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New Aromatic Compounds from the Fruiting Body of *Sparassis crispa* (Wulf.) and Their Inhibitory Activities on Proprotein Convertase Subtilisin/Kexin Type 9 mRNA Expression

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Supporting Information

ABSTRACT: Successive chromatography of EtOAc-soluble extracts of the fruiting body of *Sparassis crispa* (Wulf.) resulted in isolation of four new aromatic compounds, sparoside A (**1**) and sparalides A−C (**3**−**5**), two new naturally occurring compounds, **2** and **6**, and eight known compounds, **7**−**14**. The chemical structures were determined by interpretation of nuclear magnetic resonance and mass spectrometry spectroscopic data. Extract, solvent-soluble fractions of the extract, and all of the pure compounds isolated from the fractions were subjected to the mRNA expression assay for proprotein convertase subtilisin/kexin type 9 (PCSK9). Among them, sparoside A (**1**), hanabiratakelide A (**8**), adenosine (**11**), and 5α,6α-epoxy-(22*E*,24*R*)-ergosta-8(14),22-diene-3β,7β-diol (**14**) exhibited potent inhibitory activities on PCSK9 mRNA expression, with IC_{50} values of 20.07, 7.18, 18.46, and 8.23 μM, respectively (berberine, positive control, IC_{50} = 8.04 μM), suggesting that compounds **1**, **8**, **11**, and **14** are suitable for use in supplements to the statins for hyperlipidemia treatments.

KEYWORDS: *Sparassis crispa, proprotein convertase subtilisin/kexin type 9 (PCSK9), phthalide, sparoside A*

INTRODUCTION

Sparassis crispa (Wulf.) is an edible/medicinal mushroom belonging to the family of Sparassidaceae and is called "cauliflower mushroom" as a result of its appearance. The mushroom, which mostly grows on the stubs of coniferous trees, is distributed throughout northern temperature zones of the world.[1] The fruiting bodies of *S. crispa* produce various bioactive substances, including β-glucan,[2] benzoate derivatives,[3] sesquiterpenoids,[4] and maleic acid derivatives.[5] In particular, β-glucan is a major constituent, present in more than 40% of *S. crispa*. They have been demonstrated to show a variety of pharmacological activities, including antitumor,[6−8] hematopoietic response-enhancing,[9] wound-healing,[10] antimetastatic, antihypertensive, and antidiabetic effects.[11]

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is noted to interfere with the function of low-density lipoprotein receptor (LDLR) on the liver cell surface that transports low-density lipoprotein cholesterol (LDL-C) into the liver for metabolism, leading to high levels of LDL-C. Thus, the PCSK9 inhibitor was proposed to be a new LDL-C-lowering agent. When the PCSK9 inhibitor was used in combination with a statin, known as a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor, it has been shown to dramatically lower LDL-C levels by up to 60%. Therefore, the PCSK9 inhibitor has recently emerged as a new strategy to treat hyperlipidemia. To date, two PCSK9 inhibitors (evolocumab and alirocumab) have been approved by the U.S. Food and Drug Administration (FDA) to treat familial hypercholesterolemia, and several PCSK9 inhibitors are currently under clinical trial.[12] In addition, several natural compounds, such as berberine and curcumin, have been reported to inhibit PCSK9 mRNA expression.[13] For these reasons, more investigation is required to discover new PCSK9 inhibitors, which could be good supplements to statin treatment as a result of their effects on PCSK9 mRNA.

The extracts of the cauliflower mushroom were reported to exhibit antihypertensive and antidiabetic activities related to hyperlipidemia. To our knowledge, there are no reports on effects of cauliflower mushroom and its constituents on PCSK9 mRNA expression. We therefore examined the effects of the extracts and pure compounds on PCSK9 mRNA expression to see if their hypocholesterolemic effects could be partly explained by additional effects on PCSK9 mRNA expression. We found that some compounds from the cauliflower mushroom extracts inhibit the PCSK9 mRNA expression. Therefore, we herein report the isolation of compounds, their

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Table 1. ^{1}H and ^{13}C NMR Data of Compounds 1 and 3−5

	1[a]		3[b]		4[b]		5[a]	
number	δ_C	δ_H multi (J in Hz)	δ_C	δ_H multi (J in Hz)	δ_C	δ_H multi (J in Hz)	δ_C	δ_H multi (J in Hz)
1	111.09		168.89		168.90		172.14	
2	156.45							
3	137.22		68.98	5.28 s	66.79	5.09 s	70.46	5.15 s
3a			151.68		124.99		142.32	
4	154.57		100.49	6.82 s	135.44		104.67	6.68 s
5	112.34	6.66 s	166.11		139.19		155.49	
6	137.40		102.59	6.81 s	139.30		139.62	
7			156.23		141.94		147.44	
7a			107.05		106.25		109.50	
1′	102.64	5.73 d (4.4)	101.17	5.95 d (3.8)				
2′	73.82	4.32 dd (4.5, 6.4)	71.74	4.10 dd (4.0, 5.8)				
3′	71.23	4.10 dd (2.8, 6.5)	69.77	3.89 d (5.6)				
4′	88.46	4.16 m	87.99	4.00 dd (3.6, 5.8)				
5′	63.30	3.70 dd (3.4, 12) 3.65 dd (3.9, 12)	61.48	3.45 m				
OCH_3	61.63	3.85 s	56.19	3.85 s	61.60	3.79 s	62.65	4.01 s
CH_3	23.28	2.43 s						
$\underline{C}OOCH_3$	172.81							
$COO\underline{C}H_3$	52.68	3.93 s						

[a]Measured in CD_3OD. [b]Measured in DMSO-d_6.

structural determination, and their PCSK9 inhibitory activities from the extract of *S. crispa*.

■ MATERIALS AND METHODS

General Experimental Procedures. The high-resolution electrospray ionization mass spectrometry (HRESIMS) data were obtained on an ultrahigh-resolution electrospray ionization quadrupole time-of-flight (UHR ESI Q-TOF) mass spectrometer (Bruker, Billerica, MA). The nuclear magnetic resonance (NMR) spectra were acquired with a 300 Ultra shield spectrometer (^{1}H, 300 MHz; ^{13}C, 75 MHz, Bruker), a NMR system 500 MHz (^{1}H, 500 MHz; ^{13}C, 125 MHz, Varian, Palo Alto, CA), and a DD2 700 spectrometer (^{1}H, 700 MHz; ^{13}C, 175 MHz, Agilent Technologies, Santa Clara, CA) using the solvent signals (δ_H 2.50/δ_C 39.51 for dimethyl sulfoxide (DMSO)-d_6; δ_H 3.31/δ_C 49.15 for CD_3OD, Cambridge Isotope Laboratories, Inc., Tewksbury, MA) as internal standards; chemical shifts are indicated as δ values. Analytical high-performance liquid chromatography (HPLC) was carried out on a 1260 infinity HPLC system (Agilent Technologies) supplied with a G1311C quaternary pump, a G1329B autosampler, a G1315D photodiode array (PDA) detector, and a G1316A oven for the column. The column used was a 150 × 4.6 mm inner diameter, 5 μm, ZORBAX SB-C18 (Agilent Technologies). Semi-preparative HPLC was operated on a 600 controller (Waters, Milford, MA) with a 996 PDA detector using the column ZORBAX SB-C18 (250 × 21.2 mm inner diameter, 5 μm, Agilent Technologies). Column chromatography was operated over silica gel 60 (70−230 mesh, Merck, Darmstadt, Germany). Silica gel 60 F_{254} and RP-18 F_{254S} plates (Merck) were used for analysis by thin-layer chromatography (TLC) under detection of ultraviolet (UV) and 10% H_2SO_4 reagent to visualize the compounds. The analytical grade of solvents was used for the whole experiments.

Plant Material. Dried fruiting bodies of *S. crispa* were provided by Gyeongshin Bio Co. (Euiwang, South Korea) in August 2016. This sample was botanically identified by the corresponding author (Sang Hee Shim). A voucher was deposited at the pharmacognosy laboratory of the College of Pharmacy, Duksung Women's University (specimen NPC-16-08).

Extraction and Isolation. Dried fruiting bodies of *S. crispa* (1 kg) were extracted with 100% MeOH (3.0 L) under reflux 3 times to afford 153.0 g of the extracts. The extracts were suspended in distilled water (1.0 L) and partitioned using *n*-hexane (3 × 1.0 L), CH_2Cl_2 (3 × 1.0 L), EtOAc (3 × 1.0 L), and *n*-BuOH (3 × 1.0 L), consecutively, yielding *n*-hexane (17.6 g), CH_2Cl_2 (2.7 g), EtOAc (9.8 g), *n*-BuOH (15.0 g), and H_2O (107.9 g) layers, respectively. The EtOAc-soluble layer (9.8 g) was set apart by vacuum liquid chromatography (VLC, 40 × 9 cm) over silica gel using gradient solvents of *n*-hexane/EtOAc/MeOH (10:1:0, 2.5:1:0, 1.5:1:0, and 1:1:0.2; each 5 L), $CHCl_3$/MeOH/H_2O (10:1:0 and 5:1:0.1; each 5 L), and 100% MeOH (3 L) to obtain seven fractions (fractions E1−E7). Silica gel column chromatography was employed to fraction E2 with the elution of *n*-hexane/acetone gradient solvents (20:1 and 15:1; each 0.2 L) to afford compound **2** (8.6 mg). Fraction E4 (1.4 g) was fractionated on silica gel column chromatography (15 × 8 cm) with gradient solvents of $CHCl_3$/acetone (65:1, 20:1, 10:1, and 7:1; each 1 L) and 100% MeOH (1 L) to afford six fractions (fractions E4-1−E4-6). Fraction E4-1 was further purified with reversed-phase HPLC using a H_2O/acetonirile (60:40 → 45:55, v/v) gradient to yield compounds **12** (15 mg), **13** (18.5 mg), and **14** (1.5 mg). Compounds **1** (2.4 mg), **5** (2.9 mg), **6** (1.7 mg), **7** (1.8 mg), **8** (5.6 mg), **9** (7.3 mg), and **10** (4.1 mg) were obtained from fraction E5 using reversed-phase HPLC with a gradient of H_2O/MeOH (80:20 → 0:100, v/v). Fraction E6 was subjected to reversed-phase HPLC with gradient solvents of H_2O/MeOH (95:5 → 50:50, v/v) to furnish compounds **3** (13.0 mg), **4** (6.5 mg), and **11** (2.9 mg).

*Sparoside A (2-Hydroxy-3-methoxy-6-methylbenzoic Acid Methyl Ester 4-O-α-D-Riboside, **1**):* yellowish amorphous solid; (+) HRESIMS *m/z*, 367.1003 $[M + Na]^+$ (calcd for $C_{15}H_{20}NaO_9$, 367.1000); ^{1}H and ^{13}C NMR, see Table 1; and heteronuclear multiple-bond correlations (HMBCs, CD_3OD, H → C), H-5 → C-1, C-3, C-4, and CH_3; H-1′ → C-4, C-2′, C-3′, and C-4′; H-2′ → C-1′; H-3′ → C-1′, C-2′, C-4′, and C-5′; H_2-5′ → C-3′ and C-4′; CH_3 → C-5 and C-6; OCH_3 → C-3; and $COOC\underline{H}_3$ → $\underline{C}OOCH_3$.

*Sparalide A (5-Methoxyphthalide 7-O-α-D-Riboside, **3**):* white amorphous powder; (+) HRESIMS *m/z*, 335.0738 $[M + Na]^+$ (calcd for $C_{14}H_{16}NaO_8$, 335.0737); ^{1}H and ^{13}C NMR, see Table 1; and HMBCs (DMSO-d_6, H → C), H-3 → C-1, C-3a, C-4, C-5, and C-7a; H-4 → C-3, C-5, C-6, C-7, and C-7a; H-6 → C-1, C-4, C-5, C-7, and C-7a; H-1′ → C-3′, C-4′, and C-7′; H-3′ → C-1′; H-4′ → C-3′; H_2-5′ → C-3′ and C-4′; and OCH_3 → C-5.

*Sparalide B (6-Methoxy-4,5,7-trihydroxyphthalide, **4**):* yellow amorphous solid; (+) HRESIMS *m/z*, 235.0212 $[M + Na]^+$ (calcd for $C_9H_8NaO_6$, 235.0213); ^{1}H and ^{13}C NMR, see Table 1; and

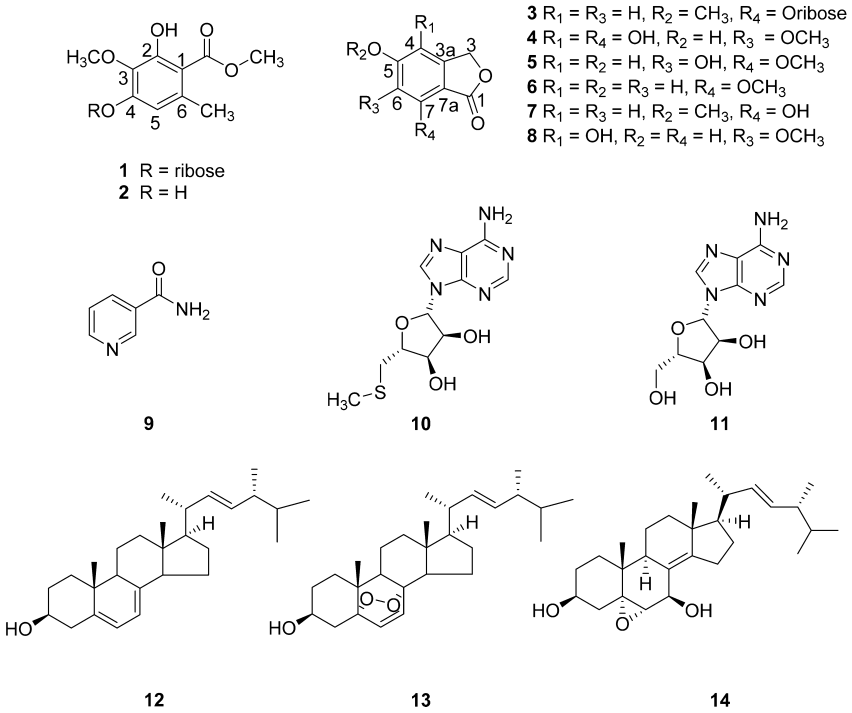


Figure 1. Structures of compounds **1**−**14** from *S. crispa*.

HMBCs (DMSO-d_6, H → C), H_2-3 → C-1, C-3a, C-4, C-5, C-7, and C-7a; and OC$\underline{H}_3$ → C-6.

Sparalide C (5,6-Dihydroxy-7-methoxyphthalide, **5***):* colorless amorphous solid; (+) HRESIMS m/z, 219.0268 $[M + Na]^+$ (calcd for $C_9H_8NaO_5$, 219.0264); ^{1}H and ^{13}C NMR, see Table 1; and HMBCs (CD_3OD, H → C), H_2-3 → C-1, C-3a, C-4, C-5, and C-7a; H-4 → C-3, C-5, C-6, and C-7a; and OC$\underline{H}_3$ → C-7.

Acid Hydrolysis of Compounds 1 and 3. Each 1 mg of compounds **1** and **3** was hydrolyzed with 1 N HCl (1 mL) at 80 °C for 2 h to afford aglycone and sugar moieties. The reaction mixtures were extracted with EtOAc to separate a sugar moiety-containing aqueous fraction from the aglycone-containing fraction. The aqueous fraction was evaporated and then analyzed on silica gel TLC plates with a gradient of acetone/H_2O for the comparison to authentic D-ribose and L-ribose (Sigma-Aldrich, St. Louis, MO).

Determination of the Absolute Configuration of Ribose. To determine the absolute configuration of ribose in compounds **1** and **3**, derivatives of the sugar moieties were analyzed.[14] Acid hydrolysis of each compound afforded aglycone and sugar moieties. The sugar moieties (each 0.5 mg) were dissolved in pyridine (100 μL), supplemented with L-cysteine methyl ester hydrochloride (0.5 mg), and placed at 60 °C for 1 h for reaction. A total of 10 μL of *o*-tolyl isothiocyanate was then added to the mixture to allow for a reaction at 60 °C for 1 h. The mixture was evaporated and subjected to reverse-phase HPLC for analysis, with a ZORBAX SB-C18 column (Agilent Technologies, 250 × 4.6 mm inner diameter, 5 μm), column temperature of 35 °C, mobile phase of H_2O/acetonitrile (75:25, v/v) for 30 min, flow rate at 0.8 mL/min, and detection wavelength at 250 nm. Both authentic D-ribose and L-ribose were reacted in the same manner as described above. The absolute configuration of ribose in compounds **1** and **3** was assigned by comparing their retention times to those of the authentic derivatives (t_R: D-ribose derivative, 12.68 min; L-ribose derivative, 8.20 min).

PCSK9 mRNA Expression Assay. The RNA extraction procedure provided by TRIzol (Life Technologies, Carlsbad, CA) was employed for total RNA extraction. In brief, cDNA was prepared by adding reverse transcriptase (200 units) and oligo-dT primer (500 ng) to total RNA (1 μg) in 50 mM Tris−HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), and 1 mM dNTPs at 42 °C for 1 h. Incubation of the solution at 70 °C for 15 min terminated the reaction, and an aliquot of the cDNA mixture (1 μL) was adopted for enzyme amplification. Polymerase chain reactions were conducted by cDNA (1 μL), master mix (9 μL) containing iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), 5 pmol of forward primer, and 5 pmol of reverse primer using a CFX384 real-time polymerase chain reaction (PCR) system (Bio-Rad) with the following conditions: 3 min at 95 °C, subsequently 40 cycles for 10 s at 95 °C, then 30 s at 55 °C, and finally plate reading. The fluorescence, which was generated using SYBR Green I DNA dye, was quantified in the course of the annealing. Specificity of the amplification was ascertained through a melting curve analysis. CFX Manager Software (Bio-Rad) was used for acquisition of data, which were presented as the cycle threshold (C_T). Then, relative abundance of an interesting gene was standardized to that of glyceraldehyde 3-phosphate dehydrogenase ($\Delta\Delta C_T$). The $2^{-(\Delta\Delta C_T)}$ method[15] was employed for calculation of mRNA abundance of the sample. Specific primer sets used in this study were as follows (5′ → 3′): GAPDH, GAAGGTGAAGGTCGGAGTCA (forward) and AATGAAGGGGTCATTGATGG (reverse); PCSK9, GGGCATTTCACCATTCAAAC (forward) and TCCAGAAAGCTAAGCCTCCA (reverse). Custom-synthesized gene-specific primers were provided by Bioneer (Daejeon, Korea).

Statistical Analyses. Data were expressed as the mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) determined the level of statistical significance, and Dunnett's *t* test was used for multiple comparison procedures. *p* values (calculated probability) less than 0.05 were regarded to be significant.

■ RESULTS AND DISCUSSION

Structural Elucidation. A series of chromatographic methods carried out on the extract of *S. crispa* led to the isolation of 14 compounds, which include four new aromatic compounds, **1** and **3**−**5**, two new naturally occurring compounds, **2** and **6**, and eight known compounds, **7**−**14** (Figure 1).

The known compounds were identified to be methyl 2,4-dihydroxy-3-methoxy-6-methylbenzoate (**2**),[16] 5-hydroxy-7-methoxyphthalide (**6**),[17] 5-methoxy-7-hydroxyphthalide (**7**),[17] hanabiratakelide A (**8**),[18] nicotinamide (**9**),[19] 5′-deoxy-5′-methylthioadenosine (**10**),[20] adenosine (**11**),[21] ergosterol

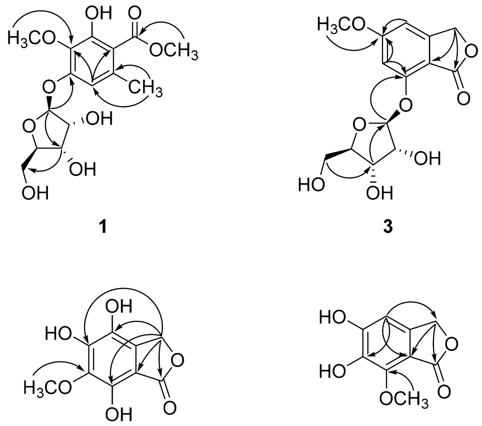
(**12**),[22] ergosterol peroxide (**13**),[23] and 5α,6α-epoxy-(22*E*,24*R*)-ergosta-8(14),22-diene-3β,7β-diol (**14**)[24] by comparing their NMR and mass spectrometry (MS) data to those in the reference. Of these, although compounds **2** and **6** have been reported as synthetic intermediates, they have been reported for the first time in nature in this study.

Compound **1** was obtained as a yellowish amorphous solid. Positive HRESIMS suggested its molecular formula to be $C_{15}H_{20}O_9$. The ^{1}H NMR spectrum of compound **1** displayed an aromatic proton at δ_H 6.66 (1H, s, H-5), one sugar unit at δ_H 5.73−3.65, two methoxyl groups at δ_H 3.93 (3H, s, COO$\underline{C}H_3$) and 3.85 (3H, s, 3-OCH$_3$), and a methyl group at δ_H 2.43 (3H, s, 6-CH$_3$). The ^{13}C NMR spectrum of compound **1** suggested existence of a carbonyl group (δ_C 172.81), six aromatic carbons (δ_C 156.45, 154.57, 137.40, 137.22, 112.34, and 111.09), one sugar unit (δ_C 102.64, 88.46, 73.82, 71.23, and 63.30), two methoxyl groups (δ_C 61.63 and 52.68), and a methyl group (δ_C 23.28). One sugar unit was confirmed to consist of a ribose by ^{1}H and ^{13}C NMR data, which was further supported by chemical reaction. Acid hydrolysis of compound **1** followed by TLC with authentic ribose supported that the sugar was ribose. Moreover, the 4.4 Hz of coupling constant for the anomeric proton at δ_H 5.73 suggested an α configuration. HMBC of the methoxyl protons at δ_H 3.93 with the carbonyl carbon at δ_C 172.81 indicated that methyl carboxylate was attached to the aromatic ring. The positions of the methoxyl, methyl group, and ribose at the aromatic ring were assigned by analysis of the HMBC spectrum. HMBCs of the methoxyl protons at δ_H 3.85 with the aromatic carbon at δ_C 137.22 and the anomeric proton at δ_H 5.73 with the aromatic carbon at δ_C 154.57 allowed for the assignment of the carbons bearing the methoxyl group and ribose. HMBC of the methyl protons at δ_H 2.43 with the aromatic methine carbon at δ_C 112.34 and the non-protonated aromatic carbon at δ_C 137.40 and the aromatic methine proton at δ_H 6.66 with the carbons at δ_C 111.09, 137.22, and 154.57 indicated the methyl, methyl carboxylate, methoxyl, and ribose were attached to the C-6, C-1, C-3, and C-4 positions, respectively. Determination of the absolute configuration of ribose was conducted by comparing the retention time of L-cysteine methyl ester and *o*-tolyl isothiocyanate derivative of acid hydrolysate to those for authentic D-/L-ribose derivatives in HPLC−UV. The derivatives of authentic D-ribose and L-ribose eluted at t_R of 12.68 and 8.20 min, respectively, on isocratic HPLC. Because the derivative of compound **1** eluted at t_R of 11.93 min, ribose in compound **1** was confirmed to have D configuration. Thus, the structure of compound **1** was determined to be 2-hydroxy-3-methoxy-6-methyl benzoic acid methyl ester 4-*O*-α-D-riboside and was named as sparoside A.

Compound **3** was obtained as a white amorphous powder. Its positive HRESIMS data suggested the molecular formula to be $C_{14}H_{16}O_8$. The ^{1}H NMR spectrum displayed two aromatic methine protons at δ_H 6.82 (1H, s, H-4) and 6.81 (1H, s, H-6), an oxymethylene at δ_H 5.28 (2H, s, H-3), one sugar unit at δ_H 5.95−3.45, and a methoxyl group at δ_H 3.85 (3H, s, 5-OCH$_3$). The ^{13}C NMR spectrum of compound **3** suggested the presence of a carbonyl group (δ_C 168.89), six aromatic carbons (δ_C 166.11, 156.23, 151.68, 107.05, 102.59, and 100.49), one pentose moiety (δ_C 101.47, 87.99, 71.74, 69.77, and 61.48), an oxymethylene (δ_C 68.98), and a methoxyl carbon (δ_C 56.19). It was presumed to be a bicyclic compound to meet seven unsaturations obtained from its molecular formula. The presence of an aromatic ring, an oxymethylene group, and carboxyl carbon indicated that compound **3** has a phthalide skeleton, which has previously been reported in *S. crispa*. Pentose was presumed to be ribose based on the carbon chemical shifts and proton resonances. The coupling constant (3.9 Hz) of the anomer proton suggested that ribose was attached to aglycone with an α configuration. The presence of ribose was verified by the direct comparison of acid hydrolysate of compound **3** with commercially available authentic ribose. The HMBCs of the oxymethylene at δ_H 5.28 with carboxyl carbon (δ_C 168.89) and aromatic carbon (δ_C 151.68 and 107.05) supported the idea that compound **3** had a γ-lactonylated aromatic compound, known as a phthalide. The singlet aromatic methine protons suggested that the methoxyl group and the ribose moiety were not attached to the adjacent carbon atoms of the aromatic ring. Their positions were confirmed by HMBC data. HMBCs of the anomeric proton (δ_H 5.95) with carbon (δ_C 156.23) and methoxyl protons (δ_H 3.85) with carbon (δ_C 166.11) indicated that ribose and methoxyl groups were attached to carbons at δ_C 156.23 and 166.11, respectively. HMBCs of the H-4 methine proton at δ_H 6.82 of phthalide with methoxylated carbon (δ_C 166.11), another aromatic methine carbon (δ_C 102.59), and non-protonated aromatic carbon (δ_C 107.05) indicated that the methoxyl group and ribose were attached to C-5 and C-7, respectively. Thus, a planar structure could be established. Assignment of the absolute configuration of ribose was conducted as described above. Because the retention time of the ribose derivative of acid hydrolysate for compound **3** was the same as that for the D-ribose derivative, the structure of compound **3** was determined as 5-methoxy-phthalide 7-*O*-α-D-riboside, named sparalide A.

Compound **4** was obtained as a yellow amorphous solid, in which its molecular formula was established as $C_9H_8O_6$ on the basis of positive HRESIMS. In its ^{1}H NMR spectrum, one oxymethylene and one methoxyl signal appeared at δ_H 5.09 (2H, s, H-3) and 3.79 (3H, s, 6-OCH$_3$), respectively. Six aromatic carbons (δ_C 141.94, 139.30, 139.19, 135.44, 124.99, and 106.25), an oxymethylene carbon (δ_C 66.79), and a carbonyl group (δ_C 168.90) shown in the ^{13}C NMR spectrum suggested that compound **4** had a phthalide skeleton, similar to compound **3**. The position of the methoxyl group was assigned to be attached to C-6 on the ground, in which the methoxyl protons (δ_H 3.79) showed HMBCs with C-6 (δ_C 139.31), while the oxymethylene protons (δ_H 5.09) showed HMBCs with C-7 (δ_C 141.94), C-5 (δ_C 139.19), C-4 (δ_C 135.44), C-3a (δ_C 124.99), and C-7a (δ_C 106.25) (Figure 2). $^4J_{CH}$ HMBCs between H$_2$-3 and C-5 and C-7 in addition to $^3J_{CH}$ HMBCs between H$_2$-3 and C-4 ascertained that the methoxyl group was attached to C-6 rather than C-4, C-5, or C-7. From these evidence, the structure of compound **4** was determined to be 6-methoxy-4,5,7-trihydroxyphthalide, named sparalide B.

Compound **5** was a colorless amorphous solid, and the molecular formula was confirmed to be $C_9H_8O_5$ by positive HRESIMS. The ^{1}H NMR spectrum of compound **5** showed an aromatic proton at δ_H 6.68 (1H, s, H-4), an oxymethylene at δ_H 5.15 (2H, s, H-3), and a methoxyl group at δ_H 4.01 (3H, s, 7-OCH$_3$). Its ^{13}C NMR spectrum exhibited six aromatic carbons (δ_C 155.49, 147.44, 142.32, 139.62, 109.50, and 104.67), an oxymethylene carbon (δ_C 70.46), a carboxyl carbon (δ_C 172.14), and a methoxyl carbon (δ_C 62.65). The ^{1}H and ^{13}C NMR data presented similarities to those for compound **4**, except for an additional aromatic proton at δ_H 6.68. Thus, it was assumed that two hydroxyl groups and a methoxyl group were attached to the aromatic ring of the phthalide skeleton,



1 **3** **4** **5**

Figure 2. Key HMBCs of compounds **1**, **3**, **4**, and **5**.

which was also supported by the observation of HRESIMS. The positions of aromatic methine carbon and methoxylated carbon were assigned to be C-4 and C-7, respectively, supported by HMBCs of H-4 (δ_H 6.68) with C-3 (δ_C 70.46), C-5 (δ_C 155.49), C-6 (δ_C 139.62), C-3a (δ_C 142.32), and C-7a (δ_C 109.50) and the methoxyl proton (δ_H 4.01) with C-7 (δ_C 147.44) (Figure 2). Using these data, the structure of compound **5** was established to be 5,6-dihydroxy-7-methoxyphthalide, named sparalide C.

Evaluation of PCSK9 mRNA Expression. The MeOH extract and polarity-based solvent-soluble layers of *S. crispa* were tested on inhibitory activity of PCSK9 mRNA expression using HepG2 cells. The EtOAc-soluble layer showed the most potent inhibitory activities at 10 μg/mL, which prompted us to elucidate the active compounds from the EtOAc-soluble layer (Figure 3). Bioactivity-guided fractionation led to isolation of 14 compounds, **1**−**14**, from this layer. All of the isolated compounds were assessed for their PCSK9 mRNA expression. The results demonstrated that compounds **1**, **8**, **11**, and **14** were found to potentially inhibit PCSK9 mRNA expression, with IC_{50} values of 20.07, 7.18, 18.46, and 8.23 μM, respectively, at the concentration of 20 μM, whereas the IC_{50} of berberine, positive control, was 8.04 μM at the same concentration as the isolated compounds. In comparison to the positive control, compound **8** (hanabiratakelide A) was found to be a stronger PCKS9 inhibitor than berberine, which is known to be one of the most potent PCSK9 inhibitors in nature thus far.

Concerning the structure−activity relationship, the results showed that the 4,5,6-trioxygenated pattern in the phthalide moiety seemed to be important for the PCSK9 inhibitory activity rather than the dioxygenation or tetraoxygenation pattern, as shown in compound **8**. In the case of simple benzoic acid derivatives (compounds **1** and **2**), the ribose moiety seemed to be important in the activity, as shown for compound **1**. The ribose moiety seemed also to play an important role in adenosine derivatives (compounds **10** and **11**), where the OH group at C-5 of the ribose moiety seemed to be effective rather than SCH_3. In the case of ergosterol derivatives, the OH group and epoxide substituents on the B ring seemed to be more effective than when they had an endoperoxide or a diene system.

The results suggest that hanabiratakelide A (**8**) and 5α,6α-epoxy-(22*E*,24*R*)-ergosta-8(14),22-diene-3β,7β-diol (**14**) as well as the extract of *S. crispa* could be good supplements to statins for the treatment of hyperlipidemia. Moreover, further studies regarding the mechanistic and *in vivo* efficacies for compounds **8** and **14** might be required.

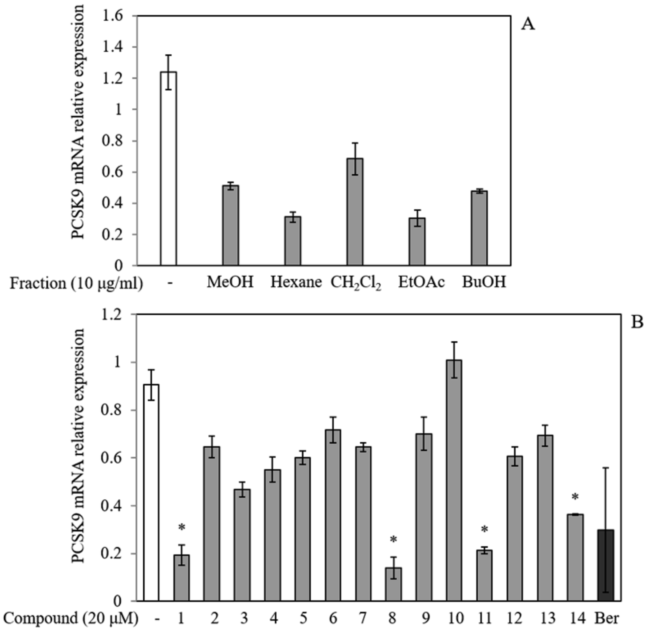


Figure 3. Effect of (A) MeOH extracts and solvent-soluble fractions of *S. crispa* and (B) isolated compounds from EtOAc-soluble fractions. Expression of PCSK9 mRNA was assayed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in cells treated with 10 μg/mL of solvent-soluble fractions and 20 μM isolated compounds.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b02657.

NMR and high-resolution mass spectrometry (HRMS) spectra of compounds **1** and **3**−**5** (PDF)

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Notes

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