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# Lignin primary structures and dirigent sites

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Although lignin is the second most abundant plant substance in vascular plants, its mode of synthesis is still the subject of much debate. However, recent progress has provided crucial evidence to support the theory that lignin primary structure is controlled at the proteinaceous level. Evidence for control over lignin assembly has been demonstrated with the discovery of monomer-invariant aryl-*O*-ether linkages in lignins that upon alkaline cleavage release the corresponding monomers in equimolar amounts, regardless of monolignol composition. Current evidence would indicate that there are only a few native lignin primary structures, the entire sequences of which now need to be fully determined. A provisional mechanistic model is proposed to account for macromolecular lignin assembly through the participation of proteins harboring arrays of dirigent (monolignol radical binding) sites.

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## Introduction

Failure to probe in detail the biochemical processes involved in lignification in vascular plants ultimately led to an unproven working hypothesis that the cell-wall reinforcing lignins, nature's second most abundant organic plant substances in the terrestrial environment, were randomly assembled [1]. Even today, some researchers still tenaciously cling to, and extravagantly expand upon, such unproven notions [2••,3–5]. However, detailed biochemical studies [6,7•,8–10] and critical analyses of various transgenic/mutant plant lines modified in the monolignol/lignin-forming pathways [11••] in various plant species, are now systematically unraveling how these biochemical coupling processes are controlled. Furthermore, related studies of the control of phenoxy radical-radical coupling have led to the discovery of monolignol (radical) binding dirigent proteins (DPs) and established their role in lignan biosynthesis [12–18,19••]. This finding clearly has broader implications for the proteinaceous control of lignification.

In this review, we describe the precarious foundation of the lignin random assembly model and the unusual lengths taken to defend this model, while summarizing the growing evidence for proteinaceous control of lignin formation and the need for lignin sequencing.

## Aspects of lignin assembly

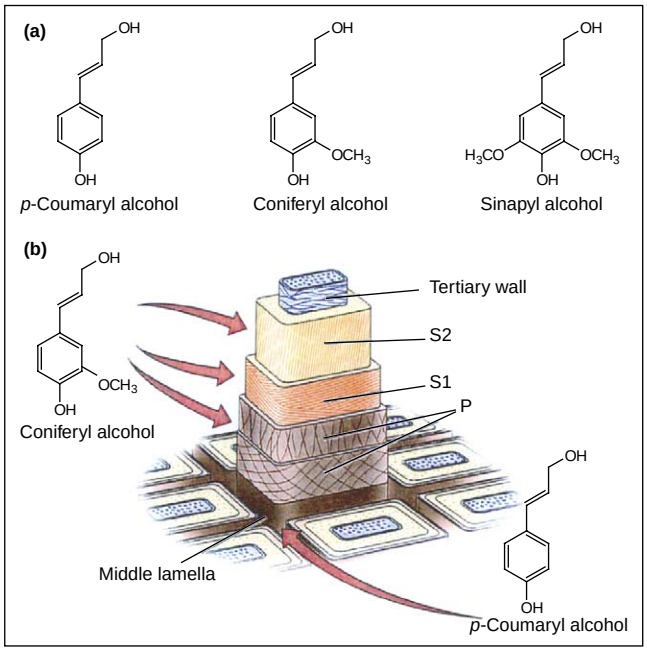
Lignins are mainly derived from the three monolignols: *p*-coumaryl, coniferyl and sinapyl alcohols (Figure 1a). The monolignols are differentially (temporally and spatially) targeted to discrete regions of various cell-wall types (Figure 1b) [20,21], where they are polymerized to afford wall-reinforcing biopolymers with distinctive biophysical properties (discussed later). The lignins are considered racemic, as evidenced by the analysis of dimeric fragments such as (±)-pinoresinols and (±)-syringaresinols (Figure 2) putatively released from lignin biopolymers [22,23]; this has to be taken into account when considering their assemblies.

### Unproven notions of random lignin assembly

In the context of modern plant biochemistry and biology, the unproven notion (actually a working hypothesis) that random phenoxy radical-radical coupling led to the formation of lignin biopolymers in vascular plants is an extraordinary incongruity spanning nearly five decades [1,2,5,24]. It was first put forward by Freudenberg [1,24,25], who supported this notion with claims that were later conclusively shown by others to be incorrect [26–31]. For example, these incorrect claims included the suggestion that synthetic and natural lignins were identical, that the hemiparasitic plant mistletoe formed host-dependent lignins, and that mosses contained lignins. Other false expectations used to support this random coupling hypothesis were also either later corrected or their limitations exposed [31–34].

One must temper some of the above criticism, with the recognition that the scientific tools available at that time were rather primitive when compared with today. Additionally, it must be remembered that there was no knowledge at that time of the existence of monolignol (radical) binding DPs that are able to dictate the outcome of phenoxy radical-radical coupling. Had this been known, it would have presumably tempered (if not eliminated) the emergence of the unproven notions of random lignin assembly. Indeed, at that time, work had only just begun on identifying the enzymes involved in monolignol formation [35,36].

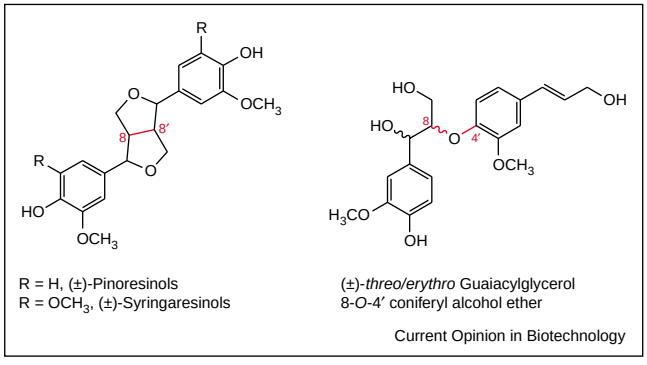
**Figure 1**




Monolignols and differential cell-wall targeting. **(a)** Chemical structures of monolignols and **(b)** telescopic representation of a conifer tracheid. *p*-Coumaryl alcohol is preferentially deposited in the middle lamella and coniferyl alcohol in the secondary wall [21]. (Figure 1b is copyrighted by the American Society of Plant Biologists and is reprinted with permission.)

Despite this precarious scientific foundation, the unproven claims of random coupling were taken to more extravagant levels. As recently as 1980, and despite lacking experimental verification, Glasser [37] speculated that 'lignin may, in fact, exist as one single molecule in its native environment' and that 'the lignin structure may never cease to grow'.

**Figure 2**




The chemical structures of dimeric racemic fragments reported to be released from lignin biopolymers. Radical-radical coupling linkages are shown in red.

More recently, others have sought to expand even further upon the unproven random (now termed 'combinatorial') coupling hypothesis [2••,3,4] to a level that is perhaps best characterized as irrational exuberance. For example, unproven assertions were made that a lignin molecule of relative molecular weight 21 500 could have as many as $10^{66}$ possible isomers, this being noted to approximate the number of atoms in the galaxy [2••,38], whereas a lignin chain composed of 20 monomers has over 17 billion possible isomers [2••]. Such unproven assertions beg the question as to how much longer such extravagant claims will continue to be made.

## A well-defined lignin primary structure

The total number of lignin primary structures throughout the plant kingdom is likely to be very small, differing from the above-mentioned numbers [2••,38] by a factor approaching either $10^{65}$ or at least a billion depending upon chain size. There are many reasons why lignins must be considered as having well-defined (i.e. protein-designated) primary structures, and some of these are given below.

### *The differential targeting of monolignols*

Lignin monomers are differentially targeted to discrete regions (lignin initiation sites) of various lignifying cell walls; for example, *p*-coumaryl alcohol is mainly targeted to the middle lamella and coniferyl alcohol to the secondary wall of the xylem elements (Figure 1b) [20,39–41]. Sinapyl alcohol, by contrast, is targeted to discrete regions in fiber-forming cell walls [42]. The physiological significance of this is apparently straightforward: differential targeting permits the construction of lignified cell walls with overall quite distinct biophysical properties. Such differences are, for example, readily apparent in the corresponding wall properties of fiber and xylem elements *in planta*.

### *Monolignol oxidation and guided assembly*

Monolignol (radical) targeting to distinct cell-wall regions has been proposed to involve, at the lignin initiation sites, both one-electron oxidation of the monomers with subsequent radical capture and lignin primary structure assembly guided by proteins harboring arrays of dirigent (monolignol radical) sites [13,16,17]; template polymerization is then considered to follow for additional replication [43,44].

There are several lines of evidence to support this. First, in terms of the one-electron oxidation of monolignols, we established that extensive downregulation of a tobacco peroxidase (TOB60) required for monolignol oxidation resulted in lignin reductions of ~50% [9]. Of course, the vasculature was significantly weakened, having much less lignin. Second, polyclonal antibodies raised against the (+)-pinoresinol-forming DP indicated the presence of putative dirigent epitopes in the regions where lignin initiation occurs [17,45]. We did not consider these

epitopes to be of the (+)-pinoresinol-forming DP, as this is a more specialized protein targeted to 8-8′ lignan (and not lignin) formation [12,16,18,19••]. Instead, we proposed that this represented the detection of protein(s) harboring array(s) of dirigent (monolignol radical) binding sites responsible for generating lignin primary structures (discussed later) [13,16,17,45]. Replication of the resulting primary lignin chain was then envisaged to occur through template polymerization, with preliminary evidence being reported from *in vitro* experiments [43,44].

### *Are lignins highly cross-linked three-dimensional biopolymers?*

Lignins are frequently described as highly cross-linked, three-dimensional biopolymers, whereas available experimental evidence suggests otherwise. For example, Dolk *et al.* [46] concluded that isolated lignins had cross-linking frequencies of less than 1 in 19 monomer units, whereas Mlynar *et al.* [47] reported there were likely to be none. Goring and colleagues also observed that isolated lignins were lamella-like [48], and Hatakeyama and coworkers reported that the molecular motion of lignins *in situ* in Japanese cedar (*Cryptomeria japonica*), camellia (*Camellia japonica*) and ginkgo (*Ginkgo biloba*) behaved much as if they were more linear polystyrene-like macromolecules [49].

Clearly, none of these data are in agreement with the unproven random coupling assembly hypothesis depicted as leading to highly cross-linked three-dimensional polymers, or even to the single molecule previously speculated by Glasser [37]. One difficulty in studying lignin primary structure is that lignin chains can self-associate very strongly [50]; this property, together with the relatively harsh treatment required for lignin-derived fragment solubilization [11••], has made analysis difficult by conventional means. Perhaps this explains why, until recently, there have been few attempts to analyze primary chain sequence information.

### *Do lignins have well-defined primary structures?*

Evidence is gathering rapidly that lignins have well-defined primary structures. In this context, it has long been known that the predominant inter-unit linkages in lignins are the 8-*O*-4′ bonds (Figure 2), the frequencies of which can reportedly vary from ~50% (gymnosperms) to ~80% (angiosperms) [15]. Convincing evidence for the existence of well-defined lignin structures recently emerged from the analysis of various lignified *Arabidopsis* lines, whether wild type, transgenic or mutant. When lignin deposition was monitored during the entire growth and development of *Arabidopsis* stems, a linear relationship was found to exist between the total amount of monomeric units, released through cleavage of the alkyl aryl ether linkages (i.e. the 8-*O*-4′ bonds) in the lignin macromolecule, and the overall amounts of lignin [10] (CL Cardenas *et al.*, unpublished). Most importantly, this linear relationship was apparently independent of lignin monomer composition: that is, whether the *Arabidopsis* lignin was guaiacyl-rich (coniferyl-alcohol-derived) or syringyl-rich (sinapyl-alcohol-derived). Yet, these distinct lignins would have been expected to give substantially different amounts of released monomers had random coupling occurred, as either one or two possible radical coupling sites at positions 3 and 5 had been removed and thus were not available for coupling. Figure 3 summarizes just one such trend in 8-*O*-4′ bond cleavage/monomer release for both the *Arabidopsis* wild type and mutants. It is evident that the total amounts of released monomers cleaved in this specific way are fully predictable at each and every stage of lignin formation, regardless apparently of either cell-type origin developmental stage or lignin monomer composition. As this accounts for by far the most abundant inter-unit linkage of the lignins (50–80%), as well as apparently signifying a monomer-invariant bonding pattern that is selectively cleaved, these data strongly indicate the designation of a well-defined primary structure or structures. Clearly, it will be instructive to ascertain if this same master curve holds for all plant species, or if there are variations on a species-by-species basis, and to identify the chemical nature of the primary sequence(s) involved.

## Are there further inconsistencies in the random coupling assembly model?

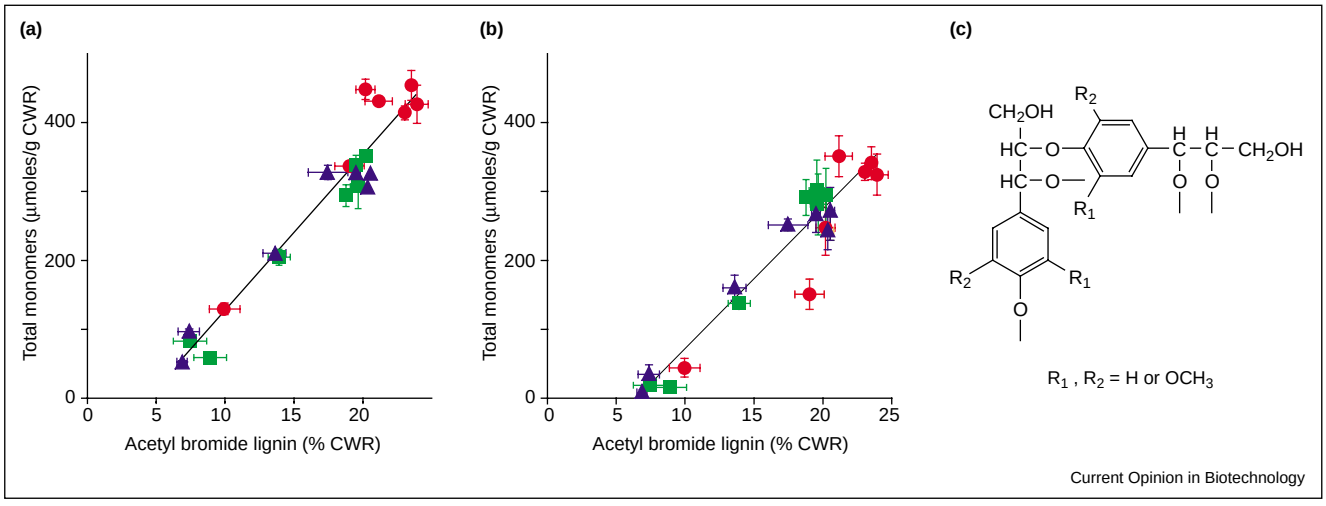
By 1970 [51], it was recognized that acidolysis (and subsequently thioacidolysis) of gymnosperm (spruce, *Picea abies*) lignins failed to release intact pinoresinol (or derivatives thereof) (Figure 2) in contradiction to earlier Freudenberg assertions [22]. This is in contrast to their facile, abundant, release from randomly coupled artificial or synthetic 'lignin' preparations. Yet, even today, no explanation for this apparent inconsistency in native lignin structure has been provided. More recently, $^{13}$C-NMR and quantitative HSQC (heteronuclear single quantum correlation) NMR spectroscopic analyses of spruce lignin-enriched preparations, isolated from 50–60 year old spruce wood stems, indicated that pinoresinol-like substructures were present, but only to the very low extent of ~2 units per 100 monomeric (lignin) residues [52]; these were speculated to be linked through the 5 position.

These putative 5-linked acid-resistant pinoresinol linkages in the lignin-enriched isolates have recently been proposed to represent obligatory linkages as part of a predetermined lignin primary chain [53••], whereas others describe their presence as a 'conundrum' [2••]. Clearly, such anomalies need to be fully explained (in a quantitative manner) and their significance determined as regards macromolecular lignin assembly and sequence.

## Lignin macromolecular assembly

The above data suggest a need for circumspection in current unproven proclamations about lignin random assembly. Emphasis now needs to be placed upon estab-

**Figure 3**

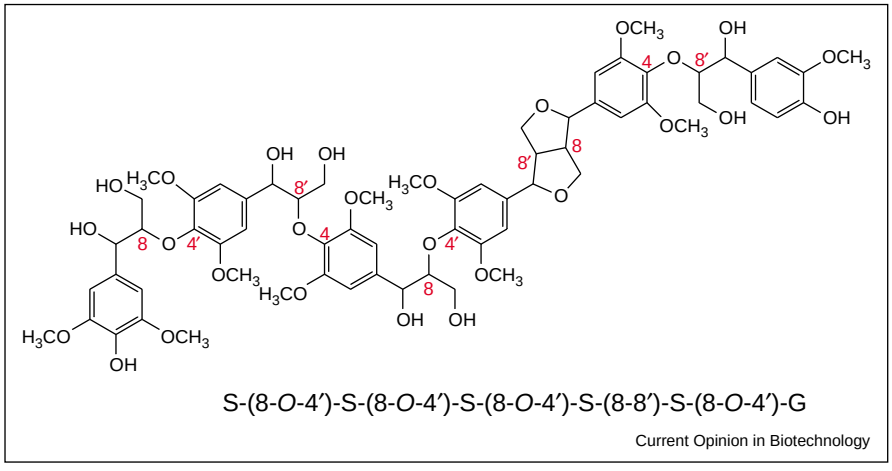



Comparison of yields of presumed lignin-derived monomeric fragments released predominantly from 8-*O*-4′ bond cleavage in lignins in intact *Arabidopsis* plant tissues during either **(a)** nitrobenzene oxidation or **(b)** thioacidolysis to that of total acetyl bromide lignin contents. Yields were measured for wild-type (red circles) and two *irx4* mutant lines repressed in their cinnamoyl CoA reductase genes with (green squares) or without trichomes (blue triangles). **(c)** The 8-*O*-4′ inter-unit lignin linkage. CWR, cell-wall residue of extractive-free plant stems.

lishing how the cell-wall proteins, harboring proposed (arrays of) dirigent (monolignol radical binding) sites, bind the corresponding monomers and how polymerization occurs to generate the resulting primary chains. Furthermore, full and unambiguous sequence data of the lignin primary chains present in distinct cell-wall regions and cell types need to be obtained. This is likely to be demanding work. However, preliminary work has already begun in this area, whereby a pentamer and two hexamers of coniferyl and sinapyl alcohol monomers (Figure 4) were reportedly sequenced from *Eucalyptus globulus* lignin-derived preparations [54••]. Even preliminary sequencing such as this, highlights the notion of billions or $10^{66}$ isomers in lignin as untenable.

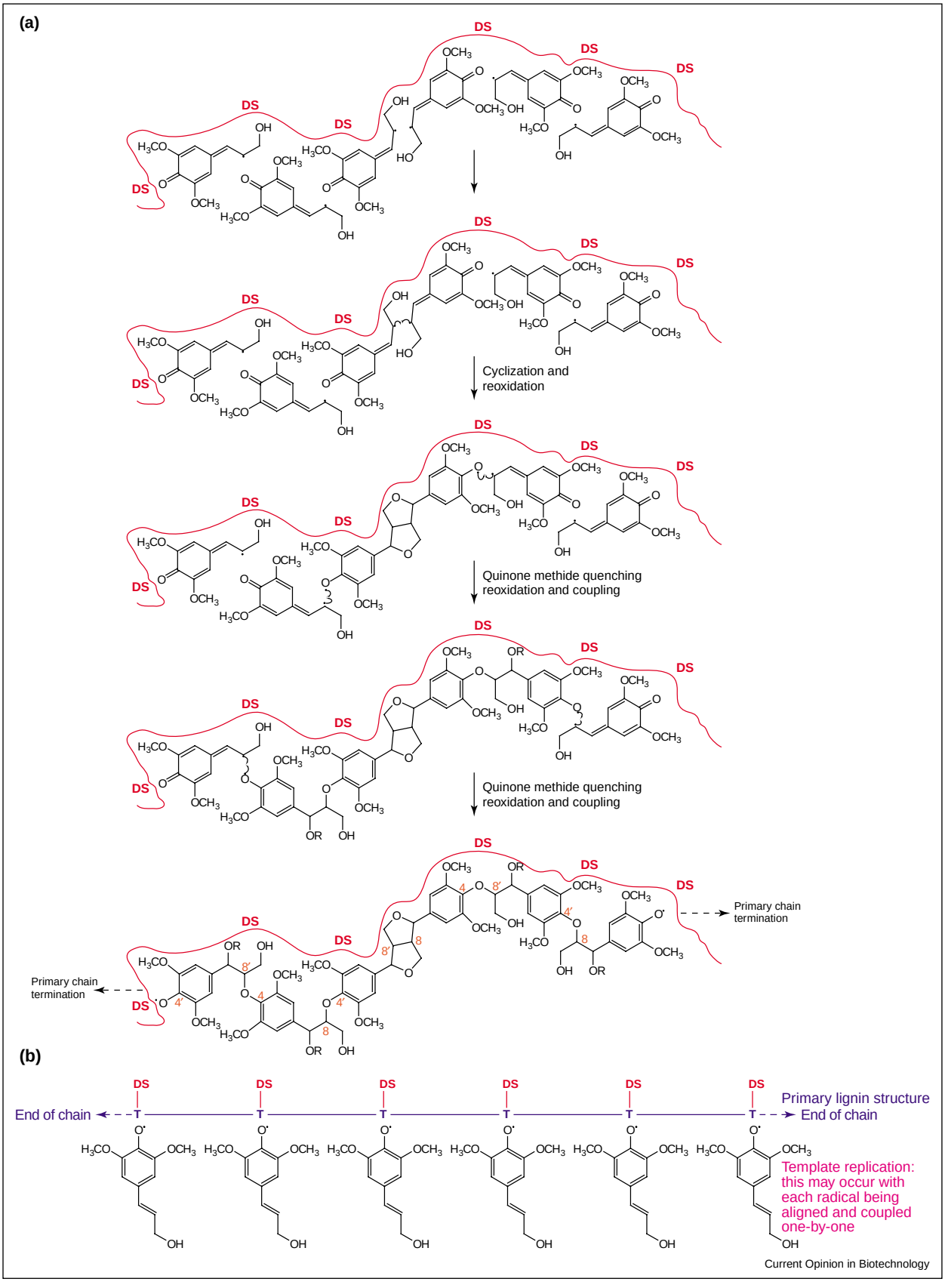
At this juncture, it is worth considering a provisional mechanistic basis for programmed lignin assembly leading to its primary structure(s). Using, for example, the putative *Eucalyptus* partial sequences, a tentative model can be proposed (Figure 5). In this specific example, the sinapyl alcohol moieties are targeted to their specific lignin initiation sites with the corresponding monomeric radicals (generated by oxidases) bound to these dirigent sites (DS) as shown. Using 8-8′ coupling as a putative

**Figure 4**




Putative primary sequence structure of a lignin-derived hexamer fragment from *Eucalyptus globulus* [54]. A pentamer of sinapyl alcohol monomers [S-(8-*O*-4′)-S-(8-*O*-4′)-S-(8-*O*-4′)-S-(8-8′)-S] and another hexamer S-(8-*O*-4′)-S-(8-*O*-4′)-S-(8-*O*-4′)-S-(8-*O*-4′)-S-(8-8′)-S] were also sequenced from *E. globulus* lignin-derived preparations. Radical-radical coupling linkages are shown in red.

**Figure 5**


(a)
DS
Cyclization and
reoxidation
Quinone methide quenching
reoxidation and coupling
Quinone methide quenching
reoxidation and coupling
Primary chain
termination
Primary chain
termination
(b)
End of chain
Primary lignin structure
End of chain
Template replication:
this may occur with
each radical being
aligned and coupled
one-by-one
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initiation point, the corresponding dimer (in either racemic or optically active form) is then re-oxidized to form a putative diradical species with chain growth continuing as shown. This continues until the primary chain is generated (ultimately in racemic form). Template polymerization, a widely documented process in the macromolecular assembly of both synthetic and natural polymers [55•], then follows. In this way, the cell wall 'organelle' maintains the assembly of the biopolymer(s).

## Can randomness in lignin structure now only be defended by strawmen?

Despite increasing the evidence for proteinaceous control of lignin primary structure, various 'strawman' models have been proposed in attempts to bolster the unproven random coupling hypothesis. These are briefly addressed below, as their basis needs to be considered fully.

### The lignin inter-unit linkage dilemma

Although cognizant of 'the dilemma of linkage specificity in lignin formation in plants', particularly with respect to the abundance of 8-*O*-4′ inter-unit linkages [5], Hatfield and Vermerris gave a quite different interpretation to our findings and hypotheses [13] as regards DPs, lignin assembly and protein harboring (arrays of) dirigent (monolignol radical) binding sites. They incorrectly assumed that we had suggested that some 50 or so different DPs, each specifying a specific (stereoselective) coupling mode, were present in lignifying cell walls, with the resulting dimers formed undergoing biopolymer assembly. Accordingly, an opinion article was devoted to why a strawman hypothesis, never proposed by ourselves, was unable to account for lignification. These researchers also advanced the notion that as only 8-8′ stereoselective coupling modes had been reported, there was no evidence for the control of 8-*O*-4′ coupling; however, this has since apparently been detected [56,57•], as had already been predicted earlier [14].

### Denying the existence of cell-wall proteins

A second paper published in 2002 [58•] attempted to deny the existence of cell-wall proteins. These researchers claimed that proteins, such as DP, could not possibly be present in cell walls, 'because lignified cell walls are so compact that proteins are too large to diffuse within them'. These researchers were apparently unfamiliar with established mechanisms of protein transport as the cell wall develops [59] and how cell-wall assembly is speculated to occur [60].

They had also incorrectly assumed that (+)-pinoresinol DP stereoselective coupling was required for lignin assembly. In its place, a model for lignification was proposed involving a diffusible $Mn^{III}$ shuttle into the cell wall for monolignol oxidation, in spite of no supporting evidence for the latter.

### Optical activity, lignins and template polymerization

The discovery of DPs and stereoselective coupling provided the first insight into how phenoxy radical radical coupling processes were controlled, even in the special case of (+)-pinoresinol formation [12]. The important point was that monolignol radical binding proteins had been discovered, and protein(s) harboring arrays of dirigent sites could thus provide the basis for the formation of lignin primary structure [13,16,17,45], including in racemic form. The latter was considered at the onset, as previous studies of lignin-derived dimeric fragments had been demonstrated to be racemic [23].

Yet, although the question of lack of optical activity in lignin formation had already been carefully addressed by ourselves [15,16,31] and others [44,53••], several proponents of random coupling re-analyzed the enantiomeric composition of various lignin-derived dimeric products; as expected they were racemic [61,62]. Apparently, it was not until 2004 that the same researchers [2••] recognized the specific role for stereoselective lignan dimer-forming DPs as being unrelated directly to lignin assembly. The same article also challenged the concept of template polymerization to account for biopolymer replication, in spite of several thousand papers describing such effects for a wide range of biopolymer and synthetic polymer applications; template polymerization is hardly a new concept.

### Challenging basic enzymology?

It has taken some decades for plant scientists to recognize that the various lignified and non-lignified cell walls in specific tissues and organs actually represent another type of 'organelle'. This is increasingly being demonstrated with the application of proteomics that has revealed the presence of hundreds of distinct cell-wall proteins of unknown physiological function (M-H Cho, unpublished) [63–65]. To most researchers, this should serve as a considerable impetus to establishing their individual functions. Such observations should also deter premature conclusions as to how cell walls are formed, and thus whether nature's second most abundant biopolymers are formed in a non-enzymatic manner.

This growing recognition of the diversity of proteins and enzymes in developing cell walls should also assist reconsideration of the unproven assertions of random lignin assembly. Yet, the view of enzymology expressed by these researchers begs some comment: that is, that 'Enzymes and proteins, including the dirigent proteins,

**(Figure 5 Legend)** Artistic rendition of a proposed programmed biochemical mechanism leading to lignin primary sequence assembly using the putative *E. globulus* lignin-derived substructure and protein(s) harbouring an array of dirigent sites. **(a)** Dirigent sites (DS) for substrate binding/polymerization and **(b)** template replication.

are optically active, have optically active binding sites, and produce optically active products' [2••]. Are these researchers really questioning, for example, whether proteins and enzymes can catalyze achiral product formation? Even the most cursory analysis of the monolignol-forming pathway dismisses that suggestion, given that beyond phenylalanine all of the enzymatic substrates and products are achiral [11••,15]. Furthermore, a failure to not understand and identify the roles of the plethora of proteins and enzymes involved in cell-wall assemblies, representing nature's most abundant store of organic carbon, would represent a departure from both 20th and 21st century science.

### Lignins do not have primary sequences?

The history of lignin structural analyses, which began on a precarious foundation, has resulted in an ever-changing depiction of provisional (i.e. uncertain) structures over the past five decades [1,37,66,67]. All of these structures are, however, liberally punctuated with artistic license and do not accurately depict lignins. Indeed, the original Freudenberg model [1] does not qualify as a precursor to a contemporary understanding of lignin structure. In large part, this situation has resulted from a focus on lignin substructure identification and on studies to estimate their relative gross frequencies, rather than focusing on biopolymer sequencing. By contrast, recent studies directed to radical-radical coupling involved in the lignan, ellagitannin and lignin biosynthetic pathways dictate a need to do otherwise. That is, they underscore the urgency for the dedicated elucidation of lignin primary structures and for the development of methods for this purpose. Thus, recent claims [2••] that there are no two identical lignin macromolecules in any plant of all plants and all species, that the number of lignin isomers is astronomical ($10^{66}$), and that lignins have no primary sequences, have no sound scientific basis. Even the most cursory consideration that a lignin-derived fragment (a hexamer) has already been sequenced [54••] might temper such claims.

## Conclusions

Recent studies, including the provisional partial sequencing of lignin-derived fragments, have obvious ramifications for our understanding of the control of lignin primary structure formation. Much effort now needs to be placed on characterizing, at the molecular level, the basis of the various (dirigent) monomer-binding sites for both lignin and lignan formation and on obtaining the primary sequences of the lignins being generated. It is clear that only through such systematic approaches at the biochemical level, will the remaining mysteries be clarified as to how nature's second most abundant organic substances are formed.

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