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ORIGINAL ARTICLE

Tomoya Okunishi · Toshiaki Umezawa · Mikio Shimada

Enantiomeric compositions and biosynthesis of *Wikstroemia sikokiana* lignans

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Abstract Thymelaeaceae plants produce dextrorotatory dibenzylbutyrolactone lignans, which are opposite enantiomers to the lignans isolated from other plants (e.g., *Forsythia* spp.). In our previous paper, (−)-pinoresinol (74% enantiomer excess), (+)-matairesinol (optically pure), and (+)-wikstromol (optically pure) were isolated from *Wikstroemia sikokiana* (Thymelaeaceae). In the present investigation, a survey of lignans and the determination of their enantiomeric compositions were continued. Four lignans, (−)-lariciresinol, (−)-secoisolariciresinol, (+)-kusunokinin, and (+)-methyltrachelogenin, were isolated from MeOH extracts of *W. sikokiana* stem. To our knowledge, we have isolated (+)-methyltrachelogenin from plants for the first time. Chiral high-performance liquid chromatographic analysis showed that (+)-kusunokinin and (+)-methyltrachelogenin were optically pure, whereas (−)-lariciresinol and (−)-secoisolariciresinol were not (39% and 45% enantiomer excess, respectively). Feeding experiments with deuterium-labeled substrates demonstrated conversion of coniferyl alcohol to the lignans and interconversion of lignans. These reaction sequences are similar to the sequence catalyzed by *Forsythia* enzymes. However, predominant enantiomers of the lignans, except for secoisolariciresinol isolated from *W. sikokiana*, have absolute configurations opposite to those of the corresponding lignans isolated from *Forsythia* spp. Based on the results of the isolation and the feeding experiments, several differences between *W. sikokiana* and *Forsythia* spp. are pointed out regarding stereochemical mechanisms for lignan biosynthesis.

T. Okunishi · T. Umezawa (✉) · M. Shimada
Wood Research Institute, Kyoto University, Uji, Kyoto 611-0011, Japan
Tel. +81-774-38-3625; Fax +81-774-38-3682
e-mail: tumezawa@kuwri.kyoto-u.ac.jp

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Key words *Wikstroemia sikokiana* · Stereochemistry of biosynthesis · Lariciresinol · Secoisolariciresinol · Dibenzylbutyrolactone lignan

Introduction

Lignan biosynthesis has been receiving widespread interest from stereochemical and biochemical points of view. During the last decade, significant advances have been made in studies of lignan biosynthesis. Thus, in 1990 Umezawa et al.[1] reported for the first time that the optically pure lignan, (−)-secoisolariciresinol [(−)-**3**] was formed enantioselectively following incubation of achiral coniferyl alcohol (**11**) with an enzyme preparation from *Forsythia intermedia* in the presence of NADPH and H_2O_2. Since then many reports[2–13] have been published, mostly by Lewis and coworkers, on lignan-synthesizing enzymes of *Forsythia* spp; and detailed enzymatic mechanisms for formation of *Forsythia* lignans including secoisolariciresinol (**3**) have been elucidated. Davin et al.[12] isolated from *Forsythia* sp. a unique protein (dirigent protein) that engendered enantioselective formation of (+)-pinoresinol [(+)-**1**] by coupling of **11** in the presence of laccase/O_2 or a single-electron oxidant. Enzymatic reduction of (+)-**1** to (−)-**3** via (+)-lariciresinol [(+)-**2**] was also well characterized.[5–7,9,11]

On the other hand, Umezawa and Shimada[14] demonstrated that cell-free extracts of *Arctium lappa* petioles catalyzed the enantioselective formation of (+)-secoisolariciresinol [(+)-**3**], which is the opposite antipode to that formed by *Forsythia* enzyme.[1–3,5–7,9,13] In addition, there are many examples[15] of naturally occurring lignans of which the absolute configuration is opposite to those isolated from *Forsythia* spp.

Literature survey[15] revealed that all the dibenzylbutyrolactone lignans isolated so far from Thymelaeaceae plants are dextrorotatory and have the same absolute configuration at C_8 and $C_{8'}$ with respect to carbon skeleton, except for (−)-matairesinol [(−)-**4**] from *Stellera chamaejasme*.[16] In marked contrast, many lignans of this

class isolated from plants of other families including *Forsythia* spp. are levorotatory, suggesting that the stereochemical mechanisms in lignan biosynthesis in Thymelaeaceae plants are different from those of other plants.

In a previous paper,[17] Umezawa and Shimada reported isolation of optically pure (+)-matairesinol [(+)-**4**] and (+)-wikstromol [(+)-**5**] as well as (−)-pinoresinol [(−)-**1** 74% e.e.] from *Wikstroemia sikokiana*. This paper reports further study of *W. sikokiana* lignans in relation to stereochemistry and biosynthesis. Some of the data reported in this paper have been presented preliminarily.[18]

Experimental

Instruments and chromatography

^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were obtained with a JNM-LA400MK FT-NMR System (Jeol Ltd.) with tetramethylsilane as an internal standard. Chemical shifts and coupling constants (J) were expressed in δ and hertz, respectively. The ^{1}H-NMR signals derived from phenolic hydroxyl groups of each compounds were not described. Gas chromatography-mass spectrometry (GC-MS) was conducted as previously.[9] The samples for GC-MS were dissolved in *N,O*-bis(trimethylsilyl)acetamide (BSA) and left standing at 60°C for 45 min; then an aliquot of the BSA solution was subjected to GC-MS analysis. Electron impact-mass spectrometry (EI-MS) and high-performance liquid chromatography (HPLC) were conducted as previously[9] but with the following elution details. The reverse-phase column used was a Waters Novapak C_{18} (150 × 3.9 mm), which was eluted with the following two solvent systems: A, CH_3CN-H_2O (23:77) at 1 ml/min; B, gradient elution at 1 ml/min by two linear gradient protocols of CH_3CN-H_2O at t = 0 to 6 min from 15:85 to 17:83, and then to 20:80 at t = 16 min, the latter composition being held for an additional 5 min. The elution conditions for chiral HPLC were as follows: lariciresinol (**2**), Chiralcel OC column (Daicel Chemical Co.; 250 × 4.6 mm) with EtOH–*n*-hexane (80:20) at 0.5 ml/min;[6] secoisolariciresinol (**3**), Chiralcel OD column (Daicel Chemical Co.; 250 × 4.6 mm) with EtOH–1% AcOH in *n*-hexane (30:70) at 0.8 ml/min; kusunokinin (**6**), Chiralcel OD column with EtOH–*n*-hexane (50:50) at 0.4 ml/min; methyltrachelogenin (**7**), Chiralpak AD column (Daicel Chemical Co.; 250 × 4.6 mm) with EtOH at 0.2 ml/min. The chiral elution condition for **3** was as reported previously[1,9] but contained AcOH to improve the separation. This did not affect the order of elution of (+)- and (−)-enantiomers. The sign for optical rotation of each enantiomer of **6** and **7** were determined by a chiral detector (JASCO, OR-990). Silica gel column chromatography employed Kieselgel 60 (Merck, 70–230 mesh). Silica gel thin-layer chromatography (TLC) employed Kieselgel 60 F_{254} (Merck, 20 × 20 cm, 0.5 and 0.25 mm). All chemicals used were of reagent grade, unless otherwise stated.

Synthesis of compounds

*(±)-[9,9,9′,9′-$^2H_4$]Pinoresinols ((±)-**1**-d$_4$)*

Ethyl ferulate prepared by acid-catalyzed esterification of ferulic acid was reduced with $LiAl^2H_4$ to give [9,9-$^2H_2$]coniferyl alcohol (**11**-d_2). (±)-[9,9,9′,9′-$^2H_4$]Pinoresinols [(±)-**1**-d_4] were synthesized from **11**-d_2 by the method of Katayama and Fukuzumi[19] but with 0.1 M potassium phosphate buffer (pH 7.0) and 0.5% H_2O_2 instead of distilled water and 3% H_2O_2, respectively.

[9,9-$^2H_2$]Coniferyl alcohol (**11**-d_2): ^{1}H-NMR ($CDCl_3$): δ 3.89 (3H, s, OCH_3), 6.20 (1H, d, J = 15.6, C_8H), 6.52 (1H, d, J = 15.8, C_7H), 6.82–6.94 (3H, m, aromatic H).

(±)-[9,9,9′,9′-$^2H_4$]Pinoresinols [(±)-**1**-d_4]: ^{1}H-NMR ($CDCl_3$): δ 3.08 (2H, dd, J = 1.3, J = 3.3, C_8H and $C_{8'}H$), 3.89 (6H, s, OCH_3 × 2), 4.73 (2H, m, C_7H and $C_{7'}H$), 6.80–6.89 (6H, m, aromatic H); MS m/z (%): 364 (5.5), 363 (29.0), 362 (M^+, 96.5), 361 (14.1), 360 (1.1), 359 (0), 358 (0), 329 (12.7), 225 (3.9), 209 (23.7), 198 (13.1), 181 (15.6), 165 (49.6), 153 (11.6), 152 (62.4), 151 (100), 137 (40.8), 133 (41.4), 124 (17.7), 109 (7.1); high-resolution MS m/z (M^+): calculated for $C_{20}H_{18}{}^2H_4O_6$: 362.1668; found: 362.1669.

*(±)-[9,9,9′,9′-$^2H_4$]Lariciresinols [(±)-**2**-d$_4$] and (±)-[9,9,9′,9′-$^2H_4$]secoisolariciresinols [(±)-**3**-d$_4$]*

(±)-[9,9,9′,9′-$^2H_4$]Lariciresinols [(±)-**2**-d_4] and (±)-[9,9,9′,9′-$^2H_4$]secoisolariciresinols [(±)-**3**-d_4] were prepared from (±)-**1**-d_4 (29.8 mg) using methods similar to those in previous reports.[6,20] The products, composed of a mixture of (±)-**2**-d_4 and (±)-**3**-d_4, as well as unreacted (±)-**1**-d_4 were submitted to preparative silica gel TLC purification (solvent: 5% MeOH-CH_2Cl_2) to afford pure (±)-**2**-d_4 (10.2 mg, 34.2%), (±)-**3**-d_4 (5.0 mg, 16.8%), and (±)-**1**-d_4 (3.5 mg, 11.7%).

(±)-[9,9,9′,9′-$^2H_4$]Lariciresinols [(±)-**2**-d_4]: ^{1}H-NMR ($CDCl_3$): δ 2.38 (1H, dd, J = 7.0, J = 7.0, C_8H), 2.52 (1H, dd, J = 10.7, J = 13.4, $C_{7'}H$), 2.67–2.73 (1H, m, $C_{8'}H$), 2.89 (1H, dd, J = 5.1, J = 13.4, $C_{7'}H$), 3.85 (3H, s, OCH_3), 3.86 (3H, s, OCH_3), 4.78 (1H, d, J = 6.6, C_7H), 6.67–6.86 (6H, m, aromatic H); MS m/z (%): 366 (5.0), 365 (27.4), 364 (M^+, 100), 363 (14.5), 362 (2.0), 361 (0.8), 360 (0.2), 240 (17.2), 225 (10.9), 223 (13.1), 208 (15.2), 195 (23.9), 194 (12.1), 193 (10.4), 182 (20.1), 177 (7.3), 166 (9.5), 153 (19.2), 151 (35.2), 137 (48.8), 125 (7.1), 122 (7.3); high-resolution MS m/z (M^+): calculated for $C_{20}H_{20}{}^2H_4O_6$: 364.1824; found: 364.1825.

(±)-[9,9,9′,9′-$^2H_4$]Secoisolariciresinols [(±)-**3**-d_4]: ^{1}H-NMR ($CDCl_3$): δ 1.84 (2H, m, C_8H and $C_{8'}H$), 2.63 (2H, dd, J = 6.5, J = 13.8, C_7H and $C_{7'}H$), 2.73 (2H, dd, J = 8.2, J = 13.8, C_7H and $C_{7'}H$), 3.80 (6H, s, OCH_3 × 2), 6.58 (2H, d, J = 1.7, aromatic H), 6.61 (2H, dd, J = 1.8, J = 7.9, aromatic H), 6.79 (2H, d, J = 7.8, aromatic H); MS m/z (%): 368 (1.8), 367 (9.3), 366 (M^+, 36.3), 365 (2.1), 364 (0.7), 363 (0.3), 362 (0.1), 348 (25.1), 196 (12.5), 193 (7.8), 192 (6.4), 137 (100), 122 (10.0); high-resolution MS m/z (M^+): calculated for $C_{20}H_{22}{}^2H_4O_6$: 366.1981; found: 366.1990.

*(±)-[arom-^{2}H]Secoisolariciresinols [(±)-**3**-d_{arom}]*

(±)-[arom-^{2}H]Secoisolariciresinols ((±)-**3**-d_{arom}) were prepared as previously described.[3]

(±)-[arom-^{2}H]Secoisolariciresinols [(±)-**3**-d_{arom}]: ^{1}H-NMR ($CDCl_3$): δ 1.86 (2H, m, C_8H and $C_{8'}$H), 2.64 (2H, dd, J = 6.6, J = 13.7, C_7H and $C_{7'}$H), 2.74 (2H, dd, J = 8.2, J = 13.8, C_7H and $C_{7'}$H), 3.56 (2H, dd, J = 4.4, J = 11.5, C_9H and $C_{9'}$H), 3.81 (6H, s, OCH_3 × 2), about 3.82 (2H, C_9H and $C_{9'}$H), 6.58 (1.5H, aromatic H), 6.80 (1.7H, aromatic H): MS *m/z* (%): 368 (0.9), 367 (4.4), 366 (15.4), 365 (29.2), 364 (28.3), 363 (7.9), 362 (1.3), 348 (3.51), 347 (7.2), 346 (7.0), 345 (2.1), 344 (0.5), 190 (11.2), 139 (74.5), 138 (100), 124 (7.6), 123 (8.1).

*[9,9,-$^2H_2$, OC^2H_3]Coniferyl alcohol (**11**-d_5), (±)-lariciresinols [(±)-**2**], and (±)-secoisolariciresinols [(±)-**3**]*

Preparation of [9,9,-$^2H_2$, OC^2H_3]coniferyl alcohol (**11**-d_5),[9] (±)-**2**,[21] and (±)-**3**[9] were reported previously.

[9,9,-$^2H_2$, OC^2H_3]Coniferyl alcohol (**11**-d_5): MS *m/z* (%): 187 (1.7), 186 (14.5), 185 (100), 184 (4.0), 183 (1.5), 182 (1.0), 181 (0.5), 180 (0.2), 141 (26.1), 140 (86.7), 133 (14.5), 128 (29.5), 127 (11.6), 121 (11.0), 120 (13.1), 105 (10.3), 93 (12.6), 92 (9.8).

*(±)-Kusunokinins [(±)-**6**] and (±)-bursehernins [(±)-**8**]*

(±)-Kusunokinins [(±)-**6**] were prepared by methylation [CH_3I, K_2CO_3 in *N*,*N*-dimethylformamide (DMF)] of (±)-haplomyrfolins [(±)-**9**] (Fig. 1b), which were synthesized by a method similar to that used for (±)-matairesinols [(±)-**4**][3] but with piperonyl alcohol instead of vanillyl alcohol as one of the starting materials.

(±)-Bursehernins [(±)-**8**] were prepared by methylation (CH_3I, K_2CO_3 in DMF) of (±)-pluviatolides [(±)-**10**] (Fig. 1b), which were synthesized by a method similar to that used for (±)-**4**[3] but with methyl 2-carboxymethyl-3-(3,4-methylenedioxyphenyl)propionate instead of methyl 2-carboxymethyl-3-(4-hydroxy-3-methoxyphenyl)propionate as one of the starting materials.

(±)-Kusunokinins [(±)-**6**]: ^{1}H-NMR ($CDCl_3$): δ 2.44–2.64 (4H, m, $C_{7'}$H × 2, C_8H, $C_{8'}$H), 2.84 (1H, dd, J = 7.1, J = 14.2, C_7H), 2.95 (1H, dd, J = 5.1, J = 14.2, C_7H), 3.82 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 3.87 (1H, dd, J = 7.3, J = 9.3, $C_{9'}$H), 4.14 (1H, dd, J = 7.1, J = 9.0, $C_{9'}$H), 5.92–5.93 (2H, m, OCH_2O), 6.47 (1H, d, J = 2.0, aromatic H), 6.55–6.59 (3H, m, aromatic H), 6.70 (1H, d, J = 7.6, aromatic H), 6.75 (1H, d, J = 8.0, aromatic H); MS *m/z* (%): 370 (M^+, 100), 235 (5.8), 178 (14.0), 177 (21.8), 152 (43.4), 151 (64.2), 135 (76.8); high-resolution MS *m/z* (M^+): calculated for $C_{21}H_{22}O_6$: 370.1416; found: 370.1417.

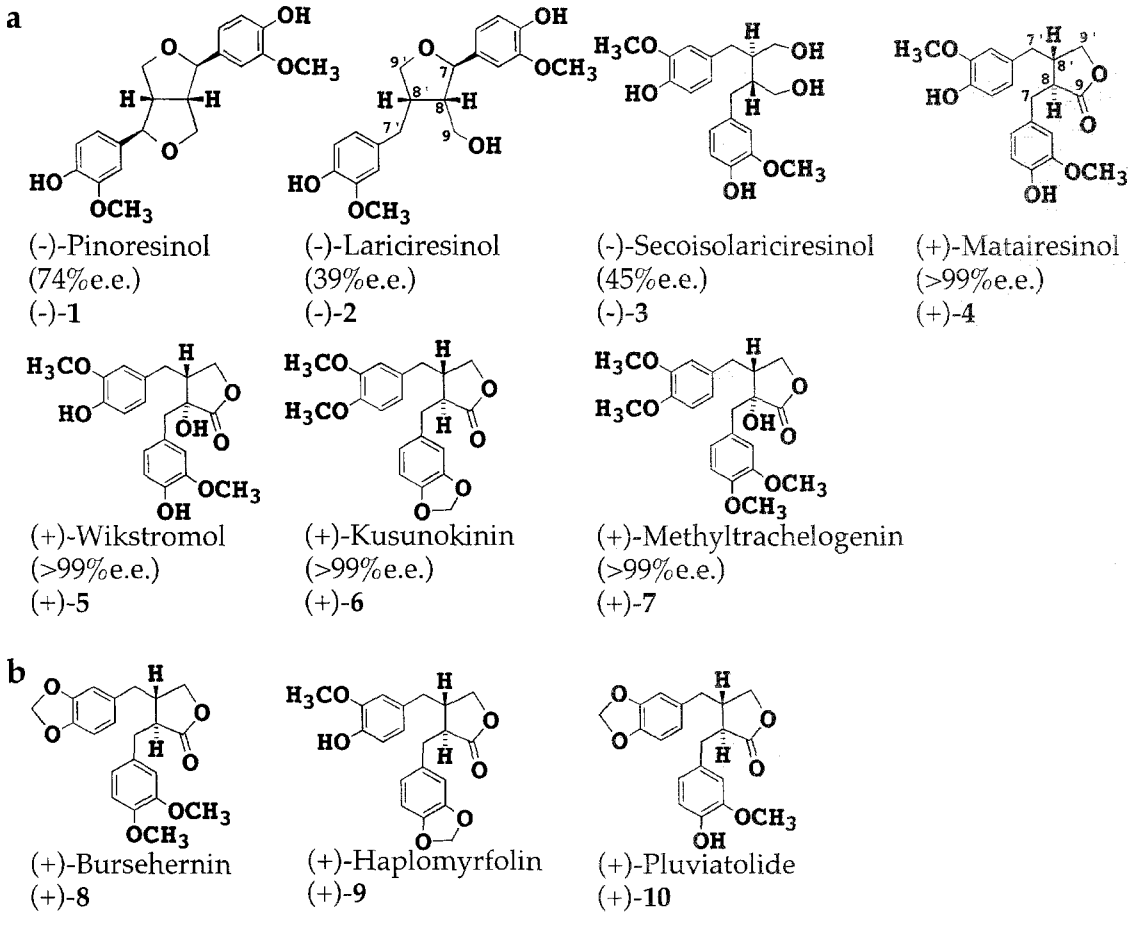

Fig. 1. Chemical structures of lignans. **a** Lignans isolated from *Wikstroemia sikokiana* and their enantiomeric compositions. **b** Related lignans

(±)-Bursehernins [(±)-**8**]: ^{1}H-NMR ($CDCl_3$): δ 2.44–2.58 (4H, m, $C_{7'}H \times 2$, C_8H, $C_{8'}H$), 2.87 (1H, dd, J = 7.1, J = 14.2, C_7H), 2.95 (1H, dd, J = 5.1, J = 14.0, C_7H), 3.82 (3H, s, OCH_3), 3.84 (3H, s, OCH_3), about 3.84 (1H, $C_{9'}H$), 4.10 (1H, dd, J = 6.8, J = 9.0, $C_{9'}H$), 5.90–5.91 (2H, m, OCH_2O), 6.41–6.45 (2H, m, aromatic H), 6.64–6.68 (3H, m, aromatic H), 6.77 (1H, d, J = 8.0, aromatic H); MS m/z (%): 370 (M^+, 98.2), 234 (18.1), 208 (7.6), 161 (7.4), 151 (100), and 135 (25.1); high-resolution MS m/z (M^+): calculated for $C_{21}H_{22}O_6$: 370.1416; found: 370.1422.

*(±)-Methyltrachelogenins [(±)-**7**]*

(±)-Methyltrachelogenins [(±)-**7**] were prepared by methylation (CH_3I, K_2CO_3 in DMF) of (±)-wikstromols [(±)-**5**], which were synthesized previously.[17]

(±)-Methyltrachelogenins [(±)-**7**]: ^{1}H-NMR ($CDCl_3$): δ 2.51 (2H, m, $C_{7'}H \times 2$), 2.92–2.97 (2H, m, C_7H and $C_{8'}H$), 3.10 (1H, d, J = 13.7, C_7H), 3.83 (3H, s, OCH_3), 3.84 (3H, s, OCH_3), 3.85 (6H, s, $OCH_3 \times 2$), 4.02 (2H, m, $C_{9'}H \times 2$), 6.63 (1H, d, J = 2.0, aromatic H), 6.66–6.68 (2H, m, aromatic H), 6.71 (1H, d, J = 1.9, aromatic H), 6.78 (1H, d, J = 8.3, aromatic H), 6.79 (1H, d, J = 8.3, aromatic H); MS m/z (%): 402 (M^+, 17.3), 151 (100); high-resolution MS m/z (M^+): calculated for $C_{22}H_{26}O_7$: 402.1678; found: 402.1686.

Plant material

Wikstroemia sikokiana Fr. et Sav. plants were collected during November 1993 in Kochi Prefecture, Japan, and used for lignan extraction. The plants were also transplanted and maintained in the experimental forest of Wood Research Institute, Kyoto University and used for feeding experiments.

Isolation of lignans

Freeze-dried *W. sikokiana* stems with bark (162.27 g) were pulverized using a Wiley mill and then extracted with hot MeOH (1100, 300, 300, 300, and 350 ml; total 2350 ml). The combined MeOH extracts (10.2978 g) were suspended in distilled water (44 ml), which was extracted with Et_2O (60 ml × 3). The combined Et_2O extracts (2.7332 g) were submitted to repeated purification by column chromatography, TLC, and reverse-phase HPLC to afford seven lignans: **1** (16.0 mg), **2** (5.2 mg), **3** (trace amount), **4** (12.4 mg), **5** (22.7 mg), **6** (2.0 mg), and **7** (trace amount) (Fig. 1a).

Lariciresinol (**2**): ^{1}H-NMR ($CDCl_3$): δ 2.37–2.43 (1H, m, C_8H), 2.54 (1H, dd, J = 10.6, J = 13.6, $C_{7'}H$), 2.68–2.77 (1H, m, $C_{8'}H$), 2.91 (1H, dd, J = 5.1, J = 13.4, $C_{7'}H$), 3.74 (1H, dd, J = 6.2, J = 8.7, $C_{9'}H$), 3.77 (1H, dd, J = 7.1, J = 11.5, C_9H), 3.86 (3H, s, OCH_3), 3.88 (3H, s, OCH_3), 3.91 (1H, dd, J = 7.1, J = 10.7, C_9H), 4.04 (1H, dd, J = 6.6, J = 8.5, $C_{9'}H$), 4.78 (1H, d, J = 6.6, C_7H), 6.68–6.87 (6H, m, aromatic H); MS m/z (%): 360 (M^+, 100), 236 (20.4), 221 (14.0), 219 (13.0), 206 (10.8), 194 (36.0), 191 (10.2), 190 (10.7), 180 (22.2), 175 (13.7), 164 (11.3), 153 (28.1), 151 (37.1), 137 (73.9), 124 (9.9), 122 (9.8); high-resolution MS m/z (M^+): calculated for $C_{20}H_{24}O_6$: 360.1573; found: 360.1573.

Secoisolariciresinol (**3**): ^{1}H-NMR ($CDCl_3$): δ 1.85 (2H, m, C_8H and $C_{8'}H$), 2.64 (2H, dd, J = 6.6, J = 13.9, C_7H and $C_{7'}H$), 2.74 (2H, dd, J = 8.1, J = 13.9, C_7H and $C_{7'}H$), 3.56 (2H, dd, J = 4.6, J = 11.2, C_9H and $C_{9'}H$), 3.81 (6H, s, $OCH_3 \times 2$), about 3.83 (2H, C_9H and $C_{9'}H$), 6.58 (2H, d, J = 2.0, aromatic H), 6.63 (2H, dd, J = 1.7, J = 8.1, aromatic H), 6.80 (2H, d, J = 7.8, aromatic H); MS m/z (%): 362 (M^+, 21.2), 344 (8.9), 194 (7.7), 189 (16.6), 137 (100), 122 (8.0); high-resolution MS m/z (M^+): calculated for $C_{20}H_{26}O_6$: 362.1730; found: 362.1734.

Kusunokinin (**6**): ^{1}H-NMR ($CDCl_3$): δ 2.45–2.66 (4H, m, $C_{7'}H \times 2$, C_8H, $C_{8'}H$), 2.84 (1H, dd, J = 7.2, J = 14.0, C_7H), 2.96 (1H, dd, J = 5.2, J = 14.2, C_7H), 3.82 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 3.88 (1H, dd, J = 7.3, J = 9.3, $C_{9'}H$), 4.14 (1H, dd, J = 7.0, J = 9.0, $C_{9'}H$), 5.92–5.93 (2H, m, OCH_2O), 6.47 (1H, d, J = 2.0, aromatic H), 6.55–6.59 (3H, m, aromatic H), 6.71 (1H, d, J = 7.8, aromatic H), 6.76 (1H, d, J = 8.3, aromatic H); MS m/z (%): 370 (M^+, 100), 235 (5.8), 177 (22.9), 152 (49.1), 151 (73.3), 135 (93.9); high-resolution MS m/z (M^+): calculated for $C_{21}H_{22}O_6$: 370.1417; found: 370.1424.

Methyltrachelogenin (**7**): ^{1}H-NMR ($CDCl_3$); δ. 2.52 (2H, m, $C_{7'}H \times 2$), 2.92–2.98 (2H, m, C_7H and $C_{8'}H$), 3.10 (1H, d, J = 13.7, C_7H), 3.84 (3H, s, OCH_3), 3.85 (3H, s, OCH_3), 3.85 (6H, s, $OCH_3 \times 2$), 4.02 (2H, m, $C_{9'}H \times 2$), 6.63 (1H, d, J = 1.7, aromatic H), 6.67–6.69 (2H, m, aromatic H), 6.71 (1H, d, J = 1.7, aromatic H), 6.79 (1H, d, J = 8.0, aromatic H), 6.79 (1H, d, J = 8.1, aromatic H); MS m/z (%): 402 (M^+, 17.2), 151 (100): high-resolution MS m/z (M^+): calculated for $C_{22}H_{26}O_7$: 402.1678; found: 402.1674.

The water layer containing glycosides were not analyzed.

Enantiomeric compositions of lignans

Racemic (±)**2**-d_4 was mixed with **2** isolated from *W. sikokiana*. The mixture was submitted to chiral HPLC separation, and both fractions, corresponding to (−)- and (+)-enantiomers, were recovered individually. Each fraction was subjected to GC-MS analysis after trimethylsilylation. Using (+)-**2**-d_4 and (−)-**2**-d_4 as internal standards, we determined the relative amounts of (+)-**2** and (−)-**2**. Determination of enantiomeric composition of **3** isolated from *W. sikokiana* was conducted in a similar way to that for **2**. Enantiomeric compositions of **6** and **7** isolated from *W. sikokiana* were determined by chiral HPLC.

Administration of deuterium-labeled coniferyl alcohol and lignans to *Wikstroemia sikokiana*

Young shoots (about 10 cm long with about 8–10 leaves) of *W. sikokiana* were cut by means of scissors, and the cut end of each shoot was placed directly in the solutions (25 mM) of the deuterium-labeled compounds (two shoots for each compound, **11**-d_5, 350 μl/shoot; (±)-**1**-d_4, 350 μl/shoot; (±)-**2**-d_4, 350 μl/shoot; (±)-**3**-d_4, 460 μl/shoot; and (±)-**3**-d_{arom}, 460 μl/shoot). Compound **11**-d_5 (1.6 mg) was dissolved in 0.1 M potassium phosphate buffer (pH 7.0, 350 μl). Com-

pounds (±)-**1**-d_4 (3.2 mg) and (±)-**2**-d_4 (3.2 mg) were dissolved in 2-methoxyethanol (70 μl) and dispersed in distilled water (280 μl) containing Tween 20 (21 mg). Compounds (±)-**3**-d_4 (4.2 mg) and (±)-**3**-d_{arom} (4.2 mg) were dissolved in MeOH (30 μl) and dispersed in 430 μl of 0.1 M potassium phosphate buffer (pH 7.0). Following uptake and metabolism for 24 h, the whole shoots including leaves were freeze-dried. The resulting dried material was hand-disintegrated using scissors and extracted with hot MeOH. The MeOH extracts were submitted directly to trimethylsilylation followed by GC-MS analysis.

To quantify the incorporation, **11**-d_5 was administered to eight shoots of *W. sikokiana* and extracted with hot MeOH as above. Then we added to the MeOH extracts the chemically synthesized lignans labeled with four deuterium atoms, (±)-**1**-d_4, (±)-**2**-d_4, and (±)-**3**-d_4, as internal standards. Aliquots of the MeOH extracts with the internal standards were then subjected to trimethylsilylation followed by GC-MS. The values of percent incorporation of deuterium atoms from **11**-d_5 into lignans **1**, **2**, and **3** were calculated by comparing the peak intensities of molecular ions of the internal standard lignans labeled with four deuterium atoms and those of the corresponding deuterium-labeled lignans formed from **11**-d_5. The percent incorporation into deuterium-labeled **4** was calculated based on a calibration curve using the molecular ion of (±)-**1**-d_4 as an internal standard.

Next, administration of **11**-d_5 was repeated, and the deuterium-labeled lignans **1** and **2** formed from **11**-d_5 were isolated after addition of (±)-**1**-d_4 and (±)-**2**-d_4 as internal standards. Their enantiomeric compositions were then determined as above.

Results

Isolation of lignans

Seven lignans were isolated from *Wikstroemia sikokiana* stems: pinoresinol (**1**), lariciresinol (**2**), secoisolariciresinol (**3**), matairesinol (**4**), wikstromol (**5**), kusunokinin (**6**), and methyltrachelogenin (**7**) (Fig. 1a). These lignans were identified by comparing their ^{1}H-NMR and mass spectral data and retention volumes on reverse-phase HPLC and chiral HPLC with those of chemically synthesized authentic samples. In addition, the possibility that the compound identified as **6** is the regioisomer bursehernin (**8**) was eliminated by comparing the spectral data with those of chemically synthesized authentic (±)-**8** (Fig. 1b).

Isolation of (−)-pinoresinol [(−)-**1**], (+)-matairesinol [(+)-**4**], and (+)-wikstromol [(+)-**5**] from *W. sikokiana* has been reported.[17] In the present investigation, the previous result was confirmed, and the precise yields were determined.

Enantiomeric compositions of lignans

Chiral HPLC analysis indicated that **6** and **7** isolated from *W. sikokiana* were optically pure and dextrorotatory (Fig. 2). Co-chromatography with authentic samples confirmed that the small peak at 9.8 ml of retention volume on the chiral HPLC chromatogram of **6** and that at 6.2 ml of retention volume on the chiral HPLC chromatogram of **7** were not (−)-enantiomers but impurities. On the other hand, **2** and **3** isolated from the plant were found to be mixtures of both enantiomers (Fig. 2), and their enantiomeric compositions were determined as 39% e.e. and 45% e.e. in favor of (−)-enantiomer, respectively.

Feeding experiments

Deuterium-labeled [9,9-$^2H_2$, OC^2H_3]coniferyl alcohol (**11**-d_5) and four lignans, (±)-[9,9,9′,9′-$^2H_4$]pinoresinols

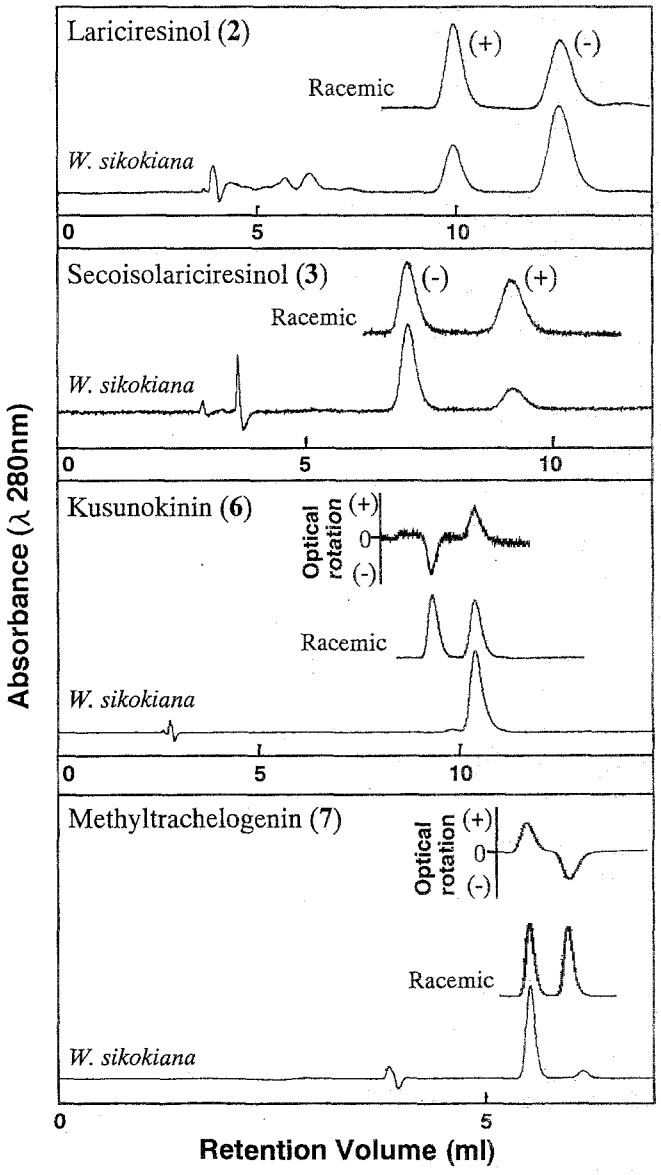

Fig. 2. Chiral high-performance liquid chromatography (HPLC) chromatograms of lignans isolated from *W. sikokiana*. Elution details are described in the Experimental section. Optical rotation was detected by a chiral detector. *W. sikokiana*: lignans isolated from *W. sikokiana*; *Racemic*: racemic authentic sample; *(+)*, *(−)*, dextrorotatory and levorotatory enantiomers, respectively

[(±)-**1**-d_4], (±)-[9,9,9′,9′-$^2H_4$]lariciresinols [(±)-**2**-d_4], (±)-[9,9,9′,9′-$^2H_4$]secoisolariciresinols [(±)-**3**-d_4], and (±)-[arom-^2H]secoisolariciresinols [(±)-**3**-d_{arom}], were administered individually to young shoots of *W. sikokiana*. The MeOH extracts were submitted to GC-MS after trimethylsilylation; and the peaks on GC corresponding to the lignans **1** (t_R = 16.9 min), **2** (t_R = 14.0 min), **3** (t_R = 10.8 min), and **4** (t_R = 15.2 min) were analyzed for deuterium incorporation. Relative intensities of molecular ion (M^+) regions and important fragment ions of the lignans are shown in Table 1. Trimethylsilylated unlabeled authentic samples of these lignans give the following M^+ and the fragment ions due to one aromatic ring formed by benzylic cleavage: (±)-**1**, *m/z* 502 (M^+) and 223; (±)-**2**, *m/z* 576 (M^+), 223 and 209; (±)-**3**, *m/z* 650 (M^+) and 209; (±)-**4**, *m/z* 502 (M^+) and 209.

When the MeOH extracts obtained following administration of **11**-d_5 were analyzed by GC-MS after trimethylsilylation, the GC peaks corresponding to the lignans gave the following extra ion peaks: t_R = 16.9 min (**1**), *m/z* 512, 511, 510, 226; t_R = 14.0 min (**2**), *m/z* 586, 585, 226, 212; t_R = 10.8 min (**3**), *m/z* 660, 659, 212; t_R = 15.2 min (**4**), *m/z* 510, 509, 212. The appearance of ion peaks at *m/z* 512 (t_R = 16.9 min, **1**), 586 (t_R = 14.0 min, **2**), 660 (t_R = 10.8 min, **3**), and 510 (t_R = 15.2 min, **4**) indicated the presence of **1**, **2**, and **3** labeled with ten deuterium atoms and of **4** labeled with eight deuterium atoms, respectively. The fragment ion peaks at *m/z* 226 and 212 are assigned to benzylic cleavage fragments labeled with three deuterium atoms at the methoxyl groups. These results clearly indicated conversion of **11**-d_5 to the lignans. The values of percent incorporation of **11**-d_5 into the lignans were **1** (0.81%), **2** (0.32%), **3** (0.09%), and **4** (0.03%) (Table 2).

Similarly, when the MeOH extracts obtained after individual administration of each lignan labeled with four deuterium atoms [(±)-**1**-d_4, (±)-**2**-d_4, or (±)-**3**-d_4] were analyzed by GC-MS, the GC peaks corresponding to **1**, **2**, and **3** gave the extra ion peaks at 506 (**1**), 580 (**2**), and 654 (**3**) (Table 1), which were not present on the mass spectra of the corresponding unlabeled authentic samples. These results indicate the occurrence of **1**, **2**, and **3** labeled with four deuterium atoms. Under the GC-MS condition employed, however, the presence of deuterium-labeled **4** was not observed (data not shown). The feeding experiment was repeated but with **3** labeled with deuterium atoms at the aromatic ring (±)-**3**-d_{arom}, and the fraction corresponding to **4** was purified. GC-MS analysis of the fraction indicated the presence of **4** labeled with deuterium atoms at aromatic ring (*m/z* 504) (Table 1, 2). The results obtained for the deuterium incorporation that were opposite from the two differently labeled precursors (±)-**3**-d_4 and (±)-**3**-d_{arom} can be explained by a primary isotope effect, which would hamper the conversion of (±)-**3**-d_4 to (±)-**4**-d_2. In the repeated experiment, incorporation into the other lignans was not examined.

The enantiomeric compositions of lignans labeled with deuterium atoms produced after administration of **11**-d_5 were as follows: [9,9,9′,9′-$^2H_4$, OC^2H_3]pinoresinol (**1**-d_{10}), 19% e.e. (−) > (+); [9,9,9′,9′-$^2H_4$, OC^2H_3]lariciresinol (**2**-d_{10}), 44% e.e. (−) > (+). The yields of [9,9,9′,9′-$^2H_4$, OC^2H_3]secoisolariciresinol (**3**-d_{10}) and [9,9,-$^2H_2$, OC^2H_3] matairesinol (**4**-d_8) were too low to determine the precise percent e.e. values.

Discussion

Umezawa and Shimada[17] reported the isolation of (−)-pinoresinol [(−)-**1**] (74% e.e.) and two optically pure dibenzylbutyrolactone lignans, (+)-matairesinol [(+)-**4**] (>99% e.e.) and (+)-wikstromol [(+)-**5**] (>99% e.e.), from *Wikstroemia sikokiana*. The predominant enantiomers of the lignans, (−)-**1** and (+)-**4**, are opposite to those of the lignans isolated from *Forsythia* plants, and the differences in stereochemical mechanisms of lignan biosynthesis between *W. sikokiana* and *Forsythia* spp. were discussed.[17]

In the present investigation, the survey of the lignans of *W. sikokiana* was continued, and the following four lignans were isolated from the plant for the first time: (−)-lariciresinol [(−)-**2**] (39% e.e.), (−)-secoisolariciresinol [(−)-**3**] (45% e.e.), and optically pure (+)-kusunokinin [(+)-**6**] (>99% e.e.) and (+)-methyltrachelogenin [(+)-**7**] (>99% e.e.). To our knowledge, this is the first report of isolation of naturally occurring dextrorotatory (+)-**7** and determination of its enantiomeric composition. Recently, methyltrachelogenin (**7**) was isolated from *Zanthoxylum lemairie* stem, but the enantiomeric composition (or the specific rotation) of the lignan was not described.[22] The isolation of the dextrorotatory dibenzylbutyrolactone lignans (+)-**4**, (+)-**5**, (+)-**6**, and (+)-**7** from *W. sikokiana* is in good accordance with the previous isolation of dextrorotatory dibenzylbutyrolactone lignans from other Thymelaeaceae plants.[15] It should be noted that these dextrorotatory dibenzylbutyrolactone lignans have the same absolute configurations at C_8 and $C_{8'}$ with respect to carbon skeletons.[15,23] In addition, the dibenzylbutyrolactone lignans isolated from *W. sikokiana* were optically pure, which accords well with the other reports of chiral HPLC analysis of this class of lignans; all the dibenzylbutyrolactone lignans of which enantiomeric compositions have so far been determined by chiral HPLC are found to be optically pure.[15]

Feeding experiments with deuterium-labeled substrates have demonstrated conversion of coniferyl alcohol (**11**) to the lignans and interconversion of lignans, as shown in Fig. 3. This is similar to the reaction sequence catalyzed by *Forsythia* enzymes.[1–3,5–7,9,13] However, the present results together with the previous report[17] revealed the stereochemical difference between lignans occurring in both *Wikstroemia* and *Forsythia* plants as follows. First, the predominant enantiomers of the *Wikstroemia* lignans are opposite to those isolated from *Forsythia* spp. except for secoisolariciresinol (**3**). The predominant enantiomers of pinoresinol (**1**), lariciresinol (**2**), and matairesinol (**4**) isolated from *W. sikokiana* are (−)-**1**, (−)-**2**, and (+)-**4**, whereas those isolated from *Forsythia* spp. are (+)-pinoresinol [(+)-**1**],[3,24,25] (+)-lariciresinol [(+)-**2**] (unpublished data), and (−)-matairesinol [(−)-**4**].[3,24–26] Similarly, the dibenzylbutyrolactone lignans isolated from *W. sikokiana*, (+)-**4**, (+)-**5**, (+)-**6**, and (+)-**7**, have absolute

Table 1. Mass tables of partially deuterated lignans (TMS ethers) isolated from *W. sikokiana* after administration of deuterium-labeled precursors

m/z	Relative intensity (%)			
	Unlabeled authentic (±)-**1**	Administered compounds		
		11-d_5[a]	(±)-**2**-d_4[a]	(±)-**3**-d_4[a]
Pinoresinol				
223	79.1	86.9	100.0	87.4
224	15.2	21.0	24.6	6.6
225	7.7	9.1	10.9	8.0
226	0.0	85.8	2.8	0.0
502	100.0	100.0	92.7	100.0
503	45.5	42.0	37.7	61.5
504	7.3	16.9	17.3	23.3
505	4.5	5.3	7.4	0.0
506	0.0	5.2	24.0	46.6
507	0.0	5.3	13.1	0.0
508	0.0	5.3	4.7	0.0
509	0.0	4.7	1.5	0.0
510	0.0	31.1	0.0	0.0
511	0.0	48.4	0.0	0.0
512	0.0	47.0	0.0	0.0
513	0.0	18.8	0.0	0.0

m/z	Relative intensity (%)			
	Unlabeled authentic (±)-**3**	Administered compounds		
		11-d_5[a]	(±)-**1**-d_4[a]	(±)-**2**-d_4[a]
Secoisolariciresinol				
209	100.0	53.0	100.0	100.0
210	48.0	28.5	45.3	42.5
211	9.2	10.0	29.1	21.3
212	3.0	54.6	8.2	5.8
650	27.6	14.1	17.9	13.7
651	14.9	0.0	10.6	7.8
652	9.1	0.0	0.0	5.3
653	4.4	0.0	0.0	0.0
654	1.9	0.0	13.9	6.9
655	0.0	0.0	8.2	5.9
656	0.0	0.0	7.3	2.3
657	0.0	0.0	0.0	0.0
658	0.0	3.4	0.0	0.0
659	0.0	8.7	0.0	0.0
660	0.0	9.5	0.0	0.0
661	0.0	5.1	0.0	0.0

m/z	Relative intensity (%)			
	Unlabeled authentic (±)-**2**	Administered compounds		
		11-d_5[a]	(±)-**1**-d_4[a]	(±)-**3**-d_4[a]
Lariciresinol				
209	50.9	35.1	51.6	31.5
210	11.7	12.1	13.0	9.3
211	4.0	5.0	5.9	8.8
212	0.9	14.6	2.2	4.4
223	59.1	42.9	52.9	54.8
224	11.1	9.1	10.9	20.6
225	4.8	5.3	7.0	7.7
226	0.9	19.8	1.2	0.0
576	52.4	39.3	37.9	16.6
577	26.9	19.7	20.6	13.1
578	13.7	11.2	11.1	5.7
579	4.3	4.6	5.3	10.7
580	1.6	6.1	11.9	19.2
581	1.1	3.5	4.8	5.7
582	0.0	2.5	4.9	0.0
583	0.0	1.9	0.0	0.0
584	0.0	5.1	0.0	0.0
585	0.0	7.4	0.0	0.0
586	0.0	12.5	0.0	0.0
587	0.0	7.7	0.0	0.0

m/z	Relative intensity (%)		
	Unlabeled authentic (±)-**4**	Administered compounds	
		11-d_5[a]	(±)-**3**-$d_{4\,arom}$[a]
Matairesinol			
209	100.0	40.7	94.9
210	28.9	11.3	61.3
211	8.5	3.7	29.3
212	1.5	6.7	13.5
502	92.7	28.6	100.0
503	38.1	10.9	25.4
504	17.3	4.4	46.4
505	4.0	2.1	36.9
506	1.1	0.9	24.8
507	0.0	1.0	0.0
508	0.3	0.6	0.0
509	0.0	2.7	0.0
510	0.5	2.9	0.0
511	0.4	2.3	0.0

[a] **11**-d_5: [9,9-$^2H_2$, OC$^2H_3$]coniferyl alcohol, (±)-**1**-d_4: (±)-[9,9,9′,9′-$^2H_4$]pinoresinols, (±)-**2**-d_4: (±)-[9,9,9′,9′-$^2H_4$]lariciresinols, (±)-**3**-d_4: (±)-[9,9,9′,9′-$^2H_4$]secoisolariciresinols, (±)-**3**-d_{arom}: (±)-[arom-^2H]secoisolariciresinols

configurations at C_8 and $C_{8'}$ opposite to those isolated from *Forsythia* spp. [e.g. (−)-**4**] with respect to carbon skeletons.[15,23] Second, the percent e.e. values of levorotatory **3** from *W. sikokiana* and *Forsythia* spp. are different. Thus, the lignans isolated from *W. sikokiana* were not optically pure, whereas optically pure (−)-secoisolariciresinol [(−)-**3**] was isolated from *Forsythia* spp.[2,3,9,25]

By considering the results of feeding experiments (Fig. 3) and the enantiomeric compositions of the lignans, the following differences between *W. sikokiana* and *Forsythia* spp. can be pointed out with respect to stereochemical mechanisms for lignan biosynthesis: First, different stereochemical mechanisms must be operating in both plants, leading to production (or accumulation) of opposite enantiomers of the lignans. This accords well with our previous conclusion.[17] Second, the metabolic step to produce optically pure lignans in *W. sikokiana* is probably different from that in *Forsythia* spp. The step may be the conversion of **3** to **4** in *W. sikokiana*, whereas in *Forsythia* spp. optically pure lignans must occur at an earlier step.

Thus, Thymeleaeceae plants, including *W. sikokiana*, are of special interest due to their producing dextroro-

Table 2. Incorporations of deuterium atoms into lignans from deuterium-labeled precursors

Administered compounds[a]	Deuterium-incorporated lignans			
	Pinoresinol	Lariciresinol	Secoisolariciresinol	Matairesinol
11-d_5	0.8%[b,c]	0.3%[b,c]	0.09%[b,c]	0.03%[b,c]
(±)-**1**-d_4	–	[c]	[c]	ND
(±)-**2**-d_4	[c]	–	[c]	ND
(±)-**3**-d_4	[c]	[c]	–	ND
(±)-**3**-d_{arom}	NA	NA	–	[c]

ND, not detected; NA, not analyzed

[a] **11**-d_5: [9,9-$^2H_2$, OC^2H_3]coniferyl alcohol, (±)-**1**-d_4: (±)-[9,9,9′,9′-$^2H_4$]pinoresinols, (±)-**2**-d_4: (±)-[9,9,9′,9′-$^2H_4$]lariciresinols, (±)-**3**-d_4: (±)-[9,9,9′,9′-$^2H_4$]secoisolariciresinols, (±)-**3**-d_{arom}: (±)-[arom-^2H]secoisolariciresinols

[b] Percent incorporation based on the amounts of **11**-d_5 administered (molar ratio)

[c] Incorporation of deuterium atoms was observed

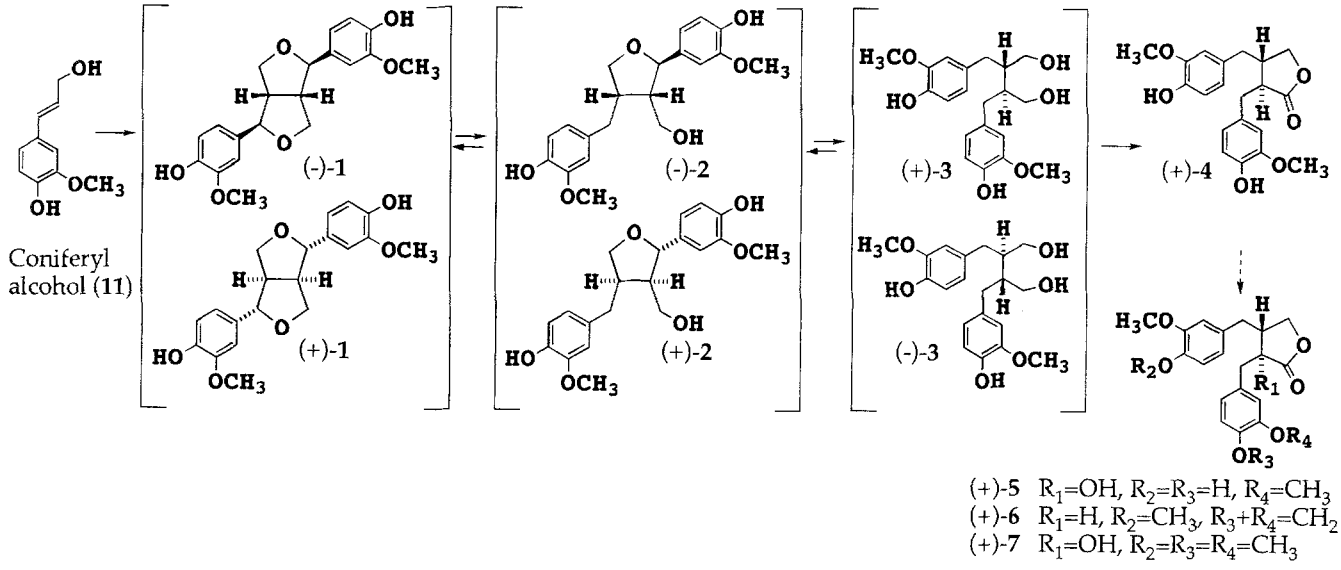

Fig. 3. Conversion of coniferyl alcohol to lignans and interconversions of lignans. *Solid arrows*, established by feeding experiments with deuterium-labeled precursors; *broken arrows*, conversion not yet established, but a putative pathway based on consideration of the chemical structures

tatory dibenzylbutyrolactone lignans, which are opposite enantiomers to those occurring in other plant species (e.g. *Forsythia* spp.). The enzymology in their biosynthesis, however, remains to be elucidated. In addition, future work must be carried out in relation to the biosynthesis of lignan glycosides, as no studies have so far been conducted on the biosynthesis of the glycosides. In conclusion, the present and previous results have revealed several differences in stereochemistry of lignan biosynthesis between *W. sikokiana* and *Forsythia* spp.

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