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Synthesis of biologically active biotinylated muramyl dipeptides

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ABSTRACT

Muramyl dipeptide (MDP) is believed to interact with an innate immune receptor, Nod2. To identify the cellular receptor for MDP, we have synthesized biotinylated MDP isomers and tested the ability of these compounds to activate Nod2 in a cell-based assay. We found that the modification of MDP does not perturb its ability to activate Nod2. These tagged versions of MDP will be useful to identify the cellular receptor of the immunostimulatory molecules.

The innate immune system is important for detecting and responding to microbial invasion.[1–3] The primary innate immune sensors, pattern recognition receptors (PRRs), are activated by pathogen associated molecular patterns (PAMPs), triggering a complex cascade of events known as the inflammatory response.[4] The Nod-like receptors (NLR) are a family of PRRs that reside in the cytosol[5] and are proposed to use their leucine rich region (LRR) to sense the presence of a pathogen.[6] However, no evidence for a direct interaction between NLRs and PAMPs has been reported.[7]

Nod2 is a member of the NLR family that is expressed in monocytes, granulocytes, dendritic, and intestinal epithelial cells.[8,9] Nod2 activation by PAMPs results in the production of immunostimulatory molecules and anti-microbial peptides through activation of the NF-κB and the mitogen activated (MAP)-kinase (p38) pathways.[10,11] The proposed ligand (or PAMP) for Nod2 is a small piece of bacterial cell wall that has long been known to have immunostimulatory properties, muramyl dipeptide (MDP) (**1**, Fig. 1).[12–14] A direct interaction between MDP and Nod2 has not been shown, so it remains unclear if MDP is sensed directly by Nod2 or by a different protein.

Sansonetti and co-workers have shown that MDP activates the Nod2 pathway in a stereo-specific manner.[15] Replacement of L-isoglutamate for D-isoglutamate, the natural stereoisomer found in MDP, eliminated the ability of MDP to stimulate Nod2 (**2**, Fig. 1). This finding suggests that the peptide portion of MDP is important for recognition. To keep the recognition region of the molecule available for interaction, we reasoned that a modification would best be made at the six-position of the carbohydrate, distant from the potential binding site (**12**, Scheme 1). In addition, we desired a version of MDP that could be easily modified with crosslinkers, biotin tags and/or fluorophores. We therefore reasoned that an amino group on the six-position of MDP would be a good chemical handle for our purposes.

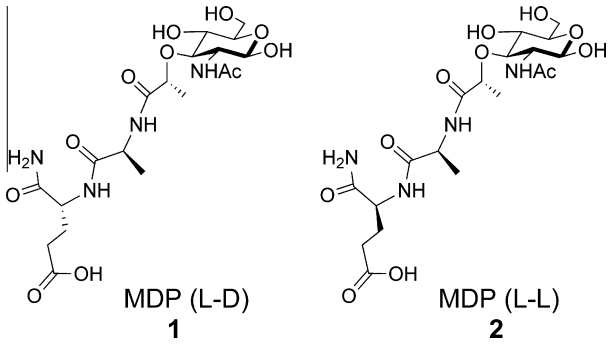

Figure 1. Muramyl dipeptide isomers: **1** is the proposed ligand for Nod2. The isomer, **2**, does not activate Nod2.

C6-Amino versions of both MDP-L-D (**12**, Scheme 1) and MDP-L-L (**13**, Scheme 1) were synthesized from protected *N*-acetyl-muramic acid (**3**, Scheme 1) in six steps, using slight modifications from a previously reported synthesis.[16,17] Our synthesis differed from the previously published protocol in two ways: (1) the peptide coupling was accomplished using the mixed anhydride method;

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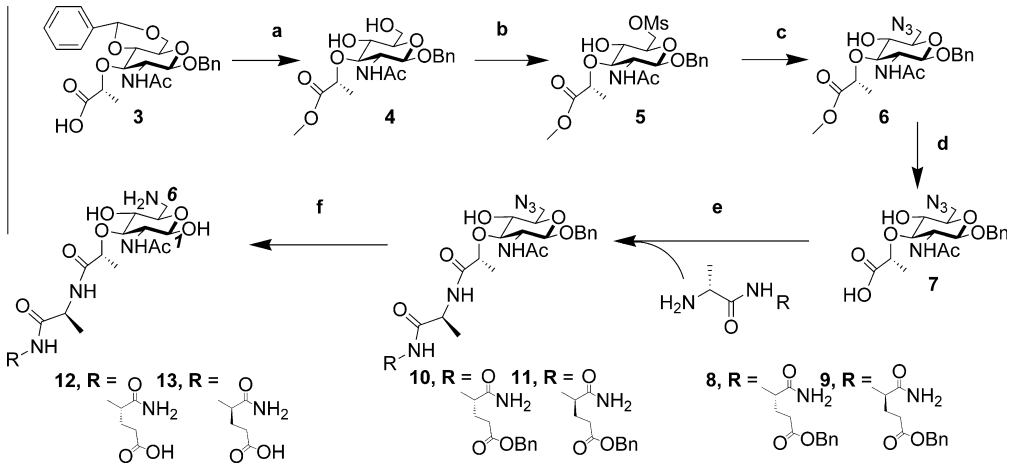


Scheme 1. Synthetic route of 6-amino MDP isomers: 12 and 13. Reagents and conditions: (a) IRA H^+ Resin, MeOH, quantitative; (b) *p*-toluene sulfonic acid, methyl sulfonyl chloride, pyridine, 92%; (c) sodium azide, DMF, 87%; (d) potassium hydroxide; (e) (i) *N*-methylmorpholine, isobutyl chloroformate, DMF; (ii) **8** or **9**, 63%; (f) H_2, Pd–C, MeOH/AcOH 82%.

and (2) the azido coupling was accomplished without prior protection of the 4-hydroxyl group. We found the protecting group on the 4-OH difficult to remove and the peptide coupling was achieved with minimal internal ester formation. Briefly, treatment with IRA H^+ resin in methanol afforded **4** in which the benzylidene group was deprotected and the methyl protecting group installed. The azido group was then installed via mesylation on the six-position of the carbohydrate in 90% yield over two steps. Following deprotection of the carboxylate, the appropriately protected dipeptide (**8** or **9**) was coupled using standard peptide coupling conditions[18] to yield the protected 6-amino muramyl dipeptide, **10** or **11** in 60% yield. Global deprotection using H_2, Pd–C yielded **12** or **13** in 41% overall yield.

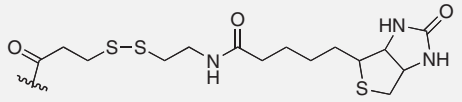
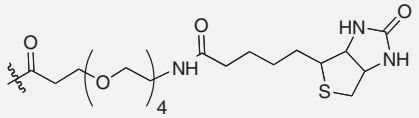
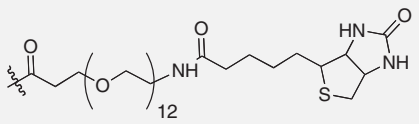
Compound **12** was reacted with a variety of succinimide-modified biotin groups to yield the biotin-6-amino-MDP-L-D derivatives (**14**, **16**, and **18**, Table 1). In addition, the biotin-6-amino-MDP-L-L series (**15**, **17**, and **19**, Table 1) was synthesized. 6-Amino muramyl dipeptide derivatives containing biotin linkers of various lengths (20, 32, and 56 Å) and one cleavable linker (**14** and **15**, Table 1) were synthesized. Table 1 represents a set of biotinylated MDP compounds to test the activation of Nod2. The L-L derivatives are an important control, as they should not activate the Nod2 pathway.

To determine if the modification at the six-position of MDP would alter Nod2 dependent NF-κB activation, the biotin MDP derivatives and the 6-amino derivatives were tested for their ability to activate Nod2 in an established NF-κB luciferase assay[9,13] (Fig. 2). Briefly, HEK-293 cells were co-transfected with Nod2 and an NF-κB driven firefly luciferase reporter. In the presence of its ligand, Nod2, will activate NF-κB which in turn stimulates expression of the luciferase reporter. As an internal control for transfection efficiency, all cells were transfected with *Renilla* luciferase.

Figure 2a indicates that all L-D isomers were able to activate Nod2, suggesting that the modification at the six-position did not interfere with the activity of MDP. In addition, none of the L-L isomers were active (Fig. 2a), indicating that the biotinylation of muramyl dipeptide does not afford any additional, non-specific biological activity. Furthermore, the ability of the modified MDPs to trigger NF-κB in a robust manner was dependent on Nod2—when Nod2 was not transfected into HEK-293 cells, only modest activity was observed (Fig. 2b). However, it should be noted that the L-D isomers also effect low level activation of NF-κB in a Nod 2 independent fashion, given the activation in HEK-293 control cells which lack Nod2.[9] The biotinylated MDPs could be useful in identifying such off target proteins.

Of all the linkers tested, compounds **14** and **16** remained the most active (Fig. 2a). The long 56 Å linker, **18**, only had minimal activity (Fig. 2a). We reasoned that the addition of the longer linker may cause the molecule to aggregate in cell culture medium or that the linker may fold in a conformation that prevents the MDP from binding to its receptor. Thus, **14** and **16** represent the MDP

Table 1
MDP probes

Compound	Stereochemistry	Biotin linker
14	L-D	
15	L-L	
16	L-D	
17	L-L	
18	L-D	
19	L-L	

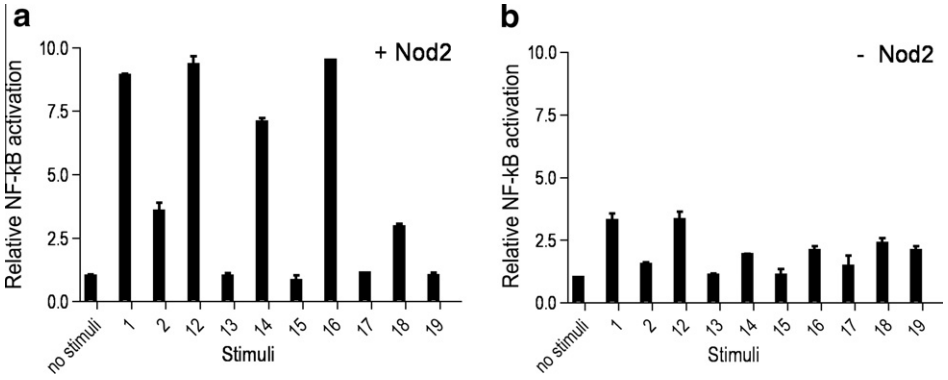


Figure 2. Nod2 dependent response to MDP: (a) Nod2 DNA was added to the transfection mixture; (b) empty vector in place of Nod2 DNA was added to the transfection mixture.

derivatives that retained the most biological activity; their respective isomers, **15** and **17**, were not active. We anticipate that these reagents will be useful tools in studies to identify the receptor for MDP, be it Nod2 or another protein.

Nod2 plays an important role in detecting the presence of bacteria. Additionally, mutations in Nod2 have been associated with two chronic inflammatory disorders: Blau syndrome and Crohn's disease.[19–22] Our synthesis of a modifiable MDP provides a tool for use in studies to better understand the molecular mechanisms by which bacteria cause inflammation. We are currently using **14**–**17** to determine if MDP interacts directly with Nod2 or other proteins and to gain knowledge at the molecular level of the initial signaling events involved in Nod2 activation.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.08.056.

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