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The Isotridecanyl Side Chain of Plusbacin-A_3 Is Essential for the Transglycosylase Inhibition of Peptidoglycan Biosynthesis

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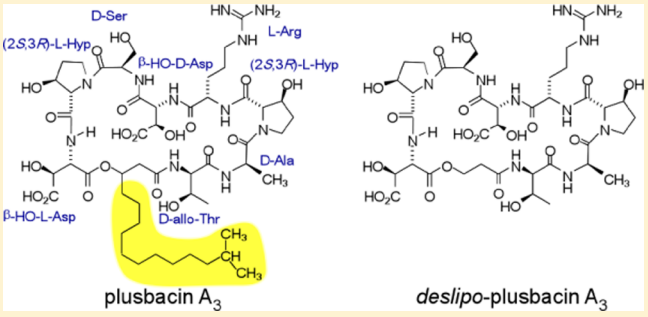
Supporting Information

ABSTRACT: Plusbacin-A_3 (pb-A_3) is a cyclic lipodepsipeptide that exhibits antibacterial activity against multidrug-resistant Gram-positive pathogens. Plusbacin-A_3 is thought not to enter the cell cytoplasm, and its lipophilic isotridecanyl side chain is presumed to insert into the membrane bilayer, thereby facilitating either lipid II binding or some form of membrane disruption. Analogues of pb-A_3, [^{2}H]pb-A_3 and *deslipo*-pb-A_3, were synthesized to test membrane insertion as a key to the mode of action. [^{2}H]pb-A_3 has an isotopically ^{2}H-labeled isopropyl subunit of the lipid side chain, and *deslipo*-pb-A_3 is missing the isotridecanyl side chain. Both analogues have the pb-A_3 core structure. The loss of antimicrobial activity in *deslipo*-pb-A_3 showed that the isotridecanyl side chain is crucial for the mode of action of the drug. However, rotational-echo double-resonance nuclear magnetic resonance characterization of [^{2}H]pb-A_3 bound to [1-^{13}C]glycine-labeled whole cells of *Staphylococcus aureus* showed that the isotridecanyl side chain does not insert into the lipid membrane but instead is found in the staphylococcal cell wall, positioned near the pentaglycyl cross-bridge of the cell-wall peptidoglycan. Addition of [^{2}H]pb-A_3 during the growth of *S. aureus* resulted in the accumulation of Park's nucleotide, consistent with the inhibition of the transglycosylation step of peptidoglycan biosynthesis.



Plusbacin-A_3 (pb-A_3) is a cyclic lipodepsipeptide (Figure 1, left) produced by *Pseudomonas* sp. PB-6250[1] that exhibits antibacterial activity with a minimal inhibitory concentration (MIC) range of 0.78−3.13 μg/mL[2] against multidrug-resistant Gram-positive pathogens: vancomycin-resistant enterococci (VRE), vancomycin intermediate-resistant *Staphylococcus aureus*, and methicillin-resistant *S. aureus* (MRSA). The activity of pb-A_3 is antagonized by the addition of cell-wall membrane particulates, and its inhibition of nascent peptidoglycan biosynthesis in a cell membrane extract suggests that pb-A_3, like vancomycin, may target the peptidoglycan precursor lipid II.[2,3] Unlike vancomycin, however, pb-A_3 is active against VRE and its activity is not affected by the addition of the tripeptide acetyl-L-Lys-D-Ala-D-Ala.[2] Thus, it appears that the binding site utilized by pb-A_3 is distinct from that of vancomycin.

The lipophilic isotridecanyl side chain of pb-A_3 is presumed to insert into the membrane bilayer, colocalizing the lipodepsipeptide at the site of peptidoglycan biosynthesis and possibly facilitating some form of drug aggregation and membrane disruption.[4] The pb-A_3 analogues, *deslipo*-pb-A_3 and [^{2}H]pb-A_3, were synthesized to test membrane insertion as a key to the mode of action. The analogues share an identical depsipeptide core structure with pb-A_3; however, *deslipo*-pb-A_3 (Figure 1, center) lacks the lipid side chain. [^{2}H]pb-A_3 (Figure 1, right) is identical to the parent compound except for the ^{2}H labels incorporated into the isopropyl subunit of the lipid side chain. Table S1 of the Supporting Information lists the minimal inhibitory concentrations (MICs) for these analogues.

To investigate in situ pb-A_3 binding, we formed a complex of [^{2}H]pb-A_3 with intact whole cells of *S. aureus* grown in a defined medium containing [1-^{13}C]glycine. The localization of [^{2}H]pb-A_3 in the cell wall was determined by ^{13}C{^{2}H} rotational-echo double-resonance (REDOR) nuclear magnetic resonance (NMR).[5] The proposed insertion of the isotridecanyl side chain into the phospholipid membrane was investigated by ^{31}P{^{2}H} REDOR NMR. These experiments measure the dipolar coupling between pairs of nuclei and therefore their internuclear separation.

Information about the in vivo mode of action of pb-A_3 was determined by adding a subinhibitory concentration of [^{2}H]pb-A_3 to *S. aureus* during the midexponential growth phase in a medium containing either [1-^{13}C]glycine and [ε-^{15}N]lysine (to label cell-wall peptidoglycan bridge links) or D-[1-^{13}C]alanine

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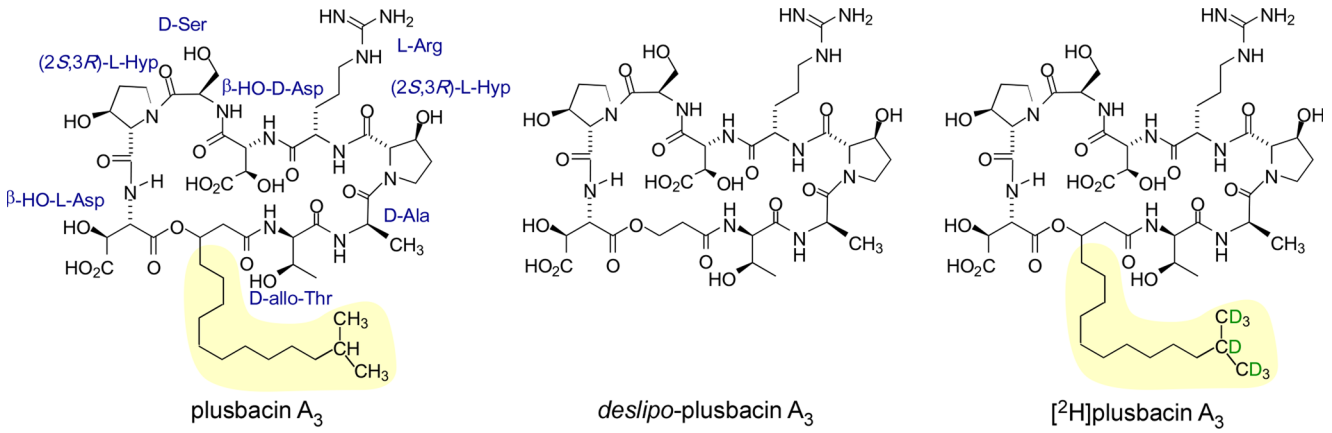

Figure 1. Chemical structures of pb-A_3, *deslipo*-pb-A_3, and [^{2}H]pb-A_3.

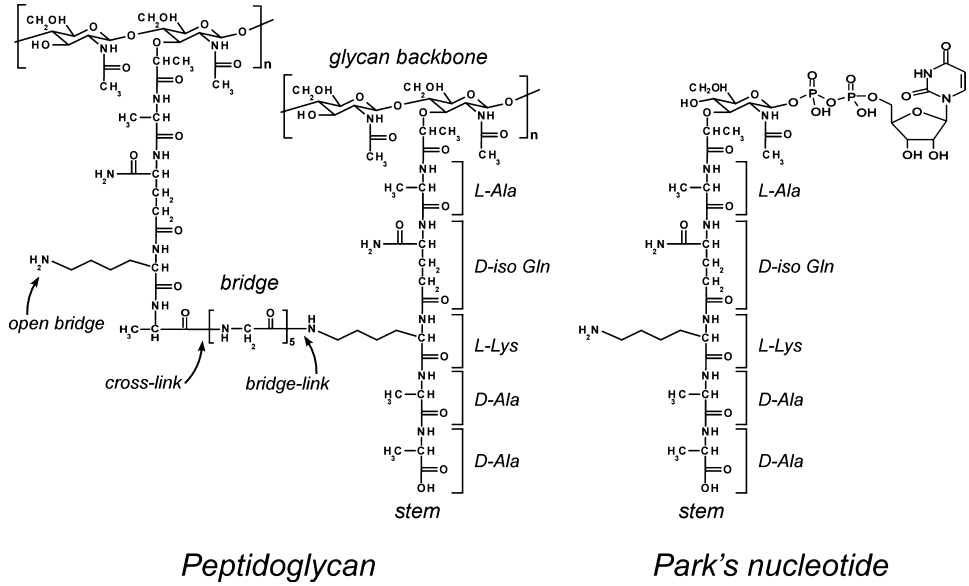

Figure 2. Chemical structure of *S. aureus* peptidoglycan. The repeat unit on the right is an un-cross-linked stem having the sequence L-Ala-D-iso-Gln-L-Lys-D-Ala-D-Ala. The pentaglycyl bridge attached to the ϵ-nitrogen of L-lysine is cross-linked to the peptide stem on the left. Chemical structure of the cytoplasmic peptidoglycan precursor Park's nucleotide.

and [^{15}N]glycine (to label cross-links) (Figure 2, left). The drug-treated *S. aureus* whole cells were harvested after being grown for 1 h, and the effects of the drug on cell-wall compositions were analyzed by ^{13}C{^{15}N} and ^{15}N{^{13}C} REDOR NMR.

MATERIALS AND METHODS

Synthesis of Plusbacin-A_3. The detailed chemical synthesis of [^{2}H]pb-A_3 is presented in the Supporting Information.

Susceptibility Testing. Minimal inhibitory concentrations (MICs) for pb-A_3, *deslipo*-pb-A_3, and [^{2}H]pb-A_3 against *Streptococcus pyogenes* M49 strain NZ131, *Streptococcus agalactiae* strain A909, MRSA (ATCC 33591), VRE (ATCC 51299), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853) were determined by the microdilution method recommended by the National Committee for Clinical Laboratory Standards (Table S1 of the Supporting Information).

Growth and Labeling of Whole Cells and Formation of the Drug Complex. A starter culture of *S. aureus* (ATCC 6538P) was grown overnight in 5 mL of trypticase soy broth at 37 °C with 250 rpm shaking and added (1% final volume) to a 1 L flask containing 335 mL of a defined medium[6] with the naturally abundant amino acid glycine replaced with isotope-labeled [1-^{13}C]glycine to incorporate the ^{13}C-specific label at the pentaglycyl cross-bridge of the peptidoglycan (Figure 2, left). *S. aureus* was harvested under stationary-phase growth (OD_{660} at 1.0) as described previously[6] and then resuspended in 7 mL of 40 mM triethanolamine buffer. In 2 mL of water was dissolved 3.9 mg of [^{2}H]pb-A_3 (MW 1165.32) that had been added dropwise to the cell suspension with gentle stirring. The bacteria/pb-A_3 mixture was equilibrated for 6 min prior to being frozen and lyophilized.

[^{2}H]pb-A_3 Addition during Growth of *S. aureus*. *S. aureus* was grown in four 1 L flasks each containing 330 mL of a defined medium[6] containing isotope-labeled [1-^{13}C]glycine and L-[ϵ-^{15}N]lysine for the ^{13}C–^{15}N pair labeling of the bridge link or D-[1-^{13}C]alanine and [^{15}N]glycine in the presence of alanine racemase inhibitor alaphosphin (5 μg/mL) for ^{13}C–^{15}N pair labeling of the cross-link of peptidoglycan (Figure 2, left). [^{2}H]pb-A_3 was added to *S. aureus* during the midexponential growth phase (OD_{660} at 0.5) to a final concentration of 11.8

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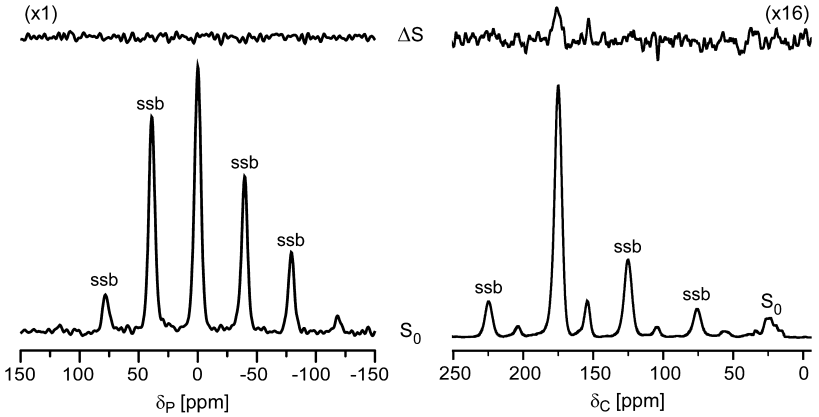

Figure 3. (Left) 202.4 MHz $^{31}P\{^{2}H\}$ REDOR spectra of $[^{2}H]$pb-A_3 complexes of whole cells of *S. aureus* after dipolar evolution of 20.0 ms. The full-echo spectrum (S_0) is at the bottom of the figure and the REDOR difference (ΔS) at the top. The spectra were the result of the accumulation of 378664 scans. Magic-angle spinning was conducted at 8000 Hz. The absence of dephasing indicates that $[^{2}H]$pb-A_3 did not embed in the membrane. (Right) 125 MHz $^{13}C\{^{2}H\}$ REDOR spectra of $[^{2}H]$pb-A_3 complexes of whole cells of *S. aureus* grown in a defined medium containing [1-^{13}C]glycine after dipolar evolution for 31.4 ms. The spectra were the result of the accumulation of 249660 scans. Magic-angle spinning was conducted at 6250 Hz . The dephasing indicates that the isotridecanonyl side chain of $[^{2}H]$pb-A_3 is found near the pentaglycyl bridge segment of *S. aureus* peptidoglycan. Spinning side bands are marked as ssb.

μg/mL. The cells were harvested by centrifugation after being grown for 1 h. The harvested cells were resuspended in 7 mL of 40 mM triethanolamine buffer and lyophilized.

Internuclear Proximities. REDOR was used to determine dipolar couplings and hence internuclear distances.[5] REDOR is a difference experiment in which two spectra are collected, S_0 (full echo) and S (dephased echo). Dipolar evolution over the rotor period in the S spectrum results in reduced (dephased) peak intensity for spin pairs that are dipolar coupled. From the difference in signal intensity (REDOR difference; $\Delta S = S_0 - S$) and the experimental dephasing time (the dipolar evolution time), the heteronuclear dipolar coupling and corresponding internuclear distance can be directly calculated.[5]

Solid-State NMR. The $^{31}P\{^{2}H\}$ and $^{13}C\{^{2}H\}$ REDOR spectra of Figure 3 were recorded using an 89 mm bore, 12 T static field (^{1}H at 500 MHz, Magnex, Agilent, Santa Clara, CA), with a Tecmag (Houston, TX) Apollo spectrometer, and four-channel transmission-line probes equipped with either 4 mm (Figure 3, left) or 5 mm (Figure 3, right) Chemagnetics/Varian stators and zirconium rotors. Radiofrequency pulses for ^{1}H were amplified first by a 50 W American Microwave Technology (AMT, Anaheim, CA) power amplifier and then by a 2 kW Creative Electronics tube amplifier. One-kilowatt AMT amplifiers were used for ^{31}P and ^{13}C pulses. For ^{2}H, a 2 kW AMT amplifier was used. The π pulse lengths were 6 μs for ^{31}P, ^{13}C, and ^{2}H. The ^{1}H decoupling field was 100 kHz throughout dipolar evolution and data acquisition with TPPM of the ^{1}H radiofrequency.[8]

The $^{13}C\{^{15}N\}$ and $^{15}N\{^{13}C\}$ REDOR spectra of Figures 4−7 and Figure S3 of the Supporting Information were recorded at a spinning speed of 5 kHz in a 4.7 T, 89 mm Oxford magnet (200 MHz) (Cambridge, England), using a Tecmag Libra pulse generator and a four-channel transmission-line probe with a Chemagnetics 7.5 mm stator. One-kilowatt amplifiers were used for all frequencies. The π pulse lengths were 10 μs for ^{13}C and ^{15}N. Cross-polarization transfers were conducted at 50 kHz for 2 ms, and ^{1}H decoupling was at 98 kHz throughout dipolar evolution and data acquisition.

All spinning rates were actively controlled to ±2 Hz. Similarly, all RF field amplitudes were under active control to eliminate long-term drifts due to component aging or changes in temperature.[9] Additionally, alternating scans of S_0 and S were acquired to compensate for short-term drift. XY-8 phase cycling[10] was used for all refocusing and dephasing pulses.[11]

Calculations. REDOR dephasing for spin-half pairs was calculated using the analytical expressions of Mueller et al.[12] REDOR dephasing for the rapidly rotating CD_3 dephaser was approximated as a single super spin centered in the triangle defined by the three ^{2}H nuclei.[13−15] The abbreviation $^{2}H_3$ is used to denote the super spin. For both these calculations, fits of $\Delta S/S_0$ as a function of dephasing time yield the dipolar coupling constant and hence the internuclear distance.

RESULTS

Antimicrobial Activities of pb-A_3, *deslipo*-pb-A_3, and $[^{2}H]$pb-A_3. The MICs of pb-A_3, *deslipo*-pb-A_3, and $[^{2}H]$pb-A_3 against Gram-positive and Gram-negative bacteria are listed in Table S1 of the Supporting Information. pb-A_3 exhibits a MIC range of 0.2−6.25 μg/mL against the Gram-positive bacteria: *St. pyogenes* M49 strain NZ131, *St. agalactiae* strain A909, MRSA (ATCC 33591), and VRE (ATCC 51299). However, it is inactive against Gram-negative organisms, *E. coli* (ATCC 25922), and *P. aeruginosa* (ATCC 27853). *deslipo*-pb-A_3 has no activity in the strains examined. The ^{2}H incorporation in $[^{2}H]$pb-A_3 did not alter any pb-A_3 antimicrobial properties.

$[^{2}H]$pb-A_3 Membrane Anchor. The 202.4 MHz $^{31}P\{^{2}H\}$ REDOR spectra of $[^{2}H]$pb-A_3 complexed with [1-^{13}C]glycine-labeled whole cells of *S. aureus* after dipolar evolution for 20 ms are shown in Figure 3 (left). The P{D} detection range was 10 Å for these experiments. This estimate is based on a $\Delta S/S_0$ of <2%, or 2 times the noise level for the sum of the centerband and first two spinning side bands (Figure 3, left), where ~20% of S_0 is due to ^{31}P in the bilayer and the remainder is mostly wall and lipoteichoic acids (see Figure 8 of ref 7). A fully extended 11-carbon side chain has a length of 8−10 Å.[16] Thus, the absence of dipolar contact between $^{2}H_3$ and the natural abundance ^{31}P of the lipid headgroups in the REDOR difference spectrum (Figure 3, top left) rules out insertion of the isotridecanyl tail of $[^{2}H]$pb-A_3 into the lipid bilayer.

$[^{2}H]$pb-A_3 Binding to [1-^{13}C]Glycine-Labeled Whole Cells. Figure 3 (right) shows 125 MHz $^{13}C\{^{2}H\}$ REDOR

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spectra after dipolar evolution for 31.4 ms. In the ΔS spectrum, the glycyl carbonyl carbon positioned at 172 ppm is dephased by 1%, indicating that [^{2}H]pb-A$_3$ is localized within the cell wall. The estimated asymptotical REDOR dephasing limit is a product of (i) drug binding site occupancy, (ii) the fraction of peptidoglycan stems with a glycyl bridge attached, and (iii) the fraction of glycine found in the cell wall.

To determine the first factor, the [^{2}H]pb-A$_3$ binding site occupancy, we estimated the total number of peptidoglycan stems found in whole cells of *S. aureus*. On the basis of a vancomycin binding assay,[7] we estimated that 14.3 μmol of peptidoglycan stems is present in whole cells of *S. aureus* grown in 335 mL of a defined medium harvested at an OD$_{660}$ of 1.0. This is a sum of un-cross-linked peptidoglycan stems terminating in D-Ala-D-Ala (6.6 μmol) and cross-linked stems (7.7 μmol). Therefore, for 3.9 mg of [^{2}H]pb-A$_3$ (3.4 μmol) complexed to whole cells, the estimated binding site occupancy is 24% (3.4 μmol/14.3 μmol).

The second factor, the fraction of stems with a pentaglycyl bridge attached, is approximately 85% for cells harvested under stationary-phase conditions.[7] The third factor, the fraction of glycine found in the cell wall of *S. aureus*, was determined from ^{13}C{^{15}N} REDOR spectra of whole cells of *S. aureus* labeled with [1-^{13}C]glycine and L-[ε-^{15}N]lysine (Figure 4, left). During a dipolar evolution of 1.6 ms, only the peptide-bonded ^{13}C−^{15}N spin pairs from the peptidoglycan bridge link, [1-^{13}C]glycine-L-[ε-^{15}N]lysine, appear at 172 ppm in the ΔS spectrum. (This ignores the minor natural abundance ^{13}C contribution to S_0.) The dephased 172 ppm intensity (13%) multiplied by 5 to account for the total number of glycines found in a pentaglycyl bridge means that 65% of the [1-^{13}C]glycine label in whole cells of *S. aureus* is found in the cell wall. Thus, the estimated maximal ^{13}C{^{2}H} REDOR dephasing limit is (0.24)(0.85)(0.65) = 13%. Depending on the position of the ^{2}H, multiple glycyl carbonyl carbons in the pentaglycyl bridge can dephase. If we assume only one carbonyl carbon dephases, the dephasing limit is 2.6% (13%/5), and the corresponding ^{13}C−^{2}H$_3$ distance for the measured ^{13}C{^{2}H} REDOR dephasing of 1% after dipolar evolution for 31.4 ms is 7 Å.

The addition of [^{2}H]pb-A$_3$ did not affect the incorporation of [1-^{13}C]glycine into the cell wall as evident by the equal 172 ppm dephasing of 13% measured for both drug-treated and untreated *S. aureus* (Figure 4). Instead, [^{2}H]pb-A$_3$-treated *S. aureus* shows a smaller-amplitude purine peak at 149 ppm in the S_0 spectrum (Figure 4, inset).

Addition of [^{2}H]pb-A$_3$ to Growing *S. aureus* Labeled with [1-^{13}C]Glycine and L-[ε-^{15}N]Lysine. Figure 5 shows

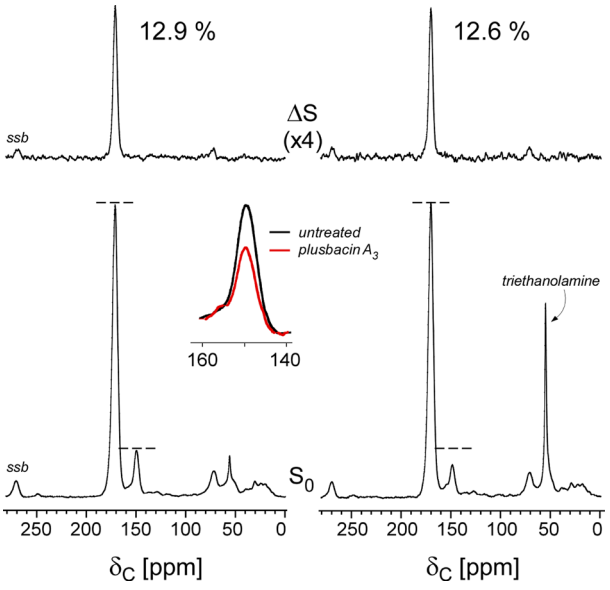

Figure 4. 50.3 MHz ^{13}C{^{15}N} REDOR spectra after dipolar evolution for 1.6 ms of whole cells of *S. aureus* grown on a defined medium containing [1-^{13}C]glycine and L-[ε-^{15}N]lysine in the absence (left) and presence (right) of [^{2}H]pb-A$_3$ (11.8 μg/mL). The full-echo spectra (S_0) are at the bottom of the figure and the REDOR differences (ΔS) at the top. Magic-angle spinning was conducted at 5000 Hz. Identical 172 ppm dephasing of 13% in the ΔS spectra for *S. aureus* grown in the absence and presence of the lipodepsipeptide indicates that [^{2}H]pb-A$_3$ did not target the *fem* factors associated with pentaglycyl bridge formation. The reduced 149 ppm peak intensity in the S_0 spectrum of *S. aureus* grown in the presence of [^{2}H]pb-A$_3$ (highlighted in the inset) was consistent with the inhibition of [1-^{13}C]glycine metabolism in purine biosynthesis. The spectra were the result of the accumulation of 10000 scans. Spinning side bands are marked as ssb.

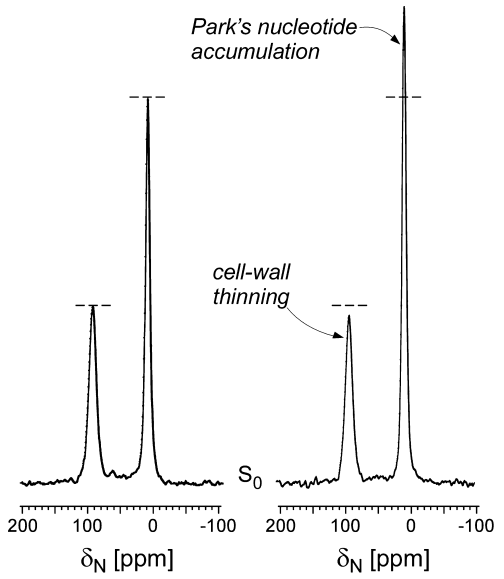

Figure 5. 20.3 MHz ^{15}N{^{13}C} REDOR full-echo spectra after dipolar evolution for 1.6 ms of whole cells of *S. aureus* grown on a defined medium containing [1-^{13}C]glycine and L-[ε-^{15}N]lysine in the absence (left) and presence (right) of [^{2}H]pb-A$_3$ (11.8 μg/mL). The drug-treated *S. aureus* shows reduced lysyl-amide peak intensity at 93 ppm (cell-wall thinning) and increased lysyl-amine peak intensity at 9 ppm (accumulation of Park's nucleotide). The spectra were normalized with respect to equal sample weight and scans and were each the result of the accumulation of 20000 scans. Magic-angle spinning was conducted at 5000 Hz.

20.3 MHz ^{15}N{^{13}C} REDOR S_0 spectra of whole cells of *S. aureus* grown in a defined medium containing [1-^{13}C]glycine and L-[ε-^{15}N]lysine without (left) and with (right) the addition of [^{2}H]pb-A$_3$ during growth. The S_0 spectra were scaled by a factor determined from ^{13}C spectra shown in Figure 4 to normalize the natural abundance lipid peak intensities found at 10−30 ppm.

The addition of [^{2}H]pb-A$_3$ during *S. aureus* growth diminished the 95 ppm lysyl amide peak intensity, consistent with [^{2}H]pb-A$_3$ inhibition of cell-wall biosynthesis, and increased the 9 ppm lysyl amine peak intensity, which indicates

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an accumulation of the cytoplasmic peptidoglycan precursor, Park's nucleotide (Figure 2, right). The inhibition of purine biosynthesis by [^{2}H]pb-A$_3$ (Figure 4) is also consistent with transglycosylase inhibition in which all available glycine is routed to peptidoglycan biosynthesis, thereby indirectly preventing [1-^{13}C]glycine incorporation in the purine biosynthetic pathway.[17,18] These results are identical to those obtained with vancomycin, a known transglycosylase inhibitor.[17,18] Thus, pb-A$_3$ also appears to be a transglycosylase inhibitor.

Addition of [^{2}H]pb-A$_3$ to Growing *S. aureus* Labeled with D-[1-^{13}C]Alanine and [^{15}N]Glycine. The D-[1-^{13}C]-alanine incorporated into the cell wall appears at 175 ppm in the S_0 spectra of the 50.3 MHz ^{13}C{^{15}N} REDOR spectra shown in Figure 6 (bottom). The alanine racemase inhibitor,

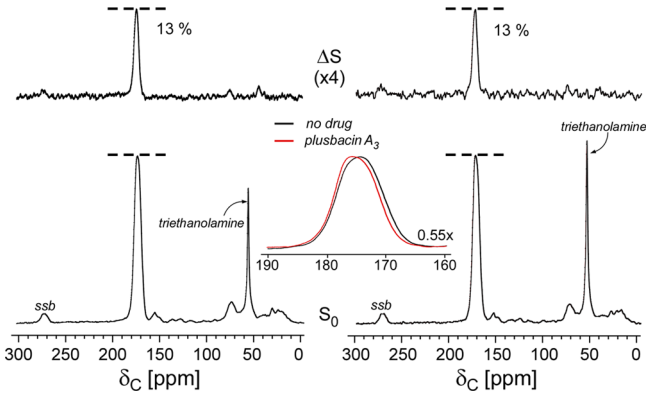

Figure 6. 50.3 MHz ^{13}C{^{15}N} REDOR spectra after dipolar evolution for 1.6 ms of whole cells of *S. aureus* grown on a defined medium containing D-[1-^{13}C]alanine and [^{15}N]glycine in the absence (left) and presence (right) of [^{2}H]pb-A$_3$. The full-echo spectra (S_0) are at the bottom of the figure and the REDOR differences (ΔS) at the top. The 175 ppm peak in the ΔS spectra is from the cross-link of the peptidoglycan in the cell wall of *S. aureus*. The addition of [^{2}H]pb-A$_3$ during the growth of *S. aureus* did not inhibit the transpeptidase activity of peptidoglycan biosynthesis in *S. aureus*, and therefore, the dephasing of the 175 ppm peak intensity was not affected. The inset is an expansion of the superimposed carbonyl carbon S_0 spectra of *S. aureus* grown in the presence (red) and absence (black) of [^{2}H]pb-A$_3$. The addition of [^{2}H]pb-A$_3$ affected the line shape of the ^{13}C carbonyl spectrum centered at 175 ppm. The spectra were the result of the accumulation of 10000 scans. Spinning side bands are marked as ssb.

alaphosphin (5 μg/mL), prevents the racemic scrambling of D-[1-^{13}C]alanine to its enantiomeric L-form.[6,7] During the short dipolar evolution period of 1.6 ms, only the peptide-bonded ^{13}C−^{15}N spin pairs from amino acids having the sequence D-[1-^{13}C]Ala-[^{15}N]Gly (peptidoglycan cross-link) appear in the ΔS spectrum. The addition of [^{2}H]pb-A$_3$ (11.8 μg/mL) did not change the ΔS 175 ppm peak intensity (Figure 6, top), and therefore, transpeptidase activity was not inhibited.

Figure S3 of the Supporting Information shows ^{15}N{^{13}C} REDOR spectra after dipolar evolution for 1.6 ms where dephasing of the [^{15}N]glycyl-amide peak, which is also directly proportional to the extent of cross-linking in whole cells, was not affected by the addition of [^{2}H]pb-A$_3$, consistent with the ^{13}C{^{15}N} results of Figure 6 for the same sample.

The superimposed enlarged carbonyl carbon regions of the full-echo (S_0) spectra of *S. aureus* cells grown with (red line) and without (black line) [^{2}H]pb-A$_3$ are shown in Figure 6 (inset). The differing line shapes in the 175 ppm region indicate differences in the cell-wall composition. Figure 7 (bottom) shows the spectral subtraction of the 175 ppm full-

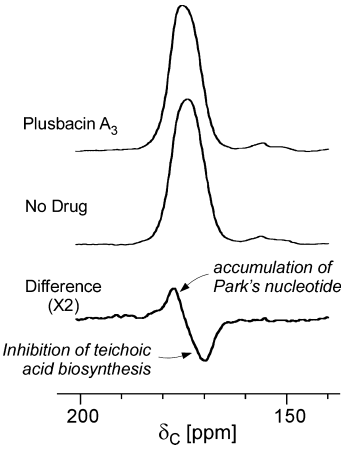

Figure 7. Enlargements of the carbonyl carbon region of the S_0 spectra shown in Figure 6. The full-echo spectrum for *S. aureus* grown in the presence of [^{2}H]pb-A$_3$ (top) is subtracted from the corresponding spectrum without the addition of drug (middle). The difference spectrum (bottom) shows a positive peak at 178 ppm due to the penultimate D-Ala carbonyl carbon of the increased level of Park's nucleotide and a negative peak at 170 ppm due to ester-linked D-Ala incorporated into teichoic acid.

echo spectrum of *S. aureus* cells grown in the presence of [^{2}H]pb-A$_3$ (Figure 7, top) from the full-echo spectrum of untreated *S. aureus* cells (Figure 7, middle). The difference spectrum shows two distinct components: a positive peak at 178 ppm and a negative peak at 170 ppm. The positive 178 ppm peak is assigned to the carboxyl terminus of the penultimate D-Ala, associated with an accumulation of Park's nucleotide in the cytoplasm caused by transglycosylase inhibition.[18] The negative 170 ppm peak corresponds to a decreased level of ester-linked D-[1-^{13}C]alanine found in cell-wall teichoic acid.[19]

DISCUSSION

The chemical structure of plusbacin consists of a cyclic depsipeptide core with a lipophilic side chain. This is a common structural motif found in numerous natural products of cyclic antimicrobial (depsi)peptides, including the calcium-dependent antibiotic complex A54145, friulimicin/amphomycin, laspartomycin, ramoplanin, fusaricidin, empedopeptin, and daptomycin.[20] The mode of action for most of these natural products is not well-known, but for daptomycin (Cubicin), the lipophilic side chain is thought to mediate the formation of drug oligomers that form a pore structure in the bacterial membrane.[4,21] The ensuing bacterial membrane depolarization is generally accepted as the killing mechanism for daptomycin, and daptomycin-related natural products. The isotridecanyl side chain of pb-A$_3$ was presumed to play a similar role in targeting the bacterial membrane.

For [^{2}H]pb-A$_3$ complexed to intact whole cells of *S. aureus*, P{D} REDOR showed that [^{2}H]pb-A$_3$ was not embedded in the membrane (Figure 3, left). This result is consistent with the absence of lipid natural abundance ^{13}C aliphatic carbon C{D} dephasing by[^{2}H]pb-A$_3$ (Figure 3, right), which would have been expected from an embedded ^{2}H-labeled side chain.[22] Therefore, at therapeutic dosage levels for intact whole cells,

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the isotridecanyl side chain of [^{2}H]pb-A$_3$ does not mediate the binding of drug to the membrane or the formation of a drug-aggregated membrane pore structure. Instead, we find [^{2}H]pb-A$_3$ localized within the cell wall. The dipolar contact between the deuterium labels of [^{2}H]pb-A$_3$ and the carbonyl carbon labels of [1-^{13}C]glycine in the peptidoglycan cross bridge (Figure 3, right) shows unambiguously that the isotridecanyl side chain is involved in cell-wall binding.

The addition of [^{2}H]pb-A$_3$ (11.8 μg/mL) to whole cells of *S. aureus* during the midexponential growth (OD_{660} at 0.5) showed no inhibition of the transpeptidation step of peptidoglycan biosynthesis (Figure 4 and Figure S3 of the Supporting Information). Although transpeptidase inhibition may occur at higher drug concentrations, the accumulation of a cytoplasmic peptidoglycan precursor (Park's nucleotide) and inhibition of purine biosynthesis (Figures 4 and 5) are consistent with [^{2}H]pb-A$_3$ acting primarily as a transglycosylase inhibitor in *S. aureus*.

We also found that [^{2}H]pb-A$_3$ inhibited the incorporation of D-[1-^{13}C]alanine into cell-wall teichoic acids (Figure 7). Similar inhibition of peptidoglycan and teichoic acid biosynthesis has been observed for *S. aureus* cells treated with vancomycin and oritavancin.[19] Such dual inhibition may be due to drug targeting of lipid II (or the membrane-associated nascent peptidoglycan) and the resulting unavailability of lipid transporter bactoprenol phosphate.[23] Because [^{2}H]pb-A$_3$ is fully active only in the presence of the intact cell wall, a conventional inhibition assay based on isolated, purified enzymes and substrates could miss the dual inhibition.

The molecular role of the isotridecanyl side chain in [^{2}H]pb-A$_3$ for transglycosylase inhibition remains unknown. However, the drug side chain is essential for antimicrobial activity. The MICs of pb-A$_3$ and *deslipo*-pb-A$_3$ (pb-A$_3$ without the isotridecanyl side chain) show *deslipo*-pb-A$_3$ has no antimicrobial activity (Table S1 of the Supporting Information). The isotridecanyl side chain may simply act as a steric blocker, preventing substrates from reaching the transglycosylase active site.[24] A similar role has been suggested[17] for the hydrophobic side chain of oritavancin, which inserts in cell-wall-free bilayers but not in the bilayers of whole cells.[22]

■ ASSOCIATED CONTENT

Supporting Information

A description of the synthesis of [^{2}H]pb-A$_3$, minimal inhibitory concentrations (micrograms per milliliter) of pb-A$_3$, *deslipo*-pb-A$_3$, and [^{2}H]pb-A$_3$ (Table S1), retrosynthetic analysis for [^{2}H]pb-A$_3$ (Figure S1), preparation of isotopically labeled depsipeptide fragment **5** (Figure S2), and 20.3 MHz ^{15}N{^{13}C} REDOR spectra after dipolar evolution for 1.6 ms of whole cells of *S. aureus* grown in a defined medium containing D-[1-^{13}C]alanine and [^{15}N]glycine in the absence (left) and presence of [^{2}H]pb-A$_3$ (right) (Figure S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

^{13}C{^{2}H} or C{D}, carbon channel observation with deuterium dephasing; ^{13}C{^{15}N} or C{N}, carbon channel observation with nitrogen dephasing; ^{15}N{^{13}C} or N{C}, nitrogen channel observation with carbon dephasing; ^{31}P{^{2}H} or P{D}, phosphorus channel observation with deuterium dephasing; REDOR, rotational-echo double resonance.

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