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[^{13}C]-Specific labeling of 8–2′ linked (−)-*cis*-blechnic, (−)-*trans*-blechnic and (−)-brainic acids in the fern *Blechnum spicant*

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Dedicated to Professor Meinhart H. Zenk on the occasion of his 70th birthday

Abstract

In vivo administration experiments using stable (^{13}C) and radio (^{14}C) labeled precursors established that the optically active 8–2′ linked lignans, (−)-*cis*-blechnic, (−)-*trans*-blechnic and (−)-*trans*-brainic acids, were directly derived from L-phenylalanine, cinnamate, and *p*-coumarate but not either from tyrosine or acetate. The radiochemical time course data suggest that the initial coupling product is (−)-*cis*-blechnic acid, which is then apparently converted into both (−)-*trans*-blechnic and (−)-*trans*-brainic acids in vivo. These findings provide additional evidence for vascular plant proteins engendering distinct but specific phenolic radical-radical coupling modes, i.e., for full control over phenylpropanoid coupling in vivo, whether stereoselective or regiospecific.

Keywords: *Blechnum orientale*; *Blechnum spicant*; Blechnaceae; Deer fern; *cis*- and *trans*- (−)-Blechnic acid; (−)-Brainic acid; Phenylpropanoid metabolism; ^{13}C NMR spectroscopy

1. Introduction

There is considerable interest in defining the biochemical processes involved in plant bimolecular phenoxy radical–radical coupling, as well as for biopolymer (e.g. lignin and suberin) assembly (Davin et al., 1997; Gang et al., 1999; Lewis and Davin, 1999; Lewis et al., 1999; Croteau et al., 2000; Davin and Lewis, 2000; Anterola and Lewis, 2002). In this context, the term dirigent protein was recently coined to describe a new class of protein(s) capable of stipulating the outcome of monolignol radical–radical coupling, the first example of which engendered formation of (+)-pinoresinol (**1a**) via intermolecular coupling of two *E*-coniferyl alcohol (**2**) derived substrate molecules (Davin et al., 1997; Gang et al., 1999). The corresponding (+)-pinoresinol (**1a**) forming dirigent protein is a homodimer (with a propensity to aggregate), and is apparently mainly of β-sheet character (Halls and Lewis, 2002). Interestingly, in western red cedar (*Thuja plicata*), there exists a multigene family encoding (+)-pinoresinol forming dirigent proteins, indicative of differential control of lignan formation in the vascular apparatus of different tissues and organs (Kim et al., 2002a,b).

Based on more recent detailed kinetic analyses, the mode of action of the (+)-pinoresinol forming dirigent protein appears to involve capture of free-radical (coniferyl alcohol (**2**) derived) moieties in open solution, with the resulting bound substrate molecules undergoing stereoselective bimolecular phenoxy radical coupling, intramolecular cyclization and product release (Halls, Davin, Kramer, and Lewis, manuscript in preparation). There is now a growing number of examples involving control of bimolecular phenoxy radical–radical coupling processes in vivo, e.g. to afford (−)-pinoresinol (**1b**) in *Linum usitatissimum* (unpublished results, this laboratory) or to afford marchantin C (**3**) from lunularic acid (**4**) in *Marchantia polymorpha* (Friederich

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et al., 1999); interesting, in the latter organism, regio- rather than stereo-selective control of phenolic radical–radical coupling apparently occurs.

The ferns (pteridophytes) are generally considered to be evolutionary forerunners of the gymnosperms and angiosperms. Of these, members of the Blechnaceae, *Blechnum orientale* and *B. spicant* (deer fern), accumulate the rather unusually 8–2′ linked, optically active, lignans, (−)-blechnic (**5**) and (−)-brainic (**6**) acids (Wada et al., 1992; Wang et al., 2001), which are presumed to be caffeic acid (**7**) derived (discussed later).

The main objective of this study was thus to clarify the biosynthetic pathway to the optically active 8–2′ linked lignans, (−)-blechnic (**5**) and (−)-brainic (**6**) acids, in *B. spicant*. As described below, following uptake and metabolism of various potential precursors of the lignans **5** and **6**, it is concluded at this time that the initial coupling product is *cis*-blechnic acid (**8**), whose further metabolism affords both **5** and **6**, respectively.

2. Results and discussion

2.1. B. spicant metabolite analysis: isolation and identification of cis-blechnic acid (8)

It was first instructive to conduct a metabolite analysis of the methanol soluble moieties extracted from *B. spicant* fronds in order to identify the phenolic components present. Thus, preparative μBondapak reversed-phase (C_{18}) HPLC afforded as the principal metabolites, the known compounds caffeic acid (**7**), 5-*O*-caffeoyl shikimate (**13**) (Veit et al., 1992), 7-*epi*-blechnic acid (**14**), (−)-*trans*-blechnic acid (**5**), (−)-*trans*-brainic acid (**6**) (Wada et al., 1992; Wang et al., 2001), and an unknown compound (**8**). The known metabolites were identified either by comparison of their mass spectral fragmentation patterns (EI-MS mode), and ^{1}H and ^{13}C NMR spectra with literature data, or in the case of (**5**) and (**6**) by HPLC co-elution with authentic standards as well.

The unknown compound (**8**), on standing in open solution (CH_3CN or H_2O) for 72 h under incandescent lighting, was found to be slowly converted into a component with the same HPLC retention volume and UV spectrum as that of (−)-*trans*-blechnic acid (**5**). Mass spectral analysis further revealed that it was isomeric with (**5**); it was also optically active ($[\alpha]_D^{20} = -128^\circ$, *c* 0.58, MeOH). Comparison of the ^{1}H and ^{13}C NMR spectra to that of (**5**), including 2D-NMR spectral analyses (^{1}H–^{1}H COSY, HMQC and HMBC), finally revealed that the unknown was *cis*-blechnic acid (**8**) as described below. That is, its ^{1}H NMR spectrum (Table 1) established the presence of five aromatic protons at δ7.25 (1H, *d*, *J* = 8.3 Hz, H-6′), 6.95 (1H, *d*, *J* = 1.7 Hz, H-2), 6.82 (1H, *dd*, *J* = 1.7, 8.3 Hz, H-6), 6.74 (1H, *d*, *J* = 8.3 Hz, H-5) and 6.72 (1H, *d*, *J* = 8.3 Hz, H-5′), as well as two *cis*-benzofuran ring protons at δ5.91 (1H, *d*, *J* = 9.5 Hz, H-7) and 4.50 (1H, *d*, *J* = 9.5 Hz, H-8) and two *cis*-double bond protons at δ6.74 (1H, *d*, *J* = 12.7 Hz, H-7′) and 5.88 (1H, *d*, *J* = 12.7 Hz, H-8′), respectively. Its ^{13}C NMR spectrum (Table 1) also established the presence of eighteen carbon resonances, with both the ^{1}H and ^{13}C NMR spectra of (**8**) and (**5**) being very similar, except for the presence of a *cis*-double bond in (**8**) [^{1}H NMR: δ6.74 (1H, *d*, *J* = 12.7 Hz, H-7′), δ5.88 (1H, *d*, *J* = 12.7 Hz, H-8′); ^{13}C NMR: δ139.7 (C-7′) and 120.2 (C-8′)] and the *trans*-double bond in blechnic acid (**5**) [^{1}H NMR: δ7.57 (1H, *d*, *J* = 16.1 Hz, H-7′), δ6.27 (1H, *d*, *J* = 16.1 Hz, H-8′); ^{13}C NMR: δ143.3 (H-7′) and 117.8 (H-8′)]. Correlations (or connectivities) for compound (**8**) were further established by 2D-NMR (^{1}H–^{1}H COSY, HMQC, HMBC) spectroscopic analyses (Table 2) conducted at low temperature (−25 °C), with additional proof of the *cis*-double bond being obtained by both NOE experiments and comparison to that of (−)-*trans*-blechnic acid (**5**). Finally, irradiation of (−)-*trans*-blechnic acid (**5**) for 10 min using an ultraviolet lamp (λ 254 nm) afforded compound (**8**) (as a mixture of (**8**):(**5**) in an ~1:7 ratio) as evidenced by HPLC, UV and mass spectral analyses (data not shown). Thus, the unknown was unambiguously identified as (−)-*cis*-blechnic acid (**8**). [Traces of a presumed *cis*-brainic acid were also detected, as evidenced by both UV and mass spectral analyses (data not shown); however, under the conditions employed it was not present in sufficient amount for unambiguous identification.]

Table 1
NMR spectroscopic data for (−)-*trans*-blechnic (**5**) and (−)-*cis*-blechnic (**8**) acids

Carbon number	(−)-*trans* Blechnic acid (**5**)		(−)-*cis* Blechnic acid (**8**)	
	^{13}C	^{1}H (Hz)	^{13}C	^{1}H (Hz)
1	129.3		129.2	
2	115.1	6.96 (*d*, 1.6)	114.7	6.95 (*d*, 1.7)
3	145.9		145.8	
4	146.5		146.2	
5	115.9	6.75 (*d*, 8.4)	115.5	6.74 (*d*, 8.3)
6	119.7	6.85 (*dd*, 1.6, 8.4)	119.3	6.82 (*dd*, 1.7, 8.3)
7	88.4	5.95 (*d*, 9.4)	88.0	5.91 (*d*, 9.5)
8	55.5	4.59 (*d*, 9.4)	55.3	4.50 (*d*, 9.5)
9	173.7		173.9	
1′	124.5		124.8	
2′	129.2		129.1	
3′	149.5		148.5	
4′	145.0		143.7	
5′	118.0	6.81 (*d*, 8.4)	116.7	6.72 (*d*, 8.3)
6′	122.6	7.12 (*d*, 8.4)	124.1	7.25 (*d*, 8.3)
7′	143.3	7.57 (*d*, 16.1)	139.7	6.74 (*d*, 12.7)
8′	117.8	6.27 (*d*, 16.1)	120.2	5.88 (*d*, 12.7)
9′	170.7		170.2	

See Experimental for spectral acquisition details.

Table 2
2D-NMR spectroscopic correlations for (−)-*cis*-blechnic (**8**) acid

Carbon number	^{1}H–^{1}H COSY	HMQC	HMBC
1			
2		H2-C2	H2-C4, H2-C6, H2-C7
3			
4			
5	H5-H6	H5-C5	H5-C3, H5-C1
6		H6-C6	H6-C4, H6-C2, H6-C7
7	H7-H8	H7-C7	H7-C9, H7-C1, H7-C6, H7-C2, H7-C8
8		H8-C8	H8-C9, H8-C3, H8-C2, H8-C1, H8-C7,
9			
1′			
2′			
3′			
4′			
5′	H5′-H6′	H5′-C5′	H5′-C3′, H5′-C1′
6′		H6′-C6′	H6′-C2′, H6′-C4′, H6′-C7′
7′	H7′-H8′	H7′-C7′	H7′-C9′, H7′-C1′
8′		H8′-C8′	H8′-C1′, H8′-C9′
9′			

2D NMR spectra (^{1}H–^{1}H COSY, HMQC and HMBC) were recorded at low temperature (−25 °C), using CD_3OD as solvent, with a Varian Mercury 300 spectrometer (see Experimental).

2.2. Uptake and metabolism of potential [^{14}C] radiolabeled precursors of Blechnum lignans

The next question was whether the optically active lignans (**5**), (**6**) and (**8**) were direct products of acetate or phenylpropanoid metabolism, since to our knowledge this had not been investigated in the pteridophytes. Thus, [2-^{14}C]NaOAc, L-[U-^{14}C]Phe (**9**), L-[U-^{14}C]Tyr (**10**), [9-^{14}C]cinnamic acid (**11**) and [8-^{14}C]-*p*-coumaric acid (**12**) were individually administered to *B. spicant* fronds for periods ranging from 4 to 84 h; these experiments utilized freshly cut young fronds (new growth, circa 10–15 cm length and ~0.5 g fresh weight each) which were individually placed into microcentrifuge tubes (1.5 ml) containing the corresponding test precursor (~200 μl solution), with incubations being carried out at ambient temperature and under continuous incandescent light. Following incubation and metabolism of each potential precursor, the fronds from each administration experiment were subjected to an extraction protocol (see Experimental) with the fractions corresponding to caffeic acid (**7**), 5-*O*-caffeoyl shikimate (**13**), (−)-*cis*-blechnic acid (**8**), (−)-*trans*-blechnic acid (**5**) and (−)-brainic acid (**6**) isolated as before and subjected to radiochemical analysis (Table 3).

As expected, neither [2-^{14}C]NaOAc nor [U-^{14}C]Tyr (**10**) were incorporated into metabolites (**5**)–(**8**) and (**13**), in accordance with them being neither directly derived from acetate nor downstream products of tyrosine ammonia lyase metabolism. On the other hand, L-[U-^{14}C]Phe (**9**), [9-^{14}C]cinnamic (**11**) and [8-^{14}C]-*p*-coumaric (**12**) acids were all efficiently incorporated into the *B. spicant* metabolites (**5**), (**8**) and (**13**). Thus, with L-[U-^{14}C]Phe (**9**), it was initially metabolized within 12 h into caffeic acid (**7**), 5-*O*-caffeoyl shikimate (**13**) and (−)-*cis*-blechnic acid (**8**), respectively, but not into either (−)-*trans*-blechnic (**5**) or (−)-*trans*-brainic (**6**) acids (see Table 3). On the other hand, L-Phe (**9**) uptake and metabolism over longer periods of time up to 84 h resulted in a steady increase in incorporation into lignans (**5**) and (**6**), as well as being incorporated into the other metabolites (**7**), (**8**) and (**13**).

Additionally, when both [9-^{14}C]cinnamic (**11**) and [8-^{14}C]-*p*-coumaric (**12**) acids were individually deployed as potential precursors, incorporation into all five possible intermediates (**5**)–(**8**) and (**13**) was again observed. These data, when taken together, thus establish that in *B. spicant*, L-Phe (**9**) was metabolized into cinnamic acid (**11**), with the latter being subsequently converted into *p*-coumaric (**12**) and caffeic (**7**) acids, 5-*O*-caffeoyl shikimate (**13**) and the lignans (**5**), (**6**) and (**8**). These findings are therefore consistent with pteridophyte PAL being monofunctional and unable to metabolize L-Tyr (**10**). The radiochemical time course incorporation data using L-Phe (**9**) also suggested that the initial lignan stereoselective coupling product is *cis* (and not *trans*)-blechnic acid (**8**), which can then be metabolized further into lignans (**5**) and (**6**); however, this tentative interpretation needs to be confirmed by demonstration of the corresponding enzymatic conversions in vitro, since **8** could also be a shunt product. Additionally, these findings raise questions about how the second aromatic hydroxyl group is introduced and when, i.e. leading to caffeic acid (**7**), 5-*O*-caffeoyl shikimate (**13**), (−)-*trans*-brainic acid (**5**) and (−)-blechnic acid (**6**), respectively. This could result from either direct hydroxylation of *p*-coumarate (**12**) or via its introduction into the corresponding quinate/shikimate ester conjugates; in other plant systems, there appears to be evidence for two such pathways (Schoch et al., 2001; Franke et al., 2002a,b; Nair et al., 2002).

2.3. Uptake and metabolism of L-[1-^{13}C], [2-^{13}C] and [3-^{13}C]phenylalanines (**9**), [2-^{13}C]-p-coumaric acid (**12**) and [2-^{13}C]caffeic acid (**7**) into the Blechnum lignans (**5**) and (**6**)

In order to confirm and extend the radiochemical incorporation data, administration experiments using the carbon-13 labeled precursors L-Phe (**9**), *p*-coumaric (**12**) and caffeic (**7**) acids, respectively, were next carried out. However, in order to identify the Phe (**9**) concentrations (and amounts) needed for optimal incorporation into the *B. spicant* lignans (**5**) and (**6**), various test solutions of 0.05, 0.1, 0.5, 1.0 and 2.0 mM natural

Table 3
Comparison of efficacy of [^{14}C-labeled] potential precursors into various *B. spicant* phenolic metabolites (% incorporation)

	Potential precursor administered (radioactivity)	Metabolic period (h)	Caffeic acid (**7**)	5-*O*-Caffeoyl shikimate (**13**)	(−)-*cis*-Blechnic acid (**8**)	(−)-*trans*-Blechnic acid (**5**)	(−)-Brainic acid (**6**)
A	[2-^{14}C]NaOAc (185 kBq)	5	0.0	0.0	0.0	0.0	0.0
		18	0.0	0.0	0.0	0.0	0.0
		26	0.0	0.0	0.0	0.0	0.0
B	[U-^{14}C]Tyr (**10**) (185 kBq)	36	0.0	0.0	0.0	0.0	0.0
C	[U-^{14}C]Phe (**9**) (148 kBq)	4	1.2	1.7	1.1	<0.1	<0.1
		8	2.3	3.4	3.0	<0.1	0.1
		12	2.3	2.5	2.6	0.1	0.4
		18	2.0	3.2	3.9	0.5	1.4
		24	2.9	4.1	2.3	0.6	1.6
		30	2.9	5.1	2.7	0.7	1.6
		36	5.0	4.7	2.0	0.7	1.9
		48	4.5	5.3	1.4	1.1	2.9
		84	2.8	3.3	1.3	2.1	4.2
D	[9-^{14}C]Cinnamic acid (**11**) (185 kBq)	24	2.9	6.9	3.5	2.0	1.5
		50	3.8	5.6	1.0	1.1	1.4
		84	2.7	5.6	1.2	1.5	1.2
E	[8-^{14}C]-*p*-Coumaric acid (**12**) (148 kBq)	24	1.0	3.0	1.4	0.5	0.7

abundance L-Phe (**9**) (each containing 4 μCi of L-[U-^{14}C]Phe (**9**)) were administered to *B. spicant* fronds for varying periods. From the results so obtained (data not shown), optimal incorporations could be obtained using 1 mM L-Phe (**9**) and a metabolism period of 5-days. Accordingly, L-[1-^{13}C], [2-^{13}C] and [3-^{13}C]Phe (**9**) (1 mM) were next administered individually to *B. spicant* fronds, with the lignans (**5**) and (**6**) purified by prep HPLC as before. The [^{13}C]-NMR spectra (Figs. 1 and 2) of the resulting isotopically enriched lignans **6** and **5** are shown in Fig. 1B–D for brainic acid (**6**), and in Fig. 2B–D for blechnic acid (**5**), respectively (discussed below). For comparison purposes, the natural abundance carbon-13 spectra of both *trans*-brainic acid (**6**) and *trans*-blechnic acid (**5**) are also included (Figs. 1A and 2A), whose assignments were based on exhaustive 2D NMR spectroscopic analyses (HMQC, HMBC and ^{1}H–^{1}H COSY) and which are in general agreement with those reported by Wada et al. (1992) [see Table 1 for *cis*-/*trans*-blechnic acids (**8**) and (**5**)].

The spectra of (−)-*trans*-brainic (**6**) (Fig. 1B–D) and (−)-*trans*-blechnic (**5**) (Fig. 2B–D) acids, following individual administration of L-[3-^{13}C], L-[2-^{13}C] and L-[1-^{13}C]Phe (**9**), established that L-Phe (**9**) was intactly incorporated into both lignan skeleta. That is, following uptake and metabolism of L-[1-^{13}C]Phe (**9**), the (–)-*trans*-brainic (**6**) and (−)-*trans*-blechnic (**5**) acids so obtained displayed carbon-13 enriched resonances for C-9 and C-9′ of (−)-*trans*-brainic acid (**6**) at δ170.5 and 170.6 ppm (Fig. 1D), and at δ173.7 and 170.7 ppm for (−)-*trans*-blechnic acid (**5**) (Fig. 2D), respectively. In a similar manner, administration of L-[2-^{13}C]Phe (**9**) gave enhanced signals for the C-8 and C8′ resonances of (−)-*trans*-brainic (**6**) acid at δ55.4 and 118.2 ppm (Fig. 1C), and at δ 55.5 and 117.8 ppm for (−)-*trans*-blechnic acid (**5**) (Fig. 2C), whereas with L-[3-^{13}C]Phe (**9**), enhanced signals were observed for C-7 and C7′ at δ88.0 and 142.7 ppm for (**6**) (Fig. 1B), and δ88.4 and 143.3 ppm for (**5**) (Fig. 2B). Note also that the relative carbon-13 enrichments were also higher (more intense) for (−)-*trans*-brainic acid (**6**) than for (−)-*trans*-blechnic acid (**5**), due in large part to the much larger endogenous levels of the latter in *B. spicant*. Additionally, the administration of [8-^{13}C]-*p*-coumaric acid (**12**) to *B. spicant* fronds afforded similar results, with, for example, carbon-13 enhancements for C-8 and C-8′ at δ 55.4 and 118.2 for (−)-*trans*-brainic acid (**6**) (data not shown). On the other hand, administration of [8-^{13}C]caffeic acid (**7**) to the *B. spicant* fronds was unsuccessful, this presumably being due to either the toxic nature of this precursor or in its failure to be transported to the site(s) of biosynthesis.

2.4. Concluding remarks

It can be inferred from these findings that the 8–2′ linked lignans, (−)-*trans*-blechnic (**5**) and (−)-*trans*-brainic (**6**) acids, result from direct oxidative coupling of

two caffeic acid (**7**) moieties, as depicted in Scheme 1, i.e. *via* stereoselective coupling of two molecules of either *cis*- or *trans*-caffeic acid (**7**) to first afford (−)-*cis*-blechnic acid (**8**). Alternatively, but less likely, stereoselective coupling could involve two molecules of *p*-coumaric acid (**12**) with subsequent hydroxylation of the aromatic ring to ultimately afford the corresponding catechols intermediates.

Interestingly, one electron oxidation of caffeic acid (**7**) in vitro using nonspecific oxidases does not apparently afford either *cis*- or *trans*-blechnic acids (**8**) and (**5**). On the other hand, when one electron oxidases, such as polyphenol oxidases (PPO), are incubated with caffeic acid (**7**), the corresponding *O*-quinone is initially formed (Brown, 1967). Furthermore, in the presence of molecules possessing nucleophilic functionalities, such as the thiols, 2-mercaptoethanol, glutathione and cysteine, the resulting *O*-quinone undergoes non-enzymatic nucleophilic attack to form various addition products linked to the aromatic ring. Of these, the 2-position is the most electrophilic, and nucleophilic addition with thiol compounds occurs there preferentially (Ito and Prota, 1977; Cheynier et al., 1986; Salgues et al., 1986), this being followed, upon reoxidation as before, by addition of nucleophiles to the 5-position (Cilliers and Singleton, 1989; Negishi and Ozawa, 2000). In the absence of thiols, the quinone radicals or corresponding quinone methides undergo oxidative coupling in a process typically described as an "enzymatically induced browning phenomenon" (Brown, 1967). It has been suggested that

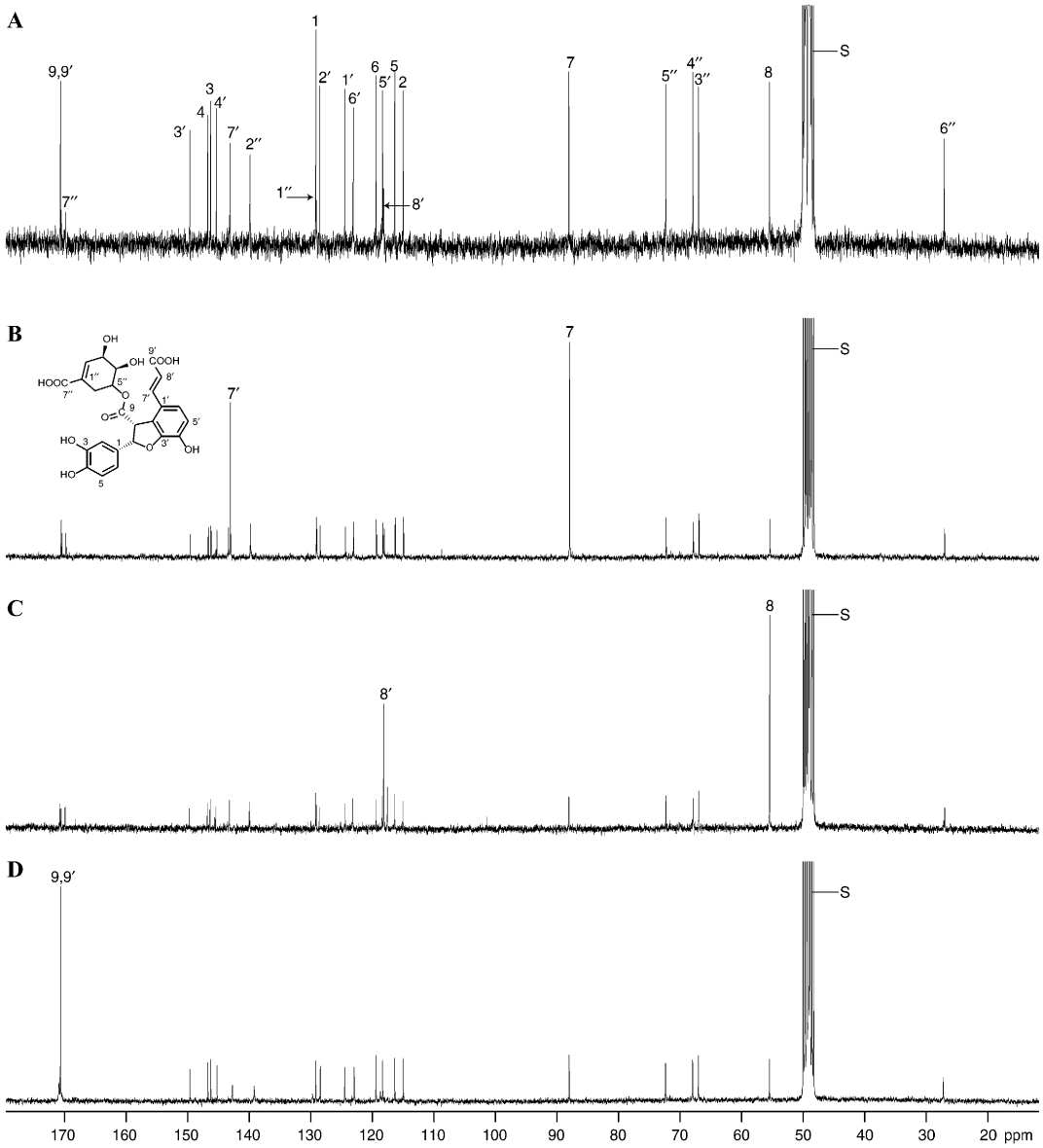


Fig. 1. ^{13}C NMR spectra of **A.** natural abundance (−)-brainic acid (**6**), as well as (−)-brainic acids (**6**) obtained following administration of **B.** [3-^{13}C], **C.** [2-^{13}C] and **D**. [1-^{13}C]-phenylalanine (**9**) (1 mM) to *B. spicant* fronds for 5 days; all spectra were recorded under identical conditions.

the products so formed are typically dimers or trimers with extended conjugation into the visible region (Cilliers and Singleton, 1989) However, even though the description and characterization of lignan products resulting from enzymatic (PPO) and non-enzymatic ($KMnO_4$, Ag_2O, O_2) oxidation of caffeic acid (**7**) in vitro has been reported (Nahrstedt et al., 1990; Cilliers and Singleton, 1991; Frías et al., 1991; Pieters et al., 1999; Rompel et al., 1999) these afford either only racemic or achiral compounds having dioxane, 8–5′ linked furan and 8–8′ linked cyclohexene bridge types. That is, no products with 8–2′ linkages have been reported through oxidative coupling of caffeic acid (**7**) in vitro. Clearly elucidating the biochemical pathway in the future to (−)-*cis*-blechnic (**8**), *trans*-blechnic (**5**) and *trans*-brainic (**6**) acids should be most instructive.

3. Experimental

3.1. Plant material

Blechnum spicant (deer fern) plants were purchased from NATS Nursery Ltd. (Surrey, British Columbia Canada) and maintained in Washington State University greenhouse facilities until needed.

3.2. General procedures

The ^1H NMR and NOE spectra of (−)-*cis* and (−)-*trans* blechnic acids (**8** and **5**) were acquired using a Varian Inova 500 spectrometer (499.86 MHz for ^1H), whereas all other NMR spectra were obtained on a Varian Mercury 300 MHz spectrometer (300.1 MHz for

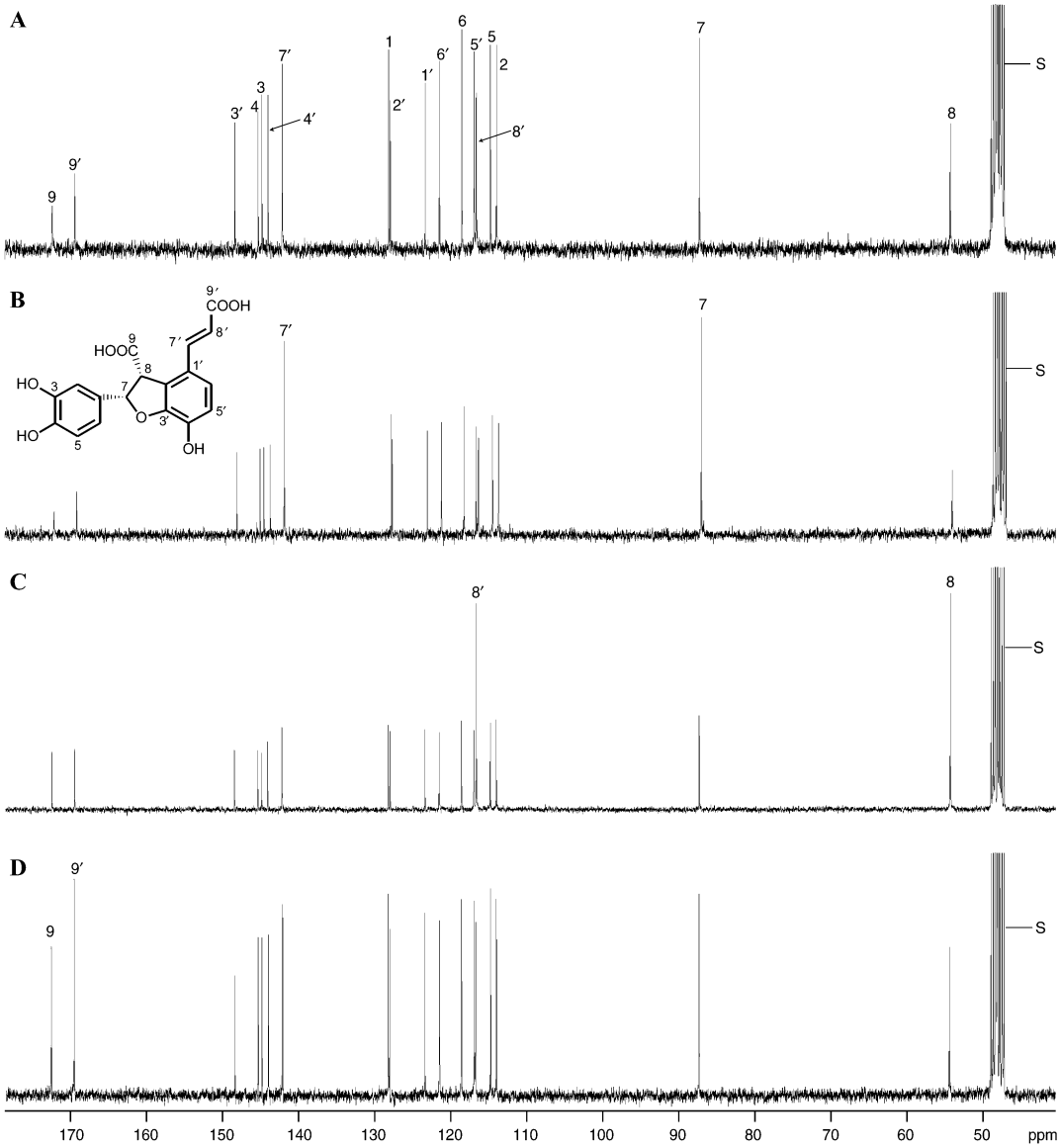
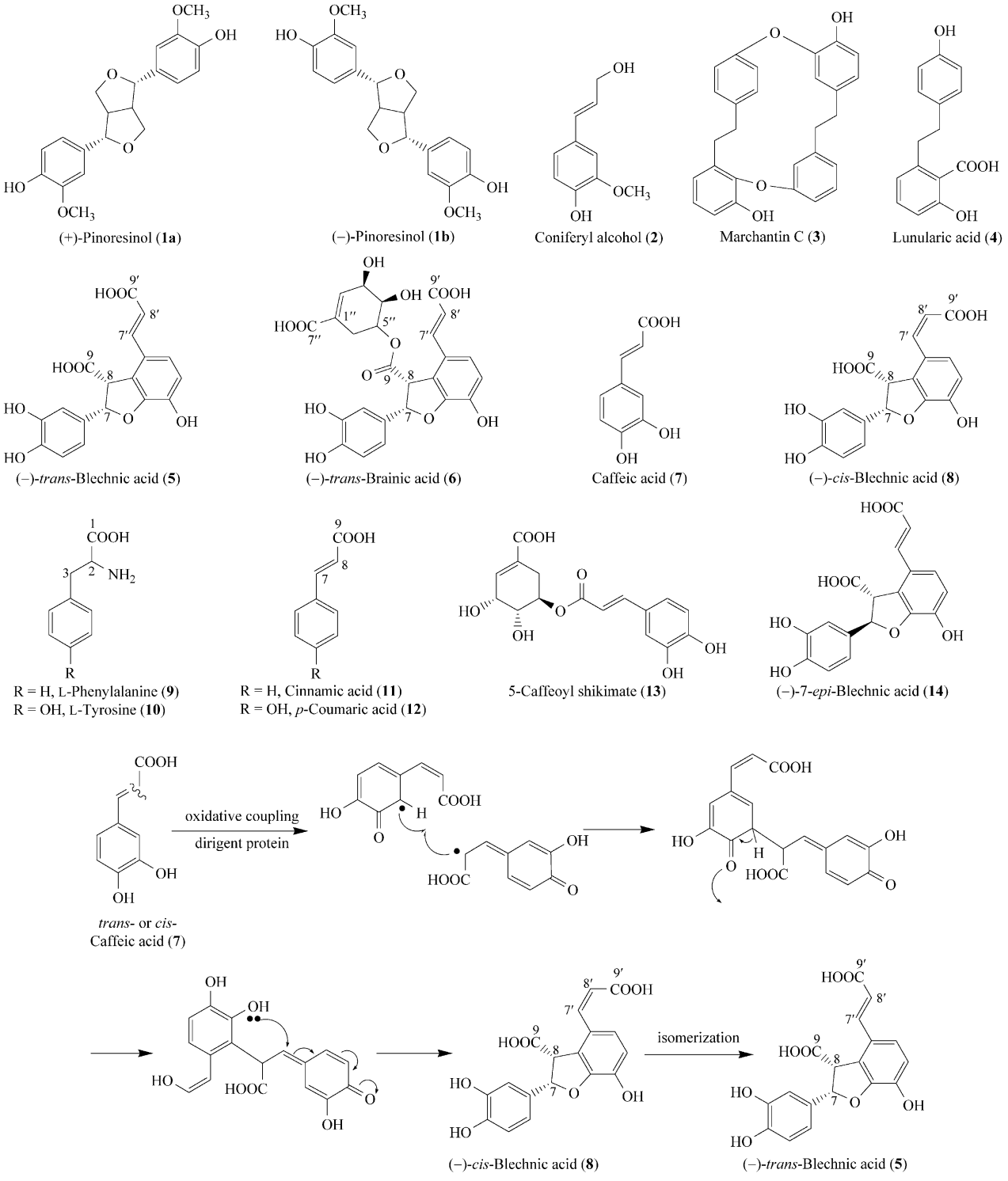


Fig. 2. ^{13}C NMR spectra of **A.** natural abundance (−)-*trans*-blechnic acid (**5**), as well as (−)-*trans*-blechnic acids (**5**) obtained following administration of **B.** [3-^{13}C], **C.** [2-^{13}C] and **D.** [1-^{13}C]-phenylalanine (**9**) (1 mM) to *B. spicant* fronds for 5 days; all spectra were recorded under identical conditions.

^{1}H and 75.5 MHz for ^{13}C, respectively). In all cases, deuterated methanol (CD_3OD, δ_c 49.15 ppm, δ_H 3.31 ppm) was used both as solvent and for referencing NMR chemical shifts, with *J* values reported in Hz. For both isotopically (^{13}C) labeled and natural abundance compounds, the ^{13}C NMR spectra were obtained using solutions of the same concentrations and recorded under the same acquisition conditions.

HMBC spectra were collected at either ambient temperature or at −25 °C on a Varian Mercury 300.1 MHz for ^{1}H and 75.5 MHz for ^{13}C. Sweep widths were 2765 and 10,264 Hz in F2 and F1 respectively with



Scheme 1. Proposed stereoselective coupling of caffeic acid (**7**) leading to (−)-*trans*-blechnic (**5**) and (−)-brainic (**6**) acids.

acquisition times of 208 and 6.2 ms in $t2$ and $t1$ respectively. A relaxation delay of 1.5 seconds was used between scans and the experiments were optimized for long-range ^{n}J ^{1}H–^{13}C couplings of 4 or 8 Hz. A 140 Hz low pass J filter was used to eliminate the ^{1}J ^{1}H–^{13}C coupling and a total of 64 scans were acquired per $t1$ point. Data were collected using the States-TPPI method (128 * 2 complex points in $t1$) and processed in the mixed F1-absorption/F2-magnitude mode (Nagayama, 1986). The data were apodixed in $t2$ with a sinebell weighting function, zero filled to 2 K points followed by Fourier transformation. The data in $t1$ were linear predicted from 128 to 256 points, apodized with a cosine function, zero filled to 2 K points and Fourier transformed.

HMQC spectra were collected at either ambient temperature or at −25 °C on a Varian Mercury 300.1 MHz for ^{1}H and 75.5 MHz for ^{13}C. Sweep widths were 2765 and 8240 Hz in F2 and F1 respectively with acquisition times of 185 ms and 7.8 ms in $t2$ and $t1$, respectively. A relaxation delay of 0.9 s was used between scans and a total of 64 scans were acquired per $t1$ point. Data were collected using the States-TPPI method (128 * 2 complex points in $t1$) and apodixed in $t2$ with a cosine weighting function, zero filled to 2K points and Fourier transformed. The data in $t1$ were linear predicted from 128 to 256 points, apodized with a cosine function, zero filled to 2 K points and Fourier transformed.

COSY spectra were collected at either ambient temperature or at −25 °C on a Varian Mercury 300 with a sweep width of 2699 Hz in F1 and F2. Data were collected with 128 increments in $t1$ and processed in the magnitude mode using sinebell weighting functions in both dimensions. Data were zero filled to 2 by 2 K after linear prediction of the $t1$ data points from 128 to 256.

Carbon-13 spectra were acquired at ambient temperature or at −25 °C on a Varian Mercury 300 at 75.5 MHz using a sweep width of 16,980 Hz. A total of 17,000 transients were recorded using a delay between scans of 1.0 s and an acquisition time of 1.75 s. A flip angle of 45° was used for ^{13}C and continuous WALTZ-16 decoupling of the protons was employed. Data were apodized with 1.0 Hz of exponential line broadening, zero filled to 128 K points and Fourier transformed.

UV spectra were obtained on a Lambda 6 UV/vis spectrophotometer (Perkin-Elmer), whereas electron impact (EI) mass spectral (MS) analyses utilized a Waters Integrity HPLC/MS system at an ionization voltage of 70 eV. Reversed phase high performance liquid chromatography (HPLC) employed a Waters HPLC system as described by Anterola et al. (1999) for either analytical or preparative separations. Analytical HPLC (System A) utilized a Waters Nova-Pak C_{18} column (4 μm, 150×3.9 mm i.d., stainless steel) eluted with MeCN/3% HOAc in H_2O (A:B) at a flow rate of 1 ml min^{-1} with detection at 280 nm as follows: A:B (2:98) from 0 to 5 min, then an A:B linear gradient from 2:98 to 30:70 between 5 min and 40 min. Preparative HPLC (System B) employed a Waters μBondapak C_{18} column, (10 μm, 300×19 mm i.d., stainless steel) eluted with MeCN/3% HOAc in H_2O (A:B) at a flow rate of 10 ml min^{-1} with detection at 280 nm as follows: A:B (5:95) from 0 to 2 min, then an A:B linear gradient from 5:95 to 19:81 between 2 and 45 min and finally from 19:81 to 30:70 between 43 and 55 min. As needed, radioactive samples were analyzed in ScintiVerse (Fischer Scientific) and measured using a liquid scintillation counter (Packard, Tricarb 2000 CA).

3.3. Chemicals

All HPLC solvents were of HPLC grade, whereas all other chemicals (except those stated below) were reagent grade or better. L-[1-^{13}C], [2-^{13}C] and [3-^{13}C]Phenylalanine (**9**) (99% atom ^{13}C) were obtained from Cambridge Isotope Laboratories, whereas L-[U-^{14}C]phenylalanine (**9**) (18.5 GBq/mmol), L-[U-^{14}C]tyrosine (**10**) (18.4 GBq/mmol), [2-^{14}C]sodium acetate (111 MBq/mmol), [9-^{14}C]-cinnamic acid (**11**) (1.89 GBq/mmol) and [2-^{14}C]malonic acid (1.48-2.22 GBq/mmol) were purchased from American Radiolabeled Chemicals, Inc. [8-^{14}C]-*p*-Coumaric acid (**12**) (6.07 MBq/mmol), [8-^{13}C]-*p*-coumaric acid (**12**) and [8-^{13}C]caffeic acid (**7**) were synthesized (Neish, 1959) by reaction of 4-hydroxybenzaldehyde/3,4-hydroxybenzaldehyde, respectively, (Sigma-Aldrich) with either [2-^{14}C]malonic acid or [2-^{13}C]malonic acid (99% atom ^{13}C, Sigma-Aldrich), in the presence of dry pyridine, aniline and piperidine, respectively.

3.4. Isolation of phenylpropanoid and lignan metabolites from *B. spicant*

B. spicant fronds (215 g dry wt.) were ground to a powder and extracted with MeOH (3×1 l). The resulting MeOH solubles were evaporated in vacuo, with the corresponding residue reconstituted in H_2O (300 ml). After partition with petrol, the aqueous solubles were dried and subjected to silica gel column chromatography (40×4.5 cm) eluted with $CHCl_3$–MeOH in ratios of 30:1, 20:1, 15:1, 10:1 and 6:1 (2 l each) and finally with $CHCl_3$–MeOH–H_2O–HOAc (30:10:1:1; 2 l).

Fractions containing **13** were combined, evaporated in vacuo, with the residue applied to a silica gel column (30×2 cm) eluted with $CHCl_3$–MeOH–H_2O–HOAc (100:10:1:1), (80:10:1:1), (60:10:1:1) and (60:10:1:1) (800 ml each). The eluate from the latter (60:10:1:1) was concentrated and further purified by silica gel column chromatography (30×1.5 cm) eluted with $CHCl_3$–MeOH–H_2O–HOAc (70:10:1:1; 1 l); fractions containing **13** were concentrated, filtered and further subjected to preparative HPLC (System B) to give 5-*O*-caffeoyl shikimate (**13**) (120 mg).

Fractions containing lignans **5**, **6**, and **14** were combined, concentrated and applied to a Sephadex LH-20 column (40×2.5 cm) eluted with H_2O, MeOH–H_2O (30:70 and 70:30, respectively) and MeOH (1 l each). The 70:30 MeOH–H_2O eluate containing lignans **5**, **6** and **14** was concentrated and further subjected to silica gel column chromatography (40×2.5 cm) eluted with $CHCl_3$–MeOH–H_2O–HOAc (40:10:1:1) (2 l) to give 7-*epi*-blechnic acid (**14**) (50 mg), (−)-*trans*-blechnic acid (**5**) (1.2 g), and (−)-*trans*-brainic acid (**6**) (180 mg).

3.5. Isolation of (−)-*cis*-blechnic acid (*8*) from *B. spicant*

B. spicant fronds (150 g, fresh wt) were ground to a powder and extracted with MeOH (400 ml) at 4 °C. The resulting MeOH solubles were next combined and evaporated to dryness in vacuo, with the residue so obtained reconstituted in cold H_2O (50 ml). Following extraction with hexanes (3×50 ml) at 4 °C, the remaining aqueous solubles were concentrated in vacuo, and subjected to silica gel column chromatography (15×2 cm) eluted with $CHCl_3$–MeOH–H_2O–HOAc (80:20:1:1; 1 l). Fractions containing **8** were combined, concentrated, filtered and further subjected to preparative HPLC separation (System B) to give (−)-*cis*-blechnic acid (**8**) (∼10 mg)

(−)-*cis*-Blechnic acid (**8**): white amorphous powder; $[\alpha]_D^{20}$ −128°, (*c* 0.58, MeOH); MALDI-MS: 381 $[M+Na]^+$; positive ion: 739 $[2M+Na]^+$, 381 $[M+Na]^+$; negative ion: 715 $[2M\text{-}1]^+$. UV (λ_{max} MeOH): 238, 280 nm. For ^1H and ^{13}C NMR spectral analyses, see Tables 1 and 2.

3.6. Cis-trans isomerization of cis- and trans-blechnic acid (*8*) and (*5*)

Trans-blechnic acid (**5**) (50 mg) was dissolved in distilled H_2O (2 ml). The resulting solution was placed in a quartz tube, and irradiated for 10 min with a Lifeguard mercury arc lamp (Philips, 400 W) whose outer shell had been removed as described in Lewis et al. (1989). HPLC analysis of the resulting reaction mixture showed two components in an ∼1:7 ratio (by UV at 280 nm) with elution volumes of 26.7 and 28.9 ml corresponding to *cis*- and *trans*-blechnic acid (**8**) and (**5**), respectively.

3.7. Administration of L-[U-^{14}C]Phe (*9*), L-[U-^{14}C]Tyr (*10*), [9-^{14}C]cinnamic acid (*11*), [8-^{14}C]*p*-coumaric acid (*12*) and [2-^{14}C]NaOAc to *B. spicant* fronds and isolation of phenolic metabolites

Freshly excised *B. spicant* fronds (new growth, ca. 10–15 cm long, ∼0.5 g fresh weight) were individually placed into Eppendorf microcentrifuge tubes (1.5 ml) containing aqueous solutions (200 μl) of each potential precursor, i.e. L-[U-^{14}C]Phe (**9**, 148 kBq), L-[U-^{14}C]Tyr (**10**, 185 kBq), [9-^{14}C]cinnamic acid (**11**, 185 kBq), [8-^{14}C]*p*-coumaric acid (**12**, 148 kBq) and [2-^{14}C]NaOAc (185 kBq), respectively. Each precursor administration was carried out at ambient temperature under continuous incandescent light, with distilled water being added to replenish the original precursor solution as needed over the duration of the experiment. Uptake and metabolism periods were as follows: 4, 8, 12, 18, 24, 30, 36, 48 and 84 h for L-[U-^{14}C]Phe (**9**), 36 h for L-[U-^{14}C]Tyr (**10**), 24, 50 and 84 h for [9-^{14}C]cinnamic acid (**11**), 24 h for [8-^{14}C]*p*-coumaric acid (**12**) and 5, 18 and 26 h for [2-^{14}C]NaOAc, respectively. For each experiment, fronds were individually frozen (liq. N_2), then ground to a fine powder in a mortar by means of a pestle, with the resulting powder being successively extracted with MeOH (3×5 ml) at ∼4 °C. [All extractions were carried out with illumination from a green safety light.] The resulting extracts from each particular precursor administration were then individually combined, concentrated in vacuo to ca. 1 ml, centrifuged (16,000 *g*), with an aliquot (100 μl) of the resulting supernatant subjected to reversed phase analytical HPLC analysis (System A). Fractions corresponding to caffeic acid (**7**), 5-*O*-caffeoyl shikimate (**16**), (−)-*cis*-blechnic (**8**), (−)-*trans*-blechnic (**5**) and (−)-brainic (**6**) acids were individually collected and subjected to scintillation counting determination (see Table 3).

3.8. Administration of L-[1-^{13}C], [2-^{13}C] and [3-^{13}C]Phe (*9*), [8-^{13}C]*p*-coumaric (*12*) and [8-^{13}C]caffeic (*7*) acids to *B. spicant* fronds and incorporation into (−)-*trans*-blechnic (*5*) and (−)-brainic (*6*) acids

Aqueous solutions (1 M, 300 ml) of L-[1-^{13}C], [2-^{13}C] and [3-^{13}C]Phe (**9**) (99% atom ^{13}C) were prepared and individually aliquoted (1 ml) into microcentrifuge tubes (1.5 ml), each of which contained a *B. spicant* frond (new growth, ca. 10–15 cm length, ∼0.5 g fresh wt, with ca. 60–70 fronds being utilized per precursor). Each experiment was carried out for a 5 day duration with continuous incandescent light, with replenishment of precursor solution as needed. Following uptake and metabolism of each precursor over 5 days, the fronds for each particular precursor administration were collected, frozen (liq. N_2), ground to a powder (mortar and pestle) and extracted with MeOH (2×250 ml) with stirring. The MeOH extracts from each experiment were next combined and evaporated to dryness in vacuo, with the resulting residues individually partitioned between hexanes and water. The aqueous solubles so obtained were individually concentrated in vacuo, and subjected to preparative HPLC (System B) to afford in each case (−)-*trans*-blechnic acid (**5**, ∼20 mg) and (−)-brainic acid (**6**, ∼3 mg), respectively. Figs. 1 and 2 depict the spectra of carbon-13 isotopically labeled and natural

abundance (−)-brainic acid (**6**) and (−)-*trans*-blechnic acid (**5**) so obtained.

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