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ORIGINAL RESEARCH

Optimization of a Benzothiazole Indolene Scaffold Targeting Bacterial Cell Wall Assembly

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Background: The bacterial cell envelope is comprised of the cell membrane and the cell wall. The bacterial cell wall provides rigidity to the cell and protects the organism from potential harmful substances also. Cell wall biosynthesis is an important physiological process for bacterial survival and thus has been a primary target for the development of antibacterials. Antimicrobial peptides that target bacterial cell wall assembly are abundant and many bind to the essential cell wall precursor molecule Lipid II.

Methods: We describe the structure-to-activity (SAR) relationship of an antimicrobial peptide-derived small molecule 7771–0701 that acts as a novel agent against cell wall biosynthesis. Derivatives of compound 7771–0701 (2-[(1E)-3-[(2E)-5,6-dimethyl-3-(prop-2-en-1-yl)-1,3-benzothiazol-2-ylidene]prop-1-en-1-yl]-1,3,3-trimethylindol-1-ium) were generated by medicinal chemistry guided by Computer-Aided Drug Design and NMR. Derivatives were tested for antibacterial activity and Lipid II binding.

Results: Our results show that the N-alkyl moiety is subject to change without affecting functionality and further show the functional importance of the sulfur in the scaffold. The greatest potency against Gram-positive bacteria and Lipid II affinity was achieved by incorporation of a bromide at the R3 position of the benzothiazole ring.

Conclusion: We identify optimized small molecule benzothiazole indolene scaffolds that bind to Lipid II for further development as antibacterial therapeutics.

Keywords: Lipid II, antibiotics, drug development, cell wall

Cell wall biosynthesis is a complex process that occurs in three stages: the cytoplasmic stage, the membrane-associated stage and at the cell wall envelope.[1,2] The main purpose of this process is the translocation of the peptidoglycan subunits N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) from the cytoplasm across the cellular membrane. These two amino sugars are coupled together by β-1,4-glycosidic bonding. Cross-linking of glycan chains occurs via amide to backbone bond formation of a pentapeptide moiety, which is attached to the MurNac sugar.[2–5] Lipid II (GlcNAc-MurNAc(pentapeptide) phosphoryl undecaprenol) is an essential intermediate in cell wall biosynthesis. Since Lipid II is partly accessible to the extracellular compartment of the cytoplasmic membrane, it is a target for antibacterial compounds.[2,5] These compounds include glycopeptides that have been in clinical use, such as vancomycin, as well as other classes of antibacterial peptides like lantibiotics, ramoplanins and defensins.[2,6–9] Based on the interaction between Lipid II and the antimicrobial peptide Human Neutrophil Peptide −1 (HNP-1),[9] we identified low molecular weight synthetic compounds that target Lipid II with high specificity and affinity.[10] In this study, we report on the structural and functional relationships of derivatives of lead compound 7771–0701.

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Materials and Methods

CADD Modeling and MD Simulations

Molecular Dynamics (MD) simulations were performed with the program CHARMM[11] using the CHARMM36 lipid[12] and protein force field[13,14] for Lipid II, the CHARMM TIP3P model[15] for water, along with the CHARMM General force field[16–18] for the ligand. Based on the previously published model from MD simulation for the BAS00127538-Lipid II complex system in aqueous solution,[10] the lead compound 7771–0701 was docked onto Lipid II by aligning its indolene group with that of BAS00127538. The system was then subjected to a short energy minimization following which a 100 ps MD simulation with a time step of 0.5 fs was carried out to further equilibrate the system. The system was then subjected to a 20 ns MD simulation run with a time step of 1 fs. Simulations were carried out in the NPT ensemble at 300 K and 1 atm with SHAKE of covalent bonds involving hydrogens, and there were no restraints in the simulations. Free energies of binding, ΔG, were estimated using the linear interaction energy (LIE) method,[19] where

$$\Delta G = \alpha\left(\left\langle E^{elec}_{bound}\right\rangle - \left\langle E^{elec}_{unbound}\right\rangle\right) + \beta\left(\left\langle E^{vdw}_{bound}\right\rangle - \left\langle E^{vdw}_{unbound}\right\rangle\right) + \gamma \quad \text{(Eq.1)}$$

in which α = 0.5, β = 0.16, γ cancels out as we only considered the relative free energies ΔΔG, and the unbound interaction energies were computed from 5 ns MD simulations of the compound alone in water. This involved manually placing one of the inhibitor benzene rings and MurNac ring in Lipid II adjacent to each other. Harmonic restraints, k(r-r0)2, were placed between the geometric centers of the above groups, where k=50 kcal/(mol Å2), r0=3 Å and r is the distance between those geometric centers. The system was then subjected to a 2000 step SD energy minimization followed by a 1 ns gas phase Langevin simulation in the presence of the restraints followed by an additional 1 ns gas phase Langevin simulation in the absence of the restraints. The resulting complex was then solvated in a 48*48*48 Å3 pre-equilibrated (45) water box for condensed phase simulations. All water molecules within 2.8 Å of the non-hydrogen atoms of the complex are removed, and two sodium ions were added to neutralize the system, which contained 10385 atoms. While all nonbonded interactions were evaluated for gas phase simulations, nonbonded interactions were truncated at 12 Å for condensed phase simulations, with a force switch smoothing from 10 to 12 Å. Simulations were performed using periodic boundary conditions with the particle mesh Ewald summation method (46) used to treat the electrostatic interactions with a real space cutoff of 12 Å. The system was minimized for 2000 SD steps and subjected to an isobaric, isothermal (NPT) MD simulation at 300 K and 1 atm. Simulations were extended for 2 ns during which the inhibitor remains in close contact with Lipid II.

3-Lipid II Purification

Short-chain water-soluble Lipid II containing a lipid tail of three isoprene units (3-Lipid II or farnesyl-Lipid II) was generated and purified essentially as described.[20] In short, M. flavus vesicles (40–80 nmol lipid-Pi) were incubated together with 200 nmol UDP-GlcNAc, 100 nmol UDP-MurNAC-pentapeptide, and 150 nmol farnesyl phosphate (3-P) in 100 mm Tris-HCl, pH 8.0, 5 mm MgCl2. The incubation lasted two hours at room temperature for 3-P. The synthesis 3-Lipid II was followed using reversed phase TLC (Merck). 3-Lipid II with RP-8 was developed in 75% methanol. For purification, the membranes were removed by centrifugation at 40,000 × g, and the supernatant was collected and loaded on a C18 HPLC column. The short chain Lipid II variant was eluted with a linear gradient from 50 mm ammonium bicarbonate to 100% methanol in 30 mins. Farnesyl-Lipid II (3-Lipid II) eluted at ~60% methanol. The identity of the Lipid II species was confirmed by mass spectroscopy.

Surface Plasmon Resonance

Surface Plasmon Resonance binding experiments were carried out on a BIAcore T100 system (BIAcore Inc., Piscataway, NY) at 25°C. The assay buffer was 10 mM HEPES, 150 mM NaCl, 0.05% surfactant P20, pH 7.4 (± 3 mM EDTA) supplemented with 10% DMSO. 3-Lipid II (50 RUs) was immobilized on CM5 sensor chips using the amine-coupling chemistry recommended by the manufacturer. For initial determination of binding, compounds were introduced into the flow-cells (30 µL/min) in the running buffer at 10 µM. Resonance signals were corrected for nonspecific binding by subtracting the background of the control flow-cell. After each analysis, the sensor chip surfaces were regenerated with 50 mM NaOH for 30 s at a flow rate 100 µL/min, and equilibrated with the buffer prior to next injection. For binding kinetics studies, binding isotherms were analyzed with manufacturer-supplied software for BIAcore T100.

Mechanism-of-Action Studies

Macromolecular synthesis inhibition by 7771–0701 was investigated using *S. aureus* MMX100 (ATCC 29213). Cells were grown at 35oC overnight on Tryptic Soy Agar Broth (Remel, Lenexa, KS), and growth from the plate was used to inoculate 15 mL of Mueller Hinton Broth. The culture was grown to early exponential growth phase (OD600 = 0.2 to 0.3) while incubating in a shaker at 35°C and 150 rpm. For each macromolecular assay, the test agent was added at either 0, 0.25, 0.5, 1, 2, or 4-fold their respective MIC values for S. aureus ATCC 29213. As positive control drugs, the following antibiotics were added at 8X MIC in order to validate each assay: Vancomycin (cell wall synthesis); ciprofloxacin (DNA synthesis), rifampin (RNA synthesis), cerulenin (lipid synthesis), and linezolid (protein synthesis).

For DNA and protein synthesis, 100 µL of cell culture reaching early exponential phase was added to triplicate wells containing various concentrations of test compound or control antibiotics (2.5 µL) at 40X the final concentration in 100% DMSO (0.1% methanol in water for Rifampicin). A 2.5% DMSO treated culture served as the "no drug" control for all experiments. Cells were added in 1.25X strength MHB to account for the volume of drug added to each reaction, or in M9 minimal medium for protein synthesis reactions. Following a 5-mins incubation at room temperature either [3H]Thymidine (DNA synthesis) or [3H] Leucine (protein synthesis) was added at 0.5–1.0 µCi per reaction, depending on the experiment. Reactions were allowed to proceed at room temperature for 15–40 min and then stopped by adding 12 µL of cold 5% trichloroacetic acid (TCA) or 5% TCA/2% casamino acids (protein synthesis). Reactions were incubated on ice for 30 min and the TCA precipitated material was collected on a 25 mm GF/1.2 µm PES 96-well filter plate (Corning). After washing five times with 200 µL per well of cold 5% TCA, the filters were allowed to dry, and then counted using a Packard Top Count microplate scintillation counter.

For cell wall synthesis, bacterial cells in early exponential growth phase were transferred to M9 minimal medium and added to 1.5 mL eppendorf tubes (100 µL/tube) containing various concentrations of test compound or control antibiotics (2.5 µL) at 40X the final concentration in 100% DMSO as described above. Following a 5-mins incubation at 37°C, [14C] N-acetyl-glucosamine (0.4 µCi/reaction) was added to each tube and incubated for 45 min in a 37oC heating block. Reactions were stopped through the addition of 100 µL of 8% SDS to each tube. Reactions were then heated at 95oC for 30 min in a heating block, cooled, briefly centrifuged, and spotted onto pre-wet HA filters (0.45 µM). After washing three times with 5 mL of 0.1% SDS, the filters were rinsed two times with 5 mL of deionized water, allowed to dry, and then counted using a Beckman LS3801 liquid scintillation counter.

For lipid synthesis, bacterial cells were grown to early exponential growth phase in MHB and 100 µL was added to 1.5 mL Eppendorf tubes (in triplicate) containing various concentrations of test compound or control antibiotics as described above. Following a 5-mins incubation at room temp., [3H] glycerol was added at 0.5 µCi per reaction. Reactions were allowed to proceed at room temperature for 40 min and then stopped through the addition of 375 µL of chloroform/methanol (1:2) followed by vortexing for 20 sec after. Chloroform (125 µL) was then added to each reaction and vortexed, followed by the addition of 125 µL dH2O and vortexing. Reactions were centrifuged at 13,000 rpm for 10 min, and then 150 µL of the organic phase was transferred to a scintillation vial and allowed to dry in a fume hood for at least 1 hr. Samples were then counted via liquid scintillation counting.

Antibacterial Activity Assay

The antibacterial activity of 7771–0701 and analogs against *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* 25922 was carried out in a 96-well turbidimetric assay essentially as described previously with the following modifications: bacteria were exposed for 30 min to compounds in 10 mM phosphate buffer containing 5% DMSO prior to addition of 2 x Muller-Hinton medium. Bacterial growth was monitored for 12 hrs and data were analyzed as described (26). Determination of the Minimal Inhibitory Concentrations (MIC) by dilution was carried out by broth dilution according to CLSI standards.[21]

Results

We used mechanism-of-action studies to assess the mode of bacterial killing by the lead 7771–0701 compound (Figure 1). 7771–0701 most potently inhibited cell wall synthesis (IC_{50} of 0.29 µg/mL vs 0.64, 0.53 and 0.85 µg/mL for DNA, protein or lipid synthesis, respectively).

We next used NMR spectroscopy to verify the interaction between Lipid II and this compound, 7771–0701. The NMR analysis (not shown), indicated a binding mode similar to that of the small molecule Lipid II binder we reported on, BAS00127538.[10] Accordingly, a CADD model of 7771–0701 in complex with soluble Lipid II

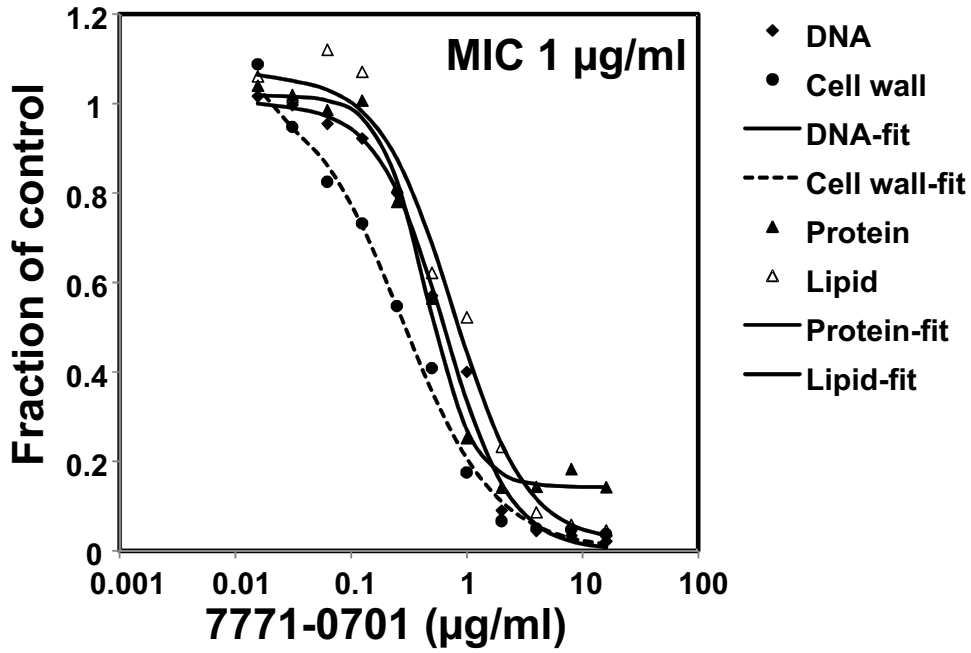


Figure 1 Mechanism of action studies of 7771–0701. Exponentially growing *S. aureus* 29213 cells were exposed to compound and comparators in triplicate using 2.5% DMSO as "no drug" control. Cells were added to Mueller-Hinton Broth or M9 medium for protein synthesis and further incubated in the presence of ^{13C}N-acetyl glucosamine (cell wall), ^{3H}glycerol (lipid), ^{3H}Thymidine (DNA), or ^{3H}Leucine (protein). Following incubation, reactions were stopped by adding TCA (DNA, protein), 8% SDS (cell wall) or chloroform/methanol (lipid) and analyzed by scintillation counting.

was generated and subjected to 20 ns MD simulations. A stable binding mode was found in the simulations with ΔΔG values relative to BAS00127538[10] of −1.0 kcal/mol. Together, these data show that compound 7771–0701 inhibits cell wall biosynthesis by binding to Lipid II.

The complex structure from MD simulation as shown in Figure 2 suggests compound 7771–0701 binds with lipid II in a similar way as BAS00127538.[10] The indolene moiety in 7771–0701 wraps around the aliphatic tail of lipid II and methylated phenyl ring in the benzothiazole group faces sugar moiety in lipid II. Positive charged nitrogen in benzothiazole group interacts with lipid II phosphate group.

To identify optimized lipid II-binding analogs and establish SAR, we synthesized 26 derivatives of the 7771–0701

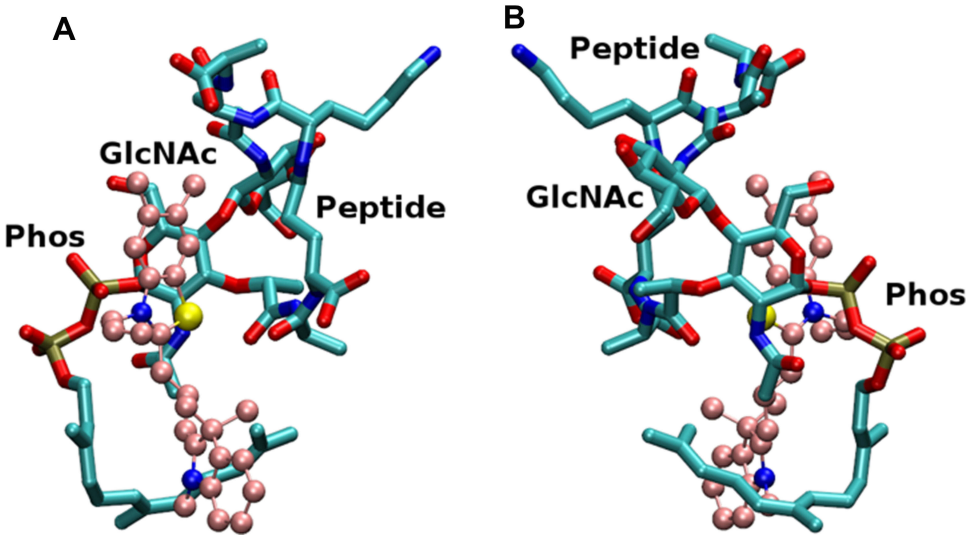


Figure 2 Model of lead compound 7771–0701 (CPK representation with carbons in pink color) in complex with lipid II (licorice representation with carbons in cyan color), based on the NMR data. The phosphate (Phos), sugar (GlcNAc) and pentapeptide (Peptide) of Lipid II are labeled. (**A**) and (**B**) panels are approximately 180 degree rotation of the complex.

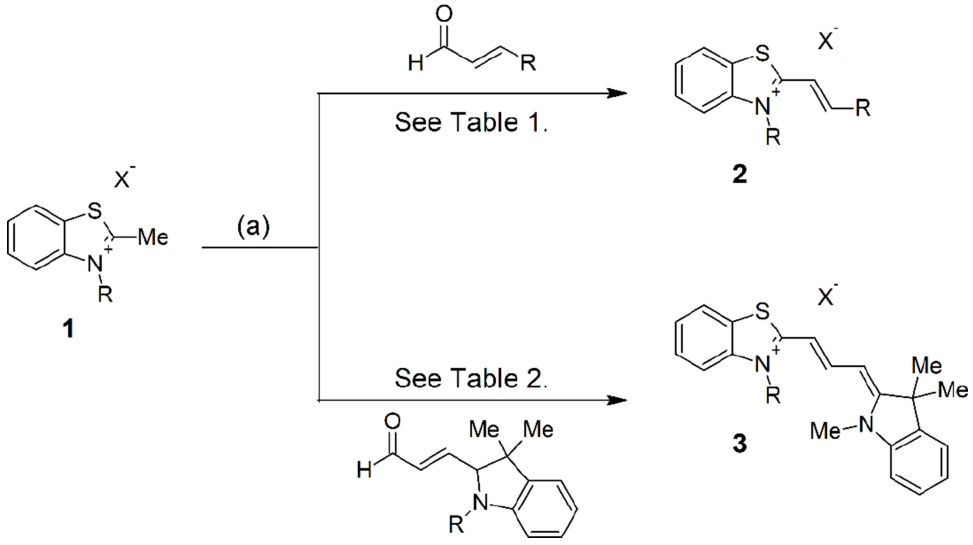
scaffold (see Scheme 1 and Supporting information), based on the CADD modeling. The first generation of molecules synthesized were to simplify the 7771–0701 scaffold that would allow access to a wide array of functionalized analogs (Scheme 1).

2-methylbenzothiazole salts 1, where R = Me, were reacted with aromatic aldehydes in acetic acid to afford alkenyl salt compounds 2 after filtration. These six compounds were tested for activity against *S. aureus, E. faecium* and were examined for Lipid II binding by surface plasmon resonance (Table 1). Reaction with dimethylamino benzaldehyde gave compound 7jc65-1 with MIC of 8, 16 and kd of 1.0 x 10–6 for S. aureus, E. faecium and Lipid II, respectively. Extending the alkene linker, 7jc65-1, gave a modest increase in activity. Enlarging the steric bulk around the 4-amino position (7jc66-2, 7jc67-1 and 7jc67-2) decreased potency drastically for all three compounds. The most potent compound of the first generation series was 7jc66-1, which gave Lipid II binding affinity of 9.0 x 10^{-7}. Based on these observations, we conclude that simplifying the scaffold did not enhance the functional parameters compared to lead scaffold.

We next generated a series of compounds which more closely resembled the lead compound and tested potency against both Gram-positive bacteria as well as determined Lipid II binding affinity. The one-step general procedure is outlined in Scheme 1, where N-alkylated benzothiazoles 1 were condensed with indolene-acetaldehyde to afford compounds 3 after filtration. All 20 compounds gave superior results compared to the first generation series (Tables 1 and 2). 7jc63 where R1 = Me gave MIC of 1, 4 and kd of 7.0 x 10^{-7} for *S. aureus, E. faecium* and Lipid II, respectively. Substituting the methyl group to allyl provided 7jc71-1 (R3 = H) which gave comparable results to lead compound 7771 (R3 = OMe).[10] Exchanging the R2 aromatic proton to halogens gave no significant increase in potency (7jc71-2 to 7jc71-4), although substituting to an acetate group (7jc73) diminished potency considerably. The importance of sulfur in the aromatic ring is demonstrated by the replacement with oxygen (7jc75-1) and drastically reduced Lipid II affinity, Kd = >64. Incorporating a chlorine in the R4 position (7jc83-1 to 7jc85-2) produced greater potency and comparable binding when compared to 7jc71-1. The introduction of a nitro group at this position (7jc91-1 to 7jc96-1) reduced the antibacterial activity 4- to 8-fold. While the N-allyl group can be replaced by ethyl (7jc81-1) or but-2-ene (7jc81-2), no significant change was observed in the activity when compared to 7jc63 or 7jc71-1. This demonstrates that the N-alkyl group is flexible to changes without affecting functionality. Finally, the greatest potency against Gram-positive bacteria and Lipid II affinity were achieved when a bromide was incorporated at the R3 position of the benzothiazole ring affording compound 7jc75-2 with MIC of 1 against *S. aureus*, 0.5 against *E. faecium* and high-affinity Lipid II binding (Kd 5.5 x 10^{-9}).

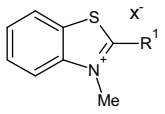
Conclusion

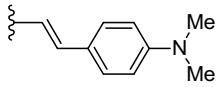
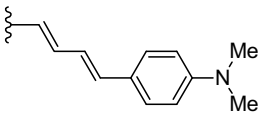
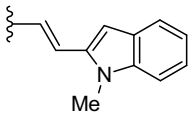
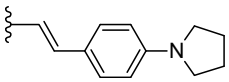
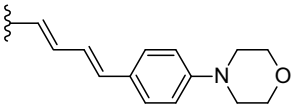
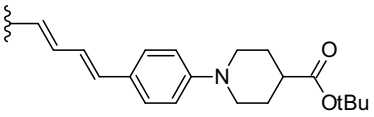
Fully synthetic compounds that bind to Lipid II have not yet been developed. Using CADD, we have identified and



Scheme 1 General synthetic route to 7771 analogs.
Note: (a) Aldehyde (1 eq.), acetic anhydride, 100 deg. C, 5 h.

Table 1 First-Generation Compounds[a]

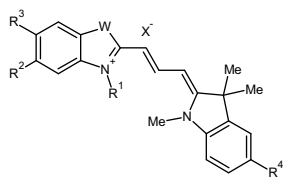


Code Number	R1	*S. aureus* MIC	*E. faecium* MIC	Lipid II K_d
7jc65-1		8	16	1.0×10^{-6}
7jc65-2		4	8	6.4×10^{-6}
7jc66-1		4	8	9.0×10^{-7}
7jc66-2		32	64	5.4×10^{-5}
7jc67-1		32	64	0.3×10^{-2}
7jc67-2		4	16	5.1×10^{-5}

Notes: [a]MIC is Minimum Inhibitory Concentration (μg/mL). K_d is the binding constant (μM).

optimized a novel scaffold that efficiently binds to this essential bacterial component of cell wall assembly. Subsequent experimental characterization of these compounds showed preferential activity against Gram-positive organisms. 7771–0701 binds to aminosugar moiety of Lipid II, thus making cross-resistance with vancomycin unlikely, since vancomycin binds to the penta-peptide. Studies like these on small molecule Lipid II-binding agents will provide insight for future development, design and synthesis of such compounds as promising therapeutic leads.

Table 2 Second-Generation Compounds[a]



Code Number	R^1	R^2	R^3	R^4	W	*S. aureus* MIC	*E. faecium* MIC	Lipid II K_d
7jc63	Me	H	H	H	S	1	4	7.0×10^{-7}
7jc71-1	Allyl	H	H	H	S	1	2	2.9×10^{-8}
7jc71-2	Allyl	Cl	H	H	S	1	2	1.1×10^{-8}
7jc71-3	Allyl	Br	H	H	S	1	4	3.4×10^{-7}
7jc71-4	Allyl	F	H	H	S	1	4	1.0×10^{-8}
7jc73	Allyl	Ac	H	H	S	4	4	2.7×10^{-6}
7jc75-1	Allyl	H	H	H	O	4	8	>64
7jc75-2	Allyl	H	Br	H	S	1	0.5	5.5×10^{-9}
7jc81-1	Ethyl	H	H	H	S	1	4	1.07×10^{-7}
7jc81-2	But-2-ene	H	H	H	S	1	4	3.6×10^{-7}
7jc83-1	Me	H	H	Cl	S	1	4	4.7×10^{-8}
7jc83-2	Allyl	H	H	Cl	S	0.5	2	0.2×10^{-8}
7jc84-1	Allyl	Cl	H	Cl	S	0.5	2	3.2×10^{-7}
7jc84-2	Allyl	Br	H	Cl	S	0.5	2	4.1×10^{-7}
7jc85-1	Allyl	F	H	Cl	S	1	4	3.2×10^{-5}
7jc85-2	Allyl	Ac	H	Cl	S	1	2	4.3×10^{-5}
7jc91-1	Allyl	H	H	NO_2	S	8	8	ND
7jc92	Allyl	F	H	NO_2	S	4	4	ND
7jc94	Allyl	Cl	H	NO_2	S	8	4	ND
7jc96-1	Allyl	Ac	H	NO_2	S	8	8	ND

Notes: [a]MIC is Minimum Inhibitory Concentration (μg/mL). K_d is the binding constant (μM).

Abbreviations

S. aureus, Staphylococcus aureus; E. faecium, Enterococcus faecium; MIC, Minimal Inhibitory Concentration; MurNAc, N-acetyl muramic acid.

Author Contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

AM Jr and EdL are inventors on US patents # 8,796,323 and # 9,351,963 to University of Maryland, Baltimore; JC, SF, AM Jr and EdL are inventors on PCT/US11/59432. AM Jr is a co-founder and CSO for SilcsBio LLC. The authors report no other conflicts of interest in this work.

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