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Phytotoxic *cis*-cinnamoyl glucosides from *Spiraea thunbergii*

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Abstract

Spiraea thunbergii Sieb. was found to contain 1-*O*-*cis*-cinnamoyl-β-D-glucopyranose and 6-*O*-(4′-hydroxy-2′-methylene-butyroyl)-1-*O*-*cis*-cinnamoyl-β-D-glucopyranose as major plant growth inhibitory constituents along with related compounds of lower phytotoxicity including 6-*O*-(*trans*-cinnamoyl)-1-*O*-(4″-hydroxy-3″-methyl-furan-2″-one)-β-D-glucopyranose, 6-*O*-(4′-hydroxy-2′-methylene-butyroyl)-1-*O*-*trans*-cinnamoyl-β-D-glucopyranose, and 1-*O*-*trans*-cinnamoyl-β-D-glucopyranose. The former three compounds were cinnamoyl glucosides.

Keywords: *Spiraea thunbergii* Sieb; Rosaceae; Thunberg spirea; Allelopathy; Plant growth inhibitors; *cis*-Cinnamoyl glucosides; *trans*-Cinnamoyl glucosides; *cis*-Spirarin; *trans*-Spirarin

1. Introduction

It is well-known that plants produce growth-regulating compounds. Some of these compounds have a role in regulating the plant's own growth, such as phyto-hormones, and others are released from plants (donors) and absorbed by other plants (acceptors), resulting in growth regulation by the donors. The latter phenomenon is defined as allelopathy, and the bioactive compounds transferred from the donor plant to the acceptor plants are termed allelochemicals. Recently, a great deal of effort have been placed on the utilization of the allelopathic potential of plants for weed control instead of herbicide application, because it would potentially be effective in reducing the risk of environmental toxicity (Rice, 1995).

To find plants with high allelopathic potential, plant leaves which were put into agar media were subjected to bioassays using lettuce (*Lactuca sativa* L.) seedlings, and it was found that *Spiraea thunbergii* Sieb, *S. cantoniensis*, and *S. pruniflora* (Rosaceae) had the highest inhibitory activity on the elongation of lettuce roots among 56 species of woody plants grown in Japan (Morita et al., 2001). In the present study, the growth-inhibiting compounds contained in *S. thunbergii* were the subject.

For the purpose of developing herbicides, compounds with high *specific activity* (biological activity per unit weight of compound) should be considered. They should have small EC_{50} (effective concentration to induce half-maximum inhibitory action) values. On the other hand, compounds with high *total activity* (biological activity per weight of compound contained in unit weight of the donor plant) should be addressed for the purpose of allelopathic research. The total activity is a function of specific activity and total content in the organism. In allelopathic research, EC_{50} values are not necessarily small. A compound with high total activity could be a chemical communication tool among organisms in the natural ecosystem. In the present study, bioassay-directed purification was conducted by monitoring total activity (inhibitory activity was plotted against amount equivalent to the original plant leaf, not to weight of extract). The causal compounds of the total activity in *S. thunbergii* were identified and their specific activities were also clarified.

2. Results and discussion

Fresh leaves of *S. thunbergii* were extracted with MeOH. The water suspension of the original MeOH extract was subjected to a liquid–liquid partition to obtain hexane, EtOAc, and water sub-fractions. Comparison of the

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inhibitory activity on lettuce root growth (total activity) revealed that the major activity of the original extract was fractionated into the EtOAc and water fractions (Fig. 1A).

The bioactive water fraction was subjected to charcoal column chromatography, and the major activity was separated into the MeOH–H_2O (9:1) eluted fraction (Fig. 1B). This was fractionated with silica gel column

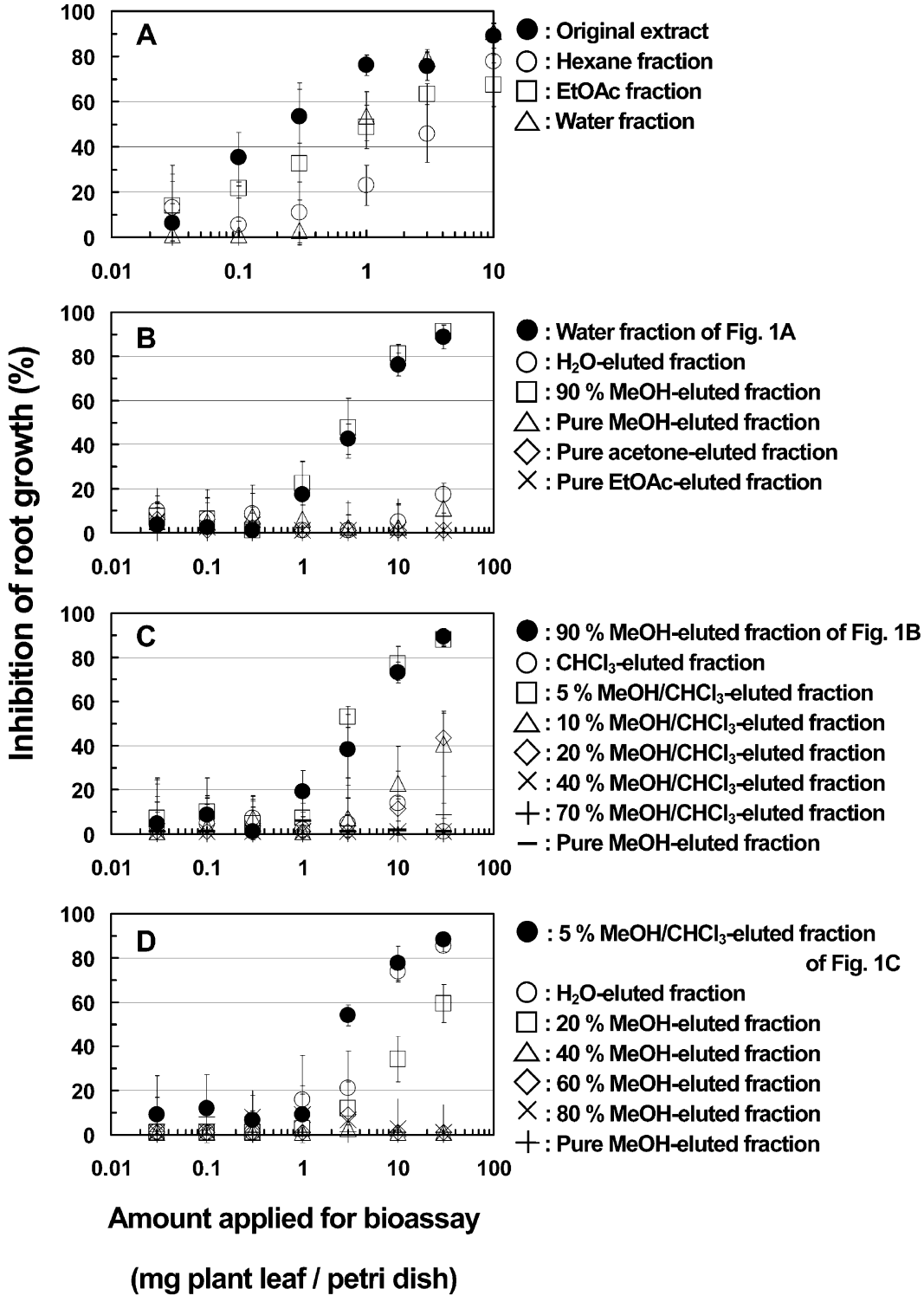

Fig. 1. Inhibitory activity of plant extract and its fractionated samples on elongation of lettuce roots (comparison on basis of total activity). (A) Original extract and its liquid-liquid partitioned fractions. (B) Water fraction of Fig. 1A and its charcoal column-separated fractions. (C) MeOH–H_2O (9:1)-eluted fraction of Fig. 1B and its silica gel column-separated fractions. (D) 5% MeOH in $CHCl_3$-eluted fraction of Fig. 1C and its reversed-phase solid-phase extraction-separated fractions. Bars indicate standard deviation ($n = 5$–6).

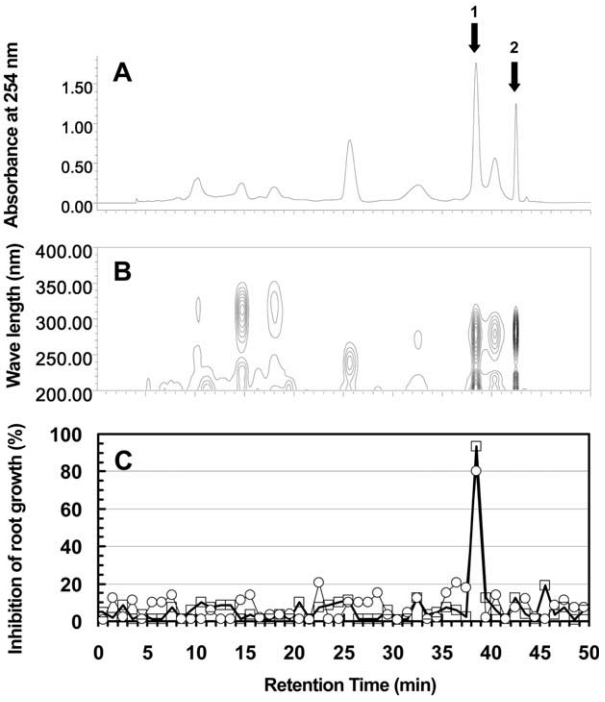

Fig. 2. Preparative HPLC chromatogram (A: detection at 254 nm, B: contour plot between 200 and 400 nm) of H_2O-eluted fraction of reversed-phase solid-phase extraction (see Fig. 1D) and inhibitory activity of these HPLC-separated fractions (each for 1 min) on elongation of lettuce roots (C). The amounts applied for the bioassay were 10 (open circles) and 50 mg (open squares) equivalent to the original fresh plant. In the HPLC analysis, the amount of sample injected was equivalent to 200 mg of fresh plant. Flow rate: 4 ml/min. MeOH–H_2O (3:7). Column temperature: 40 °C.

chromatography, and the 5% MeOH in $CHCl_3$-eluted fraction showed the highest activity (Fig. 1C). The active fraction was further purified with reversed-phase solid-phase extraction, and the activity was present in the H_2O-eluted fraction (Fig. 1D). This active fraction was then subjected to preparative HPLC, and active compound (**1**) was isolated at 38.5 min (retention time) (Fig. 2). In those purification procedures, the inhibitory activity of the original water fraction was not greatly lost (Fig. 1), and compound **1** accounted for almost all of the activity observed in the original water fraction (compound **1** contained in 10 mg equivalent to the original plant leaf inhibited 80% of elongation of lettuce roots, Fig. 2C). Therefore, it was revealed that compound **1** was the cause of the inhibitory activity of the water fraction.

The bioactive EtOAc fraction of original plant extracts (Fig. 1A) was subjected to a reversed-phase solid-phase extraction, and separated into six fractions. The main activity was fractionated into 40 and 60% MeOH in H_2O-eluted fractions (Fig. 3), and were further separated by HPLC (Fig. 4). Two major bioactive fractions were detected in the 60% MeOH-eluted fraction. These were pure compound **3** and a mixture of compounds **4** plus **5** (Fig. 4B). Although the peak intensity of compound **3** was smaller than that of compounds **4** plus **5** in the chromatogram of 60% MeOH-eluted fraction (Fig. 4B), inhibitory activity on the elongation of lettuce roots of compound **3** was almost 10-times higher than that of the mixture of compounds **4** plus **5** (Fig. 5). In the 40% MeOH-eluted fraction, compound **3** was also detected (Fig. 4A). Therefore, major inhibitory activity of the EtOAc fraction was attributed to compound **3**.

Compounds **1**, **2**, **3**, **4**, and **5** were purified in large scale with preparative HPLC. Separation of compounds **4** and **5** was achieved by HPLC eluted with 30% acetonitrile in H_2O (analytical HPLC; 12.6 and 17.0 min, preparative HPLC; 52.6 and 73.7 min for compounds **5** and **4**, respectively). These compounds were subjected to structural analyses and their UV spectra were very similar with maximum absorption observed at 279, 283, 280, 280, and 283 nm for compounds **1**, **2**, **3**, **4**, and **5**, respectively. 1D and DEPT ^{13}C NMR spectra of compound **1** indicated the presence of one pyranoside (one

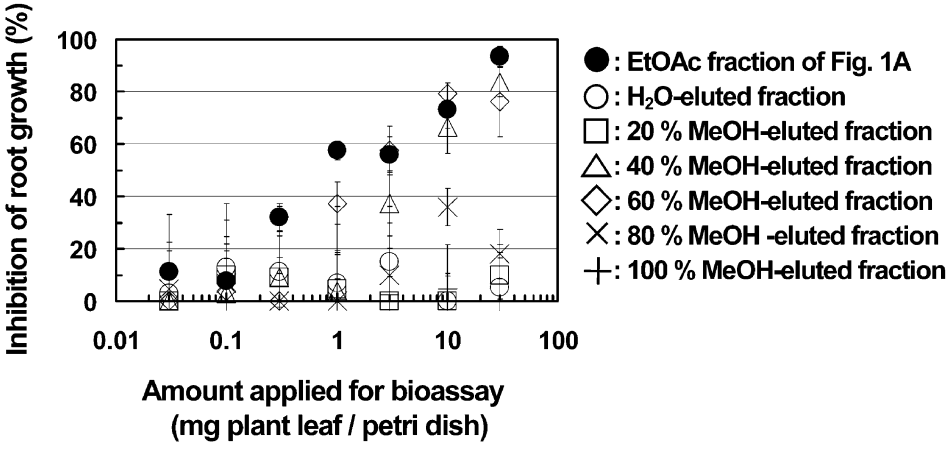

Fig. 3. Inhibitory activity of EtOAc fraction of original plant extracts (see Fig. 1A) and its reversed-phase solid-phase extraction-separated fractions on elongation of lettuce roots. Bars indicate standard deviation ($n = 5$–6).

anomeric carbon at 95.7 ppm, four –CH(OH)– carbons between 71.1 and 78.9 ppm, and one terminal $–CH_2OH$ carbon at 62.4 ppm) and one cinnamic acid (one carboxylic carbon at 166.1 ppm, one alkene (–CH = CH–) carbon-pair at 119.5 and 146.4 ppm, and one 1-substituted aromatic ring between 129.0 and 136.0 ppm) in the molecule. GC/MS analysis of the TMS-derivative of methanolysis product of compound **1** revealed that the pyranoside was glucose. Configuration of the anomeric (C-1) position of the glucose was determined as β by its ^1H-^1H coupling constant (8 Hz). To determine the C-2 configuration of the cinnamic acid moiety, ^1H–^1H coupling constant of compound **1** (13 Hz) was compared with that of reported values (12.44 and 16.03 Hz for *cis*- and *trans*-cinnamic acids, respectively; Sun et al., 2002) and determined as *cis*. A HMBC signal was observed between cinnamoyl C-1 (166.1 ppm) and glucosyl H-1 (5.53 ppm) moieties. HRMS-FAB ($[M+H]^+$, *m/z* 311.1147 calculated for $C_{15}H_{19}O_7$) and 2D NMR (^1H-^1H COSY, ^{13}C–^1H COSY, HMBC) spectral analyses confirmed that compound **1** was 1-*O*-*cis*-cinnamoyl-β-D-glucopyranose (Fig. 6). All ^{13}C NMR signals were assigned as shown in Table 1.

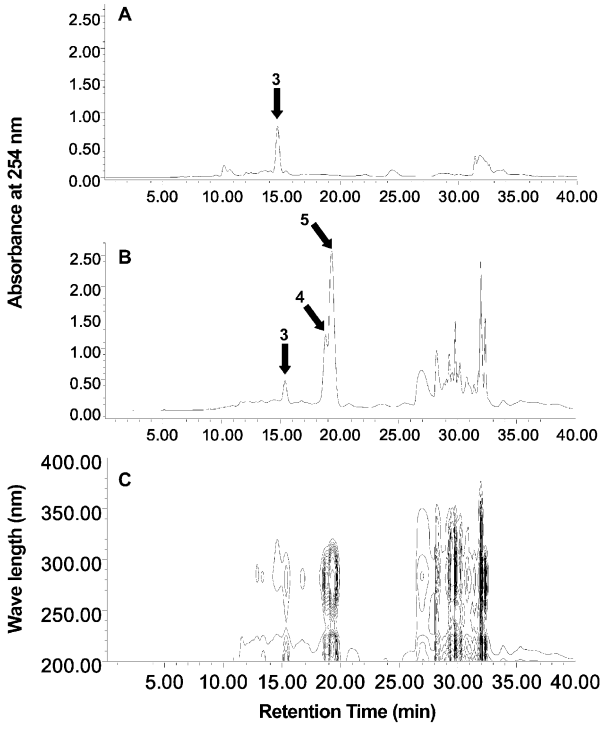

Fig. 4. Analytical HPLC chromatogram of 40% (A: detection at 254 nm) and 60% (B: detection at 254 nm, C: contour plot between 200 and 400 nm) MeOH in H_2O-eluted fractions of reversed-phase solid-phase extraction of EtOAc fraction (see Fig. 3). The amount of sample injected was equivalent to 25 mg of fresh plant for each experiment. Arrows indicate peaks with inhibitory activity. Flow rate: 0.8 ml/min. Eluent: 0–20 min; 50% MeOH in H_2O, 20–25 min; 50–80% MeOH in H_2O (linear gradient), 25–40 min; 80% MeOH in H_2O. Column temperature: 40 °C.

Compound **3** showed identical ^1H and ^{13}C NMR resonance peaks with compound **1**, with additional signals of 2-methylene butyric acid (one carboxylic carbon at 168.3 ppm, one alkene (–CH = CH–) carbon-pair at 138.7 and 128.0 ppm, one $–CH_2–$ carbon at 36.4 ppm, and one terminal $–CH_2OH$ carbon at 61.6 ppm). GC/MS analysis showed the presence of a glucopyranose moiety in compound **3**. HMBC signals were observed between the butyroyl C-1 (168.3 ppm) and glucosyl H-6 (4.49 and 4.27 ppm). HRMS-FAB ($[M+H]^+$, *m/z* 409.1529 calculated for $C_{20}H_{25}O_9$) and 2D NMR analyses revealed that compound **3** was 6-*O*-(4′-hydroxy-2′-methylene-butyroyl)-1-*O*-*cis*-cinnamoyl-β-D-glucopyranose (Fig. 6).

To clarify the specific activity of compounds **1** and **3**, the inhibitory activity of purified compounds **1** and **3** were examined on a molar basis and compared with that of (+)-2-*cis*-4-*trans*-abscisic acid and 2,4-dichlorophenoxyacetic acid (positive controls). The inhibitory activities of compounds **1** and **3** were at the same level, and the EC_{50} values were between 10^{-6} and 10^{-5} mol/l (Fig. 7). The inhibitory activities were higher than (+)-2-*cis*-4-*trans*-abscisic acid and lower than 2,4-dichlorophenoxyacetic acid. Therefore, it was clarified that compounds **1** and **3** have a very high inhibitory activity on elongation of lettuce roots. Contents of compounds **1** and **3** in *S. thunbergii*, which were determined by analytical HPLC experiments, were 0.64 and 2.95 μmol/g FW, respectively (Table 1). This result was consistent with the results of biological activities depicted in Figs. 1, 2, 3, and 5.

The 1D and DEPT ^{13}C NMR spectra of compound **4** showed the presence of one pyranose and one *trans*-cinnamoyl moiety. In addition, the presence of one –OOC–CH(CH_3)–CH(O-)–CH_2–O–(2-methyl-butanoic) structure was shown in 1D and 2D NMR spectra. The pyranose moiety was identified as glucose by GC/MS

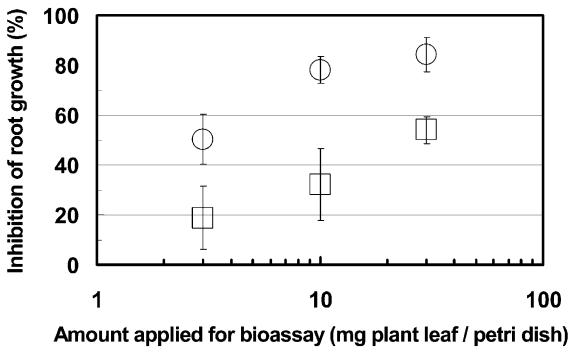

Fig. 5. Inhibitory activity of compounds **3** (open circles) and **4** plus **5** (open squares) on the elongation of lettuce roots. The amounts applied to the bioassay were on the basis of equivalent to original fresh plant weight. Compounds **3** and **4** plus **5** were prepared from the 60% MeOH in H_2O-eluted fraction of reversed-phase solid-phase extraction of EtOAc fraction by HPLC (see Fig. 4B and C). Bars indicate standard deviation ($n = 5$–6).

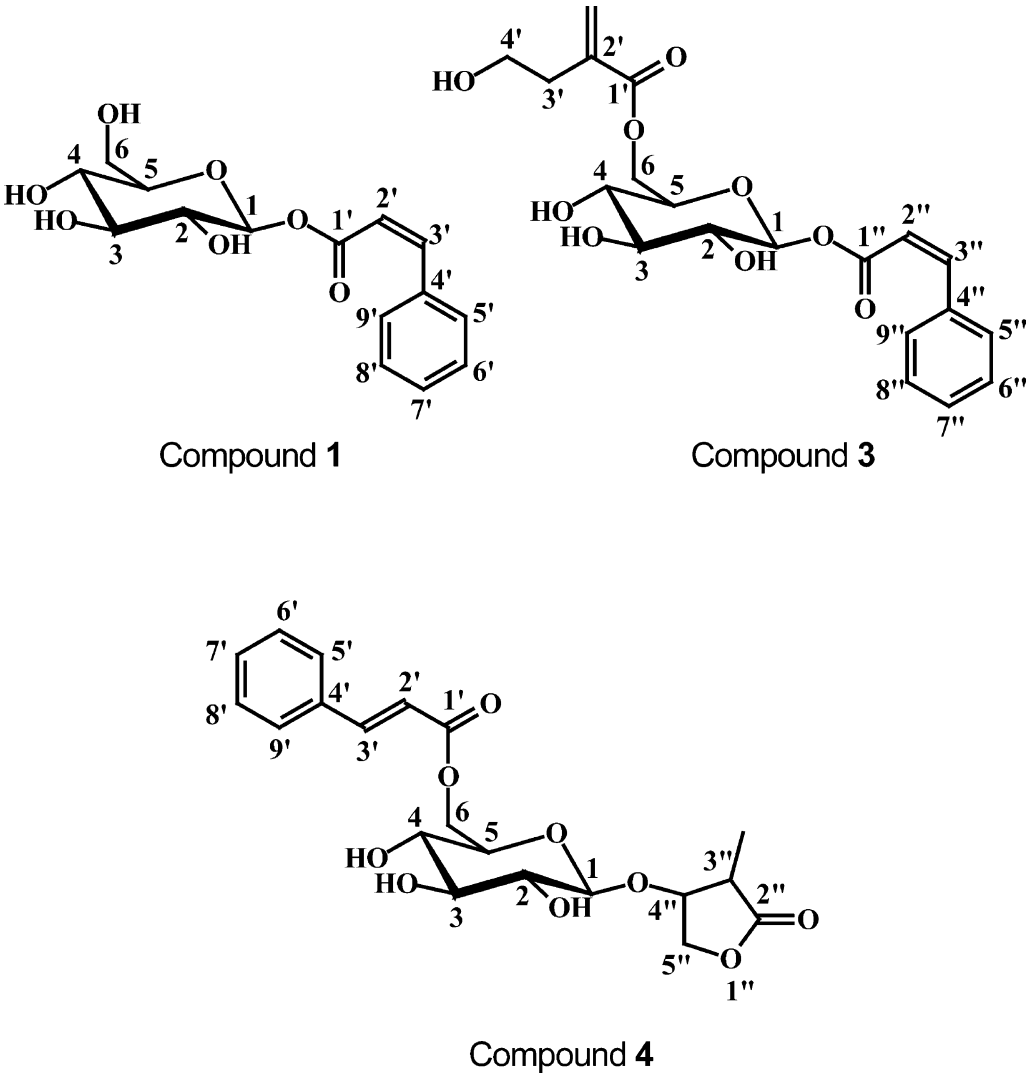

Fig. 6. Chemical structures of compounds **1**, **3** and **4**.

analysis of the TMS derivative of methanolysis product of compound **4**, and the C-1 configuration of the glucose was determined as β from the ^{1}H–^{1}H coupling constant (9 Hz). HMBC signals were observed between cinnamoyl C-1 (168.5 ppm) and glucosyl H-6 (4.55 and 4.35 ppm), indicating that the cinnamoyl moiety was bound to C-6 of the glucose. HMBC signals were also observed between glucosyl C-1 (102.8 ppm) and 2-methyl-butanoic H-3 (4.59 ppm) and 2-methyl-butanoic C-3 (77.6 ppm) and glucosyl H-1 (4.40 ppm), indicating that the 2-methyl-butanoic moiety was bound to glucosyl C-1 through O-3 of the 2-methyl-butanoic moiety. HMBC signals between C-1 (181.2 ppm) and H-4 (4.42 and 4.30 ppm) in 2-methyl-butanoic moiety revealed that it forms lactone between C-1 and C-4 (furan-2-one). HRMS-FAB analysis ($[M+H]^{+}$, m/z 409.1526

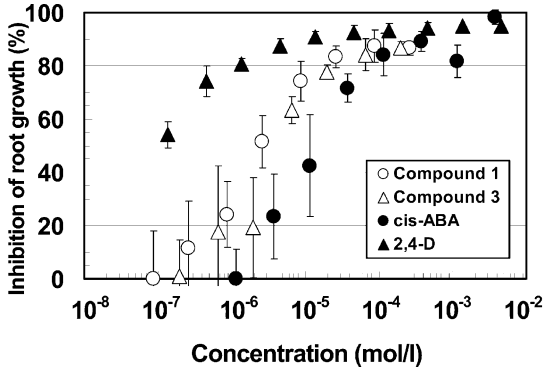

Fig. 7. Inhibitory activity of compounds **1, 3,** and positive controls [(+)-2-*cis*-4-*trans*-abscisic acid and 2,4-dichlorophenoxyacetic acid] on elongation of lettuce roots (specific activity). Bars indicate standard deviation ($n=5$–6).

Table 1
^{13}C NMR spectral data (150.8 MHz, CD_3OD) and contents of compounds **1**–**5**

C	Compound **1**	Compound **2**	Compound **3**	Compound **4**	Compound **5**
β-D-glucose moiety					
1	95.7	96.0	95.7	102.8	95.9
2	74.0	74.1	73.9	74.6	74.0
3	78.1	78.0	78.0	77.9	77.9
4	71.1	71.1	71.4	71.6	71.4
5	78.9	78.9	76.2	75.5	76.2
6	62.4	62.4	64.7	64.7	64.7
1-O-substituent					
1	166.1	167.2	166.2		167.0
2	119.5	118.3	119.4	181.2	118.2
3	146.4	147.7	146.0	40.2	147.8
4	136.0	135.6	136.0	77.6	135.6
5 (9)	131.3	129.4	131.1	73.3	129.4
6 (8)	129.0	130.1	129.1		130.1
7	130.4	131.8	130.4		131.8
3-methyl				9.1	
6-O-substituent					
1			168.3	168.5	168.3
2			138.7	118.7	138.6
3			36.4	146.6	36.3
4			61.6	135.7	61.6
5 (9)				129.3	
6 (8)				130.1	
7				131.6	
2-methylene			128.0		128.0
Content (mg/g FW)	0.20	0.13	1.21	5.34	19.4
(μmol/g FW)	0.64	0.42	2.95	13.1	47.5

calculated for $C_{20}H_{25}O_9$) revealed that compound **4** was 6-*O*-(*trans*-cinnamoyl)-1-*O*-(4″-hydroxy-3″-methyl-furan-2″-one)-β-D-glucopyranose (Fig. 6).

A literature survey revealed that compounds **1, 3** and **4** have novel structures. In the present study, *trans*-isomers of compounds **1** and **3** were also identified as compounds **2** (1-*O*-*trans*-cinnamoyl-β-D-glucopyranose) and **5** (6-*O*-(4′-hydroxy-2′-methylene-butyroyl)-1-*O*-*trans*-cinnamoyl-β-D-glucopyranose), respectively. Their chemical structures were elucidated by 1D and 2D NMR spectroscopic experiments and GC/MS analyses, as described above. All ^{13}C NMR signals and contents of compounds **1**–**5** were summarized in Table 1.

The presence of compounds **2** and **5** in *S. thunbergii* are reported by Tanabe and Kita (1980), although their plant growth inhibitory activities are not mentioned. Because they named compound **5** as spirarin, compounds **3** and **5** should be named *cis*- and *trans*-spirarin, respectively. Tanabe and Kita (1980) purified compounds **2** and **5** by crystallization. The physical states of *cis*- and *trans*-cinnamic acids are a transparent oil-type liquid and a white crystal, respectively (Sun et al., 2002). Therefore, poorly crystalline *cis*-cinnamoyl glucosides could not be isolated by following the procedure of Tanabe and Kita (1980). In the same plant, the presence of triterpenoids (glutinol and taraxerol, Tanabe et al., 1976), two hydroxycinnamoylglucosides (1-*O*-*p*-coumaryl-β-D-glucopyranose and 1-*O*-caffeyl-β-D-glucopyranose, Ina et al., 1987), antifungal methyl β,γ-dihydroxy-α-methylene-butyrate and β-hydroxy-α-methylene-butyrolactone (tulipalin B, Okuma et al., 1975), and insecticidal α-methylene-γ-butyrolactone (tulipalin A, Kim et al., 1998) and its possible precursor (6-*O*-(4-hydroxy-α-methylene-butyryl)-D-glucopyranose, 6-tuliposide A, Kim et al., 1999) have been reported, although their plant growth inhibitory activities have not been examined. Some of these compounds would be closely related with the biosynthesis of compounds **1**–**5** in *S. thunbergii*, which remains to be clarified.

The *cis*- and *trans*-cinnamoyl glucosides were well characterized by their ^1H NMR spectra of alkene protons (*cis*; 7.1 and 6.0 ppm, $J = 13$ Hz, *trans*; 7.8 and 6.6 ppm, $J = 16$ Hz). Separations between *cis*- and *trans*-isomers were well achieved by reversed-phase HPLC as shown in Figs. 2 and 4. Appreciable amounts of *trans*-isomers were present together with *cis*-isomers in the samples (Table 1). The growth inhibitory activity, however, was detected mainly in the *cis*-isomers. This implies that *cis*-isomers have stronger growth inhibitory activity than the *trans*-isomers. It is reported that

kaempferol 3-(6″-*cis*-cinnamoylglucoside) is present in *Solanum elaeagnifolium* (Chiale et al., 1991). Presence of bioactive *cis*-cinnamic acid is also reported in *Brassica parachinensis* (Yin et al., 2003). In the report, possible pathways for synthesis of *cis*-cinnamic acid are proposed: (1) a sunlight-mediated conversion from *trans*-cinnamic acid, (2) spontaneous conversion from *trans*-cinnamic acid in the presence of electron-transfer sensitizer, (3) isomerase-mediated conversion from *trans*-cinnamic acid, and (4) direct enzymatic biosynthesis from L-phenylalanine. Further study is necessary to clarify the biosynthesis pathway of *cis*-cinnamoyl glucosides in *S. thunbergii*. It would be possible that *S. thunbergii* accumulates *trans*-cinnamoyl glycosides as detoxified forms and secretes *cis*-cinnamoyl glycosides as toxic form of effective allelochemicals. To evaluate this hypothesis, the amounts present in environments should be clarified in future studies.

3. Experimental

3.1. General

1D (^{1}H, ^{13}C, and DEPT) and 2D (^{1}H–^{1}H COSY, HOHAHA, ^{13}C–^{1}H COSY, and HMBC) NMR spectra were recorded in CD_3OD-d_4 at 30 °C on a JEOL JNM α-600 spectrometer (^{1}H: 600.05 MHz, ^{13}C: 150.80 MHz) using standard JEOL software (Alpha Data System). Chemical shifts were reported in ppm relative to TMS but were recorded relative to the residual signal of the solvent (^{1}H: 3.30 ppm, ^{13}C: 49.00 ppm) and coupling constants in Hz. FAB-MS spectra were acquired using a JEOL SX102A spectrometer with samples mixed with glycerol as a matrix and analyzed using a direct inlet system. ESI-MS spectra were recorded using a triple quadrupole mass spectrometer API 300 (PE SCIEX-Applied Biosystems, Foster City, CA). For reversed-phase solid-phase extraction, chemically modified silica gel (reversed-phase, Analytichem BONDESIL C18, 40 μm, preparative grade, Varian) was filled in a glass column. HPLC was performed with a Waters 626 pump and 996 photodiode array detector, equipped with a reversed-phase column (analytical column; Inertsil® ODS-3, 3 μm, 4.6 mm i.d., 250 mm length, GL Sciences Inc., Tokyo, analytical guard column; Inertsil® ODS-3, cartridge guard column E, 5 μm, 4 mm i.d., 20 mm length, GL Sciences Inc., preparative column; Inertsil® ODS-3, 20 mm i.d., 250 mm length, GL Sciences Inc., preparative guard column; Inertsil® ODS-3, 20 mm i.d., 50 mm length, GL Sciences Inc.) at 40 °C. Flow rate of the analytical HPLC system was 0.8 and 1.0 ml/min and that of preparative HPLC system was 4 and 5 ml/min for the MeOH/H_2O and acetonitrile/H_2O elution systems, respectively. The UV spectra were recorded with the photodiode array equipped with the HPLC system on-line. 2,4-Dichlorophenoxyacetic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and (+)-2-*cis*-4-*trans*-abscisic acid (Toray Industries, Inc., Tokyo, Japan) were used as positive controls for bioassays.

3.2. Measurement of phytotoxic activity

A filter paper (27 mm ø, Type 1, Toyo Roshi Kaisha, Ltd, Tokyo) was placed in a glass petri dish (27 mm ø). Test solution was added to the filter paper in the petri dish and dried completely in vacuo at 40 °C. After addition of distilled water (0.7 ml), six pre-germinated (16 h at 20 °C in the dark) seedlings of lettuce (*Lactuca sativa* cv. Great Lakes 366) were placed on the filter paper, and incubated for 48 h at 20 °C in the dark. The inhibitory activity on root elongation was detected by measuring the length of the root and comparing the data obtained with that of untreated controls.

3.3. Isolation of phytotoxic compounds

Fresh leaves of *S. thunbergii* (110 g) were collected from the plant garden of the Fukui Prefectural University in 2002 and soaked in pure MeOH (2.6 l) and extracted for 30 days at 5 °C in the dark. The original MeOH extract was evaporated to dryness in vacuo and suspended in ca. 100 ml of water. The water suspension was partitioned three times with hexane (ca. 100 ml). The residual water fraction was then acidified to pH 2.5 with HCl and partitioned with three times with EtOAc (ca. 100 ml). The residual water fraction was neutralized with NaOH for bioassays and further purification.

The bioactive water fraction was subjected to charcoal column chromatography, with the activated charcoal (12 g, Charcoal, Activated, 031-02135, Wako Pure Chemical Industries, Ltd.) packed in a glass column (19 mm i.d., 300 mm length). The sample was then dissolved in a small amount of water and applied to the column, with successive elution using H_2O (300 ml), MeOH in H_2O (9:1, 300 ml), MeOH (300 ml), acetone (300 ml), and EtOAc (300 ml). The bioactive MeOH in H_2O (9:1)-eluted fraction was subjected to silica gel column chromatography as follows. Silica gel (18 g, Wakogel® C-300 HG, 40–60 μm, 238-01465, Wako Pure Chemical Industries, Ltd.) was suspended in $CHCl_3$ and the slurry was filled in a glass column (19 mm i.d., 300 mm length). The sample was dissolved into a small amount of MeOH and added to the slurry of the silica gel. After removal of MeOH and $CHCl_3$ in vacuo, the sample adsorbed on the silica gel was mounted on the silica gel column and eluted with 250 ml of $CHCl_3$, 250 ml of 5%, 10%, 20%, 40%, and 70% MeOH in $CHCl_3$, and then MeOH (250 ml). The bioactive 5% MeOH in $CHCl_3$-eluted fraction was subjected to a preparative reversed-phase solid-phase extraction (adsorbent; 10 g, Analy-

tichem BONDESIL C18, 40 μm, preparative grade, Varian, glass column; 10 mm i.d., 140 mm length) and eluted successively with 100 ml of H_2O then 100 ml of 20%, 40%, 60%, and 80% MeOH in H_2O and MeOH (100 ml). The bioactive H_2O-eluted fraction was subjected to analytical and preparative HPLC [eluent: MeOH in H_2O (30:70)].

The bioactive EtOAc fraction was subjected to preparative reversed-phase solid-phase extraction and eluted successively as described above. The bioactive 40 and 60% MeOH in H_2O-eluted fractions were subjected to analytical HPLC and eluted with 50% MeOH in H_2O (0–20 min), 50–80% MeOH in H_2O (20–25 min, linear gradient), and 80% MeOH in H_2O (25–40 min). Large-scale purification of compounds **3, 4,** and **5** were performed with preparative HPLC (eluent: 50% MeOH in H_2O or 30% acetonitrile in H_2O).

3.4. Glycoside analysis by GC/MS

5% HCl/MeOH (0.5 ml) was added to a cinnamoyl glycoside (approximately 0.1 mg) in a test tube, which was tightly capped and incubated for 18 h at 80 °C (methanolysis). After complete drying, pyridine (0.05 ml), hexamethyldisilazane (0.10 ml), and chlorotrimethylsilane (0.05 ml) were added to the tube. After incubating the mixture for 20 min at 80 °C, the reactant (TMS derivative) was dried with N_2 gas and extracted with hexane (0.3 ml). The TMS derivative of the methanolysis product was analyzed with GC/MS (QP-5050, Shimadzu Corp., Kyoto, Japan) equipped with a capillary column (DB-1, 0.25 mm i.d., 30 m length, 0.25 μm thickness, 122-1032, J&W) under the following conditions: injector; 250 °C, interface; 320 °C, injection; split-less mode with 30 sec of sampling time, column temperature; 80 °C for the initial 2 min followed by an increase in temperature at a rate of 2 °C / min up to 200 °C and kept at the same temperature for 8 min. The retention time and mass spectrum of the samples were compared with those of the standard TMS derivative of methyl glycosides. The retention times and mass spectra of the TMS derivatives of the methanolysis products of compounds **1- 5** were completely identical with that of TMS derivatives of the methanolysis products of authentic glucopyranose [retention time; 50.6 and 51.9 min for α and β derivatives, *m/z*; 309 (10), 217 (11), 204 (41), 147 (19), 133 (15), 89 (19), 75 (100), 74 (10), 73 (78), 59 (21), 47 (19), 45 (34), 44 (24), 43 (12)].

3.5. Quantitative determination of compounds *1–5*

trans-Cinnamic acid (60 μg) (Wako Pure Chemical Industries, Ltd.) were dissolved in CD_3OD-d_4 (0.60 ml) in a NMR tube (5 mm i.d.). Each purified compound (hydrated) was also prepared in the same way (concentration unknown). ^{1}H-NMR spectra of these samples were recorded quantitatively (acquisition time: 5.46 sec). The peak area of alkene protons in the *trans*-cinnamic acid was compared with that of alkene protons in the cinnamic acid moiety of each compound, and the concentration of each compound was determined (standard solution). By using standard solutions, concentrations of compounds **1** and **2** in the plant leaf were determined in the H_2O-eluted fraction of Fig. 1D, and concentrations of compounds **3**–**5** were determined in the 40 and 60% MeOH in H_2O-eluted fractions of Fig. 3 by analytical HPLC, and converted to total contents in the plant leaf.

3.6. Compound *1*

1-*O*-*cis*-cinnamoyl-β-D-glucopyranose. ^{1}H NMR: 7.66 (*m*, 2H, Ar H-5′ and H-9′), 7.33 (*m*, 3H, Ar H-6′, H-7′, and H-8′), 7.08 (*d*, $J=13$ Hz, 1H, H-3′), 6.01 (*d*, $J=13$ Hz, 1H, H-2′), 5.53 (*d*, $J=8$ Hz, 1H, H-1), 3.84 (*dd*, $J=2$, 12 Hz, 1H, H-6), 3.68 (*dd*, $J=5$, 12 Hz, 1H, H-6), 3.44–3.31 (*m*, 4H, H-2, H-3, H-4, and H-5). HRMS-FAB (*m/z*): $[M+H]^+$ calculated for $C_{15}H_{19}O_7$, 311.1131; found, 311.1147. FAB-MS (*m/z*): 929 ($[3M-H]^-$), 619 ($[2M-H]^-$), 309 ($[M-H]^-$) calculated for $C_{15}H_{17}O_7$. ESI-MS (*m/z*): 349 ($[M+K]^+$) calculated for $C_{15}H_{18}O_7K$, 333 ($[M+Na]^+$) calculated for $C_{15}H_{18}O_7Na$.

3.7. Compound *2*

1-*O*-*trans*-cinnamoyl-β-D-glucopyranose. ^{1}H NMR: 7.80 (*d*, $J=16$ Hz, 1H, H-3″), 7.61 (*m*, 2H, Ar H-5″ and H-9″), 7.41 (*m*, 3H, Ar H-6″, H-7″, and H-8″), 6.57 (*d*, $J=16$ Hz, 1H, H-2″), 5.56 (*d*, $J=7$ Hz, 1H, H-1), 3.85 (*dd*, $J=2$, 12 Hz, 1H, H-6), 3.69 (*dd*, $J=5$, 12 Hz, 1H, H-6), 3.46–3.34 (*m*, 4H, H-2, H-3, H-4, and H-5). FAB-MS (*m/z*): 309 ($[M-H]^-$) calculated for $C_{15}H_{17}O_7$.

3.8. Compound *3*

6-*O*-(4′-hydroxy-2′-methylene-butyroyl)-1-*O*-*cis*-cinnamoyl-β-D-glucopyranose. ^{1}H NMR: 7.62 (*m*, 2H, Ar H-5″ and H-9″), 7.32 (*m*, 3H, Ar H-6″, H-7″, and H-8″), 7.07 (*d*, $J=13$ Hz, 1H, H-3″), 6.24 (*m*, 1H, -C=CH$_2$), 6.01 (*d*, $J=13$ Hz, 1H, H-2″), 5.68 (*m*, 1H, -C=CH$_2$), 5.51 (*d*, $J=9$ Hz, 1H, H-1), 4.49 (*dd*, $J=2$, 12 Hz, 1H, H-6), 4.27 (*dd*, $J=6$, 12 Hz, 1H, H-6), 3.68–3.61 (*m*, 3H, H-5 and H-4′), 3.44 (*dd*, $J=9$, 9 Hz, 1H, H-3), 3.36 (*dd*, $J=9$, 9 Hz, 1H, H-4), 3.33 (*dd*, $J=9$, 9 Hz, 1H, H-2), 2.52 (*t*, $J=7$ Hz, 2H, H-3′). HRMS-FAB (*m/z*): $[M+H]^+$ calculated for $C_{20}H_{25}O_9$, 409.1499; found, 409.1529. FAB-MS (*m/z*): 815 ($[2M-H]^-$), 407 ($[M-H]^-$) calculated for $C_{20}H_{23}O_9$. ESI-MS (*m/z*): 447 ($[M+K]^+$) calculated for $C_{20}H_{24}O_9K$, 431 ($[M+Na]^+$) calculated for $C_{20}H_{24}O_9Na$, 409 ($[M+H]^+$) calculated for $C_{20}H_{25}O_9$.

3.9. Compound **4**

6-*O*-(*trans*-cinnamoyl)-1-*O*-(4″-hydroxy-3″-methyl-furan-2″-one)-β-D-glucopyranose. ^{1}H NMR: 7.71 (*d*, $J=16$ Hz, 1H, H-3″), 7.60 (*m*, 2H, Ar H-5″ and H-9″), 7.40 (*m*, 3H, Ar H-6″, H-7″, and H-8″), 6.56 (*d*, $J=16$ Hz, 1H, H-2″), 4.59 (*m*, 1H, H-3′), 4.55 (*dd*, $J=2$, 12 Hz, 1H, H-6), 4.42 (*d*, $J=10$ Hz, 1H, H-4′), 4.40 (*d*, $J=8$ Hz, 1H, H-1), 4.35 (*dd*, $J=6$, 12 Hz, 1H, H-6), 4.30 (*dd*, $J=4$, 10 Hz, 1H, H-4′), 3.53 (*m*, 1H, H-5), 3.37 (*m*, 2H, H-3 and H-4), 3.20 (*dd*, $J=8$, 9 Hz, 1H, H-2), 2.81 (*dq*, $J=7$, 7 Hz, 1H, H-2′), 1.18 (*d*, $J=7$ Hz, 3H, H-5′ methyl). HRMS-FAB (*m*/*z*): $[M+H]^+$ calculated for $C_{20}H_{25}O_9$, 409.1499; found, 409.1526. FAB-MS (*m*/*z*): 815 ($[2M-H]^-$), 407 ($[M-H]^-$) calculated for $C_{20}H_{23}O_9$.

3.10. Compound **5**

6-*O*-(4′-hydroxy-2′-methylene-butyroyl)-1-*O*-*trans*-cinnamoyl-β-D-glucopyranose. ^{1}H NMR: 7.80 (*d*, $J=16$ Hz, 1H, H-3″), 7.61 (*m*, 2H, Ar H-5″ and H-9″), 7.41 (*m*, 3H, Ar H-6″, H-7″, and H-8″), 6.56 (*d*, $J=16$ Hz, 1H, H-2″), 6.24 (*m*, 1H, -C=CH$_2$), 5.68 (*m*, 1H,-C=CH$_2$), 5.57 (*d*, $J=8$ Hz, 1H, H-1), 4.48 (*dd*, $J=2$, 12 Hz, 1H, H-6), 4.28 (*dd*, $J=6$, 12 Hz, 1H, H-6), 3.68–3.65 (*m*, 3H, H-5 and H-4′), 3.48 (*dd*, $J=9$, 9 Hz, 1H, H-3), 3.44 (*dd*, $J=8$, 9 Hz, 1H, H-2), 3.40 (*dd*, $J=9$, 9 Hz, 1H, H-4), 2.53 (*t*, $J=7$ Hz, 2H, H-3′). FAB-MS (*m*/*z*): 1223 ($[3M-H]^-$), 815 ($[2M-H]^-$), 407 ($[M-H]^-$) calculated for $C_{20}H_{23}O_9$.

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