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A new antiangiogenic C_{24} oxylipin from the soft coral *Sinularia numerosa*

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ABSTRACT

A new oxylipin, 15-hydroxy-tetracosa-6,9,12,16,18-pentaenoic acid (15-HTPE; **1**) was isolated as an inhibitor of tube-formation from the soft coral *Sinularia numerosa*. Its structure was elucidated by means of spectral analysis and chemical degradation. 15-HTPE inhibited tube formation of EA.hy926 cells at the concentration of 20–40 μM.

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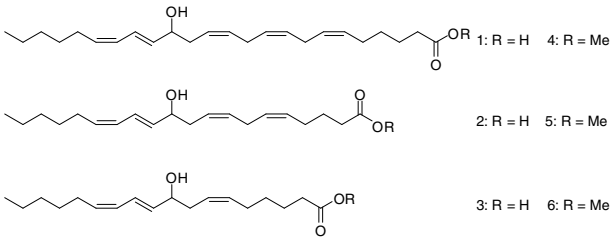
1. Introduction

Angiogenesis is a promising target for cancer chemotherapy[1,2]; the first antiangiogenic agent Avastin® was approved in 2004 by FDA for treatment of colorectal and non-small cell lung cancers[3] and numbers of antiangiogenic agents are under clinical trials.[4] To discover antiangiogenic compounds, several assay systems have been developed. Among them, the tube-formation system using endothelial cells and collagen gel[5] is one of the most popular in vitro models, representing many features of in vivo angiogenesis; activation of endothelial cells by the growth factors, degradation of basement matrices, cell migration, and formation of tube-like structure of the primary blood vessels.

In the course of our search for new antiangiogenic agents from marine invertebrates, we tested anti-tube-formation activity of 150 Japanese marine invertebrates by an assay system of the modified in vitro tube-formation model[6] using EA.hy 926 cells, a permanent human endothelial cell line.[7,8] Out of 10 active samples, the soft coral *Sinularia numerosa* collected in southern Japan showed remarkable inhibitory activity. Bioassay-guided isolation provided a new oxylipin, 15-hydroxy-tetracosa-6,9,12,16,18-pentaenoic acid (15-HTPE; **1**), along with the known 11-hydroxy-eicosa-5,8,12,14-tetraenoic acid (11-HETE; **2**), and 9-hydroxy-octadeca-6,10,12-trienoic acid (9-HOTE; **3**). This paper deals with the isolation, structural elucidation, and biological activity of **1**.



2. Results and discussion

The combined MeOH and $CHCl_3$ extracts of the frozen sample (1.0 kg, wet weight) were partitioned between H_2O and $CHCl_3$; the latter layer was further separated by the modified Kupchan procedure.[9] The $CHCl_3$ layer inhibiting tube formation was further separated by solvent partitioning with *n*-hexane/CH_2Cl_2/MeCN (10:3:7). The active lower layer was subjected to centrifugal partition chromatography (CPC) with the solvent system of *n*-heptane/EtOAc/MeCN/MeOH/H_2O (6:6:1:4:3; mobile phase: upper layer, stationary phase: lower layer) to afford three active

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fractions. The half portions of these fractions were separately purified by ODS HPLC with MeCN/H_2O (75:25) to yield a new oxylipin **1** (0.7 mg), along with the known **2** (0.5 mg) and **3** (0.4 mg). The remaining portions were treated with CH_2N_2, followed by ODS HPLC purification afforded the corresponding methyl esters, **4** (2.2 mg), **5** (2.8 mg), and **6** (2.0 mg), respectively.

The new oxylipin **1** had a molecular formula of $C_{24}H_{38}O_3$ as established by HRFABMS and NMR data, indicating six degrees of unsaturation. The ^{1}H NMR spectrum showed three olefinic protons resonating at δ 5.64 (H-16, dd, J = 6.6, 15.2 Hz), δ 6.50 (H-17, dd, J = 10.9, 15.2 Hz), and δ 5.96 (H-18, t, J = 10.9 Hz), the last of which showed a COSY cross-peak to one of seven overlapping olefinic protons resonating at δ 5.40 (H-19), thus establishing connectivities from C-16 to C-19. The geometry of $\Delta^{16,17}$ and $\Delta^{18,19}$ olefins was deduced to be *E* and *Z*, respectively, on the basis of the coupling constants (15.2 and 10.9 Hz, respectively).

Interpretation of the COSY and HOHAHA spectra led to the connectivities from C-19 to C-21 (Fig. 1). The linkage between C-19 and C-20 was also supported by HMBC cross-peaks, H-18/C-20, H-20/C-18, and H-20/C-19. The terminal propyl unit was connected to C-21 on the basis of HMBC network shown in Figure 1. A COSY cross-peak between H-16 and the oxymethine proton at δ 4.13 (H-15, dt, J = 6.6, 6.9 Hz), which was connected to non-equivalent allyl methylene protons at δ 2.31 and 2.34 (H_2-14), constructed the partial structure **a** (Fig. 1).

The remaining part of the molecule contained the terminal carboxyl group which was connected to four consecutive methylenes based on COSY and HMBC correlations (H-2/C-1 and H-3/C-1). The remaining three disubstituted olefins and two methylenes flanked by these olefins, which is a common feature of polyunsaturated fatty acids, were placed between C-14 and C-5, thus establishing the partial structure **b** (Fig. 1). Although the coupling constants could not be obtained due to the overlapping olefinic proton signals, chemical shift values for the bis-allyl methylenes (δ_C 26.7 and 26.9, δ_H 2.83 and 2.82, respectively) indicated all *E*-geometry.[10,11] HMBC cross-peaks, H-15/C-13, H_2-14/C-12, and H_2-14/C-13, connected partial structures **a** and **b** through C-12 and C-13 to complete the planar structure of **1**, that is 15-hydroxy-tetracosa-6,9,12,16,18-pentaenoic acid.

Determination of the absolute stereochemistry at C-15 was not straightforward due to the difficulty in preparing MTPA esters. Presumably, the formed MTPA ester was easily eliminated to form a conjugated tetraene unit during work-up. Instead, ozonolysis of the methyl ester of **1** followed by the reductive treatment yielded triol **7** which was converted to the tris-*p*-bromobenzoyl ester **8**. Chiral HPLC analysis of **8** afforded two peaks in a ratio of 1:1, corresponding to 2*S* and 2*R* derivatives, thus indicating the racemic nature of **1** (see scheme 1).

The new oxylipin 15-HTPE (**1**) exhibited the anti-tube-formation activity in a dose-dependent manner (20–40 μM) in the in vitro tube-formation model using EA.hy 926 cells. Although detailed evaluation was not possible due to the limited amounts of samples, similar activity was observed for both 11-HETE (**2**) and 9-HOTE (**3**).[12] It is interesting that 12(*R*)-hydroxy-5,8,14-eicosatrienoic acid (12-HETE) is known to induce in vitro tube formation of rabbit limbal microvascular endothelial (RLME) cells via expression of vascular endothelial growth factor (VEGF).[13] The opposite effects of these closely related compounds may indicate their different targets or opposite (agonistic/antagonistic) effects on the same targets.

PPARγ ligands such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), BRL49653, or ciglitazone were reported to suppress dose-dependently the proliferation and the differentiation into tube-like structures in three dimensional collagen gels of human umbilical vein endothelial cells (HUVEC). It was also revealed that 15d-PDJ_2 decreased mRNA levels of both VEGFR-1 (Flt-1) and -2 (Flk-1/KDR) in HUVEC. However, these effects were not observed for the PPARα ligand, Wy14643.[14] In contrast to this result, Meissner et al. reported that Wy14643, a PPARα ligand, suppressed VEGFR-2 protein expression and inhibited basal and VEGF-induced formation of capillary-like structures of HUVEC on Matrigel, whereas ciglitazone, a PPARγ ligand, exhibited no discernible suppression.[15] These different findings may be attributable to the different length of exposure time to the respective PPAR ligands; PPARγ ligands were presumed to require longer time (48 h) to take effects.

In our in vitro tube-formation system, EA.hy 926 cells were exposed to drugs for longer time (preincubation 12 h + incubation 24 h) than in the system used by Meissner et al. (total 12 h)[15] allowing drugs to take effects, while cells were exposed to gels or growth factors for shorter time (24 h) than in the system of Xin et al. (48 h)[14] resulting in the reduced stimulation by growth factors or gels. Therefore, our system represents the intermediate assay condition of those by Xin et al. and Meissner et al. Evaluation of inhibitory effects on the tube formation by PPARs ligands such as ciglitazone (PPARγ), Wy14643 (PPARα), and leukotriene B_4 (LTB_4; PPARα) indicated that all these ligands inhibited the tube formation in our system. This result shows that our system is capable to detect the anti-tube-formation activity of both PPARα and γ ligands, and therefore suitable for the broader search of PPARs ligands with antiangiogenic activity.

EA.hy 926 cells used in our system are known to express PPARα, δ, and γ. The results mentioned above suggest that PPARs are the possible targets of the obtained oxylipins **1**–**3**. It is also known that

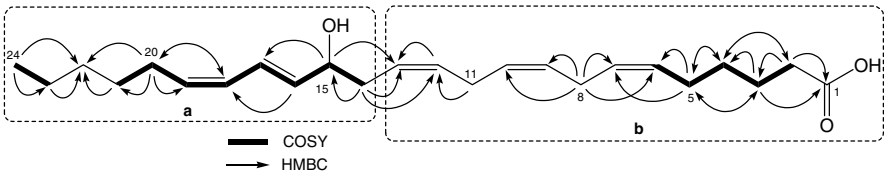
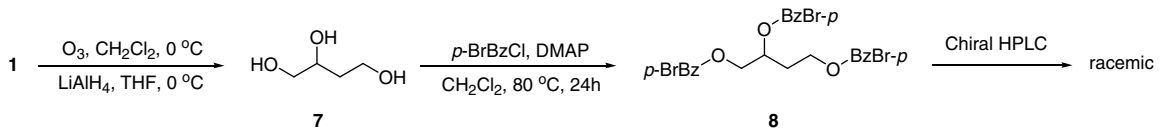


Figure 1. Key COSY and HMBC correlations and partial structures **a** and **b**.



Scheme 1. Analysis of the stereochemistry at C-15 of **1**.

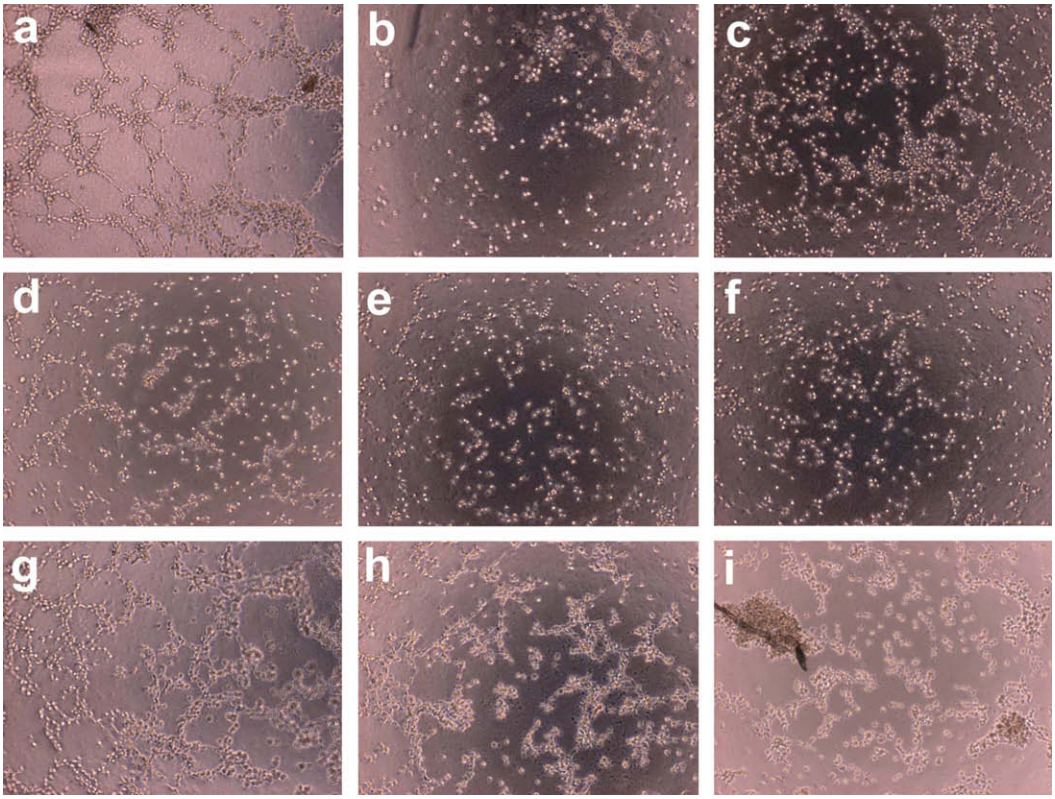
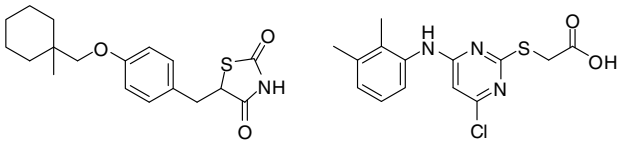


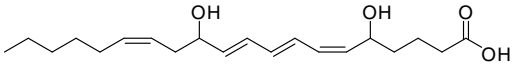
Figure 2. Anti-tube-formation activities of HTPE (**1**) and known PPARs ligands. (a) Control, (b) ciclitazone (**7**) 10 μM, (c) WY14643 (**8**) 10 μM, (d) LTB_4 (**9**) 5 μM, (e) LTB_4 (**9**) 10 μM, (f) LTB_4 (**9**) 20 μM, (g) HTPE (**1**) 10 μM, (h) HTPE (**1**) 20 μM, and (i) HTPE (**1**) 40 μM. Magnification: (a–f) 1×, (g–i) 1.6×.

IL-4 and -13 markedly increased mRNA levels of VCAM-1 in vascular endothelial cells, and administration of anti-VCAM-1 Ab blocked in vitro tubular morphogenesis of human microvascular endothelial cells induced by IL-4 and -13.[16] PPARα and γ ligands were reported to inhibit cytokine-induced VCAM-1 expression.[17] Therefore, to confirm the target of **1**–**3**, expression levels of VEGF, VEGFRs, or VCAM-1, and their direct effects on each type of PPARs, need to be evaluated (see Fig. 2).



ciglitazone (7)

WY14643 (8)



LTB_4 (9)

3. Conclusion

A new oxylipin, 15-hydroxy-tetracosa-6,9,12,16,18-pentaenoic acid (15-HTPE; **1**) having anti-tube-formation activity was isolated from the soft coral *S. numerosa* along with known 11-HETE (**2**) and 9-HOTE (**3**). 15-HTPE (**1**) exhibited the anti-tube-formation activity in a dose-dependent manner within the concentration range of 10–40 μM. From the results of anti-tube-formation assay with the known PPARs ligands, such as leukotriene B_4, ciglitazone, and Wy14643 in this in vitro tube-formation system, PPARs or VCAM-1 expression were suggested as the possible targets of **1**–**3**. However, the targets and the modes of action of these compounds are yet to be elucidated.

4. Experimental

4.1. General procedures

Ultraviolet spectra were recorded on SHIMADZU BioSpec-1600 spectrometer. FAB mass spectra were measured with a JEOL SX-102 mass spectrometer. ^1H and ^{13}C NMR spectra were recorded on JEOL JNM-A600 NMR spectrometer (recorded in CD_3OD).

4.2. Biological materials

The soft coral sample was collected on 20 July 1998 at Bounotsu, southwest coast of Kagoshima Prefecture, at the depth of 15 m. Latitude: 31°18′31.4″N; longitude: 130°13′34″E. The sample was frozen as soon as collected, and kept frozen at −20 °C until extraction.

4.3. Tube-formation assay

Tube-formation assay was carried out basically following the procedure by Oikawa et al.[6] in which EA.hy 926 cells were used instead of Human Dermal Microvascular Endothelial Cells (HDMECs). EA.hy 926 cells were preincubated at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) with the indicated concentrations of samples. Matrigel (Basement Membrane Matrix, BD Biosciences) coated plates were prepared by adding Matrigel onto 24-multiwell dish in the amount of 200 μL/well at 4 °C and allowed

to polymerize at 37 °C for 30 min. The trypsinized cells were seeded (under two cell concentrations of 1.2 and 1.4×10^5 cells/mL) onto the Matrigel coated plates in 1 mL of growth medium (DMEM with 1% FBS) containing the indicated concentration of samples. After 24 h incubation, formation of tube-like structure was observed under a phase-contrast microscope. EA.hy 926 cells with the same sample concentration were incubated on the dishes without Matrigel coating for the evaluation of cytotoxicity of the samples at the same concentration.

4.4. Isolation

The soft coral *S. numerosa* (900 g, wet weight) was extracted with MeOH and $CHCl_3$. The combined extracts was evaporated and partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer was further separated with hexane and MeOH/H_2O (9:1). The aqueous MeOH fraction was diluted with H_2O to make MeOH/H_2O (6:4) which was extracted with $CHCl_3$. The active $CHCl_3$ layer was subjected to solvent partitioning system using *n*-hexane/CH_2Cl_2/MeCN (10:3:7). The active lower layer was further separated by centrifugal partition chromatography (CPC) using *n*-heptane/EtOAc/MeCN/MeOH/H_2O (6:6:1:4:3) as a solvent system to obtain nine fractions (fr. 1–9), three of which (fr. 3–5) showed anti-tube-formation activities. Half of the active fr. 3 obtained was separated on ODS column [Inertsil (GL Science Inc.), φ 10 × 250 mm, MeCN/H_2O (75:25), flow rate: 2.0 mL/min] to yield pure compound (**1**; 0.7 mg). In the same way, each half of fr. 4 and fr. 5 was purified to yield two active compounds (**2**; 0.5 mg and **3**; 0.4 mg, respectively). The remaining halves of the fractions were treated with CH_2N_2 in ether at 0 °C prior to the final purification by ODS HPLC with MeCN/H_2O (75:25). The methyl esterification improved the yields and gave the active substances as methyl esters (**4**; 2.2 mg, **5**; 2.8 mg, and **6**; 2.0 mg, respectively).

4.5. 15-Hydroxy-tetracosa-6,9,12,16,18-pentaenoic acid (15-HTPE; 1)

Colorless solid. α_D^{27} ±0° (*c*, 0.1, MeOH); UV (MeOH) λ_{max} 236.0 nm; FABMS: $[M+Na]^+$ *m*/*z* 397, $[M-OH]^+$ *m*/*z* 357, $[M-H]^-$ *m*/*z* 373, HRFABMS: *m*/*z* 373.2736: $[M-H]^-$ (calcd for $C_{24}H_{37}O_3$, 373.2743); ^{1}H NMR (600 MHz, CD_3OD); δ 6.50 (dd, *J* = 15.2, 10.9 Hz, H-17), 5.96 (t, *J* = 10.9 Hz, H-18), 5.64 (dd, *J* = 15.2, 6.6 Hz, H-16), 5.43 (m, H-12, 13), 5.40 (dt, *J* = 10.9, 7.3 Hz, H-19), 5.37 (m, H-6), 5.36 (m, H-7), 5.34 (m, H-9, 10), 4.13 (dt, *J* = 6.6, 6.9 Hz, H-15), 2.86 (2H, m, H-11), 2.82 (2H, m, H-8), 2.34 (m, H-14a), 2.31 (m, H-14b), 2.27 (2H, t, *J* = 7.5 Hz, H-2), 2.18 (2H, dq, *J* = 7.6, 7.3 Hz, H-20), 2.10 (2H, q, *J* = 7.3 Hz, H-5), 1.62 (2H, quint., *J* = 7.5 Hz, H-3), 1.40 (2H, quint., *J* = 7.5 Hz, H-4), 1.39 (2H, quint., *J* = 7.6 Hz, H-21), 1.32 (4H, m, H-22, 23), 0.90 (t, *J* = 6.9 Hz, H-22); ^{13}C NMR (150 MHz, CD_3OD); 178.2 (C-1), 136.6 (C-16), 133.2 (C-19), 130.8 (C-12), 130.6 (C-6), 129.3 (C-18), 129.3 (C-9 or 10), 129.1 (C-7), 129.0 (C-9 or 10), 126.6 (C-17), 126.5 (C-13), 73.2 (C-15), 36.5 (C-14), 35.3 (C-2), 32.6 (C-22), 30.5 (C-21), 30.3 (C-4), 28.7 (C-20), 28.0 (C-5), 26.9 (C-11), 26.7 (C-8), 26.0 (C-3), 23.6 (C-23), 14.4 (C-24).

4.6. Stereochemistry of the hydroxy group

A portion of **1** (0.2 mg), dissolved in CH_2Cl_2, was exposed to ozone for 15 min at 0 °C. After remaining ozone was removed by N_2 bubbling, solvent was evaporated. The residue was dissolved in THF, and to this solution $LiAlH_4$ was added. After quenching the reduction by adding water, the reaction mixture was subjected to the SiO_2 column chromatography with $CHCl_3$/MeOH [(10:0), (19:1), (9:1), and (8:2)]. The fractions eluted with $CHCl_3$/MeOH (9:1) and (8:2) were shown to contain butanetriol (**7**) on the basis of TLC analysis. These fractions were dissolved in CH_2Cl_2 and combined, and then *p*-bromobezoylchloride (2.8 mg) and dimethylaminopyridine (5.6 mg) were introduced to this solution. Then, the reaction mixture was stirred overnight in the sealed tube at 80 °C. The reaction mixture was separated by SiO_2 column chromatography yielding 1,2,4-trisbromobenzoate in the *n*-hexane/EtOAc (9:1) eluting fraction, which was purified by ODS HPLC [COSMOSIL 5C_{18}-ARII (Nacalai), φ 10 × 250 mm, a linear gradient from MeOH/H_2O (9:1) to (10:0), flow rate: 2.0 mL/min]. The Chiral HPLC [Chiralcel OJ, (Daicel Chemical Industries, Ltd), φ 4.6 × 250 mm, *n*-hexane/*i*-PrOH (8:2), flow rate: 1.0 mL/min] profile of the obtained 1,2,4-tris-*p*-bromobenzoate (**8**) gave two peaks of 1:1 ratio at the retention time of 12 and 14 min corresponding to those of standard samples prepared from (*S*)- and (*R*)-butanetriols, respectively.

Acknowledgments

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