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Reversed-phase HPLC lignan chiral analysis with laser polarimetric detection

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Abstract—Vascular plants produce a plethora of lignan natural products, several of which have important pharmacological uses as antitumor, antibacterial and antiviral agents. Depending upon the plant species, the lignans are generally found either in enantiomeric excess or in enantiomerically pure (+)- or (−)-form. In this study, a reversed-phase HPLC method with UV/laser polarimetric detection was developed for the direct determination of lignan chirality and enantiomeric composition. The method was suitable for analyses of stereoselective monolignol radical-radical coupling assay mixtures containing the (+)-pinoresinol forming dirigent protein, and of lignan chirality in crude plant extracts. Over the concentration range examined, a good linear response was obtained with a detection limit of 0.4 nmol for (+)-pinoresinol.

1. Introduction

Lignans are an abundant class of plant phenol-derived natural products with important roles in defense against plant pathogens, as well as in pharmacology: in the latter case, many have significant antitumor, antiviral and antibacterial properties, with some others engendering health protective effects.[1] Several thousand lignans have been identified thus far, most of which are dimers derived (initially) via various distinct coupling modes between two achiral phenylpropanoid monomers. Typically the resulting dimers, and their downstream metabolic products, either are found in an enantiomeric excess or are enantiomerically pure. Of these, the biosynthesis of only a small subclass of lignans (mainly 8-8′ linked) has been delineated.[1]

The flowering shrub *Forsythia intermedia* has been extensively used to study 8-8′ lignan biosynthesis, particularly to the (*E*)-coniferyl alcohol **1**-derived lignans **2**–**5**. The stems of *F. intermedia*, however, mainly accumulate (−)-matairesinol **5**, (−)-arctigenin **6**, (−)-arctiin **7**, (−)-matairesinol-4-*O*-glucoside **8**, (+)-*epi*-pinoresinol **9**, (+)-*epi*-pinoresinol-4-*O*-glucoside **10**, (+)-phillygenin **11** and (+)-phillyrin **12**,[2–9] with the lignans **2**–**4** being present in very low amounts (Schemes 1 and 2). Many of the proteins and enzymes involved in their biosynthetic pathways have been identified and characterized, with the encoding genes and the corresponding functional proteins obtained.[2,10–25]

Typically, determination of the chiroptical properties of plant lignans, as well as that of other chiral metabolites, involves purification of the compound(s) of interest. This generally requires extraction of the plant material with various solvents, with the resulting extracts being subjected to numerous chromatographic procedures. The purified chiral compounds of interest are then submitted to different characterization protocols, including that of either specific rotation or circular dichroism measurements. In some instances, chiral metabolites can be separated on stationary phases to give the corresponding (+)- and (−)-enantiomeric forms, thereby allowing quantification of the amounts of each enantiomer, such as for the lignans, (±)-pinoresinols **2a**,**b**[11,15,20] and (±)-dehydrodiconiferyl alcohols **13**.[1] These procedures are, however, time consuming and require significant levels of experimental manipulation.

It was deemed instructive in this study to evaluate the use of an inline HPLC coupled laser polarimeter for the rapid analysis of various stereoselective monolignol radical–radical coupling assay mixtures, as well as for plant extracts containing chiral lignans. Initially this was employed to study dirigent protein mediated coupling in vitro, as a means of establishing the extent of stereoselective coupling engendered under various assay conditions. This in turn permitted an evaluation of the advantages and limitations of this technique, when compared with the previously reported method.[20] This

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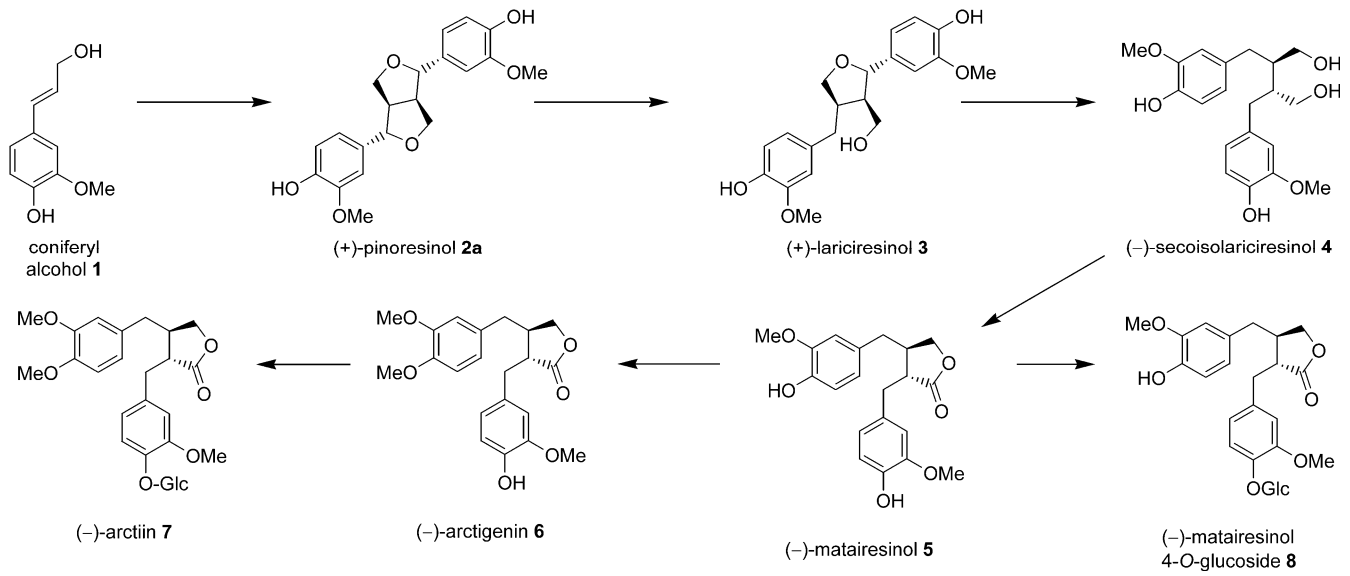
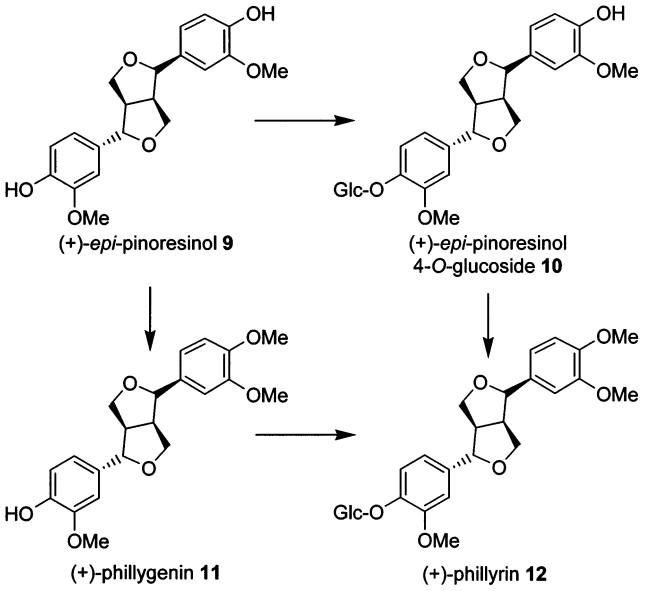


Scheme 1.



Scheme 2.

was next extended to the analysis of *F. intermedia* crude plant extracts to further examine the potential utility of this approach.

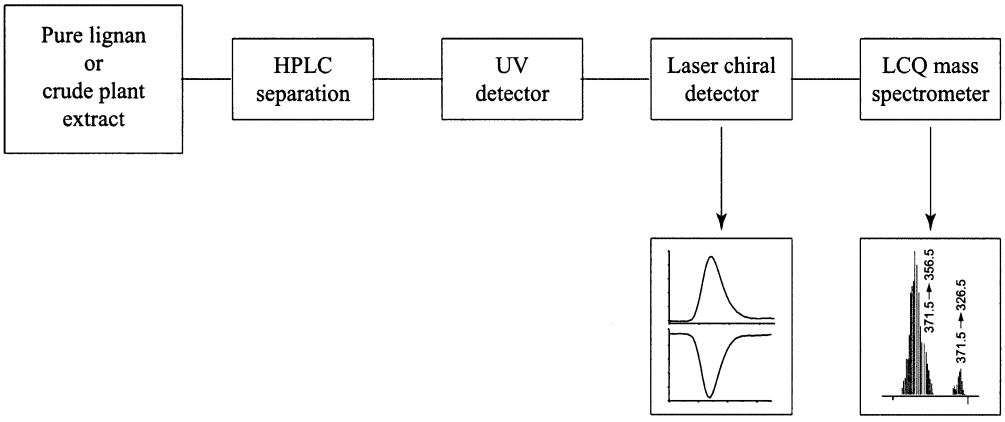
Each sample was thus subjected to initial HPLC separation with analysis of the resolved components using a UV-detector, a laser polarimeter and a mass-spectrometer (atmospheric pressure chemical ionization) linked in series (Scheme 3).

2. Results and discussion

2.1. Direct in-line chiral analysis

Entry into the 8-8′ linked lignan pathway in *F. intermedia* occurs via stereoselective coupling of the achiral monolignol, (*E*)-coniferyl alcohol **1**, to give (+)-pinoresinol **2a**[10,13,14,18,20,22] with subsequent downstream metabolism to give lignans **3**–**12**. This, in turn, represented not only the first example of stereoselective monolignol-derived radical–radical coupling in vitro, but also the discovery of the (+)-pinoresinol forming dirigent protein (Latin *dirigere*: to guide or direct).[20] The latter stipulates the outcome of this coupling, provided there is one-electron oxidative capacity present. By contrast, in the presence of a one-electron oxidant or oxidase in vitro alone, (*E*)-coniferyl alcohol **1** undergoes non-specific radical–radical coupling in open solution to form racemic (±)-dehydrodiconiferyl alcohols **13**, (±)-pinoresinols **2a**,**b**, and (±)-*erythro*/*threo*-guaiacylglycerol coniferyl alcohol ethers **14** in ratios of ~1:0.5:0.3, respectively (Scheme 4, redrawn from[25]). Thus, depending upon whether the dirigent protein (DP) is saturated (maximum activity) or not, relative to that of oxidase/oxidant capacity in the assay mixture in vitro, formation of either enantiomerically pure (+)-pinoresinol (**2a**) or an enantiomeric excess of (+)-pinoresinol (**2a**) can occur; the mechanism of the DP thus appears to involve substrate binding, of the presumed radical form, in open solution with subsequent directed (stereoselective) coupling at the protein binding site(s).

While technically very effective, the original methodology of Davin et al.[20] to assay (+)-pinoresinol **2a** formation through mediation of the (+)-pinoresinol-forming dirigent protein is fairly lengthy and time consuming. Briefly, it involves ethyl acetate extraction of the assay mixture, with the resulting dried extract (reconstituted in MeOH:H_2O (1:1)) being subjected to reversed-phase HPLC analysis. This enabled facile separation of remaining coniferyl alcohol **1** substrate from the various 8-8′-, 8-5′-, and 8-*O*-4′-linked dimers **2**, **13**, **14** (see Fig. 1(A)), during which the fraction corresponding to pinoresinol **2** was collected. The final manipulation then



Scheme 3.

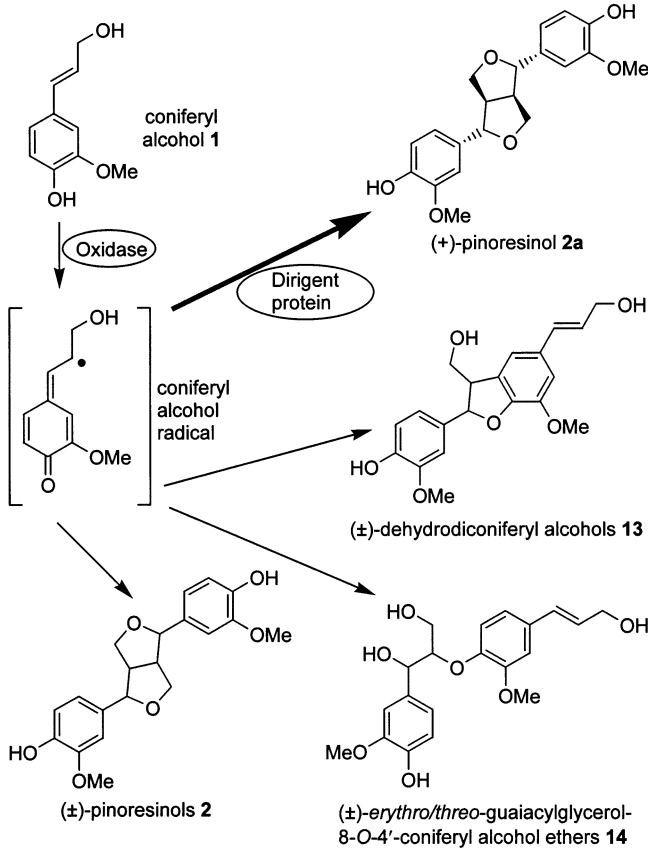
involved Chiralcel OD chromatography of the isolated pinoresinol **2** with concomitant determination of enantiomeric purity.

For radiochemical assays with [9-^{3}H] coniferyl alcohol **1**, the two HPLC procedures together with inline liquid scintillation analysis provided a convenient means not only to determine distribution of radiolabel into the various products, but also into each of the two antipodes of (+)- and (−)-[9-9′-^{3}H]-pinoresinols **2a**,**b**, respectively (see Fig. 1(B)). The chiral chromatography step, however, suffers from the disadvantage of not being able to directly establish either sign or magnitude of the specific rotation value, thereby requiring additional polarimetric and/or circular dichroism measurements. This disadvantage was further compounded by multiple HPLC manipulations, and time-consuming lyophylization protocols between chromatographic steps. On the other hand, this methodology has the advantage of a low detection limit. For example, when (+)-[9,9′-^{3}H]-pinoresinol **2a** is in a ~50% enantiomeric excess over the corresponding (−)-antipode **2b**, the detection limit by UV is ~40 pmol, whereas with inline radioactive scintillation counting (following chiral separation) it is ~12 pmol (data not shown) as in Ref. 20.

A more rapid protocol for routine (+)-pinoresinol-forming dirigent protein assays was needed, however, for performing experiments involving large numbers of assays, such as in kinetic and substrate specificity studies. [An additional rationale for a more rapid screening protocol was that comparable proteins—albeit involved in other coupling modes—await discovery, and accordingly general methodologies need to be developed.] Inline polarimetry was thus directly applied first to the Chiralcel OD separation of (+)- and (−)-pinoresinols **2a**,**b**, then to the reversed-phase HPLC analyses of various (+)-pinoresinol dirigent protein mediated coupling assay mixtures, and, finally, to the reversed phase HPLC analyses of lignan-containing plant extracts.

Accordingly, (±)-pinoresinols **2a**,**b** were next chemically synthesized in racemic form from (*E*)-coniferyl alcohol **1** by non-specific $FeCl_3$ mediated coupling.[23] The two enantiomers were separated by Chiralcel OD HPLC (as in Fig. 1(B), but now using racemic (±)-pinoresinols **2a**,**b**, with multiple separations (~500×) affording pure (+)- and (−)-antipodes **2a** and **2b**, in total amounts of ~25 mg each, respectively: for illustrative purposes, Fig. 1(C) displays the chromatograms for each of the (+)-pinoresinol **2a** and (−)-pinoresinol **2b** antipodes (~43 nmol each), using inline laser polarimetric detection.

Attention was subsequently given to direct determination of the enantiomer composition of **2** using reversed-phase HPLC with inline polarimetric detection. However, from an operational perspective, it was found necessary to restrict the HPLC elution systems to either



Scheme 4.

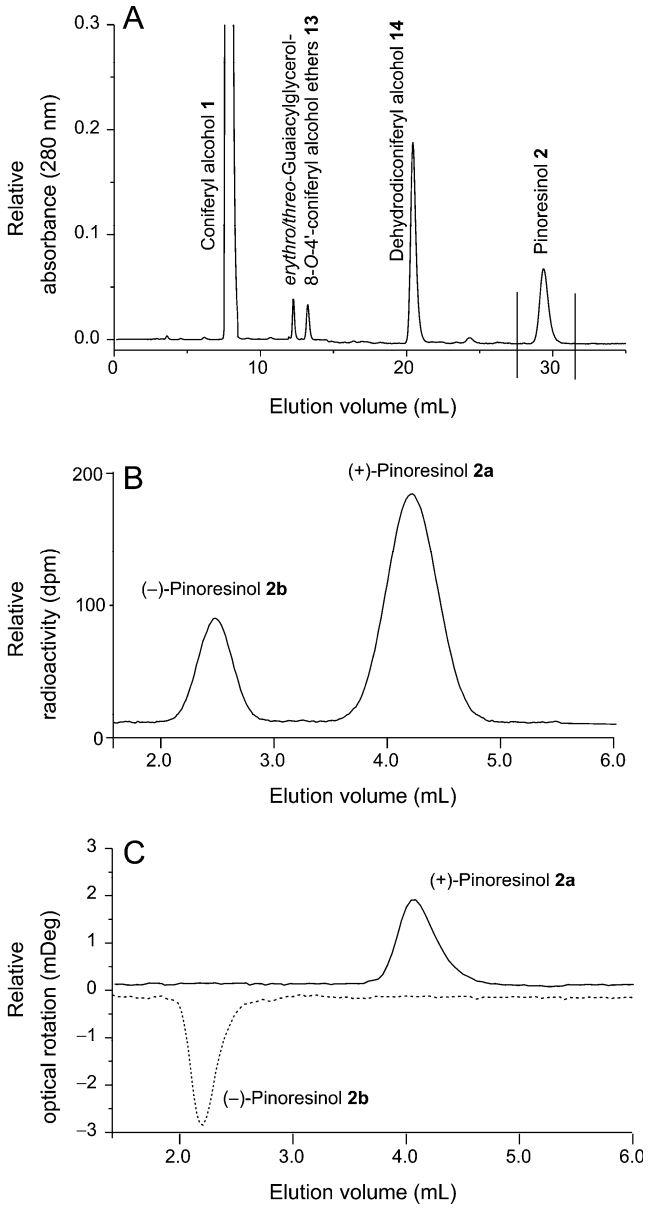


Figure 1. Representative reversed-phase and chiral HPLC analyses of various lignans resulting from (+)-pinoresinol forming dirigent protein assays using (*E*)-coniferyl alcohol **1** as substrate. (A) Reversed-phase HPLC separation of coniferyl alcohol **1**, *erythro*/*threo*-guaiacylglycerol coniferyl alcohol ethers **14**, dehydrodiconiferyl alcohols **13**, and pinoresinols **2a**,**b** with the latter being collected between 27.5 and 31 min (vertical bars) [Elution system 1, see Section 4). (B) Chiralcel OD separation of (+)-pinoresinol **2a** and (–)-pinoresinol **2b** antipodes of (±)-pinoresinols **2a**,**b**, with enantiomers in relative ratios of ~75:25 (~50% enantiomeric excess of (+)-antipode, **2a**), respectively, using radiochemical detection. (For elution conditions see Section 4.) (C) Chiralcel OD separation of pure (+)- and (–)-pinoresinols (**2a** and **2b**), with detection by inline laser polarimetry. Solid line, (+)-pinoresinol **2a**; broken line, (–)-pinoresinol **2b**. (Elution details, as for Fig. 1(B) above.)

single gradients or isocratic mixtures (data not shown), in order to improve signal to noise ratios and to reduce baseline distortions resulting from rapid solvent changes. Additionally, pressure fluctuations were an additional source of baseline instability using the PDR-chiral detector and, of the HPLC pump modules evaluated (Waters 600, 610, 710 and Alliance 2690), the Waters Alliance 2690 system gave the best results and was thus used in this study.

Acceptable operational conditions for analysis of dirigent protein assay mixtures, with inline laser polarimetric detection, were obtained using elution system 2 (see Section 4). These elution conditions markedly reduced the retention volumes of each of the components in the assay mixtures (cf. Fig. 2(A) and Fig. 1(A), respectively): coniferyl alcohol **1** and (±)-*erythro*/*threo*-guaiacylglycerol coniferyl alcohol ethers **14** were now

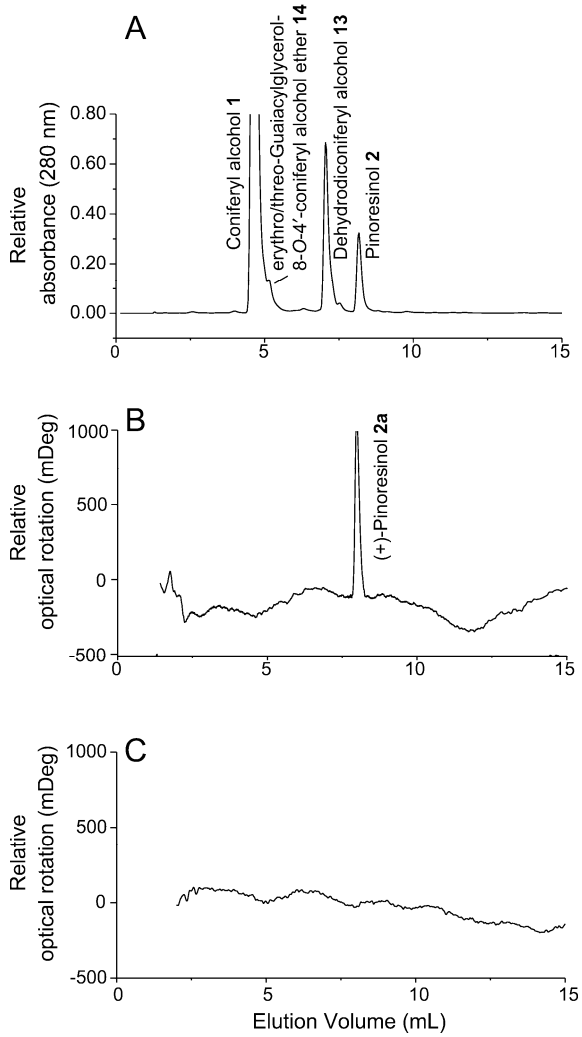


Figure 2. Representative reversed-phase HPLC with chiral analyses of products from (*E*)-coniferyl alcohol **1** coupling assays, in the presence and absence of (+)-pinoresinol forming dirigent protein (see text and Section 4). (A) Reversed-phase HPLC separation of complete assay mixture using elution system 2 with UV detection (see Section 4). (B) Laser polarimetric detection, as for A above, revealing presence of (+)-pinoresinol **2a** in enantiomeric excess. (C) Control assay, as for A above, but without dirigent protein.

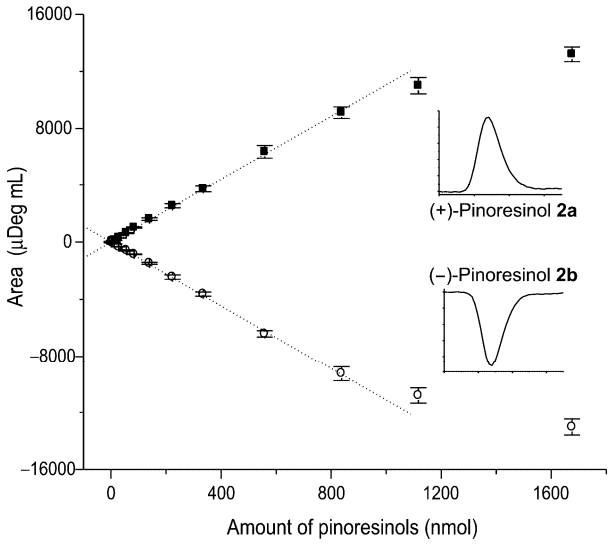


Figure 3. Calibration of inline laser polarimeter with (+)- and (−)-pinoresinols **2a** and **2b**, respectively, following reversed-phase HPLC analyses (solvent system 2). Positive values (■) depict (+)-pinoresinol **2a**, whereas negative values (○) correspond to (−)-pinoresinol **2b**. A linear curve fit (linear range ~0–800 nmol) is shown for both enantiomers. Insets illustrate representative chromatograms of (+)- and (−)-pinoresinols **2a** and **2b**.

only partially separated, whereas both pinoresinol **2** and dehydrodiconiferyl alcohol **13** remained fully resolved (Fig. 2(A)). Inline laser polarimetric analysis, however, clearly detected only the enantiomeric excess of (+)-pinoresinol **2a** as evidenced by the positive rotation (Fig. 2(B)). In contrast, control assays carried out in the absence of the dirigent protein, resulted in no detection of chiral components (Fig. 2(C)). In this case, only non-specific coupling had occurred, thus affording racemic mixtures.

For precise determination of enantiomeric composition, it was next instructive to establish both the operational calibration range and the detection limits using this HPLC procedure for the (+)-pinoresinol forming dirigent protein assays. Thus, a wide range (0.1–1700 nmol) of known concentrations of both (+)- and (−)-pinoresinols (**2a** and **2b**) were prepared, with each enantiomer being individually subjected to reversed phase HPLC analysis using dual UV and laser polarimetric detection. For the UV detection method (λ 280 nm), a linear response was obtained for pinoresinol **2** up to 120 nmol, beyond which the detector was saturated by the sample at this wavelength (data not shown). For the inline laser polarimetric detection method, however, a linear calibration range up to ~800 nmol (+)- or (−)-pinoresinols **2a** and **2b** was established, in excess of which (>800 nmol) the responses were non-linear (Fig. 3). Nevertheless, over this range, the relative responses to both antipodes displayed linear but opposite trends, with values of 11±0.08 and −11±0.11 μdeg mL nmol^{-1} for the (+)- and (−)-antipodes **2a** and **2b**, respectively (Fig. 3). Moreover, a detection limit (signal to noise ratio, $n=3$) of ~0.4 nmol pinoresinol **2** was obtained, with correlations of R >0.998 for both antipodes.

With calibration thus achieved, it was possible to determine the enantiomeric compositions of pinoresinol **2**, by comparison of the relative UV and laser polarimetric responses using a method analogous to that of Bobbitt and Linder.[26] For example, the laser polarimetric data shown in Fig. 2B corresponds to ~15.0 nmol (+)-pinoresinol **2a**, whereas that of the corresponding UV trace (Fig. 2(A)) represents ~30 nmol of pinoresinol **2**. Based on the calibration data, (+)-pinoresinol **2a** was thus present in ~50% enantiomeric excess (i.e. in a ~75:25 ratio of (+) and (−)-pinoresinols **2a**/**2b**. Thus, the inline polarimetric method not only has the advantages of both method simplification and more rapid analyses, but under these conditions it is also suitable for general purpose assays, without the need for radiolabeled substrates. Furthermore, various applications[25–29] of laser polarimetry to the chiral analysis of many different types of molecules have been reported.[30–39]

2.2. Specific rotations of lignans

The method is of considerable utility for measuring the specific rotation values of various enantiomerically pure lignan samples using the approach described by Rice et al.[40] which assumes a Gaussian peak model. Thus, known concentrations of the enantiomerically pure lignan standards **2a** and **5**–**12** (see Section 4) were subjected to reversed-phase HPLC analysis with inline laser polarimetric detection at 670 nm. From these analyses,

Table 1. Comparison of specific rotations ($[\alpha]_{670}$) of *Forsythia* lignans obtained through inline laser polarimetry with literature reported data

Lignan	$[\alpha]_{670}$	Amount (μg)	Lit. $[\alpha]_{590}$	Solvent/concentration	Plant	Ref.
(+)-Pinoresinol **2a**	+62	93	+61.6	$CHCl_3$, $c=0.26$	*F. suspensa*	4
(−)-Matairesinol **5**	55	88	50.0	MeOH, $c=1.5$	*F. intermedia*	8
(−)-Arctigenin **6**	35	46	27.5	MeOH, $c=4.5$	*F. intermedia*	8
(−)-Arctiin **7**	57	43	52.3	MeOH, $c=2.0$	*F. intermedia*	8
(−)-Matairesinol-4-*O*-glucoside **8**	46	68	43.2	MeOH, $c=1.3$	*F. intermedia*	8
(+)-*epi*-Pinoresinol **9**	+103	25	+110	MeOH, $c=5.0$	*F. intermedia*	8
(+)-*epi*-Pinoresinol-4-*O*-Glucoside **10**	+25	32	+31.4	MeOH, $c=2.0$	*F. intermedia*	8
(+)-Phillygenin **11**	+98	43	+95.7	MeOH, $c=2.0$	*F. intermedia*	8
			+120.0	MeOH, $c=0.04$	*F. suspensa*	41
(+)-Phillyrin **12**	+48	36	+43.8	MeOH, $c=2.0$	*F. intermedia*	8

the specific rotations for each lignan were determined (Table 1), the values of which were relatively favorable upon comparison with literature data.[4,8,41] For example, with (+)-pinoresinol **2a**, a specific rotation of $[\alpha]_{670}=+62$ was obtained versus the literature value of $[\alpha]_{590}=+61.6$ in $CHCl_3$.[4] [Variations between literature values and the measured specific rotations obtained in this study presumably result largely from solvent and wavelength differences, as well as from differences in enantiomeric composition in different plant systems.]

2.3. Lignan analysis in crude extracts of *Forsythia*

There is growing interest in obtaining natural (as well as synthetic) products in enantiomerically pure form, typically because the desired pharmacological properties result from a specific antipode.[42] Furthermore, with genetic manipulation of various plant biochemical pathways, there is considerable interest at present in metabolic profiling in order to comprehensively understand how metabolism can be modulated. However, to our knowledge, this has not yet been extended to the study of chiral metabolites of plants. For reasons such as these, it was useful to examine whether particular chiral metabolic components could be directly detected in a facile manner in crude plant extracts. Thus, since several enantiomerically pure lignans had previously been reported as major components of *F. intermedia* **5**–**12**,[2–9] it was a suitable plant species to evaluate the potential of this inline laser polarimeter detection method. Moreover, it permitted a comparison with both UV and MS detection methods for the same lignan **5**–**12** species.

HPLC elution conditions suitable for baseline separation (albeit with peak broadening) of each of the enantiomerically pure *F. intermedia* lignan standards **5**–**12** were obtained using solvent system 3 (see Section 4 and Fig. 4(A), UV traces). However, with inline laser polarimetry (Fig. 4(A), laser polarimeter traces), this peak broadening resulted in weaker signal-to-noise ratios and hence decreased sensitivity. Accordingly, amounts of lignans **5**–**12** needed for suitable laser polarimetric detection under these conditions (Fig. 4(A), laser polarimetry traces) typically ranged from 2.5 to 9.3 μg. Additionally, multi-stage mass spectrometry using negative ion atmospheric pressure chemical ionization was also very sensitive to detection, this engendering formation of the parent $[M-H]^-$ ions of the various lignan (glycoside)s **5**–**12**. Of the eight lignans examined, however, four sets of isobaric masses were observed (i.e. for **8** and **10**, $[M-H]^-=519.5$ m/z; for **7** and **12**, $[M-H]^-=533.5$ m/z; for **5** and **9**, $[M-H]^-=357.5$ m/z; and for **6** and **11**, $[M-H]^-=371.5$ m/z) (Fig. 4(B)). Each of these lignans could then be distinguished by additional MS/MS fragmentation (summarized in Fig. 4(B)). Thus, with (–)-matairesinol-4-*O*-glucoside **8** and (+)-*epi*-pinoresinol-4-*O*-glucoside **10**, both initially lost a glucose derived fragment to give individual charged species of 357.5 m/z $[M-H-Glc]^-$. Further fragmentation (MS^3) of the corresponding 357.5 m/z ions of each resulted in an additional loss of 44 mass units (CO_2) for the (–)-matairesinol **5** derived fragment to give the ion at 313.5 m/z, whereas that derived from the (+)-*epi*-pinoresinol **9** fragment generated the corresponding benzylic (vanillyl) ion at 151.5 m/z. [In an analogous manner, the parent aglycones (–)-matairesinol **5** and (+)-*epi*-pinoresinol **9** could also be differentiated.] The isobaric pairs, arctiin **7** and phillyrin **12** (and the corresponding aglycones **6** and **11**) were differentiated in a comparable way: thus fragmentation (MS^2) of (–)-arctiin **7** and (+)-phillyrin **12** (533.5 m/z, $[M-H]^-$) also lost a glucose-derived fragment to afford the corresponding aglycone $[M-H-Glc]^-$ derived ions at 371.5 m/z, whose further fragmentation (MS^3) gave fragments at 356.3 m/z (-CH_3) and 326.5 m/z (-CH_3 and -HCHO), respectively. In this way, both **7** and **12** could be unambiguously distinguished, as could the aglycones **6** and **11**.

Thus, application of the three detection methods (UV, inline laser polarimetry and mass spectrometry) to the crude *F. intermedia* methanolic extract gave encouraging results even at this rudimentary stage of purification (Fig. 4(C–E)). That is, UV-analysis (λ 280 nm) revealed the presence of each of the lignan (glycoside)s **5**–**8** and **11**, with **10**, **12**, and **9** being detected as minor components or as shoulders adjacent to more intense peaks (Fig. 4(C)). At this stage of purification, however, only five of the eight lignans (namely **5**–**8** and provisionally **11**) could be readily detected by laser polarimetry, whereas **9**, **10** and **12** required a higher level of purification for detection (Fig. 4(D)). On the other hand, the presence of all eight lignans could be readily confirmed by MS^2 or MS^3 fragmentation as for the corresponding standards (Fig. 4(E)).

3. Conclusion

Application of inline laser polarimetric detection of lignan chirality provides a facile means of simplifying stereoselective monolignol coupling assays, at least those affording (+)-pinoresinol **2a** in enantiomeric excess. It can be predicted that this method will also have general applicability to other phenylpropanoid coupling systems, some of which are currently under investigation. Furthermore, inline laser polarimeter chiral analysis has considerable promise even when applied to very crude plant extracts, particularly in combination with both UV and mass spectrometric methods.

4. Experimental

4.1. Instrumentation and chromatographic conditions

HPLC analyses employed an Alliance 2690 HPLC (Waters, Milford, MA, USA), equipped with a Waters Nova-Pak® C-18 column (3.9×150 mm). Elution was carried out using one of three eluant systems 1–3, each with flow rates of 1 mL min^{-1}. System 1 had a linear gradient of eluant A (3% acetic acid in H_2O, v/v) and eluant B (acetonitrile) with ratios (A:B) from 90:10 at $t=0$ to 83:17 at $t=34$ min; system 2 consisted of a linear gradient of eluants A and B in an A:B ratio of

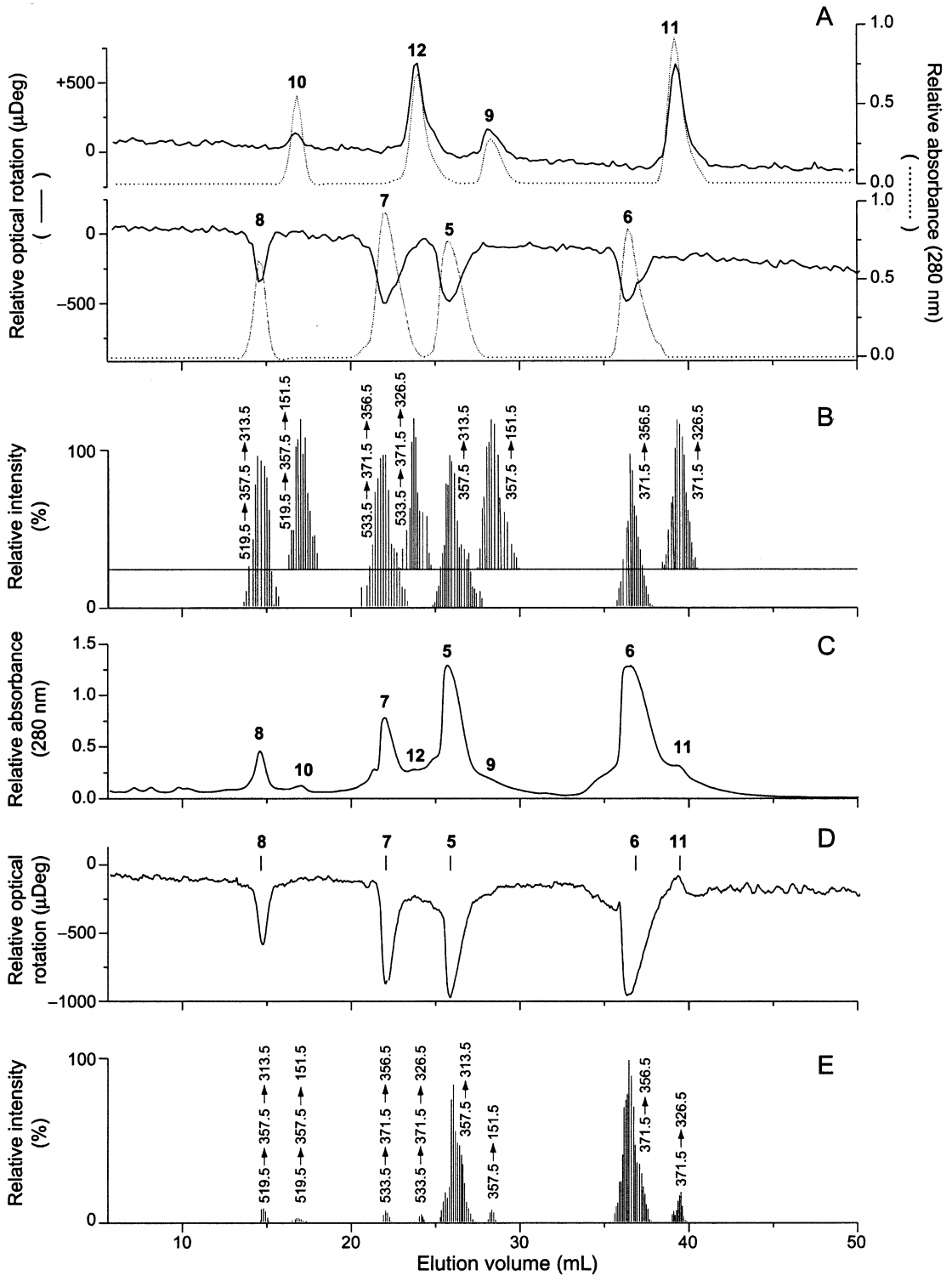


Figure 4. UV, laser polarimetric and APCI MS^2 or MS^3 detection of authentic lignans and those in a crude *F. intermedia* plant extract, following HPLC separation (solvent system 3). (A) Reversed phase HPLC separation of lignans **5**–**12** standards, with dual detection by UV (λ 280 nm, dotted lines) and inline laser polarimetry (solid lines). (B) APCI MS^2 or MS^3 detection of lignans **5**–**12** above with depiction of individual MS^2 or MS^3) fragmentation patterns. (C–E) UV, inline laser polarimetric, and MS^2 or MS^3 detection of lignans **5**–**12** in a crude methanolic extract of *F. intermedia*.

95:5 at $t=0$ to 30:70 at $t=15$ min, whereas system 3 consisted of a linear gradient of eluants A and B in an A:B ratio of 95:5 at $t=0$ to 30:70 at $t=50$ min. All eluants were dually monitored inline using an UV detector (Waters 996 photodiode array, $\lambda=280$ nm) and an Advanced Laser Polarimeter (cell volume 56 μL, length 5.17 cm) (PDR-Chiral, Palm Beach Gardens, FL, USA) connected in series.

For initial product verification using APCI MS^n, a LCQ mass spectrometer (Thermo-Finnigan, San Jose, CA, USA), equipped with atmospheric pressure chemical ionization and an ion trap, was placed downstream of the laser polarimeter. The LCQ mass spectrometer was operated with a sheath gas flow rate of 80 (arbitrary units), a vaporization temperature of 400°C in the negative ion mode, with a voltage of 5.0 kV to the capillary, and a heated capillary temperature of 200°C. All other parameters were optimized automatically using the instrument's software package for (±)-pinoresinol **2** as model compound for the lignans **5**–**12**. Additionally, the ion trap of the mass spectrometer was programmed to monitor the parent $[M-H]^-$ ions, i.e. [(−)-matairesinol-4-*O*-glucoside **8**-H]$^-$ and [(+)-*epi*-pinoresinol-4-*O*-glucoside **10**–H]$^-$, 519.5 m/z; [(−)-arctiin **7**–H]$^-$ and [(+)-phillyrin **12**–H]$^-$, 533.5 m/z; [(−)-matairesinol **5**–H]$^-$ and [(+)-*epi*-pinoresinol **9**–H]$^-$, 357.5 m/z; [(−)-arctigenin **6**–H]$^-$ and [(+)-phillygenin **11**–H]$^-$, 371.5 m/z. For APCI MS analyses, solvent system 3 was used for chromatography with mass spectral parameters set to isolate the parent ion and perform fragmentations for the isobaric masses with elution volumes of 10–20 (**8**, **10**), 20–24 (**7**, **12**), 24–33 (**5**, **9**), and 33–43 (**6**, **11**) mL, respectively. Fragmentation was performed on the parent ions selected with an 1 mass unit window with helium as the collision gas and a relative collision energy of 15%. A second fragmentation was performed on the glycosides (**8** and **10**: m/z 519.5→357.5→(MS^3 products), **7** and **12**: m/z 533.5→371.5→(MS^3 products)), again with an 1 mass unit selection window and relative collision energy of 15%.

Chiral HPLC analyses were performed using a Waters 600E HPLC equipped with a Waters 715 Ultra WISP sample processor and a Chiralcel OD column (3.5×50 mm), this being eluted with ethanol:hexanes (1:1) at a flow rate of 0.5 mL min^{-1} for 20 min. Dual UV (280 nm)/radiochemical monitoring of the effluent was achieved in series using a Waters 900 PDA detector and a Flo-one-β detector Series A-100 (Radiomatic Instruments, Meriden, CT, USA), respectively, as previously described.[20]

4.2. Monolignol and lignan standards

Unlabeled (*E*)-coniferyl alcohol **1**, [9-^{3}H]-coniferyl alcohol **1** (19.5 MBq mol^{-1}), and racemic (±)-pinoresinols **2a**,**b** were synthesized following procedures of Xia et al.[23] The (+)- and (−)-antipodes (**2a**,**b**, 25 mg each, respectively) were subsequently obtained by separation of racemic (±)-pinoresinols **2** on a Chiralcel OD HPLC column, as described by Davin et al.[13] (−)-Matairesinol **5**, (−)-arctigenin **6**, (−)-arctiin **7**, (−)-matairesinol-4-*O*-glucoside **8**, (+)-e*pi*-pinoresinol **9**, and (+)-*epi*-pinoresinol-4-*O*-glucoside **10**, were isolated from *F. intermedia* stems as previously described,[5,8] whereas (+)-phillygenin **11** and (+)-phillyrin **12** were generous gifts from Professor Sansei Nishibe (Health Sciences Univ. Hokkaido, Ishikari-Tobetsu, Hokkaido, Japan).

4.3. Polarimetric calibration and detection limits, and lignan specific rotation measurements

To determine the calibration range and detection limit for pinoresinol **2** analyses with dual UV and laser polarimetric detection systems, known concentrations of authentic samples of each (+)- and (−)- antipode (**2a**,**b**) (ranging from 100 pmol to 1700 nmol) were individually employed using reversed-phase HPLC, with solvent system 2.

Determining the lower limit of reliable detection for radiolabeled (9,9-^{3}H] pinoresinol **2** involved utilizing the pinoresinol **2** fraction (with a 50% enantiomeric excess of (+)-pinoresinol) isolated from assay mixtures by reversed phase HPLC using solvent system 1 as eluant. After lyophilizing the fractions containing pinoresinol (**2**, 50% (+)-pinoresinol enantiomeric excess), the latter was reconstituted in a minimum amount of ethanol and subsequently applied to the Chiralcel OD column (as described in Instrumentation and Chromatographic Conditions) with both UV and liquid scintillation counting detection as before. The lower limit of reliable detection was defined as the amount at which a signal to noise ratio of three was obtained for (−)-pinoresinol **2a** measurements (the least abundant antipode).

Additionally, the $[\alpha]_{670}$ values of lignans **2a** and **5**–**12** (~25–93 μg each) were measured at 22°C by subjecting these lignans individually to reversed phase HPLC (solvent system 2) with laser polarimetric detection. Calculations applied the Gaussian peak model of Rice et al.,[40] which was incorporated into the specific rotation spreadsheet supplied by PDR-Chiral Inc. (Palm Beach Gardens, FL, USA).

4.4. *F. intermedia* plant extracts

F. intermedia stems (5 g, lyophilized powder) were extracted with HPLC grade methanol (100 mL, 50°C, 1 h agitation), then filtered through a G6 glass fiber filter, with the filtrate evaporated to dryness in vacuo, and stored at −20°C until needed. The resulting residue (~240 mg) was resuspended in a minimum amount of methanol, filtered, and a portion (~1%) subjected to reversed-phase HPLC analysis (see above) using solvent system 3 for elution. The eluant was monitored by measuring changes in UV absorbance at 280 nm, as well as by laser polarimetric, and mass spectrometric detection, respectively. Further identification of the lignans in the extracts was confirmed by comparison with authentic standards of known enantiomeric purity using retention volume, UV spectral profiles, polarimetric responses, and negative ion multi-stage mass spectrometric analyses (APCI-MS^2 or APCI-MS^3), respectively.

4.5. Dirigent protein purification and assays, general procedures

The (+)-pinoresinol forming dirigent protein was purified from *F. intermedia* as described.[25] The standard

DP assay contained unlabeled (*E*)-coniferyl alcohol (**1**) (or radiolabeled [9-^{3}H]-coniferyl alcohol, **1**, 7 MBq mol^{-1} L^{-1}, for radiochemical detection) (4 mM) and flavin mononucleotide (FMN) (1 mM, Sigma-Aldrich, synthetic) in sodium 2-(*N*-morpholino)ethanesulfonic acid buffered solution (Mes, 40 mM, pH 5.0), with Na_2SO_4 (75 mM) and dirigent protein (40 nM, dimeric form[25]) to a total volume of 250 μL. Following incubation at 30°C for 4 h with overhead fluorescent lighting, the reaction was terminated by extraction with ethyl acetate (3×400 μL); the organic solubles were combined and subsequently dried in vacuo, stored at −20°C until needed, and reconstituted in H_2O:methanol (1:1, 100 μL) immediately before HPLC analysis using either radiochemical or laser polarimetric detection.

For radiochemical detection, the assay mixture extracts were subjected to reversed-phase HPLC analysis, using solvent system 1 which was optimized for separation of all assay compounds. The peak corresponding to pinoresinol **2** was then collected, with an aliquot (∼10%) removed for scintillation counting. The remainder was lyophilized, reconstituted in ethanol (100 μL) and applied to a Chiralcel OD column, eluted as described in Instrumentation and Chromatographic Conditions section, with radiochemical detection of the resulting pinoresinol (**2a**,**b**) antipodes.

For inline laser polarimetric detection, assay mixture extracts were subjected directly to reversed-phase HPLC analysis (also with UV-detection) using solvent systems 1 or 2. Of these, solvent system 2 had a signal to noise ratio of 35 for polarimetric detection, whereas the signal-to-noise ratio of solvent system 1 was only ∼5, when (+)-pinoresinol **2a** was present in ∼5 nmol quantities; as a result, solvent system 2 was used.

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