Lignin peroxidase initiates O₂-dependent self-propagating chemical reactions which accelerate the consumption of 1-(3',4'-dimethoxyphenyl)propene

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Lignin peroxidase (LiP) has been used to study the C_α–C_β cleavage of the propylene side chain in 1-(3',4'-dimethoxyphenyl)propene (DMPP) to 3,4-dimethoxybenzaldehyde (veratraldehyde, VAD). Under an air atmosphere, LiP oxidized DMPP to VAD (27.8 %) and 1-(3',4'-dimethoxyphenyl)propan-2-one (DMPA, 8.7 %), after 10 min of incubation. Dissolved O₂ was rapidly consumed during DMPP conversion, of which one-third was converted into superoxide. The remaining two-thirds of the consumed O₂ was involved in C_α–C_β cleavage of DMPP to VAD and in self-propagating chemical reactions stimulating the consumption of DMPP. The involvement of peroxy radicals, in the chemical consumption of DMPP, was confirmed by using the well-known peroxy radical reductant Mn²⁺. This metal ion severely inhibited the DMPP consumption rate under air, but did not affect the lower enzymic DMPP consumption rate under N₂. The substoichiometric requirement of LiP for H₂O₂ during DMPP oxidation could be explained in part by dismutation of

superoxide, but more importantly by direct chemical reactions of DMPP-derived peroxy radicals with fresh DMPP. Another VAD-producing route was found by incubating the DMPP oxidation product, DMPA, with LiP. Under air the molar yield of VAD was 29.7 %. In the absence of O₂, the C_α–C_β cleavage of DMPA to VAD was strongly inhibited and side-chain coupling products (dimers) were formed instead. As a whole, the results suggest two new roles of O₂ in LiP-mediated oxidation of aromatic substrates. First, O₂ is responsible for the formation of reactive peroxy intermediates, which can directly react with other substrate molecules and thereby accelerate consumption rates. Secondly, O₂ can prevent coupling reactions by lowering the pool of carbon-centred radicals accumulating during LiP catalysis.

Key words: autocatalysis, C_α–C_β cleavage, manganese, peroxy radicals, white-rot fungi.

INTRODUCTION

Lignin is a substituted phenylpropanoid polymer that is most extensively decomposed by white-rot fungi. These fungi excrete several peroxidases that depolymerize lignin by oxidation of the phenyl moieties leading to the splitting of β-O-4 linkages, and C_α–C_β bonds in the side chain. Lignin peroxidase (LiP) is largely responsible for these reactions [1], which could be deduced from the oxidation products derived from small aromatic compounds [1,2]. As a model compound for commonly occurring plant phenolics, the methyl ether of isoeugenol, 1-(3',4'-dimethoxyphenyl)propene (DMPP) and the corresponding acetyl ester [3,4] have been used. Among the DMPP oxidation products was veratraldehyde (VAD), indicating that LiP catalysed the C_α–C_β cleavage reaction of the propenyl side chain [3].

It is generally accepted that LiP oxidizes aromatic compounds by one-electron to the corresponding radical cation [1,5,6] which may undergo various non-enzymic reactions such as addition of water [1,7] or splitting off a proton to form a radical [1]. The formation of a radical enables various additional reactions to occur, such as addition of superoxide (HOO[•]) [1,8,9], radical coupling [10] or peroxy-radical formation owing to the reaction with O₂ [11]. These reactions are mainly responsible for the large variety of oxidation products.

In the present paper we describe the rapid consumption of O₂ during DMPP consumption by LiP. Some of the consumed O₂ was converted into superoxide, but the predominant part was

used in product formation, such as the C_α–C_β cleavage reaction to VAD. Therefore the role of O₂ was studied extensively, leading us to the novel finding that LiP initiates O₂-dependent self-propagating chemical reactions, which greatly stimulated substrate consumption. The involvement of peroxy radicals in this chemical-consumption pathway was inferred by the inhibitory effect of the well-known peroxy radical scavenger Mn²⁺ [11]. Furthermore, the present paper presents the first clear evidence that O₂ is important in overcoming coupling of LiP-generated carbon-centred radicals. Coupling of such radicals contributes to the repolymerization of lignin fragments.

MATERIALS AND METHODS

Chemicals

Veratryl alcohol (VA), VAD, 1-(3',4'-dimethoxyphenyl)propan-2-one (DMPA), DMPP and tetranitromethane (TNM) were obtained from Aldrich (Bornem, Belgium).

Incubation conditions

Throughout the experiments the purified LiP isoenzyme-2 from the white-rot fungus *Bjerkandera* sp. strain BOS55 was used [13]. The substrate, DMPP, was added from a 40.4 mM stock solution prepared in acetone. A standard incubation mixture (total volume

Abbreviations used: ABTS, 2,2'-azino-bis-(3-ethylbenzo-6-thiazolinesulphonic acid); DMPA, 1-(3',4'-dimethoxyphenyl)propan-2-one; DMPP, 1-(3',4'-dimethoxyphenyl)propene; DMPDD, 1-(3',4'-dimethoxyphenyl)propane-1,2-dione; IEA, isoeugenyl acetate; LiP, lignin peroxidase; SOD, superoxide dismutase; TNM, tetranitromethane; t_r, retention time; VA, veratryl alcohol; VAD, veratraldehyde.

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250 μ l) contained 0.25 mM H_2O_2 and 0.48 mM DMPP. Various buffers and LiP concentrations were used and these are described for each experiment. Each reaction was initiated by the addition of LiP and each experiment performed at least twice. At various incubation times the reaction was stopped by the addition of 500 μ l of acetonitrile. Control experiments were incubated for the maximal duration of the experiment and did not contain H_2O_2 or active LiP (inactivated by boiling).

The substoichiometric requirement for H_2O_2 was studied using 100 mM sodium tartrate, pH 3.0, containing 0.57 μ M LiP, and experiments were performed in duplicate at room temperature. The incubation time was 10 min.

In an experiment studying the O_2 and DMPP consumption in relation to superoxide and VAD production, the reaction was performed at 30 °C in 100 mM sodium succinate, pH 3.0, containing 0.053 μ M LiP. The experiments were performed in duplicate and stopped after 20, 40, 60, 90, 120, 150, 180, 240, 300 and 420 s.

The effect of Mn^{2+} under air was studied at room temperature using 100 mM sodium lactate, pH 3.0/4.0, containing 0.085 μ M LiP. Initial turnovers were calculated by using data from duplicate incubations stopped after 20, 40, 60, 80 and 100 s. The molar VAD yield, found at the Mn^{2+} concentrations studied, is presented as the average for these five duplicate experiments.

In an experiment studying the effect of complete anaerobic conditions on the reaction without Mn^{2+} , the experiment was performed in an anaerobic hood (atmosphere 96% N_2 /4% H_2). This experiment was performed in triplicate in 100 mM succinate buffer, pH 3.0, containing 0.085 μ M LiP. The reaction was stopped after 100 s.

The effect of Mn^{2+} was also studied in the anaerobic hood. This experiment was performed in duplicate in 100 mM lactate buffer, pH 3.0, containing 0.085 μ M LiP. The reaction was stopped after 100 s.

The effect of 1500 units of superoxide dismutase (SOD) from bovine erythrocytes (Sigma, Zwijndrecht, The Netherlands) was studied using 100 mM sodium lactate, pH 3.0/4.5. The incubation time was 100 s. After terminating the reaction, SOD was precipitated by the addition of acetonitrile. Samples were then subjected to centrifugation (10000 *g*) prior to HPLC analysis.

HPLC analysis

Samples were analysed using a HPLC ChemStation (Hewlett-Packard, Waldbronn, Germany) equipped with a HP1100 pumping station, an HP104M series II photodiode array detector and a HP9000-300 data processor. HPLC chromatograms were recorded at 215, 280 and 310 nm. A reversed-phase C_{18} column (200 mm long $\times$ 3 mm, int. diam., filled with ChromoSpher C_{18} -PAH 5 μ m diameter particles; Chrompack) was used. Compounds were eluted using a linear water/acetonitrile gradient, which started at 10% acetonitrile and increased to 100% acetonitrile in 15 min. The flow rate was 0.4 ml/min and the temperature was held constant at 30 °C. VAD and DMPP concentrations were determined using an external standard.

GC-MS conditions and preparation of the samples

For analysis of oxidation products, incubations were performed on a 2.5 ml scale in a test tube, applying the following conditions: 100 mM sodium succinate, pH 3.0, containing 0.085 μ M LiP, 0.25 mM H_2O_2 and 0.48 mM DMPP or 0.50 mM DMPA. The acetone content was 1.2% (v/v). After incubation for 10 min, the reaction was stopped with 5 ml of 15 mM vitamin C. Thereafter, the internal standard, 3-fluoro-4-methoxybenzal-

dehyde, was added. The mixture was then extracted twice with ethyl acetate. The combined organic layers were dried using 100% NaCl and, after separation, concentrated under a N_2 stream. Control experiments were performed using boiled LiP.

GC-MS analyses were carried out on a HP5973 quadrupole MS instrument coupled to a HP6890 gas chromatograph (Hewlett-Packard), equipped with a fused silica capillary column (HP-5MS; 30 m long $\times$ 0.25 mm int. diam.; film thickness 0.25 μ m). The carrier gas was helium and the flow rate was 1.0 ml/min. An injection temperature of 220 °C was used. The temperature program started at 70 °C and rose to 250 °C at a rate of 7 °C/min. The final temperature was maintained for 5 min. Fragment ions were obtained at an electron impact of 70 eV.

The formation of VAD and DMPA was confirmed by comparing the retention times (GC) and mass spectra with those of VAD and DMPA standards. Molar yields were calculated as mol of product formed/mol of DMPP converted.

Superoxide assay

The formation of the yellow trinitromethane anion, formed as a reaction product from TNM and superoxide, was measured spectrophotometrically at a wavelength of 350 nm (ϵ 13 500 $\text{M}^{-1}\cdot\text{cm}^{-1}$ [14]) at 30 °C.

Measurement of O_2

An incubation mixture containing 100 mM sodium succinate, pH 3.0, 0.25 mM H_2O_2 , 0.48 mM DMPP and 0.053 μ M LiP was prepared (total volume 3 ml). The O_2 consumption was measured at 30 °C using a YSI model 53 oxygen monitor (Yellow Springs Instrument Co. Inc., Yellow Springs, OH, U.S.A.).

Determination of the residual LiP activity

In order to study whether the presence of Mn^{2+} caused LiP inactivation, the residual LiP activity was measured after 100 s of incubation. This was done by transferring 100 μ l of sample from a standard incubation mixture to a mixture containing 50 mM succinate, pH 3.0, 0.5 mM H_2O_2 , and 4 mM 2,2'-azinobis(3-ethylbenzo-6-thiazolinesulphonic acid) (ABTS). The colouring of ABTS was measured spectrophotometrically at a wavelength of 420 nm at room temperature.

Mathematical procedure

When DMPP consumption was measured during the reaction, a curve was obtained. An equation of the type $y = ax^3 + bx^2 + cx + d$ was used to describe the curve. The initial DMPP consumption rate was obtained by substituting the value 0 for x in $y' = 3ax^2 + 2bx + c$.

Statistical procedure

Average values $\pm$ S.E.M. are presented.

RESULTS

Consumption of DMPP by LiP

LiP directly oxidized DMPP at a very high maximal velocity (V_{max}) of 130 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ (units $\cdot\text{mg}^{-1}$) in 100 mM sodium lactate buffered at pH 3.0. This value corresponds with a turnover frequency of 89 s^{-1} . The K_m for DMPP was 0.25 mM. VA was not required for DMPP consumption.

Scheme 1 Proposed reaction mechanism involved in the formation of VAD from DMPP catalysed by LiP

For details see text.

Figure 1 Conversion of DMPP (●) and the molar VAD yield (■) observed under air or in the complete absence of O_2 (○, □)

The experiment was performed in triplicate in 100 mM succinate buffer, pH 3.0, at room temperature.

GC-MS analysis of an ethyl acetate extract obtained from an incubation conducted under an air atmosphere revealed the presence of five discernible oxidation products. The two most predominant products (91.6% of the product peak area) were identified as VAD (**4** in Scheme 1) and DMPA (**2** in Scheme 1) and these were formed in molar yields of 27.8 and 8.1% respectively. The products not accounted for by GC-MS analysis (approx. 60%) were probably not amenable to GC analysis.

The HPLC method indicated that additional products eluted from the column after DMPP [retention time (t_r) 14.6 min] between 15 and 19 min. By acidifying the eluent (water) to pH 3.0, a total of 14 compounds were clearly detected in this region. Together these accounted for 35% of the product peak area detected at a wavelength of 215 nm. This study focuses on the GC-MS-amenable compounds.

O_2 uptake during DMPP consumption

At a LiP concentration of $0.053 \mu\text{M}$ the initial consumption rate of $0.35 \text{ nmol} \cdot \text{s}^{-1}$ is about 3-fold higher than the formation rate of VAD ($0.11 \text{ nmol} \cdot \text{s}^{-1}$). Simultaneously, uptake of O_2 occurred

Figure 2 Ratio between DMPP consumed and H_2O_2 initially added, and the molar VAD yield at various H_2O_2 concentrations

(A) Shows the ratio between consumed DMPP (nmol) and initially added H_2O_2 (●) by LiP (0.57 μM). (B) shows the DMPP conversion (●) and the molar VAD yield (○). The experiment was performed in 100 mM sodium tartrate buffer, pH 3.0, at room temperature. The total reaction time was 10 min, and the initial DMPP amount was 121 nmol (0.48 mM).

at an initial rate of $0.21 \text{ nmol} \cdot \text{s}^{-1}$. O_2 was clearly involved in the formation of DMPP oxidation products, as demonstrated by the formation of $\text{HOO}^\bullet$, which was produced at a rate of $0.12 \text{ nmol} \cdot \text{s}^{-1}$, as determined by the TNM colour test [14]. Because O_2 is consumed faster than $\text{HOO}^\bullet$ is formed, incorporation of O_2 into DMPP-derived oxidation products would probably account for the difference. When incubations were carried out under completely anaerobic conditions, the molar VAD yield was, at best, twofold lower than the molar yield under an air atmosphere (see Figure 1). This stressed the importance of O_2 for $\text{C}_\alpha\text{--C}_\beta$ cleavage. Also, the rate of DMPP consumption was clearly inhibited by the absence of O_2 . The possible involvement of $\text{HOO}^\bullet$ in VAD formation was studied by introducing superoxide scavengers [15] such as SOD into the incubation mixture. The presence of 1500 units of SOD did not affect the conversion of DMPP or the molar VAD yield (results not shown). The same effect was observed at pH 4.5 (results not shown), indicating that $\text{HOO}^\bullet$ was not involved in the $\text{C}_\alpha\text{--C}_\beta$ cleavage mechanism to VAD.

Figure 3 Turnover frequency of LiP during DMPP consumption and the molar VAD yield at various Mn^{2+} concentrations

(A) Shows the observed turnover frequency (s^{-1}) of LiP (0.085 μM) in the presence of DMPP (0.48 mM) at various Mn^{2+} concentrations. (B) shows the average ($n = 5$) molar VAD yield (%) at various Mn^{2+} concentrations. The experiment was performed in 100 mM lactate buffer, pH 3.0, at room temperature.

Effect of H_2O_2 concentration

It is well known that $\text{HOO}^\bullet$ rapidly dismutates at low pH forming H_2O_2 and O_2 [15]. This possibility was studied by introducing various amounts of H_2O_2 and allowing the reaction to run until completion. Figure 2(A) shows that the requirement for H_2O_2 was substoichiometric, as evidenced by the maximal consumption of 13 mol of DMPP/mol of H_2O_2 , which indicated that H_2O_2 was produced endogenously. Figure 2(B) shows that the molar VAD yield increases from 27% in the presence of 6.5 nmol H_2O_2 (0.026 mM) to 40% in the range from 62.5 to 125 nmol H_2O_2 (0.25–0.5 mM).

Effects of Mn^{2+}

An interesting observation was that the presence of Mn^{2+} caused a large inhibitory effect on the initial DMPP consumption rate and the molar yield of VAD. Since SOD had no inhibitory effect, the inhibition caused by Mn^{2+} could not be attributed to its $\text{HOO}^\bullet$ -scavenging activity. This finding was studied in more detail, since no comparable effect of Mn^{2+} on a LiP-catalysed reaction has been reported up until now. Figure 3(A) shows that

Table 1 The effect of several incubation conditions on DMPP conversion and the molar VAD yield

The experiments were performed in duplicate in 100 mM sodium lactate buffer, pH 3.0, at room temperature and incubated for 100 s.

Condition	Normalized to the air incubation (%)	
	DMPP conversion	Molar VAD yield
Air	100	100
Air + 1 mM Mn ²⁺	56	45
Anaerobic hood	48	54
Anaerobic hood + 1 mM Mn ²⁺	41	22

the presence of Mn²⁺ caused a sharp decrease in the observed turnover frequency, in the concentration range from 0 to 0.5 mM. From there on, only a slight decrease was noted. At a pH value of 3.0, 1 mM Mn²⁺ decreased the observed turnover frequency by a factor of 2.3. At pH 4.0, this factor was 3.8. The nature of this inhibition was studied and was found to be non-competitive as evidenced by an unchanged K_m value and a decreased V_{max} in the presence of Mn²⁺ (results not shown). To study whether the presence of Mn²⁺ caused inactivation of LiP, the residual activity in samples incubated for 100 s with and without 1 mM Mn²⁺ was measured. Inactivation of LiP could not be attributed to Mn²⁺ (results not shown). If instead of DMPP, VA (0.1–2 mM) was used as a substrate for LiP, no inhibition of VA oxidation was observed at either pH 3.0 or 4.0 in the presence of Mn²⁺ (2 mM).

Table 1 shows the effect of Mn²⁺ on the DMPP consumption rate in the presence and in the absence of air. Mn²⁺ inhibits the DMPP consumption in the air incubation, reducing the rate to a level which is comparable with that observed under anaerobic conditions. When the effect of Mn²⁺ was studied under anaerobic conditions, only very little inhibition of the DMPP consumption was observed. Therefore Mn²⁺ eliminated the stimulating effect of O₂ on the DMPP consumption. Mn²⁺ (1 mM) also affected the molar yield of VAD. The yield decreased by 50–60 % under both aerobic and anaerobic incubation conditions (see Table 1 and Figure 3B).

DMPA as an intermediate VAD precursor

Since DMPA was an intermediate of DMPP oxidation, it was tested as a substrate for LiP. Upon incubating DMPA (**2** in Scheme 1) with LiP for a total reaction time of 10 min under air, 66.7 % of the added DMPA was converted. GC–MS analysis showed that VAD was formed at a molar yield of 29.7 %. Table 2 shows that some additional side products were also formed under aerobic conditions. The most pronounced side product could be tentatively identified as 1-(3',4'-dimethoxyphenyl)propane-1,2-dione (DMPPD; **3** in Scheme 1) on the basis of its fragmentation pattern and by comparison with mass spectra in the NIST98 mass-spectral database. As shown in Table 2, product formation was clearly different under anaerobic (N₂ flushing) conditions. In this case, two DMPA dimers with a molecular ion ($[M]^+$) at an m/z of 386 were the predominant products, whereas VAD and DMPPD were only minor products. The dimers (**5** in Scheme 1), formed in a 1.96 (t_r 27.5 min) to 1 (t_r 29.9 min) ratio showed almost identical mass spectra, indicating that they were stereoisomers. The predominant fragmentation peak at an m/z of 193 is rationalized by $[M - (DMPA - H^+)]^+$, suggesting that these dimers are side-chain coupling products of DMPA.

DISCUSSION

This study on the oxidation of DMPP by the LiP isoenzyme-2 from *Bjerkandera* sp. strain BOS55 shows that the C_α–C_β cleavage reaction involved in VAD formation strongly depends on the presence of O₂. This finding was comparable with data on the VA-mediated oxidation of 1-(4'-acetoxy-3'-methoxyphenyl)propene (isoeugenyl acetate; IEA) [4]. However, several roles of O₂ in the reaction mechanism were clearly distinct in the case of DMPP. O₂ consumption resulted in the formation of superoxide and reactive peroxy-radical intermediates. The involvement of the latter intermediates in self-propagating chemical reactions accelerating the rate of DMPP consumption was inferred by inhibition studies with Mn²⁺.

Formation of superoxide

The colour reaction of TNM during consumption of DMPP under an air atmosphere clearly demonstrates HOO[•] formation.

Table 2 GC–MS analysis of compounds formed from DMPA under an air or N₂ atmosphere and the main fragment ion peaks in the MS spectrum of the tentatively identified compounds

The experiment was performed in 100 mM succinate buffer, pH 3.0, containing 0.085 μM LiP.

Compound	Product peak area (%)		m/z (electron-impact MS) of tentatively identified compounds
	Air	N ₂	
VAD	61.5	5.3	—
DMPPD	19.9	2.0	208 (2), 165 (100), 137 (10), 122 (9), 107 (6)
Dimer (t_r 27.5 min)	0	53.8	386 (8), 343 (2), 300 (6), 286 (10), 193 (100), 165 (19), 162 (15), 43 (23)
Dimer (t_r 29.9 min)	0	27.5	386 (6), 343 (3), 300 (5), 286 (8), 193 (100), 165 (23), 162 (15), 43 (22)
Unknown*	18.6	11.4	—

* Includes unknown compounds that are not shared.

The unstable $\text{HOO}\cdot$ may have dismutated, forming O_2 and H_2O_2 [15]. Endogenous H_2O_2 production may explain in part the substoichiometric requirement for H_2O_2 during DMPP consumption, as depicted in Figure 2(A).

During consumption of IEA, $\text{HOO}\cdot$ was only formed at an extremely low rate [4], whereas it was robustly formed during DMPP consumption. This difference is explained by the electron-withdrawing effect of the acetoxy group in IEA, which is not favourable for the elimination of $\text{HOO}\cdot$. In the case of DMPP, $\text{HOO}\cdot$ production does occur, owing to the electron-donating methoxy group (attached to C-4) which enhances the conversion into enol **1c** (Schemes 1 and 2).

Inhibition studies with Mn^{2+} that indicate the involvement of peroxy radicals

Mn^{2+} was found to inhibit both the DMPP consumption rate and the molar yield of VAD. This is unusual, since, in the case of the well-studied LiP substrate VA, addition of Mn^{2+} caused no inhibition (the present study) or even enhanced conversion of VA [8,15].

Mn^{2+} may play several roles in inhibiting LiP-catalysed reactions. It may reduce peroxy radicals to their corresponding hydroperoxides [11], it may function as a superoxide scavenger [15] or it may function as an alternative reducing substrate for LiP compound 1 [16]. The later two roles were excluded by experiments on the effect of SOD and the effect of Mn^{2+} on the VA oxidation rate respectively.

The reaction between Mn^{2+} and superoxide yields extra H_2O_2 [15]. An excess of H_2O_2 may inactivate LiP [1]. This, however, was not the case here, since the residual LiP activity was unaffected in the presence of 1 mM Mn^{2+} .

The inhibitory effect of Mn^{2+} on DMPP consumption was almost exclusively evident in the presence of O_2 . The extent of the inhibition reduced the activity of LiP to levels that were observed under anaerobic conditions. These observations point to Mn^{2+} -mediated reduction of intermediate peroxy radicals. These are formed owing to the extremely fast reaction between O_2 and an organic radical [1,11]. Also, the O_2 balance indicates the consumption of O_2 by DMPP-derived radical intermediates.

Intermediate peroxy radicals may undergo direct chemical reactions with DMPP such as hydrogen abstraction or addition to double bonds, ultimately regenerating the radical (self-propagating chemical reactions). In support of this hypothesis, Kapich et al. [17] demonstrated that chemical systems in which peroxy radicals are generated can directly oxidize non-phenolic lignin model compounds. Furthermore, the extreme ratio of 13 mol of DMPP consumed/mol of H_2O_2 cannot fully be accounted for by the dismutation of superoxide. The involvement of reactive intermediates directly oxidizing DMPP via self-propagating reactions would account for the difference.

The described free-radical reactions are well-known to be terminated by Mn^{2+} , since it reduces the peroxy radical to the corresponding hydroperoxide, overcoming further self-propagation chemical reactions [11]. The occurrence of these chemical reactions in the presence of O_2 results in a faster net consumption of DMPP, because it is consumed both chemically and enzymically. In other words, the presence of Mn^{2+} shifts the reaction more towards enzymic consumption of DMPP. In comparison with previously conducted research on the consumption of IEA, neither anaerobic conditions nor Mn^{2+} affected the IEA consumption rate. This indicated that IEA was solely consumed via an enzymic process.

Mn^{2+} was also found to inhibit the molar VAD yield (mol of VAD/mol of DMPP converted). Because Mn^{2+} terminates the

Scheme 2 The methoxy present at C-4 stimulates the elimination of superoxide owing to the resonance-stabilized intermediate cation derived from **1b**

discussed self-propagating chemical reactions it can be assumed that VAD has also been formed as a result of these reactions. A possible VAD-producing route owing to these reactions is discussed in the following section.

Proposed reaction mechanism involved in VAD formation

The data on DMPP suggest that the $\text{C}_\alpha\text{--C}_\beta$ cleavage mechanism is distinct from that proposed for 1-(4'-ethoxy-3'-methoxyphenyl)propene [2,12], since there is no involvement of a diol intermediate. None of the observed product peaks showed fragment ion peaks at an m/z of 212, 167 or 139, which would be expected in the case of a diol intermediate. Instead a major intermediate appeared to be DMPA.

A $\text{C}_\alpha\text{--C}_\beta$ cleavage route of DMPP is depicted in Scheme 1. DMPP (**1**) is oxidized to a radical cation intermediate (**1a**), which may react with water and O_2 to form a peroxy radical (**1b**). This radical may split off superoxide (see Scheme 2), giving the resonance-stabilized enol (**1c**) which is in equilibrium with DMPA (**2**), which we showed is used again as a substrate for LiP. DMPA is oxidized by LiP to $\text{DMPA}^{\cdot+}$. After splitting off a proton, $\text{DMPA}^{\cdot-}$ (**2a**) is formed. The latter radical may dimerize in O_2 -deficient conditions to the tentatively identified stereoisomers with structure **5**. This finding indicates that an adequate supply of O_2 is essential in preventing coupling reactions of carbon-centred radicals. The presence of O_2 results in the formation of a peroxy radical. By a radical disproportionation reaction the intermediate hydroperoxide (**2b**) is formed. Hydroperoxides have been proposed to be intermediates in the formation of side oxidation products from VA [1,8], but have also been detected as LiP oxidation products of azo dyes [18]. Spontaneous heterolytic cleavage of hydroperoxides has been proposed before [1,4,8,9]. Applying this proposal on **2b** yields two possible cleavage routes. In route a, water is cleaved off which results in the tentatively identified 1-(3',4'-dimethoxyphenyl)propane-1,2-dione. In route b, the bond between C_α and C_β is cleaved resulting in VAD (**4**). A possible way in which Mn^{2+} may interfere with VAD formation is by reducing the peroxy radical (**1b**) to the corresponding anion [11]. In this

Scheme 3 Proposed reaction mechanism involved in VAD formation during the hypothesized consumption of DMPP by O_2 -dependent free-radical reactions

manner it overcomes self-propagating chemical reactions in which **1b** couples with another DMPP molecule (see Scheme 3), or in which **1b** abstracts a hydrogen atom from a new DMPP molecule [11]. It is proposed that the O_2 -dependent self-propagating chemical reactions result in the formation of unstable organic peroxides of which a possible structure is depicted in Scheme 3. These may decompose to form VAD and ethanal.

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