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Laccase catalysed modification of lignin subunits and coupling to *p*-aminobenzoic acid

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ABSTRACT

Laccase catalysed oxidation of syringyl and guaiacyl subunits of lignin and their modification with an aromatic amine, *p*-aminobenzoic acid (PABA) were investigated. Laccase from *Galerina* sp. HC1 isolated earlier by us was used as the main catalyst, and *Trametes versicolor* laccase was used for comparison. Among the syringyl compounds, syringic acid and syringaldehyde were oxidised to 2,6-dimethoxy-1,4-benzoquinone, and in the presence of PABA yielded a cross-coupling imine product. The reaction with methyl syringol resulted in several products whose structures were determined. The possible oxidative coupling pathways were proposed for the formation of the identified products. Oxidation of syringol and the guaiacyl compounds resulted mainly in homooligomers by free radical mechanism, with a negligible tendency of reaction with the nucleophilic group of PABA. Similar treatment of Eucalyptus Kraft lignin, which is rich in syringyl moieties, showed the presence of identical products obtained with syringic acid and syringaldehyde.

1. Introduction

Lignin is the second most abundant renewable polymer after cellulose. Crude lignin is obtained in large quantities in the pulp and paper industry, mainly as Kraft lignin and lignosulfonate. The latter is used for several low value applications such as solid fuel, dispersant, additive in particle board and wood– plastic composite production, etc. The biopolymer has also been used as a starting material for the production of some bulk chemicals, e.g. vanillin and syringaldehyde [1]. One of the major reasons for the limited value addition is the complexity of lignin in terms of composition and structure, which complicates the transformation of the macromolecule to well defined products. Therefore, understanding of the mechanisms of lignin biosynthesis, chemical or enzymatic modification (e.g. oxidation), and reactivity towards different compounds is often addressed through use of monomeric or dimeric model compounds [2–4].

The composition of lignin varies depending on the botanical source and the process of extraction. There are three monolignol monomers methoxylated to different degrees: *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, which are incorporated into lignin in the form of phenylpropanoid subunits, *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) linked in randomly organised polymeric network. The S/G ratio is one of the important characteristics in the selection of wood for the manufacturing of pulp [5,6]. The structure of lignin from Eucalyptus hard wood is known to be rich in syringyl units, the S/G ratio varies between 3.2 and 6.4 in native and 4.3 and 5.2 in Kraft lignin, as revealed by pyrolysis-gas chromatography coupled to mass spectrometry (Py-GC–MS). The main pyrolysis products identified in the Eucalyptus Kraft lignin are guaiacol, syringol (or 2,6-dimethoxyphenol), 4-methylsyringol, and syringaldehyde [7,8].

In vivo, a set of oxidative enzymes, including peroxidases and laccases, catalyse the formation of monolignol radicals, which are then assumed to undergo uncatalysed coupling to form the lignin polymer [9–11]. In comparison to peroxidases, laccases have a lower redox potential and can oxidise only phenolic structures with low redox potential. The substrate spectrum of laccases is however increased by the use of laccase mediator systems (LMSs) [12]. The oxidation of Kraft lignin by laccase alone or by LMSs has long been investigated for producing upgraded compounds [13–15]. Laccase mediated modification of lignin has been used for the production of safe adhesives in place of the phenol–formaldehyde based glues [16,17], and for grafting of functional molecules to impart desired functionalities [18]. Laccase treatment is also used for bleaching of Kraft pulps as an eco-friendly alternative route to the classical chemical bleaching [19]. Laccases are now emerging as green catalysts in synthetic chemistry and some interesting applications such as synthesis of dyes, cosmetics and pharmaceutically active compounds have been demonstrated [20–25].

The present study is a continuation of our recent work on the reaction of laccase treated Eucalyptus Kraft lignin with soy protein and chitosan, respectively to yield products with adhesive properties [17]. Although a number of studies have so far been reported

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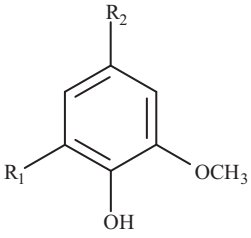
Table 1
Chemical structures of the G-type and S-type compounds used in the study. Abbreviations are written in parentheses.

Lignin monomers		Compound number	R_1	R_2	General structure
G-type	Guaiacol (G)	**1**	H	H	
	Vanillin (V)	**2**	H	CHO	
	Vanillic acid (VA)	**3**	H	COOH	
S-type	Syringol (S)	**4**	OCH_3	H	
	Syringaldehyde (SD)	**5**	OCH_3	CHO	
	Syringic acid (SA)	**6**	OCH_3	COOH	
	4-Methyl syringol (MS)	**7**	OCH_3	CH_3	

on laccase catalysed modification of different lignin model compounds and their reaction with phenolic and aromatic compounds [21,23–25], the minor differences in their molecular structures and functional groups on the compounds result in varied reactivities, reaction pathways and products formed. Herein, we investigate laccase catalysed oxidation of different guaiacyl (G)- and syringyl (S)-type subunits present in Kraft lignin and demonstrate the influence of a methoxy-substitution at *ortho*-position and also of the functional groups on the homo- and cross-coupling reactions, and the product profiles generated. The G-type compounds used were guaiacol, vanillin and vanillic acid, while the S-type compounds were syringol, 4-methyl syringol, syringaldehyde and syringic acid (Table 1). *p*-Aminobenzoic acid was chosen as a model for aromatic amines that could represent on one hand toxic pollutants in the environment and hence requiring detoxification, while on the other provide possibilities of synthesising bioactive molecules. Laccase from *Galerina* sp. HC1, isolated and characterised earlier by Ibrahim et al. [26], was used for the study, and *Trametes versicolor* laccase – a more well known enzyme – was used for comparison.

2. Materials and methods

2.1. Chemicals

The chemicals used were procured from different sources: 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), polyethylene glycol (PEG) 8000, guaiacol (G), vanillin (V), syringaldehyde (SD), deuterated chloroform ($CDCl_3$), deuterium oxide (D_2O) and ethyl acetate were from Sigma–Aldrich® (USA), syringol (2,6-dimethoxyphenol) (S) from Acros Organics (USA), vanillic acid (VA) and syringic acid (SA) from Alfa Aesar® (Germany), 4-methyl syringol (MS) from SAFC™ (USA), *para*-aminobenzoic acid (PABA), PEG 4000 from Fluka (Switzerland) and sodium azide from BDH Chemicals (England), polyethylene glycol monomethyl ether (mPEG 5000) from Shearwater polymers (USA), series of polystyrene molecular mass standards PS 10000, 30000, and 150000 from Fluka (Germany), silica gel 60 (40–63 μm), HPLC grade acetonitrile (LiChrosolv®) and acetic acid from Merck (Germany), chloroform (HiPerSolv®) from BDH Prolabo® (EC), and deuterated dimethylsulfoxide ($(CD_3)_2S{=}O$) from Amar Chemicals (Switzerland). Eucalyptus Kraft lignin was kindly provided by Innventia AB (Sweden). All the reagents used were of analytical grade.

2.2. Enzyme preparation and assay

Galerina sp. HC1 laccase was produced and partially purified as described earlier [26], while *T. versicolor* laccase was purchased from Fluka BioChemika (Switzerland) and used as such. The laccase activity was determined by following the oxidation of ABTS at 420 nm (Lambda Bio+ UV–vis spectrophotometer, PerkinElmer, USA). The reaction mixture (1 mL) contained 20 mM sodium acetate buffer pH 5, 1 mM ABTS, and a suitable amount of the enzyme sample. One unit of laccase activity was defined as the amount oxidising 1 μmol of ABTS per minute.

2.3. Oxidation and coupling reactions

For the oxidation reactions 5 mM of a model compound was allowed to react with 0.4 U/mL laccase in 20 mM sodium acetate buffer pH 5, at room temperature (~22 °C) with mixing on rocking table (Mixer 440, Swelab Instrument AB, Sweden). The reaction was stopped by adding sodium azide to the final concentration of 40 μM. The coupling of equimolar amount of PABA (5 mM) to the compounds was also investigated under similar conditions.

Oxidation of Kraft lignin (concentration 10 mg/mL) and coupling with PABA (5 mM) was also studied as above.

2.4. Analyses

A high performance liquid chromatography (HPLC) system (Jasco, Japan) consisting of two pumps (PU-980 Intelligent HPLC Pumps), a degasser (DG-980-50 3-Line Degasser), a column oven (Shimadzu CTO-10AC vp Column Oven) set at 35 °C, a UV detector (UV-975 Intelligent UV-VIS Detector) and an autosampler (AS-950-10 Intelligent Sampler) was used for the analysis of reactants and products. Samples were diluted 4 times (or 12 times for the lignin samples) in 40% (v/v) acetonitrile and injected (5 μL) into a reversed phase C18 column (ACE 5 C18-AR, 250 × 4 mm, ACE®, Scotland) equipped with a guard cartridge at the inlet. Elution was done using a mobile phase made from solutions of 0.1% acetic acid (in water) and 100% acetonitrile (ACN). Separation protocols employing different ACN gradients were developed for good resolution of reaction components obtained with each substrate (Table 2). Wavelengths that gave the highest absorbance were chosen after spectrophotometric scanning of both the substrates and products. Many common products for different substrates were later confirmed by using one HPLC protocol. It may be noted that the

Table 2
HPLC protocols used to follow the laccase catalysed modification of guaiacyl and syringyl compounds.

Substrate (abbreviation)	Flow rate (mL min^{-1})	Detection wavelength (nm)	Elution (% of ACN)
Guaiacol (G)	1	229	36–40
Vanillin (V)	1	290	40
Vanillic acid (VA)	0.5	290	20–40
Syringol (S)	0.5	269	20–40
Syringaldehyde (SD)	0.5	290	20–40
Syringic acid (SA)	0.5	290	28–40
4-methyl syringol (MS)	0.8	273	28–40

substrates and their respective oxidation products would have different molar extinction coefficients, and so the peak areas in HPLC chromatogram would not give actual product yields. However, for convenience an estimation of the amounts of the formed products is based on HPLC peak areas.

Liquid chromatography coupled to mass spectrometry (LC–MS) was used to determine the molecular mass of the products. Similar chromatographic conditions were used as for the HPLC analysis. The QSTARpulsar-i-Q-TOF tandem mass spectrometer (PE Sciex, Canada) was employed. The turbospray source was set to positive ion mode with a needle voltage +4900 V. The quadrupole system was set to scan *m*/*z* 120–1200 in TOF-MS mode and 50–700 for MS/MS with 1 second per scanning cycle.

Gel permeation chromatography (GPC) with chloroform as mobile phase was used for the determination of the average molar mass of guaiacol and syringol oxidation products. PEG 4000 and 8000, mPEG 5000, and PS 10000, 30000 and 150000 (5 mg/mL) were used as molecular mass standards. Standards and samples were dissolved in chloroform and injected (2 μL) into a GPC column (K-805, 5 μm, 300 mm × 8 mm, Shodex, Japan) at a flow rate of 0.5 mL min^{-1}. A PerkinElmer Series 200 HPLC system was used with dual detection by UV/vis detector (785A, Perkin Elmer, USA) and Evaporative Light Scattering Detector (Alltech® 3300 ELSD, Grace Davison Discovery Sciences, USA).

2.5. Isolation of reaction products for structure determination

The products obtained in the laccase catalysed reactions with various substrates were isolated from the reaction mixture by different procedures. The main oxidation product of syringic acid was extracted from 12 mL reaction volume with 24 mL ethyl acetate followed by rotary evaporation (Heidolph, UK), while the product obtained from the reaction with PABA was used directly for analysis after freeze-drying.

The major oxidation product of syringaldehyde was identified directly by LC–MS analysis of the reaction mixture without any extraction. The product of methyl syringol was isolated through extraction by two volumes of ethyl acetate from a 40 mL reaction volume followed by evaporation of the solvent under nitrogen flow. The reactions of syringaldehyde and 4-methyl syringol with PABA were run in 200 mL volume, and the resulting reaction mixture was centrifuged (25 min at 2800 × *g*). Separation (or purification) of the products from the supernatant and insoluble fraction was performed using a silica gel column (2.5 cm diameter × 80 cm length) and a solvent mixture of chloroform:methanol:water (65:31:4, v/v/v) based on preliminary screening trials on TLC plates coated by the same type of silica gel. Five millilitre fractions were collected and analysed by reversed phase HPLC using the conditions stated in Table 2. The fractions containing the same product were pooled together and dried by rotary evaporation.

The purified compounds were dissolved in 750 μL of deuterated solvent(s) and analysed by ^{1}H NMR, ^{13}C NMR, correlation spectroscopy (COSY) and heteronuclear multiple bond correlation (HMBC) using a 400 MHz NMR system (UltraShield Plus 400, Bruker, Germany). The purity of the compounds was based on HPLC determination and ranged between 80 and 98%.

3. Results and discussion

3.1. Laccase catalysed oxidation of S- and G-type compounds

Oxidation of the S- and G-type compounds catalysed by laccase, and monitoring the reaction by HPLC analysis showed the S-compounds (S, SD, SA, and MS) to be completely oxidised within 1 h while the oxidation rates of G-compounds (G, V, and VA) were much lower (6–16 h) resulting primarily in water insoluble products. As an example, a comparison of V and SD (both bearing aldehyde groups at the R_2 site) oxidation is shown in Fig. 1. The high rate of oxidation of S-compounds is attributed to the higher number of methoxy groups (Table 1), which decreases their redox potential and increases the electron density at the phenoxy group allowing them to be easily oxidised by laccase [27,28]. In case of the G-compounds, the generation of free radicals, initiated by deprotonation of the phenolic hydroxyl groups on the molecules, favours their coupling with each other thus resulting in the formation of homo-molecular polymers [24]. The products obtained after longer reaction times with laccase are dimers, oligomers or polymers; in an earlier report a range of oligomers (dimer to pentamer) were obtained on treatment of vanillic acid by a laccase from the fungus *Rhizoctonia praticola* [29].

Among the S-compounds, syringol behaved similar to the G-compounds on oxidation by laccase and was totally converted to an insoluble purple coloured product, with a molecular mass of 38.2 kDa according to GPC analysis. Treatment of syringol with a laccase from *Pycnoporus coccineus* in an organic medium reported earlier has also shown the formation of a homopolymer [30]. This preference to undergo homo-oligomerization is directly associated with the absence of substituents on the R_2 position (see Table 1) of syringol which provides an additional site for radical formation. Unlike syringol, the oxidation products of SD and SA were soluble, while those of MS were partially insoluble, and all reaction mixtures were yellowish-brown in colour. The HPLC analyses done according to Table 2 revealed one major product peak (**10**) with retention times of 11.2 min, 8.3 min and 5.2 min, in case of SD, SA and MS, respectively (Fig. 2a–c); the retention time of the product was similar when analysed using the same HPLC protocol. The product (**10**) was confirmed to be 2,6-dimethoxy-1,4-benzoquinone (2,6-DMBQ) with a molecular mass of 168.04 g mol^{-1} (cal. 168.04 g mol^{-1}) by NMR and mass spectrometric analyses (Table 3 and Supplementary Fig. S1). The HPLC–MS analyses of minor oxidation products revealed masses that corresponded to dimeric compounds of SD and SA with *m*/*z* of 319 (cal. 318.11 g mol^{-1} (**11**)) and 335 (cal. 334.10 g mol^{-1} (**12**)), respectively (Supplementary Figs. S2 and S3). The peaks separated by 18 Da appear in low *m*/*z* regions of the mass spectrum from $[M+NH_4]$ adducts. The oxidation pattern remained unchanged during 24 h reaction time. A HPLC pattern comparable to that of SD and SA was obtained for MS, with product (**13**) eluting as a broad peak at RT13.5 min (Fig. 2c).

Based on the HPLC peak areas, it is seen that the formation of putative oligomeric products upon laccase catalysed oxidation of the S-type substrates is in the order: S (100%) > MS ((**13**), 37%) > SD ((**12**), 32%) > SA ((**11**), 15%) (Fig. 2). It is clear that the presence and the nature of a substituent group at the *para* position (R_2 in Table 1) with respect to the hydroxyl group on the phenolic ring, has a drastic influence on minimising the tendency of the phenolic free radicals to couple with each other and instead to be further oxidised enzymatically to form quinonoid derivatives. SA gives the highest yield of the quinone product (**10**), while SD oxidation is slightly slower, as it would first undergo oxidation to SA, and gives higher amount of oligomer.

3.2. Laccase catalysed oxidative coupling of S- and G-type compounds with PABA

The oxidation of phenolic/aromatic substrates by laccase, followed by heteromolecular coupling can be a promising possibility for the synthesis of new compounds [18]. PABA was used as a model for aromatic amines for reaction with G- and S-compounds in the presence of laccase. Incubation of PABA alone with laccase resulted in no reaction as reported earlier even for dichloroaniline [31].

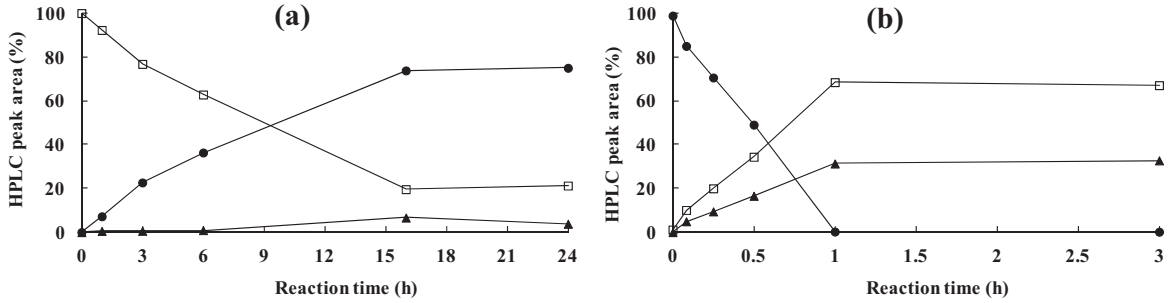
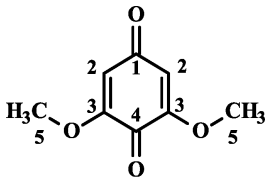


Fig. 1. Time course for the laccase catalysed oxidation of (a) vanillin (V) and (b) syringaldehyde (SD). Symbols indicate (a): (□) vanillin (**2**) with HPLC retention time (RT) of 3.4 min, (●) oxidation product (**8**) (RT 4.9), (▲) oxidation product (**9**) (RT 5.3), and (b): (●) syringaldehyde (**5**) (RT 14.1), (□) oxidation product (**10**) (RT 11.2), (▲) oxidation product (**11**) (RT 23.2). The HPLC analyses were performed according to Table 2 and Section 2.4; in case of vanillin isocratic elution with 40% acetonitrile at a flow rate of 1 mL min^{-1} was employed while a gradient of 20–40% acetonitrile at a flow rate of 0.5 mL min^{-1} was used for separation in case of syringaldehyde. The compounds were detected by absorbance at 290 nm.

Table 3
^{1}H and ^{13}C NMR chemical shifts for the oxidation product of syringic acid by *Galerina* sp. laccase.

Atom number	Assignment of chemical shifts ($(CD_3)_2S{=}O$)		Structure
	^{1}H NMR	^{13}C NMR	
1	5.97 (s[a], 2H)	187.58	
2		107.52	
3		157.73	
4		175.01	
5	3.75 (s, 6H)	56.93	(10)

[a] Singlet.

Incubation in the presence of G-molecules led to almost negligible cross-coupling reactions; V did not react at all while extremely low consumption of PABA was observed during reaction with G and VA giving 5 minor products in agreement with earlier reports involving reaction of chloroaniline with G using *R. praticola* and *T. versicolor* laccase [32] and of PABA with VA catalysed by *R. praticola* laccase [33].

Even syringol (S) did not exhibit any coupling with PABA and lower consumption of S was noted in comparison to the system without the amine. Inclusion of PABA in the reaction mixture with SD, SA and MS, respectively, showed significant reduction in the major oxidation product (**10**) and disappearance of the oligomers (**11**, **12** and **13**), while a new major product and other minor ones appeared as revealed by HPLC (Fig. 2a′–c′ and Table 4). Mikolasch and Schauer have earlier distinguished homo-molecular coupling products as well as hetero-molecular hybrid dimers during laccase catalysed reaction between substrates with different structures [34]. In our experiments, the reaction was most efficient with SA, and PABA was completely consumed within 3 h. In case of SD, more product peaks appeared on the chromatogram at the expense of the amount of the major product peak, while more 2,6-DMBQ and even PABA was left unconsumed at the end of the reaction. Reaction of PABA with MS led to the formation of several products in varying amounts; TLac displayed significantly higher reaction efficiency than GLac. The difference in catalytic efficiency of two other laccases from *M. albomyces* and *T. hirsuta* has earlier been attributed to two factors: (i) the difference in redox-potential between T1 copper of the respective laccase and the oxidised substrate, and (ii) the specificity towards that substrate (K_{cat}/K_m) [28].

When PABA was replaced with *para*-hydroxybenzoic acid, no coupling with SA was noted even at a lower reaction pH (pH 3), which highlights the importance of the stronger nucleophilicity of the amino group for coupling. Moreover, no product was detected if 2,6-DMBQ was isolated after the oxidation reaction and then incubated with PABA, or if the latter was added to the reaction mixture after oxidation of SA and the enzyme inactivated by addition of sodium azide. Hence, the presence of active laccase seemed to be

Table 4
HPLC peak area percentage of products obtained during oxidative coupling (over 3 h) of syringaldehyde (SD), syringic acid (SA) and 4-methyl syringol (MS), respectively, with *p*-aminobenzoic acid (PABA) catalysed by *Galerina* sp. laccase (GLac) or *T. versicolor* laccase (TLac). The HPLC protocols for the different substrates listed in Table 2 were used.

Substrates	Peak retention time in min (compound number)	Peak area (%)	
		GLac	TLac
SD + PABA	6.4 (PABA)	1.6	9.8
	7.1	5.8	5.7
	11.2 (**10**)	21.4	29.3
	20.6 (**14**)	30.6	22.9
	22.1	2.4	2.1
	23.4	8	3.9
	24.0 (**15**)	25	11.8
	28.7	0	11.5
SA + PABA	8.3 (**10**)	4.7	6.9
	13.5	5.2	0.2
	14.4 (**14**)	83.4	86.7
	18.4	6.1	5.8
MS + PABA	3.6	0.8	11.5
	4.0 (PABA)	57.2	5.9
	4.3	0	14.7
	6.1	14.3	0
	8.5	1.5	3
	11.0 (**17**)	10.1	0.6
	13.0 (**15**)	4.9	15.8
	16.5 (**18**)	4.1	0
	19.5	0.4	8

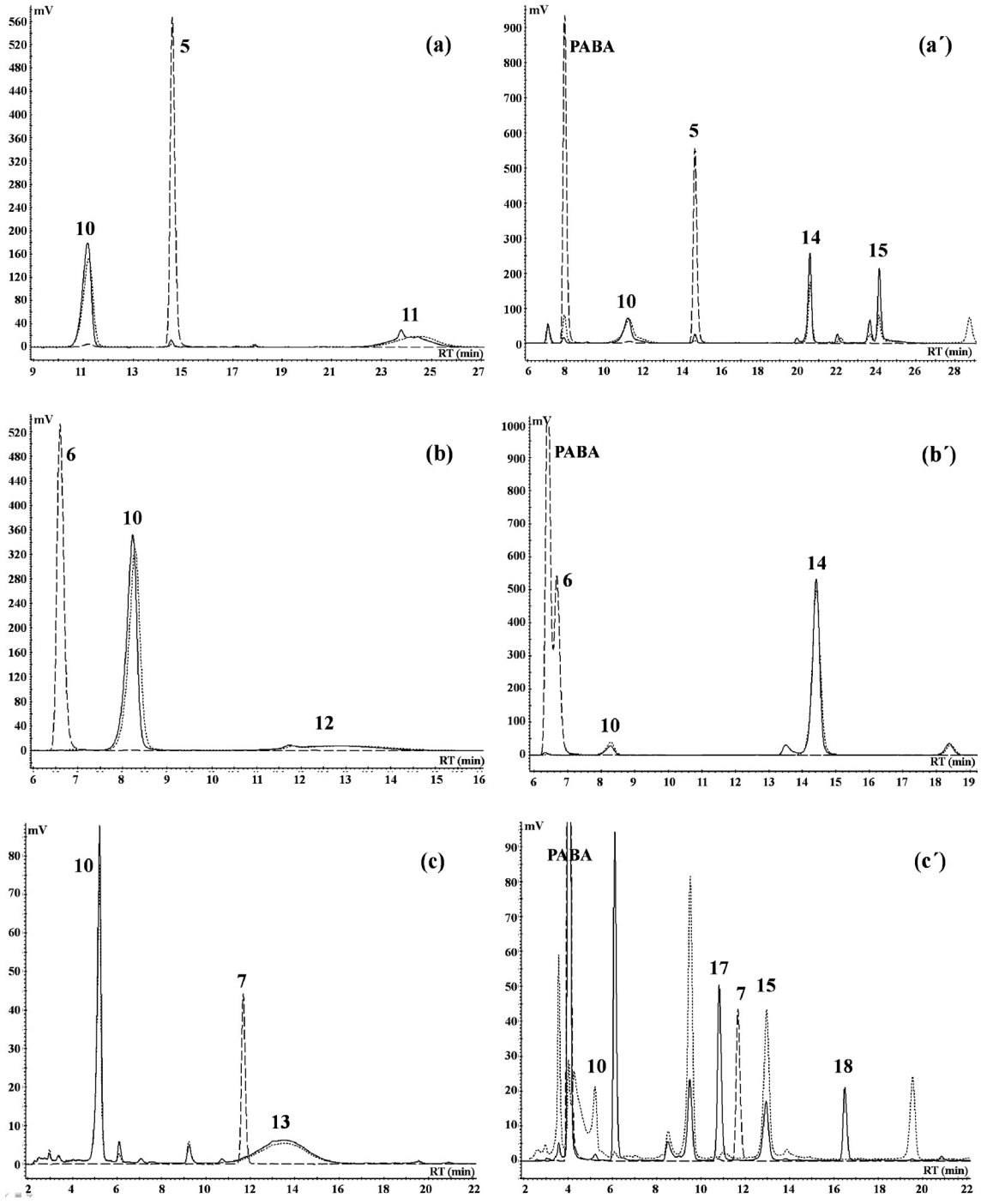


Fig. 2. HPLC chromatograms of the reaction mixtures obtained on laccase catalysed oxidation of (a) syringaldehyde, (b) syringic acid and (c) 4-methyl syringol, and their coupling to PABA (a′–c′). The HPLC protocols for the different substrates listed in Table 2 were used. The different curves correspond to pure substrate (- - -), substrate oxidised by GLac (—) and TLac (· · · · · ·). The reaction time was 3 h.

essential for the coupling to occur. These observations suggest that the aromatic amine reacts directly with the decarboxylation intermediate generated by the enzymatic reaction prior to the formation of the stable quinone product (**10**). The latter is presumably formed by the addition of water molecule to the intermediate oxidation product instead of the aromatic amine.

Table 5 presents the NMR data (chemical shifts assignments as well as short and long range homo- and hetero-nuclear coupling) and structure elucidation of the major products obtained in the laccase catalysed reactions with SD and SA, and some products from MS with PABA, and Scheme 1 proposes the reaction pathways involved leading to the formation of these products. Coupling between SA (**6**) and PABA occurs most likely by a nucleophilic attack of the latter's amino group onto the decarboxylated intermediate generated by the action of laccase. Earlier reports suggested however that the attack is made on the carbonyl group of the quinonic oxidation products, as reflected in the reaction between SA or VA with aniline [33,35]. Tatsumi et al. [33] reported on the formation of a Michael's type adduct in the reaction between aniline and protocatechuic acid which lacks an *ortho*-methoxy group.

The reaction with SD (**5**) also led to the formation of compound (**14**) and an additional compound (**15**), which appears to

Table 5
^{1}H and ^{13}C chemical shifts assignments for the main products formed (in Fig. 2) during laccase catalysed reaction of S-type compounds with PABA for 3 h. Supporting information from COSY and HMBC NMR experiments is also provided and corresponding structures elucidated.

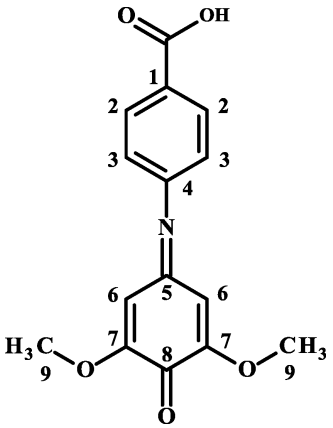
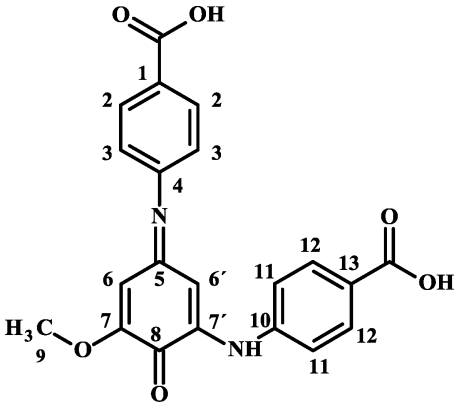
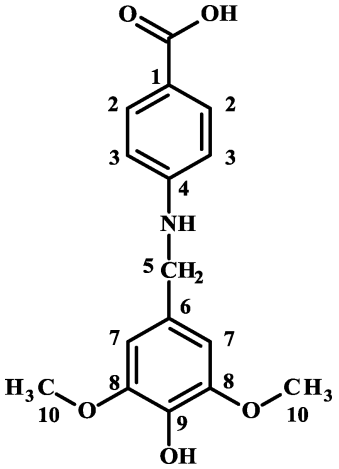
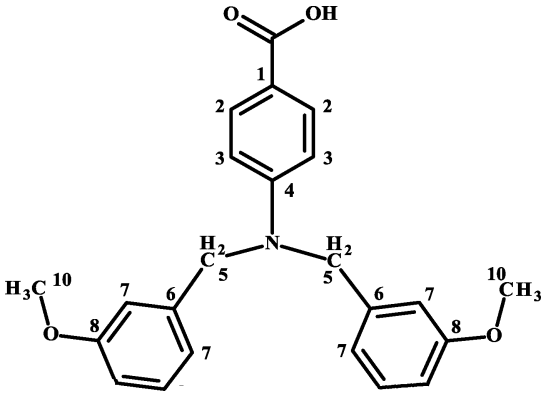
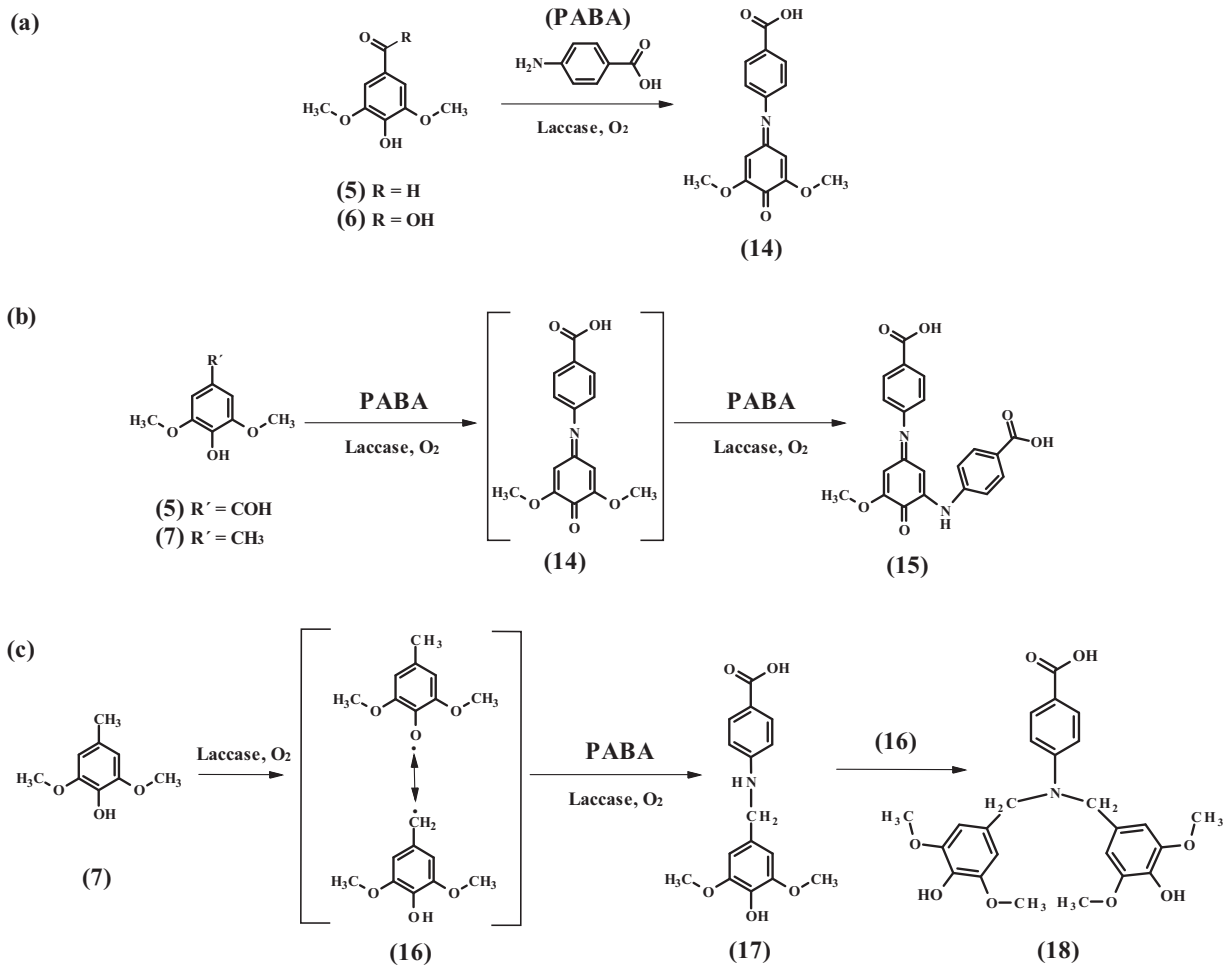
Compound number	Atom number	Assignment of chemical shifts ($(CD_3)_2S{=}O$)				Structure
		^{1}H	^{13}C	COSY	HMBC	
14	—COOH	12.72 (b[a], 1H)	167.47			
	1		127.20			
	2	7.98 (d[b], J[c]4.72 Hz, 2H)	130.94		2, 4, -COOH	
	3	6.98 (d, J4.72 Hz, 2H)	120.78		1, 3	
	4		154.34			
	5		157.55			
	6	6.52 (d, J2.16 Hz, 1H), 5.97 (d, J2.16 Hz, 1H)	111.62 98.83		6, 8	
	7		155.71, 154.96			
	8		176.09			
	9	3.82 (s, 3H), 3.59 (s, 3H)	56.56, 56.27		7	
15	—COOH		169.39			
	1		132.30			
	2	7.93 (d, J8.4 Hz, 2H)	130.53	3	4	
	3	6.87 (d, J8.4 Hz, 2H)	120.70	2	1, (3)	
	4		153.33			
	5		157.97			
	6	6.51 (s, 1H)	112.49	(6′)	8	
	6′	6.23 (s, 1H)	94.88	(6)	8	
	7		154.35			
	7′		140.11			
	8		178.01			
	9	3.86 (s, 3H)	56.37	(6)	7	
	10		141.87			
	11	7.17 (d, J8.4 Hz, 2H)	120.59	12	13, (11)	
	12	7.77 (d, J8.4 Hz, 2H)	130.34	11	10	
	13		130.93			
	—NH—	8.69 (s, 1H)				
	—COOH		163.40			

Table 5 (*Continued*)

Compound number	Atom number	Assignment of chemical shifts ($(CD_3)_2S{=}O$)				Structure
		^1H	^{13}C	COSY	HMBC	
17	—COOH		168.06			
	1		117.87			
	2	7.65 (d, J8.8 Hz, 2H)	131.44	3	2, 4, -COOH	
	3	6.61 (d, J8.8 Hz, 2H)	111.64	2	1, 3	
	4		152.86			
	5	4.20 (d, J6.0 Hz, 2H)	46.78	—NH—, (3)	4, 7	
	6		129.66			
	7	6.63 (s, 2H)	105.46		5, 7, 9	
	8		148.41			
	9	8.23 (b, 1H, low intensity) OH)	134.89			
	10	3.73 (s, 6H)	56.42		8	
	—NH—	6.86 (t[d], J5.6 Hz, 1H)		5		
18[e]	—COOH		167.86			
	1		117.93			
	2	7.69 (d, J8.8 Hz, 2H)	131.34	3	2, 4, —COOH	
	3	6.75 (d, J9.2 Hz, 2H)	111.83	2	1	
	4		152.45			
	5	4.60 (s, 4H)	54.56	(7)	4, 5, 6, 7, (9)	
	6		128,42			
	7	6.44 (s, 4H)	104.62	(5)	5, 7, 9	
	8		148,43			
	9		134.74			
	10	3.66 (s, 12H)	56.34		8	
	—OH	8.23 (b, low intensity)				

[a] Broad.
[b] Doublet.
[c] Coupling constant.
[d] Triplet.
[e] This compound was dissolved in a mixture of deuterated solvents $CDCl_3$:$(CD_3)_2S{=}O$:D_2O (25:75:15).



Scheme 1. Oxidative coupling of syringaldehyde (**5**), syringic acid (**6**), and 4-methyl syringol (**7**) with *p*-aminobenzoic acid (PABA) catalysed by laccase.

be formed by addition of a second molecule of PABA to (**14**) via demethoxylation and nucleophilic attack (Scheme 1b). In case of MS (**7**), several products were formed. The compounds (**15**), (**17**) and (**18**) were identified to be among the main products formed (Scheme 1c). Compound (**18**) would most likely be formed by addition of one PABA to the oxidation product (**16**) of MS (with an $m/z = 167$ (cal. 166.06) as detected by mass spectrometry), while coupling of another (**16**) to (**17**) results in the formation of the heterotrimer (**18**).

Among the four products (**14**, **15**, **17** and **18**), only (**17**) has been produced earlier by chemical synthesis using hazardous solvents and high temperature, and has been reported to possess activity as a hypolipidemic drug [36]. The other three compounds are completely new structures; related structures with a C–N linkage produced by laccase catalysed reaction between aromatic or cyclic amines and substituted phenolic compounds have been shown to display antibiotic activity [37–39].

Preliminary experiments were also conducted with Eucalyptus Kraft lignin in place of the S-/G-compounds. Treatment with laccase showed the presence of SA, SD and 2,6-DMBQ in the soluble fraction of the reaction mixture, indicating that a fraction of the Kraft lignin was degraded to its constitutive monomers. The reaction with PABA resulted in 4 products, with the major product being compound (**14**). Higher conversion of PABA was obtained with TLac, probably due to its higher redox potential as mentioned above.

4. Conclusion

The study shows that the difference in the number of methoxy groups in guaiacyl- and syringyl subunits of lignin results in significant difference in their reactivity on treatment with laccase. The G-type compounds with one methoxy group are prone to homo-oligomerization due to coupling of phenolic radicals, while one extra methoxy group in S-type compounds makes them (except syringol) more prone to rapid oxidation with laccase and – reaction with other aromatic molecules with a nucleophilic moiety. Furthermore, the substitution on the *para* position of the phenolic hydroxyl on the S-compounds led to the formation of diverse hetero-oligomeric products. Three new products (**14**, **15** and **17**) were identified, which would be interesting to test for bio-activity. Laccase catalysed synthesis of product (**15**), identified as a hypolipidemic agent could provide a green alternative to the conventional chemical synthesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.07.014.

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