Enzymic oxidation of vanillyl glycol

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Keywords: Lignins, Enzymes, Model compounds, Biodegradation, Oxidation, Bleaching.

SUMMARY: The tree laccase, fungal laccase, peroxidase, and tyrosinase catalysed oxidation of vanillyl glycol — 3-(4-hydroxy-3-methoxyphenyl)-1,2-propanediol — has been studied at pH 5 and 7. Tree laccase and tyrosinase were found to be inactive at pH 5.

Any enzyme- or pH-dependent differences in product composition were not observed in the experiments with laccase and peroxidase. These enzymes catalysed other types of reactions than tyrosinase. Fungal laccase and tyrosinase could oxidize the substrate to an extent corresponding to 3—5 electron abstractions/mol.

The results provide a basis for an evaluation of the potential of phenoloxidases in pulp bleaching or for the neutralization of mill effluents. The relevance of the results obtained in relation to lignin biodegradation is discussed.

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The main classes of enzymes known to catalyse phenol oxidation and coupling are the laccases, the peroxidases, and the tyrosinases (1). These types of enzymes are involved in the biosynthesis of a variety of compounds present in plants, e.g. lignins (2—5). There have been differences in opinion on the nature of the enzyme which is responsible for the formation of lignin from *p*-hydroxycinnamyl alcohols. Recent work (6) suggests that peroxidase solely is involved in the biosynthesis of lignin. White-rot fungi, which are known to decompose lignin as well as carbohydrates, produce phenoloxidases. The role of such enzymes—particularly laccases—in the biodegradation of lignin has been the subject of a considerable number of studies, but still seems to be incompletely elucidated (7—12). These enzymes may function as detoxifiers of phenolic compounds (9), have a regulatory role in the decomposition of carbohydrates as well as lignin (10), or act as "electron shuttle" in connection with the processes involved in lignin biodegradation (11).

Alternatively, phenoloxidases may be *directly* involved in the oxidation of lignin during biodegradation. Comprehensive *in vitro* studies of the laccase-catalysed oxidation of lignins have been made (13—19). Results hitherto obtained suggest that such oxidation causes only moderate changes and does not result in a far-reaching degradation.

The present paper describes a comparative study of the catalytic activity of a series of phenoloxidizing enzymes in the oxidation of a lignin-related phenol. This study aimed at elucidating the potential of phenoloxidases as catalysts for the oxidative degradation of phenolic compounds. The interest of such knowledge in connection with fungal lignin biodegradation is apparent from what has been said above. The results are also intended to provide a basis for an evaluation of the applicability of phenoloxidases in pulp bleaching or for the neutralization of mill effluents.

All experiments were performed with vanillyl glycol *1*[1] as substrate. This lignin-related phenol is exceedingly water-soluble and gives water-soluble products on enzymic oxidation. This makes it possible to use an aqueous medium for the experiments without the interference of precipitations of reaction products. Series of experiments were run with reaction media buffered at pH 5 and pH 7. A number of oxygenases [fungal laccase, tree laccase, and tyrosinase (mushroom)] and horseradish peroxidase were used in the investigations. To obtain comparable results in the experiments with different enzymes, similar experimental conditions were used. The gross differences regarding the catalytic activities of the enzymes examined in the experiments with vanillyl glycol appear from the data given in *table 1.*

To obtain a wider basis for comparisons of the catalytic properties of the enzymes, series of experiments in which vanillyl glycol was oxidized to various extents were performed and the compositions of the reaction mixtures obtained were examined by thin layer chromatography (TLC) and gel permeation chromatography (GPC). As could be expected from previous studies (1), the properties of tyrosinase were found to differ greatly from those of the other enzymes and the results from tyrosinase experiments are therefore treated in a separate section of this paper.

Experiments with laccases and peroxidase

In series of experiments with tree laccase, fungal laccase, and peroxidase vanillyl glycol *1* was oxidized in pH 7 medium to extents corresponding to an abstraction of 0.25, 0.50, and 1.0 electron/mol. Corresponding experiments were run with peroxidase and fungal laccase using a pH 5 reaction medium. The reaction mixtures were examined by TLC. The

[1] Italicized numbers designate compounds.

Table 1. Ability of a series of phenoloxidases to catalyse the oxidation of vanillyl glycol 1 as reflected by the time of reaction required for an abstraction of one electron/mol. For structures and properties of the enzymes, see (20) laccases, (21) peroxidase, and (22) tyrosinase.

Enzyme	pH 5	pH 7
Laccase A (*Polyporus versicolor*)	10 h[a]	10 h[a]
Laccase (*Rhus vernicifera*)	no reaction	200 h[a]
Peroxidase (horseradish)	<20 h[b]	<20 h[b]
Tyrosinase (mushroom)	no reaction	20 h[a]

[a] As determined by measurements of the oxygen consumption.
[b] All experiments with peroxidase were arbitrarily run for 20 h and the extent of oxidation was regulated by an initial addition of the calculated amount of hydrogen peroxide; examinations of the compositions of the reaction mixtures suggested that shorter periods of time than 20 h was required for complete reaction.

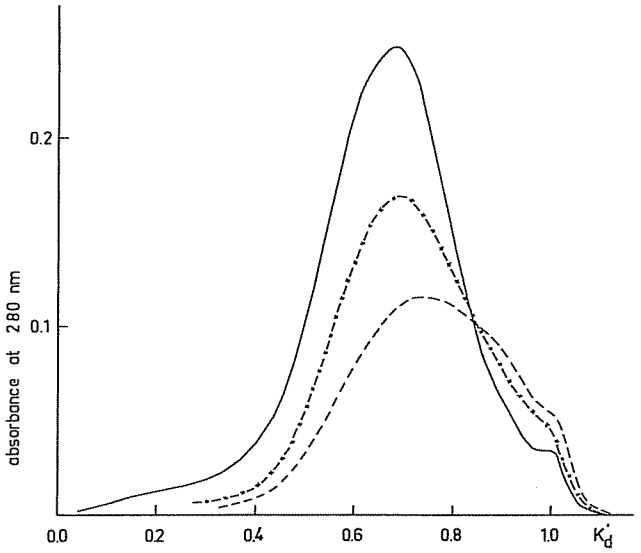


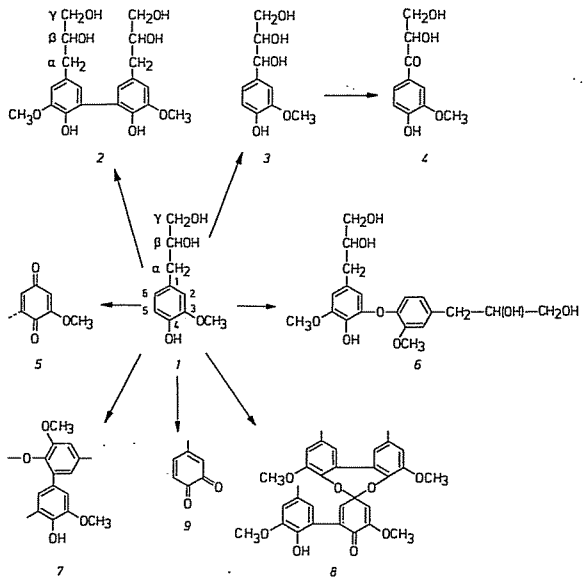
Fig. 1. GPC curves of reaction mixtures obtained on peroxidase catalysed oxidation of vanillyl glycol (1) in pH 5 medium to oxidation degrees corresponding to an abstraction of 0.25 (— — —), 0.50 (-×-×-), and 1.0 (———) electron/mol. The inflexion at $K_d' \approx 1$ was found to be artifactitious and caused by the electrolytes present in the reaction mixture.

chromatograms showed the presence of biphenyl *2* as the major reaction product and trace amounts of guaiacylglycerol *3* in addition to starting material. Besides the above mentioned compounds materials with fairly low retention values, R_F, were present in the reaction mixtures. These materials were found mostly in the R_F range 0.1—0.2 and included a component ($R_F \approx 0.13$) which gave a blue spot with 2,6-dibromo-*N*-chloroquinoneimine. This is indicative of *p*-hydroxybenzyl alcohol groups (23). Although the chromatograms were carefully examined, any enzyme- or pH-dependent differences in product composition could not be observed. Reaction mixtures with a degree of oxidation corresponding to an abstraction of one electron/mol contained small amounts of starting material; the presence of unreacted *1* is consistent with the occurrence of compounds which are oxidized to a higher degree than 1.0 electron/mol (guaiacylglycerol and in all likelihood part of the materials with a low R_F value).

GPC curves of reaction mixtures obtained with peroxidase in pH 5 medium are shown in *fig. 1*. From the GPC curves it can be concluded that the starting material is converted into dimeric and oligomeric compounds. Even in the 1.0 electron/mol experiments, no polymeric material is formed. It is also apparent from the curves in fig. 1 that the oxidation results in an increased absorbance. Within the error limits, the same GPC results were obtained on examination of reaction mixtures obtained with the other enzymes.

The calculated amount of hydrogen peroxide was added initially to the reaction mixture in the peroxidase experiments. In laccase experiments oxygen is continuously dissolved in the reaction medium and consumed in oxidation reactions. To examine as to whether the mode of addition of oxidant influenced the composition of the reaction mixtures, experiments with continuous addition of the hydrogen peroxide during a 16 h period were performed. Comparisons (GPC, TLC) of reaction mixtures obtained on initial and continuous addition of hydrogen peroxide did not reveal any differences in product composition.

The formation of biphenyl *2* and guaiacylglycerol *3* is in line with previous investigations of enzymic phenol oxidation. *Scheme 1* summarizes both reaction products shown to arise *2* and *3*, and those which could be expected from analogies and extrapolations of results with other substrates (2, 24—34).



Scheme 1

The reaction mixtures were only very slightly colored (a brownish yellow). The formation of products with absorption in the visible region (e.g. quinoid compounds) is therefore small on oxidation corresponding to an abstraction of one electron/mol or less.

In a study with fungal laccase the maximal degree of oxidation was determined. It was found that the oxygen consumption ceased after about 100 h and that the uptake at that time corresponded to an abstraction of 5 electrons/mol in pH 5 medium and 4 electrons/mol in pH 7 medium. The pH dependence is probably related to a partial precipitation of the reaction products in the pH 7 experiment. Since the same types of reaction occur at pH 5 and 7 (see above)

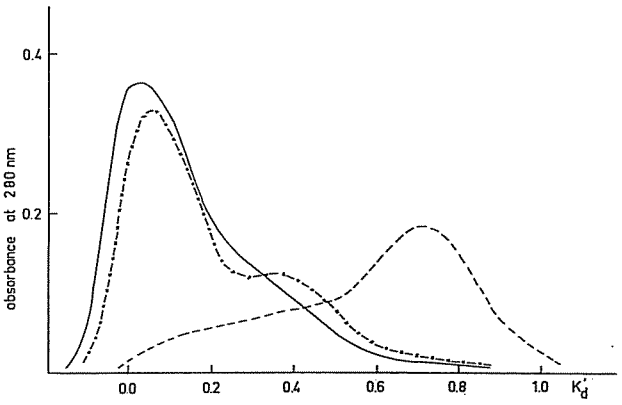


Fig. 2. GPC curves obtained on prolonged oxidation of vanillyl glycol (1) by fungal laccase (pH 5, ———; pH 7, -×-×-) and tyrosinase at pH 7 (— — —). The deactivation of the enzyme by an acid treatment (see Experimental) was omitted in experiments with exhaustive oxidation, since additional oxidation did not occur.

the precipitation is probably caused by the pH 7 buffer rather than structural differences in the composition of the reaction products. GPC experiments showed that polymerization had occurred (*fig. 2*). The reaction products had a strong red color, suggesting the formation of considerable amounts of quinoid groupings (e.g. *9*).

Experiments with tyrosinase

Vanillyl glycol *1* was oxidized in a tyrosinase catalysed reaction (pH 7 medium). Conditions were those used in the experiments with laccase and peroxidase described above. Reaction mixtures obtained in experiments with abstraction of 0.25, 0.50, and 1.0 electron/mol were examined. TLC showed the presence of considerable amounts of starting material and some materials with $R_F = 0$. Only very weak spots were otherwise apparent in the chromatograms. GPC curves of the reaction mixtures exhibit a peak corresponding to the distribution coefficient, K_d', value of vanillyl glycol (*fig. 3*). It appeared from the GPC curves that polymeric and oligomeric materials were also present in the reaction mixtures. They were colored orange red, particularly when 1.0 electron/mol had been abstracted. The color is in all likelihood due to formation of *o*-quinones of type *11*. *Scheme 2* exemplifies reactions which, by analogy (1), can be expected to occur in tyrosinase catalysed oxidation of vanillyl glycol *1*.

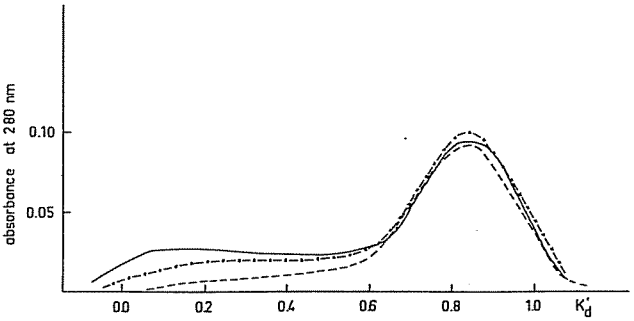
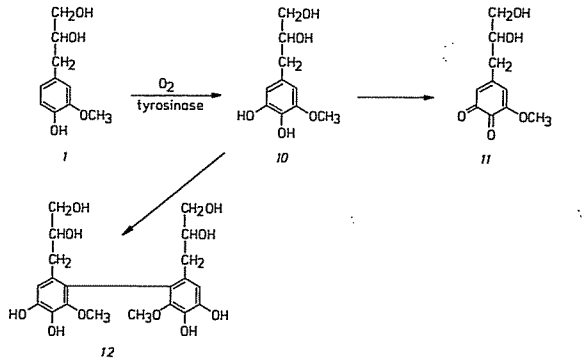


Fig. 3. GPC curves of reaction mixtures obtained on tyrosinase catalysed oxidation of vanillyl glycol (1) to degrees corresponding to an abstraction of 0.25 (— — —), 0.50 (-×-×-), and 1.0 (———) electron/mol.



Scheme 2

An alternative procedure involving an initial addition of aqueous solution of sulfur dioxide to reduce quinones was tried in order to examine whether acid treatment involved in the work-up procedure (see Experimental) caused the formation of oligomeric and polymeric products. GPC curves of reaction mixtures resembled largely those shown in fig. 3, but the amount of high molecular mass materials appeared to be somewhat smaller. Experiments with extensive oxidation showed that the rate of initial oxygen consumption was lower than in the case of fungal laccase and diminished slowly during a two week period. The final extent of oxidation corresponded to an abstraction of about 4 electrons/mol. As could be shown in GPC experiments, the final reaction mixture contained rather large amounts of low molecular mass materials (fig. 2).

Discussion

The examinations of oxidation products of vanillyl glycol *1* from experiments with fungal laccase, tree laccase, and peroxidase have not revealed any significant differences in product composition. The low rate of reaction observed in the experiments with tree laccase is probably related to the comparatively low oxidation potential of this enzyme (20). The similarity

in product composition suggests that the enzyme-substrate interactions have a general, non-specific character. This is in line with the fact that laccases and peroxidases oxidize a wide variety of substrates. It is notable in this connection that the structures and active sites of these two types of enzymes differ profoundly from each other (20, 21). Several reports (1, 31) suggest that laccase and peroxidase produce the same products on phenol oxidation. However, scattered observations have been made which suggest some differences in their mode of catalytic activity (1, 35).

The substrate specificity of laccases has been investigated and is apparently low (36, 37). The artifactitious effect of low solubility of the substrate in the reaction medium has been noted (38). On prolonged oxidation of vanillyl glycol with fungal laccase at pH 7 it was found that a precipitation of reaction products accompanied a lower degree of oxidation than was found in the corresponding pH 5 experiments (fig. 2). Diminished accessibility of precipitated materials to oxidation may be the reason for that. However, a pH dependence of the product composition has been reported (17) in other studies of the enzymic oxidation of lignin model compounds. The substrate specificity of horseradish peroxidase has been studied recently by measuring the rate of initial enzyme-substrate reaction for a variety of substrates (39). These studies suggested that not only oxidation potentials required but also structural characteristics of the substrate influenced the rate of reaction.

Tyrosinase acted very differently from the other enzymes, and this is in accord with what could be expected from earlier studies (1). It is notable that the formation of polymers occurred at a low degree of oxidation (0.25 electron/mol). On the basis of UV measurements it was concluded recently that tyrosinase catalyses the oxidation of certain lignin compounds to only a slight extent (18). Extensive oxidation occurred in the tyrosinase experiments performed in the present study. However, the oxidation does not cause any drastic changes in the UV absorbance (fig. 3) which may explain this apparent discrepancy.

In the experiments with fungal laccase it was found that about 5 electrons could be abstracted from each

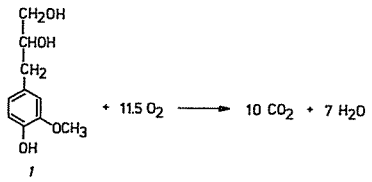


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Fig. 4. Stoichiometry of the oxidation of vanillyl glycol (1) to carbon dioxide and water. In this reaction 23 oxygen atoms change their state of oxidation from 0 to —2; i.e. 46 electron abstractions are required for complete oxidation of compound 1.

of the substrate molecules. Complete oxidation, to carbon dioxide and water of a substrate molecule requires 46 electron abstractions (*fig. 4*). This implies that the extent of enzymic oxidation obtained in the present work corresponds to about 10% of the total required for combustion. The product obtained on extensive oxidation was found to be polymeric as demonstrated by GPC experiments (fig. 2). The mode of polymerization has not been elucidated. Since abstraction of one electron/mol results in formation of primarily dimeric (and practically no polymeric) material (fig. 1) it follows that formation of a linear polyether with units attached to each other as in compound *6* can be excluded. Therefore it could be concluded that if polymerization proceeds according to known reaction routes, formation of structures *7* or *8* should be involved. Possibly quinones formed undergo addition reactions or reduction resulting in the formation of phenols which subsequently undergo oxidative phenol coupling [cf. the formation of "humic acids" on oxidation of cathecol compounds (40)].

Increased molecular mass due to phenol coupling usually occurs when phenolic lignin model compounds are subjected to enzymic oxidation (31). However, reactions which lower the molecular mass have also been encountered (26, 33). Results from experiments with lignin preparations are in accord with those obtained in model compound studies. Thus, increased molecular mass (14) as well as liberation of small fragments (15) has been observed on enzymic oxidation of lignin preparations. It should be noted that polymerization due to phenol coupling is a degradation in the sense that the number of electron abstractions needed for complete degradation is diminished.

It seems plausible to assume that phenoloxidases produced by fungi are able to oxidize phenols during biodegradation of wood to the same extent as in the above discussed model experiments, i.e. direct phenoloxidase catalyzed oxidation may be responsible for around 10% of the conversion to carbon dioxide and water. The oxidation capacity of phenoloxidases may be increased by synergistic effects of different phenoloxidases. Alternatively, increased effects may arise through conversion of initially-formed oxidation products by other enzyme systems into products which could be oxidized by phenoloxidases. Finally, it should be pointed out that the above discussion has been limited to the case of a *direct* interaction of the substrate with the enzyme. As pointed out in the introductory section the role of phenoloxidases in lignin biodegradation may be an indirect one.

Experimental

Thin layer chromatography (TLC) was performed on silica gel plates (Merck, Kieselgel 60 F_{254}). R_F values (eluent, water-saturated butanone): biphenyl *2*, 0.21; guaiacylglycerol *3*, 0.37; vanillyl glycol *1*, 0.53. The

R_F value for vanillyl glycol *1* with benzene-dioxane-acetic acid (90:25:4) as eluent was found to be 0.09. Spots were made visible by UV light and by spraying with formalin-H_2SO_4 (1:9) and subsequent heating (140 °C). Guaiacylglycerol *3* was also made visible as a blue spot by spraying with an ethanolic solution of 2,6-dibromo-*N*-chloroquinoneimine followed by dilute aqueous NaOH (23).

Gel permeation chromatography (GPC) was performed on Sephadex G-25 (30 g) with dioxane-water (1:1) as eluent. The UV absorbance of the effluent was measured by an LKB Uvicord. The distribution coefficient K_d' used in fig. 1—3 is defined in (41). $K_d'=0$ corresponds to the elution volume of lignin (a polymer) and $K_d'=1$ corresponds to the elution volume of acetone (a small molecule). K_d' values for vanillyl glycol *1* and biphenyl *2* were found to be 0.83 and 0.69, respectively. On examination of reaction mixtures from enzymic oxidation, samples were prepared by adding 3 ml dioxane to 3 ml of the reaction mixture from oxidation experiments. Separate runs with an internal standard (lignin or acetone) added were made with the samples.

Buffer solutions. A 0.1 M sodium acetate-acetic acid buffer was used in the pH 5 experiments. A pH 7 buffer was prepared by adding 0.1 M NaOH to 0.1 M KH_2PO_4 (500 ml) and subsequent dilution to 1000 ml.

Enzymes. Samples of laccase A from *Polyporus versicolor* and laccase from the laquer tree (*Rhus vernicifera*) were obtained from the Department of Biochemistry, University of Göteborg. Commercial horseradish peroxidase (Boehringer) and mushroom tyrosinase (Sigma) were used.

3- (4-hydroxy-3-methoxyphenyl)-1,2-propanediol (1) (vanillyl glycol) was prepared from eugenol acetate according to the procedure described in (42). An aqueous solution of the crude product was extracted with chloroform several times to remove contaminants. A 15 g portion of the extracted product was further purified by chromatography on silica gel (300 g) with ethyl acetate as eluent. Finally, the chromatographed product was distilled (160 °C, 1 Pa). A practically colorless oil was obtained. Some fractions of purified vanillyl glycol *1* crystallized slowly on standing, m.p. 51—53 °C.

^{1}H NMR (270 MHz, $CDCl_3$) of acetylated product: δ 2.03 (3 H, s; aliphatic acetate), 2.07 (3 H, s; aliphatic acetate), 2.30 (3 H, s; aromatic acetate), 2.88 (2 H, m; CH_2), 3.81 (3 H, s; OCH_3), 4.04 (1 H, dd, $J=6.0$ and 12 Hz; Hγ), 4.24 (1 H, dd, $J=3.0$ and 12 Hz; Hγ), 5.27 (1 H, m; Hβ), 6.78 (1 H, dd, $J=1.8$ and 8.0 Hz; H-6), 6.81 (1 H, d, $J=1.8$ Hz; H-2), 6.95 (1 H, d, $J=8.0$ Hz; H-5).

Enzymic oxidation

a. Oxidation catalysed by laccase and tyrosinase

A mixture of vanillyl glycol *1* (4 mmol) in buffer solution (80 ml), and water (100 ml, including the water used for the washing of the enzyme into the reaction mixture) was prepared in a 250 ml Erlenmayer flask. This was attached to a water seal and a funnel with a stopcock via a ground glass joint. Fungal laccase A (≈4 mg), tree laccase (≈8 mg), or tyrosinase (≈5 mg) was washed into the reaction mixture via the funnel. The water seal was in its turn connected to a gas burette containing oxygen. This arrangement made it possible to maintain an atmosphere with the composition of air in the reaction vessel during the experiment. The reaction mixture was stirred magnetically and the oxygen consumption was studied as function of time. Temperatures were in the range 20—26 °C (room temperature) during the experiments.

When the desired amount of oxygen had been consumed the enzymic oxidation was interrupted by addition of 30 ml 1.0 M hydrochloric acid. To assure a complete deactivation of the enzymes, the acidified solution was stored for 24 h at room temperature. Subsequently, 10% sodium acetate solution (pH 5 experiments) or 1.0 M K_3PO_4 solution (pH 7 experiments) was added to the reaction mixture to rise the pH to 3—4. The reaction mixtures were kept at —20 °C in polyethylene bottles. Analogous experiments with 1 or 2 mmol of vanillyl glycol *1* were performed when experiments on a smaller scale were satisfactory. Prior to examinations by TLC or GPC, the acidity of the reaction mixtures was raised to pH 2 by addition of 1 M hydrochloric acid.

b. Experiments with peroxidase

Reaction mixtures had the same composition as in the experiments described above. The calculated amount of 0.5% H_2O_2 solution was used as oxidant. After addition of hydrogen peroxide solution and enzyme the reaction mixture was set aside for 20 h in the dark (room temperature). The reaction mixtures were then treated in the same way as in the experiments with oxygenases.

Isolation and identification of biphenyl 2

Solvents were removed by film evaporation from a reaction mixture (100 ml) with enzymically (fungal laccase, pH 5) oxidized (0.5 electron/mol) vanillyl glycol *1* (0.4 g). The residue was dried over KOH in vacuo. Materials dissolved by trituration of the residue by dioxane were chromatographed on silica gel (50 g) with water-saturated butanone as eluent. The product obtained was subjected to an additional purification on silica gel (20 g) to give a chromatographically pure product (36 mg). An AEI MS 903 instrument was used for mass spectral examinations. Exact mass measurements of the molecular ion gave m/e 394.160. Calculated for $C_{20}H_{26}O_8$: m/e 394.1627. ^{1}H NMR (270 MHz, $CDC1_3$) of acetylated product: δ 2.04 (6 H, s; aliphatic acetate), 2.07 (12 H, s; aliphatic and aromatic acetate), 2.90 (4 H, m; H_α), 3.84 (6 H, s; OCH_3), 4.05 (2 H, dd, $J=6$ and 12 Hz; Hγ), 4.27 (2 H, dd, $J=3.3$ and 12 Hz; Hγ), 5.27 (2 H, m; Hβ),

6.66 (2 H, d, $J = 1.8$ Hz; aromatic protons), 6.83 (2 H, d, $J = 1.8$ Hz; aromatic protons). A splitting (1 Hz) of the signals at δ 2.04 and 4.05 is interpreted to indicate that the product is a mixture of about equal amounts of the two possible diastereomers.

Acknowledgements

The authors thank Doc. R. Brändén, Doc. B. Reinhammar and Ing. L. Strid for gifts of enzymes and valuable discussions. Financial support from Cellulosaindustriens Stiftelse för Teknisk och Skoglig Forskning samt Utbildning is gratefully acknowledged.

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(First manuscript received November 3, 1980. Revised August 1981.)