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Antibiotic That Inhibits the ATPase Activity of an ATP-Binding Cassette Transporter by Binding to a Remote Extracellular Site

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Supporting Information

ABSTRACT: Antibiotic-resistant strains of *Staphylococcus aureus* pose a major threat to human health and there is an ongoing need for new antibiotics to treat resistant infections. In a high throughput screen (HTS) of 230 000 small molecules designed to identify bioactive wall teichoic acid (WTA) inhibitors, we identified one hit, which was expanded through chemical synthesis into a small panel of potent compounds. We showed that these compounds target TarG, the transmembrane component of the two-component ATP-binding cassette (ABC) transporter TarGH, which exports WTA precursors to the cell surface for attachment to peptidoglycan. We purified, for the first time, a WTA transporter and have reconstituted ATPase activity in proteoliposomes. We showed that this new compound series inhibits TarH-catalyzed ATP hydrolysis even though the binding site maps to TarG near the opposite side of the membrane. These are the first ABC transporter inhibitors shown to block ATPase activity by binding to the transmembrane domain. The compounds have potential as therapeutic agents to treat *S. aureus* infections, and purification of the transmembrane transporter will enable further development.

Staphylococcus aureus has proven to be a highly adaptable pathogen, developing resistance almost as quickly as new antibiotics come to market.[1] Maintaining a pipeline of antibiotics with activity against *S. aureus* is necessary to stay ahead of emerging resistance.[2] The wall teichoic acid (WTA) pathway is a promising antibacterial target because WTAs, which are covalently attached to peptidoglycan, play crucial roles in cell division, antibiotic resistance, and pathogenesis.[3] WTA precursors are synthesized on a lipid carrier on the inner leaflet of the plasma membrane and then exported to the cell surface by the two-component ATP-binding cassette (ABC) transporter TarGH (Figure 1).[3b] ABC transporters are found in all domains of life and use ATP binding and hydrolysis to power conformational changes to translocate molecules across the cell membrane.[4] Although WTAs are required for infection,[3a] the first and second steps in the biosynthetic pathway, catalyzed by TarO and TarA, respectively, can be blocked genetically or pharmacologically without loss of viability; however, inhibiting subsequent steps is lethal and inhibitors of these late steps have potential as antibiotics.[5] We describe here the discovery of a promising small molecule that inhibits the wall teichoic acid pathway ABC transporter and we show that it blocks the ATPase activity of the nucleotide binding domain (NBD). Resistance mutations map the binding site to the transmembrane domain. Therefore, we propose that conformational coupling between ABC transporter subunits

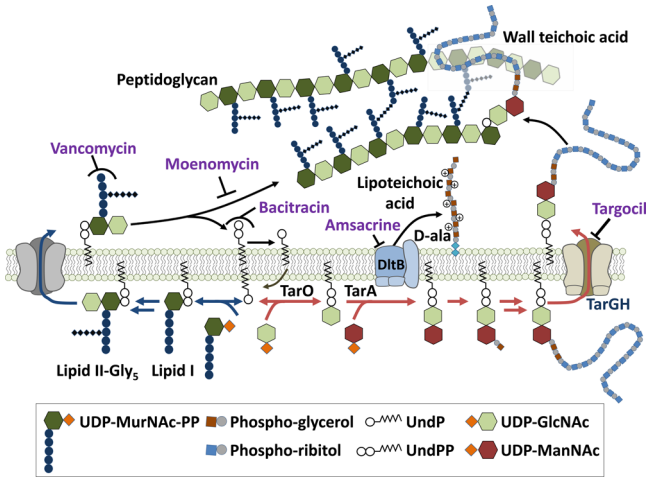

Figure 1. Schematic of cell wall biosynthetic pathways showing the sites of action of inhibitors mentioned in the text. Blue arrows denote the peptidoglycan pathway and red arrows denote the WTA pathway; these pathways use the same undecaprenyl (UndP) carrier. Antibiotic structures and legend abbreviations are explained in Figure S1.

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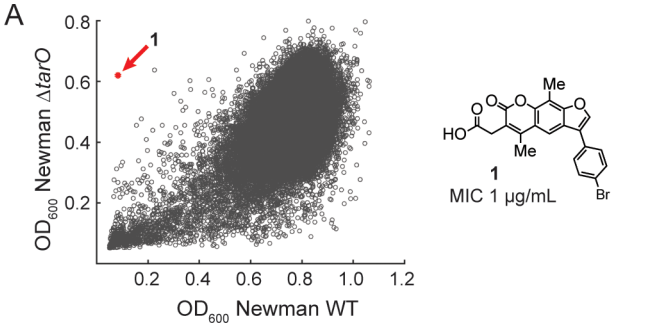
can be exploited to develop specific inhibitors that can block activity of the ATPase from a distance.

The lethal phenotype resulting from a late block in the WTA pathway, which is due to depletion of peptidoglycan precursors (see Figure 1),[2c,6] inspired us to develop a pathway-specific, whole cell assay for WTA-targeted antibiotics that involved screening a wild-type strain for growth inhibition while counterscreening a WTA null (Δ*tarO*) strain for suppression of bioactivity.[3b] As previously discovered WTA inhibitors had poor physical properties,[7] we screened 230 000 small molecules at a final concentration of ~15 μM against wild-type *S. aureus* and the Δ*tarO* knockout strain. The screen produced a single strong hit (**1**), which proved to be a furanocoumarin derivative (Figure 2A). Compound **1** was found to have a minimum



Compound	R_1	R_2	MIC μg/mL	Compound	R_1	R_2	MIC μg/mL
2	L-Pro	Cl	0.125	**6**	L-Ala	Cl	0.25
3	D-Pro	Cl	2	**7**	D-Ala	Cl	2
4	L-Pro	Br	0.125	**8**	L-Ala	Br	0.25
5	D-Pro	Br	1	**9**	D-Ala	Br	2

Figure 2. HTS screening hit led to potent anti-MRSA compounds **2** and **4**. (A) Plot of HTS results. Each circle represents the average OD_{600} of the strains in the presence of a library compound tested in duplicate. One compound (compound **1**, red circle) inhibited growth of the WT Newman strain but not Δ*tarO*. (B) Synthesized analogs of **1** with activities against *S. aureus* Newman. MICs against MRSA strains are identical (Table S1).

inhibitory concentration (MIC) of 1 μg/mL against *S. aureus* (Figure 2), including several β-lactam resistant strains (MRSA; Table S1). A literature search revealed that compound **1** had been identified as a growth inhibitor in a 2 000 000-compound screen for *S. aureus* antibiotics, but its target was not identified.[8] On the basis of structurally related compounds also reported in that large screen, we synthesized a panel of analogs. Two L-proline derivatives (**2** and **4**) were found to be especially potent inhibitors of wild-type *S. aureus* growth (0.125 μg/mL), but showed no activity against the Δ*tarO* strain (Figure 2B and Table S1). This MIC is 8-fold lower than that of targocil, a well-characterized WTA-active antibiotic.[7a] Moreover, the kinetic solubility of these compounds is two to three logs greater than targocil's, the half-lives were found to be 20−40 times longer in mouse liver microsomes, and the compounds were not cytotoxic (Table S2, Figure S2). On the basis of the promising properties of the compound, we elucidated its mechanism of action.

We first assessed the effect of the compound on pool levels of the peptidoglycan precursor, Lipid II, using a previously developed assay.[2c,6] Compounds that inhibit a late step in the wall teichoic acid pathway deplete Lipid II because this peptidoglycan precursor is biosynthesized on the same carrier lipid, undecaprenyl phosphate (UndP, Figure 1).[2c,5,6] If the UndP carrier lipid is sequestered in WTA precursors, it is not available for peptidoglycan precursor synthesis. Cultures of *S. aureus* were treated for 10 min with targocil, **2**, or three peptidoglycan synthesis inhibitors with mechanisms of action that lead either to Lipid II depletion (bacitracin, which inhibits carrier lipid recycling) or Lipid II accumulation (moenomycin and vancomycin, which inhibit peptidoglycan assembly; Figure 1, Figure 3A). Cellular lipids were extracted and the Lipid II

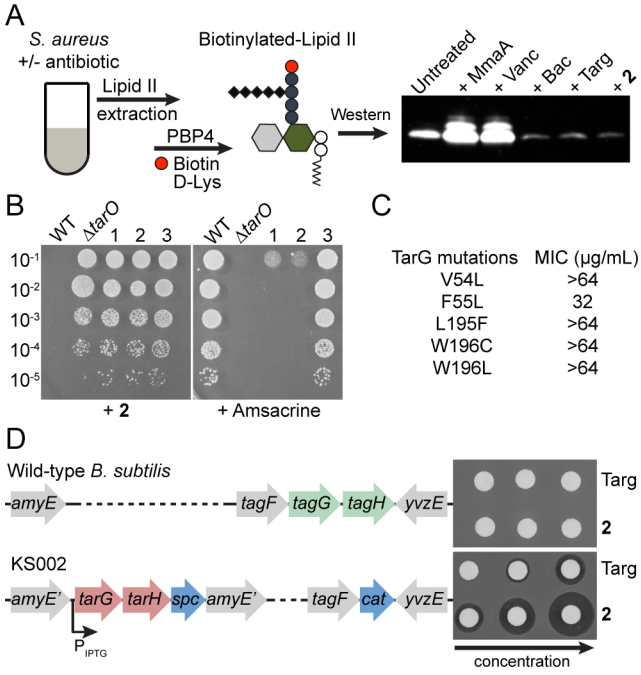


Figure 3. TarG is the target of **2**. (A) Assay to detect Lipid II abundance after antibiotic treatment, with results for control antibiotics and **2** shown. Extracted Lipid II is labeled with biotin-D-Lys using *S. aureus* PBP4 to enable detection with HRP-streptavidin. (B) Mutants resistant to **2** (lanes 1−3) were sorted into two groups by plating on amsacrine. Susceptible mutants 1 and 2 had mutations in *tarA* whereas amsacrine-resistant mutant 3 had a mutation in *tarG* (see Table S3, S4 for full list and comparison to other TarG inhibitors). (C) Substitutions in TarG that conferred high level resistance to **2**. (D) Disk diffusion assay shows that strain KS002, in which *Bacillus subtilis* TagGH was replaced with *S. aureus* TarGH, is sensitive to **2**.

present therein was labeled with biotin to enable detection by streptavidin-HRP.[2c,6] Like targocil and bacitracin, compound **2** depleted Lipid II. Combined with the suppression of bioactivity in the Δ*tarO* strain, this result confirmed inhibition of a late step in the WTA pathway.

To identify the molecular target within the WTA pathway, we selected resistant mutants on compound **2**. Twenty-seven colonies from three independent cultures were selected for evaluation. We expected two classes of mutants: those with mutations in the molecular target and those with mutations that disrupted function of TarO or TarA.[3b,5] To sort these mutants, we made use of the teichoic acid D-alanylation inhibitor, amsacrine, which prevents growth of WTA null strains.[9]

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Seventeen mutants were unable to grow on the inhibitor (Figure 3B), and all of these were found to contain null mutations in *tarO* or *tarA*; all other mutants contained point mutations that resulted in amino acid substitutions in TarG, the transmembrane component of the ABC transporter that exports WTA precursors to the cell surface (Figure 3C; Table S3). These results validated the procedure used to classify mutants and suggested that TarG is the target of **2**.

We used two different approaches to confirm TarG as the target. First, we expressed one of the resistant *tarG* alleles in a clean *S. aureus* background and found that expression conferred dominant resistance (Figure S3). Second, after verifying that **2** did not inhibit growth of *B. subtilis* (Figure S4), we made use of a previously engineered *B. subtilis* strain in which the endogenous WTA transporter genes (*tagGH*) were replaced with the *S. aureus* transporter genes at an ectopic locus.[10] Compound **2** did not show a zone of inhibition in a disk diffusion assay against wild-type *B. subtilis*, but it showed a dose-dependent inhibition zone when tested against the strain expressing the *S. aureus* transporter (Figure 3D). This gain of sensitivity to compound **2** upon heterologous expression of *S. aureus tarGH* in *B. subtilis* confirmed the *S. aureus* wall teichoic acid transporter as its target.

Several classes of compounds that inhibit WTA export have now been identified, but the class reported here is the first with solubility properties that allow mechanistic characterization.[3b,7] Elucidating how these compounds act may not only provide insight into how to improve them further, but could guide efforts to develop inhibitors of other ABC transporters. The ABC transporter family is very large and includes many possible therapeutic targets in both prokaryotes and eukaryotes, but few mechanistic studies on inhibitors have been reported.[11] P-glycoprotein inhibitors have received the most attention due to the importance of this ABC transporter in multidrug resistance in cancer.[12] Inhibitors that compete with ATP for binding to the nucleotide binding domain (NBD) or with exported substrates have been studied, but were abandoned due to lack of specificity and toxicity.[12b] The most promising P-glycoprotein inhibitors identified to date bind to the transmembrane (TM) domain in a manner that prevents substrate transport, but allows robust ATP hydrolysis.[11c,13]

To obtain information on how **2** inhibits TarGH, we coexpressed wild-type TarG with either TarH-His_6 or an ATPase-inactive TarH-His_6 mutant (E169Q), solubilized the complexes in dodecylmaltoside, purified them over an affinity column followed by size exclusion chromatography, and reconstituted them into proteoliposomes.[14] The ATPase activity of the reconstituted transporter, measured using a continuous chromogenic assay, had kinetic parameters similar to those reported for other ABC transporters (Figure S5).[15] The addition of compound **2** strongly inhibited ATPase activity with an IC_{50} of 137 nM, even though the ATP concentration was 1000-fold higher (Figure 4A, Figure S6). Additional experiments showed that the ATPase activity of the WT transporter in the presence of 1.0 μM **2** was comparable to that of a TarGH mutant containing a mutation that impairs ATP hydrolysis (TarH E169Q) (Figure S7; Figure S8).

To locate the binding site of **2** relative to the ATPase, we generated a homology model for TarGH using the human ABCG5/ABCG8 sterol transporter as the template and mapped the resistance mutations to the modeled structure (Figure 4B,C).[16] In agreement with the topology of many other ABC exporters, each TarGH dimer has 12 TM helices,[15b,17]

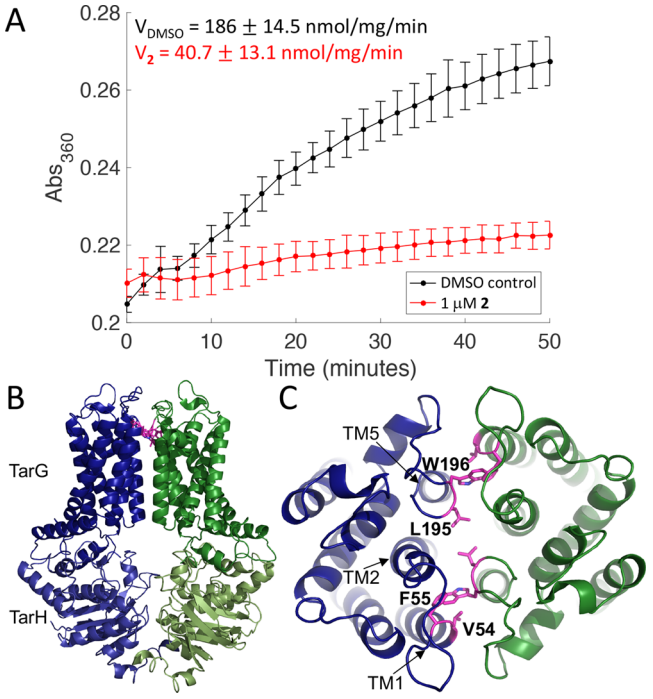

Figure 4. Compound **2** inhibits the ATPase activity of TarGH in proteoliposomes but binds in a remote location. (A) Averaged ATPase activity (n = 3; error bars = SD) of reconstituted TarGH (200 nM) in the absence (black) and presence (red) of compound **2** (1 μM). Saturating levels of ATP (1 mM) were used. (B) Homology model of TarGH. TarH is cytoplasmic and much of TarG is embedded in the membrane. (C) Top view of the TarG dimer. Mutations in residues shown in pink give high level resistance to **2**.

which are grouped such that TM helices one and two from one monomer are in close proximity to TM helix five of the other monomer. The high-level resistance mutations selected with compound **2** map near the extracellular ends of TM helices one and five. Though we cannot exclude the possibility that the resistance mutations affect the conformation of the ATPase from a distance such that it remains active but is incapable of binding inhibitor, we think it far more likely that the binding site is defined by the resistance mutations. We propose, therefore, that the binding site spans the dimer interface and, given the symmetry, that two molecules of **2** bind to the dimer. To inhibit ATP hydrolysis by binding to a remote site, the compound must lock the TM domain in a conformation that prevents the coupled interdomain structural changes required for ongoing ATP hydrolysis by the NBDs.

Compound **2**, hereafter to be called targocil-II, is the first known example of an ABC transporter inhibitor that prevents ATP hydrolysis by binding to an allosteric site in the TM domain. Given the sequence diversity of TM domains, this mode of binding would have clear advantages with respect to specificity over ATP-competitive inhibitors that bind to a very highly conserved binding pocket. Now that conditions have been developed to obtain the purified ABC transporter in active form, it may be possible to obtain structural information with inhibitor bound to facilitate development of transport inhibitors for therapeutic use.

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■ ASSOCIATED CONTENT

Supporting Information

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Methods, SI figures and tables (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) (a) Wright, G. D. *ACS Infect. Dis.* **2015**, *1*, 80. (b) Chambers, H. F.; Deleo, F. R. *Nat. Rev. Microbiol.* **2009**, *7*, 629.

(2) (a) Okano, A.; Nakayama, A.; Schammel, A. W.; Boger, D. L. *J. Am. Chem. Soc.* **2014**, *136*, 13522. (b) Bouley, R.; Kumarasiri, M.; Peng, Z.; Otero, L. H.; Song, W.; Suckow, M. A.; Schroeder, V. A.; Wolter, W. R.; Lastochkin, E.; Antunes, N. T.; Pi, H.; Vakulenko, S.; Hermoso, J. A.; Chang, M.; Mobashery, S. *J. Am. Chem. Soc.* **2015**, *137*, 1738. (c) Lee, W.; Schaefer, K.; Qiao, Y.; Srisuknimit, V.; Steinmetz, H.; Müller, R.; Kahne, D.; Walker, S. *J. Am. Chem. Soc.* **2016**, *138*, 100.

(3) (a) Weidenmaier, C.; Kokai-Kun, J. F.; Kristian, S. A.; Chanturiya, T.; Kalbacher, H.; Gross, M.; Nicholson, G.; Neumeister, B.; Mond, J. J.; Peschel, A. *Nat. Med.* **2004**, *10*, 243. (b) Swoboda, J. G.; Meredith, T. C.; Campbell, J.; Brown, S.; Suzuki, T.; Bollenbach, T.; Malhowski, A. J.; Kishony, R.; Gilmore, M. S.; Walker, S. *ACS Chem. Biol.* **2009**, *4*, 875. (c) Campbell, J.; Singh, A. K.; Santa Maria, J. P.; Kim, Y.; Brown, S.; Swoboda, J. G.; Mylonakis, E.; Wilkinson, B. J.; Walker, S. *ACS Chem. Biol.* **2011**, *6*, 106.

(4) Wilkens, S. *F1000Prime Rep.* **2015**, *7*, 14.

(5) (a) D'Elia, M. A.; Pereira, M. P.; Chung, Y. S.; Zhao, W.; Chau, A.; Kenney, T. J.; Sulavik, M. C.; Black, T. A.; Brown, E. D. *J. Bacteriol.* **2006**, *188*, 4183. (b) D'Elia, M. A.; Henderson, J. A.; Beveridge, T. J.; Heinrichs, D. E.; Brown, E. D. *J. Bacteriol.* **2009**, *191*, 4030.

(6) Qiao, Y.; Lebar, M. D.; Schirner, K.; Schaefer, K.; Tsukamoto, H.; Kahne, D.; Walker, S. *J. Am. Chem. Soc.* **2014**, *136*, 14678.

(7) (a) Lee, K.; Campbell, J.; Swoboda, J. G.; Cuny, G. D.; Walker, S. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 1767. (b) Wang, H.; Gill, C. J.; Lee, S. H.; Mann, P.; Zuck, P.; Meredith, T. C.; Murgolo, N.; She, X.; Kales, S.; Liang, L.; Liu, J.; Wu, J.; Santa Maria, J.; Su, J.; Pan, J.; Hailey, J.; Mcguinness, D.; Tan, C. M.; Flattery, A.; Walker, S.; Black, T.; Roemer, T. *Chem. Biol.* **2013**, *20*, 272.

(8) Cheng, T. J.; Wu, Y. T.; Yang, S. T.; Lo, K. H.; Chen, S. K.; Chen, Y. H.; Huang, W. I.; Yuan, C. H.; Guo, C. W.; Huang, L. Y.; Chen, K. T.; Shih, H. W.; Cheng, Y. S.; Cheng, W. C.; Wong, C. H. *Bioorg. Med. Chem.* **2010**, *18*, 8512.

(9) (a) Pasquina, L.; Santa Maria, J. P.; McKay Wood, B.; Moussa, S. H.; Matano, L. M.; Santiago, M.; Martin, S. E.; Lee, W.; Meredith, T. C.; Walker, S. *Nat. Chem. Biol.* **2016**, *12*, 40. (b) Schaefer, K.; Matano, L. M.; Qiao, Y.; Kahne, D.; Walker, S. *Nat. Chem. Biol.* **2017**, *13*, 396. (c) Matano, L. M.; Morris, H. G.; Wood, B. M.; Meredith, T. C.; Walker, S. *Bioorg. Med. Chem.* **2016**, *24*, 6307.

(10) Schirner, K.; Stone, L. K.; Walker, S. *ACS Chem. Biol.* **2011**, *6*, 407.

(11) (a) Aller, S. G.; Yu, J.; Ward, A.; Weng, Y.; Chittaboina, S.; Zhuo, R.; Harrell, P. M.; Trinh, Y. T.; Zhang, Q.; Urbatsch, I. L.; Chang, G. *Science* **2009**, *323*, 1718. (b) Jin, M. S.; Oldham, M. L.; Zhang, Q.; Chen, J. *Nature* **2012**, *490*, 566. (c) Loo, T. W.; Clarke, D. M. *Biochem. Pharmacol.* **2014**, *92*, 558. (d) Loo, T. W.; Clarke, D. M. *J. Biol. Chem.* **2015**, *290*, 29389. (e) Sherman, D. J.; Okuda, S.; Denny, W. A.; Kahne, D. *Bioorg. Med. Chem.* **2013**, *21*, 4846.

(12) (a) Szakacs, G.; Paterson, J. K.; Ludwig, J. A.; Booth-Genthe, C.; Gottesman, M. M. *Nat. Rev. Drug Discovery* **2006**, *5*, 219. (b) Crowley, E.; McDevitt, C. A.; Callaghan, R. *Methods Mol. Biol.* **2010**, *596*, 405.

(13) Falasca, M.; Linton, K. J. *Expert Opin. Invest. Drugs* **2012**, *21*, 657.

(14) Moody, J. E.; Millen, L.; Binns, D.; Hunt, J. F.; Thomas, P. J. *J. Biol. Chem.* **2002**, *277*, 21111.

(15) (a) Borths, E. L.; Poolman, B.; Hvorup, R. N.; Locher, K. P.; Rees, D. C. *Biochemistry* **2005**, *44*, 16301. (b) Perez, C.; Gerber, S.; Boilevin, J.; Bucher, M.; Darbre, T.; Aebi, M.; Reymond, J. L.; Locher, K. P. *Nature* **2015**, *524*, 433.

(16) Lee, J. Y.; Kinch, L. N.; Borek, D. M.; Wang, J.; Urbatsch, I. L.; Xie, X. S.; Grishin, N. V.; Cohen, J. C.; Otwinowski, Z.; Hobbs, H. H.; Rosenbaum, D. M.; Wang, J. *Nature* **2016**, *533*, 561.

(17) (a) Dawson, R. J.; Locher, K. P. *Nature* **2006**, *443*, 180. (b) Ward, A.; Reyes, C. L.; Yu, J.; Roth, C. B.; Chang, G. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 19005.

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