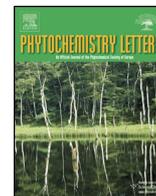




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Short communication

GSK-3 β inhibitory activities of novel dichloresorcinol derivatives from *Cosmospora vilior* isolated from a mangrove plant

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ABSTRACT

Cosmochlorins A (**1**), B (**2**), and C (**3**) were isolated from the endophytic fungus *Cosmospora vilior* IM2-155. The structures of **1**, **2**, and **3** were elucidated by a combination of extensive spectroscopic analyses, including extensive 2D NMR, HRESITOFMS, and chemical reactions. Compounds **1**, **2**, and **3** were evaluated for their biological activity. Compounds **1** and **2** partially restored the growth inhibition caused by hyperactivated Ca²⁺-signaling in mutant yeast and showed glycogen synthase kinase (GSK)-3 β inhibition activity at IC₅₀ values of 62.5 and 60.6 μ M, respectively. Further, compound **2** significantly increased osteoclast formation by more than 1.5-fold in RAW264.7 cells compared to receptor activator of nuclear factor- κ B ligand (RANKL) alone.

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1. Introduction

Marine fungi are potential sources for new pharmaceutical lead structures or biological active compounds with complex chemical structures (Hasan et al., 2015). In recent years, secondary metabolites from marine organisms, including marine fungi, are receiving increasing attention (Overy et al., 2014). In fact, thus far, many structurally and pharmacologically novel and promising bioactive secondary metabolites have been isolated from marine fungi. In search of bioactive compounds from marine microorganisms, we focused our attention on microorganisms that are associated with the mangrove plants (Shiono et al., 2013, 2015). Once isolated, the fungi were grown on unpolished rice cultures supplemented with NaCl, and their extracts were characterized by thin layer chromatography (TLC) and evaluated for antimicrobial activity. Using this methodology, *Cosmospora vilior* IM2-155 was isolated from a mangrove plant, *Sonneratia alba*, at Pagandaran, West Java, Indonesia. *S. alba* is seen in tropical and subtropical areas of Indian ocean. Three new unique halogenated compounds

(containing two chlorine atoms) were reported to be produced by this fungus. In addition, IM2-155 strain was grown on solid media containing 2% NaCl better than one without 2% NaCl. In the present investigation, we report the isolation and structural characterization of three novel compounds from IM2-155. The compounds were evaluated for their biological activities.

2. Results and discussion

C. vilior IM2-155 was grown to stationary phase at 25° C for 3 weeks in steamed unpolished rice supplemented with NaCl. The purification of these metabolites was guided by their antimicrobial activity and intense blue characteristic coloration with vanillin-sulfuric acid solution on TLC plates. The MeOH extract of the moldy, unpolished rice was evaporated to obtain an aqueous concentrate, which was then partitioned between EtOAc and H₂O. The organic layer was purified by silica gel and ODS column chromatography to afford cosmochlorins A (**1**), B (**2**), and C (**3**) (Fig. 1).

Cosmochlorin A (**1**) was isolated as an amorphous yellow powder. HRESITOFMS measurement of the negative molecular ion of **1** showed that the compound contained two chlorinated atoms since the molecular peak was composed of three signals differing by two mass units m/z 367:369:371 with an isotope ratio 10:6.5:1,

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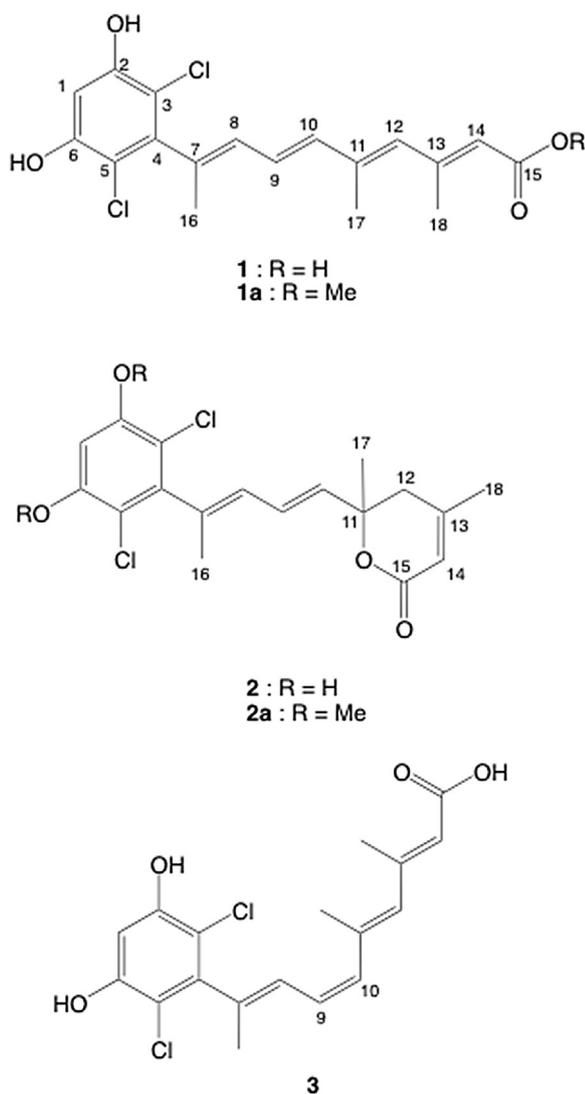


Fig. 1. Structures of **1**, **1a**, **2**, **2a** and **3**.

as expected from compound containing two chlorine atoms. The quasi-molecular peak at m/z 367.0495 [M-H⁻] matched the molecular formula, C₁₈H₁₇³⁵Cl₂O₄. The UV spectrum of **1** showed

an absorption band at 323 nm attributable to a conjugated enone system. The IR spectrum indicated bands at 3421 and 1677 cm⁻¹, indicative of hydroxyl and carboxyl groups in the chemical structure, respectively. The ¹³C NMR data (Table 1) indicates 18 signals, attributable to three methyl groups, eight methines, and seven quaternary carbons including carbonyl carbon. This outcome was further confirmed by DEPT experiments. Nine degrees of unsaturation were observed, which suggests that **1** contains an additional ring. The ¹H NMR and HMQC spectra of **1** showed proton signals indicative of a penta-substituted phenyl group [δ_{H} 6.50 (1H, s, H-1)], three methyl groups attached to olefinic carbons [δ_{H} 2.01 (3H, s, H₃-16), 2.04 (3H, s, H₃-17), 2.23 (3H, s, H₃-18)], and one disubstituted [δ_{H} 6.30 (1H, d, J = 15.1 Hz, H-10) and 6.70 (1H, dd, J = 15.1, 11.0 Hz, H-9)] and three trisubstituted double bonds [δ_{H} 5.94 (1H, d, J = 11.0 Hz, H-8) and 5.99 (1H, s, H-12), 5.73 (1H, s, H-14)]. The COSY spectra revealed the contiguous sequence of coupled signals from H-8 and H-9, and H-9 and H-10. The relationship between these fragments was determined by HMBC experiment (Table 1, Fig. 2). The olefinic methyl protons (Me-16) were correlated with C-4, C-7, and C-8; the signals Me-17 with C-10, C-11, and C-12, signals Me-18 with C-12, C-13, and C-14, and signal H-14 with C-15 and C-18 indicated the presence of 3,5,9-trimethyl-nona-2,4,6,8-tetraenoic acid. Further, the methylation of **1** afforded a methyl ester (**1a**) indicating the presence of a carboxyl group. The benzene ring-substituted pattern of **1** was deduced from the analysis of NOE difference experiments (Fig. 2). Two hydroxyl groups could be assigned as substituents at C-2 and C-6 of the penta-substituted phenyl group on the basis of observable NOE correlations between OH-2,6 and H-1. The geometries of four olefins at C-7/C-8, C-9/C-10, C-11/C-12, C-13/C-14 of tetraenoic acid moiety were found to be *E* on the basis of the ¹H-¹H coupling constant ($J_{9,10}$ = 15.1 Hz) as well as NOE correlations between Me-16 and H-9, Me-17 and H-9, H-10 and H-12, and H-12 and H-14 (Fig. 2).

The molecular formula of cosmochlorin B (**2**), C₁₈H₁₈³⁵Cl₂O₄, was determined by HRESITOFMS, indicating that **2** had the same molecular formula as **1**. The IR absorption bands at 3200 and 1685 cm⁻¹ were similar to the bands observed in case of **1**. The ¹H- and ¹³C NMR data (Table 1) for **2** were similar to that for **1** and also indicated the presence of a penta-substituted phenyl group. This was supported by the fact that the NOEs of dimethyl ether **2a** were observed between OMe-2,6 and H-1. In addition to characteristic phenyl moiety, four olefinic proton signals appeared at δ_{H} 6.32 (1H, d, J = 11.2 Hz, H-8), 7.00 (1H, dd, J = 15.6, 11.2 Hz, H-9), 5.96 (1H, d, J = 15.6 Hz, H-10), and 5.88 (1H, s, H-14), two methyl signals at δ_{H}

Table 1

¹H, ¹³C NMR and HMBC data for **1** in CD₃OD and **2** in C₅D₅N (δ in ppm, J in Hz, 400 MHz for ¹H and 100 MHz for ¹³C).

No	1			2		
	δ_{C}	δ_{H}	HMBC	δ_{C}	δ_{H}	HMBC
1	103.6 d	6.50 s	2, 3, 5, 6	104.1 d	7.01 s	2, 3, 5, 6
2, 6	153.3 s			154.3 s		
3, 5	111.9 s			111.4 s		
4	136.5 s			136.7 s		
7	144.1 s			143.5 s		
8	131.7 d	5.94 d, (11.0)	4, 10, 16	129.5 d	6.32 d, (11.2)	4, 10, 16
9	126.9 d	6.70 dd, (15.1, 11.0)	7, 11	125.3 d	7.00 dd, (15.6, 11.2)	7, 8, 11
10	139.3 d	6.30 d, (15.1)	8, 12, 17	137.2 d	5.96 d, (15.6)	8, 11, 12, 17
11	140.1 s			80.6 s		
12	135.1 d	5.99 s	10, 14, 18	39.6 t	2.34 d, (15.0) 2.35 d, (15.0)	10, 14, 17, 18 10, 14, 17, 18
13	154.9 s			155.7 s		
14	119.7 d	5.73 s	15, 18	116.6 d	5.88 s	12, 15, 18
15	170.2 s			164.4 s		
16	17.3 q	2.01 s	4, 7, 8	17.3 q	2.20 s	4, 7, 8
17	14.7 q	2.04 s	10, 11, 12	27.7 q	1.46 s	10, 11, 12
18	19.8 q	2.23 s	12, 13, 14	22.6 q	1.68 s	12, 13, 14

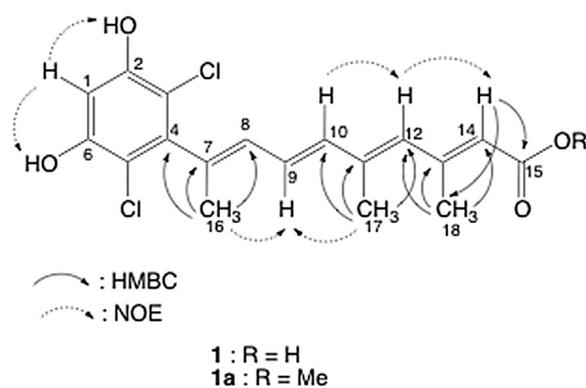


Fig. 2. Key HMBC correlations for **1** and observed NOEs for **1a**.

Table 2

^1H , ^{13}C NMR and HMBC data for **3** in CD_3OD (δ in ppm, J in Hz, 600 MHz for ^1H and 125 MHz for ^{13}C).

No	3		
	δ_{C}	δ_{H}	HMBC
1	102.4 d	6.48 s	2, 3, 5, 6
2, 6	152.4 s		
3, 5	110.5 s		
4	136.1 s		
7	142.9 s		
8	130.6 d	5.92 d, (10.1)	4, 10, 16
9	127.3 d	6.77 t, (10.1)	7, 11
10	130.7 d	6.82 d, (10.1)	8, 12, 17
11	137.2 s		
12	132.2 d	5.97 s	10, 14, 18
13	153.2 s		
14	119.2 d	5.70 s	15, 18
15	168.9 s		
16	16.1 q	2.03 s	4, 7, 8
17	20.2 q	2.03 s	10, 11, 12
18	18.4 q	2.20 s	12, 13, 14

Table 3

MIC ($\mu\text{g}/\text{ml}$) of **1** and **3**.

Microorganisms	1	3	Positive control ^a
<i>Staphylococcus aureus</i> NBRC 13276	15.6	15.6	3.9
<i>Candida albicans</i> ATCC 2019	125	125	0.48
<i>Aspergillus clavatus</i> F318a	62.5	62.5	0.12
<i>Trichoderma harzianum</i> NBRC 33016	15.6	15.6	7.8
<i>Verticillium dahliae</i> Klebahn NBRC 9470	>125	>125	2.0

^a Positive control, chloramphenicol was used for *S. aureus* NBRC 13276, amphotericin B for *C. albicans* ATCC 2019 and *A. clavatus* F318a, and thiabendazole for *T. harzianum* NBRC 33016, *V. dahliae* Klebahn NBRC 9470.

than the reference standard (Aburai et al., 2010), **2** observed the moderate active against cytotoxicity against HL60 (IC_{50} values, **1**: 73.7 μM , **2**: 53.6 μM , **3** > 100 μM).

In addition, two isolated compounds were tested for their inhibitory effect on Ca^{2+} -signaling using the mutant yeast strain *S. cerevisiae* (*zds1Δ erg3Δ pdr1Δ pdr3Δ*: YNS17 strain). Ca^{2+} is a ubiquitous second messenger that regulates diverse biological processes. The Ca^{2+} -signaling pathway for growth regulation in YNS17 strain comprises several signaling molecules such as Ca^{2+} channel, Pkc1 protein kinase C, Mpk1 MAPK, Mck1 GSK-3, and calcineurin (Mizunuma et al., 1998; Miyakawa and Mizunuma, 2007). YNS17 strain does not grow at high CaCl_2 concentrations because hyper-activated Ca^{2+} -signaling in the yeast blocks the cell-cycle progression in the G2/M phase. Cell-cycle arrest is caused by inhibition of Cdc28/Clb2 though up-regulation of Swe1. Swe1 is a kinase that specially inhibits the G2 form of Cdc28 by phosphorylating at Tyr19, and suppresses the transition from G2 to M phase. The activation of Swe1 is regulated by calcineurin and the Mpk1 MAP kinase cascade during the Ca^{2+} -dependent regulation of cell cycle and morphogenesis (Mizunuma et al., 1998). Thus, in this assay, the calcineurin inhibitor, FK506, promoted the cell growth of this mutant strain by inhibiting the Ca^{2+} -induced Swe1 activation. In this screening system (Chanklan et al., 2008; Ogasawara et al., 2008), 5 μL of two-fold serially diluted solutions of **1** or **2** were applied onto the surface of agar plates containing a lawn of growing yeast cells. Restored growth effect of **1** on YNS17 strain, caused by the diffusion from the spot of **1** into the agar plate, was detected by the appearance of a dose-dependent growth halo with an inhibition halo in the yeast lawn around the spot after 3 days at 28 °C. As shown in Fig. 3, the inhibition activity of **1** is apparently stronger than that of **2**. These phenotypes suggested that **1** and **2** inhibited Ca^{2+} -signaling of YNS17 strain through GSK-3 β inhibition, because similar phenotypic compounds, 6-(methylsulfinyl)

2.20 (3H, s, Me-16) and 1.68 (3H, s, Me-18), almost similar to the ^1H NMR data of **1**, with the difference being the lack of a singlet signal at δ_{H} 5.99 (H-12) in **1**; a singlet methyl signal at δ_{H} 2.04 (Me-17) was observed in case of **1**, and that at δ_{H} 1.46 (3H, s, Me-17) for **2**. The ^1H and ^{13}C NMR data of **2** showed signals attributable to an sp^3 methylene [δ_{H} 2.34 (1H, d, J = 15.0 Hz, H-12) and 2.35 (1H, d, J = 15.0 Hz, H-12)] and an sp^3 oxygenated quaternary carbon [δ_{C} 80.6 (C-11)] groups. Overall, the data suggested that **2** has saturated C-11/C-12 double bond in contrast to **1** in which the acidic group at C-15 was esterified with δ -lactone. This presumption was supported by the fact that **2** (290 nm) did not show UV absorption at 323 nm, which was clearly observed in case of **1**. In the HMBC spectrum of **2** (Table 1), correlations of Me-16/C-4, C-7, and C-8 and H-8/C-4, C-10, and C-16 revealed that the diene moiety group is located at C-4 and the location of dimethyl α,β -unsaturated- δ -lactone ring was determined on the basis of HMBC correlations of H-10/C-12 and Me-17/C-10 and C-12. In addition, although there is a single stereogenic center at C-11, **2** was presumably racemic mixture because of the specific rotation ($[\alpha]_{\text{D}} = 0$, c 0.33, MeOH) and the absence of any CE in the CD spectrum.

The molecular formula of cosmochlorin C (**3**), $\text{C}_{18}\text{H}_{18}^{35}\text{Cl}_2\text{O}_4$, was determined by HRESITOFMS, indicating that **3** has the same molecular formula as **1** and **2**. The UV and IR spectra of **3** closely resembled that of **1**. Thus, **3** is considered to be an isomer of **1**. The ^1H NMR spectrum of **3** was analyzed by means of COSY, HSQC, and HMBC correlations to compare with **1**. Obvious differences were found in the chemical shifts around the tetraene moiety of **1**. The coupling constant of H-9 and H-10 was found to be 10.1 Hz, whereas for **1**, it was 15.1 Hz. In the NOESY experiment, NOE interactions were recorded between H-8 and H-12 and between H-10 and Me-17; the interactions between H-8 and H-10 disappeared and the tetraene configurations of **3** were determined to be 7*E*, 9*Z*, 11*E* 13*E*. The unambiguous assignments of the signals in ^1H and ^{13}C NMR spectra of **3** were based on HMBC experiments (Table 2). Therefore, **3** exists as a *cis* isomer of **1** at the double bond between C-9 and 10.

Further, to investigate the biological activity of the characterized compounds, anti-microbial assays were performed. Compound **1** exhibited moderate antimicrobial activity against gram-positive bacteria and fungi (Shiono et al., 2005). The MIC values of **1** and **3** for *Staphylococcus aureus* NBRC 13276, *Aspergillus clavatus* F318a, *Trichoderma harzianum* NBRC 33016, and *Candida albicans* ATCC 2019 were found to be 15.6, 62.5, 15.6, and 125 $\mu\text{g}/\text{mL}$, respectively (Table 3). Compound **3** showed a similar trend of antimicrobial activity. In contrast, **2** was inactive against the tested strains. The carboxylic acid moiety played an important role in the antimicrobial activities of **1** and **2**. In the case of cytotoxicity test, although the activities of the isolated compounds were weaker

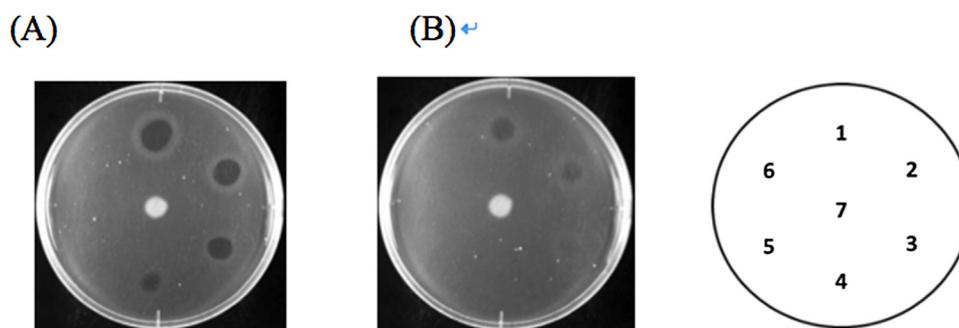


Fig. 3. Growth restored activities of **1** (A) and **2** (B) against *S. cerevisiae* YNS17 strain (*zds1Δ erg3Δ pdr1Δ pdr3Δ*) in the presence of 0.3 M CaCl₂. 1: 5 μg/spot, 2: 2.5 μg/spot, 3: 1.25 μg/spot, 4: 0.63 μg/spot, 5: 0.31 μg/spot, 6: 0.16 μg/spot, 7 (FK506): 2.5 ng/spot.

hexyl isothiocyanate (6-MSITC) (Yoshida et al., 2011) and faltarindiol (Yoshida et al., 2013) have GSK-3β inhibition activity. As expected, **1** and **2** inhibited human GSK-3β dose-dependently and to the same extent (IC₅₀ = 62.5 and 60.6 μM, respectively) (Fig. 4).

Since Ca²⁺-signaling and GSK-3β also appear to play an important role in differentiation and function of osteoclasts (Hwang and Putney, 2011; Soysa et al., 2012; Jang et al., 2011), we investigated the effects of **1** and **2** on osteoclast differentiation in RAW264.7 cells derived from Abelson murine leukemia virus-induced tumor. We induced osteoclastogenesis in RAW264.7 cells by receptor activator of nuclear factor-κB ligand (RANKL) in the presence and absence of **1** and **2** (Fig. 5). An inhibitor of GSK-3β, kenpauillone, which is an activator of osteoclastogenesis, was used as a positive control (Akiba et al., 2016). Although **1** didn't show statistically significant difference from the control (DMSO), **2** induced osteoclastic differentiation more than 1.5-fold compared to the control, which is comparable to that observed with kenpauillone. One of the molecular targets of **1** and **2** is GSK-3β and further pharmacological studies of **1** and **2** are currently underway.

In conclusion, cosmochlorins A (**1**), B (**2**), and C (**3**) were isolated from the culture of an endophytic fungus from a mangrove plant, *S. alba*, and their structures were elucidated by spectroscopic analyses. Compounds **1**, **2**, and **3** had a constrained novel structure that is unprecedented in nature and has not been reported earlier. In addition, to the best of our knowledge, **1**, **2**, and **3** are the first naturally occurring compounds containing 3-(1,5-dihydroxy-2,4-dichloro)phenyl moiety. Compounds **1** and **2** inhibited Ca²⁺-signaling in mutant yeast through GSK-3β inhibition activity

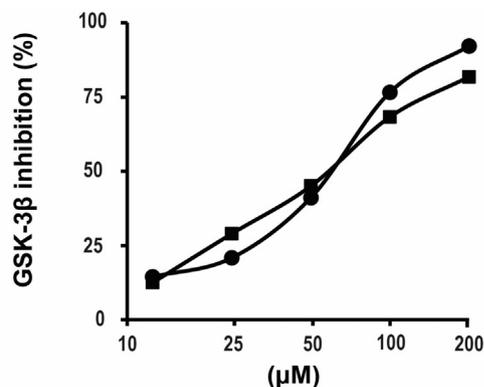


Fig. 4. GSK-3β inhibition activities of **1** and **2**. GSK-3β assay (n = 2) was performed according to references (Yoshida et al., 2011, 2013). —●—: **1**, —■—: **2**.

and **2** enhanced osteoclastic differentiation of RAW264.7 cells. We plan to carry out pharmacological studies related to the GSK-3β inhibitory activity of **1** and **2** in type-2 diabetes and Alzheimer's diseases.

3. Experimental procedures

3.1. Instrumentation

Optical rotation values were measured with a Horiba SEPA-300 polarimeter, and IR, and UV spectra were respectively recorded with Jasco J-20A, Shimadzu UV mini-1240 spectrophotometers. CD data was collected by J-820 CD spectrometer. Mass spectra were obtained with a Synapt G2 mass spectrometer instrument. NMR data were recorded on a Jeol ECZ-600 spectrometer at 600 MHz for ¹H and 150 MHz for ¹³C and an a Jeol EX-400 spectrometer at 400 MHz for ¹H and 100 MHz for ¹³C. Chemical shifts are given on a δ (ppm) scale with TMS as an internal standard. ¹H, ¹³C, COSY, HMQC and HMBC spectra were recorded using standard Jeol standard pulse sequences. Semi-preparative HPLC was carried out with Shimadzu pump and UV LC-10A detector (set at 210 nm) on Mightysil ODS column (250 × 6.0 mm i.d.) at the flow rate of 1.5 mL min⁻¹. Column chromatography was conducted on silica gel 60 (Kanto Chemical Co., Inc., Japan) and ODS (Fuji Silysia, Japan). TLC was carried out on Merck pre-coated silica gel plates (silica gel 60 F₂₅₄), and spots were detected by spraying with 10% vanillin in sulfuric acid followed by heating, or by UV irradiation. The vanillin/sulfuric acid spray reagent was prepared by dissolving 1.0 g of vanillin in 100 mL of concentrated sulfuric acid.

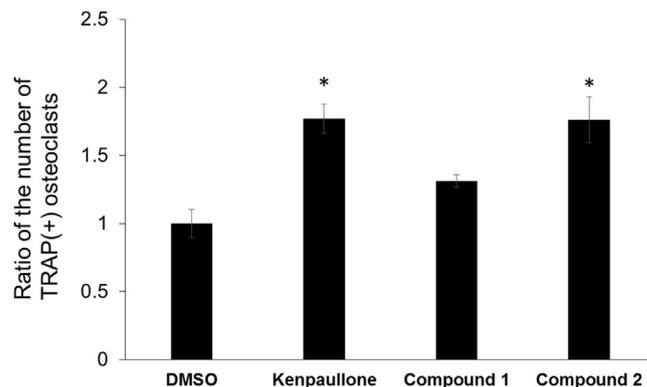


Fig. 5. The effect of **1** and **2** on osteoclastogenesis.

The number of the generated TRAP-positive multinuclear osteoclasts in the presence of the compounds, DMSO (solvent), kenpauillone (positive control, 5 μM), **1**, and **2** (5 μM), were evaluated after 4 days of the induction. The bars represent means values (±S.D., n = 4) of fold changes compared to DMSO. Statistical analysis was performed by ANOVA with Tukey's multiple comparison test (*P < 0.01).

3.2. The producing strain

The fungal strain *Cosmospora vilior* IM2-155 was isolated from the mangrove plants collected at Pagandaran (latitude: $-7^{\circ}78'$, longitude: $108^{\circ}65'$), West Java, Indonesia. The plant material (*Sonneratia alba*) was authenticated by one of authors (U.S.). A voucher specimen was deposited at Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran. The branch samples were aseptically cleaned successively with 70% EtOH for 1 min, 5% sodium hypochlorite for 5 min and 70% EtOH for 1 min, then rinsed in sterile water for two times. The aseptically clean samples were dried on sterilized paper and cut into 1 cm pieces. The pieces were placed on plates of Potato-Dextrose-Agar (PDA) containing chloramphenicol (100 mg/L). After incubation at 25°C for 7 days, the hyphal tips of the fungi on the plates were removed from the agar plates and transferred to PDA plates (slant). The strain IM2-155 was isolated and grew on slants of PDA as white colored culture. This strain was identified to be *Cosmospora vilior* by BEX. Co. LTD. Japan, using a DNA analysis of the 18S rDNA regions. This fungus has been deposited at our laboratory in the Faculty of Agriculture of Yamagata University and Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran.

3.3. Fermentation, extraction and isolation

This strain IM2-155 was cultivated on sterile steamed unpolished rice with 2% NaCl in water (total 540 g, 20 g/petri dish \times 27) at 25°C for three weeks. The moldy unpolished rice was extracted with MeOH (1.5 L), and MeOH extract was concentrated in vacuo. The resulting aqueous concentrated was partitioned into *n*-hexane layer (0.5 L), EtOAc layer (1.0 L) and aqueous layer (0.5 L). Purifications of eluates were monitored by the characteristic intense blue coloration with 10% vanillin in H_2SO_4 on pre-coated silica gel TLC plates. The EtOAc layer (2.26 g) was chromatographed on a silica gel column with stepwise elution of *n*-hexane-EtOAc (100:0-0:100) and EtOAc-MeOH (50:50, 0:100), respectively, to afford fractions 1-1 to 1-13. Fractions 1-7 (*n*-hexane – EtOAc, 40:60, 0.6 g) was rechromatographed on a silica gel column using the stepwise elution (CHCl_3 -EtOAc) to afford fractions 2-1 to 2-11. Fractions 2-5 and 2-6 (CHCl_3 -EtOAc, 60:40, 50:50, 60 mg) was further semi-preparative ODS HPLC by eluting with MeCN- H_2O (50:50) to give cosmochlorin B (**2**, 8.8 mg, $t_R = 10.5$ min, 0.00016% yield). Fractions 1-8 (*n*-hexane – EtOAc, 30:70, 0.5 g) was further separated by ODS chromatography eluted with H_2O and an increasing ratio of MeOH (100:0-0:100) to afford fractions 3-1 to 3-11. Fractions 3-7 and 3-8 (H_2O – MeOH, 40:60, 30:70, 120 mg) were combined and further separated by silica gel column chromatography eluted with CHCl_3 -MeOH (90:10) to give cosmochlorins A (**1**, 54.0 mg, 0.01% yield) and C (**3**, 1.0 mg, 0.000019% yield).

3.3.1. Cosmochlorin A (**1**)

Yellow amorphous powder; UV (MeOH) λ_{max} (log ϵ): 323 (4.2) nm; IR (KBr) ν_{max} cm^{-1} ; 3421, 2923, 2341, 1677, 1581, 1168, 960; ^1H NMR and ^{13}C NMR data see Table 1. HRESITOFMS (negative ion mode) m/z 367.0495 $[\text{M}-\text{H}]^-$ (calcd. for $\text{C}_{18}\text{H}_{17}^{35}\text{Cl}_2\text{O}_4$, 367.0499).

3.3.2. Cosmochlorin B (**2**)

White amorphous powder; UV (MeOH) λ_{max} (log ϵ): 290 (3.4), 230 (4.4) nm; IR (KBr) ν_{max} cm^{-1} ; 3200, 2981, 2931, 1685, 1581, 1172, 991; ^1H NMR and ^{13}C NMR data see Table 1. HRESITOFMS (negative ion mode) m/z 367.0526 $[\text{M}-\text{H}]^-$ (calcd. for $\text{C}_{18}\text{H}_{17}^{35}\text{Cl}_2\text{O}_4$, 367.0499).

3.3.3. Cosmochlorin C (**3**)

White amorphous powder; UV (MeOH) λ_{max} (log ϵ): 323 (4.1) nm; IR (KBr) ν_{max} cm^{-1} ; 3413, 2927, 2857, 1716, 1600, 1168, 998; ^1H NMR and ^{13}C NMR data see Table 2. HRESITOFMS (negative ion mode) m/z 367.0526 $[\text{M}-\text{H}]^-$ (calcd. for $\text{C}_{18}\text{H}_{17}^{35}\text{Cl}_2\text{O}_4$, 367.0499).

3.4. Preparations of methoxy derivatives **1a** and **2a**

Compound **1** (5 mg) was dissolved in a solution of MeOH, and trimethylsilyldiazomethane (2.0 M in diethylether, 0.05 mL), was added to the solution. The mixture was stirred at 0°C for 5 min and evaporated to dryness. The residue (11 mg) was subjected to silica gel column chromatography with mixtures of *n*-hexane-EtOAc to afford a methyl ester (**1a**, 3.0 mg). Compound **1a**: ^1H NMR (400 MHz, acetone- d_6): δ_{H} 2.06 (3H, s, Me-17), 2.07 (3H, s, Me-16), 2.26 (3H, s, Me-18), 3.59 (3H, OMe), 5.73 (1H, s, H-14), 5.98 (1H, d, $J = 11.0$ Hz, H-8), 6.09 (1H, s, H-12), 6.41 (1H, d, $J = 15.0$ Hz, H-10), 6.64 (1H, s, H-1), 6.82 (1H, dd, $J = 15.0, 11.0$ Hz, H-9), 8.88 (2H, 2,6-OH). ^{13}C NMR (100 MHz, acetone- d_6): δ_{C} 13.9 (Me-17), 16.5 (Me-16), 18.7 (Me-18), 50.3 (OMe), 102.9 (C-1), 110.8 (C-3 and C-5), 118.4 (C-14), 126.2 (C-9), 131.2 (C-8), 134.2 (C-12), 135.4 (C-4), 138.5 (C-10), 139.1 (C-11), 143.1 (C-7), 152.5 (C-2 and C-6), 153.3 (C-13), 166.5 (C-15). HRESITOFMS (positive ion mode) m/z 405.0358 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{19}\text{H}_{20}^{35}\text{Cl}_2\text{NaO}_4$, 405.0336).

Compound **2** (5 mg) was converted to dimethoxy derivative (**2a**, 3 mg) by using a method similar to that in the case of **1**. Compound **2a**: ^1H NMR (400 MHz, CDCl_3): δ_{H} 1.25 (3H, s, Me-17), 1.57 (3H, s, Me-18), 2.01 (3H, s, Me-16), 3.93 (6H, OMe), 5.72 (1H, d, $J = 15.6$ Hz, H-10), 5.84 (1H, d, $J = 11.0$ Hz, H-8), 5.86 (1H, s, H-14), 6.51 (1H, s, H-1), 6.60 (1H, dd, $J = 15.6, 11.0$ Hz, H-9). ^{13}C NMR (100 MHz, CDCl_3): δ_{C} 16.9 (Me-16), 23.2 (Me-18), 29.7 (Me-17), 39.9 (C-12), 56.5 (OMe), 80.6 (C-11), 95.9 (C-1), 113.5 (C-14), 116.5 (C-3 and C-5), 125.1 (C-9), 129.1 (C-8), 135.4 (C-4), 135.9 (C-10), 143.4 (C-7), 154.3 (C-2 and C-6), 154.9 (C-13), 164.7 (C-15). HRESITOFMS (positive ion mode) m/z 419.2787 $[\text{M} + \text{Na}]^+$ (calcd. for $\text{C}_{20}\text{H}_{22}^{35}\text{Cl}_2\text{NaO}_4$, 419.2741).

3.5. Growth restored activity of samples against YNS17 strain

Growth restored activity of **1** and **2** against mutant yeast YNS17: screening was performed according to previous described method (Chanklan et al., 2008). Each sample was dissolved in MeOH and two-fold dilutions of them were used. Difco[®] yeast-peptone-dextrose (YPD) broth and YPD agar were purchased from Becton Dickinson Biosciences (Franklin Lakes, NJ, USA). The mutant yeast, YNS17 (*MATa zds1:TRP1 erg3:HIS3 pdr1:hisG-URA3-hisG pdr3:hisG*) yeast strain was derivative of strain W303-1A. A 5 μL aliquot of samples were spotted on YPD agar medium containing YNS17 strain and 0.3 M CaCl_2 . After 3 days of incubation at 28°C , the intensity of the growth spot were observed as the result of inhibition of Ca^{2+} -signal transduction. FK506 (2.5 ng/spot) was used as a positive control. FK506 was kindly provided by Fujisawa Pharmaceutical Co., Ltd. (the present Astellas Pharma Inc., Tokyo Japan).

3.6. GSK-3 β assay

The substrate peptide (20 μM , Merck Millipore Co., Billerica, MA, USA) was mixed with human GSK-3 β (31.25 ng/well, ab60863, Abcam, Cambridge, UK) at total volume of 50 μL in the buffer [8 mM MOPS (pH 7.0), 0.2 mM EDTA, 5 μM ATP, 10 mM MgCl_2] in the presence or absence of inhibitors. Inhibitors were dissolved in dimethyl sulfoxide (DMSO) and 2 μL aliquot of the inhibitors was applied 96-well plate (12.5, 25, 50, 100, 200 μM). GSK-3 β assays were performed in a white 96-well plate and a GSK-3 β inhibitor, GSK-3 β inhibitor-I (TDZD-8: 4-benzyl-2-methyl-1,2,4-

thiadiazolidine-3,5-dione, Merck Millipore Co.), was used as a positive control.

3.7. Osteoclastogenesis assay

RAW264.7 cells were cultured in α -MEM medium containing 10% fetal bovine serum (FBS), 100 ng/ml soluble RANKL (sRANKL, Oriental Yeast), 2 mM L-glutamine, 100 units/ml penicillin, and 100 g/ml streptomycin. TRAP staining was performed after 4 days of the induction, and the multinuclear osteoclasts were counted.

3.8. Cell culture and cytotoxicity

HL60 cells (RCB0041, RIKEN BioResource Center, Tsukuba, Japan) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (BioWest, Canada) and penicillin (50 units/ml)-streptomycin (50 μ g/ml) (Gibco Corp., Carlsbad, USA) in a humidified atmosphere at 37 °C under 5% CO₂. The cytotoxicity of the compounds were examined by MTT assay, as described previously (Aburai et al., 2010). Positive control camptothecin showed an IC₅₀ value of 23.2 nM.

3.9. Antimicrobial activity

This test was performed in Petri dishes (4 cm id.) in duplicate. Each test compound was dissolved at 1 mg/mL in 10% aqueous DMSO. A suitably quantified volume of the test solution was mixed with the appropriate agar medium (2 mL) to prepare a plate with a given concentration (0–250 μ g/mL) of a test compound. Each plate was subsequently inoculated with a test microorganism (100 μ L of approximately 10⁶ CFU/mL) and incubated at 30 °C for 18–24 h for bacteria and at 25 °C for 48 h for *C. albicans* ATCC 2019 and *A. clavatus* F 318a, *T. harzianum* NBRC 33016 and *V. dahliae* Klebahn NBRC 9470. MIC is defined as the lowest concentration resulting in no visible growth after incubation. Antimicrobial assays were carried out by the method using a published protocol (Shiono et al., 2005).

Conflict of interest

The authors of the present manuscript have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data (NMR spectra of compounds 1, 2 and 3) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phytol.2016.09.007>.

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