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Mathilde Lecourt, Giorgiana Chietera, Bernard Blerot, Sylvain Antoniotti. Laccase-Catalyzed Oxidation of Allylbenzene Derivatives: Towards a Green Equivalent of Ozonolysis. *Molecules*, 2021, 26 (19), pp.6053. 10.3390/molecules26196053 . hal-03418904

HAL Id: hal-03418904

<https://hal.science/hal-03418904v1>

Submitted on 16 Nov 2021

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2 Laccase-catalyzed oxidation of allylbenzene derivatives: to- 3 wards a green equivalent of ozonolysis

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9 **Abstract:** Laccase-based biocatalytic reactions have been tested with and without mediators and
10 optimized in the oxidation of allylbenzene derivatives such as methyl eugenol taken as a model
11 substrate. The reaction primarily consisted in a hydroxylation of the propenyl side chain, either
12 upon isomerization of the double bond or not. Two pathways were then observed; oxidation of
13 both allylic alcohol intermediates could either lead to the corresponding α,β -unsaturated carbonyl
14 compound, or the corresponding benzaldehyde derivative by oxidative cleavage. Such process
15 constitutes a green equivalent of ozonolysis or other dangerous or waste-generating oxidation re-
16 actions. The conversion rate was sensitive to the substitution patterns of the benzenic ring and
17 subsequent electronic effects.

18 **Keywords:** biocatalysis; sustainability; phenylpropanoids.

20 1. Introduction

21 Oxidation processes in organic chemistry are typically waste-generating process-
22 es.[1,2] Not only that the fundamental principles of oxidation of organic substrates re-
23 quire a stoichiometric electron acceptor, but that suitable redox potentials to make these
24 transformations efficient have led to the massive use of metallic oxides eventually leav-
25 ing metallic by-products. In order to mitigate this undesired effect, combinations of cat-
26 alytic systems with benign terminal oxidants such as molecular oxygen,[3] hydrogen
27 hydroperoxide,[4] or bleach[5] have been developed. This is what Nature does for mil-
28 lions of years with enzymes evolved to achieve specific, selective, and efficient oxidation
29 reactions of organic substrates using indeed O₂ or H₂O₂ to activate metal centers (mostly
30 Cu, Fe, Mn ...) within their active sites. It is thus tempting to use these enzymes to per-
31 form oxidation reactions at laboratory or pilot and full-industrial scale in biocatalytic
32 processes.[6,7]

33 Biocatalysis is a branch of catalysis involving enzymes or cell as catalyst for a given
34 reaction. Biocatalytic processes tolerate mild operating conditions (temperature, pH,
35 pressure) and can be run in aqueous mediums as far as the substrate has a sufficient
36 solubility.[8] Enzymes can also be directly used *in vivo* by applying wild or engineered
37 cells to the substrate.[9,10] Besides that, enzymes display selectivity and / or specificity
38 which are of great interest either in fine chemical synthesis, in pharmaceutical or per-
39 fumery areas.[9,11-13] Engineered enzymes are more and more often used in industry to
40 replace late steps in a chemical synthesis where high degrees of selectivity and efficiency
41 are required and/or to improve the processes' sustainability.[14-16]

Laccases belong to the large oxidoreductase class. This family of enzymes can genuinely convert a wide range of compounds, which can be broadened by using mediators for oxidation transfer.[14,15,17,18] Laccases were investigated on various substrates such as phenols, methoxybenzenes, alkenes and polycyclic aromatic hydrocarbons.[19-22] These enzymes are employed for lignin degradation in Kraft pulp,[23-25] and in phenol depolluting processes[26,27] and are broadly considered in green oxidation catalytic systems.[14,15,28]

Methyl eugenol **1** is an allylbenzene derivative naturally produced by plants. It was identified as a product of lignin degradation in presence of laccase from *Trametes versicolor* and methyl syringate as natural mediator.[29] As **1** is an allergen and a carcinogenic agent,[30-32] it is strongly regulated in food and cosmetics.[33-35] Its metabolism by microorganisms involves cytochrome P450 which is an oxidase.[36,37] Its antifungal and cytotoxic effects were also investigated.[38,39]

Laccase has been already investigated in oxidation of phenols such as eugenol and isoeugenol and was shown to induce oxidative dimerization.[40,41] To our knowledge, laccase-catalysed oxidation of **1** and allylbenzene derivatives has never been studied. Herein, we investigated the activity of laccases on allylbenzene derivatives including **1** in a quest for alternative sustainable oxidative protocols. Intermediates and products were thus isolated, identified and engaged separately in order to better understand the mechanism of the reaction.

2. Materials and methods

Chemicals: Chemicals, organic solvents, and laccase from *Trametes versicolor* were purchased from Sigma-Aldrich (Saint-Louis, MO, USA), and used as received. Distilled water was obtained by filtration on an Elix® Reference 3 system with a Progard®S2 cartridge. Laccase M120 was obtained as a kind loan from Amano Enzyme Inc. (United Kingdom) and laccase Mrs was provided by ISM2 Laboratory, France (Pierre Rousselot Pailley, Thierry Tron, Equipe Biosciences), obtained after heterologous expression and purification. Both were stored at -18°C.

Analysis: ¹H and ¹³C NMR spectra were recorded on Bruker Avance 400 spectrometer. Chemical shifts, reported in ppm, were referenced relatively to TMS on the signals of residual solvents (¹H and ¹³C). GC/MS analysis were carried out with an Agilent 7820A chromatograph coupled to an Agilent 5977B MS detector (Agilent, California). Samples were analyzed on a capillary column SAPIENS-5MS (10 m × 0.10 mm × 0.10 μm, 5% diphenyl-95% dimethylpolysiloxane, Teknokroma, Spain). Operating conditions: carrier gas: He, constant flow, 20 mL/min; injection temp., 250 °C, injection volume, 0.2 μL, split ratio, 1:50; temp. program: 50 °C held 1 min, 50°C to 280 °C at 10 °C/min, then held isothermal (10 min), ion source temp., 230 °C; transfer line temp., 300 °C; ionization energy, 70 eV; electron ionization mass spectra were acquired over the mass range of 35–550 amu. Identification of the products was based on comparison of their MS spectra with those of commercial libraries (NIST15, Wiley), laboratory MS libraries built up from pure substances, with MS literature data, or comparison with authentic samples.

Standard procedure for the enzymatic oxidation of allylbenzene derivatives: The substrate (1 mmol, 0.25 M), the mediator (10 mol%) and laccase (5 wt%) were introduced in a 20 mL vial in a pH5 20 mM aqueous acetate buffer (4 mL). Reaction medium was incubated under an oxygen atmosphere using and incubator shaker with orbital agitation (24 h, 40 °C, 200 rpm). Work up consisted in EtOAc extraction of the aqueous phase (5×2 mL). Organic phases were pooled, dried over MgSO₄ and concentrated *in vacuo*. GC/MS and ¹H-NMR were performed to determine the reaction outcome on crude products during optimization phases and on purified material after column chromatography for synthetic purpose.

Oxidation intermediates characterization: Enzymatic oxidation was carried out on a larger scale. **1** (11.96 mmol, 0.1 M), HOBt (1.23 mmol, 166.8 mg) and laccase (46 mg) were

94 dissolved in a pH5 20 mM aqueous acetate buffer (120 mL). The mixture was stirred
95 under oxygen atmosphere in an oil bath at 40 °C. After 24 h, 1 mL of the mixture was
96 submitted to the above-described work-up and the reaction progress was estimated by
97 GC/MS analysis. In case of partial conversion, laccase and mediator addition was per-
98 formed (up to 8 additions for a total of 1292.3 mg of HOBt and 349.3 mg of laccase). Upon
99 completion of the reaction with full conversion, workup was performed as described.
100 Different products were isolated by column chromatography on silica gel (diethyl ether /
101 petroleum ether gradient as the eluent).

102 Compounds **1**, **2**, and **7** were purchased from Sigma-Aldrich (Saint-Louis, MO, USA)
103 and compound **3** was purchased from Enamine (Ukraine).

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105 1-(3,4-Dimethoxyphenyl)prop-2-en-1-one **4**: **6** (2.61 mmol) was diluted in EtOAc (0.12 M).
106 Manganese oxide (MnO₂, 4.16 eq.) was added, and the mixture was vigorously stirred at
107 room temperature. Conversion was checked by TLC and pushed up with manganese
108 oxide supplementation (8.32 eq.). After 48 h, the black suspension was filtered on a
109 Celite® pad and on silica gel before concentration *in vacuo*.

110 ¹H NMR (400 MHz, CDCl₃) δ (ppm) 7.61 – 7.56 (m, 2H, H_{Ar}), 7.19 (dd, *J* = 17.0, 10.5 Hz, 1H,
111 H₁), 6.90 (d, *J* = 8.4 Hz, 1H, H_{Ar}), 6.43 (dd, *J* = 17.0, 1.8 Hz, 1H, H₃), 5.87 (dd, *J* = 10.5, 1.8 Hz,
112 1H, H₃), 3.96 & 3.95 (s, 3H, OCH₃).

113 ¹³C NMR (100 MHz, CDCl₃) δ (ppm) 189.28, (C=O), 153.57 & 149.40 (C_{Ar}-OCH₃), 132.07,
114 (CH=), 130.56, (C_{Ar}), 129.39, (=CH₂), 123.56, (CH_{Ar}), 110.92, (CH_{Ar}), 110.11, (CH_{Ar}),
115 56.23, (OCH₃), 56.15, (OCH₃).

116 MS *m/z* (%): 192 (M⁺, 50), 165 (100), 122 (20), 107 (22), 91 (20), 79 (78), 77 (40), 63 (25), 55
117 (60), 51 (39)

118
119 1-(3,4-Dimethoxyphenyl)-2,3-epoxypropan-1-ol **5**: Isolated as a mixture of diastereomers
120 from the reaction of **1**.

121 ¹H NMR (400 MHz, CDCl₃): 3:7 mixture of 2 diastereomers. δ (ppm) 6.99 – 6.85 (m, 3H,
122 H_{Ar}), 4.87 & 4.44 (d, 1H, H₁), 3.91 & 3.81 (s, 3H, OCH₃), 3.23 (m, 1H, H₂), 2.77 – 2.97 (m, 2H,
123 H₃).

124 ¹³C NMR (100 MHz, CDCl₃): 3:7 mixture of 2 diastereomers. δ (ppm) 149.36 & 149.12
125 (C_{Ar}-OCH₃), 149.31 & 149.16 (C'_{Ar}-OCH₃), 132.99, (C_{Ar}), 132.09, (C'_{Ar}), 118.92, (C'_{HAr}),
126 118.81, (CH_{Ar}), 111.25, (CH_{Ar}), 109.68, (C'_{HAr}), 109.58, (CH_{Ar}), 109.55, (C'_{HAr}), 74.27,
127 (C-OH), 70.84, (C'-OH), 56.09 – 56.05 & 56.02, (CH, OCH₃, OCH₃), 56.09 – 56.05 & 55.17,
128 (C'_H, OC'_H₃, OC'_H₃), 45.50, (CH₂); 43.79, (C'_H₂).

129 MS *m/z* (%): diastereomer 1 (maj.) 210 (M⁺,35), 167 (100), 165 (14), 151 (54), 139 (67), 124
130 (23), 108 (19), 107 (15), 77 (25), 65 (15)

131
132 1-(3,4-Dimethoxyphenyl)prop-2-en-1-ol **6**: **2** (8.86 mmol) under a nitrogen atmosphere
133 was dissolved in distilled THF (0.11 M) and put in a –80°C bath. Vinyl magnesium bro-
134 mide (C₂H₃MgBr, 1.19 eq.) in THF was added slowly. The reaction was let warm to room
135 temperature and stirred until TLC analysis indicated that no starting material remained
136 (7 h). The reaction was quenched with a saturated aqueous NaCl solution and extracted
137 with EtOAc. The combined organic layers were washed with an aqueous solution of
138 saturated NaCl, dried on MgSO₄, filtrated, and concentrated *in vacuo* (81%).

139 ¹H NMR (400 MHz, CDCl₃) δ (ppm) 6.89 – 6.85 (m, 2H, H_{Ar}), 6.81 (m, 1H, H_{Ar}), 6.02 (ddd, *J*
140 = 17.2, 10.3, 5.9 Hz, 1H, H₂), 5.33 – 5.15 (m, 2H, H₃), 5.11 (dd, *J* = 5.9, 1.5 Hz, 1H, H₁), 3.85 &
141 3.84 (s, 3H, OCH₃). ¹³C NMR (100 MHz, CDCl₃) δ (ppm) 149.21 & 148.71 (C_{Ar}-OCH₃),
142 140.37, (CH=), 135.41, (C_{Ar}), 118.76, (CH_{Ar}), 115.01, (=CH₂), 111.13, (CH_{Ar}), 109.59,
143 (CH_{Ar}), 75.18, (CH-OH), 56.04, (OCH₃), 55.95, (OCH₃).

144 MS *m/z* (%): 194 (M⁺, 97), 165 (29), 163 (62), 151 (33), 139 (100), 138 (27), 91 (34), 79 (31),
145 77 (36), 55 (51).

146
147 3-(3,4-Dimethoxyphenyl)prop-2-en-1-ol **8**: **3** (1 mmol) was dissolved in EtOH (0.35 M)
148 and placed under a nitrogen atmosphere. NaBH₄ (1.25 eq.) was added. The mixture was

149 then stirred for 1.2 h at room temperature. Workup consisted in EtOH evaporation under
150 vacuo, solubilization in distilled water and extraction with EtOAc. Organic layers were
151 pooled, dried over MgSO₄ filtrated and concentrated *in vacuo* (100%).

152 ¹H NMR (400 MHz, CDCl₃): δ (ppm) 6.98 – 6.88 (m, 2H, H_{Ar}), 6.82 (d, *J*=8.2 Hz, 1H, H_{Ar}),
153 6.55 (d, *J*=15.9 Hz, 1H, H₃), 6.25 (dt, *J*=15.9, 5.8 Hz, 1H, H₂), 4.31 (d, *J*=5.8 Hz, 2H, H₁), 3.90
154 & 3.88 (s, 3H, OCH₃).

155 ¹³C NMR (100 MHz, CDCl₃): δ (ppm) 149.15 & 149.04 (C_{Ar}-OCH₃), 131.27 (C_{Ar}), 129.86
156 (CH=), 126.67 (=CH), 119.81 (CH_{Ar}), 111.24 (CH_{Ar}), 108.97 (CH_{Ar}), 63.95 (CH₂), 56.04
157 (OCH₃), 55.94 (OCH₃).

158 MS *m/z* (%): 194 (M⁺, 68), 165 (14), 151 (100), 138 (53), 119 (16), 107 (13), 91 (33), 79 (14), 77
159 (26), 55 (13).

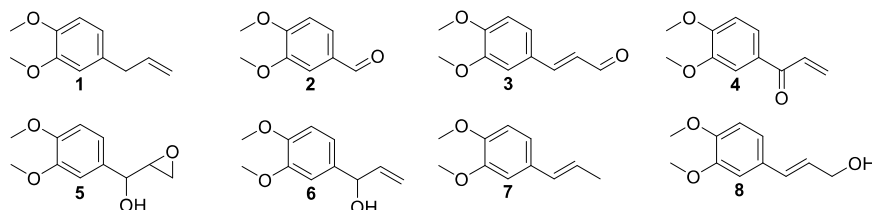
160 3. Results and discussion

161 3.1 Enzymatic reaction of methyleugenol

162 To study the reaction, **1** was taken as the model substrate for preliminary investiga-
163 tions. Parameters of **1** oxidation by laccase were screened with a focus on enzyme weight
164 ratio, mediator nature and ratio, co-solvent, pH and temperature.

165 Controls

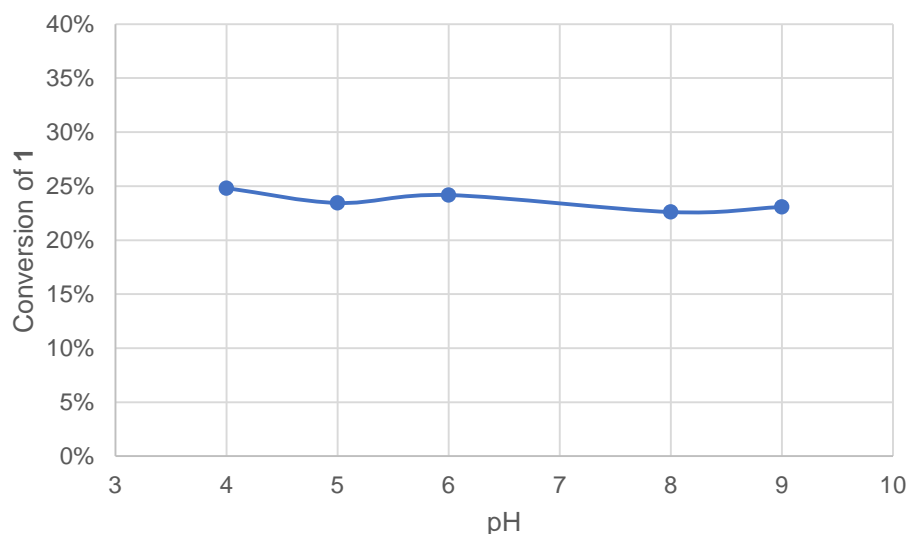
166 A couple of negative controls were performed. For the first control, **1** (1.03 mmol,
167 185.0 mg) and laccase (10% LA, 19.10 mg) were incubated in aqueous acetate buffer pH5
168 under an oxygen atmosphere for 24 h at 40 °C, before extraction with EtOAc. **1** was
169 quantitatively recovered unchanged, no conversion being noticed by GC/MS analysis. For
170 the second control performed with 2 mediators, **1** (1.03 mmol, 184.2 mg) and 1.02 mmol,
171 182.2 mg) and mediator (20% HOBT, 0.20 mmol, 27.3 mg and 20% TEMPO, 0.23 mmol,
172 35.5 mg) were incubated in aqueous acetate buffer pH5 under an oxygen atmosphere for
173 24 h at 40 °C, before extraction with EtOAc. **1** was quantitatively recovered unchanged, no
174 conversion being noticed by GC/MS neither. It can be concluded from these controls that
175 laccase is not able to oxidize **1** on its own and that the mediator alone cannot oxidize **1**
176 neither. Both mediator and laccase are involved in oxidation mechanism.



180 Figure 1. Methyl eugenol **1** and derivatives.

181 3.1.1 Effect of pH and temperature

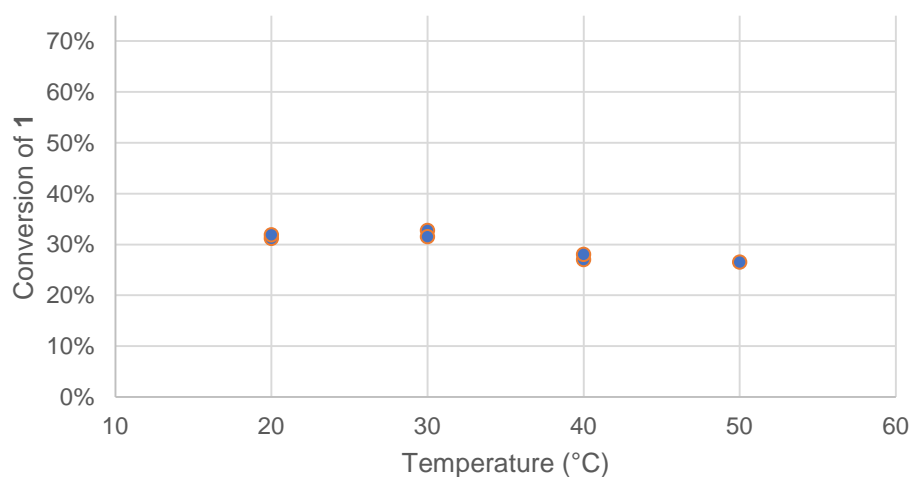
182 Laccases are known to have optimal pH ranges between 3.5 and 5 with hydrogen
183 donor substrates [15]. In order to evaluate specifically the effect of pH on our reactions of
184 interest, five different pH were tested (4-9), along with an additional test in deionized
185 water (DIW). The effect was quickly assessed by monitoring remaining **1** in the crude
reaction mixture after extraction and GC/MS analysis.



186
187
188 Figure 2. Effect of pH on conversion of **1** in the laccase-catalyzed oxidation (LA 10%_{o/w/w}, HOBT 20 mol%, buffer 0.25 M, O₂ atmosphere, incubation for 24 h, 40 °C, 200 rpm).

189 Our experiments show the stability of the laccase-mediator oxidation of **1** in the 4-9
190 pH range and in DIW (not shown).

191 Laccases optimal temperatures are known to be in the range from 50 to 70 °C but
192 they can also exhibit activity at lower temperature, such as physiological temperature
193 [15]. In order to evaluate specifically the effect of temperature on our model reaction, four
194 different temperatures were tested. Once again, the effect was quickly assessed by mon-
195 itoring remaining **1** in the crude reaction mixture after extraction and GC/MS analysis.
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200 Figure 3. Effect of temperature on conversion of **1** in the laccase-catalyzed oxidation (LA 10%_{o/w/w}, HOBT 20 mol%, acetate buffer pH5 0.25 M, O₂ atmosphere, incubation for 24 h, 200 rpm). Reactions run in duplicate.

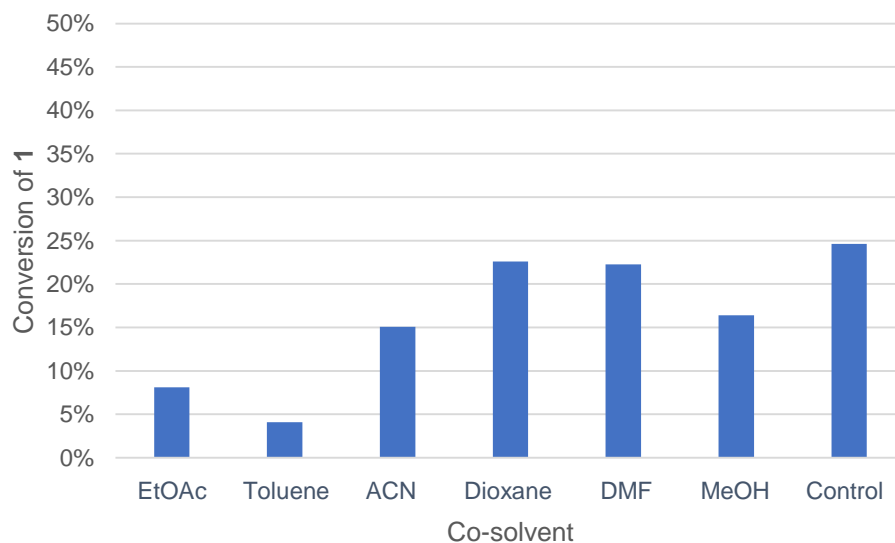
201 Optimal temperature was found to be between 20 and 30 °C for the reaction of **1**.

202 3.1.2 Effect of co-solvent

203 A co-solvent can be added to the medium to improve substrate solubility in water.
204 The positive effect of co-solvent such as EtOAc on 2,6-dimethoxyphenol dimerization by
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Trametes pubescens laccase was already reported.[42] Various co-solvents, miscible or not with water, were tested and activity was measured by GC/MS analysis (Figure 4).



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Figure 4. Effect of co-solvents on conversion of **1** in the laccase-catalyzed oxidation (LA 10%_{w/w}, HOBT 20 mol%, acetate buffer pH5 0.25 M, co-solvent 50%_{v/v} buffer, O₂ atmosphere, incubation for 24 h, 40 °C, 200 rpm).

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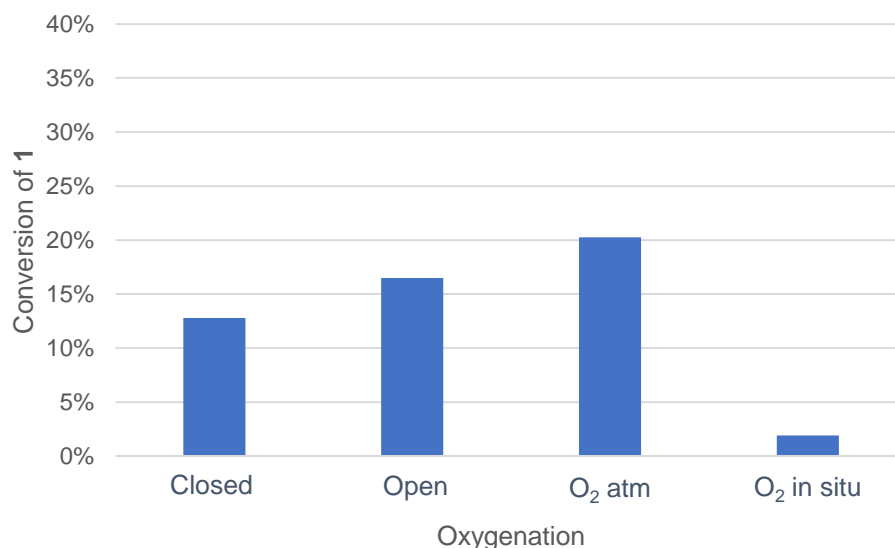
Toluene and EtOAc, that are immiscible with water at this temperature, gave the worst conversion rates. DMF and dioxane allowed for higher conversion rates.

3.1.3 Effect of oxygenation of the medium

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Medium oxygenation can be a limiting factor of an aerobic oxidation reaction as oxygen dissolution rate in water can be low. A solubility of oxygen of 39.04 mg/L in distilled water at 20 °C has been determined, for a saturated O₂ atmosphere.[43]

Various oxygenation methods were tested: closed vessel, open vessel at ambient atmosphere, vessel under an oxygen atmosphere and oxygenation by controlled in situ catalase degradation of hydrogen peroxide (Figure 5). Once again, the effect was quickly assessed by monitoring remaining **1** in the crude reaction mixture after extraction and GC/MS analysis.



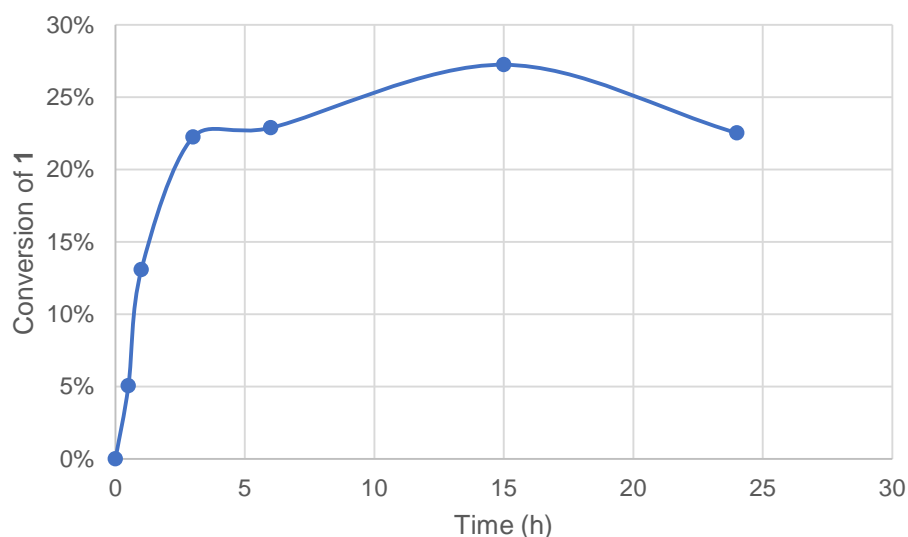
226

227 Figure 5. Effect of oxygenation on conversion of **1** in the laccase-catalyzed oxidation (LA 5%_{w/w},
228 HOBT 10 mol%, acetate buffer pH5 0.5M, DMF 5%_{v/v}, incubation for 24 h, 30 °C, 200 rpm).

229 Oxygenation with catalase – H₂O₂ system didn't improve **1** conversion. A noticeable
230 enhancement of the conversion was observed when reaction was run under an oxygen
231 atmosphere.
232

233 3.1.4 Effect of reaction time

234 Increasing reaction time usually allows a reactive system to reach the total conver-
235 sion if there is no limitation by reagents quantities. Acylation of palmarosa essential oil
236 by a lipase from *Candida rugosa* was reported to reach a 46% conversion after 24 h,
237 whereas total conversion was obtained after 96 h.[44] Effect of reaction time was meas-
238 ured by a kinetic plot and the conversion of **1** assessed by monitoring remaining **1** in the
239 crude reaction mixture after extraction and GC/MS analysis.



240
241 Figure 6. Effect of time reaction on conversion of **1** in the laccase-catalyzed oxidation (LA 10%_{w/w},
242 HOBT 20 mol%, acetate buffer pH5 0.25 M, O₂ atmosphere, incubation 40 °C, 200 rpm).

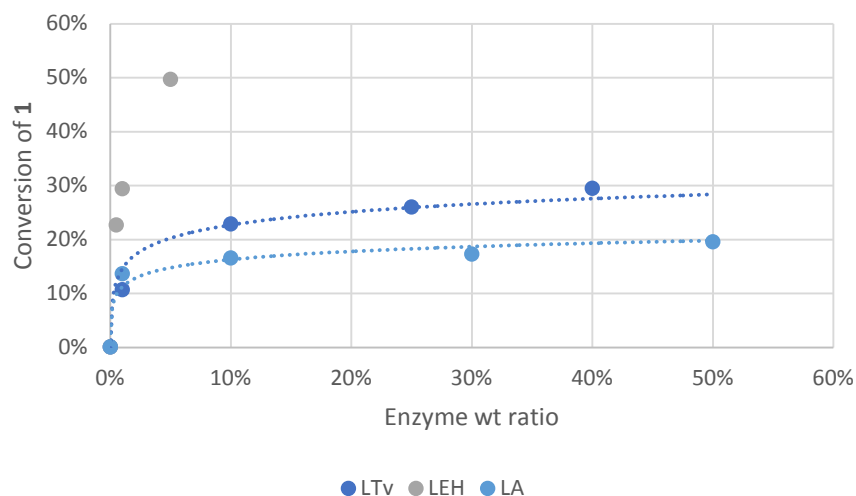
243 The assays showed that the conversion raised significantly over a 5h-period with an
244 initial reaction rate of ca. 32 mM.h⁻¹. Conversion plateau was reached at 25-30% after 10 h
245 and this might be due to the deactivation of the enzyme/mediator combination.
246 Preincubation of some components was thus investigated mostly to evaluate a possible
247 inhibiting effect of substrate **1** on the enzymatic system. The enzyme and **1** were thus
248 engaged in our conditions in the absence of mediator while in parallel, the mediator and
249 **1** were engaged similarly in the absence of the enzyme. After 24 h, the laccase-mediator
250 system was completed by the addition of the missing component and the reaction was
251 run for another 24 h. No significant differences were observed between these two ex-
252 periments and the one of Figure 6.
253

254 3.1.5 Effect of enzyme source and concentration

255 Three laccases were used in this study in order to compare their activity. Laccase
256 LTV was purchased from Sigma Aldrich (laccase from *Trametes versicolor*), laccase LA is a
257 non-GMO enzyme purchased from Amano enzyme (laccase M120) and laccase LEH was
258 obtained from ISM2 laboratory (Aix-Marseille Université) and produced by heterologous
259 expression. These variable sources exhibited different specific activities which are not
260 always precisely determined by vendors what makes difficult to design of experiments
261 for a comparison purpose. An SDS-PAGE test was performed on the enzymes, showing
262 that LA and LTV purity was rather low (see SI). In these reactivity tests, laccases were

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used in varying weight% relative to ME, and comparison was made by measuring oxidation products in the crude reaction mixture after extraction and GC/MS analysis. The effect of substrate concentration was assayed on another side and was found negligible (see SI).



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Figure 7. Effect of enzyme source on conversion of **1** in the laccase-catalyzed oxidation (laccase 1 - 50%_{ow/w}, HOBt 10 mol%, acetate buffer pH5 0.25 M, O₂ atmosphere, incubation for 24 h, 40 °C, 200 rpm).

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Increasing enzyme quantity could help to reach a better conversion into oxidation products, but the increase was somehow limited beyond 0.25 equivalent. Laccase LEH, shown to be of the highest purity, was clearly superior for this reaction with oxidation products formed with more than 50% yield with only 5 wt%. The quantity of mediator is also an important parameter in relation with the quantity of enzyme. By comparison with the conditions of Figure 7, when the reaction was performed with 4-fold HOBt as the mediator, the conversion of **1** increased by ca. 2-fold (not shown).

3.1.6 Effect of the mediator type

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Various types of mediators are already described in the literature and typically used in combination with laccases, such as 1-hydroxybenzotriazole (HOBt), TEMPO and ABTS [18]. Their role is to link the enzyme with substrates that are not compatible for steric or electronic reasons. A mediator is a molecule of low molecular weight, which is oxidizable by laccase to a high potential intermediate, and whose reduced and oxidized forms must be stable.[14] The mediator must not deteriorate during oxidation cycles, nor deactivate the enzyme. After being oxidized by laccase, the mediator oxidizes the substrate in a non-enzymatic process. The mediator returns to its initial reduced form and is ready to perform a new cycle. These mediators have redox potential compatible with the enzyme as well a broader substrate scope. All of those mediators are of synthetic origin. Natural mediators can also be used, such as violuric acid (VA), and vanillin.[28] A pool of natural and non-natural mediators was thus tested (Figure 8).

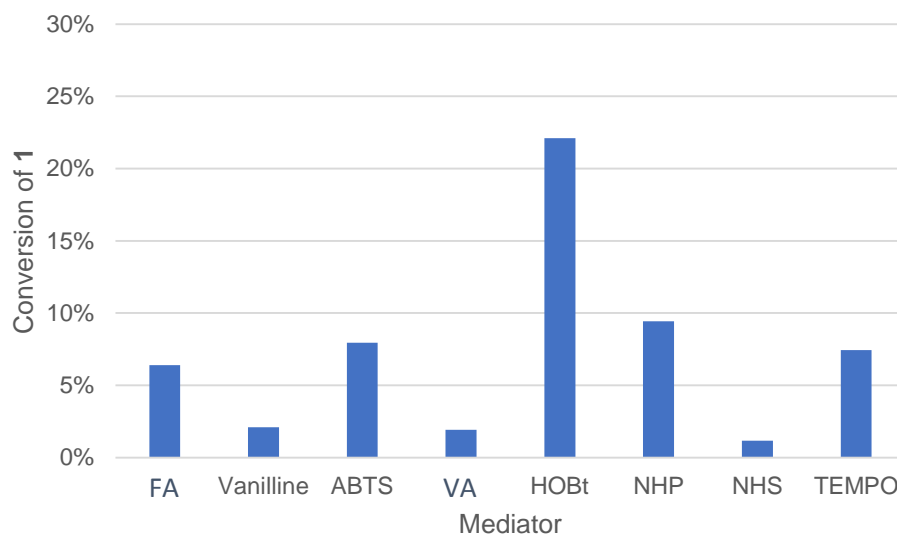


Figure 8. Effect of the mediator type on conversion of **1** in the laccase-catalyzed oxidation (LA 10%_{ow/w}, mediator 20 mol%, acetate buffer pH5 0.25 M, O₂ atmosphere, incubation for 24 h, 40 °C, 200 rpm). NPH: N-hydroxyhthalimide, NHS: N-hydroxysuccinimide, VA: violuric acid, FA: ferulic acid.

The type of mediator had an important effect on conversion, and no or very little reaction occurred in our conditions when using N-hydroxysuccinimide (NHS), vanillin, violuric acid (VA), and ferulic acid (FA). The profile of oxidation products distribution was also affected by the choice of the mediator. In the same proportion, HOBt converted more **1** than TEMPO, but HOBt produced more oxidative cleavage product such as veratraldehyde **2** and 3-(3,4-dimethoxyphenyl)prop-2-en-1-one **3** than TEMPO (Figure 9). TEMPO was also able to convert **1** into 1-(3,4-dimethoxyphenyl)prop-2-en-1-one **4**.

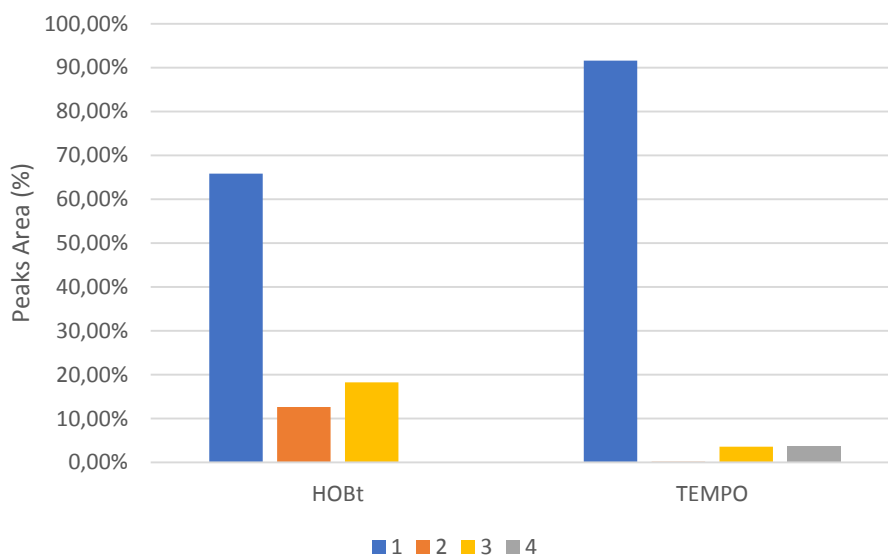


Figure 9. Comparison of oxidation products using HOBt or TEMPO as %area in the crude product determined by GC/MS (LA 10%_{ow/w}, mediator 50 mol%, acetate buffer pH5 0.25 M, O₂ atmosphere, incubation for 24 h, 40 °C, 200 rpm).

As organic molecules, mediators are extracted during work-up together with unconverted **1** and oxidation products. Using immobilized (supported) mediators could be an option as they can be easily separated from the reaction mixture by filtration. Sup-

ported HOBt and TEMPO were tested on **1** on our standard conditions but did not show any activity (not shown). In parallel, a positive control experiment was set using 4-methoxybenzyl alcohol oxidation [18]. Without mediator, no conversion was observed. Free HOBt allowed for substrate oxidation as expected with 34% and the formation of resp. 18% and 12% oxidation products such as **3** and **4**. When using supported mediator such as silica-bound TEMPO, PS-bound TEMPO and PS-bound HOBt, no reaction occurred, and the substrate was quantitatively recovered after work-up. Thus, we hypothesized that these supported mediators could not be activated by laccase within the binding pocket, enabling eventually substrate oxidation.

3.1.7 Effect of mediator concentration

Mediator in the laccase – mediator system is an intermediate that should switch between its oxidized and reduced form in a catalytic cycle. Following this scheme, each molecule of mediator should be reusable endlessly. The effect of mediator concentration on the conversion of **1** was investigated (Figure 10). Conversion was monitored by GC/MS measurement of **1** after extraction of the crude reaction mixture. With HOBt, conversion increased with the ratio reaching a plateau at ca. 50 mol%. With TEMPO however, conversion remained low regardless of its ratio.

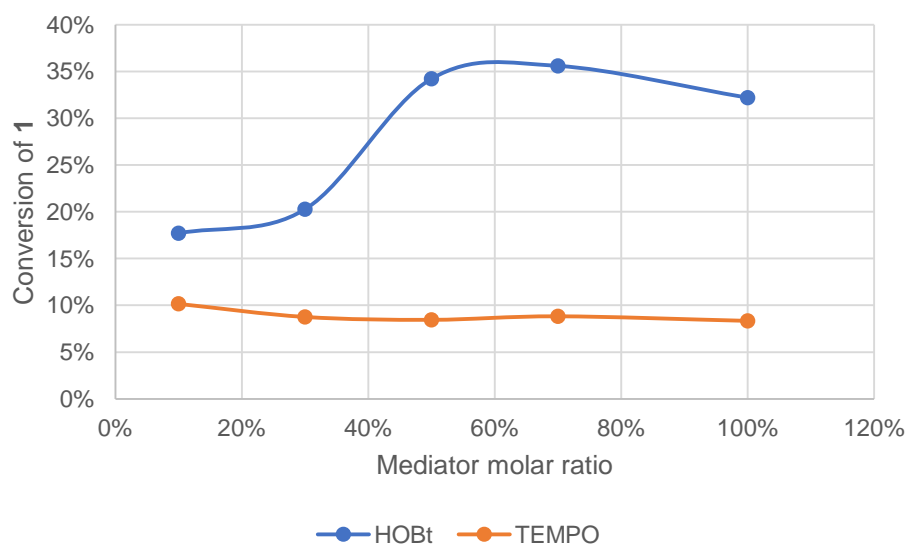


Figure 10. Effect of mediator type and ratio on conversion of **1** in the laccase-catalyzed oxidation (LA 10%_{w/w}, mediator, acetate buffer pH5 0.25 M, O₂ atmosphere, incubation for 24 h, 40 °C, 200 rpm).

3.1.8 Hypothesis of a fast deactivation and sequential supply of fresh enzyme and mediator

Previous results obtained during this study suggested a fast deactivation of the laccase-mediator system in our conditions. This hypothesis was further tested with reactions conducted with a sequential supply of fresh enzyme and mediator under time course monitoring. A total of 4 additions of 5 wt% of laccase LA was necessary to reach 100% conversion and 93% yield of **2** and **3**, favorable to the former at 54% (Figure 11). Conversely, a total of 3 additions of 10 mol% HOBt allowed a 100% conversion and 87% yield, with a 60% selectivity in favor of **3**.

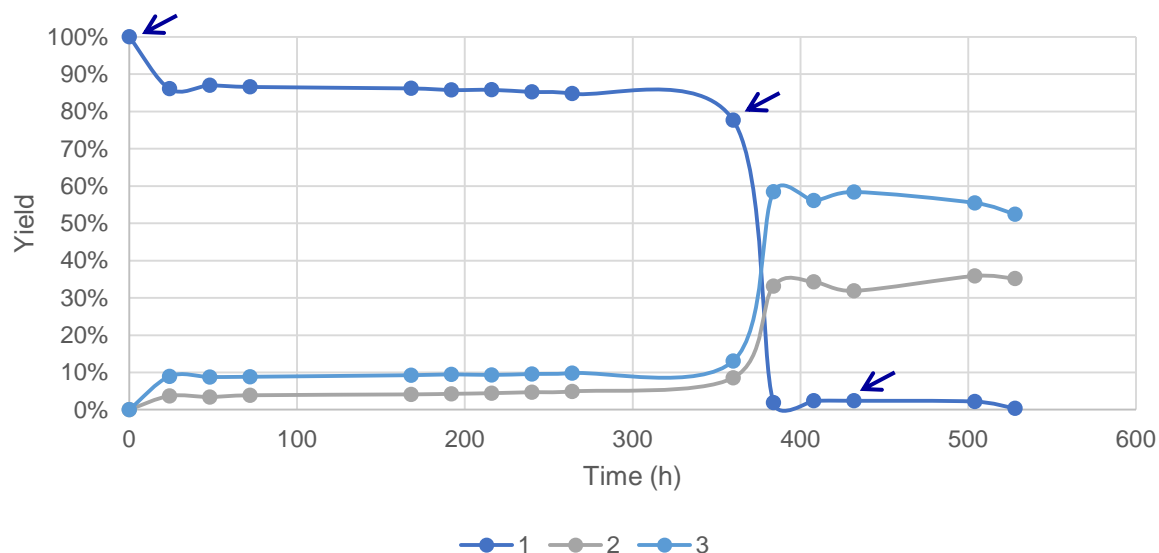
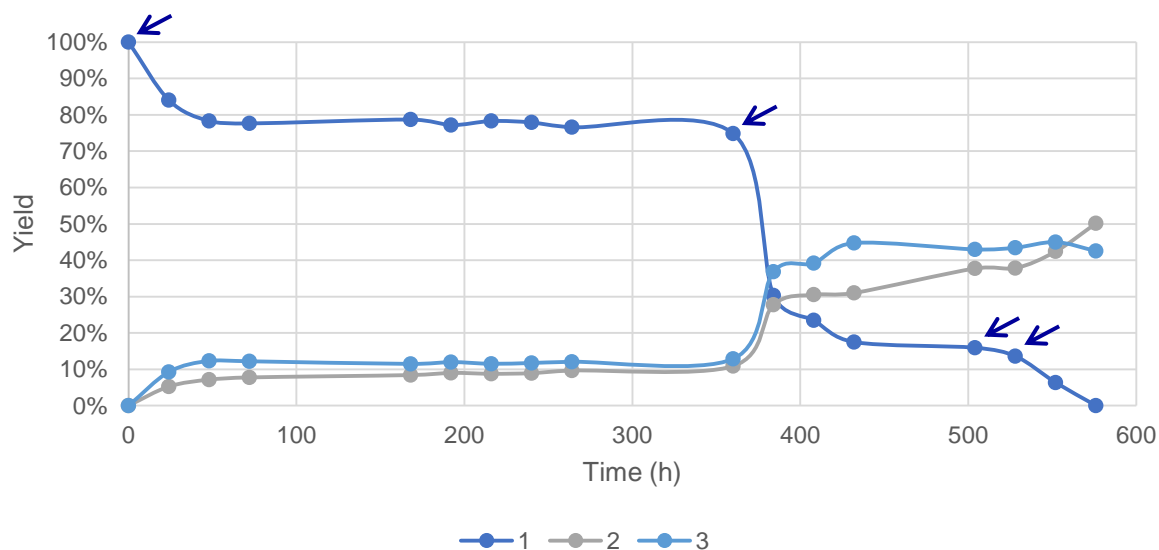


Figure 11. Sequential supply of fresh enzyme (top) and mediator (bottom) marked with arrows for each addition of material (acetate buffer pH5, 6 mmol substrate **1** 0.12 M, O₂ atmosphere, 40 °C, magnetic stirring).

These results confirm the deactivation of both the enzyme LA, already described for a laccase from *Trametes villosa* with HOBt,[24] but also the deactivation of HOBt, presumably by degradation.

2.2 Mechanistic investigations

Identification of oxidation products of **1** was possible upon daily addition of enzyme and mediator until full conversion of **1** was attained. Isolation by chromatography on silica gel and confirmation by chemical synthesis allowed to identify compounds **2**, **3**, and **4**.

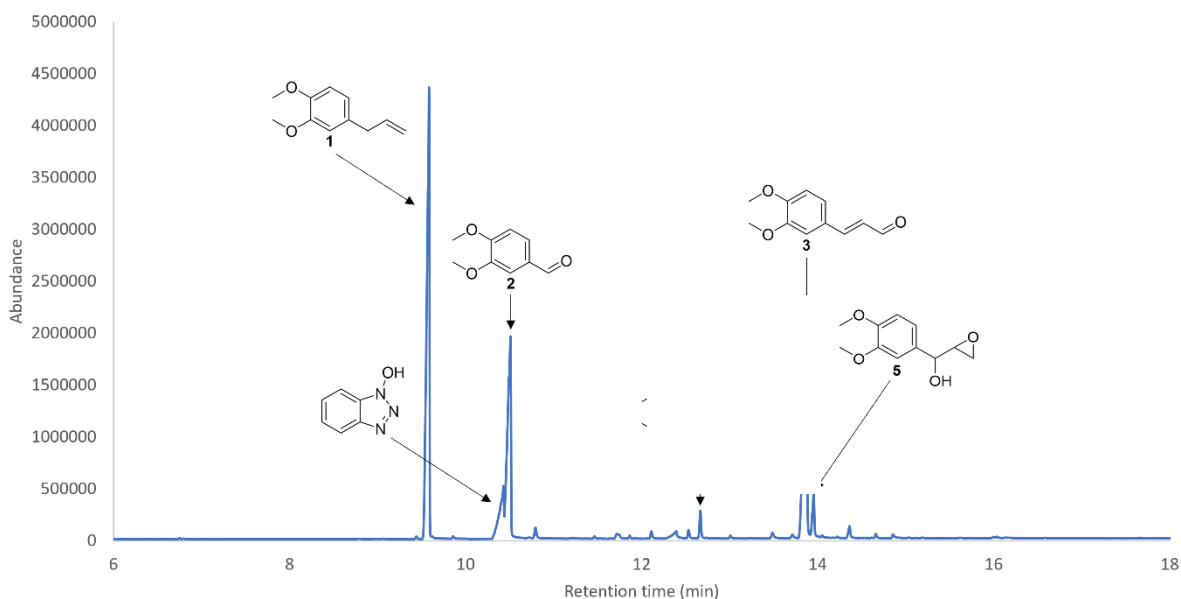
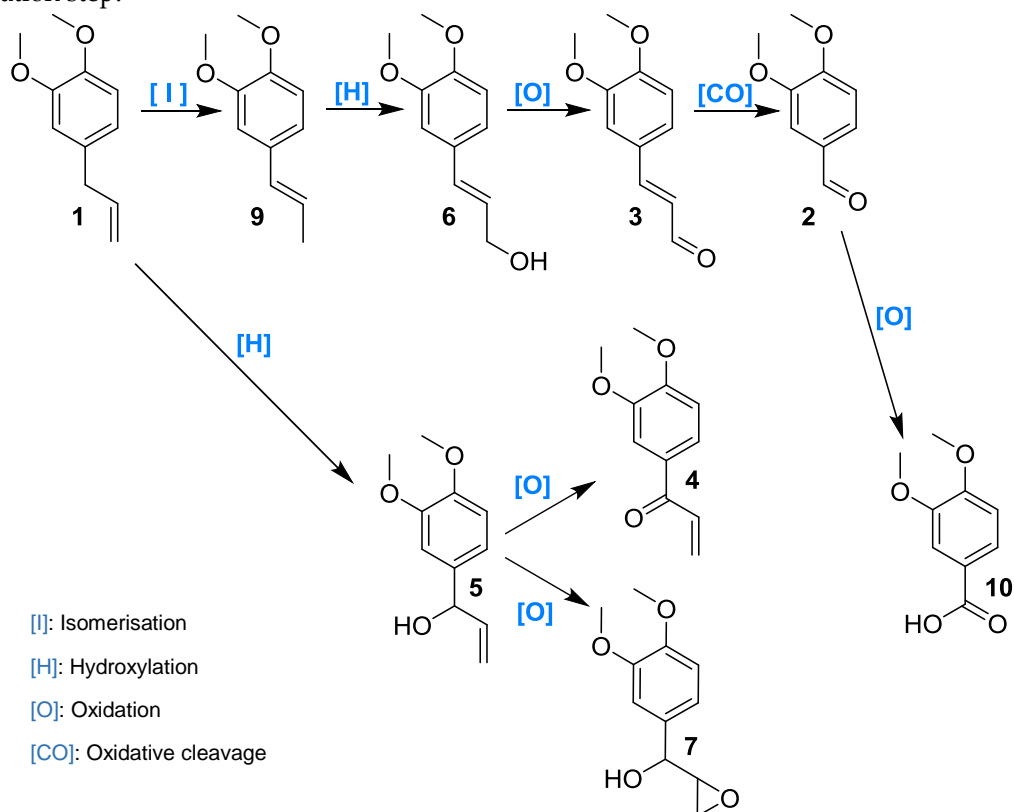


Figure 12. GC/MS chromatogram of the crude product after enzymatic reaction of **1** with HOBt as mediator. Five peaks were identified as oxidation products besides HOBt.

Reaction mechanism was hypothesized based on intermediates identified and their observed rate of formation/conversion. **1** could be either oxidized on its allylic position to **5** or first isomerized into **9** and further oxidized to **6**. A concerted isomerization/oxidation could also convert **1** into **6**. Oxidation of activated alcohol functions of **5** and **6** to their corresponding carbonyl compounds could thus take place leading to **4** and **3**, respectively. Veratraldehyde **2** could be either formed upon oxidative cleavage of all these intermediates and could eventually deliver benzoic acid derivative upon an additional oxidation step.



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Figure 13. Hypothetical mechanism of oxidation of **1** by the laccase-mediator system.

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Each step of this hypothetical mechanism was checked by submission of the corresponding intermediate, either isolated, purchased or synthesized, to the enzymatic reaction, with HOBT as mediator. Products and their proportion for each reaction are summarized in Table 1.

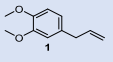
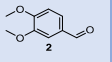
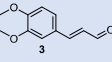
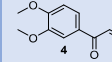
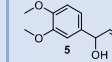
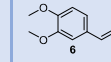
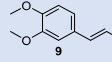
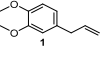
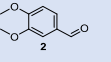
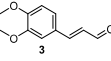
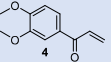
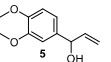
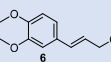
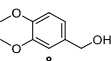
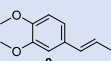
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Table 1. Cross-table of conversion of substrates and intermediates by the laccase-mediator system.

		Products						
								
Substrates		80%	3%	12%	1%	–	–	–
		–	100%	–	–	–	–	–
		–	1,3%	97%	–	–	–	–
		–	5%	–	80%	–	–	–
		–	10%	–	80%	–	–	–
		–	56%	26%	–	–	–	–
		–	93%	–	–	–	–	–
		–	28%	–	–	–	–	58%

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ME **1** is mostly converted to **2**, **3** and **4**. Aldehyde **2** is not converted by laccase – mediator system and recovered unchanged, which means that the benzoic derivative is not produced by our system. Compound **3** could not be oxidized by our system, ruling out that it could be an intermediate to **2**. Compound **4** remained mostly unconverted as well. Compound **5** could be oxidized to **4**, but only to a limited extent oxidized to **2**, ruling also out this pathway. Compound **6** was predominantly oxidized to **2** upon oxidative cleavage and to 26% of **3**. Compound **8** could be oxidized to **2** almost quantitatively and compound **9** was partially oxidized to **2** by cleavage.

Considering the data obtained from intermediates reactivity, a proposed mechanism was elaborated. **1** is oxidized at the benzylic position to **5** or both isomerized and oxidized to **6** in a concomitant manner. Then the oxidation of the alcohol function into aldehyde **3** is observed, or a direct oxidative cleavage to **2** happens. **5** could be oxidized to **4**, and these two last compounds could contribute to the formation **2** by oxidative cleavage.

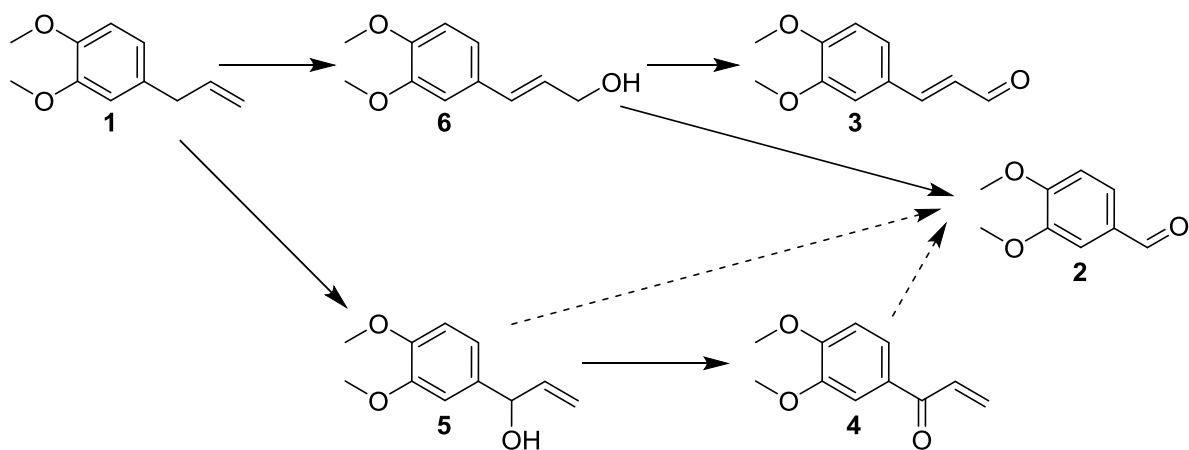


Figure 14. Proposed mechanism after data interpretation from intermediates reaction.

2.3 Scope and limitations on various allylbenzene derivatives

Reactivity of allylbenzene derivatives was investigated using commercially available substrates. Besides allylbenzene **11**, substituents on the benzenic ring exhibiting electron-withdrawing or electron-donating effects such as 1-allyl-4-(dimethylamino)benzene **12**, 1-allyl-4-methoxybenzene **13**, 1-allyl-2-methoxybenzene **14**, 1-allyl-4-(trifluoromethyl)benzene **15**, eugenyl acetate **16**, allylpentafluorobenzene **17**, anethol **18**, isoeugenyl acetate **19** and dillapiolene **20** were also tested.

Selectivity in terms of oxidation products was examined for various electronic effects (see SI). No clear trend could be identified and methyl eugenol **1** finally appeared as exhibiting the best compromise between overall electron density and electronic effects.

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5. Conclusions

The oxidation of allylbenzene derivatives by laccases and laccase-mediator systems was studied with a strong focus on methyl eugenol as model substrate. The reaction was found to be quite slow but total conversion could be obtained upon sequential addition of either laccase or mediator. The oxidation proceeded by a concerted isomerisation/hydroxylation of the propenyl side chain followed by two pathways, the former leading to cinnamaldehyde derivatives by further oxidation, and the latter to benzaldehyde derivatives by oxidative cleavage in a mild chemoenzymatic equivalent of ozonolysis. Alternatively, allylic hydroxylation could also be observed, marginally leading to cleavage products as well. The scope of the reaction was examined and was found to be dependent on substitution patterns and electronic effects; the best results being obtained with electron-rich methyl eugenol.

Supplementary Materials: Detailed spectral data, procedures and NMR spectra are available online at www.mdpi.com/xxx/s1.

Author Contributions: S.A. designed the study, M.L. performed the experiments, all authors discussed the results, S.A. and M.L. wrote the article. All authors have proofread and agreed to the published version of the manuscript.

Funding: This research was funded by CNRS, Université Cote d'Azur, and IFF-LMR.

Acknowledgments: ML's PhD fellowship is co-funded by IFF-LMR and French Région SUD. We are grateful to Thierry Tron and Pierre Rousselot-Pailley (ISM2, France) for a gift of pure laccase expressed in their laboratory, and Atsushi Tanaka (Amano Enzyme M120) for a loan of various laccase preparations.

Conflicts of Interest: The authors declare no conflict of interest.

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