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Propargylglycine-based antimicrobial compounds are targets of TolC-dependent efflux systems in *Escherichia coli*

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Abstract: A library of novel L-propargylglycine-based compounds were designed and synthesized with the goal of inhibiting the growth of Gram-negative bacteria by targeting LpxC, a highly conserved Gram-negative enzyme which performs an essential step in the lipid A biosynthetic pathway. These compounds were designed with and without a nucleoside and had varying tail structures, which modulate their lipophilicity. The synthetic scheme was improved compared to previous methods: a methyl ester intermediate was converted to a hydroxamic acid which obviated the need for a THP protecting group and improved the yields and purity of the final compounds. Antimicrobial activity was observed for non-nucleoside compounds containing a phenyl propargyl ether tail (**5**) or a biphenyl tail (**6**). An MIC of 16 µg/mL was achieved for **6** in *Escherichia coli*, but inhibition was only possible in the absence of TolC-mediated efflux. Compound **5** had an initial MIC >160 µg/mL in *E. coli*. Enhancing outer membrane permeability or eliminating efflux reduced the MIC modestly to 100 µg/mL and 80 µg/mL, respectively. These results highlight the importance of hydrophobicity of this class of compounds in developing LpxC inhibitors, as well as the design challenge of avoiding multidrug efflux activity.

Keywords: L-propargylglycine; lipopolysaccharide; tolC; antimicrobial; efflux pumps

Incidences of multidrug-resistant pathogens have increased on a global basis, with some bacterial strains now exhibiting resistance to even last-resort antibiotics.[1-9] In the US alone, at least 2 million cases of multidrug resistant bacterial infections occur each year, resulting in approximately 23,000 deaths per annum.[10] Development of new antimicrobials that are not subject to existing mechanisms of resistance is an important goal of current research.

Lipopolysaccharides (LPS) are an important structural component of the Gram-negative outer membrane, creating both a physical and charge barrier to permeability by a wide variety of compounds and antibiotics.[11-15] Each LPS molecule consists of three sections: an O-antigen, a core oligosaccharide, and a lipid A anchor embedded in the membrane. UDP-(3-*O*-((R)-3-hydroxymyristoyl))-*N*-acetylglucosamine deacetylase, or LpxC, is responsible for the first committed step of lipid A synthesis and is essential in *Escherichia coli*.[16-18] Inhibition of LpxC prevents LPS formation and enhances antibiotic sensitivity.[19-21] LpxC is conserved across Gram-negative phyla and shares no homology with mammalian enzymes, making it an attractive target for antimicrobial treatment.[22-24]

Our analysis of the crystal structure of the *Aquifex aeolicus* LpxC active site[25] found three target areas for ligand binding: a Zn^{2+} ion, a polar region, and a hydrophobic passage (Figure 1).[26-27] The LpxC natural substrate consists of a nucleoside, a diphosphate group, a glucosamine, and a long hydrocarbon tail. The strongest potential interactions can occur with the Zn^{2+} ion via ionic binding.[28] Among the LpxC inhibitors found in literature all share a hydroxamate head group (replacing the diphosphate group of the natural substrate), which binds strongly to the Zn^{2+} ion[29] forming a thermodynamically favorable five-membered ring.[30] Most inhibitor design has focused on optimizing the tail moiety, and few inhibitors include the nucleoside for binding in the polar region.[31-32] In this work, potential inhibitors of LpxC capable of targeting two or more regions in the LpxC active site were designed. The structures differ in inclusion of a nucleoside and in type of hydrophobic tail (Figure 2).

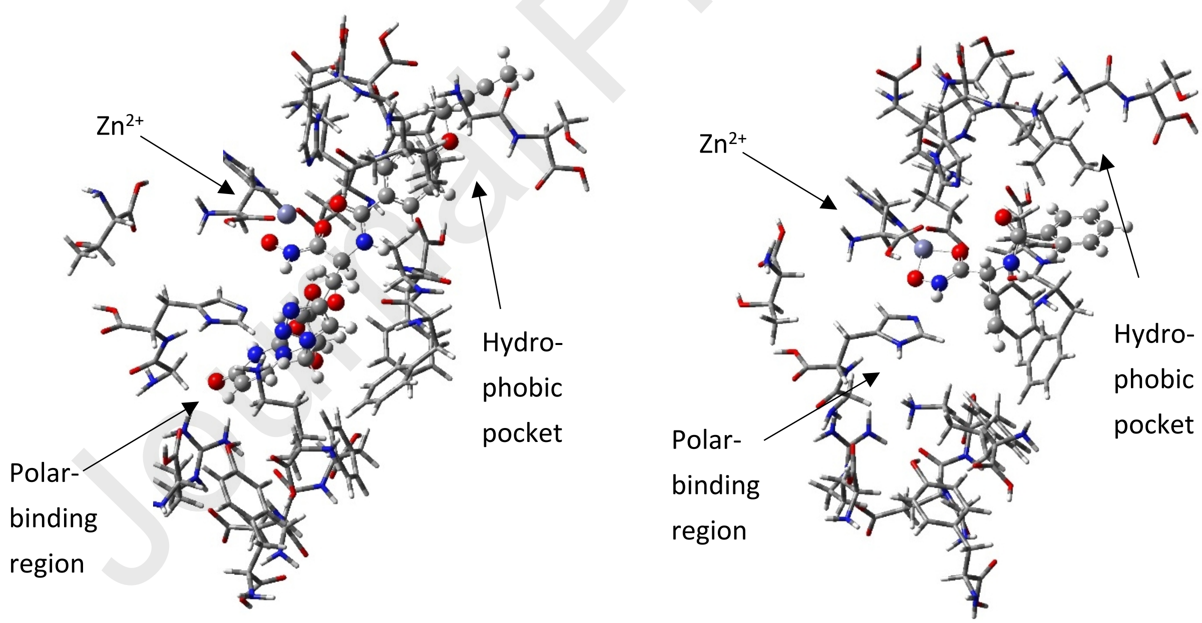


Figure 1. (Left) Compound **2** (ball and stick) optimized in the *A. aeolicus* LpxC active site (M062X/6-31G) showing the molecule interacting with the binding site via the polar binding region, the Zn^{2+} ion, and the hydrophobic pocket. (Right) Compound **4** (ball and stick) optimized in the *A. aeolicus* LpxC active site (M062X/6-31G) showing the molecule interacting with the binding site via the Zn^{2+} ion, and part of the hydrophobic pocket.[27]

Efficacy of antimicrobial compounds depends not only on their ability to bind and inhibit a specific molecular target, but also to accumulate within bacterial cells. Sufficiently lipophilic compounds can diffuse across the outer and inner membranes while other compounds enter bacterial cells via channels and porins that are selective according to size and other properties such as hydrophilicity or charge.[33-34] Once inside the cell, many antimicrobial compounds are the target of various efflux systems including those from the resistance-nodulation-division (RND), major facilitator (MF), ATP-binding cassette (ABC), small multidrug resistance (SMR) and multidrug and toxic compound extrusion (MATE) families. Of these groups, the RND family transporters have the widest range of substrates and account for the majority of intrinsic resistance in Gram negative bacteria.[15] It remains challenging to predict whether a compound will be targeted for efflux by RND transporters but transport does correlate positively with hydrophobicity.[15, 35]

Herein, we report the synthesis of a new nucleoside-containing compound **2** and the design and synthesis of novel propargylglycine-based compounds designed to inhibit LpxC activity (**4-6**, Figure 2). Additionally, a new synthetic procedure, which obviated the need for the THP-protecting group, was implemented to reduce the number of steps and improve the yield of the final hydroxamic acids. Finally, the minimal inhibitory concentrations (MICs) for compounds **1**-**6** were established for *P. aeruginosa* and *E. coli*. Improved antibacterial activity was observed in *E. coli* strains with either increased permeability or lacking TolC-dependent efflux.

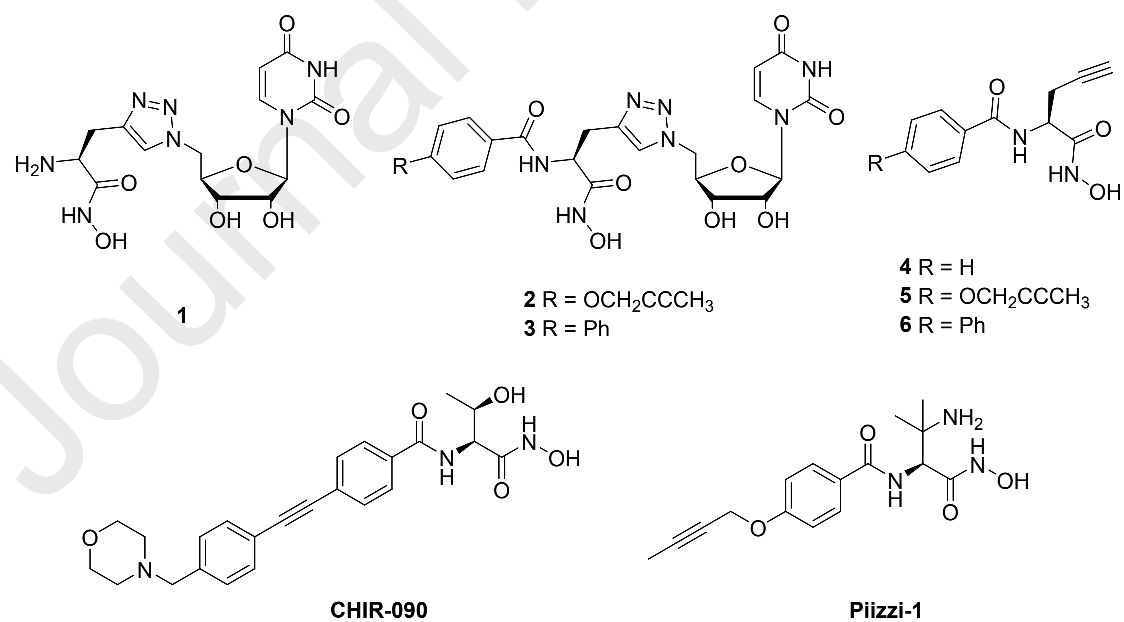


Figure 2. The structures of the propargylglycine-based compounds **1-6**, **CHIR-090** and **Piizzi-1**.

Being informed by our previous computational work,[26] we focus here on synthesizing and testing antimicrobial activity of promising compounds. Compound **1**, which does not contain a hydrophobic tail, had the most negative interaction energy at -710 kcal/mol in a solvated model.[26] However, as many key human enzymes, such as matrix metalloproteinases and zinc-dependent histone deacetylases, contain Zn^{2+} ions in their active sites, the inclusion of the hydrophobic tail should provide greater specificity for LpxC.

Incorporation of a hydrophobic tail to bind in the active site offers both design and synthetic challenges. The amino acid sequence in these tunnels differs across many Gram-negative strains, resulting in varying passage shape.[36] In order to achieve broad spectrum activity against different Gram-negative species, the tail must be able to fit within a variety of hydrophobic passages and resolve any differences. Many research groups have found that more linear tails can account for slight variations in the hydrophobic tunnel, resulting in a wide variety of tails with aromatic groups bound by acetylene or diacetylene.[37] However, many inhibitors including **CHIR-090** (Figure 2) showed low aqueous solubility as well as *in vitro* cytotoxicity.[37] In place of the diacetylene and acetylene tails, a flexible propargyl ether group was added which could specifically inhibit *P. aeruginosa* LpxC.[38] **Piizzi-1** (Figure 2), with a propargyl ether tail, showed an IC_{50} value of 0.004 μM against wild-type *P. aeruginosa* LpxC and MIC_{90} of 2 μg/mL in multidrug resistant (MDR) *P. aeruginosa*, as well as good aqueous solubility with no cytotoxic effects.[38] Due to this success, the propargyl ether tail was incorporated into our inhibitors.

The incorporation of a tail moiety allows for further modulation of the lipophilicity and solubility of the compounds, which can be observed in the calculated log P values (Table 1), giving a relative idea of the hydrophobicity of the compounds. The log P and ClogP of **CHIR-090** and **Piizzi-1** were also determined as a comparison. Compound **1**, which does not contain a hydrophobic tail, has the most negative log P value. The other nucleoside-containing compounds (**2** and **3**) are still fairly hydrophilic, despite compound **3** containing the biphenyl tail group. Although the nucleoside provides additional points for interaction in the active site, the polarity of the nucleoside decreases the relative lipophilicity, possibly providing challenges for getting into the cell and also potentially having a larger desolvation penalty when binding in the active site. Therefore, we decided to also investigate compounds that lack the nucleoside. Compounds **4**-**6**, with just the propargylglycine scaffold and various tails, have higher log P values and are more lipophilic.

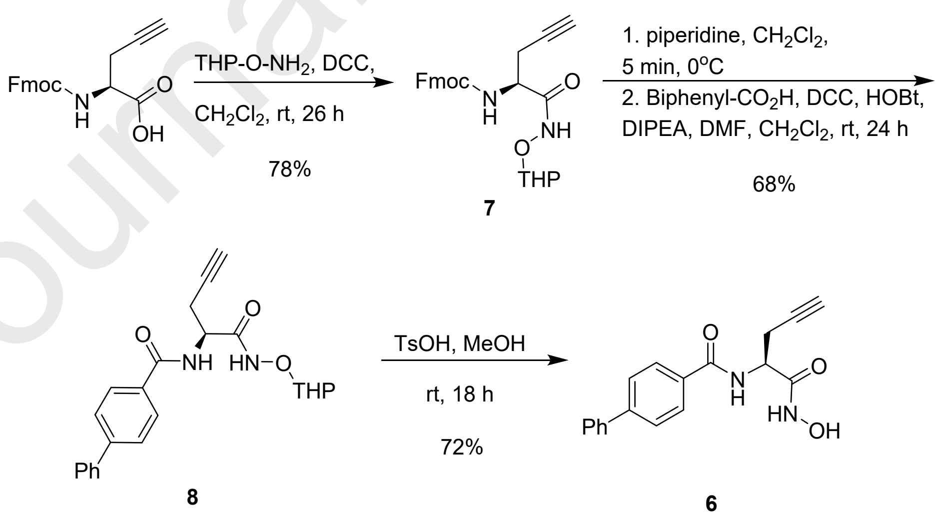
Table 1. LogP and CLogP values[1] of synthesized compounds

Compound	Log P	ClogP
Piizzi-1	0.44	0.651
CHIR-090	1.40	1.412
1	-3.52	-5.683
2	-1.69	-2.921
3	-0.01	-2.183
4	0.61	-0.227
5	1.29	0.923
6	2.28	1.661

[1] Values taken from ChemDraw Professional 18.2.

Compound **6** was initially prepared using an intermediate in the synthesis of compound **3**. Commercially available Fmoc-L-propargylglycine was converted from a carboxylic acid to a tetrahydropyran (THP)-protected hydroxamic acid in the presence of N,N'-dicyclohexylcarbodiimide (DCC) coupling agent to afford the alkyne **7** in a 78% yield.[26] The Fmoc group was then removed using piperidine and biphenyl carboxylic acid was immediately coupled using DCC to afford **8** in a 68% yield (Scheme 1). Final deprotection of the THP-protected hydroxamic acid **8** was accomplished using *p*-toluenesulfonic acid[39] to yield the final product **6** in a 72% yield (Scheme 1). However, due to difficulty in purification resulting from low solubility of **6**, this last step underwent several attempts at optimization, but did not give improved results.

Scheme 1

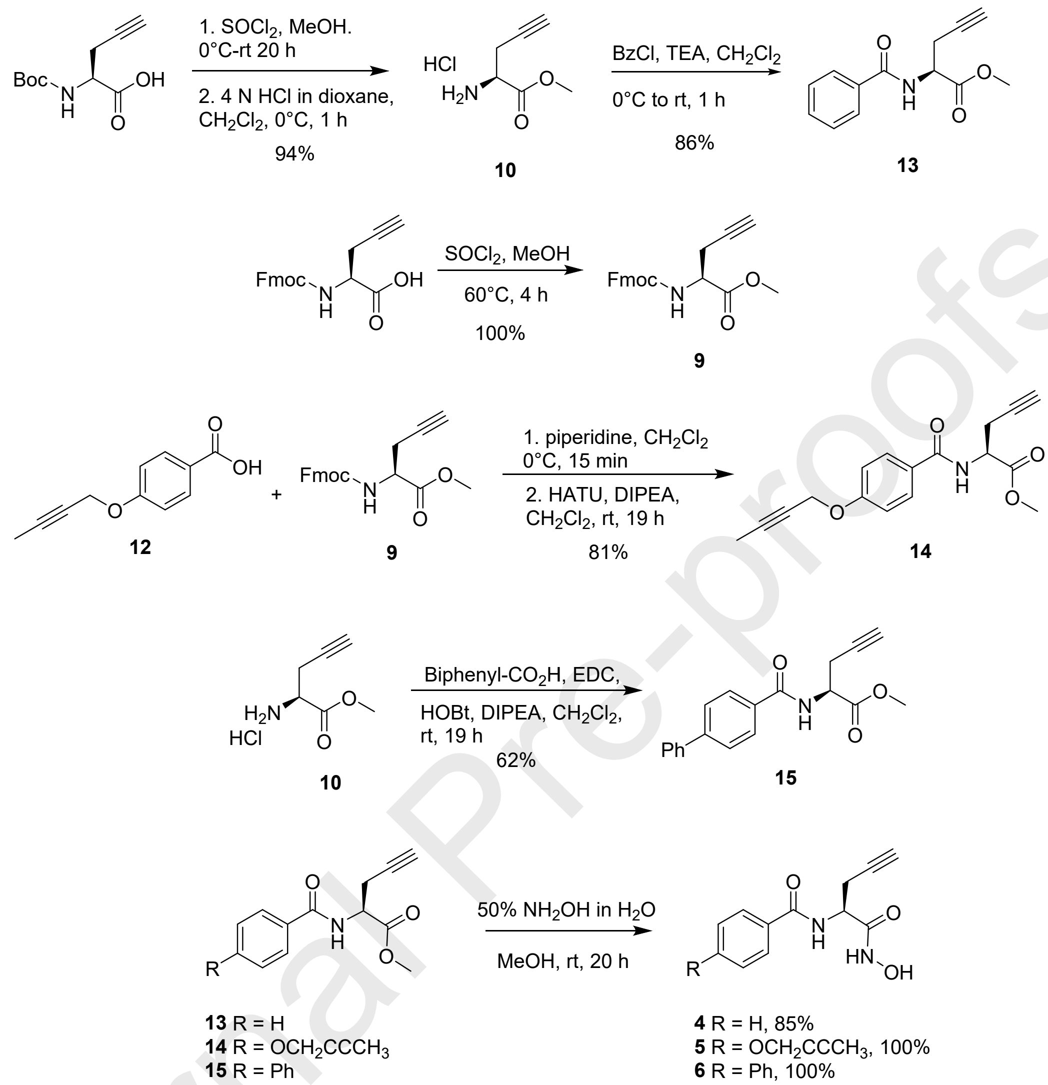


Up until this point, we had routinely used a THP-protecting group after converting the carboxylic acid to a protected hydroxamic acid to result in the alkyne derivative.[26] However, the removal of the THPprotection and purification has proven problematic, and therefore, a new synthetic scheme was created with the aim of affording the coupled product in higher yields. An approach involving converting the carboxylic acid first to a methyl ester and then converting to the hydroxamic acid in the last step of synthesis was attempted.[38, 40] In the interest of pursuing this method, Fmoc-L-propargylglycine was esterified using thionyl chloride in methanol[41] to form methyl ester **9** in a 100% yield (Scheme 2). A similar method was also employed with Boc-L-propargylglycine to afford **10** in a 94% yield. Although the Boc group was not observed in the ^{1}H and ^{13}C after esterification, HCl in dioxane was added to ensure the hydrochloride salt was available for coupling.

Compound **10** was coupled with benzoyl chloride to give alkyne **13** in an 86% yield (Scheme 2), which was significantly better than the yield obtained with the Fmoc deprotection and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/DCC coupling previously employed. The propargyl ether benzoic acid derivative **12** was synthesized as previously described by Piizzi and coworkers.[38] EDC and HATU (1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate) were explored to synthesize alkyne **14** (Scheme 2). Following Fmoc deprotection of methyl ester **9** with piperidine, acid **12** was coupled to afford alkyne **14**. The EDC procedure had been previously used by our lab, and successfully produced alkyne **14** in a 57% yield.[26] However, the HATU coupling procedure was even more successful, with alkyne **14** afforded in an 81% yield. The synthesis was carried out this way since **14** was already available, but in the future any new syntheses should start with Boc-L-propargylglycine. The biphenyl analogue **15** was also prepared using compound **10**, biphenyl carboxylic acid and EDC coupling to produce **15** in a 62% yield.

The coupled methyl esters **13**-**15** were easily converted to hydroxamic acids **4**-**6** through treatment with aqueous hydroxylamine[38] in 85-100% yields (Scheme 2). This method resulted in significantly higher yields and avoided the need for column chromatography purification. The structures of alkynes **4**-**6** were confirmed through ^{1}H NMR and ^{13}C NMR and by HR-MS. The purity was confirmed by HPLC.

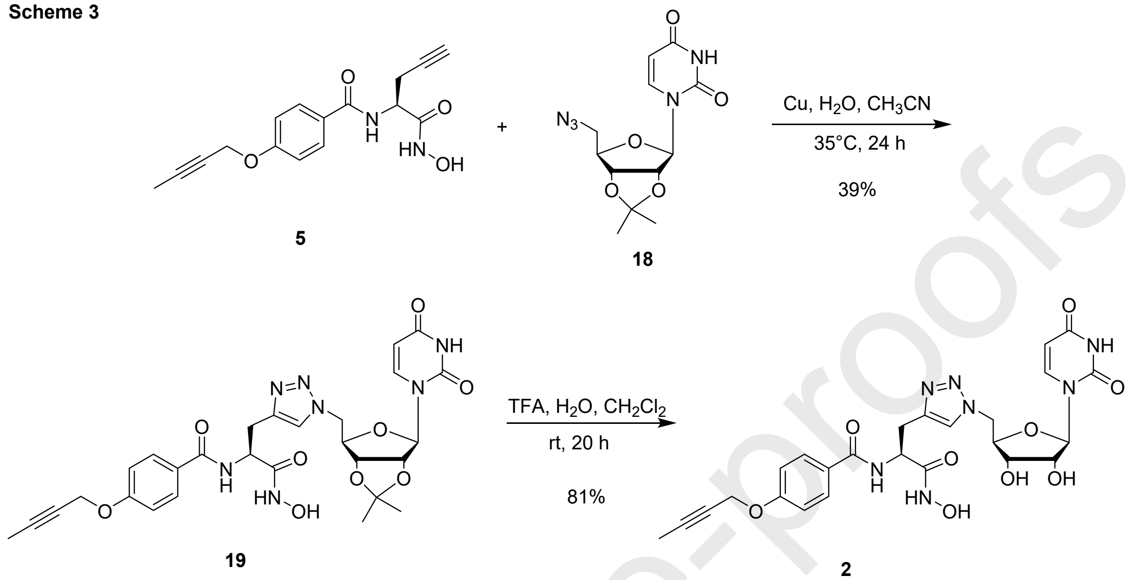
Scheme 2



Copper-catalyzed azide-alkyne cycloaddition (CuAAC) was used to synthesize triazole-linked compounds **1** and **3** with yields from 57-76% as previously described.[26] Similar methods were utilized in the synthesis of **2** (Scheme 3)**.** Synthesis of the azidonucleoside **18** was accomplished as described previously.[26] Azide **18** and alkyne **5** were dissolved in acetonitrile, after which copper powder was added to the reaction flask. Following sonication, the reaction ran for 24 hours at 35°C to afford **19** in 39% yield, which was significantly lower than yields previously obtained with the other nucleoside analogues. This reduced yield may be in part due to the presence of the hydroxamic acid.

After purification by column chromatography, compound **19** was treated with trifluoroacetic acid (TFA) in order to remove the ketal protecting group. The final compound was obtained through precipitation from a methanolic solution with diethyl ether. Compound **2** was isolated in a 81% yield, which was slightly better than literature yields of 65-70% for compounds **1** and **3** (Scheme 3).[26] Synthesis of **2** was confirmed with ^{1}H NMR and ^{13}C NMR and HR-MS. The purity was confirmed by HPLC.

Scheme 3



Purified compounds were dissolved in DMSO and the standard microbroth dilution method was used to determine the minimal inhibitory concentration (MIC) for each compound in the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*.[42] The results, which were consistent across four independent trials, are summarized in Table 2. Compounds **1**-**4** failed to show any growth inhibition for either *P. aeruginosa* or *E. coli* compared to the 1% DMSO control (Supplementary Figures 1 and 2A). Failure to inhibit growth could result from lack of activity against the intended target, but it could also be due to a low cell permeability or intrinsic resistance via multidrug efflux systems. To investigate these possibilities, three *E. coli* strains were generated.

Table 2. MICs for compounds **1-6**.

Compound	*P. aeruginosa*	*E. coli*	*E. coli* ΔCΔ4LFhuA	*E. coli* Δ*acrB*	*E. coli* Δ*tolC*
1	ND[1]	ND	ND	ND	ND
2	ND	ND	ND	ND	ND
3	ND	ND	ND	ND	ND
4	ND	ND	ND	ND	ND
5	100 µg/mL	>160 µg/mL	100 µg/mL	80 µg/mL	80 µg/mL
6	ND	ND	ND	32 µg/mL	16 µg/mL

[1] ND indicates than inhibition was not observed and an MIC was not able to be determined.

A hyperpermeable strain was constructed by creating plasmid pEF107 to constitutively express a modified version of the FhuA outer membrane porin (ΔCΔ4LFhuA) that was previously shown to enhance sensitivity to antibiotics that otherwise exhibit low rates of permeability.[43] This constitutive expression plasmid does not require antibiotic selection for stable maintenance, thus eliminating the concern that presence of either an inducer or a second antimicrobial compound will impact the observed MICs. The Δ*acrB* strain lacks function of the AcrAB-TolC multi-drug efflux system, which is responsible for the majority of efflux-based resistance in *E. coli*.[44-47] The Δ*tolC* strain lacks the outer membrane protein utilized by resistance-nodulation- cell division (RND) family efflux pumps and therefore eliminates the function of all multidrug efflux systems in this family.[44, 48-49] Neither the hyperpermeable strain (Supplementary Figure 2B) nor the efflux mutants (Supplementary Figure 2C and 2D) showed any difference in sensitivity to compounds **1-4**. Though direct interactions of these compounds with LpxC were not determined, the failure to inhibit bacterial growth suggests that the nucleoside-based structures **1-3** are not effective inhibitors of LpxC as modeling suggested, nor does the small tail structure of compound **4** result in antimicrobial activity despite its increased hydrophobicity relative to compounds **1-3**.

For *P. aeruginosa*, the MIC for compound **5** was 100 µg/mL (Figure 3). For *E. coli*, compound **5** inhibited bacterial growth but it was not possible to determine an MIC (Figure 4A). Despite sharing 57.4% sequence identity and 79.7% sequence similarity for LpxC, there are modest differences between the structures of the *P. aeruginosa* and *E. coli* enzymes that could account for this difference.[36] It is also possible that differences in outer membrane permeability or efflux activity between these species account for the observed difference. In the *E. coli* strain expressing ΔCΔ4LFhuA, the MIC for compound **5** was 100 µg/mL (Figure 4B) while in the Δ*acrB* and Δ*tolC* efflux mutants, the MIC was modestly reduced to 80 µg/mL (Figure 4C and 4D). From these results we conclude that the intermediate hydrophobicity of compound **5** limited its permeability across the *E. coli* outer membrane but was still sufficient for compound that did enter to be targeted by the AcrAB-TolC efflux pump. When permeability was enhanced or efflux eliminated, compound **5** was able to prevent *E. coli* growth.

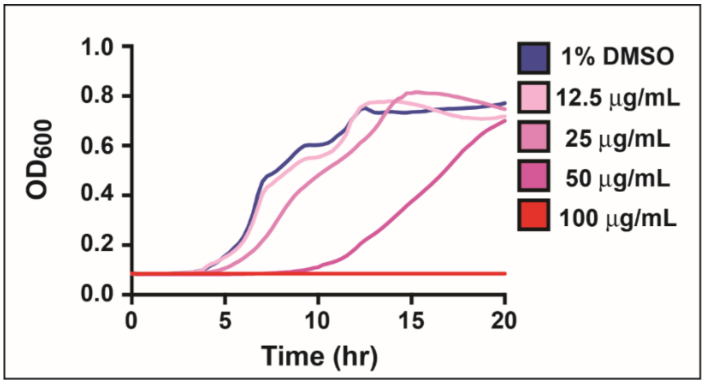


Figure 3. Compound 5 completely inhibits growth of *P. aeruginosa* at 100 µg/mL. Compound **5** delays growth of *P. aeruginosa* at 25 µg/mL (pink) and 50 µg/mL (dark pink) compared to 1% DMSO alone (blue) with complete inhibition at 100 µg/mL (red).

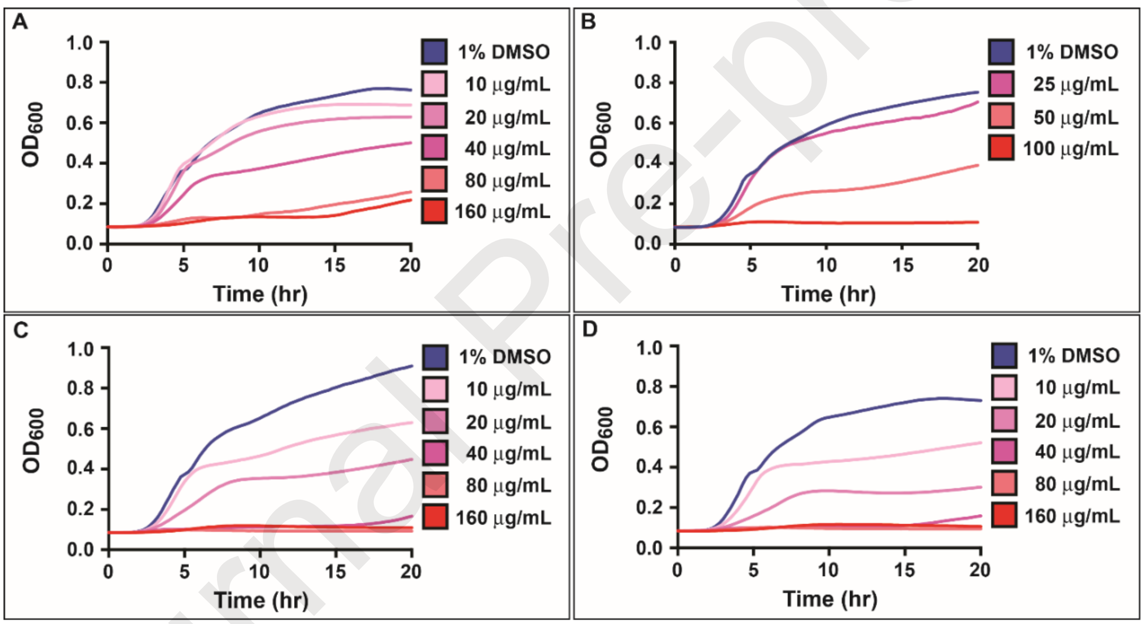


Figure 4. Compound 5 completely inhibits growth of *E. coli* when outer membrane permeability is enhanced or multidrug efflux eliminated. A) Compound **5** inhibits growth of *E. coli* in a dose dependent manner compared to 1% DMSO alone (blue) but a minor amount of growth is still observed at 160 µg/mL (red). B) Expression of the engineered outer membrane porin ΔCΔ4LFhuA results in complete inhibition of growth by compound **5** at 100 µg/mL (red). C) The Δ*acrB* strain which lacks function of the AcrAB-TolC multidrug efflux system is sensitive to compound **5** with complete inhibition of growth occurring at 80 µg/mL (orange). D) The Δ*tolC* mutant which lacks function of all RND-family efflux systems is no more sensitive to compound **5** than the Δ*acrB* strain and also exhibits complete inhibition of growth at 80 µg/mL (orange).

For compound **6**, no MIC could initially be established for either *P. aeruginosa* (Supplementary Figure 5) or *E. coli* (Figure 5A). Similarly, no MIC could be determined in the hyperpermeable *E. coli* strain expressing ΔCΔ4LFhuA (Figure 5B). It was possible to determine an MIC of 32 µg/mL in the Δ*acrB* strain

(Figure 5C) and 16 μg/mL in the Δ*tolC* strain (Figure 5D). Expressing ΔCΔ4LFhuA in combination with either the Δ*acrB* or Δ*tolC* mutation did not further alter the MICs (data not shown). These results demonstrate that compound **6**, the most hydrophobic compound tested, was not limited in its ability to enter *E. coli* cells but rather lack of inhibition was due to the activity of multiple drug efflux systems. The AcrAB-TolC multidrug efflux system was responsible for the majority of the intrinsic resistance of *E. coli* to this compound. However, compound **6** was a substrate for other RND-family efflux systems as well since deletion of *tolC* lead to a further 2-fold decrease in MIC. It is likely that we were unable to observe inhibition of *P. aeruginosa* growth by compound **6** due to similar efflux mechanisms in this organism. Intrinsic antibiotic resistance in *P. aeruginosa* is mediated primarily by the MexAB-OprM efflux system, which is also an RND-family efflux pump with a broad substrate range though, as in *E. coli*, multiple efflux systems are present in the genome.[50-53]

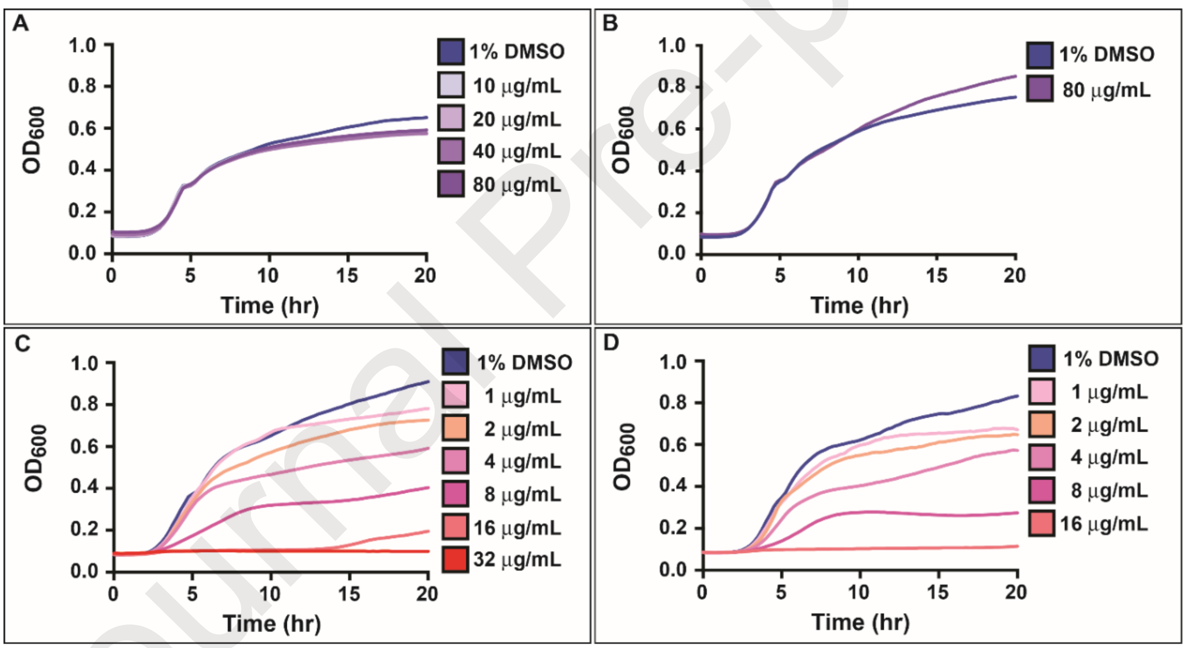


Figure 5. Compound 6 completely inhibits growth of *E. coli* when multidrug efflux is eliminated. A) Compound **6** fails to inhibit growth of *E. coli* compared to 1% DMSO alone (blue), at the solubility limit of 80 μg/mL (darkest purple). B) Expression of ΔCΔ4LFhuA does not result in growth inhibition by 80 μg/mL compound **6** (purple) compared to 1% DMSO (blue). C) A Δ*acrB* mutant lacking function of the AcrAB-TolC multidrug efflux system is inhibited by compound **6** in a dose-dependent manner compared to 1% DMSO (blue) with complete inhibition at 32 μg/mL (red). D) A Δ*tolC* mutant lacking function of all RND-family efflux systems displays increased sensitivity to compound **6** with complete inhibition at 16 μg/mL.

It is notable that both compounds **5** and **6** demonstrated some degree of bacterial growth inhibition, while compound **4** did not. While its lower hydrophobicity may have reduced its ability to enter cells, the relatively small size of this compound should allow it to readily enter the ΔCΔ4LFhuA-expressing strain. Lack antimicrobial activity by compound **4** in this genetic background suggests that a larger hydrophobic tail may be required to mediate the molecular interaction, presumably with LpxC, that results in growth inhibition. This is corroborated by the modest inhibition seen with the slightly larger tail of **5** and greatest inhibition with the largest and most hydrophobic tail of **6**. This effect may also be explained by the entropic contributions to the Gibbs energy of binding. As has been mentioned, the active site has a large polar region as well as a metal-binding region. This suggests that waters bound in this active site before ligand binding are held tightly and a large desolvation energy penalty is paid when the ligands bind, greatly reducing the enthalpic contribution to the Gibbs energy of binding.[27] The entropic contribution is thus proportionately more important. When comparing **4** with **5** and **6**, we see that the latter two have considerably more degrees of freedom in the hydrophobic tail, and thus more entropy that is lost upon binding (assuming that the hydrophobic tail assumes a somewhat rigid position upon binding). This will be explored further in future computational work.

Unfortunately, the increased hydrophobicity also seems to potentiate efflux. Little structural similarity has been observed among known substrates of RND efflux systems but one common feature appears to be hydrophobicity, with more hydrophobic compounds exhibiting increased efflux.[35, 54] Development of AcrAB-TolC inhibitors is an area of significant interest since they would function as adjuvants for a range of antimicrobial compounds but none of these inhibitors has yet entered clinical trials.[55-61] Future work must focus on developing structures that are effective inhibitors of LpxC without being targeted as substrates for efflux.

Inhibition of bacterial growth by compounds designed to target LpxC activity is a promising method of antibacterial treatment that may be useful against multidrug resistant bacteria. We investigated the use of nucleoside-containing compounds **1**-**3** designed to mimic the natural substrate of LpxC and interact with the polar region of the binding site, the Zn^{2+} ion and the hydrophobic region (in **2** and **3**). Although the nucleoside provides additional points for interaction, based on modeling, it also decreases the log P of the potential inhibitors. Therefore, we sought to increase the lipophilicity of the potential inhibitors and removed the nucleoside and subsequently increased the lipophilicity. Furthermore, the removal of the nucleoside simplifies the synthetic pathway, allowing access to the compounds in higher yields and fewer steps. Compound **2** was synthesized in 7 steps from methyl 4-hydroxybenzoate in a yield of 19%. Compound **4** was fully synthesized in 3 steps with an overall yield of 69%. Compound **5** was fully synthesized in 5 steps with an overall yield of 61%. Compound **6** was synthesized in in 3 steps in an overall

58% yield. Ultimately, the use of a methyl ester instead of the THP-protecting group resulted in higher yields and fewer steps and purifications (58% versus 38% for compound **6**).

Nucleoside-containing propargylglycine compounds **1**-**3** were not effective at inhibiting the growth of *E. coli* or *P. aeruginosa* but activity was observed for other propargylglycine compounds. The non-nucleoside compound **4** was also not effective in inhibiting the growth of either *E. coli* or *P. aeruginosa*. The most effective activity was displayed by compound **6**, which contains a large hydrophobic biphenyl tail, while modest antimicrobial activity was observed for compound **5** containing a phenyl propargyl ether tail. Interestingly, compound **5** and **Piizzi-1** (MIC = 2 μg/mL)[38] display different MICs for *P. aeruginosa* despite sharing a common tail structure. They differ in the side chain of the amino acid with compound **5** having an alkyne and **Piizzi-1** has a dimethylamino group which suggests that, while presence of the full nucleoside did not improve activity, presence of a different polar group may. The ArcAB-TolC multidrug efflux system provided significant intrinsic resistance to both of these compounds in *E. coli* with the greatest efflux-mediated resistance observed for compound **6**. The seeming requirement of a large hydrophobic tail for effective growth inhibition therefore presents a significant challenge for future inhibitor design.

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Supplementary Material: Supplementary data (full synthetic procedures, Figure S1-S3: growth curves and Figure S4-S12: ^{1}H and ^{13}C NMRs of final compounds **2**, **4-6**) associated with this article can be found, in the online version, at

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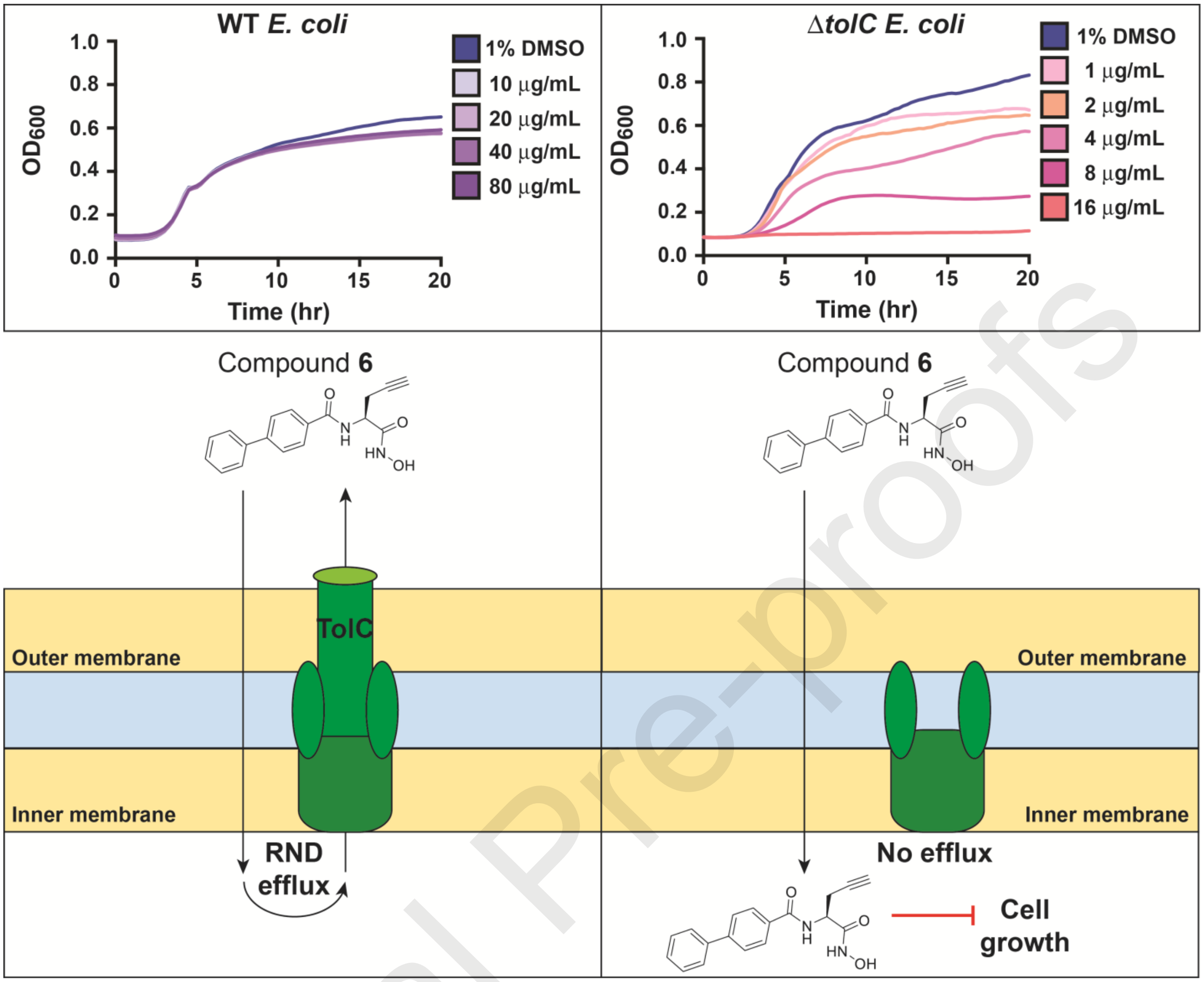

Highlights

- Novel propargylglycine-based antimicrobial compounds have been synthesized.
- Compounds with a phenyl propargyl ether or biphenyl tail exhibited antimicrobial activity.
- Enhancing outer membrane permeability or eliminating efflux in *E. coli* led to a reduced MIC.