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Antiangiogenic Effect of Chamigrane Endoperoxides from a Thai Mangrove-Derived Fungus

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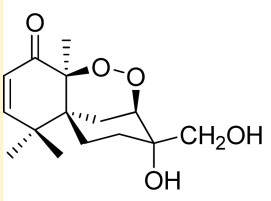
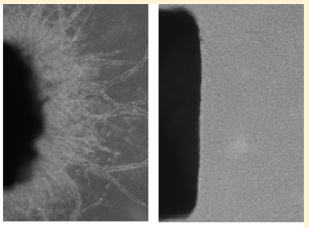
Supporting Information

ABSTRACT: As part of our ongoing efforts to investigate natural products with potential for use as cancer treatments, we have recently disclosed the cytotoxicity of unique nor-chamigrane (**1**) and chamigrane (**2**, **3**) endoperoxides from a Thai mangrove-derived fungus. Reinvestigation of this fungus in a large-scale fermentation led to the isolation of an additional new chamigrane endoperoxide (**4**) and one known analogue (**5**). Among these isolated metabolites, compound **3** (merulin C) exhibited potent antiangiogenic activity mainly by suppression of endothelial cell proliferation and migration in a dose-dependent manner, and its effect is mediated by reduction in the phosphorylation of Erk1/2. Merulin C also displayed promising activity in a rat aortic ring sprouting (ex vivo) and a mouse Matrigel (in vivo) assay.



Merulin C (**3**)



control | Merulin C (2.5 μM)

Angiogenesis is the formation of new blood vessels from pre-existing blood vessels, and it is necessary for development, reproduction, and wound repair. However, pathological angiogenesis is involved in various diseases including diabetic retinopathy, age-related macular degradation, and solid tumor growth.[1] Therefore, inhibition of angiogenesis is an important target for cancer treatment, as not only solid tumor but also metastasis are facilitated through this biological process.[2]

Endophytic fungi are known as a prolific source for the discovery of structurally interesting and biologically active secondary metabolites, some of which are promising candidates for drug development or agrochemical applications.[3,4] Endophytic fungi have also been found to produce molecules previously obtained from their host plants, for example, paclitaxel[5] and camptothecin.[6] Among plant-derived fungi, those associated with mangrove trees have received much attention from natural product researchers due to the unique ecosystem.[7−10] In addition to growing in tropical areas, which provide great biodiversity, mangroves have to deal with changing tides and broad ranges of salinity, temperature, and moisture as well as a number of other environmental factors.[11,12] It is reasonable to expect they must be home to a great variety of specific microorganisms including fungi. This prompted us to embark on the study of bioactive metabolites from Thai mangrove-derived fungi. Recently, we have disclosed the isolation of unique nor-chamigrane (**1**) and chamigrane (**2**, **3**) endoperoxides from a basidiomycete fungus isolated from *Xylocarpus granatum*.[13] As part of our studies on natural products with potential as cancer treatments, compounds **1**−**3** were evaluated for their cytotoxicity against human cancer cell lines.[13] In this paper, we report our reinvestigation of a large-scale fermentation of this mangrove-derived fungus, leading to the isolation of an additional new chamigrane endoperoxide, namely, merulin D (**4**), and one known analogue, steperoxide A (**5**),[10] in addition to merulin A (**1**, or steperoxide B) and merulins B and C (**2**, **3**). Furthermore the current study focuses on the assessment of their inhibition of angiogenesis using ex vivo, in vitro, and in vivo models, as well as on which signaling pathways are involved.

Scale-up fermentation of the fungus in liquid corn steep liquor-containing medium (20 L) and chromatographic fractionation of the EtOAc extract resulted in the isolation of a new chamigrane

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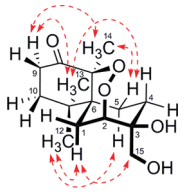
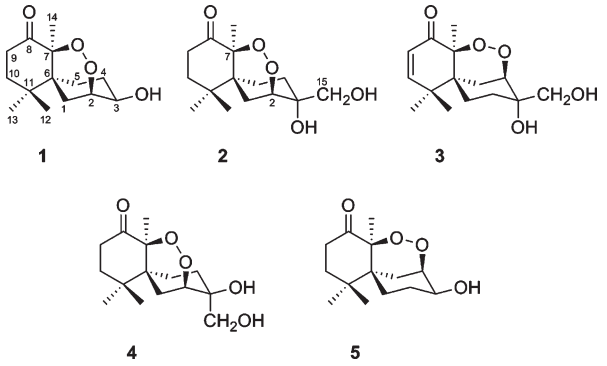


Figure 1. Key NOESY correlations of merulin D (**4**).

endoperoxide, merulin D (**4**), and four known derivatives, merulins A−C (**1**−**3**) and steperoxide A (**5**). Their structures were elucidated by 1D and 2D NMR spectroscopic data, by HRESIMS, and by comparison of the data with those previously reported.[10,13]



Merulin D (**4**) was obtained as a colorless gum. The HRESIMS data indicated a molecular formula identical to merulin B (**2**) of $C_{15}H_{24}O_5$. The NMR data of **4** were also similar to those of **2**, and analysis of 2D NMR data revealed the same gross structure as **2**, suggesting that **4** and **2** were diastereomers. In addition to slightly shifted signals, the major difference observed from their ^{1}H NMR spectra was the appearance of H_2-15 as a singlet at δ_H 3.56 in place of a pair of doublets at δ_H 3.43 and 3.94 (J = 11.1 Hz) in **2**.[13] This was probably the consequence of the different configuration at the C-3 stereocenter. Significant NOESY correlations of H-1ax/H_2-15 and H-5ax/H_2-15 as well as lack of correlation between H-4ax and H_2-15 indicated an axial position for the $-CH_2-OH$ fragment and the corresponding equatorial 3-OH group (Figure 1). Thus, compound **4** was determined to be an epimer of **2** possessing the opposite configuration at C-3 as shown.

To assess antiangiogenic effects of compounds **1**−**5**, they were first subjected to an ex vivo model, a rat aortic sprouting assay. As shown in Figure 2, merulin C (**3**) displayed strong activity with complete inhibition of microvessel sprouting from rings at a concentration of 2.5 μM, while steperoxide A (**5**) showed much weaker activity, with complete inhibition at 25 μM. Merulins A (**1**), B (**2**), and D (**4**) did not display any detectable activity at a screening dose (25 μM). Among isolated nor-chamigrane and chamigrane endoperoxides, merulin C (**3**) and steperoxide A (**5**), possessing a C-4−C-7 endoperoxide linkage, exhibited antiangiogenic activity, while derivatives sharing a C-2−C-7 endoperoxide linkage unit showed no activity. This implied that the C-4−C-7 endoperoxide might play a crucial role in the activity. Merulin C is 10 times more potent than steperoxide A, and an α,β-unsaturated ketone and/or the hydroxymethyl functionality might be partially responsible for the bioacitivity.

In order to evaluate the mechanism of action of merulin C, the following experiments were performed. Merulin C inhibited

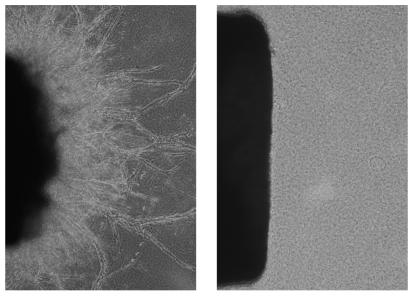


Figure 2. Suppressive effect of merulin C (**3**) in an ex vivo angiogenesis model (rat aortic sprouting assay).

human umbilical vein endothelial cell (HUVEC) proliferation in a dose-dependent manner with an IC_{50} value of 0.9 μM and about 90% inhibition at 25 μM (Figure S8a). The effect of merulin C on VEGF-induced HUVEC migration was examined using a modified Boyden chamber assay.[14] VEGF is known as a specific and critical growth factor involved in endothelial proliferation, migration, and survival during blood vessel formation.[15] As shown in Figure S8b, whereas VEGF strongly stimulated endothelial migration, merulin C could suppress VEGF-induced migration of HUVEC in a dose-dependent manner. However, the significant inhibition was observed at concentrations higher than 5 μM. Finally, the effect on HUVEC tube formation on Matrigel was investigated. Merulin C did not exhibit a significant inhibitory effect on the tubular formation of HUVECs at concentrations ranging from 2.5 to 10 μM, 50% inhibition was observed at 25 μM, and complete inhibition was detected at 50 μM (Figure S8c and S8d). It is possible that inhibition of tubular formation is caused by the cytotoxic activity. These results indicated that the antiangiogenic activity of merulin C is mainly related to the suppression of endothelial cell proliferation and migration.

The mechanism by which merulin C regulates angiogenic inhibition was investigated by Western blot analysis. Two important pathways, phosphoinositide 3-kinase/Akt and extracellular signal-regulated kinase (Erk1/2), are responsible for VEGF stimulus of endothelial cells. These pathways are involved in endothelial cell proliferation, migration, and survival.[16] Results showed that treatment of HUVECs with merulin C suppressed the phosphorylation of Erk1/2 in both a dose- and time-dependent manner (Figure 3), but did not affect the expression of phosphorylated Akt. This suggested that the antiangiogenic property of merulin C is mediated by the Erk1/2 signaling pathway. The strong suppressive effect of merulin C on the phosphorylation of Erk1/2 is in line with a previous report in which inhibitors for the Erk1/2 signaling pathway suppress endothelial cell proliferation.[17] Furthermore the weak inhibitory effect of merulin C on migration is consistent with its lack of effect on the phosphorylation of Akt, which plays an important role in endothelial cell migration.[18]

Finally, to examine whether merulin C inhibits in vivo angiogenesis, a Matrigel plug assay was carried out. FGF-containing Matrigel, with 10 μM merulin C or vehicle (DMSO), was subcutaneously implanted into male C57BL/6 mice for seven days. As shown in Figure 4, suppression of neovascularization in mice implanted with Matrigel containing merulin C was clearly observed, while analysis of the Matrigel plug of control mice

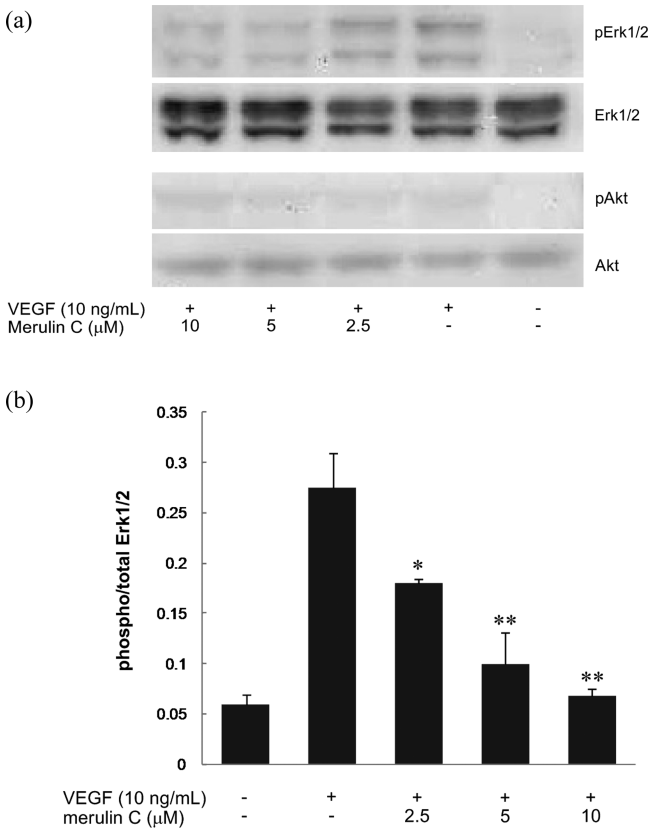


Figure 3. Effect of merulin C (**3**) on the phosphorylation of Erk1/2 and Akt in HUVECs. (a) HUVECs were pretreated with vehical and compound **3** at doses ranging from 2.5 to 10 μM for 1 h. (b) The levels of phosphorylated and total Erk1/2 protein were determined by Western blot analysis. Significantly different from control: $^{*}p < 0.05$ and $^{**}p < 0.01$.

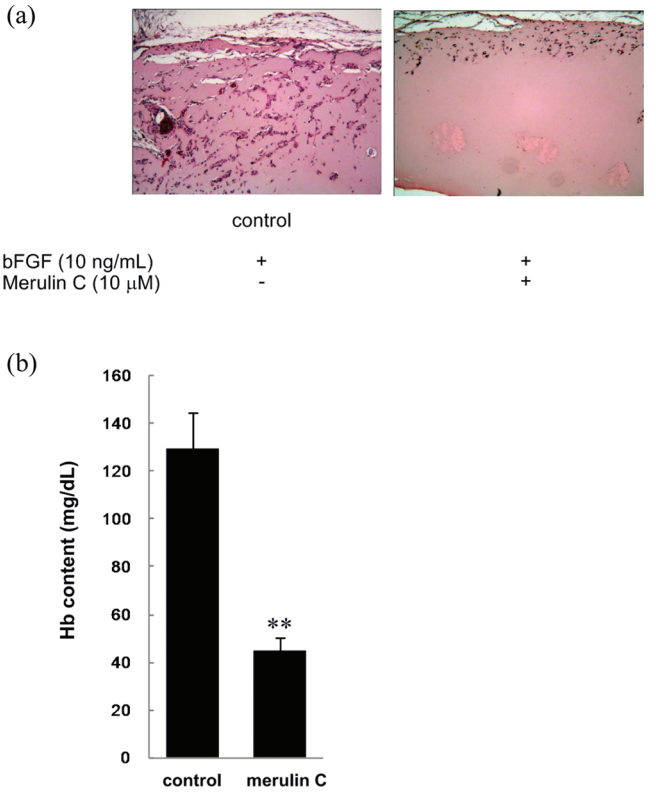


Figure 4. (a) Effect of merulin C (**3**) on bFGF-induced vessel formation in the Matrigel plug assay. (b) Measured hemoglobin content in the Matrigel plug. Significantly different from control: $^{**}p < 0.01$.

obviously indicated an angiogenic response. Furthermore the level of hemoglobin in the Matrigel plug was determined by a QuantiChrom hemoglobin assay kit. The results were in agreement with those of the histological analysis, with the hemoglobin content of the merulin C-containing gel being about one-third of that of the control one.

In conclusion, the present results indicate that merulin C, a chamigrane endoperoxide, possesses antiangiogenic activity, mainly by suppressing endothelial cell proliferation and migration, and its inhibitory effect is mediated by regulation of the Erk1/2 signaling pathway. Most importantly, merulin C even at low concentration inhibits neovessel formation in both ex vivo and in vivo assays. Taking the results together, merulin C might be a promising candidate for cancer therapy.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO DPI-370 digital polarimeter at a wavelength of 589 nm. NMR spectra were recorded with a Bruker AV400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) spectrometer using tetramethylsilane as an internal standard. HR-ESIMS were obtained from a Bruker micrOTOF mass spectrometer.

Fungal Material. The fungus (XG8D) used in this study was isolated from the leaves of *Xylocarpus granatum* collected from Samutsakorn Province, Thailand, in July 2008. On the basis of the 28S rDNA and ITS data (deposited in GenBank, accession nos. HM060640 and HM060641), the fungal strain was assigned to the family Meruliaceae (order Polyporales, subclass Incertaesedis, class Agaricomycetes, phylum Basidiomycota).[13]

Fermentation and Isolation. The fungus was grown on potato dextrose agar at room temperature for 10 days. The agar was cut into small plugs (1 cm diameter each) and inoculated in 100 Erlenmeyer flasks (1 L) containing 200 mL of corn steep liquor-containing medium (corn steep liquor 1 g, mannitol 10 g, maltose 10 g, glucose 5 g, monosodium glutamate 10 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, yeast extract 3 g, per liter). Each flask was inoculated with six plugs. After incubation at room temperature under static condition for 21 days, fungal cells were removed by filtration. The culture broth was directly extracted with EtOAc. The resulting EtOAc extract (18.90 g) was then subjected to Sephadex LH20 column chromatography and eluted with MeOH to yield seven fractions. Fraction 5 (3.06 g) was chromatographed on silica gel eluting with MeOH−CH_2Cl_2 (5:95) to yield 52 subfractions. Subfractions 33−50 (1.38 g) were combined and recrystallized from MeOH to afford merulin A (**1**, 420 mg), while subfractions 15−28 (0.86 g) were further purified by preparative HPLC (GL Science, ODS-3-A, 20 × 250 mm, flow rate 8 mL/min, MeCN−H_2O, 40:60) to yield merulin C (**3**, 130 mg). Fraction 4 (2.26 g) was subjected to flash column chromatography on silica gel (MeOH−CH_2Cl_2, 5:95), then purified further by a silica gel column (EtOAc−CH_2Cl_2, 4:1) to obtain merulin B (**2**, 45 mg). Fraction 2 (1.80 g) was fractionated by silica gel column chromatography (EtOAc−CH_2Cl_2, 4:1) to yield 30 subfractions. Subfractions 22−24 (0.32 g) were combined and purified further by silica gel column chromatography (EtOAc−CH_2Cl_2, 3:2) to afford steperoxide A (**5**, 32.0 mg). Fraction 6 (1.76 g) was subjected to silica gel column chromatography (MeOH−CH_2Cl_2, 5:95) and then rechomatographed over silica gel (acetone−hexane, 3:2) to yield merulin D (**4**, 7.4 mg).

Table 1. ^{1}H NMR (400 MHz), ^{13}C NMR (100 MHz), and HMBC Spectroscopic Data ($CDCl_3$) of Merulin D (4)

position	δ_C	δ_H, mult (J in Hz)	HMBC
1	29.1	2.04, m, H-1eq	3, 5, 6, 11
		1.70, dd (13.8, 1.8), H-1ax	5, 6, 7, 11
2	79.1	4.22, br m	3, 4, 6
3	72.7		
4	34.0	2.25, ddd (14.0, 13.2, 6.4), H-4ax	2, 3, 5, 15
		1.84, dd (14.0, 6.4), H-4eq	3, 5, 15
5	25.0	2.06, m, H-5eq	3, 4
		1.51, ddd (14.8, 14.0, 6.8), H-5ax	4, 6, 7
6	41.7		
7	90.4		
8	207.8		
9	35.6	2.68, ddd (15.4, 14.8, 6.6), H-9α	7, 8, 10
		2.44, ddd (15.4, 4.7, 2.3), H-9β	7, 8, 11
10	35.7	2.02, m, H-10β	9, 12, 13
		1.57, ddd (14.4, 6.7, 2.3), H-10α	8, 9, 13
11	37.3		
12	26.2	0.98, s	6, 10, 13
13	24.6	1.25, s	6, 10, 12
14	21.4	1.41, s	6, 7, 8
15	66.7	3.56, br s	2, 3, 4

*Merulin D (**4**):* colorless gum; $[\alpha]^{20}_D$ +160.7 (c 0.1 MeOH); ^{1}H and ^{13}C NMR, see Table 1; HRESIMS m/z 307.1519 $[M + Na]^+$ (calcd for $C_{15}H_{24}O_5Na$, 307.1521).

Cell Culture. HUVECs were purchased from Kurabo Industries (Osaka, Japan). Cells were grown in HuMedia EG2 medium containing 2% fetal bovine serum (FBS), 10 ng/mL of recombinant human epidermal growth factor, 1 μg/mL of hydrocortisone, 50 μg/mL of gentamicin, 50 ng/mL of amphotericin B, 5 ng/mL of recombinant human basic fibroblast growth factor (bFGF), and 10 μg/mL of heparin, at 37 °C in 5% CO_2. Subcultures were obtained by treating the HUVEC cultures with Hank's-based enzyme-free cell dissociation buffer solution. HUVECs at passages three to seven were used for the experiment.

Animals. Six-week-old specific pathogen-free male Wistar rats and C57BL/6 mice (Charles River Laboratory) were housed in a room with controlled temperature (25 °C) and a 12 h light/dark cycle (light on 8.00 A.M. to 8.00 P.M.). The animals were given free access to diet and deionized water. The rats and mice were maintained according to the Guide for the Care and Use of Laboratory Animals established by Hiroshima University. All experiments using animals were approved by Hiroshima University Animal Research Committee.

Ex Vivo Angiogenesis Assay. A male Wistar rat was sacrificed by bleeding from the right femoral artery under anesthesia with diethyl ether. The thoracic aorta was removed, washed with RPMI 1640 medium, turned inside out, and cut into 1 mm lengths. The aortic rings were then placed on six-well culture plates and covered with 0.5 mL of gel matrix solution (8 volume of porcine tendon collagen solution, 1 volume of 10× Eagle's MEM, and 1 volume of reconstitution buffer), then allowed to gel at 37 °C for 30 min. A 2 mL portion of RPMI 1640 medium containing 1% TIS+ with designated doses of compounds or vehicle (DMSO) was added to the wells. After incubation for 7 days at 37 °C in 5% CO_2, capillary length was estimated by phase-contrast microscopy by measuring the distance from the cut end of the aortic segment to the approximate midpoint of the capillary.

Tube Formation Assay. HUVEC tube formation assay was performed according to the method using BD Matrigel.[19] Solid gel was prepared on a 96-well tissue culture plate according to the manufacture's instructions. HUVECs (1×10^5 cells/mL) in HuMedia EG2 medium containing various doses of merulin C (**3**) or vehicle (DMSO) were seeded onto the surface on solid BD Matrigel. After incubation for 12 h at 37 °C in 5% CO_2, tube formation was observed under an inverted light microscope at 40× magnification. Microscopic fields were photographed with a digital camera (OLYMPUS DSE330-A system).

Proliferation Assay. A HUVEC suspension in HuMedia EG2 (1.5×10^4 cells/mL) was seeded onto each well of a 96-well plate (100 μL) and incubated for 24 h at 37 °C in 5% CO_2. The medium was removed and replaced with fresh HuMedia EG2 containing various doses of merulin C (**3**) or vehicle (DMSO) and incubated for 72 h at 37 °C in 5% CO_2. Cell proliferation was detected using WST-8 reagent, and the inhibition of proliferation was measured at 450 nm using a microplate reader.

Chemotactic Migration Assay. HUVEC migration assay was performed using a modified Boyden chamber.[14] A microporous membrane (8 μm) of 24-well cell culture inserts was coated with 0.1% gelatin. A HUVEC suspension in Medium 199 with 0.1% bovine serum albumin (BSA) (2.5×10^5 cells/mL) was seeded in each chamber (400 μL). The well was filled with 400 μL of Medium 199 containing 0.1% BSA and 10 ng/mL of human recombinant VEGF with or without merulin C (**3**). The assembled chamber was incubated for 6 h at 37 °C in 5% CO_2. Nonmigrated cells on the surface of the membrane were removed by scrubbing with a cotton swab. The migrated cells were fixed with methanol and stained with Diff-Quik stain (Sysmex, Kobe, Japan), then counted in three fields of each membrane under a microscope at 200× magnification.

Western Blotting. HUVECs were grown to confluence and starved for 16 h in RPMI 1640 containing 5% FBS. Cells were pretreated for 60 min with merulin C (2.5−10 μM) or vehicle and stimulated by the addition of VEGF (10 ng/mL) for 10 min. After stimulation, cells were washed with PBS twice and lysed with M-PER mammalian protein extraction reagent (Pierce, Rockford, IL, USA). Lysates were clarified by centrifugation for 15 min at 12 000 rpm at 4 °C. Protein concentration was determined using the microBCA protein assay kit (Pierce, Rockford, IL, USA) with BSA as standard. A 50 μg amount of protein was separated on SDS polyacrylamide gel under reducing conditions and transferred to a nitrocellulose membrane. After blocking, the membranes were incubated with primary antibody for phopho-Erk1/2 or phospho-Akt (Cell Signaling Technology, Tokyo, Japan) at 4 °C overnight and for Erk1/2 or Akt at room temperature, respectively. Immunoreactive bands were visualized by chemiluminescence using a WesternBreeze (Invitrogen) with a ChemiDoc XRS (BIO-RAD).

In Vivo Angiogenesis Assay. For the Matrigel plug assay, 0.5 mL of Matrigel (BD Biosciences) containing 10 μM merulin C or vehicle and bFGF (10 ng/mL) was injected subcutaneously into C57BL/6 mice. After injection, the Matrigel polymerized to form a plug. Three mice were used for each group. After 7 days, the animals were killed and the skin of each mouse was carefully pulled back to expose the Matrigel plug. The plugs were dissected out and fixed with 10% formaldehyde/phosphate-buffered saline and embedded in paraffin. The plugs were then sectioned and stained with hematoxylin−eosin stain. The amount of hemoglobin in the plugs was measured using a QuantiChrom hemoglobin assay kit (BioAssay Systems, Hayward, CA, USA). The concentration of hemoglobin was calculated from a known amount of hemoglobin assayed in parallel.

Statistical Analysis. Values are presented as means ± SEM. Data were analyzed by one-way analysis of variance followed by the Dunnet test or by Student's t test. Differences with $p < 0.05$ were considered significant.

■ ASSOCIATED CONTENT

Supporting Information. 1D and 2D NMR spectra and HRESIMS of compound **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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