Supporting Information

Chrysophaentins are competitive inhibitors of FtsZ and inhibit Z-ring formation in live bacteria

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General experimental

Protein expression was carried out using *E. coli* BL21-DE3 (Gold) transformed with an IPTG-inducible plasmid encoding *E. coli* FtsZ (EcFtsZ). Bacteria were grown in Luria-Bertani (LB) broth containing 100 μg/mL ampicillin at 37 °C with shaking (220 rpm). FtsZ expression was induced for 5 hr with 1 mM IPTG when the cell culture reached an OD_{600} of 0.4. Cells were harvested by centrifugation, lysed in Lysis buffer (50 mM Tris pH 8.0, 100 mM KCl, 1 mM EDTA, 1% benzamidine, Roche Protease Cocktail inhibitor according to the manufacturer's instructions, 0.1% β-mercaptoethanol, 1 mM $MgSO_4$, adapted from Beuria *et al.* [1]), sonicated briefly, and mechanically broken. Insoluble cell debris was removed by centrifugation. The supernatant containing soluble protein was supplemented with 5 mM $MgCl_2$ and additional protease inhibitors. The protein was precipitated with ammonium sulfate to a final concentration of 30% (w/v), and incubated at 4 °C for 4 hr. Following centrifugation, the precipitated protein pellet was resuspended in 50 mM Tris pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 10% glycerol, and dialyzed overnight against the same buffer. FtsZ was purified using a ResQ anion exchange column (GE Healthcare) eluting with a gradient of high salt buffer (50 mM Tris pH 7.4, 1 M KCl, 5 mM $MgCl_2$, 10% glycerol). Fractions containing FtsZ as determined by SDS PAGE were combined and concentrated using a GE Vivaspin concentrator prior to loading on a Superdex 75 gel filtration column. Protein was eluted from the gel filtration column in 50 mM Tris pH 7.4, 50 mM KCl, 1 mM EDTA, and 10% glycerol. FtsZ-containing fractions were pooled according to concentration and purity. Protein was

quantified by Bradford assay, according to the manufacturer's instructions, and frozen at -80 ºC until use. Recombinant *Staphylococcus aureus* FtsZ (SaFtsZ) was purified in a similar manner, except that protein expression was induced with addition of 1 mM IPTG at 30 °C for five hr. Heterologous expression of SaFtsZ in *E. coli* causes severe filamentation that leads to cell death. We found that growing the cultures at lower temperatures improved the yield of recombinant protein.

Optimization of end-point GTPase assay

The GTPase assay was optimized for EcFtsZ and SaFtsZ such that the end-point production of inorganic phosphate was similar for both proteins when the initial amount of GTP in the reaction mixture was kept constant. When 0.25 mM purified GTP from Innova Biosciences was added to 2 μM EcFtsZ in Assay buffer (50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.0, 100 mM KCl, 5 mM MgCl2), after 10 minutes at 23 °C, between 30 and 40 μmol phosphate was released and detected (P_i ColorLock Gold, Innova Biosciences). This value of P_i produced fell within the linear portion of a constructed standard curve, and corresponded to the hydrolysis of between 1.5 and 2 μmol GTP per minute per μmol EcFtsZ. A comparison of different concentrations (2 μM, 4 μM, and 6 μM) of SaFtsZ protein in the presence of 0.25 mM GTP for up to two hr showed a significantly lower rate of hydrolysis by SaFtsZ at 23 °C. These data indicated that the rate of GTP hydrolysis by SaFtsZ was much lower compared to EcFtsZ. The highest rate of hydrolysis for SaFtsZ was obtained using 6 μM SaFtsZ, and this concentration was used in all subsequent experiments. In addition, the temperature of the assay was increased to 30 °C to attempt to increase the enzymatic activity of SaFtsZ. Under these conditions, the rate of hydrolysis increased to an average of 0.4 μmol GTP per minute per μmol SaFtsZ. The conditions used in all further inhibition assays were 2 μM EcFtsZ with 0.25 mM GTP for 10 minutes at 23 °C and 6 μM SaFtsZ with 0.25 mM GTP for 20 minutes at 30 °C, both in Assay buffer. These conditions consistently gave rise to the production of >30 μmol phosphate.[2]

GTPase assay

GTPase activity of recombinant EcFtsZ and SaFtsZ was evaluated in the presence of **1** and **2**. Briefly, the assay was measured in 96-well plate format using a colorimetric assay that

measures inorganic phosphate production. Solutions containing EcFtsZ (2 μM) and SaFtsZ (6 μM) in Assay buffer (50 mM MES, pH 6.0, 100 mM KCl, and 5 mM $MgCl_2$) were treated with serial dilutions of **1** and **2** (final concentrations 0-600 μM in 5% DMSO), followed by addition of GTP (Roche or Innova Biosciences) added to a final concentration of 0.25 mM at 23 ^oC for EcFtsZ and 30 ^oC for SaFtsZ. Reactions were quenched 10 min (EcFtsZ) or 20 min (SaFtsZ) later by addition of malachite green/ammonium molybdate (P_i ColorLock Gold, Innova Biosciences) and the color developed for 30 min. GTPase inhibition by **2** was also tested against *B. subtilis* FtsZ (BsFtsZ) using the following conditions. Solutions containing BsFtsZ (10 μM) in 50 mM MES, pH 6.0, 100 mM KCl, 5 mM $MgCl_2$ were treated with serial dilutions of **2** to a final concentration of 200 μM in 2% DMSO. Hydrolysis was measured using the malachite green/ammonium molybdate read out with measurements taken between 0 to 60 min after GTP addition. The 100% activity of BsFtsZ GTPase activity was 0.75 min^{-1}, with an IC_{50} of approximately 70 μM. Inorganic phosphate was quantified by absorbance at 635 nm, and compared to a standard curve. Controls included DMSO (no significant difference in GTPase activity up to 8% DMSO); FtsZ alone in buffer plus compound; FtsZ alone in buffer; and GTP alone in buffer.

Microbroth dilution assays

Microbroth dilution assays were performed as described in the CLSI guidelines. Minimum inhibitory concentrations (MIC_{50} and MIC_{90}) were determined in 96-well format microbroth dilution assays. Antimicrobial activity of pure compounds **1** and **2** or antibiotic standards were tested by adding serial dilutions to wells containing Mueller Hinton II broth. An overnight growth of *envA1* BL21(DE3)pLysS-derivative of *E. coli*[3] was diluted in Mueller-Hinton II broth and added to the wells to a final concentration of $5x10^5$ colony forming units (CFU) per milliliter. Plates were incubated for 18 hours at 37 ^oC with shaking at 200 rpm.

After incubation, plates were read at 600 nm on a Molecular Devices plate reader. Growth curves were plotted and MIC_{50} values obtained using Kalidagraph software. Curves were fit to a simple one-site inhibition model using the equation $y=100/[1+(concentration/MIC_{50})]$, where MIC_{50} is the concentration at which the growth of bacterial cultures are reduced by 50%. MIC_{90} values were visually calculated by determining

the lowest concentration of test compound that allowed no visible growth (no difference of absorption between treated samples and blank controls).

Construction of FtsZ-RFP

To replace YFP for RFP (tdTomato) in the pFtsZ-YFP plasmid, we first inserted a SpeI restriction site between FtsZ and YFP by site-directed mutagenesis (QuikChange II Site-Directed Mutagenesis Kit, Agilent technologies). The sequences of the PAGE purified forward and reverse primers (Integrated DNA Technologies) used were:

5'–GCTGATGGATCCACTAGTATGGTGAGCAAGGGC–3'

5'–GCCCTTGCTCACCATACTAGTGGATCCATCAGC–3' .

DNA sequencing (Eurofins) using pBad forward (5'–ATGCCATAGCATTTTTATCC–3') and pBad reverse (5'–GATTTAATCTGTATCAGG–3') primers confirmed the presence of the SpeI site and an additional amino acid in the linker region, resulting in a GSTS sequence occurring between FtsZ and YFP. The gene sequence for YFP was removed using SpeI and SphI restriction enzymes (New England Biolabs), and the vector backbone was purified by gel extraction. TdTomato (Clontech) was amplified from the purchased plasmid by PCR using forward and reverse primers with respective sequences:

5'–GACTAGTATGGTGAGCAAGGGCGAG–3'

5'–ACATGCATGCATCCTACTTGTACAGCT–3'

with Pfu Ultra High Fidelity polymerase (Agilent Technologies) according to the manufacturer's instructions. The amplified tdTomato was purified (Wizard PCR preps, Promega), cut with SpeI and SphI restriction enzymes, and ligated into the vector backbone with T4 ligase. The ligation mixture was transformed by heat stock into DH5α *E. coli* (New England Biolabs). The plasmid was extracted by mini-prep protocol, and sequenced using the same primers as above. pFtsZ-RFP was electroporated into permeable *E. coli* using 25 μFD, 200 Ω, and 2.5 kV (time constant 4 ms).

Fluorescence microscopy

envA1 E. coli: The pFtsZ-YFP plasmid [4] was transformed into a permeable strain of *E. coli* (*Escherichia coli* K12 C600 leu thr lac (thi) galK lpxC::Tn10) (referred to as *envA1*)[3] via electroporation. After electroporation, colonies were screened on LB agar with 20 μg/mL

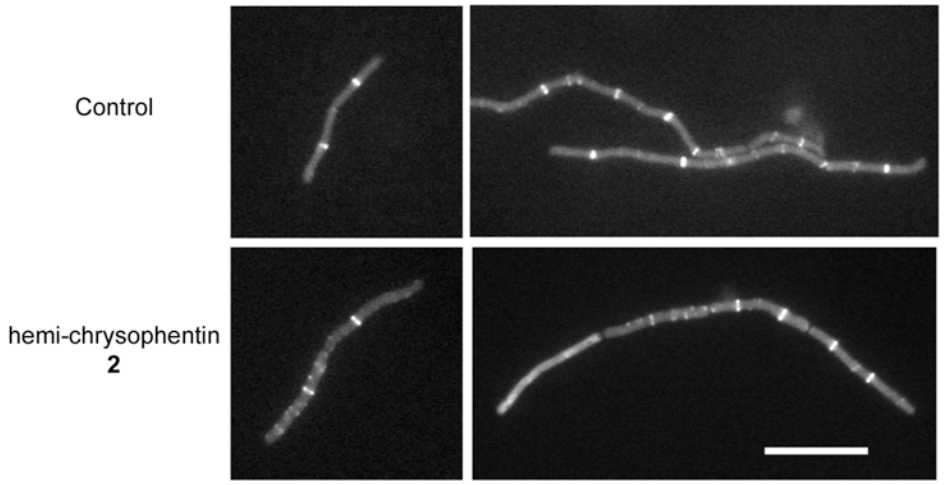
chloramphenicol for clones that contained the arabinose-inducible pFtsZ-YFP (*envA1*/pFtsZ-YFP). Replicate plates were made, and those clones that expressed yellow fluorescence at mid-cell after a 15 minute induction with 0.4% L-arabinose in LB broth supplemented with 20 μg/mL chloramphenicol were harvested and saved as glycerol stocks and frozen at -80 °C. When needed, a glycerol stock was thawed and grown in 15 mL LB broth with 20 μg/mL chloramphenicol for approximately 3 hr at 37 °C with shaking at 200 rpm, or until the OD_{600} reached ~0.2. Fluorescent protein expression was induced with 0.4% L-arabinose for 30 min. (This induction time was optimized by observing fluorescence at multiple time points post induction.) After 30 min, cells were harvested by centrifugation at 3500 rpm, and resuspended in phosphate buffered saline (PBS) (pH 7.4) with 50 μg/mL tetracycline to wash off the arabinose, and maintain selection of the *envA1* mutation for permeability. The centrifugation and wash steps were repeated three times, and after the third centrifugation, cells were washed in LB broth and centrifuged a final time. Bacteria were then resuspended in 25 mL LB broth with 20 μg/mL chloramphenicol. Small volumes (1 mL each) were removed and subjected to different treatments, including 1% MeOH or 1% DMSO (as a vehicle control), compounds **1** and **2**, or zantrin Z1, zantrin Z3, and berberine.

Cells were visualized on a polylysine coated slide with DIC and fluorescence microscopy using a Zeiss LSM700 confocal scanning laser unit with an inverted microscope (AXIO Observer.Z1) with a 100X objective, 1.3 numerical aperture lens. Experiments were carried out in duplicate on separate days. The images selected are representative of each of the experimental conditions used. Additional images as well as further controls can be seen in Ref. 2.

***B. subtilis* SU570**: We determined the effect of hemi-chrysophaentin (**2**) on Z-rings in *B. subtilis* SU570, which contains FtsZ-GFP as the only FtsZ protein. A colony of *B. subtilis* strain SU570 (168 trpC2 ftsZ::ftsZ-gfp-Spec),[5] kindly donated by Dr. Elisabeth J. Harry, was resuspended in antibiotic medium No. 3 (Pennassay broth, Becton Dickinson) with 50 μg/mL spectinomycin and grown at 30 °C to an A_{600} of 0.2. Samples of the culture were mixed with an equal volume of Medium 15 containing twice the final desired concentration of compound (24 μM to give a final concentration of 12 μM). Aliquots (10 mL) were harvested at appropriate time intervals, combined with an equal volume of pre-warmed Pennassay broth containing ~1% w/v agarose, and 10 μl of these samples were pipetted onto microscope

slides, covered, and visualized through a 100X PlanApochromatic objective with a Zeiss Axioplan fluorescence microscope equipped with a Hamamatsu 4742-95 CCD camera. Nucleoids were stained with 4,6-diamino-2phenylindole (DAPI, 0.25 μg/mL) added to the cells prior to visualization.

Figure S1. Effect of **2** on *B. subtilis* SU570.



Effect of hemi-chrys, **2** on FtsZ-GFP localization in *Bacillus subtilis* cells after 60 min treatment. Scale bar, 10 μm.

References

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