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Biosynthesis of antimalarial lignans from *Holostylis reniformis*

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ABSTRACT

Holostylis reniformis biosynthesizes 8-8′ linked lignans without 9,9′-oxygenation. To elucidate the biosynthetic pathways to these lignans, the reputed precursors [U-^{14}C]phenylalanine, [9-$^{3}H_1$]coniferyl alcohol, and [9-$^{3}H_1$]isoeugenol were administered to roots of the plant, which led to the incorporation of ^{3}H and ^{14}C into ten 2,7′ linked-lignans (aryltetralone lignans) and two 7,7′-epoxylignans (furan lignans). These administration experiments demonstrated that the lignans were propenylphenol-derived and that *H. reniformis* can exhibit regioselective control over radical–radical coupling (via isoeugenol radicals). Regiospecific control over propenylphenol-derived lignan biosynthesis was observed, together with diastereoselective control of C2–C7′ bond formation for the aryltetralone lignans (7′*R*). These experiments provide evidence that isoeugenol is a biosynthetic intermediate to the aryltetralone and furan lignans.

1. Introduction

Lignans are a very structurally diverse class of vascular plant natural products. They are typically dimers and/or higher oligomers (Moss, 2000). Lignans vary substantially in oxidation level, substitution pattern, and the chemical structure of their basic carbon framework. In addition to structural diversity, lignans show considerable diversity in terms of enantiomeric composition, biosynthesis, and phylogenetic distribution (Umezawa, 2003). Based on structural (chemotaxonomical) differences, lignans can be readily separated into distinct coupling-type subclasses (Gottlieb, 1978; Umezawa, 2003; Davin and Lewis, 2005). An examination of their corresponding structures and distribution in nature illustrates the existence of a large number of distinct phenoxyl radical–radical coupling modes, including those that are either stereoselective and/or regiospecific in coupling origin (Davin and Lewis, 2005). The first demonstration of phenoxyl radical–radical coupling control was reported during the investigation of (+)-pinoresinol formation from coniferyl alcohol in *Forsythia* species (Paré et al., 1994; Davin et al., 1997). The dirigent protein was proposed to bind and orient coniferyl alcohol-derived radicals in such a way as to enable 8,8′ coupling at the *si–si* face with subsequent intramolecular cyclization to afford (+)-pinoresinol (Davin and Lewis, 2005).

The biosyntheses of chavicol and eugenol have been shown to occur *via* the phenylpropanoid pathway to *p*-coumaryl alcohol. Activation of the side-chain alcohol of *p*-coumaryl and coniferyl alcohols, e.g. via esterification, to form a more facile leaving group *via* reductive elimination has been demonstrated (Koeduka et al., 2006; Vassão et al., 2006). This was shown to be the case using *p*-coumaryl/coniferyl esters as potential substrates for a NAD(P)H-dependent reductase to afford chavicol and eugenol. Lignans present in *Larrea tridentata* demonstrate an additional means of control of radical–radical coupling. Instead of being of monolignol origin, these lignans are derived from propenyl/allylphenols (presumably from anol) (Davin and Lewis, 2003, 2005). Pathways for the formation of aryltetralin lignans with oxygenation at C-9,9′ have been proposed. The overall pathway for the formation of podophyllotoxin, for example, has been shown to involve coniferyl alcohol, (+)-pinoresinol, (+)-matairesinol, yatein, and deoxypodophyllotoxin (Seidel et al., 2002; Fuss, 2003).

Lignans (**1**–**19**) and neolignans (**20** and **21**) have been isolated from the roots of *H. reniformis* (da Silva and Lopes, 2004, 2006; Andrade-Neto et al., 2007) (Fig. 1). Of these, lignans **6**, **12**, **13**, and **15** have attracted much interest due to their antiplasmodial activity (da Silva et al., 2004; Andrade-Neto et al., 2007). All of these lignans and neolignans are apparently derived from propenylphenols and lack the oxygenated functionalities at C-9,C-9′ which are characteristic of many other phenylpropanoids.

The aim of this work was to contribute to the elucidation of the biosynthetic pathways of lignans without oxygenation at C-9,C-9′ especially those from *H. reniformis*, by *in vivo* conversion of phenylpropanoid intermediates into aryltetralone lignans.

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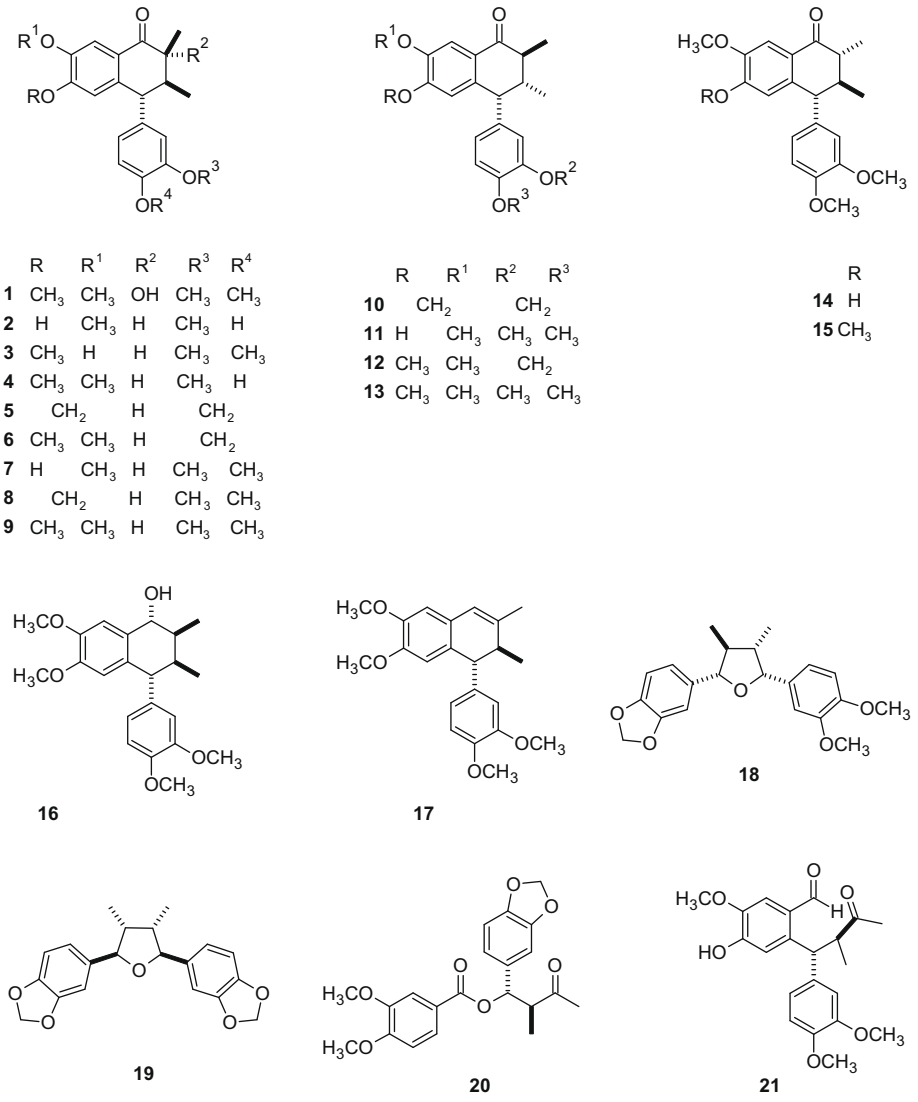

Fig. 1. Chemical structures for compounds **1**–**21**.

2. Results and discussion

Preliminary *in vivo* experiments using the potential radiolabeled precursor [9-$^3H_1$]isoeugenol **23** were carried out at two different durations (4 and 12 h) to identify satisfactory conditions for uptake and metabolism into lignans. After uptake, **9**, the most abundant aryltetralone lignan in acetone extracts (Fig. 2), was isolated by semi-preparative HPLC. Incorporation of the radiolabel into lignan **9** increased slightly with an increase in the duration of metabolism. Therefore, 12 h was chosen for the experiments using the three potential precursors [U-^{14}C]phenylalanine, [9-$^3H_1$]coniferyl alcohol **22**, and [9-$^3H_1$]isoeugenol **23** and for the "blank"/control experiment (Table 1). To verify if the radioactivity shown by the respective extracts obtained after uptake was due to the incorporation of labeled precursor into lignans, the extracts were subjected to semi-preparative HPLC, and the radioactivity of the isolated lignans was individually measured (Tables 2–4). Radiolabel was present in 10 aryltetralone lignans (**2**, **4**–**10**, **12**, and **13**) and two furan lignans (**18** and **19**), which were identified by comparison of their R_t, ESI-MS, and ^{1}H NMR spectra with those of the lignans from the blank/control experiment as well as those of authentic samples previously isolated from this plant (da Silva and Lopes, 2004, 2006; Andrade-Neto et al., 2007).

The best result of recovered activity was observed for acetone extract using [9-$^3H_1$]isoeugenol **23** (75.4%) followed by [9-$^3H_1$]coniferyl alcohol **22** (11.0%). While practically all [9-$^3H_1$]coniferyl alcohol **22** was incorporated into lignans in the ex-

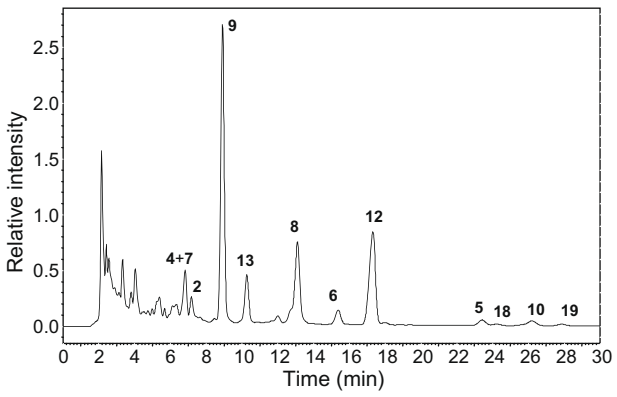

Fig. 2. Representative chromatogram of the acetone extract from the roots of *H. reniformis*. Peaks corresponding to the radiolabeled lignans are shown (C18, 150 × 3.9 mm, UV detection at 254 nm, and flow rate: 0.8 ml min^{-1}).

Table 1
Activities of precursors and extracts from *H. reniformis*.

Radiolabeled precursor	Administered activity (Bq)	Extract code	Extract mass (mg)	Extract activity (Bq)	Yield[a] (%)
[U-^{14}C]Phenylalanine	1.42×10^6	II-Hexane	36.8	6.25×10^2	0.04
		II-Acetone	546.7	1.72×10^4	1.21
[9-^{3}H$_1$]Coniferyl alcohol **22**	9.63×10^6	III-Hexane	20.9	1.20×10^4	0.12
		III-Acetone	399.6	1.06×10^6	11.01
[9-^{3}H$_1$]Isoeugenol **23**	1.28×10^5	IV-Hexane	39.8	4.75×10^3	3.71
		IV-Acetone	429.0	9.65×10^4	75.39
–		I-Hexane	32.7		
		I-Acetone	929.6		

[a] Percentage calculated in relation to total administered radioactivity (Bq).

Table 2
Lignans from *in vivo* [U-^{14}C]phenylalanine administration experiment.

Fraction[a]	Mass (mg)	Compound	Activity (Bq)	Yield[b] (%)
II-3	9.3	**2**	1.70×10^3	0.59
II-4	17.5	**9**	65	0.02
II-5	4.9	**13**	315	0.11
II-6	7.1	[c]	140	0.05
II-7	3.5	**8**	80	0.03
II-8	8.2	**6**	147	0.05
II-9	9.6	**12**	154	0.05
II-10	6.1	**5**	85	0.03
II-11	4.3	**10** + **18**	25	0.01
Total	**70.5**		$\mathbf{2.71 \times 10^3}$	**0.94**

[a] Fractions obtained from the acetone extract (110.0 mg) by HPLC.
[b] Percentage calculated in relation to total administered radioactivity (Bq).
[c] Mixture of lignans.

Table 3
Lignans from *in vivo* [9-^{3}H$_1$]coniferyl alcohol **22** administration experiment.

Fraction[a]	Mass (mg)	Compound	Activity (Bq)	Yield[b] (%)
III-2	4.3	**2** + **4** + **7**	2.80×10^5	10.57
III-3	19.7	**9**	3.05×10^3	0.12
III-4	4.4	**13**	1.05×10^3	0.04
III-5	2.7	[c]	1.02×10^3	0.04
III-6	10.1	**8**	1.67×10^3	0.06
III-7	5.5	**6**	1.04×10^3	0.04
III-8	12.2	**12**	2.00×10^3	0.08
III-9	6.6	**5**	4.83×10^2	0.02
III-10	5.4	**10** + **18**	1.65×10^3	0.06
III-11	2.0	**19**	1.66×10^2	0.01
Total	**72.9**		$\mathbf{2.92 \times 10^5}$	**11.04**

[a] Fractions obtained from the acetone extract (110.0 mg) by HPLC.
[b] Percentage calculated in relation to total administered radioactivity (Bq).
[c] Mixture of lignans.

Table 4
Lignans from *in vivo* [9-^{3}H$_1$]isoeugenol **23** administration experiment.

Fraction[a]	Mass (mg)	Compound	Activity (Bq)	Yield[b] (%)
IV-3	5.4	**2** + **4** + **7**	1.98×10^3	6.04
IV-4	10.4	**2**	78.20	0.24
IV-5	15.2	**9**	20.0	0.06
IV-6	2.8	**13**	12.49	0.04
IV-7	2.7	[c]	7.32	0.02
IV-8	7.0	**8**	11.17	0.03
IV-9	1.4	**6**	11.70	0.04
IV-10	7.5	**12**	9.67	0.03
IV-11	3.9	**5**	4.67	0.01
IV-12	2.3	**10** + **18**	8.00	0.02
IV-13	5.8	**19**	9.50	0.03
Total	**64.4**		$\mathbf{2.15 \times 10^3}$	**6.56**

[a] Fractions obtained from the acetone extract (110.0 mg) by HPLC.
[b] Percentage calculated in relation to total administered radioactivity (Bq).
[c] Mixture of lignans.

tract, the incorporation of [9-^{3}H$_1$]isoeugenol **23** into lignans represented only 8.7% of extract activity. Although the recovered activity for [U-^{14}C]phenylalanine was low (1.2%) in the extract, the fractions that contained lignans contributed to 77.7% of extract activity. The yield of 77.7% was calculated considering the total lignan activity (2.71×10^3 Bq, Table 2) in 110.0 mg, and the extract activity (1.72×10^4 Bq, Table 1) in 546.7 mg. Nonetheless, since phenylalanine and coniferyl alcohol **22** may be biosynthetic precursors of many different metabolites in *H. reniformis*, their activity decrease in the extracts could be related to other biosynthetic pathways.

Thus, administration of [U-^{14}C]phenylalanine to the roots of *H. reniformis* resulted in its conversion into the [^{14}C]aryltetralone (**2**, **5**, **6**, **8**–**10**, **12**, and **13**) and [^{14}C]furan (**18**) lignans, whereas administration of [9-^{3}H$_1$]coniferyl alcohol **22** and [9-^{3}H$_1$]isoeugenol **23** led to [9,9′-^{3}H$_1$]lignans (**2**, **4**–**10**, **12**, **13**, **18**, and **19**). These results suggest the following biosynthetic sequence: phenylalanine, coniferyl alcohol (and coniferyl ester), and isoeugenol for lignan formation in *H. reniformis* (Fig. 3), which is in accordance with that established for eugenol, isoeugenol, and anol (Reichling and Martin, 1990; Kota et al., 2004; Koeduka et al., 2006).

Rodríguez-Evora and Schepp (2005) observed that isoeugenol radical cations undergo rapid deprotonation, as well as rapid reprotonation under moderately acidic conditions in a non-protic, polar solvent. They also suggested that in a biological system, isoeugenol radical cations need not undergo a dimerization event as soon as they are produced by enzymatic oxidation; instead, the radical cations can be "stored" in their less-reactive 2-methoxy-4-vinylphenoxyl radical form, and then regenerated by enzymatic protonation when dimerization is ready to proceed. It seems that all of the lignans from *H. reniformis* have a C8–C8′ bond as a result of isoeugenol radical dimerization. All of the aryltetralone lignans have a 7′*R* configuration. Thus, *H. reniformis* lignans should result from regiospecific 8-8′ coupling, and the corresponding diastereoisomeric hydroxylated intermediates can still be diastereospecifically metabolized to produce either aryltetralol or furan lignans. Furthermore, considering the principle of the lower molecular movement to reach the transition state to form these lignans, from the 10 possible isomers (where the reactive species approach each other from their *re*–*re*, *re*–*si*, *si*–*re* or *si*–*si* faces), only seven (*re*–*re*, *si*–*re* and *re*–*si* faces) could lead to the desirable 7′*R* configuration. In addition, the participation of dirigent protein in the formation of intermediate "diarylbutanes", which in turn could give rise to aryltetralol lignans, such as **16**, by attack of radical or anion hydroxyl on the *exo* faces of quinone methide, or attack on the *endo* faces of quinone methide to yield furan lignans, should be considered. Thus, the number of the possible isomers could be reduced to three (**24**–**26**, Fig. 3). For tetralol lignan formation, an anti-periplanar addition at the double bonds in the quinone methides would result in asymmetric induction in the formation of up to two additional chiral centers (C-7′ and C-7). Therefore, independent of the mechanism, whether it involves radical or carbocation intermediates (Fig. 4), it is important that the hydroxyl group be

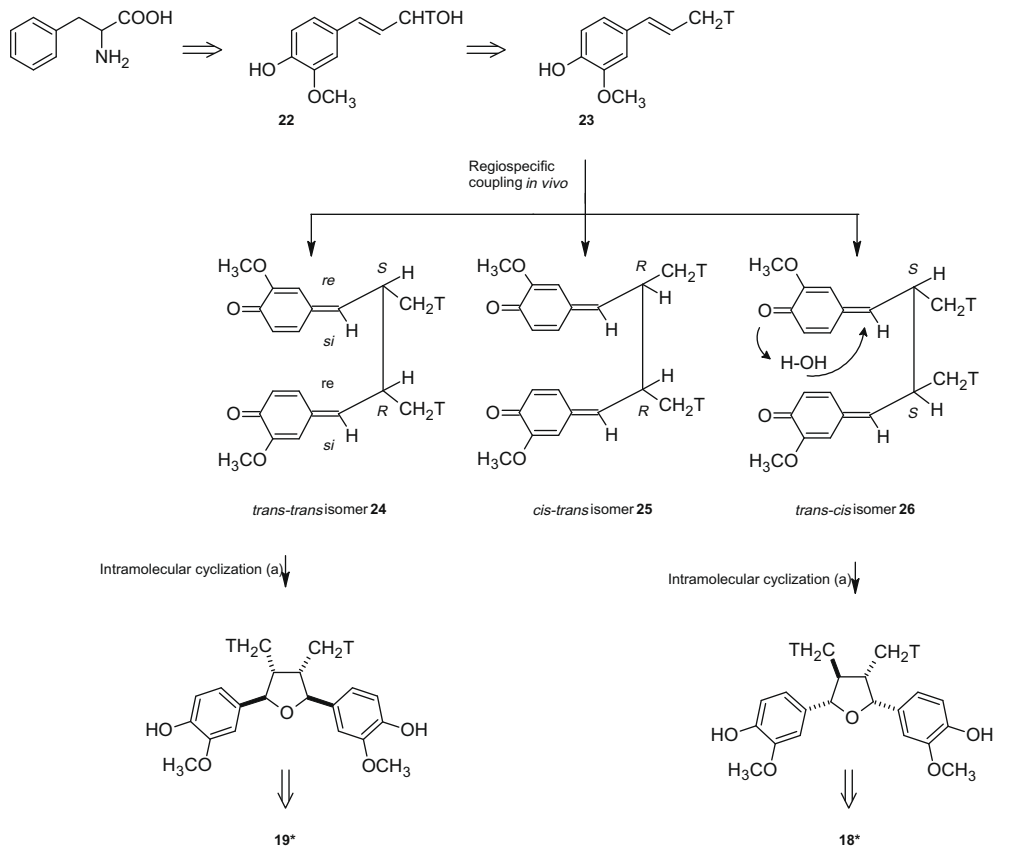

Fig. 3. Proposed biochemical pathways to lignans from [U-^{14}C]phenylalanine, [9-^{3}H$_1$]coniferyl alcohol, and [9-^{3}H$_1$]isoeugenol in *H. reniformis* roots. (*Radiolabeled lignans. [a]Possible mechanisms, which depend on the hydroxyl species attack and protonation positions as shown from intermediate **26**, for the conversion of quinone methides into epoxy lignans.)

anti-periplanar to the new C2–C7′ bond, so that it could lead to intramolecular stereoselective cyclization. Hence, it seems that the preferential configurations for the OH attack are the *re* faces. For furan lignans, such as **18** and **19**, it is important that the intermediate can adopt a conformation in which intramolecular cyclization takes place without great changes in the conformation. Therefore, the biosynthesis of all of these lignans should involve enzymes and/as proteins that are capable of stereospecifically interacting with units of *trans–trans* (**24**) and *trans–cis* (**26**) isomers with an 8*S* configuration. The former give rise to 7′*R*,8*S*,8′*S* (**2**–**9**, **16**, and **19**) and 7′*R*,8*R*,8′*S* (**1**) lignans, from which the corresponding aryltetralone lignans (**7** and **9**) could give lignans with a 7′*R*,8*R*,8′*S* (**14** and **15**, respectively) configuration *via* enolization; while 7′*R*,8*S*,8′*R* lignans (**10**–**13** and **18**) could be obtained from **26** (Figs. 3 and 4).

As previously stated, administration of the hypothetical precursors to the plant roots resulted in the incorporation of ^{3}H and ^{14}C into 2,7′-lignans (**2**, **4**–**10**, **12**, and **13**, aryltetralone lignans) and 7,7′-epoxylignans (**18** and **19**, furan lignans). Although **9** was the lignan isolated in highest concentration from the acetone extracts, the highest yields (calculated in relation to administered activity of precursor) were observed for fractions containing **2** either alone or in mixture with **4** and **7** (Tables 2–4). Quantification of these compounds was not quite accurate because their retention times were very similar and thus their peaks overlapped with that of **2**. In fact, considering the amounts of extracts subjected to semi-preparative HPLC, the production of **2** + **4** + **7** (~14%) was substantially higher in the acetone extract from [9-^{3}H$_1$]isoeugenol than in the "blank" acetone extract (~8%). Considering these results and the B ring stereochemistry of these lignans, the most feasible biosynthetic pathway for them seems to involve intermediate **24**. These administration experiments thus support the pathways proposed by Cho et al. (2003) and Vassão et al. (2007). Instead of originating from monolignol coupling, these lignans should be derived from propenylphenols, which agrees with the Gottlieb hypothesis (Gottlieb, 1978) that allyl- and/or propenylphenols are biosynthetic precursors of lignans with absence of oxygen at C-9,C-9′ (called neolignans at that time). These results also agree with *in vivo* feeding experiments using *L. tridentata* (Davin and Lewis, 2003). However, the aryltetralol lignan intermediate that gives aryltetralone differs from the results obtained in biosynthetic studies on podophyllotoxin, which involve the aryltetralin deoxypodophyllotoxin. If a similar pathway was followed by lignans in *H. reniformis*, then an anti-periplanar hydride attack on the *re* face, followed by intramolecular cyclization, should be proposed. However, in this case the loss of the stability of the intermediate carbocation (**29**) by the unshared electron pair of the oxygen would be lost. Besides, the absence of aryltetralin lignans is not in favor of this pathway in this plant.

3. Conclusions

Holostylis reniformis exhibits control over radical–radical coupling (isoeugenol radical). This control should be regioselective since the plant contains an abundant group of lignans (aryltetralone) that have been shown to be exclusively derived from 8-8′ coupling. Regiospecific control over propenylphenol-derived lignan biosynthesis was observed, together with the diastereoselective control of C2–C7′ bond for aryltetralone lignans (7′*R*). These controls suggest that an anti-periplanar OH group could promote stereoselectivity for aryltetralol formation, and a syn-periplanar orientation could lead exclusively to formation of furan lignans.

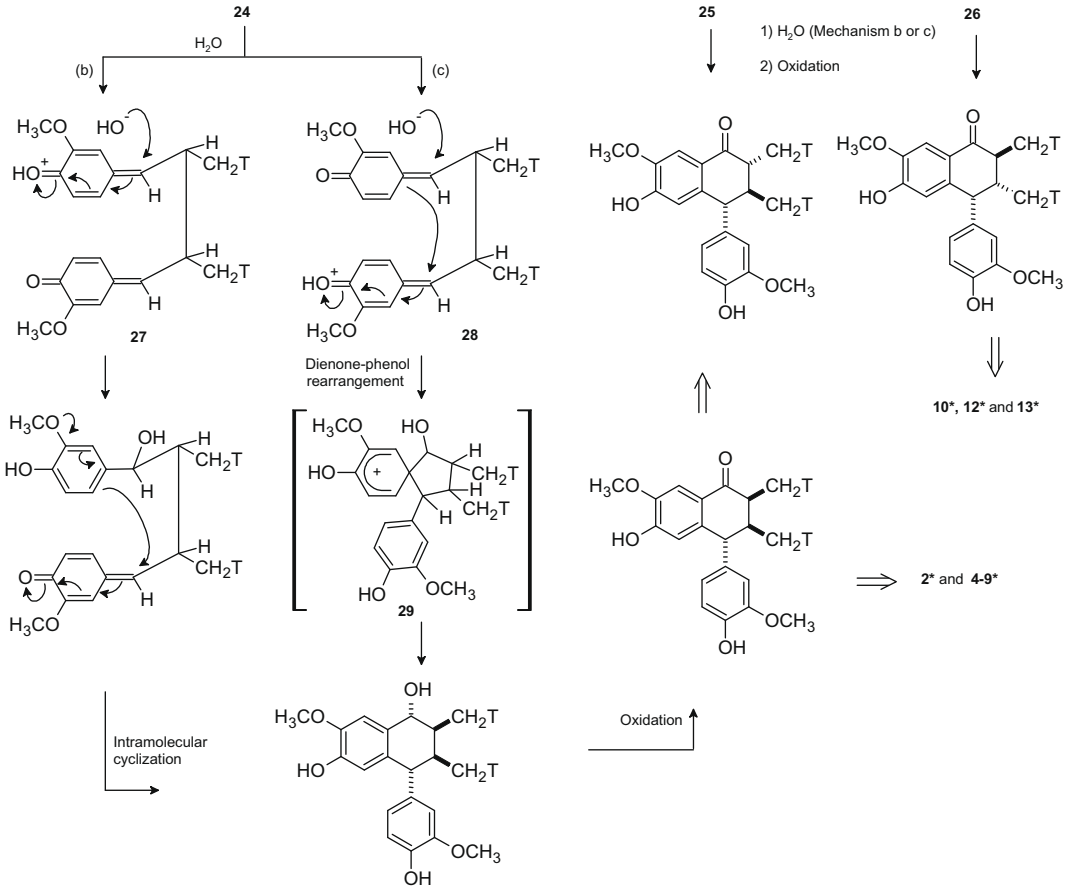

Fig. 4. Proposed biochemical pathways to aryltetralol and aryltetralone lignans from **24**–**26** intermediates in *H. reniformis* roots. (*Radiolabeled lignans. [b,c]Possible mechanisms, which depend on the hydroxyl species attack and protonation positions on **27** and **28**, for the conversion of quinone methides into aryltetralol lignans.)

4. Experimental

4.1. General experimental procedures

One-dimensional (^{1}H, ^{13}C, and *g*NOESY) and two-dimensional (^{1}H-^{1}H *g*COSY, *g*HMQC, *g*HMBC, and *g*NOESY) NMR experiments were recorded on a Varian INOVA 500 spectrometer (11.7 T) at 500 MHz (^{1}H) and 126 MHz (^{13}C), with the solvents used as internal standards. Mass spectra (LC–ESI-MS) were obtained on a Fisons Platform II, and flow injection into the electrospray source was used for LC–ESI-MS. LC analyses were carried out using C18 (MeOH–H_2O 7:3). HPLC analyses were carried out using a Shimadzu liquid chromatograph 10 Avp equipped with a UV–vis detector. Columns were RP18 (Shimadzu, C18, 150 × 3.9 mm, and flow rate: 0.8 ml min^{-1} for analytical analysis and 250 × 20 mm, and flow rate: 8 ml min^{-1} for semi-preparative analysis), and chromatograms were acquired at 254 and 280 nm. Optical rotations were measured on a Perkin Elmer 341-LC polarimeter. Thin-layer chromatography (TLC): silica gel 60 PF_{254}. Radioactive samples were analyzed in 2 ml biodegradable counting scintillant (Amersham Biosciences) and measured using a Packard Tri-carb 21000TR liquid scintillation counter.

4.2. Plant material

Seedlings of *H. reniformis* were grown in the garden at the Chemistry Institute, UNESP, SP, Brazil, using a commercial gardening soil mixture. The seeds were from plants collected in Ituiutaba, MG, Brazil, and were identified as *H. reniformis* Duch. by Dr. Condorcet Aranha and by Dr. Lindolpho Capellari Júnior. A voucher specimen (ESA 88282) was deposited at the herbarium of the Escola Superior de Agricultura, Luiz de Queiroz (ESALQ), Piracicaba, SP, Brazil. The cultivated plants were collected in February, 2006.

4.3. Materials

[U-^{14}C]*L*-Phenylalanine and NaB^{3}H$_4$ were purchased from Perkin Elmer Life. All of the other reagents were purchased from Aldrich Chemical Co., Sigma Chemical Co., or Baker Analysed, and used without purification, except where noted otherwise. All of the yields refer to chromatographically and spectroscopically (^{1}H and ^{13}C NMR) homogenous material unless otherwise stated. Radioactivity decontaminant was purchased from Sigma®/Sigma-clean®. All solvents, either HPLC- or reagent-grade, were purchased from Mallinckrodt Baker.

4.4. Chemical syntheses

All reactions were monitored by TLC analyses using UV and 10% H_2SO_4 – heat as developing agents. All reactions were carried out under an argon atmosphere with dry freshly distilled solvents under anhydrous conditions unless otherwise noted.

4.4.1. [9-$^3H_1$]Coniferyl alcohol (**22**)

[9-^{3}H$_1$]Coniferyl alcohol (**22**) was prepared based on a procedure for obtaining coniferyl alcohol **22** previously described by Nascimento et al. (2000). To coniferyl aldehyde (256.0 mg, 1.44 mmol) dissolved in MeOH (10 ml) in an ice-bath was added NaB^{3}H$_4$ (4.0 mg, 0.11 mmol, a = 7.55 GBq mmol^{-1}) along with MeOH (5 ml). The resulting reaction mixture was stirred for

30 min under an argon atmosphere, after which $NaBH_4$ (238.0 mg, 6.29 mmol) was added and the mixture was stirred for an additional 30 min. Subsequently, MeOH (5 ml), H_2O (10 ml), and a satd. soln. of NH_4Cl (3 ml) were added and the soln. was extracted with EtOAc (3 × 10 ml). The combined organic phase was washed with H_2O (5 ml), dried (dry Na_2SO_4), and concentrated to yield **22** (255.1 mg, 98.0%, $a = 1.83 \times 10^7$ Bq mmol^{-1}).

4.4.2. [9-$^3H_1$]Isoeugenol (**23**)

[9-$^3H_1$]Isoeugenol (**23**) was prepared based on a procedure for obtaining of isoeugenol previously described by Nascimento et al. (2000). To a stirred solution of [9-$^3H_1$]coniferyl alcohol (**22**, 126.0 mg, 0.70 mmol, $a = 1.83 \times 10^7$ Bq mmol^{-1}) and coniferyl alcohol (252.0 mg, 1.40 mmol) in dry CH_2Cl_2 (6 ml) were added MsCl (650 μl, 8.4 mmol) and Et_3N (1.70 ml, 12.6 mmol). After 30 min of stirring, ice water was poured into the soln., which was then extracted with CH_2Cl_2 (3 × 5 ml). The combined organic phase was washed twice with 10% HCl (5 ml), satd. $NaHCO_3$ soln. (5 ml) and brine (5 ml), and then dried (dry Na_2SO_4), and the solvent was evaporated. The resulting oil was subjected to flash chromatography (silica gel 60, CH_2Cl_2) to yield [9-$^3H_1$]coniferyl alcohol mesylate (201.9 mg, 28.6%, 0.60 mmol). The mesylate (192.8 mg, 0.57 mmol) was dissolved in dry THF (6 ml) and, in one portion by means of syringe injection, a 1 M $LiEt_3BH$ solution in THF (2.9 ml) was added to the stirring reaction mixture, which was then maintained in reflux for 9 h. Following reduction, excess hydride was quenched by the dropwise addition of MeOH and H_2O. The organoboranes were oxidized by adding 3 N NaOH (0.5 ml), followed by slow, dropwise addition of 30% H_2O_2 (0.5 ml). The reaction mixture was next poured into H_2O (7 ml), extracted with CH_2Cl_2 (3 × 7 ml), and then washed with H_2O to remove dissolved THF, dried (dry Na_2SO_4), and concentrated to give **23** (93.8 mg, 99.0%, $a = 2.27 \times 10^6$ Bq mmol^{-1}).

4.5. Feeding experiments

Roots and the adjacent parts of the stems (25.51 g) of *H. reniformis* were cut (~4 cm long) and placed directly into 20 vials, divided in four groups, containing 80 μl of distilled H_2O and the following solutions: no labeled compound (I, blank, DMSO, 20 μl), [U-^{14}C]*L*-phenylalanine (II, $a = 5.68 \times 10^4$ Bq, DMSO, 20 μl), [9-$^3H_1$]coniferyl alcohol **22** (III, $a = 3.85 \times 10^5$ Bq, DMSO, 20 μl), and [9-$^3H_1$]isoeugenol **23** (IV, $a = 5.13 \times 10^3$ Bq, DMSO, 20 μl). The addition of solns. containing each precursor in 20 μl DMSO + 80 μl H_2O to the respective vials was repeated for four times following uptake. Water was added as needed during the course of the feeding experiments (12 h). The plant materials in each group were removed and individually freeze-dried, cut, and ground. The extracts were then prepared by two extractions with hexane followed by two extractions with hot acetone, filtered, and concentrated to give eight extracts: I-hexane, I-acetone, II-hexane, II-acetone, III-hexane, III-acetone, IV-hexane, and IV-acetone (Table 1). Their activities were measured using a scintillation system. The extracts were subjected to analytical HPLC, and portions of the acetone extracts were individually subjected to semi-preparative HPLC to collect lignans from selected peaks shown in Fig. 2. Extracts I-acetone (160.0 mg), II-acetone (110.0 ± 0.5 mg), III-acetone (110.0 ± 0.5 mg), and IV-acetone (110.0 ± 0.5 mg) gave fractions I-1 to I-14, II-1 to II-11, III-1 to III-11, and IV-1 to IV-13, respectively (Tables 2–4). Each fraction was filtered through Millipore® polyvinylidene fluoride (PVDF) membranes (0.45 μm, 13.0 mm), and then analyzed by LC–ESI-MS. These fractions were also individually dissolved in MeOH (2 ml), and subjected to liquid scintillation counting (20 μl/2 ml scintillation liquid). Fraction I-3 (3.6 mg) gave a mixture comprised mainly of **2**, **4**, and **7**. Fractions I-4 to I-7 gave **7** (4.7 mg), **2** (3.9 mg), **9** (24.1 mg), and **13** (10.5 mg), respectively. Fractions I-9 to I-11, I-13, and I-14 gave **8** (12.3 mg), **6** (4.7 mg), **12** (14.0 mg), **18** (5.7 mg), and **19** (3.2 mg), respectively. This feeding procedure was also carried out using [9-$^3H_1$]isoeugenol, for 4 h and 12 h after uptake, and this was followed by isolation and determination of the activity of **9** ($a = 4.50 \times 10^2$ Bq mmol^{-1} and 4.83×10^2 Bq mmol^{-1}, respectively).

Lignans **2**, **4**, **6**–**9**, **12**, **13**, **18**, and **19** obtained from I-acetone extract were identified by comparison of their α_D, R_t (Fig. 2), and spectroscopic data (LC–ESI-MS, ^{1}H and ^{13}C NMR) with those of authentic samples (da Silva and Lopes, 2004, 2006; Andrade-Neto et al., 2007), whereas labeled lignans (**2**, **4**–**10**, **12**, **13**, **18**, and **19**) were identified by comparison of their R_t, ^{1}H NMR, and LC–ESI-MS data with those of unlabeled lignans from I-acetone extract and authentic samples.

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References

Andrade-Neto, V.F., da Silva, T., Lopes, L.M.X., do Rosário, V.E., Varotti, F.P., Krettli, A.U., 2007. Antiplasmodial activity of aryltetralone lignans from *Holostylis reniformis*. Antimicrob. Agents Chemoth. 51, 2346–2350.

Cho, M.-H., Moinuddin, S.G.A., Helms, G.L., Hishiyama, S., Eichinger, D., Davin, L.B., Lewis, N.G., 2003. (+)-Larreatricin hydroxylase, an enantio-specific polyphenol oxidase from the creosote bush (*Larrea tridentata*). Proc. Natl. Acad. Sci. USA 100, 10641–10646.

da Silva, T., Lopes, L.M.X., 2004. Aryltetralone lignans and 7,8-secolignans from *Holostylis reniformis*. Phytochemistry 65, 751–759.

da Silva, T., Lopes, L.M.X., 2006. Aryltetralol and aryltetralone lignans from *Holostylis reniformis*. Phytochemistry 67, 929–937.

da Silva, T., Krettli, A.U., Andrade-Neto, V.F., Lopes, L.M.X., Patent PI0404986-1, 2004. Lignanas, lignanas ariltetralônicas, extratos, processos de obtenção de lignanas, processo de obtenção de extratos, uso de lignanas, uso de extratos e composição farmacêutica para prevenir e tratar malária. Rev. Prop. Ind. 2004, p. 1774.

Davin, L.B., Lewis, N.G., 2003. An historical perspective on lignan biosynthesis: monolignol, allylphenol and hydroxycinnamic acid coupling and downstream metabolism. Phytochem. Rev. 2, 257–288.

Davin, L.B., Lewis, N.G., 2005. Dirigent phenoxy radical coupling: advances and challenges. Curr. Opin. Biotechnol. 16, 398–406.

Davin, L.B., Wang, H.-B., Crowell, A.L., Bedgar, D.L., Martin, D.M., Sarkanen, S., Lewis, N.G., 1997. Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. Science 275, 362–366.

Fuss, E., 2003. Lignans in plant cell and organ cultures: an overview. Phytochem. Rev. 2, 307–320.

Gottlieb, O.R., 1978. Neolignans. Fortschr. Chem. Org. Naturst. 35, 1–72.

Koeduka, T., Fridman, E., Gang, D.R., Vassão, D.G., Jackson, B.L., Kish, C.M., Orlova, I., Spassova, S.M., Lewis, N.G., Noel, J.P., Baiga, T.J., Dudareva, N., Pichersky, E., 2006. Eugenol and isoeugenol, characteristic aromatic constituents of spices, are biosynthesized via reduction of a coniferyl alcohol ester. Proc. Natl. Acad. Sci. USA 103, 10128–10133.

Kota, P., Guo, D., Zubieta, C., Noel, J., Dixon, R.A., 2004. *O*-Methylation of benzaldehyde derivatives by "lignin specific" caffeic acid 3-*O*-methyltransferase. Phytochemistry 65, 837–846.

Moss, G.P., 2000. Nomenclature of lignans and neolignans. Pure Appl. Chem. 72, 1493–1523.

Nascimento, I.R., Lopes, L.M.X., Davin, L.B., Lewis, N.G., 2000. Stereoselective synthesis of 8,9-licarinediols. Tetrahedron 56, 9181–9193.

Paré, P.W., Wang, H.-B., Davin, L.B., Lewis, N.G., 1994. (+)-Pinoresinol synthase: a stereoselective oxidase catalyzing 8,8′-lignan formation in *Forsythia intermedia*. Tetrahedron Lett. 27, 4731–4734.

Reichling, J., Martin, R., 1990. Further studies on the biosynthesis of pseudoisoeugenols in tissue cultures of *Pimpinella anisum*. Z. Naturforsch. 45c, 942–948.

Rodríguez-Evora, Y., Schepp, N.P., 2005. Reactivity of 4-vinylphenol radical cations in solution: implications for the biosynthesis of lignans. Org. Biomol. Chem. 3, 4444–4449.

Seidel, V., Windhövel, J., Eaton, G., Alfermann, A.W., Arroo, R.R.J., Medarde, M., Petersen, M., Woolley, J.G., 2002. Biosynthesis of podophyllotoxin in *Linum album* cell cultures. Planta 215, 1031–1039.

Umezawa, T., 2003. Diversity in lignan biosynthesis. Phytochem. Rev. 2, 371–390.
Vassão, D.G., Gang, D.R., Koeduka, T., Jackson, B., Pichersky, E., Davin, L.B., Lewis, N.G., 2006. Chavicol formation in sweet basil (*Ocimum basilicum*): cleavage of an esterified C9 hydroxyl group with NAD(P)H-dependent reduction. Org. Biomol. Chem. 4, 2733–2744.
Vassão, D.G., Kim, S.-J., Milhollan, J.K., Eichinger, D., Davin, L.B., Lewis, N.G., 2007. A pinoresinol-lariciresinol reductase homologue from the creosote bush (*Larrea tridentata*) catalyzes the efficient *in vitro* conversion of *p*-coumaryl/coniferyl alcohol esters into the allylphenols chavicol/eugenol, but not the propenylphenols *p*-anol/isoeugenol. Arch. Biochem. Biophys. 465, 209–218.