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Synthetic Applications of Laccase in Green Chemistry

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Abstract: Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2), multi-copper-containing oxidoreductase enzymes, are able to catalyze the oxidation of various low-molecular weight compounds, specifically, phenols and anilines, while concomitantly reducing molecular oxygen to water. Because of their high stability, selectivity for phenolic substructures, and mild reaction conditions, laccases are attractive for fine chemical synthesis. This review provides a discussion of the recent applications of this interesting enzyme in synthetic chemistry, including laccase and laccase-mediator catalyzed reactions. In addition, the review also includes a brief discussion of the distribution of laccase in nature, enzyme structure, and the catalytic mechanism which are of relevance to their applications as biocatalysts.

Keywords: biocatalysts; enzymes; laccase; oxidation; quinones

1 Introduction

Societal interest in green chemistry and advances in biotechnology have brought to the forefront the application of enzymes to address many of the challenges of modern synthetic organic chemistry. This multi-faceted challenge is being addressed by an ever-increasing suite of environmentally benign enzymes. Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.3), a blue multi-copper oxidase, is able to catalyze the oxidation of various low-molecular weight compounds, including: benzenediols, aminophenols, polyphenols, polyamines, and lignin-related molecules – while concomitantly reducing molecular oxygen to water.[1] Recently, this enzyme has garnered increasing attention as an oxidative catalyst for fine chemical synthesis due to, in part, the high stability, selectivity for phenolic substructures, and mild reaction conditions. As a biocatalyst, laccase has also been shown to have a wide variety of industrial applications, including uses in the food, pulp and paper, textile, cosmetics, and nanobiotechnology industries.[2]

This article reviews the current knowledge on the distribution of laccases in nature, their structure, catalytic mechanism and their applications in organic synthesis. To the best of our knowledge, this review has described the 'state of the art' for the laccase catalyzed reactions as of the end of 2008. The main focus of this review is on the growing number of useful synthetic applications of laccases for oxidative coupling of phenolics, deprotection of *p*-methoxyphenylamine, laccase-initiated oxidative formation of quinones, and polymerization reactions. Since the redox potential of laccase is typically about 0.5–0.8 mV *vs.* the normal

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Table 1. The redox potential (E°) of laccases at pH 5.3.[3b]

Laccase	E° (V *vs.* NHE)
Rhus vernicifera	0.44
Polyporus pinsitus	0.79
Rhizoctonia solani	0.73
Mytheliophthora thermophila	0.48
Scytalidium thermophilum	0.53

hydrogen electrode (NHE) (Table 1),[3] for the reactions where the substrate (i.e., non-phenolic compounds) to be oxidized has a redox potential higher than laccase, the presence of a low-molecular weight chemical mediator is required to facilitate oxidative reactions.[4] The application of laccase-mediator systems to oxidize a wide range of substrates, such as alcohols, sugars, ethers, alkenes, amide, and aromatic methyl groups is also discussed in this review.

2 Distribution in Nature

Laccase was first discovered by Yoshida in 1883 in the sap of the lacquer tree *Rhus vernicifera*[5a] and its biochemistry, structure and mechanism, and application have been studied ever since.[5b–d] The data for laccase from other, higher plant species are more limited and this area needs further investigation. Other plant sources of laccase include: *Rhus succedanea*,[6a] *Acer pseudoplatanus*,[6b] *Pinus taeda*,[6c,d] *Populus euramericana*,[6e] *Liriodendron tulipifera*,[6f] *Nicotiana tobacum*,[6g] *Lolium perenne*,[6h] and *Zea mays*.[6i] In plants, laccases are believed to participate in the formation of polymer lignin *via* a radical-based mechanism.[6b,7]

A few years after the discovery of plant laccase by Yoshida, fungal laccases were discovered by Bertrand in 1896.[8] The majority of laccases characterized to date have been isolated from fungi and new sources continue to be reported.[9] Until now, more than 100 laccases have been purified from fungi, and laccase from the wood-rotting white-rot Basidiomycetes are some of the most purified. The wood-rotting fungi that produce laccase are *Trametes versicolor*, *T. hirsute* (*C. hirsutus*), *T. ochracea*, *T. villosa*, *T. gallica*, *Cerrena maxima*, *Coriolopsis polyzona*, *Lentinus tigrinus*, *Pleurotus eryngii*, etc. Laccases have several roles in fungi including lignin degradation, morphogenesis, fungal plant-pathogen/host interaction, and stress defence.[1c,9]

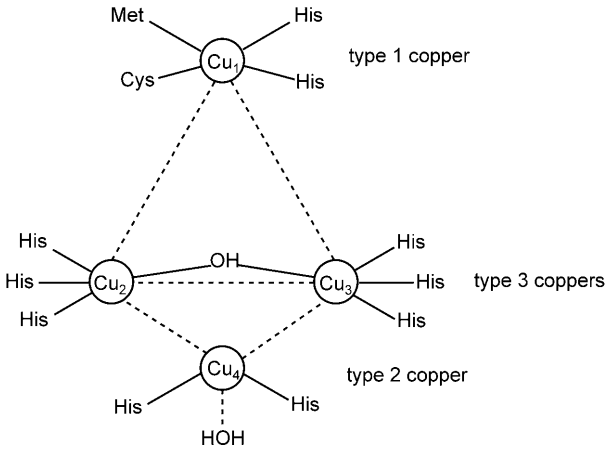
There are also some reports about laccase activity in bacteria.[10] Proteins with features typical of laccases have recently been identified in insects.[11]

3 Laccase Structure

Laccases are glycoproteins which often occur as isoenzymes that oligomerize to form multimeric complexes. The molecular weight of the monomer ranges from about 50 to 130 kD. The carbohydrate moiety of laccases typically consists of mannose, *N*-acetylglucosamine, and galactose and ranges from 10 to 45% of the protein mass. The carbohydrate moiety of laccase is believed to contribute to the stability of the enzyme.[1a,f]

The active site of laccase contains four copper atoms which are one type 1 (T_1) copper and a trinuclear cluster (T_2/T_3) consisting of one type 2 (T_2) and two type 3 (T_3) coppers. The T_1 copper atom is located at the distance of approximately 12 Å from the T_2/T_3 site, and T_2 copper atom is located at the distance of about 4 Å from T_3 copper atoms.[12] The T_1 copper has a trigonal coordination with two histidines and one cysteine, and the axial ligand of T_1 is methionine in the bacterial (CotA)[12b] and leucine or phenylalanine in fungal laccases. The T_1 copper confers the typical blue color to multicopper proteins due to the strong absorption around 600 nm. This intense absorption is caused by the covalent copper-cysteine bond. Moreover, type 1 copper is the site where substrate oxidation takes place because of its high redox potential of *ca.* +790 mV. The high redox potential is a contributing factor, but of equal importance is the accessibility of the site to the surface as well as the limited substrate access to the trinuclear cluster. Type 2 copper is coordinated by two histidines and type 3 coppers are coordinated by six histidines (each T_3 copper is coordinated to three histidine ligands). Type 2 copper shows only weak absorption in the visible region and reveals paramagnetic properties in electron paramagnetic resonance (EPR) studies. While the type 3 copper is a binuclear copper site with copper paired antiferromagnetically through a hydroxy bridge and it does not exhibit an EPR signal. The T_3 site can be characterized by an electron absorption at 330 nm (oxidized form).[6a,13] In addition, the trinuclear cluster (T_2/T_3 site) is where the reduction of molecular oxygen and release of water takes place. Scheme 1 illustrates the proposed active site of laccase CotA from *Bacillus subtilis.*

The three-dimensional structure of five fungal laccase[14] has been determined from *Coprinus cinereus* (with the T_2 copper removed),[14b] *Trametes versicolor*,[14c,d] *Pycnoporus cinnabarinus*,[14e] *Melanocarpus albomyces*[14f] and *Rigidoporus lignosus.*[12c] Moreover, the three-dimensional structure of laccase CotA from endospores of *Bacillus subtilis* has also recently been published.[12b,14g]

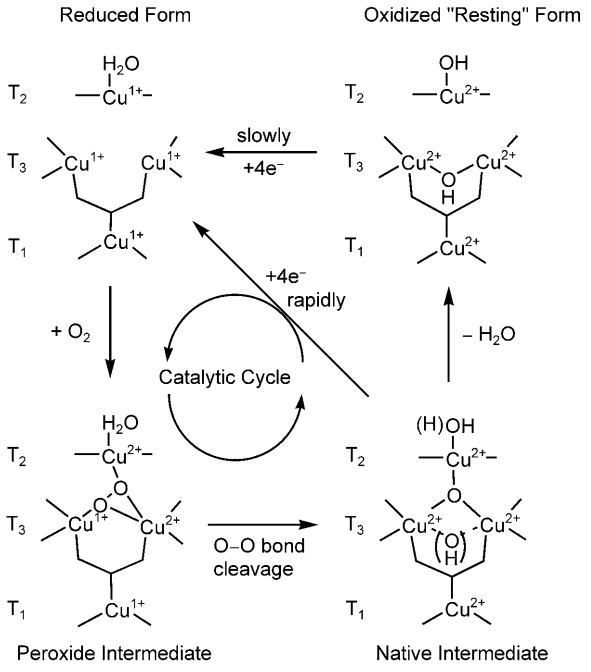


Scheme 1. Active site of laccase CotA from *Bacillus subtilis* (adapted from Enguita et al.[12b]).

4 Catalytic Mechanism and Properties

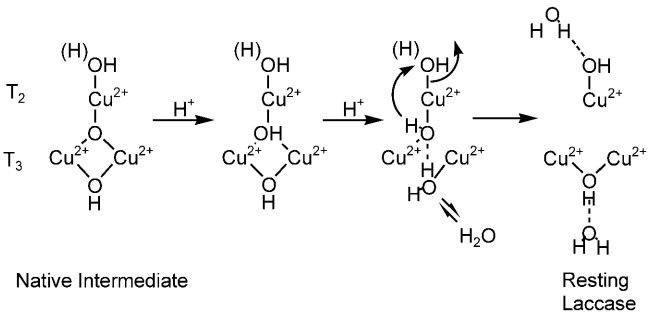
The catalytic properties of laccase have been attributed to the following three major steps.[6a,15] (i) Type 1 copper is reduced by accepting electrons from the reducing substrate. (ii) Electrons are transferred ~13 Å from type 1 copper to the trinuclear T_2/T_3 cluster. (iii) Molecular oxygen is activated and reduced to water at the trinuclear T_2/T_3 cluster.

Scheme 2 shows the catalytic mechanism of laccase involving a four-electron reduction of the dioxygen molecule to water at the enzyme copper sites.[16] The

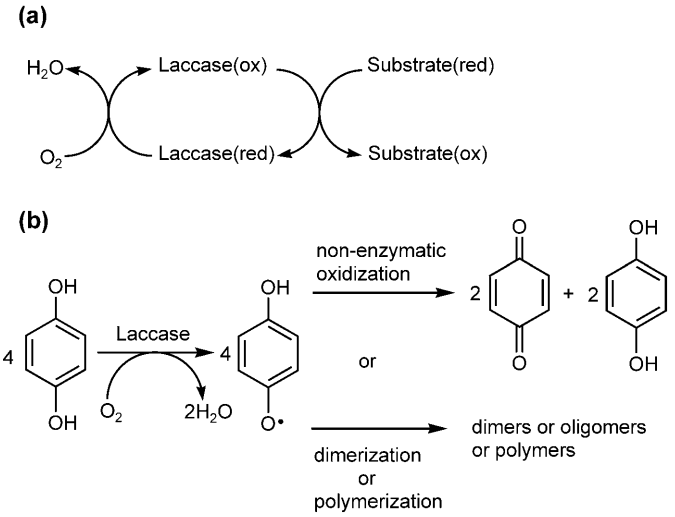


Scheme 2. Catalytic cycle of laccase showing the mechanism of four-electron reduction of a dioxygen molecule to water at the enzyme copper sites (adapted form Solomon et al.[16b])

dioxygen molecule interacts with the completely reduced trinuclear cluster (T_2/T_3) *via* a $2e^-$ process ($k \approx 2 \times 10^6\,M^{-1}s^{-1}$) to produce the peroxide intermediate which contains the dioxygen anion.[17] One oxygen atom of the dioxygen anion is bound with the T_2 and T_3 copper ions and the other oxygen atom is coordinated with another copper ion of T_3. Then, the peroxide intermediate undergoes a second $2e^-$ process ($k > 305\,s^{-1}$),[12a] and the peroxide O–O bond is ruptured to produce a native intermediate which is the fully oxidized form with the three copper centers in the trinuclear site mutually bridged by the product of full O_2 reduction with at least one Cu–Cu distance of 3.3 Å. This native, intermediate form of lacccase was confirmed by the combination of Cu K-edge X-ray spectroscopy (XAS) and magnetic circular dichroism (MCD) studied by Solomon et al.[18] Moreover, a combination of model studies and calculations has further demonstrated that the three copper centers in the trinuclear cluster are all bridged by a μ_3-oxo ligand.[19] This structure has a single μ_3-oxo ligand bridging all three coppers at the center of the cluster, with the second oxygen atom from O_2 either remaining bound or dissociated from the trinuclear site, as shown in the native intermediate structure in Scheme 2. This μ_3-oxo-bridged structure of the native intermediate provides a relatively stable structure that serves as the thermodynamic driving force for the $4e^-$ process of O_2 reduction, and also, provides efficient electron transfer (ET) pathways from the T_1 site to the other copper centers in the trinuclear cluster.[19] This efficient ET pathway leads to the fast reduction of the fully oxidized trinuclear cluster in the native intermediate to generate the fully reduced site in the reduced form for further turnover with O_2. The native intermediate can slowly convert to a completely oxidized form called "resting" laccase, which has the T_2 copper isolated from the coupled binuclear T_3 centers. The native intermediate only rearranges to the resting form in the absence of reductant and the decay of the native intermediate to the resting enzyme proceeds *via* successive proton-assisted steps is illustrated in Scheme 3.[20] The first proton binds at



Scheme 3. Proposed decay mechanism of the native intermediate to the resting laccase.[20]



Scheme 4. (a) Scheme of laccase-catalyzed redox cycles for substrate oxidation; **(b)** example of the oxidation of hydroquinone by laccase.

the μ_3-oxo center and then the second proton binds at the T_3 OH^- bridge. Finally, the three copper centers in the trinuclear cluster are uncoupled to form the resting form of laccase. The slow decay of the native intermediate is due to the rearrangement of the μ_3-oxo-bridge, the rate-limiting step, from the inside to the outside of the cluster. The T_1 site of this resting laccase can be reduced by a substrate. However, the electron-transfer rate onto the trinuclear cluster (T_2/T_3) is too low to be significant for catalysis.[6a,18]

Laccase can catalyze the oxidation of a variety of compounds, including *ortho*- and *para*-benzenediols, polyphenols, aminophenols, polyamines, lignin, aryldiamines, and a number of inorganic ions.[1] To accomplish this, laccase abstracts an electron from a substrate to produce a free radical, and reduces oxygen to water. The simplified scheme of laccase-catalyzed oxidation is illustrated in Scheme 4 with hydroquinone.

Fungal laccases typically exhibit pH optima in the range from 3.5 to 5.0 when the substrates are hydrogen atom donor compounds, and the pH-dependence curve is bell-shaped.[21] The optimum pH for phenolic compounds can actually increase at higher pH values but this is complicated by the stability of the enzyme. This pH effect results from the balance between the redox potential difference between the substrate and the inhibition of the T_2/T_3 copper site by the binding of OH^- ion.[3,22] The pH optimum for plant laccases with substrates that are donors of hydrogen atoms is different from that of fungal laccases. For example, laccase from *Rhus vernicifera* exhibited a maximal activity in neutral and weak alkaline solution.[3]

The optimal temperature for laccase-catalyzed oxidations typically ranges from 50 to 70 °C.[9b] However,

there are a few fungal laccases with an optima oxidative profile below 35 °C such as the laccase from *G. lucidum* with its highest activity observed at 25 °C.[23]

A wide spectrum of compounds has been described to inhibit laccase. These inhibitors include small inorganic anions such as azide, cyanide, fluoride and hydroxide. These ions bind with the T_2/T_3 site and this prevents the electron transfer from the T_1 site onto the T_2/T_3 site and inhibits the enzymatic activity.[3,24] Other inhibitors such as metal ions (Hg^+), fatty acids, quaternary ammonium detergents have been shown to either replace or chelate the copper centers, or denature the protein.[1e]

5 Laccase in Organic Synthesis

Due to the catalytic and electrocatalytic properties of laccase and their commercial availability, laccases have received attention over the past two decades in several industrial and biotechnological processes, including uses in the food, pulp and paper, textile, cosmetics, and nanobiotechnology industries.[1f,2b] Moreover, the catalytic oxidative potential of laccases provides intriguing opportunities in organic synthesis because of their ability to oxidize a variety of compounds.[1b] The following sections are arranged by the synthetic approaches exploiting laccases. In most cases, water was used as a part of the reaction media. Therefore, in each synthetic approach, there are examples related to the use of these enzymes in buffered solutions, in biphasic systems and even in water-saturated organic solvents.

The following section examines these growth opportunities and applications of laccase-catalyzed reaction in the absence of mediators.

5.1 Laccase-Catalyzed Transformation of Phenolic and Related Compounds

Laccase has been reported to oxidize a variety of phenolic compounds.[3,25] For example, Trejo-Hernandez and his co-workers studied the use of laccase in the crude extract of the residual compost of *Agaricus bisporus* to oxidize phenolic compounds, including guaiacol, 2,6-dimethoxyphenol, ventril alcohol, and phenol.[25a] All tested substrates formed insoluble products after being oxidized, except for ventril alcohol that was transformed to a soluble aldehyde. The relative activity of the compost extract was 2,6-dimethoxyphenol > guaiacol > phenol > ventril alcohol > aniline. This relative activity was measured in terms of the time required to oxidize the substrate. Recently, the product of the oxidation of 2,6-dimethoxyphenol by *Rhus* laccase was determined for the first time by Wan et al.[25d] The reaction was conducted in a water-organic solvent system. They found that only one product, 3,3′,5,5′- tetramethoxy,1,1′-biphenyl-4,4′-diol, was produced.

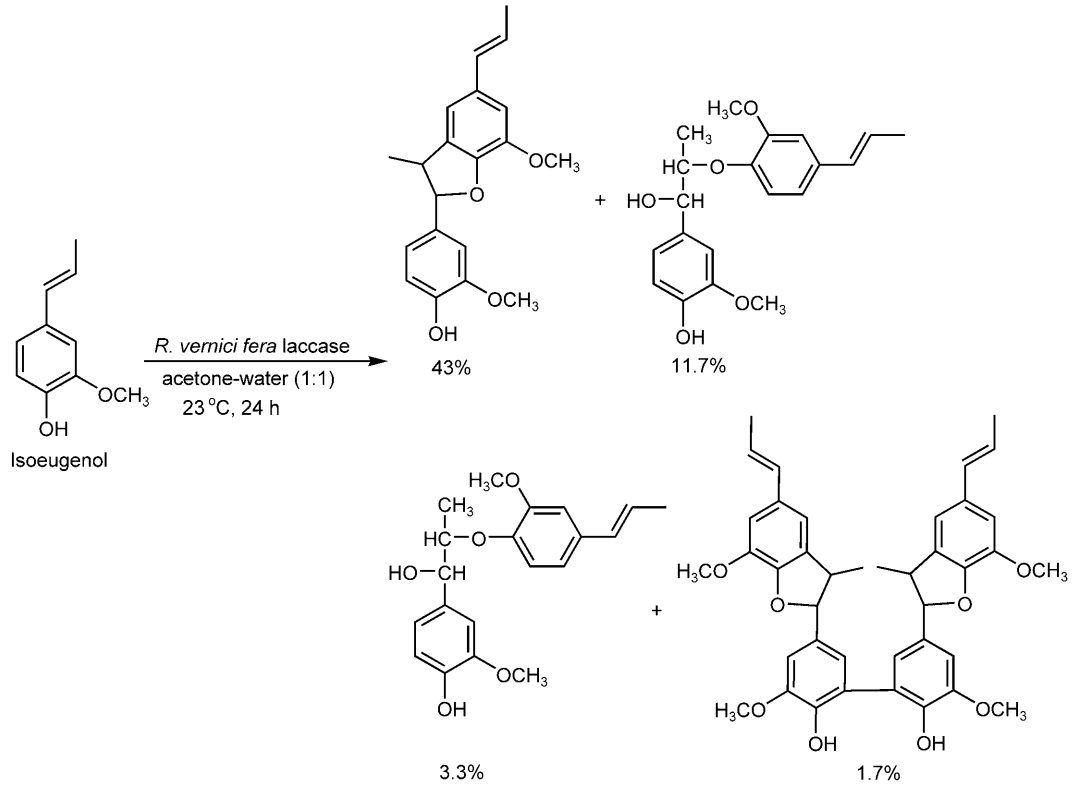
Monolignols including isoeugenol, coniferyl alcohol, and ferulic acid have also been investigated for their laccase-catalyzed oxidation reactions. Chen and co-workers studied the oxidation of isoeugenol and coniferyl alcohol by laccase from *Rhus vernicifera* (tree) and *Pycnoporus coccineus* (fungus) in acetone-water (1:1, v/v).[26] The rate of *Pycnoporus* laccase-catalyazed oxidation of isoeugenol and coniferyl alcohol is approximately 3- to 7-times faster than the rate of *Rhus* laccase-catalyzed oxidation. The rate of the oxidation depends on the nature of both the monolignol and the laccase (see Scheme 5). These studies provided information for the role of laccase in the synthesis of lignin.

Nishida and Fukuzumi examined the transformation of ferulic acid by white rot fungus, *Trametes versicolor*, in a medium containing glucose and ethanol, under aerobic conditions, as a model for the oxidative decomposition of lignin by laccase.[27] The ferulic acid was transformed into coniferyl alcohol, coniferylaldehyde, dihydroconiferyl alcohol, vanillic acid, vanillyl alcohol, 2-methoxyhydroquinone and 2-methoxyquinone. Falconnier et al. also reported the biotransformation of ferulic acid to vanillin by the white rot fungus *Pycnoporus cinnabarinus* I-937 (Scheme 6).[28]

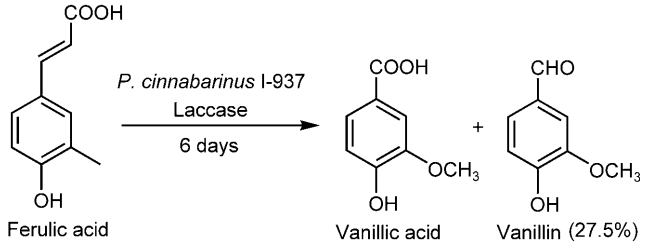
The oxidation of ferulic acid by laccase was recently used to synthesize phenolic colorants.[29] The oxidation was conducted in a biphasic system consisting of ethyl acetate and sodium phosphate buffer to generate the intermediate stable yellow products. This biphasic system facilitates the separation of the yellow product which was soluble only in the organic phase. This prevented the further polymerization of this intermediate. They suggested that this yellow-colored compound may have applications as a food colorant. The structural aspects of this yellow-colored compound remain under investigation.

Tranchimand et al. reported the synthesis of a bislactone lignan *via* laccase-initiated oxidative coupling of sinapinic acid and ferulic acid in a biphasic system (Scheme 7).[30]

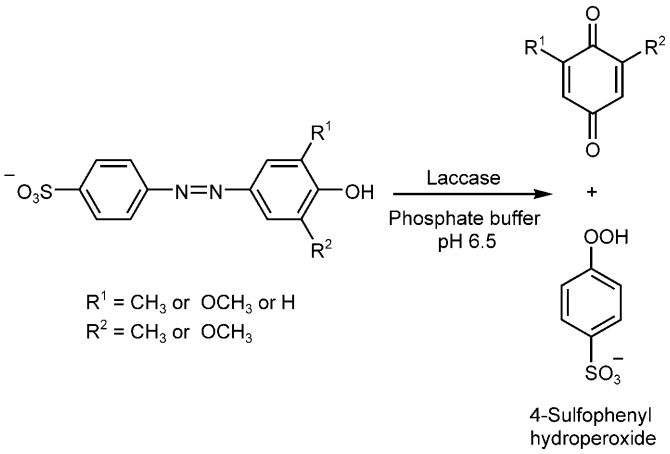
Azo dyes, the largest group of colorants used in industry, are oxidized by laccase.[31] Renganathan and Chivukula examined the oxidation of phenolic azo dyes catalyzed by laccase from *Pyricularia oryzae*.[31a] Laccase oxidized the azo dyes to 4-sulfonylhydroperoxide, a quinone compound, and other products (Scheme 8). The proposed mechanism involved laccase oxidation of the substrate to generate the corresponding phenoxy radicals which are further oxidized by laccase to produce a quinone and 4-sulfonylhydroperoxide. This study suggests that laccase oxidation can be employed for the detoxification of azo dyes. Most recently, Rehorek et al. reported a simultaneous



Scheme 5. Dimer and tetramer products from the oxidation of isoeugenol alcohol by laccase.

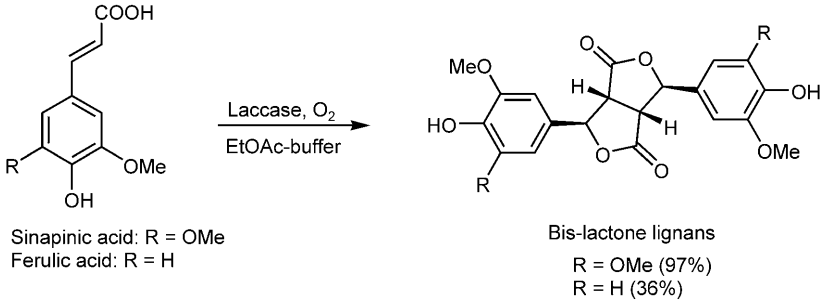


Scheme 6. Biotransformation of ferulic acid by laccase.

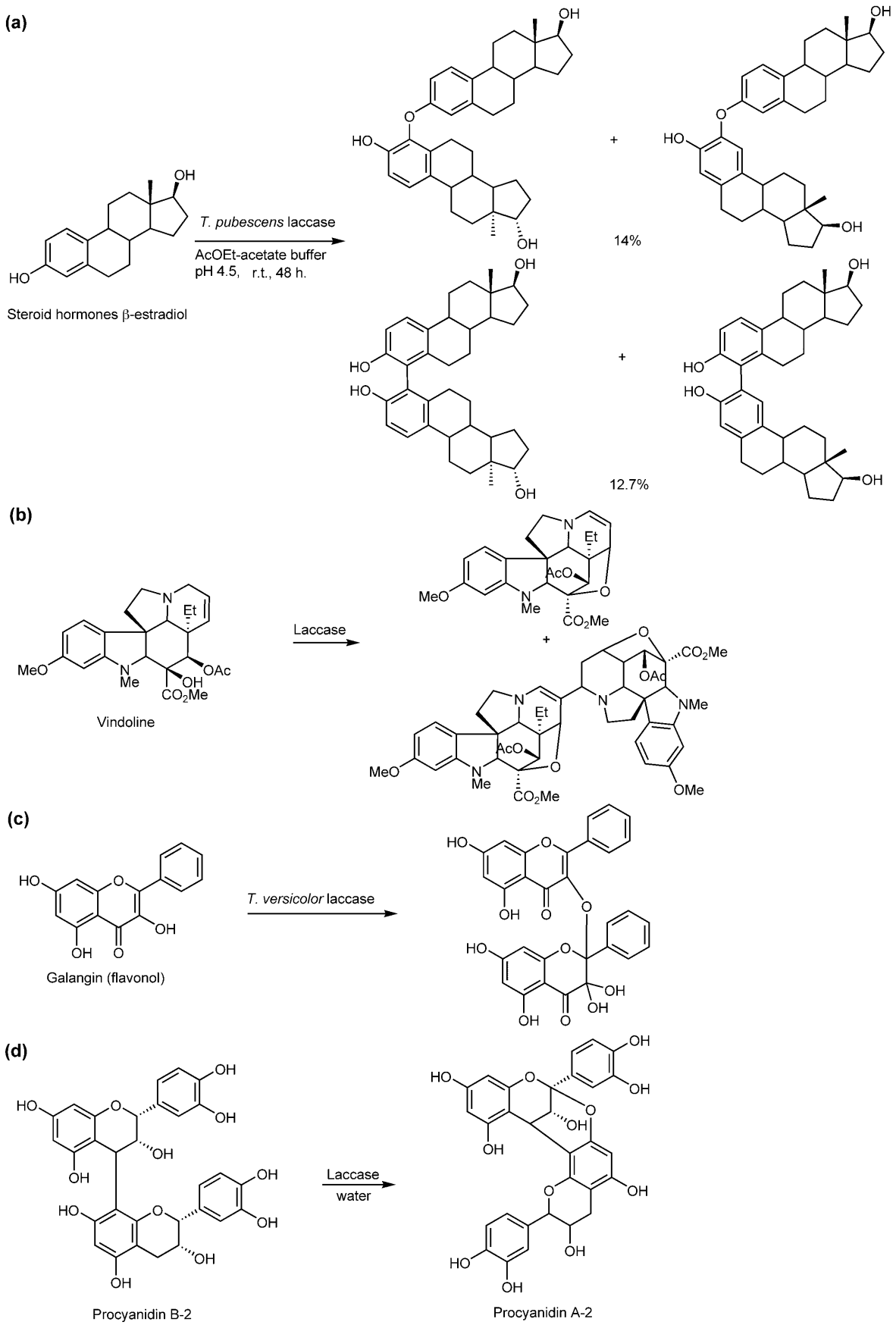


Scheme 8. The oxidation of phenolic azo dyes by laccase.

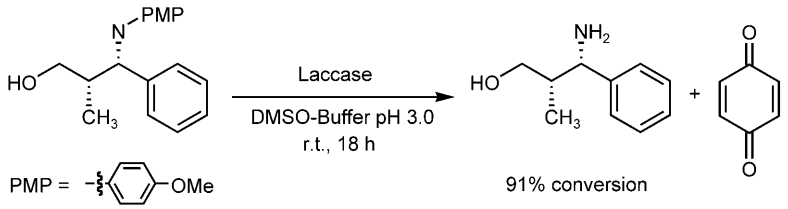
combination of laccase and ultrasound treatment in acetate buffer (pH 4.5) at 40 °C for the degradation of azo dyes, such as acid oranges and direct blue



Scheme 7. The synthesis of bis-lactone lignans.



Scheme 9. Laccase-initiated oxidative coupling of **(a)** steroid hormones, e.g., β-estradiol, **(b)** vindoline, **(c)** galangin, **(d)** procyanidin B-2.



Scheme 10. The oxidative deprotection of *p*-methoxyphenyl (PMP)-protected amines by laccase.

dyes.[31b] The degradation process was monitored by UV-Vis spectrometry and HPLC analysis. Compared to laccase or ultrasound treatment, the simultaneous treatment with laccase and ultrasound showed at least the same or higher degradation rates of the azo dyes. Besides the degradation of azo dyes, laccase was also reported to catalyze the formation of azo dyes by oxidative coupling between *o*-, *m*-, and *p*-methoxyphenols and 3-methyl-2-benzothiazolinene hydrazone.[32]

The transformation of other compounds such as steroid hormones,[33] alkaloids,[34] flavonols,[35] procyanidin B-2,[36] and *N*-(2-alkylamino-4-phenylimidazol-1-yl)-acetamides[37] have also been reported. Examples of these studies are summarized in Scheme 9.

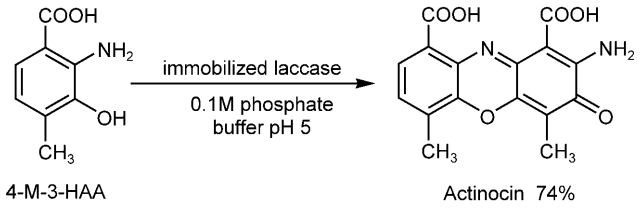
5.2 Laccase-Catalyzed Oxidative Deprotection Reactions

The use of laccase as a selective oxidative deprotection reagent for peptide synthesis has been reported. Semenov et al.[38] have shown that laccase can be used to remove phenylhydrazide protecting groups of both α- and γ-carboxyl groups. The deblocking method was performed under mild conditions in aqueous medium and at pH 7.0, in the presence of oxygen. This deprotection method leads to the modification without alteration of the amino acid side chains. Recently, Rutjes and his co-workers reported the oxidative deprotection of *p*-methoxyphenyl (PMP)-protected amines by laccase under mildly acidic conditions (Scheme 10).[39] In addition, they found that the use of mediators led to an extension of the substrate scope and increased reaction rate.

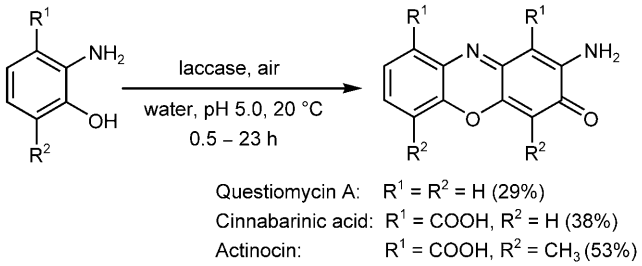
5.3 Laccase-Catalyzed Oxidative Coupling for the Synthesis of the Pharmaceutically Important Compounds

The aromatic coupling of phenolic compounds by laccase-initiated oxidation has been frequently employed as a successful route to important pharmaceutical compounds. For example, the phenoxazinone chromophores having reported antibiotic activity have been successfully been synthesized *via* laccase-catalyzed oxidative coupling reactions.[40] The synthesis of these phenoxazinone chromophores involves the formation of aminophenoxy radicals by oxidation of *o*-aminophenols by laccase at the first step. These radicals then undergo coupling and cyclocondensation reactions to form the corresponding products. However, the detailed reaction mechanism of this synthesis is still under investigation.

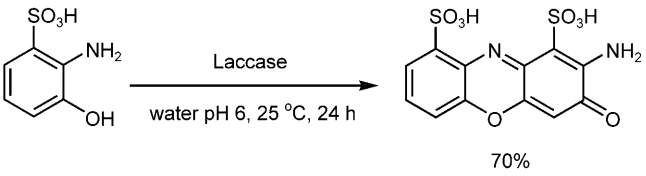
Actinocin, a key component of the actinomycin antibiotics, was synthesized by laccase-mediated oxidation of 4-methyl-3-hydroxyanthranilic acid (4-M-3-HAA) (Scheme 11).[40a] The laccase used in this study was immobilized in polyacrylamide gel. The reaction proceeded successfully in aqueous medium and in 60% acetonitrile. The use of immobilized laccase is a growing field of interest because it makes continuous



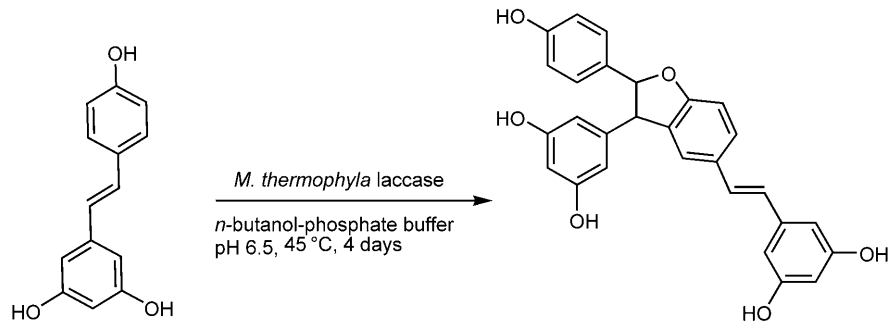
Scheme 11. The synthesis of actinocin by laccase-mediated oxidation of 4-methyl-3-hydroxyanthranilic acid.



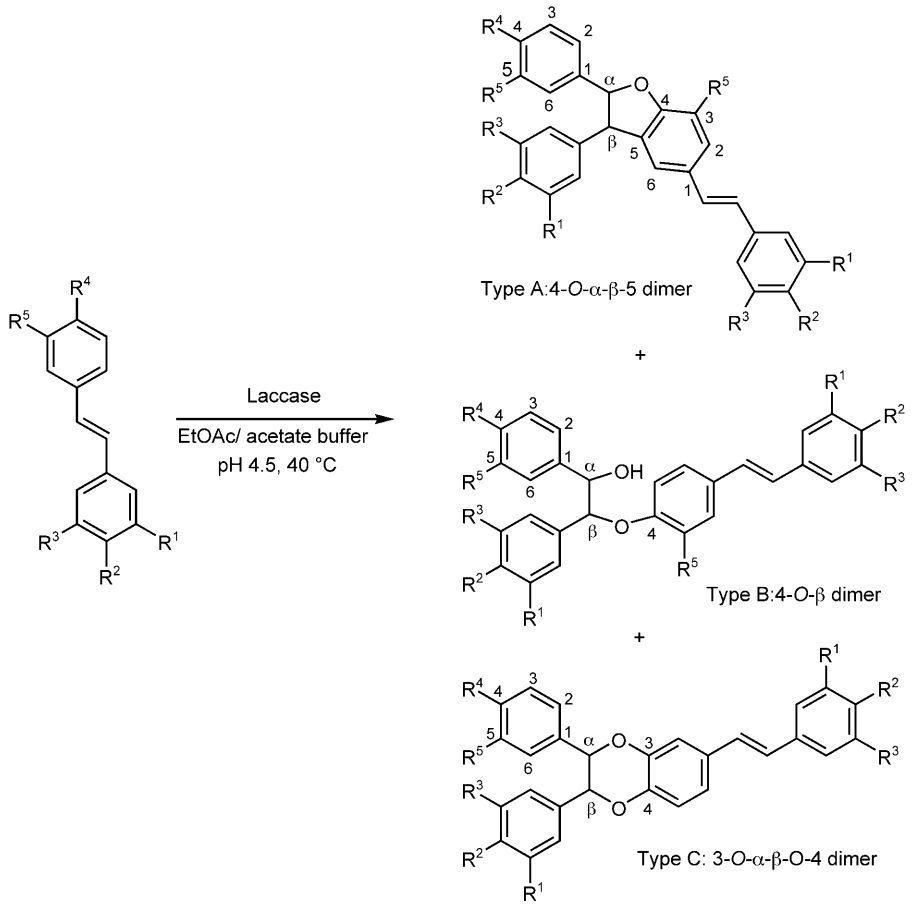
Scheme 12. The synthesis of 2-amino-3*H*-phenoxazin-3-ones.



Scheme 13. The synthesis of the sulfonate analogue of cinnabarinic acid by laccase.



Scheme 14. The transformation of *trans*-resveratrol (3,5,4′-trihydroxystilbene) by laccase.



Products

R^1	R^2	R^3	R^4	R^5	Type A	Type B	Type C
OMe	H	OMe	OH	H	59%	6%	–
H	OMe	H	OH	OH	14%	–	14%

Scheme 15. The oxidation of hydroxystilbenes by laccase.

bioreactor operations possible. This is especially useful in the production of fine chemicals and the biotreatment of industrial and agricultural wastes.[41]

Recently, Giurg et al. reported the synthesis of 2-amino-3*H*-phenoxazin-3-ones including actinocin, cinnabarinic acid, and questiomycin A by the catalytic

oxidative cycloaddition of *o*-aminophenols.[40b] These reactions were conducted in the presence of laccase and oxygen in an aqueous medium (Scheme 12). In addition, the sulfonate analogue of cinnabarinic acid was recently synthesized by the laccase-mediated oxidative dimerization of 3-hydroxyorthanilic acid (Scheme 13).[40c]

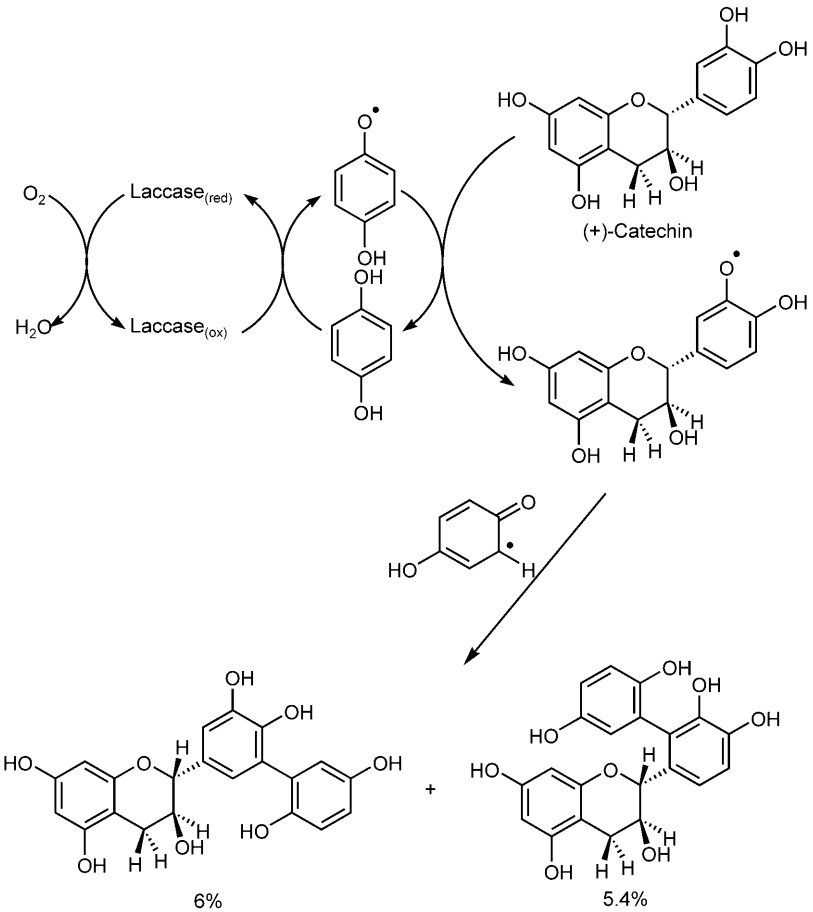
Forti and his co-workers reported the transformation of *trans*-resveratrol (3,5,4′-trihydroxystilbene) by laccase from *Myceliophtora thermophyla* and from *Trametes pubescens* to generate the dehydro dimer product that has been reported to have antioxidant properties (Scheme 14).[42] These authors recently reported the oxidation of a series of hydroxystilbenes, analogues of the phytoalexin resveratrol, by laccase from *Trametes pubescens* in an ethyl acetate/acetate buffer system.[43] In this study, three different dimeric products were identified with the main product usually being the 4-*O*-α-β-5 dimers. These products were proposed to be generated *via* radical-radical coupling dimerization reactions (Scheme 15).

Other biological active compounds have already been prepared.[44] Antioxidant gelatin-catechin conjugates have been synthesized by the laccase-catalyzed oxidation of catechin in the presence of gelatin in an aqueous medium.[44a] Moreover, the dimerizations of penicillin X,[44b] totarol,[44c] flavonolignan silybin,[44d] and salicylic ester[44e] by laccase have already been reported.

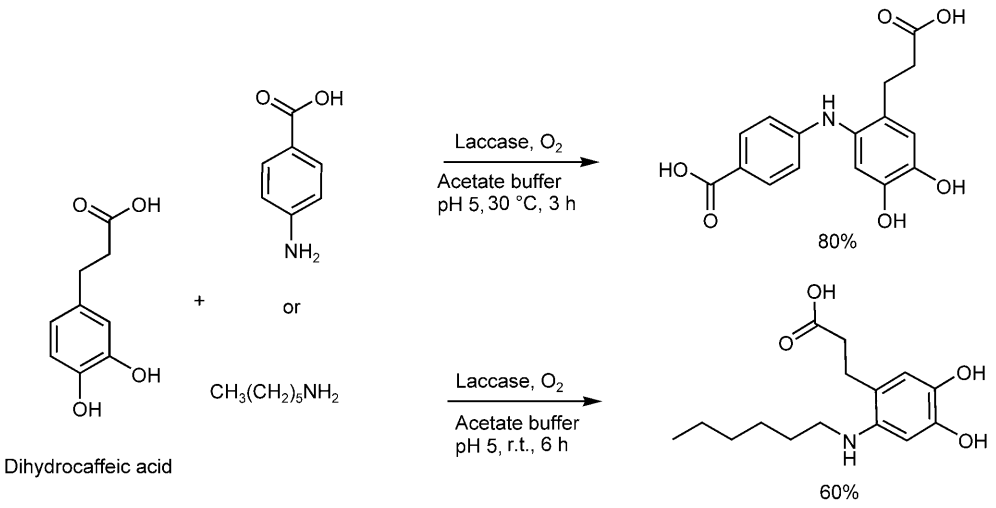
5.4 Laccase-Catalyzed Oxidative Cross-Coupling Reactions

Laccases have been shown to catalyze the oxidative cross-coupling reaction between different molecules. Oxidative coupling of hydroquinone and mithramicine or (+)-catechin have been examined.[45] In the study of the cross-coupling reaction between hydroquinone and (+)-catechin, *Rhus vernicifera* laccase catalyzed the formation of two new catechin-hydroquinone adducts (Scheme 16). In this study, hydroquinone served as both a shuttle oxidant and as a reactant, during laccase oxidations.

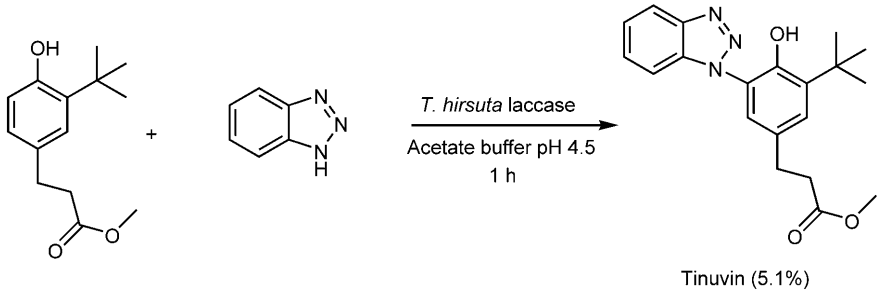
Schauer et al. reported the derivatization of the natural compound 3-(3,4-dihydroxyphenyl)-propionic acid (dihydrocaffeic acid) *via* an N-coupling reaction with amines in the presence of laccase and oxygen in



Scheme 16. Laccase-catalyzed the formation of catechin-hydroquinone adducts.



Scheme 17. Laccase-catalyzed N-coupling of dihydrocaffeic acid and amines.



Scheme 18. The synthesis of Tinuvin by a laccase-catalyzed reaction.

aqueous medium.[46] The products of these reactions were formed by an R–NH_2 attack of a cation radical of dihydrocaffeic acid (Scheme 17). Later, the authors also studied the laccase-catalyzed heteromolecular coupling of dihydrocaffeic acid with 4-aminobenzoic acid in a different reactor.[47]

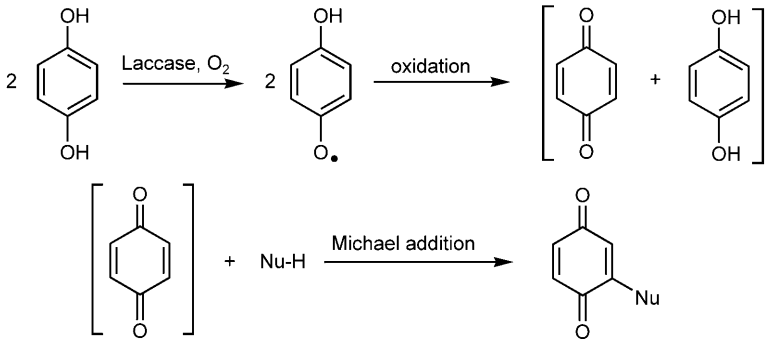
A recent example of a laccase-catalyzed cross-coupling reaction is the synthesis of Tinuvin, the benzotriazole base UV-absorber.[48] Laccase from *Trametes hirsuta* was used to catalyze the coupling reaction of 3-(3-*tert*-butyl-4-hydroxyphenyl)propionic acid methyl ester to 1*H*-benzotriazole (Scheme 18). This cross-coupling reaction occurred when 1*H*-benzotriazole was applied in four-fold molar excess.

A novel laccase-initiated cross-coupling reaction is the formation of protein-oligosaccharide conjugates.[49] The formation of hetero-cross-coupling products between the tyrosine side chain of α-casein and phenolic acid of hydrolyzed oat spelt xylan was catalyzed by *T. hirsuta* laccase. This study shows another use of laccase in the modification of the biopolymers.

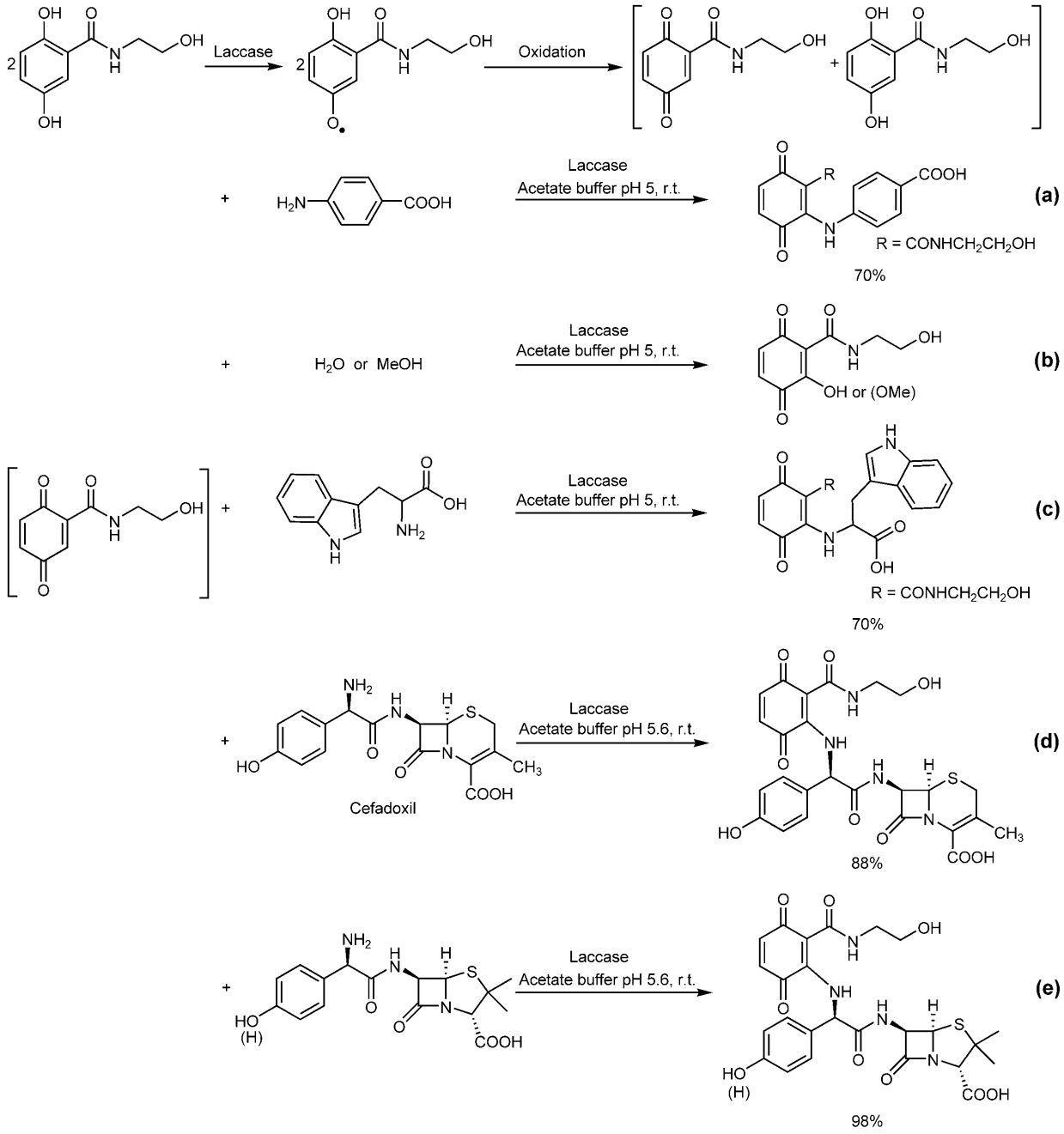
5.5 Laccase-Initiated Formation of Quinones in Organic Synthesis

An alternative approach to the use of laccase in organic synthesis is to oxidize phenolic substrates to the respective quinone structures. The quinonoid derivative is then reacted with other compounds to provide the corresponding product (Scheme 19).

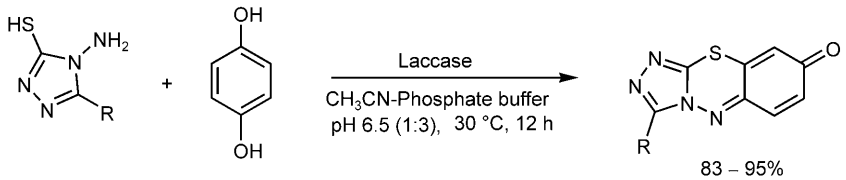
Many studies on the laccase-catalyzed synthesis of aminoquinones have been reported.[50] Aminoquinones were synthesized by nuclear amination of *p*-hydroquinones, with primary aromatic amines in the presence of fungal laccase. The mechanism of these reactions is proposed to occur *via* a Michael addition of the primary amine to the quinoniod intermediate (Scheme 20, **a**). In addition, this strategy was also used to derivatize the unprotected amino acid L-tryptophane (Scheme 20, **c**).[51] The laccase-catalyzed amination was also used in the synthesis of bioactive compounds such as β-lactam antibiotic cephalosporins (Scheme 20, **d**) and novel penicillins (Scheme 20, **e**).[52] Manda et al. showed that the quinonoid intermediate of the laccase substrate can react with solvents, such as water, methanol, and other alcohols, to form the C–O bond cross-coupling products



Scheme 19. Mechanism of the laccase-mediated formation of a quinonoid intermediate for the Michael addition reaction.

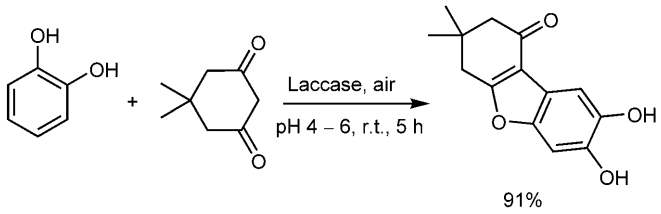


Scheme 20. Laccase-mediated amination reactions.

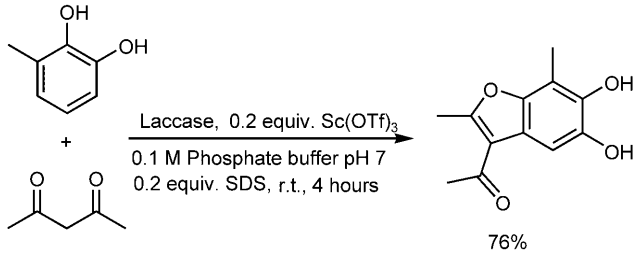


R = CH_3, C_2H_5, Ph, C_6H_4-OMe, C_6H_4-CH_3, C_6H_4-Cl, C_6H_4-Br

Scheme 21. The synthesis of 3-substituted-1,2,4-triazolo[4.3-*b*][4.1.2]benzothiadiazin-8-ones by laccase.



Scheme 22. Laccase-initiated domino reaction of cyclohexane-1,3-diones with catechols.

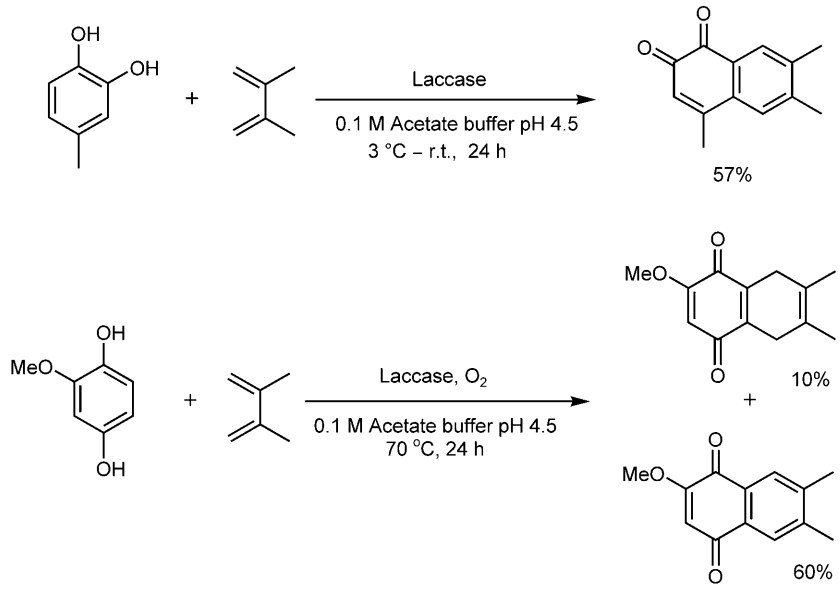


Scheme 23. Laccase-initiated domino reaction of methylcatechol and acetylacetone for the synthesis of benzofuran derivatives.

(Scheme 20, **b**).[53] Besides the laccase-catalyzed amination of *p*-hydroquinone, the laccase-catalyzed aminations of *o*-hydroquinone, such as the laccase-mediated Michael addition of ^{15}N-sulfapyridine to protocatechuic acid, have also been reported.[54]

The laccase-mediated formation of an intermediate quinone can be used in domino reactions. For example, Bhalerao et al. reported a laccase-catalyzed one-step synthesis of 3-substituted-1,2,4-triazolo[4.3-*b*]-[4.1.2]benzothiadiazine-8-ones (Scheme 21).[55] Recently, Leutbecher et al. studied the synthesis of O-heterocycles *via* a laccase-catalyzed domino reaction between 4-hydroxy-6-methyl-2*H*-pyran-2-ones with catechols.[56] Moreover, the laccase-initiated domino reaction of cyclohexane-1,3-diones with catechols for the synthesis of 3,4-dihydro-7,8-dihydroxy-2*H*-dibenzofuran-1-ones has been developed (Scheme 22).[57] The product yields ranged from 70% to 97%.

In addition, Witayakran et al. have developed a cascade synthesis of benzofuran derivatives from the reaction of catechols and 1,3-dicarbonyl compounds *via* an oxidation–Michael addition cascade reaction that is initiated by laccase. The use of $Sc(OTf)_3$



Scheme 24. An example of the synthesis of naphthoquinones *via* Diels–Alder reaction of laccase-generated quinones and dienes.

(Scheme 23) was shown to facilitate the Micheal reaction with *in situ* generated qunones.[58] This reaction was carried out in air at room temperature, in aqueous medium, and provided the benzofuran products in good yield. This catalytic system can also be recycled and reused with only a minor drop in the percentage yield of the products.

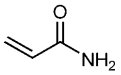
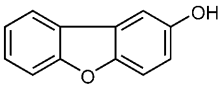
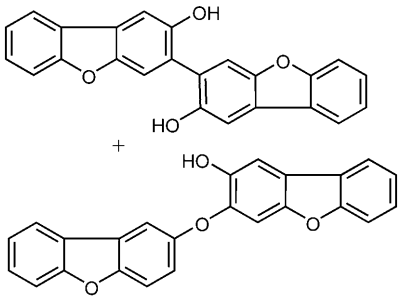
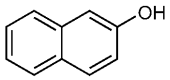
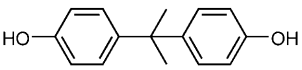
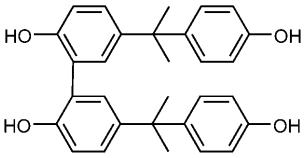
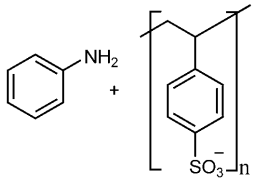
Laccase-initiated cascade reactions with *in situ* generated quinones followed by a Diels–Alder reaction have also been reported. Witayakran et al. reported the one-pot synthesis of *o*- and *p*-naphthoquinones *via* the Diels–Alder reaction of dienes with quinones generated *in situ* by laccase in an aqueous medium (Scheme 24).[59] In this reaction, hydroquinones were first oxidized by the laccase to generate intermediate quinones, and then, these quinones subsequently underwent the Diels–Alder reaction, with dienes and further oxidation to finally generate naphthoquinones, in good yields.

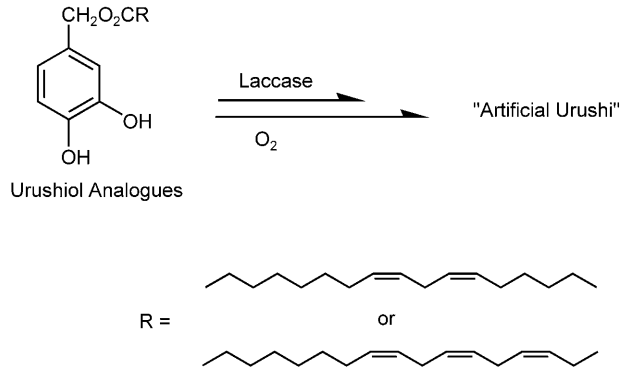
5.6 Laccase-Catalyzed Polymerization Reactions

Laccases have also been shown to catalyze the polymerization of many compounds including acrylamide, 2-hydroxydibenzofuran, phenolic pollutants, 1-naphthol, catechol, 4-chloroguaicol, bisphenol A, and aniline.[60] Some examples of these laccase-catalyzed polymerizations *via* radical coupling reactions are shown in Table 2.

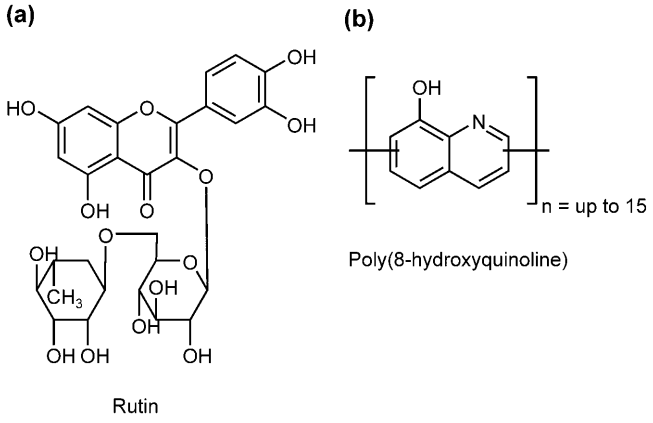
In addition, many natural or artificial natural products have been synthesized by laccase-catalyzed poly-

Table 2. Substrates, reaction conditions, and products from laccase-catalyzed polymerization *via* oxidative coupling reaction.

Substrate	Reaction Condition	Products	Refs.
 Acrylamide	Laccase, water, 65 °C, 4 h	Polyacrylamide (MW $> 6\times10^5$)	[60a]
 2-Hydroxybenzofuran	Laccase, acetate buffer pH 5, 30 °C, 3 h	Dimers such as:  + Trimers and Oligomers	[60b]
 1-Naphthol	Laccase, acetone-acetate buffer pH 5, 25 °C	Orange-colored poly(1-naphthol), average MW = 4920 Da	[60d]
 Bisphenol A	Laccase, phosphate buffer pH 6, r.t., 4 days	Dimer:  and Oligomers	[60 h]
 Aniline + Sulfonated polystyrene (SPS)	Laccase, citrate-phosphate buffer pH 3.5–4.4, 20 °C	SPS-polyaniline complex	[60k]



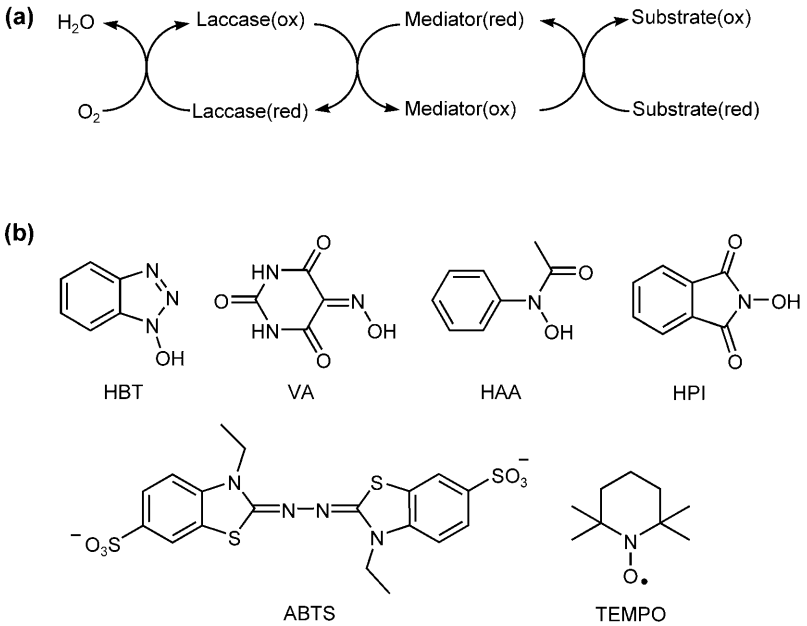
Scheme 25. The synthesis of artificial urushi by laccase-catalyzed polymerization of urushiol analogues.



Scheme 26. The structures of **(a)** rutin and **(b)** poly(8-hydroxyquinoline).

merization reactions. Kobayashi and his co-workers developed a method for the preparation of artificial urushi.[61] Urushi is an insoluble polymeric material formed by the cross-linking of urushiol monomer, whose structure is a catechol derivative, with unsaturated hydrocarbon chain consisting of monoenes, dienes, and trienes at the 3-, or 4-position of catechol. The artificial urushi in this study was prepared by laccase-catalyzed cross-linking of new urushiol analogues under mild conditions without the use of organic solvents (Scheme 25).

Rutin is one of the most famous glycosides of the flavanoid type widely present in many plants and has been reported to have biological properties, including: antioxidant, antihypertensive, anti-inflammatory, and antihemorrhagic activities (Scheme 26, **a**).[62] Kobayashi et al. synthesized poly(rutin) by laccase-catalyzed oxidative polymerization of rutin to amplify the antioxidant activity of rutin.[63] These authors also synthesized poly(catechin), a new class of flavonoid polymers, *via* the polymerization of catechin by laccase, in a mixture of acetone-acetate buffer solvent.[64] Poly(catechin) exhibited greatly amplified superoxide scavenging activity and xanthine oxidase inhibitory activity when compared with catechin. Moreover, Burton and Ncanana recently reported the laccase-catalyzed polymerization of 8-hydroxyquinoline to yield an antioxidant aromatic polymer (Scheme 26, **b**).[65] Eisenman et al. reported the use of *Cryptococcus neoformans* laccase to catalyze the synthesis of melanin from both D- and L-3,4-dihydroxyphenylalanine (DOPA).[66]

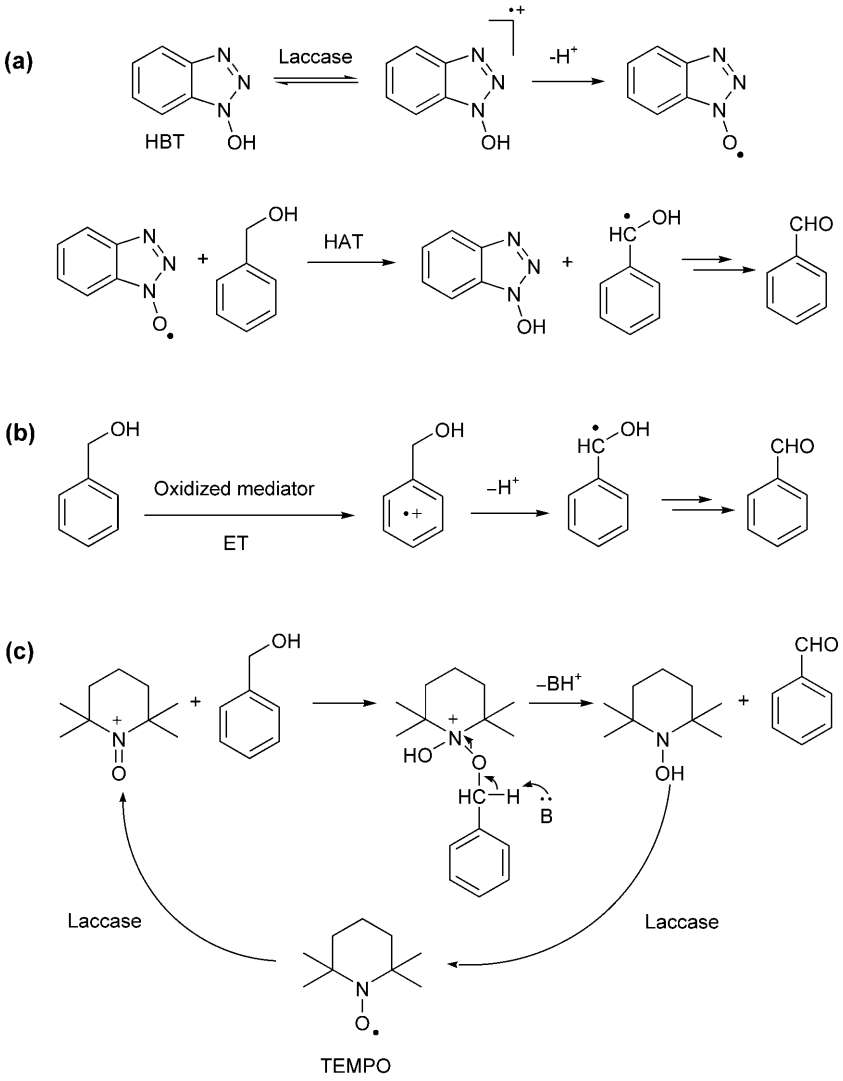


Scheme 27. (a) Schematic representation of laccase-mediator redox cycle; **(b)** chemical structures of laccase mediators.

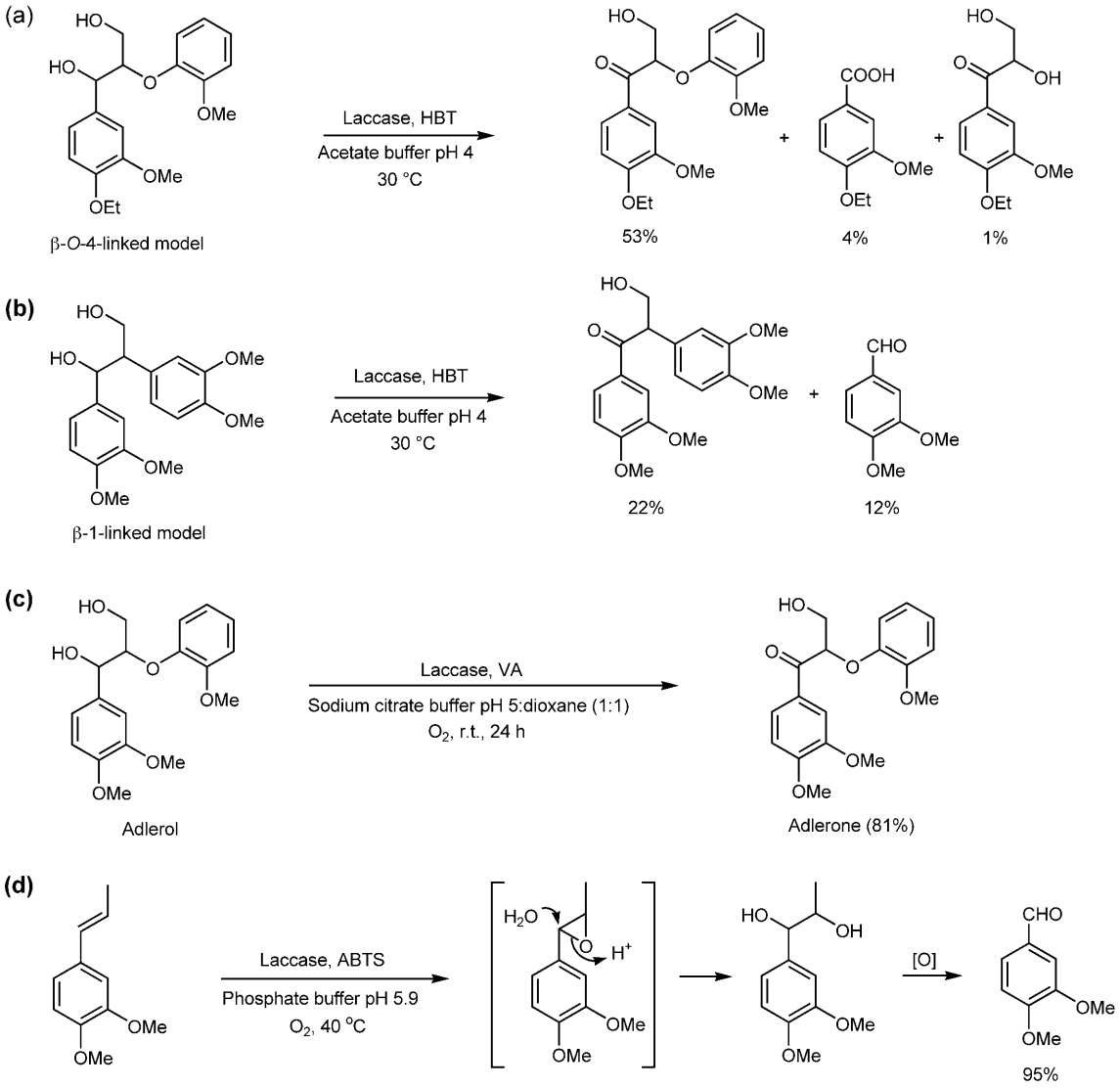
6 Laccase-Mediator Systems in Organic Synthesis

In reactions where the substrate to be oxidized has a higher redox potential than laccase, typically 0.5–0.8 mV[3] *vs.* NHE, or the substrate is too large to penetrate into the enzyme active site, the presence of a so-called 'chemical mediator' may used to facilitate the reaction. As shown in Scheme 27, **a**, the mediator first reacts with the laccase to form a reactive oxidized intermediate which then oxidizes the target substrate. The mediators that are widely used include *N*-hydroxybenzotriazole (HBT), 2,2′-azinobis-(3-ethylbenzylthiozoline 6-sulphate) (ABTS), violuric acid (VA), 3-hydroxyanthanilic acid (HAA), *N*-hydoxyphthalimide (HPI), and 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO) (Scheme 27, **b**).[4]

The mechanism of the mediator-substrate oxidation is suggested to occur *via* electron transfer (ET), radical hydrogen-atom transfer (HAT), or ionic oxidation routes, depending on the structure of the oxidized mediator.[4a,67] The mediators that have the N–OH structural feature, such as HBT, VA, HAA, and HPI, favor the HAT pathway, while ABTS reacts *via* an ET pathway. TEMPO and its analogues follow an ionic oxidation pathway. An overview of these mediator-substrate oxidation mechanisms is summarized in Scheme 28. Some recent examples of the laccase-mediator systems in synthetic applications are discussed in the following section.



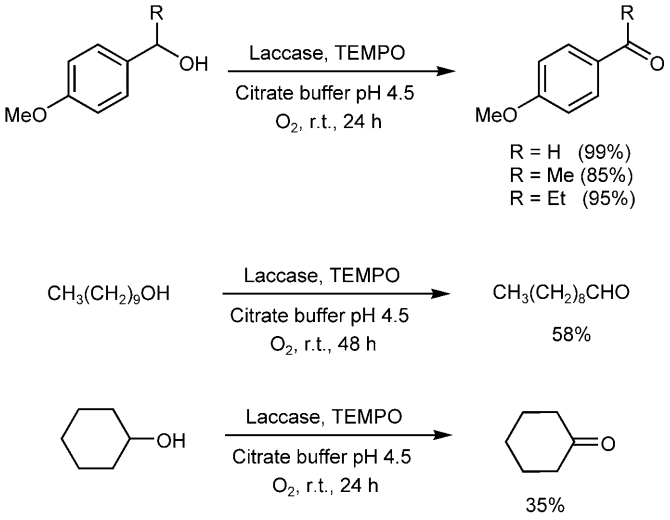
Scheme 28. Schematic representation of the mediator-substrate oxidation *via* **(a)** the HAT route, **(b)** the ET route, and **(c)** the ionic oxidation route.[4a]



Scheme 29. Laccase-mediator system catalyzes the oxidation of **(a)** β-*O*-4-linked lignin model compound, **(b)** β-1-linked lignin model compound, **(c)** Adlerol, and **(d)** 4-*O*-methylisoeugenol.

6.1 Laccase-Mediator-Catalyzed the Oxidation of Non-Phenolic Lignin Model Compounds

To date, there have been many studies that have reported the use of mediators to oxidize non-phenolic compounds. The oxidation of benzyl alcohol groups in several lignin-model studies by laccase-mediator systems has been investigated. For example, laccase/HBT have been reported to catalyze the benzylic oxidation and cleavage of β-*O*-4-linked and β-1-linked lignin model compounds (Scheme 29, **a** and **b**).[68] The laccase/VA system has been shown to efficiently oxidize Adlerol to Adlerone under reactively mild conditions (Scheme 29, **c**).[69] In addition, the oxidation of double bonds conjugated to aromatic rings in lignin model compounds, such as the oxidation of 4-*O*-methylisoeugenol by laccase/ABTS system, has also been

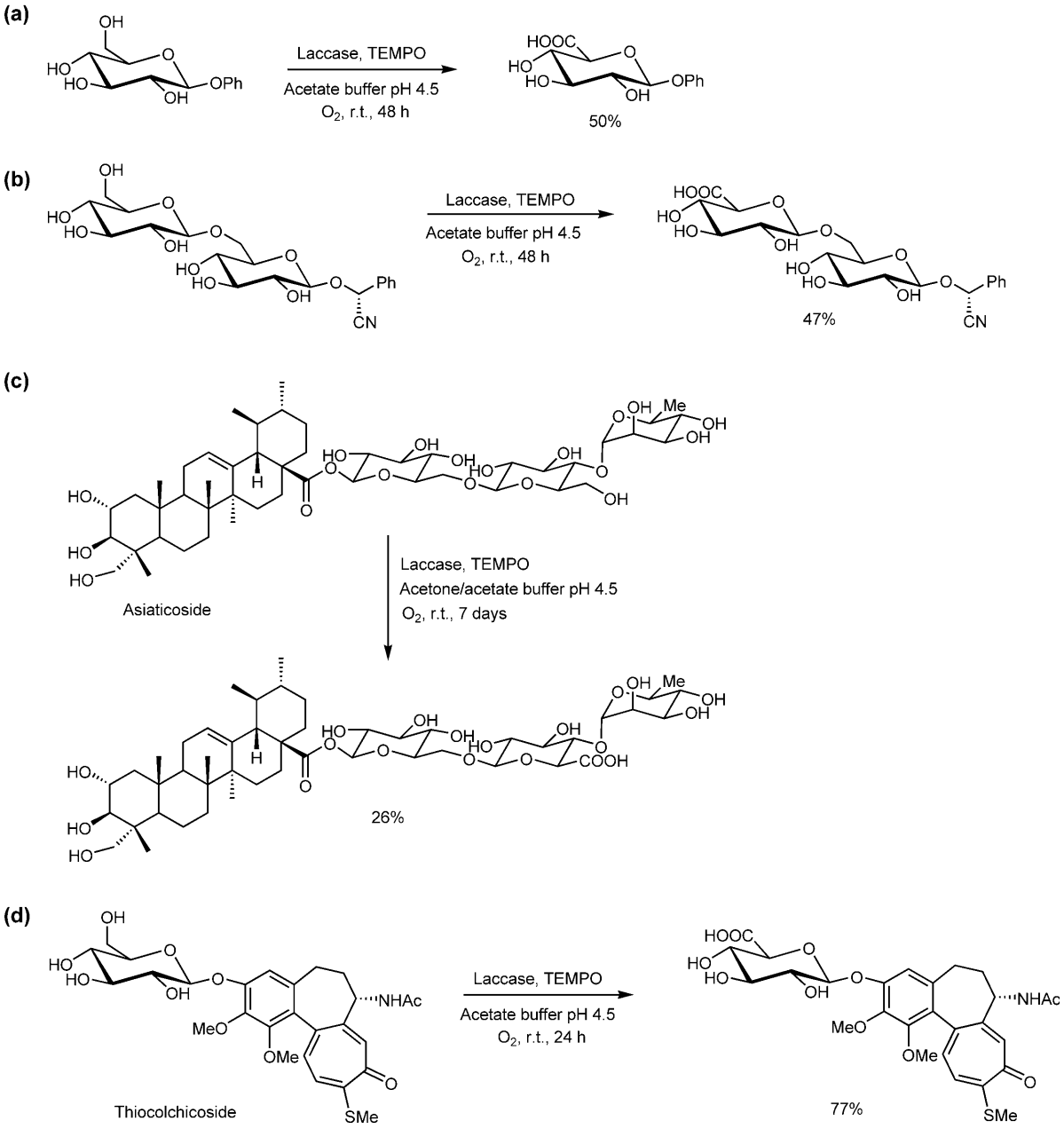


Scheme 30. The oxidation of alcohols to carbonyl compounds by laccase/TEMPO system.

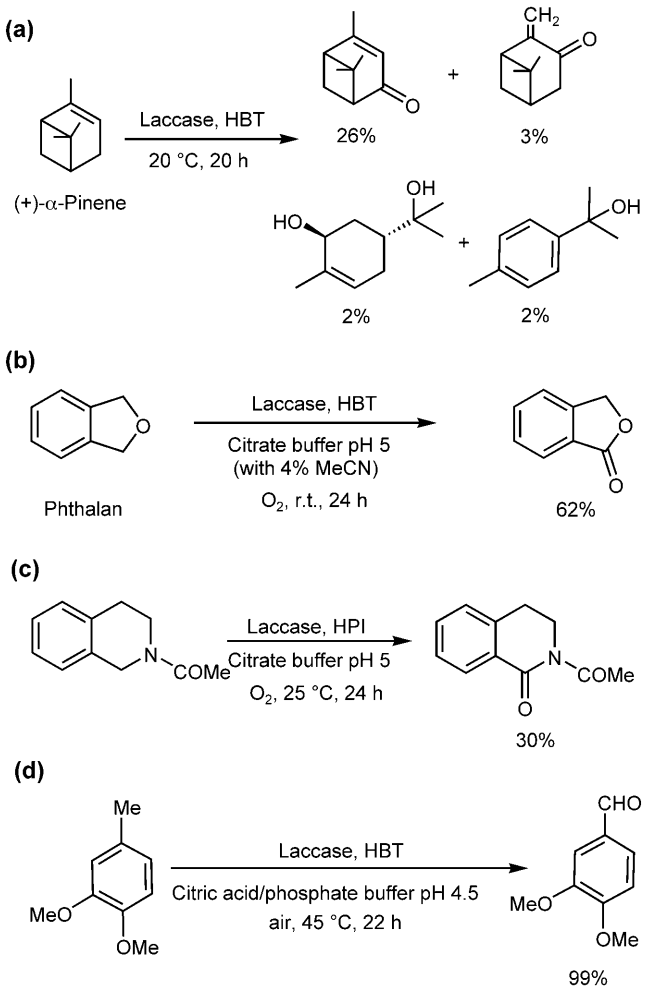
examined.[70] The major reaction in this laccase/ABTS oxidation is epoxidation of the double bond conjugated to the aromatic ring (Scheme 29, **d**).

6.2 Laccase-Mediator-Catalyzed the Oxidation of Alcohols

The studies of the laccase-mediator systems have been further extended to the oxidation of activated alcohols, including benzylic, heteroaromatic, allylic, propargylic and aliphatic alcohols, to their corresponding aldehyde or ketone products.[71] For example, Galli et al. have developed an efficient oxidation of alcohols to carbonyl compounds by the laccase/TEMPO system, at room temperature.[71a] This laccase/TEMPO system can furnish the carbonyl product in an excellent yield. Some examples of this oxidation are shown in Scheme 30. These authors also evaluated the catalytic efficiency of a variety of laccase mediators.[67b,71c] Their studies showed that, among laccase mediators, TEMPO was the most effective compound for the oxidation of alcohols, and an ionic mechanism was suggested for its action. Recently, Sheldon et al.



Scheme 31. Laccase/TEMPO-catalyzed regioselective oxidations of **(a)** a monosaccharide, **(b)** a disaccharide, **(c)** asiaticoside, and **(d)** thiocolchicoside.



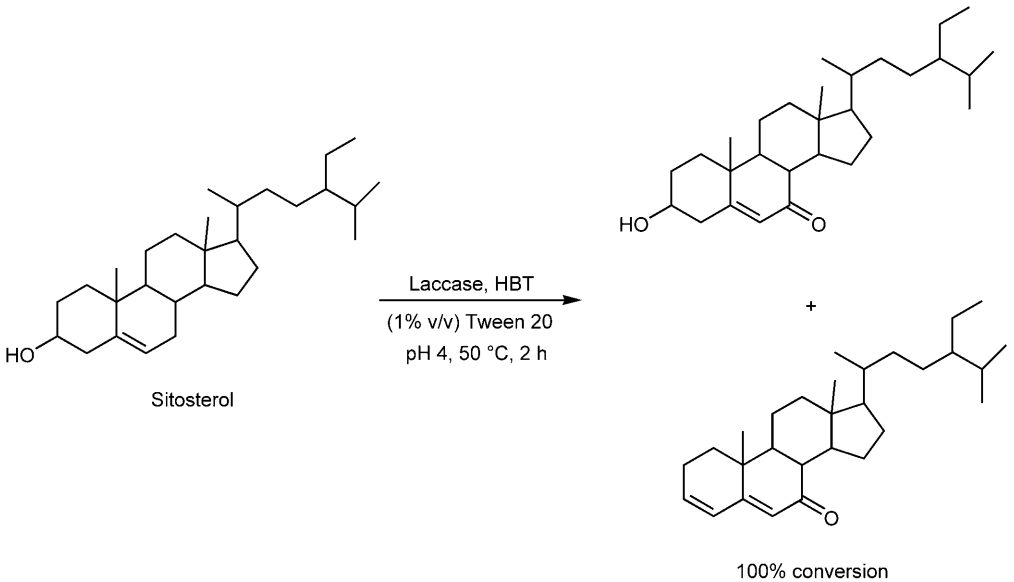
Scheme 32. Some examples of laccase-mediator-catalyzed the oxidation of **(a)** alkenes, **(b)** ethers, **(c)** amides, and **(d)** aromatic methyl groups.

extended the study of the laccase/TEMPO system for the oxidation of alcohols by evaluating the performance of a series of TEMPO derivatives as mediators of laccase in the oxidation of benzyl alcohol and 1-phenylethyl alcohol.[71d] They found that the most effective mediators are TEMPO, 4-hydroxy-TEMPO, and 4-acetylamino-TEMPO. In addition, TEMPO and its derivatives were shown to react faster with primary alcohols than with secondary alcohols.

The laccase/TEMPO system was also applied to the selective oxidation of the primary hydroxy groups of carbohydrates, including starch, cellulose, pullulan and other polysaccharides, to yield 6-aldehydes and 6-carboxylates.[72] For cellulose, a low conversion ($<5\%$) was achieved. However, the conversion yield could be improved by the activation of cellulose fibers by NaOH treatment prior to the oxidative treatment. In contrast, the oxidation of starch and pullulan by this system provided higher degrees of conversion. More recently, this laccase/TEMPO system has been used to catalyze the regioselective oxidation of other sugar derivatives, such as monosaccharides, disaccharides, asiaticoside, and natural glycosides.[73] These reactions were conducted under mild conditions, and some examples of these studies are summarized in Scheme 31.

6.3 Laccase-Mediator-Catalyzed the Oxidation of Other Functional Groups

Besides the oxidation of alcohols, the oxidations of others functional groups, such as alkenes, ethers, amide, aromatic methyl groups, and polycyclic aromatic hydrocarbons, by laccase-mediator systems



Scheme 33. Laccase-HBT-catalyzed the oxidation of a sterol (sitosterol).

have also been reported (see Scheme 32).[74] In addition, although the oxidation of phenols can be achieved by laccase oxidation, the extent and rates of this transformation were reported to be enhanced in the presence of a mediator in the reaction system.[75]

Recently, the oxidation of unsaturated lipids by laccase/HBT system was studied.[76] The major products detected from the oxidation of fatty acids were epoxy and hydroxy fatty acids while the main products obtained from the oxidation of sterols were steroid ketones (Scheme 33).

7 Conclusion

This review demonstrates the usefulness of the laccase in recent synthetic applications. Laccase or laccase-mediator systems do provide alternative, environmentally friendly, oxidation methods that can be used to replace a host of traditional chemical oxidants for a wide range of substrates. This increased application of laccase in organic synthesis will surely grow in the future as our understanding of the enzyme structure and mechanism evolve and new laccases are discovered and brought to market. It is anticipated that the reaction conditions under which laccase performs will be broadened and this will open further research opportunities. Given societies demand for green chemistry solutions and the creativity opportunities surrounding this unique oxidoreductase enzyme, we believe that the next decade will provide one of the most fruitful and promising chapters in the long history of laccase.

Acknowledgements

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