

**Supporting information for:**

**Inhibitors of bacterial tubulin target bacterial membranes in vivo**

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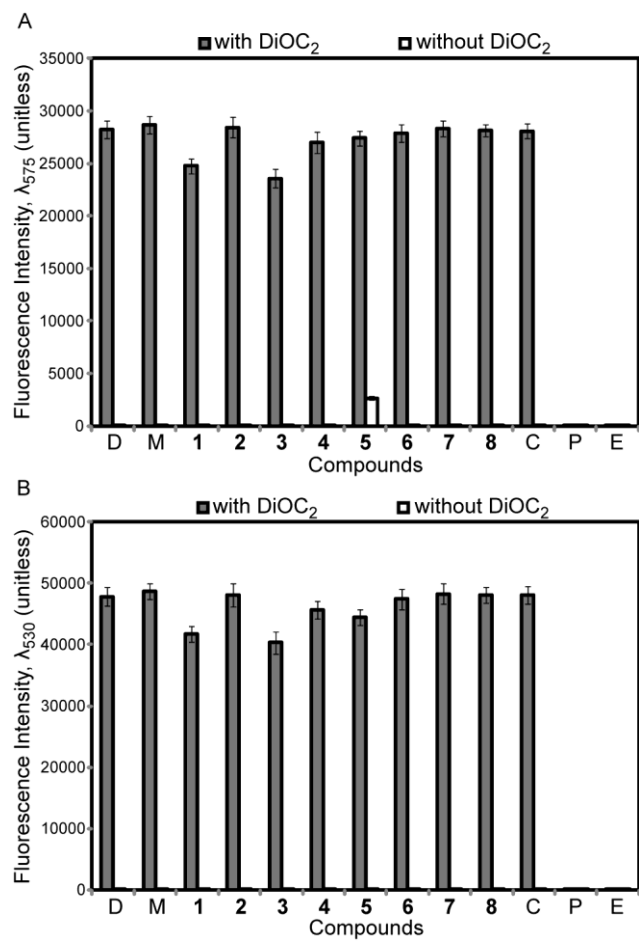
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## Supplemental Methods

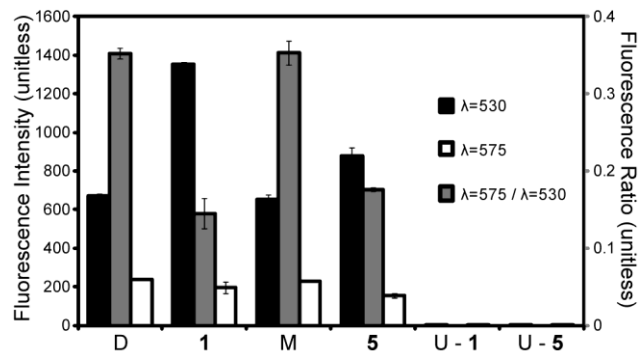
*Evaluating changes in proton transport across the membrane of bacteria treated with compounds.* We performed pH relaxation experiments in *B. subtilis* 168 by modifying the method described by Rosenthal et al. <sup>1</sup>. We grew *B. subtilis* 168 in LB to late exponential phase and harvested cells by centrifugation at 1500 rcf for 10 min at 25 °C using a Beckman Coulter GH-3.8 rotor. We rinsed the cells with 30 mL 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (TRIS) at pH 8.0 and pelleted cells by centrifugation. We subsequently rinsed the cells with 2 mL of 0.1 M TRIS pH 8.0 and harvested the cells by centrifugation at 16,000 rcf for 1 min at 25 °C with an Eppendorf 5415 D tabletop centrifuge. We rinsed the cells twice with 2 mL of salt solution consisting of 150 mM NaCl and 5 mM KCl and suspended the cells in this solution at a final density of  $\sim 3 \times 10^{10}$  cells/mL. This procedure enabled us to create a culture with significant total volume inside the cells. We placed an artificially high  $\Delta pH$  across these cells by the addition of a strong mineral acid to the un-buffered cell suspension. Before adding compounds, we adjusted the pH of the suspension to 6.0 using 0.1 M HCl. We monitored the pH of suspensions of cells using a Thermo Orion 4 Star pH meter for at least 2 min before addition of compounds. After observing the pH for 8 min after compound addition, we added CCCP to determine whether the  $\Delta pH$  remained intact or was depleted.

**Figure S1.** Fluorescence measurements of compounds used in experiments with DiOC<sub>2</sub> in the absence of cells A) Fluorescence measurements of compounds at  $\lambda_{\text{emission}}=575$  nm. B) Fluorescence measurements of compounds at  $\lambda_{\text{emission}}=530$  nm. In a 384-well plate, we measured the fluorescence intensity of 30  $\mu\text{M}$  DiOC<sub>2</sub> in 1 $\times$ PBS containing **1-8** (1 $\times$ MIC), as well as the compounds only in 1 $\times$ PBS, at  $\lambda_{\text{emission}}=575$  nm and 530 nm, and excitation at  $\lambda_{\text{excitation}}=488$  nm. The labels ‘D’, ‘M’, ‘C’, ‘P’, and ‘E’ represent DMSO, methanol, control (no treatment), 1 $\times$ PBS, and empty wells, respectively. We measured each condition in triplicate. We incubated DiOC<sub>2</sub> and compounds for 30 min before measuring fluorescence. Compound **5** emitted fluorescence at  $\lambda_{\text{emission}}=575$  nm. We performed further controls to evaluate the interference of the absorbance of **5** in DiOC<sub>2</sub> measurements (see **Figure S2**).



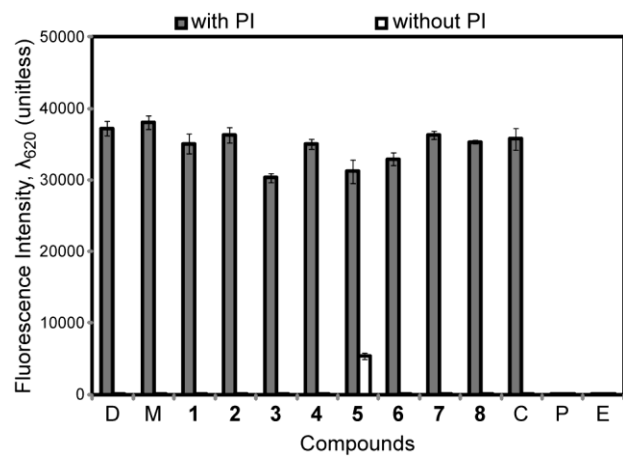
**Figure S1**

**Figure S2.** Controls to determine the interference of the fluorescence of **5** in measurements of membrane potential using the DiOC<sub>2</sub> assay. The labels ‘D’, ‘M’, and ‘U’ refer to treatments with DMSO, methanol, and unlabeled (without DiOC<sub>2</sub>), respectively. The fluorescence emission from cells treated with **5** (i.e., no DiOC<sub>2</sub>) was small in comparison to DiOC<sub>2</sub> treated cells. Cells treated with **5** only displayed an emission at  $\lambda_{575}$  of intensity  $2.6 \pm 0.1$  and  $\lambda_{530}$  of intensity  $2.6 \pm 0.1$ . The fluorescence signal in cells treated with DiOC<sub>2</sub> and methanol at  $\lambda_{575} = 231 \pm 0.7$  and  $\lambda_{530} = 653 \pm 26.9$ .



**Figure S2**

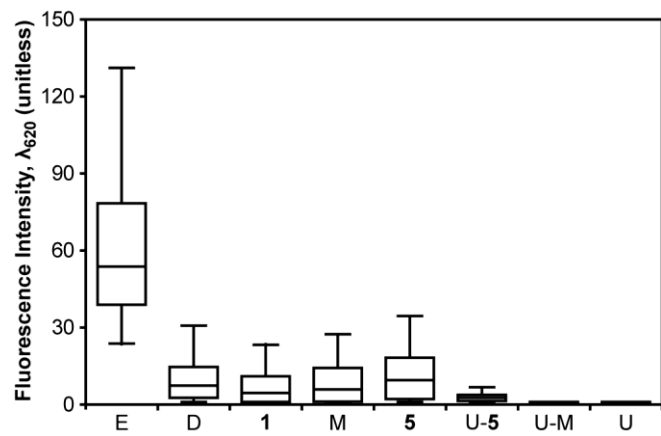
**Figure S3.** Control measurements of the fluorescence of **1-8** for experiments labeling cells with propidium iodide (PI). In a 384-well plate, we measured the fluorescence emission of 100  $\mu$ M PI in 1 $\times$ PBS containing compounds at 1 $\times$ MIC and 1 $\times$ PBS with compounds only at  $\lambda_{\text{excitation}}=488$  nm and  $\lambda_{\text{emission}}=620$  nm. The labels ‘D’, ‘M’, ‘C’, ‘P’, and ‘E’ represent DMSO, methanol, control (no treatment), 1 $\times$ PBS, and empty wells, respectively. We measured each condition in triplicate. We incubated PI and compounds for 30 min before measuring the fluorescence intensity. Sanguinarine emitted fluorescence at  $\lambda=620$  nm, and thus we performed further controls to evaluate the interference of **5** in our fluorescence-based assays (see **Figure S4**).



**Figure S3**

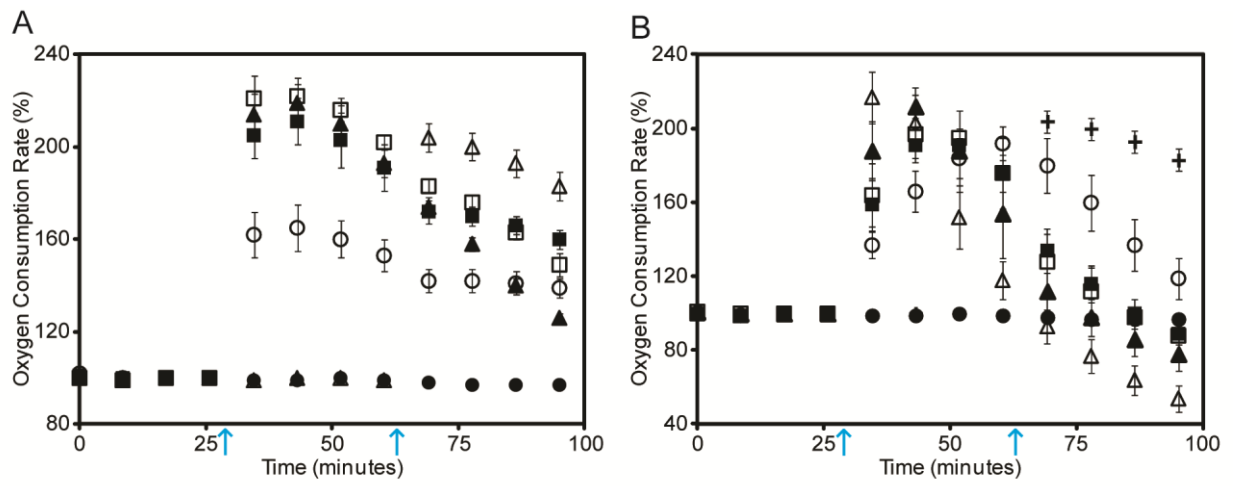


**Figure S4.** Controls for fluorescence interference of **5** in the PI assay for membrane permeability. The labels ‘E’, ‘D’, ‘M’, and ‘U’ refer to treatments with ethanol, DMSO, methanol, and unlabeled (without PI), respectively. Whisker plots represent the median in the center of the box, 25 to 75% of the population in the box, and 5 to 95% of the population between the outer whiskers. The median fluorescence value for methanol and **5** treated cells were 5.99 and 9.56, respectively. Cells treated with only **5** (i.e. no PI labeling) had a median fluorescence of 2.67. Due to significant fluorescence from **5** of approximately the intensity difference between solvent and treatment samples, the addition of **5** itself rather than permeabilization may explain the increase in signal.



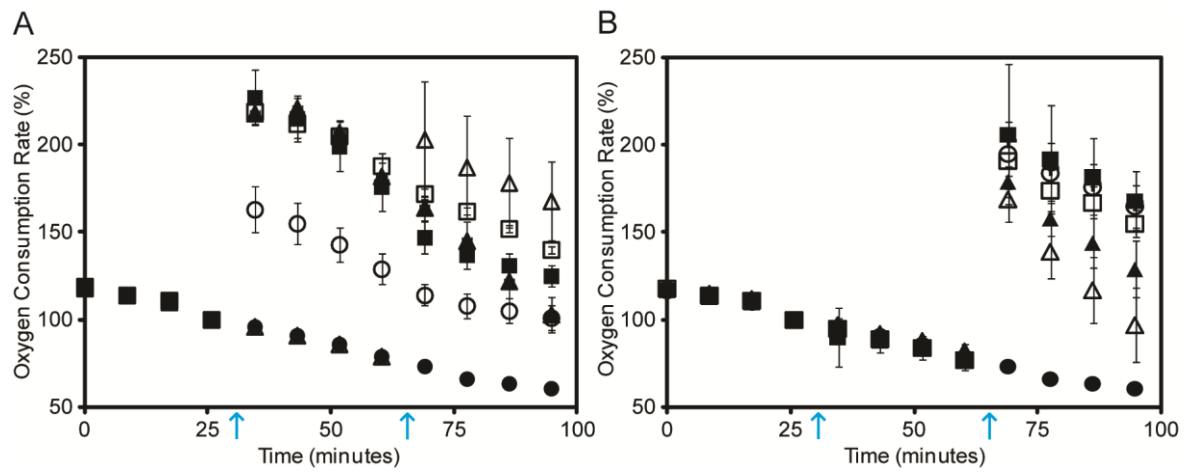
**Figure S4**

**Figure S5.** Measurement of the uncoupling activity of **1** and **2** in C2C12 myoblasts. We added compound **1** or **2** or DMSO (the solvent control) at two injection points that are designated by arrows. The following are the treatments applied in the first injection and second injection by symbol in **A**: DMSO/DMSO ●, 0.1  $\mu\text{M}$  **1**/DMSO ○, 0.2  $\mu\text{M}$  **1**/DMSO ■, 0.6  $\mu\text{M}$  **1**/DMSO □, 1.2  $\mu\text{M}$  **1**/DMSO ▲, and DMSO/0.6  $\mu\text{M}$  **1** Δ. The following are treatments applied in the first injection and second injection by symbol in **B**: DMSO/DMSO ●, 5  $\mu\text{M}$  **2**/DMSO ○, 7.5  $\mu\text{M}$  **2**/DMSO ■, 10  $\mu\text{M}$  **2**/DMSO □, 15  $\mu\text{M}$  **2**/DMSO ▲, 30  $\mu\text{M}$  **2**/DMSO Δ, and DMSO/0.6  $\mu\text{M}$  **1** +. The addition of protonophore **1** resulted in an increase in the OCR of C2C12 myoblasts. The addition of **2** also resulted in a rise in OCR, consistent with a depletion of the pmf. The maximum OCR was at 0.6  $\mu\text{M}$  of **1** and 30  $\mu\text{M}$  of **2**.



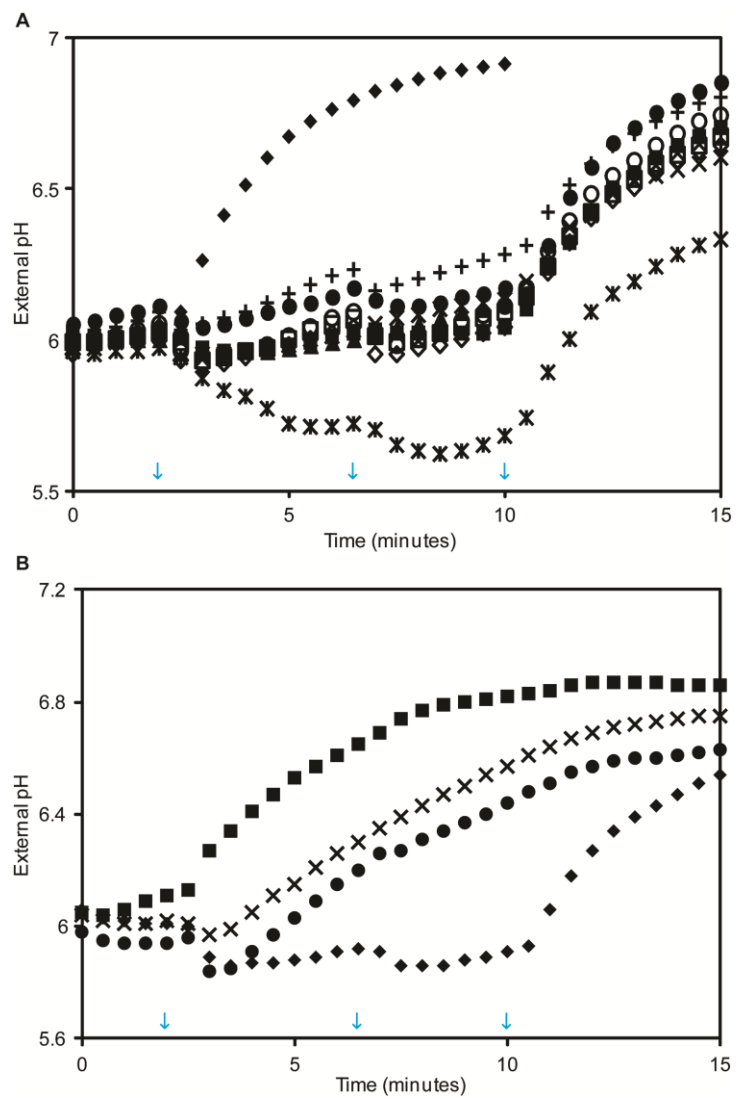
**Figure S5**

**Figure S6.** Decoupling activity of **1** and **4** on C2C12 myoblasts. We added compounds or the solvent control (i.e., DMSO) at different times indicated by the arrows. A) A list of the additives introduced in the first injection and second injection are: DMSO/DMSO ●, 0.1  $\mu\text{M}$  **1**/DMSO ○, 0.2  $\mu\text{M}$  **1**/DMSO ■, 0.6  $\mu\text{M}$  **1**/DMSO □, 1.2  $\mu\text{M}$  **1**/DMSO ▲, and DMSO/0.6  $\mu\text{M}$  **1** Δ. B) A list of the additives introduced in the first injection and second injection are: DMSO/DMSO ●, 5  $\mu\text{M}$  **4**/DMSO ○, 10  $\mu\text{M}$  **4**/DMSO ■, 15  $\mu\text{M}$  **4**/DMSO □, 30  $\mu\text{M}$  **4**/DMSO ▲, 40  $\mu\text{M}$  **4**/DMSO Δ, and DMSO/0.6  $\mu\text{M}$  **1** +. The addition of **1** resulted in an increase in the oxygen consumption rate (OCR) of C2C12 myoblasts. The addition of **4** had no affect on the OCR and thus has no significant metabolic effects on mitochondria. We observed a maximum OCR at  $\sim 0.2$   $\mu\text{M}$  of **1**.



**Figure S6**

**Figure S7.** Evaluation of the bacterial membrane as a proton barrier after treatment with **1-8**. We added compound or DMSO at two injection points designated by the first two arrows. Eight minutes after treatment with compound, we performed a third injection of 1×MIC **1** indicated by the last arrow to evaluate the presence of  $\Delta pH$ . A) A list of the additives introduced in the first and second injection are: DMSO ▲, MeOH ×, **1** ◆, **2** ■, **3** \*, **4** ●, **5** □, **6** ◇, **7** ○, **8** +. Only addition of **1** at its MIC results in relaxation of  $\Delta pH$ . The addition of **3** results in a decrease in the external pH, but does not relax  $\Delta pH$ . B) A list of the additives introduced in the first and second injection are: 10  $\mu$ M MIC **1** ■, 30  $\mu$ M **2** × and ●, and 160 nM **2** ◆. At 30  $\mu$ M of **2**, the concentration that resulted in the maximal OCR for C2C12 myoblasts, the  $\Delta pH$  for *B. subtilis* 168 is equilibrated. At a concentration of 160 nM **2**, the 1×MIC concentration, equilibration of  $\Delta pH$  does not occur. This result suggests that the effects of **2-6** are not catalytic, as is the case for **1**. Alternatively, **2-6** may require a certain lipid:compound ratio before their activity is observed.



**Figure S7**



**Figure S8.** Measurement of compound fluorescence at  $1\times$ MIC values in uninduced *B. subtilis* DS4294 cells to eliminate compounds with significant fluorescence interference for microscopy studies. The labels ‘U’ and ‘I’ represent uninduced and induced cells, respectively. The labels ‘C’, ‘Cb’, ‘Cm’, ‘D’, and ‘M’ refer to control (untreated), control cell signal at 25% cell length, control cell signal at 50% cell length, DMSO, and methanol, respectively. We measured induced cell fluorescence intensity for comparison to compound treated cells. We measured fluorescence intensity for at least three randomly selected cells using a line scan and subtracted background intensity outside the cell. We determined that fluorescence of **5** would interfere with localization studies.

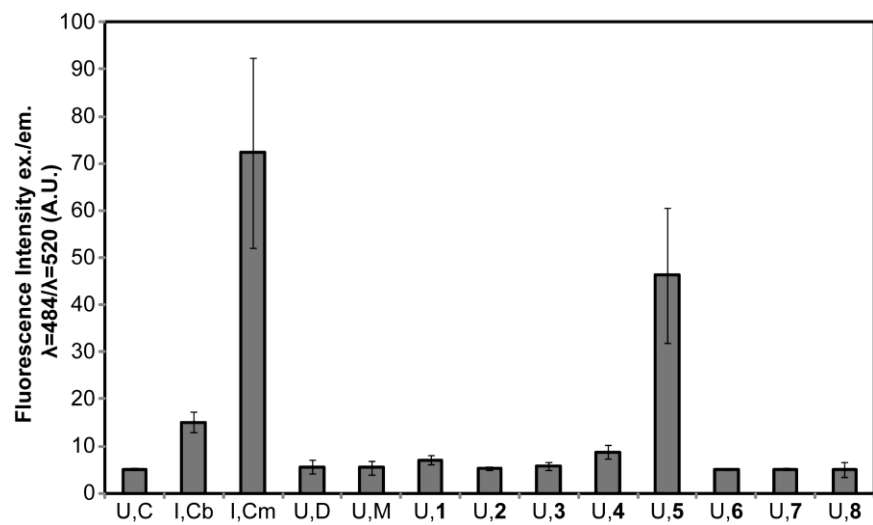


Figure S8

## References

1. K. S. Rosenthal, R. A. Ferguson and D. R. Storm, *Antimicrob Agents Chemother*, 1977, **12**, 665-672.