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A Biocatalytic One-Pot Approach for the Preparation of Lignin Oligomers Using an Oxidase/Peroxidase Cascade Enzyme System

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Abstract: Synthetic lignin was prepared biocatalytically in a one-pot, two-step reaction using an oxidase/oxidase cascade enzyme system. Using eugenol in combination with eugenol oxidase and a peroxidase, lignin-like material was produced. The cascade reaction takes advantage of the ability of the oxidase to produce coniferyl alcohol and hydrogen peroxide from eugenol and molecular oxygen. The hydrogen peroxide is used by the peroxidase for the formation of crosslinks that typify lignin. As eugenol oxidase has a broad substrate acceptance profile, also 4-allylphenol (chavicol) and 4-allyl-2,6-dimethoxyphenol could be used as precursors of the synthetic lignin. As a result, all three naturally occurring monolignols could be prepared and incorporated in the synthetic lignin. The reaction was optimized in order to achieve the highest possible yield of insoluble lignin oligomers and scaled up to 1 gram. Analysis of the water-insoluble product by gel permeation

chromatography revealed the formation of relatively small lignin oligomers (≈ 1000 dalton). By using two-dimensional heteronuclear single quantum coherence nuclear magnetic resonance spectroscopy (2D HSQC NMR) analysis it could be demonstrated that the material contained α -O-4/ β -O-4, β -O-4, β - β , β -5 linkages and dibenzodioxocin units. All these features indicate that the biocatalytically produced material closely resembles natural lignin. While 54% of eugenol was converted into water-insoluble oligomers, the remaining substrate was converted into water-soluble dimers and tetramers which are important lignin model compounds. Therefore, the presented method represents a valuable and facile biocatalytic approach for the preparation of lignin-like material and potentially valuable chemicals.

Keywords: biocatalysis; cascade reaction; lignin; oxidation; (per)oxidases; polymerization

Introduction

The demand for renewable energy is continuing to grow as more petroleum fuel is consumed and environment-related problems continue to emerge. One potent renewable resource is lignin, the second most abundant natural polymer after cellulose.^[1] Lignin is an aromatic hetero-polymer composed of three classical monolignols: coniferyl, *p*-coumaryl and sinapyl alcohols. These three monomers form the *p*-hydroxyphenyl, guaiacyl and syringyl units in lignin. These units are linked *via* among others, β -O-4, β -5, β - β , β -1, α -O-4, 4-O-5 and 5-5' linkages, of which β -O-4 gen-

erally constitutes up to 50% of total bonds in lignin (see Figure 1).^[2–6] The relative amount of the three lignols and the types and ratios of linkages in lignin are plant dependent.

Due to its complex chemical structure, processing lignin into simpler compounds is still a challenge.^[6,7] Various approaches have been reported for its valorization *via* depolymerization into smaller fragments.^[6–9] However, there is still a need for effective processes by which lignin can be used as a starting material for high-value products.

Lignin model compounds are often used for studying (bio)chemical processes by which natural lignin

Figure 1. The most common linkages found in lignin.^[2]

can be depolymerized.^[7–10] Such synthetic lignin molecules typically are short and defined oligomers (dimers, trimers, tetramers and even hexamers), in which the monolignols are linked *via* up to three different linkage types.^[4,11–17] Synthetic lignin compounds have been used to screen for microorganisms capable of producing lignin-degrading enzymes.^[18] Still, these molecules do not fully resemble natural lignin as they are highly defined molecules lacking the complexity of natural lignin. Therefore, new methodology for the production of native-like lignin model compounds is of great value for studying lignin reactivity.

The main aim of this study was to synthesize lignin starting from cheap and renewable material by a facile biocatalytic approach. Such a lignin would be a close mimic of natural lignin, harbouring various linkage types that also occur in natural lignin while containing all natural monolignols. The key biocatalyst for this study is the bacterial eugenol oxidase (EUGO) that we recently identified.^[19] This oxidase is unique in its ability to produce all three natural monolignols from the corresponding 4-allylphenols. These oxidation reactions merely require molecular oxygen as an electron acceptor (reduced into hydrogen peroxide) and water for the hydration of the quinone methide reaction intermediate to form the final monolignols. The EUGO-catalyzed conversion of 4-allylphenols (eugenol, 4-allyl-2,6-dimethoxyphenol, and chavicol, respectively) yields all three monolignols (coniferyl, sinapyl, and coumaryl alcohols, respectively) and hydrogen peroxide.^[20] These products, the monolignols and hydrogen peroxide, are precisely the requirements for a peroxidase-catalyzed reaction

that will lead to lignin formation. Similar conversions using an oxidase-laccase system starting from eugenol have resulted in the production of pinoreosinol.^[21] Peroxidases are known to oxidize monolignols into phenolic radicals which, through spontaneous free radical polymerization, form polymers (see Scheme 1).^[22]

Scheme 1. Conversion of eugenol (**1**) into coniferyl alcohol (**2**) using EUGO.^[19,20] The formed coniferyl alcohol (**2**) is oxidized by a peroxidase into lignin oligomers.

Results and Discussion

Synthesis of Coniferyl Alcohol (**2**) from Eugenol (**1**) using Eugenol Oxidase (EUGO)

To establish whether EUGO can be used for the preparation of the monolignol coniferyl alcohol (**2**), we first explored the efficiency of EUGO-catalyzed eugenol conversions. Coniferyl alcohol (**2**) is an expensive compound while its precursor eugenol (**1**) is very cheap and can be isolated from clove oil. To find optimal conditions for the production of coniferyl alcohol (**2**), various enzyme concentrations (50, 500 and 5000 nM) were tested for the conversion of 10 mM eugenol at pH 7.5, 30°C. After 20 h incubation, conversions of respectively 7.7, 37.5 and 82% were observed. The observation that the conversion was not complete, even when using a high enzyme loading, can be explained by the fact that coniferyl alcohol (**2**) is a competitive inhibitor. The formed coniferyl alcohol was extracted and compared with commercially available coniferyl alcohol using analytical HPLC revealing a high degree of purity (Supporting Information, Figure S3).

Synthesis of Lignin-Like Oligomers from 4-Allylphenols using EUGO in Combination with Peroxidases

The second step in the synthesis of lignin-like material from eugenol (**1**) is the formation of monolignol free radicals by the action of a peroxidase. We tested two sequence-unrelated peroxidases for this: (i) peroxidase from horseradish (HRP),^[23] and (ii) a DyP-type peroxidase from bacterial origin (*SviDyP*).^[24] Although the polymerisation of lignin monomers (i.e. coniferyl, coumaryl and sinapyl alcohols) using HRP has been reported previously, attempts for this conversion from eugenol using a recombinant oxidase (EUGO) coupled with a peroxidase has not been reported to the best of our knowledge.^[18,25,26] Coniferyl alcohol is a relatively expensive and labile compound while eugenol is a natural and abundant natural compound which makes it an attractive starting material for the production of lignin-like material. The use of a recombinant peroxidase (*SviDyP*) for synthesizing lignin from eugenol opens new applications for the use of this newly discovered class of peroxidases. DyP peroxidases are emerging as promising alternatives for commonly known plant and fungal peroxidases due to their robustness, higher temperature stability and ease of production.^[27] When incubating eugenol (**1**) as a substrate in the presence of EUGO and HRP or *SviDyP*, the solution quickly turned turbid which was not observed when no peroxidase was added. The insoluble material could be isolated by centrifugation and displayed a white colour and did not contain protein. Subsequently, we optimized conditions for the two enzyme combinations by monitoring the formation of turbidity through measuring the absorbance (due to light scattering) at 600 nm. This revealed that most turbidity was formed when using a temperature of 30 °C, an agitation rate of 50 rpm, a ratio of reaction volume to air headspace of 1:10, 10 mM eugenol (**1**) and a pH of 7 when using HRP and pH 6 when using *SviDyP*. The latter finding is in line with that fact that DyP peroxidases tend to have a relatively low pH optimum compared to HRP.^[27] Using these conditions, eugenol (**1**) conversion was monitored by HPLC in order to follow substrate (eugenol **1**) depletion while also measuring absorbance at 600 nm (Figure 2). This revealed that virtually all eugenol (**1**) was converted in this one-pot cascade reaction.

EUGO accepts numerous phenolic substrates.^[20] Interestingly, it also oxidizes 4-allyl-2,6-dimethoxyphenol and chavicol into their respective monolignols. Therefore we also tested EUGO/HRP and EUGO/*SviDyP* couples with these allylphenols. The use of EUGO/*SviDyP* with 4-allyl-2,6-dimethoxyphenol or chavicol resulted in only a very slight increase at 600 nm after an incubation of 98 h. EUGO/HRP gave better results when taking the absorbance at 600 nm

Figure 2. Biocatalytic conversion of eugenol (**1**) (10 mM) by EUGO (50 nM) and HRP (650 nM) in one-pot, monitored by HPLC and absorbance at 600 nm.

as a measure for the formation of lignin-like insoluble material. These data indicate that the EUGO/HRP couple can be used for the formation of lignin-like polymers that contain all three monolignols as found in natural lignin. Therefore, an experiment was performed in which a substrate mixture of all three 4-allylphenols was used. This resulted in appearance of turbidity for both enzyme cascades. In line with the results with the separate allylphenols, EUGO/HRP resulted in formation of more turbidity when compared with EUGO/*SviDyP*. While the lignin-like product obtained from the reaction with eugenol (**1**) could be characterized (*vide infra*), the product from the reactions using all three 4-allylphenols was significantly more complex to analyze. This hints to the formation of a more complex polymer that contains other monomers apart from coniferyl alcohol (**2**) and also indicates that with this biocatalytic cascade it should be possible to tune the amount of methoxy groups in the synthetic lignin-like material. This would allow us to mimic the different lignin types that are observed depending on the plant source.

Analysis of the Soluble Fraction by LC-MS and NMR

Lignin synthesis using the oxidase-peroxidase system is expected to proceed *via* the sequential formation of dimers, tetramers and even higher molecular weight oligomers. Depending on the size of such oligomers, they either remain in solution or precipitate. For a better view on the types of products formed during incubation of eugenol (**1**) with EUGO/HRP, we performed an extensive chemical analysis of the formed products. Analysis of the solution at 2, 24, and 98 h was done using analytical HPLC to follow the formation and disappearance of various compounds corre-

sponding to substrate (eugenol, **1**), intermediate (coniferyl alcohol, **2**) or products (various oligomers). This confirmed full conversion of eugenol (**1**) after 98 h, while coniferyl alcohol (**2**) could only be observed after 2 h. Mass balance analysis showed that, although eugenol (**1**) was completely converted, only about 50% is converted into water-insoluble product. The remainder of the eugenol (**1**) is converted into water-soluble products. After 24 h, formation of some pinoresinol was observed (Supporting Information, Figure S4). Analysis of the soluble fraction formed after 98 h incubation revealed that the most abundant product was a tetramer. By COSY, HMBC 2D NMR and HSQC 2D NMR measurements (see the Supporting Information, Figure S6) it could be identified as a tetramer that has two coniferyl alcohol (**2**) units in the core connected by a 5-5' linkage and each of the two coniferyl alcohol (**2**) units are connected to an eugenol (**1**) unit through a β -O-4 linkage (**3**). LC-MS also confirmed a mass of 719 in positive mode and 717 in negative mode, corresponding to the calculated tetramer mass ($M_w=718$) (see the Supporting Information, Figures S7a and S7b). The second most dominant peak in the soluble fraction was confirmed to be phenylcoumaran (**4**), a dimer known to be produced by the reaction of coniferyl alcohol (**2**) with HRP.^[28] Analysis by LC-MS confirmed a mass of 357 in negative mode (calculated $M_w=358$) (see the Supporting Information, Figure S8). A third soluble product was confirmed to be a dimer of eugenol (**1**), dieugenol (**5**) (Figure 3). Analysis by LC-MS revealed a mass of 327 in positive mode and 325 in negative mode (calculated $M_w=326$) (see the Supporting Information, Figures S9a and S9b). Figures S10–S12 in the Supporting Information show the ^1H NMR and ^{13}C NMR spectra for each of the three identified soluble oligomers. For comparison, we also performed a conversion of coniferyl alcohol to lignin using HRP and sub-

sequent HPLC analysis (Supporting Information, Figure S13). This revealed the absence of peaks corresponding to those of the tetramer and dieugenol seen when using eugenol as the starting substrate. Only peaks corresponding to retention times of phenylcoumaran and pinoresinol could be seen.

Characterization of the Lignin-Like Oligomers in the Water-Insoluble Fraction

The formed insoluble lignin product was analyzed by GPC and 2D-NMR to investigate the size of the formed oligomers as well as their chemical structure. GPC revealed the presence of oligomers in the range of 300–1100 Da relative to a polystyrene standard (M_n 690, M_w 810, d 1.2, see Figure 4). By considering the average mass of a monomer in the lignin structure, this mass would relate to dimeric to pentameric oligomers.

Figure 4. GPC analysis of lignin obtained from the biocatalytic cascade reaction with eugenol (**1**).

Figure 3. The identified structures for the major soluble products formed upon conversion of eugenol using an EUGO-HRP enzyme mixture; (**3**) tetramer, (**4**) phenylcoumaran, (**5**) dieugenol.

To confirm the presence of structural motifs that are also in natural lignin, 2D HSQC NMR analysis was performed (see Figure 5). From the obtained spectrum, clearly signals belonging to lignin α -O-4,^[29] β -O-4, β - β , β -5 and 5-5' could be observed. Also a dibenzodioxocin fragment was identified and confirmed based on previous findings.^[30] Additionally, it was found that in a conversion starting with ≈ 33 mg eugenol (**1**), α -O-4/ β -O-4, β -O-4, β -5, β - β linkages and dibenzodioxocin units were formed in a ratio of 35.8 : 12.9 : 13.4 : 13.2 : 11.4, respectively. The synthesized lignin has 48.7% α -O-4/ β -O-4 and β -O-4 linkages as compared to 45–50%, 60–62% and 74–84% in softwood, hardwood and grasses, respectively.^[6] The β -5 linkages form 13.4% of the total linkages found in the synthetic lignin. This is comparable to the amount found in natural lignin (ranging from 3 up to 12%).^[6] The amount of β - β linkages reaches 13.2% in the synthetic lignin oligomers. This is close to the range

found in natural lignin (between 1 and 12%). This is likely to originate from the preference of the enzyme mixture to produce pinoresinol-type structures. The dibenzodioxocin units represent a large fraction of the linkages found in the synthesized lignin covering 11.4% of the total linkages produced. Softwood and hardwood lignin do not exceed 7%.^[6] The material analyzed is based on a reaction using eugenol (**1**) as a starting point, resulting in *in situ* formation of only one monolignol, coniferyl alcohol (**2**). A different starting substrate (e.g. sinapyl or coumaryl alcohol) or a mixture of substrates may give different results.

By comparing the polymers formed in the soluble and insoluble fractions, it was found that only β -O-4, 5-5' and β -5 linkages were found in the soluble fraction (as shown in Figure 3). The insoluble lignin fraction showed, in addition to the aforementioned link-

ages, α -O-4, β - β linkages and a dibenzodioxocin unit (see Figure 5).

One Gram Conversion of Eugenol (**1**) into Lignin-Like Material

Lignin-like oligomer production was scaled-up by using 1 g of eugenol (**1**) in a 1 L reaction. This resulted in the production and isolation of 536 mg of insoluble lignin-like material (yield of 53.6%). The turnover number of EUGO for the reaction is equal to 120,000 [mol eugenol (**1**)/mol EUGO] and 824 (mol lignin product/mol HRP). To check the robustness of the enzyme mixture, a second conversion was carried out by adding 1 g of eugenol (**1**) to the same reaction mixture after removal of the insoluble oligo-

Figure 5. HSQC 2D NMR spectrum showing the different types of linkages formed upon biocatalytic synthesis of lignin-like material from eugenol (**1**). The method for calculating the percentage of linkages involved in the different lignin oligomers is explained in the Supporting Information under Method S2.

mers. The amount of insoluble lignin formed in the second cycle was ≈ 290 mg. As the total reaction time was 6 days, it is a clear indication that both biocatalysts are rather robust. Furthermore, downstream processing of the soluble oligomers formed from the successive conversions gave isolated yields of: 105 mg lignin tetramer, 36 mg phenylcoumaran and 2 mg dieugenol.

Conclusions

This study has successfully produced low molecular weight lignin-like oligomers using merely two enzymes and eugenol (**1**) as a substrate in a one-pot approach. It was also shown that 4-allyl-2,6-dimethoxyphenol and chavicol can be used as substrates which allows us to prepare nature-like lignin material. The products formed have been analyzed and validated using HPLC, GPC, ^1H NMR, ^{13}C NMR and 2D NMR. The ability to generate lignin *in vitro* could serve as a strong basis for future applications. Research focusing on lignin degradation may benefit from the use of defined but complex synthetic lignin that can be prepared in a facile and cheap manner.

Experimental Section

Chemicals and Reagents

Eugenol (**1**), 4-allyl-2,6-dimethoxyphenol, coniferyl alcohol (**2**), pinoresinol, DMSO- d_6 , Tris, oligonucleotide primers, and peroxidase from horseradish (HRP) were purchased from Sigma–Aldrich (St Louis, MO, USA). All media components and ampicillin antibiotic were from Fischer Scientific chemicals (Pittsburgh, PA, USA). Solvents were purchased from JT Baker (Pittsburgh, PA, USA), Lab Scan Analytical Sciences (Gliwice, Poland) and Macron Fine Chemicals (Center Valley, PA, USA). Complete Ultra tablets (protease inhibitor cocktail) were purchased from Roche (Basel, Switzerland).

Enzyme Expression, Purification and Storage

E. coli strains were grown in 5 mL LB broth medium at 37°C overnight to saturation. The cultures were diluted 100 times in terrific broth (TB) medium and grown at 37°C/135 rpm until the OD_{600} reached 0.6. Induction was performed by adding 0.02% (w/v) L-arabinose and the cells were incubated at 30°C/135 rpm overnight. *SviDyP* and EUGO were both expressed from NEB-10 β cells in 500 mL TB broth medium placed in 2.5 L flasks. *SviDyP* and EUGO were produced using pBAD-*SviDyP* and pBAD-EUGO expression vectors, respectively obtained from GECCO (Groningen, The Netherlands).

Cells were harvested by centrifugation at $6000 \times g$ (Beckman Coulter, Avanti JE centrifuge, JLA 14 rotor or Beckman Coulter, Avanti J20 XP, JLA 9.1000 rotor) for 20 min at 4°C. The pellets containing the pBAD-*SviDyP* were

washed in Buffer A containing one tablet of protease inhibitor cocktail. They were then disrupted by sonication (10 min total time with cycles of 10 s on and 10 s off at 70% amplitude) using a Sonics Vibra-Cell VCX130 probe sonicator (Newtown, CT, USA). The cell-free extract was obtained by centrifugation at $16000 \times g$ for 30 min at 4°C. The extracts were filtered using Whatman FP 30/0.45 CA-S membrane syringe filters (GE Healthcare Lifesciences, Uppsala, Sweden) to remove remaining cell debris.

Enzymes were purified by using an ÄKTApurifier (GE Healthcare Lifesciences). *SviDyP* was purified from cell-free extract using a 5 mL His-Trap HP column (GE Healthcare Lifesciences) and EUGO was purified using a 5 mL Q HP HiTrap column (GE Healthcare Lifesciences). The column was pre-equilibrated using Buffer A followed by loading of the cell-free extract. Buffer A was then used to wash unbound components from the column. Enzymes were eluted in a gradient using buffer B starting from 0 to 500 mM imidazole and 0 to 1 M KCl for each of *SviDyP* and EUGO, respectively. After purification was complete, the protein was desalted using the HiPrep 26/10 Desalting column (GE Healthcare Lifesciences) or Econo-Pac® 10DG Desalting Prepacked Gravity Flow Columns – BioRad (Hercules, CA, USA) using Buffer C. All enzymes were frozen using liquid nitrogen and stored at -20°C until further use. Buffer compositions are mentioned in the Supporting Information, Table S2.

Determination of Enzyme Concentration

EUGO and *SviDyP* enzyme concentrations were determined based on their absorbance at 441 and 280 nm, respectively. The molar extinction coefficient (ϵ) of EUGO at 441 nm = $14.2 \text{ mM}^{-1} \text{ cm}^{-1}$.^[19] *SviDyP* concentration was determined based on the predicted molecular extinction coefficient using the ProtParam tool;^[31] ϵ_{SviDyP} at 280 nm = $48.6 \text{ mM}^{-1} \text{ cm}^{-1}$. An HRP stock solution of 68 μM in 20 mM KPi, pH 7 was prepared based on its molecular weight (MW $\approx 44,000$ Da).

Biocatalytic Synthesis of Coniferyl Alcohol (2) from Eugenol (1) using EUGO

Coniferyl alcohol (**2**) was synthesized in a 2 mL reaction volume containing 20 mM KPi buffer pH 7.5, 50 nM EUGO, 5% (v/v) DMSO, 10 mM eugenol (**1**) and 50 nM catalase. The experiment was conducted at 30°C/50 rpm in an Infors HT Multitron Standard incubator (Bottmingen, Switzerland). The conversion was repeated using three different concentrations of EUGO; 50, 500 and 5000 nM. Another run was done in 25 mL volume using 20 mM eugenol (**1**) and 5000 nM of EUGO and catalase. This reaction was performed for extraction of coniferyl alcohol (**2**) using ethyl acetate to quantify the yield and purity of coniferyl alcohol (**2**) formed. For quantification of coniferyl alcohol (**2**), samples were taken and diluted 20-fold in 50 mM Tris/HCl, pH 11. The coniferyl alcohol (**2**) concentration was determined by measuring the absorbance at 313 nm using a JASCO V-660 UV/Vis spectrophotometer at different intervals using an extinction coefficient at 313 nm of $13.9 \text{ mM}^{-1} \text{ cm}^{-1}$ (see the Supporting Information for details).

Analysis of Coniferyl Alcohol by HPLC

Coniferyl alcohol (**2**) was obtained by extracting three times with an equal volume of ethyl acetate. Then, the extraction liquids were combined and the ethyl acetate was removed under vacuum using a IKA® Distilling Rotary Evaporator (Staufen im Breisgau, Germany). A sample of the liquid remaining after evaporation was taken and diluted 20-fold using MilliQ water. It was analyzed by reverse phase HPLC using a JASCO HPLC system. A 10 μ L volume of the diluted sample was injected into a Grace Altima HP C18 column (3 μ m, 4.6 $\times$ 100 mm, with a precolumn of the same material). As references, pinoresinol, coniferyl alcohol (**2**) and eugenol (**1**) (1.0 mM stock solutions) were also injected. The solvents used for the system were: a) water with 0.1% v/v formic acid and b) acetonitrile. The HPLC method was: 2 min 10% B, 30 min on a gradient of 10–100% B, 5 min 100% B followed by a 3 min decreased gradient of 100–10% B and finally re-equilibration for 7 min. Detection was done using a UV detector at 254 nm and the flow rate was maintained at 0.5 mL min⁻¹.

Biocatalytic Synthesis of Lignin-Like Oligomers from Eugenol (**1**), 4-Allyl-2,6-dimethoxyphenol and Chavicol

Lignin product was synthesized using the same mixture as for the synthesis of coniferyl alcohol (**2**) from eugenol (**1**) (see above) with some modifications. The 20 mM KPi buffer was at pH 7 for experiments done using HRP and pH 6 when *SviDyP* was used. EUGO was added as the oxidase enzyme component at 50 nM concentration. HRP or *SviDyP* were added in 680 nM concentrations. Eugenol (**1**), chavicol or 4-allyl-2,6-dimethoxyphenol were used as a substrate in 10 mM concentrations. The ratio of reaction volume to air headspace was always maintained at a ratio of 1:10. To compare the compounds produced when using eugenol as a starting substrate to those formed when using coniferyl alcohol as a starting substrate, a reaction was performed starting with 10 mM coniferyl alcohol. The hydrogen peroxide needed for the conversion was supplied by adding 10 mM glucose and 50 nM glucose oxidase. HRP was used as the peroxidase component and was added at a concentration equal to 650 nM. All other conditions are as mentioned above. For more details on the experimental conditions, see Table S1 in the Supporting Information.

Analysis of Lignin Formation

At regular intervals, samples of the lignin conversions were taken and optical density was measured using a Thermo-scientific Nanodrop 1000 (Waltham, MA, USA) at 600 nm. A conversion catalyzed by 50 nM EUGO and 650 nM HRP containing 10 mM eugenol (**1**) in a 20 mL reaction was analyzed by HPLC. The conversion was maintained in 20 mM KPi buffer pH 7 in the presence of 5% (v/v) DMSO. Samples were taken at regular intervals over 98 h and heated to 90 °C for 5 min. They were then centrifuged for 2 min and the supernatant injected into a reverse phase HPLC. The conditions were similar to those mentioned for coniferyl alcohol (**2**) analysis with some modifications. The method was: 2 min 10% B, 18 min on a gradient of 10–70% B, 3 min 70% B followed by a 10 sec decreased gradient of 70–10%

B and re-equilibration for 7 min. Detection was done at 280 nm. Standard concentrations of eugenol (**1**) and coniferyl alcohol (**2**) were mixed together in 10% acetonitrile to construct a calibration curve. The concentrations used were 0.5, 1, 2, 3 and 4 mM. Pinoresinol was also injected to analyze any significant peaks being formed.

One Gram Conversion of Eugenol (**1**)

In a 2.5 L flask, 1.0 L of KPi 20 mM pH 7, EUGO 50 nM, HRP 650 nM, in DMSO 1.5% (v/v) was prepared to which 1.0 g eugenol (**1**) was added as a final component. The flask was placed at 30 °C/50 rpm and samples were routinely taken to analyze when the eugenol (**1**) was fully consumed. The solution was filtered using a pre-weighed 47 mm, 0.45 μ m solvent filter from Waters (Milford, MA, USA) under vacuum. The filter paper carrying the lignin-like oligomers was then dried at 80 °C until constant weight and then reweighed. The difference in weight corresponds to the yield of lignin from the conversion. To test for turnover capacity of the enzymes in the conversion, the reaction was repeated using the flow-through from the first cycle. To this solution, 1.0 g eugenol (**1**) and 1.5% DMSO was added and the eugenol (**1**) consumption monitored for another 5 days. Then, 200 mL of the conversion were filtered and the residue dried in the oven at 80 °C until constant weight.

Analysis of Soluble Fraction

Acetonitrile was added to the flowthrough after the two 1.0 g eugenol (**1**) conversions were completed to make 10% (v/v) and acidified with formic acid (0.1% [v/v]). The final mixture was fractionated on a 12 g (equivalent to 18 mL) 40 μ m Reveleris C-18 cartridge (Grace, IL, USA). After sample application, the column was washed with 4 column volumes of 10% acetonitrile and eluted using a gradient of 25 column volumes to reach 80% acetonitrile. Fractions were collected and analyzed for purity using analytical HPLC as described in the LC section. Further identification of obtained products was done by LC-MS with LCQ Fleet (ThermoFisher Scientific, MA, USA) using a previously described procedure.^[32] Pure fractions for NMR analysis were extracted 3 times with ethyl acetate, dried over anhydrous MgSO₄, filtered and concentrated by rotavap.

Analysis of Insoluble Fraction

A conversion containing 20 mM KPi pH 7, 50 nM EUGO, 650 nM HRP, 33 mg eugenol (**1**) and 5% (v/v) DMSO was done in 20 mL reaction volume. The conversion was done for 72 h at 30 °C/50 rpm. 5 mL of the reaction was centrifuged for 3 min in two 5 mL Eppendorf tubes. The supernatant was discarded and 5 mL of the conversion was added again to both Eppendorf tubes. Centrifugation was repeated. The pellet was washed once with 20 mM KPi pH 7 buffer and once with demineralized water. It was then flash frozen using liquid nitrogen and freeze dried using a Christ Alpha 2–4 LDplus freeze drier (Osterode am Harz, Germany) overnight.

The freeze-dried sample was suspended in a 1:100 toluene (GPC internal standard):THF mixture to make a final concentration of 10 mg mL⁻¹. It was then sonicated at 40% amplitude until complete dissolution. Subsequently, the solu-

tion was filtered (0.2 μm syringe filter) and used for GPC analysis on a Hewlett Packard 1100 system equipped with three PL-gel 3 μm MIXED-E columns in series. The liquid phase was THF operated at 42°C at a flow-rate of 1 mL min⁻¹. Detection was accomplished at 35°C using a GBC LC 1240 RI detector. The molecular weight was calibrated using polystyrene standards of known molecular weight distribution.

The freeze-dried lignin sample was suspended in DMSO-*d*₆ and sonicated for 5 min at 40% amplitude until complete dissolution. An HSQC 2D NMR spectrum was recorded on an Agilent Technologies 400/54 Premium shielded spectrometer. Mestrenova 9.1 was used for data processing.

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