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Graphical Abstract

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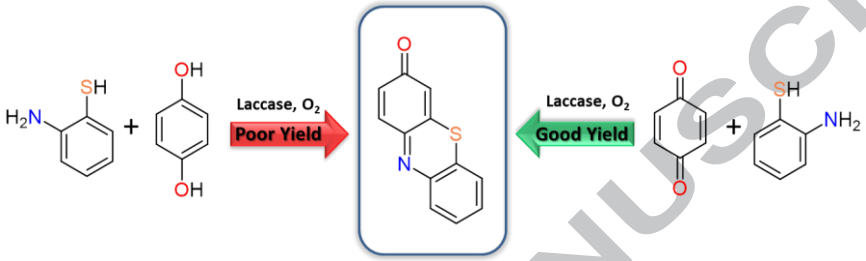

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Ecofriendly syntheses of phenothiazones and related structures facilitated by laccase – A comparative study

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ABSTRACT

The biocatalytic synthesis of phenothiazones and related compounds has been achieved in an aqueous system under mild conditions facilitated by laccase oxidation. It was found that by coupling 2-aminothiophenol directly with 1,4-quinones, the product yields could be significantly increased compared to generating the 1,4-quinones *in situ* from the corresponding hydroquinones via laccase oxidation. However, laccase still proved to be pivotal for achieving highest product yields by catalyzing the final oxidation step. Furthermore, a difference in reactivity of aromatic and aliphatic amines towards 1,4-naphthoquinone is observed. This study provides a sustainable approach to the synthesis of a biologically important class of compounds.

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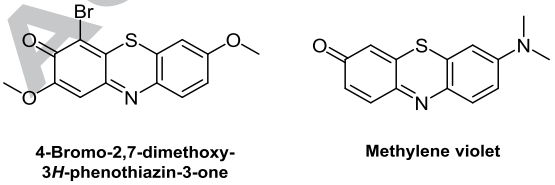
Enzyme assisted processes in chemical synthesis have proved to be a viable alternative to traditional chemical catalysis in the past few decades.[1] Their nontoxic nature, renewability and biodegradability, high activity and stability in aqueous systems, high selectivity, cost effectiveness, and general ease of use will ensure their continuing application as sustainable solutions to pollution prevention and toxic waste control within many areas of the chemical industry. Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) are a class of copper-containing enzymes of biotechnological importance that have received growing amounts of attention in previous years.[2] These multi-copper oxidases catalyze the mono-electron oxidation of four substrate molecules (typically phenolic compounds) coupled with the reduction of O_2 to $2H_2O$.[3] Their existence in nature is widespread, ranging from fungi,[4] bacteria,[5] and insects,[6] to plants[7] and algae.[8] Owing to their environmentally benign character and catalytic properties, they find significant application in a variety of fields, such as delignification and biobleaching of pulps,[9] lignocellulosic fiber modification,[10] bioremediation of industrial wastes,[11] biosensors,[12] as well as organic synthesis.[13]

Laccases have attracted increasing use as green tools for the synthesis of fine chemicals, as demonstrated by a growing number of related publications.[14] Continuing our research on laccase-catalyzed synthesis of heterocyclic compounds,[15] we focused our attention towards coupling hydroquinones with compounds containing both the thiol and amine functional groups. While laccase-catalyzed coupling reactions involving nucleophiles derived from carbon,[16] nitrogen,[17] or sulfur[15, 18] 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.[19] The laccase-catalyzed coupling of 2-aminothiophenol with hydroquinone for the synthesis of 3*H*-phenothiazin-3-one has been previously achieved;[19b] however, the process suffers from low product yield (21%). We present 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*.[20] More recently, derivatives and analogs, particularly 4-bromo-2,7-dimethoxy-3*H*-phenothiazin-3-one (Figure 1), have shown inhibitory effects on 5-lipoxygenase



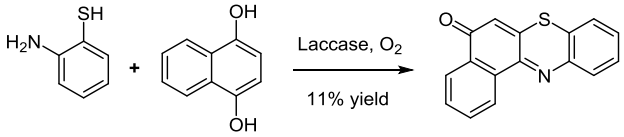
4-Bromo-2,7-dimethoxy-3*H*-phenothiazin-3-one **Methylene violet**

Figure 1. Representative structures of phenothiazones.

and mammalian leukotriene biosynthesis, thus, they find therapeutic use in treating allergies, inflammation, asthma, and cardiovascular disorders.[21] They have also displayed tuberculostatic, antibacterial, and analgesic properties and have been used to treat oxidative stress disorders.[22] Furthermore, they offer protection to mild steel from acidic corrosion, and find use in organic semiconductors and dyes (e.g. methylene violet, Fig. 1).

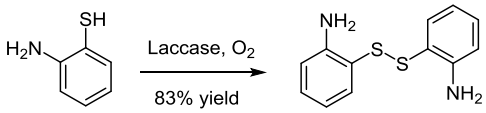
The first reported synthesis of a phenothiazone compound involved the oxidation of 3-hydroxyphenothiazine by $FeCl_3$.[23] Most early syntheses relied upon the use of stoichiometric, transition-metal-containing oxidants, such as $FeCl_3$, $K_2Cr_2O_7$, MnO_2, or ceric ammonium nitrate to oxidize phenothiazines to the corresponding phenothiazones at elevated temperatures in organic solvents.[21, 24] More contemporary syntheses involve the condensation of 2-aminothiophenol with 1,4-quinones;[21, 25] 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, we provide a green, biocatalytic approach to the synthesis of phenothiazones and related structures.

To determine if the reaction would proceed to give the desired product, an initial experiment was conducted that reacted 2-aminothiophenol (**1**) with naphthohydroquinone (**2a**) in the presence of laccase (Method A), shown in Scheme 1. The product, 5*H*-benzo[a]phenothiazine-5-one **3a**, which has shown antiproliferative activity towards human tumor cells,[26] was achieved, albeit in very low yield (11%). The low yield can be rationalized by the formation of a *S*-S dimer of **1**, which was also observed in a previous study.[19b] 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}).[27] In fact, reacting **1** with laccase alone can yield the S-S dimer product **4** (Scheme 2), which possesses antimicrobial properties and was historically used to treat syphilis,[28] in very high conversion (83%). Thus, simply reacting **1** with hydroquinones and laccase in a one-step process is not a feasible method for the synthesis of phenothiazones.



1 **2a** **3a**

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

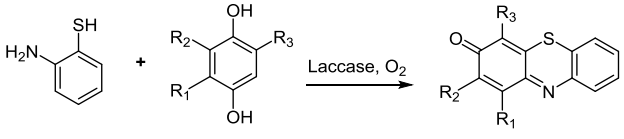


1 **4**

Scheme 2. Laccase-catalyzed dimerization of 2-aminothiophenol (**1**). Reaction conditions: 0.50 mmol 1, 50 U laccase, 6 mL 0.10 M sodium acetate buffer pH 5.0, rt, 4 h.

Table 1

Laccase-catalyzed coupling of 2-aminothiophenol (**1**) with hydroquinones (**2**)[a]



1 **2** **3**

Entry	2	Product Yield
1	**a**	**3a** 20%

2	**b**	R_1, R_2, R_3 = H	**3b**	24%	
3	**c**	R_1 = H, R_2, R_3 = OCH_3	**3c**	53%	
4	**d**	R_1, R_3 = H, R_2 = CH_3	**3d**	9%	R_1 = CH_3, R_2, R_3 = H
			3e	12%	R_1, R_3 = H, R_2 = CH_3

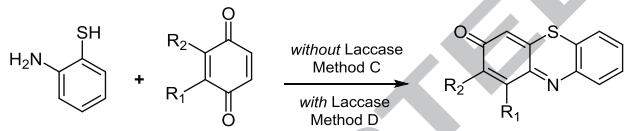
[a] Reaction conditions: 1 eq. (0.50 mmol) **1**, 1.25 eq. (0.625 mmol) **2**, 50 U laccase added at *t* = 2 h, 0.10 M sodium acetate buffer pH 5.0 with 10-15% MeOH (v:v), rt, 6 h.

Based on the aforementioned findings, we then experimented with a two-step process in which **1** is added to the reaction mixture 2 hours after the hydroquinone and laccase (Method B), similar to what has been done previously.[19b] We hypothesized that this would allow for a substantial formation of laccase-generated 1,4-quinone capable of rapidly reacting with **1** before it is oxidized by laccase. The results are shown in Table 1. As can be seen, the product yields are still quite low, with the exception of **3c** (Table 1, 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.

There seemed to be two factors contributing to low product yields: 1) oxidation of **1** by laccase, and 2) poor conversion of the hydroquinone to the corresponding 1,4-quinone by laccase. Thus, to overcome these problems, we reacted the 1,4-quinones **5** directly with **1** both without (Method C) and with (Method D)

Table 2

Coupling of 2-aminothiophenol (**1**) with 1,4-quinones (**5**) in the absence (Method C) and presence (Method D) of laccase



1 **5** **3**

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

[a] Method C reaction conditions: 1 eq. (0.50 mmol) **1**, 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) **1**, 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.

laccase. The results are displayed in Table 2. First of all, it can be seen that by using Method D, the product yields can be increased compared to employing Method B (Table 1). 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 laccase is utilized. Comparing the data in Tables 1 and 2 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 **1** with **5c** (Table 2, entry 3) is similar to that observed by Terdic, who conducted the coupling reaction in ethanol.[25c]

Analysis of the gas chromatograms for the reaction of **1** with **5b** using both Methods C and D (Fig. 2) provides a qualitative picture of the reaction systems and reveals the role laccase has in improving product yields. In Fig. 2a (Method C), the peak with m/z 213, corresponding to product **3b**, is relatively small, indicating a low product yield. In comparison, the same peak in Fig. 2b (Method D) is the predominant peak in the chromatogram, corresponding to a high product yield. Further analysis of the chromatogram in Fig. 2a shows a sizeable peak with m/z 215, which is negligible in Fig. 2b. We believe this compound to be the reduced form of product **3b**. Furthermore, allowing **1** to react with **5b** in the absence of laccase for 72 h does not significantly improve the yield of **3b**. Thus, laccase appears to be crucial for completely oxidizing the phenothiazine form to the phenothiazone form and providing greatest product yields. Scheme 3 shows the proposed reaction mechanism. Initial addition of the aromatic amino group of **1** to a carbonyl group of 1,4-quinone **5** yields the imine **6**, which is followed by addition of sulfur to an adjacent alkene carbon and subsequent tautomerization to produce the phenothiazine intermediate **7**. A final oxidation of the phenothiazine affords the phenothiazone **3**.

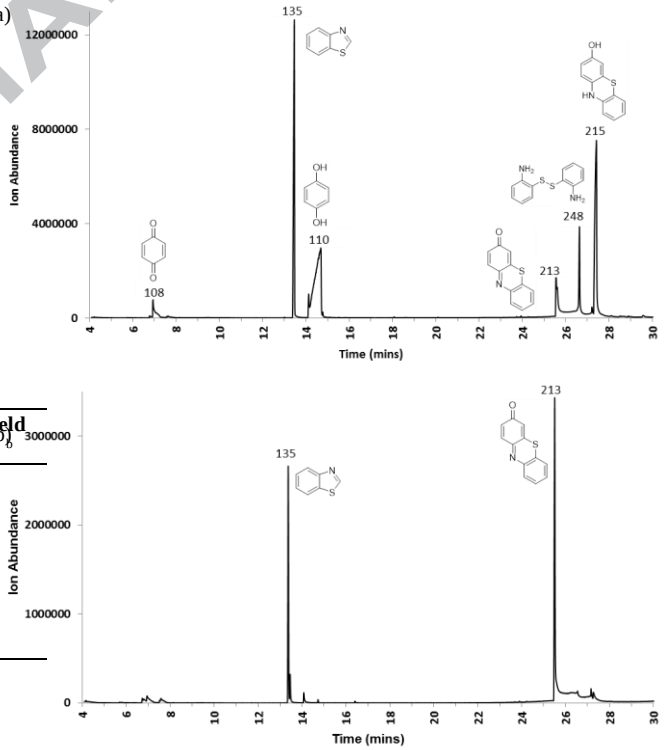
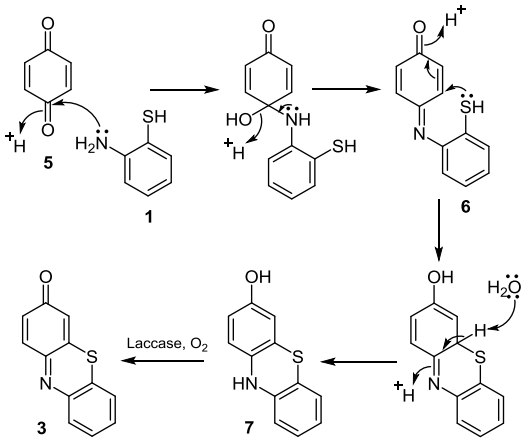


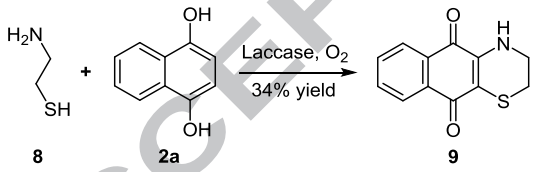
Figure 2. Qualitative gas chromatograms with m/z values of peaks for the reaction of **1** with **5b** using a) Method C and b) Method D.



Scheme 3. Reaction mechanism for the laccase-facilitated synthesis of phenothiazones.

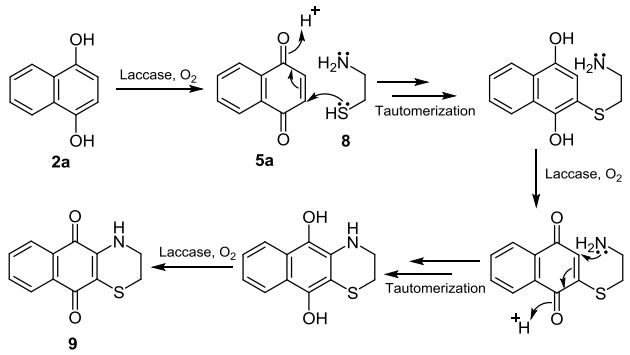
With the chemistry for the coupling of an aromatic thiol-amine with 1,4-quinones developed, we then attempted to apply the principles using a simple aliphatic thiol-amine, cysteamine (**8**). Since we observed that laccase does not directly oxidize **8**, it was possible to react this compound in one-pot with the hydroquinone **2** and laccase in one-step. Using a variety of hydroquinones and catechols,[29] 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 **8** with **2a** yielded product **9** (Scheme 4), a compound that possesses tuberculostatic potential and is also an important structural moiety present in compounds that exhibit potent antibacterial and antifungal activities.[30] A possible reason for the success in achieving a desired product when compound **2a** was used may be the increased stability of the 1,4-naphthoquinone intermediate compared to less substituted 1,4-quinones.

A proposed reaction mechanism for the laccase-catalyzed coupling of **8** with **2a** is provided in Scheme 5. First, laccase oxidizes **2a** to the corresponding 1,4-quinone **5a**, 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 **9** is reached. From this result, we observe a difference in reactivity of the aliphatic amino group of **8** and the aromatic amino group of **1** towards nucleophilic addition to 1,4-naphthoquinone. 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 **1** is 9.49, whereas the pK_b of the aliphatic amino group of **8** is 3.19.[31] Thus, in the aqueous reaction medium, the amino group of **8** is predominantly in its cationic form, rendering it less nucleophilic and less reactive than the aromatic amino group of **1**, which is mostly in its neutral form. The increased nucleophilicity of the amino group of **1** is what allows it to rapidly react with the carbonyl carbon of a 1,4-naphthoquinone, whereas for **8**, the sulfur adds first to an alkene carbon.



8 **2a** **9**

Scheme 4. Laccase-catalyzed coupling of cysteamine (**8**) with naphthohydroquinone (**2a**). Reaction conditions: 5 eq. (2.50 mmol) **8**, 1 eq. (0.50 mmol) **2a**, 50 U laccase, 8.5 mL 0.10 M sodium acetate buffer pH 5.0 : 1.5 mL MeOH, rt, 12 h.



2a **5a** **8** **9**

Scheme 5. Reaction mechanism for the laccase-catalyzed coupling of naphthohydroquinone (**2a**) with cysteamine (**8**).

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,[32] such as waste prevention, atom economy, catalysis (laccase), 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 **1** with **5b** (Method D) for the synthesis of **3b** as an example, green chemistry can be quantified. The E factor, which measures kg of waste produced per kg of desired product, was calculated to be 17.7, which falls toward the lower limit expected for fine chemical syntheses.[33] The ratio of the incorporation of all reactant atoms into the desired product (i.e. the atom economy)[34] 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}.

In summary, we have developed an environmentally friendly approach for the synthesis of phenothiazones and related structures facilitated by laccase. By coupling 1,4-quinones with 2-aminothiophenol in an aqueous reaction medium in the presence of laccase, the yields of phenothiazones can be substantially increased compared to when laccase is 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 towards 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.

Acknowledgment

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at

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Ecofriendly syntheses of phenothiazones and related structures facilitated by laccase – A comparative study

Highlights

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- Biocatalytic synthesis of phenothiazones in one-pot under mild conditions.
- Laccase-catalyzed coupling of 2-aminothiophenol with hydroquinones.
- Increased yields achieved when 1,4-quinones are used compared to hydroquinones.
- Difference in reactivity of aromatic and aliphatic amine towards nucleophilic addition.
- Favorable green chemistry metrics, such as E factor and atom economy.