Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

SUPPORTING INFORMATION

Selective inhibition of p97 by chlorinated analogues of dehydrocurvularin

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1. Recombinant protein expression and purification

1.1. p97. *E. coli* BL21 (DE3) cells containing plasmid 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 buffer (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, 1M KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, 20mM imidazole), and eluted with elution buffer (50 mM HEPES, pH 7.4, 1M KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, 250mM 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 an 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.

1.2. p97-Cys0. Site directed mutagenesis was performed using wild type pET14b-p97 as a template. Ten out of 12 cysteine residues in p97 (C69, C77, C104, C174, C184,

C415, C535, C572, C691, and C695) were mutated to valine and the remaining 2 cysteine residues (C209 and C522) were mutated to alanine using a modified quick change procedure that employs offset oligonucleotide harboring the desired mutation [1]. All mutations were confirmed by DNA sequencing. Expression and purification of the p97-Cys0 construct was carried out as for wild-type p97.

1.3.p97-C522A. Site directed mutagenesis was performed using wild type pET14b-p97 as a template. A cysteine 522 residue was mutated to alanine and mutagenesis was carried out as for p97-Cys0 construct. A mutation was confirmed by DNA sequencing. Expression and purification of p97-C522A was performed as for wild-type p97 then further purified through a Superose 6 10/300 GL preparative grade column (GE Healthcare) equilibrated with 20 mM HEPES pH 7.4, 150 mM KCl, 1 mM $MgCl_2$, 5% glycerol, and 1 mM DTT. Fractions containing p97-C522A were pooled, snap frozen, and stored at -80°C until needed.

2. Malachite green assay. 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 **1**-**4** at the desired concentrations (0.137 µM, 0.411 µM, 1.23 µM, 3.70 µM, 11.1 µM, 33.3 µM, 66.7 µM, 100 µM, and 200 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 $(NH_4)_2MoO_4$, 1 M HCl, 10% tween 20). After 5 min, 10 µl of 34% sodium citrate was added and the OD_{670} was read on a GEN5 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).

3. Covalent interaction of dehydrocurvularins and p97. The dehydrocurvularins (1 mM) and 1 μM p97 were incubated at 4°C for 12 h, 21°C for 6 h, or 37°C for 2 h in assay buffer (50 mM Tris, 150 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.4). 1% DMSO was run as control. Then the reaction was dialyzed into assay buffer for 2 h using Slide-A-Lyzer® Dialysis Cassette (Thermo Scientific) and the buffer was exchanged for fresh buffer 3 times every 2 h. The ATPase assay was then performed. To initiate the reaction 2 mM ATP was added. At 10 min, 20 min, 30 min, 40 min, 50 min and 60 min, a 10 μL aliquot was taken and added to 800 μL of malachite green solution (9.3 μM malachite green, 53 mM $(NH_4)_2MoO_4$, 1 M HCl, 10% tween 20). After 5 min, 100 μl of 34% sodium citrate was added and the OD_{660} was read on GENESIS 10S VIS Spectrophotometer (Thermo Scientific), converted to nmol inorganic phosphate from a standard curve, and plotted as a function of time.

4. Cellular studies.

4.1 Cytotoxicity analyses

Cells were seeded at a density of 10,000 cells in 90 μL of DMEM containing 10% FBS in a 96-well plate format. After 12 h incubation at 37°C in a 5% CO_2 environment, the cells were treated with compounds 1-4 in DMEM to the desired final concentrations in a serial dilution gradient (200 μM, 100 μM, 66.6 μM, 33.3 μM, 11.1 μM, 3.70 μM, 1.23 μM, 0.411 μM, and 0.137 μM) for 48 h. Negative controls were conducted using cells without any additional materials. Analysis was conducted by preparing a 2 mg/mL Thiazoyl Blue Tetrazolium Bromide-MTT (Amresco) stock solution in 1X PBS. 20 μL of MTT stock solution was added to each well. After 2 h of incubation, medium was removed and 100 μL of solubilization solution (0.4M HCl in isopropanol) was added to the plate to dissolve crystals. After 10 min of incubation with gentle shaking, the plate was analyzed using a

GEN5 plate reader (BioTek Synergy 2) at 570 nm. Negative 'no-cell' control absorbance wells were prepared with culture media ± compound only to account for background interference. The data were corrected with the absorbance of compounds in the negative 'no-cell' control and normalized to the 'not-treated' control to obtain the relative absorbance intensity. Absorbance values were averaged from technical triplicate samples to generate individual biological replicates. LD_{50} values were determined from the plot of relative absorbance intensity versus compound concentration with Prism software (GraphPad) using non-linear regression modelling.

4.2 Western blot analysis

Cells were cultured with the ascribed compound in DMEM supplemented with 10% FBS for 24 h in 1.9 cm^2 culture dishes (Greiner Bio-One). The media was removed and the cells were harvested in sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, 0.1% bromophenol blue) and lysed via sonication followed by clearance of the cell debris by centrifugation. These were then applied to 4-20% gradient SDS PAGE gels (Invitrogen) and transferred to Nitrobind Nitrocellulose transfer membrane (Maine Manufacturing) using BoltTM Miniblot Module (Life Technologies) gel box. The blots were blocked in 5% milk for 1 h. Primary antibodies were applied in 5% milk at 1:1000 rabbit anti-GFP (GeneTex), 1:1000 mouse anti-β-Actin (Santa Cruz), 1:1000 mouse anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Santa Cruz), 1:1000 anti-ubiquitin (Sigma), or 1:1000 anti-HA epitope (Covance). The blots were washed three times at 10 min intervals with wash buffer (1X PBS, 0.1% Tween 20). Secondary anitbodies were applied in 5% milk at 1:3000 goat anti-mouse (Santa Cruz) or 1:3000 goat anti-rabbit (Santa Cruz). The blots were washed four times at 10 min intervals with wash buffer, incubated in Supersignal West Pico, Dura, or Femto Substrate (Thermo Scientific), and imaged using ChemiDocTM XRS (Bio-Rad). The blots were analyzed using Quantity One 1-D Analysis software (Bio-Rad).

5. Molecular docking study

Create ligand and receptor docking files. Compounds **1** and **4** were created with ChemOffice 2014 Chem3D software. Their energy-minimized conformations were output with pdb format. Torsion parameters for the two compounds were set in AutoDockTools to give pdbqt format. The coordinates for p97 (PDBID 3CF3) were downloaded from the Protein Data Bank. Then flexible and rigid p97 files were created in AutoDockTools by setting Cys522, Lys524 and Asp577 as flexible residues. The resulting flexible and rigid p97 files were output as pdbqt format.

Create a grid file. The ligand, flexible, and rigid 3CF3 pdbqt files were opened with AutoDockTools and then the autogrid box was set. The xyz-dimensions of the autogrid box were set as 50, 35, 35, respectively and the center grid box were set as 94.082, 124.104, 305.073, respectively. Spacing was set as 0.375 Å.

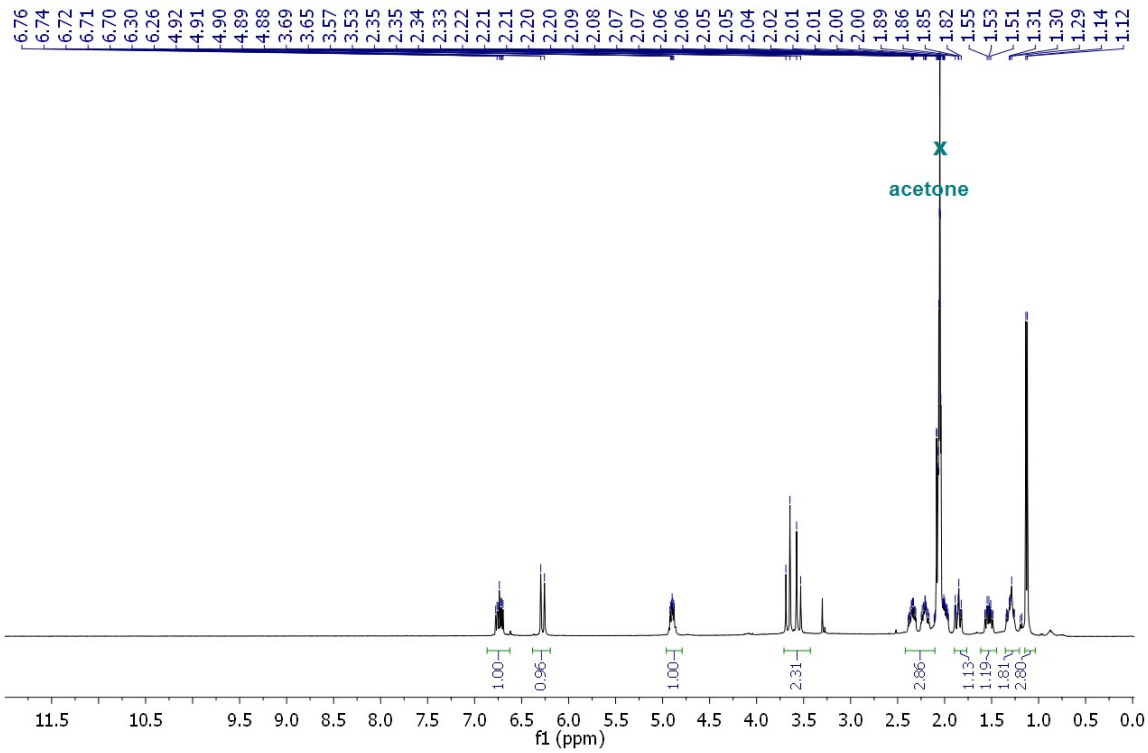
Autodock. The ligand, flexible, and rigid 3CF3 pdbqt files were opened in AutoDockTools, then these molecules were chosen as rigid macromolecules or flexible macromolecules. The Genetic Algorithm was chosen as the search parameter. The maximum number of evaluations was set as short (250,000) and the other parameters were set as default. The docking parameters were set as the default parameters.

Analyse docking results. The interesting conformations were output as pdbqt file and open it to save as pdb file. The binding model were analysed in MacPyMOL(1.3).

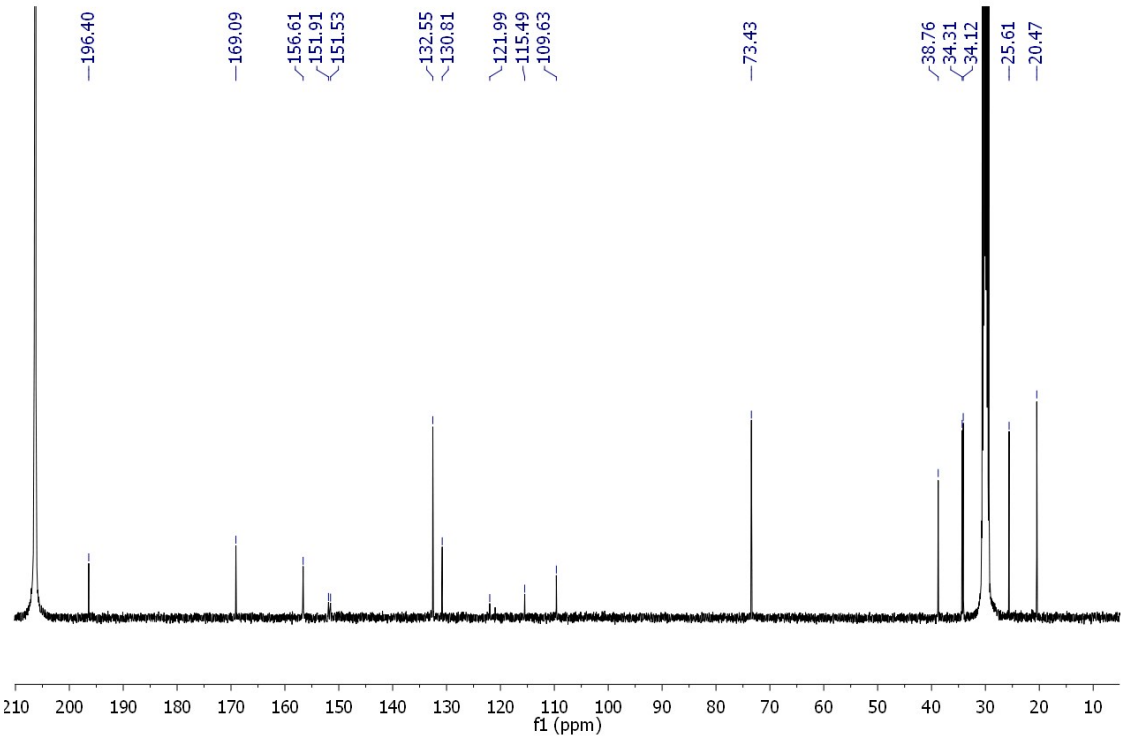
6. 4,6-Dichloro-10,11-dehydrocurvularin (4)

White amorphous solid; UV (CH3OH) λmax (log ε) 363.5 (3.89); 208.0 (4.68) nm; 1H NMR: δ 6.74 1H, (ddd, J = 15.7, 9.9, 5.9), 6.28 (1H, d, J = 15.7, 1H), 4.90 (1H, qdd, 6.3, 6.3, 2.4), 3.67 (1H, d, J = 17.5), 3.55 (1H, d, J = 17.5), 2.34 (1H, m), 2.21 (1H, m), 1.98 (1H, m), 1.85 (1H, m), 1.53 (1H, m), 1.29 (1H, m), and 1.20 (3H, d, J = 6.3); 13C NMR: 196.4 (C), 169.1 (C), 156.6 (HC), 151.9 (C), 151.5 (C), 151.5 (C), 132.5 (CH),

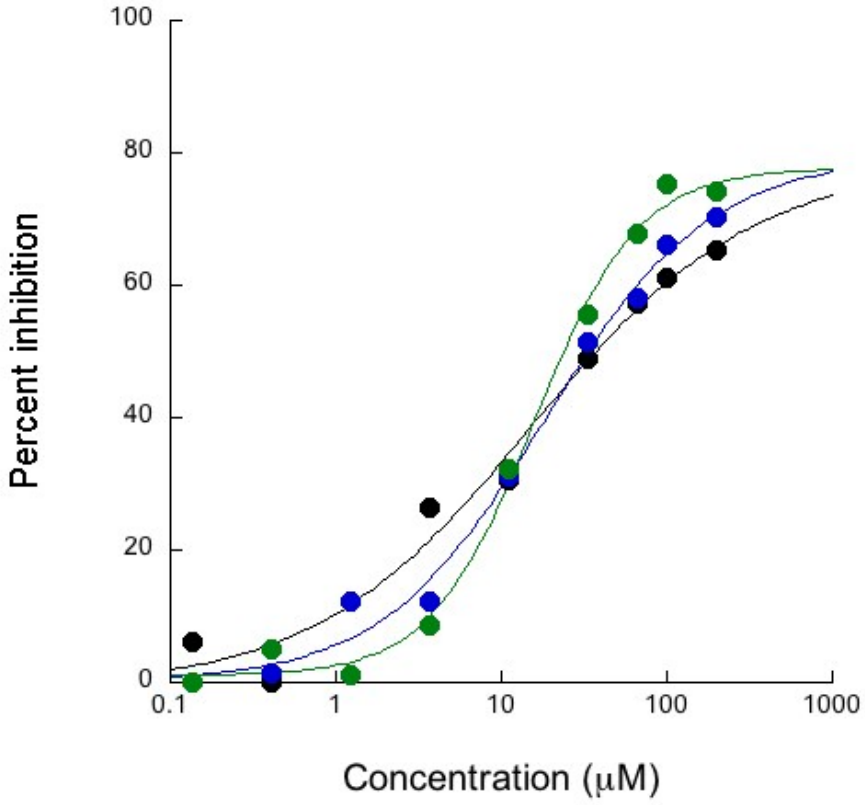
130.8 (C), 122.0 (C), 115.5 (C), 109.6 (H), 73.4 (CH), 38.7 (CH2), 34.3 (CH2), 34.1 (CH2), 25.5 (CH2), 20.5 (CH3); HRESIMS: m/z 357.0297 [M-H]- (calcd for C16H15Cl2O5, 357.0302).



S1. ^{1}H NMR spectrum (400 MHz) of 4,6-dichloro-10,11-dehydrocurvularin (4) in acetone-d_6.

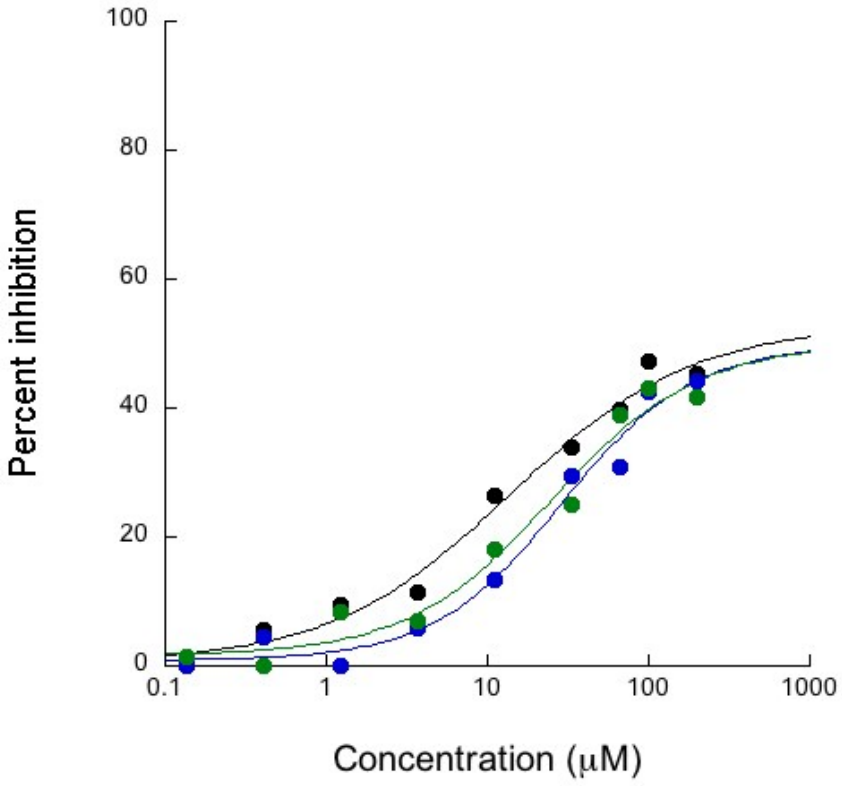


S2. ^{13}C NMR spectrum (100 MHz) of 4,6-dichloro-10,11-dehydrocurvularin (4) in acetone-d_6.



S3. IC_{50} curves for compound 2. Values determined at varying ATP concentrations from 100 μM (black), 500 μM (blue), and 1000 μM (green).

S4. IC_{50} curves for compound 3. Values determined at varying ATP concentrations from 100 μM (black), 500 μM (blue), and 1000 μM (green).



S5. IC_{50} curves for compound 3. Values determined at varying ATP concentrations from 100 μM (black), 500 μM (blue), and 1000 μM (green).

Compound	p97 (100)	p97 (500)	p97 (1000)	Cys0	C209A	C522A
1	>200 μM	>200 μM	>200 μM	>200 μM	>200 μM	>200 μM
2	15.3 ± 9.9 μM (65%)	18.4 ± 5.4 μM (70%)	15.8 ± 1.9 μM (72%)	>200 μM	16.0 ± 2.09 μM	>200 μM
3	24.3 ± 10.8 μM (42%)	44.1 ± 9.3 μM (32%)	40.2 ± 5.1 μM (36%)	>200 μM	19.8 ± 9.2 μM	>200 μM
4	13.9 ± 6.4 μM (46%)	29.2 ± 11.9 μM (43%)	25.0 ± 13.3 μM (43%)	>200 μM	22.9 ± 2.2 μM	>200 μM

Table S1. IC_{50} values for curvularin and analogues. The percent number indicates highest percentage inhibition.

	1	2	3	4
LD_{50}	>100 μM	7.9 ± 1.1 μM	4.47 ± 0.8 μM	8.3 ± 0.9 μM

Table S2. LD50 values for curvularin and analogues. HEK293 cells were incubated in the presence of compounds for 48 h and cell viability was assessed using an MTT colorimetric assay.

Compound	Calculated K_i (μM)	Calculated ΔG (kcal/mol)
1	300.33	-4.81
4	2.61	-7.61

Table S3. Calculated K_i values and ΔG for compound **1** and **4**.

Supplementary References

1. Zheng L, Baumann U, Reymond JL. An efficient one-step site directed and site saturation mutagenesis protocol. *Nucleic Acids Res.* **2004**, 32, e115

2. Klock H.E. and Lesley. S. A. The Polymerase Incomplete Primer Extension (PIPE) method applied to high-throughput cloning and site-directed mutagenesis. *Methods Mol Biol*. **2009**, 498, 91-103