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Bioorganic Chemistry 31 (2003) 206–215
BIOORGANIC CHEMISTRY
www.elsevier.com/locate/bioorg

Radical formation and coupling of hydroxycinnamic acids containing 1,2-dihydroxy substituents

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Received 12 December 2001

Abstract

Hydroxycinnamic acids involved in the deposition and cross-linking of plant cell-wall polymers do not usually contain 1,2-dihydroxy substituents, despite the presence of both 3,4-dihydroxycinnamic acid and 4,5-dihydroxy-3-methoxycinnamic acid as intermediates in the biogenesis of lignin. Since the *O*-methyl transferases, enzymes catalysing methylation, are targets for the genetic manipulation of lignin biosynthesis, the potential incorporation of these 1,2-dihydroxated substrates becomes increasingly significant. Using EPR spectroscopy, it was observed that 1,2-dihydroxy substituents did not have an inhibitory effect on radical formation. Increasing the extent of hydroxylation and methoxylation, resulted in an increased ease of substrate oxidation. Despite formation of the parent radicals, coupling did not proceed, under conditions that generally result in phenylpropanoid polymerisation. It is postulated that intermolecular radical-coupling reactions are inhibited due to rapid conversion to the *o*-quinone. In contrast, when methoxylated at C3, as in 4,5-dihydroxy-3-methoxycinnamic acid, radical coupling proceeds with the major product resulting from 8-O-3 radical coupling and formation of a substituted 2,3-dihydro-1,4-dioxin ring.

Keywords: Hydroxycinnamic acid; EPR; *O*-Methyl transferase; Lignin biosynthesis; Caffeic acid; Catechol

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0045-2068/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0045-2068(03)00042-7

1. Introduction

Substituted hydroxycinnamic acids involved in the deposition and cross-linking of plant cell-wall polymers are produced by the phenylpropanoid pathway. Cinnamic acid (Fig. 1, **1**) undergoes a succession of hydroxylation and methylation reactions to provide a series of intermediate acids (Fig. 1, **2**–**6**). In theory, any of these acids may be reduced via the aldehyde (Fig. 1, **8**a–c) to the corresponding alcohol (Fig. 1, **9**a–c) but, in vivo, this appears to be restricted to acids which do not contain 1,2-dihydroxy substituents (Fig. 1, **2**, **4**, and **6**).

The *O*-methyl transferases (*O*MT), the enzymes responsible for methylation of the hydroxyl groups at C3 and C5, are targets for genetic manipulation of lignin biosynthesis. Genes encoding *O*MT have been characterised from a number of species and RNA antisense and sense strategies have been successfully used to modulate *O*MT expression [1–4]. Significant reduction in lignin content without apparent detrimental consequences for the plant have been observed in several

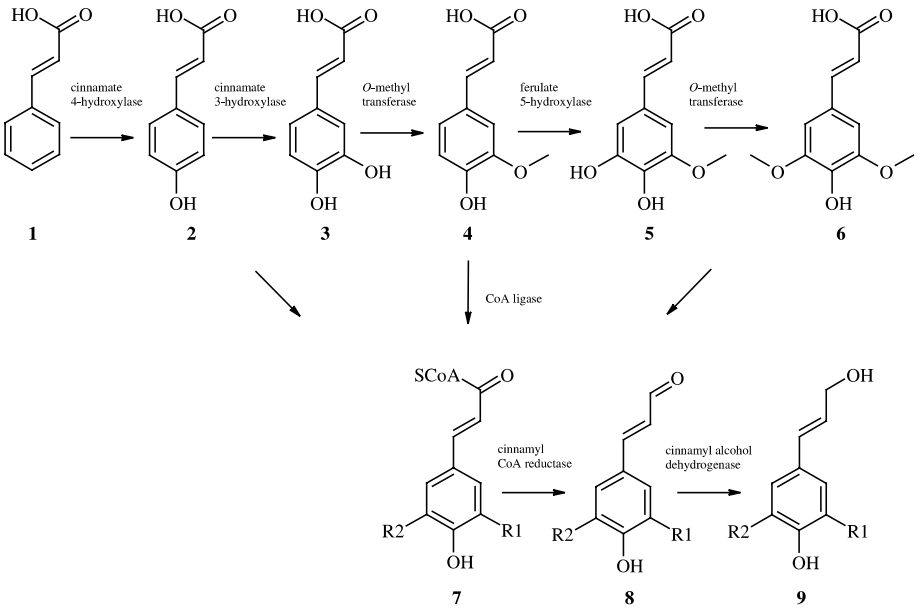

Fig. 1. Phenylpropanoid pathway; cinnamic acid (**1**), 4-hydroxycinnamic acid (**2**), 3,4-dihydroxycinnamic acid (**3**), 4-hydroxy-3-methoxycinnamic acid (**4**), 4,5-dihydroxy-3-methoxycinnamic acid (**5**), 3,5-dimethoxy-4-hydroxycinnamic acid (**6**), R1 and R2 = H CoA thioester of 4-hydroxycinnamate (**7a**), R1 = H and R2 = OCH_3 CoA thioester of 4-hydroxy-3-methoxycinnamate (**7b**), R1 and R2 = OCH_3 CoA thioester of 3,5-dimethoxy-4-hydroxycinnamate (**7c**), R1 and R2 = H 4-hydroxycinnamyl aldehyde (**8a**), R1 = H and R2 = OCH_3 4-hydroxy-3-methoxycinnamyl aldehyde (**8b**), R1 and R2 = OCH_3 3,5-dimethoxy-4-hydroxycinnamyl aldehyde (**8c**), R1 and R2 = H 4-hydroxycinnamyl alcohol (**9a**), R1 = H and R2 = OCH_3 4-hydroxy-3-methoxycinnamyl alcohol (**9b**), and R1 and R2 = OCH_3 3,5-dimethoxy-4-hydroxycinnamyl alcohol (**9c**).

constructs [5]. The presence of a 4,5-dihydroxy-3-methoxylated unit was identified by thioacidolysis for lignins from both transgenic poplar and tobacco, although no accumulation of the 3,4-dihydroxylated compound was observed [6]. These results apparently are not consistent with the view that angiosperm *O*MT are non-specific with regard to *ortho* methylation of the 3,4-dihydroxylated substrate and its 4,5-dihydroxy-3-methoxy analogue. Whether this observation is actually due to a difference in enzyme specificity, the result of an inability to detect the 3,4-dihydroxylated compound, or to the selective incorporation of these compounds into the polymer, is uncertain. In this paper we address the likelihood of parent phenylpropanoid radicals containing 1,2-dihydroxy substituents participating in polymer cross-linking reactions and being incorporated into the lignin macromolecule.

Parent radical generation is catalysed by iron-containing peroxidases and possibly monooxygenases [7–9]. The redox cycling capacity of peroxidase can be monitored by electron paramagnetic resonance (EPR) spectroscopy. Addition of hydrogen peroxide to peroxidase results in formation of compound I, and the concomitant loss of the signal at $g = 6.277$ as Fe^{III} is oxidised to Fe^{IV}. Therefore, the ability of substrates to reduce peroxidase compounds I and II back to the resting state of peroxidase can be used as a guide to their ease of oxidation and hence their potential for further participation in coupling reactions.

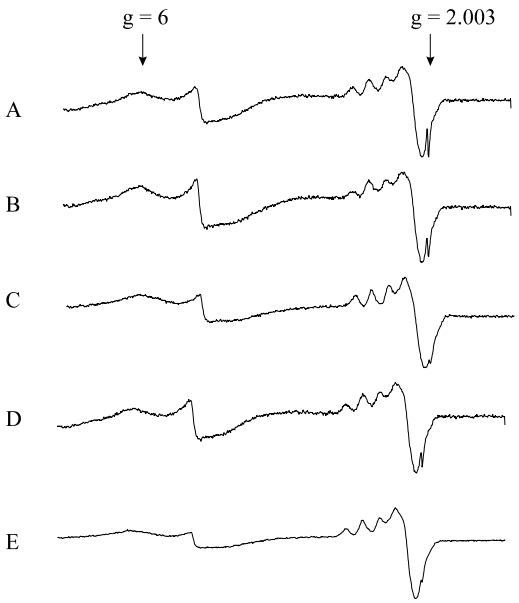

Fig. 2. Low temperature EPR spectra of peroxidase, compound I in the presence of 4-hydroxycinnamic acid (A), 3,4-dihydroxycinnamic acid (B), 3,4,5-trihydroxycinnamic acid (C), 4-hydroxy-3-methoxycinnamic acid (D), and 3,4-dihydroxy-5-methoxycinnamic acid (E). Spectral width = 3500 Gauss.

2. Materials and methods

2.1. Synthesis of 4,5-dihydroxy-3-methoxycinnamic acid (3-(4,5-dihydroxy-3-methoxyphenyl) prop-2-enoic acid)

A solution containing 3,4,5-trihydroxybenzaldehyde (500 mg, 3.2 mmol) and malonic acid (2 g) dissolved in pyridine (10 mL) was heated to 55 °C until complete dissolution occurred. Aniline and piperidine (both freshly distilled; 0.1 mL) were added to this solution and the reaction stirred at room temperature for 1 h. The mixture was left to stand overnight and then neutralised with HCl (3 M). The pyridine was removed by co-distillation with toluene and the oily residue was then redissolved in ethyl acetate. The organic layer was washed with saturated aq. NaCl, left to stand over Na_2SO_4 (anhydrous), filtered, and the solvent evaporated to afford 3,4,5-trihydroxycinnamic acid as a yellow solid. Yield 445 mg (70%); NMR δ_H ($(CD_3)_2SO$) 6.11 (1H, d, *J* 15, C(7)H), 6.59 (2H, s, C(2,6)H), 7.35 (1H, d, *J* 15, C(8)H) ppm and δ_C ($(CD_3)_2SO_4$) 107.5 (C2,6), 115.2 (C8), 124.7 (C1), 136.2 (C4), 145.0 (C3,5), 146.2 (C7), 167.8 (C9) ppm. Subsequently, 3,4,5-trihydroxycinnamic acid (105 mg, 0.5 mmol) was dissolved in aqueous sodium tetraborate (5% w/v; 80 mL) and stirred under nitrogen at room temperature. Dimethyl sulphate (3 mL) and NaOH (5 mL, 7.5 M) were added over a period of ca. 3 h. The solution was then left to stand at room temperature overnight. The solution was acidified to pH 1 with H_2SO_4 (20% v/v) and partitioned between ethyl acetate and saturated aq. NaCl. The organic layer was left to stand over Na_2SO_4 (anhydrous), filtered, and the solvent evaporated under reduced pressure. The product was then purified by flash chromatography on silica [Merck 109385], eluting with ethyl acetate/hexane 2:3 (33 × 7 mL), ethyl acetate (11 × 7 mL), and methanol (33 × 7 mL). Yield 31.5 mg (30%); NMR δ_H ($(CD_3)_2SO_4$) 3.79 (3H, s, OCH_3) 6.24 (1H, d, *J* 15.9, C(7)H), 6.69 (1H, d, *J* 1.7 C(6)H), 6.81 (1H, d, *J* 1.7, C(2)H), 7.39 (1H, d, *J* 15.9, C(8)H) ppm and δ_C ($(CD_3)_2SO_4$) 56.0 (OCH_3), 104.0 (C2), 109.7 (C6), 115.8 (C8), 124.7 (C1), 137.0 (C4), 144.8 (C7), 145.8 (C5), 148.6 (C3), 167.9 (C9) ppm. The correlation of methoxyl protons to C3 was confirmed by an heteronuclear multiple bond connectivity (HMBC) NMR experiment.

Alternatively, 4,5-dihydroxy-3-methoxycinnamic acid can be prepared by the following method. 3,4-Dihydroxybenzaldehyde (2.25 g, 16.3 mmol) was dissolved in acetic acid (60 mL) and bromine (0.7 mL) was added over a period of 2 h. The solubility increased initially with the addition of bromine, but began to precipitate as reaction neared completion. The reaction mixture was then poured into an ice-water mixture (35 mL) and the light brown precipitate collected and dried. Yield 2.4 g (67%). Sodium (freshly cut; 0.9 g) was dissolved in methanol (anhydrous; 15 mL) and the volume of methanol reduced to 5 mL. 5-Bromo-3,4-dihydroxybenzaldehyde (1.3 g, 6 mmol) and copper(II) chloride (0.32 g) were dissolved in *N*,*N*-dimethylformamide (anhydrous; 6 cm^3) and this solution was added to the sodium methoxide solution and refluxed for 2 h. After most of the methanol was removed, water (15 mL) was added and the solution acidified with HCl (3 M). This solution was then extracted into ethyl acetate and washed with sodium hydrogen sulphite (10% w/v).

The aqueous layer was then acidified with sulphuric acid (3 M) and extracted into ethyl acetate. The organic layer was left to stand over anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure to afford 4,5-dihydroxy-3-methoxy-benzaldehyde. Yield 0.72 g (71%). Condensation with malonic acid was achieved by treatment of 4,5-dihydroxy-3-methoxy-benzaldehyde (0.54 mg, 3.2 mmol) in the same way as indicated above for 3,4,5-trimethoxybenzaldehyde. The product was then purified by dry-column chromatography on silica [Merck 1.07736], eluting with ethyl acetate/hexane 3:2 + 1% acetic acid (11 × 7 mL) and ethyl acetate (11 × 7 mL). Yield 0.63 g (94%); The NMR spectrum corresponded to that reported above.

2.2. EPR spectroscopy

Solutions were prepared to contain the substrate (5.0 μmol), peroxidase (0.25 mmol), and hydrogen peroxide (1.0 mmol) in zinc acetate buffer (pH 5.5; 0.2 M; 2 mL). These solutions were applied by autosampler and the spectra (X-band) recorded at 298 K in a Bruker EPR cell positioned in the cavity of a Bruker E106 spectrometer using the following instrument settings: modulation frequency, 100 kHz; centre field, 3471.5 Gauss; sweep width, 50 Gauss; time constant, 40.96 ms; sweep time 81.92 s and power, 20 mW. Continuous-flow EPR spectroscopy was performed as reported previously [10]. Computer simulations of spectra, giving the hydrogen hyperfine coupling constants (a_H), were performed using the SIMEPR program [11], which sequentially varies all the parameters for each radical species until a minimum in the error surface is located. The best fit was determined by visual comparison and as a minimum in the sum of the squared residuals.

2.3. Treatment of substrates with peroxidase and hydrogen peroxide

3,4-Dihydroxycinnamic acid (100.8 mg, 0.56 mmol) was dissolved in phosphate buffer (88 mL; pH 6.5; 25 mM) to give solution A. Solution B was prepared by dissolving hydrogen peroxide (0.076 mL, 0.67 mmol, 1.2 eq) in phosphate buffer (88 mL; pH 6.5; 25 mM). Both A and B were added to a solution of horseradish peroxidase (275 U, 2.4 mg) in phosphate buffer (24 mL; pH 6.5; 25 mM) at a rate of 2 mL h^{-1} under nitrogen with continuous stirring. After 24 h a further aliquot of peroxidase was added (125 U, 1.1 mg). The pH was reduced with HCl (3 M) and partitioned between ethyl acetate and saturated aq. NaCl. The organic layer was left to stand over Na_2SO_4, filtered, and evaporated to dryness. Yield based on molecular weight of starting material, 93.7 mg (93%). 4,5-Dihydroxy-3-methoxycinnamic acid was treated in the same way. Yield based on molecular weight of starting material, 108.2 mg (92%). NMR (major product) δ_H ($(CD_3)_2SO_4$) 3.71 (3H, s, $AOCH_3$), 3.81 (3H, s, $BOCH_3$) 5.06 (1H, d J 5.5, A(7)H), 5.22 (1H, d, J 5.5 A(8)H), 6.41 (1H, d, J 15.6, B(8)H), 6.45 (1H, d, J 2.3, A(2)H), 6.54 (1H, d, J 2.3, A(6)H), 6.90 (1H, d, J 1.8, B(6)H), 6.95 (1H, d, J 1.8, B(2)H), and 7.45 (1H, d, J 15.6, B(7)H) ppm and δ_C ($(CD_3)_2SO_4$) 55.74 ($AOCH_3$), 55.86 ($BOCH_3$), 74.67 (A8), 74.86 (A7), 102.87 (A2), 104.58 (B2), 107.98 (B6), 110.27 (A6), 117.58 (B8), 125.80 (A1), 126,86 (B1), 133.78 (B4), 134.42 (A4), 143.79 (B7), 145.49 (A5), 145.53 (A3),

148.01 (B3), 148.38 (B5), 167.71 (B9), and 168.67 (A9) ppm. All correlations were confirmed by heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC) experiments.

3. Results and discussion

The presence of the high-spin iron (III) signal ($g \sim 6$) indicates the regeneration of the resting state of the enzyme following the oxidation of the substrates by its H_2O_2 activated forms, compound I and compound II, both of which contain iron (IV). The signal observed at $g = 2.003$ was similar to that observed at 4 K (data not shown). At the lower temperature this signal is comprised of two components, one of which is the porphyrin π-cation radical of compound I [12]. The other component is believed to be a free radical located on the protein. At this temperature (125 K), this signal is considered more likely to be solely from the latter component. It is, however, interesting to note that this signal decreases with increasing extent of hydroxylation and methoxylation and is representative of reduction by compounds with increasing oxidation potential. For all substrates studied, the iron (III) signal was regenerated. This demonstrated that replacement of a hydroxy substituent with a methoxyl group had little effect on the ability of parent radical formation. From this observation it appeared that the catalytic action of peroxidase to oxidise phenylpropanoid substrates was not impaired by the presence of a catechol functional group.

Since it appeared that radical formation was not inhibited by the presence of 1,2-dihydroxy substituents, it was necessary to characterise the electron-spin distribution, as this has been shown previously to determine the product of phenylpropanoid coupling [10]. Using Ce^{IV} as an oxidant, in the continuous-flow system used previously for similar substrates [10], failed to generate a stable radical from 3,4-dihydroxycinnamic acid and no EPR spectrum was observed. However, incubation of this substrate with peroxidase and hydrogen peroxide in the presence of Zn^{II} ions permitted the observation of an EPR spectrum showing good signal-to-noise ratio (Fig. 3). In this system, Zn^{II} serves a spin stabilisation agent [13]. In order to simulate the spectrum, two radical species were required. Species A, which made up 27% of the signal and species B, which represented 73% of the signal. This gave an r^2 value of 0.988 and from this simulation, hyperfine coupling constants were obtained (Table 1). Due to the presence of two radical species, distribution of the unpaired electron was difficult to assign and therefore structures generated computationally to represent the unpaired electron distribution were compared (Fig. 4).

The structure representing the radical generated on the hydroxyl at C4 showed potential coupling positions at C5, C8 and through the oxygen at C4, as indicated in green. When the radical is generated on the hydroxyl at C3 the potential coupling positions become C6, C7 and through the oxygen at C3. The same patterns are observed when the compound is methoxylated at C3. Therefore, to determine the actual coupling regiochemistry both compounds were incubated with peroxidase and hydrogen peroxide.

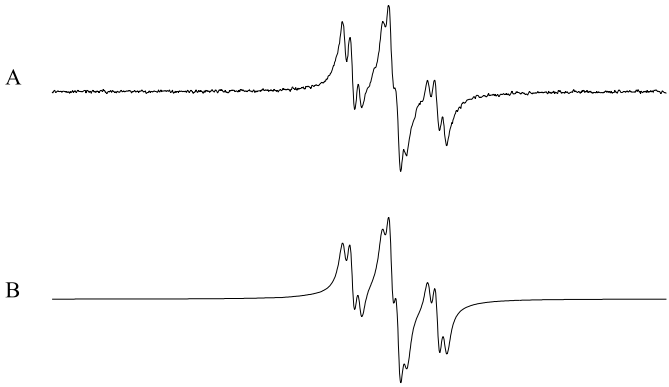

Fig. 3. Zinc-stabilised, EPR spectrum (A) and simulation (B) of 3,4-dihydroxycinnamic acid in the presence of peroxidase and hydrogen peroxide. The centre field is at 3479 Gauss with a spectral width of 40 Gauss.

Table 1
Hyperfine coupling constants (a_H) derived from the computer-simulated zinc stabilised EPR spectrum of 3,4-dihydroxycinnamic acid for the two radical species A and B

	a_H	a_H	a_H	a_H
A	3.214	3.702	0.628	0.555
B	2.348	2.275	0.018	0.018

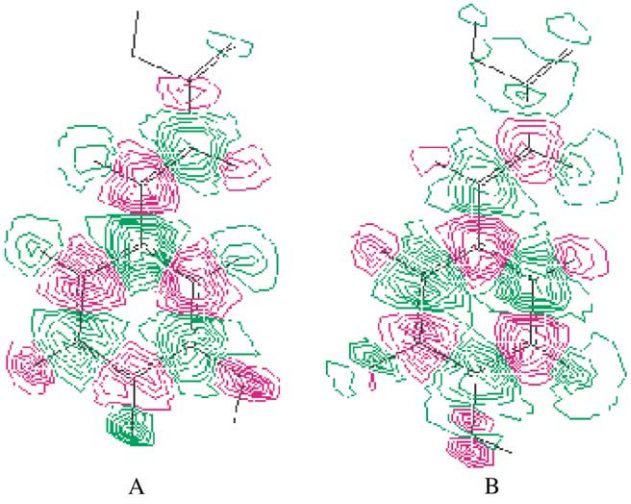

Fig. 4. Graphical distribution of unpaired-electron density calculated for geometrically optimised structures calculated by AM1 for 3,4-dihydroxy cinnamic acid with the radical generated on the hydroxyl at C4 (A) and with the radical generated on the hydroxyl at C3 (B). Positive spin densities are represented in green.

Incubation of 3,4-dihydroxycinnamic acid with peroxidase and hydrogen peroxide, under the conditions generally used for the production of dehydrogenation polymers [14], failed to give any coupling products and the starting material was recovered unchanged. Although the catechol functional group did not prevent radical formation as shown by EPR spectroscopy, coupling of the parent radical did not proceed. However, it should be noted that this observation occurs only under enzyme-catalysed oxidative coupling conditions, as this is in contrast to results observed under acidic or pyrolysis conditions [15] and in systems modelling the non-enzymic auto-oxidation of 3,4-diydoxycinnamic acid [16]. This may also explain the possible presence of dimers in suspension-cultured cell walls [17]. The failure to detect radicals from 3,4-dihydroxycinnamic acid in the continuous-flow EPR system, under conditions suitable for detection of phenylpropanoids which do not contain 1,2-dihyroxyl substituents, suggests that any radicals formed are rapidly removed from the system. One possible explanation is that the radicals are rapidly converted into the *o*-quinone, as previously observed by electrochemical methods and pulse radiolysis [18]. Recording of an EPR spectrum in the presence of zinc indicated formation of two different radical species. Whether the mechanism of *o*-quinone formation is by two-step oxidation of 3,4-dihydroxycinnamic acid (Eq. (1)) or disproportionation of the *o*-semiquinone (Eq. (2)) is uncertain and almost certainly dependent on reaction conditions. Although this reaction is reversible, formation of the semiquinone radical is not thermodynamically favourable [19]. Therefore, intermolecular radical-coupling reactions are unlikely to occur.

$$\mathrm{Ph(OH)_2} \rightarrow \mathrm{Ph(OH)O^{\cdot}} \rightarrow \mathrm{Ph(O^{\cdot})_2} \rightarrow \mathrm{Ph(O)_2} \tag{1}$$

$$2\mathrm{Ph(OH)_2} \rightarrow 2\mathrm{Ph(OH)O^{\cdot}} \rightarrow 2\mathrm{PhO^{-}O^{\cdot}} \rightarrow \mathrm{Ph(O)_2} + \mathrm{Ph(OH)_2} \tag{2}$$

EPR demonstrated that further substitution with hydroxyl groups increases the ability of the compounds to reduce peroxidase compound I, as did additional methoxyl substitution (Fig. 2). When 4,5-dihydroxy-3-methoxycinnamic acid was incubated with peroxidase and hydrogen peroxide, radical coupling was observed. The major product was a dimer, which resulted from 8-O-3 radical coupling and formation of a substituted 2,3-dihydro-1,4-dioxin ring (Fig. 5). This demonstrates that coupling regiochemistry favours the pattern of positive spin density observed for the parent radical generated on the hydroxyl at C4 (Fig. 4). Incorporation of these units into lignin would result in formation of this linkage type and ^{13}C NMR spectroscopy of lignin extracted from *O*MT downregulated plants, showed the presence of unassigned signals in the same spectral region [20,21] and later in caffeic acid OMT deficient plants [22]. The minor products were not identified. However, their high solubility in ethyl acetate suggested they were not polymeric in nature. As well as increasing the ease of oxidation, additional methoxyl substituents will increase the stability of the parent radical. These features may account for the ability of 4,5-dihydroxy-3-methoxycinnamic acid to participate in radical coupling reactions. It also explains the detection of this unit and not 3,4-dihydroxylated units in the cell walls of plants in which *O*MT activity has been reduced. This indicates the significant effect substrate structure, as well as mechanism of dimerisation, has on the incorporation

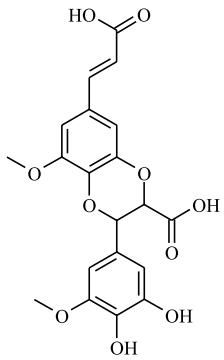

Fig. 5. Structure of dimer resulting from 8-O-3 radical coupling of 4,5-dihydroxy-3-methoxycinnamic acid and formation of a 2,3-dihydro-1,4-dioxin ring.

of phenylpropanoids into the plant cell wall. It also highlights the importance for polymer formation, of hydroxyl methylation at C3 and C5 prior to reduction and incorporation into lignin.

Acknowledgments

Financial support for this work was provided by the Scottish Executive, Rural Affairs Department (formally the Scottish Office, Agriculture, Environment and Fisheries Department).

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