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ORIGINAL ARTICLE

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Novel stilbenoids isolated from the heartwood of *Shorea laeviforia*

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Abstract Two novel stilbenoids, laevifonol (an ε-viniferin-ascorbic acid hybrid compound) and laevifoside (an *O*-glucoside of ampelopsin A) were isolated from the heartwood of *Shorea laeviforia*. Their structures were elucidated by nuclear magnetic resonance spectroscopic evidence including HMQC, HMBC, and NOESY. The novel compounds and other known stilbenoids from *S. laeviforia* were evaluated for inhibitory activity against rat liver 5α-reductase. Positive inhibitory activities were observed in resveratrol dimers and tetramers. No inhibitory activity was detected in laevifonol and ampelopsin A glucosides, laevifoside, or hemsleyanoside, whereas inhibitory activity was seen in their aglycon. These results suggest that the hydrophilic moiety in these compounds may inhibit action with the hydrophobic active site of the enzyme.

Key words *Shorea laeviforia* · ε-Viniferin–ascorbic acid hybrid · Ampelopsin A *O*-glucoside · Oligostilbenes · Rat liver 5α-reductase inhibitor

Introduction

Testosterone is converted to a potent androgen 5α-dihydrotestosterone (DHT) by steroid 5α-reductase, and excessive production of DHT is responsible for androgen-dependent diseases. A potent 5α-reductase inhibitor may be able to remedy or prevent this type of disease (e.g., male-pattern baldness, benign prostatic hyperplasia, acne, female hirsutism). Many steroidal 5α-reductase inhibitors have been investigated. One, finasteride showed strong inhibitory activity;[1] it decreases the serum concentration of DHT and increases that of testosterone.[2] Clinical effectivenesses for the treatment of benign prostatic hyperplasia and male-pattern baldness have been shown for this compound.[3,4] Recently, finasteride was approved by the U.S. Food and Drug Administration (FDA) for the treatment of symptomatic benign prostatic hyperplasia, and it became available commercially for therapy of these androgen-dependent diseases. Some adverse effects (e.g., loss of libido, loss of erection, ejaculatory dysfunction, hypersensitivity reactions, gynecomastia, severe myopathy), however, have been reported.[5] It is preferable to develop therapeutic agents without such serious adverse effects.

We previously reported on rat liver 5α-reductase inhibitory compounds from tropical commercial wood species. The methanol extracts of *Shorea* species showed potent inhibitory activity, and four trimeric stilbenes were isolated as inhibitory compounds from melapi (*Shorea* sp.) heartwood.[6] In a previous report, dimeric stilbenes synthesized by a horseradish peroxidase/H_2O_2 system showed strong 5α-reductase inhibitory activity.[7] In this paper we report the structural determinations of the novel compounds laevifonol and laevifoside and evaluate the 5α-reductase inhibitory activity of oligostilbenes and their derivatives isolated from the heartwood of *Shorea laeviforia* (Dipterocarpaceae).

Materials and methods

Sample wood

A block of *Shorea laevifolia* was collected in Indonesia by Dr. Wasrin Syafii (Bogor Agricultural University, Indonesia) and was preserved at the herbarium of the Department of Forest and Forest Products Sciences, Kyushu University, Japan. The block was ground in a Wiley mill, and the meal was extracted with methanol (MeOH) for 24 h at room temperature.

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Extraction and separation of extractives of *Shorea laevifolia*

A methanol extract of milled heartwood (500 g) of *S. laeviforia* was concentrated. After adding the same volume of water, the mixture was partitioned against *n*-hexane, diethyl ether, and *n*-butanol (*n*-BuOH). The *n*-BuOH-soluble fraction (64 g) was subjected to column chromatography on Sephadex LH-20 using ethanol (EtOH) as the eluent. Further purification was performed by silica gel column chromatography using a $CHCl_3$-MeOH (2:1) system and by reverse-phase preparative high-performance liquid chromatography (HPLC) (GL Science) (C-18 column Inertsil PREP-ODS: 20 mm i.d. × 250 mm) using an acetonitrile-0.1% trifluoroacetic acid (TFA) gradient (15%–50% acetonitrile) as the eluent. Hemsleyanoside F (**7**: 53 mg), laevifoside (**2**: 48 mg), laevifonol (**1**: 620 mg), (−)-ampelopsin A (**3**: 32 mg), (−)-ε-viniferin (**4**: 7 mg), (−)-hopeaphenol (**5**: 37 mg), and (+)-isohopeaphenol (**6**: 46 mg) were isolated.

Isolated compounds from *S. laeviforia*

The ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra, ^1H-^1H correlated spectroscopy (COSY), heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple bond correlation (HMBC), and nuclear Overhauser effect (NOE) NMR experiments were performed in MeOH-*d*4 (**1**, **2**, **7**) or acetone-*d*6 (**3**, **4**, **5**, **6**), using a 400 MHz NMR spectrometer. Complete assignment of all protons and carbons in ^1H and ^{13}C spectra of compounds were confirmed by HMQC spectra. The molecular weights were measured by fast atom bombardment-mass spectrometry (FAB-MS) using a glycerol matrix.

Compounds **3**,[8] **4**,[9] **5**,[10] **6**,[11] and **7**[12] are known from the literature and are identified through comparison of spectroscopic data.

Laevifonol (**1**) (Fig. 1): brown solid: FAB-MS *m/z* 627 $(M-H)^-$. $[\alpha]_D$-124°, (*c* 0.496, MeOH). UV (MeOH) λ_{max} ($\log\varepsilon$): 278 (3.91), 284 (3.92), 298 (3.78) nm. ^1H NMR (400 MHz, MeOH-*d*4) δ_H 3.15 (brd, *J* = 7.1, H-8′), 3.22 (d, *J* = 11.0, H-8), 3.98 (*dd*, *J* = 10.3, *J* = 4.3, H-6″), 4.09 (dd, *J* = 10.0, *J* = 2.0, H-6″), 4.20 (*m*, H-5″), 4.36 (brs, H-4″), 5.03 (d, *J* = 8.0, H-7′), 5.29 (d, *J* = 11.3, H-7), 5.86 (d, *J* = 2.0, 2H, H-10′, H-14′), 6.14 (t, *J* = 2.0, H-12′), 6.17 (d, *J* = 2.0, H-12), 6.71 (m, 6H, H-3, H-5, H-2′, H-3′, H-5′, H-6′), 6.92 (d, *J* = 8.5, H-2, H-6), 7.13 (brs, H-14); ^{13}C NMR (100 MHz, MeOH-*d*4) δ_C 57.1 (C-8), 57.1 (C-8′), 74.6 (C-5″), 75.9 (C-6″), 81.3 (C-2″), 89.6 (C-4″), 90.8 (C-7), 94.8 (C-7′), 102.5 (C-12′), 107.5 (C-10″, C-14″), 111.0 (C-14), 115.8 (C-3′, C-5′), 116.1 (C-3, C-5), 119.1 (C-3″), 123.3 (C-10), 128.2 (C-2, C-6), 129.0 (C-2′, C-6′), 129.8 (C-9), 131.7 (C-1), 132.4 (C-1′), 145.9 (C-9′), 158.2, 158.8 (C-4′ and C-4), 159.0 (C-13), 159.6 (C-11′, C-13′), 161.3 (C-11), 173.6 (C-1″).

Laevifoside (**2**) (Fig. 1): brown solid: FAB-MS *m/z* 631 $(M-H)^-$. $[\alpha]_D$-157°, (c 0.560, MeOH). UV (MeOH) λ_{max} ($\log\varepsilon$): 283 (3.94) nm. ^1H NMR (400 MHz, MeOH-*d*4) δ_H 3.40 (d, *J* = 8.6, H-3″), 3.43 (dd, *J* = 5.4, *J* = 2.2, H-5″), 3.47 (m, H-2″), 3.47 (m, H-4″), 3.69 (dd, *J* = 12.2, *J* = 5.4, H-6″), 3.88 (dd, *J* = 12.2, *J* = 2.0, H-6″), 4.08 (d, *J* = 11.4, H-8′), 4.94 (d, *J* = 7.3, H-1″), 5.39 (d, *J* = 3.4, H-7), 5.46 (d, *J* = 3.4, H-8), 5.75 (d, *J* = 10.8, H-7′), 6.12 (d, *J* = 1.2, H-14′), 6.34 (d, *J* = 1.8, H-12′), 6.43 (d, *J* = 2.2, H-12), 6.58 (d, *J* = 8.6, H-3, H-5), 6.70 (d, *J* = 8.5, H-3′, H-5′), 6.82 (d, *J* = 8.8, H-2, H-6), 6.83 (d, *J* = 2.2, H-14), 7.02 (d, *J* = 8.8, H-2′, H-6′); ^{13}C NMR (100 MHz, MeOH-*d*4) δ_C 44.0 (C-7), 49.9 (C-8′), 62.5 (C-6″), 71.3 (C-8), 71.5 (C-3″), 74.8 (C-2″), 77.8 (C-4″), 78.0 (C-5″), 89.1 (C-7′), 98.8 (C-12), 101.7 (C-12′), 102.1 (C-1″), 105.3 (C-14′), 112.1 (C-14), 115.5 (C-3, C-5), 116.1 (C-3′, C-5′), 118.9 (C-10′), 122.6 (C-10), 128.8 (C-2, C-6), 129.9 (C-2′, C-6′), 130.7 (C-1), 132.8 (C-1′), 139.6 (C-9), 142.8 (C-9′), 156.0 (C-4), 157.2 (C-11′), 158.6 (C-4′), 159.1 (C-13′), 159.5 (C-13), 160.1 (C-11).

Acid hydrolysis of laevifoside (**2**)

Acid hydrolysis of laevifoside (**2**) and analysis of the hydrolysis products were performed as described by Hara et al.[13] Laevifoside (20 mg) was heated in 1 ml of 1 N HCl at 60°C for 2 h. The reaction mixture was cooled to 0°–5°C,

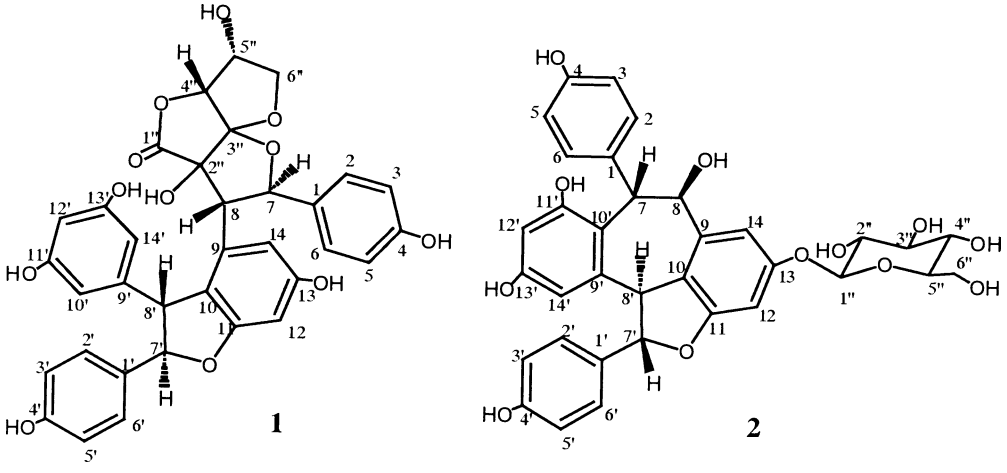


Fig. 1. Structures of laevifonol (**1**) and laevifoside (**2**)

and the precipitate (7 mg) was collected by filtration. The residue was purified by silica gel column chromatography using ethyl acetate-*n*-hexane (2:1) eluent to afford the aglycon, brown solid: ^{1}H NMR (400 MHz, acetone-d6) δ_H 4.14 (d, J = 11.4), 5.40 (d, J = 4.9), 5.42 (d, J = 4.9), 5.74 (d, J = 11.4), 6.13 (d, J = 2.0), 6.22 (d, J = 1.9), 6.43 (d, J = 2.1), 6.63 (d, J = 1.9), 6.63 (d, J = 8.7, 2H), 6.75 (d, J = 8.7, 2H), 6.78 (d, J = 8.7, 2H), 6.89 (d, J = 8.8, 2H), 7.10 (d, J = 8.8, 2H).

The supernatant was neutralized with Ag_2CO_3. After centrifugation, the supernatant was concentrated in vacuo to give a sugar fraction. Pyridine solution (1 ml) of the sugar fraction (7 mg) and 10 mg of L-cysteine methyl ester hydrochloride (Wako Pure Chemicals, Tokyo, Japan) were mixed and warmed at 60°C for 1 h. Hexamethyldisilazane-trimethylchlorosilane (HMDS-TMCS) (Wako) was added, and warming at 60°C was continued for another 30 min. The supernatant (1 μl) was subjected to gas chromatography-mass spectrometry (GC-MS) analysis, which was conducted on a GC-17A (Shimadzu, Kyoto, Japan) gas chromatograph equipped with a Neutra Bond-5 (30 m × 0.25 mm; film thickness 0.4 μm; GL Science, Tokyo, Japan) coupled to a QP-5000 quadruple mass spectrometer injector. The mass spectrometer was operated in the electron impact mode at 70 eV. Helium was used as the carrier gas with a flow rate of 0.8 ml/min. The first oven temperature was 200°C, which was increased to 300°C at a rate of 10°C/min. The absolute configuration of sugar was determined by comparison of the GC-MS data of D- or L-glucose thiazolidine derivative derived in a manner similar to that described above.

Preparation of rat liver microsomes

Sprague-Dawley female rat livers were removed and homogenized in medium A (0.32 M sucrose, 1 mM dithiothreitol, and 20 mM sodium phosphate, pH 6.5) as described in a previous report.[7] The homogenate was centrifuged at 10 000 g for 10 min at 0°C. The resulting pellets were suspended in medium A and centrifuged again.

The microsomes were obtained as a precipitate from further ultracentrifugation of the preceding supernatants (105 000 g for 1 h at 0°C). The washed microsomes were suspended in 10 ml of medium A, and dispersion of the microsomes was achieved using a syringe with 18-, 23-, 26-gauge needles in succession. The microsome suspension was divided into small aliquots and stored at −80°C. The microsomes were diluted with medium A just before use.

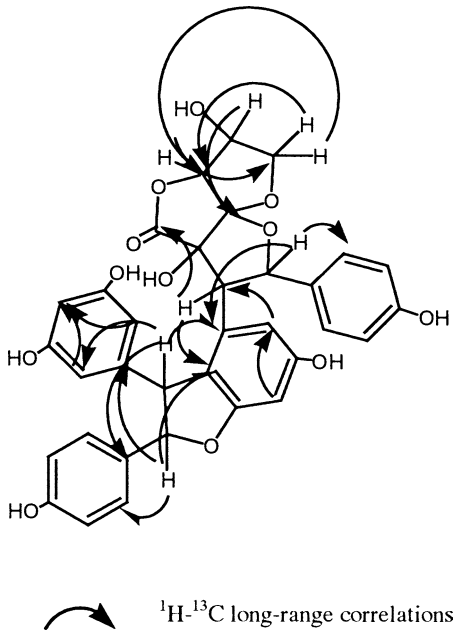
Determination of 5α-reductase inhibitory activity

The standard reaction mixture, in a final volume of 0.3 ml, contained microsomes, 150 μM testosterone in 10 μl ethanol containing 0.7 μM [4-^{14}C] testosterone, 167 μM NADPH, and medium A, with or without the indicated amount of a sample in 10 μl of dimethylsulfoxide (DMSO). The amount of the enzyme fraction was adjusted to set the rate of conversion of testosterone to DHT at around 70%. The reaction was started by adding microsomes to the preheated reaction solution in a tube. After 10 min the incubation was terminated by adding 10 μl of 3 M NaOH. To extract metabolites, 1 ml of diethyl ether was added, and the tubes were capped and shaken. The organic phase was applied to a silica plate (Kieselgel 60 F_{254}), and the plate was developed in ethyl acetate-*n*-hexane (7:3) at room temperature. The radioactivity profile was determined with an imaging analyzer (Fuji Film Co.).

Results and discussion

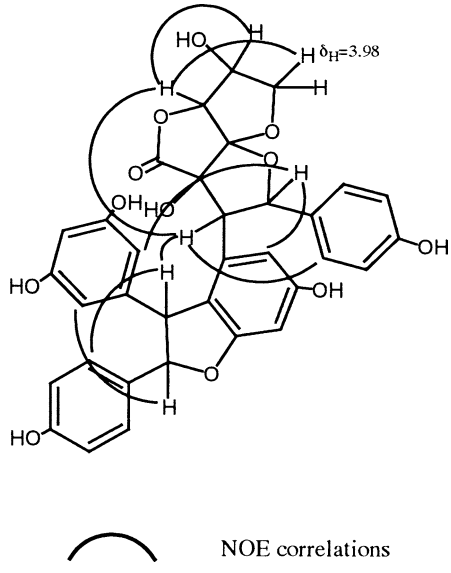
One-dimensional NMR and FAB-MS measurements of compound **1** indicated the molecular formula to be $C_{34}H_{28}O_{12}$ and showed a specific rotation of −91.4°. The FAB-MS spectral data revealed a molecular ion $[M\text{-}H]^-$ peak at *m/z* 627. The ^{1}H-NMR spectrum showed four sets of AX-type hydrogen at δ_H 6.71 (6H, m), 6.92 (2H, d, J = 8.5); one set of AX_2-type hydrogen δ_H 5.86 (2H, d, J = 2.0), 6.14 (t, J = 2.0); *meta*-coupled aromatic hydrogen δ_H 6.17 (d, J = 2.0), 7.13 (brs); and eight aliphatic signals. Of those, the COSY spectrum showed that the signal at δ_H 3.22 (d, J = 11.0) correlated with that at δ_H 5.29 (d, J = 11.3), and the signal at δ_H 3.15 (brd, J = 7.1) correlated with that at δ_H 5.03 (d, J = 8.0). These spectrum data indicated that this compound has a dimeric stilbene skeleton as part of its structure. Complete assignment of all protons and carbons were confirmed by HMQC. The remaining four aliphatic proton signals and the attached carbon signals were in good agreement with the signal pattern of bicyclic ascorbic acid or its derivative data.[14] Significant correlations determined by HMBC of H-7 with C-2 and C-9; H-8 with C-1, C-14, C-10, and C-1″; H-7′ with C-2′, C-9′, and C-10; and H-8′ with C-1′, C-9, and C-10/14 established that the planar structure can be drawn as laevifonol, as shown in Fig. 2. The configurations between H-7 and H-8, and that between H7′ and H-8′ given by their coupling constants are proof of the *trans*-conformation. The relative configuration of laevifonol, which can be drawn as shown in Fig. 3, resulted in NOE interactions between H-2/6 and H-8, H-7 and H-14, H-7 and H-10′/14′, H-8 and H-4″, H-8 and H-8′, H-2′/6′ and H-8′, H-7′ and H-10′/14′, and H-4″ and H-5″. The biosynthesis of this compound is throught to involve the oxidative cross-coupling of ascorbic acid with a dimeric stilbene, (−)-ε-viniferin.

One-dimensional NMR and FAB-MS measurements of **2** indicated the molecular formula to be $C_{34}H_{32}O_{12}$ and showed specific rotation of −57.1°. The FAB-MS spectral data revealed a molecular ion $[M\text{-}H]^-$ peak at *m/z* 631. The ^{1}H- and ^{13}C-NMR spectra showed that this compound contains the β-glucopyranosyl moiety, and the spectrum of aglycon moiety showed similarities to those of (−)-ampelopsin A. Compared with the ^{13}C-NMR spectrum data of (−)-ampelopsin A, which was also isolated from *S. laeviforia*, downfield shifts were observed at 2.9 ppm for C-10, 1.3 ppm for C-12, 0.6 ppm for C-13, and 1.4 ppm for C-14.



^{1}H-^{13}C long-range correlations

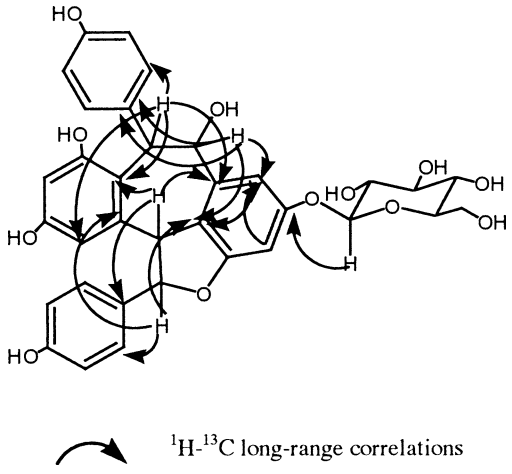
Fig. 2. Significant heteronuclear multiple bond correlation (HMBC) spectrum correlations of **1**



NOE correlations

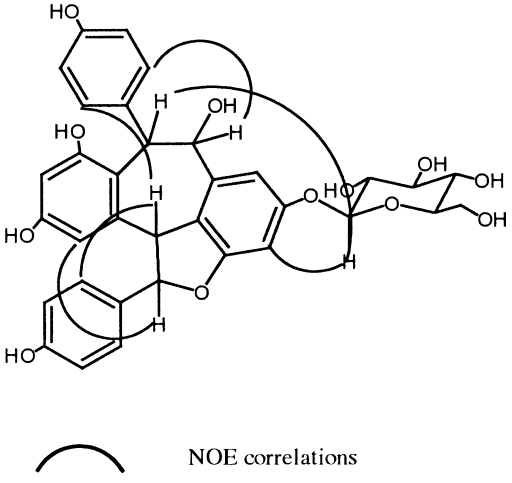
Fig. 3. Significant nuclear overhauser effect spectroscopy (NOESY) spectrum correlations of **1**. *NOE*, nuclear overhauser effect

An HMBC correlation of anomeric proton with C-13 was established (Fig. 4). The absolute configuration of the sugar moiety was determined to be β-D-glucopyranoside, evaluated by derivation of methyl 2-(polyhydroxyalkyl)-thiazolidine-4(*R*)-carboxylate; and ^{1}H-NMR data of the aglycone was in accord with that of (−)-ampelopsin A. These data indicated that the planar structure of laevifoside was ampelopsin A-13-*O*-β-D-glucopyranoside. The relative configuration of laevifoside, which can be drawn as shown in Fig. 5, resulted in NOE interactions between H-8 and H-2/6, H-8′ and H-3′/5′, H-7′ and H-14′, H-1″ and H-12, and H-1″ and H-7.



^{1}H-^{13}C long-range correlations

Fig. 4. Significant HMBC spectrum correlations of **2**



NOE correlations

Fig. 5. Significant NOESY spectrum correlations of **2**

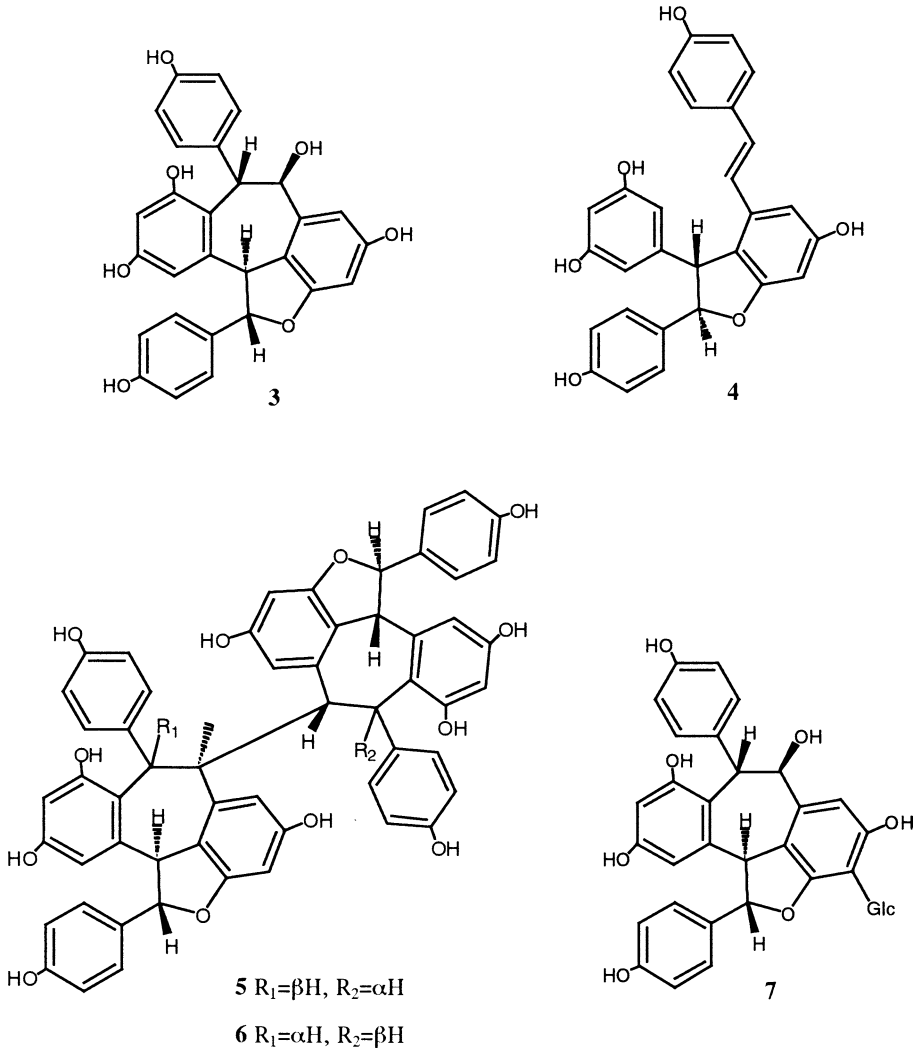
Table 1. IC_{50} values of stilbenoids isolated from *S. laeviforia* against rat liver 5α-reductase

Compound	IC_{50} (μm)
Laevifonol (**1**)	>>796.0
Laevifonoside (**2**)	>>791.0
(−)-Ampelopsin A (**3**)	48.8
(−)-ε-Viniferin (**4**)	20.1
(−)-Hopeaphenol (**5**)	78.2
(+)-Isohopeaphenol (**6**)	102.4
Hemsleyanoside (**7**)	>>791.0

Five known stilbenoids – **3**,[8] **4**,[9] **5**,[10] **6**,[11] and ampelopsin A-12-*C*-β-glucopyranoside **7**[12] – were also isolated from *S. laeviforia* (Fig. 6). Many stilbenoids have been reported from *Shorea* species.[12,15–20] These five known compounds have already been isolated from *Shorea* species, but there have been no reports about 5α-reductase inhibitory activity of these stilbenoids.

Rat liver 5α-reductase inhibitory activity was evaluated using rat liver microsomes as a 5α-reductase crude enzyme. The inhibitory activities of the oligostilbenes **3**, **4**, **5**, and **6**,

Fig. 6. Known stilbenoids from *S. laeviforia*



were recognized as the concentration of 50% inhibition (IC_{50}) values of 48.8, 20.1, 78.2, and 102.4μM, respectively (Table 1). Brief structure–activity relations among dimers were described in a previous paper.[7] Inhibitory activity of tetramers **5** and **6** was weak compared with that of the dimers. On the other hand, a comparison of the inhibitory activities of ampelopsin A glucopyranosides (**2**, **7**) with that of their aglycon (**3**) revealed no inhibitory activity of **2** and **7**, whereas positive inhibitory activity was revealed in compound **3**. Furthermore, compounds **1**, **2**, and **7**, which exhibit no inhibitory activity, have a hydrophilic moiety as part of their structure: glucoside or ascorbate. These results suggest that the hydrophilic moiety in these compounds may inhibit action with the hydrophobic active site of the enzyme. Though inhibitory activity was not shown in the novel compounds from *S. laeviforia*, it is possible that laevifoside acts as a prodrug in vivo, as the inhibitory activity of its aglycon, ampelopsin A, is relatively strong.

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