Supporting Information

Cell Morphology Profiling of a Natural Product Library Identifies Bisebromoamide and Miuraenamide A as Actin Filament Stabilizers

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Experimental Procedures

Cell culture and screening

HeLa cells (RBRC-RCB0007, Riken BRC Cell Bank) were maintained in minimal essential medium, supplemented with 10% fetal bovine serum, at 37 ˚C under 5% CO_2. For morphology-based screening, HeLa cells were seeded on 96-well tissue culture plates at 5 x 10^3 cells per well. Cells were incubated for 24 h, then the medium was exchanged to include the test molecule at a specific concentration. The cells were incubated for 1 h at 37 ˚C, then bright-field images were captured by differential interference contrast (DIC) microscopy. Compounds which induced nuclear protruded morphology in >50% of the cells in the well were considered as screening hits. The experiment was repeated twice.

Actin polymerization/depolymerization assay

The actin polymerization/depolymerization assay was performed using Actin Polymeization Biochem Kit (Cytoskeleton, Inc.). For actin polymerization, 100 μL of pyrene labeled G-actin (0.05 mg mL^{-1}) in buffer (5 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP) was mixed with various concentrations of test compound dissolved in 1 μL DMSO. After 3 min, polymerization was initiated by adding polymerization buffer to give a final concentration of 5 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 50 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 1% DMSO, 0.2 mg mL^{-1} actin, and the test compound at a specific concentration. The time course of polymerization was monitored by fluorimeter (excitation at 365 nm, emission at 405 nm). Prior to beginning the actin depolymerization assay, G-actin was mixed with polymerization buffer to give a final concentration of 5 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 125 mM KCl, 5 mM $MgCl_2$, 5 mM ATP and 1 mg mL^{-1} actin. The mixture was incubated for 1 h at room temperature to obtain a 1 mg mL^{-1} F-actin stock. Depolymerization was initiated by diluting 10 μL of F-actin stock with 90 μL of a solution consisting of 5 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 1% DMSO, and the test compound at a specific concentration. The time course of depolymerization was monitored by fluorimeter (excitation at 365 nm, emission at 407 nm). Each assay was repeated twice and the representative results are shown.

Viability assay

HeLa cells were seeded on a 96-well tissue culture treated plate at a density of 5 x 10^3 cells/well. Cells were incubated for 24 h then the media was exchanged to a medium containing test molecule. The cells were incubated for another 24 h, and then viability was measured using Cell Counting Kit (DOJINDO). Briefly, the cells were incubated in a medium containing 10% WST-8 cell counting kit solution. After 1 h incubation at 37 ˚C, the absorbance of 450 nm was measured. Cell viability was calculated by defining DMSO treated cells as 100 % viable.

Chemicals

Wiskostatin and (-)-blebbistatin were purchased from Calbiochem. Actinomycin D, bleomycin hydrochloride, 5-fluorouracil, irinotecan hydrochloride trihydate, methotrexate, olomoucine, trichostatin A, thapsigargin, tunicamycin were purchased from Wako Pure Chemicals.

Isolation of pectenotoxin-2

Unidentified sponge (1.2 kg. wet wt) was collected at a depth of 0-3 m off the coast of the Maeda Promontory, Okinawa, Japan, in 2010. The specimens were extracted with MeOH (2 L). The extract was concentrated to give an aqueous mixture (20 mL), which was partitioned between EtOAc (500 mL x 3) and H_2O (500 mL). The extracts in EtOAc were concentrated, and the residue (1.5 g) was further partitioned between 90% MeOH (250 mL x 3) and hexane (200 mL). The aqueous MeOH portion (0.25 g) was chromatographed on silica gel, eluting with $CHCl_3$ and then 50 : 1 $CHCl_3$/MeOH. The fraction (15 mg) eluted with 50:l $CHCl_3$/MeOH was chromatographed on ODS (Cosmosil 75C_{18}-OPN, Nacalai tesque, step gradient eluting with 60%, 70%, 80%, 90%, 100% MeOH). The fraction (5.0 mg) eluted with 70-90% MeOH was separated by preparative HPLC (Inertsil ODS-3, 20 x 50 mm, gradient eluting with 60-100 %MeOH) to afford a fraction (1.2 mg, 1/2000 of the fraction induced nuclear protrusion of HeLa cells) containing pectenotoxin-2. The fraction was further separated by successive preparative HPLC (Inertsil ODS-3, 20 x 50 mm, gradient eluting with 70-100% MeOH containing 0.1% TFA; Inertsil ODS-3, 4.6 x 150 mm, 70% MeOH 0.1% TFA; Inertsil ODS-3, 4.6 x 150 mm, 75% MeOH 0.1% TFA) to give pure pectenotoxin-2 (0.1 mg from the wet animals in a yield of 8.3 x 10^{-6}%) as a colorless oil: t_R = 22.2 min [Inertsil ODS-3, 4.6 x 150 mm, 70% MeOH 0.1% TFA,

flow rate 1.0 mL/min. detection at 215 nm]; ESIMS *m/z* 881 $[M+Na]^+$, HRFABMS *m/z* $[M+H]^+$ 859.4813 (calcd for $C_{47}H_{70}O_{14}$+H 859.4844). ^{1}H NMR and ^{13}C NMR data, see Yasumoto, T., Murata, M., Oshima, Y., Sano, M., Matsumoto, G. K., and Clardy, J. (1985) Diarrhetic shellfish toxins, *Tetrahedron 41*, 1019–1025.

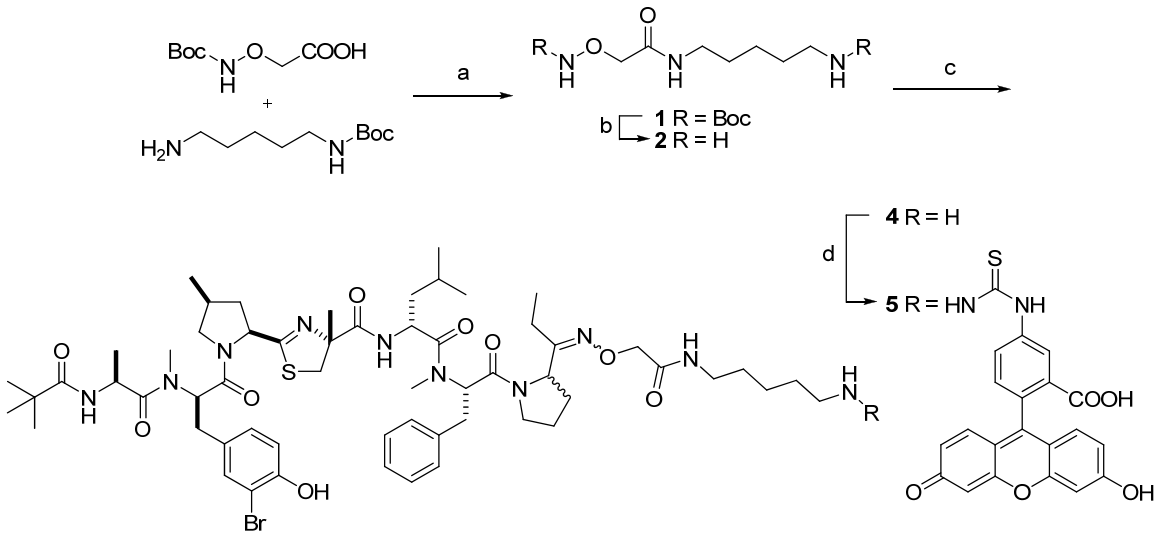
Isolation of lyngbyabellin C

Unidentified sponge (1.5 kg. wet wt) was collected at a depth of 0-3 m off the coast of the Bise Promontory, Okinawa, Japan, in 2010. The specimens were extracted with MeOH (2 L). The extract was concentrated to give an aqueous mixture (30 mL), which was partitioned between EtOAc (500 mL x 3) and H_2O (500 mL). The extracts in EtOAc were concentrated, and the residue (3.5 g) was further partitioned between 90% MeOH (200 mL x 3) and hexane (200 mL). The aqueous MeOH portion (1.0 g) was chromatographed on silica gel, eluting with $CHCl_3$ and then 20:1 $CHCl_3$/MeOH. The fraction (23 mg) eluted with 20:l $CHCl_3$/MeOH was chromatographed on ODS (Cosmosil 75C_{18}-OPN, Nacalai tesque, step gradient eluting with 60%, 70%, 80%, 90%, 100% MeOH). The fraction (10.2 mg) eluted with 70% MeOH was separated by preparative HPLC (Inertsil ODS-3, 20 x 50 mm, gradient eluting with 50-80% MeOH) to afford a fraction (1.2 mg, 1/2000 of the fraction induced nuclear protrusion of HeLa cells) containing lyngbyabellin C. The fraction was further separated by successive preparative HPLC (YMC-Pack ODS-A, 150 x10 mm, gradient eluting with 75-100% MeOH containing 0.1% trifluoroacetic acid; Inertsil ODS-3, 4.6 x 150 mm, 60% MeOH 0.1% TFA) to give pure lyngbyabellin C (0.02 mg from the wet animals in a yield of 1.3 x 10^{-6}%) as a colorless oil: t_R = 22.1 min [Inertsil ODS-3, 4.6 x 150 mm, 60% MeOH 0.1% TFA, flow rate 1.0 mL/min. detection at 215 nm]; ESIMS *m/z* 609/611/613 (100:75:20, $[M+H]^+$ ion cluster), *m/z* 631/633/635 (100:75:20, $[M+Na]^+$ ion cluster), HRFABMS *m/z* $[M+H]^+$ 609.0892, 611.0868, 613.0875 (calcd for $C_{24}H_{30}Cl_2N_2O_8S_2$+H, 609.0899, 611.0874, 613.0854).^{1}H NMR and ^{13}C NMR data, see Luesch, H., Yoshida, W. Y., Moorea, R. E., and Paul, V. J. (2002) Structurally diverse new alkaloids from Palauan collections of the apratoxin-producing marine cyanobacterium *Lyngbya* sp., *Tetrahedron 58*, 7959–7966.

Synthesis of bisebromoamide-fluorescein conjugate

General Procedures.

The solvents used for chemical synthesis were dried prior to use. Thin-layer chromatography was carried out with glass TLC plates precoated with Merck silica gel 60 F254. Column chromatography was performed with Fuji Silysia Chemical silica gel BW820MH. Proton nuclear magnetic resonance spectra were recorded in deuterated solvents at 300 MHz. High-resolution mass spectra were obtained with a JEOL JMS-700 spectrometer.



Scheme Conditions: (a) DCC, Et_3N, THF, rt. 10 h (b) TFA, $CHCl_3$, rt. 1 h
(c) bisebromoamide, MeOH, 80 °C, 0.5 h (d) fluorescein isothiocyanate, Et_3N, rt, 1 h

Synthesis of 1

To a solution of Boc-aminooxy acetic acid (0.20 g, 1.1 mmol) and triethylamine (0.5 mL) in THF (10 mL) were added *N*,*N'*-dicyclohexylcarbodiimide (0.26 g, 1.3 mmol) and mono-*N*-Boc-1,5-pentanediamine (0.23 g, 1.2 mmol). This solution was stirred at room temperature overnight, diluted with brine, and extracted with ethyl acetate (3 × 50 mL). The combined extracts were dried over Na_2SO_4 and then concentrated in vacuum. The residue was purified by column chromatography on silica gel with Hexane/Ethyl acetate mixtures to give the corresponding amide **1** (0.22 g, 57%) as a colorless oil. ^{1}H NMR (CD_3OD, 300 MHz) δ_H 4.23 (s, 2H), 3.25 (t, *J*=6.2 Hz, 2H), 3.02 (dd, *J*=5.2 11.4 Hz, 2H), 1.61-1.32 (m, 24H); HRMS (FAB) Exact mass calcd for $C_{17}H_{33}N_3O_6$ + H requires *m/z* 376.2448 Found *m/z* 376.2447

2

To a solution of **1** (0.21 g, 0.56 mmol) in $CHCl_3$ (3 mL) was added TFA (3 mL). This solution was stirred at room temperature for 1 h and then concentrated in vacuum to give **2** (0.23 g, quant) as a trifluoroacetic acid salt, which was used without further purification for the next reaction. ^1H NMR (CD_3OD, 300 MHz) δ_H 4.53 (s, 2H), 3.27 (t, *J*=6.4 Hz, 2H), 2.92 (t, *J*=7.2 Hz, 2H), 1.73-1.53 (m, 4H), 1.47-1.40 (m, 2H); HRMS (FAB) Exact mass calcd for $C_7H_{17}N_3O_2$ + H requires *m/z* 176.1399 Found *m/z* 176.1397

4

To a solution of Bisebromoamide (**3**) (5.0 mg, 4.9 μmol) in MeOH (3 drops) was added amine **2** (39 mg, 100 μmol). The reaction mixture was stirred at 80 ºC for 0.5 h, and then diluted with water (0.5 ml). This reaction mixture was purified by reversed-phase column chromatography (Cosmosil 75C_{18}-OPN, 20%~100% MeOH) to give **4** (5.0 mg, 87 %) as a mixture of four stereoisomers (isomers of α position of ketone and *E/Z* stereoisomers of ketoxime). The purity of compound **4** was confirmed by reverse-phase HPLC analysis. The identity was verified by mass spectral analysis of the isolated compound. HRMS (FAB) Exact mass calcd for $C_{58}H_{87}BrN_{10}O_9S$ + H requires *m/z* 1179.5640, 1181.5635 Found *m/z* 1179.5637, 1181.5636

5

To a solution of compound **4** (1.0 mg, 0.85 μmol) and triethylamine (0.05 mL) in DMSO (0.2 mL) was added fluoresceinisothiocyanate (0.50 mg, 1.3 μmol). This solution was stirred at room temperature for 1h, and purified by reversed-phase HPLC (ODS, 80% CH_3CN in 0.1% TFA) to give **5** (0.86 mg, 55%) as a mixture of four stereoisomers (isomers of α position of ketone and *E/Z* stereoisomers of ketoxime). The purity of compound **5** was confirmed by reverse-phase HPLC analysis. The identity was verified by mass spectral analysis of the isolated compound. HRMS (FAB) Exact mass calcd for $C_{79}H_{98}BrN_{11}O_{14}S_2$ + H requires *m/z* 1568.5998, 1570.5977, 1569.6031, 1571.6011 Found *m/z* 1568.6366, 1570.6323, 1569.6376, 1571.6396

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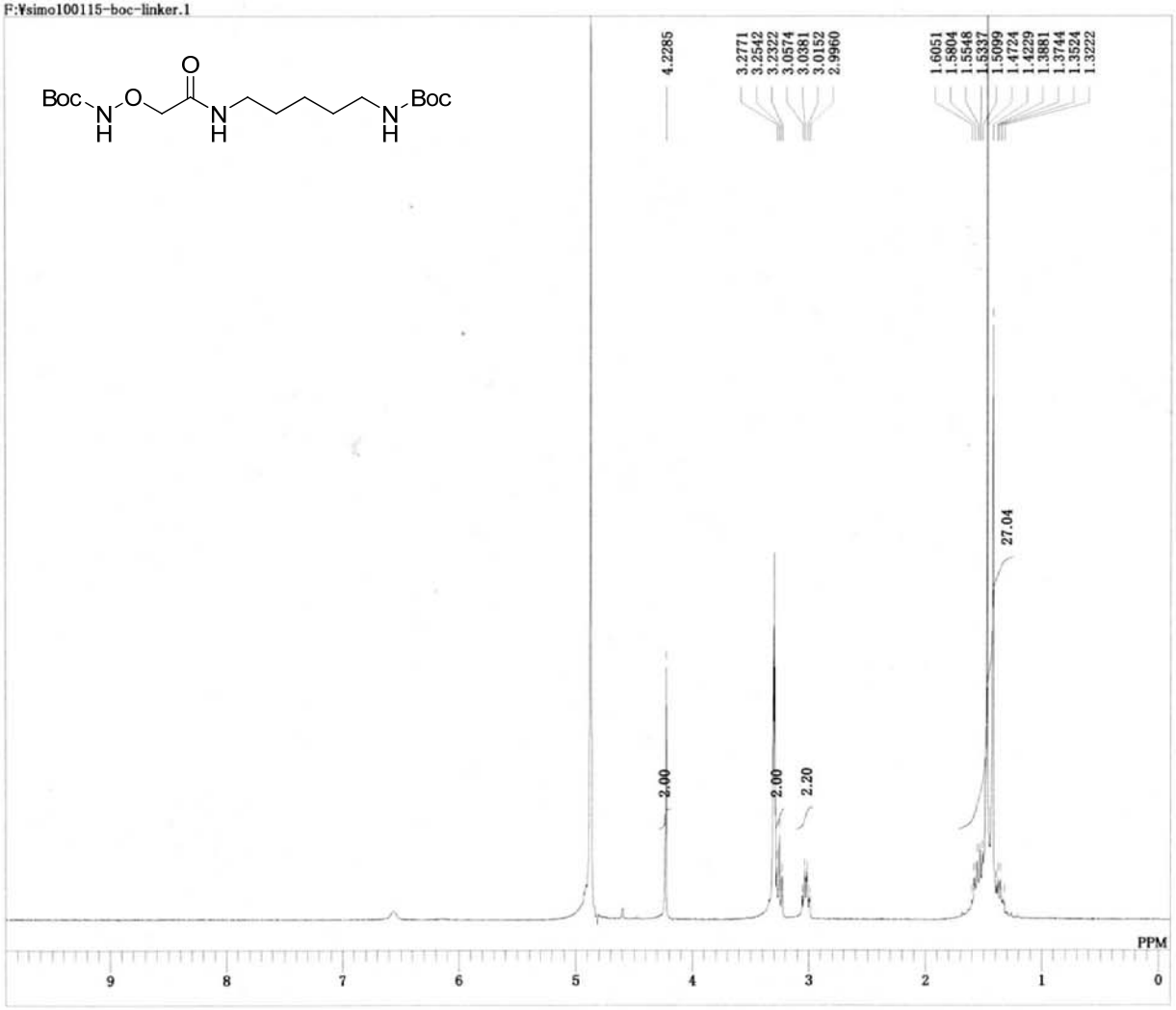

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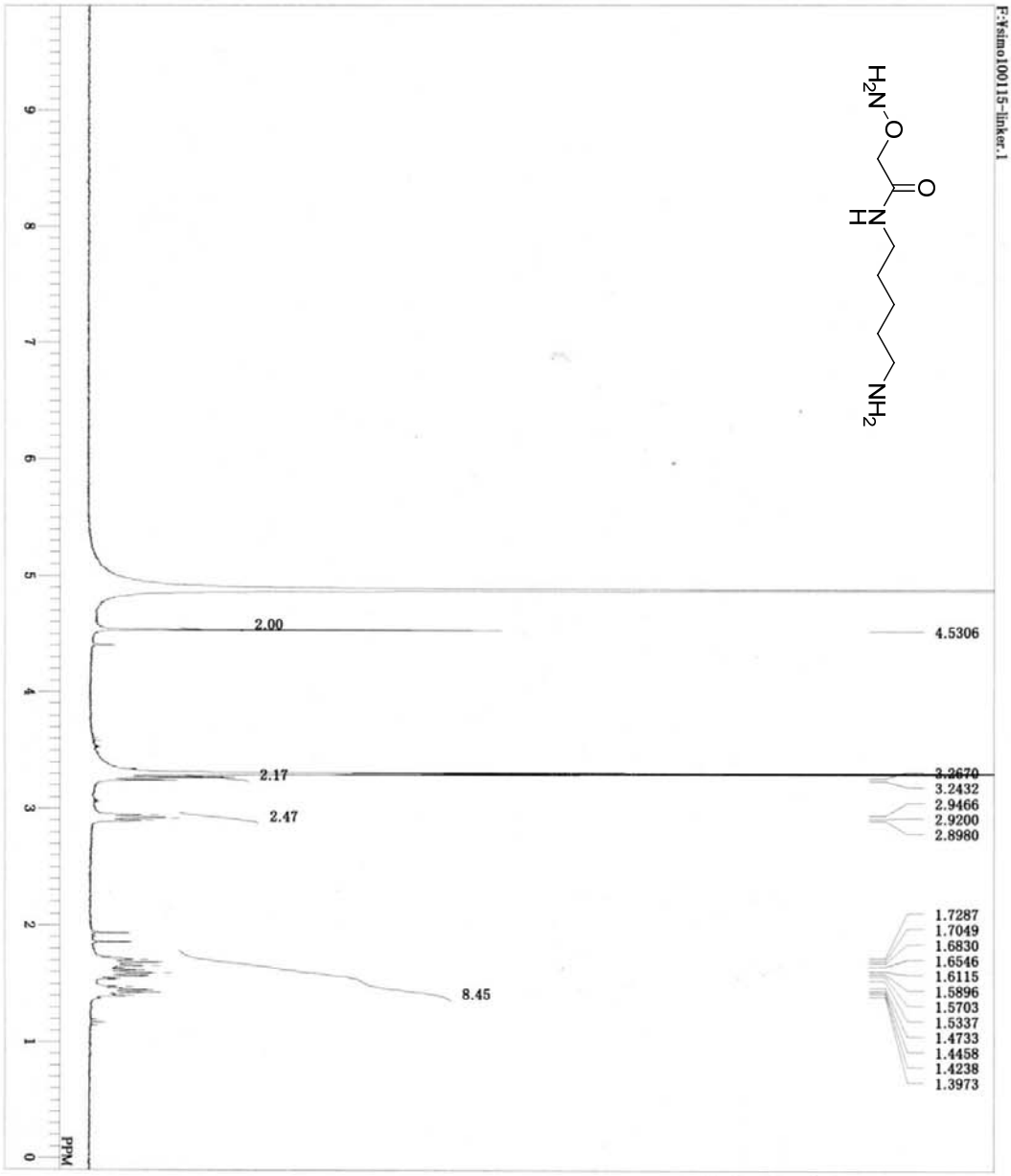
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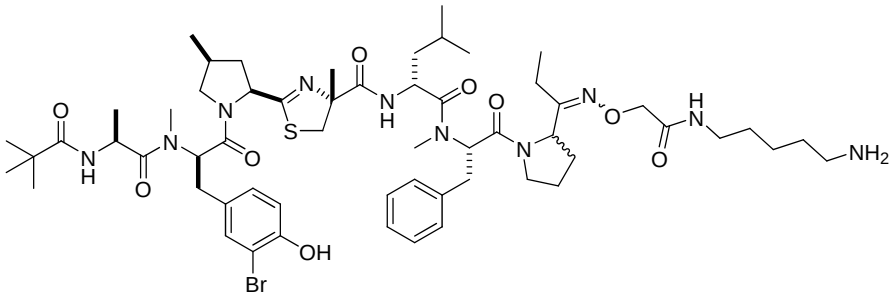

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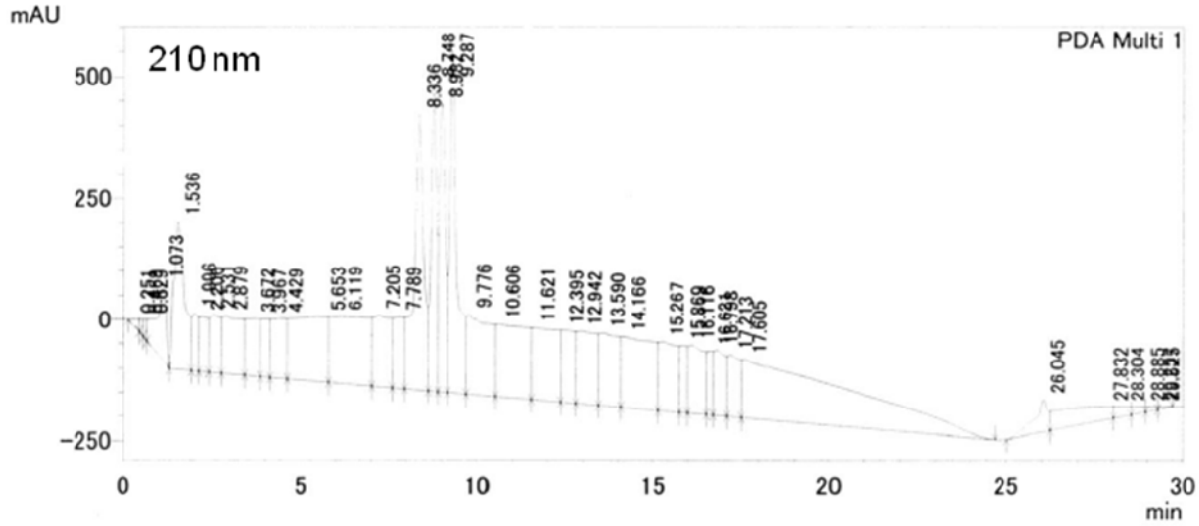

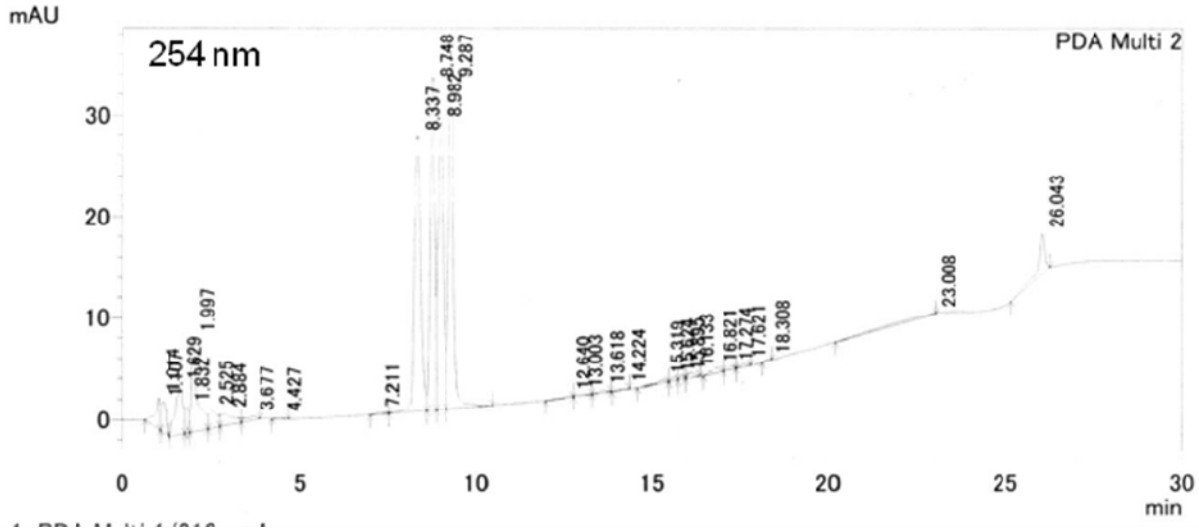

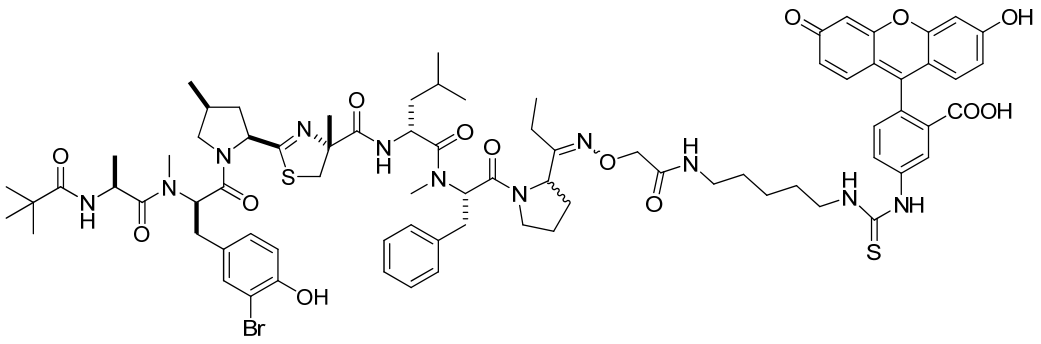

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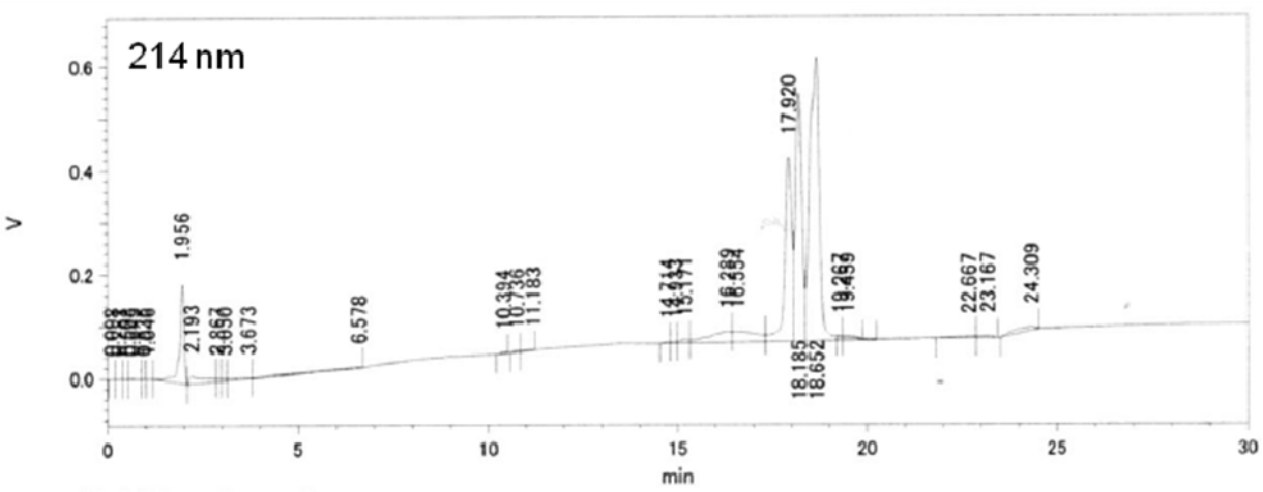

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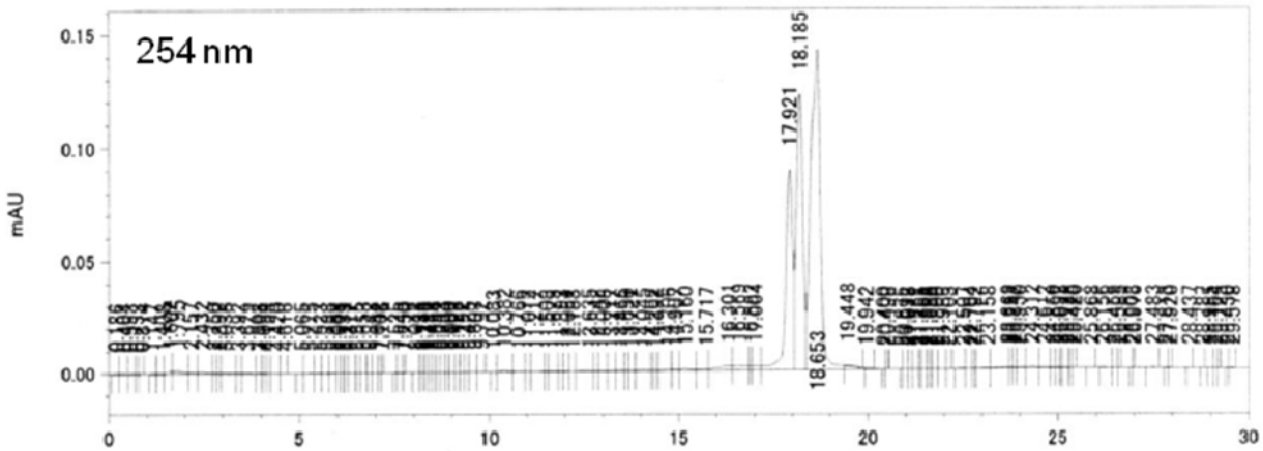

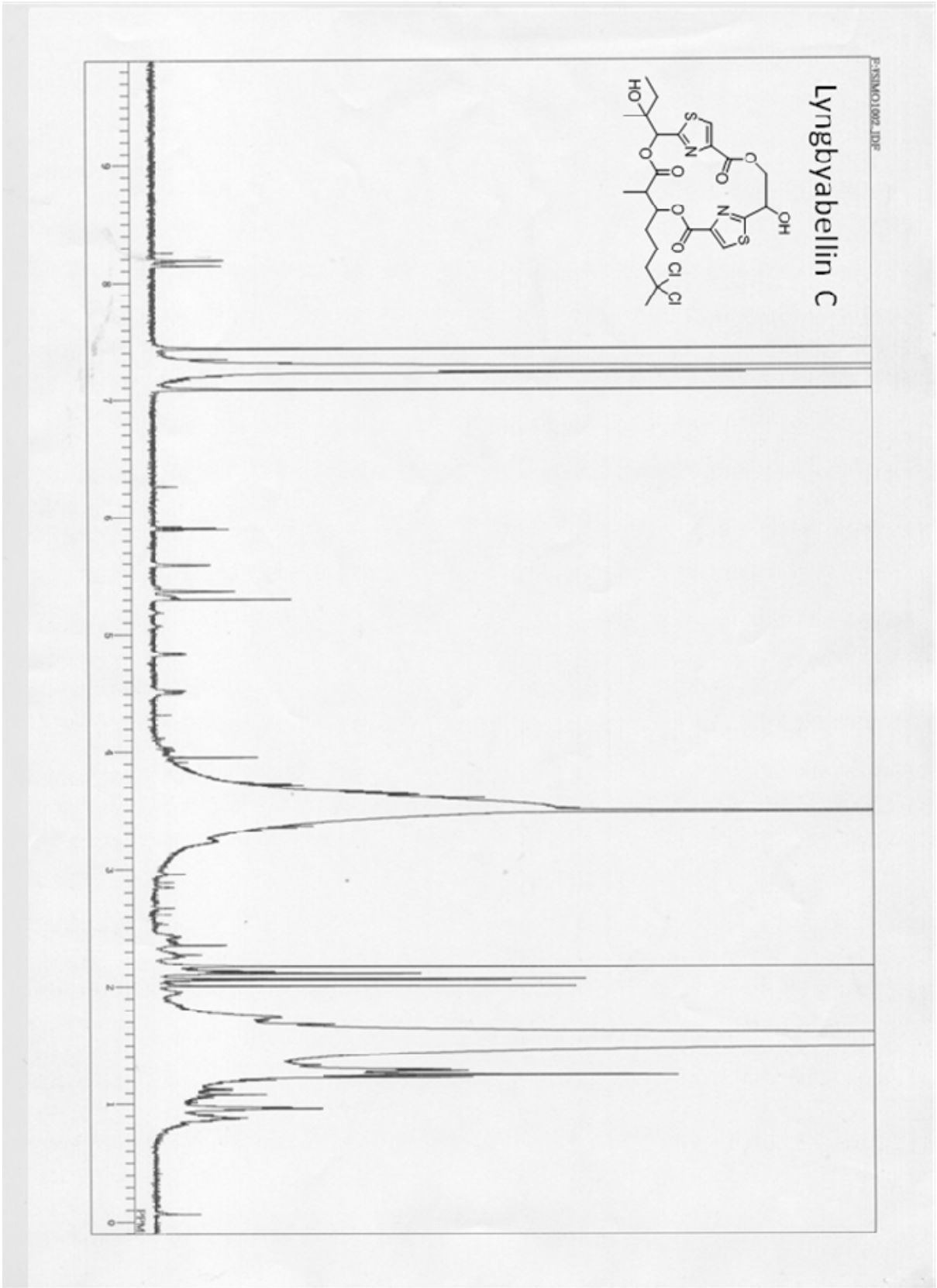
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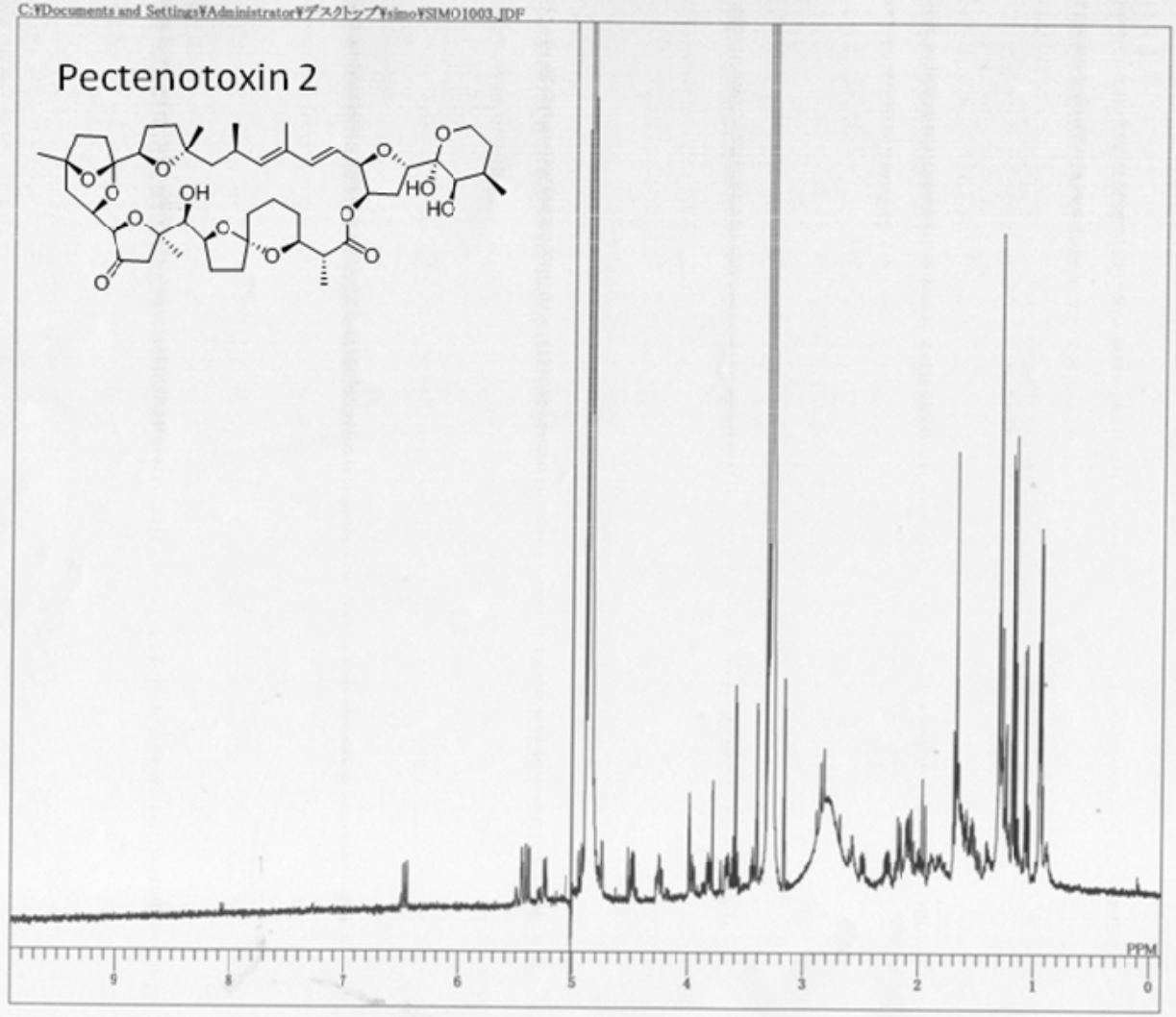

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Supplementary Figure Legends

Supplementary Figure S1. DIC and fluorescent images of HeLa cells treated with cytotoxic compounds. HeLa cells were treated with 100 nM mycalolide B, 100 nM swinholide A, 1 μM doliculide, 10 μM phalloidin, 100 nM seragamide A, 1 μM actinomycin D, 10 μM blebbistatin, 100 μg/mL bleomycin, 100 μM colchicine, 100 μM 5-fluorouracil, 100 μM irinotecan, 1 mM methotrexate, 100 μM oloumoucin, 100 μM paclitaxel, 1 μM trichostatin A, 1 μM thapsigargin, 10 μM tunicamycin for 1 h. Then the cells were immunostained with anti-α-tubulin antibody (shown in green) and co-stained with Hoechst 33342 (shown in blue) and phalloidin-rhodamine (shown in red). Lower panel shows magnified images of the boxed area. Scale bar = 20 μm.

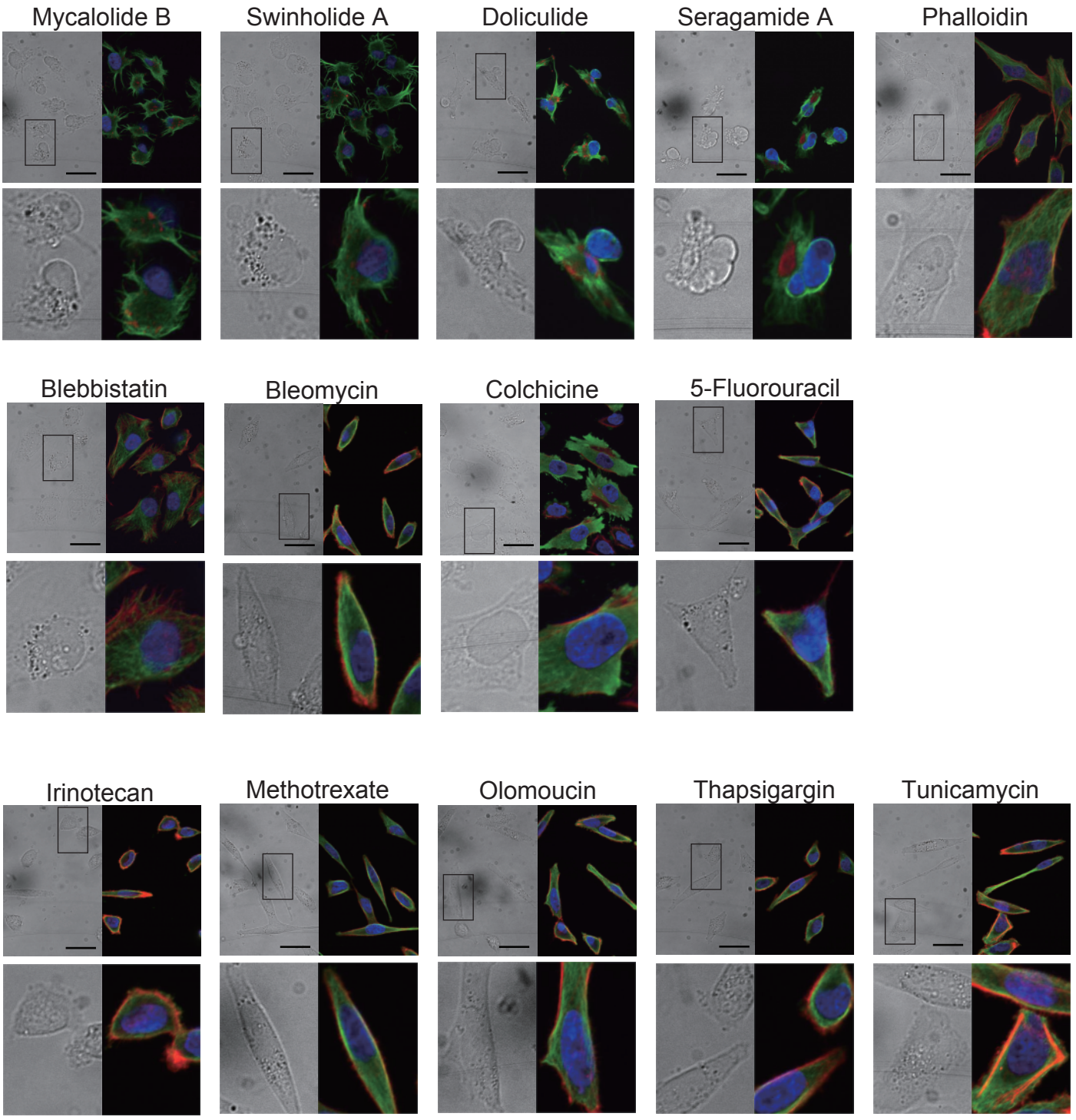
Supplementary Figure S2. Characterization of morphological alteration induced by jasplakinolide.

a) Dose response of cells exhibiting morphological alteration. HeLa cells were treated with 15.6, 62.5, 125, 250 or 500 nM jasplakinolide for 1 h. The morphological alteration was categorized into 3 types and the number of cells which belong to each type was counted by eye. Type 1: No morphological change or weak blebbing of the plasma membrane. Type 2: Nuclear-protruded morphology. Type 3: Completely retracted cytosol, and only the nucleus is visible. For each concentration 4 images containing a total of >100 cells were analyzed. Illustration and DIC image of an example of cell morphology type 1-3 is shown. b,c) Dose-response curves of cells treated with jasplakinolide analyzed by automated cell image analysis. HeLa cells were treated with 15.6, 62.5, 125, 250 or 500 nM jasplakinolide for 1 h. Distance between centroids of nucleus and cell (b) and the area of cytoplasm (c) was measured. Each plot represents mean ± S.D. 10 images, each containing 40 cells on average, was analyzed. 100 nM, in which the cells show large nucleus-cell centroid distance, was employed for the cell image analysis in Figure 1 panel b.

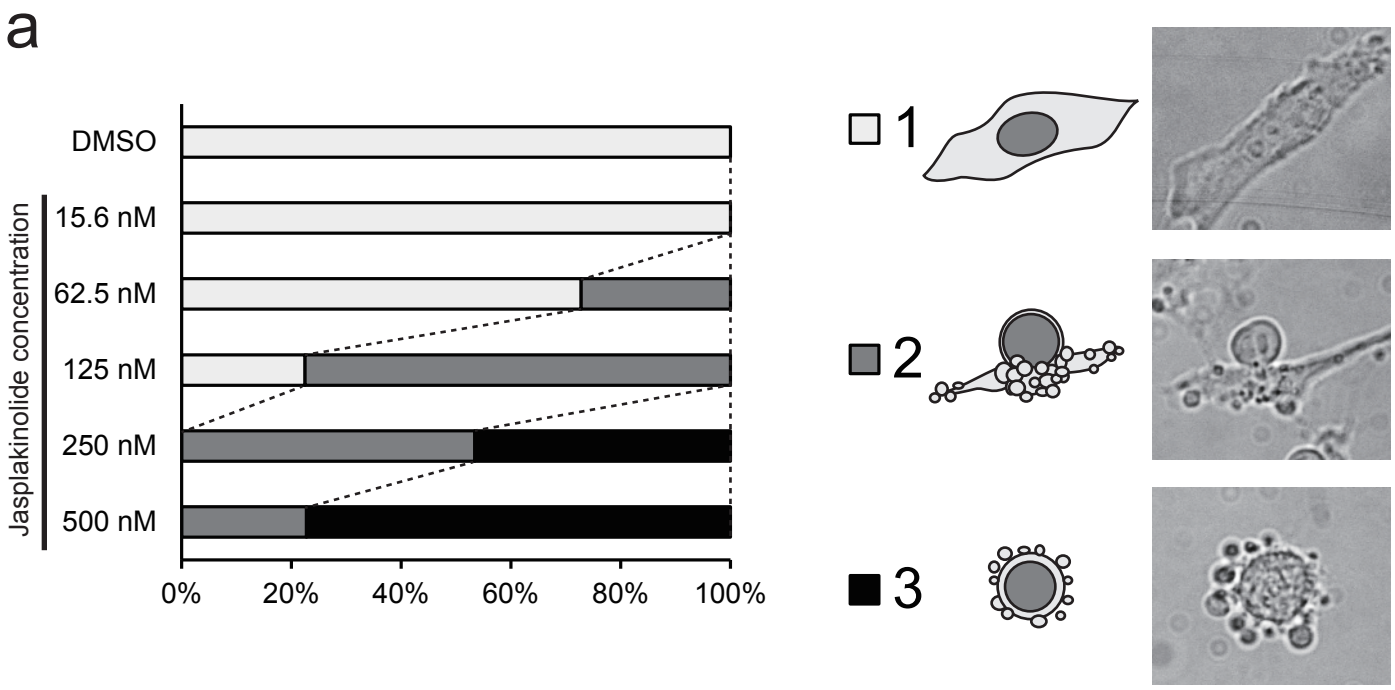
Supplementary Figure S3. Bioactivity of bisebromoamide derivatives.

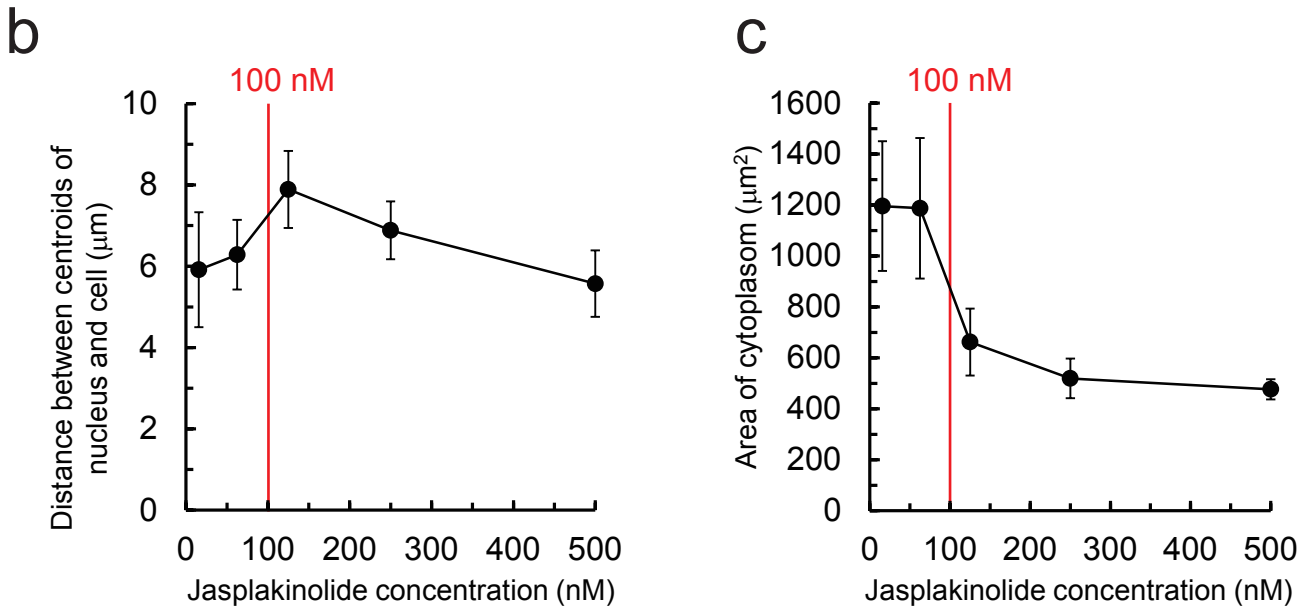
a) HeLa cells were treated with bisebromoamide or Bise-Flu at the indicated concentrations for 24 h. Cell viability was measured by the WST-8 method. Data are represented as mean of three

independent experiments ± SD. b) Spectra of pyrene-labeled G-actin mixed with Bise-Flu. Ex: 365 nm c) Spectra of pyrene-labeled F-actin mixed with Bise-Flu. Ex: 365 nm. d,e) Effect of Bise-linker on *in vitro* actin polymerization (d) and depolymerization (e). f) Structure of Bise-linker. g) C2C12, HEK293, SK-BR-3 and NIH/3T3 cells were fixed, permeablized and co-stained with 10 nM Bise-Flu and 165 nM rhodamine phalloidin (Phal-Rhod). Green: Bise-Flu; red: rhodamine phalloidin (Phal-Rhod); ψελλοω: μεργεδ ιμαγε. Σχαλε βαρ = 20μm.

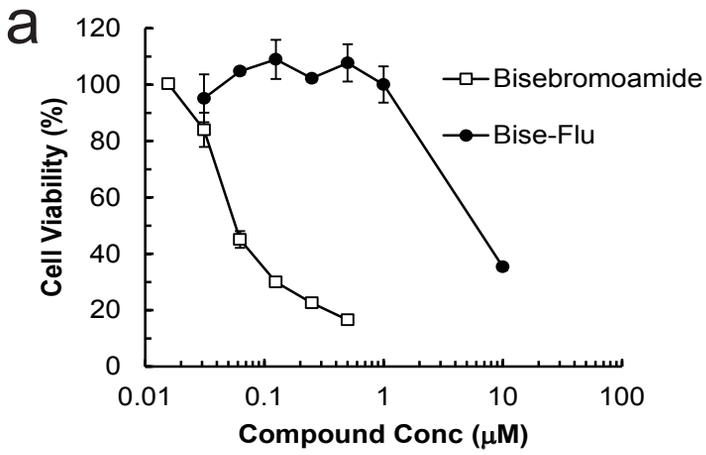


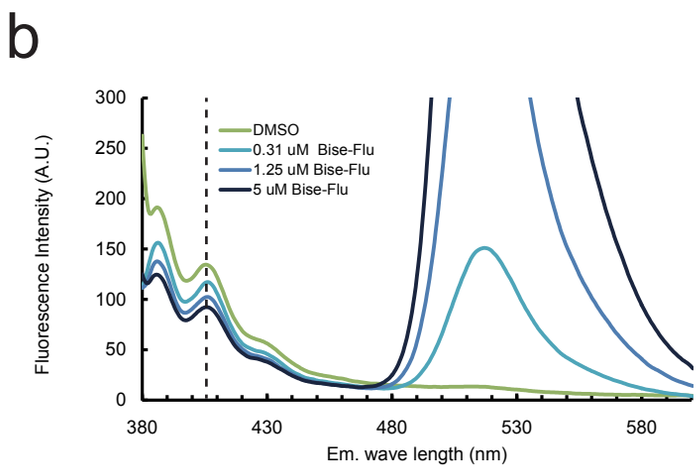
Supplementary Figure S1

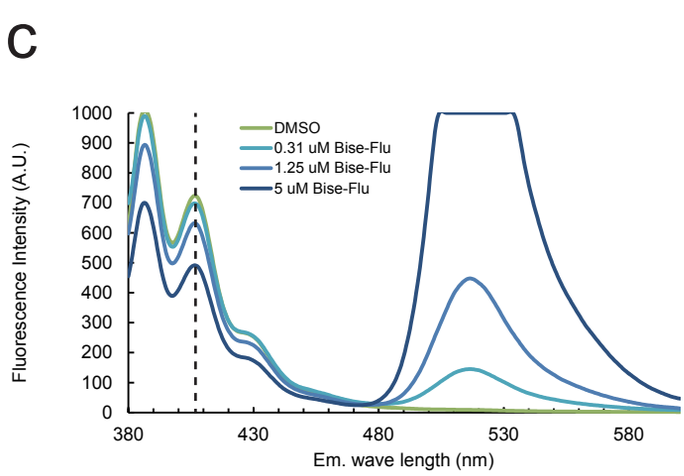


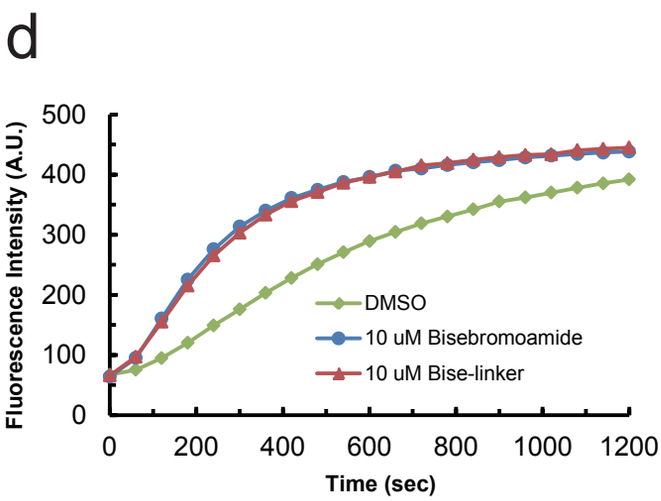


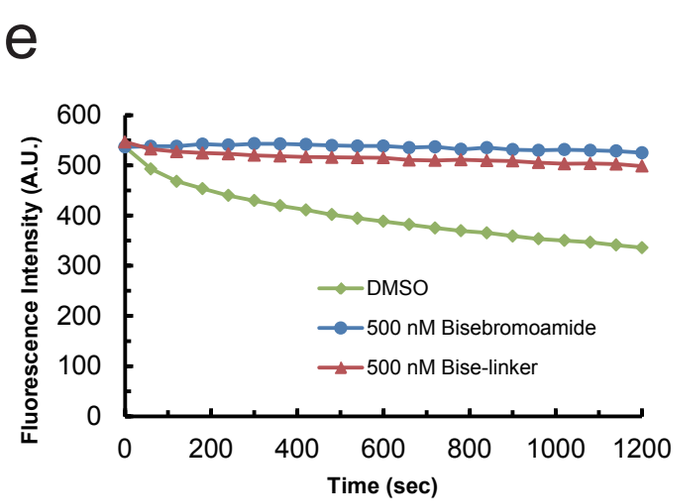
Supplementary Figure S2

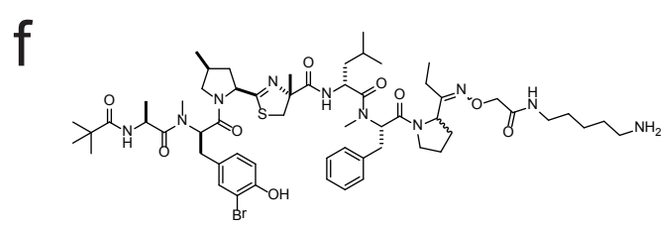












Supplementary Figure S3

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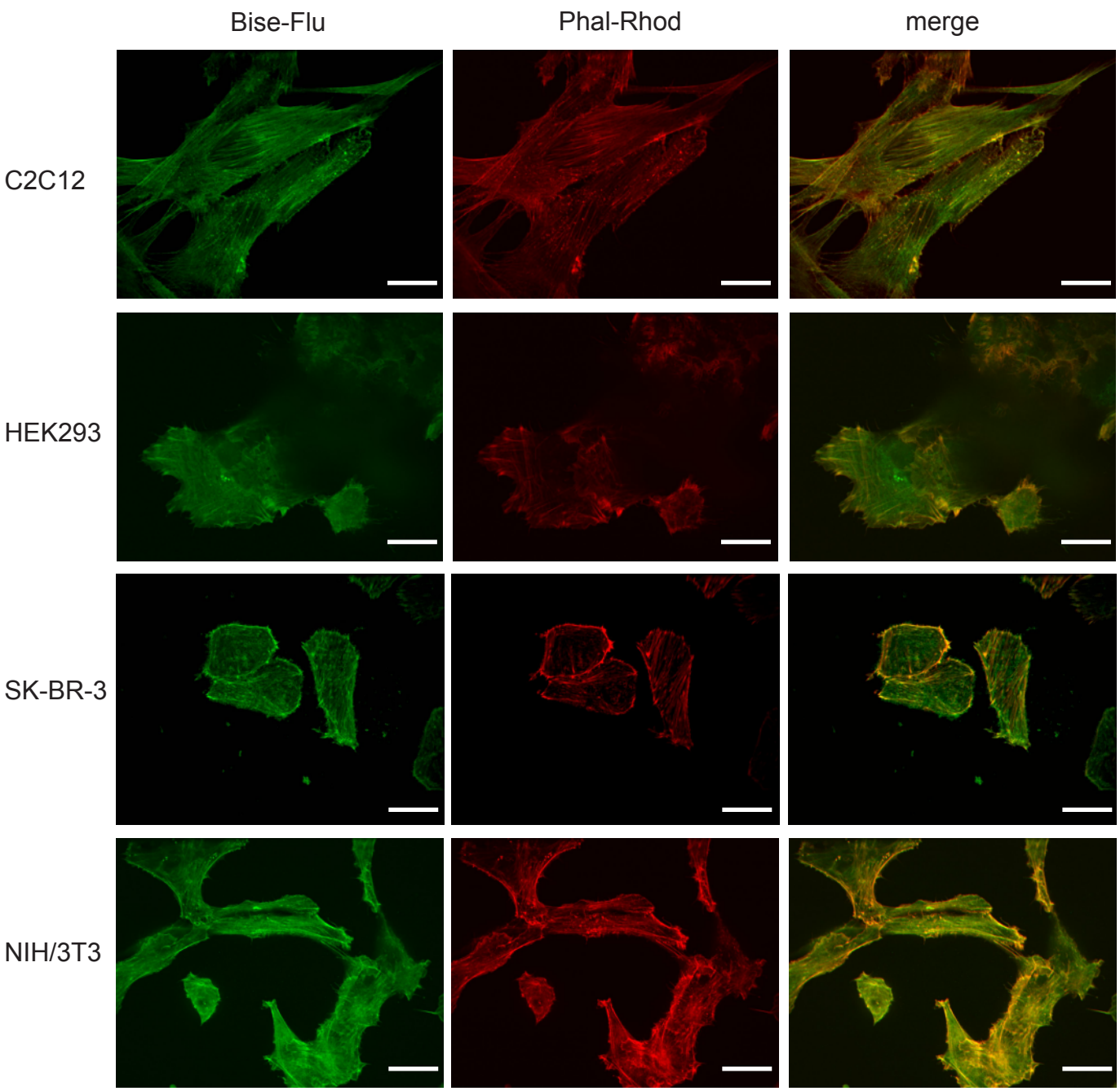

Supplementary Figure S3