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Synthesis and Application of Methyl *N,O*-Hydroxylamine Muramyl Peptides

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Abstract

The innate immune system's interaction with bacterial cells plays a pivotal role in a variety of human diseases. Carbohydrate units derived from a component of bacterial cell wall, peptidoglycan (PG), are Known to stimulate an immune response. Nonetheless, access to modified late-stage peptidoglycan intermediates is limited due to their synthetic complexity. A method to rapidly functionalize PG fragments is needed to better understand the natural host–PG interactions. Here methyl *N,O*-hydroxylamine linkers are incorporated onto a synthetic PG derivative, muramyl dipeptide (MDP). The modification of MDP maintained the ability to stimulate a nuclear factor Kappa-light-chain-enhancer of activated B cells (NF-κB) immune response dependent on the expression of nucleotide-binding oligomerization domain-containing protein 2 (Nod2). Intrigued by this modification's maintenance of biological activity, several applications were explored. Methyl *N,O*-hydroxylamine MDP was amendable to *N*-hydroxylsuccinimide (NHS) chemistry for bioconjugation to fluorophores as well as a self-assembled monolayer for Nod2 surface plasmon resonance analysis. Finally, linker incorporation was applicable to larger PG fragments, both enzymatically generated from *Escherichia coli* or chemically synthesized. This methodology provides rapid access to PG probes in one step and allows for the installation of a variety of chemical handles to advance the molecular understanding of PG and the innate immune system.

Graphical Abstract

Make a change: A facile approach to specifically modify fragments of bacterial peptidoglycan (PG) and its derived immunostimulatory molecules is presented. *N,O*-hydroxylamine linkers are

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Conflict of Interest

The authors declare no conflict of interest.

Supporting information and the ORCID identification numbers for the authors of this article can be found under https://doi.org/10.1002/cbic.201800731: experimental procedures, spectral data, NF-κB assay conditions, and detailed E. coli experiment and mass spectrometry analysis.

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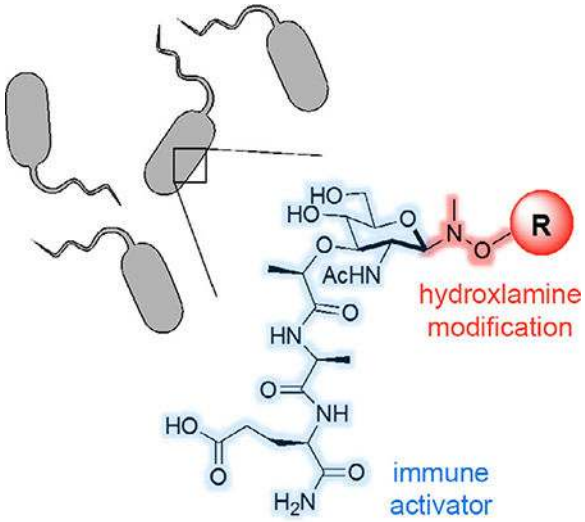
incorporated onto a synthetic PG derivative, muramyl dipeptide (MDP). This methodology provides rapid access to PG probes in one step.



Keywords

methyl *N,O*-hydroxylamine; muramyl dipeptide (MDP); NF-kappaB; Nod2

The innate immune system is the first line of defense against an array of pathogenic organisms, while simultaneously maintaining a symbiotic relationship with commensal organisms.[1] Through detection of pathogen associated molecular patterns (PAMPs) by pattern-recognition receptors (PRRs), the immune system is able to identify pathogens and initiate the proper immune response.[2] For bacteria, PAMPs can consist of pieces of flagellum or cell wall, which is includes peptidoglycan (PG). PG is comprised of alternating sugar units of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) with a cross-linked peptide chain (Scheme 1 A).[3] It is hypothesized that a combination of host and pathogen enzymes cleaves the PG to produce an assortment of fragments, some of which are immunostimulatory.[3a,4] Several PRRs, such as PG recognition proteins (PGRPs), cell surface toll-like receptors (TLRs), and cytosolic NOD-like receptors (NLRs), utilize these fragments to initiate a mechanism of host defense.[3a,5]

The human NLR, Nod2, has long been known to sense fragments of PG to produce an immune response through NF-κB and the p38 mitogen-activated protein kinase (MAPK) pathways.[3] In 2003, muramyl dipeptide (MDP), a synthetic component of the bacterial cell wall, originally proposed in 1974 by Lederer et al., was identified as the PG ligand to be sensed by Nod2 to produce an immune response (Scheme 1 A).[6,7] Moreover, mutations in Nod2 result in a loss of Nod2-dependent recognition of MDP and PG.[6,8] Consequently, both the misrecognition of cell wall fragments, as well as Nod2 mutations, are thought to play an important role in the development of Crohn's disease.[8] In order to determine whether MDP directly bound to Nod2, synthetic probes were needed to develop appropriate binding assays: biotinylated MDP[9] and 6-amino MDP[10] (Scheme 1 B). These probes were invaluable in demonstrating the binding specificities of Nod2; however, they come with

some limitations as they require lengthy chemical transformations (that is, 13+ chemical steps) and expert knowledge in muramic acid chemistry. Here, a simple and approachable method is developed that incorporates an amine linker onto unprotected muramyl peptides ranging from small monosaccharides to larger naturally occurring lysed saccharides which are otherwise synthetically challenging[4,11] (Scheme 1 C).

Previously, direct amine functionalization of unprotected PG carbohydrates at the C1-position (i.e., the anomeric carbon) relied heavily on reductive amination, resulting in the open ring conformer.[12] We were inspired by elegant chemistry developed by Peri and co-workers that introduced methyl *N,O*-hydroxylamine linkers as alternatives for incorporation of amine functionality onto unprotected glycans.[13] Unlike reductive amination, hydroxylamine incorporation is able to maintain the biologically relevant closed ring structure, while yielding high anomeric β selectivity.[12] Several groups have successfully used the N-alkylhydroxylamines to conjugate glycans onto biomacromolecules, microarrays and potential drug candidates;[14] this chemistry is sometimes referred to as neoglycosylation.[14h] To date, this chemistry has not been used for the functionalization of PG-based fragments and given the biological importance of these compounds we saw a tremendous utility in this methodology. In this study, methyl *N,O*-hydroxylamine linkers are incorporated onto MDP and other PG fragments for development of accessible probes for anyone interested in studying host-PG interactions.

In designing the appropriate linkers, a short hydrophobic chain with at least three carbons was incorporated to decrease false negatives,[15] as well as a long hydrophilic linker to decrease hydrophobicity, in protein binding assays (Scheme 1 C). Using a modified synthesis of a two-carbon bifunctional linker originally developed by Blixt et al., syntheses of both linkers were designed (Scheme 2).[12, 14a] For the shorter linker **10** (Scheme 2 A), **8** was reacted with *N*-Boc-*N*-methylhydroxylamine using sodium hydride to yield protected **9**, which was subsequently deprotected using trifluoracetic acid to yield **10**. In a similar manner, starting from commercially available Bromo-PEG_3-BocAmine, the PEG_3 linker **12** was synthesized and isolated (Scheme 2B). We note occasional decomposition was observed during deprotection of **12** and the conversion of fresh Bromo-PEG_3-BocAmine directly to product is recommended.

With both linkers in hand, attachment to synthesized MDP was investigated. Such incorporation was also envisioned on commercially available MDP, thereby greatly reducing the number of steps to obtain amine functionalized MDP for studies with Nod2, or other innate immune receptors. MDP was dissolved in sodium acetate buffer (pH 4.5) followed by addition of the linker (**10** or **12**; Scheme 3). Coupled compounds were purified by reversed-phase chromatography to obtain the final compounds in moderate yield and only one chemical step. Validation of attachment to the anomeric position was confirmed by HMBC between the NCH_3 of the linker and the C-1 of MDP (see the Supporting Information, Section XI). Moreover, stereoselectivity for the one anomer was observed and confirmed by NMR analysis. With a HMBC coupling between H-2 and C-1 of MDP (Supporting Information, Section XI) and observation of coupling between H-1 and C-5,[14a] thus suggesting β-anomer selectivity. This simple synthesis provides the non-specialist access to well-defined, modifiable PG fragments.

To demonstrate that the modification at the C1-position of MDP did not affect Nod2 dependent NF-κB activation, an established NF-κB luciferase reporter assay was utilized.[6, 9b, 16] Briefly, if Nod2 senses the modified ligands **1** or **2**, NF-κB will be activated and stimulate the expression of luciferase. Cells were treated with 20 μM of ligand for 5 h. **1** and **2** were able to stimulate a Nod2 dependent immune response as seen with the positive control, MDP (20 μM; Figure 1). As the linker length increased, a decrease in activity was observed. The longer PEG linker may cause the ligand to aggregate or fold, ultimately affecting MDP's ability to properly bind to its cellular receptor. Additionally, the long linker could affect cellular transport into the cell. The data suggest that the receptor does accommodate modifications at the C1 position, with preference for shorter alkyl linkers, in agreement with previous reports.[17] With confirmation that incorporation of an amine linker onto MDP maintains biological activity, we sought to explore applications for this amine modification of MDP.

To begin, the methyl *N,O*-hydroxylamine MDP (**1**) was subjected to a surface plasmon resonance (SPR) binding assay with the innate immune receptor, Nod2 previously established by our laboratory.[10] The amine functionalized PG derivatives were appended to a self-assembled monolayer which allows the binding affinity for Nod2 to be assessed. MDP is linked to the surface via the C-1 and the previously established C-6 position (Figure 2A), allowing us to directly compare binding affinities for the site of modification. A concentration gradient of Nod2 LRR[10b] was applied to the surface and the binding affinities for both compounds were determined. Nod2 LRR binds to 6-amino MDP with low nanomolar binding affinity (95(±9) nM) in agreement with the literature (Figure 2 B).[10b, 18a] When (**1**) was attached to the surface via the C-1 position, binding decreased to high nanomolar affinity (700(±100) nM; Figure 2 C). These data suggest that the orientation of the carbohydrate is important for maximal binding to be achieved. In addition, these data support the cellular activation studies (Figure 1) in which longer linker lengths diminished NF-κB activation, suggesting that additional steric bulk decreases binding affinity. Thus, the modification of the C-1 position has allowed for a survey of the binding pocket of the LRR and its PG ligand. These binding studies are essential to understanding the carbohydrate orientation within the Nod2 binding pocket[10b] in the absence of a crystal structure of the human LRR and its ligand.

The *N,O*-hydroxylamine MDP serves a useful handle to expand the chemical biology toolbox for other biological assays in addition to SPR. For example, rapid bioconjugation[19] to fluorophores, affinity tags, or cross-linking moieties can be subsequently attached via the amine of the *N,O*-hydroxylamine MDP. To explore this application, a near-infrared wavelength range Cy5 dye was coupled to **1** and successfully installed by using *N*-hydroxylsuccinimide (NHS) chemistry (Scheme 4). This Cy5 MDP, **1A**, conjugate will be an interesting probe for several biological investigations such as fluorescent microscopy[6, 20] fluorescent polarization (FP)[18] and/or incubation with microarrays.

While the *N,O*-hydroxylamine linker demonstrates utility for the development of biological applications for MDP like fragments, we also wanted to assure that the chemistry was amendable to larger PG compounds as access to functionalized derivatives is extremely challenging[4] Therefore, a method to functionalize naturally derived fragments would

greatly expand the ability to study the interactions of PG in the microbiome field. Before testing the methyl *N,O*-hydroxylamine linker on isolated PG, incorporation was tested on a disaccharide fragment **21** (Scheme 5 A). Utilizing a reported procedure[21] starting material **21** was prepared with minor deviations. With this intermediate, the acetyl and 2-(trimethylsilyl)-ethanol (TMSE) protecting groups were simultaneously removed and the intermediate was subsequently subjected to hydrogenation. The fully deprotected disaccharide was then coupled directly to the methyl *N,O*-hydroxylamine linker in good yield. These data demonstrate that the methodology is amenable to larger, unprotected PG fragments. Compound **3** was tested for the ability to activate a Nod2 dependent NF-κB response; this compound did not activate NF-κB signaling as it is lacking the peptide (Figure S2). Encouraged by the incorporation on **3**, labeling larger, enzymatically derived PG fragments was investigated.

Bacterial cells are naturally subjected to lytic enzymes derived from their own biology and human hosts that serve to break down PG.[2, 3, 22] One such human protein is lysozyme, an enzyme commonly found in saliva and tears[23] This enzyme naturally cleaves PG to form a disaccharide unit (Scheme 5 B).[2, 3] We and others desired a method to chemically modify this carbohydrate unit for fluorescent visualization, binding assays or affinity purification[24] This work utilizes the methyl *N,O*-hydroxylamine linker chemistry to modify pools of PG fragments generated from lysozyme cleavage. To achieve this, isolated lysed PG from *Escherichia coli*,[24a, 25] was dissolved in a solution of linker **10** and sodium acetate buffer (pH 4.5; Scheme 5 B). The reaction mixture was subjected to high-resolution mass spectrometry-liquid chromatography (HRMS-LC).[24a, 25, 26] Two experimental controls were performed: 1) PG in buffer without linker, and 2) linker in buffer without PG. In the experimental treatment, multiple disaccharide PG fragments (**A1**, **B1**, **C1**) including the expected lysozyme product (**B1**)[24a, 25] functionalized with the methyl *N,O*-hydroxylamine linkers were observed (Table S1 in the Supporting Information, entries 1–3). The PG fragments that were not subjected to linker attachment conditions revealed the corresponding non-linker parents without the methyl *N,O*-hydroxylamine linker moiety, suggesting specific linker attachment to the PG (Table S2, entries 1–6). We note the addition of the linker, which contains a primary amine, appears to increase the ionization efficiency of select carbohydrate fragments when electrospray ionization (ESI) is used. For example, pentapeptide disaccharide (**C2**), was not observed without linker addition (Table S2, entry 6) and upon linker addition, product was more readily observed (Table S1, entry 6), suggesting this method will be useful in identifying lower abundant fragments isolated from biological samples and/or confirming the presence of a PG fragment. The former could also include isotopes to allow for the development of an "isotag".[27] A noteworthy fragment was identified as GlcNAc-MurNac-tripeptide(iE-DAP) **A1** as a lysozyme product, with a mass of 955.45580 (9.61331 ppm) with linker, as well as it is (M/2)+H of 478.23175. Interestingly, this fragment was also identified by Philpott and co-workers as a major product released upon muramidase treatment of isolated peptidoglycan.[28]

Innate immune receptors such as Nod2 are vital in detecting pathogenic bacteria, and misrecognition of these organisms is thought to lead to a variety of illnesses, such as Crohn's disease.[8] Therefore, methyl *N,O*-hydroxylamine linker incorporation onto to PG

fragments will allow for the advancement of synthetic and naturally derived PG chemical probes to better understand the extent of host-PG interactions through immobilization of ligands to a variety of surfaces and bioconjugation of PG fragments. Finally, this methodology provides access to a PG framework for the development of immunomodulators and adjuvants.

Experimental Section

Synthesis of C1-linker peptidoglycan derivatives

This is a general synthesis (for the detailed synthesis of **1**, **2**, **3** see the Supporting Information). Muramyl peptide (1 equiv) and methyl *N,O*-hydroxylamine (6–10 equiv) were dissolved in 1M sodium acetate buffer pH 4.5 and mixed for 48 h. The reaction was concentrated, purified by using HPLC (Auto Purification System 2767 Sample Manager with HPLC and SQD2 MS), and characterized with NMR spectroscopy and HRMS.

Synthesis of Cy5 MDP

See the Supporting Information for experimental details, NMR characterization and HRMS.

NF-κB luciferase assay

An established assay was used.[9b, 10a, c] See the Supporting Information for additional details/statistical analysis.

Surface plasmon resonance (SPR) assay

An assay previously developed and used in our lab was utilized.[10] See the Supporting Information for additional details/analysis of data.

C1-linker and isolated *E. coli* peptidoglycan studies

See the Supporting Information for specific protocols.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Biography

Catherine Leimkuhler Grimes received her PhD in Chemistry from Harvard University in 2006 working with Prof. Daniel Kahne and completed a Cancer Research Institute funded postdoctoral fellowship working with Prof. Erin O'Shea (Harvard) and Dr. Dan Podolsky (MGH). She began her independent career in 2011 in the Department of Chemistry and Biochemistry at the University of Delaware. Her laboratory uses carbohydrate chemistry, innate immunology, biochemistry and molecular biology to investigate the molecular mechanisms the body uses to sense and respond the presence of bacterial peptidoglycans.

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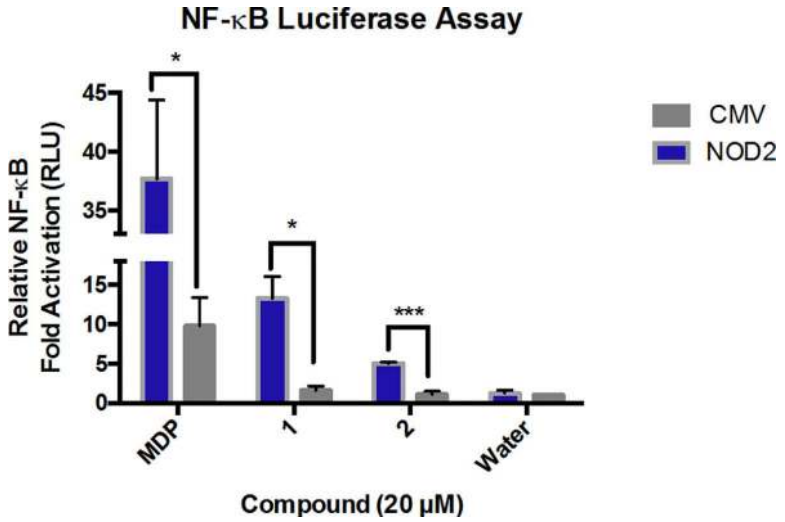


Figure 1.

NF-κB activation for PG derivatives: HEK293T cells were transfected using Lipofectamine with (±) Nod2 plasmid, NF-κB reporter, and a renilla control for 16 h. The cells were treated with stimuli for 5 h, harvested, and tested for luciferase. All compounds were tested at 20 μM. The data represent three independent experiments (n=3) on separate occasions with their three biological replicates averaged. Results are depicted as the mean±SEM. An unpaired two-tailed student t-test was performed to determine significance between the two groups (Nod2 and CMV) using GraphPad Prism 6. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ with a confidence level of 95%. Procedures and individual replicates can be found in the Supporting Information.

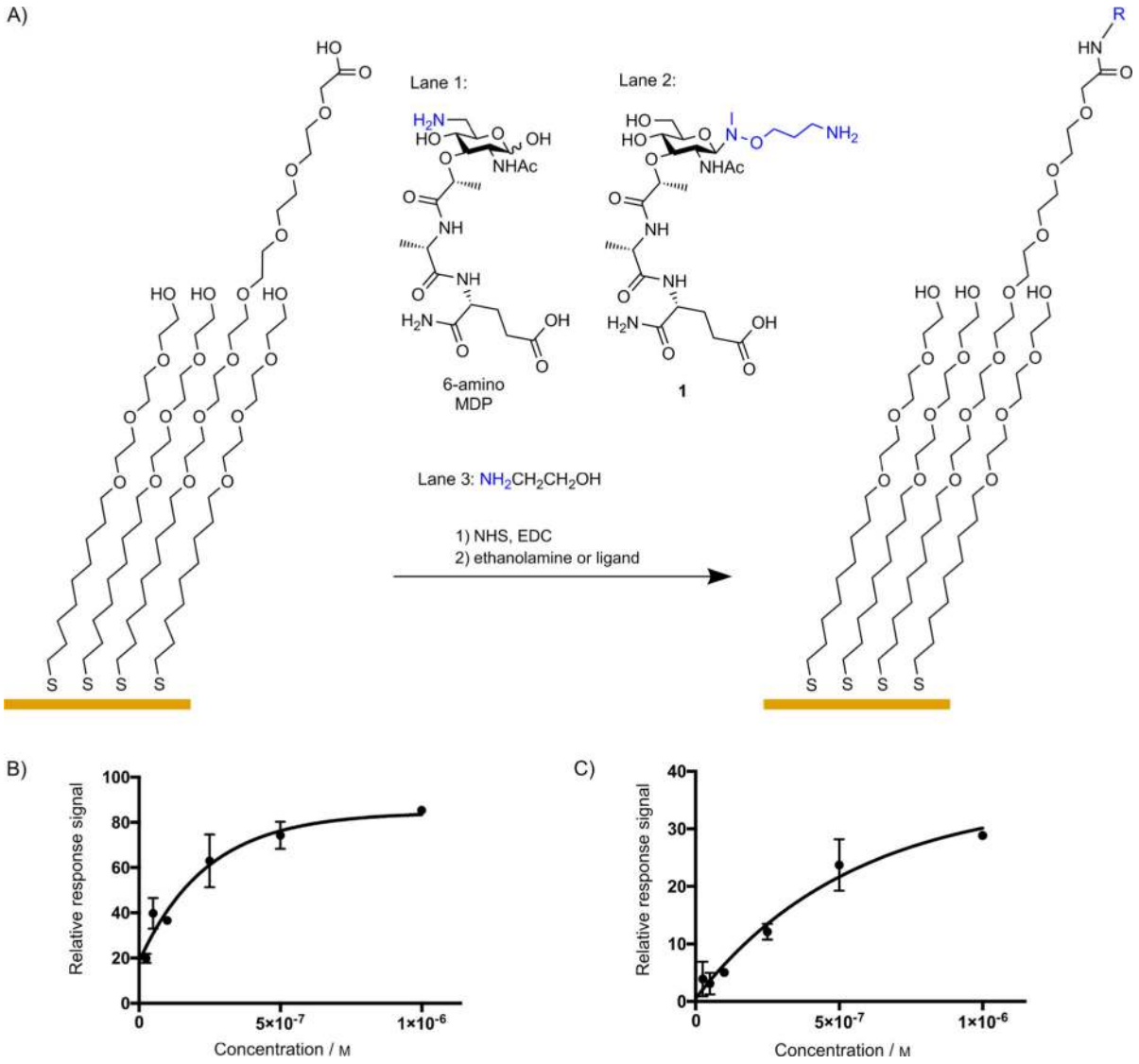
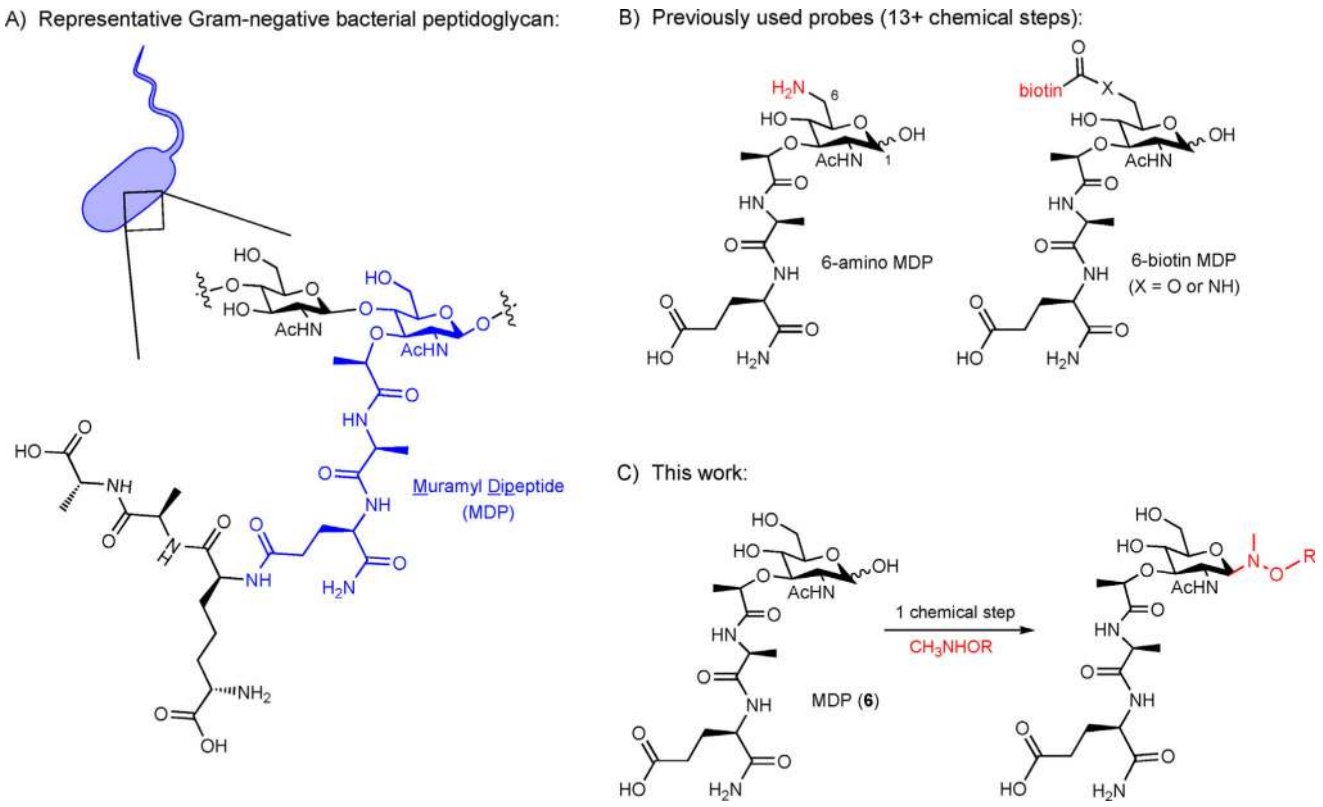


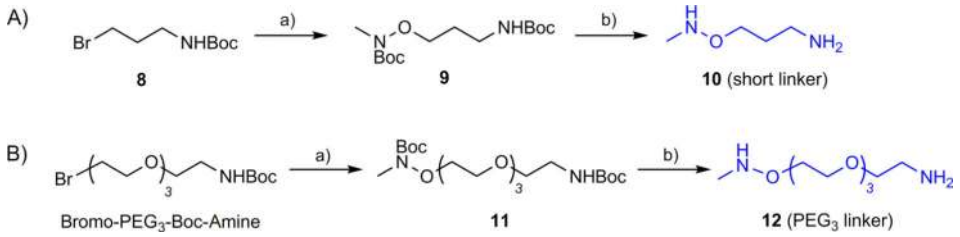
Figure 2.

SPR analysis of C-1 and C-6 functionalized MDP derivatives. A) Surface functionalization occurs through EDC, NHS coupling resulting in the formation of an amide bond at the C-6 or C-1 of the carbohydrate. Binding curve of Nod2 LRR to B) 6-amino MDP and C) compound **1**. Nod2 LRR at pH 6.5 was applied to the chip at a flow rate of 3 μl min^{-1}, and the resonance signal was recorded after 20 min. Nod2 bound both ligands with nanomolar affinity after subtraction from the ethanolamine control (lane 3). Using Prism 6 (GraphPad) the data were fitted to a standard one-site binding model by nonlinear regression analysis; error bars represent the standard deviation of each data point.



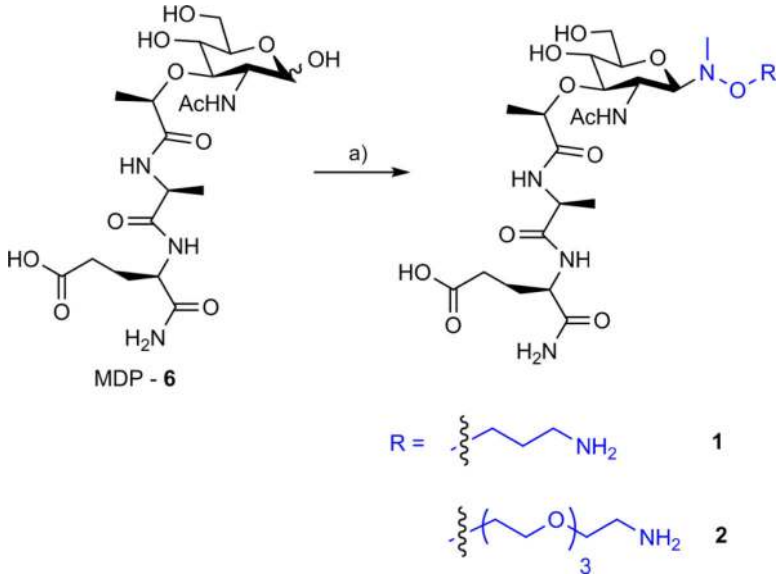
Scheme 1.

Bacterial peptidoglycan fragment modifications: A) Bacterial cell wall PG composition with MDP, an immunostimulatory fragment (blue). B) Previously synthesized probes for understanding the MDP–Nod2 interaction with modifications at the C6 position of MDP. C) Methyl *N,O*-hydroxylamine linker (red) modified unprotected MDP selectively at the C1 (anomeric) position in one chemical transformation.



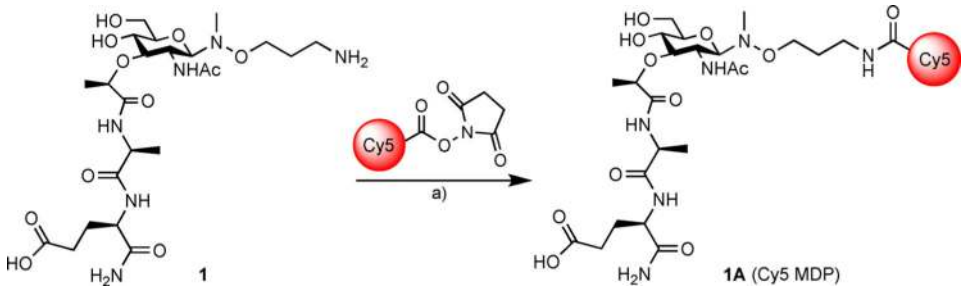
Scheme 2.

Methyl *N,O*-hydroxylamine linker synthesis: A) Short linker **10**: a) *N*-Boc-*N*-methylhydroxylamine, NaH, DMF (78%); b) TFA, CH_2Cl_2 anhydrous, quantitative. B) PEG_3 linker **12**; reagents and conditions as in (A); yields: a) 98%, b) quantitative.



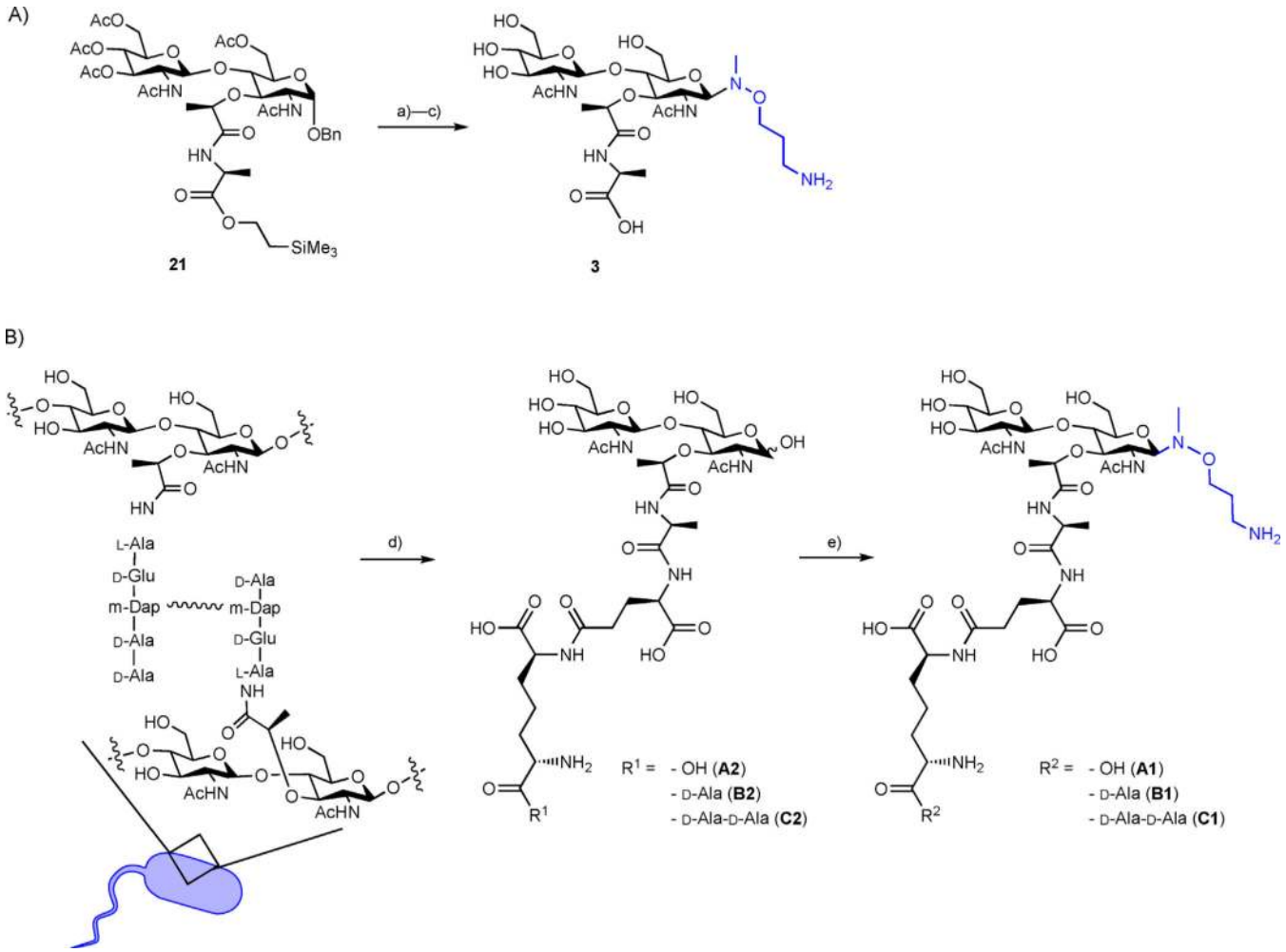
Scheme 3.

Synthesis of MDP methyl *N,O*-hydroxylamine coupled derivatives **1** and **2**. a) Sodium acetate buffer (1M, pH 4.5), linker **10** or **12**, 13–27%.



Scheme 4.

Synthesis of Cy5-labeled MDP. a) Na_2CO_3, MeOH, 53%.



Scheme 5.

Preparation of large methyl *N,O,* -hydroxylamine PG Fragments: A) Synthesis of disaccharide methyl *N,O*-hydroxylamine derivative **3**. a) LiOH, ACN/H_2O; b) $Pd(OH)_2$, H_2, THF/H_2O/acetic acid; c) linker **10**, sodium acetate buffer (1M, pH 4.5), 46% over 3 steps. B) Modification of isolated PG. d) Lysozyme; e) linker **10**, sodium acetate buffer (1M, pH 4.5).