Supporting Information

Cancer-Specific Dual-factor Cascade Recognition Fluorescent Probe for Imaging and tumor diagnosis

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Synthesis of Probe S1-F.

To a suspension of compound **S1** (662 mg, 2.0 mmol) in 30 mL anhydrous acetonitrile, 3-aminopropan-1-ol (751 mg, 10.0 mmol) were added at room temperature, and the mixture was further stirred at room temperature for 1 h. Then, the acetonitrile was removed under reduced pressure, and the crude was purified on silica gel (CH_2Cl_2: CH_3OH = 15:1) to give intermediate product **1** with a yield of 15 %.

To a suspension of intermediate product **1** (138 mg, 0.3 mmol) in 10 mL anhydrous acetonitrile, 2,4-dinitrobenzenesulfonyl chloride (133 mg, 0.5 mmol) and triethylamine (91 mg, 0.9 mmol) were added at 0 ℃, and the mixture was further stirred at room temperature for 6 h. Then, the solvent was removed under reduced pressure, and the crude was purified on silica gel (CH_2Cl_2: CH_3OH = 30:1) to give the final product **S1-F** with a yield of 46 %. **S1-F**, yellow solid. HRMS (ESI): calculated for $C_{27}H_{24}N_5O_8S_2^+$ $[M+H]^+$ 610.1061, and found to be 610.1066. ^{1}H-NMR (500 MHz, DMSO-d_6) δ 9.01 (s, 1H), 8.85 (d, J = 8.6 Hz, 1H), 8.43 (d, *J* = 8.2 Hz, 1H), 8.40-8.35 (m, 3H), 8.31 (d, *J* = 8.2 Hz, 1H), 7.76 (t, *J* = 7.4 Hz,1H), 7.29 (d, *J* = 8.4 Hz, 1H), 4.03 (t, *J* = 6.8 Hz, 2H), 3.44 (t, *J* = 6.4 Hz, 4H), 3.31 (t, *J* = 6.2 Hz, 4H), 2.83 (t, *J* = 6.5 Hz, 4H), 1.69-1.67 (m, 2H).

Materials and Instruments

All reagents involved in the experiments were purchased from commercial suppliers and directly used without any further purification. NMR spectra were recorded using a Bruker 500 spectrometer. Mass spectra were obtained with HP1100LC/MSD MS and LC/Q-TOF-MS instruments. UV-vis spectra were measured on a PerkinElmer Lambda 750s spectrophotometer. Fluorescence spectra were recorded by using a Hitachi F-7000 fluorescence spectrophotometer. Fluorescence imaging was measured with an Olympus FV-1000 inverted fluorescence microscope.

Water Solubility

As previously reported, water solubility was measured at pH=7 using a shake-flask method and HPLC.[1] Excessive (10 mg) **S1-F** was spiked into purified water (1 mL). The mixture was shaken for 24 h with a Hangzhou Aosheng Instrument Co., Ltd. constant temperature incubation shaker shaken at 220 rpm at room temperature and left to sit for 1 h. The saturated solution was filtrated through a filter membrane (pore size = 0.22 μm) and transferred to other Eppendorf tubes for analysis by HPLC. The sample was performed in duplicate. For quantification, a model HP1100 HPLC (Agilent) system was used with a Kromasil 100-5C18 column (250 mm × 4.6 mm, 5 μm) and eluant is acetonitrile/water (60%-95%). The flow rate was 1.0 mL/min, and the injection

volume was 15 μL. Aqueous concentration was determined by comparison of the peak area of the saturated solution with a standard curve plotted peak area versus known concentrations, which were prepared by solutions of compound **S1-F** in an aqueous solution containing 2% Tween 20 at 2000, 400, 80, 16, and 3.2 μM. The formula for compound concentration and peak area is determined as follows:

$y = 0.0482x - 0.8769$, x = peak area, y = concentration (μg/mL), $R^2 = 0.98$

Spectral Measurements

A stock solution (10 mM) of the probe in DMSO was prepared. During the measurement, a sample solution was prepared by mixing an appropriate amount of the stock solution of the probe with the proper amounts of analytes (Cys, Hcy, GSH, and so on), respectively, and finally diluted by PBS (10 mM, pH 7.4) to obtain the desired concentration. The mixtures were incubated at 37 °C for an appropriate time, and then fluorescence intensities of the probes were measured with a slit width of 5 nm × 10 nm at 37 °C.

Fluorescence Polarization Assay (FPA)

For the competitive binding assay for the Mcl-1 and Bcl-2 protein, FAM-Bid peptide (10 nM), Mcl-1 protein (55 nM) and Bcl-2 protein (140 nM) were pre-incubated in the assay buffer (100 mM potassium phosphate, pH 7.5; 100 μg/mL bovine gamma globulin; 0.02% sodium azide). Next, serial dilutions of compounds were added. After a 10-minute incubation, the polarization values were measured using the Spectra Max M5 Detection System in a black 96-well plate. For each assay, the FP values of blank controls (FAM-Bid only) were recorded as P_{min}; the FP values of negative controls (FAM-Bid and proteins) were recorded as P_{max}, and the FP values of test wells (compounds, FAM-Bid, and protein) were recorded as P_{test}. The inhibition rates of the compounds at each concentration point were calculated using the equation as follows: inhibition rate (%) = $[1 - (P_{test} - P_{min})/(P_{max} - P_{min})] \times 100\%$, and the IC_{50} values were calculated using GraphPad Prism 6.0 software (Figure S1). Saturation experiments determined that FAM-Bid binds to the Mcl-1 and Bcl-2 proteins with K_d values of 1.9 nM and 8 nM, respectively. The K_i values were calculated as previously reported.[2]

Molecular Docking

The 3D structures of the human Mcl-1 (PDB ID: 2NLA) and Bcl-2 (PDB ID: 7JMT) were obtained from the RCSB Protein Data Bank. The 3D structures of the inhibitors were generated using Chembio3D Ultra 11.0 followed by energy minimization. The AutoDock 4.0 program equipped with ADT was used to perform the automated

molecular docking. Grid maps covering the canonical BH3 binding groove of the Mcl-1 and Bcl-2 protein were defined for all inhibitors in the AutoDock calculations using a grid spacing of 0.375 Å. The GA-LS algorithm was adopted using default settings. For each docking job, 100 hybrid GA-LS runs were carried out. A total of 100 possible binding conformations were generated and grouped into clusters based on a 1.0 Å cluster tolerance. The docking models were analyzed and the detailed interactions between compounds and residues were represented using Discovery Studio.

Cellular Imaging

MCF-7, Hela, NCI-H23, HEK-293 and MCF10A cells were cultured in DMEM/high glucose and RPMI1640 (Hyclone, Beijing, China) supplemented with 10% fetal bovine serum (Gibco Company, USA), respectively. All cells were cultured at 37 °C in 5% CO_2. Cells were grown to 80-90% confluency in confocal dishes. Then, probe **S1-F** (5 μM) was added for 1 h and washed with PBS three times. An Olympus FV-1000 inverted fluorescence microscope with a 20 × objective lens was used in order to conduct the fluorescence imaging. The probe **S1-F** was excited at 485 nm, and the emission spectra were collected at 550-570 nm.

Co-localization

MCF-7 and NCI-H23 cells were cultured and treated with **S1-F** (5 μM) using the same method as cellular imaging. The cells were subsequently fixed for 30 min at 4 °C with 4% formaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS for 15 min, followed by simple wash and blocking with 5% BSA, 10% normal goat serum, and 0.1% Triton X-100 in PBS at room temperature for 30 min. Subsequently, the cells were treated with anti-Bcl-2 and anti-Mcl-1 overnight at 4 °C, respectively, and washed three times with PBST. After incubation with Alexa Fluor 645 anti-Rabbit IgG (diluted 1:100) and a subsequent wash, the cells were finally imaged.

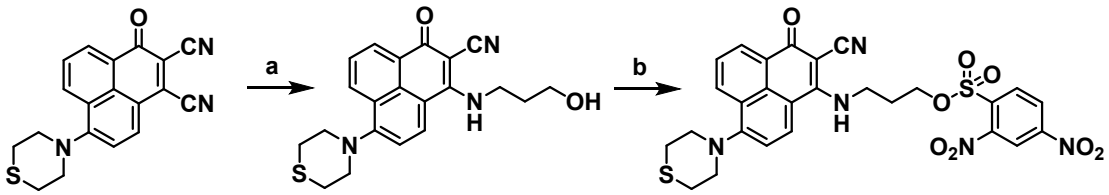
An Olympus FV-1000 inverted fluorescence microscope with a 60 × objective lens was used in order to conduct the fluorescence imaging. The probe **S1-F** was excited at 485 nm, and the emission spectra were collected at 550-570 nm. Alexa Fluor 645 anti-Rabbit IgG was excited at 605 nm, and the emission spectra were collected at 635-655 nm. Fluorescence imaging of all the groups was performed under the same experimental conditions. Fluorescence imaging of all the groups was performed under the same experimental conditions. Image analysis was performed using FV10-ASW 3.0 and ImageJ software.

Fluorescence Imaging of Surgically Resected Cancer and Benign Tissues

Human surgical tissue slices of 20 μm were prepared from liver cancer tissues and benign thyroid nodule tissues using freezing microtome (LEICA CM1860 UV). Next, these tissues were incubated with **S1-F** (10 μM, 37 °C) for 30 min, followed by washing thrice with phosphate buffer saline (0.01 M, pH 7.4). Under the confocal fluorescence microscope (Olympus FV1000-IX81) with a 10 × objective lens, probe **S1-F** was excited at 485 nm, and the emission spectra were collected at 550-570 nm. Image analysis was performed using FV10-ASW 3.0 and ImageJ software.

Statistical analysis

Statistical analysis was performed using IBM SPSS software (version 20.0, IBM, Armonk, NY) and all data were presented as mean ± SD. Significant differences among the groups were analyzed using the unpaired Student's t-test. A value of $P < 0.01$ was considered to be significant.



Scheme 1. Synthetic route of **S1-F.** (a) $H_2NCH_2CH_2CH_2OH$, CH_3CN, rt, 2 h; (b) 2,4-dinitro-benzenesulfonyl chloride, DIEA, THF, rt, 12 h.

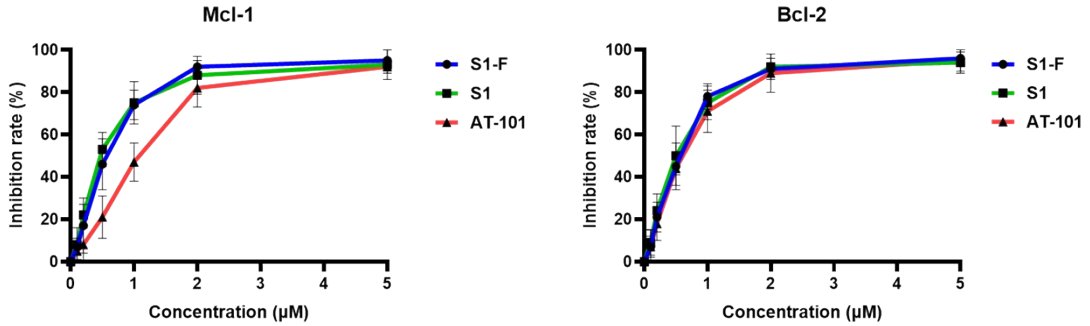


Fig. S1 Competitive binding curves of **S1-F**, **S1**, and **AT-101** to Mcl-1 and Bcl-2 proteins as determined in our fluorescence-polarization-based binding assay.

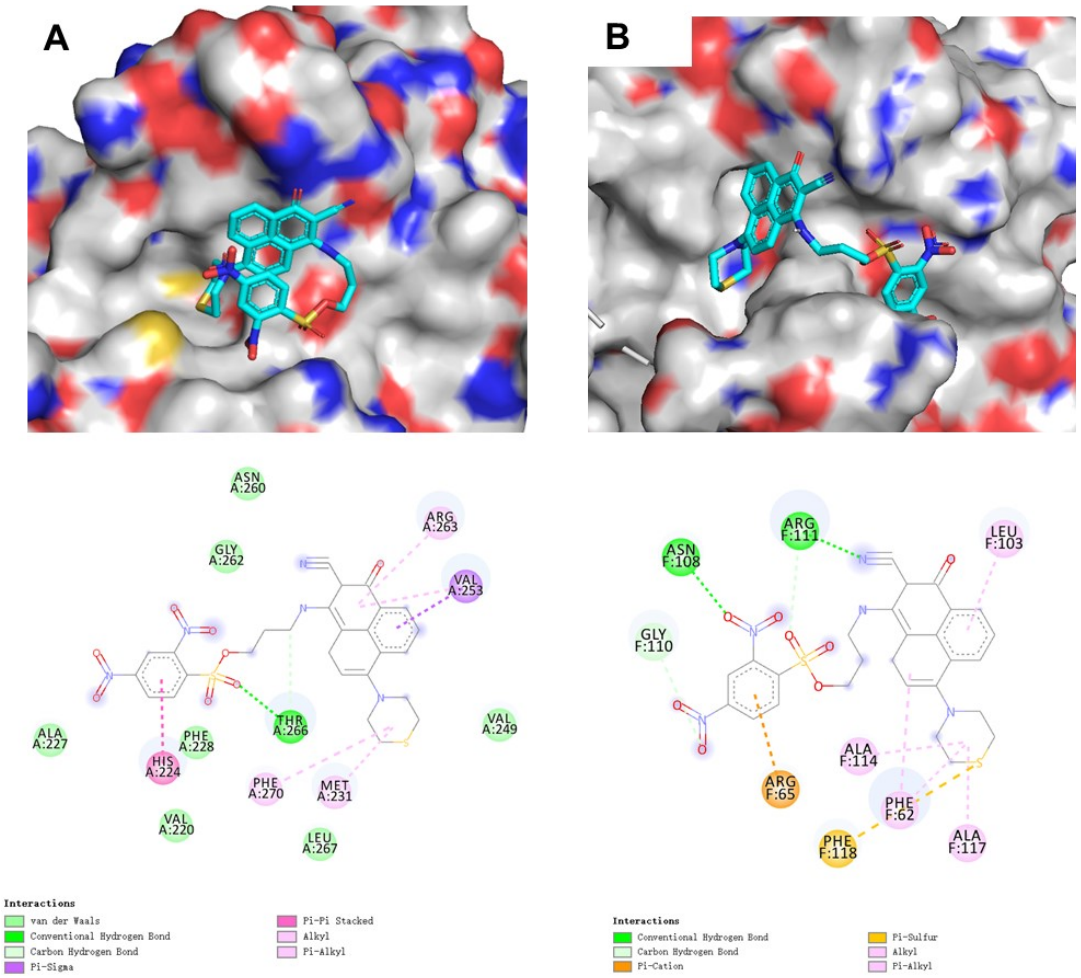


Fig. S2 Proposed binding mode of **S1-F** to (A) Bcl-2 and (B) Mcl-1 by molecular docking.

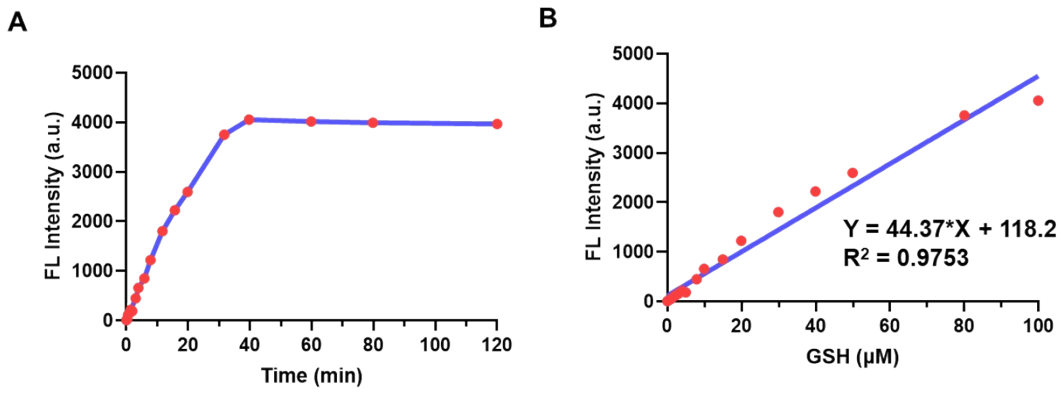


Fig. S3 (A) The calibration plot of time-dependent fluorescence changes of **S1-F** (10 μM) after GSH addition (100 μM) in PBS. (B) The calibration plot of fluorescence intensity changes of **S1-F** with 0-100 μM of GSH.

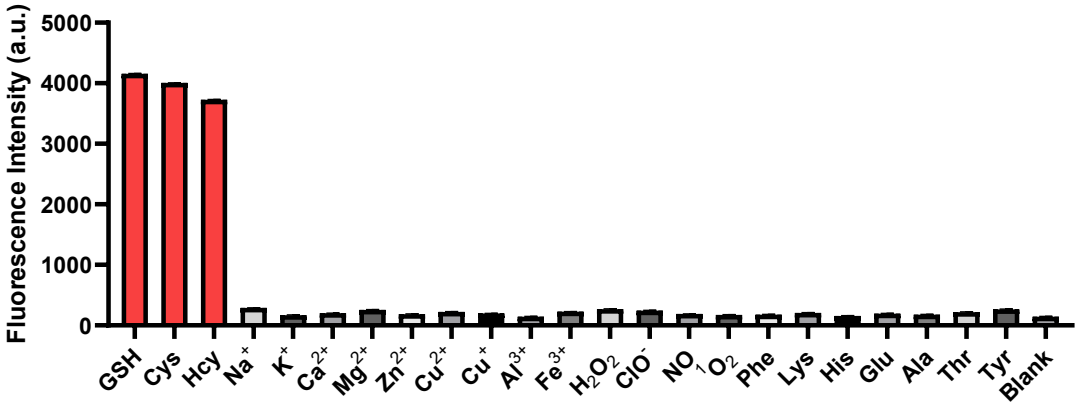


Fig. S4 Fluorescence response of 10 μM **S1-F** to various ROS, RNS, metal ions, and amino acids.

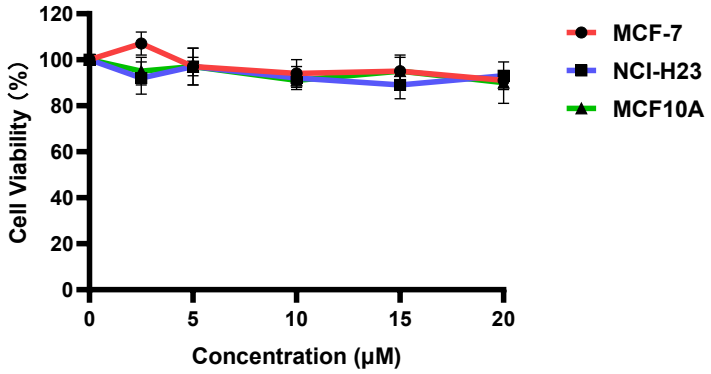


Fig. S5 Antiproliferative activity of **S1-F** measured using MTT assays at 6 h. Values are the mean ± SD for n = 3.

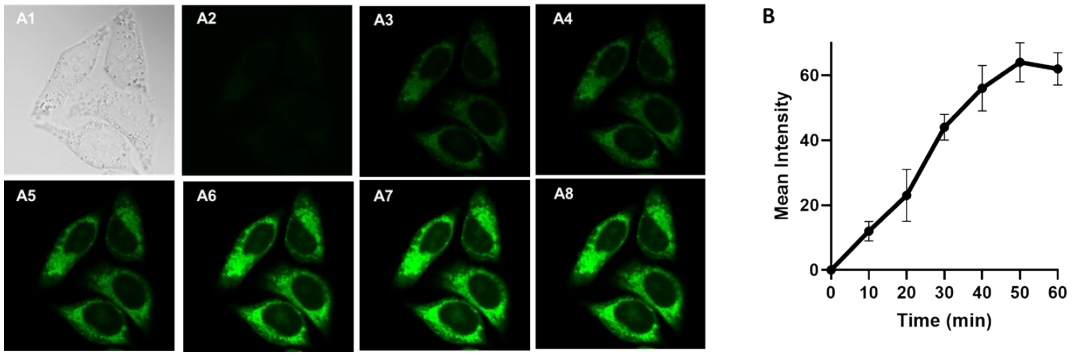


Fig. S6 Real-time monitoring performance of probe in HeLa cells. (A) fluorescence images of the probe from 0 to 60 min. (B) the quantitative data of probe's fluorescence signals in (A). All figures represent the results from n = 3 independent samples.

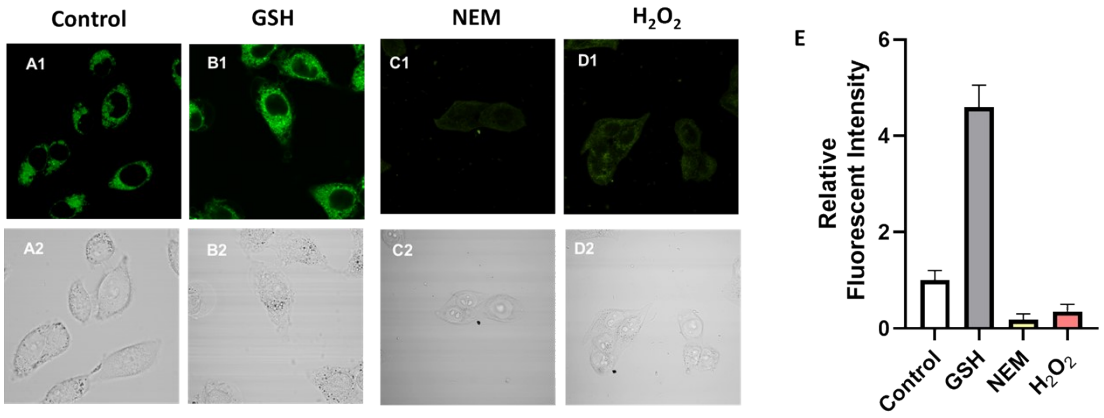


Fig. S7 (A-D) Fluorescence images of HeLa cells treated with PBS (A), 1 mM GSH (B), 1 mM NEM (C) or 100 μM H_2O_2 (D). (E) the quantitative data of the probe's fluorescence signals in (A-D). The concentration of probes is 5 μM. Fluorescence channel (λex = 435 nm, λem = 540-570 nm). All figures represent the results from n = 3 independent samples.

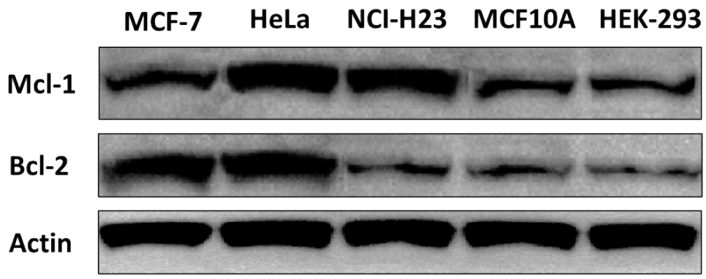


Fig. S8 Expression levels of Bcl-2 and Mcl-1 in MCF-7, HeLa, NCI-H23, MCF10A, and HEK-293, determined by western blotting. All figures represent the results from n = 3 independent samples.

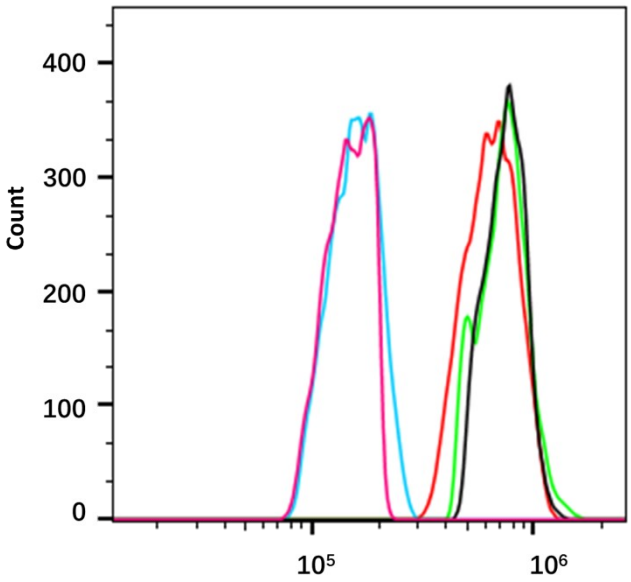


Fig. S9 Fluorescence intensity of 5 μM probe **S1-F** in cancer cells (MCF-7, HeLa, and NCI-H23 cells) and normal cells (MCF10A and HEK-293 cells) measured by FCM (Red: MCF-7; Black: NCI-H23; Green: HeLa; Blue: MCF10A; Pink: HEK-293).

Reference

[1] M. Jiang, H. Zhang, Y. Song, F. Yin, Z. Hu, X. Li, Y. Wang, Z. Wang, Y. Li, Z. Wang, Y. Zhang, S. Wang, S. Lu, G. Xu, T. Song, Z. Wang, Z. Zhang, Discovery of Biphenyl Derivatives to Target Hsp70-Bim Protein-Protein Interaction in Chronic Myeloid Leukemia by Scaffold Hopping Strategy. *J. Med. Chem.*, **2024**, 67, 12068-12084.

[2] Z. Zhang, X. Li, T. Song, Y. Zhao, Y. Feng, An anthraquinone scaffold for putative, two-face Bim BH3 alpha-helix mimic. *J. Med. Chem.*, **2012**, 55, 10735-10741.