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ACS Infect. Dis., **Just Accepted Manuscript** • DOI: 10.1021/acsinfecdis.8b00105 • Publication Date (Web): 01 May 2018

Just Accepted

ACS Publications

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Total Syntheses of Bulgecins A, B and C and Their Bactericidal Potentiation of the β-Lactam Antibiotics

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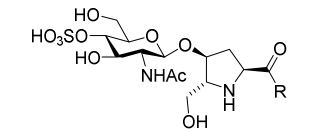
The bulgecins are iminosaccharide secondary metabolites of the Gram-negative bacterium *Paraburkholderia acidophila* and inhibitors of lytic transglycosylases of bacterial cell-wall biosynthesis and remodeling. The activities of the bulgecins are intimately intertwined with the mechanism of a co-biosynthesized β-lactam antibiotic. β-Lactams inhibit the penicillin-binding proteins, enzymes also critical to cell-wall biosynthesis. The simultaneous loss of the lytic transglycosylase (by bulgecin) and penicillin-binding protein (by β-lactams) activities results in deformation of the septal cell wall, observed microscopically as a bulge preceding bacterial cell lysis. We describe a practical synthesis of the three naturally occurring bulgecin iminosaccharides and their mechanistic evaluation in a series of microbiological studies. These studies identify potentiation by the bulgecin at sub-minimum inhibitory concentrations of the β-lactam against three pathogenic Gram-negative bacteria, and establish for the first time that this potentiation results in a significant increase in the bactericidal efficacy of a clinical β-lactam.

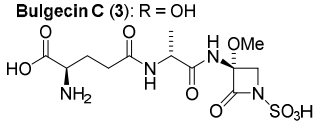
Keywords: Bacteria, Cell Wall, Lytic Transglycosylases, Antibiotic Resistance, β-Lactam Antibiotics

The appearance of extensively drug-resistant bacterial infections in both the hospital and the community has sustained debate as to whether the era of successful monochemotherapy of bacterial infections is fading. An alternative approach, with precedent in cancer chemotherapy, is the combination of complementary agents. The combination of an antibiotic with another potentially synergistic antibiotic, or with a compound that would potentiate the activity of the antibiotic, is attractive in the light of the dearth of clinical options. While such combinations can have extraordinary medical value (as exemplified by the combination of β-lactams with β-lactamase inhibitors),[1–4] every aspect to the selection and implementation of successful combination therapy is a scientific challenge.[5–10] With respect to this challenge, useful guidance may be provided by Nature.

Imada et al.,[11, 12] of the antibiotic discovery group at Takeda, reported the isolation of bulgecins A–C (**1–3** of Figure 1) in 1982. Each is a simple variation on a glycosylated iminosaccharide, and each biosynthesized by a Gram-negative bacterium (annotated then as *Pseudomonas mesoacidophilia*, but recently reassigned as *Paraburkholderia acidophila*).[13, 14] Additional studies with this bacterium by Imada et al. led to the isolation of a monobactam-class β-lactam antibiotic, sulfazecin (**4**).[15, 16] Combination of bulgecin (itself bereft of antibacterial activity) with sulfazecin significantly improved the latter's antibacterial activity toward other Gram-negative bacteria.[17, 18] All doubts that this potentiation was fortuitous were dispelled by the observation of an intimate connection of the genes encoding the biosynthetic enzymes for sulfazecin and bulgecin.[14, 19, 20] Hence, Nature selected co-production of an antibacterial and a potentiator within the same producer organism.



Bulgecin A (1): R = $NHCH_2CH_2SO_3H$
Bulgecin B (2): R = $NHCH_2CH_2CO_2H$
Bulgecin C (3): R = OH



Sulfazecin (4)

Figure 1. Chemical structures of the bulgecins and of sulfazecin. Bulgecins are glycopeptides comprised of a common β-sulfo-GlcNAc linked to a disubstituted L-proline. Bulgecin A is the most abundant of the bulgecin natural products. Sulfazecin is a monobactam β-lactam antibiotic that is structurally distinguished by the sulfamate functional group.

The morphological effect resulting from the complementarity of these two agents is a characteristic mid-cell bulge (hence "bulgecin") that precedes lysis of the bacterium. A biochemical basis for this effect was provided by Templin et al.,[21] who confirmed the original observations of Imada et al. that the bulgecin effect extended to other β-lactam antibiotics, and determined that the bulge was a result of bulgecin inhibition of a periplasmic enzyme, the Slt lytic transglycosylase. As optimal potentiation with bulgecin was found with β-lactams that inactivated the penicillin-binding proteins (PBPs) of septal cell-wall formation, the hypothesis was presented that the simultaneous loss of the catalytic activity of these PBPs by β-lactams, and of the lytic transglycosylase(s) (LTs) by bulgecin, results in a structurally defective cell-wall septum. This hypothesis has experimental support.[22]

The lytic transglycosylases of Gram-negative bacteria share key attributes with the PBPs. Both are found as families of enzymes within Gram-negative bacteria. For example, the Gram-negative pathogen *Pseudomonas aeruginosa* has eight different PBPs and eleven distinct LTs. Within each family there is redundancy of function, but inability to tolerate overall loss of all function. In comparison to the PBPs (where there is a credible hypothesis for the function of each family member) the LT family is both more structurally and more functionally diverse. At present within the LT family there are few guiding hypotheses as to the function of its individual members.[23, 24] The understanding of LT function is (even 25 years after their discovery) a frontier at the nexus of bacterial shape, bacterial cell-wall biosynthesis, and antibiotic mechanism.

Given our ambitions to explore critically this nexus,[24–28] access to bulgecins (which have proven invaluable for LT study)[29–32] was required and we opted for total synthesis. We report here practical syntheses of bulgecins A, B and C. Bulgecin A was accomplished in 34 steps and 3% overall yield from

D-serine as the starting material. Furthermore, we revisit the biological activities of these compounds by modern methodology to disclose the unique potentiating activity of bulgecins.

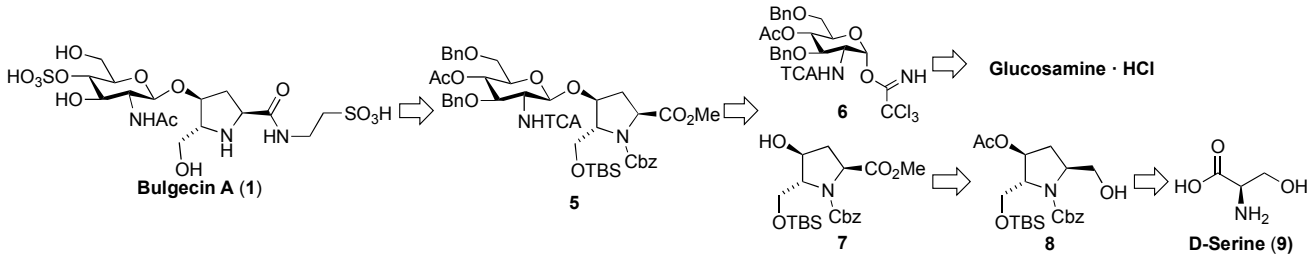
RESULTS and DISCUSSION

Synthesis of the Bulgecins. Bulgecin A (**1**) is a glycopeptide comprised of three segments: a sulfo-GlcNAc saccharide, a 3*R*-hydroxy-4*S*-(hydroxymethyl)-L-proline (numbering from C_α) core (named bulgecinine), and a taurine in amide linkage to the bugecinine core. Bulgecin A is the most abundant of the three bulgecins found as natural products (Figure 1: bulgecin B has a β-alanine amide replacing the taurine; bulgecin C lacks amide functionalization of its proline carboxylate: structures **2** and **3**, respectively). While several syntheses of bulgecinine were reported in the decades following the discovery of the bulgecins, only two syntheses of bulgecins have been reported. The first synthesis reported was that of bulgecin A by a group from Osaka University (without experimental details).[33] Their synthesis was followed by the synthesis (with experimental details) of bulgecin C by Barrett and Pilipauskas.[34, 35] The key decision points for our own synthetic plan (as guided by the useful disclosures in these previous syntheses) were the selection of a concise and high-yielding preparation of a protected bulgecinine, and the choice of a protected and activated GlcNAc glycosyl donor for the β-selective glycosylation. In both respects our own choices diverged from those of Wakamiya et al.[33, 36] and Barrett and Pilipauskas.[34, 35]

For the synthesis of protected bulgecinines, we were drawn to a classical method for the stereoselective synthesis of substituted pyrrolidines, that of a intramolecular aminomercuration of a γ-alkene to a carbamate-protected amine, followed by the retentive O_2-induced oxidative cleavage of the intermediate carbon-mercury bond to give a secondary alcohol.[37] The value of this methodology for the synthesis of bulgecinine was established by Khalaf et al.[38] and confirmed by Wang et al.[39] In this route the single stereocenter of the D-serine starting material sets the absolute stereochemistry of the two additional stereogenic carbons of the pyrrolidine. We therefore envisioned **5** as the key intermediate, disassembled retrosynthetically into the known GlcNAc donor **6** and the protected bulgecinine **7**, which in

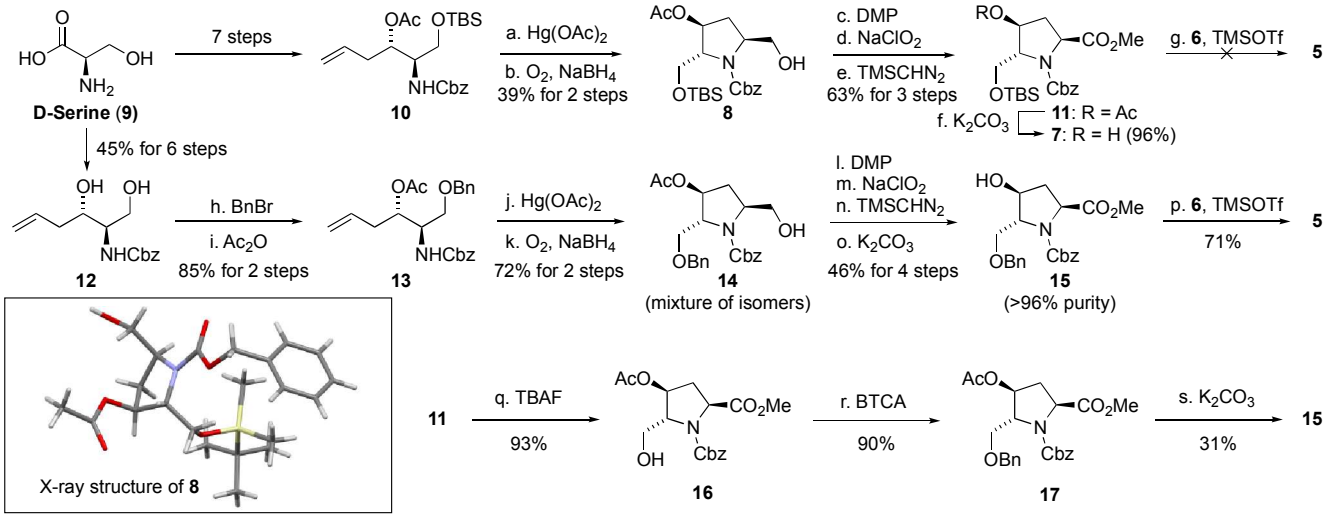
turn would be synthesized from D-serine (**9**) through Wang's trisubstituted pyrrolidine **8**. The synthetic plan is shown in Scheme 1.

Scheme 1. Synthetic plan for bulgecin A (1)



The GlcNAc donor **6** was prepared using a reported procedure.[40] Allyl carbamate **10** (Scheme 2) was prepared as described by Wang et al.[39] Its transformation to the protected pyrrolidine **8** (with confirmation of its structure by X-ray crystallographic analysis; see box in Scheme 2 and Figure S1), by successive aminomercuration and oxidative demercuration with oxygen gas, used a modified adaptation of Wang's procedure. Selective oxidation of the primary alcohol of 8 to the carboxylic acid used the sequential combination of Dess-Martin and Pinnick oxidations, with the resulting carboxylic acid transformed to the methyl ester (**11**) with $TMSCHN_2$. Zemplen-type removal of the *O*-acetyl group afforded the key protected bulgecinine derivative **7**.

Scheme 2. Synthesis of 5 from D-serine (9)[a]



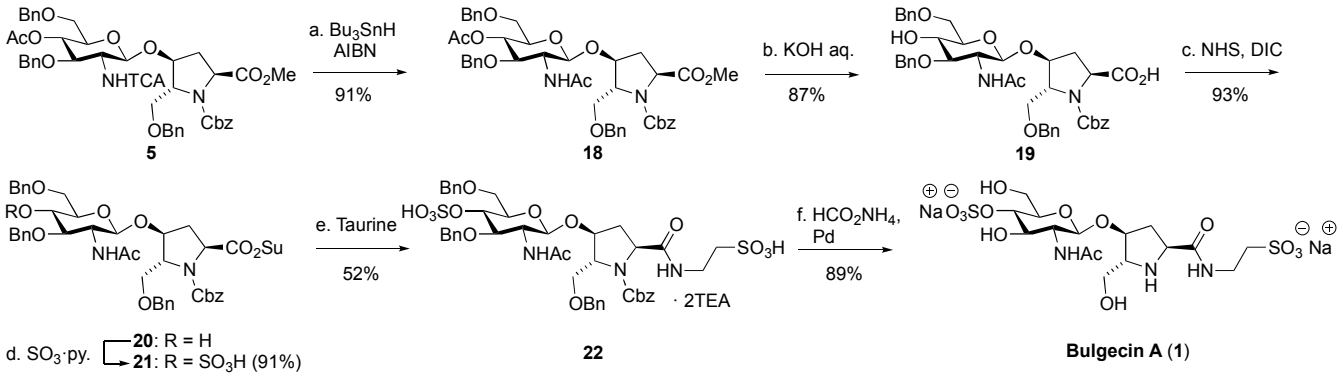
[a]Reagents and conditions: (a) $Hg(OAc)_2$, MeCN, 0 ºC, 1.5 h; EtOAc, brine, 2 h; (b) $NaBH_4$, O_2, DMF, 3 h; (c) DMP, DCM, 0 ºC to rt, O/N; (d) 2 M 2-methyl-2-butene in THF, *t*-BuOH, $NaClO_2$-NaH_2PO_4 in H_2O, 3 h; (e) 2 M $TMSCHN_2$ in hexane, toluene, MeOH, 2 h; (f) K_2CO_3, MeOH, 1.5 h, 96%; (g) **6**, TMSOTf, MS4A, DCM, 0 ºC, 3 h; (h) BnBr, Ag_2CO_3, toluene, O/N; (i) Ac_2O, pyridine, 0 ºC to rt, O/N; (j) $Hg(OAc)_2$, MeCN, 0 ºC to rt, O/N; EtOAc, brine, 1.5 h; (k) $NaBH_4$, O_2, DMF, 3 h; (l) DMP, DCM, 0 ºC to rt, 1.5 h; (m) 2 M 2-methyl-2-butene in THF, *t*-BuOH, $NaClO_2$-NaH2PO4 in H2O, 1 h; (n) 2 M $TMSCHN_2$ in hexane, toluene, MeOH, 1.5 h; (o) K_2CO_3, MeOH, 1 h; (p) **6**, TMSOTf, MS4A, DCM, 0 ºC, 3 h; (q) 1 M TBAF in THF, AcOH, O/N; (r) BTCA, TfOH, DCM, O/N; (s) K_2CO_3, MeOH, 3 h.

Glycosylation of **7** with GlcNAc donor **6** using TMSOTf activation gave a complex mixture of byproducts. Presuming that the TBS protecting group is not preserved under the acidic conditions, we decided to use the *O*-benzyl bulgecinine derivative **15** as the acceptor (Scheme 2). We derived the necessary *O*-benzylated allylcarbamate **13** by selective benzylation of the primary alcohol, followed by acetylation of diol **12**. Aminomercuration and successive oxidative demercuration gave **14**. The yield (72%) was comparable to the yield reported previously.[39] Oxidation and protecting group manipulation of **14** (as described for **7**) gave **15**. To confirm that the stereochemistry of **15** is identical to that of **7**, we also synthesized **15** from **11** (desilylation with TBAF, followed by *O*-benzylation using benzyl trichloroacetimidate with TfOH activation; third row of Scheme 2). In contrast to the glycosylation of **7**, glycosylation of **15** was a clean reaction that proceeded with satisfactory yield (71%).

The reductive dechlorination (Bu_3SnH, AIBN) of the non-participating *N*-trichloroacetyl group of **5** was uneventful, giving **18** in a 91% yield (Scheme 3). Mindful of the possibility of C_α epimeriza-

tion of the bulgecinine core, milder saponification conditions (compared to previous reports)[35] were used(aq KOH in *i*PrOH). The resulting carboxylic acid **19** was converted to *O*-succinimidyl active ester **20** and its secondary alcohol sulfated to afford **21**. The overall yield for these four steps from **5** was excellent (67%). The remaining two steps provided challenge. Acyl transfer to taurine occurred in moderate yield (52%), in part due to the difficult isolation and purification of **22**.

Scheme 3. Synthesis of bulgecin A (**1**)[a]

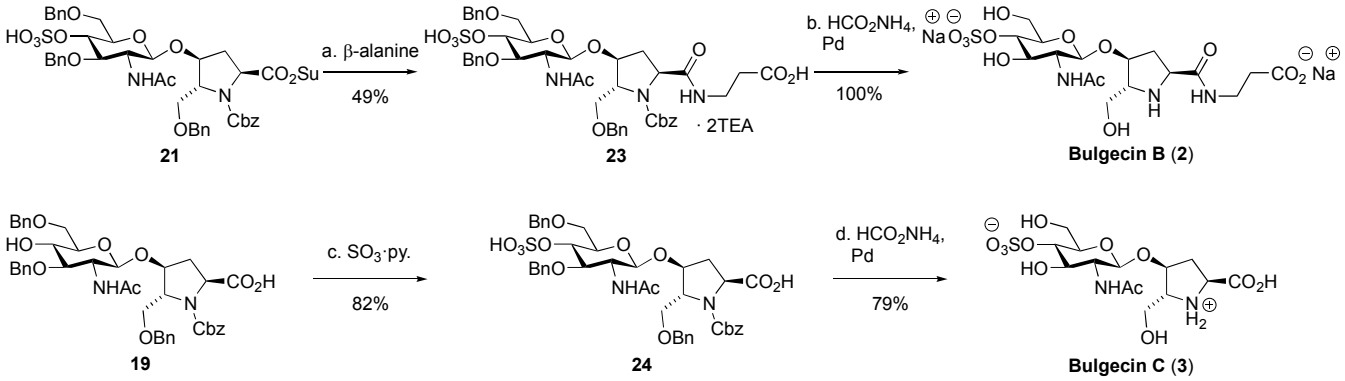


[a]Reagents and conditions: (a) Bu_3SnH, AIBN, toluene, rt to 90 ºC, O/N; (b) 4 M KOH aq., *i*-PrOH, 3 d; (c) NHS, DIC, DCM, 0 ºC to rt, O/N, (d) SO_3·py., DMF, 2 h; (e) taurine, TEA, H_2O, MeCN, O/N; (f) HCO_2NH_4, Pd black, MeOH, O/N.

In both previous syntheses of bulgecins difficulties were encountered with the hydrogenolysis required for the final deprotections.[33–35] We confirmed this difficulty. In our hands as well, transfer-hydrogenation conditions, as identified by Barrett and Pilipauskas, was the superior choice. However, while this procedure in our hands achieved full deprotection to bulgecin A (**1**), the sample had 15% of a by-product that could not be removed without substantial loss of product. Based on an observation that ammonium formate was a superior reducing agent compared to formic acid under transfer-hydrogenation conditions (increased yield of *O*-benzyl deprotection and reduced impurity levels),[41] we finally obtained pure bulgecin A (**1**) in excellent yield (89%) for this last step. We further observed that use of Na^+-form cation-exchange resin in the reaction work-up was superior to the use of H^+-form strongly acidic cation-exchange resin. This latter resin also gave an impurity. These observations sug-

gest that bulgecin A is not stable to strong acid. Bulgecin A was obtained from D-serine as the starting material in 34 synthetic steps and with a 3% overall yield. Syntheses of bulgecins B and C were accomplished from the intermediates used in the synthesis of bulgecin A (Scheme 4).

Scheme 4. Synthesis of bulgecin B (**2**) and C (**3**)[a]



[a]Reagents and conditions: (a) β-alanine, TEA, H_2O, DCM, O/N; (b) HCO_2NH_4, Pd black, MeOH, O/N; (c) SO_3·py., DMF, 3 h; (d) HCO_2NH_4, Pd black, MeOH, O/N.

Coupling of **21** with β-alanine gave precursor **23**, which was converted quantitatively into bulgecin B (**2**). Compound **24** was synthesized by *O*-sulfation of **19**. Transfer hydrogenation using ammonium formate gave bulgecin C (**3**) in good yield (79%).

Bulgecin A potentiation with a clinical β-lactam antibiotic as assessed by bacterial growth-curves. We assessed bulgecin A, B, and C in microbiological assays. We performed growth-curve assays[14, 31] for *P. aeruginosa*. These experiments were conducted in the presence of 2- to 8-fold below minimal-inhibitory concentration (sub-MIC) of the β-lactam antibiotic ceftazidime, in the absence of ceftazidime, in the presence of bulgecin (A, B, or C), and in the presence of bulgecin (A, B, or C) and sub-MIC of ceftazidime (Figure S2). Each synthetic bulgecin analog demonstrated comparable ability to potentiate ceftazidime against *P. aeruginosa*. For this reason, we continued our additional analyses with bulgecin A.

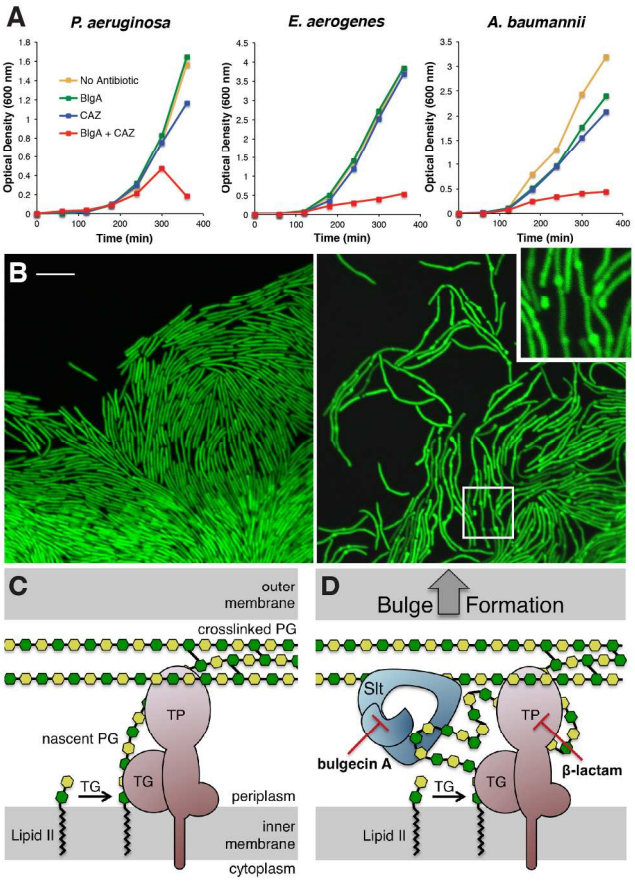


Figure 2. Bacterial growth-curve assays and bacterial bulge-formation as visualized by microscopy. (a) Growth-curve assays of *P. aeruginosa*, *E. aerogenes* and *A. baumannii*. The results for no antibiotic (yellow curve), 50 μg/mL bulgecin A (green curve), ceftazidime (CAZ; blue curve) and 50 μg/mL bulgecin A + CAZ (red curve) are shown. The sub-MIC concentrations of CAZ used for *P. aeruginosa*, *E. aerogenes* and *A. baumannii* were 0.28 μg/mL, 8 μg/mL, and 2 μg/mL, respectively. (b) Confocal microscopy demonstrating that GFP-labeled *P. aeruginosa* grown in the presence of sub-MIC of CAZ exhibits elongation without bulges (on the left) and those grown in the presence of CAZ and bulgecin A exhibit elongation with bulges (on the right). The boxed area is expanded to highlight the bulges. A 10-μm scale bar is given in the top left corner. (c) In cell-wall homeostasis, lipid II is polymerized to the nascent peptidoglycan chain, comprised of repeats of the *N*-acetylglucosamine (NAG, light-green hexagons)-*N*-acetylmuramylpentapeptide (NAM, dark-green hexagons) disaccharide, by the transglycosylase (TG). The transpeptidase (TP) crosslinks the nascent peptidoglycan to the growing edge of the cell wall. (d) β-Lactam antibiotics inhibit the TP activity, resulting in the accumulation of non-crosslinked nascent peptidoglycan, which serves as substrate for the lytic transglycosylase Slt in *P. aeruginosa.* Bulgecin A inhibits the Slt activity. Dual inhibition of Slt and TP results in the formation of the bulge, leading to the breached structural integrity of the cell wall.[22]

Next, we performed growth-curve assays for the four Gram-negative members of the ESKAPE panel of bacterial pathogens ("KAPE": *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* species). The ESKAPE panel members comprise the most problematic bacterial pathogens.[42–45] Figure 2 documents the potentiation of the ceftazidime activity for *P. aeruginosa*, *Enter-*

obacter aerogenes, and *A. baumannii*. The results for *K. pneumoniae* indicated no potentiation (data not shown), possibly implying lack of penetration of bulgecin A into the periplasmic space of this organism. The bacteria grew in the absence of the antibiotic, in the presence of sub-MIC of ceftazidime, or in the presence of bulgecin A by itself. However, potentiation by bulgecin A was seen at sub-MIC of ceftazidime for these three bacteria (Figure 2A). In the case of *P. aeruginosa*, bulgecin A caused rapid cell lysis once bacteria reached the mid-logarithmic phase of growth (approximately $OD_{600} = 0.5$) in the presence of ceftazidime. In contrast, bulgecin A and sub-MIC of ceftazidime caused sustained and early inhibition of growth of *E. aerogenes* and *A. baumannii*.

We visualized these events using a PAO1 strain of *P. aeruginosa* containing a mini-Tn7 chromosomal, constitutive GFP-expressing gene insertion, which fluoresces upon excitation at 488 nm with light emission at 525 nm. We performed swarm-motility assays (as described previously).[46, 47] Bulgecin A was combined with *P. aeruginosa* planktonic culture and spot inoculated at the plate center and ceftazidime was placed at a second site 20 mm away. These placements allowed bacteria to be exposed to bulgecin A prior to swarming towards ceftazidime. A control experiment omitted bulgecin A. Bacteria were imaged at the swarm colony edge. We saw the elongated phenotype in both cases due to bacterial exposure to sub-MIC of ceftazidime.[48, 49] However, in the presence of bulgecin A, we saw extensive and periodic bulge formation in elongated bacteria. The bacteria in this group also demonstrated an increase in girth. In the control experiment in the absence of bulgecin A, these bulges were absent (Figure 2B). The formation of bulges was documented previously by Imada and Kintaka by scanning electron microscopy and is the origin of the name of the natural product.[11, 18] The bulges are at the sites of cell division (mid-cell), whereby failure of the organism to divide in the presence of ceftazidime is coupled with the formation of the bulges (Figure 2B). We also showed that cells grown in the presence of bulgecin A, but without antibiotic, displayed no alteration in the phenotype (not shown). This observation is likely due to the multiplicity of LTs—11 are known in *P. aeruginosa*—with overlapping redundant activities.[27] Not all of the 11 LTs are expected to be inhibited by bulgecin A.[50]

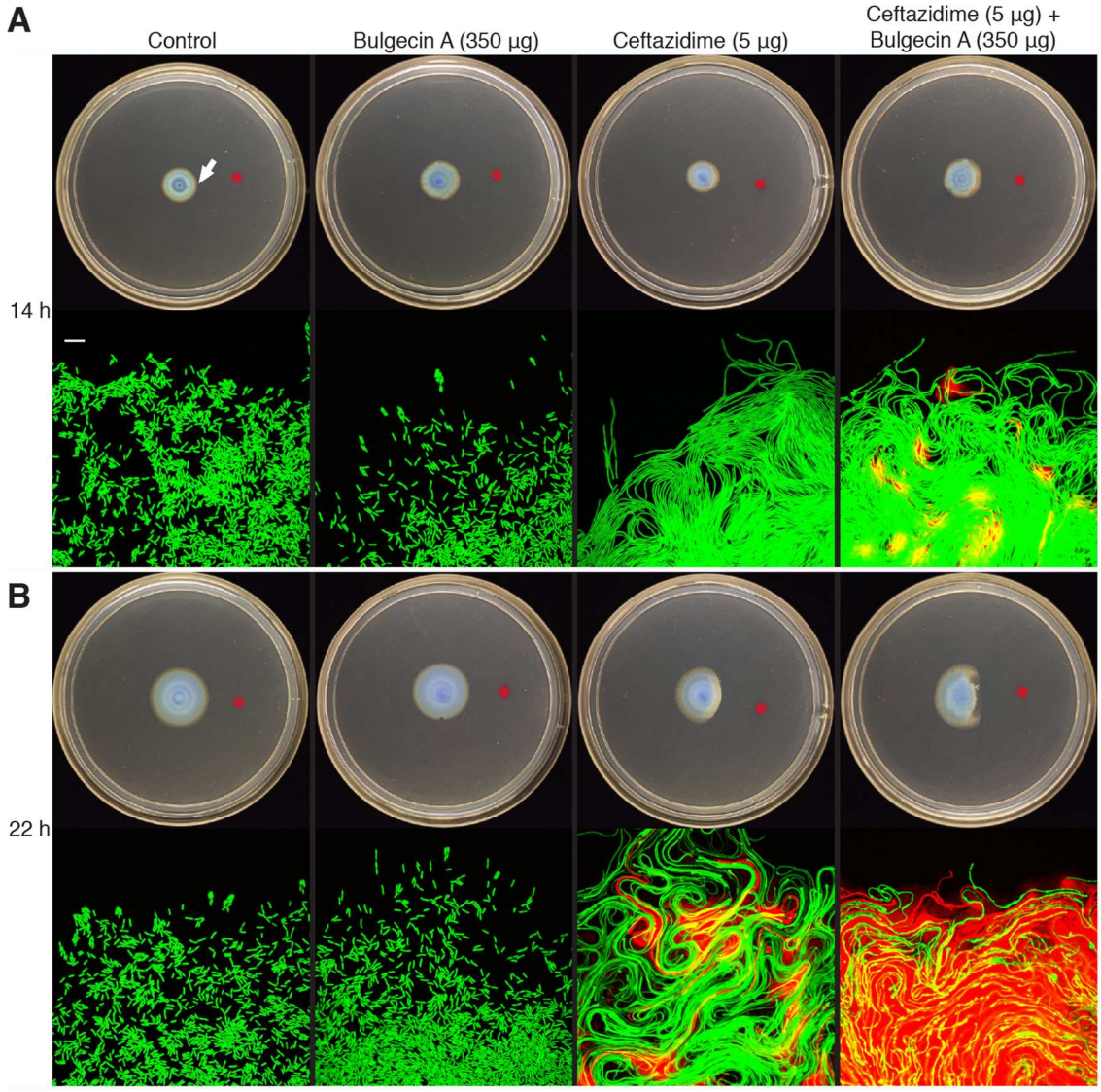


Figure 3. Bulgecin potentiation of the bactericidal activity of ceftazidime. GFP-labeled *P. aeruginosa* was spotted at the center of a 100 mm swarm plate and 350 μg bulgecin A and/or 5 μg CAZ were/was spotted at the red dot. Bacteria were imaged at (a) 14 hours and (b) 22 hours post-inoculation. The fluorescent images (100× magnification) were captured at the growing edge. As a representative example, an arrow marks the imaging site for the top left plate. Red fluorescence indicates cell lysis. A 10-μm scale bar is given in the left-most panel.

Bulgecin A potentiation of a clinical β-lactam antibiotic as visualized by microscopy. A distinct and reproducible feature of *P. aeruginosa* growth in the presence bulgecin A and sub-MIC of ceftazidime is that the rate of growth was largely unperturbed until the mid-log phase, when cell lysis occurs. The reason for this lag, not seen with the other two organisms, is not known. We analyzed this event using the swarm assays, with one exception. The bacteria grown to mid-log phase were placed at

one position on an agar plate and a mixture of bulgecin A and ceftazidime was placed at a second position on the plate at a distance of 20 mm. This permutation was intended to mimic the effect of encounter of bacteria with two drugs in combination (bulgecin A and ceftazidime). At the conclusion of the experiment propidium iodide (λ_{exci} 561 nm, λ_{emi} 595 nm) was imaged to visualize DNA liberated by cell lysis.[51] We imaged the swarm colony edge (white arrow, top left panel) for each case (Figure 3A) at 14 hours, and at 22 hours, after inoculation. In the case of control bacteria and that of bacteria in the presence of bulgecin A alone, we saw no effect. In the presence of ceftazidime alone, we saw an elongated cell phenotype after 14 hours (Figure 3A). Cell lysis was not detected. We began to see cell lysis by 22 hours (Figure 3B) in the presence of ceftazidime alone. In contrast, when bulgecin A and ceftazidime were present at 14 hours, the elongated-bulged bacteria had already begun to lyse (Figure 3A, bottom right corner). Significant cell death at 22 hours is seen only with the combination of the two (Figure 3B, bottom right corner).

CONCLUSIONS

The prescient discovery of Imada et al. of the bulgecins was made in an era when clinical options for treatment of infections were not as limited as the present. Some infections by Gram-negative bacteria may be treated currently with only a single antibiotic and certain infections could be fatal by more than 50%, such that a return to the pre-antibiotic era has become subject of discussion.[52–55] We revisited bulgecins with this clinical backdrop. Our practical syntheses of the bulgecins, and our microbiological proof that their potentiation is *bactericidal at sub-MIC of ceftazidime*—a β-lactam used clinically to treat Gram-negative ESKAPE infections—merit a fresh look at bulgecins. We hasten to add that the mixture of bulgecin A and sub-MIC of ceftazidime results in early onset of bactericidal activity, which is critical for rapid reduction of bacterial load in an infection. Bulgecins as potentiators of antibacterial activity hold great promise. This study is not a culmination for the bulgecins, but emphatic evidence supporting their further mechanistic study.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Figure S1–S4, Table S1 and S2, X-ray structure details of 8, experimental details of biological assays, synthetic procedures and characterization data, NMR spectra of newly synthesized compounds and key compounds (PDF)

Crystallographic structure of 8 (CIF)

Abbreviations

PBP, penicillin-binding protein; LT, lytic transglycosylase; GlcNAc, *N*-acetyl glucosamine; BlgA, bulgecin A; CAZ, ceftazidime; MIC, minimum-inhibitory concentration; NAG, *N*-acetylglucosamine; NAM, *N*-acetylmuramic acid; GFP, green fluorescent protein.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

This work was supported by grants GM61629 (SM) and AI113219 (JDS) from the NIH. ST was supported by a postdoctoral fellowship from the Uehara Memorial Foundation. DAD was supported by NIH Training Grant T32GM075762 and by the ECK Institute for Global Health.

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