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Lignin Chemistry: Biosynthetic Study and Structural Characterisation of Coniferyl Alcohol Oligomers Formed In Vitro in a Micellar Environment

Samantha Reale,[a] Francesca Attanasio,[b] Nicoletta Spreti,[a] and Francesco De Angelis*[a]

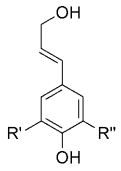
Abstract: Model coniferyl alcohol lignin (the so-called dehydrogenative polymerisate, DHP) was produced in water under homogeneous conditions guaranteed by the presence of a micellised cationic surfactant. A complete study of the activity of the enzymatic system peroxidase/H_2O_2 under our reaction conditions was reported and all the reaction products up to the pentamer were characterised by ^{1}H NMR spectroscopy and ESI mass spectrometry. Our system, and the molecules that have been generated in it, represent a closer mimicry of the natural microenvironment since an enzyme, under micellar conditions, reproduces the cell system better than in buffer alone. On the basis of the oligomers structures a new biosynthetic perspective was proposed that focused attention on a coniferyl alcohol dimeric quinone methide as the key intermediate of the reaction. A formal, strictly alternate sequence of a radical and an ionic step underlines the reaction, thus generating ordered oligolignols structures. Alternatively to other model lignins, our olignols present a lower degree of radical coupling between oligomeric units. This offers a closer biosynthetic situation to the observation of a low rate of radical generation in the cell wall.

Keywords: coniferyl alcohol · lignin · mass spectrometry · polymers · surfactants

Introduction

Lignin, the most abundant organic substance on earth after cellulose, is a structural polymer present in all woody plants. Over the past 170 years of research,[1] lignin has proven to be quite an intractable macromolecule, and many questions still exist about its formation, structure, occurrence and commercial utilisation.

Concerning lignins structural elucidation, the main problem is related to the difficulty of isolating it from the other wood components without damaging its structure. In addition, in contrast to proteins and nucleic acids which, by simple hydrolysis, give rise to their constituent molecules, no degradation methods have been developed for generating the monomeric building blocks—of which there are only three (*p*-hydroxycoumaryl, coniferyl (**1**) and sinapyl alcohols)—of the lignin polymer in a structurally unaltered



R' = R" = H	*p*-Hydroxycoumaryl alcohol
R' = OCH_3; R"= H	Coniferyl alcohol (**1**)
R' = R"= OCH_3	Sinapyl alcohol

form. The high number and variegated forms of linkages that occur among the three monomeric components and the resistance to degradation of the ether bonds that frequently occur limit the extent to which analytical and degrading procedures can be used to elucidate the lignin structure. It is also worth observing that the lignin macromolecule exists in different structural assemblies, depending on its distribution in a plethora of vegetable species, its age and plant distribution loci. It is, in fact, more appropriate to refer to the polymer as "lignins", rather than using the singular form.

Owing to the problems associated with the degradation and analytical studies, investigations on the growth course of lignin by starting from its precursors—*p*-hydroxycinnamyl

[a] Dr. S. Reale, Prof. N. Spreti, Prof. F. De Angelis
Department of Chemistry, Chemical Engeeneering and Material
University of L'Aquila, Via Vetoio
I-67100 Coppito, L'Aquila (Italy)
Fax: (+39) 0862433753
E-mail: francesco.deangelis@univaq.it

[b] Dr. F. Attanasio
Tumor Cell Invasion Laboratory, Consorzio Mario Negri Sud
Via Nazionale 8 A, I-66030 S. Maria Imbaro, Chieti (Italy)

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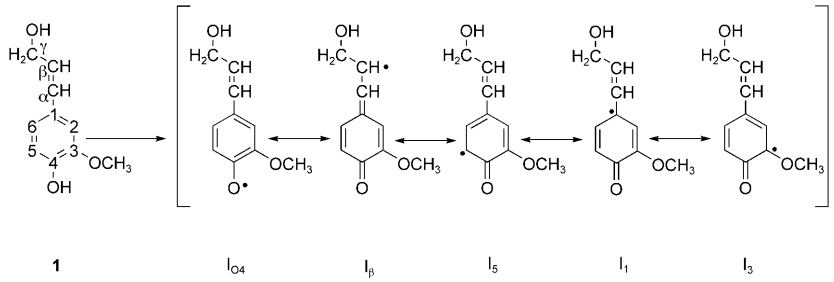
alcohols—have been used extensively in the attempt to elucidate the lignin structure. However, since the polymerisation reaction of the monomeric lignin precursors cannot be studied in vivo, many theories on lignin structure and biosynthesis still rely upon in vitro experiments.[2–5]

A major milestone in lignin chemistry was Freudenberg's success, almost sixty years ago, in polymerising coniferyl alcohol into a lignin-like dehydrogenative polymer (DHP) by using a fungal laccase and other oxidative enzymes.[6–8] Only coniferyl alcohol, the most abundant lignin constituent, was used as the starting material to simplify the structural investigations on the dehydrogenation products. In fact the number of products would increase and they would be largely more complex, if the other two monomeric precursors of lignin (*p*-hydroxycoumaryl and sinapyl alcohols) were also present in the reaction mixture.

In the presence of peroxidase and hydrogen peroxide, as well as in the presence of laccase, coniferyl alcohol (**1**) undergoes dehydrogenation by losing its phenolic hydrogen atom to form a phenoxy radical, stabilised by resonance according to the mesomeric forms shown in Scheme 1.[8]



Scheme 1. Enzymatic dehydrogenation of coniferyl alcohol (**1**) and related phenoxy radicals.

The reaction continues through the radical coupling of two of these mesomeric forms to give a dilignol molecule, and then proceeds by radical and/or ionic steps to form higher lignols.

Some problems, however, are connected with Freudenberg's oligolignols and DHP synthesis. From the biological point of view, lignins appear to be synthesised by a non-enzymatic process occurring through/outside the living cell membrane in a polysaccharide gelified environment, finally resulting in the plant cell wall lignification.[9] This particular issue has been addressed in the last few years by Monties and co-workers, who succeeded in the preparation of lignin polymeric models under conditions that mimic the cell-wall lignification environment. They studied the β-glucosidase/peroxidase-triggered polymerisation of coniferin (coniferyl alcohol glucoside)[10,11] and coniferyl alcohol polymerisation in the presence of pectin.[12,13] In both cases it has been demonstrated that the structures of the obtained polymers approximate those of native lignins more closely than the pure coniferyl lignin model, whereas the latter leads to the isolation of pectin-DHP complex.

As to, in particular, the evaluation of the type of intermolecular linkages that are involved in "artificial" lignins (DHP) devoid of the saccharide moieties, the in vitro studies are based, in general, on the very first procedure described by Freudenberg himself in 1958 for the polymerisation of coniferyl alcohol.[14] According to this procedure, coniferyl alcohol is reacted in phosphate buffer at pH 6.5, in the presence of horseradish peroxidase and hydrogen peroxide. The main problem associated with this procedure is the instantaneous separation of the growing oligomers and the consequent formation of a heterogeneous reaction medium for which the polymerisation process is practically over. A series of technical solutions to the problem have been put forward[15–17] for trying to keep in solution the polymerisation products while the reaction proceeds, but they have only the effect of retarding the unavoidable precipitation of the growing polymer.

For example, Kirk and Brunow proposed a procedure in which a solution containing both coniferyl alcohol and the enzyme and a solution containing H_2O_2 were added simultaneously over a long period of time to a stirred solution of vanillyl alcohol or guaiacyl glycerol.[16] These two substances, following the suggestion of Adler,[17] serve as water-soluble sites of polymerisation and would retard, but not inhibit, the precipitation of the starting material and intermediates or of the oligomers and polymer itself.

It is also worth pointing out that, although DHPs were found to be good models for lignins, their structures, which are strongly dependent on the polymerisation conditions, may be quite different from the natural polymer,[2–5,18] principally because the wood cell environment, in which the native lignins are assembled, is dramatically different from the in vitro environment. In addition, as we have already pointed out, the oligolignols once formed in vitro subtract themselves very rapidly from a possibly higher degree of polymerisation, thus conferring to DHPs very low degrees of polymerisation.

From the picture outlined above it follows that a major achievement in the study of lignins and synthetic DHPs would be the concept of a reaction medium in which the starting material and the growing polymer would dissolve, still preserving the catalytic activity of horseradish peroxidase. In a former preliminary study we have reported on the in vitro reaction conditions generated by a cationic surfactant in phosphate buffer, able to maintain in solution the growing DHP even for weeks, still permitting horseradish peroxidase to exert its catalytic activity.[19] Cetyltrimethylammoniumsulfate $[(CTA)_2SO_4]$ was used,[20] at a concentration higher than the critical micelle concentration (c.m.c.), to allow the formation of a novel in vitro model of lignin. The

polymerisation process was shown to be an alternate, ordinate sequence of radical and ionic steps. On the other hand, it is worth nothing that the already cited pectin-associated DHP[12] shows solubility characteristics that are not dissimilar to our DHP formed in the presence of surfactant.

In the present paper, we investigate in detail the polymerisation process of coniferyl alcohol catalysed by horseradish peroxidase, in the presence of cetyltrimethylammoniumsulfate: 1) The catalytic activity of the enzyme, under these conditions, was studied. 2 and 3) The coniferyl alcohol oligomers were analysed by HPLC, isolated and fully characterised by ^{1}H NMR spectroscopy and ESI mass spectrometry. 4) Finally, a possible reaction pathway for coniferyl alcohol polymerisation under our biosynthetic conditions was proposed.

Results and Discussion

Peroxidase characterisation in the presence of cetyltrimethylammoniumsulfate and optimisation of the reaction conditions for coniferyl alcohol polymerisation: A surfactant, in principle, should be able to allow the solubilisation in water of a lipophylic compound, such as coniferyl alcohol, the starting material we have chosen for studying in vitro lignin biosynthesis, as well as its polymerisation products. On the other hand, its presence should not interfere with the stability and activity of horseradish peroxidase, the enzyme chosen to catalyse the polymerisation reaction.

To investigate this latter issue, we have examined a series of commercially available and synthetic surfactants with different head-group charge (i.e. anionic, cationic and zwitterionic) at various concentrations,[21] looking for the stability/activity of horseradish peroxidase against *o*-phenylendiamine, a water-soluble model substrate that is oxidised by hydrogen peroxide in the presence of the enzyme. The reaction course was followed by UV spectroscopy (see the Experimental Section).

Finally, $(CTA)_2SO_4$ was selected as the surfactant of choice. It is actually a surfactant synthesised from commercial CTABr.[20] CTABr, in fact, is not suitable when used in the presence of an oxidative reagent as it can also act as a substrate under such reaction conditions.

The influence of the surfactant on the enzyme activity was evaluated, in a phosphate buffer, at different surfactant concentrations (3×10^{-3}, 9×10^{-3} and 27×10^{-3} M—c.m.c. being 2.7×10^{-4} M). It is worth noting that the minimum surfactant concentration that is able to guarantee a clear solution of coniferyl alcohol is 1.0×10^{-4} M. This concentration, however, is not sufficient to avoid the precipitation of the polymerisation products. A buffered solution of *o*-phenylendiamine was used as a reference for the evaluation of the enzyme activity, following the substrate oxidation by UV spectroscopy. It appears that the enzyme works for over 24 h at any surfactant concentration. Its activity is at a maximum at the minimum surfactant concentration, whereas the activity is reduced to 50% at the highest surfactant concentration. It is worth emphasising also that at $[(CTA)_2SO_4]=27\times10^{-3}$ M, both coniferyl alcohol and its polymerisation products are kept in solution (precipitation is never observed), thus creating a homogeneous reaction environment (see below).

To rule out either a spontaneous coniferyl alcohol polymerisation under oxidative conditions or any micellar catalysis, two series of control experiments were carried out. In the first of them, an acetone solution of coniferyl alcohol was slowly added to a phosphate buffer solution of hydrogen peroxide (at three different concentrations: 0.1, 0.8 and 1.6%), in the absence of the enzyme. The solution was then monitored by TLC for over four days; during this period of time coniferyl alcohol was absolutely stable. In the second series of experiments $(CTA)_2SO_4$ (same concentrations listed above) was added, in the absence of the enzyme to exclude any possible micellar catalysis in the polymerisation process. Also in these cases the coniferyl alcohol polymerisation does not take place at all.

In our initial experiments, carried out on 5 mg of coniferyl alcohol each, we decided to follow the substrate polymerisation, catalysed by horseradish peroxidase/hydrogen peroxide in the presence of the surfactant, by TLC (eluent: $CHCl_2$/MeOH 20:1). The reaction medium is constituted by 5 mM phosphate buffer at pH 6.5, hydrogen peroxide (0.1%), and 1.75 U of horseradish peroxidase (Sigma type II 200 U mg^{-1}), in the presence of $(CTA)_2SO_4$ at 3×10^{-3} M, the minimum surfactant concentration able to maintain both coniferyl alcohol and all the incoming oligomerisation products in solution. A control reaction in buffer solution alone, without the surfactant, was also carried out. It is worth noting that, soon after the enzyme addition, the pure buffer reaction medium becomes immediately heterogeneous with the formation of a precipitate, whereas the solution created in the presence of the surfactant remains clear due to the surfactants ability to aid the solubilisation of the starting material and of all the incoming polymerisation products.

To evaluate the effect of the surfactant concentration on the kinetics of the coniferyl alcohol polymerisation, the reaction course was followed by UV spectroscopy. Accordingly, three experiments at increasing surfactant concentration (see above) were carried out. The reaction progress was monitored by following the increase of relative maxima in the range from 300 to 500 nm (in particular at 330 and 390 nm), which are clearly related to the reaction products. Incidentally, the absorption maximum at 330 nm is due to pinoresinol (a coniferyl alcohol dimer) or the pinoresinyl moiety present in any of the produced oligomers eventually (our own measurements on reference samples). The coniferyl alcohol disappearance could not be followed, since its absorption maximum ($\lambda_{max}=260$ nm) is in the same absorption range of the enzyme and hydrogen peroxide.

In contrast to the test reactions carried out by using *o*-phenylendiamine as the substrate, with coniferyl alcohol the maximum velocity is registered at a 9×10^{-3} M surfactant concentration.

A clear picture came out when the reaction was followed by RP-HPLC. At the lowest surfactant concentration (i.e., 3×10^{-3} M), after only 1 min, eight well-separated peaks are present in the chromatographic profile (see the Experimental Section for the chromatographic details). At the same reaction time, with the surfactant concentration at 27×10^{-3} M, only three peaks are present, the one related to coniferyl alcohol being the most abundant. On the other hand, under these last conditions (which we considered safer as they allow a perfect solubility of the reaction products) the substrate is practically totally consumed after 5 min of reaction time. A nice series of products, well-separated by HPLC, is produced (Figure 1). This chromatographic profile, however, fully reproduces that obtained after 1 min at a surfactant concentration of 3×10^{-3} M.

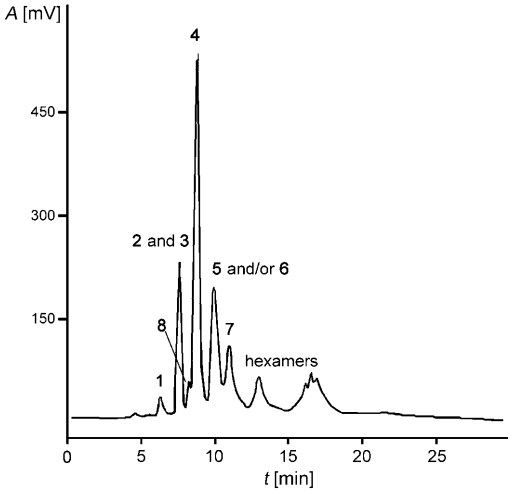
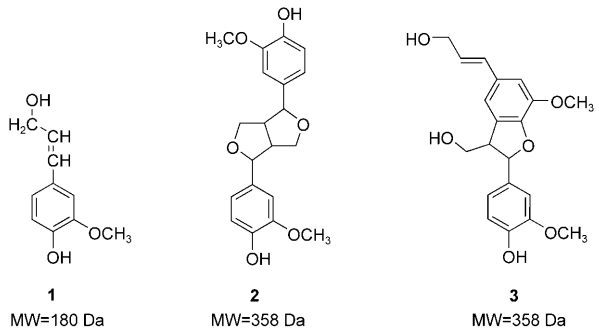


Figure 1. RP-HPLC profile of the reaction mixture after 5 min (numbers refer to structures **1**–**8**).

Thus, to guarantee the homogeneity of the reaction mixture and at the same time to preserve an adequate enzyme activity for the subsequent experiments, the following reaction conditions were chosen: Phosphate buffer (5 mM) at pH 6.5, $[(CTA)_2SO_4]=27\times10^{-3}$ M, coniferyl alcohol (5 mg mL^{-1}), hydrogen peroxide (0.1 %), horseradish peroxidase (1.75 UE).

Separation and identification of coniferyl alcohol polymerisation products: The coniferyl alcohol (**1**) polymerisation reaction (see above for conditions) was followed by RP-HPLC. After 5 min, the coniferyl alcohol is no longer present and the reaction was stopped by the addition of a few drops of 5 % $Na_2S_2O_7$. A first separation attempt was carried out by using TLC (a number of chromatographic runs, combining the bands with the same R_f). Six well-separated bands are in fact present on the chromatographic plate. Unfortunately, once these products are re-analysed by TLC, or even by HPLC, they are revealed to be an even more complicated mixture than the starting reaction mixture itself. This allows the hypothesis of a substantial product instability and interconversion phenomenon (de-polymerisation and re-polymerisation).

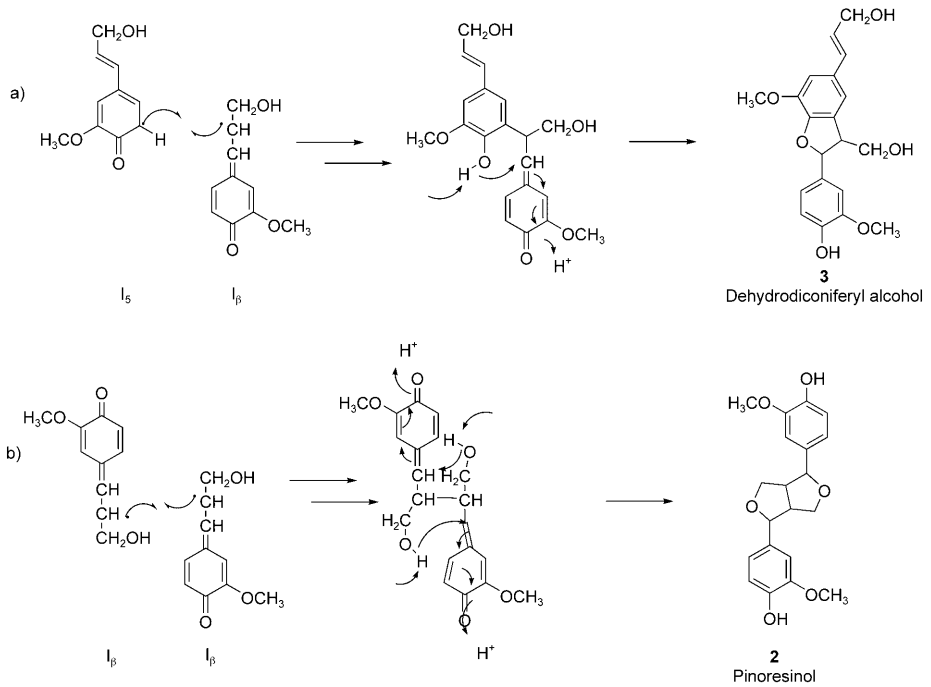
The reaction products (the analytical HPLC profile is shown in Figure 1) were then separated by using semi-preparative RP-HPLC. Five well-separated abundant peaks are in fact present in the chromatographic profile, together with some other minor peaks. However, also in this case an interconversion reaction occurs when concentrating the solutions of the products, always leading again to complex mixtures in which, finally, two main products could be identified. They, even if already present, were not largely abundant in the original reaction mixture but appear to be generated as the separation and workup procedures are carried out. These two "end-products", which were revealed to be stable, have been finally isolated by preparative TLC, purified and characterised by ^{1}H NMR spectroscopy as pinoresinol (**2**) and dehydrodiconiferyl alcohol (**3**) (see the Supporting Information for their ^{1}H NMR spectra).



They are both well-known coniferyl alcohol dimers.[22] Pinoresinol (**2**) is characterised by a β–β structure, since it is formed by a β-radical/β-radical coupling reaction, after coniferyl alcohol oxidation (see Schemes 1 and 2). The dehydrodiconiferyl alcohol (**3**) has the so-called β-5 structure, originating from the coupling of a β-radical from one coniferyl alcohol unit with a 5-radical formed from a second coniferyl alcohol molecule.

To isolate and identify also the other coniferyl alcohol polymerisation products, it was imperative to stabilise them. The reaction mixture was thus treated with acetic anhydride and pyridine so that all of the hydroxy groups present on the coniferyl alcohol polymerisation products were acetylated. Their high reactivity, in fact, would depend on the presence of the free OH groups, the easily oxidisable phenolic ones in particular.

The HPLC analysis of the reaction mixture after acetylation (Figure 2) gives rise to a chromatographic profile that parallels that obtained for the non-acetylated reaction mixture, the main difference being a positive shift in the retention times. The co-eluting free alcohol oligomers were separated after acetylation as a result of the different degrees/nature of acetylation (free phenolic vs. hydroxyl groups—vide infra).



Scheme 2. Dimer formation by one radical step. a) Radical coupling between a coniferyl alcohol radical at the 5-position (I_5) and one at the β-position, which gives rise to a dehydrodiconiferyl alcohol dimer. b) Radical coupling between two coniferyl alcohol radicals at the β-position to give a pinoresinol dimer.

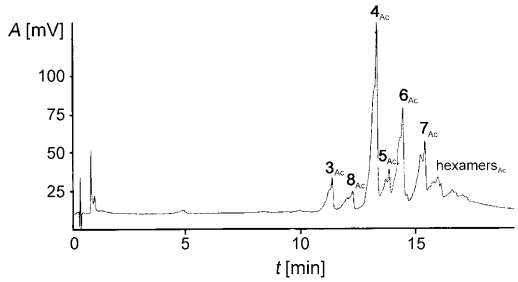


Figure 2. RP-HPLC profile of the solvent extracted/acetylated reaction mixture after 5 min of reaction (numbers refer to structures **3**–**8**).

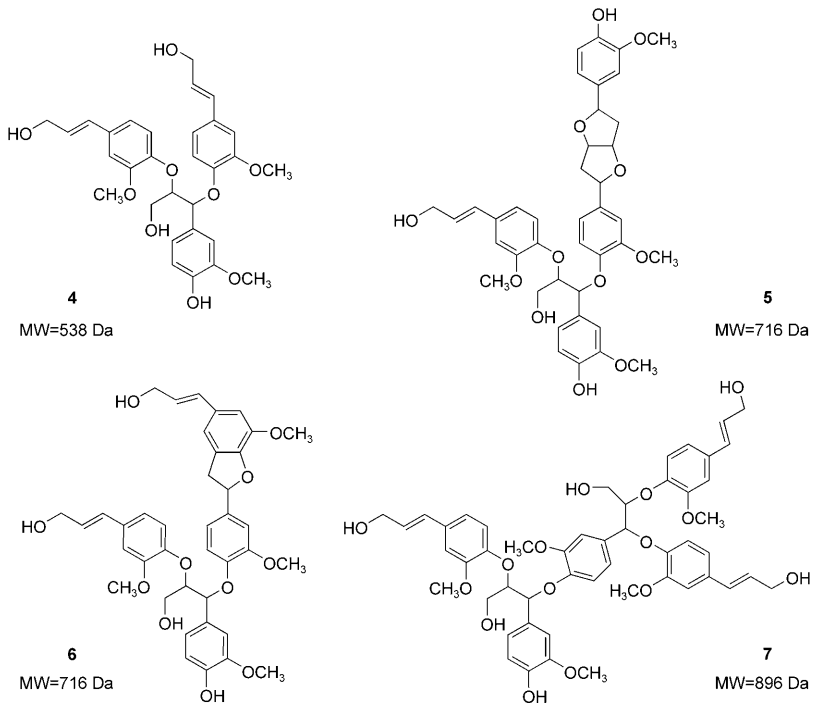
The separation of the acetylated products was carried out by semi-preparative HPLC, giving rise to six pure products: each of them appears as a single peak once re-analysed by analytical HPLC.

The characterisation of the acetylated products of coniferyl alcohol polymerisation was carried out by ^{1}H NMR spectroscopy. Acetylated dehydrodiconiferyl alcohol was easily identified (see the Supporting Information for the ^{1}H NMR spectrum). Its spectrum together with those of the non-acetylated dimers (**2** and **3**) were used as references for the study of the spectra of the more complex molecules (the spectrum of acetylated coniferyl alcohol is also reported in the Supporting Information as a reference datum). At first examination it is apparent that in the high-order oligomerisation products the pinoresinyl and/or dehydrodiconiferyl alcohol moieties are also present. It is important to consider that, according to a well-established biosynthetic process for lignin generation,[23] the polymerisation process would proceed through an oxidative phenol coupling reaction and an addition reaction to an intermediate quinone methide moiety (vide infra).

First of all, for each product, the polymerisation degree was established by counting the methoxy group content, in the ^{1}H NMR spectra. Monomeric coniferyl alcohol only possesses one methoxy group, which is obviously repeated *n* times in the *n*-mer. In addition, the count of the acetyl groups gives the number of the free hydroxy functions present in the non-acetylated molecule. Three acetylated oxygen atoms in a dimer, for instance, means that one of the two hydroxy groups of one coniferyl alcohol unit is engaged in an ether linkage. Moreover, by examining the chemical shifts of the acetoxy groups, it is also possible to attribute their aliphatic or aromatic nature. Finally, by considering that three aromatic protons are present in coniferyl alcohol, any aromatic proton missing in an oligomer implies that one extra aromatic position is involved in an interunit linkage.

The examination of the olefinic proton region gives information on how many propenoic side-chain moieties survive after oligomerisation. Protons that are lacking in the overall olefinic balance are now on sp^3 carbon atoms, involved in β–β, β–O-4, β-5 or α-O-4 interunit linkages, and should be looked for in the corresponding spectral regions. Accordingly, also the methylene groups adjacent to the oxyacetyl moieties produce chemical shifts relevant to their molecular environment, depending whether they are at an allylic position.

All the above considerations have been used to identify the coniferyl alcohol oligomers, which have been isolated by semi-preparative RP-HPLC as fully acetylated derivatives. Besides the dehydrodiconiferyl alcohol (**3**), α-O-4/β-O-4 coniferyl alcohol trimer **4**, two tetramers (**5** and **6**; one including a pinoresinyl and the other a dehydrodiconiferyl alcohol unit) and a pentamer with α-O-4/β-O-4 interunit bonds (**7**) have been fully characterised, again, as peracetylated compounds (the ^{1}H NMR spectra of their acetylated congeners, together with signal attributions, are reported in the Supporting Information). It could be appreciated that the structural complexity of the coniferyl alcohol oligomers increases with the degree of polymerisation, as does the appearance of the proton spectra of their acetyl esters.



Mass spectrometry: To collect more information on the coniferyl alcohol oligomers, a series of ESI mass spectrometric experiments have been carried out.

We already reported on the ESI mass spectrometric analysis of the polymerisation products, as obtained by direct infusion of the reaction mixture, after doping with ammonium acetate to get a better response in the positive-ion mode.[19] All the oligomers up to the octamer were revealed, their masses differing one from the other, alternatively, by 178 and 180 Da. This was interpreted in the light of a regular reaction mechanism that operates for the coniferyl alcohol polymerisation, under our reaction conditions (see the following for more details).

We are now able to give more information on the oligomers structures, looking also at their ESIMS spectra (both in the positive- and in the negative-ion modes) obtained after HPLC separation, as well as of their peracetylated derivatives. Table 1 reports the molecular weights and the mass spectrometric data of the oligomers **1**–**9** (besides coniferyl alcohol itself), together with their fully acetylated derivatives.

The mass spectra (in positive- and negative-ion modes) obtained by direct infusion of the whole reaction mixture are reported in Figure 3a and b (see also Table 1 for peaks attributions). Dimers appear mainly as protonated or deprotonated ions in the positive and negative modes, respectively. Conversely the higher oligomers form ammonium clusters in the positive-ion spectrum, whereas they appear as deprotonated ions in the negative-ion one. It is worth observing that signals up to the octamer (not listed in Table 1) are present in both spectra. The satellite peaks at higher masses with respect to the $M\mathrm{NH_4}^+$ ions (5 Da increment) are due to sodiated ions, as demonstrated also by the CID (collision induced dissociation) spectra obtained by increasing the orefice potential. The sodiated ions, in fact, being more stable than the ammoniated ones, fragment to a lower extent and survive the dissociation process more easily.

It is worth observing that in the CID negative-ion spectrum the small satellite peaks at 2 mass units lower than those relevant to the main oligomers are selected by the induced fragmentation process (see inset in Figure 3b, in which the case of tetramers is shown). The corresponding molecules, which are shown to be more robust than the relevant high-mass congeners, could be possibly formed by a further dehydrogenation—cyclisation process to give trimer **8** and tetramer **9** (tentative structures are shown). It is well-known that cyclic ions are more resistant to fragmentation than the corresponding acyclic ones.

All the results described above were confirmed by the

Table 1. MS data for the coniferyl alcohol oligomers (bold numbers refer to structures **1**–**9**, roman numerals refer to the polymerisation degree—pedix "P" indicates the presence of a pinoresynil moiety in the structure and pedix "D" indicates the presence of a dehydrodiconiferyl moiety—and numbers in italic indicate that the relevant peaks are not present in the mass spectra).

	MW	$M\mathrm{H}^+$[a]	$M\mathrm{NH_4}^+$[a]	$[M-\mathrm{H}]^-$[b]	$[M+\mathrm{AcO}]^-$[b]	$M_{\mathrm{Ac}}\mathrm{NH_4}^+$[a,c]	$[M_{\mathrm{Ac}}+\mathrm{AcO}]^-$[b,c]
1	180	181	198	179	*239*	240	281
2 $\mathrm{II_P}$	358	359	376	357	*417*	460	501
3 $\mathrm{II_D}$	358	359	376	357	*417*	502	543
4 III	538	539	556	537	*597*	724	765
8 $\mathrm{III_P}$[d]	536	537	554	535	*595*	680	721
5 $\mathrm{IV_P}$	716	717	734	715	*775*	902	943
6 $\mathrm{IV_D}$	716	717	734	715	*775*	944	985
9 IV[d]	714	715	732	713	*773*	942	983
V[d]	894	895	912	893	*953*	1122	1163
$\mathrm{V_P}$	896	897	914	895	*955*	1122	1163
7 $\mathrm{V_D}$	896	897	914	895	*955*	1167	1208
$\mathrm{VI_P}$	1074	1075	1092	1073	*1133*	1344	1385
$\mathrm{VI_D}$	1074	1075	1092	1073	*1133*	1386	1427

[a] Positive-ion mode. [b] Negative-ion mode. [c] Peracetylated products. [d] Further dehydrogenated product.

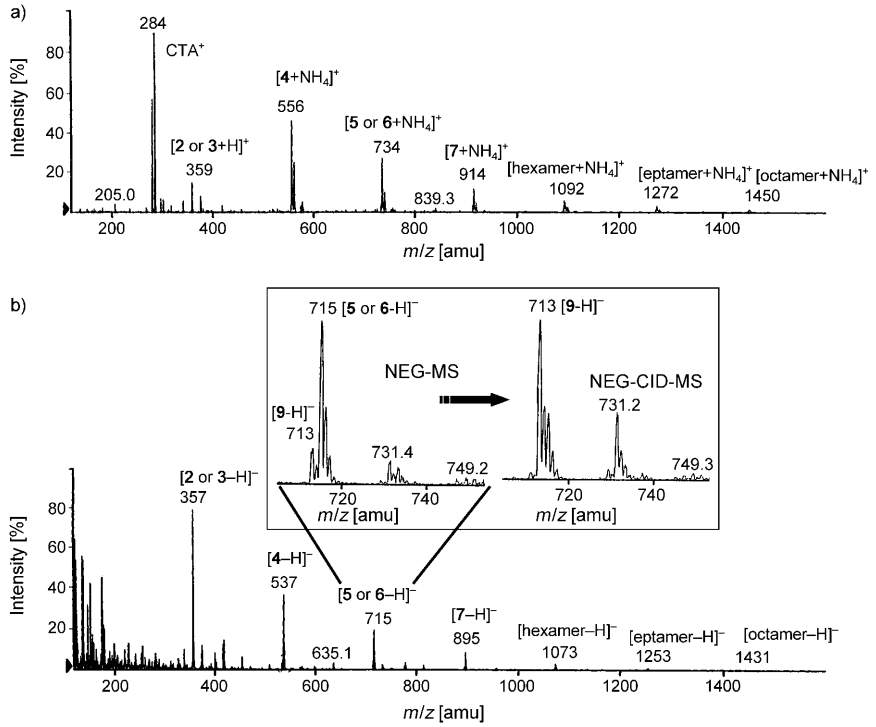
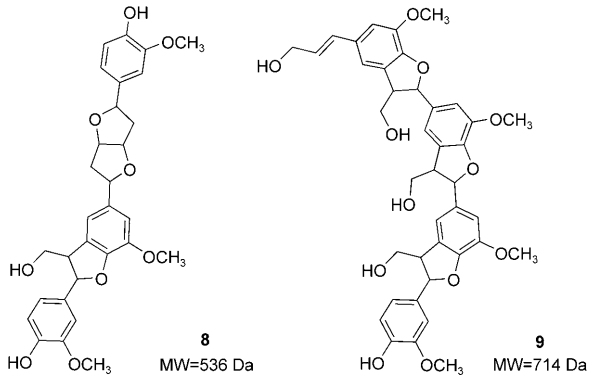


Figure 3. ESIMS spectra in the positive- (a) and negative-ion (b) mode of the reaction mixture. In the inset of the negative-ion spectrum a comparison between the mass spectrum of the tetramers and the relevant CID-MS is shown.



RP-HPLC–ESIMS analysis (the relevant HPLC profile, together with the mass spectra (insets) are reported in Figure 4). Eluent doping with ammonium acetate was employed for a more sensitive mass spectrometric response, thus giving rise to the formation of pronounced clusters with the ammonium cations in the positive-ion mode and with the acetate anions in the negative-ion mode.

The analysis of the mass spectra of the fully acetylated oligomers, obtained after RP-HPLC separation (see Figure 2 for the HPLC profile—refer to Table 1 for the mass peaks attributions) give further structural information, in particular with respect to the differentiation between the oligomers with the same polymerisation degree which comprise, in their molecular structures, a pinoresinyl or, alternatively, a dehydrodiconiferyl moiety. They are, in fact, isobaric molecules that, however, possess a different number of hydroxy functions (see structures **1**–**3**). It follows that their fully acetylated derivatives differ by one acetyl unit and, accordingly, their molecular weights are separated by 42 Da (see Table 1), the one which includes the pinoresinyl unit with a lower molecular weight.

The ESI mass spectra of the separated acetylated oligomers of coniferyl alcohol are reported in the Supporting Information. The molecules always appear cationised by the ammonium ion (i.e., 18 mass units higher than the molecular weight) in the positive-ion spectra, whereas in the negative-ion spectra they get the negative charge by virtue of being complexed by the acetate anion (i.e., 59 mass units higher than the molecular weight). The oligomers up to the tetramer also form dimeric clusters, cationised by the ammonium ion in the positive or by the acetate anion in the negative-ion mode, respectively.

Acetylated dehydrodiconiferyl alcohol is the first compound to be eluted after HPLC separation (structure **3**, for the free alcohol). Its cationised molecular ion appears at *m*/*z*: 502, corresponding to the free alcohol molecular weight (358 Da) plus three acetyl moieties (42 Da × 3) plus the ammonium cation (18 Da). Also the cationised dimeric cluster appears at *m*/*z*: 986. In the negative-ion mode, the relevant peaks (clusters with the acetate anion) are at *m*/*z*: 543 and 1028, respectively. It is worth noting that the acetylated derivative of pinoresinol (its cationised molecular ion should be at *m*/*z*: 460) does not appear to be separated under these chromatographic conditions.

The acetylated trimer (compound **4** is the free alcohol) presents the cationised molecular ion at *m*/*z*: 724 (the free alcohol plus four acetyl moieties and the ammonium ion—*m*/*z*: 765 in the negative-ion mode). The relevant chromatographic peak is preceded by a small signal corresponding to a new trimer that, according to the cationised molecular-ion signal that occurs at *m*/*z*: 680, should comprise the pinoresinyl moiety (only three acetyl moieties are in fact present) and must also be formed by way of a further dehydrogenation process (two mass units less than expected). These types of compounds have also been revealed by the CID-MS experiments, performed on the direct infusion of the

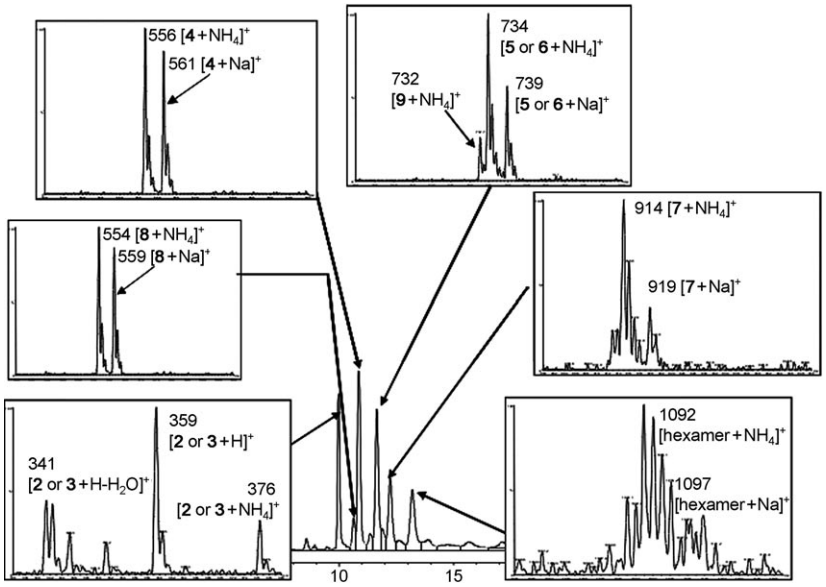


Figure 4. RP-HPLC–ESIMS profile of the coniferyl alcohol polymerisation reaction mixture.

whole reaction mixture (vide supra). We have assigned to this compound the tentative structure of **8**, as a free alcohol. It should be emphasised that oligomers with a higher oxidation degree, with respect to those we are studying herewith, have already been reported by us in a study by MALDI-MS of the coniferyl alcohol polymerisation products, carried out in buffer alone.[24]

The case of the two tetramers, the one comprising the pinoresinyl unit and the other the dehydrodiconiferyl one, is paradigmatic (see structures **5** and **6** for the corresponding free alcohols). The former, in fact, takes four acetyl groups (at *m*/*z*: 902 once cationised by the ammonium ion—*m*/*z*: 943 in the negative-ion mode), whereas the latter is acetylated five times (*m*/*z*: 944 and 985, respectively). The mass difference between the two acetylated isomers is of 42 mass units. The acetylated trimer to which we assigned the tentative structure **9** (*m*/*z*: 942 in the positive-ion spectrum) comprises the dehydrodiconiferylic subunit, and it is further dehydrogenated with respect to **6**.

Only one acetylated pentamer was then revealed (**7** is the corresponding alcohol), its cationised molecular ion occurring at *m*/*z*: 1167 (*m*/*z*: 1208 in the negative-ion mode).

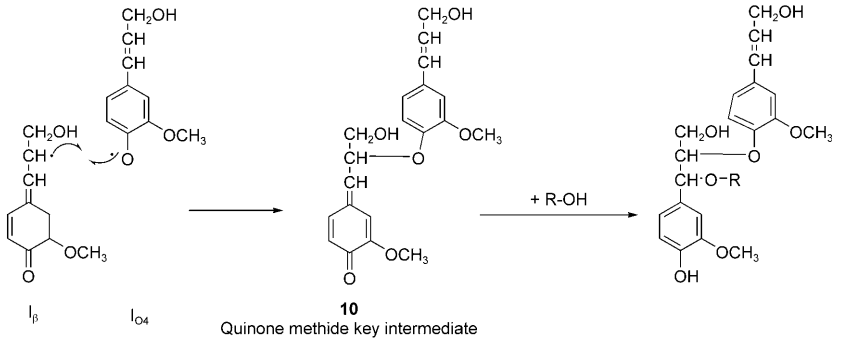
Finally, the signals of two acetylated hexamers also occur, as a non-resolved chromatographic peak. The one including the pinoresinyl moiety is at *m*/*z*: 1345 and the other with the dehydrodiconiferyl unit at *m*/*z*: 1386. In general, however, the acetylated oligomers that possess the pinoresinyl substructure present a lower chromatographic retention time than the corresponding congeners with the dehydrodiconiferyl substructure.

Biosynthesis: On the grounds of the structural information obtained by the ^{1}H NMR spectroscopic analysis and the ESI mass spectrometric investigations of the coniferyl alcohol oligomers, formed by horseradish peroxidase/H_2O_2 oxidative polymerisation in a surfactant doped reaction medium, it is possible to draw a reasonable hypotheses on the biosynthetic pathways leading to their formation.

In general, it should be considered that the reaction conditions we have developed allow the contemporary presence, in solution, of all the reactive species and reaction intermediates. No precipitation occurs. Also the already just-formed oligomeric molecules are present in solution, behaving then as building blocks for the growing polymer.

As to the two dimeric lignols, the mechanism for their formation is already well established,[23] and reported in Scheme 2. Pinoresinol (**2**), in fact, derives from a β–β radical coupling (see Scheme 1 for radical locations). Dehydrodiconiferol (**3**) is formed by way of a radical coupling reaction, involving position 5 of one coniferyl alcohol unit and the β radical of a second monomer, with successive O4-α ring closure by way of an intramolecular electrophilic addition. In both cases, one radical step is involved.

The bond formation between a β-radical and an O4-radical, on two different coniferyl alcohol molecules, produces the reactive key intermediate quinone methide **10**, which then undergoes conjugate electrophillic addition by the other alcohols that are present in solution (Scheme 3).[25]



Scheme 3. Formation of a quinone methide intermediate by radical coupling of a β- radical and an O4-radical to give the so-called β-O4 substructure. The addition to the quinone methide intermediate of a water molecule (with R = H) gives rise to the β-O4 dimer, whereas the addition of a coniferyl alcohol molecule or oligomer will give the trimer **4** or higher oligomers (see structures **4**–**7** and the text).

The addition of a new coniferyl alcohol unit gives rise to the trimer **4**, with an ionic step that follows the radical one. **2** and **3** additions to quinone methide generate the two isomeric tetramers **5** and **6**. In total, two radical steps and an ionic one are involved in this latter case. In all the cases, a new α-O4 bond is formed.

According to this hypothesis, the addition of the trimer to **10** forms the only pentamer **7** (in total, two radical and two ionic steps). On the contrary, two isomers are formed by the addition of the two tetramers giving rise to the hexamers (three radical and two ionic steps are overall involved).

The pathway that has been outlined above firmly relies upon the mass spectral data. The oligomeric units differ from each other by 178 and 180 mass units, alternatively. With the molecular weight of coniferyl alcohol at 180 Da, the coupling reaction of two radicals, that is, one radical step, would give a mass increment of 178 mass units with respect to the starting material. This is the case, for the formation of the two dimeric alcohols and of the quinone methide **10**. Conversely, the addition of one coniferyl unit (i.e., an ionic step) to **10** would result in a 180 mass unit increment. As the polymerisation goes on, one radical step and then an ionic one follow, formally alternating each other and following a regular fashion. This will produce, finally, a mixture of oligomers that, as demonstrated by their molecular weights, differ alternatively by 178 and 180 mass units, according to their increasing degree of polimerisation.

The same considerations hold for the fully acetylated derivatives. They differ from each other by 220 and 222 mass units corresponding, respectively, to 178 plus 42 and 180 plus 42 mass units; 42 Da, again, is the mass increment due to the presence of one extra acetyl moiety. As already observed, however, the presence in the molecule of a pinoresinyl unit would introduce a defect in the mass increment of 42 Da.

On this basis it can be proposed that in our reaction medium radical coupling mainly occurs for the formation of the dimeric structures, that is, pinoresinol (**2**), dehydrodiconiferyl alcohol (**3**) and the quinone methide (**10**). The polymerisation reaction then proceeds through the addition of coniferyl alcohol itself or of the oligomers already present in the reaction medium to the quinone methide **10**. Since no water addition to the quinone methide is observed, it is conceivable that the addition of the coniferyl alcohol oligomers is favoured over the water molecule by the micellar lipophilic environment from which water is excluded. Moreover, as already pointed out by Brunow and co-workers,[4] our working (almost neutral) pH favours the formation of benzyl aryl ethers over the benzylic alcohols, as products of the phenolic addition to the quinone methide.

The decomposition reaction that all the oligomers, with the exception of the dimers, easily undergo also merits consideration. Preparative TLC and solvent evaporation to isolate the products after HPLC separation produces a thorough decomposition that finally leads to the principal formation of pinoresinol and dehydrodiconiferyl alcohol. All the oligomers from **4** to **7**, just to cite only those that have been fully characterised, can form a radical at the phenolic oxygen atom of the former quinone methide moiety (see Scheme 3), which then stabilises itself by releasing the group located at the α-carbon atom. The quinone methide **10** is formed again, whereas the unpaired electron is carried away by a phenolic fragment that in most cases will produce the two stable dimers, eventually. This decomposition reaction, in principle, could be also promoted by acidic or basic catalysis.

Conclusion

Model coniferyl alcohol lignins can be created in water under homogeneous conditions guaranteed by the presence of a suitable cationic surfactant at a concentration higher than c.m.c. The reaction takes place in minutes. No precipitation occurs even by prolonging to hours the reaction time. In the past, many in vitro lignins have been produced, but in all cases the immediate precipitation of the growing polymer was the major drawback to be overcome. A complete study of the activity of the enzymatic catalyst, under our reaction conditions, has been carried out, and all the reaction products up to the pentamer have been characterised by ^{1}H NMR spectroscopy and ESI mass spectrometry. This soft ionisation technique, which usefully complements the well-established pyrolysis mass spectrometry, only recently has been used in lignin studies opening new intriguing perspectives for the structural elucidation and biosynthesis of this molecule.[26]

Based on the oligomers structures that we have identified in the reaction mixture, a new biosynthetic point of view has been proposed now, which focuses attention on the quinone methide **10**, as the key intermediate of the reaction progress. Addition reaction of the other phenolic oligomers that are created in the reaction mixture on this conjugated olefin allows the production of new oligomers with a higher degree of polymerisation, according to a regularly defined chain-reaction module. A formal, strictly alternate sequence of a radical and an ionic step underlines the reaction progress, thus generating ordered oligolignol structures formed by coniferyl alcohol subunits (according to the generally accepted practice of using coniferyl alcohol as the only starting material for the in vitro experiments[4]). This consideration marks an important difference with what is generally reported in the current literature, for which lignin formation is known to proceed in a random fashion by radical and ionic steps.[27,28] Our experiments, in fact, are more in favour of some alternative points of view[29,30] that assume an ordered structure for the natural lignins.

We have also demonstrated that oligomeric lignins, as produced under our reaction conditions, are not stable molecules, at least when attempts for their isolation are carried out. This observation fully parallels all those reported in the literature,[5] in which natural lignins are described as chemically and enzymatically highly unstable molecules, a situation that generally makes their isolation as unaltered mole-

cules difficult and prevents their complete structural characterisation.

In our in vitro model the lignin growth to higher polymers appears to be limited by the coniferyl alcohol supply. This, of course, is not the case of lignin formation in the natural, wooden cells. A common observation, in both cases, is that our oligomers, and the natural polymer, are "alive" molecules, ready to restart a new polymerisation process according to the environmental, natural or even laboratorial conditions.

Finally, three points are to be underlined.[19] It has been considered that an enzyme, under micelle conditions, reproduces better the cell system than in buffer alone.[31,32] As a consequence, we think that our system represents a closer mimicry of the natural microenvironment with respect to the in vitro experiments that have been proposed in the past. Secondly, the lack of water addition to quinone methide-like intermediates, as is the case for our model, marks a difference with the other in vitro studies. Last, but not least, alternatively to other model lignins, our olignols present a lower degree of radical coupling between oligomeric units. This offers a biosynthetic circumstance closer to the observation of a low rate of radical generation in the cell wall,[33–36] thus reproducing in an in vitro experiment what is generally observed in the in vivo lignifications process.

We would also comment, however, that in such a complicated "lignin world" our biosynthetic approach and the results we have obtained usefully complement all the other related in vitro studies. It might be interesting, in fact, to look at the effect of carbohydrates and cellulose, for instance, in the biosynthetic outcome in the presence of a micellised cationic surfactant.

Experimental Section

Materials: Horseradish peroxidase type II, 200 U mg^{-1} (Sigma); coniferyl alcohol HPLC purity grade (Aldrich); HPLC grade solvents (Riedel-deHaën); $(CTA)_2SO_4$ synthesised according to reference [10].

Methods

Test for horseradish peroxidase activity in the presence of $(CTA)_2SO_4$: H_2O_2 (0.03%) and horseradish peroxidase (3.5 mUE) were sequentially added to phospate buffer (1 mL, 9 mM at pH 6.5) containing *o*-phenilendiammine (8.8×10^{-5} M) and $(CTA)_2SO_4$ (3 mM or 9 mM or 27 mM). The reaction mixture was maintained at 30 °C and the UV absorbance at 492 nm (Shimadzu UV 160 A double ray spectrophotomer) was measured after 2,5 min.

TLC: Eluent mixture: CH_2Cl_2/CH_3OH 20:1

Coniferyl alcohol polymerisation in the presence of $(CTA)_2SO_4$: Coniferyl alcohol (5 mg) was dissolved in sodium phosphate buffer (1.0 mL, 5 mM, pH 6.5) containing $(CTA)_2SO_4$ ($2.7\cdot10^{-2}$ M). H_2O_2 (10 μL, 10%) and buffer (5 μL) containing two purpurogallin units of horseradish peroxidase (Type II, 200 U mg^{-1}, Sigma, St. Louis MO) were sequentially added to this solution. The clear solution was vigorously stirred over a period of 5 min, the reaction was then stopped by the addition of one drop of $Na_2S_2O_7$ (5%) and the mixture extracted with ethyl acetate. The organic layer was washed with brine, dried and the solution evaporated under vacuum.

HPLC and HPLC–ESIMS analysis: The sample, dissolved in a solution of ammonium acetate (0.1%)/acetonitrile 50:50 at a concentration of about 2 mM, was eluted (by an HPLC HP1100, Agilent) by a gradient of ammonium acetate (0.1%) (eluent A) and acetonitrile (eluent B) with B ramping from 0 up to 40% within 15 min, flow rate = 0.2 mL min^{-1} in a RP-18 (5 μm) packaged 30–2 mm chromatographic column (Luna, Phenomenex). The column eluent was then split and a 5 μL min^{-1} flow was analysed by the ESI mass spectrometer (Quattro LC-Z-SPRAY ESI source-triple quadrupole, Micromass) with the following source parameters: capillary: 2.87 V, cone voltage: 20 and 35 V for in source fragmentation experiments, extractor: 3 V; source temperature: 120 °C, desolvation temperature: 120 °C. Also the DAD spectra were acquired.

^{1}H NMR analysis: The DHP fractions collected after HPLC separation were dissolved in $CDCl_3$ and analysed in a Bruker 200 MHz NMR spectrometer.

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