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Phytochemistry Letters



Short communication

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



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ARTICLE INFO

ABSTRACT

Article history:

Received 11 May 2016

Received in revised form 10 September 2016

Accepted 20 September 2016 Available online xxx

2 partially restored the growth inhibition caused by hyperactivated Ca²⁺-signaling in mutant yeast and showed glycogen

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 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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Keywords:

Mangrove

Endophyte

*Cosmospora vilior*Ca²⁺-signaling

Osteoclast formation

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 micro-organisms, 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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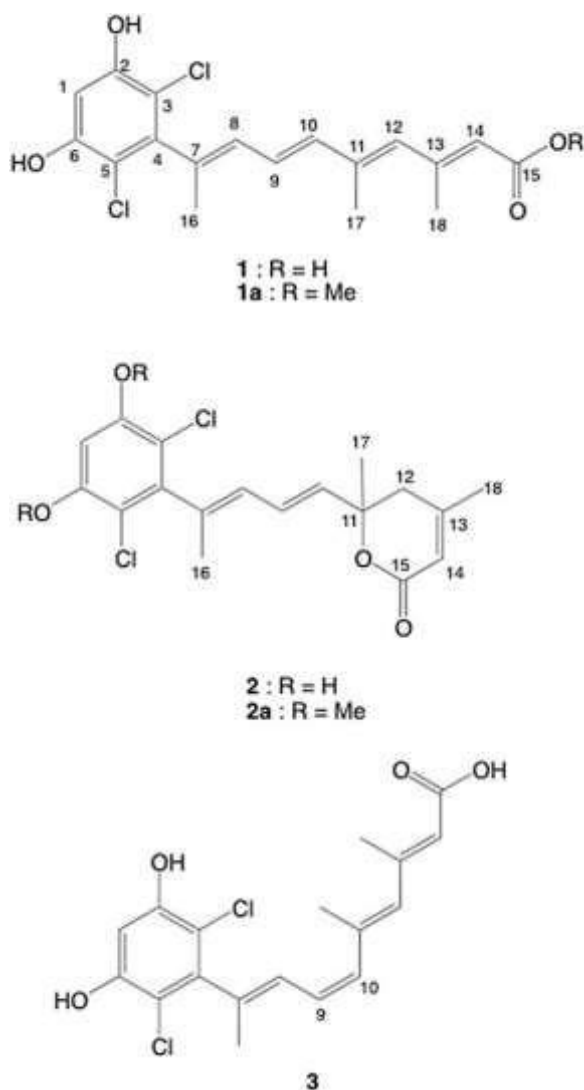


Fig. 1. Structures of 1, 1a, 2, 2a and 3.

as expected from compound containing two chlorine atoms. The quasi-molecular peak at m/z 367.0495 [M-H]⁻ matched the molecular formula, C₁₈H₁₇Cl₂O₄. The UV spectrum of 1 showed

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 pentasubstituted phenyl group [d_H 6.50 (1H, s, H-1)], three methyl groups attached to olefinic carbons [d_H 2.01 (3H, s, H₃-16), 2.04 (3H, s, H₃-17), 2.23 (3H, s, H₃-18)], and one disubstituted [d_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 [d_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 pentasubstituted 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), C₁₈H₁₈Cl₂O₄, 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 pentasubstituted 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 d_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 d_H

Table 1

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

No	1			2		
	d_C	d_H	HMBC	d_C	d_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)	10, 14, 17, 18
					2.35 d, (15.0)	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

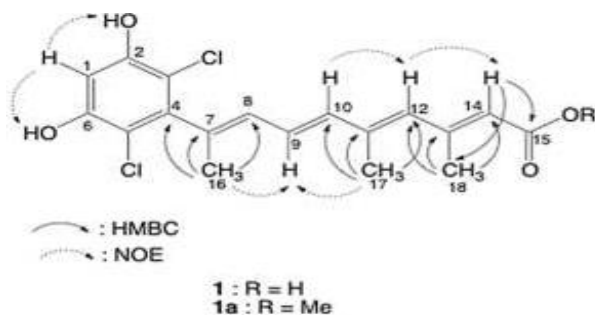


Fig. 2. Key HMBC correlations for 1 and observed NOEs for 1a.

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. The ^1H and ^{13}C NMR data of 2 showed signals attributable to an sp³ methylene [δ_{H} 2.34 (1H, d, $J_3 = 15.0$ Hz, H-12) and 2.35 (1H, d, $J = 15.0$ Hz, H-12)] and an sp³ 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 α -lactone. This presump-

tion 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 α -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), C₁₈H₁₈³⁵Cl₂O₄, 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 7E, 9Z, 11E, 13E. 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. Com-

parisons were made for Staphylococcus aureus NBRC 13276, Aspergillus clavatus F318a, Trichoderma harzianum NBRC 33016, and Candida albicans

ATCC 2019 were found to be tested although the activities of the isolated compounds were weaker

^{13}C NMR and HMBC data for 3 in CD₃OD (δ in ppm, J in Hz, 600 MHz for ^1H and 125 MHz for ^{13}C).

Carbon	Chemical Shift (ppm)	Assignment	Correlations
1	102.4	d	2,3,5,6
2, 6	152.4	s	3
3, 5	110.5	s	
4	136.1	s	
7	142.9	s	
8	130.6	d	5.92 d, (10.1)
9	127.3	d	6.77 t, (10.1)
10	130.7	d	6.82 d, (10.1)
11	137.2	s	
12	132.2	d	5.97 s
13	153.2	s	
14	119.2	d	5.70 s
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 (mg/ml) of 1 and 3.

Microorganisms	1	3	Positive control
Staphylococcus aureus NBRC 13276	15.6	15.6	3.9
Candida albicans ATCC 2019	125	125	0.48
Aspergillus clavatus F318a	62.5	62.5	0.12
Trichoderma harzianum NBRC 33016	15.6	15.6	7.8
Verticillium dahliae 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 (IC₅₀ values, 1: 73.7 μM , 2: 53.6 μM , 3 > 100 μM).

In addition, two isolated compounds were tested for their inhibitory effect on Ca²⁺-signaling using the mutant yeast strain S. cerevisiae (zds1D erg3D pdr1D pdr3D: YNS17 strain). Ca²⁺ is a ubiquitous second messenger that regulates diverse biological processes. The Ca²⁺-signaling pathway for growth regulation in YNS17 strain comprises several signaling molecules such as Ca²⁺ 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 CaCl₂ concentrations because hyper-activated Ca²⁺-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 Ca²⁺-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 Ca²⁺-induced Swe1 activation.

In this screening system (Chanklan et al., 2008; Ogasawara et al., 2008), compound 1 exhibited moderate growth inhibitory activity against growing yeast cells. Restored growth effect of 1 on YNS17 strain,

ATCC 2019 were found to be caused by the diffusion from the spot of 1 into the agar plate, was tested. As shown in Fig. 3, the inhibition activity of 1 is apparently weaker than that of the reference standard strains. The carboxylic acid

group, because similar phenotypic compounds, 6-(methylsulfinyl)

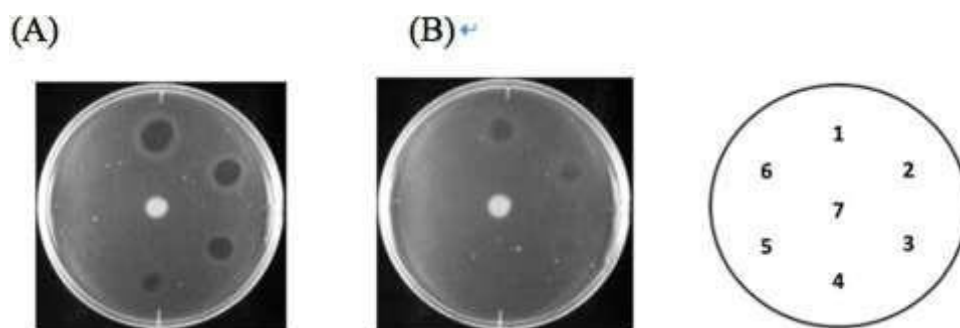


Fig. 3. Growth restored activities of 1 (A) and 2 (B) against *S. cerevisiae* YNS17 strain (*zds1D* 1.25 *erg3D pdr1D pdr3D*) in the presence of 0.3 M CaCl_2 . 1: 5 mg/spot, 2: 2.5 mg/spot, 3: 1 mg/spot, 4: 0.63 mg/spot, 5: 0.31 mg/spot, 6: 0.16 mg/spot, 7 (FK506): 2.5 ng/spot.

hexyl isothiocyanate (6-MSITC) (Yoshida et al., 2011) and faltarindiol (Yoshida et al., 2013) have GSK-3 β inhibition activity. As expected, 1 and 2 inhibited human GSK-3 β dose-dependently and to the same extent (IC_{50} = 62.5 and 60.6 μM , respectively) (Fig. 4).

Since Ca^{2+} -signaling and GSK-3 β 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-3 β , 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-3 β 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 Ca^{2+} -signaling in mutant yeast through GSK-3 β inhibition activity

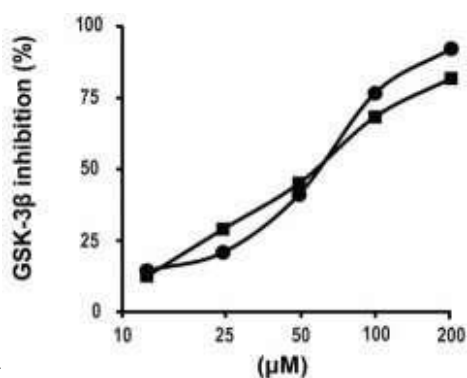


Fig. 4.

GSK-3 β assay ($n = 2$) was performed according to references (Yoshida et al., 2011, 2013). —

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 CFU/mL) and

*—: 1, —&—: 2. < 0.01).

and 2 enhanced osteoclastic differentiation of RAW264.7 cells. We plan to carry out pharmacological studies related to the GSK-3 β 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 ^{13}C and a Jeol EX-400 spectrometer at 400 MHz for ^1H and 100 MHz for ^{13}C . Chemical shifts are

given on a δ (ppm) scale with TMS as an internal standard. ^1H , ^{13}C , 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 \times 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.

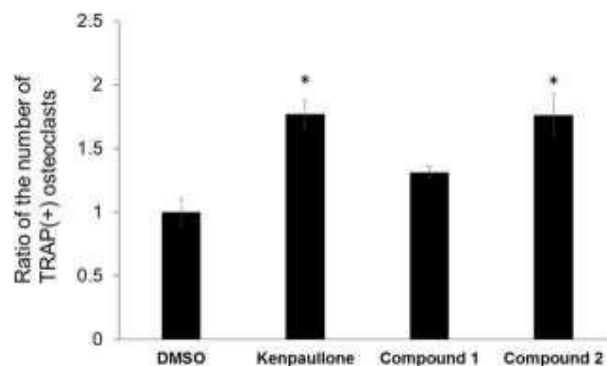


Fig. 5. The effect of 1 and 2 on osteoclastogenesis.

3.2. The producing strain

The fungal strain *Cosmospora vilior* IM2-155 was isolated from the mangrove plants collected at Pagandaran (latitude: 7 78, longitude: 108 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 x 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 H₂SO₄ 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 (CHCl₃-EtOAc) to afford fractions 2-1 to 2-11. Fractions 2-5 and 2-6 (CHCl₃-EtOAc, 60:40, 50:50, 60 mg) was further semi-preparative ODS HPLC by eluting with MeCN H₂O (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 H₂O and an increasing ratio of MeOH (100:0:0:100) to afford fractions 3-1 to 3-11. Fractions 3-7 and 3-8 (H₂O MeOH, 40:60, 30:70, 120 mg) were combined and further separated by silica gel column chromatography eluted with CHCl₃-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) $\tilde{\nu}_{\max}$ cm⁻¹; 3421, 2923, 2341, 1677, 1581, 1168, 960; ¹H NMR and ¹³C NMR data see Table 1. HRESITOFMS (negative ion mode) m/z 367.0495 [M-H] (calcd. for C₁₈H₁₇³⁵Cl₂O₄, 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) $\tilde{\nu}_{\max}$ cm⁻¹; 3200, 2981, 2931, 1685, 1581, 1172, 991; ¹H NMR and ¹³C NMR data see Table 1. HRESITOFMS (negative ion mode) m/z 367.0526 [M-H] (calcd. for C₁₈H₁₇³⁵Cl₂O₄, 367.0499).

3.3.3. Cosmochlorin C (3)

White amorphous powder; UV (MeOH) λ_{\max} (log ϵ): 323 (4.1) nm; IR (KBr) $\tilde{\nu}_{\max}$ cm⁻¹; 3413, 2927, 2857, 1716, 1600, 1168, 998; ¹H NMR and ¹³C NMR data see Table 2. HRESITOFMS (negative ion mode) m/z 367.0526 [M-H] (calcd. for C₁₈H₁₇³⁵Cl₂O₄, 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-d₆): 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). ¹³C NMR (100 MHz, acetone-d₆): 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 [M + Na] (calcd for C₁₉H₂₀³⁵Cl₂NaO₄, 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: H NMR (400

MHz, CDCl₃): 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). ¹³C NMR (100 MHz, CDCl₃): 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 [M + Na]⁺ (calcd. for C₂₀H₂₂³⁵Cl₂ Na O₄, 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 mL aliquot of samples were spotted on YPD agar medium containing YNS17 strain and 0.3 M CaCl₂. After 3 days of incubation at 28 C, the intensity of the growth spot were observed as the result of inhibition of Ca²⁺-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 mM, 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 mL in the buffer [8 mM MOPS (pH 7.0), 0.2 mM EDTA, 5 mM ATP, 10 mM MgCl₂] in the presence or absence of inhibitors. Inhibitors were dissolved in dimethyl sulfoxide (DMSO) and 2 mL aliquot of the inhibitors was applied 96-well plate (12.5, 25, 50, 100, 200 mM). 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 mg/mL) (Gibco Corp., Carlsbad, USA) in

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 mg/mL) of a test compound. Each plate was subsequently inoculated with a test microorganism (100 μ l) 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.

Acknowledgements

We would like to acknowledge the kind cooperation of the collecting plants materials by Dr. Yenny Febriani Yun and Dr. Lilis Siti Aisyah, Faculty of Mathematics and Natural Science, Jenderal Achmad Yani University, Indonesia. We thank Mr. Yuki Kiyokawa and Mr. Naoyuki Yamashita of Division of Dental Pharmacology, Niigata University Graduate School of Medical and Dental Sciences, Japan for assistance with the osteoclast differentiation assay. We also thank Mr. Tetsuaki Kawamura of Department of Biological

Chemistry and Food Science, Iwate University, Japan for YNS17 strain assay, and SCADS (Screening Committee of Anticancer Drugs) supported by Grant-in-Aid for Scientific Research on Innovative Areas, Scientific Support Programs for Cancer Research, from The Ministry of Education, Culture, Sports, Science and Technology Japan for providing deposited chemical library.

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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