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INVESTIGATION OF LIGNIFICATION BY MEANS OF FLUORINATED ANALOGUES OF FERULIC ACID AND CONIFERYL ALCOHOL

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; oxidation; peroxidase; fluorinated cinnamic compounds; lignin.

Abstract—2-Fluoro-analogues of ferulic acid and coniferyl alcohol were synthesized from vanillin acetate. These new compounds were used as hydrogen donors by tobacco peroxidases, giving rise to lignin-like compounds. In the presence of standard peroxidase substrates, the fluorinated analogues behaved as competitive inhibitors and could therefore be used both *in vivo* and *in vitro* as antimetabolites of peroxidase-catalysed reactions.

INTRODUCTION

Synthetic lignin-like compounds are obtained *in vitro* upon incubation of monolignols in the presence of peroxidase and hydrogen peroxide. The first reaction of the polymerization process produces a phenolic dimer made up of two cinnamic alcohol molecules linked through the β-carbon of one with the phenolic hydroxyl of the other [1, 2]. The further progress of the polymerization process is also mediated by peroxidative activities. However, substantial differences were noticed in the bonding patterns of *in situ* and dehydropolymerized lignins [2, 3]. These results raise the question of the exact function of the acidic cell wall isoperoxidases which are generally supposed to catalyse *in vivo* the final steps of lignin biosynthesis [4, 5]. We attempted, therefore, to investigate, by histochemical and biochemical methods, the cell wall peroxidase activities assumed to be involved in lignin polymerization. To this end, fluorinated analogues of ferulic acid and coniferyl alcohol were synthesized and checked as inhibitors of peroxidase reactions. Fluorine is similar in size to hydrogen but very different in reactivity [6]. The exchange of hydrogen for a fluorine in a reactive position of a substrate can give rise to fluorinated analogues which act as antimetabolites towards the fluorine-free parents [7]. Thus, we chose to introduce the fluorine atom into the 2 position of the propane chain in order to get an interaction with the site of coupling of the radicals. We present here the first data from preliminary investigations carried out with structural analogues of cinnamic acids and alcohols. Experiments were performed with tobacco stems, a material already investigated in our laboratory [8, 9]. Cell wall peroxidases were isolated from two distinct areas of tissue, bark (B) and xylem (X), and investigated separately.

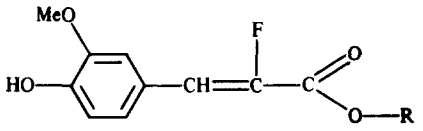
RESULTS AND DISCUSSION

Nature of the fluorinated substances

Three cinnamic compounds carrying a fluorine atom on the β-carbon were used in the assays (Fig. 1). Two were salts of 2-fluoroferulic acid respectively, i.e. 2-propanamine, (*Z*)-2-fluoro-3-(4-hydroxy-3-methoxyphenyl)-2-propenoate (1:1) (**1**) and (*Z*)-2-fluoro-3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid, monosodium salt (**2**). The third compound was the 2-fluoroanalogue of coniferyl alcoholic (*Z*)-2-fluoro-4-(3-hydroxy-1-propenyl)-2-methoxy-phenol.

Inhibition of peroxidase activities by fluorinated aromatic compounds

Histochemical investigations. Transverse sections of tobacco stems were incubated with hydrogen peroxide and several peroxidase substrates (guaiacol; tetramethylbenzidine: TMB; *p*-phenylenediaminepyrocatechol: PPD-PC; pyrogallol) in the presence or absence of the analogues (Table 1). In the absence of the analogues, the greatest oxidative polymerization occurred, whatever the



1 R = NH—$CHMe_2$
2 R = Na

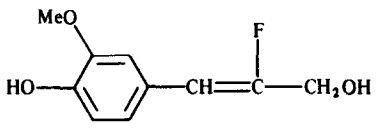


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Fig. 1. Structure of the fluorinated substances, **1**–**3** used in this study.

Table 1. Relative intensity of the staining obtained on tobacco sections with different peroxidase substrates

Substrate	Bark			Xylem	
	Parenchyma	Sclerenchyma	Phloem	Lignifying walls	Lignified walls
Guaiacol	+	++	+	++	+/−
+1	+	+	+	+	+/−
+2	+/−	+	−	+	−
+3	+	++	+	++	+
TMB	+	++	+	+	−
+1	−	−	−	−	−
+2	−	−	−	−	−
+3	−	−	−	−	−
PPD–PC	+/−	++	−	++	+/−
+1	+/−	+	−	+	+/−
+2	−	+	−	++	+
+3	−	+	−	++	+
Pyrogallol	−	+/−	−	++	+/−
+1	−	+/−	−	++	+/−
+2	−	+/−	−	++	+/−
+3	−	+/−	−	++	+/−

Transverse sections were incubated with H_2O_2 and a hydrogen donor alone or with **1**, **2** or **3**.

substrate, in lignifying cell walls (young sclerenchyma cells and differentiating xylem). Phloem and parenchyma cells reacted only with guaiacol and TMB. The analogues inhibited completely TMB oxidation but were almost ineffective with the other substrates.

Biochemical assays. Electrophoretograms of the different enzymatic extracts (Fig. 2) revealed that all the solubilized fractions contained several isoforms. The covalently bound fractions (CB) were completely free of cationic isoforms. Bark fractions generally contained more isoforms than the xylem. Moreover, mostly anionic isoforms which, according to Schloss *et al.* [10], are involved in the polymerization process, could be detected in xylem fractions.

According to the histochemical observations, the three fluorinated compounds strongly inhibited TMB oxidation. The kinetic patterns obtained from a Dixon plot [11] with 2-fluoroconiferyl alcohol (**3**) indicated competitive inhibition. The K_i values are reported in Table 2. The two other analogues (**1** and **2**) gave non-linear Dixon plots which excluded K_i estimations. The competitive inhibition observed with 2-fluoroconiferyl alcohol established that this compound could be used as a substrate by tobacco peroxidases. This process was further investigated.

Oxidation of fluorinated compounds by tobacco peroxidases

In the presence of hydrogen peroxide, all enzymatic fractions were able to oxidize the three fluorinated compounds, giving rise to a pink product with maximum absorbance at 520 nm (Fig. 3). This coloured substance disappeared very quickly but it was still possible to estimate the oxidation rate of the reaction by recording the absorbance at 520 nm as the initial velocity was maintained for nearly 30 sec. After several hours a pre-

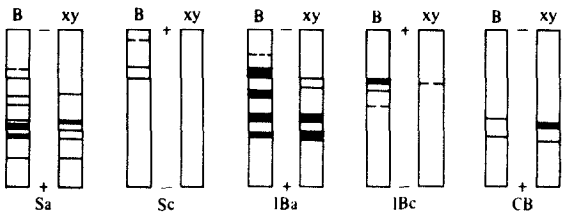

Fig. 2. Isoenzyme patterns of the peroxidase fractions. Peroxidases were separated by polyacrylamide disc gel electrophoresis. S, soluble fraction; IB, ionically bound fraction; CB, covalently bound fraction. Sa, IBa, CB, separation of anionic isoperoxidases; running buffer: Tris–HCl, pH 8.2. Sc, IBc, separation of cationic isoperoxidases; running buffer: acetic acid–β-alanine, pH 4.5.

Table 2. K_i values for **3**

	Bark	Xylem
IB_A	9	17
IB_C	1	−
CB	25	2

The inhibition constants were estimated from Dixon plots. Substrate: TMB; enzymatic fractions: cell wall fractions IB_A, IB_C (anionic and cationic ionically bound peroxidases) and CB (covalently bound peroxidases), isolated from bark and xylem tissues; K_i as $M \times 10^4$

cipitate appeared in the assay medium. This product was insoluble in common organic solvents and may have a 'lignin-like' structure. The nature of this polymer is under investigation by using the standard techniques of lignin chemistry, in particular thioacidolysis [12].

pH profiles for peroxidation of the fluorinated analogue are shown in Fig. 4. Optima are obtained at relatively high pH values and with **1** and **3** two optima are observed. This might be due to the action of different isoforms.

K_m values were estimated for fluorinated and non-fluorinated substrates (Table 3). Affinities for **1** and **2** were slightly lower than those obtained with routine synthetic hydrogen donors like TMB and pyrogallol. The high K_m value obtained for **3** might result from the presence of ethanol in the incubation medium (added because of the low solubility of **3**). Naturally occurring compounds such as ferulic acid, vanillin and coniferyl alcohol were also used as sbstrates. With ferulic acid, linear or non-linear Lineweaver–Burk plots were observed depending on the nature of the enzymatic fraction (Fig. 5). With vanillin and coniferyl alcohol, non-linear plots were obtained for all cell wall fractions. Furthermore, a lag time was observed for oxidation of vanillin and coniferyl alcohol but not for ferulic acid. Similar results have been previously reported for oxidation of scopoletin and ferulic acid [13, 14]. The influence of the presence of a fluorine atom on the active carbon of the hydrogen donor was further investigated by comparing the distribution of peroxidase activities between the different cell fractions when fluorinated and non-fluorinated phenolic compounds were used as substrates. In all cases, the soluble fraction of the bark was the most active. In the bark, the cell wall activities were very low whereas in

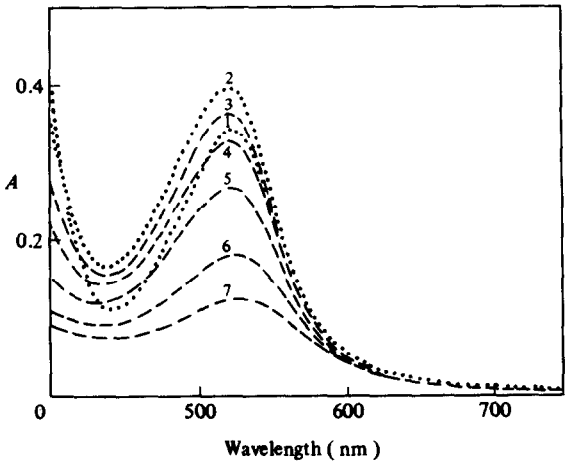

Fig. 3. Absorption spectrum of oxidized **2** in Tris buffer, pH 7.2, recorded 30 sec (1), 1 min 20 sec (2), 2 min 15 sec (3), 3 min 15 sec (4), 5 min 30 sec (5), 10 min (6) and 20 min (7) after the addition of peroxidase and hydrogen peroxide.

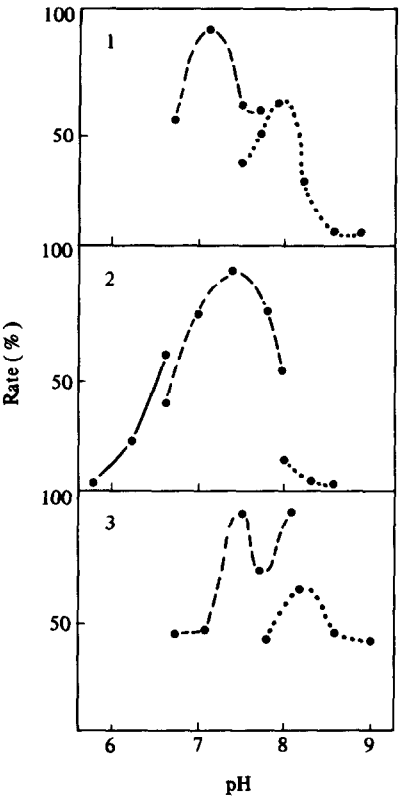

Fig. 4. Effect of pH on the oxidation rate of **1** (1), **2** (2) and **3** (3). ●———●, citrate-phosphate buffer; ●------●, Na-K phosphate buffer; ● . . . . . ●, Tris–HCl buffer.

Table 3. Michaelis constants ($M \times 10^4$) for fluorinated and non-fluorinated phenolic substrates of bark and xylem cell wall peroxidases

	Bark			Xylem		
Substrate	IB_A	IB_C	CB	IB_A	IB_C	CB
TMB	2.1	1.3	1.2	1.2	0.9	1.1
Pyrogallol	1.1	0.6	0.7	—	—	0.5
Ferulic acid	—	—	12	—	—	4
1	7	8	16	6	15	5
2	4	8	9	6	6	6
3	50	38	330	—	—	42

TMB, tetramethylbenzidine; IB_A and IB_C respectively anionic and cationic ionically bound peroxidases; CB, covalently bound peroxidases.

the xylem about 40% of the total activity was recovered in the cell wall fractions (Fig. 6). Thus, it was found that the presence of fluorine did not change significantly the cell distribution of peroxidase activities.

In conclusion, the data presented here, show that 2-fluoro-substituted cinnamic compounds can be used as proton donors for peroxidative reactions. Moreover, the polymerization process proceeds in spite of the presence of a fluorine atom bound to the active β-carbon of the propane side chain. This process apparently gives rise to 'lignin-like' material. We observed also that peroxidases can act on phenolic acids as well as on alcohols. These data suggest that mostly monolignols or monolignol conjugates are available in the apoplasm for lignin polymerization. Fluorinated analogues of conjugated monolignols possibly involved in transport through the cytoplasmic membrane are now being synthesized in order to test this hypothesis.

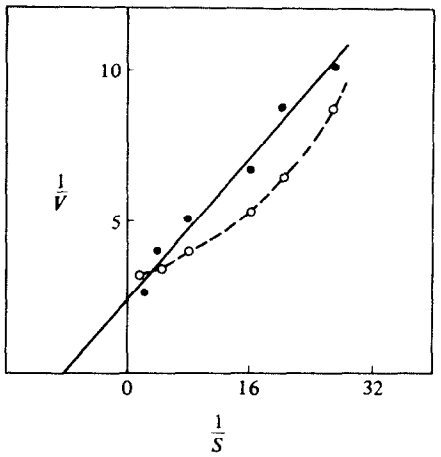

Fig. 5. Double reciprocal plot of the oxidation of ferulic acid by bark cell wall fractions. ●————●, CB fraction; ○- - - - - -○, IBa fraction; S as 10^{-2} M; v as arbitrary units.

EXPERIMENTAL

Five-to-six-month-old tobacco plants (*Nicotiana tabaccum var. Samsum*) were used. They were grown in a glass house at 20°.

Histochemical techniques. Fresh hand-made sections were incubated at room temp. in appropriate media. Peroxidative activities were visualized using H_2O_2 with one of the following substrates: guaiacol [15], PPD-PC [16], TMB [9] and pyrogallol [17]. **1** and **2** were dissolved in the buffer used for peroxidase detection. **3** was dissolved in EtOH and then added to the incubation medium.

Biochemical assays. 10 cm long stem segments were peeled to remove the epidermis and outer cortical layers then split longitudinally. The pith was discarded and the remaining tissues were separated at the cambial level into two fractions respectively: bark B (inner cortical parenchyma, phloem and fibers) and xylem X. Each tissue fraction was ground first in liquid N_2 then further in 0.1 M Na–K–Pi buffer, pH 6.0, with a Polytron. Enzymatic extractions were performed as previously described [18]. Soluble peroxidases S, were recovered first. Cell wall enzymes were fractionated into weakly and strongly bound enzymes, the so-called ionically (IB) and covalently (CB) bound enzymes, by 1M NaCl and pectinase-cellulase treatments. Activities still remaining in residual cell walls, r, were also taken into account. Ionically bound enzymes were further fractionated into anionic and cationic enzymes (IBa and IBc) by ion-exchange chromatography on DEAE-Sepharose CL 6B (Pharmacia, Sweden) columns (20 × 1.7 cm) equilibrated with 0.01M Na–K-Pi buffer, pH 7.1. IBc were eluted with this buffer and IBa with NaCl 0.5M in 0.01M Pi buffer, pH 7.1. All enzymatic extracts were reduced to a small vol. in an Amicon ultra-filtration cell (Membrane PM 10; Amicon, Lexington USA). Spectrophotometric assays were performed as in ref. [9]. Electrophoretic assays: isoperoxidases were separated by electrophoresis on polyacrylamide disc gel as previously described [18].

Synthesis of fluorinated compounds. Fluorinated compounds **1** and **2** were synthesized by Wittig–Horner reaction of ethyl (diethylphosphono)fluoroacetate [19] with vanillin acetate. The *E* isomer, the major product, was then converted to the *Z* thermodynamic isomer using Br_2 in CCl_4. Hydrolysis and treatment with the required base (2-propanamine or NaOH) gave **1** or **2** respectively. Reduction with $LiAlH_4$ in THF afforded the desired alcohol, **3**. All products gave analytical and spectral data

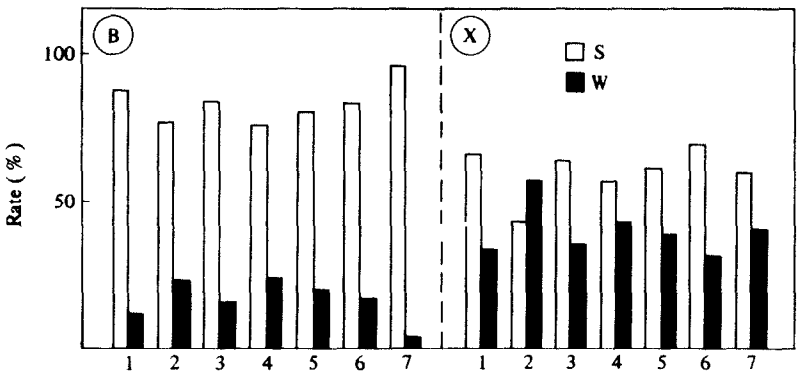

Fig. 6. Level of soluble and wall bound activities in bark (B) and xylem (X) with different hydrogen donors. Soluble (S) and cell wall (W) activities were added and normalized to 100. 1 to 4, non-fluorinated hydrogen donors: 1, TMB; 2, pyrogallol; 3, PPD-PC; 4, guaiacol; 5, **1**; 6, **2**; 7, **3**.

(NMR ^{1}H, ^{13}C, ^{19}F; IR and MS) in agreement with the proposed structures. Full chemical experimental conditions are published elsewhere [20].

REFERENCES

1. Sarkanen, K. V. (1971) in *Lignins* (Sarkanen, K. V. and Ludwig, C. H. eds) pp. 95–163. Wiley, New York.
2. Higuchi, T. (1985) in *Biosynthesis and biodegradation of Wood Components*, pp. 141–159. Academic Press, New York.
3. Lewis, N. G., Newman, J., Rej, R. N., Inst, G. and Ripmeister, J. (1987) *Macromolecules* (in press).
4. Harkin, J. M. and Obst, J. R. (1973) *Science* **180**, 296.
5. Mäder, M., Meyer, Y. and Bopp, M. (1975) *Planta* **122**, 250.
6. Schlosser, M. (1978) *Tetrahedron* **34**, 3.
7. Welch, J. T. (1987) *Tetrahedron* **43**, 3123.
8. Goldberg, R., Le, T. and Catesson, A. M. (1985) *J. Exp. Botany* **36**, 503.
9. Imberty, A., Goldberg, R. and Catesson, A. M. (1984) *Plant Sci. Letters* **35**, 103.
10. Schloss, P., Walter, C. and Mäder, M. (1987) *Planta* **170**, 225.
11. Dixon, M. (1953) *Biochem. J.* **55**, 161.
12. Lapierre, C., Monties, B. and Rolando, C. (1986) *Holzforschung* **40**, 113.
13. Reigh, D. L., Wender, S. H. and Smith, E. C. (1975) *Physiol. Plant.* **34**, 44.
14. Kim, S. S., Wender, S. H. and Smith, E. C. (1980) *Phytochemistry* **19**, 165.
15. Fielding, J. L. and Hall, J. L. (1978) *J. Exp. Botany* **29**, 969.
16. Hanker, J. J., Yates, P. E., Metz, C. B. and Rustioni, A. (1977) *Histochem. J.* **9**, 789.
17. Siegel, S., Frost, P. and Porto, P. (1960) *Plant Physiol.* **35**, 163.
18. Goldberg, R., Imberty, A. and Chu Ba, J. (1986) *Phytochemistry* **24**, 1271.
19. Elkik, E. and Francesch, C. (1986) *Bull. Soc. Chim. Fr.* 423.
20. Elkik, E., Francesch, C., Rolando, C. and Goldberg, R. (1988) *J. Org. Chem.* (in press).