Regioselectivity in lignin biosynthesis. The influence of dimerization and cross-coupling

Kaisa Syrjänen and Gösta Brunow *

Department of Chemistry, Laboratory of Organic Chemistry, PO Box 55, FIN-00014 University of Helsinki, Finland

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We have studied the regioselectivity of oxidative phenol coupling in lignin formation using an oxidation system that distinguishes between dimerization reactions and cross-coupling reactions. We found that the regioselectivity of coupling was different in the two reactions. For instance, in coniferyl alcohol dimerization the formation of β–5 coupling product has a slight prevalence over the formation of β–*O*-4 product; in cross-coupling the β–*O*-4 mode is favoured in a ratio of ≈10 : 1. This ratio is higher than that found in isolated softwood lignins. The degree of cross-coupling was influenced only to a small extent by changes in the rates of conventional addition of coniferyl alcohol (Zulauf *versus* Zutropf conditions). We found that diffusion through a dialysis membrane did effectively suppress the dimerization of coniferyl alcohol. Of the different oxidants investigated, manganese triacetate in acetic acid yielded the highest proportion of cross-coupling product.

Introduction

The final phases of the formation of lignin in the cell walls of vascular plants involve oxidative coupling of *p*-hydroxycinnamyl alcohol precursors (such as coniferyl alcohol) to phenolic structures on the growing polymer chain. The coupling is visualized as a more-or-less random process without enzymic control of the regioselectivity of the process.[1,2] The validity of this concept has been tested many times by oxidation of precursors with enzymes and with inorganic oxidants. The dehydrogenation polymers (DHPs) have been found to contain most of the structural units found in natural lignins; complete identity between synthetic and natural lignins has, however, not been achieved. The problems with reproducing lignin biosynthesis *in vitro* has recently led some investigators to challenge the whole concept of random polymerization.[3] We feel that the experimental evidence known so far still is compatible with the original idea proposed by Freudenberg and Adler:[1,4] that the polymerization itself is a purely chemical process and that the enzymic control extends only to the rates of feeding of the monomers into the reaction zone and the generation of the phenoxyl radicals. This argument is supported by the results of a recent study where it was established that lignins are not optically active.[5] A concept that has become increasingly important in this process is that of *cross-coupling*, where the monomer lignol radical, instead of dimerizing, reacts with phenolic structures on the growing chain. When lignin formation is reproduced *in vitro*, the main difficulty is to achieve the necessary degree of cross-coupling whilst suppressing the dimerization. In an effort to find practicable means of promoting cross-coupling at the expense of dimerization, we have studied model systems with *p*-hydroxycinnamyl precursors (monolignols) and phenols representing structures of the polymer chain. We found recently that bond formation in oxidative phenol coupling is governed by a combination of factors such as oxidation potential and radical reactivity.[6,7] Phenols which have similar oxidation potentials, such as coniferyl alcohol and a syringyl β–*O*-4 dimer, yielded cross-coupling products in good yield. It is more difficult to achieve cross-couplings that are not favoured by similarity of oxidation potentials. In our experiments with hydrogen peroxide and horseradish peroxidase, oxidation of equimolar mixtures of coniferyl alcohol **1** and guaiacyl glycerol-β-guaiacyl ether **2** did not yield any cross-coupling products (Tables 1 and 2, Exp. 1). The coniferyl alcohol, with its lower oxidation potential, reacts by dimerization. (Similarly, sinapyl alcohol dimerizes and does not couple with a syringyl dimer.[7]) The abundance of arylglycerol-β-aryl ether structures in natural guaiacyl lignins shows that cross-coupling between coniferyl alcohol and guaiacyl groups in the polymer does occur in spite of the oxidation-potential difference.

We have carried out oxidation experiments with coniferyl alcohol **1** to find conditions under which cross-coupling between phenols of unequal redox potentials can be achieved. Different modes of addition of reagent, such as fast (Zulauf) and slow (Zutropf), and different oxidants were tested. The two competing reactions, cross-coupling and dimerization, are shown in Scheme 1. To simplify the analysis of reaction products we replaced the β-ether model compound, which was used in our previous experiments, with apocynol **3**. We found that the regioselectivity of coupling can be controlled by controlling the rate of addition of monolignol.

Results

In the oxidation experiments, we kept the pH lower than is customary in work on lignin synthesis. We have shown that lower pH favours the formation of dimers and suppresses polymerization.[8] Low pH also seems to yield a higher content of β–*O*-4 structures and a low content of β–β coupling products.[9] In each experiment we used equimolar amounts of apocynol **3** and coniferyl alcohol **1**. The products (**1**, **3**, **5**–**7** and **9**) were identified with the aid of authentic compounds. The structure of compound **4** was determined by MS and NMR. The dimeric β–*O*-4 compound **8** was identified by NMR using published data.[10] In the following discussion the coupling products are classified as *dimers* when they are formed from two identical phenols and *cross*-products when they are products of coupling of two different phenols.

The product mixtures were analysed by HPLC and flash chromatography. For quantitative analysis the products were separated into three fractions A, B and C as shown in Fig. 1, I. The fractions were weighed and the proportions of the individual compounds were calculated from the integrals of signals in the ^{1}H NMR spectra. In the first fraction, **A**, there was only

Table 1 Reaction conditions for experiments 1–10. For details see Experimental section

Exp.	Starting materials	Oxidant	Solvent	Zulauf	Zutropf
1	**1**, **2**	H_2O_2–HRP	Acetone, 20% buffer (pH 3.5)	x	
2	**1**			x	
3	**3**			x	
4	**1**, **3**			x	
5	**1**, **3**				5.5 h
6	**1**, **3**				21 h
7	**1**	$Mn(OAc)_3$	Glacial acetic acid	x	
8	**1**, **3**			x	
9	**1**, **3**	MnO_2	Acetone 20%, buffer (pH 3.5)	x	
10	**1**, **3**	$FeCl_3$	Distilled water	x	

Table 2 Yields of products in experiments 1–10

	Monomer recovery (%)		Dimers (%)							
Exp.	**1**	**2/3**	**4** cross-β–5	**5** dimeric β–5	**6** 5–5	**7** cross-β–*O*-4	**8** dimeric β–*O*-4	**9** β–β	Total yield	Oligomers (%)
1		44		18			16	8	42	14
2	36			24			16	12	52	12
3		50			34				34	3[a]
4		45		15	3	5	14	8	45	8
5	4	31	1.5	10	3	10	8	8	40.5	14.5
6	3	31	1	9	2	9	11	6	39	12
7	11			22			50	2	74	15
8		23	1	9	1	18	26	1	56	14
9		28		15	2	5	5	6	33	33
10	4	44		10		5	18	9	42	10

[a] A tetrameric dioxepine (*cf.* ref. 11) was also formed (13%).

unchanged starting material, compounds **1** and **3**, which are easily identified from the spectra. Hα′ of apocynol gives a signal at δ 5.90 (q) and Hα′, Hβ′ and Hγ′ of coniferyl alcohol are at δ 4.75 (d), 6.27 (dt) and 6.64 (d), respectively. The second fraction, **B**, consists of cross-β–5 (**4**), dimeric β–5 (**5**) and the 5–5 dimer from apocynol (**6**). Hα′ of compound **5** is at δ 6.64 (d), but Hα′ of **4** and Hα′ of **6** overlap at δ 5.88 (m). Peak heights of compounds **4** and **6** in the HPLC chromatogram I are roughly 1:2 and the yields are in this case estimated from the HPLC curve taking into account the estimated absorbances at 280 nm according to Pew and Connors.[11] The third fraction, **C**, consists of cross-coupled β–*O*-4 (**7**), dimeric β–*O*-4 (**8**) and β–β from coniferyl alcohol (**9**). Hα′ of compound **8** is at δ 6.62 (d), Hα′ of compound **7** is at δ 5.85 (m) and Hβ′ of pinoresinol (**9**) is at δ 3.14 (br s). The results of the quantitative estimations are collected in Table 2.

Oxidation with H_2O_2–HRP

Coniferyl alcohol was first oxidized alone with 0.5 equivalents of oxidant (Exp. 2). The β–5 (**5**), β–*O*-4 (**8**) (mixture of *erythro* and *threo* isomers) and β–β (**9**) dimers were formed in 24, 16 and 12% yield, respectively, which is well in accord with earlier results.[1,2] Some coniferyl alcohol remained (36%) and 12% was oxidized further to oligomeric products. When the oxidation was repeated with addition of an equimolar amount of β–*O*-4 model compound **2** (Exp. 1), the result was very similar. Apart from coupling products **5**, **8** and **9**, only dimeric products from coniferyl alcohol were recovered together with unchanged **2**.

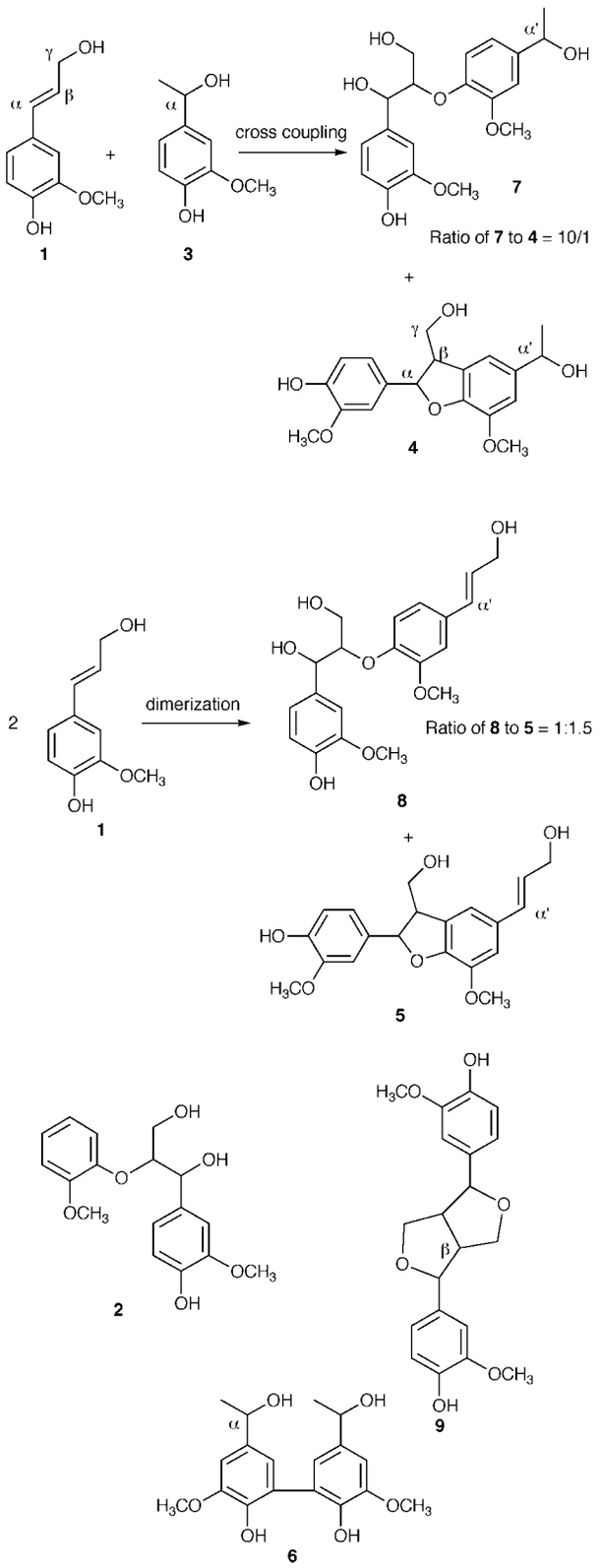
Cross-coupling was then attempted with equimolar amounts of coniferyl alcohol and apocynol. Again the main products were the coniferyl alcohol dimers **5**, **8** and **9** together with some dimer **6** from apocynol. [Oxidation of apocynol **3** alone gave the biphenyl **6** (34%) together with some of the corresponding dioxepine.[11,12]] A small amount of cross-coupling product (≈5%) was isolated and identified as the β–*O*-4 structure **7** by comparison with an authentic sample. Erickson and Miksche[13] have reported a β–*O*-4 cross-coupling product as a main product in the oxidation of coniferyl alcohol with a guaiacyl model compound, but no details were reported. In order to increase the amount of cross-coupling by slow addition of oxidant and coniferyl alcohol (Zutropf) the addition was extended over 5.5 h and, further, to 21 h. This almost doubled the amount of cross-coupling product and, furthermore, a small amount of cross-coupled β–5 product **4** was identified for the first time. The amount of **4** was only one-tenth that of **7**, which means that the regioselectivity of cross-coupling strongly favours β–*O*-4 structures over β–5 structures. A still slower addition of coniferyl alcohol was achieved by letting the coniferyl alcohol diffuse through a dialysis membrane as suggested by Tanahashi and Higuchi.[14] In this case the coniferyl alcohol did not form a dimer on oxidation; instead, it coupled with apocynol to give a cross-coupled β–*O*-4 product **7** (Fig. 1, III). This again demonstrates the strong tendency of monolignols to form β–*O*-4 products in cross-coupling.

Manganese triacetate oxidations

Manganese triacetate in glacial acetic acid has been used to produce oligomeric lignols from coniferyl alcohol. In our experiment, $Mn(OAc)_3$ was added to an equimolar mixture of **1** and **3** all at once (Exp. 8). In the dimer fraction, 18% was β–*O*-4 cross-coupling product **7**. The *erythro*:*threo* ratio was 65:35. Coniferyl alcohol was also oxidized alone (Exp. 7) with $Mn(OAc)_3$ and the proportions of β–5, β–*O*-4 and β–β dimers (22:50:2) are similar to those values observed by Landucci and Ralph.[15] The manganese triacetate–acetic acid system clearly favours the formation of β–*O*-4 structures. The results of Exp. 8 are presented in Fig. 1, II.

MnO_2 and $FeCl_3$

The manganese dioxide and the ferric chloride oxidations (Exp. 9 and 10) gave similar results to the hydrogen peroxide–HRP oxidations.



Scheme 1 Dimerization and cross-coupling products.

In all experiments there was an oligomeric fraction. This material was similar to DHPs. From ^{13}C NMR spectra it could be seen that in HRP–H_2O_2 oxidations there were more β–*O*-4 than β–5 linkages in Zutropf experiments, while in Zulauf experiments equal amounts of β–5 and β–*O*-4 linkages were found. The oligomeric fraction in $Mn(OAc)_3$ oxidations consisted mainly of β–*O*-4 structures with some β–5 and β–β structures present.

Discussion

It has long been presumed that, in the preparation of synthetic lignin, a slow rate of addition of monomer gives a more 'lignin-like' product.[16] Experimental results supporting this idea were found by Lai and Sarkanen.[17] They found that slow addition of monomer produced a synthetic lignin with a larger proportion of β–*O*-4 structures. This was interpreted as the result of 'end-wise' polymerization which constitutes cross-coupling of the monomer with phenols on the polymer chain. This effect has so far only been observed in polymerization experiments under conditions that are notoriously difficult to reproduce in a satisfactory manner. The present experiments reveal the actual coupling step and make it possible to determine the regioselectivity of the cross-coupling reaction without interference from other reactions as in the formation of oligomeric DHPs.

Using H_2O_2–HRP and fast addition of coniferyl alcohol (the Zulauf method) we obtained 5% cross-coupling product. With slow addition (Zutropf) the yield of cross-coupled β–*O*-4 dimer rose to ≈10% and a small amount (≈1%) of cross-β–5 dimer was detected. Diffusion through a dialysis membrane[14] proved to yield a slow enough rate of addition. In a very slow reaction, coniferyl alcohol formed only cross-coupling product. This demonstrates that it is possible to regulate the degree of cross-coupling by controlling the rate of addition of monolignol.

The oxidation potential of the oxidant may also influence the rate of cross-coupling. In experiments with $Mn(OAc)_3$–acetic acid, Landucci and Ralph[15] have reported that oxidation of coniferyl alcohol with $Mn(OAc)_3$ gives mainly β–*O*-4 dimers. In our experiments a large amount (50%) of β–*O*-4 dimer from coniferyl alcohol was formed but also a remarkable amount (18%) of cross-β–*O*-4 dimer, and only 1% of cross-β–5 dimer (Exp. 8). In this case both the oxidant and the solvent polarity were different from those in the HRP oxidations. Solvent polarity has been shown to influence the regioselectivity of coupling.[8,18]

In conclusion, it is evident from these studies that the ratio of cross-coupling to dimerization of monolignols to a large extent determines the outcome of lignin biosynthesis. This in turn is determined mainly by relative oxidation potentials of monolignols and the rates of addition of the monolignols. The role of the oxidant still remains to be determined.

Experimental

General

All mps were measured on an Electrothermal (digital melting point) apparatus in open capillary tubes and are uncorrected. The buffered aqueous solution was obtained using citric acid (0.01 M)–phosphate (0.02 M) buffer (pH 3.5). Horseradish peroxidase (EC 1.11.1.7) was from Serva, activity 450 U mg^{-1}. 30% aq. hydrogen peroxide (Merck) was diluted to give a 3% solution (≈0.8 mol dm^{-3}) before use. Silica gel for column chromatography was Merck Kieselgel 60 (230–400 mesh). TLC was performed on silica gel plates (Merck Kieselgel 60 F_{254}). Spots were made visible with UV light. In the dialysis experiment regenerated cellulose tubular membrane (Cellu Sep T2, nominal MWCO 6000–8000) was used. ^1H NMR and ^{13}C NMR spectra were recorded at 200 MHz with a Varian Gemini instrument. Deuteriochloroform was used as solvent. Mass spectra were recorded on a JEOL JMS-SX102 instrument. HPLC was performed using a Waters 600 pump, LiChrospher Si 60 (5 μm) columns (0.4 × 25 cm and 1 × 25 cm) and Waters 996 UV spectrophotometric detector with detection at 280 nm. Hexane–ethyl acetate (11 : 9) was used as eluent. The injection volume was 20 or 500 mm^3. Evaporations were conducted under reduced pressure at a temperature less than 40 °C. Products were acetylated with dry acetic anhydride and pyridine (1 : 1) overnight at room temperature.[19] THF was purified by distillation over sodium.

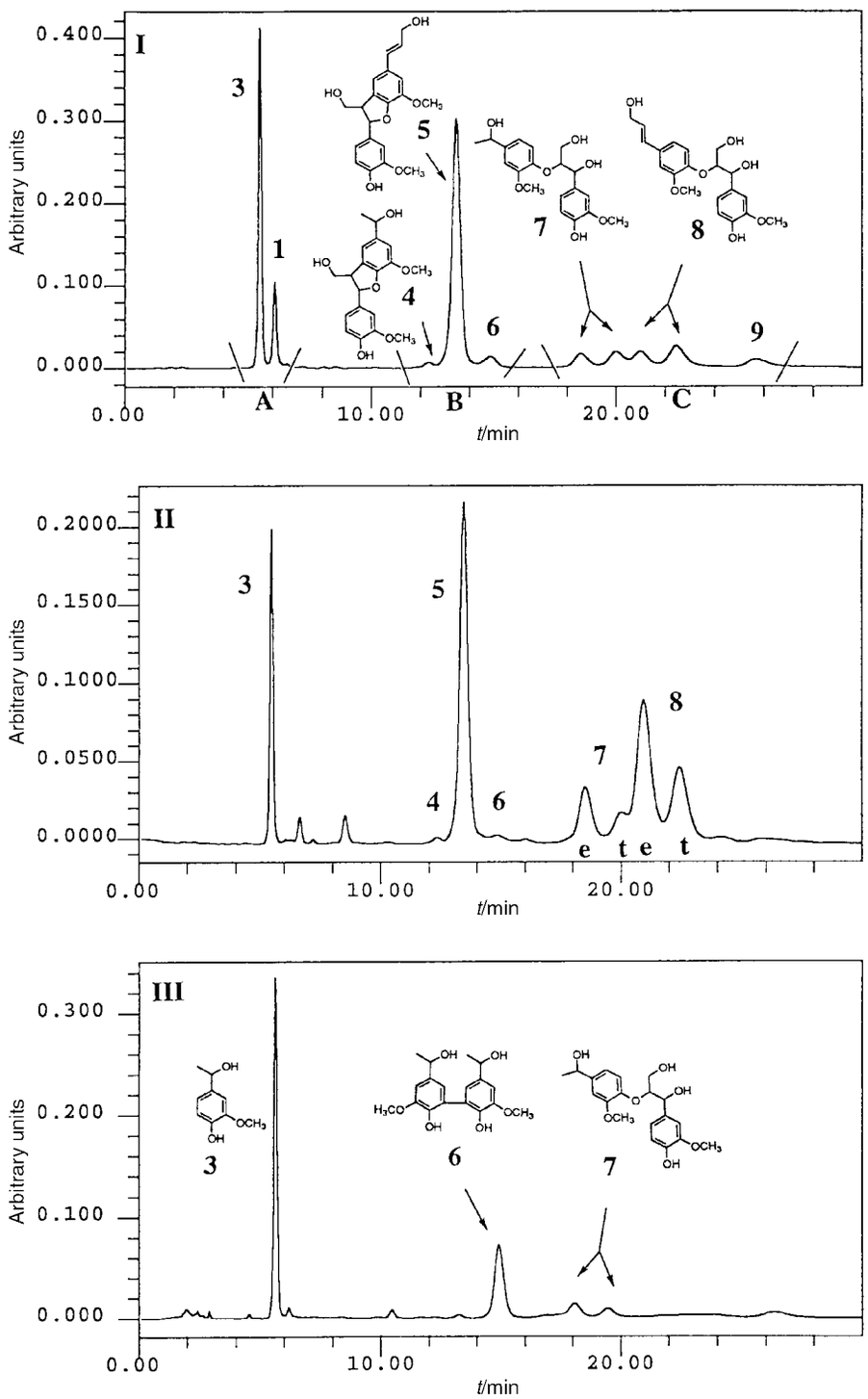


Fig. 1 HPLC chromatograms: I, H_2O_2–HRP oxidation, Zutropf. II, $Mn(OAc)_3$ oxidation, Zulauf. III, H_2O_2–HRP oxidation, addition through dialysis membrane. (The *erythro* and *threo* forms of **7** and **8** are marked in chromatogram II.)

Materials

Coniferyl alcohol **1** was prepared from vanillin (commercial grade, Fluka). A Knoevenagel reaction with vanillin and malonic acid[20] followed by esterification with ethanol–sulfuric acid gave ethyl ferulate. Reduction of ethyl ferulate to coniferyl alcohol was done with DIBAL-H as described by Quideau and Ralph.[21] Apocynol **3** was prepared from acetovanillone (commercial grade, Aldrich) as described by Bailey and Dence.[22] Compound **4** has not been reported previously. A sample was isolated from an oxidation experiment (Exp. 5) and identified by ^{1}H NMR, MS and HRMS. Spectral data for the *triacetate of compound* **4**: δ_H 1.57 (3 H, CH_3), 2.09 (3 H, $COCH_3$), 2.12 (3 H, s, $COCH_3$), 2.35 (3 H, s, $COCH_3$), 3.86 (3 H, s, OCH_3), 3.96 (3 H, s, OCH_3), 3.90 (1 H, m, Hβ′), 4.28–4.56 (2 H, m, Hγ′), 5.57 (1 H, d, *J* 6.6 Hz, Hα′), 5.87 (1 H, q, Hα′), 6.86–7.06 (5 H, ArH); *m/z* 472 (M^+, 1%), 412 (45), 352 (15), 310 (100), 295 (27), 278 (14), 195 (7) and 175 (8) (Found: M^+, 472.1726. $C_{25}H_{28}O_9$ requires M, 472.1733).

Compounds **5** and **9** were isolated from the oxidation mixture of coniferyl alcohol (Exp. 2) and identified by comparison of retention time and NMR spectra with those of authentic compounds.[23,24] Compound **6** was prepared from acetovanillone according to published procedures.[25,26] The β–*O*-4 cross-coupling product **7** was synthesized for HPLC identification. The synthesis is based on the procedure developed by Nakatsubo and co-workers, substituting carbonyl-protected acetovanillone for guaiacol.[27–31] Mp 62–65 °C; *m/z* 364 (M^+,

5%), 346 (19), 328 (8), 316 (12), 298 (24), 269 (7), 239 (34), 194 (88), 176 (100), 150 (95); HRMS (Found: M^+, 364.1510. $C_{19}H_{24}O_7$ requires *M*, 364.1522). Compound **7** has been synthesized earlier by a different route and the NMR spectrum has been published by Landucci and Ralph.[32]

In experiment 3 some tetrameric dioxepine from apocynol (*cf.* ref. 11) was isolated. Spectral data for the *dioxepine tetraacetate*: δ_H 1.56 and 1.65 (together 9 H, 2 d, *J* 6.6 Hz, 3 × CH_3), 2.10, 2.15, 2.16 and 2.21 (each 3 H, s, $COCH_3$), 3.73, 3.88, 3.92 and 3.94 (each 3 H, 4 s, 4 × OCH_3), 5.85–6.25 (4 H, m, Hα′ and quinone H), 6.81–7.17 (7 H, ArH and quinone H); δ_C 21.2 (Ar-CO*C*H_3), 22.1 and 23.0 (CO*C*H_3 and CH_3), 56.1 (OCH_3), 56.7 (OCH_3), 109.9–153.5 (arom.), 168.8 (*C*OCH_3), 170.8 (*C*OCH_3) and 178.9 (C=O); *m*/*z* 788 (M^+, 3%), 728 (1), 668 (9), 608 (12), 568 (9), 548 (15), 504 (12), 488 (32), 462 (17), 446 (46), 341 (46), 298 (68), 281 (100) (Found: M^+, 788.2667. $C_{42}H_{44}O_{15}$ requires *M*, 788.2680).

Oxidation of coniferyl alcohol and apocynol

All oxidation experiments were done under an argon atmosphere. Reaction conditions are summarized in Table 1. The ethyl acetate extract was washed with water, dried with Na_2SO_4 and evaporated. The crude product was acetylated and fractionated with flash chromatography (eluent: ethyl acetate–hexane 9 : 11).

Oxidations with H_2O_2–HRP

Zulauf method—general procedure.—Apocynol (0.28 g, 1.66 mmol) and coniferyl alcohol (0.30 g, 1.66 mmol) were dissolved in acetone (25 cm^3) and horseradish peroxidase (10 mg; 450 U mg^{-1}) in buffer solution (100 cm^3) was then added. H_2O_2 (0.83 mmol), diluted to 5 cm^3 with buffer, was added to the solution over 30 min. The mixture was stirred for an additional 30 min and then extracted with ethyl acetate.

Zutropf method.—Apocynol (0.28 g, 1.66 mmol) and horseradish peroxidase (10 mg; 450 U mg^{-1}) were dissolved in acetone (1 cm^3)–buffer (4 cm^3). H_2O_2 (0.83 mmol) diluted to 15 cm^3 with buffer, and coniferyl alcohol (0.30 g, 1.66 mmol), dissolved in acetone (6 cm^3)–buffer (9 cm^3), were added gradually to the solution *via* syringe pump over 5.5 h (Exp. 5). The mixture was then stirred for an additional 30 min and extracted with ethyl acetate. In Exp. 6, addition was done over 21 h with peristaltic pumps following the procedure described by Kirk and Brunow.[33]

Oxidation with $Mn(OAc)_3$

Apocynol (0.28 g, 1.66 mmol) and coniferyl alcohol (0.30 g, 1.66 mmol) were dissolved in glacial acetic acid (30 cm^3), and solid $Mn(OAc)_3$ (0.67 g, 2.5 mmol) was added to the solution. The mixture was stirred for 30 min, poured into water, and extracted with ethyl acetate. The ethyl acetate layer was also washed several times with freshly prepared 10% aq. $NaHCO_3$.

Oxidation with MnO_2

Apocynol (0.17 g, 1.0 mmol) and coniferyl alcohol (0.18 g, 1.0 mmol) were dissolved in acetone (15 cm^3)–buffer (60 cm^3) and the mixture was added to dry MnO_2[34] (0.43 g, 5.0 mmol). The mixture was stirred for 15 min, then was filtered through a bed of Celite and extracted with ethyl acetate.

Oxidation with $FeCl_3$

Apocynol (0.17 g, 1.0 mmol) was dissolved in acetone (10 cm^3)–distilled water (55 cm^3). A solution of $FeCl_3$ (0.16 g, 1.0 mmol) in 5 cm^3 of water, and a solution of coniferyl alcohol (0.18 g, 1.0 mmol) in 5 cm^3 of acetone, were added in ten portions to the solution over 30 min. The mixture was then stirred for an additional 30 min and extracted with ethyl acetate.

Dialysis experiment

Apocynol (0.17 g, 1.0 mmol) dissolved in acetone (1 cm^3), and HRP (20 mg) in buffer (10 cm^3), were put into the dialysis tube. Coniferyl alcohol (0.18 g, 1.0 mmol) as a solution in acetone (1 ml), and H_2O_2 (0.5 mmol), were added to the buffer solution (250 ml) and poured into the conical flask. The tube was then placed into the solution and stirred with a magnetic stirrer for 17 h. The contents of the tube and the outer buffer solution were extracted separately with ethyl acetate, and the organic layers were dried (Na_2SO_4) and evaporated.

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