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Modeling suberization with peroxidase-catalyzed polymerization of hydroxycinnamic acids: Cross-coupling and dimerization reactions

Daniel Arrieta-Baez, Ruth E. Stark *

College of Staten Island Department of Chemistry, City University of New York Graduate Center and Institute for Macromolecular Assemblies, 2800 Victory Boulevard Room 6S-235, Staten Island, NY 10314-6600, USA

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Abstract

An anionic potato peroxidase (EC 1.11.1.7, APP) thought to be involved in suberization after wounding was isolated from slices of *Solanum tuberosum* in order to elucidate the first steps of dehydrogenative polymerization between pairs of different hydroxycinnamic acids (FA, CafA, CA and SA) present in wound-healing plant tissues. Use of a commercial horseradish peroxidase (HRP) – H_2O_2 catalytic system gave the identical major products in these coupling reactions, providing sufficient quantities for purification and structural elucidation. Using an equimolar mixture of pairs of hydroxycinnamic acid suberin precursors, only caffeic acid is coupled to ferulic acid and sinapic acid in separate cross-coupling reactions. For the other systems, HRP and APP reacted as follows: (1) preferentially with ferulic acid in a reaction mixture that contained *p*-coumaric and ferulic acids; (2) with sinapic acid in a mixture of *p*-coumaric and sinapic acids; (3) with sinapic acid in a mixture of ferulic and sinapic acids; (4) with caffeic acid in a reaction mixture of *p*-coumaric and caffeic acids. The resulting products, isolated and identified by NMR and MS analysis, had predominantly β-β-γ-lactone and β-5 benzofuran molecular frameworks. Five cross-coupling products are described for the first time, whereas the β-O-4 dehydrodimers identified from the caffeic acid and sinapic acid cross-coupling reaction are known materials that are highly abundant in plants. These reactivity trends lead to testable hypotheses regarding the molecular architecture of intractable suberin protective plant materials, complementing prior analysis of monomeric constituents by GC–MS and polymer functional group identification from solid-state NMR, respectively.

Keywords: Anionic potato peroxidase; Horseradish peroxidase; Hydroxycinnamic acids; Dehydrodimers; Radical cross-coupling reaction; Suberin

1. Introduction

Suberin is a complex, intractable biopolymer found in specialized plant cell walls (e.g., mature roots, tubers, stolons, rhizomes, bark), thought to be comprised of a phenolic domain attached to the cell wall and aliphatic components that are probably attached to the phenolic domain (Kolattukudy, 1978, 1980). Its chemical constituents include long-chain fatty acids as the aliphatic component (Kolattukudy, 1980, 1984) and phenolic derivatives as the aromatic component (Cottle and Kolattukudy, 1982; Bernards and Razem, 2001; Bernards, 2002). Whereas the nature of the phenolic matrix is incompletely defined, the evidence to date shows that in potato (*Solanum tuberosum* L.) tubers it is comprised primarily of hydroxycinnamic acids and their derivatives, hydroxycinnamoyl alcohols, and glycerol (Moire et al., 1999). Solid-state ^{13}C NMR of the intact material has provided detailed information on the composition of suberized potato cell wall (Garbow et al., 1989) and, in particular, clear-cut evidence for the occurrence of hydroxycinnamic acid and alcohol structural moieties (Bernards et al., 1995; Yan and Stark, 2000). The isolation of specific peroxidases (Espelie and Kolattukudy, 1985; Bernards et al., 1999; Quiroga et al., 2000) has prompted the suggestion that polymerization of these phenolic acids to form the suberin aromatic network occurs via a peroxidase/H_2O_2-mediated free radical coupling process

* Corresponding author. Tel.: +1 718 982 3894; fax: +1 718 982 3745.
E-mail address: stark@mail.csi.cuny.edu (R.E. Stark).

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(Razem and Bernards, 2002) analogous to lignification (Kolattukudy, 1980). In spite of these advances, neither the identity of the inter-unit bonds nor the molecular structure of suberin's phenolic core is currently known.

Model systems designed to elucidate the role of peroxidase enzymes (EC 1.11.1.7) in lignin biosynthetic pathways have often used horseradish peroxidase (Syrjänen and Brunow, 1998, 2000), generating dehydrogenative polymers of hydroxycinnamoyl alcohols, hydroxycinnamoyl aldehydes and hydroxycinnamic acids. The type of product is influenced by the rate at which substrates (phenolic compounds and H_2O_2) are added to the enzyme, and the proportion of dimers as compared with polymeric products depends on pH (Syrjänen and Brunow, 2000; Larsen et al., 2001). The ferulic acid dimers in particular have been compared with the structures deduced from solid-state ^{13}C NMR of suberized potato tissues (Bernards et al., 1995; Yan and Stark, 2000).

Ferulic acid (**1**) (FA), caffeic acid (**2**) (CafA), coumaric acid (**3**) (CA), and sinapic acid (**4**) (SA) are known to accumulate in potato tubers during wound healing and have been identified as monomers in the suberin structure (Kolattukudy, 1980; Bernards et al., 1995; Bernards and Lewis, 1998). A variety of regioisomeric dehydrohomodimers of these acids (Fig. 1) have been identified as products of β-β′ (**5**–**7**), 5-5′ (**8**), β-5 (**9**), 4-O-5 (**10**) and β-O-4 (**11**) radical coupling mediated by peroxidases (Ralph et al., 1994; Larsen et al., 2001). Ferulate trimers have been reported recently in maize cells, where they are believed to tighten the cell wall; higher oligomers of FA are also evident during polymerization with horseradish peroxidase (HRP) (Rouau et al., 2003). The final step in the formation of the suberin network within cell walls is thought to involve peroxidase-initiated dehydrogenative polymerization of these phenolic compounds with substituted hydroxycinnamic acids and hydroxycinnamoyl alcohols (Bernards et al., 1995; Bernards and Razem, 2001), but the polymerization itself is viewed as occurring without enzymatic control over the type or distribution of structural units. Moreover, the presence of suberin monomers other than FA raises the possibility that cross-coupling reactions mediated by a peroxidase could produce *p*-hydrophenyl, guaiacyl and syringyl subunits. Although the likelihood of cross-coupling reactions during lignin biosynthesis is supported by recent dimerization studies (Fournand et al., 2003), no analogous observations have been documented for suberin monomers.

In the current study, the anionic potato peroxidase (APP) associated with the suberization response in potato

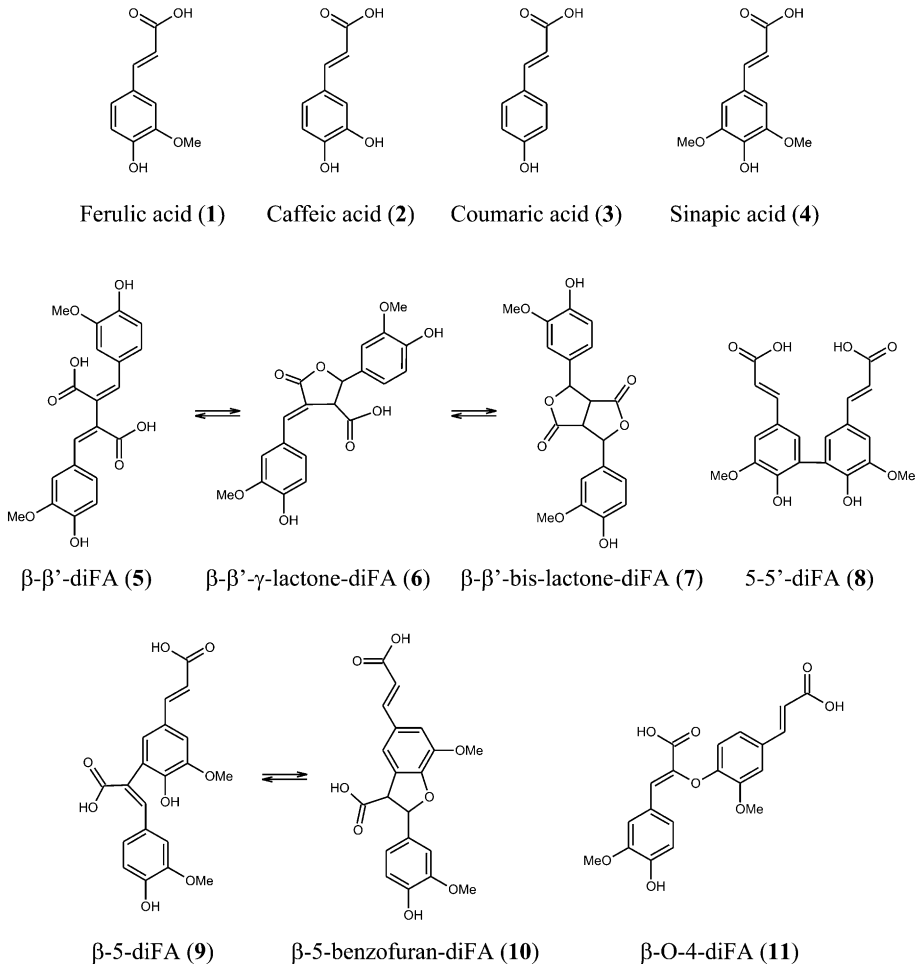


Fig. 1. Chemical structures of the major phenolic monomers and dehydrodimers isolated from plants.

tissues and a commercially available HRP were used in parallel to investigate the products of the first steps of dehydrogenative polymerization between pairs of hydroxycinnamic acids present in suberized tissues (FA (**1**), CafA (**2**), CA (**3**) and SA (**4**)). Using equimolar mixtures of these hydroxycinnamic acid monomers, only caffeic acid (**2**) was found to couple to ferulic acid (**1**) and sinapic acid (**4**) in separate cross-linked reactions. The resulting products, which were formed with either choice of peroxidase enzyme, were isolated and identified by NMR and mass spectral analysis as β-β-γ-lactone and β-5-benzofuran systems. Five new cross-coupling products are reported. The types and relative frequencies of inter-unit bonds found in these dimers establish patterns of reactivity that may prevail during suberin biosynthesis.

2. Results

2.1. Isolation of the wound-induced anionic peroxidase

Anionic potato peroxidase (APP) was obtained from a 7-day-old wound site in potato tubers using a protocol that combines size-exclusion and anion-exchange chromatography (Espelie and Kolattukudy, 1985; Bernards et al., 1999). Preliminary screening showed that the peroxidase activity was near maximal after 7 days of wound-healing in the thin periderm layer; this material was used as the enzyme source. Enzyme activity toward guaiacol and ferulic acid substrates was used to select peroxidase-containing fractions at each purification step. Both SDS–PAGE yielding a molecular mass of 45 kDa and an optimal enzyme activity at pH 4.5 were consistent with the presence of the anionic peroxidase (Bernards et al., 1999).

2.2. Dehydrogenative dimerization of hydroxycinnamic acids

When any particular hydoxycinnamic acid was used as the unique substrate for the peroxidase-H_2O_2 system (HRP or APP), complex mixtures were detected by HPLC analysis. The major products were purified and compared with those reported in the literature (Ralph et al., 1994; Syrjänen and Brunow, 2000; Larsen et al., 2001; Bunzel et al., 2003). The observed interunit linkages included β-5 (**9**), β-O-4 (**11**) and β-β′ (**5**) bonds, but no coupling modes producing 4-O-5 or 5-5′ (**8**) bonds were evident. These results (Table 1) demonstrate the dominance of coupling modes that produce a covalent bond at the β-carbon of at least one of the participating radicals, as reported previously (Syrjänen and Brunow, 2000; Larsen et al., 2001) and may be attributed to the high reactivity of β-radicals.

2.3. Dehydrogenative dimerization and cross-coupling of hydroxycinnamic acid mixtures

When equimolar mixtures of two hydroxycinnamic acids were oxidized using the peroxidase-H_2O_2 system (HRP or APP), only two mixtures were found to undergo cross-coupling reactions. Caffeic acid (**2**) reacted with both ferulic (**1**) and sinapic (**4**) acid in separate reactions to form heterodimers. With the other hydroxycinnamic acid mixtures, one of the substrates underwent peroxidase-mediated oxidation almost exclusively: (1) ferulic acid (**1**) in a reaction mixture that also contained *p*-coumaric acid (**3**); (2) sinapic acid (**4**) in a mixture that also contained *p*-coumaric acid (**3**); (3) sinapic acid (**4**) in a mixture that also contained ferulic acid (**1**); and (4) caffeic acid (**2**) in a reaction mixture that also contained *p*-coumaric acid (**3**) (Table 2). These latter kinetic preferences are in accord with those reported previously (Takahama, 1995). The same chromatographic peaks were observed from both HRP- and APP-mediated reactions, indicating the formation of identical products in the cross-linking reactions and validating the use of HRP as a readily available enzyme for the investigation of suberin polymerization.

2.3.1. Products from caffeic acid–ferulic acid reactions

When the peroxidase-mediated oxidation products of caffeic acid (**2**) and ferulic acid (**1**) were subjected to reversed-phase HPLC, numerous peaks were obtained (Fig. 2(a)). A preliminary separation of these materials was made on a chromatographic column, and final purification was achieved by reversed-phase HPLC. Four major compounds were identified as shown in Fig. 3: three new cross-coupling products (**12**–**14**) and β-5-benzofuran-diCafA (caffeic acid dimerization product, **15**).

Table 2
Oxidative coupling reactions with mixtures of hydroxycinnamic acids

Mixture of hydoxycinnamic acids	Type of reaction[a]
Ferulic acid + caffeic acid	Dimerization and cross-coupling
Ferulic acid + coumaric acid	Ferulic acid dimerization
Ferulic acid + sinapic acid	Sinapic acid dimerization
Caffeic acid + coumaric acid	Caffeic acid dimerization
Caffeic acid + sinapic acid	Dimerization and cross-coupling
Coumaric acid + sinapic acid	Sinapic acid dimerization

[a] Reaction conditions are described in the text.

Table 1
Dehydrogenative (oxidative) dimerization products of hydroxycinnamic acids

Hydroxycinnamic acid	Products[a]	References
Ferulic acid	β-β′-γ-lactone-diFA, 5-5′-diFA, β-5′-benzofuran-diFA	Larsen et al. (2001)
Caffeic acid	β-β′-γ-lactone-diCafA, β-β′-γ-lactone-diCafA derivatives, β-5′-benzofuran-diCafA	Tazaki et al. (2001)
Coumaric acid	β-β′-bis-lactone-DiCA and 5-5′-diCA	This work
Sinapic acid	β-β′-γ-lactone-diSA	Ralph et al. (2000); Bunzel et al. (2003)

[a] Reaction conditions are described in the text.

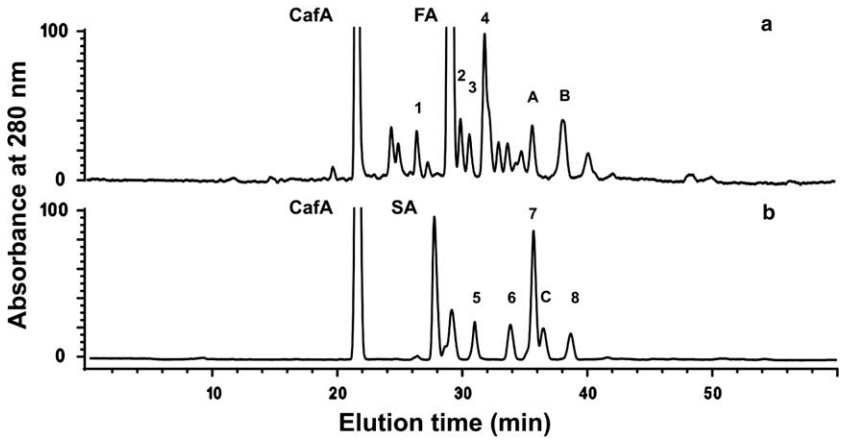
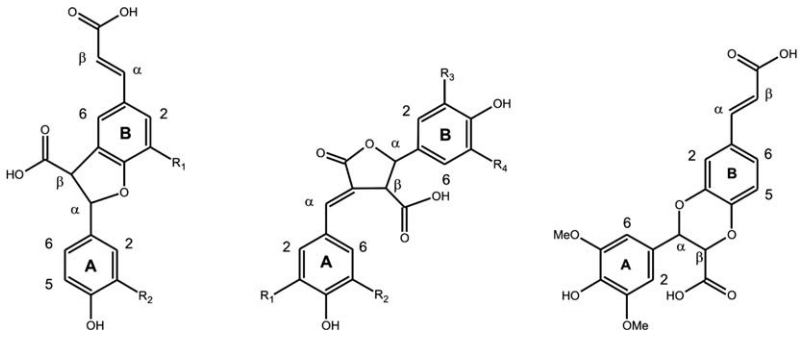


Fig. 2. A typical chromatogram obtained from reversed-phase HPLC analysis of the reaction products obtained from (a) horseradish peroxidase-hydrogen peroxide oxidation of a mixture of FA (**1**) and CafA (**2**); (b) HRP–H_2O_2 oxidation of a mixture of SA (**4**) and CafA (**2**).

The structures of these compounds were determined by ^{1}H NMR, ^{13}C NMR, 2D ^{1}H–^{13}C HMQC, 2D ^{1}H–^{13}C HMBC, and HR-MS, in an analogous fashion to previous reports of lignin homodimers (Ralph et al., 1994, 2004).

In the positive HR FAB mass spectrum using glycerol as a matrix, the $[M + H]^+$ of **12** was observed at *m/z* 373.0925, establishing the molecular formula as $C_{19}H_{17}O_8$. The ^{1}H NMR spectrum displayed several features consistent with the dimeric structure proposed in Fig. 3: an AMX aromatic system (corresponding to A2H, A5H and A6H at δ 7.20, 6.84 and 7.17 ppm, respectively), an AB aromatic system bearing two protons *meta* to one another (B2H and B6H at δ 6.67 ppm) and implicating substitution at position B5. In addition, the scalar coupling constants supported the presence of two *trans* olefinic protons (BαH and BβH at δ 7.90 and 6.19 ppm, respectively; $J = 15.46$ Hz), and two methine protons (AαH and AβH at δ 4.39 and δ 4.48 ppm, respectively). The covalent connection of the B ring to one of these CH groups (Aβ) was confirmed by HMBC cross-peaks (AβH at δ 4.48 ppm with B6C at δ 110.85 ppm). The chemical shift of AαC at δ 66.07 ppm suggested bonding to an oxygen; together these observations indicated a β-5-benzofuran dimer structure. The



Compound **12** R_1= OH, R_2= OCH_3

Compound **13** R_1= OCH_3, R_2= OH

Compound **15** R_1= OH, R_2= OH

Compound **14** R_1= OCH_3, $R_{2,4}$= H, R_3= OH

Compound **16** $R_{1,2}$= OCH_3, R_3= OH, R_4= H

Compound **18** $R_{1,3}$= OH, $R_{2,4}$= H

Compound **19** (α,β *trans*) $R_{1,2,3,4}$=OCH_3

Compound **20** (α,β *gauche*) $R_{1,2,3,4}$=OCH_3

Compound **17**

Fig. 3. Chemical structures of the major products isolated from the peroxidase-hydrogen peroxide coupling and dimerization reactions.

Table 3
^{1}H and ^{13}C NMR (600 and 150 MHz in CD_3OD) data for cross-coupling products **12**–**14**

Position	**12**		**13**		**14**	
	^{1}H NMR, δ (ppm), J (Hz)	^{13}C NMR, δ (ppm)	^{1}H NMR, δ (ppm), J (Hz)	^{13}C NMR, δ (ppm)	^{1}H NMR, δ (ppm), J (Hz)	^{13}C NMR, δ (ppm)
A2	7.20 (1H, d, 2.13)	110.37	7.18 (1H, d, 2.05)	117.37	7.26 (1H, d, 2.17)	113.26
A5	6.84 (1H, d, 8.11)	116.17	6.96 (1H, d, 8.20)	118.16	6.84 (1H, d, 8.39)	115.97
A6	7.17 (1H, dd, 2.13, 8.11)	120.36	7.14 (1H, dd, 2.05, 8.20)	123.13	7.12 (1H, dd, 2.17, 8.39)	126.56
B2	6.67 (1H, s)	114.75	6.84 (1H, s)	119.78	6.71 (1H, d, 1.91)	112.66
B5	–	[a]	–	[a]	6.74 (1H, d, 7.98)	115.91
B6	6.67 (1H, s)	110.85	6.99 (1H, s)	110.30	6.65 (1H, dd, 1.91, 7.98)	117.69
Aα	4.39 (1H, d, 4.80)	66.07	6.00 (1H, d, 7.57)	88.40	7.64 (1H, s)	141.22
Aβ	4.48 (1H, d, 4.80)	47.35	4.28 (1H, d, 7.57)	56.69	–	[a]
Bα	7.90 (1H, d, 15.46)	138.99	7.57 (1H, d, 16.01)	145.88	5.62 (1H, d, 2.63)	82.11
Bβ	6.19 (1H, d, 15.46)	118.58	6.32 (1H, d, 16.01)	117.31	4.12 (1H, d, 2.63)	54.24
OMe A3	3.94 (3H, s)	56.20			3.87 (3H, s)	56.30
OMe B3			3.82 (3H, s)	56.40		

[a] These carbons do not give signals in HMQC or HMBC NMR spectra.

methoxy protons at δ 3.94 ppm and A5H proton at δ 6.84 ppm showed HMBC interactions with A3C at δ 148.8 ppm, verifying the substitution of the methoxy group on that benzene ring. On the basis of these spectroscopic data (Table 3), compound **12** (8 mg) was identified as 5-[(*E*)-2-carboxyvinyl]-7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-2,3-dihydro-1-benzofuran-3-carboxylic acid (β-5-benzofuran-FA-CafA dimer).

Compound **13** (Fig. 3) is also a new cross-coupling product. FABMS did not yield a molecular ion, but the observed fragments were consistent with the molecular formula $C_{19}H_{16}O_8$: m/z 329 $[372 - COOH]^+$, 316 $[372 - 2CO]^+$, and 194 $[372 - CafA]^+$. NMR spectra of this compound displayed the same ring systems as **12** (Fig. 3): an AMX aromatic system (corresponding to A2H, A5H and A6H at δ 7.18, 6.96 and 7.14 ppm, respectively), an AB aromatic system bearing two protons *meta* to one another (B2H and B6H at δ 6.84 and 6.99 ppm, respectively) and thus substitution at position B5 of the ring. The spectrum of **13** also showed two *trans* olefinic protons (BαH and BβH at δ 7.57 and 6.32 ppm, respectively; $J = 16.01$ Hz) and two methine protons (AαH and AβH at δ 6.00 and 4.28 ppm, respectively). Through observations and reasoning similar to that described above, it was deduced from an HMBC experiment that protons Aα and Aβ were coupled with the B ring in a β-5-benzofuran configuration. In contrast to **12**, the methoxy protons at δ 3.82 ppm showed HMBC interactions with a carbon at δ 149.10 ppm; however, there is no interaction between A5H and this carbon, implying substitution of the methoxy group on the B aromatic ring. On the basis of these spectroscopic results (Table 3), it was concluded that compound **13** (9 mg) is 5-[(*E*)-2-carboxyvinyl]-2-(3,4-dihydroxyphenyl)-7-methoxy-2,3-dihydro-1-benzofuran-3-carboxylic acid (β-5-benzofuran-CafA-FA dimer).

The molecular formula $C_{19}H_{17}O_8$ of the compound **14** was determined by HR FABMS analysis of the peak at m/z 373.0923 ($[M + H]^+$). The ^{1}H NMR spectrum of compound **14** (Fig. 3) displayed two AMX aromatic spin systems, indicating that neither of these rings is fused to another ring, but rather they belong to the ferulic acid (**1**) and caffeic acid (**2**) constituents, respectively. The observation of an olefinic proton at δ 7.64 ppm and two methine protons at δ 5.62 and 4.12 ppm suggested the presence of a β-β′-γ-lactone. An HMBC experiment showed interactions of the olefinic proton (AαH at δ 7.64 ppm) with A2C (δ 113.26 ppm) and A6C (δ 126.56 ppm) as well as with BβC (δ 54.24 ppm) in the lactone ring. These data confirm the presence of a β-β′-γ-lactone. The methoxy protons at δ 3.87 ppm and A5H at δ 6.84 showed an HMBC interaction with A3C (δ 148.8 ppm), confirming the presence of a ferulic moiety in the A ring. These data (Table 3) confirm the identification of **14** (7 mg) as (4*E*)-2-(3,4-hydroxyphenyl)-2-(4-hydroxy-3-methoxybenzylidene)-5-oxotetrahydrofuran-3-carboxylic acid (β-β′-γ-lactone-FA-CafA dimer).

Compound **15** (isolated from peak 4; Fig. 2(a)) was the major product from this reaction (16 mg) and was characterized as a β-5-benzofuran-diCafA (**15**) based on NMR results similar to those reported previously (Tazaki et al., 2001; Table 4). Compound **15** showed a molecular ion at m/z 359.0766 ($[M + H]^+$) in positive-mode HR FABMS, consistent with the molecular formula $C_{18}H_{15}O_8$.

Additional HPLC peaks displayed in Fig. 2(a) (peaks **A** and **B**) contained a mixture of FA dimers with trimer and/or higher oligomeric products. These materials are currently under analysis in our laboratory.

2.3.2. Products from caffeic acid–sinapic acid reactions

Caffeic acid (**2**) and sinapic acid (**4**) were the second pair of monomers that dimerize via oxidative cross-coupling. As before, preliminary separation was carried out using column chromatography; final purification using reversed-phase HPLC yielded six peaks (Fig. 2(b)). Five major products were identified: two new cross-coupling products (**16** and

17) and three homodimers (**18**–**20**). The structures of these compounds were determined by ^{1}H NMR, ^{13}C NMR, 2D ^{1}H–^{13}C HMQC, 2D ^{1}H–^{13}C HMBC, and HR-MS.

The molecular formula of compound **16** was determined by positive HR FABMS analysis as $C_{20}H_{19}O_9$ ($[M+H]^+$ m/z 403.1027). The ^{1}H NMR spectrum showed two aromatic systems: an A_2 system (A2H and A6H at δ 6.96 ppm) assigned to the sinapic unit, and an AMX system (B2H, B5H, and B6H at δ 6.71, 6.75 and 6.65, respectively) attributed to the caffeic acid. The observation of an olefinic proton (AαH at δ 7.64 ppm) and two methine protons (BαH at δ 5.61 ppm and BβH at δ 4.12 ppm, respectively) suggested the presence of a β-β′-γ-lactone. The structure was confirmed with HMBC and HMQC experiments, which displayed through-bond connectivities similar to those described for compound **14**. These data (Table 5) permitted identification of **16** (10 mg) as (4*E*)-2-(3,4-dihydroxyphenyl)-4-(4-hydroxy-3,5-dimethoxybenzylidene)-5-oxotetrahydrofuran-3-carboxylic acid (β-β′-γ-lactone-SA-CafA dimer).

Peak 8 (Fig. 2(b)) consisted of two major products that were subsequently separated and purified by HPLC: a new cross-linking product (**17**, Fig. 3) of SA (**4**) and CafA (**2**) and a β-β′-γ-lactone-diSA (**20**, Fig. 3) from the sinapic acid (**4**) dimerization reaction. HR FABMS of compound **17** gave a molecular ion at m/z 403.1028 ($[M+H]^+$), consistent with the molecular formula $C_{20}H_{19}O_9$. The aromatic proton resonances appeared as follows: an A_2 system (A2H and A6H at δ 6.70 ppm) assigned to the sinapic unit, and an AMX system (B2H, B5H, and B6H at δ 7.28, 7.58 and 7.16 ppm, respectively) that is connected to two olefinic protons (BαH and BβH at δ 7.58 and 6.34 ppm, respectively) from the caffeic moiety. The similar chemical shifts of the methine protons at δ 5.49 and 5.02 ppm (AαH and AβH) suggested that the hydroxyl groups of caffeic acid (**2**) are bound to the Aα and Aβ carbons of the sinapic acid (**4**). HMQC and HMBC cross-peaks confirmed the identification of **17** (5 mg, Table 5) as 6-[(*E*)-2-carboxyvinyl]-3-(4-hydroxy-3,5-dimethoxyphenyl)-2,3-dihydro-1,4-benzodioxane-2-carboxylic acid.

Compound **18** (Fig. 3, 9 mg), isolated from peak 6 (Fig. 2(b)), was identified as a β-β′-γ-lactone-diCafA (**18**) in accord with prior NMR spectroscopy data (Tazaki et al., 2001). This structure was confirmed from ES-MS, which showed ions at m/z 359.06 ($[M+H]^+$), 315.07 ($[M+H-CO_2]^+$), and 125.05 ($[M+H-234]^+$), corresponding to a molecular formula of $C_{18}H_{15}O_8$.

Compound **19** (Fig. 3), isolated from Peak 7 (Fig. 2(b)), was the most abundant product isolated from this reaction (22 mg). The ^{1}H NMR spectrum showed two singlet aromatic resonances at δ 6.91 and 6.74 ppm (A2H/A6H and B2H/B6H, respectively), one olefinic proton at δ 7.59 ppm, and two aliphatic methine protons at δ 5.73 and 4.57 ppm ($J = 7.2$ Hz). A similar pattern of chemical shifts was observed for compound **20**, which was isolated from peak 8: two singlet aromatic peaks at δ 6.95 and 6.59 ppm (A2H/A6H and B2H/B6H, respectively), one olefinic proton at δ 7.65 ppm, and two aliphatic methine protons at δ 5.70 and 4.17 ppm ($J = 3$ Hz). Taken together, these data (Table 4) confirmed the presence of two β-β′-γ-lactone-diSA isomers, one with a *trans* configuration of BαC and BβC (compound **19**, 22 mg) and the other with a *gauche* configuration (compound **20**, 6 mg). Compounds **19** and **20** each displayed ions at m/z 447.1291 ($[M+H]^+$) in their positive HR FAB mass spectra, consistent with a molecular formula of $C_{22}H_{23}O_{10}$.

3. Discussion

By using a model system to examine the fundamental chemistry of suberin biosynthesis, the foregoing studies complement prior work that identified molecular groupings and domain architecture in suberin from potato wound periderm using solid-state NMR (Stark et al., 1994; Bernards et al., 1995; Yan and Stark, 2000). The molecular structures herein identified from dimerization reactions of hydroxycinnamic acids, including five novel cross-coupling dehydrodimers, can guide our interpretation of spectral data collected for intact potato suberin and test hypotheses regarding cross-linking structures in these protective plant polymers. The current work also provides a rationale for the formation of complex heteropolymeric structures from hydroxycinnamic acids present in suberizing plant tissues and lays the groundwork for exploring the impact of parameters such as number and abundance of various substrates, sequence of substrate addition, and pH on these biosynthetic processes.

When a single hydroxycinnamic acid and H_2O_2 are added simultaneously as substrates for the HRP or APP peroxidases, the rates of reaction for the various substrates are related as follows: feruloyl > caffeoyl > *p*-coumaroyl ≈ syringyl, as demonstrated in prior studies (Bernards et al., 1999; Quiroga et al., 2000). However, when an equimolar mixture of hydroxycinnamic acids is subjected to oxidation by the peroxidase-H_2O_2 system, only caffeic acid (**2**) couples to ferulic acid (**1**) and sinapic acid (**4**) in separate cross-reactions. For instance, ferulic acid (**1**) dimerizes more readily than *p*-coumaric acid (**3**) but reacts competitively with caffeic acid (**2**) to form both homodehydrodimers and cross-coupling products.

For the other mixtures, only dimers derived from a single monomer are formed. Because the initial step in the oxidation of phenolic monomers by peroxidases is the formation of phenoxyl radicals, the homodimers could be formed as follows: *p*-CA (**3**) is oxidized more rapidly than FA (**1**); the resulting *p*-CA radical oxidizes FA (**1**) to form the corresponding radicals (and regenerates unreactive *p*-CA, **3**); the FA phenoxyl radicals then give rise to FA dehydrodimers (**5**–**11**). The relative paucity of *p*-coumaric acid dimers in reaction products of hydroxycinnamic mixtures is also in accord with our finding that these functional groupings are nearly absent in fully formed potato suberin (Yan and Stark, 2000).

Table 4
^{1}H and ^{13}C NMR (600 and 150 MHz in CD_3OD) data for homodimer products **15**, **18**, **19** and **20**

Position	**15**		**18**		**19**		**20**	
	^{1}H NMR, δ (ppm), J (Hz)	^{13}C NMR, δ (ppm)	^{1}H NMR, δ (ppm), J (Hz)	^{13}C NMR, δ (ppm)	^{1}H NMR, δ (ppm), J (Hz)	^{13}C NMR, δ (ppm)	^{1}H NMR, δ (ppm), J (Hz)	^{13}C NMR, δ (ppm)
A2	6.81 (1H, d, 1.95)	113.39	7.55 (1H, d, 1.90)	113.12	6.91 (1H, s)	108.42	6.95 (1H, s)	108.53
A5	6.75 (1H, d, 7.99)	115.93	6.80 (1H, d, 8.04)	116.01	–	148.71	–	148.52
A6	6.74 (1H, dd, 1.95, 7.99)	118.21	7.01 (1H, dd, 1.90, 8.04)	126.56	6.91 (1H, s)	108.42	6.95 (1H, s)	108.53
B2	7.13 (1H, s)	117.39	6.71 (1H, d, 2.4)	111.95	6.74 (1H, s)	104.04	6.59 (1H, s)	103.08
B5	–	[a]	6.75 (1H, d, 8.4)	115.90	–	148.16	–	148.73
B6	7.00 (1H, s)	116.62	6.64 (1H, dd, 2.4, 8.4)	116.85	6.74 (1H, s)	104.04	6.59 (1H, s)	103.08
Aα	5.95 (1H, d, 7.22)	88.38	7.55 (1H, s)	141.13	7.59 (1H, s)	139.23	7.65 (1H, s)	141.19
Aβ	4.22 (1H, d, 7.22)	56.50	–	[a]	–	[a]	–	[a]
Bα	7.55 (1H, d, 15.90)	146.17	5.62(1H, d, 2.4)	81.36	5.73 (1H, d, 7.2)	80.89	5.70 (1H, d, 3.00)	81.93
Bβ	6.25 (1H, d, 15.90)	115.95	4.12 (1H, d, 2.4)	54.85	4.57 (1H, d, 7.2)	52.99	4.17 (1H, d, 3.00)	54.30
OMe A3	–	–	–	–	3.87 (6H, s)	55.91	3.85 (6H, s)	55.97
OMe B3	–	–	–	–	3.84 (6H, s)	55.91	3.80 (6H, s)	55.97

[a] These carbons do not give signals in HMQC or HMBC NMR spectra.

Although the most abundant dehydrodimer identified previously in plant material is β-O-4 diFA (**11**) (Ralph et al., 1994; Ralph et al., 2000), the primary dehydrodimers generated in vitro from ferulic acid utilizing our peroxidase-hydrogen peroxide system are β-5-coupled products (Fig. 1) (Larsen et al., 2001). The latter trend is confirmed in the present work: β-5-benzofuran and β-β′-γ-lactone systems are found to be the main products isolated in these peroxidase-mediated oxidative-coupling reactions of suberin monomers, irrespective of whether HRP or APP enzymes are employed to oxidize the substrate under a common set of conditions.

Although some authors have reported that particular peroxidases exhibit differing reactivity and form different FA (**1**) oxidation products (Ward et al., 2001), others have claimed very similar product profiles based on more complete product analysis (Wallace and Fry, 1999; Ward et al., 2001). In any case, our studies reveal product formation that is independent of whether APP or HRP is employed. For instance, the HPLC chromatograms from oxidative coupling of FA catalyzed by either enzyme showed a dozen identical products. Analogous observations were made for dimerization and cross-coupling reactions of the other suberin monomers. A rationale for obtaining common products from diverse peroxidases comes from the X-ray structures of HRP-FA complexes, in which loose enzyme–substrate association is thought to result when the phenoxyl radical vacates the catalytic site and polymerizes with other radicals to form dimeric and oligomeric products (Henriksen et al., 1999). That is, the last step in the formation of the main dehydrodimers (β-5-benzofuran and β-β′-γ-lactone systems) may not be enzymatically controlled; rather it may be governed by the chemical character of the radical (e.g., oxidation potential and radical reactivity) and its environment (Syrjänen and Brunow, 1998; Henriksen et al., 1999). Since β-radicals are the most reactive of the possible free radicals, coupling modes that produce a covalent bond at the β-carbon of one or both of the participating radicals are thus expected to predominate.

It is also noteworthy that HPLC monitoring of the peaks corresponding to the β-β-γ-lactone and β-5 benzofuran systems shows increases in size as these products form during the first minutes of reaction; however, their intensity levels off even as the substrate(s) are consumed at the end of the reaction. This pattern of product formation suggests that the dimers are intermediates in oxidative-coupling reactions that may form higher oligomers under our experimental conditions. Some of the oligomers are present in peaks A and B as noted above, though others may remain undetected in our experiments because they are sparingly soluble or present in small amounts.

The isolation of a modest amount of compound **17** from sinapic–caffeic acid cross-coupling also implicates reactions that proceed through the β-O-4 conformation. The formation of β-O-4 dehydrodimers is distinctive in its requirement of water addition to an intermediate quinone

Table 5
^{1}H and ^{13}C NMR (600 and 150 MHz in CD_3OD) data for cross-coupling products **16** and **17**

Position	**16**		**17**	
	^{1}H NMR, δ (ppm), J (Hz)	^{13}C NMR, δ (ppm)	^{1}H NMR, δ (ppm), J (Hz)	^{13}C NMR, δ (ppm)
A2	6.96 (1H, s)	109.20	6.70 (1H, s)	117.37
A6	6.96 (1H, s)	109.20	6.70 (1H, s)	117.37
B2	6.71 (1H, d, 2.10)	112.99	7.28 (1H, d, 1.9)	119.78
B5	6.75 (1H, d, 8.24)	116.36	7.58 (1H, d, 8.44)	116.24
B6	6.65 (1H, dd, 2.10, 8.24)	117.50	7.16 (1H, dd, 1.9, 8.44)	110.30
Aα	7.64 (1H, s)	141.82	5.49 (1H, d, 2.5)	88.40
Aβ	–	[a]	5.02 (1H, d, 2.5)	86.50
Bα	5.61 (1H, d, 3.12)	82.48	7.58 (1H, d, 16.23)	145.88
Bβ	4.12 (1H, d, 3.12)	54.83	6.34 (1H, d, 16.23)	117.31
OMe A3 OMe A5	3.86 (6H, s)	56.30	3.77 (6H, s)	56.20

[a] These carbons do not give signals in HMQC or HMBC NMR spectra.

methide (Sipila and Brunow, 1991), so this cross-coupling pattern would be expected to be favored kinetically if acidic pH values prevail in vivo. Such conditions could be achieved by the diffusion of hydroxycinnamic acid monomers from the cytosol to the cell wall, and low pH conditions should also activate the anionic peroxidase that is believed to catalyze suberization.

The presence of suberized polysaccharides in our potato wound periderm preparations, even after exhaustive enzymatic removal of carbohydrates and extraction with organic solvents, allows for only limited spectroscopic and structural comparisons with the hydroxycinnamic acid dehydrodimers identified herein. Nevertheless, a group of ^{1}H NMR signals observed at δ 7.7 and 7.8 ppm from DMSO-swelled suberin samples (S. Tian, unpublished observations) suggests the presence of vinylic protons coupled to an aromatic ring. Their downfield chemical shifts and lack of J-couplings to aromatic or vinyl protons are both consistent with the inclusion of these protons in β-β′-, β-β′-γ-lactone, β-5-, β-5-benzofuran and/or β-O-4-dehydrodimer molecular frameworks analogous to those reported in lignins (Ralph et al., 2004). The validity of these dehydrodimers as models for the suberin aromatic domain is reinforced by NMR analysis of potato suberin KOH degradation products (W. Wang, unpublished observations), which shows the same distinctive ^{1}H resonances and J-coupling patterns as well as additional ^{1}H–^{13}C gHMQC correlations at (δ 7.7, 140.08 ppm) and (δ 7.8, 141.20 ppm).

4. Conclusions

The findings reported herein have spurred several additional investigations that can bring us closer to an understanding of the fundamental chemistry that occurs during suberization in potato wound periderm. For instance, experiments are ongoing in our laboratory to delineate chemical bonding patterns in higher oligomers from the peroxidase-H_2O_2 reactions, to monitor the course of these reactions to include formation of insoluble products, and to develop model systems for suberin association or bonding with plant cell walls. Ultimately, a molecular-level understanding of this biosynthetic process should elucidate the detailed nature of the suberin protective polymer and guide our efforts to design a more robust defense against pathogens that compromise the agricultural viability of food crops.

5. Experimental

5.1. General

NMR spectra were acquired on a Varian ^{UNITY}INOVA spectrometer operating at ^{1}H and ^{13}C frequencies of 599.95 and 150.87 MHz, respectively. Hydroxycinnamic acid dimers were dissolved in CD_3OD and contained 1% tetramethylsilane as a chemical shift standard (Aldrich). Typical experimental conditions included 16 repetitions and 1.0 s between successive acquisitions. Data processing and ^{1}H integration were performed using VNMR software. Several two-dimensional experiments (e.g., gmqCOSY, gHMBC, and gHMQC) were used to establish through-bond connectivities as described previously (Hurd, 1990; Fang et al., 2001). HR FAB-MS (positive) and FAB-MS were done using JEOL AX-505 double focusing and JEOL HX-110 double focusing mass spectrometers (MSU Mass Spectrometry Facility) with Gly and NBA as matrices. ES-MS was done using an Agilent 1100 LC/MSD instrument. UV spectra were recorded on a UV–Vis Helios spectrophotometer.

5.2. Chemicals

Ferulic acid (FA, **1**), caffeic acid (CafA, **2**), coumaric acid (CA, **3**) and sinapic acid (SA, **4**) were purchased from Sigma–Aldrich Chemical Co. (Milwaukee, WI). Horserad-

ish peroxidase type II and X were purchased from Sigma Chemical Co. (St. Louis, MO). The internal standards 3,4,5-trimethoxybenzoic acid and hydrogen peroxide (35 wt% solution) were obtained from Fluka (St. Louis, MO). Reaction buffers were prepared from sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$) and disodium hydrogen phosphate ($Na_2HPO_4 \cdot 10H_2O$).

5.3. Anionic peroxidase isolation and partial purification

Potatoes (*S. tuberosum* L.) were peeled, cut into discs, and suberized for 7 days (Stark et al., 1994). For isolation of the anionic peroxidase, all steps were performed at 4 °C or on ice. The suberized tissue was removed from the outer surface of each disc and mechanically homogenized with 10 mM sodium phosphate, pH 6.5. The mixture was filtered and centrifuged at 10,000*g* for 15 min. Ammonium sulfate was added slowly to the supernatant until 40% saturation was achieved, and the mixture was kept stirring for 30 min. After centrifugation at 10,000*g* for 15 min, the supernatant was brought to 80% saturation with solid ammonium sulfate and recentrifugated at 15,000*g* for 15 min. The precipitate was resuspended and dialyzed overnight against the same buffer. The extract was concentrated by ultrafiltration (YM 10 membrane, Amicon), loaded onto a Sephadex G-100 column pre-equilibrated with 25 mM Bis-Tris (pH 7.1) in twenty 3- to 5- mL batches, and eluted with 25 mM Bis-Tris (pH 7.1).

Fractions from Sephadex G-100 were assayed for peroxidase activity as described below; active fractions were pooled and concentrated to 8 mL by ultrafiltration (YM 10 membrane, Amicon). This extract was loaded onto a DEAE chromatographic column equilibrated with 25 mM Bis-Tris (pH 7.1) and eluted with a salt gradient (0–300 mM NaCl in 25 mM Bis-Tris, pH 7.1). Fractions containing peroxidase activity were pooled, desalted, and concentrated by ultrafiltration (YM 10 membrane, Amicon) into 25 mM Bis-Tris (pH 7.1).

5.4. Peroxidase activity and SDS–PAGE analysis

Peroxidase activity was determined by measuring the increase in absorbance at 470 nm after addition of anionic peroxidase (1 nM), guaiacol (0.15 mM) and hydrogen peroxide (4 mM) to 60 mM potassium acetate at pH 6.5. All reactions were performed in triplicate. SDS–PAGE was performed on 14% acrylamide gels essentially as described previously (Bernards et al., 1999), though only one isoform was observed.

5.5. Oxidative phenolic coupling assay

For test reactions run on an analytical scale, oxidative polymerization of *p*-hydroxycinnamic acids was conducted at 25 °C using a 1.3-mL reaction mixture in 60 mM phosphate buffer (pH 6.1), 1 mM H_2O_2, 4 μM *p*-hydroxycinnamic acid and 20 μL of APP or HRP (130 μg/mL). Cross-coupling was then attempted with equimolar amounts of two *p*-hydroxycinnamic acids at a concentration of 4 mM and 10 μL of 4 mM H_2O_2. The extents of the enzymatic reactions were determined through the decrease in HPLC peak area at 280 nm for the *p*-hydroxycinnamic acids. All reactions were carried out in triplicate.

The reaction products were separated by HPLC (Hewlett–Packard 1090 series II/L) using a reversed-phase column (3.9 × 300 mm, μBondapak C_{18}, Waters) at 27 °C and gradient elution with solvent A (phosphate buffer, 0.01 M, pH 3) and solvent B (CH_3CN) using the following elution profile: 0–50 min linear gradient from 90% A: 10% B to 65% A: 45% B: linear gradient from 50–60 min to 40% A: 60% B and continuing isocratically at 40% A: 60% B for 10 additional minutes. Flow rate: 1 mL min^{-1}. Injection volume: 20 μL.

To obtain a sufficient quantity of products for structural identification, HRP and H_2O_2 were added to 500-mL reaction mixtures containing 150 mg of, e.g., ferulic acid (**1**) with an equimolar amount of caffeic acid (**2**). Reactions were repeated for each pair of different hydroxycinnamic acids. The reactions were quenched after 5 min by addition of aqueous HCl (2.0 M, 20 mL). The resulting product 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.

5.6. Product isolation and purification by HPLC

Two systems were used for product separations: column chromatography and preparative TLC with $CHCl_3$/MeOH (9:1, v/v). The main products were subjected to HPLC purification performed with a reversed-phase column (1.3 × 24 cm C_{18} Zorbax, Agilent) at 27 °C, using gradient elution as described above for the analytical-scale reactions. Flow rate: 1.5 mL min^{-1}. Injection volume: 50–100 μL.

5.6.1. 5-[(E)-2-carboxyvinyl]-7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-2,3-dihydro-1-benzofuran-3-carboxylic acid (β-5-benzofuran-FA-CafA dimer, Compound **12**)

Amorphous orange solid. UV λ_{max} (MeOH) nm: 222, 232, 290, 320. HR FAB-MS (positive): *m/z* 373.0925 $[M + H]^+$ (Calc. for $C_{19}H_{17}O_8$, *m/z* 373.0845). ^{13}C NMR (150.87, CD_3OD): δ 66.07 (C-Aα), 47.35 (C-Aβ), 138.99 (C-Bα), 118.58 (C-Bβ), 56.20 (OMe). ^1H NMR (599.95, CD_3OD): δ 4.39 (1H, d, $J_{A\alpha-A\beta} = 4.8$, H-Aα), 4.48 (1H, d, $J_{A\alpha-A\beta} = 4.8$, H-Aβ), 7.9 (1H, d, $J_{B\alpha-B\beta} = 15.46$, H-Bα), 6.19 (1H, d, $J_{B\alpha-B\beta} = 15.46$, H-Bβ), 3.94 (3H, s, OMe).

5.6.2. 5-[(E)-2-carboxyvinyl]-2-(3,4-dihydroxyphenyl)-7-methoxy-2,3-dihydro-1-benzofuran-3-carboxylic acid (β-5-benzofuran-CafA-FA dimer, Compound **13**)

Amorphous red solid. UV λ_{max} (MeOH) nm: 282, 290, 460. HR FAB-MS *m/z*: 329 $[C_{19}H_{16}O_8 - COOH]^+$, 316 $[372 - 2\ CO]^+$, and 194 $[372 - CafA]^+$. ^{13}C NMR

(150.87, CD_3OD): δ 88.4 (C-Aα), 56.69 (C-Aβ), 145.88 (C-Bα), 117.31 (C-Bβ), 56.40 (OMe). ^1H NMR (599.95, CD_3OD): δ 6.0 (1H, d, $J_{A\alpha-A\beta} = 7.57$, H-Aα), 4.28 (1H, d, $J_{A\alpha-A\beta} = 7.57$, H-Aβ), 7.57 (1H, d, $J_{B\alpha-B\beta} = 16.01$, H-Bα), 6.32 (1H, d, $J_{B\alpha-B\beta} = 16.01$, H-Bβ), 3.82 (3H, s, OMe).

*5.6.3. (4E)-2-(3,4-hydroxyphenyl)-2-(4-hydroxy-3-methoxybenzylidene)-5-oxotetrahydrofuran-3-carboxylic acid (β-β′-γ-lactone-FA-CafA dimer, Compound **14**)*

Amorphous orange powder. UV λ_{max} (MeOH) nm: 208, 235, 290(sh), 305(sh), 338. HR FAB-MS (positive): *m/z* 373.0923 $[M + H]^+$ (Calc. for $C_{19}H_{17}O_8$, *m/z* 373.0845). ^{13}C NMR (150.87, CD_3OD): δ 141.22 (C-Aα), 82.11 (C-Bα), 54.24 (C-Bβ), 56.30 (OMe). ^1H NMR (599.95, CD_3OD): δ 7.64 (1H, s, H-Aα), 5.62 (1H, d, $J_{B\alpha-B\beta} = 2.63$, H-Bα), 4.12 (1H, d, $J_{B\alpha-B\beta} = 2.63$, H-Bβ), 3.87 (3H, s, OMe).

*5.6.4. β-5-Benzofuran-diCafA, Compound **15***

Compound **4** was characterized as a β-5-benzofuran-diCafA based on NMR results similar to those reported previously (Tazaki et al., 2001).

*5.6.5. (4E)-2-(3,4-dihydroxyphenyl)-4-(4-hydroxy-3,5-dimethoxybenzylidene)-5-oxotetrahydrofuran-3-carboxylic acid (β-β′-γ-lactone-SA-CafA dimer, Compound **16**)*

Amorphous red powder. UV λ_{max} (MeOH) nm: 206, 238, 292(sh), 339. HR FAB-MS (positive): *m/z* 403.1027 $[M + H]^+$ (Calc. for $C_{20}H_{19}O_9$, *m/z* 403.0951). ^{13}C NMR (150.87, CD_3OD): δ 141.82 (C-Aα), 82.48 (C-Bα), 54.83 (C-Bβ), 56.30 (OMe). ^1H NMR (599.95, CD_3OD): δ 7.64 (1H, s, H-Aα), 5.61 (1H, d, $J_{B\alpha-B\beta} = 3.12$, H-Bα), 4.12 (1H, d, $J_{B\alpha-B\beta} = 3.12$, H-Bβ), 3.86 (6H, s, OMe).

*5.6.6. 6-[(E)-2-carboxyvinyl]-3-(4-hydroxy-3,5-dimethoxyphenyl)-2,3-dihydro-1,4-benzodioxane-2-carboxylic acid, Compound **17***

Amorphous powder. UV λ_{max} (MeOH) nm: 241, 252(sh), 297, 318. HR FAB-MS (positive): *m/z* 403.1028 $[M + H]^+$ (Calc. for $C_{20}H_{19}O_9$, *m/z* 403.0951). ^{13}C NMR (150.87, CD_3OD): δ 88.4 (C-Aα), 86.50 (C-Aβ), 145.88 (C-Bα), 117.31 (C-Bβ), 56.20 (OMe). ^1H NMR (599.95, CD_3OD): δ 5.49 (1H, d, $J_{A\alpha-A\beta} = 2.5$, H-Aα), 5.02 (1H, d, $J_{A\alpha-A\beta} = 2.5$, H-Aβ), 7.58 (1H, d, $J_{B\alpha-B\beta} = 16.23$, H-Bα), 6.34 (1H, d, $J_{B\alpha-B\beta} = 16.23$, H-Bβ), 3.77 (6H, s, OMe).

*5.6.7. β-β′-γ-lactone-diCafA, Compound **18***

Compound **18** was identified as a β-β′-γ-lactone-diCafA in accord with prior NMR data (Tazaki et al., 2001).

*5.6.8. β-β′-γ-lactone-diSA, Compounds **19** and **20***

Compounds **19** and **20** each displayed ions at *m/z* 447.12 ($[M + H]^+$) in their positive HR FAB mass spectra, consistent with a molecular formula of $C_{22}H_{23}O_{10}$. The NMR data were in accord with those previously reported (Bunzel et al., 2003).

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