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Supplementary Information

C-Terminal Modification of Polytheonamide B Uncouples Its Dual Functions in MCF-7 Cancer Cells

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Methods

General remarks. All reactions sensitive to air or moisture were carried out under argon (Ar) atmosphere in dry solvents, unless otherwise noted. DMF and *i*-Pr_2NEt were distilled from $MgSO_4$ and KOH, respectively. All other reagents were used as supplied unless otherwise noted. LRMS spectra were recorded on a micrOTOF II (Bruker Daltonics) electrospray ionization time-of-flight (ESI-TOF) mass spectrometer. High-performance liquid chromatography (HPLC) experiments were performed on an 1100 HPLC system (Agilent).

Quantification of peptides. The quantification of **2** and **3** was carried out using HPLC (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 = 10/90 to 20/80 over 20 min, then 20/80 to 50/50 over 30 min, flow rate: 0.600 mL/min, detection: UV 220 nm, temperature: 45 °C). HPLC chromatograms were recorded under the UV detection, and the peak areas of the compounds (UV 220 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 polytheonamide B (**1**, UV 220 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 = 25/75 to 50/50 over 35 min, flow rate: 0.600 mL/min, detection: UV 220 nm, temperature: 45 °C).

Compound 2. A solution of **1** (50.3 μg, 10.0 nmol) in *n*-PrOH was transferred to a seal tube and dried under a stream of Ar. To the dried **1** was added DMF (50.0 μL). To the solution of **1** in DMF were added a solution of HOAt (1.39 mg, 10 μmol) in DMF (50.0 μL), a solution of PyBOP (5.31 mg, 10 μmol) in DMF (50.0 μL), and a solution of 2,4,6-colldine (5.40 μL, 40 μmol) in DMF (50.0 μL). The reaction mixture was stirred at room temperature for 5 min. To the resultant mixture was added a solution of amine **4** (3.86 mg, 20 μmol) in DMF (50.0 μL). After being stirred at room temperature for 48 h, the resultant mixture was purified by reversed-phase HPLC (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 = 30/70 to 50/50 over 20 min, then 50/50 to 90/10 over 30min, flow rate: 0.600 mL/min, detection: UV 220 nm, temperature: 45 °C) to give **2** (t_R = 12.1–12.9 min, 3.37 nmol, 34%): LRMS (ESI-TOF) calcd for $C_{225}H_{390}N_{62}O_{71}S$ $[M+3H]^{3+}$ 1711.29, found 1711.10.

Compound 3. A solution of **1** (50.3 μg, 10.0 nmol) in *n*-PrOH was transferred to a seal tube and dried under a stream of Ar. To the dried **1** was added DMF (40.0 μL). To the solution of **1** in DMF were added a solution of HOAt (1.38 mg, 10 μmol) in DMF (40.0 μL), a solution of PyBOP (5.28 mg, 10 μmol) in DMF (40.0 μL), and a solution of 2,4,6-colldine (5.37 μL, 40 μmol) in DMF (40.0 μL). The reaction mixture was stirred at room temperature for 5 min. To the resultant mixture was added a solution of amine **5** (1.72 mg, 10 μmol) in DMF (40.0 μL). After being stirred at room temperature for 48 h, the resultant mixture was purified by reversed-phase HPLC (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 = 30/70 to 50/50 over 20 min, then 50/50 to 90/10 over 30min, flow rate: 0.600 mL/min, detection: UV 220 nm, temperature: 45 °C) to give **3** (t_R = 13.2–13.8 min, 6.99 nmol, 70%): LRMS (ESI-TOF) calcd for $C_{229}H_{401}N_{62}O_{71}S$ $[M+3H]^{3+}$ 1729.98, found 1729.88.

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.

MCF-10A cells were obtained from ATCC. The cells were maintained with mammary epithelial cell growth medium [MEBM (CC3151, Lonza) supplemented with MEGM SingleQuots (CC-4136, Lonza), cholera toxin (100 ng/mL), 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 d to reach 70–90% cell confluence.

Plasma membrane depolarizing activity assay. 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 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (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 bis(1,3-dibutylbarbituric acid)trimethine oxonol ($DiBAC_4(3)$)]. 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 **1** (25 nM) as 100% and by addition of vehicle (DMSO) as 0%. The plasma membrane depolarization activity of each compound was evaluated as half maximal 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.

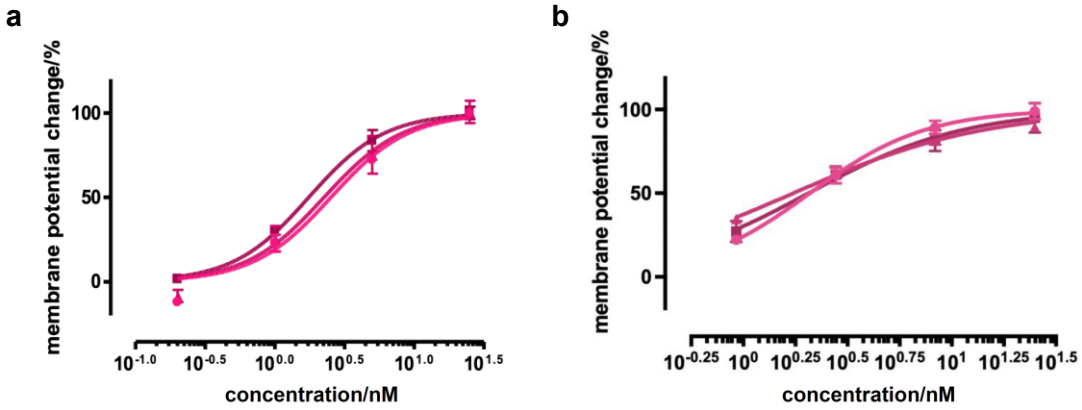


Figure S1. Concentration-response curves of (a) **2** and (b) **3** (three independent experiments). The cells were incubated for 1 h in the presence of each compound, and the membrane potential change (%) was evaluated. Each plot is displayed as mean ± SD of three replicates.

Evaluation of lysosomal pH neutralization using LysoTracker Red DND-99. 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**, **2**, or **3** 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 (2 h, 4 h, or 8 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). The number of particles was analyzed by Analyze Particles function using ImageJ.[S1]

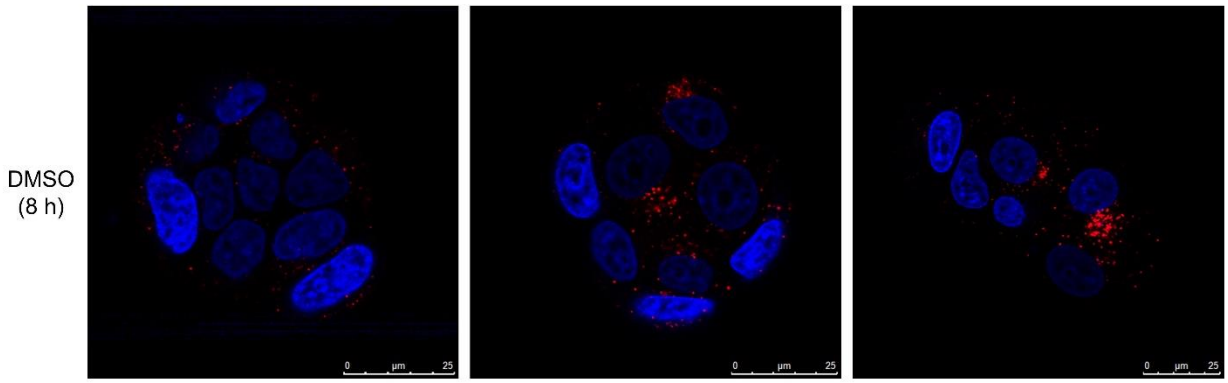


Figure S2. Three images obtained after addition of DMSO for the analysis of stained particle numbers. The nuclei were stained with Hoechst 33342. Scale bar represents 25 μm.

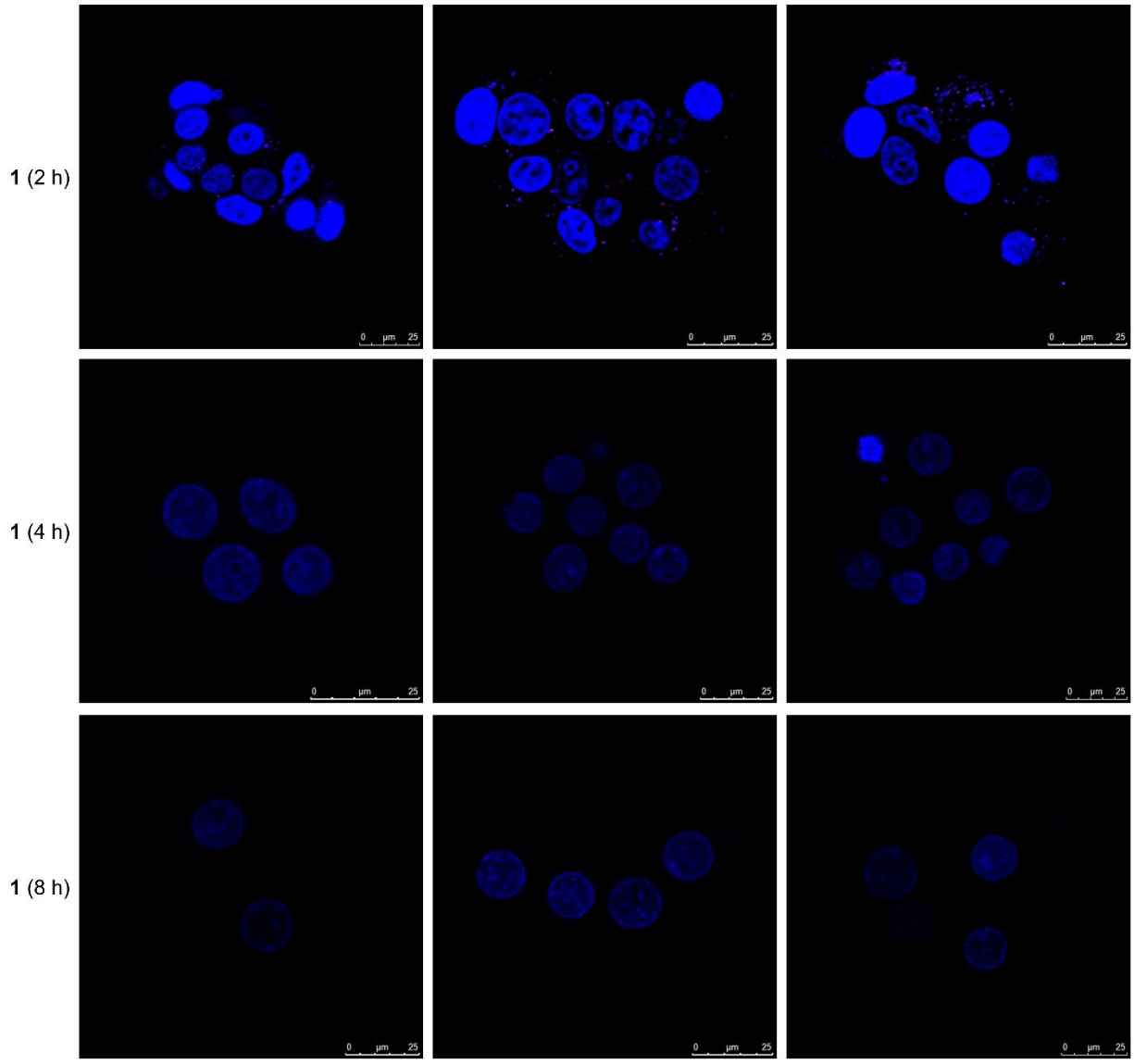


Figure S3. Three images obtained after addition of **1** for the analysis of stained particle numbers. The nuclei were stained with Hoechst 33342. Scale bar represents 25 μm.

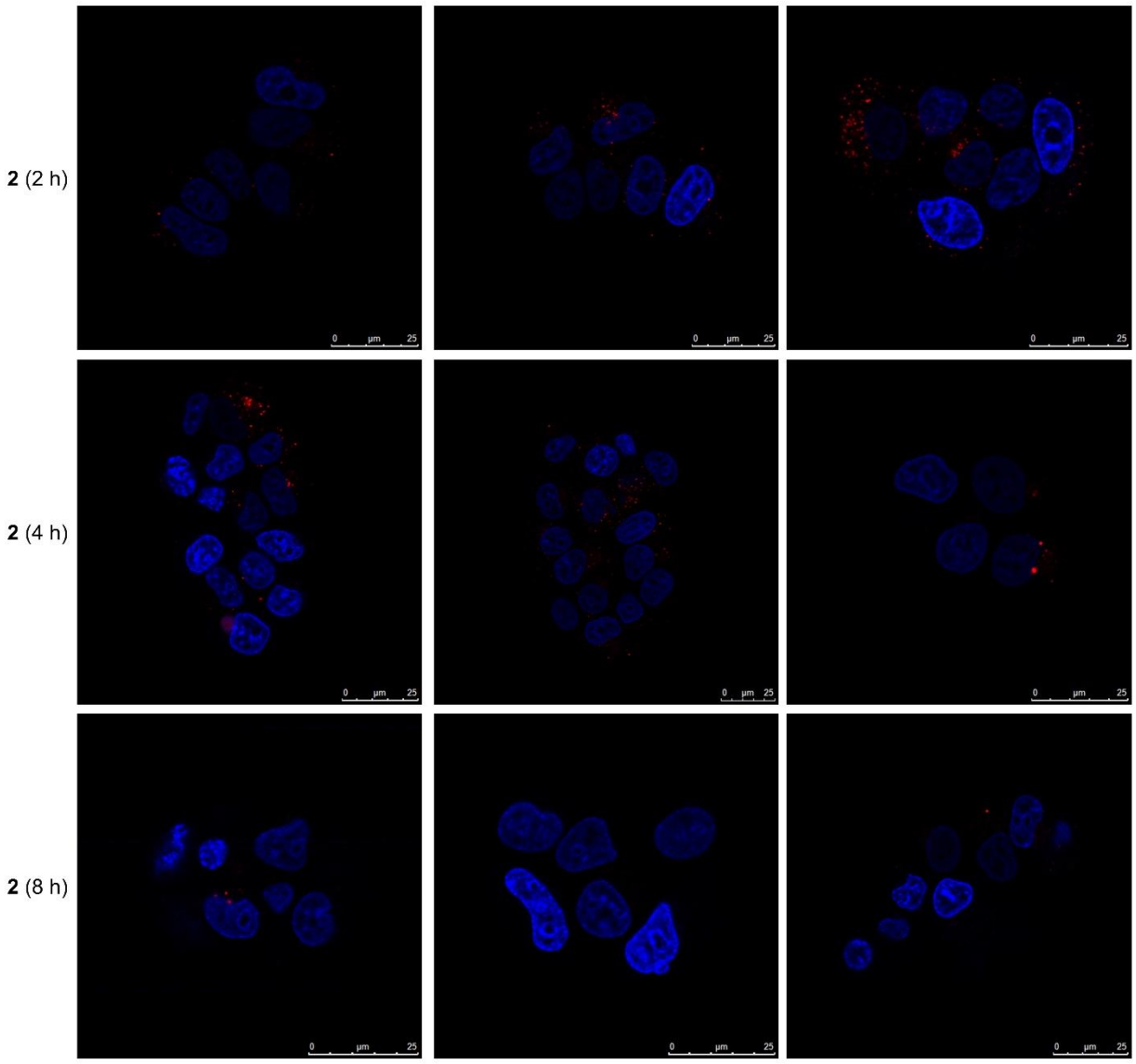


Figure S4. Three images obtained after addition of **2** for the analysis of stained particle numbers. The nuclei were stained with Hoechst 33342. Scale bar represents 25 μm.

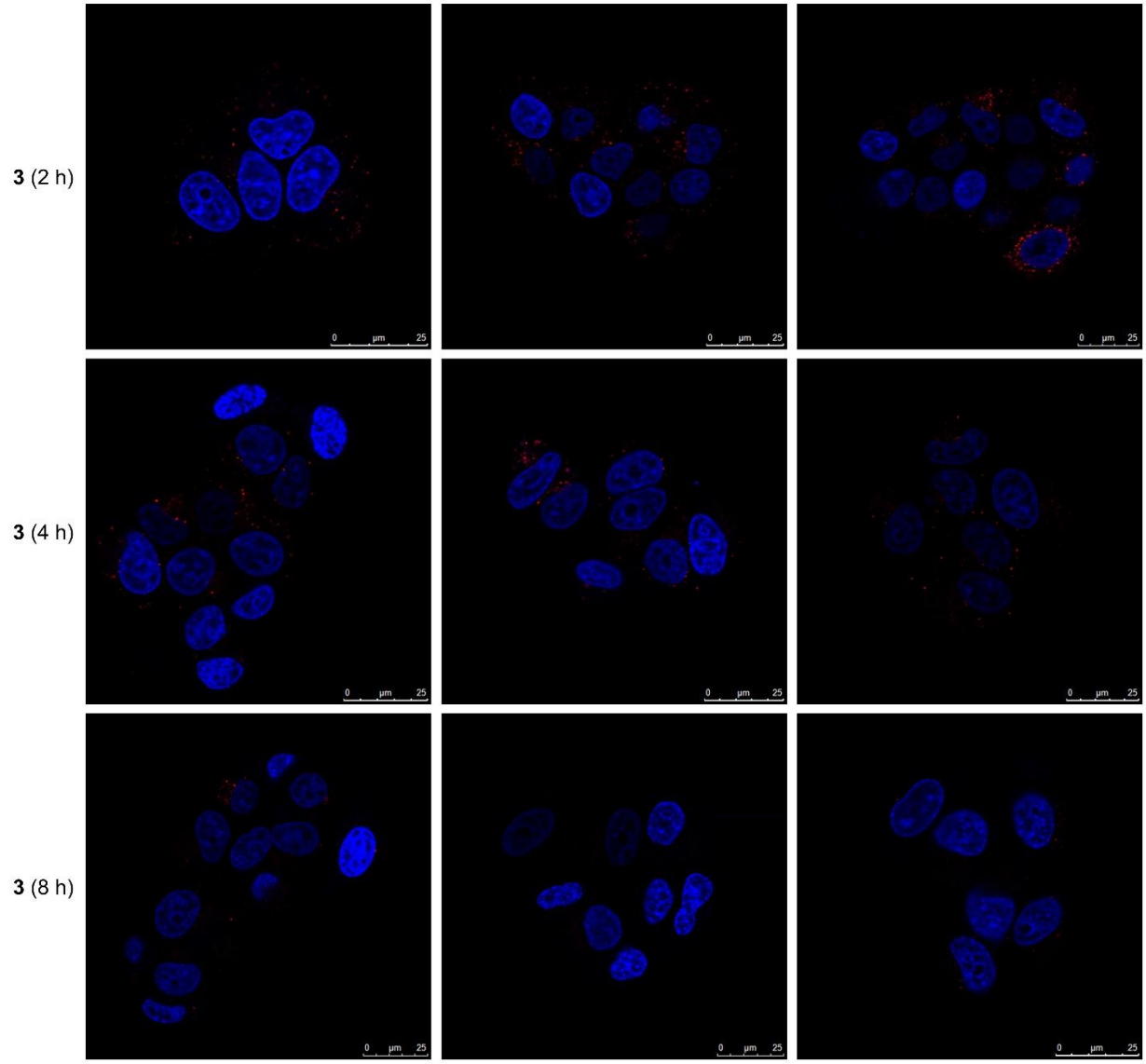


Figure S5. Three images obtained after addition of **3** for the analysis of stained particle numbers. The nuclei were stained with Hoechst 33342. Scale bar represents 25 μm.

Sulforhodamine B assay. Various concentrations of **2** or **3** in the growth medium containing 2% DMSO were prepared by 5-fold serial dilutions. MCF-7 or MCF-10A cells were cultured in 6 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 (MCF-7 cells) or 2.00×10^4 cells/mL (MCF-10A cells). The cell suspension (100 μL/well) was seeded into the black polystyrene flat-bottom 96-well plate (Greiner Bio-One). The cells 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 plate. The final concentration of the compounds ranged from 0.64 pM to 50 nM for **2**, and 0.32 pM to 25 nM for **3**. The final concentration of cells and DMSO were 6.25×10^5 cells/mL and 1%, respectively. The plate was incubated at 37 °C under atmosphere of 5% CO_2 for 48 h. To the cells in the plate was added an ice-cold solution of 30 w/v% trichloroacetic acid in H_2O (100 μL/well). The plate was incubated at 4 °C for 60 min, washed with H_2O (× 4), and dried. To each well of the plate was added a solution of sulforhodamine B in AcOH/H_2O (1/99, 0.57 mg/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 plate was vortexed at room temperature for 10 min. The fluorescence (Ex. 485 nm/Em. 585 nm) of each well was measured on a Spectra Max Gemini EM microplate reader (Molecular Devices). The cytotoxicity of each compound was evaluated as half maximal inhibitory concentration (IC_{50}, nM) by means of three replicates. Sigmoidal curve fittings were performed on Prism 4 (Graphpad Software). The cell viability was normalized against untreated cells as 100% and the growth medium as 0%. IC_{50} value of each compound was determined by three independent experiments.

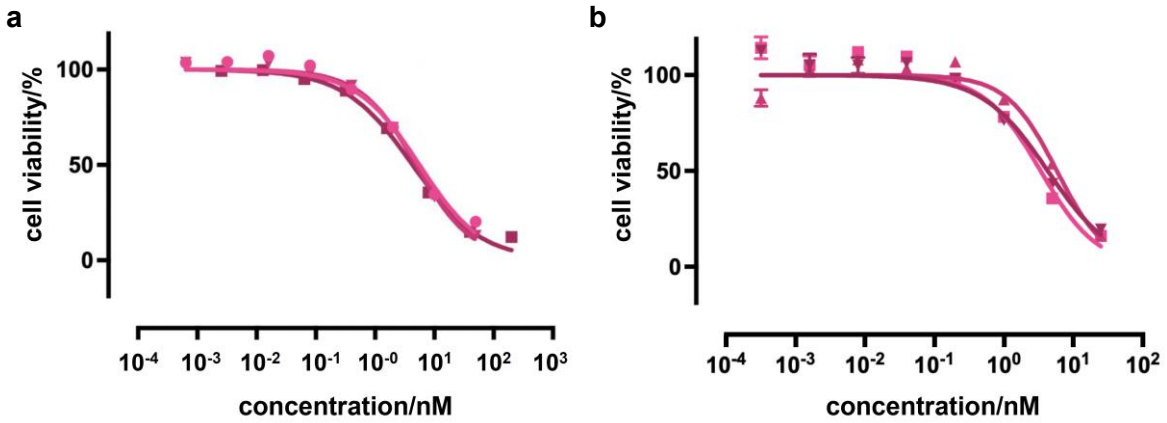


Figure S6. Concentration-response curves of (a) **2** and (b) **3** against MCF-7 cells (three independent experiments). The cells were incubated for 2 d in the presence of each compound, and the cell viability (%) was evaluated. Each plot is displayed as mean ± SD of three replicates.

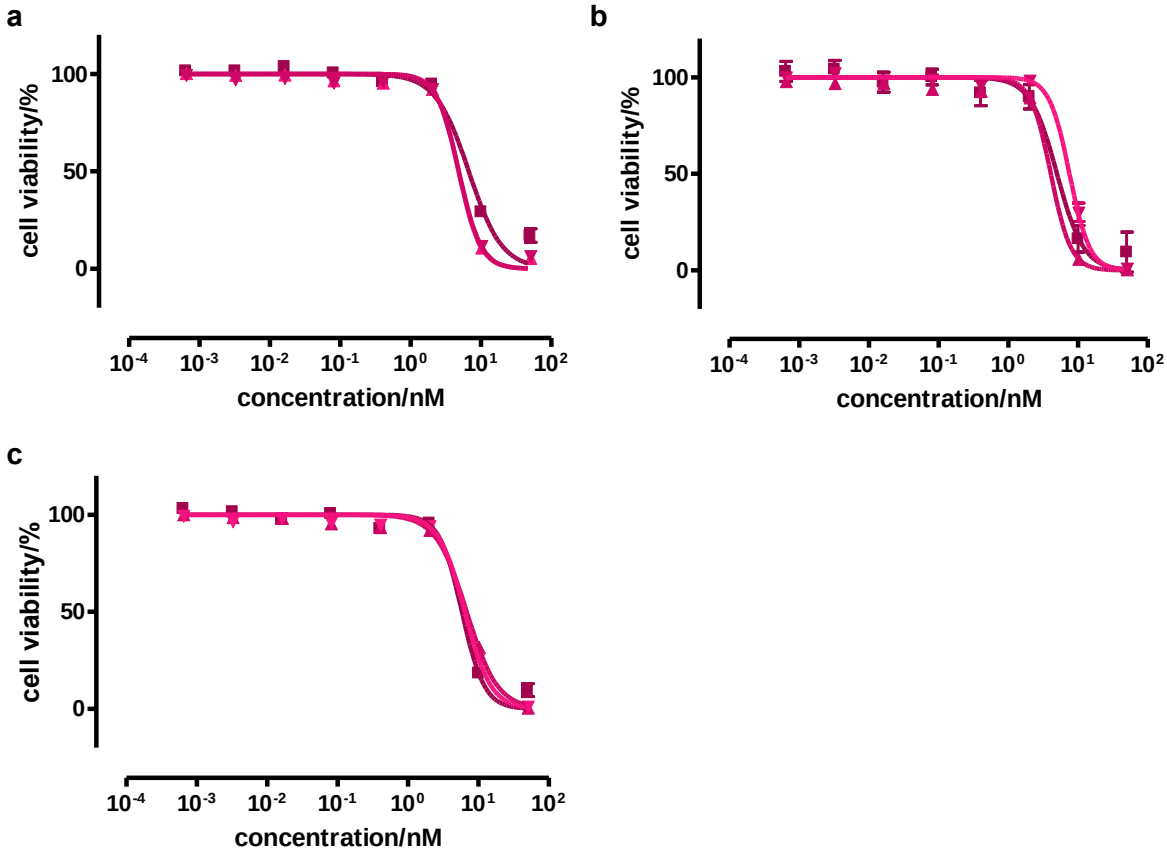


Figure S7. Concentration-response curves of (a) **1**, (b) **2**, and (c) **3** against MCF-10A cells (three independent experiments). The cells were incubated for 2 d in the presence of each compound, and the cell viability (%) was evaluated. Each plot is displayed as mean ± SD of three replicates.

Table S1. Cytotoxicity against MCF-10A of **1**–**3**.

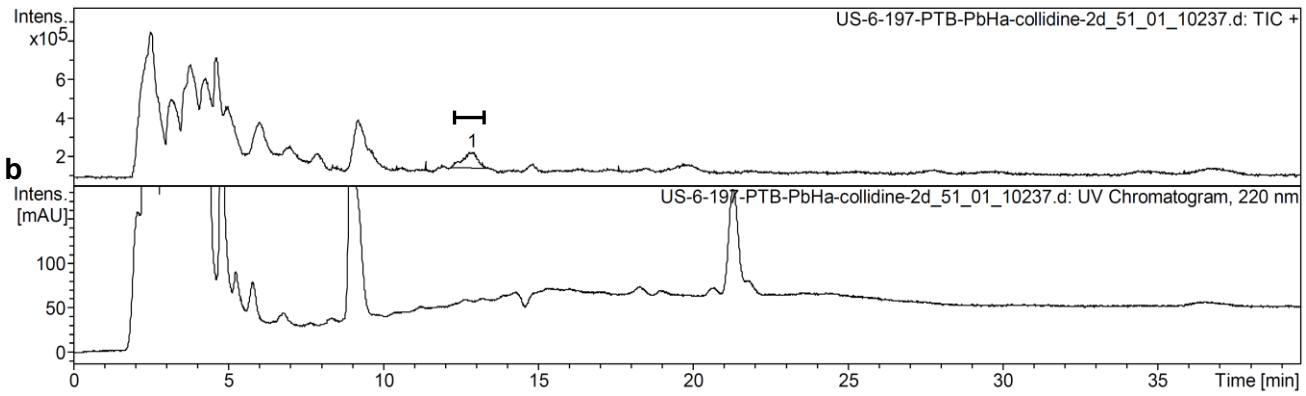
compounds	cytotoxicity against MCF-10A cells (IC_{50}, nM)[a]
1	5.5 ± 0.9
2	5.6 ± 1.5
3	6.4 ± 0.4

[a]Data are displayed as mean ± SD from three independent experiments.

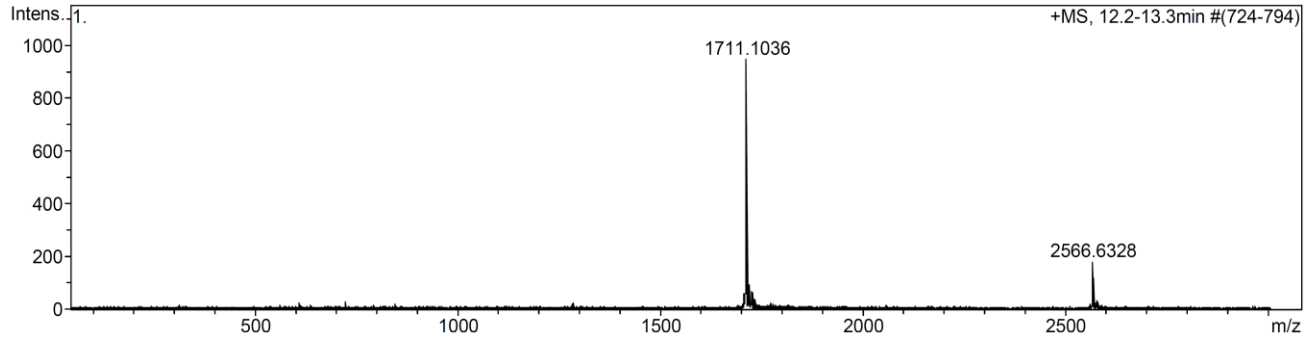
HPLC Charts for Purification of Synthetic Compounds

a

b



c



d

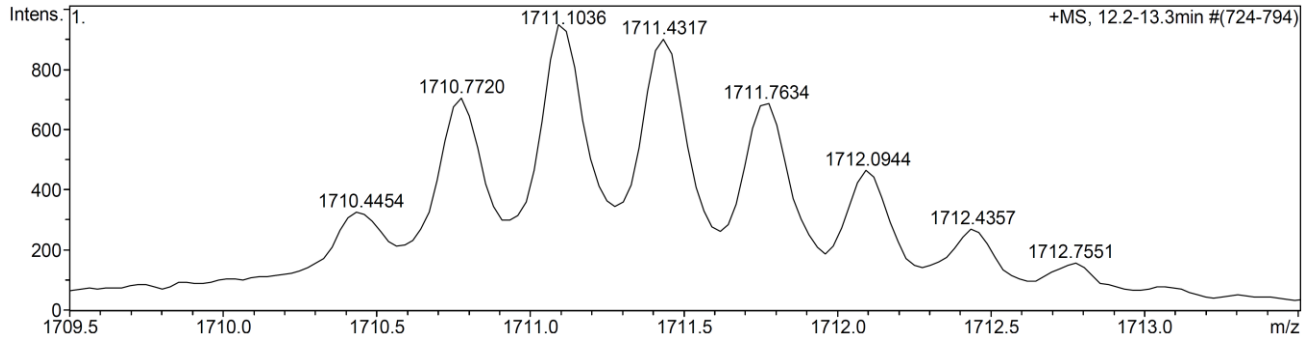


Figure S8. HPLC chart for purification of **2**. (a) Total ion chromatogram and (b) UV chromatogram of the crude material. Column: Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H_2O + 0.05% TFA, linear gradient: A/B = 30/70 to 50/50 over 20 min, then 50/50 to 90/10 over 30 min flow rate: 0.600 mL/min, detection: UV 220 nm, temperature: 45 ℃. (c) MS signals of peak 1 in Figure S8a (m/z = 0–3000) and (d) (m/z = 1709.5–1713.5).

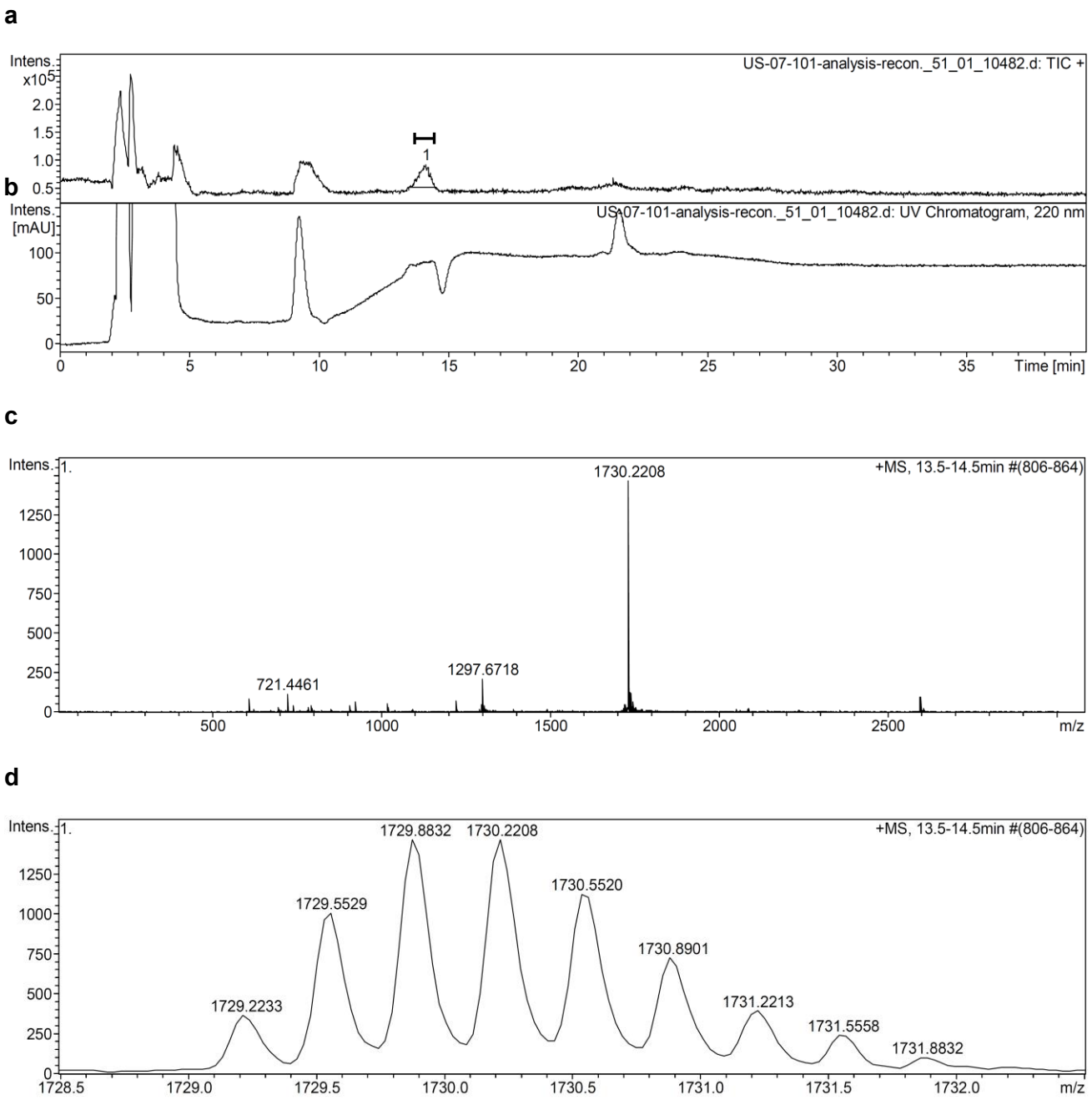


Figure S9. HPLC chart for purification of **3**. (a) Total ion chromatogram and (b) UV chromatogram of the crude material. Column: Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H_2O + 0.05% TFA, linear gradient: A/B = 30/70 to 50/50 over 20 min, then 50/50 to 90/10 over 30 min flow rate: 0.600 mL/min, detection: UV 220 nm, temperature: 45 ℃. (c) MS signals of peak 1 in Figure S9a (*m*/*z* = 0–3000) and (d) (*m*/*z* = 1728.5–1732.5).

HPLC Charts for Analysis of Purified 2 and 3

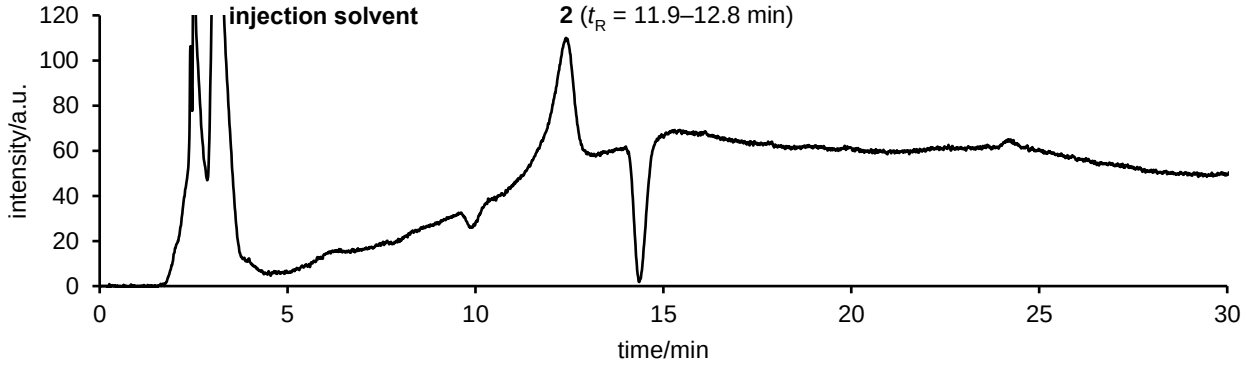


Figure S10. HPLC chart for analysis of purified **2**. Column: Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H_2O + 0.05% TFA, linear gradient: A/B = 30/70 to 50/50 over 20 min, then 50/50 to 90/10 over 30 min flow rate: 0.600 mL/min, detection: UV 220 nm, temperature: 45 ℃.

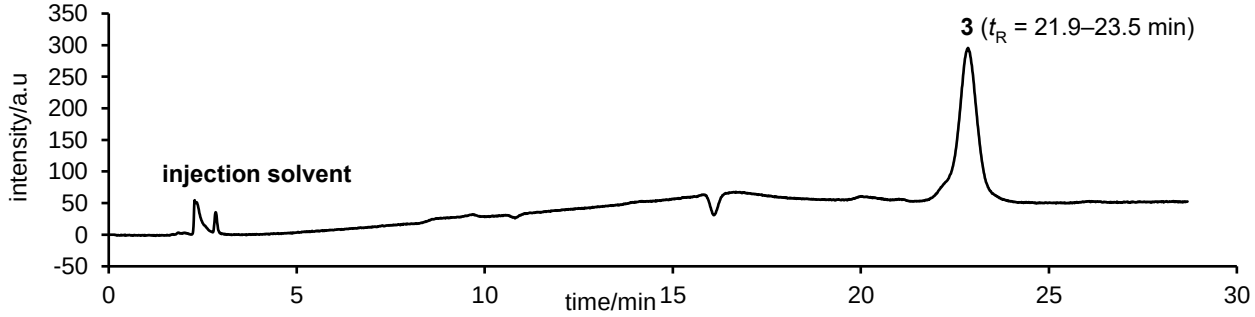


Figure S11. HPLC chart for analysis of purified **3**. Column: Inertsil C8-3 4.6 × 150 mm, eluent A: *n*-PrOH + 0.05% TFA, eluent B: H_2O + 0.05% TFA, linear gradient: A/B = 20/80 to 50/50 over 30 min, flow rate: 0.600 mL/min, detection: UV 220 nm, temperature: 45 ℃.

References

S1. Schneider, C. A.; Rasband, W. S.; Eliceiri, K. W. *Nat. Methods* **2012**, *9*, 671–675.