Note

Sesquiterpenoids from the Rhizomes of *Curcuma phaeocaulis* and Their Inhibitory Effects on LPS-Induced TLR4 Activation

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Received January 26, 2016; accepted March 22, 2016

Two new guaiane-type (2, 6) and one new furanogermacrane-type (11) sesquiterpenoids have been isolated along with twelve known compounds from an EtOAc-soluble extract of *Curcuma phaeocaulis* rhizomes. The structures of the isolated compounds were elucidated using a combination of NMR, MS, and circular dichroism (CD) spectra. The inhibitory effects of each compound on lipopolysaccharide (LPS)-induced Toll-like receptor 4 (TLR4) activation in THP-1-Blue cells were assessed, and compound 4 showed more potent inhibitory activity against LPS-stimulated TLR4 activation.

Key words *Curcuma phaeocaulis*; Zingiberaceae; sesquiterpenoid; Toll-like receptor 4 (TLR4); lipopolysaccharide (LPS)

Curcuma phaeocaulis Valeton, a member of the family Zingiberaceae, is distributed across Korea, China, and Japan.[1)] Its rhizomes have been broadly prescribed as a traditional remedy for reducing blood stasis and alleviating pain symptoms.[2)] This plant has continually attracted attention from numerous scientists due to its various pharmacological activities, which include antiinflammatory,[3)] antioxidative,[4)] antitumor,[5)] and hepatoprotective effects.[6)] Prior phytochemical studies[4,7)] have indicated that *C. phaeocaulis* contains diverse sesquiterpenoids and diarylheptanoids that contribute to the pharmacological effects of its rhizomes.[7,8)]

Toll-like receptors (TLRs) have important functions in the innate immune system, which is responsible for eliminating infectious microorganisms, including bacteria, fungi, protozoa, and viruses.[9,10)] However, unregulated TLR stimulation is a pathogenic mechanism of autoimmune[11] and inflammatory diseases.[12)] In particular, TLR4 is recognized by lipopolysaccharide (LPS), which is found in the cell walls of Gram-negative bacteria. The activation of TLR4 leads to the induction of nuclear factor kappa B (NF-κB) signaling cascades that initiate the production of pro-inflammatory molecules, including tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, IL-8, and IL-12.[13)] This pathway is biologically important for regulating host homeostasis; however, the inability to regulate excessive TLR4 activation causes abnormal inflammatory responses.

Results and Discussion

In our ongoing studies of TLR4 inhibitors from traditional medicinal resources, we have determined that a 95% EtOH extract of *C. phaeocaulis* rhizomes has a half maximal inhibitory concentration (IC_{50}) value of 8.8 μg/mL. This extract was progressively fractionated with EtOAc, *n*-BuOH, and H_2O, and the EtOAc-soluble fraction was subjected to various chromatographic experiments, which allowed us to isolate new guaiane-type (**2**, **6**), and furanogermacrane-type (**11**) sesquiterpenoids, as well as twelve known compounds: procurcumenol (**1**),[14)] procurcumadiol (**3**),[14)] zedoarondiol (**4**),[15)] phaeocaulisine E (**5**),[7)] wenyujinin L (**7**),[16)] phacadinane B (**8**),[17)] curcumenolactone A (**9**),[6)] curcumenolactone B (**10**),[6)] zedoarofuran (**12**),[18)] curcolonol (**13**),[8)] 4α-hydroxy-8,12-epoxyeudesma-7,11-diene-1,6-dione (**14**),[19,20)] and neolitacumone A (**15**)[21)] (Fig. 1). These known compounds were elucidated by comparing their spectroscopic profiles with previously published data.

Compound **2** was isolated as an amorphous white powder. This compound's molecular formula of $C_{15}H_{22}O_3$ was deduced from high resolution-electrospray ionization (HR-ESI)-MS data (273.1461 $[M+Na]^+$, Calcd for 273.1467). Its IR spectrum included absorption bands for hydroxy (3412 cm^{-1}) and conjugated carbonyl (1650 cm^{-1}) groups. ^{1}H-NMR spectral data (Table 1) for **2** revealed signals from four methyl groups [δ_H 1.78 (s, H_3-12), 1.75 (t, J=1.2 Hz, H_3-13), 1.36 (s, H_3-14), and 1.95 (d, J=1.2 Hz, H_3-15)], one methine proton [δ_H 2.23 (dd, J=11.4, 4.8 Hz, H-5)], and one olefinic proton [δ_H 5.85 (d, J=1.2 Hz, H-9)].^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) data included 15 carbon signals produced by four methyl carbons (δ_C 22.6, 21.4, 26.6, 20.5), four olefinic carbons (δ_C 135.6, 130.2, 152.6, 137.8), three methylene carbons (δ_C 35.8, 39.1, 22.7), and one carbonyl carbon (δ_C 198.5). One dimensional (1D)-NMR spectroscopic data for **2** were highly similar to NMR data for 1,4-dihydroxy-7(11),9(10)-guaiandien-8-one, aerugidiol,[22)] which was isolated from *C. aeruginosa* in 1991. A guaiane skeleton that includes two hydroxyl groups and one conjugated ketone group was demonstrated by the H_3-14/C-4, H_3-15/C-1 and C-9, and H_3-12/C-8 heteronuclear multiple bond connectivity (HMBC) correlations (Fig. 2), suggesting that the planar structure of **2** was identical to that of aerugidiol. Similar nuclear Overhauser effect (NOE) correlations were observed between H_2-6α and H_3-14; between H_2-3α and H_3-14 for **2** and aerugidiol (Fig. 3); however, the optical rotation of **2** was estimated to be $[\alpha]_D^{20}$ +267.4 (aerugidiol: lit.[22)] $[\alpha]_D^{26}$ −17.0), and its circular dichro-

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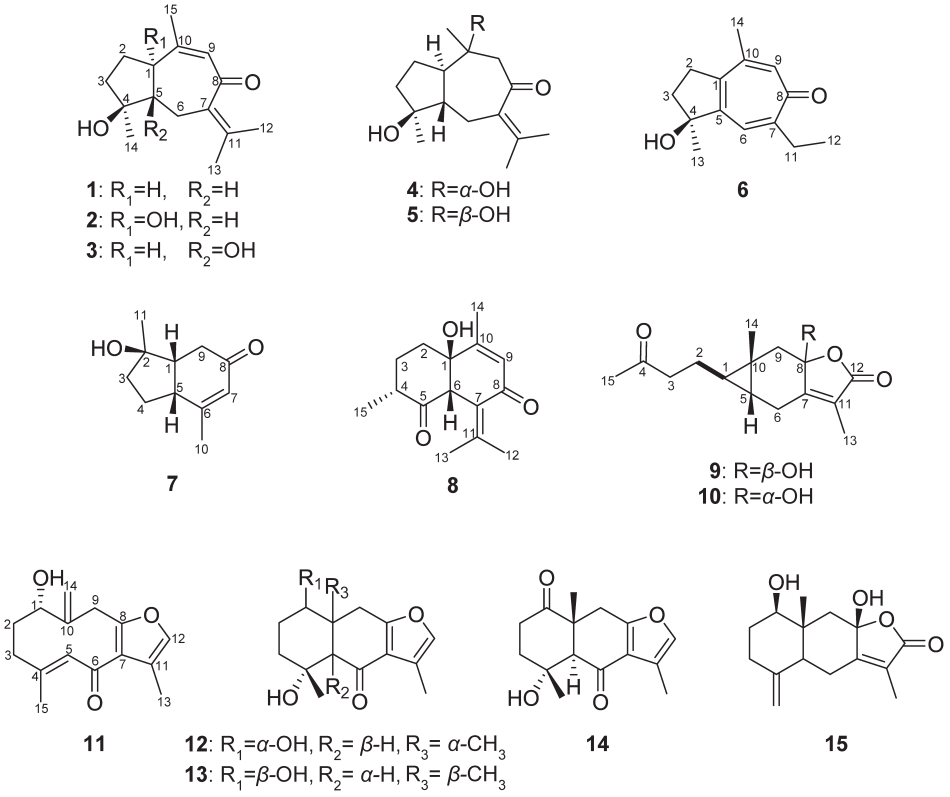

Fig. 1. Chemical Structures of the Isolated Compounds **1**–**15**

Table 1. ^{1}H- and ^{13}C-NMR Spectroscopic Data of Compounds **2**, **6**, and **11**[a)]

Position	**2**		**6**		**11**	
	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	83.2	—	147.5	—	77.0	4.20, dd (9.0, 3.6)
2	35.8	1.95, m	32.7	2.91, ddd (17.4, 9.0, 3.0)	34.6	2.19, m
				2.79, dt (17.4, 7.8)	—	2.08, m
3	39.1	2.00, m	40.1	2.19, ddd (12.6, 7.8, 3.0)	33.9	2.43 ddd (14.4, 7.2, 2.4)
		1.80, m		2.05, m	—	2.04, m
4	81.1	—	84.7	—	146.2	—
5	55.8	2.23, dd (11.4, 4.8)	152.3	—	128.8	6.40, s
6	22.7	2.46, br d (11.4)	133.6	7.50, s	194.6	—
		1.74, m			—	—
7	135.6	—	156.0	—	123.3	—
8	198.5	—	187.4	—	165.7	—
9	130.2	5.85, d (1.2)	140.1	7.06, s	33.3	3.80, d (17.4)
					—	3.50, d (17.4)
10	152.6	—	149.1	—	151.1	—
11	137.8	—	29.6	2.67, q (7.2)	123.1	—
12	21.4	1.78, s	13.9	1.19, t (7.2)	140.4	7.24, s
13	22.6	1.75, t (1.2)	27.6	1.42, s	10.2	2.18, d (0.6)
14	26.6	1.36, s	25.1	2.32, s	112.8	5.08, s
	—	—	—	—	—	4.48, s
15	20.5	1.95, d (1.2)	—	—	20.0	1.57, s

a) ^{1}H- and ^{13}C-NMR spectroscopic data were recorded at 600 and 150 MHz, respectively. The spectrum of compound **2** was obtained in $CDCl_3$, and the spectrum of compounds **6** and **11** were obtained in CD_3OD.

ism (CD) cotton effects, which were observed at 239 nm ($\Delta\varepsilon$ −33.2), 279 nm ($\Delta\varepsilon$ +15.7), and 346 nm ($\Delta\varepsilon$ +8.5), were opposed to those of aerugidiol,[22)] indicating that **2** is a stereoisomer of aerugidiol. Likewise, completely different CD spectra pattern have been observed for **1** and epiprocurcumenol, which is similar to **2** and aerugidiol due to a difference of these compounds' configurations at C-1.[14)] The CD spectrum of **2** suggested that it had the same absolute configuration as those of **1**. Therefore, **2** and aerugidiol might be epimers at C-1, and **2** was determined to be (1*S*,4*S*,5*R*)-1,4-dihydroxy-

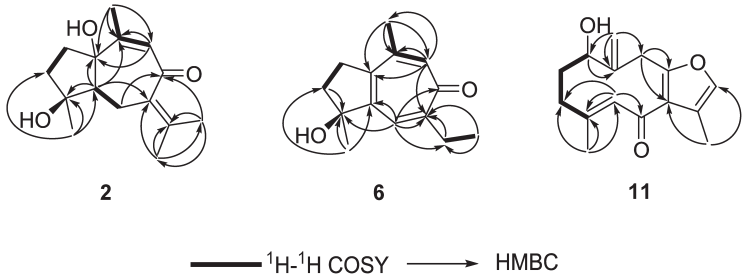

Fig. 2. Key ^{1}H–^{1}H COSY and HMBC Correlations of Compounds **2**, **6**, and **11**

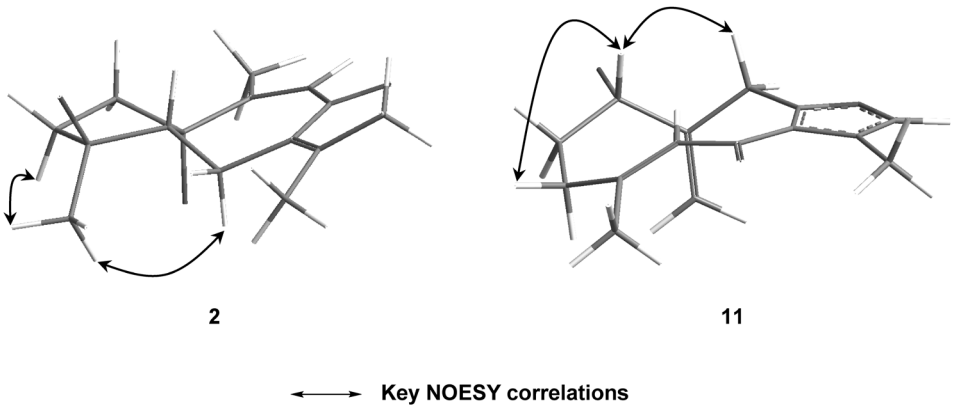

Fig. 3. Key NOESY Correlations of Compounds **2** and **11**

7(11),9(10)-guaidien-8-one; this compound was named 1-*epi*-aerugidiol.

Compound **6** was obtained as a yellow syrup, and its molecular formula of $C_{14}H_{18}O_2$ was determined based on a pseudo-molecular ion peak in the HR-ESI-MS spectrum at *m/z* of 219.1380 ([M+H]$^+$, Calcd for 219.1385). Its IR spectrum indicated the presence of a hydroxyl group (3403 cm^{-1}) and a conjugated carbonyl group (1667 cm^{-1}). Its ^{1}H-NMR spectrum (Table 1) included three methyl signals [δ_H 1.19 (t, J=7.2 Hz, H_3-12), 1.42 (s, H_3-13), 2.32 (s, H_3-14)] and two olefinic proton signals [δ_H 7.50 (s, H-6), 7.06 (s, H-9)]. ^{13}C-NMR spectroscopic data, including DEPT spectra, contained 14 carbon signals, which included six olefinic carbons (δ_C 147.5, 152.3, 133.6, 156.0, 140.1, 149.1) and one conjugated carbonyl group (δ_C 187.4). With respect to NMR data, **6** was similar to a previously isolated guaiane-type sesquiterpenoid, phaeocaulisin D,[7] but differed due to the absence of one methyl group and one hydroxy group. This conclusion was further supported by the HMBC correlations between H-6 and C-5, C-8, and C-11; between the other olefinic proton H-9 and C-1, C-7, and C-14; and between the ethyl group (H_2-11, H_3-12) and C-7. Finally, the oxygenated quaternary carbon C-4 was correlated with the methylene proton H_2-3, the olefinic proton H-6, and the methyl proton H_3-13 (Fig. 2). Accordingly, **6** was an analog of phaeocaulisin D; thus, this compound possessed a guaiane sesquiterpenoid moiety and a chiral center at C-4. Using the empirical bulkiness rule developed by Frelek and Szczepek,[23] the absolute configuration of **6** at C-4 was determined to be *S* based on the positive Cotton effect at approximately 340 nm ($\Delta\varepsilon$ +0.13) in the $Rh_2(OCOCF_3)_4$-induced CD spectrum.[7,23] This result was in good agreement with CD data for Rh-complexes of the analogous compound phaeocaulisin D.[7] Thus, **6** was determined to be (4*S*)-7-ethyl-4-hydroxy-1(5),6(7),9(10)-guaiatrien-8-one; this compound was named phaeocaulisin R.

Compound **11** was obtained as a yellow syrup, and its molecular formula was determined to be $C_{15}H_{18}O_3$ by HR-ESI-MS data (247.1326 [M+H]$^+$, Calcd for 247.1334). The IR spectrum displayed absorption bands for hydroxyl (3423 cm^{-1}), conjugated carbonyl group (1643 cm^{-1}), and double bonds (1535). Its ^{1}H-NMR spectrum (Table 1) showed signals for two methyl protons [δ_H 2.18 (d, J=0.6 Hz, H_3-13) and δ_H 1.57 (s, H_3-15)], one oxygenated methine proton [δ_H 4.20 (dd, J=9.0, 3.6 Hz, H-1)], a pair of olefinic protons on *exo*-cyclic double bonds [δ_H 5.08 (s, H_2-14a), 4.48 (s, H_2-14b)], and another olefinic proton [δ_H 7.24 (s, H-12)]. The ^{13}C-NMR and DEPT spectra of **11** indicated the presence of 15 carbons, including eight olefinic carbons (δ_C 146.2, 128.8, 123.3, 165.7, 151.1, 123.1, 140.4, 112.8), three methylene carbons (δ_C 34.6, 33.9, 33.3), two methyl carbons (δ_C 20.0, 10.2), one oxygenated secondary carbon (δ_C 77.0), and one carbonyl carbon (δ_C 194.6). The connectivity of C-1 to C-3 was established by ^{1}H–^{1}H correlation spectroscopy (COSY) correlations between H_2-2 and H-1, H_2-3, and the HMBC correlations from H-1 to C-10, from H-5 to C-3, C-4, and C-6, from H_2-9 to C-8 and C-7, from H_3-13 to C-7 and C-12, from H_2-14 to C-1, C-9, and C-10, and from H_3-15 to C-3, C-4, C-5 were supportive of the structure shown in Fig. 1. The partial moieties of **11** adjacent to C-1 were similar to those of 1-*epi*-dihydrochrysanolide,[24] which comprised those of the secondary hydroxyl group and the *exo*-cyclic double bond at C-1 and C-14, and lactone moieties instead of conjugated furan group of **11**. NOE correlations between H-1 and H_2-3β (δ_H 2.04, m) and H_2-9β [δ_H 3.50, d (17.4)] were observed on the nuclear Overhauser effect spectroscopy (NOESY) spectrum of **11**, suggesting that H-1, H_2-3β, and H_2-9β were in the same orientation (Fig. 3). The modified Mosher's ester method (Fig. 4) were used to determine the absolute configurations

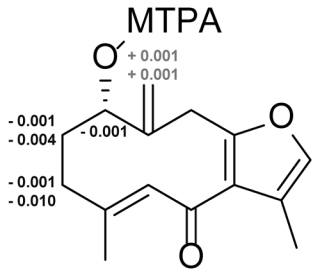

Fig. 4. $\Delta\delta$ Values (δ_S–δ_R) in ppm of the MTPA Esters [(*R*)-MPTA (**11a**), (*S*)-MTPA (**11b**)]

of **11**,[25] and it was thus identified as (1*S*)-1-hydroxy-8,12-epoxygermacra-4(5),7(8),10(14),11(12)-tetraen-6-one; this compound was named (1*S*)-1-hydroxy-isofuranodienone.

The THP-1-Blue cell line, which stably expresses NF-κB- and AP-1-inducible reporter genes such as secreted embryonic alkaline phosphatase (SEAP) upon LPS stimulation, was used to evaluate the inhibitory effects of our isolated compounds **1-15** on TLR4 activation.[26] THP-1-Blue cells were stimulated with LPS (50 ng/mL) for 18 h in the presence of each compounds **1**–**15**. LPS-induced TLR4 activation was inhibited by **4**, **8**, and **9** [with an IC_{50} value of 22.5±1.0 μM (**4**), 54.8±1.2 μM (**8**), 91.0±6.3 μM (**9**)] relative to the positive control luteolin (IC_{50} value: 2.6±0.8 μM),[27] whereas the remaining compounds showed little to no inhibition. Interestingly, **5**, a 10-epimer of **4** that exhibits a β-orientation at OH-10, produced no inhibitory effects (IC_{50}>100 μM), suggesting that in guaiane-type sesquiterpenoids, the configuration at OH-10 may affect the inhibition of TLR4 activity.

In this study, three new and twelve known sesquiterpenoids were isolated from the EtOAc-soluble fraction of *C. phaeocaulis*, and the inhibitory effects of each isolated compound **1**–**15** on LPS-induced TLR4 activation were assessed. Recent studies have indicated that zedoarondiol (**4**) inhibits pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, in LPS-stimulated murine macrophages by suppressing NF-κB transcriptional activity.[28] Taken together, these findings suggest that sesquiterpenoids from *C. phaeocaulis* may be promising as inhibitors for TLR4-mediated inflammatory disease. Additional research is needed to evaluate relatively effective new candidate compounds that could target LPS-stimulated TLR4 activity.

Experimental

General Procedures Optical rotation was measured on a JASCO P-2000 (Jasco Co., Tokyo, Japan) polarimeter. UV spectra were determined on a Spectramax M_2^e (Molecular Devices, Sunnyvale, CA, U.S.A.) spectrophotometer. CD spectra were recorded on a JASCO J-710 (Jasco Co.) spectropolarimeter. IR spectra data were measured on a Spectrum GX (Perkin-Elmer, Wellesley, MA, U.S.A.) Fourier transform (FT)-IR spectrometer. ^{1}H-, ^{13}C-, and 2D-NMR spectroscopic data were recorded on a JEOL JNM-ECA600 or JEOL JNM-EX400 (JEOL, Tokyo, Japan) instrument using tetramethyl silane (TMS) as a reference. HR-ESI mass spectrum data were obtained on Bruker maXis 4G (Bruker, Bremen, Germany) spectrometer. Column chromatography was performed with silica gel (Kieselgel 60, 230–400 mesh, Merck, Darmstadt, Germany), and silica gel 60 F_{254} and RP-18 F_{254}s (Merck) were used for TLC profiling. Medium pressure liquid chromatography (MPLC) was performed on a Combiflash RF (Teledyne Isco, Lincoln, NE, U.S.A.), and preparative HPLC was carried out on a Shimadzu LC-6AD (Shimadzu Co., Kyoto, Japan) instrument equipped with a SPD-20 A detector using a Phenomenex Luna C_{18} (250 mm×21.2 mm, 5 μm, Phenomenex Luna, Torrance, CA, U.S.A.). (*R*)- and (*S*)-α-Methoxy-α-(trifluoromethyl)-phenylacetyl (MTPAs) were purchased from TCI, Co., Ltd. (Tokyo, Japan).

Plant Material *C. phaeocaulis* rhizomes were purchased from an herbal store in Jeongeup, Korea, in April 2014. One of the authors (M.-C. Rho) performed botanical identification, and a voucher specimen (KRIBB-KR2014-700) has been deposited at the laboratory of Eco-friendly Material Research Center, Jeonbuk Branch of the Korea Research Institute of Bioscience and Biotechnology.

Extraction and Isolation Dried and pulverized *C. phaeocaulis* rhizomes (4 kg) were extracted at room temperature with 95% EtOH (40 L×3), and the filtrate was evaporated *in vacuo* to obtain an EtOH extract (283 g). This extract was suspended in H_2O and progressively partitioned with EtOAc (151 g) and BuOH (46 g). The EtOAc-soluble extract was chromatographed on a silica gel column using a stepwise gradient of a hexane–EtOAc (1 : 0–0 : 1) solvent system to generate 38 sub-fractions (CPE1–CPE38). CPE14 (5.2 g) was separated by C_{18} MPLC to produce 17 sub-fractions (CPE14A–CPE14Q) using a gradient solvent system composed of H_2O and MeOH (30–100%), and SPE14D (815 mg) was re-chromatographed by C_{18} MPLC to generate 4 fractions (CPE14D1–CPE14D4). CPE14D1 (39.3 mg) was further purified by preparative HPLC (40% MeCN, 6 mL/min) to produce **9** (3.2 mg) and **10** (1.6 mg). Compounds **11** (15.8 mg) and **12** (83.2 mg) were separated from CPE14D3 (379 mg) by using preparative HPLC (25% MeCN, 6 mL/min). Compounds **1** (263.1 mg) and **14** (4.4 mg) were isolated from CPE14I (540 mg) by preparative HPLC (50% MeCN, 6 mL/min). CPE19 (2.2 g) was chromatographed by C_{18} MPLC and eluted with H_2O and MeOH (20–100%). Among the resulting sub-fractions (CPE19A–CPE19P), CPE19D (101.2 mg) was separated by preparative HPLC (25% MeCN, 6 mL/min) to produce **2** (33.4 mg), **4** (8.3 mg), **7** (2.8 mg), and **8** (8.7 mg). Furthermore, CPE19H (155.8 mg) was separated by preparative HPLC (25% MeCN, 6 mL/min) to yield **3** (2.9 mg), **5** (21.4 mg), **6** (1.6 mg), and **15** (17.1 mg). In addition, **13** (143.9 mg) was isolated from CPE19I (240 mg) by preparative HPLC (25% MeCN, 6 mL/min).

1-Epiaerugidiol (**2**)

Amorphous white powder, $[\alpha]_D^{20}$ +267.4 (c=0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 239 nm (1.91) and 317 nm (1.46); CD ($CHCl_3$) λ_{max} ($\Delta\varepsilon$) 239 nm (−33.2), 279 nm (+15.7), and 346 nm (+8.5); IR (KBr) ν_{max} 3412, 2968, 2920, 2853, 1650, 1442, 1376, 1306, 1225, 1043, 922, and 759 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 1; HR-ESI-MS *m/z* 273.1461 ($C_{15}H_{22}O_3Na$, Calcd for 273.1467).

Phaeocaulisin R (**6**)

Yellow syrup, $[\alpha]_D^{20}$ +45.0 (c=0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 239 nm (2.02) and 276 nm (1.84); $Rh_2(OCOCF_3)_4$-induced CD ($CHCl_3$) λ_{max} ($\Delta\varepsilon$) 340 nm (+0.1); IR (KBr) ν_{max} 3403, 2967, 2928, 2856, 1667, 1507, 1461, 1193, 1163, 859, 786, and 738 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 1; HR-ESI-MS *m/z* 273.1380 ($C_{14}H_{19}O_2$, Calcd for 219.1385).

1-Hydroxy-isofuranodienone (**11**)

Yellow syrup, $[\alpha]_D^{20}$ +6.0 (c=0.1, CH_3OH); UV (CH_3OH)

λ_{max} (log ε) 203 nm (2.14) and 256 nm (1.70); IR (KBr) ν_{max} 3423, 2927, 2886, 1643, 1535, 1409, 1384, 1262, 1027, and 893 cm^{-1}; ^{1}H- and ^{13}C-NMR data, see Table 1; HR-ESI-MS *m/z* 247.1326 ($C_{15}H_{19}O_3$, Calcd for 247.1334).

Preparation of (*R*)- and (*S*)-MTPA Ester of 11 (11a, b) Compounds **11** (4 mg) and 4-dimethylaminopyridine (4-DMAP) were dissolved in anhydrous pyridine (1 mL), and transfer into each vials for both *R*- and *S*-MTPA acylation reactions. After being dried in a N_2 gas stream for 3 h, bis-(*R*)- and (*S*)-MTPA-Cl (25 mg) were added, and each mixture was kept at 40°C in a water bath for 5 h. The MTPA derivatives were purified by semi-preparative HPLC using a C_{18} column, eluting with a gradient solvent system composed of H_2O and acetonitrile (35–100%). (*R*)-MTPA ester (**11a**, 0.4 mg): amorphous powder; ^{1}H-NMR (600 MHz, pyridine-d_5), δ: 7.32 (1H, s, H-12), 6.50 (1H, s, H-5), 5.14 (1H, s, H_2-14a), 4.82 (1H, s, H_2-14b), 4.75 (1H, s, H-1), 4.03 (1H, d, J=17.4 Hz), 3.76 (1H, d, J=17.4 Hz), 2.38 (5H, m, H_2-3a), 2.35 (5H, s, H_3-13), 2.29 (1H, m, H_2-2a), 2.29 (1H, m, H_2-2b), 2.14 (2H, m, H_2-3b), 1.58 (3H, s, H_3-15). (*S*)-MTPA ester (**11b**, 0.5 mg): amorphous powder; ^{1}H-NMR (600 MHz, pyridine-d_5), δ: 7.32 (1H, s, H-12), 6.50 (1H, s, H-5), 5.14 (1H, s, H_2-14a), 4.82 (1H, s, H_2-14b), 4.75 (1H, s, H-1), 4.03 (1H, d, J=17.4 Hz), 3.76 (1H, d, J=17.4 Hz), 2.37 (5H, m, H_2-3a), 2.35 (5H, s, H_3-13), 2.28 (1H, m, H_2-2a), 2.13 (1H, m, H_2-2b), 2.05 (2H, m, H_2-3b), 1.58 (3H, s, H_3-15).

Measurement of Inhibitory Effect on TLR4 Activation THP-1-Blue cells were purchased from a commercial supplier (InvivoGen Corp., San Diego, CA, U.S.A.). TLR4 activity was evaluated according to the manufacturer's protocol, with the modifications described in our previous studies.[27)]

Acknowledgments This research was a part of the project titled 'Development of feed additive for fishery immune stimulation,' funded by the Ministry of Oceans and Fisheries and was supported by a Grant from the KRIBB Research Initiative Program, Republic of Korea (KGM2221622).

Conflict of Interest The authors declare no conflict of interest.

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