TITLE:

Synthesis of Masarimycin, a Small Molecule Inhibitor of Gram-Positive Bacterial Growth.

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Peptidoglycan, metabolism, autolysin, chemical biology, inhibitor, cell wall

SUMMARY:

A detailed protocol is presented for preparing the bacteriostatic diamide masarimycin, a small molecule probe that inhibits the growth of *Bacillus subtilis* and *Streptococcus pneumoniae* by targeting cell wall degradation. Its application as a chemical probe is demonstrated in synergy/antagonism assays and morphological studies with *B. subtilis* and *S. pneumoniae*.

ABSTRACT:

Peptidoglycan (PG) in the cell wall of bacteria is a unique macromolecular structure that confers shape, and protection from the surrounding environment. Central to understanding cell growth and division is the knowledge of how PG degradation influences biosynthesis and cell wall assembly. Recently, the metabolic labeling of PG through the introduction of modified sugars or amino acids has been reported. While chemical interrogation of biosynthetic steps with small molecule inhibitors is possible, chemical biology tools to study PG degradation by autolysins are underdeveloped. Bacterial autolysins are a broad class of enzymes that are involved in the tightly coordinated degradation of PG. Here, a detailed protocol is presented for preparing a small molecule probe, masarimycin, which is an inhibitor of N-acetylglucosaminidase LytG in *Bacillus subtilis,* and cell wall metabolism in *Streptococcus pneumoniae*. Preparation of the inhibitor *via* microwave-assisted and classical organic synthesis is provided. Its applicability as a tool to study Gram-positive physiology in biological assays is presented.

INTRODUCTION:

Peptidoglycan (PG) is a mesh-like polymer that delineates cell shape and structure in both Gram-positive and Gram-negative bacteria[1,2]. This heteropolymer is a matrix of amino sugars cross-linked by short peptides[3–6] with a backbone composed of β-(1,4)-linked alternating N-

acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues (**Figure 1**)[1]. Attached to the C-3 lactyl moiety of MurNAc is the stem peptide. The metabolism of PG involves a tightly coordinated system of biosynthetic and degradative enzymes to incorporate new material into the cell wall[7,8]. Degradation of PG is carried out by enzymes collectively referred to as autolysins[9] and further classified based on the specificity of the bond cleaved. Autolysins participate in many cellular processes including cell growth, cell division, motility, PG maturation, chemotaxis, protein secretion, genetic competence, differentiation, and pathogenicity[10,11]. Unraveling the specific biological functions of individual autolysins can be daunting, due in part to functional redundancy. However, recent biophysical[8,12,13] and computational studies[12] have provided new insight into their roles in PG metabolism. In addition, recent reports have provided further insight into the synthesis[14] and membrane-mediated[15–17] steps in PG metabolism. A thorough understanding of the relationship between degradative and synthetic pathways of PG metabolism could give rise to previously untapped antibiotic targets.

While there have been significant advances in methodology to study glycobiology in eukaryotes, bacterial glycobiology and, in particular, PG metabolism has not advanced at a similar rate. Current chemical approaches to study PG metabolism include fluorescently labeled antibiotics[18], fluorescent probes[19,20], and metabolic labeling[21–24]. These new approaches are providing new ways to interrogate bacterial cell wall metabolism. While some of these strategies are capable of labeling PG *in vivo,* they can be species-specific[19], or only work in strains lacking a particular autolysin[25]. Many PG labeling strategies are intended for use with isolated cell walls[26] or with *in vitro* reconstituted PG biosynthesis pathways[20,27,28]. The use of fluorescently labeled antibiotics is currently limited to biosynthetic steps and transpeptidation[18].

The current knowledge of bacterial autolysins and their role in cell wall metabolism comes from genetic and *in vitro* biochemical analysis[11,29–32]. While these approaches have provided a wealth of information on this important class of enzymes, deciphering their biological role can be challenging. For instance, due to functional redundancy[33], deletion of an autolysin in most cases does not result in halting bacterial growth. This is despite their implied role in cell growth and division[7,12]. Another complication is that genetic deletion of bacterial autolysins can give rise to meta-phenotypes[34]. Meta-phenotypes arise from the complex interplay between the pathway affected by the genetic deletion and other interconnected pathways. For instance, a meta-phenotype can arise *via* a direct effect such as the lack of an enzyme, or an indirect effect such as a disruption of regulators.

Currently, there are only a few inhibitors of glycosidase autolysins such as N-acetylglucosaminidases (GlcNAcase) and N-acetylmuramidases, which can be used as chemical probes to study the degradation of PG. To address this, diamide masarimycin (previously termed as fgkc) has been identified and characterized[35] as a bacteriostatic inhibitor of *Bacillus subtilis* growth that targets the GlcNAcase LytG[32] (**Figure 1**). LytG is an *exo*-acting GlcNAcase[36], a member of cluster 2 within glycosyl hydrolase family 73 (GH73). It is the major active GlcNAcase during vegetative growth[32]. To our knowledge, masarimycin is the first inhibitor of a PG-acting GlcNAcase that inhibits cellular growth. Additional studies of masarimycin with *Streptococcus pneumoniae* found that masarimycin likely inhibits cell wall metabolism in this organism[37]. Here,

the preparation of masarimycin is reported for use as a chemical biology probe to study physiology in the Gram-positive organisms *B. subtilis,* and *S. pneumoniae.* Examples of morphological analysis of sub-minimum inhibitory concentration treatment with masarimycin, as well as a synergy/antagonism assay are presented. Synergy and antagonism assays using antibiotics with well-defined modes of action can be a useful way to explore connections between cellular processes[38–40].

PROTOCOL:

1. General methods

NOTE: All compounds were purchased from standard suppliers and used without further purification.

1.1 Carry out thin-layer chromatography (TLC) on an aluminum plate precoated with silica gel XG F254. Detect spots under a UV lamp, by immersion in *p*-anisaldehyde stain, or by exposing to I_2 vapor.

1.2 Record all nuclear magnetic resonance (NMR) spectra on a 400 MHz spectrometer.

NOTE: ^{1}H- NMR and ^{13}C-NMR spectra were referenced to residual solvent peaks. Coupling constants are given in [Hz] and chemical shifts in [ppm].

1.3 Record atmospheric pressure chemical ionization (APCI) mass spectrometry spectra of masarimycin on a compact mass spectrometer equipped with an atmospheric solids analysis probe.

2. General procedure for preparation of masarimycin

NOTE: Perform the below steps in a fume hood.

2.1 Prepare a 0.1 M solution in methanol of each reactant: cyclohexylamine, cyclohexyl carboxaldehyde, *o*-iodobenzoic acid, and cyclohexyl isocyanide[35].

CAUTION: Cyclohexylamine, cyclohexyl isocyanide, and cyclohexyl carboxaldehyde are flammable. They can cause skin corrosion and induce oral, dermal, respiratory, or reproductive toxicity. Keep compounds away from open flames, hot surfaces, and ignition sources. Wear appropriate skin and eye protection, work in a well-ventilated area and avoid inhalation of vapors or mist. For storage, keep bottles tightly closed and store them in a cool, dry place. Store cyclohexyl carboxaldehyde in a desiccator under an N_2 atmosphere.

2.2 Mix 5 mL of cyclohexylamine (0.1 M solution in methanol) and 5 mL of cyclohexyl carboxaldehyde (0.1 M in methanol) in a capped round bottom flask and stir the solution using a

magnetic stir bar on a stir/hot plate for 30 min at 40 °C in a sand bath. Monitor temperature using a thermometer placed approximately 1 cm below the sand surface.

2.3 After 30 min, add 5 mL of cyclohexyl isocyanide (0.1 M solution in methanol) to the solution from step 2.2 and stir for an additional 20 min at 50 °C. Lastly, add 5 mL of *o*-iodobenzoic acid (0.1 M solution in methanol) to the reaction mixture and continue stirring at 55 °C for 3–5 h.

2.4 Monitor the progress of the reaction periodically by TLC approximately every hour after the above reaction mixture had been stirred for 3 h.

2.5 Cut a 3 cm x 6 cm strip of aluminum-backed TLC plate. Using a #2 pencil, draw a line approximately 1 cm from the bottom. Using a glass microcapillary, spot approximately 5 µL of the reaction mixture onto the TLC plate and allow it to dry.

2.6 To a 150 mL beaker, add enough mobile phase (90:10 hexane: isopropanol) to cover the bottom of the beaker. Using a pair of tweezers, carefully place the above TLC plate into the beaker ensuring that the TLC plate enters the mobile phase evenly. Cover the top of the beaker with a piece of tinfoil.

NOTE: Ensure that the mobile phase does not cover the line and spotted sample.

2.7 Allow the mobile phase to travel up the TLC plate until it is approximately 1 cm below the top of the plate. Remove the TLC plate and using a pencil, draw a line indicating the distance traveled by the mobile phase. Allow the TLC plate to dry in a fume hood.

2.8 Once dried, place the TLC plate in a beaker containing a small amount of solid I_2 and cover the beaker with a piece of tin foil. Monitor the TLC for the development of yellow/brown spots. Once developed, remove the TLC plate and mark the location of the spots using a pencil (**Supplementary Figure 1**).

NOTE: If I_2 spots are not marked, the stain will dissipate over time. Spots can also be visualized on the TLC plate by UV-light, *p*-anisaldehyde staining, or potassium permanganate staining (see **Supplementary Information**).

2.9 Calculate R_f values for all visualized spots using the following formula:

$$R_f = \frac{distance\ travelled\ by\ spot\ (mm)}{distance\ travelled\ by\ mobile\ phase\ (mm)}$$

2.10 Consider the reaction complete when only one spot with $R_f = 0.3$ is visible on the TLC plate. Remove the solvent in a rotatory evaporator under reduced pressure and dry the crude product (obtained as a yellowish-brown oil) under a high vacuum until all methanol is evaporated.

2.11 Dissolve the dried crude product in 30 mL of ethyl acetate and transfer it to a separatory funnel. Extract ethyl acetate sequentially with 1 M HCl (2 x 30 mL), H_2O (30 mL), saturated

$NaHCO_3$ solution (2 x 30 mL), H_2O (30 mL) and saturated NaCl solution (2 x 30 mL). Discard the aqueous layers.

NOTE: The ethyl acetate layer is the top layer in each of the extractions. For each extraction, vigorously shake the separatory funnel containing the ethyl acetate and aqueous solution (HCl, H_2O, $NaHCO_3$, or NaCl) and allow the layers to fully separate.

2.12 Remove the ethyl acetate layer from the separatory funnel and collect it in an Erlenmeyer flask. Add a spatula full of Na_2SO_4 (anhydrous) to remove residual water from ethyl acetate.

NOTE: The ethyl acetate solution is considered dry when Na_2SO_4 in the flask runs freely and does not clump. If Na_2SO_4 is clumping, an additional spatula of Na_2SO_4 can be added.

2.13 Filter the dried ethyl acetate solution through #1 filter paper to remove Na_2SO_4. Wash the filter paper with a small amount of ethyl acetate. Take the filtered ethyl acetate into a round bottom flask and remove the solvent on a rotatory evaporator under reduced pressure to obtain masarimycin as oil once all the ethyl acetate is removed.

2.14 Dissolve the masarimycin oil obtained above in a minimal amount (1–2 mL) of 9:1 hexane: isopropanol and stir on a magnetic stir plate until all the compound is dissolved.

2.15 Purify the dissolved masarimycin by flash chromatography using a 12 g normal phase silica flash column.

2.15.1 Equilibrate the flash column with 10 column volumes of mobile phase (99:1 hexane: isopropanol) with the instrument set at a flow rate of 15 mL/min.

NOTE: After equilibration is completed, stop the flow and disconnect the top of the column from the system.

2.15.2 Draw the dissolved masarimycin using a 5 mL syringe. Connect the syringe directly to the top of the equilibrated flash column and inject the solution into the column. Reconnect the loaded column to the flash chromatography system and initiate the gradient elution.

2.15.3 Elute masarimycin from the column using gradient elution to a final mobile phase concentration of 10:90 hexane: isopropanol over 12 column volumes. Monitor the elution of masarimycin *via* absorption at 230 and 254 nm.

2.15.4 Collect the compounds eluted from the column by a fraction collector that collects 20 mL of solvent per fraction.

NOTE: If a flash chromatography system is not available, purification of masarimycin can be performed *via* a gravity silica column with a 3:1 (hexane: ethyl acetate) mobile phase. Fractions

containing masarimycin can be identified by TLC using the same mobile phase. Visualization of TLC spots was done with either UV light, I_2 vapor, or potassium permanganate staining.

2.15.5 Identify fractions containing masarimycin by TLC (steps 2.5–2.9) or mass spectrometry on a compact mass spectrometer equipped with an atmospheric solids analysis probe. Dry the final product under vacuum (~0.3 mbar).

NOTE: Masarimycin is routinely obtained as a colorless oil or solid with a yield of 55%–70% with respect to mmol of cyclohexyl carboxaldehyde added to the reaction. Calculate the final yield of masarimycin by obtaining the mass of the purified masarimycin and calculating the theoretical yield of the reaction using the following formula:

$$\% \text{ yield} = \frac{mass\ of\ purified\ product}{theoretical\ mass\ of\ product} \times 100\%$$

2.16 Confirm the structure of masarimycin by NMR.

2.16.1 Dissolve ~10 mg of masarimycin sample in 0.5 mL of $CDCl_3$. Using a Pasteur pipet, transfer the solution to a 5 mm NMR tube and cap the tube. Place the NMR tube in the spectrometer.

2.16.2 Acquire ^{1}H and ^{13}C NMR spectra using manufacturer preset experiments. Chemical shift assignments and representative spectra are provided in **Supplementary Figures 3–4**.

2.17 Store masarimycin dry or dissolved in DMSO (25 mM final concentration) at -20 °C until use.

3. Microwave procedure for preparation of masarimycin

3.1 Prepare 0.6 M solutions of cyclohexylamine, cyclohexyl carboxaldehyde, cyclohexyl isocyanide, and o-iodobenzoic acid in acetonitrile.

3.2 Add a stir bar and 10 mL of acetonitrile to a glass microwave reaction vial.

3.3 Add 2 mL of cyclohexylamine (0.6 M in acetonitrile), 2 mL of cyclohexyl carboxaldehyde (0.6 M in acetonitrile), and 7 mL of acetonitrile to the vial.

3.4 Place the microwave reaction vial in the microwave carousel. Stir the mixture, heat it for 30 min at 50 °C at a power setting of 400 W, and allow it to cool to room temperature.

3.5 Add 2 mL of o-iodobenzoic acid (0.6 M in methanol) and 2 mL of cyclohexyl isocyanide (0.6 M in acetonitrile) to the vial. Stir the mixture, heat it to 100 °C in the microwave for 40 min at a power setting of 400 W and allow it to cool to room temperature.

3.6 Monitor the progress of the reaction by TLC (90:10 hexane: isopropanol) using I_2 vapor after the completion of step 3.5.

NOTE: If TLC shows that the reaction is incomplete (i.e., multiple spots on TLC), place the reaction vial back in the microwave and set the microwave conditions described in step 3.5.

3.7 Once the reaction is complete, pour the solution into a 100 mL round-bottom flask and evaporate it to dryness using a rotary evaporator.

3.8 Follow steps 2.6–2.16 above to complete the aqueous workup, purification, and characterization of masarimycin.

4. Synergy and antagonism assay

4.1 Grow *Streptococcus pneumoniae R6* on Mueller-Hinton (MH) agar plates containing 5% (v/v) sheep blood at 37 °C under anaerobic conditions. In all experiments, use second passage cells grown in 5 mL of MH broth at 37 °C under anaerobic conditions until OD_{600} is ~0.4.

4.2 Subject the inhibitors masarimycin and optochin to serial 1:2 dilutions in respective solvents, with the resulting concentrations flanking the minimum inhibitory concentration (MIC) values of each inhibitor.

4.2.1 Make the initial dilution of masarimycin in dimethyl sulfoxide (DMSO) until a concentration of 100 µM was reached. From this point, make masarimycin dilutions in MH broth. Prepare optochin stock solution (3.5 mM) by dissolving commercially available optochin (see **Table of Materials**) in sterile MH broth.

NOTE: Masarimycin stock solutions were made at 25 mM in DMSO.

4.3 To a sterile 96-well microtitre plate, add 2 µL aliquots of each optochin dilution to each row of the plate. To the same plate, add 2 µL aliquots of each masarimycin dilution to each column to create an array of optochin and masarimycin concentrations on the plate (**Figure 2**).

4.4 Add sterile MH broth (93 µL) to each well containing the above inhibitors. Inoculate the microtitre plates with 5 µL of culture (OD_{600} ~0.4) from step 4.1.

NOTE: Inoculation of the 96-well plate is typically done under anaerobic conditions in an anaerobic workstation. The final volume in the well is 100 µL.

4.5 Grow cultures for 18 h at 37 °C under anaerobic conditions, followed by the addition of 30 µL of 0.01% (m/v) solution of resazurin sodium salt. Incubate the plate at room temperature for 15 min to allow the formation and stabilization of color.

NOTE: Resazurin solution is prepared by dissolving the compound in distilled water and can be stored at 4 °C for up to two weeks.

4.6 Directly read the concentration values from the plate and assign the lowest inhibitor concentration for which no bacterial growth is observed (blue color) as [X] (see step 4.7.1), i.e., the lowest inhibitory concentration of the drug in the presence of the co-drug.

NOTE: Positive bacterial growth is identified in the wells by the resazurin dye turning pink. MIC values for each drug alone (i.e., in the absence of co-drug) are determined in a similar manner using the resazurin MIC assay[35] with each drug separately (**Supplemental Figure 5**). MICs in *S. pneumoniae* are 7.8 µM and 15.85 µM for masarimycin and optochin, respectively.

4.7 Determine the fractional inhibitory concentration (FIC) and FIC index (FIC_I) using the following equations.

4.7.1 $FIC = [X]/MIC_x$, where [X] (from step 4.6) is the lowest inhibitory concentration of the drug in the presence of the co-drug, and MIC_x is the lowest inhibitory concentration of the drug in the absence of the co-drug.

4.7.2 $FIC_I = FIC_{masarimycin} + FIC_{antibiotic}$

NOTE: $FIC_I < 0.5$ = synergistic, $0.5 < FIC_I < 1$ = additive, $1 < FIC_I < 4$ = indifferent, $FIC_I > 4$ = antagonistic.

5. Morphological study

5.1 Grow *Bacillus subtilis 11774* on Luria-Bertani (LB) agar plates (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) containing 1.5% Bacto agar at 37 °C. In all experiments, use second passage cells grown in 5 mL of LB broth at 37 °C until $OD_{600} = 1$. Grow *S.pneumoniae* in the same manner as in step 4.1.

5.2 After obtaining a cell culture density with $OD_{600nm} = 1$ for *B. subtilis*, or $OD_{600nm} = 0.4$ for *S. pneumoniae*, add masarimycin using a pipette to the culture tube labeled "treated" to a final concentration of 3.8 µM (0.75x MIC for *B.subtilis*), or 5.85 µM (0.75x MIC for *S.pneumoniae*). To the second culture tube labeled "control", add an equivalent volume of DMSO.

5.3 For *B.subtilis,* place the samples in an incubator at 37 °C for 90 min with shaking at 150 rpm. For *S. pneumoniae,* incubate the cells without shaking under anaerobic conditions.

5.4 After 90 min, chemically fix the cultures in a 1:10 mixture (v/v) of culture media and fixing buffer (20 mM HEPES, 1% formaldehyde (pH 6.8)) at 4 °C overnight. After fixing is complete, apply 10–20 µL of samples to glass microscope slides using a pipette and allow them to air dry. Fix the air-dried samples by heating the glass slides using a Bunsen burner.

5.5 After heat-fixing, stain samples with the addition of 100 µL of 0.1% (m/v) methylene blue (solution in 20% (v/v) ethanol). Incubate the stained slides for 10 min and wash away the excess dye with dH_2O. Then, gently heat the stained slides to 60 °C in an oven for 15–20 min to bring

cells to a common focal plane.

5.6 Seal the stained samples by placing a microscope coverslip over the stained cells. Then, seal the edges using microscope slide cement. Place the sealed microscope slide on the microscope stage and bring the image into focus at 100x magnification using bright-field microscopy.

5.7 Place a drop of immersion oil on the microscope slide and bring the field of view to focus using 1000x magnification. Acquire micrographs using a camera attached to the microscope and its associated software. Acquire images using the auto white balance and aperture settings on the software.

NOTE: Alternatively, images can be processed using the open-source ImageJ software.

REPRESENTATIVE RESULTS:

Masarimycin is a small molecule bacteriostatic inhibitor of *B. subtilis* and *S. pneumoniae* and has been shown to inhibit the exo-acting GlcNAcase LytG in *B. subtilis*[35,37] and target the cell wall in *S. pneumoniae*[37]. Masarimycin can be efficiently prepared either by the classical or microwave-assisted organic synthesis with yields in the 55%–70% range. Microwave-assisted synthesis has the advantage of a significant reduction in time to synthesize the compound. Microwave-assisted synthesis shortens the synthesis from 5–6 h (traditional synthesis) to 2–3 h while maintaining comparable yields. Flash chromatography provides a rapid purification of masarimycin in high purity (**Supplementary Figures 1**–**2**). Structural assignments from ^{1}H and ^{13}C NMR spectra along with representative spectra are provided in **Supplementary Figures 3–4**.

Synergy and antagonism screens can be a useful tool to reveal functional connections among cellular components (synergy) and to investigate genetic networks and mechanisms of drug action (antagonism)[40]. Evaluation of synergy/antagonism with the ATPase inhibitor optochin in *S. pneumoniae* is presented in **Figure 2**. Resazurin microtitre plate assay[41] provides an easy readout of the growth/non-growth of the organism. The lowest concentration of compound to inhibit bacterial growth (blue color) is taken as the MIC value in the presence of a co-drug. Wells with bacterial growth will be pink in color. The relationship between masarimycin and optochin was determined by calculating the fractional inhibitor concentration index (FIC_I) using equations in protocol step 4.7. The FIC_I value for the masarimycin-optochin interaction is calculated to be 1.5, indicating an indifferent relationship based on published standards[42]. Phenotypic assays using masarimycin in *B. subtilis* at sub-MIC concentrations presented a sausage-like phenotype (**Figure 3B**) which differs from reported phenotypes of the Δ*lytG* mutant in the literature[32] and more closely resembles multiple autolysin knockouts[29]. Phenotypic analysis of *S. pneumoniae* with masarimycin at sub-MIC concentrations presented a clumping phenotype (**Figure 3D**). This clumping phenotype is distinct from those reported for *S. pneumoniae* cell-wall-acting GlcNAcases[43–45].

FIGURE AND TABLE LEGENDS:

Figure 1: Structure of peptidoglycan showing the cleavage site of the *exo*-acting *N*-acetyl glucosaminidase LytG from *Bacillus subtilis*. Inset shows the structure of the LytG inhibitor

masarimycin.

Figure 2: **Synergy/Antagonism assay to explore antagonistic/synergistic relationships with masarimycin and optochin in *S. pneumoniae*.** Blue or purple color indicates no bacterial growth, while pink color indicates bacterial growth. The MIC in the presence of co-drug is taken as the lowest concentration that shows no bacterial growth (blue color).

Figure 3: **Morphological analysis.** Morphological changes to *B. subtilis* (**A,B**) and *S. pneumoniae* (**C,D**) when treated with 0.75x MIC ($MIC_{B.subtilis}$ = 3.8 µM and $MIC_{S.pneumoniae}$ = 7.8 µM) masarimycin. Cells were fixed and stained with 0.1% (m/v) methylene blue and visualized by bright field microscopy under oil immersion at 1000x magnification. This figure has been modified from [35].

Supplementary Figure 1: Representative thin layer chromatography of masarimycin post aqueous workup. The mobile phase is 90:10 hexane: isopropanol and iodine vapor is used for staining spots. R_f = 0.3 for masarimycin.

Supplementary Figure 2: Representative flash chromatogram for the purification of masarimycin. The peak at approximately 1.2 column volumes contains masarimycin.

Supplementary Figure 3: Representative ^{1}H NMR of masarimycin dissolved in $CDCl_3$ and recorded on a 400 MHz NMR spectrometer. Spectrum is referenced to residual $CHCl_3$ solvent peak at δ = 7.26. Numbers in green above chemical shifts indicate proton assignments at the corresponding positions in the structure of masarimycin (see inset).

Supplementary Figure 4: Representative ^{13}C NMR spectrum of masarimycin dissolved in $CDCl_3$ at 100 MHz. Spectrum referenced to residual $CHCl_3$ solvent peak at δ = 77.36. Numbers in green above chemical shifts indicate carbon atom assignments at the positions in the structure of masarimycin (see inset).

Supplementary Figure 5: Representative resazurin MIC assay of masarimycin against *B. subtilis*.

DISCUSSION:

Masarimycin is a single micromolar bacteriostatic inhibitor of *B. subtilis*[35] *and S. pneumoniae*[37] growth. In *B. subtilis,* masarimycin has been shown to inhibit the GlcNAcase LytG[35], while the precise molecular target in the cell wall of *S. pneumoniae* has not been identified[37]. Synthesis of masarimycin using either the classical organic synthesis or microwave procedure provides the inhibitor in good yield and high purity. Low yields of masarimycin can typically be attributed to the oxidation of the cyclohexyl carboxaldehyde. To overcome this, it is recommended to store cyclohexyl carboxaldehyde under an inert atmosphere in a desiccator. Oxidation of the aldehyde to the corresponding carboxylic acid can be seen as a white solid in the bottle. Purchasing small quantities of cyclohexyl carboxaldehyde without storing it for extended periods greatly reduces this problem.

NMR assignment of masarimycin structure is complicated by the presence of a mixture of *cis* and

trans forms of the amide bond as well as atropisomers around the *o*-iodophenyl ring that results in multiple peaks. This can result in a proton chemical shift spread over 1 ppm thereby complicating assignments[35]. As a result, partial assignment of NMR chemical shifts for both ^{1}H and ^{13}C NMR spectra along with representative spectra are provided in **Supplementary Figures 3–4**. If there is difficulty in assigning ^{1}H and ^{13}C chemical shifts for masarimycin due to the mixture of isomers, 2-dimensional NMR experiments can be used. Correlated spectroscopy (COSY) can be used to identify proton spin systems, while heteronuclear single quantum coherence spectroscopy (HSQC) NMR experiments can be used to identify proton-carbon single bond correlations. Once purified, masarimycin can be stored at -20 °C as oil or dissolved in DMSO to a concentration of 25 mM until needed. It is recommended to store in small aliquots to reduce the number of freeze-thaw cycles. After repeated freeze-thaw cycles of the compound, the masarimycin stock solution should be checked by TLC to monitor for any degradation.

Synergy and antagonism screens can be an effective strategy to identify pathway interactions and can be used to understand the mode of action of small molecules. **Figure 2** shows an example of a synergy/antagonism assay with *S. pneumonia* R6 using masarimycin and the ATPase inhibitor optochin (note that the synergy/antagonism screening in *B. subtilis* is still an ongoing investigation). For reproducibility, second passage cells were used and grown to an OD_{600nm} of no more than 0.4. A FIC_I of 1.5 was observed for the interaction between masarimycin and optochin, indicating an indifferent relationship between the antibiotic pair. The indifferent relationship between masarimycin and optochin indicates no apparent interaction between the pathways these antibiotics target. While these assays can provide useful information about drug interactions, it is important to note that synergy/antagonism assays should be run with biological replicates and use of the more conservative cutoffs as described by Odds[42]. This helps to prevent the interpretation of observed minor synergistic or antagonistic relationships.

Phenotypic analysis of *B. subtilis* cells treated with sub-MIC masarimycin (**Figure 3B**) indicates a phenotype that differs from phenotypes reported for genetic deletion of *lytG*[32] and more closely resembles phenotypes of *B. subtilis* strains with multiple autolysin deletions[29]. This discrepancy in phenotype is intriguing because while *in vitro* inhibition of LytG has been demontrated[35], a *ΔlytG* mutant has no observable phenotype[32]. This discrepancy can in part be explained by differences in genetic and chemical inactivation[46,47]. The observed differences in the chemical or genetic inactivation of LytG is an intriguing question that is currently under investigation. *S. pneumoniae* cells treated with masarimycin presented a phenotype (**Figure 3D**) distinct from the genetic deletion of the corresponding GlcNAcase (GH73, cluster 2) LytB[37,43,44,48]. This morphological discrepancy highlights the challenges in assigning the mode of action or attributing the biological target of small molecule inhibitors. Morphological phenotypes can arise from a more complex set of interactions other than a single genetic deletion or chemical inactivation of a cell-wall acting enzyme. These meta-phenotypes[34] can arise from complex interactions *via* direct (lack of an enzyme(s)) or indirect (loss of regulators) mechanisms.

To the best of our knowledge, masarimycin is the first inhibitor of a bacterial autolysin that demonstrates inhibition of bacterial growth (**Supplementary Figure 5**). It is a narrow-spectrum bacteriostatic inhibitor of growth in *B. subtilis* and *S.pneumoniae.* This narrow spectrum is a

limitation for multi-species comparative studies of cell-wall metabolism between Gram-positive and Gram-negative organisms. This narrow spectrum is in part due to differences in some of the glycosyl hydrolase autolysins used during vegetative growth between Gram-positive (GlcNAcase) and Gram-negative (lytic transglycosylase) organisms. Using small-molecule inhibitors such as masarimycin for inhibiting PG autolysins, in particular, GlcNAcases can provide an orthogonal approach to traditional genetics for elucidating autolysin function. Masarimycin has a distinct advantage over some chemical biology methods, in that it can be used in more than one species (*B. subtilis* and *S. pneumoniae*). It can allow for comparative studies of cell wall metabolism between rod-shaped (*B.* subtilis) and coccoid (*S. pneumoniae*) species. The less coregulated cell wall metabolism and division in *S. pneumoniae* provides a counter-point in the more tightly regulated system of rod-shaped species[49,50]. Future applications of this technique will be to identify the molecular target in *S.pneumoniae* and explore the differences between genetic and chemical inactivation of autolysins in *S.pneumoniae* and *B. subtilis.*

Critical Steps in the Protocol

It is important to pay attention to the effective concentration of masarimycin in biological and biochemical assays. Due to its hydrophobic nature, concentrations above 250 µM (65x MIC in *B. subtilis*) can result in solubility and aggregation issues that can impact the interpretation of biological data. Properly controlling for the effect of vehicle (i.e*.,* DMSO) in all experiments is essential.

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DISCLOSURES:

Reid, C. W. has intellectual property involving specific applications of masarimycin.

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