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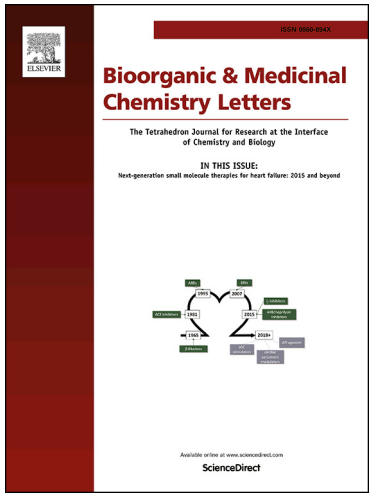

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First-generation structure-activity relationship studies of 2,3,4,9-tetrahydro-1*H*-carbazol-1-amines as CpxA phosphatase inhibitors

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ABSTRACT

Genetic activation of the bacterial two component signal transduction system, CpxRA, abolishes the virulence of a number of pathogens in human and murine infection models. Recently, 2,3,4,9-tetrahydro-1*H*-carbazol-1-amines were shown to activate the CpxRA system by inhibiting the phosphatase activity of CpxA. Herein we report the initial structure-activity relationships of this scaffold by focusing on three approaches 1) A-ring substitution, 2) B-ring deconstruction to provide N-arylated amino acid derivatives and 3) C-ring elimination to give 2-ethylamino substituted indoles. These studies demonstrate that the A-ring is amenable to functionalization and provides a promising avenue for continued optimization of this chemotype. Further investigations revealed that the C-ring is not necessary for activity, although it likely provides conformational constraint that is beneficial to potency, and that the (*R*) stereochemistry is required at the primary amine. Simplification of the scaffold through deconstruction of the B-ring led to inactive compounds, highlighting the importance of the indole core. A new lead compound **26** was identified, which manifests a ~30-fold improvement in CpxA phosphatase inhibition over the initial hit. Comparison of amino and *des*-amino derivatives in bacterial strains differing in membrane permeability and efflux capabilities demonstrate that the amine is required not only for target engagement but also for permeation and accumulation in *Escherichia coli*.

Keywords: two-component system, sensory kinase, antibacterial, drug discovery, medicinal chemistry, CpxRA, permeability, efflux

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The urgency to develop new modalities to treat drug-resistant infections is unquestionable, especially for those caused by multi- and extensively-drug resistant Gram-negative pathogens. Reports of infectious strains resistant to all known clinically approved antibiotics have surfaced and raise the

possibility that common infections may soon become untreatable if new therapeutic advancements are not developed in a timely fashion.[1]

All currently available antibiotics inhibit a limited number of enzymes and processes that have essential functions for bacterial growth.[2] The dogma in the field is that only compounds that completely inhibit cell growth will prove to be clinically useful. Despite intensive efforts, few new targets or drug classes have been identified for Gram-negative bacteria in the past 50 years.[3] In fact, the ability to address resistance has resorted in large part to structural modification of known classes.[4] This approach typically provides only short-lived solutions, as new derivatives are susceptible to the rapid evolution of cross-resistance.[5] The reality is that bacterial resistance now outpaces our ability to derivatize known scaffolds and advance the new entities to the clinic. Thus, new targets, antibacterial chemotypes, and approaches are desperately required. In contrast to traditional approaches, one strategy that is gaining significant traction is to render organisms vulnerable to host immune clearance by targeting virulence determinants or processes that control the expression of virulence factors.[6]

In this context, bacterial two-component signal transduction systems (2CSTS) represent attractive targets for antibacterial drug discovery. In contrast to 2CSTS, that act usually at histidine and aspartate residues, mammalian signal transduction systems utilize kinases and phosphatases that act at tyrosine, serine, or threonine residues.[7] Because 2CSTS are ubiquitous in bacteria, are not found in mammals, and perform phosphorelay on different amino acids, they are excellent antimicrobial targets. These systems consist of a sensory kinase (SK) and a response regulator (RR). In response to a stimulus (1, **Figure 1**), the SK autophosphorylates a conserved histidine (2, **Figure 1**), and a cognate RR catalyzes the transfer of this phosphate group to itself on an aspartic acid residue (3, **Figure 1**).[8] Phosphorylation of the RR leads to a biological response, usually resulting in changes to gene transcription. Some SKs also possess phospho-RR phosphatase activity (4, **Figure 1**) and are thus critical to signaling duration.[7] Loss of SK phosphatase activity leads to persistent RR phosphorylation and thus constitutive activation of the pathway.

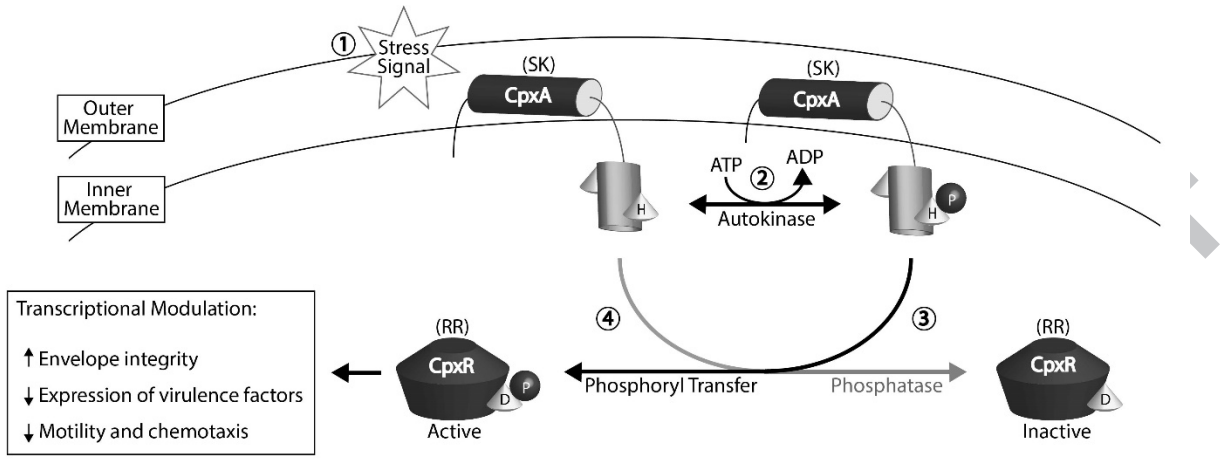
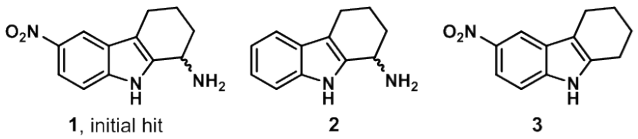


Figure 1. CpxRA signaling overview.[9-11] In response to membrane stress (1), CpxA (SK) undergoes autophosphorylation at a conserved His residue in an ATP-dependent manner (2). CpxR (RR) then catalyzes the transfer of the phosphate group to a cognate Asp residue (3), resulting in CpxR-P and activation. CpxA exhibits phosphatase activity and can dephosphorylate CpxR-P to deactivate the system (4).

CpxRA, (SK = CpxA; RR = CpxR) is a 2CSTS found in many drug-resistant Gram-negative bacteria, including all members of the *Enterobacteriaceae*, *Neisseria gonorrhoeae,* and *Haemophilus ducreyi*. A major function of CpxRA is to alleviate membrane stress by reducing protein traffic through the cell membrane.[8] Activation of CpxRA reduces the expression of virulence factors, which must traverse the cytoplasmic membrane to reach the cell surface. Uncontrolled, genetic activation of CpxRA abolishes the virulence of *Salmonella enterica* serovar Typhimurium in mice.[12] Additionally, genetic activation of CpxRA by deletion of *cpxA* and loss of phosphatase activity abolishes the ability of *H. ducreyi* to cause disease in human volunteers,[13] of *N. gonorrhoeae* to infect mice,[14] and impairs the ability of uropathogenic *Escherichia coli* (UPEC) to cause pyelonephritis in mice.[15] Thus, activation of CpxRA impairs the ability of multiple extracellular and intracellular pathogens to infect their experimental or natural hosts.

Most of what is known about 2CSTS has been produced through genetic manipulation of the pathway constituents or by the development of a few small molecule inhibitors of these systems.[16-17] As such, the development of chemical probes to selectively modulate these systems is a valuable pursuit, as these tools could be used to advance the understanding of these pathways and provide a better picture of therapeutic utility. Recently, compound **1** (**Figure 2**) was shown to inhibit the phosphatase activity of

CpxA, resulting in accumulation of CpxR-P and thus activation of the CpxRA system (**Figure 1**) as measured by a phospho-CpxR sensitive *lacZ* reporter in *E. coli* cells.[18] Herein, we report our initial progress in advancing the structure-activity relationships (SAR) for this 2,3,4,9-tetrahydro-1*H*-carbazol-1-amine scaffold.



Analog	EC_{50} (μM)
1	24.8 ± 1.4
2	30.2 ± 1.1
3	>160

Figure 2. Initial SAR reported on the 2,3,4,9-tetrahydro-1*H*-carbazol-1-amine chemotype.[18]

Results reported in the initial discovery paper indicate that the 6-nitro functionality is not required for CpxA phosphatase inhibition (**2**, **Figure 2**), but that removal of the primary amine abolishes activity (**3**, **Figure 2**). To advance the SAR on this chemotype, we focused on three major approaches to reveal structural motifs important for CpxA inhibition: 1) A-ring substitution, 2) B-ring deconstruction to provide N-arylated amino acid derivatives and 3) C-ring removal to give 2-alkylamino substituted indoles (**Figure 3**). Compounds were initially evaluated for their ability to activate a CpxR-responsive *lacZ* reporter in a modified *E. coli* strain.[18]

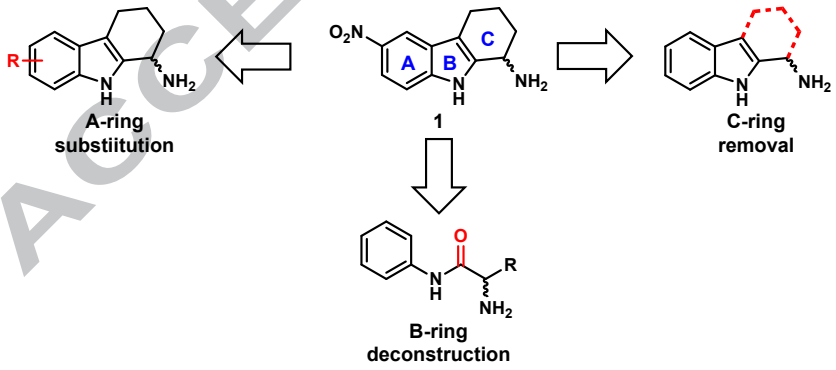
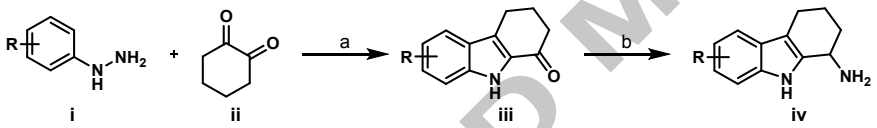


Figure 3. SAR strategy and rationale for the targeted analogs.

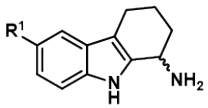
A-Ring Substitution. Due to previous observations that substitution is allowed on the 5-position of the indole and may provide a means to alter the potency of this class, we evaluated a number of

substituents that ranged broadly in size, polarity, and hydrogen bonding capabilities. Analogs were prepared in straight forward fashion via a two-step process employing Fischer-indole methodology[19] followed by reductive amination (**Scheme 1**). As shown in **Figure 4**, the presence of a chlorine or fluorine at the 5-position (**5**-**6**) provided the best activity, favoring the smaller, fluorine (**6**). While incorporation of bromine at this position was also evaluated, evidence of bacterial cytotoxicity and general activation of the stress response in the reporter strain was noted and thus the level of CpxRA activation could not be determined. Installation of a trifluoromethyl group at the 5-position (**7**) also provided equipotency to compound **1**. Incorporation of more electron rich substituents like a methoxy (**8**) or methyl (**9**) group was detrimental to activity. Inclusion of various other resonance mediated electron withdrawing groups such as a methyl ester (**10**), carboxylic acid (**11**), sulfonamide (**12**), or nitrile (**13**) failed to induce reporter activity beyond baseline, indicating no CpxA phosphatase inhibition for these analogs.



Scheme 1. General synthetic approach to **1–13**. Reagents and conditions: (a) 1 M HCl, H_2O, 120 °C, 32-66%; (b) NH_4OAc, $NaCNBH_3$, MeOH, 60 °C, 73-94%.

In hopes of revealing activity trends based on basic physicochemical Hansch parameters (e.g., lipophilicity (π), size (E_s), and electronics (σ_p)), –log (EC_{50}) values were plotted versus individual descriptors. Unfortunately, no obvious linear free energy relationship correlations are observed.

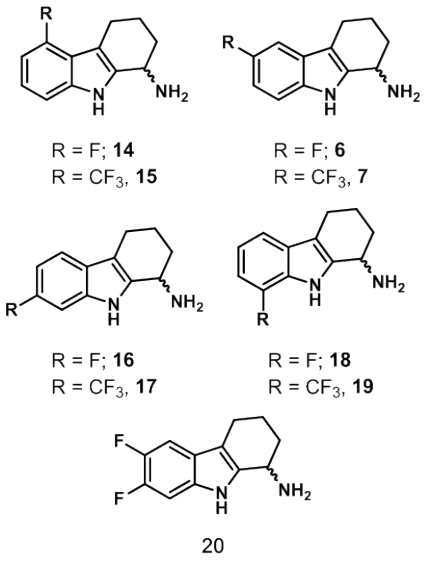


1, $R^1 = NO_2$
2, $R^1 = H$
4, $R^1 = Br$
5, $R^1 = Cl$
6, $R^1 = F$
7, $R^1 = CF_3$
8, $R^1 = OMe$
9, $R^1 = Me$
10, $R^1 = COOMe$
11, $R^1 = COOH$
12, $R^1 = SO_2NH_2$
13, $R^1 = CN$

Analog	EC_{50} (μM)
1	24.8 ± 1.4
2	30.2 ± 1.1
4	ND[a]
5	10.9 ± 3.4
6	7.7 ± 0.2
7	23.1 ± 7.7
8	>100
9	34.1 ± 1.8
10	>100
11	>100
12	>100
13	>100

Figure 4. β-galactosidase activity of A-ring substituted analogs. [a]Evidence of stress response activation due to cytotoxicity. Results are provided as the mean ± SEM of at least three separate experiments performed in triplicate. ND = not determined

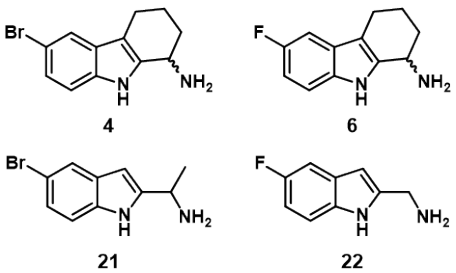
To further the interrogation of A-ring substitution, we performed a "substituent walk" to evaluate the optimal positioning for the fluoro and trifluoromethyl substituents. The observation that the 6-fluoro analog **16** exhibited near equipotency to **6** led us to synthesize and evaluate the 5,6-difluoro analog **20** (**Figure 5**). Gratifyingly, this substituent combination proved to be additive and improved activity ~3-fold from compound **6** and ~5-fold from compound **16** (**Figure 5**). Overall, in comparison to the initial hit **1** and the unsubstituted derivative **2**, compound **20** exhibits ~9-fold and ~11-fold improvements in cellular potency, respectively.



Analog	EC_{50} (μM)
14	65.9 ± 8.6
15	>100[a]
6	7.7 ± 0.2
7	23.1 ± 7.7
16	11.9 ± 0.9
17	ND[b]
18	56.8 ± 10.8
19	>100
20	2.7 ± 0.0

Figure 5. Results of "substituent-walk" around the A-ring. [a]Low levels of agonism did not allow for EC_{50} calculation. [b]General activation of stress response due to cytotoxicity. Results are provided as the mean ± SEM of at least three separate experiments performed in triplicate.

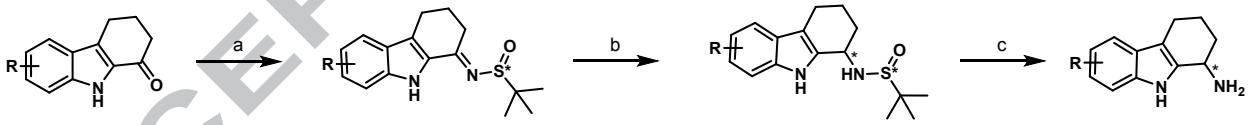
C-Ring Deconstruction. In parallel to the A-ring studies, two derivatives lacking the intact C-ring were assessed for CpxRA agonism and thus provide insight into the importance of this feature. As shown in **Figure 6**, simplification of the 2,3,4,9-tetrahydro-1*H*-carbazol-1-amine scaffold to provide compound **21** resulted in an apparent decrease in bacterial cytotoxicity, as no evidence of cell death was observed at the concentrations evaluated, thus allowing for calculation of an EC_{50} value. . Comparison of **6** and **22**, reveals a 10-fold loss in activity when the C-ring is eliminated. These initial results suggest that the C-ring is not necessary for target engagement, but likely provides beneficial conformational bias that improves ligand-target engagement. It is also worth noting that **21** showed no signs of general activation of the stress response, a complication observed for the related compound, **4**.



Analog	EC_{50} (μM)
4	N.D.[a]
21	44.2 ± 7.7
6	7.7 ± 0.2
22	79.5 ± 6.7

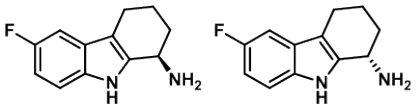
Figure 6. Effects of C-ring deconstruction. Results are provided as the mean ± SEM of at least three separate experiments performed in triplicate.

In addition to C-ring deconstruction, we were interested in probing the requirements associated with the primary amine. As mentioned previously, initial studies revealed the amine to be essential for activity (**Figure 2**). It was not known, however, if stereochemical preference exists at this position and/or if the amino group was simply providing hydrogen bond interactions that could be replenished with different functionalities. To probe these questions, we synthesized **23**-**25** (**Figure 7**). Access to enantiopure amines was achieved through the use of Ellman's sulfinamides as chiral auxillaries to induce stereoselective reductive amination (**Scheme 2**). As shown in **Figure 7**, only one of the two enantiomers is active, with the (*R*)-enantiomer **23** being eutomer and the (S)-enantiomer **24** the distomer.

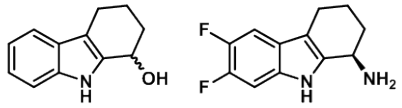


Scheme 2. Synthesis of compounds **23** and **24**. Reagents and conditions: (a) *t*-butanesulfinamide, $Ti(OEt)_4$, toluene, reflux, 84%; (b) $NaBH_4$, THF, -78 °C; (c) HCl▪dioxane, MeOH, 23 °C 99%.

Additionally, replacement of the primary amine with an alcohol produces an analog that is inactive up to 100 μM in the β-galactosidase assay (**25**, **Figure 7**). As demonstrated by **26**, incorporation of the 5,6-difluoroindole with the (*R*) stereocenter produces the same enhancement in activity as noted with the racemic series and provides the most active compound identified in this series to date.



23 24

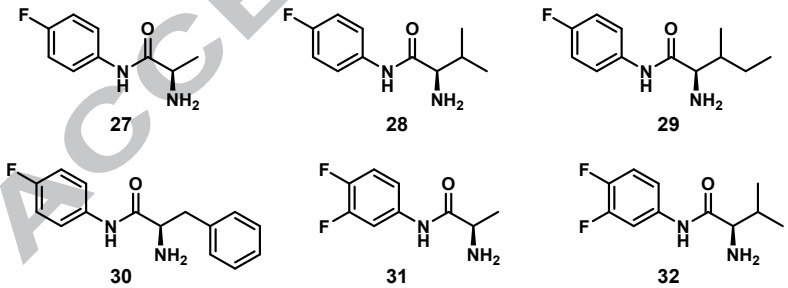


25 26

Analog	EC_{50} (μM)
23	3.5 ± 0.2
24	>100
25	>100
26	1.1 ± 0.1

Figure 7. Assessment of amine substitution and stereochemical requirements. Results are provided as the mean ± SEM of at least three separate experiments performed in triplicate.

Considering the seemingly dispensable C-ring, the requirement for a primary amine, and necessity of (*R*) stereochemistry, we were intrigued by the possibility of simplifying this scaffold even further through the deconstruction of the B-ring to arrive at more synthetically tractable N-arylated amino acids. As shown in **Figure 3** this chemotype keeps the requisite A-ring and maintains the sp^2 hybridization of the carbon alpha to the "indole" nitrogen. The resulting amide is expected to adopt the more energetically favored *trans* geometry,[20] thus providing a reasonable isostere for the parent indole, while alleviating the rigidity of the parent heterocycle. Unfortunately, all analogs synthesized in this series (**Figure 8**) were inactive at concentrations up to 80 μM.



27 28 29

30 31 32

Figure 8. *seco*-amide analogs synthesized and assessed for CpxRA modulation.

As mentioned previously, **1** is believed to indirectly activate the CpxRA 2CSTS through the inhibition of CpxA phosphatase activity, thus rendering CpxR phosphorylated for an indefinite period of time.[18] To confirm whether the results of the *lacZ* reporter assay observed for the more potent derivatives

were consistent with mechanisms reported for **1**, we evaluated the ability of **23** to inhibit CpxA phosphatase activity through Phos-Tag methodology, gel visualization, and densitometry quantification of CpxR phosphorylation levels, exactly as described.[18] As is seen **Figure 9** and as reported previously,[18] both the Δ*cpxA* mutant and the compound-treated wild type expressed more CpxR than the untreated wild type, likely because CpxR-P positively autoregulates its transcription. Similar to compound **1**, treatment with compound **23** (10 uM) caused a statistically significant increase in the ratio of CpxR-P to CpxR relative to the untreated wild type and the Δ*cpxA* mutant. In contrast, activation in the Δ*cpxA* mutant increased the accumulation of CpxR-P but did not cause a statistically significant increase in the ratio of CpxR-P to CpxR compared to the wild type. Taken together, the data suggest that pharmacological activation of CpxRA by inhibition of CpxA phosphatase activity yields different results than activation via deletion of *cpxA*, perhaps because CpxA kinase activity is retained in the pharmacological approach.

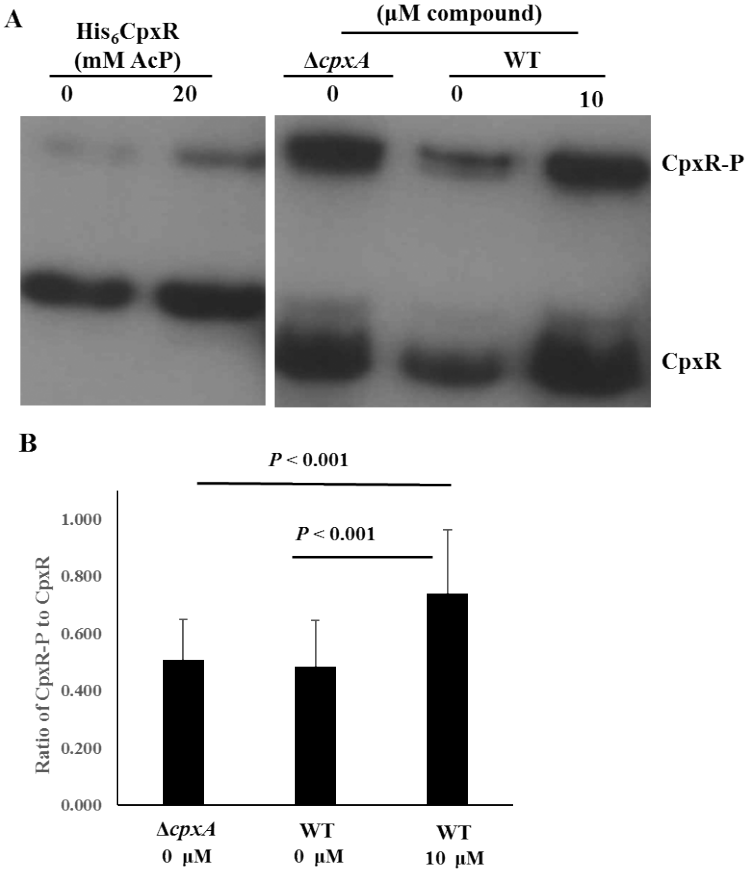


Figure 9. Compound **23** induces phospho-CpxR accumulation. A) Composite representative Western blot of His_6CpxR incubated with 0 or 20 mM AcP, and the Δ*cpxA* mutant and WT grown in media with 0.4% glucose and 0 or 10 μM compound. Note that

migration of His_6CpxR is slower than native CpxR. B) The ratio CpxR-P to CpxR was determined using densitometry; average and standard deviation are from 5 independent experiments. P-values were calculated with one-way ANOVA.

The dependence of compound activity on the stereochemistry of the primary amine is indicative of interaction with a 3-dimensional binding pocket within CpxA. Additionally, our SAR indicates that the amine is essential for activity. In light of the recent work by the Hergenrother lab[21] that highlights the importance of primary amines in porin mediated transport, we were curious if the primary amine was also involved in permeation and/or accumulation of the 2,3,4,9-tetrahydro-1*H*-carbazol-1-amine chemotype in Gram-negative bacteria. To evaluate this, we developed a mass spectrometry method to determine the accumulation of compounds **6** (a derivative from our studies) and **33** (a commercially available *des*-amino comparator) in isogenic strains of *E. coli* with different efflux efficiencies and controlled permeability of the outer membrane (**Figure 10**). WT-Pore cells contain the wild-type repertoire of efflux pumps and the arabinose-controlled large pore that when expressed, permeabilizes the outer membrane to antibiotics as

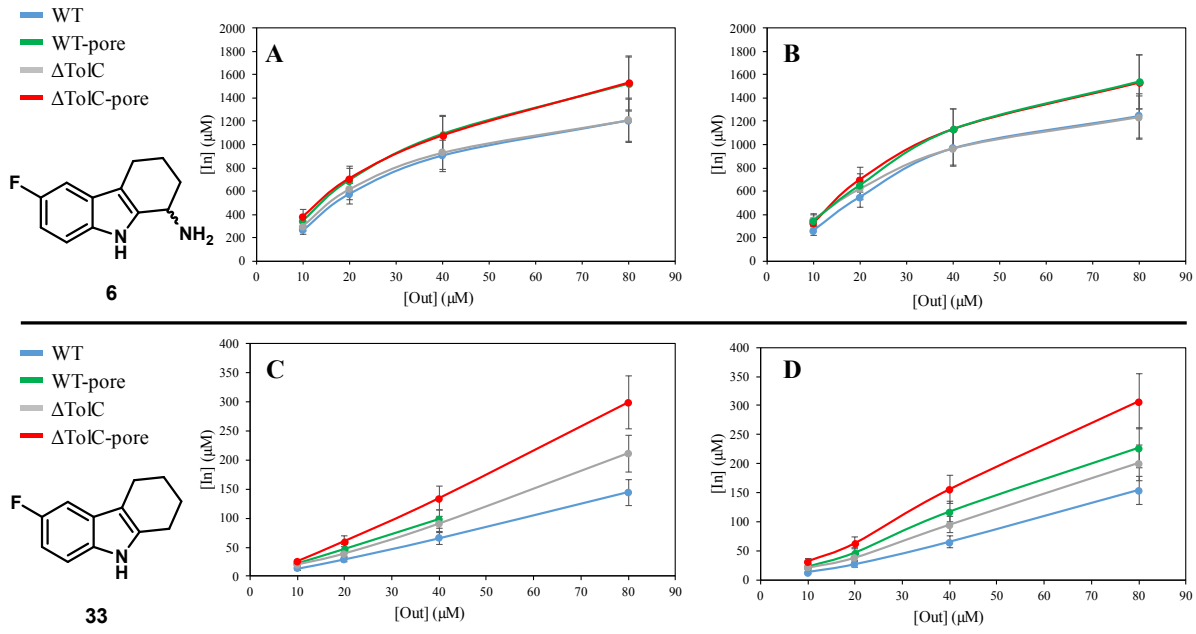


Figure 10. Intracellular uptake of compounds **6** and **33** into isogenic *E. coli* strains that exhibit different efflux efficiencies and controlled permeability of the outer membrane. WT = *E.coli* BW25113; WT-Pore = BW25113 *att*Tn7:: *att*Tn7::mini-Tn7T-Km^{rr} -*araC*-P_{araBAD}-*fhuAΔCΔ4L* ; ΔTolC = BW25113 Δ*tolC-ygiBC*; ΔTolC-Pore = ΔTolC *att*Tn7::mini-Tn7T-Km^{r} -*araC*-P_{araBAD}-*fhuAΔCΔ4L*.[22]

large as vancomycin.[22] The ΔTolC cells are efflux-deficient derivatives lacking the outer membrane channel TolC required for the activity of various efflux pumps of *E. coli.* Cells with intact and hyperporinated (induced) outer membranes were incubated with increasing concentrations of compounds **6** and **33** and the intracellular accumulation was analyzed after 1 and 40 minutes of incubation with compounds. As seen in **Figure 10**, compound **33**, which lacks the primary amine, is susceptible to efflux and suffers from poor permeability across the outer membrane. Compound **6** on the other hand contains the primary amine, accumulates at much higher intracellular levels overall, is still sensitive to efflux, albeit to a lesser extent, and accumulates less in wild-type than in efflux deficient *E. coli*. Additionally, **6** accumulates equally well in the cells with intact and hyperporinated outer membrane. These results clearly demonstrate that the primary amine is not only involved in target engagement but is also imperative to the permeation and accumulation in a Gram-negative organism.

Given that conventional approaches to develop new antibiotics have yielded few new drugs, the potential reward of exploring novel targets and mechanisms is very high. Bacterial 2CSTS are major players in organismal pathogenicity, fitness, communication, biofilm formation, and antibacterial resistance making them attractive targets. Unfortunately, most of what we know about these systems has been revealed through genetic manipulation or small molecule inhibitors of such systems, which is not always indicative of therapeutic potential. As such, there is an increasing interest in developing small molecule probes that can be used to activate these systems to uncover new biology that may translate to new antibacterial approaches. Along these lines, we aimed to improve the activity of **1**, a newly identified CpxA phosphatase inhibitor that indirectly activates the CpxRA signaling pathway through increasing the lifetime of CpxR-P. Leveraging three different structural modification approaches, we identified compound **26** (EC_{50} = 1.1 μM), which exhibits ~30-fold improvement in CpxA phosphatase inhibition over the initial hit **1** as determined in a cellular *lacZ* reporter assay. The stereochemical and conformational requirements found for the primary amine of this chemotype illustrates its importance in target engagement and involvement in membrane permeation and protection against efflux in *E. coli*. Studies are ongoing to determine the CpxRA selectivity (or lack thereof) of this chemotype and to assess

its efficacy in animal models of infection. Nonetheless, this work provides useful SAR and a foundation for which to advance the utility of 2,3,4,9-tetrahydro-1*H*-carbazol-1-amines as chemical probes to uncover new biology in bacterial two-component signal transduction systems.

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SUPPLEMENTARY DATA

Supplemental data [synthetic procedures, biological methods, and characterization information for new compounds **23**, **24**, and **26**] to this article can be found online at xxxxxx.

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Table of Contents Graphic

First-generation structure-activity relationship studies of 2,3,4,9-tetrahydro-1*H*-carbazol-1-amines as CpxA phosphatase inhibitors

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