Hydroxystilbenes are Monomers in Palm Fruit Endocarp Lignins

José Carlos del Río,§ Jorge Rencoret,§ Ana Gutiérrez, Hoon Kim, and John Ralph*

Instituto de Recursos Naturales y Agrobiología de Sevilla (IRNAS), CSIC, Av. Reina Mercedes, 10, 41012-Seville, Spain (J.C.d.R., J.Re., A.G.); Department of Energy Great Lakes Bioenergy Research Center, the Wisconsin Energy Institute, University of Wisconsin-Madison, Madison, WI 53726, USA (H.K., J.Ra.); Department of Biochemistry, University of Wisconsin-Madison, Madison, WI 53706, USA (H.K., J.Ra.)

§ Co-first authors [Contributed equally to the work performed and the writing of this paper]

* Corresponding authors

ORCID IDs: 0000-0002-3040-6787 (J.C.d.R.); 0000-0003-2728-7331 (J.Re.); 0000-0001-7230-7309 (AG); 0000-0001-7425-7464 (H.K.); 0000-0002-6093-4521 (J.Ra.).

One Sentence Summary: Lignin polymers in the tough outer skin (endocarp) of palm fruits are produced, in part, from a new class of monomers, hydroxystilbenes, including the valuable resveratrol and piceatannol.

Author Contributions

J.C.d.R and J.Re., who contributed equally, obtained the initial samples, prepared lignins, ran DFRC, NMR and GPC analyses, made original assignments (including the discovery of the 3 hydroxystilbenes) and wrote the first draft of the paper. A.G. contributed to the experimental
design and the discussion of the results and critically reviewed the manuscript. H.K. ran the 700 MHz NMR experiments resulting in the figures here, contributed to the experimental design, performed the in vitro biomimetic radical coupling reactions of piceatannol itself to create and structurally validate the authentic dimers, and of piceatannol with synthesized coniferyl and sinapyl alcohols to produce authentic crossed dimers and oligomers. J.Ra. contributed to the experimental design and the in vitro biomimetic cross-coupling reactions, confirmed the structural assignments, and (with all authors) prepared and reviewed the final manuscript. The authors all declare no conflicts of interest.

Funding Information

J.C.d.R, J.Re., and A.G. were funded by the Spanish projects AGL2014-53730-R and CTQ2014-60764-JIN (co-financed by FEDER funds), and the CSIC project 2014-40E-097; J.Ra. and H.K. were funded by the DOE Great Lakes Bioenergy Research Center (DOE BER Office of Science DE-FC02-07ER64494).

Address correspondence to: delrio@irnase.csic.es or jralph@wisc.edu

ABSTRACT

Lignin, the plant cell wall polymer that binds fibers together but makes processing difficult, is traditionally formed from three monomers, the so-called monolignols (p-coumaryl, coniferyl, and sinapyl alcohols). Recently we discovered, in grass lignins, a phenolic monomer that falls outside the canonical lignin biosynthetic pathway, the flavone tricin. As we show here, palm fruit (macaúba, carnauba, and coconut) endocarps contain lignin polymers derived in part from a previously unconsidered class of lignin monomers, the hydroxystilbenes, including the valuable compounds piceatannol and resveratrol. Piceatannol could be released from these lignins upon DFRC, a degradative method that cleaves β-ether bonds, indicating that at least a fraction is incorporated through labile ether bonds. NMR spectroscopy of products from the copolymerization of piceatannol and monolignols confirms the structures in the natural polymer, and demonstrates that piceatannol acts as an authentic monomer participating in coupling and cross-coupling reactions during lignification. Palm fruit endocarps therefore contain a new class of ‘stilbenolignin’ polymers, further expanding the definition of lignin and implying that
compounds such as piceatannol and resveratrol are potentially available in what is now essentially a waste product.

**Keywords:** Lignification, lignin biosynthesis, radical coupling, piceatannol, resveratrol, stilbenolignin

**INTRODUCTION**

Lignin has long been considered to be a complex phenylpropanoid polymer derived essentially from the oxidative radical coupling of three \( p \)-hydroxycinnamyl alcohols ("monolignols") differing in their degree of methoxylation, \( p \)-coumaryl, coniferyl, and sinapyl alcohols, that form the \( p \)-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively, when incorporated into the lignin polymer (Boerjan et al., 2003; Ralph et al., 2004; Morreel et al., 2010). Once synthesized in the cytoplasm, the monolignols are transported to the cell wall where they are oxidized and polymerized. The oxidation/dehydrogenation reaction is initiated by one-electron oxidation of a phenolic monolignol to its phenoxy radical by plant peroxidases and laccases. Lignification then proceeds by radical coupling of two phenoxy radicals, between positions dictated by resonance delocalization of the single-electron density, and usually in an endwise manner between a monomer and the growing lignin chain, giving rise to variously interconnected monomer-derived units characterized by their interunit ether and carbon-carbon linkages.

It is now increasingly appreciated that lignins also derive from monomeric units beyond the traditional monolignols. As has been reviewed (Ralph, 2010; Vanholme et al., 2012; Mottiar et al., 2016), several other phenolic compounds, all deriving from the shikimate-derived monolignol biosynthetic pathway, have been found to behave as lignin monomers in many plants, participating in radical coupling reactions during lignification and resulting in cross-coupled structures. Several monomers derived from truncated monolignol biosynthesis, such as the hydroxycinnamaldehydes that are the immediate precursors of monolignols, are also especially prevalent in various mutant and transgenic plants. Incompletely methylated monomers, caffeyl and 5-hydroxyconiferyl alcohol, are found in OMT-deficient mutants and transgenics and can be found as the sole lignin monomers involved in seed coat lignification in several plants (Chen et al., 2012; Chen et al., 2013). Monolignol ester conjugates (with acetate,
$p$-coumarate, and $p$-hydroxybenzoate) are also used as lignin monomers in a variety of natural plants (Ralph, 2010). We recently exploited the clear metabolic malleability of lignification by extending the monolignol conjugates to include monolignol ferulates. Introducing an exotic feruloyl-CoA monolignol transferase gene/protein into poplar and arabidopsis, in a Nature-inspired manner, was recently shown to successfully introduce readily chemically cleavable ester linkages into the lignin backbone, facilitating its depolymerization during pretreatments or pulping (Wilkerson et al., 2014). It was subsequently shown, via more sensitive analytical methods, that Nature has in fact been biosynthesizing lignins with low levels of these conjugates all along, in a variety of plant species but not universally (Karlen et al., 2016).

In all of the cases above, however, the monomers have been derived from the monolignol biosynthetic pathway. The recent discovery of the flavone tricin in the lignins from grasses and other monocots was therefore unanticipated, but is now well established (del Río et al., 2012b; Rencoret et al., 2013; Lan et al., 2015; Lan et al., 2016a; Lan et al., 2016b; Eloy et al., 2017). Tricin, unlike monolignols that originate from the shikimate biosynthetic pathway, is biosynthesized from a combination of the shikimate and acetate/malonate-derived polyketide pathways. Tricin’s only mode of incorporation is via $4’-O-\beta$-coupling with a monolignol, and can therefore only appear at the initiating end of the lignin chain.

All these discoveries indicate that lignification is a flexible mechanism and that the plant is capable of using a variety of phenolic compounds for the formation of the lignin polymers. The discovery of ‘non-conventional’ phenolic precursors, different from the three canonical monolignols, illustrates the high metabolic plasticity of lignification and reveals that any phenolic compound that is transported to the cell wall may be oxidized and incorporated into the lignin polymer during lignification via radical coupling reactions, subject exclusively to simple chemical compatibility (Boerjan et al., 2003; Ralph et al., 2004; Ralph, 2006; Ralph et al., 2008; Vanholme et al., 2008; Morreel et al., 2010; Ralph, 2010; Vanholme et al., 2012; Mottiar et al., 2016). In this study, we report the occurrence of a second class of polyphenolic compounds, hydroxystilbenes, also arising from outside the monolignol biosynthetic pathway, in the lignins of palm fruit endocarps.

**Hydroxystilbenes, Stilbenolignans, and the Possibility of Stilbenolignins**
Hydroxystilbenes are a class of non-flavonoid polyphenolics that, like the flavonoids, are metabolic hybrids resulting from a combination of the shikimate-derived phenylpropanoid and the acetate/malonate-derived polyketide pathways. Hydroxystilbenes, as with monolignols, can be oxidized to form radicals that are resonance-stabilized, as shown for piceatannol $\text{I}$ in Fig. 1A. Hydroxystilbene (dehydro)dimerization (e.g., Fig. 1B and Fig. 1C) or the cross-coupling of different hydroxystilbenes produces a wide variety of dimers and higher oligomers. Illustrating their latent chemical compatibility with lignification, hydroxystilbenes can cross-couple with monolignols $\text{M}$ (e.g., Fig. 1D and Fig. 1E); several stilbenolignans have been identified in a variety of plants from different families (Kobayashi et al., 1996; Lee et al., 2001; Yao et al., 2006; Begum et al., 2010). It is therefore reasonable to speculate that phenolic stilbenoids present in the cell wall could also cross-couple with monolignols and the growing lignin polymer to become integrally incorporated into the lignin structure, as occurs with other non-traditional monomers. We propose that such polymers be classed as stilbenolignins, and the low-molecular-mass oligomers as stilbenolignols, as for the recently coined terms flavonolignins and flavonolignols (Lan et al., 2015; Lan et al., 2016a; Lan et al., 2016b).

RESULTS AND DISCUSSION

Release of Hydroxystilbenes by Derivatization Followed by Reductive Cleavage

Lignins isolated from macaúba ($\text{Acrocomia aculeata}$), carnauba ($\text{Copernicia prunifera}$), and coconut ($\text{Cocos nucifera}$) palm fruit endocarps were analyzed by derivatization followed by reductive cleavage (DFRC), the degradation method that cleaves β-ether bonds in the lignin polymer but leaves γ-esters intact (Lu and Ralph, 1997a; Wilkerson et al., 2014; Lu et al., 2015; Karlen et al., 2016). The chromatogram of the DFRC degradation products from macaúba, Fig. 2, and from all three palms, Fig. S1, shows the released $\text{cis}$- and $\text{trans}$-isomers of guaiacyl ($\text{cG}$ and $\text{tG}$), and syringyl ($\text{cS}$ and $\text{tS}$) lignin monomers (as their acetylated derivatives) arising from normal units in lignin, as well as peaks corresponding to $\gamma$-$p$-hydroxybenzoylated syringyl ($\text{cSzB}$ and $\text{tSzB}$) lignin units as usually noted from the lignins from palms (Ralph and Landucci, 2010; Rencoret et al., 2013; Lu et al., 2015). More interesting was a strong peak, released from both macaúba and carnauba lignins, and at low levels from coconut lignin, that we had not observed previously and was identified as the hydroxystilbene piceatannol $\text{I}$ (Fig. 2 and Fig. S1). Its identity was confirmed by comparison with an authentic piceatannol standard that presented
exactly the same retention time and mass spectrum as the released compound. Two other related hydroxystilbenes, isorhapontigenin 2 and resveratrol 3, were also released from these lignins upon DFRC, although in lower amounts. The relative areas from the GC-MS-TIC peaks are
given on the Figures. For reasons that will become evident below, we note that neither of the products from other catechols, caffeyl or 5-hydroxyconiferyl alcohol, that have been observed in lignins from various O-methyltransferase-deficient plants was evident beyond the trace levels
that are always seen from demethylation of G and S units via DFRC (and are always in proportion to the normal G and S monomer levels) (Lu and Ralph, 1998).

Piceatannol, isorhapontigenin, and resveratrol have not been detected previously in native lignins. Such hydroxystilbenes, like the lignin monomers themselves, can only be released via DFRC from polymer units that are present in β-ether-linked structures. Unlike tricin, which has only one possible mode of incorporation, piceatannol would be expected to couple and cross-couple with other piceatannol molecules through different types of linkages forming a variety of dimeric [e.g., \( P_b \) (Fig. 1B) and \( P_c \) (Fig. 1C)] and oligomeric structures. Indeed, such compounds, often optically active, are known from various plant extractives (Baba et al., 1994; Iliya et al., 2002; Li et al., 2005; Xiang et al., 2005; Morikawa et al., 2010; Quideau et al., 2011). Moreover, piceatannol can also cross-couple with monolignols (and oligolignols) in a variety of ways, two of which are shown in Fig. 1D and Fig. 1E. That such cross-coupling reactions can occur is evidenced by the array of stilbenolignans, including aiphanol \( V \), and kompasinol A or maackolin \( K \), that are the radical coupling products of piceatannol and sinapyl alcohol (Kobayashi et al., 1996; Lee et al., 2001; Banwell et al., 2005; Begum et al., 2010). Although lignification and lignan formation are distinct processes separated in time and space (Ralph et al., 1999; Umezawa, 2003), the presence of such stilbenolignans reveals that piceatannol is also compatible with the radical coupling reactions that typify lignification. The actual amounts of piceatannol monomers being incorporated into these lignins is logically, due to the coupling modes available to it, higher than the level released by DFRC. An assessment of the extent to which the polymer derives from piceatannol (and the other hydroxystilbenes), and the types of structures produced, is most readily gained from NMR studies.

**NMR Examination of the Lignins for Coupling and Cross-coupling Products of Piceatannol**

The lignins isolated from macaúba, carnauba, and coconut palm fruit endocarps were analyzed by 2D HSQC NMR in DMSO-\( d_6 \)/pyridine-\( d_5 \) (4:1) (Fig. 3 and Fig. 4); in order to assess the product levels in the entire material, the unfractionated cell wall material was also analyzed via previously described whole-cell-wall-NMR methods (Mansfield et al., 2012) (Fig. S2). Large differences were observed in the spectra of these lignins with respect to the spectra of other ‘typical’ lignins, particularly in the signals observed in the aromatic region (Fig. 3). The main aromatic correlation peaks corresponded to the different lignin (guaiacyl \( G \) and syringyl \( S \)) units...
as well as the pendant \( p \)-hydroxybenzoates (\( pB \)). The most striking feature was the presence of a previously unreported group of strong signals (labelled \( P_b, P_c \)) appearing at 100-107/5.8-6.8 ppm (\( \delta_C/\delta_H \)) that we assign here to piceatannol-derived units. Likewise, the oxygenated-aliphatic

![Figure 3. Aromatic regions of the 2D HSQC NMR spectra. (A) Macaruba fruit endocarp MWL (MWL = milled wood lignin). (B) Caraba fruit endocarp MWL. (C) Coconut endocarp MWL. (D) Dimers and oligomers from biomimetic coupling of piceatannol. (E) Piceatannol and coniferyl alcohol cross-coupled polymers from biomimetic coupling. (F) Piceatannol and sinapyl alcohol cross-coupled polymers from biomimetic coupling. The piceatannol-derived peaks in the palm endocarp lignins are well matched with those from the biomimetic in vitro polymerization and dimerization products. Note that, as neither the piceatannol stilbene double-bond \( P_f \) and \( P_s \) peaks, nor the corresponding \( P_f \) and \( P_s \) peaks from the piceatannol dimers \( P_b \) and \( P_c \), or the cross-dimer \( \mathbf{V} \), are evident in the lignins (A–C) or the cross-coupled synthetic lignins (E and F), the initial coupling of piceatannol at its 8-position must be highly dominant; the piceatannol-containing structures shown are to label the moieties from the various homo-coupled and cross-coupled entities, but do not imply that such intact units are in the polymer, i.e., they are clearly further coupled in the polymer. All spectra were run in DMSO-\( d_6 \)/pyridine-\( d_5 \), 4:1. Analogous spectra from unfractionated whole-cell-wall samples and the coniferyl alcohol + piceatannol biomimetic cross-coupling, showing most of the same spectral features, are in Fig. S2. Compositional percentages are from volume-integration and are on an \( S+G+H+P \) basis; the \( p \)-hydroxybenzoate, because it is pendant on the lignin and because, as a more slowly relaxing unit, it is significantly over-represented, is not included in this total but expressed as a % of that total.]

![Figure 3]
region of the spectra, Fig. 4, also showed signals other than those commonly observed from conventional lignin structures (β-aryl ethers A, phenylcoumarans B, resinols C, or cinnamyl alcohol end-groups I). These correlation peaks, labelled $P_b$ and $P_c$ are assigned here to structures
involving piceatannol units. Definitive assignments of these signals were achieved by HSQC-TOCSY (Fig. S3) and HMBC (Fig. S4) experiments and by comparing with piceatannol polymerization products and in vitro biomimetic cross-coupling reaction results, panels D-F in Fig. 3 and Fig. 4. Thus, $P_b$ was identified as a benzodioxane structure formed via 8–O–4′-type radical coupling of a piceatannol unit at its 8-position with another piceatannol unit (at its O–4′ position) followed by internal trapping of the quinone methide intermediate by the 3′-hydroxyl group (Fig. 1B); $P_c$ was identified as a phenylcoumaran structure formed by the radical coupling of a piceatannol unit (at its 8-position) with another piceatannol unit (at its 10′-position) followed by a subsequent 11′–O–7 bonding during rearomatization of the quinone methide intermediate (Fig. 1C). Assignment details are described in the Supplemental Material.

In addition to the radical dehydrodimerization structures from two piceatannol monomers, an important structure $V$, arising from the cross-coupling of piceatannol and monolignols, was also identified in the lignins of all three palm fruit endocarps. As anticipated for cross-coupling of a monomer with a catechol unit, characteristic benzodioxane structures are clearly observed in the NMR spectra of Fig. 4. The $C_\alpha/H_\alpha$ ($V_\alpha$) and $C_\beta/H_\beta$ ($V_\beta$) correlations were observed at $\delta_C/\delta_H$ 75.8/4.97 and 78.0/4.20 in DMSO-$d_6$/pyridine-$d_5$ (4:1), whereas the $C_\gamma/H_\gamma$ correlations appear at around $\delta_C/\delta_H$ 60.1/3.44 & 3.63, superimposed on other signals. All these signals are in the same coupling network, as seen in the HSQC-TOCSY spectrum, Fig. S3. These correlation signals match those for benzodioxane structures found in other lignins from the catechol monomers caffeyl alcohol or 5-hydroxyconiferyl alcohol, and particularly with the trans-isomer (Chen et al., 2012; Chen et al., 2013; Tobimatsu et al., 2013), and are clearly different from the benzodioxane structure of homo-coupled piceatannols $P_b$. The benzodioxane structures do not result from the incorporation of caffeyl alcohol, nor from the related 5-hydroxyconiferyl alcohol, both of which may be evidenced in the spectra of lignins from O-methyltransferase-deficient plants (Ralph et al., 2001; Wagner et al., 2011), as the expected monomers from such involvement in lignins were not observed here by DFRC and the characteristic aromatic signals from such catechols were also not evident; all of the peaks in the HSQC spectra (Fig. 3A and Fig. 3B and Fig. 3C) are fully consistent with those from the incorporation of piceatannol and with the piceatannol homo-coupled dimers/oligomers (Fig. 3D) and, for structures $V$ in particular, its cross-coupling products with monolignols (Fig. 3E and Fig. 3F). The cross-coupled benzodioxane structures found in the lignins from palm fruit endocarps are therefore uniquely
formed via radical coupling of a monolignol (at its β-position) and the catechol moiety of piceatannol (at its O-4′ position) followed by internal trapping of the quinone methide intermediate by the 3′-hydroxyl group in the piceatannol unit to form the benzodioxane structure V (Fig. 1D). A similar cross-coupling product of piceatannol and sinapyl alcohol, the stilbenolignan aiphanol, having a benzodioxane bridge, has been found in the seeds of *Aiphanes aculeata* from the Arecaceae family (Lee et al., 2001). Biomimetic cross-coupling reactions between piceatannol and *p*-hydroxyccinnamyl alcohols successfully proved that benzodioxane structures V could be easily formed during the radical reaction; both coniferyl alcohol (Fig. 4E) and sinapyl alcohol (Fig. 4F) produced respectable levels of benzodioxane structures V in which the chemical shifts of the unique peaks are well matched with the correlations appearing in the lignins here (Fig. 4). The occurrence of these benzodioxane structures in the lignins from macaúba, carnauba, and coconut palm fruit endocarps compellingly demonstrates that the stilbene piceatannol acts as an authentic monomer participating in coupling and cross-coupling reactions during lignification of these tissues.

Further strong evidence for the cross-coupling of piceatannol to monolignol or lignin components is the complete absence of P7 and P8 peaks from the piceatannol end-group in cross-coupling reactions and in the lignin samples. Such P7 and P8 peaks of the piceatannol end-group were detected and assigned from *in vitro* piceatannol homo-coupling dimerization/oligomerization reactions (Fig. 3D). Normally monolignol end-groups, including the double bonds in cinnamyl alcohol sidechains (Fig. 3E, peaks for structure I), can be easily found in the spectra from synthetic lignins and also at lower levels in isolated lignin polymers. However, we could not detect the piceatannol end-group peaks (P7 and P8) in the spectra from any of the lignin samples (Fig. 3A and Fig. 3B and Fig. 3C) and nor from the products of the *in vitro* cross-coupling experiments (Figs. 3E and Fig. 3F), yet the piceatannol had clearly integrated into these polymers. It is apparent that most of the piceatannol must radically couple to monolignols or the phenolic end of the growing lignin polymer, by coupling at P8 before further polymerization at its catechol and/or resorcinol ends. As neither the piceatannol stilbene double-bond P7 and P8 peaks, nor the corresponding P7 and P8 peaks from the piceatannol dimers Pb and Pc or the crossed-dimer V, are evident in the lignins (Fig. 3A and Fig. 3B and Fig. 3C) or the cross-coupled synthetic lignins (Fig. 3E and Fig. 3F), the initial coupling of
piceatannol at its 8-position must be highly dominant. Elucidating piceatannol’s mode of incorporation into the lignin polymer will require significantly more sophisticated studies.

Mechanisms for dehydrodimerization of piceatannol, and its cross-coupling with monolignols and the growing lignin polymer

As occurs with monolignols, piceatannol (as for other hydroxystilbenes) is also oxidized by peroxidases and/or laccases to form a radical that is stabilized by resonance (Fig. 1A). These radicals can couple and cross-couple with other stilbenoids forming a variety of dimers and higher oligostilbenes (e.g., Fig. 1B and Fig. 1C) (Quideau et al., 2011; Keylor et al., 2015). In addition, piceatannol can cross-couple with monolignols via radical coupling reactions generating a variety of stilbenolignans (e.g., Fig. 1D and Fig. 1E); chiral compounds of this type are presumably used in plant defense (Lee et al., 2001; Begum et al., 2010). It is obvious from the polymers analyzed here that piceatannol (and also its dimers and higher oligomers) can also cross-couple with monolignols and the growing lignin polymer, in the typically chemically controlled fashion of lignification, to be integrally incorporated into the racemic lignin polymer. As we have concluded previously, any phenolic component present in the cell wall during lignification can be incorporated into the polymer, simply subject to its chemical compatibility with the radical coupling reactions involving the components in that zone (Ralph et al., 2008).

The implication here is that, for whatever reason, these palms are both synthesizing and transporting hydroxystilbenes to the cell wall for polymerization to produce these previously unknown polymers.

The mechanisms for the formation of the different structures involving piceatannol units identified in the lignins from palm fruit endocarps are detailed in Fig. 1. Piceatannol dimerization can produce various structures characterized by their different inter-unit linkages, including the 8–O–4’ and 8–10’ structures found in the lignins from palm fruit endocarps. The 8–O–4’ (cassigarol E type) benzodioxane structure is formed via 8–O–4’-type radical coupling of a piceatannol unit at its 8-position (a position equivalent to the β-position of a monolignol) with another piceatannol unit (at its O–4’ position) followed by internal trapping of the quinone methide intermediate by the 3’-hydroxyl group forming a benzodioxane structure (Fig. 1B), as in structures Pb in the lignins. The mechanism for formation of the 8–10’ (scirpusin A type) phenylcoumaran structure (Pc in the lignin) involves the radical coupling of a piceatannol unit (at
its 8-position) with another piceatannol unit (at its 10′-position) followed by a subsequent 11′–
O–7 bonding during rearomatization of the quinone methide intermediate producing the
phenylcoumaran structure, as shown in Fig. 1C. Such a coupling reaction is possible due to the
highly extended conjugation in these stilbene systems in which single-electron density from the
radical produced by abstraction of the H from the 4-OH of piceatannol extends all the way out to
C10 (and in fact C12) (Fig. 1A). The mechanism for β–O–4′ cross–coupling of a p-
hydroxycinnamyl alcohol (at its β-position) and a piceatannol (as its 4′–O position) is similar to
that for the formation of the benzodioxane structure from two piceatannols shown above, and
also involves the subsequent 3′–O–α′ bonding during quinone methide rearomatization
producing the benzodioxane structure V found in the lignins of the selected palm fruit endocarps
(Fig. 1D).

In addition to the structures identified in the lignins from palm fruit endocarps, and due to the
large variety of radical coupling products that can potentially form from the highly conjugated
piceatannol and p-hydroxycinnamyl alcohols, several other structures coupled at different
positions could also be formed by homo- and cross-coupling. Among these structures, we can
refer to the stilbenolignans kompasinol A (isolated from Koompassia malaccensis, from the
Fabaceae) and maackolin (isolated from Maackia amurensis, from the Fabaceae) (Kobayashi et
al., 1996; Begum et al., 2010). The key ring structure in kompasinol A is formed by radical
coupling of sinapyl alcohol at its β-position with the 8′-position of piceatannol followed by
internal trapping of the quinone methide on the piceatannol moiety by the γ-OH and
rearomatization of the sinapyl alcohol-derived moiety’s quinone methide by nucleophilic attack
at its α-position by the electron-rich 10′-position of the piceatannol, Fig. 1E. Although these β–8′
structures were not detected in the lignins here, they and other linkage types may exist and the
unambiguous identification of any of them would provide additional evidence for piceatannol’s
being incorporated into the lignin polymer.

Molecular Weight Distributions, and the Role of Stilbenolignins

The three lignins exhibited similar molecular weight distributions, around 5500–6500 g mol⁻¹
with relatively narrow polydispersity, with M_w/M_n ~ 1.61–1.84 (Fig. S5, Table S2), which
appears to indicate that the lignin polymer in the selected palm fruit endocarps is quite
homogeneous, and therefore does not include simple stilbenes, dimers or higher oligostilbenes
mixed with the lignin. These data therefore support our contention that the lignin polymer in palm fruit endocarps includes hydroxystilbenes, mostly piceatannol, fully integrated into the polymeric structure.

We can only speculate on the role of these stilbenolignin polymers. Palm fruits are drupes that contain an extremely hard lignified endocarp surrounding the seed. Endocarp lignification therefore plays an important role in seed protection. The incorporation of hydroxystilbenes into the lignin polymer may allow the production of higher amounts of lignin (by using other phenolic compounds present in the lignification zone) and appears to contribute to endocarp hardening. The piceatannol-derived components could also provide additional antioxidant properties to the endocarp, contributing to seed protection. Although piceatannol has been identified in the lignins of the fruit endocarps from the three palm species selected for this study, the analysis of a broader collection of palm species (and beyond) is required in order to establish the phylogenic range of the occurrence of such stilbenolignins.

CONCLUSION

It is evident from the three palm endocarp lignin polymers analyzed here that piceatannol can cross-couple with monolignols and the growing lignin polymer, in the typically chemically controlled fashion of lignification, to be integrally incorporated into the racemic lignin polymer. As we have concluded previously, any phenolic component present in the cell wall during lignification can be incorporated into the polymer, simply subject to its chemical compatibility with the radical coupling reactions involving the components in that zone (Ralph et al., 2008). The implication here is that, for whatever reason, these palms are both synthesizing and possibly transporting hydroxystilbenes to their fruit endocarp cell walls for polymerization to produce these previously unknown copolymers; we cannot rule out the possibility that small stilbenolignols are produced in the cytoplasm in the same way as oligolignols are also implicated (Dima et al., 2015), and that it is these that are transported to the wall. The incorporation of non-conventional monomers, not usually present in the lignins of other plants, as is the case for the piceatannol described here, can open up new ways to design and engineer the lignin structure to produce polymers and plant-based biomaterials with altered properties. A whole new generation of modified lignin polymers can be envisioned via introducing hydroxystilbenes into plant lignification pathways, as already anticipated with other phenolic compounds (Grabber et al.,...
2010; Elumalai et al., 2012; Grabber et al., 2012; Tobimatsu et al., 2012; Vanholme et al., 2012; Grabber et al., 2015), and as already achieved with the introduction of monolignol ferulates and *p*-coumarates into plants that do not normally possess them (Wilkerson et al., 2014; Smith et al., 2015; Sibout et al., 2016). Additionally, hydroxystilbenes such as piceatannol and, in particular, resveratrol, are quite valuable (with the cheapest prices being ~$US80 and $280 per kg on alibaba.com), and their potential availability in bulk quantities from lignins will spur additional interest into deriving value from lignins (Rinaldi et al., 2016).

**MATERIALS AND METHODS**

**Samples**

Macaúba (*Acrocomia aculeata*) and carnauba (*Copernicia prunifera*) palm fruits were collected from native populations located in the municipality of Mirabela, Minas Gerais, Brazil. The coconut (*Cocos nucifera*) sample originated from India and was supplied from Bonnysa Agroalimentaria Co. (Alicante, Spain). The endocarps of the fruits were manually separated using a knife and subsequently dried in a forced-air oven at 40 °C until reaching constant mass. The dried samples were milled using a knife mill (1 mm screen) and successively extracted for 8 h with acetone (200 mL) and hot water (100 mL, 3 h at 100 °C) in a Soxhlet apparatus to purify the cell walls. Klason lignin content was estimated as the residue after sulfuric acid hydrolysis of the pre-extracted material according to the TAPPI method T222 om-88 (TAPPI, 2004, 2006; http://www.tappi.org/content/SARG/T222.pdf), and further corrected for ash and protein content. Three replicates were used for each sample. The data indicated that the selected palm fruit endocarps contained extremely high lignin contents, accounting for 39.8, 38.8, and 33.2% in macaúba, carnauba, and coconut respectively.

**Lignin isolation and purification**

The lignin preparations were obtained from extractive-free samples according to the classical ‘milled wood lignin’ (MWL) procedure (Björkman, 1956). Around 40 g of extractive-free material were finely ball-milled in a Retsch PM100 planetary ball mill (Retsch, Haan, Germany) for 25 h at 400 rpm using a 500 mL agate jar and agate ball bearings (20 x 20 mm). The ball-milled material was then extracted with dioxane-water, 96:4 (v/v) (20 mL of solvent/g of milled
fiber), and the isolated lignin was subsequently purified as described previously (del Río et al., 2012a). The final yields were ~15% of the original Klason lignin content.

**Analyses**

**Derivatization Followed by Reductive Cleavage (DFRC)**

DFRC degradation was performed according to the developed protocol (Lu and Ralph, 1997a; Lu and Ralph, 1997b) using the detailed procedure previously described (del Río et al., 2012a). The acetylated lignin degradation products were then analyzed by GC/MS on a Saturn 4000 (Varian, Walnut Creek, CA) instrument fitted with a medium-length high-temperature capillary column (DB5-HT, 15 m × 0.25 mm i.d., 0.1 μm film thickness; from J&W Scientific). Helium was used as carrier gas at a rate of 2 mL min⁻¹. The samples were injected with an autoinjector (Varian 8200) directly onto the column using a septum-equipped programmable injector system, that was programmed from 120 °C (0.1 min) to 340 °C at a rate of 200 °C min⁻¹ and held at the maximum temperature until the end of the analysis. The oven temperature was programmed from 120 °C (1 min) to 340 °C (10 min) at a rate of 10 °C min⁻¹. The temperature of the transfer line was set at 300 °C during the analysis.

Chromatograms of the DFRC products from each of the palm endocarp samples are shown in Fig. 2 and Fig. S1.

**Nuclear Magnetic Resonance (NMR) Spectroscopy**

Multidimensional NMR spectra (2D HSQC, 2D HMBC, 2D HSQC-TOCSY, 3D TOCSY-HSQC) experiments from lignin samples (~40 mg) were acquired, in parallel, in DMSO-\textit{d}_6 (0.75 mL) on an AVANCE III 500 MHz instrument (Bruker, Karlsruhe, Germany) and in DMSO-\textit{d}_6/pyridine-\textit{d}_5 (4:1) on a Bruker Biospin (Billerica, MA, USA) AVANCE 700 MHz spectrometer, both fitted with cryogenically cooled 5 mm gradient probes with inverse geometry (proton coils closest to the sample). NMR of polymerization/dimerization products of piceatannols and \textit{p}-hydroxycinnamyl alcohols were also analyzed in DMSO-\textit{d}_6/pyridine-\textit{d}_5 (4:1) on the 700 MHz NMR instrument. Whole-cell-wall samples were analyzed in DMSO-\textit{d}_6/pyridine-\textit{d}_5 (4:1) on the 700 MHz NMR instrument based on the gel-NMR method previously described (Kim and Ralph, 2010). The central residual DMSO peak was used as internal reference (δ\textit{C}/δ\textit{H} 39.5/2.49). All NMR experiments used Bruker’s standard pulse programs:
HSQC experiments used “hsqcetgpsisp2.2” (adiabatic-pulsed version), the HMBC experiments used “hmbcgplpndqf” with long-range $J$-coupling evolution times of 62.5 ms (and/or 80 ms when required), the HSQC-TOCSY experiments used “hsqcdietgpsisp.2”, and the 3D TOCSY-HSQC experiments (not shown) used “mlevhsqcetgp3d”. The detailed NMR experimental conditions have been described elsewhere (del Río et al., 2012a). Integrals are from volume-integration of contours from C/H pairs that are in similar coupling environments. Thus, for the aromatics, Fig. 3, the peaks used are $S_{2/6}$, $G_2$, $H_{2/6}$, and $P_{c2}$; $P_{b2}$ was not resolved, so its expected integral was calculated from $P_{c2}$ via the ratio of the resolved $P_{b6}$ to $P_{c6}$ peaks; the $pB_{2/6}$ peak was used for the $p$-hydroxybenzoates that are not included in the lignin background total and are expressed simply as a % of that total. In Fig. 4, the various units were relatively quantified via the volume integrals of the $A_\alpha$, $B_\alpha$, $C_\alpha$, $C'_\alpha$, $P_{b7}$, $P_{c7}$, and $V_\alpha$ correlation peaks.

The NMR spectra had the following parameters for the lignins: spectra were acquired from 11.5 to -0.5 ppm in F2 ($^1$H) using 3366 data points for an acquisition time (AQ) of 200 ms, an interscan delay (D1) of 1 s, 215 to -5 ppm in F1 ($^{13}$C) using 620 increments (F1 acquisition time 8 ms) of 32 scans, with a total acquisition time of 7 h. For the in vitro polymerization products, 16 scans per increment were performed with a total acquisition time of 3.5 h. Processing used typical matched Gaussian apodization (GB = 0.001, LB = -0.5) in F2 and squared cosine-bell in F1. Interactive integrations of contours in 2D HSQC plots were carried out using Bruker’s TopSpin 3.5 (Mac) software, as was all data processing. Spectra from whole-cell-wall samples, Fig. S2, were run under similar conditions but acquired from 11.5 to -0.5 ppm in F2 ($^1$H) with 1682 data points (acquisition time 100 ms), 215 to -5 ppm in F1 ($^{13}$C) with 620 increments (F1 acquisition time 8 ms) of 56 scans with a 500 ms interscan delay; the $d_{24}$ delay was set to 0.86 ms ($1/8J$, $J$ = 145 Hz). The total acquisition time for each was 6 h.

**Gel-Permeation Chromatography (GPC)**

GPC was performed on a Shimadzu Prominence-i LC-2030 3D GPC system (Shimadzu, Kyoto, Japan) equipped with a photodiode array (PDA) detector, using the following conditions: column, PLgel 5 µm MIXED-D, 7.5 x 300 mm (Agilent Technologies, United Kingdom); eluent, tetrahydrofuran (THF); flow rate, 0.5 mL min$^{-1}$; temperature, 40 °C; sample detection, PDA response at 280 nm. The data acquisition and computation used LabSolution GPC software.
version 5.82 (Shimadzu). The molecular weight calibration was via polystyrene standards (Mw range from $5.8 \times 10^2$ up to $3.24 \times 10^6$ Da, Agilent Technologies).

**Dimerization of Piceatannol and Polymerization with $p$-Hydroxycinnamyl Alcohols**

Piceatannol 1 (20 mg, 0.082 mmol) was dissolved in acetone:water (1:10, v/v, 11 mL). Horseradish peroxidase (5 mg; EC 1.11.1.7, 173 purpurogallin units per mg solid, type II) was added to the reaction solution directly and stirred. Excess hydrogen peroxide (30%, 0.5 mL) was added at once into the reaction solution while the solution was stirred. The color changed to dark red immediately. The solution was stirred for 15 min at room temperature for the short-time dimerization reaction, or stirred 1.5 h for the extended polymerization reaction. The crude products were extracted with EtOAc and washed with sat. aq. NH$_4$Cl solution and water. The EtOAc fraction was dried over anhydrous MgSO$_4$ and evaporated.

Cross-coupling polymerization reactions between piceatannol (20 mg, 0.082 mmol) and coniferyl alcohol (14.8 mg, 0.082 mmol), or between piceatannol 1 (20 mg, 0.082 mmol) and sinapyl alcohol (17.2 mg, 0.082 mmol) were performed as above, but stirred for 17 h.

**Supplemental Data**

The following Supplemental Materials are available in the online version of this article.

**Additional Details, and Assignment Discussion**

**Supplemental Figure S1.** Hydroxystilbenes released from lignins by reductive cleavage.

**Supplemental Figure S2.** 2D HSQC NMR spectra of whole cell walls in DMSO-$d_6$/pyridine-$d_5$.

**Supplemental Figure S3.** 2D HSQC-TOCSY and HMBC NMR spectra of isolated lignins in DMSO-$d_6$ showing diagnostic $P_b$, $P_c$, and $V$ correlations.

**Supplemental Figure S4.** 2D HMBC NMR spectra of isolated lignins in DMSO-$d_6$ showing main correlations for piceatannol-derived units.
Supplemental Figure S5. Molecular weight distribution of the lignins from the fruit endocarps of macaúba, carnauba, and coconut palm.

Supplemental Data S1. Assignments of the $^1$H/$^{13}$C correlation signals of structures involving piceatannol units in the HSQC spectra (in DMSO-$d_6$/pyridine-$d_5$) of the lignins from palm fruit endocarps.

Supplemental Data S2. Weight-average ($M_w$) and number-average ($M_n$) molecular weights (g mol$^{-1}$), and polydispersity ($M_w/M_n$) of the MWLs isolated from macaúba, carnauba, and coconut fruit endocarps.

ACKNOWLEDGEMENTS

We thank Anderson B. Evaristo (University of Viçosa, Brazil) for providing the carnauba and macaúba palm fruits, and Dr. Manuel Angulo for performing the preliminary NMR analyses that were acquired on a Bruker AVANCE III 500 MHz instrument from the NMR facilities of the General Research Services of the University of Seville (SGI-CITIUS). We are grateful to Gautham Ramapriya and Christos Maravelias (U. Wisconsin) for helping us find bulk prices for the hydroxystilbenes.

Figure Legends

Figure 1. Piceatannol radical, and radical coupling reactions. (A) The most stable phenolic radical is that from dehydrogenation of the 4-OH. Resonance forms show how coupling can occur at the 4–O-, 5-, 8- and 10-positions, among others. (B) Piceatannol dehydrodimerization by 8–O–4´-coupling to give P$_b$, Cassigarol E (Li et al., 2005). (C) Piceatannol dehydrodimerization by 8–10´-coupling to give P$_c$, Scirpusin B (Nakajima et al., 1978). (D) Cross-coupling of monolignols M, sinapyl and coniferyl alcohol, with piceatannol 1 via β–O–4´-
coupling to give the stilbenolignols V, aiphanol (Lee et al., 2001; Begum et al., 2010) and its
guaiacyl analogue. (E) Cross-coupling of monolignols M with piceatannol I via β-8’-coupling to
give the stilbenolignols K, Kompasinol A or Maackolin (Kobayashi et al., 1996; Lee et al., 2001;
Yao et al., 2006; Begum et al., 2010) and their guaiacyl analogues. Note that the names are often
given for dimeric hydroxystilbenes or stilbenolignans that may be optically active; here we are
referring to the achiral compounds produced during lignification; stereochemical rendering on
Pb, Pc, V, and K is to show the trans-nature of the rings and does not imply optical activity – the
other enantiomer is equally present in the racemates. These structures, other than K (for which
no indication can be found), are evidenced in the lignins from macaúba, carnauba, and coconut
palm endocarps.

Figure 2. Hydroxystilbenes released from lignins by reductive cleavage. Total-ion
chromatogram (TIC) of the DFRC degradation products released from the lignin from macaúba
fruit endocarps, showing the presence of stilbenoid compounds (I: piceatannol; 2:
isorhamptigenin; 3: resveratrol, as their acetate derivatives). cG, tG, cS and tS are the normal
cis- and trans-coniferyl (G) and sinapyl (S) alcohol monomers (as their acetate derivatives). cSρB
and tSρB are the cis- and trans-sinapyl p-hydroxybenzoates (as their acetate derivatives). Below:
Electron-impact (EI) mass spectrum from peak 1 matches that of an authentic standard of
piceatannol I (acetylated). The chromatograms from all three palm endocarps examined here
(macaúba, carnauba, and coconut) are shown in Fig. S1. Relative peak areas are given, with all
identified lignin-derived aromatic components, including those from the released
hydroxystilbenes, totaling 100%. [See Fig. S1 for the traces from all 3 palm samples].

Figure 3. Aromatic regions of the 2D HSQC NMR spectra. (A) Macaúba fruit endocard MWL
(MWL = milled wood lignin). (B) Carnauba fruit endocard MWL. (C) Coconut endocard MWL.
(D) Dimers and oligomers from biomimetic coupling of piceatannol. (E) Piceatannol and
coniferyl alcohol cross-coupled polymers from biomimetic coupling. (F) Piceatannol and sinapyl
alcohol cross-coupled polymers from biomimetic coupling. The piceatannol-derived peaks in the
palm endocard lignins are well matched with those from the biomimetic in vitro polymerization
and dimerization products. Note that, as neither the piceatannol stilbene double-bond P7 and P8
peaks, nor the corresponding P7’ and P8’ peaks from the piceatannol dimers Pb and Pc or the
crossed-dimer \( V \), are evident in the lignins (A–C) or the cross-coupled synthetic lignins (E and F), the initial coupling of piceatannol at its 8-position must be highly dominant; the piceatannol-containing structures shown are to label the moieties from the various homo-coupled and cross-coupled entities, but do not imply that such intact units are in the polymer, i.e., they are clearly further coupled in the polymer. All spectra were run in DMSO-\( d_6 \)/pyridine-\( d_5 \), 4:1. Analogous spectra from unfractionated whole-cell-wall samples and the coniferyl alcohol + piceatannol biomimetic cross-coupling, showing most of the same spectral features, are in Fig. S2. Compositional percentages are from volume-integration and are on an S+G+H+P=100% basis; the \( p \)-hydroxybenzoate, because it is pendant on the lignin and because, as a more slowly relaxing unit, it is significantly over-represented, is not included in this total but expressed as a % of that total.

**Figure 4.** Oxygenated-aliphatic regions of the 2D HSQC NMR spectra. (A) Macaúba fruit endocarp MWL (MWL = milled wood lignin). (B) Carnauba fruit endocarp MWL. (C) Coconut endocarp MWL. (D) Piceatannol dimers and oligomers showing both \( P_b \) and \( P_c \) structures with correlations that are well matched with peaks in the three lignins. (E) Piceatannol and coniferyl alcohol cross-coupled polymers. (F) Piceatannol and sinapyl alcohol cross-coupled polymers. The piceatannol-monolignol polymers provided evidence of the cross-coupling reactions to produce benzodioxane structures \( V \). Both trans and cis configurations are evidenced in the in vitro polymerization products, but only the trans-form can be found in the lignins. Again, analogous whole-cell-wall spectra are in Fig. S2. Structures are with \( R = H \) (\( G \) unit) or \( R = \text{OMe} \) (\( S \) unit), and are labelled as \( X \) (\( R' = H \)) or \( X' \) (\( R' = \text{pB} \)) where \( X \) is generic for \( A \), \( B \), \( D \), and \( I \); \( C' \) is a special case that gets its own structure. Aromatic rings are also designated as \( G \) or \( S \) (or \( G/S \) in the case of either being allowed), with a label’s color intensity signifying the main types; for example, structures \( C \) and \( C' \) are largely syringyl (\( S \)) based, with such \( G \) units being minor. Percentages for the various units are from volume-integration and total 100%.
Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only; Title Only; Author and Title

Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only; Title Only; Author and Title

Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only; Title Only; Author and Title

Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only; Title Only; Author and Title

Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only; Title Only; Author and Title

Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only; Title Only; Author and Title

Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only; Title Only; Author and Title

Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only; Title Only; Author and Title

Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only; Title Only; Author and Title

Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only; Title Only; Author and Title

Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only; Title Only; Author and Title

Pubmed: Author and Title
CrossRef: Author and Title
Google Scholar: Author Only; Title Only; Author and Title

Pubmed: Author and Title


composition in Pinus radiata. The Plant Journal 67: 119-129


