SUPPORTING INFORMATION

Functional chromatography reveals three natural products that target the same protein with distinct mechanisms of action

MinJin Kang,[†[a]] Tongde Wu,[†[a]] E. M. Kithsiri Wijeratne,[[b]] Eric C. Lau,[[a]] Damian J. Mason,[[a]] Celestina Mesa,[[a]] Joseph Tillotson,[[a]] Donna D. Zhang,[[a]] A. A. Leslie Gunatilaka,[[b]] James J. La Clair,[[c]] and Eli Chapman*[[a]]

[a] College of Pharmacy, Department of Pharmacology and Toxicology, University of Arizona Tuscon, AZ 85721-0207, United States

[b] Southwest Center for Natural Products Research and Commercialization, School of Natural Resources and the Environment, College of Agriculture and Life Sciences University of Arizona Tuscon, AZ 85706-6800, United States

[c] Xenobe Research Institute, P. O. Box 3052, San Diego, CA 92163-1052, United States

Corresponding Author: chapman@pharmacy.arizona.edu

Author Contributions: †These authors contributed equally to this study.

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A. General Methods. Unless otherwise noted, all reagents and chemicals were purchased from Alfa Aesar, Fisher Biosciences, Strem Chemicals, Sigma-Aldrich or TCI and used without further purification. NMR spectra were recorded on a 500 MHz DRX-500 (Bruker) and 400 MHz Avance III (Bruker). FID files were processed using MestRenova version 8.1 (MestreLab Research). NMR spectra were referenced relative to residual solvent peaks according to S. Budavari, M.J. O'Neil, A. Smith, P.E. Heckelman, The Merck Index, an Encyclopedia of Chemicals, Drugs, and Biologicals, Eleventh Edition, Merck Co., Inc. Rahway, NJ, 1989. Mass spectral data was collected on a 9.4 T Apex-Qh FTICR (Bruker), operated at the University of Arizona Mass Spectrometry Facility. LC/MS was performed using a Hewlett Packard Series 1100 with a C18 Waters Symmetry Column (3.5 µm, 4.6 × 75 mm) under the following conditions:

Solvents: A = 2.5% aq. CH_3CN containing 0.1% formic acid
B = 99.9% CH_3CN containing 0.1% formic acid
Flow rate: 0.3 mL/ min
Injection Volume: 80-100 µL
Method:
0-5 min = 5% B
5-30 min = 5% B to 95% B
30-45 min = 95% B
45-46 min = 95% B to 5% B
46-61 min = 5% B

B. Recombinant protein expression and purification.

B.1. p97. *E. coli* BL21(DE3) cells containing pET14b-p97 were grown in Luria Broth (LB) medium containing 100 µg/mL ampicillin at 37°C to an OD_{600} of 0.8, followed by induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 37°C. Cells were collected by centrifugation (3000 × g for 10 min), resuspended in 40 mL lysis buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM β-mercaptoethanol (BME), one complete EDTA-free protease inhibitor cocktail per 50 mL of media (Roche)), and lysed by single passage through an M110-T microfluidizer (Microfluidics). The lysate was clarified by centrifugation (118,834 × g, 1 h, 4°C) and the resulting supernatant was incubated for 1 h at 4°C with Talon Metal Affinity Resin (Clontech) in 50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol. The resin and supernatant were then loaded into a 25 mL disposable column (BioRad), washed with 10 column volumes of wash buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, 5 mM imidazole), followed by 10 column volumes of stringent wash buffer (50 mM HEPES, pH 7.4, 1 M KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, 20 mM imidazole), and eluted with elution buffer (50 mM HEPES, pH 7.4, 1 M KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, 250 mM imidazole). Fractions were analyzed by 12% SDS PAGE and those containing p97 were pooled and dialyzed into storage buffer (20 mM HEPES, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, pH 7.4), concentrated with a 30 kDa Ultra-15 centrifugal filter (Amicon) to yield a solution of pure p97 protein at 12 mg/mL, which was aliquoted, frozen in liquid nitrogen, and stored at -80°C until needed.

B.2. GroEL. *E. coli* GroEL was cloned into a pTrc99a vector and expressed in *E. coli* DH5α cells. Transformed bacteria were grown in LB medium containing 100 µg/mL ampicillin at 37°C until the OD_{600} was 0.8. The culture was then induced with 0.5 mM IPTG for 3 h at 37°C. The cells were recovered by centrifugation (3000 × g for 10 min).

Cell pellets from 4 L of culture were resuspended in 50 mL of buffer A (50 mM Tris, pH 7.4, 1 mM DTT). The cells were lysed by passage through an M110-T microfluidizer (Microfluidics) and the lysate was clarified by centrifugation (118,834 × g, 1 h, 4°C) in a Ti45 rotor (Beckman). The cleared lysate was applied to a 75 mL FFQ column (Pharmacia) equilibrated in buffer A plus 20% buffer B (50 mM Tris, pH 7.4, 1 M NaCl, 1 mM DTT) and eluted with a gradient to 50% B over 10 column volumes. The fractions were analyzed by 12% SDS PAGE and GroEL containing fractions were pooled and $(NH4)_2SO_4$ was added slowly to 1.2 M. This sample was then applied to a 25 mL Source ISO150 hydrophobic interaction column (Pharmacia) equilibrated in buffer C (50 mM Tris pH 7.4, 1 mM DTT, 1.2 M $(NH_4)_2SO_4$). GroEL containing fractions were pooled and dialyzed into storage buffer (50 mM Tris pH 7.4, 50 mM KCl, 1 mM DTT) and concentrated with a 30 kDa Ultra-15 centrifugal filter (Amicon) to 40 mg/mL.

B.3. ClpX. ClpX was cloned into a pET14b vector and the protein was expressed in *E. coli* BL21(DE3) cells. Transformed bacteria were grown in LB plus 100 µg/mL of ampicillin at 37°C until the OD_{600} was 0.6 and cooled down to 21°C for 1 h. Protein production was induced with 0.5 mM IPTG for 6 h at 21°C. The cells were recovered by centrifugation (3000 × g for 10 min) and frozen at -80°C. Cell pellets were resuspended in 20 mL of lysis buffer (25 mM HEPES-KOH, pH 7.4, 100 mM KCl, 400 mM NaCl, 10% glycerol, 2 mM BME, and complete EDTA-free protease inhibitor cocktail tablets (Roche)). The cells were lysed by passage through an M110-T microfluidizer (Microfluidics). The lysate was clarified by centrifugation (118,834 × g, 1 h, 4°C) in a Beckman Ti45 rotor and the resulting supernatant was loaded onto 3 mL of Talon resin, pre-equilibrated with 25 mM HEPES-KOH, pH 7.4, 100 mM KCl, 400 mM NaCl, and 10% glycerol. After incubation at 4°C with stirring for 1 h, the column was washed with 60 mL of wash buffer (25 mM HEPES-KOH, pH 7.4, 100 mM KCl, 400 mM NaCl, 10% glycerol, 2 mM BME, 20 mM imidazole), and the ClpX protein was eluted with elution buffer (25 mM HEPES-KOH, pH 7.4, 100 mM KCl, 400 mM NaCl, 10% glycerol, 2 mM BME, 250 mM imidazole). Fractions were analyzed by 12% SDS PAGE and those containing ClpX were pooled, dialyzed into storage buffer (50 mM Tris, pH 7.4, 50 mM KCl, 1 mM DTT), concentrated with a 10 kDa Ultra-15 centrifugal filter (Amicon), flash frozen, and stored at -80°C.

B.4. NSF. A clone containing the gene for Chinese hamster (*Cricetulus griseus*) *N*-ethylmaleimide sensitive factor (NSF) was kindly provided by Dr. Zev Bryant (Stanford University). The NSF protein was expressed in *E. coli* Rosetta 2 (DE3) cells. Transformed bacteria were grown in LB medium plus 50 µg/mL kanamycin and 25 µg/mL chloramphenicol at 37°C until the OD_{600} was 0.8. Protein expression was then induced with 0.5 mM IPTG for 4 h. The cells were recovered by centrifugation (3000 × g for 10 min) and stored at -80°C. Cell pellets were resuspended in 40 mL of lysis buffer (50 mM HEPES-KOH, pH 7.4, 400 mM KCl, 10% glycerol, 2 mM BME, 1 mM PMSF and complete EDTA-free protease inhibitor cocktail tablets (Roche)). The cells were lysed by passage through an M110-T microfluidizer (Microfluidics). The lysate was clarified by centrifugation (118,834 × g, 1 h, 4°C) in a Ti45 rotor (Beckman) and the resulting supernatant was loaded onto 2 mL of Talon resin pre-equilibrated with 50 mM HEPES, pH 7.4, 400 mM KCl, and 10% glycerol. After incubation at 4°C while stirring for 1 h, the column was washed with 20 mL of wash buffer (lysis buffer with 20 mM imidazole). The NSF protein was eluted in elution buffer (lysis buffer with 250 mM imidazole). Fractions containing NSF were pooled and dialyzed into dialysis buffer (20 mM HEPES, pH 7.4, 150 mM KCl, 1 mM $MgCl_2$, 5% glycerol, 1 mM DTT) and concentrated with a 30 kDa Ultra-15 centrifugal filter (Amicon) to reduce the volume to

2 mL. The protein was then run through a HiLoad 26/600 Superdex 200 preparative grade column (GE Healthcare) with 20 mM HEPES, pH 7.4, 150 mM KCl, 1 mM $MgCl_2$, 5% glycerol, and 1 mM DTT. Fractions containing NSF were pooled, snap frozen, and stored at -80°C until needed.

C. Preparation of extracts. The following procedures were used to prepare the extracts reported in this study.

C.1. Culturing and extraction of *Chaetomium globosum*. *C. globosum* was cultured in forty 500 mL tissue culture flasks (T-flasks) each containing potato dextrose agar (PDA) (135 mL) coated on five sides of the T-flask (total surface area ca. 460 cm^2) for 28 d at 27°C. MeOH (200 mL) was added to all T-flasks, the flasks were briefly sonicated, and the resulting mixture was filtered through Whatman No. 1 filter paper and a layer of Celite 545. The filtrate was concentrated to one-fourth of its original volume and extracted with EtOAc (5 × 500 mL). Evaporation under reduced pressure afforded the EtOAc extract (2.30 g).

C.2. Culturing and extraction of *Corynespora* sp. BA-10763. A seed culture of the fungus was grown on PDA for two weeks and was used for inoculation. Mycelia were scraped out and mixed with sterile potato dextrose broth (PDB) (150 mL) and filtered through a 100 µm filter to separate spores from the mycelia. The OD_{600} of the spore solution was measured and adjusted to between 0.3 - 0.4 with PDB. This spore solution (10 mL) was used to inoculate ten 500 mL Erlenmeyer flasks, each containing 200 mL of PDB. After incubation at 160 rpm and 28°C for 4 d, the glucose level in the medium was monitored using glucose strips (Uriscan), and on day 12, the strip gave a green color for the glucose test, indicating a very low level of glucose in the medium. Mycelia were separated from the supernatant by filtering through Whatman No. 1 filter paper. The supernatant (pH 4.6) was neutralized to pH 7 with 0.5 M NaOH and extracted with EtOAc (10 × 500 mL), and the combined EtOAc extracts were evaporated under reduced pressure to afford a dark brown semisolid (1.1 g).

C.3. Culturing and extraction of *Phoma* sp. NRRL 46751. A seed culture of the fungus grown on PDA for two weeks was used for inoculation. Mycelia were scraped out and vortexed with sterile PDB (90 mL) and filtered through a 100 µm filter to separate spores from the mycelia. The OD_{600} of the spore solution was measured and adjusted to 0.6 by adding PDB. This spore solution was used to inoculate nine 2 L Erlenmeyer flasks, each containing 1 L of the sterile medium (24 g of PDB and 6 g of sucrose in distilled H_2O) and incubated at 28°C with shaking at 160 rpm. The glucose level in the medium was monitored using glucose strips (Uriscan), and on day 21 the glucose test indicated complete depletion of glucose in the medium. Mycelia were separated from the supernatant by filtration through Whatman No.1 filter paper, freeze dried, and extracted with 1:1 $CHCl_3$:MeOH (4 × 700 mL). The combined extracts were evaporated under reduced pressure, and the resulting residue was suspended in H_2O (200 mL) and extracted with EtOAc (4 × 200 mL). The combined EtOAc extracts were washed with H_2O (3 × 200 mL), dried over anhydrous Na_2SO_4, and evaporated under reduced pressure to give the EtOAc extract (4.4 g) as a dark brown oil.

D. Isolation of rheoemodin (1) by functional chromatography. A solution of *Chaetomium globosum* extract (1 mg/mL) in PBK (1 mL) was added to 150 µL of resin bearing 5 mg/mL of p97 in a 2 mL Eppendorf tube and shaken on an inversion rotator at 4°C. After 12 h, the supernatant was discarded and the resin washed three times with 500 µL of PBK (50 mM KPhos, 150 mM KCl, pH 7.4). After the third wash, bound

molecules were eluted with 95% EtOH. The EtOH fraction was transferred to a glass vial, dried by airflow, and analyzed via HPLC followed by high-resolution mass spectroscopy (HRMS). Control experiments were run using resin bearing 5 mg/mL of FtsZ under identical procedures as that used for p97. A sample of the peak highlighted in Fig. 2 was purified by reverse phase HPLC (rp-HPLC) and characterized spectroscopically.

Rheoemodin (1): orange solid; m.p. 291°C (dec); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.30 (brs, 2H), 7.06 (d, *J* = 2.4 Hz, 2H), 6.50 (d, *J* = 2.4 Hz, 2H); APCIMS (+) ve mode *m/z* 273 $[M+H]^+$.

E. Isolation of 1-hydroxydehydroherbarin (2) by functional chromatography. A solution of *Corynespora sp.* extract (1 mg/mL) in PBK (1 mL) was added to 150 µL of resin bearing 5 mg/mL of p97 in a 2 mL Eppendorf tube and shaken on an inversion rotator at 4°C. After 12 h, the supernatant was discarded and the resin was washed three times with 500 µL of PBK. The bound compounds were eluted with 95% EtOH. The EtOH fraction was transferred to a glass vial, dried by airflow, and analyzed via HPLC followed by HRMS. Control experiments were run using resin bearing 5 mg/mL of FtsZ under identical procedures as that used for p97. A sample of the peak highlighted in Fig. 3 was purified by rp-HPLC and characterized spectroscopically.

Table S1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectroscopic data of **2** in $CDCl_3$.

Position	δ_C	δ_H	*J* in Hz
1	93.1	4.73	ddd (18.8, 3.4, 1.9)
		4.66	dt (18.8, 3.7)
3	160.2		
4	114.6	2.80	ddd (17.6, 3.7, 1.9)
		2.52	dt (17.6, 3.4)
4a	135.7		
5	181.0		
5a	133.8		
6	104.6	7.22	d (2.4)
7	164.3		
8	103.5	6.68	d (2.4)
9	161.7		
9a	123.6		
10	183.3		
10a	135.7		
OMe	56.4	3.93	s
OMe	55.9	3.92	s
Me-3	28.4	1.59	s

1-Hydroxydehydroherbarin (2): orange, amorphous solid; $[\alpha]_D^{25}$ -12.1 (*c* 0.06, $CHCl_3$); UV (EtOH) λ_{max} (log ε) 447 (4.21), 421 (4.13), 337 (4.29), 278 (4.82) nm; IR ν_{max} 3450, 2920, 1680, 1585, 1323, 1161, 1064, 837 cm^{-1}; for ^{1}H NMR and ^{13}C NMR data, see Table S1; HRFABMS *m/z* 303.0867 $[M+H]^+$ (calcd. for $C_{16}H_{15}O_6$, 303.0869).

F. Isolation of phomapyrrolidone A (3) by functional chromatography. A solution of *Phoma* sp. NRRL 4675 extract (1 mg/mL) in PBK (1 mL) was added to 150 µL of resin in a 2 mL Eppendorf tube and shaken on an inversion rotator at 4°C. After 12 h, the supernatant was removed and the resin was washed three times with 500 µL of PBK. After the third wash, bound compounds were eluted with 95% EtOH. The EtOH fraction was transferred to glass vial, dried by airflow, and analyzed via HPLC followed

by HRMS. Control experiments were run using resin bearing 5 mg/mL of FtsZ under identical procedures as that used for p97. A sample of the peak highlighted in Fig. 3 was purified by rp-HPLC and characterized spectroscopically.

Table S2. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectroscopic data of **3** in $CDCl_3$

Position	δ_C	δ_H	*J* in Hz
1	43.2	2.93	q (4.8)
2	142.5		
3	130.8	4.40	brs
4	54.0		
5	144.4		
6	128.8		
7	49.3	2.02	t (11.9)
8	41.2	1.67	m
9	40.4	2.12	brd (16.0)
		0.68	ddd (16.0, 11.6, 4.4)
10	32.1	1.56	m
11	44.6	0.67	ddd (16.0, 11.6, 4.4)
		1.86	m
12	31.1	1.92	m
13	58.2	1.05	m
14	91.9	4.30	dd (7.4, 2.4)
15	59.3	1.67	d (11.6)
16	60.8	2.97	d (11.6)
17	201.6		
18	60.2	2.95	d (5.6)
19	171.6		
20	18.6	0.97	d (6.8)
21	14.7	1.49	s
22	28.4	1.18	s
23	15.3	1.81	s
24	22.5	0.94	d (6.8)
25	19.9	1.14	d (6.8)
1'	43.8	3.83	m
2'	177.3		
3'	34.6	2.43	dd (13.2, 11.2)
		3.67	dd (13.2, 5.6)
4'	133.5		
5'	133.1	6.97	dd (8.4, 2.0)
6'	126.3	6.68	dd (8.4, 2.4)
7'	159.0		
8'	120.9	7.70	dd (8.4, 2.0)
9'	131.5	7.14	dd (8.4, 2.4)
NH		7.66	s

Phomapyrrolidone A (3): white solid; m.p. 218-220°C; $[\alpha]_D^{25}$ +256 (*c* 1.0, MeOH); UV (EtOH) λ_{max} (log ε) 223 (3.27) nm; IR (KBr) ν_{max}3271, 2925, 1778, 1712, 1502, 1342, 1232, 1178 cm^{-1}; for ^{1}H NMR and ^{13}C NMR data, see Table S1; HRFABMS *m/z* 526.2966 $[M-H]^-$ (calcd. for $C_{34}H_{40}NO_4$ 526.2963).

G. Malachite green assay. The following procedures were used for ATPase activity.

G.1. p97. Assay buffer (100 µL) containing 50 nM p97 protein in buffer (50 mM Tris, 150 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.4,) was dispensed into each well of a 96 well plate. Test compounds at the desired concentrations (0.0004 µM, 0.002 µM, 0.004

μM, 0.02 μM, 0.04 μM, 0.2 μM, 2 μM, 10 μM, 20 μM, 40 μM, 60 μM, and 100 μM) dissolved in DMSO (2 μL) were added to each well. DMSO (2 μL) and 50 mM EDTA (final concentration) were used as negative and positive controls, respectively. Following incubation at 21°C for 10 min, the ATPase assay was initiated by adding 100 μM ATP to each well followed by incubation at 21°C. At 30 min, 60 min, 90 min and 120 min, a 20 μL aliquot was taken and added into 50 μL of malachite green solution (9.3 μM malachite green, 53 mM $(NH_4)_2MoO_4$, 1M HCl, 10% Tween 20). After 5 min, 10 μL of 34% sodium citrate was added and the OD_{670} was read on a GEN5 plate reader (BioTek Synergy 2). The IC_{50} values were calculated by fitting the percentage inhibition at a given compound concentration plotted on semi-log scale using KaleidaGraph (Synergy Software). Data from this assay and ATP dose response studies are presented in Fig. 4a.

G.2. GroEL. Assay buffer containing GroEL (50 mM Tris pH 7.4, 150 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 50 nM GroEL) was dispensed into each well of a 96 well plate, and the assay was run as described above and was reported in Fig. 4b.

G.3. ClpX. Assay buffer containing ClpX (50 mM Tris pH 7.4, 150 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 50 nM ClpX) was dispensed into each well of a 96 well plate, and the assay was run as described above and was reported in Fig. 4b.

G.4. NSF. Assay buffer containing NSF (50 mM Tris pH 7.4, 150 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 200 nM NSF) was dispensed into each well of a 96 well plate, and the assay was run as described above and was reported in Fig. 4b.

H. TCRα-GFP microscopy. HEK293 cells stably expressing Ub^{G76V}GFP or TCRα-GFP were kindly provided by Dr. Ron Kopito (Stanford University). For live cell imaging, cells were seeded (2×10^6 cells) on 35-mm glass-bottom dishes (BioExpress), grown 18 h, and treated with 5 μM of **1**, **2** or **3** in DMSO (0.1% final DMSO concentration). DMSO (0.1%) was used as a negative control. The cells were imaged in phenol-red free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Images were captured with an Observer Z1 microscope (Zeiss) by using the Slidebook 5.0 software (Intelligent Imaging Innovations, Inc.). Data from this study is provided in Fig. 5a.

I. Ub^{G76V}GFP and TCRα-GFP degradation assay. HEK293 cells stably expressing Ub^{G76V}GFP and TCRα-GFP were seeded on 35 × 10 mm dishes (2×10^6 cells) and grown for 18 h before being treated with DMEM containing 4 μM MG132 for 1h. The cells were then washed three times with 37°C phosphate buffered saline (PBS) (1 mL). DMEM containing 10% FBS, 106.6 μM cycloheximide and 2% DMSO or test compound in DMSO (10 μM, 20 μM, and 50 μM were each screened) was added to each plate and incubated for 1 or 2 h. Cells were trypsinized for 2 min, neutralized with DMEM media (1 mL), washed with PBS (2 × 1 mL), and resuspended in PBS (1 mL). The fluorescence intensity was determined by flow cytometry on a FACScan flow cytometer (BD Biosciences) equipped with an air-cooled 15 mW argon ion laser tuned to 488 nm. The emission fluorescence of GFP was detected and recorded through a 530/30 bandpass filter. List mode data files consisting of 10,000 events gated on FSC (forward scatter) versus SSC (side scatter) were acquired and analyzed using CellQuest PRO software (BD Biosciences) at a rate of 200-400 events per second. Data from these studies are provided in Fig. 5b and Fig. 5c.

J. Wesern blot analysis. Rabbit anti-GFP (GeneTex), anti-caspse 3 (Cell Signaling), anti-eIF2α [pS52] (Invitrogen); mouse anti-GAPDH (glyceraldehyde-3-phosphate

dehydrogenase; Santa Cruz), anti-ubiquitin (Sigma), anti-XBP1s (Biolegend), anti-HA epitope (Covance), and mouse anti-LC3 I/II (Sigma), were purchased from the indicated commercial sources. To detect protein expression in total cell lysates, cells were harvested in sample buffer (50 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate (SDS), 10% glycerol, 100 mM dithiothreitol (DTT), 0.1% bromophenol blue), electrophoresed through 4-20% gradient SDS-polyacrylamide gels, and subjected to immunoblot analysis with indicated antibodies (Fig. 5d).

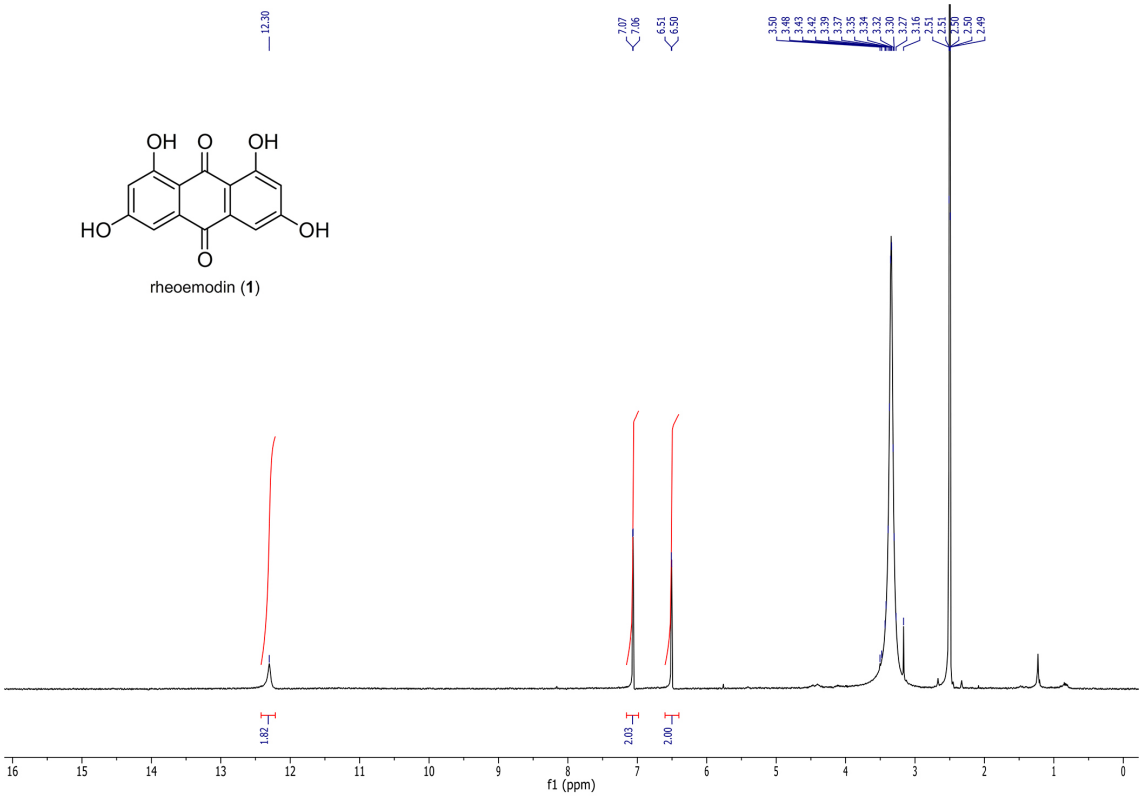

Figure S1. ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of rheoemodin (**1**)

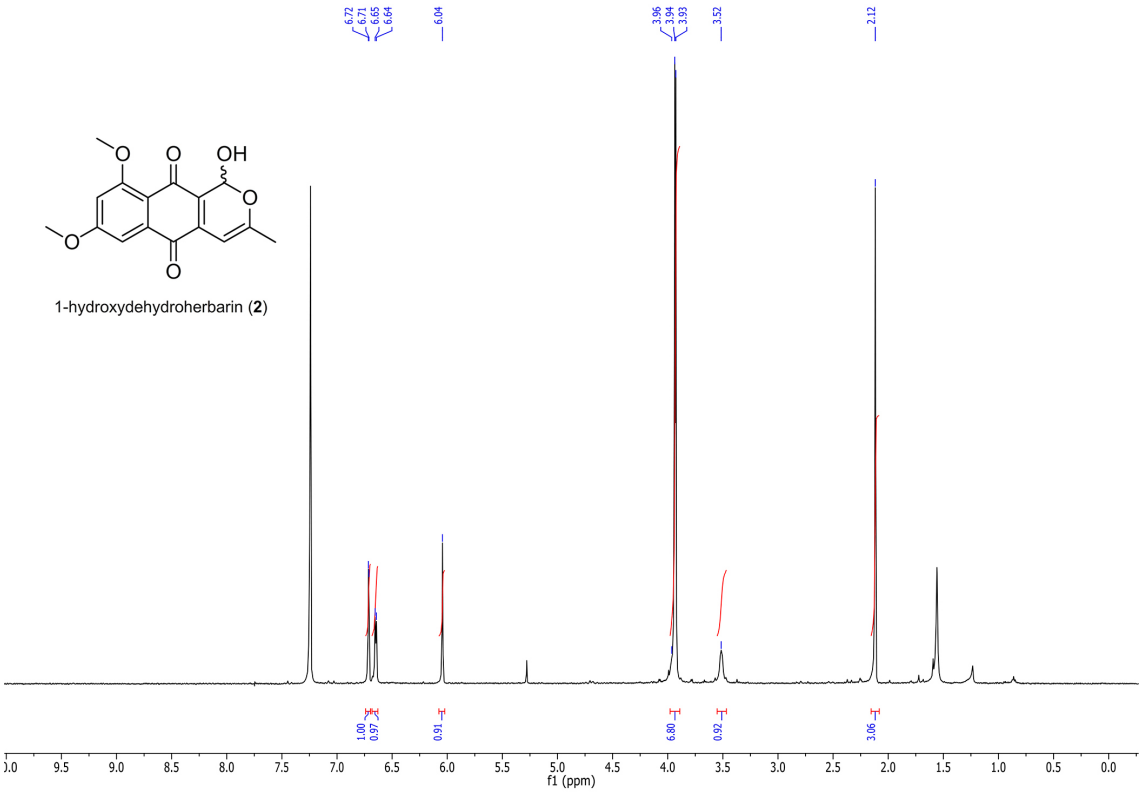

Figure S2. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of 1-hydroxyherbarin (**2**)

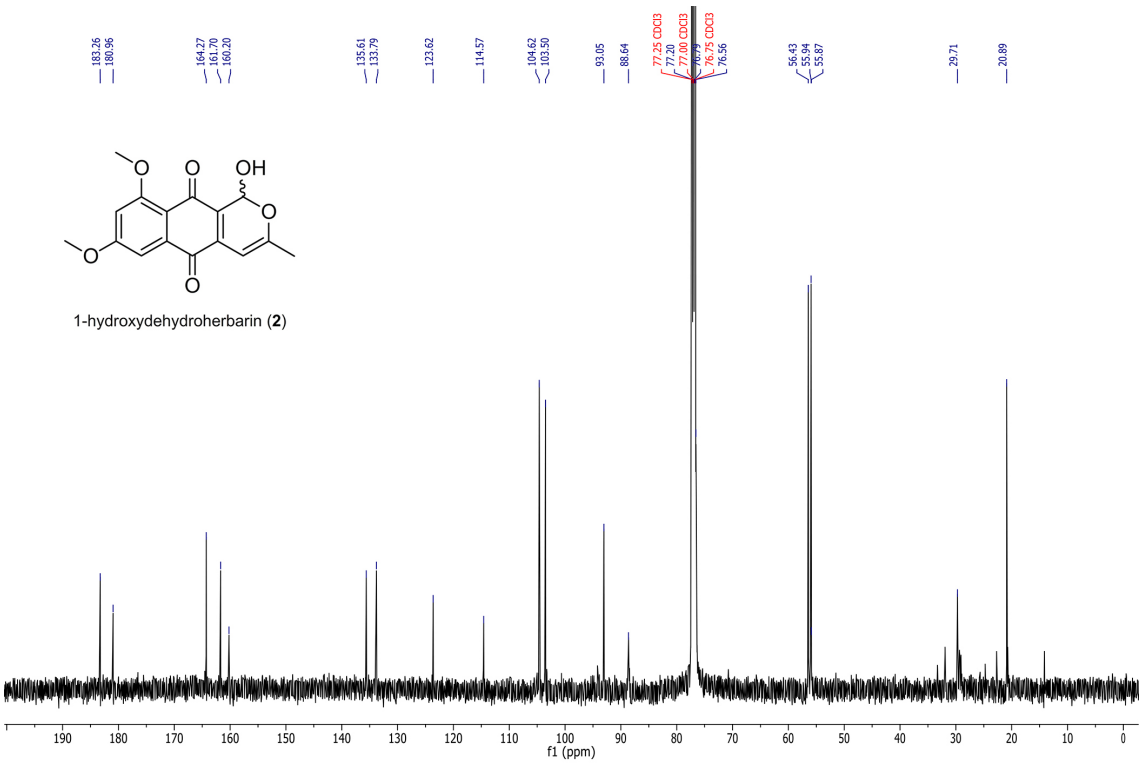

Figure S3. ^{13}C NMR spectrum (125 MHz, $CDCl_3$) of 1-hydroxyherbarin (**2**)

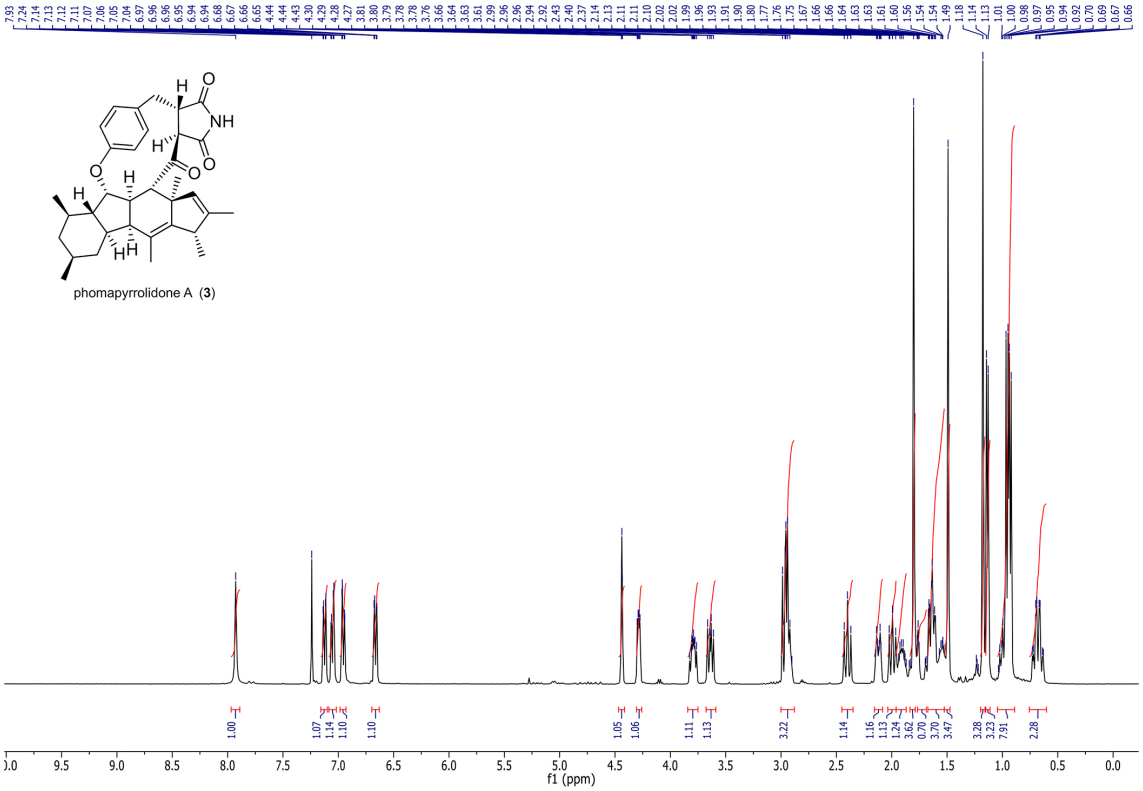

Figure S4. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of phomapyrrolidone A (**3**)

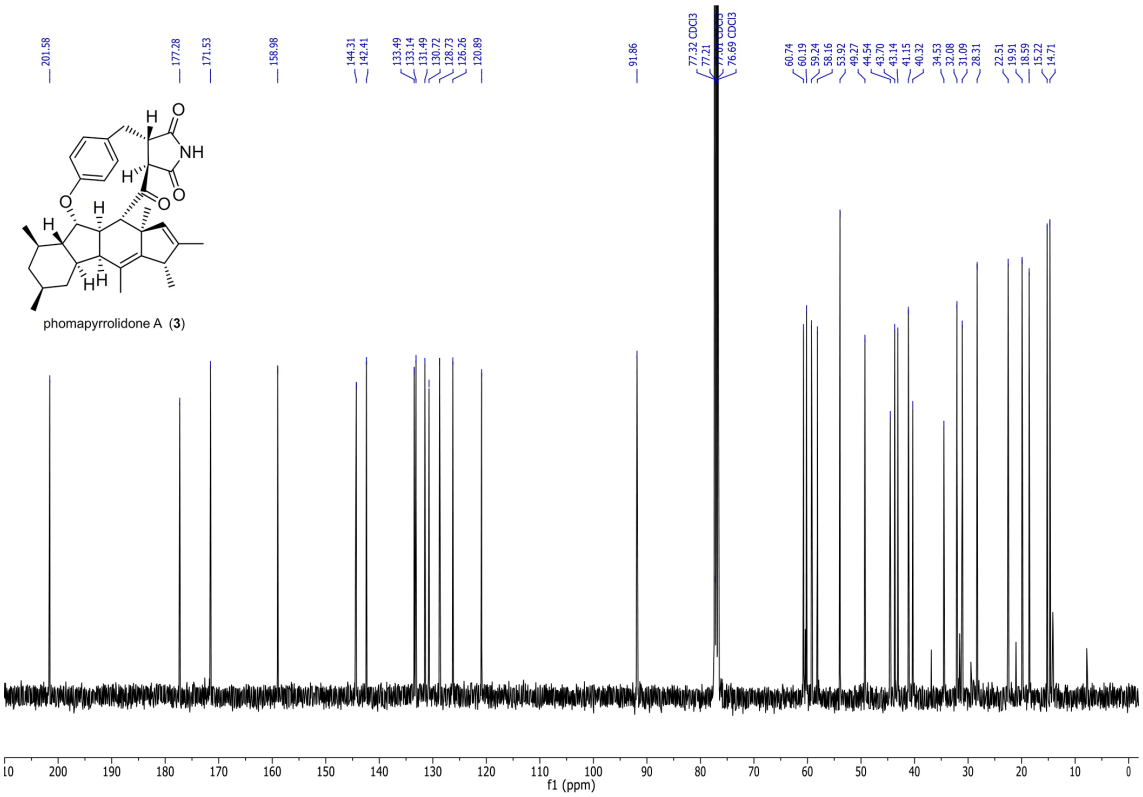

Figure S5. ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of phomapyrrolidone A (**3**)

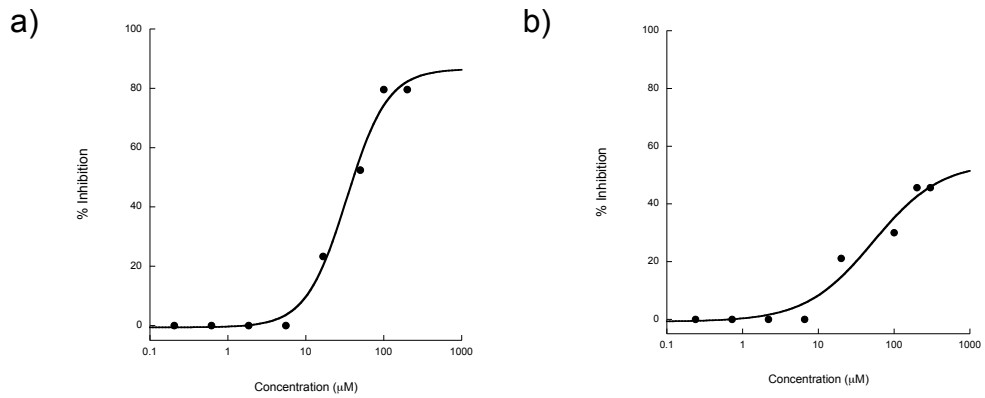

Figure S6. Compound **1** IC_{50} curves. a) 100 μM ATP. b) 500 μM ATP

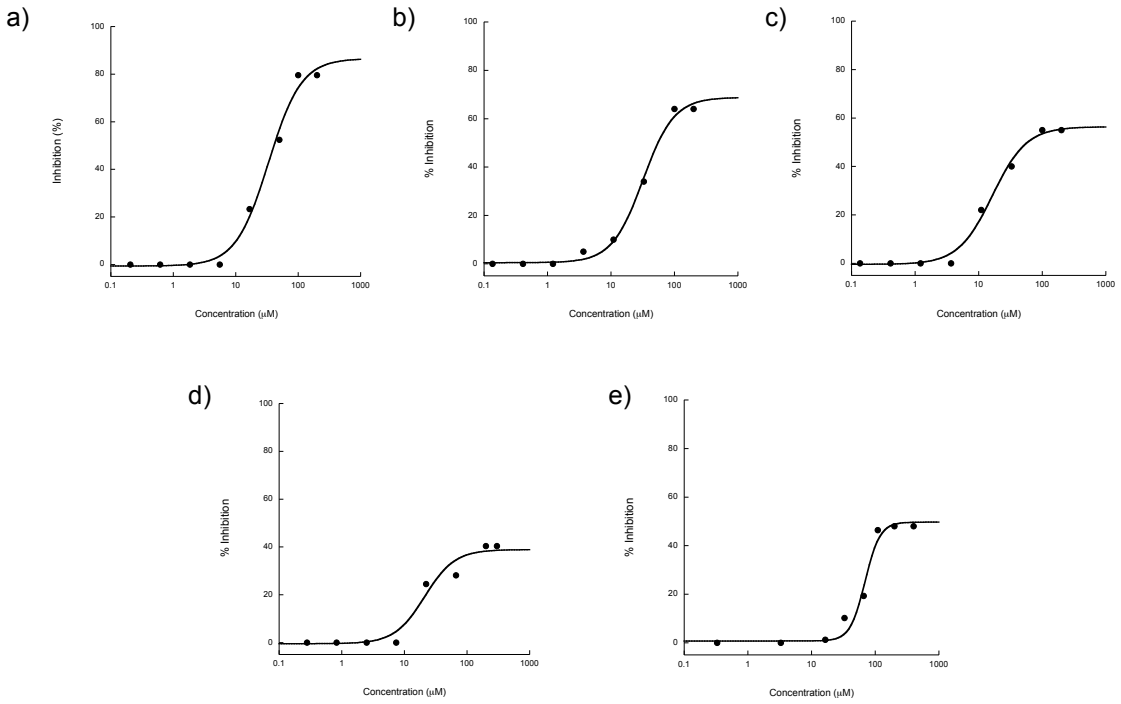

Figure S7. Dose-response curves for compound **2**. a) 100 μM ATP. b) 500 μM ATP. c) 1 mM ATP. d) p97-Cys0. e) ClpX.

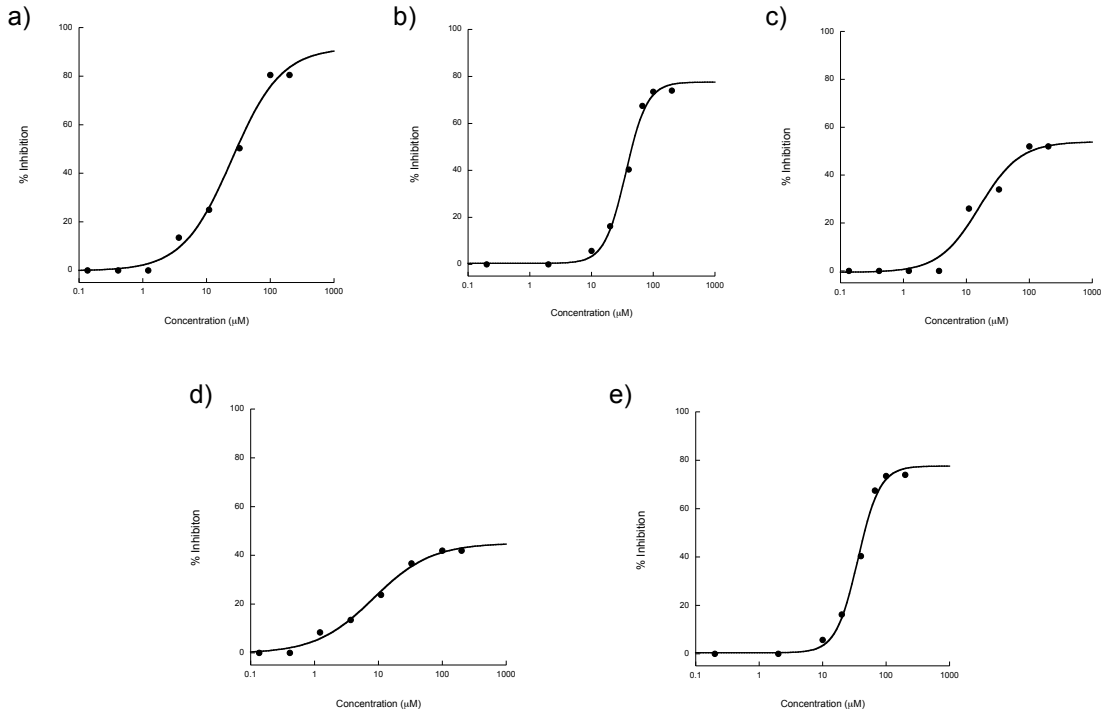

Figure S8. Dose response for compound **3**. a) 100 μM ATP. b) 500 μM ATP. c) 1 mM ATP. d) p97-Cys0. e) ClpX.