THCz: Small molecules with antimicrobial activity that block cell wall lipid intermediates

Elisabeth Reithuber,a,b, Torbjörn Wixe,b,1, Kevin C. Ludwig,c,1, Anna Müller,b, Hanna Uvell,b, Fabian Grein,c,d, Anders E. G. Lindgren,b,e, Sandra Muschiold,e,f, Priyanka Nannapaneni,f, Anna Erikskob,1, Tanja Schneider,c,2, Staffan Normark,a,b,2, Birgitta Henriques-Normark,a,b,c,d, Fredrik Almqvist,c,d,e,2,3, and Peter Mellroth,e,3

“Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet 171 77 Stockholm, Sweden; “Institute for Pharmaceutical Microbiology, University Hospital Bonn, University of Bonn, Bonn 53115, Germany; “German Center for Infection Research (DZIF), partner site Bonn-Cologne, Bonn 53115, Germany; “Laboratories for Chemical Biology Umeå (LCBU), Umeå University, Umeå 90736, Sweden; and “Clinical Microbiology, Karolinska University Hospital Solna 171 76 Stockholm, Sweden

Contributed by Staffan Normark, October 1, 2021 (sent for review May 4, 2021; reviewed by Patrice Courvalin and Leiv Sigve Håvarstein)

Emerging antibiotic resistance demands identification of novel antibacterial compound classes. A bacterial whole-cell screen based on pneumococcal autolysin-mediated lysis induction was developed to identify potential bacterial cell wall synthesis inhibitors. A hit class comprising a 1-amin-substituted tetrahydrocarbazole (THCz) scaffold, containing two essential amine groups, displayed bactericidal activity against a broad range of gram-positive and selected gram-negative pathogens in the low micromolar range. Mode of action studies revealed that THCz inhibit cell envelope synthesis by targeting undecaprenyl pyrophosphate-containing lipid intermediates and thus simultaneously inhibit peptidoglycan, teichoic acid, and polysaccharide capsule biosynthesis. Resistance did not readily develop in vitro, and the ease of synthesizing and modifying these small molecules, as compared to natural lipid II–binding antibiotics, makes THCz promising scaffolds for development of cell wall–targeting antimicrobials.

Streptococcus pneumoniae | antibiotic resistance | antimicrobials | cell wall biosynthesis | tetrahydrocarbazole

Since the discovery of penicillin by Alexander Fleming (1) in 1928, antibiotics have greatly improved the health quality and life expectancy of mankind. However, multidrug resistance among most microbial pathogens is reaching alarming levels, and the World Health Organization foresees a postantibiotic era where common bacterial infections may become life-threatening again due to the lack of adequate treatment regimens (2). Many of the most commonly used antibacterial drugs today, ~50% of all antibiotic prescriptions, and over 70% of intravenous applications in clinical settings, rely on inhibitors of cell wall biosynthesis (3). The essentiality of the bacterial cell wall for structural integrity and growth, and the lack of a similar structure in mammalian cells, makes the cell wall biosynthesis machinery a most attractive antibiotic target. Inhibition of cell wall synthesis can be accomplished by two main mechanisms: either by inhibition of enzyme function, for example, by beta-lactam antibiotics targeting the penicillin-binding proteins (PBPs), or by binding and blocking access to essential cell wall precursors such as the ultimate peptidoglycan building block lipid II. While a wide range of beta-lactam derivatives of different classes are continuously optimized to bypass bacterial resistance development (4), the highly conserved lipid II molecule constitutes an attractive target, as resistance development is intrinsically limited (5, 6). Lipid II is a disaccharide pentapeptide peptidoglycan subunit linked to an undecaprenyl lipid vehicle via a pyrophosphate group. It is synthesized in the cytoplasm and flipped over to the outer leaflet of the plasma membrane to provide cell wall building blocks for PBPs (7). On the outside, the ultimate peptidoglycan precursor is readily accessible for antibiotics. Presently, lipid II–binding antibiotics of at least five chemical classes are known, comprising glycopeptides (e.g., vancomycin) (8), lantibiotics (e.g., nisin), defensins (e.g., plecactin), lipopeptides (e.g., epedempeptin), and depsipeptides (e.g., teixobactin) (9, 10). More recently, the last-resort antibiotic daptomycin was further shown to target undecaprenyl-containing lipid intermediates (11). In common for these agents is that binding to lipid II sequesters the molecule and makes it unavailable for peptidoglycan biosynthesis. However, antibiotic activities can vary substantially depending on the binding site on the lipid II molecule (6). Vancomycin, for example, binds to the terminal D-alanyl-D-alanine residue of the lipid II stem peptide, a part of the molecule that is altered in resistant strains. In contrast, resistance development to compounds that recognize the pyrophosphate moiety as minimal binding motif, which is present in several cell wall intermediates from different pathways (i.e., peptidoglycan, wall teichoic acid, and capsule biosynthesis), is strongly hampered. This structural feature is highly conserved among bacteria, and direct target modifications have not been observed (7).

The vast majority of lipid II–binding antibiotics described so far mainly act on gram-positive bacteria, since the outer membrane of gram-negative bacteria restricts target access mainly due to the large size of these compounds. Notably, no small-molecule inhibitor (<500 Da) targeting lipid II has been identified so far.

Here, we developed a bacterial whole-cell screening platform aimed to identify small molecules with cell wall synthesis inhibition activity against a broad range of gram-positive and selected gram-negative pathogens, as well as THcz. This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

The authors declare no competing interest.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

Author contributions: E.R., T.S., S.N., B.H.-N., F.A., and P.M. designed research; E.R., T.W., K.C.L., A.M., H.U., F.G., A.E.G.L., S.M., P.N., and A.E. performed research; E.R., F.A., and P.M. contributed new reagents/analytic tools; E.R., T.W., K.C.L., A.M., H.U., F.G., A.E.G.L., S.M., P.N., A.E., T.S., S.N., B.H.-N., F.A., and P.M. analyzed data; and E.R., T.S., S.N., B.H.-N., F.A., and P.M. wrote the paper.

Reviews: P.C., Institut Pasteur; and L.S.H., Norges miljø- og biovitenskapelige universitet.

Significance

Considering the alarming emergence of resistance to most antibiotics and the need for new antibiotics, the finding here of a small-molecule class, THCz, that displayed bactericidal activity against gram-positive and selected gram-negative bacteria, is of the greatest importance. We found that THCz target the cell envelope synthesis and can easily be synthesized and modified, and resistance did not readily develop in vitro. Thus, THCz are promising scaffolds for development of bacterial cell wall inhibitors.

PNAS 2021 Vol. 118 No. 47 e2108244118 https://doi.org/10.1073/pnas.2108244118 | 1 of 12

Published November 16, 2021.
inhibiting activity. The screen used the induction of autolysin-mediated lysis as a phenotypic readout for cell wall inhibition. It is well established that cell wall targeting agents, in addition to stalling cell wall synthesis, can also trigger activation of endogenous bacterial autolysins that facilitate cell wall degradation, leading to bacterial lysis (12). From the screen, a 1-amino substituted tetrahydrocarbazole (THCz) hit class was identified that was found to be bactericidal at low micromolar concentrations. Comprehensive structure–activity relationship (SAR) studies of a series of synthesized THCz analogs identified the two central amine groups as essential for antibacterial activity. Mode of action studies revealed that THCz simultaneously inhibit different cell envelope biosynthesis pathways by targeting lipid II (peptidoglycan), lipid IIIWTA (wall teichoic acid), and lipid Icap (capsule) as well as the central lipid carrier undecaprenyl pyrophosphate (C55-PP). Compared to natural lipid II inhibitors, THCz are relatively easy to synthesize and modify, and thus represent promising scaffolds for antibiotic drug development.

Results

Screening for Cell Wall Inhibitors. A whole-cell high-throughput screening (HTS) procedure was developed using the major respiratory tract pathogen Streptococcus pneumoniae as the bacterial target organism. The screen utilized the induction of pneumococcal autolysis to score for compounds with potential cell wall synthesis inhibiting activity. The decrease in optical density (OD600nm) following compound treatment was used as an indicator of bactericidal activity and provided an easy and powerful readout for hit selection. For the main screen, we used the nonencapsulated strain Tigr4R (T4R) that is generally more sensitive to most treatments than the encapsulated parental Tigr4 (T4) strain (Fig. 1A). Bacterial cultures in the early logarithmic growth phase were challenged with a compound library (50 μM per substance) comprising 17,500 substances (http://www.cbcs.se/), and the OD600nm was measured at timed intervals. For 99.1% of the compounds, an increase in OD600nm following compound addition was recorded, suggesting that no substantial growth inhibition occurred. Furthermore, no compound had a ΔOD600nm value near zero at 120 min after challenge that would indicate bacteriostatic activity. Compounds yielding a negative ΔOD600nm value at 120 min after treatment (156 compounds, 0.9%) were scored as hits. Out of these compounds, 71 also exhibited comparable activity against the encapsulated T4 strain and were validated on the LytA-deficient T4 derivative strain, resulting in a hit rate of 0.4% for the total screen (Fig. 1A).

Characterization of THCz Analogs. The present study characterizes three hit compounds from the screen (THCz-1, THCz-2, and THCz-3) (Fig. 1A) and related synthesized derivatives. These initial THCz screening hits shared a central tetrahydrocarbazole scaffold with a short 1-amino substituted linker (–NH–CH2CH2–) and had different substitutions in positions R1 and R2 (Fig. 1). The minimum inhibitory concentration (MIC) of the hit compounds against S. pneumoniae was determined to 1 μg/mL (Tables 1 and 2 and SI Appendix, Tables S1–S3). Clinical pneumococcal isolates from the Pneumococcal Molecular Epidemiology Network strain collection, resistant to one or several conventional antibiotics, were equally as sensitive to THCz-1 as the wild-type strain (SI Appendix, Table S4), showing that common acquired resistance mechanisms did not confer decreased sensitivity to THCz-1. Further testing of THCz-1 against a panel of clinically relevant pathogens (Table 1) revealed antimicrobial activity against a broad range of gram-positive bacteria,

![Fig. 1. Autolysin-mediated lysis screening identified THCz as an antibacterial hit class. (A) Schematic illustration of the autolysin-mediated lysis screen. Pneumococcal cultures grown in multiwell plates were challenged with the chemical compound library, and OD was recorded and used for evaluation of lysis induction and hit selection. Hit compounds were validated and classified. The three hit compounds from the tetrahydrocarbazole compound class (THCz-1, THCz-2, and THCz-3) are depicted. (B) Structure of THCz-1, where molecular moieties relevant for the structure activity and toxicity relationship investigations (R^1 and R^2) are highlighted with dashed lines. Also indicated is the central diamino motif that was identified as essential for activity shown in the protonation state likely encountered at physiological pH.](https://doi.org/10.1073/pnas.2108244118)
Table 1. Antibacterial spectrum of THCz-1

| Species                  | Strain                  | MIC THCz-1, μg mL⁻¹ |
|--------------------------|-------------------------|---------------------|
| *S. pneumoniae*          | ATCC 903                | 0.3                 |
| *Streptococcus parasanguinis* | ATCC 6051              | 1                   |
| *S. pyogenes*             | ATCC 29213              | 1                   |
| *S. aureus*               | ATCC 25923              | 1                   |
| *N. gonorrhoeae*          | ATCC 43617              | 8                   |
| *M. bovis*                | ATCC 35734              | 16                  |
| *M. smegmatis*            | ATCC 70084              | 8                   |
| *M. catarrhalis*          | ATCC 43617              | 8                   |
| *E. coli*                 | ATCC 11775              | >41                 |
| *P. aeruginosa*           | ATCC 10145             | >41                 |
| *N. gonorrhoeae*          | ATCC 10145             | >41                 |

THCz-1 sensitivity was determined in cation-adjusted MHB. All strains are characterized in SI Appendix, Table S8.

*THCz-1 sensitivity was determined in supplemented C-Y medium.

†THCz-1 sensitivity was determined in THY medium.

‡THCz-1 sensitivity was determined in TWEEN80 supplemented cation-adjusted MHB.

§Outer membrane hyperpermeable and efflux deficient.

Depending on the medium.

including drug-resistant strains, such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-intermediate *S. aureus* (VISA), and vancomycin-resistant *Enterococcus faecium* (VRE). Importantly, THCz-1 was also active against gram-negative pathogens such as *Neisseria gonorrhoeae* and *Moraxella catarrhals*, with MIC values in the range of 1 μg/mL to 8 μg/mL, and against mycobacteria (*Mycobacterium bovis* and *Mycobacterium smegmatis*, 8 μg/mL to 16 μg/mL). Moreover, the substance displayed activity against an *Escherichia coli* strain with a defective outer membrane, but not against wild-type *E. coli* or *Pseudomonas aeruginosa*, indicating that the outer membrane of certain gram-negative species may provide protection against THCz-1. Interestingly, Su et al. (13) have previously recorded activity against an *E. coli* strain of a related THCz analog but with a 2,4-diaminopyrimidine substituent in position R².

As the hit compound THCz-1 was obtained from an HTS assay that employed pneumococcal autolysis as the readout, lysis and killing kinetics were compared to penicillin and tetracycline that inhibits peptidoglycan and protein synthesis, respectively. Treatment of pneumococci with THCz-1 induced a more rapid bacteriolytic effect than penicillin, and no viable colonies could be recorded 9 h after treatment (Fig. 2 A and B and SI Appendix, Fig. S1). The contribution of cell wall hydrolyase activity to the lytic and bactericidal response was assayed using an isogenic pneumococcal strain deficient in the major autolysin LytA (T4ΔlytA) grown in an elevated (110 mM) concentration of choline chloride that causes cell wall dissociation and functional inactivation of other choline-binding cell wall hydrolyases. In the absence of hydrolyase activity, reduced lysis was observed following treatment of pneumococci with THCz-1 and penicillin (Fig. 2C), yet the bactericidal effect was retained, albeit with delay (Fig. 2 B and D). Tetracycline caused a growth-inhibitory phenotype without any prominent lysis and displayed a considerably slower killing curve that was not affected by the activity of cell wall hydrolyases (Fig. 2 A–D).

Transmission electron microscopy (TEM) of THCz-1–treated unencapsulated T4R cells confirmed that lysis was exerted through the action of choline-binding cell wall hydrolyases, since bacteria remained intact in a strain lacking the major autolysin *lytA* (T4ΔlytA) grown in elevated choline chloride concentrations (Fig. 2 E–H). THCz-1–treated *S. pneumoniae* (T4R) displayed cell wall ruptures in close proximity to the equatorial plane (Fig. 2F) correlating to the site where nascent peptidoglycan, the putative substrate of LytA, has been suggested to be incorporated during cell wall synthesis (14, 15). Together, these data confirmed that THCz-1 treatment, as for penicillin, caused induction of autolysin-mediated lysis, although the bactericidal activity is not solely explained by autolysin activation.

**SAR.** We next explored the molecular context of the THCz compound class in relation to the antibacterial activity. For this, we developed a synthesis scheme for substituted THCz and oxygen analogs (SI Appendix, Figs. S2 and S3) that allowed an exploration of the SAR of THCz derivatives and the investigation of the impact on toxicity of different substitutions on cultured human cell lines. The following SAR description is supported by a more extensive analysis provided in SI Appendix, Tables S1–S3. Most substitutions in position R¹ had little or no effect on the antibacterial activity, and an unsubstituted analog (THCz-45) maintained activity. However, a carbamate in R² (THCz-42) was found to have a marked negative effect on the antibacterial activity, suggesting that an anionic substituent in this position was not suitable. Sterically demanding substituents in R¹ (THCz-43 and THCz-44) did have an adverse effect on toxicity, leaving a methyl group (i.e., THCz-39 and THCz-40) or an unsubstituted R¹ (THCz-19 and THCz-45) the most favorable of the herein tested R¹ substitutions. In position R², it was important for antibacterial activity that a sterically demanding substituent, such as an adamantyl or a phenyl (i.e., THCz-3 and THCz-36 vs. THCz-24), was linked to the central scaffold by at least two carbons (THCz-36, THCz-37, and THCz-38 vs. THCz-35). Substitutions in the phenyl ring of R² were only favorable in the para position in the context of toxicity. A hydroxyl or bromo substituent (THCz-39 and THCz-40) caused a twofold to fourfold reduction in MIC and a corresponding increase in IC₅₀ in comparison to the original screening hit THCz-1 (Table 2).
The THCz analogs contain a stereocenter (SI Appendix, Fig. S5 A and B), and the initial screen and the SAR were carried out with racemic mixtures of the two enantiomers. We therefore separated the enantiomers from a subset of THCz analogs (THCz-1, THCz-39, and THCz-40) (SI Appendix, Fig. S5 C–I) and tested their antibacterial activities separately. Our data showed that both the R and S forms displayed similar MIC (SI Appendix, Table S5) in comparison to each other and to the racemic mixture. Combined, the untargeted investigation on modifications of the THCz scaffold showed that the central diamino motif was essential, and sterically demanding substitutions on a not less than two-carbon chain linker in R² were required, while modifications in R¹ were dispensable for antibacterial activity.

Mode of Action Studies. As THCz analogs showed an autolysin-inducing bacteriolytic effect in the early logarithmic phase, a well-known feature for cell wall targeting agents, we reasoned that THCz might interfere with cell wall synthesis. To verify this hypothesis and to approach target identification, a set of pathway-specific Bacillus subtilis bioreporter strains (16, 17) was treated with selected active analogs, THCz-1, THCz-39, and THCz-40 and compared to the inactive derivative THCz-5 (Table 2 and SI Appendix, Table S5). Indeed, THCz-1, THCz-39, and THCz-40, but not THCz-5, specifically induced the cell wall responsive reporter strain, while reporter strains indicative for interference with DNA, RNA, and protein biosynthesis were not activated (Fig. 3A). Furthermore, treatment of B. subtilis with THCz-1, THCz-39, and THCz-40 induced characteristic cell shape deformations, as visualized through phase-contrast microscopy, revealing the formation of cell membrane blebbing, indicative of inhibition of peptidoglycan biosynthesis and autolysin activation. Membrane blebbing was also observed following control treatment with vancomycin, nisin, or bacitracin, but not with the inactive THCz-5 or with antibiotics having targets other than the cell wall, such as clindamycin, ciprofloxacin, and rifampicin (Fig. 3B). To narrow down the target within the peptidoglycan biosynthesis pathway, we investigated the effect of THCz analogs on the LiaRS stress response using a B. subtilis luciferase bioreporter. The LiaRS two-component system is known to be sensitively induced in response to antibiotics that target lipid II or C55-PP, for example, vancomycin or bacitracin (18). Monitoring Pliai-lux bioluminescence over time revealed a strong induction for all THCz analogs tested, except for the inactive analog THCz-5 (Fig. 3C). Similar induction of the LiaRS reporter was also observed for the separated enantiomers of the active analogs, validating that the S and R forms have the same mode of action (SI Appendix, Fig. S6 A–C).

Corroborating interference with the lipid II biosynthesis cycle, treatment of S. aureus whole cells resulted in accumulation of the ultimate soluble peptidoglycan precursor UDP-MurNAc-pentapeptide (Fig. 3D), indicating that a late-stage membrane-associated peptidoglycan biosynthesis step was inhibited. Higher THCz concentrations (>5× MIC) impeded cytoplasmic accumulation due to increased lysis, as a result of induction of the autolytic system, or due to effects related to membrane interaction.

### Table 2. SAR and toxicity of a selection of THCz analogs

| THCz- | R¹   | X       | R²   | MIC [μM (μg mL⁻¹)]¹ | IC₅₀ [μM (μg mL⁻¹)] [μ]² |
|-------|------|---------|------|---------------------|-------------------------|
| 16    | NH   | NH      |      | 3.1 (1.3)           | 12.1 (4.9) ± 3.0 (1.2) [6] |
| 25    | NH   | NH      |      | 3.1 (1.1)           | 17.5 (7.1) ± 1.9 (0.8) [3] |
| 5     | NH   | NH      |      | >100 (>29.1)        | 140.8 (41.0) ± 16.9 (4.9) [4] |
| 6³    | NH   | O       |      | 50 (18.4)           | 134.1 (49.3) ± 42.4 (15.6) [5] |
| 19    | NH   | NH      |      | 6.3 (1.9)           | 22.2 (6.8) ± 1.8 (0.6) [3] |
| 39    | Me   | NH      |      | 6.3 - 12.5 (2.0 - 4.0) | 33.4 (10.7) ± 5.9 (1.9) [7] |
| 40    | Me   | NH      |      | 6.3 (2.4)           | 28.0 (10.7) ± 4.1 (1.6) [7] |

*Cy, cyclohexyl; Ph, phenyl; Me, methyl.

¹Minimum inhibitory concentration of THCz analogs for S. pneumoniae T4 in supplemented C+Y medium. Most abundant MIC is given. An overview of the observed MIC distribution is given in SI Appendix, Fig. 5A.

²IC₅₀ values of 10⁵ A549 cells/mL challenged with a serial titration of the respective THCz analogs. Average ± SD are given, and the number of biological replicates is noted in brackets. In the presence of 10% fetal bovine serum, mimicking cell culture conditions, the plasma protein binding affinity of THCz analogs caused an overall fourfold increase in MIC (SI Appendix, SI Text).

³Original hit compounds from the screen; see also Fig. 1 for structures.

⁴For simplicity, we refer to all compounds studied here as THCz, although this compound is an oxa-tetrahydrofuran.

**The THCz analogs** contain a stereocenter (**SI Appendix, Fig. S5 A and B**), and the initial screen and the SAR were carried out with racemic mixtures of the two enantiomers. We therefore separated the enantiomers from a subset of THCz analogs (THCz-1, THCz-39, and THCz-40) (**SI Appendix, Fig. S5 C–I**) and tested their antibacterial activities separately. Our data showed that both the R and S forms displayed similar MIC (**SI Appendix, Table S5**) in comparison to each other and to the racemic mixture. Combined, the untargeted investigation on modifications of the THCz scaffold showed that the central diamino motif was essential, and sterically demanding substitutions on a not less than two-carbon chain linker in R² were required, while modifications in R¹ were dispensable for antibacterial activity.

**Mode of Action Studies.** As THCz analogs showed an autolysin-inducing bacteriolytic effect in the early logarithmic phase, a well-known feature for cell wall targeting agents, we reasoned that THCz might interfere with cell wall synthesis. To verify this hypothesis and to approach target identification, a set of pathway-specific *Bacillus subtilis* bioreporter strains (16, 17) was treated with selected active analogs, THCz-1, THCz-39, and THCz-40 and compared to the inactive derivative THCz-5 (**SI Appendix, Table S5**). Indeed, THCz-1, THCz-39, and THCz-40, but not THCz-5, specifically induced the cell wall responsive reporter strain, while reporter strains indicative for interference with DNA, RNA, and protein biosynthesis were not activated (Fig. 3A). Furthermore, treatment of *B. subtilis* with THCz-1, THCz-39, and THCz-40 induced characteristic cell shape deformations, as visualized through phase-contrast microscopy, revealing the formation of cell membrane blebbing, indicative of inhibition of peptidoglycan biosynthesis and autolysin activation. Membrane blebbing was also observed following control treatment with vancomycin, nisin, or bacitracin, but not with the inactive THCz-5 or with antibiotics having targets other than the cell wall, such as clindamycin, ciprofloxacin, and rifampicin (Fig. 3B). To narrow down the target within the peptidoglycan biosynthesis pathway, we investigated the effect of THCz analogs on the LiaRS stress response using a *B. subtilis* luciferase bioreporter. The LiaRS two-component system is known to be sensitively induced in response to antibiotics that target lipid II or C55-PP, for example, vancomycin or bacitracin (18). Monitoring Plia-lux bioluminescence over time revealed a strong induction for all THCz analogs tested, except for the inactive analog THCz-5 (Fig. 3C). Similar induction of the LiaRS reporter was also observed for the separated enantiomers of the active analogs, validating that the S and R forms have the same mode of action (**SI Appendix, Fig. S6 A–C**).

Corroborating interference with the lipid II biosynthesis cycle, treatment of *S. aureus* whole cells resulted in accumulation of the ultimate soluble peptidoglycan precursor UDP-MurNAc-pentapeptide (Fig. 3D), indicating that a late-stage membrane-associated peptidoglycan biosynthesis step was inhibited. Higher THCz concentrations (>5× MIC) impeded cytoplasmic accumulation due to increased lysis, as a result of induction of the autolytic system, or due to effects related to membrane interaction.
THCz did not induce the formation of pores in comparison to the lantibiotic nisin which is an established pore former (SI Appendix, Supplementary Materials and Methods and Fig. S7) (9). However, THCz treatment resulted in the delocalization of GFP-MinD in *B. subtilis*, indicating membrane depolarization. The cell division inhibitor MinD is bound to the membrane via a C-terminal amphipathic helix and requires the presence of the membrane potential for its specific cellular localization pattern. MinD localizes to newly formed cell poles, thereby directing FtsZ to midcell guiding division septum placement (19). Compared to untreated control cells, GFP-MinD delocalized in cells treated with THCz, resulting in irregular dispersion of GFP-MinD within
5 min. MinD delocalization was also observed for the inactive THCz-5 variant, suggesting that the induced membrane effects are not the primary cause of killing (SI Appendix, Fig. S8).

In search of the molecular target, we next investigated the impact of THCz-1 on peptidoglycan biosynthesis reactions in vitro using purified S. aureus enzymes and substrates. Quantitative analysis of PBP2-mediated transglycosylation of lipid II revealed a dose-dependent inhibition (Fig. 3E). Almost complete inhibition was observed at a twofold molar excess of THCz-1 with respect to lipid II, suggesting that THCz-1 forms a stoichiometric complex with the substrate rather than inhibiting the enzyme. Similarly, lipid II synthesis catalyzed by the MurG glycosyltransferase was inhibited (SI Appendix, Fig. S9).

Furthermore, THCz-1 was also found to inhibit dephosphorylation of C55-PP to C55-P, a crucial step in the recycling of the lipid carrier conducted by undecaprenyl pyrophosphate phosphatase YbjG. A twofold molar excess of THCz-1 caused a full inhibition of YbjG-mediated dephosphorylation of C55-PP. THCz were added in molar ratios of 0.5 to 2 with respect to the amount of the substrate lipid II or C55-PP used in the individual test system. Inhibitions of PBP2- and YbjG-catalyzed reactions were quantified by the relative amount formed of C55-PP and C55-P, respectively. The error bars represent the SD from the triplicate runs. (E and F) Antagonistic effect of purified cell wall intermediates on antimicrobial activity of THCz. THCz compounds were exposed to selected purified cell wall precursors for 10 min at indicated molar ratios prior to incubation with M. luteus cells; +, antagonization of antimicrobial activity; −, no antagonization; ND, not determined. Results of three independent experiments are shown.

![Fig. 3](https://doi.org/10.1073/pnas.2108244118) THCz: Small molecules with antimicrobial activity that block cell wall lipid intermediates

---

**Fig. 3.** THCz analogs with intact diamino motif interact with C55-PP and C55-PP–linked cell wall precursors. (A) Impact of THCz-1 and analogs (THCz-40, THCz-39, and THCz-5) on major biosynthesis pathways in B. subtilis. B. subtilis bioreporter strains with selected promotor-lacZ gene fusions were used to identify interference with DNA (P_{promot}), RNA (P_{ppp}), protein (P_{prom}), and cell wall (P_{prom}) biosynthesis. Induction of a specific stress response results in expression of β-galactosidase indicated by a blue halo surrounding the inhibition zone. Antibiotics vancomycin, ciprofloxacin, clindamycin, and rifampicin were used as positive controls. (B) Treatment of B. subtilis with THCz induces severe cell shape deformations as visualized by phase-contrast microscopy. (Scale bar for all images: 2 μm.) (C) Induction of the LiaRS bioreporter by THCz compared to vancomycin, indicating interference with the lipid II biosynthesis cycle. (D) Intracellular accumulation of the ultimate soluble cell wall precursor UDP-MurNAc-pentapeptide in vancomycin-treated and THCz-1–treated cells of S. aureus as analyzed by means of reverse-phase HPLC. (E) Impact of THCz on individual peptidoglycan biosynthesis reactions. THCz inhibits the PBP2-catalyzed transglycosylation of lipid II and the YbjG-catalyzed dephosphorylation of C55-PP. THCz were added in molar ratios of 0.5 to 2 with respect to the amount of the substrate lipid II or C55-PP used in the individual test system. Inhibitions of PBP2- and YbjG-catalyzed reactions were quantified by the relative amount formed of C55-PP and C55-P, respectively. The error bars represent the SD from the triplicate runs. (E and F) Antagonistic effect of purified cell wall intermediates on antimicrobial activity of THCz. THCz compounds were exposed to selected purified cell wall precursors for 10 min at indicated molar ratios prior to incubation with M. luteus cells; +, antagonization of antimicrobial activity; −, no antagonization; ND, not determined. Results of three independent experiments are shown.
THCz-39 and THCz-40 that contained an intact diamino motif, in contrast to the inactive THCz-5 (Fig. 3G). In accordance with the in vitro data and the SAR profile, THCz-39 was less efficiently antagonized by lipid II compared to THCz-1 and THCz-40 (Fig. 3G), and fourfold higher concentrations of THCz-39 were required for full inhibition of the PBP2c-catalyzed reaction (SI Appendix, Fig. S10). In agreement with the in vitro activity of the racemic mixture, both enantiomers of THCz-40 similarly inhibited lipid II transglycosylation (SI Appendix, Fig. S6 D and E). Combined, these data indicate that the pyrophosphate moiety represents the essential motif for target interaction, and that the diamino motif of THCz analogs is required for this interaction. In agreement, the MurT/GatD-catalyzed amidation of lipid II was unaffected in the presence of THCz-1, strongly suggesting that the stem peptide of lipid II is not involved in binding (SI Appendix, Fig. S11). Furthermore, THCz analogs did not interfere with the PBP4-mediated carbboxypeptidation, releasing the terminal D-Ala residue from the pentapeptide stem (SI Appendix, Fig. S9B), suggesting that interactions with the lipid II stem peptide are less relevant and that THCz do not sterically hinder enzyme interaction with that region of the target molecule. Compared to natural lipid II binders, for example, vancomycin, THCz do not form extraction-stable complexes with lipid II (SI Appendix, Fig. S12), pointing to decreased binding affinity.

Together, these data reveal that THCz specifically binds to multiple undecaprenyl pyrophosphate–coupled cell wall precursors, and with C55-PP as such, thereby simultaneously inhibiting several cell wall biosynthetic pathways, including the recycling of the C55-P carrier utilized by all these pathways. A recent study reported that fungal P-type ATPases can be inhibited by similar THCz analogs, and a complex with a THCz analog and a mammalian Ca\(^{2+}\) ATPase (SERCA) was shown in a cocystal structure (20). However, in our study, neither single-deletion mutants of the four genes annotated as P-type ATPases in the S. pneumoniae T4 strain (SP0729, SP1551, SP1623, and SP2101) (SI Appendix, Tables S7 and S8) nor the quadruple mutant displayed decreased sensitivity to THCz-1, suggesting that P-type ATPases are not essential bacterial, or at least pneumococcal, THCz targets (SI Appendix, Table S6). The sequence similarity of these pneumococcal P-type ATPases with rabbit/human SERCA is only between 21% and 38% by identity, suggesting that the binding interfaces might be different.

THCz-1 Affects Capsule Production. More recently, dual targeting of similar cell wall precursors by teixobactin was correlated with a limited propensity to develop resistance (5). Resistance to THCz-1 also did not readily develop in vitro, as no resistant mutants could still grow at this concentration, contrary to the unexposed continuously THCz-1 exposed isolate #22 was affected by 2x MIC, whereas the unencapsulated T4R and marginally more than the less encapsulated T4cpsE\(^{G134MC}\)-Erm (BHN1691) (Fig. 4E). Thus, decreased or abolished capsule production caused a marginal desensitization to THCz-1. Interestingly, a short passage of S. pneumoniae in sub-MIC concentrations of THCz-1 (SI Appendix, Supplementary Materials and Methods) led to a decrease in the phosphorylcholine amount compared to the untreated strains (SI Appendix, Supplementary Materials and Methods and Fig. S15) as well as a decrease in the sensitivity of T4ΔlptA to externally added LytA (SI Appendix, Supplementary Materials and Methods and Fig. S16), indicating that THCz-1 also targets teichoic acid synthesis.

Discussion

In the present study, we developed a screening procedure where small-molecular compounds were screened for their ability to trigger autolysin-mediated lysis of pneumococci in order to find potential cell wall inhibitors. Although discovered in the 1970s, the underlying molecular mechanisms behind how cell wall–targeting antibiotics trigger autolysin activation still remain elusive (12). It has been suggested that an active cell wall synthesis machinery would sequester a potential autolysin substrate and that autolysin misplacement on teichoic acids would be part of the triggering event (14, 23–27). Our screening procedure for pneumococcal autolysin activation provided an uncomplicated and powerful protocol for identification of potential cell wall inhibitors. The herein described THCz compound class was one of the most active identified in the screen. Related THCz analogs with antimicrobial activity have been reported, but few targets have been proposed (13, 20, 28). Our mode of action studies revealed that THCz analogs stalled cell wall biosynthesis by targeting lipid II and other undecaprenyl pyrophosphate–containing lipid precursors involved in teichoic acid and capsule synthesis. The interaction required the pyrophosphate moiety (C55-PP) as minimal motif, and THCz relied on the diamino motif for potent antibacterial activity. It seems therefore likely that the two amino groups of THCz interact with the two negatively charged phosphate groups of the target. Supporting this, THCz analogs are expected to be positively charged at physiological pH, since the amino group in R\(^2\) has a calculated pK\(_a\) of 9.8 (for THCz-1). Furthermore, the requirement of a \(-\text{NH}-\text{CH}_2\text{CH}_2-\) linker-connected hydrophobic group in position R\(^2\) supports a model for target interaction in which this group is inserted into the plasma membrane to facilitate the diamino–pyrophosphate interaction (Fig. 5). The comparable activities of the tested THCz enantiomers could possibly be understood by such a model. If the role of the hydrophobic part of R\(^2\) is to anchor THCz into the plasma membrane, the relative stereo conformation would not be crucial for the
diamino–pyrophosphate interaction. This would thus contrast many antibiotics where stereoisomers display stereoselectivity due to protein binding constraints (29).

THCz analogs displayed a broad antibacterial spectrum and were active against all gram-positive Firmicutes species tested as well as Actinobacteria, including mycobacterial species. Notably, they were also active against gram-negative \textit{N. gonorrhoeae} and \textit{M. catarrhalis}, possessing lipooligosaccharides instead of lipopolysaccharides, which provides higher permeability across the outer membrane (30, 31), as has been observed for cationic and amphiphilic antimicrobial peptides, for example, the defensin Plectasin and the lantibiotic nisin (32). This is probably attributable to their relatively small molecular mass that allows for translocation across the outer membrane of certain species. THCz did not exhibit antimicrobial activity against wild-type \textit{E. coli}, while an \textit{E. coli} mutant with a hyperpermeable outer membrane was shown to be susceptible, indicating that THCz, despite their small size, are unable to pass the water-filled porins of gram-negatives. In addition to the broad antimicrobial spectrum, the low propensity of resistance development and absence of cross-protective resistant phenotypes are attributable to the target. The ubiquitous and highly conserved undecaprenyl pyrophosphate constitutes a keystone lipid scaffold for the synthesis and subcellular translocation not only of peptidoglycan but also for teichoic acids and polysaccharide capsule precursors, and is therefore hard for bacteria to modify. In accordance, the slightly decreased THCz-1 sensitivity of the obtained spontaneous \textit{cpsE} mutant with decreased capsule

Fig. 4. Decreased capsular polysaccharide production confers reduced sensitivity to THCz. Growth curves of strains (A) \textit{BHN1368} and (B) \textit{BHN1364} in THY medium challenged with a titration series of THCz-1, solvent control (DMSO) in comparison to untreated growth control. (C) Quantification of the capsular polysaccharide amount of the wild-type \textit{T4} (\textit{BHN842}), the unencapsulated \textit{T4R} (\textit{BHN659}), an isolate of \textit{T4} with reduced THCz-1 sensitivity (\textit{BHN1364}), unexposed control strain of \textit{T4} with retained THCz-1 sensitivity (\textit{BHN1368}), point mutant \textit{T4cpsE}^{G394C}-\textit{erm} (\textit{BHN1691}) with an \textit{erm} open reading frame (ORF) inserted in the capsular operon after \textit{cps4E} ORF, the \textit{cpsE} knockout strain (\textit{BHN1690}), and the control wild-type strain \textit{T4-erm}(\textit{BHN1692}) with an \textit{erm} ORF inserted in the capsular operon after \textit{cps4E} ORF. (D) Growth curve and exposure to a serial dilution of THCz-1 of \textit{T4}. (E) Bacterial killing after 1 h of exposure to 2x MIC THCz-1 (1.5 \text{\mu}M, 0.6 \text{\mu}g/mL in THY medium) of wild-type \textit{T4} (\textit{BHN842}), the unencapsulated \textit{T4R} (\textit{BHN659}), and the mutant \textit{T4cps}^{G394C} (\textit{BHN1691}). Paired experiments (each experiment was carried out in technical triplicates) are depicted with the same symbol. Average and SD are given.
did not affect sensitivity to THCz-1, arguing against these proteins as antibacterial targets. The low sequence similarity (<39%) between the pneumococcal and mammalian orthologs indicates that the binding interface might be different. Thus, knowledge of the structural requirements for C55'-PP interaction of THCz and the constraints of mammalian P-type ATPases binding should facilitate further modifications of THCz analogs to reduce cytotoxicity without affecting the antibacterial activity. In conclusion, the presented THCz analogs represent a class of small (<500 Da) synthetic molecules that inhibit bacterial cell wall synthesis by binding to lipid II and other undecaprenyl pyrophosphate–containing lipid precursors, targets conserved and present in all bacteria. This bacterial target interaction is highly attractive, and the small size and relative ease by which tetrahydrocarbazoles can be synthesized and modified could constitute a molecular platform for development of novel bacterial cell wall inhibitors.

Materials and Methods

Chemicals. A subset of 17,500 compounds from the Chemical Biology Consortium Sweden Primary Screening Set collection were screened in the whole-cell HTS assay. These compounds are chemically diverse and have lead-to-drug-like properties with respect to parameters such as molecular weight, hydrogen bond accepting and donating groups, lipophilicity, and polar surface area. Compounds THCz-1, THCz-2, THCz-3, THCz-24, and THCz-35 corresponding to ChemBridge ID nos. S279631, S272685, S256343, S279631, and S277652, respectively, were purchased from ChemBridge. All other compounds were synthesized as described in SI Appendix for general chemistry and synthesis of THCz analogs. The separation of enantiomers, circular dichroism spectroscopy, and the synthesis of the stereoselective compound (+)-(R)-AL682 are also given in SI Appendix. THCz analogs were dissolved in anhydrous dimethyl sulfoxide (DMSO) (Invitrogen).

Bacterial Growth Conditions. Pneumococci were cultured overnight on blood agar plates at 37 °C with 5% CO2. For pneumococcal suspension cultures, C+Y medium (SI Appendix) supplemented with 9% Glucose Bouillon (1% glucose added to 25 g/L Nutrient broth No. 2, Oxoid) and 1% horse serum (Haltunab) or Todd Hewitt broth (Sigma-Aldrich-T1438) with 0.5% yeast extract (THY medium) were used as indicated. For experiments with T4ΔlytA (SI Appendix, Table SB), the growth medium was supplemented with 110 mM choline chloride (Sigma), to inhibit choline-binding cell wall hydrolases. Growth conditions for other bacterial species are specified in the respective sections.

HTS Procedure. The unencapsulated S. pneumoniae strain T4R (SI Appendix, Table SB), an isogenic T4-derived strain, was used for the primary antibiotic screen. S. pneumoniae was grown in C+Y medium to an optical density at 600 nm OD600nm = 0.6 and sedimented by centrifugation (5 min, 5,000 rcf, 4 °C). The cells were resuspended in a 1:10 volume (thus 10-fold concentrated) in C+Y medium containing 20% glycerol and frozen at −80 °C. For screening, a 0.5-mL aliquot was thawed and diluted by mixing with 49.5 mL of prewarmed C+Y medium. The bacterial suspension was distributed into 96-well tissue culture plates (200 μL per well) of a Honeycomb plate (Oy Lysis Kinetics. For comparative lysis kinetics precultures of T4 or T4ΔlytA grown in supplemented C+Y medium (with an additional 110 mM choline chloride for the autolysin mutant) to midlog phase (OD600nm ≈ 0.5 (~5 to 6 × 109 colony-forming units (cfu)/mL)) were diluted to OD600nm = 0.05 in fresh medium and distributed into wells (400 μL per well) of a Honeycomb plate (Oy Growth Curves AB Ltd). A Bioscreen C plate reader (Oy Growth Curves AB Ltd) was used to record the growth kinetics. Cultures were grown to early log-phase (OD600nm ≈ 0.15 (~1 to 2 × 108 cfu/mL)), and treatments were added to a
Determination of the antimicrobial susceptibility testing from the Clinical and Laboratory Standards Institute (CLSI, formerly known as the National Committee for Clinical Laboratory Standards (NCCLS)). Compounds were administered in 100-μL stock solutions in 4-μL volumes to each well followed by thorough mixing. One percent (vol/vol) DMSO-treated cells and untreated controls were included. Sterile media samples were used to blank the measurement values. Viability determination of T4 and T4ΔΔ+ΔT+ was performed at 30 min, 3 h, 6 h, 9 h, and 12 h following challenge by serial dilutions and spreadings on blood agar plates from colonies that were counted after overnight incubation at 37 °C at 5% CO2. Where appropriate, 100 μL of the undiluted sample was plated, providing a detection limit of 10 cfu/mL.

TEM. Pneumococcal strains T4R and T4ΔΔ+ΔT+ (SI Appendix, Table S3) were prepared as for the lysis kinetic experiment. Untreated cells and cells treated with 10 μg/ml vancomycin were centrifuged, washed with PBS, and resuspended and fixed for 20 min with a 400-μL mixture of paraformaldehyde (2%) and glutaraldehyde (0.05%) in phosphate-buffered saline (PBS). Cells were then washed once with 1 mL of PBS and resuspended in 40 μL of PBS. Samples of 10 μL were placed for 2 min on carbon-coated grids (Oxford Instruments), and negative staining was performed with 2% uranyl acetate in water. Specimens were examined in a Tecnai 12 Spirit Bio TWIN TEM (FEI Company) operated at 100 kV, and digital images were recorded using a Veleta camera (Olympus Soft Imaging Solutions, GmbH).

Minimal Inhibitory Concentration Determinations. Determination of the minimal inhibitory concentration (MIC) was performed by the standardized microdilution procedure in accordance with the Performance Standards for Antimicrobial Susceptibility Testing of the Clinical and Laboratory Standards Institute (CLSI) (33) with the following modifications: For S. pneumoniae strains, the assay was done in supplemented C5 medium and in THY medium for comparison with other bacterial species. MICs against mycobacteria were determined in cation-adjusted Mueller Hinton broth (MHB) (Oxoid) supplemented with 0.05% Tween80 (Sigma-Aldrich). N. gonorrhoeae was grown in tryptone soy broth (Merck) supplemented with IsoVitalex (Becton Dickinson) according to the manufacturer’s instructions. All other strains were tested using cation-adjusted MHB. Briefly, the inoculum was prepared from a liquid preculture grown to midlog phase and diluted to reach a concentration of ~5 × 10^6 cfu/mL. Two microliters of the 100-fold concentrated serial dilutions of chemicals were added to the wells. The plates were incubated overnight at ambient atmosphere and 37 °C. For S. aureus, P. aeruginosa, and B. subtilis MIC determination in THY medium, the plates were incubated with shaking (~200 rpm). The MICs were determined as the lowest concentrations where no visible growth was observed. For determination of the plasma protein binding capacity of the THCz-1 compounds, bacteria were grown in C5 medium supplemented with 10% HyClone Fetal Bovine Serum (GE Healthcare).

Cytotoxicity Assay. Lung epithelial A549 cells (ATCC CCL-185) were grown in 1x RPMI medium 1640 (Gibco) with 9% HyClone Fetal Bovine Serum (GE Healthcare) and 1x Penicillin/Streptomycin solution (Gibco). Cells were trypsinized and seeded at 1 × 10^5 cells/mL for MIC determination (Table 2) and 1 × 10^6 cells/mL for SAR/STR structure toxicity relationship (STR) investigation (SI Appendix, Tables S1–S3) in 100 μL of medium per well of 96-well flat bottom plates (Sarstedt). The plates were incubated with shaking (~200 rpm). After 24 h, the medium containing the compounds was removed, and the cells were washed with PBS. Subsequently, 100 μL of antibiotic-free medium containing the compounds in a serial dilution for MIC determination (Table 2), and concentrations of 100, 50, 25, and 12.5 μM in 1% DMSO (final concentration) for SAR/STR investigation (SI Appendix, Tables S1–S3) were added to the wells. After 19 h, resazurin sodium salt (Sigma) (20 μL of 440 μg/mL in H2O) was added to each well, and the plates were further incubated for 4 h. Samples (80 μL per well) were transferred to micro test plates for immunoanalytics (Sarstedt), and absorbance was measured at 590 nm (SpectraMax Plus, Molecular Devices). Absorbance values were blanked with resazurin-containing medium, and the percentage of viable cells was calculated in comparison to solvent-treated cells. The reciprocal number for nonviable cells is given in SI Appendix, Tables S1–S3 for the estimation of cytotoxicity. Negative toxicity values or toxicity values below 100% were set to zero. 100% toxicity was considered as 0% survival. Cell culture calculation was performed using nonlinear fit log(1000−x)/log(100−x) versus response calculation with variable slope and constraints of 0% and 100% toxicity and top box constraints respectively in GraphPad Prism 5.04.

β-Galactosidase Reporter Assays. B. subtilis 168 amyE:pacC6 cultures with the promoter fusions Pwacυat-lox (cell wall), Pwacυat-lox (DNA), Pwacυat-lox (RNA), and Pwacυat-lox (protein) (34) were grown in MHB containing 5 μg/mL chloramphenicol at 30 °C to an OD600 of 0.5. Subsequently, Mueller Hinton agar was inoculated with 1 × 10^7 cfu/mL of the respective reporter strain. The agar was further supplemented with 5 μg/mL chloramphenicol, and X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at final concentrations of 75 μg/mL (cell wall), 125 μg/mL (DNA), and 250 μg/mL (RNA and protein). After pouring and solidification of the inoculated agar, 10 μg of THcz-1, 20 μg of THcz-40, 80 μg of THcz-39, and 100 μg of THc2-5 were spotted. Three micrograms of clindamycin served as positive control for the protein reporter and as negative control for the cell wall, DNA, and RNA reporter; 6 μg of vancomycin was used as positive control for the cell wall reporter and as negative control for the protein reporter; 0.3 μg of ciprofloxacin served as positive control for the DNA reporter; and 6 μg of rifampicin was used as positive control for the RNA reporter. Results were documented after 20 h of incubation at 30 °C.

Luciferase Reporter Assays. B. subtilis luciferase reporter assays were conducted as previously described (35). Briefly, B. subtilis 168 sacA:pCH101 was grown in MHB containing 5 μg/mL chloramphenicol at 30 °C to an OD600 of 0.5. Cells were added to 96-well white wall microtiter plates containing antibiotics (2 μg/mL THcz-1, 128 μg/mL THcz-5, 2 μg/mL vancomycin, 2 μg/mL bacitracin, 0.5 μg/mL lincomycin, 128 μg/mL ciprofloxacin, 128 μg/mL rifampicin, 128 μg/mL lysisone, or DMSO and incubated overnight. After 30 min of antibiotic exposure (treatment with lysisone was shortened to 10 min), 200-μL culture samples were fixed in 1 mL of a 1:3 mixture of acetic acid and methanol. Five microliters of fixed cells were immobilized on a thin film of 1% wt/vol agarose containing 0.9% (wt/vol) NaCl supported on a microscope slide. Imaging was performed by phase contrast microscopy on a Zeiss Axio Observer Z1 microscope (Zeiss) equipped with an HXP 120-V light source and an Axio Cam MR3 camera. Images were acquired with ZEN 2 software (Zeiss) and analyzed and postprocessed using ImageJ v1.45s software (NIH) (37).

Antagonization Assays. Antagonism assays were performed as previously described (5). Briefly, antagonizing the antibiotic activity of THcz by potential target molecules was conducted by a MIC-type assay setup in microtiter plates. THcz-1 (5 μg/mL) was mixed with potential antagonists (C55-P, C55-PP, UDP-N-acetylmuramic acid pentapeptide [UDP-MurNAc-pentapeptide], lipid I, lipid II, lipid IIIβ/α, and lipid Iβ/α in 0.5- to 10-fold molar excess with respect to the antibiotic. M. luteus DSM1790 (5 × 10^6 cfu/mL) was added, and samples were examined for visible growth after 20-h incubation. Experiments were performed in triplicate.

Quantification of Intracellular UDP-MurNAc-ppe. To analyze the cytoplasmic nucleotide pool, we adapted the protocol of Kohlrausch and Höltje (38). S. aureus SGS11 was grown in 15 mL of MHB at 37 °C to an OD600 of 0.6 and incubated with 130 μg/mL chloramphenicol for 15 min. THcz-1 was added at 1x, 2x, 5x, and 10x MIC and incubated for another 30 min. Lipid II-complexing for the protein reporter (THcz-1) was used as positive control. Extraction of nucleotide-linked peptidoglycan precursors and their analysis was performed by high-pressure liquid chromatography (HPLC) as described previously (39).

Impact of THcz on Membrane-Bound Peptidoglycan Biosynthesis Reactions In Vitro. Peptidoglycan synthesis reactions were reconstituted using purified proteins and substrates in vitro. PBP2-His6 and YbgJ-His6 were purified as described earlier (5, 39), except that PBP2 was solubilized with 0.06% Triton X-100, and additional immobilized metal ion affinity chromatography purification steps were performed.

Transglycosylation by PBFP2 was performed by incubating 2 nmol of lipid II in 20 mM 2-(N-morpholino)ethanesulfonic acid, 2 mM MgCl2, 2 mM CaCl2, 0.04% Triton X-100, pH 5.5 in a total volume of 50 μL. The reaction was initiated by the addition of 8 μg of PBFP2-His6 and incubated for 2 h at 37 °C. Vancomycin-containing products were extracted from the reaction mixtures with an equal volume of l-butanol/pyridine acetate, pH 4.2 (1:2, vol/vol), and analyzed by thin-layer chromatography using chloroform/methanol/water/ammonia.
1. A. Fleming, On the bacteriolytic action of cultures of a penicillium, with special reference to their use in the isolation of β-lactam. Br. J. Exp. Pathol. 10, 226 (1929).

2. World Health Organization, Antimicrobial Resistance: Global Report on Surveillance (World Health Organization, 2014).

3. H. Krömer, M. J. Boley, H. Rovini, The antibiotic drugs market. Nat. Rev. Drug Disc. 6, 19–20 (2007).

4. K. Bush, P. A. Bradford, β-Lactams and -lactamase inhibitors: An overview. Cold Spring Harb. Perspect. Med. 6, a025247 (2016).

5. L. L. Ling et al., A new antibiotic kills pathogens without detectable resistance. Nature 517, 455–459 (2015).

6. A. Muller, A. Klockner, T. Schneider, Targeting a cell wall biosynthesis hot spot. Nat. Prod. Rep. 34, 909–932 (2017).

7. A. J. F. Egan, J. Errington, W. Vollmer, Regulation of peptidoglycan synthesis and remodelling. Nat. Rev. Microbiol. 18, 446–460 (2020).

8. I. G. Boneca, G. Chiosis, Vancomycin resistance: Occurrence, mechanisms and strategies to combat it. Expert Opin. Ther. Targets 7, 311–328 (2003).

9. F. Grein, T. Schneider, H. G. Sahil, Docking on lipid II: A widespread mechanism for potent bacterioidal activities of antibiotic peptides. J. Mol. Biol. 431, 3520–3530 (2019).

10. J. Medema-Silva, S. Jekkmanne, E. Breukink, M. Weinigart, Towards the native binding modes of antibiotics that target lipid II. ChemBioChem 20, 1731–1738 (2019).

11. F. Grein et al., Ca2+–Daptomycin targets cell wall biosynthesis by forming a tripartite complex with undecaprenyl-coupled intermediates and membrane lipids. Nat. Commun. 11, 14550 (2020).

12. A. Medinas, S. Wals, Mechanism of action of defensins: Triggering of the peptidoglycan autolytic enzyme by inhibitors of cell wall synthesis. Proc. Natl. Acad. Sci. U.S.A. 72, 4162–4166 (1975).

13. L. Su et al., Design, synthesis and evaluation of hybrid of tetrahydrocarbazole with 2,4-diaminopyrimidine scaffold as antibacterial agents. Eur. J. Med. Chem. 162, 203–211 (2019).

14. P. Melloth et al., LytA, major autolysin of Streptococcus pneumoniae, requires access to nascent peptidoglycan. J. Biol. Chem. 287, 11018–11029 (2012).

15. A. Tymaps, M. Banazafi, C. A. Gross, W. Vollmer, From the regulation of peptidoglycan synthesis to bacterial growth and morphology. Nat. Rev. Microbiol. 10, 123–136 (2011).

16. A. Urban et al., Novel whole-cell antibiotic biosensors for compound discovery. Appl. Environ. Microbiol. 73, 6436–6443 (2007).

17. D. A. Wirtz et al., Biosynthesis and mechanism of action of the cell wall targeting antibiotic hypetpin. Angew. Chem. Int. Ed. Engl. 60, 13579–13586 (2021).

18. T. Mascher, S. L. Zimmer, T. A. Smith, J. D. Helmann, Antibiotic-inducible promoter governed by the cell envelope stress-sensing two-component system LysA of Bacillus subtilis. Antimicrob. Agents Chemother. 48, 2888–2896 (2004).

19. H. Strahl, L. W. Hamaone, Membrane potential is important for bacterial cell division. Proc. Natl. Acad. Sci. U.S.A. 107, 12281–12286 (2010).

20. J. Ellsbury et al., Tetrahydrocarbazoles are a novel class of potent P-type ATPase inhibitors with antifungal activity. PLoS ONE 13, e0188260 (2018).

21. R. T. Carter, W. T. Forsee, M. H. Bender, K. D. Ambrose, J. Ytterberg, CypA from type 2 Streptococcus pneumoniae catalyses the reversible addition of glucose-1-phosphate to a polypropyl phosphate acceptor, initiating type 2 capsule repeat unit formation. J. Bacteriol. 187, 7425–7432 (2005).

22. J. C. Paton, C. Trappetti, Streptococcus pneumoniae capsular polysaccharide. Microb. Spectr., 10.1128/microbiolspec.GPP3-0019-2018 (2019).

23. J. Flores-Kim, G. S. Dobhal, A. Fenton, D. Z. Rudner, T. G. Bernhardt, A switch in surface polymer biogenesis triggers growth-phase-dependent and antibiotic-induced bacterial lysis. eLife 8, e44912 (2019).

24. T. Homma et al., Dual targeting of cell wall precursors by teixobactin leads to cell lysis. Antimicrob. Agents Chemother. 60, 6510–6517 (2016).

25. P. Melloth et al., Structural and functional insights into peptidoglycan access for the lytic amidase LytA of Streptococcus pneumoniae. Mol. Biol, 601120–e13 (2014).

26. T. Sandalova et al., The crystal structure of the major pneumococcal autolysin LytA in complex with a large peptidoglycan fragment reveals the pivotal role of glycans for lytic activity. Mol. Microbiol. 101, 954–967 (2016).

27. M. Schlag et al., Role of staphylolysin wall teichoic acid in targeting the major autolysin LysA. Mol. Microbiol. 78, 864–873 (2010).

28. T. V. Akalaeva et al., Antitubercular, antifungal, and antibacterial activity in vitro of 1-phenethyliaminol-1,2,3,4-tetrahydrocarbazoles. Pharm. Chem. J. 34, 826–829 (1990).

29. A. J. Hutt, J. O’Grady, Drug chirality: A consideration of the significance of the stereochemistry of antimicrobial agents. J. Antimicrob. Chemother. 37, 7–32 (1996).
30. P. Edebrink et al., Structural studies of the O-polysaccharide from the lipopolysaccharide of Moraxella (Branhamella) catarrhalis serotype A (strain ATCC 25238). Carbohydr. Res. 257, 269–284 (1994).
31. T. Schneider et al., Plectasin, a fungal defensin, targets the bacterial cell wall precursor Lipid II. Science 328, 1168–1172 (2010).
32. C. Brunati et al., Expanding the potential of NAI-107 for treating serious ESKAPE pathogens: Synergistic combinations against Gram-negatives and bactericidal activity against non-dividing cells. J. Antimicrob. Chemother. 73, 414–424 (2018).
33. Clinical Laboratory Standards Institute, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard (Clinical and Laboratory Standards Institute, Wayne, PA, ed. 9, 2012).
34. H. Harms et al., Antimicrobial dialkylresorcins from marine-derived microorganisms: Insights into their mode of action and putative ecological relevance. Planta Med. 84, 1363–1371 (2018).
35. S. Tan, K. C. Ludwig, A. Müller, T. Schneider, J. R. Nodwell, The lasso peptide siamycin-I targets lipid II at the Gram-positive cell surface. ACS Chem. Biol. 14, 966–974 (2019).
36. M. Wenzel et al., Proteomic response of Bacillus subtilis to lantibiotics reflects differences in interaction with the cytoplasmic membrane. Antimicrob. Agents Chemother. 56, 5749–5757 (2012).
37. C. A. Schneider, W. S. Rasband, K. W. Eliceiri, NIH Image to Image: 25 years of image analysis. Nat. Methods 9, 671–675 (2012).
38. U. Kohlrausch, J. V. Höltje, Analysis of murein and murein precursors during antibiotic-induced lysis of Escherichia coli. J. Bacteriol. 173, 3425–3431 (1991).
39. T. Schneider et al., The lipopeptide antibiotic Friulimicin B inhibits cell wall biosynthesis through complex formation with bactoprenol phosphate. Antimicrob. Agents Chemother. 53, 1610–1618 (2009).
40. P. D. Rick et al., Characterization of the lipid-carrier involved in the synthesis of enterobacterial common antigen (ECA) and identification of a novel phosphoglyceride in a mutant of Salmonella typhiurium defective in ECA synthesis. Glycobiology 8, 557–567 (1998).
41. T. Schneider et al., In vitro assembly of a complete, pentaglycine interpeptide bridge containing cell wall precursor (lipid II-Gly5) of Staphylococcus aureus. Mol. Microbiol. 53, 675–685 (2004).
42. M. Rausch et al., Coordination of capsule assembly and cell wall biosynthesis in Staphylococcus aureus. Nat. Commun. 10, 1404 (2019).
43. G. Rouser, S. Fkeischer, A. Yamamoto, Two dimensional then layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. Lipids 5, 494–496 (1970).
44. P. García, M. P. González, E. García, R. López, J. L. García, LytB, a novel pneumococcal murein hydrolase essential for cell separation. Mol. Microbiol. 31, 1275–1281 (1999).
45. R. G. Kansal, A. McGeer, D. E. Low, A. Norrby-Teglund, M. Kotb, Inverse relation between disease severity and expression of the streptococcal cysteine protease, SpEL, among clonal M1T1 isolates recovered from invasive group A streptococcal infection cases. Infect. Immun. 68, 6362–6366 (2000).