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Metabolic engineering of novel lignin in biomass crops

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Contents

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Summary

Lignin, a phenolic polymer in the secondary wall, is the major cause of lignocellulosic biomass recalcitrance to efficient industrial processing. From an applications perspective, it is desirable that second-generation bioenergy crops have lignin that is readily degraded by chemical pretreatments but still fulfill its biological role in plants. Because plants can tolerate large variations in lignin composition, often without apparent adverse effects, substitution of some fraction of the traditional monolignols by alternative monomers through genetic engineering is a promising strategy to tailor lignin in bioenergy crops. However, successful engineering of lignin incorporating alternative monomers requires knowledge about phenolic metabolism in plants and about the coupling properties of these alternative monomers. Here, we review the current knowledge about lignin biosynthesis and the pathways towards the main phenolic classes. In addition, the minimal requirements are defined for molecules that, upon incorporation into the lignin polymer, make the latter more susceptible to biomass pretreatment. Numerous metabolites made by plants meet these requirements, and several have already been tested as monolignol substitutes in biomimetic systems. Finally, the status of detection and identification of compounds by phenolic profiling is discussed, as phenolic profiling serves in pathway elucidation and for the detection of incorporation of alternative lignin monomers.

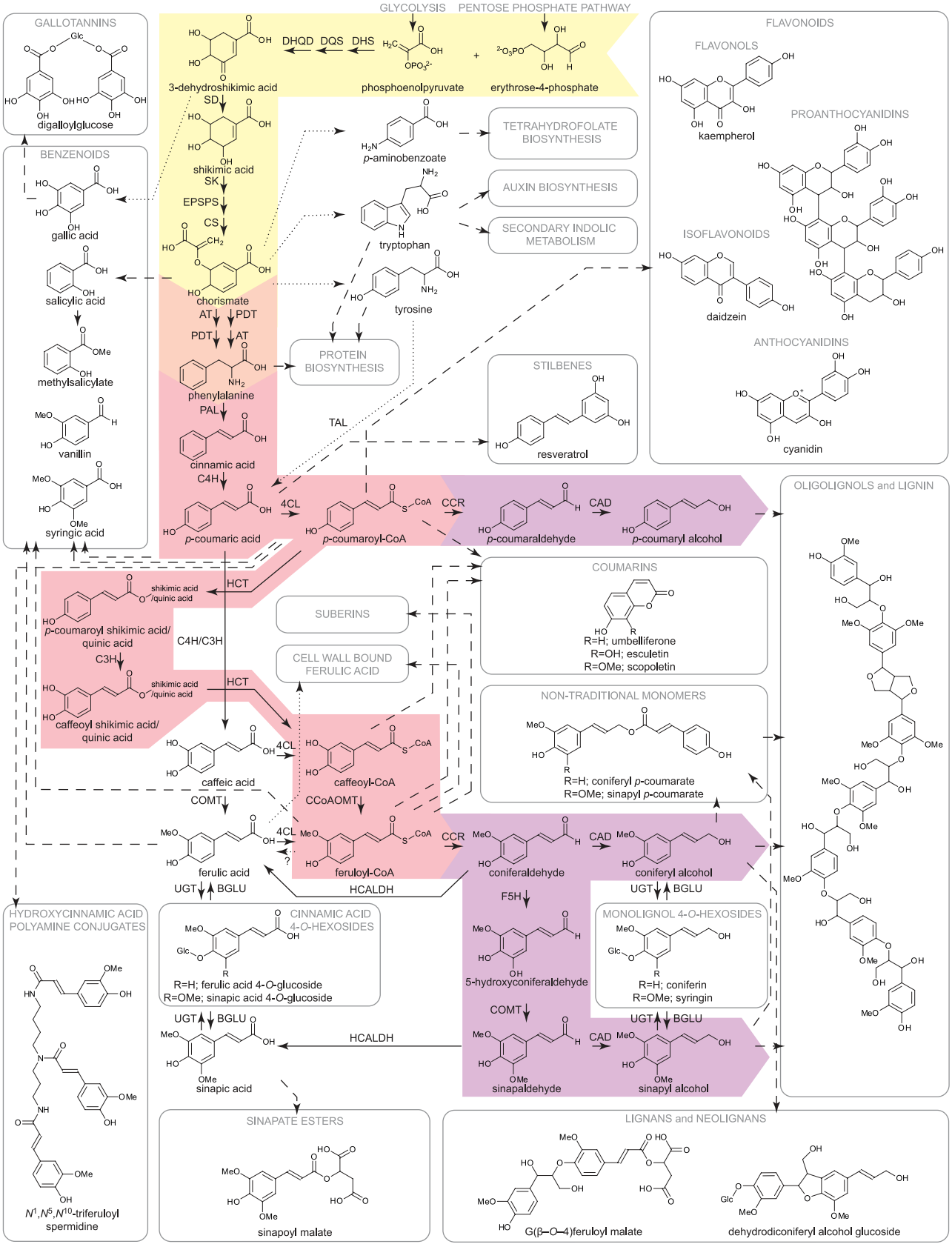
I. Introduction

The development of lignin was a keystone event in the evolution of vascular land plants because lignin provided the necessary strength and hydrophobicity to fiber and vessel cell walls to allow plants to grow tall in a gravitropic environment and to transport water and nutrients in their vascular system (Rogers & Campbell, 2004; Weng & Chapple, 2010). Lignin is a complex aromatic polymer in which the cell wall polysaccharides (mainly cellulose and hemicelluloses) and cell wall glycoproteins are embedded. It is synthesized from the oxidative coupling of *p*-hydroxycinnamyl alcohol monomers and related compounds (Boerjan *et al.*, 2003; Ralph *et al.*, 2004b; Vanholme *et al.*, 2010a). The main units in the polymer, *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, are derived from the monolignols *p*-coumaryl, coniferyl and sinapyl alcohol, monomers differing in the number of methoxyl substituents on the aromatic ring. The relative abundance of these main units varies among plant species, tissues, cell types, and developmental stages. Research on lignin biosynthesis has become a primary focus since it became clear that lignin is the major factor in lignocellulosic biomass recalcitrance to efficient processing. Lignin hinders the release of monosaccharides during enzymatic hydrolysis of cell wall polysaccharides – a process called saccharification – which is necessary for the production of second-generation biofuels and materials from biomass-derived sugars (Chen & Dixon, 2007; Yoshida *et al.*, 2008). More specifically, lignin negatively affects the saccharification process by immobilizing cellulases (and associated enzymes) and blocking them from reaching their polysaccharide substrates (Chang & Holtzapple, 2000; Nakagame *et al.*, 2011). In order to facilitate saccharification, mechanical, thermal and chemical pretreatments have been developed to disrupt cell wall structure, rendering the lignocellulosic material more accessible to the polysaccharidases (Carvalheiro *et al.*, 2008; Kristensen *et al.*, 2008; Hendriks & Zeeman, 2009; Chundawat *et al.*, 2011). With the recognition that lignin is a problem, it has been appealing to consider reducing lignin contents via genetic engineering, a strategy that would reduce the input of chemicals and energy during the pretreatment reactions (Chen & Dixon, 2007). Drastic reductions in the biosynthesis of lignin, however, have negative effects on plant growth and development (Li *et al.*, 2010; Gallego-Giraldo *et al.*, 2011b; Voelker *et al.*, 2011). By contrast, plants can tolerate large shifts in lignin composition, often without visible effects on plant development and morphology (Ralph *et al.*, 1997; Meyer *et al.*, 1998; Marita *et al.*, 1999; Franke *et al.*, 2000; Stewart *et al.*, 2009; Vanholme *et al.*, 2012). In fact, plants also show a remarkable ability to augment their polymer make-up by incorporating novel phenolic monomers, as is particularly evident when pathway gene down-regulations, limiting the flux to the traditional monolignols, lead to the build-up of products from pathway intermediates, for example, hydroxycinnamaldehydes, and 5-hydroxyconiferyl alcohol (Ralph *et al.*, 2001b). Advances in analytical techniques have revealed that numerous phenolic metabolites act as natural lignin monomers in wild-type plants – examples include acylated hydroxycinnamyl alcohols, hydroxybenzaldehydes and dihydrohydroxycinnamyl alcohols (Ralph *et al.*, 1997, 2008a; Vanholme *et al.*, 2008). The incorporation of atypical monomers that are rare, or even absent, in lignin of wild-type plants (hereafter called alternative lignin monomers) can be accomplished through genetic engineering (Jackson *et al.*, 2008; Ralph *et al.*, 2008b; Eudes *et al.*, 2012). Therefore, research is now also focusing on the biosynthesis and incorporation of alternative monomers into lignin to alter the structure of the lignin polymer to facilitate lignin removal from lignocellulosic biomass by chemical pretreatments or to improve the penetration and action of hydrolytic enzymes (Simmons *et al.*, 2010; Chundawat *et al.*, 2011). Various modified lignin polymers might be envisioned to maintain the biological role of lignin in the plant while permitting more efficient conversion of lignocellulosic biomass for industrial saccharification.

II. Phenolic metabolism

The lignin polymer is the product of oxidative coupling of phenolic metabolites, normally *p*-hydroxycinnamyl alcohol monomers (the so-called monolignols). Steering the pathway to produce alternative monomers therefore requires a fundamental knowledge of phenolic metabolism, that is, the enzymes and metabolites involved in the pathways, and how these pathways are regulated. Although many phenolics are specific for one or a few plant species, several major classes of phenolics are found throughout the plant kingdom. The biosynthetic route towards these major classes has been studied via metabolic and genomic tools, mostly in Arabidopsis. In this section, the current knowledge of the main phenolic pathways in Arabidopsis and in other model species is briefly presented as a prelude to describing alternative monomers for lignification (Fig. 1).

The shikimate pathway, which is present in bacteria, yeasts and plants, but not in animals, is the entry pathway towards a plethora of phenolic compounds. This plastid-localized pathway is highly transcriptionally and post-translationally controlled (Chen *et al.*, 2006b; Tzin & Galili, 2010). In seven enzymatic steps, the glycolytic intermediate phosphoenol pyruvate and the pentose phosphate pathway intermediate erythrose-4-phosphate are metabolized into chorismate via 3-dehydroshikimate as an intermediate (Herrmann & Weaver, 1999). Although the biosynthetic route is not yet fully elucidated, 3-dehydroshikimate also serves as a precursor for gallic acid and, thus, gallotannin biosynthesis (Dewick & Haslam, 1969; Werner *et al.*, 2004). Chorismate serves as the precursor for *p*-aminobenzoate (an intermediate in tetrahydrofolate biosynthesis; Basset *et al.*, 2004) and the aromatic amino acids phenylalanine, tyrosine and tryptophan (Knaggs, 2003). Phenylalanine is produced from chorismate by the action of two enzymes, a dehydratase and an aminotransferase, the exact order of action being unknown (Cho *et al.*, 2007; Yamada *et al.*, 2008; Corea *et al.*, 2012). Tryptophan is necessary for the production of auxin and secondary indolic metabolites, such as indolic glucosinolates and camalexin (Malitsky *et al.*, 2008). In addition, chorismate is the main precursor for salicylic acid in Arabidopsis, although salicylic acid can also be produced from benzoic acid (Léon *et al.*, 1995; Wildermuth *et al.*, 2001; Métraux, 2002; Strawn *et al.*, 2007).



The general phenylpropanoid pathway uses phenylalanine as an entry substrate and results, after seven steps, in feruloyl-CoA (Humphreys & Chapple, 2002; Boerjan *et al.*, 2003). Following deamination of phenylalanine to cinnamate by phenylalanine ammonia-lyase (PAL), hydroxylation of the aromatic ring leads to *p*-coumarate, a reaction catalyzed by cinnamate 4-hydroxylase (C4H). In grasses, a PAL isozyme catalyzing the deamination of both phenylalanine (PAL activity) and tyrosine (tyrosine ammonia-lyase activity) *in vitro* might be implicated in the direct conversion of tyrosine to *p*-coumarate *in vivo* (Rösler *et al.*, 1997). Activation of the acid to a thioester by 4-coumarate: CoA ligase (4CL) yields *p*-coumaroyl-CoA. The subsequent 3-hydroxylation of *p*-coumaroyl-CoA to caffeoyl-CoA involves three enzymatic steps, at least in dicots. First, *p*-coumaroyl-CoA is transesterified to its quinic or shikimic acid ester derivative by hydroxycinnamoyl-CoA: shikimate/quinate hydroxycinnamoyltransferase (HCT). *p*-Coumaroyl shikimate or quinate is then hydroxylated by *p*-coumarate 3-hydroxylase (C3H, named when it was assumed that *p*-coumarate was the direct substrate) and then transesterified again by HCT to caffeoyl-CoA. Recently, an alternative 3-hydroxylation route has been found; the poplar (*Populus trichocarpa*) heterodimeric C4H/C3H protein complex efficiently converts *p*-coumaric acid to caffeic acid (Chen *et al.*, 2011), after which 4CL might convert caffeic acid into caffeoyl-CoA. Further methylation of the 3-hydroxyl group by caffeoyl-CoA *O*-methyltransferase (CCoA*O*MT) yields feruloyl-CoA. Various pathways branch off from the general phenylpropanoid pathway, including the monolignol-specific pathway and the pathways towards flavonoids, benzenoids, coumarins, and sinapate and ferulate esters.

The monolignol-specific pathway includes four well-studied enzymatic steps that convert feruloyl-CoA into the monolignols coniferyl alcohol and sinapyl alcohol (Humphreys & Chapple, 2002; Boerjan *et al.*, 2003). First, feruloyl-CoA is reduced to coniferaldehyde by cinnamoyl-CoA reductase (CCR). Hydroxylation at the 5-position is catalyzed by ferulate 5-hydroxylase (F5H), which is also now often called coniferaldehyde 5-hydroxylase (CAld5H) to reflect its preferred substrate, to produce 5-hydroxyconiferaldehyde (Humphreys *et al.*, 1999; Osakabe *et al.*, 1999). The subsequent methylation of the newly introduced 5-hydroxyl group is catalyzed by caffeic acid *O*-methyltransferase (COMT), whose preferred substrate is also now known to be the aldehyde (Li *et al.*, 2000; Parvathi *et al.*, 2001), to provide sinapaldehyde. Further reduction to their corresponding alcohols, coniferyl alcohol and sinapyl alcohol, is catalyzed by cinnamyl alcohol dehydrogenase (CAD). A specific sinapyl alcohol dehydrogenase (SAD) involved in the reduction to sinapyl alcohol has been proposed in aspen (*Populus tremuloides*) based on *in vitro* enzymatic assays (Li *et al.*, 2001), but an *in vivo* role of SAD in monolignol biosynthesis has never been convincingly demonstrated (Guo *et al.*, 2010; Barakate *et al.*, 2011). Although the depicted pathway is thought to occur in many species (certainly Arabidopsis, tobacco (*Nicotiana tabacum*) and poplar), an alternative sequence of reactions for sinapyl alcohol production probably occurs in *Medicago* (Lee *et al.*, 2011) and the pathways in grasses are currently being evaluated (Withers *et al.*, 2012). In *Medicago*, the flux towards coniferyl and sinapyl alcohol bifurcates after caffeoyl-CoA production (Lee *et al.*, 2011). Caffeoyl-CoA destined for sinapyl alcohol synthesis is reduced by CCR to caffealdehyde, which is then converted to sinapaldehyde by the sequential actions of COMT, F5H and COMT.

The monolignols that result from the above-described monolignol-specific pathway are used for at least three different product classes: oligolignols/lignin, monolignol 4-*O*-hexosides and (neo) lignans. Oligolignols are racemic radical coupling products of monolignols that arise during lignin polymerization (Morreel *et al.*, 2004a, 2010a,b; Ralph *et al.*, 2004b). Lignans are formed by the initial stereospecific β-β coupling (see Fig. 2 for nomenclature) of two monolignol radicals (Umezawa, 2003). Secondary metabolites arising from two monolignol radicals that are stereospecifically β-*O*-4- or β-5-coupled are called neolignans (Umezawa, 2003). Stereospecific coupling reactions in (neo)lignan biosynthesis appear to be assisted by dirigent proteins (Davin *et al.*, 1997; Umezawa, 2003; Beejmohun *et al.*, 2007; Pickel *et al.*, 2010). Because of their antioxidant properties, (neo)lignans are believed to be involved in defense responses (Davin *et al.*, 1997). Some may also have a hormonal function; dehydrodiconiferyl alcohol glucoside (DCG) has been associated with cell division-promoting activities (Binns *et al.*, 1987; Teutonico *et al.*, 1991; Li *et al.*, 2010). The third metabolic class derived from monolignols includes the 4-*O*-glucosylated monolignols (e.g. coniferin and syringin). Several glucosyl transferases involved in their biosynthesis have been described in Arabidopsis, as well as β-glucosidases that convert the monolignol 4-*O*-glucosides back to their respective aglycones (Lim *et al.*, 2005; Escamilla-Treviño *et al.*, 2006; Lanot *et al.*, 2006). The biological role of monolignol 4-*O*-hexosides has not been unequivocally defined, but they could serve as storage forms for their aglycones (Lim *et al.*, 2005; Vanholme *et al.*, 2012). This hypothesis is supported by the finding that monolignol

Fig. 1 Phenolic metabolism in plants. The phenolic metabolite classes are given (in gray frames), as well as pathways and metabolic sinks that use phenolic metabolites or shikimate pathway intermediates as substrates. Representative metabolites are given for phenolic classes. Not every phenolic metabolic class shown is present in every plant species. The major route towards the monolignols *p*-coumaryl, coniferyl and sinapyl alcohol is given in color; the shikimate pathway (yellow), phenylalanine biosynthesis (orange), general phenylpropanoid pathway (pink) and monolignol-specific pathway (purple). Arrows with dashed lines designate known routes that involve multiple enzymatic steps; for simplicity, the individual enzymatic steps are not shown. Arrows with dotted lines designate unknown or unauthenticated routes. Arrows with a question mark are routes that have been suggested in the literature. DHS, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; DQS, 3-dehydroquinate synthase; DHQD, 3-dehydroquinate dehydratase; SD, shikimate dehydrogenase; SK, shikimate kinase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; CS, chorismate synthase; AT, amino transferase; TAL, tyrosine ammonia-lyase; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate: CoA ligase; HCT, hydroxycinnamoyl-CoA: shikimate/quinate hydroxycinnamoyltransferase; C3H, *p*-coumarate 3-hydroxylase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid *O*-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; UGT, UDP-glucosyltransferase; HCALDH, hydroxycinnamaldehyde dehydrogenase; BGLU, β-glucosidase.

4-*O*-glucosides are sequestered into the vacuoles of Arabidopsis, whereas monolignol aglycones are transported to the apoplast (Miao & Liu, 2010; Alejandro *et al.*, 2012).

The general phenylpropanoid- and monolignol-specific pathways also provide hydroxycinnamic acids, which include *p*-coumaric, caffeic, ferulic and sinapic acids. Hydroxycinnamic acids can be esterified or amidated by a variety of moieties such as malate, quinate, glucose, sucrose, choline, putrescine, spermidine, hydroxyanthranilate and tyramine; this can differ between plant species and plant tissues (Dimberg *et al.*, 1993; Martin-Tanguy, 1997; Schmidt *et al.*, 1999; Mahesh *et al.*, 2007; Milkowski & Strack, 2010). In Arabidopsis, the largest portion of the ferulic and sinapic acid pool is made from coniferaldehyde and sinapaldehyde via hydroxycinnamaldehyde dehydrogenase (HCALDH) (Nair *et al.*, 2004). The presence of ferulate and sinapate esters in *hcaldh* and *ccr1* mutants, however, proves the existence of an alternative pathway probably involving feruloyl-CoA (Nair *et al.*, 2004; Chen *et al.*, 2006a; Mir Derikvand *et al.*, 2008; Vanholme *et al.*, 2012). Alternatively, ferulic acid might be made from *p*-coumaric acid via 3-hydroxylation and 3-*O*-methylation by C3H/C4H and COMT (Chen *et al.*, 2011). Sinapate esters (e.g. sinapoyl glucose and sinapoyl malate) are putatively important as UV-protectants in

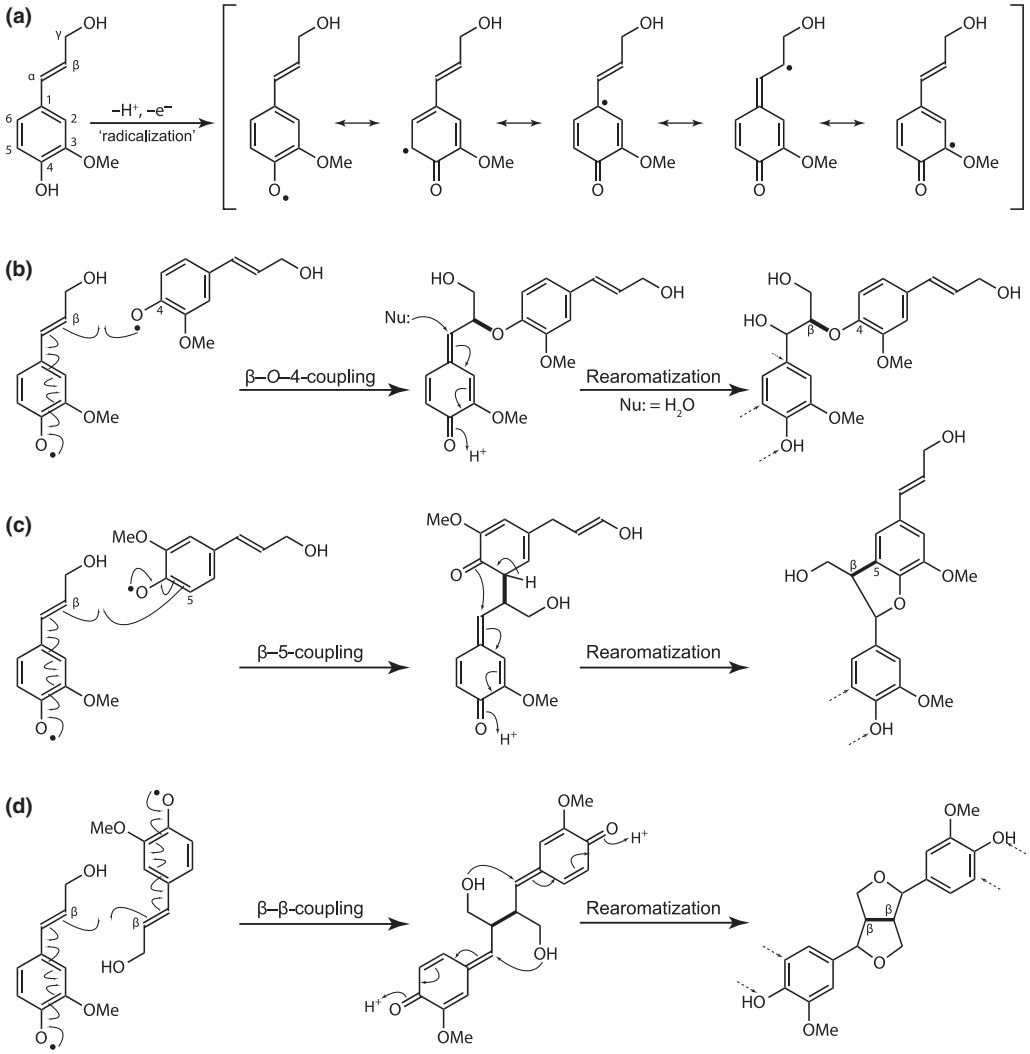


Fig. 2 Oxidative radicalization (i.e. dehydrogenation) and coupling of the traditional lignin monomer, coniferyl alcohol. (a) The 4-*O*-localized radical formed upon dehydrogenation is stabilized via delocalization, as shown by the five resonance forms. By convention, α, β and γ, and 7, 8 and 9 are used to indicate the aliphatic carbon positions in lignin monomers/polymers and (neo)lignan/oligolignols, respectively. To ensure consistent nomenclature in the text, α, β and γ are used for both lignins and (neo)lignan/oligolignols. (b) β-*O*-4 coupling of two coniferyl alcohol monomers. An external nucleophile (Nu:) provides the pathway for re-aromatization of the quinone methide intermediate. In the (common) case where the nucleophile is water, this results in the hydroxylation at the Cα position. However, if the nucleophile is a hydroxyl function from a hemicellulosic sugar moiety, a covalent ether bond between lignin and the hemicellulose is formed (see Fig. 4). (c) β-5 coupling of two coniferyl alcohol monomers. Re-aromatization of the quinone methide intermediate is via internal trapping (via the formal 4-OH that can also be considered to result from keto-enol tautomerization of the intermediate shown), and a phenylcoumaran structure is formed. (d) β-β coupling of two coniferyl alcohol monomers. The γ-hydroxyl groups serve as nucleophiles for internal trapping of the quinone methide, resulting in two re-aromatization reactions (that are likely to be sequential, and not necessarily concerted as shown here), and the formation of a resinol structure. The bond formed during the radical coupling reaction is the one used to describe the various dehydrodimerization reactions and is shown in bold. Lignification proceeds mainly via endwise addition of new monolignols to the free-phenolic end, following renewed radicalization, at the 4-O- or 5-positions (and also, to a small extent, at the 1-position) shown by the dotted arrows.

Brassicaceae and the genes involved in their biosynthesis are well described in Arabidopsis (Lorenzen *et al.*, 1996; Fraser *et al.*, 2007; Sinlapadech *et al.*, 2007). By contrast, the biological function and biosynthesis of ferulate esters are poorly understood. Ferulate esters are likely intermediates in the production of cell wall-bound ferulates (Rohde *et al.*, 2004; Mir Derikvand *et al.*, 2008) and of suberins (Bernards *et al.*, 1995; Franke & Schreiber, 2007; Soler *et al.*, 2007; Molina *et al.*, 2009; Rautengarten *et al.*, 2012). Ferulate-polysaccharide esters have well-established roles in polysaccharide–polysaccharide and lignin–polysaccharide cross-linking in grasses (Ralph *et al.*, 1994a,b, 1998; Hatfield *et al.*, 1999; Grabber *et al.*, 2000; Ralph, 2010), and may even function as nucleation sites for lignification (Ralph *et al.*, 1995; Grabber *et al.*, 2002). Ferulate esters are also found in neolignan oligomers (e.g. G(β-*O*-4)feruloyl malate) for which the biological role is as yet unknown (Rohde *et al.*, 2004; Böttcher *et al.*, 2008; Meißner *et al.*, 2008; Huis *et al.*, 2012; Vanholme *et al.*, 2012). Hydroxycinnamic acid polyamine conjugates are present in numerous plant species (Martin-Tanguy, 1997) and several of their biosynthetic genes have been characterized in Arabidopsis (Fellenberg *et al.*, 2009; Grienenberger *et al.*, 2009; Matsuno *et al.*, 2009). Hydroxycinnamic acids are also 4-*O*-glucosylated, resulting in metabolites that share characteristics with 4-*O*-glucosylated monolignols and hydroxycinnamaldehydes (Lim *et al.*, 2005). Finally, sinapyl and coniferyl *p*-coumarate esters serve as (nontraditional) monolignols of lignin (Lu & Ralph, 1999; Ralph, 2010).

Via the action of polyketide synthases, several phenolic pathways diverge from phenylpropanoid biosynthesis (Yu & Jez, 2008). For instance, the flavonoid biosynthetic pathway derives mainly from *p*-coumaroyl-CoA. The flavonoid biosynthetic pathway can be further divided into the general flavonoid, flavonol, isoflavonoid, anthocyanin and proanthocyanin (condensed tannin) pathways (Tanner *et al.*, 2003; Lapčík, 2007; Yonekura-Sakakibara *et al.*, 2008). Structural genes of the general flavonoid pathway are well characterized in Arabidopsis (Yonekura-Sakakibara *et al.*, 2008), but enzymatic steps involved in flavonoid modification (e.g. glycosylation and acylation) are poorly understood. The committed step towards flavonoids, catalyzed by the polyketide synthase chalcone synthase (CHS), condenses *p*-coumaroyl-CoA with three acetyl-CoA molecules to produce naringenin chalcone. Although aromatic hydroxylations can occur within the flavonoid pathway itself, in some species CHS may accept multiple hydroxycinnamoyl-CoA substrates to create a diverse set of hydroxylated and methoxylated flavonoids after the first step in flavonoid biosynthesis (Dao *et al.*, 2011). For example, sinapoyl, caffeoyl and *p*-coumaroyl-CoA are at least formal precursors of various flavones, such as tricin (found in grasses), luteolin and apigenin. Flavonoid biosynthesis and monolignol biosynthesis are even more intertwined; for example, Arabidopsis COMT also carries out its methoxylation reaction on flavonols to convert quercetin to isorhamnetin (Muzac *et al.*, 2000; Goujon *et al.*, 2003), and tricin has just been implicated as a monomer in grass lignins (del Río *et al.*, 2012). In specific plant species, hydroxycinnamoyl-CoAs are the substrates for other polyketide reactions. For instance, stilbene synthase catalyzes the biosynthesis of stilbenes in pine (*Pinus sylvestris* and *Pinus densiflora*), grape (*Vitis vinifera*), peanut (*Arachis hypogaea*) and sorghum (*Sorgum bicolor*) (Hammerbacher *et al.*, 2011).

Benzenoids are characterized by a C6-C1 skeleton. Some benzenoids, such as benzoic, *p*-hydroxybenzoic and vanillic acids, are made via chain-shortening of the C6-C3 skeleton of phenylpropanoids, whereas others, such as gallic acid, are synthesized from 3-dehydroshikimate. As described in the second paragraph of Section II, salicylic acid can be synthesized via chorismate or from benzoic acid. Two possible routes have been proposed for the biosynthesis of benzoic acid from cinnamic acid: the CoA-independent (non-β-oxidative) route via benzaldehyde and the CoA-dependent (β-oxidative) route via benzoyl-CoA (Hertweck *et al.*, 2001; Boatright *et al.*, 2004; Ibdah *et al.*, 2009). Except for an aldehyde oxidase involved in the CoA-independent biosynthesis of benzoic acid in seeds of Arabidopsis (Ibdah *et al.*, 2009) and a cinnamate:CoA ligase (CNL) and a 3-ketoacyl-CoA thiolase involved in the CoA-dependent biosynthesis of benzoic acid in petunia (*Petunia hybrida* BA) flowers (Van Moerkercke *et al.*, 2009; Klempien *et al.*, 2012), none of the genes involved in these pathways have been described. Because of similarities between fatty acid catabolism and the CoA-dependent chain-shortening of phenylpropanoids to benzenoids, the respective enzymes of these routes are expected to be (distant) homologs (Hertweck *et al.*, 2001). *p*-Hydroxybenzoates acylate lignins in *Populus*, *Salix* and *Palmae* species (Ralph, 2010), and benzenoids such as methylsalicilate, methylbenzoate, benzylbenzoate and benzylacetate, are volatiles and part of the floral scent (Dudareva *et al.*, 2004).

Another biosynthetic route that branches from the general phenylpropanoid biosynthetic pathway leads to coumarins. Following C2-hydroxylation of the hydroxycinnamoyl-CoA esters, lactonization of the side-chain produces the corresponding coumarins, which are often stored as glycosides. This yields umbelliferone, esculetin and scopoletin as the coumarin products of *p*-coumaric, caffeic and ferulic acids, respectively. The enzyme responsible for the first step, that is, the C6′ hydroxylation of feruloyl-CoA, has been described in Arabidopsis (Kai *et al.*, 2008).

Although much information about phenolic metabolism has been gathered over recent decades, the low proportion of identified metabolites in phenolic profiling studies (Morreel *et al.*, 2010a; Vanholme *et al.*, 2010c) underscores the complexity of these pathways and the need for methods to speed up structural characterization. Certainly, knowledge gaps in metabolic products and pathways will hamper attempts to bioengineer new types of lignin using alternative monomers from the phenylpropanoid metabolism.

III. Lignin biosynthesis and structure

After their biosynthesis, monolignols are translocated to the apoplast via a largely unresolved mechanism probably involving ATP-binding cassette (ABC) transporters (Miao & Liu, 2010). Recently, an Arabidopsis ABC transporter (AtABCG29) has been identified that is capable of transporting *p*-coumaryl alcohol when expressed in yeast, whereas Arabidopsis mutated for this transporter contained less lignin and was more sensitive to *p*-coumaryl alcohol

(Alejandro *et al.*, 2012). Upon entering the cell wall matrix, monolignols are oxidized by peroxidases and/or laccases to monolignol radicals that eventually polymerize into the lignin macromolecule via combinatorial radical–radical coupling reactions (Fig. 2). Laccases and peroxidases are encoded by large gene families and the individual members have overlapping activities (Vanholme *et al.*, 2010a). Two laccases involved in lignification have been identified (Berthet *et al.*, 2011). Direct contact between the peroxidase/laccase and the substrate is not needed – radical-transfer reactions can also pass the radical from one molecule to another. All radical coupling reactions in lignification are termination events; thus, continued lignification of cell walls requires new hydrogen-abstraction of monomers and the growing lignin oligomer following each coupling reaction. It is assumed, but not proven, that a monolignol radical can transfer its single electron to the growing polymer, but other transfer agents may be involved. For instance, the *p*-coumarate moiety in sinapyl *p*-coumarate, or manganese oxalate, may act as radical shuttles (Takahama *et al.*, 1996; Takahama & Oniki, 1997; Önnerud *et al.*, 2002; Ralph *et al.*, 2004a; Hatfield *et al.*, 2008). Because radical coupling is a purely chemically driven process, independent of control by any proteinaceous agent, any phenolic molecule having the proper chemical kinetic and thermodynamic radical-generation and cross-coupling propensities can couple into the lignin polymer (Harkin, 1967; Lu & Ralph, 1999, 2002, 2008; Boerjan *et al.*, 2003; Morreel *et al.*, 2004a; Ralph *et al.*, 2004b; Ralph, 2006; Vanholme *et al.*, 2010a).

The monolignol radical is resonance-stabilized, having various sites of enhanced single-electron density in the molecule (Fig. 2a). Mutual coupling of monolignols (dimerization) and cross-coupling with the growing polymer lead not only to the characteristic H, G and S units, but also to various inter-unit linkage types (Fig. 2b–d). At least 60% of the inter-unit linkages in dicots are β-aryl ethers arising from β-*O*-4 coupling of a monolignol at its β-position to the 4-*O*-position of the growing oligomer. These β-aryl ether linkages can, unlike other prevalent interunit linkages, be cleaved by harsh alkaline or acidic pretreatment of the lignocellulosic biomass (Sarkanen & Ludwig, 1971). The two other major inter-unit linkages in lignin are phenylcoumarans and resinols formed by β-5 and β-β coupling, respectively. Both are carbon–carbon linkages (among the so-called 'condensed linkages') that can only be broken under extremely harsh conditions that would also degrade the polysaccharides. For the three major types of linkages, the incoming monolignol radical reacts exclusively at its β-position, enabling the resulting 4-*O*-phenolic function produced after re-aromatization of the quinone methide intermediate to enter another coupling reaction. Although the β-*O*-4, β-5, and β-β couplings yield a linear polymer, branching can occur whenever the 4-*O*- or 5-position of one lignin oligomer or polymer couples with the 5-position of another lignin oligomer or polymer, producing 5-5 and 4-*O*-5 linkages. The coupling reactions involved in lignification have been previously reviewed in detail (Ralph *et al.*, 2004b).

Which primary units are present in the lignin polymer depends largely on the taxon of the plant. In general, gymnosperm lignins are rich in G units, with small amounts of H units, but no S units. Lignins from dicots are composed of both G and S units with only traces of H units, whereas lignin of monocot grasses also contains G and S units with modest levels (typically < 5%) of H units. It should be pointed out that H unit proportions in grasses are often overestimated because *p*-coumarate units acylating lignin are often mistakenly quantified as H units (Boerjan *et al.*, 2003; del Río *et al.*, 2012). Nevertheless, it should be stressed that the lignin unit composition is highly variable, not only between species, but also between tissue and cell types, and even within a single cell wall. In addition, any phenolic molecule entering the cell wall region may be oxidized and incorporated into the lignin polymer (Harkin, 1967; Lu & Ralph, 1999, 2002, 2008; Boerjan *et al.*, 2003; Morreel *et al.*, 2004a; Ralph *et al.*, 2004b; Ralph, 2006; Vanholme *et al.*, 2010a). Many alternative monomers are found in the lignin of wild-type plants. For example, traditional monolignols are often acylated at their γ-position with acetate, *p*-hydroxybenzoate or *p*-coumarate (Lu & Ralph, 1999, 2002, 2008; Morreel *et al.*, 2004a). Such acylated units can even be highly abundant; for example, coniferyl and sinapyl acetate may constitute 50% or more of the units in lignin from kenaf (*Hibiscus cannabinus*) (Ralph, 1996; Del Río *et al.*, 2007). Also, dihydro-hydroxycinnamyl alcohols, hydroxybenzaldehydes and hydroxycinnamic acids and products from an incomplete monolignol biosynthesis, such as hydroxycinnamaldehydes, are found in lignins of wild-type plants (Baucher *et al.*, 1996; Ralph *et al.*, 1997, 2008a; Sibout *et al.*, 2002; Boerjan *et al.*, 2003).

Lignin composition can be steered via genetic engineering. For example, F5H-deficient plants produce lignin composed almost entirely of G units rather than the normal complement of both S and G units (Meyer *et al.*, 1998; Marita *et al.*, 1999). Conversely, *F5H* up-regulation can lead to plants with extremely high proportions of S units (Meyer *et al.*, 1998; Marita *et al.*, 1999; Franke *et al.*, 2000; Stewart *et al.*, 2009). Plants deficient in *COMT* produce elevated amounts of 5-hydroxyconiferyl alcohol, an alternative monomer that has a high propensity to undergo β-*O*-4 coupling, producing novel benzodioxane structures within the lignin (Marita *et al.*, 2001; Ralph *et al.*, 2001a,b; Jouanin *et al.*, 2004; Morreel *et al.*, 2004b; Lu *et al.*, 2010). These inter-unit linkages are below or close to the detection limit in wild-type plants (Atanassova *et al.*, 1995; Morreel *et al.*, 2004b; Lu *et al.*, 2010; Huis *et al.*, 2012). In an extreme case, an Arabidopsis *comt* mutant with concomitant *F5H* overexpression produced a lignin with over 90% of its units linked by benzodioxane structures (Vanholme *et al.*, 2010b; Weng *et al.*, 2010). The coupling of alternative monomers into lignins was also enhanced in plants with reduced CAD or CCR activity. Dicots that are deficient in CAD accumulate lignin units derived from hydroxycinnamaldehydes (Kim *et al.*, 2000, 2002, 2003; Lapierre *et al.*, 2004; Ralph *et al.*, 2004b; Sibout *et al.*, 2005; Leplé *et al.*, 2007) and plants deficient in CCR are characterized by lignins containing small amounts of ferulic acid-derived units (Dauwe *et al.*, 2007; Leplé *et al.*, 2007; Mir Derikvand *et al.*, 2008; Ralph *et al.*, 2008b). The observation that plants readily incorporate alternative monomers to form lignins with altered physicochemical properties opens up the possibility of bioengineering various phenolic pathways to produce phenolic monomers that can be exported to the cell wall to create new types of lignins designed for efficient industrial processing of biomass.

IV. Alternative lignin monomers for biofuel applications

Alternative lignin monomers must meet certain criteria to confer increased susceptibility of biomass to pretreatments. As mentioned above, the monomer must meet the minimal requirement for a molecule to be radicalized and to couple into the lignin polymer. Given all available data about molecules that are polymerized into lignin, both *in vivo* in wild-type and genetically engineered plants and *in vitro* in synthetic lignins (dehydrogenation polymers (DHPs)), this minimal requirement is the presence of a phenolic function (Harkin, 1967; Lu & Ralph, 1999, 2002, 2008; Boerjan *et al.*, 2003; Morreel *et al.*, 2004a; Ralph *et al.*, 2004b; Ralph, 2006; Vanholme *et al.*, 2010a). As a consequence of incompatibilities in radical coupling reactions, *p*-hydroxyphenyl moieties fare less well than guaiacyl or syringyl moieties, at least when incorporating into guaiacyl-syringyl lignins, but other phenolics have not been well studied. Phenolic molecules with an accessible β-position (i.e. a side-chain conjugated to the phenol) allowing for so-called 'endwise' β-*O*-4 coupling are also considered ideal (Ralph, 2006). Although the ability to efficiently cross-couple with monolignols is a prerequisite, the ultimate utility of alternative monomers is determined by their abilities to lessen the inherent inhibitory effects of lignin on cell wall saccharification or to render lignin easier to remove by chemical pretreatments. For this purpose, we envision that five types of phenolics could prove useful as alternative monomers for lignification. These include (1) monomers that directly produce a readily cleavable functionality in the polymer, (2) hydrophilic monomers, (3) difunctional monomers and monomer conjugates linked via a readily cleavable functionality, (4) monomers that minimize lignin–polysaccharide cross-linking and (5) monomers that give rise to shorter lignin polymers. As described in the one but last paragraph of this Section (IV), alternative monomers can possess one or several of these characteristics, which may be of value for enhancing the conversion of biomass into fermentable sugars. In addition to the above-mentioned restrictions, molecules composed of only carbon, oxygen and hydrogen are most attractive as alternative monolignols for biofuel applications. This is because molecules containing other elements, such as nitrogen and phosphorous, although they might result in added-value degradation products from lignin, would probably increase the need to provide plants with increased nitrogen- and phosphorus-containing fertilizer for their biosynthesis. In some cases, greater fertilizer use might be justified, but as a general rule this would undesirably increase the financial and environmental cost of growing biomass for biofuel production.

Monomers that directly produce a readily cleavable functionality in the lignin polymer Ferulic acid (**11G**) is an example of a monomer that produces a cleavable functionality upon incorporation in the lignin polymer, in this case an acetal that can be readily cleaved under mildly acidic conditions (Ralph *et al.*, 2008b). By β-*O*-4 coupling of ferulic acid with the growing polymer, decarboxylation to form a side-chain truncated unit and β-*O*-4 coupling again with a monomer or the polymer, an acetal is formed – the reaction mechanism is depicted in Fig. 3. Ferulic acid is incorporated into the lignin of *CCR*-down-regulated plants (Dauwe *et al.*, 2007; Leplé *et al.*, 2007; Mir Derikvand *et al.*, 2008; Ralph *et al.*, 2008b). Heavily *CCR*-down-regulated plants (with lower lignin contents) are compromised, usually having stunted growth and collapsed vessels, but it is unlikely that this is solely attributable to the incorporation of low amounts of ferulic acid into the lignins – it is probably a result of the reduced lignin contents and other metabolic and structural changes. Finding the means to incorporate ferulic acid or other related monomers might still prove to be fruitful. In principle, other monomers with carboxylic acid side-chains could form acetals too, but they must be capable of undergoing the double β-*O*-4 coupling reactions. An alternative strategy to introduce acetal-type inter-unit linkages in the lignin

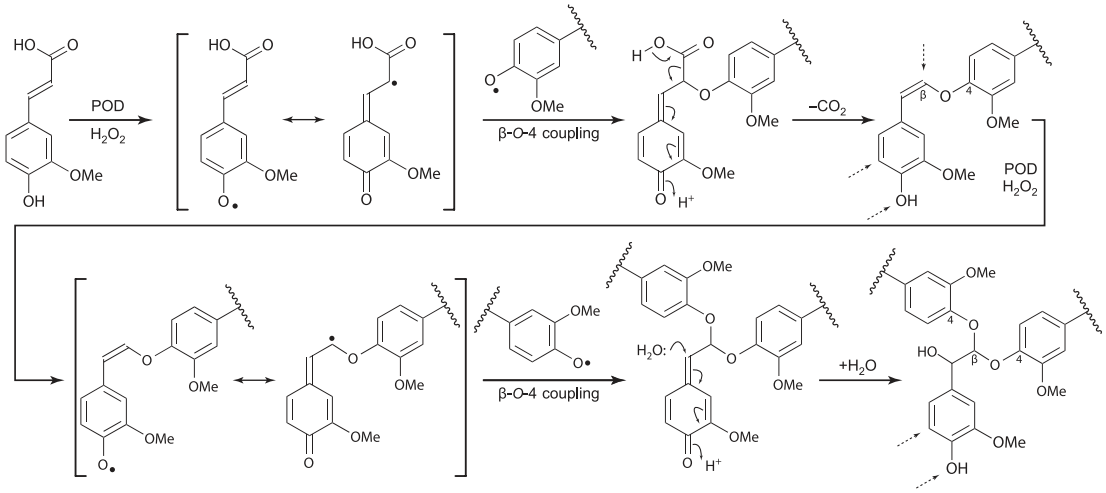


Fig. 3 Formation of acetal functionalities in lignin via the incorporation of ferulic acid. Aromatization of the quinone methide intermediate of ferulic acid β-O-4 coupled to a phenolic end group (of a generic guaiacyl lignin unit here) is via decarboxylation of the Cγ carboxylic acid function. The newly generated α-β double bond allows the ferulic acid-derived unit to enter a second β-O-4 coupling reaction via its β-position, ultimately creating the acetal functionality after the usual re-aromatization of the quinone methide. The dotted arrows indicate the positions where lignification can proceed.

polymer is via alternative lignin monomers that bear a hydroxyl or ether function at the β-position (as in compounds **16**, **24**, **26**, **32**, **48**, **57**–**59** and **79**). In this case, β-*O*-4 coupling will directly lead to the formation of an acetal.

Hydrophylic monomers Incorporation of hydrophilic monomers into lignin could enhance the penetration and, therefore, the hydrolysis of the lignocellulosic biomass by saccharifying enzymes, even without pretreatment. Lignin hydrophobicity could be modulated by the incorporation of phenolics with extensive side-chain or aromatic ring hydroxylation (e.g. monomers such as guaiacylglycerol (**4G**)) or substitution with hydrophilic groups (e. g. feruloyl quinate (**28G**), feruloyl glucose (**35G**) or isoconiferin (**34G**)). Hydrophilic groups attached by ester and glycosidic linkages would probably be cleaved under alkaline or acidic conditions; thus, the value of alternative monomers containing such groups would be diminished under many pretreatment conditions. However, hydroxyl or carboxylic acid groups remaining after pretreatment could enhance the extractability of lignin fragments into aqueous media, aiding delignification. The effect of hydrophilic lignin on plant growth and development is not obvious at this stage. Hydrophobic lignin may facilitate water transport in vessel elements but, while this has often been speculated to be the case, this requirement has never been demonstrated experimentally. If hydrophobic lignins are indeed required for water transport, hydrophilic monomers could be targeted toward fibers or other nonconducting tissues.

Difunctional monomers and monomer conjugates linked via a readily cleavable functionality Introducing monomers or conjugates with compatible phenolic groups at both 'ends' of the molecule allows lignification to proceed in both directions to incorporate the monomer. If coupling at the side-chain β-position is possible, such monomers can form important branch points in the polymer. More importantly, if these units contain bonds that are readily cleaved during anticipated processing, they introduce labile groups into the polymer backbone, allowing it to be readily 'unzipped'; delignification can therefore be achieved under less stringent conditions, releasing the polysaccharides with lower energy requirements and higher yields for enzymatic saccharification or other uses. A wide array of phenolics meet these criteria, but the simplest examples include coniferyl ferulate (**7GG**) (Grabber *et al.*, 2008; Ralph, 2010) linked by an alkali- and acid-labile ester bond, 3-methoxytyramine ferulate (**8GG**) linked by a somewhat acid-labile amide bond and compounds such as disinapoyl glucose (**78SS**) and diferuloyl sucrose (**81GG**), where two phenolic units are linked by labile ester bonds to a core 'spacer'. All possess guaiacyl or syringyl type moieties compatible with oxidative coupling with monolignols and are therefore expected to become integral components of lignin. Monomers with one or more *p*-hydroxyphenyl moieties might also be utilized, but any molecule with a lower propensity to undergo radical coupling might hinder full incorporation into lignin. *ortho*-Diphenol (catechol) and 1,2,3-triphenol (pyrogallol) monomers are also prevalent phenolic metabolites and can profitably be considered. Unlike monomers that upon incorporation render lignin more hydrophilic, such difunctional monomers could maintain the hydrophobicity of lignin that may be required for water transport or plant defense responses. Their incorporation into hydrophobic polymers should also shield their ester, amide or glycosidic linkages from attack by hydrolytic enzymes produced by pathogenic fungi or bacteria. Thus, these monomer substitutes are a way of introducing 'zips' into lignin that can be readily cleaved during processing while maintaining the functional properties of lignin required by plants.

Monomers that minimize lignin–polysaccharide cross-linking Monomers that minimize lignin–polysaccharide cross-linking should enhance the inherent degradability of lignocellulosic biomass by saccharifying enzymes. The adverse effects of lignin–polysaccharide cross-linking on wall polysaccharide digestibility have been demonstrated in grasses (Grabber *et al.*, 1998a,b, 2000, 2002; Ralph *et al.*, 1998; Hatfield *et al.*, 1999; Grabber, 2005), where cross-linking is mediated by ferulates on arabinoxylans (Fry, 1986; Fry & Miller, 1989; Yamamoto *et al.*, 1989; Ralph *et al.*, 1998, 2004a; Hatfield *et al.*, 1999; Ralph, 2010). As a result of this finding, efforts are underway to attack this cross-linking mechanism by targeting the putative transferase that acylates arabinoxylans with ferulate (Yoshida-Shimokawa *et al.*, 2001; Mitchell *et al.*, 2007; Buanafina, 2009; Piston *et al.*, 2010). In grasses and all other plants, lignin–polysaccharide cross-linking also apparently results from polysaccharides adding to the quinone methide intermediates produced during lignification and such cross-linkings also appear to limit fiber saccharification (Grabber *et al.*, 2003; Grabber & Hatfield, 2005). During β-*O*-4 coupling, re-aromatization of the quinone methide intermediate occurs mainly via the proton-assisted nucleophilic attack of water at the α-position (Fig. 2). Actually, any nucleophile present in the neighborhood can participate in the reaction instead of water, an example being hemicellulosic alcohol (or acid) groups (Ralph *et al.*, 2004b; Simmons *et al.*, 2010). In the latter case, lignin becomes covalently linked to the hemicellulosic network, rendering its removal more difficult. Quantifying these benzyl ether and ester cross-links in cell walls is problematic and limited to a small fraction of lignin that can be extracted from cell walls (Balakshin *et al.*, 2008, 2011). Nevertheless, judicious choice of lignin monomers can minimize/eliminate this cross-linking mechanism in plants. Alternative monomers with *ortho*-diphenol structures, such as caffeyl alcohol (**1C**), 5-hydroxyconiferyl alcohol (**1F**) or epicatechin (**73C**), and with 1,2,3-triphenol structures, such as ethyl gallate and epigallocatechin (**73L**), readily form benzodioxane structures; rapid internal trapping of the quinone methide, which is produced following a monolignol's β-*O*-4 coupling with such *o*-diphenols, precludes any possibility of benzyl ether and ester cross-linking of hemicellulosic alcohol or acid groups with those units (Fig. 4a). Lignins derived solely from caffeyl alcohol have been discovered in seed coats (Chen *et al.*, 2012); lignins incorporating caffeyl alcohol have been observed in CCoAOMT-deficient gymnosperm cell cultures (Wagner *et al.*, 2011). Lignins incorporating 5-hydroxyconiferyl alcohol derive from various COMT-deficient dicots and monocots (Van Doorsselaere *et al.*, 1995; Lapierre *et al.*, 1999; Marita *et al.*, 2001; Ralph *et al.*, 2001a,b; Morreel

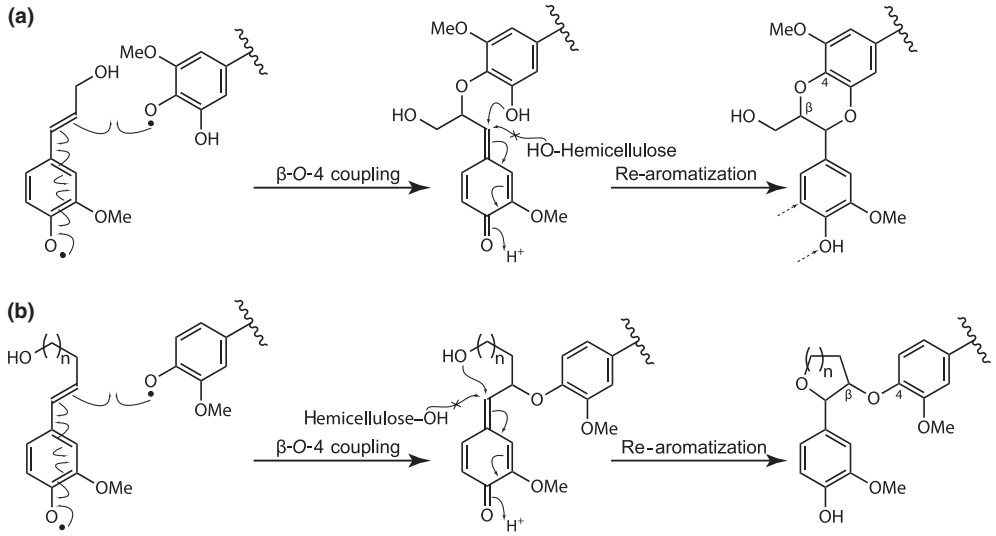


Fig. 4 An interesting way to avert lignin–polysaccharide cross-linking during β-*O*-4 coupling. (a) The quinone methide intermediate formed via β-*O*-4 coupling is trapped via an intra-molecular reaction of the novel C5-phenolic function, thereby precluding the possibility of inter-molecular nucleophilic attack by hemicellulose (indicated by a crossed arrow). (b) Similarly, the intra-molecular trapping via an alcoholic function on the δ ($n = 1$) or ε ($n = 2$) position of suitable alternate lignin monomers also avoids the inter-molecular nucleophilic attack by hemicellulose (crossed arrow). The dotted arrows indicate the positions where lignification can proceed.

et al., 2004b; Lu *et al.*, 2010; Vanholme *et al.*, 2010b; Weng *et al.*, 2010). Where they have been tested, the cell walls from these materials have enhanced digestibilities (Guo *et al.*, 2001a,b; Fu *et al.*, 2011). The effect of introducing rosmarinic acid (**49CC**) into lignins has recently been evaluated (see Section V) and found to be effective at improving saccharification, even without pretreatment (Tobimatsu *et al.*, 2012). Other candidate alternative monolignols, such as guaiacyl butenol, provide a side-chain-based intramolecular pathway to trap lignin quinone methides that are formed from the monomer itself following β-*O*-4, β-β, or β-5 coupling (e.g. **25**, **27**, **43**–**47**). Trapping by the δ- or ε-hydroxyl group would result in tetrahydrofuran (oxolane) or tetrahydropyran (oxane) structures (Fig. 4b).

Monomers that give rise to shorter lignin polymers Decreasing the average length of the lignin polymers should also enhance the extractability of lignin. Alternative monomers that, upon polymerization, end up as an aliphatic or phenolic end group, rather than becoming an internal unit, might serve as polymerization initiation or termination monomers, respectively. Augmenting their availability at the lignification site might lead to a higher rate of lignin initiation or termination reactions, probably yielding a higher number of shorter lignin molecules, that is, lignins with lower degrees of polymerization. Monomers that initiate polymerization, for example dihydroconiferyl alcohol (**2G**) (Ralph *et al.*, 1997) or benzenoids (**9**, **20**), enter only into a single coupling reaction, that is, at the 4-*O*- or 5-position, thus consuming the phenolic function during initial coupling. A proof of principle is apparent in a study in which the bacterial hydroxycinnamoyl-CoA hydratase-lyase (*HCHL*) was expressed in Arabidopsis (Eudes *et al.*, 2012). *p*-Hydroxybenzaldehyde (**9H**) and *p*-hydroxybenzoate (**20H**) were incorporated into the lignin of HCHL engineered plants, which resulted in lignin with a reduced molecular weight and an improved saccharification of pretreated stem cell walls (Eudes *et al.*, 2012). Importantly, total lignin and biomass yield were not affected. Alternatively, co-polymerization with a monomer possessing a rather high oxidation potential enhances the termination of polymerization and reduces the average length of the lignin polymers. Among the traditional monolignols, *p*-coumaryl alcohol has the highest oxidation potential (Syrjänen & Brunow, 1998). Consequently, phenolic profiling (see Section VI) of flax (*Linum usitatissimum*) stem tissues showed that H units were preferentially phenolic end groups of oligolignols (Huis *et al.*, 2012) and thioacidolysis shows that a high fraction of H-units are free-phenolic (Lapierre *et al.*, 1988; Pitre *et al.*, 2007; Lapierre, 2010). In part, this is thought to be attributable to radical transfer (to more stable units) in the radical-limited system – a similar phenomenon occurs with *p*-coumarate esters, which also remain uncoupled (Ralph *et al.*, 1994a, 2004a; Hatfield *et al.*, 2008). Furthermore, lignins from transgenic or mutant plants that contain a high level of H units are of lower molecular weight (Ziebell *et al.*, 2010) and are enriched in β-5 and β-β linkages as compared with wild-type lignin (Ralph *et al.*, 2006; Wagner *et al.*, 2007). The relative decrease in β-*O*-4 coupling reactions, which are the main lignin polymer elongation reactions, suggests the presence of shorter lignins in these transgenic and mutant plants.

Finally, alternative monomers combining several of the above-mentioned mechanisms for altering lignin properties would be especially attractive as genetic engineering targets. For example, disinapoyl glucose (**78SS**) features two readily incorporated sinapate moieties attached to a hydrophilic moiety by readily cleavable ester linkages. Rosmarinic acid (**49CC**, noted above), epigallocatechin gallate (**74LL**) and dicaffeoyl quinate (**72CC**) all possess two cross-link-preventing *o*-diphenol functionalities connected by labile ester linkages, and in addition the latter molecule also contain a hydrophilic moiety. Finally, gallotannins

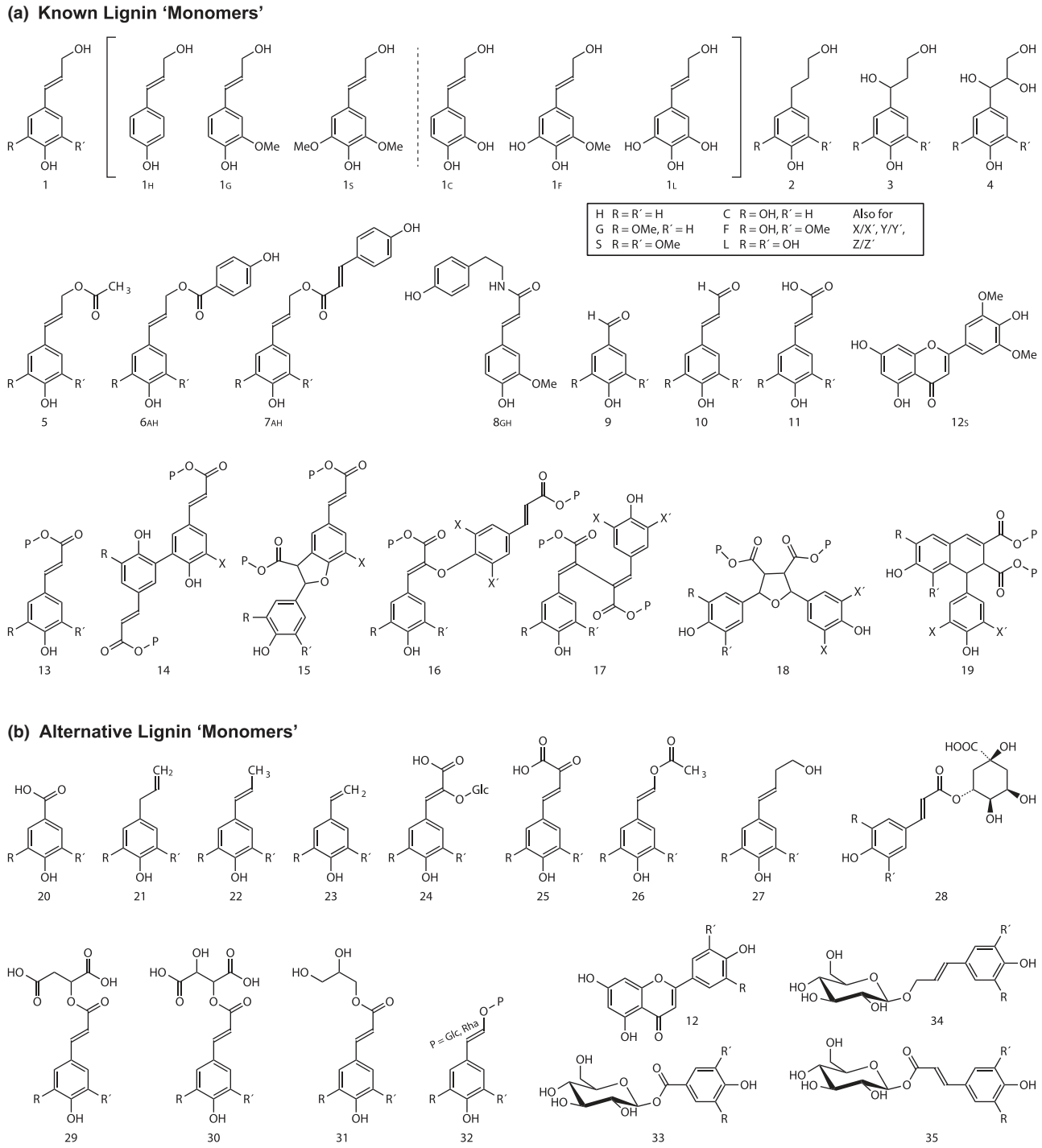
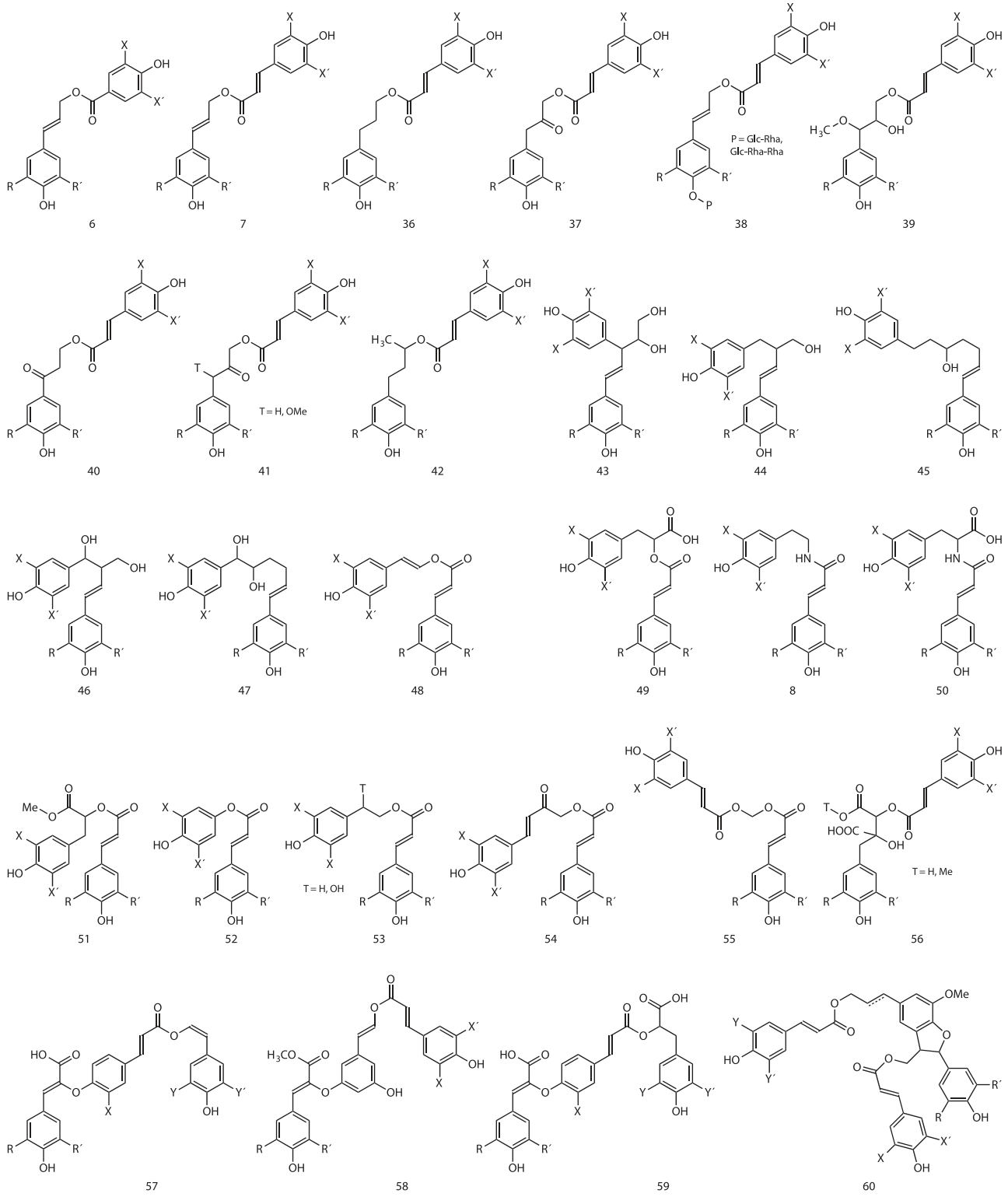


Fig. 5 Compounds found within the plant kingdom that (potentially) satisfy criteria for alternative lignin monomers. (a) Monomers that have already been authenticated or implicated in lignification. (b) Alternative monomers that, upon incorporation into the lignin polymer, potentially make the lignin more susceptible to biomass pretreatment. Aromatic ring units are all phenols, invariably p-hydroxy-aryl units here. Substituents are labeled R/R′ for the 'first' aromatic ring, X/X′ for the second, Y/Y′ for the third and Z/Z′ for the fourth. In all cases, the descriptor notation uses the compound number followed by the defined rings, in the order described as: H (*p*-hydroxyphenyl), G (guaiacyl), S (syringyl), C (caffeyl), F (5-hydroxyguaiacyl), or L (gallyl) with A being used for any or all (generic) units. The convention is illustrated with compounds 7 as follows: 7SG, sinapyl ferulate (R = R′ = OMe, X = OMe, X′ = H); 7AH, general hydroxycinnamyl *p*-coumarate (R,R′ = H/OH/OMe; X = X′ = H). Where necessary, other variable substituents are used (P, T) and a variable single or double bond in some structures is designated with one solid and one dashed bond line. The names of the compounds, the plant species in which the compounds are found and references to the literature are available in Supporting Information Notes S1.

(**86**) contain multiple pyrogallol units connected by labile ester linkages and the extensive hydroxylation of this molecule could make an altered lignin more hydrophilic.

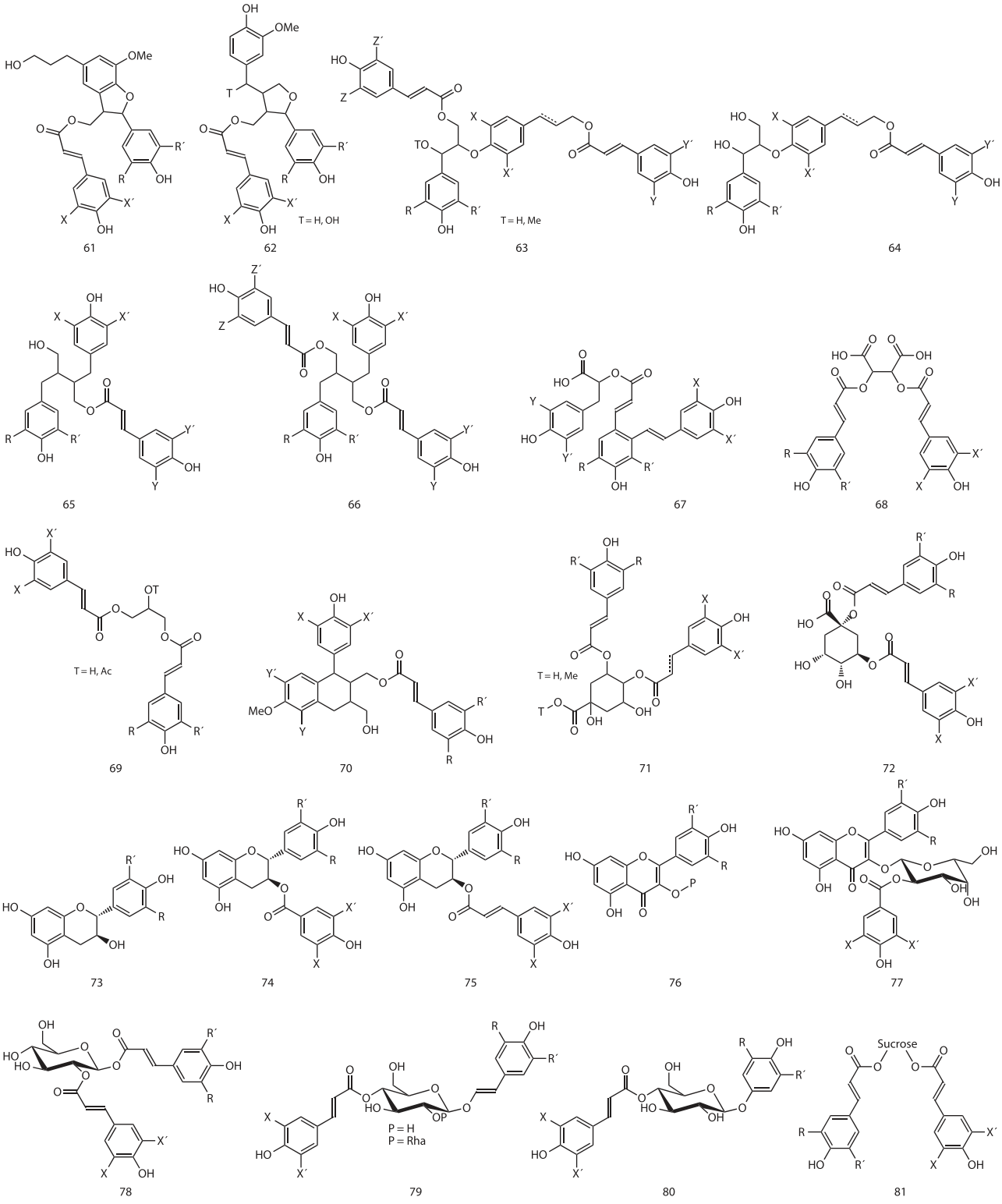
To exploit the biochemical variation of potential alternative monolignols, we screened the SciFinder Scholar database (CAS; http://www.cas.org/products/scifindr/index.html) for plant metab-

(b) Alternative Lignin 'Monomers' *(ctd)*



olites that fulfill one of the above-mentioned criteria, that is, the metabolites must have a phenolic function, upon incorporation in the lignin polymer they must render it easier to degrade by chemical pretreatments, and they must be composed of carbon, oxygen and hydrogen only. Over 160 plant metabolites satisfying the above-mentioned criteria for promising alternative lignin monomers were identified (Fig. 5). Given the fact that only a small proportion of plant metabolites have been characterized thus far, the list of

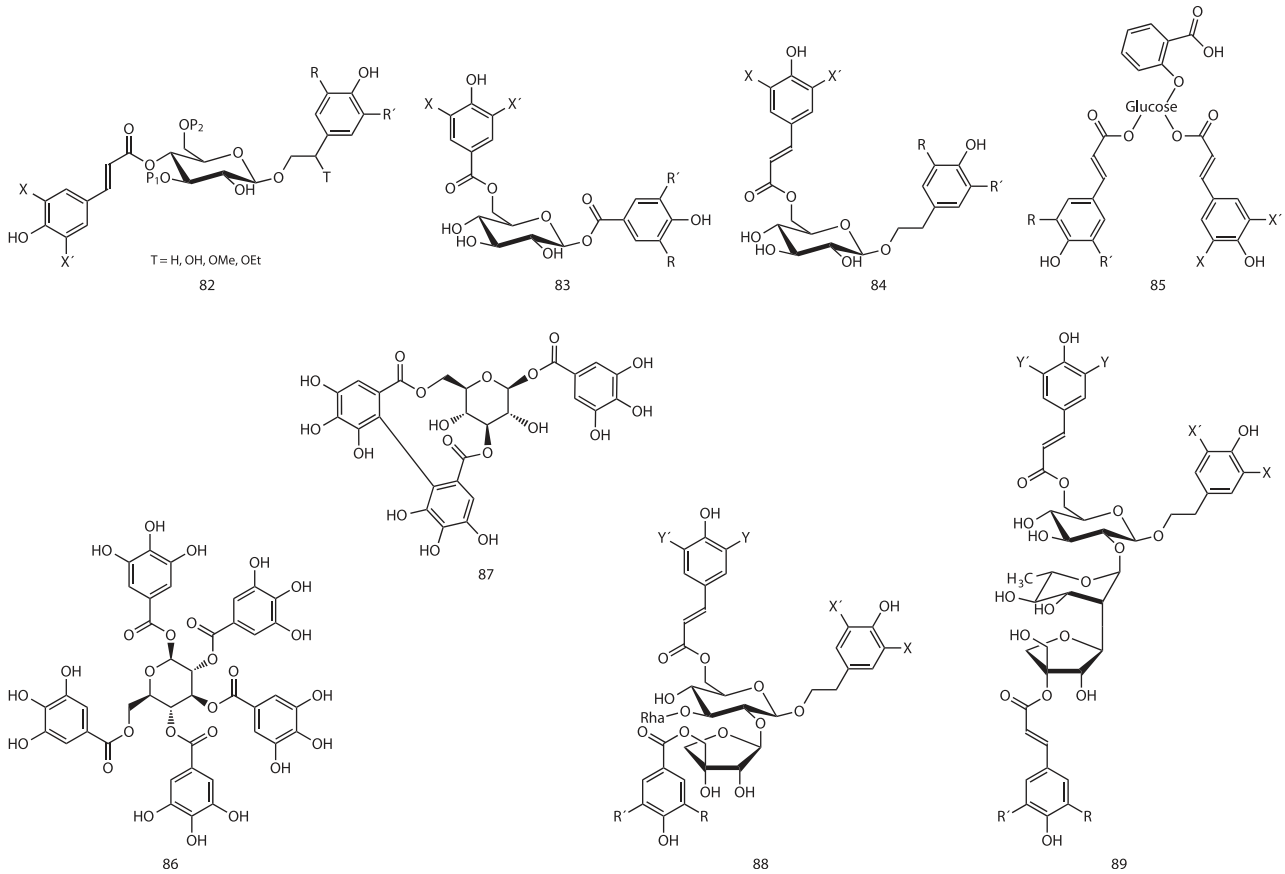
(b) Alternative Lignin 'Monomers' *(ctd)*



candidate alternative lignin monomers will grow further as targeted phenolic profiling studies are being carried out. Bacteria and fungi also contain attractive enzymatic activities for other types of phenolic compounds that might be exploited for modifying plant lignin (Masai *et al.*, 2007; Merali *et al.*, 2007; Eudes *et al.*, 2012).

In principle, such lignin modifications can be accomplished in lignocellulosic biomass crops by cloning and expressing alternative monomer biosynthetic pathways in conjunction with appropriate tissue-specific promoters. *In vitro* testing is necessary to cut down this large number of the 'candidate' alternative lignin monomers

(b) Alternative Lignin 'Monomers' *(ctd)*



(Grabber *et al.*, 2008, 2010; Tobimatsu *et al.*, 2012). Such *in vitro* tests should at least include the co-polymerization of the proposed alternative monomers with traditional monolignols in DHPs or biomimetic *in vitro* lignified cell walls followed by analysis of the DHPs and lignified cell walls for the effects of pretreatment on delignification and by analysis of the lignified cell walls for the saccharification potential. In addition, DHPs and *in vitro* lignified cell walls should be analyzed for the incorporation of the alternative monomer by phenolic profiling (Morreel *et al.*, 2004a) and nuclear magnetic resonance (NMR) (Grabber *et al.*, 2010; Tobimatsu *et al.*, 2012). These tests are to determine whether the proposed alternative lignin monomers do incorporate in sufficient amounts in the lignin polymer, and also to what extent the efficiency of incorporation of the monomers depends on the competing phenolics present in the cell wall matrix.

V. Candidate alternative monolignols in biomimetic systems

Plant genetic engineering studies will, of course, ultimately determine the feasibility and utility of modified lignins and their compatibility with plant growth and development. The engineering of plants will, however, be much more efficient if *in vitro* DHP studies and biomimetic cell wall lignification studies are first carried out to test the compatibility of the various monolignol substitutes with lignification, and to determine their potential effects on cell wall delignification and saccharification. In the latter case, for example, isolated maize (*Zea mays*) cell walls containing bound peroxidases are stirred in water or buffer solutions and artificially lignified by slowly adding separate solutions of lignin precursors and dilute hydrogen peroxide (Grabber *et al.*, 1996b, 1998c). Candidate monolignol substitutes are typically added with normal monolignols to comprise 35–45% of the weight of the precursor mixture, potentially yielding a shift in lignin composition comparable to that observed in some mutant or transgenic plants with altered lignin biosynthesis. The effects of alternative monomer on lignin formation and the susceptibility of cell walls to chemical pretreatments and saccharification are compared with lignified controls prepared with normal monolignols (Grabber *et al.*, 2008, 2010, 2012; Tobimatsu *et al.*, 2012).

Thus far the work with the DHPs and lignified cell wall model systems has mainly focused on difunctional monomers or monomer conjugates linked via a readily cleaved functionality and monomers that minimize lignin–polysaccharide cross-linking. For instance, incorporation of coniferyl ferulate (**7GG**) facilitated lignin depolymerization and increased lignin extractability by up to twofold in aqueous NaOH, providing an avenue for producing fiber with less lignin contamination and delignifying at lower temperatures or lower chemical consumption (Grabber *et al.*, 2008; Ralph, 2010).

As alluded to in Section IV, more recent model studies have demonstrated the utility of rosmarinic acid (**49CC**), an ester

conjugate with two catechol moieties (Tobimatsu *et al.*, 2012). In *in vitro* DHP experiments, rosmarinic acid readily underwent peroxidase-catalyzed copolymerization with monolignols to form polymers with benzodioxane inter-unit linkages, suggesting that fewer lignin–carbohydrate cross-links could be formed via lignin quinone methide intermediates. Incorporation of rosmarinic acid permitted extensive depolymerization of *in vitro* lignified cell walls by mild alkaline hydrolysis, via cleavage of ester linkages within the rosmarinic acid moiety (in the lignin) itself. Copolymerization of rosmarinic acid with monolignols modestly depressed lignification of cell walls and promoted subsequent cell wall saccharification by fungal enzymes after mild alkali pretreatment. Incorporating rosmarinic acid also improved cell wall saccharification by fungal enzymes and by rumen microflora even without alkaline pretreatments, possibly by modulating lignin hydrophobicity and/or limiting cell wall cross-linking.

In other studies (Grabber *et al.*, 2010, 2012), epigallocatechin gallate (**74LL**), epicatechin gallate (**74CL**), epicatechin vanillate (**74CG**), epigallocatechin (**73L**), galloyl hyperin (**77CL**) and pentagalloyl glucose (**86**) formed wall-bound lignin at moderate to high concentrations and their incorporation increased *in vitro* ruminal fiber fermentability by 20 to 33% relative to lignified controls. By contrast, ethyl gallate and corilagin (**87**) severely depressed lignification and increased fermentability by *c.* 50%. Thus, addition of these units probably acted indirectly to improve fermentability through severely reducing lignin content. Regardless of the mechanism, ethyl gallate and corilagin would probably be of limited value as target monomers, because they severely disrupted cell wall lignification. Such reductions in lignin content, also already attained by down-regulating enzymes in the general phenylpropanoid and monolignol pathway, often reduce plant fitness (Gallego-Giraldo *et al.*, 2011b; Voelker *et al.*, 2011). Improvements in fermentability with flavan-3-ols were associated with increased hydroxylation, but this response was not necessarily caused by increased lignin hydrophilicity because flavonol glycosides and gallate esters with more extensive hydroxylation (e.g. hyperoside (**76C**, P=Gal), galloyl hyperin (**77CL**), and pentagalloyl glucose (**86**)) had less pronounced effects on cell wall fermentability. Among flavan-3-ols (**73**), gains in cell wall fermentability were related to the presence of gallate and pyrogalloyl units. Furthermore, the copolymerization of monolignols with epicatechin gallate (**74CL**), epigallocatechin gallate (**74LL**) and, to a lesser degree, pentagalloyl glucose (**86**) reduced the proportion of ferulates that underwent cross-linking with lignin (Grabber *et al.*, 2010, 2012). These reductions in ferulate–lignin cross-linking should contribute to the improved cell wall fermentability (Grabber *et al.*, 2009). From these experiments, epigallocatechin gallate (**74LL**) appeared a promising target for incorporation into lignin for improving the delignification and saccharification of biomass crops (Grabber *et al.*, 2012).

In addition to studying the incorporation of the alternative monomers in lignin and the resulting cell wall properties, the biomimetic systems are also interesting systems with which to reveal peroxidase inactivation. For instance, partial substitution of coniferyl alcohol with coniferyl ferulate (**7GG**) tended to accelerate peroxidase inactivation and reduce cell wall lignification and cross-linking of feruloylated xylans to lignin (Grabber *et al.*, 2008). Analogous effects were seen with sinapyl *p*-coumarate (**7SH**), which is, however, a monomer conjugate that is heavily implicated as being successfully incorporated into grass lignins (Ralph *et al.*, 1994a; Grabber *et al.*, 1996a; Lu & Ralph, 1999, 2008; Hatfield *et al.*, 2009; Ralph, 2010; Withers *et al.*, 2012). Notably, because non-bound apoplastic peroxidases were removed before artificial lignification in these experiments, peroxidase inactivation would be more markedly manifested in the model system than in plants. Nevertheless, such observations need to be tracked when genetically altered plants are developed.

VI. From phenolic profiling to lignomics

Phenolic profiling is a technique ideally suited for identifying new alternative lignin monomers and pathway intermediates, and in addition for verifying their incorporation into *in vivo* or *in vitro* lignins and for monitoring plant responses to phenolic pathway engineering. Basically, this technique includes the identification of phenolic metabolites by reversed-phase (ultra) high-pressure liquid chromatography coupled to mass spectrometry (UHPLC-MS). This technique permits the detection and quantification of essentially every small phenolic molecule with a molecular mass below 1500 Da at concentrations in the micromolar to millimolar range. An important bottleneck in the field of phenolic profiling is the structural characterization of detected compounds in the UHPLC-MS chromatogram. The identification of unknown compounds might be revealed via NMR upon compound purification (Nakabayashi *et al.*, 2009). However, purification is often impossible because of co-eluting impurities, compound degradation or extremely low compound concentrations. In such cases, retention times and MS data (including high mass accuracy MS, from which the chemical formula can be determined, and MS^n spectra) are often the only means for resolving the structure.

Concerning the identification of alternative monomers, the study and identification of metabolites that make up the lignome are of special interest. The lignome is defined as the ensemble of all phenolics for which the biosynthesis is co-regulated with lignin biosynthesis and includes the oligolignol (small lignin polymers) pool (Morreel *et al.*, 2010a). The oligolignol pool in model species such as poplar and Arabidopsis has been well characterized (Morreel *et al.*, 2004a, 2010b). Although the main lignome components have been elucidated in these species, the identity of many compounds, which are potentially useful as alternative monomers, remains unknown. Important progress in the structural elucidation of oligolignols was made when it was discovered that various lignin units (i.e. G and S) and linkage types (i.e. β-*O*-4, β-5 and β-β) show characteristic MS fragmentation patterns (Morreel *et al.*, 2010a,b). Given the characteristic MS fragmentation, an algorithm was developed allowing the sequencing of oligolignols, that is, determining the order of the monolignols in the polymer, as well as the bonds connecting them (Morreel *et al.*, 2010a). This approach greatly simplifies the identification of oligolignols and aids the discovery of alternative lignin units. The algorithm allowed the complete sequencing of 36 of the 134 oligolignols present in

poplar xylem extracts; for the remaining compounds only partial sequences were obtained because of the presence of unidentified units (bonds or monomers) (Morreel *et al.*, 2010a). These partially identified compounds are therefore prime targets for further structural elucidation, which can help in finding new monomers and elucidating their coupling propensities. For example, an MS fragmentation pattern associated with the presence of an arylglycerol unit was frequently encountered when sequencing small poplar lignin polymers (Morreel *et al.*, 2010a). Four G(β-*O*-4)S(β-5) $G^{glycerol}$ isomers were detected. Intriguingly, the arylglycerol was always β-5-, but never β-*O*-4-coupled, suggesting that the glycerol monomer has different coupling propensities than coniferyl alcohol. An algorithm for annotating oligolignols is particularly useful for studying the lignome of plants that are genetically modified to synthesize alternative monomers and for sequencing the oligolignol fraction of DHPs made with alternative monomers. Thus far, the lignome of many taxa such as grasses and gymnosperms remains largely unexplored and may contain new types of alternative lignin monomers that may prove to be useful for modifying lignin properties.

Our Scifinder-based search resulted in over 160 reported plant metabolites that fit the criteria to be used as alternative monomers (see Section IV). Nevertheless, as only a fraction of the secondary metabolites are currently known (Hadacek, 2002; Saito & Matsuda, 2010), many more alternative monomers are waiting to be identified together with their biosynthetic pathways. Structural elucidation of plant phenolics is thus of prime interest. Given the above-mentioned limitations for isolating and identifying phenolics, many researchers have invested heavily in annotating metabolites by MS^2 spectral information, often leading to the construction of species-specific databases (Moco *et al.*, 2006; Farag *et al.*, 2007; Böttcher *et al.*, 2008; Matsuda *et al.*, 2009, 2010, 2011). In addition to the use of MS^2 spectral libraries, information about other phenolic metabolites within the same plant extract can be used for structural elucidation. This is because the substrates and products of well-known enzymatic conversions, such as methylation or glycosylation, are often observed in the same chromatogram (Iijima *et al.*, 2008). To search for these 'candidate substrate product pairs' (CSPPs), an algorithm was developed that uses the input of mass differences corresponding to particular enzymatic conversions (K. Morreel *et al.*, unpublished). This tool is expected to significantly accelerate the identification of alternative monomers and their pathway intermediates.

VII. Phenolic pathway engineering towards alternative monolignols

According to the strategy outlined in this paper, pathway engineering in lignocellulosic biomass crops should entail the biosynthesis of the most promising alternative monomers and their incorporation in the lignin polymer. In some cases, the cloning of a biosynthetic pathway might need to be combined with the mutation of (an) endogenous gene(s) to re-direct the flux into the newly established pathway to reduce the competition for substrates at the level of biosynthesis of the monomers, their transport and their polymerization.

Pathway engineering of lignocellulosic biomass crops implies the cloning of biosynthetic pathways. Unfortunately, for most of the candidate alternative lignin monomers depicted in Fig. 5, the biosynthetic enzymes are unknown. Nevertheless, the occurrence of these candidate alternative lignin monomers in plant species opens up the possibility of elucidating their biosynthetic pathways. The few exceptions whose pathways have been well elucidated are sinapate esters (**29S**, **35S**, **78SS**) (Nair *et al.*, 2004; Niggeweg *et al.*, 2004; Fraser *et al.*, 2007; Sinlapadech *et al.*, 2007; Liu, 2010) and rosmarinic acid (**49CC**) (Ellis & Towers, 1970; Matsuno *et al.*, 2002; Petersen *et al.*, 2009; Liu, 2010). Recently, enzymes capable of synthesizing ferulate conjugates (**7AG**) have been obtained (Wilkerson *et al.*, 2011), and the putative *p*-coumarate analog in grasses has been identified (Withers *et al.*, 2012). In addition, an enzyme that is likely to catalyze the first committed step towards phenylbutanoids has been isolated from rhubarb (*Rheum palmatum*; Abe *et al.*, 2001). This enzyme, benzalacetone synthase (BAS), belongs to the polyketide synthase family and makes the phenylbutanoid *p*-hydroxybenzalacetone from malonyl-CoA and *p*-coumaric acid (Shimokawa *et al.*, 2010). For some other alternative lignin monomers that are found in plants, an enzymatic activity might be conjectured. For example, for the biosynthesis of monomer conjugates linked by ester or amide functionalities, at least one acylation reaction might be suggested to take place. Acyltransferases, currently known to be responsible for the transfer of hydroxycinnamic acids to recipient molecules, fall into three families. They all generally depend on CoA- or glucose-conjugated phenylpropanoids (Steffens, 2000; D'Auria, 2006; Kang *et al.*, 2006; Liu, 2010) and they produce *O*- or *N*-linked products depending on the specific acyltransferase. Hydroxycinnamoyl-CoA-dependent BAHD (BEAT, AHCT, HCBT and DAT – after the first four members characterized) superfamily acyltransferases and the 1-*O*-acylglucose ester-dependent serine carboxypeptidase-like proteins (SCPLs) catalyze both *O*- and *N*-transacylation, while the general control non-depressible 5 (GCN5)-related N-acyltransferases (GNAT) catalyze only *N*-transacylation (Milkowski & Strack, 2004; Vetting *et al.*, 2005; D'Auria, 2006). Searching BAHD, SCPL and GNAT-coding expressed sequence tags (ESTs) in the tissue where the alternative lignin monomer is also found might therefore result in candidate genes that can further be tested via feeding assays and other reverse genetic tools. In addition, phenolic profiling can also help in pathway elucidation, as it might identify molecules that are structurally related to alternative lignin monomers. Given a pool of structurally related molecules, enzymatic conversions can be proposed for their synthesis, which again enables the search for ESTs.

The exact subcellular location of the biosynthesis and storage of most candidate alternative monomers is unknown. Several monomers might be recognized *in planta* as 'true' monomers, because of their structural similarity to them, and therefore transported by default to the cell wall. That this is indeed the case is proven by the increase of ferulic acid, 5-hydroxyconiferyl alcohol and cinnamaldehydes in the lignin of CCR-, COMT- and CAD-deficient plants, respectively (Atanassova *et al.*, 1995; Ralph *et al.*, 2001b; Kim *et al.*, 2003; Morreel *et al.*, 2004b; Sibout *et al.*,

2005; Dauwe *et al.*, 2007; Mir Derikvand *et al.*, 2008), and by the incorporation of hydroxybenzaldehyde and hydroxybenzoate in HCHL engineered plants (Eudes *et al.*, 2012). However, alternative lignin monomers that bear a glucose, malate or quinate moiety are more prone to be stored in the vacuole, both in their endogenous species and when produced in lignocellulosic biomass crops (Wink, 1997; Bartholomew *et al.*, 2002; Dean *et al.*, 2003). This subcellular localization has been proven via vacuolar isolation followed by phenolic profiling for sinapoyl glucose (**35S**) and sinapoyl malate (**29S**) in *Raphanus sativus* and for chlorogenic acid (**28C**) in *Catharanthus roseus* (Sharma & Strack, 1985; Ferreres *et al.*, 2011). For these monomers, rerouting to the cell wall is needed.

An important issue with genetic engineering of the lignin pathway is that plants with perturbed lignification often show unwanted pleiotropic effects (Chen & Dixon, 2007; Li & Chapple, 2010; Li *et al.*, 2010; Vanholme *et al.*, 2010b; Gallego-Giraldo *et al.*, 2011b). Although the exact cause(s) of these effects is not fully known, they have been attributed to impaired water transport (Jones *et al.*, 2001; Franke *et al.*, 2002), altered levels of dehydrodiconiferyl alcohol glucoside (DCG) that might influence cell proliferation and expansion (Abdulrazzak *et al.*, 2006; Li & Chapple, 2010), the lack of cell wall integrity or the release of elicitors that trigger responses at the level of gene expression (Li & Chapple, 2010; Seifert & Blaukopf, 2010), and the accumulation of phenylpropanoid pathway intermediates or products (Vanholme *et al.*, 2010b; Gallego-Giraldo *et al.*, 2011a). In this respect, the recent finding that reduced growth in Arabidopsis plants with impaired lignin biosynthesis is correlated with increased concentrations of salicylic acid is of particular interest (Gallego-Giraldo *et al.*, 2011a; Lee *et al.*, 2011). While HCT-down-regulated Arabidopsis plants were severely affected in growth, reducing salicylic acid biosynthesis by crossing in a mutation in the isochorismate synthase (ICS) gene could partially restore plant growth (Gallego-Giraldo *et al.*, 2011a). This is an interesting observation because it shows that growth defects are (partly) caused by the unintended accumulation of phenolics, and that these defects can be (partly) complemented by redirecting the flux. How the phenolic steady state will react to pathway engineering will differ case by case and is difficult to predict. In the ideal case, the pathway engineering would only alter lignin biosynthesis and not plant growth and performance, but if it does, possible solutions are to engineer the new pathway in specific cell types only; for example, the use of fiber-specific promoters might restrict the altered lignin to be deposited in supportive but not conductive tissues. Alternatively, suppressor screens might be carried out to identify the molecular causes of the pleiotropic effects and to identify the genes to mitigate the unwanted phenotypes (Halpin, 2010).

It has been noted that the main building blocks of lignin seem relatively conserved over different taxonomic clades (Weng & Chapple, 2010). However, this observation may result from the fact that only few plant species have been investigated with current analytical (including powerful NMR) methods able to detect and identify 'novel units' and often the novelty is missed by failing to recognize that the lignins may have derived from, for example, acylated monolignols. There are numerous examples of lignins, in both 'natural' and transgenic plants, being partially to substantially derived from monomers that are not the three classical monolignols; these include the conjugates: monolignol acetates in many plants, monolignol *p*-coumarates in all grasses, monolignol *p*-hydroxybenzoates in *Salix*, *Populus*, and *Palmae*; but also catechol-type monomers such as caffeyl and 5-hydroxyconiferyl alcohol, double-bond-reduced monomers such as dihydroconiferyl alcohol, the hydroxycinnamaldehydes, etc. – as all noted in Sections I and III (Ralph *et al.*, 1997, 2008a; Lu & Ralph, 1999; Lu *et al.*, 2004; Morreel *et al.*, 2004a; Vanholme *et al.*, 2008; Stewart *et al.*, 2009; Ralph, 2010; Chen *et al.*, 2012; del Río *et al.*, 2012). It is currently not clear whether these nontraditional lignins confer different properties to the plants that have made evolution to select these lignins over the classical HGS-type lignins, but they clearly have their niches. It also will remain to be tested how plants respond to the incorporation of high amounts of the proposed alternative monomers in terms of growth and development, biotic and abiotic stresses and mechanical properties. In cases such as introducing readily cleavable bonds into the backbone of the lignin polymer by utilizing various ester conjugates as monomer replacements, however, it may be sufficient to introduce these at reasonably low levels that will not greatly alter the structural properties but will render the polymer dramatically easier to cleave into smaller fragments during pretreatments. Evidence suggests that modifications such as some of the ones proposed here could lead to significantly improved plant materials (from the point of view of biomass conversion) and that some of the suggested modifications are likely to prove game-changing for plant cell wall utilization. Interesting times are ahead as researchers strive to introduce some of these new traits into important biomass crops.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Notes S1 The compounds, the plant species in which the compounds are found, and references used in the compilation of Fig. 5.

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Supporting Information Notes S1

(Full legend to Fig. 5 in the main text) Compounds found within the plant kingdom that (potentially) satisfy criteria for alternative lignin monomers. (a) Monomers that have already been authenticated or implicated in lignification. (b) Alternative monomers that, upon incorporation into the lignin polymer, potentially make the lignin more susceptible to biomass pretreatment.

Naming conventions. Aromatic ring units are all phenols, invariably *p*-hydroxy-aryl units here. Substituents are labeled R/R´ for the 'first' aromatic ring, X/X´ for the second, Y/Y´ for the third and Z/Z´ for the fourth. In all cases, the descriptor notation uses the compound number followed by the defined rings, in the order described as: **H** (*p*-hydroxyphenyl), **G** (guaiacyl), **S** (syringyl), **C** (caffeyl), **F** (5-hydroxyguaiacyl), or **L** (gallyl) with **A** being used for any or all (generic) units. The convention is illustrated with compounds **7** as follows…

7HH – *p*-coumaryl *p*-coumarate (R = R´ = X = X´ = H)
7GH – coniferyl *p*-coumarate (R = OMe, R´ = X = X´ = H)
7SH – sinapyl *p*-coumarate (R = R´ = OMe, X = X´ = H)
7GG – coniferyl ferulate (R = OMe, R´ = H, X = OMe, X´ = H)
7SG – sinapyl ferulate (R = R´ = OMe, X = OMe, X´ = H)
7SS – sinapyl sinapate (R = R´ = X = X´ = OMe)
7AH – general hydroxycinnamyl *p*-coumarate (R,R´ = H/OH/OMe; X = X´ = H)
7SA – sinapyl general-hydroxycinnamate (R = R´ = OMe; X,X´ = H/OH/OMe)
… etc.

Where necessary, other variable substituents are used (P, T) and designated directly in the structure caption; a variable single-or-double bond in some structures is designated as such directly in the structure caption (here) also.

1 The hydroxycinnamyl alcohols: **1H**, **1G**, and **1S** are the traditional monolignols:
1H *p*-Coumaryl alcohol: the precursor of H-units in lignin (Boerjan *et al.*, 2003),
1G Coniferyl alcohol: the precursor of G-units in lignin (Boerjan *et al.*, 2003),
1S Sinapyl alcohol: the precursor of S-units in lignin (Boerjan *et al.*, 2003),
1C Caffeyl alcohol: produces catechyl units (usually as benzodioxanes) in lignins (Wagner *et al.*, 2011),
1F 5-Hydroxyconiferyl alcohol: produces 5-hydroxyguaiacyl units (usually as benzodioxanes) in lignins of COMT-deficient plants (Lu *et al.*, 2010),
1L 3,4,5-Trihydroxycinnamyl alcohol: would produce gallyl units in lignins (this compound has not been found in plants and is hypothetical only).
2 Dihydro-hydroxycinnamyl alcohols: dihydroconiferyl alcohol **2G** is found in softwoods, particularly in a loblolly pine *cad* mutant (Ralph *et al.*, 1997).
3 Arylpropane-1,3-diols: in particular, **3G** is found in softwoods (Ralph *et al.*, 1999).
4 Arylglycerols: previously thought to derive from β-ethers during lignin isolation, but may be monomers derived from hydroxycinnamyl alcohols via H_2O_2 (Hammel *et al.*, 1994; Morreel *et al.*, 2010).
5 Hydroxycinnamyl acetates: Monolignol conjugates directly used in lignification in kenaf (Ralph, 1996), some grasses, and many other plant species (and apparently as minor components in hardwood lignification (Ralph, 1997).
6 Hydroxycinnamyl *p*-hydroxybenzoates: Monolignol conjugates directly used in lignification especially in *Salix*, *Palmae* and *Populus* species (Lu *et al.*, 2004; Morreel *et al.*, 2004; Stewart *et al.*, 2009). Conjugate **6SH** appears predominant.
7 Hydroxycinnamyl *p*-hydroxycinnamates: coniferyl *p*-coumarate **7GH**, sinapyl *p*-coumarate **7SH** and possibly *p*-coumaryl *p*-coumarate **7HH** are used directly in lignification in all (C3 and C4) grasses (Lu & Ralph, 1998). Coniferyl ferulate **7GG** is found in *Rhizoma chuanxiong* (Kong *et al.*, 2006; Li *et al.*, 2006), Apiaceae: *Angelica sinensis* (Zschocke *et al.*, 1998) and *Lomatium californicum* (Chou *et al.*, 2006).
8 Hydroxycinnamoyl tyramines conjugates: N-*trans*-feruloyl tyramine **8G** is found in tobacco (Negrel & Martin, 1984) and other *Solanaceae* (Turnock *et al.*, 2001; Liu *et al.*, 2011; Sun *et al.*, 2011).
9 Hydroxybenzaldehydes: vanillin **9G** and syringaldehyde **9S** are incorporated at low levels into most lignins (Boerjan *et al.*, 2003), **9H** is found in many plant species.
10 Hydroxycinnamaldehydes: particularly coniferaldehyde **10G** and sinapaldehyde **10S** are incorporated at low levels into most lignins (Boerjan *et al.*, 2003).
11 Hydroxycinnamic acids: *p*-coumaric acid **11H**, ferulic acid **11G**, sinapic acid **11S** and caffeic acid **11C** are found in many plant species (Clifford, 1999; Gonthier *et al.*, 2003), 5-hydroxyferulic acid **11F** accumulates in COMT-deficient plants (Dauwe *et al.*, 2007), 3,4,5-trihydroxycinnamic acid **11L** is found in *Aspalathus linearis* (Rabe *et al.*, 1994). Products of **11G** are found in the lignin of CCR-deficient plants (Dauwe *et al.*, 2007; Leplé *et al.*, 2007; Mir Derikvand *et al.*, 2008).
12 Flavanols: apigenin **12H**, chrysoeriol **12G**, tricin **12S** and luteolin **12C** are found in many plant species (Goławska *et al.*, 2010; Xu *et al.*, 2010; Wu *et al.*, 2011). Units of **12S** have been identified in grass lignins (Mouri & Laursen, 2011).
13 Hydroxycinnamate esters (P = polysaccharide): Ferulate-polysaccharide esters **13G** are found incorporated into grass lignins, possibly acting as lignin nucleation sites (Ralph *et al.*, 1995; Marcia, 2009).
14 5–5-Dehydro-hydroxycinnamates: **14GG** is a major ferulate dehydrodimer in grasses (Ralph *et al.*, 1994) and is also found in *Eleocharis dulcis* (Parr *et al.*, 1996).

15 β–5-Dehydro-hydroxycinnamates: **15GG** is a major ferulate dehydrodimer in grasses (Ralph *et al.*, 1994) and is also found in *Eleocharis dulcis* (Parr *et al.*, 1996).

16 β–*O*–4-Dehydro-hydroxycinnamates: **16GG** is a major ferulate dehydrodimer in grasses (Ralph *et al.*, 1994) and is the major ferulic acid dimer in *Eleocharis dulcis* (Parr *et al.*, 1996).
17 β–β-Dehydro-hydroxycinnamates (open form): **17GG** is a major ferulate dehydrodimer in grasses (Ralph *et al.*, 1994).
18 β–β-Dehydro-hydroxycinnamates (THF form): **18GG** is a major ferulate dehydrodimer in grasses (Ralph *et al.*, 1994).
19 β–β-Dehydro-hydroxycinnamates (cyclic form): **19GG** is a major ferulate dehydrodimer in grasses (Ralph *et al.*, 1994). Note that the 4–*O*–5- and another β–β-derived dehydrodimer (not shown) have also been found in grasses (Ralph *et al.*, 1994).
20 *p*-Hydroxybenzoic acids: *p*-hydroxybenzoic acid **20H**, vanillic acid **20G**, syringic acid **20S**, protocatechuic acid **20C** and gallic acid **20L** are common to many plant species (Chrzanowski *et al.*, 2011; Wang *et al.*, 2011; Skrzypczak-Pietraszek & Pietraszek, 2012).
21 Allylphenols: chavicol **21H** is found in *Ocimum basilicum* (Politeo *et al.*, 2007), eugenol **21G** is common to many plant species (Amma *et al.*; Dinh *et al.*, 2012; Singh *et al.*).
22 Propenylphenols: isoeugenol **22G** is common to many plant species (Vassão *et al.*, 2006).
23 Vinylphenols: 4-vinylphenol **23H** is made by yeast from **11H** (Buron *et al.*, 2011).
24 Lespedezate **24H** is found in *Lespedeza cuneata* (Shigemori *et al.*, 1990).
25 *p*-Hydroxyphenyl acrylic acids; **25H** is found in *Citrus medica* (He *et al.*, 1988).
26 2-*p*-Hydroxyphenylvinyl acetate **26H** is found in *Fraxinus uhdei* (Perez-Castorena *et al.*, 1997).
27 Guaiacyl butanol **27G** is found in *Zingiber cassumunar* (Masuda & Jitoe, 1995).
28 *p*-Hydroxycinnamoyl quinic acid conjugates: *p*-coumaroyl quinic acid **28H** is found in *Coffea* species (Alonso-Salces Rosa Maria *et al.*, 2009) and chlorogenic acid **28C** and feruloyl quinic acid **28G** are found in many plant species (Clifford *et al.*, 2007; Dauwe *et al.*, 2007; Jaiswal *et al.*, 2010).
29 *p*-Hydroxycinnamoyl malic acid conjugates: *p*-coumaroyl malic acid **29H**, caffeoyl malic acid **29C** and feruloyl malic **29G** are found in *Thunbergia alata* (Housti *et al.*, 2002) and *Phaseolus vulgaris* (Tanguy & Martin, 1972)**,** sinapoyl malic acid **29S** is found in Brassicaceae (Ruegger *et al.*, 1999; Do *et al.*, 2007).
30 *p*-Hydroxycinnamoyl tartaric acid conjugates: *p*-coumaroyl tartrate **30H** is found in *Vitis vinifera* (Ferrandino & Guidoni, 2010) and caffeoyl tartrate (caftaric acid) **30C** is found in *Vitis vinifera* (Gunata *et al.*, 1987) and *Syringodium filiforme* (Nuissier *et al.*, 2010).
31 *p*-Hydroxycinnamoyl glycerol conjugates: *p*-coumaroyl glycerol **31H** is found in *Zea mays* (Fenz & Galensa, 1989) and *Juncus effusus* (Shima *et al.*, 1991), 1-*O*-feruloyl glycerol is found in *Lilium auratum* (Shimomura *et al.*, 1987).
32 Dehydrosalidroside **32H** (P = Glc) is found in *Betula pendula* (Vainiotalo *et al.*, 1991) and *Ononis vaginalis* (1-β-D-glucopyranosyl-2-(4´-hydroxyphenyl)-ethene) (Abdel-Kader, 1997), **32H** (P = Rha) is found in *Joannesia princeps* (2-(4-hydroxyphenyl)ethenyl-α-L-rhamnopyranosides) (Achenbach & Benirschke, 1997).
33 *p*-Hydroxybenzoyl glucose: 1-*O*-galloyl-β-D-glucose **33L** is found in many plant species (Gómez-Caravaca *et al.*, 2011; Salem *et al.*, 2011; Puppala *et al.*, 2012).
34 *p*-Hydroxycinnamyl alcohol-γ-glucosides: triandrin **34H** is found in *Salix viminalis* (Minakhmetov *et al.*, 2002), isoconiferin **34G** is found in many plant species (Lewis *et al.*, 1988; Mei *et al.*, 2008; Lu *et al.*, 2012).
35 *p*-Hydroxycinnamoyl glucoses: *p*-coumaroyl glucose **35H** is found in *Ipomoea batatas* (Kojima & Villegas, 1984),1-feruloyl-β-D-glucose **35G** is found in *Nicotiana tabacum* (Runeckles & Woolrich, 1963), sinapoyl glucose **35S** is found in *Brassicaceae* (Milkowski *et al.*, 2004).
36 Dihydro-*p*-hydroxycinnamyl *p*-hydroxycinnamates; dihydroconiferyl ferulate **36GG** and dihydrosinapyl ferulate **36SG** are found in *Peganum nigellastrum* (Ma *et al.*, 2000) and *Relhania* species (Tsichritzis & Jakupovic, 1990), dihydrosinapyl *p*-coumarate **36SH** is found in *Eremanthus glomeratus* (Bohlmann et al., 1981), dihydrosinapyl caffeate **36SC** is found in *Relhania* species (Tsichritzis & Jakupovic, 1990), dihydro-*p*-coumaryl caffeate **36HC** is found in *Cassinia* and *Ozothamnus* species (Wollenweber *et al.*, 2008).
37 Petasiphenol **37CC** is found in *Petasites japonicum* (Iriye *et al.*, 1992).
38 Solargin I **38SG** (P = Glc-Rha), solargin II **38SC** (P = Glc-Rha), solargin III **38SG** (P = Glc-Rha-Rha) and solargin IV **38SC** (P = Glc-Rha-Rha) are found in *Solenostemma argel* (Kamel, 2003).
39 Angiferulate **39GG** is found in *Angelica sinensis* (Deng *et al.*, 2006).
40 ***p*-**Coumaroyl hydroxydimethoxy phenyl propanone **40SH** is found in *Sasa quelpaertensis* (Sultana & Lee, 2009).
41 Cimiracemate B **41CG** (R=H) and cimiracemate D **41CG** (R=H) are found in *Cimicifuga racemosa* (Chen *et al.*, 2002).
42 1**-**Methyl-3-(4´-hydroxyphenyl)-propyl caffeate **42HC** and 1-methyl-3-(3´,4´-dihydroxyphenyl)-propyl caffeate **42CC** are found in *Zuccagnia punctate* (Svetaz *et al.*, 2004).
43 Agatharesinol **43HH** is found in *Cryptomeria japonica* (Imai *et al.*, 2006a; Imai *et al.*, 2006b) and *Sequoiadendron gigantea* (Henley-Smith & Whiting, 1976), sequosempervirin B **43HG** and sequosempervirin C **43HS** are found in *Sequoia sempervirens* (Zhang *et al.*, 2005) and metasequirin D **43GG** is found in *Metasequoia glyptostroboides* (Dong *et al.*, 2011).
44 Imperanene **44GG** is found in *Imperata cylindrica* (Matsunaga *et al.*, 1995).

45 Diarylheptanoids **45HH** and **45HC** are found in *Curcuma* species (Kaewamatawong *et al.*, 2009; Li J *et al.*, 2010).
46 Yateresinol **46HH** is found in *Libocedrus yateensis* (Erdtman & Harmatha, 1979) and *Cryptomeria japonica* (Takahashi *et al.*, 1983).
47 Galanganol B **47HH** is found in *Alpinia galanga* (Kaur *et al.*, 2010).
48 Nepetoidin B **48CC** is found in *Plectranthus caninus* (Lukhoba *et al.*, 2006).
49 Isorinic acid **49CH** is found in *Anthoceros agrestis* (Vogelsang *et al.*, 2006) and *Helicteres isora* (Satake *et al.*, 1999) and rosmarinic acid **49CC** commonly found in species of the Boraginaceae and the subfamily Nepetoideae of the Lamiaceae (Petersen *et al.*, 2009).
50 Hydroxycinnamoyl tyrosines: caffeoyl-*N*-tyrosine **50CH** is found in *Coffea canephora* (Alonso-Salces R. M. *et al.*, 2009), deoxyclovamide **50HH** and clovamide **50CC** are found in *Theobroma cacao* (Sanbongi *et al.*, 1998).
51 **51GG** is found in *Ehretia obtusifola* (Iqbal *et al.*, 2005).
52 4-Hydroxy-3-methoxyphenyl ferulate **52GG** is found in *Hypericum hookeranum* (Wilairat *et al.*, 2005).
53 *p*-Hydroxyphenethyl ferulate **53GH** (T=H) is found in *Angelica sinensis* (Deng *et al.*, 2006) and *Sida spinosa* (Darwish & Reinecke, 2003), decursidate **53GH** (T=OH) is found in *Peucedanum decursivum* (Kong & Yao, 2000; Yao *et al.*, 2001).
54 Calebin A **54GG** is found in *Curcuma longa* (Park & Kim, 2002).
55 *p*-Coumaroyl feruloyl methane **55GH** is found in *Curcuma longa* (Gupta & Ghosh, 1999).
56 2**-**feruloyl piscidic acid **56HG** (T=H), 2-feruloyl fukiic acid (cimicifugic acid A) **56CG** (T=H), 2-caffeoyl piscidic acid **56HC** (T=H), caffeoyl fukiic acid **56CC** (T=Me) and cimicifugic acid G **56CC** (T=H) are found in *Cimicifuga* species (Takahira *et al.*, 1998; Nuntanakorn *et al.*, 2006).
57 Sebestenoid A **57GGG** is found in *Cordia sebestena* (Dai *et al.*, 2010).
58 Sebestenoid B **58CC** is found in *Cordia sebestena* (Dai *et al.*, 2010).
59 Salvianolic acid H **59CCC** is found in *Salvia cavaleriei* (Zhang & Li, 1994).
60 Boehmenan C **60GGG** (single bond) and boehmenan D **60SGG** (single bond) are found in *Ochroma lagopus* (Paula *et al.*, 1995). **60GGG** (single bond), **60SGG** (single bond) and boehmenan K **60GGH** (double bond) are found in *Hibiscus cannabinus* (Seca *et al.*, 2001), boehmenan X **60GHG** (single bond) is found in *Durio carinatus* (Rudiyansyah *et al.*, 2010).
61 Methylcedrusin *p*-coumarate ((7*R*,8*S*)-3´-*O*-methylcedrusin 9-*p*-coumarate) **61GH** is found in *Larix olgensis* (Yang *et al.*, 2005).
62 **(+)-**Lariciresinol 9´-caffeinate **62GC** (T=H), (-)-7-hydroxylariciresinol 9´-*p*-coumarate **62GH** (T=OH) and lariciresinol 9´-*p*-coumarate **62GH** (T=H) are found in *Larix olgensis* (Yang *et al.*, 2005).
63 Carolignan E **63GGGG** (single bond, T=H), carolignan X **63GFGH** (single bond, T=H) and carolignan Y **63GFGH** (single bond, T=Me) are found in *Durio* species (Rudiyansyah *et al.*, 2010). Carolignan F **63GSGG** (single bond, T=H), carolignan K; **63GGHG** (double bond, T=H) and **63GGGG** (single bond, T=H) are found in *Hibiscus cannabinus* (Silva *et al.*, 2002), carolignan M **63GLGG** (single bond, T=H) is found in *Sambucus williamsii* (Yao *et al.*, 2005), dadahol A **63GSHH** (double bond, T=H) and dadahol B **63GGHH** (double bond, T=H)) are found in genus *Artocarpus* (Hakim, 2010).
64 Carolignan H **64GGG** (single bond) is found in *Hibiscus cannabinus* (Silva *et al.*, 2002).
65 Hanultarin **65GGG** is found in *Berberis amurensis* (Park *et al.*, 2009) and *Trichosanthes kirilowii* (Moon *et al.*, 2008; Lee *et al.*, 2011) and (-)-(2*R*,3*R*)-1-*O*-feruloyl-8,8´-bisdihydrosiringenin **65SSG** is found in *Hypericum petiolulatum* (Zhao *et al.*, 2009).
66 Diferuloyl secoisolariciresinol **66GGGG** is found in *Antidesma membranaceum* (Buske *et al.*, 1997), *Penthorum chinense* (Zhang *et al.*, 2007) and *Betula* species (Fuchino *et al.*, 1995). 9,9´-*O*-di-(E)-sinapoyl-meso-dimethoxysecoisolariciresinol **66SSSS** and 9,9´-*O*-di-feruloyl-meso-5,5´-dimethoxysecoisolariciresinol **66SSGG** are found in *Lindera obtusiloba* (Lee *et al.*, 2010).
67 Salvianolic acid A **67CCC** is found in *Salvia miltiorrhiza* (Lai *et al.*, 2011).
68 Caffeoyl *p*-coumaroyl tartaric acid **68CH** (Mulinacci *et al.*, 2001), caffeoyl feruloyl tartaric acid **68CG** and chicoric acid **68CC** are found in *Cichorium* species (Mulinacci *et al.*, 2001; Shaikh *et al.*, 2010). **68CC** is also found in *Syringodium filiforme* (Nuissier *et al.*, 2010), and **68GG** and **68CG** are found in *Echinacea angustifolia* (Becker & Hsieh, 1985).
69 di-*p*-Hydroxycinnamoyl glycerol conjugates: 3-*O*-Caffeoyl-1-*O*-feruloyl glycerol **69GC** (T=H), 1,3-*O*-dicaffeoyl glycerol **69CC** (T=H) and 3-*O*-caffeoyl-1-*O*-*p*-coumaroyl glycerol **69HC** (T=H) are found in *Tillandsia streptocarpa* (Delaporte *et al.*, 2006), 1-*O*-*p*-coumaroyl-3-*O*-feruloyl glycerol **69HG** (T=H) is found in *Tillandsia streptocarpa* (Delaporte *et al.*, 2006), *Asparagus offiinalis* (Zhouxuan *et al.*, 2009), *Sparganium stoloniferum* (Shirota *et al.*, 1996) and *Lilium* species (Luo *et al.*, 2012), 1,3-*O*-diferuloyl glycerol **69GG** (T=H) is found in *Lilium henryi* (Shimomura *et al.*, 1988) and *Sparganium stoloniferum* (Shirota *et al.*, 1996) (the structural related 1-*O*-*p*-coumaroyl-2-*O*- feruloyl glycerol, 1,2-*O*-diferuloyl glycerol and 2-*O*-*p*-coumaroyl-1-*O*-*p*-feruloyl glycerol are found in *Lilium henryi* (Shimomura *et al.*, 1988)). Lasiocarpin A **69HH** (T=H), lasiocarpin B **69HG** (T=H) and lasiocarpin C **69GG** (T=H) are found in *Populus lasiocarpa* (Asakawa *et al.*, 1977).
70 Isolariciresinol *p*-coumarate **70HGG** is found in *Larix olgensis* (Yang *et al.*, 2005).
71 Caffeoyl dihydrocaffeoyl quinic acid **71HH** (T=H, single bond) and salicornate **71HH** (T=Me, double bond) are found in *Salicornia herbacea* (Kim *et al.*, 2011).
72 1,3-Dicaffeoyl quinic acid (Cynarine) **72CC** is found in *Cynara* species (Trajtemberg *et al.*, 2006; Sałata & Gruszecki). Homologues are found in many plant species, e.g., the 1,5-homologue (caftaric acid) is found in

Asteraceae (Slanina *et al.*, 2001; Binns *et al.*, 2002), the 4,5- homologue is found in *Pteris multifida* (Harinantenaina *et al.*, 2008) and the 3,5- homologue in *Artemisia gmelinii* (Könczöl *et al.*, 2012), many homologues are also found in *Ilex paraguariensis* (Jaiswal *et al.*, 2010; Hussein *et al.*, 2011).

73 (Epi)catechin **73C** is found in *Dimocarpus longan* (Sudjaroen *et al.*, 2012), **73C** and (epi)gallocatechin **73L** are found in *Camelia sinensis* (Hilal & Engelhardt, 2007; Song *et al.*, 2012) and *Theobroma cacao* (Payne *et al.*, 2010).

74 Epigallocatechin gallate **74LL**, epicatechin gallate **74CL**, epicatechin 3-*O*-(3´-*O*-methyl) gallate **74CF** and epiafzelechin gallate **74HL** are found in *Camelia sinensis* (Manir *et al.*, 2012).

75 (-)-epigallocatechin 3-*O*-*p*-coumaroate **75LH** is found in *Camelia sinensis* (Manir *et al.*, 2012).

76 Flavonol glycosides like kaempherol glycosides **76H** (P=Gly) and quercetin glycosides **76C** (e.g. hyperoside (P=Gal)) are found in many plant species (Bravo, 1998; Monagas *et al.*, 2006; Segawa *et al.*, 2006).

77 Astragalin 2″-gallate **77HL**, and the homologues astragalin 6″-gallate and astragalin 2″,6″-digallate are found in *Loropetalum chinense* (Romussi & Sancassan, 1983), astragalin 6´´-gallate is also found in *Quercus ilex* (Romussi & Sancassan, 1983), quercetin-3-β-D-galactopyranoside gallates (galloyl hyperin) **77CL** is found in Euporbiacea (Nahrstedt *et al.*, 1974; Li R *et al.*, 2010).

78 Disinapoylglucose **78SS** is found in Brassicaceae (Baumert *et al.*, 2005; Ferreres *et al.*, 2007) and *Raphanus sativus* (Dahlbender & Strack, 1984).

79 Dehydroacteoside **79CC** (P=H) and isodehydroacteoside **79CC** (P=Rha) are found in *Monochasma savatieri* (Yahara *et al.*, 1986).

80 **80GG** is found in *Alpinia speciosa* (Masuda *et al.*, 2000).

81 3,6´-*O*-diferuloylsucrose **81GG** is found in *Lilium henryi* (Shimomura *et al.*, 1988).

82 Calceolarioside A **82CC** (P1=H, P2=H,T=H) is found in *Calceolaria hypericina* (Capasso *et al.*, 1993) and *Fraxinus* species. (Chen *et al.*, 2009), syringalide C **82CG** (P1=Rha, P2=H,T=H) is found in *Syringa vulgaris* (Kikuchi *et al.*, 1988), leucosceptoside A **82HG** (P1=Rha, P2=H,T=H) is found in *Leucoseptrum japonicum* (Miyase *et al.*, 1982), cistanoside D **82GG** (P1=Rha, P2=H,T=H) is found in *Cistanchis herba* (Kobayashi *et al.*, 1984), betonyoside A **82CG** (P1=Rha, P2=H,T=OH) is found in *Stachys officinalis* (Miyase et al., 1996), campneoside I **82CC** (P1=Rha, P2=H,T=OMe) and campneoside II **82CC** (P1=Rha, P2=H,T=OH) are found in *Campsis chinensis* (Imakura *et al.*, 1985), ilicifolioside 1 **82CC** (P1=Rha, P2=H,T=OEt) is found in *Acanthus ilicifolius* (Wu *et al.*, 2003), globusintenoside **82CC** (P1= Rha-Glu-feruloyl, P2=H,T=H) is found in *Globularia sintenisii* (Kırmızıbekmez *et al.*, 2004), buddleoside A **82CC** (P1=Rha, P2=Xyl-feruloyl, T=H) is found in *Buddleia lindleyana* (Lu *et al.*, 2005) and forsythoside C **82CC** (P1=H, P2=Rha, T=OH) and the methyl ether (S-suspensaside methyl ether) are **82CC** (P1=H, P2=Rha, T=OMe) found in *Forsythia* species. (Endo & Hikino, 1982; Cui *et al.*, 2010). Similar compounds are found *Monochasma savatieri* (Li *et al.*, 2012).

83 Gallotannins are found in many plant species (Barbehenn & Constabel, 2011) for instance, (1-*O*-(3-methoxy-4-hydroxyphenyl)-6-*O*-galloyl-β-D-glucopyranoside **83GL** and (1-*O*-(3,5-dimethoxy-4-hydroxyphenyl)-6-*O*-galloyl glucopyranoside **83SL** are found in *Laguncularia racemosa* (Shi *et al.*, 2010).

84 Acteoside **84LL** is found in *Plantago psyllium* (Li et al., 2005) and *Clerodendron* species (Nagao *et al.*, 2001).

85 Salicylic acid 2-*O*-β-D-(3´,6´-dicaffeoyl)-glucopyranoside **85CC** is found in *Merremia umbellate* (Yan *et al.*, 2010).

86 Gallotanins like penta-1,2,3,4,6-*O*-galloyl-β-D-glucose **86** are found in many plant species (Gross, 2008; Zhang *et al.*, 2009).

87 Corilagin, an ellagitanin found in *Punica granatum* (Nawwar *et al.*, 1994) and *Dimocarpus longan* (Sudjaroen *et al.*, 2012).

88 Newbouldioside B **88SHF** is found in *Newbouldia laevis* (Gormann *et al.*, 2006).

89 Newbouldioside C **89FHS** is found in *Newbouldia laevis* (Gormann *et al.*, 2006).

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