Supporting Information

The impact of lignin sulfonation on its reactivity with laccase and a laccase/HBT system

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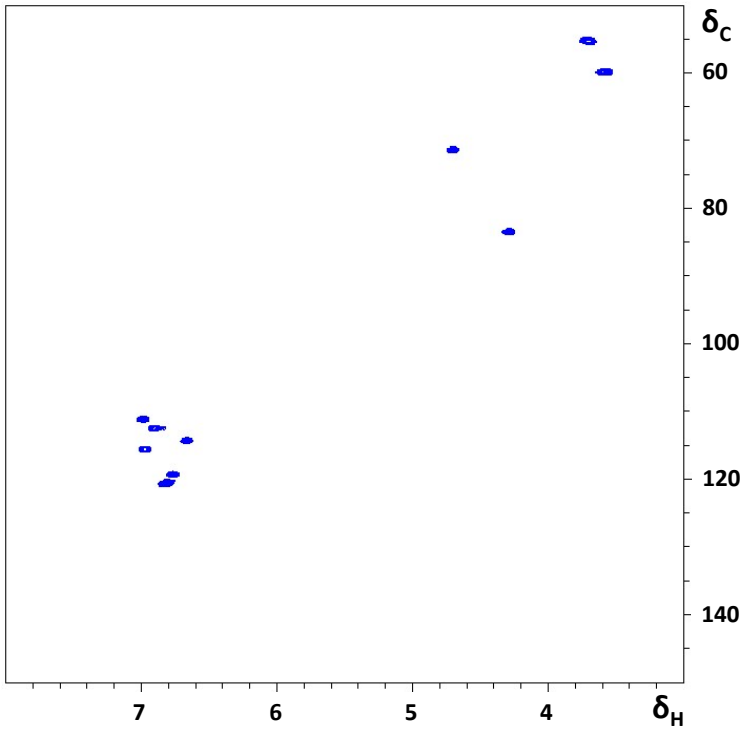
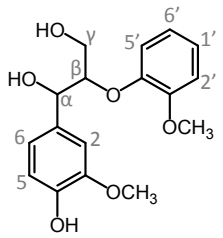
Purification of sulfonated lignin model compounds

After the sulfonation step in the microwave reactor, the incubation mixtures were cooled down to room temperature and desalted by using solid phase extraction. Supelclean ENVI-Carb cartridges (Sigma Aldrich, St. Louis, MO, USA) were activated with 80% ACN and washed with water. Subsequently, the incubation mixture was applied onto the cartridge and the cartridges were washed with 10 column volumes of water to remove salts. The model compounds were then eluted using 3 column volumes of 100% ACN, and dried under a nitrogen flow. The residue was dissolved in 1 mL water and was further purified by using a Reveleris Flash system (Grace Davison Discovery Sciences, Columbia, MD, USA), equipped with a 4 g Reveleris RP Flash cartridge, ELSD detector and UV detector. The eluents used were water (eluent A) and ACN (eluent B), both containing 1% (v/v) formic acid. After activation of the cartridge with eluent B, and washing with 5 column volumes of eluent A, the desalted reaction mixtures were injected. The sulfonated model compounds were then separated from byproducts using the following gradient profiles: For SGBG, 0-1 min at 3% B (isocratic), 1-9.3 min from 3 to 16% B (linear gradient), 9.3-9.6 min from 16 to 100% B (linear gradient), 9.6-11 min at 100% B (isocratic); For SVBG: 0-1 min at 5% B (isocratic), 1-9.3 min from 5 to 18% B (linear gradient), 9.3-9.6 min from 18 to 100% B (linear gradient), 9.6-11 min at 100% B (isocratic). The flow was set at 18 mL min^{-1} and fractions of 4 mL were collected. The resulting fractions were diluted 10 times with water and analyzed by using RP-UHPLC-PDA-MS. Fractions that contained the sulfonated model compounds and that were free of byproducts were pooled. Remaining ACN was evaporated under reduced pressure, after which the model compound solutions were freeze-dried and stored in a desiccator.

2D HSQC NMR analysis of the lignin model compounds

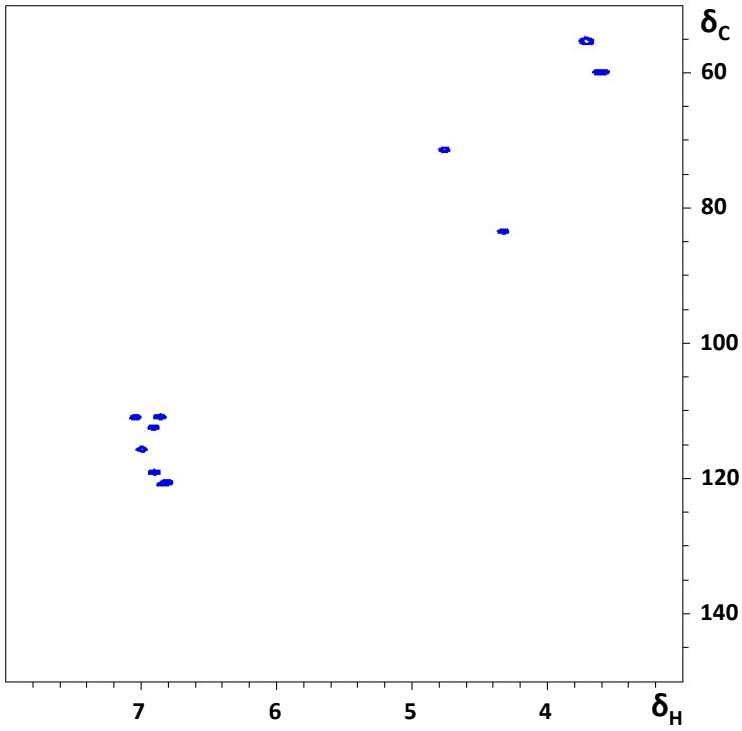
In order to verify that the model compounds SGBG and SVBG were sulfonated at the Cα position, the lignin model compounds were analyzed by using 2D NMR. For GBG and VBG, approximately 1 mg was dissolved in 500 µL DMSO-d_6, and for SGBG and SVBG, approximately 150 µg was dissolved in 450 µL DMSO-d_6. The NMR experiments were recorded at 25 ºC by using hsqcetgpsisp2.2 and hmbcgpndqf pulse sequences on a Bruker AVANCE III 600 MHz NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a 5 mm cryo-probe. The internal temperature of the probe was set at 298 K. Spectral widths were 6,000 Hz (10-0 ppm) for the ^{1}H-dimension and 25,000 Hz (165-0 ppm) for the ^{13}C dimension. The solvent peak (DMSO-d_6) was used as an internal reference (δ_C 39.5 ppm; δ_H 2.49 ppm).

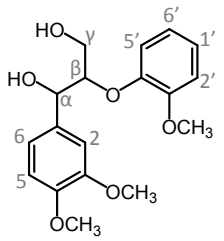




δ_C/δ_H (ppm)	assignment
55.2/3.70	C-H in methoxyl groups
59.7/3.58	C_γ-H_γ
71.3/4.70	C_α-H_α
83.4/4.28	C_β-H_β
111.1/6.98	C_2-H_2
112.3/6.89	$C_{2'}$-$H_{2'}$
114.3/6.66	C_5-H_5
115.5/6.97	$C_{5'}$-$H_{5'}$
119.2/6.76	C_6-H_6
120.5/6.81	$C_{1'}$-$H_{1'}$ and $C_{6'}$-$H_{6'}$

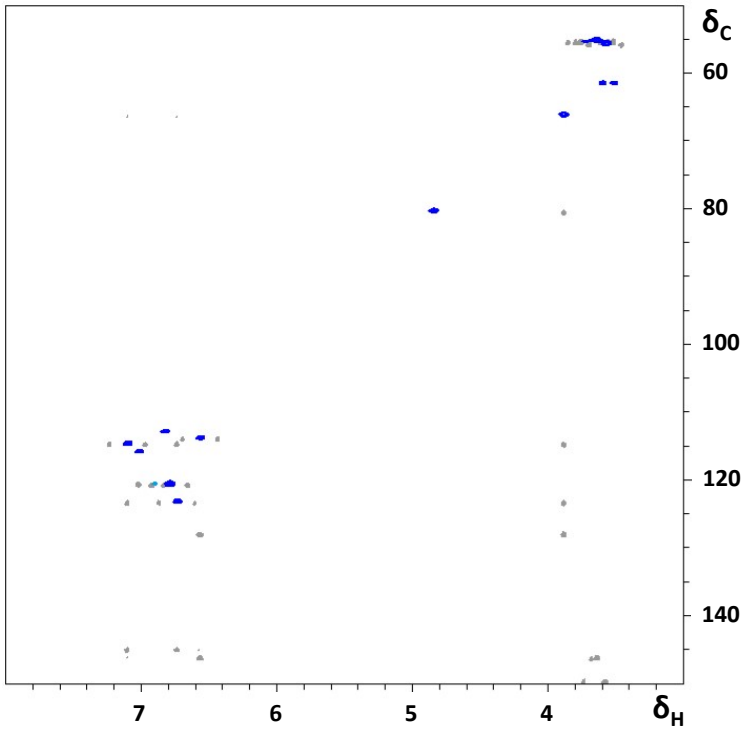
Fig. S1 2D HSQC NMR spectrum and peak annotations of the model compound GBG. Both the side chain and aromatic region are included. Peak assignment was based on Ralph et al.[1]

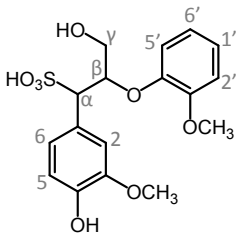




δ_C/δ_H (ppm)	assignment
55.2/3.71	C-H in methoxyl groups
59.8/3.60	C_γ-H_γ
71.3/4.76	C_α-H_α
83.3/4.33	C_β-H_β
110.8/6.86	C_5-H_5
110.9/7.04	C_2-H_2
112.4/6.91	$C_{2'}$-$H_{2'}$
115.6/6.99	$C_{5'}$-$H_{5'}$
119.0/6.91	C_6-H_6
120.6/6.82	$C_{1'}$-$H_{1'}$ and $C_{6'}$-$H_{6'}$

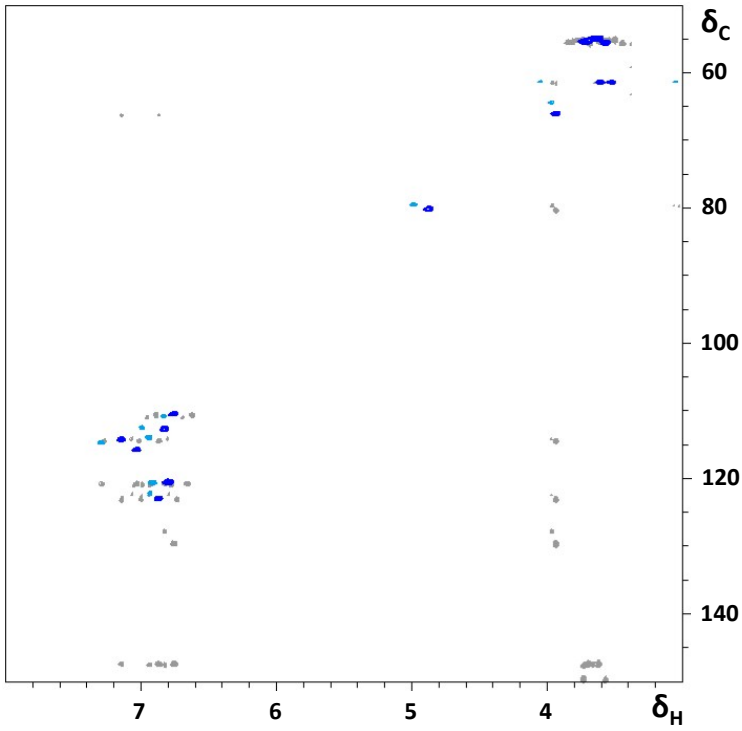
Fig. S2 2D HSQC NMR spectrum and peak annotations of the model compound VBG. Both the side chain and aromatic region are included. Peak assignment was based on Ralph et al.[1]

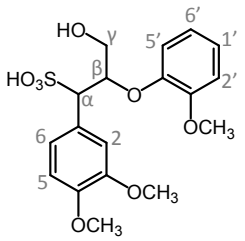




δ_C/δ_H (ppm)	assignment
55.1/3.63	C-H in methoxyl groups
61.3/3.51 and 3.59	C_γ-H_γ
66.0/3.87	C_α-H_α
80.2/4.83	C_β-H_β
112.7/6.82	$C_{2'}$-$H_{2'}$
113.7/6.56	C_5-H_5
114.5/7.09	C_2-H_2
115.7/7.00	$C_{5'}$-$H_{5'}$
120.4/6.78	$C_{1'}$-$H_{1'}$ and $C_{6'}$-$H_{6'}$
123.1/6.73	C_6-H_6

Fig. S3 2D HSQC (blue) and HMBC (grey) NMR spectrum and peak annotations of the model compound SGBG. Both the side chain and aromatic region are included. Peak assignment was done based on the combination of HSQC and HMBC data, and by comparison of the chemical shifts with the chemical shifts of sulfonated lignin models reported by Lutnaes et al.[2] The peak in light blue corresponds to the least abundant diastereomer of SGBG ($C_{1'}$-$H_{1'}$ and $C_{6'}$-$H_{6'}$).





δ_C/δ_H (ppm)	assignment
54.9/3.63	C-H in methoxyl groups
61.3/3.52 and 3.61	C_γ-H_γ
66.0/3.93	C_α-H_α
80.1/4.88	C_β-H_β
110.4/6.76	C_5-H_5
112.6/6.82	$C_{2'}$-$H_{2'}$
114.2/7.15	C_2-H_2
115.7/7.03	$C_{5'}$-$H_{5'}$
120.4/6.80	$C_{1'}$-$H_{1'}$ and $C_{6'}$-$H_{6'}$
122.8/6.87	C_6-H_6

Fig. S4 2D HSQC (blue) and HMBC (grey) NMR spectrum and peak annotations of the model compound SVBG. Both the side chain and aromatic region are included. Peak assignment was done based on the combination of HSQC and HMBC data, and by comparison of the chemical shifts with the chemical shifts of sulfonated lignin models reported by Lutnaes et al.[2] In the table, only the chemical shifts of the most abundant diastereomer are included. In the table, only the chemical shifts of the most abundant diastereomer are included. The peaks of the other diastereomer are indicated in light blue.

1. S. A. Ralph, J. Ralph, L. Landucci and L. Landucci, *US Forest Prod. Lab., Madison, WI (http://ars. usda. gov/Services/docs. htm*, 2004.
2. B. F. Lutnaes, B. O. Myrvold, R. A. Lauten and M. M. Endeshaw, *Magnetic Resonance in Chemistry*, 2008, **46**, 299-305.

RP-UHPLC-PDA analysis

Reaction products were separated by using an Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, degasser, autosampler and photodiode array (PDA) detector. Samples (5 μL) were injected onto an Acquity UPLC BEH C18 column (150 x 2.1 mm, particle size 1.7 μm) (Waters, Milford, MA, USA). The flow rate was 400 μL min^{-1} at 45 °C. Water (A) and acetonitrile (B) were used as eluents, both acidified with 0.1% (v/v) formic acid. The following gradient was used: 0-1.5 min at 5% B (isocratic), 1.5-32 min from 5 to 35% B (linear gradient), 32-33 min from 35 to 100% B (linear gradient), 33-38 min at 100% B (isocratic), 38-39 min from 99 to 5% B (linear gradient) and 39-44 min at 5% B (isocratic). The PDA detector was set to record wavelengths between 200 and 700 nm.

Electrospray Ionization – Ion Trap Mass Spectrometry (ESI-IT-MS)

Mass spectrometric data were obtained with an LTQ Velos Pro mass spectrometer (Thermo Scientific) equipped with a heated ESI probe coupled to the UHPLC system. Nitrogen was used as sheath gas and auxiliary gas. Data were collected in both positive and negative ionization mode over the *m/z* range 120-2,000. Data dependent MS^2 analysis was performed on the most intense ion by using collision-induced dissociation with a normalized collision energy of 35%. To gain MS^2 spectra of the second and third most abundant ions, dynamic exclusion was used, with a repeat count of six MS^2 spectra per parent ion within a time frame of 15 s. The most intense ion was selected The system was tuned with LTQ Tune Plus 2.7 (Thermo Scientific) upon direct injection of GBG in both positive and negative ionization mode. The ion transfer tube temperature was 300 °C,

source heater temperature was 250 °C and the source voltage was 3.5 kV. Data were processed with Xcalibur 2.2 (Thermo Scientific).

Electrospray Ionization – Fourier Transform Mass Spectrometry (Accurate mass determination)

For accurate mass determination, reaction products were separated using a Vanquish UHPLC system (Thermo Scientific). The same samples were used as for ESI-IT-MS, after a 10-fold further dilution in MilliQ water. The injection volume was 1 µL. The column, eluents and gradient were identical to those described for RP-UHPLC-PDA analysis. The column compartment heater was set to 45 °C, the eluent preheater was set to 45 °C and the post-column cooler was set to 40 °C. A Thermo Q Exactive Focus hybrid quadrupole-orbitrap mass spectrometer (Thermo Scientific) equipped with a heated ESI probe coupled to the Vanquish RP-UHPLC system was used to acquire accurate mass data. Half of the flow was directed toward the MS. Full MS data were recorded in both negative and positive ionization mode over a range of *m/z* 100-1,500 at a resolution of 70,000. The mass spectrometer was calibrated in both positive and negative mode using Tune 2.8 software (Thermo Scientific) by direct infusion of Pierce LTQ ESI positive and negative ion calibration solutions (Thermo Scientific). Nitrogen was used as sheath gas (30 arbitrary units) and auxiliary gas (20 arbitrary units). The capillary temperature was 320 °C; the probe heater temperature was 280 °C; the source voltage was 2.8 kV; and the S-lens RF level was 50. Data processing was done using Xcalibur 2.2 (Thermo Scientific) and Compound Discoverer 2.0 (Thermo Scientific). Molecular formulas of reaction products were determined with a set of requirements unique for each incubation. In incubations with GBG or VBG and laccase alone, the determination of molecular formulas was restricted to C, H and O atoms, and a maximum mass error of 5 ppm. In the case of GBG or VBG with laccase and HBT, also nitrogen atoms were allowed, with a maximum of 6 N atoms per molecule. The requirements for reaction products of the

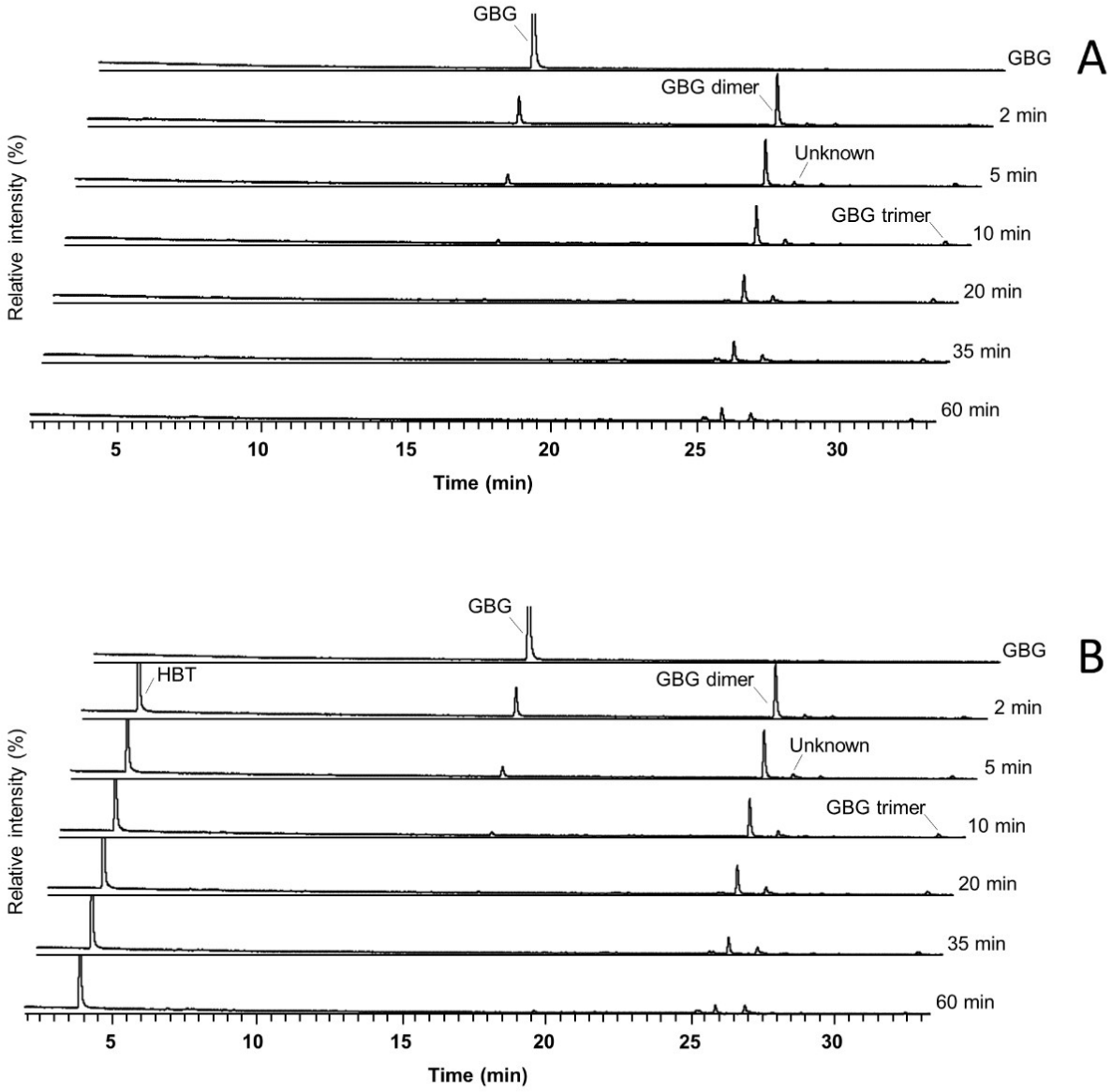
sulfonated model compounds were the same, with the adaptation that 8 sulfur atoms were allowed in all cases. For every peak, this led to a list of candidates, with only one plausible formula.

Matrix-Assisted Laser Desorption Ionization – Time Of Flight Mass Spectrometry (MALDI-TOF-MS)

Prior to analysis, a cation exchange resin (AG 50W-X8 DOWEX) was added to the samples for at least 3 h. Under continuous flow of a hairdryer, 1 µL of 25 mg mL^{-1} 2,5-dihydroxybenzoic acid (DHB) was mixed with 1 µL sample on a MTP 384 ground steel target plate (Bruker Daltonics). Mass spectra (*m/z* 500-3,500) were acquired using a Bruker UltraFlextreme MALDI-TOF (Bruker Daltonics) instrument equipped with a Smartbeam2 nitrogen laser (337 nm) operated in reflector mode with an acceleration voltage of 25 kV. Ion voltages were set to 20.00 and 17.90 kV, reflector voltages to 20.80 and 10.90 kV and the lens voltage to 7.85 kV. Ionization in positive mode was carried out with a laser beam intensity of 20-30% at 500 Hz. Each mass spectrum (*m/z* 500 – 3,500) was obtained from four additions of 250 laser shots to a total of 1,000 shots. Calibration was carried out with a 1 mg/ml maltodextrin solution (DP 20; Mw 400 – 3,500 Da). Data was processed using FlexAnalysis 3.3 (Bruker Daltonics).

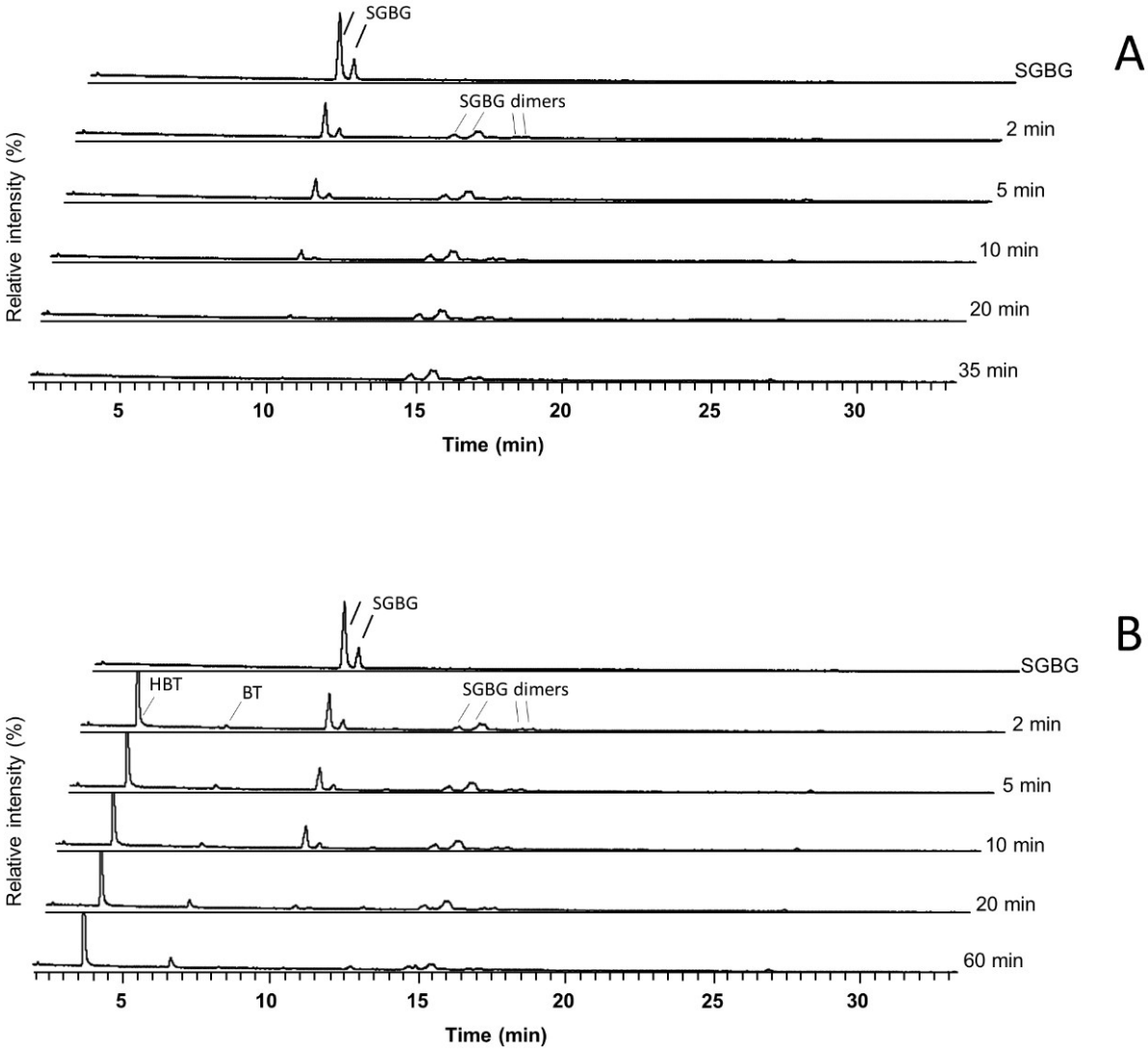
Oxygen consumption

Oxygen consumption was measured with an Oxytherm System (Hansatech Kings Lynn, UK). Lignin model compounds and mediators were used as substrates at 0.4 mM in a sodium phosphate buffer (50 mM) at pH 4. Also in incubations containing both a model compound and a mediator, the individual concentrations were 0.4 mM. After equilibration, laccase was added to obtain an activity of 1 U mL^{-1}. Here, a higher laccase activity was used than in the incubations described in the article, in order to obtain a more clear decrease in O_2 concentration within the short time frame of the measurement. Incubations were performed in a total volume of 1 mL at 25 °C. Data were acquired by using Oxygraph Plus software (Hansatech).



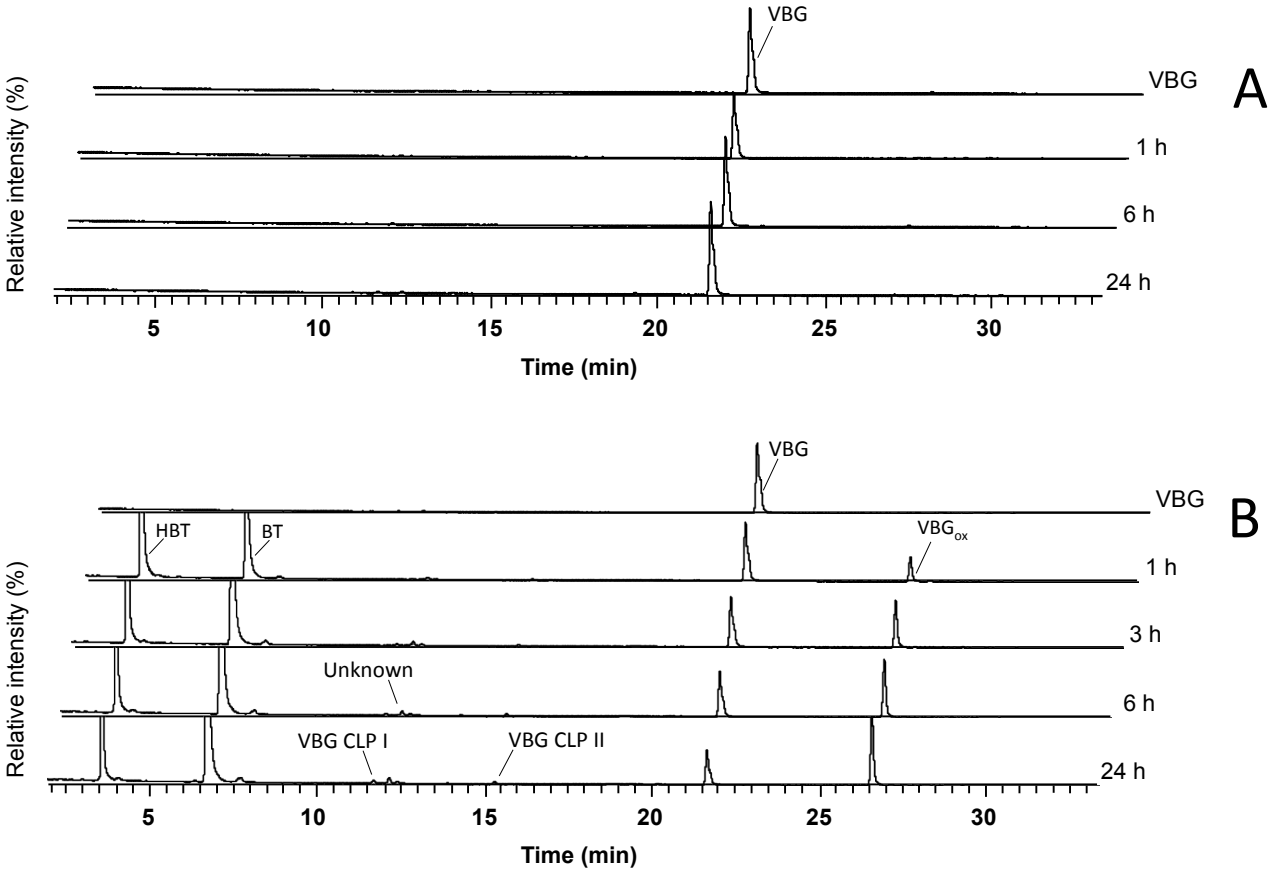
150

151 **Fig. S5** RP-UHPLC-UV_{280} chromatograms in time of GBG incubated with laccase (A) and laccase/HBT
152 (B).



153

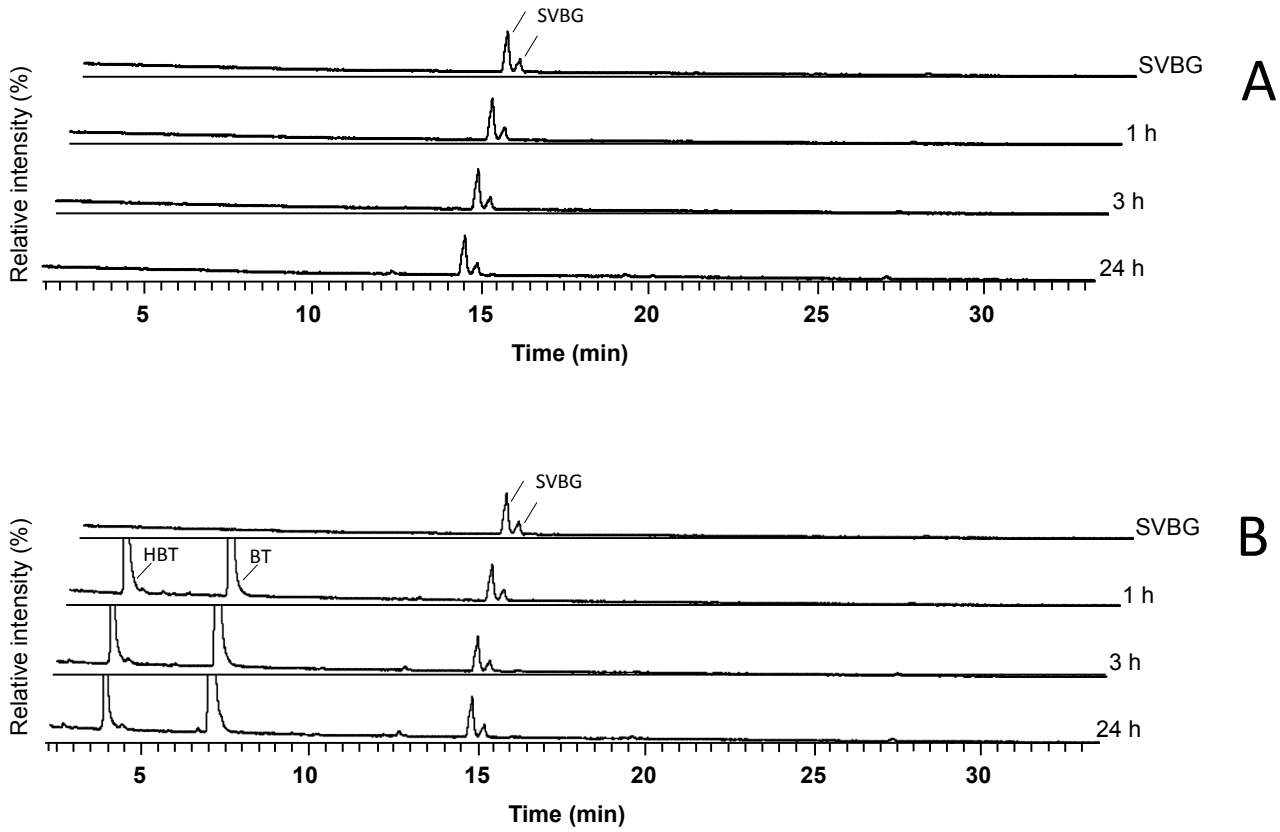
154 **Fig. S6** RP-UHPLC-UV_{280} chromatograms in time of SGBG incubated with laccase (A) and laccase/HBT
155 (B).



157

158 **Fig. S7** RP-UHPLC-UV$_{280}$ chromatograms in time of VBG incubated with laccase (A) and laccase/HBT
159 (B).

160



161

162 **Fig. S8** RP-UHPLC-UV_{280} chromatograms in time of SVBG incubated with laccase (A) and laccase/HBT
163 (B).

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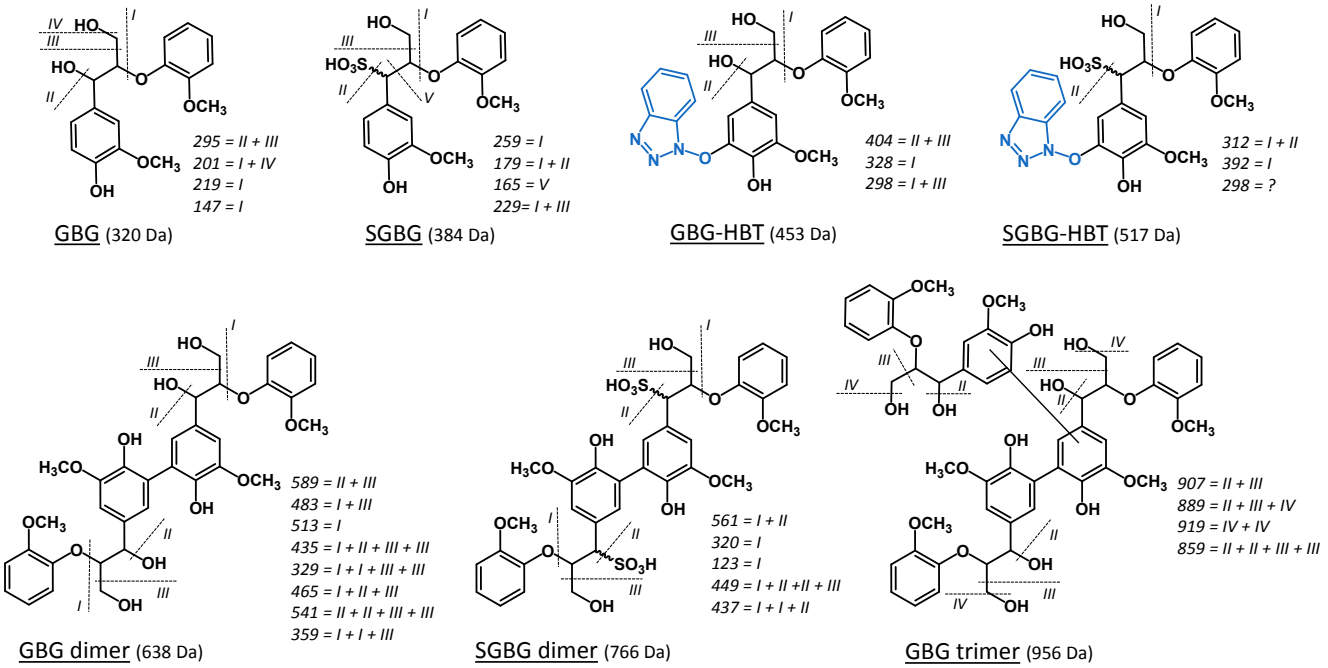
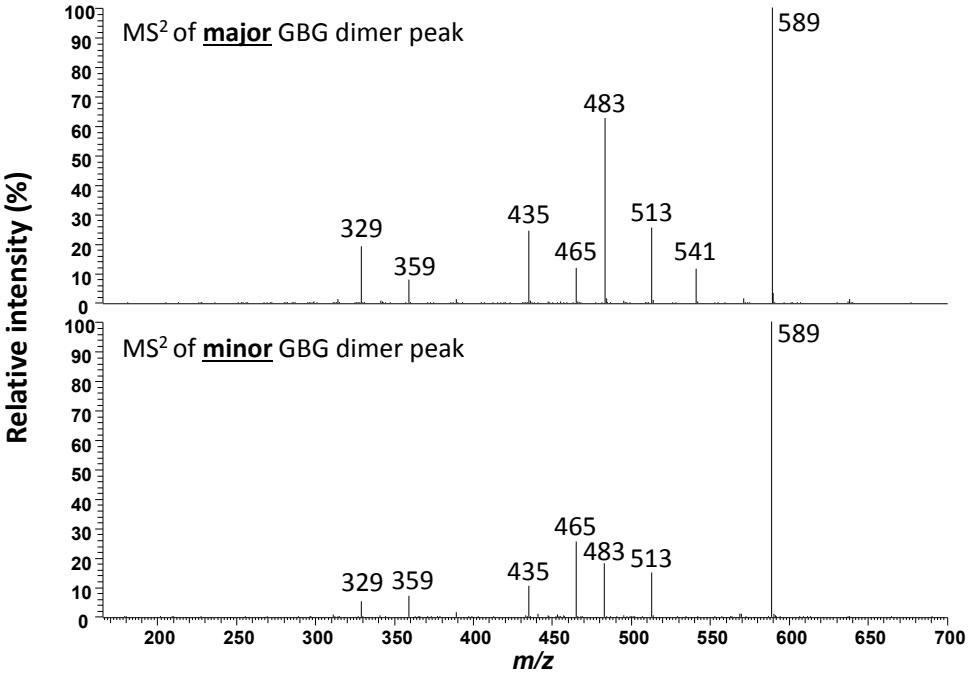


Fig. S9 Proposed fragmentation patterns of GBG, SGBG and their reaction products formed after incubation with laccase and laccase/HBT. The dotted lines represent the proposed fragmentation pattern, resulting in the MS^2 fragments reported in Table 1. The patterns correspond to fragmentation of parent ions $[M+Na]^+$ for GBG, $[M-2H]^{2-}$ for SGBG dimer, and $[M-H]^-$ for all other molecules.



171

172 **Fig. S10** MS^2 fragmentation patterns of the major GBG dimer (Rt=16.0 min) and minor GBG dimer
173 (Rt=17.9 min) formed upon incubation with laccase. Both fragmentation patterns originate from a parent
174 ion of *m/z* 637 in negative mode.

175

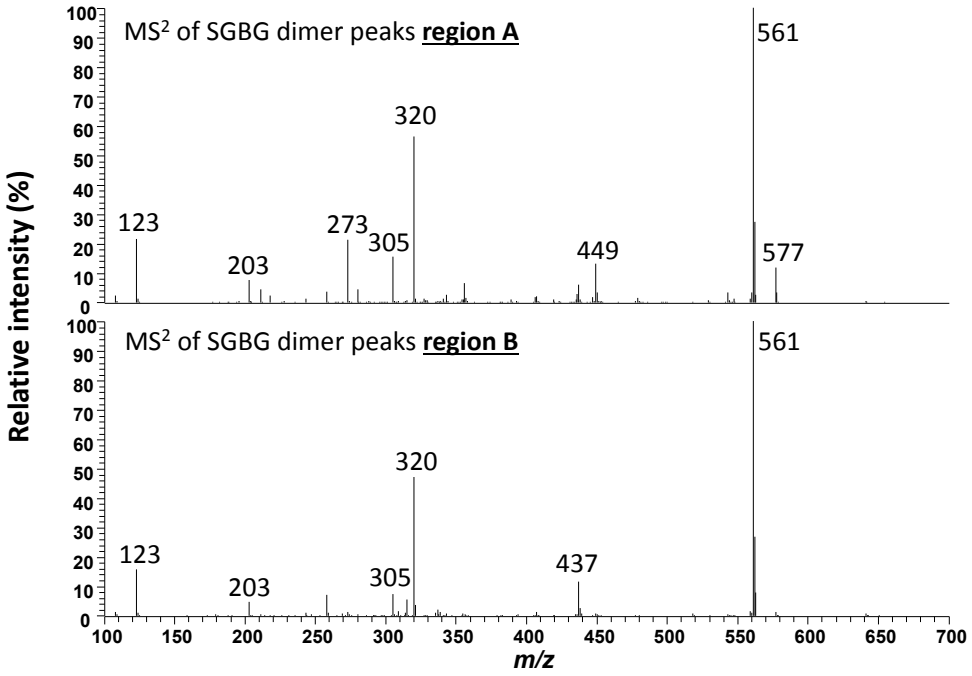
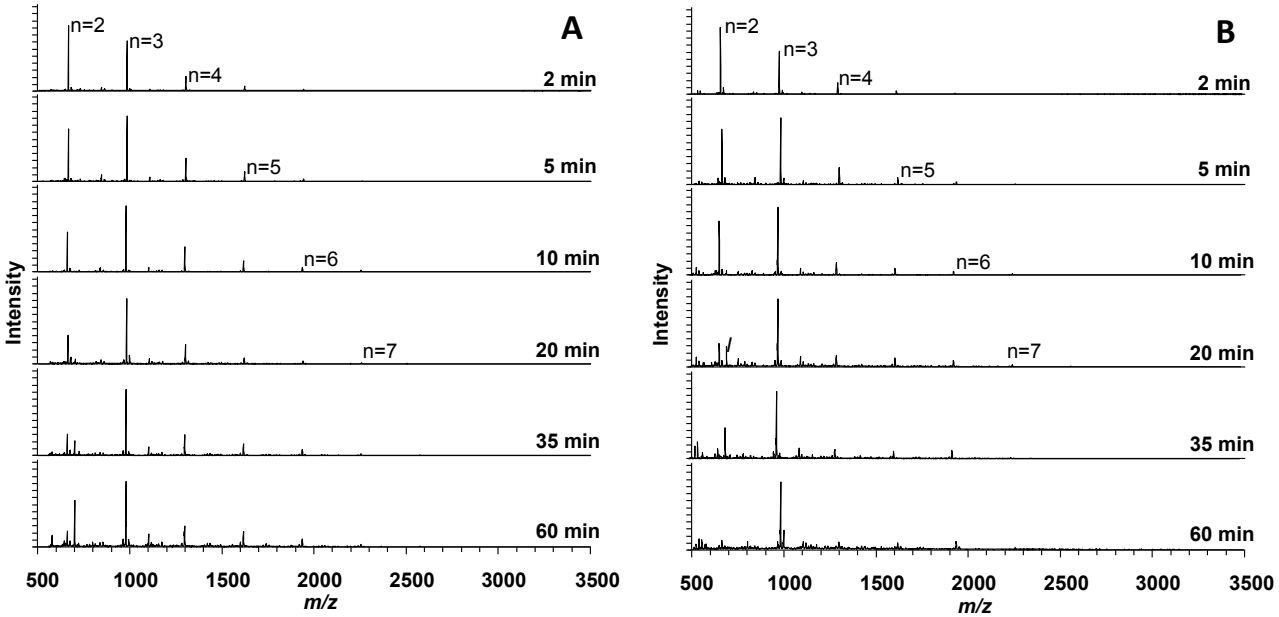
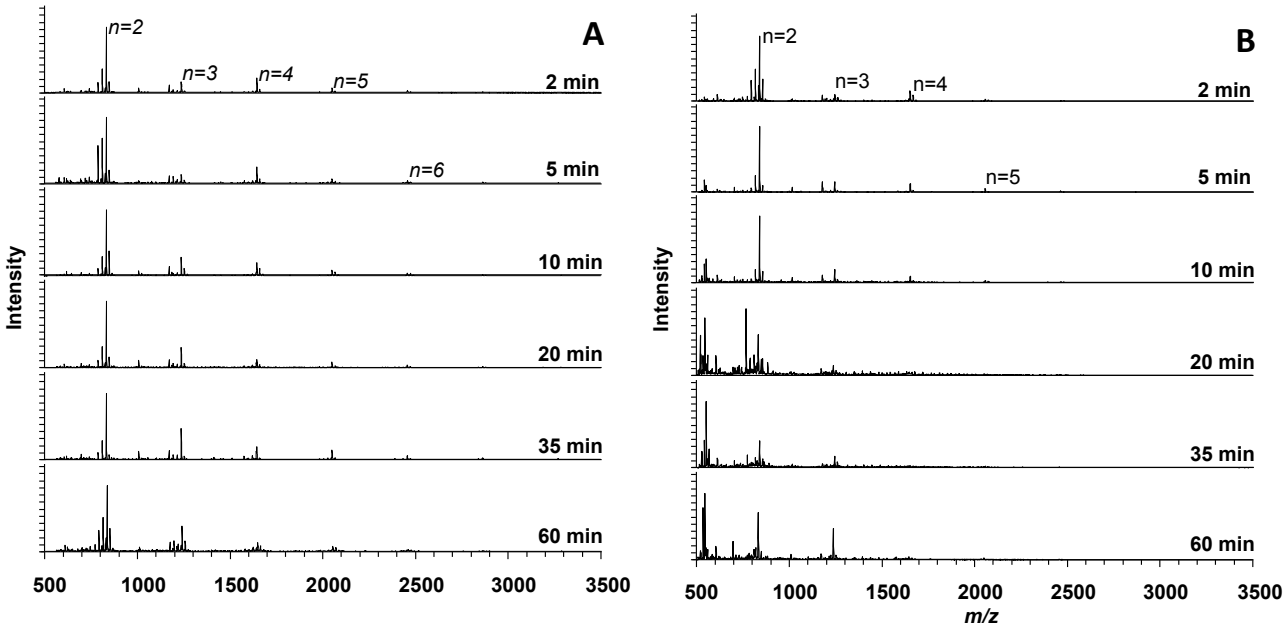


Fig. S11 MS^2 fragmentation patterns of the two regions of SGBG dimers (region A and B) shown in Fig. 2. Both fragmentation patterns originate from a parent ion of *m/z* 382 (=$[M\text{-}2H]^{2-}$) in negative mode.



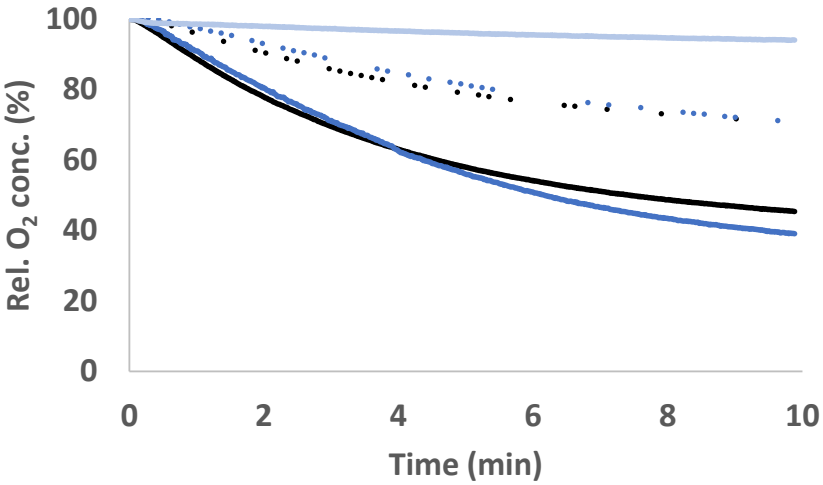
Peak	Tentative annotation	Ionization	*m/z*	Observed in incubation
n=2	GBG dimer	$[M+Na]^+$	661.2	Lac; lac/HBT
n=3	GBG trimer	$[M+Na]^+$	979.3	Lac; lac/HBT
n=4	GBG tetramer	$[M+Na]^+$	1297.4	Lac; lac/HBT
n=5	GBG pentamer	$[M+Na]^+$	1615.6	Lac; lac/HBT
n=6	GBG hexamer	$[M+Na]^+$	1933.7	Lac; lac/HBT
n=7	GBG heptamer	$[M+Na]^+$	2251.8	Lac; lac/HBT

Fig. S12 MALDI-TOF-MS spectra in time of GBG incubated with laccase (A) and laccase/HBT (B). The table shows the observed *m/z* values and their corresponding tentative annotations.



Peak	Tentative annotation	Ionization	*m/z*	Observed in incubation
-	SGBG dimer	$[M+Na]^+$	789.2	Lac; lac/HBT
-	SGBG dimer	$[M-H+2Na]^+$	811.2	Lac; lac/HBT
n=2	SGBG dimer	$[M-2H+3Na]^+$	833.2	Lac; lac/HBT
-	SGBG dimer	$[M-3H+3Na+K]^+$	849.1	Lac; lac/HBT
-	SGBG trimer	$[M+Na]^+$	1171.2	Lac; lac/HBT
-	SGBG trimer	$[M-H+2Na]^+$	1193.2	Lac; lac/HBT
-	SGBG trimer	$[M-2H+3Na]^+$	1215.2	Lac; lac/HBT
n=3	SGBG trimer	$[M-3H+4Na]^+$	1237.2	Lac; lac/HBT
-	SGBG trimer	$[M-3H+3Na+K]^+$	1253.2	Lac; lac/HBT
-	SGBG tetramer	$[M-H+2Na]^+$	1575.2	Lac; lac/HBT
-	SGBG tetramer	$[M-3H+4Na]^+$	1619.2	Lac; lac/HBT
n=4	SGBG tetramer	$[M-4H+5Na]^+$	1641.2	Lac; lac/HBT
n=5	SGBG pentamer	$[M-5H+6Na]^+$	2045.3	Lac; lac/HBT
n=6	SGBG hexamer	$[M-4H+2Na+3K]^+$	2454.3	Lac; lac/HBT

Fig. S13 MALDI-TOF-MS spectra in time of SGBG incubated with laccase (A) and laccase/HBT (B). The table shows the observed *m/z* values and their corresponding tentative annotations.



189

190 **Fig. S14** Oxygen consumption of lignin model compounds, mediators and combinations thereof in the
191 presence of laccase: GBG (solid, black); SGBG (dotted, black); GBG+HBT (solid, blue); SGBG+HBT
192 (dotted, blue); HBT (solid, light blue).

193

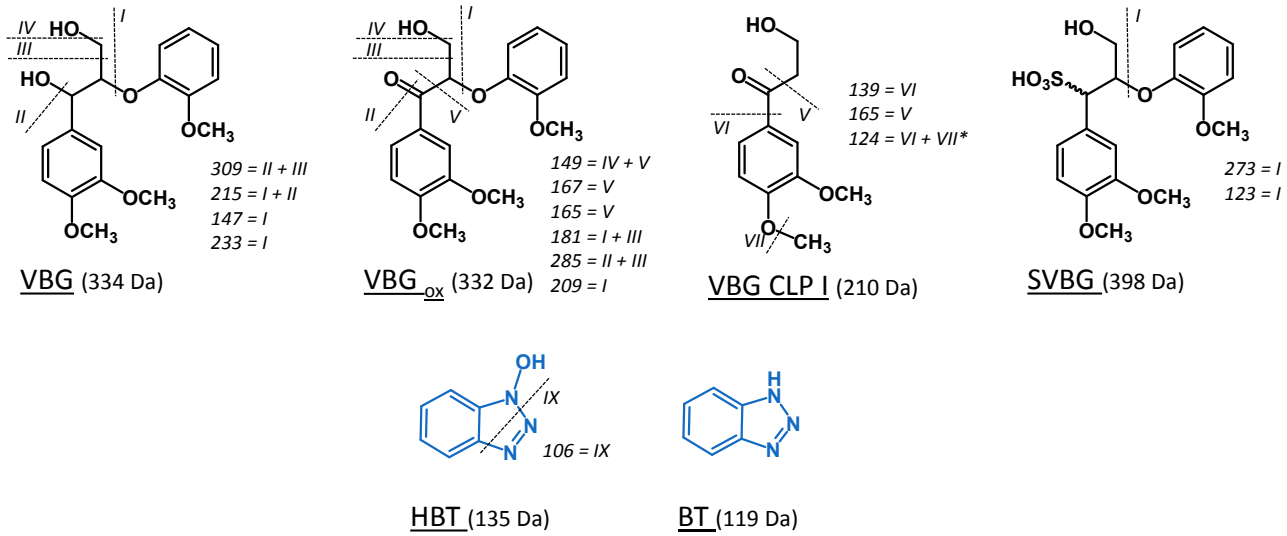


Fig. S15 Proposed fragmentation patterns of VBG, SVBG, HBT and of reaction products of VBG formed in laccase/HBT incubations. The dotted lines represent the proposed fragmentation pattern, resulting in the MS^2 fragments reported in Table 2. The patterns correspond to fragmentation of parent ions $[M+Na]^+$ for VBG, $[M+H]^+$ for VBG_{ox}, VBG CLP I and BT, and $[M-H]^-$ for SVBG and HBT.
* This fragmentation is suggested to be a radical fragmentation.