EXPLOITING THE OXIDIZING CAPABILITIES OF LACCASES FOR SUSTAINABLE CHEMISTRY

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EXPLOITING THE OXIDIZING CAPABILITIES OF LACCASES FOR SUSTAINABLE CHEMISTRY

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LIST OF SYMBOLS AND ABBREVIATIONS

ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonate)
ACS	American Chemical Society
Asn	Asparagine
Asp	Aspartic acid
ATR	Attenuated total reflectance
ATRP	Atom transfer radical polymerization
C_p	Heat capacity
CEO	Chief executive officer
CLEAs	Cross-linked enzyme aggregates
Cys	Cysteine
δ	Chemical shift
DSC	Differential scanning calorimetry
DEPT	Distortionless enhancement by polarization transfer
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
E^o	Redox potential
E_{act}	Activation energy
EC	Enzyme Commission
EDTA	Ethylenediaminetetraacetic acid
ee	Enantiomeric excess
EPA	Environmental protection agency

EPT	Electron/proton transfer
ES	Enzyme-substrate complex
EtOH	Ethanol
EP	Enzyme-product complex
eq	Equivalents
ESI	Electrospray ionization
ESR	Electron spin resonance
ET	Electron transfer
EtOAc	Ethyl acetate
EWG	Electron-withdrawing group
FTIR	Fourier transform infrared
G	Gibbs free energy
GC-MS	Gas chromatography – mass spectrometry
GCI	Green Chemistry Institute
Gln	Glutamine
Glu	Glutamic acid
GPC	Gel permeation chromatography
h	Planck's constant
H	Enthalpy
HAT	Hydrogen atom transfer
His	Histidine
HIV	Human immunodeficiency virus
HBT	*N*-Hydroxybenzotriazole

HMBC	Heteronuclear multiple bond correlation
HOMO	Highest-occupied molecular orbital
HPI	*N*-Hydroxyphthalimide
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
IUBMB	International Union of Biochemistry and Molecular Biology
J	Coupling constant
K	Equilibrium constant
K_M	Michaelis constant
k	Rate constant
k_B	Boltzmann constant
κ	Transmission coefficient
LCHCs	Lignin-core hyperbranched copolymers
Leu	Leucine
LMS	Laccase-mediator system
LUMO	Lowest-unoccupied molecular orbital
Lys	Lysine
M_n	Number average molecular weight
M_w	Weight average molecular weight
Met	Methionine
MeOH	Methanol
mp	Melting point
MS	Mass spectrometry

MSD	Mass selective detector
MW	Molecular weight
$NAD(P)^+$	Nicotinamide adenine dinucleotide phosphate
NaOEt	Sodium ethoxide
NHA	*N*-Acetyl-*N*-phenylhydroxylamine
NHE	Normal hydrogen electrode
NHND	*Endo*-*N*-hydroxy-5-norbornene-2,3-dicarboximide
NI	Native intermediate
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
PDB	Protein data bank
PDI	Polydispersity index
PEGMA	Poly(ethylene glycol) methyl ether methacrylate
PEI	Polyethylenimine
Phe	Phenylalanine
PI	Peroxy intermediate
ppm	Parts per million
PSS	Polymer Standards Service
PTFE	Polytetrafluoroethylene
QM/MM	Quantum mechanical / molecular mechanical
R	Gas constant
RI	Refractive index
rt	Room temperature

S	Entropy
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
Ser	Serine
STY	Space time yield
T	Temperature
$t_{1/2}$	Reaction half-time
T_d	Decomposition temperature
T_g	Glass transition temperature
T_p	Peak derivative temperature
TEMPO	2,2,6,6-Tetramethyl-1-piperidinyloxy
TGA	Thermogravimetric analysis
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
TMDP	2-Chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane
TNC	Trinuclear cluster
Trp	Tryptophan
TS	Transition-state
TST	Transition-state theory
U	Enzyme unit
USA	United States of America
UV-Vis	Ultraviolet-visible
v	Reaction velocity or rate

$\tilde{\nu}$	Vibrational frequency
V_{max}	Maximum reaction velocity
ν	Frequency of crossing transition-state
v:v	Volume:volume
VLA	Violuric acid

SUMMARY

Enzyme catalyzed processes are rapidly becoming a viable means to accomplish chemical transformations in the field of synthetic chemistry. In an era where concern about the current and future state of the environment is at its peak, biocatalysts offer many advantages over conventional chemical catalysts, such as low toxicity, renewability, biodegradability, high selectivity, and high activity and stability in aqueous solvents at ambient temperature and neutral pH.

The current dissertation research focuses on one particular class of enzyme: laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2). These enzymes belong to the sub-class of enzymes known as multi-copper oxidases. Attributing to the fact that they oxidize a variety of electron-rich organic compounds while concomitantly catalyzing the four electron reduction of O_2 to $2H_2O$, they have received increasing use in recent decades as green catalytic oxidants within a variety of industries, including pulp and paper, bioremediation, textiles, food, cosmetics, and pharmaceuticals.

Within the field of organic synthesis, laccases have been employed to catalyze a multitude of radical-radical coupling, cross-coupling, and cascade reactions. Part one of this dissertation research has focused on harnessing the ability of laccases to generate reactive *para*-quinones in situ from the corresponding hydroquinones, followed by reaction with a variety of nucleophiles to perform novel carbon-carbon, carbon-nitrogen, and carbon-sulfur bond forming reactions for the synthesis of new and existing compounds. All reaction protocols are conducted in one-pot in an aqueous solvent system at ambient to slightly elevated temperatures and neutral to slightly acidic pH. As an

example of laccase-catalyzed carbon-carbon bond forming reactions, which is the topic of Chapter 4, the model compound benzoylacetonitrile was coupled with a variety of substituted hydroquinones via a laccase-catalyzed α-arylation reaction to afford benzylic nitriles. Focus was then geared toward using laccases to synthesize heterocyclic bis-sulfide compounds, such as 2,3-ethylenedithio-1,4-quinones, via coupling 1,2-ethanedithiol to hydroquinones in a laccase-catalyzed carbon-sulfur bond forming reaction, which is discussed in Chapter 5. Continuing on with laccase-catalyzed syntheses of heterocycles, Chapter 6 presents a comparative study for the synthesis of phenothiazones that examines different methods of coupling 2-aminothiophenol with both hydroquinones and *para*-quinones under laccase catalysis.

In part two of this dissertation, the fundamental laccase-catalyzed coupling chemistry developed in part one was applied to functionalize the surface of kraft lignin. In Chapter 7, it is demonstrated how a simple hydroquinone can be employed as a bridging reagent along with a trithiol that acts as a branching reagent to synthesize lignin-core hyperbranched polymers and polymer networks. This work provides a novel route for the synthesis of lignin-based biomaterials as well as a means for lignin valorization within the biorefinery.

The content in the final chapters of this dissertation will attempt to tie together the major findings of the research conducted, as well as provide guidance for future work in the field of laccase-catalyzed coupling reactions.

CHAPTER 1. INTRODUCTION

Sustainability, the ability of a process to continuously operate indefinitely without depleting natural resources, is the core concept influencing all aspects of modern society: sustainable manufacturing processes, sustainable living conditions, and sustainable business operations to name a few. In fact, in a recent study by the United Nations Global Compact, which is the largest global corporate sustainability initiative with over 8,000 member companies from 161 countries, 96% of CEOs surveyed believe that sustainability should be incorporated into a company's strategy and operations.[1] Thus, it is fair to say we are currently experiencing the birth of an era of sustainability.

The chemical and forest products industries have acknowledged and championed sustainability concerns and are at the forefront position to make a significant and lasting effect on environmental sustainability. An initial step in achieving this goal in the United States was the establishment of the Pollution Prevention Act of 1990, which identified reducing waste production at the source as a key strategy in alleviating the harmful effects of chemical waste on the environment.[2] Seemingly spurred on by the Act, a whole new field of science was energized: green chemistry. The concept, coined in 1991 by Paul Anastas, aims at promoting the design and development of chemical products and processes that reduce or eliminate the generation of hazardous materials.[3] Since its inauguration, numerous initiatives have evolved to promote an awareness of green chemistry and the environmental and economic benefits its implementation can provide to contemporary society.

Potentially the most pioneering work to date in the field of green chemistry was the establishment of the 12 Principles of Green Chemistry. Devised by Anastas and John Warner, these principles aim to guide chemists in designing safer and environmentally benign chemical products and processes.[4] Central to the 12 Principles is the idea of waste prevention, rather than waste treatment, as many of the principles are based on reducing or eliminating waste generation. This can be achieved by such strategies as using benign solvents or conducting solvent-free reactions, employing catalysts, eliminating the need for derivatization, and using and manufacturing biodegradable materials. The standard metric for assessing waste generation is the E Factor, which measures kg of waste produced per kg of desired product formed.[5] Thus, reducing the E Factor of chemical processes is of high priority. As an example, pharmaceutical giant Pfizer, who is a member of the ACS GCI Pharmaceutical Roundtable,[6] used the principles of green chemistry to reduce the E Factor for the synthesis of Lyrica, an anticonvulsant drug, from 86 to 9.[7] Not only does this have a beneficial impact on the environment, but it also yields economic benefits in the form of cost savings related to waste treatment, which provides any company with a competitive edge, especially in the fiercely competitive pharmaceutical industry. It is clear that green chemistry practices have been adopted – for example, in the US, hazardous waste generation has dropped from 278 million tonnes to 35 million tonnes in the past two decades.[7]

Catalysis is largely viewed as a “foundational pillar” of green chemistry.[8] By employing catalysts rather than stoichiometric reagents to perform chemical transformations, both the amount of waste generated and the amount of reagent required are significantly reduced. Thus, catalysis achieves both environmental end economic

benefits simultaneously. Biocatalysts, or enzymes, take the benefits of catalysis to new heights. These naturally derived catalysts are highly active and stable in aqueous solvents at ambient temperature and pressure, thus eliminating the need for reactions to be conducted in organic solvents or in the presence of auxiliary energy requirements. They are also highly selective, thus reducing the formation of unwanted side products. And given that they are found in biological systems, biocatalysts are relatively inexpensive, renewable, nontoxic, and biodegradable. Attributing to revolutionary advances in the field of protein engineering,[9] the use of biocatalysts within various sectors of the chemical industry is at its highest.[10-12] One such class of biocatalysts that have received much use over the years in a variety of industrial applications are laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2).[13] Owing to their environmentally benign nature, in that they require O_2 as their only co-substrate and produce H_2O as the sole by-product, they have found much use as green catalytic oxidants in the field of synthetic chemistry.[14-15]

Guided by the 12 Principles of Green Chemistry, chemists hold the ability to create a sustainable industry and make a difference. The ability to harness the power of nature is key to providing a sustainable society whilst also providing significant economic savings. The current dissertation aims to exploit the oxidizing capabilities of laccases for the development and implementation of sustainable chemistry. The objectives of the current research have been divided into two parts. Part one deals with the applications of laccases in organic synthesis, with the specific goals:

- to provide a green solution in organic synthesis;
- to develop fundamental laccase-catalyzed coupling chemistry;

- to catalyze novel carbon-carbon, carbon-nitrogen, and carbon-sulfur bond forming reactions;
- to synthesize new and existing compounds under environmentally benign conditions (i.e. aqueous solvent, ambient to slightly elevated temperatures, and neutral to slightly acidic pH).

Part two of the research deals with the laccase-assisted functionalization of lignin, with the specific objectives:

- to apply the fundamental laccase-catalyzed coupling chemistry to graft small molecules onto the surface of lignin;
- to develop novel lignin-based biomaterials / lignin-core hyperbranched copolymers;
- to establish an alternative route for lignin valorization within the biorefinery.

CHAPTER 2. LITERATURE REVIEW[I]

2.1 Green Chemistry

Modern society is becoming increasingly aware of the negative environmental impact posed by the chemical industry. With this increased awareness, the field of green chemistry has been steadily gaining momentum within the greater chemical industry as chemists and chemical engineers alike strive to alleviate the harm that has been inflicted on the environment and reduce any further damage that may be incurred in the future. Although green chemistry processes have been in place for centuries, the term "green chemistry" was only recently coined, in 1991 by then US EPA chemist Paul Anastas, who defined it as "the design, development, and implementation of chemical products and processes to reduce or eliminate the use and generation of substances hazardous to human health and the environment."[3]

2.1.1 The 12 Principles of Green Chemistry

Perhaps the most pioneering work to date in the field of green chemistry was the establishment of the 12 Principles of Green Chemistry. Devised by Anastas and John Warner, these principles aim to guide chemists in designing safer and environmentally

[I] A portion of the content written in this chapter originated from published manuscripts. The manuscript titled "Two decades of laccases: advancing sustainability in the chemical industry" was published in *The Chemical Record* (**2017**, *17(1)*, 122-140) and was reproduced with permission from John Wiley and Sons. The manuscript titled "Conversion of lignin into value-added materials and chemicals via laccase-assisted copolymerization" was published in *Applied Microbiology and Biotechnology* (**2016**, *100(20)*, 8685-8691) and was reproduced with permission from Springer. The other author in both manuscripts is Arthur J. Ragauskas, who is affiliated with Georgia Institute of Technology. The copyright license agreements are provided in Appendix B.

benign chemical products and processes.[4, 16] The 12 Principles of Green Chemistry are stated below and are summarized in Figure 1.

1. It is better to prevent waste than to treat or clean up waste after it is formed.
2. Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product.
3. Wherever practicable, synthetic methodologies should be designed to use and generate substances that possess little or no toxicity to human health and the environment.
4. Chemical products should be designed to preserve efficacy of function while reducing toxicity.
5. The use of auxiliary substances (e.g. solvents, separation agents, etc.) should be made unnecessary wherever possible and innocuous when used.
6. Energy requirements should be recognized for their environmental and economic impacts and should be minimized. Synthetic methods should be conducted at ambient temperature and pressure.
7. A raw material of feedstock should be renewable rather than depleting wherever technically and economically practicable.
8. Unnecessary derivatization (blocking group, protection/deprotection, temporary modification of physical/chemical processes) should be avoided whenever possible.
9. Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.

10. Chemical products should be designed so that at the end of their function they do not persist in the environment and breakdown into innocuous degradation products.
11. Analytical methodologies need to be further developed to allow for real-time, in –process monitoring and control prior to the formation of hazardous substances.
12. Substances and the form of a substance used in a chemical process should be chosen so as to minimize the potential for chemical accidents, including releases, explosions, and fires.

Figure 1. The 12 Principles of Green Chemistry Pocket Guide.[17]

While every attempt was made to abide by all the 12 Principles of Green Chemistry in this dissertation research, some of them were not applicable. Thus, the principles that have been addressed in the current work are: 1. Prevent waste; 2. Atom economy; 3. Less hazardous synthesis; 5. Benign solvents and auxiliaries; 7. Use of

renewable feedstocks; 9. Catalysis; and 12. Inherently benign chemistry for accident prevention.

2.1.2 Green Chemistry Metrics

It is not sufficient enough to simply state that a reaction process or synthetic protocol is green. The greenness of a reaction must be quantified by using metrics such as the E factor, atom economy, and space time yield, and if catalysts are involved, both the turnover number and turnover frequency should also be determined, if possible. The E Factor, a concept first published by Roger Sheldon in 1992,[18] is the mass ratio of waste produced to desired product (Equation 1) and is a useful tool to assess waste management

$$E\ Factor = \frac{Mass\ of\ waste}{Mass\ of\ desired\ product} = \frac{\Sigma\ Mass\ of\ reactants - Mass\ of\ desired\ product}{Mass\ of\ desired\ product} \quad (1)$$

in chemical processes. Typical E Factors within different areas of the chemical industry are provided in Table 1.[19] The atom economy is commonly used as a quantitative measure of the efficiency of the synthetic process and is defined as the percentage ratio of the incorporation of reactant atoms into the desired product (Equation 2).[20] The space

$$Atom\ Economy = \frac{MW\ of\ desired\ product}{\Sigma\ MW\ of\ reactants} \times 100\% \quad (2)$$

time yield (STY) of a synthetic process quantifies the amount of desired product formed per unit volume and time, calculated using Equation 3, thus can have units such as $\frac{mol}{L \cdot h}$.

$$STY = \frac{Amount\ of\ desired\ product}{Volume \times Time} \quad (3)$$

Although more applicable to industrial scale synthetic processes, the STY is also useful in assessing production efficiency of lab scale synthetic processes. For synthetic processes involving catalysts, which comprise many transformations nowadays, the

turnover number, calculated as the molar ratio of substrate consumed to amount of catalyst used (Equation 4), and the turnover frequency, simply the turnover number divided by time (Equation 5), are informative quantities used to assess the efficiency of the employed catalyst.

$$Turnover\ Number = \frac{mol\ of\ substrate\ consumed}{mol\ of\ catalyst\ used} \quad (4)$$

$$Turnover\ Frequency = \frac{Turnover\ Number}{Time} \quad (5)$$

Table 1. Typical E Factors within different areas of the chemical industry.[19]

Industry	Product Mass (tons)	E Factor
Oil refining	10^6-10^8	<0.1
Bulk chemicals	10^4-10^6	<1-5
Fine chemicals	10^2-10^4	5-50
Pharmaceuticals	10-10^3	25-100

2.1.3 Adoption of Green Chemistry within the Chemical Industry

Since the establishment of the 12 Principles of Green Chemistry, a concerted global effort to adhere to the principles and unite in developing a more sustainable society is evident. In the United States, such initiatives as the US Green Chemistry Program and the American Chemical Society Green Chemistry Institute (ACS GCI), as well as the US Presidential Green Chemistry Challenge Award, have dramatically increased the adoption of green chemistry practices into existing operations.[16] Green chemistry has also received a warming welcome across the globe, with over 20 countries, including powerhouse nations such as Japan, the United Kingdom, and Australia, also participating in the movement, establishing their own green chemistry programs and initiatives..[16]

2.2 Chemistry in Water

Syntheses in the field of organic chemistry have classically been performed in organic solvents, such as toluene, dichloromethane, and acetonitrile to name a few. These traditional organic solvents are far from ideal when discussing the topic of a green and sustainable chemical industry as they are not renewable or biodegradable, costly to dispose of, usually flammable, potentially explosive, and toxic to both humans and the environment. Water on the other hand is abundant, inexpensive, safe to use, recyclable, and completely benign to both humans and the environment, which are some key reasons why synthetic chemists are focusing their attention more and more toward using water as a solvent in organic synthesis.[21] The types of reactions that can occur in water are vast, ranging from simple nucleophilic substitutions to complex pericyclic reactions, and the products that can be achieved have extensive diversity and functionality. After all, all biological reactions that occur in nature and all complex molecules that are synthesized within humans and other organisms occur in an aqueous medium. In addition, new types of reactions have been discovered in which water is a vital component. It is believed that water acts as a catalyst in certain situations, lowering the activation energies of bond cleavage and formation, allowing for reactions to occur that would otherwise not take place in organic solvents.[21]

A core principle of synthetic chemistry is a battle of competing rates. For example, in nucleophilic addition reactions, the nucleophile must attack the electrophile at a faster rate than say the rate of decomposition of that electrophile if the nucleophilic addition reaction is to succeed. Thus, accelerating the rate of a desired reaction to the point in which it is kinetically favorable is of enormous importance for high yielding

processes. Conducting organic synthesis reactions employing water as a solvent has shown to increase the rates of particular reactions immensely (by a factor of 79,300 for a particular Diels-Alder reaction),[22] even to the point of catalyzing reactions that would otherwise not occur in organic solvents. Furthermore, the use of water as a solvent can greatly influence the stereoselectivity of transformations, providing >99% enantiomeric excess (ee) in many instances.

2.2.1 The Michael Addition Reaction

Conjugate 1,4-additions (also known as Michael additions) involve the nucleophilic addition of carbanions and other suitable nucleophiles to the *β*-position of *α*,*β*-unsaturated compounds, such as *α*,*β*-unsaturated ketones, aldehydes, esters, and nitriles (Figure 2). Substantial developmental research in conjugate 1,4-additions was

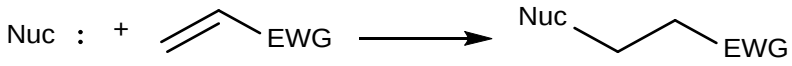


Figure 2. The Michael addition.

conducted by the American chemist Arthur Michael in the 1880's, for who the reaction is named after.[23] Breakthrough work by Michael was conducted in 1887 when he reacted diethylmalonate and ethyl cinnamate in ethanol under basic conditions to yield the corresponding 1,4-addition product (Figure 3).[24] In contemporary organic synthesis, the Michael addition is one of the most important tools for achieving carbon-carbon, carbon-nitrogen (aza-Michael addition), and carbon-sulfur (thiol-Michael addition) bond formations and is the key bond forming reaction that has been used in the current work.

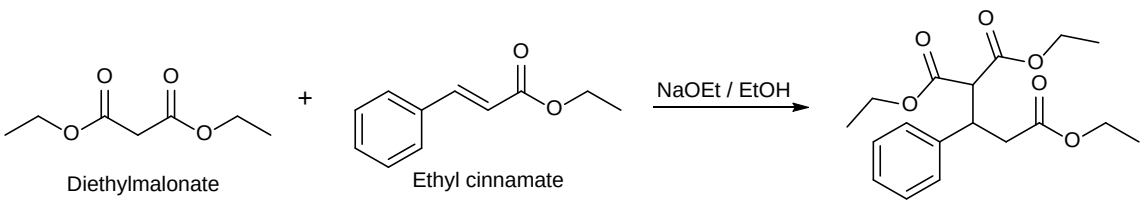


Figure 3. Michael addition of diethylmalonate with ethyl cinnamate.[24]

There are multiple positions where nucleophilic addition can take place on *α*,*β*-unsaturated compounds: the *α*-carbon, the *β*-carbon, the carbonyl carbon for carbonyl compounds, and the nitrile carbon in nitrile compounds. Generally speaking, the *β*-carbon is more electrophilic than the *α*-carbon, as it participates in resonance stabilization of the partial positive charge on the carbonyl carbon in *α*,*β*-unsaturated carbonyls,[25] resulting in an increased likelihood of nucleophilic attack. Furthermore, the *β*-carbon is "softer" than the *α*-carbon, rendering water, a "hard" nucleophile, a suitable medium to conduct the Michael addition reaction because water does not compete or interfere with other "softer" nucleophiles adding to the *β*-carbon of *α*,*β*-unsaturated compounds.[21] The following section will discuss the primary forces impacting the reaction kinetics at both the ground states and transition states of reactions conducted using water as a solvent, paying particular attention to Michael addition reactions where appropriate.

2.2.2 Induced Effects of Water on Chemical Transformations

When discussing the use of water a as solvent in organic synthesis, it is important to distinguish between three broad classes of reaction phenomena that can occur, simultaneously in many cases: 1) reactions that occur "in water", in which the reactants are soluble in water; 2) reactions that occur within hydrophobic cavities within water,

usually between insoluble reactants; and 3) reactions that occur "on water", or more strictly speaking at the water-oil interface, in which the reactants are insoluble in water.[26] The following sections will discuss the types of effects that are present in each phenomenon.

2.2.2.1 Hydrogen-Bonding Effects – Transformations "In Water"

The main phenomena responsible for rate enhancements for reactions conducted in water in which reactants are soluble are hydrogen-bonding and polarity effects. If one or multiple of the reactants possess hydrogen-bond donor or acceptor sites, then those reactants are able to participate in hydrogen-bonding interactions with water, resulting in stabilization/lowering in energy of that compound's molecular orbitals. The origin of this energy lowering is due to less electron density and interorbital repulsion among the impacted molecular orbitals.[26] Depending on which molecular orbitals are taking part in the bond forming reaction, the hydrogen-bonding induced lowering in energy of molecular orbitals may increase or decrease the rate of the reaction. For example, in the nucleophilic addition reaction of a general nucleophile with a general electrophile depicted in Figure 4, which involves the highest-occupied molecular orbital (HOMO) of the nucleophile interacting with the lowest-unoccupied molecular orbital (LUMO) of the electrophile,[27] hydrogen-bonding interactions between water and the nucleophile will lower the frontier molecular orbital energies of the nucleophile, increasing the $HOMO_{Nuc}$ – $LUMO_{Elec}$ gap, resulting in a larger activation energy of reaction (E_{act}) and consequently decreasing the reaction rate. On the other hand, if the electrophile forms hydrogen-bonds with water, the frontier molecular orbital energies of the electrophile will

be stabilized and the $HOMO_{Nuc} - LUMO_{Elec}$ gap will be narrowed, resulting in a smaller E_{act} and a subsequently increased rate of reaction.

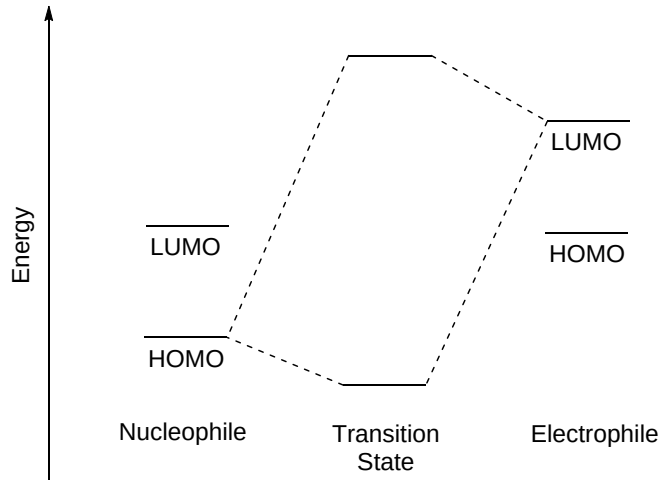


Figure 4. Molecular orbital depiction of nucleophilic addition reaction.

To assess the effect hydrogen-bonding interactions have on transition state stabilization and rates of certain chemical transformations in water, many studies have focused on pericyclic reactions such as the 4π + 2π Diels-Alder cycloaddition and 1,3-dipolar cycloadditions, as well as the Claisen rearrangement. For example, quantum mechanical/molecular mechanical (QM/MM) computations have been employed to determine the contribution of the hydrogen-bonding effect in stabilizing the transition state for the Diels-Alder cycloaddition reaction of cyclopentadiene with methyl vinyl ketone (a hydrogen-bond acceptor) in water compared to the gas phase (Figure 5). The computations unveiled a significant stabilization of the transition state (-3.5 kcal/mol) for the cycloaddition reaction conducted in water, due in part to hydrogen-bonding interactions.[28] A more recent study utilizing QM/MM computations also demonstrated

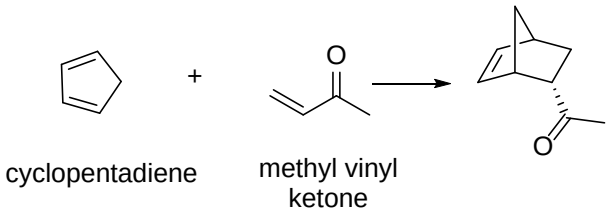


Figure 5. Diels-Alder cycloaddition of cyclopentadiene with methyl vinyl ketone.[28]

that the transition state energy for the cycloaddition of cyclopentadiene with methyl vinyl ketone increases in going from water, to acetonitrile, to methanol as a solvent, further validating the stabilizing effects of hydrogen-bonding interactions.[29] Experimental data for the Claisen rearrangement of a water soluble allyl vinyl ether derivative in a variety of solvents, shown in Figure 6, revealed that the rate of the rearrangement is 214 times faster in water than in cyclohexane.[30]

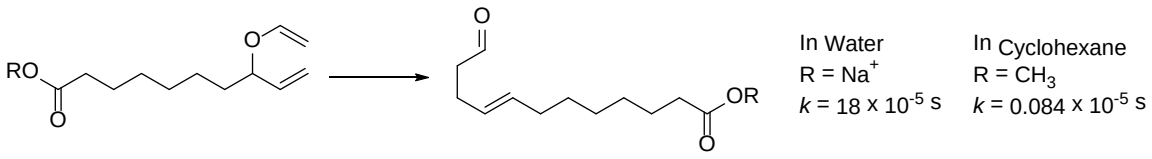


Figure 6. Claisen rearrangement of an allyl vinyl ether.[30]

It has also been postulated that the hydrogen-bonding effect is not just localized to the hydrogen-bonds between water and reactants (i.e. the primary water-solvation shell), but also extends to secondary bridging hydrogen-bonding of structured water clusters in the vicinity. Butler and co-workers compared the 1,3-dipolar cycloaddition reaction of pyridazinium-dicyanomethanide 1,3-dipole with various dipolarophiles in water and in organic solvents, such as acetonitrile, acetone, methanol, ethanol, and *tert*-butyl alcohol (Figure 7). They noted that by increasing the water content in water-organic solvent mixtures, the rate of the cycloaddition reaction increases dramatically. Theoretical

calculations unveiled an extended hydrogen-bonding effect: E_{act} for the cycloaddition reactions were reduced when a four-water cluster was bound to the carbonyl group of the dipolarophile compared to when only one water molecule was bound. Furthermore, adding an acetonitrile molecule that is able to interact with the dipolarophile carbonyl group to the complex containing the four-water cluster indeed raises the E_{act} once again.[31]

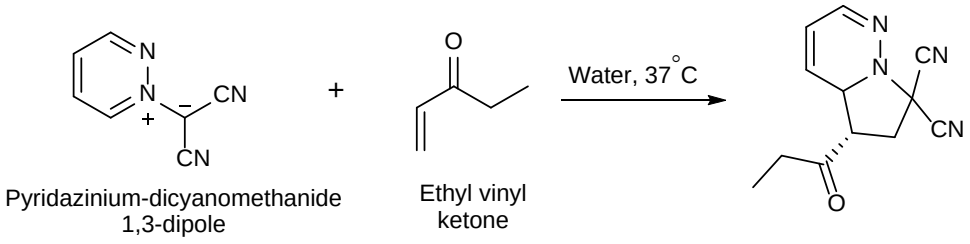


Figure 7. 1,3-Dipolar cycloaddition reaction of pyridazinium-dicyanomethanide 1,3-dipole with ethyl vinyl ketone.[31]

Regarding the Michael addition reaction in water, the discovery chemistry team at the Bristol-Myers Squibb Pharmaceutical Research Institute in Wallingford, CT, USA, observed significantly enhanced rates in water compared to methanol for the Michael addition of amines and thiols to the α,β-unsaturated carbonyl moiety of dehydroalanine amides (Figure 8).[32] The reaction did not proceed at all in DMF or THF, however, when one equivalent of water was added, the reaction occurred, albeit, at a slower rate than in pure water. The authors proposed a hydrogen-bonding stabilization of the transition state for the increased reaction rate. In a follow up study by the same research group, nocathiacins, a class of cyclic thiazolyl peptide antibiotics containing the dehydroalanine amide side chain, were used as the acceptor molecule for the Michael addition of thiols and amines in an effort to introduce functionality and increase the water solubility of the

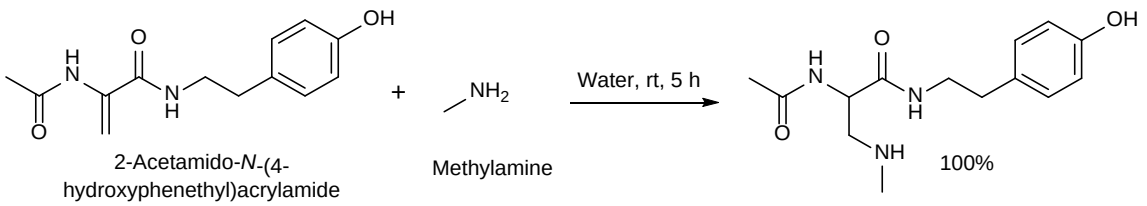


Figure 8. Michael addition of methylamine to a dehydroalanine amide in water.[32]

antibiotics.[33] Mild reaction conditions are necessary to maintain structural integrity of nocathiacins; furthermore, the use of water as a solvent enhances the selectivity of the addition, which can be further enhanced by lowering the reaction temperature, even to as low as -20°C, so that the reaction is essentially conducted in frozen water (i.e. ice). More recently, the hydrogen-bonding enhanced rate acceleration was observed in the Michael addition of 1,3-dicarbonyl compounds with *α,β*-unsaturated nitro compounds in brine using cinchona-based organocatalysts (Figure 9).[34] The stereoselectivity of the carbon-carbon bond-forming reaction was also enhanced in water. Evidence for hydrogen-bonding enhanced rate acceleration is inferred from the reduced rate of addition when the reaction is conducted in D_2O.

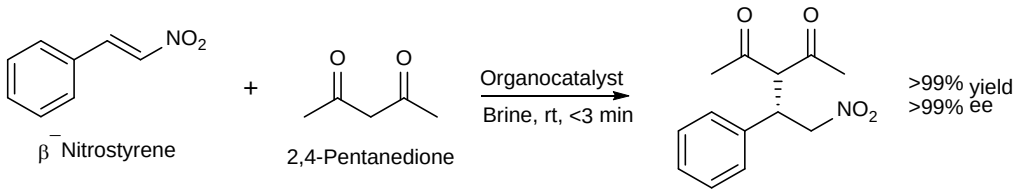


Figure 9. Michael addition of 2,4-pentanedione with *β*-nitrostyrene.[34]

2.2.2.2 <u>The Hydrophobic Effect – Transformations within Hydrophobic Cavities in Water</u>

Although widely considered an "in water" effect, I believe that the hydrophobic effect is deserving of its own category because the transformations that it promotes do not occur in solution, rather the transformations take place in hydrophobic cavities within the solution. Generally speaking, the hydrophobic effect is the forced aggregation of nonpolar substances within an aqueous medium to form nonpolar aggregates and micelles. It is of extreme importance in biological systems, dictating the folding of proteins and the formation of membranes.[21] The effect is also of paramount significance in synthetic reactions taking place in an aqueous medium, as the forced aggregation of nonpolar compounds leads to a greater frequency of physical collisions between molecules and consequently enhanced rates of reaction. Furthermore, the forced aggregation heightens the ground state energy of the reactants without affecting the transition state energy significantly, thus lowering E_{act}, also resulting in an increased reaction rate.[35-36]

The hydrophobic effect derives its origins from thermodynamics. The solvation of a nonpolar compound by water requires an ordering of water molecules on the surface of the nonpolar compound, resulting in a decrease in entropy, *S*, and a corresponding increase in the Gibbs free energy, *G*, of the system, based on Equation 6. When nonpolar

$$\Delta G = \Delta H - T\Delta S \qquad (6)$$

compounds aggregate, the surface area exposed to water is reduced, which releases water molecules allowing them to diffuse freely, resulting in an increase in entropy and a corresponding decrease in Gibbs free energy of the system (Figure 10).[21] Additionally, a common misconception about the cause of the hydrophobic effect is that it is due to the attraction of nonpolar compounds to each other in aqueous media; however, this is not

accurate. In actuality, it is the extremely large cohesive energy density of water (550.2 cal/cm^3 - the largest cohesive energy density of all known solvents)[37] that causes water molecules to attract each other, thus essentially forcing the nonpolar compounds into close proximity with each other.[21]

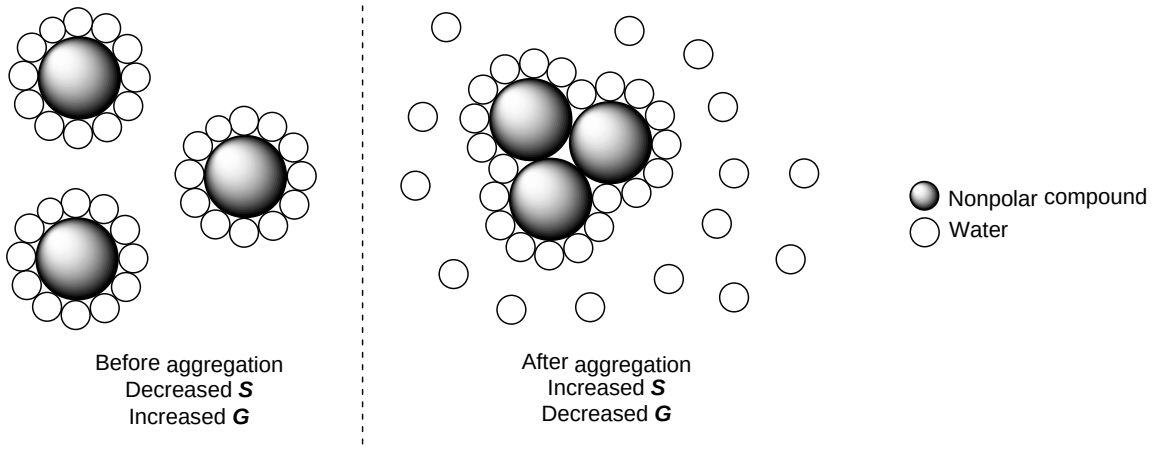
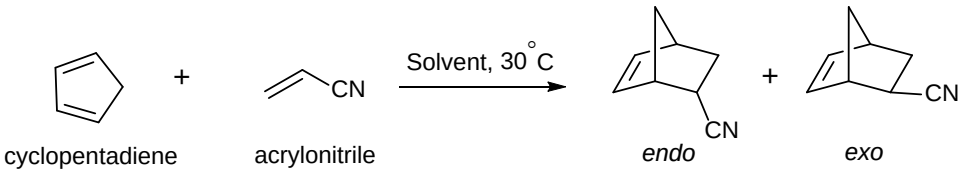


Figure 10. Thermodynamic origin of the hydrophobic effect.[21]

The use of salts has been applied extensively to probe the hydrophobic effect in aqueous solutions. Depending on the salt employed, it can either increase the internal pressure of the solution, forcing organic compounds out of solution and to aggregate (i.e. salting-out), or alternatively, the salt may decrease the internal pressure of the solution, allowing more of the organic compound into the solution phase (i.e. salting-in). Generally speaking though, the vast majority of salts increase the internal pressure of the solution due to a decrease in volume caused by the solvation of the salt ions by water molecules – a phenomenon known as electrostriction.[21] Thus, the increase in internal pressure and augmentation of the hydrophobic effect lead to an increase in reaction rates for nonpolar reactants in aqueous media. The increase in pressure also has an effect on the

stereochemistry of specific reactions, such as the Diels-Alder cycloaddition, typically yielding the *endo*-stereoisomer product rather than the *exo*-stereoisomer due to its more compact structure.[38-39]

Ronald Breslow has studied the impact of the hydrophobic effect on the Diels-Alder reaction in water extensively, his work providing much insight for synthetic chemists.[40] In Breslow and Darryl Rideout's pioneering work published in 1980, clear evidence for the hydrophobic effect rate enhancement was observed for the Diels-Alder reaction of cyclopentadiene with either methyl vinyl ketone or acrylonitrile (Figure 11).[41] Initially, reactions were carried out in water with *β*-cyclodextrin, based on the hypothesis that *β*-cyclodextrin could provide a hydrophobic cavity for the nonpolar reactants to aggregate and undergo a cycloaddition with one another. Indeed, the cycloaddition reactions proceed at rates 2-3 orders of magnitude greater than when the reactions were performed in the nonpolar organic solvent isooctane. The reactions conducted in pure water also showed a significant rate enhancement compared to when



Solvent	***k* x 10^5 $M^{-1}s^{-1}$**
Isooctane	1.9
MeOH	4.0
H_2O	59.3
H_2O (10mM β-cyclodextrin)	537.0

Figure 11. Diels-Alder cycloaddition of cyclopentadiene with acrylonitrile in different solvents.[41]

isooctane was used, although not to the extent as the reactions carried out with added *β*-cyclodextrin. Furthermore, to eliminate the perception of any polarity or hydrogen-bonding induced rate enhancements, the reactions were also conducted in methanol for comparison, with the polar organic solvent also providing inferior rates compared to water. Thus it became clear that the increased reaction rates were due to the hydrophobic effect. Evidence for the roles salts play in the hydrophobic effect were also uncovered: adding the salting-out reagent LiCl more than doubled the reaction rate for the Diels-Alder reaction of cyclopentadiene with methyl vinyl ketone compared to pure water, while adding the salting-in reagent guanidinium chloride slightly reduced the rate compared to pure water.[41]

The hydrophobic effect has also proven to be responsible for rate accelerations of particular Michael addition reactions conducted in aqueous media. A study by Lubineau and Augé in 1992 on the Michael addition reaction of nitromethane and nitroethane with methyl vinyl ketone uncovered significant rate enhancements when the reactions were performed in water compared to CH_2Cl_2, THF, or toluene, as well as polar organic solvents, such as methanol and DMSO (Figure 12).[42] Additionally, the reaction rates were increased even further when aqueous sugar solutions were used as solvent. The authors proposed that the increased rates were due to a negative volume of activation and increased internal pressure brought about by the hydrophobic effect. Similar reactions utilizing primary and secondary nitroalkanes as the Michael donors and *α,β*-unsaturated nitriles, esters, and ketones as the Michael acceptors have afforded the Michael adducts in high yields in short reaction times using water as a solvent.[43-44] In another study, the

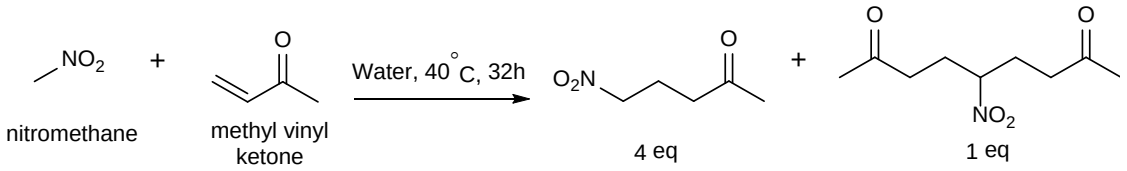


Figure 12. Michael addition of nitromethane with methyl vinyl ketone in water.[42]

rate enhancement for the Michael addition of primary and secondary amines with *α,β*-unsaturated nitriles in water compared to acetonitrile has been attributed to the increased internal pressure brought on by the hydrophobic effect and has been likened to similar rate enhancements observed when an applied external pressure is present.[45] Evidence for the hydrophobic effect has also been observed for the organocatalyzed Michael addition of 2,4-pentanedione with *β*-nitrostyrene (Figure 9). The reaction conducted in brine, which induces a salting-out effect, requires less than 10 minutes to achieve a 99% conversion, whereas when the reaction medium is composed of the salting-out agent $LiClO_4$ dissolved in pure water, less than 2% conversion is reached in 30 minutes.[34] Not only has the water induced hydrophobic effect proved to increase the rate of the Michael addition reaction, but it has also shown to improve the enantioselectivity of addition as well. In a recent study, the Michael addition of acetylacetone with cyclohex-2-enone in water using a ytterbium(III) triflate complex with an *α*-amino acid ligand as the Lewis acid catalyst (Figure 13) discovered that the stereoselectivity of addition can be controlled by adjusting the temperature of the reaction. The authors propose that water induces a large entropic factor, which has been termed *stereospecific aqueous solvation*, that can be used to tailor the enantioselectivity of addition.[46]

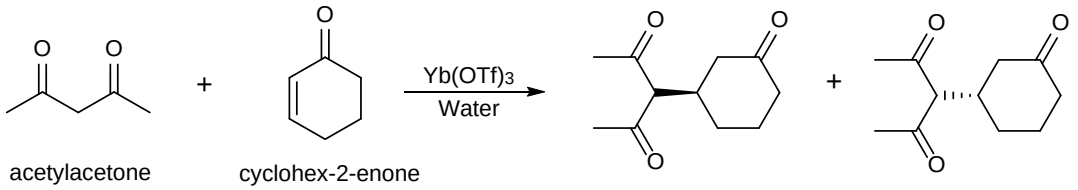
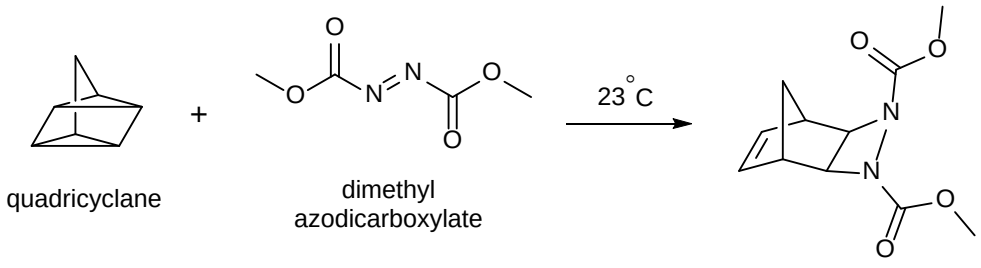


Figure 13. Michael addition of acetylacetone with cyclohex-2-enone in water catalyzed by a ytterbium(III) triflate Lewis acid catalyst.[46]

2.2.2.3 Trans-Phase Hydrogen-Bonding – Transformations "On Water"

A third important effect in which water can influence chemical reactions is through trans-phase hydrogen-bonding effects. Although lesser known than "in water" hydrogen-bonding effects and hydrophobic effects, the more recently considered trans-phase hydrogen-bonding effects can be used to explain the rate enhancements and stereoselectivities obtained for reactions performed using water as a solvent when the reactants are insoluble in water.

Sharpless and colleagues were the first to notice significant enhancements in reaction rates for systems in which the organic reactants are insoluble in water, so that the reactions take place in a heterogeneous aqueous suspension, hence the term "on water."[47] In their initial studies in 2005, the researchers noticed an immense increase in reaction rate for the $2\sigma + 2\sigma + 2\pi$ cycloaddition of the strained hydrocarbon quadricyclane and dimethyl azodicarboxylate, shown in Figure 14, when the reaction was conducted in water compared to when it was performed in organic solvents such as toluene, acetonitrile, or methanol, or when the reaction was performed under neat conditions (i.e. solvent-free). The reaction conducted using water as a solvent was completed in only 10 minutes, affording the 1,2-diazetidine in 82% yield. Thus, it was



Solvent	Time to completion
Toluene	>120 h
Acetonitrile	84 h
MeOH	18 h
Neat (solvent-free)	48 h
H_2O	10 min

Figure 14. Cycloaddition of quadricyclane with dimethyl azodicarboxylate in a variety of solvents.[47]

postulated that the heterogeneity of the reaction system, in which the organic reactants are dispersed as oil droplets or solid suspensions within the aqueous phase, was critical for the superior reaction rates. Furthermore, based on the observation that the cycloaddition reaction proceeded slowly in perfluorohexane (a solvent which the reactants were also insoluble in) and less accelerated in D_2O, the criterion of heterogeneity as a sole factor contributing to the enhanced rates was inadequate, thus the researchers proposed a second criterion that water must also be present. It was also realized that vigorous stirring of the reaction mixture was key, as it promoted the formation of finer organic particles/droplets and consequently increased the interfacial surface area of the organic phase with the aqueous phase. Other transformations that experienced similar enhancements in reaction rates in an aqueous suspension included the Diels-Alder cycloaddition, the Claisen rearrangement, and nucleophilic opening of an epoxide. While the authors proposed plausible reasons as to the origins of the rate enhancements, they conceded that the exact mechanism by which the rates of the

reactions were increased was still unclear and that further research was required to identify the governing cause. It had been proposed that because the transition state complexes of the reactions performed by Sharpless and coworkers were probably more efficient at participating in hydrogen-bonding interactions than the ground states were, leading to a decreased E_{act} and increased reaction rate, the primary hydration layer at the organic-aqueous interface may provide vital information.[48]

About a decade earlier, Du, Freysz, and Shen studied the interfacial structure of water at the organic-aqueous boundary via vibrational spectroscopy.[49] They discovered a sharp peak at 3680 cm^{-1} in the infrared spectrum, attributing the signal to free hydroxyl groups at the organic-aqueous interface. At the organic-aqueous interface, water molecules will arrange themselves into a network that minimizes the surface free energy by maximizing the total number of hydrogen bonds for each water molecule. The study uncovered that such an arrangement involves one in every four water molecules having an unbound hydroxyl group, which penetrates into the organic phase (Figure 15). In 2007, Jung and Marcus applied this knowledge in an attempt to explain the "on water" rate improvements experienced by Sharpless and colleagues a couple years earlier.[50] In their publication, calculated data from kinetic models for the reaction in Figure 14 conducted "on water" and under solvent-free conditions were compared to the experimental data. The theoretical calculations provide a 3×10^5-fold rate enhancement for the reaction carried out "on water" as opposed to solvent-free conditions, which is in good agreement with the experimentally derived value of 1.5×10^5. Transition state theory computations yielded a lowering of E_{act} by approximately 7 kcal/mol for the "on water" reaction compared to the neat reaction. The authors concluded that the underlying

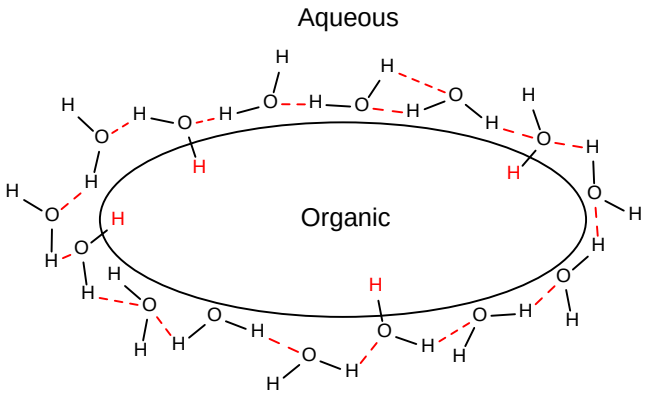
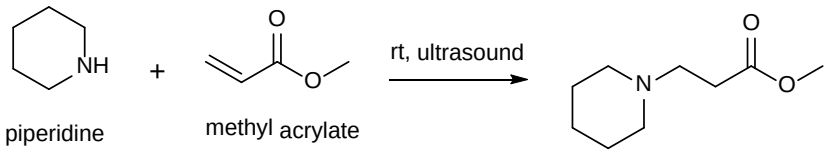


Figure 15. Trans-phase hydrogen-bonding at the organic-aqueous interface.[49-50]

mechanism for the reduction in E_{act} and hence remarkable rate enhancements experienced by Sharpless and colleagues in their "on water" reactions was due to hydrogen-bonding interactions between the transition state complex and the interfacial hydroxyl groups of water. Thus, the trans-phase hydroxyl groups essentially provide a source of catalytic protons.

"On water" rate enhancements have been observed for several Michael addition protocols in recent years. Keller and Feringa have employed a ytterbium(III) triflate catalyst to promote the Michael addition of *β*-keto esters and *α*-nitroesters with *α,β*-unsaturated ketones and aldehydes using water as a solvent.[51-52] They noticed that the reaction mixture need not be homogeneous for the reaction to occur, and that reaction mixtures in which one or both of the reactants were suspended in water proceeded at a faster rate. Little or no reaction took place in organic solvents, such as THF, CH_2Cl_2, and dioxane. Exceptional rate enhancements have been achieved under ultrasound conditions for the aza-Michael addition of a variety of amines with *α,β*-unsaturated ketones, esters, and nitriles in water compared to several common organic solvents as well as solvent-free

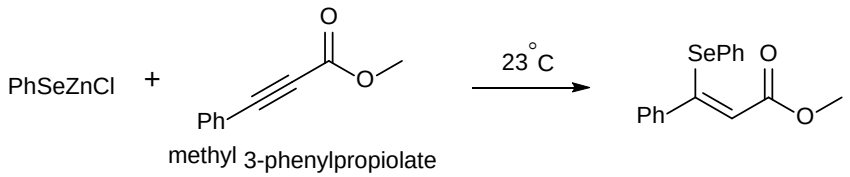
conditions.[53] The reaction of piperidine with methyl acrylate afforded the Michael adduct in 98% yield in only 5 minutes under ultrasound compared to 93% yield in 15 minutes for solvent-free conditions and 45% yield in one hour for the reaction performed in THF (Figure 16). It can be thought that the use of ultrasound disperses the organic reactants within the aqueous medium so as to increase the interfacial surface area between the aqueous and organic phases, thus increasing the trans-phase hydrogen-



Solvent	Time	Yield
THF	60 mins	45%
Neat (solvent-free)	15 mins	93%
H_2O	5 mins	98%

Figure 16. Comparison of the Michael addition reaction of piperidine with methyl acrylate in THF, neat, and water.[53]

bonding effect. In a rare seleno-Michael addition reaction, a phenyl substituted zinc selenolate was used as the Michael donor to react with carbonyl conjugated alkynes in water or THF (Figure 17).[54] Due to the insolubility of one or both reactants in water, the reactions conducted using water as a solvent were in the form of an aqueous suspension. Generally, the reactions performed “on water” achieved almost quantitative yields of the vinylic selenides within 2 hours compared to the reactions performed in THF, which took 24 hours to reach comparable yields. Furthermore, stereoselectivity of addition was enhanced "on water" with products consistently favoring the *Z* isomer.



methyl 3-phenylpropiolate

Solvent	Time	Yield	*Z/E* Ratio
THF	24 h	35%	100:0
H_2O	2 h	99%	100:0

Figure 17. Seleno-Michael addition of a phenyl substituted zinc selenolate with methyl 3-phenylpropiolate.[54]

2.2.3 Oxidation of Phenols and Hydroquinones in Water for the Synthesis of para-Quinones

Phenol and quinone chemistries are ubiquitous in nature. Phenols are abundant in plant materials and many natural products and serve as a source of powerful, naturally derived antioxidants, such as the flavonoids resveratrol and catechin (Figure 18), the former exhibiting strong cancer chemopreventive activity.[55] Quinones too are found all throughout nature in plants, bacteria, fungi, arthropods, and echinoderms,[56] with perhaps the most well-known and important *para*-quinone being coenzyme Q (Figure 18), which plays a key role in oxidative phosphorylation and in the electron-transport chain within the mitochondrial membrane, and is thus vital for human and animal life.[57] Quinones are also present in many biologically active compounds,[58] and owing to their intense colors, are widely used as organic dyes and pigments.[59]

The relationship between phenols and quinones is governed by redox chemistry. For example, a phenol such as hydroquinone, can be converted to *para*-benzoquinone via the loss of two electrons and two protons, a process known as oxidation, while the reverse is also possible, the reduction of *para*-benzoquinone to hydroquinone via the

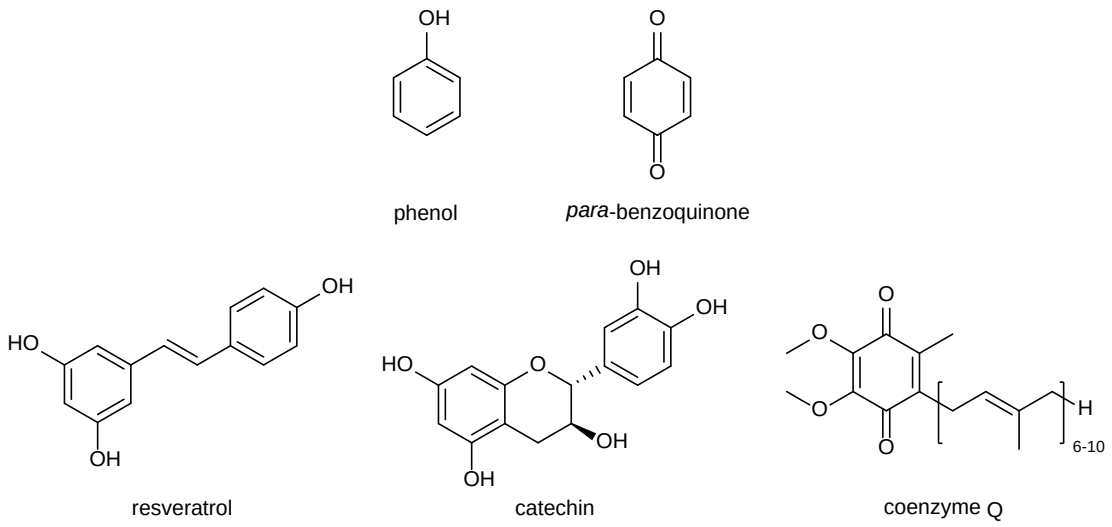


Figure 18. Basic structure of a phenol and a *para*-benzoquinone, as well as more complex phenols resveratrol and catechin, and the *para*-benzoquinone coenzyme Q.[55, 57]

acquisition of two electrons and two protons (Figure 19). By and large, the majority of strategies used to convert phenols into *para*-benzoquinones involve stoichiometric oxidants or transition-metal catalysts, and are conducted in organic solvents, such as toluene and acetonitrile.[58] This is not ideal from a green chemistry perspective. Methods that are conducted in aqueous solvents do exist, such as the use of peroxides, bleach (NaClO), or Frémy's salt ($K_2NO(SO_3)_2$);[60] however, these are all stoichiometric oxidants, thus are not atom economical and contribute largely to hazardous waste.

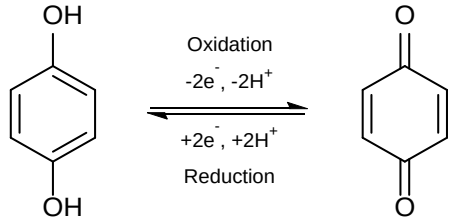


Figure 19. Redox reaction between hydroquinone and *para*-benzoquinone.

Enzymatic oxidation using various oxidase enzymes mimics the process that occurs in natural systems and is a much more sustainable approach, as it employs naturally derived catalysts which are nontoxic, renewable, biodegradable, and operate under mild conditions in aqueous solvents. Furthermore, the *para*-quinones can be generated and used for subsequent reactions in one-pot without any need for isolation and purification. This enzymatic approach forms the basis of the current dissertation research - the *in situ* generation of *para*-quinones via enzymatic oxidation of the corresponding hydroquinones in water.

2.2.4 Michael Addition to para-Quinones in Water

Water is a suitable solvent to conduct the Michael addition reaction of various nucleophilic compounds with *para*-quinones for reasons such as hydrogen-bonding and hydrophobic effects. Once the nucleophile attacks the *para*-quinone at one of its olefinic carbon atoms, the adduct will tautomerize to the hydroquinone form, and depending on the electronic properties of the new substituent and if an oxidant, such as atmospheric oxygen is present, may or may not re-oxidize back to the *para*-quinone form (Figure 20). This section is not meant to be exhaustive, rather it is meant to be representative of how

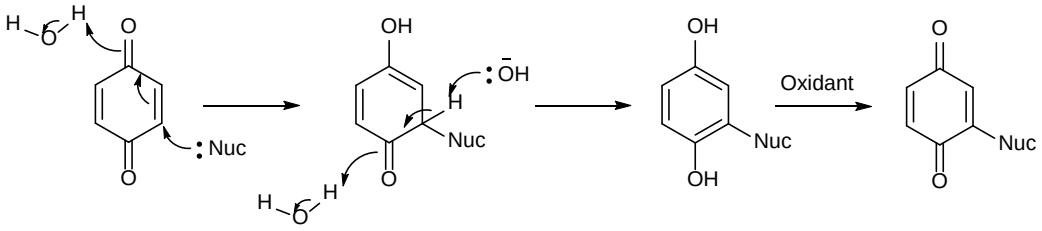


Figure 20. Michael addition to *para*-quinones in water.

this synthetic methodology can be used to create carbon-nitrogen and carbon-sulfur bonds under environmentally friendly conditions.

In a simple and efficient reaction protocol, Yadav and coworkers demonstrated that the Michael addition of *para*-benzoquinone, 1,4-naphthoquinone, and 2,6-dimethoxyquinone proceeded smoothly with ethanethiol, thiophenol, and 4-methylthiophenol in water at room temperature to yield the corresponding thio-benzene-1,4-diol adducts in excellent yields (≥ 82%) within 15 minutes of reaction (Figure 21).[61]

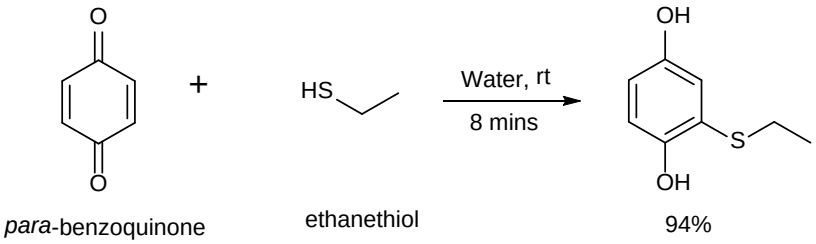


Figure 21. Michael addition of ethanethiol with *para*-benzoquinone in water.[61]

It is believed that the extremely fast reaction rates achieved in water are the result of hydrogen-bonding effects induced by the interaction of both reactants with water. More specifically, hydrogen-bonding between water and the carbonyl oxygen of the quinone increases the electrophilic character of the conjugated olefinic carbon atom, while hydrogen-bonding between water and the sulfhydryl group of the Michael donors increases the nucleophilicity of the thiol group, both factors contributing to the accelerated reaction rates. Similar yields and reaction times were achieved when aliphatic and aromatic amines were used as the Michael donors instead of thiols, and the underlying phenomena responsible for the increased reaction rates in water compared to organic solvents were rationalized in a similar fashion to the reactions involving thiols (i.e. hydrogen-bonding effects).[62] The main difference between the reactions involving

amines and the those involving thiols as the Michael donors is that the reactions involving amines undergo a final oxidation to yield the *para*-quinone (Figure 22), whereas the reactions involving the thiols remain in the hydroquinone state. Also, the amine-substituted *para*-quinones precipitate out of solution, allowing for a much more facile product separation. The "on water" rate enhancements of both of these reaction protocols were assessed in another study and it was found that Michael addition reactions of amines and thiols with *para*-quinones in which one or both reactants were insoluble in water (i.e. carried-out in suspension) proceeded much more rapidly than did the same reactions conducted in organic solvents.[63] It was proposed that hydrophobic effects play a prominent role in the rate accelerations for the reactions conducted in aqueous suspension.

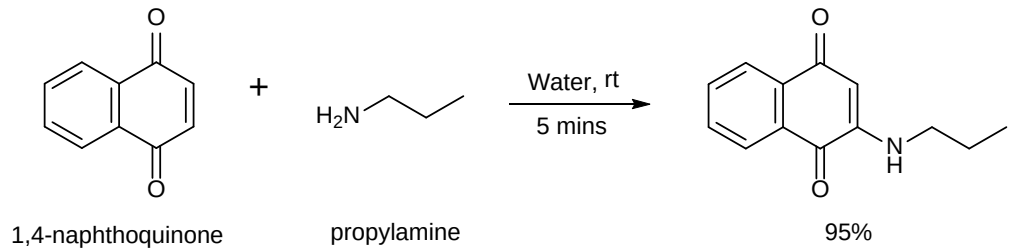


Figure 22. Michael addition of propylamine with 1,4-naphthoquinone in water.[62]

While the above mentioned synthetic methodologies present an efficient and green approach for the Michael addition using *para*-quinones as the Michael acceptors, they are not without limitations. For example, given that the *para*-quinones are the starting reactant, they must be stable enough so that they can be isolated from the reaction mixture in which they were synthesized or have long enough shelf-life to be purchased from a commercial source. The current dissertation work overcomes this challenge by

generating the *para*-quinones from the corresponding hydroquinones via enzymatic oxidation, and then consuming the *para*-quinone shortly after it is formed in a subsequent Michael addition in the same reaction vessel. Thus, not only are hydroquinones, which originate from a renewable feedstock, used as the *para*-quinone precursors, but *para*-quinones that are too unstable to be isolated can also be used as Michael acceptors because they are consumed immediately after their generation.

2.3 Biocatalysis

Biocatalysis entails the entire spectrum from using purified enzymes to perform chemical transformations all the way through to fermentations with microorganisms. The use of biocatalytic systems within many areas of the chemical industry has been rapidly increasing in recent decades as a result of an industry wide effort toward greener processes. Biocatalysts possess many advantages over conventional chemical catalysts such as being renewable and biodegradable, nontoxic, highly active and stable in aqueous environments under mild conditions, highly selective, relatively inexpensive, all the while providing significant productivity.[64-65] They also transform many renewable materials and natural products, thus providing an avenue to develop a bio-based chemical industry. Furthermore, with technological advances in the fields of genetics, molecular biology, and protein engineering, biocatalysts and biocatalytic networks are being engineered to convert an ever-increasing range of substrates in shorter reaction times.[9] Given that this dissertation research uses a single enzyme to catalyze chemical reactions, the remainder of this section will focus primarily on single enzyme processes and will

only briefly address biocatalytic networks and fermentations as they pertain to prominent industrial processes.

2.3.1 Enzymes

Enzymes are catalytically active proteins found in nature and are responsible for the chemical transformations that take place in biological systems. The word "enzyme" is a Greek term for "in yeast", referring to the observation that there was a substance, or substances, *in* yeast that were responsible for carrying out the chemical transformations associated with fermentations.[57]

The International Union of Biochemistry and Molecular Biology (IUBMB) has developed a systematic way of naming enzymes based on the enzyme's substrate and the type of reaction it catalyzes. Each enzyme is also assigned an Enzyme Commission (EC) classification that consists of four numbers, each separated by a period. The first number represents the primary enzyme class, while the following three numbers successively represent the subclass, sub-subclass, and serial number.[57] Table 2 lists the six primary classes of enzymes along with the type of transformation each class catalyzes. By and large, the vast majority of industrially relevant biocatalytic transformations are carried

Table 2. Enzyme classification.[57]

Primary EC Number	Enzyme Classification	Reaction Catalyzed
1	Oxidoreductases	Redox reactions
2	Transferases	Transfer functional groups
3	Hydrolases	Hydrolysis reactions
4	Lyases	Group elimination and addition
5	Isomerases	Isomerizations
6	Ligases	Bond formation coupled with ATP hydrolysis

out using the class of enzymes known as hydrolases. The use of oxidoreductases to catalyze industrially important redox processes has been steadily rising over the years and these enzymes are regarded as the second most used enzyme class for industrial applications after hydrolases. The enzyme used in this dissertation research belongs to the class known as oxidoreductases.[10]

2.3.2 Origins of Enzyme Catalysis

The manner in which an enzyme (E) performs a chemical transformation is by first associating with a suitable substrate (S) molecule in what can be described as a "lock-and-key" model (Figure 23), a concept put forward by Emil Fisher in 1894.[57] This essentially means that a particular enzyme will bind a substrate if the geometric shape of the enzyme's active site (i.e. the part of the protein where catalysis takes place) and substrate are complementary. However, it was later proposed by John Haldane that the binding of enzyme and substrate in the ground state is not an exact fit, but that the enzyme binds more tightly with the substrate in the transition-state (TS), which

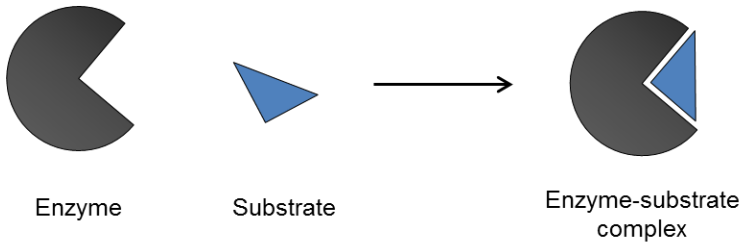


Figure 23. "Lock-and-key" model of enzyme-substrate interaction.

contributes enormously to the rate enhancements induced by enzymes,[66] as will be discussed in the following paragraphs. In any case, the substrate binds reversibly in the active site of the enzyme via either covalent or non-covalent interactions, such as

hydrogen-bonding and hydrophobic interactions, to form what is known as the enzyme-substrate (ES) complex. The chemical transformation then occurs, resulting in the conversion of substrate to product (P), which is then expelled from the enzyme to yield free product and free enzyme. The binding of substrate by the enzyme not only brings the substrate within close proximity of the enzyme's catalytic center, but also aligns the substrate in the correct orientation to promote efficient interaction, both of which are crucial for catalysis.[57]

Arguably the most remarkable feature of enzymes is the tremendous rate accelerations they are capable of effecting on chemical transformations. The rate acceleration or rate enhancement can be defined as the ratio of the rate constant for the catalyzed reaction to that of the uncatalyzed reaction, as shown in Equation 6. Thus, the

$$Rate\ enhancement = k_{cat}/k_{uncat} \quad (6)$$

lower bound is the rate of the uncatalyzed reaction, k_{uncat}, and the upper bound is set by the rate of diffusion in a solution, which is approximately 10^9 s^{-1} M^{-1}.[67] As an example of the impressive catalytic power of enzymes, the uncatalyzed decarboxylation of amino acids has a k_{uncat} of approximately 10^{-17} s^{-1} which corresponds to a reaction half-time ($t_{1/2}$) on the order of one billion years, compared to the catalytic rate constant k_{cat} of the decarboxylation reaction catalyzed by arginine decarboxylase, which is approximately 10^3 s^{-1}, which corresponds to a reaction half-time on the order of seconds.[68] Thus, enzymes are capable of increasing the rates of reactions immensely, approximately twenty orders of magnitude for the case of arginine decarboxylase, achieving a chemical transformation that would otherwise take a billion years to occur within seconds. As a comparison, the best chemical catalysts available to synthetic chemists can provide rate

enhancements of about six to eight orders of magnitude, yet another reason why biocatalysts are superior to conventional chemical catalysts.

So how do enzymes induce such immense reaction rate accelerations? A thermodynamic argument suggests that a decrease in the free energy of the transition state ($\Delta G^{\ddagger}$), resulting from a reduction in the enthalpy of activation (H_{act}), is a key contributing factor to the enhanced reaction rates for single substrate reactions.[67] This TS stabilization, which effectively lowers the E_{act}, is the underlying force responsible for the increased reaction rates induced by not only enzymes, but all types of catalysts. The reduction in H_{act}, which is essential to offset the unfavourable decrease in entropy upon substrate binding with enzyme, can be explained by invoking concepts from transition-state theory (TST). It is suggested that the enzyme binds the substrate more tightly in the TS than it does in the ground-state as a result of increased enzyme-substrate interactions (e.g. hydrogen-bonding) in the TS compared to the ground-state, which results in an increase in the effective concentration of activated substrate and subsequently increased reaction rate. In essence, the geometries of the enzyme and the activated substrate are precisely complementary.[69] The reaction system is represented pictorially as a reaction coordinate diagram in Figure 24. As illustrated, the reaction pathway can progress through two routes, a higher energy uncatalyzed path or a lower energy catalyzed path, both of which originate from the same reactant(s) and culminate in the formation of the same product(s). The rate enhancement can be calculated using Equation 7 below:

$$Rate\ enhancement = e^{\Delta\Delta G\ddagger/\mathrm{RT}} \quad (7)$$

where $\Delta\Delta G^{\ddagger}$ is the difference in free energy of TS complex between the catalyzed and

uncatalyzed reactions (equivalent to ΔE_{act} between the catalyzed and uncatalyzed reactions), R is the gas constant, and T is the temperature.[57]

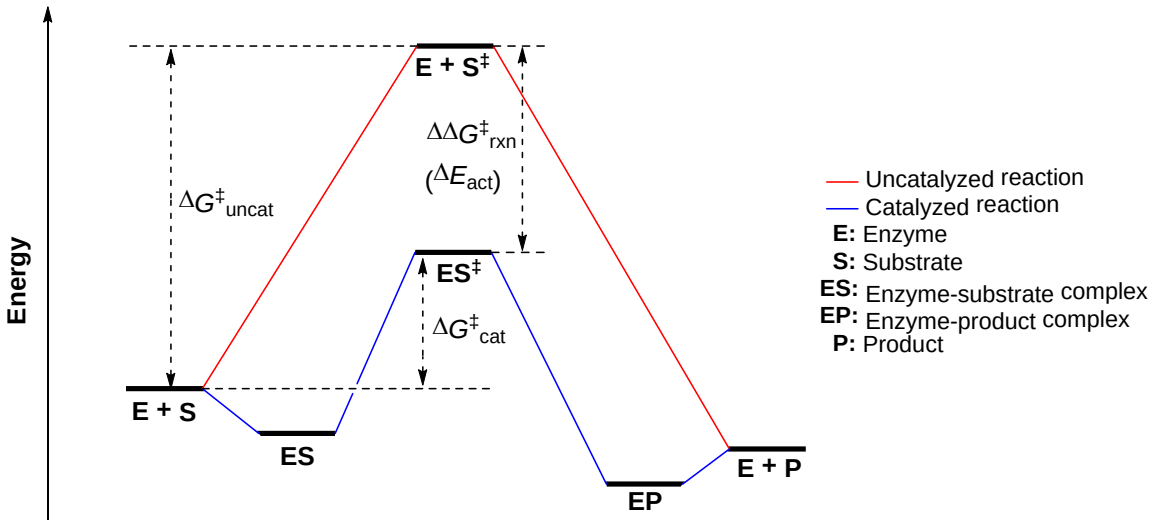


Figure 24. Reaction coordinate diagram comparing uncatalyzed reaction versus enzyme catalyzed reaction.[67]

In 1963, Joseph Kurz developed a kinetic model based on a thermodynamic cycle that could be used to explain TS stabilization induced by a generic catalyst.[70] For the scenario in which an enzyme reacts with a single substrate to produce a single product, as in Equation 8, the corresponding thermodynamic cycle is depicted in Figure 25.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \tag{8}$$

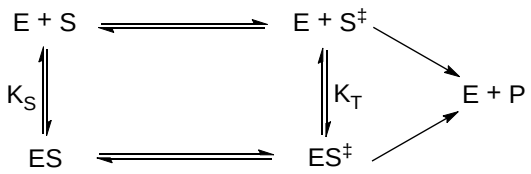


Figure 25. Thermodynamic cycle illustrating transition-state stabilization in enzymatic catalysis.[70]

The rate constant of a reaction can be expressed in the form of Equation 9:

$$k = \kappa \cdot \nu \cdot K^{\ddagger} \quad (9)$$

where k is the rate constant, κ is the transmission coefficient, ν is the frequency of crossing the TS, and $K^{\ddagger}$ is the TS equilibrium constant. Assuming that the transmission coefficient and the frequency of crossing the transition state are near unity, Equation 9 simplifies to Equation 10. From the thermodynamic cycle, an expression can be derived

$$k = K^{\ddagger} \quad (10)$$

that equates the rate enhancement of a catalyzed process compared to the uncatalyzed process to the ratio of association constants of enzyme with ground-state substrate (K_S) and activated substrate (K_T) (Equation 11).[70] All of the above mentioned work

$$Rate\ enhancement = k_{cat}/k_{uncat} = K_S/K_T \quad (11)$$

corroborates the hypothesis made by Linus Pauling in the 1940s, which stated that for an enzyme to successfully accomplish a chemical transformation in a short period of time, the TS must be stabilized.[71]

2.3.3 Enzyme Kinetics

Kinetic data of enzyme catalyzed transformations is extremely important, not only for industrial purposes in determining the time period of a biocatalytic process, but also to offer insight into the reaction mechanism, which is of immense worth in drug design, specifically in the area of enzyme-small molecule interactions. The study of enzyme kinetics dates back to as early as 1902 when Adrian Brown investigated the hydrolysis of sucrose into fructose and glucose by invertase.[72] However, perhaps the most pioneering work in the field of enzyme kinetics was conducted by Leonor Michaelis and Maud

Menten in 1913 when they applied the principles of thermodynamics to the case of sucrose hydrolysis.[73] Michaelis and Menten are nowadays widely regarded as the founders of the field of enzyme kinetics and are whom the governing equation is named after.

Before delving into the derivation of the rate equation for enzymatic reactions, it is important to note that the rate constant of a reaction, *k*, has origins grounded in TST. Using the principles of thermodynamics, an expression for the rate constant can be derived (Equation 12):

$$k = (k_B T/h) e^{-(\Delta G^{\ddagger}/RT)} \quad (12)$$

where k_B is the Boltzmann constant, *h* is Planck's constant, R is the gas constant, T is temperature, and $\Delta G^{\ddagger}$ is free energy of TS.[57] This expression takes into account intrinsic values such as κ and *v* that were discussed in the preceding section.

The enzymatic reactions in the current dissertation research abide by the reaction model depicted in Equation 8, in which the decomposition of ES to E + P is irreversible. Additionally, when the substrate concentration is much greater than the enzyme concentration (i.e. [S] >> [E]), which is typically the case for enzyme catalyzed processes both in industrial applications and in biological systems, the reaction rate is independent of the [S], that is the reaction rate is zeroth order in [S], but first order in [E]. Thus the second reaction where ES decomposes to E + P is the rate-limiting step, and the reaction rate can be expressed in the form of Equation 13, where *v* is the reaction rate or velocity.

$$v = \frac{d[P]}{dt} = k_2[ES] \quad (13)$$

However, this equation does not suffice because [ES] is a transient species that cannot be quantified. Therefore, for the rate expression in Equation 13 to have practical value, [ES]

must be expressed in terms of measurable species. The change in concentration of [ES] with respect to change in time can be modeled as in Equation 14. To this equation,

$$\frac{d[\mathrm{ES}]}{dt} = k_1[\mathrm{E}][\mathrm{S}] - k_{-1}[\mathrm{ES}] - k_2[\mathrm{ES}] \quad (14)$$

Michaelis and Menten applied an argument based on the assumption that $k_{-1} >> k_2$, which is usually incorrect. Haldane and George Briggs, on the other hand, applied the assumption of a steady state based on the hypothesis that [ES] increases at the start of a reaction and then levels off (i.e. reaches equilibrium) as time progresses, so that the rate of change of [ES] throughout the course of a reaction is equal to zero (Equation 15).[74]

$$\frac{d[\mathrm{ES}]}{dt} = 0 \quad (15)$$

This assumption is valid for instances where [S] >> [E]. Applying the steady state assumption, along with the additional assumption that S is converted to P only, Equation 14 can be re-written in the form of Equation 16. Applying the enzyme mass balance, in

$$k_1[\mathrm{E}][\mathrm{S}] = k_{-1}[\mathrm{ES}] + k_2[\mathrm{ES}] \quad (16)$$

which the total enzyme concentration $[\mathrm{E}]_\mathrm{T}$, a measurable quantity, is equal to the sum of free enzyme [E] and [ES] (Equation 17), and rearranging yields Equation 18, where

$$[\mathrm{E}]_\mathrm{T} = [\mathrm{E}] + [\mathrm{ES}] \quad (17)$$

$$(([\mathrm{E}]_\mathrm{T} - [\mathrm{ES}])[\mathrm{S}])/[\mathrm{ES}] = (k_{-1} + k_2)/k_1 = K_M \quad (18)$$

K_M is the Michaelis constant. Rearranging, solving for [ES] and then multiplying each side by k_2 yields an expression for the initial reaction rate or velocity, v_o, also known as the Michaelis-Menten equation (Equation 19), where V_max is the maximum reaction rate

$$v_\mathrm{o} = \frac{d[\mathrm{P}]}{dt} = k_2[\mathrm{ES}] = (k_2[\mathrm{E}]_\mathrm{T}[\mathrm{S}])/(K_M + [\mathrm{S}]) = (V_\mathrm{max}[\mathrm{S}])/(K_M + [\mathrm{S}]) \quad (19)$$

or velocity, and is equal to the product of k_2 and $[E]_T$. Although Equation 19 oversimplifies the real life situation by not taking into consideration effects such as enzyme deactivation and inhibition, it is still the governing equation for enzyme kinetics and can be manipulated to take into account the above mentioned effects.

The Michaelis-Menten equation (Equation 19) can be plotted to depict v_o as a function of [S] (Figure 26).[57] The curve represents a hyperbola which has an asymptote equal to V_{max}. The [S] corresponding to the point on the curve where v_o is equal to half V_{max} is the Michaelis constant, K_M. Thus, the K_M, which is temperature and pH dependent, is a measure of the affinity of an enzyme for a given substrate and is therefore unique for each enzyme-substrate pair. From the plot, it can be visually deduced that as the enzyme becomes completely saturated with substrate (i.e. at very large [S]), the rate of the reaction approaches V_{max}, but never reaches it. On the other end of the spectrum at very low [S] (i.e. [S] << K_M), the rate becomes first order in both [S] and [E], and v_o can be expressed as in Equation 20. In this equation, the value k_{cat}/K_M represents the catalytic

$$v_o = (k_{cat}/K_M)[E][S] \quad (20)$$

efficiency of the enzyme. Furthermore, for the simple one substrate reaction shown in Equation 8, $k_{cat} = k_2$, which essentially equates to the turnover number of the enzyme,

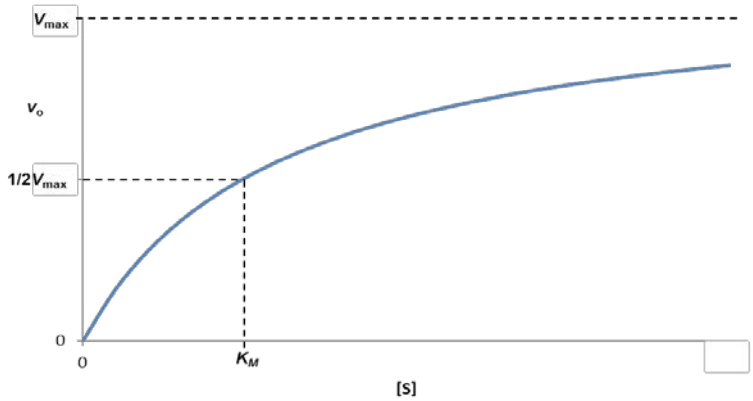


Figure 26. A generic Michaelis-Menten plot.[57]

which is the number of substrate molecules each enzyme converts per unit time (usually one second).

It is often difficult to obtain accurate values for K_M and V_{max} from Michaelis-Menten plots. In 1934, Hans Lineweaver and Dean Burk developed an alternative method to experimentally determine K_M and V_{max} by plotting the reciprocal plot of the Michaelis-Menten equation, which were later named Lineweaver-Burk plots.[75] This equation is linear with a slope equal to K_M/V_{max}, a $1/v_o$ intercept equal to $1/V_{max}$, and a 1/[S] intercept equating to $-1/K_M$. Thus, this allowed for K_M and V_{max} to be determined with more confidence. However, Lineweaver-Burk plots have more or less become redundant nowadays due to advances in computational methods.

2.3.4 Enzyme Activity

Unlike conventional chemical catalysts, which can be applied to reaction systems using molar amounts or mole percentages just like any other reagent, the use of enzymes is a little more complex. Owing to a multitude of influences, such as enzyme source, stability, and pH and temperature of reaction medium to name a few, each enzyme will

likely have a different catalytic activity. The standard unit of enzyme activity is enzyme units, U, and can be determined via Equation 21. There are a few variations of enzyme

$$1\text{U} = \frac{1\ \mu\text{mol product generated (or substrate consumed)}}{\text{minute}} \tag{21}$$

activity. For example, specific activity is the nomenclature given when pure protein is used and enzyme activity is expressed as U/mg pure protein. Another variation is the volumetric activity, which is U/mL of crude enzyme solution, which is the appropriate enzyme activity in the current dissertation research.

Enzyme activity is determined by conducting an enzyme assay experiment. This experiment is dependent on either the formation of a product or the consumption of a substrate and on an analytical method in which to quantify the amount of depleted substrate or generated product over time. Spectroscopic techniques, such as ultraviolet-visible (UV-vis) spectroscopy and fluorescence spectroscopy, have found much use in enzyme assay experiments. The enzyme activity is highly dependent on parameters such as temperature, pH, solvent, and ionic strength of the reaction medium, therefore, the reaction conditions under which the enzyme assay was performed must also be appended when providing enzyme activity data.

2.3.5 Enzyme Inhibition

As was discussed in section 2.3.3, the classical Michaelis-Menten equation does not take into consideration the effects enzyme inhibition have on enzyme kinetics. The effect of enzyme inhibition is caused by an inhibitor (I), which can be defined as any molecule that is able to interact with an enzyme in such a way so as to reduce or halt the catalytic activity of the enzyme. Enzyme inhibitors are important in the field of

therapeutic drug design as they provide a means to temporarily or permanently retard enzymes that are crucial to the survival and proliferation of bacteria, fungi, and viruses, as well as adverse metabolic pathways within the human body. For example, the HIV therapeutic indinavir (trade name Crixivan), developed by Merck in the 1990s, is able to bind to HIV protease, an enzyme that is essential for viral reproduction, and inhibit its activity, thus reducing the proliferation of HIV.[76]

Enzyme inhibition can be reversible or irreversible and take the form of one of three modes: competitive inhibition, uncompetitive inhibition, or mixed inhibition (Figure 27).[57] Competitive inhibition involves the inhibitor binding at the substrate binding site, thus competing with the enzyme's native substrate for active site binding and effectively reduces the concentration of free enzyme available for the native substrate to bind with, an effect that will make the K_M appear larger than it really is. Such inhibitors typically resemble the structure of the native substrate, but can also include the product of the catalytic reaction, an effect known as product inhibition. Based on the knowledge that the enzyme binds more tightly to the TS species, the class of inhibitors known as TS analogs have proved to be extremely potent inhibitors. Uncompetitive inhibition involves the inhibitor binding to the ES complex; thus, this type of inhibitor does not compete with the substrate for binding at the enzyme's active site, hence, will underestimate the actual K_M and $V_{\max}$ values. An uncompetitive inhibitor induces its inhibition effect by distorting the protein in such a way so that its activity is reduced. Finally, mixed inhibition is competitive and uncompetitive inhibition combined, so the inhibitor induces its effects by binding to both the free enzyme and the ES complex. This type of inhibition results in an underestimation of $V_{\max}$.

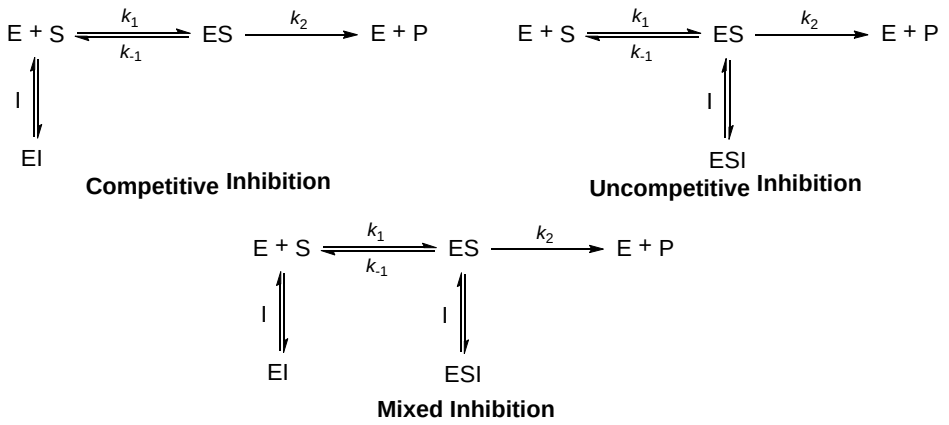


Figure 27. Reaction models of enzyme inhibition modes.[57]

2.3.6 Enzyme Stability and Deactivation

The topics of enzyme stability and deactivation are of great significance in biocatalysis. These factors must be taken into consideration when deploying an industrial scale biocatalytic process as they will have a dramatic influence on process parameters such as temperature, residence time, solvent, and amount of enzyme to be used.

Protein folding and unfolding is a fascinating natural phenomenon. The final step in protein synthesis involves the folding of the elongated polypeptide chain into the three-dimensional architecture of the native enzyme conformation, which follows a potential energy landscape where the native protein is the energy sink.[77] The forces responsible for stabilizing proteins and holding them in their native conformation are mostly hydrophobic in nature.[78] The thermodynamic stability of a protein can be modeled by Equation 22:

$$T_s = T_m e^{-[\Delta H_m/(T_m \cdot \Delta C_p)]} \quad (22)$$

where T_s is the temperature of maximum stability, T_m and H_m are the melting temperature (i.e. temperature at which protein is half unfolded) and enthalpy, respectively, and C_p is the heat capacity.[78] The curve of this equation resembles an inverse parabola, with the global maximum indicating the T_s. T_s is not necessarily the optimum temperature at which a biocatalytic process should be conducted at because the enzyme usually exhibits greater catalytic activity closer to T_m; however, protein denaturation can occur very rapidly in the vicinity of T_m, thus the true optimum process temperature (T_{opt}) is located somewhere between the temperature that produces the greatest catalytic rate and the temperature of maximum enzyme stability (i.e. $T_s < T_{opt} < T_m$), as demonstrated in Figure 28.

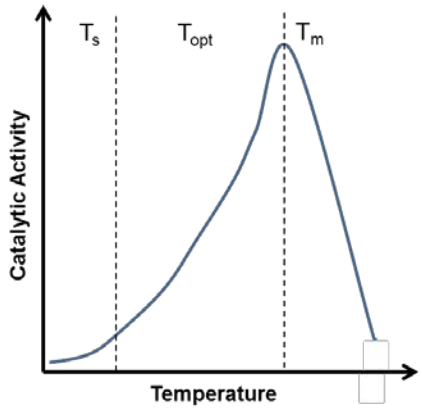


Figure 28. Catalytic activity as a function of temperature for a generic biocatalytic process.

Enzyme deactivation is detrimental to the catalytic activity of the enzyme. There are two main processes that can lead to enzyme deactivation: conformational processes, such as inter- and intramolecular aggregation; and covalent processes, such as hydrolysis of labile bonds, deamidation of asparagine (Asn) or glutamine (Gln) residues, and reduction of disulfide bonds.[78] There are many underlying causes responsible for

deactivation. Research strongly suggests that covalent processes are more likely to occur at elevated temperatures, an effect known as thermal deactivation.[78] Stirring and agitation has also shown to deactivate enzymes, proposedly as a result of collisions between active and inactive enzymes, with the observed deactivation rate constant, $k_{d,obs}$, proportional to stirring power.[79] Enzyme deactivation has also shown to occur in the presence of gas bubbles, presumably due to liquid/gas interfacial interactions.[80] A similar interfacial enzyme deactivation process is also evident in aqueous/organic solvent systems.[81]

Kinetics of enzyme folding/unfolding and deactivation can be represented by the Lumry-Eyring model, named after Rufus Lumry and Henry Eyring for their pioneering research on the topic.[82] In the model, depicted in Equation 23, an equilibrium exists

$$\mathrm{N} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{U} \xrightarrow{k_d} \mathrm{I} \tag{23}$$

between the native, catalytically active, folded state (N) and the unfolded, inactive state (U) such that unfolding from N to U is a reversible process, and an irreversible process that converts U to a permanently inactive state (I). In this model, k_d represents the intrinsic deactivation rate constant from U to I. Combining $k_{d,obs}$ with the observed catalytic rate constant $k_{cat,obs}$, one can calculate the turnover number of the enzyme taking into account deactivation effects (Equation 24).

$$Turnover\ Number = k_{cat,obs}/k_{d,obs} \tag{24}$$

2.4 Laccases

The enzymes used in this dissertation research are laccases. Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) belong to the broader family of

multicopper oxidases – proteins that contain four copper ions in their active site.[13] Their discovery dates way back to 1883 where they were identified as a component of the sap of the Japanese lacquer tree *Rhus vernicifera*.[83] A little over a decade later, the first fungal derived laccases were discovered in various species of *Russula* fungi.[84] In nature, laccases catalyze the one electron oxidation of a variety of organic and inorganic substrates whilst concomitantly reducing molecular oxygen to water. Attributing to their environmentally benign character and their broad substrate range, their use as biotechnological tools in a variety of industries is continuing to prosper.

Baldrian has compiled data of molecular weight, isoelectric point, temperature and pH optima, and K_M for common substrates, for over 100 fungal laccases isozymes.[85] In general, fungal laccases exhibit highest activity between 50 and 70°C, while the pH optimum is highly substrate dependent. Given that their exists much diversity among different laccase isozymes with respect to molecular weight, quaternary structure, and spectroscopic properties, an enzyme can be defined as a laccase by the substrates it can oxidize. Thus, one defining feature of a laccase is an enzyme that is able to oxidize syringaldazine, but cannot oxidize tyrosine.[86] Furthermore, a more current definition can be proposed based on amino acid sequence homology. Multicopper oxidases contain four highly conserved regions, denoted L1-L4, that encompass the copper-coordinating residues in the active site of the enzyme. The L1 and L3 regions however, are distinct for fungal laccases, a feature which assists in differentiating them within the broader class of multicopper oxidases.[87] The following sections will discuss the distribution of laccases in nature, their structure, catalytic and redox properties, typical substrates and inhibitors, as well as the applications of laccase-mediator systems (LMS).

2.4.1 Natural Sources

Historically, laccases have been isolated predominantly from plants and fungi. However, in recent years, the laccase toolbox has been expanding, with the enzymes also being detected in bacteria, insects, and algae.[88-90] The physiological functions of laccases from different sources are highly diverse.

Fungal laccases are derived from numerous species of basidiomycetous and ascomycetous fungi as both extracellular and intracellular enzymes (Figure 29).[85] In fungi, laccases are responsible for fruiting body formation, pigmentation, morphogenesis, plant pathogenesis, fungal virulence, the synthesis of humic substances in soil, and lignin degradation.[88, 91-92] Plant laccases, on the other hand, are partially responsible for the biosynthesis of lignin.[93] The antagonistic roles of lignin polymerization and degradation

Figure 29. Wood-rot fungi *Trametes versicolor*: a common source of laccases.[94]

between plant and fungal laccases, respectively, have been studied via molecular docking and dynamic simulation methods utilizing lignin model compounds and have revealed that differences in enzyme structure and in substrate binding mode are contributing factors.[95] Bacterial laccases play a role in morphogenesis, in the synthesis of melanin, and in the production of the brown spore pigment, which protects the spore

coat against UV light and hydrogen peroxide.[96-98] In general, bacterial laccases possess higher thermostability and increased tolerance of alkaline pH and high salt concentrations, as well as a larger binding cavity, compared to fungal and plant laccases.[88] Enzymes with laccase activity have also been isolated from bovine rumen microflora, where it is believed they aid in the digestion of ryegrass lignin.[99] Laccases are also found in insects, where they are believed to play a role in tanning, a process that hardens the newly secreted exoskeleton, by catalyzing the cross-linking reaction between structural proteins in the insect and catechol derivatives.[90] More recently, laccases have been isolated from soil algae, where it is proposed that they contribute to the turnover of soil organic matter.[89]

Multiple laccase isozymes can be produced by a single source due to the presence of multiple laccase genes.[100] For example, the fungus *Pleurotus ostreatus* produces at least eight laccase isozymes.[85] The reasoning for this gene multiplicity may be due to the different physiological functions proposed for laccases from a given source,[100] as discussed in the preceding paragraph. The remaining sections will focus primarily on fungal laccases as these enzymes have been studied extensively and are widely used for biotechnological purposes due to their higher redox potentials compared to plant and bacterial laccases.

2.4.2 Structure

Details of the three-dimensional structures of laccases can be obtained from purified crystals of the enzymes. A recent review surveying all the currently available crystal structures of laccases has been presented by Hakulinen and Rouvinen.[101] Fungal laccases are typically monomeric proteins with molecular weight of 60-70 kDa,[85]

although oligomeric forms of laccases do exist. For example, *Trametes villosa* laccase isozymes exist as homodimers.[102] It is believed that the oligomeric architecture may aid in stabilizing the protein. Laccases are glycoproteins with an extent of glycosylation usually between 10 to 25%.[85] The carbohydrates are linked to the polypeptide chain via *N*-linkages,[103] and have many roles including structural, protection against proteolytic degradation,[104] and increasing thermostability.[105] The three-dimensional structure of a *Trametes versicolor* laccase is presented in Figure 30.

The active site of laccases contain four copper ions, categorized based on spectroscopic features as Type 1 Cu (T1), Type 2 Cu (T2), and binuclear Type 3 Cu (T3) (Figure 31).[106] Substrate oxidation occurs at the T1 Cu while oxygen reduction takes place at the T2 and T3 Cu sites, which are collectively known as the trinuclear cluster (TNC). Fungal laccases have three sequentially arranged cupredoxin-like domains, each of which contains Greek key β-barrel topology. T1 Cu is located in domain 3, and TNC is entrenched between domains 1 and 3.[100] In fungal laccases, the T1 Cu possesses trigonal planar geometry coordinated with the S atom of a Cys residue and the Nδ1 atoms of two His residues.[100] In laccases from nonfungal origins, a fourth coordinating ligand, usually an axial Met, may be present. The absence of an axial ligand at the T1 Cu site in most fungal laccases is compensated for by a stronger Cu-S_{Cys} bond.[107] The $S_{Cys} \rightarrow$ Cu charge transfer, which gives an absorption at 614 nm , manifests itself visually as the deep blue color of the enzymes.[108] The three Cu ions comprising the TNC possess a triangular geometric arrangement. The antiferromagnetically coupled T3 Cu ions are coordinated with three His residues each and are bridged via a hydroxide ligand, while the T2 Cu ion is coordinated with two His ligands and one water molecule.[109]

The substrate binding cavity is primarily lined with hydrophobic residues along with a highly conserved His and acidic residue, as depicted in Figure 32.[110] Mutagenic and structural studies have shown that this acidic residue (either an Asp or Glu) is crucial for the oxidation of substrates containing phenolic or aromatic amine functional groups. It is believed that this residue, in its carboxylate form, is able to hydrogen bond with OH or NH_2 groups of substrates, aiding in deprotonation of the substrate.[110-111] Thus, pH control is important to ensure the residue is present in its carboxylate form (typically

Figure 30. Three-dimensional structure of a *Trametes versicolor* laccase. Protein backbone shown in ribbon representation (blue), copper ions as spheres (magenta), and carbohydrates as lines (green/blue/red). Image created with open-source *PyMol* from PDB file 1GYC.[94]

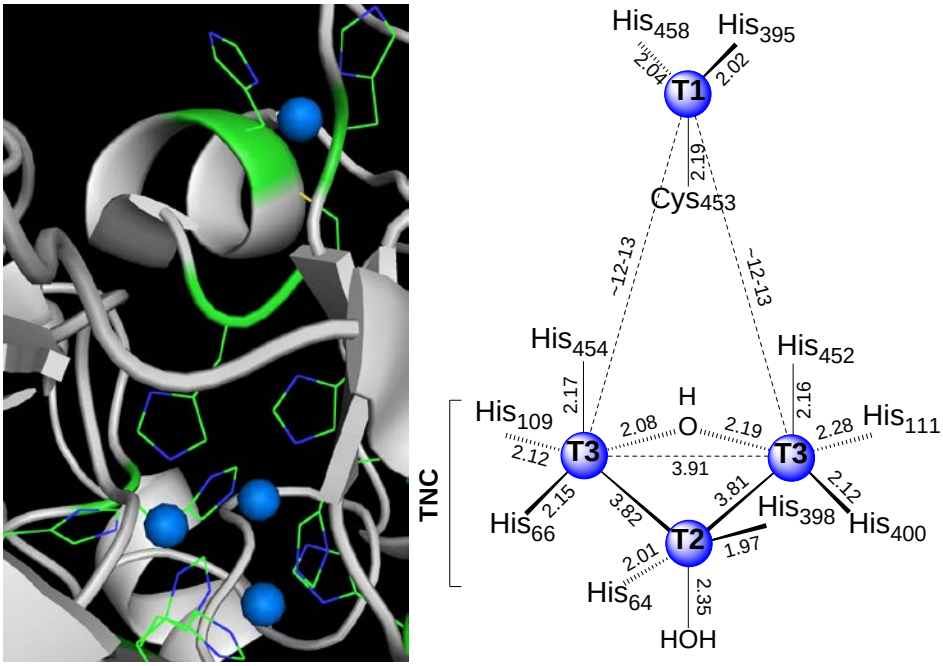


Figure 31. Left: 3D representation of laccase active site. Image created with open-source *PyMol* from the crystal structure of a *Trametes versicolor* laccase (PDB file 1GYC). Right: schematic representation of the active site of the same laccase showing coordinating residues and interatomic distances (Å).[94]

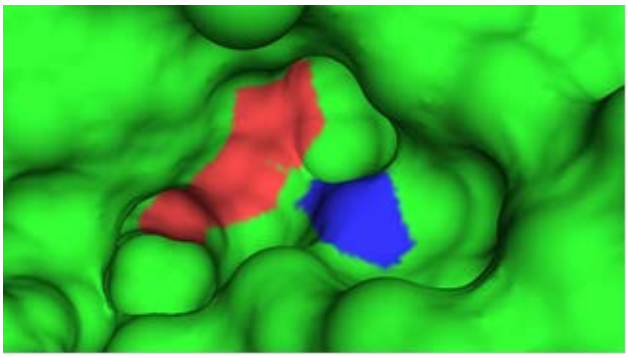


Figure 32. Substrate binding cavity of a *Trametes versicolor* laccase depicted as an electrostatic potential surface displaying the conserved His (blue) and acidic (red) residues. Image created with open-source *PyMol* from PDB file 1GYC.[94]

around pH 5 or higher). Furthermore, the pH optimum for specific substrates can be altered via replacing this acidic residue with non-acidic residues.[112]

Recently, a variety of computational techniques have been used to shed light on the potential pathway of oxygen diffusion into the TNC, providing evidence for the existence of a purposefully constructed channel.[113] Structures of some fungal laccase isozymes exhibit an extended C-terminal, which may block this channel, significantly impairing the catalytic efficiency of the enzyme.[114-115] A recent study by Hu and coworkers showed that the activity of a laccase from the fungus *Pleurotus florida* could be increased substantially when its C-terminal was truncated by 13 residues.[116] Substrate accessibility to the active site also affects the catalytic efficiency of the enzyme. Sometimes this can be hindered by bulky, hydrophobic residues at the entrance of the active site.[100]

2.4.3 Redox Properties

The redox potentials ($E°$) of laccases play a paramount role in the overall energetics and kinetics of electron transfer during the oxidation of substrate by the T1 Cu. In fact, kinetic analyses show that the difference in $E°$ between the T1 Cu and substrate determines the rate of electron transfer, and that this reaction is the rate-limiting step of the entire catalytic cycle.[117] Fungal laccase redox potentials vary from one source to another, ranging between 0.44 and 0.79 V versus NHE,[118] and are generally considerably higher than laccases from plant and bacterial origins.

Structural studies of fungal laccases have deduced that the greater the coordination distances of ligands to the T1 Cu, the higher the $E°$.[109, 119] In high $E°$ laccases, the T1 Cu-N distance of a ligating His residue is elongated. This increased distance is believed to be caused by a hydrogen bond between highly conserved Glu and Ser residues, the former located on the same helix as the ligating His residue, thus pulling this His further away from the T1 Cu. It is postulated that the increased Cu-N distance renders the Cu ion more electron deficient due to less of a contribution of the lone pair from N_{His}, which may destabilize higher oxidation states, thus increasing the $E°$.[109] The same logic may also be used to explain why the absence of a fourth axial ligand in fungal laccases correlates with a markedly higher redox potential. Site-directed mutagenesis of the axial ligand shows that when this ligand is changed from a noncoordinating residue, such as Leu or Phe, to a coordinating Met residue, the redox potential of the T1 Cu is lowered substantially.[120-121] This theory has been further validated by QM/MM molecular dynamics simulation results.[122] Other factors, including solvent accessibility, dipole orientation, and hydrogen bonding, also contribute to the differences in redox

potential and substrate oxidation.[120] Protein engineering techniques, such as site-directed mutagenesis and directed evolution, are used to modify the structure of the enzymes in the hope of improving redox properties, with the ultimate intention of fabricating tailor-made laccases for specific industrial purposes.[123]

2.4.4 Substrates and Inhibitors

A comprehensive list of fungal laccase substrates and inhibitors has been assembled by Baldrian.[85] Owing to their $E°$, which are typically in the range of 0.6 to 1.2 V vs NHE,[124] phenols have demonstrated themselves to be suitable substrates for laccases. Thus, it should come as no surprise that lignin, an irregular phenolic polymer that is one of the main constituents of wood, is a natural substrate of laccases. Representative laccase substrates include: phenols, *para*- and *ortho*-diphenols, polyphenols, aromatic and polyamines, thiophenols, and some metal ions.

True laccase inhibitors are small molecules that are capable of binding to the Cu ions of the TNC, thereby interrupting the internal electron transfer process. Inhibitors include: sodium azide,[125] small halides,[117, 126] heavy metals, and EDTA to name a few. Bulky organic compounds, such as medicarpin, have also shown to inhibit laccase activity. The inhibitory effect is due to medicarpin blocking both the solvent channel where oxygen enters and the substrate binding site.[127] Recently, it has been suggested that mono-carboxylates, such as propionate and butyrate, are able to substantially reduce the activity of some fungal laccases.[128] This has important implications on the selection of a suitable buffer system to carry out laccase-catalyzed reactions.

2.4.5 Catalytic Mechanism

Mono-electron oxidation of substrate, which occurs at the T1 Cu site, is the first step in the catalytic cycle (Figure 33). Galli and coworkers have studied both wild type and mutant laccases to gain insight into the oxidative mechanism. Via the Hammett approach and Marcus analysis, they were able to provide unambiguous evidence for a concerted electron/proton transfer (EPT) mechanism in the oxidation of phenolic substrates by laccases.[129] A concerted EPT mechanism is an energetically efficient route as it avoids the formation of a high-energy charged intermediate, as would be generated if either electron or proton were transferred individually, and is commonplace in many biological systems.[130] It is believed that the Nδ1 of a highly conserved T1 Cu coordinating His residue in the substrate binding site may be the initial electron acceptor from the substrate.[111]

Once the T1 Cu ion has gained an electron form the substrate, the electron is then shuttled to the TNC via one of two routes: through-bond via a highly conserved Cys-His motif, or through-space.[111] The transfer of electrons from the T1 Cu ion to the TNC is greatly affected by pH. It is known that the $E°$ of both laccases and substrates vary with pH, and that the $E°$ of a phenolic substrate will decrease more significantly compared to the $E°$ of a laccase at increased pH, allowing for more energetically favorable electron transfer from substrate to the T1 Cu ion. However, at higher pH values, internal electron transfer from the T1 Cu ion to the TNC is inhibited by the binding of a hydroxide anion to the T2 and T3 Cu ions, which severely hinders the catalytic efficiency of the enzyme.[131] Hence the significance of pH control in laccase-catalyzed processes.

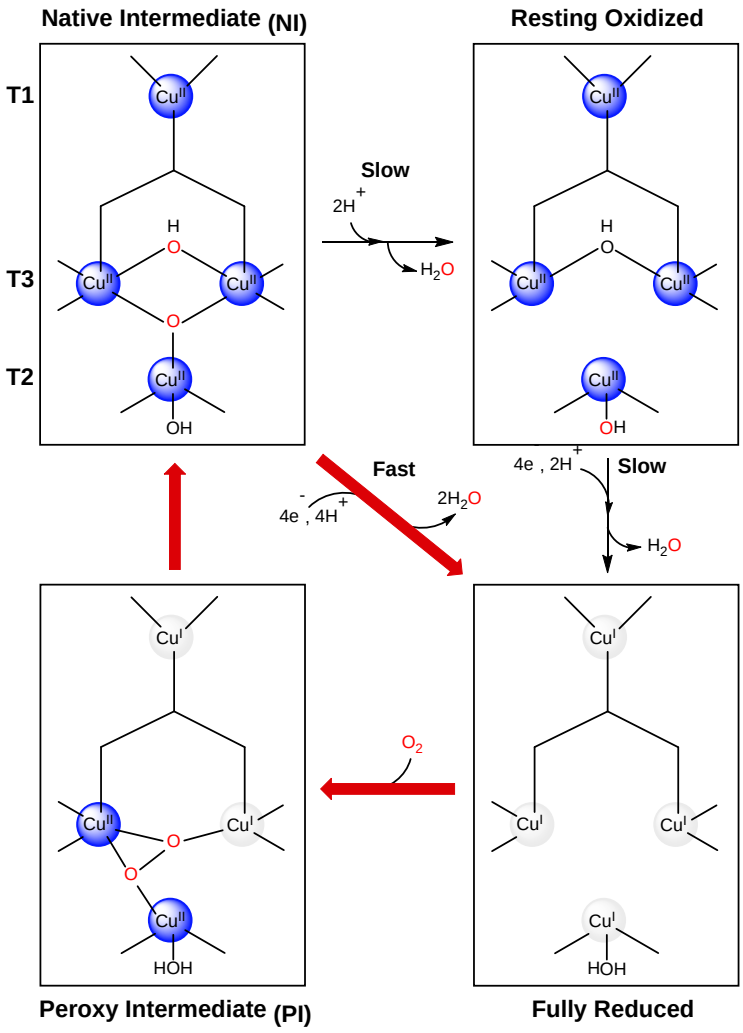


Figure 33. Laccase catalytic cycle.[94]

Through use of a variety of spectroscopic techniques, Solomon and his coworkers have elucidated the catalytic cycle, including geometric and electronic structures of intermediates. The native intermediate (NI) is a fully oxidized form of the enzyme with all three Cu ions of the TNC connected via a μ_3-oxo bridging ligand.[108] This ligand allows for electron transfer to all Cu ions of the TNC. In the presence of a reducing substrate, the NI will gain four electrons, reducing all Cu^{II} ions to Cu^{I} ions, whilst concomitantly releasing two molecules of water with the aid of four protons.

Spectroscopic data suggests that one of the T3 Cu ions is reduced first, followed by the T2 Cu ion, then the other T3 Cu ion, and then finally, with nowhere to relay the electron, the T1 Cu ion remains reduced.[132]

The reaction of fully reduced enzyme with dioxygen occurs via two sequential two-electron steps. First, dioxygen binds in the TNC, followed by instantaneous two-electron transfer, one electron from the T2 Cu ion and one from a T3 Cu ion, to the bound oxygen molecule to generate the peroxy intermediate (PI).[133] On further transfer of two more electrons to the PI, one electron from the other T3 Cu ion and one from the T1 Cu ion, the O-O bond is cleaved and the NI is reached, thus completing the catalytic cycle. Experimental and computational data suggest that a highly conserved carboxylate residue (typically Asp) in the vicinity of the T2 Cu ion may assist in stabilizing the PI and is vital for O-O bond cleavage.[134]

On the occasion that there is no reducing substrate present, the NI decays to an oxidized resting form of the enzyme. Given that the rate of decay is orders of magnitude slower than the turnover rate of the enzyme, it is suggested that this resting form of the enzyme is not part of the catalytic cycle.[132] A more in depth discussion of the catalytic cycle and the reduction of oxygen to water, including experimental and computational data, is provided by Solomon and coworkers.[135]

2.4.6 Laccase-Mediator System (LMS)

The scope of laccase-catalyzed oxidations can be expanded to include atypical laccase substrates, such as those with $E°$ too high for laccases to oxidize or too bulky to enter the active site of the enzyme, via use of the laccase-mediator system (LMS). This system employs small molecules that are readily oxidized by laccases to act as electron

shuttles between the enzyme and the target substrate (Figure 34). Once oxidized by laccase, the small molecule mediator must be stable enough to diffuse into the bulk reaction medium and react with the final substrate. The first report of an LMS was described by Bourbonnais and Paice in 1990. They established that a system containing

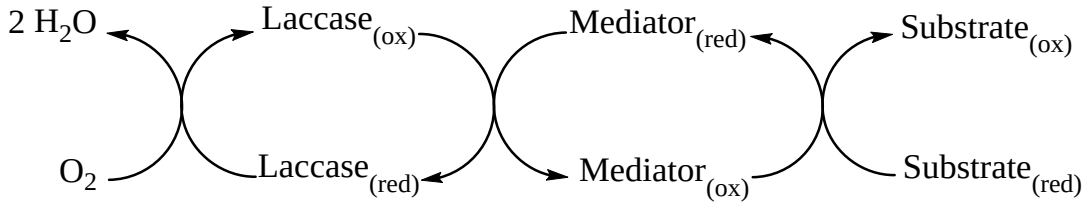


Figure 34. Laccase-mediator system (LMS).[94]

both a laccase from *Trametes versicolor* and the common laccase enzyme assay substrate 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) could oxidize non-phenolic lignin model compounds, such as veratryl alcohol.[136] Since this pioneering work, LMS have been employed for a variety of industrial applications, such as delignification, bleaching of kraft pulps, and in the degradation of dyes and xenobiotic compounds.[137] Furthermore, the system has been used to perform a variety of synthetically useful chemical transformations.[138] For example, in the conversion of aromatic methyl groups and benzylic alcohols into the corresponding benzaldehydes,[139-140] for the conversion of diols to lactones,[141] in the deprotection of *para*-methoxyphenyl and *N*-benzyl protected amines,[142-143] and for the regeneration of the coenzyme $NAD(P)^+$.[144]

Laccase mediators can be either natural or synthetically derived. Structurally, natural mediators resemble lignin degradation products, such as acetosyringone, syringaldehyde, and *para*-coumaric acid.[145] The fact that these lignin derived compounds have proved to be efficient mediators in the LMS delignification of kraft pulp supports the role of fungal

laccases as delignifying enzymes in the environment.[146] Some common synthetic mediators include: ABTS, the *N*-hydroxy type mediators, such as violuric acid (VLA), *N*-acetyl-*N*-phenylhydroxylamine (NHA), *N*-hydroxybenzotriazole (HBT), and *N*-hydroxyphthalimide (HPI), and the radical 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) (Figure 35).[146] Depending on the structure of the mediator, the mechanism by which the LMS functions differs. Research suggests that ABTS follows an electron transfer (ET) mechanism, and that the N-OH type mediators follow a hydrogen atom transfer (HAT) route,[147] while TEMPO operates via an ionic mechanism (Figure 36).[148] Whether to use natural or synthetic mediators in LMS processes has been of much debate in recent years. While natural mediators have the advantages of being less toxic and less expensive than their synthetic counterparts, they are generally less effective.[149] The reasoning for this is that nitroxyl radicals formed via the oxidation of *N*-hydroxy compounds are more stable than the corresponding phenoxy radicals formed via the oxidation of phenols,[150] meaning that nitroxyl radicals will persist for a longer period of time and react more selectively.

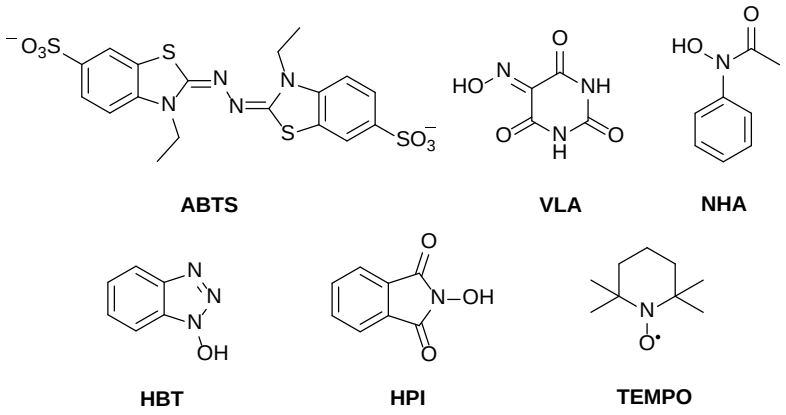


Figure 35. Chemical structures of common synthetic mediators used in LMS.[94]

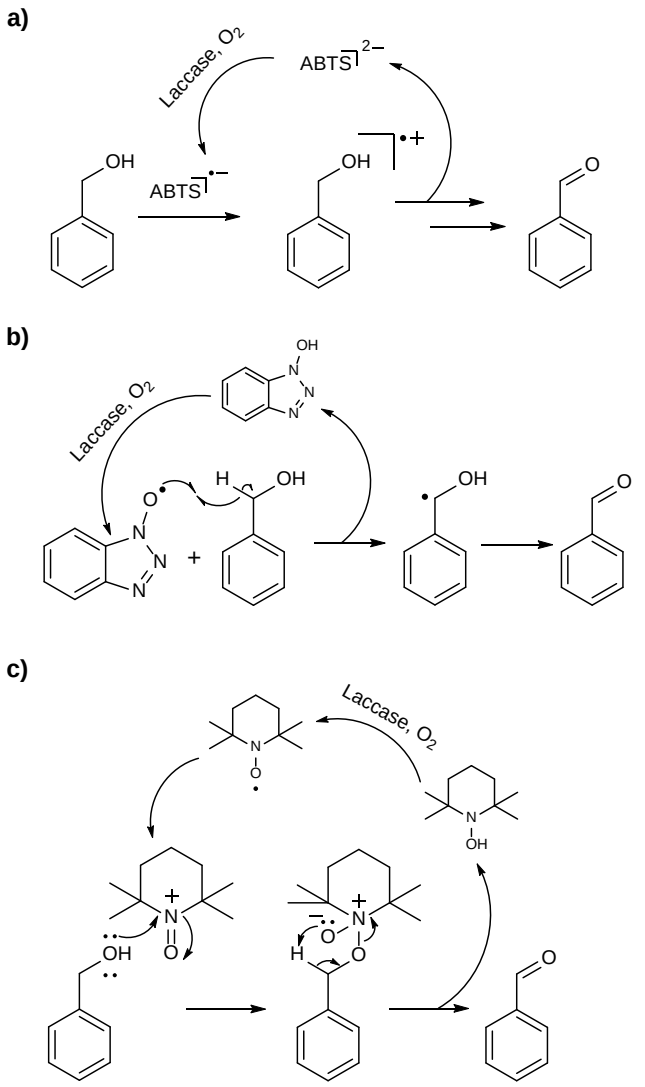


Figure 36. LMS routes. a) Electron transfer (ET) mechanism; b) Hydrogen atom transfer (HAT) mechanism; c) Ionic mechanism.[94]

2.4.7 Applications of Laccases in Organic Synthesis

Laccases have proved to be exciting catalysts in the field of synthetic organic chemistry in recent decades. They offer significant advantages over traditional chemical oxidants and transition metal catalysts, such as being renewable, biodegradable, relatively inexpensive, highly active in aqueous solvents and under mild conditions, and having a

broad substrate range which can be further expanded via the use of LMS. The versatility of these enzymes in organic synthesis is exemplified by the variety of chemical transformations they are capable of effecting. In section 2.4.5, the laccase catalytic mechanism, consisting of substrate oxidation and oxygen reduction, was discussed in detail. The resultant laccase-generated phenoxy radicals that are produced upon oxidation of phenolic compounds can undergo a variety of reactions, including: radical-radical coupling reactions of monomers for the synthesis of dimers, oligomers, and polymers; radical cross-coupling reactions; and *in situ* generation of *ortho*- and *para*-quinones from the corresponding catechols and hydroquinones, respectively, via disproportionation (Figure 37).[151]

In the following sections, examples of how laccases and LMS have been applied to effect carbon-carbon and carbon-heteroatom (O, N, and S) bond forming reactions under environmentally benign conditions will be discussed. Given that there exists over 200 published accounts on the use of laccases for organic synthesis purposes at the point in time this dissertation was written, the following is not meant to be exhaustive, rather it is intended to be illustrative of the types of reactions that can be afforded and a means to gauge and compare how the current dissertation work is different and original from previous research. For comprehensive accounts of laccase-catalyzed reactions, the reader is encouraged to consult recent reviews.[14-15]

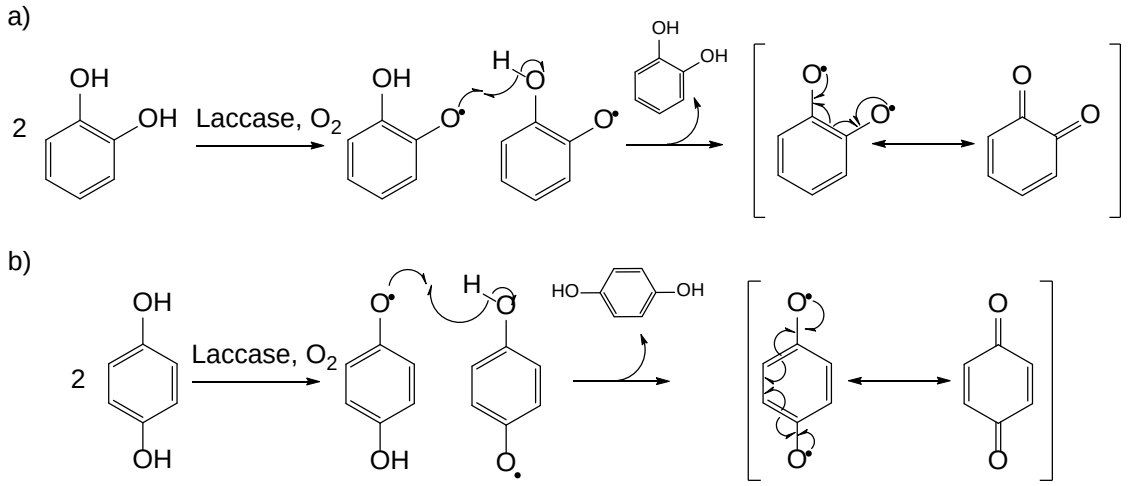


Figure 37. Laccase-initiated disproportionation of a) catechols into *ortho*-quinones, and b) hydroquinones into *para*-quinones.

2.4.7.1 Laccase-Catalyzed Radical-Radical Coupling, Dimerization, and Polymerization Reactions

The laccase-catalyzed coupling of naturally occurring bioactive compounds has been the focus of much research. Two different fungal laccases have been successfully employed in the dimerization of *trans*-resveratrol in aqueous solvent under mild conditions (Figure 38). Resveratrol is a phenolic compound present in dietary plants as well as red wine that exhibits strong antioxidant activity and is believed to have positive implications in cancer and heart disease prevention. The dimer also exhibits strong antioxidant properties in assays with 2,2-diphenyl-1-picrylhydrazyl free radical.[152] Subsequent studies focused on broadening the scope of the laccase-catalyzed dimerization of resveratrol were performed by reacting laccases with a glycosylated derivative of resveratrol, namely piceid,[153] and various hydroxystilbene analogs of resveratrol.[154] All dimers were produced via radical-radical couplings between carbon-

carbon radicals and carbon-oxygen radicals. In a follow up study a decade later, the laccase-synthesized resveratrol dimers were further oxidized using the chemical oxidant 2,3-dichloro-5,6-dicyano-*para*-benzoquinone to afford the benzofuran framework, which is present in many biologically active molecules.[155]

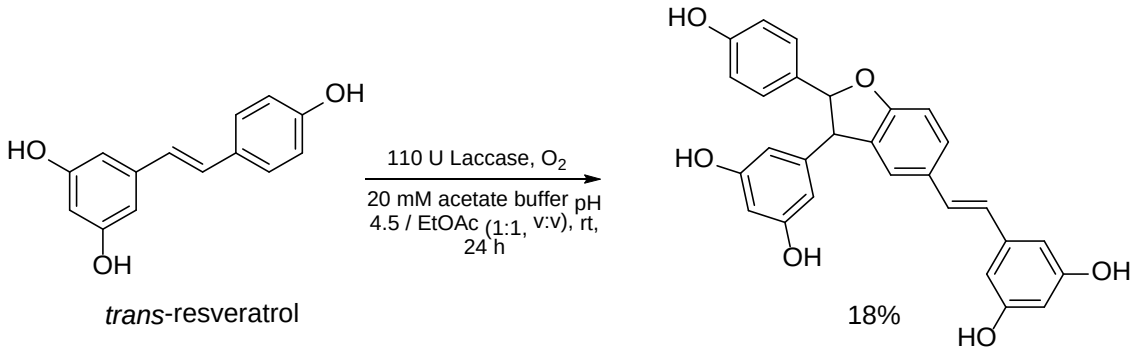


Figure 38. Laccase-catalyzed dimerization of *trans*-resveratrol.[152]

Laccases from the fungus *Trametes pubescens* have been used to dimerize totarol in an aqueous/organic solvent system (1:1, v:v) in high conversions, the extent to which was highly dependent on the organic cosolvent (Figure 39). Totarol is a diterpenoid that possesses potent antibacterial effects and has found much use in cosmetic and personal care products. The use of laccases to synthesize the dimers, which were formed via either a C-C or C-O linkage, was far more superior in regards to product yields compared to using chemical oxidants, such as MnO_2 and $FeCl_3$.[156] Similar conditions have been used to synthesize C-C and C-O dimers of the flavonolignan silybin. Crucial to successful dimerization was selectively protecting specific hydroxyl groups on silybin so as to inhibit polymerization. The dimers exhibit good radical scavenging abilities.[157] The dimerization of silymarin flavonolignans was also achieved in a similar fashion.[158] Very recently, *Trametes versicolor* laccases have been employed in a radical-radical

dimerization reaction for the synthesis of dihydrobenzofuran neolignanamides under mild conditions in short reaction times. The products displayed potent antiproliferative activity towards a few different cancerous human cells, such as colon, mammary, and prostate.[159] Laccases from *Coriolus versicolor* have been employed in the dimerization of penicillin X ester derivatives via a radical-radical coupling mechanism. The penicillin X dimers exhibited good antibacterial activity.[160]

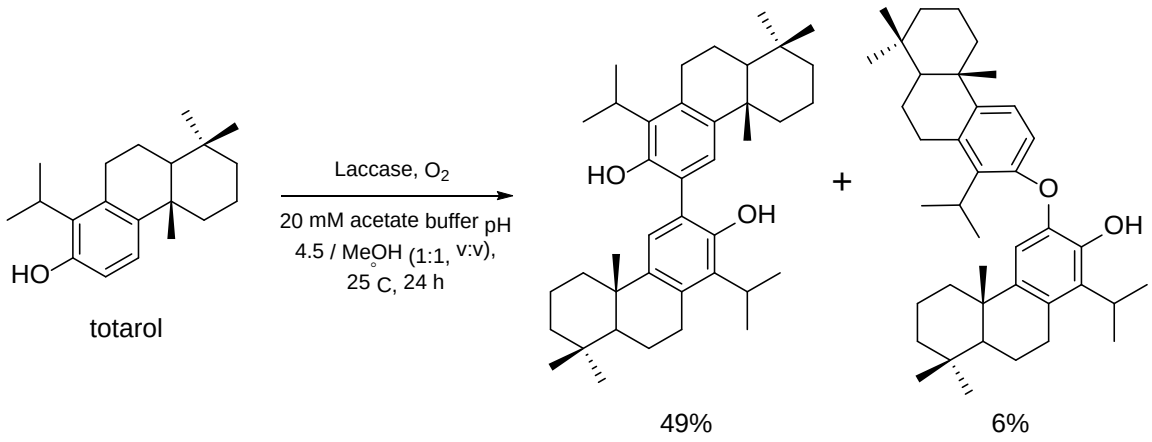


Figure 39. Laccase-catalyzed dimerization of totarol.[156]

Laccase-catalyzed heteromolecular radical coupling of catharanthine and vindoline was employed to synthesize the bisindole alkaloid anhydrovinblastine, a natural product that possesses anti-tumor and anti-cancer properties. The synthesis exploits the aromatic amine groups on both reactants, which are susceptible to laccase oxidation, to produce resonance-stabilized radicals that can couple with one another. A final reduction with $NaBH_4$ affords the final product in 56% yield.[161]

Laccase-catalyzed dimerizations are not only applicable to phenolic compounds, but can also be used for the dimerization of aromatic amines. For example, it has been demonstrated that 2,4–diaminodiphenylamine can be dimerized to yield the

benzocarbazole framework in good yield under mild reaction conditions utilizing a bacterial laccase.[162] In a similar way, the laccase-catalyzed dimerization of *ortho*-aminophenols has led to the synthesis of novel phenoxazinone dyes. The dimerization of 4-methyl-3-hydroxyanthranilic acid catalyzed by laccases immobilized in polyacrylamide gel resulted in the synthesis of actinocin (Figure 40), which is a chromophore present in actinomycin antibiotics.[163] Related head-to-tail dimers with tuneable water solubility and good antioxidant properties have been synthesized using sulfonated derivatives of 3-hydroxyanthranilic acid.[164-167] Laccases have also been useful in the trimerization of indole and sesamol.[168-169]

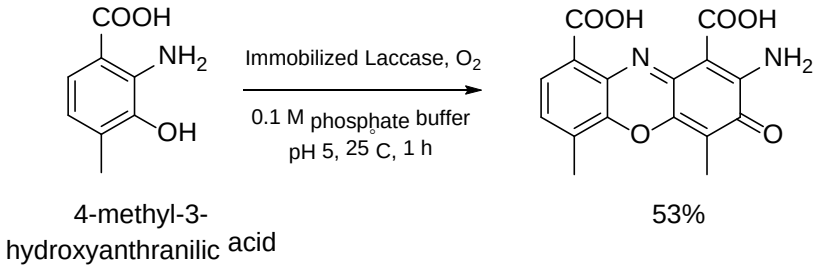


Figure 40. Laccase-catalyzed dimerization of 4-methyl-3-hydroxyanthranilic acid.[163]

Greener fluorination methods have been identified by the ACS GCI as a key research focus area in the field of organic synthesis in the coming years because fluorinated compounds represent a class of important building blocks for many pharmaceuticals and advanced materials. Toward this, Simon and coworkers have developed an elegant approach to the trifluoromethylation of phenols by combining either Langlois' reagent ($NaSO_2CF_3$) or Baran's zinc sulfonate ($Zn(SO_2CF_3)$) with laccases from *Agaricus bisporus* (Figure 41).[170] The trifluoromethyl-substituted phenols are synthesized via a radical-radical coupling reaction between the laccase-generated

phenoxy radical and the peroxide-generated trifluoromethyl radical. Products are afforded in good yield with high regioselectivity under mild conditions, and the reaction protocol is tolerant of most common functional groups present on the phenol.

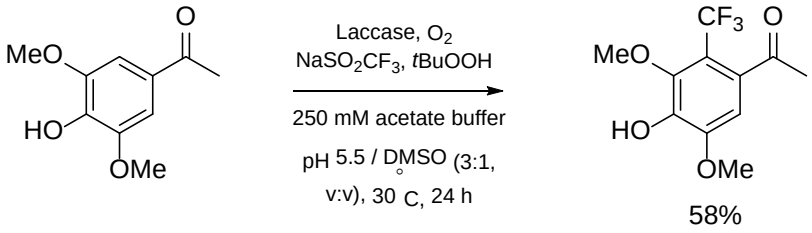


Figure 41. Laccase and Langlois' reagent catalyzed trifluoromethylation of phenols.[170]

Kurisawa and coworkers have studied the laccase-induced polymerization of two major flavonoids present in many plants, catechin and rutin, the latter being a glycosylated flavonoid.[171-172] Both polyphenol monomers efficiently polymerized using *Myceliophthora* laccases in an aqueous/organic solvent system at room temperature. Electron spin resonance (ESR) analyses of both polymers indicated the presence of a radical species in each polymer, which may be responsible for the increased radical scavenging and antioxidant activities compared to the catechin and rutin monomers. In a subsequent study by another group, laccase-generated poly(catechin) was coupled with the antibiotics trimethoprim and sulfamethoxazole using *N*,*N*′-disuccinimidyl carbonate as the coupling reagent.[173] The poly(catechin)–antibiotic conjugates were then coated onto the surface of silicone and polyurethane catheters via laccase-catalyzed coupling. The poly(catechin)–antibiotic functionalized catheters significantly reduced bacterial adhesion to the catheters, thus present a promising strategy to reduce catheter-associated infections. Laccase-mediated polymerizations of *N*-vinylimidazole,[174] *m*-

phenylenediamine,[175] urushiol,[176] taxifolin,[177] 8-hydroxyquinoline,[178] and catechol have also been documented.[179]

2.4.7.2 Laccase-Catalyzed C-C Bond Forming Reactions Involving Catechols and Hydroquinones

Carbon-carbon bond forming reactions are arguably the most important type of chemical transformations in organic synthesis. The use of carbon based nucleophiles to perform laccase-catalyzed cross-coupling reactions provides a green method for C-C bond formation between hydroquinones or catechols and compounds containing an acidic methylene or methine proton.[180] The first reaction of this type was described in 2005 when the activated methylene group of heterocyclic 1,3-dicarbonyls were employed as the nucleophiles to react with substituted catechols for the synthesis of benzofuran ring systems via a laccase-catalyzed domino reaction that proceeded via way of a laccase-generated *ortho*-quinone intermediate (Figure 42).[181] The products of interest possess a benzofuran core, which is an important structural motif present in many pharmacologically active compounds exhibiting antioxidant, antimicrobial, and anti-HIV-1 properties.[182]

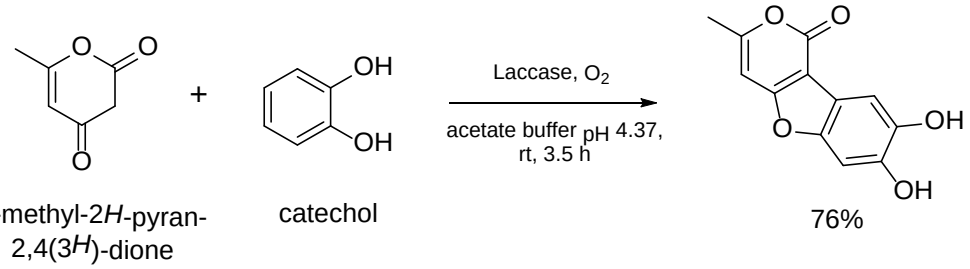


Figure 42. Laccase-catalyzed coupling of catechol with a heterocyclic 1,3-dicarbonyl.[181]

Our group has studied the oxidation-Michael addition cascade of aliphatic 1,3-dicarbonyls with catechol and 3-methylcatechol. The addition of a water stable Lewis acid, such as $Sc(OTf)_3$ or $Yb(OTf)_3$, provided an increase in product yields, presumably by coordinating with the carbonyl groups of 1,3-dicarbonyls to promote the Michael addition step (Figure 43).[183] This reaction highlighted the opportunity to combine classical inorganic catalysis with biocatalysis, an exciting field of future research. In a follow up study, we called upon the aptitude of a lipase to assist in promoting the Michael addition step.[184] This cocatalytic enzyme system was regarded as a greener alternative compared to the previous system where $Sc(OTf)_3$ was employed as it eliminated hazardous waste associated with the transition metal catalyst; however, the product yields were generally slightly lower. A common feature of the two aforementioned cocatalytic systems is the recyclability of the reaction medium.

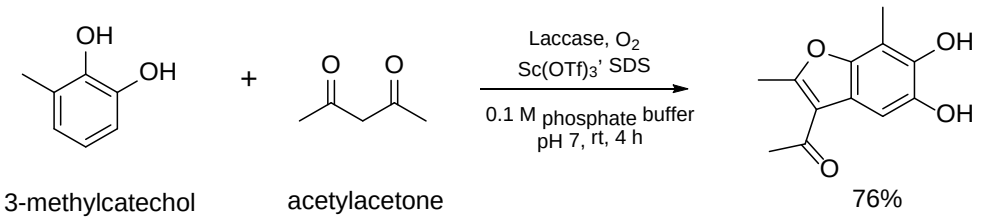


Figure 43. Laccase-catalyzed coupling of 3-methylcatechol with a 1,3-dicarbonyl.[183]

Since these initial studies, several original research articles have been published on the laccase-catalyzed oxidation-Michael addition of catechols with aliphatic, cyclic, and heterocyclic 1,3-dicarbonyls for the synthesis of a variety of benzofuran products,[185-189] 3-arylated 4-oxochromanes,[190] α-arylated cyclic β-dicarbonyl compounds,[191] and substituted *para*-benzoquinones.[192] These studies exemplify the versatility of this enzyme initiated cascade reaction as a legitimate synthetic tool for organic chemists.

The acidic methylene group of pyrazolinones has also been exploited to effect laccase-catalyzed C-C bond forming reactions. The coupling of 3-methyl-1-phenyl-pyrazolin-5-one or 3-methyl-pyrazolin-5-one with both catechols and hydroquinones utilizing laccases from *Pycnoporous cinnabarinus* yielded the benzofuro[2,3-c]pyrazole derivatives in good yields under mild reaction conditions.[193] This approach has also been used to couple 3-*tert*-butyl-1*H*-pyrazol-5(4*H*)-one with several substituted catechols.[194] Along similar logic, the laccase-initiated coupling of 3-substituted oxindoles and catechols has been achieved in high yields and high regioselectivity in a phosphate buffer/acetonitrile (2:1, v:v) solvent system.[195] The transformation effectively adds an aryl group at the C-3 position of oxindoles by exploiting the reactivity of the methine carbon.

Another C-C bond forming reaction that has been catalyzed by laccases is a Diels-Alder reaction. Drawing upon the previously discussed work of Rideout and Breslow for inspiration,[41] our group has developed the first and only laccase-catalyzed Diels-Alder reaction to date. In our aqueous one-pot protocol, laccases were employed to couple both catechols and hydroquinones with assorted dienes for the synthesis of 1,2-naphthoquinones and 1,4-naphthoquinones, respectively (Figure 44).[196-197] The idea is similar to other reactions involving catechols and hydroquinones in that laccases are employed to generate the reactive quinone intermediate which can then go on to react with the diene in a 4+2 cycloaddition. Depending on the hydroquinone and diene employed, two types of products were formed: in the majority of cases, complete oxidation occurred to yield the aromatized 1,4-naphthoquinone products, however, when

the dienes possess terminal alkyl substituents, complete oxidation did not occur and the final products lack aromaticity.

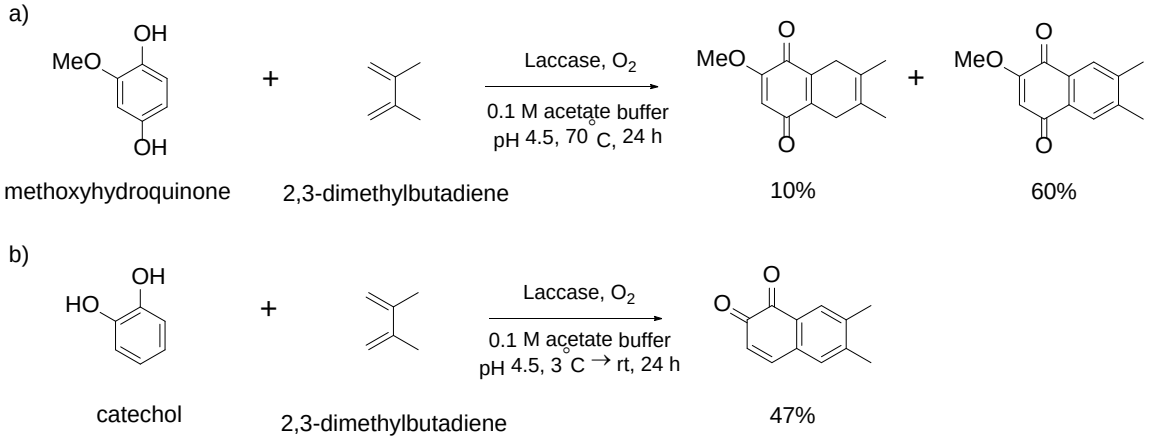
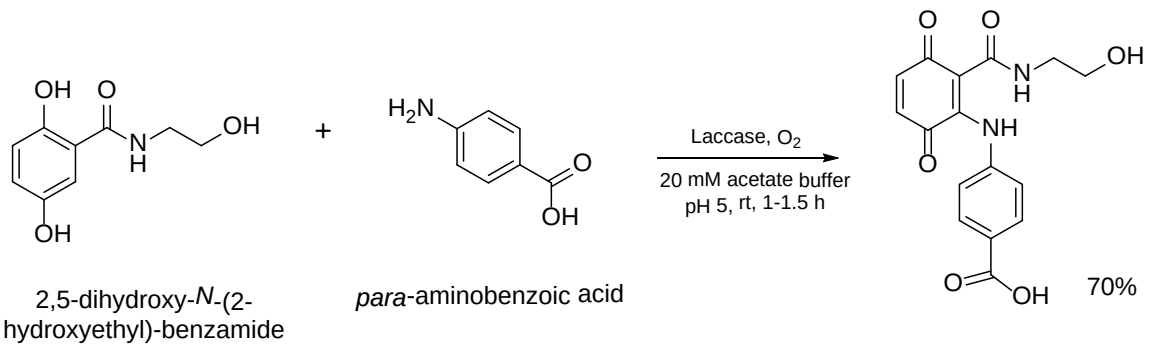


Figure 44. Laccase-catalyzed Diels-Alder reaction between dienes and a) hydroquinones or b) catechols.[196-197]

2.4.7.3 Laccase-Catalyzed C-N Bond Forming Reactions Involving Catechols and Hydroquinones

Laccase-catalyzed carbon-nitrogen bond forming reactions occur via Michael addition of amines to laccase-generated *ortho*- and *para*-quinones. One of the first accounts of such a reaction was described by Niedermeyer et al. in 2005 when fungal laccases were used to couple variously substituted hydroquinones with a variety of *para*-substituted anilines (Figure 45).[198] The reactions proceeded in an aqueous buffer at room temperature to provide very good yields of mono- and diaminated *para*-quinones.

Nuclear amination of 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide or 2,5-dihydroxybenzoic acid methyl ester with *para*-aminobenzoic acid catalyzed by laccases from *Trametes villosa* yielded the mono-aminated *para*-quinones in high yields.[199] In a later study by the same group, amination of hydroquinones with various alkyl, alkoxy,



2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide

para-aminobenzoic acid

Figure 45. Laccase-catalyzed coupling of 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide with *para*-aminobenzoic acid.[198]

and halo substituents was achieved by reaction with *para*-aminobenzoic acid in the presence of fungal laccases at room temperature in a pH 7 citrate-phosphate buffer.[200] Products were generally formed in good yield as the mono- or diaminated adducts, with the regioselectivity of Michael addition to the *para*-quinone ring dictated by the nature of the substituent. Furthermore, product yields achieved using laccases as the oxidant were greater than when sodium iodate was used as the oxidant. *para*-Aminobenzoic acid has also been used to aminate the naturally occurring catechol dihydrocaffeic acid via laccase-catalysis. The authors postulate that the reaction does not occur via an *ortho*-quinone intermediate, rather through a radical coupling mechanism. The reaction also proceeds with the aliphatic amine *n*-hexylamine.[201] Laccase-catalyzed coupling of 1,4-dihydroxy-2-naphthoic acid with aniline and substituted anilines via amination was achieved in a succinate-lactate buffer/DMF (1:1, v:v) solvent system at room temperature. The mono-aminated naphthoquinones were synthesized in good to excellent yields. The products exhibited potent cytostatic effects against a number of cancer cell lines, including melanoma, breast, and renal.[202]

The Michael addition of amino acids with laccase-generated *para*-quinones has proved to be a valuable method for the derivatization of several amino acids, such as L-phenylalanine and L-tryptophan (Figure 46).[203-204] Hahn and colleagues compared the biocatalytic derivatization of amino acids using laccases with the chemical route that employs sodium iodate.[205] In this study, both L-Phe and L-Trp were reacted with hydroquinone and substituted hydroquinones in the presence of laccase or sodium iodate. As was observed in the reactions of *para*-hydroquinones with *para*-aminobenzoic acid, product yields of the mono- or diaminated *para*-quinones were much greater when laccase was employed, indicating the biocatalytic route is superior in this amino acid derivatization protocol. In a later study by the same group, L-lysine was reacted with hydroquinones under laccase catalysis to yield Lys substituted *para*-quinones.[206] Structural characterization of the products revealed that Lys could bond to the laccase-generated *para*-quinone via either the α- or ε-amino group to yield a complex mixture of products. The study was extended further to the reactions of a lysine-tyrosine dipeptide and oligopeptides with dihydroxylated aromatic compounds. The products mimic the naturally occurring mussel adhesive proteins, thus, this synthetic methodology is regarded as a means to produce functional biomaterials. In another recent study, the alanine-histidine dipeptide carnosine was coupled with ferulic acid via laccase catalysis in an aqueous solution under mild conditions. The synthesized hydroxycinnamoyl-peptides exhibited significantly enhanced antioxidant and antiproliferative properties compared to carnosine.[207]

Just as amino acids have been derivatized by laccase-catalyzed couplings with hydroquinones and catechols, so to have various antibiotics. Mikolasch and coworkers

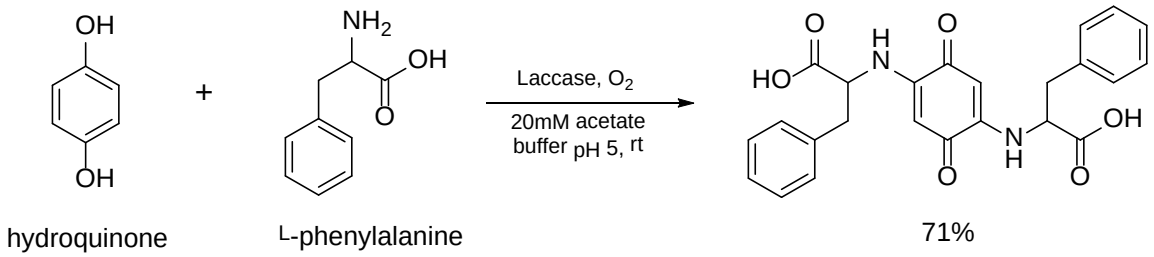


Figure 46. Laccase-catalyzed coupling of hydroquinone with L-phenylalanine.[203]

successfully coupled a variety of *β*-lactam antibiotics, such as ampicillin, amoxicillin, cefadroxil, cefalexin, cefaclor, and loracarbef with derivatives of 2,5-dihydroxybenzoic acid and methyl substituted catechols using laccases from *Trametes spec.* or *Myceliophthora thermophila* in aqueous solvent at room temperature (Figure 47).[208-210] The newly synthesized antibiotics inhibited the growth of several strains of gram positive bacteria. A later study identified methylhydroquinone and 2,3-dimethylhydroquinone as laccase substrates that exhibit the greatest antibacterial activity out of thirty-eight screened hydroquinones and catechols.[211] Thus, these two hydroquinones were then cross-coupled with the previously mentioned *β*-lactam antibiotics via laccase-catalysis. The derivatized *β*-lactam antibiotics exhibited very promising antibacterial properties. In another study by the same research group, *N*-analogous corollosporines, which are antimicrobial natural products found in the marine fungus *Corollospora maritima*, were derivatized by coupling with 2,5-dihydroxybenzoic acid derivatives. As it turns out, the derivatized *N*-analogous corollosporines exhibited greater antimicrobial properties than did the parent corollosporines.[212] Novel morpholine derivatives with biological activity have also been synthesized in a similar fashion.[213]

A similar synthetic protocol was used to derivatize 1-aminobenzotriazole by coupling it with methyl and ethyl esters of 2,5-dihydroxybenzoic acid mediated by

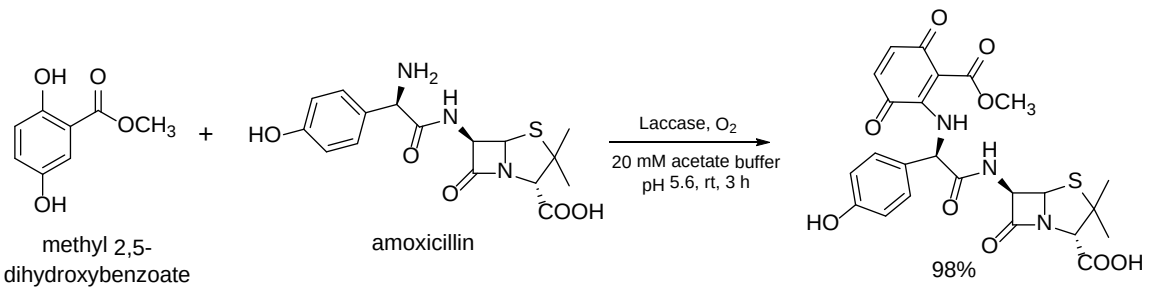


Figure 47. Laccase-catalyzed coupling of methyl 2,5-dihydroxybenzoate with amoxicillin.[208]

laccase catalysis, albeit, in poor yields.[214] Earlier, this methodology had been used for the synthesis of tinuvin, a UV-absorber that is a component of many polymer formulations, specifically by coupling 1*H*-benzotriazole with 3-(3-*tert*-butyl-4-hydroxyphenyl) propionic acid methylester via laccase catalysis.[215]

The laccase-catalyzed coupling of 2,5-dihydroxybenzoic acid and 2,5-dihydroxy-*N*-(2-hydroxyethyl)benzamide with compounds containing multiple nucleophilic amine moieties has been studied.[216] Amines such as 4-aminoimidazole-5-carboxamide and 3-aminopyrazole-4-carboxamide first added to an olefinic carbon atom of the laccase-generated *para*-quinone via the aromatic amino group, and then, following a second laccase-catalyzed oxidation, the aliphatic amino group added to the neighboring carbonyl group, yielding the cyclized adducts. When diaminopyridines with adjacent aromatic amino groups were used, phenazine products were formed.

The coupling of several primary aliphatic amines with 3-methylcatechol was achieved using both native and recombinant laccases. The products were formed via a laccase-generated *ortho*-quinone intermediate to which regioselective Michael addition of amines occurred at the C-5 position. A final oxidation resulted in the mono-aminated *ortho*-quinone products in moderate yields within 1-2 hours.[217] The laccase-mediated

coupling of catechols and hydroquinones with *N*,*N*′-dimethylethylenediamine has resulted in the synthesis of the heterocyclic quinoxalines in good yields (Figure 48).[218] A phosphate buffer at pH 7 proved to be the optimal reaction medium. This is the first time this important class of pharmaceutical intermediates has been enzymatically synthesized and this study further adds to the growing list of enzyme generated heterocyclic compounds.

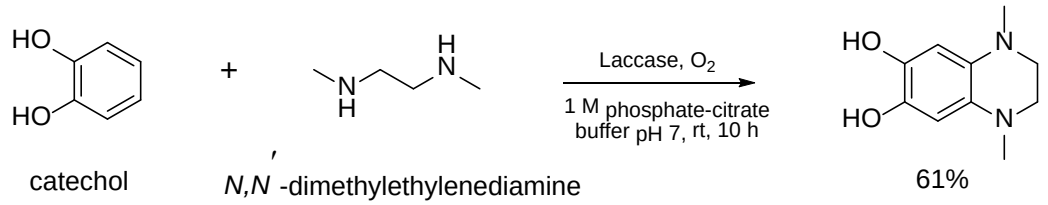


Figure 48. Laccase-catalyzed coupling of catechol with *N*,*N*′-dimethylethylenediamine.[218]

2.4.7.4 Laccase-Catalyzed C-S Bond Forming Reactions Involving Catechols and Hydroquinones

Accounts of laccase-catalyzed carbon-sulfur bond forming reactions have been far less studied than the analogous C-C and C-N bond forming reactions, possibly due to theories that suggest small sulfhydryl compounds are inhibitors of laccases. Despite this notion, studies entailing the successful laccase-catalyzed coupling of sulfhydryl compounds with hydroquinones and catechols do exist.

The laccase-catalyzed coupling of naphthohydroquinone and 1,4-dihydroxy-2-naphthoic acid with alkyl and aryl thiols for the synthesis of 1,4-naphthoquinone-2,3-bis-sulfides has been achieved in an aqueous/organic solvent mixture at slightly elevated temperature (Figure 49). Product yields were moderate, however, but the reaction

protocol eliminates the need for a chemical oxidant and sodium salts of alkyl thiols.[219] In a follow up study by the same group, the carboxylate moiety of 1,4-dihydroxy-2-naphthoic acid was first reduced with borane to yield the benzylic alcohol moiety before reaction with alkyl and aryl thiols and laccase. In this scenario, both mono- and dithiolated 1,4-napthoquinones were produced, the latter being the major product. Formation of the dithiolated products are thought to proceed via a rare laccase-generated *ortho*-quinone methide intermediate. Furthermore, C-C dimers of the monothiolated 1,4-napthoquinones were also identified in reasonable amounts, most likely formed via a radical-radical coupling mechanism.[220]

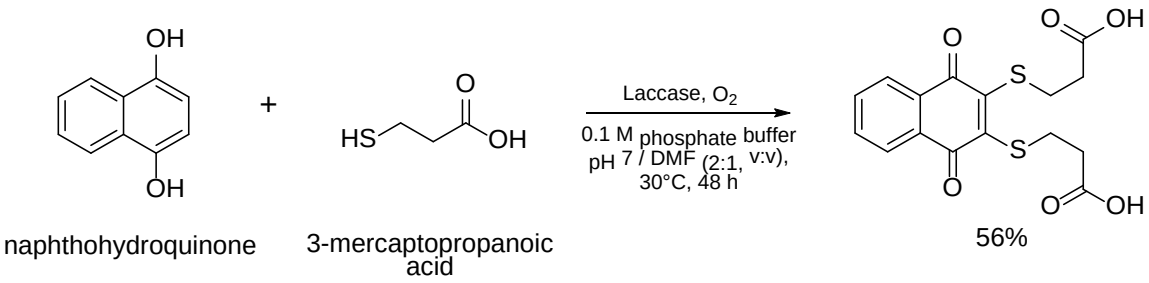


Figure 49. Laccase-catalyzed coupling of naphthohydroquinone with 3-mercaptopropanoic acid.[219]

The laccase-catalyzed coupling of catechols with thiols for the synthesis of catechol thioethers has been achieved. Abdel-Mohsen et al. reacted catechol and C-3 or C-4 substituted catechols with 2-mercaptobenzoxazole, 2-mercaptobenzothiazole, and 4,5-dihydrothiazole-2-thiol in a phosphate buffer/methanol solvent system using laccases from *Agaricus bisporus* (Figure 50).[221] The catechol thioethers were synthesized in up to 96% yield, however, the addition of thiols to laccase-generated *ortho*-quinones was not regioselective in many cases, with two regioisomers forming in many reactions.

Nonetheless, this study presents a mild, ecofriendly approach for the synthesis of an important class of biologically active molecules. A laccase-catalyzed domino reaction between catechol or 3-methylcatechol and 6-substituted 1,2,3,4-tetrahydro-4-oxo-2-thioxo-5-pyrimidinecarbonitriles that involves sequential C-N and C-S bond formations has been accompished.[222] The pyrimidobenzothiazole products were synthesized in excellent yields, but typically as regioisomeric mixtures.

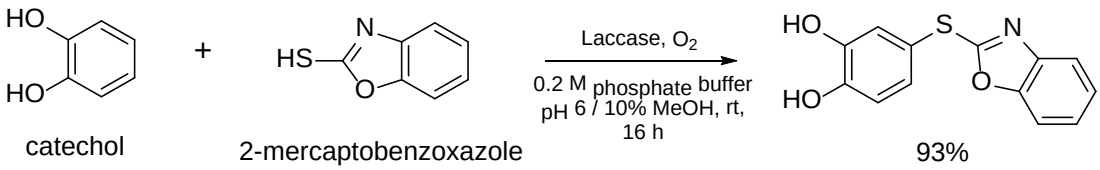


Figure 50. Laccase-catalyzed coupling of catechol with 2-mercaptobenzoxazole.[221]

An assortment of thiolated compounds have been synthesized in the laccase-mediated coupling of several hydroquinones with three aromatic thiols.[223] Depending on how many substituents were present on the hydroquinone, mixtures of mono-, di-, and trithiolated *para*-quinones were afforded under environmentally benign conditions. Furthermore, S-S dimer products were detected in the reactions of laccases with the aromatic thiols, indicating that laccases are capable of performing oxidative dimerizations of benzenethiols. Similar results have been obtained for the dimerization of heterocyclic thiols using LMS_{ABTS}.[224]

2.4.7.5 Laccase and LMS Catalyzed Oxidation of Alcohols and C-H Bonds

The oxidative versatility of laccases and LMS is exemplified by the abundance of oxidative transformations that have been documented. For example, LMS_{TEMPO} using laccase from the fungus *Trametes pubescens* has been used to oxidize the hydroxymethyl

group of mono- and disaccharides in a slightly acidic aqueous buffer at room temperature to yield the corresponding carboxylic acids in moderate yields (Figure 51).[225] A follow up study demonstrated that the same laccases immobilized onto the surface of Eupergit C250L could be used in the LMS_{TEMPO} oxidation of natural glycosides to provide increased conversion and in addition, the immobilized biocatalysts could be reused in up to ten subsequent reaction cycles without loss of activity.[226]

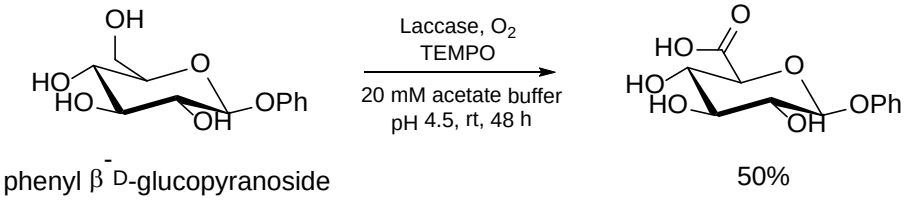


Figure 51. LMS_{TEMPO} oxidation of phenyl *β*-D-glucopyranoside.[225]

Laccases from *Trametes villosa* in combination *N*-hydroxy mediators (HBT and HPI) have been successfully employed in the bioconversion of benzylic and cyclic ethers into benzoate esters and lactones, respectively. Reactions proceeded via a HAT mechanism, and the *N*-hydroxy mediators proved to be superior than using LMS_{TEMPO} in regards to product yields.[227] The same LMS was employed for the oxidation of alkyl substituted amides and lactams for the synthesis of imides in reasonable yields. As in the case of ethers, product formation occurs via a HAT mechanism.[228] The chemoselective C-4 oxidation of catechins as well as regioselective oxidation of certain steroids have also been achieved using $LMS_{HBT.}$[229-230] Additionally, LMS using laccases from *Trametes versicolor*, either free or immobilized, have been used for the oxidation of various polyaromatic hydrocarbons resulting in the introduction of carbonyl groups onto the hydrocarbon skeleton.[231-233]

LMS have been used extensively for the selective, mild oxidation of benzylic alcohols into benzaldehydes. Potthast et al. were the first to describe such a method that employed LMS_{ABTS} to convert benzylic alcohols into benzaldehydes in almost quantitative yields (Figure 52). For this reaction protocol to work however, the benzylic alcohol must have atleast one free *ortho* position.[140] These results were supported a few years later by the work of another gorup.[234] An LMS_{TEMPO} catalytic system has also been applied to the selective oxidation of benzylic and aliphatic alcohols to yield the corresponding aldehyde products. The product yields are much greater when benzylic alcohols are reacted compared to when the aliphatic alcohols are used.[235] Very recently, this catalytic system has evolved to incorporate laccases immobilized onto magnetic nanoparticle supports.[236] The immobilized laccases exhibited greater catalytic activity and greater temperature and pH stability compared to the free enzymes, and in combination with TEMPO, could be used to convert benzylic alcohols into the corresponding benzaldehydes in almost quantitative yields. Furthermore, the immobilized enzyme system could be reused in six subsequent cycles without much loss in activity. Laccases immobilized in Na-Alginate matrix have been used for the oxidation of glycerol to produce glyceric acid utilizing TEMPO as a mediator.[237]

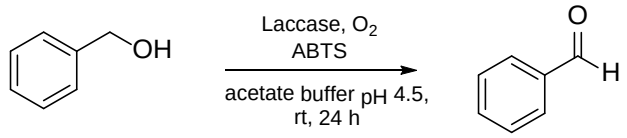


Figure 52. LMS_{ABTS} oxidation of benzyl alcohol to benzaldehyde.[140]

LMS have been utilized widely for the selective oxidation of aromatic methyl groups to yield benzaldehydes, with no further oxidation to benzoic acids. The first account of such a reaction protocol was described in 1995 by Potthast and coworkers, who used LMS_{ABTS} to convert toluene and substituted toluenes into the corresponding aldehydes in excellent yields under mild conditions (Figure 53).[139] Since this initial account, similar studies have been published on the subject.[238-239] Subsequent studies utilizing HBT or HPI as the mediator also proved to be successful in converting aromatic methyl groups into aldehydes, as well as allylic alcohols into the corresponding aldehydes.[240] Very recently, novel yellow laccases have been iosolated from new strains of the fungi *Coriolopsis floccosa*, *Trametes hirsuta*, *Daedalea flavida,* and *Fomes durissimus* and used to oxidize aromatic methyl groups to aldehydes in excellent yields ($\geq$ 87%) in less than two hours in the absence of a mediator.[241-244]

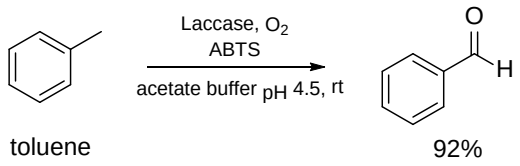
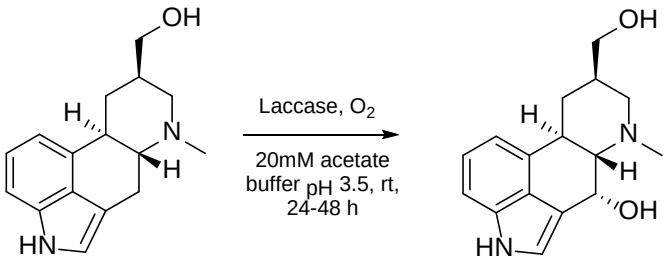


Figure 53. LMS_{ABTS} oxidation of toluene to benzaldehyde.[139]

Stereoselective C-4 hydroxylation or ergot alkaloids, a class of important bioactive natural compounds, has been achieved using laccases from *Trametes versicolor* (Figure 54). Modification at the C-4 position of ergot alkaloids had yet to be achieved with other methods. Product formation is proposed to proceed through a laccase-generated enimminium intermediate which is then subjected to a nucleophilic attack by water to yield the final product.[245]



lysergol 35%

Figure 54. Laccase-mediated stereoselective C-4 hydroxylation of lysergol.[245]

2.5 Lignin Valorization

In the early 1900s, it was common practice to produce fuels, materials, and chemicals for industrial purposes from terrestrial plants and trees. However, midway through the century, this reliance upon biomass as a source for the production of these necessary commodities had shifted well in favor of petroleum resources.[246] And it's not hard to understand why: petroleum was cheap, abundant, and, contrary to biomass, inexpensively and effortlessly processed into marketable products. Therefore, it seemed logical, and for many companies was a sound business strategy. Fast-forward ahead to the 21st century, petroleum supplies are dwindling, and the public is becoming increasingly aware of the impact fossil fuels have on contemporary society's carbon footprint and the associated negative environmental consequences. Thus, for life on Earth as we know it to be sustainable, the paradigm needs to be shifted once again, reverting back to renewable, carbon-neutral biomass as the primary source for fuels, chemicals, and materials. Introducing the biorefinery, the biomass analog of the long standing oil refinery. In an idealized model, the biorefinery will separate biomass into its basic

chemical constituents and transform each individual component into a marketable product, maximizing yield and complete use of this renewable resource.[247]

2.5.1 Woody Biomass

Woody biomass consists of an intertwined matrix of three main constituents – cellulose, hemicellulose, and lignin (Figure 55) – and minor amounts of extractives and

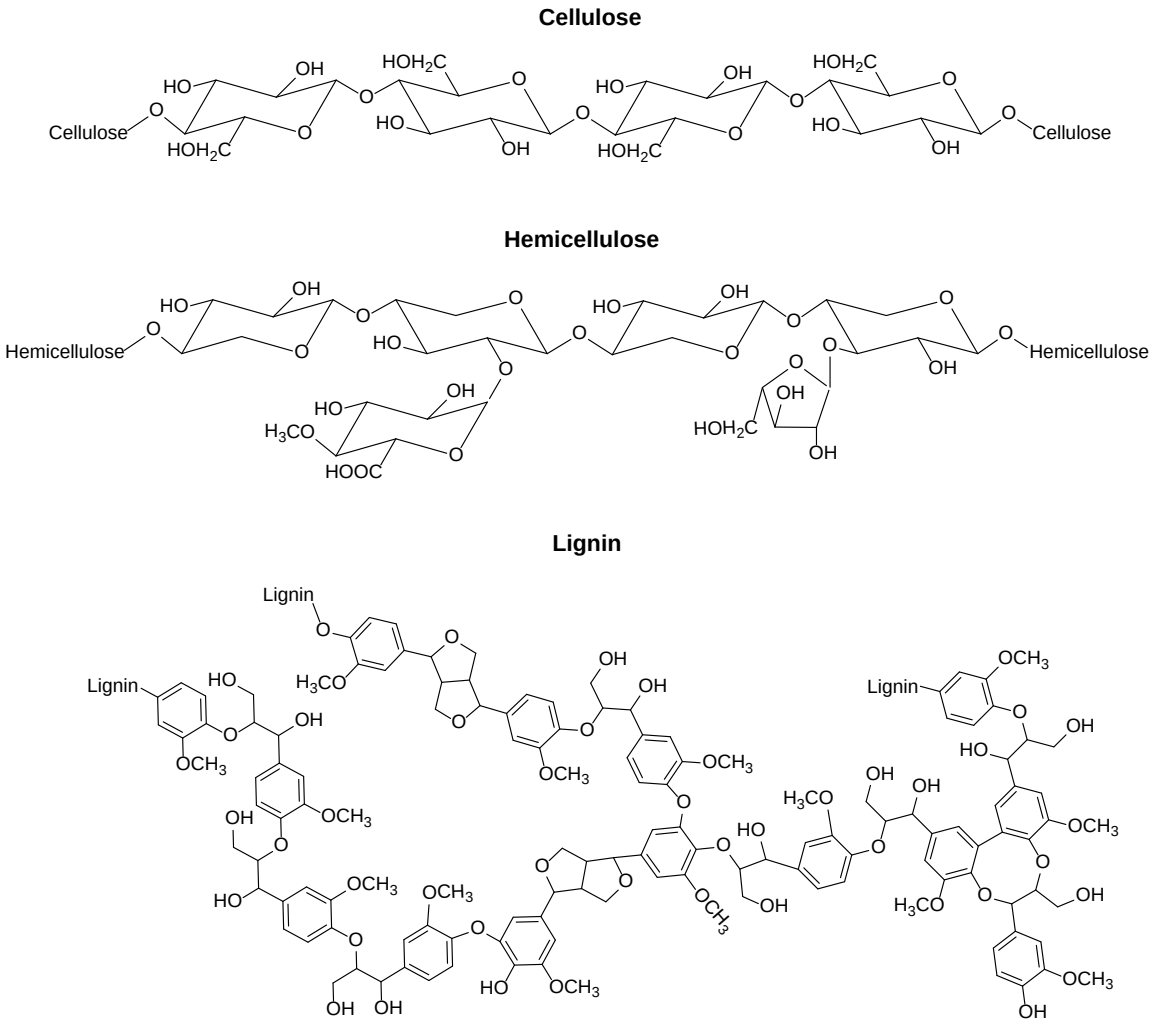


Figure 55. The chemical structures of cellulose, hemicellulose, and softwood lignin.[248]

minerals. While the relative proportion of each varies from source to source, woody biomass generally contains 35-50% cellulose, 25-30% hemicellulose, and 15-30% lignin.[247] The vast majority of the polysaccharides present in woody biomass are used to produce paper products and, more recently, enzymatically hydrolyzed and subsequently fermented to produce bioethanol, while a smaller portion is used for the production of fine chemicals.[247] Utilization of lignin, on the other hand, has not been as prosperous, thus it is largely regarded as a waste material.

2.5.2 Lignin

Lignin is a highly irregular, amorphous polymer of oxidatively coupled 4-hydroxyphenylpropanoid units (Figure 55) and is the second most abundant terrestrial biopolymer on Earth, surpassed only by cellulose. In woody biomass, lignin provides mechanical support, a means for water conduction throughout the tree or plant, and resistance to microbial degradation. The three main monolignols which comprise lignin are *p*-coumaryl alcohol (H), coniferyl alcohol (G), and sinapyl alcohol (S), all of which are derived from phenylalanine (Figure 56). The type and amount of monolignol that the

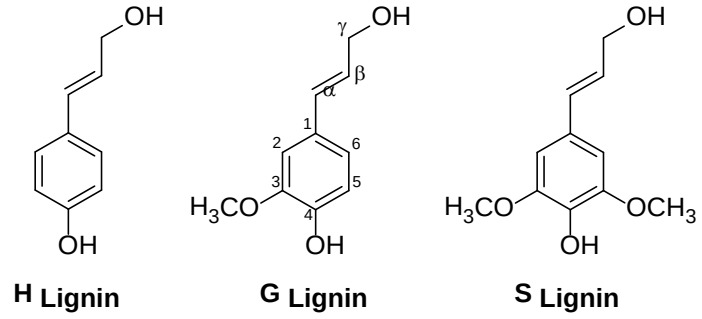


Figure 56. Structures of monolignols: *p*-coumaryl alcohol (H), coniferyl alcohol (G), and sinapyl alcohol (S).[248]

lignin macromolecule is composed of depends on the nature of the woody biomass. In general, hardwoods contain primarily S and G lignin, whereas softwoods consist of predominantly G units with a minor fraction of H units.[249] The biosynthesis of the lignin polymer occurs *in situ* via the action of oxidative enzymes, such laccases and peroxidases, which generate phenoxy radicals on the monolignols that are then able to undergo radical-radical couplings to produce C-C and C-O bond linkages. The lignin polymer chain thus grows by one unit at a time via radical-radical couplings in a process known as endwise coupling.[249] There are several types of lignin structural linkages that are synthesized via this enzymatic radical coupling. For example the β-O-4 structural unit (Figure 57), which is the most prevalent structural linkage accounting for approximately 45-50% of the linkages in softwood lignin,[250] is generated via radical coupling between a phenoxy radical and a carbon radical at the β position.

Production of lignin on an industrial scale has historically occurred as a by-product of kraft pulping in the paper making process. Briefly, the kraft pulping process

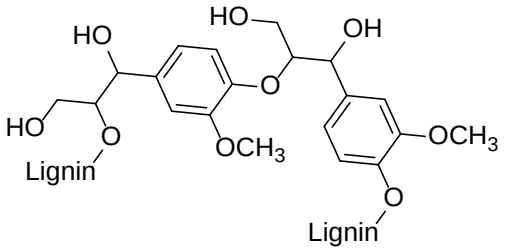


Figure 57. The β-O-4 linkage in softwood lignin.

involves separating cellulose fibers from lignin by employing highly alkaline conditions that promote the selective degradation and solubilization of the lignin component of wood without degrading cellulose to a large extent. What results are high quality

cellulose fibers which can then be used in the fabrication of assorted paper products, and a solution of dissolved lignin, known as black liquor (Figure 58). The solubilized lignin can be precipitated by increasing the acidity of the solution to produce solid kraft lignin via a process known as the LignoBoost process (Figure 59).[251] In this way, lignin is produced on the order of 50 million tons a year, approximately only 2% of which is used for value-added products, such as dispersants and binders, while the remainder is burned as an inefficient fuel to power the pulping process.[252-254] In recent decades, the increased demand for second generation biofuels (i.e. lignocellulosic derived ethanol) and the associated advent of industrial cellulosic ethanol plants has resulted in an overwhelming increase in lignin production as a co-product of different biomass conversion technologies. It has been forecasted that approximately another 60 million tons/year of lignin will be produced within the next decade in the US alone.[255] Thus, it is imperative that robust methods for lignin valorization be developed for the fully integrated biorefinery to be implemented.[256]

Figure 58. Black liquor from kraft pulping process.[248]

Figure 59. Precipitated kraft lignin.[248]

2.5.3 Laccase-Assisted Functionalization of Lignin

One strategy that has proved to be highly successful assisting in the conversion of lignin into marketable products is enzymatic modification.[257-258] Using enzymes to modify the structure of lignin provides environmental and economic advantages over chemical or thermal processes, such as mild reaction conditions, the use of renewable and inexpensive biocatalysts, and reduction in the use of toxic chemicals. In essence, it is a completely biotechnological approach. Laccases have received much use in the field of lignocellulosic fiber modification to create paper products with increased strength, hydrophobicity, and antimicrobial properties.[259] Regarding the use of laccases to modify lignin to create value-added materials and chemicals, much success has been achieved in the generation of resin free particle boards, such as medium-density fiberboard, and wood composites that have comparable strength and mechanical properties to those containing artificial resins.[260] This is particularly important given the growing concerns and knowledge on the negative health and environmental impacts of formaldehyde emissions from typical phenol-formaldehyde resins currently used as binders in particle boards.[261]

The binding phenomenon observed upon laccase treatment is due to laccase-generated phenoxy radicals on the lignin component of the fiber surface, which are capable of undergoing radical-radical coupling reactions with one another that lead to an increase in bonding among fibers.[262-263] Thus, this method of lignin modification mimics the very process that occurs in nature.

Following similar principles, novel lignin copolymers can be synthesized utilizing laccases to graft molecules onto the surface of lignin, via either a radical-radical coupling mechanism or a nucleophilic addition via a quinone methide intermediate (Figure 60). In this way, novel lignin-derived biomaterials possessing biodegradable properties can be crafted. In the past, Milstein and coworkers were able to demonstrate the copolymerization of kraft lignin and organosolv lignin with low molecular weight compounds, such as vanillic acid, 4,4′-methylenediphenyl diisocyanate, and acrylamide,

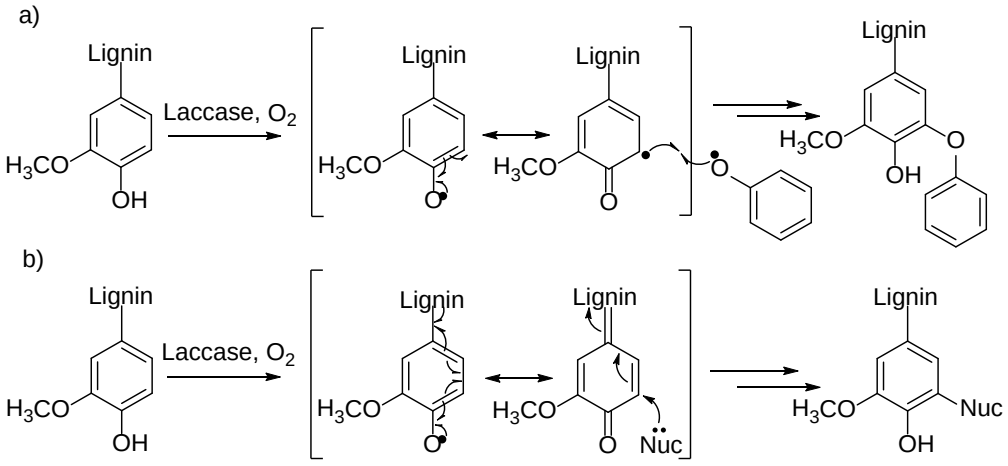


Figure 60. Laccase-catalyzed grafting reactions: a) Radical-radical coupling mechanism; b) Nucleophilic addition mechanism.[248]

in the presence of a fungal laccase.[264] It has also been previously established that a fungal laccase is able to graft water soluble phenols, such as guaiacol sulfonate and 4-

hydroxyphenylacetic acid, onto the surface of kraft lignin, increasing the water solubility of the lignin macromolecule.[265]

2.5.3.1 Formulation of Novel Lignin-Based Biomaterials

As mentioned previously, much research has been dedicated to the formulation of lignin based resins so as to reduce or eliminate the use of formaldehyde based resins. Ibrahim et al. have demonstrated the use of a fungal laccase to graft polyethylenimine (PEI), chitosan, and soy protein onto hardwood kraft lignin.[266] The adhesive properties of the formulations were tested via tensile strength measurements using a loading machine. It was discovered that the formulation prepared by laccase treated lignin, followed by $NaBH_4$ reduction and mixed with soy protein yielded an adhesive with greater than 50% of the strength of commercial polyurethane adhesive and good water resistance properties. Furthermore, the incorporation of lignin renders the adhesive with antimicrobial properties. The authors concluded that the created adhesives may find suitable use in the binding of paper and cardboard boxes. In another study, it was established that lignin may serve as a viable base material to replace synthetic latex in the formulation of an adhesive used for wool floor coverings. Aracri and coworkers used a fungal laccase to copolymerize a variety of technical lignins with gallic acid, tannic acid, and dopamine in an attempt to increase reactive quinonoid moieties on the surface of lignin that are capable of undergoing nucleophilic addition by amino groups present in wool to form covalent linkages between lignin and wool.[267] Based on loop withdrawal force measurements, the lignin based adhesives exhibited good flexibility and comparable strength performance to that of the synthetic latex adhesives.

Over the years, the research group of Mai has investigated the chemo-enzymatic grafting of acrylic compounds onto the surface of lignin. Incorporating a lignin backbone into synthetic acrylic polymers, such as polyacrylamide and polyacrylic acid, has proved to be a successful approach in creating novel engineering plastics, thickeners, fillers, and adsorbents with biodegradable properties. Initial studies demonstrated that a fungal laccase in combination with an organic peroxide, such as dioxane peroxide, were able to successfully copolymerize acrylamide with softwood organosolv lignin.[268-269] Additional studies showed that a variety of technical lignosulfonates could be copolymerized with acrylamide and acrylic acid in the presence of laccase and *t*-butylhydroperoxide, and that this system was more effective in promoting copolymerization than a Fenton-like system consisting of ferrous ion and *t*-butylhydroperoxide.[270] A follow up exploration into the mechanism of the grafting and copolymerization reactions revealed the roles of laccase and organic peroxides. It was proposed that laccase initially oxidizes lignin to generate phenoxy radicals, which then go on to oxidize peroxides to produce peroxy radicals that are capable of initiating a homopolymerization of acrylic monomers. The living polymerization of acrylic monomers is eventually quenched by radical-radical coupling reactions between the living ends of the homopolymers and lignin radicals (Figure 61).[271]

Extensive research has been conducted over the past two decades on the laccase-catalyzed synthesis of conducting polyaniline and its applications.[272-273] Very recently, Zhang and coworkers synthesized a polyaniline-lignosulfonate complex via laccase catalysis.[274] The lignosulfonate acted as a template for the synthesis of linear polyaniline. The conductive complex was able to be successfully immobilized onto the

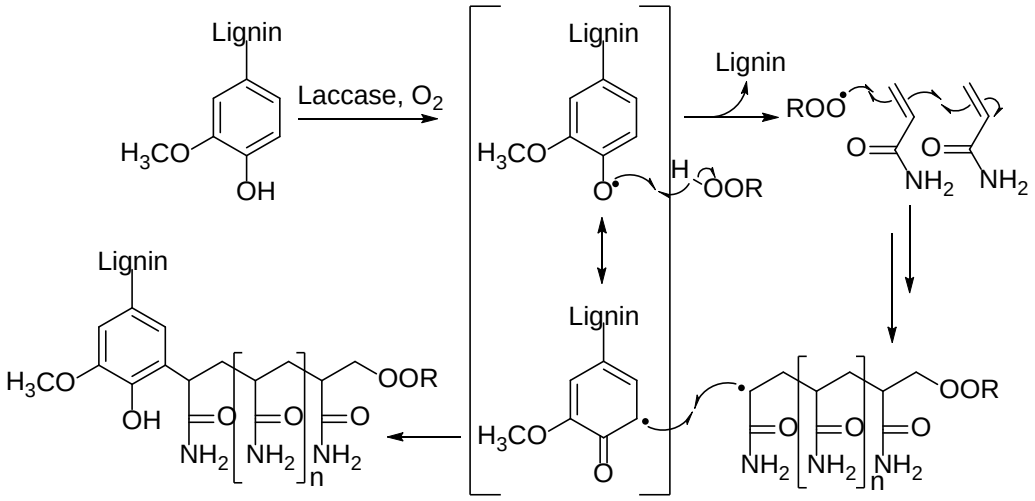


Figure 61. Reaction mechanism for the laccase-initiated copolymerization of lignin with acrylamide (adapted from Mai et al. 1999).[248, 269]

surface of cotton and therefore, may find potential use as a textile in electronic devices. Also in a recent report, the laccase-catalyzed grafting of hydrophilic compounds, such as glucosamine and the tripeptide glycil-tyrosyl-glycine, to hardwood organosolv lignin and alkali pretreated wheat straw lignin was achieved.[275] The synthesized lignin-carbohydrate and lignin-peptide conjugates provide a framework for further functionalization and formulation of materials with distinct properties.

2.5.3.2 Mechanistic Insights into Laccase-Induced Lignin Functionalization

Equally as important as the applications of lignin-based copolymers are the molecular mechanisms by which they are synthesized. Kudanga et al. have studied the mechanisms of lignin functionalization by using laccases to couple small phenolic molecules to lignin model compounds. Using a bacterial laccase, the researchers were able to successfully couple the phenolic compound tyramine to the lignin model syringylglycerol β-guaiacylether.[276] Subsequent studies with another common structural

unit found in lignin, dibenzodioxocin, demonstrated that a variety of phenolic compounds could be coupled to this lignin model using laccases.[277-278] Based on the structures of the reaction products from the coupling reactions, it can be inferred that phenolic compounds form covalent linkages with lignin model compounds via radical-radical coupling reactions (Figure 62). In the case of syringylglycerol *β*-guaiacylether, a model of S type lignin, coupling occurs via a phenoxy radical to form a C-O bond, whereas for dibenzodioxocin, a model for G type lignin, coupling occurs exclusively at the vacant position 5 of the aromatic ring to yield a C-C adduct. These results provide great

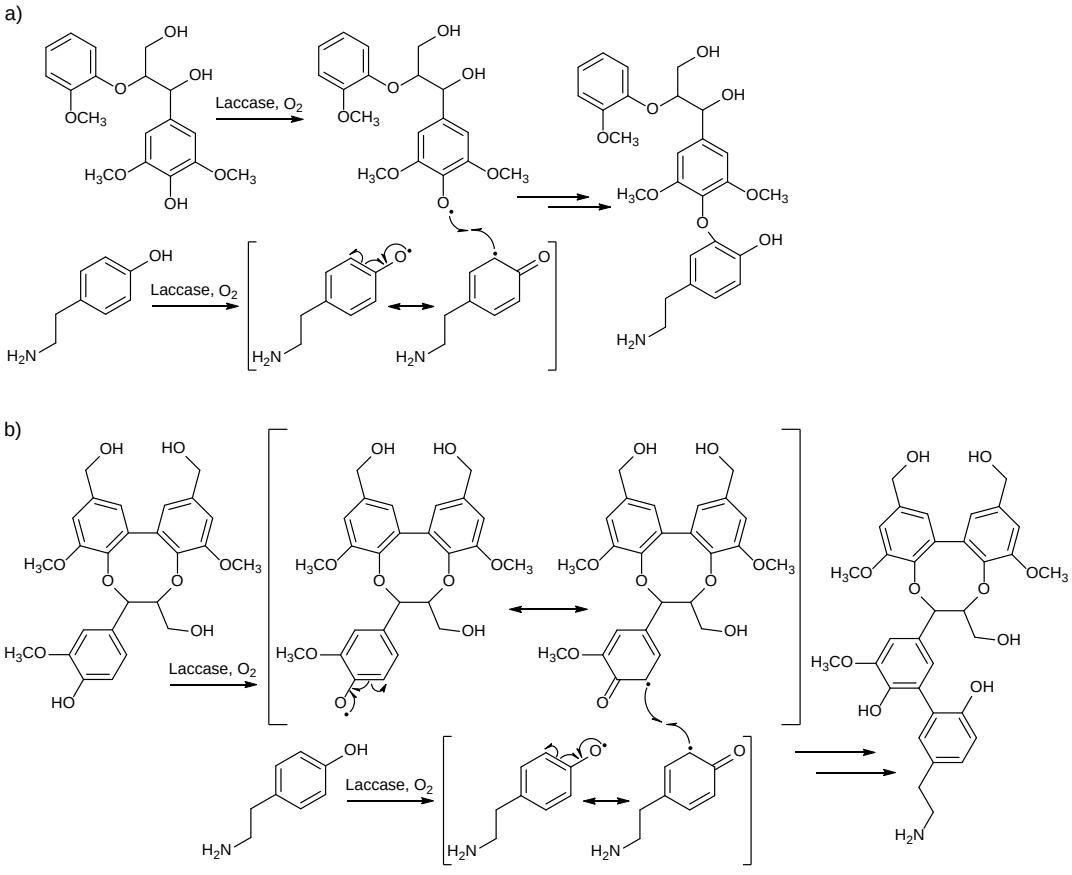


Figure 62. Reaction mechanism for the laccase-catalyzed coupling of tyramine with a) syringylglycerol *β*-guaiacylether (adapted from Kudanga et al. 2009a),[276] and b) dibenzodioxocin.[248]

mechanistic insight into laccase-mediated couplings of phenolic compounds onto the surface of lignin and guidance for future functionalization strategies.

CHAPTER 3. EXPERIMENTAL

3.1 Materials

3.1.1 Chemicals, Solvents and Materials

All reagents and solvents were purchased from either Sigma-Aldrich or VWR and were used as received, except for *tert*-butylhydroquinone, which was a product of Acros Organics, and *endo*-*N*-hydroxy-5-norbornene-2,3-dicarboximide (NHND), which was purchased from Alfa Aesar. Chloroform and dioxane were distilled just prior to use. Aluminum sheets pre-coated with silica gel 60 (EMD Chemicals) were used for thin-layer chromatography (TLC) experiments. Silica gel 60 (EM Separations Technology) was used as the stationary phase for column chromatography. Glass plates coated with silica gel (20 x 20 cm, 2000 μm) were used for preparative layer chromatography. Nitrogen and helium gas were purchased from Airgas. Nitrogen gas was dried using a Drierite™ gas-drying unit (Sigma-Aldrich).

3.1.2 Enzyme

Laccases from the white-rot fungus *Trametes villosa* expressed in an *Aspergillus* host (NOVO NS51002) were appreciatively donated by Novo Nordisk Biochem (now Novozymes), Franklinton, North Carolina, USA, and used as received. This fungal laccase is of high $E°$ (~0.79 V vs NHE),[118] and according to the manufacturer, possesses a pH optimum of 4.5 and an optimum temperature of 45°C,[279] making it highly suitable for industrial biotechnology applications. The enzyme solution is kept frozen at -20°C in small vials until it is required in an effort to preserve enzyme activity.

3.1.3 Lignin

Southern pine softwood kraft lignin, isolated via the LignoBoost process, was donated by Domtar, Plymouth, USA, and was purified following standard methods prior to use.

3.2 Experimental Procedures

3.2.1 Enzyme Assay

Laccase activity was determined according to standard literature procedures which involve the oxidation of ABTS dianion to ABTS radical anion (Figure 63).[280] The oxidation of 3.50 mL of solution consisting of 50 μM ABTS in 0.10 M sodium acetate buffer (pH 5.0) by 8.0 x 10^{-5} mL laccase solution was observed spectrophotometrically at room temperature (22°C) via a UV-vis spectrophotometer by following the absorbance increase at 420 nm (ε_{420} = 3.6 x 10^4 M^{-1} cm^{-1}). Volumetric laccase activity is expressed in units (U) per mL where U = μmol ABTS oxidized per minute. The assay was run in triplicate and the enzyme activity used in subsequent experiments is the average of the three experiments. Images of the reaction solution before the addition of laccase and 10 minutes after the addition of laccase are presented in Figure 63.

Based on the data obtained from the enzyme assay experiment, an absorbance vs time curve can be constructed. Figure 64 displays the absorbance vs time curve for one of the experiments. From the data, it is possible to calculate the activity of the enzyme. An example calculation from experiments one is detailed below.

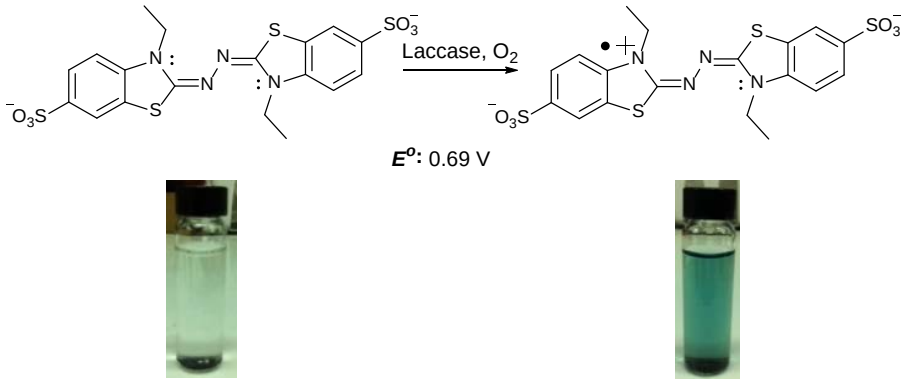


Figure 63. Laccase-catalyzed oxidation of ABTS dianion to ABTS radical anion with accompanying images of enzyme assay solution before the addition of laccase (left vial) and 10 minutes after the addition of laccase (right vial).

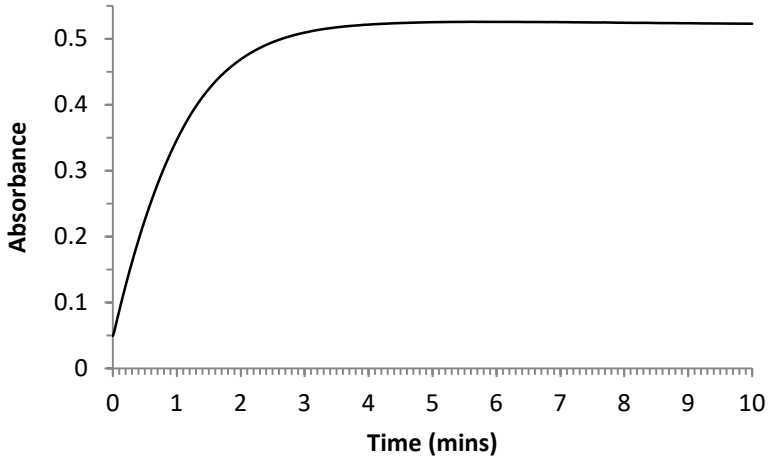


Figure 64. Absorbance vs time curve for the laccase-catalyzed oxidation of ABTS dianion to ABTS radical anion.

In 3.5 mL of reaction solution, there is:

$$3.5 \times 10^{-3} L \left(\frac{50\ \mu mol\ ABTS}{L}\right) = 0.175\ \mu mol\ ABTS$$

Assuming that maximum absorbance indicates ABTS dianion has been completely oxidized to ABTS radical anion, then an absorbance of 0.5256 indicates that 0.175 µmol ABTS has been oxidized. Given that the greatest rate of change in absorbance occurs

between 0-10 s (0-0.166 min), which corresponds to an absorbance change of 0.0638, then:

$$\frac{0.0638}{0.5256} \times 100\% = 12.14\%\ ABTS\ oxidized\ \times 0.175\ \mu mol\ ABTS$$

$$= 0.0212\ \mu mol\ ABTS\ oxidized$$

The volumetric activity can then be calculated:

$$\frac{U}{mL} = \frac{\mu mol\ ABTS\ oxidized}{\text{min}\times\ mL\ enzyme\ solution} = \frac{0.0212\ \mu mol\ ABTS\ oxidized}{0.166\ \text{min}\times\ (8.0\times10^{-5}\ mL)} = 1596\ \frac{U}{mL}$$

Experiment 1: 1596 U/mL

Experiment 2: 1452 U/mL

Experiment 3: 1482 U/mL

Average enzyme activity = 1510 U/mL

Standard deviation = 62 U/mL

3.2.2 General Procedure for the Laccase-Catalyzed Synthesis of Benzylic Nitriles

Benzoylacetonitrile (1 mmol) was added to a 250 mL round bottom flask equipped with a stir bar followed by 30 mL of 0.1 M sodium phosphate buffer (pH 7.0) and the solution was stirred vigorously and heated to 60°C in an oil bath. Once the solid had dissolved (~1 h) the substituted hydroquinone (0.25 mmol) was introduced, followed by the desired amount of laccase stock solution. The reaction mixture was stirred in the presence of air at 60°C for 24 h. The reaction progress was monitored by TLC using silica gel coated on aluminum sheets as the stationary phase, 10% MeOH in DCM (v:v) as the mobile phase, and iodine vapor as the staining agent. In each reaction, all the starting hydroquinone had been consumed after the 24 h period, as judged by TLC and GC-MS data. After this time, the reaction mixture was allowed to cool to room

temperature and then filtered via gravity filtration to capture any solids, if present. The solids were left to dry in a fume hood overnight. The liquid fraction was acidified to pH 3.5 with conc. H_2SO_4, extracted with EtOAc (3 x 30 mL), dried over $MgSO_4$, and the solvent removed via rotary evaporation. The crude solid and extract portions were combined and purified via column chromatography using silica gel as the stationary phase and 10% MeOH in DCM (v:v) as the mobile phase to obtain the desired products. The products were characterized by HRMS, ^{1}H NMR, ^{13}C NMR, ^{1}H-^{13}C HMBC NMR, and FTIR methods.

3.2.3 General Procedure for the Laccase-Catalyzed Synthesis of 2,3-Ethylenedithio-1,4-quinones

The hydroquinone (0.50 mmol) was added to a 50 mL round bottom flask equipped with a stir bar followed by 15 mL of solvent and the mixture was stirred. Once the solid had dissolved, 1,2-ethanedithiol (2.50 mmol) was introduced, followed by 50 U of laccase. The reaction mixture was stirred at room temperature (22°C) for 16 h whilst O_2 was bubbled through. The reaction progress was monitored by TLC using silica gel coated on aluminum sheets as the stationary phase, 1:1 EtOAc/hexane (v:v) mixture as the mobile phase, and iodine vapor as the staining agent. Once the reaction was complete, the reaction mixture was extracted with EtOAc (3 x 20 mL), dried over $MgSO_4$, and the solvent removed via rotary evaporation. The crude extract was purified via column chromatography using silica gel as the stationary phase and 1:1 EtOAc/hexane (v:v) mixture as the mobile phase to obtain the desired products. The products were characterized by HRMS, ^{1}H NMR, ^{13}C NMR, and FTIR.

3.2.4 General Procedure for the Laccase-Catalyzed Synthesis of Phenothiazones and Related Structures

The synthetic procedures for the laccase-catalyzed synthesis of phenothiazones and related compounds differ depending on the starting reagents, and multiple synthetic routes were experimented with. Chapter 6.2.3 provides detailed experimental procedures for the synthesis of each individual compound.

3.2.5 Lignin Purification

The lignin purification procedure followed standard methods developed previously with some slight modifications.[281-282] Firstly, treatment of lignin with EDTA-$2Na^+$ was performed to remove trace metals. This process involved suspending 5.0180 g of lignin in 95 mL of deionized water in a 250 mL beaker and stirring vigorously. To this, 0.4996 g of EDTA-$2Na^+$ was added and the mixture was allowed to stir at room temperature for 1.5 h, at which time the pH of the mixture was adjusted to 3.0 with 2 M H_2SO_4. The mixture was then frozen overnight (15 h), thawed at room temperature, and then vacuum filtered through a sintered glass funnel. The solid was collected and re-suspended in cold deionized water and stirred vigorously for 20 mins in an ice bath at 0°C and then vacuum filtered through a sintered glass funnel. This final step was repeated two more times and then the solid was dried in a vacuum oven at 45°C overnight (19 h). Mass of lignin recovered: 4.7463 g (94.6% recovery).

The next step involved Soxhlet extraction of lignin to remove any remaining extractives. 4.7463 g of lignin was placed in a cellulose thimble and then Soxhlet

extracted with 100 mL of toluene for 6.5 h. The lignin was then allowed to dry in a fume hood overnight. Mass of lignin recovered: 4.4131 g (93% recovery).

The final step involved a dioxane/water extraction to separate the lignin from residual carbohydrates. Approximately 1 g of lignin was added to a 25 mL vial and dissolved in 10 mL distilled dioxane : deionized water solution (9:1, v:v) and stirred for 20 h. After this time, the mixture was centrifuged, the supernatant collected, and the solvent removed via rotary evaporation. The remaining solid was then dissolved in 10 mL distilled dioxane : deionized water solution again and allowed to stir for 70 h, after which the mixture was centrifuged, the supernatant collected, and the solvent removed via rotary evaporation. Approximately 4 mL of deionized water was added to the remaining lignin, the mixture frozen, and then freeze-dried for 48 h. The pure kraft lignin was stored in a refrigerator until further use. The purified kraft lignin was analyzed via GPC, ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, FTIR, TGA, DSC, and SEM.

3.2.6 Synthesis of Tris(2-mercaptoethyl)amine

The synthesis of *tris*(2-mercaptoethyl)amine was based on a combination of two previously published syntheses.[283-284] 6.637 mL (7.46 g, 50 mmol) of triethanolamine was dissolved in 12.5 mL distilled chloroform and added dropwise, over the course of an hour, into a solution of thionyl chloride (12.765 mL, 20.82 g, 175 mmol) in 20 mL distilled chloroform, which was stirring in a 100 mL round bottom flask. The reaction was carried out at ambient temperature while stirring until gas evolution stopped, then, the reaction mixture was refluxed at 60-65°C for 4 h. The mixture was then allowed to cool to room temperature and then the white precipitate was vacuum filtered and washed

with 3 x 30 mL distilled chloroform and dried under vacuum at room temperature overnight (15 h). Yield of *tris*(2-chloroethyl)amine hydrochloride: 10.4000 g (86.3%).

In the next step, 10.4000 g of *tris*(2-chloroethyl)amine hydrochloride and 9.8551 g (0.13 mol) of thiourea were added to a 250 mL round bottom flask equipped with a stir bar. 50 mL of 200 proof ethanol was added to dissolve the solids and the mixture was gently stirred and refluxed at 80°C for 5 h. After this time, the mixture was allowed to cool to room temperature and the white solid was vacuum filtered (low pressure), washed with 50 mL ethanol, and dried under vacuum at room temperature overnight (15 h). Yield of *tris*(ethylisothiouronium)amine chloride: 17.5750 g (86.8%).

In the final step, 9.3917 g of *tris*(ethylisothiouronium)amine chloride was added to 13 mL of deionized water that had been purging with nitrogen in a three-necked round bottom flask equipped with a stir bar. The mixture was vigorously stirred under nitrogen atmosphere. Once the solid had dissolved, 17 mL of 4.7 M NaOH was added and the solution was heated and stirred at 80°C for 15 mins under nitrogen atmosphere. The mixture was then cooled rapidly in an ice bath under nitrogen atmosphere and then extracted with 3 x 20 mL distilled chloroform, the organic phases combined, dried over $MgSO_4$, and solvent removed via rotary evaporation. The final product was purified via micro distillation. Yield of *tris*(2-mercaptoethyl)amine: 5.8925 g (79.8%). The product was characterized by GC-MS, ^{1}H NMR, and ^{13}C NMR.

3.2.7 Laccase-Mediated Synthesis of Lignin-Core Hyperbranched Copolymers (LCHCs)

Approximately 100 mg of purified kraft lignin was added to a 50 mL round bottom flask equipped with a stir bar. 2 mL of dioxane was then added, followed by 8 mL of 0.1

M sodium phosphate buffer pH 8.0 and the mixture was stirred and heated to 50°C for approximately 10 mins to dissolve the lignin. Then, 496.56 mg (4.0 mmol) of methylhydroquinone was added to the mixture followed by 333 μL (394.77 mg, 2.0 mmol) of *tris*(2-mercaptoethyl)amine. Finally, 200 U of laccase was added and the reaction mixture was allowed to stir for 20 h at 50°C. After this time, the mixture was allowed to cool to room temperature, centrifuged, the supernatant decanted, and the remaining brown sludge washed with 3 x 15 mL deionized water followed by 3 x 15 mL dioxane. The brown paste was then dried in a vacuum oven at 30°C for 24 h. Yield: 524 mg. The final material was analyzed via elemental analysis, ^{1}H NMR, ^{13}C NMR, ^{1}H-^{13}C HMBC NMR, ^{13}C DEPT-135 NMR, FTIR, TGA, DSC, and SEM.

3.3 Analytical Procedures

3.3.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

All NMR experiments were conducted on a Bruker Avance DRX 400 MHz spectrometer using a 5 mm PABBO BB-1H/D Z-Grad probe at room temperature unless stated otherwise. All chemical shifts are given in ppm relative to TMS and multiplicities are designated as s (singlet), d (doublet), t (triplet), and m (multiplet). Data analysis was performed using MestReNova software.

3.3.1.1 ^{1}H NMR Experiments

Quantitative ^{1}H NMR experiments were conducted at a spectrometer frequency of 400 MHz with a 90° pulse width, a 15 s relaxation delay, with 16 scans.

3.3.1.2 ^{13}C NMR Experiments

^{13}C NMR experiments are proton decoupled and were conducted at a spectrometer frequency of 100 MHz with a 90° pulse width, a 1 s relaxation delay, with 8192 scans.

3.3.1.3 ^{13}C DEPT-135 NMR Experiments

^{13}C DEPT-135 NMR experiments were conducted at a spectrometer frequency of 100 MHz with a 135° pulse width, a 3 s relaxation delay, with 4096 scans.

3.3.1.4 ^{1}H-^{13}C HMBC NMR Experiments

^{1}H-^{13}C HMBC NMR experiments were conducted at a spectrometer frequency of 400 MHz in regards to the ^{1}H nucleus and 100 MHz in regards to the ^{13}C nucleus with a 90° pulse width, a 1.5 s relaxation delay, with 32 scans.

3.3.1.5 ^{31}P NMR Experiments

The method and solvent preparation are based on experiments developed by Granata and Argyropoulos (1995),[285] and Zawadzki and Ragauskas (2001).[286] In brief, 15 mL of anhydrous pyridine was mixed with 10 mL of $CDCl_3$, followed by the addition of 25 mg of chromium (III) acetylacetonate (relax reagent) and 100 mg of the internal standard NHND. 20 mg of purified kraft lignin was then dissolved in 0.5 mL of the above solution, followed by the addition of 70 μL of the lignin phosphitylation reagent 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP) just prior to NMR analysis. Quantitative ^{31}P NMR experiments were conducted at a spectrometer frequency of 162 MHz with a 90° pulse width, a 25 s relaxation delay, with 64 scans.

3.3.2 Fourier Transform Infrared (FTIR) Spectroscopy

FTIR analyses were carried out on a PerkinElmer Spectrum 100 spectrometer using the attenuated total reflectance (ATR) method. 128 scans were used for all analyses. Data was gathered using Spectrum 10 software and analyzed using Microsoft Excel.

3.3.3 Ultraviolet-Visual (UV-Vis) Spectroscopy

UV-Vis spectroscopy was used for the enzyme assay experiments. Analyses were performed on a PerkinElmer LAMBDA 35 UV-Vis spectrophotometer. Absorption wavelength was fixed at 420 nm and absorbance data points were collected every 2 s. Data was gathered with UV WinLab software and analyzed using Microsoft Excel.

3.3.4 Gas Chromatography – Mass Spectrometry (GC-MS)

GC–MS experiments were run using an Agilent Technologies 7890A GC system equipped with a HP-5MS column coupled with a 5975C inert MSD with triple-axis detector. Samples were completely dissolved in an appropriate solvent to give a final concentration of 1 mg/mL. The sample was injected into the column via the automatic liquid sampler (ALS) accessory with an injection volume of 1 μL. Helium was used as the carrier gas at a flow pressure of 10 psi. Initial oven temperature was 50°C for all experiments, while the final oven temperature and the rate at which the oven temperature was increased varied depending on the analysis. Data was collected and analyzed using Agilent ChemStation software installed with an NIST library.

3.3.5 Mass Spectrometry (MS)

High-resolution mass spectrometry (HRMS) analyses were performed by the Georgia Institute of Technology Bioanalytical Mass Spectrometry Facility on a Micromass AutoSpec M spectrometer using the electron ionization method. Molecular weight data are accurate to within 5 ppm.

3.3.6 Gel Permeation Chromatography (GPC)

A Polymer Standards Service (PSS) SECurity 1200 system equipped with Agilent Technologies high-performance liquid chromatography (HPLC) parts, such as UV (270 nm) and refractive index (RI) detectors, was used for molecular weight distribution analysis of purified kraft lignin. The sample was dissolved in THF that had been degassed and then filtered through a polytetrafluoroethylene (PTFE) membrane (pore size of 0.45 μm) to yield a final sample concentration of 1 mg/mL. Degassed and ultrafiltered THF was used as the mobile phase and a Waters Styragel® HR 2 column was used as the stationary phase. The sample was injected into the column via the ALS accessory with an injection volume of 30 μL. The experiment was run at 30°C at a pump flow rate of 1 mL/min. Data was calibrated against a polystyrene standard calibration curve, and collected and analyzed with PSS WinGPC Unity software.

3.3.7 Melting Point Determination

A Barnstead International Mel-Temp® apparatus was used to determine melting temperature ranges for all synthesized products. Approximately 3 mg of compound was loaded into a glass capillary tube which was then placed into the melting point

temperature apparatus equipped with a thermometer. The temperature was ramped from room temperature at a rate of approximately 5°C per minute until the compound had completely melted.

3.3.8 Elemental Analysis

Elemental analysis experiments were conducted by Atlantic Microlab, Inc., Norcross, USA. All values were determined via combustion and are reported as percent weight.

3.3.9 Thermogravimetric Analysis (TGA)

TGA analyses were conducted on purified kraft lignin and LCHCs using a PerkinElmer Pyris 1 thermogravimetric analyzer. Approximately 15 mg of sample was loaded into the sample holder and the experiments were conducted under nitrogen (flow rate of 50 mL/min) with the following temperature profile: heat from 50°C to 600°C at a rate of 20°C/min, and holding at 600°C for 5 mins. One data point was collected per second. Data was recorded as mass %. Data was exported and analyzed using Microsoft Excel.

3.3.10 Differential Scanning Calorimetry (DSC)

DSC analyses were conducted on purified kraft lignin and LCHCs using a TA Instruments Q200 differential scanning calorimeter. Approximately 5 mg of sample was placed in the sample pan and the experiments were conducted under nitrogen (flow rate of 50 mL/min) with the following temperature profile: hold at 0°C for 5 mins, heat from 0°C to 160°C at 20°C/min, hold at 160°C for 5 mins, cool to 0°C at a rate of 20°C/min,

hold at 0°C for 5 mins, heat from 0°C to 160°C at 20°C/min, hold at 160°C for 5 mins, cool to 25°C at a rate of 20°C/min. Data was collected during the second heating cycle. Heat flow was reported in mW. Data was exported and analyzed using Microsoft Excel.

3.3.11 Scanning Electron Microscopy (SEM)

Surface morphology of pure kraft lignin and LCHCs was analyzed on a Zeiss LEO 1530 Gemini field emission scanning electron microscope. The samples were sputter coated with gold prior to analysis to provide a thin conducting layer on the sample surface.

CHAPTER 4. LACCASE-CATALYZED ALPHA-ARYLATION OF BENZOYLACETONITRILE WITH SUBSTITUTED HYDROQUINONES[II]

4.1 Introduction

The formation of benzylic nitriles via α-arylation of the corresponding primary or secondary nitrile is a key C-C bond forming reaction in organic synthesis. Compounds possessing a benzylic nitrile functionality are of importance to the pharmaceutical industry as this structural moiety is present in biologically active compounds such as anastrozole (estrogen-dependent breast cancer), verapamil and gallopamil (antiarrhythmic), cilomilast (anti-inflammatory), levocabastine (allergic conjunctivitis), piritramide (postoperative pain), and diphenoxylate (diarrhea).[287] Not only are benzylic nitriles present in the final active pharmaceutical ingredient, but they are also important synthetic intermediates for many other bioactive compounds,[288-292] for example, in the preparation of Ibuprofen,[293] as well as compounds exhibiting antimicrobial and antifungal properties.[294] Their versatility as synthetic intermediates in organic synthesis is exemplified by the wide array of structural classes that can be formed upon their transformation, including pyridines, carboxylic acids, primary amines, bicyclic amidines, aldehydes, esters, β-lactams, and lactones.[295-297]

Several strategies exist for the synthesis of benzylic nitriles. One of the earliest methods involves nucleophilic aromatic substitution of aryl halides and heteroaryl halides

[II] This manuscript, titled "Laccase-catalyzed α-arylation of benzoylacetonitrile with substituted hydroquinones," was published in *Chemical Engineering Research and Design* (**2015**, *97*, 128-134). The other author is Arthur J. Ragauskas, who is affiliated with Georgia Institute of Technology. The manuscript was reproduced with permission from Elsevier; the copyright license agreement is provided in Appendix B.

with nitrile carbanions.[298-303] The nucleophilic substitution approach has also been applied to the displacement of benzylic halides via the use of a cyanide nucleophile.[304] There are numerous accounts involving transition metal catalysis (predominantly utilizing palladium complexes) to perform cross-coupling reactions of various primary and secondary nitriles with substituted aryl halides.[295, 305-316] The limitations to these methods are that they can only be applied to benzylic or aryl halides and the reactions must be performed in an organic solvent at high temperatures in the presence of a transition metal catalyst or strong base, which, for the latter, eliminates the possibility of carrying out the α-arylation reaction in the presence of base sensitive functional groups on the aromatic ring (e.g. hydroxy group). Approaches aimed at performing α-arylation reactions in the absence of halogen substituents on the aromatic ring have been developed, such as direct aryne insertion into α-cyanocarbonyl compounds via a benzyne intermediate,[317] a Friedel-Crafts type reaction,[318] and photochemical methods.[319-320] However, all these methods, with the exception of a solvent-free method recently developed by Yoshida et al.,[319] require the reaction to be carried out in an organic solvent. Thus, employing laccases to catalyze such a transformation under environmentally benign conditions is of great appeal.

At the time this study was undertaken, the vast majority of synthetic reactions catalyzed by laccases involved radical-coupling reactions of phenolic monomers and cross-coupling reactions of substituted catechols and hydroquinones with nitrogen based nucleophiles via *in situ* generated *ortho*- and *para*-quinones. Recently however, the cross-coupling approach has expanded to include carbon derived nucleophiles, and this has proved to be a useful tool in forming C-C bonds, which was reviewed in depth in

Chapter 2.4.7.2. Much of the laccase-catalyzed C-C bond forming reactions focused on combining catechols and hydroquinones with 1,3-dicarbonyl compounds in a laccase-catalyzed domino reaction, which exploits the acidic methylene protons of 1,3-dicarbonyls.[181, 183] To the best of our knowledge, there are no accounts on the use of methylene groups substituted with a nitrile used in laccase-catalyzed cross-coupling reactions with substituted catechols or hydroquinones. Herein, the green synthesis of benzylic nitriles via the laccase-catalyzed cross-coupling reaction of benzoylacetonitrile (**1**) with substituted hydroquinones (**2**) is disclosed.

4.2 Experimental

4.2.1 Materials

All compounds were purchased from Sigma-Aldrich except *tert*-butylhydroquinone, which was a product of Acros Organics. Laccases from the white-rot fungus *Trametes villosa* expressed in an *Aspergillus* host (NOVO NS51002) were appreciatively donated by Novo Nordisk Biochem (now Novozymes), Franklinton, North Carolina, USA. All compounds, solvents, and enzyme were used as received without further purification. Aluminum sheets pre-coated with silica gel 60 (EMD Chemicals) were used for thin-layer chromatography (TLC) experiments. Silica gel 60 (EM Separations Technology) was used as the stationary phase for column chromatography.

4.2.2 Enzyme Assay

Full experimental procedures regarding laccase activity measurements are detailed in Chapter 3.2.1.

4.2.3 General Procedure for the Laccase-Catalyzed Reaction of Benzoylacetonitrile with Substituted Hydroquinones

Full experimental procedures regarding the laccase-catalyzed synthesis of benzylic nitriles are provided in Chapter 3.2.2.

4.2.4 Product Characterization Data

NMR and MS spectra of new compounds are given in Appendix A. All NMR experiments were conducted using DMSO-d_6 as the solvent.

2-(2′,5'-Dihydroxy-4'-methoxyphenyl)-3-oxo-3-phenylpropanenitrile (**6a**)

Yield: 37%; dark-brown solid; mp: 106-111°C; IR: $\tilde{\nu}$ 3289, 2228, 1624, 1473, 1354, 1315, 1189, 1115, 1020, 902, 815, 750, 699 cm^{-1}; ^1H NMR: δ 3.72 (s, 3H, OCH_3), 5.74 (s, 1H, CH), 6.12 (s, 1H, Ar-H), 7.04 (s, 1H, Ar-H), 7.54 (m, 5H, Ar-H), 8.44 (s, 1H, Ar-OH), 8.51 (s, 1H, Ar-OH) ppm; ^{13}C NMR: δ 55.0, 56.5, 92.5, 96.6, 105.0, 118.6, 126.9, 128.5, 130.4, 141.4, 143.4, 166.9, 188.2 ppm; HRMS (ESI): $C_{16}H_{13}NO_4$ calculated 283.0845, found 283.0843.

2-(2',5'-Dihydroxyphenyl)-3-oxo-3-phenylpropanenitrile (**6b**)

Yield: 24%; dark-yellow solid; mp: 95–100°C; IR: $\tilde{\nu}$ 3302, 2220, 1637, 1594, 1459, 1201, 1167, 998, 915, 807, 750, 700, 667 cm^{-1}; ^1H NMR: δ 6.05 (s, 1H, CH), 6.37 (d, ^3J = 8.6 Hz, 1H, Ar-H), 7.09 (d, ^3J = 8.6 Hz, 1H, Ar-H), 7.55 (m, 5H, Ar-H), 8.54 (s, 1H, Ar-OH), 8.95 (s, 1H, Ar-OH) ppm; ^{13}C NMR: δ 92.7, 104.6, 108.4, 110.0, 126.9, 128.5, 130.5, 141.4, 142.3, 153.8, 167.5, 188.7 ppm; HRMS (ESI): $C_{15}H_{11}NO_3$ calculated 253.0739, found 253.0732.

2-(2',5'-Dihydroxy-4'-methylphenyl)-3-oxo-3-phenylpropanenitrile (**6d**) and 2-(2',5'-Dihydroxy-3'-methylphenyl)-3-oxo-3-phenylpropanenitrile (**7d**)

Yield: 26%; yellow/orange solid; mp: 95-100°C; IR: $\tilde{\nu}$ 3289, 2237, 1646, 1626, 1472, 1167, 972, 907, 802, 754, 698 cm^{-1}; **6d**: ^{1}H NMR: δ 2.25 (s, 3H, CH_3), 5.88 (s, 1H, CH), 6.22 (s, 1H, Ar-H), 6.24 (s, 1H, Ar-H), 7.53 (m, 5H, Ar-H), 8.51 (s, 1H, Ar-OH), 8.81 (s, 1H, Ar-OH) ppm; ^{13}C NMR: δ 14.7, 93.1, 102.3, 110.0, 119.6, 126.6, 126.9, 128.4, 130.4, 141.4, 153.7, 167.3, 188.8 ppm; **7d**: ^{1}H NMR: δ 2.08 (s, 3H, CH_3), 5.87 (s, 1H, CH), 6.23 (s, 1H, Ar-H), 7.02 (s, 1H, Ar-H), 7.56 (m, 5H, Ar-H), 8.45 (s, 1H, Ar-OH), 8.80 (s, 1H, Ar-OH) ppm; ^{13}C NMR: δ 16.2, 92.7, 104.3, 111.1, 117.6, 124.4, 127.1, 128.4, 130.5, 142.2, 151.7, 167.2, 188.3 ppm; HRMS (ESI): $C_{16}H_{13}NO_3$ calculated 267.0895, found 267.0892.

- **Note:** NMR data for compounds **6d** and **7d** were obtained from the spectrum of the 60:40 regioisomeric mixture of **6d** and **7d**.

2-(3',6'-Dihydroxy-2',4'-dimethoxyphenyl)-3-oxo-3-phenylpropanenitrile (**6h**)

Yield: 25%; brown solid; mp: 175-180°C; IR: $\tilde{\nu}$ 3415, 3298, 2937, 2833, 2212, 1646, 1626, 1473, 1364, 1272, 1146, 898, 846, 747, 698, 663 cm^{-1}; ^{1}H NMR: δ 2.90 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3), 6.89 (s, 1H, Ar-H), 7.45 (m, 5H, Ar-H), 8.13 (s, 1H, Ar-OH), 8.20 (s, 1H, Ar-OH) ppm; ^{13}C NMR: δ 56.6, 58.6, 92.1, 112.1, 127.4, 127.8, 130.3, 136.7, 139.5, 141.6, 141.9, 144.7, 166.3, 189.0 ppm; HRMS (ESI): $C_{17}H_{15}NO_5$ calculated 313.0950, found 313.0961.

2-(3'-*tert*-Butyl-2',5'-dihydroxyphenyl)-3-oxo-3-phenylpropanenitrile (**7c**)

Yield: 46%; yellow solid; mp: 228-233°C; IR: $\tilde{\nu}$ 3370, 2954, 2237, 1641, 1599, 1473, 1346, 1285, 1163, 920, 846, 811, 737, 693 cm^{-1}; ^{1}H NMR: δ 1.36 (s, 9H, *t*-Bu), 5.75 (s, 1H, CH), 5.90 (s, 1H, Ar-H), 6.32 (s, 1H, Ar-H), 7.52 (m, 5H, Ar-H), 8.46 (s, 1H, Ar-OH), 8.81 (s, 1H, Ar-OH) ppm; ^{13}C NMR: δ 29.6, 30.0, 33.7, 92.6, 102.5, 106.3, 126.9, 128.5, 130.4, 133.1, 141.4, 153.4, 166.7, 188.8 ppm; HRMS (ESI): $C_{19}H_{19}NO_3$ calculated 309.1365, found 309.1354.

2-(2'-Acetyl-3',6'-dihydroxyphenyl)-3-oxo-3-phenylpropanenitrile (**8f**)

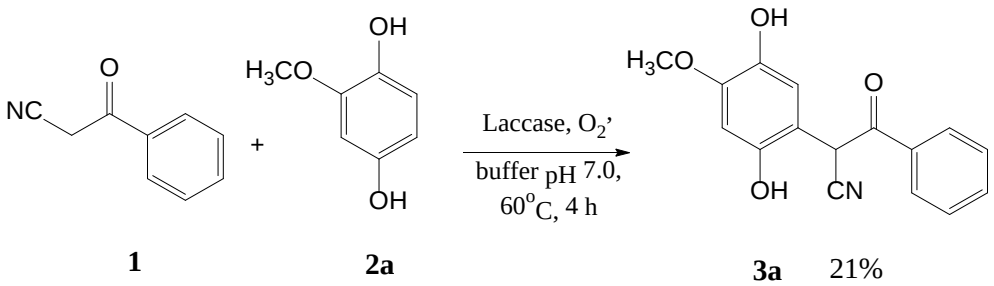
Yield: 89%; brown solid; mp: 106-111°C; IR: $\tilde{\nu}$ 3302, 2228, 1616, 1572, 1454, 1411, 1350, 1207, 1189, 998, 907, 854, 798, 737, 693, 672 cm^{-1}; ^{1}H NMR: δ 1.84 (s, 3H, CH_3), 5.75 (s, 1H, CH), 6.54 (d, ^{3}J = 8.7 Hz, 1H, Ar-H), 7.24 (d, ^{3}J = 8.7 Hz, 1H, Ar-H), 7.43 (m, 5H, Ar-H), 7.73 (s, 1H, Ar-OH), 9.74 (s, 1H, Ar-OH) ppm; ^{13}C NMR: δ 31.2, 92.8, 101.5, 108.9, 112.4, 118.9, 127.5, 128.7, 131.1, 141.1, 142.2, 152.4, 165.9, 189.2, 201.7 ppm; HRMS (ESI): $C_{17}H_{13}NO_4$ calculated 295.0845, found 295.0855.

Ethyl 2-(1'-cyano-2'-oxo-2'-phenylethyl)-3,6-dihydroxybenzoate (**8g**)

Yield: 80%; orange solid; mp: 76-81°C; IR: $\tilde{\nu}$ 3315, 2982, 2235, 1627, 1572, 1457, 1415, 1197, 1020, 920, 802, 693, 667, 616 cm^{-1}; ^{1}H NMR: δ 0.96 (t, ^{3}J = 7.1 Hz, 3H, CH_3), 3.30 (q, ^{3}J = 7.1 Hz, 2H, CH_2), 5.74 (s, 1H, CH), 6.57 (d, ^{3}J = 8.6 Hz, 1H, Ar-H), 7.44 (m, 5H, Ar-H), 7.58 (d, ^{3}J = 8.6 Hz, 1H, Ar-H), 7.93 (s, 1H, Ar-OH), 9.63 (s, 1H, Ar-OH) ppm; ^{13}C NMR: δ 13.5, 55.0, 60.0, 93.2, 107.6, 108.9, 114.2, 127.4, 128.5, 131.5, 139.8, 141.8, 154.6, 166.3, 167.5, 188.8 ppm; HRMS (ESI): $C_{18}H_{15}NO_5$ calculated 325.0950, found 325.0946.

4.3 Results and Discussion

The initial reaction for the laccase-catalyzed *α*-arylation of benzoylacetonitrile (**1**) was conducted using methoxyhydroquinone (**2a**) (Figure 65). The reaction was performed at 60 °C to aid in the solubility of **1** in the aqueous solution, in which it was only slightly soluble at room temperature, and in a 0.1 M sodium phosphate buffer pH 7.0 to increase the concentration of the conjugate base of **1**. The reaction product **3a** was formed as a single regioisomer in moderate yield (21%).



1 **2a** **3a** 21%

Figure 65. Initial reaction of benzoylacetonitrile (**1**), 1 mmol, with methoxyhydroquinone (**2a**), 0.25 mmol.

Interestingly, addition of **1** to **2a** yielded only the C-C bond adduct and not the substituted benzofuran product that would be expected from the cyclizing O-C bond formation that has been seen in the majority of these types of reactions. Beifuss et al. also experienced this same trend in their study involving the addition of 3-*tert*-butyl-1H-pyrazol-5(4*H*)-one to substituted catechols in which only the C-C adduct was formed.[194] This trend may be due to the electron-withdrawing nature of the newly formed substituent on the hydroquinone or catechol, which could raise the redox potential of the substrate to a value where oxidation by the current laccases used is not possible.

A major side-reaction that occurred, which may be responsible for the moderate product yield, was oxidative degradation of **1**. GC-MS analysis of the crude extract from the reaction mixture showed a significant amount of benzoic acid formation. It is thought that this degradation pathway is caused by **2a** acting as a laccase-mediator, as the mixture of just laccase and **1** did not provide the desired reaction. A proposed reaction mechanism for the formation of the potential 1,2-dicarbonyl intermediate **4** and its hydrolysis to benzoic acid and isocyanic/cyanic acid is shown in Figure 66. Compound **2a** is oxidized by laccases and then abstracts a hydrogen atom from the methylene carbon of **1** to produce a resonance-stabilized radical. This radical combines with oxygen and is then hydrolyzed to the corresponding intermediate **4**, which is then further hydrolyzed to benzoic acid. It must be noted that **4** is an unstable intermediate that was unable to be isolated.

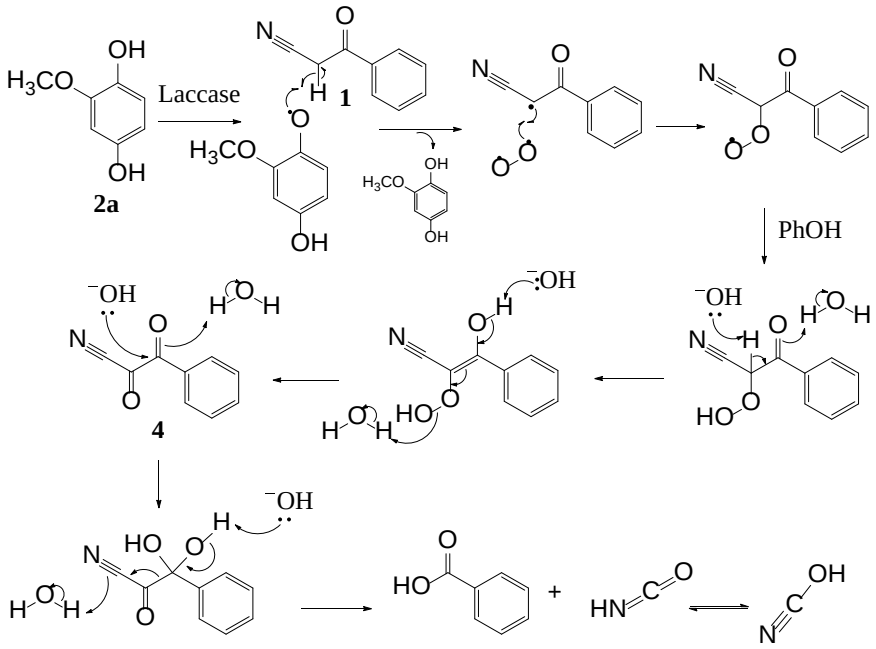


Figure 66. Proposed mechanism for the oxidative degradation of **1** to form benzoic acid and isocyanic/cyanic acid via 1,2-dicarbonyl intermediate **4**.

The next step was focused on increasing the yield of product **3a** by modifying the reaction conditions so that the reaction could be of synthetic value. Parameters such as the **1**:**2a** ratio, laccase dose, pH of buffer solution, temperature, and time were all varied to determine the reaction conditions that would provide the greatest product yield. The results of reaction optimization are displayed in Table 3. As can be seen, yield of **3a** seems to be independent of the amount of laccase used (entries 1 and 2). 10 U of laccase was shown to be an ample amount of enzyme to completely oxidize 0.25 mmol of **2a** to the corresponding *para*-quinone under the given reaction conditions. Bubbling O_2 through the reaction mixture, a method employed to increase laccase activity, actually decreased the product yield (entry 3), probably due to an increase in the oxidative degradation route. Increasing the ratio of **1**:**2a** provided an increase in product yield (entries 1, 4, and 5). A 4:1 ratio of **1**:**2a** seemed to be the most feasible option as a 10:1 ratio didn't increase the yield substantially (24% compared to 21%), probably due to the low solubility with the larger amount of **1**. The reaction time seemed to have the largest impact on the product yield (entries 1, 6, 7, and 8); the longer the reaction time, the greater the yield. This suggests that the coupling reaction is likely to be the rate-limiting step, not the oxidation reaction. The pH of the buffer solution was also shown to have a major impact on product yield. It was found that by lowering the pH to 5.0 (entry 9), the oxidative degradation pathway of **1** could be retarded; however, no product was formed, probably due to the very low amount of the enolate form of **1** being present. Increasing the pH to 7.5 and 8.0 (entries 10 and 11, respectively) showed a minor decrease in product yield. As was expected, temperature played a crucial role in the formation of the desired product as the reaction conducted at room temperature (entry 12) yielded no

product. From these results, it was concluded that the optimum reaction conditions for the formation of **3a** are those listed in entry 8 of Table 3.

Table 3. Results of reaction optimization for the laccase-catalyzed cross-coupling reaction of benzoylacetonitrile (**1**) with methoxyhydroquinone (**2a**).

Entry	1:2a[a]	Laccase (U)	pH[b]	Temp. (°C)	Time (h)	Yield (%) 3a
1	4:1	10	7.0	60	4	21
2	4:1	100	7.0	60	4	21
3[c]	4:1	10	7.0	60	4	11
4	2:1	10	7.0	60	4	16
5	10:1	10	7.0	60	4	24
6	4:1	10	7.0	60	2	9
7	4:1	10	7.0	60	12	26
8	**4:1**	**10**	**7.0**	**60**	**24**	**37**
9	4:1	10	5.0	60	24	0
10	4:1	10	7.5	60	24	34
11	4:1	10	8.0	60	24	33
12	4:1	10	7.0	22	24	0

[a] 0.25 mmol of **2a** used in all experiments
[b] 30 mL of 0.1 M sodium acetate buffer was used for pH 5.0 and 0.1 M sodium phosphate buffer was used for pH 7.0
[c] O_2 bubbled through the reaction mixture

The optimized conditions were then employed to react **1** with a series of substituted hydroquinones to determine the effect the substituent has on the product yield and regioselectivity of the reaction. The reaction scheme is shown in Figure 67. Firstly, laccase oxidizes the substituted hydroquinone substrates **2**, which are then further oxidized to the *para*-quinone intermediates **5**. The enolate of **1** is then able to undergo nucleophilic attack on the *para*-quinone. This can occur at three possible positions of *para*-quinone intermediates **5**: carbon 3, 5, or 6. The products were formed as either single regioisomers or a mixture of products **6** and **7**.

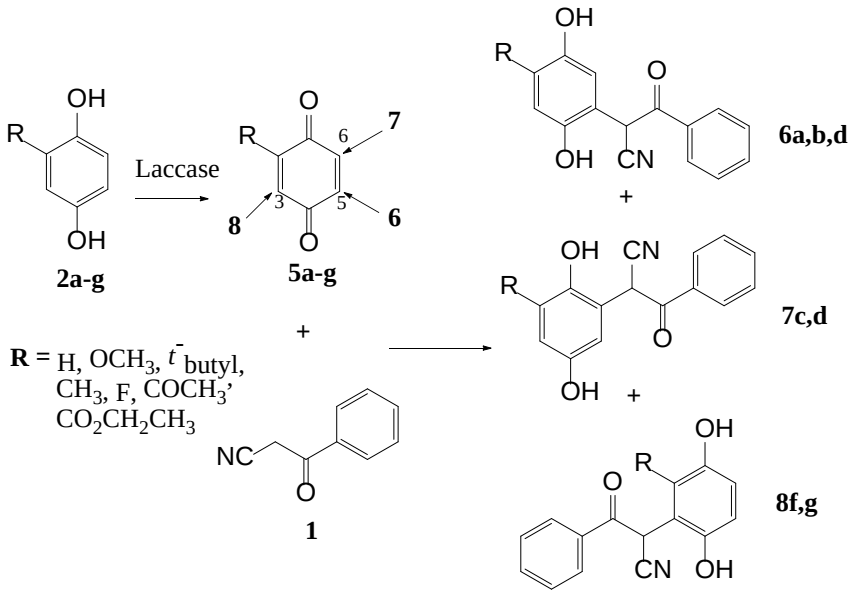


Figure 67. The laccase-catalyzed reaction of benzoylacetonitrile (**1**) with a variety of substituted hydroquinones (**2**).

The results for the reaction of **1** with a series of substituted hydroquinones **2** are shown in Table 4. Firstly, the amount of laccase employed to completely oxidize the hydroquinone **2** to the corresponding quinone **5** is highly substituent dependent. Electron-donating substituents (i.e. OCH_3, *t*-butyl, CH_3) on the hydroquinone required only 10 U of laccase to be completely oxidized (as determined by TLC and GC-MS data) while those hydroquinones containing electron-withdrawing substituents (i.e. F, $COCH_3$, $CO_2CH_2CH_3$) required a much greater amount of laccase to achieve the same result. This is consistent with data showing that the redox potential of the hydroquinone increases when electron-withdrawing substituents are present compared to when electron-donating substituents are present and that the rate of oxidation by laccase is dependent on the redox potential of the substrate, with substrates having a lower redox potential exhibiting a greater rate of oxidation.[150, 321]

Table 4. Product yields and ratios for the laccase-catalyzed reaction of benzoylacetonitrile (**1**) with substituted hydroquinones (**2**)[a]

Entry	2	R	Laccase (U)	Product	Ratio 6:7	Yield (%) 3a
1	**a**	OCH_3	10	**6a**	-	37
2	**b**	H	50	**6b**	-	24
3	**c**	*t*-butyl	10	**7c**	-	46
4	**d**	CH_3	10	**6d, 7d**	60:40	26
5	**e**	F	50	-	-	-
6	**f**	$COCH_3$	100	**8f**	-	89
7	**g**	$CO_2CH_2CH_3$	100	**8g**	-	80
8	**h**	[b]	10	**6h**	-	25

[a] Reaction conditions: 1 mmol **1**, 0.25 mmol **2**, 60°C, 24 h, 30 mL 0.1 M sodium phosphate buffer pH 7.0
[b] Compound **2h** is 2,6-dimethoxyhydroquinone

With regards to product yields, it can be seen from Table 4 that the two hydroquinones containing a conjugated carbonyl within their substituent, **2f** and **2g** (entries 6 and 7), by far provided the highest yielding products (**8f** and **8g**). This can be rationalized by the stability of the initial radical that is formed upon laccase oxidation, as the carbonyl group allows for further delocalization via resonance, thus making these radicals more stable compared to those where radical delocalization into the substituent is not possible. A more stable radical would decrease the likelihood of hydrogen atom abstraction from **1**, thus decreasing the extent of oxidative degradation of **1** (Figure 66). This same argument can be used to explain the other product yields. Hydroquinones **2a** and **2c** (entries 1 and 3) have substituents with greater electron-donating power than **2d** (entry 4), allowing for a more stable radical via induction, thus producing greater product yields. **2b** provided the lowest yielding product (entry 2), while **2e**, which has a highly electron-withdrawing fluorine substituent, provided no coupling product due to complete oxidative degradation of **1** (entry 5). Compound **2h** provided a lower than expected product yield based on the preceding argument; however, this is likely due to the added

steric hindrance of the second methoxy substituent. It must be noted that the reaction of **1** with phenylhydroquinone produced a coupling product in good yield (63%); however, the product could not be fully characterized due to overlapping signals in the ^{1}H and ^{13}C NMR spectra, due to the formation of non-isolable regioisomers (data for product from reaction of **1** with phenylhydroquinone: yield: 63%; dark-brown solid; mp: 105-110°C; IR: $\tilde{\nu}$ 3307, 2237, 1638, 1598, 1472, 1167, 1024, 998, 907, 698 cm^{-1}; HRMS (ESI): $C_{21}H_{15}NO_3$ calculated 329.1052, found 329.1048).

The structures of the products formed by the laccase-catalyzed reaction of **1** with substituted hydroquinones **2** were determined based on ^{1}H, ^{13}C, and ^{1}H-^{13}C HMBC NMR spectroscopic data. The structures of the compounds in which different regioisomers could exist are shown in Figure 68. Product **6a** was formed as a single regioisomer from the reaction of **1** with **2a**. The methoxy protons were assigned to the singlet at δ = 3.72 ppm in the ^{1}H NMR spectrum. This signal exhibited only one correlation in the HMBC spectrum, that being a ^{3}J correlation with C-4′ (δ = 143.4 ppm). C-4′ showed four correlations in the HMBC spectrum, including a ^{3}J with the singlet at δ = 7.04 ppm, corresponding to 6′-H, thus ruling out products that would be formed via addition at positions 3 and 6 of **5a**. Product **7c**, on the other hand, which was formed by the reaction of **1** with **2c**, was found to be produced via addition at carbon 6 of **5c**. C-3′ (δ = 133.1 ppm), which was assigned based on a ^{3}J HMBC correlation with the *t*-butyl protons at δ = 1.36 ppm, provided only three HMBC correlations, thus providing evidence for the proposed structure.

The reaction of **1** with **2d** yielded a mixture of two products, **6d** and **7d** in a 60:40 ratio, which could not be separated via column chromatography. The product **6d**, C-4′ (δ

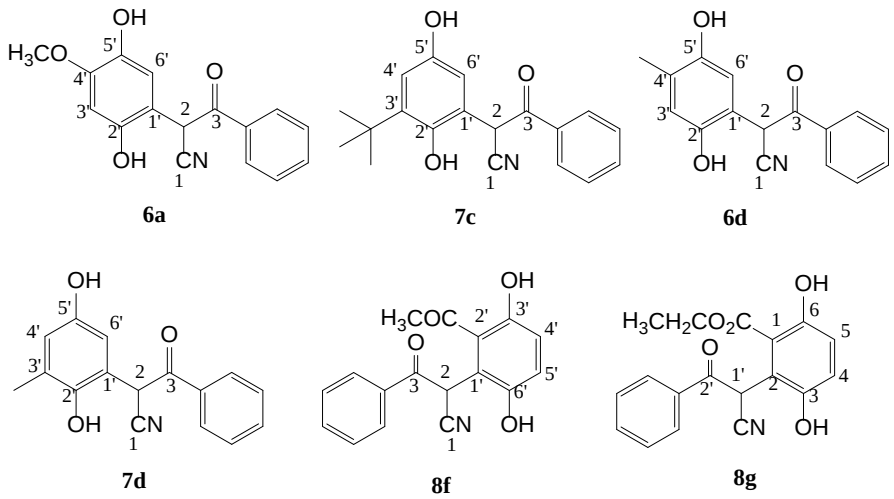


Figure 68. Structures of the products formed by the laccase-catalyzed reaction of benzoylacetonitrile (**1**) with substituted hydroquinones (**2**).

= 141.4 ppm) showed a ^{3}J HMBC correlation with 5′-OH (δ = 8.81 ppm) as well as correlations with 3′-H, 6′-H, and CH_3. On the other hand, C-3′ in **7d** exhibited only three correlations in the HMBC spectrum, ^{2}J with CH_3 and 4′-H and ^{3}J with 2′-OH.

The regiochemistry of products **8f** and **8g** was rather unexpected, given the steric hindrance of nucleophilic attack at carbon 3 of **5f** and **5g** supplied by the substituent. Nevertheless, the NMR data provides clear evidence for the proposed structures. The ^{1}H NMR spectrum of **8f** clearly shows two sets of doublets in the aromatic region corresponding to the 4′-H (δ = 6.54 ppm) and 5′-H (δ = 7.24 ppm) with a coupling constant of ^{3}J = 8.7 Hz. Similarly, the ^{1}H NMR spectrum of **8g** also shows two sets of doublets in the aromatic region, a signal at δ = 6.57 ppm and another at δ = 7.58 ppm, with a coupling constant of ^{3}J = 8.6 Hz.

The regioselectivity of the reaction products can be explained based on both steric and electronic effects, with the latter seemingly having a larger influence. For hydroquinones containing electron-donating substituents (i.e. **2a,c,d**), addition

preferentially takes place on the side of the *para*-quinone opposite the substituent, which is favorable based on steric effects. Also, for these compounds, C5 is the most electrophilic carbon, further aiding in addition to this position. The reason for addition to C6 when an alkyl substituent is present, as in the cases for **2c** and **d** (minor regioisomer), is unexpected given that addition to the more electrophilic C5 preferentially takes place when a methoxy substituent is present (**2a**) and is the major product formed when a methyl substituent is present (**2d**). However, a similar trend has been observed in the laccase-catalyzed addition of thiols to *ortho*-quinones whereby addition of the thiol takes place at a different carbon of the *ortho*-quinone depending on whether the catechol precursor is substituted with a methoxy or an alkyl substituent.[221] For hydroquinones containing an electron-withdrawing substituent (i.e. **2f, g**), it seems that electronics dictate the regiochemistry of addition as nucleophilic attack occurs solely at the more electrophilic, yet more hindered, C3 to yield products **8f** and **8g**.

4.4 Conclusions

A green, one-pot method for the α-arylation of a primary nitrile has been developed. It employs laccases to oxidize substituted hydroquinones to generate reactive *para*-quinones *in situ*, which are then able to undergo a cross-coupling reaction with benzoylacetonitrile to produce the corresponding benzylic nitrile products. The reaction conditions were optimized to provide the greatest yield of product, which was found to occur under mild conditions (60°C, pH 7.0). The product yields range from good to excellent with hydroquinones possessing a substituent with a conjugated carbonyl providing the highest yields, probably due to a more stable radical species. The

regioselectivity of the reaction products can be explained via electronic effects, in that nucleophilic attack is more likely to occur on the more electrophilic carbon.

CHAPTER 5. LACCASE-CATALYZED SYNTHESIS OF 2,3-ETHYLENEDITHIO-1,4-QUINONES[III]

5.1 Introduction

To date, there had been much work published on laccase-catalyzed C-C and C-N bond forming reactions; however, similar reported reactions involving sulfur based nucleophiles for laccase-catalyzed C-S bond formations were few.[219-222, 322] The focus of the current study was to employ laccases to perform the cross-coupling reaction of various substituted hydroquinones (**2**) with a small dithiol, namely 1,2-ethanedithiol (**9**). This study leverages the recent study conducted by Kidwai et al. whereby a successful laccase-catalyzed addition of a diamine to both hydroquinones and catechols was achieved for the synthesis of novel quinoxalines.[218] The current study was conducted in spite of research that suggests some small sulfhydryl compounds (e.g. cysteine) are potent inhibitors of laccases from particular fungal species.[323]

The reaction products of the laccase-catalyzed addition of **9** with substituted hydroquinones contain the 2,3-ethylenedithio-1,4-quinone substructure (Figure 69). Compounds containing quinones are present all throughout nature and in many biologically active natural products.[58] The compounds synthesized in this study are no different. For example, 3′,4′-(ethylenedithio)avarone (Figure 70), a synthetic derivative of the marine sponge sesquiterpene quinone avarone, is a compound that contains the 2,3-ethylenedithio-1,4-quinone structural moiety and has shown to exhibit antiproliferative

[III] This manuscript, titled "Laccase-catalyzed synthesis of 2,3-ethylenedithio-1,4-quinones," was published in *Journal of Molecular Catalysis B: Enzymatic* (**2015**, *119*, 85-89). The other author is Arthur J. Ragauskas, who is affiliated with Georgia Institute of Technology. The manuscript was reproduced with permission from Elsevier; the copyright license agreement is provided in Appendix B.

activity towards tumor cells.[324] Another compound with similar structural features, dithianon (Figure 70), has also been shown to possess cancerostatic properties as well as fungicidal activity.[325]

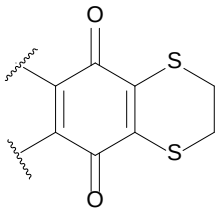


Figure 69. 2,3-Ethylenedithio-1,4-quinone substructure.

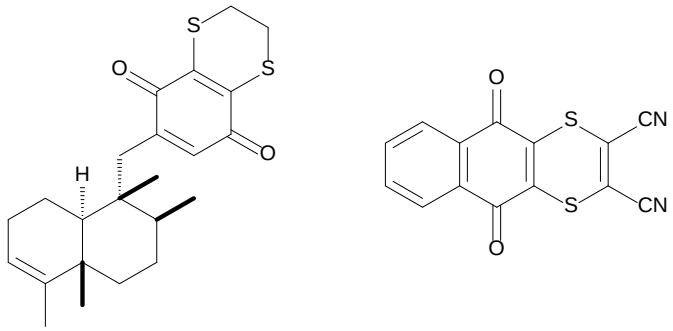


Figure 70. Compounds containing the 2,3-ethylenedithio-1,4-quinone substructure.

Traditional syntheses of sulfide-substituted 1,4-quinones involve the nucleophilic addition of thiol to 2,3-dichloro-1,4-quinone derivatives.[326] These reactions are usually conducted in ethanol and require heat. In addition, alkylthiols only undergo substitution to the quinone once; thus, sodium salts of the alkyl thiols must be prepared and used for the substitution reaction to occur at both position 2 and 3 of 2,3-dichloro-1,4-quinone derivatives. Furthermore, prior synthetic steps must be performed to arrive at the 2,3-dichloro-1,4-quinone intermediate that require harsh conditions and a chemical oxidant (cerium ammonium nitrate).[219] Thus, the laccase-catalyzed addition of **9** to substituted

hydroquinones conducted in this study is a simple, one-step, green alternative to the synthesis of 2,3-ethylenedithio-1,4-quinones.

5.2 Experimental

5.2.1 Materials

All compounds were purchased from Sigma-Aldrich except *tert*-butylhydroquinone, which was a product of Acros Organics. Laccases from the white-rot fungus *Trametes villosa* expressed in an *Aspergillus* host (NOVO NS51002) were appreciatively donated by Novo Nordisk Biochem (now Novozymes), Franklinton, North Carolina, USA. All compounds, solvents, and enzyme were used as received without further purification. Aluminum sheets pre-coated with silica gel 60 (EMD Chemicals) were used for thin-layer chromatography (TLC) experiments. Silica gel 60 (EM Separations Technology) was used as the stationary phase for column chromatography.

5.2.2 Enzyme Assay

Full experimental procedures regarding laccase activity measurements are detailed in Chapter 3.2.1.

5.2.3 General Procedure for the Laccase-Catalyzed Reaction of 1,2-Ethanedithiol with Substituted Hydroquinones

Full experimental procedures regarding the laccase-catalyzed coupling of 1,2-ethanedithiol with substituted hydroquinones are provided in Chapter 3.2.3.

5.2.4 Product Characterization Data

NMR, FTIR, and MS spectra of new compounds are given in Appendix A. All NMR experiments were conducted using $CDCl_3$ as the solvent.

6-Methoxy-2,3-dihydrobenzo[*b*][1,4]dithiine-5,8-dione (**10a**)

Yield: 64%; purple solid; mp: 203-208°C; IR: $\tilde{\nu}$ 1616 (C═O) cm^{-1}; ^1H NMR: δ 3.25 (s, 4H, $(CH_2)_2$), 3.82 (s, 3H, OCH_3), 5.89 (s, 1H, C═CH) ppm; ^{13}C NMR: δ 27.00, 27.55, 56.80, 107.16, 135.11, 139.99, 159.34, 175.81, 181.73 ppm; HRMS (ESI): $C_9H_8O_3S_2$ calculated 227.9915, found 227.9922.

6-Methyl-2,3-dihydrobenzo[*b*][1,4]dithiine-5,8-dione (**10b**)

Yield: 57%; brown solid; mp: 170-175°C; IR: $\tilde{\nu}$ 1636 (C═O) cm^{-1}; ^1H NMR: δ 2.04 (s, 3H, CH_3), 3.22 (s, 4H, $(CH_2)_2$), 6.56 (s, 1H, C═CH) ppm; ^{13}C NMR: δ 15.65, 26.33, 26.51, 132.78, 137.36, 137.69, 145.70, 180.38, 180.71 ppm; HRMS (ESI): $C_9H_8O_2S_2$ calculated 211.9966, found 211.9960.

6-(*tert*-Butyl)-2,3-dihydrobenzo[*b*][1,4]dithiine-5,8-dione (**10c**)

Yield: 68%; dark purple solid; mp: 112-117°C; IR: $\tilde{\nu}$ 1635 (C═O) cm^{-1}; ^1H NMR: δ 1.29 (s, 9H, $C(CH_3)_3$), 3.24 (s, 4H, $(CH_2)_2$), 6.58 (s, 1H, C═CH) ppm; ^{13}C NMR: δ 26.20, 26.76, 28.80, 35.18, 123.96, 131.19, 132.03, 155.93, 180.20, 181.08 ppm; HRMS (ESI): $C_{12}H_{14}O_2S_2$ calculated 254.0435, found 254.0439.

6-Phenyl-2,3-dihydrobenzo[*b*][1,4]dithiine-5,8-dione (**10d**)

Yield: 71%; black solid; mp: 72-77°C; IR: $\tilde{\nu}$ 1633 (C═O) cm^{-1}; ^1H NMR: δ 3.29 (s, 4H, $(CH_2)_2$), 6.84 (s, 1H, C═CH), 7.46 (m, 5H, Ar-H) ppm; ^{13}C NMR: δ 26.36, 26.69, 128.09, 128.83, 129.79, 130.45, 132.16, 133.11, 135.35, 145.72, 179.67, 180.45 ppm; HRMS (ESI): $C_{14}H_{10}O_2S_2$ calculated 274.0122, found 274.0130.

2,3-Dihydronaphtho[2,3-*b*][1,4]dithiine-5,10-dione (**10e**)

Yield: 74%; purple solid; mp: 215-220°C; IR: $\tilde{\nu}$ 1643 (C=O) cm^{-1}; ^{1}H NMR: δ 3.31 (s, 4H, $(CH_2)_2$), 7.69 (m, 2H, Ar-H), 8.08 (m, 2H, Ar-H) ppm; ^{13}C NMR: δ 26.63, 126.50, 131.20, 133.33, 140.38, 178.18 ppm; HRMS (ESI): $C_{12}H_8O_2S_2$ calculated 247.9966, found 247.9966.

5.3 Results and Discussion

To investigate whether the laccase-catalyzed reaction between **9** and substituted hydroquinones **2** would proceed to yield the desired product, an initial experiment was conducted that reacted **9** with methoxyhydroquinone (**2a)** in the presence of laccases (Figure 71). As was postulated, the reaction proceeded to yield 2,3-ethylenedithio-1,4-quinone **10a** as determined by spectral data. The reaction product is likely achieved via the laccase generated *in situ* 1,4-quinone intermediate.

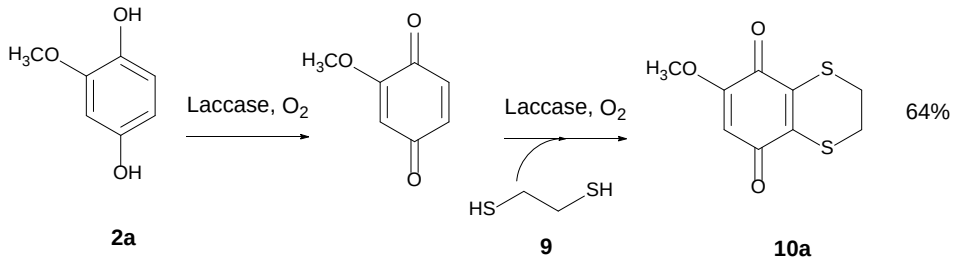


Figure 71. Laccase-catalyzed reaction of 1,2-ethanedithiol (**9**) with methoxyhydroquinone (**2a**). Reaction conditions: 2.50 mmol **9**, 0.50 mmol **2a**, 50 U laccase, 15 mL 0.10 M sodium acetate buffer pH 5.0, rt, 24 h.

In order to make the synthesis of practical value, follow up studies were conducted to determine the conditions under which optimal product yield could be obtained. Parameters that were experimented with include: the ratio of **9**:**2a**, laccase

amount, pH of aqueous solvent, temperature, and time. The results of the reaction optimization are displayed in Table 5. Given that the amount of substrate (i.e. methoxyhydroquinone **2a**) used in each reaction was 0.50 mmol, the first step was to determine the appropriate amount of laccase that would ensure complete oxidation. Comparing entries 1-3 (Table 5), it can be seen that 50 U of laccase (entry 1) was sufficient for complete oxidation as 100 U (entry 3) had no additional impact on product yield and 20 U (entry 2) did not provide complete oxidation (as determined by GC-MS data). Next, the ratio of **9**:**2a** was experimented with. It was found that having a low **9**:**2a** ratio significantly impacted product yields as only trace amounts of desired product were detected under these conditions (entries 4 and 5). This suggests that a large excess of **9** must be present to assist in preventing undesirable 1,4-quinone polymerization and degradation reactions that are known to occur. However, a ratio of 10:1 (entry 6) did not significantly increase product yield compared to a 5:1 ratio (entry 1). Increasing temperature (entry 7) had a negative impact on product yield, probably due to an increase in quinone polymerization and degradation reactions, as did increasing the pH (entry 8), which is likely due to the reduced activity of laccases at the higher pH.[85] Reaction time was also experimented with and as can be seen in Table 5 a reaction time of 16 h (entry 10) was sufficient enough to complete the reaction as 8 h (entry 11) did not give a complete reaction and 48 h (entry 9) did not increase the product yield substantially. Finally, a control reaction was conducted that reacted **9** with **2a** in the absence of laccases (entry 12), which gave no reaction. Thus, the reaction conditions in entry 10 were taken to be the optimal conditions for the laccase-catalyzed addition of **9** to **2a**.

Table 5. Reaction optimization results for the laccase-catalyzed reaction of 1,2-ethanedithiol (**9**) with methoxyhydroquinone (**2a**).

Entry	1:2a Ratio[a]	Laccase (U)	pH[b]	Temp. (°C)	Time (h)	Yield (%) 3a
1	5:1	50	5.0	rt	24	64
2	5:1	20	5.0	rt	24	ir
3	5:1	100	5.0	rt	24	63
4	1:1.25	50	5.0	rt	24	trace
5	2:1	50	5.0	rt	24	trace
6	10:1	50	5.0	rt	24	65
7	5:1	50	5.0	50	24	37
8	5:1	50	7.0	rt	24	49
9	5:1	50	5.0	rt	48	66
10	**5:1**	**50**	**5.0**	**rt**	**16**	**64**
11	5:1	50	5.0	rt	8	ir
12	5:1	-	5.0	rt	16	nr

ir: incomplete reaction; nr: no reaction

[a] 0.50 mmol of **2a** used in all experiments

[b] 15 mL of 0.10 M sodium acetate buffer was used for pH 5.0 and 0.10 M sodium phosphate buffer was used for pH 7.0

Following the reaction optimization, the optimal reaction conditions were then employed to react **9** with other substituted hydroquinones in the presence of laccases (Figure 72). Substrates included hydroquinones substituted with a methyl, methoxy, *t*-butyl, or aryl group, as well as 1,4-naphthohydroquinone **2j** and 2,6-dimethoxyhydroquinone **2h**. The product yields are given in Table 6. In general, the yields tend to increase with increasing size of the substituent. This is likely due to the fact that a bulky substituent on the hydroquinone will reduce the probability of competing quinone polymerization and degradation reactions from occurring, thus allowing for the desired thiol addition reaction to take place. Furthermore, the reaction did not proceed to yield any desired product with unsubstituted hydroquinone, when electron-withdrawing substituents were present (e.g. fluorohydroquinone and chlorohydroquinone), or when catechols were used, as was observed in the study documented in Chapter 4.[327] Thus, for

a thiol addition reaction to occur, the results suggest that a stable 1,4-quinone intermediate is mandatory, which is achieved with a bulky electron-donating substituent. Interestingly, the product resulting from the reaction of **9** with **2h** (Table 6, entry 6) was the identical product to that resulting from the reaction of **9** with **2a** (Table 6, entry 1), the product being **10a**, albeit in a lower yield. This implies that the dithiol undergoes addition to the *in situ* 1,4-quinone twice in the presence of a substituent that can be readily eliminated, that being methanol in the current example.

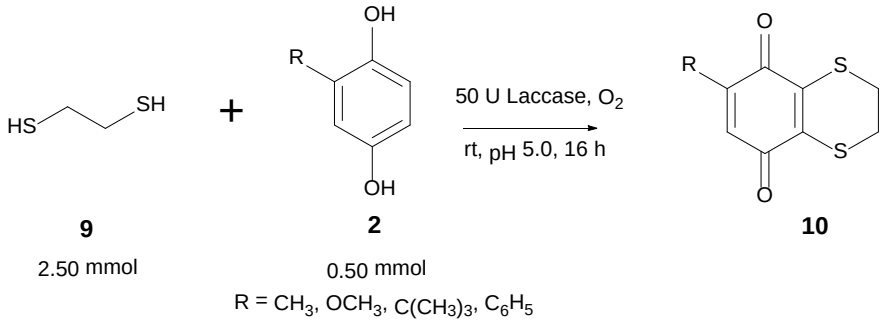


Figure 72. Laccase-catalyzed reaction of 1,2-ethanedithiol (**9**) with substituted hydroquinones (**2**).

Table 6. Products and yields for the laccase-catalyzed addition of 1,2-ethanedithiol (**9**) to substituted hydroquinones (**2**).[a]

Entry	Substrate	R	Product	Yield (%)
1	**2a**	OCH_3	**10a**	64
2	**2d**	CH_3	**10b**	57
3	**2c**	$C(CH_3)_3$	**10c**	68
4	**2i**	C_6H_5	**10d**	71
5	**2j**[b]		**10e**	74
6	**2h**[c]		**10a**	44

[a] Reaction conditions: 2.50 mmol **9**, 0.50 mmol **2**, 50 U laccase, rt, 16 h. **2a,d** were reacted in 15 mL 0.10 M sodium acetate buffer pH 5.0 and **2c,h-j** were reacted in 15 mL 0.10 M sodium acetate buffer pH 5.0 containing 10% (v:v) methanol to aid solubility

[b] Compound **2j** is 1,4-naphthohydroquinone

[c] Compound **2h** is 2,6-dimethoxyhydroquinone

The structures of all the reaction products were unambiguously determined via spectroscopic and spectrometric data. Interestingly, the protons of the methylene groups in the products, which derive from **9**, appear as a singlet in the ^{1}H NMR spectra, regardless of the substituent on the quinone moiety. The current data is consistent with data from another compound containing the 2,3-ethylenedithio-1,4-quinone substructure, namely 3′,4′-(ethylenedithio)avarone (Figure 70), whose two adjacent methylene group's protons also appear as a singlet in the ^{1}H NMR spectrum.[324]

The products of the laccase-catalyzed addition of **9** to **2** are proposed to be formed via a sequential oxidation-addition-oxidation-addition-oxidation mechanism (Figure 73). Laccases oxidize the starting hydroquinone **2** into the 1,4-quinone **5**. This highly reactive intermediate undergoes nucleophilic addition by **9**, and followed by tautomerization back to the aromatic form, the product of a single sulfur addition, **11**, is reached. Due to the presence of laccase, oxidation occurs yet again to give the quinone intermediate **12**, which undergoes a second nucleophilic addition by the other sulfur atom of the dithiol to yield the double addition product **13** after another tautomerization. After a final laccase-catalyzed oxidation, the final product **10** is achieved. The sequential oxidations and additions that are proposed in the current mechanism have also been witnessed in other laccase-catalyzed reactions involving sulfur nucleophiles as well as nitrogen-derived nucleophiles.[199, 205, 219-220, 328] The subsequent oxidations are made possible by the electron-donating nature of the sulfur and nitrogen substituents whose pi electrons add electron density to the aromatic ring allowing for a more favorable oxidation compared to substituents that withdraw electron density form the aromatic ring. It must be noted that, given the appropriate amount of laccases are used, the reaction will go to completion (i.e.

product **10**) and that no other products resulting from a single addition or incomplete oxidation (i.e. **11**, **12**, or **13**) were observed at the end of the reaction period.

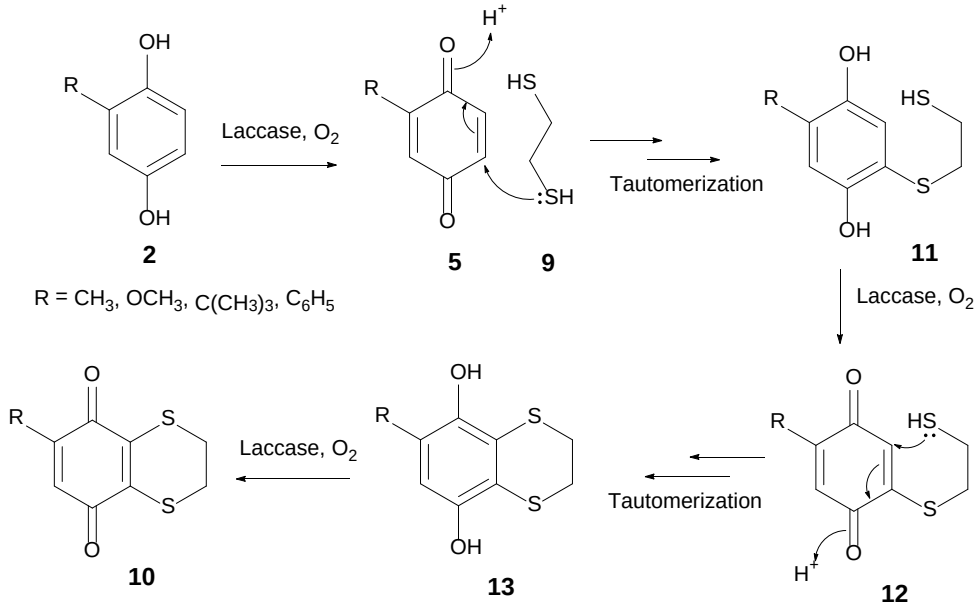


Figure 73. Proposed reaction mechanism for the laccase-catalyzed reaction of 1,2-ethanedithiol (**9**) with substituted hydroquinones (**2**) to produce 2,3-ethylenedithio-1,4-quinones (**10**).

Worthy of a mention is the occurrence of side-reactions that produce small cyclic sulfides. Based on GC-MS data of the crude extracts of the laccase-catalyzed reactions of **9** with **2**, small amounts of both 1,2,3-trithiolane and 1,2,5,6-tetrathiocane (Figure 74) were detected. It is thought that these products are formed via a radical-coupling mechanism. Interestingly, the reaction of **9** with laccase alone did not provide any reaction; however, adding the laccase-mediator violuric acid into the reaction mixture afforded the cyclic sulfides. Thus, it is proposed that, in the laccase-catalyzed reaction of **9** with **2**, the hydroquinones act as the laccase-mediators in that the initial phenoxy radicals generated via laccase oxidation are able to oxidize **9** to produce sulfur

radicals,[125] which can then undergo coupling reactions with one another to yield the cyclic sulfides.

Figure 74. Side products of the laccase-catalyzed reaction of 1,2-ethanedithiol (**9**) with substituted hydroquinones (**2**).

The presented synthetic protocol fulfills many of the principles of green chemistry. Using the reaction of **9** with **2j** as an example, the atom economy of this synthetic scheme is 92%, which indicates a highly efficient reaction. Regarding waste generation, the E factor for the reaction of **9** with **2j** was calculated to be 64.1. This may seem rather high, but is standard for chemical syntheses within the pharmaceutical industry.[19] The E factor could be decreased significantly by reducing the amount of EtOAc used in the extraction stage, which, as can be seen in Table 7, contributes more than 90% to the E factor. The reactions are conducted in an aqueous acetate buffer solution, employing methanol as a cosolvent on some occasions, which are environmentally benign solvents. Furthermore, the substitution of a dichloromethane based solvent with an EtOAc/hexane solvent system for chromatography completely eliminates chlorinated solvent waste, which is of high priority in the field of organic synthesis due to its toxicity and high cost of disposal.[329] The reactions are run at ambient temperature and pressure, thus, the operating conditions are safe and there are no auxiliary energy demands. The process employs a catalytic oxidant (i.e. laccases), rather than a stoichiometric oxidant, which dramatically reduces waste production. Furthermore,

the biocatalyst is renewable, non-toxic, and biodegradable and the enzyme substrates (i.e. hydroquinones) are derived from a renewable feedstock (i.e. lignin). The space time yield of the synthetic process, using the reaction of **9** with **2j** as an example once again, was calculated to be 0.002 mol × L^{-1} × h^{-1}. This is rather low, but could be increased by lowering the solvent volume or increasing the rate of reaction via use of cross-linked enzyme aggregates (CLEAs) of laccases.[224, 330]

Table 7. Contribution of each chemical to the E factor for the reaction of **9** with **2j**.

Chemical	Mass (mg)	% Contribution
9	235.5	3.9
2j	80.1	1.3
Laccase	39.7[a]	0.7
CH_3COOH	29.2	0.5
NaOAc	70.9	1.2
CH_3OH	118.7[b]	2.0
EtOAc	5400[b]	90.4

Note: for convenience, column chromatography was used to purify the products. However, a variety of purification methods can be used, thus materials used in chromatography were not factored into the E factor calculations

[a] Mass of laccase solution

[b] Taking into account a loss of 10% of solvent used

5.4 Conclusions

An eco-friendly method for the synthesis of novel 2,3-ethylenedithio-1,4-quinones has been presented. The method utilizes the oxidizing ability of laccases to produce 1,4-quinones *in situ* that undergo nucleophilic addition by 1,2-ethanedithiol followed by subsequent oxidation and addition steps to afford the 2,3-ethylenedithio-1,4-quinone products in good yields. The reactions are carried out under mild conditions in one-pot in an aqueous solvent. The stability of the *in situ* generated 1,4-quinones proved

to be a vital factor in determining whether the cross-coupling reaction would occur, which was achieved by bulky electron-donating substituents on the hydroquinones. The presence of small cyclic sulfide compounds was detected, probably formed by competing radical reactions. This study is evidence that laccase-catalyzed additions involving small thiols are possible in spite of research that suggests small thiols are inhibitors of laccases.

CHAPTER 6. ECOFRIENDLY SYNTHESES OF PHENOTHIAZONES AND RELATED STRUCTURES FACILITATED BY LACCASES – A COMPARATIVE STUDY[IV]

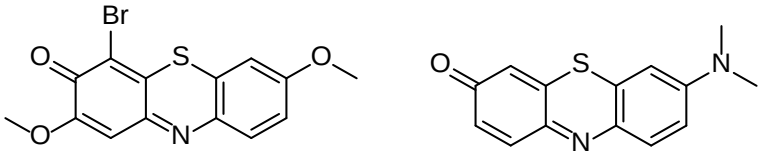
6.1 Introduction

Building on the chemistry developed in Chapter 5, the study presented in this chapter was focused toward coupling hydroquinones with compounds containing both the thiol and amine functional groups. While laccase-catalyzed coupling reactions involving nucleophiles derived from carbon, nitrogen, or sulfur have been widely studied, the use of compounds containing two nucleophilic centers capable of forming multiple bonds to yield cyclic products is much less explored.[222, 322, 331] The laccase-catalyzed coupling of 2-aminothiophenol with hydroquinone for the synthesis of 3*H*-phenothiazin-3-one has been previously achieved;[331] however, the process suffers from low product yield (21%). The current study presents an alternative methodology that significantly increases product yields of several synthesized phenothiazones.

The phenothiazones are an important class of compounds that possess a variety of biological activities and practical use. Early studies demonstrated that these compounds exhibit lethal effects on liver fluke as well as paralytic effects on the human parasitic worm *Ascaris lumbricoides*.[332-333] More recently, derivatives and analogs, particularly 4-bromo-2,7-dimethoxy-3*H*-phenothiazin-3-one (Figure 75), have shown inhibitory effects on 5-lipoxygenase and mammalian leukotriene biosynthesis, thus, they find therapeutic

[IV] This manuscript, titled "Ecofriendly syntheses of phenothiazones and related structures facilitated by laccase – a comparative study," was published in *Tetrahedron Letters* (**2016**, *57*, 3749-3753). The other author is Arthur J. Ragauskas, who is affiliated with Georgia Institute of Technology. The manuscript was reproduced with permission from Elsevier; the copyright license agreement is provided in Appendix B.

use in treating allergies, inflammation, asthma, and cardiovascular disorders.[334-335] They have also displayed tuberculostatic, antibacterial, and analgesic properties and have been used to treat oxidative stress disorders.[336-337] Furthermore, they offer protection to mild steel from acidic corrosion, and find use in organic semiconductors and dyes (e.g. methylene violet, Figure 75).



4-Bromo-2,7-dimethoxy-3*H*-phenothiazin-3-one

Methylene violet

Figure 75. Representative structures of phenothiazones.

The first reported synthesis of a phenothiazone compound involved the oxidation of 3-hydroxyphenothiazine by $FeCl_3$.[338] Most early syntheses relied upon the use of stoichiometric, transition-metal-containing oxidants, such as $FeCl_3$, $K_2Cr_2O_7$, MnO_2, or ceric ammonium nitrate to oxidize phenothiazines to the corresponding phenothiazones at elevated temperatures in organic solvents.[334-335, 339-342] More contemporary syntheses involve the condensation of 2-aminothiophenol with 1,4-quinones;[334-335, 343-346] however, these reactions are all conducted in organic solvents. Thus, there lacks a method that is conducted both in an aqueous solvent system and free of stoichiometric, transition-metal oxidants. Herein, a green, biocatalytic approach to the synthesis of phenothiazones and related structures is reported.

6.2 Experimental

6.2.1 Materials

All reagents and solvents were purchased from either Sigma-Aldrich or VWR. Laccases from the white-rot fungus *Trametes villosa* expressed in an *Aspergillus* host (NOVO NS51002) were appreciatively donated by Novo Nordisk Biochem (now Novozymes), Franklinton, North Carolina, USA. All compounds, solvents, and enzyme were used as received without further purification. Aluminum sheets pre-coated with silica gel 60 (EMD Chemicals) were used for thin-layer chromatography (TLC) experiments. Glass plates coated with silica gel (20 x 20 cm, 2000 μm) were used for preparative layer chromatography.

6.2.2 Enzyme Assay

Full experimental procedures regarding laccase activity measurements are detailed in Chapter 3.2.1.

6.2.3 Synthetic Procedures for the Laccase-Facilitated Synthesis of Phenothiazones

5*H*-Benzo[a]phenothiazin-5-one (**15a**)

Method A: 100.1 mg (0.625 mmol) of 1,4-naphthohydroquinone (**2j**) was dissolved in 1.5 mL MeOH and added dropwise to 8.5 mL 0.10 M sodium acetate buffer pH 5.0 that was stirring in a 50 mL round-bottom flask. Then, 53.5 μL (62.6 mg, 0.50 mmol) of 2-aminothiophenol (**14**) was added to the mixture, followed by 33.1 μL (50 U) of laccase solution. The resulting mixture was allowed to stir at rt for 6 h. After this time, the mixture was centrifuged and the supernatant decanted. The remaining solid was washed

with deionized water (3 x 5 mL) and dried overnight in a vacuum oven at 30°C. The supernatants were combined and extracted once with 10 mL EtOAc to recover any soluble product, dried over $MgSO_4$, and the solvent removed via rotary evaporation. The crude solid and crude extract were combined and the product was purified via preparative layer chromatography using silica gel coated on glass plates as the stationary phase and dichloromethane containing 1% MeOH (v:v) as the mobile phase. Yield of **15a**: 14.5 mg (11%).

Method B: 100.1 mg (0.625 mmol) of **2j** was dissolved in 1.5 mL MeOH and added dropwise to 8.5 mL 0.10 M sodium acetate buffer pH 5.0 containing 33.1 μL (50 U) of laccase solution while stirring at rt in a 50 mL round-bottom flask. After 2 h, 53.5 μL (62.6 mg, 0.50 mmol) of **14** was added to the mixture, and the reaction was allowed to stir for an additional 4 h. Work-up and purification as in Method A. Yield of **15a**: 26.3 mg (20%).

Method C: 98.8 mg (0.625 mmol) of 1,4-naphthoquinone (**5j**) was suspended in 1.5 mL MeOH and added to 8.5 mL 0.10 M sodium acetate buffer pH 5.0 that was stirring in a 50 mL round-bottom flask. Then, 53.5 μL (62.6 mg, 0.50 mmol) of **14** was added to the mixture and the reaction was allowed to stir for 6 h at rt. Work-up and purification as in Method A. Yield of **15a**: 57.9 mg (44%).

Method D: 98.8 mg (0.625 mmol) of **5j** was suspended in 1.5 mL MeOH and added to 8.5 mL 0.10 M sodium acetate buffer pH 5.0 that was stirring in a 50 mL round-bottom flask. Then 53.5 μL (62.6 mg, 0.50 mmol) of **14** was added to the mixture and the reaction was allowed to stir for 2 h at rt. After this time, 33.1 μL (50 U) of laccase

solution was added and the reaction was allowed to stir for an additional 4 h. Work-up and purification as in Method A. Yield of **15a**: 68.5 mg (52%).

3*H*-Phenothiazin-3-one (**15b**)

Method B: 68.8 mg (0.625 mmol) of hydroquinone (**2b**) was dissolved in 1 mL MeOH and added dropwise to 9 mL 0.10 M sodium acetate buffer pH 5.0 that was stirring in a 50 mL round-bottom flask. Then, 33.1 μL (50 U) of laccase solution was added and the mixture was allowed to stir at rt for 2 h. After this time, 53.5 μL (62.6 mg, 0.50 mmol) of **14** was added to the mixture, and the reaction was allowed to stir for an additional 4 h. After this time, the mixture was centrifuged and the supernatant decanted. The remaining solid was washed with deionized water (3 x 5 mL) and dried overnight in a vacuum oven at 30°C. The supernatants were combined and extracted once with 10 mL EtOAc to recover any soluble product, dried over $MgSO_4$, and the solvent removed via rotary evaporation. The crude solid and crude extract were combined and the product was purified via preparative layer chromatography using silica gel coated on glass plates as the stationary phase and dichloromethane containing 5% MeOH (v:v) as the mobile phase. Yield of **15b**: 25.6 mg (24%).

Method C: 67.6 mg (0.625 mmol) of *p*-benzoquinone (**5b**) was dissolved in 1 mL MeOH and added dropwise to 9 mL 0.10 M sodium acetate buffer pH 5.0 that was stirring in a 50 mL round-bottom flask. Then, 53.5 μL (62.6 mg, 0.50 mmol) of **14** was added to the mixture and the reaction was allowed to stir for 6 h at rt. Work-up and purification as in Method B. Yield of **15b**: 9.7 mg (9%).

Method D: 67.6 mg (0.625 mmol) of **5b** was dissolved in 1 mL MeOH and added dropwise to 9 mL 0.10 M sodium acetate buffer pH 5.0 that was stirring in a 50 mL round-bottom flask. Then, 53.5 μL (62.6 mg, 0.50 mmol) of **14** was added to the mixture and the reaction was allowed to stir for 2 h at rt. After this time, 33.1 μL (50 U) of laccase solution was added and the reaction was allowed to stir for an additional 4 h. Work-up and purification as in Method B. Yield of **15b**: 65.0 mg (61%).

2,4-Dimethoxy-3*H*-phenothiazin-3-one (**15c**)

Method B: 106.4 mg (0.625 mmol) of 2,6-dimethoxyhydroquinone (**2h**) was dissolved in 1 mL MeOH and added dropwise to 9 mL of 0.10 M sodium acetate buffer pH 5.0 that was stirring in a 50 mL round-bottom flask. Then, 33.1 μL (50 U) of laccase solution was added and the mixture was allowed to stir at rt for 2 hours. After this time, 53.5 μL (62.6 mg, 0.50 mmol) of **14** was added to the mixture, and the reaction was allowed to stir for an additional 4 h. After this time, the mixture was centrifuged and the supernatant decanted. The remaining solid was washed with deionized water (3 x 5 mL) and dried overnight in a vacuum oven at 30°C. The supernatants were combined and extracted once with 10 mL EtOAc to recover any soluble product, dried over $MgSO_4$, and the solvent removed via rotary evaporation. The crude solid and crude extract were combined and the product was purified via preparative layer chromatography using silica gel coated on glass plates as the stationary phase and dichloromethane containing 2.5% MeOH (v:v) as the mobile phase. Yield of **15c**: 72.4 mg (53%).

1-Methyl-3*H*-phenothiazin-3-one (**15d**) and 2-Methyl-3*H*-phenothiazin-3-one (**15e**)

Method B: 77.6 mg (0.625 mmol) of methylhydroquinone (**2d**) was dissolved in 1 mL MeOH and added dropwise to 9 mL 0.10 M sodium acetate buffer pH 5.0 that was stirring in a 50 mL round-bottom flask. Then, 33.1 μL (50 U) of laccase solution was added and the mixture was allowed to stir at rt for 2 hours. After this time, 53.5 μL (62.6 mg, 0.50 mmol) of **14** was added to the mixture, and the reaction was allowed to stir for an additional 4 h. After this time, the mixture was centrifuged and the supernatant decanted. The remaining solid was washed with deionized water (3 x 5 mL) and dried overnight in a vacuum oven at 30°C. The supernatants were combined and extracted once with 10 mL EtOAc to recover any soluble product, dried over $MgSO_4$, and the solvent removed via rotary evaporation. The crude solid and crude extract were combined and the products were purified via preparative layer chromatography using silica gel coated on glass plates as the stationary phase and dichloromethane containing 5% MeOH (v:v) as the mobile phase. Yield of **15d**: 10.2 mg (9%). Yield of **15e**: 13.6 mg (12%).

Method C: 76.3 mg (0.625 mmol) of methyl-*p*-benzoquinone (**5d**) was dissolved in 1 mL MeOH and added dropwise to 9 mL 0.10 M sodium acetate buffer pH 5.0 that was stirring in a 50 mL round-bottom flask. Then, 53.5 μL (62.6 mg, 0.50 mmol) of **14** was added to the mixture and the reaction was allowed to stir for 6 h at rt. Work-up and purification as in Method B. Yield of **15d**: 10.0 mg (9%). Yield of **15e**: 14.1 mg (12%).

Method D: 76.3 mg (0.625 mmol) of **5d** was dissolved in 1 mL MeOH and added dropwise to 9 mL 0.10 M sodium acetate buffer pH 5.0 that was stirring in a 50 mL round-bottom flask. Then, 53.5 μL (62.6 mg, 0.50 mmol) of **14** was added to the mixture and the reaction was allowed to stir for 2 h at rt. After this time, 33.1 μL (50 U) of laccase

solution was added and the reaction was allowed to stir for an additional 4 h. Work-up and purification as in Method B. Yield of **15d**: 27.3 mg (24%). Yield of **15e**: 33.0 mg (29%).

6.2.4 Laccase-Catalyzed Dimerization of 2-Aminothiophenol

53.5 μL (62.6 mg, 0.50 mmol) of **14** was added to 6 mL 0.10 M sodium acetate buffer pH 5.0 that was stirring in a 50 mL round-bottom flask, followed by 33.1 μL (50 U) of laccase solution. The reaction was allowed to stir at rt for 4 h. The mixture was extracted with EtOAc (3 x 6 mL), organic phases combined, dried over $MgSO_4$, and the solvent removed via rotary evaporation. The product was purified via preparative layer chromatography using silica gel coated on glass plates as the stationary phase and dichloromethane containing 5% MeOH (v:v) as the mobile phase. Yield of **16**: 51.5 mg (83%).

6.2.5 Laccase-Catalyzed Coupling of 1,4-Naphthohydroquinone and Cysteamine

192.9 mg (2.50 mmol) of cysteamine (**19**) was added to a 50 mL round-bottom flask equipped with a stir bar. 8.5 mL 0.10 M sodium acetate buffer pH 5.0 was added followed by 33.1 μL (50 U) of laccase solution and the mixture was stirred. Then, 80.1 mg (0.50 mmol) of **2j** was dissolved in 1.5 mL MeOH and added dropwise to the mixture. The reaction mixture was allowed to stir at rt for 12 h. The precipitate was then filtered, washed with deionized water, and left to dry in a fume hood overnight. The product was purified via preparative layer chromatography using silica gel coated on glass plates as the stationary phase and dichloromethane containing 1% MeOH (v:v) as the mobile phase. Yield of **20**: 39.3 mg (34%).

6.2.6 Product Characterization Data

NMR, FTIR, and MS spectra of new compounds are given in Appendix A. All NMR experiments were conducted using $CDCl_3$ containing 10% CD_3OD (v:v) as the solvent unless stated otherwise.

5*H*-Benzo[a]phenothiazin-5-one (**15a**)

Red-orange solid; mp: 163-164°C; IR: $\tilde{\nu}$ 1591 (C=N), 1628 (C=O) cm^{-1}; ^1H NMR: δ 6.69 (s, 1H, C=CH), 7.33 (m, 3H, Ar-H), 7.64 (m, 2H, Ar-H), 7.78 (d, ^3J = 7.5 Hz, 1H, Ar-H), 8.15 (d, ^3J = 5.8 Hz, 1H, Ar-H), 8.71 (d, ^3J = 7.1 Hz, 1H, Ar-H) ppm; ^{13}C NMR: δ 120.01, 122.98, 124.77, 125.69, 125.78, 127.89, 130.14, 131.43, 131.80, 132.57, 133.47, 134.33, 138.32, 138.63, 144.66, 180.62 ppm; m/z 264 (M+1, 19%), 263 (M^+, 100), 235 (57), 203 (10), 190 (9), 117 (12).

3*H*-Phenothiazin-3-one (**15b**)

Red-purple solid; mp: 160-161°C; IR: $\tilde{\nu}$ 1599 (C=N), 1627 (C=O) cm^{-1}; ^1H NMR: δ 6.69 (s, 1H, C=CH), 6.87 (d, ^3J = 9.4 Hz, 1H, C=CH), 7.42 (m, 3H, Ar-H), 7.56 (d, ^3J = 9.8 Hz, 1H, C=CH), 7.84 (m, 1H, Ar-H) ppm; ^{13}C NMR: δ 119.80, 123.91, 125.13, 128.17, 131.26, 134.10, 135.14, 135.84, 139.21, 140.03, 146.33, 182.81 ppm; m/z 215 (M+2, 10%), 214 (M+1, 14), 213 (M^+, 89), 185 (100).

2,4-Dimethoxy-3*H*-phenothiazin-3-one (**15c**)

Red-orange solid; mp: 191-192°C; IR: $\tilde{\nu}$ 1596 (C=N), 1626 (C=O) cm^{-1}; ^1H NMR: δ 3.89 (s, 3H, OCH_3), 4.01 (s, 3H, OCH_3), 6.78 (s, 1H, C=CH), 7.37 (t, ^3J = 7.5 Hz, 1H, Ar-H), 7.43 (t, ^3J = 7.6 Hz, 1H, Ar-H), 7.50 (d, ^3J = 7.6 Hz, 1H, Ar-H), 7.80 (d, ^3J = 7.8 Hz, 1H,

Ar-H) ppm; ^{13}C NMR: δ 56.47, 59.74, 109.40, 121.33, 123.29, 125.62, 127.91, 129.56, 132.75, 139.74, 144.79, 146.21, 157.34, 171.36 ppm; m/z 275 (M+2, 21%), 273 (M^+, 100), 258 (33), 255 (41), 244 (22), 230 (50), 159 (71).

1-Methyl-3*H*-phenothiazin-3-one (**15d**)

Purple solid; mp: 144-145°C; IR: $\tilde{\nu}$ 1594 (C=N), 1623 (C=O) cm^{-1}; ^{1}H NMR: δ 2.13 (s, 3H, CH_3), 6.58 (s, 1H, C=CH), 6.71 (s, 1H, C=CH), 7.37 (m, 3H, Ar-H), 7.82 (d, ^3J = 5.5 Hz, 1H, Ar-H) ppm; ^{13}C NMR: δ 18.56, 119.45, 124.14, 124.87, 127.82, 130.97, 133.33, 134.30, 135.80, 138.87, 146.52, 148.01, 182.83 ppm; m/z 229 (M+2, 30%), 228 (M+1, 21), 227 (M^+, 100), 199 (90), 198 (79), 167 (25), 154 (16).

2-Methyl-3*H*-phenothiazin-3-one (**15e**)

Red-purple solid; mp: 180-181°C; IR: $\tilde{\nu}$ 1600 (C=N), 1626 (C=O) cm^{-1}; ^{1}H NMR: δ 2.13 (s, 3H, CH_3), 6.69 (s, 1H, C=CH), 7.39 (m, 3H, Ar-H), 7.43 (s, 1H, C=CH), 7.81 (d, ^3J = 6.7 Hz, 1H, Ar-H) ppm; ^{13}C NMR: δ 16.52, 119.59, 123.45, 125.10, 127.96, 130.60, 133.68, 135.02, 136.56, 139.28, 144.34, 146.55, 182.92 ppm; m/z 229 (M+2, 14%), 228 (M+1, 17), 227 (M^+, 100), 199 (73), 198 (38), 167 (12), 154 (8).

2,2'-Dithiobis-benzenamine (**16**)

Yellow solid; mp: 90-91°C; IR: $\tilde{\nu}$ 3294 (N-H), 3374 (N-H) cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 4.13 (s, 4H, NH_2), 6.51 (m, 2H, Ar-H), 6.64 (m, 2H, Ar-H), 7.09 (m, 4H, Ar-H) ppm; ^{13}C NMR ($CDCl_3$): δ 115.39, 118.39, 118.90, 131.74, 136.96, 148.73 ppm; m/z 248 (M^+, 55%), 124 (100), 80 (36).

3,4-Dihydro-2*H*-naphtho[2,3-*b*][1,4]thiazine-5,10-dione (**20**)

Purple solid; mp: 218-219°C; IR: $\tilde{\nu}$ 1657 (C=O), 3347 (N-H) cm^{-1}; ^{1}H NMR: δ 2.94 (t, ^{3}J = 4.5 Hz, 2H, CH_2), 3.70 (t, ^{3}J = 4.2 Hz, 2H, CH_2), 6.11 (s, 1H, NH), 7.52 (t, ^{3}J = 7.3 Hz, 1H, Ar-H), 7.59 (t, ^{3}J = 7.3 Hz, 1H, Ar-H), 7.91 (d, ^{3}J = 7.4 Hz, 1H, Ar-H), 7.97 (d, ^{3}J = 7.5 Hz, 1H, Ar-H) ppm; ^{13}C NMR: δ 23.87, 41.65, 111.18, 126.24, 126.42, 130.39, 132.45, 133.23, 134.39, 141.19, 178.10, 179.59 ppm; m/z 232 (M+1, 16%), 231 (M^+, 100), 230 (35), 216 (29), 198 (15), 104 (13), 76 (14).

6.2.7 Calculation of Green Metrics

Using the reaction of **14** with **5b** (Method D) for the synthesis of **15b** as an example.

Atom Economy

$$\% \text{ Atom Economy} = 100\% \times \left(\frac{\text{Molecular weight of desired product}}{\sum \text{Molecular weights of reactants}}\right)$$

MW **14**: 125.19 g/mol

MW **5b**: 108.09 g/mol

MW ½O_2: 16.00 g/mol

MW **15b**: 213.26 g/mol

$$\% \text{ Atom Economy} = 100\% \times \left(\frac{213.26}{108.09 + 125.19 + 16.00}\right)$$

$$= 85.6\%$$

E Factor

$$\text{E Factor} = \frac{\text{Mass of waste}}{\text{Mass of desired product}} = \frac{\text{Mass of reactants} - \text{Mass of desired product}}{\text{Mass of desired product}}$$

Mass of **14**: 62.6 mg

Mass of **5b**: 67.6 mg

Mass of laccase: 39.7 mg

Mass of MeOH: 79.1 mg (assuming loss of 10% of solvent used)

Mass of CH_3COOH: 19.5 mg

Mass of NaOAc: 47.3 mg

Mass of EtOAc: 900 mg (assuming loss of 10% of solvent used)

Mass of **15b**: 65.0 mg

$$\text{E Factor} = \frac{62.6 + 67.6 + 39.7 + 79.1 + 19.5 + 47.3 + 900 - 65.0}{65.0}$$

$$= 17.7$$

Space Time Yield

$$\text{Space Time Yield} = \frac{\text{Amount of desired product (mol)}}{\text{Volume (L)} \times \text{Time (h)}}$$

$$= \frac{0.000305 \text{ mol}}{(0.01 \text{ L}) \times (6\text{h})}$$

$$= 0.0051 \frac{\text{mol}}{\text{L} \cdot \text{h}}$$

6.3 Results and Discussion

To determine if the reaction would proceed to give the desired product, an initial experiment was conducted that reacted 2-aminothiophenol (**14**) with 1,4-naphthohydroquinone (**2j**) in the presence of laccases (Method A), shown in Figure 76. The product, 5*H*-benzo[a]phenothiazine-5-one **15a**, which has shown antiproliferative

activity towards human tumor cells,[347] was achieved, albeit in very low yield (11%). The low yield can be rationalized by the formation of a S-S dimer of **14**, which was also observed in a previous study.[331] Studying the k_{cat} values for laccase oxidation of phenols and their thiol analogs, it can be seen that benzenethiols are oxidized at a significantly greater rate than phenols (e.g. k_{cat} catechol = 3300 min^{-1}, k_{cat} 1,2-benzenedithiol = 45000 min^{-1}).[117] In fact, reacting **14** with laccases alone can yield the S-S dimer product **16** (Figure 77) – which possesses antimicrobial properties and was historically used to treat syphilis –[348-349] in very high conversion (83%). Thus, simply reacting **14** with hydroquinones and laccases in a one-step process is not a feasible method for the synthesis of phenothiazones.

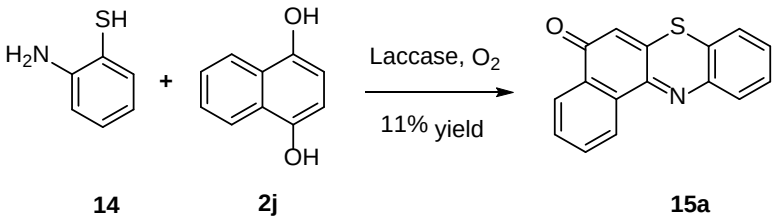


Figure 76. Laccase-catalyzed coupling of 2-aminothiophenol (**14**) with naphthohydroquinone (**2j**). Reaction conditions: 1 eq. (0.50 mmol) **14**, 1.25 eq. (0.625 mmol) **2j**, 50 U laccase, 8.5 mL 0.10 M sodium acetate buffer pH 5.0 : 1.5 mL MeOH, rt, 6 h.

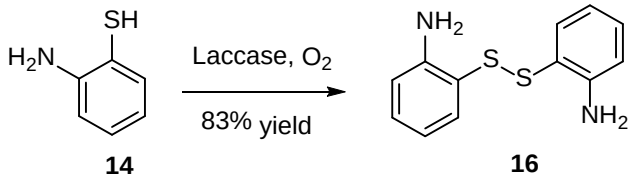
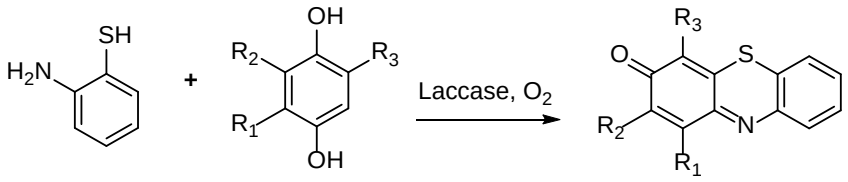


Figure 77. Laccase-catalyzed dimerization of 2-aminothiophenol (**14**). Reaction conditions: 0.50 mmol **14**, 50 U laccase, 6 mL 0.10 M sodium acetate buffer pH 5.0, rt, 4 h.

Based on the aforementioned findings, we then experimented with a two-step process in which **14** is added to the reaction mixture 2 hours after the hydroquinone and laccases (Method B), similar to what has been done previously.[331] We hypothesized that this would allow for a substantial formation of laccase-generated 1,4-quinone capable of rapidly reacting with **14** before it is oxidized by laccases. The results are shown in Table 8. As can be seen, the product yields are still quite low, with the exception of **15c** (Table 8, entry 3). These results indicate that this is a viable procedure for the synthesis of 2,4-disubstituted phenothiazones, which are produced via the highly stable laccase-generated 2,6-disubstituted-1,4-quinone intermediate. However, for the remaining hydroquinones examined, this is still not a practical synthetic method.

Table 8. Laccase-catalyzed coupling of 2-aminothiophenol (**14**) with hydroquinones (**2**).[a]



14 **2** **15**

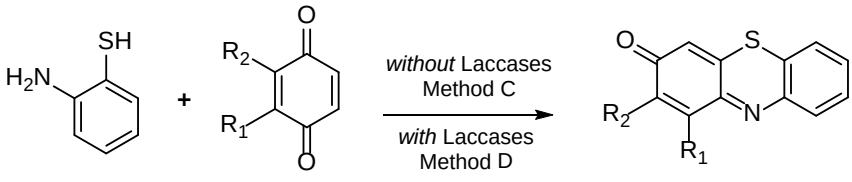
Entry	2		Product Yield		
1	**j**		**15a**	20%	
2	**b**	R_1, R_2, R_3 = H	**15b**	24%	
3	**h**	R_1 = H, R_2, R_3 = OCH_3	**15c**	53%	
4	**d**	R_1, R_3 = H, R_2 = CH_3	**15d**	9%	R_1 = CH_3, R_2, R_3 = H
			15e	12%	R_1, R_3 = H, R_2 = CH_3

[a] Reaction conditions: 1.25 eq. (0.625 mmol) **2**, 50 U laccase, 1 eq. (0.50 mmol) **14** added at t = 2 h, 0.10 M sodium acetate buffer pH 5.0 with 10-15% MeOH (v:v), rt, 6 h

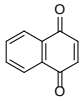
There seemed to be two factors contributing to low product yields: 1) oxidation of **14** by laccases, and 2) poor conversion of the hydroquinone to the corresponding 1,4-

quinone by laccases. Thus, to overcome these problems, we reacted the 1,4-quinones **5** directly with **14** both without (Method C) and with (Method D) laccases. The results are displayed in Table 9. First of all, it can be seen that by using Method D, the product yields can be increased compared to employing Method B (Table 8). Thus, it seems as though the aforestated problems can be reduced or eliminated by using this methodology. Furthermore, when comparing the results of Methods C and D, it is noticed that the product yields can be substantially increased when laccases are utilized. Comparing the data in Table 8 and Table 9 for the synthesis of phenothiazones using different methods, we can see that when employing Method D, the product yields can be increased on average by 2.5 fold compared to using Method B, and up to 6.8 fold compared to when Method C is used. For comparison, the regioselectivity of addition for the reaction of **14** with **5d** (Table 9, entry 3) is similar to that observed by Terdic, who conducted the coupling reaction in ethanol.[345]

Table 9. Coupling of 2-aminothiophenol (**14**) with 1,4-quinones (**5**) in the absence (Method C) and presence (Method D) of laccases.



14 **5** **15**

Entry	5		Product Yield Method C [a]		Product Yield Method D [b]	
1	**j** 	**15a**	44%		**3a**	52%
2	**b** $R_1, R_2 = H$	**15b**	9%		**3b**	61%
3	**d** $R_1 = H, R_2 = CH_3$	**15d**	9%	$R_1 = CH_3, R_2 = H$	**3d**	24%
		15e	12%	$R_1 = H, R_2 = CH_3$	**3e**	29%

[a] Method C reaction conditions: 1 eq. (0.50 mmol) **14**, 1.25 eq. (0.625 mmol) **5**, 0.10 M sodium acetate buffer pH 5.0 with 10-15% MeOH (v:v), rt, 6 h
[b] Method D reaction conditions: 1 eq. (0.50 mmol) **14**, 1.25 eq. (0.625 mmol) **5**, 50 U laccase added at t = 2 h, 0.10 M sodium acetate buffer pH 5.0 with 10-15% MeOH (v:v), rt, 6 h

Analysis of the gas chromatograms for the reaction of **14** with **5b** using both Methods C and D (Figure 78) provides a qualitative picture of the reaction systems and reveals the role laccases have in improving product yields. In the top chromatogram in Figure 78 (Method C), the peak with m/z 213, corresponding to product **15b**, is relatively small, indicating a low product yield. In comparison, the same peak in the bottom chromatogram of Figure 78 (Method D) is the predominant peak in the chromatogram, corresponding to a high product yield. Further analysis of the top chromatogram shows a sizeable peak with m/z 215, which is negligible in the bottom chromatogram. It is believed that this compound is the reduced form of product **15b**. Furthermore, allowing **14** to react with **5b** in the absence of laccases for 72 h does not significantly improve the yield of **15b**. Thus, laccases appear to be crucial for completely oxidizing the phenothiazine form to the phenothiazone form and providing greatest product yields.

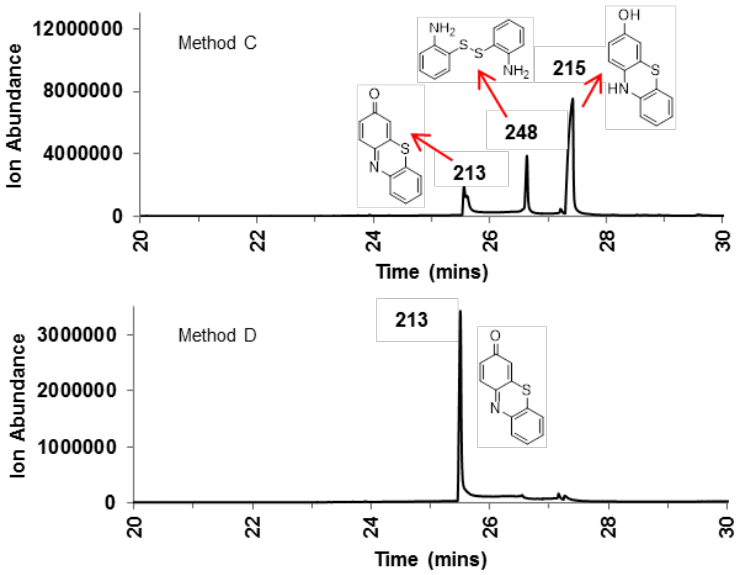


Figure 78. Qualitative gas chromatograms with m/z values of peaks for the reaction of **14** with **5b** using Method C (top) and Method D (bottom).

Figure 79 shows the proposed reaction mechanism. Initial addition of the aromatic amino group of **14** to a carbonyl group of 1,4-quinone **5** yields the imine **17**, which is followed by addition of sulfur to an adjacent alkene carbon and subsequent tautomerization to produce the phenothiazine intermediate **18**. A final oxidation of the phenothiazine affords the phenothiazone **15**.

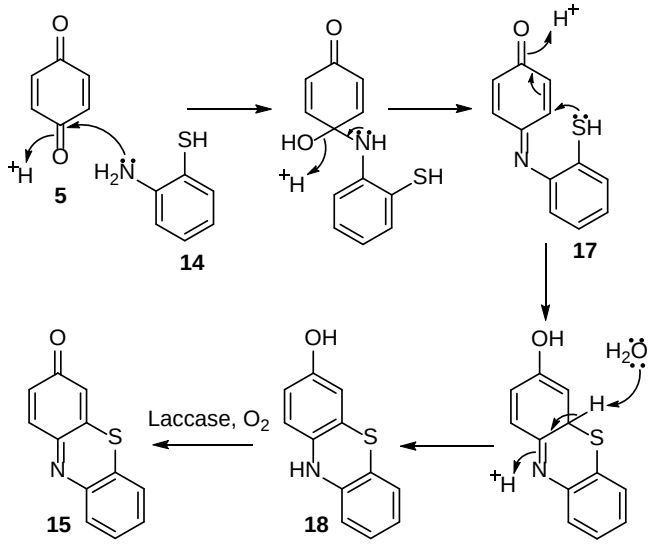


Figure 79. Reaction mechanism for the laccase-facilitated synthesis of phenothiazones.

With the chemistry for the coupling of an aromatic thiol-amine with 1,4-quinones developed, the principles were then applied to a simple aliphatic thiol-amine, cysteamine (**19**). Since it was observed that laccases do not directly oxidize **19**, it was possible to react this compound in one-pot with the hydroquinone **2** and laccases in one-step. Using a variety of hydroquinones and catechols – hydroquinone, methylhydroquinone, methoxyhydroquinone, *t*-butylhydroquinone, phenylhydroquinone, fluorohydroquinone, chlorohydroquinone, bromohydroquinone, 2′,5′-dihydroxyacetophenone, ethyl 2,5-dihydroxybenzoate, 3-methylcatechol, 4-methylcatechol, 3-methoxycatechol, and 1,2-dihydroxynaphthalene – the results of these reactions were generally unsuccessful, providing a vast mixture of products (based on GC-MS and TLC analyses). One reaction was successful, however; the laccase-catalyzed coupling of **19** with **2j** yielded product **20** (Figure 80), a compound that possesses tuberculostatic potential and is also an important structural moiety present in compounds that exhibit potent antibacterial and antifungal activities.[350-351] A possible reason for the success in achieving a desired product when

compound **2j** was used may be the increased stability of the 1,4-naphthoquinone intermediate compared to less substituted 1,4-quinones.

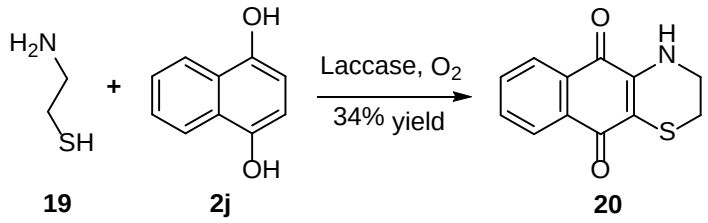


Figure 80. Laccase-catalyzed coupling of cysteamine (**19**) with 1,4-naphthohydroquinone (**2j**). Reaction conditions: 5 eq. (2.50 mmol) **19**, 1 eq. (0.50 mmol) **2j**, 50 U laccase, 8.5 mL 0.10 M sodium acetate buffer pH 5.0 : 1.5 mL MeOH, rt, 12 h.

A proposed reaction mechanism for the laccase-catalyzed coupling of **19** with **2j** is provided in Figure 81. First, laccases oxidize **2j** to the corresponding 1,4-quinone **5j**, which is followed by addition of sulfur to an alkene carbon. Following subsequent tautomerization and oxidation, the nitrogen then adds to the neighboring alkene carbon, forming a six-membered heterocyclic ring. After another tautomerization and final oxidation, product **20** is reached. From this result, a difference in reactivity of the aliphatic amino group of **19** and the aromatic amino group of **14** towards nucleophilic addition to 1,4-naphthoquinone is observed. This difference in reactivity may be rationalized by the differences in basicity of the amino groups. The pK_b of the aromatic amino group of **14** is 9.49, whereas the pK_b of the aliphatic amino group of **19** is 3.19.[352] Thus, in the aqueous reaction medium, the amino group of **19** is predominantly in its cationic form, rendering it less nucleophilic and less reactive than the aromatic amino group of **14**, which is mostly in its neutral form. The increased nucleophilicity of the amino group of **14** is what allows it to rapidly react with the carbonyl carbon of a 1,4-naphthoquinone, whereas for **19**, the sulfur adds first to an alkene carbon.

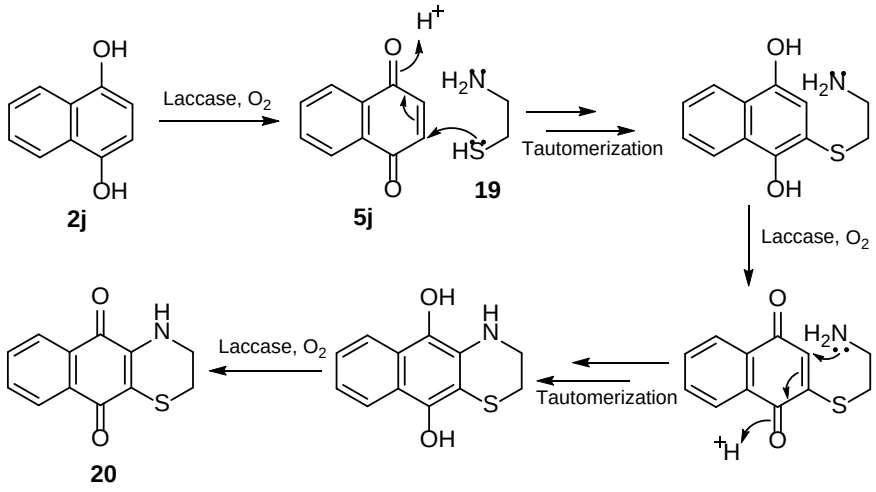


Figure 81. Reaction mechanism for the laccase-catalyzed coupling of naphthohydroquinone (**2j**) with cysteamine (**19**).

The presented synthetic protocol for the preparation of phenothiazones offers an ecofriendly alternative to the synthesis of these important compounds, further progressing sustainability within the field of chemical synthesis. The process addresses many of the principles of green chemistry, such as waste prevention, atom economy, catalysis (laccases), benign solvents (aqueous solvent system), renewable feedstocks (1,4-quinones and hydroquinones are biomass derived), and a safe and energy efficient synthetic procedure. Using the reaction of **14** with **5b** (Method D) for the synthesis of **15b** as an example, green chemistry can be quantified. The E factor was calculated to be 17.7, which falls toward the lower limit expected for fine chemical syntheses,[19] while the atom economy of the reaction is also very good, calculated as 86%, as is the space time yield, calculated to be 0.005 mol x L^{-1} x h^{-1}.

6.4 Conclusions

In summary, an environmentally friendly approach for the synthesis of phenothiazones and related structures facilitated by laccases has been developed. By coupling 1,4-quinones with 2-aminothiophenol in an aqueous reaction medium in the presence of laccases, the yields of phenothiazones can be substantially increased compared to when laccases are not present or the 1,4-quinones are generated *in situ* via laccase-catalyzed oxidation of the corresponding hydroquinone. Furthermore, a difference in reactivity between aromatic and aliphatic amines toward nucleophilic addition to 1,4-naphthoquinone was also observed. This study adds to the ever-growing toolkit of enzyme assisted processes in chemical synthesis, aiding in the increasing global effort of promoting sustainability within the field.

CHAPTER 7. LACCASE-MEDIATED SYNTHESIS OF LIGNIN-CORE HYPERBRANCHED COPOLYMERS

7.1 Introduction

Modern society has traditionally been based around a linear economy. The vast majority of chemicals and materials used to manufacture consumer products are derived from non-renewable petroleum resources, and once these commodities have been consumed, they are more often than not simply discarded into overflowing landfills or incinerated, the latter being a contributor to greenhouse gas emissions. However, due to the ever-growing concerns about the future welfare of the environment in which we inhabit, there has been a collaborative global effort to develop a more sustainable circular economy. Under this paradigm, raw materials and chemicals used for the manufacture of consumer goods will originate from renewable resources and be designed for reuse, recycle, or biodegradability.[353] In this manner, the entire process, from manufacture to degradation, is almost carbon neutral and will thus only contribute a minor portion to society's carbon footprint.

The concept of utilizing lignocellulosic biomass, and in particular lignin, as an alternative sustainable raw material for the production of chemicals, materials, and fuels was discussed in Chapter 2.5, Also discussed was the application of laccases in the copolymerization of lignin with a variety of monomers and polymers for the synthesis of novel biomaterials that have found applications in adhesives, fillers, and plastics.

Over the past few years, there has been an increase in research within the field of hyperbranched polymers due to their desirable properties, such as high functionality, high

solubility, and low viscosity.[354] Relating to this, using lignin as a core polymer and branching out on its surface provides a promising strategy to synthesize novel biomaterials from lignin. Kai and colleagues (2016) have developed a series of lignin-core supramolecular hyperbranched polymers by functionalizing the surface of lignin with poly(ethylene glycol) methyl ether methacrylate (PEGMA) via atom transfer radical polymerization (ATRP) followed by combination with α-cyclodextrin.[252] The produced hydrogels exhibit many promising properties and may have potential application within the biomedical field. The current study aims to develop a lignin-core hyperbranched copolymer by employing laccases to continuously graft small molecules onto the surface of kraft lignin. Building on the research developed in the previous three chapters, particularly Chapter 2, where the successful coupling of dithiols and hydroquinones via a laccase-catalyzed thiol-Michael addition was achieved,[355] the current study applies this fundamental laccase-catalyzed coupling chemistry for the synthesis of lignin-core hyperbranched copolymers (LCHCs) by combining kraft lignin with methylhydroquinone and a trithiol mediated by laccase catalysis.

7.2 Experimental

7.2.1 Materials

Laccases from the white-rot fungus *Trametes villosa* expressed in an *Aspergillus* host (NOVO NS51002) were appreciatively donated by Novo Nordisk Biochem (now Novozymes), Franklinton, North Carolina, USA. Southern pine softwood kraft lignin, isolated via the LignoBoost process, was donated by Domtar, Plymouth, USA, and was purified prior to use. *Endo-N*-hydroxy-5-norbornene-2,3-dicarboximide (NHND) was

purchased from Alfa Aesar. All other reagents and solvents were purchased from Sigma-Aldrich and were used as received, except chloroform and dioxane, which were distilled just prior to use. *Tris*(2-mercaptoethyl)amine was synthesized. Nitrogen gas was purchased from Airgas and was dried using a Drierite™ gas-drying unit (Sigma-Aldrich).

7.2.2 Enzyme Assay

Full experimental procedures regarding laccase activity measurements are detailed in Chapter 3.2.1.

7.2.3 Lignin Purification

Full experimental procedures regarding lignin purification are detailed in Chapter 3.2.5.

7.2.4 Synthesis and Characterization of Tris(2-mercaptoethyl)amine

Full experimental procedures regarding the synthesis of *tris*(2-mercaptoethyl)amine are detailed in Chapter 3.2.6. Characterization data of *tris*(2-mercaptoethyl)amine is consistent with that reported in the literature. Colorless viscous liquid; ^{1}H NMR (DMSO-d_6): δ 2.33 (s, 3H, SH), 2.57 (t, 6H, CH_2), 2.67 (t, 6H, CH_2) ppm; ^{13}C NMR (DMSO-d_6): δ 22.01, 56.41 ppm; m/z 197, 150 (100%), 90, 61.

7.2.5 Laccase-Mediated Synthesis of Lignin-Core Hyperbranched Copolymers

Full experimental procedures regarding the laccase-mediated synthesis of lignin-core hyperbranched copolymers (LCHCs) are detailed in Chapter 3.2.7.

7.2.6 Analytical Procedures

The purified kraft lignin and synthesized LCHCs were subjected to the following analyses: ^{1}H NMR, ^{13}C NMR, ^{1}H-^{13}C HMBC NMR, ^{13}C DEPT-135 NMR, ^{31}P NMR, FTIR, GPC, TGA, DSC, SEM, and elemental analysis. Detailed procedures, including experimental parameters, for each of these analyses are provided in Chapter 3.3.

7.3 Results and Discussion

The use of laccases for the copolymerization of lignin with both low molecular weight compounds and other polymers has proved to be a successful strategy for lignin functionalization in recent years. Furthermore, the use of lignin as a core macromolecular scaffold for which hyperbranched polymers can be constructed upon has only just been established in the past few years and presents an exciting new route for lignin valorization.[252, 356] With both these in mind, it was viewed as a great opportunity to synthesize LCHCs via laccase-assisted grafting of small molecules onto lignin.

For this study to be successful, it was important to select an appropriate lignin that would be conducive to laccase-assisted functionalization. An industrial softwood kraft lignin was decided upon for two main reasons: 1) kraft lignin is highly abundant as it is produced in the millions of tons by the pulp and paper industry as a by-product of the kraft pulping process, and 2) softwood lignins are composed of primarily guaiacyl units,[249] which, unlike syringyl units found in hardwood lignins, contain a vacant position on the aromatic ring, which is essential for significant grafting to occur.

7.3.1 Purified Kraft Lignin Molecular Weight Data and Structural Characterization

The obtained industrial softwood kraft lignin was treated prior to use to remove any trace metals, extractives, and remaining carbohydrates. Molecular weight distribution data for the purified kraft lignin was obtained via GPC. Values for number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity index (PDI) are given in Table 10. The M_n and M_w were quite small, with values of 993 g/mol and 2,352 g/mol, respectively, with the latter equating to approximately 13 monolignol units per lignin polymer, while the PDI was also quite narrow, yielding a value of 2.37.

Table 10. Molecular weight distribution data of purified kraft lignin.[a]

M_n	M_w	PDI
993	2,352	2.37

[a] M_n = number average molecular weight (g/mol); M_w = weight average molecular weight (g/mol); PDI = polydispersity index

Structural features of the purified kraft lignin were elucidated via various spectroscopic techniques. The ^{1}H and ^{13}C NMR spectra (Figure 82) show significant amounts of methoxyl protons and carbons at chemical shifts of 3.79 ppm and 55.64 ppm, respectively, which are typical of softwood kraft lignins.[357] The ^{1}H NMR spectrum revealed signals for aromatic protons centered around 6.77 ppm and a broad peak for phenolic protons centered around 8.64 ppm. The FTIR spectrum (Figure 83) also displays a prominent O-H stretching absorption in the range of 3150-3550 cm^{-1}. Quantitative ^{31}P NMR spectroscopy is another invaluable tool to analyze the abundance of different types of hydroxyl groups present in lignin following derivatization with TMDP according to the reaction shown in Figure 84. The quantitative ^{31}P NMR spectrum is displayed in

a)

b)

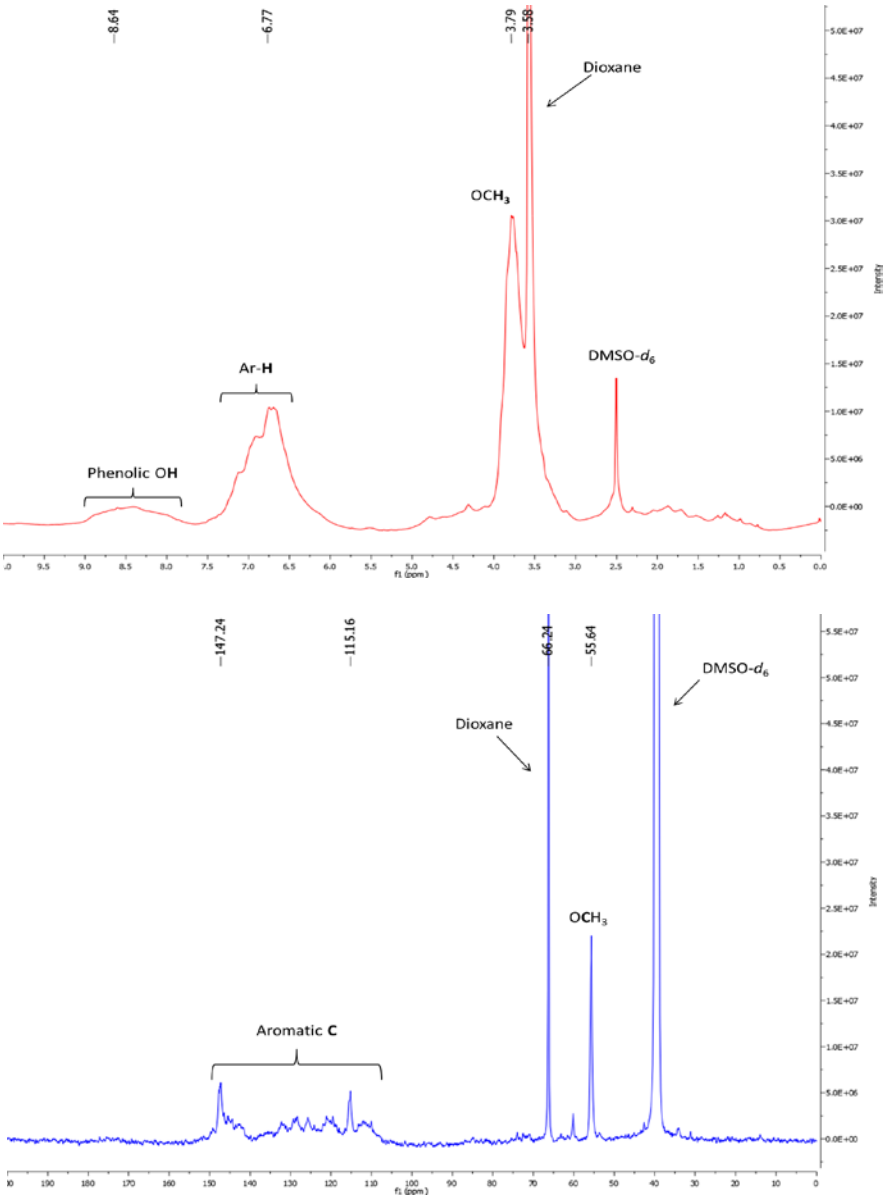


Figure 82. a) ^{1}H NMR spectrum and b) ^{13}C NMR spectrum of purified kraft lignin.

Figure 85 and the various types of hydroxyl groups have been quantified using NHND as an internal standard (Table 11). As can be seen in Table 11, the guaiacyl hydroxyl group is the most abundant with 2.06 mmol/g lignin, while there were also substantial amounts of C5 condensed and aliphatic hydroxyl groups, which are characteristic of alkali-processed softwood lignins.[358]

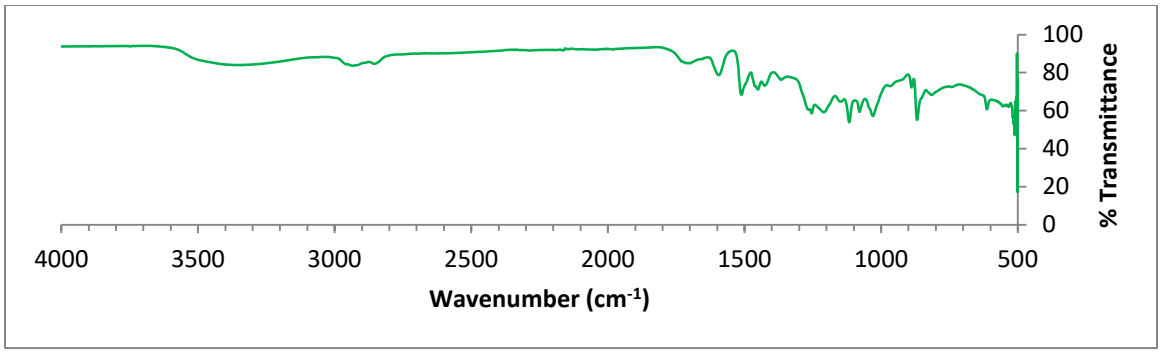


Figure 83. FTIR spectrum of purified kraft lignin.

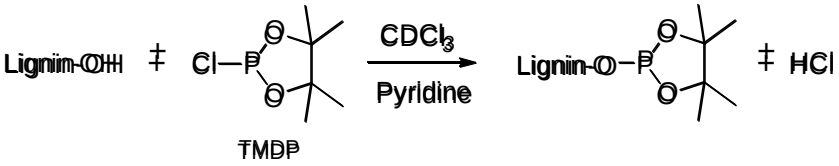


Figure 84. Phosphitylation of lignin with TMDP.

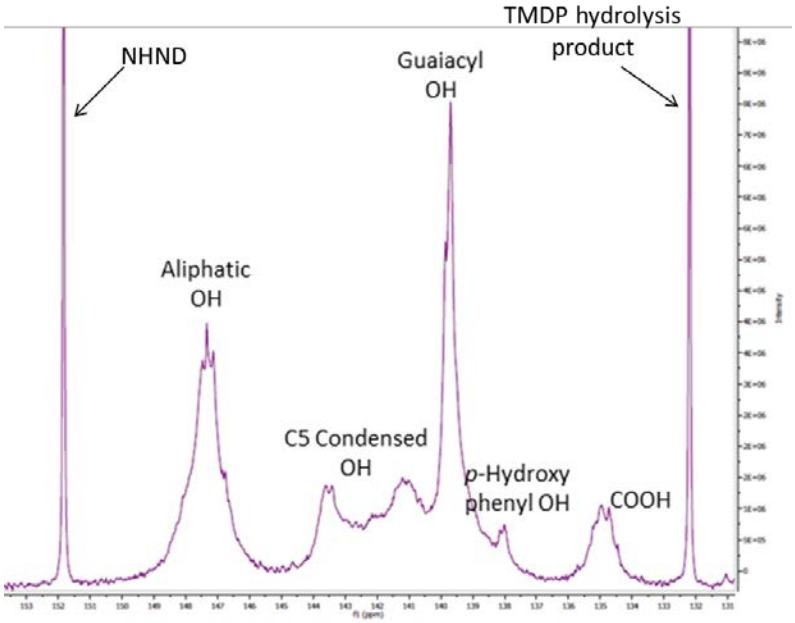


Figure 85. ^{31}P NMR spectrum of purified kraft lignin.

Table 11. Quantitative hydroxyl group content for purified kraft lignin based on ^{31}P NMR analysis.

Hydroxyl Group	mmol/g Lignin
COOH	0.31
p-Hydroxy phenyl OH	0.12
Guaiacyl OH	2.06
C5 Condensed OH	1.84
Aliphatic OH	1.99

7.3.2 Laccase-Mediated Synthesis of Lignin-Core Hyperbranched Copolymers

Based on research conducted in the previous chapters, thiols were identified as good nucleophiles for the Michael-addition reaction with laccase-generated *para*-quinones. Thus, the synthesis of LCHCs was attempted utilizing this fundamental laccase-catalyzed coupling chemistry. Methylhydroquinone (**2d**) was selected as the quinone precursor because it contains three vacant positions for potential nucleophilic addition and the methyl group allows for facile detection of the hydroquinone monomer within the copolymer via NMR spectroscopic analysis. A trithiol was selected as a prime bridging reagent for the synthesis of LCHCs because the thiol groups add rapidly and reliably to *in situ* generated *para*-quinones. Thus, the synthesis of LCHCs proceeded using purified kraft lignin, methylhydroquinone (**2d**), and *tris*(2-mercaptoethyl)amine (**21**) as the components and laccases as the catalysts.

The reaction was conducted at 50°C in an aqueous phosphate buffer at pH 8.0 with 20% dioxane (v:v) as cosolvent to ensure solubility of the lignin. A proposed mechanism for the formation of LCHCs is provided in Figure 86. As can be seen, laccases oxidize both lignin and **2d** to generate phenoxy radicals, which can couple with

one another via either C-C or C-O bond formation. This is followed by laccase-catalyzed oxidation of the methylhydroquinone moiety to produce a *para*-quinone, which subsequently undergoes nucleophilic addition by **21**. The remaining free thiols are then capable of reacting with laccase-generated *para*-quinones that may be present in the reaction mixture, and the laccase-catalyzed polymerization continues. While the presented reaction mechanism represents an idealized scenario, it must be mentioned that other mechanistic routes may occur, such as radical-radical C-C or C-O couplings between **2d** radicals or sulfur-sulfur couplings between laccase-mediator generated sulfur radicals.

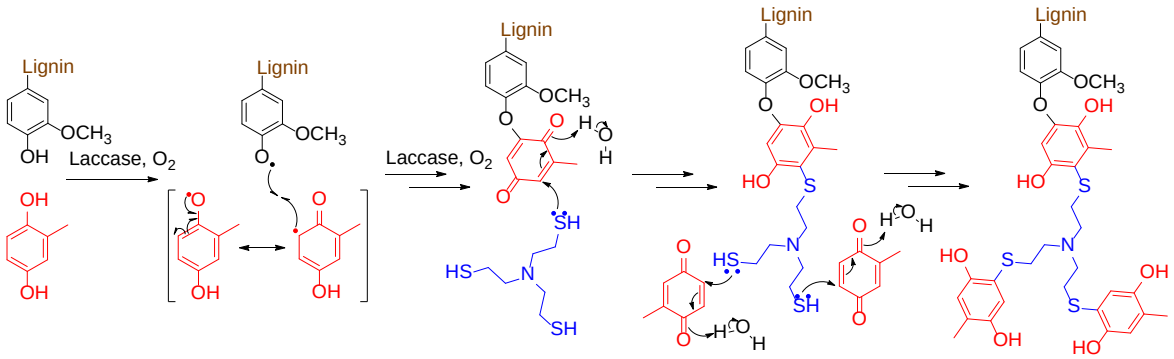


Figure 86. Proposed reaction mechanism for the laccase-mediated synthesis of lignin-core hyperbranched copolymers.

The product that precipitated out of the reaction mixture was in the form of a thick brown paste, which was subsequently washed with deionized water and then dioxane to remove any unreacted lignin that may have also precipitated. Following drying in a vacuum oven at 30°C for 24 hours, the paste had hardened into a solid glossy material that was insoluble in organic solvents. It is likely that curing took place during the drying process, possibly due to the presence of reactive free thiol end groups, which has been known to occur for hyperbranched polymers synthesized with **21** and ethylene

glycol diacrylate.[283] Thus, it is likely that a network of LCHCs exists, such as that illustrated in Figure 87, rather than discrete LCHCs. This is the likely cause of the lack of solubility of the synthesized LCHCs in organic solvents, which is not uncommon for lignin containing copolymers.[356, 359-360] It is due to this insolubility that molecular weight data for the synthesized LCHCs could not be obtained via GPC analysis.

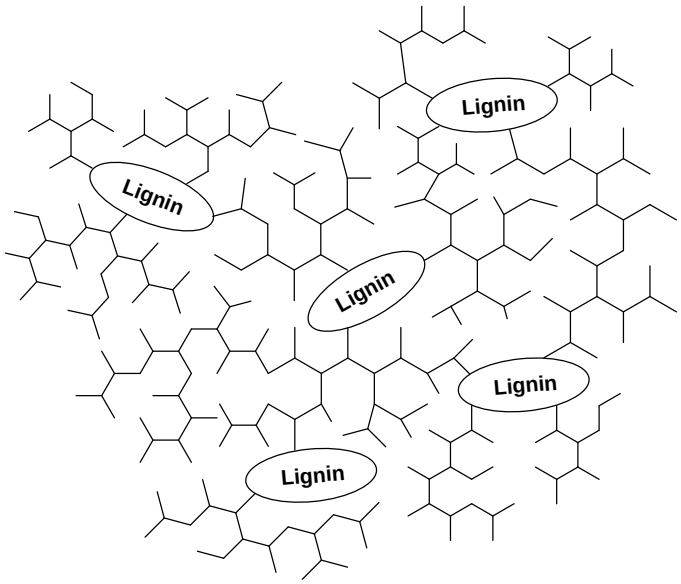


Figure 87. Schematic of lignin-core hyperbranched copolymer network.

7.3.3 Structural Characterization of Lignin-Core Hyperbranched Copolymers

The lack of solubility of the copolymeric material in any organic solvent tested posed a serious challenge for structural analysis at the molecular level. However, upon grinding up the material and vigorously stirring in DMSO-d_6 at 50°C for 6 hours, a dark cloudy mixture that was suitable for NMR analysis was obtained. NMR spectroscopic data provided insight into the possible structure of the copolymeric material, which is presented in Figure 88. Comparing the ^{1}H NMR spectrum of the copolymeric material

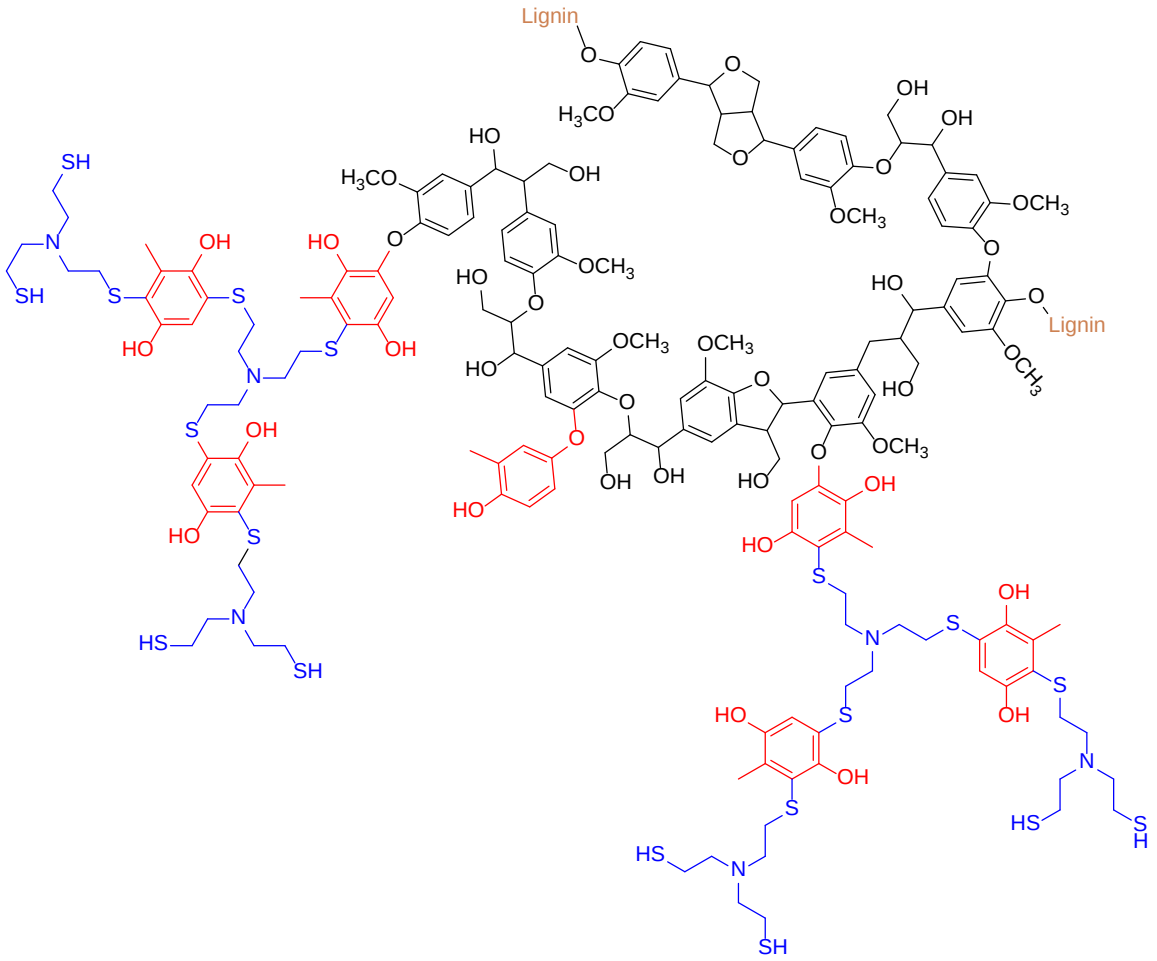


Figure 88. Proposed structure of lignin-core hyperbranched copolymers.

(Figure 89) with the ^{1}H NMR spectra of its constituents – lignin (Figure 82), methylhydroquinone, and *tris*(2-mercaptoethyl)amine (Figure 90) – the structural features of each component could be identified and a full assignment of the spectrum was achieved. Furthermore, a broadening of the peaks in the ^{1}H NMR spectrum, particularly of the phenolic protons dispersed around 8.31 ppm, aromatic protons in the range of 6.45-6.70 ppm, the methylene protons at 2.85 ppm, and the methyl protons between 2.05-2.10 ppm, is indicative of polymerization. The methoxy protons originating from lignin are still present at 3.72 ppm, albeit at a rather low intensity, while the S-H peak at 2.33 ppm

in Figure 90b is no longer present, indicating the successful Michael-addition of the thiol group.

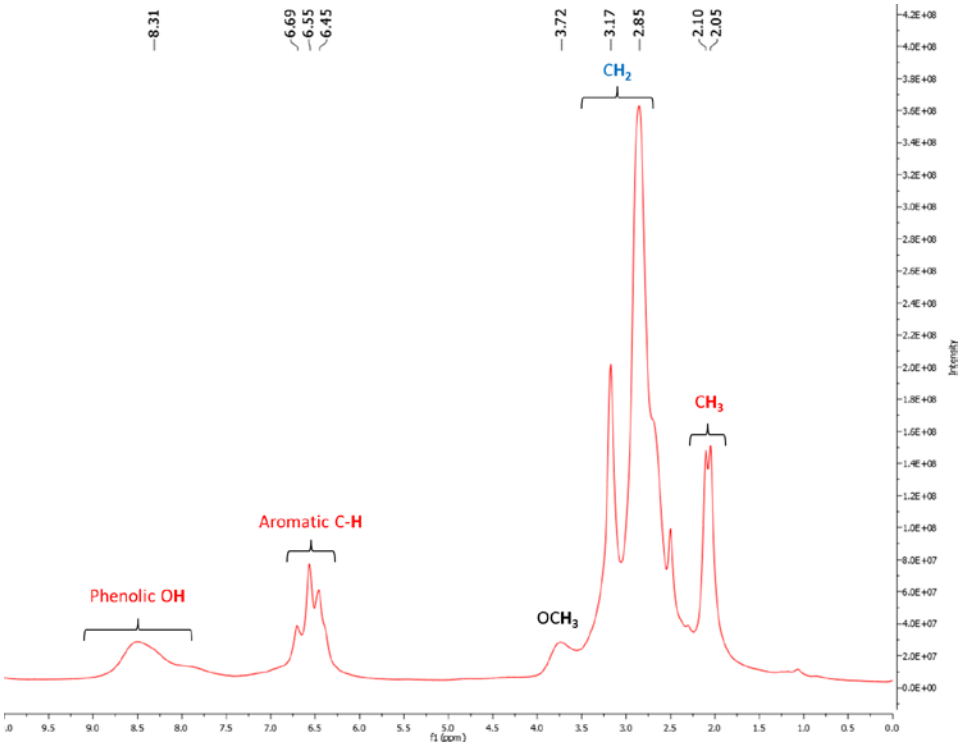


Figure 89. ^{1}H NMR spectrum of lignin-core hyperbranched copolymers.

Analysis of the ^{13}C NMR data further aids in confirming the various structural features of the copolymeric material. As can be seen from the ^{13}C NMR spectrum in Figure 91a, and by comparison with the ^{13}C NMR spectra of the individual components (Figure 92), all the structural features of the various constituents can be identified and assigned. The degree of substitution of the carbon atoms, and therefore their origin, were confirmed via ^{13}C DEPT-135 NMR analysis (Figure 91b).

While the 1D NMR results provide evidence that all three components exist in polymer form within the copolymeric material, they do not provide any information about covalent linkages between each component, which is important to decipher whether

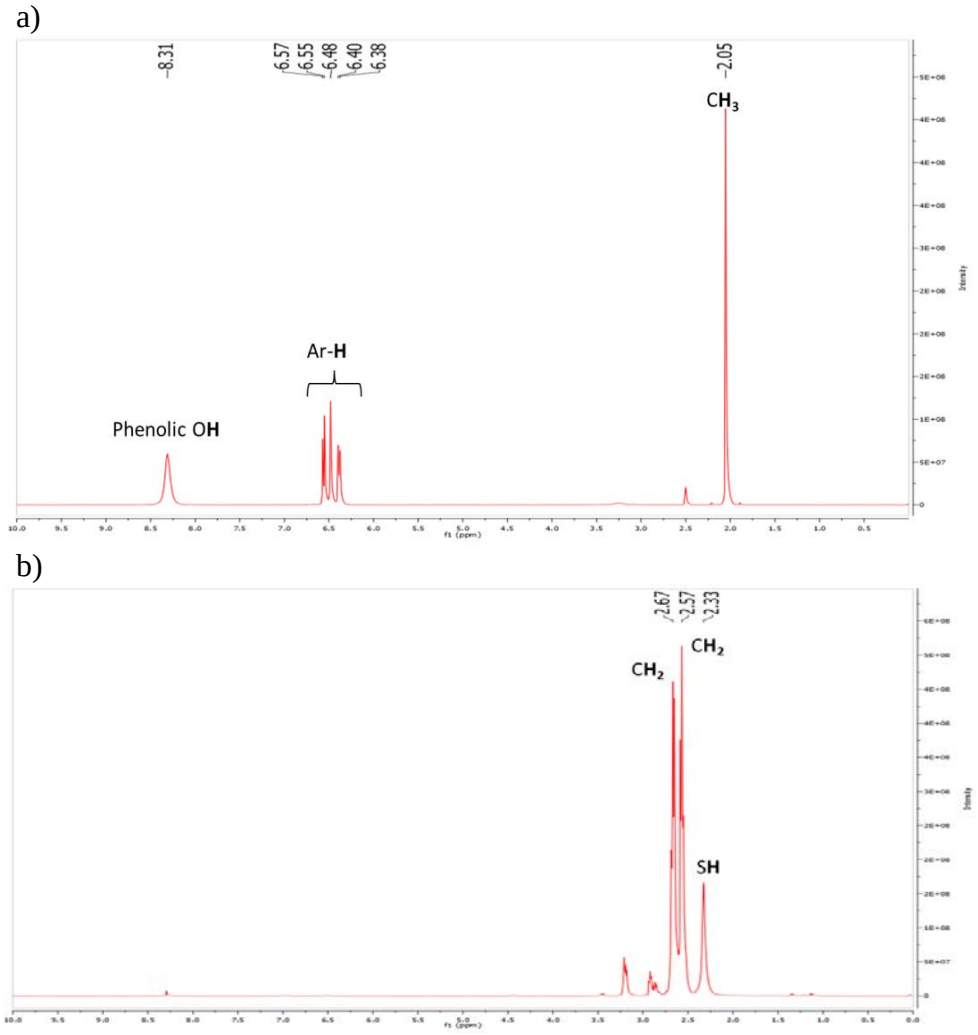


Figure 90. ^{1}H NMR spectrum of a) methylhydroquinone, and b) *tris*(2-mercaptoethyl)amine.

the material is just a simple blend of polymers or if it is a true copolymer. Therefore, it was necessary to analyze the copolymeric material via correlation spectroscopy, such as ^{1}H-^{13}C HMBC NMR. The HMBC spectrum of the copolymeric material (Figure 93a) displays a signal that correlates the proton peak at 2.91 ppm and the carbon peak at 121.80 ppm, which is attributed to a ^{3}J correlation between methylene protons of **21** with a quaternary aromatic carbon of **2d**. Thus, this signal provides evidence of a covalent linkage between **21** and **2d**. This assignment was further supported by evidence from the

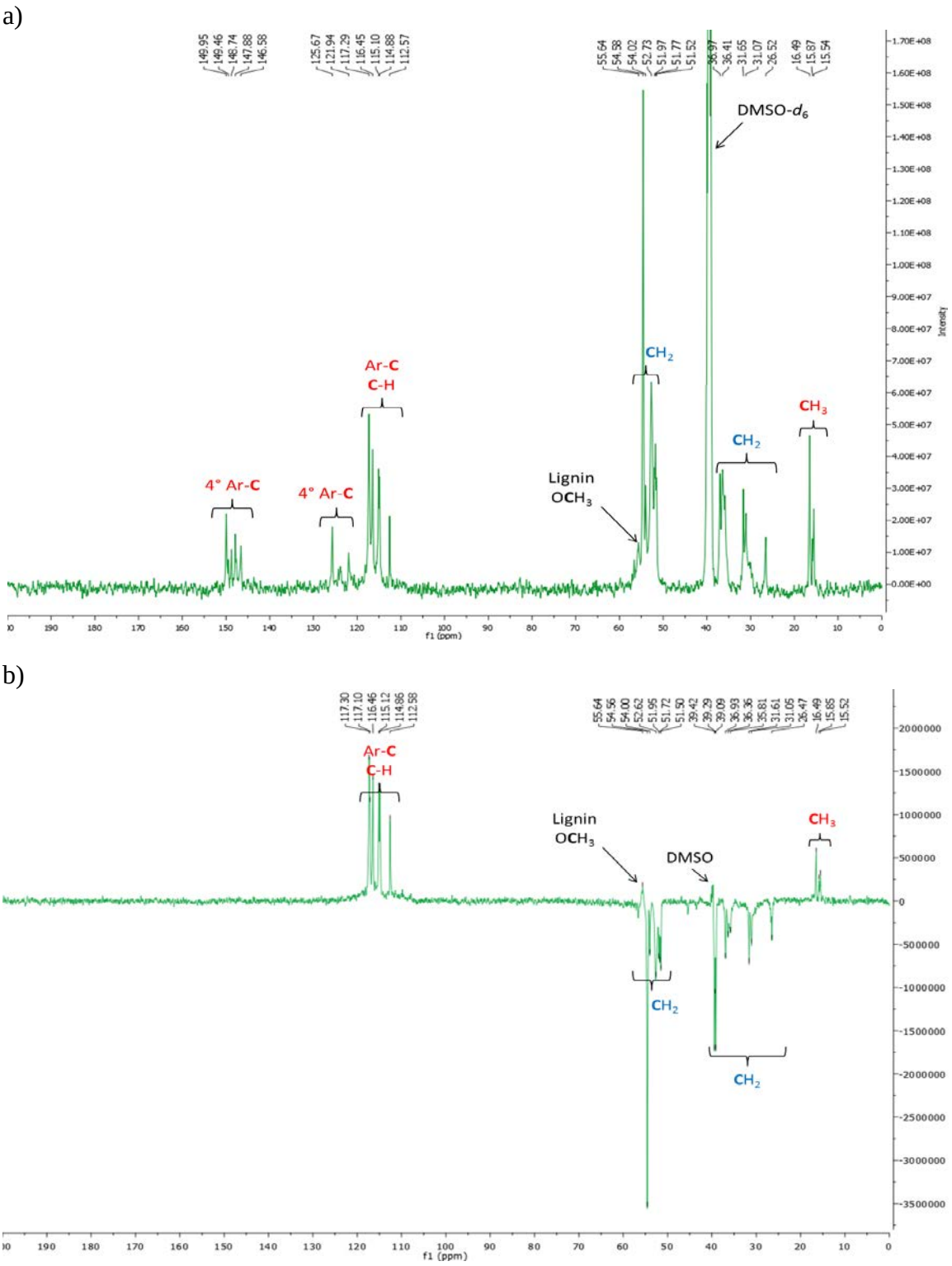
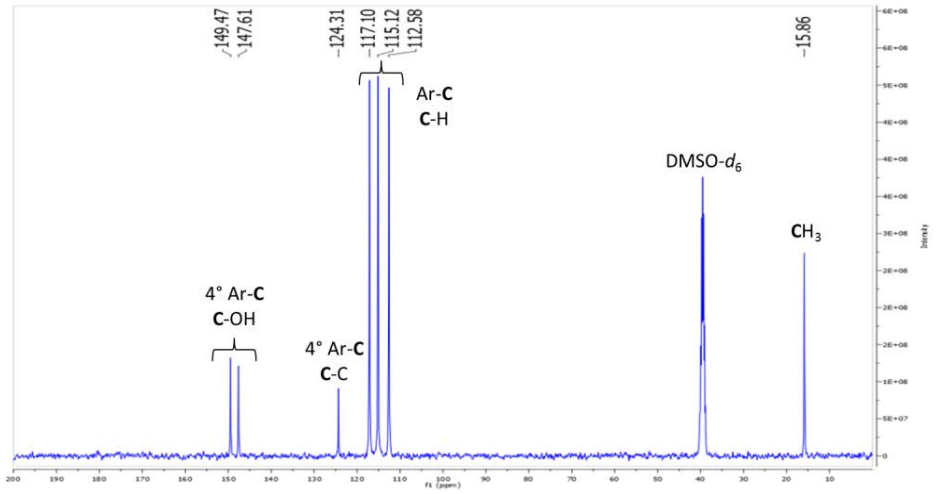


Figure 91. a) ^{13}C NMR spectrum, and b) ^{13}C DEPT-135 NMR spectrum of lignin-core hyperbranched copolymers.

a)



b)

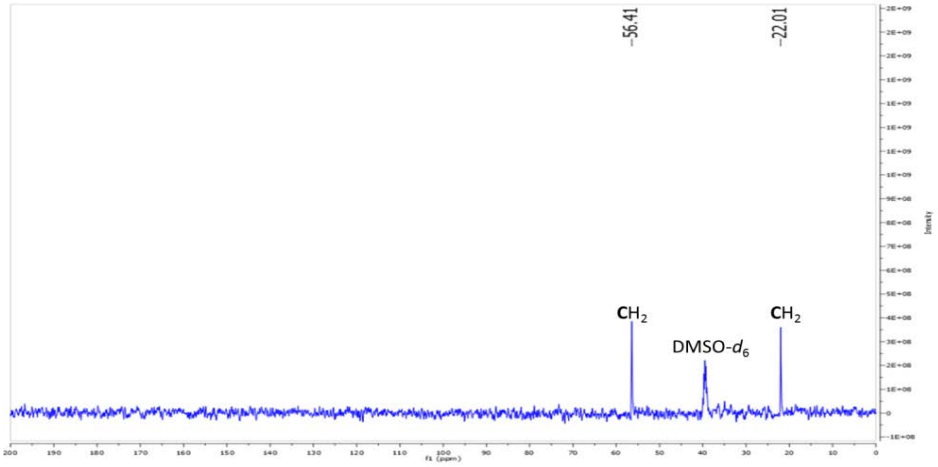


Figure 92. ^{13}C NMR spectrum of a) methylhydroquinone, and b) *tris*(2-mercaptoethyl)amine.

^{1}H-^{13}C HMBC spectrum of the product of the reaction of **21** with methyl-1,4-quinone (**5d**) in the absence of laccases (Figure 93b), which displays the same signal. Unfortunately however, no covalent linkages between lignin and either of the other two components could be identified from the ^{1}H-^{13}C HMBC NMR spectrum, possibly due to the low amount of lignin in the copolymeric material or the ^{1}H and ^{13}C correlations were too great a range to be detected in an observable quantity by the HMBC experiment.

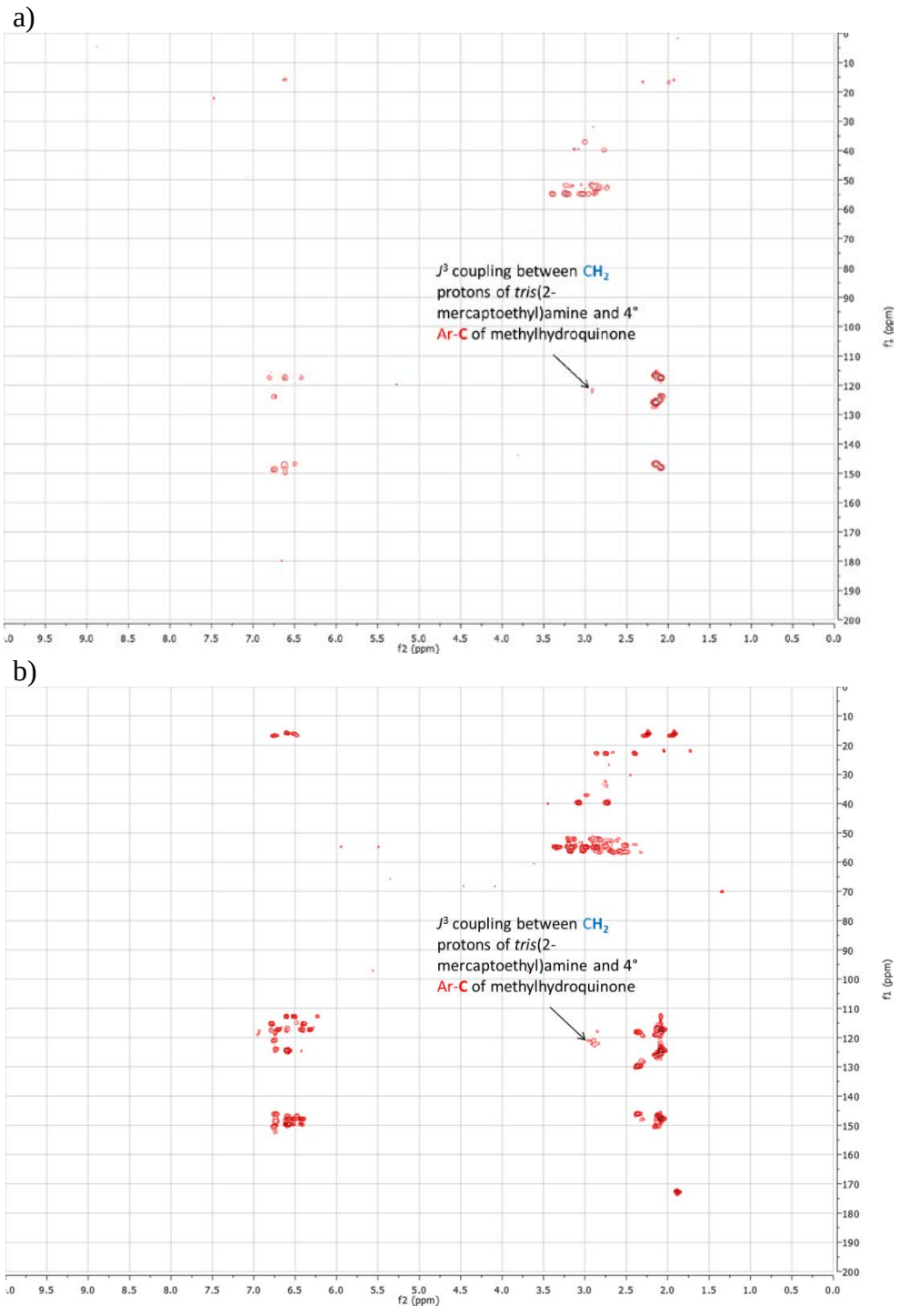


Figure 93. ^{1}H-^{13}C HMBC spectrum of a) lignin-core hyperbranched copolymers, and b) product from the reaction of methyl-1,4-quinone with *tris*(2-mercaptoethyl)amine.

7.3.4 Thermal Analysis

Thermal analysis of the copolymeric material via TGA not only provides useful information regarding the thermal properties of the material, but in this scenario, may also provide structural insight. By inspecting the TGA curves in Figure 94, it can be noticed that the curve for the copolymeric material is very clean, in that the material exhibits a distinct onset temperature for thermal degradation. A clean curve such as this would imply that the material is a uniform copolymer network with covalent linkages rather than a blend of polymers, as the latter would most likely display multiple degradation temperatures for each of the individual components. Furthermore, the curve for kraft lignin and the curve for the copolymeric material are quite different, and no sign of any free lignin within the copolymeric material can be detected.

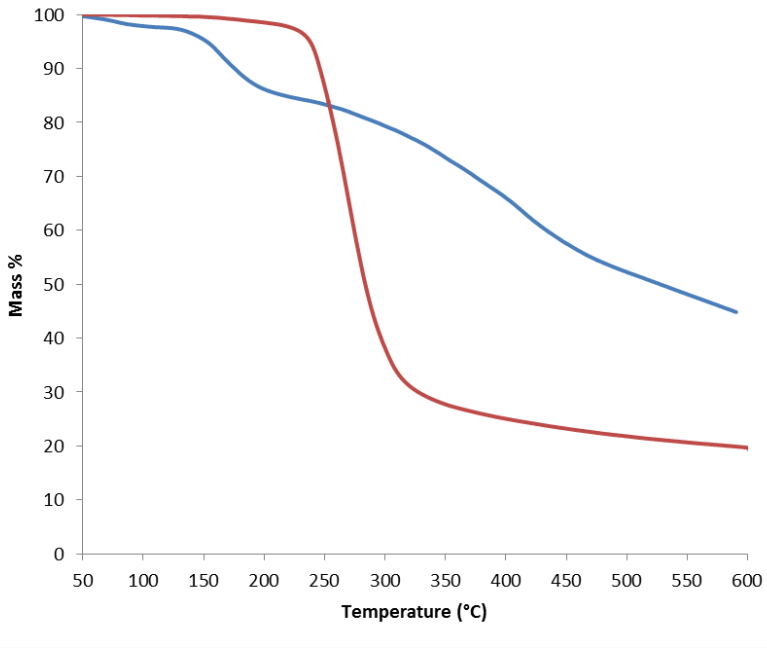


Figure 94. TGA curve of pure kraft lignin (blue) and copolymeric material (red).

Pertinent data extracted from the curves are given in Table 12. Based on the decomposition temperature, T_d, and peak derivative temperature, T_p (temperature at which greatest mass loss occurs), it can be inferred that the copolymeric material possesses greater thermostability compared to the purified kraft lignin, with T_d and T_p values of 238°C and 266°C, respectively, for the copolymeric material, which are approximately 100°C higher than those obtained for the purified kraft lignin. Comparison of these values with other LCHCs synthesized elsewhere, such as lignin copolymerized with PEGMA, reveals that the copolymeric material synthesized in this study is less thermally stable, with T_p values for the lignin-PEGMA hyperbranched copolymers approximately 150°C higher than the value obtained for the material produced in the current study.[252] However, it must be noted that the M_w of the starting lignin used for the synthesis of lignin-PEGMA hyperbranched copolymers is an order of magnitude larger than the M_w of the lignin used in this study, which may be a likely cause for the increased thermostability of the lignin-PEGMA hyperbranched copolymers. The mass percent remaining at 500°C (i.e. char yield) was significantly less for the copolymeric material compared to the purified kraft lignin, 22% as opposed to 52%, which is expected due to the lower lignin content in the copolymeric material.

Table 12. Thermal analysis data of purified kraft lignin and copolymeric material.

	T_d (°C)[a,b]	T_p (°C)[a]	T_g (°C)[c]	% Mass at 500°C[a]
Pure kraft lignin	152	166	-	52
Copolymeric material	238	266	50-60	22

[a] Determined via TGA; [b] T_d was measured as temperature at which mass % is 5% less than measured at 50°C; [c] Determined via DSC

The glass transition, T_g, range for the copolymeric material obtained from the DSC curve (Figure 95) was measured to be 50-60°C, while no T_g range could be determined accurately for the purified kraft lignin based on DSC measurements, which is not surprising as it has been documented that while lignin in its native state within wood exhibits a T_g range, kraft lignin in its purified form does not exhibit a distinctive T_g.[254] The T_g range obtained for the copolymeric material is comparable to T_g values obtained for lignin based thermal-responsive elastomers that were synthesized by copolymerizing lignin with hyperbranched poly(ester-amine-amide).[356]

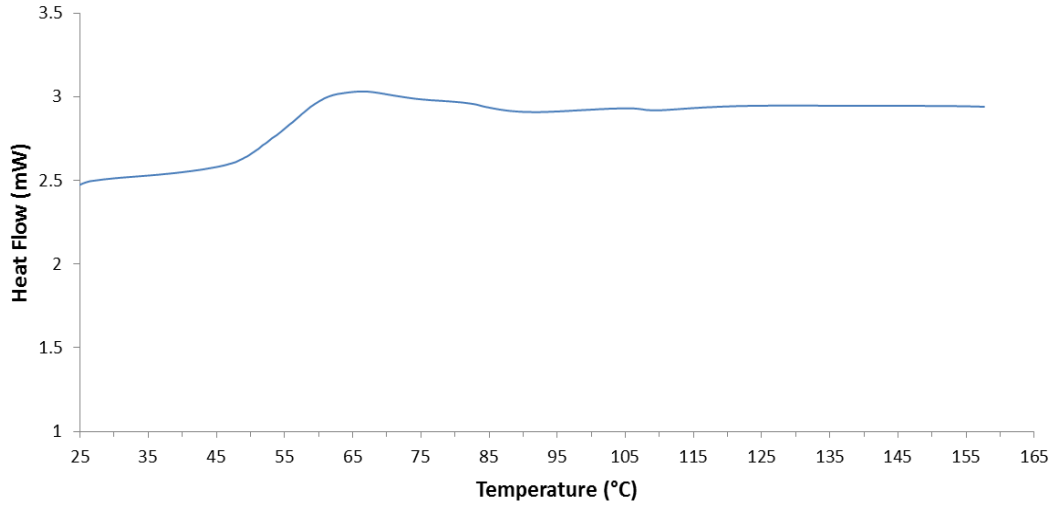


Figure 95. DSC curve of copolymeric material.

7.3.5 Elemental Analysis of Lignin-Core Hyperbranched Copolymers

The results for C, H, N, and S content of LCHCs obtained via elemental analysis are presented in Table 13. Oxygen content was calculated by difference. Based on the nitrogen content, the *tris*(2-mercaptoethyl)amine mass component of the copolymeric material was calculated as follows:

$$Mass\ \%\ N\ in\ tris(2-mercaptoethyl)amine$$

$$= \frac{atomic\ mass\ of\ N\ (\frac{g}{mol})}{molecular\ weight\ of\ tris(2-mercaptoethyl)amine\ \left(\frac{g}{mol}\right)} \times 100\%$$

$$= \frac{14.00}{197.39} \times 100\% = 7.09\%$$

Assuming that all nitrogen in the copolymeric material originates from *tris*(2-mercaptoethyl)amine, then:

$$Mass\ \%\ tris(2-mercaptoethyl)amine\ in\ copolymer$$

$$= \frac{mass\ \%\ N\ in\ copolymer}{mass\ \%\ N\ in\ tris(2-mercaptoethyl)amine} \times 100\% = \frac{4.64}{7.09} \times 100\%$$

$$= 65.44\%$$

Thus, the remaining 35% of the material is composed of lignin and methylhydroquinone; however, it is not possible to distinguish between the two based on elemental analysis.

Table 13. Elemental analysis data of lignin-core hyperbranched copolymer material.

Element	Mass %
C	48.86
H	6.15
N	4.64
S	30.17
O	10.18[a]

[a] Calculated by difference

7.3.6 Surface Characterization

By visual inspection of the copolymeric material it can be seen that the material possesses a glossy surface (Figure 96a), which has also been observed for other lignin containing copolymers.[356, 359] Additionally, it is noticed that the material takes on the characteristic dark brown color of lignin. Delving further into the fine details of the surface structure, examination of the SEM images (Figure 96) uncovers a smooth

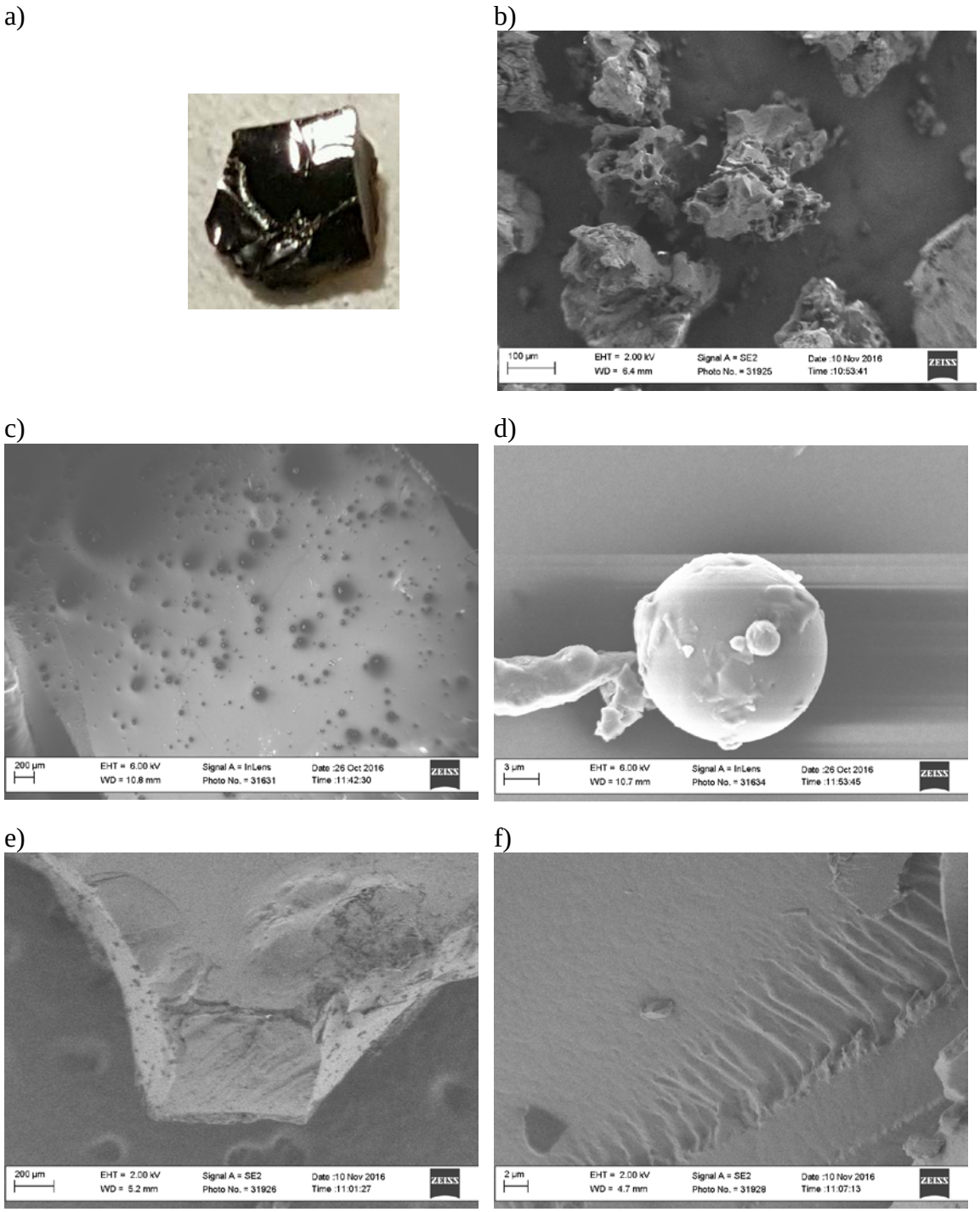


Figure 96. a) Image of the copolymeric material; b-f) SEM images of pure kraft lignin (b), and copolymeric material - top view (c,d), fracture line (e,f).

homogeneous surface with crater like structures dispersed throughout, while an extreme close-up view reveals the perfectly spherical shape of one such crater (Figure 96d), which

seems to be protruding from the surface. The homogeneity of the material seems to extend throughout the bulk material, as witnessed by SEM images of the fracture line (Figure 96e,f). This is interesting given the porous nature of the kraft lignin (Figure 96b), which correlates well with the role of native lignin in wood as a means for water conduction. The lack of resemblance of any porous structure within the copolymeric material may be due to the low overall lignin content, which, given that *tris*(2-mercaptoethyl)amine accounts for 65% of the material's mass (based on elemental analysis), realistically may only account for about 10% of the material's mass (the exact amount of lignin could not be accurately determined because it is impossible to differentiate between methylhydroquinone and lignin based on elemental analysis data). The morphology of the material, which lacks any order or orientation, is in contrast to a laccase-generated lignin-isocyanate copolymer, which shows a distinct fibrillar structure with the fibers oriented in a uniform direction.[264]

7.4 Conclusions

This study demonstrates the potential to synthesize LCHCs via laccase-catalyzed coupling chemistry. It has been stated previously that the vast majority of hyperbranched polymers synthesized to date are of laboratory interest rather than commercial usage, but value added products are gradually being developed.[354] The current study is a preliminary study, and future work aimed at developing practical materials utilizing this chemistry may focus on different monomer selection so as to produce a material with better thermal or mechanical properties. Addition of an external plasticizer into the formulation, such as polybutadiene, which has been combined with lignin in the past for the production of lignin-based thermoplastic, is one potential method that could be used

to improve the physical properties of the material.[360] Also, given that the material synthesized in this study exists as a paste before drying, it may be worthwhile evaluating its adhesives properties, as it has been demonstrated in the past that lignin copolymerized with other small molecules or polymers via laccase catalysis produces resins that can be used as adhesives for particleboards and wool floor coverings.[266-267] Nevertheless, this study provides evidence that laccases can be employed for the synthesis of LCHCs under sustainable conditions and provides yet another novel route for lignin valorization.

CHAPTER 8. OVERALL CONCLUSIONS

To recap, the main objectives of this dissertation research were divided into two parts: part one was aimed at developing fundamental laccase-catalyzed coupling chemistry via novel C-C, C-N, and C-S bond forming reactions for the synthesis of new and existing compounds under environmentally benign conditions so as to provide a green solution in organic synthesis, while part two of the dissertation research focused on applying the fundamental laccase-catalyzed coupling chemistry to the functionalization of lignin for the development of novel lignin-based biomaterials and to establish an alternative route for lignin valorization within the biorefinery. The content in Chapters 4-6 are devoted to developing the fundamental laccase-catalyzed coupling chemistry, while Chapter 7 deals with the application of the developed laccase-catalyzed coupling chemistry to the functionalization of lignin.

Along the way of developing the new laccase-catalyzed bond forming reactions, many novel and existing compounds were synthesized. Some of the compounds are known to be important biologically active molecules, such as the phenothiazones (Chapter 6), which were synthesized in an aqueous solvent system for the first time, while others, such as the benzylic nitriles and 2,3-ethylenedithio-1,4-quinones (Chapters 4 and 5, respectively), are prominent structural moieties in active pharmaceutical ingredients and present as building blocks in the synthesis of therapeutic compounds. Furthermore, regarding the synthesis of 2,3-ethylenedithio-1,4-quinones and phenothiazones, as well as the small cyclic disulfides that were inadvertently synthesized

in Chapter 5, this dissertation research has made significant pioneering contributions to the laccases-catalyzed syntheses of heterocyclic compounds.

Much knowledge was gained throughout the course of this dissertation research regarding differences in reactivities of *in situ* generated *ortho*- and *para*-quinones and how this is a vital factor in determining the success of a reaction. No products were observed for any of the reactions performed in this dissertation research that involved coupling the selected nucleophiles with catechols under laccase catalysis. Given that the reactions were successful in forming coupling products when hydroquinones were reacted with the same nucleophiles under laccase catalysis, there is an obvious difference in reactivity between the laccase-generated *ortho*-quinones and *para*-quinones. Given that in all chemical transformations there exists a battle of competing rates, such that in the current examples a successful reaction is contingent upon the nucleophile being able to react rapidly enough with the *in situ* generated quinone before the quinone decomposes or polymerizes, it may be that the laccase-generated *ortho*-quinones decompose or polymerize at such rapid rates that they are not present in the reaction medium for a long enough period to allow for nucleophilic addition. In comparison, based on the current research, the *para*-quinones seem to be much more stable than the *ortho*-quinones as they persist in the reaction medium for an ample period to react with the tested nucleophiles and form coupling products.

A similar argument can be made for how the steric and electronic properties of the hydroquinone substituents affect the stability of the *in situ para*-quinone and subsequent product yields. Hydroquinones bearing electron-withdrawing substituents, such as halogens, generally provided very low product yields or no coupling products, with the

exception of the carbonyl-substituted hydroquinones 2,5-dihydroxyacetophenone and ethyl 2,5-dihydroxybenzoate in the laccase-catalyzed reactions with benzoylacetonitrile, while hydroquinones bearing electron-donating substituents generally provided coupling products in good yields. It seems as though the electron-withdrawing nature of certain substituents destabilizes the laccase-generated *para*-quinones, which may be the cause of low product yields. In contrast, hydroquinones containing bulky electron-donating substituents invariably provided moderate to good product yields, particularly 1,4-naphthohydroquinone. The electron-donating nature of these substituents allow for a more stable *in situ para*-quinone, while the added steric hindrance of bulky substituents may reduce the likelihood of competing polymerization reactions occurring.

Much insight into how certain reaction conditions and electronic properties of hydroquinone substituents impacted the rate and extent of laccase catalysis was also gained during the course of this research. Firstly, conditions such as temperature, solvent, and pH of solvent, greatly impacted laccase catalysis. By analyzing reaction mixtures via TLC and GC-MS at various periods during the course of the reactions, it was noticed that, for a given laccase substrate, the extent and rate at which substrate was consumed was highly dependent on the aforementioned reaction conditions. Invariably, substrate oxidation by laccases occurred at a much more rapid rate at slightly elevated temperatures (40-50°C), in an aqueous buffer at mildly acidic pH values (5.0), which is consistent with the reaction conditions for optimum catalytic efficiency for fungal laccases.[85] At alkaline pH values and at ambient temperature, substrate oxidation generally took longer or a greater amount of laccases were required to achieve the same extent of oxidation. Regarding hydroquinone substituent effects, hydroquinones

containing electron-donating substituents were oxidized much more rapidly or required lesser amount of laccases to be completely oxidized compared to hydroquinones bearing electron-withdrawing substituents (refer to the results in Table 4). This is consistent with trends on how the electronic properties of substituents affect the redox potentials of hydroquinones and how substrate redox potentials affect the rate and extent to which they are oxidized by laccases: as the electron-withdrawing nature of the substituent increases, so too does the redox potential of the hydroquinone, and as the redox potential of the substrate increases, oxidation by laccases becomes less energetically favorable, resulting in a decreased rate or extent of reaction.[117]

Insights into the potential modes of inhibition of laccases were unraveled, as the results of Chapter 5 demonstrate that the laccase-catalyzed couplings of hydroquinones with a small sulfhydryl compound can be successfully achieved in spite of research that suggests small sulfhydryl compounds are inhibitors of laccases. In past laccase catalytic studies involving thiols, it seems as though laccases are not oxidizing the substrate, which can be easily mistaken for inhibition, proposedly by binding to the TNC and inhibiting internal electron transfer; however, in actuality, this is not the case. The current dissertation research supports the findings of Johannes and Majcherczyk (2000) who discovered that small sulfhydryl compounds are not true inhibitors of laccases, rather, small sulfhydryl compounds are easily oxidized by laccase-generated radicals, which act as mediators to oxidize the sulfhydryl compounds, in turn regenerating the initial laccase substrate.[125] Based on classical enzyme activity assays that measure either the consumption of substrate or accumulation of product, it is easy to understand how this misconception came about.

LMS chemistry proved to be ubiquitous in laccase-catalyzed processes, as witnessed in the degradation of benzoylacetonitrile (Chapter 4) and the S-S coupling reactions that resulted in the formation of small cyclic sulfides (Chapter 5). This should come as no surprise given the highly reactive nature of radicals and it seems as though this issue cannot be circumvented given that laccases generate phenoxy radicals upon oxidation of phenolic compounds. The question is whether the LMS is a friend or foe? In the case of the laccase-catalyzed syntheses of benzylic nitriles in Chapter 4, LMS chemistry lead to the degradation of benzoylacetonitrile, which significantly reduced product yields of benzylic nitriles. The same can be said for the reactions performed in Chapter 5, as the radical-radical coupling of sulfur radicals of 1,2-ethanedithiol also reduced product yields of 2,3-ethylenedithio-1,4-quinones; however, that study demonstrated that it is possible to synthesize small cyclic sulfides via LMS. Thus, it seems as though in some situations LMS chemistry can be detrimental to the productivity of the reaction protocol, while in others, it can be a useful way to synthesize novel compounds. It comes down to experimenting with the reaction conditions to devise a set of conditions that will provide the desired outcome.

The developed fundamental laccase-catalyzed coupling chemistry was able to be successfully utilized for the functionalization of purified kraft lignin. The laccase-mediated synthesis of LCHCs utilizing laccase-catalyzed couplings between lignin, methylhydroquinone, and a trithiol provides another route for lignin valorization and a means to synthesize novel lignin-derived biomaterials. Evidence seems to suggest the formation of a copolymeric network rather than distinct copolymers, resulting in the creation of a brittle, glossy material. These preliminary results indicate that it is possible

to functionalize lignin using this type of chemistry; however, additional research needs to be conducted into developing a material that is suitable for use in a specific application.

In closing, laccases have proved themselves to be important biotechnological tools with widespread applicability. The types of chemical transformations that can be accomplished are vast and the types of products and materials that can be produced are of practical value, all of which is achieved under environmentally benign conditions. The use of laccases as a foundation for building this dissertation research has provided a truly remarkable and fruitful journey and has greatly aided in accomplishing the research goal of developing and promoting a more sustainable chemical industry.

CHAPTER 9. RECOMMENDATIONS FOR FUTURE WORK

While this dissertation research has aided in expanding the synthetic utility of laccases to advance sustainability within the chemical industry, there still exists the opportunity to build on this research even further. Regarding the types of chemical transformations that can be effected, new laccase-catalyzed bond-forming reactions are consistently being reported in the literature. Perhaps one method to aid in expanding the scope of laccase-catalyzed processes is to combine the oxidizing capabilities of laccases with transition metal catalysts. For example, very recently and for the first time, laccases have been paired with palladium and ruthenium transition metal complexes for the selective oxidation of benzylic alcohols and olefins, respectively.[361-362] This pioneering work is just one example of the potential novel uses of laccases in the field of synthetic chemistry, thus it will be exciting to witness innovative developments in this field in the near future.

Although the use of laccases as biotechnological tools for industrial purposes is promising, there exist challenges facing the commercialization of laccase-mediated processes, such as the lack of availability of affordable, highly active enzymes, and separation of enzyme from the reaction medium after process completion. To combat these problems, advances in protein engineering have made it possible for the manufacture of thermostable laccases that are tolerant to organic solvents and ionic liquids, which enhances their suitability for industrial applications.[363-364] Also, recent research has shown that by combining laccases with Au nanoparticles to formulate laccase-Au hybrids, the laccase activity can be increased dramatically.[365] Advances in

enzyme immobilization technology, such as cross-linked enzyme aggregates and adsorption onto multi-walled carbon nanotubes,[330, 366] provide enhanced enzyme operational and storage stabilities, as well as provide a facile means to separate and reuse the enzyme.[367-368] Furthermore, combining laccases with sonochemistry has shown to reduce the consumption of chemicals and energy, thereby providing a cost effective means to upscale laccase-catalyzed processes to an industrial scale.[369] These examples are just a few of the many ways scientists and engineers are working to provide industrially practical laccase biotechnology.

Knowing that laccase-catalyzed processes inherently involve radicals, particularly phenoxy radicals, another relevant research topic may involve thermodynamic and kinetic studies on the fate of the laccase-generated phenoxy radicals. Aspects to consider might include the relevant likelihood of the phenoxy radicals undergoing disproportionation, dimerization, polymerization, or hydrogen-atom abstraction of another compound. Given that the success of laccase-catalyzed coupling reactions involving hydroquinones and catechols is critically dependent on the laccase-generated phenoxy radicals undergoing disproportionation to yield quinones, information regarding the likelihood of disproportionation occurring as opposed to competing processes, such as dimerization, polymerization, or hydrogen-atom abstraction, and under what conditions, would be extremely useful to the synthetic chemist when devising a synthetic plan and in assessing the potential outcome of such a synthesis.

Enzymatic processes, particularly on an industrial scale, are typically accompanied by well-defined kinetic analyses; however, kinetic studies on laccase-catalyzed processes involving hydroquinones and catechols are anything but trivial.

Kinetic studies rely on the ability to measure a change in concentration of a chemical species over time, typically either the depletion of a substrate or the accumulation of a product. In the case of laccase-catalyzed processes involving hydroquinones and catechols, the product of laccase oxidation is a phenoxy radical, which is not stable enough to be quantified. Moreover, upon reacting further, the phenoxy radical does not just produce a single product; it could react to form a dimer or polymer, or undergo disproportionation with another phenoxy radical to yield a quinone and regenerate the original hydroquinone or catechol. Thus, neither the hydroquinone or catechol substrates, nor the phenoxy radical product generated upon laccase oxidation, can be quantified with a high degree of accuracy. Therefore, future research may focus on developing reliable methods to perform kinetic analyses on the abovementioned laccase-catalyzed processes.

Regarding the laccase-mediated synthesis of LCHCs, future work should involve collaborating with materials scientists and engineers to develop a material that has superior physical and mechanical properties and is tailored for a particular application, such as an adhesive resin or a thermoplastic. The current dissertation research provides a pioneering framework on which to build on and proves that laccases can be used to successfully functionalize lignin and synthesize LCHCs. Whether the material produced in this dissertation is of practical value remains to be seen; however, its use as an adhesive in particleboard production should be assessed.

The abovementioned recommendations for future research on the topic of laccase-catalyzed processes highlight only a few of the potential research avenues, but the list could go on. Thus, laccases are truly enzymes that keep giving, and it will be exciting to

witness innovative ways in which laccases can continue to advance sustainability in the chemical industry and beyond.

APPENDIX A

NMR, FTIR, AND MASS SPECTRA OF NEW COMPOUNDS

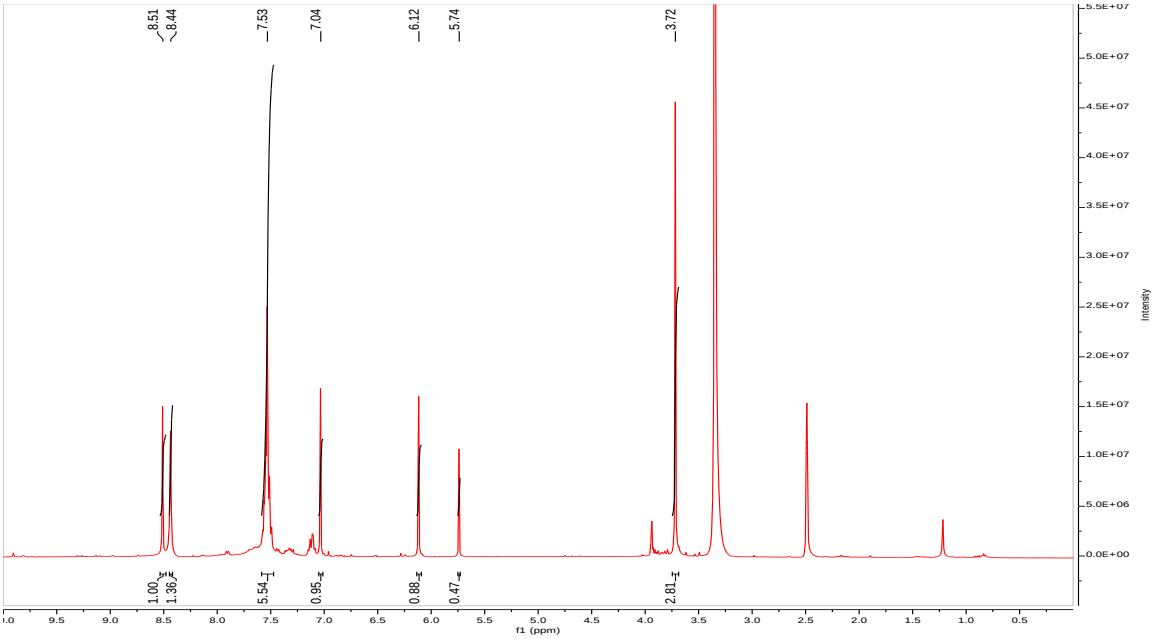


Figure A1. ^{1}H NMR spectrum of compound **6a**.

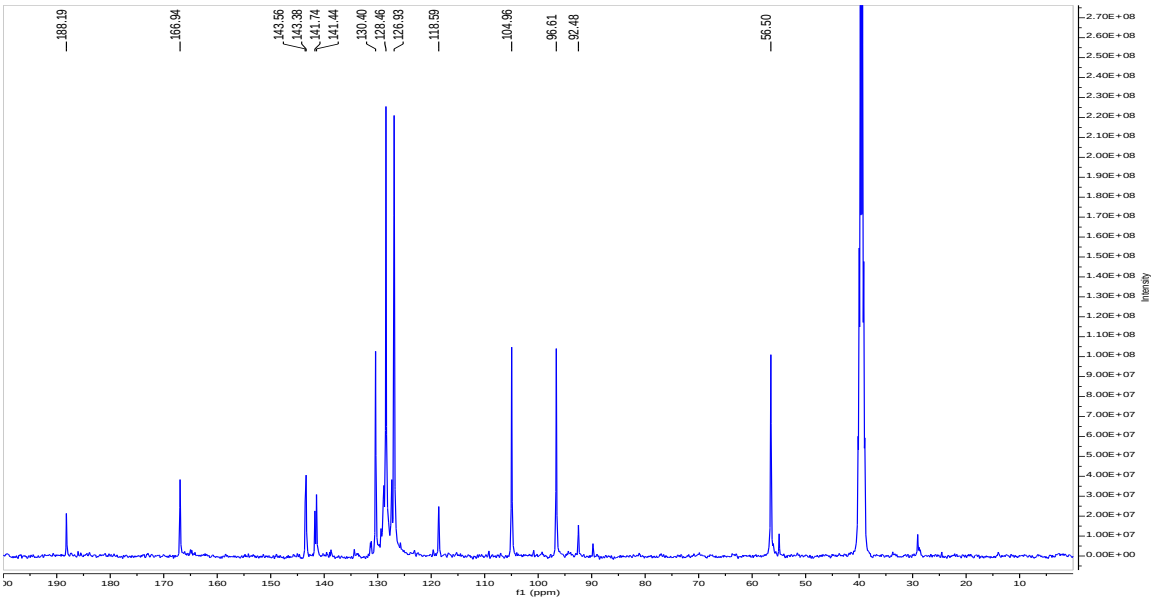


Figure A2. ^{13}C NMR spectrum of compound **6a**.

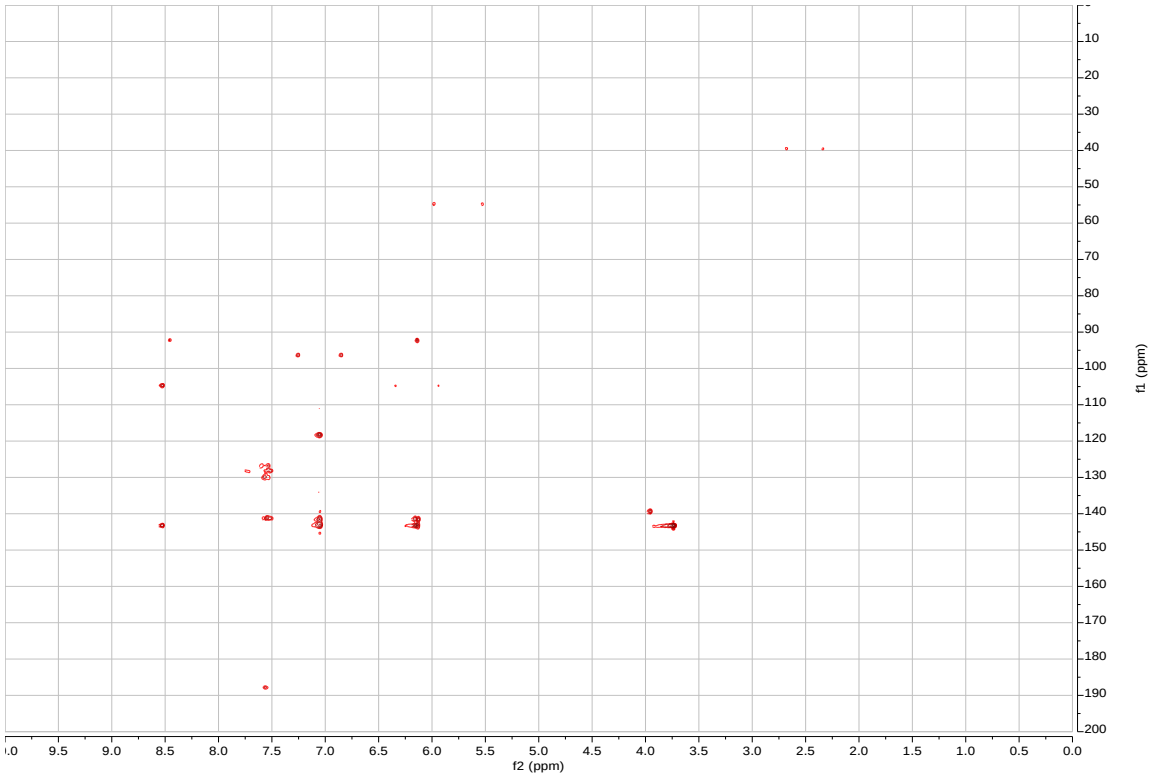


Figure A3. ^{1}H-^{13}C HMBC NMR spectrum of compound **6a**.

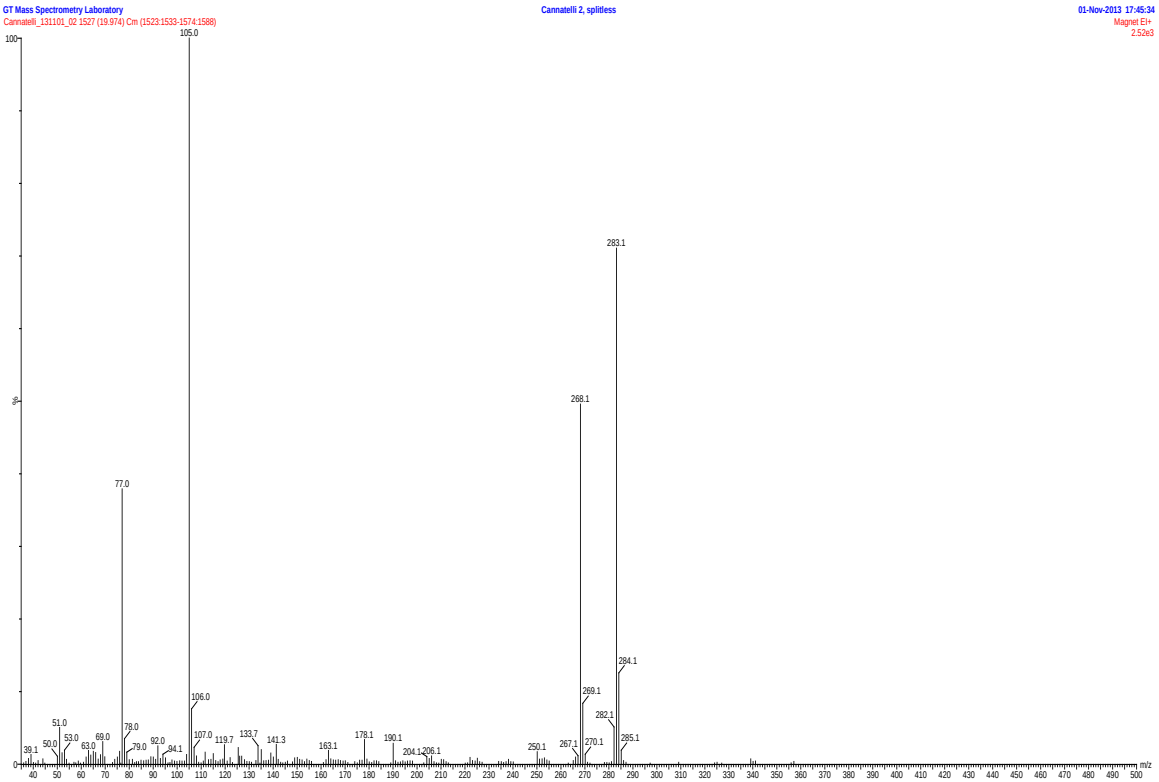


Figure A4. Mass spectrum of compound **6a**.

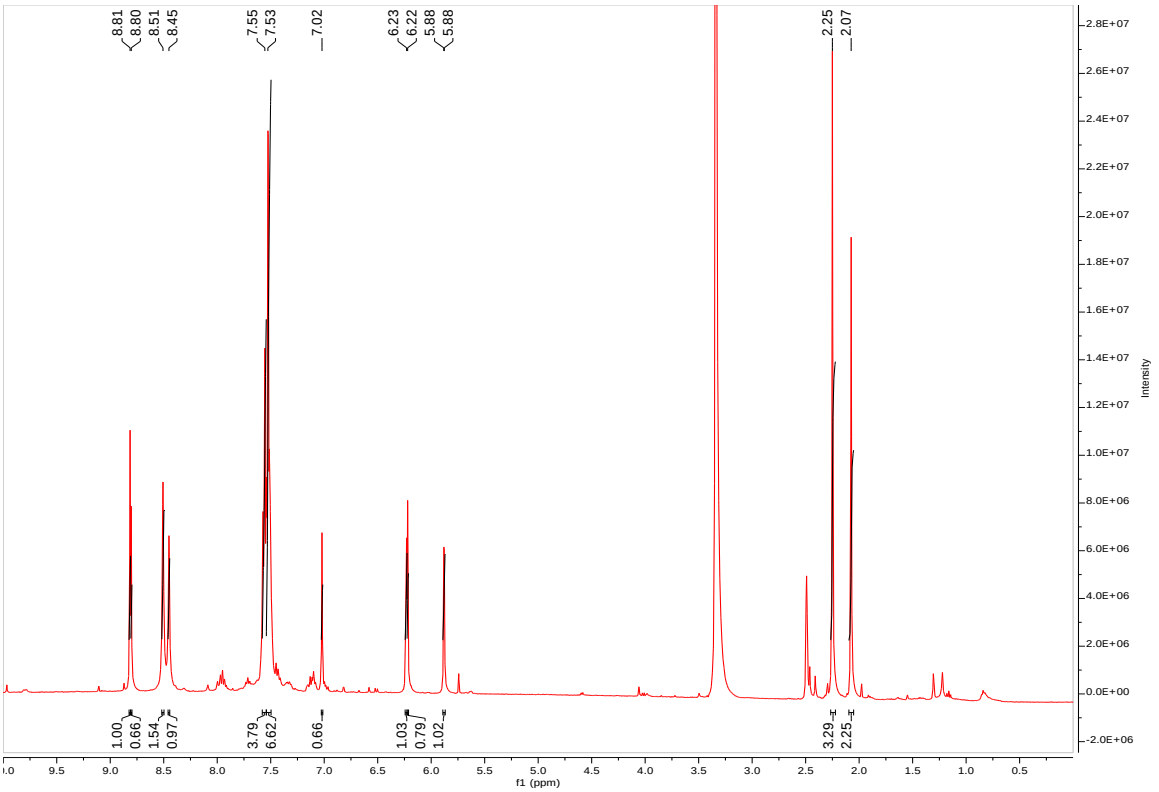


Figure A5. ^{1}H NMR spectrum of regioisomeric mixture of compounds **6d** and **7d**.

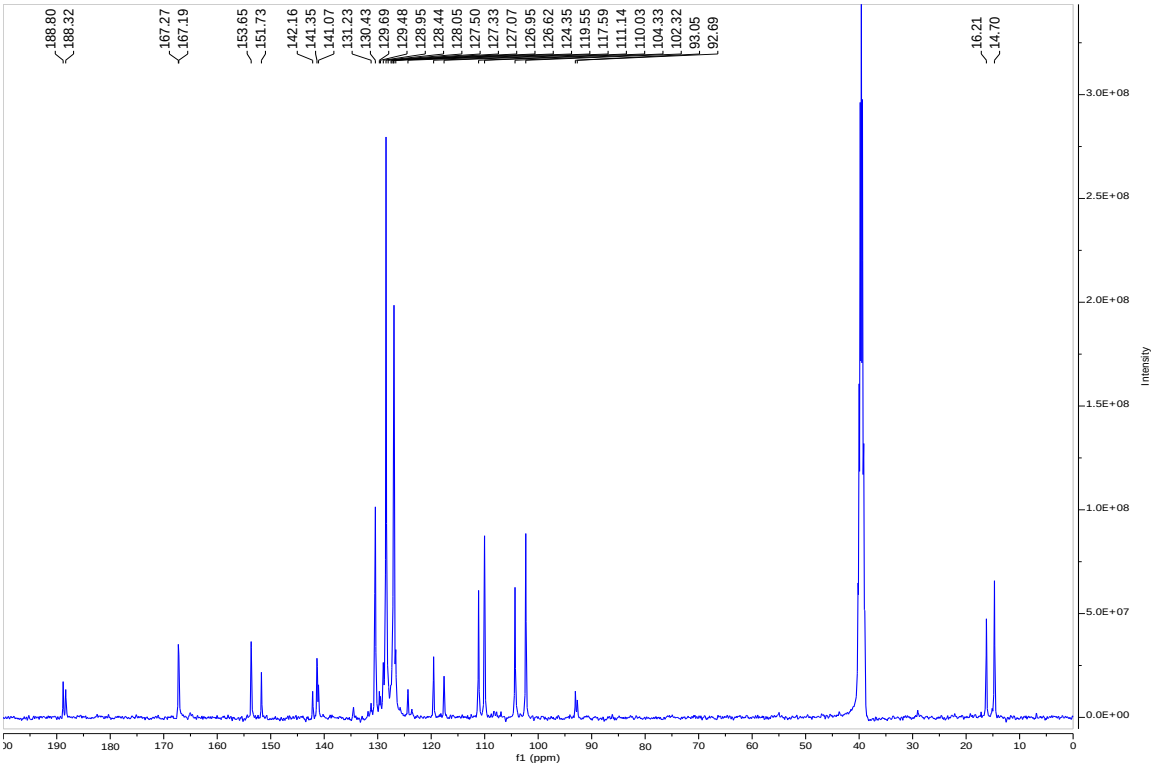


Figure A6. ^{13}C NMR spectrum of regioisomeric mixture of compounds **6d** and **7d.**

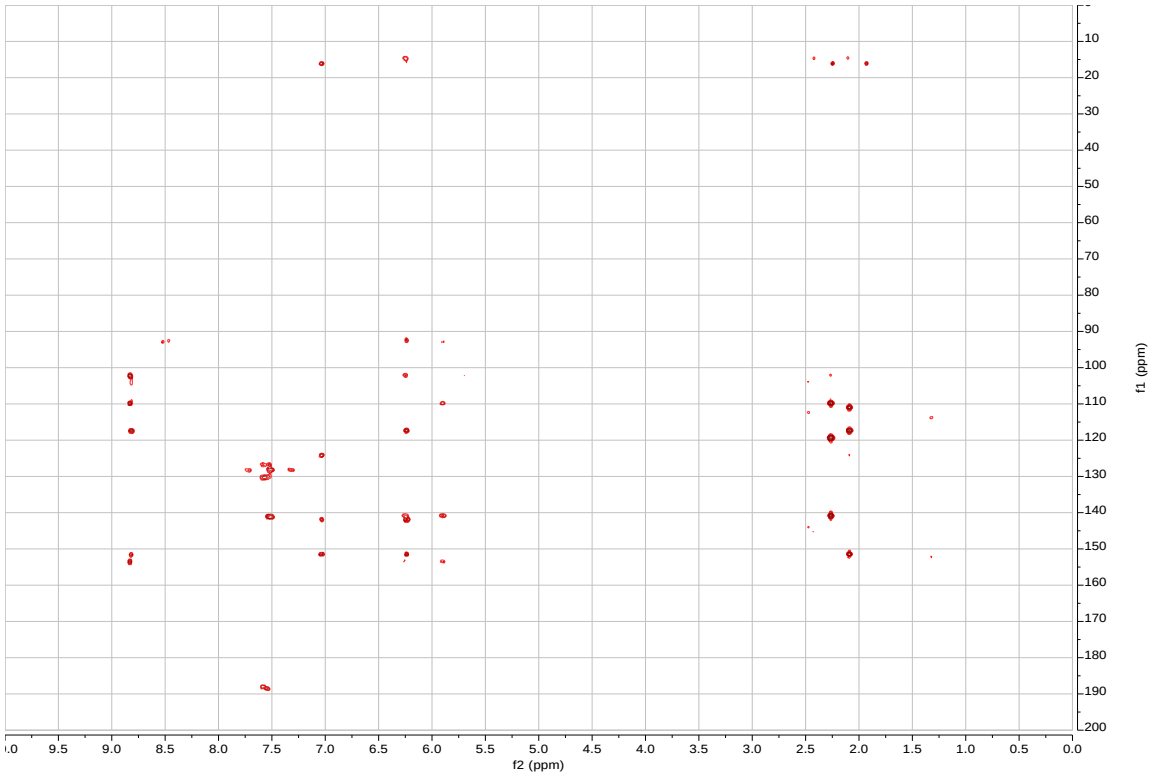


Figure A7. ^{1}H-^{13}C HMBC NMR spectrum of regioisomeric mixture of compounds **6d** and **7d**.

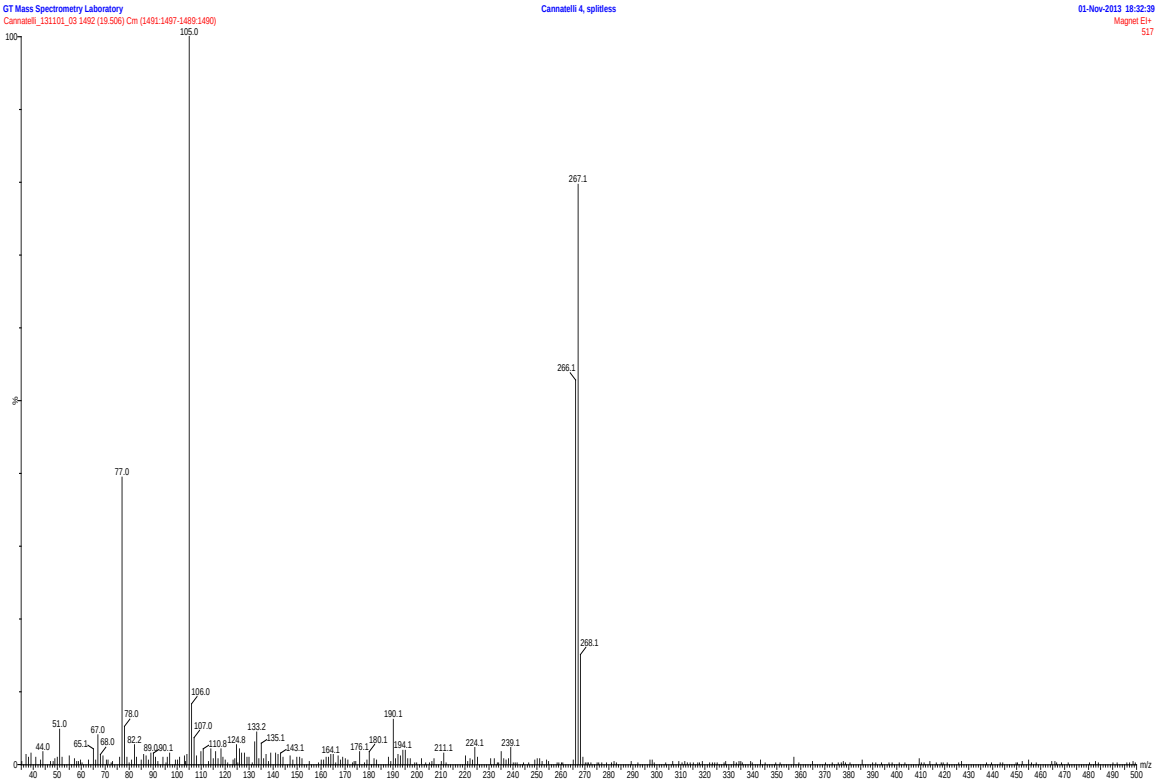


Figure A8. Mass spectrum of regioisomeric mixture of compounds **6d** and **7d**.

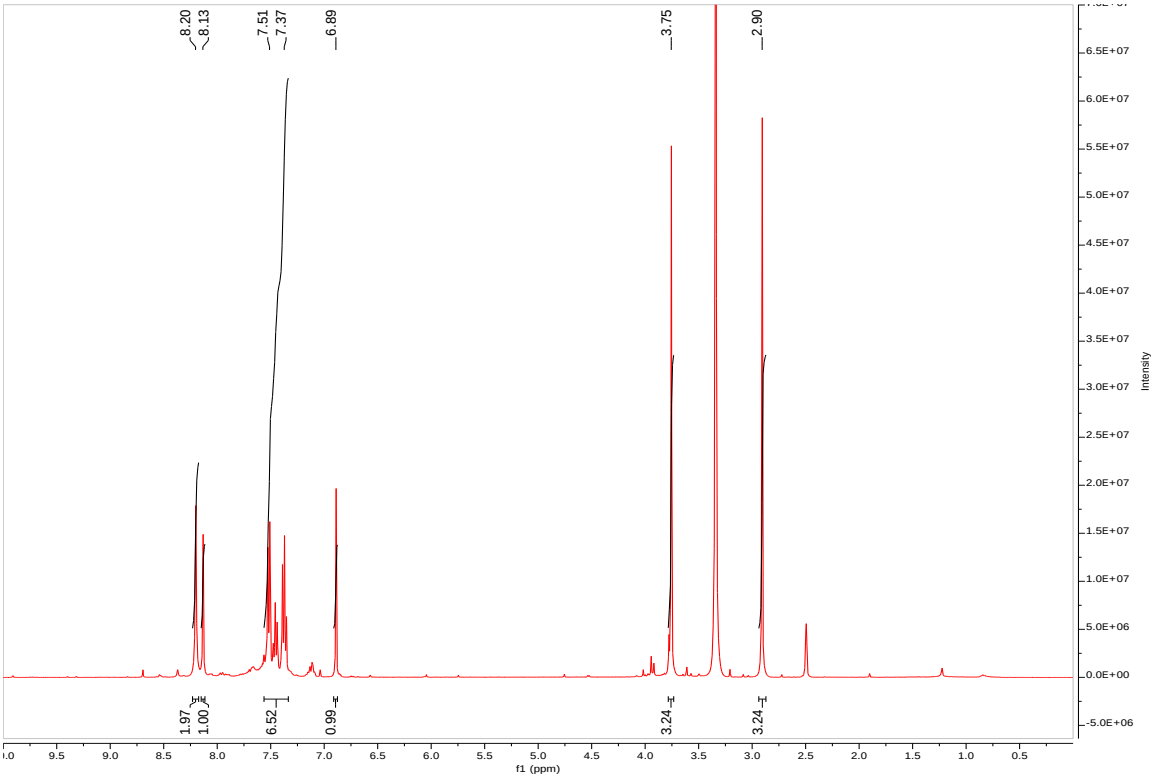


Figure A9. ^{1}H NMR spectrum of compound **6h**.

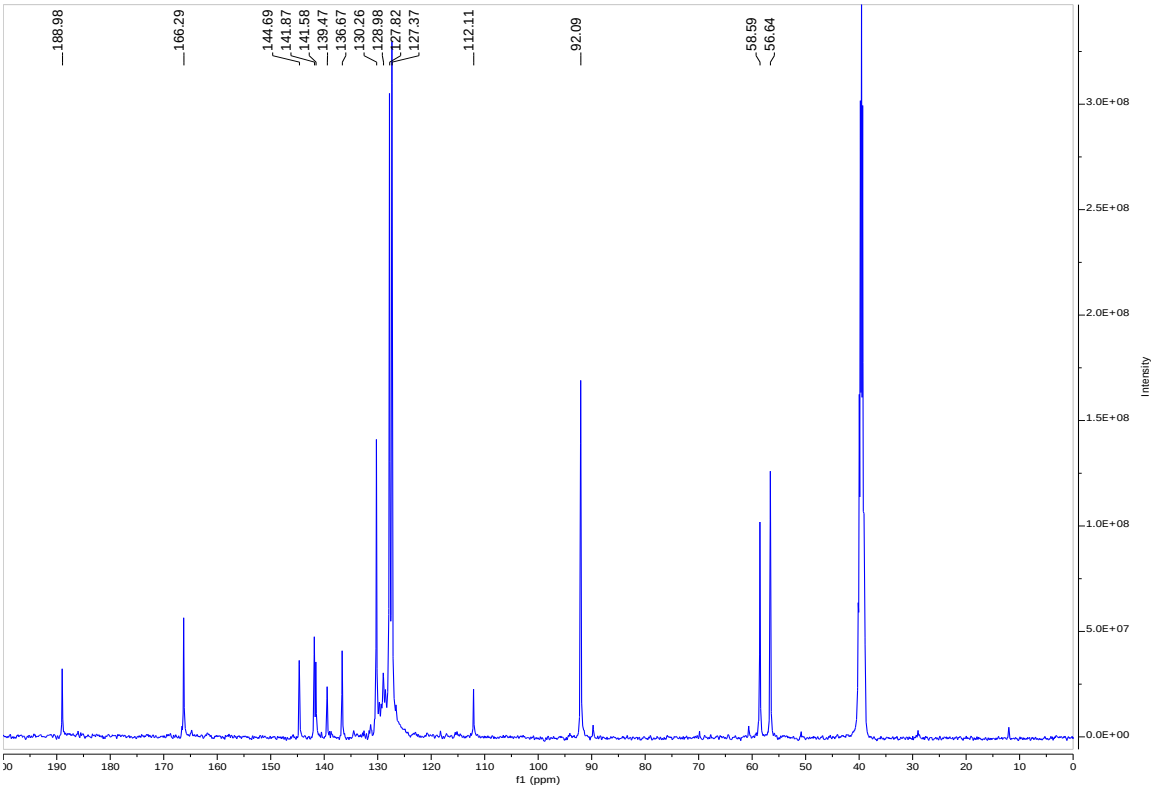


Figure A10. ^{13}C NMR spectrum of compound **6h.**

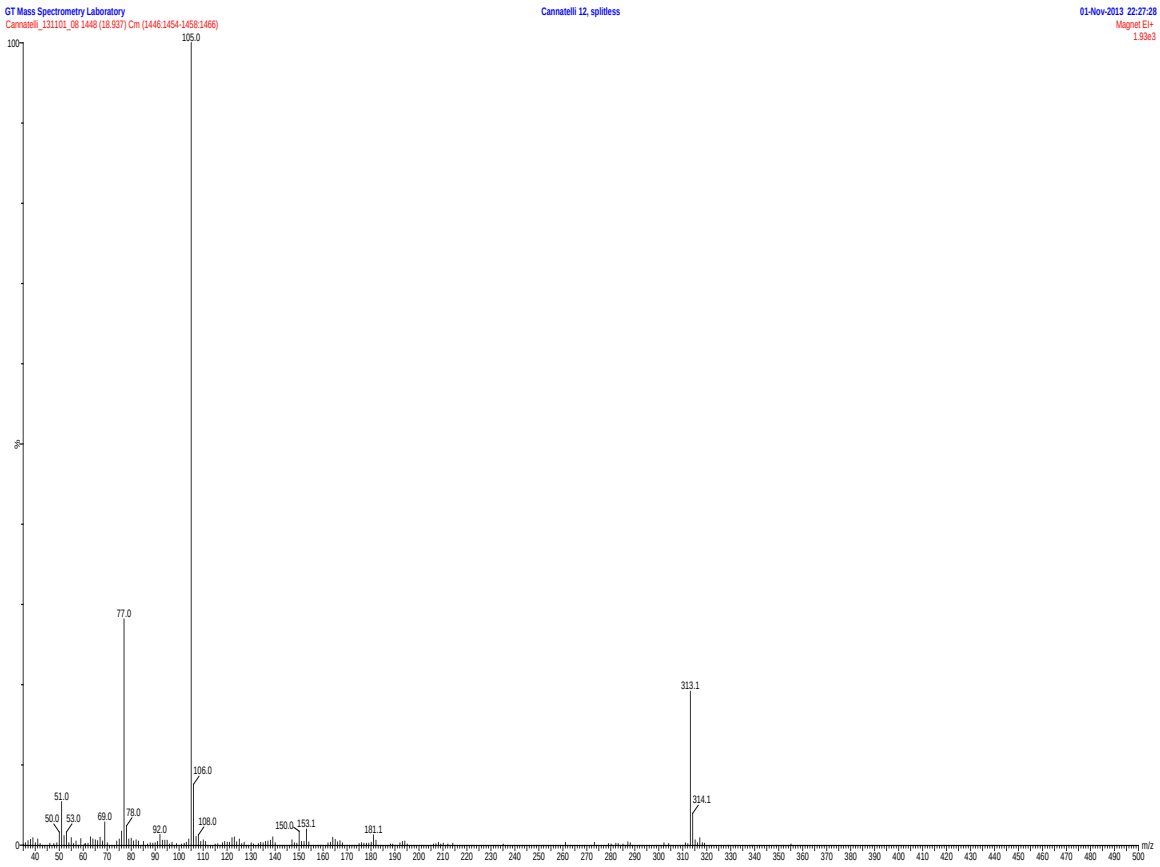


Figure A11. Mass spectrum of compound **6h**.

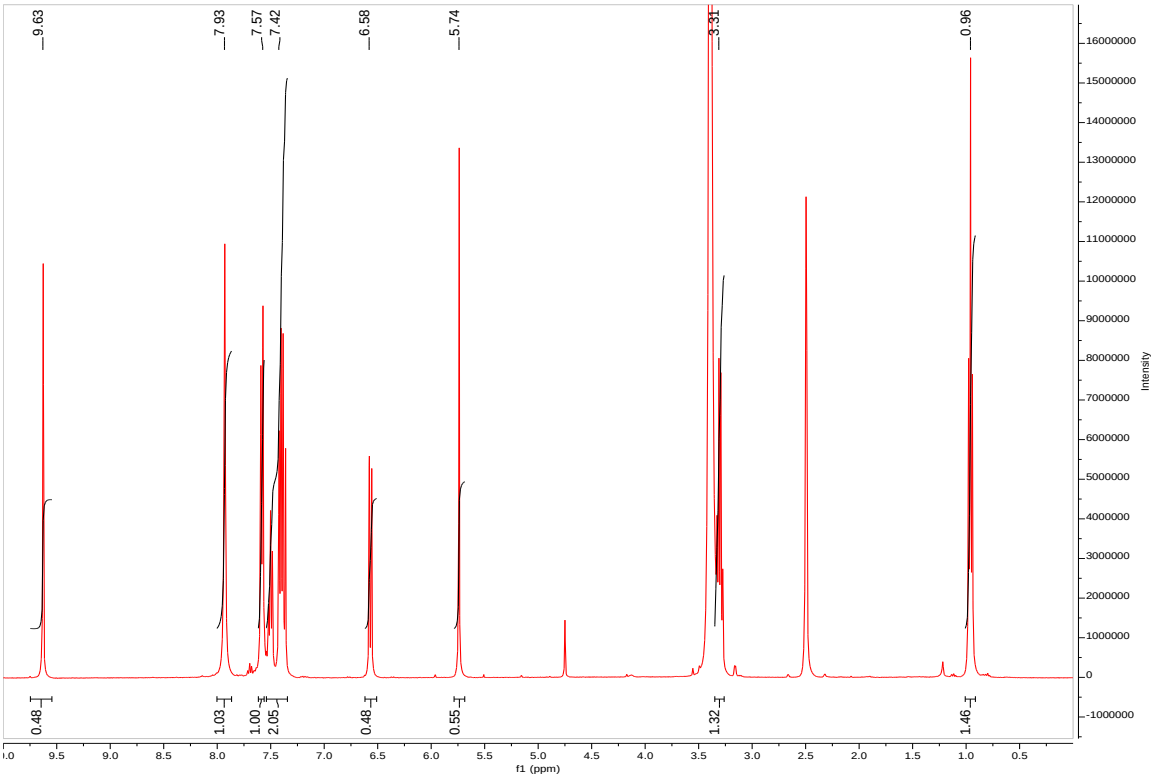


Figure A12. ^{1}H NMR spectrum of compound **8g**.

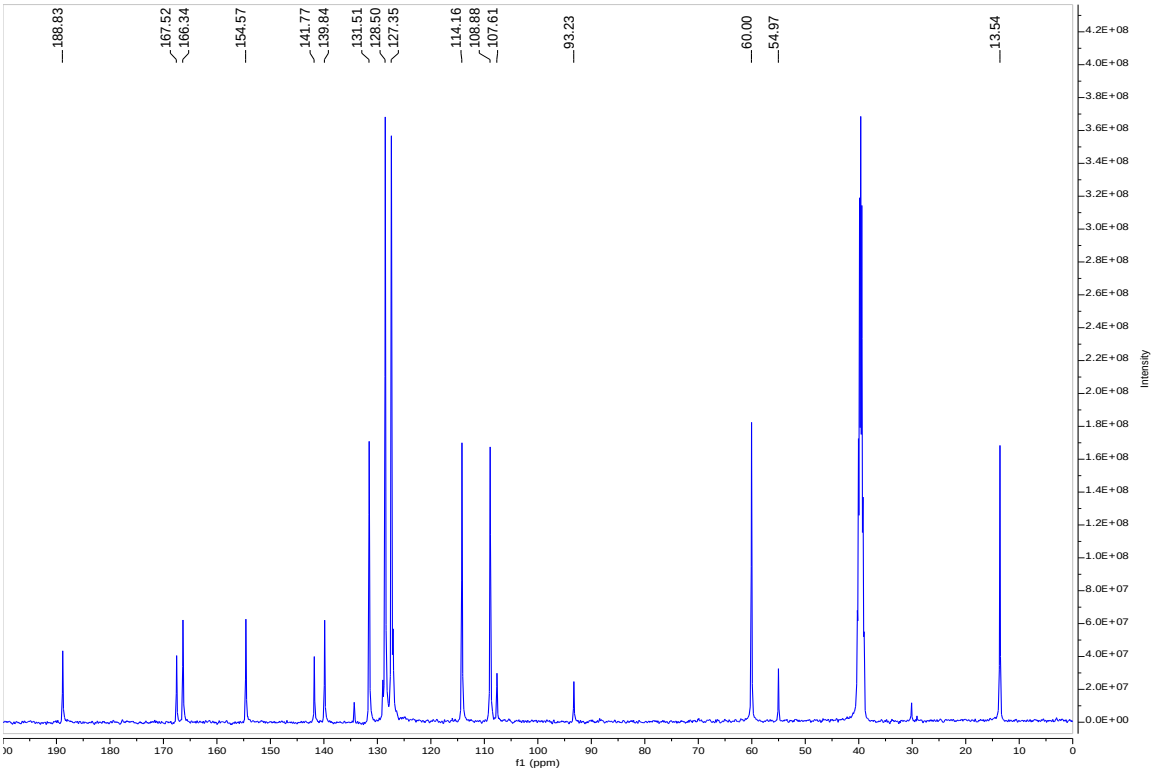


Figure A13. ^{13}C NMR spectrum of compound **8g**.

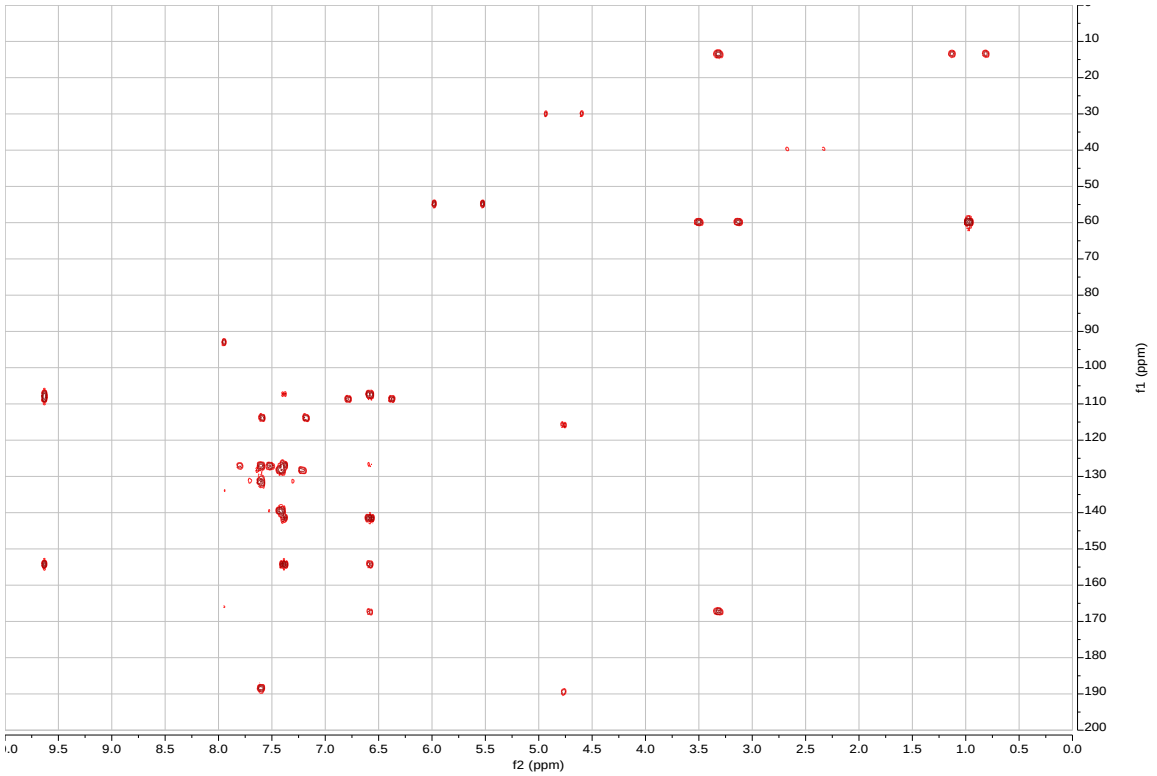


Figure A14. ^{1}H-^{13}C HMBC NMR spectrum of compound **8g**.

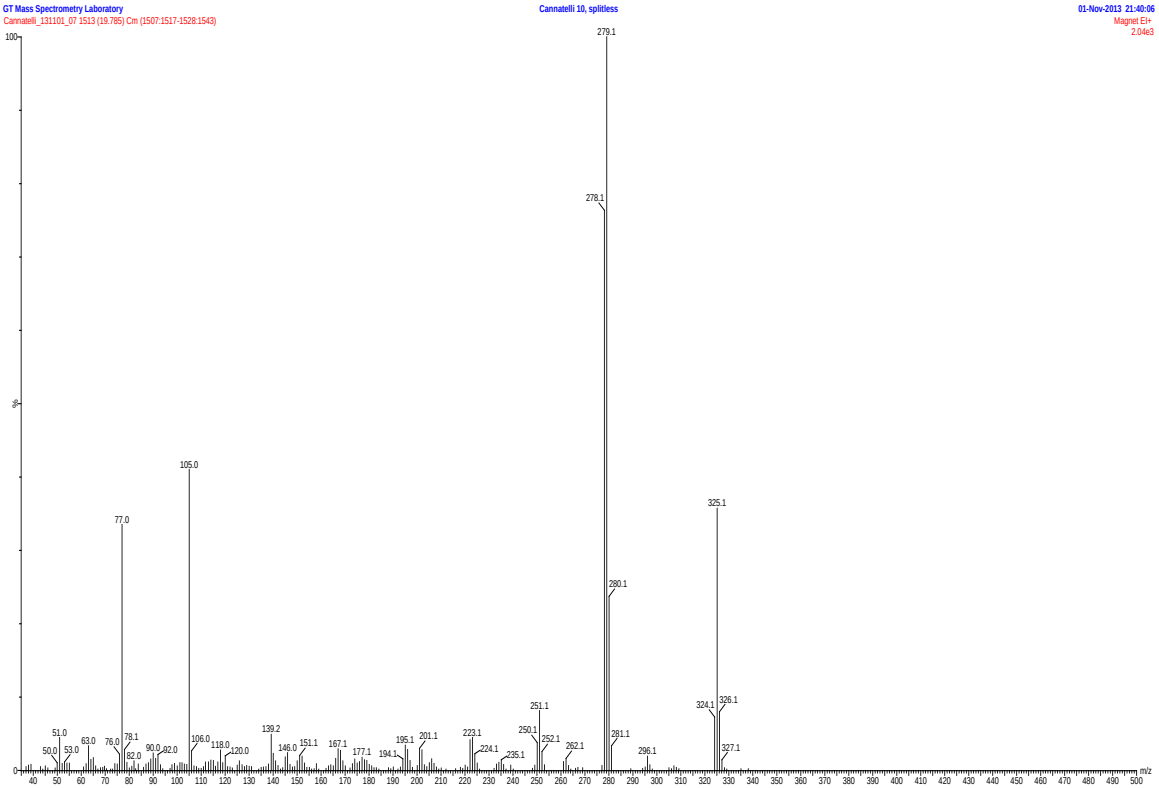


Figure A15. Mass spectrum of compound **8g**.

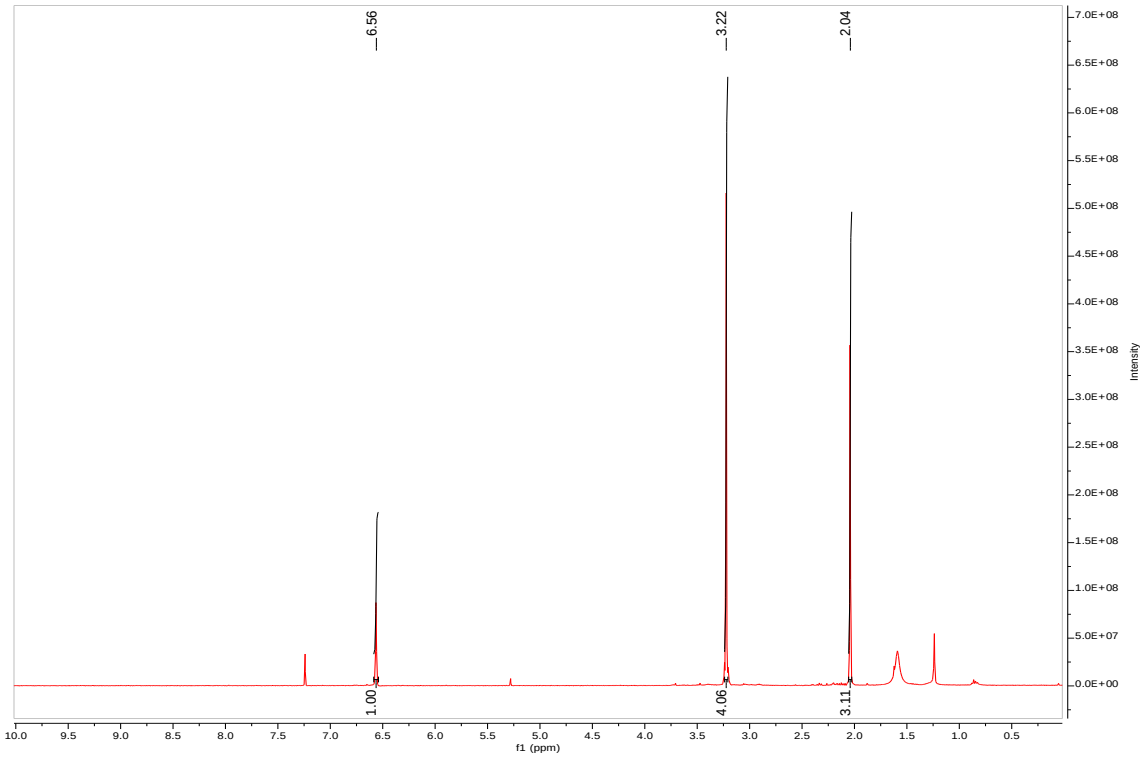


Figure A16. ^{1}H NMR spectrum of compound **10b**.

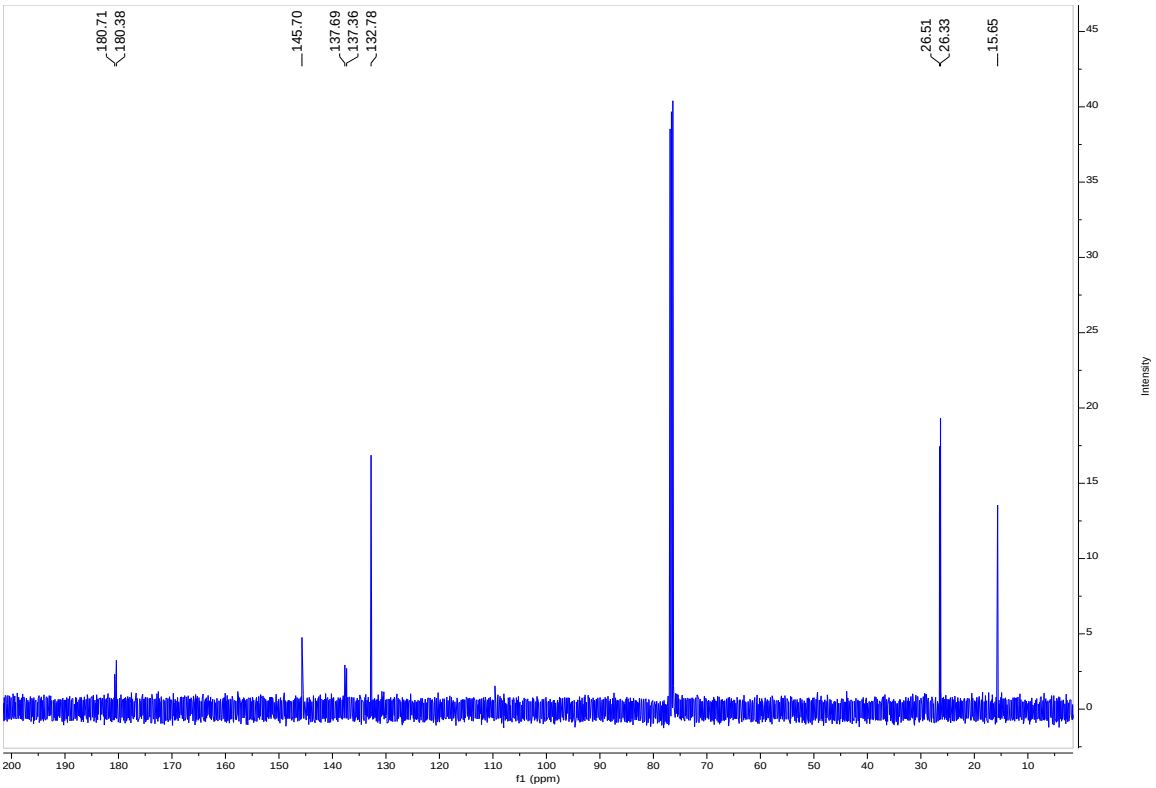


Figure A17. ^{13}C NMR spectrum of compound **10b**.

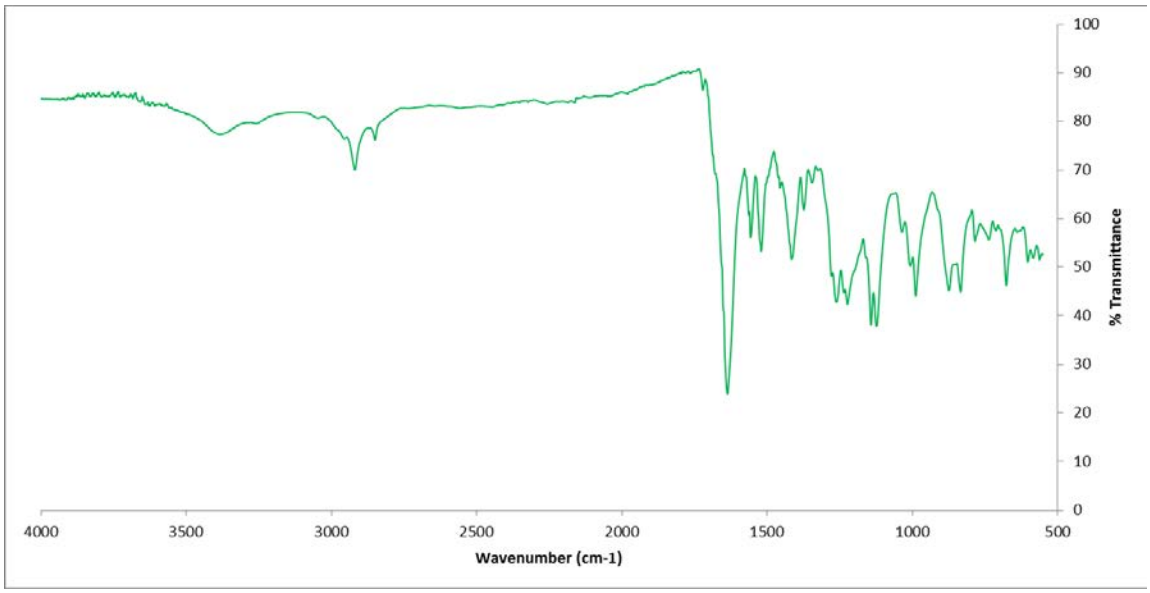


Figure A18. FTIR spectrum of compound **10b**.

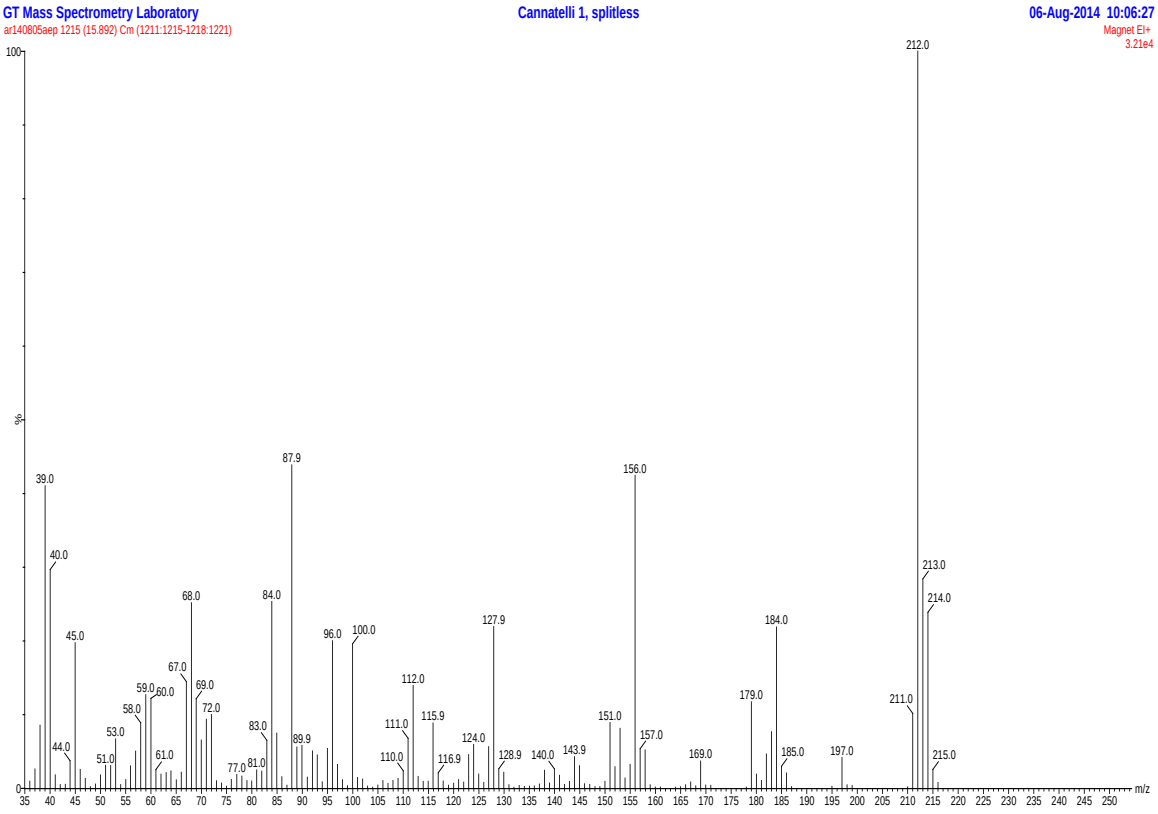


Figure A19. Mass spectrum of compound **10b**.

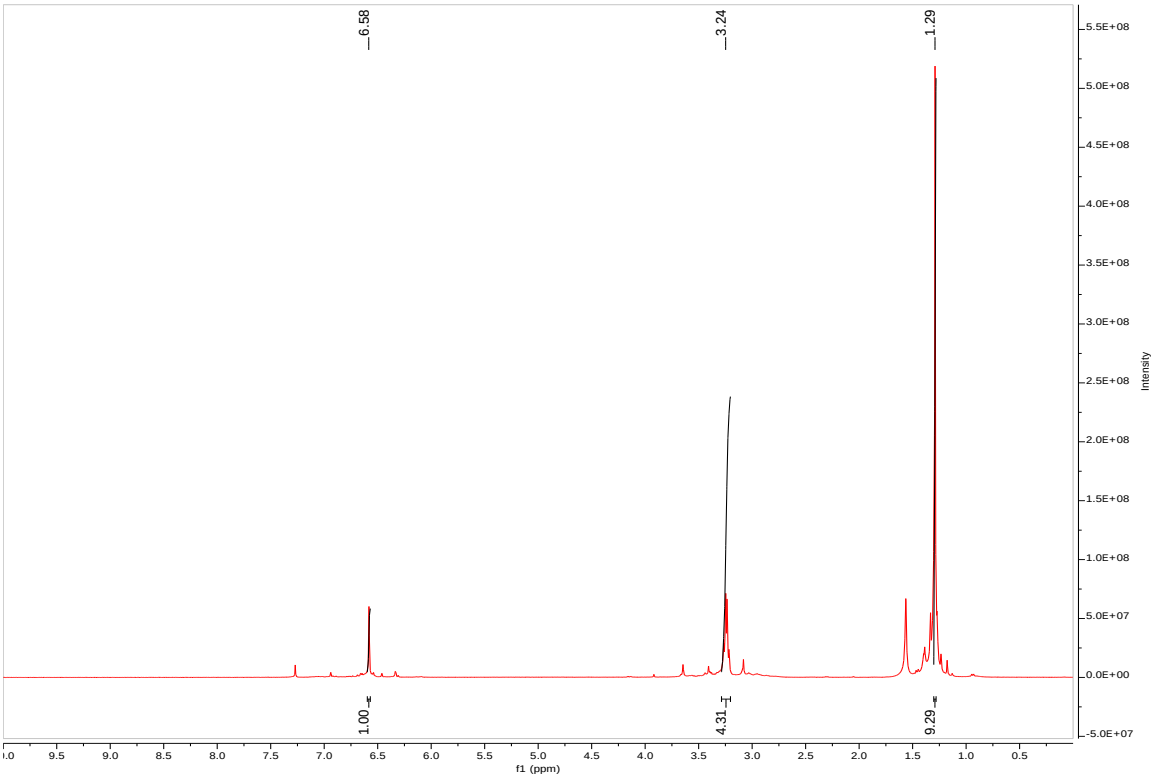


Figure A20. ^{1}H NMR spectrum of compound **10c**.

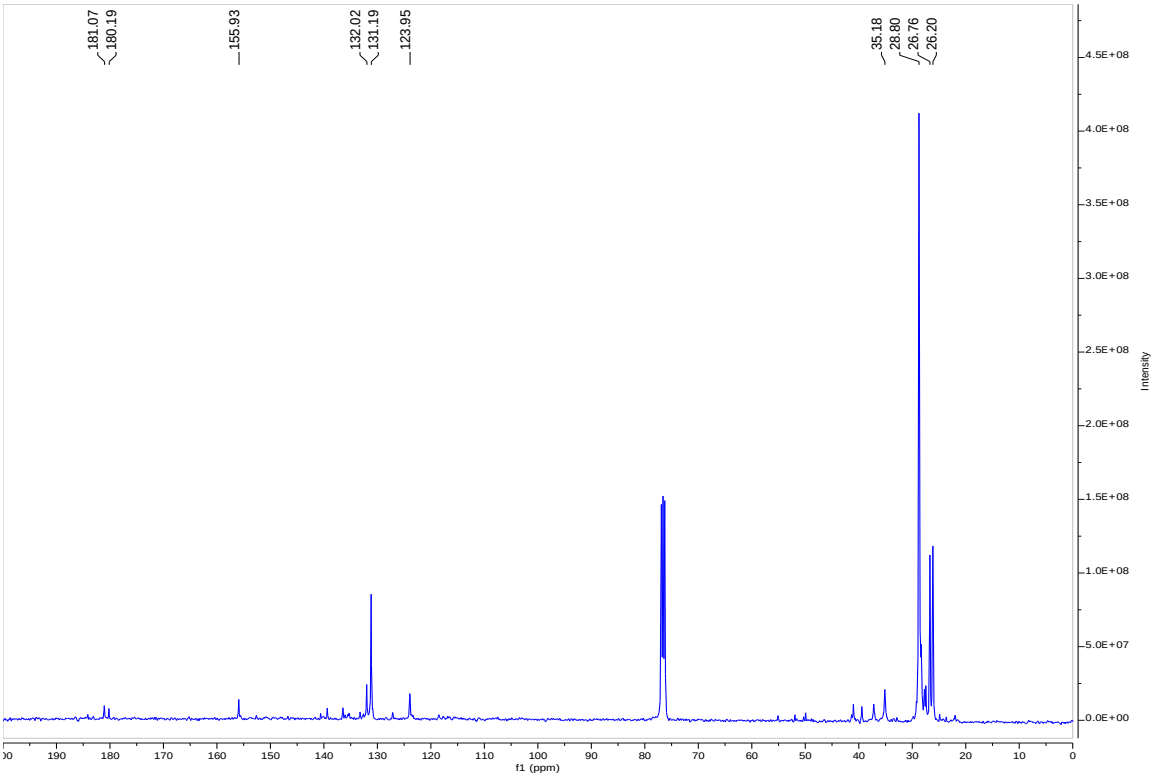


Figure A21. ^{13}C NMR spectrum of compound **10c**.

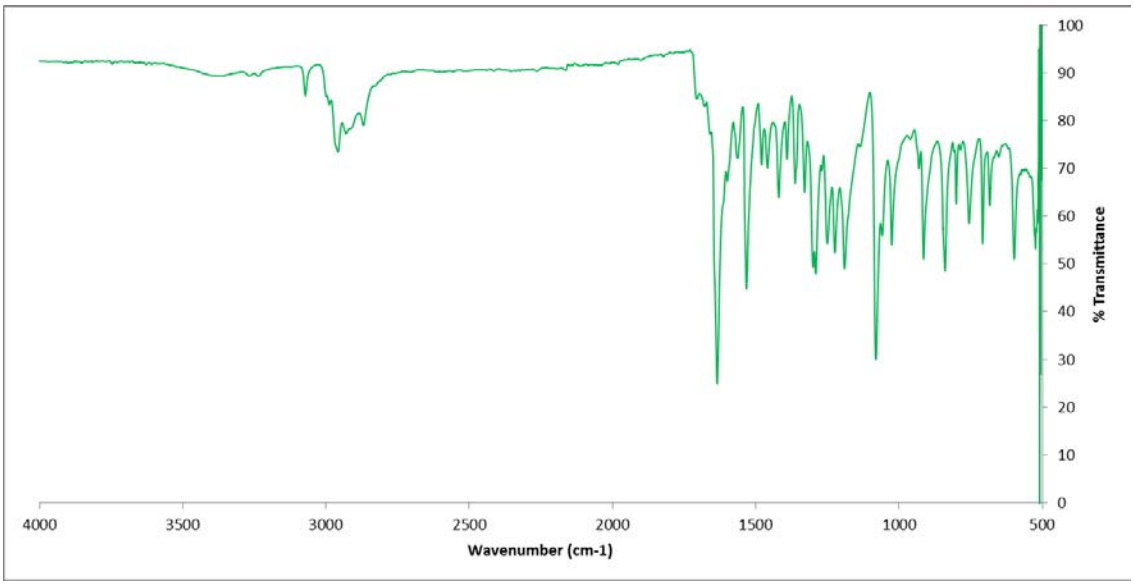


Figure A22. FTIR spectrum of compound **10c**.

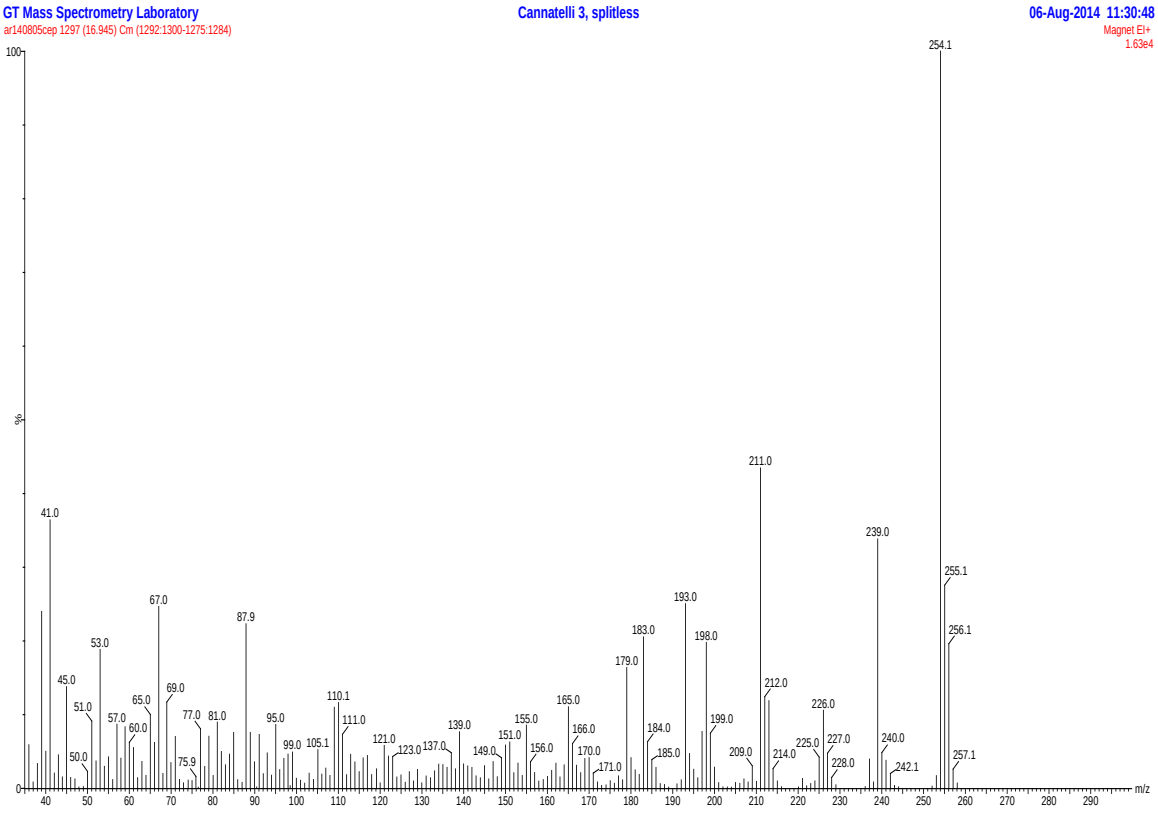


Figure A23. Mass spectrum of compound **10c**.

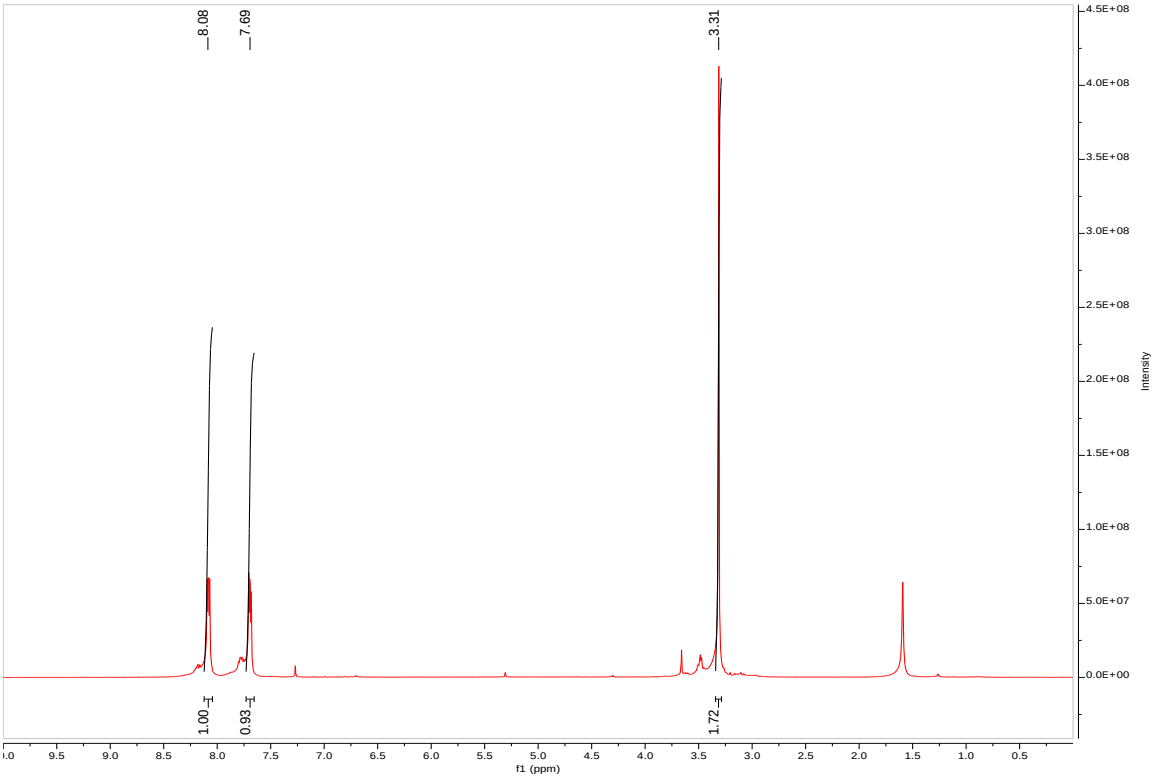


Figure A24. ^{1}H NMR spectrum of compound **10e**.

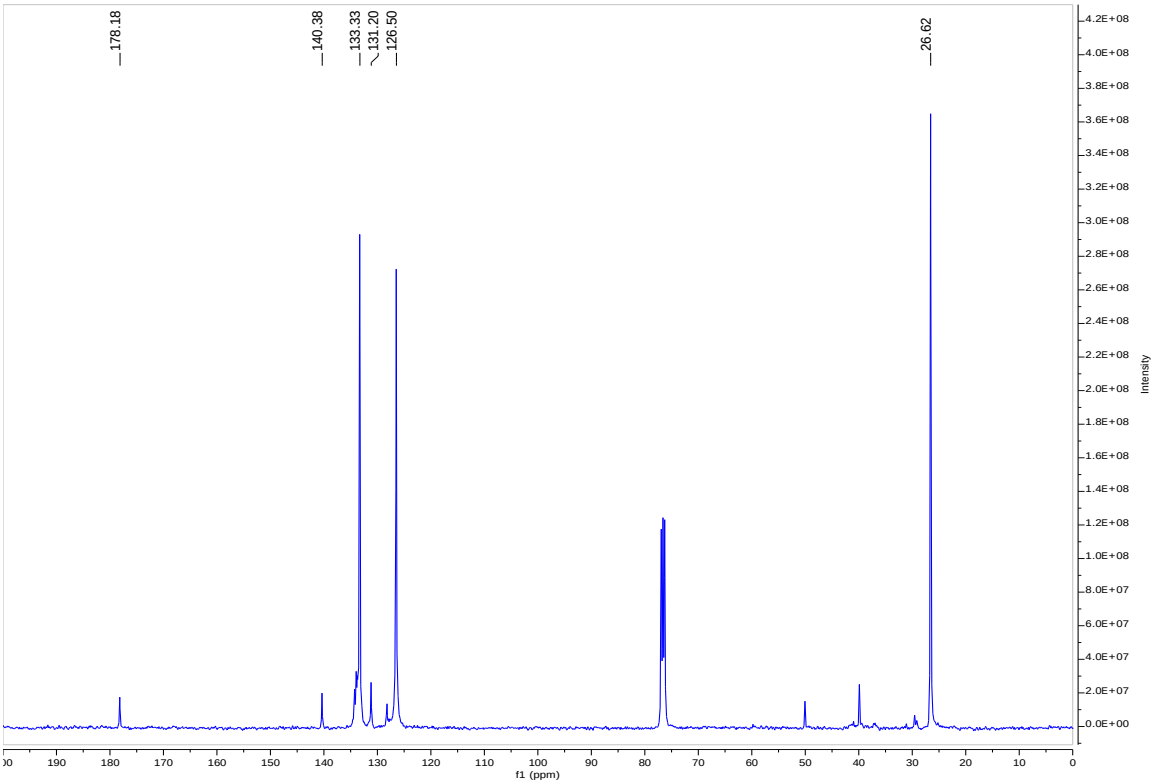


Figure A25. ^{13}C NMR spectrum of compound **10e**.

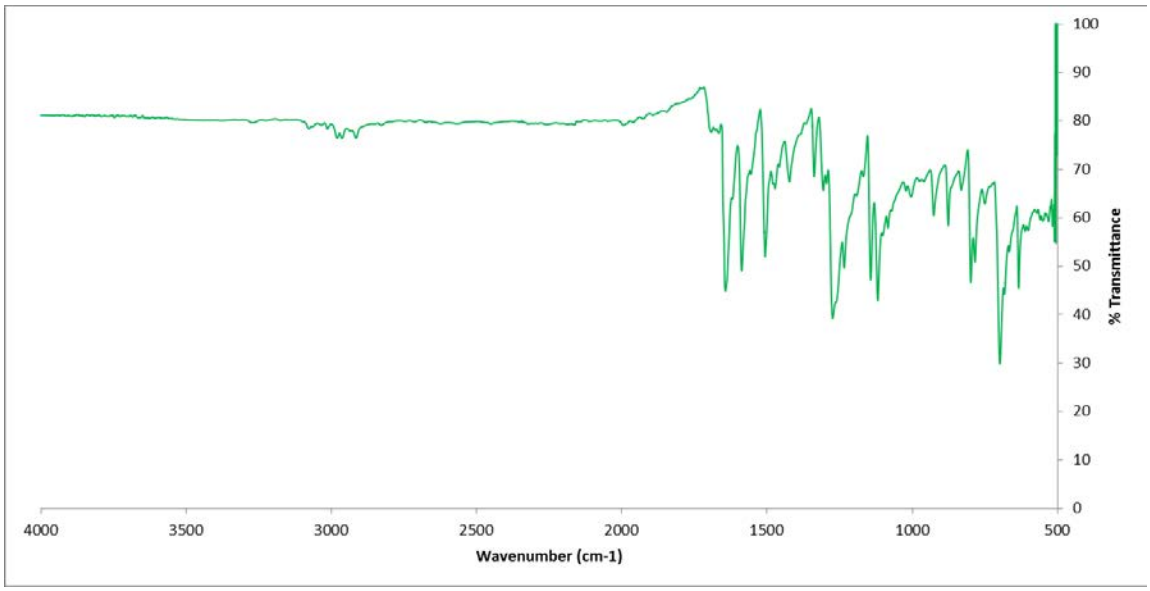


Figure A26. FTIR spectrum of compound **10e**.

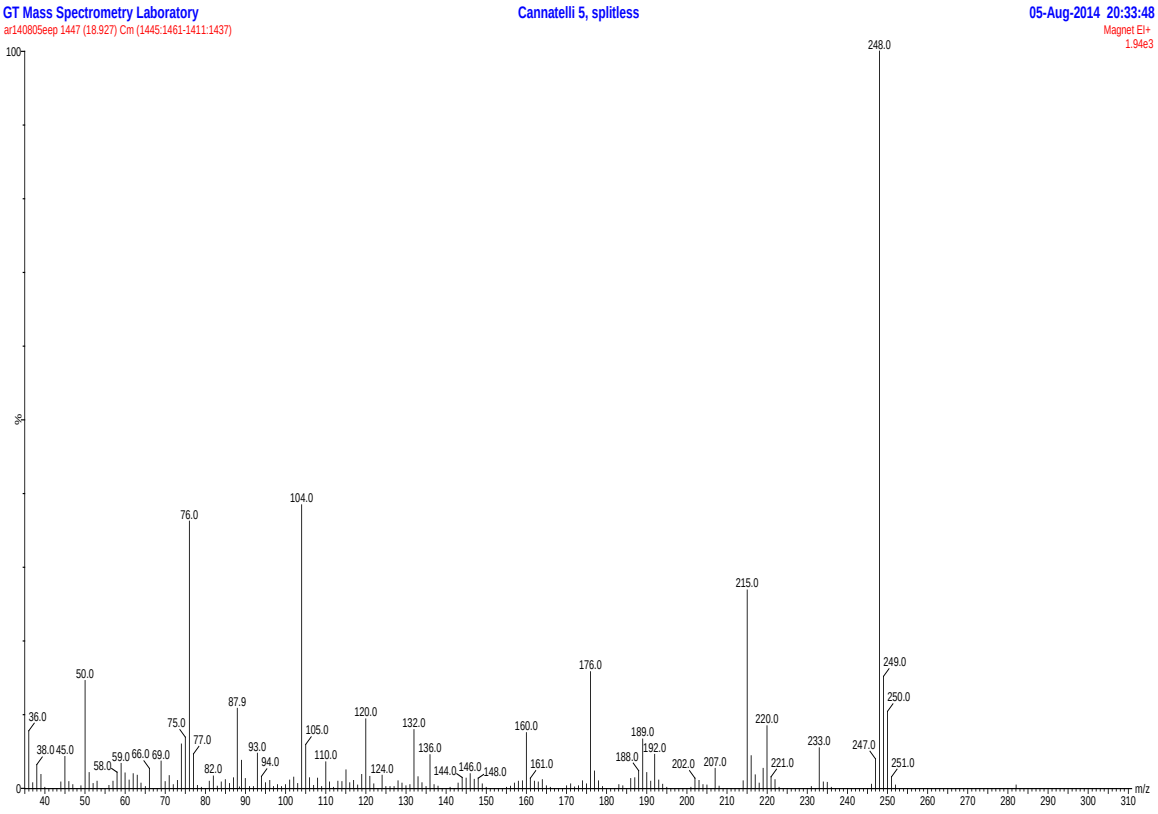


Figure A27. Mass spectrum of compound **10e**.

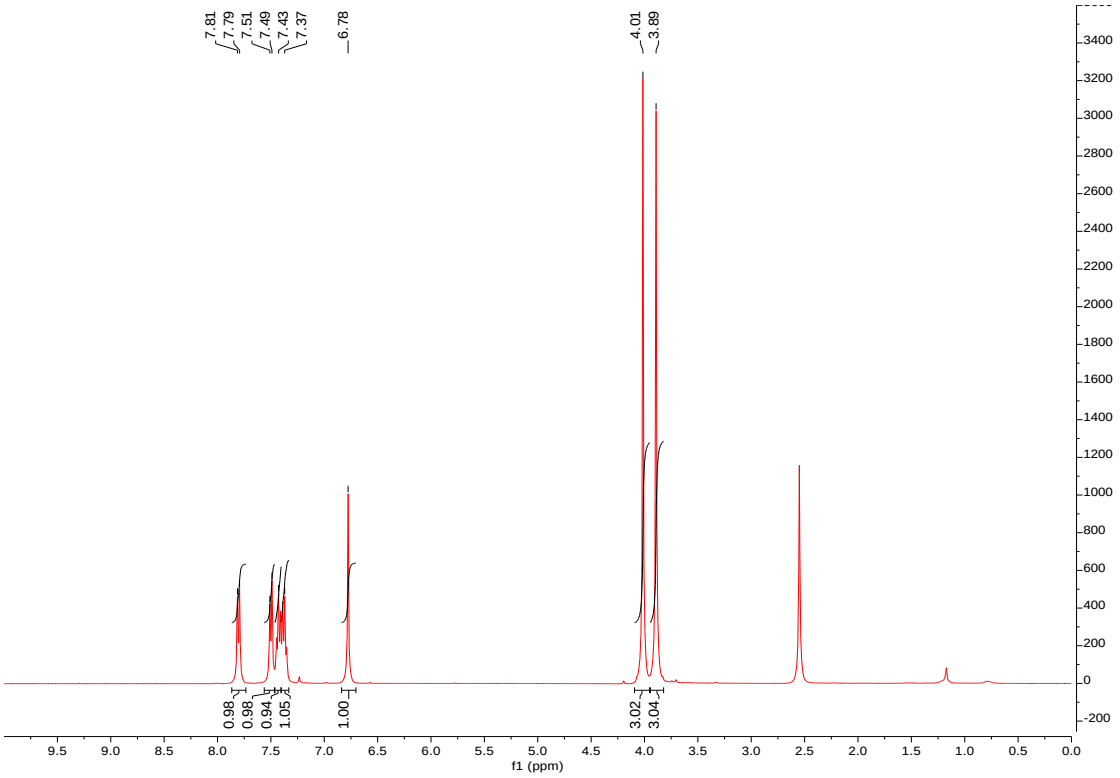


Figure A28. ^{1}H NMR spectrum of compound **15c**.

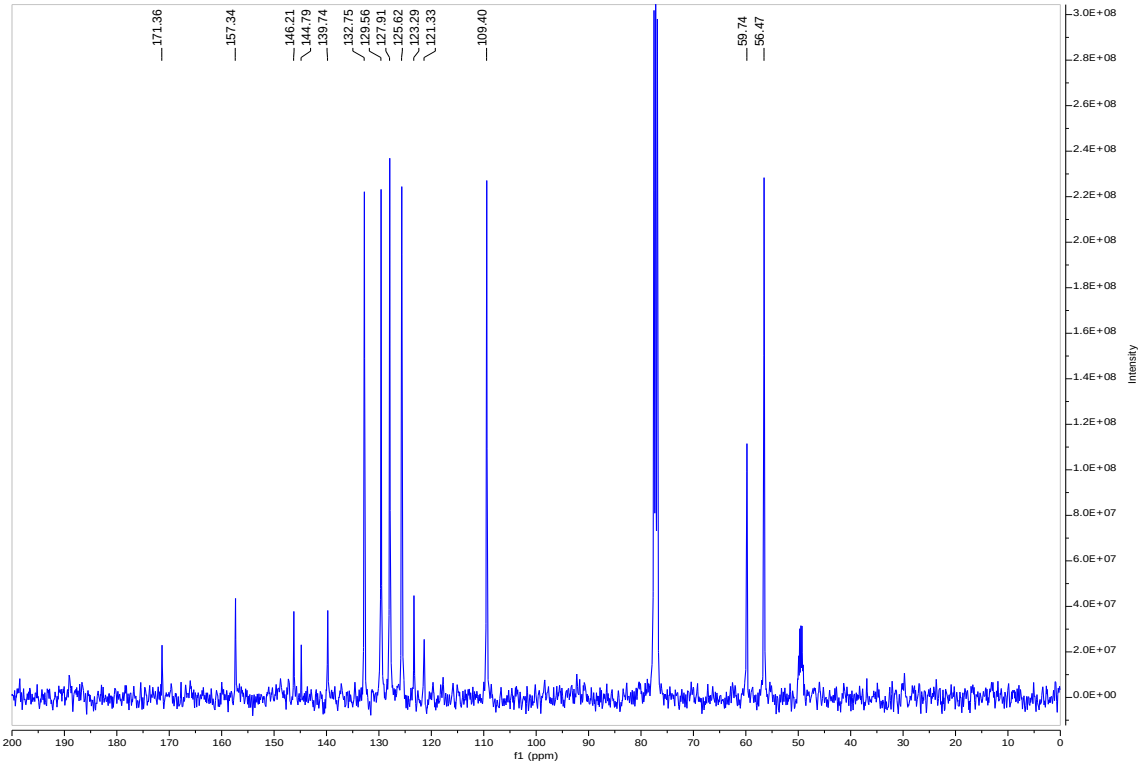


Figure A29. ^{13}C NMR spectrum of compound **15c**.

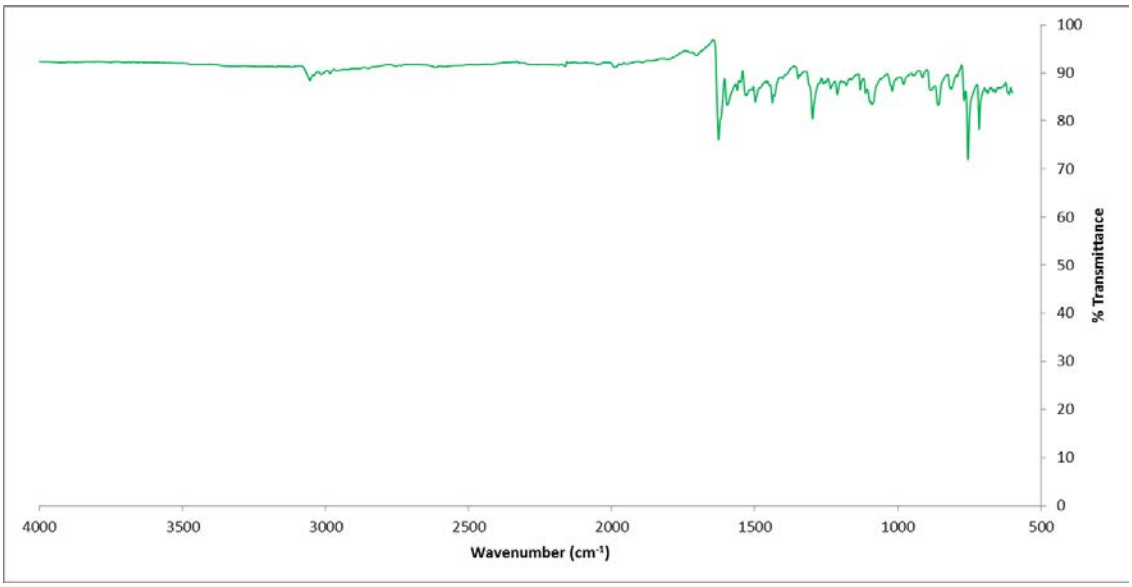


Figure A30. FTIR spectrum of compound **15c**.

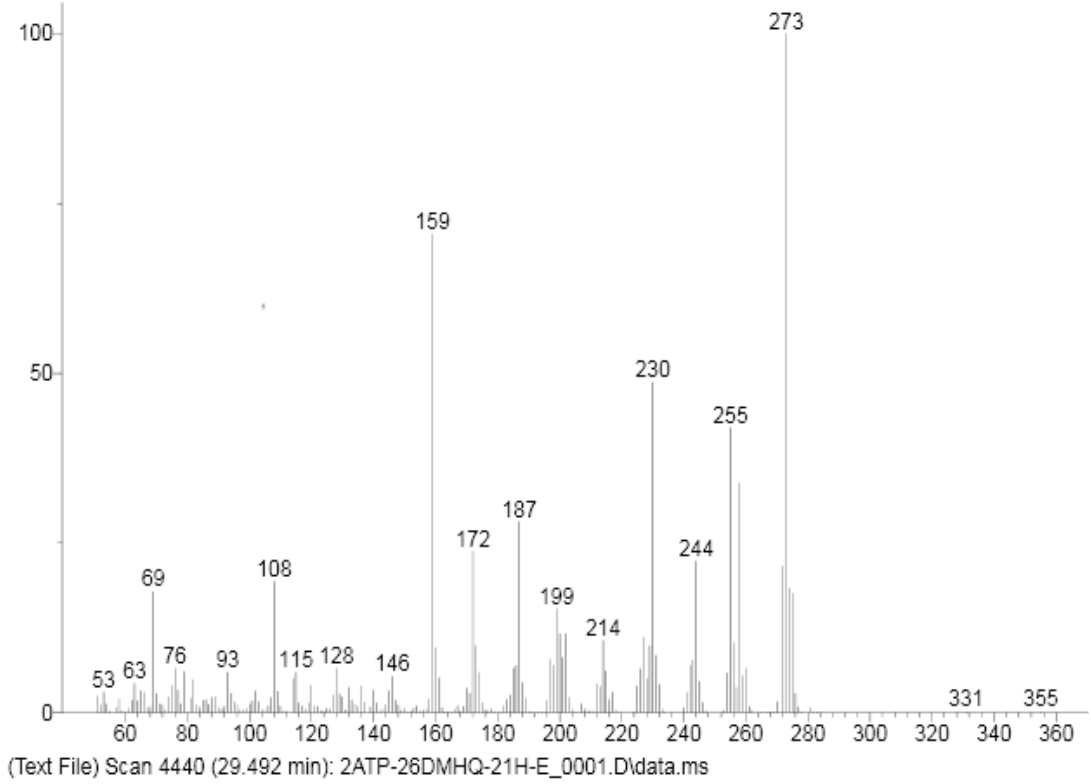


Figure A31. Mass spectrum of compound **15c**.

APPENDIX B

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