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Derivatives of Ribosome-Inhibiting Antibiotic Chloramphenicol Inhibit the Biosynthesis of Bacterial Cell Wall

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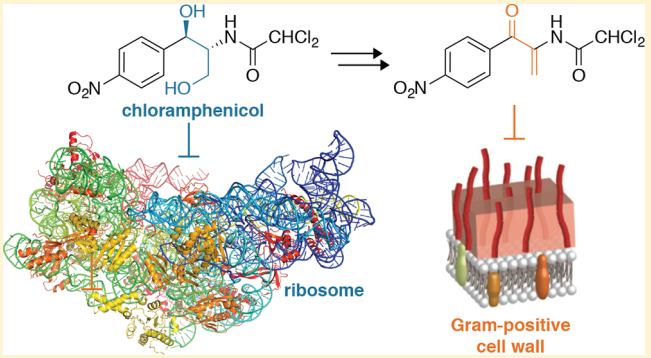
Supporting Information

ABSTRACT: Here, we describe the preparation and evaluation of α,β-unsaturated carbonyl derivatives of the bacterial translation inhibiting antibiotic chloramphenicol (CAM). Compared to the parent antibiotic, two compounds containing α,β-unsaturated ketones (**1** and **4**) displayed a broader spectrum of activity against a panel of Gram-positive pathogens with a minimum inhibitory concentration range of 2−32 μg/mL. Interestingly, unlike the parent CAM, these compounds do not inhibit bacterial translation. Microscopic evidence and metabolic labeling of a cell wall peptidoglycan suggested that compounds **1** and **4** caused extensive damage to the envelope of *Staphylococcus aureus* cells by inhibition of the early stage of cell wall peptidoglycan biosynthesis. Unlike the effect of membrane-disrupting antimicrobial cationic amphiphiles, these compounds did not rapidly permeabilize the bacterial membrane. Like the parent antibiotic CAM, compounds **1** and **4** had a bacteriostatic effect on *S. aureus*. Both compounds **1** and **4** were cytotoxic to immortalized nucleated mammalian cells; however, neither caused measurable membrane damage to mammalian red blood cells. These data suggest that the reported CAM-derived antimicrobial agents offer a new molecular scaffold for development of novel bacterial cell wall biosynthesis inhibiting antibiotics.



KEYWORDS: *antibiotics, bacterial resistance, chloramphenicol, cell envelope, in vitro translation*

Consisting of the three ribosomal ribonucleic acids (rRNAs), 16S, 23S, and 5S, and over 50 proteins, the bacterial ribosome, which carries out the translation process, contains the target sites for many clinically used antibiotics.[1−5] One such class of antibiotics is the phenicols, which includes the natural product chloramphenicol (CAM) and its two semisynthetic derivatives thiamphenicol (TAM) and florfenicol (Figure 1).[6]

CAM was discovered through its isolation from *Streptomyces venezuelae* and *Streptomyces phleochromogenes* var. *chloromyceticus* in 1947.[7] Notably, CAM is one of the few natural products discovered to date that contains both a nitrophenyl ring and a halogen functional group.[8] CAM inhibits translation by binding at the peptidyltransferase center of the 50S subunit of the bacterial ribosome in a position that overlaps with the aminoacyl moiety of the A-site tRNA.[9,10] An X-ray crystal structure of CAM bound to the 50S subunit of the bacterial ribosome revealed that its primary and secondary alcohols as well as the carbonyl of its dichloroacetamide functionality form hydrogen bonds with nucleotides of the 23S rRNA (Figure 1A).[11,12]

As is the fate of all antibiotics, the percentage of bacteria that have evolved resistance to CAM is on the rise. The most common mechanism of resistance is deactivation through enzymatic modifications including *O*-acetylation by acetyltransferases or, in some cases, *O*-phosphorylation by CAM phosphotransferases.[13−15] Resistance to CAM is also acquired by mutations or modifications of nucleotides in the 23S rRNA, through decreased cell permeability, and through expression of multidrug efflux proteins that reduce its intracellular concentration.[8] In addition to emergence of resistance, the potent antibacterial activity of CAM is overshadowed by its reversible bone marrow depressing effect that can, in severe cases, lead to lethal aplastic anemia and is therefore a serious obstacle that limits its use in cases of systemic infections.[16]

The search for phenicols with improved therapeutic properties has so far yielded two clinically useful CAM derivatives. TAM (Figure 1B), also known as thiophenicol, is a *p*-methylsulfonylphenyl derivative of CAM that possesses a similar spectrum of activity.[17,18] To address the emerging resistance to both CAM and TAM, conversion of the primary alcohol of TAM to the corresponding fluorine functionality resulted in florfenicol (Figure 1B), a semisynthetic phenicol that is particularly effective against CAM-resistant bacteria and

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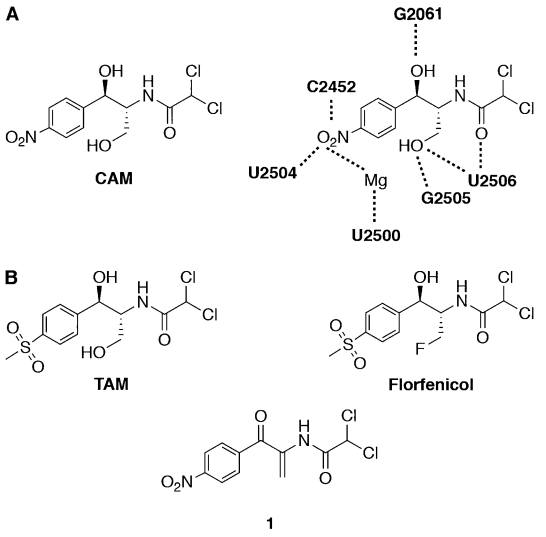

Figure 1. (A) Structure of CAM and its interactions with 23S rRNA nucleotides. (B) Structures of synthetic members of the phenicol class of antibiotics and of the antibacterial CAM derivative **1**.

is used solely in veterinary medicine.[19,20] Not surprisingly, evolution of resistance did not skip florfenicol, and of the known CAM resistance genes, a limited number also confer resistance to florfenicol.[21,22]

Among the numerous CAM analogues generated to date that have not reached the clinic, our attention was drawn to the unique structure of the CAM derivative *α*-dichloroacetamido-*p*-nitroacrylophenone (compound **1**, Figure 1B) that was reported in 1969 by Kono et al.[23,24] This CAM derivative displayed potent antibacterial activity against a CAM-resistant *Staphylococcus aureus*. This activity was attributed to the fact that compound **1** lacks the primary and secondary alcohols of CAM that serve as the target for enzymatic inactivation by acetyl- and phosphotransferases. Since the essential roles of the primary and secondary alcohols of CAM in facilitating binding to the peptidyltransferase center of the bacterial ribosome was unraveled decades after the discovery of **1**, we questioned its function as an inhibitor of bacterial translation.[11,12] Herein, we report a structure−activity relationship (SAR) and a mode of action study of this CAM derivative.

RESULTS AND DISCUSSION

Synthesis. We first developed a robust synthetic route for the generation of enone and enal analogues of compound **1** for SAR studies. The enone and enal derivatives were generated from commercially available CAM and TAM (compounds **1a** and **2a**, respectively, Scheme 1A) or from the unnatural CAM derivatives **3a**−**5a**. Compounds **3a**−**5a** were prepared via acylation of commercially available (1*R*,2*R*)-(−)-2-amino-1-(4-nitrophenyl)-1,3-propanediol (also known as CAM base). The enone derivatives **1**−**5** were synthesized in two steps from starting materials **1a**−**5a**. Selective esterification of the primary alcohol of **1a**−**5a** afforded compounds **1b**−**5b** with a primary alcohol protected by a pivaloyl ester. Dess-Martin oxidation of the secondary alcohol of **1b**−**5b** (35−100% isolated yields) followed by chromatography on silica gel, which catalyzed the elimination of the *O*-pivaloyl ester, gave the desired enone derivatives **1**−**5** in 81−100% isolated yields (Scheme 1A).

Enal derivatives **6**−**9** were prepared from CAM (**1a**), TAM (**1b**), and their analogues **3a**−**4a** in a four-step sequence (Scheme 1B). The primary alcohols of compounds **1a**−**4a** were selectively protected with a *tert*-butyldimethylsilyl (TBDMS) group to afford compounds **6a**−**9a** in 34−100% isolated yields. The secondary alcohol of compounds **6a**−**9a** was protected with a methoxymethyl (MOM) group to afford compounds

Scheme 1. Synthesis of CAM-Derived Enone and Enal Analogues

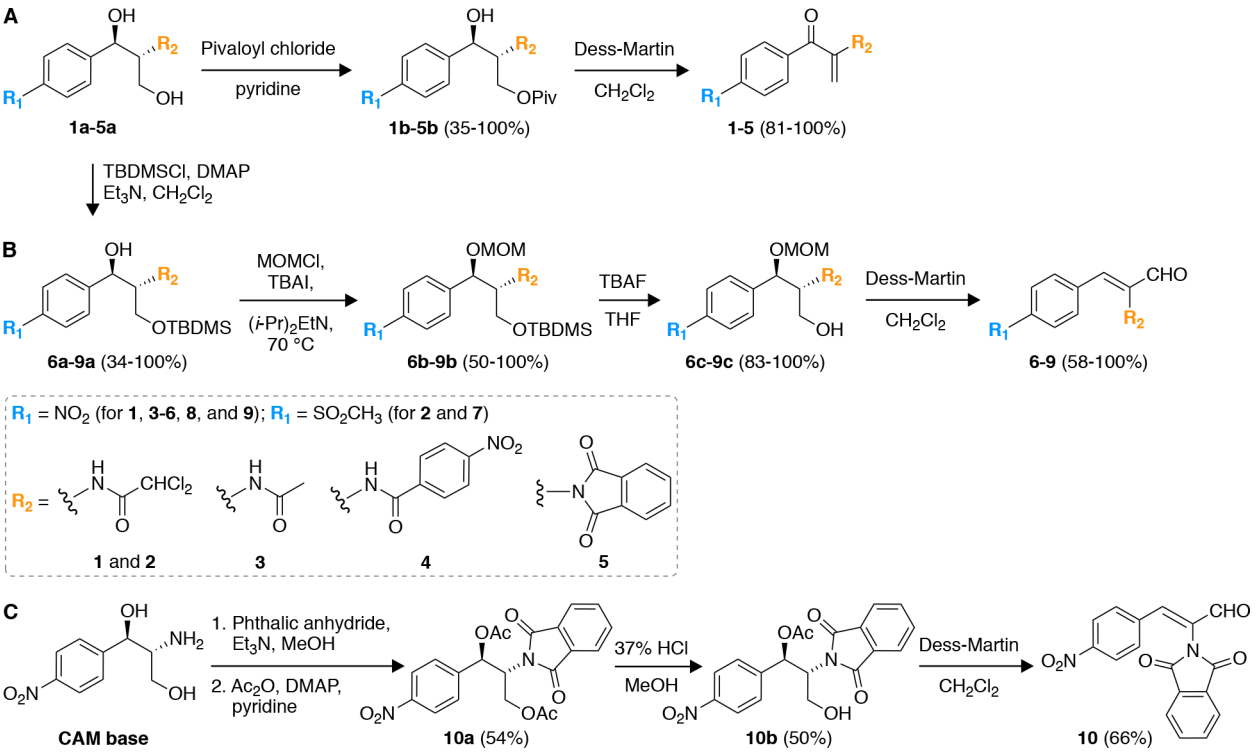

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Table 1. Minimal Inhibitory Concentration (MIC) Values in μg/mL[a]

bacterial strain[b]		compound											
		1	2	3	4	5	6	7	8	9	10	CAM	TAM
Gram-positive	A	8	64	32	8	>64	>64	>64	>64	>64	>64	4	>64
	B	8	32	32	8	>64	>64	>64	>64	>64	>64	64	>64
	C	8	64	16	8	>64	>64	>64	>64	>64	>64	64	>64
	D	4	16	16	4	>64	>64	>64	>64	64	>64	4	32
	E	4	64	16	4	>64	>64	>64	>64	>64	>64	4	16
	F	4	32	16	4	64	64	>64	>64	64	>64	4	8
	G	2	32	16	4	>64	64	>64	>64	64	>64	16	8
	H	8	32	16	8	>64	>64	>64	>64	>64	>64	8	16
	I	8	32	32	8	>64	>64	>64	>64	>64	>64	8	8
	J	8	64	32	8	>64	>64	>64	>64	>64	>64	8	16
	K	8	16	32	8	64	64	>64	>64	64	>64	2	2
	L	32	64	64	32	>64	>64	>64	>64	>64	>64	8	8
	M	8	32	16	4	>64	>64	>64	>64	>64	>64	2	4
	N	16	64	32	16	>64	>64	>64	>64	>64	>64	>64	>64
	O	32	>64	64	16	>64	>64	>64	>64	>64	>64	4	8
Gram-negative	P	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	4	>64
	Q	64	64	32	>64	>64	>64	>64	>64	>64	>64	4	32
	R	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	4	32
	S	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
	T	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	4	64
	U	64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
	V	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	8	>64

[a]MIC values were determined using the double-dilution method. The MIC for each compound was determined in triplicate in two independent sets of experiments. [b]MIC values were determined against the following strains. Gram-positive: **A** = *Staphylococcus aureus* ATCC 33592; **B** = *S. aureus* ATCC BAA-43; **C** = *S. aureus* ATCC 33591; **D** = *S. aureus* ATCC 43300; **E** = *S. aureus* USA600; **F** = *S. aureus* G1; **G** = *S. aureus* C2; **H** = *S. aureus* ATCC 29213; **I** = *S. aureus* Cowan; **J** = *Listeria monocytogenes* ATCC 19115; **K** = *Streptococcus pyogenes* ATCC 14289; **L** = *Enterococcus faecalis* ATCC 29212; **M** = *Bacillus megaterium* ATCC 14945; **N** = *Enterococcus feacalis* ATCC 51299; **O** = *Enterococcus faecium* ATCC 19434. Gram-negative: **P** = *Escherichia coli* ATCC 25922; **Q** = *E. coli* NR698; **R** = *Salmonella enterica* ATCC 14028; **S** = *Pseudomonas aeruginosa* ATCC 27853; **T** = *Klebsiella pneumoniae* ATCC 27736; **U** = *Acinetobacter baumannii* ATCC 19606; **V** = *Enterobacter cloacae* ATCC 13047.

6b–9b in 50–100% isolated yields, followed by removal of the TBDMS group to afford compounds **6c–9c** (83–100% isolated yields). Using a strategy similar to that developed for the generation of the enone derivatives **1–5**, Dess-Martin oxidation of the primary alcohol of **6c–9c** followed by elimination of the resulting oxidation product during flash chromatography on silica gel afforded the desired enal derivatives **6–9** in 58–100% isolated yields.

Finally, enal **10** was generated in three steps from CAM base (Scheme 1C). Selective removal of the acetyl ester from the primary hydroxyl of the di-*O*-acetyl-*N*-phthalimide CAM derivative **10a** under acidic conditions afforded the mono-acetylated compound **10b** in 50% isolated yield. Dess-Martin oxidation of **10b** followed by elimination of the resulting oxidation product during flash chromatography on silica gel afforded enal **10** in 66% isolated yield. The structures of all final compounds were confirmed and characterized by ^{1}H and ^{13}C NMR (Figures S1–S20) as well as high-resolution mass spectrometry.

Antibacterial Activity. The antibacterial activity of compounds **1–10** against a panel of 22 bacterial strains was evaluated using the broth double-dilution method.[25] As control antibiotics, we tested the phenicols CAM and TAM from which compounds **1–10** were derived. The chosen panel of Gram-positive pathogens was composed of 15 bacterial strains (A–O). Of nine strains of *S. aureus* (strains A–I), seven (strains A–G) are methicillin-resistant *S. aureus* (MRSA). Notably, the panel of Gram-positive bacteria included vancomycin-resistant *E. faecalis* and *E. faecium* (strains N and O, respectively), drug-resistant Gram-positive pathogens for which new antibiotics are needed. To investigate the antimicrobial activity of compounds **1–10** against Gram-negative bacteria, we chose a panel of seven representative bacterial pathogens (P–V). To exclude the possibility of poor anti-Gram-negative activity due to permeability limitations, activity was evaluated against the *Escherichia coli* (*E. coli*) NR698 (LptD4213 mutant, strain Q), which has an outer membrane with increased permeability.[26]

The minimum inhibitory concentration (MIC) values of the parent CAM and TAM and their derivatives **1–10** are presented in Table 1. Of the tested enones **1–5**, compounds **1** and **4** exhibited antibacterial activity against all Gram-positive strains in the panel with MIC values in the range of 2–32 μg/mL. Notably, these two compounds were potent against Gram-positive strains B, C, and N that were highly tolerant to the parents CAM and TAM (MICs $\geq$ 64 μg/mL). All of the tested Gram-negative strains, including LptD4213 mutant *E. coli* strain that has a more permeable outer membrane, were tolerant to all derivatives, including compounds **1** and **4** (MICs $\geq$ 64 μg/mL). These data suggest that limited outer membrane permeability is unlikely to account for the lack of anti-Gram-negative activity of compounds **1** and **4** and that it is possible that the target(s) of these antibacterial agents may exist only in Gram-positive bacteria or differ in structure from the corresponding target(s) in Gram-negative bacteria.

Analysis of the antibacterial activity experiments provided several interesting SAR insights. None of the tested enals **6–10** displayed antibacterial activity. More specifically, unlike enones **1** and **4**, which displayed the most potent antibacterial activity,

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the corresponding enals **6** and **9**, respectively, were inactive, indicating that the enone structure is necessary for antibacterial activity. Enones **1** and **2** differ structurally solely by the substitution on their phenyl ring (*p*-nitro and *p*-methylsulfonyl, respectively); however, whereas **1** displayed the highest antibacterial potency against the tested panel of bacteria, **2** exhibited modest to poor antibacterial activity. These data indicate that the *para*-positioned nitro group contributes significantly to the interactions of compounds **1** and **4** with their target(s). Enones **1** and **3** differ structurally only by the substitution on the amide moiety (dichloroacetamide and acetamide, respectively). The antimicrobial activity of **3** was modest compared to that of **1** indicating that the dichloroacetamide group is important for the antibacterial activity of compound **1**. Nevertheless, enones **4** and **1** that differ solely by their amide substitution (*p*-nitrobenzamide and dichloroacetamide, respectively) displayed a similar antibacterial activity potency and spectrum, suggesting that there is some tolerance to structural changes at the amide substituent.

Time-Kill Kinetic Test. To investigate whether compounds **1** and **4** exert a bactericidal or bacteriostatic effect, we studied growth kinetics in an assay with *S. aureus* ATCC 29213 (strain H, a quality control strain). Briefly, bacterial cultures were treated with CAM, compound **1**, compound **4**, and the cationic amphiphile 6′-*S*-tetradecyl tobramycin (S-14)[27−30] at concentrations 2-fold higher than their MICs. S-14 is known to be bactericidal and served as a control. The culture was incubated at 35 °C. After 0, 1, 2, 4, 6, and 24 h, 20 μL of the suspension was serially diluted and inoculated on agar plates. After 24 h of incubation at 35 °C, the numbers of viable bacteria colonies were counted, and the results are summarized in Figure 2.

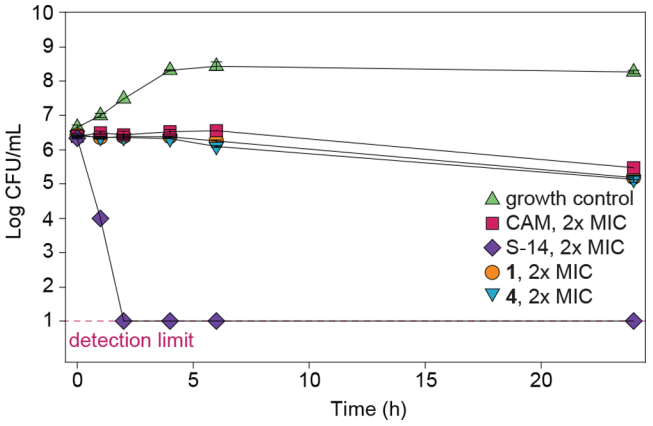

Figure 2. Time-kill kinetics of *S. aureus* ATCC 29213 (strain H) with compound **1**, compound **4**, CAM, and S-14 at 2× MIC. After 0, 1, 2, 4, 6, and 24 h, an aliquot (20 μL) was diluted (10^2-, 10^3-, 10^4-, and 10^5-fold) into saline and 10 μL of each dilution was plated in duplicate on Mueller-Hinton agar plates. After 24 h (starting from each tested time point) of incubation at 35 °C, the number of colonies on each plate was counted. Time-kill assays were analyzed by determining the reductions in viable count (CFU/mL).

No viable bacteria were detected in the sample treated with the membrane disrupting antimicrobial cationic amphiphile S-14 after 2 h. In contrast, no significant changes in numbers of viable colonies of compounds **1** and **4** and of the parent antibiotic CAM were observed over the first 6 h of incubation. Moreover, less than 1 order of magnitude decreases were observed in number of viable colonies after 24 h of incubation with compounds **1**, **4**, or CAM. These results suggest that, similarly to CAM and as opposed to the rapidly acting membrane disrupting cation amphiphile S-14, compounds **1** and **4** exert a bacteriostatic antibacterial effect even at 2-fold over their MIC.

***In Vitro* Resistance Selection Experiment.** Bacteria evolve resistance to antimicrobial agents, and it is desirable that resistance does not evolve rapidly. To study the potential of compounds **1** and **4** to induce resistance compared to CAM, we exposed *S. aureus* ATCC 29213 (strain H) to subinhibitory concentrations of these agents during 12 successive subcultures.[31] Briefly, the tested bacterium was exposed to subinhibitory concentrations (0.5× MIC) of compounds **1**, **4**, and CAM. The ratios of the measured MIC values for each of the 12 passages were then determined. Data are presented in Figure 3.

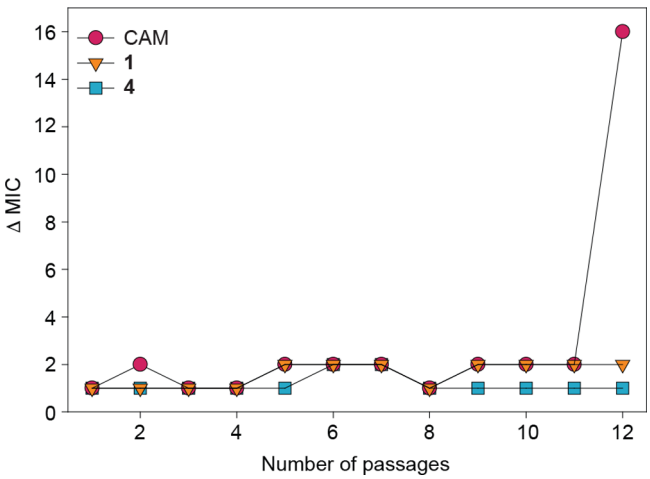

Figure 3. Comparative study on the emergence of resistance in *S. aureus* ATCC 29213 (strain H) after 12 serial passages in the presence of compound **1**, compound **4**, and CAM. Relative (Δ) MIC is the normalized ratio of MIC obtained for a given subculture to MIC obtained upon first exposure.

The bacteria developed resistance to the parent antibiotic CAM: After 12 passages, the MIC value for CAM was 16-fold higher than it was after the first passage. In contrast, this bacterium showed low propensity to develop resistance to compounds **1** and **4** with no increase in MIC value for **4** and only a 2-fold increase for **1** after 12 passages. These results suggest that compounds **1** and **4** are less prone than CAM to induce the evolution of resistance in *S. aureus*.

Inhibition of Bacterial Translation. To study the mode of action of the CAM-derived enones, we first tested the possibility that, like the parent phenicol CAM, these compounds act by binding to the bacterial ribosome, thereby inhibiting bacterial translation. We evaluated the effect of CAM as well as that of enones **1** and **4** on translation in commercially available cell-free extracts from *E. coli*[32] (Table 2 and Figure S21). Enones **1** and **4** were more than 2 orders of magnitude less potent than the parent CAM as inhibitors of *in vitro* prokaryotic translation. These results suggest that, whereas the parent antibiotic CAM inhibits bacterial translation, neither compound **1** nor **4** does so efficiently. Thus, these CAM derivatives likely exert their antibacterial activity via a different mode of action.

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Table 2. Prokaryotic *in Vitro* Translation 50% Inhibitory Concentration (IC_{50}) Values[a]

compound	IC_{50} (μg/mL)
1	168 ± 57
4	86 ± 10
CAM	0.80 ± 0.09

[a]Inhibition of luciferase translation was quantified in a coupled transcription/translation assay using *E. coli* S30 extracts. Experiments were performed in duplicate.

Transmission Electron Microscopy-Based Study of Effects on Bacterial Cell Shape. A large percentage of the antibiotics in clinical use, including CAM, target the bacterial ribosome. Since *in vitro* translation experiments suggested that neither compound **1** nor **4** likely target the bacterial ribosome, we next explored the possibility that they may affect bacterial cell envelope assembly. The complex multilayered structure bacterial cell envelope and the biosynthetic pathways involved in its biogenesis are targets for numerous antibiotics, including β-lactams, glycopeptides, and lipopeptides. Transmission electron microscopy (TEM) allows visualization of morphological changes of the membrane and cell wall ultrastructure under native conditions that are indicative of perturbation of cell envelope biosynthesis or assembly. We obtained TEM images of *S. aureus* ATCC 29213 (strain H) cells that were untreated or treated with either compound **1** or **4** (Figures 4 and S22). As positive controls, cells were treated with the membrane-disrupting antimicrobial cationic amphiphile S-14 and oxacillin, a β-lactam that interferes with the synthesis of peptidoglycan.[33]

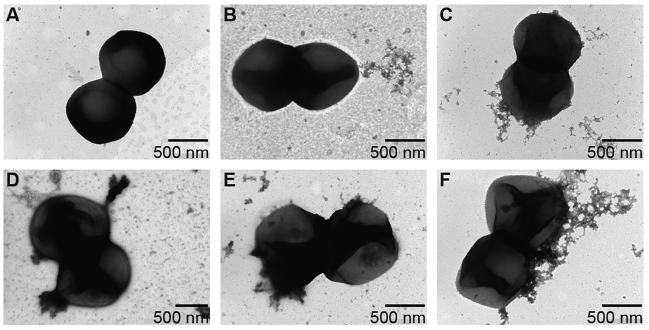

Figure 4. TEM images of *S. aureus* ATCC 29213 (strain H) incubated for 1 h at 37 °C with compounds at 2× MIC. (A) Untreated cells. (B) Cells treated with CAM. (C) Cells treated with S-14. (D) Cells treated with oxacillin. (E) Cells treated with compound **1**. (F) Cells treated with compound **4**.

The cell envelopes of untreated cells and CAM-treated cells were intact (Figure 4A,B, respectively). This observation is in agreement with the fact that the antimicrobial activity of CAM results from its bacterial translation inhibiting activity and not from direct perturbation of the bacterial cell wall or cell membrane assembly. As expected, the bacterial cells treated with the membrane disrupting cationic amphiphile S-14 (Figure 4C) appeared damaged with extensive ruptures. Finally, significant deformations and decomposition were evident in the cell envelope of bacteria treated with the peptidoglycan transpeptidase inhibitor oxacillin (Figure 4D). Interestingly, unlike the parent antibiotic CAM, the surfaces of cells treated with CAM-derived compounds **1** and **4** were deformed and ruptured (Figure 4E,F, respectively). Taken together with the dramatic reduction in the inhibition of translation compared to the parent CAM, evidence from the TEM images suggest that, unlike the parent antibiotic CAM, compounds **1** and **4** exert their antibacterial activity through perturbation of the bacterial cell envelope biosynthesis or assembly.

Evaluation of Bacterial Cell Membrane Perturbation Effects. Unlike membrane disrupting antibiotics such as lipopeptides[34] and several classes of synthetic antimicrobial cationic amphiphiles derived from amino sugars that have been reported to date,[35−37] compounds **1** and **4** are not chemically defined as cationic amphiphiles. Nevertheless, the extensive deformation that these compounds caused to the tested Gram-positive cells after a short 1 h incubation at concentrations 2-fold higher than their respective MIC values suggested that they may act by perturbation of the bacterial membrane integrity.

To investigate the possible effects of compounds **1** and **4** on bacterial membrane integrity, we performed a propidium iodide (PI) test with *S. aureus* ATCC 29213 (strain H) treated with these CAM derivatives.[38] Cells with damaged membranes become permeable to PI and are stained with this red fluorescent dye. For the PI cell permability experiments, bacterial cells were incubated with either compound **1** or **4** at MIC for 1 h, after which the samples were stained with PI and visualized by fluorescence microscopy (Figure 5). As controls, we tested the effects of CAM, S-14, and oxacillin on bacterial cell permeability.

Untreated and CAM-treated *S. aureus* ATCC 29213 cells (strain H) were not stained with PI under the experimental conditions (Figure 5A−C,D−F, respectively). This result can be rationalized by the fact that CAM, which inhibits bacterial protein synthesis, has no direct effect on the permeability of the bacterial cell within the incubation period used here. In contrast, as expected, almost all of the bacteria in the sample that was treated with the membrane disrupting antimicrobial cationic amphiphile S-14 were stained with PI (Figure 5G−I). Compared to the untreated bacteria, no significant increase in PI stained cells was observed in the sample treated with the cell wall biosynthesis inhibitor oxacillin (Figure 5J−L), indicating that the inhibition of cell wall biosynthesis did not cause an increase in the bacterial membrane permeability under the experiment conditions. Like oxacillin, neither compound **1** nor **4** significantly increased the permeability of the bacterial cells to PI (Figure 5M−O,P−R, respectively). Taking into account the observed bacteriostatic effect of compounds **1** and **4** on *S. aureus* ATCC 29213 cells, the results of the PI assay further support that these compounds do not directly interfere with the bacterial membrane integrity and likely cause the cell shape damage observed in the TEM images through perturbation of bacterial cell wall biosynthesis.

Inhibition of Peptidoglycan Biosynthesis. To test if the bacterial cell wall is targeted by compounds **1** and **4**, we investigated whether or not these compounds alter peptidoglycan biosynthesis. It was previously demonstrated that metabolic incorporation of *R*-2-amino-3-azidopropanoic acid, an azido-D-alanine derivative, followed by attachment of a fluorescent dye using a copper-free click reaction enables *in vivo* visualization of peptidoglycans. This method was successfully implemented for visualization of the effects of different cell wall biosynthesis inhibiting antibiotics.[39] Treatment of bacteria with fosfomycin, a drug that inhibits one of the early stages of peptidoglycan biosynthesis, completely abrogates the incorporation of the azido-D-alanine derivative. In contrast, penicillin, which inhibits transpeptidation of the peptidoglycan chains in

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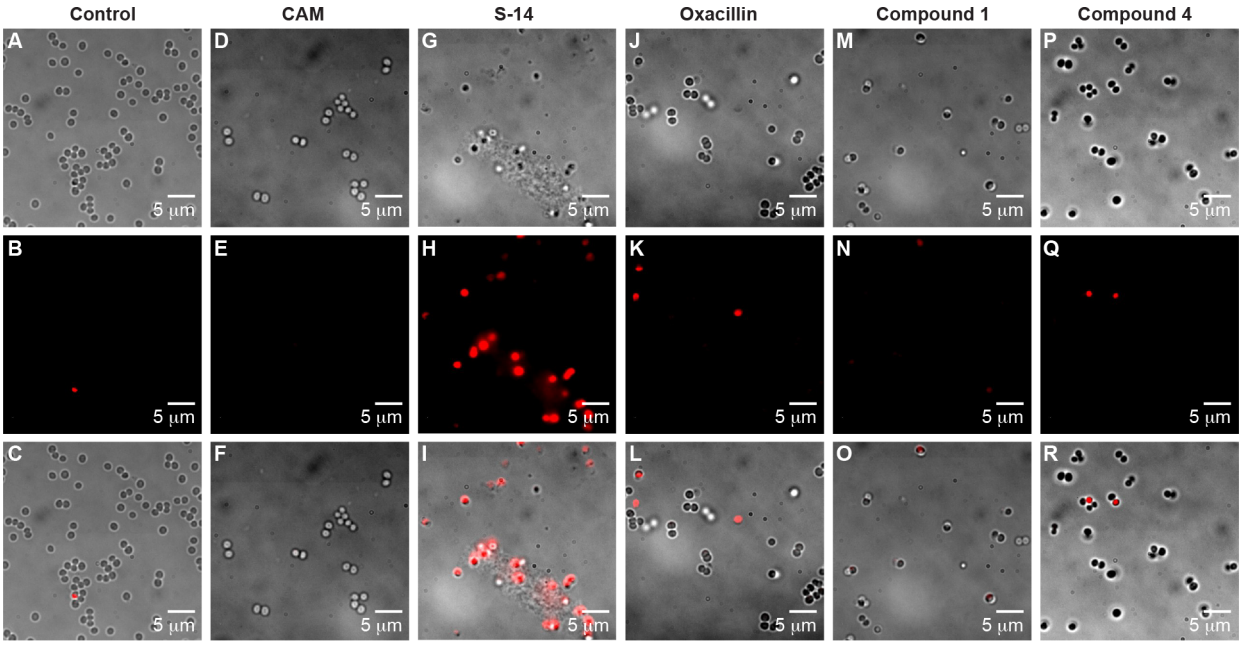

Figure 5. Bright field (top), fluorescence images of PI (537/26 nm excitation and 607/36 nm emission, red; middle), and merged images (bottom) of cultures of *S. aureus* ATCC 29213 (strain H) incubated for 1 h at 37 °C and 1× MIC. (A–C) Untreated cells. (D–F) Cells treated with CAM. (G–I) Cells treated with S-14. (J–L) Cells treated with oxacillin. (M–O) Cells treated with compound **1**. (P–R) Cells treated with compound **4**.

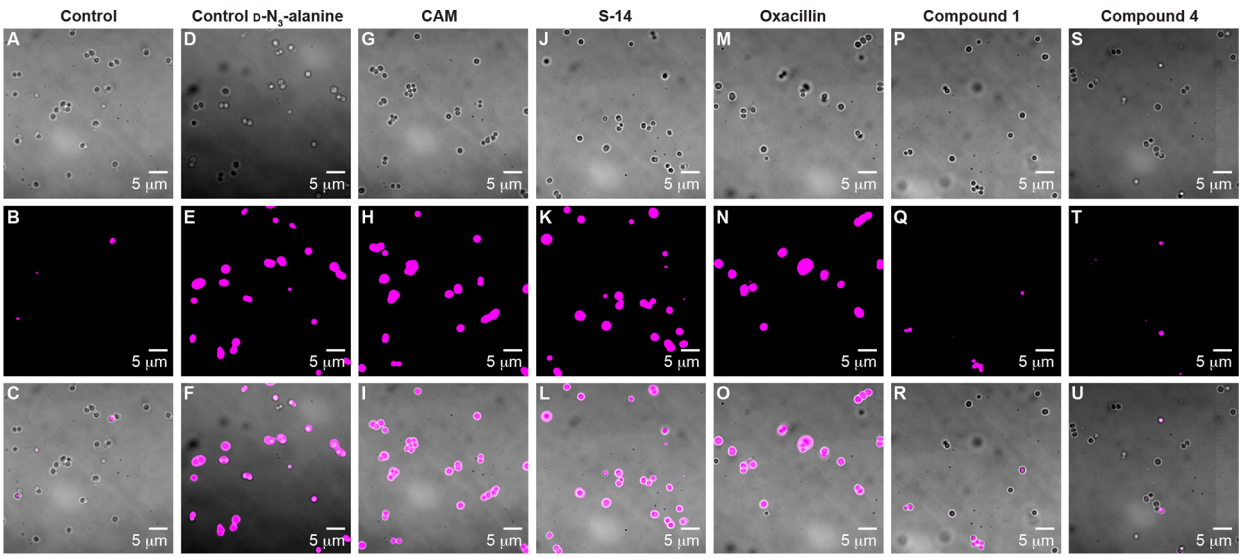

Figure 6. Bright field (top), fluorescence images of TAMRA-DBCO (537/26 nm excitation and 607/36 nm emission, magenta; middle), and merged images (bottom) of cultures of *S. aureus* ATCC 29213 (strain H) incubated for 1 h at 37 °C without or with compounds at 2× their MIC values. (A–C) Untreated cells. (D–F) Cells treated only with azido-D-alanine. (G–I) Cells treated with CAM. (J–L) Cells treated with S-14. (M–O) Cells treated with oxacillin. (P–R) Cells treated with compound **1**. (S–U) Cells treated with compound **4**.

the later stage of cell wall biosynthesis, has a modest effect on the incorporation of azido-D-alanine, presumably since the incorporation occurs during the early stage of the peptidoglycan biosynthesis.

On the basis of this labeling method, we evaluated the incorporation of azido-D-alanine into the cell wall of *S. aureus* ATCC 29213 (strain H). We compared the incorporation in untreated bacteria to cultures treated with oxacillin, the membrane disrupting cationic amphiphile S-14, CAM, compound **1**, and compound **4** (Figure 6). No fluorescent staining was observed for bacteria that were not preincubated with azido-D-alanine and then incubated with TAMRA-DBCO dye ruling out the possibility of nonspecific staining of untreated bacteria (Figure 6A–C). In contrast, bacteria that were preincubated with azido-D-alanine and untreated with an antibacterial agent were effectively stained by the fluorescent dye (Figure 6D–F). Similar to the untreated bacteria that were preincubated with azido-D-alanine, the samples that were treated with CAM (Figure 6G–I) were labeled. This was expected since this antibiotic inhibits translation and does not directly affect the cell wall biosynthesis process. In cultures treated with the membrane disrupting cationic amphiphile S-14, all of the cells were fluorescently labeled (Figure 6J–L). Most of the cells treated with oxacillin were labeled by the fluorescent dye presumably because this antibiotic inhibits a late stage of the peptidoglycan biosynthesis and therefore does not prevent

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the incorporation of azido-D-alanine. Interestingly, in samples treated with either compound **1** or **4** (Figure 6P–R and S–U, respectively) most of the cells were unlabeled by the fluorescent dye. These results support our hypothesis that, unlike the parent antibiotic CAM, compounds **1** and **4** perturb the integrity of the bacterial cell wall by inhibiting early stages of peptidoglycan biosynthesis.

Mammalian Cell Plasma Membrane and Cytotoxic Effects. The observed bacterial cell deformation effects of CAM-derived **1** and **4** raised the question of whether these compounds might also disrupt mammalian cell membranes. We therefore evaluated the effect of **1** and **4** on membranes of rat red blood cells using a hemolysis experiment (Figure 7), a commonly used assay to evaluate mammalian plasma membrane damage.[27] Notably, up to a concentration of 128 μg/mL, which is 4- to 32-fold higher than the MIC values of these compounds against the tested panel of Gram-positive bacteria, no to very limited hemolysis was measured.

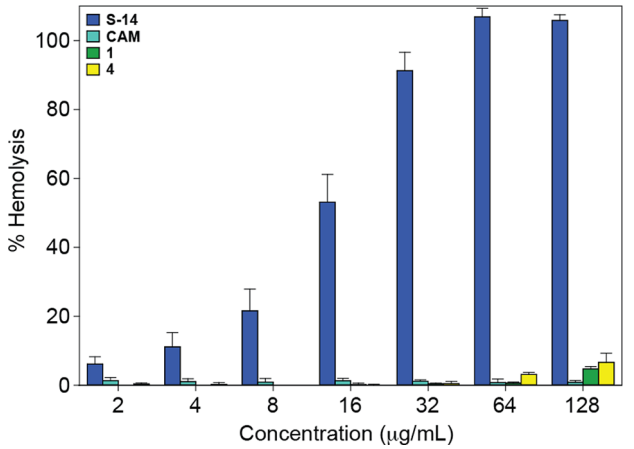

Figure 7. Dose-dependent rat erythrocytes hemolysis of compounds **1** and **4**. CAM and S-14 were used as negative and positive control, respectively.

Since compounds **1** and **4** contain an electrophilic α,β-unsaturated carbonyl that can pose an inherent risk of nonspecific reactions with numerous nucleophilic residues in mammalian cells,[40] we evaluated the potential mammalian cell toxicity[41] of these antibacterials. We used compound **3**, which displayed less potent antibacterial activity (Table 1), and CAM for comparison. Two nucleated immortalized mammalian cells lines were evaluated: the human lung carcinoma epithelial cells A549 and the normal human bronchial epithelial cells BEAS-2B. The cells were incubated with the test compounds for 24 h, after which cell viability was evaluated using a colorimetric assay with resazurin (Figure 8). The parent antibiotic CAM did not have any measurable effect on the tested mammalian cell lines up to 32 μg/mL. However, incubation of the cells with the antibacterial CAM-derived **1** and **4** at a low concentration (2 μg/mL) resulted in significant reduction in cell viability. The toxicity of compounds **1** and **4** to mammalian cell lines at the same concentration range as that needed for inhibition of bacterial growth limits their therapeutic value. Therefore, chemical modification of the CAM-derived α,β-unsaturated carbonyl scaffold of these compounds is necessary to reduce toxicity and develop novel and clinically useful cell wall targeting antibiotics. Similar data were observed with the less active compound **3**. These results indicate that these

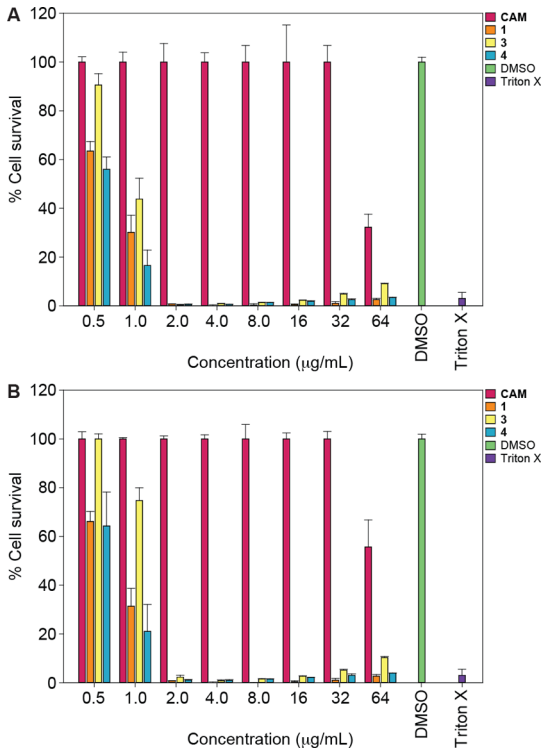

Figure 8. Mammalian cell toxicity of CAM as well as compounds **1**, **3**, and **4** against (A) A549 cell line and (B) BEAS-2B cell line. Note: DMSO (1.25% final concentration) and Triton X were used as negative and positive controls, respectively.

antibacterial agents are toxic to mammalian cells, but that their toxicity does not result from direct perturbation of mammalian cell membrane as is evident from the results of the hemolysis assay.

CONCLUSIONS

We developed a short synthetic route for the generation of a unique type of derivative of the bacterial translation inhibiting antibiotic CAM. Two of the derivatives, compounds **1** and **4**, which contain an enone group, displayed broad spectrum anti-Gram-positive activity including that against strains with high tolerance to the parent antibiotic CAM. Interestingly, through bacterial *in vitro* translation experiments, we showed that, unlike the parent antibiotic CAM, compounds **1** and **4** are poor inhibitors of translation and therefore exert their antibacterial activity via a different mechanism. Like CAM, compounds **1** and **4** exerted a bacteriostatic effect on *S. aureus* cells. Propidium iodide-based cell permability experiments indicated that compounds **1** and **4** did not rapidly increase the membrane permeability of bacterial cells further indicative of the bacteriostatic nature of these antibacterial agents. TEM images of bacteria that were pretreated with compounds **1** and **4** showed that they caused extensive damage to the bacterial cell envelope. Fluorescent labeling of *S. aureus* cells that were preincubated with azido-D-alanine, which is metabolically incorporated into the cell wall peptidoglycan, indicated that compounds **1** and **4** likely exert their antibacterial activity by inhibiting the early stage of cell wall biosynthesis. As there is an alarming increase in resistance to the currently available repertoire of cell wall biosynthesis inhibiting antibiotics including β-lactams, glycopeptides, and fosfomycin, the reported CAM-derived antibacterial enones offer a novel

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molecular scaffold for development of new cell wall targeting antibiotics.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.8b00078.

Detailed synthetic procedures, ^{1}H and ^{13}C NMR assignments for all of the intermediates and final compounds **1**−**10**, MS data, ^{1}H and ^{13}C NMR spectra of compounds **1**−**10** (Figures S1−S20), detailed biochemical and biological experimental procedures, a figure with IC_{50} values (Figure S21), and a figure with TEM images (Figure S22) (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CAM, chloramphenicol; *E. coli*, *Escherichia coli*; MIC, minimum inhibitory concentration; MOM, methoxymethyl; PI, propidium iodide; rRNA, ribosomal ribonucleic acid; SAR, structure−activity relationship; *S. aureus*, *Staphylococcus aureus*; TAM, thiamphenicol; TEM, transmission electron microscopy; TBDMS, *tert*-butyldimethylsilyl

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