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# Biosynthesis of tetrahydrobenzofuran neolignans in somatic embryos of *Ocotea catharinensis*

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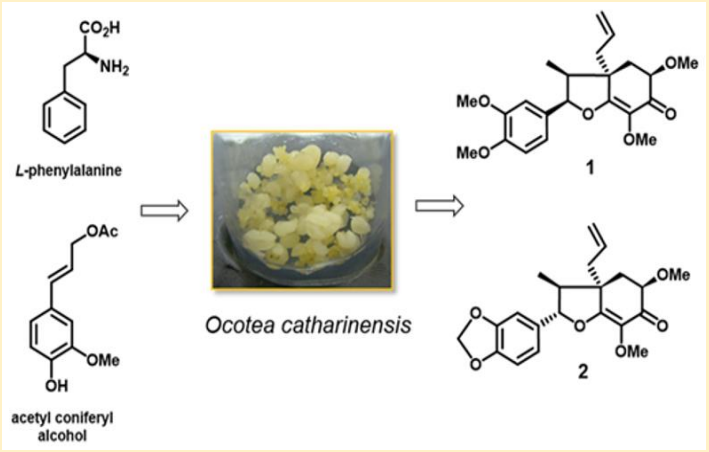
**ABSTRACT:** Somatic embryos of *Ocotea catharinensis* were used as a model to investigate the biosynthetic pathway of tetrahydrobenzofuran neolignan formation by means of feeding $^{13}$C-labelled precursors followed by analysis using MS and $^{13}$C NMR. Isotopomers of *L*-[$^{13}$C]-phenylalanine administered to embryos were incorporated into tetrahydrobenzofuran neolignans and the analysis of $^{13}$C NMR clearly revealed the enriched position of precursors. While feeding a series of putative intermediate including [8–$^{13}$C]-ferulic acid, [8–$^{13}$C]-glycoferulic acid, and [8–$^{13}$C]-coniferyl alcohol were not successful in incorporation to the neolignans, the [8–$^{13}$C]-coniferyl acetate was detected as an intermediate in the biosynthesis of the neolignan 5'-methoxy-porosin. In the bioconversion assay using the protein fraction from the embryogenic cultures, only the substrate coniferyl acetate was converted into isoeugenol, which together with eugenol, is one of the putative precursors of neolignan formation. These findings support that the tetrahydrobenzofuran neolignans are derived from the oxidative coupling between units of *E*- isoeugenol and 5'-methoxy-eugenol leading to a regio- and stereospecific products.





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## 1. Introduction

Lauraceae is a large family of higher plants, comprising about 50 genera with approximately 3000 species. Fossil records show that the first Lauraceae species appeared during the middle of the Cretaceous period and for this reason this plant family is considered one of the most primitive families of Angiosperms belonging to the Magnoliidae clade (Doyle and Endress, 2000; Endress and Doyle, 2009). Some species of this family are widely commercialized and appreciated for their culinary uses, such as cinnamon (*Cinnamomum zeylanicum* and *C. cassia*) (Anand *et al.*, 2016) and avocado (*Persea americana*) (Cervantes–Paz and Yahia, 2021; Jimenez *et al.*, 2021). The genus *Ocotea*, one of the Brazilian genera with wide occurrence in the tropics, provide high quality wood with species like *Ocotea catharinensis* and *O. acutifolia* often been used in construction and carpentry (Montagna *et al.*, 2018). Furthermore, species from the Lauraceae family like *Cinnamomun camphora* in Asia, popularly known as camphor and *Aniba rosiodora*, source of rosewood in South America, are rich in essential oils and are used worldwide in perfumery (Amaral *et al.*, 2017; Amazonas *et al.*, 2020).

The secondary metabolites most frequently found in Lauraceae are phenylpropanoids and their dimeric compounds neolignans (Aiba *et al.*, 1975; 1977; Funasaki *et al.*, 2009; Rozo–Lugo *et al.*, 2018), in addition to flavonoids (David *et al.*, 1994; Rossi *et al.*, 1997), pyrones (Nehme *et al.*, 2008; Rossi *et al.*, 1997), and alkaloids (Gottlieb, 1972; Teles *et al.*, 2019). *Ocotea catharinensis* is a native species to the Atlantic Forest of Brazil, found in the states of Paraná, Santa Catarina, Rio Grande do Sul and São Paulo. Due to the high quality of its wood and difficulty in propagation, however, its natural population has decreased significantly, resulting in its inclusion in the list of endangered species (Montagna *et al.*, 2018). Therefore, a somatic embryogenesis system has been developed with the aim of increasing cultivation and propagation. Phytochemical studies of *O. catharinensis* somatic embryos, stems and leaves have shown that neolignans, especially those of a tetrahydrobenzofuranoid and bicyclooctanoid type, constitute the main secondary metabolites produced by this species (Funasaki *et al.*, 2009).

From a biosynthetic point of view, lignans, neolignans and related compounds are interconnected through the general phenylpropanoid pathway that has the amino acid *L*–phenylalanine as a primary precursor. Then, by a series of enzymatic steps the formation of cinnamyl alcohols is achieved deriving propenylbenzenes and allylbenzenes, which are supposedly the direct precursors of neolignans in *Ocotea* and other plant species (Anterola and Lewis, 2002; Ferrer *et al.*, 2008; Sartorelli *et al.*, 2001; Suzuki and Umezawa, 2007).

The key step involved in the formation of neolignans is the oxidative coupling reaction in which the phenoxide radicals produced by an oxidase may undergo the dimerization reaction followed by further intramolecular polar or by pericyclic reactions determining the main neolignan sub–classes to be formed (Gottlieb, 1972). Examples of these neolignans are the tetrahydrobenzofuran and bicyclooctane [3.2.1] neolignans frequently found in Lauraceous species (Alvarenga *et al.*, 1977; Coy–Barrera *et al.*, 2009; Salleh and Ahmad, 2017). Specifically, tetrahydrobenzofuran–6(2*H*)– and dihydrobenzofuran–6(2*H*)–neolignans such as porosin (Aiba *et al.*, 1976), and mirandin (Aiba *et al.*, 1977) were characterized from Lauraceae species. Nevertheless, to date, the biosynthetic pathways leading to neolignan formation are restricted to the steps required for the formation of allyl and propenylphenols and only one case was addressed to investigate the neolignan formation in *Piper* species (Sartorelli *et al.*, 2001). Interestingly, the acetylation of *p*–coumaric alcohol to form an intermediate prone to be reduced by a reductase, suggest that the leaving group is required for the elimination of the alcohol. This step was demonstrated using enzymatic conversion from cell cultures of basil (*Ocimun basilicum*), for which the specific enzyme eugenol synthase (EGS) requires coniferyl acetate and NADPH to form eugenol (Vassão *et al.*, 2006). In the case of *Petunia* flower cultures, an enzyme homologous to that found in basil, isoeugenol synthase (IGS), also uses coniferyl acetate and NADPH as substrates to catalyse the formation of *E*–isoeugenol. Two mechanisms proposed for the elimination of the acetate group for the formation of allyl and propenylphenols involves a direct attack of the hydride from NADPH at the C–9 carbon of the side chain, leading to the formation of isoeugenol or the attack of the hydride to the C–7 carbon leading to the conjugated elimination of acetyl group giving rise to the eugenol (Koeduka *et al.*, 2006; Vassão *et al.*, 2006). For this reason, herein, we used the somatic embryos and a series of potential $^{13}$C–labeled substrates to investigate what the biosynthetic precursors and intermediates of tetrahydrobenzofuran neolignans are in *Ocotea catharinensis*.


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## 2. Experimental

### *2.1 Reagents*

Pyridine and piperidine were, respectively, distilled under reduced pressure in the presence of KOH (25 g $L^{-1}$) and, stored with a 4 Å molecular sieve, in a vacuum desiccator protected from light. Ethyl ether ($Et_2O$) and tetrahydrofuran (THF), separately, were dried using metallic sodium and distilled over benzofuran for synthetic preparations. The dichloromethane, spectroscopic degree, was left under anhydrous calcium chloride ($CaCl_2$) for 12 h after which it was filtered and distilled under reduced pressure in the presence of calcium hydride ($CaH_2$). It was stored on a 4 Å molecular sieve, protected from light in a dry $N_2$ (g) atmosphere. HPLC grade toluene was used without prior treatment. Chromatographic and spectroscopic analysis used analytical grade solvents (Merck®, Tedia® and J.T. Baker®) and purified water in the Milli–Q system (Millipore®). The reagents were obtained commercially with a high degree of purity, were used without previous purification.

### *2.2 High Performance Liquid Chromatography (HPLC)*

Chromatographic analyses were carried out using a Shimadzu chromatograph model SCL–10Avp, equipped with two analytical pumps model LC–10AD, connected to a diode array detector model SPD–M10Avp with an automatic injector model SIL–9A, controlled by a communication module SCL–10AVP. The samples were solubilized in MeOH: $H_2O$ (8:1 v/v), filtered through a 0.45 μm Millex® membranes and injected on a Phenomenex® reverse phase C–18 column (Luna C–18 150 x 4.6 mm, 5 μm). The data was analysed using the program Class–VP version 6.10 program. The solvent system used in the HPLC analysis consisted of a mixture of $CH_3CN$:$H_2O$ (2:3) for 18 min; $CH_3CN$:$H_2O$ (4:1) for 2 min; $CH_3CN$:$H_2O$ (2:3) for 6 min and the detector was set at λ 280 nm.

### *2.3 Analytical and preparative thin layer chromatography*

TLC analyses were performed on Merck® plates, silica gel 60, with fluorescence indicator F254, with aluminum support thickness 0.2 mm. Prep–TLC purification were performed on 20 x 20 cm glass plates with 1.0 mm thick silica gel 60 from Merck® and fluorescence indicator $F_{254}$. The plates were developed under UV 254 and 366 nm or after being sprayed with a solution of sulfuric vanillin, followed by heating. For preparative thin layer radial chromatography, round glass plates were used, covered with a 2.0 mm thick layer of Kieselgel 60 $F_{254}$ were used. The samples (about 100 mg) were solubilized in hexane:EtOAc (1:1) and recovered according to the UV visible bands.

### *2.4 Column chromatography*

The purification of compounds using column chromatography was performed on glass chromatographic column, with length and diameter of the column determined according to the sample masses to be fractionated. Merck® 0.063 – 0.200 mm (70–230 mesh ASTM) silica gel 60 and C18 reverse phase silica were used as the stationary phases. The proportion of silica used for the column was approximately 20 times the mass of the sample to be purified.

### *2.5 High performance liquid chromatography coupled to the mass spectrometer (HPLC–ESI)*

The extracts from the somatic embryos fed with precursors were analysed by high performance liquid chromatography coupled to a mass spectrometry detector (HPLC–ESIMS). Mass spectrometry analysis was performed on a Bruker, Esquire 2000 plus in a positive electrospray mode, capillary voltage 4.5 kV and skimmer 70 V.

### *2.6 Electronic Ionization Mass spectrometry*

The mass spectra with electron impact ionization, were recorded on a Shimadzu mass spectrometer model GCMS–QP2010, equipped with a capillary column DB–5 (30 m x 0.25 mm, i.d. 0.25 μm).

### *2.7 Nuclear Magnetic Resonance*

The nuclear magnetic resonance analyses were performed at the Analytical Center of the Institute of Chemistry of the University of São Paulo (IQ – USP). The spectra were recorded on Bruker AC200 (200 MHz), Varian Gemini 200 (200 MHz), Varian Inova 300 (300 MHz) and Bruker DRX 500 (500 MHz) spectrometers. The samples were solubilized in $CDCl_3$ (Isotec® Inc. and Aldrich®). The chemical shifts (d) of the obtained spectra are described as ppm in relation to the TMS signal, used as an internal reference standard in all samples (0.05%).


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### 2.8 Embryos of Ocotea catharinensis

Fruits of *Ocotea catharinensis* Mez. (Lauraceae) were collected in the Serra da Cantareira State Park of the Forest Institute of São Paulo, São Paulo–SP. To obtain the somatic embryos, the seeds were treated and inoculated according to the methodology described (Moura–Costa *et al.*, 1993). The embryos were kept in WPM culture medium (Wood Plant Medium) (Lloyd and McCown, 1980), supplemented with sucrose (20 g/L), sorbitol (22 g $L^{-1}$), glutamine (0.4 g $L^{-1}$) and phytagel (Phytagel, Sigma Co., USA) (2 g $L^{-1}$). The pH of the medium was adjusted to 5.8 with NaOH before adding the phytagel. Then, the media were distributed in test tubes (8 mL/tube), closed, and autoclaved at 120 ºC for 15 min, at 1.1 kgf $cm^{-2}$. After 21 weeks of cultivation, the somatic embryos in the globular stage were inoculated in a new culture medium, for the maintenance of embryogenic cultures *in vitro*.

### 2.9 Extraction and purification of neolignans from somatic embryos of Ocotea catharinensis

The analysis of the content of embryos of *O. catharinensis* was investigated using fresh embryos (130 g) crushed in $N_2$ (l) and extracted with MeOH:$H_2O$ (9:1) using an Ultraturrax apparatus (T25 basic, IKA Labortechnik®) at 11,000 rpm. The solution was extracted twice with chloroform solutions, which were combined and dried yielding 480 mg of a $CDCl_3$ fraction. The $CHCl_3$ fraction was then submitted to a chromatographic column, with flash silica, eluted with $CH_2Cl_2$:EtOAc (1:2), followed by $CH_2Cl_2$:Acetone (1:1) (Funasaki *et al.*, 2009). The fractions were pooled after TLC analysis and samples containing the major neolignans 5'–methoxyporosin and armenin B were subjected to further separation by prep–TLC. Standard neolignans were used as control samples to monitor the purification of $^{13}C$–labelled compounds after incorporations of substrates.

### 2.10 HPLC profile of somatic embryo extracts

The chromatographic profile of the extracts from the embryos was analyzed by HPLC using the standards compounds isolated from *O. catharinensis* embryos. The extracts were filtered through a 0.45 µm Millipore filter and a Sep–Pack C–18 filter eluted in MeOH: $H_2O$ (9:1) to eliminate the lipophilic matrix. The chromatographic analysis was carried out as described (See item 2.2).

### 2.11 Feeding of L–[1–$^{13}C$]–, L–[2–$^{13}C$]– and L–[3–$^{13}C$]–phenylalanine in embryos of O. catharinensis

*In vitro* culture of 21 days–old somatic embryos with were transferred to Petri dishes containing two sheets of filter paper. The embryos were left on the plates for 30 min, at room temperature, in a laminar flow hood in order to dehydrate. Solutions containing 0.5 and 1.0 mg of *L*–[1–$^{13}C$]–phenylalanine in 100 µL of Milli–Q Water were administered to 1.0 g of embryos in Petri dishes. Solutions of each concentration were fed in triplicate for 12, 24, 48, 72 and 96 h, in a culture room, in the presence of incandescent light, at 25 °C, with replacement of the precursor solution when required. As a control, 1.0 g of embryos was used without the addition of the labeled precursor. After each incubation time, the samples were crushed in liquid nitrogen, extracted with MeOH:$H_2O$ (9:1) and the solutions were partitioned with chloroform. The chloroform was evaporated under an $N_2$ stream, the fractions were resuspended in MeOH:$H_2O$ (9:1), filtered through a 0.45 µm Millipore filter and analyzed using HPLC–ESI, in order to quantify the incorporation of the $^{13}C$–labelled precursor into the neolignans. Then, the optimum incubation time was defined as 72 h and a concentration of 1.0 mg/100 µL solutions of *L*–[2–$^{13}C$]–phenylalanine and *L*–[3–$^{13}C$]–phenylalanine were fed under the same condition, with the experiments being carried out in triplicate and analyzed by HPLC–ESI. In the case of the incubation of the embryos with the respective *L*–phenylalanine isotopomers, 8.0 g of embryos divided into Petri dishes with 4.0 g each were incubated and 800 µL of the solution (1 mg $mL^{-1}$) were administered to the embryos of each plate for 72 h, with replenishment of the solution (800 µL) every 24 h. The neolignans isolated were subjected to $^{1}H$ and $^{13}C$ NMR analysis.

### 2.12 Administration of [8–$^{13}C$]–ferulic acid

For the preparation of [8–$^{13}C$]–ferulic acid and all $^{13}C$–labelled compounds, see Supplementary Material. The incubation of somatic embryos with [8–$^{13}C$]–ferulic acid was carried out under the same conditions as the *L*–phenylalanine isotopomers. In the first assay for the incorporation, [8–$^{13}C$]–ferulic acid was solubilized in 0.5 µL of DMSO, 0.5 µL of 0.5% NaOH solution with the remaining volume filled with Milli–Q Water. Each of the concentrations, 0.25, 0.5 and 1.0 mg of the acid in 100 µL of the solution were tested for 12, 24, 48, 72 and 96 h. The quantification of the incorporation was determined by HPLC–ESI analysis. In the second assay, [8–$^{13}C$]–ferulic acid was solubilized in $H_2O$:acetone


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(4:1) and was tested under the same conditions as the first incubation with the experiments being carried out in triplicate. The embryos were incubated in larger quantities by solubilizing [8–$^{13}$C]–ferulic acid in $H_2O$:acetone (4:1) at 0.5 mg in 100 µL, for 48 h, with replacement of the precursor solution every 24 h. The obtained extracts were subjected to purification and the neolignans were analyzed by $^{1}$H and $^{13}$C NMR analysis.

### *2.13 Administration of [8–$^{13}$C]–glycoferulic acid*

Due to the low solubility of [8–$^{13}$C]–ferulic acid in aqueous medium, the alternative approach was to prepare the [8–$^{13}$C]–glycoferulic acid. The embryos were incubated under the same conditions as the previous precursors, with the tested concentrations being 0.25, 0.5 and 1.0 mg of [8–$^{13}$C]–glycoferulic acid solubilized in 100 µL of Milli–Q water. Each concentration was tested for the same timeframe of 12–96 h. The experiments were carried out in triplicate. The crude extracts of the incubated embryos and the control samples were analyzed using CG–MS (EI) analysis.

### *2.14 Administration of [8–$^{13}$C]–coniferyl alcohol*

The [8–$^{13}$C]–coniferyl alcohol was administered to embryos under the same conditions as [8–$^{13}$C]–ferulic acid, with concentrations at 0.25, 0.5 and 1.0 mg in 100 µL of $H_2O$:acetone (4:1). Each solution at various concentrations was incubated with the embryos for the timeframe of 12–96 h. The obtained extracts were subjected to the HPLC–ESI analysis as described. Embryos were also incubated with [8–$^{13}$C]–coniferyl alcohol at large scale, under the same conditions as [8–$^{13}$C]–ferulic acid, to obtain the neolignans for NMR analysis.

### *2.15 Administration of [8–$^{13}$C]–coniferyl acetate*

The embryos were incubated with the respective test concentrations of 0.25; 0.5 and 1.0 mg in 100 µL of Milli–Q Water solution containing 0.5 µL of methanol. The solutions were tested separately for the timeframe of 12–96 h. The extracts were prepared as before, and the fractions were subjected to GC–MS analysis. An amount of approximately 4.0 g of embryos were incubated with 800 µL of the solution containing [8–$^{13}$C]–coniferyl acetate solubilized in water (1 mg.100 µL$^{-1}$) for 72 h, with replacement of the precursor solution every 24 h. The neolignans were isolated and subjected to $^{1}$H and $^{13}$C NMR analysis.

### *2.16 Preparation of extracts from embryos incubated with $^{13}$C–precursors*

After optimization conditions for incorporation, larger quantities (8.0 g) of the embryos were incubated with $^{13}$C–precursors. The extracts incubated with the labelled precursors were prepared accordingly, and the chloroform fractions (100 mg) were subjected to prep–TLC fractionation using hexane:EtOAc (7:3), 3 elutions, yielding about 6.0 mg of the pure neolignans.

### *2.17 Extraction of soluble proteins from somatic embryos of O. catharinensis*

Approximately 8.0 g of 21–day old embryos grown *in vitro* were ground in liquid nitrogen using a mortar and pestle with 100 mL of 0.1 g L$^{-1}$ sodium phosphate buffer, pH 7.0 and 10% (w/w) (800 mg) of PVPP. The extract was centrifuged for 30 min at 9,000 rpm (4 °C). The supernatant was filtered through a miracloth and the residue was stored at (4 °C) for later extraction of cell wall proteins. The obtained supernatant contains soluble proteins, which were precipitated by the addition of 80% $(NH_4)_2SO_4$ solution. The supernatant was discarded, and the residue was resuspended in 3.0 mL of phosphate buffer and stored at –80 °C for further assays.

### *2.18 Extraction of proteins contained in cellular microsomes*

The residue obtained after the second centrifugation was resuspended in 10 mL of 0.1 mol L$^{-1}$ Tris–HCl buffer, pH 7.5 and homogenized with a mortar and pestle after which the solution was centrifuged for 90 min at 48,000 rpm (4 °C). The obtained supernatant was precipitated by the addition of 80% $(NH_4)_2SO_4$ solution and was then centrifuged for 90 min at 48,000 rpm (4 °C). The supernatant was discarded, and the residue was resuspended in 3.0 mL of 0.1 mol L$^{-1}$. Tris–HCl buffer, pH 7.5. The solution corresponds to the proteins contained in cellular microsomes and was stored at –80 °C, before use.

The amount of protein present in the enzymatic extract of the embryos was determined by classical method (Bradford, 1976). For each assay, a standard curve of bovine serum albumin (BSA) was determined, as BSA was used as the standard protein. The content of soluble proteins obtained in the enzymatic extract was 8.32 mg mL$^{-1}$ whereas for the fraction corresponding to the proteins contained in cellular microsomes it was 4.21 mg mL$^{-1}$.


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### *2.19 Incubation assays of coniferyl acetate with enzyme extracts*

The enzymatic conversion assays were developed according to published procedures (Lu *et al.*, 2004; Vassão *et al.*, 2006). The coniferyl acetate was tested with several enzymatic fractions, obtained from the somatic embryos of *O. catharinensis*. The solution for the enzymatic conversion had 100 µL of enzymatic extract, a solution of 20 µL of 25 mmol $L^{-1}$ coniferyl acetate in methanol, 76 µL of 40 mmol $L^{-1}$ MES buffer (pH 5.0) and 4 µL of 50 mmol $L^{-1}$ NADPH yielding a final volume of 200 µL. After 30 min of incubation at 28 °C, the reaction was terminated by extraction with EtOAc (2 x 500 µL). As a control, only the precursor and the enzymatic preparation were incubated in 40 mmol $L^{-1}$ MES buffer (pH 5.0). In the enzymatic conversion assay, the standards were incubated under the same conditions as the reactions, without adding the enzymatic fractions. The enzymatic fractions, the precursor coniferyl acetate and the possible products of the reaction (eugenol and *E*–isoeugenol) were analyzed by HPLC in a Phenomenex® reverse phase C–18 column (Luna C–18 150 x 4.6 mm, 5 µm), with a flow of 1 mL $min^{-1}$, a detector at λ 280 nm and, using the eluent $H_2O$ (A) and $CH_3CN$ (B) as follow: initial time 0 min (A:B, 4:1), 27 min (A:B, 1:1), 28.5 min (A:B, 0:1), 31.5 min (A:B, 0:1), 33–55 min (A:B, 4:1).

### *2.20 Incubation assays of E–isoeugenol and 5'–methoxy–eugenol with enzymatic extracts*

The unlabeled phenylpropanoids *E*–isoeugenol and 5'–methoxy–eugenol were incubated together with the enzymatic fractions from embryos under different conditions to determine optimal conversion to the corresponding neolignans 5'–methoxy–porosin and armenin B. The enzymatic conversion reaction was carried out with 100 µL of enzymatic extract, 10 µL of 25 mmol $L^{-1}$ E–isoeugenol and 10 µL of 25 mmol $L^{-1}$ 5'–methoxy–eugenol in methanol, 5 µL of 1 mg $mL^{-1}$ of horseradish peroxidase, 5 µL of 10 mmol.$L^{-1}$ $H_2O_2$, 5 µL of 50 mmol $L^{-1}$ NADPH, 110 µL of 40 mmol $L^{-1}$ MES buffer (pH 5.0) and 5 µL of 50 mmol $L^{-1}$ NADPH, for a final volume of 250 µL. After 1.0 h of incubation at 28 °C, the reaction was terminated by extraction with EtOAc (2x 500 µL). The precursors, the possible products of the reaction, the control and the enzymatic fractions were analyzed by HPLC as previously described.

## 3. Results and discussion

### *3.1 Analysis of somatic embryo extracts from O. catharinensis*

The content of the embryos of *O. catharinensis* had been previously described to contain the neolignans 5'–methoxy–porosin (**1**) and armenin B (**2**) (Fig. 1) (Funasaki *et al.*, 2009). For this study, the HPLC analysis of the embryos and the identification of these neolignans was consistent with previous data allowing the incorporations of $^{13}C$–labeled precursors using EI–MS, ESI and $^{13}C$ NMR analysis.

The neolignans 5'–methoxy–porosin (**1**) and armenin–B (**2**) (Fig. 1) were purified and used as chromatographic standards in the $^{13}C$–precursor incorporation experiments. These two neolignans were confirmed by analysis of $^{1}H$ NMR, $^{13}C$ NMR and analysis of EI–MS data and matched with those described by Ishige *et al.* (1991).

The mass spectra obtained by EI–MS of the 5'–methoxy–porosin neolignan, showed peaks according to the fragmentation proposal described and comparison with the published data (Ishige *et al.*, 1991). Armenin B was identified by comparing the $^{1}H$ and $^{13}C$ NMR data with the data described (Aiba *et al.*, 1978; Felicio *et al.*, 1986; Ishige *et al.*, 1991).

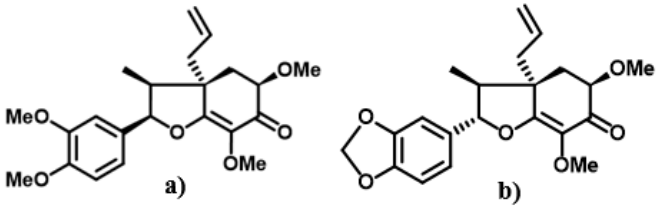


**Figure 1.** Chemical structures of neolignans 5'–methoxy–porosin **(a)** and armenin B **(b)** from somatic embryos of *Ocotea catharinensis.*
**Source:** Adapted from Funasaki *et al.* (2009).

### *3.2 Analysis of the incorporations of L–[1–$^{13}C$]–, L–[2–$^{13}C$]– and L–[3–$^{13}C$]–phenylalanine*

The experiments with $^{13}C$–isotopomers of *L*–phenylalanine were performed to determine the optimal incubation time to reach the maximum incorporation of $^{13}C$–precursors into the neolignans 5'–methoxy–porosin (MM= 388 Da) and armenin B (MM= 372 Da) (Fig. 2). The percentage of $^{13}C$ incorporations in the neolignans was determined by means of HPLC–ESI analysis of the crude extracts from embryos fed with the substrates and compared with the isotopic contribution to M+1 and M+2 in natural abundance neolignans (Tab. 1).


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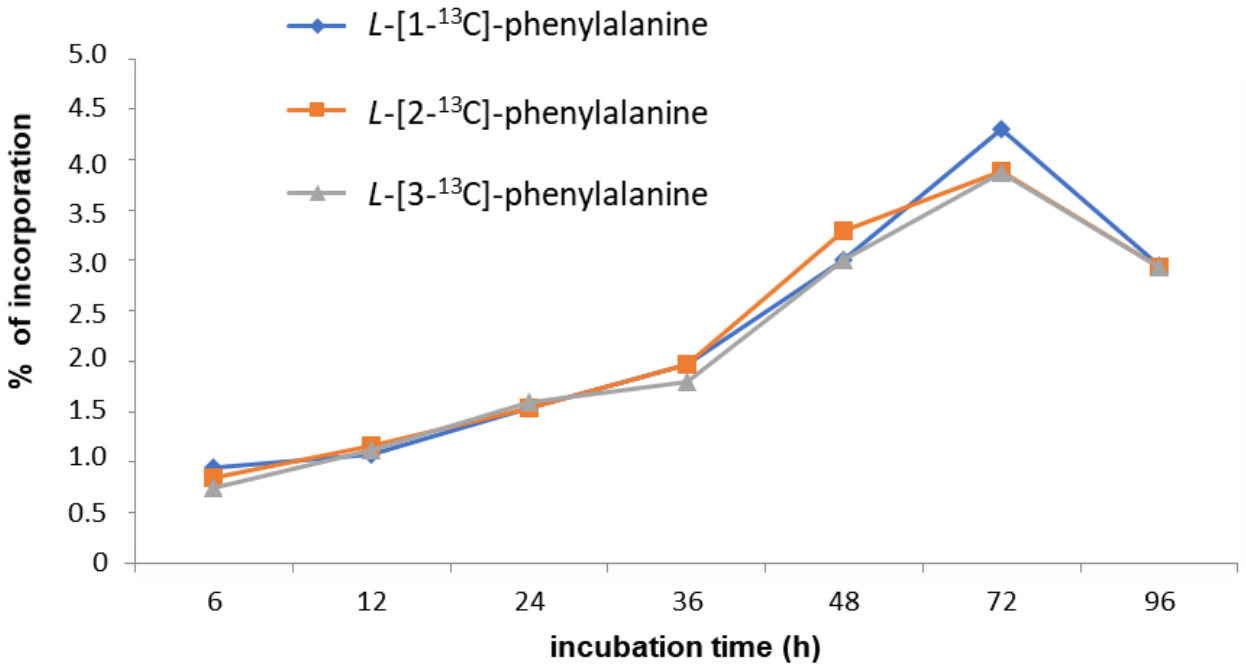


**Figure 2.** Time course of incorporation of isotopomers of *L*–phenylalanine into 5'–methoxy–porosin (**1**).

**Table 1.** ESI data showing incorporation of isotopomers of *L*–phenylalanine into 5'–methoxy–porosin (**1**) and armenin B (**2**) in embryos of *Ocotea catharinensis*.

| ***L*–[1–$^{13}$C]–phenylalanine** | | | | | | |
|---|---|---|---|---|---|---|
| | 5'–methoxy–porosin (**1**) | | | armenin B (**2**) | | |
| **Ions** | $[M+H]^+$ | $[M+H+1]^+$ | $[M+H+2]^+$ | $[M+H]^+$ | $[M+H+1]^+$ | $[M+H+2]^+$ |
| | 389 | 390 | 391 | 373 | 374 | 375 |
| **% of incorporation** | 100 | 9.0 | 2.2 | 100 | 7.2 | 1.2 |
| ***L*–[1–$^{13}$C]–phenylalanine** | | | | | | |
| | 5'–methoxy–porosin (**1**) | | | armenin B (**2**) | | |
| **Ions** | $[M+H]^+$ | $[M+H+1]^+$ | $[M+H+2]^+$ | $[M+H]^+$ | $[M+H+1]^+$ | $[M+H+2]^+$ |
| | 389 | 390 | 391 | 373 | 374 | 375 |
| **% of incorporation** | 100 | 8.6 | 1.98 | 100 | 6.6 | 1.1 |
| ***L*–[1–$^{13}$C]–phenylalanine** | | | | | | |
| | 5'–methoxy–porosin (**1**) | | | armenin B (**2**) | | |
| **Ions** | $[M+H]^+$ | $[M+H+1]^+$ | $[M+H+2]^+$ | $[M+H]^+$ | $[M+H+1]^+$ | $[M+H+2]^+$ |
| | 389 | 390 | 391 | 373 | 374 | 375 |
| **% of incorporation** | 100 | 8.8 | 2.0 | 100 | 6.8 | 1.4 |

In the mass spectrum of unlabeled 5'–methoxy–porosin the peaks at *m/z* 389 $[M+H]^+$, 390 $[M+H+1]^+$ and 391 $[M+H+2]^+$ indicated the expected molecular ions and the ions resulting from incorporation of one or two $^{13}$C atoms. In the case of unlabeled armenin B, the peaks of molecular ions were observed at *m/z* 373 $[M+H]^+$, 374 $[M+H+1]^+$ and 375 $[M+H+2]^+$.

Considering the natural abundance of $^{13}$C in the neolignans, the calculations to obtain the percentage of $^{13}$C incorporated were performed by comparing the relative intensity of the molecular mass ($[M+H]^+$), ($[M+H+1]^+$), and ($[M+H+2]^+$) of the unlabeled neolignans with that of the enriched neolignans. The highest incorporation of *L*–phenylalanine isotopomers in both neolignans was observed at 72 h. The neolignans isolated from the extracts obtained after the incubation period of 72 h were subjected to $^{13}$C NMR analysis to characterize the enrichment position after incorporation of *L*–phenylalanine isotopomers into the structures of the 5'–methoxy–porosin (**1**) and armenin B (**2**). The comparison of the $^{13}$C NMR spectrum of the unlabeled 5'–methoxy–porosin (6.0 mg) (control), with the spectrum of the labelled (6.0 mg) with *L*–[1–$^{13}$C]–phenylalanine, showed an increase in the intensity of the peaks corresponding to the C–9 (δ 11.7 ppm) and C–9'


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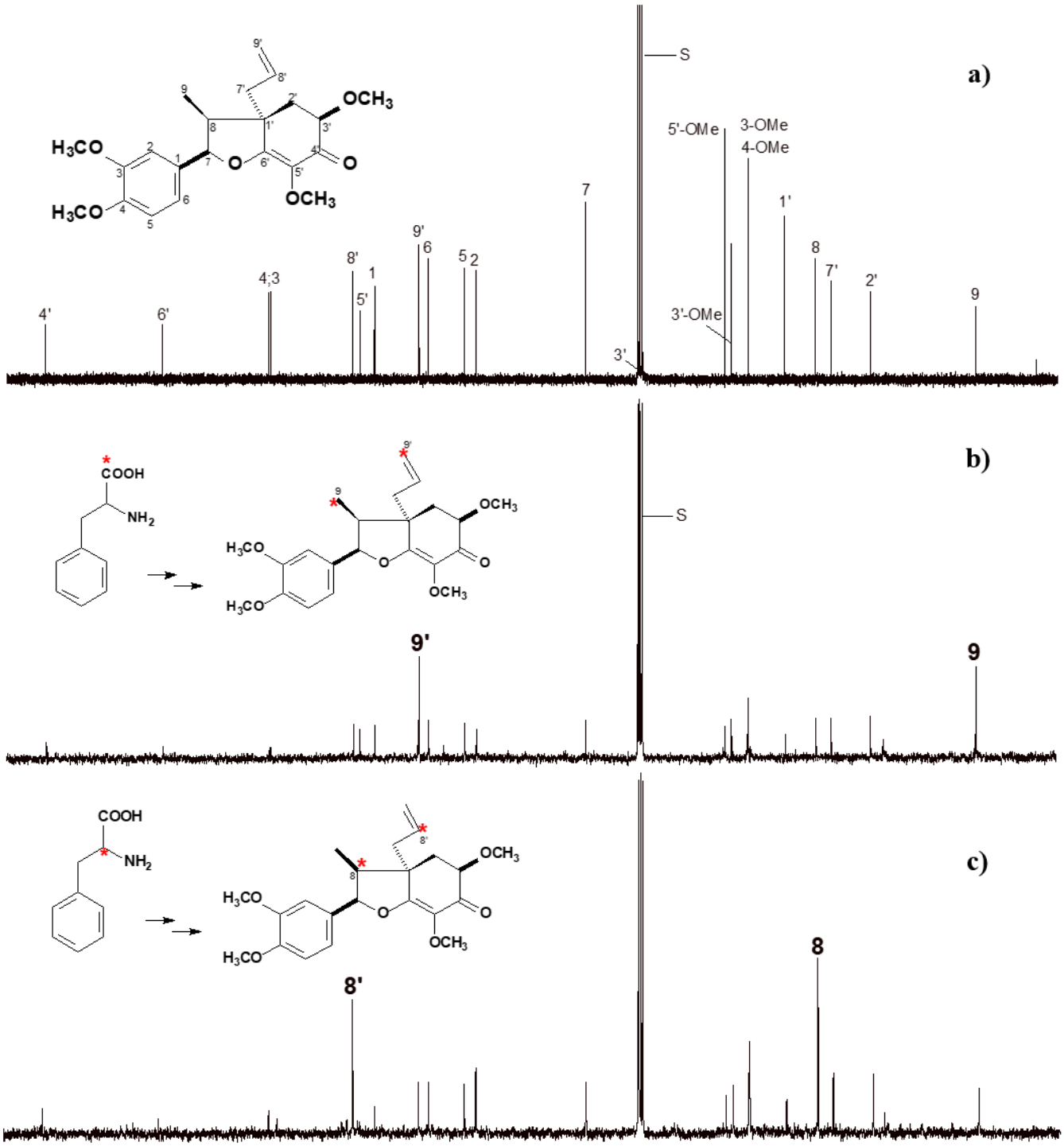
(δ 119.9 ppm) carbons (Fig. 3). Likewise, administration of *L*–[2–$^{13}$C]–phenylalanine resulted in a product with $^{13}$C NMR signals enriched at positions C–8 (δ 42.7 ppm) and C–8' (δ 132.7 ppm). The spectrum of the neolignan incubated with *L*–[3–$^{13}$C]–phenylalanine had the positions C7 (δ 87.5 ppm) and C7' (δ 39.8 ppm) enriched (Fig. 3).





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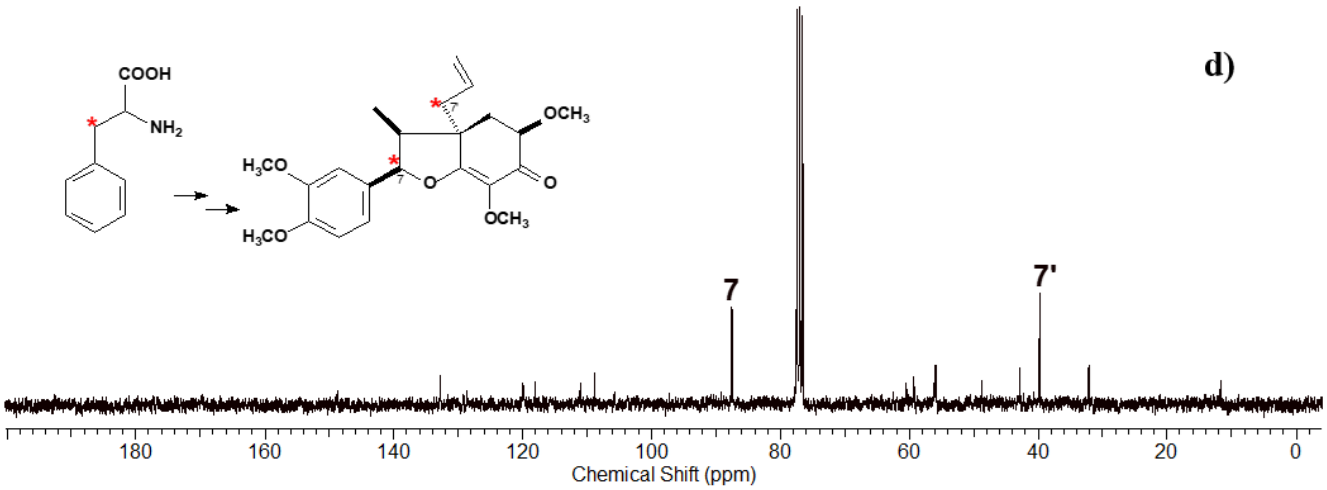
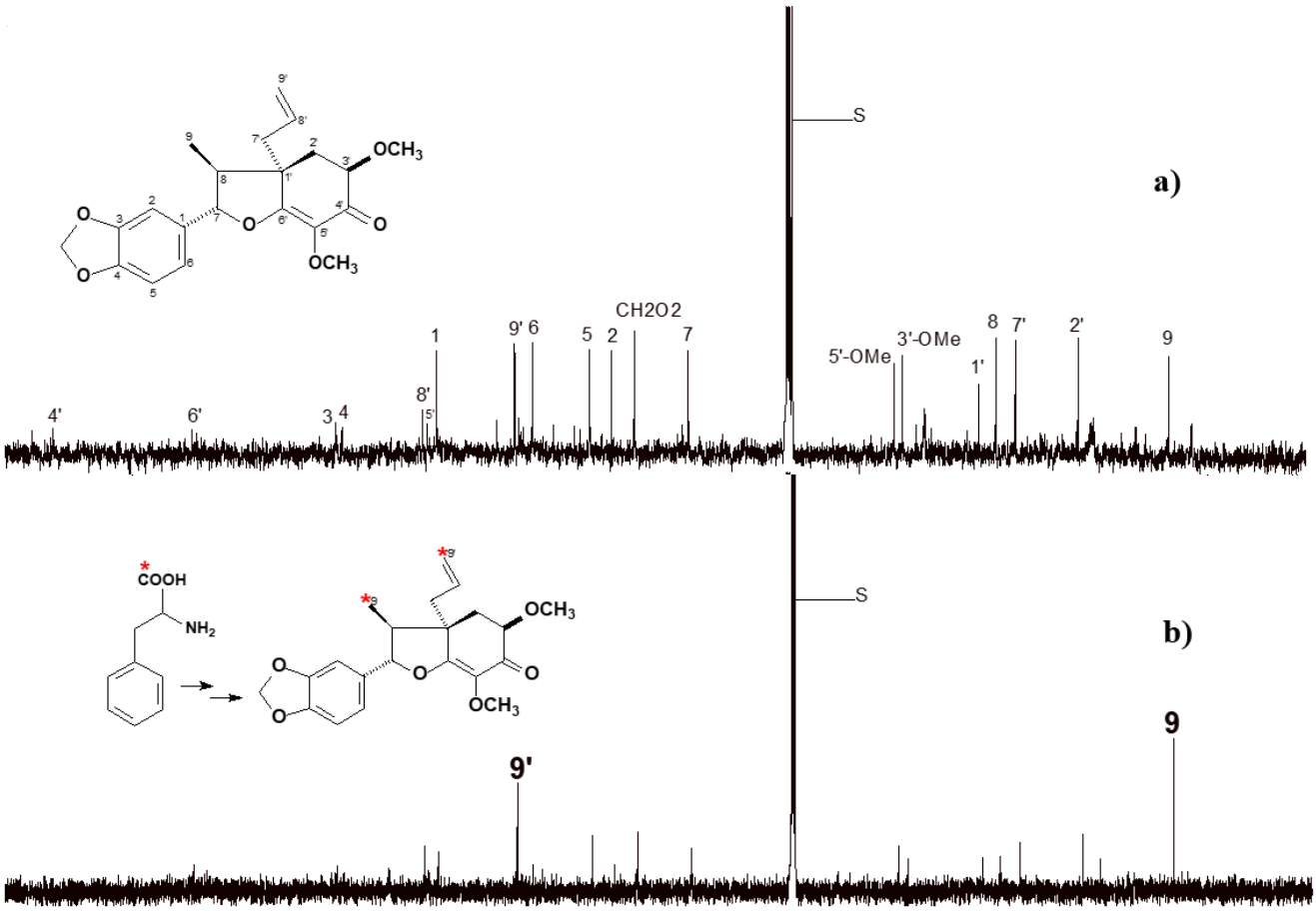



**Figure 3.** $^{13}C$ NMR spectra of: **(a)** natural abundance 5'–methoxy–porosin; **(b)** Incorporation with *L*–[1–$^{13}C$]–phenylalanine; **(c)** Incorporation with *L*–[2–$^{13}C$]–phenylalanine and **(d)** incorporation with *L*–[3–$^{13}C$]–phenylalanine. S: $CDCl_3$.

The $^{13}C$ NMR analysis for armenin B (5.0 mg) showed a similar profile of increased signals as that of 5'–methoxy–porosin. The incubation with *L*–[1–$^{13}C$]–phenylalanine led to an increase in the intensity of the C9 (δ 17.5 ppm) and C9' (δ 120.0 ppm) carbons (Fig. 4). While the one enriched with *L*–[2–$^{13}C$]–phenylalanine showed an increase in signal intensity in C8 (δ 44.6 ppm) and C8' (δ 132.5 ppm) carbons. The administration of *L*–[3–$^{13}C$]–phenylalanine, meanwhile, showed signals enriched at positions C–7 (δ 93.8 ppm) and C–7' (δ 40.8 ppm) (Fig. 4).





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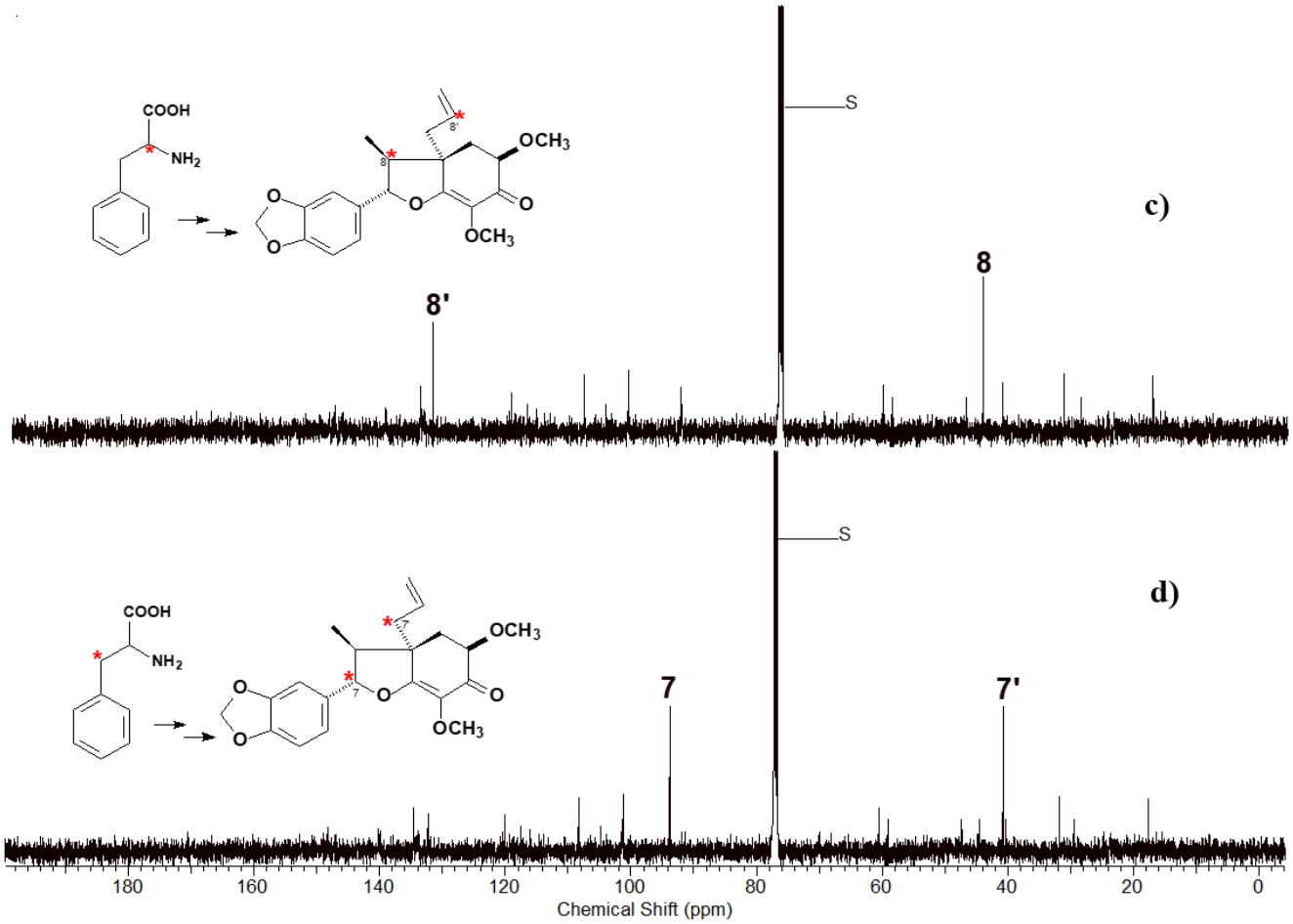


**Figure 4.** $^{13}C$ NMR of natural abundance armenin B **(a)**; Incorporation with *L*–[1–$^{13}C$]–phenylalanine **(b)**; Incorporation with *L*–[2–$^{13}C$]–phenylalanine **(c)** and incorporation with *L*–[3–$^{13}C$]–phenylalanine **(d)**. S: $CDCl_3$.

The first isotopic labelling studies involving the biosynthetic study of the phenylpropanoid anethole using *Pimpinella anisum* with $^{14}C$–labeled *L*–phenylalanine resulted in the incorporation of the intact side chain of the amino acid into anethole (Manitto *et al.*, 1974). While the HPLC–ESI analysis showed the incorporation of *L*–phenylalanine isotopomers in the structure of neolignans in *O. catharinensis*, the $^{13}C$ NMR spectra confirmed that the carbon incorporation pattern of $^{13}C$ with *L*–[1–$^{13}C$], *L*–[2–$^{13}C$]– and *L*–[3–$^{13}C$]–phenylalanine. The data is consistent with the proposed formation of neolignans by the phenylpropanoid pathway, with the intact incorporation of the two *L*–phenylalanine molecules into the structure of neolignans, without rearrangement of the amino acid side chain. The first isotopic labelling studies involving the biosynthetic study of the phenylpropanoid anethole using *Pimpinella anisum* with $^{14}C$–labeled *L*–phenylalanine resulted in the incorporation of the intact side chain of the amino acid into anethole (Manitto *et al.*, 1974). While the HPLC–ESI analysis showed the incorporation of *L*–phenylalanine isotopomers in the structure of neolignans in *O. catharinensis*, the $^{13}C$ NMR spectra confirmed that the carbon incorporation pattern of $^{13}C$ with *L*–[1–$^{13}C$], *L*–[2–$^{13}C$]– and *L*–[3–$^{13}C$]–phenylalanine. The data is consistent with the proposed formation of neolignans by the phenylpropanoid pathway, with the intact incorporation of the two *L*–phenylalanine molecules into the structure of neolignans, without rearrangement of the amino acid side chain.

### *3.3 Analysis of [8–$^{13}C$]–ferulic acid incorporation*

The HPLC–ESI analysis of the crude extracts of the somatic embryos of *O. catharinensis* incubated with [8–$^{13}C$]–ferulic acid together with the control, did not show incorporation of the precursor to the neolignans. Extracts of the embryos were prepared after 12, 24, 48, 72 and 96 h of incubation. The embryos were incubated in large scale with [8–$^{13}C$]–ferulic acid for 48 h, as during this time the embryos were less oxidized. The purified 5'–methoxy–porosin (5.0 mg) and armenin B (5.0 mg) neolignans were subjected to $^{1}H$ and $^{13}C$ NMR analysis. In the case of incorporation with [8–$^{13}C$]–ferulic acid,


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supposedly the enrichment with carbon thirteen would be in the positions of C8 and C8' of the corresponding neolignans. The $^{13}C$ NMR spectra of the control samples, 5'–methoxy–porosin and armenin B (Fig. 4) were compared with those resulting from the incubations and no detectable changes were observed in the peaks intensity corresponding to C8 and C8' of the neolignans (data not shown).

Previous biosynthetic studies have demonstrated the incorporation of 0.053% of [8–$^{14}C$]–ferulic acid in the podophyllotoxin lignan, isolated from young plants of *Podophyllum hexandrum* (Jackson and Dewick, 1984). However, methylchavicol, isolated from *Ocimum basilicum* leaves after incubation with *p*–[8–$^{14}C$]–coumaric acid, similarly to the incorporation of ferulic acid, did not show any incorporation in its structure (Vassão *et al.*, 2006). Supposedly, these results may be a consequence of the toxic nature of the precursor to the plant in this case, to the embryos of *O. catharinensis* because, after 48 h the embryos were completely oxidized. The low solubility of ferulic acid in aqueous solution may also have caused difficulties in transporting it to the biosynthesis site (Davin *et al.*, 2003; Vassão *et al.*, 2006).

### *3.4 Analysis of [8–$^{13}C$]–glycoferulic acid incorporation*

The biosynthetic studies looking at the origin of the lignans arctin and phillyrin lignans in *Forsythia* shoots were carried out using ferulic acid, coniferyl alcohol and glycosylated coniferaldehyde, labelled with $^{3}H$ and $^{14}C$ (Stöckigt and Klischies, 1977). Therefore, the glycosylation of the phenolic hydroxyl would contribute to minimize the oxidation of the acid, due to the higher solubility of the substrate in water and, consequently, improve its absorption in the plant. Based on this principle, [8–$^{13}C$]–glycoferulic acid was administered to somatic embryos of *O. catharinensis*, at different time intervals and different concentrations to determine optimal conditions for its incorporation into the neolignans. Therefore, extracts with natural abundance and those incubated with [8–$^{13}C$]–glycoferulic acid were analyzed by mass spectrometry (EI). From the proposed fragmentation of 5'–methoxy–porosin, it was observed that the peaks at *m/z* 178, 319 and 358 Da of the control sample, should show the peaks enriched with carbon thirteen at *m/z* in 179, 320 and 360 Da, respectively. The embryos showed better appearance and longer incubation time at 72 h and, therefore, from these experiments, extracts were obtained and subjected to the separation of the neolignans by prep–TLC. According to the hypothesis suggested for the pathway of formation of neolignans (Fig. 5), there should be incorporation in the positions of C8 and C8', originating from [8–$^{13}C$]–ferulic acid. However, the comparison of the relative intensities of $^{13}C$ NMR peaks of naturally abundant neolignans with those incubated with [8–$^{13}C$]–glycoferulic acid did not show any increase in intensity at the C8 and C8' positions (data not shown).

### *3.5 Analysis of the incorporation of [8–$^{13}C$]–coniferyl alcohol*

*In vitro* administrations of [8–$^{13}C$]–coniferyl alcohol in *O. catharinensis* embryos were performed at different concentrations, time intervals (12, 24, 48, 72 and 96 h) and, in the absence and presence of light, to identify optimal conditions for absorption and metabolism in neolignans **1** and **2**.

The comparison of the HPLC–ESI spectra of the crude extracts of the somatic embryos of *O. catharinensis* incubated with [8–$^{13}C$]–coniferyl alcohol with the control samples, showed that this substrate was not incorporated into the 5'–methoxy–neolignans porosin and armenin B. According to the biosynthetic proposal for the formation of neolignans, we expected to observe the incorporation of one alcohol molecule at the position of C–8 and the other at C–8 'in the structure of neolignans **1** and **2**. Nevertheless, similarly to the case of ferulic acid and its glycosylated version embryos incubated with [8–$^{13}C$]–coniferyl alcohol for 48 h, time interval with embryos were not fully oxidized and submitted to ESI, EI or $^{1}H$ and $^{13}C$ NMR analysis did not show any increase in carbon intensity at positions C–8 and C–8' when compared to the control (data not shown). It is assumed that the rapid degradation of coniferyl alcohol prior to its entry into the biosynthetic path, coupled with its low solubility in an aqueous medium did not result in the enrichment with carbon thirteen of the corresponding neolignans **1** and **2**, as expected. In a contrasting experiment using enzymatic conversion, the biosynthesis of the lignan (–)–secoisolariciresinol was investigated in *Forsythia intermedia*. The precursor [9–$^{2}H_2$]–coniferyl alcohol in that study served as a precursor to the coupling reactions in the presence of NADPH and $H_2O_2$ (Katayama *et al.*, 1992).


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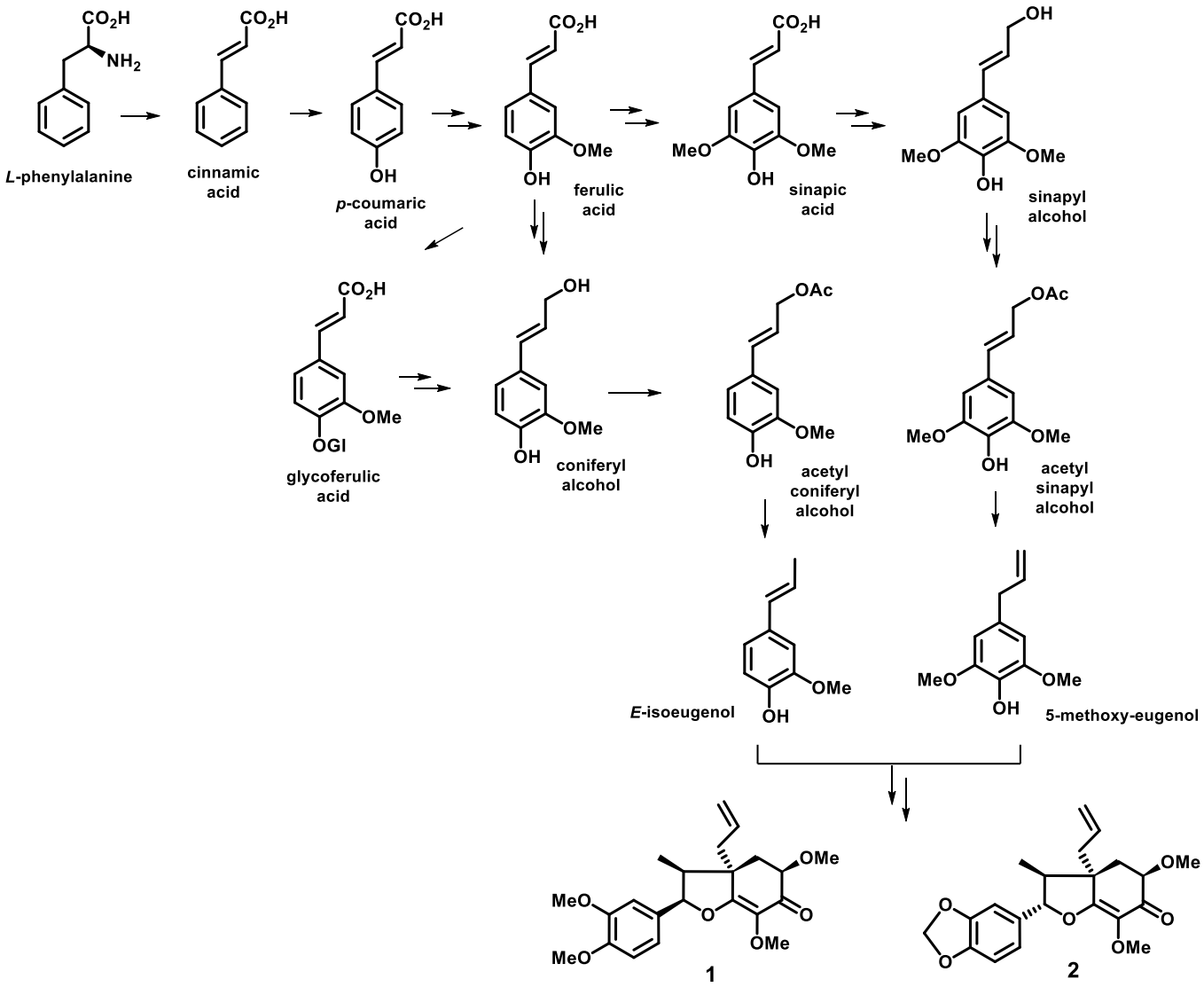


**Figure 5.** Biosynthetic pathway of tetrahydrobenzofuran neolignans in embryogenic cultures of *O. catharinensis*.

### *3.6 Analysis of the incorporation of [8–$^{13}$C]–coniferyl acetate*

The [8–$^{13}$C]–coniferyl acetate was next chosen because it presents a good leaving group, whose exit is carried out by reductive elimination. This phenylpropanoid precursor would have the additional stage of activation by acetylation and was recently described as an important biosynthetic intermediate for lignans (Ferrer *et al.*, 2008; Koeduka *et al.*, 2013; Vassão *et al.*, 2006). Therefore, similarly to the previous precursors, the incubation of [8–$^{13}$C]–coniferyl acetate in embryos of *O. catharinensis* in the time frame of 12–96 h was tested. Mass spectrometry (EI) was used to analyze the crude extract of the fractions resulting from incorporation and control, to determine the percentage of $^{13}$C incorporation into the neolignans. The incorporation of the substrate occurred for 72 h of incubation.

The mass spectrum of 5'–methoxy–porosin revealed fragment ions in *m/z* 179 and *m/z* 320 Da with an increased intensity when compared with the spectra of the control samples. The level of incorporations were 5.48% and 5.77%, respectively, while in the fragment ion at *m/z* 358 Da, $^{13}$C incorporations of 3.20% were observed in the $[M+1]^+$ 359 Da and of 2.12% in the $[M+2]^+$ 360 Da (Figure S14). Therefore, incorporation of the two [8–$^{13}$C]–coniferyl acetate molecules could be observed in 5'–methoxy–porosin. Curiously, similar incorporation to armenin B was not observed in the mass spectra of the enriched crude extract.

According to the biosynthetic proposal for the formation of neolignans, in the case of incorporation with [8–$^{13}$C]–coniferyl acetate, supposedly enrichment


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with carbon thirteen would also be in the positions of the C8 and C8' of the neolignans between the $^{13}C$ NMR spectra of the 5'–methoxy–porosin control sample as compared to the incubated one. The spectra showed an increase in the intensity of C8' (δ 132.7 ppm) and in C8 (δ 42.8 ppm) (Fig. 6). Armenin B was obtained in a very small amounts and did not allow a good quality $^{13}C$ NMR spectrum.

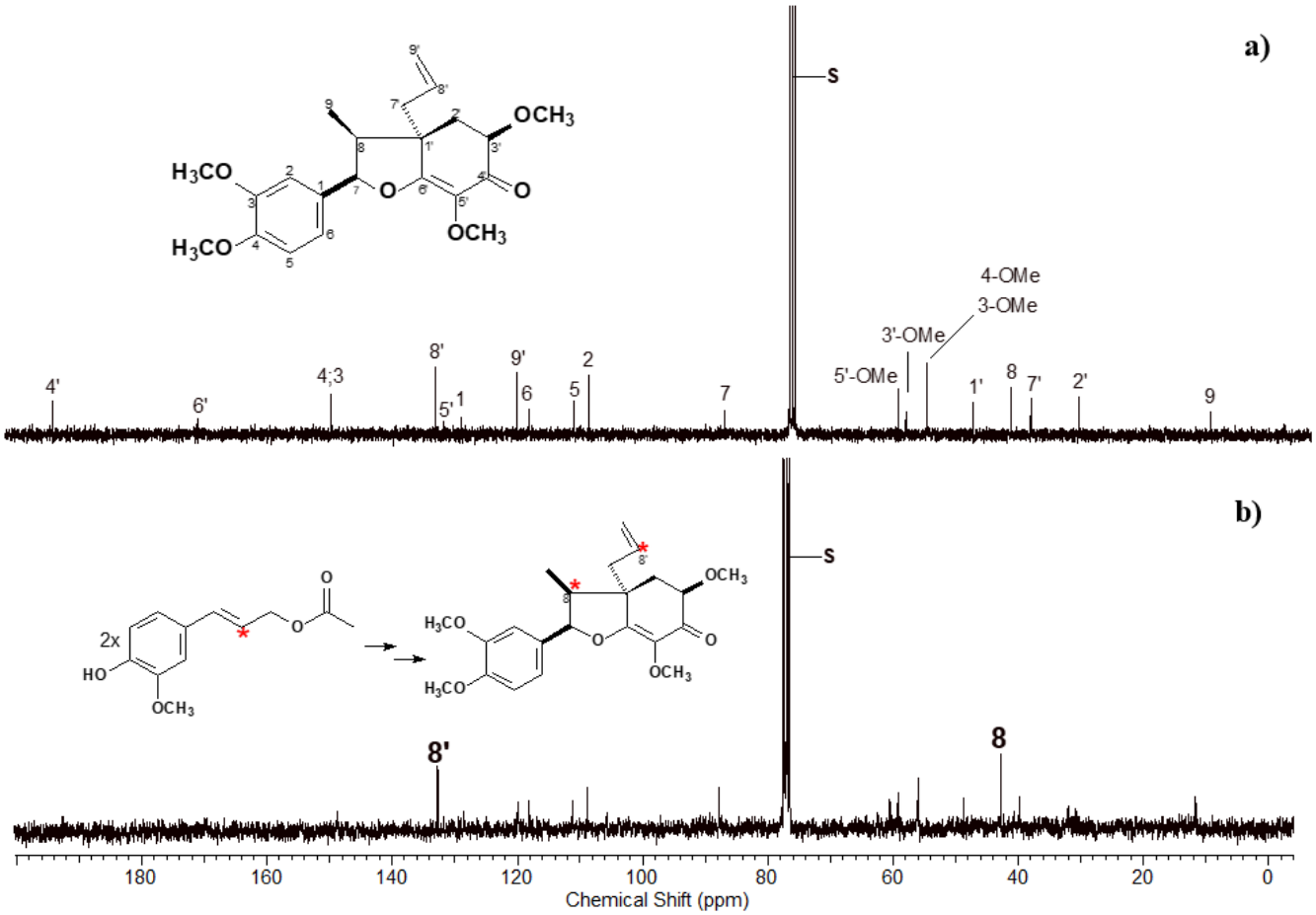


**Figure 6.** $^{13}C$ NMR of 5'–methoxy porosin at natural abundance **(a)** and of 5'–methoxy porosin after incubation with [8–$^{13}C$]–coniferyl acetate **(b)**.

The positions of the hydrogens directly linked to the $^{13}C$–enriched carbon atom were confirmed through the HSQC spectrum, in which it was possible to observe the correlations of hydrogen at δ 2.56 (H–8), with δ 42.8 (C–8), as well as of hydrogens at δ 5.95 (H–8 '), with δ 132.8 (C–8'). The HMBC contour map showed correlations of δ 2.56 (H–8) with δ 11.7 (C–9), δ 39.8 (C–7 ') and with δ 169.9 (C–6 '). The hydrogens at δ 5.95 (H–8') directly linked to C–8' correlate with δ 39.8 (C–7') as well as with δ 48.8 (C1').

The results obtained from mass spectra (EI), $^{13}C$ NMR, HSQC and HMBC contour maps, confirmed that the entire 5'–methoxy–porosin skeleton was formed by the condensation of two molecules of [8–$^{13}C$]–coniferyl acetate, which was transformed by a sequence of enzymatic steps.

### *3.7 Enzymatic conversion with intermediate phenylpropanoid substrates*

The conversion of precursors using enzymatic fractions obtained from embryos of *O. catharinensis* were analyzed by HPLC. The crude extracts obtained from various incubation times allowed us to determine the ability to convert coniferyl acetate into eugenol or *E*–isoeugenol. The comparison of the analysis by HPLC of the control (enzymatic extract and MES buffer) (Fig. 7a), with the enzyme fractions and the respective standards, allowed us to identify the conversion of coniferyl acetate into *E*–isoeugenol as an enzyme conversion product (Fig. 7b).

For the elimination of the acetate group and the formation of *E*–isoeugenol it was demonstrated that if the nucleophilic attack of the hydride, originating from


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NADPH, occurs at the C–9 carbon of the side chain of the coniferyl acetate, it would lead to the formation of propenylphenol. If the hydride attack occurs at C–8, it would lead to the formation of eugenol (Koeduka *et al.*, 2006; 2013; Vassão *et al.*, 2006).

These results, combined with the incorporation of two molecules of [8–$^{13}$C]–coniferyl acetate to 5'–methoxy–porosin, corroborate the pathway in which *E*–isoeugenol is required for the formation of the 5'–neolignans methoxy–porosin and armenin B.

Similarly, the conversion of *E*–isoeugenol and 5–methoxy–eugenol in 5'–methoxy–porosin or in armenin B was attempted. According to the proposed biosynthesis scheme, the substrate would undergo oxidative coupling by the action of enzymes (dirigent protein) leading to the production of tetrahydrobenzofuran neolignans. The analysis of the enzymatic conversion of the precursors *E*–isoeugenol and 5–methoxy–eugenol to neolignan 5'–methoxy–porosin was done by HPLC and no coupling reaction between of *E*–isoeugenol and 5–methoxy–eugenol in tetrahydrobenzofuran neolignans was observed. Only the signals referring to the compounds of the enzymatic fraction and the precursors were observed.

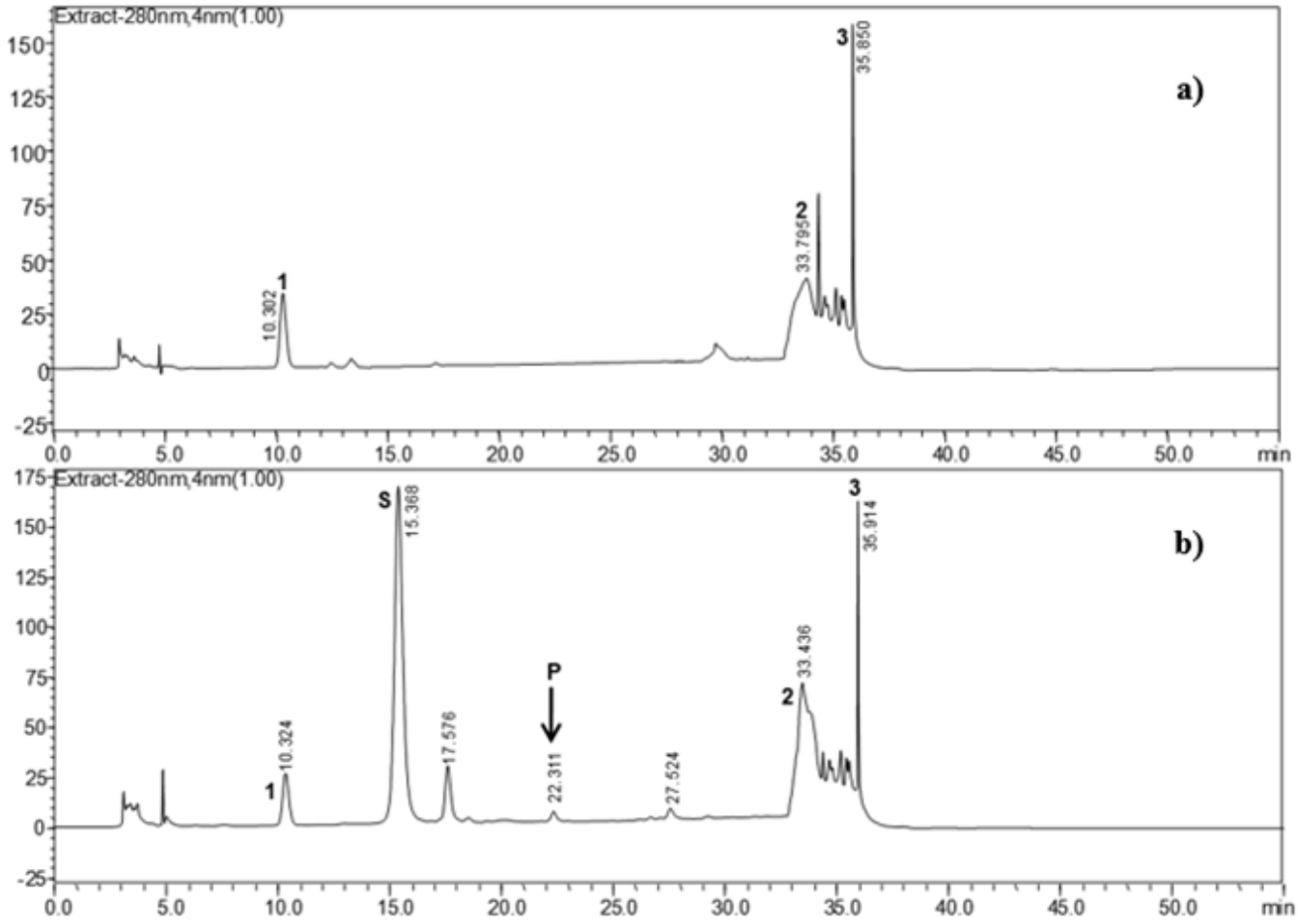


**Figure 7.** HPLC analysis of the conversion of substrates by enzymatic fraction of *O. catharinensis* embryos. **(a)** Control indicating the presence of endogenous compounds in the enzyme fraction; **(b)** Conversion of coniferyl acetate to *E*–isoeugenol. (P) *E*–isoeugenol formed by the reaction; (S): substrate coniferyl acetate.

## 4. Conclusions

The embryos of *O. catharinensis* was shown to contain revealed to be a unique source of tetrahydrobenzofuran neolignans as indicated by the HPLC–PDA analysis. Therefore, it was used as a model for the biosynthetic studies because of the easy protocol to feed labelled precursors and chemical composition without many interferences. The analysis of enriched crude extracts using $^{13}$C *L*–phenylalanine isotopomers as substrates and mass spectrometric analysis of the products indicated $^{13}$C enrichment in the tetrahydrobenzofuran neolignans 5'–methoxy–porosin and armenin B. The $^{13}$C NMR analysis confirmed


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unequivocally the labelling of the $^{13}C$ position in the structures of the neolignans. The use of various isotopomers of *L*–phenylalanine showed an intact incorporation of the side chain of two phenylalanine molecules in the respective neolignans without any rearrangement.

Biosynthetic investigation involving a more specific precursor in the pathway showed the incorporation of two molecules of [8–$^{13}C$]–coniferyl acetate in 5'–methoxy–porosin. This conversion was confirmed using enzymatic conversion assays, with conversion of coniferyl acetate into *E*–isoeugenol which is considered an intermediate precursor for the formation of tetrahydrobenzofuran neolignans.

These results support the hypothesis that tetrahydrobenzofuran neolignans would result from the oxidative coupling between radical units produced from *E*–isoeugenol and 5–methoxy–eugenol. Since the neolignans are optically active, the key enzymatic dimerization reaction would be responsible for the regio– and stereospecificity of the coupling reaction.

## Authors' contribution

**Conceptualization:** Kato, M. K.
**Data curation:** Santos, E. L.; Kato, M. K.; Floh, E. I. S.
**Formal Analysis:** Santos, E. L.; Kato, M. K.; Floh, E. I. S.
**Funding acquisition:** Kato, M. K.; Floh, E. I. S.
**Investigation:** Santos, E. L.
**Methodology:** Santos, E. L.
**Project administration:** Kato, M. K.; Floh, E. I. S.
**Resources:** Kato, M. K.; Floh, E. I. S.
**Software:** Not applicable.
**Supervision:** Kato, M. K.; Floh, E. I. S.
**Validation:** Kato, M. K.; Floh, E. I. S.
**Visualization:** Santos, E. L.
**Writing – original draft:** Santos, E. L.; Kato, M. K.; Floh, E. I. S.
**Writing – review & editing:** Santos, E. L.; Kato, M. K.; Floh, E. I. S.

## Data availability statement

All data sets were generated or analyzed in the current study.

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