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The Reactivity of Phenolic and Non-Phenolic Residual Kraft Lignin Model Compounds with Mn(II)-peroxidase from *Lentinula edodes*

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Abstract—Three phenolic model compounds representing bonding patterns of residual kraft lignin were incubated with manganese peroxidase from *Lentinula edodes*. Extensive degradation of all the phenolic models, mainly occurring via side-chain benzylic oxidation, was observed. Among the tested model compounds the diphenylmethane α-5 phenolic model was found to be the most reactive, yielding several products showing oxidation and fragmentation at the bridging position. The non-phenolic 5-5′ biphenyl and 5-5′ diphenylmethane models were found unreactive.

Introduction

Unbleached kraft pulp contains residual highly modified lignin. Removal of this residual lignin, to make a bleached white product, is commercially performed through multistage bleaching processes.[1,2] Environmental concerns have prompted the development of alternative totally chlorine-free (TCF) bleaching processes.[3] The use of enzymes as mild and environmentally benign bleaching agents is being considered by the pulp and paper industry.[3,4]

Among these are Mn-peroxidases from white-rot fungi and it has been shown that MnP is able to oxidize, demethylate and delignify kraft pulp.[5,6] This enzyme, which contains one iron protoporphyrin IX prosthetic group,[7] acts by oxidizing Mn(II) to Mn(III), which, in turn, if suitably chelated, is a freely diffusible oxidizing species with a high redox potential (Fig. 1).[7–10] In fact, chelation of Mn(III) by organic acids such as malonate and lactate stabilizes Mn(III) at a high redox potential (0.9–1.2 V).[10,11] The lignin oxidation process is thought to occur via the formation of a phenoxy radical through the oxidation of lignin phenolic subunits by Mn(III) chelates.[7–10]

Such mechanism hypothesis is strongly supported by several studies reported on lignin model compounds.[12,16] More specifically, MnP-catalyzed oxidation of phenolic arylglycerol β-aryl ether[14,15] and diarylpropane[12] dimeric lignin models has been extensively investigated. These dimeric products represent the most abundant lignin subunits. However, the residual lignin present in kraft pulps has a modified structure with significant amounts of condensed moieties such as 5-5′, α-5′ and diphenylmethane subunits.[17–20]

To date, there are no literature studies dealing with the behavior of MnP toward such lignin moieties, despite the fact that their presence is very significant within the kraft lignin backbone. The white-rot fungus *Lentinula edodes* has been reported to produce MnP both under solid state and submerged fermentation conditions.[16,21] In a previous paper we reported the isolation and purification of this enzyme from solid-state cultures of *L. edodes*.[21] We also reported the oxidation of veratryl alcohol by purified MnP.[21] The oxidation of such a non-phenolic lignin model compound has been also reported by other investigators.[16]

Keywords: Mn(II)-peroxidase; *Lentinula edodes*; lignin degradation; lignin models.

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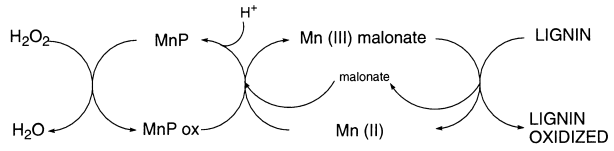


Figure 1. Catalytic cycle of manganese peroxidase. The resting enzyme is oxidized by H_2O_2 and in turn oxidizes Mn(II) to Mn(III). Mn(III), suitably chelated, performs the oxidation of lignin.

The aim of this work was to elucidate the reactivity of *L. edodes* MnP toward condensed lignin model compounds. A series of model compounds resembling the fundamental bonding patterns of residual kraft lignin, namely 5-5′-biphenyl, diphenylmethane and α-5 model compounds, were incubated with the enzyme isolated from *L. edodes*. To elucidate the ability of this enzyme in the oxidation of non-phenolic systems, both phenolic and non-phenolic model compounds were studied.

Results and Discussion

To test the capability of MnP in the oxidation of residual kraft lignin, three different phenolic model compounds were selected. The 5-5′ biphenyl dihydrocresol (**1**), 3,3′-dihydroxy-4,4′-dimethoxy-6,6′-dimethyl diphenylmethane (**2**), and the -5 model 2,4-dihydroxy-3,3′-dimethoxy-5-methyl diphenylmethane (**3**). MnP was also tested in the oxidation of the non-phenolic analogues of **1** and **2**, dihydromethyl veratrole (**4**) and 3,3′,4,4′-tetramethoxy-6,6′-dimethyl diphenylmethane (**5**), respectively.

Biodegradation of dihydrocresol (1)

When the biphenyl model compound **1** was submitted to MnP-catalyzed oxidation in the presence of hydrogen peroxide as an oxygen donor, extensive degradation was observed. After 7 h incubation only 17.2% of **1** was still present in the reaction mixture, as measured by GC quantitative analysis[22] (Table 1).

The oxidation products detected are reported in Scheme 1. Compounds **6**, **7** and **8** are all side-chain oxidation products to form benzyl alcohol, aldehyde and carboxylic acid respectively. Compound **6** was recovered as the main product in 63.8% yield (Table 1). Compounds **7** and **8** were recovered in 7.9 and 1.9% yield, respectively. In addition, trace amounts of a demethoxylation product, **9**, were also found (0.62% yield).

Biodegradation of 3,3′-dihydroxy-4,4′-dimethoxy-6,6′-dimethyl diphenylmethane (2)

In a similar way the diphenyl methane **2** was found almost completely degraded upon MnP/H_2O_2 oxidation during 7 h, the residual substrate recovery being 7.7% (Table 1). The metabolites detected were all side-chain oxidation products to benzyl alcohol (**10**, 0.30%) aldehyde (**11**, 0.34%), dialdehyde (**13**, 31.2%) and carboxylic acid (**12**, 30.4%) (Scheme 2).

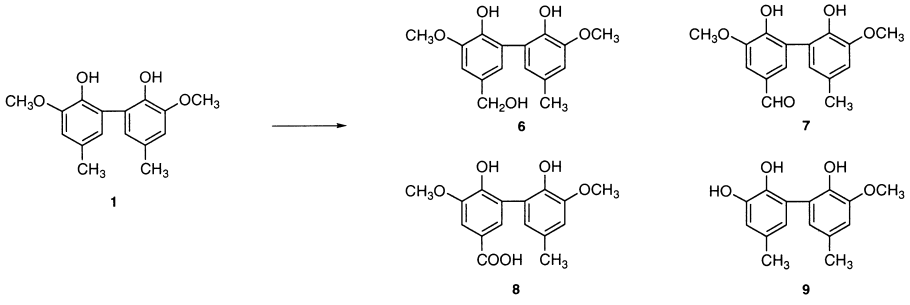
Carboxylic acid (**12**) and dialdehyde (**13**) were the main metabolites detected (31.2 and 30.4%, respectively), while **10** and **11** were present only in low amounts. Neither oxidation at the bridging carbon nor products coming from fragmentation in that position were detected.

Biodegradation of 2,4-dihydroxy-3,3(-dimethoxy-5-methyl diphenylmethane (3)

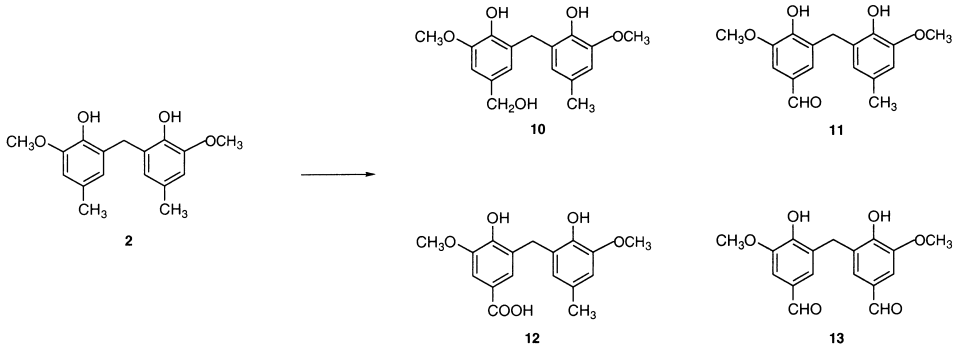
The α-5 model compound **3** was the most reactive residual kraft lignin substructure among the three tested, upon MnP/H_2O_2 oxidation. After 7 h incubation, only 1.1% of the initial product was recovered (Table 1). The isolated metabolites were again side-chain oxidation products (Scheme 3). More specifically, besides the formyl

Table 1. Substrate/product mass balance

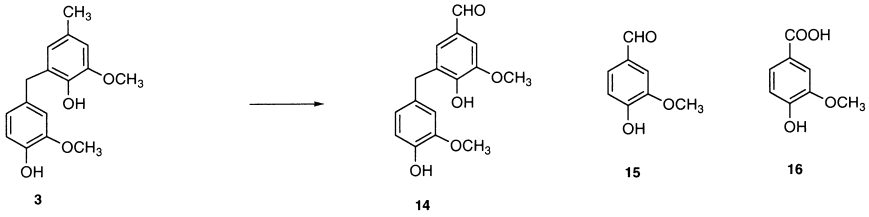
Compound	Conversion (%)	Products yield (%)	Unrecovered products (%)
1	82.8	**6** 63.8; **7** 7.9; **8** 1.9; **9** 0.6	8.6
2	92.3	**10** 0.30; **11** 0.34; **12** 31.2; **13** 30.4	30.06
3	98.9	**14** 0.30; **15** 1.1; **16** 2.6.	94.9
4	0	0	0
5	0	0	0



Scheme 1. Manganese peroxidase biodegradation of the 5-5′ condensed model dihydrocresol **1**.



Scheme 2. Manganese peroxidase biodegradation of the diphenylmethane condensed model 3,3′-dihydroxy-4,4′-dimethoxy-6,6′-dimethyl diphenylmethane **2**.



Scheme 3. Manganese peroxidase biodegradation of the α-5 condensed model 2,4-dihydroxy-3,3′-dimethoxy-5-methyl diphenylmethane **3**.

derivative (**14**) (0.3%), vanillin (**15**) (1.1%) and vanillic acid (**16**) (2.6%) were detected.

Compounds **15** and **16** are clearly products derived from the oxidation of **3** at the carbon bridging position and subsequent fragmentation. In principle the oxidation at the bridging position could yield 5-carbocyclic compounds. Under our experimental conditions, such products were not formed. This is in accord with the hydrogen peroxide oxidation pattern of the same product previously reported.[22] The regioselectivity displayed by the MnP/H_2O_2 oxidation of the diphenylmethane units is worth noting. The position *para* to the phenolic moiety appears to be more reactive. It could be hypothesized that in this case the lower inductive effect exerted by the OH group makes the carbon center more nucleophilic and lowers its redox potential. Moreover, spin density considerations taking into account the stability of the resulting radicals after cleavage at the benzylic carbon center indicate the same reaction pathway.[22]

Thus, in **2**, only the methyl groups are oxidized, while in **3**, where both the methyl and methylene groups are *para* to the OH, the oxidation occurs in both the positions. This behavior could account for the higher reactivity of α-5 versus 5-5′ units.

However, the higher susceptibility to oxidation of the bridging position of the α-5 substructure than of the diphenylmethane is in accord with a previous study on the degradation of residual kraft lignin models by laccase.[23] In that case, only the α-5 model compound yielded a bridge oxidation product. Also, more substrate was oxidized by MnP than by laccase under comparable reaction conditions.[23] Analogously, α-5 model compounds showed a somewhat higher reactivity than diphenylmethane (**2**) toward H_2O_2 oxidation in the presence of metal ions.[22]

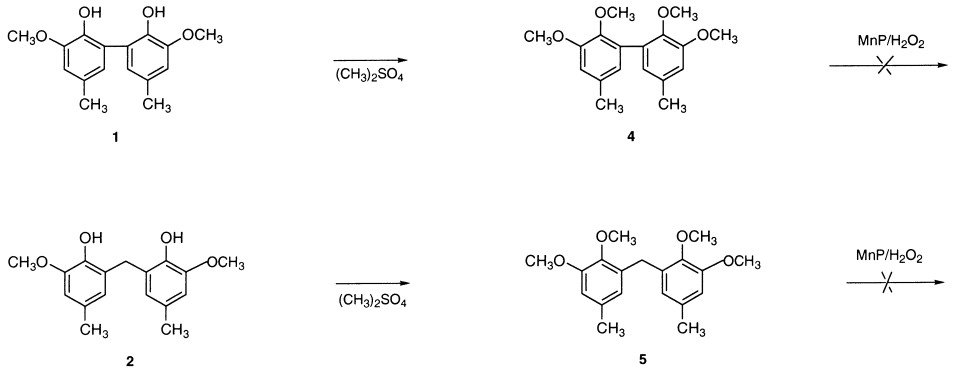
It is worth noting that the metabolites detected were in a low amount with respect to the initial substrate. This implies that extensive degradation may have occurred yielding the formation of water soluble fragments.

Oxidation of non-phenolic biphenyl and diphenylmethane model compounds 4 and 5

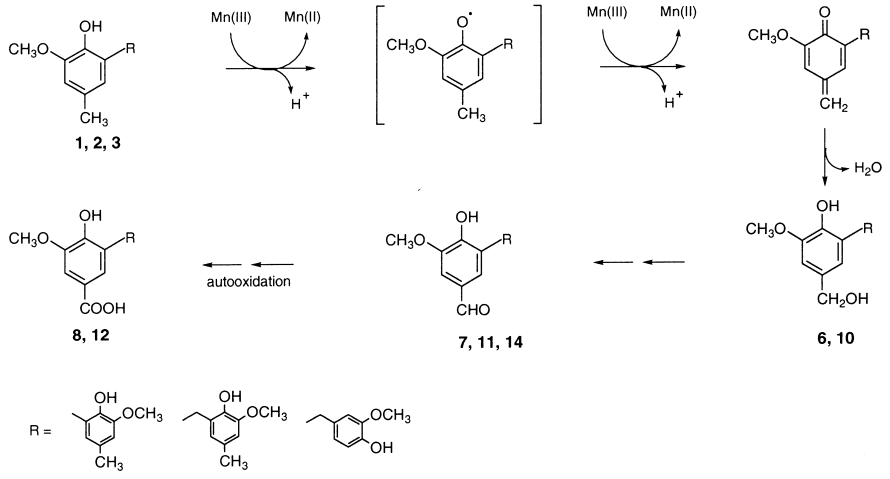
In order to verify the ability of MnP to oxidize non-phenolic residual kraft lignin model compounds, dihydrocresol (**1**) and 3,3′-dihydroxy-4,4′-dimethoxy-6,6′-dimethyl diphenylmethane (**2**) were methylated with dimethylsulfate under alkaline conditions (Scheme 4). The non-phenolic derivatives obtained **4** and **5**, respectively, were submitted to treatment with MnP/H_2O_2 under the same experimental conditions selected for **1**, **2**, and **3**. Compounds **4** and **5** were completely unreactive under these conditions; in fact, after 7 h incubation they were recovered unchanged from the reaction mixture (Table 1).

Conclusions

Phenolic residual kraft lignin model compounds were extensively oxidized by MnP/H_2O_2 treatment. In spite of different results obtained by MnP oxidation of poplar native lignin,[24] no quinones were found in the reaction medium. However, due to their volatility they could have been lost during the reaction work up.[24] The biphenyl model compound **1** showed the lowest reactivity among the tested models, being degraded at 82.8% extent. Diphenylmethane units **2** and **3** were found more reactive. Among these the α-5′ model compound **3** was oxidized at the benzylic bridging position yielding monomeric fragments. Only a small part of the products were detected by GC–MS techniques. This probably



Scheme 4. Synthesis and manganese peroxidase biodegradation of the non-phenolic 5-5′ biphenyl and diphenylmethane model compounds **4** and **5**.



Scheme 5. Proposed reaction pathway for the formation of side-chain oxidation products from condensed model compounds upon incubation with manganese peroxidase from *Lentinula edodes*.

implies the formation of small fragments from over-oxidation processes.

The formation of the products detected can be rationalized on the basis of the previously hypothesized mechanism for MnP.[7–16] The Mn(III) chelate generated by MnP is able to produce a phenoxy radical, in turn further oxidized to quinone methide intermediate which would undergo the formation of the side-chain oxidation products, as shown in Scheme 5.

In spite of the fact that *L. edodes* MnP is able to oxidize veratryl alcohol, the non-phenolic 5-5′ and diphenylmethane model compounds **4** and **5**, respectively, were found completely unreactive.

Experimental

^{1}H and ^{13}C NMR spectra were recorded on a Varian XL 300 spectrometer. Mass spectroscopy (MS) was performed with a Hewlett–Packard 5971 mass-selective detector on a Hewlett–Packard 5890 gas chromatograph. All solvents were ACS reagent grade and were redistilled and dried according to standard procedures. Chromatographic purifications were performed on columns packed with Merck silica gel 60, 230–400 mesh for flash technique. Thin layer chromatography was carried out using a Merck platten Kieselgel 60 F254.

Organism and culture conditions. *Lentinula edodes*, strain SC-495, was kindly provided from Professor T. H. Quimio (Los Banos, Philippine). The mycelium was maintained and routinely subcultured on potato-dextrose agar slants. The inoculum was prepared as previously described.[21] Solid-state fermentation was performed on corn straw (*Zea mais* L.) as previously described.[21]

Enzyme extraction, clarification and concentration. The colonized substrate (25-day-old cultures) was squeezed with the aid of a hydraulic press, generating a pressure of 200 atm, which was able to extrude around 75% of the fluid out of the substrate. The resulting liquid was filtered through glass wool and centrifuged at 11,000 *g* for 30 min. The pale-brown supernatant was clarified by polyethyleneimine (PEI) addition. Proteins in the PEI-clarified extract were precipitated by $(NH_4)_2SO_4$ addition up to 85% saturation and centrifuged (11,000 *g*, 30 min). The precipitate was resuspended in 10 mM

imidazole–HCl buffer pH 6.0 (buffer A), concentrated on a stirred cell, equipped with a Diaflo membrane (cut-off 10 KDa) and diafiltered against the same buffer. The retentate was stored at –20 °C in small aliquots for further purification.

Chromatographic fractionation. The retentate was applied to a 2.5 × 45 cm Q-Sepharose fast flow column, equilibrated with buffer A at a flow rate 2 mL/min, and washed with 2 bed volumes of starting buffer. Mn-peroxidase activity was eluted by a two-step NaCl gradient (0–0.4 M in 130 mL, 0.4–0.6 M in 180 mL). The pooled active fractions were concentrated, dialyzed against buffer A and loaded onto a 10 × 1.2 cm Concanavaline-A-Sepharose equilibrated with buffer A containing 0.5 M NaCl, 1 mM $MnCl_2$ and $CaCl_2$ at a flow rate 0.1 mL/min. The activity was eluted by a 15 mL gradient 0–0.2 M of α-D-methyl-mannopyranoside. The active fractions were pooled, dialifiltrated against buffer A and loaded onto a 8 × 1.5 cm column AH-Sepharose 4B equilibrated with bufffer A at a flow rate 0.2 mL/min and the enzyme was eluted by a NaCl gradient with the following program (0–0.3 M in 20 mL). The last chromatographic step was performed with a Sephacryl S-200 column equilbrated with imidazole buffer pH 6.0 at a flow rate of 0.6 mL/min. The column was calibrated with creatine phosphatase (81 KDa), bovine serum albumine (66 KDa) and ovalbumine (45 KDa).

Electrophoresis. Polyacrylamide (10%) sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) was performed according to the Laemmli method.[25] Molecular mass determination was performed by using the prestained low-molecular weight standards (Bio-Rad Laboratories, USA). Proteins were visualized by using the Coomassie Blue R-250 and/or the silver staining procedure.

Enzyme activity. Mn-peroxidase activity was assayed spectrophotometrically at 30 °C by the method of Waarishi et al.[26] The assay mixture (1 mL) contained 0.5 mM $MnSO_4$, 75 μM H_2O_2 in 50 mM malonate buffer at pH 4.5 and the formation of Mn(III)–malonate complex was kinetically followed at 270 nm (ε_{270} = 11,590 M^{-1} Cm^{-1}) One international unit (IU) was defined as the amount of enzyme required to produce one micromole manganic chelate per minute per milliliter. The activity of MnP was also assayed in the presence of dioxane. Assays of enzymatic activity of MnP kept 7 h in dioxane:water = 1:3 as a solvent mixture showed residual 90% activity. Protein was quantitated by the Bradford method using bovine serum albumin as standard.[27]

Residual kraft lignin model compounds. The model compounds **1**, **2** and **3** were synthesized by known methods.[28,29] Non-phenolic models dihydromethyl veratrole **4** and (3,3′4,4′-tetramethoxy-6,6′-dimethyl) diphenylmethane **5** were synthesized by methylating the corresponding phenols **1** and **2** using dimethylsulfate (Me_2SO_4) under alkaline conditions.[22] Phenols **1** and **2** (0.1 mol) were dissolved in 10 mL of NaOH 30% (aqueous solution) under magnetic stirring, and Me_2SO_4 (0.2 mol) was added at 60 °C during 1 h. After 2 h the reaction mixtures were cooled, neutralized with ammonium chloride, and extracted with ethyl acetate. The organic solvent was evaporated under reduced pressure, and the crude products were crystallized from hexane–ethyl acetate to give **3** and **4** in quantitative yield.

Dihydromethyl veratrole 3. ^{1}H NMR ($CDCl_3$) δ 2.33 (s, 6H, CH_3), 3.64 (s, 6H, OCH_3), 3.88 (s, 6H, OCH_3), 6.64–6.74 (m, 4H, CH); ^{13}C NMR ($CDCl_3$) δ 21.26 (CH_3), 55.79 (OCH_3), 60.64 (OCH_3), 112.58 (CH), 123.53 (CH), 132.59 (C) 132.81 (C), 144.60 (C), 152.30 (C). Mass spectrometry data are reported in Table 2.

(3,3′4,4′-Tetramethoxy-6,6′-dimethyl) diphenylmethane 4. ^{1}H NMR ($CDCl_3$) δ 2.24 (s, 6H, CH_3), 3.76 (s, 6H, OCH_3), 3.85 (s, 6H, OCH_3), 3.96 (s, 2H, CH_2), 6.52–6.60 (m, 4H, CH); ^{13}C NMR ($CDCl_3$) δ 21.30 (CH_3), 29.07 (CH_2), 55.62 (OCH_3), 60.37 (OCH_3), 111.21 (CH), 122.87 (CH), 133.29 (C) 134.46 (C), 144.88 (C), 152.66 (C). Mass spectrometry data are reported in Table 2.

Oxidation of residual kraft lignin model compounds. Due to the low solubility of kraft lignin model compounds in water, their oxidation was performed in a 1:3 (v/v)

Table 2. Mass spectrometric data

Product	Derivative	MS (*m*/*z*) data (%)
1	—[a]	274 (M, 100), 241 (24), 227 (21), 199 (19)
1	-Si$(CH_3)_3$[b]	418 (M, 38), 403 (M-15, 18), 388 (M-30, 26), 287 (14), 179 (11), 73 (100)
2	—	288 (M, 84), 255 (21), 241 (8), 151 (33), 138 (100), 123 (7)
2	-Si $(CH_3)_3$	432 (M, 56), 417 (M-15, 12), 402 (M-30, 24), 73 (100)
3	—	274 (M, 95), 241 (12), 151 (28), 150 (100), 149 (22), 124 (18), 121 (13)
3	-Si $(CH_3)_3$	419 (M + 1, 26), 418 (M, 100), 403 (M-15, 26), 388 (M-30, 91), 358 (23), 343 (18), 179 (20), 73 (81)
4	—	302 (M, 100), 287 (M-15, 16), 272 (M-30, 21) 256 (93), 241 (25), 213 (23), 128 (28)
5	—	316 (M, 100), 301 (M-15, 11), 269 (23), 255 (21), 165 (26), 151 (99), 135 (78)
6	-Si $(CH_3)_3$	506 (M, 28), 329 (100), 147 (8), 73 (87)
7	-Si $(CH_3)_3$	432 (M, 21), 417 (M-15, 26), 301 (8), 73 (100)
8	-Si $(CH_3)_3$	520 (M, 8), 431 (5), 417 (3), 343 (32), 269 (6), 147 (5), 73 (100)
9	-Si $(CH_3)_3$	476 (M, 16), 373 (7), 358 (10), 147 (14), 73 (100)
10	-Si $(CH_3)_3$	520 (M, 9) 505 (M-15, 5), 417 (7), 343 (22), 73 (100)
11	-Si $(CH_3)_3$	446 (M, 28), 431 (M-15, 62), 357 (6), 269 (8), 179 (8), 73 (100)
12	-Si $(CH_3)_3$	534 (M, 14), 519 (11), 415 (10), 357 (24), 297 (10), 147 (12), 73 (100)
13	-Si $(CH_3)_3$	460 (M, 12), 446 (18), 445 (71), 371 (8), 73 (100)
14	-Si $(CH_3)_3$	432 (M, 22), 414 (M-15, 13), 402 (M-30, 16), 193 (16), 73 (100)

[a]Underivatized.
[b]Trimethylsilylated with *N,O*-bis(trimethylsilyl)-acetamide.

dioxane:water solvent mixture. 5 mM of substrate dissolved in dioxane: malonate buffer pH 4.5 0.05 M (1 mL, 1:3 v/v), in the presence of $MnSO_4$ (1 mM), were treated with purified manganese peroxidase isolated from *L. edodes* 2.5 U/mL and 75 M H_2O_2 at 35 °C. Every 2 h a new batch of H_2O_2 was added. After 7 h the reactions were halted by heating the mixtures in boiling water. A suitable amount of 4-propyl benzoic acid acting as internal standard was then added. The reaction mixture was diluted with water and extracted in ethyl acetate. The organic layer was separated, dried over anhydrous $MgSO_4$ and the organic solvent was evaporated under reduced pressure. In order to analyze the reaction products the residues were dissolved in 1 mL of pyridine and submitted to silylation by addition of *N*,*O*-bis(trimethylsilyl)-acetamide. After 30 min the mixtures were subjected to gas chromatography (GC) and gas chromatography–mass spectrometric (GC–MS) analyses.

Characterization of metabolites. Gas chromatography and gas chromatography–mass spectrometry of the reaction products were performed using a DB1 column (30 m × 0.25 mm and 0.25 mm film thickness), and an isothermal temperature profile of 100 °C for the first 2 min, followed by a 20 °C/min temperature gradient to 300 °C and finally an isothermal period at 300 °C for 10 min. The injector temperature was 280 °C. Chromatography grade helium was used as the carrier gas. The fragmentation patterns (Table 2) were compared to those of authentic samples.[21,28–34]

Acknowledgements

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