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Dirigent Proteins Guide Asymmetric Heterocoupling for the Synthesis of Complex Natural Product Analogues

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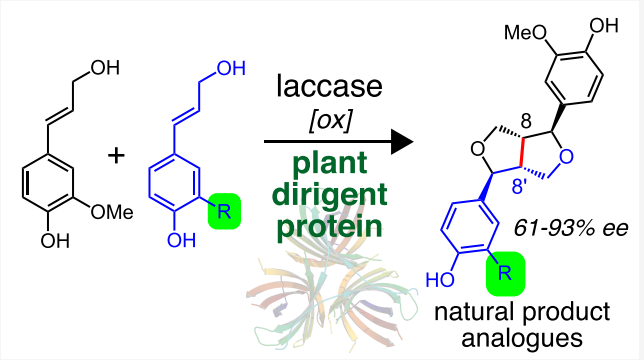
ABSTRACT: Phenylpropanoids are a class of abundant building blocks found in plants and derived from phenylalanine and tyrosine. Phenylpropanoid polymerization leads to the second most abundant biopolymer lignin while stereo- and site-selective coupling generates an array of lignan natural products with potent biological activity, including the topoisomerase inhibitor and chemotherapeutic etoposide. A key step in etoposide biosynthesis involves a plant dirigent protein that promotes selective dimerization of coniferyl alcohol, a common phenylpropanoid, to form (+)-pinoresinol, a critical C_2 symmetric pathway intermediate. Despite the power of this coupling reaction for the elegant and rapid assembly of the etoposide scaffold, dirigent proteins have not been utilized to generate other complex lignan natural products. Here, we demonstrate that dirigent proteins from *Podophyllum hexandrum* in combination with a laccase guide the *hetero*coupling of natural and synthetic coniferyl alcohol analogues for the enantioselective synthesis of pinoresinol analogues. This route for complexity generation is remarkably direct and efficient: three new bonds and four stereocenters are produced from two different achiral monomers in a single step. We anticipate our results will enable biocatalytic routes to difficult-to-access non-natural lignan analogues and etoposide derivatives. Furthermore, these dirigent protein and laccase-promoted reactions of coniferyl alcohol analogues represent new regio- and enantioselective oxidative heterocouplings for which no other chemical methods have been reported.



INTRODUCTION

Ubiquitous phenylpropanoid monomers in plants (termed monolignols) are the starting point for the biosynthesis of two very different classes of molecules: polymeric lignin, the structural component of the lignified plant cell wall, and lignans, bioactive dimeric natural products that typically contain multiple chiral centers and aryl ethers (see Scheme 1A for common modes of coupling). While nonselective polymerization of monolignol radical species results in the synthesis of lignin,[1] regio- and enantioselective coupling routes are thought to lead to diverse optically active dimeric lignans that accumulate in the tissue of both edible and medicinal plants.[2−4] One important example is etoposide, an anticancer drug that is semisynthetically produced from a lignan natural product, (−)-podophyllotoxin, found in *Podophyllum* spp. (Scheme 1A).

Etoposide and related drugs (etopophos and teniposide) act as reversible topoisomerase inhibitors by intercalating at the interface of the topoisomerase IIβ and DNA cleavage complex and stabilizing the double stranded break, thereby inducing apoptosis of cancer cells.[5] However, one liability of etoposide is the propensity of the drug to undergo demethylation by human liver enzyme CYP3A4 to generate a quinone with an altered activity profile (Scheme 1A, highlighted in green).[6−10] We hypothesized that an analogue of etoposide bearing E-ring substituents less prone to oxidation might limit unwanted hepatic metabolism.

Etoposide is currently manufactured using a semisynthetic approach that requires sourcing a related lignan, (−)-podophyllotoxin, from the medicinal plant *Podophyllum* spp. Recently, we established a complete biosynthetic pathway for 4′-demethyl (−)-epipodophyllotoxin, or etoposide aglycone (EA) through heterologous expression of previously known[11−16] and newly identified[17] *Podophyllum hexandrum* (*Ph*) genes in *Nicotiana benthamiana* (*Nb*). Given the modular nature of EA biosynthesis, we considered that analogous couplings of alternative monolignol building blocks could enable efficient modification of the etoposide E ring for the formation of analogues.

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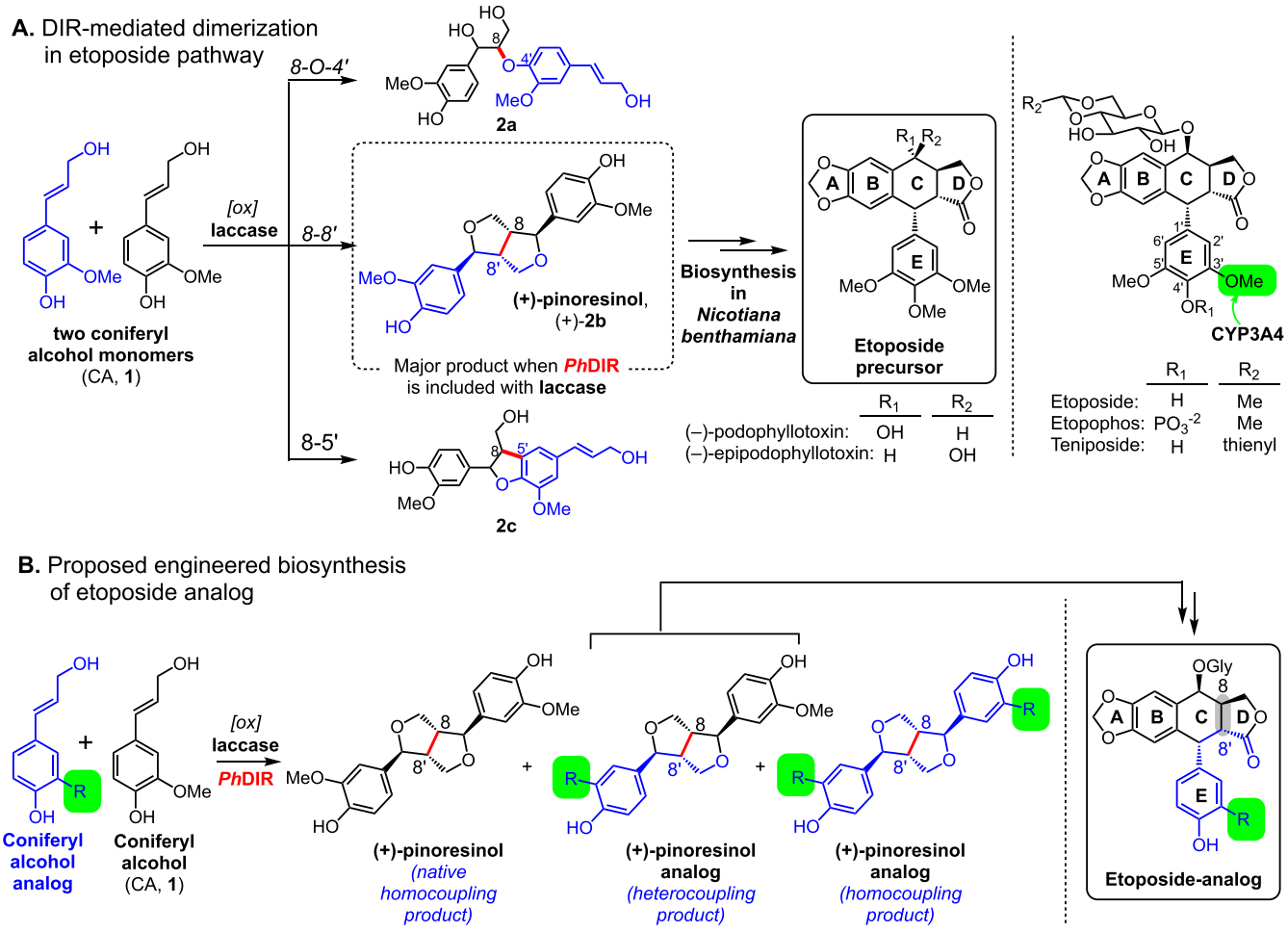
Scheme 1. Proposed Enantioselective Production of Hetero-(+)-Pinoresinol Analogues for Biosynthesis of Etoposide Analogues[a]



[a]**A.** Laccase oxidizes coniferyl alcohol and generates CA radical species that undergo three unique modes of coupling.[13,18,19] (Note on the coupling product naming convention: coupling products of the same monomer combination are collectively referred by their assigned compound number, and specific coupling modes are denoted by lowercase letters: **a**, 8-O-4′; **b**, 8-8′; and **c**, 8-5′.) In presence of *Ph*DIR, oxidized radical species of coniferyl alcohol form predominantly 8-8′ coupling products enantioselectively. The 8-8′ coupling product (+)-pinoresinol can be subsequently processed by enzymes involved in the etoposide aglycone (EA) biosynthetic pathway. The methoxy group of etoposide highlighted in green is prone to oxidation by human CYP3A4 and therefore is a target for modification using an engineered biosynthesis route. **B.** For each coupling mode (8-8′ coupling mode shown here; see Figure S1 for all other possible products), there are three possible types of coupling products when a mixture of two monomers is used: one heterocoupling and two distinct homocoupling products. We propose that the use of coniferyl alcohol analogues for (+)-pinoresinol analogue synthesis will lead to etoposide variants with modifications at the E ring.

The EA pathway involves convergent coupling of two coniferyl alcohol (CA) monomers for rapid complexity generation (route shown in Scheme 1A shows the native biosynthetic pathway that begins with dimerization of two CA monomers).[13,18,19] This key step involves oxidation of CA monomers to their corresponding radical species by a peroxidase or a laccase.[19,20] Dirigent proteins (DIRs), described from *Podophyllum* and other plant species, then direct the resulting radicals toward a remarkable stereoselective C_2 symmetric dimerization.[13,21,22] DIRs are not known to affect the rate of monolignol oxidation, nor are they thought to impact the net rate of dimerization. However, DIRs do change the relative ratio of different coupling products. In the absence of DIRs, monolignol radicals undergo spontaneous and nonselective couplings. This mechanism is supported empirically such that stereoselectivity is observed only in the presence of both an oxidizing agent (e.g., enzymatic or inorganic) and a DIR.[13,23] In contrast, multiple racemic coupling products are formed if only an oxidizing agent is present (for examples of other coupling modes, see Scheme 1A).

Comparison of crystal structures of enantiocomplementary dirigent proteins (a (+)-pinoresinol-forming DIR from *Pisum sativum* [PsDRR206][24] and a (−)-pinoresinol-forming DIR from *Arabidopsis thaliana* [AtDIR6][25]) suggests that the active sites exhibit contrasting conformations, likely serving as "molds" for the differential binding of two radical substrates for the *si-si* or *re-re* coupling, respectively. The ability of dirigent proteins to mediate a highly regio- and enantioselective coupling reaction to generate (+)-pinoresinol raises the intriguing possibility that these proteins could couple other monolignols, including those not typically found in nature. To date, (+)-pinoresinol-forming DIRs have exhibited high substrate specificity toward CA;[13] however, their ability to guide *hetero*coupling reactions (beneficial for the desired

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etoposide analogue generation described here) has not been explored. Stereoselectivity determined at this *Ph*DIR-mediated (+)-pinoresinol-forming step that funnels the supply of CA into the EA pathway is crucial, because several of the remaining pathway enzymes are known to be enantiospecific.[15,26] This proposed engineered route could yield valuable non-natural and *hetero*coupled lignan analogues that would enable specific modifications of the E ring of etoposide.

To determine the inherent substrate flexibility of DIRs and expand the utility of this direct complexity-generating reaction, we investigated whether DIRs could mediate stereoselective heterocoupling of various analogues of the natural substrate, coniferyl alcohol (Scheme 1B). Here, we show that *Ph*DIR is capable of promoting the coupling of synthetic and natural monolignols to form non-natural heterocoupled lignans. The resulting non-natural heterocoupling products retain the necessary stereochemistry to be further processed by the rest of the EA pathway enzymes for etoposide analogue biosynthesis. We anticipate the complex lignan scaffolds we produced will have direct utility in a biosynthetic route to etoposide analogues that are less prone to E-ring metabolism and formation of the reactive etoposide quinone (Scheme 1B, highlighted in blue).

RESULTS AND DISCUSSION

CA Analogue Design and Preparation. In choosing substrate designs to test for dirigent protein mediated coupling, we considered previous structure−activity relationships that had been established for the parent drug etoposide. For example, it has been shown that replacement of both the 3′- and 5′-methoxy groups by hydrogens in the E ring results in only a moderately decreased topoisomerase inhibition, and replacement of only the 5′-methoxy group by a hydrogen results in comparable topoisomerase inhibition as the parent compound.[27,28] In contrast, methylation of the 4′-hydroxy group resulted in the loss of drug activity, consistent with recent evidence from the ternary crystal structure of topoisomerase IIβ, DNA, and etoposide that suggests an important favorable interaction between the functional group and topoisomerase residue D479.[5] These data, in addition to the crystal structure of the DNA cleavage complex, suggested that activity would not be compromised by replacement of the E ring methoxy substituents with functional groups at the 3′ and 5′ positions anticipated to prevent the unwanted metabolism. With this hypothesis in mind, analogues of **1** (**3**-**11**) were prepared, containing phenolic substituents with varying degrees of electronegativity (Figure 1). Compounds **3** and **4** are naturally occurring monolignols produced by plants that differ by the number of methoxy substituents ortho to the phenol. Monomers **5**, **6**, and **7** were designed to explore significantly altered electronic states of the aromatic ring, relative to coniferyl alcohol, with the strongly electron-withdrawing trifluoromethoxy, fluorine, and bromine groups replacing the methoxy group, respectively. Additionally, the bromine group of **7** could serve as a synthetic handle for functionalization of the coupling product to diversify the product profile post-coupling. Compounds **8** and **9** replace the methoxy group with mildly electron-donating methyl groups. Both **10** and **11** retain the alkyl aryl ethers at the 3′ position. For **10**, the electronic state of the phenolic ring is expected to be altered by the presence of the 5′-fluorine substitution compared to **1**. Monomer **11** probes the effects of a bulkier alkoxy group substitution.

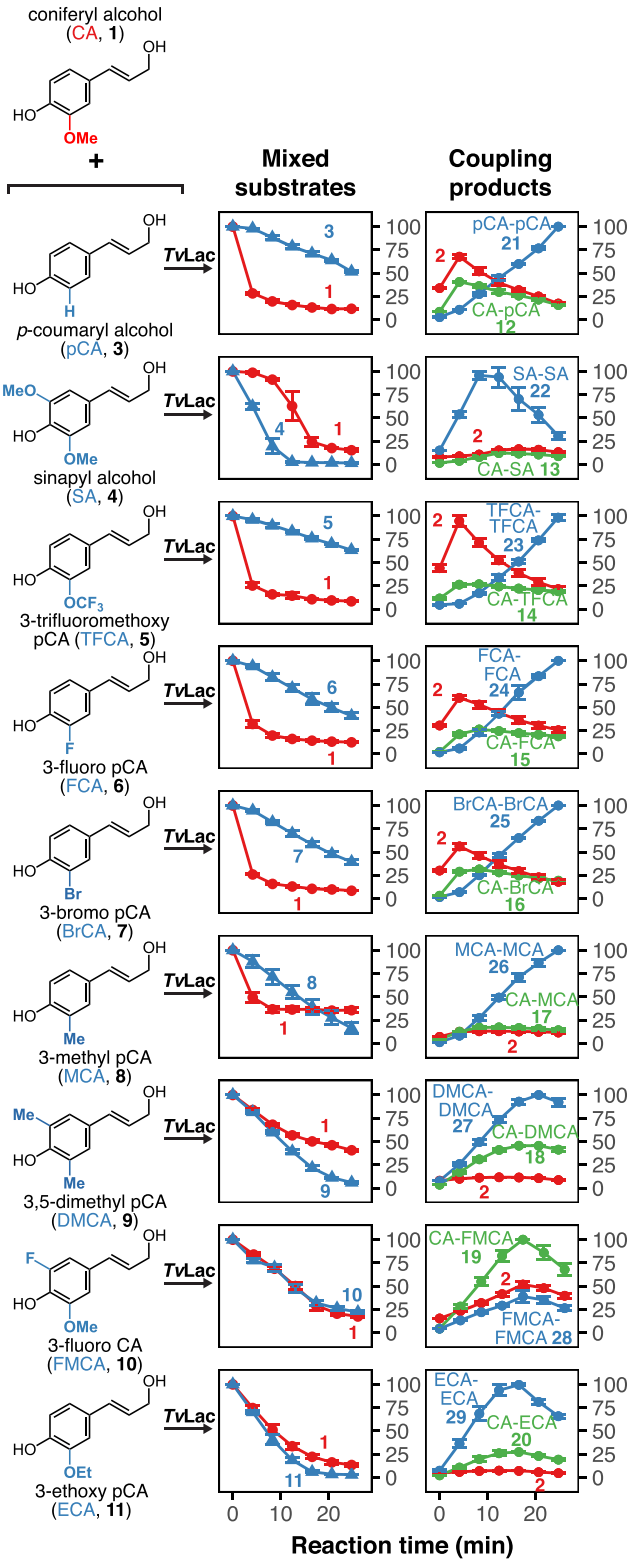

Figure 1. Relative rates of monolignol oxidation and coupling product formation catalyzed by *Tv*Lac. Each monomer is present as an equimolar mixture with **1**. **1**, **3**, and **4** are naturally occurring monolignols; all others are synthetic. For substrate oxidation, data points indicate the mean of peak integrations for the corresponding monomer species (m/z of $[M\text{-}H_2O+H]^+$) detected by LC-MS; EIC integrations are normalized to the peak integration at the initial reaction time points per species. For coupling product formation, data points indicate the mean of peak integrations for the corresponding

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Figure 1. continued

regioisomeric products detected by LC-MS (m/z of $[M\text{-}H_2O+H]^+$, a common ion to all regioisomeric products **a**–**c**); EIC integrations are normalized to the maximum product EIC integration observed over the entire time course; relative ratios of products are unchanged from the raw data. CA-CA (**2**) denotes the natural dimer, CA-X the heterocoupling product, and X-X the non-CA dimer, where X denotes the non-CA analogue. Error bars show standard deviations of data from triplicate reactions. Consumption of coupling products (e.g., **2** past ∼5 min) is likely due to nonselective *Tv*Lac activity resulting in oligomerization.

Oxidation Rates of Monolignol Analogues. Dirigent proteins are thought to harness reactive monolignol radicals and direct selective dimerization after an initial oxidation event. In these transformations, several oxidizing reagents, inorganic or enzymatic, have been used to catalyze the initial oxidation of coniferyl alcohol.[13,29] We anticipated that efficient heterocoupling between **1** and a different monolignol analogue, necessary for the E-ring modified etoposide analogue, would only occur if the oxidation rates of each were closely matched. To probe this, we first examined relative rates of oxidation of our panel of monomers in vitro, using a fungal laccase from *Trametes versicolor* (*Tv*Lac) that is known to catalyze single electron oxidation of naturally occurring monolignols (**1**, **3**, and **4**). Time-course of each substrate oxidation in the presence of **1** was investigated using liquid chromatography–mass spectrometry (LC-MS) to track substrate consumption during the reaction. For naturally occurring monolignols, **4** exhibited the fastest oxidation rate with *Tv*Lac, followed by **1**, and **3** with the slowest rate of oxidation (Figure 1, mixed substrates). As previously reported,[29] the decreasing trend of oxidative rates with decreasing number of methoxy substituents attested to the importance of electron-donating nature of the methoxy group for single-electron oxidation mechanism of laccases. In line with these observations, compounds **5**, **6**, and **7**, which lack the electron-donating aromatic substituents, exhibited noticeably slower oxidation rates in comparison to **1**. Compound **8**, with one methyl substituent, showed slower oxidation compared to **1**, while compound **9** with two methyl groups showed a more closely matched oxidation trend as **1**. More importantly, the rates of *Tv*Lac oxidation of **10** and **11** are sufficiently matched with that of **1**, and both of these monomers would be particularly useful for generating E-ring modified etoposide variants. As anticipated, in the corresponding coupling reactions, appreciable levels of the desired heterocoupling products accumulate over the course of the assay (Figure 1, coupling products [heterocoupling products in green]; see Figure S1 for enumeration of all possible coupling products). In contrast, slower-to-oxidize electron-deficient monolignols (relative to **1**) do not couple as efficiently with **1** to generate heterocoupling products. Taken together, a trend emerges where the relative rates of *Tv*Lac oxidation for the two monomers present in a mixture determine the identity of the major product formed. In this model, the collision of oxidized monolignols dictates identity of the coupling product, a process that is controlled by the relative concentration of these monolignol radical species. These data confirm that efficient formation of the desired heterocoupling products requires a close match in monomer oxidation rates.

Expression and Extraction of Functional *Ph*DIR. To test if dirigent proteins could influence the regio- and enantioselectivity of these heterocoupling reactions, we first needed sufficient supply of *Ph*DIR for in vitro assays. *Ph*DIR was expressed in *N. benthamiana* leaves using *Agrobacterium*-mediated transient expression as described previously.[17] While DIRs have previously been successfully expressed in heterologous hosts including *Pichia pastoris* and plant cell cultures,[30−32] for plant leaf expression, the apoplastic fluid wash method (APW) has been employed as a secretion and extraction method for heterologous protein expression in *N. benthamiana*, without the need for further processing of the extracted proteins.[25,33,34] Since many annotated dirigent proteins, including *Ph*DIR, are computationally predicted (by

Table 1. Reaction Yields of 8-5′ and 8-8′ Coupling Products and Enantiomeric Excess of (+)-Pinoresinol Analogues

monomers	coupling	oxidant	plant extract[a]	8-5′, **b**(μM)[b]	[%][c]	8-8′, **c**(μM)[b]	[%][c]	8-8′ regioisomeric content (%)[d]	% *ee*[e]
1	CA-CA (**2**)	*Tv*Lac	EV	250 ± 47	[50]	122 ± 26	[24]	33	2
			*Ph*DIR	57 ± 10	[11]	446 ± 29	[89]	89	88
1 + **8**	CA-MCA (**17**)	*Tv*Lac	EV	86 ± 11	[17]	33 ± 5	[7]	28	2
			*Ph*DIR	55 ± 2	[11]	83 ± 3	[17]	60	91
	MCA-MCA (**26**)	*Tv*Lac	EV	62 ± 14	[12]	15 ± 5	[3]	19	0
			*Ph*DIR	41 ± 3	[8]	11 ± 2	[2]	21	31
1 + **10**	CA-FMCA (**19**)	*Tv*Lac	EV	136 ± 19	[27]	24 ± 5	[5]	15	1
			*Ph*DIR	10 ± 2	[2]	145 ± 4	[29]	94	94
	FMCA-FMCA (**28**)	*Tv*Lac	EV	N/A[f]		49 ± 8	[10]	N/A[f]	−2
			*Ph*DIR	N/A[f]		129 ± 3	[26]	N/A[f]	82
1 + **11**	CA-ECA (**20**)	*Tv*Lac	EV	182 ± 12	[36]	<10[g]	[<2]	<5	0
			*Ph*DIR	54 ± 11	[11]	88 ± 16	[18]	62	89
	ECA-ECA (**29**)	*Tv*Lac	EV	168 ± 11	[34]	<10[g]	[<2]	<6	−1
			*Ph*DIR	147 ± 23	[29]	20 ± 10	[4]	12	47

[a]Plant extracts obtained with APW from leaves expressing empty vector (EV) or *Ph*DIR. [b]Reaction yield was estimated from a standard curve using EIC peak integration as analyzed by LC-MS on C18 chromatography. For **17** and **26**, standard curves of **2b** and **2c** were used to estimate yields. Error values are standard deviations of triplicates. [c]100% theoretical yield corresponds to 500 μM product generated from coupling of 500 μM each substrate (1 mM total substrate concentration). [d]8-8′ regioisomeric content was calculated by the following formula: [8-8′, **b**]/([8-8′, **b**]+[8-5′, **c**])*100%. [e]*ee*: enantiomeric excess of (+)-pinoresinol or analogue over (−)-pinoresinol or analogue, calculated using the EIC peak integration ratios on chiral chromatography, averaged from triplicate reactions. [f]Not applicable; 8-5′ coupling product was not observed with the fluorine substituent. [g]Below the limit of linearity of 10 μM.

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SignalP) to contain N-terminal signal peptides that direct the proteins to the apoplast, the extracellular space in plants,[35,36] we applied a similar apoplastic extraction strategy to the *N. benthamiana* leaves expressing *Ph*DIR without adding an extrinsic signal peptide and carried out activity assays of the protein without further purification. To control for different environmental factors such as salt concentration and presence of native *N. benthamiana* laccases and peroxidases, the APW protocol was performed in parallel using plants transformed with an empty vector (EV), and the resulting extracts were used as negative controls. To validate dirigent protein expression, **1** was subjected to oxidation by *Tv*Lac in the presence of plant extract from leaves expressing *Ph*DIR. As expected, on the basis of previously characterized activity of a (+)-pinoresinol-forming DIR,[13] the regioisomeric profile was nearly inverted compared to that in the absence of *Ph*DIR, with the (+)-pinoresinol (**2b**) regioisomeric content increasing from 33% (EV control) to 89% (*Ph*DIR extract) (Figure S2a and Table 1; reaction scheme shown in Scheme 1A).

Altered Regioselective Product Distributions Mediated by *Ph*DIR. With functional *Ph*DIR in hand, we next tested its capacity to direct the outcome of oxidative *hetero*coupling of CA and each of the alternative monomers shown in Figure 1. In our initial analysis of the reaction outcomes, we observed that the addition of *Ph*DIR to reactions catalyzed by *Tv*Lac did not alter the ratio of all homo- and heterocoupling products compared to *Tv*Lac alone (see Figure 2: unfilled bars in the graphs indicating all products from homocoupling, e.g., **2** and **21**-**29**, or heterocoupling, e.g., **12**-**20**). This observation is in accordance with previous literature findings that dirigent proteins do not possess catalytic activity; accordingly, the rate of formation of the three coupling product types is dictated only by the *Tv*Lac oxidation step.

We next quantified the impact of the *Ph*DIR addition on the distribution of regioisomers formed for each coupling type. This analysis revealed that the addition of *Ph*DIR significantly influences the regioselectivity of the heterocoupling reaction, shifting the major product to the 8-8′ regioisomer for heterocoupling of all monolignol analogues with **1** (Figures 2 and S2, filled, slash-patterned columns for products **12**-**20**; and Table 1). More specifically, the desired 8-8′ heterocoupling product **19b** comprised 94% of the quantified CA-FMCA heterocoupling products **19b**-**c** (6.2-fold increase compared to the negative control, i.e., reactions with *Tv*Lac and EV extract, at 15%). Similarly, in reactions with *Ph*DIR, **20b** constitutes 62% of the quantified CA-ECA heterocoupling products **20b**-**c** (~12-fold increase from <5% in control reaction). In addition, couplings of **1** with **3**-**9** each resulted in a similar trend of regioselectivity increase in heterocoupling reaction (Figures 2c,d and S2b−f for products **12**-**18**), revealing remarkable flexibility in the ability of *Ph*DIR to couple **1** to both natural and non-natural monolignols. These data show promise that the E ring modification of etoposide could be accomplished through a biosynthetic route from production of (+)-pinoresinol analogues. Moreover, in the context of plant metabolism, it is notable that **1**, **3**, and **4** are common and abundant metabolites found throughout the plant kingdom. Our in vitro results raise the possibility that naturally occurring optically active heterocoupled lignans, e.g., (+)-demethoxypinoresinol (CA-pCA 8-8′ heterocoupling product, **12b**) from *Gladiolus segetum*[37] and (+)-medioresinol (CA-SA 8-8′ heterocoupling product, **13b**) from *Eucommia ulmoides*,[38] originate with pinoresinol-like couplings catalyzed by an

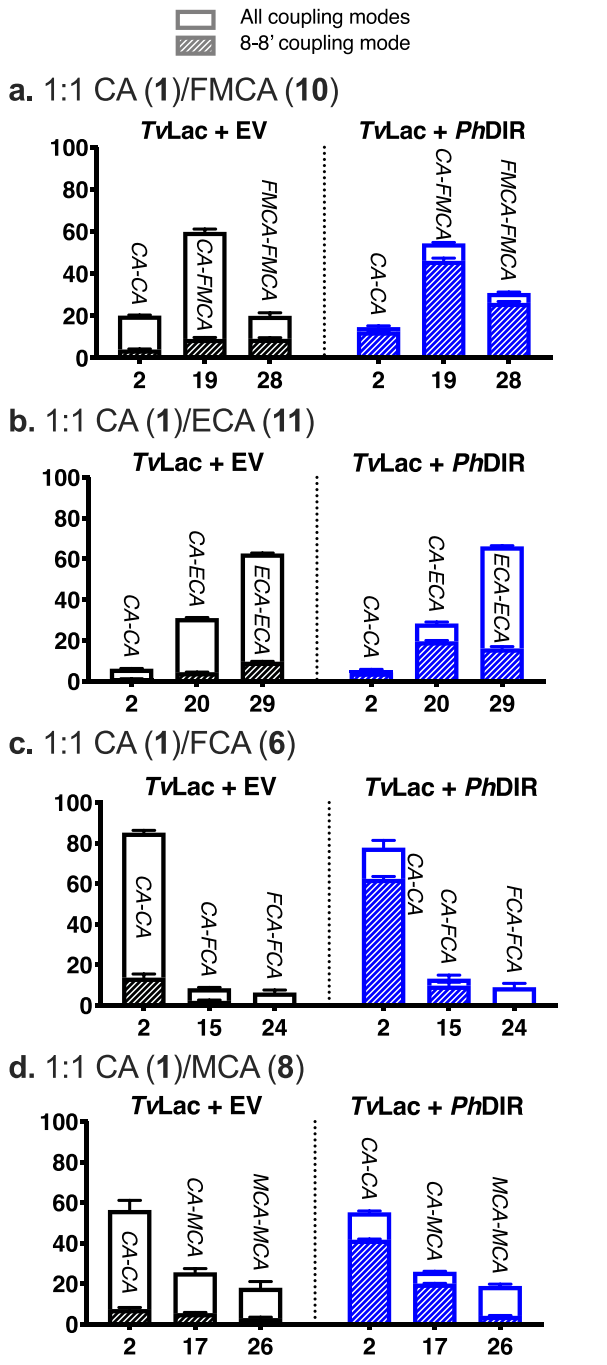

Figure 2. End-point coupling product distributions in the presence and absence of*Ph*DIR. Couplings of equimolar (a) **1** and **10**, (b) **1** and **11**, (c) **1** and **6**, and (d) **1** and **8**. Total content of the three coupling product types for each reaction was estimated by summing up peak integrations of the corresponding ion species (m/z of $[M\text{-}H_2O+H]^+$) detected by LC-MS, and each coupling product composition (type and coupling mode) was normalized to the total integrations. Values reflect only relative product concentrations to compare reactions. Colors indicate reactions carried out with empty vector control (black, on the left) or *Ph*DIR (blue, on the right) extracts. Filled, slash-patterned columns indicate levels of 8-8′ coupling products. Bar heights indicate the mean of triplicates, and error bars show standard deviations.

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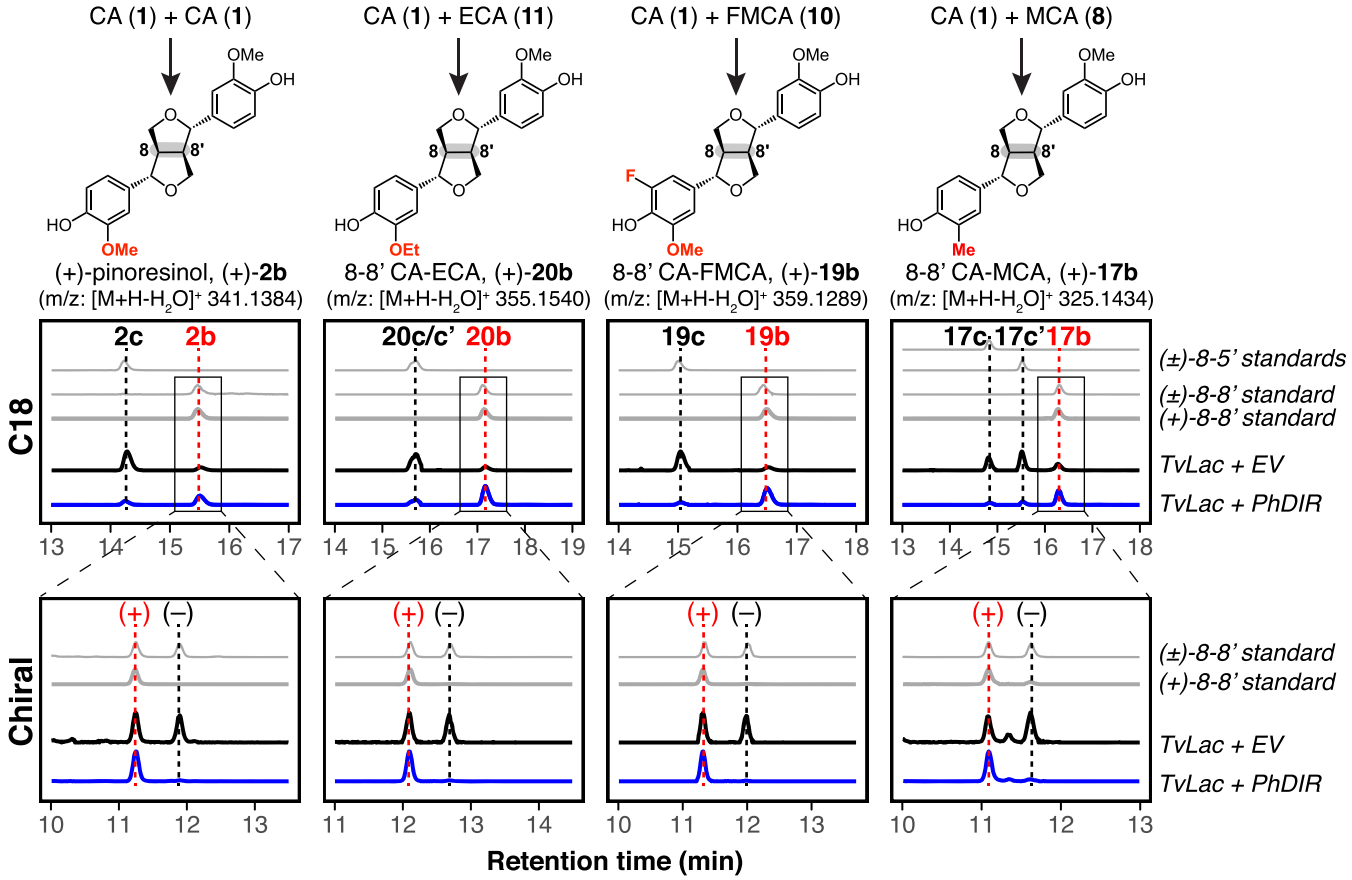

Figure 3. *Ph*DIR-mediated regio- and stereoselective heterocoupling of coniferyl alcohol and selected analogues. EICs as analyzed by LC-MS with C18 and chiral chromatography show separation of regioisomers and enantiomers, respectively, of end-point *Tv*Lac reaction products in the absence and presence of *Ph*DIR extracts (black and blue lines, respectively) from an equimolar substrate mixture. Authentic standards, purchased [(+)-**2b**] or purified from preparatory-scale reactions with and without *Ph*DIR (all other standards), are shown in gray. (For **17** and **20**, the **c**/**c′** suffix denotes the 5-8′/8-5′ regioisomeric coupling products.) Control reactions were supplemented with an equal volume of plant extracts transformed with an empty vector (EV). C18 traces are to scale, and chiral traces are normalized per reaction. Chromatographic peaks corresponding to the (+)-pinoresinol analogues (structures shown above) are denoted with red dotted lines, and their regioisomers (C18) and enantiomers (chiral) are denoted with black dotted lines. Relative stereochemistry assignments are supported by the identical ^{1}H NMR spectra of purified racemic 8-8′ coupling products isolated from the *Tv*Lac-only control reactions and enantioenriched 8-8′ coupling products from *Tv*Lac-*Ph*DIR reactions (Figure S11). Regioisomeric products are distinguishable based on MS/MS fragmentation pattern (Figure S12).

oxidase and mediated by dirigent proteins. However, the biosynthetic origins of these molecules have not been described to date.

Unlike the high regioselectivity observed for *Ph*DIR-mediated *hetero*coupling reaction, *homo*coupling of CA analogues was not as selective. Regioselectivity observed for homocouplings of **10** and **11** only moderately increased compared to the striking heterocoupling regioselectivity increase (e.g., ∼2-fold increase for **29b** vs ∼12-fold increase for **20b**). In the coupling reaction of **1** and **6**, the addition of *Ph*DIR did not increase the apparent percent composition of 8-8′ homocoupled product FCA-FCA, **24b** (Figure S6). More generally, we did not observe any increase in regioselectivity in the formation of non-natural dimers derived from **3**-**9** (Figures S3−S9). In line with these results, dirigent proteins have previously been shown to not impact the oxidative dimerization of **3** and **4**.[13] It is possible that the larger increase in regioselectivity observed in heterocoupling reaction compared to non-CA dimerization (when it occurs) can be explained by substrate specificity in the binding pocket of *Ph*DIR. Although speculative, *Ph*DIR binding pocket is thought to take two units of monolignol radicals to facilitate coupling; one hypothesis is that the pocket preferentially binds an initial CA monolignol but is tolerant of CA analogues as the second monomer.

Asymmetric Heterocoupling Guided by *Ph*DIR. Encouraged by the increased regioselectivity observed in the *Ph*DIR-mediated heterocoupling of CA with all monolignol analogues, we next investigated enantiomeric ratios of the heterocoupled 8-8′ products formed in our assays. In the dimerization of **1**, the native substrate of *Ph*DIR, the major product was confirmed to be (+)-pinoresinol with an enantiomeric excess (*ee*) >85% compared to <5% ee in control experiments without *Ph*DIR (Figure 3 and Table 1). Notably, for the heterocoupling products formed at the highest levels, e.g., **19b** and **20b**, we determined that the 8-8′ heterocoupling products formed selectively in the presence of *Ph*DIR were also produced in high enantioselectivity with 94% *ee* and 89% *ee*, respectively. In addition, heterocoupling products formed (albeit at lower levels) in the coupling reactions of **1** with each of **3**-**9** also proceeded with high enantioselectivity (71−93% *ee*) in the presence of *Ph*DIR (Table 2 and Figures S3−S9). These results reveal the remarkable flexibility of dirigent proteins for guiding the

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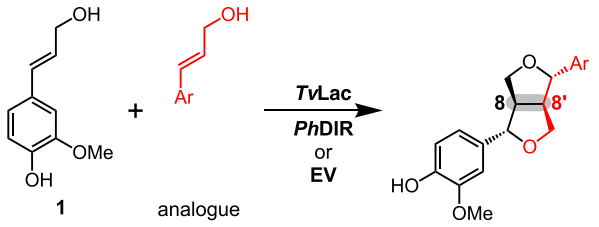
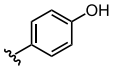
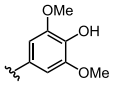
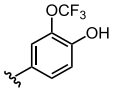
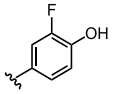
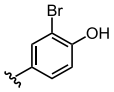
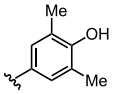
Table 2. Selective Heterocoupling Mediated by *Ph*DIR



analogue	product	Ar	plant extract[a]	yield (%)[b]	*ee* (%)[c]
3	**12b**		EV	<2	<5
			*Ph*DIR	4	77
4	**13b**		EV	3	<5
			*Ph*DIR	22	79
5	**14b**		EV	<2	<5
			*Ph*DIR	3	71
6	**15b**		EV	<2	<5
			*Ph*DIR	5	89
7	**16b**		EV	<2	<5
			*Ph*DIR	3	88
9	**18b**		EV	18	<5
			*Ph*DIR	60	93

[a]See Table 1 footnote. [b]See Table 1 footnote; reaction yield was estimated using the standard curve of **2b**. [c]See Table 1 footnote.

enantioselective formation not only of C_2 symmetric dimers but also for 8-8′ heterocoupling products bearing four contiguous stereocenters.

Relative to the 8-8′ heterocoupling products, we observed lower regio- and enantioselectivity for the formation of the corresponding non-natural 8-8′ homocoupling products in the presence of *Ph*DIR (Table 1 and Figure S10). Of these products, the highest *ee* was obtained for **28b** (82% *ee*), suggesting that *Ph*DIR is capable of accepting the monolignols that closely resemble **1** in structure (e.g., **10**). In line with this hypothesis, monomer **11**, bearing a bulky ethoxy substituent, was converted to its 8-8′ dimer (**29b**) with only modest selectivity in the presence of *Ph*DIR (47% *ee*), and homocoupling of **8**, with a methyl group substituent, showed only a modest increase in enantioselectivity in the presence of *Ph*DIR (31% *ee* for **26b**); in these reactions, the 8-5′ regioisomer was still the major product. Moreover, in the coupling of **1** with **3**-**7** and **9**, no enantioselectivity increase was observed for the homocouplings of the monolignol analogues (Figures S3−S9), further suggesting that the promiscuity of *Ph*DIR is specific to the asymmetric *hetero*coupling mechanism.

Optimization of Oxidation Reaction for Heterocoupling. For the formation of some of the desired pinoresinol analogues (e.g., **15b** and **17b**), yield was limited by the slow oxidation of the monolignol analogue relative to **1**. To address this, different reaction parameters were considered. In an effort to better couple the oxidation rates of monolignol substrates, we compared the activities of various oxidants: *Tv*Lac, horseradish peroxidase (HRP), and ammonium persulfate (APS). When we subjected an equimolar mixture of **1** and **6** to a free coupling reaction in the presence of each of these oxidants, we observed that the relative oxidation rate differences observed between **1** and **6** were similar regardless of the oxidant used (Figures 4a), where **6** was much slower to oxidize compared to **1**. We also examined the effect of varying pH on the reaction. Because the pK_a of the phenols vary depending on the substituent identity, we hypothesized that the pH of *Tv*Lac oxidation reactions could impact the relative rate of monomer oxidations. While *Tv*Lac activity was comparable at either pH 4 and the enzyme's optimal pH of 5.5, relative oxidation rates did not differ at different pH conditions (Figure S13).

As an alternative approach to improve heterocoupling product yields, we tested whether superstoichiometric amounts of **6** compared to **1** would result in higher accumulation of heterocoupled products **15** (and homocoupling products **24**) (Figure 4b,c and S14). While CA homocoupling products (**2**) remained as the major product, this strategy was effective for increasing the formation of **15** from the coupling of **1** and **6**. Altered stoichiometry was even more beneficial in the coupling of **1** and the methyl analogue **8** (Figure 4d) and enabled the product isolation described below. However, these yield improvements are maximal at a 1:3 ratio of **1** and **8**; at higher ratios, enantioselectivity begins to drop when DIR is included in the reaction (Figure S15). Additionally, increasing the amount of *Ph*DIR in the assay improved regioselectivity while the absolute yield of **17b** remained constant.

Preparatory-Scale Biosynthesis of Synthetic Lignan Analogues Using *Ph*DIR Plant Extracts. Careful optimization of in vitro *Tv*Lac-*Ph*DIR reactions allowed isolation and characterization of desired non-natural (+)-pinoresinol analogues (Table 3). Time-courses of *Tv*Lac-EV and *Tv*Lac-*Ph*DIR reactions were taken to gain more insight into this coupled enzyme-protein reaction prior to product isolation and to avoid further apparent *Tv*Lac-catalyzed polymerization of the desired coupling products (Figure S16). In assays containing *Ph*DIR, accumulation of the desired products was highest at a point coinciding with full consumption of monolignol substrates. These reaction conditions were employed for preparatory-scale in vitro coupling reactions of **1** with **10** or **11**. For coupling of **1** and **8**, a molar ratio of 1:3 (**8** in excess) was used, while the amount of *Ph*DIR extract was kept consistent as other coupling reactions. The 18% isolated yield (29% reaction yield, Table 1) of the CA-FMCA 8-8′ heterocoupling product **19b** (Table 3) exceeds the yields of racemic (±)-pinoresinols (natural coupling product; at most 20%) obtained from coupling reactions promoted by either laccase alone or a synthetic oxidant, as previously reported in literature.[35,39] This is an exciting proof of concept for the robustness and flexibility of using plant chemistry for the direct enantioselective synthesis of natural product analogues not readily obtained through chemical methods.

CONCLUSIONS

Taken together, our results show that plant-derived dirigent protein can be used to promote the regio- and stereoselective coupling of non-natural monolignols and CA to make functionalized variants of (+)-pinoresinol, a precursor to clinically used chemotherapeutics. The transformation involves coupling of achiral precursors and formation of three new

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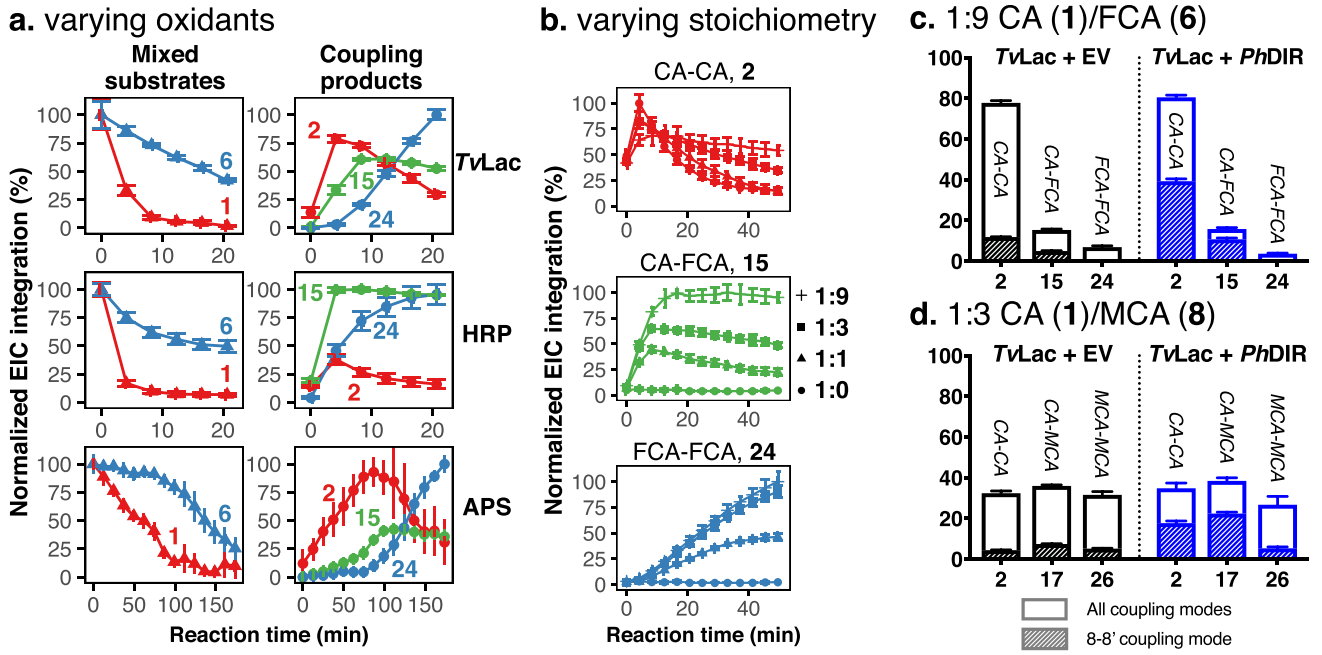

Figure 4. Optimization of heterocoupling product formation. (a) Relative oxidation rates and product formation in a coupling reaction of **1** and **6**, with *Tv*Lac, HRP, and APS as oxidants. For data normalization, see Figure 1 caption. (b) Coupling product formation in a *Tv*Lac oxidation of 0.5 mM **1** and 0, 0.5, 1.5, or 4.5 mM **6** (1:0, 1:1, 1:3, and 1:9 ratios, respectively). Data points indicate the mean of EIC peak integration (m/z of $[M\text{-}H_2O+H]^+$) normalized to the maximum product level per coupling product type. (c and d) End-point product distributions of (part c) **1** and **6** at a molar ratio of 1:9 (500 μM and 4.5 mM, respectively) and (part d) **1** and **8** at a molar ratio of 1:3 (500 μM and 1.5 mM, respectively). See Figure 2 caption for data normalization.

Table 3. Isolated Yields of the (+)-Pinoresinol Analogues

product	isolated yield (mg)	*Ph*DIR extract used (ml)	yield (%)[a]	*ee* (%)[b]
8-8′ CA-MCA, **17b**	0.2	3	4	73
8-8′ CA-FMCA, **19b**	1.2	3.5	18	93
8-8′ CA-ECA, **20b**	0.7	4	9	93
8-8′ FMCA-FMCA, **28b**	1.2	3.5	19	82
8-8′ ECA-ECA, **29b**	0.5	2.9	9	61

[a]100% theoretical yield corresponds to 500 μM product generated from 500 μM limiting monomer (**1**) concentration (1:1 molar ratio of **1** and **10** or **11**, and 1:3 molar ratio of **1** and **8**). [b]*ee*: enantiomeric excess of (+)-pinoresinol analogue over (−)-pinoresinol analogue, calculated using the EIC peak integration ratios on chiral chromatography.

bonds and four stereocenters in a single step. Importantly, there is no analogous synthetic method for this direct coupling and complexity-generating reaction. We anticipate that the expanded substrate versatility of DIRs described here, combined with future protein engineering and optimization efforts to overcome the limitations of the initial oxidation step, will enable the production of etoposide analogues with altered substituents that still function as topoisomerase inhibitors but are resistant to degradation by human liver cytochrome P450s.

While in vitro biochemical characterization of dirigent proteins has begun to shed light on their role in selective coupling of monolignols, our observation that heterocoupling products can be formed suggests the potential for previously unrealized activities of dirigent proteins in plants, including those characterized to date that mediate (+)-pinoresinol or (−)-pinoresinol-forming reactions.[13,30,35,40,41] In addition, a related protein from cotton, the (+)-gossypol-forming dirigent protein, promotes stereoselective aryl−aryl bond formation.[34,42] Notably, low concentrations of both (+)-gossypol and gossypol-6-methyl ether (which can be viewed as a heterocoupling product of hemigossypol and hemigossypol-6-methyl ether) have been found in the native plants, suggesting the possibility of other dirigent-protein-mediated heterocoupling reactions occurring in nature.[43]

The typical plant genome, e.g., *Arabidopsis thaliana*, contains on the order of 20−50 putative DIR homologues, the vast majority of which have not been characterized, and it is unclear whether they are associated with natural product biosynthesis.[44,45] However, the diversity of known optically active lignans found in plants suggests additional members of this class of proteins could also promote alternative stereoselective coupling reactions.[46,47] In our study, we demonstrate an expanded substrate versatility of DIRs promoting stereoselective heterocoupling of natural and non-natural monolignol analogues. Furthermore, we showcase synthesis and isolation of optically active coupling products derived from a broad substrate scope using crude plant extracts containing functional DIRs. These results show promise for combining conventional synthetic chemistry and biosynthesis engineering techniques to access synthetic analogues of complex natural products that can be optimized for use in the clinic.

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EXPERIMENTAL SECTION

Transient Expression of *P. hexandrum* Genes by Agro-Infiltration. *Agrobacterium tumafaciens* (GV3101, gentamicin^R; agrobacteria or agro) transformants with *P. hexandrum* genes in pEAQ-HT (kanamycin^R) vectors were prepared as described previously.[17] Agrobacteria (gent^R and kan^R) grown on LB plates supplemented with gentamicin and kanamycin were resuspended in LB media and centrifuged at 8000 x *g* for 5 min. Supernatant was discarded, and the cell pellets were resuspended in 500 μL of 10 mM MES buffer at pH 5.6 with 10 mM $MgCl_2$ and 150 μM acetosyringone for 1−2 h induction at room temperature. The induced cell suspension was further diluted with induction media to the desired inoculum level measured by optical density measurement at 600 nm. The agrobacterial suspension was infiltrated on the underside of the *N. benthamiana* leaves with a needleless 1-ml syringe. Plants were 5−6 weeks old at the time of infiltration, grown under a 16-h light cycle. Biological replicates consisted of leaves from different *N. benthamiana* plants from the same batch, unless otherwise noted.

Apoplastic Fluid Wash Protocol (APW). Plant extracts were obtained from *N. benthamiana* leaves by APW as described previously.[25,48] Briefly, *Ph*DIR-harboring or empty pEAQ-HT vector was transiently expressed in *N. benthamiana* by agro-infiltration ($OD_{600\ nm}$ = 0.1−0.3) for 5 days. Leaves were vacuum-infiltrated with 0.1 M MES and 0.3 M NaCl at pH 5.5 through three cycles of pulling vacuum to 150 Torr and slow release to atmospheric pressure. The infiltrated leaves were layered on top of a piece of parafilm and wrapped around a pipet tip and placed inside a syringe in place of a plunger. The syringe assembly was placed in a conical tube and was centrifuged at 1500 x *g* for 15 min at 4 °C. The extracts collected at the bottom of the conical tubes were transferred to clean tubes and centrifuged at 14000 x *g* for 10 min at 4 °C to clear undesired cellular debris. The supernatants were ultrafiltrated (Amicon Ultra-0.5 mL 10-kD) at 14000 x *g* for 10 min at 4 °C, and the retentate was aliquoted, flash-frozen in liquid nitrogen, and stored at −80 °C until use at 5-fold dilution.

Time-Course of *Tv*Lac Oxidation on LC-MS. Enzyme assays contained a final concentration of 1 mM equimolar mixture (1% MeOH in water) and 0.1 mU/μL *Tv*Lac in 1.5 mM MES buffer at pH 5.5. When *Tv*Lac was substituted with HRP or APS (Figure 4a), 5 pg/μL HRP and 10 mM H_2O_2, or 1 mM APS was used. When the molar ratio of the mixed substrates was varied (Figure 4b−d, S14 and S15), the amount of the limiting monomer (often **1**) was kept constant at 500 μM with the final total substrate concentration ranging 0.5−5 mM. (E.g., for a molar ratio of 1:3, the final concentrations of the limiting and excess monomers were 500 μM and 1.5 mM, respectively, with the final total substrate concentration of 2 mM.) For the varied pH study (Figure S13), citrate buffer was used for pH 2 and 4, HEPES buffer at pH 7, and Tris buffer at pH 8.5. (Because there is no chromatography in this analysis, to minimize salt contamination in the mass spectrometer, it was important to use organic buffer reagents and ammonium hydroxide or sulfuric acid for adjusting pH.) For standards (Figure S17), bovine serum albumin was supplemented instead of *Tv*Lac at 152 ng/μL. Reactions were initiated by addition of 2 mM substrate stock solutions (equi-volume dilution to a final concentration of 1 mM). Substrates were added to the reaction at ∼1 min prior to the first injection of each sample. Real-time reaction samples were analyzed on the LC-MS on the positive ion mode through direct injection. Three samples were analyzed within the same worklist at a time, and reaction replicates were randomized over different worklists. Time points were recorded every 1.5−1.6 min, which accounts for the time of data collection as well as sample injection performed by the instrument.

Time-Course of In Vitro *Tv*Lac-EV vs *Tv*Lac-*Ph*DIR Assays on LC-MS. Enzyme assays contained a final concentration of 1 mM equimolar substrate mixture, 1% MeOH, 0.333 mU/μL *Tv*Lac, and 20% v/v *Ph*DIR or EV plant extract (transformed with agrobacteria at $OD_{600\ nm}$ = 0.3) in 20 mM MES buffer at pH 5.5. Reactions were initiated by addition of substrate stock solutions at 1.875 mM (1.875% DMSO in water). Substrates were added to the reaction at 1 min prior to the first injection of each sample. Reaction replicates were analyzed on the LC-MS on the positive ion mode at the same time. Time points were recorded every 1.5−1.6 min (from the timestamps of the data files), which accounts for the duration of data collection as well as sample injection performed by the instrument.

In-Vitro LC-MS End-Point Analysis with C18 and Chiral Chromatography. For assays involving *Ph*DIR apoplastic plant extract, the protocols by Gasper et al. were followed with modifications.[25] Enzyme assays were prepared by diluting the plant extract of leaves expressing either *Ph*DIR or EV (transformed with agrobacteria at $OD_{600\ nm}$ = 0.1) 5-fold in 0.1 M MES buffer containing 0.3 M NaCl and 0.333 mU/μL *Tv*Lac at pH 5.5. Substrate stock solutions were prepared from 100 mM solutions in DMSO for **1** and **8**-**11** and in MeOH for **1** and **3**-**7**. (In our hands, use of methanol stock solutions resulted in increased production of coupling products derived from **3**-**7** (CA-X and X-X) in comparison to that when DMSO stock solutions were used.) Reactions were initiated by addition of 1.875 mM equimolar substrate solutions in 1.875% DMSO or MeOH for a final total substrate concentration of 1 mM at 30 °C. After 20 min for assays containing **4** and 40 min for the rest, the coupling reactions were quenched by addition of an equal volume of acetonitrile. The reactions were centrifuged at 15000 rpm for 10 min, and the supernatants were filtered for LC-MS anaylsis on the positive ion mode.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c13164.

General methods and materials, synthetic procedures, supplementary figures, supplementary table, NMR spectra, and supplementary references (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

LC-MS, liquid chromatography−mass spectrometry; EIC, extracted ion chromatogram; HPLC, high-performance liquid chromatography; Lac, laccase; EV, empty vector (control); DIR, dirigent protein; CYP, cytochrome P450

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