Supplementary Information

Gramicidin A Accumulates in Mitochondria, Reduces ATP Levels, Induces Mitophagy, and Inhibits Cancer Cell Growth

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25 pages

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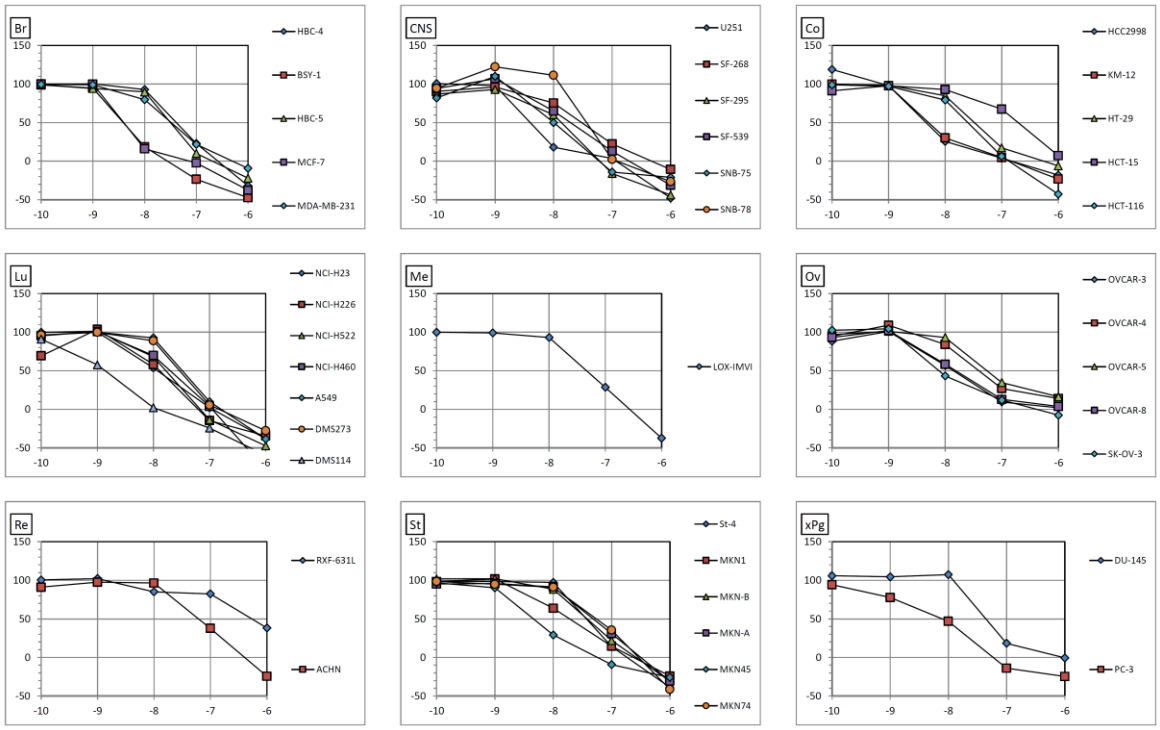
Supporting Figures

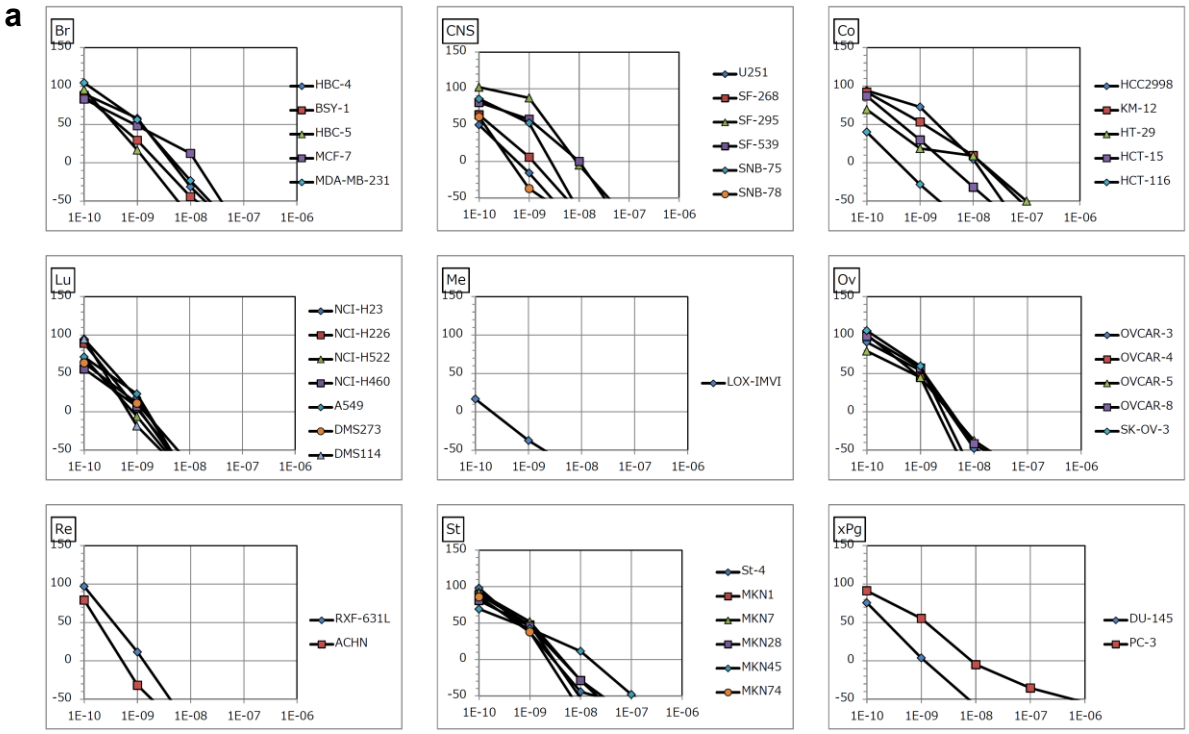
a



b

	CELL LINE	Log GI50	Log TGI	Log LC50
Br	*			
	HBC-4	-7.39	-6.58	-6.00
	BSY-1	-8.41	-7.56	-6.00
	HBC-5	-7.50	-6.69	-6.00
	MCF-7	-8.40	-7.12	-6.00
	MDA-MB-231	-7.49	-6.30	-6.00
CNS	*			
	U251	-8.40	-6.93	-6.00
	SF-268	-7.52	-6.32	-6.00
	SF-295	-7.87	-7.21	-6.00
	SF-539	-7.71	-6.70	-6.00
	SNB-75	-8.00	-7.22	-6.00
	SNB-78_	-7.44	-6.92	-6.00
Co	*			
	HCC2998	-8.34	-6.83	-6.00
	KM-12	-8.29	-6.83	-6.00
	HT-29	-7.48	-6.26	-6.00
	HCT-15	-6.71	-6.00	-6.00
	HCT-116	-7.60	-6.87	-6.00
Lu	*			
	NCI-H23_	-7.93	-6.95	-6.00
	NCI-H226	-7.89	-7.20	-6.00
	NCI-H522	-7.78	-7.17	-6.00
	NCI-H460	-7.70	-6.95	-6.34
	A549	-7.49	-6.79	-6.00
	DMS273	-7.53	-6.83	-6.00
	DMS114	-8.86	-7.92	-6.27
Me	*			
	LOX-IMVI_	-7.33	-6.57	-6.00
Ov	*			
	OVCAR-3	-7.86	-6.00	-6.00
	OVCAR-4	-7.40	-6.00	-6.00
	OVCAR-5	-7.26	-6.00	-6.00
	OVCAR-8	-7.82	-6.00	-6.00
	SK-OV-3	-8.11	-6.38	-6.00
Re	*			
	RXF-631L_	-6.27	-6.00	-6.00
	ACHN	-7.21	-6.39	-6.00
St	*			
	St-4_	-7.43	-6.74	-6.00
	MKN1	-7.72	-6.62	-6.00
	MKN-B	-7.42	-6.58	-6.00
	MKN-A	-7.31	-6.50	-6.00
	MKN45	-8.34	-7.24	-6.00
	MKN74	-7.26	-6.54	-6.00
xPg	*			
	DU-145	-7.36	-6.03	-6.00
	PC-3	-8.11	-7.23	-6.00
	*			
MG-MID		-7.69	-6.69	-6.02
Delta		1.17	1.23	0.33
Range		2.60	1.92	0.34

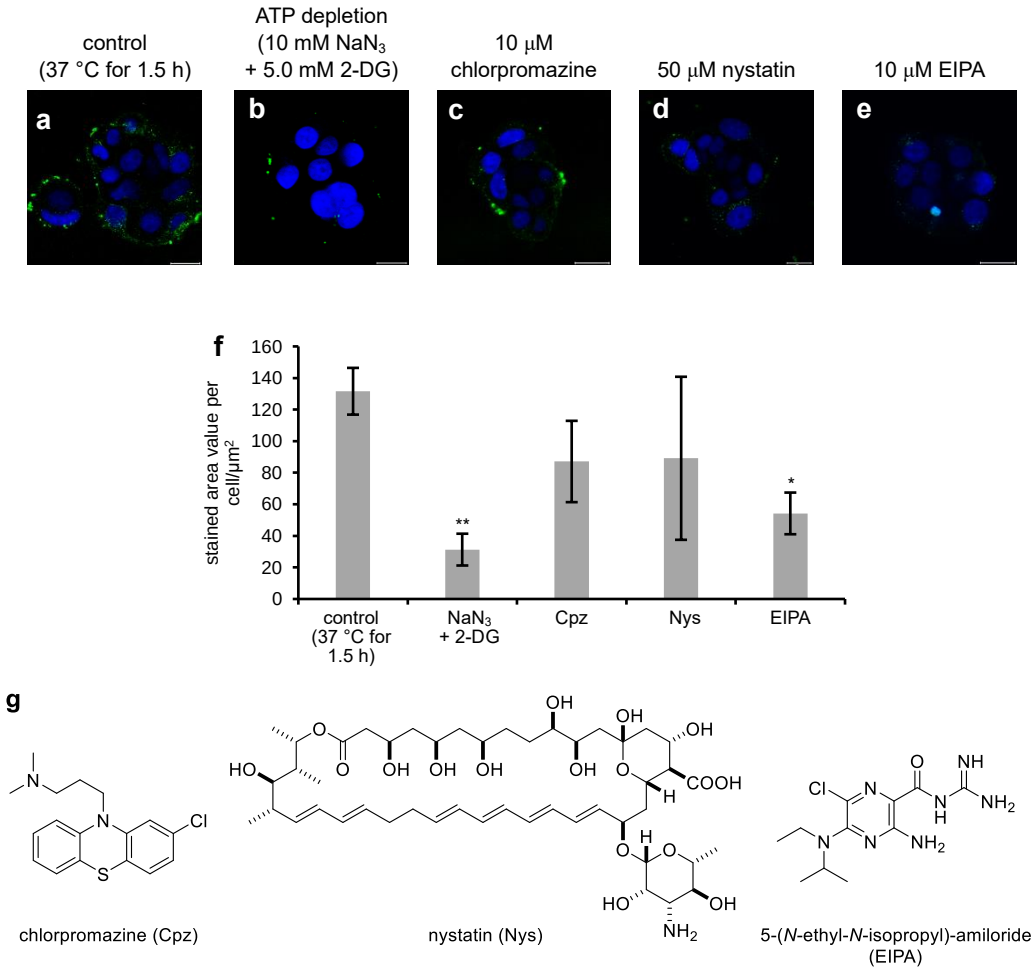
Figure S1. JFCR39 cancer cell panel assay of gramicidin A (**1**). (a) Concentration-response curves obtained by sulforhodamine B assays. The cells were incubated for 2 d in the presence of **1**, and the cell growth (%) was evaluated. Each plot is displayed as mean of two replicates. (b) Obtained $\log GI_{50}$, logTGI (total growth inhibition), and $\log LC_{50}$ values of **1**. MG-MID: mean of obtained $\log GI_{50}$, Delta: difference between $\log GI_{50}$ of the cell line exhibiting the highest sensitivity and MG-MID, Range: difference between $\log GI_{50}$ values of the cell lines exhibiting the highest sensitivity and lowest sensitivity, TGI: concentrations that cause cell growth (%) = 0%.



b

	CELL LINE	Log GI50	Log TGI	Log LC50
Br	*			
	HBC-4	-8.83	-8.27	-7.65
	BSY-1	-9.28	-8.52	-7.81
	HBC-5	-9.35	-8.73	-8.16
	MCF-7	-8.97	-7.81	-7.34
	MDA-MB-231	-8.85	-8.22	-7.56
CNS	*			
	U251	-9.92	-8.20	-8.48
	SF-268	-9.68	-8.85	-8.19
	SF-295	-8.52	-7.98	-7.33
	SF-539	-8.78	-7.92	-7.42
	SNB-75	-8.90	-8.49	-8.07
	SNB-78	-9.81	-7.78	-8.65
Co	*			
	HCC2998	-8.59	-7.88	-7.37
	KM-12	-8.87	-7.78	-7.03
	HT-29	-9.54	-7.77	-6.92
	HCT-15	-9.28	-8.44	-7.61
	HCT-116	-9.92	-7.68	-8.56
Lu	*			
	NCI-H23	-9.33	-8.75	-8.28
	NCI-H226	-9.46	-8.87	-8.29
	NCI-H522	-9.65	-8.69	-8.40
	NCI-H460	-9.80	-8.85	-8.33
	A549	-9.48	-8.72	-8.28
	DMS273	-9.66	-8.78	-8.15
	DMS114	-9.53	-8.41	-8.48
Me	*			
	LOX-IMVI	-9.92	-6.84	-8.63
Ov	*			
	OVCAR-3	-8.89	-8.39	-7.87
	OVCAR-4	-9.01	-8.61	-8.26
	OVCAR-5	-9.07	-8.37	-7.65
	OVCAR-8	-8.85	-8.34	-7.74
	SK-OV-3	-8.85	-8.50	-8.15
Re	*			
	RXF-631L	-9.37	-8.81	-8.31
	ACHN	-9.66	-8.06	-8.65
St	*			
	St-4	-9.13	-8.46	-7.63
	MKN1	-8.99	-8.45	-7.95
	MKN7	-8.90	-8.28	-7.60
	MKN28	-9.05	-8.31	-7.50
	MKN45	-9.21	-7.73	-6.89
	MKN74	-9.17	-8.57	-8.11
xPg	*			
	DU-145	-9.56	-8.86	-8.06
	PC-3	-8.83	-8.00	-6.15
	*			
MG-MID		-9.24	-8.31	-7.88
Delta		0.68	1.08	0.77
Range		1.40	1.88	2.50

Figure S2. JFCR39 cancer cell panel assay of polytheonamide B (**5**). (a) Concentration-response curves obtained by sulforhodamine B assays. The cells were incubated for 2 d in the presence of **5**, and the cell growth (%) was evaluated. Each plot is displayed as mean of two replicates. (b) Obtained $\log GI_{50}$, logTGI (total growth inhibition), and $\log LC_{50}$ values of **5**. MG-MID: mean of obtained $\log GI_{50}$, Delta: difference between $\log GI_{50}$ of the cell line exhibiting the highest sensitivity and MG-MID, Range: difference between $\log GI_{50}$ values of the cell lines exhibiting the highest sensitivity and lowest sensitivity, TGI: concentrations that cause cell growth (%) = 0%.



compounds	concentration (μM)	function[S1]
chlorpromazine (Cpz)	10	inhibitor of clathrin-dependent endocytosis
nystatin (Nys)	50	inhibitor of lipid raft/caveolae pathway
5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA)	10	selective inhibitor of Na^+/H^+ exchanger and blocker of macropinocytosis

Figure S3. Cellular uptake of active fluorescent probe **3** in the presence of endocytosis and metabolic inhibitors. (a) Cellular uptake of **3** (50 nM) after incubated at 37 °C for 1.5 h. (b) Inhibition assay of cellular uptake of **3** (50 nM) in the presence of 10 mM NaN_3 and 5.0 mM 2-deoxy-D-glucose (2-DG), (c) 10 μM chlorpromazine (Cpz), (d) 50 μM nystatin (Nys), and (e) 10 μM 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA). Scale bar represents 20 μm. (f) Stained area value per cell. $*P < 0.05$ and $**P < 0.01$ determined by Dunnett's test. Data are displayed as mean ± SD of five images. (g) Structures, tested concentrations, and functions of endocytosis inhibitors.

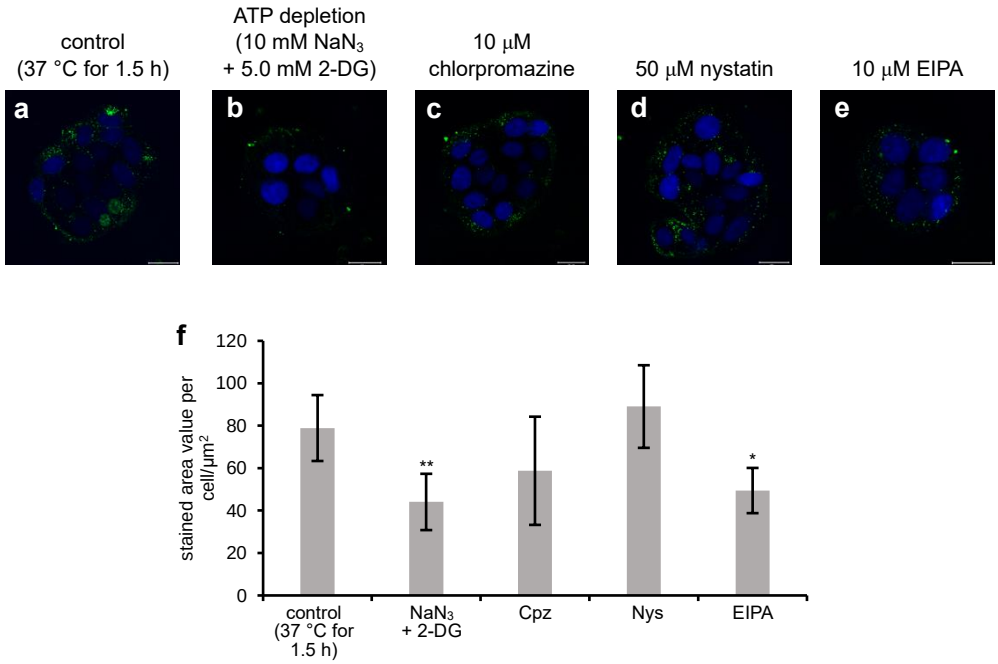


Figure S4. Cellular uptake of inactive fluorescent probe **4** in the presence of endocytosis and metabolic inhibitors. (a) Cellular uptake of **4** (50 nM) after incubated at 37 °C for 1.5 h. (b) Inhibition assay of cellular uptake of **4** (50 nM) in the presence of 10 mM NaN_3 and 5.0 mM 2-deoxy-D-glucose (2-DG), (c) 10 μM chlorpromazine (Cpz), (d) 50 μM nystatin (Nys), and (e) 10 μM 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA). Scale bar represents 20 μm. (f) Stained area value per cell. $*P < 0.05$ and $**P < 0.01$ determined by Dunnett's test. Data are displayed as mean ± SD of five images.

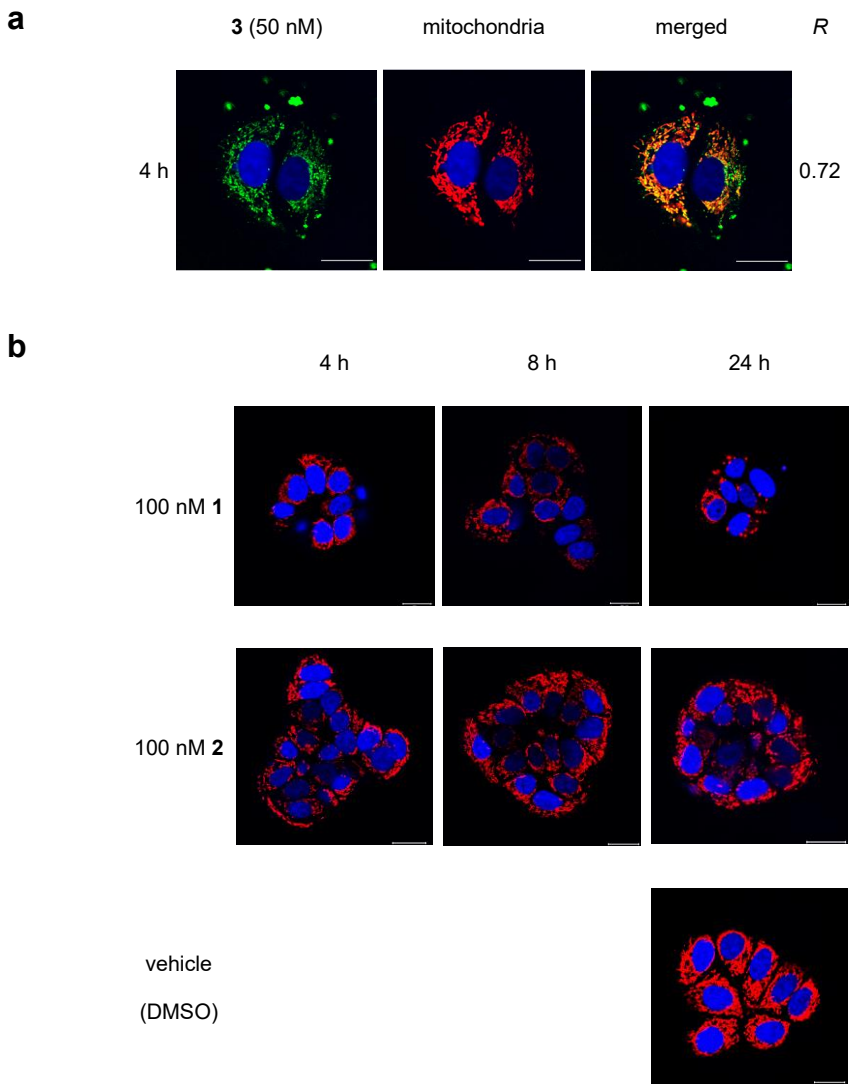


Figure S5. (a) Colocalization of active fluorescent probe **3** with tetramethylrhodamine methyl ester (TMRM). The probe (50 nM) was incubated for 4 h. (b) Confocal fluorescent images obtained using TMRM after the incubation with 100 nM gramicidin A (**1**) or 100 nM inactive analogue (**2**) for 4 h, 8 h, and 24 h. Vehicle (DMSO) was added and incubated for 24 h. Scale bars represent 20 μm.

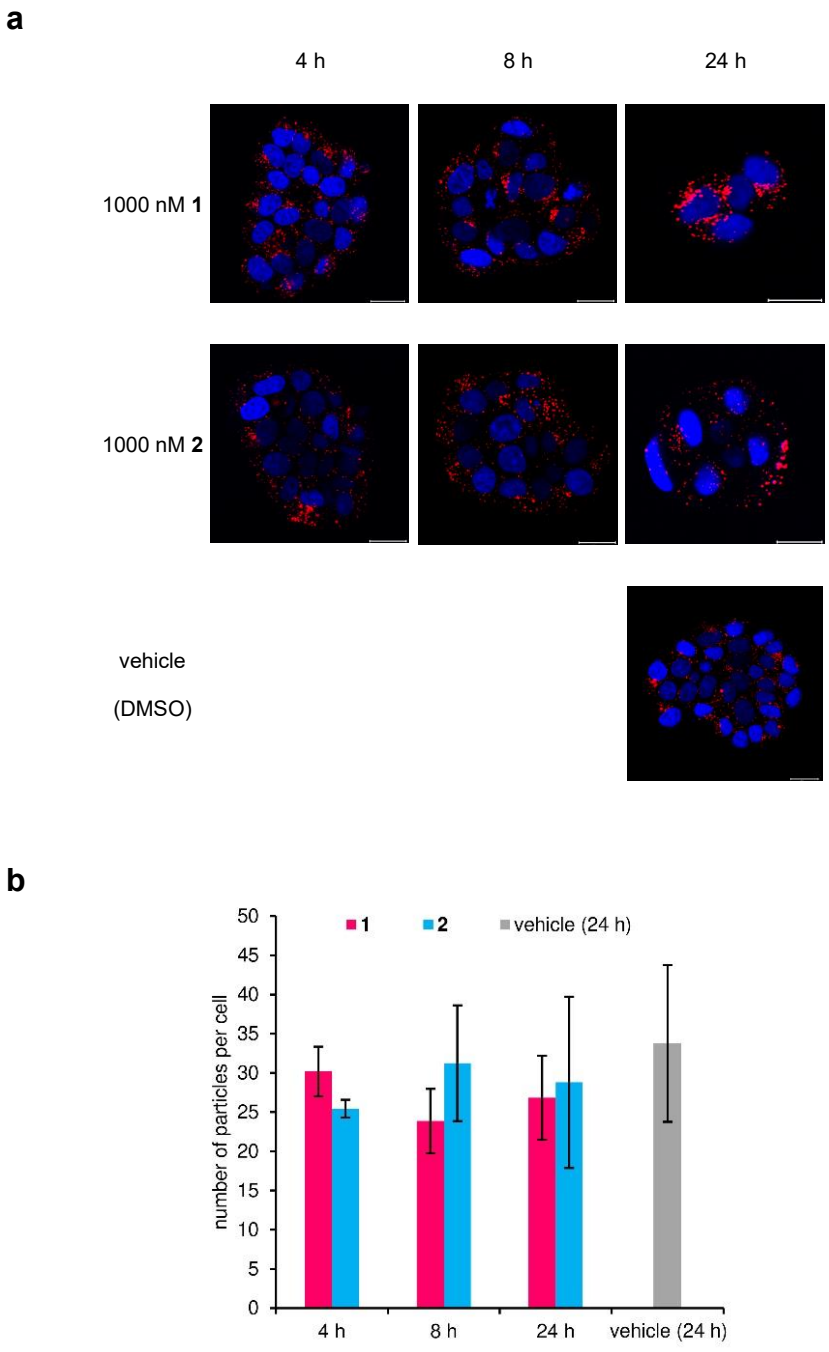


Figure S6. (a) Confocal fluorescent images obtained using LysoTracker Red DND-99 after the incubation with 1000 nM gramicidin A (**1**) or 1000 nM inactive analogue (**2**) for 4 h, 8 h, and 24 h. Vehicle (DMSO) was added and incubated for 24 h. Scale bars represent 20 μm. (b) Time-dependent change of fluorescence from LysoTracker Red DND-99. The number of particles of each confocal image was determined by image analysis. Each plot was made from three images as mean ± SD. The difference was not significant in comparison of each plot with that of vehicle (DMSO, 24 h) by Dunnett's test.

Methods

General Remarks. All reactions sensitive to air or moisture were carried out under argon (Ar) atmosphere in dry solvents, unless otherwise noted. CH_2Cl_2, DMF, Et_2O, and THF were purified by Glass Contour solvent dispensing system (Nikko Hansen). *i*-Pr_2NEt was distilled from KOH. All other reagents were used as supplied unless otherwise noted. Solid-phase peptide synthesis (SPPS) was performed on a microwave-assisted peptide synthesizer MWS-1000 (EYELA) using a sealed reaction vessel, a reaction temperature of which was monitored by an internal temperature probe, or an automated peptide synthesizer Initiator + Alstra (Biotage). Optical rotations were measured on a P-2200 polarimeter (JASCO) at room temperature using sodium D line. Infrared (IR) spectra were recorded on FT/IR-4100 spectrometer (JASCO) as a thin film on CaF_2. ^1H NMR spectra were recorded on a JNM-ECS-400 spectrometer (JEOL). Chemical shifts were denoted in δ (ppm) relative to a residual solvent peak as an internal standard (CD_2HOD in CD_3OD, ^1H δ 3.31). HRMS spectra were recorded on a micrOTOF II (Bruker Daltonics) electrospray ionization time-of-flight (ESI-TOF) mass spectrometer. UV absorbance was measured on a UV-1800 UV-VIS spectrophotometer (Shimadzu). High-performance liquid chromatography (HPLC) experiments were performed on a HPLC system equipped with a PU-4180 RHPLC pump (JASCO). Ultrahigh-performance liquid chromatography (UHPLC) experiments were performed with an Extrema system (JASCO).

Quantification of peptides. The quantification of **3** and **4** was carried out using HPLC (column: Inertsil C8-3 4.6 × 150 mm, eluent A: MeCN + 0.05% TFA, eluent B: H_2O + 0.05% TFA, linear gradient: A/B = 35/65 to 100/0 over 40 min, flow rate: 0.500 mL/min, detection: UV 500 nm, temperature: 45 °C). HPLC chromatograms were recorded under the UV detection, and the peak areas of the peptides (UV 500 nm) were calculated using HyStar LC integration software (Bruker Daltonics). The quantity of each compound was determined from its peak area using the peak area of compound **12** (UV 500 nm) as the reference (column: Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H_2O + 0.05% TFA, linear gradient: A/B = 15/85 to 35/65 over 30 min, flow rate: 0.600 mL/min, detection: UV 500 nm, temperature: 45 °C).

Preloaded resin 6. To trityl-OH-ChemMatrix resin (143 mg, 0.40–0.65 mmol/g) in 5 mL LibraTube (Hipep Laboratories) was added $SOCl_2/CH_2Cl_2$ (1/51, 2.75 mL) at room temperature. After being stirred at room temperature for 12 h, the reaction mixture was filtered. The same treatment was repeated (× 1). The resultant resin was washed with CH_2Cl_2 (2.00 mL × 5) and *N*-methylmorpholine/CH_2Cl_2 (2.00 mL × 5).

To the above resin were added a solution of Fmoc-L-Trp(Boc)-OH (294 mg, 0.558 mmol) in CH_2Cl_2 (1.00 mL) and a solution of *N*-methylmorpholine (114 μL, 1.04 mmol) in CH_2Cl_2 (1.00 mL) at room temperature. After being stirred at room temperature for 12 h, the reaction mixture was filtered. The same treatment was repeated (× 1). To the resultant resin was added *N*-methylmorpholine/MeOH (1/3, 114 μL) at room temperature. After being stirred at room temperature for 1 h, the resultant mixture was filtered, and washed with CH_2Cl_2 (2.00 mL × 3).

To the above resin was added Ac_2O/CH_2Cl_2 (1/3, 2.00 mL) at room temperature for the capping of remaining hydroxy groups. After being stirred at room temperature for 15 min, the reaction mixture was filtered, washed with CH_2Cl_2 (2.00 mL × 5), DMF (2.00 mL × 5), MeOH (2.00 mL × 5), and Et_2O (2.00 mL × 5), and dried under vacuum to give preloaded resin **6** (169 mg). The loading rate was determined to be 0.230 mmol/g by the following method.

Determination of loading rate. Fmoc-protected resin was treated with piperidine/NMP (1/4, 1.00 mL) at room temperature for 15 min and the supernatant was collected. The same treatment was repeated (× 1). The resin was washed with NMP (2.00 mL × 4) and the supernatant was collected. UV absorption at 301 nm of the combined supernatants was measured. The background absorbance was canceled by subtracting the control absorbance obtained from a solution of piperidine/NMP (1/24). The loading rate (x mmol/g) was determined by the following Equation S1, where a is the weight of Fmoc-protected resin (mg), and b is absorbance at 301 nm.

$$x = (10000 \times b) / (7800 \times a) \quad \text{(S1)}$$

Procedures for solid-phase peptide synthesis (SPPS). The resin-bound 15-mer peptide was prepared as an intermediate of **3** on a peptide synthesizer MWS-1000 (EYELA). Standard operation was shown as follows:
Step 1: The solid supported N_α-Fmoc peptide was deprotected with piperidine/NMP (1/4, 60 °C, 10 min).
Step 2: The 5 mL LibraTube containing the resin was washed with NMP (2.00 mL, 30 sec × 5).

Step 3: N_α-amino acid (4.0 eq) was activated by a solution of *O*-(7-aza-1*H*-benzotriazole-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HATU, 4.0 eq, 0.45 M)/1-hydroxy-7-azabenzotriazole (HOAt, 4.0 eq, 0.45 M) in NMP. To the solution of activated Fmoc-amino acid was added a solution of *i*-Pr_2NEt (8.0 eq, 2.0 M) in NMP. The resultant mixture was transferred to the 5 mL LibraTube.

Step 4: The activated amino acid was coupled with the peptide on the resin (60 °C, 100 W, 20 min) and the 5 mL LibraTube containing the resin was washed with NMP (2.00 mL, 30 sec × 5)

Steps 1−4 were repeated and amino acids were condensed on the solid support. Steps 1, 2, and 4 were carried out under a stream of N_2. Step 3 was carried out under atmosphere of Ar.

Procedure for automated solid-phase peptide synthesis (automated SPPS). The resin-bound 15-mer peptide was prepared as an intermediate of **4** on an automated peptide synthesizer Initiator + Alstra (Biotage). Standard operation was shown as follows:

Step 1: The solid supported N_α-Fmoc peptide was deprotected with piperidine/NMP (3.00 mL, 1/4, 40 °C, 5 min).

Step 2: The resin in a reaction vessel (10 mL filter tube) was washed with NMP (2.60 mL, 60 sec × 4).

Step 3: A solution of N_α-Fmoc-amino acid (4.0 eq, 0.50 M) in NMP was activated by *O*-(7-azabenzotriazole-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HATU, 4.0 eq, 0.45 M)/1-hydroxy-7-azabenzotriazole (HOAt, 4.0 eq, 0.45 M) in NMP. To the solution of activated N_α-Fmoc-amino acid was added *i*-Pr_2NEt (8.0 eq). The resultant mixture was transferred to the reaction vessel.

Step 4: The activated N_α-Fmoc-amino acid was coupled with the peptide on the resin (40 °C, 20 min) and the reaction vessel containing the resin was washed with NMP (3.00 mL, 60 sec × 4).

Steps 1−4 were repeated and amino acids were condensed on the solid support. Steps 1−4 were carried out under a stream of N_2.

Active fluorescent probe 3. Preloaded resin **6** (38.9 μmol, loading rate: 0.230 mmol/g) in 5 mL LibraTube was washed with CH_2Cl_2 (2.00 mL × 3) and NMP (2.00 mL × 3). The resin was subjected to 14 cycles of the SPPS protocol to give the resin-bound 15-mer peptide.

The N_α-Fmoc group of the above resin-bound 15-mer peptide was removed by steps 1 and 2 of the standard SPPS protocol. The resin-bound peptide was washed with NMP (2.00 mL × 3), CH_2Cl_2 (2.00 mL × 3), MeOH (2.00 mL × 3), and Et_2O (2.00 mL × 3), and dried under vacuum to give the dried resin-bound peptide.

To the above resin-bound peptide was added a solution of *p*-nitrophenyl formate (32.5 mg, 194 μmol) and *N*-methylmorpholine/DMF (0.4/99.6, 2.50 mL). After being stirred at room temperature for 16 h, the mixture was filtered, washed with DMF (2.00 mL × 3), CH_2Cl_2 (2.00 mL × 3), MeOH (2.00 mL × 3), and Et_2O (2.00 mL × 3), and dried under vacuum to give resin-bound peptide **7a** (188 mg).

The above resin-bound peptide **7a** (92.3 mg, 19.1 μmol) was washed with CH_2Cl_2 (2.00 mL × 3). To the resultant resin was added $(CF_3)_2CHOH/CH_2Cl_2$ (1/3, 1.00 mL) at room temperature. After being stirred at room temperature for 1 h, the reaction mixture was concentrated under a stream of Ar to give the crude protected peptide **8a**. The crude peptide **8a** was dissolved in *i*-$PrOH/H_2O$ (1/1), and filtered through 0.20 μm PTFE filter.

The filtrate was purified by reversed-phase HPLC (column: Inertsil C8-3 10 × 250 mm, eluent A: MeOH+ 0.05% HCO_2H, eluent B: H_2O + 0.05% HCO_2H, linear gradient: A/B = 70/30 to 100/0 over 40 min, flow rate: 1.50 mL/min, detection: UV 280 nm, temperature: 40 °C) to give the crude **8a** (t_R = 57.8–62.7 min, 13.8 mg), which was used for the next reaction without further purification. For characterization, the crude **8a** was subjected to MS analysis: HRMS (ESI-TOF) calcd for $C_{117}H_{167}N_{19}NaO_{25}$ $[M+Na]^+$ 2262.2307, found 2262.2288.

To the above crude peptide **8a** (1.29 mg, 0.580 μmol) were added a solution of amine **9**[S2] (735 μg, 1.73 μmol) in THF (50.0 μL), a solution of 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DEPBT, 518 μg, 1.73 μmol) in THF (50.0 μL), and a solution of 2,4,6-collidine (458 nL, 3.46 μmol) in THF (50.0 μL) at room temperature. After being stirred at room temperature for 4 h, the reaction mixture was concentrated under a stream of Ar to give the crude peptide **10a**. The crude peptide **10a** was dissolved in *i*-PrOH, and filtered through 0.20 μm PTFE filter. The filtrate was purified by reversed-phase HPLC (column: Inertsil C8-3 10 × 250 mm, eluent A: *i*-PrOH + 0.05% HCO_2H, eluent B: H_2O + 0.05% HCO_2H, linear gradient: A/B = 60/40 to 100/0 over 40 min, flow rate: 1.00 mL/min, detection: UV 280 nm, temperature: 40 °C) to give the crude **10a** (t_R = 36.7–39.0 min, 0.760 mg), which was used for the next reaction without further purification. For characterization, the crude **10a** was subjected to MS analysis: HRMS (ESI-TOF) calcd for $C_{136}H_{205}N_{21}Na_2O_{32}$ $[M+2Na]^{2+}$ 1345.7439, found 1345.7439.

To the above crude peptide **10a** (216 μg, 81.6 nmol) was added TFA/CH_2Cl_2/*i*-Pr_3SiH (24/24/1, 200 μL) at 0 °C. After being stirred at 0 °C for 0.5 h and at 30 °C for 1 h, the reaction mixture was concentrated under a stream of Ar and dried under vacuum to give the crude peptide **11a**.

To carboxylic acid **12**[S3] (100 μg, 246 nmol) were added a solution of PyBOP (130 μg, 246 nmol) in DMF (20.0 μL), a solution of HOAt (33.0 μg, 246 nmol) in DMF (20.0 μL), and a solution of *i*-Pr_2NEt (85.7 nL, 492 nmol) in DMF (20.0 μL) at room temperature. After being stirred at room temperature for 5 min, the reaction mixture was transferred with DMF (50.0 μL) to the above crude peptide **11a**. After being stirred at room temperature for 24 h, the reaction mixture was filtered through 0.20 μm PTFE filter. The filtrate was purified by reversed-phase HPLC (column: Inertsil C8-3 4.6 × 150 mm, eluent A: MeCN + 0.05% TFA, eluent B: H_2O + 0.05% TFA, linear gradient: A/B = 35/65 to 100/0 over 40 min, flow rate: 0.500 mL/min, detection: UV 500 nm, temperature: 45 °C) to give **3** (t_R = 27.4–29.1 min, 127 μg, 10% over 34 steps): $[\alpha]_D^{21}$ +11.7 (*c* 0.0545, MeOH); IR (film) 3292, 2963, 2360, 2337, 1680, 1653, 1541, 1522, 1205, 1136 cm^{-1}; HRMS (ESI-TOF) calcd for $C_{131}H_{191}BF_2N_{24}O_{24}$ $[M+2H]^{2+}$ 1267.2274, found 1267.2276.

Inactive fluorescent probe 4. Preloaded resin **6** (30.8 μmol, loading rate: 0.178 mmol/g) in the reaction vessel (10 mL filter tube) was washed with CH_2Cl_2 (2.00 mL × 3) and NMP (2.00 mL × 3). The resin was subjected to 14 cycles of the automated SPPS protocol to give the resin-bound 15-mer peptide.

The N_α-Fmoc group of the above resin-bound 15-mer peptide was removed by steps 1 and 2 of the standard automated SPPS protocol. The resin-bound peptide was washed with NMP (2.00 mL × 3), CH_2Cl_2 (2.00 mL × 3), MeOH (2.00 mL × 3), and Et_2O (2.00 mL × 3), and dried under vacuum to give the dried resin-bound peptide.

To the above resin-bound peptide was added a solution of *p*-nitrophenyl formate (25.7 mg, 148 μmol) and *N*-methylmorpholine/DMF (0.4/99.6, 2.50 mL). After being stirred at room temperature for 16 h, the mixture was filtered, washed with DMF (2.00 mL × 3), CH_2Cl_2 (2.00 mL × 3), MeOH (2.00 mL × 3), and Et_2O (2.00 mL × 3), and dried under vacuum to give the resin-bound peptide **7b**.

The above resin-bound peptide **7b** (7.93 μmol) was washed with CH_2Cl_2 (2.00 mL × 3). To the resultant resin was added $(CF_3)_2CHOH/CH_2Cl_2$ (1/3, 1.00 mL) at room temperature. After being stirred at room temperature for 1 h, the reaction mixture was concentrated under a stream of Ar to give the crude protected peptide **8b**. The crude peptide **8b** was dissolved in *i*-PrOH/H_2O (1/1), and filtered through 0.20 μm PTFE filter. The filtrate was purified by reversed-phase HPLC (column: Inertsil C8-3 10 × 250 mm, eluent A: MeOH + 0.05% HCO_2H, eluent B: H_2O + 0.05% HCO_2H, linear gradient: A/B = 70/30 to 100/0 over 40 min, flow rate: 1.00 mL/min, detection: photodiode array detector 190–400 nm, UV chromatogram: 280 nm, temperature: 40 °C) to give the crude **8b** (t_R = 56.5–75.8 min, 8.41 mg), which was used for the next reaction without further purification. For characterization, the crude **8b** was subjected to MS analysis: HRMS (ESI-TOF) calcd for $C_{120}H_{173}N_{19}Na_2O_{26}$ $[M+2Na]^{2+}$ 1171.6309, found 1171.6285.

To the above crude peptide **8b** (2.77 mg, 1.21 μmol) were added a solution of amine **9**[S2] (1.54 mg, 3.63 μmol) in THF (100 μL), a solution of 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DEPBT, 1.09 mg, 3.63 μmol) in THF (100 μL), and a solution of 2,4,6-collidine (961 nL, 7.26 μmol) in THF (100 μL) at room temperature. After being stirred at room temperature for 4 h, the reaction mixture was concentrated under a stream of Ar to give the crude peptide **10b**. The crude peptide **10b** was dissolved in *i*-PrOH, and filtered through 0.20 μm PTFE filter. The filtrate was purified by reversed-phase HPLC (column: Inertsil C8-3 10 × 250 mm, eluent A: *i*-PrOH + 0.05% HCO_2H, eluent B: H_2O + 0.05% HCO_2H, linear gradient: A/B = 60/40 to 100/0 over 40 min, flow rate: 1.50 mL/min, detection: photodiode array detector 190–400 nm, UV chromatogram: 280 nm, temperature: 40 °C) to give the crude **10b** (t_R = 23.1–25.2 min, 1.64 mg), which was used for the next reaction without further purification. For characterization, the crude **10b** was subjected to MS analysis: HRMS (ESI-TOF) calcd for $C_{139}H_{211}N_{21}NaO_{33}$ $[M+Na]^+$ 2726.5404, found 2726.5397.

To the above crude peptide **10b** (308 μg, 114 nmol) was added TFA/CH_2Cl_2/*i*-Pr_3SiH (24/24/1, 200 μL) at 0 °C. After being stirred at 0 °C for 0.5 h and at 30 °C for 1 h, the reaction mixture was concentrated under a stream of Ar and dried under vacuum to give the crude peptide **11b**.

To carboxylic acid **12**[S3] (139 μg, 342 nmol) were added a solution of PyBOP (181 μg, 342 nmol) in DMF (20.0 μL), a solution of HOAt (45.9 μg, 342 nmol) in DMF (20.0 μL), and a solution of *i*-Pr_2NEt (119 nL, 684 nmol) in DMF (20.0 μL) at room temperature. After being stirred at room temperature for 5 min, the reaction mixture was transferred with DMF (50.0 μL) to the above crude peptide **11b**. After being stirred at room temperature for 24 h, the reaction mixture was filtered through 0.20 μm PTFE filter. The filtrate was purified by reversed-phase HPLC (column: Inertsil C8-3 4.6 × 150 mm, eluent A: MeCN + 0.05% TFA, eluent B: H_2O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 0.500 mL/min, detection: UV 500 nm, temperature: 45 °C) to give **4** (t_R = 17.8–18.7 min, 218 μg, 17% over 34 steps): $[\alpha]_D^{22}$ = +15.7 (*c* 0.0478, MeOH); IR (film) 3316, 2962, 2871, 1678, 1536, 1445, 1202, 1138 cm^{-1}; HRMS (ESI-TOF) calcd for $C_{130}H_{187}BF_2N_{24}Na_2O_{25}$ $[M+2Na]^{2+}$ 1290.1989, found 1290.1967.

Cell Culture. MCF-7 cells were obtained from the American Type Culture Collection (ATCC). The cells were maintained with growth medium [Dulbecco's Modified Eagle's Medium (DMEM)-low glucose (Sigma) supplemented with 10% heat-inactivated fetal bovine serum, penicillin G (100 units/mL), and streptomycin (100 μg/mL)] under atmosphere of 5% CO_2 at 37 °C. The growth medium was refreshed every 2 or 3 d to reach 70–90% cell confluence.

Sulforhodamine B Assay (Table 1). Growth inhibitory activities of the tested compounds were evaluated according to the literature.[S4] Cell growth (%) was defined as follows:

$$\text{cell growth (\%)} = \frac{FL(sample) - FL(day\ 0)}{FL(control) - FL(day\ 0)} \times 100 \qquad \{FL(sample) \geq FL(day\ 0)\}$$

$$\text{cell growth (\%)} = \frac{FL(sample) - FL(day\ 0)}{FL(day0)} \times 100 \qquad \{FL(day\ 0) > FL(sample)\}$$

where *FL* = mean of the fluorescence intensity (excitation wavelength 485 nm/emission wavelength 585 nm), *day* 0 = time of addition of the tested compound as serial dilutions, and *control* = control wells treated by vehicle (DMSO).

Various concentrations of compounds in the growth medium containing 2% DMSO were prepared by serial dilutions. MCF-7 cells were cultured in 6.0 cm cell culture dishes filled with the growth medium and harvested by trypsinization at 37 °C for 5–10 min. The collected cells were resuspended into the growth medium at 1.25 × 10^5 cells/mL. The cell suspension (100 μL/well) was seeded into the black polystyrene flat-bottom 96-well plates (sample plates, Greiner Bio-One). For calculating *FL*(*day* 0), the same cell suspension (100 μL/well) was seeded into an independent 96-well plate (*day* 0 plate). The sample plate and *day 0* plate were incubated at 37 °C under atmosphere of 5% CO_2 for 24 h. Aliquots of the former medium (100 μL) containing compounds were added to each well of the sample plate. The growth medium containing 2% DMSO (100 μL) was added to the wells for calculating *FL*(*control*) of the sample plate and the wells for calculating *FL*(*day* 0) of the *day 0* plate. To the cells in *day* 0 plate was added an ice-cold solution of 30 w/v% trichloroacetic acid in H_2O (100 μL/well). The *day 0* plate was incubated at 4 °C for 60 min, washed with H_2O (× 4), dried, and stored at room temperature. The sample plate was incubated at 37 °C under atmosphere of 5% CO_2 for 48 h. To the cells in the sample plate was added an ice-cold solution of 30 w/v% trichloroacetic acid in H_2O (100 μL/well). The sample plate was incubated at 4 °C for 60 min, washed with H_2O (× 4), and dried. To each well of the *day* 0 plate and sample plate was added a solution of sulforhodamine B in H_2O (570 μg/mL, 100 μL/well). The fixed cells were stained at room temperature for 30 min in the dark. The cells were washed with AcOH/H_2O (1/99 × 4) and dried. To the stained cells was added a solution of 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) in H_2O (10 mM, 200 μL/well). The plates were vortexed at room temperature for 10 min. The fluorescence (excitation wavelength 485 nm/emission wavelength 585 nm) of each well was measured on a Spectra Max Gemini EM microplate reader (Molecular Devices). Growth inhibitory activity of each compound was evaluated as GI_{50} (nM) by means of three replicates. Sigmoidal curve fittings were performed on Prism 4 (Graphpad Software). The median growth inhibition concentration

(GI_{50}, concentration showing 50% cell growth) and the median lethal concentration (LC_{50}, concentration showing −50% cell growth) values of **1**, **2**, **3**, and **4** were determined by three independent experiments.

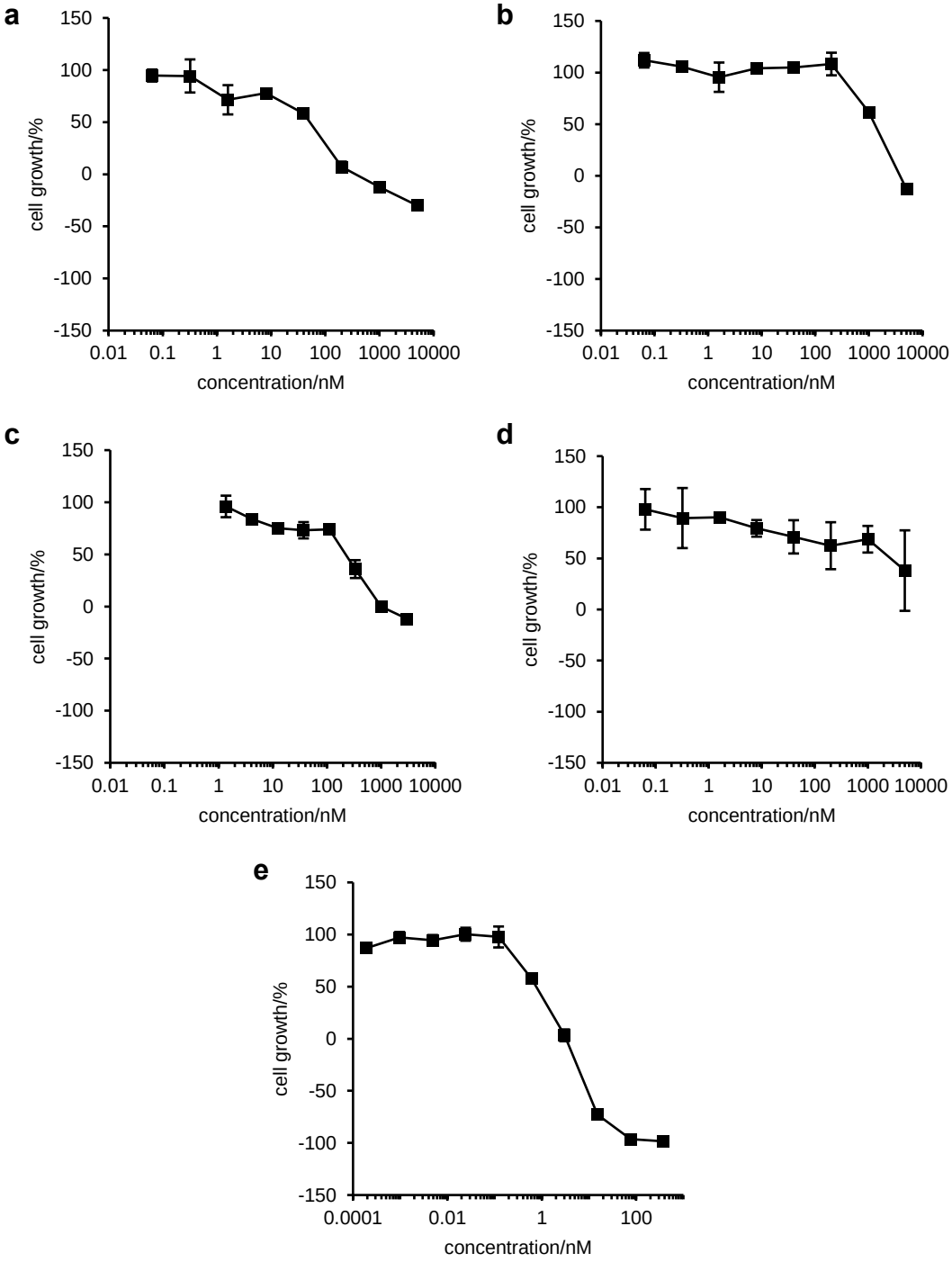


Figure S7. Representative concentration-response curves of (a) gramicidin A (**1**), (b) inactive analogue **2**, (c) active fluorescent probe **3**, (d) inactive fluorescent probe **4**, and (e) polytheonamide B (**5**)[S5] against MCF-7 cells. The cells were incubated for 2 d in the presence of each compound, and the cell growth (%) was evaluated. Each plot is displayed as mean ± SD of three replicates.

Plasma membrane depolarizing activity assay (Figure 2, Table 2). Various concentrations of compounds in DMSO were prepared by 5-fold serial dilutions. MCF-7 cells were cultured in the growth medium at 37 °C

and harvested by trypsinization at 37 °C for 5–10 min. The collected cells were resuspended into the growth medium at 1.25 ×10^5 cells/mL. The cell suspension (100 μL/well) was seeded into a black polystyrene flat-clear-bottom 96-well plate (Greiner Bio-One). The cells were incubated at 37 °C under atmosphere of 5% CO_2 for 2 d, and then each well was washed (200 μL × 1) and filled (198 μL) with the buffer [20 mM HEPES, 120 mM NaCl, 2.0 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM glucose, 1.0 μM $DiBAC_4(3)$]. The cells were incubated at 37 °C under atmosphere of 5% CO_2 for 1 h. Aliquots of DMSO solution containing the compounds (2.0 μL/well) were added to each well. The fluorescence (excitation wavelength 485 nm/emission wavelength 530 nm) of each well was measured at every 5 min for 60 min on a Spectra Max Gemini EM microplate reader. The background drift from DMSO addition and non-specific interaction between the tested compounds and $DiBAC_4(3)$ were canceled by subtracting the control traces obtained from vehicle (DMSO) and compounds with $DiBAC_4(3)$ without cells, respectively. The fluorescence intensities were evaluated by means of four replicates. Each fluorescence intensity at 60 min was transformed to membrane potential change (%) by normalization against the fluorescence intensity at 60 min by addition of **5** (25 nM) as 100% and by addition of vehicle (DMSO) as 0%. The plasma membrane depolarization activity of each compound was evaluated as 50% effective concentration (EC_{50}, nM) by means of three independent experiments. Sigmoidal curve fittings were performed on Prism 4 (Graphpad Software). EC_{50} value of each compound was determined by three independent experiments for **1**, two independent experiments for **2**, and one experiment for **3** and **4**.

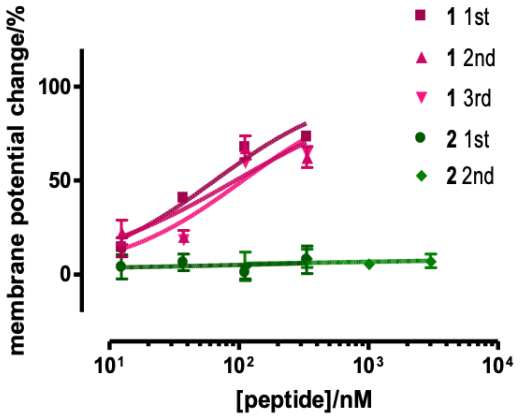


Figure S8. Concentration-response curves of gramicidin A (**1**) and inactive analogue **2**. Each plot is displayed as mean ± SD of three replicates.

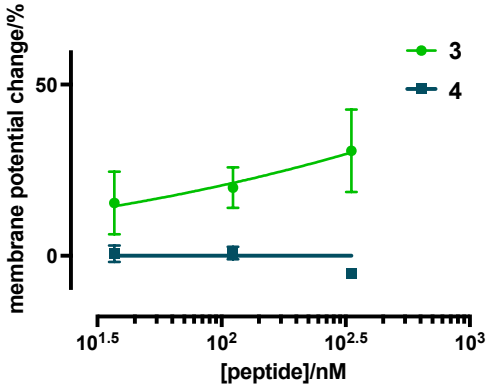


Figure S9. Concentration-response curves of active fluorescent probe **3** and inactive fluorescent probe **4**. Each plot is displayed as mean ± SD of three replicates.

Cellular uptake analysis (Figures S3 and S4). MCF-7 cells were cultured in the growth medium at 37 °C and harvested by trypsinization at 37 °C for 5–10 min. The collected cells were resuspended into the growth medium at 5.0×10^4 cells/mL. The cell suspension (1.0 mL) was seeded to a 3.5 cm poly-L-lysine coated glass-bottom dish (Matsunami Glass Ind.). The cells were preincubated at 37 °C under atmosphere of 5% CO_2 for 1 d, and then the medium was replaced with the fresh growth medium (1.0 mL). The cells were incubated at 37 °C under atmosphere of 5% CO_2 for 1 d. A solution of the endocytosis or metabolic inhibitor [a solution of NaN_3 and 2-deoxy-D-glucose in phosphate buffered saline with calcium and magnesium (NaN_3: 1.0 M, 10 μL, final concentration = 10 mM; 2-deoxy-D-glucose: 0.5 M, 10 μL, final concentration = 5.0 mM), a solution of chlorpromazine in DMSO (2.0 mM, 5.0 μL, final concentration = 10 μM), a solution of nystatin in DMSO (10 mM, 5.0 μL, final concentration = 50 μM), or a solution of 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA) in DMSO (2.0 mM, 5.0 μL, final concentration = 10 μM)] was added to the cells, and then the cells were incubated at 37 °C under atmosphere of 5% CO_2 for 30 min. A solution of **3** or **4** in DMSO (10 μM, 5.0 μL, final concentration = 50 nM) was added to the cells, and then the cells were incubated at 37 °C under atmosphere of 5% CO_2 for 50 min. A solution of Hoechst 33342 in H_2O (1.0 mM, 10 μL, final concentration = 10 μM) was added to the cells, and then the cells were incubated at 37 °C under atmosphere of 5% CO_2 for 10 min. The dish was washed (1.0 mL × 3) and filled (1.0 mL) with Hank's Balanced Salt Solution with calcium and magnesium (no phenol red), and subsequently subjected to confocal fluorescence microscopic analysis on LSM510-META equipped with a 63× oil-immersion objective lens (Carl Zeiss). The stained area value by **3** or **4** were analyzed by Measure function of ImageJ.[S6]

Colocalization analysis of fluorescent probes with MitoTracker Red CMXRos (Figures 3c and 3e). MCF-7 cells were cultured in the growth medium at 37 °C and harvested by trypsinization at 37 °C for 5–10 min. The collected cells were resuspended into the growth medium at 5.0×10^4 cells/mL. The cell suspension (1.0 mL) was seeded to a 3.5 cm poly-L-lysine coated glass-bottom dish (Matsunami Glass Ind.). The cells were preincubated at 37 °C under atmosphere of 5% CO_2 for 1 d, and then the medium was replaced with the fresh growth medium (1.0 mL). The cells were incubated at 37 °C under atmosphere of 5% CO_2 for 1 d. A solution of **3** or **4** in DMSO (10 μM, 5.0 μL, final concentration = 50 nM) was added to the cells, and then the cells were incubated at 37 °C under atmosphere of 5% CO_2 for different period (4 h, 8 h, and 24 h). A solution of MitoTracker Red CMXRos (Thermo Fisher Scientific) in DMSO (20 μM, 5.0 μL, final concentration = 100 nM) was added to the cells, and then the cells were incubated at 37 °C under atmosphere of 5% CO_2 for 5 min. A solution of Hoechst 33342 in H_2O (1.0 mM, 10 μL, final concentration = 10 μM) was added to the cells, and then the cells were incubated at 37 °C under atmosphere of 5% CO_2 for 10 min. The dish was washed (1.0 mL × 3) and filled (1.0 mL) with Hank's balanced salt solution with calcium and magnesium (no phenol red), and subsequently subjected to confocal fluorescence microscopic analysis on TCS SP5 II equipped with a 63× oil-immersion objective lens (Leica Microsystems). Pearson's correlation coefficients were calculated by Costes' analysis using ImageJ with JACoP Plug-in.

Colocalization analysis of fluorescent probes with LysoTracker Red DND-99 (Figures 3d and 3f). MCF-7 cells were cultured in the growth medium at 37 °C and harvested by trypsinization at 37 °C for 5–10 min. The collected cells were resuspended into the growth medium at 5.0×10^4 cells/mL. The cell suspension (1.0 mL) was seeded to a 3.5 cm poly-L-lysine coated glass-bottom dish (Matsunami Glass Ind.). The cells were preincubated at 37 °C under atmosphere of 5% CO_2 for 1 d, and then the medium was replaced with the fresh growth medium (1.0 mL). The cells were incubated at 37 °C under atmosphere of 5% CO_2 for 1 d. A solution of **3** or **4** in DMSO (10 μM, 5.0 μL, final concentration = 50 nM) was added to the cells, and then the cells were incubated at 37 °C under atmosphere of 5% CO_2 for different period (4 h, 8 h, and 24 h). A solution of LysoTracker Red DND-99 (Thermo Fisher Scientific) in DMSO (10 μM, 5.0 μL, final concentration = 50 nM) was added to the cells, and then the cells were incubated at 37 °C under atmosphere of 5% CO_2 for 50 min. A solution of Hoechst 33342 in H_2O (1.0 mM, 10 μL, final concentration = 10 μM) was added to the cells, and then the cells were incubated at 37 °C under atmosphere of 5% CO_2 for 10 min. The dish was washed (1.0 mL × 3) and filled (1.0 mL) with Hank's balanced salt solution with calcium and magnesium (no phenol red), and subsequently subjected to confocal fluorescence microscopic analysis. Pearson's correlation coefficients were calculated by Costes' analysis using ImageJ with JACoP Plug-in.

Mitophagy detection assay (Figures 4c and 4d). MCF-7 cells were cultured in the growth medium at 37 °C and harvested by trypsinization at 37 °C for 5–10 min. The collected cells were resuspended into the growth medium at 5.0×10^4 cells/mL (Matsunami Glass Ind.). The cell suspension (1.0 mL) was seeded to 3.5 cm poly-L-lysine coated glass-bottom dish. The cells were preincubated at 37 °C under atmosphere of 5% CO_2 for 1 d, and then the medium was replaced with the fresh growth medium (1.0 mL). The cells were incubated at 37 °C under atmosphere of 5% CO_2 for 1 d. The dish was washed (1.0 mL × 2) with serum-free growth medium, and filled with the serum-free growth medium (1.0 mL) containing Mtphagy dye (final concentration = 100 nM, Dojindo Molecular Technologies). The cells were incubated at 37 °C under atmosphere of 5% CO_2 for 30 min, and then the dish was washed (1.0 mL × 2) and filled (1.0 mL) with the growth medium. A solution of **1** or **2** in DMSO (20 μM, 5.0 μL, final concentration = 100 nM), a solution of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) in DMSO (2.0 mM, 5.0 final concentration = 10 μM), or vehicle (DMSO, 5.0 μL) was added to the cells, and then the cells were incubated at 37 °C under atmosphere of 5% CO_2 for different period [4 h, 8 h, and 24 h for **1** and **2**, 24 h for CCCP and vehicle (DMSO)]. The dish was washed (1.0 mL × 2) with the serum-free growth medium, and filled (1.0 mL) with the serum-free growth medium containing Lyso dye (final concentration = 1.0 μM, Dojindo Molecular Technologies). The cells were incubated at 37 °C under atmosphere of 5% CO_2 for 20 min. A solution of Hoechst 33342 in H_2O (1.0 mM, 10 μL, final concentration = 10 μM) was added to the cells, and then the cells were incubated for 10 min. The dish was washed (2.0 mL × 2), filled (2.0 mL) with Hank's balanced salt solution with calcium and magnesium (no phenol red), and subsequently subjected to confocal fluorescence microscopic analysis on TCS SP5 II equipped with a 63× oil-immersion objective lens (Leica Microsystems). Pearson's correlation coefficients were calculated by Costes' automated threshold analysis using ImageJ with JACoP Plug-in.

Colocalization analysis of fluorescent probes with Mtphagy dye (Figure 4b). MCF-7 cells were cultured in the growth medium at 37 °C and harvested by trypsinization at 37 °C for 5–10 min. The collected cells were resuspended into the growth medium at 5.0×10^4 cells/mL. The cell suspension (1.0 mL) was seeded to 3.5 cm poly-L-lysine coated glass-bottom dish (Matsunami Glass Ind.). The cells were preincubated at 37 °C under atmosphere of 5% CO_2 for 1 d, and then the medium was replaced with the fresh growth medium (1.0 mL). The cells were incubated at 37 °C under atmosphere of 5% CO_2 for 1 d. The dish was washed (1.0 mL × 2) with the serum-free growth medium, and filled (1.0 mL) with the serum-free growth medium containing Mtphagy dye (final concentration = 100 nM, Dojindo Molecular Technologies). The cells were incubated at 37 °C under atmosphere of 5% CO_2 for 30 min. Then, the dish was washed (1.0 mL × 2) and filled (1.0 mL) with the growth medium. A solution of **3** or **4** in DMSO (10 μM, 5.0 μL, final concentration = 50 nM) was added to the cells, and then the cells were incubated at 37 °C under atmosphere of 5% CO_2 for 24 h. A solution of Hoechst 33342 in H_2O (1.0 mM, 10 μL, final concentration = 10 μM) was added to the cells, and then the cells were incubated for 10 min. The dish was washed (2.0 mL × 2), filled (2.0 mL) with Hank's balanced salt solution with calcium and magnesium (no phenol red), and subsequently subjected to confocal fluorescence microscopic analysis on TCS SP5 II equipped with a 63× oil-immersion objective lens (Leica Microsystems). Pearson's correlation coefficients were calculated by Costes' automated threshold analysis using ImageJ with JACoP Plug-in.

Evaluation of inner mitochondrial membrane potential change using tetramethylrhodamine methyl ester (TMRM) (Figures 5b and S5). MCF-7 cells were cultured in the growth medium at 37 °C and harvested by trypsinization at 37 °C for 5–10 min. The collected cells were resuspended into the growth medium at 5.0×10^4 cells/mL. The cell suspension (1.0 mL) was seeded to 3.5 cm poly-L-lysine coated glass-bottom dish (Matsunami Glass Ind.). The cells were preincubated at 37 °C under atmosphere of 5% CO_2 for 1 d, and then the medium was replaced with the fresh growth medium (1.0 mL). The cells were incubated at 37 °C under atmosphere of 5% CO_2 for 1 d. A solution of **1** or **2** in DMSO (20 μM, 5.0 μL, final concentration = 100 nM), or vehicle (DMSO) was added to the cells, and then the cells were incubated at 37 °C under atmosphere of 5% CO_2 for different period [4 h, 8 h, or 24 h for **1** and **2**, 24 h for vehicle (DMSO)]. A solution of tetramethylrhodamine methyl ester (TMRM) in DMSO (50 μM, 5.0 μL, final concentration = 250 nM) was added to the cells, and then the cells were incubated for 20 min. A solution of Hoechst 33342 in H_2O (1.0 mM, 10 μL, final concentration = 10 μM) was added to the cells, and then the cells were incubated for 10 min. The dish was washed (2.0 mL × 2), filled (2.0 mL) with Hank's balanced salt solution with calcium and magnesium (no phenol red), and subsequently subjected to confocal fluorescence microscopic analysis on LSM510-META equipped with a 63× oil-immersion objective lens (Carl Zeiss). Mitochondrial area stained by TMRM were analyzed by Measure function of ImageJ.

Evaluation of lysosomal pH neutralization using LysoTracker Red DND-99 (Figure S6). MCF-7 cells were cultured in the growth medium at 37 °C and harvested by trypsinization at 37 °C for 5–10 min. The collected cells were resuspended into the growth medium at 5.0×10^4 cells/mL. The cell suspension (1.0 mL)

was seeded to a 3.5 cm poly-L-lysine coated glass-bottom dish (Matsunami Glass Ind.). The cells were preincubated at 37 °C under atmosphere of 5% CO_2 for 1 d, and then the medium was replaced with the fresh growth medium (1.0 mL). The cells were incubated at 37 °C under atmosphere of 5% CO_2 for 1 d. A solution of **1** or **2** in DMSO (200 μM, 5.0 μL, final concentration = 1000 nM) or vehicle (DMSO) was added to the cells, and then the cells were incubated at 37 °C under atmosphere of 5% CO_2 for different period (4 h, 8 h, or 24 h). A solution of LysoTracker Red DND-99 in DMSO (10 μM, 5.0 μL, final concentration = 50 nM) was added to the cells, and then the cells were incubated for 50 min. A solution of Hoechst 33342 in H_2O (1.0 mM, 10 μL, final concentration = 10 μM) was added to the cells, and then the cells were incubated for 10 min. The dish was washed (2.0 mL × 2), filled (2.0 mL) with Hank's balanced salt solution with calcium and magnesium (no phenol red), and subsequently subjected to confocal fluorescence microscopic analysis on TCS SP5 II equipped with a 63× oil-immersion objective lens (Leica Microsystems) or LSM510-META equipped with a 63× oil-immersion objective lens (Carl Zeiss). The number of particles was analyzed by Analyze Particles function using ImageJ.

Mitochondrial ATP Production Assay (Figure 6b). Various concentrations of compounds in DMSO were prepared by serial dilutions. MCF-7 cells were suspended in reaction buffer [5.0 mM KH_2PO_4/K_2HPO_4 (pH 7.4), 210 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 3.0 mM $MgCl_2$, 5.0 mM disodium succinate, 2.5 mM glucose, 2 U/mL hexokinase (Roche), 2 U/mL glucose-6-phosphate dehydrogenase (Fujifilm Wako Pure Chemical), 0.25 mM nicotinamide adenine dinucleotide phosphate ($NADP^+$), 25 μM P1,P5-di(adenosine-5')pentaphosphate peptasodium salt (Ap5A), 3.0 μM rotenone, 0.006% DMSO] at a concentration of 2.5×10^5 cells/mL, and kept on ice. Aliquots of the DMSO (1.5 μL) containing the compounds were added to each well of a clear flat-bottom 96-well plate (TrueLine). For calculating 100% or 0% control, DMSO (1.5 μL) or a solution of oligomycin A in DMSO (1 mM, 1.5 μL) were added to each well of the plate. A suspension of digitonin (10 mg/mL, 1.5 μL/well) was added to all the wells of the plate. The suspension of the cells in the reaction buffer (300 μL/well) was added to all the wells of the plate. The plate was shaken at 37 °C for 5 min, and then a solution of ADP in H_2O (100 mM, 1.5 μL/well) was added to all the wells of the plate. The UV absorbance of each well at 340 nm was measured at 37 °C every 1 min with shaking by using the kinetic protocol of Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific). The rate of change of UV absorbance was calculated from the slope of linear approximation. The linear approximation was performed in the time range where the line showed $r^2 > 0.99$. All the experiments were conducted in triplicate. Each slope was plotted against the concentration of the compound, and then sigmoidal curve fittings were performed on Prism 4 (GraphPad Software). EC_{50} values of **1** and oligomycin A were determined by three independent experiments.

Cell cycle analysis (Figure 7). MCF-7 cells were cultured in the growth medium at 37 °C and harvested by trypsinization at 37 °C for 5–10 min. The collected cells were resuspended into the growth medium at 1.25×10^5 cells/mL. The cell suspension (2.0 mL) was seeded to 6.0 cm cell culture dish filled with the growth medium (4.0 mL). The cells were preincubated at 37 °C under atmosphere of 5% CO_2 for 2 d, and then the

medium was replaced with the fresh growth medium (5.0 mL). A solution of **1** in DMSO (500 μM, 5.0 μL, final concentration = 500 nM, 100 μM, 5.0 μL, final concentration = 100 nM, or 20 μM, 5.0 μL, final concentration = 20 nM) was added to the cells, and the cells were incubated at 37 °C under atmosphere of 5% CO_2 for 24 h or 48 h. The cells were harvested by trypsinization at 37 °C for 5–10 min. The collected cells were resuspended into the growth medium at 2.0×10^5 cells/mL. The cell suspension (1.0 mL) was transferred to 1.5 mL microtube. The cells were collected by centrifugation at 65 × *g* for 3 min at 4 °C. The pellet was washed with phosphate buffered saline (× 2). The pellet was resuspended into phosphate buffered saline (500 μL). To the suspension was added Cell Cycle Assay Solution Deep Red (5.0 μL, Dojindo Molecular Technologies) at room temperature. After being incubated at 37 °C for 5 min in the dark, the suspension was subjected to BD FACSAria II cell sorter (Becton Dickinson). The data were analyzed with ModFit LT 5.0 (Verity Software).

Table S1. Percentage of the phase distribution treated with gramicidin A (**1**) for 48 h

concentration of **1** (nM)	phase distribution (%)[a]		
	G1	S	G2/M
0 (untreated)	53.0 ± 1.04	25.4 ± 2.02	21.6 ± 0.99
20	52.1 ± 0.94	23.1 ± 1.71	24.7 ± 2.54
100	56.8 ± 2.32	17.0 ± 0.81	26.1 ± 1.64
500	65.4 ± 0.79	9.72 ± 0.15	24.9 ± 0.67

[a]The values are displayed as mean ± SD of three independent experiments.

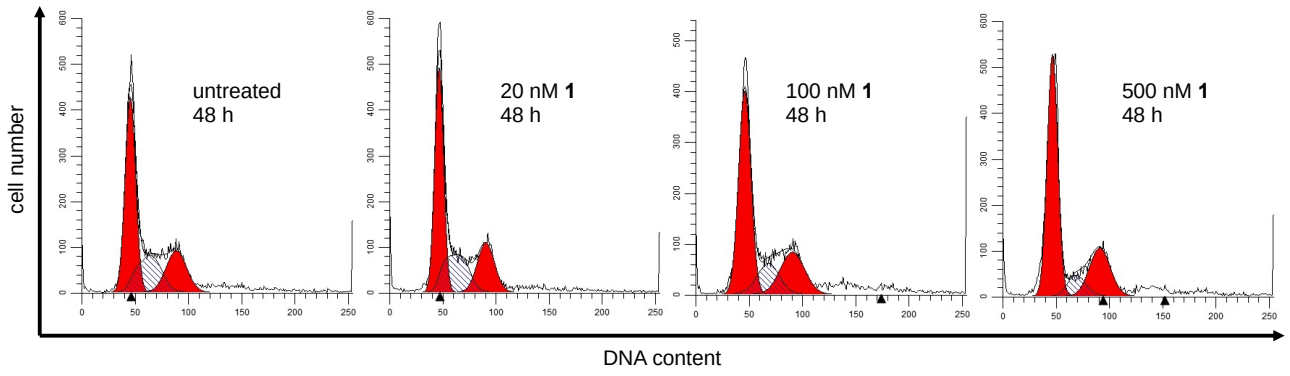


Figure S10. Representative cell cycle distribution after 48-h incubation with gramicidin A (**1**).

HPLC Charts for Purification of Synthetic Compounds

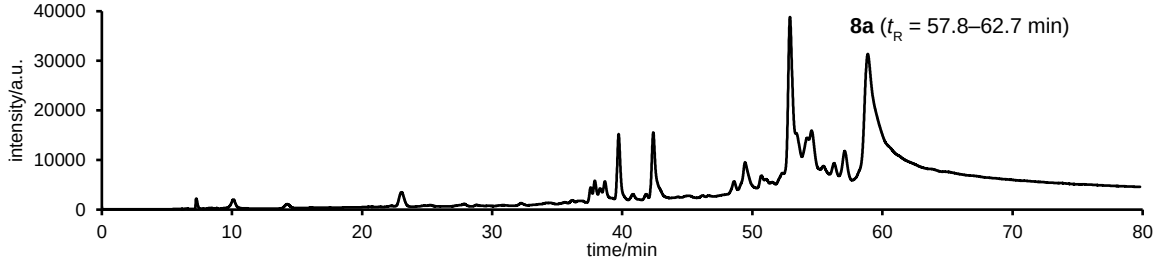


Figure S11. HPLC chart for purification of **8a**. Column: Inertsil C8-3 10 × 250 mm, eluent A: MeOH + 0.05% HCO_2H, eluent B: H_2O + 0.05% HCO_2H, linear gradient: A/B = 70/30 to 100/0 over 40 min, flow rate: 1.50 mL/min, detection: UV 280 nm, temperature: 40 °C.

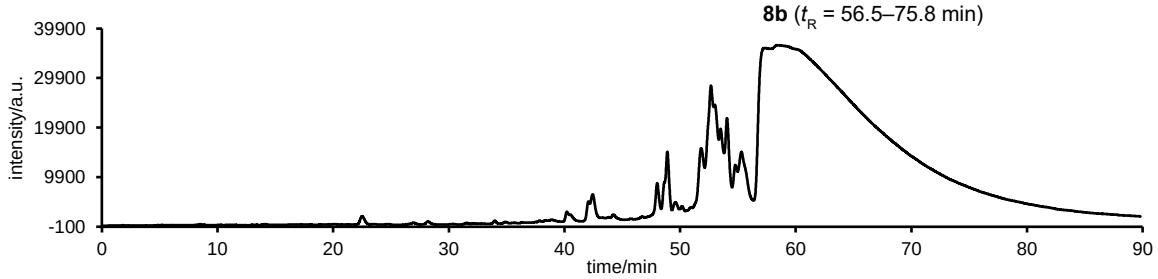


Figure S12. HPLC chart for purification of **8b**. Column: Inertsil C8-3 10 × 250 mm, eluent A: MeOH + 0.05% HCO_2H, eluent B: H_2O + 0.05% HCO_2H, linear gradient: A/B = 70/30 to 100/0 over 40 min, flow rate: 1.50 mL/min, detection: UV 280 nm, temperature: 40 °C.

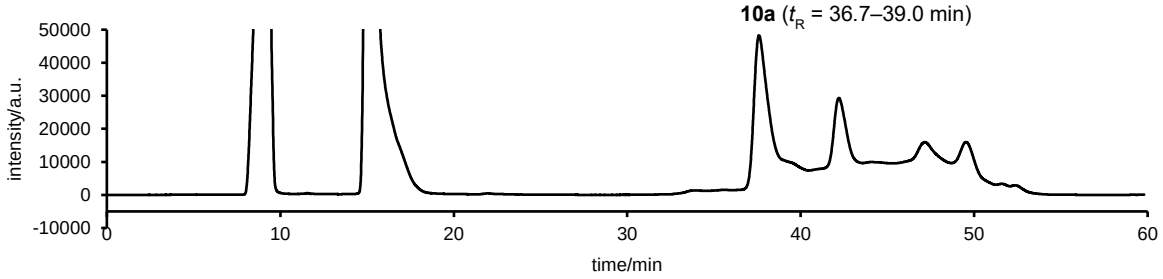


Figure S13. HPLC chart for purification of **10a**. Column: Inertsil C8-3 10 × 250 mm, eluent A: *i*-PrOH + 0.05% HCO_2H, eluent B: H_2O + 0.05% HCO_2H, linear gradient: A/B = 60/40 to 100/0 over 40 min, flow rate: 1.50 mL/min, detection: UV 280 nm, temperature: 40 °C.

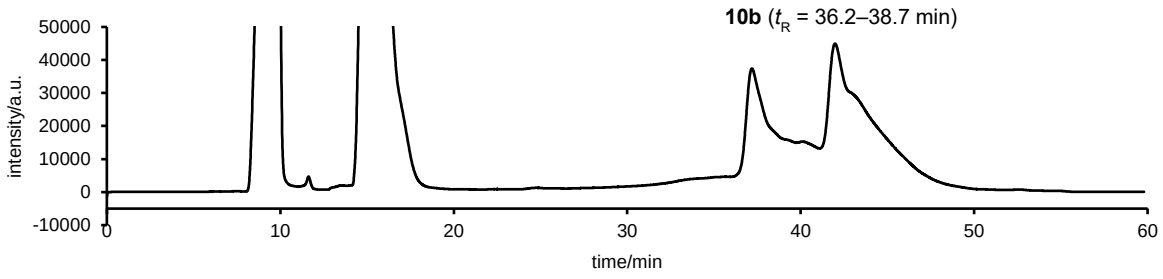


Figure S14. HPLC chart for purification of **10b**. Column: Inertsil C8-3 10 × 250 mm, eluent A: *i*-PrOH + 0.05% HCO_2H, eluent B: H_2O + 0.05% HCO_2H, linear gradient: A/B = 60/40 to 100/0 over 40 min, flow rate: 1.50 mL/min, detection: UV 280 nm, temperature: 40 °C.

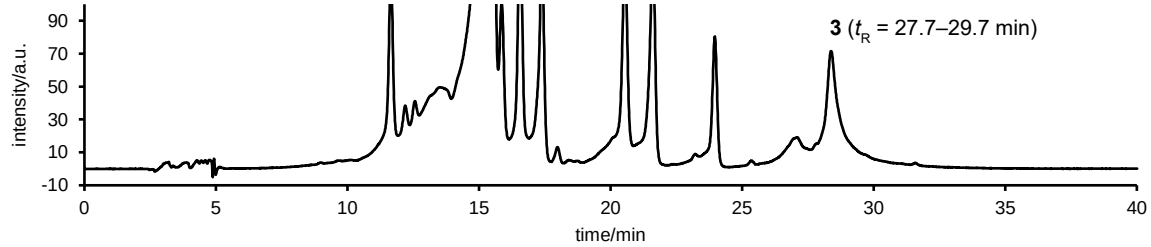


Figure S15. HPLC chart for purification of **3**. Column: Inertsil C8-3 4.6 × 150 mm, eluent A: MeCN + 0.05% TFA, eluent B: H_2O + 0.05% TFA, linear gradient: A/B = 35/65 to 100/0 over 40 min, flow rate: 0.500 mL/min, detection: UV 500 nm, temperature: 40 ℃.

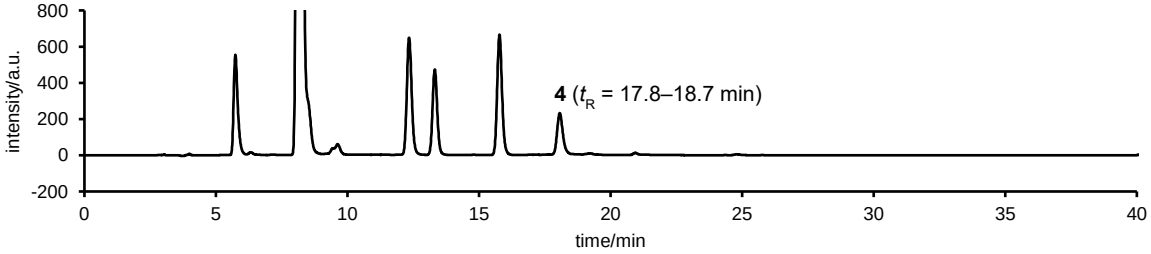


Figure S16. HPLC chart for purification of **4**. Column: Inertsil C8-3 4.6 × 150 mm, eluent A: MeCN + 0.05% TFA, eluent B: H_2O + 0.05% TFA, linear gradient: A/B = 50/50 to 100/0 over 40 min, flow rate: 0.500 mL/min, detection: UV 500 nm, temperature: 40 ℃.

UHPLC Chart for Analysis of Purified 3 and 4

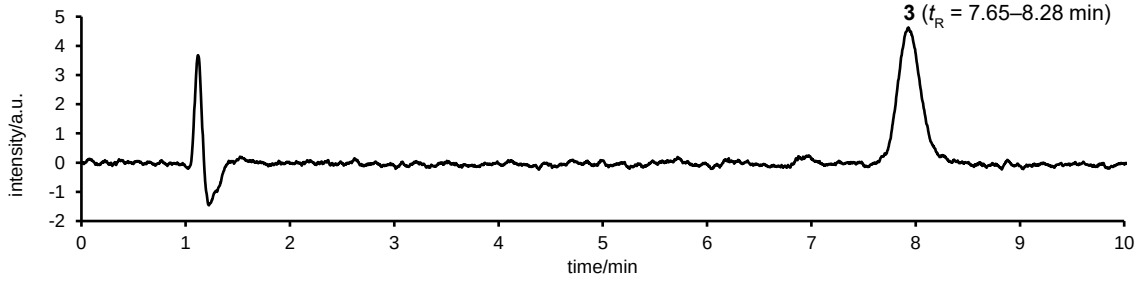


Figure S17. UHPLC chart for analysis of purified **3**. Column: Accucore C18 2.1 × 150 mm, eluent A: MeOH + 0.05% TFA, eluent B: H_2O + 0.05% TFA, linear gradient: A/B = 79/21, flow rate: 0.300 mL/min, detection: photodiode array detector 200–648 nm (UV chromatogram: 500 nm), temperature: 40 ℃.

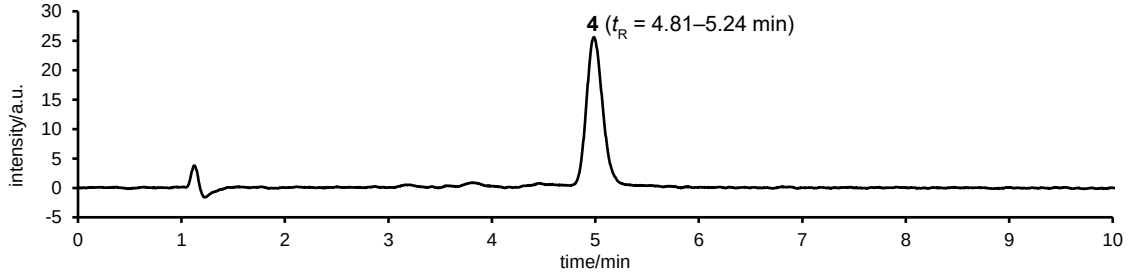


Figure S18. UHPLC chart for analysis of purified **4**. Column: Accucore C18 2.1 × 150 mm, eluent A: MeOH + 0.05% TFA, eluent B: H_2O + 0.05% TFA, linear gradient: A/B = 79/21, flow rate: 0.300 mL/min, detection: photodiode array detector 200–648 nm (UV chromatogram: 500 nm), temperature: 40 ℃.

NMR Spectra

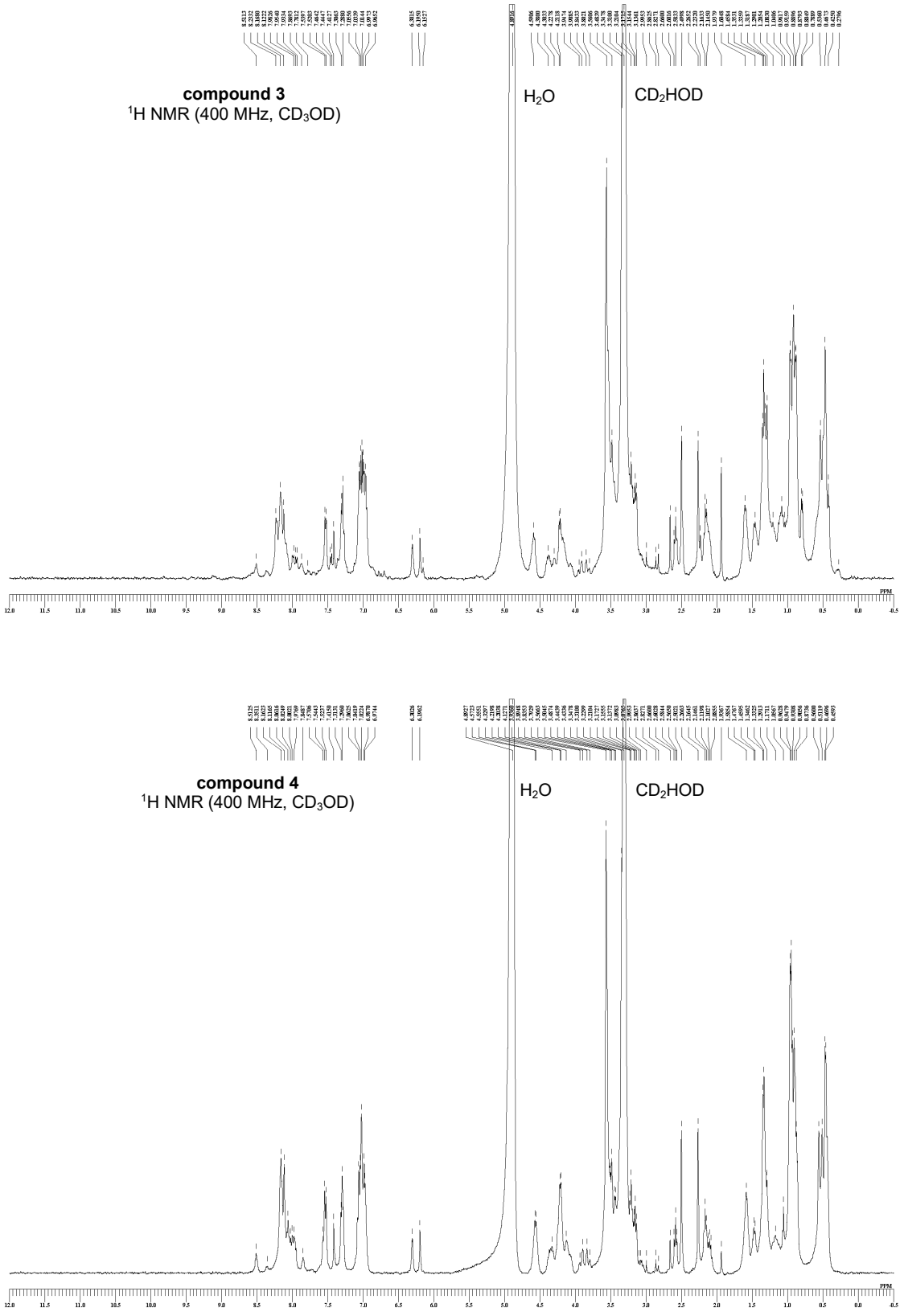


Figure S19. ^{1}H NMR spectra of compounds **3** and **4**.

References

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