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FURANOFURAN LIGNAN METABOLISM AS A FUNCTION OF SEED MATURATION IN *SESAMUM INDICUM*: METHYLENEDIOXY BRIDGE FORMATION

IN HONOUR OF PROFESSOR G. H. NEIL TOWERS 75TH BIRTHDAY

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Key Word Index—*Sesamum indicum*; Pedaliaceae; seed development; methylenedioxy bridge formation; lignans; enantiospecificity; pinoresinol; piperitol; sesamin; sesamolin; antioxidants

Abstract—*Sesamum indicum* seeds accumulate the antioxidant lignans, (+)-sesamin and (+)-sesamolin. It was established that seeds of 8-week old *S. indicum* plants catalyzed distinct lignan transformations (methylenedioxy bridge formation in piperitol and sesamin, as well as oxygen insertion in sesamolin) depending upon the stage of seed maturity, i.e. lignan formation was developmentally regulated. The most mature seeds at the eight week developmental stage efficiently converted (+)-[3,3′-O^{14}CH$_3$] pinoresinol into (+)-piperitol and (+)-sesamin, whereas younger seeds had a higher conversion into (+)-sesamolin; the corresponding (−)-[3,3′-O^{14}CH$_3$] pinoresinol antipode was not metabolized. Microsomal preparations obtained from *S. indicum* seeds catalyzed methylenedioxy bridge formation via an O_2/NADPH/cytochrome P-450 dependent transformation of (+)-pinoresinol into (+)-piperitol. Preliminary characterization of the enzyme established its cytochrome P-450 dependence.

INTRODUCTION

The sesame lignans (Figure 1, **1–4**) have important roles in plant defense, as well as being commercially employed as potent antioxidants (e.g. sesamolin **4**) [1]. They can also function either as synergists with pyrethroids in insecticidal preparations [2, 3], or as cyclic AMP phosphodiesterase [4] and Δ-5 desaturase inhibitors in mammalian species [5]. Lignans are also of rapidly growing interest because of their dietary role(s) in significantly reducing the incidence rates of breast and prostate cancers [6] and in helping lower cholesterol levels [7–9].

For the above reasons, establishing the biosynthetic pathway to the sesame lignans that are primarily found in the seeds of *Sesamum indicum* is of interest as the first step in biotechnologically manipulating their levels *in vivo*. The plant grows to maturity over about a 12 week period during normal growth and development, with seed and seed pod initiation, formation and maturation occurring continually after *ca.* 6 weeks. In a previous study [10], the metabolic fate of (±)-[3,3′-O^{14}CH$_3$] pinoresinols **1a/1b** was investigated in mature seeds of *S. indicum*, where it was found that only the (+)-enantiomer **1a** was metabolized *in vivo*, giving radiolabeled (+)-piperitol **2a**, (+)-sesamin **3a**, and (+)-sesamolin **4a** Fig. 1. The objective of this investigation was to define the enzymology involved in methylenedioxy bridge formation, specifically that involved in (+)-piperitol **2a** biosynthesis. There are only two previous reports of aryl methylenedioxy bridge formation in plants, namely that affording benzylisoquinoline alkaloids [11, 12] and isoflavonoids [13], respectively. This contribution identifies the role of an enantiospecific, NADPH-dependent cytochrome P-450 involved in (+)-piperitol **2a** formation, in general accordance with the enzymology previously established for the alkaloids and isoflavonoids [11–13].

RESULTS AND DISCUSSION

Label incorporation of pinoresinol into furanofuran lignans

It was first instructive to ascertain whether sesame lignan **2–4** formation in seeds was developmentally regulated or not. Thus, three groups of *S. indicum*

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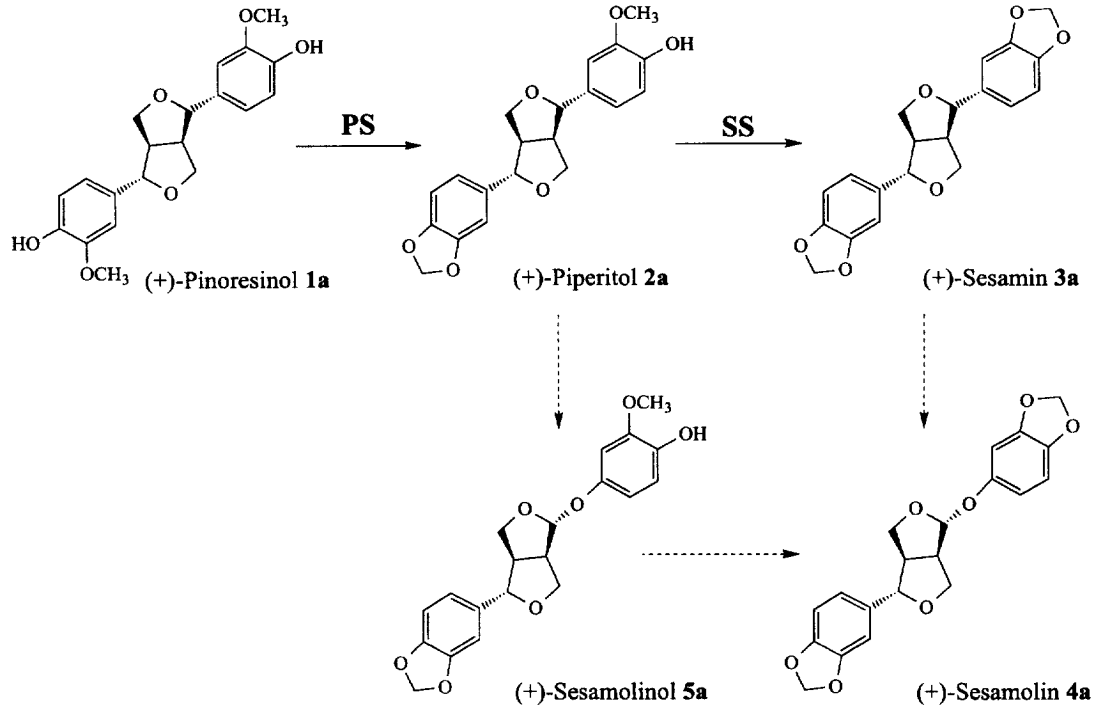

Fig. 1. Conversion of (+)-pinoresinol **1a** into (+)-piperitol **2a** and (+)-sesamin **3a**, with possible subsequent metabolic steps to afford (+)-sesamolin **4a** in *Sesamum indicum*; PS: (+)-piperitol synthase; SS: (+)-sesamin synthase.

seeds were harvested at different plant developmental stages, these being trivially characterized as young (6 week), intermediate (8–10 week) and mature (12–14 week) plants, respectively. Each seed group was individually administered radiolabeled (±)-[3,3′-$O^{14}CH_3$] pinoresinols **1a/1b** (156 μg, 50 kBq mg^{-1}, 200 μl) in 5% DMSO and 5% Tween 80. After incubation at 25° for 2 h, the seeds were extracted with EtOAc, and following partial purification by silica gel column chromatography, the radiolabeled lignans were reconstituted in MeOH (100 μl), containing (+)-sesamin **3a** (~20 μg), and (+)-sesamolin **4a** (~20 μg) as radiochemical carriers; (+)-piperitol **2a** was not added as it was present in good abundance in the seeds. This mixture was then subjected to reversed-phase HPLC analysis with radiochemical detection, and subsequent chiral column HPLC analysis revealed only the presence of the (+)-antipodes. For young, intermediate and mature plants, respectively, this experiment gave precursor incorporations of 0.16, 0.54 and 0.29% into (+)-[3′-$O^{14}CH_3$, 3-$O^{14}CH_2$] piperitol **2a** and 0.18, 0.62 and 0.37% into (+)-[3, 3′-$O^{14}CH_2$] sesamin **3a**. Based upon these results, two month old (intermediate) plants were used for the remainder of the study.

Developmental regulation of lignan biosynthesis

In order to determine subsequent standard assay conditions, 8–10 week old seeds were next administered (±)-[3,3′-$O^{14}CH_3$] pinoresinols **1a/1b** (156 μg, 50 kBq mg^{-1}, 200 μl) as before, but with incubation times of 0.25, 0.5, 1 and 4 h, and then every 4 h up to 44 h, respectively. Over this time frame, absolute incorporations ranging from 1.6 to 4.6% for (+)-piperitol **2a**, and 0.86 to 2.24% for (+)-sesamin **3a** (see Experimental). were observed. All subsequent experiments used an one hour incubation period.

Seeds from intermediate (8 week old) *S. indicum* plants were then arbitrarily segregated into six different stages of maturity (see Fig. 2(A)), with each group being administered (±)-[3,3′-$O^{14}CH_3$] pinoresinols **1a/1b** (156 μg, 50 kBq mg^{-1}, 200 μl). It should be noted that for the 8-week old plant, the seed pods at stage 6 had begun to emerge at about 1 week after initial flowering, and the remaining stages 1–5 emerged subsequently and sequentially at approximately 3–4 day intervals. As can be seen (Figure 2(B)), the relative formation of (+)-piperitol **2a**, (+)-sesamin **3a** and (+)-sesamolin **4a** was directly correlated with the developmental stage of the maturing seeds. Thus, seeds at the very earliest point (stage 6) only afforded a small conversion of (+)-[3,3′-$O^{14}CH_3$] pinoresinols **1a** into (+)-piperitol **2a**, whereas at stages 4 and 5, the conversion into (+)-sesamin **3a** and (+)-sesamolin **4a** had reached a maximum. As the seed (pod)s matured further, the ratios of metabolic end-products from (+)-pinoresinol **1a** also changed significantly. At stages 1 and 3, the conversion of (+)-pinoresinol **1a** into (+)-piperitol **2a** now became dominant, although (+)-sesamin **3a** was also formed in good amount. In contrast with stages 4 and 5, however, only a relatively

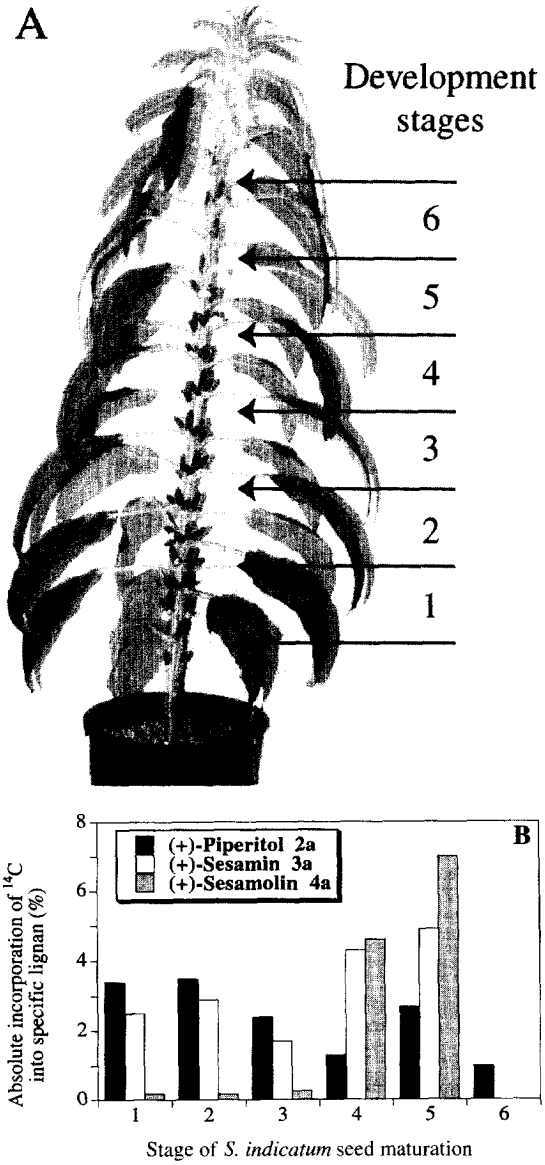

Fig. 2. (A) *S. indicum* plant showing the six stages of seed maturation arbitrarily chosen; and (B) Radiochemical content of (+)-[3′-O^{14}CH$_3$, 3-O^{14}CH$_2$] piperitol **2a**, (+)-[3,3′-O^{14}CH$_2$] sesamin **3a**, and [3,3′±O^{14}CH$_2$] (+)-sesamolin **4a**, respectively, following incubation of *S. indicum* seeds with [3,3′-O^{14}CH$_3$] pinoresinols **1a/1b**. *S. indicum* plants used were grown for 8 weeks, with seeds removed at different stages of maturation (1–6) as shown, and incubated with precursor at 25° for 1 hour under white light.

small conversion into (+)-sesamolin **4a** was observed. Taken together, this data clearly suggest a direct correlation between seed maturation stage and specific lignan formation.

In order to unambiguously establish that these radiochemical data were correct, experiments were next conducted using deuterated (±)-[9,9′-^{2}H$_2$; 3,3′-OC^{2}H$_3$] pinoresinols **1a/1b** (156 μg, 200 μl) as precursors, these being incubated with mature sesame seeds at stages 1 and 2. The enzymatically synthesized (+)-piperitol **2a** so obtained was then subjected to reversed-phase HPLC/mass spectroscopic analysis. This gave a molecular ion ($M^+ + 9$) at *m/z* 365, corresponding to the presence of nine deuterium atoms in the enzymatically formed (+)-[9,9′-^{2}H$_2$, 3′-OC^{2}H$_3$, 3-OC^{2}H$_2$] piperitol **2a**. Thus (+)-pinoresinol **1a** was intactly incorporated into (+)-piperitol **2a**.

The first and second stages of *S. indicum* seed development were next used to identify the cofactor requirement of the stereoselective enzyme catalyzing methylenedioxy bridge formation. Assays were individually carried out by incubation of (±)-[3,3′-O^{14}CH$_3$] pinoresinols **1a/1b** (25 mM, 10 μl, 36.9 kBq mg^{-1}) with either crude protein extracts or microsomal preparations from *S. indicum*, in the presence of various redox cofactors, FAD, FMN, NAD$^+$, NADP$^+$, NADH and NADPH (1 mM, final concentration) at pH 7.5 (see Experimental). The lignans were then extracted from the assay mixture with EtOAc, and purified by reversed-phase HPLC with radiochemical detection as before. Formation of radiolabeled piperitol **2** was detected only when the microsomal preparation was incubated with (±)-[3,3′-O^{14}CH$_3$] pinoresinols **1a/1b** in the presence of NADPH; none of the other co-factors were effective in helping catalyze this conversion. Interestingly, no conversion into either sesamin **3** or sesamolin **4** was observed, in contrast to the results of precursor incubation with the intact sesame seeds.

Chiral column HPLC analyses of the purified piperitol **2** (see Experimental) revealed that the microsomal preparations also catalyzed the enantiospecific formation of piperitol **2** (Figure 3(A)–(C)). That is, following incubation with the NADPH-dependent piperitol synthase microsomal preparation, only the (+)-antipode **2a** was radiolabeled (Figure 3(B)). A further confirmation of this result was obtained by examination of the relative ratio of the (+)- and (−)-antipodes of pinoresinols **1a/1b** remaining following incubation with the (+)-piperitol synthase. As can be seen in Fig. 3(C), only the (+) antipode is depleted, with the ratio of (+)- to (−)-enantiomers now having dropped to 5:7, relative to that of the original substrate (1:1). For illustrative purposes, Fig. 3(A) and (D) show the facile separation of the (+)- and (−)-enantiomers of [3,3′-O^{14}CH$_3$] pinoresinol **1a/1b** and synthetic [3′-O^{14}CH$_3$] piperitols **2a/2b**, respectively.

Enzymatic conversion of pinoresinol **1** *to piperitol* **2**

For final verification of the identity of the (+)-piperitol synthase *in vitro* product, ten assays were next simultaneously conducted using (±)-[9,9′-^{2}H$_2$; 3,3′-OC^{2}H$_3$] pinoresinols **1a/1b** (25 mM, 10 μl) as substrate in the presence of 1 mM NADPH and the microsomal preparation. The assay mixture was then submitted to reversed-phase HPLC column chromatography/mass spectrometry analysis as before and the piperitol **2** isolated gave a molecular ion at *m/z*

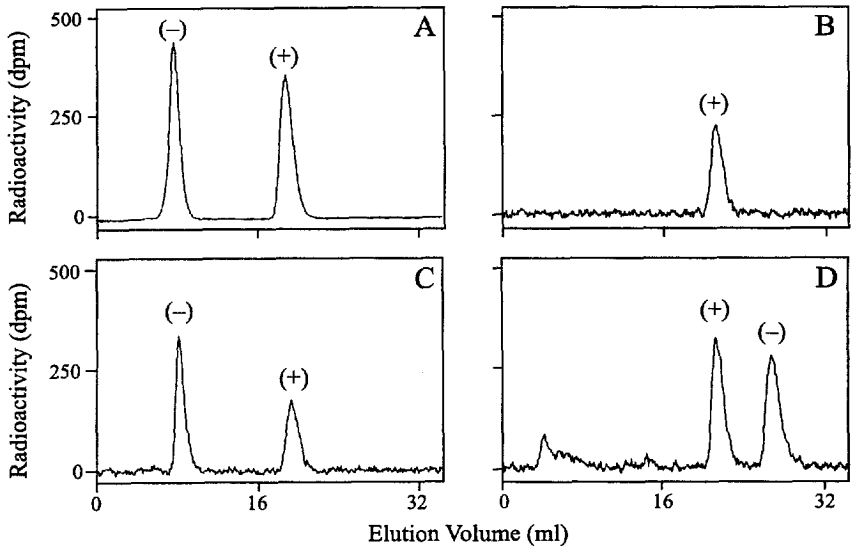

Fig. 3. Chiral HPLC separations: (A) (+)- and (−)-antipodes of (±)-[3,3′-O^{14}CH$_3$] pinoresinols **1a/1b**; (B) Determination of optical purity of pre-purified (+)-[3′-O^{14}CH$_3$, 3-O^{14}CH$_2$] piperitol **2a**, formed following incubation of (±)-[3,3′-O^{14}CH$_3$] pinoresinols **1a/1b** with *S. indicum* seed microsomal preparation; (C) Enantiomeric composition of [3,3′-O^{14}CH$_3$] pinoresinols **1a/1b**, remaining after incubation with piperitol synthase, where only the (+)- antipode is depleted; and (D) Chiral separation of (+)- and (−)-antipodes of synthetic (±)-[3′-O^{14}CH$_3$] piperitols **2a/2b**.

365 ($M^+ + 9$), corresponding to the presence of nine deuterium atoms. Thus, the NADPH-dependent microsomal preparation unambiguously catalyzed conversion of (+)-pinoresinol **1a** into (+)-piperitol **2a**, in accordance with the enantiospecific transformation noted for the intact sesame seeds.

Having established the authenticity of the enzymatic transformation, a time-course analysis was next carried out (Figure 4(A)). As can be seen, this was approximately linear over a 30 min time-frame reaching a maximum level within *ca.* 1 h. Assays were also conducted at various pH values, ranging from pH 6.5 to 9.8 (see Experimental) which gave an optimum at pH 7.5 (Figure 4(B)); the temperature dependence of the reaction was also investigated, and a maximum activity at 40° was observed (Figure 4(C)).

Characteristics of (+)-piperitol synthase

As discussed earlier, NADPH (1 mM) was essential for (+)-piperitol **2a** formation by the *S. indicum* microsomal-catalyzed preparation. However, a NADPH regenerating system ($NADP^+$ (1 mM), glucose-6-P (1.5 mM), glucose-6-P-dehydrogenase (10 units)) also enhanced product formation by *ca.* 13%, and addition of $MgCl_2$ (10 mM) in the presence of NADPH (1 mM) resulted in a 111% further increase compared to NADPH alone. On the other hand, the simultaneous addition of NADPH (1 mM) and NADH (1 mM) had no additional effect on further increasing piperitol **2a** formation.

Given the enantiospecific conversion catalyzed by the *S. indicum* microsomal preparation and its NADPH requirement, this strongly suggested the possible involvement of an O_2-requiring cytochrome P-450 for methylenedioxy bridge formation leading to (+)-piperitol **2a**, as observed previously for the formation of the alkaloid, canadine [12], and the isoflavonoid, pseudobaptigenin [13]. To establish that this was indeed correct, experiments were next carried out by incubation of the microsomal preparation with several established P-450-specific inhibitors. The results shown in Table 1 demonstrate that clotrimeizol (300 μM), miconazol (300 μM) and cytochrome C (170 μM) resulted in complete inhibition of (+)-piperitol synthase, whereas the others displayed a strong inhibition.

Accordingly, it was next instructive to establish whether this conversion was light reversible and inhibited by carbon monoxide, since this is diagnostic for cytochrome P-450 monooxygenases [14]. As can be seen in Table 2, incubation of the microsomal preparation with substrate in a CO/O_2 (9:1) atmosphere in the dark led to a 90% inhibition of (+)-piperitol synthase, which could be partially reversed by incubation under white light. Under these assay conditions, O_2 is not a limiting factor in the reaction as shown by a control experiment with N_2/O_2 (9:1) (Table 2).

Thus, the (+)-piperitol synthase microsomal preparation from mature seeds of *S. indicum* required both O_2 and NADPH, and was strongly inhibited by CO, cytochrome c, and various P-450-specific inhibitors. Given that CO inhibition could also be partially reversed under white light, these data clearly establish that methylenedioxy bridge formation affording (+)-

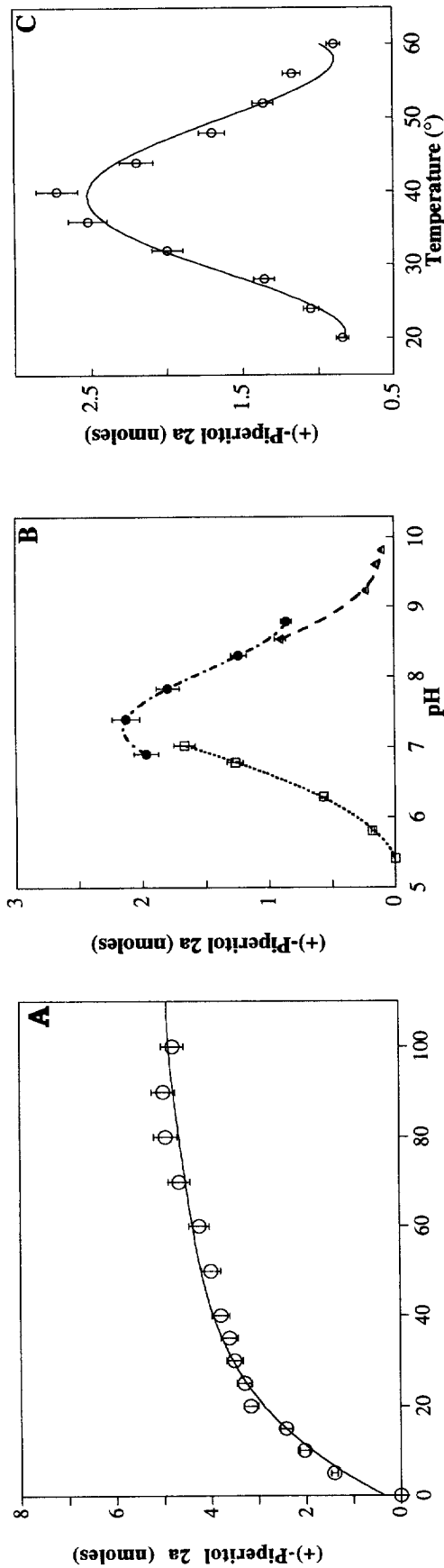

Fig. 4. Characterization of the (+)-piperitol synthase conversion: (A) Time-course of formation of (+)-[3′-O^{14}CH$_3$, 3-O^{14}CH$_2$] piperitol **2a** catalyzed by (+)-piperitol synthase; (B) pH optimum (□: MES-NaOH buffer, ●: Tris–HCl buffer and △: Glycine–NaOH buffer); and (C) temperature optimum Buffer concentration: 100 mM; assays used (±)-[3,3′-O^{14}CH$_3$] pinoresinols **1a**/**1b** (25 mM, 10 μl, 36.9 kBq mg^{-1}) in the presence of NADPH (1 mM).

Table 1. Effect of various cytochrome P-450 enzyme inhibitors on the activity of microsomal bound (+)-piperitol synthase from *S. indicum*

Cytochrome P-450 inhibitor	Inhibitor concentration (μM)	(+)-Piperitol **2a** formation (nmol)	Inhibition (%)
None	0	5.29	0
Clotrimeizol	300	0	100
	35	0	100
Metyrapone	300	2.84	46.4
	35	3.86	26.8
Miconazol	300	0	100
	35	1.58	71.2
Tropolone	300	1.81	65.5
	35	2.34	54.4
Cytochrome c	170	0	100
	17	2.51	52.4[1]

For assay conditions, see Experimental.

Table 2. Effect of carbon monoxide and light on the activity of microsomal bound (+)-piperitol synthase from *S. indicum*

Assay condition	Enzyme activity (%)
Air	100
N_2/O_2 (9:1) dark	97
N_2/O_2 (9:1) white light	95
CO/O_2 (9:1) dark	9
CO/O_2 (9:1) white light	42

For assay conditions, see Experimental.

piperitol **2a** from (+)-pinoresinol **1a** in sesame seeds is a cytochrome P-450-dependent reaction.

In summary, when (±)-[3,3′-O^{14}CH$_3$] pinoresinols **1a**/**1b** were administered to eight week old sesame seeds at their most advanced stage of maturation, (+)-[3,3′-O^{14}CH$_3$] pinoresinol **1a** was metabolized into (+)-[3′-O^{14}CH$_3$, 3-O^{14}CH$_2$] piperitol **2a**, (+)-[3,3′-O^{14}CH$_2$] sesamin **3a** and (+)-[3,3′-O^{14}CH$_2$] sesamolin **4a**, respectively. However, when (±)-[3,3′-O^{14}CH$_3$] pinoresinols **1a**/**1b** were incubated with microsomal preparations at the same sesame seed developmental stage, only (+)-[3′-O^{14}CH$_3$, 3-O^{14}CH$_2$] piperitol **2a** forming capacity was observed, but not that affording either (+)-[3,3′-O^{14}CH$_2$] sesamin **3a** or (+)-[3,3′-O^{14}CH$_2$] sesamolin **4a**. This suggests that methylenedioxy bridge formation of both (+)-sesamin **3a** and (+)-sesamolin **4a** may involve two distinct consecutive O_2/NADPH-requiring cytochrome P-450 dependent enzymatic steps. Future work will examine both enzymatic conversions leading to **2a** and **3a** in greater detail, as well as elucidating the mechanism of oxygen insertion step to give (+)-sesamolin **4a**, together with defining their substrate specificities.

EXPERIMENTAL

Plant material

Sesamum indicum plants were grown from seed to maturity (12 weeks) in Washington State University greenhouse facilities, and were pollinated by hand.

Materials

All solvents and reagents were of reagent or HPLC grade.

Instrumentation

High performance liquid chromatography (HPLC) was carried out as described in [15] using either reversed-phase (Waters, Novapak C18, 150 × 3.9 mm i.d.), or chiral (Daicel, Chiralcel, OD, 240 × 4.6 mm i.d.) columns with detection at 280 nm. For reversed phase HPLC, lignans were separated using a (MeCN-3% HOAc in H_2O, (v/v)) linear gradient solvent system from 2:8 to 19:1 in 60 min at a flow rate of 1.0 ml min^{-1}. Chiral HPLC separation (flow rate 0.8 ml min^{-1}) employed EtOH-hexanes (1:1) for (+)- and (−)-pinoresinols **1a**/**1b** and (1:4) for (+)- and (−)-piperitols **2a**/**2b**, respectively. Radioactive samples were analyzed using a radio-chromatography detector (Radiomatic Series A-100), with Flo-Scint II. EIMS analyses employed a HPLC/MS (Waters Integrity), equipped with a reversed phase column (Waters C_{18} Novapak, 150 × 2 mm i.d., 4 μm) eluted with a linear solvent gradient as follows: MeCN-3% HOAc in H_2O (v/v) from 2:8 to 3:7 in 10 min, then to 1:1 in 25 min and held at this ratio for an additional 10 min (flow rate: 0.25 ml min^{-1}). For MS analyses, TIC (total intensity chromatogram) were recorded from m/z 80 to 700 at a rate of 1 scan s^{-1}, with an optimal nebulizer temperature of 80°. All HPLC sam-

ples were filtered prior to analysis (ACRO LC3S disposable filter, Gelman Science, 0.45 μm).

Chemical synthesis

(±)-[3,3′-O^{14}CH$_3$] pinoresinols **1a**/**1b** and (±)-[3′-O^{14}CH$_3$] piperitols **2a**/**2b** were synthesized as described in [10] and (±)-[9,9′-^{2}H$_2$; 3,3′-OC^{2}H$_3$] pinoresinols **1a**/**1b** were prepared as in [16].

Administration of (±)-[3,3′-*O*^{14}*CH*$_3$] *pinoresinols* **1a**/**1b** *to* S. indicum *seeds from six to fourteen week-old plants*

Three groups of *S. indicum* pods were harvested at different plant developmental stages, namely young (6 week), intermediate (8–10 week) and mature (12–14 week) plants, respectively. Each seed group (~400 mg) was then individually administered radiolabeled (±)-[3,3′-O^{14}CH$_3$] pinoresinols **1a**/**1b** (156 μg, 200 μl, 50 kBq mg^{-1}) in 5% DMSO and 5% Tween 80. After 2 h incubation, the seeds were frozen (liq. N_2), freeze-dried, then ground in a mortar with a pestle and extracted with EtOAc (2 ml) containing (+)-sesamin**3a** (~20 μg), and (+)-sesamolin **4a** (~20 μg) as radiochemical carriers. The EtOAc solubles were concentrated *in vacuo* to give a final volume of *ca.* 500 μl, and applied to a silica gel column (2.5 × 1 cm i.d.), eluted successively with hexanes-EtOAc (1:9, 2 ml) and EtOAc (4 ml). The EtOAc fractions were combined, concentrated in *vacuo*, reconstituted in MeOH (100 μl), with an aliquot (50 μl) subjected to reversed-phase HPLC with radiochemical detection of the eluent. Absolute incorporation levels of (±)-[3,3′-O^{14}CH$_3$] pinoresinols were 0.16, 0.54 and 0.29% into (+)-[3′-O^{14}CH$_3$, 3-O^{14}CH$_2$] piperitol and 0.18, 0.62 and 0.37% into (+)-[3, 3′-O^{14}CH$_2$] sesamin for young intermediate and mature plants, respectively.

Administration of (±)-[3,3′-*O*^{14}*CH*$_3$] *pinoresinols* **1a**/**1b** *to* S. indicum *seeds from eight week-old plants*

Pods were harvested from eight week-old plants at six different stages of maturation (see Fig. 2(A)). The corresponding seeds were then removed and *ca.* 400 mg from each stage (1–6) were placed directly into a solution of (±)-[3,3′-O^{14}CH$_3$] pinoresinols **1a**/**1b** (156 μg, 200 μl, 50 kBq mg^{-1}) in 5% DMSO and 5% Tween 80. Following uptake and metabolism under constant white light at 25° for 0.25, 1 and 4 h, and then every 4 h up to 44 h, respectively, the seeds were removed, frozen (liq. N_2) and freeze-dried. For each time-point, the dried seeds were ground in a mortar with a pestle, the lignans extracted and analyzed by reversed-phase HPLC with radiochemical detection of the eluent, as described above. Fractions corresponding to pinoresinol **1** and piperitol **2** were also individually collected, freeze-dried, then redissolved in MeOH (100 μl) with an aliquot (50 μl) subjected to chiral column (Chiralcel OD) chromatography with radioactivity of eluent measured as before. Absolute incorporation levels of (±)-[3,3′-O^{14}CH$_3$] pinoresinols **1a**/**1b** into (+)-[3′-O^{14}CH$_3$, 3-O^{14}CH$_2$] piperitol **2a** were 1.6, 2.3, 3.4, 2.8, 3.1, 2.9, 4.3, 3.3, 2.0, 2.0, 4.6, 3.5 and 3.0%, respectively for 0.25 h, 1 h, 4 h, and subsequent 4 h increments. In an analogous manner, incorporation into (+)-[3, 3′-O^{14}CH$_2$] sesamin **3a** were 0.86, 1.1, 1.8, 1.5, 1.8, 2.1, 2.2, 1.8, 0.7, 1.1, 2.2, 2.2 and 2.3%, respectively. Accordingly, all subsequent assays were of a 1 h duration.

Administration of (±)-[9,9′-^{2}*H*$_2$, 3,3′-*OC*^{2}*H*$_3$] *pinoresinols* **1a**/**1b** *to* S. indicum *seeds*

To *S. indicum* seeds (400 mg) from 8 week-old plants, at stages 1 and 2 of development (See Fig. 2(A)), were administered (±)-[9,9′-^{2}H$_2$; 3,3′-OC^{2}H$_3$] pinoresinols **1a**/**1b** (156 μg, 200 μl) in 5% DMSO and 5% Tween 80. After 1 h incubation, the seeds were frozen (liq. N_2), freeze-dried and extracted as described above. The EtOAc fractions were combined, concentrated in *vacuo*, reconstituted in MeOH (100 μl), with an aliquot (50 μl) subjected to reversed-phase HPLC/MS. The (+)-[9,9′-2H$_2$, 3′-OC^{2}H$_3$, 3-OC^{2}H$_2$] piperitol **2a** so obtained was then directly analyzed by mass spectral fragmentation. EIMS *m/z* (rel. int.): 365 $(M+9)^+$ (61.8), 332 (9.8), 209 (15.8), 165 (37.6), 154 (81.6), and 151 (100).

Preparation of S. indicum *microsomal fractions*

All manipulations were carried out at 4°. Fresh sesame seeds (20 g) from 8 week-old plants, at stages 1 and 2 of development, were frozen (liq. N_2) and ground (mortar and pestle). The resulting powder was homogenized with PVPP (10% w/w) and Tris–HCl buffer (0.1 M, pH 7.5, 20 ml) containing 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMFS) and 0.5 M sucrose. The homogenate was filtered through Miracloth (Calbiochem) and the filtrate centrifuged (10,000 *g*, 10 min). Microsomes were sedimented by centrifugation (100,000 *g*, 60 min), resuspended in Tris–HCl buffer (0.1 M, pH 7.5, 4 ml), homogenized in a glass–glass homogenizer and then used directly for assaying piperitol **2** formation.

Incubation of (±)-[3,3′-*O*^{14}*CH*$_3$] *pinoresinols* **1a**/**1b** *with* S. indicum *seed microsomal preparations*

Standard assay procedure. Each assay mixture (250 μl) consisted of (±)-[3,3′-O^{14}CH$_3$] pinoresinols **1a**/**1b** (25 mM, 10 μl, 36.9 kBq mg^{-1}), NADPH (25 mM, 10 μl), $MgCl_2$ (250 mM, 10 μl) and the microsomal preparation (220 μl), with the reaction initiated by addition of NADPH (25 mM, 10 μl). (Controls were performed using denatured enzyme, where the microsomal preparation was boiled (96°, 10 min)). After 30 min incubation at 40° with shaking, the reaction mixture was extracted with EtOAc (400 μl) containing (+)-sesamin **3a** (~20 μg) and (+)-

sesamolin **4a** (~20 μg) as radiochemical carriers, and 3-methoxy-4-phenylmethoxybenzaldehyde-4-*O*-benzyl-vanillin (2.42 μg) as an internal standard. After centrifugation (14,000 *g*, 2 min), the EtOAc solubles were removed and the extraction procedure was repeated with EtOAc (400 μl). For each assay, the EtOAc solubles were combined and evaporated to dryness *in vacuo*. The resulting residue was reconstituted in MeOH (100 μl), filtered, with an aliquot (50 μl) subjected first to reversed-phase HPLC to obtain the purified piperitol **2**, and then chiral column chromatography to establish the optical purity. (For determining the cofactor requirement, 10 μl of a 25 mM solution of NADH, NAD^+, $NADP^+$, FMN, and FAD were individually added to the assay mixture in place of NADPH (25 mM). Assays were also conducted using 25 mM $NADP^+$ (10 μl), 50 mM glucose-6-P (10 μl) and glucose-6-P-dehydrogenase (10 μl, 10 units) as a NADPH regenerating system [11]).

Incubation of (±)-[9,9′-$^2H_2$, 3,3′-OC^2H_3] *pinoresinols* **1a**/**1b** *with* S. indicum *microsomal preparations*

(±)-[9,9′-$^2H_2$; 3,3′-OC^2H_3] Pinoresinols **1a**/**1b** (25 mM, 10 μl) were incubated with the microsomal preparation (230 μl) and NADPH (25 mM, 10 μl) exactly as described above. Ten assays were conducted in parallel. After 30 min incubation with shaking, the reaction mixture was extracted with EtOAc, with EtOAc solubles from each assay being combined, evaporated to dryness *in vacuo*, reconstituted in MeOH (100 μl) and submitted to reversed-phase HPLC/MS. The (+)-[9,9′-$^2H_2$, 3-OC^2H_2, 3′-OC^2H_3] piperitol **2a** so obtained, analyzed by mass spectral fragmentation gave: EIMS *m/z* (rel. int.): 365 $(M+9)^+$ (42), 332 (6.2), 209 (14.2), 165 (33.9), 154 (100), 151 (83.9).

pH and temperature optima

Standard assay conditions were used to determine the pH optimum of the (+)-piperitol synthase, except that the buffer was either MES–NaOH (0.1 M, pH 5.0–7.0), Tris–HCl (0.1 M, pH 7.0–9.0) or glycine–NaOH (0.1 M, pH 9.0–10.5). Temperatures between 0° and 64° were evaluated for their capacity to engender (+)-piperitol **2a** formation under the standard assay conditions.

Incubation of microsomal preparation from S. indicum *with* (±)-[3,3′-$O^{14}CH_3$] *pinoresinols* **1a**/**1b** *in the presence of carbon monoxide*

The effects of carbon monoxide and light on microsomal-bound (+)-piperitol synthase were examined using the standard assay conditions conducted in an atmosphere of carbon monoxide–oxygen (9:1) or nitrogen–oxygen (9:1) both in the dark and under white light.

Incubation of microsomal preparation from S. indicum *with* (±)-[3,3′-$O^{14}CH_3$] *pinoresinols* **1a**/**1b** *in the presence of cytochrome-P-450 inhibitors*

To determine the effect of cytochrome-P-450 inhibitors [11, 12], standard assays conditions were employed but in the presence of clotrimeizol, metyrapone, miconazol, tropolone (300 and 35 μM, final concentration) or cytochrome c (170 and 17 μM, final concentration).

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