Chemical Constituents from the Roots and Rhizomes of *Sophora tonkinensis* and Their Effects on Proprotein Convertase Substilisin/Kexin Type 9 Expression

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ABSTRACT: This study was conducted to further investigate bioactive molecules from *Sophora tonkinensis* that can inhibit proprotein convertase substilisin/kexin type 9 (PCSK9) expression. After interpreting NMR spectroscopic data and MS spectral data of all isolates, a new naturally occurring compound, 6-hydroxy-vitexin-2″-*O*-rhamnoside (**7**), was identified along with 30 known compounds. The calculation of the gauge-including atomic orbital (GAIO) and electronic circular dichroism (ECD) proposed the absolute configuration of **17** as (2*S*,3*R*)-methyl-2-(4-hydroxybenzyl)tartrate by comparing the calculated ECD with experimental data. All isolates were tested for their inhibitory effects on PCSK9 mRNA expression. Of the tested compounds, (+)-isolariciresinol (**12**) inhibited PCSK9 expression via down-regulation of HNF1α and SREBPs.
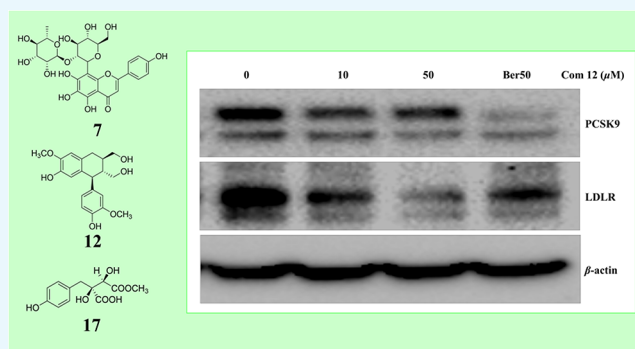
■ INTRODUCTION

Sophora tonkinensis Gapnep. (Leguminosae) is widely distributed in China, Korea, and Vietnam. Its dried roots and rhizomes, known as "Shan-Dou-Gen" in China, have been used in traditional herbal medicine to treat abdominal pain, asthma, fever, throat inflammation, tumors, dermatitis, gastrointestinal hemorrhage, and throat swelling.[1−4] Secondary metabolites such as prenylated flavonoids[5−7] and alkaloids[8−10] have been reported from *S. tonkinensis*. Additionally, various pharmacological studies have documented the 5AMP-activated protein kinase activation ability,[5] anti-inflammatory,[11,12] antiviral,[9] and cytotoxic activities[10] of *S. tonkinensis*.

A previous study reported that prenylated flavonoids can reduce the expression of proprotein convertase substilisin/kexin type 9 (PCSK9), which facilitates the degradation of the low-density lipoprotein receptor (LDLR), hindering cellular uptake of cholesterol.[5] Since the decrease of LDLR on the cell surface due to PCSK9-mediated LDLR degradation hampers the lowering of cholesterol levels, inhibition of PCSK9 expression may be advantageous for lowering blood cholesterol levels. Several natural products such as berberine and curcumin inhibit PCSK9 expression and lower cholesterol levels.[13] Hence, to discover diverse natural products that could inhibit PCSK9 expression, further investigation on *S. tonkinensis* was conducted. In this study, the structure elucidations of one previously undescribed flavonoid glycoside (**7**) and 30 known compounds from the ethanolic extract of *S. tonkinensis* have

been reported (Figure S1). In addition, the stereochemistry of the known structure **17** was proposed using gauge-including atomic orbital (GAIO) and electronic circular dichroism (ECD) calculations, and the effects of all isolated compounds on PCSK9 and LDLR mRNA expression were evaluated.

■ RESULTS AND DISCUSSION

The molecular formula of **7** ($C_{27}H_{30}O_{15}$) was deduced from the protonated ion peak at *m/z* 595.1678 $[M + H]^+$ from the HRESIMS data. The ^1H NMR data of **7** displayed the signals of a 1,4-disubstituted aromatic ring system at δ_H 8.00 (2H, d, *J* = 8.8 Hz, H-2′ and 6′) and 6.94 (2H, d, *J* = 8.8 Hz, H-3′ and 5′), one olefinic proton signal at δ_H 6.60 (1H, s, H-3), and two anomeric proton signals at δ_H 5.03 (1H, d, *J* = 9.9 Hz, H-1) and 5.09 (1H, brs, H-1‴). The ^{13}C NMR spectroscopic data of **7** exhibited 24 carbon signals that were derived from a ketone carbon at δ_C 182.7 (C-4), 1,4-disubstituted aromatic ring carbons at δ_C 161.4 (C-4′), 134.0 (C-2′/6′), 122.1 (C-1′), and 115.8 (C-3′/5′), one aromatic ring at δ_C 163.7 (C-7), 161.3
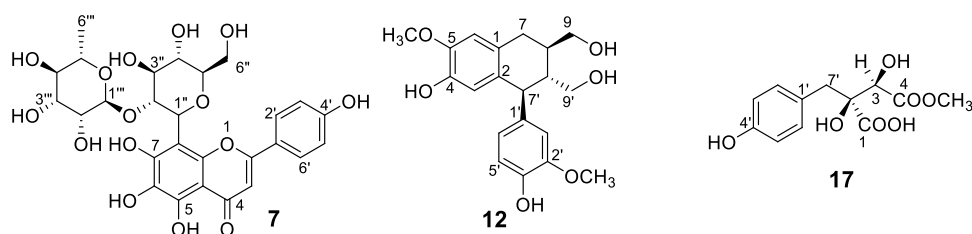
Figure 1. Representative compounds **7**, **12**, and **17** isolated from *S. tonkinensis*.

(C-5), 156.7 (C-9), 135.4 (C-6), and 104.4 (C-8/10), one *C*-glucopyranose at 72.3 (C-1″), 76.7 (C-2″), 80.2 (C-3″), 71.0 (C-4″), 81.4 (C-5″), and 61.5 (C-6″), one *O*-rhamnose at 101.1 (C-1‴), 73.7 (C-4‴), 70.8 (C-2‴), 70.4 (C-3‴), 68.5 (C-5‴), and 16.6 (C-6‴).

Based on the ¹H and ¹³C-NMR spectroscopic data, the structure of **7** was assumed to be similar to that of vitexin 2″-rhamnoside (**9**),[17,18] except for the disappearance of one aromatic proton signal at C-6 in **9**. In addition to this difference, when comparing the molecular formula of **7** with that of **9**, one additional oxygen atom was present in **7**, and thus, a hydroxy group was assumed to be attached to C-6 (Figure 1).

Further HMBC correlations of δ_H 5.09 (H-1‴) to δ_C 76.7 (C-2″), 70.8 (C-2‴), and 68.5 (C-5‴) and δ_H 4.26 (H-2″) to δ_C 101.1 (C-1‴) suggested that α-rhamnose was connected to C-2″ in the glucose. The HMBC correlations of δ_H 5.03 (H-1″) to δ_C 163.7 (C-7), 156.7 (C-9), 104.4 (C-8), and 76.7 (C-2″) indicated that the glucose was attached to C-8 (Figure 2).

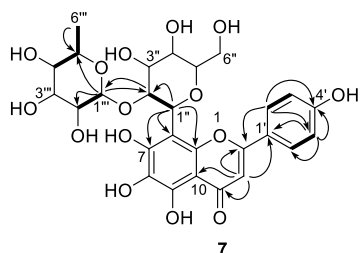**Figure 2.** Key HMBC correlations (→) and ¹H-¹H COSY correlations; (—) of compound **7**.

HPLC analysis using an arylthiocarbamoyl-thiazolidine derivative revealed that the absolute configuration of rhamnose was L-form (Figure S15B). Therefore, the structure of compound **7** was elucidated as scutellarein-8-*C*-[α-L-rhamno-pyranosyl-(1 → 2)]-β-D-glucopyranoside, and it was named 6-hydroxy-vitexin-2″-*O*-rhamnoside.

The ¹H and ¹³C NMR data of **17** [[α]$_D$²⁰ = +23.5 (*c* 0.74, MeOH)] were almost identical to those of (2*R*,3*S*)-methyl-2-(4-hydroxybenzyl)tartrate.[19] However, the absolute configuration of (2*R*,3*S*)-methyl-2-(4-hydroxybenzyl)tartrate was suggested by simply comparing its optical rotation value [[α]$_D$²⁵ = +46.9 (*c* 0.12, MeOH)] with a similar compound, piscidic acid.[20] In order to resolve the absolute configuration of **17**, the NMR chemical shifts calculations were performed by the GIAO method for DP4⁺ analysis for the possible structures **17A** (2*R*, 3*S*) and its diastereomer, **17C** (2*R*, 3*R*). As a result, structure **17A** (2*R*, 3*S*) showed the highest probability of 99.95% compared to structure **17C** (0.05%) (Figure S13). Furthermore, the calculated ECD curves of enantiomer structures **17A** (2*R*, 3*S*) and **17B** (2*S*, 3*R*) were compared with experimental data. As shown in the result (Figure 3), the calculated ECD curve of the structure **17B** (2*S*, 3*R*) exhibited a similar pattern to that of the experimental data.[21] From these results, compound **17** was determined to be (2*S*,3*R*)-methyl 2-(4-hydroxybenzyl)tartrate (Figure 3).

The structures of 29 known compounds were confirmed as bersimoside I (**1**),[22] abrisaponin L (**2**),[23] kuzusapogenol A methyl ester (**3**),[24] subproside I methyl ester (**4**),[25] subproside IV methyl ester (**5**),[26] subproside I (**6**),[25] sophoraflavone A (**8**),[27] vitexin-2″-*O*-rhamnoside (**9**),[17] bayin (**10**),[6] lyoniresinol (**11**),[28] (+)-isolariciresinol (**12**),[29] maltol-*O*-β-D-glucopyrano-side (**13**),[30] licoagroside B (**14**),[31] 5″-methyl-licoagroside B (**15**),[31] oxymatrine (**16**),[32] dimethyl (2*S*,3*R*)-(+)-2-(4-hydroxybenzyl)tartrate (**18**),[20] 2-(4-hydroxybenzyl) tartrate (**19**),[33] 4-methyl eucomate (**20**),[34] dimethyl eucomate (**21**),[34] 5-methyluracil (**22**),[35] uracil (**23**),[35] 1,4-dihydroxy-2-methoxybenzene 4-*O*-β-D-glucopyranoside (**24**),[36] 4-hydroxybenzoic acid 4-*O*-β-D-glucopyranoside (**25**),[37] gallic acid (**26**),[38] 4-hydroxybenzoic acid (**27**),[39] trimethyl citrate (**28**),[40] 3-hydroxy-3-methyl-1,5-dimethyl ester (**29**),[41] ethyl-β-D-gluco-pyranoside (**30**),[42] and 1-*O*-methyl-inositol (**31**)[43] (Figure S1)
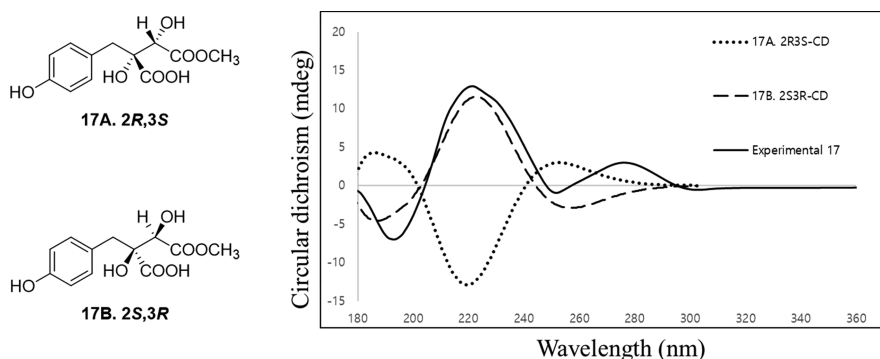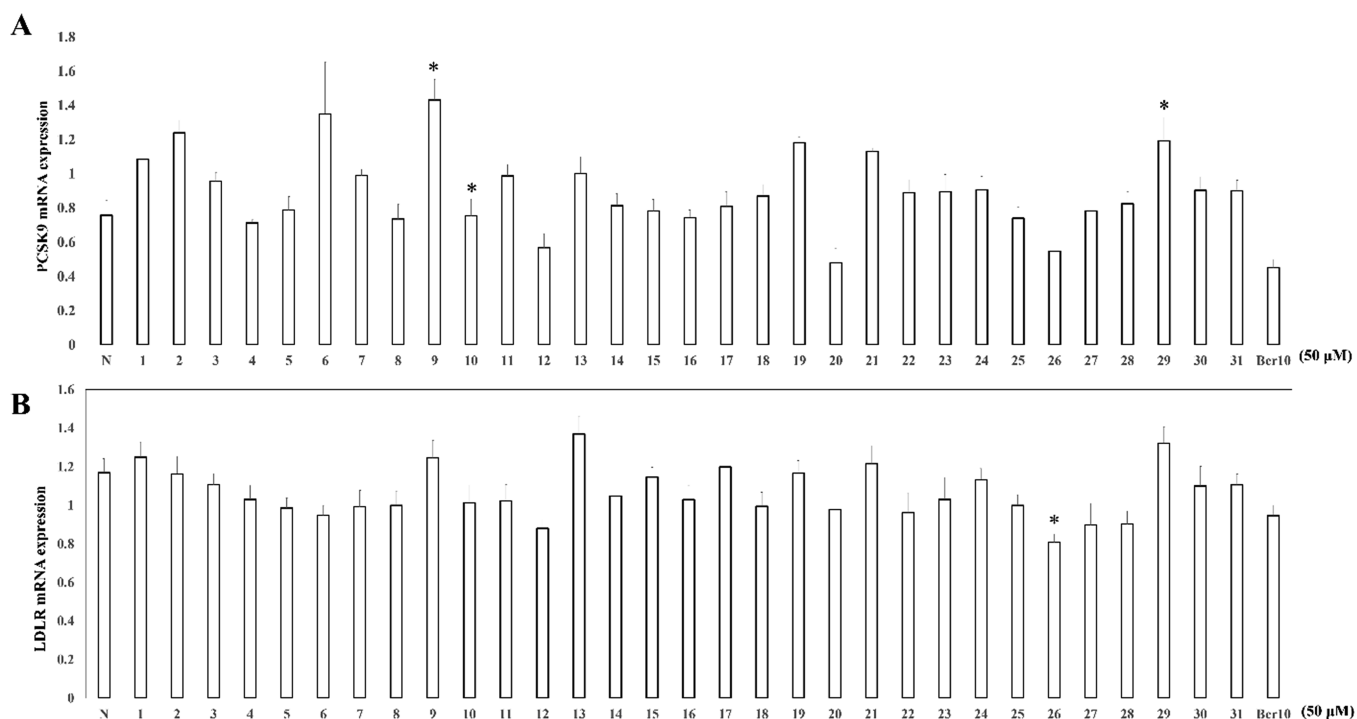

Figure 3. Comparisons of calculated ECD spectra of **17A** and **17B** and experimental ECD curve of **17**.

Figure 4. Effects of compounds **1−31** on the PCSK9 and LDLR mRNA expressions in the HepG2 cells. (A) Expression of PCSK9 mRNA was assayed by qRT-PCR in cells treated with compounds **1−31** (50 μM), and berberine·HCl 10 μM (Ber10) for 24 h. (B) Expression of LDLR mRNA was assayed by qRT-PCR in cells treated with compounds **1−31** (50 μM), and berberine·HCl 10 μM (Ber10) for 24 h. *$p < 0.01$ as compared to the non-treated group by Dunnett's t test.
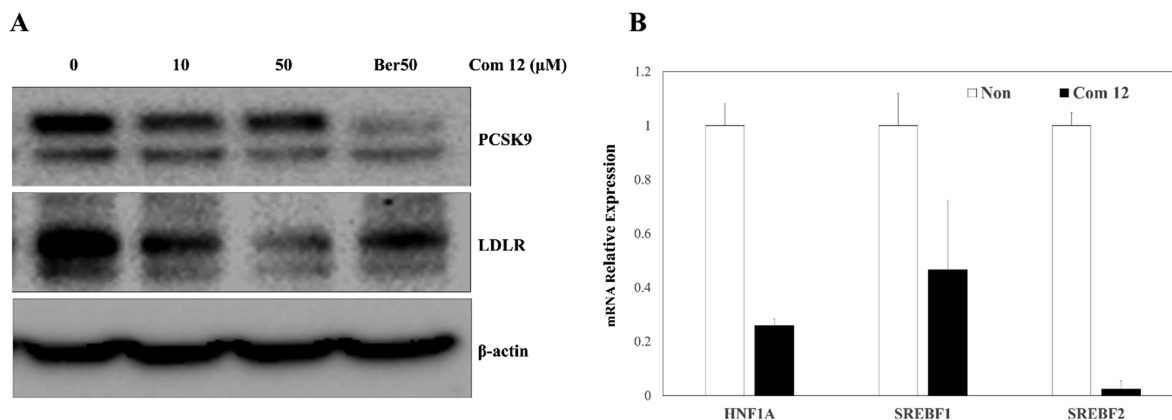
Figure 5. Effect of (+)-isolariciresinol (**12**) on the PCSK9 and LDLR protein expressions and HNF1αSREBF1 and SREBF2 in the HepG2 cells. (A) Protein expression of PCSK9, LDLR, and β-actin was assayed by western blotting in cells treated with (+)-isolariciresinol (**12**) for 24 h. (B) Effect of transcription factor changes in HepG2 cells of (+)-isolariciresinol (**12**) (50 μM) was confirmed by qRT-PCR. *$p < 0.05$ as compared to the non-treated group by Dunnett's t test.

by comparing the measured spectroscopic data with the published values.

To examine the effects of all compounds (**1−31**) on PCSK9 and LDLR expression, qRT-PCR was performed in HepG2 cells (Figure 4A), and the results indicated that PCSK9 expression was significantly downregulated by (+)-isolariciresinol (**12**) as compared to the vehicle.

Next, the effects of (+)-isolariciresinol (**12**) on PCSK9 and LDLR protein expression in HepG2 cells were tested. In western blotting, PCSK9 protein expression was suppressed by (+)-isolariciresinol (**12**). Additionally, LDLR protein was decreased by 10 and 50 μM (+)-isolariciresinol (**12**) (Figure 5A). According to previous studies, lignan-type compounds, such as sauchinone and schinlignan D, inhibit PCSK9

expression and concomitantly increase LDLR expression, similar to the effect of berberine, a positive control,[43,44] but (+)-isolariciresinol (**12**) used in the present study inhibited both PCSK9 and LDLR expression. As documented, PCSK9 transcription is regulated by transcriptional factors such as HNF1α and SREBPs.[45−47] The upregulation of SREBP transcriptional factor activates both PCSK9 and LDLR transcription. In the case of (+)-isolariciresinol (**12**), both HNF1α and SREBP mRNA expressions were downregulated (Figure 5B), partially explaining the suppression of PCSK9 and LDLR protein expression.

In the present study, a new flavonoid C-glycoside, 6-hydroxy-vitexin-2″-O-rhamnoside (**7**), and 30 known compounds were isolated. Among the active compounds in the

PCSK9 expression inhibitory assay, (+)-isolariciresinol (12) could potentially inhibit PCSK9 expression via downregulation of the transcriptional factors HNF1α and SREBPs. These data support the possibility that (+)-isolariciresinol (12) can be used to lower cholesterol levels. However, further *in vivo* studies assessing the concentrations of LDL cholesterol and PCSK9 in the plasma and liver may be required to support the potential of this compound as a cholesterol lowering agent.

■ EXPERIMENTAL SECTION

General Experimental Instruments. Nuclear magnetic resonance (NMR) was measured on a Varian 400 spectrometer (Varian, CA, USA) and a Varian 800 spectrometer (Varian, CA, USA). High-resolution mass spectrometric data were obtained from a Waters Xevo G2 Q-TOF mass spectrometer (Waters, MA, USA). The measurements of ECD and UV spectra were performed using a Chirascan Plus circular dichroism spectrometer at 180 to 400 nm with the scanning rate at 1 nm (APL, Surrey, UK) using a 1 mm cuvette (Hellma, Jena, Germany), methanol was used to set the baseline after the records of all samples, and finally the data were smoothed by the Savitzky−Golay method with a smooth window size of 10 points. Fourier transform infrared (FT-IR) on a ThermoFisher Scientific, NicoletTM iSTM 5 FT-IR spectrometer (ThermoFisher Scientific, Madison, WI, USA) was used. Semi-preparative high-performance liquid chromatography (HPLC) was performed on a Gilson 321 pump and Gilson 172 diode array detector (Gilson, Madison, WI, USA) with HPLC columns [YMC-pack Ph, 250 × 20 mm] or [YMC-pack Ph, 250 × 10 mm] (YMC, Kyoto, Japan). Water purified by a Milli-Q system (Waters Corporation, Milford, MA, USA) was used. A Sephadex LH-20 (GE Healthcare, Uppsala, Sweden) C-18 reversed-phase silica gel and a silica gel (Cosmosil, Kyoto, Japan) were used for column chromatography. Silica gel 60F$_{254}$ plates (Merck, Darmstadt, Germany) were used for TLC analysis, and a spraying agent of 10% aqueous H$_2$SO$_4$ was used to visualize the spots.

Cell Culture and Chemical Reagents. The HepG2 human hepatocellular liver cell line (provided by the Korea Research Institute of Bioscience and Biotechnology, South Korea) was cultured according to the previous publication.[48] Eagle's minimum essential medium, penicillin, streptomycin of Hyclone (Logan, UT, USA), and bovine serum albumin from Sigma-Aldrich (St. Louis, MO, USA) were used for cell culture. Antibodies (PCSK9, LDLR, and β-actin) were purchased from Abcam, Inc. (Cambridge, MA, USA). Primers for PCSK9, LDLR, HNF1a, SREBF1, SREBF2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Bioneer Corp. (Daejeon, South Korea). Solvents for extraction and isolation (ethanol, methanol, chloroform, etc.) were provided by SK Chemicals (Seoul, Korea). HPLC-grade solvents, acetonitrile and methanol (SK Chemicals, Seoul, South Korea), were used for HPLC. NMR solvents were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

Extraction and Isolation Method. The roots and rhizomes of *S. tonkinensis* were collected in September 2009 from Mashan City, Guanxi Province of the People's Republic of China. A voucher specimen (SKC04) was deposited at Herbarium of Medicinal Plant Garden, College of Pharmacy, Seoul National University, Republic of Korea.

The raw materials (600.0 kg) were extracted twice with 4200 L of 50% ethanol at a temperature of 80 °C. The crude extract was evaporated and partitioned with *n*-butanol to afford 24.0 kg of butanol-soluble fraction. The butanol-soluble fraction (500.0 g) was chromatographed on Diaion HP-20 using MeOH−H$_2$O as the solvent system (50:50, 70:30, 90:10, and 100:0) and then finished with acetone to give five fractions (SK50, SK70, SK90, SK100, and SKA) (Ahn et al., 2019).[5]

SK50 (64.0 g) was chromatographed over Diaion HP-20 using MeOH−H$_2$O as the solvent system (20:80, 30:70, 40:60, and 50:50) and then 100% acetone to give 5 subfractions (SK50-20, SK50-30, SK50-40, SK50-50, and SKA). SK50-20 (15.6 g) was chromatographed on a silica gel column using a gradient mixture of chloroform and MeOH (100:0 to 3:1) and then 100% MeOH, to give 15 subfractions (SK50-20-1 to SK50-20-15). SK50-20-5 (116.8 mg) was subjected to HPLC (MeOH−H$_2$O, 35:65, 3 mL/min, isocratic elution mode), affording 23 (t_R 18.4 min, 2.9 mg) and 24 (t_R 19.5 min, 2.9 mg). SK50-20-10 (1.3 g) was separated using normal-phased MPLC silica gel-column chromatography (100 g) with a gradient mixture of *n*-hexane−EtOAc solvent system (90:10 to 0:100), giving two pure compounds 17 (7.4 mg) and 30 (9.2 mg) as well as 7 subfractions (SK50-20-10A to SK50-20-10G). The aforementioned MPLC separation method was used for the purification of SK50-20-11 (2.7 g), which provided 16 subfractions (SK50-20-11A to SK50-20-11P). SK50-20-11B (99.6 mg) was chromatographed on Sephadex LH-20 using 100% MeOH, affording 7 subfractions (SK50-20-11B1 to SK50-20-11B7) including 18 (25.5 mg) and 27 (4.4 mg). Compound 25 (5.3 mg) was obtained from the separation of SK50-20-11G (235.1 mg) by MPLC RP-C$_{18}$ silica gel-column chromatography (50 g) using a binary solvent system of MeOH−H$_2$O (5:95 to 40:60). SK50-20-13 (5.2 g) was chromatographed on a silica gel column using a gradient solvent of chloroform-MeOH (20:1 to 3:1) to give 7 subfractions (SK50-20-13A to SK50-20-13G). SK50-20-13C (2.1 g) was further purified by Sephadex LH-20 column chromatography (100% MeOH), yielding 4 subfractions (SK50-20-13C1 to SK50-20-13C4) including 13 (2.5 mg). SK50-20-13D (1.7 g) was purified by MPLC RP-C$_{18}$ silica gel-column chromatography (100 g) with a solvent mixture of MeOH−H$_2$O (5:95 to 60:40) to give 3 subfractions (SK50-20-13D1 to SK50-20-13D3). SK50-20-13D2 (1.1 g) was separated by silica column chromatography using chloroform−MeOH (10:1 to 1:1), giving 6 subfractions (SK50-20-13D2A to SK50-20-13D2F) along with 31 (9.5 mg). Compounds 26 (4.4 mg) and 16 (12.3 mg) were isolated from SK50-20-13E (886.5 mg) using Sephadex LH-20 (100% MeOH).

The fraction SK50-30 (10.2 g) was chromatographed on a silica gel column using *n*-hexane:EtOAc (90:10 to 0:100) followed by a gradient mixture of chloroform−MeOH (10:1 to 1:1) and finally fractionated into 17 subfractions (SK50-30-1 to SK50-30-17). SK50-30-3 (124.3 mg) was applied to an HPLC (MeOH−H$_2$O, 33:67, 3.0 mL/min, isocratic elution) to yield 28 (t_R 25.3 min, 2.0 mg). Compounds 14 (t_R 19.1 min, 6.6 mg), 10 (t_R 20.36 min, 14.4 mg), and 7 (t_R 22.2 min, 4.6 mg) were isolated from a semi-preparative HPLC separation (MeOH−H$_2$O, 20:80 to 70:30, 2.0 mL/min, gradient elution) of SK50-30-12 (521.3 mg). SK50-30-14 (325.9 mg) was purified by HPLC using a gradient solvent of MeOH−H$_2$O (20:80, 2.0 mL/min) for 15 min and then MeOH−H$_2$O (30:70, 2.0 mL/min) for 20 min to obtain 8 (t_R 35.4 min, 10.0 mg).

The fraction SK50-40 (5.9 g) was chromatographed on a silica gel column using n-hexane−EtOAc (10:1 to 3:1) and then chloroform−MeOH (30:1 to 3:1) to obtain 16 subfractions (SK50-40A to SK50-40P). SK50-40A (45.2 mg) was purified by HPLC (MeCN-H_2O, 70:30, 2.0 mL/min, isocratic elution) to yield **30** (t_R 23.2 min, 1.8 mg). Compound **29** (t_R 18.5 min, 25.0 mg) was furnished from HPLC separation (MeCN−H_2O, 15:85, 2.0 mL/min, isocratic elution) of SK50-40C (85.0 mg). From subfractions SK50-40F (69.3 mg) and SK50-40H (72.0 mg), **22** (t_R 15.0 min, 1.8 mg) and **19** (t_R 19.6 min, 15.7 mg) were separated by HPLC purification (MeCN−H_2O, 20:80, 2.0 mL/min, isocratic elution). SK50-40J (859.3 mg) was separated on a Sephadex LH-20 column using 100% MeOH, giving 11 subfractions (SK50-40J1 to SK50-40J11), along with **11** (2.4 mg) and **12** (1.1 mg). Nine subfractions (SK50-40M1 to SK50-40M9), including **15** (16.4 mg), were separated from SK50-40M (523.1 mg) by a Sephadex LH-20 column using 100% MeOH. Further, SK50-40M7 (224.3 mg) was purified by HPLC (MeCN-H_2O, 15:85, 2.0 mL/min, isocratic elution), affording **21** (t_R 14.1 min, 4.4 mg) and **20** (t_R 17.5 min, 10.9 mg). From SK50-40N (615.3 mg), compound **9** (t_R 17.5 min, 4.1 mg) was isolated by Sephadex LH-20 (100% MeOH) purification followed by HPLC (MeCN-H_2O, 10:90, 2.0 mL/min, isocratic elution).

SK70 (38.5 g) was chromatographed by MPLC NP-C_{60} silica gel-column chromatography (300 g), with a gradient mixture of EtOAc−MeOH (70:30 to 0:100) providing 40 subfractions (SK70-1 to SK70-40). SK70-19 (12.4 g) was applied to a MPLC RP-C_{18} (100 g) with MeOH−H_2O (45:55 to 100:0), giving 15 subfractions (SK70-19-1 to SK70-19-15). SK70-19-12 (15.0 mg) was purified by HPLC (MeCN-H_2O, 65:35, 2.0 mL/min, isocratic elution), yielding **4** (t_R 27.5 min, 1.3 mg). SK70-37 and SK70-38 (8.8 g) were subjected to an RP silica gel C_{18}-MPLC with MeOH−H_2O (20:80 to 100:0) to give 20 subfractions (SK70-3738-1 to SK70-3738-20). Compounds **2** (t_R 26.3 min, 2.4 mg) and **3** (t_R 29.8 min, 3.4 mg) were isolated from SK70-3738-7 (97.6 mg) by using HPLC (MeCN−H_2O, 15:85, 2.0 mL/min, isocratic elution). From SK70-3738-9 (90.5 mg), **1** (t_R 19.5 min, 7.0 mg) was purified by an HPLC method (MeCN−H_2O, 15:85, 2.0 mL/min, isocratic elution). SK70-3738-18 (100.0 mg) was purified by HPLC (MeCN-H_2O, 22:78, 2.0 mL/min, isocratic elution), yielding **5** (t_R 12.1 min, 10.5 mg) and **6** (t_R 14.6 min, 8.0 mg).

ECD Calculations. Three 3D structures (**A**−**C**) from ChemBio3D Ultra13.0 in which the energies were minimized at the MMFF94 level were used for conformational analyses in Spartan'16 software. All conformers (7 conformers for each) were optimized at the mPW1PW91 function with 6-311+G (d, p) basis set by the density functional theory (DFT) method (Tables S3−S7). All of the optimized structures were checked to ascertain the absence of imaginary frequencies. The NMR chemical shifts of diastereomer structures **17A** (2R, 3S) and **17C** (2R, 3R) were calculated by the GIAO method using mPW1PW91/6-311+G (d, p) with PCM mode and methanol as the solvent. The ECD calculations of enantiomer structures **17A** (2R, 3S) and **17B** (2S, 3R) were also conducted at the mPW1PW91/6-311+G, (d, p) level using the TDDFT method with the CPCM model using methanol as the solvent with the consideration of 50 excitations in the Gaussian 16 software. Lastly, the calculated ECD curves were plotted using Boltzmann population-weighted calculation. Additionally, the calculated UV curves of the calculated structures were compared with the experimental **17** (Figure S14).

Sugar Analysis of Compounds 1 and 7. Compounds **1** and **7** (2.0 mg each) were dissolved in 2 N HCl (2 mL) and heated in a water bath at 90 °C for 2 h. The hydrolyzed product was dried using a stream of N_2. The dried residue was treated with pyridine (0.1 mL) and L-cysteine methyl ester hydrochloride (0.7 mg) and heated at 60 °C for 1 h. Then, a solution of o-tolyl isothiocyanate in pyridine (0.2 mL) was added to the reaction mixture and then was heated at 60 °C for 1 h. The authentic sugars, D-glucuronic acid, D-galactose, D-glucose, and L-rhamnose, were derivatized as the aforementioned method. Finally, all solutions were filtered and subjected to HPLC using the YMC-Pack-ODS-A column (250 × 4.6 mm, 5 μm) with MeCN−H_2O (25:75), 0.8 mL/min, by isocratic for 40 min and MeCN 100% for 5 min. The absolute configuration of sugars in each compound was established by comparison of the retention times with those of the authentic sugar derivatives.[14−16] The obtained retention time of authentic sugar derivatives were as follows: D-galactose derivative at t_R 13.98 min; D-glucuronic acid derivative at t_R 15.09 min; D-glucose derivative at t_R 15.74 min; and L-rhamnose derivative at t_R 24.30 min. (Figure S15).

Quantitative Real-Time RT-PCR. Total cellular RNA was isolated using a Trizol RNA extraction kit according to the manufacturer's instructions and previous publication.[48]

Immunoblot Analysis. The protein expression was assessed by western blotting according to previous publication.[49]

6-Hydroxy-vitexin-2″-O-rhamnoside (7). Yellowish powder, $[\alpha]_D^{20} = -55.1$ (c 0.46, MeOH); UV (MeOH) λ_{max} (log ε): 212.0 (3.19), 254.0 (2.81), 331.0 (3.08) nm; CD (MeOH) λ_{max} ($\Delta\varepsilon$): 228.0 (+ 0.23), 250.0 (−0.35), 280.0 (+ 0.13) nm. FT-IR (ATR) v_{max}: 3388, 1649, 1573, 1352, 1238, 1041 cm^{-1}. ^{1}H-NMR (CD$_3$OD, 400 MHz): δ_H 8.00 (2H, d, J = 8.8 Hz, H-2′ and 6′), 6.94 (2H, d, J = 8.8 Hz, H-3′ and 5′), 6.60 (1H, s, H-3), 5.03 (1H, d, J = 9.9 Hz, H-1″), 5.09 (1H, brs, H-1‴), 4.26 (1H, t, J = 9.5 Hz, H-2″), 3.95 (1H, d, J = 12.0 Hz, H-6a″), 3.84 (1H, brs, H-3‴), 3.79 (1H, dd, J = 12.0, 5.9 Hz, H-6b″), 3.62 (2H, overlapped, H- 3″ and 4″), 3.45 (1H, m, H-5″), 3.41 (1H, dd, J = 9.7, 3.3 Hz, H-2‴), 3.11 (1H, t, J = 9.5 Hz, H-4‴), 2.46 (1H, qu, J = 7.2 Hz, H-5‴), 0.65 (3H, d, J = 6.1 Hz). ^{13}C-NMR (CD$_3$OD, 100 MHz): δ_C 182.7 (C-4), 165.2 (C-2), 163.7 (C-7), 161.4 (C-4'), 161.3 (C-5), 156.7 (C-9), 135.4 (C-6), 134.0 (C-2'/6'), 122.1 (C-1'), 115.8 (C-3'/5'), 104.4 (C-8/10), 102.1 (C-3), 101.1 (C-1‴), 81.4 (C-5″), 80.2 (C-3″), 76.7 (C-2″), 73.7 (C-4‴), 72.3 (C-1″), 71.0 (C-4″), 70.8 (C-2‴), 70.4 (C-3‴), 68.5 (C-5‴), 61.5 (C-6″), 16.6 (C-6‴). HRESIMS m/z [M + H]$^+$ 595.1678 (calcd for $C_{27}H_{31}O_{15}$ 595.1663).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c01676.

> The structures of compounds **1**−**31** of *S. tonkinensis*, UV, CD, HRESIMS, IR, 1D and 2D NMR spectra for compounds **7**, and **12**, DP4$^+$ analysis, calculated and experimental ECD and UV, and sugar analysis (PDF)

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Notes

The authors declare no competing financial interest.

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