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PII: S1381-1177(15)30132-6
DOI: http://dx.doi.org/doi:10.1016/j.molcatb.2015.12.012
Reference: MOLCAB 3302

To appear in: *Journal of Molecular Catalysis B: Enzymatic*

Received date: 20-8-2015
Revised date: 30-12-2015
Accepted date: 31-12-2015

Please cite this article as: Max Schlippert, Annett Mikolasch, Veronika Hahn, Frieder Schauer, Enzymatic thiol Michael addition using laccases: Multiple CS bond formation between p-hydroquinones and aromatic thiols, Journal of Molecular Catalysis B: Enzymatic http://dx.doi.org/10.1016/j.molcatb.2015.12.012

Enzymatic thiol Michael addition using laccases: Multiple C-S bond formation between *p*-hydroquinones and aromatic thiols

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Electronic Supplementary Information (ESI) available: Experimental methods; ^{1}H NMR, ^{13}C NMR, HSQC, HMBC data, retention time, UV-vis, and MS-data.

Graphical abstract

Enzymatic thiol Michael addition using laccases: Multiple C-S bond formation between *p*-hydroquinones and aromatic thiols,
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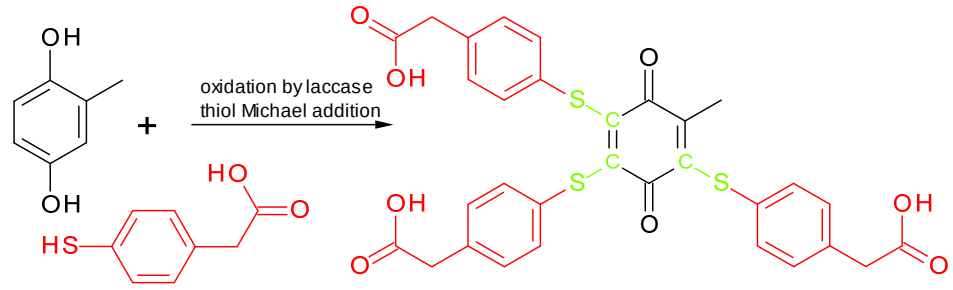

Highlights

- Laccase created C-S bonds between *p*-hydroquinones and aromatic thiols.
- We discovered different thiolated products, ranging from dimers to tetramers.
- Laccase created S-S bonded dimers from aromatic thiols.
- The use of laccase is a convenient way for catalysis under eco-friendly conditions.

Abstract

Laccases create C-C, C-O or C-N bonds and have been investigated intensively as catalysts for green chemistry and white biotechnology. However, little is known about C-S bond formation in laccase-catalyzed reactions. We have used the laccases from *Pycnoporus cinnabarinus* and *Myceliophthora thermophila* to create heteromolecular reaction products from *p*-hydroquinones and aromatic thiols via C-S bond formation. We discovered a broad assortment of different thiolated products, ranging from dimers to tetramers. During laccase-catalysis of the aromatic thiols without *p*-hydroquinones homomolecular dimers were formed and these were always linked by S-S bonds. The use of laccase is a convenient way to form heteromolecular thiolated products and homomolecular S-S bonds under eco-friendly environments.

Abbreviations: ABTS (2,2'azino-bis(3-ethylbenzothioazoline-6-sulfonic acid)diammonuim salt), HPLC (high performance liquid chromatography), LC-MS (liquid chromatography-mass spectrometry), API-ES (atmospheric pressure ionization electrospray), NMR (nuclear magnetic resonance), MeOH (methanol), 2MPD (2-mercaptopyrimidine), 4MBA (4-mercaptobenzoic acid), MPAA (4-mercaptophenylacetic acid)

Keywords: laccase, C-S linkage, aromatic thiol, SH-containing compound, *p*-hydroquinone

1 Introduction

Enzyme-catalyzed oxidations using atmospheric oxygen as a non-toxic oxidant are of considerable general interest. Laccases (E.C. 1.10.3.2, benzendiol:dioxygen oxidoreductase) are phenol oxidases, which contain four copper atoms in their catalytic center [1]. They are used for a wide range of organic syntheses with a broad spectrum of reactants, including monophenols, anilines and dihydroxylated substrates [2]. They use atmospheric oxygen to oxidize hydroxyl groups and abstract the hydrogen, thus creating radicals. These radicals can either undergo bonding reactions with another compound or transfer the radical character onto another reactant and act as a radical mediator [3].

Laccases are widely distributed in fungi and plants, but can also be found in insects and bacteria [4, 5]. Their natural functions include the synthesis and degradation of lignin and aromatic compounds [6], the synthesis of pigments in fungi [7] and the sclerotization of insects [8]. Laccases find applications in the disposal of soil pollutants [9] such as pentachlorophenol and TNT [10]. They are also used in the paper industry for the preparation of pulp [11], in the food industry for the removal of unwanted phenols and in the textile industry for the treatment of waste water [12, 13]. The oxidation of suitable substrates often yields radicals, which can undergo various reactions such as the coupling of two different reaction partners. In the case of phenolic compounds bond formations like C–C [14, 15], C–O [16] and C=C [17] or in case of reactions with amines C–N [18-20] or C=N [17-19] may result. Laccase-catalyzed bond formation can be used in green chemistry for the synthesis of many different substances, e.g. the formation of benzofurans and benzofuran-1-ones [21-23], naphthoquinones [24, 25], aminobenzoquinones[19, 20, 26-31], substituted azoles [32, 33], 3-substituted 1,2,4-triazolo[4,3-b][4.1.2]benzothiadiazine-8-ones [34], phenazines [35], and phenoxazinone derivatives [36-38], as well as the dimerization of salicylic esters [39] and penicillins [40]. It has been shown, that the modification of existing antibiotics with laccases further improves their inhibiting abilities, even against MRSA-strains [20, 27].

All the above mentioned reactions create bonds by using C-C, C-O or C-N interlinkages [19, 35, 41] and have been investigated in depth. In contrast C-S bond formation has so far not been dealt with to any great extent in the relevant literature [42-47] although it is of great interest both for green chemistry and white biotechnology.

To broaden the range of application of laccases in fine-chemical synthesis, we exploited the laccases of *Pycnoporus cinnabarinus* and *Myceliophthora thermophila* for reactions involving C-S bond formation. These fungal laccases catalyzed the reactions of several *p*-hydroquinones with three different thiols. The products were analyzed by MS and NMR spectroscopy to gain more insight into their properties and structures.

2 Experimental

2.1 Enyzmes. The laccases used were obtained from the white rot fungi *Pycnoporus cinnabarinus* and *Myceliopthora thermophila.*

Pycnoporus cinnabarinus was isolated in northern Germany and is deposited at the strain collection of the Department of Biology of the University in Greifswald, from where it can be obtained. The fungus was

cultivated on malt agar plates for 7 days at 30 °C and then maintained at 4 °C. Three 1 cm^2 agar fragments were placed in a nitrogen-rich medium [48] and incubated without shaking at 30°C for 7 days. This preculture was homogenized and 6 mL of homogenate was incubated with 40 mL medium. To stimulate laccase production, 10 mM veratryl alcohol was added and then agitated in a water bath for 3 days at 30 °C and 160 rpm. To purify the laccase from the medium the culture was filtered through a glass fiber filter and stirred with DEAE-Sephacel (Sigma-Aldrich, Steinheim, Germany) for 1h. Afterwards the enzyme was eluted with 20 mM sodium acetate buffer (pH 5) and 70 mM NaCl. To desalt the laccase-containing buffer a PD10 column (Sephadex™; GE Healthcare, Buckinghamshire, UK) with 20 mM sodium acetate buffer (pH 5) was used. The purified laccase was stored at -20 °C and used at room temperature in sodium acetate buffer (pH 5) because of its pH optimum of around pH 5.0 (ABTS) [49-51].

Laccase from *Myceliophthora thermophila* (expressed in a genetically modified *Aspergillus* sp.) was obtained from Novozymes (Bagsvaerd, Denmark). It was used as received (activity 1000 $U*g^{-1}$; substrate: syringaldazine) in citrate phosphate buffer (CPB, 18 mM citrate, 165 mM phosphate) at its pH optimum of pH 7.0 [50, 52].

2.2 Measurement of laccase activity. The activity of laccase was determined spectrophotometrically at 420 nm with ABTS as substrate using the method described by Jonas [51].

2.3 Experimental procedures. For analytical experiments one thiol (1-5 mM) and one *p*-hydroquinone (1 mM) were incubated with laccase (activity 1 U) in a final volume of 4 ml of the respective buffer (sodium acetate buffer, pH 5 for *Pycnoporus cinnabarinus* and citrate phosphate buffer, pH 7 for *Myceliophthora thermophila,* both stored at 4 °C and used at room temperature for reactions). Reaction mixtures were incubated with agitation at 200 rpm at room temperature in brown-glass-bottles. After incubation for 20 min, 2 h, or 24 h the mixture was analyzed by HPLC, LC-10AT VP system (Shimadzu, Germany) consisting of a FCV-10AL VP pump, SPD-M10A VP diode array detector, and a SCL-10A VP control unit controlled by Class-VP version 6.12 SP5. The separation of substances was achieved on a RP18 column at a flow rate of 1 mL min^{-1}. The solvent system consisted of methanol (eluent A) and 0.1 % phosphoric acid (eluent B), starting from an initial ration of 10% A and 90% B and reaching 100% methanol within 14 min.

The isolated products were characterized using a LC-MS system. The API mass spectrometry experiments were performed on an Agilent Series 1200 HPLC system with diode array detector and an Agilent 6120 quadrupole mass spectrometer (Waldbronn, Germany). The MS was run with ES ionization source in positive and negative mode (dry and nebulizer gas: nitrogen; nebulizer pressure, 45 psig; drying gas flow, 10 L min^{-1}; drying gas temperature, 350 °C; capillary voltage, 4 kV; fragmentor voltage, 75 V). HPLC separation was performed on a Zorbrax SB-C18 (2.1×50 mm, 1.8 µm) column (Agilent, Waldbronn, Germany), at a flow rate of 0.3 mL min^{-1}. A solvent system consisting of acetonitrile (eluent A) and 0.1 % aqueous ammonium formate (eluent B), starting from an initial ratio of 10 % A and 90 % B and reaching 100 % acetonitrile within 7 min, holding this until 13 min, was used.

Product isolation was achieved using solid phase extraction on a RP18 silica gel column (6 mL, 40-64 µm, LiChrolut, 2000 mg, Merck, Darmstadt). Product **3d** was isolated from the reaction mixture (40 mL) after an incubation period of 24 h (**1a** : **2d** 2 mM : 1 mM assay). After charging the column with 6 mL reaction mixture, 6 mL of a methanol-distilled water (60 % : 40 % v/v) mixture was used to remove undesired impurities. Elution of the red product fraction was performed with 2 mL methanol. Product **3g** was isolated from the reaction mixture (50 mL) after an incubation period of 20 min (**1b** : **2b** 1 mM : 1 mM assay). After charging the column with 6 mL reaction mixture, the orange product fraction was eluted with 2 mL methanol. Product **3h** was isolated from the reaction mixture (50 mL) after an incubation period of 20 min (**1b** : **2c** 1 mM : 1 mM assay). After charging the column with 6 mL reaction mixture, the orange product fraction was eluted with 2 mL methanol. Product **3j** was isolated from the reaction mixture (50 mL) after an incubation period of 20 min (**1b** : **2d** 1 mM : 1 mM assay). After charging the column with 6 mL reaction mixture, the orange product fraction was eluted with 2 mL methanol. Product **3o** was isolated from the reaction mixture (60 mL) after an incubation period of 20 min (**1c** : **2d** 1 mM : 1 mM assay). After charging the column with 6 mL reaction mixture, the orange-yellow product fraction was eluted with 2 mL methanol. Product **4i** was isolated from the reaction mixture (50 mL) after an incubation period of 2 h (**1b** : **2e** 5 mM : 1 mM assay). After charging the column with 6 mL reaction mixture, 6 mL of a methanol-distilled water (60 % : 40 % v/v) mixture was used to remove undesired impurities. Elution of the dark red product product fraction was performed with 2 mL methanol. Product **4j** was isolated from the reaction mixture (80 mL) after an incubation period of 2 h (**1c** : **2a** 1 mM : 1 mM assay). After charging the column with 6 mL reaction mixture, 6 mL of a methanol-distilled water (10 % : 90 % v/v) mixture was used to remove undesired impurities. Elution of the brown product fraction was performed with 2 mL methanol. Product **4m** was isolated from the reaction mixture (80 mL) after an incubation period of 20 min (**1c** : **2d** 5 mM : 1 mM assay). After charging the column with 6 mL reaction mixture, 4 mL of methanol-distilled water (50 % : 50 % v/v) was used to remove undesired impurities. Elution of the red product fraction was performed with 8 mL of a methanol-distilled water (60 % : 40 % v/v) mixture. **4n** was isolated from the reaction mixture (40 mL) after an incubation period of 2 h (**1c** : **2e** 1 mM : 1 mM assay). After charging the column with 6 mL reaction mixture, the brown product fraction was eluted with 2 mL methanol.

The NMR spectra were obtained at 600 MHz (^{1}H, ^{1}H^{1}H COSY, HSQC, HMBC) and at 150 MHz (^{13}C) on a Bruker Avance 600 instrument (Rheinstetten, Germany). The solvent used was deuterated methanol. Chemical shifts expressed in δ (ppm) calibrated on the resonaces of the residual nondeuterated solvent. For NMR spectroscopy the isolated products were dried by lyophilization.

2.4 Analytical data of products

Atom numbering for each isolated product is shown in Electronic Supplementary Information.

(3a). R_f (LC-MS) 6.9 min. UV-vis (MeOH) λ_{max} 225, 251, 360 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 219, AP-ESI: pos. Mode $[M+H]^+$ 221.

(3b). R_f (LC-MS) 11.6 min. UV-vis (MeOH) λ_{max} 254, 384 nm. MS *m/z* AP-ESI neg. mode $[2M-H]^-$ 465, AP-ESI: pos. Mode $[M+H]^+$ 235.

(3c). R_f (LC-MS) 15.3 min. UV-vis (MeOH) λ_{max} 249, 310 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 247, AP-ESI: pos. Mode $[M+H]^+$ 249.

(3d). R_f (LC-MS) 11.6 min. UV-vis (MeOH) λ_{max} 260, 295 nm. MS *m/z* AP-ESI neg. mode $[2M-H]^-$ 495.

(3e). R_f (LC-MS) 13.1 min. UV-vis (MeOH) λ_{max} 234, 331 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 306, AP-ESI: pos. Mode $[M+H]^+$ 308.

(3f). R_f (LC-MS) 8.5 min. UV-vis (MeOH) λ_{max} 223, 261 nm, 435 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 259, AP-ESI: pos. Mode $[M+H]^+$ 261.

4-(4-Methyl-3,6-dioxocyclohexa-1,4-dien-1-yl)sulfanyl)benzoic acid (3g). Synthesis and isolation as described above. Orange solid. Yield 66.6 % (8.89 mg). ^{1}H NMR: δ 2.02 (s(broad), 3H, H-7'), 5.81 (s(broad), 1H, H-2'), 6.72 (s(broad), 1H, H-5'), 7.55 (d, J = 8.3 Hz, 2H, H-3/H-5), 8.09 (d, J = 8.3 Hz, 2H, H-2/H-6). ^{13}C NMR: δ 14.3 (C-7'), 120.7(C-2'), 117.7 (C-5)', 134.6 (C-3/C-5), 130.6 (C-2/C-6), 129.7 (C-4), 140.3 (C-1), 172.5 (C-7), 183.9 (C-6'), 184.5(C-3'). ^{1}H ^{1}H COSY: H-3/H-5 (H-2/H-6); H-2/H-6 (H-3/H-5). HMBC: H-3/H-5 (C-2/C-6, C-3/C-5, C-1), H-2/H-6 (C-4, C-2/C-6, C-3/C-5, C-7). R_f (HPLC) 12.1 min. UV-vis (MeOH) λ_{max} 230, 280, 425 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 273, AP-ESI: pos. Mode $[M+H]^+$ 275.

4-(4,5-Dimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl)sulfanyl)benzoic acid (3h). Synthesis and isolation as described above. Orange solid. Yield 72 % (10.2 mg). ^{1}H NMR: δ 2.00, s, 3H, H-7', 2.07, s, 3H, H-8', 5.78, s, 1H, H-2', 7.55, d, J = 8.2 Hz, 2H, H-3/H-5, 8.08, d, J = 8.2 Hz, 2H, H-2/H-6. ^{13}C NMR: δ 10.8 C-7', 10.9 C-8', 125.5 C-2', 134.6 C-3/C-5, 130.6 C-2/C-6, 129.7 C-4, 140.2 C-1/C-5', 141.5 C-4', 153.4 C-1', 172.5 C-7, 183.7 C-6', 184.2 C-3'. ^{1}H ^{1}H COSY: H-7' (H-3/H-5); H-8' (H-3/H-5); H-3/H-5 (H-7', H-8', H-2/H-6); H-2/H-6 (H-3/H-5). HMBC: H-7' (C-5', C-3'); H-8' (C-4', C-6'); H-2' (C-4', C-1', C-6'); H-3/H-5 (C-2/C-6, C-3/C-5, C-1); H-2/H-6 (C-4, C-2/C-6, C-3/C-5, C-7). R_f (HPLC) 13,2 min. UV-vis (MeOH) λ_{max} 219, 287, 428 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 287, AP-ESI: pos. Mode $[M+H]^+$ 289.

4-(4-Methoxy-3,6-dioxocyclohexa-1,4-dien-1-yl)sulfanylbenzoic acid (3i). Synthesis and isolation as described above. Orange-brown solid. Yield 79.3 % (11.2 mg). ^{1}H NMR: δ 3.86, s(broad), 3H, H-7', 5.71, s(broad), 1H, H-2', 6.11, s(broad), 1H, H-5', 7.56, d, J = 8.3 Hz, 2H, H-3/H-5, 8.09, d, J = 8.5 Hz, 2H, H-2/H-6. ^{13}C NMR: δ 55.6 C-7', 123.2 C-2', 106.1 C-5', 134.5 C-3/C-5, 130.7 C-2/C-6, 129.1 C-4, 140.4 C-1, 159.9 C-4', 72.5 C-7, 178.7 C-3', 183.8 C-6' . ^{1}H ^{1}H COSY: H-3/H-5 (H-3/H-5), H-2/H-6 (H-3/H-5). HMBC: H-7' (C-4'), H-3/H-5 (C-2/C-6, C-3/C-5, C-1), H-2/H-6 (C-4, C-2/C-6, C-3/C-5, C-7). R_f (HPLC) 10.8 min. UV-vis (MeOH) λ_{max} 217, 319, 444 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 289, AP-ESI: pos. Mode $[M+H]^+$ 291.

(3j). R_f (LC-MS) 5.9 min. UV-vis (MeOH) λ_{max} 285 nm, 486 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 319, AP-ESI: pos. Mode $[M+H]^+$ 321.

(3k). R_f (LC-MS) 1.3 min. UV-vis (MeOH) λ_{max} 226 nm, 262 nm, 343 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 362, AP-ESI: pos. Mode $[M+H]^+$ 364.

(3l). R_f (HPLC) 10.8 min. UV-vis (MeOH) λ_{max} 210 nm, 310 nm, 433 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 273, AP-ESI: pos. Mode $[M+H]^+$ 275.

(3m). R_f (HPLC) 12.5 min. UV-vis (MeOH) λ_{max} 220 nm, 270 nm, 433 nm. MS *m/z* AP-ESI neg. mode $[M-COOH]^-$ 243, AP-ESI: pos. Mode $[M+H]^+$ 289.

(3n). R_f (LC-MS) 2.9 min. UV-vis (MeOH) λ_{max} 229 nm, 260 nm, 346 nm. MS *m/z* AP-ESI neg. mode $[M+OH-H]^-$ 376, AP-ESI pos. Mode $[M+OH+H]^+$ 378.

2-[4-(4-Methoxy-3,6-dioxo-cyclohexa-1,4-dien-1-yl)sulfanylphenyl]phenylic acid (3o). Synthesis and isolation as described above. Orange solid. Yield 63.7 % (12.04 mg). ^{1}H NMR: δ 3.72, s, 2H, H-7; 3.85, s, 3H,

H-7'; 5.73, s, 1H, H-2'; 6.11, s, 1H, H-5'; 7.50, d, J = 8.5 Hz, 2H, H-2/H-6; 7.52, d, J = 8.5 Hz, 2H, H-3/H-5. ^{13}C NMR: δ 40.6 C-7; 55.8 C-7'; 123.2 C-2'; 106.3 C-5'; 131.2 C-2/C-6; 135.2 C-3/C-5; 125.6 C-4, 138.2 C-1, 156.2 C-1', 159.9 C-4', 173.7 C-8, 178.6 C-3', 183.9 C-6'. ^{1}H ^{1}H COSY: H-7 (H-2/H-6), H-7' (H-5', H-3/H-5), H-5' (H-7'), H-2/H-6 (H-7), H-3/H-5 (H-7'). HMBC: H-7 (C-2/C-6, C-1, C-8), H-7' (C-5', C-4'), H-2' (C-1', C-4', C-6'), H-5' (C-1', C-4', C-3', C-6'), H-2/H-6 (C-7, C-4, C-2/C-6), H-3/H-5 (C-4, C-3/C-5, C-1). R_f (HPLC) 10,5 min. UV-vis (MeOH) λ_{max} 250, 303, 450 nm. MS *m/z* AP-ESI neg. mode $[M-COOH]^-$ 259, AP-ESI: pos. Mode $[M+H]^+$ 305.

(3p). R_f (HPLC) 10.4 min. UV-vis (MeOH) λ_{max} 268 nm, 294 nm, 492 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 333, AP-ESI: pos. Mode $[M+H]^+$ 335.

(3q). R_f (HPLC) 11 min. UV-vis (MeOH) λ_{max} 230 nm, 309 nm, 384 nm. MS *m/z* AP-ESI neg. mode $[M+OH-H]^-$ 376, AP-ESI: pos. Mode $[M+OH+H]^+$ 378.

(4a). R_f (LC-MS) 6.3 min. UV-vis (MeOH) λ_{max} 239 nm, 304 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 329, AP-ESI pos. Mode $[M+H]^+$ 331.

(4b). R_f (LC-MS) 13.3 min. UV-vis (MeOH) λ_{max} 226 nm, 269 nm, 321 nm. MS *m/z* AP-ESI pos. Mode $[M+H]^+$ 345.

(4c). R_f (LC-MS) 14.2 min. UV-vis (MeOH) λ_{max} 250 nm, 287 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 357, AP-ESI pos. Mode $[M+H]^+$ 359.

(4d). R_f (LC-MS) 14.2 min. UV-vis (MeOH) λ_{max} 240 nm, 340 nm. MS *m/z* AP-ESI pos. Mode $[M+H]^+$ 416.

(4e). R_f (LC-MS) 11.7 min. UV-vis (MeOH) λ_{max} 230 nm, 271 nm, 374 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 411, AP-ESI pos. Mode $[M+H]^+$ 413.

(4f). R_f (HPLC) 14.7 min. UV-vis (MeOH) λ_{max} 230 nm, 280 nm, 410 nm. MS *m/z* AP-ESI pos. Mode $[M+H]^+$ 427.

(4g). R_f (LC-MS) 11.6 min. UV-vis (MeOH) λ_{max} 275 nm, 488 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 439, AP-ESI pos. Mode $[M+H]^+$ 441.

(4h). R_f (HPLC) 13.1 min. UV-vis (MeOH) λ_{max} 282 nm, 475 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 441, AP-ESI pos. Mode $[M+H]^+$ 443.

(4i). R_f (HPLC) 13.2 min. UV-vis (MeOH) λ_{max} 288 nm, 405 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 471, AP-ESI pos. Mode $[M+H]^+$ 473.

2-[4-[4-[4-(Carboxymethyl)phenyl]sulfanyl-2,5-dihydroxyphenyl]sulfanylphenyl]acetic acid (4j). Synthesis and isolation as described above. Brown solid. Yield 29.5 % (10.4 mg). ^{1}H NMR: δ 3.58, s, 4H, H-7, 5.89, s(broad), 1H, H-2'/H-5', 7.45, d, J = 8.1 Hz, 4H, H-3/H-5, 7.51, d, J = 8.1 Hz, 4H, H-2/H-6. ^{13}C NMR: δ 44.7 C-7, 124.2 C-2'/C-5', 134.9 C-3/C-5, 131.1 C-2/C-6, 124.1 C-4, 141.4 C-1, 177.7 C-8. ^{1}H ^{1}H COSY: H-7 (H-2/H-6); H-3/H-5 (H-2/H-6); H-2/H-6 (H-7, H-3/H-5). HMBC: H-7 (C-2/C-6, C-1, C-8), H-3/H-5 (C-3/C-5, C-1), H-2/H-6 (C-7, C-4, C-2/C-6). R_f (HPLC) 12.8 min. UV-vis (MeOH) λ_{max} 219, 370 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 439, AP-ESI: pos. Mode $[M+H]^+$ 441.

(4k). R_f (HPLC) 13.5 min. UV-vis (MeOH) λ_{max} 250 nm, 292 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 453, AP-ESI pos. Mode $[M+NH_4]^+$ 472.

(4l). R_f (HPLC) 14.1 min. UV-vis (MeOH) λ_{max} 204 nm, 247 nm, 325 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 467, AP-ESI pos. Mode $[M+H]^+$ 469.

2-[4-[2-[4-(Carboxymethyl)phenyl]sulfanyl-4-methoxy-3,6-dioxocyclohexa-1,4-dien-1-yl]sulfanylphenyl]acetic acid (4m). Synthesis and isolation as described above. Darkblue solid. Yield 32.9 % (12.37 mg). ^{1}H NMR: δ 3.61, s, 8H, C-7, 3.81, s, 3H, H-7', 6.07, s, 1H, H-5', 7.28, m, 12H, 7.47, m, 4H. ^{13}C NMR: δ 40.2 C-7, 55.9 C-7', 107.8 C-5', 129.9 C-, 127.9 C-, 148.1 C-1', 159.8 C-4', 175.7 C-3', 180.7 C-6'. ^{1}H ^{1}H COSY: H-7' (H-5'); H-5' (H-7'); 7.28 (HC-7); 7.47 (H-7). HMBC: C-7 (C-8); H-7' (C-4'); H-5' (C-1', C-4', C-3'). R_f (HPLC) 12 min. UV-vis (MeOH) λ_{max} 251, 339, 491 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 469, AP-ESI: pos. Mode $[M+H]^+$ 471.

2-[4-[5-[4-(Carboxymethyl)phenyl]sulfanyl-2,4-dimethoxy-3,6-dioxocyclohexa-1,4-dien-1-yl]sulfanylphenyl]acetic acid (4n). Synthesis and isolation as described above. Darkbrown solid. Yield 29.4 % (9.6 mg). ^{1}H NMR: δ 3.58, s, 4H, H-7, 3.89, s, 6H, H-7'/H-8', 7.27, d, J = 8.3 Hz, 4H, H-2/H-6, 7.45, d, J = 8.3 Hz, 4H, H-3/H-5. ^{13}C NMR: δ 41.1 C-7, 60.2 C-7'/C-8', 129.8 C-2/C-6, 127.9 C-3/C-5, 157.4 C-2'/C-4', 175.8 C-3', 180.7 C-6'. ^{1}H ^{1}H COSY: H-7 (H-2/H-6, H-3/H-5), H-2/H-6 (H-7, H-3/H-5), H-3/H-5 (H-7, H-2/H-6). HMBC: H-7 (C-2/C-6, C-1, C-8), H-7'/H-8' (C-2'/C-4'), H-2/H-6 (C-7, C-3/C-5, C-2/C-6, C-1), H-3/H-5 (C-3/C-5, C-1). R_f (HPLC) 12,5 min. UV-vis (MeOH) λ_{max} 249, 310 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 499, AP-ESI: pos. Mode $[M+NH_4]^+$ 518.

(4o). R_f (LC-MS) 3.5 min. UV-vis (MeOH) λ_{max} 227 nm, 255 nm, 384 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 526, AP-ESI pos. Mode $[M+H]^+$ 528.

(5a). R_f (LC-MS) 11.6 min. UV-vis (MeOH) λ_{max} 247 nm, 341 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 439, AP-ESI pos. Mode $[M+H]^+$ 441.

(5b). R_f (LC-MS) 12.2 min. UV-vis (MeOH) λ_{max} 251 nm, 339 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 453, AP-ESI pos. Mode $[M+H]^+$ 455. **(5c)**. R_f (LC-MS) 11.3 min. UV-vis (MeOH) λ_{max} 229 nm, 271 nm, 401 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 563, AP-ESI pos. Mode $[M+H]^+$ 565.

(5d). R_f (LC-MS) 5.9 min. UV-vis (MeOH) λ_{max} 227 nm, 255 nm, 384 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 577, AP-ESI pos. Mode $[M+H]^+$ 579.

(5e). R_f (HPLC) 13.9 min. UV-vis (MeOH) λ_{max} 216 nm, 254 nm, 389 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 605.

Product mixture: **2-[4-[4,5-bis[[4-(carboxymethyl)phenyl]sulfanyl]-2-methyl-3,6-dioxocyclohexa-1,4-dien-1-yl]sulfanylphenyl]acetic acid** (**5f**), 2-[4-[[4-(carboxymethyl)phenyl]disulfanyl]phenyl]acetic acid (**6c**). Synthesis and isolation as described above. Darkbrown solid. Yield 18.8 % (9.34 mg). ^{1}H NMR: δ 2.18, s, 3H, H-7', 3.59, s, 2H, H-7, 3.61, s, 2H, H-7, 3.616, s, 2H, H-7, 3.619, s, 2H, H-7, 7.13, d, J = 8.5 Hz, 2H, 7.16, d, J = 8.5 Hz, 2H, 7.18, d, J = 8.3 Hz, 2H, 7.22, d, J = 8.3 Hz, 2H, 7.27, d, J = 8.3 Hz, 4H, 7.34, d, J = 8.3 Hz, 2H, 7.47, d, J = 8.3 Hz, 2H. ^{13}C NMR: δ 14.26 C-7', 40.00 C-7, 40.09 C-7, 40.12 C-7, 129.86, 130.80 129.89, 130.56, 129.91, 130.87, 127.89, 175.84 C-6', 178.59 C-3'. ^{1}H ^{1}H COSY: H-7' (3.59, 3.61, 7.13, 7.16, 7.47), H-7 (H-7'), 7.13 (H-7', H-7, 7.16), 7.16 (H-7', 7.13), 7.18 (3.616/3.619, 7.22), 7.22 (7.18), 7.27 (3.616/3.619, 7.34, 7.47), 7.34 (7.27), 7.47 (H-7', 3.61, 7.27), HMBC: H-7' (131.54 C-,143.62 C-, 147.59 C-, C-3'), H-7 (129.86, 129.89, 129.91, 129.92); H-7 (134.33, 134.45, 134.58, 134.68), H-7 (173.68, 173.70, 173.73, 173.79), 7.13 (129.86, 130.56), 7.16 (130.80, 134.45), 7.18 (129.89, 131.55), 7.22 (130.56, 134.33), 7.27 (130.87, 135.10), 7.34 (130.87, 134.58), 7.47 (127.89, 134.68), R_f (HPLC) 14.2 min. UV-vis (MeOH) λmax 250 nm, 412 nm. MS m/z AP-ESI neg. mode $[M-H]^-$ 619, AP-ESI pos. Mode $[M+NH_4]^+$ 638.

(**5g**). R_f (HPLC) 13.7 min. UV-vis (MeOH) λmax 251 nm, 410 nm. MS m/z AP-ESI neg. mode [M-H]- 635, AP-ESI pos. Mode $[M+NH_4]^+$ 654.

(**5h**). R_f (LC-MS) 6.1 min. UV-vis (MeOH) λmax 250 nm, 420 nm. MS m/z AP-ESI neg. mode [M-H]- 692, AP-ESI pos. Mode $[M+H]^+$ 694.

2-(Pyrimidin-2-yldisulfanyl)pyrimidin (**6a**). White solid. Yield 22 % (12 mg). ^{1}H NMR: δ 7.27, t, J = 4.9 Hz, 1H, H-5, 8.62, d, J = 4.9 Hz, 2H, H-4/H-6. ^{13}C NMR: δ 118.5 C-5, 158.0 C-4/C-6, 169.0 C-2. ^{1}H ^{1}H COSY: H-5 (H-4/H-6); H-4/H-6 (H-5). HMBC: H5 C-5 (C-4/C-6, C2); H-4/H-6 C-4/C-6 (C-5, C-4/C-6, C-2). R_f (HPLC) 8,3 min. UV-vis (MeOH) λ_{max} 237 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 221, AP-ESI: pos. Mode $[M+H]^+$ 223.

4-[(4-Carboxyphenyl)disulfanyl)]benzoic acid (**6b**). Yellow solid. Yield 71.8 % (10.7 mg). ^{1}H NMR: δ 7.51, d, J = 8.4 Hz, 2H, H-2/H-6, 7.92, d, J = 8.4 Hz, 2H, H-3/H-5. ^{13}C NMR: δ 126.1 C-2/C-6, 129.7 C-3/C-5, 136.9 C-4, 138.8 C-1, 172.9 C-7. ^{1}H ^{1}H COSY: H-2/H-6 (H-3/H-5), H-3/H-5 (H-2/H-6). HMBC: H-2/H-6 (C-2/C-6, C-3/C-5, C-4, C-1, C-7), H-3/H-5 (C-2/C-6, C-3/C-5, C-1, C-7). R_f (HPLC) 13.7 min. UV-vis (MeOH) λ_{max} 273 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 305, AP-ESI: pos. Mode $[M+Na]^+$ 239.

2-[4-[[4-(Carboxymethyl)phenyl]-disulfanyl]phenyl]acetic acid (**6c**). White solid. Yield 88.2 % (8.7 mg). ^{1}H NMR: δ3.48, s, 2H, H-7, 7.29, d, J = 8.3 Hz, 2H, H-3/H-5, 7.41, d, J = 8.3 Hz, 2H, H-2/H-6. ^{13}C NMR: δ 44.4 C-7, 129.7 C-3/C-5, 128.1 C-2/C-6, 134.2 C-1, 137.9 C-4, 178.1 C-8. ^{1}H ^{1}H COSY: H-7 (H-3/H-5, H-2/H-6); H-3/H-5 (C-7, H-3/H-5, H-2/H-6), H-2/H-6 (C-7, H-3/H-5, H-2/H-6). HMBC: H-7 (H C-3/C-5, C-4, C-8); H-3/H-5 (C-7, C-2/C-6, C-3/C-5, C-1), H-2/H-6 (C-2/C-6, C-3/C-5, C-4). R_f (HPLC) 14.1 min. UV-vis (MeOH) λ_{max} 245 nm. MS *m/z* AP-ESI neg. mode $[M-H]^-$ 333, AP-ESI: pos. Mode $[M+NH_4]^+$ 352.

3 Results

3.1 General observations. The three thiols used in this study, 2MPD, 4MBA and MPAA, consist of six-membered aromatic or hetero-aromatic rings carrying a mercapto group. They are all able to form a homomolecular product during the laccase-catalyzed reaction with both laccases in the same way. The dimers formed were all linked by S-S bonds (see Scheme 1). These reactions could also be analyzed without laccase, but the reaction time was shortened by more than tenfold by adding laccase.

The *p*-hydroquinones used in this study can be described as alkyl- or methoxy-substituted and are themselves laccase substrates (for structures see Table 1). All *p*-hydroquinones were converted to their respective quinones, as described previously [19, 29]. Nearly every combination of thiols and *p*-hydroquinones resulted in at least one heteromolecular product. Depending on the amount of thiol in the reaction, the products were dimers (see Table 1), trimers (see Table 2) or tetramers (see Table 3). The reactions of the laccase from *Pycnoporus cinnabarinus* were basically the same as those of *Myceliophthora thermophila*.

3.2 Reactions of 2MPD. In the reaction mixtures of 2MPD (**1a**) and the *p*-hydroquinones (**2a**-**2f**) the formation of heteromolecular products was detected by HPLC/UV-vis and LC-MS analysis. The reaction rate was slower than for the other two thiols. Product formation took place in the first few hours, but most of the products were highly unstable and could no longer be detected after 24 h. After 24 h there was still an excess of 2MPD, demonstrating the slow reaction rate of the heteromolecular C-S formation. With HPLC and LC-MS analyses we found 5 dimers (Table 1), 4 trimers (Table 2) and 2 tetramers (Table 3). Only one of these products was stable

enough to be isolated. The dimer **3d**, consisting of one molecule 2MPD and one molecule methoxyhydroquinone, was isolated by solid phase extraction and shown to have a yield of 28.3 % using the laccase of *Pycnoporus cinnabarinus*. The reaction mixture changed color from light yellow to colorless. Heteromolecular C-S product formation only took place in the presence of laccase.

3.3 Reactions of 4MBA. In contrast to the reactions of 2MPD, the reaction of 4MBA (**1b**) and *p*-hydroquinones (**2a-2f**) were much more promising. In every reaction mixture C-S heteromolecular products were detected (Table 1, 2, 3).

Laccase-catalyzed reactions of 4MBA with *p*-hydroquinones (**2a-2f**) generally proceeded very fast. Using laccase from *Myceliophthora thermophila*, the reactants were consumed within 20 min. The products seemed to be stable in the reaction solution (as example, see figure S1 in Electronic Supplementary Information for the formation of **3h**). If equimolar concentrations of compounds were used, the formation of dimers was favored. Three have been isolated (**3g-3i**) after using the laccase of *Myceliophthora thermophila*. In reaction mixtures with an excess of 4MBA (2-5 : 1 mM), the formation of trimers (Table 2) and tetramers (Table 3) was observed. It was possible to isolate the trimer **4i** by solid phase extraction after using the laccase of *Pycnoporus cinnabarinus* for the transformation.

The formation of the homomolecular product of 4MBA was detected in all reactions as side product. It was present in smaller amount in the equimolar reaction mixtures and in higher amount in the reaction mixtures with an excess of 4MBA. The retention time of the homomolecular products only differed slightly from those of the C-S heteromolecular products and in consequence the isolations were difficult to accomplish for heteromolecular trimers and tetramers. The reaction mixtures changed color from clear to orange or dark red. The products formed were stable in solution and could still be detected after weeks, when stored at 4° C.

3.4 Reactions of MPAA. As in the reactions of 4MBA, MPAA (**1c**) also showed promising results. In every reaction mixture the formation of stable hetermolecular products was demonstrated. The amount of MPAA influenced the type and the yields of the resulting products. In equimolar reaction mixtures, dimers were favored. Excess MPAA favoured the formation of trimers, of tetramers and also of the undesired homomolecular product of MPAA. All newly formed links between the reactants involved C-S-bonds. As shown above, the reactions were very fast, with the reactants being consumed within 20 min for equimolar reactions and within 2h for higher concentrations of MPAA. The formation of products was analyzed over this time scale (as example, see figure S2 in Electronic Supplementary Information for the formation of tetramer **5f**).

For all products formed, it was possible to derive the structure from the HPLC and LC-MS analysis. We detected in total 6 C-S coupled dimers (Table 1), 6 C-S coupled trimers (Table 2) and 4 C-S coupled tetramers (Table 3) from MPAA (**1c**) and *p*-hydroquinones (**2a-2f**). With MPAA it was easier to separate the transformation product from undesired impurities and from the homomolecular product of MPAA. As a result, 1 dimer (**3o**), 3 trimers (**4j, 4m, 4n**) and 1 tetramer (**5f**) were isolated by solid phase extraction. All these reactions were catalyzed by the

laccase of *Pycnoporus cinnabarinus.* The reaction mixtures changed color from clear to red or brown-red. The products formed were stable in solution and could still be detected after weeks, when stored at 4° C.

3.5 Detailed Structural Characterization of 3h, 4n, 5f.

In the UV-vis spectrum **3h** showed similar absorption maxima to monoaminated aminoquinones[20, 26], such as the weak maximum between 400 and 500 nm and two maxima under 300 nm, indicating the formation of a monothiolated quinone.

LC-MS analyses with AP-ESI in both positive and negative modes showed the molecular mass of **3h** to be 288. This mass could be attributed to the thiolation of **2c** with **1b** under loss of four hydrogen atoms. ^{1}H NMR spectral data of **3h** showed characteristic signals for both compounds (Table 4). Multiplicity of H-2' suggests the thiolation having taken place at C-1'. ^{13}C NMR showed typical signals for quinones in the range of 180 ppm, an indication for the quinoid character of **3h**. The HMBC spectrum showed correlations between the proton H-2'and the quinone carbonyl carbon C-6' and additional between the protons H-7'and H-8' and the quinone carbonyl carbons, unambiguously proving **3h** to be a thiolquinone and being substituted at C-1'. The bond formation between **1b** and **2c** was also confirmed by cross-signals from the methyl protons H-7'and H-8' with H-3 and H-5 in the ^{1}H^{1}H COSY. All results led to the identification of **3h** as 4-(4,5-dimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl)sulfanyl)benzoic acid (Figure 1A).

The UV-vis spectrum of **4n** showed maxima only under 400 nm in accordance with those of known diaminated quinines [26] indicating the formation of a dithiolated quinone.

LC-MS with API-ESI in both positive and negative modes showed the molecular mass of **4n** to be 500. This mass could be attributed to the thiolation of **2e** with two molecules of **1c** accompanied by the loss of six hydrogen atoms. ^{1}H NMR spectral data of **4n** showed the presence of two MPAA residues (Table 4). No protons of the aromatic system of **2e** remained. ^{13}C NMR showed again typical signals for quinones. This showed **4n** to be a dithiolated quinone substituted at C-1' and C-5'. All results led to the identification of **4n** as 2-[4-[5-[4-(carboxymethyl)phenyl]sulfanyl-2,4-dimethoxy-3,6-dioxocyclohexa-1,4-dien-1-yl]sulfanylphenyl]acetic acid (Figure 1B).

The UV-vis spectrum of **5f** showed maxima only under 400 nm indicating the formation of a trithiolated quinone. LC-MS analyses with AP-ESI in both positive and negative modes showed the molecular mass of **5f** to be 620. This mass could be attributed to the thiolation of **2b** with three molecules of **1c** accompanied by the loss of eight hydrogen atoms. ^{1}H NMR spectral data of **5f** showed the presence of three MPAA residues. No protons of the aromatic system of **2b** remained. ^{13}C NMR showed again typical signals for quinones. The bond formation between **1c** and **2b** was also confirmed by cross-signals from the methyl protons H-7' with H-3 and H-5 in the ^{1}H^{1}H COSY. All together this showed **5f** to be a trithiolated quinone substituted at C-1', C-4' and C-5' and led to the identification of **5f** as 2-[4-[4,5-bis[[4-(carboxymethyl)-phenyl]sulfanyl]-2-methyl-3,6-dioxocyclohexa-1,4-dien-1-yl]sulfanylphenyl]acetic acid (Figure 1C).

4 Discussion

Structural characterization of three homomolecular reaction products showed that in the course of laccase-catalyzed reactions, the three selected thiols act as laccase substrates. The homomolecular reaction products were formed as dimers with S-S links in all three experiments (Scheme 1). No higher oligomers or other monomers have been detected. The reaction rate was high for 4MBA and MPAA with the reactants being consumed within 20 min. 2MPD reacted slower in all reactions. Even after 24 h, we could detect a moderate amount of 2MPD. Still, the data show that thiols can be laccase substrates, and that they have a reaction rate similar to that of the classic phenolic laccase substrates.

In heteromolecular reactions of three selected thiols *p*-hydroquinones can be nuclear thiolated, because thiols can also be used as heteroatomic donors in the Michael addition [53]. Depending on the substituents of the *p*-hydroquinone, the properties of the aromatic thiol, and the concentration of the compounds in solution, the course of the laccase-catalyzed reaction and the properties of the resulting products differed to a considerable extent. The thiolation of *p*-hydroquinones proceeded in one step without the detection of the corresponding quinones and much faster than aminations of *p*-hydroquinones, where in a first step a quantitative formation of the corresponding quinone took place [19, 29]. This observation can be explained with a general higher nucleophilicity of thiols compared with amines. This may also be the reason for the fast formation of trimers and tetramers. The thiolate anion is often the active species in Michael additions [53]. The mechanism underlying the reaction is likely to be an intermediate laccase-catalyzed formation of the corresponding quinones (**7**) and subsequent thiolation with aromatic thiols by Michael addition (Scheme 2). After a second oxidation the monothiolated products **3**, which were isolated for **3d, g, h, i** and **o**, are formed. Products **3** undergo a second Michael addition, resulting in the formation of intermediates **9**, which are comparable to the intermediates **8**. After a third oxidation the dithiolated products **4**, which were isolated for **4i, j, m** and **n**, are produced. The position of the thiolations at the aromatic ring is determined by the substituents of the aromatic or heteroaromatic thiol partner and by the substitution of the laccase substrate used. The chemical synthesis of trimers like **4** has also been described for aminoquinones [19, 30]. Depending on the number of substituents at the laccase substrates (**2a-f**) a third Michael addition could be shown resulting in the formation of intermediates **10**, which are comparable to the intermediates **8** and **9** and undergo a fourth oxidation to the trithiolated quinones **5**.

The use of laccase for fine chemistry has been well studied. The coupling of *p*-hydroquinones and amines have been described several times [19, 26, 30, 41, 54] forming C-N linked heteromolecular compounds. However, to date little has been reported about laccase-catalyzed reactions with thiols generating C-S linkages.

The heteromolecular reaction approaches resulted in a multitude of transformation products. All of them were characterized by HPLC and LC-MS and some were isolated in higher yields and analyzed by NMR. All compounds are linked by C-S bonds. Oligomers consisted of one molecule *p*-hydroquinone and one to three molecules of the thiol used. 4MBA and MPAA had rapid reaction rates and formed stable C-S heteromolecular products with *p*-hydroquinones. Overall, we detected 39 heteromolecular transformation products with C-S bonds. As stated above, we used two different laccases in these experiments. The laccase of *Pycnoporus cinnabarinus* has a pH optimum of 5 [49-51] and the laccase of *Myceliophthora thermophila* a pH optimum of 7 [50, 52]. We did not detect any differences in the spectrum of products formed though there were differences in the amount and stability of the products. This is most likely caused by the different pH values. The thiol Michael addition reaction rates increase with pH due to the increased concentration of the thiolate anion [53].

C-S-linkage in a traditional chemical pathway is only possible under extreme reaction conditions. For the bonding process of thiols with alkyl halides, it is necessary to maintain a reaction temperature of 200 °C and the polar solvent hexamethylphosphoramide [55]. Another coupling of thiols and alkyls described requires a temperature of 90 °C and a solvent like ethanol or dimethyl sulfoxide [56]. Other copper-catalyzed reactions also have the disadvantage of requiring high temperatures and large amounts of copper salts [57]. Therefore, the use of laccase to form C-S-linkages is economic and the reactions take place under mild conditions, room temperature, fast reaction rate, low resource input and an aqueous environment, making them a prime example for green chemistry.

5 Conclusion

In this study we present new transformation products of thiols and *p*-hydroquinones. The formation of laccase induced S-S and C-S bonds is described. We demonstrate the use of thiols as laccase substrates, thus increasing the potential of laccases in organic synthesis and green chemistry. By using laccase we can synthesize products in environmentally friendly conditions.

6 Acknowledgments

The authors thank Michael Lalk for providing NMR data and Robert Jack for reviewing the manuscript.

7 Literature

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Figure captions

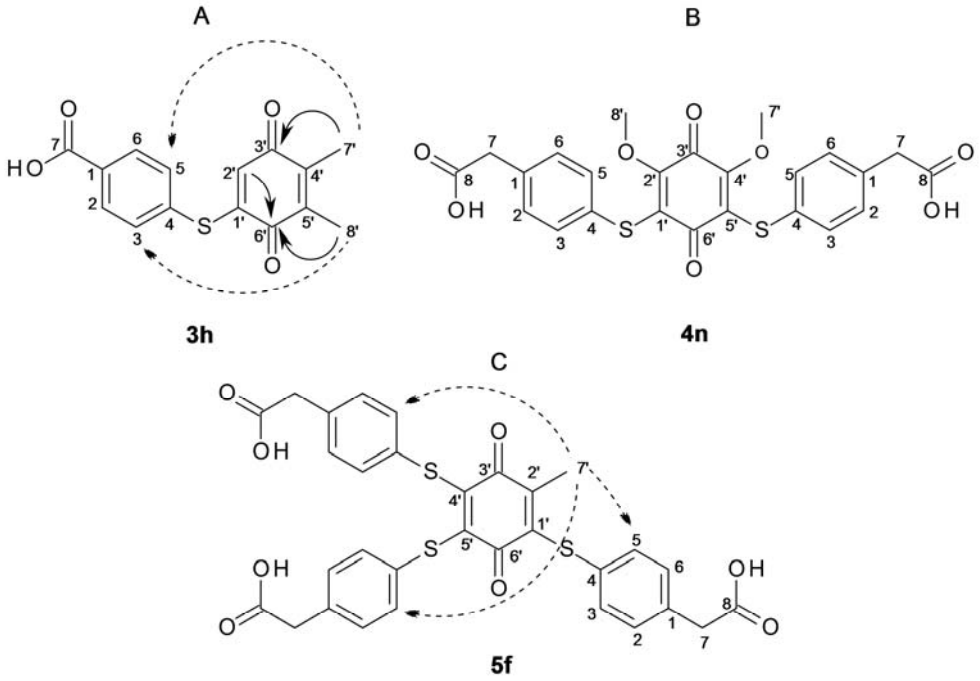
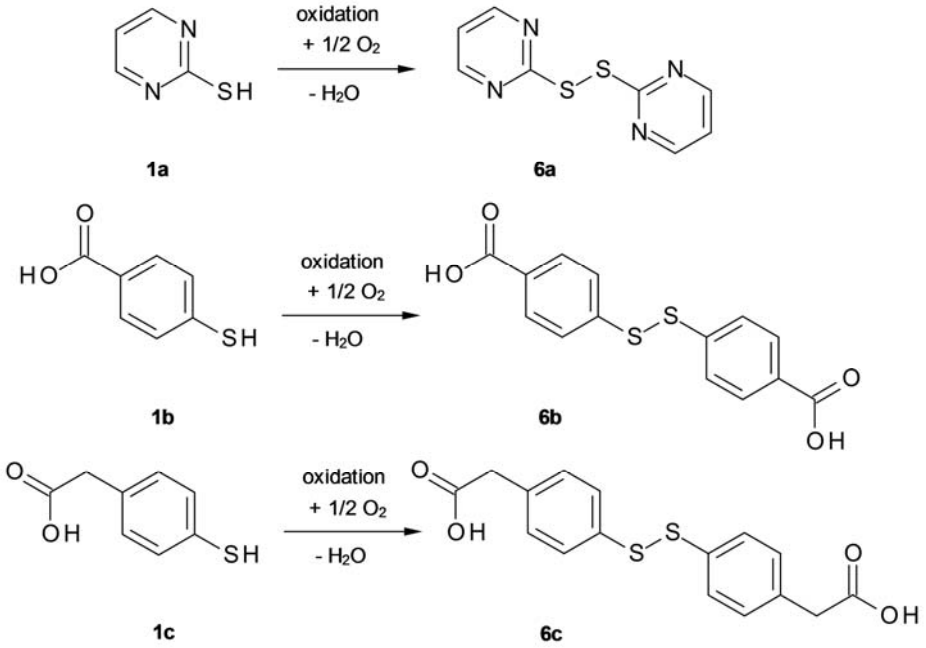
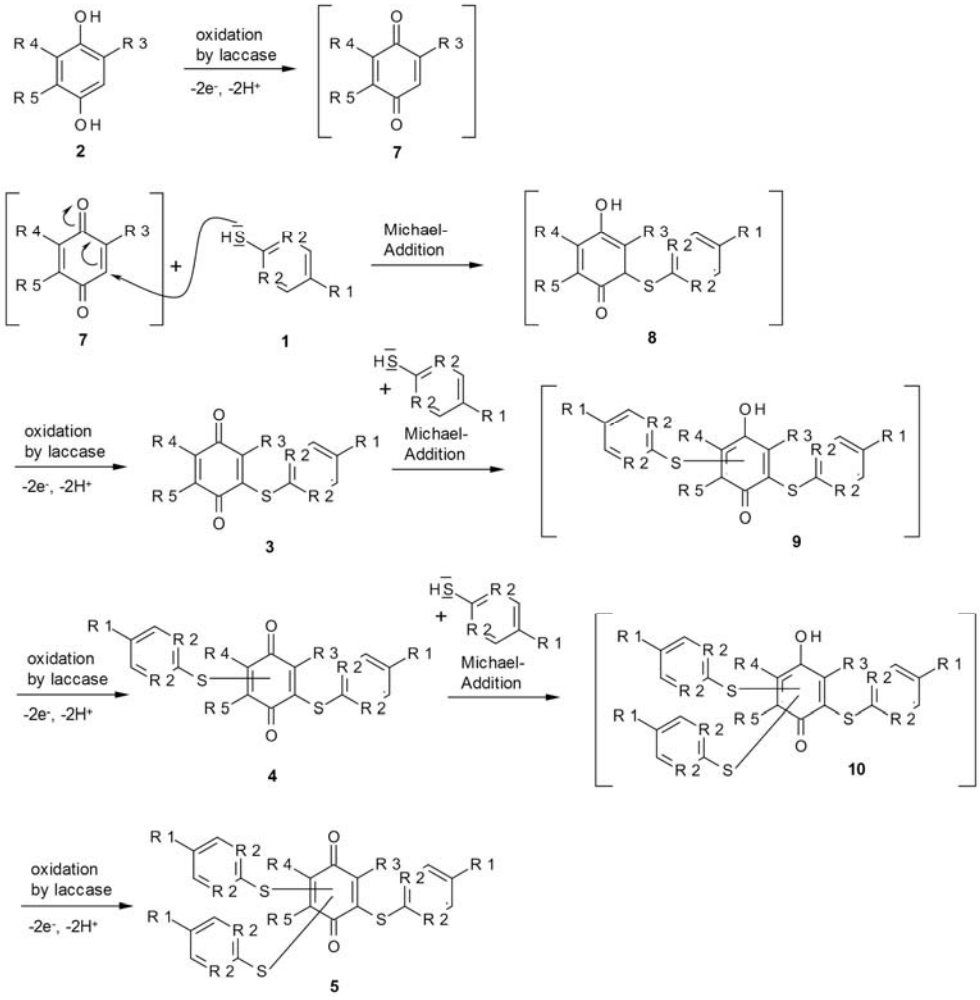


Figure 1: Atom numbering and important ^{1}H-^{13}C (HMBC) (H ⟶► C) and ^{1}H-^{1}H (COSY) correlations (H ---► C) of (A) **3h**, (B) **4n**, (C) **5f**

Scheme captions



Scheme 1: Homomolecular laccase-catalyzed reactions of 1a (2MPD), 1b (4MBA) and 1c (MPAA)



Scheme 2: Possible reaction mechanism. Describtion of R1 to R5 see table 1 to 3.

Tables

Table 1: *p*-Hydroquinones and thiols used in laccase-catalyzed biotransformation and synthesized dimeric C-S coupling products (yield in parentheses, n.d. = not determined; *Pc*l = *Pycnoporus cinnabarinus*-laccase, *Mt*l = *Myceliophthora theromphila*-laccase)

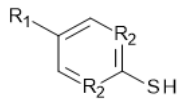
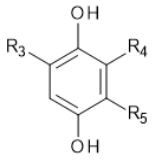
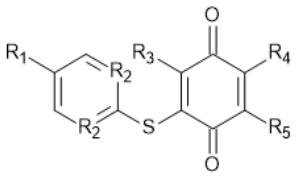
Sulfur compound	*p*-Hydroquinone	Dimeric product
		
1a: R_1 = H; R_2 = N	**2a**: R_3=R_4=R_5=H	**3a**: (n.d.)
1a	**2b**: R_3= R_5=H; R_4= CH_3	**3b**: (n.d.)
1a	**2c**: R_3= H; R_4=R_5= CH_3	**3c**: (n.d.)
1a	**2d**: R_3= R_5=H; R_4= OCH_3	**3d**: (28.3% *Pc*l)
1a	**2f**: R_3= R_5=H; R_4= $CONHCH_2CH_2OH$	**3e**: (n.d.)
1b: R_1 = COOH; R_2 = CH	**2a**	**3f**: (n.d.)
1b	**2b**	**3g**: (66.6% *Mt*l)
1b	**2c**	**3h**: (72.0% *Mt*l)
1b	**2d**	**3i**: (79.3% *Mt*l)
1b	**2e**: R_5= H; R_3=R_4= OCH_3	**3j**: (n.d.)
1b	**2f**	**3k**: (n.d.)
1c: R_1 = CH_2COOH; R_2 = CH	**2a**	**3l**: (n.d.)
1c	**2b**	**3m**: (n.d.)
1c	**2c**	**3n**: (n.d.)
1c	**2d**	**3o**: (63.7% *Pc*l)
1c	**2e**	**3p**: (n.d.)
1c	**2f**	**3q**: (n.d.)

Table 2: *p*-Hydroquinones and thiols used in laccase-catalyzed biotransformation and synthesized trimeric C-S coupling products (yield in parentheses, n.d. = not determined; *Pc*l = *Pycnoporus cinnabarinus*-laccase, *Mt*l = *Myceliophthora theromphila*-laccase)

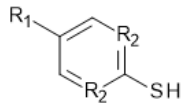
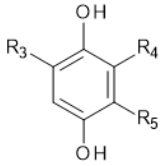
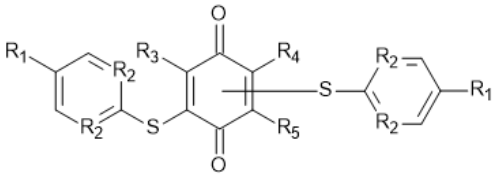
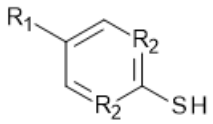
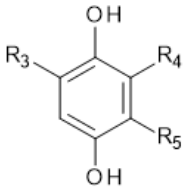
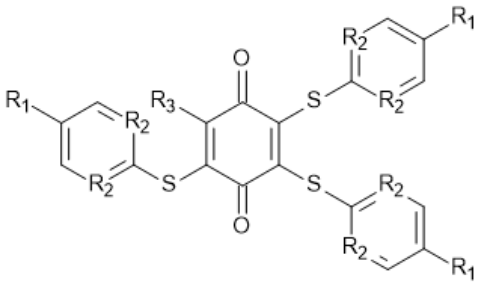
Sulfur compound	*p*-Hydroquinone	Trimeric product
		
1a: R_1 = H; R_2 = N	**2a**: R_3=R_4=R_5=H	**4a**: (n.d.)
1a	**2b**: R_3= R_5=H; R_4= CH_3	**4b**: (n.d.)
1a	**2c**: R_3= H; R_4=R_5= CH_3	**4c**: (n.d.)
1a	**2f**: R_3= R_5=H; R_4= $CONHCH_2CH_2OH$	**4d**: (n.d.)
1b: R_1 = COOH; R_2 = CH	**2a**	**4e**: (n.d.)
1b	**2b**	**4f**: (n.d.)
1b	**2c**	**4g**: (n.d.)
1b	**2d**: R_3= R_5=H; R_4= OCH_3	**4h**: (n.d.)
1b	**2e**: R_5= H; R_3=R_4= OCH_3	**4i**: (26.2% *Pc*l)
1c: R_1 = CH_2COOH; R_2 = CH	**2a**	**4j**: (29.5% *Pc*l)
1c	**2b**	**4k**: (n.d.)
1c	**2c**	**4l**: (n.d.)
1c	**2d**	**4m**: (32.9% *Pc*l)
1c	**2e**	**4n**: (29.4% *Pc*l)
1c	**2f**	**4o**: (n.d.)

Table 3: *p*-Hydroquinones and thiols used in laccase-catalyzed biotransformation and synthesized tetrameric C-S coupling products (yield in parentheses, n.d. = not determined; , *Pc*l = *Pycnoporus cinnabarinus*-laccase, *Mt*l = *Myceliophthora theromphila*-laccase)

Sulfur compound	*p*-Hydroquinone	Tetrameric product
		
1a: R_1 = H; R_2 = N	**2a**: R_3=R_4=R_5=H	**5a**: (n.d.)
1a	**2b**: R_4= R_5=H; R_3= CH_3	**5b**: (n.d.)
1b: R_1 = COOH; R_2 = CH	**2a**	**5c**: (n.d.)
1b	**2b**	**5d**: (n.d.)
1c: R_1 = CH_2COOH; R_2 = CH	**2a**	**5e**: (n.d.)
1c	**2b**	**5f**: (18.8% *Pc*l)
1c	**2d**: R_4= R_5=H; R_3= OCH_3	**5g**: (n.d.)
1c	**2f**: R_4= R_5=H; R_3= $CONHCH_2CH_2OH$	**5h**: (n.d.)