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Facile Synthesis and Metabolic Incorporation of *m*-DAP Bioisosteres Into Cell Walls of Live Bacteria

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Abstract

Bacterial cell walls contain peptidoglycan (PG), a scaffold that provides proper rigidity to resist lysis from internal osmotic pressure and a barrier to protect cells against external stressors. It consists of repeating sugar units with a linkage to a stem peptide that becomes highly crosslinked by cell wall transpeptidases (TP). While synthetic PG fragments containing L-Lysine in the 3rd position on the stem peptide are easier to access, those with *meso*-diaminopimelic acid (*m*-DAP) pose a severe synthetic challenge. Herein, we describe a solid phase synthetic scheme based on widely available building blocks to assemble *meso*-cystine (*m*-CYT), which mimics key structural features of *m*-DAP. To demonstrate proper mimicry of *m*-DAP, cell wall probes were synthesized with *m*-CYT in place of *m*-DAP and evaluated for their metabolic processing in live bacterial cells. We found that *m*-CYT-based cell wall probes were properly processed by TPs in various bacterial species that endogenously contain *m*-DAP in their PG. Additionally, we have used hybrid Quantum Mechanical/Molecular Mechanical (QM/MM) and Molecular Dynamics (MD) simulations to explore the influence of *m*-DAP analogs on the PG crosslinking. The results showed that crosslinking mechanism of transpeptidases occurred through a concerted process. We anticipate that this strategy, which is based on the use of inexpensive and commercially available building blocks, can be widely adopted to provide greater accessibility of PG mimics for *m*-DAP containing organisms.

Introduction

Peptidoglycan (PG) is a structurally integral part of the bacterial cell wall that provides cellular rigidity and prevents lysis from osmotic stress. The primary monomeric unit of PG includes a disaccharide of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) sugars, with linkage to a stem peptide, typically a pentamer with the sequence L-Ala-iso-D-Glu-L-Lys-D-Ala-D-Ala or with *meso*-diaminopimelic acid (*m*-DAP) in place of L-Lys at the 3rd position.¹ Nascent PG units are imbedded within the existing PG scaffold by

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the transglycosylation of the disaccharides and the crosslinking of neighboring stem peptides, carried out by transpeptidase (TP) enzymes.²

There are considerable similarities in PG structure across different types of bacteria and, yet, slight structural variations can alter cell wall physiochemical properties and can tune interaction with neighboring cells including host organisms. Today, we know that fragments of PG are released into surrounding environments as by-products of cell wall metabolism or as a component of signaling systems to modulate the response from a neighboring bacteria or a host organism.¹⁻³ A prominent difference in PG primary structure occurs at the 3rd position on the stem peptide, which is also the site for PG crosslinking and Braun's lipoprotein attachment (Scheme 1A).^{4, 5} The difference of a single carboxylic acid between *m*-DAP and L-Lys can play a major role in how PG is sensed. As an example, vegetative *Bacillus subtilis* (*B. subtilis*) cells release PG fragments to communicate the initiation of a synchronized germination event from spores using the sensor protein PrkC.^{6, 7} The specificity in this communication is dictated by the complete lack of PrkC response to L-Lys containing PG fragments, which are nonnative to the *m*-DAP based PG of *B. subtilis*.⁸ Similarly, bacteria release biomacromolecules (e.g., PG) that are collectively known as pathogen-associated molecular patterns (PAMPs), which can be sensed by human immune cells indicating that there may be an infection.⁹ NOD1, from the family of PAMP receptors, is known to be activated by the minimal agonist D-Glu-*m*-DAP (iE-DAP).¹⁰ It was recently demonstrated that several bacterial species amidate *m*-DAP to evade NOD1 activation.^{11, 12} Together, these examples highlight how alterations to the PG stem peptide structure can regulate sensing and metabolism of PG fragments.

A large number of pathogenic and commensal bacteria have *m*-DAP based PG. Therefore, it is critically important to be able to synthesize homogeneous *m*-DAP based PG fragments (as opposed to heterogenous isolated PG fragments from live bacteria) to systematically dissect the biological consequences of alterations to the PG stem peptide found in these organisms.^{13, 14} While Fmoc-protected L-Lys building blocks are readily available and highly compatible with standard solid phase peptide chemistry, *m*-DAP is not commercially available and this poses a significant synthetic barrier. The internal symmetry of *m*-DAP coupled with the need to have differentiated, orthogonally protected stereocenters, makes its synthesis and that of its protected counterparts highly challenging.¹⁵⁻²⁰ Instead of the difficult-to-synthesize *m*-DAP, an alternative strategy involves the utilization of *m*-DAP analogs that mimic the key components and are functionally similar to *m*-DAP. Examples of such analogs include *meso*-lanthionine (*m*-LAN)²¹⁻²³ and *meso*-oxa-DAP.²⁴⁻²⁷ While both of these analogs recapitulate aspects of *m*-DAP as demonstrated by their processing by enzymes that recognize *m*-DAP residues, their synthesis still involves several solution phase chemistry steps and they are not readily adaptable to Fmoc-based solid phase chemistry. We envisioned that we could use widely available Fmoc-protected L-Cys as a precursor to the synthesis of *meso*-cystine (*m*-CYT) analogs (Scheme 1B). *m*-CYT preserves critical structural features at the terminal end of the sidechain, and this building block can easily be diversified using commercially available amino acids to build homogenous PG fragments and their naturally occurring derivatives. We leveraged our synthetic strategy to access variants of *m*-DAP bioisosteres and showed that naturally existing modifications to the PG structure can alter their metabolism and processing by cell wall biosynthetic machinery.

We anticipated that we could leverage the mimicry of *m*-DAP by *m*-CYT to study PG crosslinking, which is an essential step in the biosynthesis of properly structured bacterial cell walls. PG crosslinking is mainly carried out by Penicillin Binding Protein transpeptidases (D,D-TP) and L,D-transpeptidases (Ldts) to generate distinct connectivity patterns (Scheme 2A). The importance of these two TP classes of enzymes is illustrated by the general lack of sensitivity of Ldts against most β -lactam antibiotics (e.g., penicillins and cephalosporins). A large number of clinically relevant antibiotics interfere with PG crosslinking and, therefore, probes that track cell wall crosslinking dynamics can potentially reveal novel targets for drug discovery. Single D-amino acid PG probes have now become essential tools to image PG biosynthesis.^{28–33} Yet, single D-amino acid PG probes cannot interrogate how the stem peptide structure (or its many chemical modifications) can control PG crosslinking or processing. We^{34, 35}, and others^{36–39}, have recently demonstrated that fluorescently tagged structural mimics of nascent TP substrates can be crosslinked into the growing PG scaffold of live cells, thus hijacking the cell wall machinery (Scheme 2B). Indeed, we have shown that penta-, tetra-, and tri-peptides that are analogs of the PG stem peptide are accepted by TPs to varying extents.^{34, 35} However, to this point we had been unable to investigate the role of *m*-DAP (and its modifications) because of the challenge in building *m*-DAP containing peptides.

At first, we set out to establish a potential route to build *m*-CYT containing PG fragments that involved a post-cleavage modification with the crude peptide. Fmoc-L-Cys(Trt)-OH was coupled into the 3rd position of the pentapeptide using conventional peptide coupling conditions. Following the concomitant global deprotection and release from the solid support, the crude material was treated with D-cystine to promote the installation of D-Cys onto the free sulfhydryl of the L-Cys containing peptide *via* disulfide exchange to generate *m*-CYT. The lack of cysteine residues in naturally existing PG will ensure the chemoselective modification of L-Cys in synthesizing *m*-CYT containing PG fragments. Tripeptides were chosen to establish the recognition of the sidechain since tripeptides cannot act as acyl-donors in the TP reaction (they lack the D-Ala necessary for the acyl-donor step) and, instead, operate solely as acyl-acceptors. In this configuration, the amino group on the *m*-CYT tripeptide acts as the nucleophile in the PG crosslinking reaction (Scheme 2B). Fluorescein was conjugated to the *N*-terminus to track incorporation into the PG *via* TPs and fluorescence can be readily quantified using flow cytometry (Scheme 2C). A panel of probes was assembled based on analogs of the PG stem peptide that incorporated naturally existing chemical modifications to the backbone and sidechain (Figure 1A).

We first chose to evaluate the metabolic labeling of *Mycobacterium smegmatis* (*M. smegmatis*), whose PG biosynthesis tracks similarly with the pathogenic *Mycobacterium tuberculosis*, the causative agent of Tuberculosis (TB).^{40–42} TB continues to impose a tremendous health burden across the globe and the unique composition of the *Mycobacterium* cell wall provides an opportunity for the elucidation of novel drug targets. For this set of experiments, *M. smegmatis* growing in early lag-phase were treated with exogenously supplied probes and incubated overnight. Supplementation of *M. smegmatis* cells with **TriE***m*CYT (a tripeptide probe that contains iso-D-Glu on the second position and *m*-CYT on the 3rd position) resulted in cellular fluorescence levels that were ~50-fold higher

than control cells, a clear indication that *m*-CYT satisfactorily mimics *m*-DAP and is recognized by the native TP (Figure 1B). While PG precursors are initially biosynthesized with an iso-D-Glu in the 2nd position of the stem peptide, it is now established that for several Gram-positive organisms, iso-D-Glu is converted to iso-D-Gln.^{43–45} In fact, the gene encoding the tandem enzyme MurT-GatD, responsible for iso-D-Glu amidation, is essential in *Staphylococcus aureus* and *Streptococcus pneumoniae*.^{46, 47} It was demonstrated, *in vitro*, that absence of iso-D-Glu amidation resulted in undetectable levels of PG crosslinking.^{43, 44} Yet, PG crosslinking reactions utilize two PG strands and it was not established if iso-D-Glu amidation was required on both acyl-acceptor and acyl-donor strands. Our group recently showed that iso-D-Glu amidation plays an important role in controlling PG crosslinking in the acyl-donor strand of *M. smegmatis* and *M. tuberculosis*.³⁵ Amidation of the second position to iso-D-Glu (**TriQ_mCYT**) resulted in the doubling of PG incorporation as evident by the increase in cellular fluorescence, which indicates a preference for amidated iso-D-Glu but not an absolute requirement. Our new results indicate that iso-D-Glu amidation in the acyl-acceptor strand may not be an absolute requirement for PG crosslinking in *M. smegmatis*.

The sidechain of the 3rd position (L-Lys or *m*-DAP, Scheme 2) in naturally existing PG contains the critical amine nucleophile and it is, theoretically, possible that PG strands lacking the carboxylic acid could be competent acyl-strand acceptors in the TP crosslinking reactions. To test the essentiality of the carboxylic acid moiety in *m*-CYT, two control probes were designed and synthesized: **TriQLLys** and **TriQCystamine**. However, our results showed that the carboxylic acid moiety is essential for the acyl-acceptor strand to crosslink into the PG scaffold as the analogous probes lacking the carboxylic acid group led to minimal cellular fluorescence regardless of the linker composition (methylene and disulfide, Figure 1A). Critically, these results also indicate that the increase in cellular fluorescence is not purely due to disulfide exchange at the cell surface based on the low labeling levels with **TriQCystamine**. A major advantage of our strategy is the ability to access derivatives of *m*-DAP that are known to be naturally occurring such as amidation of the carboxylic acid. Currently, there is little known about *m*-DAP amidation in *M. smegmatis* and *M. tuberculosis*, whereas it was previously reported that the PG of *Mycobacterium abscessus* contains about 20 percent of amidated *m*-DAP.⁴⁸ Some plausible biological roles of *m*-DAP amidation have been proposed. In a transposon screen for lysozyme potentiation in a *m*-DAP containing organism, *Rhodococcus erythropolis*, a gene was identified that is related to *m*-DAP amidation.⁴⁹ Later, the same gene was found to play a role in β -lactam antibiotic sensitivity in *Corynebacterium glutamicum* (*C. glutamicum*)⁵⁰ and *B. subtilis*.⁵¹ The absence of live cell PG probes has hampered our ability to establish the effect of *m*-DAP amidation on PG crosslinking. To gain insight into this open question, we investigated how *m*-DAP amidation impacts TP crosslinking levels by building **TriE_mCYT_{NH2}** and **TriQ_mCYT_{NH2}**. Interestingly, amidation of the *m*-DAP crossbridge in combination with iso-D-Glu (**TriE_mCYT_{NH2}**) led to similar TP-based incorporation as **TriQ_mCYT**. These results suggest the possibility that *m*-DAP amidation serves as a compensatory modification to the lack of iso-D-Glu amidation. The double amidation in **TriQ_mCYT_{NH2}** led to fluorescence levels equivalent to with **TriE_mCYT**. 3D lattice light sheet (ref - see below comment) imaging revealed that fluorescence was concentrated at the cell boundaries, but

could also be observed to be concentrated at the cell poles and division septa (Figure 1C). Reversing the peptide sequence of the stem peptide analog led to a dramatic decrease in labeling levels (Figure S1).

Having demonstrated that tripeptide probes displaying *m*-CYT residues are functional in live bacterial cells, we next performed several additional experiments to further define the mode of probe incorporation into the bacterial PG scaffold. Incorporation of tripeptide-based probes can potentially be processed by two distinct classes of TPs. We attempted to determine if an individual TP class contributed more prominently to the crosslinking of the *m*-CYT probe into the PG scaffold by using antibiotics to interfere with specific TP function. As a point of comparison, we also assessed incorporation of two probes that we had previously disclosed to be D,D-TP specific (**PentaQLLys**) and Ldt-specific (**TetraQLLys**).³⁵ Co-incubation of ampicillin, a β -lactam that does not inhibit Ldts⁵², with **TriQmCYT** led to a slight increase in cellular labeling in a concentration dependent manner consistent with Ldt-mediated PG incorporation and similar to the Ldt-specific **TetraQLLys** (Figure 2). The increase in cellular fluorescence may be interpreted as a response mechanism to the inactivation of parts of the cell wall biosynthetic machinery, a feature that we previously observed with *Enterococcus faecium*³⁵ and we are exploring further. As expected, ampicillin treatment led to a decrease in cellular labeling with the D,D-TP specific **PentaQLLys**. Treatment with sub-lethal concentrations of meropenem, an inhibitor of both TP classes⁵³, resulted in a decrease of **TriQmCYT** labeling. The mechanism of PG incorporation was further interrogated by using Ldt-deletion strains. Whereas deletion of two individual Ldts did not impact cellular labeling, the deletion of the five primary Ldts led to near background cellular fluorescence (Figure S2). Together, these results principally established that *m*-CYT is a functional substitute for native *m*-DAP in TP processing in live bacterial cells.

We next sought to use computational simulations to assess the mimicry of *m*-CYT for *m*-DAP in the context of their recognition in the active site of Ldts. Initially, it should be pointed out that a classification of Ldts enzymes from *M. tuberculosis* and *M. smegmatis* was proposed taking into account the homology sequence of Ldts into 18 mycobacterial genomes.⁵⁴ A phylogenetic and biochemical study to distinguish mycobacterial Ldts was recently carried out by Zandi and co-workers in which the class 2 (LdtB) of Ldt_{Msm} was identified as homologue of Ldt_{Mt2}.⁵³ They established a theoretical model of Ldt_{Msm} (LdtB) by a homology modelling approach using the SWISS-MODEL server.^{55, 56} Here, we applied a similar procedure to the theoretical model of Ldt_{Msm} which has 70% of sequence identity with the selected template (Ldt_{Mt2}, PDB CODE: 5K69). This model had a RMSD of 0.064 Å after by overlapping template (Ldt_{Mt2}) and target (Ldt_{Msm}) structures, consistent with what was observed by Zandi and co-workers.⁵³ Therefore, our theoretical model of Ldt_{Msm} was appropriately used for classical and mechanical-quantum simulations. The details of our homologue model are available on SI.

Our previous QM/MM computational studies revealed that catalytic mechanism of Ldt_{Mt2} starts with a proton transfer from Cys346 to His328, where the ionic-pair is more stable than neutral form.^{57, 58} Here, the computational simulations start from product state of Ldt-PG reaction with ionic-pair of Cys364 and His328 catalytic residues. To explore the influence of

m-DAP analogs during PG crosslinking three systems were built: (I) Ldt-*m*-DAP, (II) Ldt-L-Lys, and (III) Ldt-*m*-CYT. The structural and energetic parameters obtained from QM/MM umbrella sampling simulations are summarized on Table S1. Our results for the 2D-FES obtained by the RCX versus RCY for the systems I-III are shown in Figure 3. The crosslinking mechanism of Ldt_{Msm} occurs *via* a concerted reaction, which agree with catalytic mechanism of Ldt_{Mt2} proposed on our previous computational studies. Interestingly, the theoretical free energy barrier obtained for the synthetic PG (*m*-CYT) are close to the value obtained to native PG (*m*-DAP) (about 3 kcal/mol higher), making the reaction between *m*-CYT and Ldt kinetically favorable. These results confirm that *m*-CYT can adopt the proper configuration to mimic the endogenous *m*-DAP.

Bacteria use modifications to the primary sequence of the PG stem peptide as an adaptation mechanism that is, likely, specific to its needs and niche.^{6, 59} As such, it is possible that amidation of D-iGlu and *m*-DAP may not have the same consequence in all bacteria with *m*-DAP based PG. We became interested in leveraging our probes to investigate naturally occurring PG modifications in the probiotic organism *Lactobacillus plantarum* (*L. plantarum*).⁶⁰ Despite our lack of understanding of all the ways that probiotics modulate host immune responses, it is clear that PG fragments play an integral part *via* the modulation of NOD receptors.^{61, 62} Chemical modifications to the PG stem peptide can, in turn, alter the level of activation by PG sensors as is evidenced by the muted response by NOD1 receptors when treated with PG fragments containing amidated D-iGlu⁶³ and *m*-DAP.⁶⁴ In fact, deletion of the gene responsible for *m*-DAP amidation in *L. plantarum* results in impaired cell growth and filamentation⁶⁵ and it is lethal in *B. subtilis*.⁵¹ Incorporation of tripeptide based probes into the PG of *L. plantarum* was tested by supplementing the probes during cell culture (Figure 4). Similar to *M. smegmatis*, the carboxylic acid within the sidechain in the 3rd position of the stem peptide was critical for high cellular labeling levels. In contrast to *M. smegmatis*, there is less pronounced selectivity for D-iGlu amidation and, instead, *m*-DAP amidation alone is sufficient to yield high levels of PG incorporation (Figure 4). Interestingly, the supplementation of **TriQmCYT_{NH2}** in *L. plantarum* led to the highest labeling levels our laboratory has ever observed with any cell wall probe and showed the expected labeling pattern (Figure S3). The high cell surface labeling levels observed could prove to be helpful in grafting epitopes (in place of fluorescein) on the surface of *L. plantarum* to promote specific adhesion to abiotic surfaces or targeted binding to pathogenic mammalian cells.

To confirm that the fluorescent signal in the flow cytometry experiments was, in fact, emitted from within the PG scaffold, a series of additional experiments were performed. First, *L. plantarum* cells were labeled with the tripeptide probe **TriQmCYT_{NH2}**, fixed with formaldehyde to preserve the basic cellular structure, and subjected to a treatment with mutanolysin, which is expected to selectively depolymerize the PG scaffold.⁶⁶ We found that fluorescent signals were reduced in a time-dependent manner, consistent with embedment of the probes within the PG scaffold (Figure S4). As a control experiment, labeled cells were treated with lysozyme and no reduction in cellular fluorescence was observed, as *L. plantarum* is inherently resistant to lysozyme due to *O*-acetylation of GlnNAc.⁶⁶ As a complement to PG digestion, which releases the entire scaffold regardless of what the stem

peptide structure is, we took advantage of the disulfide bond to delineate probe incorporation. *L. plantarum* cells were incubated with **TriQmCYT_{NH2}** and subsequently treated with dithiothreitol (DTT), which is expected to reduce the disulfide bond in the crosslinked *m*-CYT thus releasing the part of the probe containing fluorescein from the remaining PG scaffold (Figure S5). The observed decrease in cellular fluorescence is consistent with the proposed mode of incorporation and further suggests that the tripeptide probe is primarily incorporated as an acyl-acceptor strand because acyl-donor strands would not result in probe release from the PG. Similarly, pre-treatment of the probes with DTT prior to incubation with bacterial cells abolished labeling of cells with **TriQmCYT_{NH2}** but did not alter the labeling levels of the control D-Lys(FITC), which is a clear indication that the D-Cys fragment of *m*-CYT is necessary for incorporation into the PG (Figure S6). Finally, we performed PG analysis of **TriQmCYT_{NH2}** treated *L. plantarum* cells and found evidence of dimeric crosslinked PG fragments that are entirely consistent with Ldt-mediated incorporation and no evidence of PBP-mediated incorporation (Figure S7).

Appreciating that the disulfide bond in *m*-CYT provides a unique chemical handle, we hypothesized that we could selectively assess the mode of probe incorporation by treatment with a reducing agent. Transpeptidases catalyze the crosslinking of two neighboring stem peptides, with one stem peptide acting as the acyl-donor and the other acting as the acyl-acceptor strand. We previously showed that tetramer and pentamer stem peptide analogs can potentially act as either acyl-donor or -acceptor strands owing to the inclusion of the nucleophilic crossbridge and the sacrificial C-terminal D-alanine.^{35, 67} We reasoned that DTT reduction of the *m*-CYT containing probes after their metabolic incorporation within the PG scaffold would selectively release probes that were installed *via* the acyl-acceptor mode. On the other hand, probes should be retained if they were incorporated into the PG scaffold *via* the acyl-donor mode. To test this idea, we treated *L. plantarum* cells with **PentaQmCYT** or **PentaQmCYT_{NH2}** overnight and then treated them with DTT. Our results showed that there was increased labeling upon amidation of the crossbridge to carboxamide and that *m*-CYT is also recognized by the PG machinery when installed onto a pentameric stem peptide analog (Figure S7). Moreover, there was a considerable decrease in cellular fluorescence following DTT treatment of cells labeled with **PentaQmCYT_{NH2}** but no change in cells labeled with **D-Lys(FITC)**. The decrease in fluorescence levels likely points to **PentaQmCYT_{NH2}** as a primary acyl-acceptor strand with the remaining fluorescence potentially describing the portion of incorporation as acyl-donor strands. Finally, PG incorporation was verified using standard PG analysis from cells treated with **TriQmCYT_{NH2}**. Two additional bacterial species were tested for *m*-CYT labeling including *Listeria monocytogenes* (*L. monocytogenes*) and *Lactobacillus casei* (*L. casei*). *L. monocytogenes* possess an *m*-DAP type of PG structure while *L. casei* is a L-Lys type bacteria that typically contains a D-Asn crossbridge.⁶⁸ It was observed that *L. monocytogenes* labeled in a manner consistent with other *m*-DAP-based organisms (Figure S9) but, as expected, *L. casei* showed little labeling with the probe displaying *m*-CYT (Figure S10).

There is increasing interest in understanding the cell wall biosynthetic machinery in live bacterial cells. The principal driving force for *in vivo* analysis is the emerging paradigm of

extensive protein-protein interactions that control components of the cell wall biosynthetic machinery to modulate the growth and remodeling of PG. Our group and others have previously described a class of L-Lys-based PG probes that operates by mimicking components of the PG stem peptide to metabolically label the PG of live cells by TP-mediated incorporation, thus providing a direct signal that reports on PG biosynthesis. And yet, the lack of facile synthetic routes to *m*-DAP building blocks or their derivatives to assemble PG analogs of *m*-DAP containing organisms has hampered fundamental studies using live bacteria. We describe a strategy that makes use of the simple and widely available Fmoc-protected L-Cys to build *m*-CYT, which recapitulates key structural features of *m*-DAP. After demonstrating the ease of synthesizing *m*-CYT containing peptides, we showed that TPs accepted *m*-CYT in place of *m*-DAP by labeling live bacterial cells. More specifically, we installed *m*-CYT in a PG probe that can only be crosslinked into the bacterial PG scaffold by mimicking *m*-DAP. We anticipate that *m*-CYT will complement emerging tools to better understand cell wall biosynthesis in *m*-DAP containing organisms by providing a reliable route to a bioisostere that is biologically functional.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Chemical structures for tripeptide-based probes. Fl = carboxyfluorescein. (B) Flow cytometry analysis of *M. smegmatis* treated overnight with 100 μ M of tri-peptide probes. Data are represented as mean + SD (n = 3). (C) XY slice through a 3D fluorescence image of *M. smegmatis* treated overnight with 100 μ M of TAMRA modified **Tri_EmCYT_{NH2}**. Scale bar = 5 μ m. The full 3D image is available in the Supporting information.

Figure 2. Flow cytometry analysis of *M. smegmatis* treated overnight with 100 μM of each probe and increasing concentrations of ampicillin or meropenem. Data are represented as mean $\pm$ SD (n=3).

Figure 3.

Plots of 2D-FES calculated at DFTB/MM level (left) and combinations of RCX and RCY coordinates (right), describing the PG crosslinking mechanism of (A) Ldt-*m*-DAP, (B) Ldt-L-Lys, and (C) Ldt-*m*-CYT. QM atoms for each system are in blue color. The energy values are reported in kcal/mol.

Figure 4.

Flow cytometry analysis of *L. plantarum* treated overnight with 100 μ M of each probe. Data are represented as mean + SD (n=3).

Scheme 1.

(A) Chemical structure of the basic unit of PG (disaccharide attached to the stem pentapeptide). A major difference between bacterial species occurs at the 3rd position (L-Lys and *m*-DAP). This schematic diagram shows how the bioisostere *m*-CYT mimics key structural features of *m*-DAP. (B) Proposed synthetic route to assemble *m*-CYT containing PG fragments.

Scheme 2.

(A) Cartoon representation of the two primary modes of PG crosslinking carried about by D,D- and L,D-transpeptidases. (B) Snapshot representation of the acyl-intermediate of the TP reaction showing the hijacking of this step in the installation of the fluorescently tagged PG probe into the existing PG scaffold. (C) Probe incorporation on the cell surface can be readily measured using flow cytometry cell analysis.