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Letter

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Human Microbiome Inspired Antibiotics with Improved β-Lactam Synergy against MDR *Staphylococcus aureus*

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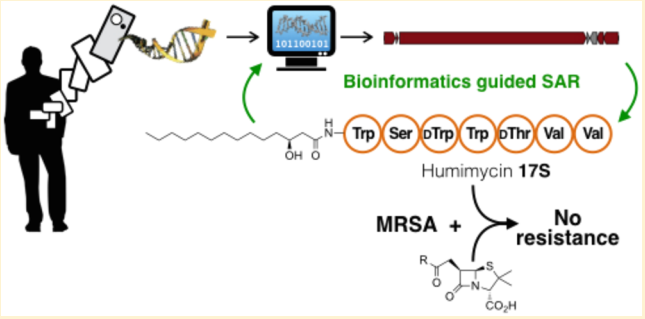
Supporting Information

ABSTRACT: The flippase MurJ is responsible for transporting the cell wall intermediate lipid II from the cytoplasm to the outside of the cell. While essential for the survival of bacteria, it remains an underexploited target for antibacterial therapy. The humimycin antibiotics are lipid II flippase (MurJ) inhibitors that were synthesized on the basis of bioinformatic predictions derived from secondary metabolite gene clusters found in the human microbiome. Here, we describe an SAR campaign around humimycin A that produced humimycin **17S**. Compared to humimycin A, **17S** is a more potent β-lactam potentiator, has a broader spectrum of activity, which now includes both methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus faecalis* (VRE), and did not lead to any detectable resistance when used in combination with a β-lactam. Combinations of β-lactam and humimycin **17S** provide a potentially useful long-term MRSA regimen.



KEYWORDS: *Syn-BNP, MRSA, antibiotics, β-lactam, NRPS*

Over 50 years after their introduction, β-lactams remain a critical component of antibiotic therapy. Unfortunately, β-lactam resistant bacteria, in particular methicillin resistant *Staphylococcus aureus* (MRSA), are of growing concern in hospital settings around the world.[1−3] The recently identified MRSA active humimycin antibiotics are inhibitors of the *Staphylococcus* MurJ homologue,[4] an essential protein responsible for transporting the cell wall intermediate lipid II from the cytoplasm to the outside of the cell.[5−7] As seen with other flippase inhibitors, the humimycins potentiate and restore the activity of β-lactams against MRSA,[8−10] making them potentially useful for restoring the clinical utility of β-lactams.[11,12] Potentiation of β-lactam activity with MurJ inhibitors is thought to arise from the fact that these antibiotics target different steps in the cell wall biosynthesis pathway. Here, we describe a systematic structure−activity relationship (SAR) study to improve humimycin A (**1S**).

The humimycins were identified using a novel bioactive small molecule discovery approach, whereby structures encoded by gene clusters found in bacterial (meta)genome sequence data are bioinformatically predicted and then chemically synthesized.[4,13] We have called the molecules generated using this new approach syn-BNPs (Synthetic-Bioinformatic Natural Products). Syn-BNPs are not intended to be exact copies of NPs but instead analogs that are structurally close enough to the natural metabolite to facilitate the identification of bioactive molecules without the traditional requirement of isolating a NP from a fermentation broth.

The humimycins are *N*-acylated linear heptapeptides whose structures were bioinformatically predicted from nonribosomal peptide synthetase (NRPS) gene clusters found in *Rhodococcus* species, including *Rhodococcus erythropolis*, which is observed in ∼20% of sequenced human oral and nasal microbiomes.[14−16] Our efforts to improve on the activity of the humimycins began with a systematic exploration of the *N*-acyl substituent (Figure 1a), as current bioinformatic algorithms cannot predict its exact structure on an NRP.[17] We began by making drastic changes to the lipid, including a peptide with a free *N*-terminus (**10**) as well as analogs containing aromatic substituents: 2,3-dihydroxybenzoyl (**11**), 2-naphthoyl (**12**), and 6-phenylhexanoyl (**13**). Each peptide was assayed for antibiosis against MRSA USA300, the most common strain seen in community-acquired *Staph* infections in the United States.[18] All four analogs were inactive at the highest concentration tested (128 μg/mL). We continued with less dramatic changes to the *N*-terminal lipid

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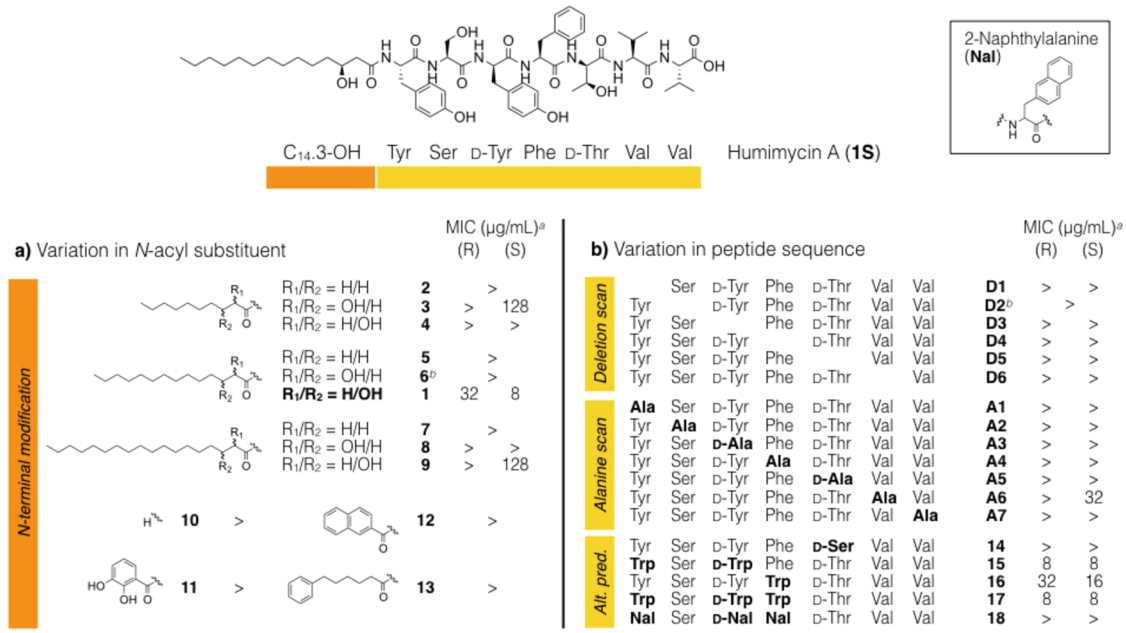

Figure 1. Humimycin analogs with variations in (a) *N*-acylation and (b) peptide sequence. [a]Separate MIC values are reported for analogs containing a (*R*)- and (*S*)-hydroxylated fatty acid as the *N*-substituent. ">" denotes that the MIC is greater than the highest concentration tested (128 μg/mL). [b]The diastereomers were tested as a mixture as they were inseparable by HPLC.

starting with examining the importance of *β*-hydroxylation. Antibiosis in all analogs missing the hydroxyl moiety [C10 decanoyl (**2**), C14 myristoyl (**5**), and C18 stearoyl (**7**)] was abrogated. Next, we produced a collection of analogs containing different length lipids with either *β* (**1**, **4**, **9**) or *α* (**3**, **6**, **8**) oxidation. Regardless of lipid length, shifting the hydroxyl group by even one carbon (*β* to *α*) dramatically reduced antibiosis (**3**, **6**, **8**). In the case of different length *β*-hydroxyl lipids, only *β*-hydroxylmyristoyl showed any significant antibiosis, with the *S*-isomer being more active than the *R*-isomer (Figure S1). The near absolute requirement for the (*S*)-*β*-hydroxylmyristoyl substituent suggests that the lipid moiety in humimycin does not simply play a generic hydrophobic role but instead is likely required for its specific interaction with MurJ.

Our SAR study of the peptide portion of humimycin involved the synthesis of both a set of one amino deletions and an alanine scan[4] across the peptide. Each peptide was capped at the *N*-terminus with a *β*-hydroxymyristic acid. The first group, the *Deletion Scan* (**D1**−**D6**), consisted of six analogs each lacking one amino acid (Figure 1b). As the two *C*-terminal residues are both valine, the deletion of the sixth or seventh residues yields the same structure. The second group, the *Alanine Scan* (**A1**−**A7**), consisted of seven lipopeptides each replacing one amino acid in humimycin with alanine. When assayed for antibiosis, with the exception of **A6S** (32 μg/mL), none of these analogs were active against *S. aureus* USA300.

While not active alone, the ability of humimycins to potentiate the activity of *β*-lactams allows us to explore the SAR of the alanine analogs in greater detail using a potentiation assay. The minimum inhibitory concentration (MIC) of each alanine replacement analog was determined in the presence of 8 μg/mL of carbenicillin, where humimycin has an MIC of 1 μg/mL (Figure 2). Under these conditions, we observed functional differences between alanine substitution analogs, with the Tyr3 (**A3S**), Phe4 (**A4S**), and Thr5 (**A5S**) alanine replacements showing the most dramatic losses in antibiosis (128, 64, and

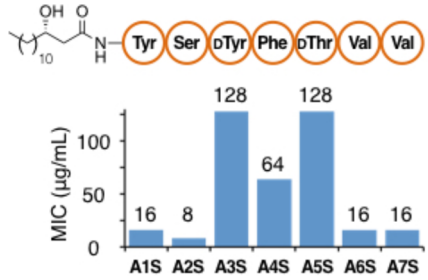

Figure 2. *S*-isomer of each analog of the *Alanine Scan*[4] collection was tested in the presence of 8 μg/mL of carbenicillin against MRSA USA300.

128 μg/mL, respectively). This suggests that, while the entire heptapeptide is important for antibiosis, the central three residues are least tolerant of change.

This initial SAR work indicated that only minor modifications could be made to any part of the humimycin structure without abrogating its antibacterial properties. As the chemical space around a heptapeptide is very large, we sought to gain intelligent insights into what additional minor structural variants would be reasonable to synthesize. As mentioned above, humimycin A was originally synthesized on the basis of a bioinformatic prediction derived from an NRPS gene cluster. The three NRPS prediction algorithms we used to analyze this gene cluster (Stachelhaus, NRPSPred2, and Minowa)[19−21] generated some alternative structural predictions that differ from humimycin A by (i) tryptophan at positions 1 and 3 instead of tyrosine, (ii) tryptophan at position 4 instead of phenylalanine, or (iii) a D-serine at position 5 instead of D-threonine. Interestingly, the bioinformatically variable positions largely correlate with the residues we identified as being most important in the alanine scan experiment. We therefore continued our SAR study by synthesizing the bioinformatically predicted variants.

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The first alternative bioinformatic prediction we synthesized was the peptide containing a D-serine instead of D-threonine at the fifth position (**14**). Surprisingly, this minor change completely abrogated the antibiosis of the peptide. The two analogs where tryptophan replaced either tyrosine at positions 1 and 3 (Trp1/D-Trp3) (**15**) or phenylalanine at position 4 (Trp4) (**16**) both showed antibiosis activity; however, only the former showed comparable activity to humimycin. Replacing all three aromatic residues with tryptophan (Trp1/D-Trp3/Trp4) (**17**) also yielded an antibiotic that exhibited the same potency as humimycin. On the basis of this observation, we synthesized an analog where 2-naphthylalanine replaced tryptophan at positions 1, 3, and 4 (**18**). The relative minor change from indole to naphthalene, where a H-bond donor is removed with a concomitant slight increase in ring size, abrogated antibiosis.

To better evaluate the antibiosis of these new tryptophan analogs, humimycin and **15**–**17** were tested for activity against a panel of antibiotic resistant *S. aureus* clinical isolates (Figure 3

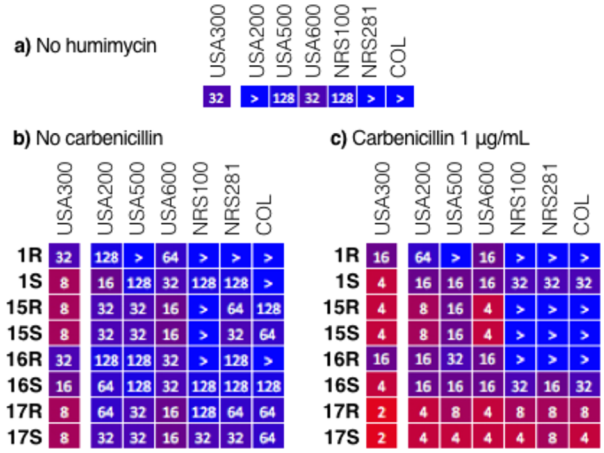

Figure 3. MIC of (a) carbenicillin or humimycin derivatives against MRSA strains in the presence of (b) 0 or (c) 1 μg/mL of carbenicillin. MIC of humimycin derivatives in the presence of 2, 4, or 8 μg/mL of carbenicillin can be found in Figure S7. ">" denotes greater than 128 μg/mL. In addition to β-lactams, USA600, NRS100, and NRS281 are resistant to vancomycin, tetracycline, and erythromycin, respectively.

and Table S1a). **17S** showed the broadest and most potent antibiosis both when tested alone and when tested in combination with carbenicillin. In fact, it was the only analog that showed activity against all of the *S. aureus* strains in the panel. In addition, when assayed against a phylogenetically diverse collection of bacteria, unlike humimycin, **17S** showed activity against vancomycin resistant *Enterococcus faecalis* (VRE) (MIC 8 μg/mL), another pathogen for which resistance to the current arsenal of antibiotics is of growing concern (Table S1b).[22,23] On the basis of its superior antibacterial properties, we carried out a more extensive analysis of **17S**.

One of the most important characteristics in determining the utility of future antibiotics is the rate at which resistance arises (Figure S5). To compare resistance rates for humimycin A (**1S**) and **17S**, *S. aureus* USA300 was plated separately on 2.5× MIC of each antibiotic (20 μg/mL). On the basis of the number of resistant mutants that appeared in these experiments, **17S** shows a greater than 50-fold reduction in resistance rate compared to **1S** (1.8×10^{-9} and 1.0×10^{-7}, respectively). The less common **17S** resistant strains all show cross-resistance to **1S** (Table S1c).

In our initial characterization of the humimycins, we identified 18 humimycin resistant *S. aureus* strains, each containing a single nonsynonymous point mutation in MurJ.[4] We tested these strains for cross-resistance to **17S**. In contrast to humimycin, which is inactive against all resistant strains at 16 μg/mL, **17S** is active against the entire collection at this concentration (Figure 4a). In the presence of 4 μg/mL of

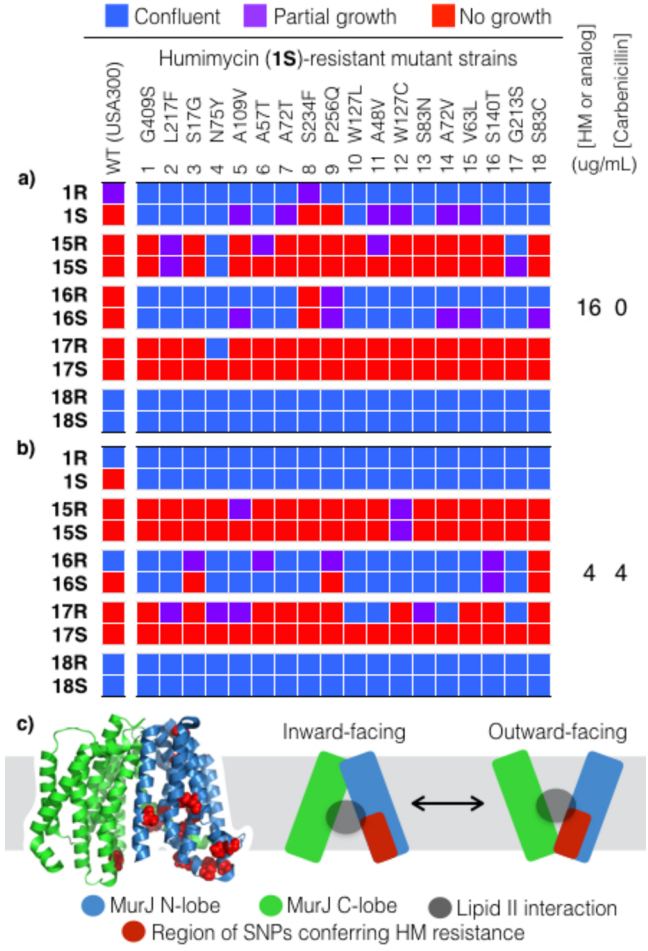

Figure 4. Humimycin-resistant mutants containing nonsynonymous SNP in MurJ were tested against humimycin and its analogs (**15**–**18**) at (a) 16 μg/mL without carbenicillin and (b) 4 μg/mL with carbenicillin (4 μg/mL). (c) SNPs that confer humimycin resistance cluster in the cytoplasmic side of the *N*-lobe and partially overlap with the modeled lipid II binding region of MurJ based on the MOP flippase structure from *T. africanus*.

carbenicillin, the growth of all MurJ point mutants are inhibited with as little as 4 μg/mL of **17S** (Figure 4b). The rare **17S** resistant strains we raised in this study also remain susceptible to **17S** when potentiated with 4 μg/mL of carbenicillin (MIC ≤ 4 μg/mL, Table S1c). While these observations suggest that **1S** and **17S** have the same mode of action, additional experiments are required to confirm this hypothesis.

No structure of a Gram-positive flippase is available; however, the crystal structure of the flippase from the Gram-negative bacterium *Thermosipho africanus* was recently solved (PDB: 5T77).[24] Although flippase proteins from Gram-negative and Gram-positive bacteria diverged quite some time ago (~20% identity), this structure provides a model to begin to understand the interaction between the humimycin

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antibiotics and MurJ. We mapped each mutation in *S. aureus* flippase onto the *T. africanus* flippase structure based on a ClustalW alignment of the two proteins. Interestingly, the vast majority of humimycin resistance mutations cluster in the *N*-lobe in a region near the bottom of the cleft that is thought to open and close during lipid II transportation across the membrane (Figure 4c).

In addition to evaluating the rate at which resistance develops against **17S** in isolation, we also looked for resistance rates when it was used in combination with a β-lactam. In the presence of even a small amount of carbenicillin, we were unable to detect resistance by direct plating on the **17S**/β-lactam mixture (20 and 8 μg/mL, corresponding to 2.5× and 0.25× of their respective MIC). In comparison, direct plating on the **1S**/β-lactam mixture under identical conditions showed a resistance rate of 0.22×10^{-9}. Furthermore, even after serial passage of *S. aureus* USA300 for 14 days in the presence of a sublethal mixture of **17S**/β-lactam (2 μg/mL of each), we did not detect any resistant mutants.

Our initial SAR study through *N*-substituent modifications, an *Alanine Scan*, and a *Deletion Scan* indicated that humimycin could tolerate very little structural change and still retain its antibiosis, suggesting an intimate interaction between the antibiotic and its flippase target. Ultimately, the identification of humimycin **17S** was guided by a more detailed bioinformatics analysis of humimycin biosynthetic gene cluster, highlighting the utility of the syn-BNP approach for not only the initial discovery of new bioactive small molecules but also their subsequent optimization. **17S** shows potent β-lactam potentiation, activity against MRSA and VRE, low rate of resistance alone, and no detectable resistance when exposed to the **17S**/β-lactam mixture. Taken together, **17S**/β-lactam combination therapy could provide an alternative MRSA treatment regimen. The systematic application of a syn-BNP approach to the rapidly expanding collection of sequence data derived from bacterial genomes and environmental metagenomes should provide an increasing number of naturally inspired small molecules that can be assessed not only for activity against drug resistant bacterial pathogens but also more broadly for activities with diverse biomedical relevance.

METHODS

Wang resins with preloaded amino acids were purchased from Matrix Innovation. Coupling reagents benzotriazol-1-yl-oxy-tripyrrolidinophosphonium hexafluorophosphate (PyBOP) and 6-chloro-1-hydroxy-benzotriazole (Cl-HOBt) were purchased from P3 BioSystems. Fmoc/side-chain protected amino acids were purchased from P3 BioSystems, ChemPep, and Chem-Impex International. Hydroxylated fatty acids were purchased from Matreya, TCI America, and Santa Cruz Biotechnology. All other chemicals and solvents were purchased from Sigma-Aldrich. Consumables were purchased from Fisher Scientific and VWR International. Luria-Bertani (LB) medium was prepared directly from premixed powder purchased from Becton Dickinson (BD) & Co. Sheep blood in tryptic soy agar plates were purchased from Hardy Diagnostics.

Humimycin derivatives were synthesized either manually or using an automated microwave peptide synthesizer (Alstra Initiator plus, Biotage). Peptide syntheses were typically performed on a 50 to 100 μmol scale on preloaded Wang resins (designated as 1 equiv). In each cycle, the *N*-terminal Fmoc protecting group was removed by two rounds of treatment with 20% (v/v) piperidine in dimethylformamide (DMF) for 10 min each and washing with DMF. Amino acid (4 equiv) couplings were conducted in DMF using PyBOP (3.95 equiv), Cl-HOBt (8 equiv), and DIEA (8 equiv) for activation. For manual synthesis, double couplings were carried out at room temperature for 1 h per coupling; for automated microwave syntheses, each coupling lasted 15 min at 70 °C. After completion of syntheses, resin-bound peptides were washed and cleaved using a trifluoroacetic acid (TFA) cocktail (4 mL) composed of TFA supplemented with 2.5% (v/v) water and 2.5% (v/v) triisopropylsilane. The resulting solution was then added into cold *tert*-butyl methyl ether and centrifuged at 4000*g* at 4 °C for 15 min. The supernatant was discarded, and the pellet was dissolved in 50% MeCN (v/v) for HPLC purification. Note that all hydroxylated fatty acids were purchased as racemic mixtures and used as is in *N*-acylation reactions. The resulting diastereomers were then separated by HPLC and verified by high-resolution mass spectrometry (HRMS, Table S2). All pure peptides were dissolved in DMSO (12.8 mg/mL) and stored at −20 °C.

In most cases (22 out of 24), the resulting mixture of diastereomers was readily resolved by reversed-phase HPLC and characterized as separate entities. The remaining two diastereomer pairs (**6** and **D2**) were inseparable, and each was tested as a mixture. The MIC values of these two pairs were reported as a single value. Absolute stereochemistry assignment of humimycin A (**1S**) and the most potent derivative (**17S**) was accomplished by small-scale synthesis using pure (*R*)- and (*S*)-3-hydroxymyristic acid for *N*-acylation (Figure S1 and Reference S1).[4] These enantiopure peptides were used to identify the absolute stereochemistry of their counterparts resulting from bulk syntheses through coinjections on reversed-phase HPLC. For these two sets of lipopeptides, the (*R*)- and (*S*)-isomers consistently eluted as the less hydrophobic and more hydrophobic compound, respectively, on the basis of HPLC retention time. We therefore assigned the stereochemistry of all other humimycin derivatives on the basis of their respective HPLC retention times.

Standard susceptibility assays were performed in LB medium in 96-well microtiter plates to determine the MIC by using the broth microdilution method in accordance to protocols recommended by the Clinical and Laboratory Standards Institute. LB medium was first added to all wells (50 μL). Antibiotic stock solutions and LB broth (50 μL) were then added to the first well of each row and diluted serially across the microtiter plate. The last two wells were reserved for positive (antibiotic-free) and negative (bacteria-free) controls. Overnight bacterial cultures were diluted 5000-fold and used as the inoculum (50 μL per well) to yield a final volume of 100 μL in each well. In a typical setup, the final antibiotic concentrations ranged from 128 to 0.25 μg/mL. MIC values were determined by visual inspection after static incubation at 37 °C for 24 h. All assays were done in duplicate. Potentiated susceptibility assays were performed similarly, except that the LB medium for setting up the potentiated assays contain β-lactam (carbenicillin) at 2× of the final concentration. Overnight cultures of MRSA were diluted with β-lactam-free LB medium and used as the inoculum. Note that MRSA strains such as USA300 and COL grow significantly slower in the presence of carbenicillin at 4 μg/mL or higher, and the corresponding MIC values were determined after 48 h of static incubation at 37 °C.

To raise antibiotic-resistant mutants (Figure S5), a single *S. aureus* USA300 colony from a freshly streaked plate was first inoculated into LB medium (antibiotic-free). The overnight

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culture was spread onto LB agar plates in triplicate to determine the density of viable cells based on the resulting colony forming unit (CFU/mL). For mutant selection by direct plating, the same overnight culture (200 μL) was mixed with LB medium (100 mL) containing humimycin A (**1S**) or **17S** at 20 μg/mL (2.5× of its MIC value), and the resulting mixture was distributed into a microtiter plate (200 μL per well). The number of wells with bacterial growth (N) was recorded after static incubation at 37 °C after 24 h. The mutation rate was reported as (N ÷ viable cells). For mutant selection by serial passage, the overnight culture (40 μL) was passaged by mixing with LB medium (4 mL, 100× dilution) containing **17S** and carbenicillin (2 μg/mL of each, 0.5× of their respective potentiated MICs) and incubated at 37 °C at 220 rpm. The grown culture was passaged daily. The resulting culture after 14 days was screened by direct plating for resistant mutants in the presence of **17S** (20 μg/mL) and carbenicillin (8 μg/mL).

S. aureus USA300 strains with mutations that confer resistance to humimycin A were raised previously.[4] These mutants have been fully sequenced and shown to carry a single nonsynonymous point mutation in MurJ and no more than one other mutation throughout their genomes. They were inoculated into LB media, grown overnight, and tested in the presence of the indicated carbenicillin and humimycin (or its derivatives) concentrations. Growth was visually inspected after static incubation at 37 °C for 24 h and recorded as confluent (blue), partial growth (purple), or no growth (red).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.7b00056.

Materials and methods, bacterial strain information, supporting figures and tables, analytical data (NMR and HRMS), and supporting references (PDF)

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Notes

The authors declare no competing financial interest.

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