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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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J. Am. Chem. Soc., **Just Accepted Manuscript** • DOI: 10.1021/jacs.8b03304 • Publication Date (Web): 09 Jul 2018

ACS Publications

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Synthesis of functionalized *N*-acetyl muramic acids to probe bacterial cell wall recycling and biosynthesis

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ABSTRACT: Uridine diphosphate *N*-acetyl muramic acid (UDP NAM) is a critical intermediate in bacterial peptidoglycan (PG) biosynthesis. As the primary source of muramic acid that shapes the PG backbone, modifications installed at the UDP NAM intermediate can be used to selectively tag and manipulate this polymer via metabolic incorporation. However, synthetic and purification strategies to access large quantities of these PG building blocks, as well as their derivatives, are challenging. A robust chemoenzymatic synthesis was developed using an expanded NAM library to produce a variety of 2-*N* functionalized UDP-NAMs. In addition, a synthetic strategy to access biorthogonal 3-lactic acid NAM derivatives was developed. The chemoenzymatic UDP synthesis revealed that the bacterial cell wall recycling enzymes MurNAc/GlcNAc anomeric kinase (AmgK) and NAM α-1 phosphate uridylyl transferase (MurU) were permissive to permutations at the two and three positions of the sugar donor. We further explored the utility of these derivatives in the fluorescent labeling of both Gram (-) and Gram (+) PG in whole cells using a variety of bioorthogonal chemistries including the tetrazine ligation. This report allows for rapid and scalable access to a variety of functionalized NAMs and UDP NAMs, which now can be used in tandem with other complementary bioorthogonal labeling strategies to address fundamental questions surrounding PG's role in immunology and microbiology.

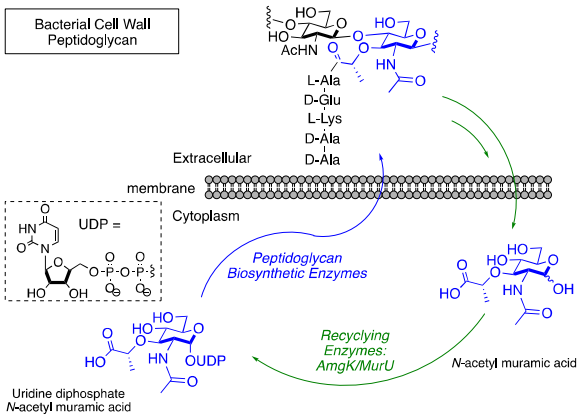
Introduction

Organophosphates are among the essential molecular components for life. In addition to their role in the scaffold of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), energy storage and post-translational modifications of proteins, these phosphate groups hold valuable roles in glycobiology.[1-2] From the polymerization of energy storing molecules such as glycogen to glycosylation of lipids on the surface of cells, the addition of a unique sugar molecule onto hetero-atom scaffolds via phosphate chemistry can tune the chemical and physical properties of the biomolecule.[3-8] While many phosphate shuttling biomolecules are key intermediates for signaling, they also serve as carriers in many essential biosynthetic pathways.

Uridine diphosphates (UDPs) are organophosphate carriers that play a critical role in the biosynthesis of bacterial peptidoglycan (PG), a coat that surrounds each bacterial cell to protect it from environmental changes and stresses.[9-10,11] The strength of the bacterial cell wall is directly correlated to its molecular composition; a complex network of peptides and two carbohydrates, *N*-acetyl glucosamine (NAG) and *N*-acetyl muramic acid (NAM), which are stitched together through a conserved biosynthetic pathway that utilizes UDP sugar carriers of NAG and NAM carbohydrates (Scheme 1).[10]

NAM serves as a core structural element exclusively for bacterial cell wall. While bacterial PG is dynamic, constantly changing as bacterial cells grow and divide, the NAM building blocks of this material are conserved across all bacterial species.[10] Synthetic and naturally occurring small molecule units derived from bacterial PG with the NAM glycan core are known to activate human innate immune responses.[12-13] Interestingly, extending or truncating the peptide portion of these intermediates results in a tunable immune response.[14-16]

Scheme 1. Peptidoglycan Recycling and Biosynthetic Pathways



The ability to monitor the natural production and breakdown of this polymer at both the glycan and peptide levels is critical for deciphering how human hosts are recognizing and responding to bacteria at the molecular level.[17-20] The development of a variety of small molecule NAM probes as well

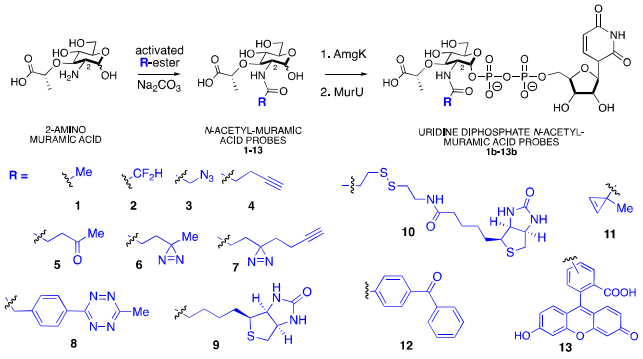
as methodology to selectively label the NAM core of PG in whole cells could help tease out the molecular fingerprint of these interactions to reveal how different bacteria present their PG to host cells or how host cells process this material.

All bacteria naturally build their PG utilizing a series of conserved enzymatic steps beginning with the production of UDP NAM through biosynthetic enzymes MurA and MurB.[21] Recently, our laboratory developed methodology to selectively install azide or alkyne bioorthogonal tags onto the NAM backbone of bacterial PG in whole cells for fluorescent visualization via copper catalyzed azide alkyne cycloaddition (CuAAC).[22-25] This strategy was achieved by taking advantage of the alternative formation of UDP NAM via the NAM lactol building block and cell wall recycling enzymes MurNAc/GlcNAc anomeric kinase (AmgK) and NAM α-1 phosphate uridylyl transferase (MurU)[26] (Scheme 1). Here, we further explored the chemical space of AmgK and MurU substrates to expand both the NAM and UDP NAM tools available to probe bacterial PG biosynthesis and recycling. We developed the necessary synthetic routes to show that the system is amenable to a variety of permutations and demonstrate the utility of the method in labeling bacterial PG.

Results

The investigation began with the design and synthesis of an expanded library of NAM derivatives to probe the substrate specificity of AmgK. The modular synthesis of the previously reported *N*-acetyl azido and alkyne NAM derivatives (**3, 4**) gave access to large quantities of the precursor, 2-amino muramic acid (Scheme 2). This molecule was poised to functionalize using mild amide bond coupling conditions with a variety of activated ester derivatives in order to generate a library of NAM compounds (Scheme 2, Supporting Information). This library includes multiple bioorthogonal NAMs[27] (Scheme 2, Table 1) ranging from ketone condensation intermediates[28] (**5**), to inverse electron demand diels alder (IEDDA) compounds[29-32] (**8, 11**). Photoactivatable intermediates with diazirine[33-37] and benzophenone functionality[38-40] (**6, 7, 12**) were also synthesized in addition to di-fluoro methyl[41-43] (**2**) fluorescein (**13**) and biotin[44-46] tags (**9, 10**). To assess if modifications around the carbohydrate were tolerated, the starting material, 2-amino-muramic acid and a NAM derivative with an azide modification at the 6-position, 6-azido-NAM, were synthesized (Supporting Information).

Scheme 2. Library of 2- *N* NAM and UDP NAM probes



With the library of NAM derivatives in hand, the substrate specificity of the bacterial cell wall recycling enzyme AmgK was probed (Table 1 and 2). Each of the NAM derivatives were treated with purified AmgK[26] and product formation was monitored by high resolution mass spectrometry (HRMS) (Supporting Information Table 1). The corresponding mono-phosphate NAM products were observed in almost all of the intermediates with the exception of cyclopropene derivative **11,** benzophenone **12** and fluorescein derivative **13** (Table 1, Supporting Information Table 1), suggesting that these modifications were not accepted by the kinase. 6-azido-NAM (Supporting Information, Scheme 3) and 2-amino-muramic acid showed no conversion into the monophosphate, indicating that they are not substrates.

Bioorthogonal Reaction or Chemical Utility	Substrate	AmgK	MurU
Copper Catalyzed Azide-Alkyne Cycloaddition	3, 4, 14, 6-azido NAM	Y, Y, Y, N	Y, Y, Y, N
Ketone Condensation	5	Y	Y
Photoactivating Crosslinker	6, 7, 12	Y, Y, N	Y, Y, ---
Inverse Electron-Demand Diel's-Alder	8, 11	Y, N	Y, ---
NMR Characterization	2	Y	Y
Streptavidin Affinity Purification	9, 10	Y, Y	N, N
Fluorescence Visualization	13	N	---
Other	1, amino	Y, N	Y, ---

Table 1. AmgK and MurU substrate specificity. Each compound was grouped based on common bioorthogonal reactivity or utility. Turnover of AmgK and MurU are reported with either (Y) representing formation of corresponding product, (N) representing no formation of product and (---) representing untested substrate due to no conversion of AmgK. Product formation was monitored by high-resolution mass spectrometry (HRMS).

In order to compare AmgK's substrate preferences, the kinetic profile of AmgK with the NAM derivatives was measured according to the ATPase enzyme coupled assay used by Mayer and co-workers.[47] For **1**, apparent K_m and k_{cat} were 30.41 ± 5.75 μM and 6.58 ± 0.21 s^{-1}, respectively (Table 2, Supporting Information). These data agree with the values reported by Mayer and coworkers[26]. For substrates **3** and **4**, AmgK showed ~10 fold less efficiency than it had turning over **1**. The catalytic efficiencies were ~100 fold less with other derivatives as substrates (Table 2, Supporting Information). Apparent k_{cat}/K_m values were acquired for substrates whose rates did not plateau by fitting the experimental kinetic parameters to a double reciprocal plot (Table 2, Supporting Information). **5**, **6**, **7, 8** and **9** appear to have high K_m values based on this analysis, suggesting that the enzyme has low affinity for these larger modifications (Supporting Information).

The monophosphate NAM intermediates were then subjected to uridylyl transferase MurU[48] to generate the UDP-sugar (Scheme 2). The production of the UDP NAM derivatives was monitored by HRMS (Table 1, Supporting Information Table 1). MurU converted the monophosphate intermediates of **1**-**8** to their respective UDP products as observed by HRMS. Furthermore, intermediates **1, 3**-**5** were converted in milligram quantities (i.e. > 10 mgs) and isolated by preparatory mass directed HPLC for complete NMR characterization (Supporting Information).

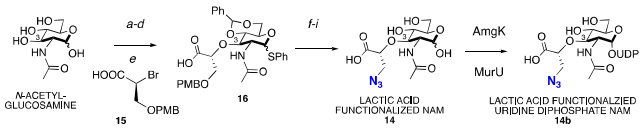
Table 2. Kinetic Characterization of AmgK with NAM derivatives as substrates.

Substrate	k_{cat}(s^{-1})	K_m(mM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
1[a]	6.58 ± 0.21	(3.04 ± 0.58) X 10^{-2}	(2.16 ± 0.42) X 10^5
3[a]	14.07 ± 0.72	1.12 ± 0.16	(1.26 ± 0.19) X 10^4
4[a]	9.74 ± 0.45	0.55 ± 0.09	(1.8 ± 0.3) X 10^4
14[a]	4.67 ± 0.14	(3.03 ± 0.54) X10^{-2}	(1.54 ± 0.28) X 10^5
5[b]	-	-	3.45 X 10^3
6[b]	-	-	1.55 X 10^3
7[b]	-	-	1.02 X 10^3
8[b]	-	-	2.25 X 10^3
9[b]	-	-	2.95 X 10^3

([a], kinetic parameters were calculated by fitting the experimental data into the Michaelis-Menten equation using program GraphPad Prism-6. [b], Apparent k_{cat}/K_m values were calculated by fitting the experimental data to the Lineweaver-Burk equation). Standard Error (SE) were calculated based on 3 technical replicates of each sample concentration

In addition to the 2-*N* functionalized NAM derivatives, modification at the 3-OH lactic acid portion of the small molecule was desired (Scheme 3). It is known that some bacteria including *Mycobacterium tuberculosis* (Mtb), naturally modify their bacterial PG to a *N*- glycolyl moiety at the 2-*N* position via NamH.[49-50] In order to avoid the loss of a 2-*N* biorthogonal probe through this pathway, we moved to install the functionality at another location of the NAM sugar. As these derivatives have not been made before, a synthesis of lactic acid functionalized NAMs was designed and implemented (Scheme 3, Supporting Information).

To efficiently derivatize the 3-OH position, the synthesis of a novel azide modified lactic acid precursor, which would be added to the NAG core, was developed (Scheme 3, Supporting Information). We found that the addition reaction proceeded most efficiently with the brominated lactic acid scaffold **15.** The *para*-methoxybenzyl (PMB) protecting group was selected over other groups due to ease of installation and removal. **15** was readily coupled to the suitably protected NAG 1-thioglycoside to yield the derivatized NAM. Subsequent methyl esterification, PMB deprotection with DDQ revealed the free alcohol, which was activated for azido installation.



Scheme 3. Synthesis of lactic acid N_3 functionalized NAM

a. Ac_2O, DMAP, pyridine (85%), b. PhSH, $SnCl_4$, DCM, reflux (54%), c. NaOMe, MeOH, r.t. (quant), d. $PhCH(OMe)_2$, TsOH, DMF, 70°C (72%), e. NaH, **15**, DMF (57%), f. K_2CO_3, MeI, DMF, r.t. (80%), g. DDQ, DCM/H_2O (quant), h. MsCl, pyridine/DCM (80%), i. 1. NaN_3, DMF, 70°C; 2. IRA H^+, H_2O, 95°C; 3. TCCA (12% over 3 steps).

To generate the final unprotected NAM lactic acid derivative, a mild deprotection strategy was utilized. Notably, the thiol-protecting group was removed under oxidative conditions with trichloroisocyanuric acid (TCCA)[51], an environmentally friendly chemical reagent. . Global deprotection revealed compound **14**. **14** was shown to be accepted by recycling enzymes AmgK and MurU to generate the corresponding UDP NAM intermediate **14b** by HRLCMS (Table 1 and Supplementary Table 1). Kinetic characterization with AmgK was performed and the data show that the kinase accepted the lactic acid modification with the same efficiency as the natural NAM (Table 2).

With the formation of the UDP-NAM derivatives at the 2 and 3 position of the carbohydrate confirmed, the promiscuity of the bacterial PG biosynthetic enzymes MurC-F was explored (Scheme 1, Supplemental Scheme 1). All of the UDP NAM derivatives subjected to MurC-F were able to be converted into the desired pentapeptide Park's nucleotide derivatives and were observed by HRMS (Supporting Information Table 1). The relaxed substrate specificity of the PG recycling and biosynthetic enzymes motivated the expansion of the PG glycan labeling method of whole bacterial cells. In order to improve this methodology, three key areas were explored: modification at the 3-postion of the NAM, click-chemistry utility and UDP-NAM-carrier tolerance

First the lactic acid modified NAM derivative **14** was tested for incorporation in whole bacterial cells. *E. coli QKU*, a MurQ[52] knockout *E. coli* strain that contains the recycling enzymes AmgK and MurU, cells were pulsed with **14** for two doubling times (45 minutes) in the presence of fosfomycin and subsequently treated with CuAAC click chemistry conditions as previously reported[22]. Cells were visualized with superresolution Structured Illumination Microscopy (SIM)[53] (Fig 1, Supplementary Fig 1, Supplemental Material). Fluorescent signal was observed at the septal division ring of the bacterial cells as compared to the positive control cells treated with **3** and the negative control cells treated with **1** in which only background fluorescence was observed (Supplementary Fig. 2 and 3A, respectively). This positive labeling result suggests that NAM derivative **14** can be utilized by the bacterial PG biosynthetic enzymes, which is consistent with the *in vitro* enzymatic conversion study (Supporting information Table 1).

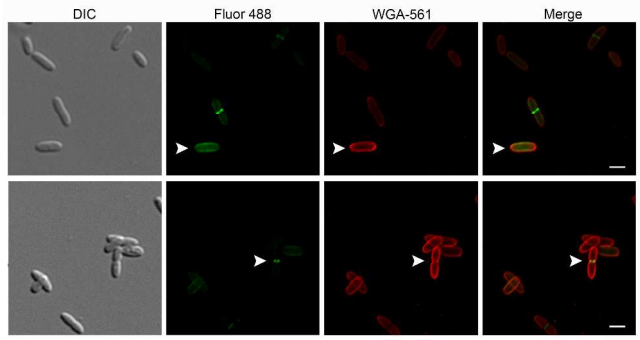


Figure 1. *E. coli QKU* cells fluorescently modified with the lactic acid NAM derivative 14 and WGA co-staining. DIC and maximum intensity projection 2D images from super-resolution SIM Z-stacks of *E. coli* QKU cells treated with **14**, fosfomycin, IPTG for 45 min and clicked with Alk488 (green) and then stained with tetramethylrhodamine WGA 561 (red) of whole cell (white arrow, top) and dividing cell (white arrow, bottom) (scale bars, 10 μm). Images are representative of a minimum of three fields viewed per replicate with at least two technical replicates and the experiment was conducted in three biological replicates.

Other bioorthogonal "click" chemistries were probed for tolerance by the NAM-PG labeling method. Previously, copper catalyzed azide-alkyne cycloaddition (CuAAC) in whole cells was performed. Here the tetrazine-transcyclooctene (TCO) ligation, the fastest click reaction to date was assessed.[32, 54] *E. coli* QKU cells were treated with either **1** or NAM derivative **8** and IEDDA was performed with TCO-TAMRA (Supporting Information). Cells were visualized with superresolution SIM. The positive control cells were labeled with **3** and treated with CuAAc to ensure *E. coli* QKU labeling competence (Supplementary Fig 4). The optimal probe pulse length of **8** was assessed (Supplementary Fig. 5A) in the presence and absence of fosfomycin. It was determined that 20 min pulse lengths were optimal without fosfomycin (Fig 2). It appeared that fluorescent labeling was only obtained in select cells per field of view when cells were treated with **8** (Fig 2, Supplementary Figure 5B), indicating that the uptake of the tetrazine probe was not uniform throughout cells or that a sub-population of the cells were capable of accepting the tetrazine modification. Cells that were treated with **1**, the unlabeled control, showed no fluorescence (Supplementary Fig 3B), suggesting that this limited labeling is not due to the fluorophore non-specifically associating to the bacteria.

Traditionally, we and others have utilized mass spectrometry evidence for probe PG incorporation.[22, 55-56] However, due to the low fluorescent probe incorporation with **8** and **14**, we desired a method other than mass spectrometry to confirm PG labeling. Wheat germ agglutinin (WGA) co-labeling, a technique commonly used to stain the NAG backbone of the bacterial PG was used.[57-58] If co-localization was observed with the TCO-TAMRA probe, then modification of the PG could be considered. Co-localization with tetramethylrhodamine-WGA stain, in *E. coli* QKU cells treated with **14** was observed suggesting labeling of the PG (Fig. 1, Supplementary Figure 1). No co-localization with 488-WGA stain in *E. coli* QKU cells treated with **8** was observed (Supplementary Fig 6). Cells that incorporated **8** appeared to have degraded cell morphologies, indicating that tetrazine incorporation is not well tolerated and thus has low WGA signal.

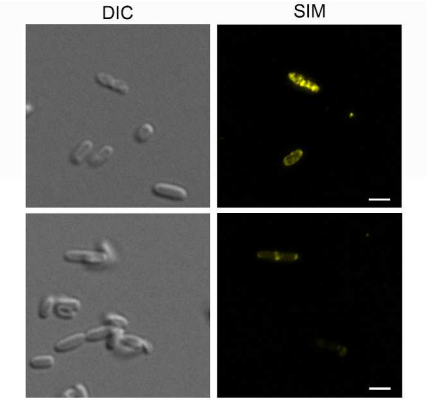


Figure 2. *E. coli QKU* cells modified with 8 for tetrazine-TCO ligation. DIC and 2D images from super-resolution SIM Maximum Intensity Projection Images Z-stacks of *E. coli* QKU cells treated with **8** and IPTG for 20 min and clicked with TCO-TAMRA (yellow) (scale bars, 2 μm). Images are representative of a minimum of three fields viewed per replicate with at least two technical replicates and the experiment was conducted in at least three biological replicates.

Finally, UDP-NAM modifications were tested for whole cell incorporation. To date, two methods have been used to incorporate NAM derivatives into the PG of whole cells. One involves the genetic manipulation of the bacterial cell to include the recycling enzymes AmgK and MurU[22] . The second method involved a laborious 15 step synthesis of uridine diphosphate muramyl tetrapeptide fluorescein derivative[59]. The latter was a tour de force that demonstrated the unnatural UDP peptide fluorophore derivative could be taken up and embedded into the PG of Gram + lactic acid bacteria. However, the method was limited due to the demanding chemical synthesis of the UDP probe. Therefore, we took advantage of the scalable chemoenzymatic production of the uridine diphosphate sugar derivatives **3b** and **4b** containing the azide and alkyne functionality, respectively (Scheme 2). This method was amendable to milligram scale synthesis; the phosphate and uridylyl steps were performed subsequently without purification of the phosphate intermediate (Supporting Information). Yields for the multiple enzymatic transformations ranged from 19% to 89%. Whole cell bacterial labeling with these derivatives was explored using compound **3b**.

UDP sugars exist as charged species at physiological pH (Scheme 1 and 2), and diffusion through the bacterial cell membrane could be challenging. Therefore, the whole cell studies began with the bacterium *Lactobacillus acidophilus,* a commensal[60] Gram + organism commonly found in yogurt and previously used by Nishimura and coworkers to label bacterial PG using UDP fluorescein lysine derivatives.[59] After incubation with *L. acidophilus* and the azido UDP NAM **3b**, SIM imaging revealed fluorescent labeling as compared to the control cells which were incubated with the natural UDP NAM compound **1b** (Supplementary Fig. 7 A and B). *L. acidophilus* cells that were treated with **3b** and CuAAC were then stained with WGA-561 and PG co-localization was observed (Fig. 3). These data imply that the other UDP derivatives isolated on scale (**4b-6b**) could be incorporated. We note that

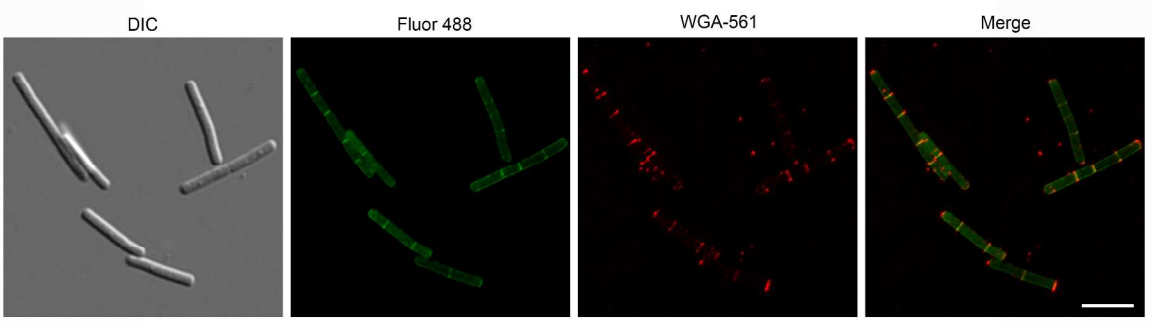


Figure 3. *L. acidophilus* labeling with azido UDP NAM 3b and WGA costaining. DIC and 2D Maximum Intensity Projection images from super-resolution SIM Z-stacks *L. acidophilus* cells treated with **3b**, clicked with Alk488 (green) and treated with WGA-561 (red) (scale bars, 10 μm). Images are representative of a minimum of three fields viewed per replicate with at least two technical replicates and the experiment was conducted in at three biological replicates.

bacterial labeling was only achieved while bacteria were actively growing (Supplementary Fig 8A,B).

As the growth conditions are challenging for *L. acidophilus*, other bacteria species were assayed for UDP NAM **2b** labeling. *S. aureus*, *B. subtilis* and two strains of *E. coli: E. coli DH5a* and *E. coli* RMF795, an *E. coli* mutant strain with a semi-permeable outer membrane.[61] *S. aureus*, *B. subtilis, and* the *E. coli* strains were treated with the azido UDP NAM **3b**. SIM imaging revealed no fluorescent labeling after click incorporation of fluorophore as compared to the control cells which were incubated with the natural UDP NAM **1b** (Supplementary Figure 9A-D). We hypothesize that a subset of species may be capable of UDP NAM uptake or tolerant to modification on NAM residues. These probes could help define which bacteria have this capability.

Discussion

Traditionally, the synthesis of UDP and its sugar cargos has been extremely demanding, and therefore underexplored. As UDP sugars are critical for nearly every step of the biosynthesis of bacterial PG, access to these phosphate intermediates is valuable to understanding the vitality of this polymer. The modular synthesis of 2-*N* functionalized NAMs allowed for the development of a library of NAM derivatives to include substrates beyond the azide and alkyne compounds (**3, 4**). We chose to construct the library at the 2-*N*-postion (Scheme 1) as modification at the 6 position was not tolerated by AmgK (Table 1, entry 1). We have expanded the NAM probes to include derivatives with capabilities of Staudinger ligations, IEDDA, photo activating, affinity tagging, as well as fluorine NMR spectroscopy analysis (Scheme 2). With these derivatives in hand, we were able to assess the substrate specificity and kinetic profile of the recycling enzyme AmgK, a key enzyme in the production line of uridine diphosphate NAM sugars (Table 1 and Table 2). AmgK appears to tolerate modifications at the 2 and 3 positions of the carbohydrate, converting modifications such as levulinic acid, biotin, tetrazine, and diazirine to the respective phosphor-sugars. Compounds that lacked the acetyl (Table 1, entry 8) or displayed a quaternary center alpha to the carbonyl of the amide (**11-13**) were not accepted by the enzyme (Table 1). As there is no crystal structure for this kinase, we propose that AmgK "reads" the carbonyl state of the NAM. Although AmgK was able to turnover substrates containing atomistically large modifications at the 2-position, kinetic characterization revealed that not all modifications were well tolerated. Bulky modification to the 2-position resulted in a ~100-fold decrease in the catalytic efficiency of the enzyme (**5-9**, Table 2). Yet, modification at the 3-position was comparable to the natural substrate (Table 2), suggesting that the enzyme is more tolerant to modification at this position. Further experiments are necessary to test the steric limitations of this position on the carbohydrate.

We further explored the chemoenzymatic synthesis of UDP NAM derivatives, of which until this work were previously limited through synthetic methodology and no UDP NAM derivatives with modifications on the carbohydrate have been reported. We optimized the purification and compound characterization of these UDP derivatives and further explored their utility in the conversion to the pentapeptide intermediates of PG biosynthesis known as Park's nucleotide (Scheme 1). Substrates that AmgK converted to the mono-phosphate were next used to assess substrate specificity for the other PG recycling enzyme, MurU, and the four PG biosynthetic enzymes (MurC-F) (Scheme 1). The data show that all modifications tolerated by AmgK were accepted by the subsequent enzymes, except the biotin modifications, **9** and **10**, which was not turned over by MurU (Table 1, Supporting Information Table 1). This is the first report of a large collection of Park's nucleotide derivatives and will be valuable probes in studying bacterial cell wall biosynthesis in a variety of organisms.

Other types of bio-orthogonal chemistry were assayed; the tetrazine NAM derivative **8** was subjected to the whole cell labeling methodology in which genetically engineered *E. coli* cells containing the recycling enzymes AmgK and MurU were subjected to IEDDA with TCO TAMRA. While few cells were labeled using this methodology, the ones that were fluorescent have different morphologies (Fig 2) with what appears to be a thinner cell wall compared to the healthier cells in the field of view. While it appears that the smaller bioorthonoal functionality is more desirable to study global whole cell NAM labeling, the tetrazine modification upon optimization could prove useful in studying live-cell UDP NAM production and shuttling.

In order to test if the PG labeling method was amenable to modification at the 3-postion of the NAM, a new synthesis of a modified 3-muramic acid was developed. The synthesis of the 3- azido NAM derivative **14** was designed and optimized to correctly set the muramic acid stereochemistry while incorporating the unnatural bioorthogonal azide handle (Scheme 3). Biochemical assays indicated that both the recy-

cling and early PG biosynthetic enzymes are permissive to the azide modification (Table 1, 2 and Supporting Table 1). When **14** was used in the whole cell labeling of *E. coli* QKU cells, labeling was concentrated to the PG septal division ring (Figure 1, Supplementary Fig 1). A small population of cells appears to be labeled beyond the septal division ring (Figure 1, top row white arrow). This preliminary observation suggests that the modification at the lactic acid is incorporated into lipid linked PG intermediates[62] and/or new PG but may fail to incorporate effectively into the growing PG polymer, suggesting that different transglycosylases have different substrate preferences (Fig 1 and Supplemental Fig 1). Further detailed mass spectrometry analysis is necessary to quantify the pools of PG that are modified. **14** and derivatives thereof will be extremely valuable in studying the PG of bacteria that are known to modify the 2-position, such as Mtb, which is known to *N*-glycolylate this position to evade an immune response[49, 63-66]. NAM derivatives containing modification at the 3-position could be used to probe this resistance mechanism.

This work expands and improves the methodology to label bacterial PG at the NAM residue. Previously, this methodology was limited to the genetic manipulation of organisms to include the recycling enzymes *amgK* and *murU* as well as lengthy synthesis of UDP peptide probes. In order to eliminate this genetic editing step and make the synthesis scalable and accessible, we took advantage of the chemoenzymatic production of the azide UDP NAM derivatives and developed the methodology to produce these analogues. PG co-labeling was only achieved in *L. acidophilus* as observed by superresolution microscopy (Figure 3). The result complements the previous work of Nishimura and coworkers where fluorescein was synthetically attached to the lysine of UDP-NAM pentapeptide to label the PG of *L. acidophilus*.[59] With the application of superresolution microscopy, new details about the localization of the NAM residues in *L. acidophilus* along the PG-division ring and sidewall have been observed *(Fig. 3)*.

It is worth noting, that the growth conditions of the bacterial cells resulted in different labeling efficiencies for *L. acidophilus* (Supplemental Figure 8A,B). *L. acidophilus* is a facultative anaerobe and is notoriously difficult to grow under laboratory conditions. We qualitatively note that when cells were actively growing, labeling efficiency of the PG appeared to be higher than when cells were dormant (Supporting Information, Supplementary Figure 8A,B), indicating that the cells may have been under different stresses, and those that were more actively growing and dividing are more likely to uptake the UDP NAM derivative. We note that these NAM probes may be used to explore bacterial nucleotide sugar transportation. To date, there are not any known UDP-NAM transporters that could allow for this event to occur. However, transporters for UDP galactose (UGT), UDP NAG, GDP fucose (GFT) and CMP sialic acid (CST) exist.[67] The identification of UDP NAM sugar transporters could be potential sources for selective antibiotic targets.

In conclusion, we have developed two critical synthetic methods: chemoenzymatic synthesis of UDP-NAM sugar derivatives and modification chemistry for the 3-position of the NAM lactic acid. These syntheses were used to explore the substrate specificity of the PG recycling and biosynthetic enzymes and revealed relaxed specificity at the 2 and 3 positions of the NAM. These probes allowed for new biological applications of the PG-glycan-labeling method to be developed: bioorthogonal tetrazine ligation and lactic acid modification. The robust chemoenzymatic synthesis of the 2-azido UDP NAM derivative allowed a Gram-positive commensal to be labeled on the sugar backbone at unprecedented resolution. The NAM-PG labeling method and the expanded substrate scope will be used in tandem with other complementary bioorthogonal labeling strategies[55-56, 59, 68-78] to illuminate fundamental aspects of PG biosynthesis and immune processing. These tools will allow the study of NAM production and breakdown of a variety of pathogen and commensal bacteria to reveal novel antibiotic targets and immune recognition elements.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Supplementary Information includes supplementary figures and tables, biochemical methods, synthetic procedures and compound characterization and is available as a single PDF file.

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The authors declare no competing financial interest.

AUTHOR CONTRIBUTION

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENT

We thank Professor Joseph Fox and the Fox Laboratory, specifically Yi Li, the Boyd Laboratory at the University of Delaware, specifically Nathan McDonald. We thank Professor Burnaby Munsen at the University of Delaware. We thank Dr. Papa Nii Asare-Okai, the director of the MS facility as well as Dr. Yang in the Chemistry and Biochemistry Department at the University of Delaware, with assistance in MS. We thank Dr. Jeffrey Caplan, the Director of Bioimaging and the Delaware Biotechnology Institute for assistance with superresolution microscopy. For financial support, this project was supported by the NIH U01 Common Fund program with grant number U01CA221230-01; Delaware COBRE program, with a grant from the National Institute of General Medical Sciences-NIGMS P20GM104316-01A1. C.L.G. is a Pew Biomedical Scholar and Cottrell Scholar, and thanks the Pew Foundation and the Research Corporation for Science Advancement. K.E.D. and M.R.J thank the NIH for support through a CBI training grant: 5T32GM008550. For instrumentation support, the Delaware COBRE and INBRE programs supported this project with a grant from the National Institute of General Medical Sciences–NIGMS (5 P30 GM110758-02, P20GM104316-01A1 and P20 GM103446) from the National Institutes of Health.

ABBREVIATIONS

AmgK, MurNAc/GlcNAc anomeric kinase; CMP, cyotidine monophosphate; CMP sialic acid transporter, CST; CuAAC, copper catalyzed azide alkyne cycloaddition; DNA, Deoxyribonucleic acid; GDP, guanosine diphosphate; GFT, GDP fucose transporter; HPLC, High-Performance Liquid Chromatography HRMS, High resolution mass spectrometry; IEDDA, Inverse electron demand diels al-der; Mtb, Mycobacterium tuberculosis; MurU, NAM α-1 phosphate uridylyl; NAG, N-acetylglucosamine; NAM, N-acetylmuramic acid; PG, Peptidoglycan; PMB, para-methoxybenzyl; RNA, Ribonucleic acid; SIM, Structured illumination microscopy; TCO, trans-cyclooctene; TCCA, trichloroisocyanuric acid; TLC, thin layer chromatography; UDP, Uridine diphosphate; UDP galactose transporter, UGT; WGA, Wheat Germ Agglutinin.

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