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NOTE

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Survey and enzymatic formation of lignans of *Anthriscus sylvestris*

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Abstract Gas chromatography – mass spectrometry analysis of the β-glucosidase-treated MeOH extracts of *Anthriscus sylvestris* showed, based on comparison of the mass spectra and retention times with those of authentic samples, the presence of lignans, yatein, secoisolariciresinol, lariciresinol, matairesinol, hinokinin, and pluviatolide. The existence of small amounts of bursehernin was suggested by mass chromatography. In addition, nemerosin and deoxypodophyllotoxin were tentatively identified by comparing the mass spectra with those reported in the literature. Enzyme preparations from *A. sylvestris* catalyzed the formation of secoisolariciresinol and lariciresinol from coniferyl alcohol. Furthermore, the enzyme preparation catalyzed the formation of lariciresinol from (±)-pinoresinols and the formation of secoisolariciresinol from (±)-lariciresinols. Thus, pinoresinol/lariciresinol reductase (PLR) activity was detected. Chiral high-performance liquid chromatography analysis showed selective formation of (+)-lariciresinol and (−)-secoisolariciresinol from (±)-pinoresinols with the *A. sylvestris* PLR preparation, indicating that the stereochemical property of *A. sylvestris* PLR-catalyzed reduction was similar to those of *Forsythia* PLR and *Arctium lappa* ripening seed PLR.

Key words Lignan · *Anthriscus sylvestris* · Biosynthesis

Introduction

Biosynthesis of lignans has been studied from many aspects because this class of compounds has peculiar stereochemical properties[1–3] and various biological activities (e.g., antitumor, antimitotic, antiviral[2–8]). Moreover, biosynthesis of some lignans is closely related to heartwood formation, which is a metabolic event specific to woody plants but not to herbaceous plants.[2,9]

An aryltetralin lignan, podophyllotoxin, isolated from *Podophyllum* plants[6–8] is well known to have antitumor activity. The lignan isolated from *Podophyllum hexandrum* is exploited commercially as a source of the semisynthetic anticancer drugs etoposide and teniposide.[2–8,10] However, owing to the limited supply of the plant, much attention has been focused on the availability and biosynthesis of the lignan.[8] Several woody plants (e.g., *Juniperus savina*, *Thujopsis dolabrata*, *Callitris drummondii*, *Hernandia ovigera*) have been known to produce podophyllotoxin or its congeners.[7] Thus, biotechnological production of the antitumor lignan by the woody plants is a challenging subject in the field of wood chemistry and biochemistry. As the first step, it is critically important to understand the biosynthetic mechanisms. However, much remains unknown about the biosynthesis of podophyllotoxin.

In addition to these woody plants and *Podophyllum* spp., some herbaceous plants such as *Linum* spp. (especially those belonging to the section *Syllinum*, including *Linum flavum* and *Linum album*)[5,11,12] and *Anthriscus sylvestris*[13–19] have been known to produce significant amounts of podophyllotoxin congeners. Studies of lignan biosynthesis in *Linum* spp.,[20] which produce principally 5-methoxypodophyllotoxin in addition to podophyllotoxin,[21] have been reported. Although isolation of podophyllotoxin from *A. sylvestris* has not yet been reported, angeloyl podophyllotoxin has been isolated from the species.[22] In addition, this species produces not only the precursor lignans of podophyllotoxin, deoxypodophyllotoxin (= desoxypodophyllotoxin, anthricin)[23] and yatein,[24] but also the typical heartwood lignans yatein and hinokinin,[17] which are found specifically in the heartwood region in the conifers *Libocedrus yateensis*[25] and *Chamaecyparis obtusa*,[9] respectively. Thus, knowledge of the lignan biosynthetic mechanism in *A. sylvestris* can be applied to biotechnological production of podophyllotoxin in woody and herba-

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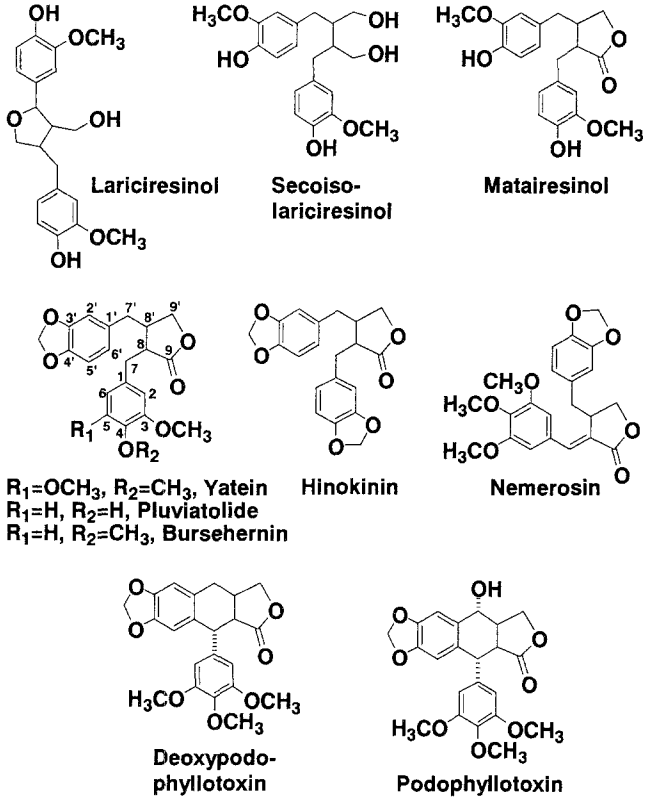

Fig. 1. Chemical structures of lignans

ceous plants and to studies of heartwood lignan biosynthetic mechanisms. In addition, this species exhibits good growth behavior and can be maintained easily in laboratories.

Altogether, *A. sylvestris* is probably a good plant material for lignan biosynthesis studies to access mechanisms involved in the formation of antitumor and heartwood lignans. As the first step, it is necessary to characterize the precursor lignans of yatein and deoxypodophyllotoxin in *A. sylvestris*. We report here the preliminary survey of lignans in this species and the enzyme activities catalyzing formation of lariciresinol and secoisolariciresinol, which are upstream lignans in the biosynthetic pathway (Fig. 1).

Materials and methods

Instrumentation

^{1}H-nuclear magnetic resonance (NMR) spectra were obtained with a JNM-LA400MK FT NMR system (JEOL) using tetramethylsilane as an internal standard. Chemical shifts and coupling constants (J) are given in δ and Hertz, respectively. Gas chromatography-mass spectrometry (GC-MS) was conducted as previously described.[26] Reversed-phase high-performance liquid chromatography (HPLC) was conducted as previously described[27] with the following elution conditions: The column was a Waters Novapak C_{18} (150 × 3.9 mm), which was eluted with two solvent systems: (1) Solvent system A was used for gradient elution at 1 ml/min by a linear solvent gradient protocol of CH_3CN-H_2O at t = 0 (23:77) to 15 min, then to 50:50 at t = 20 min, and held at this ratio for an additional 5 min. (2) Solvent system B was used for gradient elution at 1 ml/min by a linear solvent gradient protocol of CH_3CN-H_2O at t = 0–6 min from 15:85 to 17:83; at t = 6–16 min from 17:83 to 20:80; 20:80 at t = 16–20 min; at t = 20–26 from 20:80 to 50:50; and held at this ratio for an additional 5 min. Chiral HPLC separation of lariciresinol and secoisolariciresinol was conducted exactly as previously described.[26] Silica gel thin-layer chromatography (TLC) and silica gel column chromatography employed Kieselgel 60 F_{254} (Merck) and Kieselgel 60 (Merck), respectively. All solvents and reagents used were of reagent or HPLC grade unless otherwise mentioned.

Compounds

[9,9-$^2H_2$, OC$^2H_3$]Coniferyl alcohol,[27] (±)-[9,9,9′,9′-$^2H_4$]pinoresinols,[28] (±)-pinoresinols,[27] (±)-[9,9,9′,9′-$^2H_4$]lariciresinols,[28] (±)-lariciresinols,[29] (±)-[9,9,9′,9′-$^2H_4$] secoisolariciresinols,[28] (±)-secoisolariciresinols,[27] (±)-matairesinols,[30] (±)-hinokinins,[9] (±)-pluviatolides,[9] and (±)-bursehernins[28] were prepared previously. (±)-Yateins were prepared from (±)-4-demethylyatein benzyl ethers (a gift of Dr. Shingo Kawai) by debenzylation (10% Pd/C, H_2) followed by methylation (diazomethane).

(±)-Yateins, ^{1}H-NMR ($CDCl_3$): 2.43–2.63 (3H, m, $C_{7;8,8'}$H), 2.88 (1H, dd, J = 13.8 and 6.3, C_7H), 2.92 (1H, dd, J = 13.8 and 5.4, C_7H), 3.817 (3H, s, OCH_3), 3.819 (6H, s, OCH_3 × 2), 3.87 (1H, dd, J = 9.0 and 7.6, C_9H), 4.17 (1H, dd, J = 9.1 and 7.2, C_9H), 5.92–5.93 (2H, m, -OCH_2O-), 6.35 (2H, s, $C_{2,6}$H), 6.45–6.47 (2H, m, $C_{2',6'}$H), 6.69 (1H, dd, J = 0.8 and 7.6, $C_{5'}$H).

Plant materials

Anthriscus sylvestris (L.) Hoffm. (Umbelliferae) plants were grown from seeds collected in a suburb of Brussels, Belgium in 1996 or at Kyoto University Forest in Ashiu, Kyoto in 1997. The plants were maintained in the experimental garden of the Wood Research Institute, Kyoto University for about 6 months.

Lignans in *Anthriscus sylvestris*

Aerial parts (including leaves, petioles, racemes) and roots of *A. sylvestris* were individually harvested and washed with tap and distilled water. Each sample (0.7–1.6 g) was frozen (liquid N_2), freeze-dried, disintegrated with scissors, and then extracted with hot MeOH (11 ml). An aliquot (5 mg) of the MeOH extract was treated with β-glucosidase (from almonds; Sigma G-0395) (10 units in 0.5 ml of a 0.1 M NaOAc buffer at pH 5.0) for 24 h at 37°C. The reaction mixture was extracted with CH_2Cl_2 (0.5 ml × 2), and the combined solution was evaporated off. The resulting residue was subjected to GC-MS analysis after derivatization with 7 μl of *N,O*-bis(trimethylsilyl) acetamide (BSA) for 45 min at 60°C.

In a separate experiment, aerial parts of *A. sylvestris* (from University Forest in Ashiu) were harvested and freeze-dried as above. The dried materials (48.7 g) were ground with a Waring blender and extracted with hot MeOH (100 ml × 10); then the solvent was evaporated off. The MeOH extract (12.3 g) was dispersed in Et_2O (50 ml × 10), and the Et_2O-soluble materials were removed. The Et_2O-insoluble residue (10.3 g) was suspended in 180 ml of 0.1 M NaOAc buffer (pH 5.0) containing 2544 units of β-glucosidase, and the solution was incubated for 24 h at 37°C. Then the solution was extracted with EtOAc (80 ml × 4), and the solvent was evaporated off. The EtOAc extract (721 mg) was fractionated into six fractions by silica gel column (φ30 × 80 mm) chromatography. Each fraction was subjected to GC-MS analysis after trimethylsilyl (TMS) derivatization.

Enzyme preparation

An ammonium sulfate precipitate (0%–70% saturation) was prepared from cell-free extracts of young leaves (including petioles) of *A. sylvestris* (from seeds harvested in Belgium) exactly as previously described[31] and used as an enzyme preparation after desalting with a Sephadex G-25 (Pharmacia) column preequilibrated and eluted with a 0.1 M potassium phosphate buffer (pH 7.0). The protein content of the enzyme preparation was 0.55–8.65 mg/ml, being measured by the method of Bradford[32] using bovine serum albumin as a standard.

Enzymatic conversion of [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol

The reaction mixture was composed of 50 μl of 25 mM [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol in a 0.1 M potassium phosphate buffer (pH 7.0), 50 μl of 50 mM NADPH in the same buffer, 25 μl of 7.6 mM H_2O_2 in the same buffer, and 120 μl of the cell-free extracts from *A. sylvestris*. The reaction was initiated by adding H_2O_2. After incubation for 1 h at 30°C, the reaction mixture was extracted with EtOAc containing unlabeled (±)-pinoresinol, (±)-lariciresinol, and (±)-secoisolariciresinol as internal standards. The EtOAc extract was dried under high vacuum and dissolved in 7 μl of BSA. After standing at 60°C for 45 min, an aliquot (0.8 μl) of the BSA solution was subjected to GC-MS analysis, and the lignans formed were identified and quantified (Table 1).

Enzymatic conversion of (±)-[9,9,9′,9′-$^2H_4$]pinoresinols and (±)-[9,9,9′,9′-$^2H_4$]lariciresinols

The reaction mixture consisted of 150 μl of 50 mM of (±)-[9,9,9′,9′-$^2H_4$]pinoresinols in MeOH, 1 ml of 50 mM NADPH in a 0.1 M potassium phosphate buffer (pH 7.0), and 16 ml of the cell-free extracts from *A. sylvestris* leaves. The reaction was initiated by adding (±)-[9,9,9′,9′-$^2H_4$]pinoresinols. After incubating for 1 h at 30°C, the reaction mixture was extracted with EtOAc containing unlabeled (±)-lariciresinol and (±)-secoisolariciresinol as internal standards. An aliquot of the EtOAc extract was subjected to GC-MS analysis, and [$^2H_4$]lariciresinol and [$^2H_4$]secoisolariciresinol were identified. The remainder was submitted to silica gel TLC [solvent, MeOH-CH_2Cl_2 (3:97)] followed by reversed-phase HPLC (solvent system A for the lariciresinol purification, and solvent system B for the secoisolariciresinol purification) to give [$^2H_4$]lariciresinol and [$^2H_4$]secoisolariciresinol. Enantiomeric compositions of the deuterium-labeled lignans were determined as previously described using the corresponding unlabeled (±)-lariciresinols and (±)-secoisolariciresinols as internal standards, respectively.[26–28]

In a separate experiment, the results shown in Table 2 were obtained under assay conditions similar to those described above but with the following scaled-down constituents: 5 μl of 25 mM of (±)-[9,9,9′,9′-$^2H_4$]pinoresinols in

Table 2. Enzymatic formation of [$^2H_4$]lariciresinol from (±)-[9,9,9′,9′-$^2H_4$]pinoresinols

Cofactor	[$^2H_4$]Lariciresinol formation (nmol h^{-1} mg^{-1} protein)
Complete assay	
NADPH	6.18
Control assay[a]	
None	0
Denatured enzyme/NADPH	0

[a] Control assay refers to a complete assay but with omission of either a cofactor or the denatured enzyme (boiled for 10 min)

Table 1. Enzymatic formation of [$^2H_{10}$]pinoresinol, [$^2H_{10}$]lariciresinol, and [$^2H_{10}$]secoisolariciresinol from [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol

Cofactor	[$^2H_{10}$]Pinoresinol formation[b]	[$^2H_{10}$]Lariciresinol formation[b]	[$^2H_{10}$]Secoisolariciresinol formation[b]
Complete assay			
H_2O_2/NADPH	20.7	13.6	36.0
Control assay[a]			
H_2O_2	144.0	0	0
NADPH	11.1	10.7	16.9
Denatured enzyme/ H_2O_2/NADPH	8.2	0	0

[a] Control assay refers to a complete assay but with omission of either cofactors or the denatured enzyme (boiled for 10 min)
[b] Expressed in nanomoles per hour per milligram of protein

MeOH, 50 μl of 50 mM NADPH in a 0.1 M potassium phosphate buffer (pH 7.0), and 200 μl of the enzyme preparation.

(±)-[9,9,9′,9′-$^2H_4$]Lariciresinols were incubated under the same assay conditions as above, except that 5 μl of 25 mM (±)-[9,9,9′,9′-$^2H_4$]lariciresinols were used as substrates.

Results and discussion

The GC-MS analysis of the β-glucosidase-treated MeOH extracts of the aerial parts and roots of *A. sylvestris* showed the presence of the lignans yatein and secoisolariciresinol, which were identified by comparing of the mass spectra and retention times on GC with those of authentic samples.

Yatein: MS *m/z* (%): 400([M]$^+$, 100), 368(20), 239(10), 223(10), 181(88), 135(31)

Secoisolariciresinol (TMS ether): MS *m/z* (%): 650([M]$^+$, 20), 368(43), 560(10), 470(22), 261(52), 209(100), 179(21)

In addition, two GC peaks, **A** and **B**, were detected.

A: MS, *m/z* (%): 398([M]$^+$, 23), 263(100), 235(4), 207(18), 176(12), 161(5), 135(55), 209(100), 179(21)

B: MS, *m/z* (%): 398([M]$^+$, 100), 283(5), 230(13), 185(18), 181(30), 173(21)

The mass spectra of peaks **A** and **B** essentially matched data in the literature for the mass spectra of nemerosin[16] and deoxypodophyllotoxin,[33] respectively, which were previously isolated from *Anthriscus* spp.[13–19]

When partially fractionated MeOH extracts after glucosidase treatment from aerial parts of *A. sylvestris* by silica gel column chromatography were analyzed by GC-MS, the lignans lariciresinol, matairesinol, hinokinin, and pluviatolide were identified by comparing the mass spectra and retention times with those of authentic samples.

Lariciresinol (TMS ether): *m/z* (%): 576([M]$^+$, 41), 561 (10), 486(26), 324(31), 277(31), 223(46), 209(45), 179(19)

Matairesinol (TMS ether): MS *m/z* (%): 502([M]$^+$, 94), 293(4), 209(100), 179(37)

Hinokinin: MS *m/z* (%): 354([M]$^+$, 58), 135(100)

Pluviatolide (TMS ether): MS *m/z* (%): 428([M]$^+$, 85), 179(28), 135(26)

In addition, the presence of small amounts of bursehernin was suggested by comparing mass chromatograms with those of the synthesized authentic sample. Secoisolariciresinol, lariciresinol, matairesinol, and pulviatolide were identified for the first time in *Anthriscus* spp.

Recently, it has been found that there is great stereochemical diversity in upstream steps of lignan biosynthesis, and it is important to examine the stereochemical property of enzymatic reactions of the upstream steps in *A. sylvestris*. When [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol was incubated with the *A. sylvestris* enzyme preparation in the presence of NADPH and H_2O_2, the lignans [$^2H_{10}$]pinoresinol, [$^2H_{10}$]lariciresinol, and [$^2H_{10}$]secoisolariciresinol were formed (Table 1, Fig. 2). They were identified by comparing their mass spectra as TMS ethers and the retention times

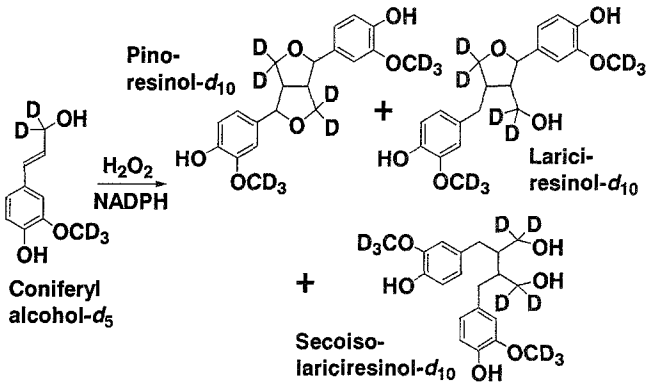

Fig. 2. Enzymatic conversion of [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol to [$^2H_{10}$]pinoresinol, [$^2H_{10}$]lariciresinol, and [$^2H_{10}$]secoisolariciresinol

Table 3. Enzymatic formation of [$^2H_4$]secoisolariciresinol from (±)-[9,9,9′,9′-$^2H_4$]lariciresinols

Cofactor	[$^2H_4$]Secoisolariciresinol formation (nmol h^{-1} mg^{-1} protein)
Complete assay	
NADPH	47.3
Control assay[a]	
None	3.2
Denatured enzyme/NADPH	11.8

[a] Control assay refers to a complete assay but with omission of either a cofactor or the denatured enzyme (boiled for 10 min)

by GC with those of unlabeled authentic samples, as previously reported.[26,27,34] Dehydrogenated dimers other than pinoresinol were not investigated. Next, we incubated (±)-[9,9,9′,9′-$^2H_4$]pinoresinols with the enzyme preparation in the presence of NADPH because pinoresinol/lariciresinol reductase (PLR) has been known to be involved in the production of lariciresinol and secoisolariciresinol from pinoresinol.[35] GC-MS analysis indicated that [$^2H_4$]lariciresinol was produced during the incubation (Table 2). [$^2H_4$]Lariciresinol was not formed with the omission of NADPH or with denatured enzyme, demonstrating that this reaction is enzymatic and requires NADPH as a cofactor. [$^2H_4$]Secoisolariciresinol was not detected in this assay, probably due to the low enzyme activity. Therefore, we next incubated (±)-[9,9,9′,9′-$^2H_4$]lariciresinols with the preparation in the presence of NADPH, which afforded [$^2H_4$]secoisolariciresinol (Table 3). Control assays resulted in insignificant specific activity, demonstrating that this reaction is enzymatic and needs NADPH as a cofactor. These results demonstrate pinoresinol/lariciresinol reductase (PLR) activity in this species. In a separate experiment, when we carried out the scaled-up incubation of (±)-[9,9,9′,9′-$^2H_4$]pinoresinols with the enzyme preparation in the presence of NADPH, both [$^2H_4$]lariciresinol and [$^2H_4$]secoisolariciresinol were formed, and their enantiomeric compositions were determined as follows: [$^2H_4$]lariciresinol, 93% *e.e.* in favor of (+)-enantiomer; [$^2H_4$]secoisolariciresinol, 95% *e.e.* in favor of (−)-enantiomer (Fig. 3).

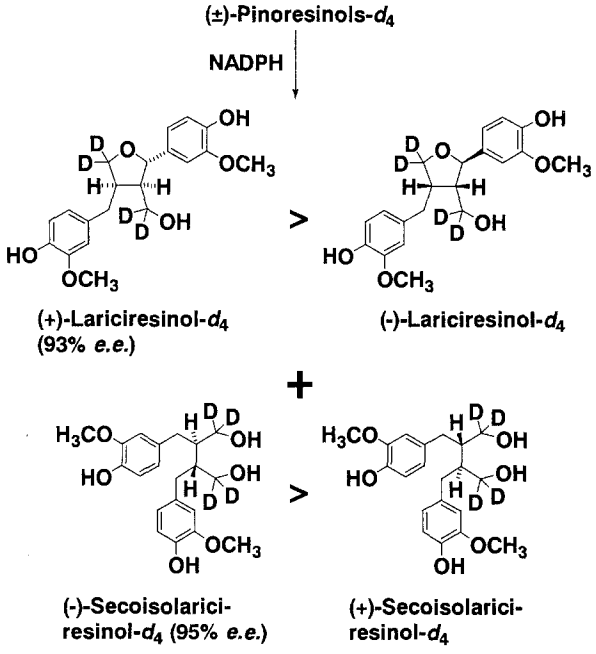

Fig. 3. Enantiomeric compositions of [$^2H_4$]lariciresinol and [$^2H_4$]secoisolariciresinol formed enzymatically from (±)-[9,9,9′,9′-$^2H_4$]pinoresinols

The PLR activity together with enzymatic formation of the lignans from coniferyl alcohol accorded well with those with *Arctium lappa*[26,38] and *Forsythia* spp.[27,35–39] In addition, the PLR-catalyzed selective formation of (+)-lariciresinol and (−)-secoisolariciresinol from (±)-pinoresinols with the *A. sylvestris* enzyme preparation suggested that the stereochemical property of *A. sylvestris* PLR-catalyzed reduction was similar to that of *Forsythia* PLR[35] and *A. lappa* ripening seed PLR.[26]

Lignan formation by the *Anthriscus* enzyme preparation along with the detection of lariciresinol and secoisolariciresinol from the plant suggests strongly that the conversion pinoresinol → lariciresinol → secoisolariciresinol is operating in *A. sylvestris*, as in *Forsythia* spp.[2,40] Although Dewick et al. reported the in vivo conversion of matairesinol to podophyllotoxin via yatein and deoxypodophyllotoxin,[23,24,41] a detailed pathway after secoisolariciresinol to yatein via matairesinol awaits enzymatic experiments in *A. sylvestris*, which are underway in our laboratory.

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