Regioselective Dimerization of Ferulic Acid in a Micellar Solution

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Dehydrodimers of hydroxycinnamates play an important role in the cross-linking of plant cell walls. An aqueous solution of quaternary ammonium salts with a long aliphatic chain is known to spontaneously organize itself into micelles with the ionic part at the outer sphere. It is shown that regioisomeric ferulic acid dehydrodimers can be obtained in one step from *trans*-ferulic acid after attachment to these micelles and using the biomimetic peroxidase−H_2O_2 system. The surfactant hexadecyltrimethylammonium hydroxide yielded *trans*-4-(4-hydroxy-3-methoxybenzylidene)-2-(4-hydroxy-3-methoxyphenyl)-5-oxotetrahydrofuran-3-carboxylic acid (25%), (*E*,*E*)-4,4′-dihydroxy-5,5′-dimethoxy-3,3′-bicinnamic acid (21%), and *trans*-5-[(*E*)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran-3-carboxylic acid (14%), whereas the surfactant tetradecyltrimethylammonium bromide gave 4-*cis*, 8-*cis*-bis(4-hydroxy-3-methoxyphenyl)-3,7-dioxabicyclo[3.3.0]octane-2,6-dione (18%) as the main product. The use of micelles appears to be not only a new way to synthesize regioisomeric ferulic acid dehydrodimers but may also help to understand the regiospecificity of dimeric hydroxycinnamate formation in vivo.

Keywords: *Ferulic acid; ferulic acid dehydrodimers; micelles; peroxidase; hydrogen peroxide; surfactant*

INTRODUCTION

Hydroxycinnamates have been recognized as important components of plant cell walls, and it has been shown that they are present in significant quantities mainly ester-linked to polysaccharides in Graminaceous monocot and Caryophylaceous dicot cell walls (*1*, *2*). Ferulic acids esterified to polysaccharides, e.g. arabinoxylans, pectins, and xyloglucans, can form dimers through a dimerization reaction. Two mechanisms have been reported. One is an oxidative coupling reaction which can be catalyzed by various systems, such as peroxidase−hydrogen peroxide (*3*−*5*) or polyphenol oxidase, including laccases (*4*), as well as some purely chemical systems (*3*, *5*, *6*), leading to the formation of various ferulic acid dehydrodimers. The other mechanism is a photoinduced cycloaddition between the ethylene carbons of two phenolic acids leading to the formation of cyclobutane type dimers such as truxillic and truxinic acids (*7*−*10*). Thus, ferulic acid dehydrodimers are then able to cross-link different polysaccharide chains and, in some cases, polysaccharide and lignin (*11*). Cross-linking of cell wall components is expected to have a marked influence on numerous cell wall properties such as accessibility, extensibility, plasticity, digestibility, and adherence (*12*).

The 8-O-4-diFA, 8-5-diFA, 8-5-benzofuran-diFA, 5-5-diFA, 8-8-aryl-diFA, and 8-8-diFA are the main dehydrodimers so far identified in plant materials (Figure 1), with 8-O-4-diFA often being the most abundant (*3*, *13*−*17*). In contrast, the primary ferulic acid dehydrodimer generated in vitro from ferulic acid esters is the 8−5-coupled product, 8-5-benzofuran-diFA, when utilizing the biomimetic peroxidase−hydrogen peroxide system (*5*, *16*, *18*, *19*) or a range of single-electron oxidants (*3*, *5*). Obviously, some kind of control must be utilized in the cell in order to produce regiospecific dehydrodimers. Recently it was discovered that "guiding" proteins were involved in the lignin and lignan biosynthesis (*20*) and a similar guiding principle might be operating in the biosynthesis of dehydrodimers.

It is known that photodimerization of cinnamic acids can be controlled by the action of quaternary ammonium or amine *N*-oxide surfactants (*7*, *10*). These surfactants form large aggregates, e.g. micelles, in aqueous solutions with the ionic part at the surface to the water. In the presence of these aggregates, the carboxylic acids will be organized around them with the acid part pointing inward. With *trans*-ferulic acid and a strongly basic surfactant a similar organization is expected to occur as the stronger acid moiety (K_a of carboxylic acid $\gg K_a$ of phenol) will be oriented toward the aggregates (Figure 2). With an acidic surfactant this orientation is still expected as the undissociated carboxylic acid moiety will have a greater polarity compared to that of a phenolic group. The aim of this study was to investigate whether oxidative coupling of *trans*-ferulic acid can be regioselectively controlled using surfactants.

MATERIALS AND METHODS

Chemicals. *trans*-Ferulic acid and peroxidase (EC 1.11.1.7, type VI-A, 1000 units/mg, from horseradish) were purchased from Sigma (Deisenhofen, Germany) and 30% aqueous hydrogen peroxide (H_2O_2), *ortho*-phosphoric acid (purity > 99%), acetic acid (purity > 99.8%), sodium hydroxide, and anhydrous sodium sulfate (Na_2SO_4) were purchased from Merck (Darmstadt, Germany). The surfactant tetradecyltrimethylammonium bromide and 10% aqueous hexadecyltrimethylammonium hydroxide were purchased from Aldrich (Steinheim, Germany) and Fluka (Buchs, Switzerland), respectively. Tri-

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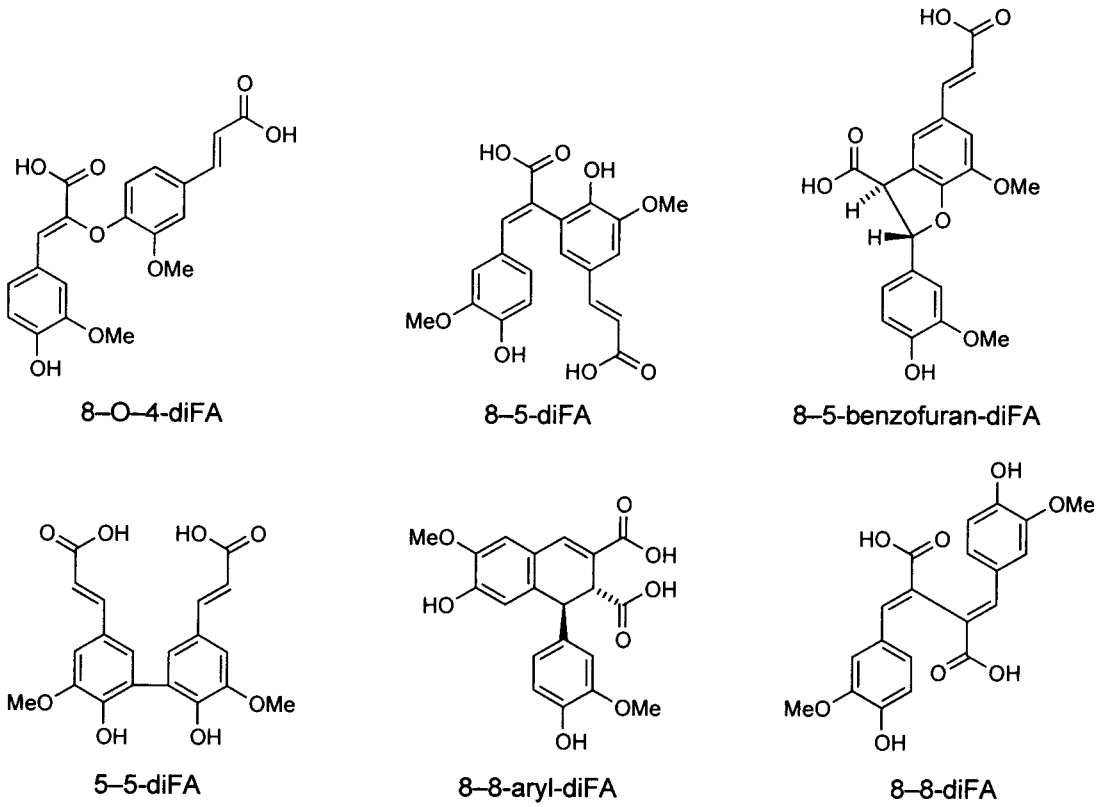

Figure 1. Chemical structures of the main ferulic acid dehydrodimers isolated from plants.

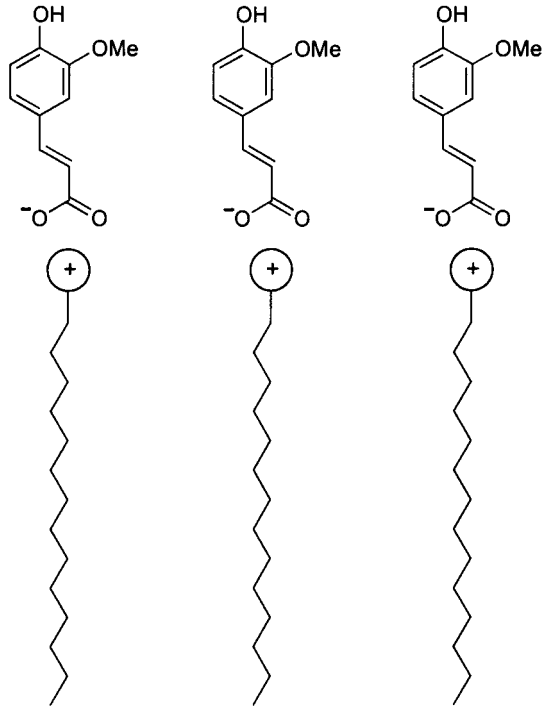

Figure 2. Expected organization of a strongly basic surfactant and *trans*-ferulic acid in an aqueous solution.

fluoroacetic acid (TFA), ethyl acetate (EtOAc), and methanol (MeOH) were all obtained from Aldrich and was of HPLC grade.

Dimerization of Ferulic Acid in Micellar Solutions. *trans*-Ferulic acid (230 mg) was dissolved in water (110 mL) at 60 °C. After the solution cooled (to ~ 40 °C), aqueous hexadecyltrimethylammonium hydroxide (0.3388 M, 3.5 mL, molar ratio to *trans*-ferulic acid 1:1) or tetradecyltrimethylammonium bromide (800 mg, molar ratio to *trans*-ferulic acid 2:1) was added to the stirred solution followed by addition of 30% aqueous H_2O_2 (0.08 mL) and peroxidase (0.1 mg in 0.22 mL). The pH in the former reaction media was 7.0 before addition of peroxidase and 8.0 after addition 5 min later. With tetradecyltrimethylammonium bromide as surfactant the pH remained constant at 3.0. The reactions were quenched after 5 min by addition of aqueous HCl (2.0 M, 20 mL). The reaction mixtures were extracted twice with EtOAc (2 × 50 mL), and the combined EtOAc extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure at 35 °C.

Dimerization of Ferulic Acid in Buffered Solutions. *trans*-Ferulic acid (230 mg) was dissolved in either an acetate buffer (0.1 M, 110 mL, pH 7.5) or a phosphate buffer (0.1 M, 110 mL, pH 3.0) at 60 °C. After the solution cooled (to ~ 40 °C), aqueous 30% H_2O_2 (0.08 mL) was added followed by peroxidase (0.1 mg in 0.22 mL). The reactions were quenched after 5 min by addition of aqueous HCl (2.0 M, 20 mL). The reaction mixtures were extracted twice with EtOAc (2 × 50 mL), and the combined EtOAc extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure at 35 °C.

Isolation, Quantification, and Identification of Ferulic Acid Dehydrodimers. The residue from the EtOAc extract was dissolved in MeOH (10 mL) and filtered through a 0.45-μm nylon filter (Osmonics Cameo syringe filters, Frisenette ApS, Ebeltoft, Denmark) before separation by preparative HPLC. For preparative HPLC a Merck L-6200 intelligent pump and a Merck L-4200 UV−Vis detector were used. Separations were performed at 35 °C on a Develosil ODS-HG-5 (RP-18, 250 × 20 mm i.d., Nomura Chemical Co., Japan) column protected with a guard cartridge (50 × 20 mm i.d.) packed with the same material as the column. The following gradient was used: 300 mL of 32% MeOH + 1‰ TFA; 300 mL of 34% MeOH + 1‰ TFA, 300 mL of 36% MeOH + 1‰ TFA; 300 mL of 38% MeOH + 1‰TFA; 300 mL of 40% MeOH + 1‰ TFA, and 300 mL of 80% MeOH. Compounds were detected at 280 nm. Flow rate was 7 mL min^{-1} and injection volume was 10 mL. The purity of the isolated ferulic acid dehydrodimers was determined by reversed-phase analytical

Table 1. Yields of Regioisomeric Dehydrodimers from Ferulic Acid Prepared in Micellar and Buffered Solutions

reaction conditions[a]	compound, (yields in %)[b,c]			
	2	**3**	**4**	**5**
i	25 ± 3	21 ± 2	14 ± 2	0
ii	25 ± 3	0	38 ± 3	0
iii	2 ± 1	0	1 ± 1	18 ± 3
iv	0	0	0	0

[a] (**i**) Hexadecyltrimethylammonium hydroxide (molar ratio to *trans*-ferulic acid 1:1), peroxidase, and H_2O_2 (pH 7.0−8.0); (**ii**) *trans*-ferulic acid, peroxidase, and H_2O_2 in 0.1 M acetate buffer solution (pH 7.5); (**iii**) tetradecyltrimethylammonium bromide (molar ratio to *trans*-ferulic acid 2:1), peroxidase, and H_2O_2 (pH 3.0); (**iv**) *trans*-ferulic acid, peroxidase, and H_2O_2 in 0.1 M phosphate buffer solution (pH 3.0). [b] Means ± standard deviation ($n = 2$). [c] Compound **2**, 8-8-γ-lactone-diFA; compound **3,** 5-5-diFA; compound **4**, 8-5-benzofuran-diFA; compound **5**, 8-8-bis-lactone-diFA.

HPLC on a LiChrospher 100 RP-18 (5 μm; 244 × 4 mm i.d., Merck, Darmstadt, Germany) column according to the method of Andreasen et al. (*15*, *21*). *trans*-Ferulic acid (**1**), 8-8-γ-lactone-diFA (**2**), 5-5-diFA (**3**), 8-5-benzofuran-diFA (**4**), and 8-8-bis-lactone-diFA (**5**) were quantified directly in the reaction mixtures by reversed-phase analytical HPLC (**1**, $R_t = 69.7$ min; **2**, $R_t = 92.2$ min; **3**, $R_t = 103.7$ min; **4**, $R_t = 104.9$ min; **5**, $R_t = 95.6$ min) using external standards of **1**−**5** (Table 1).

The purified ferulic acid dehydrodimers (**2**−**5**) were identified by UV, ^{1}H and ^{13}C NMR, and electrospray mass spectroscopy (ESI−MS). ESI−MS (negative ion) of all isolated dehydrodimers showed molecular ion peaks at m/z 385 $[M-H]^-$ in accordance with their structure.

8-8-γ-Lactone-diFA (**2**): amorphous solid, UV λ_{max} (MeOH); 235, 285 (shoulder), 334 nm and NMR (*3*) data in accordance with literature values.

5-5-DiFA (**3**): mp > 350 °C (literature, (*22*) > 350 °C), and UV (*13*, *16*) and NMR (*3*) data in accordance with literature values.

8-5-Benzofuran-diFA (**4**): mp 166−170 °C with decomposition (literature, (*3*) 169−174 °C), and UV (*13*, *16*) and NMR (*3*) data in accordance with literature values.

8-8-Bis-lactone-diFA (**5**): mp 211−213 °C (literature, (*23*) 213.8−214.7 °C), and UV (*24*) and NMR (*23*, *24*) data in accordance with literature values.

RESULTS AND DISCUSSION

The reaction of ethyl ferulate with H_2O_2 and peroxidase according to the method of Ralph et al. (*19*) gave primarily the 8-5-benzofuran-diFA and 8-O-4-diFA, 8-5-diFA, and 5-5-diFA (Figure 1) as minor products (*16*),

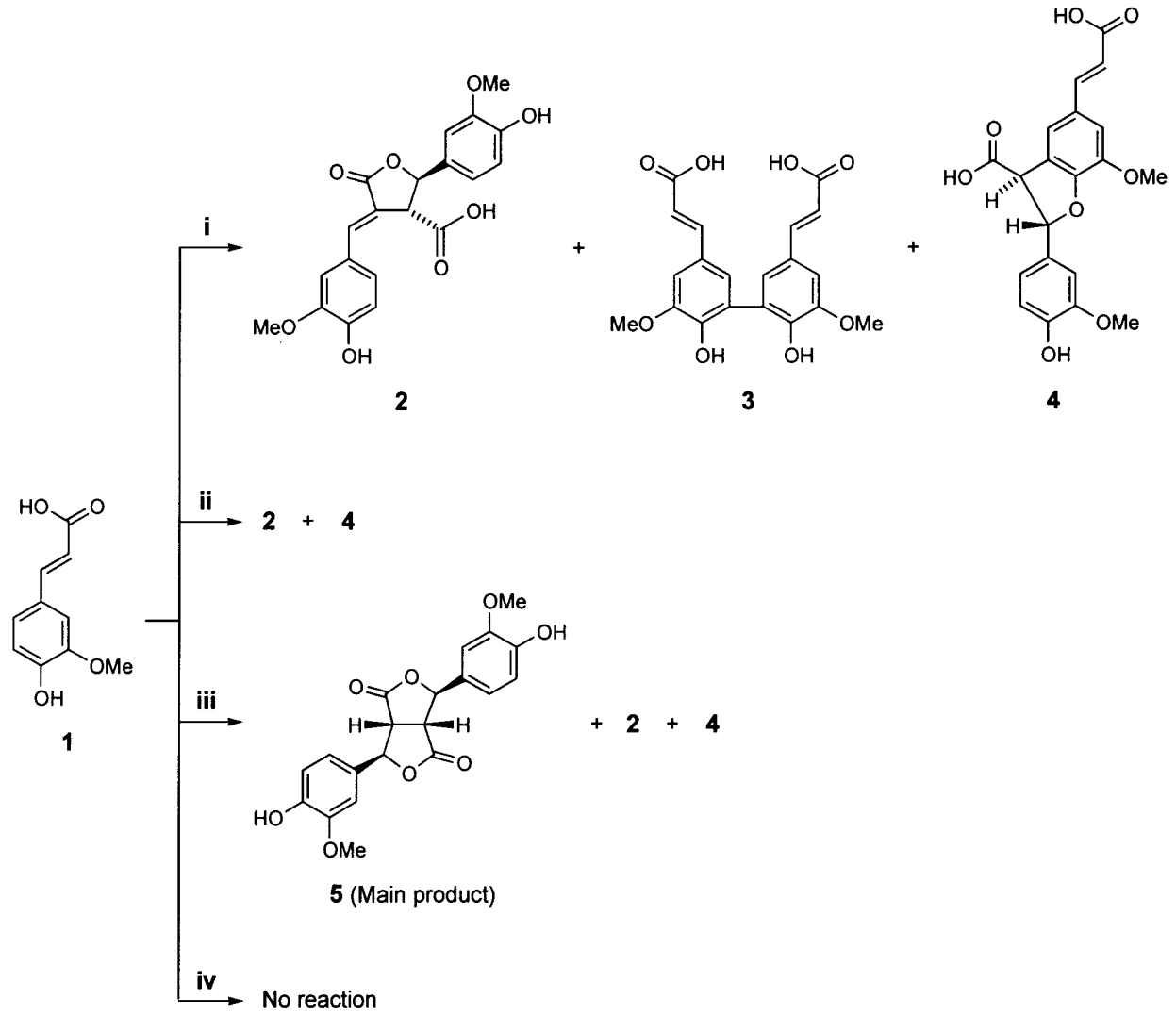

Figure 3. Dimerization of *trans*-ferulic acid in a micellar solution with hexadecyltrimethylammonium hydroxide and tetradecyltrimethylammonium bromide as the surfactants, respectively, and the corresponding dimerizations in nonmicellar buffered solutions. (**i**) Hexadecyltrimethylammonium hydroxide, peroxidase, and H_2O_2 (pH 7.0−8.0). (**ii**) Peroxidase, H_2O_2, and 0.1 M acetate buffer solution (pH 7.5). (**iii**) Tetradecyltrimethylammonium bromide, peroxidase, and H_2O_2 (pH 3.0). (**iv**) Peroxidase, H_2O_2, and 0.1 M phosphate buffer solution (pH 3.0).

whereas we observed no reaction in an unbuffered solution using *trans*-ferulic acid as the substrate. On the other hand, we found that the combination of *trans*-ferulic acid and the strongly basic surfactant hexadecyltrimethylammonium hydroxide in a molar ratio of 1:1 gave a complete reaction within 5 min. The distribution of products was quantified by analytical HPLC and was 25% 8-8-γ-lactone-diFA (**2**), 21% 5-5-diFA (**3**), and 14% 8-5-benzofuran-diFA (**4**) (Figure 3 and Table 1). During the reaction, the pH of the solution changed from 7.0 to 8.0. Repeating this experiment in a buffered solution (pH 7.5) but without surfactant gave a different product distribution, as no 5-5-diFA (**3**) was produced. The major products were 8-8-γ-lactone-diFA (**2**, yield 25%) and 8-5-benzofuran-diFA (**4**, yield 38%). Compounds **2**−**4** were obtained in a pure state by preparative HPLC (see Materials and Methods). Unidentified polymeric or higher oxidized products accounted for the remainder in both the micellar and buffered solutions, as an almost complete conversion of *trans*-ferulic acid was observed by analytical HPLC. 8-8-γ-Lactone-diFA (**2**) probably arises from an 8−8-coupled dehydrodimer followed by an intramolecular attack of one of the carboxylic acid moieties, whereas 5-5-diFA (**3**) and 8-5-benzofuran-diFA (**4**) are the well-known 5−5- and 8−5-coupled ferulic acid dehydrodimers, respectively (*3*). With other substrates (*trans*-*p*-coumaric and *trans*-sinapic acid) a similar reaction pattern was observed yielding a complex mixture of dimers (data not shown).

Changing the surfactant to tetradecyltrimethylammonium bromide gave 18% 8-8-bis-lactone-diFA (**5**) and only small amounts of 8-8-γ-lactone-diFA (**2**) and 8-5-benzofuran-diFA (**4**) (Figure 3 and Table 1) but with a substantial amount of polymeric or higher oxidized compounds. The pH remained constant at 3.0 during the reaction. Repeating this experiment in a buffered solution (pH 3.0) but without surfactant gave no reaction. Similar surfactants such as hexadecyltrimethylammonium hydrogencarbonate or hexadecyltrimethylammonium bromide gave almost identical reaction yields compared to that from tetradecyltrimethylammonium bromide (data not shown). The ratio of these surfactants to *trans*-ferulic acid was found to be important as a ratio close to 1 gave rise to additional amounts of byproducts. A molar excess of surfactant (50−100%) gave the cleanest reactions and with similar yields. 8-8-Bis-lactone-diFA (**5**) closely resembles the lignan pinoresinol, which has recently been stereoselectively prepared from coniferyl alcohol by means of a "guiding" protein (*20*). Compound **5** probably arises from an 8−8-coupled dehydrodimer followed by intramolecular attack of both of the carboxylic acid moieties, and it was obtained in a pure state by preparative HPLC (see Materials and Methods). With other substrates (*trans*-*p*-coumaric or *trans*-sinapic acid) only trace amounts of dimer products were observed (data not shown).

In general, for all the reactions mentioned, the absence of peroxidase or H_2O_2 gave no reaction, and the amount of peroxidase and reaction time could be increased 10 times without changing the product distribution and yield. The micellar reactions worked equally well at room temperature, but a reaction temperature around 40 °C was preferable to minimize reaction times and the risk of precipitation. The use of micelles for the synthesis of 5-5-diFA (**3**) might be an alternative way to obtain this compound because of the relatively simple reaction condition and because the yields are comparable to those obtained by existing synthetic methods (*16*, *22*, *25*).

From the present results it can be concluded that the outcome of radical dimerization of hydroxycinnamic acids is dependent on both the geometric and electronic nature of a controlling agent, and this seems to offer a new way to produce dehydrodimers of hydroxycinnamic acids on a preparative scale. Although micelles might not be involved in the biosynthetic assembly of these dehydrodimers, the present results indicate that a controlling agent might be present in vivo, e.g. "guiding" proteins as in the case of the biosynthesis of lignins and lignans.

ABBREVIATIONS USED

8-O-4-diFA, (*Z*)-β-{4-[(*E*)-2-carboxyvinyl]-2-methoxyphenoxy-4-hydroxy-3-methoxycinnamic acid; 8-5-diFA, (*E*,*E*)-4,4′-dihydroxy-3,5′-dimethoxy-β,3′-bicinnamic acid; 8-5-benzofuran-diFA, *trans*-5-[(*E*)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxyphenyl)-7-methoxy-2,3-dihydrobenzofuran-3-carboxylic acid; 5-5-diFA, (*E*,*E*)-4,4′-dihydroxy-5,5′-dimethoxy-3,3′-bicinnamic acid; 8-8-aryl-diFA, *trans*-7-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-6-methoxy-1,2-dihydronaphthalene-2,3-dicarboxylic acid; 8-8-γ-lactone-diFA, *trans*-4-(4-hydroxy-3-methoxybenzylidene)-2-(4-hydroxy-3-methoxyphenyl)-5-oxotetrahydrofuran-3-carboxylic acid; 8-8-bis-lactone-diFA, 4-*cis*,8-*cis*-bis(4-hydroxy-3-methoxyphenyl)-3,7-dioxabicyclo[3.3.0]octane-2,6-dione; 8-8-diFA, 4,4′-dihydroxy-3,3′-dimethoxy-β,β′-bicinnamic acid.

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