PUBLICATION III

Experiences of kraft lignin functionalization by enzymatic and chemical oxidation

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Experiences of Kraft Lignin Functionalization by Enzymatic and Chemical Oxidation

Anna Kalliola,* Martta Asikainen, Riku Talja, and Tarja Tamminen

Linear hydrophilic derivatives are expected to soften lignin and improve its utilization in composite applications. Oxidation by means of laccase in the presence of oxygen was employed in an attempt to functionalize commercial kraft lignin by vanillic acid-PEG ester and ether derivatives. *Thielavia arenaria* and *Melanocarpus albomyces* laccases at pH 6 and 8 were used. According to O_2 consumption and the increase in molar mass, the tested laccases were active toward the lignin and the vanillic acid derivatives and also formed corresponding phenoxyl radicals. However, homogenous polymerization instead of cross-coupling and functionalization took place. As an alternative, lignin functionalization by the ester derivative by chemical oxidation under alkali-O_2 conditions was also tested. Efficient lignin polymerization was observed. However, functionalization was not detected. Interestingly, a clear decrease in lignin glass transition temperature was obtained by an isolation procedure that included freeze-drying. This suggests that functionalization may not be necessary to induce the desired softening effect.

Keywords: Kraft lignin; Vanillic acid; Polyethylene glycol; Composite; Glass transition temperature; Laccase; Oxygen; Oxidation

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INTRODUCTION

The production of bio-composites containing lignin as the thermoplastic component is a promising application for this industrially significant biomass side stream material (Gosselink *et al.* 2004; Schorr *et al.* 2014). The problem, however, is that lignin itself has poor thermal stability (Fenner and Lephardt 1981) and melt-flow (softening) behavior under the elevated temperatures in composite processing. The latter is partly due to crosslinking between the macromolecules *via* intermolecular hydrogen bonding. Polymer blending (Kadla and Kubo 2004; Kubo and Kadla 2005) or chemical modifications (Glasser and Jain 1993a, b; Lora and Glasser 2002) of lignin by linear substituents, *e.g.*, alkylene, (poly)ethylene, propylene oxide, and butylene oxide have been studied as means to block this type of crosslinking. This internal plasticization of lignin is often measured as the reduced glass transition temperature (T_g), which indicates the ability of the material to undergo softening at reduced temperature. Lignin plasticization by chemical modification enhances the glass transition and reduces the brittleness of lignin derived polymers, both characteristics desirable *e.g.* in polymer coating and composite applications (Hult *et al.* 2013; Laurichesse and Avérous 2014).

Laccase enzymes catalyze the oxidation of a wide range of aromatic substrates, especially phenols, with the simultaneous reduction of molecular oxygen to water. Phenolic substrates are oxidized to phenoxyl radicals, which, depending on the reaction conditions, can spontaneously polymerize *via* radical coupling or react further leading to quinones, Cα

oxidation, or cleavage of Cα–Cβ bonds or aromatic rings (Giardina *et al*. 2010). However, the main reaction pathway of the phenoxyl radicals generated by laccase is considered to be spontaneous polymerization. The other reaction pathways are not that likely to occur due to slow kinetics of oxygen addition to the phenoxyl radical species (Crestini *et al*. 2010).

Laccases have been widely used for lignin modification. Recently, in comparing different types of technical and emerging lignins, higher lignin-laccase (*Trametes hirsute*) reactivity was found to correlate with lower lignin molecular mass and higher amounts of monomeric phenolics (West *et al*. 2014). Laccases have also been applied to provide a route for the addition of desired functionalities onto lignin, as the phenoxyl radicals are potential sites for coupling reactions with the functionalizing reactant (phenolic) radicals. Lund and Ragauskas (2001) investigated the incorporation of water-soluble phenols with carboxylic or sulfonic acid groups onto kraft lignin by laccase (*Trametes villosa*) catalysis in dioxane. The oxidative coupling between lignin and a phenol was most strongly illustrated by the incorporation of guaiacol sulfonate onto lignin, which made the lignin water-soluble at a pH of 2.4. A chemo-enzymatic polymerization pathway has been reported as a means to graft acrylamide and acrylic acid onto lignosulfonates. In this process, the role of laccase (*Trametes versicolor*) appeared to catalyze the formation of phenoxyl radicals in lignin, which further induced the formation of organic peroxide-derived radicals required as initiators in the co-polymerization (Mai *et al*. 2000a,b, 2001, 2002). In the investigations by Hüttermann *et al.* (2000) laccase activated lignosulfonate owing relative high radical density was shown to react as a crosslinking agent after mixing into a kraft lignin solution. The activated lignosulphonate was also postulated to react with nucleophiles such as cellulose and starch via covalent bonds.

The objective of this study was to find ways to soften lignin to enhance its utilization for composite applications. The primary route studied was *via* the introduction of hydrophilic functionality into the lignin structure. Phenolic derivatives with polyether type hydrophilic side chain were synthesized and applied for this purpose. Laccase-catalyzed oxidation was studied as a plausible method to induce crosslinking between lignin and the hydrophilic derivative.

EXPERIMENTAL

Materials

Lignin

A commercial kraft lignin, Indulin® AT (MeadWestvaco; USA), was used in this study.

Laccases

Three laccases were tested: *Thielavia arenaria* Lcc1 (TaLcc1) and Lcc2 (TaLcc2) (Paloheimo *et al.* 2006a, b) produced as recombinant enzymes in *Trichoderma reesei* by Roal Oy (Rajamäki, Finland) and *Melanocarpus albomyces* (r-MaL) (overproduced as recombinant enzyme in *Trichoderma reesei* by VTT).

The TaLcc1 and TaLcc2 are most active in mild acidic conditions, whereas r-MaL, in slightly alkaline conditions. Enzyme activity measurements were carried out according to Kalliola *et al.* (2011).

Methods

Synthesis of vanillic acid derivatives

Vanillic acid was derivatized with polyethylene glycol mono methyl ether (PEG-OMe, Mn 550). Two derivatives were prepared: (1) Ester derivative with vanillic acid and PEG-OMe (Ester V-PEG) was prepared by esterification with *p*-toluenesulfonic acid as a catalyst (Fig. 1) and (2) ether derivative was prepared by first brominating vanillyl alcohol (Fig. 2), followed by etherification with PEG-OMe (Fig. 3).

The analytical data associated with the structures of vanillic acid PEG ester, bromovanillate and vanillic acid PEG ether are shown in Appendix.

1. Ester derivative (Ester V-PEG)

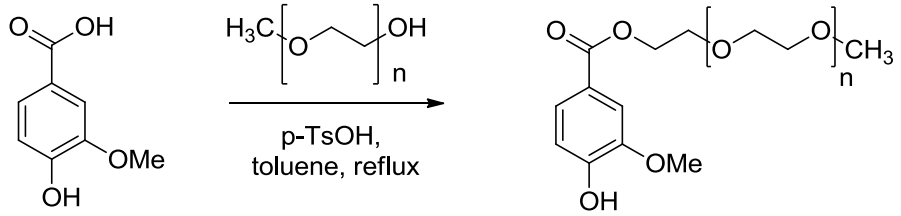

Fig. 1. Synthesis of vanillic acid PEG ester

The reaction flask was charged with vanillic acid (5.0 g, 29 mmol, 100 mol-%) and poly(ethylene glycol) methyl ether (16.3 g, 29 mmol, 100 mol-%, Mn 550). Toluene was added (50 mL), and the reaction mixture was stirred at room temperature until a smooth suspension formed. Then, *p*-TsOH (1.1 g, 6 mmol, 20 mol-%) was added. The reaction mixture was heated up to reflux for 70 h. The solvent was evaporated on a rotary evaporator and the residue was purified with column chromatography (EtOAc to remove the vanillic acid residues, then 5 % MeOH in CH_2Cl_2). The product was obtained as slightly brown oil (12.5 g, 18 mmol, 60 %).

2. Ether derivative (Ether V-PEG)

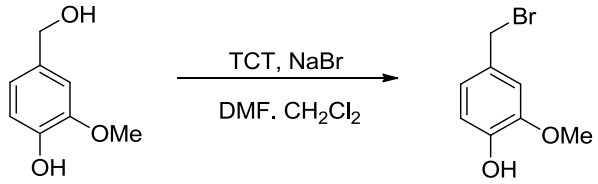

Fig. 2. Synthesis of bromovanillate (Nieddu *et al.* 2008)

Trichlorotriazine (TCT) (0.92 g, 5 mmol, 106 mol-%) was charged in the reaction flask under argon and DMF (1.5 mL, 19.4 mmol, 412 mol-%) was added. After 2 h, TCT had reacted and no solvents remained. CH_2Cl_2 (12 mL) was added, and the reaction mixture was stirred vigorously for 1 h before the addition of NaBr (0.98 g, 9.5 mmol, 202 mol-%). After 20 h, 4-hydroxy-3-methoxy benzyl alcohol (0.72 g, 4.7 mmol, 100 mol-%) was added and the reaction mixture was let to stir at room temperature for another 24 h. The reaction was quenched by adding 10 mL of water and 10 mL of 1N HCl; the resulting mixture was filtrated through a pad of Celite with a CH_2Cl_2 wash, and the organic layer was collected

from the filtrate and concentrated in vacuum. The residue was purified with flash column chromatography to yield bromovanillate (0.26 g, 1.2 mmol, 26 %), which was a slightly yellow oil. The product compound decomposed quickly.

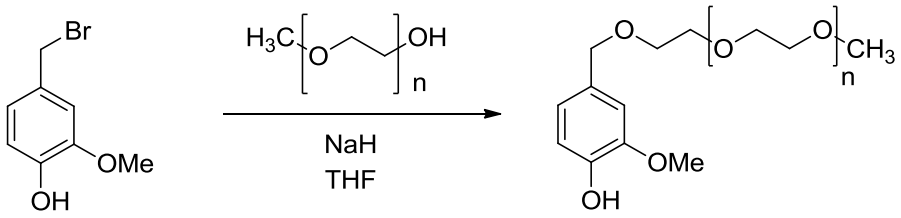

Fig. 3. Synthesis of vanillic acid PEG ether

NaH (60 w-%, 60 mg, 1.4 mmol, 120 mol-%) was charged in the reaction flask under argon and washed twice with 1 mL of hexane to remove any oil. Tetrahydrofuran (THF) (1 mL) was added, followed by the addition of poly(ethylene glycol) methyl ether (0.66 g, 1.2 mmol, 100 mol-%, Mn 550) in 2 mL of THF. After 15 min, bromovanillate was added over 30 min in 3 mL of THF. The reaction was let to stir at room temperature overnight before the addition of water (0.05 mL). The reaction mixture was filtered through a pad of Celite with a THF wash. The filtrate was collected and solvents were evaporated in vacuum to yield the product as brown oil (0.72 g, 1.1 mmol, 87 %).

Laccase-catalyzed oxidation

Lignin was first dissolved in 0.1 M NaOH, followed by gradual adjustment of pH to the desired level of 6 or 8 using 1 M HCl. Thereafter, the derivative (liquid at r.t.) was added to the system, followed by the addition of laccase. The total dry solids of the two-component system, containing lignin and the derivative, were set to 2.5 w-%.

The dosing of the lignin and the derivative was based on the phenolic hydroxyl group (PhOH) content in the substrates, as the PhOHs are the reactive sites in laccase catalyzed oxidation. The content of phenolics in Indulin® AT and in the ester/ether derivative was 4.3 (Hult *et al.* 2013) and 1.4 mmol/g, respectively. The molar ratio of lignin: derivative was set to 3:1, which equals a mass ratio of 1:1. In the experiments, laccases were dosed as nkat/g substrate, *i.e.*, lignin or derivative. Laccase treatments were also performed with one-component systems containing only lignin or the derivative as references.

Alkali-O_2 oxidation

Alkali-O_2 oxidation at high substrate concentration, 25 w-% dry solids, was performed as a chemical method to functionalize the lignin. Two-thirds of the substrate concentration included lignin and 1/3 of Ester V-PEG. Reference oxidations without the derivative were conducted at lignin dry solids of 25 and 16.7 w-% (2/3 of the 25 w-%). Analogously to the laccase experiments, dissolution of lignin in NaOH was done first, followed by the addition of the ester derivative in the case of the two-component system. The initial pH of the two-component system was set to 12.2 to avoid the hydrolysis of the ester derivative. In the case of the reference experiments conducted at lignin dry solids of 25 and 16.7 w-%, the initial pH was set to 13.3 and 12.2, respectively. The oxidation was performed in a 1-L Parr reactor applying 20 bars of initial O_2 excess for the reaction

solution (total 300 g). The initial temperature was set to 40 °C, and the reaction time was 30 min.

The oxygen consumed in the experiments was calculated from the reactor pressure data by applying the ideal gas law equation and the values of water vapor pressure. The dissolved oxygen was taken into account. A molar mass (Mw) of 180 g/mol was used for Indulin® AT.

After the oxidation, the reaction solution was dialyzed against ion exchanged water using tubes from the cellulose ester with molecular weight cut-offs of 1000 and 3500 Da (Spectra/Por Biotech) to remove the free Ester V-PEG from the large lignin macromolecules, which was targeted to be at least partly functionalized during the oxidation. The dialyzed sample solution was freeze-dried.

Analysis

The reactivity of laccases toward the one- and two-component systems was evaluated by monitoring oxygen consumption in the reaction solution just after preparing the solutions. Monitoring was performed with an Oxy-10 mini sensor oxygen meter (PreSens, Germany) in a closed 1.9-mL vessel based on dynamic luminescence quenching. Parallel treatments were also performed.

Average molar masses (M_n, M_w) of the material in the reaction solutions and in the dialyzed and freeze-dried samples were measured by size exclusion chromatography (SEC), using PSS MCX 1000 and 100,000 columns in 0.1 M NaOH eluent (25 °C) with UV detection (280 nm). The molar mass distributions and average molar masses were calculated in relation to polystyrene sulfonate standards using Waters Empower 2 software.

The glass transition temperature (T_g) of the dialyzed and freeze-dried samples was measured by differential scanning calorimetry (DSC; Mettler Toledo DSC820, STARe SW 12.10, Mettler Toledo GmbH, Switzerland). First, 5 to 8 mg of sample was weighed into a standard aluminum crucible with a volume of 40 µL, which had been oxidized at 500 °C prior to use. The crucible was closed by cold-pressing and the lid was pricked to allow evaporation of volatile substances during the measurement. The DSC was used at a heating rate of 10 °C/min, and a flow of dry N_2 (50 mL/min) was used to purge the measurement cell. The drying of the lignin sample was carried out at dynamic temperature range from 25 to 105 °C followed by isothermal conditioning at 105 °C for 20 min. The thermogram of the lignin sample was recorded across a temperature range from 25 to 250 °C. The thermogram of the Ester-PEG was recorded twice over the temperature range from -80 to 50 °C. The T_g was determined as a midpoint temperature of the baseline shift at the glass transition region.

RESULTS AND DISCUSSION

Laccase-Catalyzed Oxidation

O_2 consumption

Laccase-catalyzed oxidation forms phenoxyl radicals in substrates simultaneously with the reduction of oxygen to water. Thus, monitoring the consumption of dissolved oxygen in the reaction solution served as a means to evaluate the reactivity of the laccases towards the one- and two-component systems. Figures 4a-e show the dissolved O_2 consumption as a function of time in the various one- and two-component systems experimented with lignin and the derivatives at pH 6 and 8.

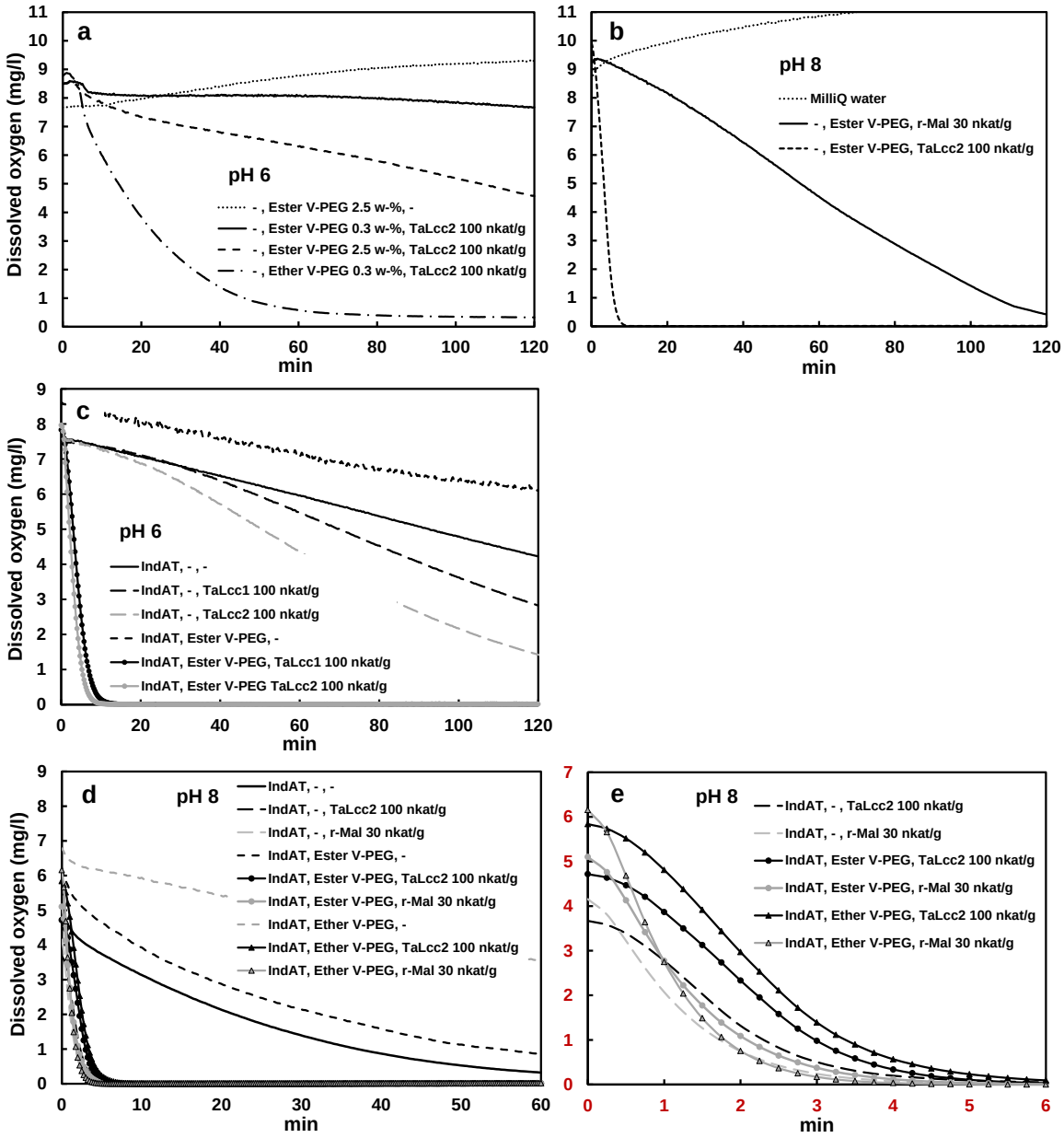

Fig. 4. (a) TaLcc2 reactivity toward ester and ether derivatives at pH 6; (b) r-MaL and TaLcc2 reactivity toward ester derivative at pH 8; (c) TaLcc1 and TaLcc2 reactivity towards Indulin® AT without or with ester derivative at pH 6; (d) r-MaL and TaLcc2 reactivity towards Indulin® AT without or with ester or ether derivative at pH 8; (e) r-MaL and TaLcc2 reactivity toward Indulin® AT without or with ester or ether derivative at pH 8, focus on the first 6 minutes

At pH 6, the reduction of dissolved O_2 in the reaction solution indicated that Ester V-PEG derivative is a substrate for TaLcc2 (Fig. 4a). Without the laccase, there were no O_2 consuming reactions. The O_2 consumption was remarkably enhanced when using the ether derivative as a substrate for TaLcc2 (comparison of the ester and ether derivative experiments conducted at 0.3 w-% dry solids). This was likely because the ether type of the derivative resembles lignin structure more than the oxidized ester type. At pH 8, in addition to r-MaL (30 nkat/g) having optimum activity in slightly alkaline conditions, the TaLcc2 (100 nkat/g) was functioning well towards the ester derivative (Fig. 4b).

Because TaLcc2 was detected to be clearly more reactive against the ether than the ester derivative, lignin functionalization at pH 6 was studied only using the ester to avoid strong homogeneous polymerization of the derivative. TaLcc1 and TaLcc2, both favoring mild acidic conditions, were active towards Indulin® AT at pH 6 (Fig. 4c). However, they were clearly more reactive in the system when replacing half of the lignin with the Ester V-PEG derivative.

Auto-oxidation of Indulin® AT took place already at pH 8, as a clear reduction of dissolved O_2 was detected (Fig. 4d). The auto-oxidation of kraft lignin under slightly alkaline conditions has also been observed in previous studies (Kalliola *et al*. 2011). Again, at pH 8, both r-MaL (30 nkat/g) and TaLcc2 (100 nkat/g) were functioning well towards Indulin® AT with or without the ester or ether derivative (Fig. 4d). When observing the first minutes of the reaction period more closely, it was seen that the two-component system containing the ether derivative consumed O_2 faster than the system containing the ester derivative (Fig. 4e).

Molar mass distribution

Monitoring the O_2 consumption in the reaction solution of the two-component system did not reveal if lignin actually was functionalized, *i.e.*, if the radical coupling between lignin and the derivative had taken place. Therefore, the molar mass distributions and the average molar masses (M_n, M_w) of part of the samples were determined (Fig. 5a-c, Table 1).

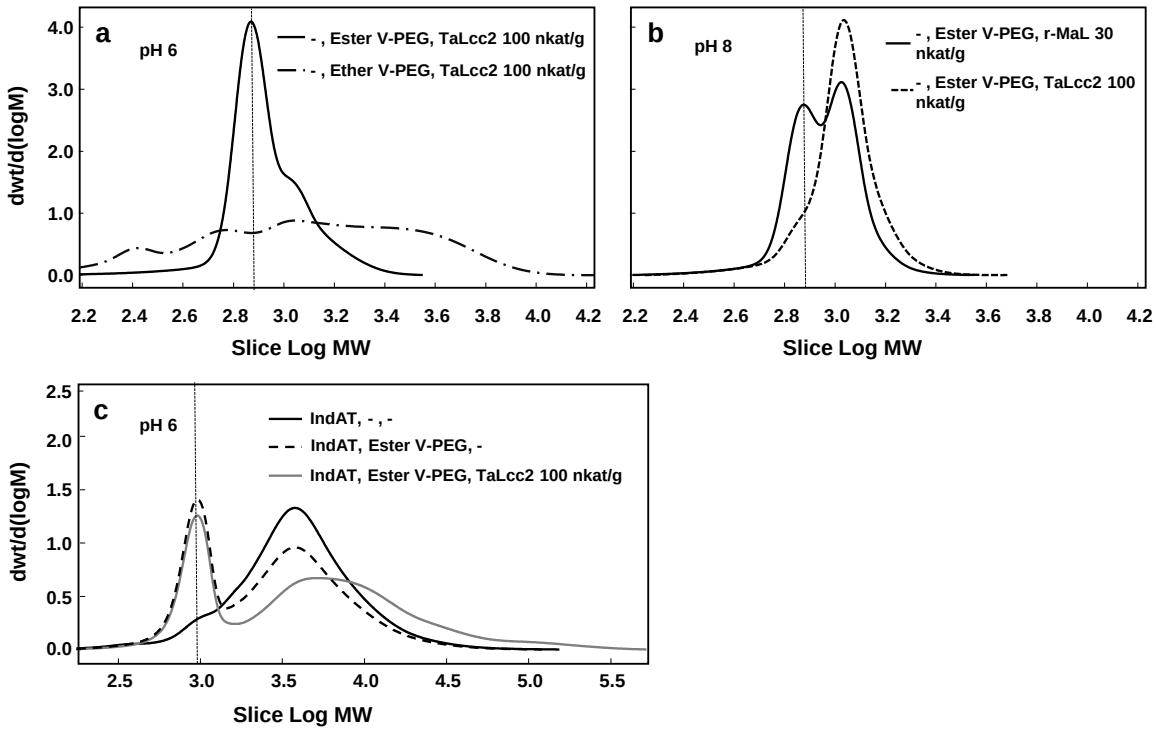

Fig. 5. (a) Molar mass distributions (MMDs) of ester and ether derivatives treated in the presence of TaLcc2 at pH 6; (b) MMDs of ester derivative treated in the presence of r-MaL and TaLcc2 at pH 8; (c) MMDs of Indulin® AT with or without ester derivative in the presence or absence of TaLcc2 at pH 6

Table 1. Average Molar Masses of One- or Two-Component Systems Treated in the Presence or Absence of Laccases at pH 6 and pH 8 (MMDs Shown in Fig. 5)

Lignin	Derivative	pH conditions	Laccase, dose (nkat/g)	M_n (g/mol)	M_w (g/mol)	PD
-	Ester V-PEG	-	-	780	865	1.1
-	Ester V-PEG	6	TaLcc2, 100	775	895	1.2
-	Ether V-PEG	6	TaLcc2, 100	790	1730	2.2
-	Ester V-PEG	8	r-MaL, 30	795	910	1.2
-	Ester V-PEG	8	TaLcc2, 100	905	1035	1.1
Indulin® AT	-	-	-	2 600	5 500	2.1
Indulin® AT	Ester V-PEG	6	-	1 700	4 400	2.6
Indulin® AT	Ester V-PEG	6	TaLcc2, 100	2 100	13 600	6.5

The results indicated that at pH 6, TaLcc2 catalyzed minor dimerization of the ester and clear polymerization of the ether derivative (Fig. 5a). The behavior was consistent with the detected O_2 consumption. At pH 8, dimerization of the ester derivative was induced by both r-MaL and TaLcc2 (Fig. 5b).

When comparing the molar mass distributions of the 2-component system treated in the presence and absence of TaLcc2 at pH 6, it was seen that the signal caused by the ester derivative was present in both distributions. In the presence of TaLcc2, the lignin part of the distribution was wider, representing homogenous polymerization of Indulin® AT (Fig. 5c). The results indicate that no or only very minor functionalization took place at pH 6 even though TaLcc2 was observed to be active against both components, lignin and Ester V-PEG, separately.

Alkali-O_2 Oxidation

Because the lignin functionalization by laccase-catalyzed oxidation was not successful, alkali-O_2 oxidation was tested as an alternative chemical method. Oxygen is an environmentally friendly oxidizing agent and widely utilized in modern pulp bleaching technology to degrade residual lignin. At an alkaline pH, molecular oxygen is able to react with phenolic lignin without an enzyme or other catalyst. Recently, odor of kraft lignin, originating from small-molecular volatile phenolics, was reduced by oxygen oxidation at alkaline pH at room temperature under conditions that induce polymerization of phenolics in an analogous manner to the laccase-catalyzed reaction (Kalliola *et al.* 2012).

Lignin reactions with oxygen in alkaline conditions start with the formation of a phenoxyl radical through an electron transfer from the ionized phenolic group (pKa 10 to 11) to molecular oxygen (Fig. 6). The subsequent steps include a reaction with the superoxide anion radical ($O_2{\cdot}^-$) forming a hydroperoxide anion structure and its rearrangement to the primary oxidation products (oxirane, muconic acid ester, or carbonyl structures) (Chang and Gratzl 1980; Gierer 1982; Sixta *et al*. 2006). The hydroperoxide structure, with a pKa value of 12 to 13, plays a crucial role, and has lately been shown to be the key intermediate in the course of phenolic lignin oxidation (Ji *et al*. 2009a,b). Below pH 12, the hydroperoxide intermediates tend to protonate and decompose homolytically back to phenoxyl radicals, which may spontaneously combine without re-oxidation caused by $O_2{\cdot}^-$. Thus, the pH in the reaction solution determines whether the intermediate reacts further, leading to degradation (Route A in Fig. 6), or induces radical coupling (Route B in Fig. 6). The primary reaction products arising from the hydroperoxide anion can react further to acidic low molecular weight reaction products (Gierer and Imsgard 1977a,b;

Chang and Gratzl 1980; Gellerstedt and Agnemo 1980), which have contribution to pH (Kalliola *et al.* 2011).

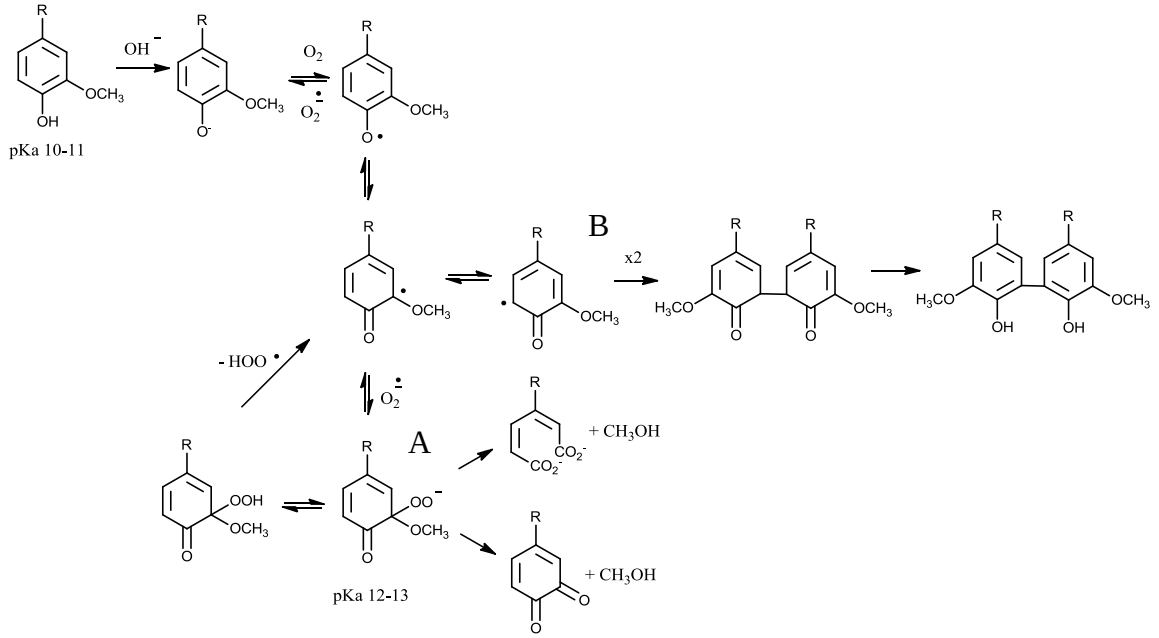

Fig. 6. Reaction mechanism of lignin with oxygen (adopted from Chang and Gratzl 1980; Ji *et a*l. 2009a,b). Route A: degradation, Route B: coupling

High lignin concentration in alkali-O_2 oxidation may favor coupling reactions because the phenoxyl radicals are close to each other. Secondly, the dissolution and diffusion of oxygen is hindered in viscous lignin-alkali solutions, which restricts the availability of oxygen species taking part in the degradation reactions.

O_2 consumption

According to the pressure drop in the reactor during the alkali-O_2 oxidation, the oxidation reactions at lignin dry solids of 25 w-% consumed much oxygen (Fig. 7a, solid, black curve). The reactions were exothermic, as can be seen from the temperature increase (Fig. 7, solid, grey curve). The corresponding behavior in the experiment, wherein 1/3 of the lignin was replaced with the ester derivative, indicated clearly less reactions. The behavior of the second reference experiment, which only contained lignin at 16.7 w-% (2/3 of 25 w-%) was similar. The results indicate that only Indulin® AT reacted, Ester V-PEG being relatively inert. Only the ester derivative was studied by the chemical oxidation route.

The computed oxygen consumption was 0.83 mol O_2/ mol lignin in the experiment with high lignin content, 25 w-%, but less than half of that in the experiments having 16.7 w-% of lignin (Fig. 7b). The initial pH, 13.3, and the increased temperature during the oxidation accelerated the oxidation reactions leading to higher O_2 uptake in the case of 25 w-% than in the case of 16.7 w-% of lignin. With the 2-component system (and its reference), the initial pH was set to 12.2 to avoid the hydrolysis of the ester derivative. The final pH after the oxidations conducted at 25 w-% and 16.7 w-% of lignin (2-component system and its reference) were 9.9 and 10.2, respectively. The greater pH drop during the oxidation in the case of 25 w-% also indicated higher reactivity and formation of acidic degradation products than in the case of 16.7 w-%.

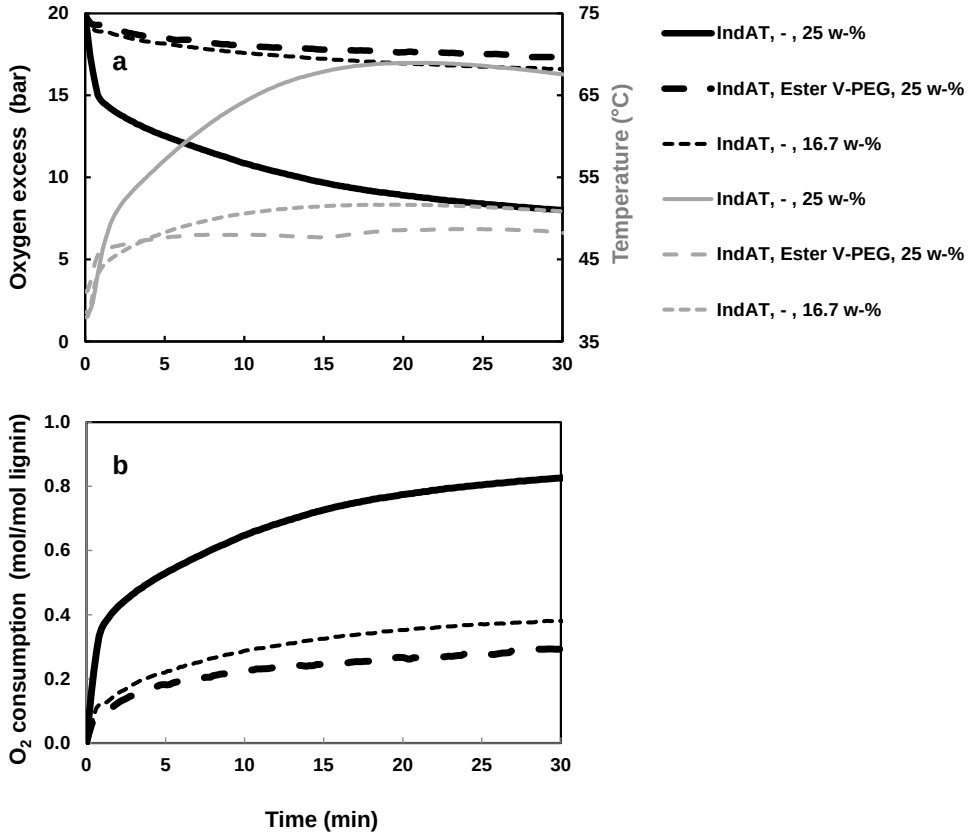

Fig. 7. (a) Pressure drop (black curves) and increase in the solution temperature (grey curves) during the alkali-O_2 oxidations of Indulin® AT without or with ester derivative; (b) Consumed oxygen during the alkali-O_2 oxidations

Molar mass distribution and Tg

The MMDs of Indulin® AT, the ester derivative, and the alkali-O_2 oxidized samples are shown in Fig. 8.

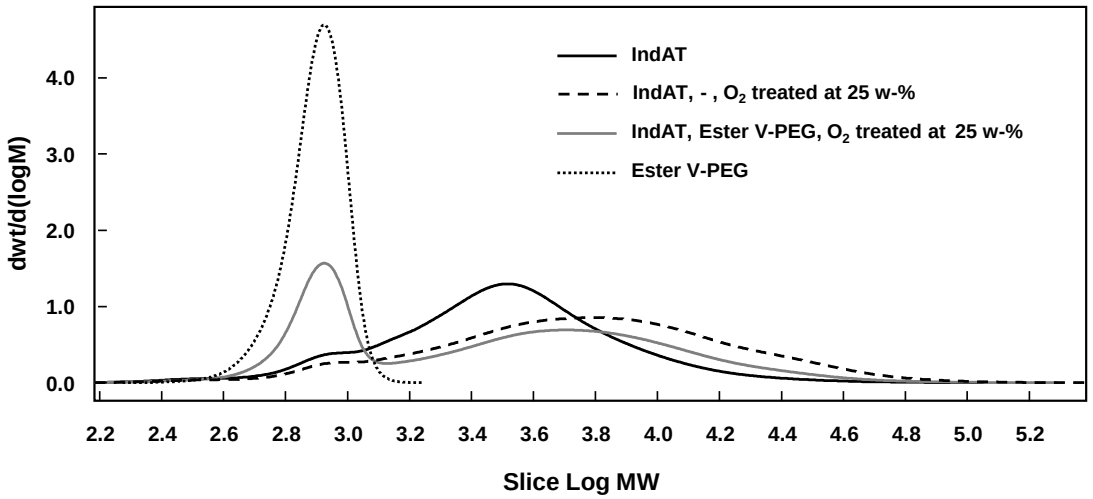

Fig. 8. MMDs of Indulin® AT, ester derivative, and alkali-O_2 oxidized Indulin® AT without or with the derivative

The alkali-O_2 oxidation caused strong condensation of Indulin® AT at 25 w-%, most likely via the 5-5 radical coupling, and increased the molar mass from 4500 to 9700 g/mol (Table 2). Hence, chemical oxidation is a promising, simple method to increase the molar mass of lignin via polymerization. The 5-5 coupling reactions are probably more pronounced with softwood kraft lignin than with hardwood kraft lignin because the latter is rich in syringyl type lignin units containing two methoxyl groups in the aromatic ring.

The signal caused by Ester V-PEG was present in the MMD of the two-component system (Fig. 8), indicating that no major functionalization occurred. In addition, there was no or only very minor homogenous polymerization of the ester derivative. These conclusions are also supported by the results of dialysis yield and glass transition temperature (Table 2).

Table 2. Analysis Results of Indulin® AT, Ester Derivative, and Alkali-O_2 Oxidized Indulin® AT Without or With the Derivative (MMDs Shown in Fig. 8). Cut-Off of Dialysis Membrane Marked in the Case of Dialysis Purification

Lignin, derivative, d.s. (w-%)	Dialysis CO, Da	Yield after dialysis	Computed lignin yield	M_n (g/mol)	M_w (g/mol)	PD	T_g (°C)	ΔC_p (Jg^{-1} K^{-1})
IndAT	-	-	-	2 100	4 500	2.1	144	0.329
IndAT, - , 25%*	1000	75	75	3 300	6 200	1.9	92	0.222
	3500	79	79	3 200	5 900	1.8	99	0.126
IndAT, - , O_2 at 25%	-	-	-	3 100	9 700	3.1	-	-
	1000	78	78	4 400	10 300	2.3	116	0.108
	3500	74	74	4 400	10 100	2.3	117	0.159
IndAT, ester, O_2 at 25%	-	-	-	1 600	5 600	3.5	-	-
	1000	50	75	3 500	7 800	2.2	99	0.19
	3500	54	81	3 300	7 500	2.3	95	0.132
Ester V-PEG**	-	-	-	760	800	1.1	-56	0.791
* Indulin® AT dissolved in NaOH at 25 w-% dry solids, un-oxidized								
** Melting temperature, Tm 5.4 °C; ΔH -62 J g^{-1}								

Alkali-O_2 oxidation for the conversion of softwood black liquor (Mathias *et al.* 1995) and softwood kraft lignins (Fargues *et al.* 1996; Araújo *et al.* 2010; Rodrigues Pinto *et al*. 2011) to phenolic aldehydes, especially to vanillin has been carried out at very aggressive pH conditions. In these batch oxidations, high yield on vanillin, up to 10% of the initial lignin has been obtained using lignin content of *ca.* 6 w-%, pH close to 14, temperatures near 150 °C, and O_2 partial pressure of 3 to 5 bars (constant). It has been observed that independent on the lignin source, the control of pH is the most important variable of the vanillin production. High alkaline pH is required both for the production of vanillin and to remain its yield, since at lower values of pH (<11.5) the vanillin oxidizes *i.e.* degrades at a considerable rate. These findings and the results of the present study highlight the importance of the hydroperoxide intermediate (pKa value of 12 to 13) and the prevailing pH conditions in the formation of the primary oxidation products during the alkali-O_2 oxidation. By maintaining the pH below the pKa value of the hydroperoxide intermediate mostly condensation (by radical coupling) or secondary oxidation reactions of the already formed structures take place.

The yield after the dialyses in the samples that only contained Indulin® AT (un-oxidized and oxidized) was in the range of 74 to 79 %, showing that the dialysis membranes passed *ca.* 25 % of the lignin material. In the sample containing 2/3 of indulin® AT and 1/3 of the derivative, the yield was as low as 50 to 54 %, indicating that in addition to the 25% of the lignin material also all of the derivative (M_w 800 g/mol) was free, *i.e.*, un-grafted to lignin and passed through the membranes with cut-offs of 1000 and 3500 Da.

Typically, plasticization (functionalization) provides lignin with lower T_g. When comparing the T_g values of the dialyzed, un-oxidized Indulin® AT (92; 99 °C) to that of dialyzed, oxidized two-component sample (99*;* 95 °C), there is no clear difference. This indicates that the alkali-O_2 oxidation did not induce functionalization.

By DSC, T_g for Indulin® AT has been determined to be between 135 and 142 °C (Penkina *et al.* 2012). Here, interestingly, a clear decrease in T_g for Indulin® AT, from 144 to 92 to 99 °C, was obtained by the reference procedure, including lignin dissolution in NaOH, followed by dialysis purification and freeze-drying. Here, the internal bonding of dissolved lignin, namely the intra-molecular hydrogen bonds, was probably disabled by the freeze-drying. When disabling the (re)arrangement of lignin, the mobility of the molecules is retained, providing lower T_g. This suggests that if aiming at a moderate reduction of lignin T_g, no modification for the internal plasticization is needed if using an isolation procedure hindering the internal bonding of lignin. The results of thermogravimetric analyses (Schorr *et al.* 2014) have shown that kraft lignins begin to degrade at temperatures around 160 °C, the extrusion temperature being generally around 160 to 165 °C. By using lignin with lower T_g, the composite processing temperatures might also be reduced, which hinders the thermal degradation of lignin.

Although T_g generally increases with increasing molar mass, it has been established that T_g increases with the degree of condensation, expressed as the fraction of phenylpropanoid units involved in C–C linkages (Baumberger *et al.* 2002). Also, in the present study, it was observed that when lignin was more condensed by the alkali-O_2 oxidation, it possessed higher T_g (116; 117 °C) than the less condensed form. However, in the case of the condensed Indulin® AT with M_w higher than 10,000 g/mol (dialysis purified), the T_g was clearly lower than that measured from the un-dialyzed Indulin® AT.

CONCLUSIONS

1. Functionalization of Indulin® AT with vanillic acid-PEG ester- and ether-type derivatives was studied using laccases from *Thielavia arenaria* and *Melanocarpus albomyces*, to soften lignin. Functionalization could not be verified. However, homogenous polymerization of lignin and the derivatives took place, indicating that the laccases were active in the reaction system. The ether derivative was a more preferable substrate for laccase than the ester derivative.
2. Molecular oxygen reacts with lignin and other phenolic compounds that are in a form of phenolates, generating phenoxyl radicals. The formed phenoxyl radicals were shown to either degrade or condensate by radical coupling analogously to the reactions taking place after the laccase catalyzed oxidation.
3. Alkali-O_2 oxidation was tested as an alternative method to functionalize Indulin® AT by the ester derivative. No functionalization was detected, nor did the alkaline oxidation have any effect on the ester derivative. However, significant condensation of

Indulin® AT occurred under the selected conditions of alkali-O_2 oxidation, suggesting that chemical oxidation is a potential means to increase lignin molecular weight prior to further modification for polymeric and material applications.

4. The results also indicate that if aiming at a moderate reduction of lignin T_g, functionalization of lignin is not necessary. Alternatively, a procedure including freeze-drying, whereby the internal bonding in lignin is suppressed, can be applied. However, the contribution of the lowered T_g toward improving the thermoplastic properties of lignin under the composite processing conditions should be verified by experiments.

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APPENDIX

Vanillic acid PEG ester had the following analytical data associated with its structure: ^{1}H NMR (500 MHz, DMSO-d6) δ 9.98 (s, 1H, OH), 7.48 (dd, 1H, J=8.3, 2.0 Hz), 7.44 (d, 1H, J=2.0 Hz), 6.89 (d, 1H, J=8.3 Hz), 4.34-4.33 (m, 2H), 3.82 (s, 3H), 3.74-3.72 (m, 2H), 3.60-3.58 (m, 2H), 3.52-3.49 (m, 45H), 3.44-3.42 (m, 2H); ^{13}C NMR (125 MHz, DMSO-d6) δ 165.6, 151.6, 147.4, 123.5, 120.5, 115.2, 112.5, 72.4, 71.3, 69.9, 69.6, 68.4, 63.6, 58.0, and 55.6; FT-IR (thin film, cm^{-1}) ν_{max} of 3310, 2871, 1710, 1284, and 1103.

From the PEG-OMe (Mn 550) distribution, the main vanillic ester products were expected to be $C_{33}H_{58}O_{16}$ and $C_{31}H_{54}O_{15}$. HRMS analysis gave $C_{31}H_{55}O_{15}$ [M+H] with accuracy of 4.5 ppm.

Bromovanillate had the following analytical data associated with its structure: ^{1}H NMR (500 MHz, DMSO-d6) δ 8.50 (s, 1H, OH), 7.26 (d, 1H, J=0.95 Hz), 7.17 (d, 1H, J=8.1 Hz), 7.06 (dd, 1H, J=8.1, 0.95 Hz), 4.77 (s, 2H), 3.80 (s, 3H); 13C NMR (125 MHz, DMSO-d6) δ 160.6, 138.4, 137.0, 122.7, 121.3, 113.5, 55.9, and 45.8.

Vanillic acid PEG ether had the following analytical data associated with its structure: ^{1}H NMR (500 MHz, DMSO-d6) δ 8.53 (s, 1H), 6.64-6.63 (m, 1H), 6.54-6.52 (m, 1H), 6.44-6.43 (m, 1H), 4.24 (s, 2H), 3.67 (s, 3H), 3.50-3.48 (m, 47H), 3.44-3.40 (m, 4H); ^{13}C NMR (125 MHz, DMSO-d6) δ 121.7, 117.3, 115.8, 113.3, 112.2, 72.9, 72.4, 71.3, 69.8, 69.6, 68.2, 60.2, 58.1, 55.2; FT-IR (thin film, cm^{-1}) ν_{max} of 3461, 2870, 1457, 1106, and 1036.