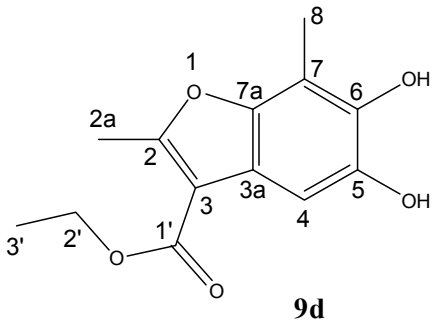
[a]Isolated yield; [b]Reaction time is 1 hour.

The data in Table 18 show that the reaction depends on the reactivity of the *in situ*-generated *o*-quinones. The very reactivity quinones, such as 3-methoxy-1,2-benzoquinone and 3-fluoro-1,2-benzoquinone, which have rich electron donating group (OMe) or a strong electron withdrawing group (F), respectively, did not provided any desired products (entries 7 and 8). This reactivity pattern may be caused by side reactions of these highly reactive quinones. In contrast, the reaction of catechols, such as 3-

methylcatechol and catechol with laccase generated moderately reactive quinones that gave excellent yields of the corresponding benzofuran products as shown in entries 1-6. Moreover, the reactivity of 1,3-dicarbonyl compounds also have an effect on the reaction. When we used 1,3-dicarbonyl compounds that had an electron withdrawing group (Cl) at alpha-position, the reaction time was only 1 hour. The shorter reaction time caused by the increase of alpha-proton acidity of these 1,3-dicarbonyl compounds that make it easier to deprotonate and ready to react with in situ-generated o-quinone in the reacion solution. Besides 3-substituted catechols, 4-substituted catechols, such as 4-chlorocatechol and 3,4-dihydroxy benzoic acid, can also be used for the synthesis of polyhydroxylated benzofurans (entries 9 and 10). However, the yield of the product is low.

In addition, we observed that this reaction system gave only one isomer from potential products that could occur. This could be explained by the existence of a substituent at the C-3 position of catechols that probably causes the Michael acceptors, *in situ* generated *o*-quinones, to be attacked by 1,3-dicarbonyl compounds only at less hindered C-5 position to yield the observed product (see Figure 108). Most of the products from this study are known compounds. Only product **9d** is unknown. Therefore, the structure of **9d** was confirmed by the $^{1}$H NMR, $^{13}$C NMR and HMBC correlations as shown in Table 19.

**Table 19.** $^{1}H$ and $^{13}C$ assignment and HMBC correlations for compound **9d**[a]




**9d**

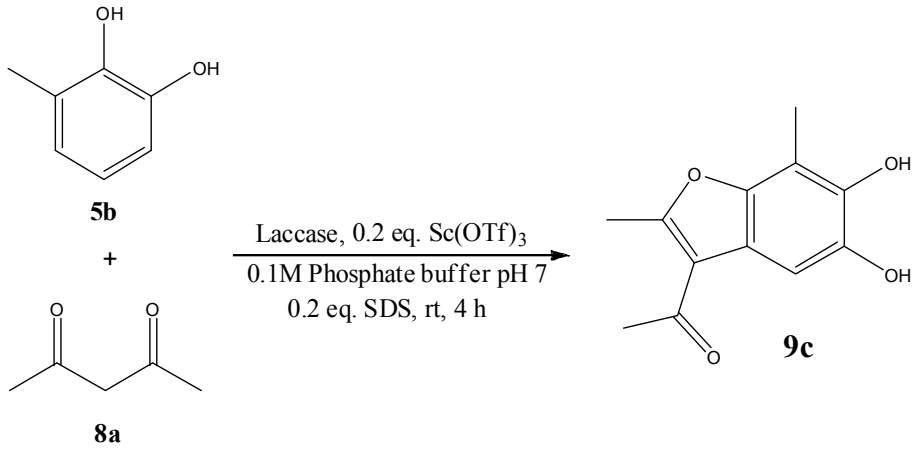
| Carbon | $^{13}C$ ($\delta$) | $^{1}H$ ($\delta$) | $^{1}H$-$^{13}C$ Correlations |
|---|---|---|---|
| 2a | 14.2 | 2.66, s, 3H | C2, C3 |
| 4 | 102.8 | 7.13, s, 1H | C3, C5, C6, C7a |
| 8 | 8.9 | 2.25, s, 3H | C6, C7, C7a |
| 2’ | 59.8 | 4.29, q, 2H (7) | C1’, C3’ |
| 3’ | 14.2 | 1.35, t, 3H (7) | C2’ |
| OH (5) | | 9.31, s, 1H | C4, C5 |
| OH (6) | | 8.42, s, 1H | C6, C7 |

[a]Measure in DMSO-$d_6$ at 125 ($^{13}C$) or 500 MHz (1H, $J$ (Hz) values in parentheses). Chemical shifts are expressed in $\delta$(ppm). The HMBC spectrum is shown in Appendix A.3.

### 6.3.5 The Recyclability of the Laccase/Sc(OTf)$_3$-Catalytic System

Next, we examined the recyclability of the two-component catalytic system, laccase/Sc(OTf)$_3$, for the synthesis of benzofuran 3a by the reaction of **5b** and **8a** in 0.10 M phosphate buffer pH 7.0 and 20 mol% SDS. Due to the product **9c** precipitated during the reaction, we can directly reuse this catalytic system after product filtration. The results shown in Table 20 demonstrate that this catalytic system was readily recyclable for three runs, with approximately a 10% drop of the product yield/reaction.

**Table 20.** Recycling of the laccase/$Sc(OTf)_3$ catalytic system for the synthesis of 3-acetyl-5,6-dihydroxy-2,7-dimethylbenzofuran (**9c**)




| Run | Yield[a] of **9c** (%) |
|---|---|
| 1 | 76 |
| 2 | 62 |
| 3 | 51 |

[a]Isolated yield.

## 6.4 Conclusions

In conclusion, this study provides an efficient green chemistry synthesis of benzofuran derivatives from the reaction of catechols and β-dicarbonyl compounds using a catalytic system of laccase and $Sc(OTf)_3$ in surfactant aqueous medium. This reaction is regioselective providing only one isomer product and the first example of a two component catalytic system employing laccase and a lanthanide Lewis acid catalyst. The yield of the products from reaction depended on both the reactivity of catechols and β-dicarbonyl compounds. For this reaction system, catechols with moderate reactivity yield benzofuran products in excellent yield. In addition, the newly developed catalytic system could also be recycled and reused for two additional runs, with only a minor drop in product yields.

# CHAPTER 7

# CO-CATALYTIC ENZYME SYSTEM FOR THE MICHAEL ADDITION REACTION OF IN SITU-GENERATED ORTHO-QUINONES[iv]

## 7.1 Introduction

In recent years, the advances in genomics and biotechnology have dramatically broadened the availability of low-cost enzymes. In turn, this has increased the potential application of enzymes for organic synthesis while also addressing the challenges of green chemistry [32]. A growing field of interest in this field is the application of enzyme-initiated domino reactions [1,113-115]. Under optimized reaction conditions it has been shown that several biocatalytic reactions can be carried out in a single reactor [137-146]. For example, Kroutil and his co-workers [148] recently developed the one pot, two step, two enzyme cascade reaction for the synthesis of enantiopure epoxide. Herein, we report on the use of two enzymes, laccase and lipase, in the domino reaction of *in situ*-generated *o*-quinones followed by enzyme catalyzed Michael addition.

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[iv] This manuscript was submitted to European Journal of Organic Chemistry, 2008. It is entitled as **"**Co-catalytic enzyme system for the Michael addition reaction of in situ-generated ortho-quinones". The other author is Dr. Arthur J. Ragauskas from the School of Chemistry and Biochemistry at the Georgia Institute of Technology

Although lipases (triacylglycerol hydrolase, EC 3.1.1.3) have been known to catalyze the hydrolysis and the synthesis of esters formed from alcohols and acids [304,305,318], recent studies have reported the ability of lipases to catalyze Michael addition reactions [321,322,325]. For example, Torre et al. [321] provided the initial demonstration that lipase was able to catalyze the Michael addition of secondary amines to acrylonitrile. This reaction is clearly different from the natural process this enzyme is usually associated with. Berglund et al. [325] has reported the Michael addition of 1,3-dicarbonyl compounds to α,β-unsaturated carbonyl compounds catalyzed by a *C. antarctica* lipase B mutant. Moreover, Wang et al. [322] recently established that lipase M from *Mucor javanicus* was able to catalyze the Michael addition reaction of pyrimidine with a disaccharide acrylate.

According to Chapter 6, an aqueous cascade synthesis of benzofuran derivatives from the reaction of catechols and 1,3-dicarbonyl compounds via an oxidation-Michael addition sequence catalyzed by laccase and $Sc(OTf)_3$/SDS was successfully developed [404]. Depending on the exact substrates, one-pot yields of benzofurans averaged 50-79% and in the absence of $Sc(OTf)_3$, these yields decreased to 45-65%. Hence, the use of an aqueous Lewis acid was critical for efficient synthesis of the desired compounds. In regards to environmental concern, this system still produces some hazardous waste from the metal catalyst. Therefore, the development of alternative methodologies to replace the lanthanide metal catalyst in this synthesis is a high priority to enhance the overall green chemistry aspect of this one-pot synthetic reaction. This Chapter presents the use of enzyme, lipase, as an alternative catalyst in conjunction with laccase for the synthesis of benzofuran derivatives. In addition, in this study, this lipase/laccase co-catalytic system

was also used to catalyze the Michael addition of *in situ*-generated *o*-quinones and aromatic amines.

## 7.2 Experimental Section

### 7.2.1 General Information

All chemicals were used as received without further purification. Laccase (EC 1.10.3.2) from *Trametes villosa* was donated by Novo Nordisk Biochem, North Carolina. Lipases were purchased from Aldrich. Unit definition of each lipase is different depending on the method that Aldrich used to measure lipase activity. The enzymes were kept frozen until used. $^{1}H$ and $^{13}C$ NMR spectra were recorded on a Bruker-400 spectrometer operating at 400 MHz for $^{1}H$ and 100 MHz for $^{13}C$ in $d_6$-DMSO or $CDCl_3$ using tetramethylsilane (TMS) as the internal standard. All reactions were monitored by TLC. TLC was performed on aluminum sheets precoated with silica gel 60 F254 (EMD Chemicals). Column chromatography was performed on Combiflash Companion instrument (Teledyne Isco company) using RediSep normal-phase flash columns. Mass spectra were carried out in The Georgia Institute of Technology Bioanalytical Mass Spectrometry Facility.

### 7.2.2 Enzyme Assay

Laccase activity measurement is described in Chapter 3 (Experimental Materials and Procedures).

### 7.2.3 General Procedure of the Synthesis of Benzofuran Derivatives Using Laccase-Lipase Co-Catalytic System.

The detail of the reaction procedure is described in Chapter 3 (Experimental Materials and Procedures).

### 7.2.4 Procedure for the Study of the Reaction of 5a and 8a (with and without Lipase PS)

In a 250-mL round-bottom flask, 40 ml of 0.10 M phosphate buffer pH 7.0 and **5a** (2 mmol, 0.2202 g) were mixed together. Next, 200 U of laccase was added to reaction mixture and then, **8a** (0.4004 g, 410 µl, 4 mmol ) and 1848 U of lipase PS (or no lipase) were added. The reaction was then stirred at room temperature in a flask open to the atmosphere for 4.5 h. A 3 ml aliquot of the reaction mixture was taken every 30 minutes during the reaction and extracted with 10 ml of EtOAc. The organic phase was then dried over $MgSO_4$, filtered and concentrated under reduced pressure. The resulting crude product was submitted to quantitative $^1$H NMR analysis to measure the formation of product **9a** using 0.5 ml of 0.20 M 1,3,5-trioxane in $d_6$-DMSO as internal standard. Figure 109 illustrates $^1$H-NMR spectra of the crude mixture that show the formation of product **9a** during the reaction. Ar-H peaks of **9a** are used to calculate the yield of **9a**.

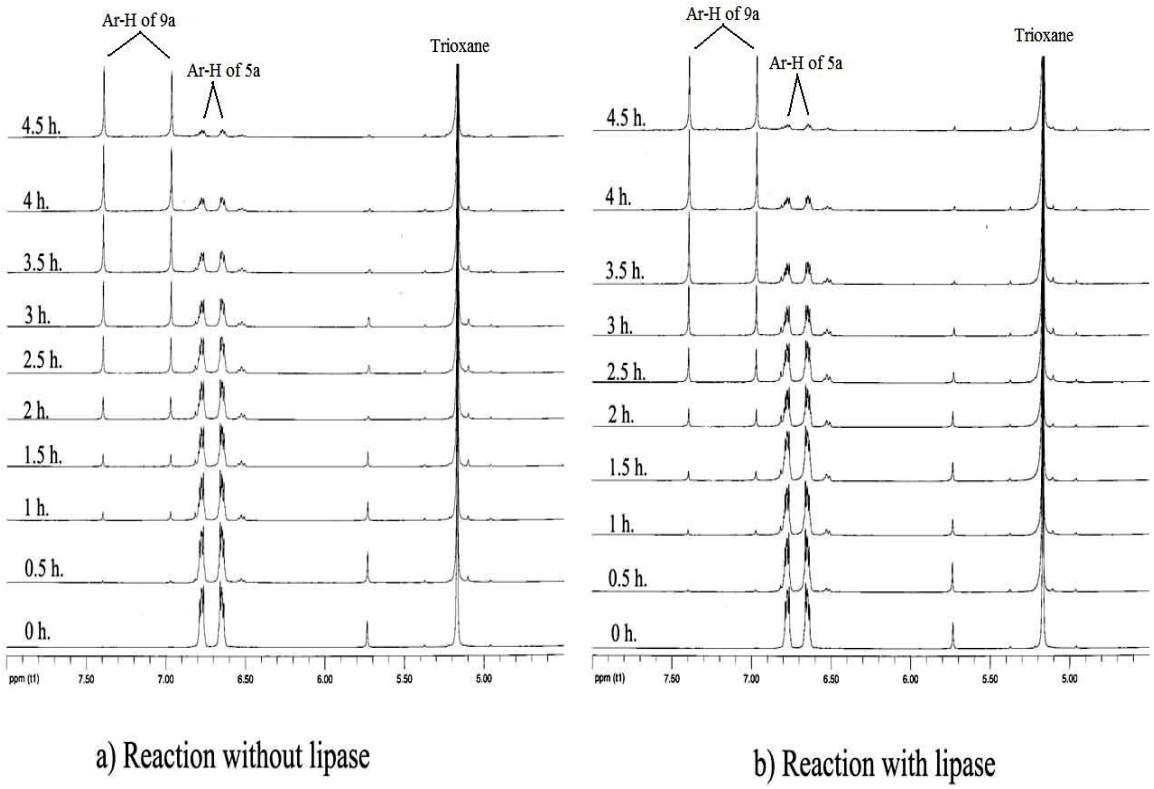



**Figure 109.** $^{1}$H-NMR of crude mixture from the laccase-catalyzed the reaction of **5a** and **8a** with and without lipase. These spectra demonstrate the formation of **9a** and the decrease of starting material **5a** during the reaction.

### 7.2.5 General Procedure for the Reaction of Catechols and Anilines Catalyzed by Laccase-Lipase Co-Catalytic System.

The detail of the reaction procedure is described in Chapter 3 (Experimental Materials and Procedures).

### 7.2.6 Product Characterization

Compond **9a** [394], **9b** [395], **9c** [394], **9d** [404], and **11a** [405] are known compounds and our $^{1}H$ and $^{13}C$ NMR data are consistent with those in the literature. Structure **11b**, **11c**, and **11d** are, to the best of our knowledge, new compounds. $^{1}H$ and $^{13}C$ assignments and HMBC correlation for compound **11b**, **11c**, and **11d** are summarized in Table 21. These NMR spectra are shown in Appendix A.4.

Compound **11a**

Red solid: Yield: 87 mg (30%). m.p. 193-195 ºC. $^{1}H$ NMR (400 MHz, $CDCl_3$): $\delta$ = 8.59 (br s, 1H, OH), 7.55 (br s, 1H, OH), 7.42 (t, $J$ = 9 Hz, 4 H, 4 × CH arom.), 7.22 (t, $J$ = 9 Hz, 2 H, 2 × CH arom.), 7.12 (br s, 4 H, 4 × CH arom.), 6.10 (s, 2 H, 2 × CH) ppm. $^{13}C$ NMR (100 MHz, $CDCl_3$): $\delta$ = 96.0, 121.9, 125.7, 129.4 ppm. MS (EI): *m/z* = 290 ($M^+$, 70%), 261 (26), 144 (15), 77 (23), 51 (8). HRMS (EI): calcd. for $C_{18}H_{14}N_2O_2$ 290.1055; found 290.1038.

Compound **11b**

Red solid. Yield: 129.5 mg (37%). m.p. 161-162 ºC. $^{1}H$ NMR (400 MHz, $CDCl_3$): $\delta$ = 8.50 (br s, 1 H, OH), 7.56 (br s, 1 H, OH), 7.08 (d, $J$ = 7 Hz, 4 H, 4 × CH arom.), 6.94 (d, $J$ = 8 Hz, 4 H, 4 × CH arom.), 6.07 (s, 2 H, 2 × CH), 3.84 (s, 6 H, 2 × $OCH_3$) ppm. $^{13}C$ NMR (100 MHz, $CDCl_3$): $\delta$ = 157.4, 151.9, 135.3, 123.9, 114.2, 95.1, 55.1 ppm. IR (KBr): $\upsilon_{max}$ = 3293 (s), 3246 (s), 3040 (w), 2834 (w), 1739 (w), 1654 (w), 1606 (s), 1580 (s), 1525 (s), 1511 (s), 1411 (s), 1330 (m), 1286 (m), 1244 (s), 1217 (s), 1199 (s), 1173 (m), 1033 (m), 840 (m)$cm^{-1}$. MS (EI): *m/z* = 350 ($M^+$, 86%), 319 (100), 291 (12), 174 (15), 146 (12), 92 (7), 77 (9). HRMS (EI): calcd. for $C_{20}H_{18}N_2O_4$ 350.1266; found 350.1247.

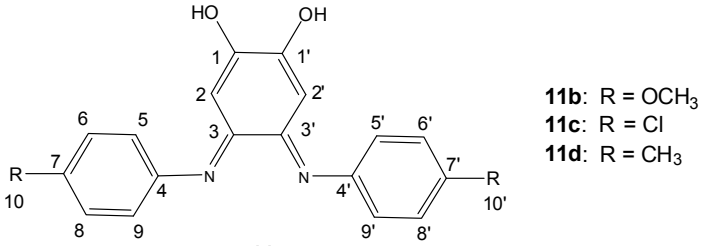
Compound **11c**

Red solid. Yield: 182.6 mg (51%). m.p. 219-221 ºC. $^{1}$H NMR (400 MHz, DMSO-$d_6$): $\delta$ = 9.24 (br s, 2 H, 2 × OH), 7.44 (d, $J$ = 7 Hz, 4 H, 4 × CH arom.), 7.19 (br s, 4 H, 4 × CH arom.), 5.81 (s, 2 H, 2 × CH) ppm. $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ = 152.0, 142.6, 129.1, 128.9, 123.7, 97.5 ppm. IR (KBr): $\nu_{max}$ = 3298 (s), 3031 (w), 1736 (w), 1660 (w), 1606 (m), 1573 (s), 1536 (s), 1493 (s), 1480 (s), 1415 (s), 1334 (s), 1221 (s), 1188 (s), 1087 (m), 1007 (m), 830 (m) cm$^{-1}$. MS (EI): *m/z* = 358 ($M^+$, 42%), 323 (80), 288 (8), 178 (18), 144 (15), 127 (100), 84 (57), 65 (18), 49 (75). HRMS (EI): calcd. for $C_{18}H_{12}N_2O_2Cl_2$ 358.0275; found 358.0266.

Compound **11d**

Red solid. Yield: 159 mg (50%). m.p. 194-196 ºC. $^{1}$H NMR (400 MHz, $CDCl_3$): $\delta$ = 8.55 (br s, 1 H, OH), 7.55 (br s, 1 H, OH), 7.20 (d, $J$ = 7 Hz, 4 H, 4 × CH arom.), 7.02 (br s, 4 H, 4 × CH arom.), 6.09 (s, 2 H, 2 × CH), 2.37 (s, 6 H, 2 × $CH_3$) ppm. $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ = 152.2, 135.5, 129.9, 122.0, 95.7, 20.9 ppm. IR (KBr): $\nu_{max}$ = 3297 (s), 3031 (w), 2917 (w), 1739 (m), 1663 (w), 1600 (s), 1572 (s), 1533 (s), 1511 (s), 1488 (s), 1413 (s), 1337 (s), 1219 (s), 1189 (s), 1153 (s), 897 (m), 814 (m), 732 (m) cm$^{-1}$. MS (EI): *m/z* = 318 ($M^+$, 42%), 303 (100), 275 (15), 158 (13), 130 (8), 91 (14), 65 (8), 49 (11). HRMS (EI): calcd. for $C_{20}H_{18}N_2O_2$ 318.1368; found 318.1348.

**Table 21.** $^{1}H$ and $^{13}C$ assignments and HMBC correlation for compound **11b**, **11c**, and **11d**.[a]




**11**

**11b**: R = $OCH_3$
**11c**: R = Cl
**11d**: R = $CH_3$

| Compound **11b** | | | |
|---|---|---|---|
| Carbon[b] | $^{13}C$ ($\delta$) | $^{1}H$ ($\delta$) | $^{1}H$-$^{13}C$ correlation |
| 2, 2’ | 95.1 | 6.07, s, 2H | C3, C3’ |
| 5, 5’, 9, 9’ | 123.9 | 7.08, d, 4H (7) | C4, C4’, C6, C6’, C7, C7’, C8, C8’ |
| 6, 6’, 8, 8’ | 114.2 | 6.94, d, 4H (8) | C4, C4’, C5, C5’, C7, C7’, C9, C9’ |
| 10, 10’ | 55.1 | 3.84, s, 6H | C7, C7’ |
| OH (1, 1’) | | 7.56, br s, 1H | |
| | | 8.50, br s, 1H | |

| Compound **11c** | | | |
|---|---|---|---|
| Carbon[c] | $^{13}C$ ($\delta$) | $^{1}H$ ($\delta$) | $^{1}H$-$^{13}C$ correlation |
| 2, 2’ | 97.5 | 5.81, s, 2H | C3, C3’ |
| 5, 5’, 9, 9’ | 129.1 | 7.44, d, 4H (7) | C4, C4’, C6, C6’, C8, C8’ |
| 6, 6’, 8, 8’ | 123.7 | 7.19, br s, 4H | C5, C5’, C7, C7’, C9, C9’ |
| OH (1, 1’) | | 9.24, br s, 2H | |

| Compound **11d** | | | |
|---|---|---|---|
| Carbon[d] | $^{13}C$ ($\delta$) | $^{1}H$ ($\delta$) | $^{1}H$-$^{13}C$ correlation |
| 2, 2’ | 95.7 | 6.09, s, 2H | C3, C3’ |
| 5, 5’, 9, 9’ | 122.0 | 7.02, br s, 4H | C6, C6’, C7, C7’, C8, C8’ |
| 6, 6’, 8, 8’ | 129.9 | 7.20, d, 4H (7) | C5, C5’, C9, C9’, C10, C10’ |
| 10, 10’ | 20.9 | 2.37, s, 6H | C6, C6’, C7, C7’, C8, C8’ |
| OH (1, 1’) | | 7.55, br s, 1H | |
| | | 8.55, br s, 1H | |

[a]Measure in $CDCl_3$ or DMSO-$d_6$ at 100 MHz ($^{13}C$) or 400 MHz ($^{1}H$, $J$ (Hz) values in parentheses). Chemical shifts are express in $\delta$ (ppm); [b]Compound **11b**: $^{13}C$ ($\delta$) of C-3/3’, C-4/4’ and C7/7’ = 151.9, 135.3, and 157.4 ppm; [c]Compound **11c**: $^{13}C$ ($\delta$) of C-3/3’, C-4/4’ and C7/7’ = 152.0, 142.6, and 128.9 ppm; [d]Compound **11d**: $^{13}C$ ($\delta$) of C-3/3’ and C7/7’ = 152.2 and 135.5 ppm.

## 7.3 Results and Discussion

### 7.3.1 Laccase-Lipase Co-Catalytic System for the Reaction of Catechols and 1,3-Dicarbonyl Compounds

In this study, laccase first catalyzed the oxidation of catechols to the corresponding *o*-quinones which were reacted in-situ with 1,3-dicarbonyl compounds *via* a Michael addition reaction. The Michael addition step was catalyzed by lipase and the resulting addition product undergoes a subsequent intramolecular cyclization to form benzofuran derivative products (see Figure 110).

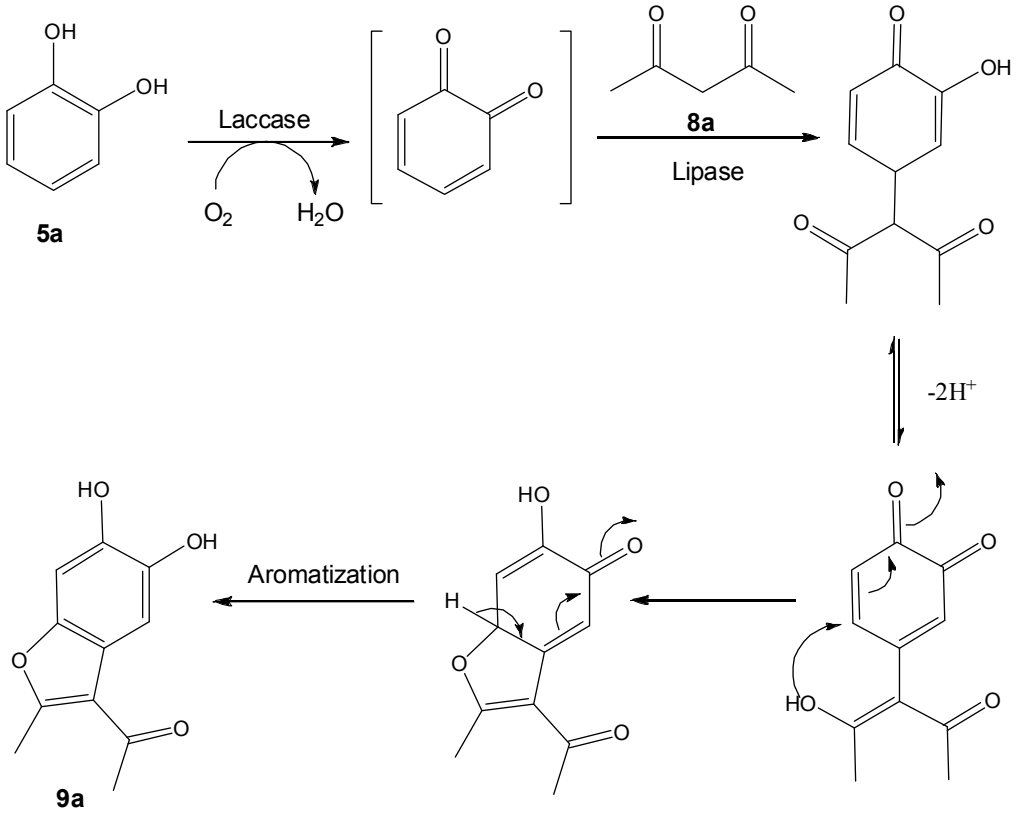


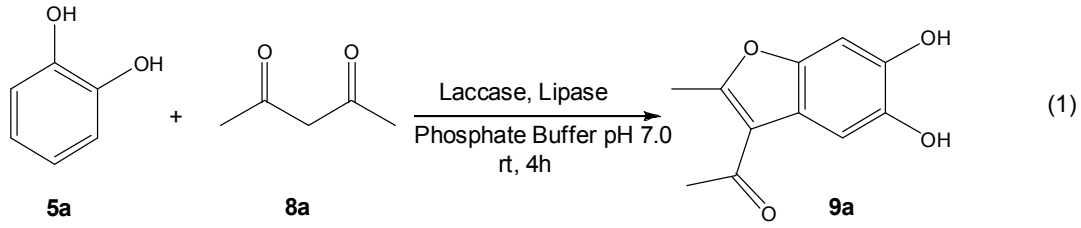

**Figure 110.** Proposed reaction pathway of laccase/lipase catalytic system for the synthesis of compound **9a**.

In our initial studies, the reaction of catechol (**5a**) and acetylacetone (**8a**) in the presence of laccase and lipase from *Candida rugosa* (lipase CR, 60,000U/mg) was investigated. The reaction was carried out under atmospheric conditions at room temperature (23 ˚C) in an aqueous buffered solution for 4 hours. We found that the optimal yield of the product (**9a**) of 60% was achieved when conducting the reaction of **5a** and **8a** in 1:2 molar ratio at pH 7.0, and using 100 U of laccase and 10 mg (600,000 U) of lipase CR per 1 mmol of **5a**. Because of the high activity of *in situ*-generated quinone, some side products (e.g. from the polymerization of the quinone) were also observed but in this study we did not separate and indentify them. For the control reaction when no laccase and lipase was added, no product was formed. When this reaction was preformed using only lipase no product was formed, and in the presence of laccase only, the product **9a** was formed in only 33% yield.

After this preliminary study, the next phase was to examine a variety of lipases for this reaction system. The esterases studied included lipase CR (60,000U/mg), lipase from *Pseudomonas cepacia* (lipase PS, 46.2U/mg), and lipase B *Candida antarctica* (CALB, 10.8U/mg). The activity of these lipases was measured by Aldrich methods which are different for each lipase. The catalytic properties of these lipases were investigated by reacting 8a with catechols, 5a and 3-methylcatechol (5b), in the presence of laccase, as summarized in Table 22. This study established that the optimal amount of each lipase to provide the highest yield of the product was different. The optimal amount of lipase CR, lipase PS and CALB for the reaction conditions used was 600,000 U, 924 U, and 54 U per 1 mmol of catechol, respectively. The data in Table 22 shows that the yield of the product usually increased when lipase was added to the reaction. Lipase PS

and lipase CR gave a high yield of the products for both reactions while CALB was good only for reaction 2. In addition, lipase PS activity used in the reaction was much less than of lipase CR. Therefore, lipase PS was chosen for further study. In order to verify whether the lipase reaction is indeed catalyzed by the active site of lipase PS and not by the protein, the reactions using inactivated lipase PS were conducted. The results in Table 22 show that the inactivated lipase PS showed no catalytic activity for these reactions.

**Table 22.** Reaction of catechol (**5a**) and acetylacetone (**8a**) in the presence of laccase with a variety of lipases.

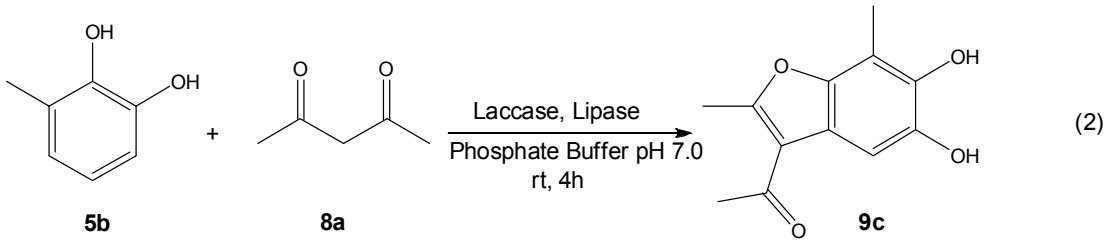



**5a** **8a** **9a** (1)

| Lipase | Yield (%)[a] |
|---|---|
| No lipase | 33 |
| Inactivated lipase from *Pseudomonas cepacia* | 31 |
| Lipase from *Candida rugosa* (Lipase CR) | 60 |
| Lipase from *Pseudomonas cepacia* (Lipase PS) | 58 |
| Lipase B *Candida antarctica* (CALB) | 41 |

[a]Isolated yield.

**Table 22.** (Continued)




(2)

| Lipase | Yield (%)[a] |
|---|---|
| No Lipase | 53 |
| Inactivated lipase from *Pseudomonas cepacia* | 50 |
| Lipase from *Candida rugosa* (Lipase CR) | 56 |
| Lipase from *Pseudomonas cepacia* (Lipase PS) | 60 |
| Lipase B *Candida antarctica* (CALB) | 62 |

[a]Isolated yield.

To further define the catalytic benefits of lipase PS, the reaction of **5a** and **8a** in the presence of laccase with and without lipase PS were carried out. Sample aliquots were taken every 30 minutes during the reaction and a quantitative analysis of product **9a** was measured by $^{1}$H-NMR spectroscopy using 1,3,5-trioxane as an internal standard. The calculated yield of the product **9a** is higher than the isolated yield shown in Table 22 in both cases (with and without lipase PS). This can be explained by the losing of product yield during the isolation process. However, in the end of reaction, the yield difference between the reaction with and without lipase is about the same which is approximately 25%. Figure 111 shows that in the beginning of the reaction, the rate and yield of **9a** from both reactions were almost the same. This can be explained by the predominance of laccase-catalyzed oxidation of catechol at the beginning of the reaction. At this stage, catechol was gradually oxidized by laccase which led to a low concentration of *o*-

quinone. After 2 hours of the reaction (when the concentration of laccase-generated quinone was high enough), the reaction with lipase PS was predominant and provided a higher rate of the reaction and higher yield of the product than the reaction without lipase PS. Therefore, lipase PS can enhance the overall yield for this reaction system.

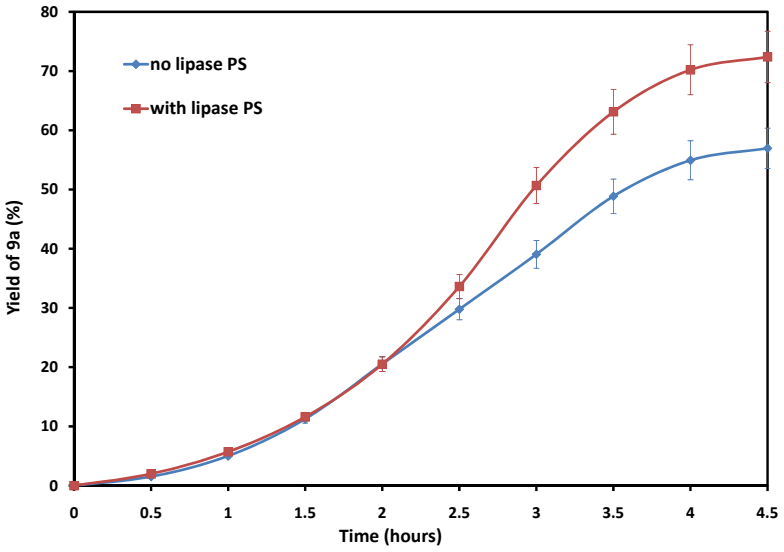


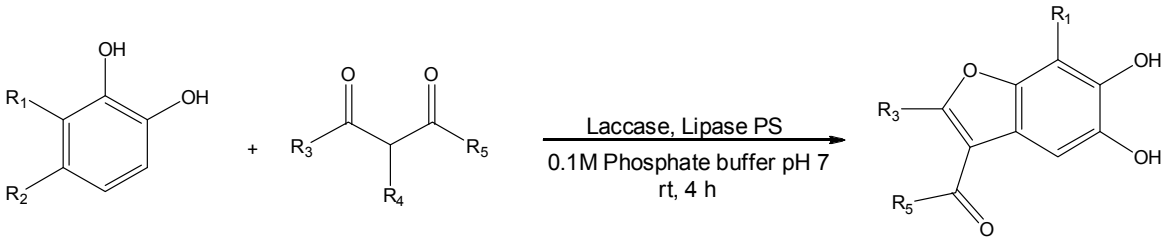

**Figure 111.** The formation of compound **9a** from the reaction of **5a** and **8a** in the presence of laccase. The percent yield of **9a** was measured by $^{1}$H-NMR spectroscopy.

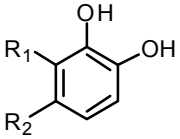
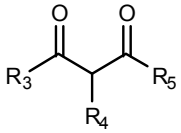
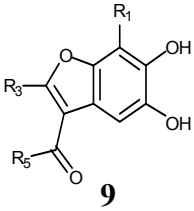
Following these optimization studies, we evaluated the breadth of this laccase-lipase co-catalytic system for the synthesis of benzofuran derivatives using a variety of catechols and 1,3 dicarbonyl compounds. The results summarized in Table 23 clearly suggest that the inactivated lipase has no catalytic effect on the reactions. In addition, the reactivity of the 1,3-dicarbonyl compound employed also has an effect on efficiency of

this two-enzyme system. When we used 1,3-dicarbonyl compounds that had an electron withdrawing group (Cl) at the alpha-position, the reaction was complete in 1.5-2 hours. The shorter reaction time was ascribed to the increased acidity of the alpha-proton of these substituted 1,3-dicarbonyl compounds. The proposed mechanism of the elimination of Cl atom is illustrated in Figure 112. Besides 3-substituted catechols, 4-substituted catechols, such as 4- chlorocatechol, can also be used for the synthesis of polyhydroxylated benzofurans (entry 11). However, the yield of the product is low. In addition, we observed that this reaction system gave only one isomer form of the possible benzofuran products.

Next, we examined the recyclability of this two-enzyme catalytic system for the synthesis of benzofuran **9c**. The product **9c** is relatively insoluble in the aqueous reaction mixture and readily precipitates out of solution. Simple filtration of the product mixture facilitates reuse of the lipase/laccase reaction system. The results shown in Table 24 demonstrate that this catalytic system can be reused for a second reaction, but for the third treatment only a low yield of the product was formed. The decrease of product yield after the third experiment resulted from the presence of laccase inhibitor, $Cl^-$, in the reaction mixture that led to the decrase of laccase activity [198].

**Table 23.** The study of the laccase/lipase catalyzed reaction of catechols and 1,3-dicarbonyl compounds in aqueous medium




| Entry | Catechol | 1,3-Dicarbonyl compound | Product (%yield)[a] |
|---|---|---|---|
| |  **5** |  **8** |  **9** |
| 1 | **5a**: $R_1 = R_2 = H$ | **8a**: $R_3 = R_5 = Me$, $R_4 = H$ | **9a** (58%) |
| | | | **9a** (31%)[b] |
| 2 | **5a** | **8b**: $R_3 = R_5 = Me$, $R_4 = Cl$ | **9a** (51%)[c] |
| | | | **9a** (40%)[b] |
| 3 | **5a** | **8c**: $R_3 = Me$, $R_4 = H$, $R_5 = OEt$ | **9b** (11%) |
| 4 | **5a** | **8d**: $R_3 = Me$, $R_4 = Cl$, $R_5 = OEt$ | **9b** (53%)[c] |
| | | | **9b** (26%)[b] |
| 5 | **5b**: $R_1 = Me$, $R_2 = H$ | **8a** | **9c** (60%) |
| | | | **9c** (50%)[b] |
| 6 | **5b** | **8b** | **9c** (72%)[c] |
| | | | **9c** (52%)[b] |
| 7 | **5b** | **8c** | **9d** (13%) |
| 8 | **5b** | **8d** | **9d** (66%)[c] |
| | | | **9d** (39%)[b] |
| 9 | **5c**: $R_1 = OMe$, $R_2 = H$ | **8a** | - |
| 10 | **5d**: $R_1 = F$, $R_2 = H$ | **8a** | - |
| 11 | **5e**: $R_1 = H$, $R_2 = Cl$ | **8a** | **9a** (8%) |

[a]Isolated yield; [b]Isolated yield from the reaction using inactivated lipase PS; [c]Reaction time is 1.5-2 hours.

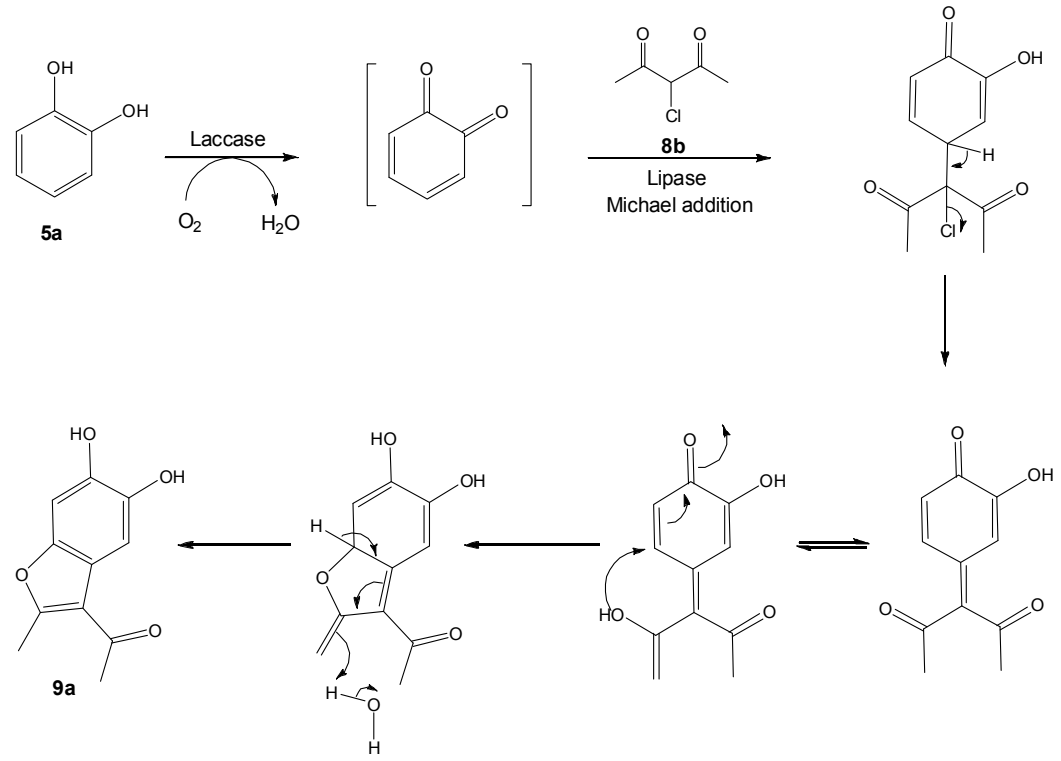
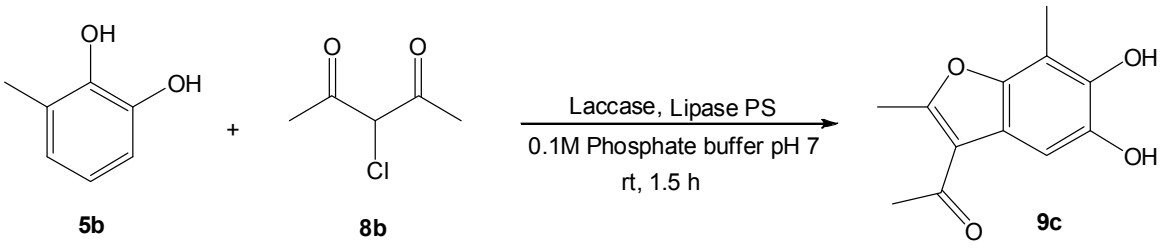



**Figure 112.** The proposed mechanism of the elimination of Cl atom from the laccase/lipase catalyzed reaction of catechol and 8b in aqueous medium.

**Table 24.** Recycling of the laccase/lipase co-catalytic system for the synthesis of 3-acetyl-5,6-dihydroxy-2,7-dimethylbenzofuran (**9c**)




| Run | Yield[a] of **9c** (%) |
|---|---|
| 1 | 72 |
| 2 | 62 |
| 3 | 5 |

[a]Isolated Yield.

### 7.3.2 Laccase-Lipase Co-Catalytic System for the Reaction of Catechols and Anilines

Next, we explored the feasibility of the laccase-lipase co-catalytic system for the reaction between catechol and aromatic amines, anilines. Lalk and his co-worker have demonstrated the ability to synthesize aminoquinones by laccase initiated oxidation of *p*-hydroxyquinones followed by Michael addition of primary aromatic amines in a good to excellent yields [11]. In contrast, herein, this nuclear animation reaction with the reactive 1,2-catechols was reported to yield the corresponding products less than 35%. In the presence of lipase, we had hypothesized that thus enzyme could catalyze the Michael addition step of the reaction between the laccase-generated *o*-quinone and anilines thereby improving the overall yields. We first conducted the reaction of catechol (**5a**) and aniline (**10**) in the presence of laccase, with or without lipase PS, in phosphate buffer pH 7.0 at room temperature for 3.5 hours. The ratio of catechol and aniline was 1 to 2, and 100U of laccase and 924U of lipase per 1 mmol of catechol were used. An insoluble red color product precipitated out of solution during the reaction. Therefore, the product was readily collected by filtration completion of reaction. The results show that the yield of the reaction with lipase PS increased by ~30% when compare to the yield of the reaction without lipase PS. Next, the amount of lipase PS used in the reaction was increased from 924U to 1848U per 1 mmol of catechol to study the effect of lipase dose on the reaction system. This result suggests that the increase of lipase dose did not provide a significant improvement for this reaction system (Table 25). Characterization of the product by NMR and mass spectrum indicated that the product was composed of a 1:2 ratio of 1,2-benzoquinone and aniline ($M^+/Z$ = 290). Moreover, the product was not a quinone

structure because the carbonyl carbon signal was not observed in $^{13}$C-NMR spectrum. The proposed reaction pathway for this reaction and the structure of product (**11a**) was illustrated in Figure 113. Compound **11a** are known compounds and our $^{1}$H and $^{13}$C NMR data are consistent with those in the literature [405]. In our and literature's $^{13}$C-NMR spectrum, peak of carbon that connect to nitrogen atom is not observed. This may be due to the effect of nitrogen atom that broaden the peak and make it too weak to observe.

After the preliminary study, the reaction between catechol and other anilines was conducted. The results of these studies are summarized in Table 25. The reaction of catechol and anilines in the presence of laccase and lipase PS provided a higher yield than the reaction in presence of laccase only. The yield of the product in the reaction with lipase PS increased up to 70% compare to the reaction without lipase PS (Table 25, Entry 4). Therefore, the overall yield of the product of this reaction system can be enhanced by lipase PS.

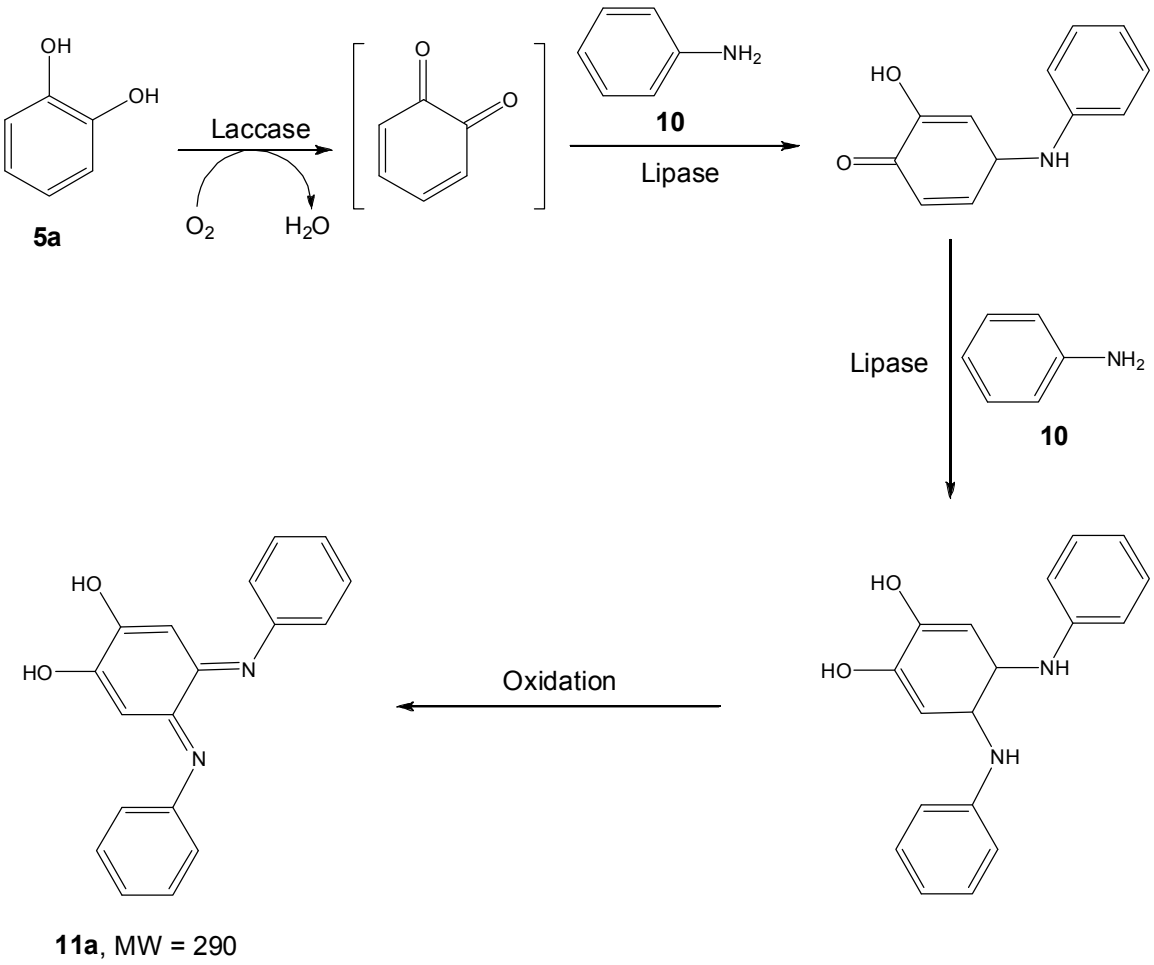
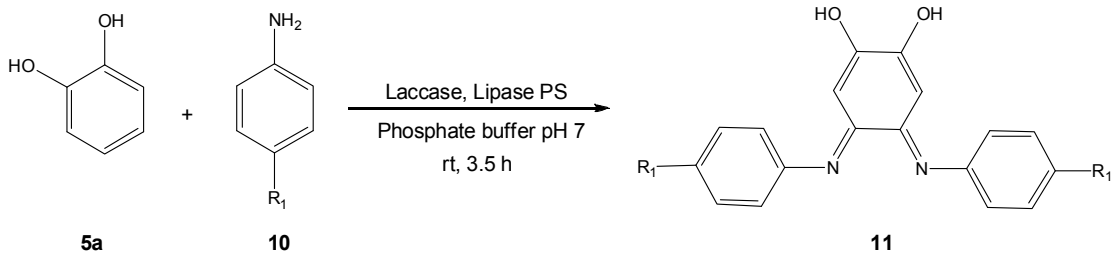



**Figure 113.** Proposed reaction pathway of laccase/lipase catalytic system for the reaction between catechol (**5a**) and aniline (**10**).

**Table 25.** Reactions of catechol and anilines in the presence of laccase,with (or without) lipase PS in aqueous medium.




**5a** **10** **11**

**11a**: $R_1$ = H
**11b**: $R_1$ = $OCH_3$
**11c**: $R_1$ = Cl
**11d**: $R_1$ = $CH_3$

| Entry | $R_1$ | Yield[a] of Product (%) | |
|---|---|---|---|
| | | Without Lipase | With Lipase |
| 1 | H | 23 | 30 |
| 2 | H | 23 | 28[b] |
| 3 | $OCH_3$ | 25 | 37 |
| 4 | Cl | 30 | 51 |
| 5 | $CH_3$ | 32 | 50 |

[a] Isolated yield; [b] Used 1848U of lipase PS.

## 7.4 Conclusions

In conclusion, this study demonstrates the potential of using lipase to catalyze Michael addition reaction, and presents a new co-catalytic enzymatic system employing laccase and lipase for green chemistry synthesis. Lipase was found to catalyze the addition reaction between laccase-generated *o*-quinones and 1,3-dicarbonyl compounds in aqueous medium. In this reaction, the catalytic system of laccase and lipase PS was regioselective, providing only one isomer product and is the first example of a two enzyme catalytic system for the synthesis of benzofurans. The yields of the products from reaction depend on the reactivity of the starting catechols and β-dicarbonyl compounds. Based on our experimental results, catechols with moderate reactivity yield benzofuran products in excellent yield. Moreover, lipase was also shown to catalyze the addition reaction between laccase-generated *o*-quinone and aromatic amines. In the presence of lipase and laccase, the yield of the final products increased in the range from 30 to 70% when compared to the reaction in the presence of laccase alone. Therefore, this paper illustrates a unique aqueous-based two-enzyme system for green chemistry synthesis and future applications are under study.

# CHAPTER 8

# MODIFICATION OF HIGH-LIGNIN CONTENT SOFTWOOD KRAFT PULP WITH LACCASE AND AMINO ACIDS[v]

## 8.1 Introduction

The interest in modifying cellulosic fibers especially with the assistance of enzymes is a growing field of research and interest [262]. A variety of enzymes are available for the surface modification of lignocellulosics fibers [263,264]. Compared to chemical treatments which involve harsh reaction conditions, loss of desirable components, and potential use of hazardous chemicals, enzymatic treatment conditions are often milder, less damaging to the fiber, and are environmentally friendly. Enzymatic surface modifications of fibers can be accomplished with glucohydrolysis and oxidative enzymes [263]. One of these oxidoreductases is laccase (benzenediol :oxygen oxidoreductase, EC 1.10.3.2) which is a multi-copper-containing oxidoreductase enzyme widely distributed in plants and fungi [3]. The majority of fungi that produce laccase belong to the class of white rot fungi involved in lignin degradation and can mineralize this substrate. Laccase can catalyze the oxidation of various substrates including phenols,

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[v]This manuscript was accepted for publication in Enzyme and Microbial Technology, 2008. It is entitled as **"**Modification of high-lignin content softwood kraft pulp with laccase and amino acids". The other author is Dr. Arthur J. Ragauskas from the School of Chemistry and Biochemistry at the Georgia Institute of Technology

benzenediols, aminophenols, polyphenols, polyamines, and lignin-related molecules, with concomitant reduction of oxygen to water [4-10].

Laccase applications in pulp and paper technology have been reported for biopulping, biobleaching, deinking, mill process water and effluent treatment, and fiber modification [20]. Recently, laccase research studies have shifted toward fiber modification. Laccase has been used to catalyze biografting of a variety of substrates to technical lignins. For example, Lund and Ragauskas demonstrated that laccase catalyzed the grafting of guaiacol sulfonate to lignin which enhanced its water solubility [22]. Huttermann et al. reported that laccase can catalyze the reaction of lignin with cellulose yielding a product in which the lignin was covalently bounded to cellulose [23]. Furthermore, Mai et al. grafted lignin with synthetic polymers derived from acrylic and acrylamide to create a new class of engineered plastics [24-27]. In addition, laccase has been shown to have the potential to biograft low-molecular-weight compounds to lignin-rich cellulosic fibers. Viikari et al. [28] recently modified the fiber surfaces of thermomechanical pulp (TMP) by laccase and tyramine. This modification is a two-stage functionalization method consisting of enzymatic activation of fiber surfaces followed by addition of radicalized compounds reacting preferentially by radical coupling. Chandra et al. reported the grafting of phenolic acids, including 4-hydroxyphenylacetic acid (PAA) [30], 4-hydroxybenzoic acid (4-HBA) [31], and gallic acid [29] to high-lignin content softwood kraft fibers. The grafting of these charged phenolics via a laccase generated phenolate radical was shown to lead to improved tensile and burst strength for the resulting paper. The paper strength improvements were ascribed to the capacity of carboxyl groups to promote fiber-fiber bonding and fiber swelling [406-412].

Laccase is also attractive for fine chemical synthesis because of its high stability, selectivity for phenolic substructures, and mild reaction conditions [11-14,18,19,244,366,379,380,404,413]. For instance, Michałek and Szarkowska [413] studied the reaction between laccase generated *p*-quinones and amino acids to produce quinone-amino acid complexes. The propensity of laccase to catalyze the oxidation polyphenolic has been reported by Chakar and Ragauskas [366] and Lalk et al. [11] has reported a laccase catalyzed nuclear animation reaction with *p*-diphenols and aromatic amines. According to the studies in Chapter 4-7, laccase has also been shown to initiate a cascade synthesis of naphthoquinone derivatives via Diels-Alder reaction between benzenediols and dienes [379,380] as well as the synthesis of benzofuran derivatives via oxidation-Michael addition between *o*-benzenediols and 1,3-dicarbonyl compounds [404]. Based on these results, it was apparent that laccase can be employed to generate reactive quinoidal structures in lignin-rich fibers that could then be reacted with amino acids to generate enhanced fiber charge as shown in Figure 114. This Chapter examines the optimal grafting conditions with respect to fiber charge and its impact on sheet strength properties.

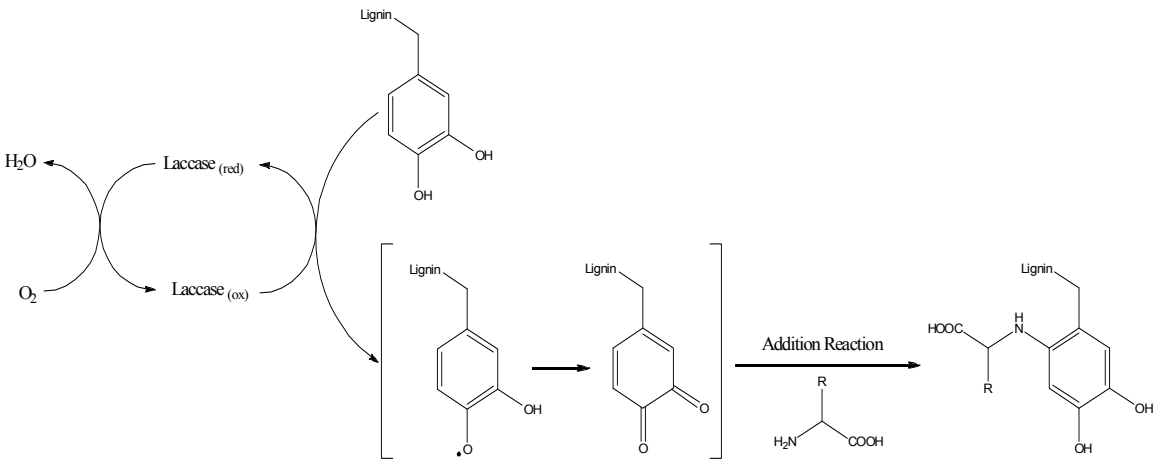



**Figure 114.** Propose mechanism for the grafting treatment of high-lignin content softwood kraft pulp with laccase and amino acids.

## 8.2 Experimental Section

### 8.2.1 Materials

All chemicals were obtained from Aldrich and used as received without further purification. Laccase (EC 1.10.3.2) from *Trametes villosa* was donated by Novo Nordisk Biochem, North Carolina and frozen till used. A commercial linerboard softwood kraft pulp (17% of lignin content) was obtained from a southeastern U.S.A manufacturing facility. The pulp was exhaustively washed until the filtrate was pH neutral and colorless. Pulp was air dried and soxhlet extracted for 24 hours with acetone with subsequent washing with water prior to all treatments.

### 8.2.2 Enzyme Assay

Enzyme activity measurement is described in Chapter 3 (Experimental Materials and Procedures).

### 8.2.3 Pulp Treatment

Laccase (80 U/1 o.d. g pulp) and an amino acid (3.2 mmol/1 o.d. g pulp) were added with stirring to a 5% consistency aqueous suspension of linerboard pulp buffered to pH 7 with 0.10 M sodium phosphate solution. The resulting slurry was stirred for 4 h at room temperature and then left stand 20 h. After treatment, the pulp sample was filtered, washed with deionized water until the filtrate was colorless and air-dried. Typically, pulp mass recovery was 95%.

### 8.2.4 Bulk Acid Group Measurment

Conductrometric titration for bulk acids was based on the work of Katz [328]. In brief, pulp (1.50 g o.d.) was stirred in 300.00 ml of 0.10 M HCl for 1 hour followed by rinsing in a fine fritted funnel with deionized water. The sample was then re-suspended in 250.00 ml of 1 mM NaCl solution, spiked with 1.50 ml of 0.10 M HCl and titrated against 0.05 M NaOH at 0.25 ml increments, recording the conductivity at each increment. The titration data was plotted as conductivity vs. volume to determine the milli-equivalent of acid groups per g of pulp. The reported results were the average of two measurements which typically differed by less than 3%.

### 8.2.5 Paper Testing

Treated pulps and control were disintegrated for 30,000 revolutions and then were refined for 3,000 revolutions according to TAPPI Standard T 248 [327]. Handsheets (3 g) were formed according to TAPPI Standard T 205 [327] and TAPPI conditioned (23 ˚C, 50% relative humidity) for at least 24 hours before physical testing.

Apparent density, tensile strength, tearing resistance, and wet tensile strength were determine according to TAPPI methods T 210, T 494, T 414, and T 456 [327]. The results of each physical testing were the average of five measurements with error less than 3%. Nitrogen content was analyzed by elemental microanalysis (Huffman Laboratories, Inc., Golden, CO) and the results are reported on a dried sample basis. The SEM pictures of handsheets were taken using a Hitachi S-800 FE-SEM. The handsheet sample was stuck on the SEM sample holding stub by the conductive double sides sticky carbon film and then was coated with alloy of Au/Pt prior to analysis.

## 8.3 Results and Discussion

### 8.3.1 Preliminary Study of the Grafting Condition

To determine the optimal condition for the modification of the linerboard pulp, a preliminary study was conducted with laccase and glycine (Gly). In this modification, the linerboard pulp was first stirred at 5% consistency in a pH 7.0 phosphate buffer solution with laccase (80 U/1g pulp) and Gly (0.8 mmol/1g pulp) for 4 h at room temperature and

then left unstirred for an additional 20 h. The treated pulp was washed, filtered, air dried, and then analyzed for bulk fiber charge. The results of analysis for laccase-Gly treated pulp (Lac/Gly), laccase-treated pulp (Lac) Gly-treated pulp (Gly) and control pulp are shown in Fig. 2a. These results demonstrate that laccase treated pulp provided a higher yield of acid groups compared to the control pulp due to the oxidation of lignin by laccase. Gly-treated pulp gave the similar acid content compared to the control pulp. This result suggested that Gly itself did not react with the lignin in the pulp fibers under the reaction conditions employed. However, when the pulp was treated with both laccase and Gly, the treated pulp gave the highest yield of carboxyl groups. This increase of carboxyl groups indicated that laccase-treated fibers facilitated the grafting of Gly onto the fiber lignin. Then, to determine the effect of the treatment conditions on grafting, the pH of the treatment was changed from 7.0 to 4.5 which is known to be the optimal pH for laccase [365,366]. The result shows that the treatment at pH 4.5 provided a reduced content of acid groups than the treatment at pH 7.0. This was attributed to the higher pH requirements needed for Micheal addition of amino acids to lignin quinonoid compounds (Figure 115 (top)). The requirement of using higher pH, pH 7, for the Micheal addition catalyzed by laccase was also reported by Michałek et. al. [413] and Ragauskas et al. [404].

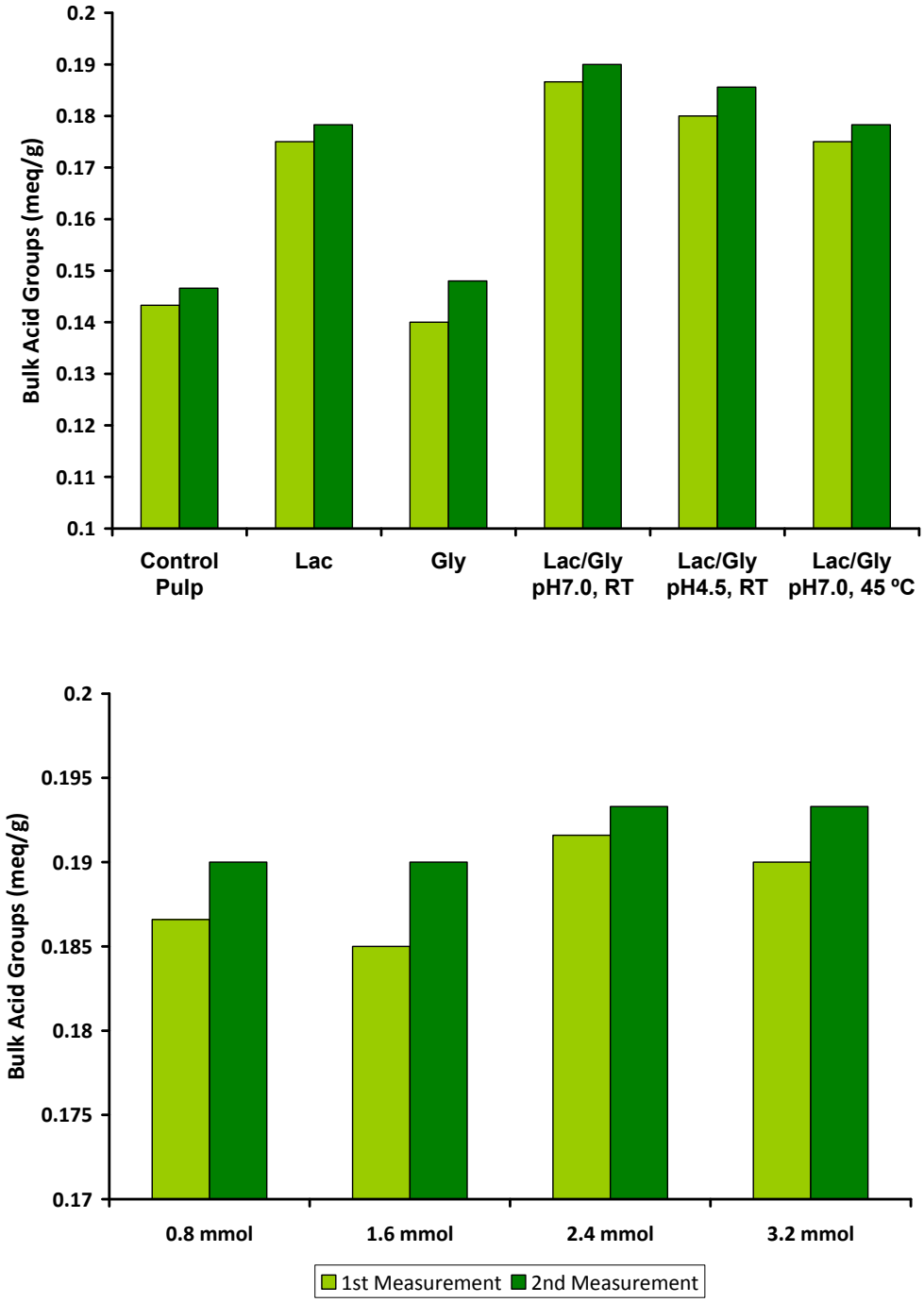



**Figure 115.** (top) Bulk acid group content of control pulp, laccase treated pulp (Lac), glycine treated pulp (Gly), and laccase-glycine treated pulp (Lac/Gly) at different conditions (The control pulp, laccase treated pulp and Gly-treated pulp were treated in the same condition as laccase-Gly treated pulp but no laccase and Gly, no Gly, and no laccase, respectively); (bottom) bulk acid group content of pulps treated with laccase and different amount of glycine at pH 7.0 and room temperature.

The effect of the reaction temperature on this grafting procedure was also examined. The pulp was treated at pH 7.0 and at 45 ˚C instead of at room temperature. The result of this treatment showed that the increase in temperature did not increase the acid group content of the fibers (Figure 115(top)). Therefore, the optimal condition of this fiber modification was the treatment at pH 7.0 and at room temperature. The effects of differing charges of Gly were also evaluated as shown in Figure 115(bottom). These results shows that the pulp treated with 1.6 mmol of Gly showed similar amount of bulk acid content when compare with 0.8 mmol Gly-treated pulp. However, the acid content increased when the pulp was treated with 2.4 mmol and 3.2 mmol of Gly/1 g fiber.

### 8.3.2 The Effect of Amino Acids on the Modifying Fibers

After this preliminary study, the next phase was to examine the effect of differing amino acids for laccase initiated fiber grafting. Softwood linerboard kraft pulp was treated with laccase (80 U/1g pulp) and amino acid in phosphate buffer pH 7.0 at room temperature. A variety of amino acids were used for this study including Gly, phenylalanine (Phe), serine (Ser), aspartic acid (Asp), histidine (His), arginine (Arg), and alanine (Ala). The properties of amino acids mainly depend on the pH of the surrounding environment. The amino acids can become more positively or negatively charged due to the loss and gain of protons ($H^+$) at a given pH. In general, the pK values of the α-carboxylic acid groups of amino acids lie in a small range around 2.2 so that above pH 3.5 these groups are almost entirely in their carboxylate forms. The α-amino groups all have pK values near 9.4 and are therefore almost entirely in their ammonium ion forms below pH 8.0 [414]. Therfore, at the experimental pH (pH 7.0), both the carboxylic acid

and the amino groups of α-amino acids are ionized. When the amino acids have charged polar side chains, the pK values of the side chain groups have to be considered. In this study, histidine side chain, an imidazolium moiety (pK = 6.0), was deprotonated at pH 7.0. Therfore, the histidine side chain can participate in the addition reaction with the laccase-oxidized fibers at this pH. The results illustrated in Figure 116a show that His gave the highest acid content compared to the other amino acids. This result was ascribed to the enhanced nucleophilic property of the nitrogen of imidazole side chain of His. Moreover, when considered the isoelectric point (pI) of the amino acids with nonpolar or uncharged side chains, including Gly (pI = 6.06), Ala (pI = 6.01), Phe (pI = 5.49), and Ser (pI = 5.68), their pI are all below 7. Therefore, at the pH above their pI (pH 7.0), some of ammonium ions of these amino acids were deprotonated which led to the liberation of some free amino groups that can react with the oxidized fibers. As a consequence, the acid groups of the fibers increased in some content after the treatment with these amino acids and laccase at pH 7.0 (Figure 116a).

In addition, different amounts of each amino acid (i.e., 1.6, 2.4, and 3.2 mmol/1g pulp) were examined to find the optimal amount of amino acid for modifying fibers. The results in Figure 116a also indicate that the greater the amount of amino acid employed the greater increase in fiber charge for most amino acids. The acid group content reached the maximum yield when the amount of amino acids was 3.2 mmol/1g pulp. Therefore, 3.2 mmol/1g pulp was chosen as an optimal amount of amino acids for this treatment system.

Next, the interaction between amino acids and pulp fibers was investigated. The pulp was treated with an amino acid (3.2 mmol/1g pulp) only and compared the acid

content with control pulp, laccase-treated pulp and laccase-amino acid treated pulp. Figure 116b demonstrates that the amino acid-treated pulp provided a 10-25% increase of carboxyl group content compared to control pulp. These results indicate that some of amino acid can react with pulp fibers presumably due to quinonoid structures present in kraft pulps [415]. However, the carboxyl group content of the amino acid treatments was still 11-20% less than of the laccase-amino acid treatments. Therefore, the highest acid group content was obtained when the linerboard pulp was treated with both laccase and amino acid.

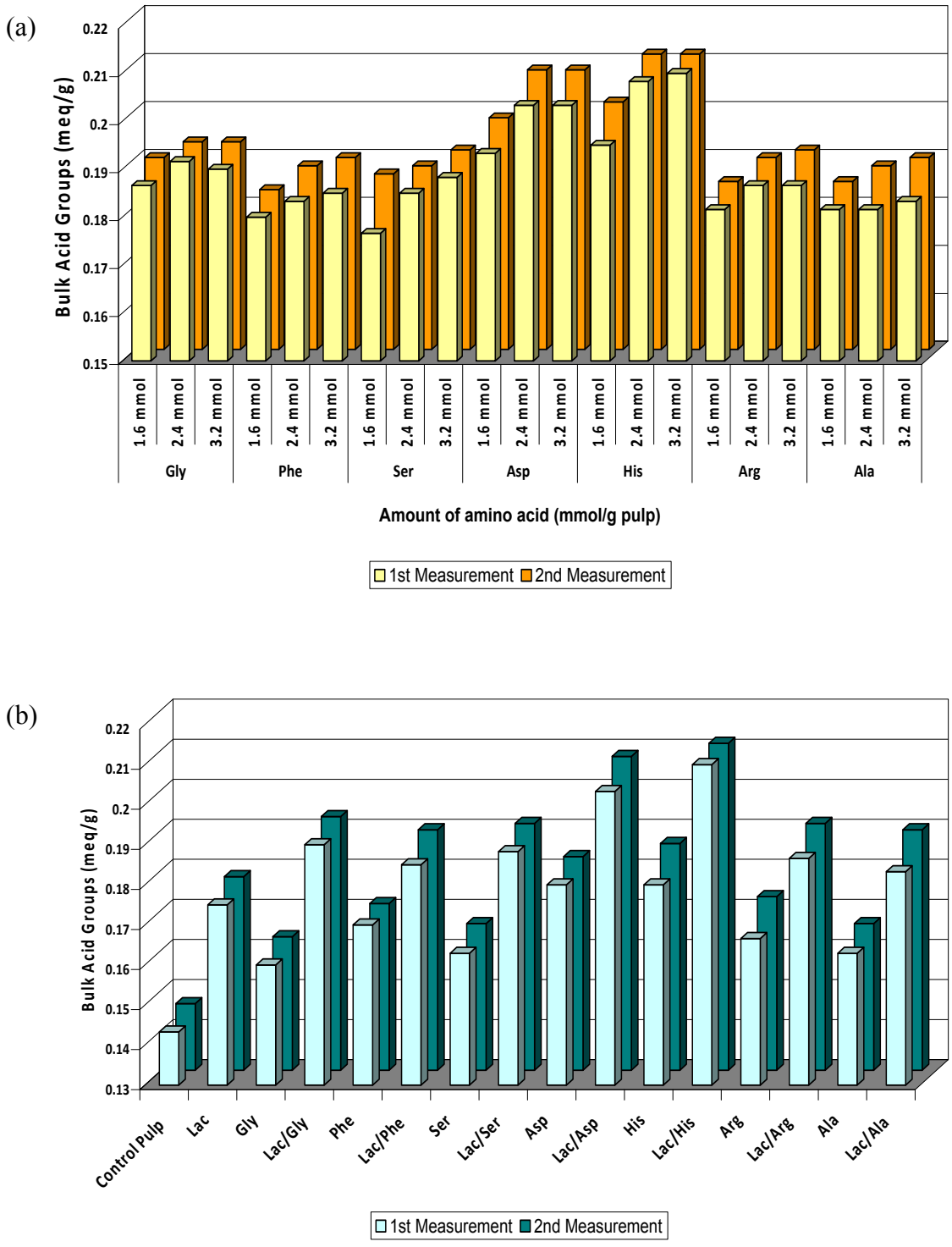



**Figure 116.** Bulk acid group content of (a) linerboard pulps treated with a variety of amino acids in the presence of laccase (80 U/1g pulp); (b) linerboard pulps treated with a variety of amino acids (3.2 mmol/ 1g pulp) in the presence and absence of laccase.

### 8.3.3 The Effect of Laccase Dose

After optimizing the treatment condition, the next study was to determine the effect of laccase dose on the modifying fibers. The experiments were conducted by treating linerboard pulp with different amount of laccase which are 20, 40, 60, 80, and 100U/1g pulp in the presence of His (3.2 mmol/1g pulp) in phosphate buffer pH 7.0 at room temperature. Figure 117 demonstrates that the carboxyl group content increased when the amount of laccase increased. The carboxyl group content reached the highest amount when the amount of laccase was 80 U/g pulp. Therefore, the optimal amount of laccase for this modification was 80 U/g pulp.

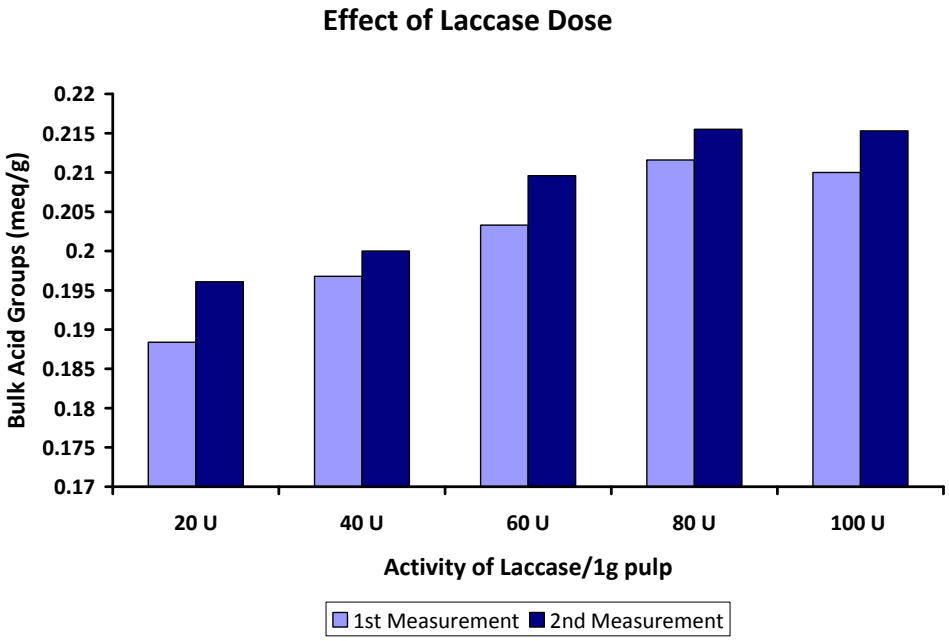



**Figure 117.** Bulk acid group content of linerboard pulps that were treated with histidine (3.2 mmol/ 1g pulp) and different amount of laccase.

### 8.3.4 Nitrogen Content of Laccase-His Treated Pulp

The laccase-His grafting treatment conditions which provided the best yield of bulk fiber acid groups were selected for further study. The linerboard pulp was treated with laccase and histidine using the optimal condition as described in experimental section 8.2.3. Then, the pulp samples were sent for nitrogen analysis. Nitrogen content of laccase-His treated pulp was measured and compare with nitrogen content of control and laccase treated pulp. The nitrogen content of laccase-His treated pulp was 120-140% higher than of control and laccase treated pulp as shown in Figure 118. These results show that His was bonded with pulp fibers after the grafting treatment which led to the increase of nitrogen content of the fibers.

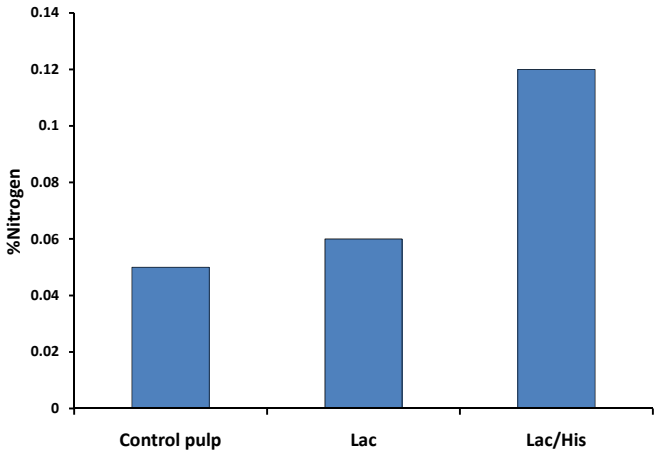



**Figure 118.** Nitrogen content of control pulp, lacccase treated pulp (Lac), and laccase-His treated pulp (Lac/His).

### 8.3.5 Paper Strength Properties

The objective of this section is to evaluate the effects of the laccase-amino acid grafting treatment on paper strength properties. The physical properties of handsheets made from laccase-His treated pulp were compared to the physical properties of the handsheets made from control pulp and laccase treated pulp. The results of the paper testing are illustrated in Figure 119. The strength properties of the handsheets were examined including tensile strength, tearing resistance, and wet tensile strength. These results indicate that the handsheets made from laccase-His treated pulp gave the highest strength properties in comparison to handsheets made from control and laccase treated pulp. The ratio of wet/dry strength is about 5.2 for the laccase-His treated pulp. Although it has been suggest that the minimum ratio of wet/dry strength about 15 is required for the wet-strength paper [416], this study is a good start for the modification of lignocellulosic fibers by laccase via oxidation-Michael addition. Therefore, in the future, the further investigation to improve the wet tensile strength of resulting paper for this modification system has to be conducted. The improvement of wet tensile strength of unbleached kraft pulp by the combination of lacccase with mediator and a heat treatment has been reported by Lund and Felby [297]. The wet/dry strength ratio of laccase, laccase-mediator, and laccase-mediator with heat treatment is 3.5, 6.7, and 14.7, respectively. This shows that heat treatment has a tremendous effect on the increase of wet strength property. Compared to Lund and Felby's study, our wet/dry strength ratio is comparable to those results without heat treatment. Therefore, our fiber modification system could be further improved by using laccase in combination of mediator or heat treatment to increase the wet tensile strength of the modified fibers.

Moreover, the images of the handsheet surface of the control, laccase treated, and laccase-His treated pulp were taken by the scanning electron microscope (SEM). SEM images in Figure 120 show that the laccase-His treated fibers are more collapse than control and laccase treated fibers which led to form better bonding between fibers in handsheet resulting in the increase of the paper strength of laccase-His treated pulp.

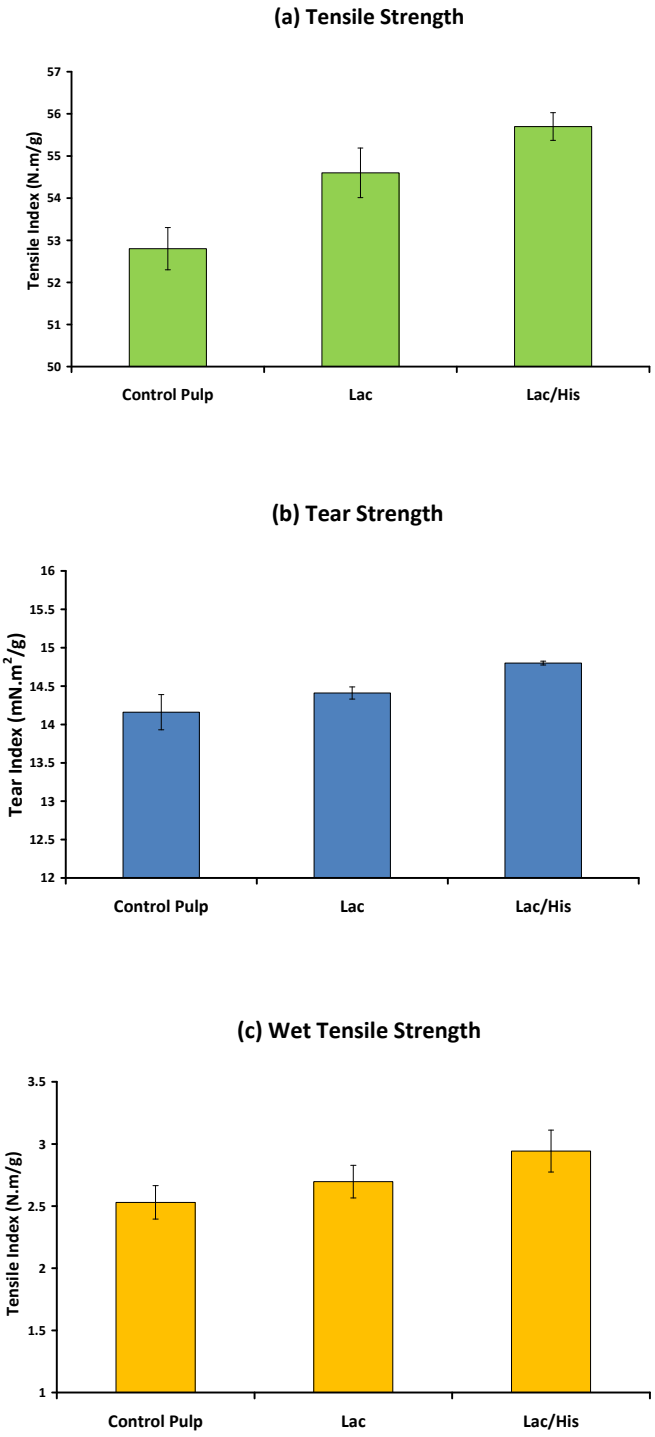



**Figure 119.** Physical paper properties of handsheets made from control pulp, laccase treated pulp (Lac), and laccase-histidine treated pulp (Lac/His); (a) tensile strength; (b) tear strength; (c) wet tensile strength.

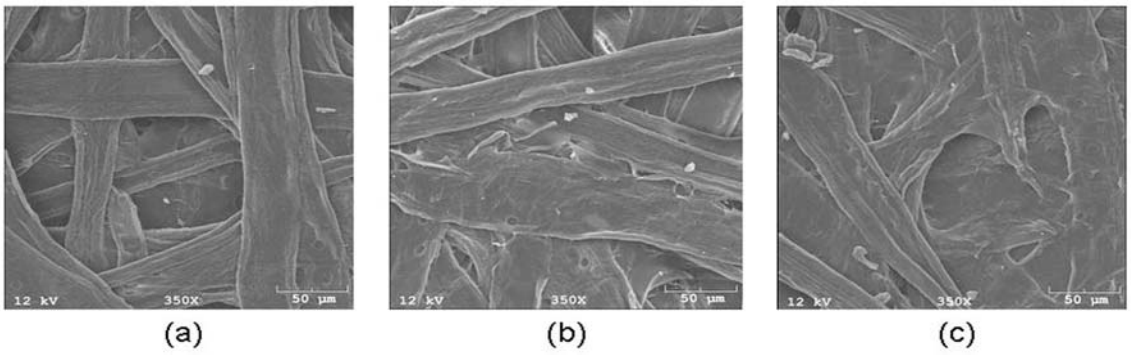



**Figure 120.** Scanning electron microscope (SEM) images of handsheets made from (a) control pulp; (b) laccase treated pulp; (c) laccase-histidine treated pulp.

## 8.4 Conclusions

This study presents a new environmentally-friendly method for modifying lignin-rich fibers. This modification employed laccase to oxidize lignin in the fibers, and then the carboxyl groups were introduced to pulp fibers by an addition reaction between the oxidized fibers and amino acids. The condition for this treatment was pH 7.0 at room temperature. Laccase-amino acid treatment of fibers resulted in an increase in carboxyl group content of the fibers that enhanced the strength properties of the resulting paper, including tensile strength, tearing resistance, and wet tensile strength. The SEM images show that the laccase-amino acid treated fibers are more collapse than control and laccase-treated fibers which led to form better bonding between fibers in handsheet. In this study, among the several different amino acids studied, the treatment of pulp with laccase and His provided the best result in increasing carboxyl group content and paper properties. The ability to use laccase selectively graft amino acids to lignin rich pulp fibers provides a new and unique fiber modification technology which will have many future opportunities. The improvement of this fiber modification system to increase the strength properties of the modified paper is under investigated.

# CHAPTER 9

# OVERALL CONCLUSIONS

The original idea about using laccase for this study was inspired by various interesting applications of laccase as biocatalysts. Laccase has been known to have applications in many industrial areas, expecially in the pulp and paper industry. However, the applications of laccase have recently shifted toward fine chemical synthesis because of its high stability, selectivity for phenolic substructures, and mild reaction conditions. This study utilized the oxidative potential of laccase to convert hydroquinones to quinones *in situ*. Since the quinonoid compounds have a wide spectrum of chemistry, various possible reactions of the *in situ*-generated quinones can be investigated. First, the property of quinoniod compounds as good dienophiles for the Diels-Alder reactions attracted our interest. Moerover, many studies showed that the Diels-Alder reactions performed in an aqueous medium showed beneficial effects on the reaction rate, reactivity, and selectivity of Diels-Alder reaction. Therefore, the study of the laccase-triggered Diels-Alder reaction in aqueous media was conducted first. This reaction methodology provides a unique green chemistry synthesis.

In Chapter 4, the *para*-quinones were generated *in situ* by the laccase oxidation of the corresponding 1,4-hydroquinones and subsequently underwent the Diels-Alder reaction with dienes, and further oxidation to finally generate 1,4-naphthoquinones, in good yields. However, the reactivity of the reaction depends on the substrate specificity of laccase and the reactivity of both generated quinones and dienes. Temperature also has

an important impact on the formation of the final products. To obtain the naphthoquinones as major products, the reactions have to perform at 70 $^{o}$C. At the lower temperature, 25 $^{o}$C, the major products showed to be the Diels-Alder adducts. This successful synthesis of p-naphthoquinones catalyzed by laccase led to the further study of the laccase-triggered Diels-Alder reaction for *o*-naphthoquinones synthesis in Chapter 5. This study has to deal with the very reactive *in situ*-generated *o*-quinones that easily undergo dimerization and polymerization. Therefore, the reactions were conducted at a low temperature (3-25 $^{o}$C) to lower the rate of those side reactions and a high excess of dienes were used to push the reaction toward Diels-Alder reaction. In addition, these reactions were carried out in an aqueous medium and yielded *o*-naphthoquinones up to 80%, depending on the exact structure of the starting hydroquinone and diene.

Besides Diels-Alder reactions, Michael addition reactions of *in situ*-generated *o*-quinones were also investigated. In Chapter 6, the cascade synthesis of benzofuran derivatives was conducted from the reaction of catechols and 1,3-dicarbonyl compounds via oxidation-Michael addition in the presence of laccase and $Sc(OTf)_3$/SDS. In this procedure, *ortho*-quinones, generated *in situ* from the oxidation of catechols by laccase, underwent the Michael addition reaction with 1,3-dicarbonyl compounds, and then underwent intramolecular cyclization to benzofuran derivatives. This reaction was carried out under air at room temperature, in an aqueous medium, and provided benzofuran products in 50 – 79% yield. In addition, this reaction system showed recyclability. Although the use of an aqueous Lewis acid was critical for efficient synthesis of the desired compounds, this system still produced a hazardous waste from the transitional metal catalyst. Therefore, to enhance the overall green chemistry aspect, the use of lipase

as an alternative catalyst in conjunction with laccase as an alternative methodology for the synthesis of benzofuran derivatives was developed in Chapter 7. This laccase/lipase reaction system was carried out under air at room temperature, in an aqueous medium, and provided benzofuran products in good yields. Moreover, this laccase/lipase co-catalytic system was also used to catalyze the Michael addition of *in situ*-generated *o*-quinones and anilines. In the presence of lipase and laccase, the yield of the final products increased in the range from 30 to 70% when compare to the reaction in the presence of laccase alone. Therefore, this study illustrates a unique aqueous-based two-enzyme system for green chemistry synthesis.

In the last phase of this research, the interest shifted toward another interesting application of laccase, which is fiber modification. Laccase has been reported to facilitate the grafting of a variety of compounds to lignin or lignocellulosic fibers. Chapter 8 demonstrates the potential of laccase-facilitated grafting of amino acids to high lignin content pulps to improve their physical properties in paper products. These physical properties can be enhanced by increasing ionic fiber charges. In an effort to increase carboxylic acid groups, a unique two-stage laccase grafting protocol, in which fibers were initially treated with laccase followed by grafting reactions with amino acids was developed. The condition for this treatment was pH 7.0 at room temperature. In this study, a variety of amino acids, including glycine, phenylalanine, serine, arginine, histidine, alanine, and aspartic acid, were examined. The results show that histidine provided the best yield of acid groups on pulp fiber and was used for the preparation of handsheets for physical strength testing, including tensile, tear, and wet tensile strength properties. Laccase-histidine treated pulp showed an increase in strength properties of the

resulting paper. Moreover, this study presents a new environmentally-friendly method for modifying lignin-rich fibers.

# CHAPTER 10

# RECOMMENDATIONS FOR FUTURE WORK

Several other studies might be conducted to further explore other applications of laccase, both in organic synthesis and in fiber modification. Some particularly attractive options are as follows:

- To address the environmental concern, immobilized laccase would be used in the reaction. The immobilized laccase could be reused and would reduce waste from the reaction.
- The use of laccase alone in the reaction limits the scope of substrates. The addition of laccase mediators, such as ABTS, HBT, and TEMPO, into the reaction system would broaden the scope of the substrates and would lead to the discovery of new green synthetic chemistry.
- According to this research, reaction conditions, such as temperature and pH, affect the formation of the reaction products. Therefore, conducting the reaction at different conditions could provide different final products and could lead to the discovery of new compounds.
- Laccase could be used to facilitate the grafting of other compounds to high-lignin content pulp fibers to improve the properties of existing products and create new product platforms.
- Future research programs should focus on large-scale laccase-biografting technology.

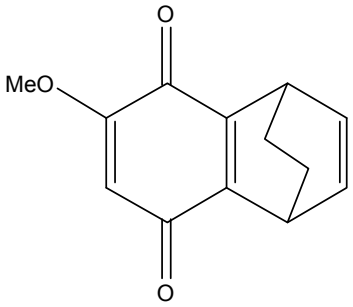
# APPENDIX A

# NMR AND IR SPECTRA OF NEW COMPOUNDS

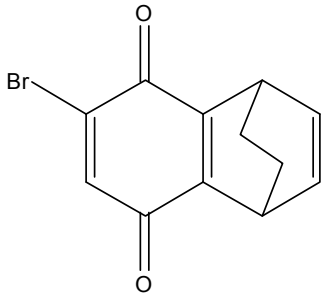
## A.1 NMR and IR Spectra of New Compounds in Chapter 4

There are two new compounds obtained from the experiments in Chapter 4:

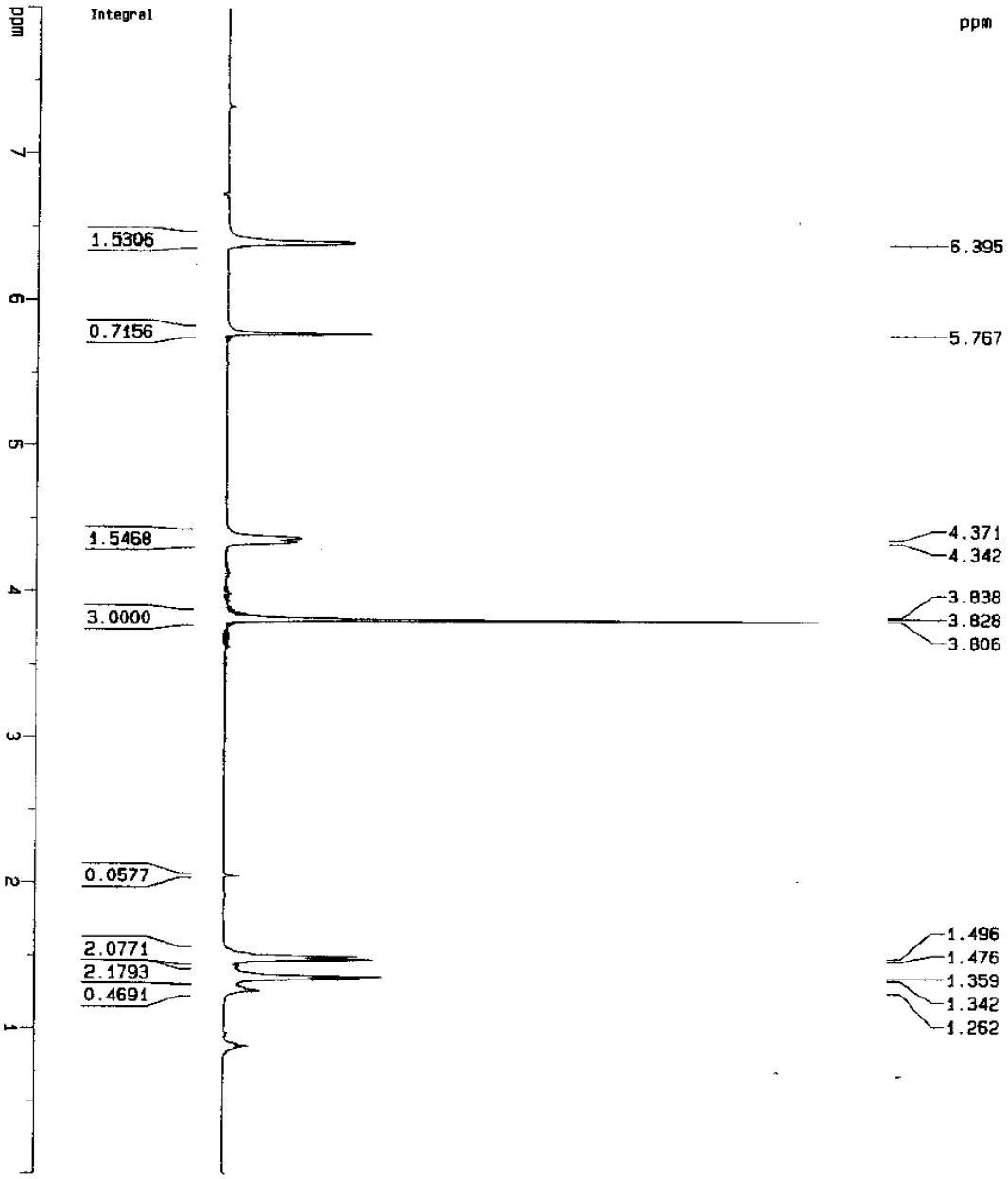
- 1,4-Dihydro-6-methoxy-1,4-ethanonaphthalene-5,8-dione (**3f**)




- 1,4-Dihydro-6-bromo-1,4-ethanonaphthalene-5,8-dione (**3h**)



## A.1.1 $^{1}$H-NMR Spectrum of compound 3f




Current Data Parameters
NAME Hi-5-HY39
EXPNO 1
PROCNO 1

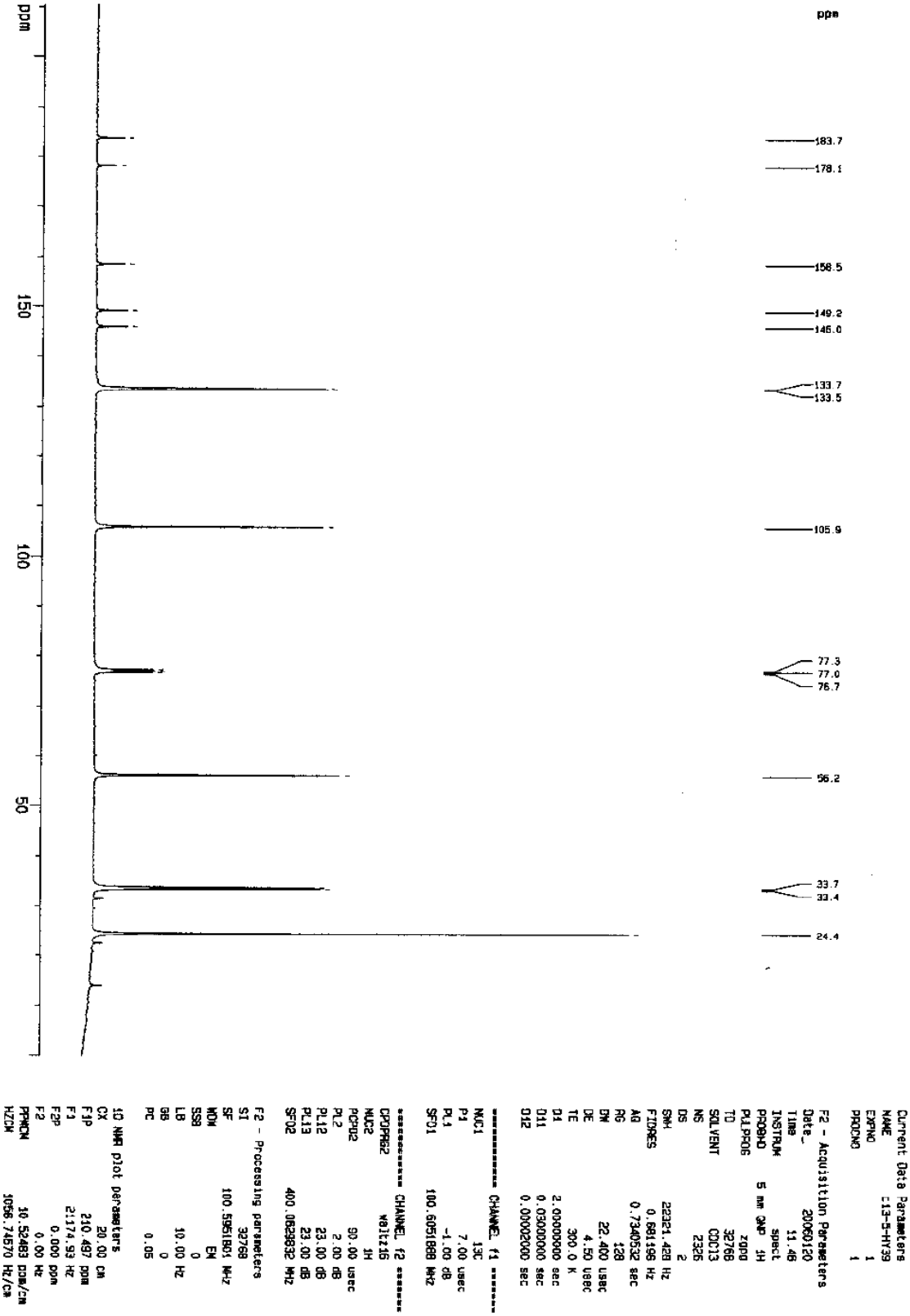
F2 - Acquisition Parameters
Date_ 20060120
Time 11.37
INSTRUM spect
PROBHD 5 mm QNP 1H
PULPROG zg
TD 4096
SOLVENT CDCl3
NS 100
DS 0
SWH 8169.935 Hz
FIDRES 1.994613 Hz
AQ 0.2507252 sec
RG 64
DW 61.200 usec
DE 4.50 usec
TE 300.0 K
D1 3.00000000 sec

========== CHANNEL f1 ========
NUC1 1H
P1 9.00 usec
PL1 2.00 dB
SFO1 400.0625780 MHz

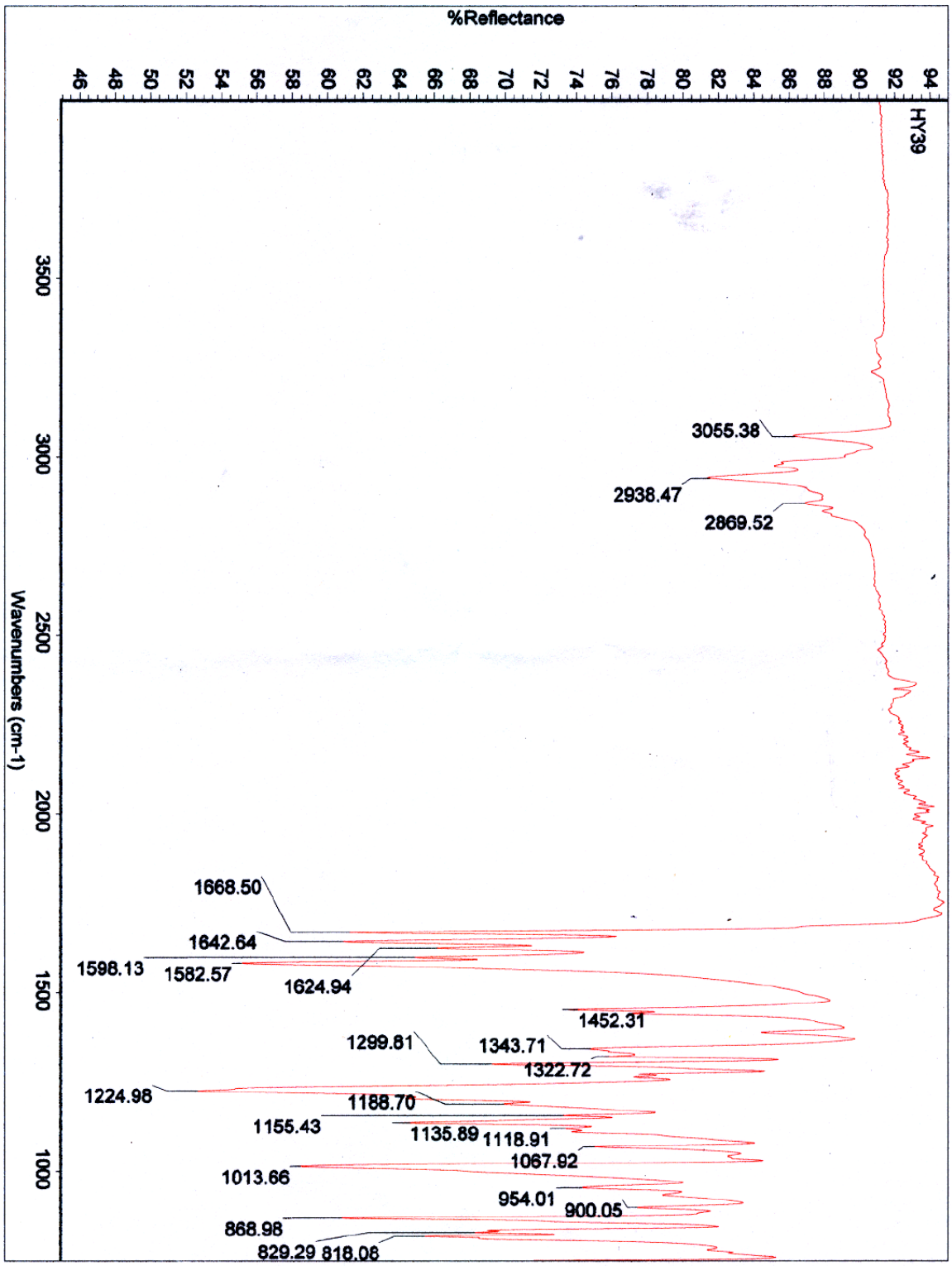
F2 - Processing parameters
SI 4096
SF 400.0599973 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.00

1D NMR plot parameters
CX 20.00 cm
F1P 8.000 ppm
F1 3200.48 Hz
F2P 0.000 ppm
F2 0.00 Hz
PPMCM 0.40000 ppm/cm
HZCM 160.02400 Hz/cm

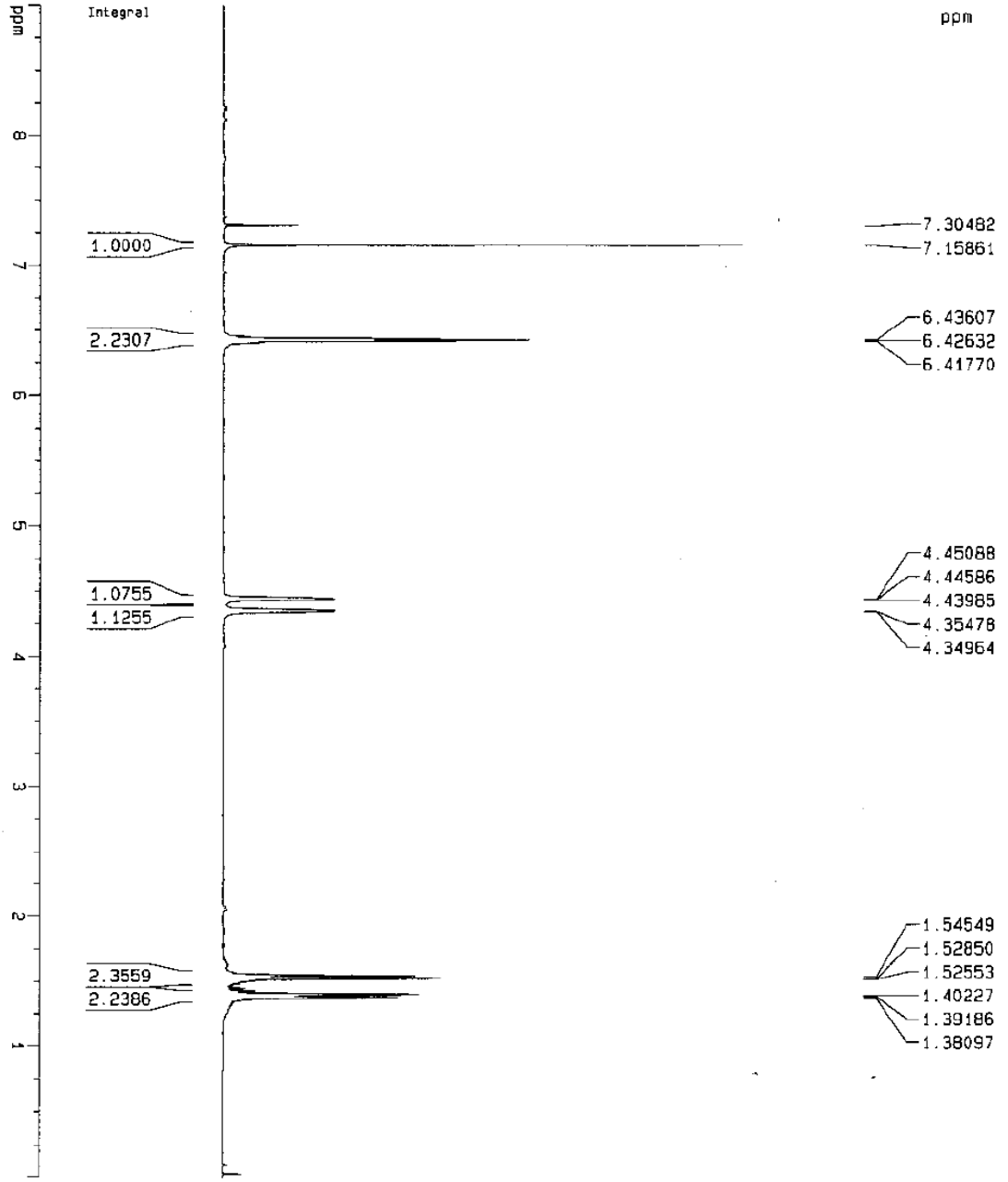
## A.1.2 $^{13}$C-NMR Spectrum of Compound 3f



### A.1.3 IR Spectrum of Compound 3f



## A.1.4 $^{1}$H-NMR Spectrum of Compound 3h



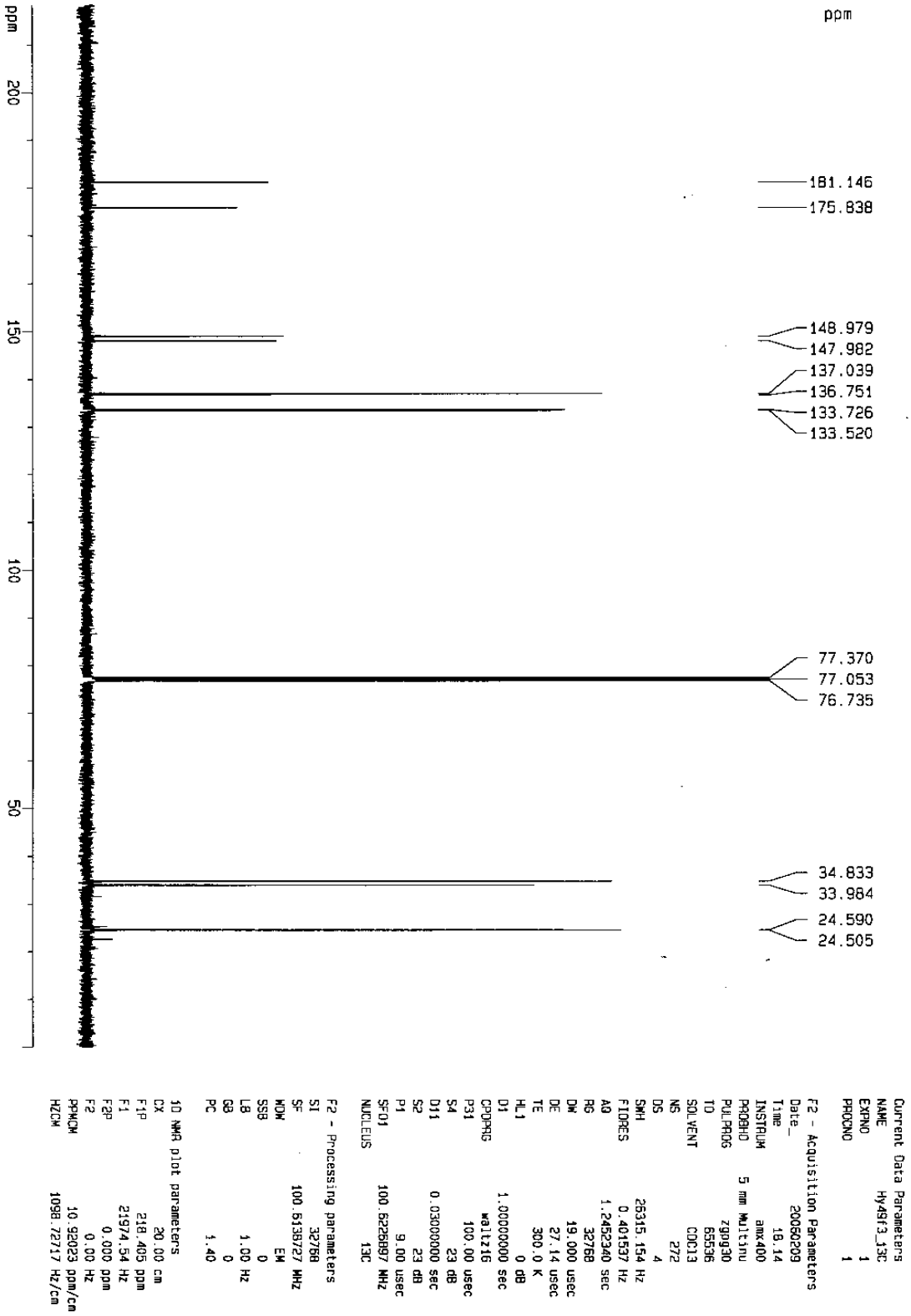

Current Data Parameters
NAME Hy4913_H
EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
Date_ 20060209
Time 18.00
INSTRUM amx400
PROBHD 5 mm Multinu
PULPROG zg30
TD 65536
SOLVENT CDCl3
NS 30
DS 2
SWH 8333.333 Hz
FIDRES 0.127157 Hz
AQ 3.9322100 sec
RG 512
DW 60.000 usec
DE 85.71 usec
TE 300.0 K
HL1 1 dB
D1 1.00000000 sec
P1 10.00 usec
SFO1 400.1368230 MHz
NUCLEUS 1H

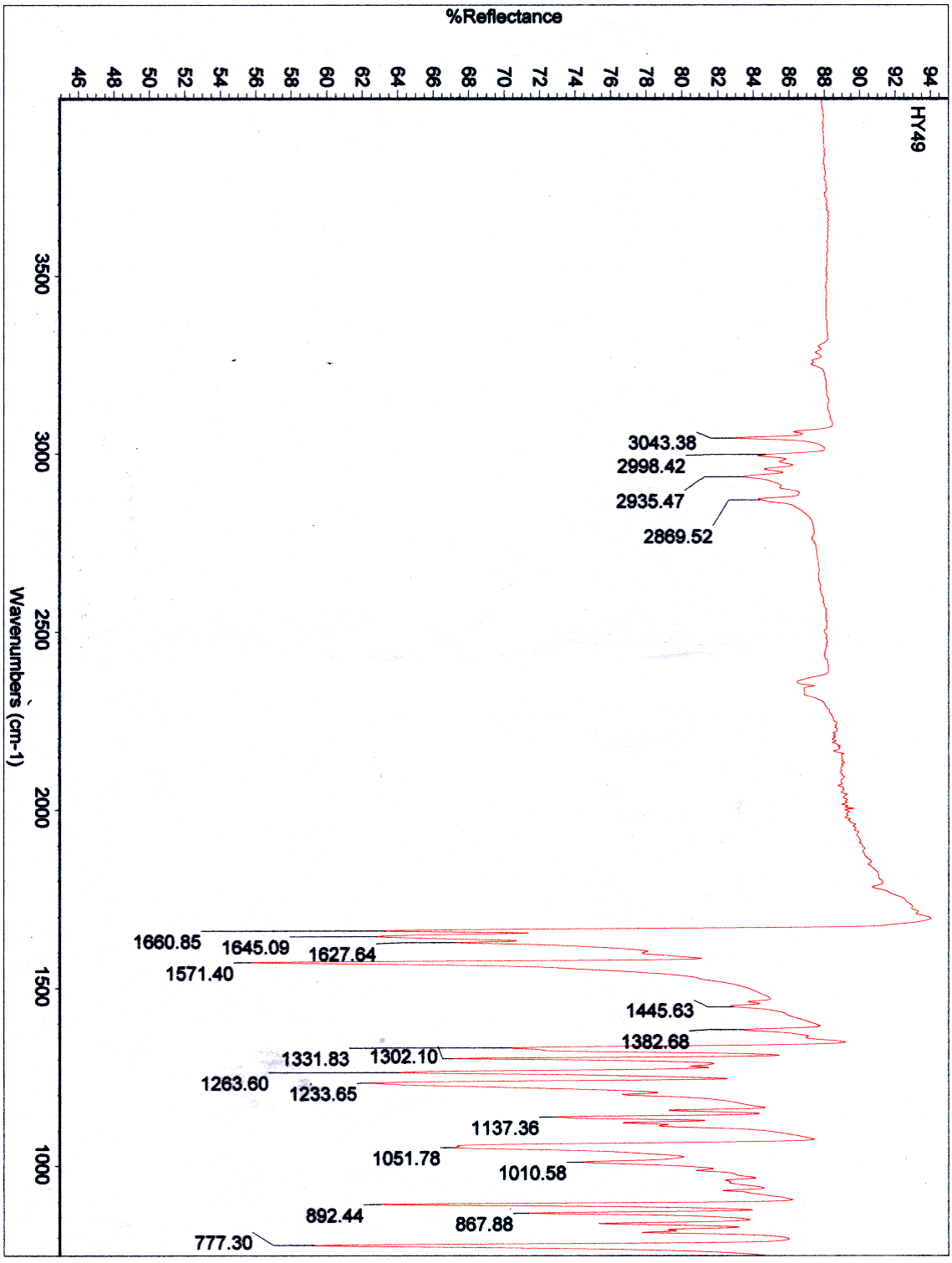
F2 - Processing parameters
SI 32768
SF 400.1343753 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00

1D NMR plot parameters
CX 20.00 cm
F1P 9.000 ppm
F1 3601.21 Hz
F2P 0.000 ppm
F2 0.00 Hz
PPMCM 0.45000 ppm/cm
HZCM 180.06047 Hz/cm

## A.1.5 $^{13}$C-NMR Spectrum of Compound 3h



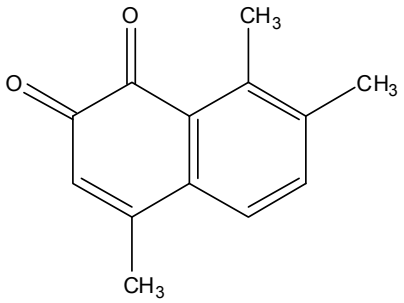
## A.1.6 IR Spectrum of Compound 3h



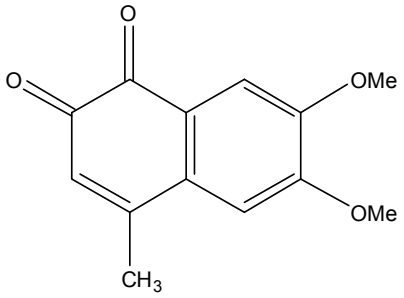
## A.2 Spectra of New Compounds in Chapter 5

There are two new compounds obtained from the experiments in Chapter 5:

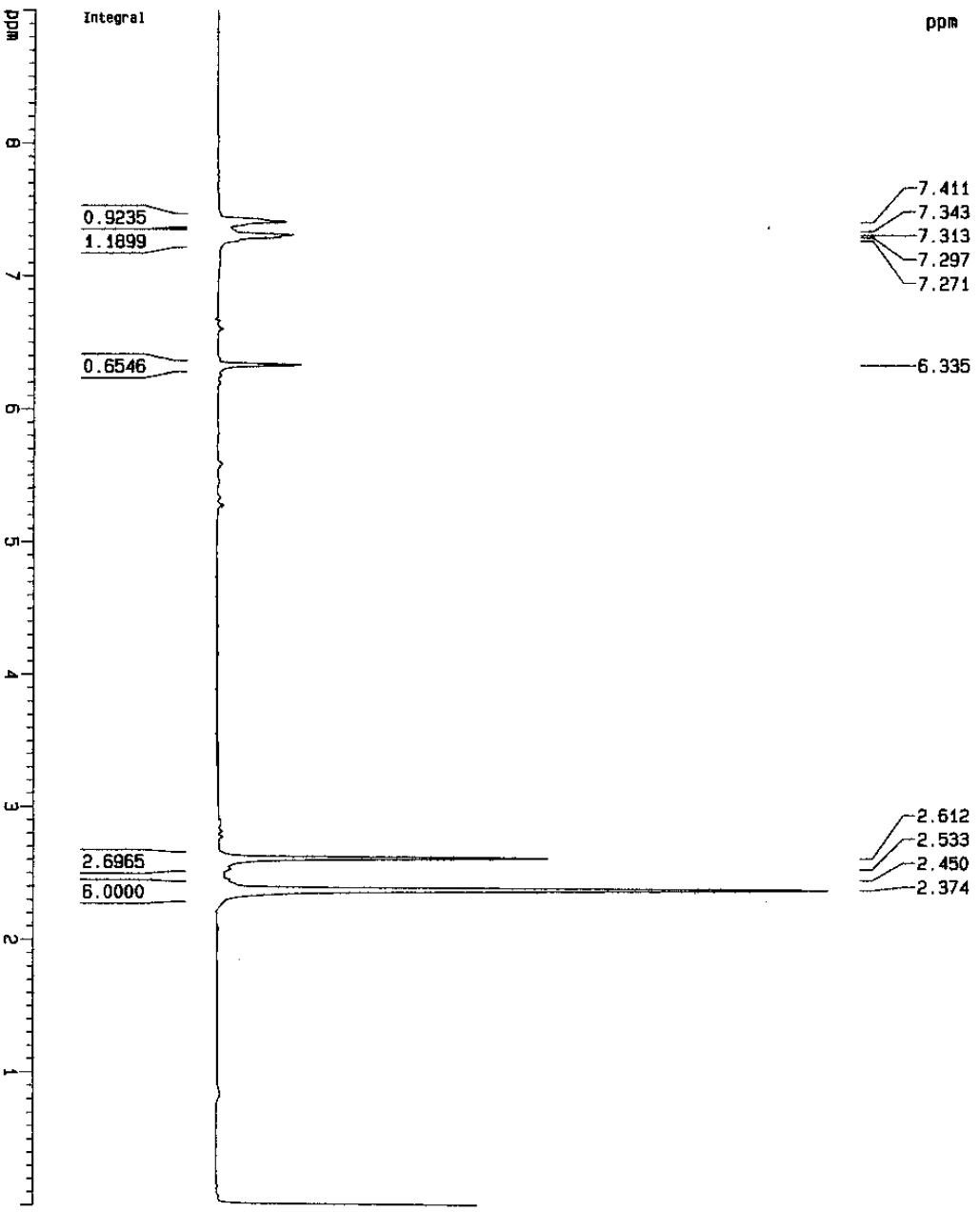
- 4,7,8-trimethyl-1,2-naphthoquinone (**6e**)



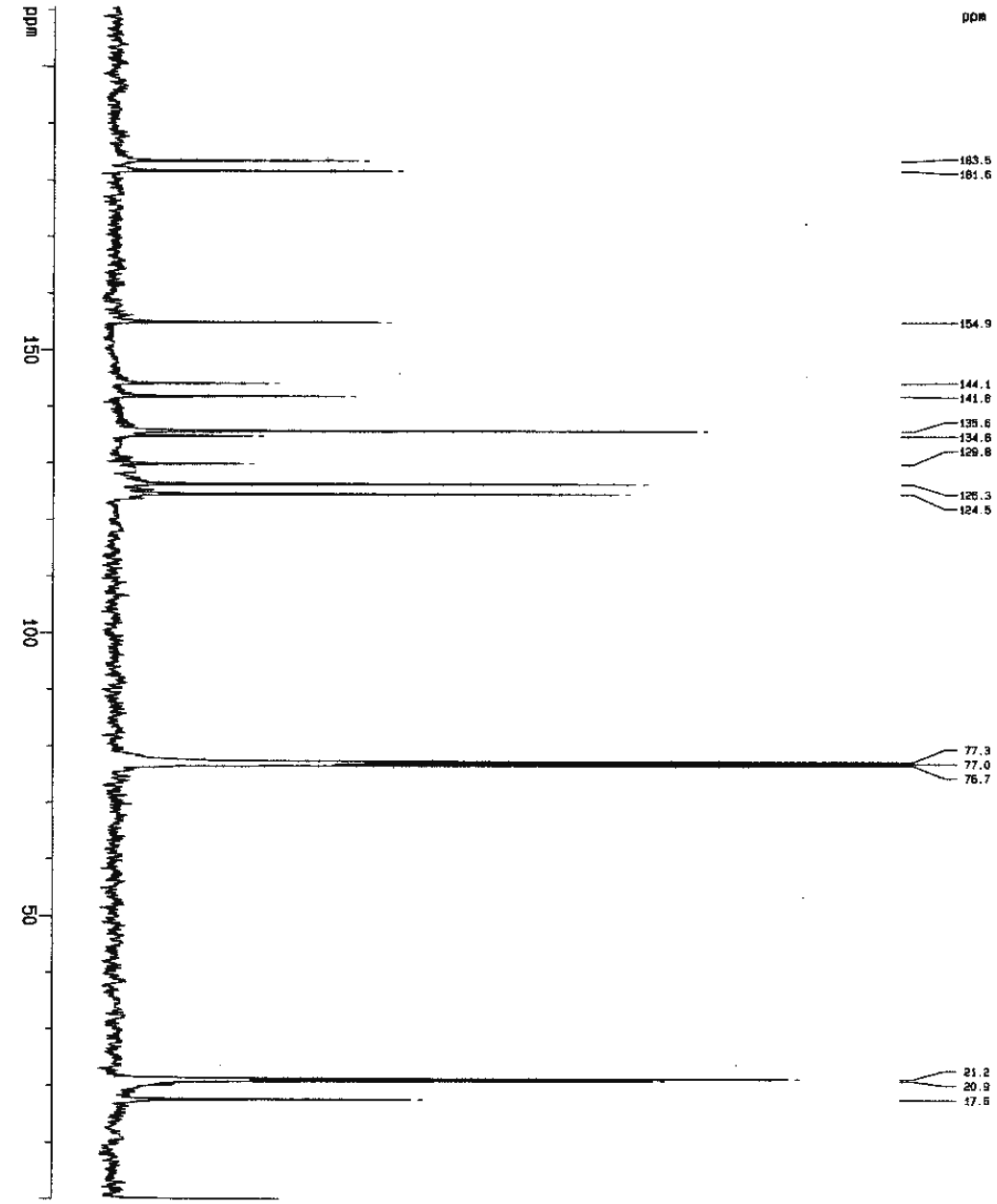

- 4-methyl-6,7-dimethoxy-1,2-naphthoquinone (**6f**)



## A.2.1 $^{1}$H-NMR Spectrum of Compound 6e



## A.2.2 $^{13}$C-NMR Spectrum of Compound 6e

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EXPNO                         1
PROCNO                        1

F2 - Acquisition Parameters
Date_              20080706
Time                   15.49
INSTRUM                spect
PROBHD     5 mm QNP 1H
PULPROG                zgpg
TD                     32768
SOLVENT                CDCl3
NS                      4000
DS                         2
SWH                22321.428 Hz
FIDRES              0.681196 Hz
AQ                  0.7340532 sec
RG                        128
DW                     22.400 usec
DE                      4.50 usec
TE                     300.0 K
D1                  2.00000000 sec
D11                 0.03000000 sec
D12                 0.00002000 sec

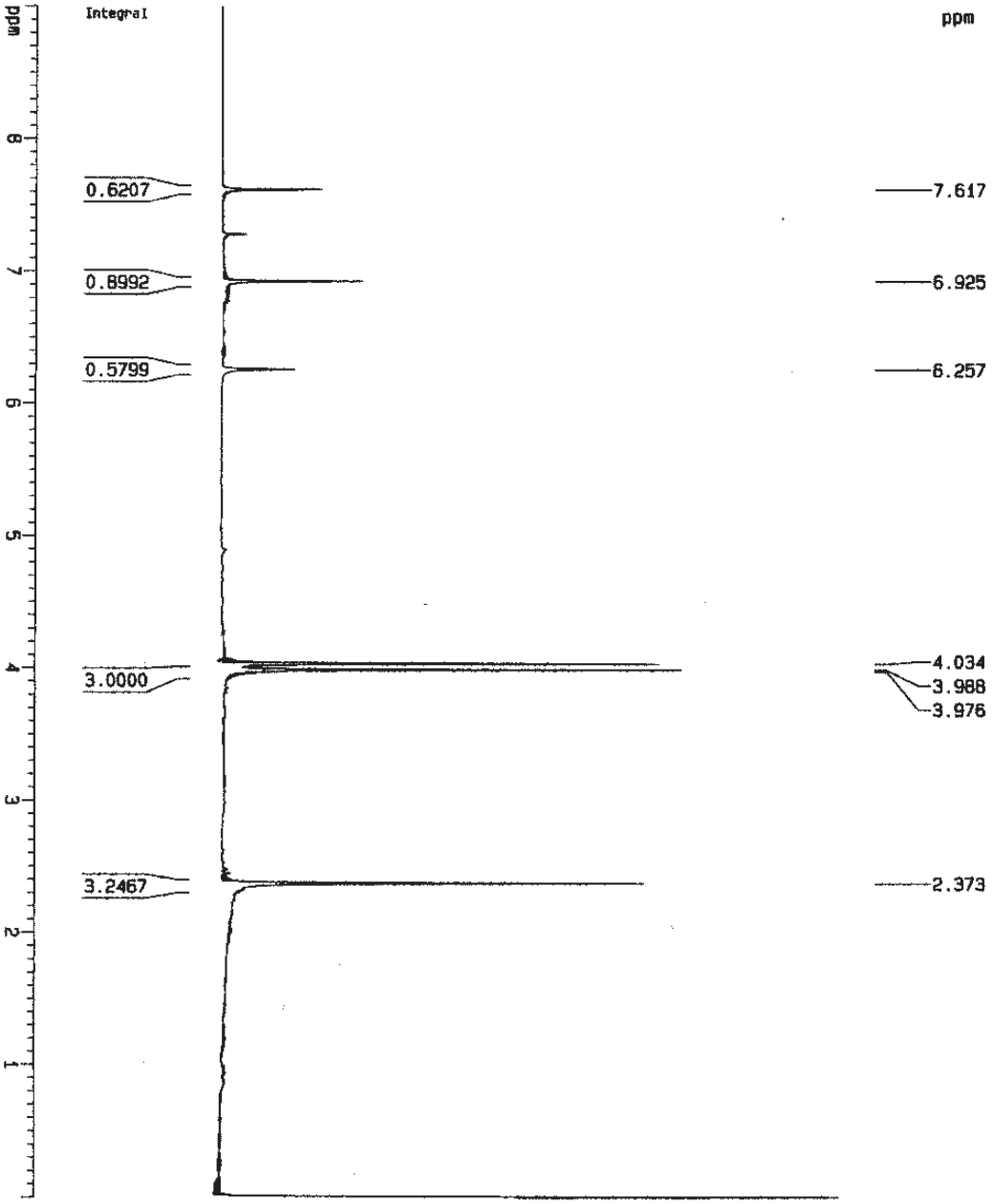
============== CHANNEL f1 ============
NUC1                      13C
P1                       7.00 usec
PL1                     -1.00 dB
SFO1           100.6051888 MHz

============== CHANNEL f2 ============
CPDPRG2                waltz16
NUC2                       1H
PCPD2                  90.00 usec
PL2                     2.00 dB
PL12                   23.00 dB
PL13                   23.00 dB
SFO2           400.0629832 MHz

F2 - Processing parameters
SI                        32768
SF             100.5951712 MHz
WDW                          EM
SSB                           0
LB                     10.00 Hz
GB                            0
PC                         0.05

1D NMR plot parameters
CX                       20.00 cm
F1P                    210.530 ppm
F1                   21178.33 Hz
F2P                    0.000 ppm
F2                     0.00 Hz
PPMCM                 10.52632 ppm/cm
HZCM                1058.91553 Hz/cm
```

## A.2.3 $^{1}$H-NMR Spectrum of Compound 6f




Current Data Parameters
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EXPNO 1
PROCNO 1

F2 - Acquisition Parameters
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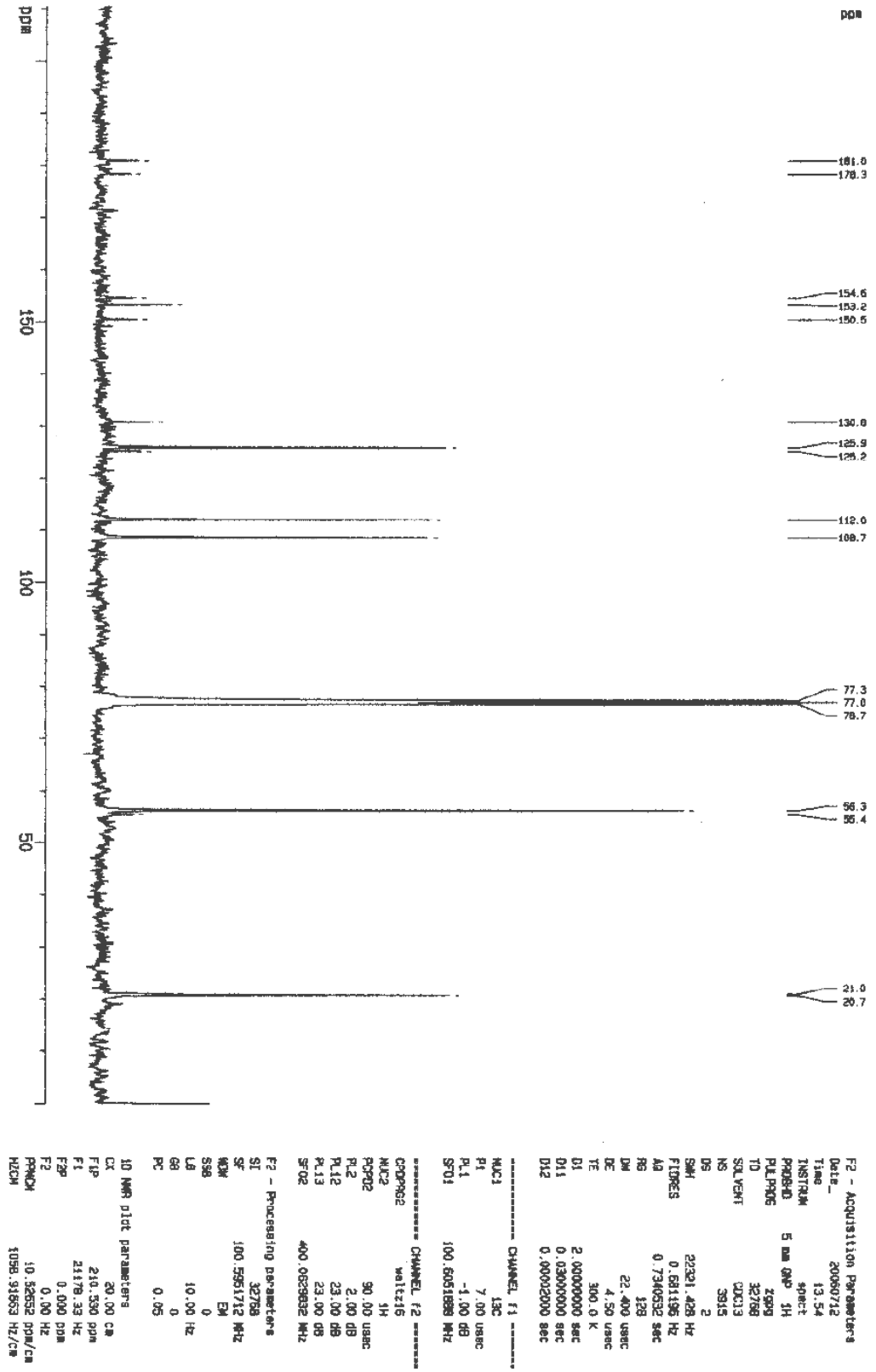
Time 13.41
INSTRUM spect
PROBHD 5 mm QNP 1H
PULPROG zg
TD 4096
SOLVENT CDCl3
NS 400
DS 0
SWH 8169.935 Hz
FIDRES 1.994613 Hz
AQ 0.2507252 sec
RG 512
DW 61.200 usec
DE 4.50 usec
TE 300.0 K
D1 3.00000000 sec

========== CHANNEL f1 ==========
NUC1 1H
P1 8.50 usec
PL1 0.00 dB
SFO1 400.0626780 MHz

F2 - Processing parameters
SI 4096
SF 400.0600052 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.00

1D NMR plot parameters
CX 20.00 cm
F1P 9.000 ppm
F1 3600.54 Hz
F2P 0.000 ppm
F2 0.00 Hz
PPMCM 0.45000 ppm/cm
HZCM 180.02699 Hz/cm

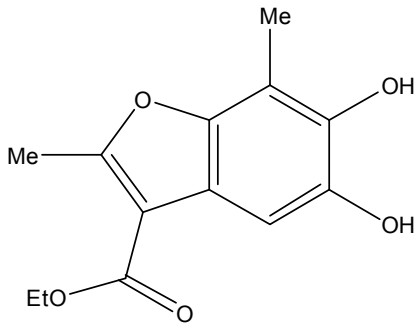
## A.2.4 $^{13}$C-NMR Spectrum of Compound 6f



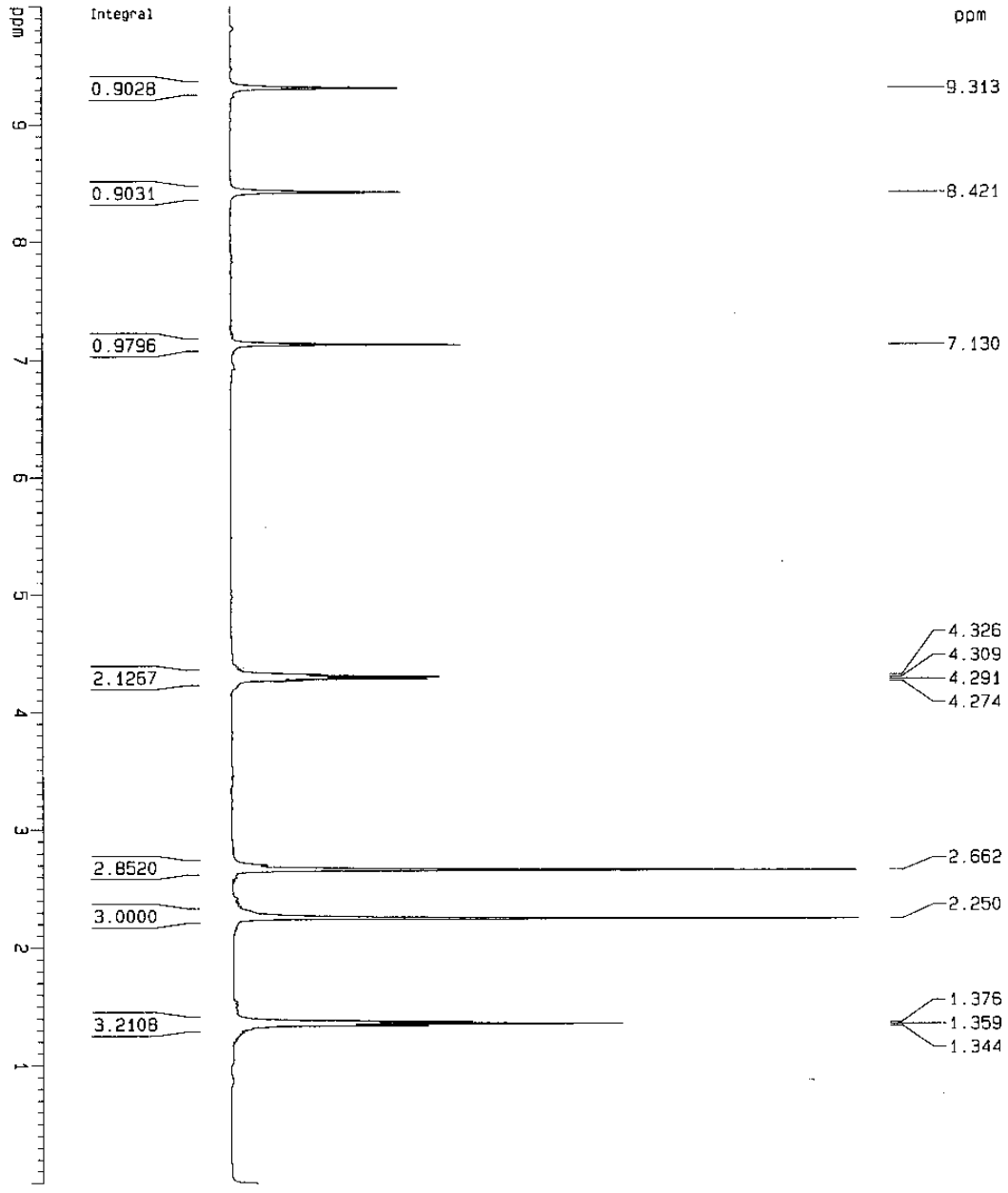
## A.3 Spectra of New Compounds in Chapter 6

There is one new compound obtained from the experiments in Chapter 6:

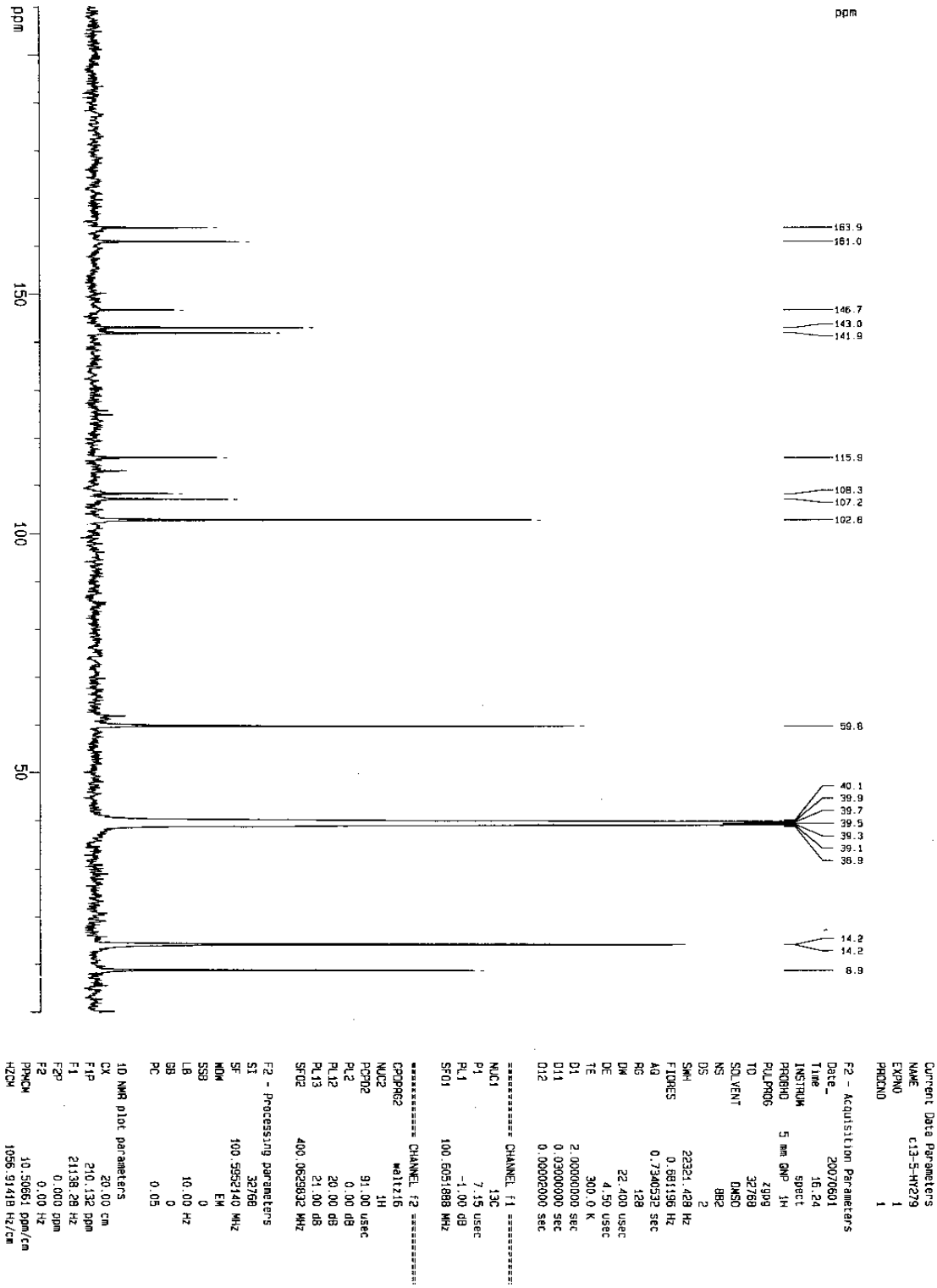
- Ethyl-5,6-dihydroxy-2,7-dimethyl-3-benzofuran carboxylate (**9d**).



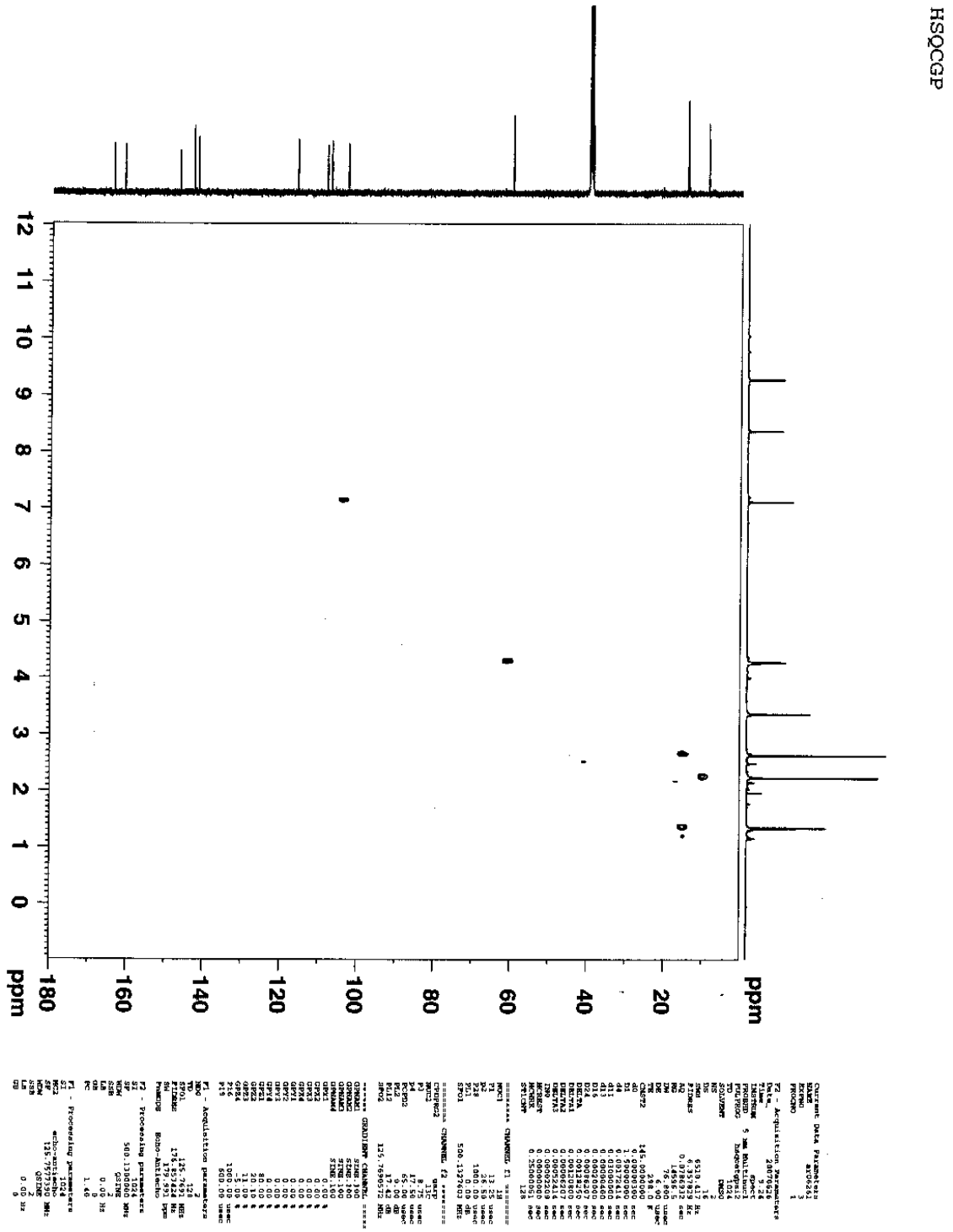
## A.3.1 $^{1}$H-NMR Spectrum of Compound 9d



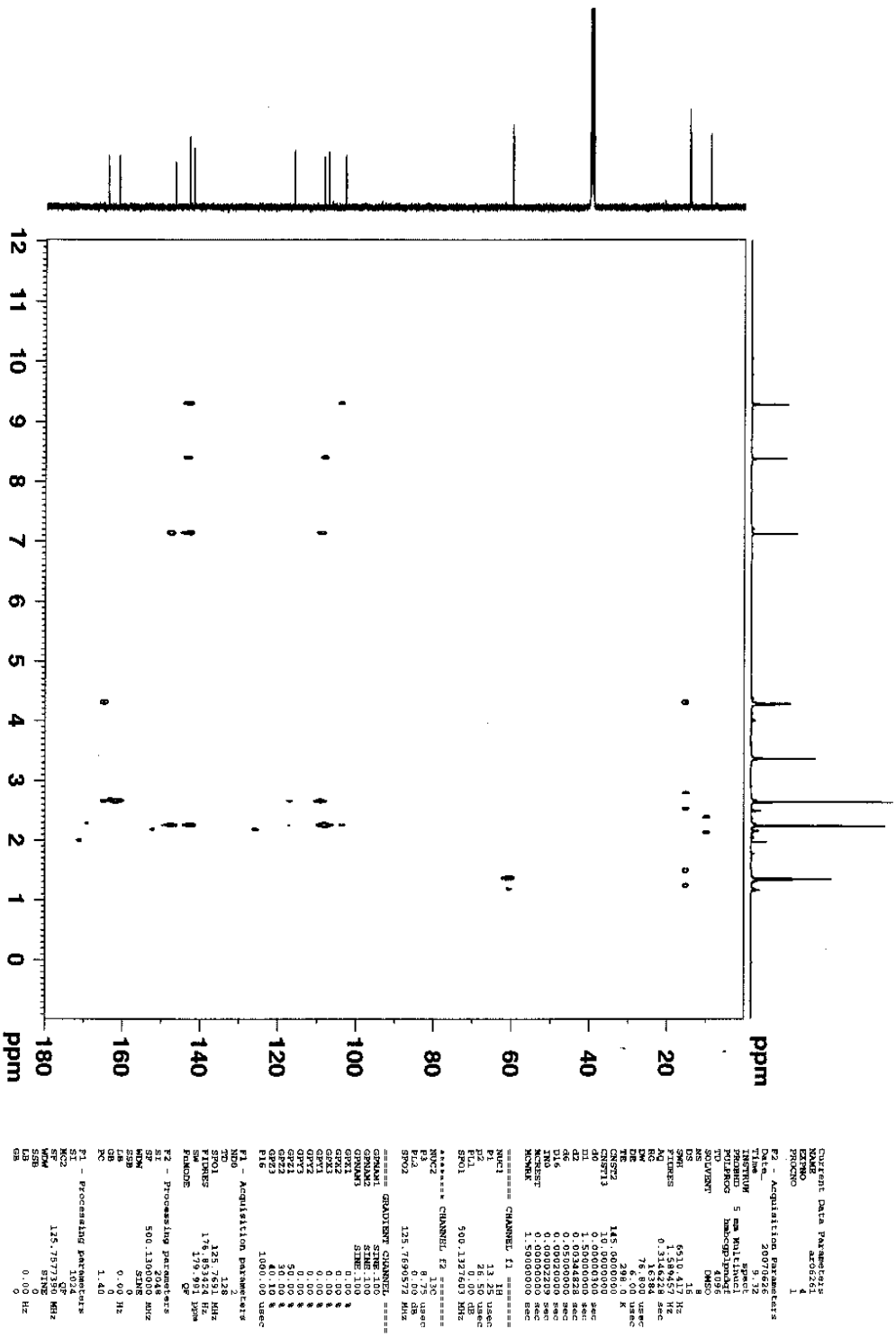
## A.3.2 $^{13}$C-NMR Spectrum of Compound 9d



## A.3.3 HSQC Spectrum of Coumpound 9d



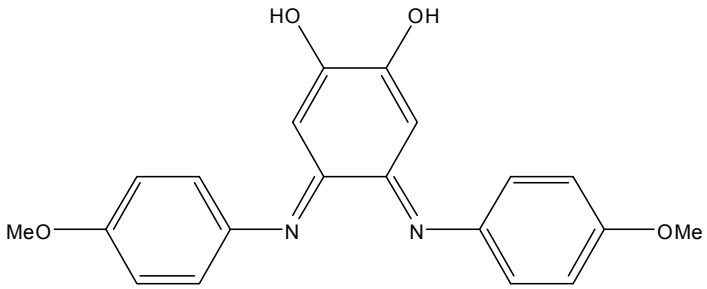
## A.3.4 HMBC Spectrum of Compound 9d



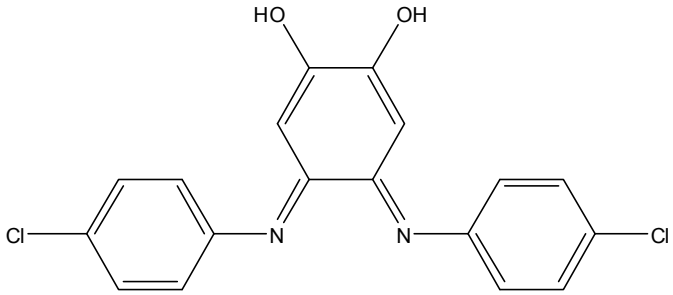
## A.4 Spectra of New Compounds in Chapter 7

There are three new compounds obtained from the experiments in Chapter 7:

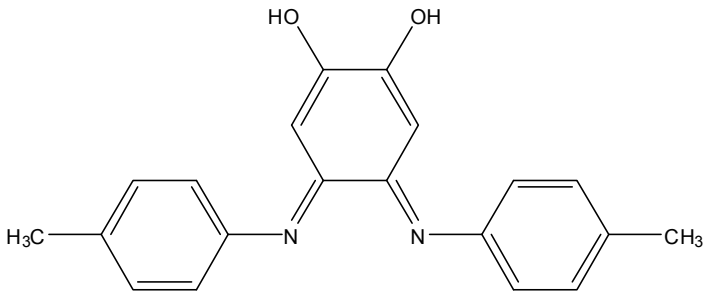
- Compound **11b**



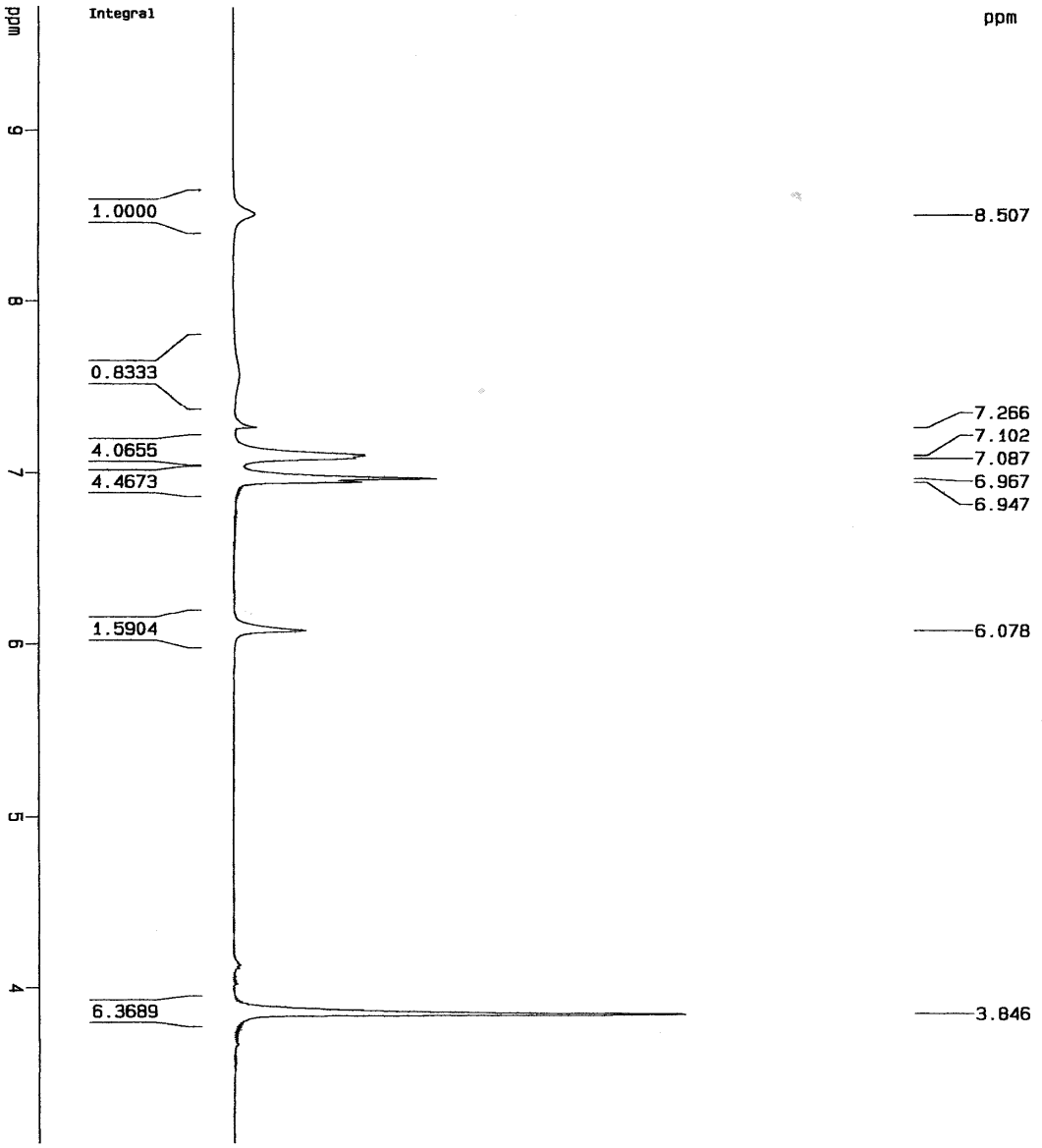

- Compound **11c**




- Compound **11d**



## A.4.1 $^{1}$H-NMR Spectrum of Compound 11b

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Current Data Parameters
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EXPNO                         2
PROCNO                        1

F2 - Acquisition Parameters
Date_              20080618
Time                      13.21
INSTRUM                   spect
PROBHD     5 mm QNP  1H
PULPROG                   zg
TD                        4096
SOLVENT                   DMSO
NS                          48
DS                           0
SWH                8169.935 Hz
FIDRES             1.994613 Hz
AQ                 0.2507252 sec
RG                         256
DW                 61.200 usec
DE                  4.50 usec
TE                     300.0 K
D1                 3.00000000 sec

======== CHANNEL f1 ========
NUC1                       1H
P1                   8.40 usec
PL1                   0.00 dB
SFO1          400.0626780 MHz

F2 - Processing parameters
SI                        4096
SF            400.0600092 MHz
WDW                       EM
SSB                        0
LB                   1.00 Hz
GB                         0
PC                      1.00

1D NMR plot parameters
CX                    20.00 cm
F1P                  9.722 ppm
F1                  3889.43 Hz
F2P                  3.091 ppm
F2                  1236.63 Hz
PPMCM              0.33155 ppm/cm
HZCM              132.64026 Hz/cm
```
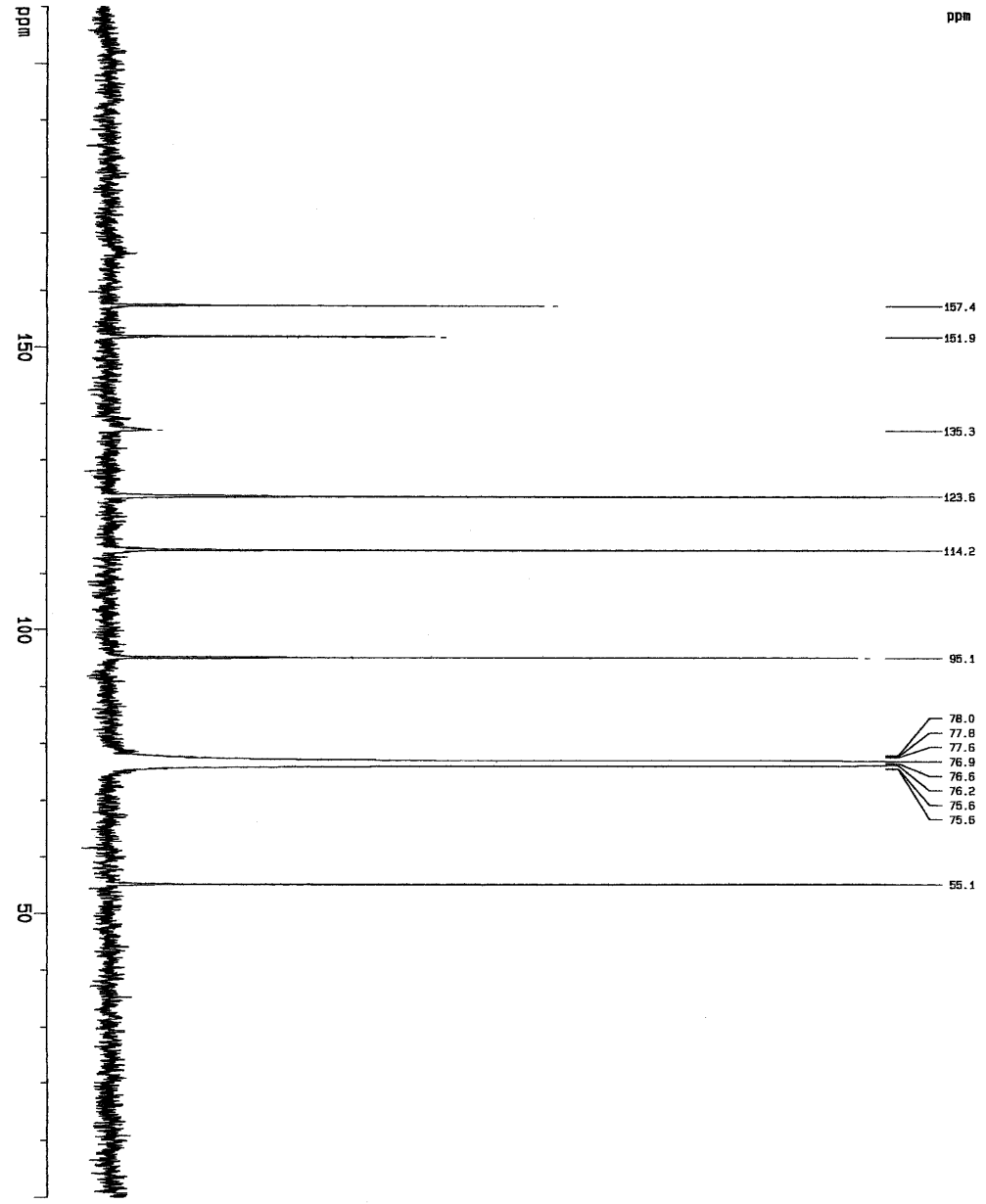

## A.4.2. $^{13}$C-NMR Spectrum of Compound 11b

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Current Data Parameters
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EXPNO                            1
PROCNO                           1

F2 - Acquisition Parameters
Date_                     20080722
Time                         11.45
INSTRUM                       spect
PROBHD          5 mm QNP 1H
PULPROG                       zgpg
TD                           32764
SOLVENT                      CDCl3
NS                            2048
DS                               0
SWH                      22321.428 Hz
FIDRES                    0.681279 Hz
AQ                        0.7339636 sec
RG                           16384
DW                          22.400 usec
DE                           4.50 usec
TE                          300.0 K
D1                     25.00000000 sec
D11                    0.03000000 sec
D12                    0.00002000 sec

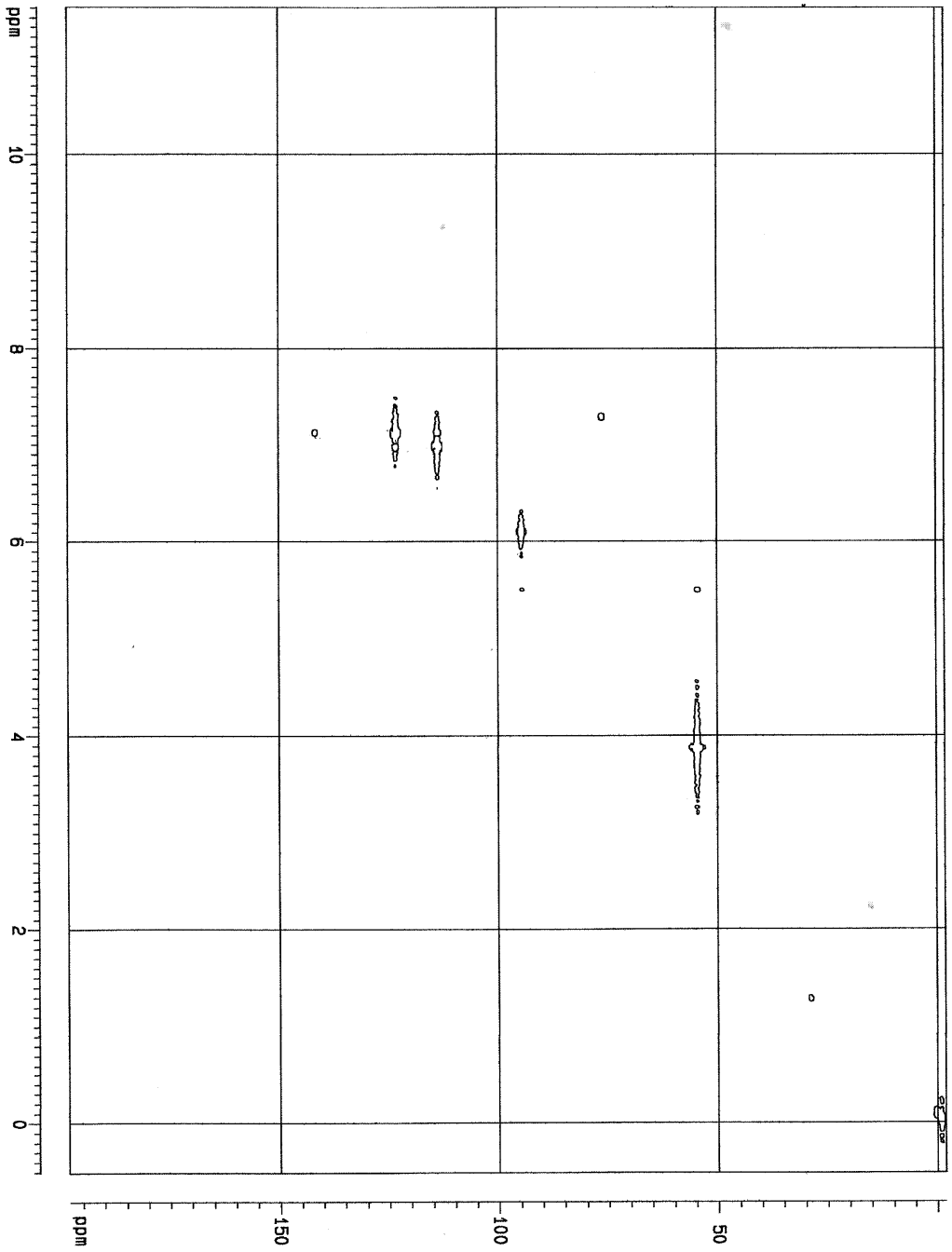
================ CHANNEL f1 =======
NUC1                          13C
P1                           8.40 usec
PL1                         -1.00 dB
SFO1                  100.6051898 MHz

================ CHANNEL f2 =======
CPDPRG2                   waltz16
NUC2                          1H
PCPD2                      95.00 usec
PL2                          0.00 dB
PL12                        20.00 dB
PL13                        21.00 dB
SFO2                  400.0629832 MHz

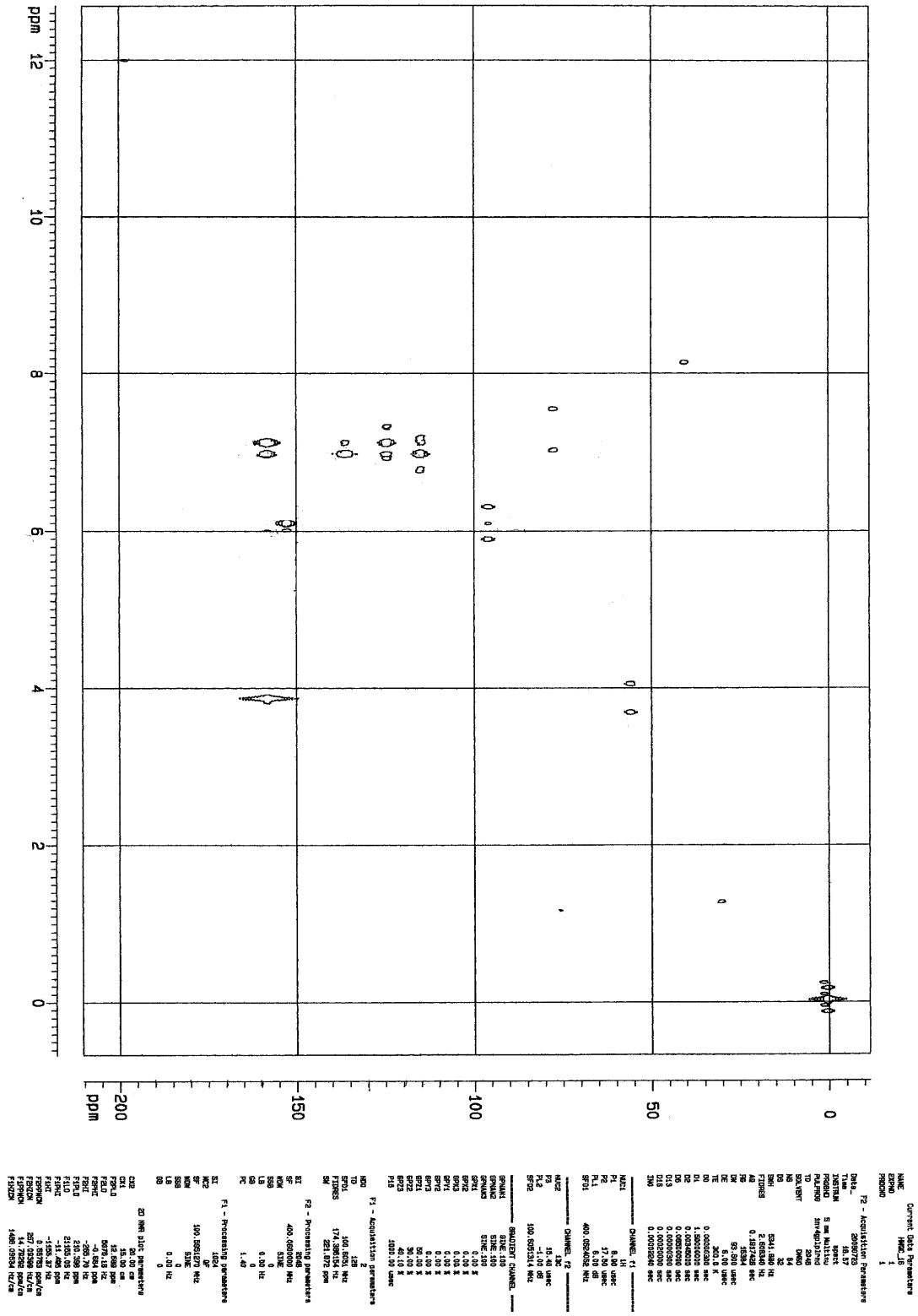
F2 - Processing parameters
SI                           32768
SF                    100.5952160 MHz
WDW                             EM
SSB                              0
LB                            5.00 Hz
GB                               0
PC                            0.05

10 NMR plot parameters
CX                           20.00 cm
F1P                         210.132 ppm
F1                        21138.28 Hz
F2P                           0.000 ppm
F2                            0.00 Hz
PPMCM                        10.50661 ppm/cm
HZCM                      1056.91418 Hz/cm
```

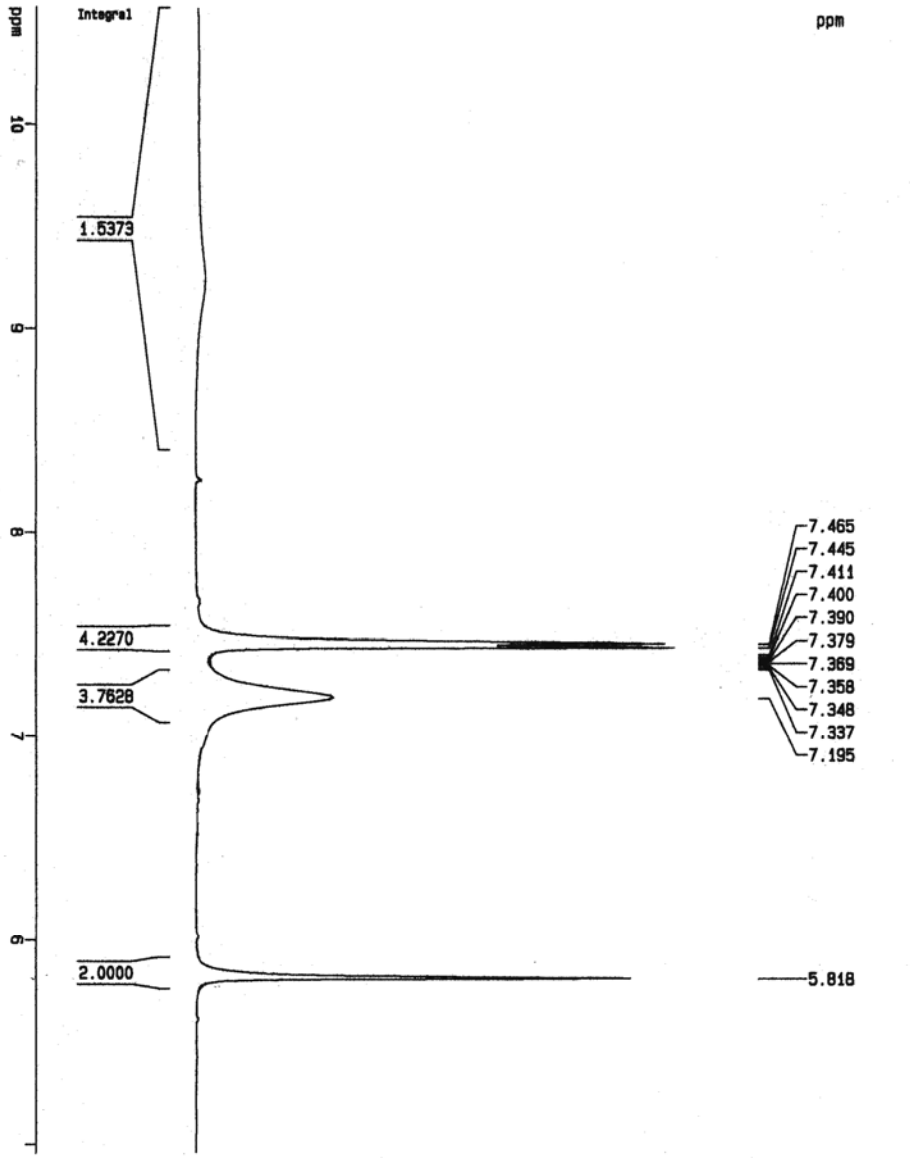
## A.4.3 HMQC Spectrum of Compound 11b



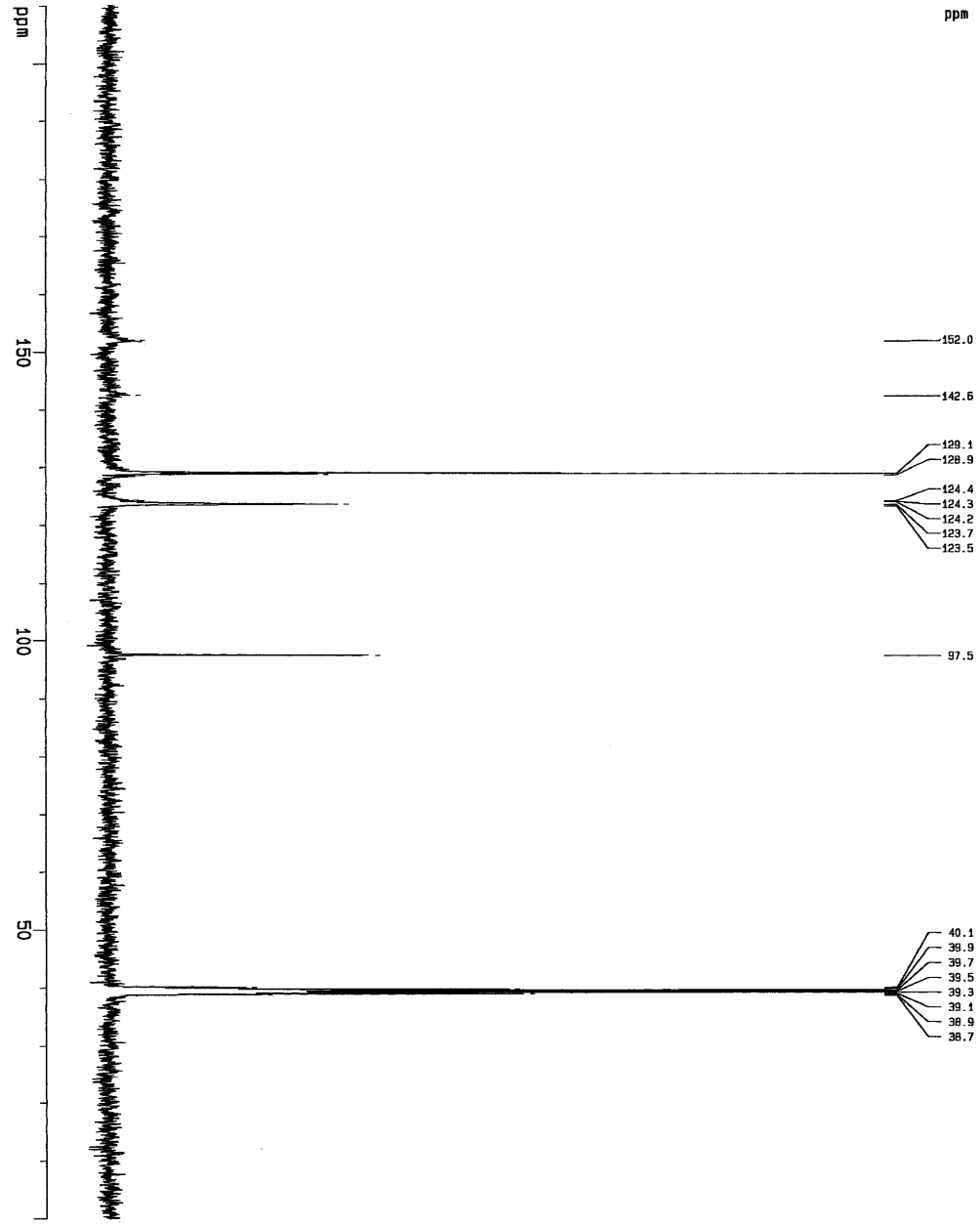
## A.4.4 HMBC Spectrum of Compound 11b



## A.4.5 $^{1}$H-NMR Spectrum of Compound 11c



## A.4.6 $^{13}$C-NMR Spectrum of Compound 11c

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EXPNO                            2
PROCNO                           1

F2 - Acquisition Parameters
Date_                     20080730
Time                          15.58
INSTRUM                        spect
PROBHD          5 mm Multinu
PULPROG                       zgpg
TD                           32764
SOLVENT                       DMSO
NS                              169
DS                                2
SWH                       22321.428 Hz
FIDRES                     0.681279 Hz
AQ                         0.7339536 sec
RG                               64
DW                         22.400 usec
DE                          4.50 usec
TE                           300.0 K
D1                      5.00000000 sec
D11                     0.03000000 sec
D12                     0.00002000 sec

======== CHANNEL f1 ========
NUC1                            13C
P1                         15.10 usec
PL1                        -1.00 dB
SF01                  100.6051988 MHz

======== CHANNEL f2 ========
CPDPRG2                    waltz16
NUC2                            1H
PCPD2                      100.00 usec
PL2                          6.00 dB
PL12                        26.50 dB
PL13                        26.50 dB
SFO2                  400.0629832 MHz

F2 - Processing parameters
SI                           32768
SF                    100.5952160 MHz
WDW                             EM
SSB                              0
LB                          5.00 Hz
GB                               0
PC                            0.05

1D NMR plot parameters
CX                           20.00 cm
F1P                      210.132 ppm
F1                       21138.28 Hz
F2P                        0.000 ppm
F2                          0.00 Hz
PPMCM                   10.50661 ppm/cm
HZCM                    1056.91418 Hz/cm
```
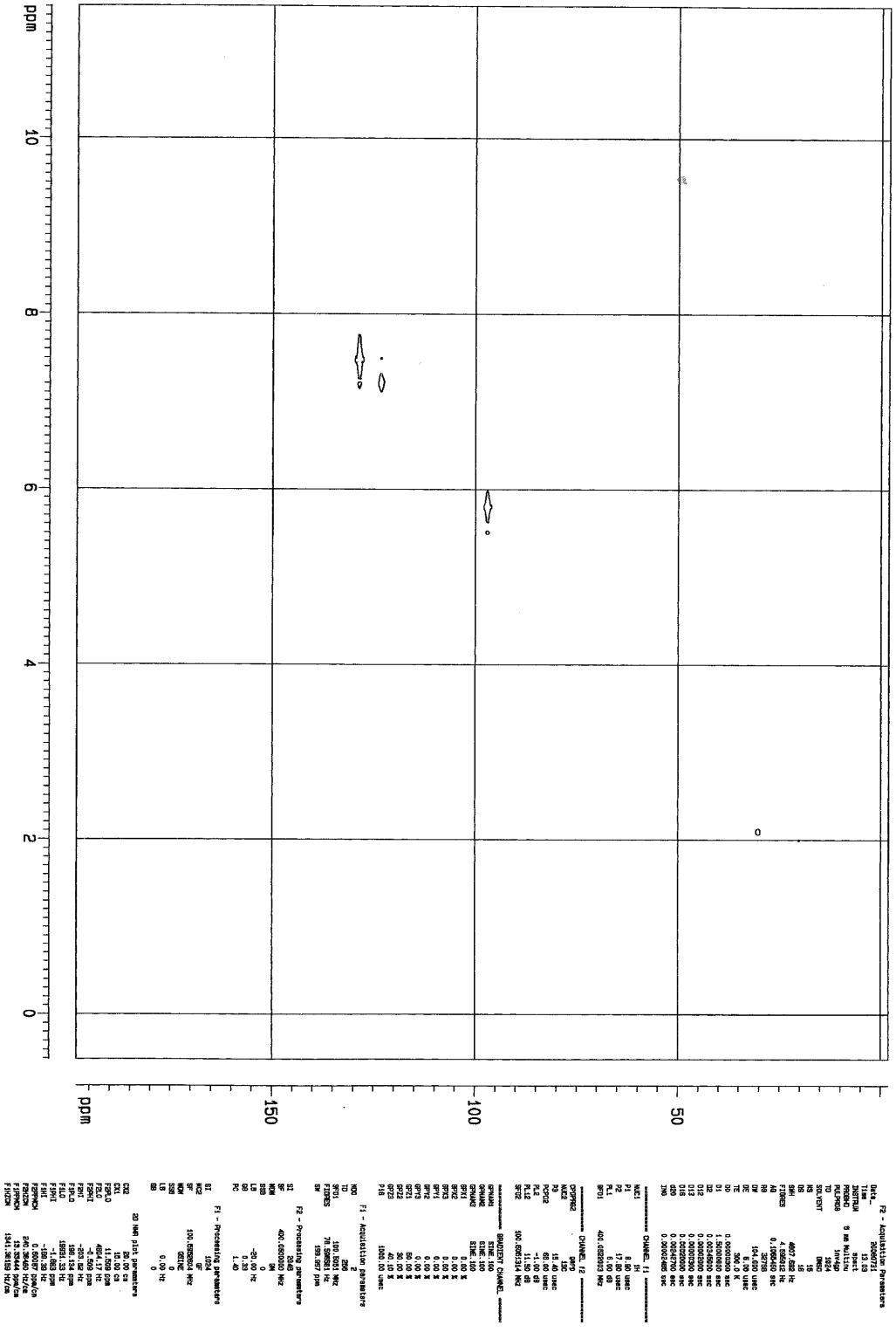
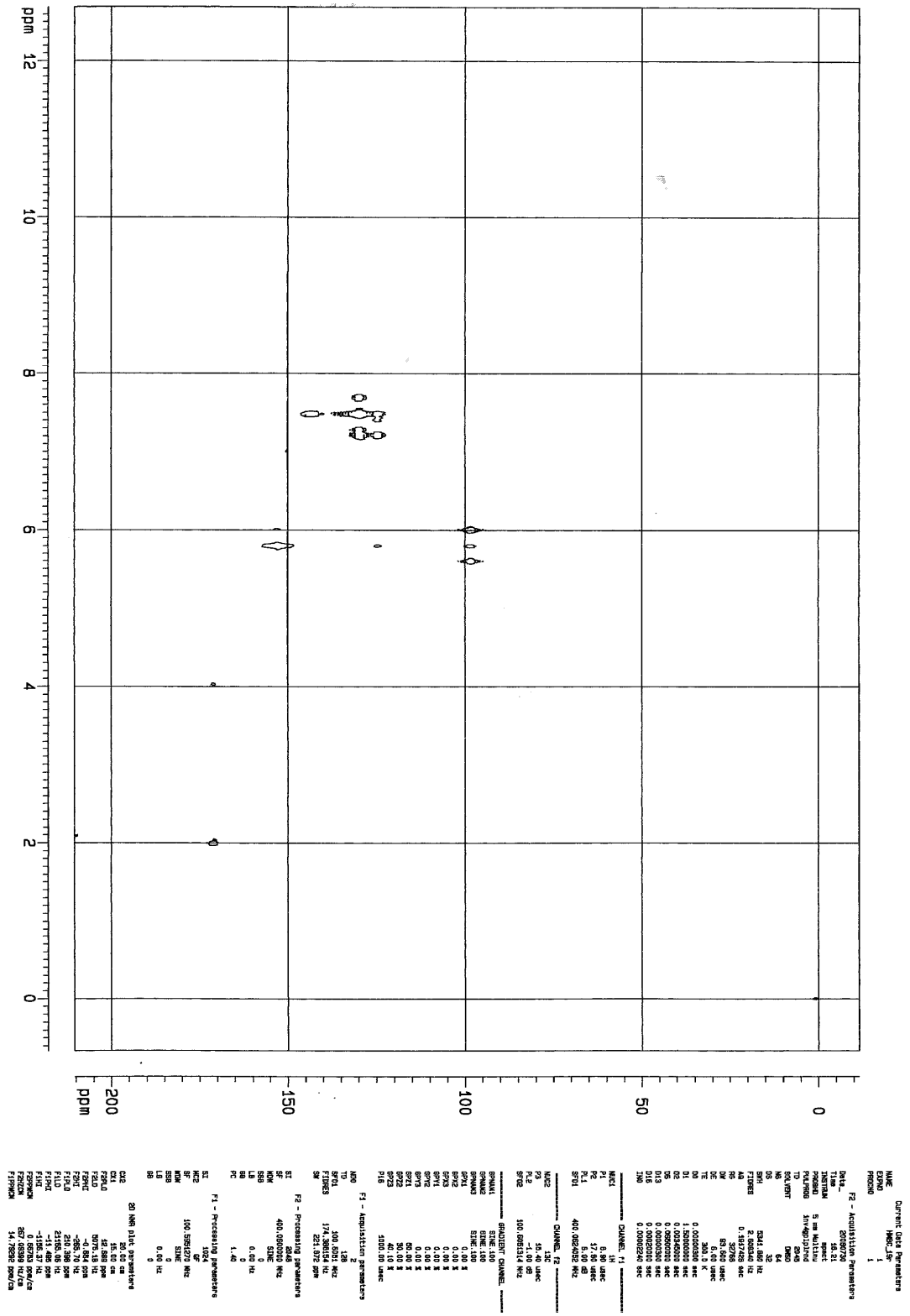
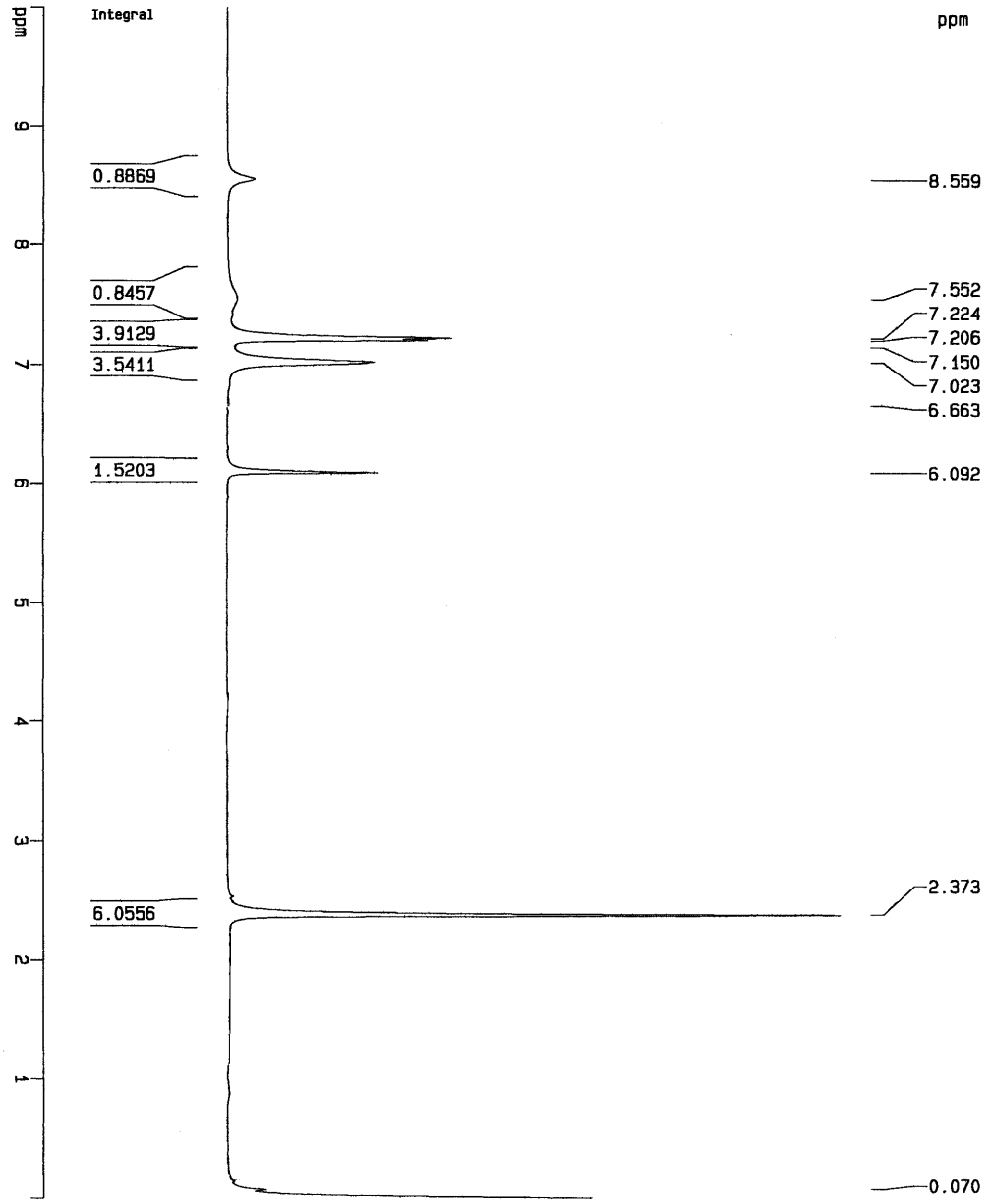

## A.4.7 HMQC Spectrum of Compound 11c



## A.4.8 HMBC Spectrum of Compound 11c



## A.4.9 $^{1}$H-NMR Spectrum of Compound 11d

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Current Data Parameters
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EXPNO                         1
PROCNO                        1

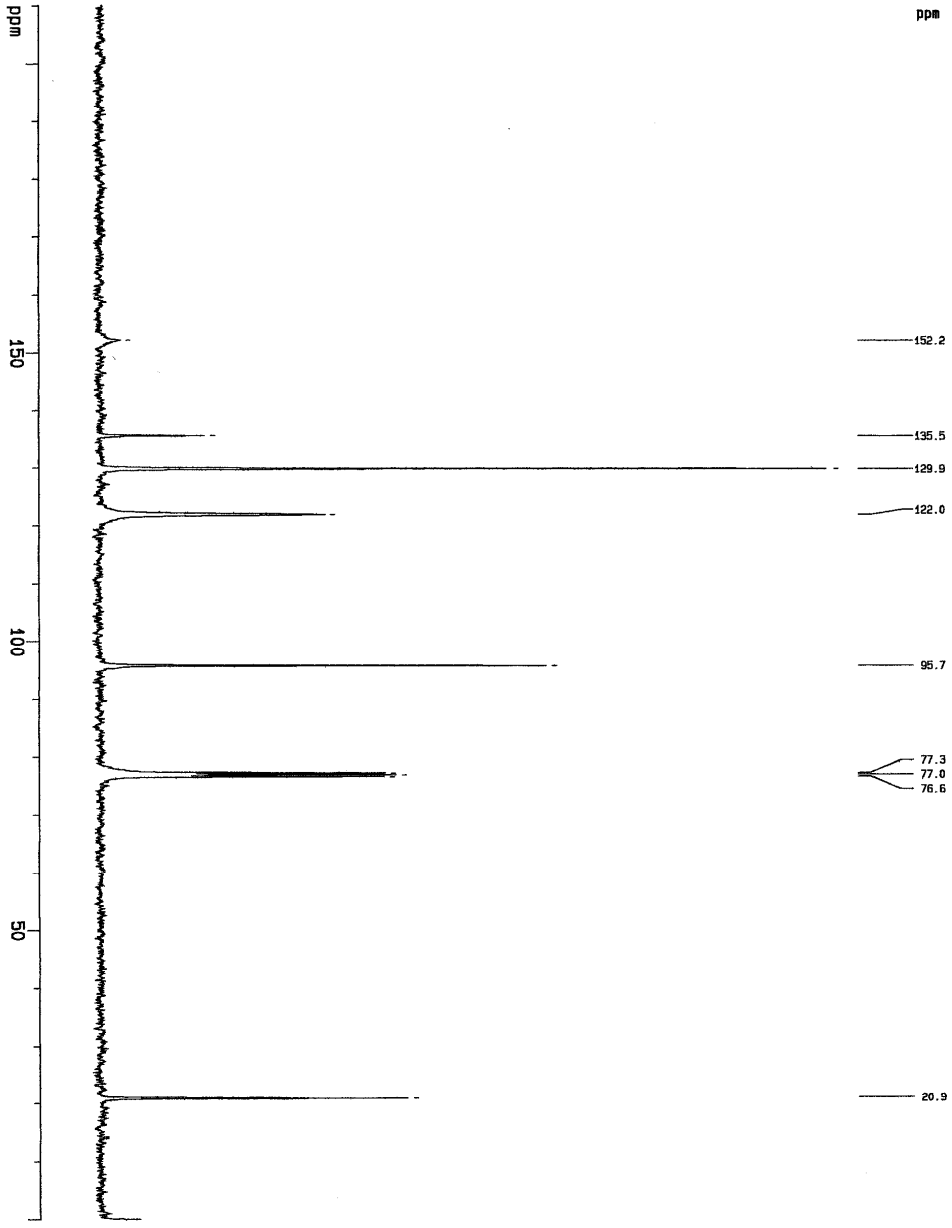
F2 - Acquisition Parameters
Date_              20080616
Time                   15.48
INSTRUM                spect
PROBHD     5 mm QNP 1H
PULPROG                 zg
TD                    4096
SOLVENT               CDCl3
NS                       24
DS                        0
SWH                8169.935 Hz
FIDRES             1.994613 Hz
AQ                 0.2507252 sec
RG                      256
DW                 61.200 usec
DE                  4.50 usec
TE                  300.0 K
D1                 3.00000000 sec

======== CHANNEL f1 ========
NUC1                      1H
P1                     8.40 usec
PL1                    0.00 dB
SFO1           400.0626780 MHz

F2 - Processing parameters
SI                        4096
SF             400.0600132 MHz
WDW                       EM
SSB                        0
LB                   1.00 Hz
GB                         0
PC                      1.00

1D NMR plot parameters
CX                   20.00 cm
F1P                10.000 ppm
F1                 4000.60 Hz
F2P                 0.000 ppm
F2                     0.00 Hz
PPMCM            0.50000 ppm/cm
HZCM           200.03001 Hz/cm
```

## A.4.10 $^{13}$C-NMR Spectrum of Compound 11d

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Current Data Parameters
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EXPNO                          1
PROCNO                         1

F2 - Acquisition Parameters
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Time                       16.02
INSTRUM                    spect
PROBHD     5 mm QNP 1H
PULPROG                    zgpg
TD                         32768
SOLVENT                    CDCl3
NS                          2181
DS                             2
SWH                    22321.428 Hz
FIDRES                 0.681196 Hz
AQ                     0.7340532 sec
RG                           64
DW                        22.400 usec
DE                         4.50 usec
TE                        300.0 K
D1                     2.00000000 sec
D11                    0.03000000 sec
D12                    0.00002000 sec

======== CHANNEL f1 ========
NUC1                         13C
P1                         8.40 usec
PL1                       -1.00 dB
SFO1                 100.6051888 MHz

======== CHANNEL f2 ========
CPDPRG2                  waltz16
NUC2                         1H
PCPD2                     95.00 usec
PL2                        0.00 dB
PL12                      20.00 dB
PL13                      21.00 dB
SFO2                 400.0629832 MHz

SI                         32768
SF                   100.5951773 MHz
WDW                           EM
SSB                            0
LB                         10.00 Hz
GB                             0
PC                          0.05

1D NMR plot parameters
CX                         20.00 cm
F1P                      210.132 ppm
F1                     21138.28 Hz
F2P                        0.000 ppm
F2                         0.00 Hz
PPMCM                  10.50661 ppm/cm
HZCM                   1056.91382 Hz/cm
```
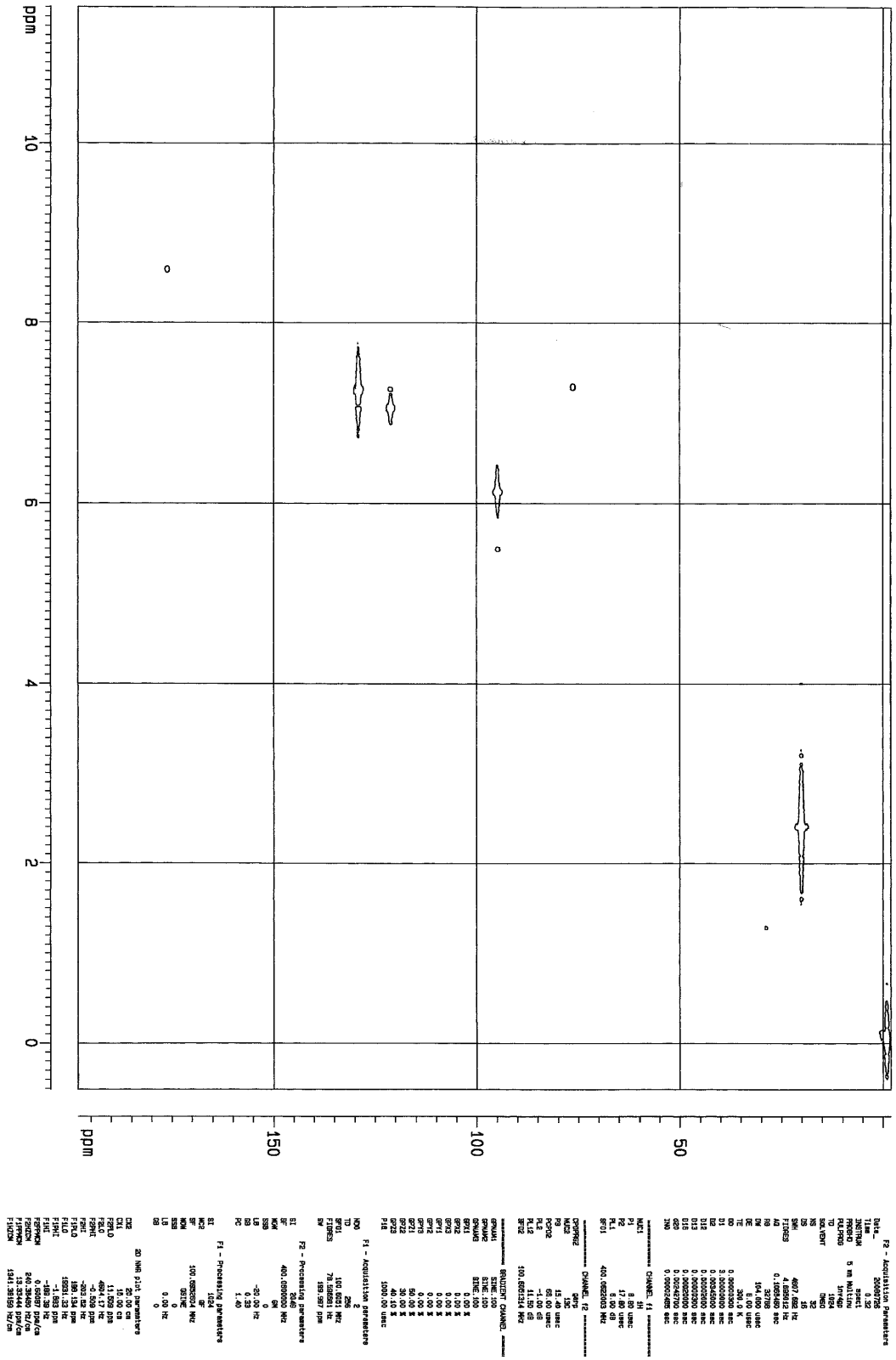
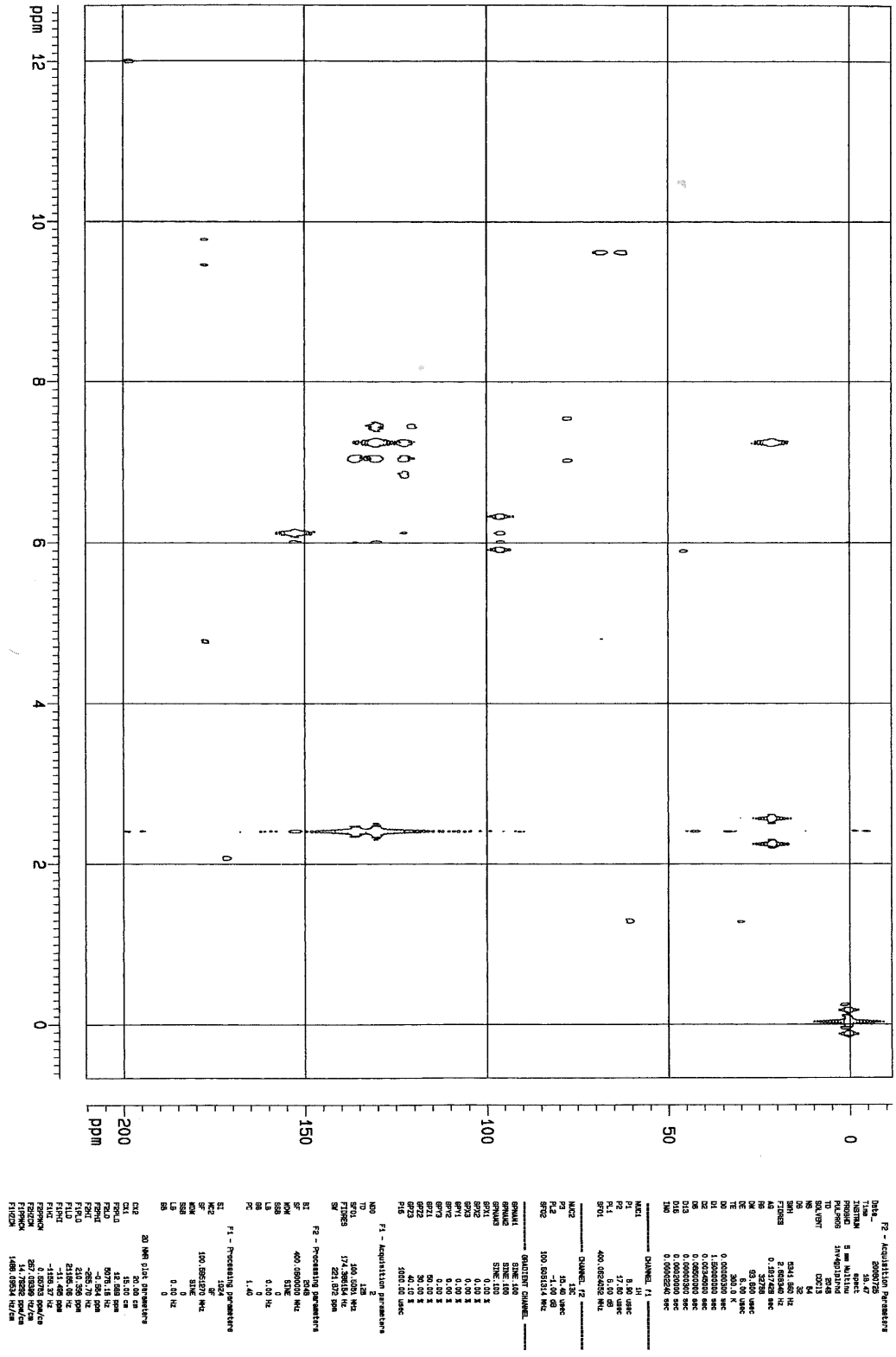
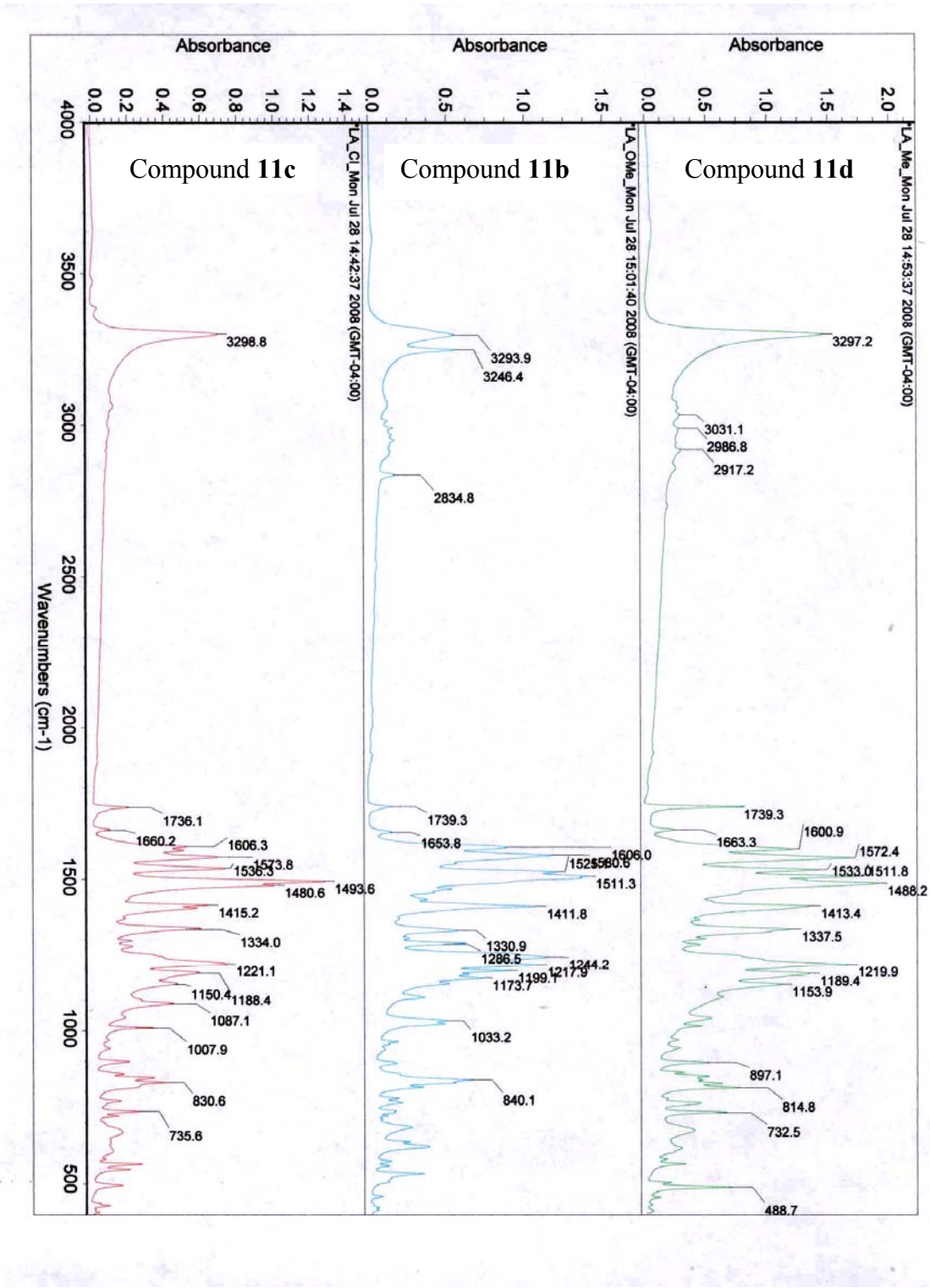

## A.4.11 HMQC Spectrum of Compound 11d



## A.4.12 HMBC Spectrum of Compound 11d



## A.4.13 IR Spectra of compound 11b, 11c and 11d



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# APPENDIX C

# TENSILE AND TEAR STRENGTH

## C.1 Tensile Strength

The tensile strength of paper sheets is especially complex as many variables play a role in controlling the magnitude of this property. Tensile strength is dependent on both the fiber strength properties and the bonding that occurs between fibers. The tensile strength theory that has attracted the most attention has been that of Page. The "Page" equation (Equation 4) was shown in a publication in 1969 [417] and remains a fixture in paper physics discussions. The equation represents a comprehensive account of the variables encountered in attempting to predict tensile strength from the properties of the fiber and for bonds between fibers. The equation (Equation 4) also attempts to calculate "bondstrength" from all of these variables affecting tensile strength

$$(1/T) = (9/8Z) + [(12g \times C)/(P \times l \times b \times RBA)]$$

Where:
l = fiber length (length)
b = fiber-fiber bond strength ($N/m^2$)
RBA = relative bonded area (unit less)
g = gravitational constant -(length/second$^2$ = 9.8 $m/s^2$)
T = tensile breaking length (length)
Z = zero span tensile (length)
C = fiber coarseness (weight/length)
P = fiber perimeter (length)

**Equation 4.** The Page equation.

Equation 4 shows that the inverse of tensile strength should be linearly proportional to the inverse of fiber strength, fiber length, and RBA. The tensile strength predictions of the Page equation are illustrated in Figure 121.

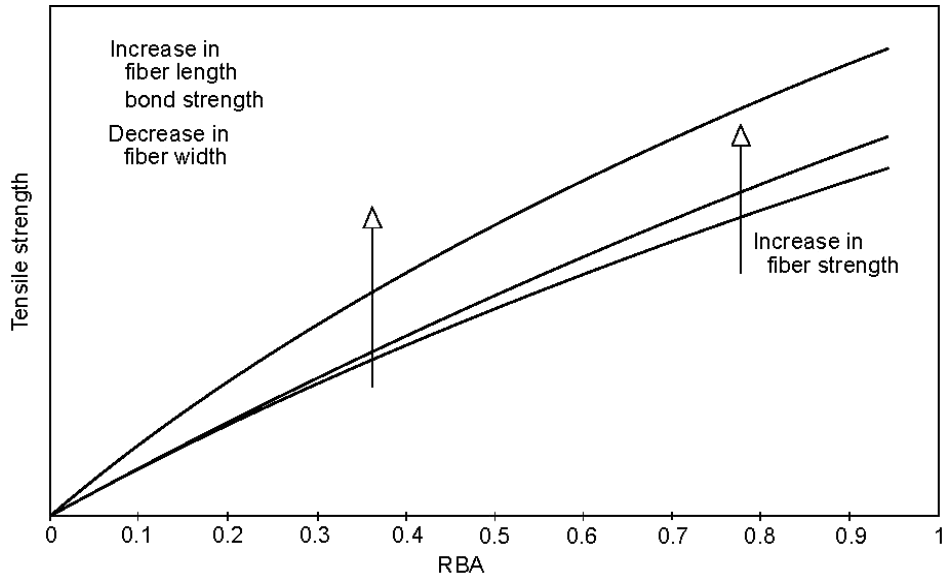


**Figure 121.** Predictions from Page equation for tensile strength of paper vs. relative bonded area together with the qualitative effect of increasing fiber properties.

The relative bonded area (RBA) in Page's equation is a measure of the contact area between fibers in the sheet [417].This is measured by light scattering co-efficient or through nitrogen absorption measurements. Increases in bonded area can be achieved by increasing wet-pressing pressure. With subsequent testing of a strength property (such as tensile strength) and scattering coefficient, the sheet strength can be extrapolated to zero sheet strength. The result of this extrapolation is an estimate of the scattering coefficient of unbonded fibers ($S_o$) that can be used to calculate the relative bonded area [417].

Equation 5 shows the relationship between relative bonded area and light scattering coefficient.

$$RBA = (S_o - S)/S_o$$

Where:
$S_o$ = scattering co-efficient of the unbonded sheet ($m^2/kg$)
S = scattering co-efficient for a paper sheet ($m^2/kg$)

**Equation 5.** Page's equation for computing relative bonded area.

In the Page equation, most of the variables are measurable except for b, the fiber to fiber bond strength or "shear strength" of the fiber bonds [417]. Once all of the measurable variables are obtained, the Page parameter ([1/T – 9/(8Z)]-1) can be plotted against the light scattering coefficient (S) (Equation 6). This plot can be used to obtain the bond strength (b) and the scattering coefficient of the unbound fibers ($S_o$) from the slope and intercept respectively.

$$[(1/T) - (9/8Z)]-1 = b \times [(1/\gamma) - (S/(\gamma \times S_o))]$$

$$\gamma = [(12g \times C)/ (P \times l)]$$

**Equation 6.** Parameters to plot for obtaining bond strength using the Page equation.

The Page equation is only valid for sheets made with good formation, free from kinks or curls [418]. This is because sheets with poor formation fail earlier due to uneven concentrations of stress in areas of low basis weight. Kinks and curls cause changes in the

fiber length variable in the equation. The kinks and curls also decrease the number of load-bearing elements in the sheet.

In the physical testing of paper, tensile strength is determined by measuring the force required to break a narrow strip of paper where both the length of the strip and the rate of loading are closely specified [285]. The amount of stretch at rupture may be determined at the same time. Some modern testers provide a plot of the stress/strain curve and compute the area under the curve which is referred to as tensile energy absorption, a measure of paper toughness. These testers also provide for measurement of creep under various tensile loading.

## C.2 Wet Tensile Strength

Paper is a layered mat consisting of a network of cellulose fibers held together by intermolecular forces (van der Waals and hydrogen bonding) which are very sensitive to water. The extent of bonding steadily decreases as the water content of the paper increases. The water wets the fibers, and then, the bonds are broken leaving somewhere between 3% and 10% of the original dry strength (at 50% relative humidity). The residual strength of wet paper results from remaining covalent fiber-fiber bonds. Therefore, there is a need for paper products to retain some strength when subjected to high humidity or when soaked in water. Many applications have been developed to improve the wet strength of paper [416].

The way to determine wet strength of the paper is to measure its burst or tensile strength when wet. There are useful Standard Methods for the determination of wet

strength (e.g. TAPPI Method T456), although many non-standard tests have been developed over the years. In the TAPPI Method, a strip of paper is completely wetted before applying a breaking force. The paper is immersed in water or, if it is too weak, it is mounted in the jaws of a tensile tester and wet midway over a distance of 2.54 cm. The load required to break the paper is then recorded. The result reported as percent wet strength (wet strength as a percentage of the dry strength).

### C.3 Tear Strength

Tearing resistance is the total energy per tear length consumed when a specimen of a given geometry undergoes tearing. Tearing resistance therefore has the units of load and is sometimes called tear strength, although it is energy, not stress, that one measures. Tearing strength is normally determined with the Elmendorf apparatus which uses a falling pendulum to continue a tear in the paper sample when the force is applied perpendicular to the plane of the sheet; the loss of energy, measured by the height of swing of the pendulum, is related to the force required to continue the tear [285]. The Elmendorf tear test is recognized as a good measure of fiber strength within the sheet. Apparatus for carrying out in-plane tear testing is available, but the procedure is not widely utilized. In the in-plane tear measurement, load is applied in the plane of paper, often at a 2 x 6° angle as Figure 122(a) shows. In the out-of-plane tear test or Elmendorf tear of Figure 122(b), load is in the out-of-plane direction.

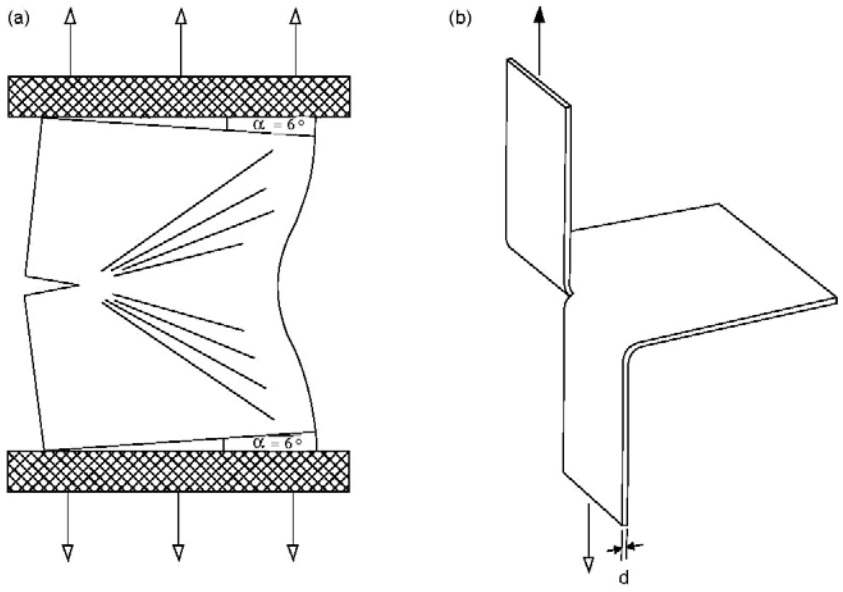


**Figure 122.** (a) The in-plane tear test; (b) the out-of-plane or Elmendorf tear test.

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