Stereospecificity in enzymic and non-enzymic oxidation of β-*O*-4 lignin model compounds

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The degradation of the *erythro* and *threo* isomers of the non-phenolic lignin model compound 2-(2,6-dimethoxyphenoxy)-1-(3,4-dimethoxyphenyl)-1,3-propanediol was examined. Enzymic and non-enzymic oxidation of the diastereomers was performed with *Trametes versicolor* lignin peroxidase and cerium(IV) ammonium nitrate, respectively. Mixtures of approximately equal amounts of the diastereomers were partially degraded and subsequently analyzed with TLC and ^{1}H-NMR. Analysis of reaction mixtures from enzymic as well as non-enzymic oxidation, revealed a preferential degradation of the *threo* form. Preliminary analyses of enzymic reaction mixtures of either the *erythro* or *threo* isomer suggest they yield in part different products. The observations made would have implications for the understanding of how enzymes attack lignins. They should also be taken into consideration in experiments where model compounds are being used to mimic native lignin.

Lignin model compound; Stereospecificity; Lignin peroxidase isozyme; *Trametes versicolor*; Cerium(IV) ammonium nitrate; ^{1}H-NMR

1. INTRODUCTION

The biodegradation of lignins is a process of great importance and complexity [1]. Lignins have a polymeric structure and consist of phenylpropane units [2]. The most abundant intermonomeric linkage is the arylglycerol-β-aryl ether bond (cf. Fig. 1). Two adjacent phenylpropane units that attach to each other by such an ether bond constitute a structural element of the so called β-*O*-4 type. This structure occurs in the form of diastereomers (*erythro* and *threo* forms) as has recently been demonstrated [3-5]. Cleavage of dimeric β-*O*-4 lignin model compounds has been extensively studied using purified lignin degrading enzymes and fungal cultures [1,6]. However, so far, very little attention has been devoted to the question how diastereoisomerism affects the susceptibility of the β-*O*-4 structure.

In this contribution we focus the attention on the possible significance of substrate stereochemistry by examining the susceptibility of diastereomers of a non-phenolic lignin model compound, 2-(2,6-dimethoxyphenoxy)-1-(3,4-dimethoxyphenyl)-1,3-propanediol (here referred to as compound 1) (Fig. 1), towards oxidation. The reaction was investigated using ultraviolet-visible absorption spectroscopy, ^{1}H-NMR spectroscopy and thin-layer chromatography. As oxidants we used lignin peroxidase isozymes isolated from the white-rot fungus *Trametes versicolor* [7], or an inorganic outer-sphere oxidant, cerium (IV) ammonium nitrate [8,9].

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Abbreviations: NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TMS, tetramethylsilane

2. MATERIALS AND METHODS

2.1. *Lignin model compounds*

The *erythro* and *threo* isomers of 2-(2,6-dimethoxyphenoxy)-1-(3,4-dimethoxyphenyl)-1,3-propanediol (compound 1) were synthesized as described in [10]. ^{1}H-NMR data for the diastereomers of compound 1 are given in [11].

2.2. *Enzyme*

Lignin peroxidase isozymes were purified from cultures of *Trametes versicolor* (PRL 572) as described previously [7]. All enzymic reactions were performed with only one highly purified isozyme present (isozyme A, B, or C, as described in [12]). One unit of enzyme activity is defined as in [7].

2.3. *Oxidation of compound 1 with lignin peroxidase*

A mixture of the diastereomers of compound 1 was dissolved in 95% ethanol to a concentration of 15 mg/ml. As a reference, a portion of this solution (containing 5.0 mg of the substrate) was mixed with water and 0.40 M sodium tartrate buffer (pH 2.9) to a final volume of 10 ml and a buffer concentration of 50 mM. The solution obtained was then extracted with chloroform. The extracted material was acetylated and examined by ^{1}H-NMR spectral analysis as described below.

The enzymic reaction was performed at room temperature (21°C) with magnetic stirring. A determination of the time required for the reaction was obtained by TLC analysis of aliquots (taken at intervals of 1 min) of a reaction mixture, prepared as described below (with addition of hydrogen peroxide solution every other min).

A portion of the substrate solution (containing 10 mg substrate) was mixed with water, 0.40 M sodium tartrate buffer (pH 2.9) and 8 U of lignin peroxidase to a final volume of 25 ml and a buffer concentration of 50 mM. The reaction was started by addition of 100 μl 0.30% hydrogen peroxide solution. After 2 min, additional hydrogen peroxide was added (100 μl 0.30% solution). After 3 min, the reaction was interrupted by extraction with chloroform (20 + 2 × 10 ml). The

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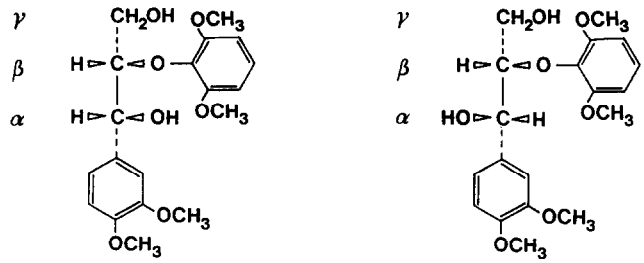

1a 1b

Fig. 1. Lignin model compound 1a (*erythro*) and 1b (*threo*).

extract was dried over Na_2SO_4. The solvent was removed by film evaporation and the residue was, after acetylation, examined by ^{1}H-NMR.

2.4. *Oxidation of compound 1 with cerium(IV) ammonium nitrate ($Ce(NO_3)_4 \cdot 2\ NH_4NO_3$)*

A mixture of the diastereomers of compound 1 (19 mg) was dissolved in acetic acid (0.29 ml) and water (37 ml). A portion of the solution (5 ml) was subjected to the work-up procedures described below and the isolated product was used as reference in TLC examinations as well as in the ^{1}H-NMR spectrometric determinations (after acetylation).

Cerium(IV) ammonium nitrate (19 mg) was dissolved in 6.5 ml water and added to the solution of the lignin model compound (magnetical stirring). TLC examination after 0.5 h indicated only moderate changes of the composition of the reaction mixture. A second portion of the oxidant (19 mg in 6.5 ml water) was added 1.5 h after the first addition of cerium reagent. The reaction mixture was set aside for 1 h and then examined by TLC. The results showed that only traces of the *threo* form were left in the reaction mixture while the amount of *erythro* form seemed to be essentially unchanged. The reaction mixture was neutralized with saturated $NaHCO_3$ solution and extracted with chloroform (40 + 2 × 20 ml). The extract was dried over Na_2SO_4 and solvents removed by film evaporation. The residue was acetylated and examined by ^{1}H-NMR.

2.5. *Thin-layer chromatography*

TLC was performed on silica gel plates (Merck, Kieselgel 60 F_{254}) with toluene-dioxane-acetic acid (90:25:4) (R_F values: 1a, 0.24; 1b, 0.27; 3,4-dimethoxybenzyl alcohol, 0.28; 3,4-dimethoxybenzaldehyde, 0.47) and water-saturated 2-butanone (R_F value: 1-(3,4-dimethoxyphenyl)-1,2,3-propanetriol, 0.37) as eluents. Spots were made visible by ultraviolet light and by spraying with formalin-H_2SO_4 (1:9) and subsequent heating.

2.6. *^{1}H-NMR spectra*

^{1}H-NMR spectra were recorded at 270 MHz with a Bruker WH270 instrument (temperature approximately 300 K). Deuteriochloroform was used as a solvent (internal reference, TMS). Quantitative estimates were performed with hexamethylbenzene (^{1}H-NMR spectrum: δ 2.23 (18H, singlet; CH_3)) as internal standard.

2.7. *^{1}H-NMR spectrometric determination of the amounts of* erythro *and* threo *isomers of compound 1*

The reaction product was treated with a mixture of acetic anhydride (1.5 ml) and pyridine (1.5 ml) overnight (25 ml flask). The reaction was quenched by addition of ethanol. Acetic acid and pyridine were released by sequential addition and removal (film evaporation) of ethanol (15–20 ml) 6–7 times. The products were analyzed by ^{1}H-NMR spectrometry and the amounts of the diastereomers of compound 1 were determined from integrations of the signals from acetate groups and benzylic protons.

2.8. *Spectroscopy in the ultraviolet-visible region*

Reaction mixtures consisted of a 0.10 M sodium tartrate solution (pH 3.0) which was 0.40 mM with respect to H_2O_2 and 2.0 mM with respect to compound 1. Reactions were started by the addition of lignin peroxidase and monitored at room temperature (21°C) using a Shimadzu model UV-160 double-beam recording spectrophotometer. The reference cuvette had the same content as the reaction mixture but with no enzyme added. Scannings between 200 and 600 nm were performed at intervals of 2 min.

3. RESULTS

Table I shows the results of experiments where mixtures of diastereomers of compound 1 have been oxidized. In the enzymic oxidation, performed with lignin peroxidase isozyme C, we noted a change of the *erythro/threo* ratio from 46:54 to 87:13. Quantitative ^{1}H-NMR spectrometric analysis showed that about 80% of the initial amount of *erythro* form was left in the sample. The amount of *threo* form remaining should then be around 10%. This finding shows that the *erythro* and *threo* forms of compound 1 differ significantly in susceptibility. In addition, several small-scale experiments were performed with isozymes A, B, or C, respectively, and analyzed with TLC. Visual examinations of TLC plates revealed a distinctly selective oxidation of the *threo* form in all experiments.

The oxidation with cerium(IV) ammonium nitrate resulted in a change of the *erythro/threo* ratio from 45:55 to 83:17. In this case, approximately 70% of the initial amount of *erythro* form could be recovered.

Preliminary studies of the degradation of diastereomers of a closely related *β-O*-4 model compound, 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol, by cultures of the white-rot fungus *Phanerochaete chrysosporium* also suggest a preferen-

Table I

Oxidation of diastereomers of 2-(2,6-dimethoxyphenoxy)-1-(3,4-dimethoxyphenyl)-1,3-propanediol (compound 1)

	Isomer ratio (*erythro:threo*)	Amounts in reaction mixture (% of initial values)		Oxidizing agents
		erythro	*threo*	
Initial	46:54	100	100	Lignin peroxidase/H_2O_2
After reaction	87:13	80	10	
Initial	45:55	100	100	Cerium(IV) ammonium nitrate
After reaction	83:17	70	12	

tial degradation of the *threo* form (Kirk, T.K., Mozuch, M., Lundquist, K. and Von Unge, S., unpublished data). It is noteworthy in this context that an attempt to detect specificity in degradation of enantiomers of a β-1 lignin model compound by the same fungus did not prove successful [13].

Enzymic and non-enzymic reactions were in some experiments continued until essentially all the substrate had been degraded. The products obtained were examined by TLC. In these experiments, only one of the diastereomers at a time was present in the reaction mixture. Evidence for the presence of 3,4-dimethoxybenzaldehyde as a major product in the reaction mixtures, was obtained by TLC examinations. Reduction with $NaBH_4$ yielded a compound displaying the same R_F value and colour reaction as 3,4-dimethoxybenzyl alcohol. TLC with water-saturated 2-butanone as eluent indicated the presence of another product, 1-(3,4-dimethoxyphenyl)-1,2,3-propanetriol, in the reaction mixture. A similar cleavage product (arylglycerol derivative) has earlier been identified in enzymic degradation of a β-syringyl ether compound, for instance by Umezava et al. [14]. However, in their study no significant amounts of aromatic aldehyde were obtained. This could be due to the fact that oxidation has not been pursued to a large extent in the experiments described in [14], and compounds isolated may not represent final oxidation products.

Comparisons of enzymic reaction mixtures of individual diastereomers indicate that not only common reaction products are formed but also substances that are specific for the stereoisomer used as starting material. Fig. 2 illustrates the oxidation of *erythro* and *threo* forms of compound 1 with lignin peroxidase recorded by ultraviolet-visible difference spectroscopy. The spectral differences developing during the reaction are not identical for the *erythro* and *threo* forms. The most easily recognized difference occurs around 300 nm which is a region for strong absorption of 3,4-dimethoxybenzaldehyde. That this compound would be a more predominant product for the *erythro* form than for the *threo* form at an early stage of the reaction is also supported by the analyses of enzymic reaction mixtures with TLC. In the TLC analyses a product unique for the *erythro* form was observed at R_F value 0.21 while with the *threo* form a substance with R_F value 0.15 could be noted.

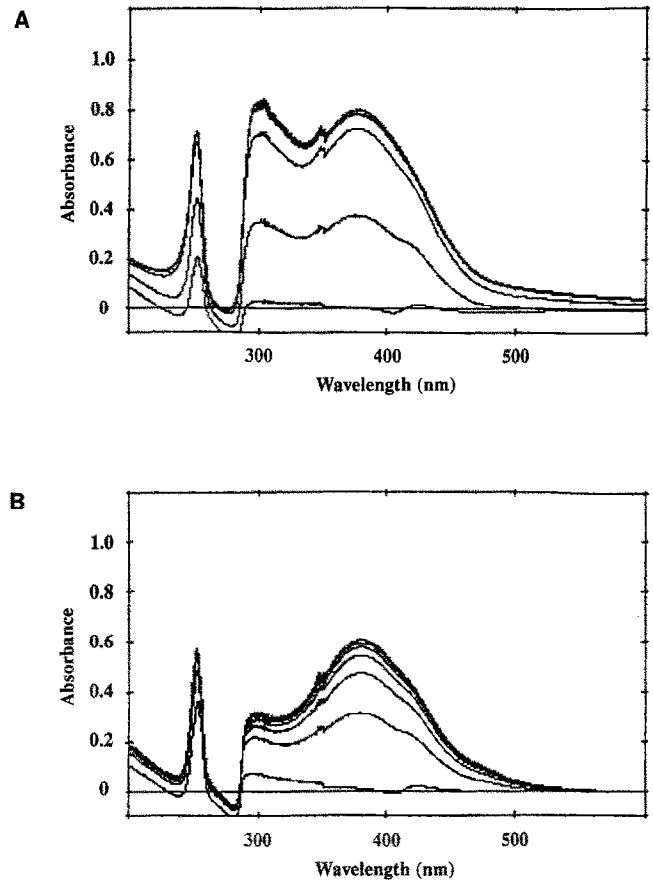

Fig. 2. Difference absorption spectra of enzymic oxidations of compounds 1a (A) and 1b (B). For experimental details, see section 2.8.

4. DISCUSSION

In this study, we have tried to take stereochemical aspects of lignin degradation into consideration. Utilizing a stereochemically defined dimeric model compound (compound 1), we investigated the susceptibility of individual diastereomers (the *erythro* and *threo* forms of compound 1) towards purified isozymes of lignin peroxidase and cerium(IV) ammonium nitrate.

The *threo* form of compound 1 was found to be more susceptible to attack in both the enzymic and the non-enzymic system, a phenomenon observed with two independent methods of analysis (^{1}H-NMR and TLC). The preferential degradation of the *threo* form could be observed with all the 3 isozymes of lignin peroxidase examined, as well as with the cerium reagent. A primary conclusion drawn from these experiments is that the stereochemistry of the dimeric substrate does have a profound influence upon its degradation.

Previous studies (reviewed in [1,6]) of lignin model compounds of the β-*O*-4 type have shown that important steps in degradation could be C_α-C_β cleavage, β-ether bond cleavage and aromatic ring cleavage. The experiments with TLC and the recordings of difference absorption spectra (Fig. 2) suggest that final reaction products and intermediates may be different for the *erythro* and *threo* forms of compound 1. For instance, certain discrepancies were noted in the appearance of 3,4-dimethoxybenzaldehyde, a product presumably created by cleavage at the diastereomeric center (C_α-C_β) of compound 1. At an early stage of reaction, the aromatic aldehyde appears to be a more dominating product for the *erythro* form, the less susceptible of the two isomers, than for the *threo* form. However, the

question whether the *erythro* and *threo* forms follow different pathways in oxidative degradation cannot be definitively answered without further studies.

In conclusion, the *threo* isomer of β-*O*-4 lignin model compounds have, in all systems studied so far, been found to be degraded at a substantially higher rate than the *erythro* isomer. This finding would have implications for several areas such as: (i) understanding of how lignin is attacked and of the pathways by which it is degraded enzymically as well as non-enzymically [4,15], (ii) characterizations of ligninolytic enzymes or organisms using model compounds for lignin and (iii) studies of reaction patterns of model compounds should preferably be carried out using stereochemically defined substrates.

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