



## Redox potentials, laccase oxidation, and antilarval activities of substituted phenols

Keshar Prasain<sup>a</sup>, Thi D.T. Nguyen<sup>a</sup>, Maureen J. Gorman<sup>b</sup>, Lydia M. Barrigan<sup>a</sup>, Zeyu Peng<sup>b</sup>, Michael R. Kanost<sup>b</sup>, Lateef U. Syed<sup>a</sup>, Jun Li<sup>a</sup>, Kun Yan Zhu<sup>c</sup>, Duy H. Hua<sup>a,\*</sup>

<sup>a</sup> Department of Chemistry, Kansas State University, Manhattan, KS 66506, United States

<sup>b</sup> Department of Biochemistry, Kansas State University, Manhattan, KS 66506, United States

<sup>c</sup> Department of Entomology, Kansas State University, Manhattan, KS 66506, United States

### ARTICLE INFO

#### Article history:

Received 15 November 2011

Revised 3 January 2012

Accepted 11 January 2012

Available online 21 January 2012

#### Keywords:

*Anopheles gambiae*

Anti-larval activity

Halogenated di- and trihydroxybenzenes

Laccases

Mosquito larvicides

Redox potential

Substituted di-*tert*-butylphenols

### ABSTRACT

Laccases are copper-containing oxidases that are involved in sclerotization of the cuticle of mosquitoes and other insects. Oxidation of exogenous compounds by insect laccases may have the potential to produce reactive species toxic to insects. We investigated two classes of substituted phenolic compounds, halogenated di- and trihydroxybenzenes and substituted di-*tert*-butylphenols, on redox potential, oxidation by laccase and effects on mosquito larval growth. An inverse correlation between the oxidation potentials and laccase activity of halogenated hydroxybenzenes was found. Substituted di-*tert*-butylphenols however were found to impact mosquito larval growth and survival. In particular, 2,4-di-*tert*-butyl-6-(3-methyl-2-butenyl)phenol (**15**) caused greater than 98% mortality of *Anopheles gambiae* larvae in a concentration of 180 nM, whereas 2-(3,5-di-*tert*-butyl-4-hydroxyphenyl)-2-methylpropanal oxime (**13**) and 6,8-di-*tert*-butyl-2,2-dimethyl-3,4-dihydro-2*H*-chromene (**33**) caused 93% and 92% mortalities in concentrations of 3.4 and 3.7  $\mu$ M, respectively. Larvae treated with di-*tert*-butylphenolic compounds died just before pupation.

© 2012 Elsevier Ltd. All rights reserved.

### 1. Introduction

Mosquitoes, the most important arthropods affecting human health, are vectors of human diseases. The control of mosquitoes with insecticides is currently in jeopardy, since there have been no new public health insecticides developed for mainstream vector control in the past 30 years. Laccases, copper-containing oxidases, are present in fungi, plants, bacteria, and insects and have been implicated in a variety of physiological processes and various applications including bioremediation, bio-fuel cells, pigmentation, cell wall synthesis, detoxification, wound healing, and biosensors.<sup>1</sup> Substituted *o*-, *m*-, and *p*-phenols, polyphenols, and aromatic amines and *N*-hydroxamates are oxidized by laccases with a concomitant reduction of oxygen to water.<sup>2–7</sup> The involvement of laccases in cuticle sclerotization or tanning is essential to insect survival.<sup>8,9</sup> Laccase inhibitors are limited and few useful organic compounds have been found.<sup>10–12</sup> Our studies aimed at identifying laccase substrates that would be oxidized to produce compounds potentially toxic to mosquito larvae, through covalent modification of laccase<sup>13,14</sup> or other molecular targets within the insect, pursued two approaches: (1) production of methine quinone intermediates for covalent linkage to nucleophilic protein moieties, represented

by class 1 compounds in Figure 1, and (2) formation of phenoxy free radical intermediates in the laccase active site, represented by class 2 compounds in Figure 1. These two approaches led us to synthesize these two classes of substituted phenolic compounds (Fig. 1) and to study their oxidation by laccase, redox potentials and antilarval activities. The phenolic compounds are laccase substrates and do not appear to inhibit laccase, however, several class 2 compounds possess potent antilarval activity. The results may shed light onto future design of environmentally compatible laccase substrates and mosquito larvicides.

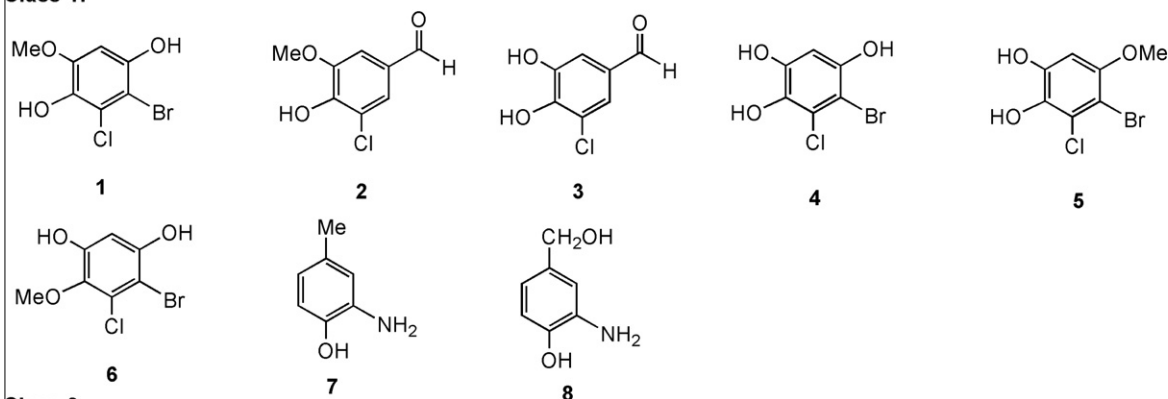
### 2. Results and discussion

Our initial plan was to position a leaving group such as bromine or chlorine into phenolic molecules. Upon oxidation by laccase, the resulting cyclic haloenone is a reactive molecule that undergoes addition–elimination reaction with a nucleophile such as the amino function of lysine or hydroxyl function of serine or tyrosine of laccase or some other nearby insect proteins. If such covalent linkage reaction could occur, halogenated phenols may lead to an inhibition of protein function and result in toxicity. Figure 2 depicted a proposed oxidation of *p*-dihydroquinone **1** to *p*-quinone **16** followed by an addition–elimination reaction with a nucleophile of protein. To test this notion, we treated *p*-quinone **16** (vide infra; for synthesis) with *L*-alanine benzyl ester (**17**; as a *p*-toluenesulfonic acid salt)

\* Corresponding author. Tel.: +1 785 532 6699; fax: +1 785 532 6666.

E-mail address: duy@ksu.edu (D.H. Hua).

## Class 1:



## Class 2:

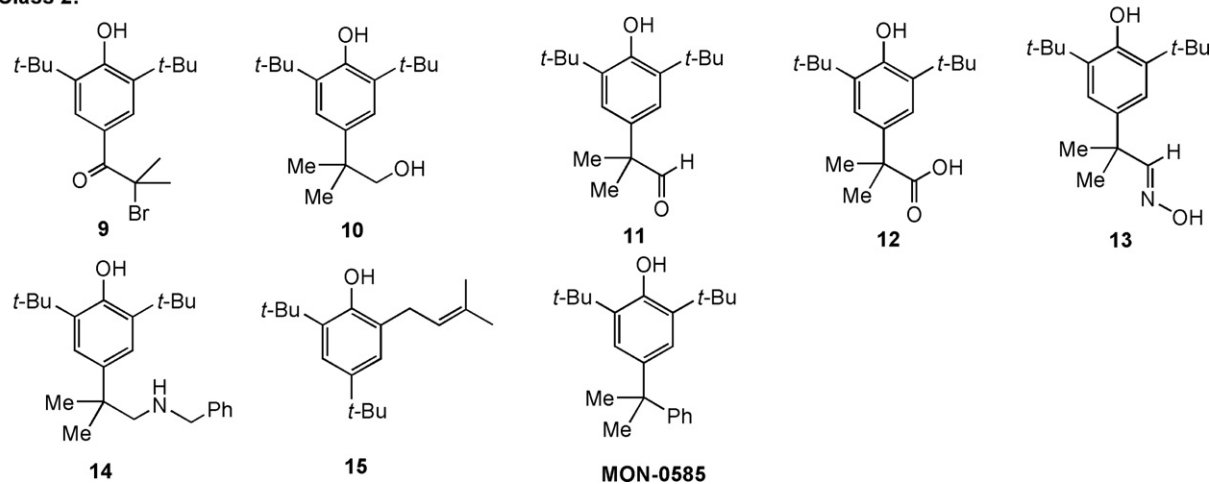


Figure 1. Synthesized halogenated polyphenols, aminophenols and di-*tert*-butyl substituted phenols.

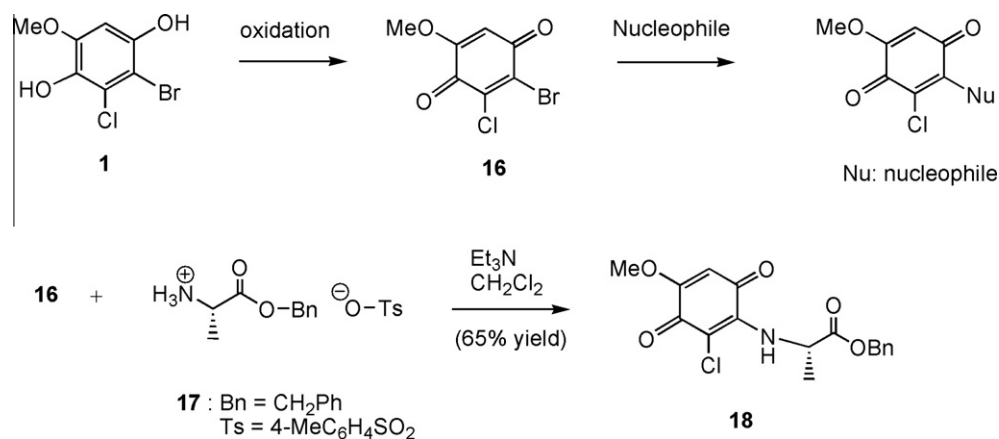


Figure 2. Proposed covalent linkage of *para*-quinone 2 with nucleophile and reaction of quinone 2 with L-alanine.

and 2 equiv of triethylamine in dichloromethane at 25 °C (Fig. 2). The amino function of L-alanine serves as a nucleophile. As expected, addition product **18** was isolated in 65% yield. The quinone structure of **18** was revealed by its IR spectrum in which the quinone C=O and C=C absorptions appear at 1663 and 1580 cm<sup>-1</sup>, respectively. This encouraging result prompted us to synthesize class 1 molecules containing halogenated phenols and aminophenols such as compounds **1–8** (Fig. 1). Oxidation of 3-chloro-4-hydroxy-5-methoxybenzaldehyde (**2**) by laccase may provide a hydroxymethine quinone, which should have similar reactivity to that of the quinone derived from **1**. Compounds **3–5** will afford

either reactive *ortho*- or *para*-quinones. 4-Bromo-5-chloro-6-methoxybenzene-1,3-diol (**6**) possessing *meta*-dihydroxy functions should not produce quinone. *o*-Aminophenols, such as compounds **7** and **8**, were investigated since the resulting iminoquinones could be more reactive than the quinone analogs and may complex with the copper in the laccase binding site (T1 site).<sup>13,14</sup>

Since substituted di-*tert*-butyl phenols including MON-0585 (Fig. 1) were implicated as insect growth regulators,<sup>15–17</sup> and they can be oxidized by Co(II)-Schiff base complex and oxygen to produce 4-substituted 2,6-di-*tert*-butyl-6-hydroperoxy-2,4-cyclohexadienones,<sup>18,19</sup> we therefore envision that substituted

di-*tert*-butylphenols may undergo oxidation with laccase catalyzed by coppers and oxygen in the active site to provide di-*tert*-butylhydroperoxy-2,4-cyclohexadienones that are toxic to insects through their reaction with endogenous nucleophiles. Hence, class 2 compounds including di-*tert*-butylphenols **9–15** were synthesized and their redox potentials, oxidation by laccase, and anti-larval activities determined. MON-0585, 2,6-di-*tert*-butyl-4-( $\alpha$ ,  $\alpha$ -dimethylbenzyl)phenol, reported by Monsanto Co.,<sup>16</sup> was prepared and used as a control.

## 2.1. Synthesis

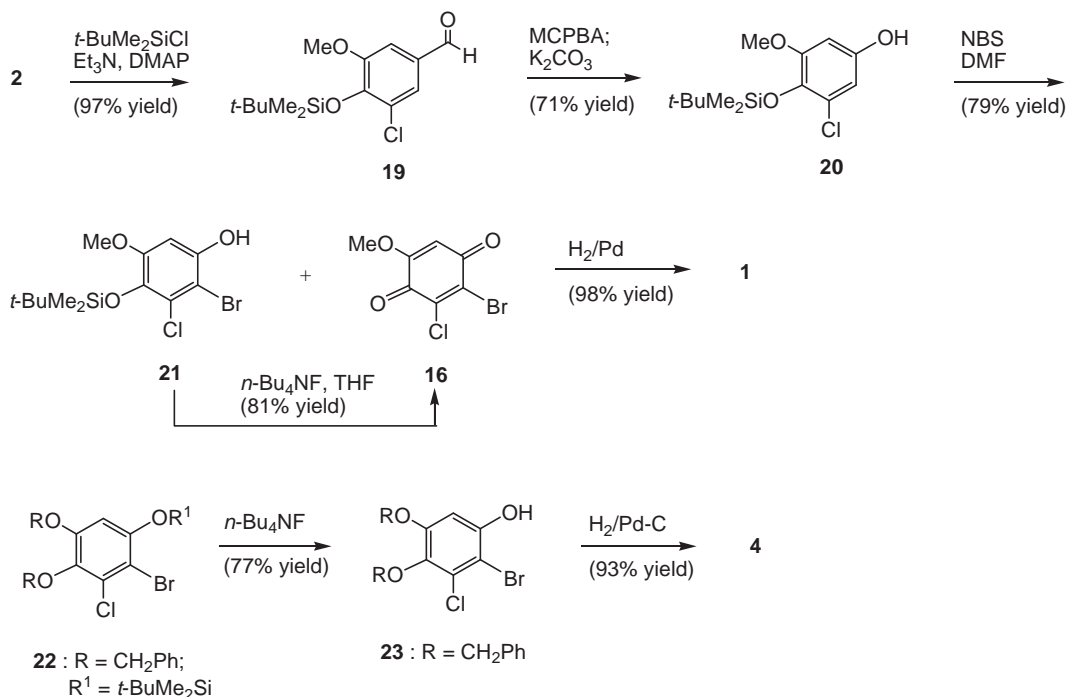
In our reported total synthesis of (+)-chloropuuphenone,<sup>20</sup> chlorinated phenols such as compounds **2** and **3** were prepared from vanillin. Utilizing these two chlorinated phenolic compounds, class 1 compounds, **1** and **4–6**, were synthesized via a sequence of protection, Baeyer–Villiger oxidation, regioselective bromination, and deprotection reactions. Compounds **7** and **8** were readily available from the reduction of 4-hydroxy-3-nitrobenzaldehyde (**31**). *p*-Dihydroxybenzene **1** was synthesized from 3-chlorovanillin (**2**) by a sequence of reactions depicted in Scheme 1: (i) protection of C4-hydroxyl function with *t*-butyldimethylsilyl chloride; (ii) Baeyer–Villiger oxidation<sup>21</sup> of the aldehyde function with *m*-chloroperbenzoic acid (MCPBA); (iii) basic methanolysis of the resulting formate moiety with K<sub>2</sub>CO<sub>3</sub> in methanol; (iv) regioselective bromination with *N*-bromosuccinimide in *N,N*-dimethylformamide (DMF); (v) deprotection of the silyl ether moiety; and (vi) reduction of the quinone moiety with hydrogen and palladium/carbon in ethanol. In the bromination reaction of phenol **20** with *N*-bromosuccinimide (NBS) in DMF, compounds **21** and **16** were isolated in 31% and 48% yield, respectively. Regioselective bromination onto the less hindered C2 of phenol **20** has been reported previously.<sup>20</sup> Compound **16** likely derived from a hydrolytic cleavage of the silyl ether function of **21** from HBr generated through NBS and the phenol and/or a trace amount of water presence in the reaction mixture followed by oxidation to the quinone. The infrared spectrum of quinone **16** does not display hydroxyl absorption, but quinone stretch at 1683 cm<sup>-1</sup>, and the spectrum

of dihydroquinone **1** shows strong hydroxyl absorption at 3303 cm<sup>-1</sup>. Desilylation of compound **21** with tetra-*n*-butylammonium fluoride gave quinone **16**. Oxidation of dihydroquinone to benzoquinone under the NBS reaction conditions has been reported,<sup>22</sup> however, oxidation under tetra-*n*-butylammonium fluoride reaction conditions is unexpected.

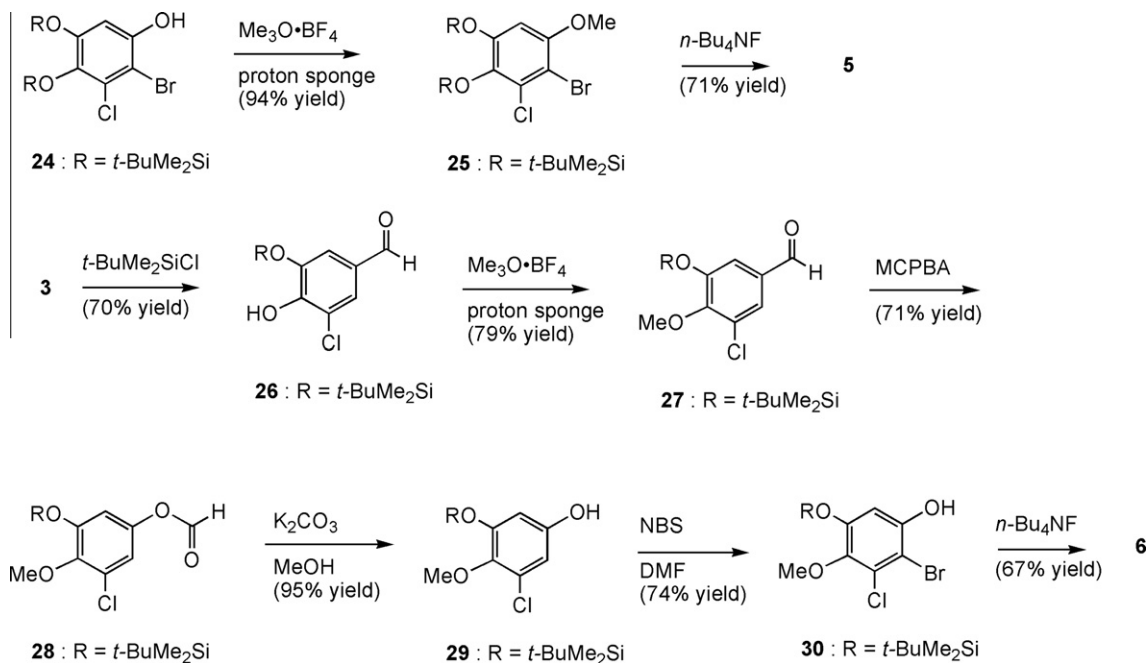
The *t*-butyldimethylsilyl and two benzyl protecting groups of compound **22**<sup>20</sup> were readily removed by a sequential treatment with tetra-*n*-butylammonium fluoride and hydrogen-palladium/carbon to give triol **4** in a 72% overall yield. Triol **4** is soluble in water and readily oxidized in air to give the corresponding quinone, which gradually decomposes to produce black solids. Hence, the compound is stored in a dry box under nitrogen atmosphere.

*o*-Dihydroxybenzene **5** was readily synthesized from compound **24**<sup>20</sup> by methylation of the phenolic hydroxyl moiety with trimethylxonium tetrafluoroborate followed by desilylation with tetra-*n*-butylammonium fluoride (Scheme 2). Compound **5** does not undergo oxidation to give the corresponding *o*-quinone as that of *p*-dihydroxybenzene **1** (vide supra), which evidence from its IR spectrum showing strong hydroxyl absorption bands at  $\nu$  3436 and 3219 cm<sup>-1</sup>. Since the less hindered C5-hydroxyl moiety of 3-chloro-4,5-dihydroxybenzaldehyde (**3**)<sup>20</sup> should react faster with an electrophile than the more hindered C4-hydroxy, protection of C5-OH followed by methylation of C4-OH, and subsequent functional group manipulation would lead to *m*-dihydroxybenzene analog **6** (Scheme 2). Thus, silylation of **3** with *t*-butyldimethylsilyl chloride, 4-dimethylaminopyridine (DMAP), and triethylamine gave a 7:1 ratio of silyl ether **26** and the C4-silyl ether byproduct, which were separated by silica gel column chromatography. Methylation of C4-hydroxy moiety of **26** with trimethylxonium tetrafluoroborate, followed by Baeyer–Villiger oxidation of the aldehyde function with MCPBA, and basic methanolysis of the resulting formate afforded phenol **29**. Regio-selective bromination of **29** with NBS followed by removal of the silyl ether protecting group produced *m*-dihydroxybenzene **6**.

*o*-Aminophenols **7**<sup>23</sup> and **8**<sup>24</sup> were readily prepared from the reduction of both nitro and aldehyde functions of 4-hydroxy-3-nitrobenzaldehyde (**31**) using a catalytic amount of palladium



Scheme 1. Synthesis of compounds **1** and **4**.



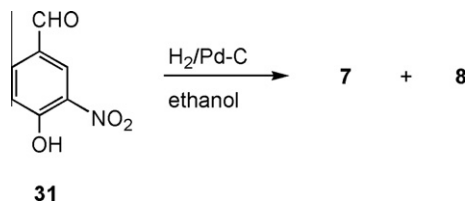
Scheme 2. Synthesis of compounds 5 and 6.

under 30 psi of hydrogen in ethanol (Scheme 3). The two products (1:1) were separated by silica gel column chromatography. Apparently, compound **7** derived from a reduction of the benzylic hydroxyl moiety of compound **8** under the hydrogenation reaction conditions.

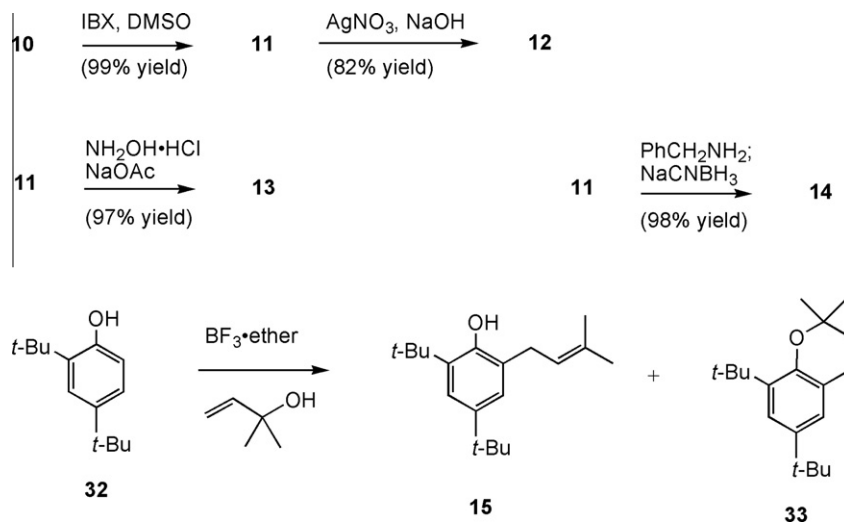
Various halogenated polyphenols and substituted aminophenols were synthesized starting from 3-chlorovanillin (**2**) and

4-hydroxy-3-nitrobenzaldehyde (**31**), respectively. The reaction sequences are simple and should be applicable to the synthesis of various substituted polyphenols.

The synthesis of class 2 compounds mainly utilized Friedel–Crafts reaction of di-*tert*-butylphenols. Following the reported procedure,<sup>25</sup> compounds **9** and **10** were prepared by an acylation reaction of 2,6-di-*t*-butylphenol with isobutyryl chloride and aluminum chloride followed by bromination with cupric bromide in dichloromethane and ethyl acetate (to give compound **9**), and reduction with lithium aluminum hydride in ether (to produce compound **10**). Oxidation of alcohol **10** with *o*-iodoxybenzoic acid (IBX) in DMSO afforded aldehyde **11**, further oxidation with silver nitrate and sodium hydroxide gave acid **12**<sup>19</sup> (Scheme 4). Treatment of aldehyde **11** with hydroxylamine produced oxime **13**, and a reductive amination reaction of **11** with benzylamine followed by sodium cyanoborohydride furnished amine **14**. Compound **15** was synthesized in 46% yield from a Friedel–Crafts



Scheme 3. Synthesis of compounds 7 and 8.



Scheme 4. Synthesis of compounds 11–15, and 33.

alkylation reaction of 2,4-di-*tert*-butylphenol (**32**) with 2-methyl-3-buten-2-ol and BF<sub>3</sub>·ether in dichloromethane at 25 °C. A 10% yield of benzopyran **33** was also isolated, which likely derived from an acid-catalyzed ring closing reaction of **15**. MON-0585 or 2,6-di-*tert*-butyl-4-cumylphenol was prepared by a Friedel–Crafts alkylation reaction of 4-cumylphenol and isobutylene in dichloromethane in the presence of a catalytic amount of sulfuric acid in a sealed tube at 80 °C for 6 h.<sup>26</sup>

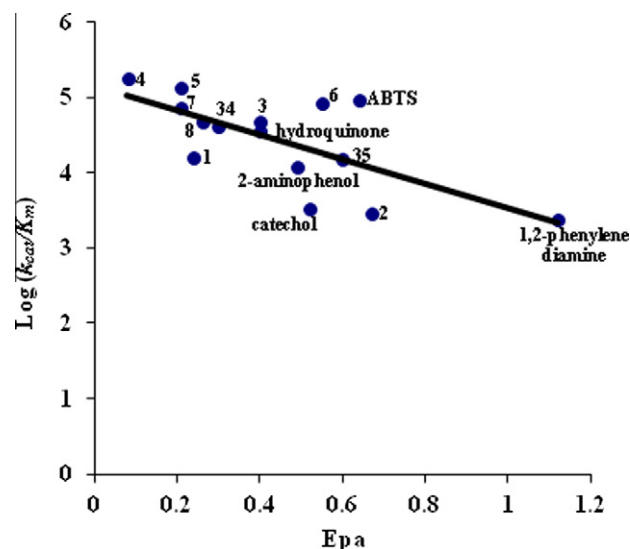
## 2.2. Redox potentials and laccase activities

Dopamine, *N*-acetyldopamine (NADA) and related catecholamines are oxidized by laccase in insects during sclerotization and pigmentation of cuticle.<sup>27,28</sup> Insects with decreased laccase function develop serious cuticle defects and die;<sup>29</sup> therefore, an inhibitor of laccases might function as a novel type of insecticide. Alternatively, oxidation of phenolic compounds by laccase may produce compounds that are toxic to insects through their reaction with nucleophilic groups in proteins. As vertebrate animals possess tyrosinase (or monophenol monooxygenase),<sup>30,31</sup> compounds that are selectively oxidized by laccase but not tyrosinase may serve as pro-insecticides. Selective laccase inhibitors or substrates generating toxic oxidized products might have useful properties of toxicity to insects but not vertebrates. An electrochemical study based on cyclic voltammetry (CV) was carried out to understand the inherent redox properties of the substrates. We tested compounds **1–8**, **34**, and **35** for evidence of irreversible inhibition of fungal laccase but did not observe inhibition (data not shown). However, these compounds were oxidized by laccase (vide infra).

Various known laccase substrates such as hydroquinone, catechol, 2-aminophenol, 1,2-phenylenediamine, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 3-amino-4-hydroxybenzoic acid (**34**), 4-amino-3-hydroxybenzoic acid (**35**), and our synthesized materials, compounds **1–15**, **18**, **33**, and MON-0585 were studied for their redox potentials (Table S1) and ability to be oxidized by fungal laccase (Table 1).

Most compounds present some redox properties as listed in Table S1. Interestingly, even though the CV features varied dramatically, the main oxidation potential of the substrates showed a good correlation with the laccase activity. Besides the oxidation potential, these compounds also showed different electrochemical oxidation/reduction reversibility which is indicated by the separation between the peak potentials of the oxidation and reduction waves in CV, that is,  $\Delta E_p$ .<sup>32</sup> In general, we divided the compounds into four groups shown in Figures S1–S4. The first group of compounds **4**, **5**, **7**, **8**, **9**, **34**, and **35** all present at least one pair of redox

waves with a small peak separation ( $\Delta E_p$ ) ranging from ~60 mV to 100 mV, indicating that they are electrochemically active with nearly reversible redox properties. The second group consists of ABTS, compounds **1** and **3**, and MON-0585, which show a peak separation between 140 mV and 290 mV. These compounds are not as active as the first group but still show quasi-reversible redox properties. The third group includes hydroquinone, catechol, 2-aminophenol, 1,2-phenylenediamine and compound **6**, which show nearly irreversible redox waves as indicated by the large peak separation between the redox waves (with  $\Delta E_p = 370$ – $420$  mV) or complete absence of the reduction waves corresponding to the oxidation waves at electropotential above 0.0 V. The fourth group either only shows one of the redox waves or has an enormous peak separation (>540 mV), including compounds **2**, **10–15**, **18**, and **33**. Interestingly, compounds **4** and **5** have very similar structures and thus both show reversible redox properties but the redox potentials were shifted. Compounds **2** and **3** also have very close structures but compound **2** is much more irreversible. Compound **33** did not show measurable redox signal besides the large peak due to electrolysis of water at 2.0 V, which is likely due to the low solubility.



**Figure 3.** Inversion correlation of laccase catalytic efficiency and oxidation potential of compounds **1–8**, **34**, **35**, ABTS, 2-aminophenol, catechol, hydroquinone, and 1,2-phenylenediamine.

**Table 1**

Kinetic constants of fungal laccase with known laccase substrates and substituted phenols

Substrate	$k_{\text{cat}}$ (min <sup>-1</sup> )	$K_m$ (mM)	$k_{\text{cat}}/K_m$ (min <sup>-1</sup> mM <sup>-1</sup> )	$\epsilon$ (M <sup>-1</sup> cm <sup>-1</sup> ) <sup>a</sup>	Wavelength (nm)
Hydroquinone	7130	0.02	35,650	17,252 <sup>34</sup>	248
Catechol	2010	0.60	3350	2211 <sup>34</sup>	450
2-Aminophenol	2250	0.19	11,840	17,865 <sup>35</sup>	434
1,2-Phenylenediamine	1040	0.43	2420	13,000 <sup>36</sup>	440
ABTS	8360	0.09	92,890	36,000 <sup>34</sup>	414
Compound <b>1</b>	1100	0.07	15,710	2590	400
Compound <b>2</b>	6310	2.20	2870	762	410
Compound <b>3</b>	1900	0.04	47,500	3600	440
Compound <b>4</b>	8680	0.05	173,600	1700	500
Compound <b>5</b>	5380	0.04	134,500	876	450
Compound <b>6</b>	5760	0.07	82,290	2280	350
Compound <b>7</b>	7870	0.11	71,550	5180	390
Compound <b>8</b>	3780	0.08	47,250	9840	390
Compound <b>34</b>	2930	0.07	41,860	14,480	440
Compound <b>35</b>	2550	0.17	15,000	13,760	440

<sup>a</sup> The extinction coefficients ( $\epsilon$ ) are for the oxidized products. Extinction coefficients for oxidized hydroquinone, catechol, 2-aminophenol, 1,2-phenylenediamine, and ABTS have been reported previously.<sup>34–36</sup>

It is noted that compounds **9–15**, **18**, and **33** are the most water-insoluble materials. This causes unreliable CV measurements in aqueous PBS buffer solution containing 20% ethanol as reported in this study. They either present high oxidation potentials ( $>0.68$  V) or do not show oxidation waves within the applicable potential range in aqueous solution (with an upper limit at  $\sim+0.8$  to 1.2 V in most experiments). These compounds likely absorb into larvae and produce larvae-killing activity (vide infra).

To determine how well fungal laccase can oxidize the water-soluble compounds, we determined the kinetic constants of fungal laccase using compounds **1–8**, **34**, and **35** along with known

laccase substrates such as hydroquinone, catechol, 2-aminophenol, 1,2-phenylenediamine, and ABTS.<sup>33</sup> Results are summarized in Table 1. The known laccase substrates serve as positive controls and for comparison. A relationship between catalytic efficiency ( $k_{cat}/K_m$ ) and redox potential is depicted in Figure 3.

As shown in Figure 3, there is an inverse correlation between laccase catalytic efficiency and oxidation potential of the water-soluble compounds **1–8**, **34**, **35**, ABTS, 2-aminophenol, catechol, hydroquinone, and 1,2-phenylenediamine. These results are consistent with previous studies.<sup>37–39</sup> These compounds show either nearly reversible or quasi-reversible redox properties except 1,2-phenylenediamine. The lower the peak potential for oxidation (i.e.  $E_{pa1}$  in Table S1), the higher the laccase activity is. 1,2-Phenylenediamine shows a very high oxidation potential and thus gives the lowest laccase activity. However, no clear correlation was found between the electrochemical reaction rate and reversibility of the compounds, both of which are reflected by the peak separation redox waves (i.e.  $\Delta E_p = E_{pa} - E_{pc}$ ). Compounds **9–15**, **33**, and MON-0585 are insoluble in water, and their oxidation by laccase could not be detected.

### 2.3. Anti-mosquito larval activities

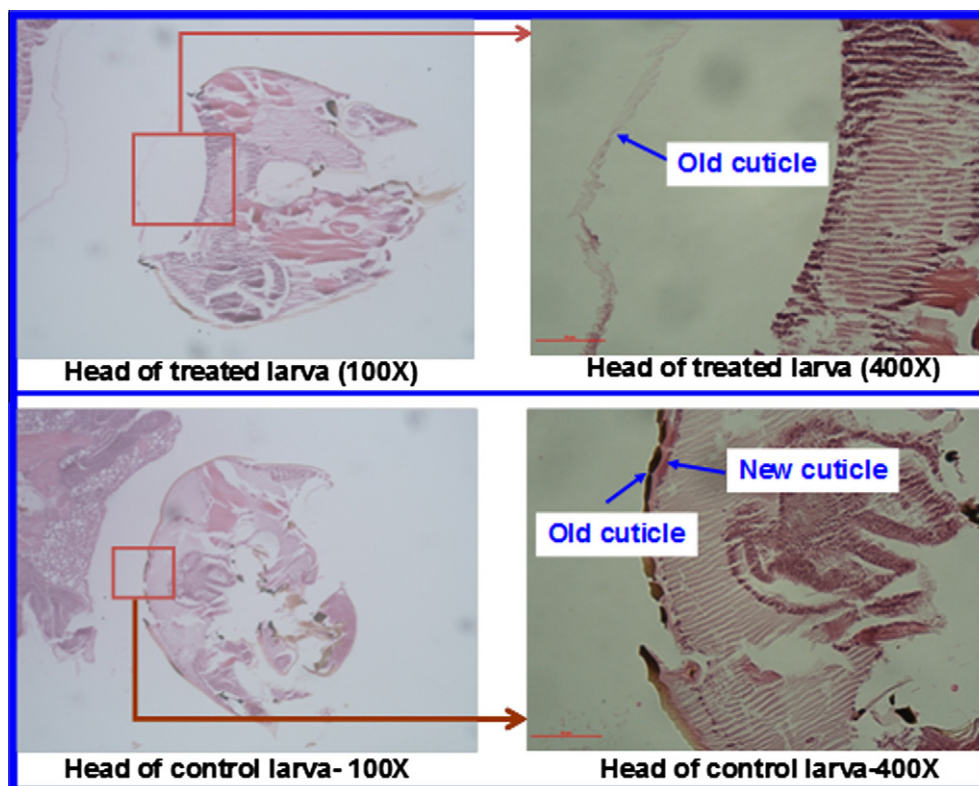
Since laccases oxidize phenolic compounds in their function in insect cuticle sclerotization,<sup>9</sup> we investigated potential anti-mosquito larval activities of laccase substrates such as compound **4** and dihydroquinone, di-*t*-butylphenols such as compounds **9–15**, compound **18**, and **33**. MON-0585 was used as a positive control. Table 2 summarizes toxicities of substituted phenols to third-instar larvae of *Anopheles gambiae* in a three-day bioassay. The water-soluble samples such as dihydroquinone and triol **4** were dissolved in water and used, while other water-insoluble materials were dissolved in acetone and used for the treatment of mosquito larvae in aqueous solution. Laccase substrates such as dihydroquinone

**Table 2**

Toxicities of substituted phenols to third-instar larvae of *Anopheles gambiae* in a three-day bioassay

Compound	% Mortality (mean $\pm$ SE)	
	50 $\mu$ g/L	1000 $\mu$ g/L
Control	0 $\pm$ 0 b	3.50 $\pm$ 3.50 d
Dihydroquinone	1.25 $\pm$ 1.25 b	5.38 $\pm$ 1.80 d
Compound <b>4</b>	0 $\pm$ 0 b	11.4 $\pm$ 3.85 d
Compound <b>9</b>	0 $\pm$ 0 b	60.0 $\pm$ 11.5 c
Compound <b>10</b>	0 $\pm$ 0 b	46.7 $\pm$ 3.25 c
Compound <b>11</b>	0 $\pm$ 0 b	65.0 $\pm$ 5.00 c
Compound <b>12</b>	5.50 $\pm$ 3.45 b	12.3 $\pm$ 1.35 d
Compound <b>13</b>	5.41 $\pm$ 3.28 b	93.1 $\pm$ 3.99 ab
Compound <b>14</b>	3.71 $\pm$ 2.14 b	7.70 $\pm$ 3.14 d
Compound <b>15</b>	98.1 $\pm$ 1.93 a	98.3 $\pm$ 1.68 ab
Compound <b>18</b>	5.00 $\pm$ 3.18 b	6.66 $\pm$ 2.71 d
Compound <b>33</b>	5.77 $\pm$ 3.69 b	91.7 $\pm$ 6.31 b
MON-0585	93.3 $\pm$ 4.71 a	100 $\pm$ 0 a

Results are mean  $\pm$  standard errors (SE) of four replicates ( $n = 4$ ); each with 12–15 third-instar larvae. Means followed by the same letter in the same column are not significantly different ( $P > 0.05$ ) according to Fisher's LSD multiple comparison test after arcsine square root transformation of the percentage data (ProStat; Poly Software International, 2002).



**Figure 4.** Microscopic comparisons of *An. gambiae* larvae in the control and killed by compound **15**.

and compound **4** and quinone **18** were not toxic at the concentrations tested, however, di-*t*-butylphenols including benzopyrane **33** (without phenolic OH function) produced significant mortality at 1000 µg/mL concentration. Most strikingly, compound **15** possesses similar anti-larval activity to that of MON-0585 at 50 µg/mL concentration. The trifluoroacetic acid salt of amine **14** and sodium salt of carboxylic acid **12** are water-soluble and they do not possess anti-larval activity. Hence, the water-soluble compounds may not have been absorbed by larvae and consequently lacked anti-larval activity. Water-insoluble materials such as compound **15** likely absorb into larvae and produce larvae-killing activity. Compound **15** appears to be much more toxic to mosquito larvae than methoprene, which caused only about 60% mortality at 100 µg/L in 3 days against the third-instar larvae of *Culex molestus*.<sup>40</sup> Larvae treated with di-*tert*-butylphenolic compounds died just before pupation. Because the larval mortality occurred over the course of 3 days, it suggests that the target does not involve the neurological system typical of most insecticides. We examined microscopic sections of larvae from the control and compound **15** treated groups, and found that the cuticle was thin in compound **15** treated larvae when compared to the control larvae (Fig. 4). The treated larvae appeared to start molting by separating the larval cuticle, but failed to remove the old cuticle. There was very little apparent synthesis of new pupal cuticle in the treated larvae. This was observed not only in the head, but also in the thorax and abdomen of the insects killed by compound **15**. Thus, it appears that these compounds affect the development of the cuticle, a target of the mosquito that is different from the neurological targets of currently existing insecticides. However, the exact target and mechanism of action of these compounds remain to be studied.

### 3. Conclusion

Oxidation of phenols by laccases led us to synthesize various phenolic compounds containing hydroxyl, halide, aldehyde, amino, and di-*tert*-butyl moieties. The redox potential, laccase activity, and antilarval activity were investigated. Phenolic compounds containing hydroxyl, halide, aldehyde, and amino functions were found to be laccase substrates but not inhibitors. The redox potentials and laccase activities of various phenolic compounds were correlated. It was found that a decrease of oxidation potential leads to higher laccase activity. A number of di-*tert*-butylphenolic compounds such as compounds **13**, **15** and **33** were found to have potent anti-larval activity. Their mechanism of action appears to be different from that of MON-0585 and remains to be investigated.

## 4. Experimental section

### 4.1. General methods

Nuclear magnetic resonance spectra were obtained at 400 MHz for <sup>1</sup>H and 100 MHz for <sup>13</sup>C in deuteriochloroform, unless otherwise indicated, and reported in ppm. Infrared spectra were taken from a Nicolet 380 FT-IR instrument (Thermo Scientific) in solid forms and are reported in wave numbers (cm<sup>-1</sup>). Low-resolution mass spectra were taken from an API 2000-triple quadrupole ESI-MS/MS mass spectrometer (from Applied Biosystems). High-resolution Mass spectra were obtained from a LCT Premier (Waters Corp., Milford MA) time of flight mass spectrometer. The instrument was operated at 10,000 resolution (W mode) with dynamic range enhancement that attenuates large intensity signals. Mass correction for exact mass determinations was made automatically with the lock mass feature in the MassLynx data system. A reference compound in an auxiliary sprayer is sampled every third cycle by toggling a 'shutter' between the analysis and reference needles.

The reference mass is used for a linear mass correction of the analytical cycles. Cyclic voltammetric experiments were performed on a CHI400A potentiostat (CH Instruments, TX) with a three-electrode setup consisting of a 3-mm diameter glassy carbon disk electrode embedded in epoxy as a working electrode, a Ag/AgCl (saturated KCl) as the reference electrode, and a coiled Pt wire as the counter electrode. 4-Hydroxy-3-nitrobenzaldehyde, 3-amino-4-hydroxybenzoic acid, 4-amino-3-hydroxybenzoic acid, and 2-methyl-3-buten-2-ol were purchased from Aldrich Co., and *L*-alanine benzyl ester *p*-toluenesulfonic acid salt was from Advanced CHEMTECH. *Trametes versicolor* (fungal) laccase was purchased from Sigma (product # 53739). The compounds, **1–15**, **33**, and MON-0585 used in biological studies are ~98% pure as indicated by HPLC analysis (see Supplementary data for HPLC graphs).

### 4.2. *O*-Benzyl *N*-(6-Chloro-2,5-dioxo-4-methoxy-1,4-cyclohexadienyl)-*L*-alanine (**18**)

A solution of 50 mg (0.20 mmol) of quinone **16**, 42 mg (0.2 mmol) of *L*-alanine benzyl ester *p*-toluenesulfonic acid salt, and 40 mg (0.40 mmol) of triethylamine in 5 mL of dichloromethane under argon was stirred at 25 °C for 12 h. The reaction solution was diluted with dichloromethane and washed with water and brine, dried (MgSO<sub>4</sub>), concentrated, and column chromatographed on silica gel using a gradient mixture of hexane and ethyl acetate as eluant to give 45 mg (65% yield) of compound **18**. IR (neat)  $\nu$  3300, 3100 (w), 2939, 1735, 1663, 1580, 1500, 1445, 1381, 1258, 1190, 1123, 1004, 829, 694; <sup>1</sup>H NMR  $\delta$  7.37 (s, 5H, Ph), 5.78 (s, 1H, =CH), 5.21 (s, 2H, CH<sub>2</sub>Ph), 5.20 (pentet, *J* = 8 Hz, 1H, CHN), 3.85 (s, 3H, OMe), 1.56 (d, *J* = 8 Hz, 3H, Me); <sup>13</sup>C NMR  $\delta$  185.2, 180.9, 172.3 (2C), 161.3, 141.8, 135.1, 128.9 (2C), 128.8 (2C), 128.5, 103.2, 67.8, 57.1, 52.0, 20.4; MS *m/z* 372.2 (M+Na<sup>+</sup>), 350.2 (M+1); HRMS calcd for C<sub>17</sub>H<sub>16</sub>ClNNaO<sub>5</sub><sup>+</sup> (M+Na<sup>+</sup>) 372.0609, found 372.0614.

### 4.3. 4-(*tert*-Butyldimethylsilyloxy)-3-chloro-5-methoxybenzaldehyde (**19**)

To a solution of 0.30 g (1.61 mmol) of aldehyde **2**<sup>20</sup> in 5 mL of dichloromethane at 0 °C under argon, were added 0.27 ml (1.93 mmol) of triethylamine, 39 mg of 4-dimethylaminopyridine (0.32 mmol), and 0.29 g (1.93 mmol) of *t*-butyldimethylsilyl chloride. The solution was stirred at 25 °C for 8 hours, diluted with 50 mL of diethyl ether, washed with water and brine, dried (MgSO<sub>4</sub>), concentrated, and column chromatographed on silica gel using a mixture of hexane and diethyl ether (4:1) as eluant to give 0.47 g (97% yield) of compound **19**. <sup>1</sup>H NMR  $\delta$  9.76 (s, 1H), 7.45 (d, *J* = 2.0 Hz, 1H), 7.27 (d, *J* = 2.0 Hz, 1H), 3.85 (s, 3H), 1.01 (s, 9H), 0.21 (s, 6H); <sup>13</sup>C NMR  $\delta$  190.0, 151.9, 147.7, 130.0, 126.6, 126.3, 108.4, 55.6, 25.8 (3C), 19.0, -3.8 (2C); HRMS calcd for C<sub>14</sub>H<sub>22</sub>ClO<sub>3</sub>Si (M+H<sup>+</sup>) 301.1021, found 301.1028.

### 4.4. 4-(*tert*-Butyldimethylsilyloxy)-3-chloro-5-methoxyphenol (**20**)

To a solution of 0.20 g (0.66 mmol) of compound **19** in 10 mL of dichloromethane under argon at 25 °C, was added 0.25 g (1.0 mmol) of *m*-chloroperbenzoic acid (70% purity), and the solution was heated to reflux for 8 h. The reaction solution was cooled to 25 °C, diluted with aqueous sodium thiosulfate, and extracted three times with diethyl ether. The organic layers were combined, washed with saturated aqueous NaHCO<sub>3</sub>, water, and brine, dried (MgSO<sub>4</sub>), concentrated, and column chromatographed on silica gel using a mixture of hexane and diethyl ether (2:1) as eluant to give 0.21 g (98% yield) of 4-(*tert*-butyldimethylsilyloxy)-3-chloro-5-methoxyphenyl formate (**20A**). <sup>1</sup>H NMR  $\delta$  8.26 (s, 1H, OCHO),

6.78 (d,  $J = 2.9$  Hz, 1H), 6.58 (d,  $J = 2.9$  Hz, 1H), 3.80 (s, 3H), 1.03 (s, 9H), 0.20 (s, 6H);  $^{13}\text{C}$  NMR  $\delta$  159.3, 151.8, 143.2, 140.4, 125.9, 114.4, 104.1, 55.7, 26.0, 19.0,  $-3.9$ ; HRMS calcd for  $\text{C}_{14}\text{H}_{22}\text{ClO}_4\text{Si}$  ( $\text{M}+\text{H}^+$ ) 317.0976, found 317.0968.

A solution of 0.21 g (0.65 mmol) of formate **20A** and 0.45 g (3.2 mmol) of  $\text{K}_2\text{CO}_3$  in 10 mL of methanol was stirred at 25 °C for 4 h, and the solution was diluted with 10 mL of aqueous  $\text{NH}_4\text{Cl}$ . The solution was extracted with ethyl acetate twice, and the combined extract was washed with brine, dried ( $\text{MgSO}_4$ ), concentrated, and column chromatographed on silica gel using a mixture of hexane and diethyl ether (2:1) as eluant to give 0.14 g (72% yield) of phenol **20**:  $^1\text{H}$  NMR  $\delta$  6.43 (d,  $J = 2.8$  Hz, 1H), 6.32 (d,  $J = 2.8$  Hz, 1H), 3.77 (s, 3H), 1.03 (s, 9H), 0.17 (s, 6H);  $^{13}\text{C}$  NMR  $\delta$  152.2, 149.8, 135.8, 125.7, 108.2, 99.2, 55.5, 26.1 (3C), 19.0,  $-4.0$  (2C); HRMS calcd for  $\text{C}_{13}\text{H}_{22}\text{ClO}_3\text{Si}$  ( $\text{M}+\text{H}^+$ ) 289.1027, found 289.1000.

#### 4.5. 2-Bromo-4-(tert-butyldimethylsilyloxy)-3-chloro-5-methoxyphenol (21) and 2-bromo-3-chloro-5-methoxy-1,4-benzoquinone (16)

To a solution of 84 mg (0.29 mmol) of phenol **20** in 2 mL of DMF under argon was added 57 mg (0.32 mmol) of *N*-bromosuccinimide, and the solution was stirred at 25 °C for 12 h. The reaction solution was diluted with diethyl ether and washed with water and brine, dried ( $\text{MgSO}_4$ ), concentrated, and column chromatographed on silica gel using a gradient mixture of hexane and diethyl ether as eluant to give 27 mg (31% yield) of bromophenol **21** and 35 mg (48% yield) of *p*-benzoquinone **16**. Bromophenol **21**:  $^1\text{H}$  NMR  $\delta$  6.56 (s, 1H), 5.38 (br s, 1H, OH), 3.78 (s, 3H), 1.03 (s, 9H), 0.18 (s, 6H);  $^{13}\text{C}$  NMR  $\delta$  151.5, 147.4, 136.8, 125.8, 101.5, 98.4, 55.6, 26.1, 19.0,  $-3.9$ ; HRMS calcd for  $\text{C}_{13}\text{H}_{19}\text{BrClO}_3\text{Si}$  ( $\text{M}-\text{H}$ ) 364.9975, found 365.0076;  $\text{C}_{13}\text{H}_{20}\text{BrClO}_3\text{SiNa}$  ( $\text{M}+\text{Na}^+$ ) 388.9951, found 388.9934. *p*-Benzoquinone **16**: light yellow solids; Mp. 167–170 °C; IR (neat)  $\nu$  2953, 2913, 2839, 1683, 1638, 1613, 1556, 1454, 1229, 1168  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR  $\delta$  6.16 (s, 1H), 3.89 (s, 3H);  $^{13}\text{C}$  NMR  $\delta$  177.5, 172.4, 158.9, 137.4, 107.9, 107.2, 57.3; HRMS calcd for  $\text{C}_7\text{H}_5\text{BrClO}_3$  ( $\text{M}+\text{H}^+$ ) 250.9105, found 251.0593.

#### 4.6. Conversion of silyl ether 21 to *p*-benzoquinone 16

A solution of 25 mg (68  $\mu\text{mol}$ ) of silyl ether **21** and 68  $\mu\text{L}$  (68  $\mu\text{mol}$ ) of *n*- $\text{Bu}_4\text{NF}$  (1 M in THF) in 2 mL of THF was stirred under argon from 0 °C for 30 min., diluted with 10 mL of aqueous  $\text{NH}_4\text{Cl}$ , and extracted with ethyl acetate twice. The combined extract was washed with brine, dried ( $\text{MgSO}_4$ ), concentrated and column chromatographed on silica gel using a mixture of hexane/diethyl ether (1:1) as eluant to give 14 mg (81% yield) of *p*-benzoquinone **16**, which spectral data are identical with that described above.

#### 4.7. 2-Bromo-3-chloro-5-methoxy-1,4-dihydroxybenzene (1)

A mixture of 0.10 g (0.40 mmol) of quinone **16** and 20 mg of 10% palladium over carbon in 10 mL of ethanol under 1 atm. of hydrogen was stirred at 25 °C for 15 min., filtered through Celite, and washed with ethanol. The filtrate was concentrated to give 99 mg (98% yield) of dihydroxybenzene **1**: IR (neat)  $\nu$  3303, (s), 2935, 2851, 1601, 1497, 1441, 1210, 1071, 1046, 994, 864, 842, 825  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR  $\delta$  6.60 (s, 1H), 5.50 (br s, 1H, OH), 5.30 (br s, 1H, OH), 3.89 (s, 3H, OMe);  $^{13}\text{C}$  NMR  $\delta$  148.2, 147.7, 136.7, 121.1, 100.2, 99.4, 56.0; HRMS calcd for  $\text{C}_7\text{H}_6\text{BrClO}_3$  ( $\text{M}^+$ ) 251.9189, found 252.0427.

#### 4.8. 2-Bromo-3-chloro-4,5-dibenzoyloxyphenol (23)

A solution of 0.20 g (0.37 mmol) of silyl ether **22**<sup>20</sup> and 0.37 mL of *n*- $\text{Bu}_4\text{NF}$  (1 M solution in THF) in 4 mL of THF was stirred under

argon at 0 °C for 1 h. The solution was diluted with 30 mL of saturated aqueous NaCl solution, extracted twice with ethyl acetate (30 mL each), and the organic extract was dried ( $\text{MgSO}_4$ ), filtered, concentrated to dryness, and column chromatographed on silica gel using a gradient mixture of hexane and diethyl ether as eluant to give 0.12 g (77% yield) of compound **23** as white solids, mp 108–110 °C;  $^1\text{H}$  NMR  $\delta$  7.40–7.20 (m, 10H), 6.66 (s, 1H), 5.07 (s, 2H), 4.96 (s, 2H);  $^{13}\text{C}$  NMR  $\delta$  153.1, 149.9, 139.8, 137.0, 136.1, 129.2, 128.8, 128.5, 128.44, 128.40, 127.6, 102, 100.6, 75.4, 71.3; HRMS calcd for  $\text{C}_{20}\text{H}_{17}\text{BrClO}_3$  ( $\text{M}+\text{H}^+$ ) 419.0044, found 419.0051.

#### 4.9. 5-Bromo-6-chlorobenzene-1,2,4-triol (4)

To a solution of compound **23** (25 mg, 0.06 mmol) in 1 mL of ethanol was added 2.5 mg of 10% palladium over carbon, and the mixture was stirred under 1 atmosphere of hydrogen at 25 °C for 3 h. The reaction mixture was filtered through Celite, concentrated to dryness to give 13 mg (93% yield) of compound **4**: Mp. >350 °C; IR (neat)  $\nu$  3382 (br s, OH stretch), 2932, 2843, 1614, 1437, 1285, 1170, 1070  $\text{cm}^{-1}$ ; UV (in methanol)  $\lambda$  209.6 ( $\epsilon_{\text{max}} = 30,300$ ), 291.8 (1250; likely derived from a partial oxidation of the polyphenol functions), 332.5 (4640), 397.1 (1785) nm;  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  6.66 (s, 1H, Ar), 5.58 (br s, 1H, OH), 5.27 (br s, 1H, OH), 5.21 (br s, 1H, OH);  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ )  $\delta$  149.4, 147.6, 137.4, 123.1, 103.2, 100.6; HRMS calcd for  $\text{C}_6\text{H}_5\text{BrClO}_3$  ( $\text{M}+\text{H}^+$ ) 238.9105, found 238.9111.

#### 4.10. 2-Bromo-4,5-bis-(tert-butyldimethylsilyloxy)-3-chloro-1-methoxybenzene (25)

To a solution of 0.10 g (0.21 mmol) of **24**<sup>20</sup> in 2 mL of dichloromethane at 0 °C under argon, were added 55 mg (0.26 mmol) of proton sponge 38 mg (0.26 mmol) of trimethyloxonium tetrafluoroborate, and the mixture was stirred at 0 °C for 8 h. It was diluted with water and extracted twice with diethyl ether, the combined extract was washed with water and brine, dried ( $\text{MgSO}_4$ ), concentrated, and column chromatographed on silica gel using a mixture of hexane and diethyl ether (10:1) as eluant to give 67 mg (94% yield based on reacted **24**) of **25** along with 31 mg of recovered **24**.  $^1\text{H}$  NMR  $\delta$  6.43 (s, 1H), 3.80 (s, 3H), 1.03 (s, 9H), 0.98 (s, 9H), 0.23 (s, 6H), 0.17 (s, 6H);  $^{13}\text{C}$  NMR  $\delta$  151.0, 147.5, 138.9, 128.9, 104.7, 104.5, 56.9, 26.3 (6C), 18.9, 18.8,  $-3.3$  (2C),  $-3.5$  (2C); HRMS calcd for  $\text{C}_{19}\text{H}_{35}\text{BrClO}_3\text{Si}_2$  ( $\text{M}+\text{H}^+$ ) 481.0996, found 481.0951.

#### 4.11. 4-Bromo-3-chloro-5-methoxybenzene-1,2-diol (5)

To a solution of 51 mg (0.10 mmol) of **25** in 2 mL of THF under argon at 0 °C, was added 0.20 mL (0.20 mmol) of *n*- $\text{Bu}_4\text{NF}$ , and the solution was stirred for 30 min. It was diluted with diethyl ether, washed with water and brine, dried ( $\text{MgSO}_4$ ), concentrated, and column chromatographed on silica gel using a gradient mixture of dichloromethane and methanol as eluant to give 19 mg (71% yield) of **5**. IR (neat)  $\nu$  3436, 3219 (broad & s), 2917, 2851, 1580, 1462, 1417, 1315, 1188, 1070, 984, 820  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR  $\delta$  6.59 (s, 1H), 5.60 (br s, 1H, OH), 5.24 (br s, 1H, OH), 3.83 (s, 3H);  $^{13}\text{C}$  NMR  $\delta$  151.4, 144.4, 134.2, 121.8, 101.7, 99.7, 57.1; HRMS calcd for  $\text{C}_7\text{H}_7\text{BrClO}_3$  ( $\text{M}+\text{H}^+$ ) 252.9267, found 252.9254.

#### 4.12. 3-(tert-Butyldimethylsilyloxy)-5-chloro-4-hydroxybenzaldehyde (26)

To a solution of 3.2 g (19 mmol) of aldehyde **3**, 2.9 mL (22 mmol) of triethylamine, and 0.45 g (3.7 mmol) of 4-dimethylaminopyridine in 60 mL of dichloromethane at 0 °C under argon, was added 3.4 g (22 mmol) of *t*-butyldimethylsilyl chloride, and the solution was stirred at 25 °C for 8 h. It was diluted with diethyl



ether, washed with water and brine, dried (MgSO<sub>4</sub>), concentrated, and column chromatographed on silica gel using hexane and dichloromethane (1:1) as eluant to give 3.7 g (70% yield) of compound **26** and 0.84 g (11% yield) of 5-chloro-3,4-bis-(*t*-butyldimethylsilyloxy)benzaldehyde. Compound **26** was crystallized from diethyl ether to provide single crystals and its structure was unequivocally identified by a single-crystal X-ray analysis. <sup>1</sup>H NMR δ 9.76 (s, 1H, CHO), 7.51 (d, *J* = 2.8 Hz, 1H), 7.27 (d, *J* = 2.8 Hz, 1H), 6.24 (s, 1H, OH), 1.03 (s, 9H), 0.32 (s, 6H); <sup>13</sup>C NMR δ 189.9, 149.9, 144.2, 129.4, 126.8, 120.7, 115.9, 25.8, 18.4, -4.2; HRMS calcd for C<sub>13</sub>H<sub>20</sub>ClO<sub>3</sub>Si (M+H<sup>+</sup>) 287.0870, found 287.0858.

5-Chloro-3,4-bis-(*t*-butyldimethylsilyloxy)benzaldehyde: <sup>1</sup>H NMR δ 9.78 (s, 1H), 7.50 (d, *J* = 2 Hz, 1H), 7.29 (d, *J* = 2 Hz, 1H), 1.05 (s, 9H), 0.99 (s, 9H), 0.27 (s, 6H), 0.24 (s, 6H); <sup>13</sup>C NMR δ 190.1, 149.9, 149.4, 130.3, 128.0, 126.0, 119.0, 26.3 (3C), 26.2 (3C), 19.0, -3.2, -3.4; HRMS calcd for C<sub>19</sub>H<sub>34</sub>ClO<sub>3</sub>Si<sub>2</sub> (M+H<sup>+</sup>) 401.1735, found 401.1747.

#### 4.13. 3-(*tert*-Butyldimethylsilyloxy)-5-chloro-4-methoxybenzaldehyde (**27**)

To a solution of 0.17 g (0.59 mmol) of compound **26** in 3 mL of dichloromethane at 0 °C under argon, were added 0.26 g (1.2 mmol) of proton sponge and 0.18 g (1.2 mmol) of trimethylxonium tetrafluoroborate and the mixture was stirred at 0 °C for 8 h. The reaction mixture was diluted with water and extracted twice with diethyl ether. The combined extract was washed with water and brine, dried (MgSO<sub>4</sub>), concentrated, and column chromatographed on silica gel using a mixture of hexane and diethyl ether (10:1) as eluant to give 0.35 (79% yield) of **27**: <sup>1</sup>H NMR δ 9.67 (s, 1H, CHO), 7.31 (d, *J* = 2 Hz, 1H), 7.06 (d, *J* = 2 Hz, 1H), 3.69 (s, 3H), 0.81 (s, 9H), 0.02 (s, 6H); <sup>13</sup>C NMR δ 190.1, 153.4, 150.9, 132.8, 129.8, 125.5, 119.9, 60.7, 25.8, 18.4, -4.4; HRMS calcd for C<sub>14</sub>H<sub>22</sub>ClO<sub>3</sub>Si (M+H<sup>+</sup>) 301.1027, found 301.1421; negative ion detection mode: C<sub>14</sub>H<sub>20</sub>ClO<sub>3</sub>Si (M<sup>-</sup>) 299.0870, found 298.9978.

#### 4.14. 3-(*tert*-Butyldimethylsilyloxy)-5-chloro-4-methoxyphenyl formate (**28**)

To a solution of 0.21 g (0.70 mmol) of aldehyde **27** in 2 mL of dichloromethane under argon, was added 0.18 g (1.1 mmol) of *m*-chloroperbenzoic acid (70%), and the solution was heated to reflux for 8 h. The reaction solution was diluted with 5 mL of aqueous sodium thiosulfate and extracted twice with diethyl ether. The combined extract was washed with saturated aqueous NaHCO<sub>3</sub>, water, and brine, dried (MgSO<sub>4</sub>), concentrated, and column chromatographed on silica gel using a mixture of hexane and diethyl ether (10:1) as eluant to give 0.16 g (71% yield) of **28**. <sup>1</sup>H NMR δ 8.23 (s, 1H, OCHO), 6.83 (d, *J* = 2.6 Hz, 1H), 6.59 (d, *J* = 2.6 Hz, 1H), 3.81 (s, 3H), 1.01 (s, 9H), 0.21 (s, 6H); <sup>13</sup>C NMR δ 158.9, 150.7, 146.6, 145.4, 129.1, 115.8, 113.6, 60.7, 25.8 (3C), 18.4, -4.5; HRMS calcd for C<sub>14</sub>H<sub>22</sub>ClO<sub>4</sub>Si (M+H<sup>+</sup>) 317.0976, found 317.1355.

#### 4.15. 3-(*tert*-Butyldimethylsilyloxy)-5-chloro-4-methoxyphenol (**29**)

A solution of 0.16 g (0.50 mmol) of compound **28** and 0.34 g (2.50 mmol) of K<sub>2</sub>CO<sub>3</sub> in 5 mL of methanol was stirred at 25 °C for 4 h and diluted with diethyl ether. The mixture was washed with water and brine, dried (MgSO<sub>4</sub>), concentrated, and column chromatographed on silica gel using a mixture of hexane and diethyl ether (10:1) as eluant to give 0.14 g (95% yield) of **29**: <sup>1</sup>H NMR δ 6.48 (d, *J* = 2.6 Hz, 1H), 6.29 (d, *J* = 2.6 Hz, 1H), 3.75 (s, 3H), 1.01 (s, 9H), 0.20 (s, 6H); <sup>13</sup>C NMR δ 152.0, 150.7, 142.6, 128.1, 110.8,

108.6, 60.7, 25.8 (3C), 18.4, -4.5; HRMS calcd for C<sub>13</sub>H<sub>22</sub>ClO<sub>3</sub>Si (M+H<sup>+</sup>) 289.1027, found 289.1041.

#### 4.16. 2-Bromo-5-(*tert*-butyldimethylsilyloxy)-3-chloro-4-methoxyphenol (**30**)

To a solution of 90 mg (0.31 mmol) of **29** in 2 mL of DMF under argon was added 61 mg (0.34 mmol) of *N*-bromosuccinimide, and the solution was stirred at 25 °C for 12 h. It was diluted with diethyl ether, washed with water and brine, dried (MgSO<sub>4</sub>), concentrated, and column chromatographed on silica gel using a mixture of hexane and diethyl ether (10:1) as eluant to give 85 mg (74% yield) of **30**: Mp 44–45 °C; <sup>1</sup>H NMR δ 6.55 (s, 1H), 5.42 (s, 1H, OH), 3.77 (s, 3H), 1.01 (s, 9H), 0.20 (s, 6H); <sup>13</sup>C NMR δ 150.2, 149.6, 143.1, 124.2, 107.2, 103.0, 60.8, 25.8 (3C), 18.5, -4.5; HRMS calcd for C<sub>13</sub>H<sub>21</sub>BrClO<sub>3</sub>Si (M+H<sup>+</sup>) 367.0132, found 367.0142.

#### 4.17. 4-Bromo-5-chloro-6-methoxybenzene-1,3-diol (**6**)

To a solution of 50 mg (0.14 mmol) of **30** in 2 mL of THF under argon at 0 °C, was added 0.14 mL (0.14 mmol) of *n*-Bu<sub>4</sub>NF (1 M solution in THF), and the solution was stirred at 0 °C for 30 min. It was diluted with dichloromethane, washed with water and brine, dried (MgSO<sub>4</sub>), concentrated, and column chromatographed on silica gel using a gradient mixture of dichloromethane and methanol as eluants to give 23 mg (67% yield) of **6**. IR (neat) ν 3321 (br s), 3260, 2917, 1589, 1421, 1241, 984, 800; <sup>1</sup>H NMR δ 6.65 (s, 1H), 3.87 (s, 3H); <sup>13</sup>C NMR δ 150.5, 150.0, 138.7, 127.5, 101.8, 101.7, 61.5; HRMS calcd for C<sub>7</sub>H<sub>7</sub>BrClO<sub>3</sub> (M+H<sup>+</sup>) 252.9267, found 252.9279.

#### 4.18. 2-Amino-4-methylphenol (**7**) and 2-amino-4-(hydroxymethyl)phenol (**8**)

A mixture of 0.30 g (1.8 mmol) of 4-hydroxy-3-nitrobenzaldehyde (**31**) and 0.15 g of 10% Pd/C in 30 mL of ethanol was shaken on a hydrogenator under 30 psi atmosphere of hydrogen for 4 h. The reaction mixture was filtered through Celite and carefully washed with ethyl acetate. The filtrate was concentrated and column chromatographed on silica gel using a mixture of dichloromethane and methane (9:1) as eluant to give 91 mg (41% yield) of **7**<sup>23</sup> and 0.10 g (41% yield) of **8**.<sup>24</sup> Compound **7**: IR (neat) ν 3370 (sharp, m), 3301 (sharp, m), 2921, 1601, 1519, 1458, 1388, 1286, 878, 800 cm<sup>-1</sup>; <sup>1</sup>H NMR δ 6.62 (d, *J* = 7.6 Hz, 1H), 6.58 (d, *J* = 1.6 Hz, 1H), 6.48 (dd, *J* = 7.6, 1.6 Hz, 1H), 2.21 (s, 3H); <sup>13</sup>C NMR δ 141.9, 134.4, 131.2, 120.0, 118.1, 115.4, 20.9. Compound **8**: IR (neat) ν 3387 (sharp, m), 3313 (sharp, m), 3047 (broad), 2802, 1605, 1515, 1454, 1364, 1286, 1221, 1155, 1008, 816 cm<sup>-1</sup>; <sup>1</sup>H NMR (D<sub>2</sub>O) δ 6.88 (d, *J* = 1.2 Hz, 1H), 6.85 (d, *J* = 8 Hz, 1H), 6.77 (dd, *J* = 8, 1.2 Hz, 1H), 4.49 (s, 2H); <sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>) δ 142.9, 136.1, 133.5, 114.9, 113.8, 113.4, 63.3.

#### 4.19. 2-(3,5-Di-*tert*-butyl-4-hydroxyphenyl)-2-methylpropanal (**11**)

A solution of 0.17 g (0.61 mmol) of 2,6-di-*t*-butyl-4-(1,1-dimethyl-2-hydroxyethyl)phenol (**10**)<sup>25</sup> and 0.21 g (0.73 mmol) of IBX in 3 mL of DMSO was stirred under argon at 25 °C for 12 h, diluted with dichloromethane, washed with water and brine, dried (MgSO<sub>4</sub>), and concentrated to give 0.17 g (99% yield) of aldehyde **11**. Spectral data and TLC indicated the compound is pure and was used in subsequent reactions without purification. IR (neat) ν 3603 (OH), 2952, 2900, 2700 (C–H aldehyde), 1714 (C=O aldehyde), 1435, 1360, 1230, 1141, 1120, 903, 826, 744; <sup>1</sup>H NMR δ 9.45 (s, 1H, CHO), 7.06 (s, 2H, Ar), 5.22 (s, 1H, OH), 1.44 (s, 24H, Me); <sup>13</sup>C NMR δ 202.8 (C=O), 153.1, 136.2 (2C), 131.4, 123.6 (2C),

50.4, 34.8, 30.4 (*t*-Bu), 22.7; MS negative mode:  $m/z$  275.6 ( $M-1$ ); positive mode:  $m/z$  299.4 ( $M+Na^+$ ).

#### 4.20. 2-(3,5-Di-*tert*-butyl-4-hydroxyphenyl)-2-methylpropanoic acid (**12**)<sup>19</sup>

A silver oxide solution was prepared from 0.50 g (2.94 mmol) of silver nitrate and 0.24 g (5.88 mmol) of NaOH in 2 mL of water. To it, a solution of 0.16 g (0.59 mmol) of aldehyde **11** in 10 mL of 1,4-dioxane was added, and the solution was stirred at 25 °C for 12 h. The reaction solution was acidified with 1 N HCl to pH ~2 and extracted with diethyl ether twice. The combined ether extract was washed with water and brine, dried (MgSO<sub>4</sub>), concentrated, and filtered through a short silica gel column to give 0.14 g (82% yield) of carboxylic acid **12**.<sup>19</sup> IR (neat)  $\nu$  3200 (v. broad), 2953, 2864, 1646 (s, C=O), 1593 (C=C aromatic), 1454, 1360, 1307, 1237, 918, 874 cm<sup>-1</sup>; <sup>1</sup>H NMR  $\delta$  6.52 (s, 1H, Ar), 1.29 (s, 18H, *t*-Bu), 1.25 (s, 6H, Me); <sup>13</sup>C NMR  $\delta$  189.3, 187.9, 158.0 (2C), 137.3, 130.3 (2C), 35.7 (2C, Me), 30.3, 29.5 (6C, *t*-Bu), 22.4; MS  $m/z$  293.2 ( $M+1$ ).

#### 4.21. 2-(3,5-Di-*tert*-butyl-4-hydroxyphenyl)-2-methylpropanal oxime (**13**)

To a solution of 40 mg (0.14 mmol) of aldehyde **11** in 3 mL of acetonitrile and water (2:1), were added 25 mg (0.35 mmol) of NH<sub>2</sub>OH·HCl and 60 mg (0.43 mmol) of sodium acetate, and the solution was stirred at 25 °C for 1.5 h. The reaction solution was diluted with water and extracted twice with diethyl ether. The combined extract was concentrated to give 41 mg (97% yield) of oxime **13**: <sup>1</sup>H NMR  $\delta$  7.49 (s, 1H, CH=N), 7.12 (s, 2H, Ar), 1.47 (s, 6H, Me), 1.44 (s, 18H, *t*-Bu); <sup>13</sup>C NMR  $\delta$  158.7 (C=N), 152.5, 135.9, 135.7, 122.9 (2C), 41.0, 34.7, 30.5 (6C), 26.9; MS  $m/z$  314 ( $M+Na^+$ ).

#### 4.22. 4-[(2-Benzylamino)-1,1-dimethylethyl]-2,6-di-*tert*-butylphenol (**14**)

A solution of 47 mg (0.17 mmol) of aldehyde **11** and 18 mg (0.17 mmol) of benzylamine in 2 mL of methanol was stirred under argon at 25 °C for 12 h. To it, 16 mg (0.26 mmol) of NaCNBH<sub>3</sub> was added, and the resulting solution was stirred for 2 h, diluted with water, and extracted twice with diethyl ether. The combined extract was washed with water and brine, dried (MgSO<sub>4</sub>), concentrated to give 60 mg of amine **14** (97% yield). <sup>1</sup>H NMR  $\delta$  7.35–7.18 (m, 5H, Ph), 7.13 (s, 2H, Ar), 5.08 (broad s, 1H), 3.72 (s, 2H, CH<sub>2</sub>N), 2.68 (s, 2H, CH<sub>2</sub>N), 1.43 (s, 18H, *t*-Bu), 1.33 (s, 6H, Me); <sup>13</sup>C NMR  $\delta$  151.92, 140.4, 137.8, 135.4, 128.5, 128.1, 127.0, 122.7, 61.5, 54.2, 38.6, 34.7, 30.6, 27.9; MS  $m/z$  368.5 ( $M+1$ ).

#### 4.23. 2,4-Di-*tert*-butyl-6-(3-methyl-2-butenyl)phenol (**15**) and 6,8-di-*tert*-butyl-2,2-dimethyl-3,4-dihydro-2H-chromene (**33**)

To a cold (-78 °C) solution of 0.12 g (1.4 mmol) of 2-methyl-3-buten-2-ol in 3 mL of dichloromethane under argon was added 0.20 g (1.4 mmol) of BF<sub>3</sub>•ether followed by 0.20 g (0.96 mmol) of 2,4-di-*tert*-butylphenol. The solution was warmed to 25 °C and stirred for 1 h, diluted with aqueous NaHCO<sub>3</sub>, and extracted twice with diethyl ether. The combined extract was washed with water and brine, dried (anhydrous Na<sub>2</sub>SO<sub>4</sub>), concentrated, and column chromatographed on silica gel using 1% diethyl ether in hexane as eluant to give 0.12 g (46% yield) of **15** and 26 mg (10% yield) of **33**. Compound **15**: <sup>1</sup>H NMR  $\delta$  7.22 (d,  $J=3$  Hz, 1H, Ar), 6.99 (d,  $J=3$  Hz, 1H, Ar), 5.34 (t, heptet,  $J=7, 1$  Hz, 1H, =CH), 3.37 (d,  $J=7$  Hz, 2H, CH<sub>2</sub>), 1.85 (s, 3H, Me), 1.80 (s, 3H, Me), 1.43 (s, 9H, *t*-Bu), 1.31 (s, 9H, *t*-Bu); <sup>13</sup>C NMR  $\delta$  151.5, 142.3, 135.9, 135.7, 126.0, 125.0, 122.6, 122.3, 35.1, 34.4, 31.9 (3C), 31.7, 30.0 (3C), 26.0, 18.2; MS negative mode:  $m/z$  273.8 ( $M-1$ ).

Compound **33**: <sup>1</sup>H NMR  $\delta$  7.14 (d,  $J=3$  Hz, 1H, Ar), 6.93 (d,  $J=3$  Hz, 1H, Ar), 2.79 (t,  $J=7$  Hz, 2H, CH<sub>2</sub>), 1.79 (t,  $J=7$  Hz, 2H, CH<sub>2</sub>), 1.39 (s, 9H, *t*-Bu), 1.36 (s, 6H, Me), 1.30 (s, 9H, *t*-Bu); <sup>13</sup>C NMR  $\delta$  150.3, 141.0, 136.9, 124.2, 121.8, 119.9, 73.9, 35.2, 34.3, 33.0, 31.9, 30.0, 27.3, 23.5; MS positive mode:  $m/z$  275.2 ( $M+1$ ).

#### 4.24. Cyclic voltammetry

Cyclic voltammetric experiments<sup>8,27</sup> were carried out with a three-electrode setup at a scan rate of 100 mV/s as described in General Methods. The glassy carbon electrode was polished with an alumina paste on a clean microcloth prior to the experiment. The electrochemical studies were performed in a 20% ethanol aqueous PBS buffer solution, and a concentration of 10 mM of the substrate was used. Ethanol is needed to dissolve some of the substrates. Experiments at pH 7.0 and 5.5 (acetic acid was added to adjust the pH to 5.5) were conducted. The solution was deoxygenated with nitrogen prior to the addition of the substrate. Cyclic voltammetric experiments for each substrate were carried out in triplicate and consistent results were obtained.

#### 4.25. Laccase assays

Laccase purified from the fungus *Trametes versicolor* was purchased from Sigma (product #53739). Laccase was dissolved in 50 mM ammonium acetate (pH 5), 50% glycerol, and was stored at -20 °C. We used peptide mass fingerprinting to determine that the most abundant protein in the laccase preparation was *T. versicolor* laccase IIIb, a 53 kDa enzyme (accession number AAL93622, data not shown). Product literature from Sigma stated that laccase was 10% of the total protein content. This was used to calculate the laccase concentration for determination of  $k_{cat}$  values. The most abundant protein was laccase, which was confirmed by peptide mapping.

Extinction coefficients for the oxidized products of compounds **1**, **3–8**, **34**, and **35** were estimated by fully oxidizing 100  $\mu$ M compound with 25 ng laccase, using a spectrophotometer to measure absorbance of the product at a particular wavelength (see Table 1), and converting the units to M<sup>-1</sup> cm<sup>-1</sup>.

Enzymatic reactions used to determine kinetic constants contained 0.58 nM laccase plus various concentrations of substrate in 100 mM sodium citrate, pH 5, in a volume of 200  $\mu$ L. The substrate concentrations used were typically 0.01, 0.02, 0.1, 0.2, 0.4, 0.6, 1, 2, and 4 mM. A spectrophotometer was used to monitor product formation (using wavelengths listed in Table 1). GraphPad Prism 4 was used to fit data to the Michaelis–Menten equation or a substrate inhibition equation. The Michaelis–Menten equation is:

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

The substrate inhibition equation is:

$$v = \frac{V_{max}[S]}{K_m + [S](1 + [S]/K_{si})}$$

Akaike's Information Criterion was used to select the equation with a better fit to the data. The appropriate equation was then used to estimate two kinetic constants,  $K_m$  and  $k_{cat}$ .

#### 4.26. Anti-larval assay

##### 4.26.1. Mosquito

A laboratory colony of *Anopheles gambiae* was initially obtained from the Malaria Research and Reference Reagent Resource Center (MR4) (Manassas, VA) and was cultured in the Department of Entomology at Kansas State University (Manhattan, KS) based on the method of Benedict<sup>41</sup> with modifications.<sup>42,43</sup>

#### 4.26.2. Bioassay of chemicals

The toxicities of the chemicals were screened using third-instar mosquito larvae at 25 °C as described.<sup>43</sup> After a group of 12–15 larvae was transferred into a glass beaker containing 99.5 mL of distilled water and 0.5 mL of larval food,<sup>41</sup> the stock solution of each chemical was added to the beaker to obtain final concentrations of 50 or 1000 µg/L. Each bioassay also included controls under the same conditions except that the same volume of acetone or water depending on the solvent used to dissolve the chemical was added to each beaker. All the treatments and controls were repeated four times and larval mortalities were assessed at 72 h.

#### Acknowledgment

This work was supported by a grant from the National Institutes of Health (AI070864). We thank Ms. Sharon R. Starkey for her technical assistance in carrying out the anti-larval studies, Mr. Stewart Gardner for his technical assistance in carrying out some of the lacase activity assays, Dr. Yasuaki Hiromasa for peptide mass fingerprinting, and Dr. Sundeep Rana for technical assistance in the synthesis.

#### Supplementary data

Supplementary data (Table S1, Figures S1–S4, and HPLC graphs of compounds 1–15) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2012.01.021.

#### References and notes

- Kunamneni, A.; Camarero, S.; Garcia-Burgos, C.; Plou, F. J.; Ballesteros, A.; Alcalde, M. *Microb. Cell Factories* **2008**, *7*, 32.
- Quarantino, D.; Federici, F.; Petruccioli, M.; Fenice, M.; D'Annibale, A. *Antonie van Leeuwenhoek* **2007**, *91*, 57.
- Baldrian, P. *FEMS Microbiol. Rev.* **2006**, *30*, 215.
- Rebrikov, D. N.; Stepanova, E. V.; Koroleva, O. V.; Budarina, A. I.; Zakharova, M. V.; Yurkova, T. V.; Solonin, A. S.; Belova, O. V.; Pozhidayeva, Z. A.; Leont'evsky, A. A. *Appl. Biochem. Microbiol.* **2006**, *42*, 564.
- Smirnov, S. A.; Koroleva, O. V.; Gavrilova, V. P.; Belova, A. B.; Klyachko, N. L. *Biochemistry* **2001**, *66*, 774.
- Xu, F.; Deussen, H.-J. W.; Lopez, B.; Lam, L.; Li, K. *Eur. J. Biochem.* **2001**, *268*, 4169.
- Garzillo, A. M. V.; Colao, M. C.; Caruso, C.; Caporale, C.; Celletti, D.; Buonocore, V. *Appl. Microbiol. Biotechnol.* **1998**, *49*, 545.
- Kramer, K. J.; Kanost, M. R.; Hopkins, T. L.; Jiang, H.; Zhu, Y. C.; Xu, R.; Kerwin, J. L.; Turecek, F. *Tetrahedron* **2001**, *57*, 385.
- Dittmer, N. T.; Kanost, M. R. *Insect Biochem. Mol. Biol.* **2010**, *40*, 179.
- Couto, S. R.; Toca, J. L. *Curr. Enzyme Inhib.* **2006**, *2*, 343.
- Johannes, C.; Majcherczyk, A. J. *Biotechnol.* **2000**, *78*, 193.
- Paterson, R. R. M.; Meon, S.; Zainal Abidin, M. A.; Lima, N. *Curr. Enzyme Inhib.* **2008**, *4*, 172.
- Lyashenko, A. V.; Bento, I.; Zaitsev, V. N.; Zhukhlistova, N. E.; Zhukova, Y.; Gabdoulkhakov, A. G.; Morgunova, E. Y.; Voelter, W.; Kachalova, G. S.; Stepanova, E. V.; Koroleva, O. V.; Lamzin, V. S.; Tishkov, V. I.; Betzel, C.; Lindley, P. F.; Mikhailov, A. M. *J. Biol. Inorg. Chem.* **2006**, *11*, 963.
- Giardina, P.; Faraco, V.; Pezzella, C.; Piscitelli, A.; Vanhulle, S.; Sannia, G. *Cell Mol. Life Sci.* **2010**, *67*, 369.
- Walton, B. T.; Sanborn, J. R.; Metcalf, R. L. *Pesticide Biochem. Physiol.* **1979**, *12*, 23.
- Semensi, V.; Sugumar, M. *Pesticide Biochem. Physiol.* **1986**, *26*, 220.
- Biggers, W. J.; Laufer, H. *Biol. Bull.* **2004**, *206*, 13.
- Nishinaga, A.; Shimizu, T.; Matsuura, T. *Tetrahedron Lett.* **1981**, *22*, 5293.
- Lai, J. T. *Tetrahedron Lett.* **2001**, *42*, 557.
- Hua, D. H.; Huang, X.; Chen, Y.; Battina, S. K.; Tamura, M.; Noh, S. K.; Koo, S. I.; Namatame, I.; Tomoda, H.; Perchellet, E. M.; Perchellet, J.-P. *J. Org. Chem.* **2004**, *69*, 6065.
- Godfrey, I. M.; Sargent, M. V.; Elis, J. A. *J. Chem. Soc., Perkin Trans. I* **1974**, 1353.
- Barakat, M. Z.; El-Wahab, M. F. A.; El-Sadr, M. M. *J. Am. Chem. Soc.* **1955**, *77*, 1670.
- Kikugawa, Y.; Tsuji, C.; Miyazawa, E.; Sakamoto, T. *Tetrahedron Lett.* **2001**, *42*, 2337.
- Touzeau, F.; Arrault, A.; Guillaumet, G.; Scalbert, E.; Pfeiffer, B.; Rettori, P.; Merour, J.-Y. *J. Med. Chem.* **1962**, *2003*, 46.
- Schwartz, L. H.; Flor, R. V. *J. Org. Chem.* **1969**, *34*, 1499.
- Masagutov, R. M.; Tolstikov, G. A.; Kirichenko, G. N.; Grigor'eva, N. G.; Tsypysheva, L. G. *Neftekhimiya* **1985**, *25*, 481.
- Kramer, K. J.; Nuntnarumit, C.; Aso, Y.; Hawley, M. D.; Hopkins, T. L. *Insect Biochem.* **1983**, *13*, 475.
- Brunet, P. C. J. *Insect Biochem.* **1980**, *10*, 467.
- Arakane, Y.; Muthukrishnan, S.; Beeman, R. W.; Kanost, M. R.; Kramer, K. J. *PNAS* **2005**, *102*, 11337.
- Hearing, V. J., Jr.; Ekel, T.; Montague, P. M.; Nicholson, J. M. *Biochim. Biophys. Acta* **1980**, *611*, 251.
- Hearing, V. J.; Jimenez, M. *Int. J. Biochem.* **1987**, *19*, 1141.
- Bard, A. J.; Faulkner, L. R. *Electrochemical Methods: Fundamental and Applications*, 2nd ed.; Wiley: New York, 2001.
- Osman, A. M.; Wong, K. K. Y.; Fernyhough, A. *Biochem. Biophys. Res. Commun.* **2006**, *346*, 321.
- Eggert, C.; Temp, U.; Eriksson, K.-E. L. *Appl. Environ. Microbiol.* **1996**, *62*, 1151.
- Fu, P. K.-L.; Abuzakm, S.; Turro, C. *Photochem. Photobiol.* **2005**, *81*, 89.
- Giurg, M.; Piekalska, K.; Gebala, M.; Ditkowski, B.; Wolanski, M.; Peczyńska-Czoch, W.; Mlochowski, J. *Synth. Commun.* **2007**, *37*, 1779.
- Xu, F. *Biochemistry* **1996**, *35*, 7608.
- Xu, F.; Shin, W.; Brown, S. H.; Wahleithner, J. A.; Sundaram, U. M.; Solomon, E. I. A. *Biochim. Biophys. Acta* **1996**, *1292*, 303.
- Tadesse, M. A.; D'Annibale, A.; Galli, C.; Gentili, P.; Sergi, F. *Org. Biomol. Chem.* **2008**, *6*, 868.
- Spafford, H.; Jardine, A.; Carver, S.; Tarala, K.; van Wees, M.; Weinstein, P. J. *Am. Mosquito Control Assoc.* **2007**, *23*, 304.
- Benedict, M. Q. In *The Molecular Biology of Insect Disease Vectors*; Crampton, J. M., Beard, C. B., Louis, C., Eds.; Chapman and Hall: London, 1997; pp 3–12.
- Zhu, K. Y.; Heise, S.; Zhang, J.; Anderson, T. D.; Starkey, S. R. *J. Med. Entomol.* **2007**, *44*, 1047.
- Zhang, J.; Zhu, K. Y. *Insect Biochem. Mol. Biol.* **2006**, *36*, 712.