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Author(s)	Wang, Qianqian
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Synthesis and Function of *Mycobacterium* Cell Wall Peptidoglycan Fragments

（結核菌ペプチドグリカンフラグメントの合成と機能）

Qianqian Wang (倩倩 王)

Graduate School of Science
Osaka University

Index

List of Abbreviations

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Ac	Acetyl
Ac_2O	Acetyl acetate
AcOH	Acetic acid
Ala	Alanine
Alloc	Allyloxycarbonyl
Ar	Aryl
BLI	Bio-layer Interferometry
Bn	Benzyl
Boc	*t*-Butyloxycarbonyl
t-Bu	*t*-Butyl
CARD	Caspase recruitment domain
CLRs	C-type lectin receptors
CRs	Complement receptors
DAMPs	Damage-associated molecular patterns
DAP	Diaminopimelic acid
DMEM	Dulbecco's Modified Eagle's Medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DMT-MM	4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium Chloride
EEA1	Early endosome antigen-1
FBS	Fetal bovine serum
Fmoc	9-Fluorenylmethyloxycarbonyl
GlcNAc	*N*-acetyl glucosamine
Gln (Q)	Glutamine
Glu (E)	Glutamic acid
Glyc	Glycolyl
GST	Glutathione S-transferase
HEK	Human embryonic kidney
HOBt	1-Hydroxybenzotriazole
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
iE-DAP	γ-D-glutamyl-*meso*-DAP
IFN	Interferon
IKK	Inhibitor of NF-κB (IκB)-kinase

LIT	Linear ion trap
LM	Lipomannan
LP	Lipoprotein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTA	Lipoteichoic acid
Lys	Lysine
mAGP	Mycolic acid-Arabinogalactan-Peptidoglycan
Man-LAM	Mannose-capped lipoarabinomannan
MAPK	Mitogen-activated protein kinase
MDP	Muramyl dipeptide
MRs	Mannose receptors
MS4Å	Molecular sieves 4Å
MurNAc	*N*-acetylmuramic acid
MurNGlyc	*N*-glycolylmuramic acid
MyD88	Myeloid differentiation factor 88
NCA	*N*-carboxyanhydride
NF-κB	Nuclear factor-κB
NHS	*N*-hydroxysuccinimide
NLRs	NOD-like receptors
NMR	Nuclear magnetic resonance
NOD	Nucleotide-binding oligomerization domain
PAMPs	Pathogen-associated molecular patterns
PASTA domain	Penicillin-binding protein and serine/threonine kinase associated domain
PBS	Phosphate buffered saline
$Pd(PPh_3)_4$	Tetrakis(triphenylphosphine)palladium(0)
$Pd(OH)_2$	Palladium hydroxide
PEG	Polyethylene glycol
PEPT1	Oligopeptide transporter 1
PFT	Pore forming toxin
PGN	Peptidoglycan
Ph	Phenyl
PIM	Mannosyl-phosphatidyl-myo-inositol-based glycolipid
PRRs	Pattern-recognition receptors
quant.	Quantitative
RICK	Receptor-interacting serine/threonine kinase

RIG-I	Retinoic acid-inducible gene I
RLRs	Retinoic acid-inducible gene (RIG)-I-like receptors
RNIs	Reactive nitrogen intermediates
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS Polyacrylamide gel electrophoresis
SPR	Surface plasmon resonance
SRs	Scavenger receptors
STPKs	Ser/Thr protein kinases
TB	Tuberculosis
TEA	Triethylamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLRs	Toll-like receptors
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
Troc	2,2,2-Trichloroethoxycarbonyl
Tween-20	Polyoxyethylene (20) sorbitan monolaurate
WSCD•HCl	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide monohydrochloride
Z	Carboxylbenzyl
ZOSu	*N*-(Benzyloxycarbonyloxy)succinimide

Chapter I Introduction

Mycobacterium tuberculosis (*M. tuberculosis*) is the pathogen for Tuberculosis (TB). TB is a major global health problem, which ranks as the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV).[1] There is a clear and urgent need for a deeper understanding of the pathogenesis and physiology of the causative organism, *M. tuberculosis,* in order to look for more effective vaccines and antibiotics.

The cell wall of *M. tuberculosis* possesses unique compositions. Mycolic acid, arabinogalactan, and peptidoglycan, called mAGP complex, are the main components, which play important roles in the bacterial host survival and subsequent pathogenesis. The bacterial cell wall is also one of the main targets of current TB drugs, for example Isoniazid[2] and Ethionamide[3] block the synthesis of mycolic acid and Ethambutol[4] disrupts arabinogalactan synthesis by inhibiting the enzyme arabinosyl transferase. However, the function of *Mycobacterium* peptidoglycan has not been well elucidated. Therefore, a series of *Mycobacterium* peptidoglycan fragments were prepared by organic synthesis by the author and their recognition by the host innate immune system and their contribution to the growth of the bacterium itself were studies.

Innate immune system

Humans are living in an environment that is full of various microorganisms, but usually prevent infection with the highly effective defense system. The innate immune system serves as the first line of defense against the invading microbes. This host-defense system is evolutionarily ancient and found in all multicelluar organisms, such as vertebrates, insects[5] and plants.[6] The innate immunity is primarily mediated by phagocytes (or antigen presenting cells) including macrophages and dendritic cells and is important for the activation of adaptive immunity.[7]

The innate immune system recognizes conserved microbial structures, pathogen-associated molecular patterns (PAMPs) or microbial-associated molecular patterns (MAMPs), via a limited number of germline-encoded pattern-recognition receptors (PRRs). Two main classes of PRRs have been identified (Fig. I-1): one is the transmembrane proteins such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs); and the other is the cytoplasmic proteins such as NOD-like receptors (NLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs). Classical PAMPs/MAMPs include lipopolysaccharides (LPS), peptidoglycan (PGN), lipoproteins (LP), acids (LTA), and microbial RNA and DNA.[8] Recent evidence manifested that PRRs also recognize host-derived danger signals released from damaged cells, termed damage-associated molecular patterns (DAMPs).[9] Upon ligand recognition, PRRs trigger a multitude of intracellular signaling cascades that rapidly induce the expression of a variety of overlapping and unique genes involved in the inflammatory and immune responses.[10]

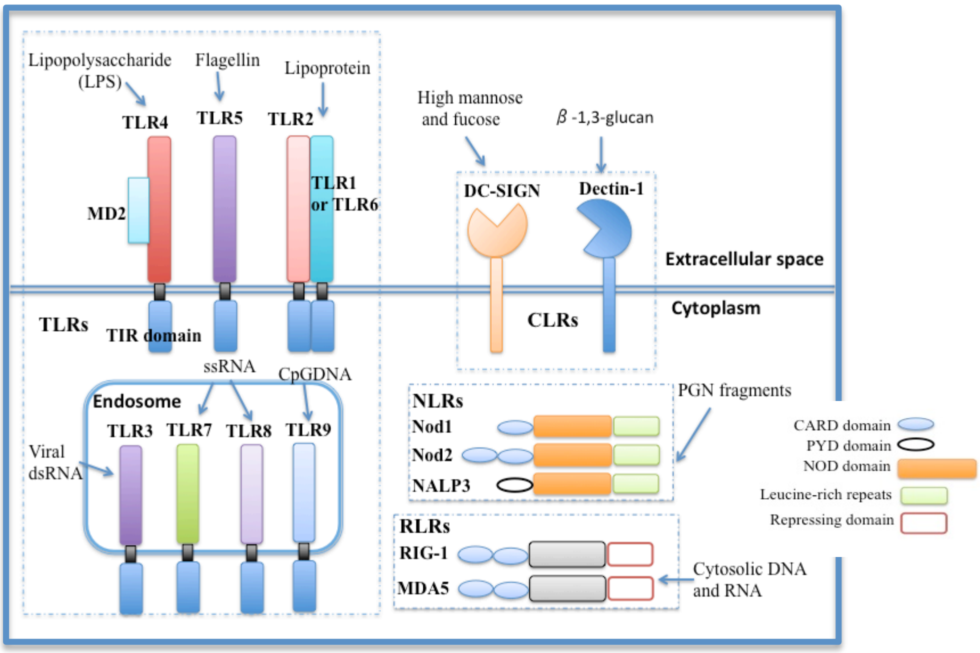


Figure I-1. Pattern Recognition Receptors and their ligands.

TLRs are the first discovered group of PRRs and have been extensively studied.[11] They sense the invaders on the cell surface and in the intracellular endosomes and lysosomes. TLRs are type I transmembrane proteins with extracellular ligand binding leucine-rich repeats (LRRs); transmembrane domains; and cytoplasmic signaling Toll–interleukin 1 (IL-1) receptor (TIR) domains.[12] To date, 10 TLRs have been identified in human and 12 in mice, they recognize components from a wide range of microbes: bacteria, viruses, parasites, and fungi. Basically, TLR2 (with TLR1 or TLR6), TLR4, and TLR5 localize on the plasma membrane and recognize lipoproteins, LPS and flagellin, whereas TLR3, TLR7, TLR8, and TLR9 recognize dsRNA, ssRNA, and CpG DNA on the intracellular vesicular compartments, respectively.[13]

CLRs comprise another transmembrane receptor family, possessing at least one carbohydrate recognition domain, which recognize mannose, fucose and glucan carbohydrate structures on viruses, bacteria or fungi. Recognition by CLRs leads to the internalization of the pathogen, its degradation and subsequent antigen presentation.[14]

The types and functions of CLRs are described in detail by Geijtenbeek and Gringhuis.[15]

NLRs are the largest group of cytoplasmic receptors and have been considered as the key sensors of intracellular microbes and other dangerous signals. The NLR family is composed of 22 proteins in human. They share similar structures: a variable *N*-terminal protein-protein interaction domain, called caspase recruitment domain (CARD), pyrin domain (PYD) or baculovirus inhibitor repeat (BIR); a central nucleotide-binding oligomerization domain (NOD), which regulates self-oligomerization when being activated; a C-terminal leucine-rich repeat (LRR) that recognizes PAMPs.[16] Among them, Nod1 and Nod2 have been widely studied, they recognize bacterial molecules derived from the hydrolysis and synthesis of peptidoglycan,[17] which is an important component in the bacterial cell wall. The details of Nod1 and Nod2 are discussed later.

As another family of cytoplasmic PRRs, RLRs, including retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2), recognize various types of RNA and DNA viruses and induce type I IFN production through mitochondrial antiviral signaling protein (MAVS) and IRF3.[18] They are characterized by a central helicase domain with ATPase catalytic activity, which is responsible for binding of viral dsRNA; and C-terminal repressing domain (RD) (CTD), that is crucial for autoregulation and binding with the 5’-triphosphate end of the viral genome.[19]

***Mycobacterium tuberculosis* and PRRs**

Tuberculosis (TB) infection begins with the inhalation of the tubercle bacilli to the lung of the host. Here, the bacteria interact with many immune cells, but their favored one is alveolar macrophages. Macrophages contain two kinds of PRRs: one is mainly involved in bacterial phagocytosis, such as complement receptors (CRs), scavenger receptors (SRs) and mannose receptors (MRs), which facilitate their entry to the phagocytic cells; the other one is responsible for the immune recognition, sensing specific PAMPs of *Mycobacteria* and activating intracellular signaling pathways to induce cytokine production. TLRs are the key receptors for the recognition of *M. tuberculosis* antigens and activation of immune cells.[20] Various *Mycobacterial* components are able to be recognized by TLRs, especially TLR2, TLR4 and TLR9, their interaction could activate the secretion of proinflammatory cytokines such as IL-1, IL-8, IL-12 and TNF-α, mainly through myeloid differentiation factor 88

(MyD88)-dependent pathway.[21] TLR2 recognizes lipomannan (LM), 19-kDa lipoprotein (LP), phosphatidylinositol mannoside (PIM) and mannosylated lipoarabinomannan (Man-LAM). TLR4 binds to 38 kDa LP (PhoS1)[22] and heat shock protein 65, 70[23], whereas *Mycobacterial* DNA is the agonist of phagosomal TLR9.[24] However, TLRs are not the only PRRs for *M. tuberculosis* components, Nod2 has been suggested to play a role for the intracellular cytokine production.[25] Dectin-1, a member of CLRs, might also recognize the α-glucan expressed on the bacterial surface.[26]

Although, the invading *M. tuberculosis* can induce host innate and protective adaptive immune response, sometimes the responses cannot efficiently eliminate the bacteria. In many cases, *M. tuberculosis* are able to survive in modified macrophages by evading host immunity to establish persistent or latent infection. Multiple evasion mechanisms are employed, including 1) inhibition of phagolysosomal fusion; 2) resistance to reactive oxygen and nitrogen intermediates; 3) interference with antigen presentation. TLRs are found to have some impact on these aspects. Man-LAM, an important TLR2 agonist, inhibits the acquisition of early endosome antigen-1 (EEA1) that is crucial for phagolysosomal fusion by different strategies.[27] Man-LAM is also shown to induce the production of anti-inflammatory cytokines such as IL-4 and IL-10 to inhibit the activity of nitric oxide (NO) synthase,[28] an important enzyme for the production of reactive nitrogen intermediates (RNIs). RNIs provide another killing mechanism in active macrophage. Another TLR2 agonist, 19-kDa lipoprotein, activates innate immune functions at early stage, but prolonged stimulation leads to TLR2-dependent inhibition of MHC class II expression and antigen processing.[29] Prolonged signaling by TLR2 ligands also brings about the production of IL-4 and IL-10 to antagonize the pro-inflammatory cytokine response. IL-4 and IL-10 are proved to inhibit IFN-γ-induced macrophage activation,[30] which may promote bacteria evasion of T-cell responses and persistence of chronic infection. However, up to now, the contribution of Nod proteins to the *Mycobacterial* immune evasion has been rarely studied, the author initiated this kind of research by investigation the interaction between *M. tuberculosis* PGN fragments and Nod1 and Nod2. The results were shown in Chapter III.

Bacterial cell wall and PGN

Based on the Gram stain, bacteria are roughly divided into two categories: Gram-positive and Gram-negative, they have similar internal, but different external structures (Fig. I-2).

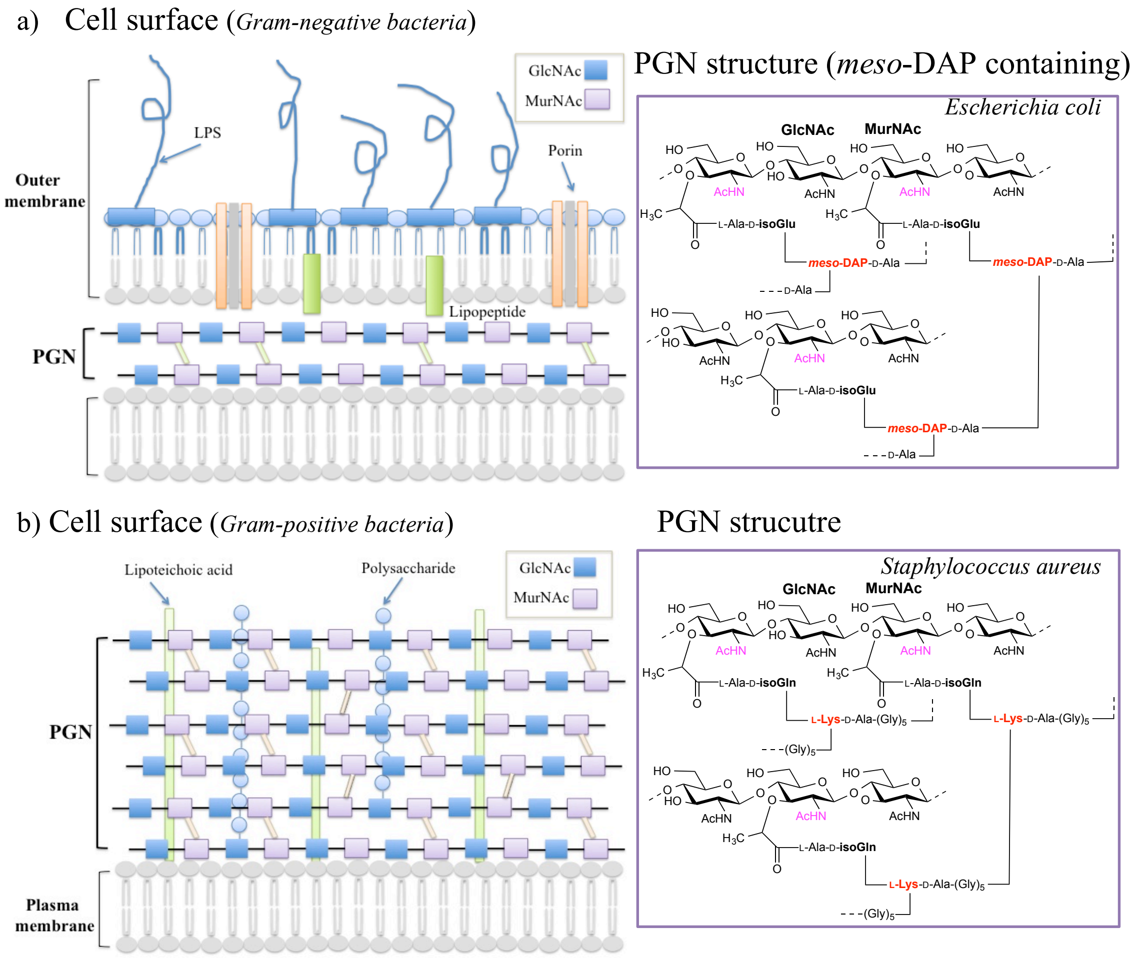
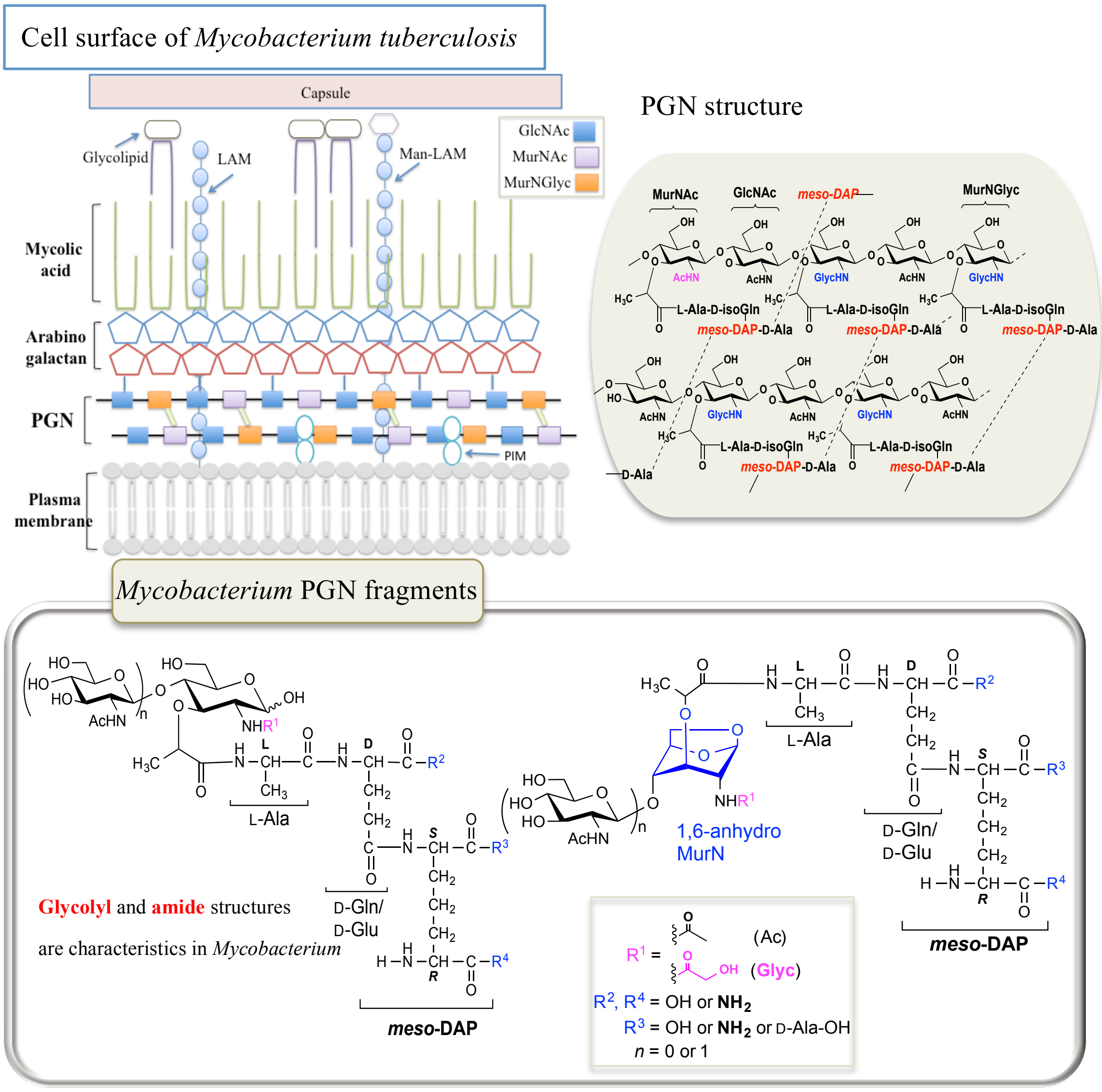


Figure I-2. The cell surface and peptidoglycan (PGN) structures of bacteria.[16] a) for Gram-negative bacteria, such as *E. coli*; b) for Gram-positive bacteria, such as *Staphylococcus aureus*. Reprinted with permission from *Annu. Rev. Pathol. Mech. Dis.* **2009**, *4*, 365-398. Copyright © (2009) Annual Reviews.

Gram-positive bacteria have thick, and multilayered cell wall, with peptidoglycan (PGN) surrounds the cytoplasmic membrane. Gram-positive cell walls also contain other structures, such as polysaccharides and lipoteichoic acids. Lipoteichoic acids have a fatty acid and are anchored in the cytoplasmic membrane.[31] Gram-negative cell walls are usually more complicated than Gram-positive cell walls. Very adjacent to the cytoplasmic membrane is a thin PGN layer, which accounts for 5% to 10% by weight. Outside of the PGN layer is the outer membrane, which is unique to Gram-negative bacteria. The outer membrane has an asymmetric bilayer structure. The inner layer contains phospholipids while the outer layer is mainly composed of lipopolysaccharide (LPS), also known as endotoxin. LPS is a powerful stimulator of immune responses.[32] Porins and lipoprotein are also observed in high concentration but not teichoic or lipoteichoic acids. *M. tuberculosis* has unique cell wall compositions, which have been greatly linked to its drug-resistance and pathogenesis (Fig. I-3). Peptidoglycan (PGN), arabinogalactan, and mycolic acid are the core structures of the whole cell wall. The capsule is composed of free lipids with different length of fatty acids and free carbohydrates. Interspersed are mannosyl-phosphatidyl-*myo*-inositol-based glycolipids (PIM), related lipomannan (LM), mannose-capped lipoarabinomannan (Man-LAM) and cell-wall proteins.[33] The special lipid-complexes are found in abundant quantities in the cell wall and sensed by various PRRs to regulate the host immune response, as described above.



Cell surface of *Mycobacterium tuberculosis*

PGN structure

Mycobacterium PGN fragments

Figure I-3. The cell surface and PGN structures of *Mycobacterium tuberculosis*. Abbreviation: PIM, Mannosyl-phosphatidyl-*myo*-inositol-based glycolipids; LM, lipomannan; Man-LAM, mannose-capped lipoarabinomannan.

Peptidoglycan (PGN) is an important element in the cell wall of all the bacteria, providing both shape and structural integrity to the cell.[34] In general, PGN comprises long glycan chains with repeating β(1-4) disaccharide of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) that are linked via peptide bridges to form mesh-like network. The stem peptide crosslinks with other fragments through usually the third amino acid. The third amino acid is usually a diaminocarboxylic acid, such as L-Lys (in Gram-positive bacteria) or *meso*-diaminopimelic acid (*meso*-DAP, in Gram-negative bacteria and some Gram-positive bacteria, e.g.: *Bacillus subtilis* and *Mycobacteria*). PGN has conserved structure in most bacteria, but modifications are observed in some cases.[35] The PGN layer in *M. tuberculosis* has modifications in both glycan backbone and peptide stem (Fig. I-3). In the glycan chain, besides the conventional MurNAc, *N*-glycolylmuramic acid (MurNGlyc) is also generated catalyzed by an enzyme, *N*-acetyl-muramic acid hydroxylase (NamH).[36] In the peptide, carboxylic acids of D-Glu and *meso*-DAP are extensively amidated. The glycolylated residue exhibited increased resistance to β-lactam antibiotics and lysozyme, and it has also been hypothesized to stabilize the cell wall with hydrogen bonding.[36] The amidation may keep the bacteria from recognizing by Nod1, which used to be observed in *B. subtilis*,[37] representing an immune evasion mechanism by *M. tuberculosis*. The 1,6-anhydro structures as well as other modifications were obtained from the LC-MS analysis of *M. tuberculosis* muropeptides (Fig. I-4).[38]

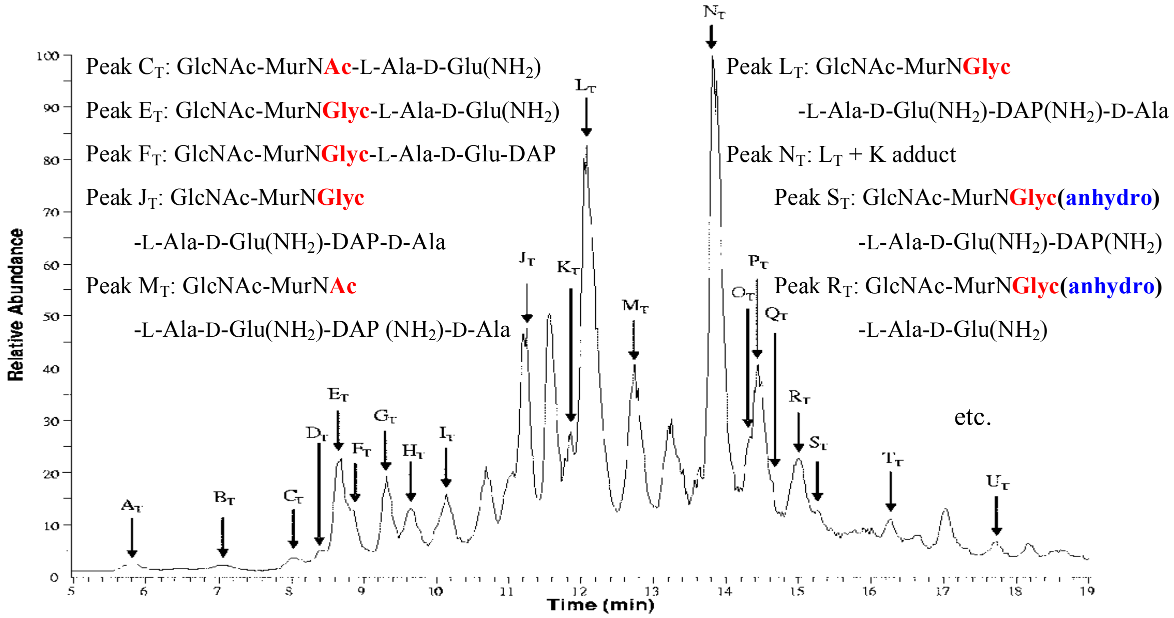


Figure I-4. LC-MS analysis of the muropeptides obtained from *M. tuberculosis* PGs.[38] PGs were digested with *Chalaropsis* sp. Muramidase. Reprinted with permission from *J. Bacteriol.* **2008**, *190*, 655-661. Copyright © (2008) American Society for Microbiology.

Nod1 and Nod2

Nod1 (also known as CARD4) and Nod2 (also known as CARD15) are two best characterized cytoplasmic proteins among NLRs. Nod1 contains one CARD domain while Nod2 contains two CARD domains. Upon interaction with their ligands, Nod1 and Nod2 activate the adaptor protein RICK via homotypic CARD-CARD interaction to trigger both nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways (Fig. I-5).[39,40] Nod1 is common in a wide variety of cell types, however, the expression of Nod2 is confined only to leukocytes and epithelial cells.[41] It is well established that the ligands of Nod1 and Nod2 are PGN-derived peptides, but not the whole PGN. γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP) is the minimum ligand for Nod1,[42] and muramyl dipeptide, MurNAc-L-Ala-D-isoGln (MDP), is for Nod2.[43] However, whether Nod1 and Nod2 sense the PGN motifs through direct binding or some other intermediate proteins was not clear until recently. Direct binding of iE-DAP to the LRR domain of Nod1 was observed utilizing surface plasmon resonance (SPR) and atomic force microscopy.[44] The direct binding between MDP and Nod2 was also reported in two papers: one used SPR technique,[45] the other applied a biotinylated-MDP pull-down assay.[46] Moreover, addition of the PGN ligand to the purified monomeric Nod1 could induce oligomerization.[47] These results confirmed the previous studies about the functions of Nod1 and Nod2.

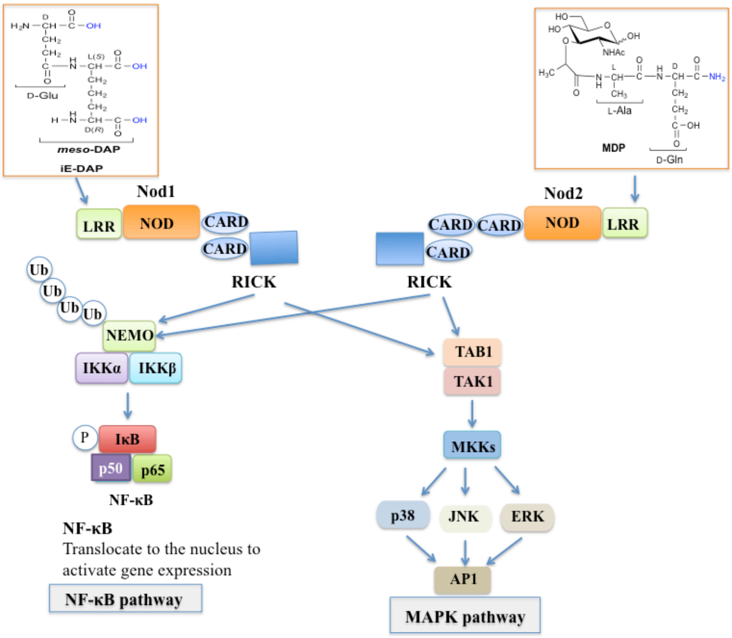


Figure I-5. Activation of nuclear transcription factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways through Nod1 and Nod2 recognition.[40] a) NF-κB signaling pathway:

Nod1/Nod2 recognize iE-DAP and MDP respectively to initiate protein oligomerization and recruitment and activation of RICK via CARD-CARD interaction. Activation of RICK leads to lysine[63]-linked polyubiquitination of NEMO and the activation of IKK complex, which further result in the phosphorylation of IκB (the NF-κB inhibitor). Then the phosphorylated IκB is degraded and NF-κB is released to translocate to the nucleus to activate gene expression; b) MAPK signaling pathway: active RICK also recruits TAB/TAK1 complex and regulates the activation of MAPKs (JUK, p38, ERK) through MKKs. The phosphorylated kinases translocate to the nucleus and mediate the phosphorylation of AP-1 transcription factors to activate the expression of target genes. The TAK1 can also affect the IKK complex. Abbreviations: RICK, receptor-interacting serine/threonine kinase; CARD, caspase recruitment domain; NEMO, NF-κB essential modulator; IKK, the inhibitor of NF-κB (IκB)-kinase; TAK1, transforming-growth-factor-b-activated kinase 1; TAB, TAK1-binding protein; JNK, c-Jun amino-terminal kinase; ERK, extracellular signal-regulated kinase; p38, p38 kinases; MKKs, MAPK kinases; AP-1, activator protein-1.

Because of the cytosolic localization, Nod1 and Nod2 recognize the intracellular bacterial components. Peptidoglycan enters the cytosol in both invasive and non-invasive ways (Fig. I-6). The immune cells such as macrophages and dendritic cells (DCs) generate the PGN ligands by ingesting the whole bacteria and digesting them in phagolysosome. On the other hand, bacterial secretion systems create pores through pathogens to enable the soluble debris to go to the cytosol to activate Nod1 and Nod2. The bacterial type III (T3SS or TTSS) and IV (T4SS or TFSS) secretion systems play important role in the non-invasive entry for Gram-negative bacteria. For example, *H. pylori* used T4SS to inject peptidoglycan degradation products directly to the intestinal epithelial cells.[48] The pore forming toxins (PFT) pneumolysin produced from *S. pneumonia* and anthrolysin O from *B. antbracis* can also internalize PGN fragments to facilitate their interaction with NLRs.[49] During bacterial cell growth and division, peptidoglycan is hydrolyzed and recycled in both Gram-positive and Gram-negative bacteria. So certain amount of the PGN hydrolyzed products are released to the host extracelluar environment.[50] The PGN fragments can also be taken up to the cytoplasm either by endocytosis[51] or oligopeptide transporter 1 (PEPT1).[52]

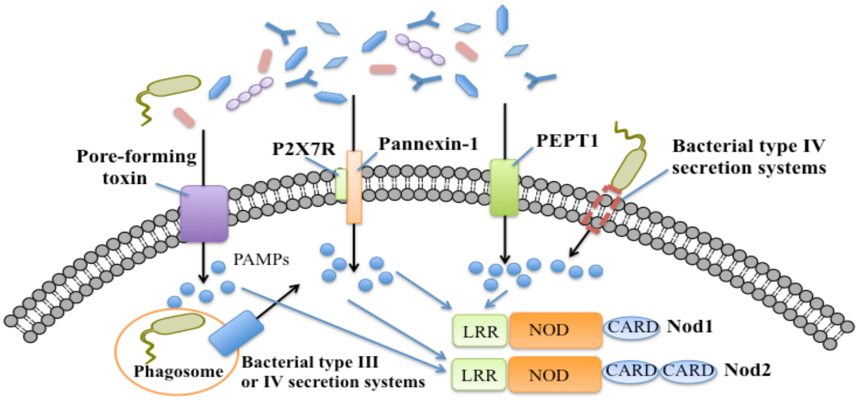


Figure I-6. The cytosolic entry of microes for Nod1 and Nod2 recognition. The microbial molecules go into the cytosol through phagocytosis, pore-forming toxins, ATP-mediated activation of Panexin-1 pore, oligopeptide transporter 1 (PEPT1) and bacterial type III or IV secretion systems.

Mycobacterium tuberculosis serine/threonine kinase PknB

The reversible protein phosphorylation plays an important role in signal transduction and bioactivity regulation, which provides a class of promising pharmaceutical targets. Bacteria mainly use the two-component phosphotransfer systems to transmit signals, involving a histidine protein kinase and a response regulator protein.[53] While Ser/Thr/Tyr-specific protein phosphorylation is a key post-translational modification with regulatory and signaling potential in eukaryotes.[54] Genomics has revealed the presence of eukaryotic-like protein kinase in bacteria as well, Ser/Thr protein kinases (STPKs) are the most distributed ones.[55] In fact, *Mycobacterial* genomes contain relatively lower amount of two-component systems when compared with other bacterial genomes in the similar size. The deficiency appears to be compensated by the alternative STPKs signal transduction mechanism. *M. tuberculosis* possess genes encoding 11 STPKs, namely PknA-PknL, excluding PknC. The gene encodes PknB is located in an operon containing five genes (Fig. I-7a): pstP (protein phosphatase), rodA (involved in cell shape control), pbpA (involved in peptidoglycan synthesis), and pknA and pknB,[56] and the start codon of each gene overlaps with the stop condon of the previous one, implying coupling of transcription and translation,[57] so PknB is implicated to be a regulator of cell growth and division. Genes coding PknB-like protein kinases are also found in many other Gram-positive bacteria.[58] PknB is a reporter-like transmembrane protein consisting of kinase domain, a juxtamembrane region, a short transmembrane domain and the extracellular domain (Fig. I-7b).[59]

a) Structure of the *pstP-pknB* gene cluster

pstP, phosphoserine/threonine protein phosphatase; *rodA*, cell division protein; *pbpA*, peptidoglycan biosynthesis protein; *pknA-pknB*, Ser/Thr protein kinases.

b) Structure of STPK PknB

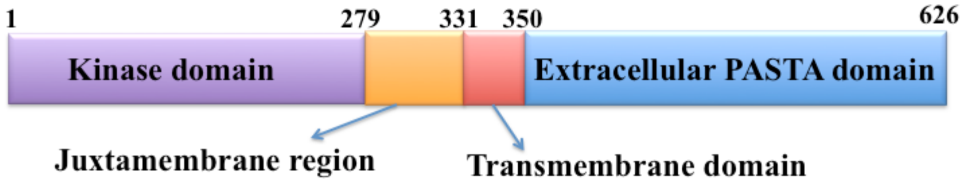


Figure I-7. Schematic representation of gene *pknB* and STPK PknB from *M. tuberculosis*. a) Structure of the *pstP-pknB* gene cluster; b) Structure of STPK PknB.

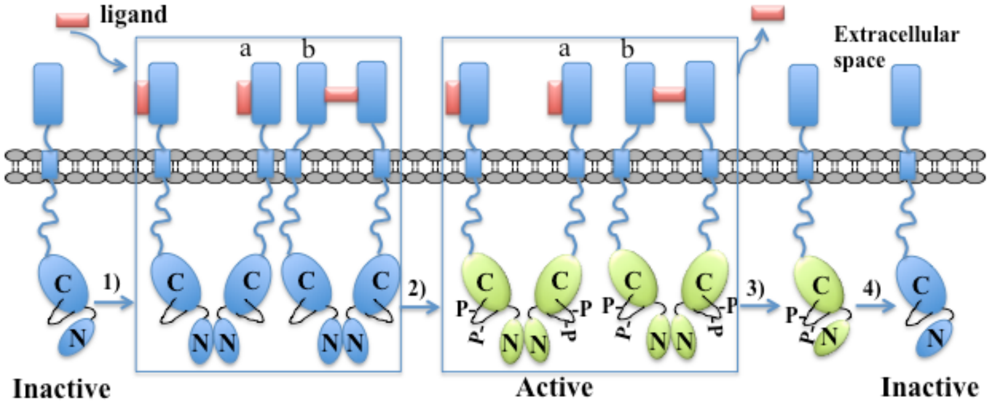


Figure I-8. Model of *M. tuberculosis* PknB Regulation. 1) Dimerization of the kinase domains of inactive PknB monomers, possibly mediated by the extracellular binding of ligand. The dimer adopts a conformation that facilitate ATP binding and the following phosphorylation. Ligand could a) bind to one PknB molecule[71] or b) bind to two PknB molecules[61]; 2) Activation of kinase domain through autophosphorylation and subsequent transphosphorylation of substrates; 3) Release of active autophosphorylated monomer to amplify the signal; 4) Dephosphorylation by PstP phosphatase to regenerate the inactive monomer.

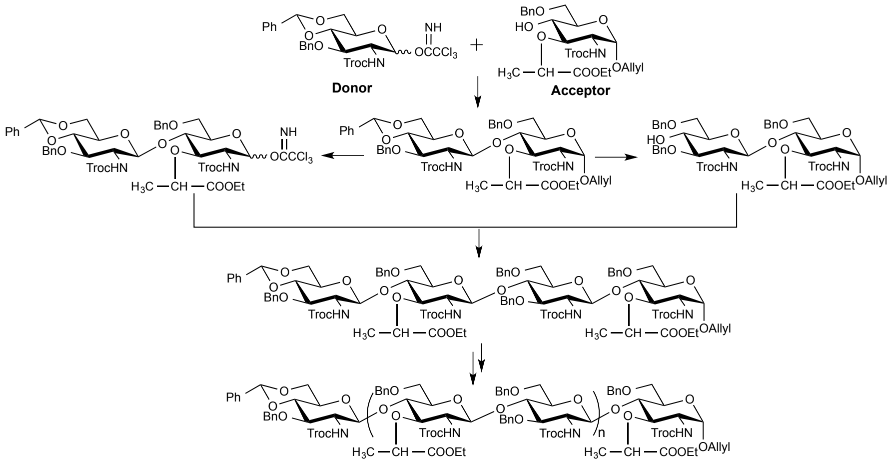
The crystal structure of PknB kinase domain displayed a two-lobed structure (C- and N-lobes), implying conservation of protein fold and universal catalytic mechanism of STPKs in prokaryotes and eukaryotes.[60] The activation of PknB kinase domain relies on the dimerization of the N-lobe interface via a distinctive allosteric mechanism (Fig. I-8).[61]

A number of substrates of PknB have been identified. Of these, Wag31 (DivIVA) is involved in the cell division and cell shape.[62] The beta-ketoacyl AcpM synthases KasA and KasB are involved in mycolic acid biosynthesis.[63] PBPA and GlmU are two important enzymes for peptidoglycan biosynthesis.[64] PknB can also phosphorylate virulence factors SigH and RshA.[65] Besides, PknB has autophosphorylation properties as well as cross-phosphorylates PknA[57] and PknG.[66] However, the understanding of how bacterial PknB responds to extracellular signals remains in a nascent stage. The extracellular domain of PknB is made up of four PASTA domains (penicillin-binding protein and serine/threonine kinase associated domain) which is a conserved region in many Gram-positive bacteria, consisting of three β strands and an α helix, with a loop region of variable length between the first and second β strands.[67] The four PASTA domains exhibit a linear organization,[68] and is predicted to bind to unlinked peptidoglycan,[67] based on the crystal structure analysis of PASTA containing penicillin binding protein PBP2x from *Streptococccus pneumonia* bound to two molecules of penicillin-type antibiotics.[69] And the three PASTA repeats containing extracellular domain of STPK PrkC from *Bacillus subtilis* has been displayed to bind to peptidoglycan fragments.[70] PrkC serves as the receptor for peptidoglycan fragments during bacterial spores germination. Recently, direct interaction between PASTA domains of PknB and muropeptides was shown by using surface plasmon resonance method.[71]

Development of chemical synthesis of PGN

Because of the difficulty of isolating highly pure bacterial glycoconjugates, including PGN fragments, from natural sources, it becomes very necessary to get synthetic bioactive compounds for collecting precise and accurate information on the relationships between recognition structure and biological functions. With regards to PGN, the synthesis of both Lys type PGN partial structures with mono-, di-, tetra- and octasaccharide[72-74] and *meso*-DAP type PGN fragments with mono-, di-, and tetrasaccharide have been achieved.[75-77] For the construction of glycan chain, mainly two kinds of methods have been developed as shown in Fig. I-9. In Fukase group in Osaka University, the repeating glycans of PGN was accomplished by coupling of a key disaccharide glucosaminyl-β(1–4)-muramic acid unit. Stereoselective glycosylation of disaccharide units was realized by the neighboring group participation effect of *N*-Troc (Troc = 2,2,2-trichloroethoxycarbonyl) group and proper reactivity of *N*-Troc-glucosaminyl trichloroacetimidate.[73,78] Mobashery group synthesized tetrasaccharide by 2+1+1 strategy, they used *N*-dimethylmaleoyl (DMM) protective group to form β(1-4) glycoside bond and the lactate residue was introduced at a late stage.[79] Researchers have also done a lot of work on the synthesis of orthogonally protected *meso*-DAP, which is described in detail in Chapter II.

A. Glycan synthesis by Fukase group



B. Glycan systhesis by Mobashery group

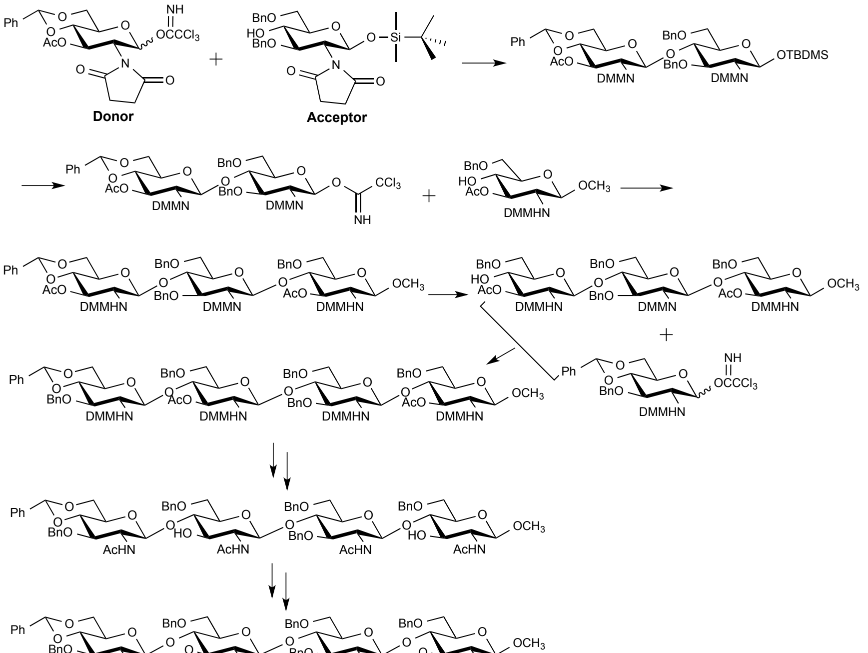


Figure I-9. Construction of Glycan chain.

Previous studies of human Nod1 (hNod1) and Nod2 (hNod2) with synthetic PGN ligands

a) hNod1 activation with synthetic PGN fragments

In 2003, Inohara group (collaborating with Fukase group) and Philpott group independently elucidated that the intracellular protein Nod1 was a receptor for PGN fragments derived primarily from Gram-negative bacteria.[42] Fukase group[42a] manifested that the core structure recognized by Nod1 was iE-DAP (γ-D-glutamyl-*meso*-DAP) using both highly purified and chemically synthesized compounds (Fig. I-10) while Philpott group[42b] showed that hNod1 specifically detected a DAP-containing GlcNAc-MurNAc-tripeptide (L-Ala-γ-D-Glu-*meso*-DAP) purified from Gram-negative bacteria. In the same paper, Fukase group reported that iQ-DAP (γ-D-glutaminyl-*meso*-DAP) also stimulated Nod1 with a little weaker potency than iE-DAP. Later, tripeptide A-iE-DAP (L-Ala-γ-D-glutamyl-*meso*-DAP) was discovered to show stronger activity than iE-DAP whereas tripeptide A-iQ-DAP (L-Ala-γ-D-glutaminyl-*meso*-DAP) had even less activity than iQ-DAP (Fig. I-10),[80] implying that the –COOH group of glutamic acid was important for Nod1 recognition. Fukase group also explored the effect of DAP configuration on hNod1 activation. All the four synthetic iE-DAP stereoisomers: iE-(2*R*,6*R*)-DAP, iE-(2*R*,6*S*)-DAP, iE-(2*S*,6*R*)-DAP (natural *meso* form), and iE-(2*S*,6*S*), showed Nod1 activation, the natural form iE-(2*S*,6*R*)-DAP was the most potent one. The stereoisomeric DAP mixture displayed similar activity to iE-*meso*-DAP.[81] A library of *N*-acylated iE-DAP[81,82] was synthesized from commercially available DAP racemate, and their Nod1 stimulating efficiency was investigated. The derivatives with longer hydrophobic chains showed stronger activation than iE-DAP, and KF-1B (*N*-myristoyl-iE-DAP) was several hundred folds more active than iE-DAP, which could be used for in vivo functional study of Nod1. The improved activity was explained by the increased interaction with the cell membrane and transfer into the host cell. David[83] group also constructed a series of racemic DAP containing iE-DAP derivatives to study structure-activity relationships and found another potent hNod1 agonist C12 *N,N*-dialkyl- iE-DAP.

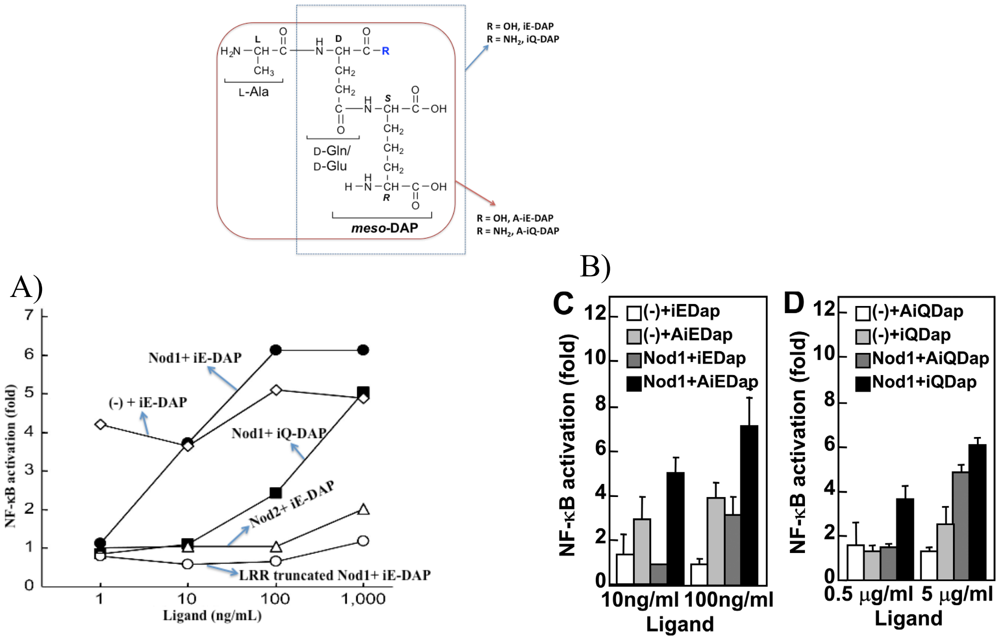


Figure I-10. Human Nod1 stimulatory activity of synthetic di/tripeptides. A)[42a]; B)[80]. Ref. 42a was reprinted by permission from Macmillan Publishers Ltd: [*Nat. Immunol.*] (*Nat. Immunol.* **2003**, *4*, 702-707), copyright © (2003) Nature Publishing Group. Ref. 80 was reprinted with permission from *J. Exp. Med.* **2006**, *203*, 203-213. Copyright © (2006) The Rockefeller University Press.

In addition to the DAP-containing peptides, Fukase group also succeeded in the synthesis of glycopeptide structures of PGN, including mono-, and disaccharide in the combination with tri- and tetrapeptide (Fig. I-11).[76] For the hNod1 stimulation study, MS-3P$_{(DAP)}$ and DS(anh)- 3P$_{(DAP)}$ exhibited very potent hNod1 activation, which were comparable to KF1B, but their tetrapeptides containing analogues showed extremely weak activities, suggesting that the free carboxyl group at 2-position of DAP is favorable for hNod1 recognition.

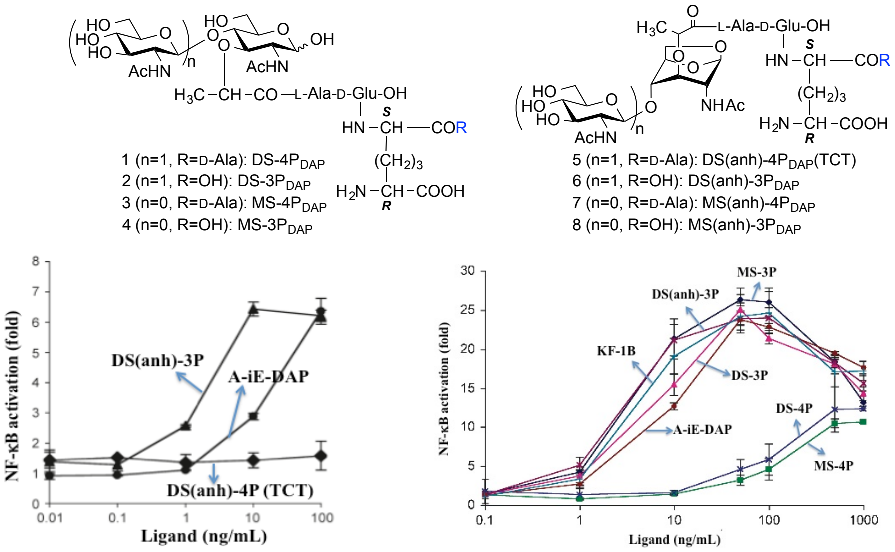
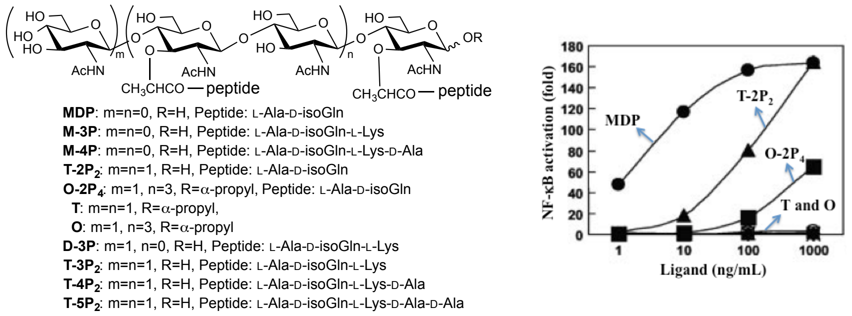


Figure I-11. Human Nod1 stimulation by *meso*-DAP containing PGNs.[76] Reprinted with permission from *Chem. Eur. J.* **2008**, *14*, 10318-10330. Copyright © (2008) WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

b) hNod2 activation with synthetic PGN fragments

Although MDP (muramyl dipeptide: MurNAc-L-Ala-D-isoGln) had been suggested to be the minimum partial structure for immunostimulation in the middle of 1970s,[84] the mechanism of immunostimulation was not revealed until 2003, when the cytosolic protein Nod2 was reported as the receptor of PGN.[43] Nod2 is believed to elicit broad recognition of bacteria, because its minimum ligand MDP is presenting universally in the PGNs from both Gram-positive and Gram-negative bacteria. Nod2 not only detects MDP, but also MDP containing structures which have L-lysine or L-ornithine at the third amino acid.[37] In order to fully understand the structure-activity relationships between PGN fragments and Nod2, Fukase group initially synthesized a series of PGN partial structures containing mono-, di-, tetra- and octasaccharide in combination with di-, tri-, and tetrapeptide (Fig. I-12).[73] The tetra- and octasaccharide containing fragments, T–2P$_2$ and O–2P$_4$, showed Nod2 dependent immunostimulatory activity, whereas only glycan chains T and O did not, indicating that the peptide residues were essential for the Nod2 stimulation.[43a] The most potent ligand was MDP, and that longer glycan chains showed lower Nod2 activation. The Nod2 activations of monosaccharides with different peptide lengths were also investigated. MDP (containing dipeptide) was the stronger stimuli than longer peptide containing structures, and MurNAc-L-Ala-D-isoGln-L-Lys also showed relatively lower but definite activity.

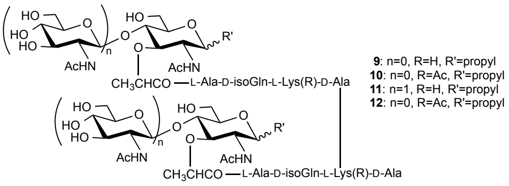
Lysine-type cross-linked PGN fragments were also successfully synthesized (Fig. I-13a),[85] and the linked PGN structures showed no Nod2 activity in hNod2-transfected HEK293T but showed weak immunostimulating activity in human peripheral mononuclear cells, suggesting the linked structures somehow degraded by enzymes in the latter case. On the other hand, the non-linked pentapeptide containing structures was not active in both cases.



MDP: m=n=0, R=H, Peptide: L-Ala-D-isoGln
M-3P: m=n=0, R=H, Peptide: L-Ala-D-isoGln-L-Lys
M-4P: m=n=0, R=H, Peptide: L-Ala-D-isoGln-L-Lys-D-Ala
T-2P$_2$: m=n=1, R=H, Peptide: L-Ala-D-isoGln
O-2P$_4$: m=1, n=3, R=α-propyl, Peptide: L-Ala-D-isoGln
T: m=n=1, R=α-propyl,
O: m=1, n=3, R=α-propyl
D-3P: m=1, n=0, R=H, Peptide: L-Ala-D-isoGln-L-Lys
T-3P$_2$: m=n=1, R=H, Peptide: L-Ala-D-isoGln-L-Lys
T-4P$_2$: m=n=1, R=H, Peptide: L-Ala-D-isoGln-L-Lys-D-Ala
T-5P$_2$: m=n=1, R=H, Peptide: L-Ala-D-isoGln-L-Lys-D-Ala-D-Ala

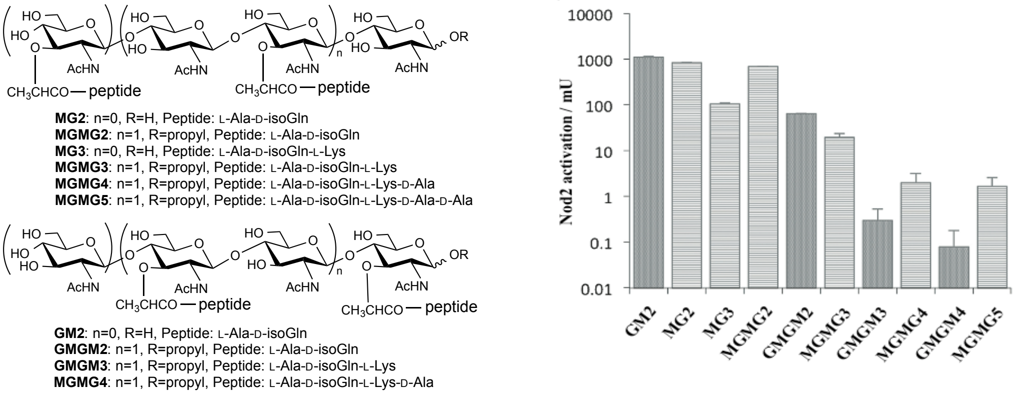
Figure I-12. Synthesis of PGN fragments with different length of glycans and peptides and their hNod2 activity evaluation.[73] Reprinted with permission from *Org. Biomol. Chem.* **2006**, *4*, 232-242. Copyright © (2006) Royal Society of Chemistry.

a) Synthesis of Lysine-type cross-linked PGN fragments



9: n=0, R=H, R'=propyl
10: n=0, R=Ac, R'=propyl
11: n=1, R=H, R'=propyl
12: n=0, R=Ac, R'=propyl

b) Synthesis of PGN fragments with two kinds of glycan sequence



MG2: n=0, R=H, Peptide: L-Ala-D-isoGln
MGMG2: n=1, R=propyl, Peptide: L-Ala-D-isoGln
MG3: n=0, R=H, Peptide: L-Ala-D-isoGln-L-Lys
MGMG3: n=1, R=propyl, Peptide: L-Ala-D-isoGln-L-Lys
MGMG4: n=1, R=propyl, Peptide: L-Ala-D-isoGln-L-Lys-D-Ala
MGMG5: n=1, R=propyl, Peptide: L-Ala-D-isoGln-L-Lys-D-Ala-D-Ala

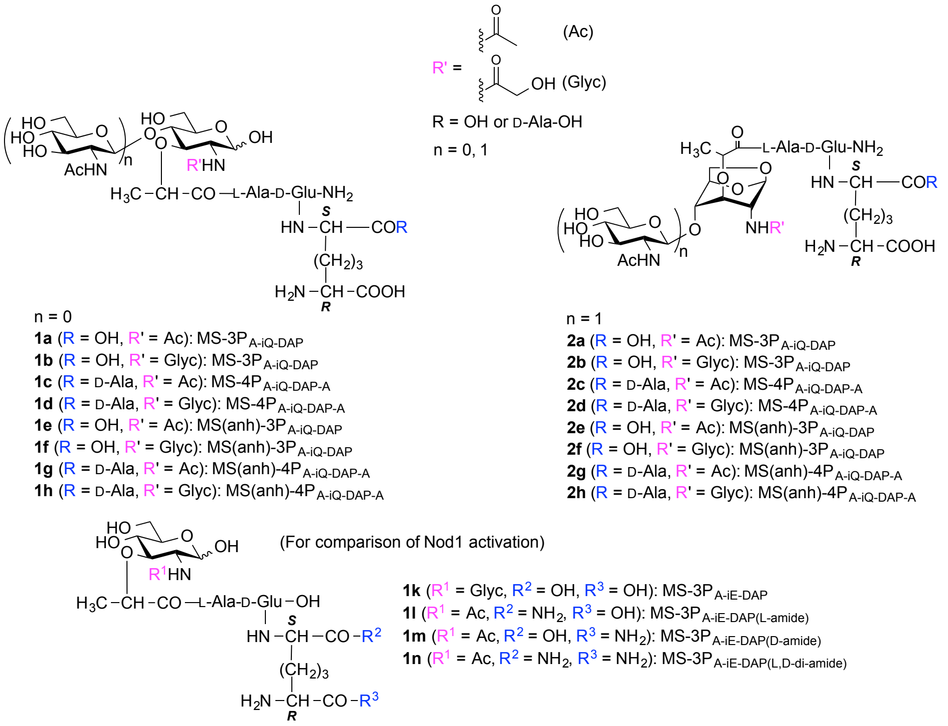
GM2: n=0, R=H, Peptide: L-Ala-D-isoGln
GMGM2: n=1, R=propyl, Peptide: L-Ala-D-isoGln
GMGM3: n=1, R=propyl, Peptide: L-Ala-D-isoGln-L-Lys
MGMG4: n=1, R=propyl, Peptide: L-Ala-D-isoGln-L-Lys-D-Ala

Figure I-13. PGNs with cross-linked structure[85] and two kinds of glycan sequence.[74] Ref. 74 was reprinted with permission from *ChemBioChem* **2013** *14*, 482-488. Copyright © (2013) WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. Ref. 85 was reprinted with permission from *Tetrahedron Lett.* **2009**, *50*, 3631–3634. Copyright © (2009) Elsevier Ltd.

Recently, various tetrasaccharide containing PGN partial structures were also synthesized in Osaka Univ. group. They contained two kinds of glycan sequence: GlcNAc-MurNAc and MurNAc-GlcNAc.[74] The MGMG $(\text{MurNAc-GlcNAc})_2$ sequences (obtained through cleavage by *N*-acetylglucosaminidase) showed much stronger hNod2 activities than GMGM $(\text{GlcNAc-MurNAc})_2$ sequences (cleaved by muramidase, including lysozyme) (Fig. I-13b), indicating that the enzymatic degradation process might affect the immunomodulation in human.

Synthesis and functions of *Mycobacterium* cell wall peptidoglycan fragments

One of major part of the consequent pathogenesis of *M. tuberculosis* is from the unique composition of the bacterial cell wall, and the biological activities of *Mycobacterium* PGN fragments have not been well elucidated. In this thesis, the author successfully synthesized a series of *meso*-DAP containing *Mycobacterium* PGN fragments (Fig. I-14), including mono- and disaccharides, along with tripeptide (L-Ala-D-isoGln-*meso*-DAP) and tetrapeptide (L-Ala-D-isoGln-*meso*-DAP-D-Ala) as well as glycolylation and amidation modifications. To start the synthesis, an efficient and highly stereoselective synthesis for *meso*-diaminopimelic acid (DAP) derivatives was developed by using chemoenzymatic reaction. The detailed synthesis strategy was shown in Chapter II.



n = 0
1a (R = OH, R' = Ac): MS-3P$_{\text{A-iQ-DAP}}$
1b (R = OH, R' = Glyc): MS-3P$_{\text{A-iQ-DAP}}$
1c (R = D-Ala, R' = Ac): MS-4P$_{\text{A-iQ-DAP-A}}$
1d (R = D-Ala, R' = Glyc): MS-4P$_{\text{A-iQ-DAP-A}}$
1e (R = OH, R' = Ac): MS(anh)-3P$_{\text{A-iQ-DAP}}$
1f (R = OH, R' = Glyc): MS(anh)-3P$_{\text{A-iQ-DAP}}$
1g (R = D-Ala, R' = Ac): MS(anh)-4P$_{\text{A-iQ-DAP-A}}$
1h (R = D-Ala, R' = Glyc): MS(anh)-4P$_{\text{A-iQ-DAP-A}}$

n = 1
2a (R = OH, R' = Ac): MS-3P$_{\text{A-iQ-DAP}}$
2b (R = OH, R' = Glyc): MS-3P$_{\text{A-iQ-DAP}}$
2c (R = D-Ala, R' = Ac): MS-4P$_{\text{A-iQ-DAP-A}}$
2d (R = D-Ala, R' = Glyc): MS-4P$_{\text{A-iQ-DAP-A}}$
2e (R = OH, R' = Ac): MS(anh)-3P$_{\text{A-iQ-DAP}}$
2f (R = OH, R' = Glyc): MS(anh)-3P$_{\text{A-iQ-DAP}}$
2g (R = D-Ala, R' = Ac): MS(anh)-4P$_{\text{A-iQ-DAP-A}}$
2h (R = D-Ala, R' = Glyc): MS(anh)-4P$_{\text{A-iQ-DAP-A}}$

1k (R^1 = Glyc, R^2 = OH, R^3 = OH): MS-3P$_{\text{A-iE-DAP}}$
1l (R^1 = Ac, R^2 = NH_2, R^3 = OH): MS-3P$_{\text{A-iE-DAP(L-amide)}}$
1m (R^1 = Ac, R^2 = OH, R^3 = NH_2): MS-3P$_{\text{A-iE-DAP(D-amide)}}$
1n (R^1 = Ac, R^2 = NH_2, R^3 = NH_2): MS-3P$_{\text{A-iE-DAP(L,D-di-amide)}}$

Figure I-14. Targeted *Mycobacterium* PGN fragments.

The author then evaluated the function of *Mycobacterium* PGN fragments in two aspects: in host and in *M. tuberculosis.* In the view of host's protective system, the author studied their immune stimulation via human Nod1 and Nod2 recognition (Chapter III). This was the first study to investigate the immunostimulating ability of *Mycobacterium* PGN fragments with chemically synthesized PGN fragments, which contain *meso*-DAP and *N*-glycolyl (Glyc) muramic acid (MurNGlyc) residues. The mechanism study will contribute to the understanding of the pathogenesis of *Mycobacterium*-related diseases and reveal the effects of *N*-glycolyl group to the survival of *Mycobacteria*-related bacteria in host.

In the view of *M. tuberculosis* biosynthetic system*,* the author investigated their binding affinity to the extracellular PASTA domains of bacterial Ser/Thr kinase PknB (Chapter IV). Compared with the previous report,[71] the author explored more diverse and specific structures, which will help to accumulate more accurate knowledge about the functional mechanism of PknB and design more potent antibiotics.

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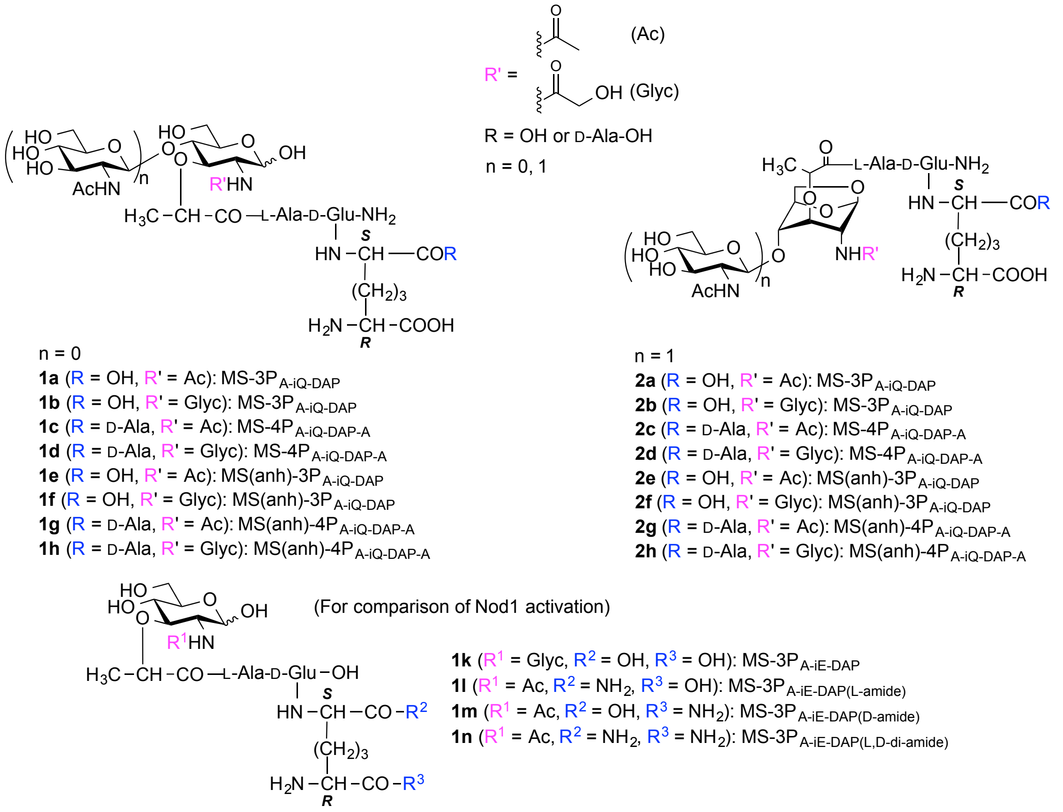
Chapter II Synthesis of (2*S*,6*R*)-diaminopimelic acid (*meso*-DAP) containing *Mycobacterium* Peptidoglycan (PGN) fragments

In order to synthesize the *Mycobacterium* PGN fragments shown in Fig. II-1, the author firstly planned to construct the orthogonally protected *meso*-DAP derivatives by newly developed chemoenzymatic method. Then *meso*-DAP containing tripeptide (L-Ala-D-isoGln-*meso*-DAP) and tetrapeptide (L-Ala-D-isoGln-*meso*-DAP-D-Ala) were obtained. Meanwhile, the preparation of glycan parts including monosaccharides and disaccharides, especially the *N*-glycolyl containing ones were developed. The coupling of the peptides and glycans could lead to the target structures as shown in Fig. II-1.



n = 0

1a (R = OH, R' = Ac): MS-3P$_{\text{A-iQ-DAP}}$

1b (R = OH, R' = Glyc): MS-3P$_{\text{A-iQ-DAP}}$

1c (R = D-Ala, R' = Ac): MS-4P$_{\text{A-iQ-DAP-A}}$

1d (R = D-Ala, R' = Glyc): MS-4P$_{\text{A-iQ-DAP-A}}$

1e (R = OH, R' = Ac): MS(anh)-3P$_{\text{A-iQ-DAP}}$

1f (R = OH, R' = Glyc): MS(anh)-3P$_{\text{A-iQ-DAP}}$

1g (R = D-Ala, R' = Ac): MS(anh)-4P$_{\text{A-iQ-DAP-A}}$

1h (R = D-Ala, R' = Glyc): MS(anh)-4P$_{\text{A-iQ-DAP-A}}$

n = 1

2a (R = OH, R' = Ac): MS-3P$_{\text{A-iQ-DAP}}$

2b (R = OH, R' = Glyc): MS-3P$_{\text{A-iQ-DAP}}$

2c (R = D-Ala, R' = Ac): MS-4P$_{\text{A-iQ-DAP-A}}$

2d (R = D-Ala, R' = Glyc): MS-4P$_{\text{A-iQ-DAP-A}}$

2e (R = OH, R' = Ac): MS(anh)-3P$_{\text{A-iQ-DAP}}$

2f (R = OH, R' = Glyc): MS(anh)-3P$_{\text{A-iQ-DAP}}$

2g (R = D-Ala, R' = Ac): MS(anh)-4P$_{\text{A-iQ-DAP-A}}$

2h (R = D-Ala, R' = Glyc): MS(anh)-4P$_{\text{A-iQ-DAP-A}}$

1k (R^1 = Glyc, R^2 = OH, R^3 = OH): MS-3P$_{\text{A-iE-DAP}}$

1l (R^1 = Ac, R^2 = NH$_2$, R^3 = OH): MS-3P$_{\text{A-iE-DAP(L-amide)}}$

1m (R^1 = Ac, R^2 = OH, R^3 = NH$_2$): MS-3P$_{\text{A-iE-DAP(D-amide)}}$

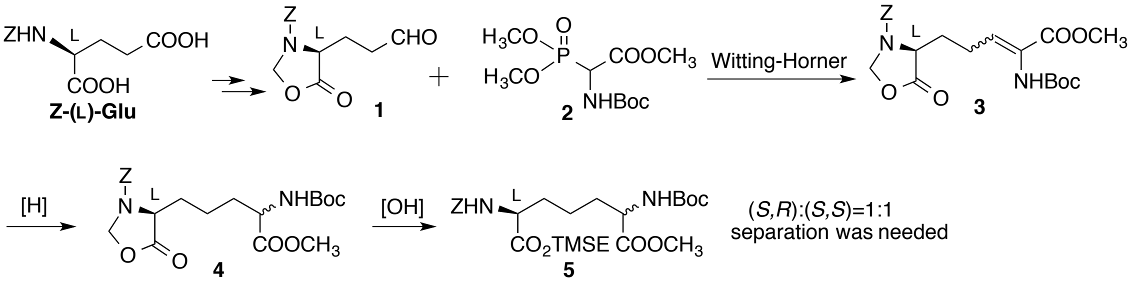
1n (R^1 = Ac, R^2 = NH$_2$, R^3 = NH$_2$): MS-3P$_{\text{A-iE-DAP(L,D-di-amide)}}$

Figure II-1. Target *Mycobacterium* PGN fragments.

II-1 Synthesis of (2*S*,6*R*)-diaminopimelic acid (*meso*-DAP)

II-1-a Introduction

The *meso*-DAP moiety is the core amino acid of Nod1 ligands. As iE-DAP being the minimum ligand, some of its derivatives have been shown to be potent Nod1 agonists as described in Chapter I. The synthesis of orthogonally protected *meso*-DAP have been reported by several groups,[1-6] and some were used for the functionalization of DAP to construct PGN fragments. Schmidt group[2] utilized Wittig–Horner reaction with L-glutamate aldehyde and a phosphoryl glycine derivative to produce the C-7 dicarboxylic acid derivative, after C=C double bond reduction and protecting group manipulation to afford the desired compounds (Fig. II-2).

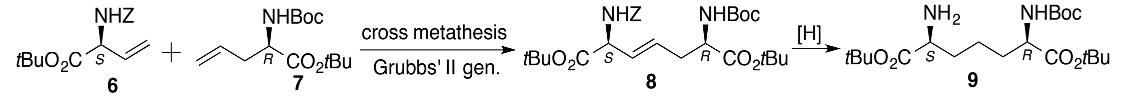


(*S*,*R*):(*S*,*S*)=1:1
separation was needed

Figure II-2. Synthesis of *meso*-DAP derivatives by Schmidt group.[2]

Boons group[3] obtained *meso*-DAP by connecting two chiral amino acids (allyl glycine and vinyl glycine derivatives) with cross metathesis, followed by reduction of the double bond of the resulting compound (Fig. II-3). Fukase group[4] used Kocienski-modified Julia olefination with the D-serine derivatives at low temperature to avoid epimerization (Fig. II-4). Mobashery group[5] applied L-aspartic acid in two Wittig reactions, followed by Sharpless epoxidation of allyl alcohol with ethyl L-tartarate and its selective epoxide ring opening with azide to construct the *meso*-DAP analogue. Recently, Takahata group[6] prepared *meso*-DAP by a cross metathesis between the readily available Garner aldehyde-derived vinyl glycine and protected allyl glycine (Fig. II-3).

a) By Boons group



b) By Takahata group

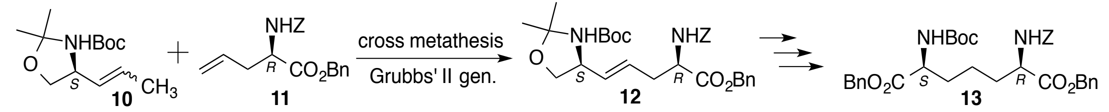


Figure II-3. Synthesis of *meso*-DAP derivatives through cross metathesis.

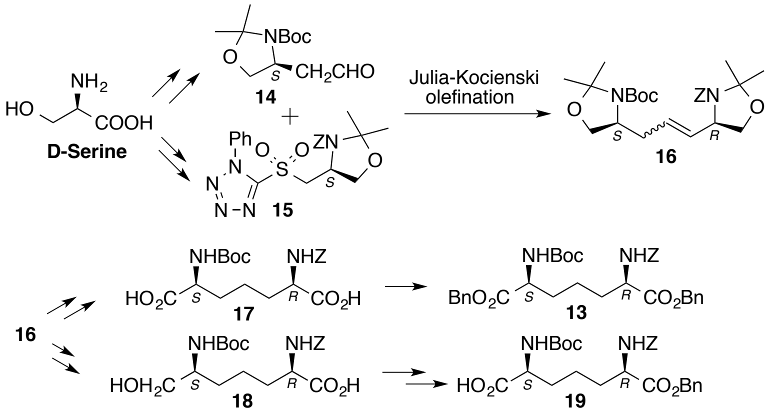


Figure II-4. Synthesis of *meso*-DAP derivatives by Fukase group.

II-1-b Synthesis of *meso*-DAP with chemoenzymatic method

All the reported methods for orthogonally protected chiral *meso*-DAP synthesis as shown in the previous section, however, have several disadvantages: multi-step, harsh conditions, inaccessible starting materials, low yields, and epimerization. Herein, the author initially developed an enzymatic method for the orthogonally protected *meso*-DAP synthesis (Fig. II-5), which is advantageous because of mild conditions, short steps, and high stereoselectivity. Moreover, the reactions could be done in large scale. The L- and D-aminoacylase can hydrolyze the *N*-acetyl groups in L- and D-configuration respectively with very high selectivity. The commercially available racemic 2,6-diaminopimelic acid **20** was firstly acetylated to give **21**. After hydrolyzing the L-*N*-acetyl of **21** by L-aminoacylase (Acylase Amano), without any purification, the free amino group was protected with *tert*-butyloxycarbonyl (Boc) group. Herein, the author obtained a mixture with three compounds: L-*N*-Boc-D-*N*-acetyl-DAP (**23a**, desired compound), L,L-di-*N*-Boc-DAP (**23b**) and **21**, which were completely separated only by extraction with ethyl acetate (EA) at different pH values (Fig. II-6):

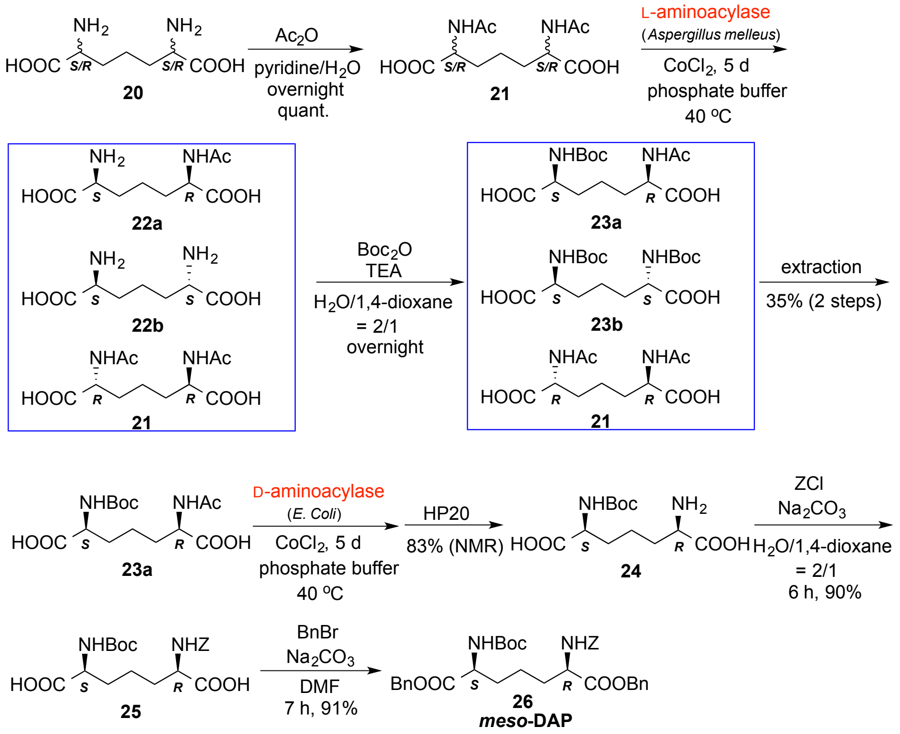


Figure II-5. Synthesis of orthogonally protected ***meso*-DAP**.

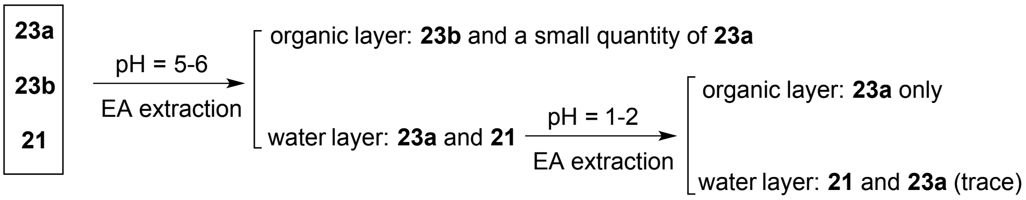


Figure II-6. Workflow for **23a** purification.

In order to further improve the optical purity and facilitate the final global deprotection, *N*-acetyl group in D-configuration of **23a** was cleaved by D-aminoacylase to give **24** and roughly purified by HP20 before being substituted with carboxybenzyl (Z) group in **25**. The two carboxylic acids were protected with benzyl group to give the orthogonally protected **26** (***meso*-DAP**). For the introduction of Z group, *N*-(benzyloxycarbonyloxy)succinimide (ZOSu) was first used as the Z group source, but the products were contaminated with large amount of *N*-hydroxysuccinimide (NHS) which was hard to be separated out. So, the reagent for the formation of benzyl carbamate was changed to benzyl chloroformate (ZCl) from ZOSu, and it was found that the reaction proceeded well in H_2O/1,4-dioxane 2:1 with Na_2CO_3 as the base.

The enzymatic reactions were conducted under very mild conditions (40 ^{o}C in 0.05 M phosphate buffer) and the existence and concentration of cobalt (II) ion were very important to accelerate the reaction. After the examination of reaction conditions, the author found the best conditions as follows; xx mM for cobalt (II), 1 mM for L-aminoacylase, and 10 mM for D-aminoacylase. From the commercially available racemic DAP to the alternatively protected *meso*-DAP, the whole procedure was reduced to 6 steps, and all the conditions could be easily achieved. The resulting protected chiral *meso*-DAP **26** showed 100% optical purity as judged by chiral HPLC.[7]

II-1-c Synthesis of *meso*-DAP-D-Ala via *N*-carboxyanhydride formation

In order to realize the selective coupling between D-Ala and the carboxyl group at the L-configuration of DAP, a triphosgene mediated cyclization reaction was employed to afford the *N*-carboxyanhydride (NCA) ring (Fig. II-7). The ring-opening polymerization of amino acid *N*-carboxyanhydrides (NCAs) is the most economical and expedient process for the synthesis of polypeptide-containing block copolymers.[8] Free α-amino acids can be selectively activated and used for the peptide coupling by NCA method. The author thus utilized the NCA method for the synthesis of dipeptide **29**. Boc group of **25** was cleaved with trifluoroacetic acid (TFA), and the deprotected product **27** was reacted with triphosgene to form *N*-carboxyanhydride (NCA) in **28**.[9] The coupling of **28** with HCl•H-D-Ala-OBn was carried out by using triethylamine (TEA) at 0 °C for 0.5 h to afford the desired dipeptide **29**, which was then protected with Boc group to give **30**. In order to reduce the polymerization by-products, 3-equivalents of HCl•H-D-Ala-OBn was used against **28**. Benzylation of **30** gave the fully protected dipeptide **31** (***meso*-DAP-D-Ala**).

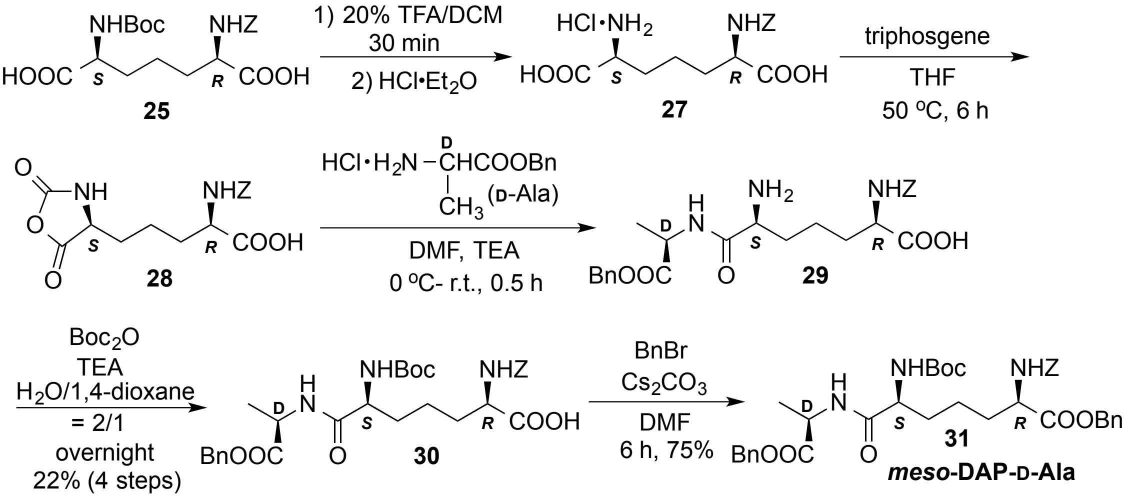


Figure II-7. Synthesis of ***meso*-DAP-D-Ala**.

The synthesis of *meso*-DAP containing tripeptide (**H-L-Ala-D-isoGln-*meso*-DAP**) and tetrapeptide (**H-L-Ala-D-isoGln-*meso*-DAP-D-Ala**) were then successfully achieved in large amounts (Fig. II-8). The Boc group of **26** and **31** were deprotected with TFA, and the liberated amino groups were coupled to the side-chain carboxyl group of Boc-L-Ala-D-isoGln using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (WSCD•HCl), 1-hydroxybenzotriazole (HOBt), and triethylamine (TEA) in THF to give fully protected tripeptide **32** and tetrapeptide **33**, respectively. Subsequently, deprotection of the Boc group to give ready-to-use tripeptide **34** (**H-L-Ala-D-isoGln-*meso*-DAP**) and tetrapeptide **35** (**H-L-Ala-D-isoGln-*meso*-DAP-D-Ala**).

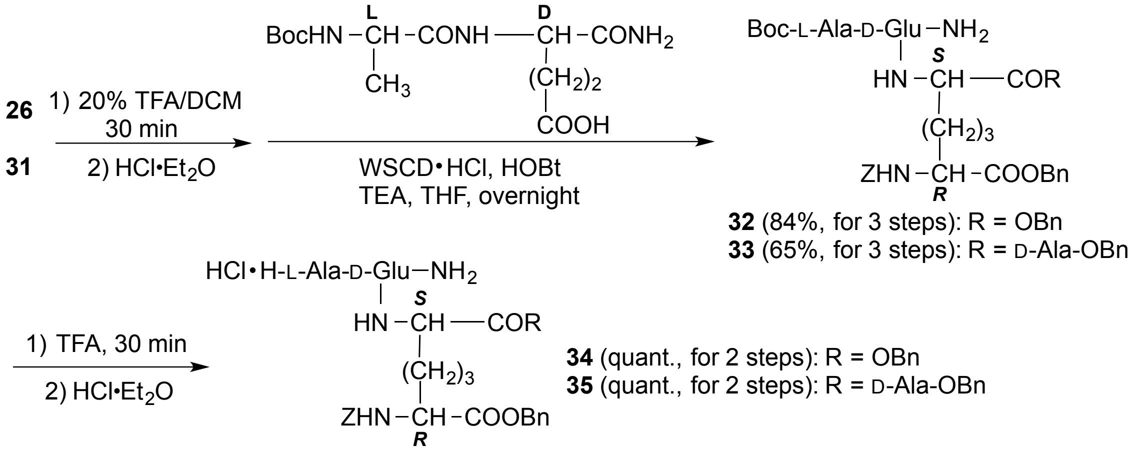


Figure II-8. Synthesis of *meso*-DAP containing tripeptide **34** and tetrapeptide **35**.

II-1-d Synthesis of *meso*-DAP derivatives with amidation modification

Because of the extensive existence of amidation in the *meso*-DAP moiety of natural *Mycobacterium* PGN, the author also prepared three *meso*-DAP derivatives with amidation at different sites: L-amidated **50**, D-amidated **51**, and L,D-di-amidated **52** (Fig. II-9). For the synthesis of L-amide-*meso*-DAP **38**, NCA in **28** was reacted with anhydrous NH_3 (5 equiv.) in 1,4-dioxane to give the L-amidated **36**. After protection of the free amino and carboxyl group, **38** was obtained successfully. The D-amide-*meso*-DAP **42** was also prepared from **24** in a similar way. The two –COOH groups in **25** were amidated by 28% ammonia aqueous solution with WSCD•HCl, HOBt and TEA to give L,D-di-amide-*meso*-DAP **43**. The amidated *meso*-DAPs were then reacted with the side-chain carboxyl group of Boc-D-Glu-OBn and Boc-L-Ala consecutively to afford the tripeptides. After Boc-deprotection, the appropriately protected key intermediates, tripeptides **50**, **51**, and **52**, were successfully synthesized.

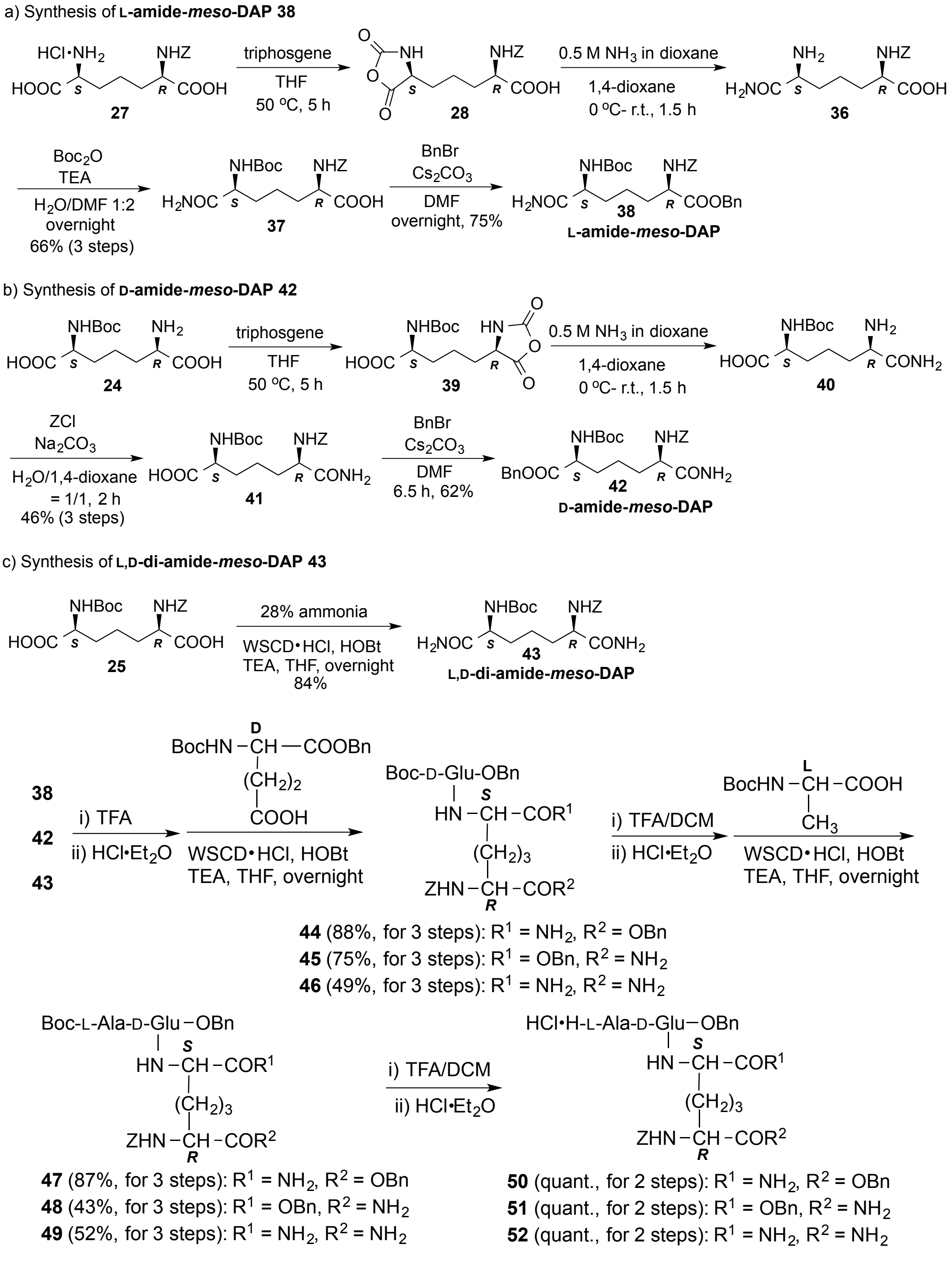


Figure II-9. Synthesis of *meso*-DAP derivatives with amidation modification: **50**, **51** and **52**.

II-2 Synthesis of *Mycobacterium* PGN fragments with monosaccharides

The preparation of *N*-acetyl muramyl group containing PGN fragments released from *E. coli* were reported by Fukase group in 2008.[4] Based on their synthesis strategy, both *N*-glycolyl and *N*-acetyl muramyl group containing structures were synthesized with some modifications.

II-2-a Synthesis of PGN fragments with *N*-glycolyl muramyl group

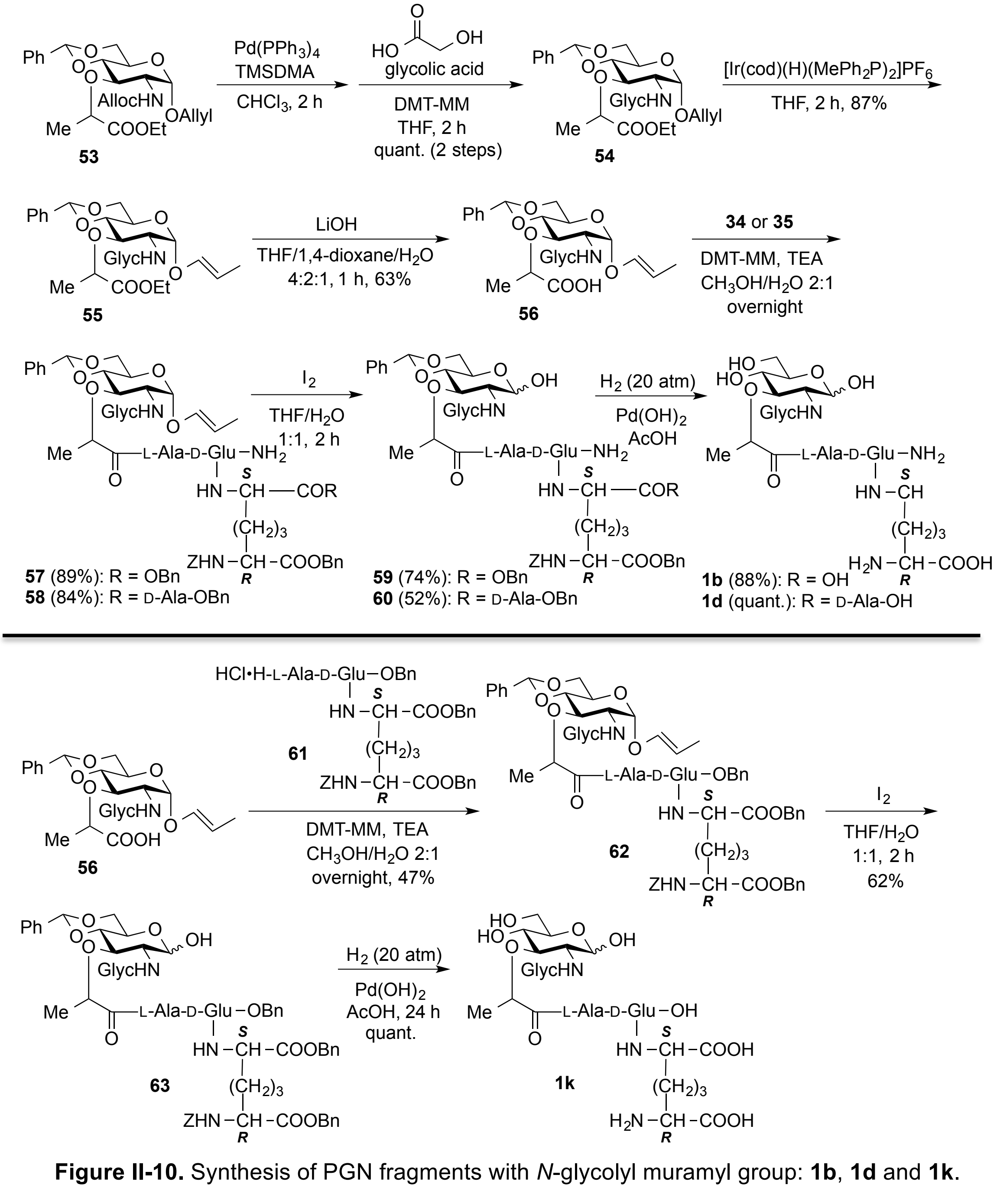


Figure II-10. Synthesis of PGN fragments with *N*-glycolyl muramyl group: **1b**, **1d** and **1k**.

The author first synthesized the PGN fragments with *N*-glycolyl muramyl group and tripeptide (L-Ala-D-**isoGln**-*meso*-DAP) **1b** or tetrapeptide (H-L-Ala-D-**isoGln**--*meso*-DAP-D-Ala) **1d** (Fig. II-10, upper part). The Alloc group in **53**[10] was removed by $Pd(PPh_3)_4$, and then the liberated amino group was glycolylated to give **54**. For the glycolylation reaction, unprotected glycolic acid was used to reduce reaction steps, and DMT-MM [4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride] was used to prevent the side-reaction caused by the hydroxyl group in glycolic acid. DMT-MM has been proved to be powerful coupling reagent to enable the direct condensation of carboxylic acids and amines in protic solvents such as methanol, ethanol, 2-propanol, or water, as well as a mixture of these solvents.[11] Prior to treatment with DMT-MM, mixing the carboxylic acid and amine for several minutes was found to be essential in this reaction.[12] The isomerization of the allyl group in **54** to propenyl group was realized by H_2 activated $[Ir(cod)(MePh_2P)_2]PF_6$ (cod = 1,5-cyclooctadiene) to give **55**. The ethyl ester in **55** was deprotected with LiOH in THF/1,4-dioxane/H_2O 4:2:1. The resulting **56** was condensed with tripeptide **34** or tetrapeptide **35** using DMT-MM in CH_3OH/H_2O 2:1 to afford **57** and **58**, respectively. The removal of propenyl group with iodine and water gave **59** and **60**. All the remaining protecting groups (4,6-benzylidene group, benzyl group and Z group) were removed completely by hydrogenation with $Pd(OH)_2$ in acetic acid to generate **1b** and **1d**. The author also synthesized **1k** with *N*-glycolyl muramyl group and tripeptide **61** (L-Ala-D-**isoGlu**-*meso*-DAP) (Fig. II-10, lower part), whose hNod1 activation was evaluated as well (Chapter III).

II-2-b Synthesis of PGN fragments with *N*-glycolyl 1,6-anhydro-muramyl group

In regard to the preparation of PGN fragments with *N*-glycolyl 1,6-anhydro-muramyl group (Fig. II-11), the azide group in **64**[4] was reduced with Zinc powder in THF/AcOH, and the resulting amino group was glycolylated to give **65**. Subsequently, **65** underwent ethyl ester cleavage, peptide coupling, and final deprotection to afford the target compounds **1f** and **1h**. In hydrogenation reaction, THF/H_2O/AcOH was used instead of AcOH for preventing the cleavage of the 1,6-anhydro-ring.

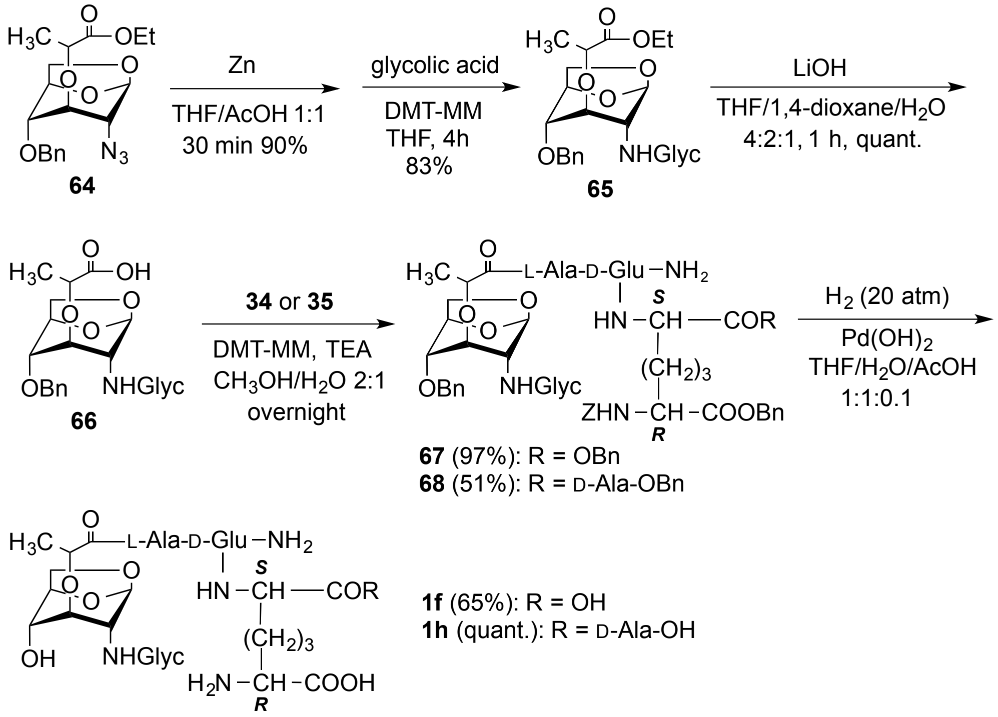


Figure II-11. Synthesis of PGN fragments with *N*-glycolyl 1,6-anhydro-muramyl group **1f** and **1h**.

II-2-c Synthesis of PGN fragments with *N*-acetyl muramyl and *N*-acetyl 1,6-anhydro-muramyl group

For comparing the effect of the modification at the 2-amino group (acetyl vs glycolyl), PGN fragments with *N*-acetyl muramyl group (**1a** and **1c**) and *N*-acetyl 1,6-anhydro-muramyl group (**1e** and **1g**) were also prepared from **69**[4] and **64**, respectively (Fig. II-12). In order to compare the effect of amidation modification on the host immune stimulation, PGN fragments **1l**, **1m** and **1n** with peptides, **50**, **51** and **52**, were also synthesized (Fig. II-12). In all the carboxylic acid and amine condensation reactions (**69** with **34**, **69** with **35**, **75** with **34**, and **75** with **35**), WSCD·HCl was used as the coupling reagent. For the final deprotection of **83**, palladium reagents (10% Pd/C and $Pd(OH)_2$ were first used with various H_2 pressures and reduction times, but the reaction were not succeeded. Presumably because the two adjacent amide groups might affect on the Pd-catalysis and deactivate the catalytic efficacy. The author then changed the cleavage reaction to a strong acidic condition. The reaction was performed with TMSOTf/TFA/thioanisole/anisole in *m*-cresol,[13] the deprotected compound **1n** was obtained successfully.

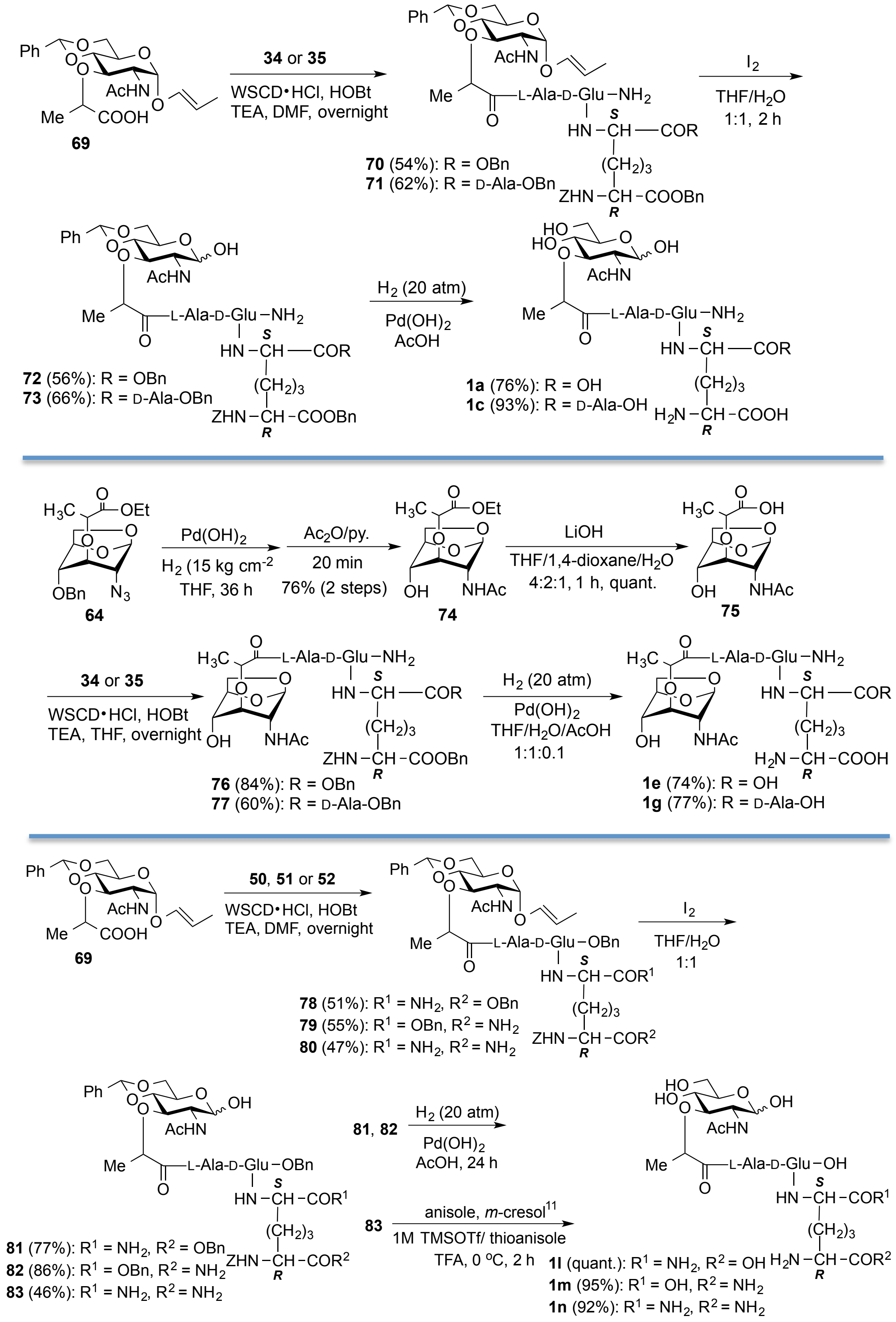


Figure II-12. Synthesis of *N*-acetyl monosaccharide containing PGN fragments.

II-3 Synthesis of *Mycobacterium* PGN fragments with disaccharides

II-3-a Preparation of Disaccharides

Based on the methods for monosaccharide-containing PGN fragments developed in the previous section (II-2), the disaccharide-containing PGN fragments from *M. tuberculosis* were also synthesized.

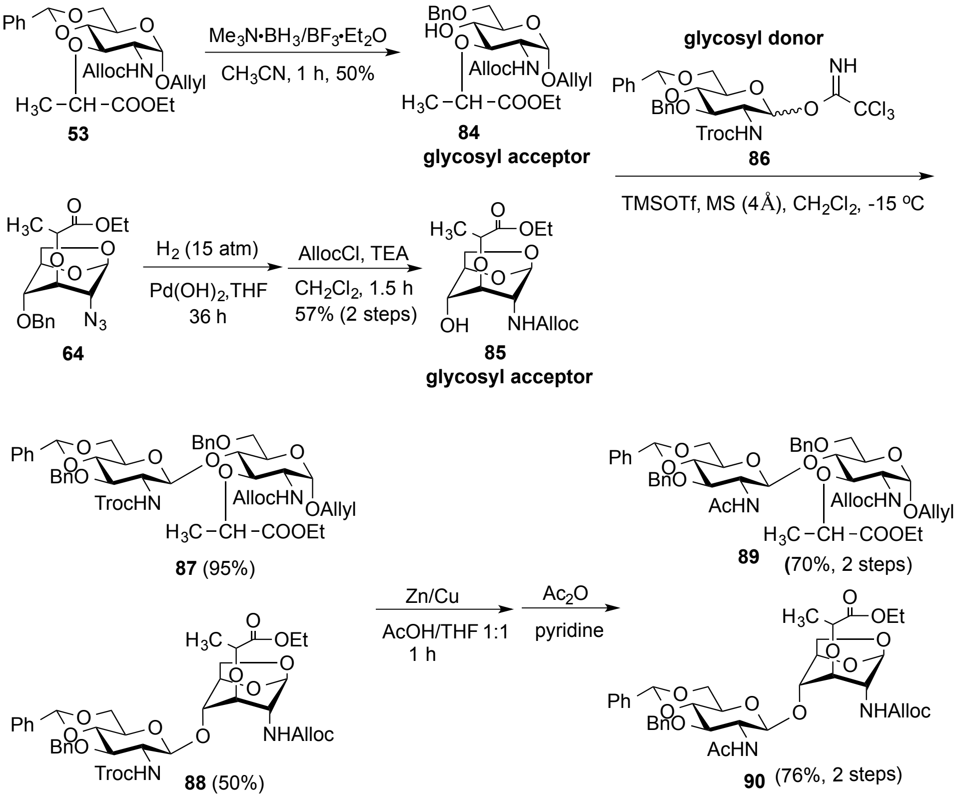


Figure II-13. Synthesis of disaccharides **89** and **90**.

Two disaccharide units **89** and **90** were synthesized from **53** and **64**, respectively, as shown in Fig. II-13; The compound **89** has regular muramyl group, and **90** has 1,6-anhydro-muramyl group. The glycosyl acceptor **84** was prepared from the regioselective ring opening of 4,6-*O*-benzylidene of **53** to form 6-*O*-benzyl derivative by using $BH_3 \cdot Me_3N/BF_3 \cdot Et_2O$.[10] On the other hand, the synthesis of glycosyl acceptor **85** was initiated from hydrogenation of both benzyl group and azide group in **64**, and Alloc group was then introduced to the free amino group. The *N*-trichloroethoxycarbonyl (Troc)-glucosaminyl trichloroacetimidate **86**[10] was chosen as the glycosyl donor because of its excellent β-selective glycosylation ability due to the neighboring participation of Troc group. The glycosylation proceeded smoothly in the presence of MS 4Å and by utilizing TMSOTf as the catalyst in dry CH_2Cl_2 under Argon atmosphere. *N*-Troc group of **87** and **88** was removed by Zn-Cu couple in AcOH, the free amino group was then acetylated with Ac_2O and pyridine to afford **89** and **90**. For easier experimental procedure, one-pot reaction by mixing of Ac_2O, AcOH, THF 1:1:1 was performed and gave the target compounds in comparable yield with the two-step reactions. The disaccharides **89** and **90** were the key intermediates for the following reactions. The Alloc groups were removed and then either glycolylated (to give the glycolyl group containing PGN fragments) or acetylated (to give the acetyl group containing PGN fragments) were performed to obtain the following compounds described in the next sections.

II-3-b Synthesis of PGN fragments with GlcNAc-MurNGlyc and GlcNAc-MurNAc

For the synthesis of *Mycobacterium* PGN fragments with GlcNAc-MurNGlyc and GlcNAc-MurNAc, the disaccharide **89** was derived for the further condensation reactions. First of all, the Alloc group of **89** was cleaved by $Pd(PPh_3)_4$, and the liberated amino group was then glycolylated to give **91** in two steps. The amino group derived from **89** was also acetylated to give **92** in one step (Fig. II-14). Afterwards, disaccharide containing PGN fragments were prepared in the similar manner to their corresponding monosaccharides as shown in Fig. II-15. Isomerizaiton of the Allyl group with Ir-complex, deprotection of ethyl ester, peptide coupling with **34** or **35**, cleavage of propenyl group with I_2 in water, and final hydrogenation to afford the fully deprotected compounds **2a**-**2d**.

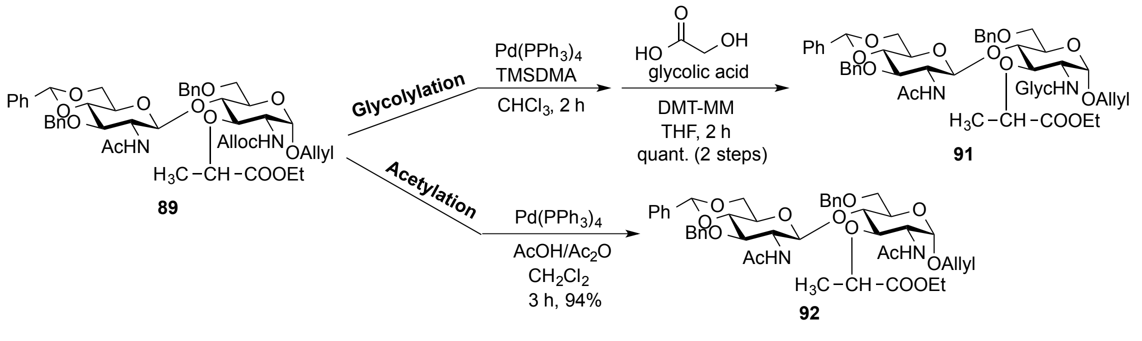


Figure II-14. Synthesis of disaccharides **91** and **92**.

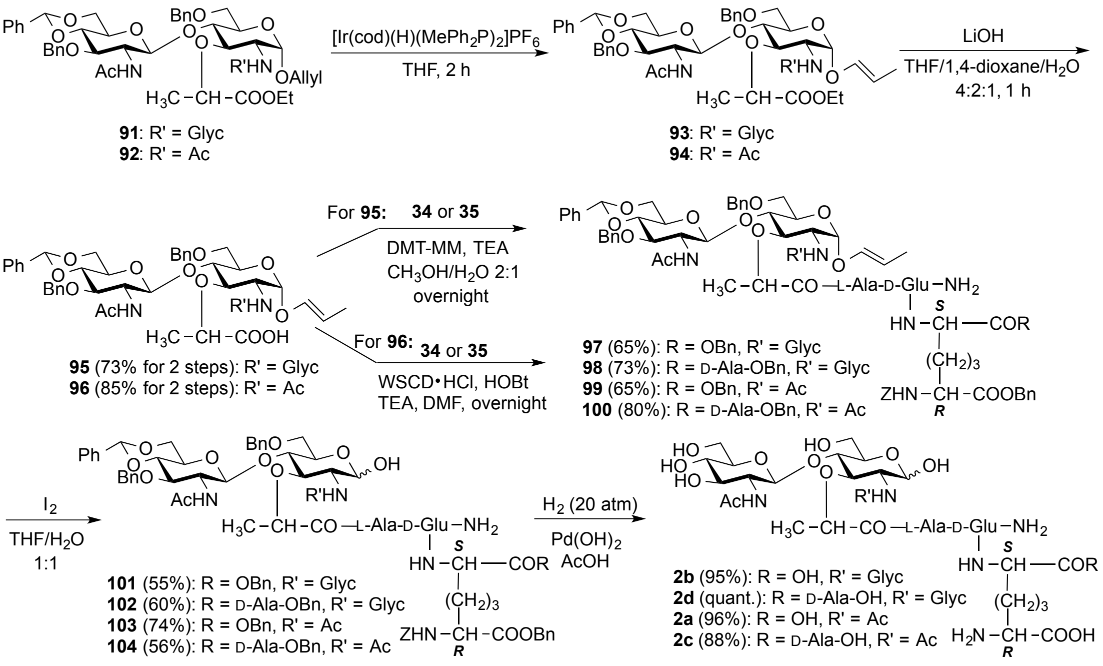


Figure II-15. Synthesis of PGN fragments with **GlcNAc-MurNGlyc** and **GlcNAc-MurNAc**.

II-3-c Synthesis of PGN fragments with GlcNAc-(1,6-anhydro)MurNGlyc and GlcNAc-(1,6-anhydro)MurNAc

For the synthesis of *Mycobacterium* PGN fragments with GlcNAc-(1,6-anhydro)MurNGlyc and GlcNAc-(1,6-anhydro)MurNAc, 1,6-anydro-muramyl group containing disaccharides **105** and **106** were also prepared in the same way as **91** and **92**, as described in the previous section. The Alloc group in **90** was replaced with glycolyl group for **105** and with acetyl group for **106** (Fig. II-16).

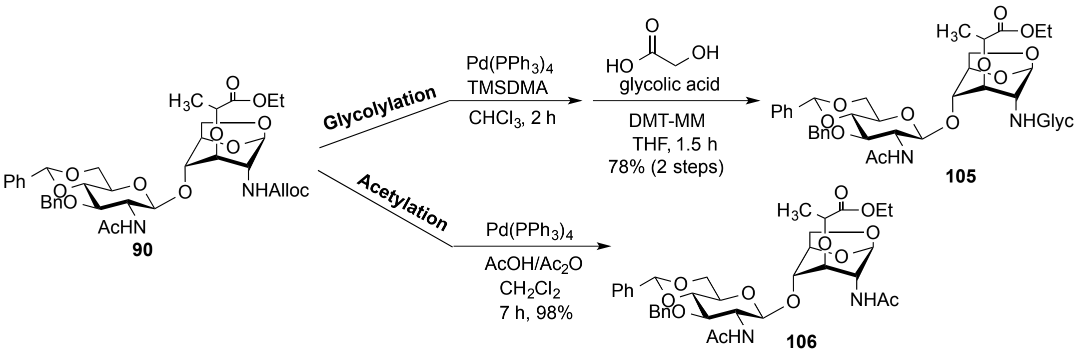


Figure II-16. Synthesis of disaccharides **105** and **106**.

Utilizing these disaccharides **105** and **106**, ethyl ester at the muramic acid moiety was hydrolyzed to give **107** or **108**, and peptide condensation with tripeptide or tetrapeptide gave the fully protected compounds **109**, **110**, **111** and **112**. The final deprotection of these compounds were carried out to give the target compounds **2e-2h** (Fig. II-17).

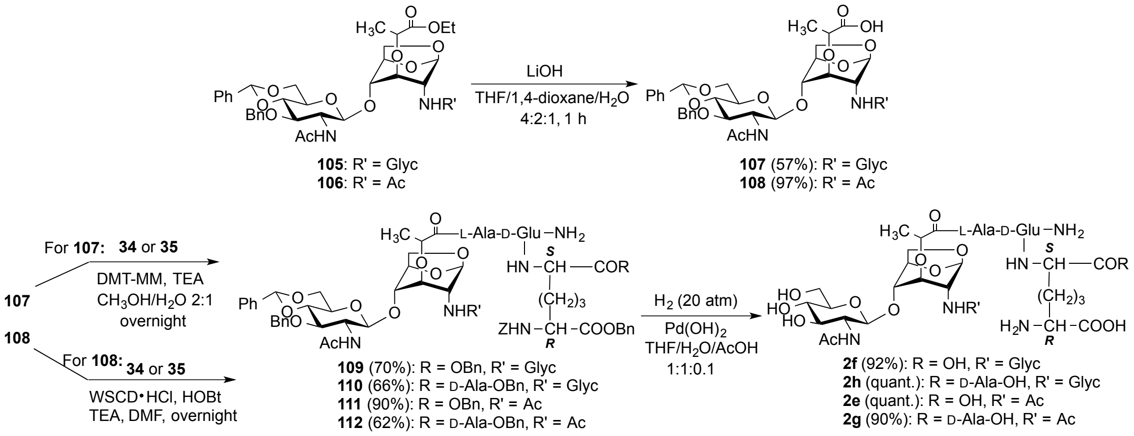


Figure II-17. Synthesis of PGN fragments with **GlcNAc-(1,6-anhydro)MurNGlyc** and **GlcNAc-(1,6-anhydro)MurNAc**.

II-4 Summary

In this Chapter, an efficient and highly stereoselective synthesis for *meso*-diaminopimelic acid (DAP) derivatives has been developed by using chemoenzymatic reaction. The resulting *meso*-DAP derivatives were applied for the synthesis of oligopeptides, including tripeptide (L-Ala-D-isoGln-*meso*-DAP) and tetrapeptide (L-Ala-D-isoGln-*meso*-DAP-D-Ala). By coupling the peptides with monosaccharides and disaccharides including MurNGlyc and 1,6-anhydro-MurNGlyc, a series of *Mycobacterium* peptidoglycan fragments were obtained successfully. The author also synthesized mono-amidated or di-amidated *meso*-DAP containing PGN fragments to compare the amidation effect on the bioactivities.

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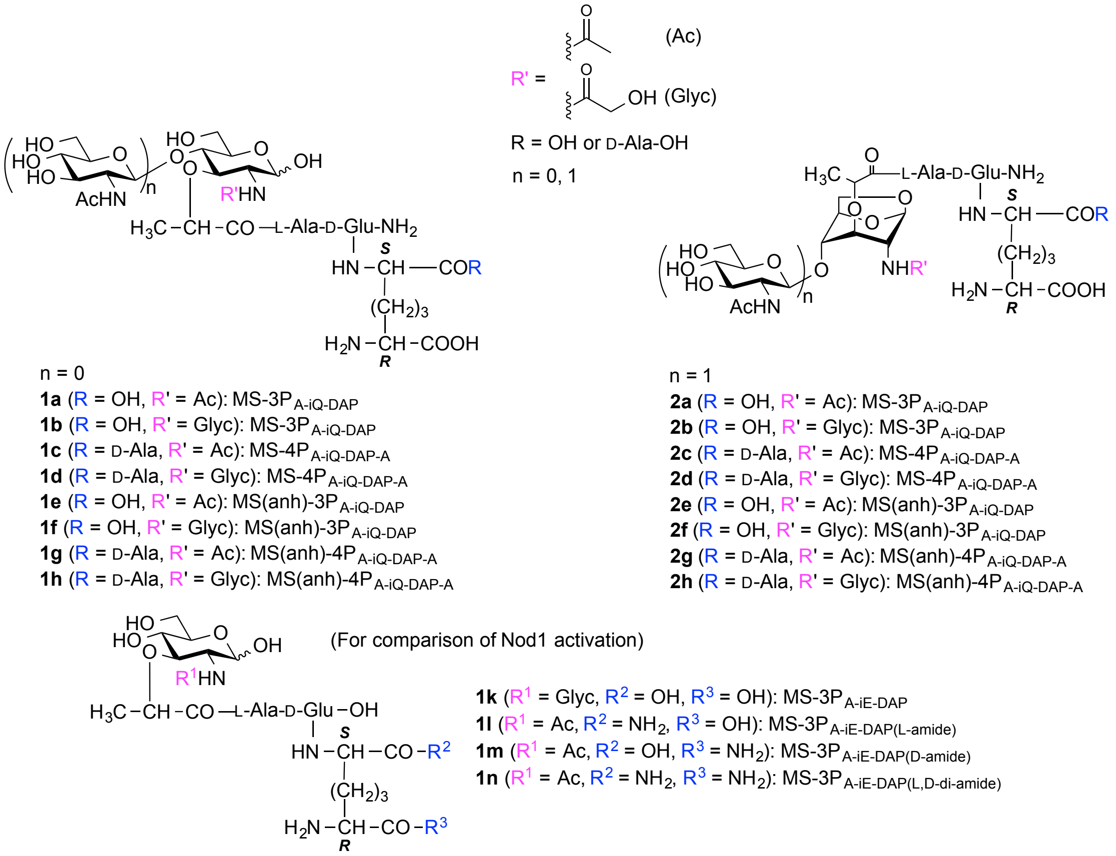
Chapter III Human immune stimulation via hNod1 and hNod2 recognition by *Mycobacterium* PGN fragments

Nod1 and Nod2 are two main cytosolic receptors for PGN fragments, and the minimum ligand for Nod1 is iE-DAP,[1] while MDP[2] is the ligand for Nod2. Investigations have been done on the innate immune stimulation of regular PGN fragments through interaction with human Nod1 and Nod2 as described in Chapter I. However, the immunostimulating abilities of *meso*-DAP containing *Mycobacterium* PGN fragments have been rarely reported. The unique glycolylation and amidation modifications in the *Mycobacterium* PGN have been correlated to the bacterial immune evasion abilities, but no direct evidence had been obtained so far. The author synthesized a series of *Mycobacterium* PGN fragments in Chapter II as shown in Fig. III-1, and also evaluated their hNod1 and hNod2 activation with cell assays utilizing HEK293T based conditions; hNod1 assay was done with stably human *Nod1* transfected cells (HEG1G), whereas hNod2 assay was conducted with transiently human *Nod2* transfected cells, to find out what kind of structural motifs would affect on the innate immune modulation.



n = 0

1a (R = OH, R' = Ac): $\text{MS-3P}_{\text{A-iQ-DAP}}$
1b (R = OH, R' = Glyc): $\text{MS-3P}_{\text{A-iQ-DAP}}$
1c (R = D-Ala, R' = Ac): $\text{MS-4P}_{\text{A-iQ-DAP-A}}$
1d (R = D-Ala, R' = Glyc): $\text{MS-4P}_{\text{A-iQ-DAP-A}}$
1e (R = OH, R' = Ac): $\text{MS(anh)-3P}_{\text{A-iQ-DAP}}$
1f (R = OH, R' = Glyc): $\text{MS(anh)-3P}_{\text{A-iQ-DAP}}$
1g (R = D-Ala, R' = Ac): $\text{MS(anh)-4P}_{\text{A-iQ-DAP-A}}$
1h (R = D-Ala, R' = Glyc): $\text{MS(anh)-4P}_{\text{A-iQ-DAP-A}}$

n = 1

2a (R = OH, R' = Ac): $\text{MS-3P}_{\text{A-iQ-DAP}}$
2b (R = OH, R' = Glyc): $\text{MS-3P}_{\text{A-iQ-DAP}}$
2c (R = D-Ala, R' = Ac): $\text{MS-4P}_{\text{A-iQ-DAP-A}}$
2d (R = D-Ala, R' = Glyc): $\text{MS-4P}_{\text{A-iQ-DAP-A}}$
2e (R = OH, R' = Ac): $\text{MS(anh)-3P}_{\text{A-iQ-DAP}}$
2f (R = OH, R' = Glyc): $\text{MS(anh)-3P}_{\text{A-iQ-DAP}}$
2g (R = D-Ala, R' = Ac): $\text{MS(anh)-4P}_{\text{A-iQ-DAP-A}}$
2h (R = D-Ala, R' = Glyc): $\text{MS(anh)-4P}_{\text{A-iQ-DAP-A}}$

1k (R^1 = Glyc, R^2 = OH, R^3 = OH): $\text{MS-3P}_{\text{A-iE-DAP}}$
1l (R^1 = Ac, R^2 = NH_2, R^3 = OH): $\text{MS-3P}_{\text{A-iE-DAP(L-amide)}}$
1m (R^1 = Ac, R^2 = OH, R^3 = NH_2): $\text{MS-3P}_{\text{A-iE-DAP(D-amide)}}$
1n (R^1 = Ac, R^2 = NH_2, R^3 = NH_2): $\text{MS-3P}_{\text{A-iE-DAP(L,D-di-amide)}}$

Figure III-1. The synthetic *Mycobacterium* PGN fragments.

III-1 Human Nod1 activation of *Mycobacterium* PGN fragments

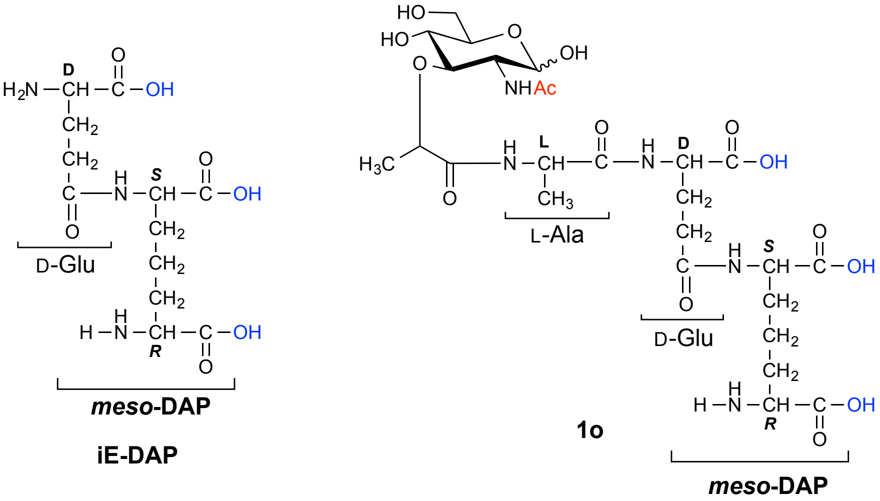
III-1-a HEG1G bioassay for human Nod1 activation

The human Nod1 assay for the synthesized PGN fragments of *Mycobacterium* was done with stably human *Nod1* transfected cells (HEG1G). The HEG1G cells were gifts from N. Inohara (University of Michigan Medical School, Michigan, US). HEG1G cells were HEK293T cells constitutively expressing human Nod1-FLAG and NF-κB-dependent GFP reporter plasmid, which were generated by transfection of pMSCV-puro-Nod1-FLAG and NF-κB-eGFP followed by antibody selection as described.[3]

III-1-b Human Nod1 activation by *Mycobacterium* PGN fragments

Although comprehensive study of the structure-activity relationships for Nod1 activation with the PGN fragments of *Mycobacterium* has not been performed, some related analysis were reported. Especially about the effect of the peptide structure, previous studies in our group have demonstrated that tripeptide A-iE-DAP (L-Ala-γ-D-glutamyl-*meso*-DAP) showed stronger hNod1 activity than A-iQ-DAP (L-Ala-γ-D-glutaminyl-*meso*-DAP),[4] indicating that the –COOH of glutamic acid was important for Nod1 recognition. Girardin group found that the amidation of the carboxyl group on the D-asymmetric carbon of DAP suppressed the Nod1 stimulation by using hydrolyzed PGN fragments from *B. subtilis*,[5] suggesting the modification relates to its immune evasion. Our group discovered that GlcNAc-(anh)MurNAc-L-Ala-D-isoGln-*meso*-DAP (L,D-di-amidated DAP) lost its Nod1 activity.[6] So the amidation of either Glu or DAP would not be tolerated with Nod1 activation. It was further shown that hNod1 recognizes tripeptide-containing (A-iE-DAP) natural PGN fragments having disaccharide (GlcNAc-(anh)MurNAc) or monosaccharide (MurNAc), but very weakly responses to their corresponding tetrapeptide-containing ones.[7] *Mycobacterium* PGN fragments have the combination of these structural modifications, and the comprehensive analysis would reveal one side of the PGN functions of the pathogenic bacteria.

In the assay, **iE-DAP** and **1o**, regularly obtained in Gram-negative bacteria such as *Escherichia coli*, were used as the representative Nod1 ligands for the reference. Their structures are shown in Fig. III-2. Compound **1o** had been shown to have more potent activity than **iE-DAP** with the transiently h*Nod1* transfected cells.[7]



iE-DAP **1o**

Figure III-2. Structures of **iE-DAP** and **1o**. (PGN fragment structures in regular Gram-negative bacteria such as *Escherichia coli*).

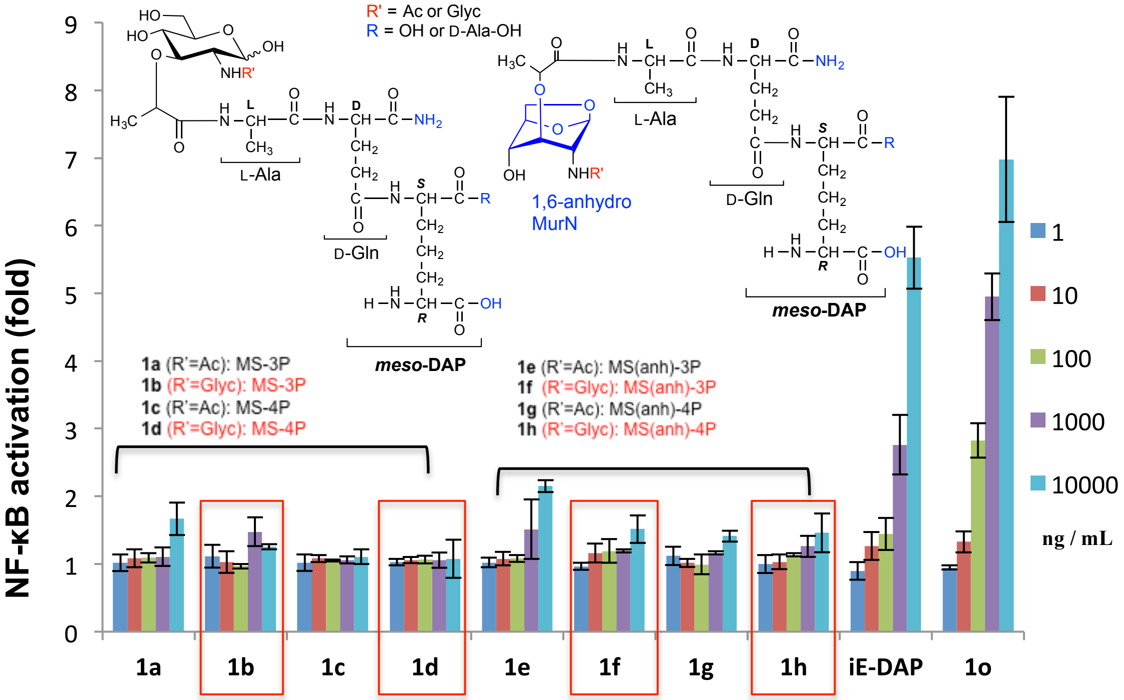


Figure III-3. Stimulation of hNod1 by *Mycobacterium* PGN fragments with monosaccharides. HEK293T cells were stably transfected with human-*Nod1*, and the indicated amount of each compound was added to the cells and the ability of each compound to activate NF-κB was determined spectrophotometrically at 535 nm.

Fig. III-3 displayed the result of hNod1 activation with monosaccharides containing PGN fragments. The compounds **1a-1d** have regular muramyl structure, and

1e-**1f** have 1,6-anhydro-muramyl group. In comparison with **iE-DAP** and **1o**, the second amino acid, D-Glu, of all the compounds was amidated, and they showed reduced hNod1 activation. The compounds containing glycolyl group and tripeptide (**1b** and **1f**), exhibited slightly lower activity than the corresponding acetyl ones (**1a** and **1e**), while the ones containing glycolyl group and tetrapeptide (**1d** and **1h**) did not show clear difference from their acetyl counterparts (**1c** and **1g**). For the acetyl-muramyl compounds (**1a** vs **1c, 1e** vs **1g**), it was shown that the tripeptide bearing **1a** and **1e** had stronger activity than their tetrapeptide containing **1c** and **1g** at 1000 ng/mL and 10000 ng/mL, but this tendency was not so obvious for the glycolyl-muramyl structures. The author also found that the anhydro-structures were slightly more potent than regular muramic acid structures. The above results demonstrated that amidation of D-Glu in *Mycobacterium* PGN fragments strongly weakened their hNod1 recognition, and glycolyl modification at the muramic acid slightly reduced the hNod1 activity.

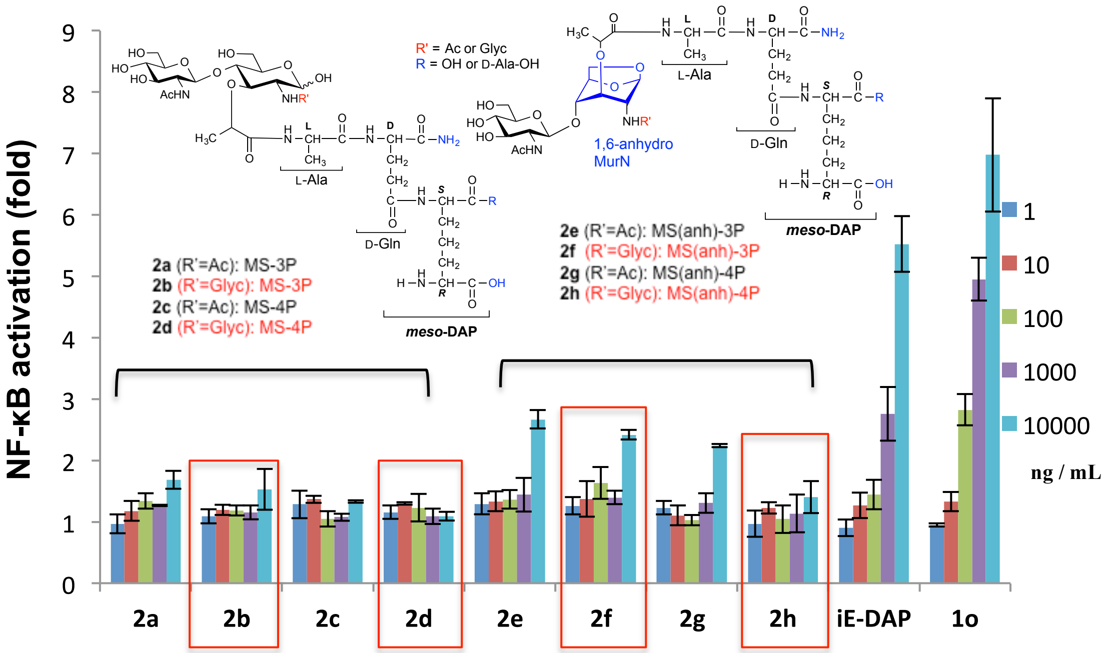


Figure III-4. Stimulation of hNod1 by *Mycobacterium* PGN fragments with disaccharides. HEK293T cells were stably transfected with human-*Nod1*, and the indicated amount of each compound was added to the cells and the ability of each compound to activate NF-κB was determined spectrophotometrically at 535 nm.

Fig. III-4 showed the result of hNod1 activation with disaccharide containing PGN fragments. **2a**-**2d** contain GlcNAc-MurN structure, and **2e**-**2f** have GlcNAc-(1,6-anhydro)MurN motif. They manifested similar tendency to monosaccharide containing fragments, and the amidation at D-Glu reduced the hNod1 activation. Glycolyl group containing structures (**2b**, **2d**, **2f** and **2h**), enclosed in red boxes, showed slightly weaker potency than the corresponding acetyl ones (**2a**, **2c**, **2e** and **2g**). It was very obvious that the tripeptide containing compounds, either with acetyl group or glycolyl group, were better ligands for huam Nod1 than the tetrapeptide containing ones. Similarly, the 1,6-anhydro-structure could improve their recognition by hNod1. When taking the compounds with different lengths of glycan chain into comparison, as shown in Fig. III-5 (**1a** vs **2a**, **1b** vs **2b**, **1e** vs **2e** and **1f** vs **2f**), the author observed that elongation of the glycan chain with GlcNAc could enhance the hNod1 recognition for the 1,6-hydro-muramyl carrying structures (**2e** and **2f**), but had little impact on the regular muramyl containing ones (**2a** and **2b**).

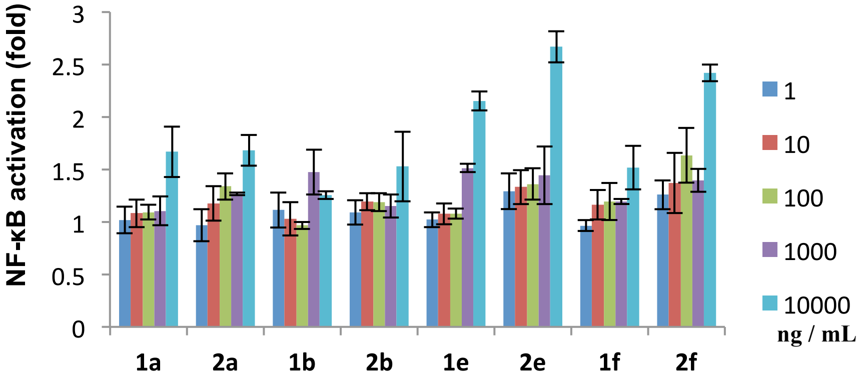


Figure III-5. hNod1 activation by *Mycobacterium* PGN fragments with different lengths of glycan chain. HEK293T cells were stably transfected with human-*Nod1*, and the indicated amount of each compound was added to the cells and the ability of each compound to activate NF-κB was determined spectrophotometrically at 535 nm.

In order to elucidate the effect of amidation modifications on hNod1 activation, the author continued to study the interaction between compounds **1l**, **1m**, **1n** and hNod1. The activity of **1k** (MurNAc-L-Ala-D-isoGlu-*meso*-DAP) was also evaluated. For easier comparison, the results were summarized as shown in Fig. III-6. Changing the acetyl group (**1o**) to glycolyl group (**1k**) could slightly reduce the hNod1 activation. Compared with **1o**, the amidated *meso*-DAP containing monosaccharides **1l**, **1m** and **1n** showed weaker activities with different reduced extent. The potency order was L-amidated > D-amidated > L,D-amidated, implying that the D-COOH was more important than the L-COOH for hNod1 sensing. Taking **1a** together, it was very evident that the amidations at either the second amino acid (D-Glu) or the third amino acid (*meso*-DAP) entity were not favorable for hNod1 stimulation.

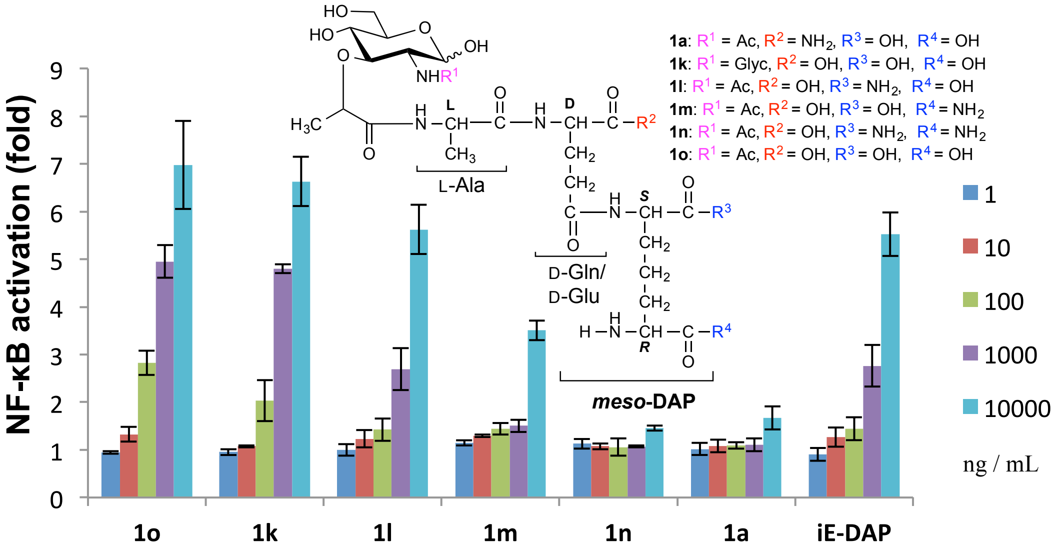


Figure III-6. Stimulation of hNod1 by *Mycobacterium* PGN fragments with various modifications. HEK293T cells were stably transfected with human-*Nod1*, and the indicated amount of each compound was added to the cells and the ability of each compound to activate NF-κB was determined spectrophotometrically at 535 nm.

III-2 Human Nod2 activation of *Mycobacterium* PGN fragments

III-2-a HEK293T bioassay for human Nod2 activation

The human Nod2 activity of the synthesized PGN fragments of *Mycobacterium* was analyzed with using the transiently transfected human embryonic kidney (HEK) 293T cells with human *Nod2*.

III-2-b Human Nod2 activation by *Mycobacterium* PGN fragments

Nod2 is believed to elicit broad recognition of bacteria, because its minimum ligand MDP is presenting universally in the PGNs from both Gram-positive and Gram-negative bacteria. Nod2 also detects MDP containing structures that have L-lysine or L-ornithine at the third amino acid, but the activity decreased as the glycan and peptide chain lengths increased.[5,8] Behr group showed that the synthetic *N*-glycolyl MDP was more potent than *N*-acetyl MDP in hNod2 activation both *in vitro* and *in vivo*,[9] while Coggeshall group observed that *N*-glycolyl MDP exhibited slightly reduced hNod2 activation than *N*-acetyl MDP at 0.1 μg/ml with stably human *Nod2* transfected cells.[10] However, there were no reports about the hNod2 actvition of *Mycobacterium* PGN fragments with D-isoGln and *meso*-DAP, as the major fragment structures released to the environment in the biosynthetic system. The author's work would reveal the immunomodulatory circumstances of DAP type PGN-containing bacteria including *Mycobacterium*.

In the assay, **MDP** and **1j** (MurNAc-L-Ala-D-isoGln-L-Lys) were used as the representative Nod2 ligands as the references. The structures of **MDP**, *N*-glycolyl **MDP** and **1j** are shown in Fig. III-7. The synthetic *meso*-DAP containing *Mycobacterium* PGN fragments (**1a**-**1d**) showed very weak stimulating ability to hNod2, firmly illustrating that *meso*-DAP moiety decreases the Nod2 activation dramatically. Moreover, the 1,6-anhydro muramyl group containing structures had no activation to hNod2 (Fig. III-8).

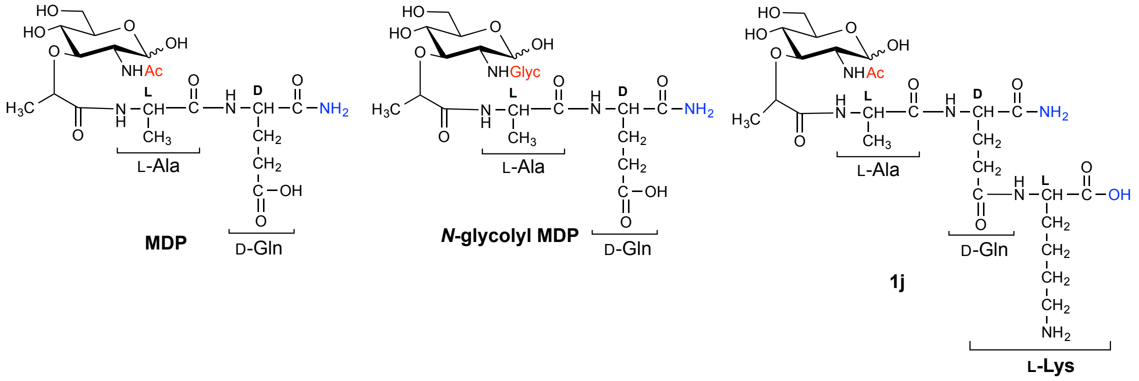


Figure. III-7. Structures of **MDP**, *N*-glycolyl **MDP** and **1j**.

As for the impact of glycolylation (compounds in the red boxes in Fig. III-8), *N*-glycolyl MDP showed lower hNod2 activity than MDP, which was not consistent with the reported results from Behr group,[9] and similar to the results from Coggeshall group.[10] In *meso*-DAP containing structures, *N*-glycolyl mutant with monosaccharide (**1b**) showed slightly reduced hNod2 activities than its corresponding *N*-acetyl one (**1a**). The tripeptide containing structures manifested a little stronger potency than the tetrapeptide containing ones (**1a** vs **1c**, **1b** vs **1d**). The same tendencies were found in disaccharide containing structures as well as shown in Fig. III-9, decreased performance of glycolyl group was also observed from the tripeptide-containing fragments (**2a** vs **2b**). In the released fragment structures during PGN biosynthesis, tri- or tetrapeptide-containing fragments often appeared more than dipeptide-containing fragments, and the high potency of the dipeptide-containing fragment such as MDP and *N*-glycolyl MDP may be compromised by others *in vivo*. Thus these *meso*-DAP containing bacteria such as *Mycobacterium* modulate the host defense via hNod2 activation.

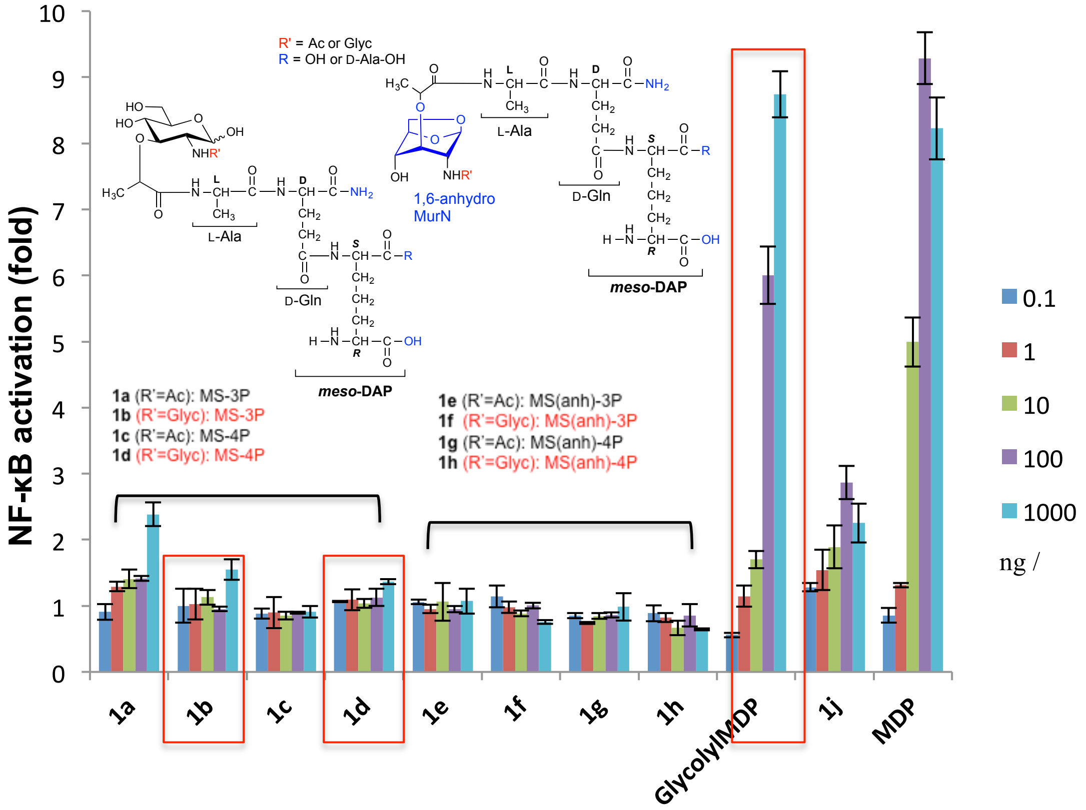


Figure III-8. Stimulation of hNod2 by *Mycobacterium* PGN fragments with monosaccharides. HEK293T cells were transiently transfected with human-*Nod2*, and indicated amount of each compound was added to the cells and the ability of each compound to activate NF-κB was determined by luciferase reporter assay.

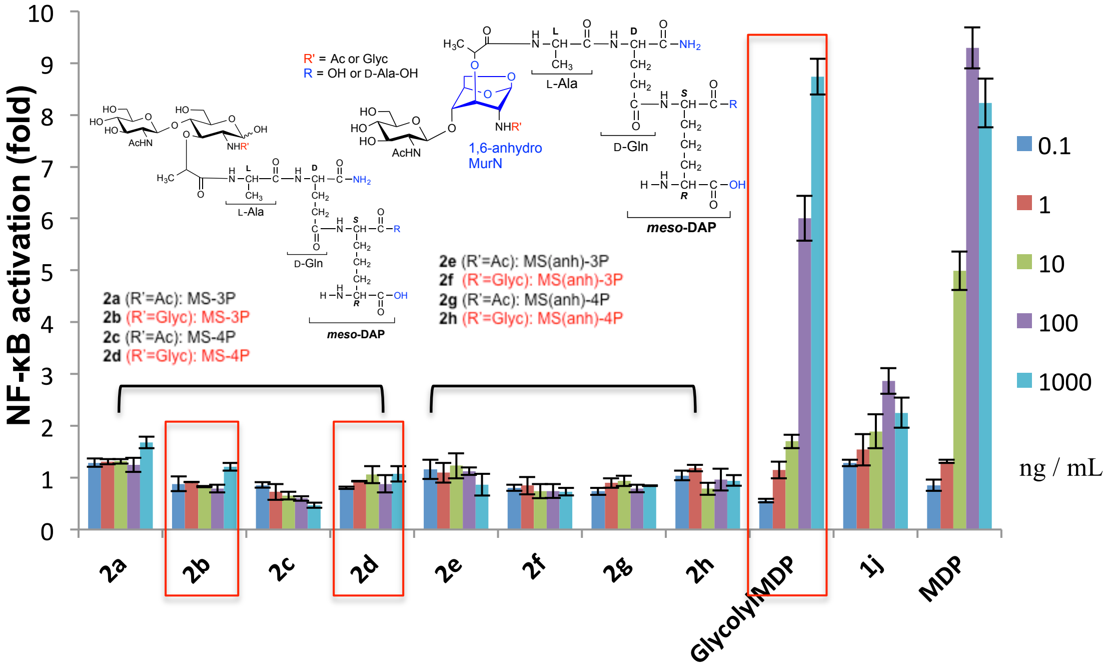


Figure III-9. Stimulation of hNod2 by *Mycobacterium* PGN fragments with disaccharides. HEK293T cells were transiently transfected with human-*Nod2*, and indicated amount of each compound was added to the cells and the ability of each compound to activate NF-κB was determined by luciferase reporter assay.

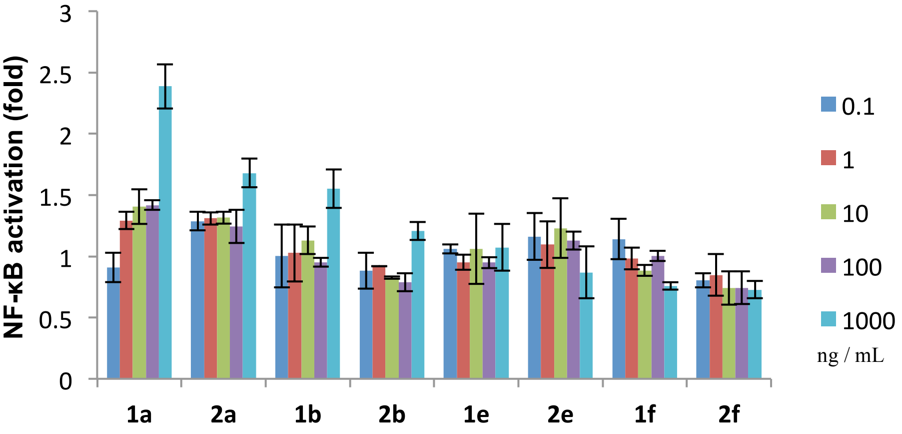


Figure III-10. Stimulation of hNod2 by *Mycobacterium* PGN fragments with different lengths of glycan chain (Mono- and Disaccharides). HEK293T cells were transiently transfected with human-*Nod2*, and indicated amount of each compound was added to the cells and the ability of each compound to activate NF-κB was determined by luciferase reporter assay.

The mono- and disaccharides with the same peptide part were also compared to see the effect of glycan chain on hNod2 activation (Fig. III-10). For the regular muramyl group containing compounds, the prolonged glycan chain showed reduced activity (**1a** vs **2a**, **1b** vs **2b**), whereas no hNod2 activation was observed in the 1,6-anhydro muramyl group containing structures (**1e**, **1f**, **2e** and **2f**).

III-3 Summary

In this chapter, the author analyzed the modulation of human innate immune system via the cytosolic receptor proteins hNod1 and hNod2 by the synthesized *Mycobacterium* PGN fragments.

In the hNod1 and hNod2 stimulation assays, most of the comprehensively prepared *Mycobacterium* PGN fragment structures with mono- and disaccharides, which can be released to the environment of the bacteria, showed weaker activities.

for hNod1: Amidation at either D-Glu or *meso*-DAP residues reduced hNod1 activation and glycolylation also slightly suppressed hNod1 recognition, while 1,6-anhydro containing structures and longer glycan chains showed slightly higher hNod1 activation.

for hNod2: DAP moiety and glycolylation reduced the hNod2 activation,
longer glycan chains also showed weaker hNod2 stimulating abilities,
1,6-anhydro containing structures were not hNod2 ligands.

This is the first demonstration that *Mycobacterium* PGN fragments exhibited very weak hNod1 and hNod2 activation, which provides a proof for the cytosolic immune evasion of *M. tuberculosis*. Amidation is responsible for the reduced hNod1 responses, which is consistent with previous results of the PGN fragments with similar structural motif. On the other hand, the glycolylation modification did not show significant effect on the immunity in the presence of *meso*-DAP, and glycolylated PGN fragments showed only slightly weaker activities than the acetylated ones. The glycolyl group perhaps contributes more to maintain the integrity of the cell wall and improve the resistance to lysozyme, which is also an important element for bacterial survival.

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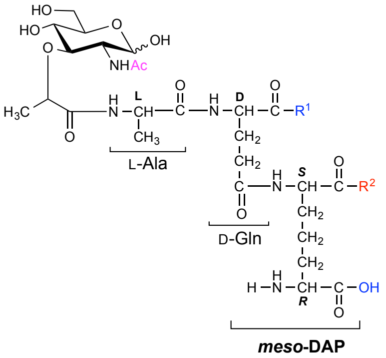
Chapter IV Binding affinity of peptidoglycan to the extracellular PASTA domains of *Mycobacterial* Ser/Thr protein kinase PknB

PknB is one of the most widespread eukaryote-like Ser/Thr protein kinases in *M. tuberculosis*, which is in the regulation of cell growth and cell division through phosphorylation of proteins involved in these processes as described in Chapter I. The kinase function of PknB is triggered by the stimuli recognized by the extracellular domain of the kinase. The extracellular region is composed of four PASTA domains, which was proved to strongly bind to *meso*-DAP containing chemically synthetic muropeptides (Fig. IV-1) by Husson group.[1] They suggested that this interaction was required for localizing PknB to the sites where active PGN hydrolysis and synthesis occur in *M. tuberculosis*. This was the first evidence for the direct binding between muropeptides and PASTA domains of PknB.



	K_D (μM)
5: **R**^{1} = NH_2, **R**^{2} = OH	21.8
6a: **R**^{1} = NH_2, **R**^{2} = D-Ala-OH	12.7
6b: **R**^{1} = OH, **R**^{2} = D-Ala-NH_2	>100
6c: **R**^{1} = NH_2, **R**^{2} = D-Ala-NH_2	14.9
6d: **R**^{1} = OH, **R**^{2} = D-Ala-OH	53.6

Figure IV-1. Structures and K_D values of muropeptides used in previous binding assay.[1]

The previous binding assay was done by using PGN fragments with monosaccharide (Fig. IV-1), having very limited variations. The K_D values were obtained with SPR (Surface Plasmon Resonance) method. Husson group and our group have collaborated to investigate the binding affinity of PknB extracellular PASTA domains with more diverse PGN structures. The author thus started the synthesis of various *Mycobacterium* PGN structures as shown in Chapter II, which include disaccharide muropeptides, 1,6-anhydromuramyl containing muropeptides and especially the unusual glycolyl modified muropeptides (Fig. IV-2). The author then conducted the binding assay with Octet red system based on BLI (Bio-Layer Interferometry).

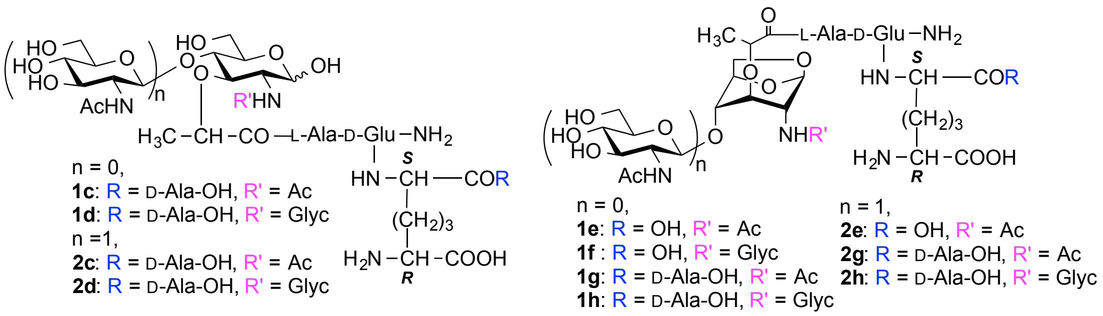


Figure IV-2. Structures of PGN fragments used in the binding assay with PknB.

IV-1 BLI (Bio-Layer Interferometry)[2]

BLI is an optical analytical technique that measures interference patterns between waves of light. White light goes down the fiber-optic biosensor to two interfaces separated by a thin layer at the tip of the sensor: a biocompatible layer on the surface and an internal reference layer (Fig. IV-3). Light is reflected from each of the two layers, and two reflected beams interfere constructively or destructively with each other. This interference pattern is detected at the CCD array detector. When the tip of a biosensor is dipped into either ligands (the first binder) or analytes (the second binder), thickness of the biocompatible layer is increased, and consequently, the interference pattern of the reflected lights is changed. This change is monitored at the detector, and reported on a sensorgram as a change in wavelength (nm shift). In other words, the response intensity

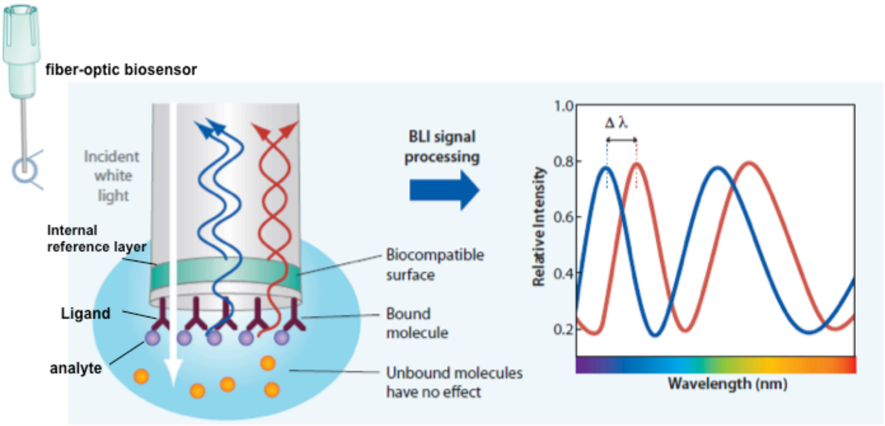
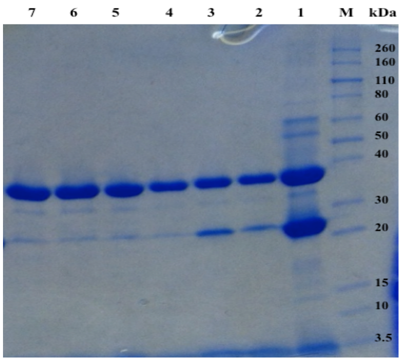


Figure IV-3. Working mechanism of BLI.[2] Reprinted with permission from FortéBio *Application Note 14*. Copyright © (2013) Pall Corporation.

of BLI is proportional to the size of ligands and analytes. The same as SPR, BLI is a label free analysis method, and a variety of biosensors are available. Because of the small molecular weight of the analytes (synthetic PGN fragments), high density “Super Streptavidin” (SSA) biosensor and biotinylated protein were chosen in the author’s experiment, utilizing the strong affinity between streptavidin and biotin.

IV-2 Preparation of Extracellular Domain of PknB (ED-PknB)

First, the extracellular domain of PknB (ED-PknB), which contains four PASTA domains, was prepared for the binding analysis with PGN fragment structures of *Mycobacteria*. Its purity was checked by SDS-PAGE (Fig. IV-4). The ED-PknB was then biotinylated with biotinylation reagents (EZ-Link NHS-PEG$_4$-Biotin) in 1:1 molar ratio to the protein, in order to avoid overbiotinylation, because there are 11 lysine residues in the ED-PknB as shown in Fig. IV-5.



M: Marker (Novex® Sharp Unstained Protein Standard);

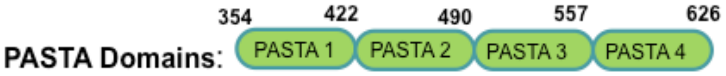
Lane 1: after Thrombin digestion;

Lane 2-3: 1st time GST removal;

Lane 4-5: 2nd time GST removal;

Lane 6-7: 3rd time GST removal (pure ED-PknB)

Figure IV-4. SDS-PAGE gel of purification of ED-PknB.



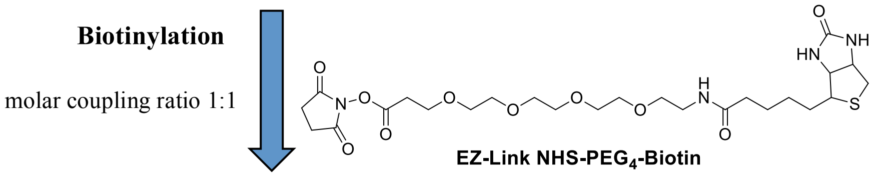
GS ^{354}GGITRDVQVPDVRGQSSADAIATLQNRGFKIRTLQKPDSTIPPDHVIGTDPAANTS

VSAGDEITVNVS ^{422}TGPEQREIPDVSTLTYAEAVKKLTAAGFGRFKQANSPSTPELVGK

VIGTNPPANQTSAITNVVIIIVG ^{490}SGPATKDIPDVAGQTVDVAQKNLNVYGFTKFSQAS

VDSPRPAGEVTGTNPPAGTTVPVDSVIELQVS ^{557}KGNQFVMPDLSGMFWVDAEPRLRA

LGWTGMLDKGADVDAGGSQHNRVVYQNPPAGTGVNRDGIITLRFG ^{626}Q



Biotinylated ED-PknB

Figure IV-5. Preparation of Biotinylated ED-PknB.

IV-3 BLI binding assays and kinetic analysis

Binding affinity between ED-PknB (PASTA domains) and PGN fragments were performed with BLI system on Octet RED384 instrument (ForteBio). Double references were used to obtain precise data; 1) non-protein loading reference biosensors with the same protocol as the protein-loaded biosensors to remove systematic artifacts, 2) protein-loaded biosensors with only the buffer solution instead of the analytes solution to correct signal drift.[3] The raw data was processed with "double reference subtraction" before data analysis. The binding constants of all compounds were calculated by using the 2:1 (Heterogeneous Ligand) Model in the "ForteBio Data Analysis 8.1".

IV-4 Binding affinity of PGN fragments for the ED-PknB (PASTA domains)

The BLI analysis of the synthetic PGN fragments was performed and the K_D values were shown in Fig. IV-6 and IV-7, and their sensorgrams are shown in the last part of this chapter. Fig. IV-6 gave the K_D values of regular muramyl group containing PGN fragments. Glycolyl group slightly reduced the affinity for ED-PknB in monosaccharides (**1c** vs **1d**), whereas resulted in 14-fold decrease weaken in disaccharides (**2c** vs **2d**). When taking the *N*-acetyl muramyl group containing compounds **1c** and **2c** into comparison, addition of the *N*-acetyl glucosamine

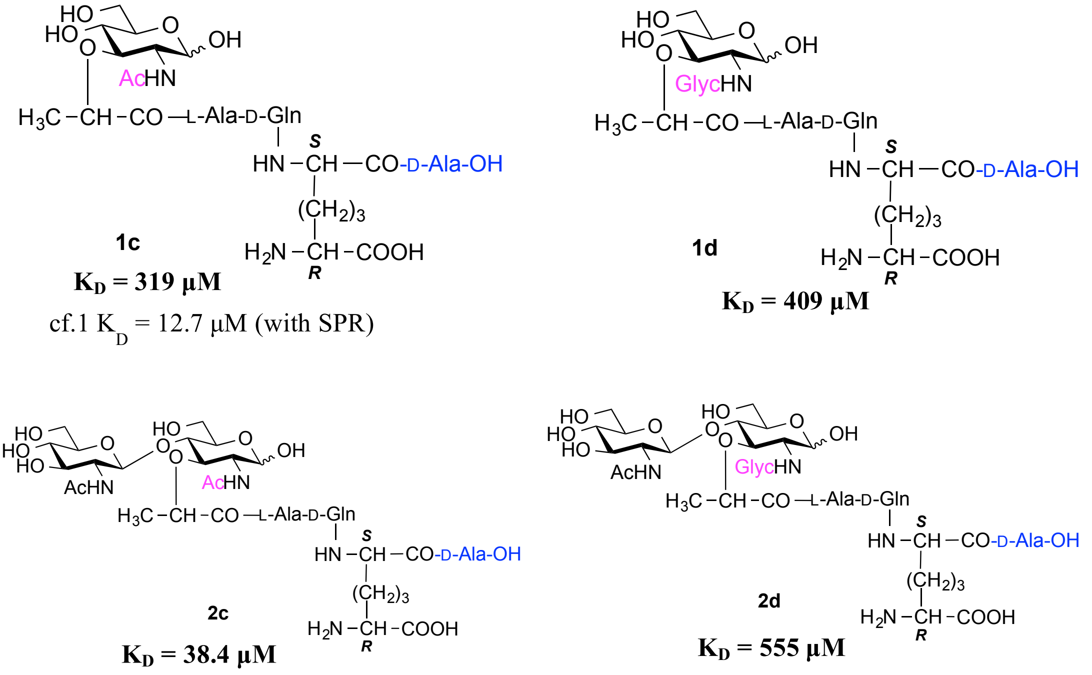


Figure IV-6. K_D values of PGN fragments with muramyl group.

in the glycan chain led to 8-fold increase in binding. The author also evaluated the ED-PknB binding affinity of 1,6-anhydro muramyl group containing structures as shown in Fig. IV-7, and found that the 1,6-anhydro moiety showed stronger interaction with the PASTA domains when compared with the non-anhydro analogues: **1g** bound 9-fold stronger than **1c**, and **2g** increased the binding by 3-fold of **2c**. It was also observed that the tripeptide containing structures exhibited much weaker binding abilities than their corresponding tetrapeptide containing ones: **1e** vs **1g**, **1f** vs **1h**, **2e** vs **2g**, indicating that D-Ala, the fourth amino acid in the peptide, was very important for the recognition between PGN fragments and protein. It was also revealed that the elongation of the glycan chain enhanced the binding, and **2g** showed the strongest affinity. In (anh)MurN containing structures, similar to the MurN containing compounds, the glycolylated ones also showed much more reduced affinity than the acetylated ones, **1f**, **1h** and **2h** in contrast to **1e**, **1g** and **2g**, respectively. Because the (anh)MurN structure is produced and used at certain stages of the biosynthetic process of the PGN, PknB might recognize the structural motif to control its functions.

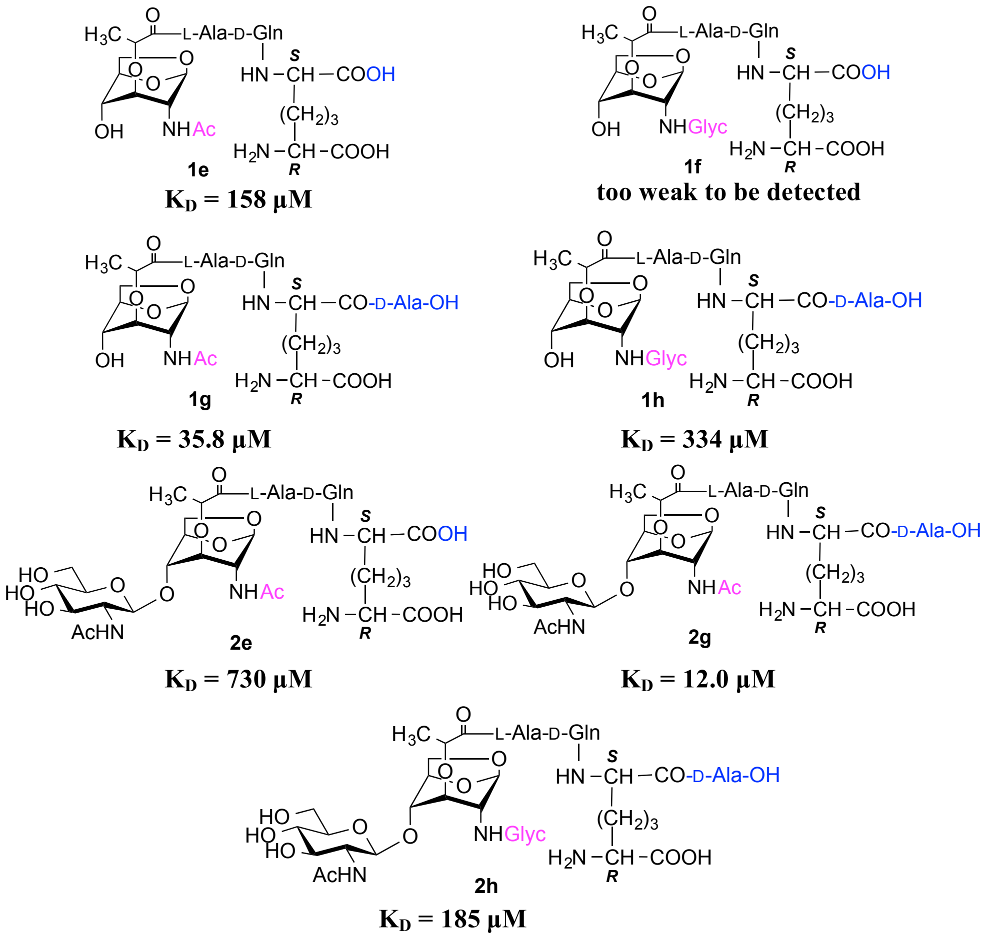


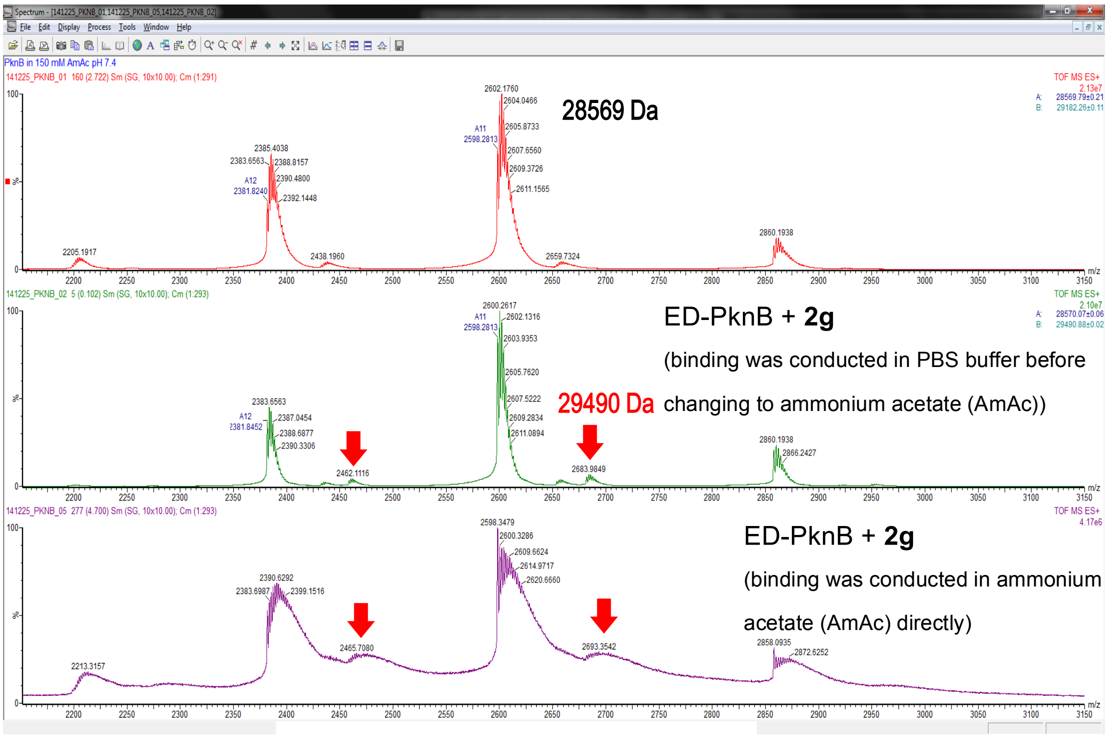
Figure IV-7. K_D values of PGN fragments with 1,6-anhydro muramyl group.

From the results of this assay with BLI method, MurNAc-L-Ala-D-Glu(NH_2)-*meso*-DAP **1c** showed K_D of 319 μM, although the K_D determined with SPR previously was 12.7 μM. A little different protein immobilization methods were used (for BLI, protein binds to the sensors via non-covalent bond between Streptavidin and Biotin, whereas for SPR, the interaction between protein and sensor chip was covalent amide bond), but the reason for the difference of the data is not clarified. The subsequent examinations are undertaken.
(*The sensorgrams for the compounds tested are given at the end of this Chapter*)

IV-5 Binding character analysis of 2g with ED-PknB (PASTA domains) by native Mass Spectrometry (nMS)

Native mass spectrometry (nMS) allows to detect proteins and protein-ligand complexes in their native or near-native states in solution,[4] which provides information about the composition, stoichiometry, and structural properties of protein complexes.[5] The theoretically unrestricted mass range of nMS allows detection of both small subunit and large complex.[6] Therefore, native MS has become a powerful structural biology technique.

For nMS analysis, in order to preserve the intact state and non-covalent interaction between protein and ligand, the protein complex are dissolved in aqueous solutions, mostly in ammonium acetate, and directly sprayed by ESI under highly controlled pressure by the system. The author collaborated with Dr. Ishii and Dr. Uchiyama from Okazaki Institute for Integrative Bioscience (OIIB)/Osaka Univ. to conduct the nMS analysis of ED-PknB and the complex of **2g**-ED-PknB as showed in Fig. IV-8. The molecular weight of **2g** is 920.9240 Da, and the molecular weight of protein is 28569 Da, the red arrow pointed out the complex peak, whose molecular weight was 29490 Da, suggesting that the binding molar ratio between **2g** and ED-PknB was 1:1.



Binding ratio ED-PknB : **2g** = 1:1

Figure IV-8. Native MS of ED-PknB (0.78 μM) and **2g**-ED-PknB (data from Dr. Ishii and Uchiyama (OIIB). The MS measurement proceeded in 150 mM ammonium acetate (pH 7.4) with SYNAPT G2-Si HDMS (WATERS).

IV-6 Summary

The synthetic *Mycobacterium* PGN fragments showed affinities to the PASTA domains of PknB from *Mycobacterium tuberculosis.* 1,6-Anhydro MurNAc and longer glycan chains had relatively higher binding potency, while MurNGlyc form showed reduced abilities than the regular MurNAc. D-Ala, as the fourth amino acid at the peptide stem, is one of the important structural motifs for the binding.

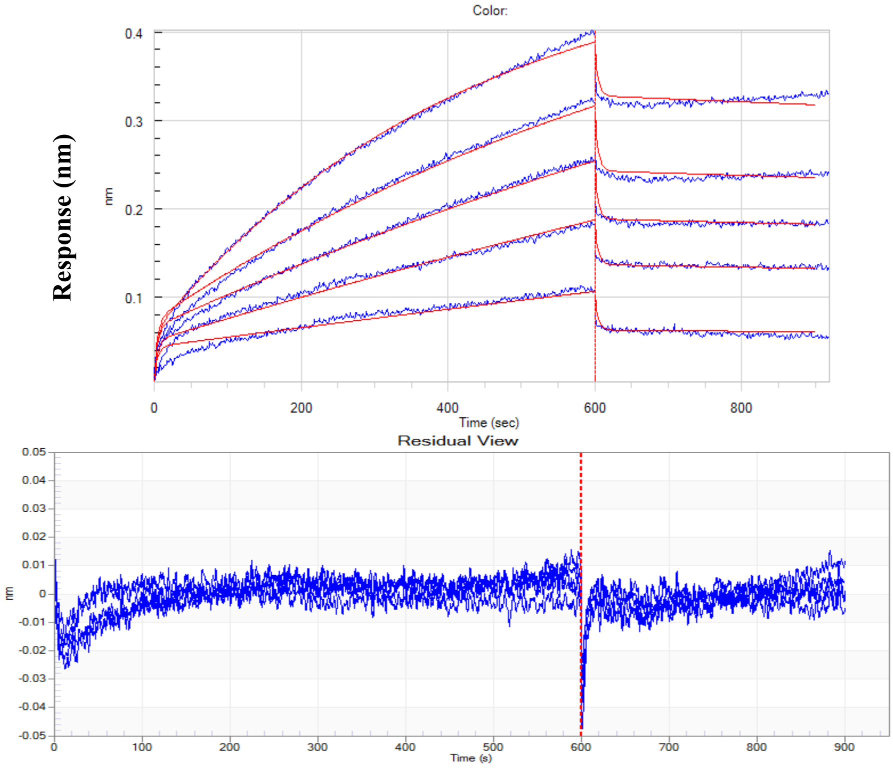
High ratio of glycolylation on MurN has been found in *Mycobacterium tuberculosis*, and on the other hand, PknB showed lower affinity to the glycolyl MurN containing structure than acetylated MurN (MuNAc). This difference might link to the function of PknB during the biosynthetic pathway of *Mycobacterium tuberculosis* PGN.

It was also obvious that the 1,6-anhydro MurNAc showed higher affinity to PknB than the regular MurNAc fragments. Because the 1,6-anhydro MurNAc structure is an important motif during the cell wall construction, the results suggested that PknB might modulate or recognize certain state of the cell wall synthesis.

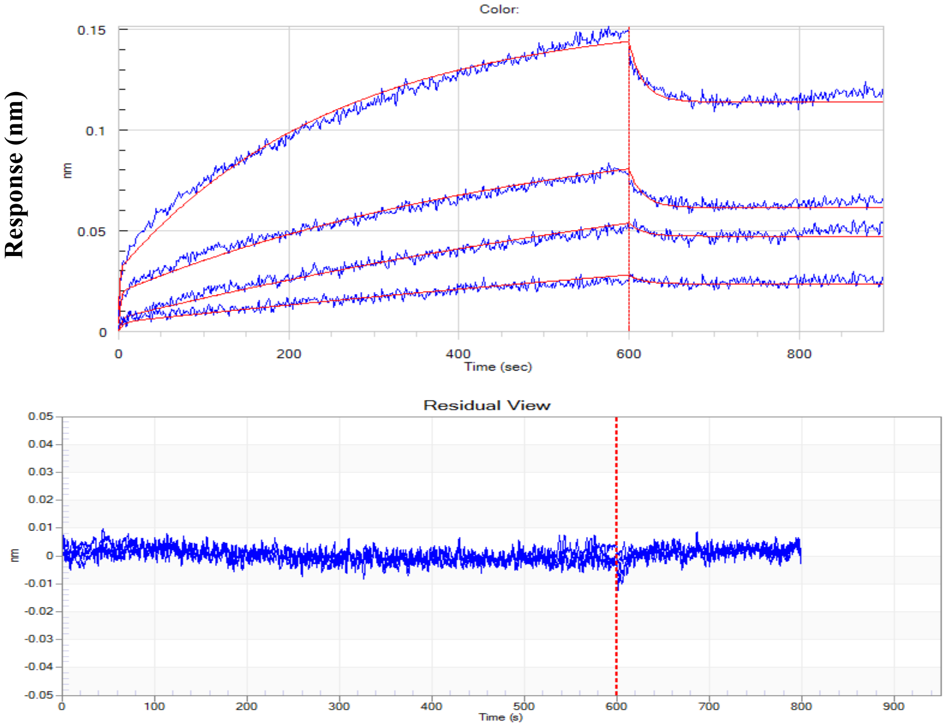
The genes coding PknB-like protein kinases are also found in many other Gram-positive bacteria,[7] and the results obtained in this research would also contribute to understand these similar proteins in bacteria.

The sensorgrams for the compounds tested

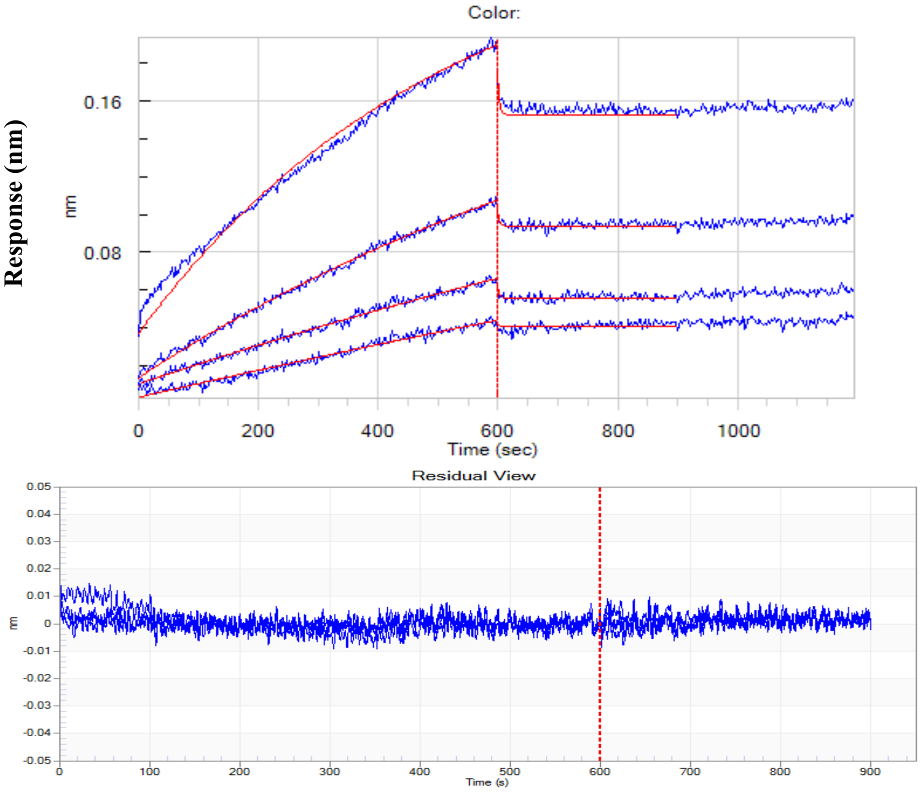
1) Compound **1c**



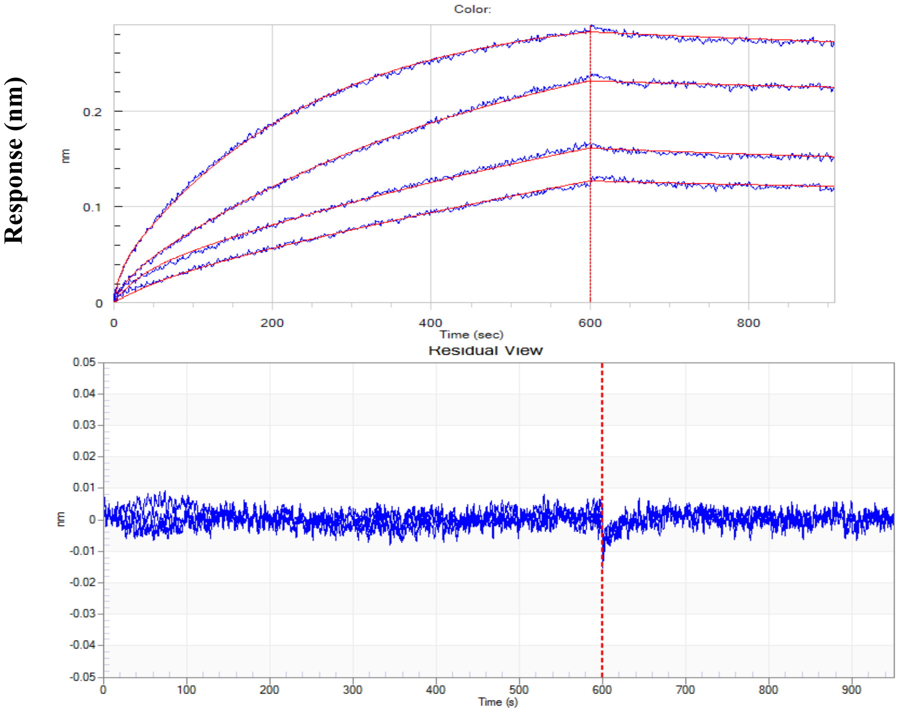
2) Compound **1d**



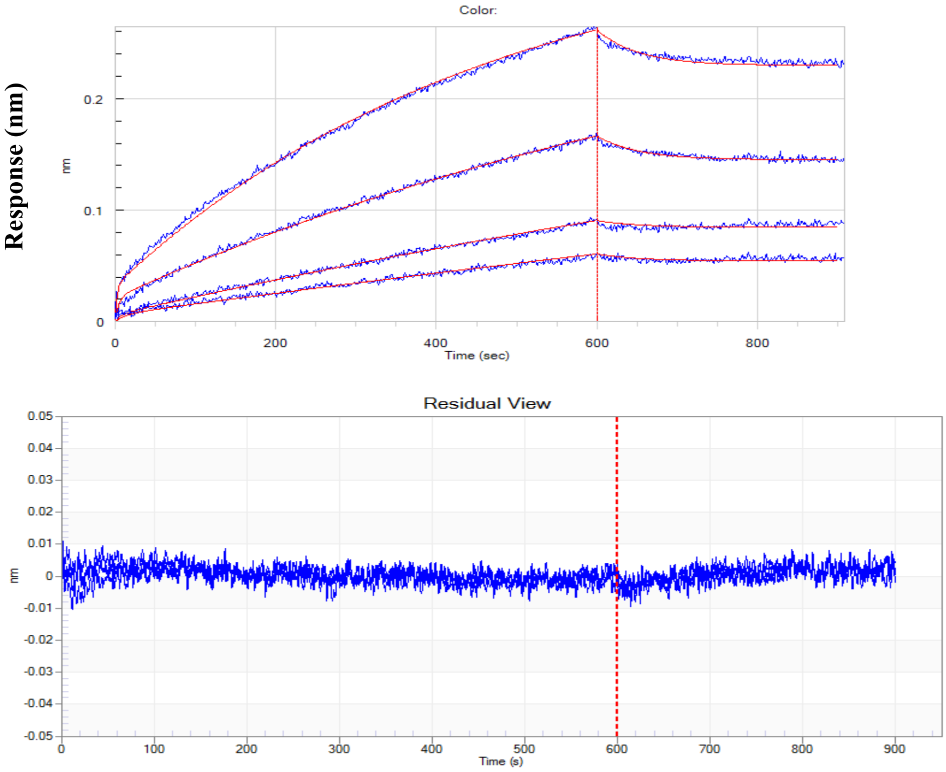
3) Compound **1e**



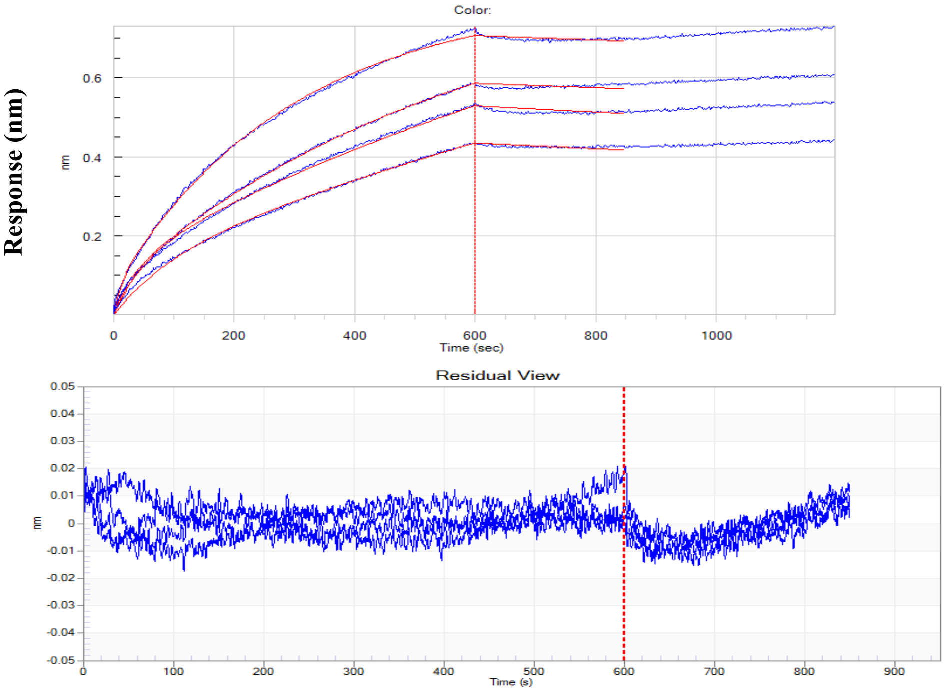
4) Compound **1g**



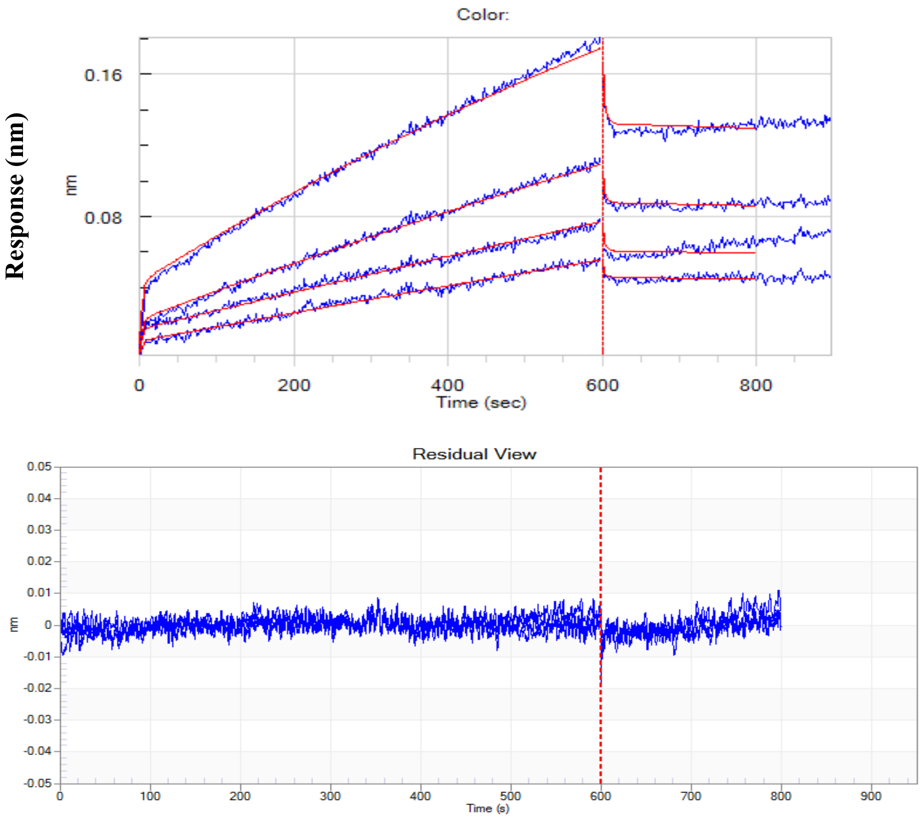
5) Compound **1h**



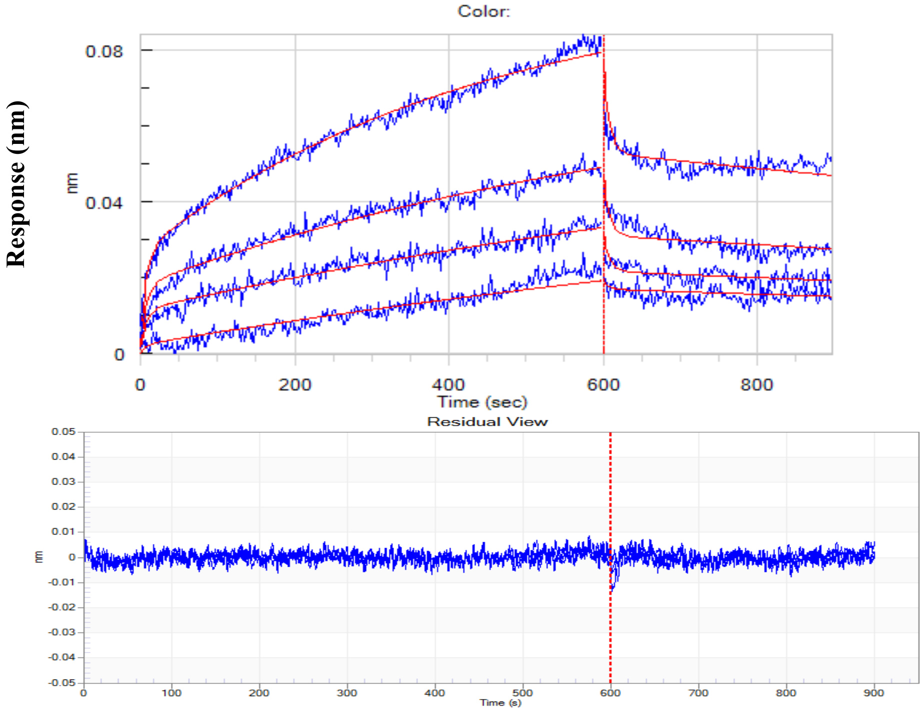
6) Compound **2c**



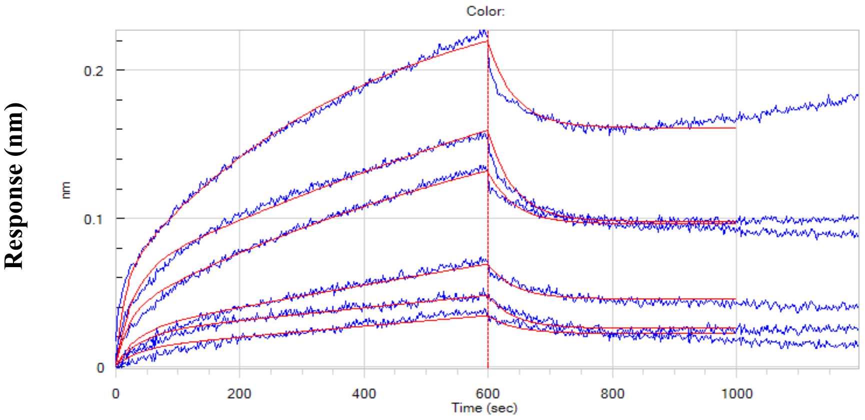
7) Compound **2d**

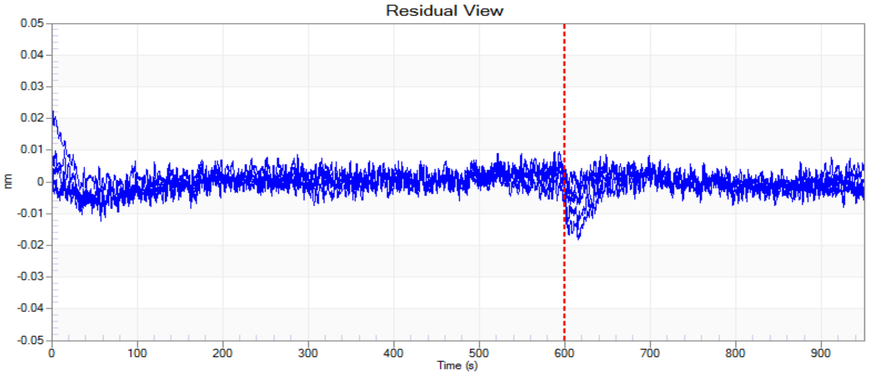


8) Compound **2e**

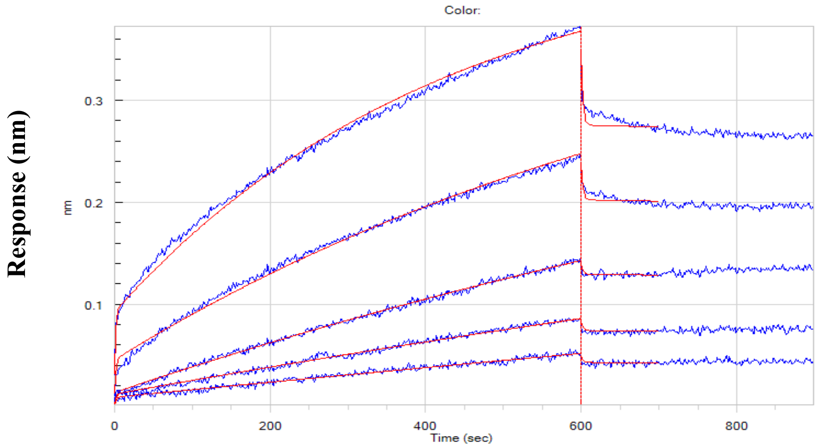


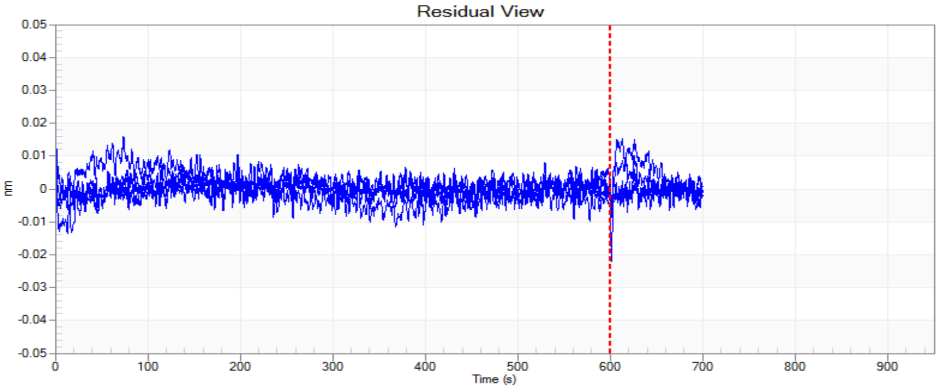
9) Compound **2g**





10) Compound **2h**





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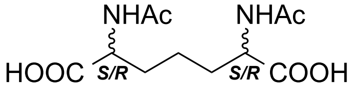
Experimental Section

General procedures: ^{1}H NMR and ^{13}C spectra were recorded in indicated solvents by using a JEOL ECS 400, or a JEOL ECA 500, or a Varian INOVA 700 spectrometers. The chemical shifts in $CDCl_3$ are given in δ values from tetramethylsilane as an internal standard. HRMS measurement was carried out by using Thermo Fisher Scientific ESI-LIT-orbitrap mass spectrometry. Silica-gel column chromatography was carried out by using Kieselgel 60 (Merck, 0.040 - 0.063 mm) at medium pressure (2-4 kg/cm^2). Reverse phase chromatography was carried out using Sephadex HP20 at atmospheric pressure. TLC analysis was performed on Silica-gel 60 F254 (Merck) and compound visualized by UV (254 nm), phosphomolybdic acid solution (5.0% in EtOH), 0.03% *p*-methoxybenzaldehyde in EtOH-conc. H_2SO_4-acetic acid buffer or 0.2% ninhydrin in EtOH-collidine-acetic acid buffer. Anhydrous CH_2Cl_2 was prepared by distillation from calcium hydride. Non-aqueous reactions were carried out under argon atmosphere unless otherwise noted. All other reagents and solvents used were purchased from commercial sources. Molecular sieves 4 Å were activated with microwave 1 min × 3 times before use. All reactions were performed at room temperature, unless otherwise stated.

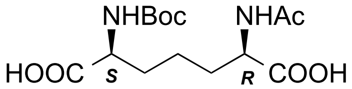


2,6-diacetamidoheptanedioic acid (21) 2,6-diaminopimelic acid **20** (20 g, 105.15 mmol) was dissolved in H_2O (1 L) and pyridine (180 mL). Then, acetic anhydride (150 mL) was added at 0 ^{o}C, the resulting clear solution was stirred overnight at room temperature. H_2O and most of the pyridine were evaporated in vacuo. The remaining pyridine and some other impurities were removed by flowing though the Dowex 50W × 8 strong acidic resin. The solution was neutralized with 2 M aqueous NH_3, followed by lyophilizing to give compound **21** as white solid (quant.).

^{1}H NMR (400 MHz, D_2O): δ 4.03-4.00 (2H, m; α$\underline{H}$ × 2), 1.93 (6H, s; -NHCOC$\underline{H}_3$ × 2), 1.77-1.51 (4H, m; 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.36-1.20 (2H, m; 4-C$\underline{H}_2$).

^{13}C NMR (100 MHz, D_2O): δ 180.0, 179.9, 174.3, 174.2, 55.9, 55.8, 31.9, 31.9, 22.8, 22.6, 22.5.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{11}H_{18}N_2O_6Na$: 297.1057 [M+Na]$^+$; found: 297.1060.



(2*S*,*6R*)-6-acetamido-2-((*tert*-butyloxycarbonyl)amino)heptanedioic acid (23a)

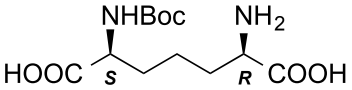
Compound **21** (27 g, 98 mmol) was dissolved in phosphate buffer (pH = 8.2, 0.05 M) to a final concentration as 0.05 M, heated to 40 °C in water bath, then L-aminoacylase (5.4 g) and $CoCl_2 \cdot 6H_2O$ (466 mg, 10^{-3} M) were added. The brown solution was stirred at 40 °C for 5 days. Activated carbon was added after cooling to room temperature, then warmed to 70 °C for 10 minutes. After filtration, the filtrate was evaporated to gain grey red solid.

Without any purification, the red solid was dissolved in H_2O (200 mL) and triethylamine (60 mL). Boc_2O (70 mL,) was dissolved to 1,4-dioxane (100 mL) before dropping into the water solution at 0 °C, stirred at room temperature and monitored by TLC until complete reaction. The mixture was adjusted to pH 5-6 with 2 M aqueous HCl and extracted with ethyl acetate to remove di-butoxycarbonated compound. Further adjusted to pH 2-3 and extracted with a large amount of ethyl acetate to get pure **23a**. The ethyl acetate layer was dried over Na_2SO_4 and concentrated in vacuo. The oily compound was resuspended to 50 mL water and neutralized with 2 M aqueous NH_3, then the solution was lyophilized to get compound **23a** as white solid (11.40 g, 35%, 2 steps).

^{1}H NMR (400 MHz, D_2O): δ 4.06 (1H, dd, J = 4.8 Hz, 8.7 Hz; -C$\underline{H}$(NHAc)-), 3.82 (1H, br s; -C$\underline{H}$(NHBoc)-), 1.94 (3H, s; -NHCOC$\underline{H}_3$), 1.78-1.48 (4H, m; 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.37-1.29 (11H, m; -OC(C$\underline{H}_3$)$_3$, 4-C$\underline{H}_2$).

^{13}C NMR (100 MHz, D_2O): δ 180.1, 179.5, 174.3, 158.3, 81.7, 56.2, 55.5, 32.0, 31.7, 28.4, 22.6.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{14}H_{24}N_2O_7Na$: 355.1476 $[M+Na]^+$; found: 355,1480.

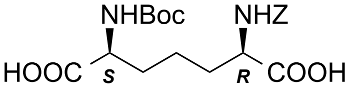


(2*S*,6*R*)-6-amino-2-((*tert*-butyloxycarbonyl)amino)heptanedioic acid (24)

D-aminoacylase (5.65 g) was dissolved to phosphate buffer (pH = 8.2, 0.05 M) in a conical flask, heated to 40 °C in water bath. After adding $CoCl_2 \cdot 6H_2O$ (1.62 g, 6.81 mmol), the flask was shaked by hand until the solid was dissolved completely, then **23a** (11.3 g) was added to the mixture. Without stirring, the mixture was reacted at 40 °C for 7 days. After that, activated carbon was added after cooling to room temperature, then warmed to 70 °C for 10 minutes. After filtration, the filtrate was concentrated, the residue was purified with HP20 to give compound **24** as purple solid (7.90 g, 80%).

^{1}H NMR (500 MHz, D_2O): δ 4.18 (0.2H, br s; -CH(NHAc)-), 3.93 (1H, br s; -CH(NHBoc)-), 3.78 (0.8H, m; -CH(NH_2)-), 2.02-1.67 (4H, m; 3-CH_2, 5-CH_2), 1.52-1.49 (11H, m, -OC$(CH_3)_3$, 4-CH_2).

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{12}H_{22}N_2O_6Na$: 313.1370 [M+Na]$^+$; found: 313.1372.

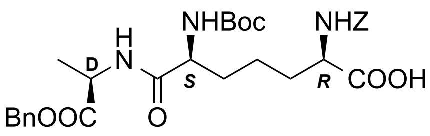


(2*S*,6*R*)-6-(((benzyloxy)carbonyl)amino)-2-((*tert*-butyloxycarbonyl)amino)heptanedioic acid (25) Compound **24** (7.5 g) and sodium carbonate (10.95 g) were dissolved to 250 mL water. Benzyl chloroformate was first dissolved in 1,4-dioxane (125 mL), and dropped to the reacting flask at 0 °C, reacted at 0 °C for 1h, then warmed to room temperature for another 3.5 h. The solution was extracted by ether once, the water layer was acidified to pH 2-3, and extracted with ethyl acetate, dried over Na_2SO_4. After concentration, the crude compound was purified by silica-gel flash column chromatography ($CHCl_3$:CH_3OH:CH_3COOH = 12:1:0.1) to give compound **25** as white solid (8.77 mg, 86%).

^{1}H NMR (400 MHz, CD_3OD): δ 7.36-7.25 (5H, m; ArH), 5.08 (2H, s; -CH_2Ph-), 4.15 (1H, dd, J = 4.6 Hz, 8.4 Hz; -CH(NHZ)-), 4.07 (1H, dd, J = 4.6 Hz, 8.2 Hz; -CH(NHBoc)-), 1.93-1.60 (4H, m; 3-CH_2, 5-CH_2), 1.54-1.42 (11H, m; -OC$(CH_3)_3$, 4-CH_2).

^{13}C NMR (100 MHz, CD_3OD): δ 176.1, 175.8, 158.6, 158.1, 138.2, 129.4, 128.9, 128.7, 80.5, 67.6, 55.2, 54.7, 32.4, 32.3, 28.7, 23.3.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{20}H_{28}N_2O_8Na$: 447.1738 [M+Na]$^+$; found: 447.1744.



(2*R*,6*S*)-7-(((*R*)-1-(benzyloxy)-1-oxopropan-2-yl)amino)-2-(((benzyloxy)carbonyl)amino)-6-((*tert*-butoxycarbonyl)amino)-7-oxoheptanoic acid (30)

Compound **25** (2.0 g, 4.71 mmol) was dissolved in 50% TFA in CH_2Cl_2 (30 mL) and the resulting reaction mixture was stirred for 30 min. After this time, the reaction mixture was concentrated and then co-evaporated with toluene. Addition of 1M HCl ether solution gave **27** as white solid, which was lyophilized in 1,4-dioxane. The white solid was dissolve in dry THF at 50 °C, followed by addition of triphosgene (1.54 g, 5.18 mmol) in dry THF. The clear solution was reacted at 50 °C for 6 h. After cooling to room temperature, the solvent was evaporated quickly, the oily solid **28** was washed by dry hexane twice. Without purification, the next step was proceeded immediately.

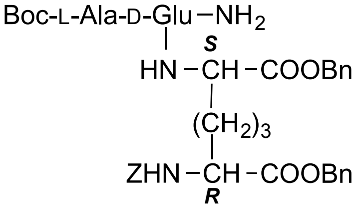
The above compound **28** was dissolved in dry DMF (60 mL) at 0 °C. HCl·H-D-Ala-OBn (3.05 g, 14.13 mmol) and triethylamine (2.6 mL) was dissolved into dry DMF (30 mL) before charging to the reacting flask. The mixture was reacted at 0 °C for 0.5 h, then was allowed to react at room temperature for 0.5 h. After removing DMF, the next step was proceeded directly.

The compound was dissolved in H_2O/ 1,4-dioxane (1:1, 80 mL), and triethylamine (4.8 mL) was added at 0 °C. Afterwards, di-*tert*-butyl dicarbonate (13 mL) was dropped into the solution. The solution was reacted at room temperature overnight. Then it was acidified to pH 2-3 and extracted with ethyl acetate, dried over Na_2SO_4. After concentration, the crude compound was purified twice by silica-gel flash chromatography, first eluted with $CHCl_3$:MeOH:CH_3COOH = 15:1:0.1, then eluted with $CHCl_3$:MeOH = 10:1 to give the pure compound **30** (606.8 mg, 22% for 5 steps).

^{1}H NMR (400 MHz, CD_3OD): δ 7.36-7.24 (10H, m; Ar$\underline{H}$), 5.17-5.08 (4H, m; -O-C$\underline{H}_2$-Ph × 2), 4.41 (1H, q, J = 7.3 Hz; D-Ala-α$\underline{H}$), 4.12 (1H, dd, J = 4.8 Hz, 9.0 Hz; -C$\underline{H}$(NHZ)-), 4.03 (1H, t, J = 4.8 Hz; -C$\underline{H}$(NHBoc)-), 1.88-1.42 (15H, m; DAP 3-C$\underline{H}_2$, 4-C$\underline{H}_2$, 5-C$\underline{H}_2$, -OC(C$\underline{H}_3$)$_3$), 1.37 (3H, d, J = 7.3 Hz; D-Ala-βC$\underline{H}_3$).

^{13}C NMR (100 MHz, CD_3OD): δ 175.9, 174.8, 173.7, 158.7, 157.7, 138.2, 137.3, 129.6, 129.5, 129.3, 129.2, 129.0, 128.8, 80.7, 67.9, 67.6, 55.8, 55.3, 33.2, 32.4, 28.7, 23.2, 17.4.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{30}H_{40}N_3O_9$: 586.2759 $[M+H]^+$; found: 586.2764.



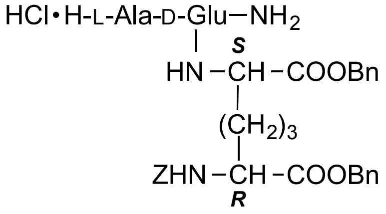
Fully-protected L-Ala-D-isoGln-*meso*-DAP (32)

Compound **26** (1.0 g, 1.65 mmol) was dissolved in 50% TFA in CH_2Cl_2 (20 mL) and the resulting reaction mixture was stirred for 30 min. After this time, the reaction mixture was concentrated and then co-evaporated with toluene. Addition of 1M HCl ether solution gave a white solid. The supernatant was decanted to give Boc-deprotected intermediate. WSCD·HCl (477 mg, 2.47 mmol), HOBt (334 mg, 2.47 mmol), and Boc-L-Ala-D-isoGln-COOH (785 mg, 2.47 mmol) were added to the above flask, and dissolved with anhydrous THF. Under Argon atmosphere, triethylamine (689 µL, 4.95 mmol) was syringed to the solution at 0 °C. The mixture was reacted overnight at room temperature. 10% citric acid solution was added to the mixture and extracted with ethyl acetate, the resulting organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 20:1) to give **32** as white solid (1.11g, 84%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.72 (1H, s; NH), 7.36–7.30 (16H, m; ArH, NH), 6.66 (1H, m; NH), 5.77 (1H, d, J = 7.0 Hz; NH), 5.55 (1H, m; NH), 5.16-5.06 (7H, m; -CH_2-Ph × 3, NH), 4.83 (1H, s; Gln-αH), 4.51 (1H, dd, J = 7.4 Hz, 12.5 Hz; DAP 2-H), 4.40 (1H, dd, J = 7.2 Hz, 12.2 Hz; DAP 6-H), 4.11 (1H, s; Ala-αH), 2.35-2.24 (2H, m; Gln-γCH_2), 2.10-2.05 (1H, m; Gln-βCH), 1.92 (1H, br s; Gln-βCH), 1.87-1.66 (4H, m; DAP 3-CH_2, 5-CH_2), 1.52-1.43 (11H, m; DAP 4-CH_2, -OC(CH_3)$_3$), 1.23 (3H, d, J = 6.9 Hz; Ala-βCH_3).

^{13}C NMR ($CDCl_3$, 125 MHz): δ 173.5, 173.1, 172.1, 156.1, 155.5, 136.2, 135.3, 135.2, 128.7, 128.6, 128.5, 128.5, 128.3, 128.2, 80.3, 67.4, 67.1, 67.1, 53.7, 52.6, 51.4, 50.8, 32.6, 31.7, 31.0, 30.5, 28.3, 21.2, 18.0.

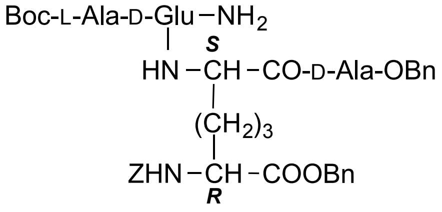
HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{42}H_{53}N_5O_{11}Na$: 826.3634 [M+Na]$^+$; found: 826.3646.



$$\begin{array}{l} \text{HCl}\cdot\text{H-L-Ala-D-Glu}-\text{NH}_2 \\ \qquad\qquad\quad | \;\; \textbf{\textit{S}} \\ \qquad\qquad \text{HN}-\text{CH}-\text{COOBn} \\ \qquad\qquad\qquad | \\ \qquad\qquad\quad (\text{CH}_2)_3 \\ \qquad\qquad\qquad | \\ \qquad\qquad \text{ZHN}-\text{CH}-\text{COOBn} \\ \qquad\qquad\qquad \textbf{\textit{R}} \end{array}$$

Protected H-L-Ala-D-isoGln-*meso*-DAP (34)

Compound **32** (800 mg, 1 mmol) was dissolved in TFA (3 mL) and the resulting reaction mixture was stirred for 30 min. After this time, the reaction mixture was concentrated and co-evaporated in toluene. Addition of 1M HCl ether solution gave a white solid. The supernatant was decanted to give Boc-deprotected intermediate **34**. The compound was used without further purification.



$$\begin{array}{l} \text{Boc-L-Ala-D-Glu}-\text{NH}_2 \\ \qquad\quad | \;\; \textbf{\textit{S}} \\ \qquad \text{HN}-\text{CH}-\text{CO-D-Ala-OBn} \\ \qquad\qquad | \\ \qquad\quad (\text{CH}_2)_3 \\ \qquad\qquad | \\ \qquad \text{ZHN}-\text{CH}-\text{COOBn} \\ \qquad\qquad \textbf{\textit{R}} \end{array}$$

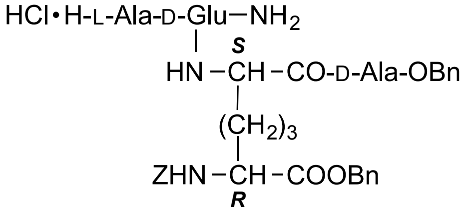
Protected L-Ala-D-isoGln-*meso*-DAP-D-Ala (33)

Compound **33** was synthesized from compound **31** with similar method to compound **32**. The crude product purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 30:1) to give **33** as white solid (450 mg, 65%).

^{1}H NMR (500 MHz, CD_3OD): δ 7.34–7.26 (15H, m; Ar$\underline{H}$), 5.18-5.04 (6H, m; -C$\underline{H}_2$-Ph × 3), 4.43 (1H, q, 7.3 Hz; D-Ala-α$\underline{H}$), 4.33 (1H, dd, J = 3.2 Hz, 10.9 Hz; Gln-α$\underline{H}$), 4.26 (1H, dd, J = 5.5 Hz, 7.6 Hz; DAP 2-$\underline{H}$), 4.17 (1H, m; DAP 6-$\underline{H}$), 4.00 (1H, q, 7.2 Hz; L-Ala-α$\underline{H}$), 2.32-2.22 (3H, m; Gln-βC$\underline{H}$, γC$\underline{H}_2$), 1.84-1.42 (16H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$, 4-C$\underline{H}_2$, -OC(C$\underline{H}_3$)$_3$), 1.37 (3H, d, J = 7.3 Hz; D-Ala-βC$\underline{H}_3$), 1.28 (3H, d, J = 7.2 Hz; L-Ala-βC$\underline{H}_3$).

^{13}C NMR ($CDCl_3$, 98.5 MHz): δ 174.2, 173.9, 173.7, 173.4, 172.6, 172.3, 156.5, 156.2, 137.9, 135.5, 135.4, 128.7, 128.6, 128.6, 128.5, 128.4, 128.2, 128.1, 128.1, 80.7, 67.2, 67.1, 67.0, 54.0, 53.5, 51.2, 50.7, 48.4, 31.6, 31.2, 31.1, 29.9, 28.3, 21.7, 17.7, 16.9.

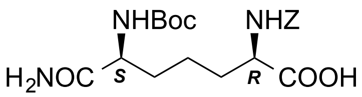
HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{45}H_{58}N_6O_{12}Na$: 897.4005 [M+Na]$^+$; found: 897.4016.



$$\begin{array}{l} \mathrm{HCl{\bullet}H\text{-}L\text{-}Ala\text{-}D\text{-}Glu{-}NH_2} \\ \qquad\qquad\qquad\;\; | \;\; \boldsymbol{S} \\ \qquad\qquad\quad \mathrm{HN{-}CH{-}CO\text{-}D\text{-}Ala\text{-}OBn} \\ \qquad\qquad\qquad\qquad | \\ \qquad\qquad\qquad\;\; \mathrm{(CH_2)_3} \\ \qquad\qquad\qquad\qquad | \\ \qquad\qquad\quad \mathrm{ZHN{-}CH{-}COOBn} \\ \qquad\qquad\qquad\qquad \boldsymbol{R} \end{array}$$

Protected H-L-Ala-D-isoGln-*meso*-DAP-D-Ala (35)

Compound **35** was synthesized from compound **33** with similar method to compound **34**. The compound was used without further purification.



7-amino-(2*R*,6*S*)-2-(((benzyloxy)carbonyl)amino)-6-((*tert*-butoxycarbonyl)amino)-7-oxoheptanoic acid (37) Compound **27** (80 mg, 0.222 mmol) was dissolve in dry THF at 50 °C, followed by addition of triphosgene (73 mg, 0.246 mmol) in dry THF. The clear solution was reacted at 50 °C for 5 h. After cooling to room temperature, the solvent was evaporated quickly, the oily solid **28** was washed by dry hexane twice. Without purification, the next step was proceeded immediately.

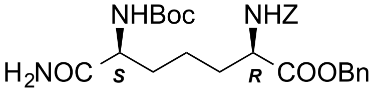
The above compound **28** was dissolved in dry 1,4-dioxane (4 mL) at 0 °C, then 0.5 M ammonia solution in 1,4-dioxane (2 mL) was added at 0 °C. The mixture was allowed to react at 0 °C for 30 min to give **36**. After removing solvent, the next step was proceeded directly.

Compound **36** was dissolved in H_2O/ DMF (1:2, 7.5 mL), and triethylamine (0.37 mL) was added at 0 °C. Afterwards, Di-*tert*-butyl dicarbonate (0.92 mL) was dropped into the solution. The solution was reacted at room temperature overnight. Then it was acidified to pH 2-3 and extracted with ethyl acetate, dried over Na_2SO_4. After concentration, the crude compound was purified by silica-gel flash chromatography, eluted with $CHCl_3$:MeOH:CH_3COOH = 15:1:0.1 to give the pure compound **37** (62 mg, 66% for 3 steps).

^{1}H NMR (400 MHz, CD_3OD): δ 7.37-7.26 (5H, m; ArH), 5.16-5.04 (2H, m; -O-CH_2-Ph), 4.13 (1H, dd, J = 4.8 Hz, 8.9 Hz; -CH(NHZ)-), 4.00 (1H, dd, J = 4.6 Hz, 8.5 Hz; -CH(NHBoc)-), 1.87-1.42 (15H, m; DAP 3-CH_2, 4-CH_2, 5-CH_2, -OC(CH_3)$_3$).

^{13}C NMR (100 MHz, CD_3OD): δ 177.8, 176.1, 158.7, 157.9, 138.2, 129.5, 129.0, 128.8, 80.6, 67.6, 55.6, 55.4, 33.1, 32.5, 28.7, 23.4.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{20}H_{29}N_3O_7Na$: 446.1898 $[M+Na]^+$; found: 446.1906.



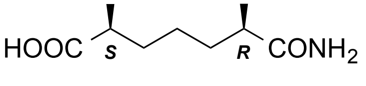
7-amino-benzyl-(2*R*,6*S*)-2-(((benzyloxy)carbonyl)amino)-6-((*tert*-butoxycarbonyl)amino)-7-oxoheptanoate (L-amide-*meso*-DAP) (38)

Compound **37** (55 mg, 0.13 mmol) and cesium carbonate (25 mg, 0.077 mmol) were dissolved to dry DMF (6 mL). 20 min later, benzyl bromide was added to the reacting flask at 0 °C, reacted at 0 °C for 1h, then reacted at room temperature overnight. The reation was quenched by water and extracted with ethyl acetate, dried over Na_2SO_4. After concentration, the crude compound was purified by silica-gel flash column chromatography ($CHCl_3$:CH_3OH = 35:1) to give compound **38** as white solid (36.5 mg, 55%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.36-7.29 (10H, m; ArH), 6.10 (1H, s; NH), 5.47 (1H, d, J = 7.6 Hz; NH), 5.41 (1H, s; NH), 5.21-5.06 (5H, m; -O-CH_2-Ph × 2, NH), 4.40 (1H, d, J = 6.2 Hz; -CH(NHZ)-), 4.05 (1H, s; -CH(NHBoc)-), 1.83-1.39 (15H, m; DAP 3-CH_2, 4-CH_2, 5-CH_2, -OC(CH_3)$_3$).

^{13}C NMR (100 MHz, $CDCl_3$): δ 174.2, 172.1, 156.0, 155.8, 136.2, 135.3, 128.7, 128.6, 128.4, 128.2, 128.1, 80.2, 67.2, 67.1, 53.6, 53.4, 32.2, 31.8, 28.3, 21.2.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{27}H_{35}N_3O_7Na$: 536.2367 $[M+Na]^+$; found: 536.2375.



7-amino-(2*S*,6*R*)-6-(((benzyloxy)carbonyl)amino)-2-((*tert*-butoxycarbonyl)amino)-7-oxoheptanoic acid (41)

Compound **24** (34 mg, 0.117 mmol) was dissolve in dry THF at 50 °C, followed by addition of triphosgene (38 mg, 0.129 mmol) in dry THF. The clear solution was reacted at 50 °C for 5 h. After cooling to room temperature, the solvent was evaporated quickly, the oily solid **39** was washed by dry hexane twice. Without purification, the next step was proceeded immediately.

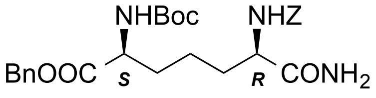
The above compound **39** was dissolved in dry 1,4-dioxane (4 mL) at 0 °C, then 0.5 M ammonia solution in 1,4-dioxane (1.2 mL) was added at 0 °C. The mixture was allowed to react at 0 °C for 30 min to give **40**. After removing solvent, the next step was proceeded directly.

Compound **40** and sodium carbonate (50 mg, 0.468 mmol) were dissolved to H_2O (3 mL). Benzyl chloroformate was first dissolved in 1,4-dioxane (3 mL), and dropped to the reacting flask at 0 °C, reacted at 0 °C for 1 h, then warmed to room temperature for another 1 h. The solution was extracted by ether once, the water layer was acidified to pH 2-3, and extracted with ethyl acetate, dried over Na_2SO_4. After concentration, the crude compound was purified by silica-gel flash column chromatography ($CHCl_3$:CH_3OH:CH_3COOH = 15:1:0.1) to give compound **41** as white solid (100 mg, 46% for 3 steps).

^{1}H NMR (400 MHz, CD_3OD): δ 7.36-7.26 (5H, m; ArH), 5.12-5.04 (2H, m; -O-CH$_2$-Ph), 4.09-4.01 (2H, m; -CH(NHBoc)-, -CH(NHZ)-), 1.83-1.42 (15H, m; DAP 3-CH$_2$, 4-CH$_2$, 5-CH$_2$, -OC(CH$_3$)$_3$).

^{13}C NMR (100 MHz, CD_3OD): δ 177.7, 177.4, 158.5, 158.1, 138.1, 129.5, 129.0, 128.9, 80.4, 67.7, 55.1, 33.0, 32.8, 28.7, 23.3.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{20}H_{29}N_3O_7Na$: 446.1898 $[M+Na]^+$; found: 446.1904.



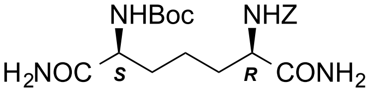
7-amino-benzyl-(2*S*,6*R*)-6-(((benzyloxy)carbonyl)amino)-2-((*tert*-butoxycarbonyl)amino)-7-oxoheptanoate (D-amide-*meso*-DAP) (42)

Compound **42** was synthesized from compound **41** with similar method to compound **38**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 30:1) to give compound **42** as white solid (70 mg, 62%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38-7.26 (10H, m; ArH), 6.11 (1H, s; NH), 5.40 (1H, s; NH), 5.32 (1H, s; NH), 5.21-5.10 (5H, m; -O-CH$_2$-Ph × 2, NH), 4.33 (1H, d, J = 5.9 Hz; -CH(NHBoc)-), 4.14 (1H, d, J = 4.8 Hz; -CH(NHZ)-), 1.88-1.38 (15H, m; DAP 3-CH$_2$, 4-C$\underline{H}_2$, 5-C$\underline{H}_2$, -OC(C$\underline{H}_3$)$_3$).

^{13}C NMR (100 MHz, CDCl$_3$): δ 173.6, 172.4, 156.3, 155.6, 136.2, 135.3, 128.7, 128.6, 128.4, 128.2, 128.1, 127.8, 80.2, 67.2, 54.1, 52.7, 32.4, 31.7, 28.3, 21.2.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{27}H_{35}N_3O_7Na$: 536.2367 [M+Na]$^+$; found: 536.2375.



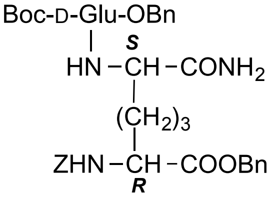
(2*S*,6*R*)-2-(((benzyloxy)carbonyl)amino)-6-((*tert*-butoxycarbonyl)amino)-heptanediamide (L,D-di-amide-*meso*-DAP) (43)

Compound **43** was synthesized from **25** and 28% ammonia with similar method to the synthesis of **32**. The crude product was purified by silica-gel flash column chromatography (CHCl$_3$/MeOH 15:1) to give compound **43** as white solid (67 mg, 84%).

^{1}H NMR (400 MHz, CD$_3$OD): δ 7.28-7.17 (5H, m; Ar$\underline{H}$), 5.02-4.96 (2H, m; -O-C$\underline{H}_2$-Ph), 3.99 (1H, dd, J = 5.0 Hz, 9.0 Hz; -C$\underline{H}$(NHZ)-), 3.90 (1H, dd, J = 4.6 Hz, 8.0 Hz; -C$\underline{H}$(NHBoc)-), 1.70-1.34 (15H, m; DAP 3-C$\underline{H}_2$, 4-C$\underline{H}_2$, 5-C$\underline{H}_2$, -OC(C$\underline{H}_3$)$_3$).

^{13}C NMR (100 MHz, CD$_3$OD): δ 177.8, 177.5, 158.5, 157.9, 138.1, 129.5, 129.0, 128.9, 80.6, 67.7, 56.1, 55.6, 33.1, 33.0, 28.7, 23.4.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{20}H_{30}N_4O_6Na$: 445.2058 [M+Na]$^+$; found: 445.2075.



$$\begin{array}{l} \text{Boc-D-Glu-OBn} \\ \quad | \quad \mathbf{\textit{S}} \\ \text{HN}-\text{CH}-\text{CONH}_2 \\ \qquad | \\ \qquad (\text{CH}_2)_3 \\ \qquad | \\ \text{ZHN}-\text{CH}-\text{COOBn} \\ \qquad \mathbf{\textit{R}} \end{array}$$

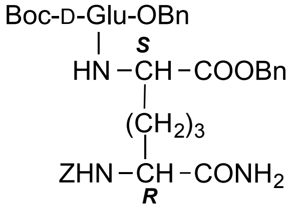
Fully protected D-isoGlu-*meso*-DAP(L-amide) (44)

Compound **44** was synthesized from compound **38** and Boc-D-Glu-OBn with similar method to compound **32**. The crude product was purified by silica-gel flash column chromatography (CHCl$_3$/MeOH 30:1) to give compound **44** as white solid (100 mg, 88%).

^{1}H NMR (500 MHz, CDCl$_3$): δ 7.37-7.26 (15H, m; Ar$\underline{H}$), 6.63 (1H, s; N$\underline{H}$), 6.36 (1H, d, J = 5.4 Hz; N$\underline{H}$), 5.49 (1H, d, J = 6.9 Hz; N$\underline{H}$), 5.35-5.33 (2H, m; N$\underline{H}$ × 2), 5.19-5.06 (6H, m; -O-C$\underline{H}_2$-Ph × 3), 4.39-4.33 (3H, m; DAP 2-$\underline{H}$, 6-$\underline{H}$, Glu-α$\underline{H}$), 2.27-2.22 (3H, m; Gln-γC$\underline{H}_2$, Gln-βC$\underline{H}$), 1.88-1.59 (5H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.41 (11H, br s; DAP 4-C$\underline{H}_2$, -OC(C$\underline{H}_3$)$_3$).

^{13}C NMR (100 MHz, CDCl$_3$): δ 173.8, 172.2, 172.1, 172.0, 156.1, 155.9, 136.2, 135.2, 128.7, 128.6, 128.6, 128.5, 128.3, 128.2, 128.0, 80.3, 67.3, 67.1, 53.4, 52.7, 52.5, 32.3, 31.9, 31.0, 28.8, 28.3, 21.2.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{39}H_{48}N_4O_{10}Na$: 755.3263 [M+Na]$^+$; found: 755.3271.



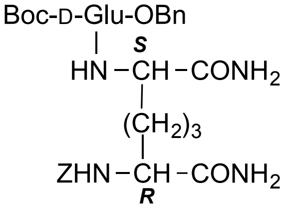
Fully protected D-isoGlu-*meso*-DAP(D-amide) (45)

Compound **45** was synthesized from compound **42** with similar method to compound **32**. The crude product was purified by silica-gel flash column chromatography (CHCl$_3$/MeOH 35:1) to give compound **45** as white solid (64 mg, 75%).

^{1}H NMR (500 MHz, CDCl$_3$): δ 7.40-7.31 (15H, m; ArH), 6.56 (1H, s; NH), 6.44 (1H, s; NH), 5.50 (1H, s; NH), 5.33 (2H, br s; NH × 2), 5.25-5.07 (H, m; -O-CH$_2$-Ph × 3), 4.62 (1H, dd, J = 7.8 Hz, 13.2 Hz; DAP 2-H), 4.35 (1H, d, J = 6.2 Hz; Glu-αH), 4.17 (1H, d, J = 6.1 Hz; DAP 6-H), 2.32-2.20 (2H, m; Gln-γCH$_2$), 2.10-2.00 (2H, m; Gln-βCH$_2$), 1.88-1.58 (4H, m; DAP 3-CH$_2$, 5-CH$_2$), 1.41 (11H, br s; DAP 4-CH$_2$, -OC(CH$_3$)$_3$).

^{13}C NMR (100 MHz, CDCl$_3$): δ 173.7, 172.3, 172.1, 171.7, 156.3, 155.7, 136.3, 135.3, 128.7, 128.6, 128.5, 128.5, 128.3, 128.2, 128.0, 80.3, 67.2, 67.0, 53.9, 52.8, 51.5, 32.2, 32.1, 31.7, 28.8, 28.3, 21.2.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{39}H_{48}N_4O_{10}Na$: 755.3263 [M+Na]$^+$; found: 755.3283.



Fully protected D-isoGlu-*meso*-DAP(L,D-di-amide) (46)

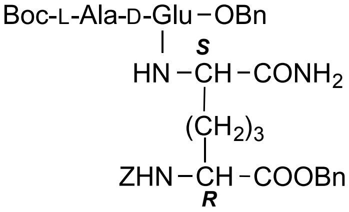
Compound **46** was synthesized from compound **43** with similar method to compound **32**. The crude product was purified by silica-gel flash column chromatography (CHCl$_3$/MeOH 17:1) to give compound **46** as white solid (126 mg, 49%).

^{1}H NMR (400 MHz, DMSO-d$_6$): δ 7.81 (1H, d, J = 8.0 Hz; NH), 7.34-7.26 (13H, m; ArH, NH × 3), 7.20 (1H, d, J = 8.2 Hz; NH), 6.93 (2H, s; NH × 2), 5.15-4.97 (4H, m;

-O-CH$_2$-Ph × 2), 4.12 (1H, dd, J = 7.3 Hz, 13.3 Hz; DAP 2-H), 3.99 (1H, dd, J = 8.7 Hz, 13.1 Hz; Glu-αH), 3.87 (1H, dd, J = 8.2 Hz, 13.1 Hz; DAP 6-H), 2.22-2.19 (2H, m; Gln-γCH$_2$), 1.99-1.91 (1H, m; Gln-βCH), 1.79-1.70 (1H, m; Gln-βCH), 1.65-1.43 (4H, m; DAP 3-CH$_2$, 5-CH$_2$), 1.36-1.26 (11H, m; DAP 4-CH$_2$, -OC(CH$_3$)$_3$).

^{13}C NMR (100 MHz, DMSO-d$_6$): δ 173.8, 173.6, 172.2, 171.1, 155.9, 155.5, 137.0, 135.9, 128.3, 128.2, 127.9, 127.6, 127.6, 78.2, 65.7, 65.3, 54.4, 53.4, 52.2, 31.7, 31.5, 28.1, 26.5, 21.9.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{32}H_{43}N_5O_9Na$: 664.2953 [M+Na]$^+$; found: 664.2968.



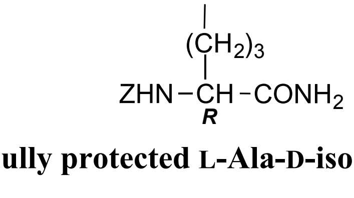
Fully protected L-Ala-D-isoGlu-*meso*-DAP(L-amide) (47)

Compound **47** was synthesized from compound **44** and Boc-L-Ala-OH with similar method to compound **32**. The crude product was purified by silica-gel flash column chromatography (CHCl$_3$/MeOH 30:1) to give compound **47** as white solid (81 mg, 87%).

^{1}H NMR (400 MHz, CDCl$_3$): δ 7.33-7.26 (15H, m; ArH), 7.22 (1H, d, J = 8.0 Hz; NH), 6.89 (1H, s; NH), 5.56 (1H, d, J = 7.6 Hz; NH), 5.49 (1H, s; NH), 5.17-5.05 (7H, m; -O-CH$_2$-Ph × 3, NH), 4.48 (1H, s; Glu-αH), 4.37-4.29 (2H, m; DAP 2-H, 6-H), 4.10 (1H, t, J = 6.6 Hz; Ala-αH), 2.26-2.13 (2H, m; Gln-γCH$_2$), 1.93-1.67 (6H, m; Gln-βCH$_2$, DAP 3-CH$_2$, 5-CH$_2$), 1.48-1.41 (11H, br s; DAP 4-CH$_2$, -OC(CH$_3$)$_3$), 1.29 (3H, d, J = 7.1 Hz; Ala-βCH$_3$).

^{13}C NMR (100 MHz, CDCl$_3$): δ 174.5, 173.4, 172.4, 172.1, 171.6, 156.1, 155.8, 136.2, 135.3, 135.3, 128.7, 128.6, 128.6, 128.5, 128.3, 128.2, 128.1, 80.7, 67.3, 67.2, 67.1, 53.6, 53.2, 51.0, 32.0, 31.5, 31.0, 28.3, 27.6, 21.5, 17.9.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{42}H_{53}N_5O_{11}Na$: 826.3634 [M+Na]$^+$; found: 826.3646.



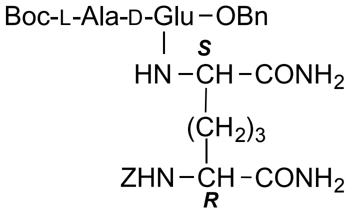
Fully protected L-Ala-D-isoGlu-*meso*-DAP(D-amide) (48)

Compound **48** was synthesized from compound **45** with similar method to compound **32**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 35:1) to give compound **48** as white solid (26 mg, 43%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40-7.30 (15H, m; ArH), 7.13 (1H, s; NH), 7.02 (1H, s; NH), 6.41 (1H, s; NH), 5.57 (1H, s; NH), 5.25-5.10 (6H, m; -O-CH_2-Ph × 3), 4.61-4.56 (2H, m; DAP 2-H, Glu-αH), 4.14 (2H, s; Ala-αH, DAP 6-H), 2.23 (3H, br s; Gln-γCH_2, βCH), 1.89-1.56 (5H, m; DAP 3-CH_2, 5-CH_2, βCH), 1.47-1.42 (11H, m; DAP 4-CH_2, -OC(CH_3)$_3$), 1.28 (3H, d, J = 6.7 Hz; Ala-βCH_3).

^{13}C NMR (100 MHz, $CDCl_3$): δ 175.4, 173.9, 172.4, 172.1, 170.7, 156.9, 156.2, 136.2, 135.3, 128.9, 128.8, 128.7, 128.6, 128.3, 128.1, 80.5, 67.4, 67.2, 67.1, 54.1, 52.3, 51.8, 51.5, 32.1, 31.8, 31.2, 28.3, 21.4, 20.9.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{42}H_{53}N_5O_{11}Na$: 826.3634 $[M+Na]^+$; found: 826.3646.



Fully protected L-Ala-D-isoGlu-*meso*-DAP(L,D-di-amide) (49)

Compound **49** was synthesized from compound **43** with similar method to compound **32**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 15:1) to give compound **49** as white solid (126 mg, 49%).

^{1}H NMR (400 MHz, DMSO-d_6): δ 8.21 (1H, d, J = 7.8 Hz; NH), 7.81 (1H, d, J = 8.0 Hz; NH), 7.38-7.27 (10H, m; ArH), 6.94 (1H, s; NH), 6.80 (1H, d, J = 7.1 Hz; NH), 5.15-4.97 (4H, m; -O-CH_2-Ph × 2), 4.26 (1H, dd, J = 5.0 Hz, 9.2 Hz; Glu-αH), 4.12-4.09 (1H, m; DAP 2-H), 4.03-4.00 (1H, m; Ala-αH), 3.86 (1H, dd, J = 5.0 Hz, 8.9 Hz; DAP 6-H), 2.19-2.15 (2H, m; Gln-γCH_2), 2.04-1.95 (1H, m; Gln-βCH), 1.85-1.76 (1H, m; Gln-βCH), 1.66-1.41 (4H, m; DAP 3-CH_2, 5-CH_2), 1.36-1.23 (11H, m; DAP 4-CH_2, -OC(CH_3)$_3$), 1.17 (3H, d, J = 7.1 Hz; Ala-βCH_3).

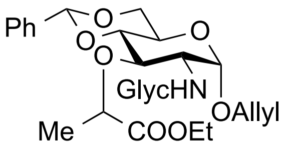
^{13}C NMR (100 MHz, DMSO-d_6): δ 173.7, 173.5, 172.9, 171.5, 171.0, 155.8, 154.9, 137.0, 135.8, 128.3, 128.2, 127.9, 127.7, 127.6, 78.0, 65.9, 65.3, 54.4, 52.1, 51.5, 49.6, 31.6, 31.2, 28.1, 27.9, 26.8, 21.9, 18.4.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{35}H_{48}N_6O_{10}Na$: 735.3324 $[M+Na]^+$; found: 735.3320.

Protected H-L-Ala-D-isoGlu-*meso*-DAP(L-amide) (50),

H-L-Ala-D-isoGlu-*meso*-DAP(D-amide) (51),

H-L-Ala-D-isoGln-*meso*-DAP(L,D-amide) (52)
Compounds **50**, **51** and **52** were synthesized from compound **47**, **48** and **49**, respectively, with similar method to the synthesis of **34**. The compounds were used without further purification.

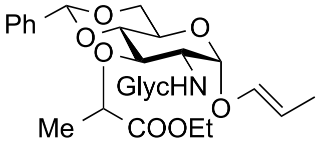


1-Allyl-2-glycolylamino-4,6-*O*-benzylidene-2-deoxy-3-*O*-[(*R*)-1-(ethoxycarbonyl)ethyl]-α-D-glucopyranoside (54) Under Argon protection, $\mathrm{Pd(PPh_3)_4}$ (188 mg, 0.163 mmol) and (dimethylamino)trimethylsilane (528 uL, 3.26 mmol) were added to the $\mathrm{CHCl_3}$ solution (20 mL) of compound **53** (400 mg, 0.814 mmol), the resulting mixture was stirred at room temperature for 2 h. The reaction was quenched with $\mathrm{H_2O}$, and extracted with $\mathrm{CHCl_3}$. The organic layer was washed with saturated aqueous $\mathrm{NaHCO_3}$, brine and dried over $\mathrm{Na_2SO_4}$, and then concentrated in vacuo. Without any purification, the residue was reacted with glycolic acid (93 mg, 1.22 mmol), DMT-MM (450.5 mg, 1.63 mmol) in THF (10 mL) at room temperature. After 2 h, the reaction was quenched with $\mathrm{H_2O}$, and extracted with $\mathrm{CHCl_3}$. The organic layer was washed with saturated aqueous $\mathrm{NaHCO_3}$, brine and dried over $\mathrm{Na_2SO_4}$, and then concentrated in vacuo. The residue was purified by silica-gel flash column chromatography (AcOEt/toluene 1:1.5) to give compound **54** as yellow solid (378.9 mg, quant.).

^{1}H NMR (500 MHz, $\mathrm{CDCl_3}$): δ 7.57–7.35 (6H, m; $\mathrm{N\underline{H}}$, $\mathrm{Ar\underline{H}}$), 5.91-5.83 (1H, m; $\mathrm{\text{-}O\text{-}CH_2\text{-}C\underline{H}{=}CH_2}$), 5.58 (1H, s; $\mathrm{Ph\text{-}C\underline{H}{=}O_2}$), 5.26 (1H, ddd, J = 1.6 Hz, 3.2 Hz, 17.3 Hz; $\mathrm{\text{-}O\text{-}CH_2\text{-}CH{=}C\underline{H}H}$), 5.20-5.17 (2H, m; $\mathrm{\text{-}O\text{-}CH_2\text{-}CH{=}CH\underline{H}}$, H-1), 4.48 (1H, q, J =7.1 Hz; Lac-α$\underline{\mathrm{H}}$), 4.27 (1H, dd, J = 4.6 Hz, 10.0 Hz; H-6’), 4.23-4.07 (6H, m; $\mathrm{\text{-}COOC\underline{H}_2CH_3}$, $\mathrm{\text{-}NHCOC\underline{H}_2OH}$, $\mathrm{\text{-}O\text{-}C\underline{H}H\text{-}CH{=}CH_2}$, H-2), 4.00 (1H, ddt, J = 1.3 Hz, 6.0 Hz, 12.9 Hz; $\mathrm{\text{-}O\text{-}CH\underline{H}\text{-}CH{=}CH_2}$), 3.88-3.69 (4H, m; H-5, H-3, H-6, H-4), 3.33 (1H, t, J = 6.3 Hz; $\mathrm{\text{-}NHCOCH_2O\underline{H}}$), 1.39 (3H, d, J = 7.0 Hz; Lac-β$\mathrm{C\underline{H}_3}$), 1.26 (3H, t, J =7.15 Hz; $\mathrm{\text{-}COOCH_2C\underline{H}_3}$).

^{13}C NMR ($\mathrm{CDCl_3}$, 125 MHz): δ 174.9, 172.5, 137.2, 133.6, 132.9, 132.1, 132.1, 132.0, 129.0, 125.8, 101.3, 97.0, 83.3, 75.4, 75.3, 68.9, 68.8, 62.9, 62.2, 61.3, 53.3, 18.8, 14.0.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $\mathrm{C_{23}H_{31}NO_9Na}$: 488.1891 [M+Na]$^+$; found: 488.1892.

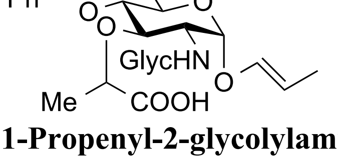


1-propenyl-2-glycolylamino-4,6-*O*-benzylidene-2-deoxy-3-*O*-[(*R*)-1-(ethoxycarbnyl) ethyl]-α-D-glucopyranoside (55) $[Ir(cod)(MePh_2P)_2]PF_6$ (21.8 mg, 0.0256 mmol) was activated by H_2 in dry THF (2 mL) and added to the dry THF solution (3 mL) of compound **54** (300 mg, 0.644 mmol). The mixture was allowed to stir at room temperature for 2 h, then H_2O was added and extracted with ethyl acetate. The organic layer was washed with saturated aqueous $NaHCO_3$, brine and dried over Na_2SO_4, and then concentrated in vacuo. The residue was purified by silica-gel flash column chromatography ($CHCl_3$/acetone 20:1) to give compound **55** as pale yellow solid (261 mg, 87%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.65 (1H, d, J = 1.2 Hz; N$\underline{H}$), 7.46-7.37 (5H, m; Ar$\underline{H}$), 6.10 (1H, dq, J = 1.6 Hz, 12.2 Hz; -O-C$\underline{H}$=CH-), 5.58 (1H, s; Ph-C$\underline{H}$=O_2), 5.37 (1H, d, J = 3.4 Hz; H-1), 5.16-5.09 (1H, m; -O-CH=C$\underline{H}$-), 4.49 (1H, q, J = 7.0 Hz; Lac-α$\underline{H}$), 4.28-4.06 (6H, m; H-2, H-6, -COOC$\underline{H}_2$CH$_3$, -NHCOC$\underline{H}_2$OH), 3.86-3.70 (4H, m; H-3, H-5, H-4, H-6'), 3.09 (1H, dd, J = 5.2 Hz, 6.9 Hz; NHCOCH$_2$O$\underline{H}$), 1.54 (3H, dd, J = 1.6 Hz, 6.9 Hz; -CH=CH-C$\underline{H}_3$), 1.41 (3H, d, J = 7.0 Hz; Lac-βC$\underline{H}_3$), 1.27 (3H, t, J = 7.2 Hz; -COOCH$_2$C$\underline{H}_3$).

^{13}C NMR (125 MHz, $CDCl_3$): δ 175.0, 172.7, 142.8, 137.2, 129.0, 128.3, 125.8, 105.3, 101.4, 96.9, 83.1, 75.3, 75.1, 68.8, 63.1, 62.1, 61.4, 53.2, 18.8, 14.1, 12.3.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{23}H_{31}NO_9Na$: 488.1891 $[M+Na]^+$; found: 488.1896.



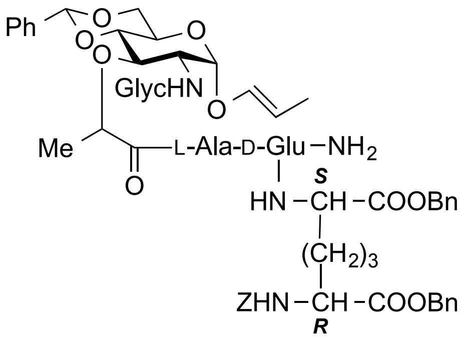
1-Propenyl-2-glycolylamino-4,6-*O*-benzylidene-2-deoxy-3-*O*-[(*R*)-1-(carbonyloxy)ethyl]-α-D-glucopyranoside (56) Compound **55** (255 mg, 0.548 mmol) and LiOH·H_2O (25.3 mg)were dissolved in THF/1,4-dioxane/H_2O 4:2:1 (7 mL) and stirred at room temperature for 1 h. Then the solution was neutralized with Dowex H^+ and filtrated to get clear solution. After evaporation, the residue was purified by by silica-gel flash column chromatography ($CHCl_3$/MeOH/AcOH 15:1:0.1) to give compound **56** as white solid (151 mg, 63%).

^{1}H NMR (400 MHz, CD_3OD): δ 7.47-7.33 (5H, m; Ar$\underline{H}$), 6.16 (1H, dd, J = 1.6 Hz, 12.4 Hz; -O-C$\underline{H}$=CH-), 5.63 (1H, s; Ph-C$\underline{H}$=O_2), 5.39 (1H, d, J = 3.2 Hz; H-1), 5.17-5.09

(1H, m; -O-CH=C$\underline{H}$-), 4.44 (1H, q, *J* = 6.8 Hz; Lac-α$\underline{H}$), 4.16-3.90 (5H, m; H-2, H-6, H-5, -NHCOC$\underline{H}_2$OH), 3.81-3.74 (3H, m; H-3, H-4, H-6’), 1.53 (3H, dd, *J* = 1.6 Hz, 6.9 Hz; -CH=CH-C$\underline{H}_3$), 1.34 (3H, d, J = 6.9 Hz; Lac-βC$\underline{H}_3$).

^{13}C NMR (100 MHz, CD_3OD): *δ* 178.9, 175.8, 144.5, 139.0, 129.9, 129.2, 127.2, 105.7, 102.8, 98.1, 84.3, 77.6, 75.4, 69.7, 68.1, 64.7, 62.7, 55.1, 19.4, 12.4.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{21}H_{27}NO_9Na$: 460.1578 $[M+Na]^+$; found: 460.1580.



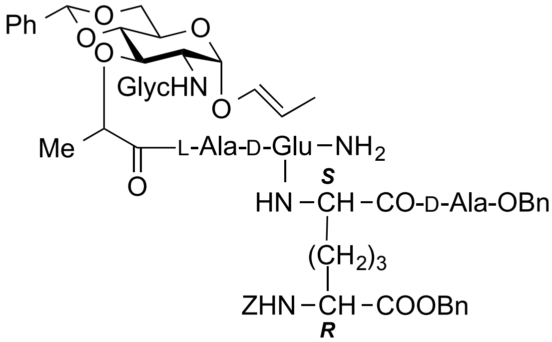
Protected monosaccharide containing *N*-glycolylmuramyl group and tripeptide (57)

Compound **56** (46 mg, 0.107 mmol) and **34** (50 mg, 0.071 mmol) were dissolved to MeOH/H_2O 2:1 (18 mL), then triethylamine (30 μL, 0.213 mmol) was added. The mixture was stirred at room temperature for 10 min before DMT-MM was added, allowed to react overnight. After dissolving the white solid to H_2O, the mixture was extracted with $CHCl_3$. The organic layer was washed with 10% citric acid solution, saturated aqueous $NaHCO_3$, brine and dried over Na_2SO_4, and then concentrated in vacuo. The residue was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 20:1) to give compound **57** as white solid (71 mg, 89%).

^{1}H NMR (500 MHz, $CDCl_3$:CD_3OD 10:1): *δ* 7.46-7.30 (20 H, m; Ar$\underline{H}$), 6.11 (1H, d, *J* = 12.3 Hz; -O-C$\underline{H}$=CH-), 5.57 (1H, s; Ph-C$\underline{H}$=O_2), 5.19-5.05 (8H, m; -O-CH=C$\underline{H}$-, H-1, -C$\underline{H}_2$Ph × 3), 4.49-4.43 (2H, m; Gln-αH, DAP 2-$\underline{H}$), 4.33-4.18 (5H, m; H-2, H-6, Lac-α$\underline{H}$, Ala-α$\underline{H}$, DAP 6-$\underline{H}$), 4.02 (2H, d, *J* = 5.3 Hz; -NHCOC$\underline{H}_2$OH), 3.86-3.67 (4H, m; H-3, H-4, H-5, H-6’), 2.32-2.24 (2H, m; Gln-γC$\underline{H}_2$), 2.11 (1H, br s; Gln-βC$\underline{H}$), 1.91 (1H, br s; Gln-βC$\underline{H}$), 1.83-1.60 (4H, m; DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.54 (3H, d, *J* = 6.9 Hz; -CH=CH-C$\underline{H}_3$), 1.39-1.34 (8H, m; Lac-βC$\underline{H}_3$, Ala-βC$\underline{H}_3$, DAP 4-C$\underline{H}_2$).

^{13}C NMR (125 MHz, $CDCl_3$:CD_3OD 10:1): *δ* 174.5, 174.4, 174.0, 173.7, 173.1, 172.4, 156.5, 142.4, 137.1, 136.3, 135.3, 129.2, 128.7, 128.6, 128.6, 128.4, 128.3, 128.3, 128.1, 127.9, 126.0, 106.1, 101.7, 97.5, 81.6, 78.0, 68.8, 67.3, 67.3, 67.1, 63.3, 62.0, 53.9, 53.8, 52.6, 52.5, 52.3, 52.1, 32.1, 31.8, 31.0, 28.7, 21.6, 19.4, 17.3, 12.3.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{58}H_{70}N_6O_{17}Na$: 1145.4690 $[M+Na]^+$; found: 1145.4686.

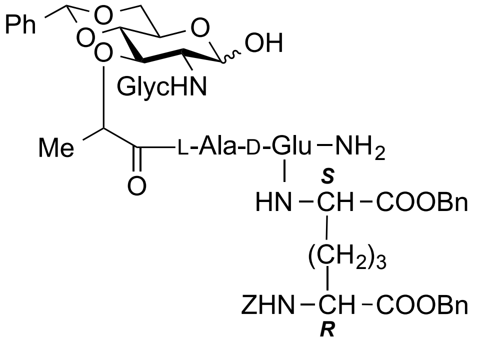


Protected monosaccharide containing *N*-glycolylmuramyl group and tetrapeptide (58) Compound **58** was synthesized from **56** and **35** with similar method to the synthesis of **57**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 20:1) to give compound **58** as white solid (31 mg, 84%).

^1H NMR (400 MHz, $CDCl_3$:CD_3OD 10:1): *δ* 7.47-7.29 (20 H, m; ArH), 6.12 (1H, dd, *J* = 1.6 Hz, 12.4 Hz, ; -O-CH=CH-), 5.57 (1H, s; Ph-CH=O_2), 5.22-5.04 (8H, m; -O-CH=CH-, H-1, -CH_2Ph × 3), 4.53 (1H, q, *J* = 7.1 Hz; D-Ala-αH), 4.40-4.15 (7H, m; Gln-αH, DAP 2-H, H-2, H-6, Lac-αH, Ala-αH, DAP 6-H), 4.00 (2H, s; -NHCOCH_2OH), 3.86-3.68 (4H, m; H-3, H-4, H-5, H-6'), 2.29-2.18 (3H, m; Gln-γCH_2, βCH), 1.83-1.64 (5H, m; Gln-βCH, DAP 3-CH_2, 5-CH_2), 1.55 (3H, dd, *J* = 1.2 Hz, 6.8 Hz; -CH=CH-CH_3), 1.40-1.32 (11H, m; Lac-βCH_3, Ala-βCH_3, D-Ala-βCH_3, DAP 4-CH_2).

^{13}C NMR (100 MHz, $CDCl_3$:CD_3OD 10:1): *δ* 174.4, 173.9, 173.7, 173.2, 172.8, 172.6, 172.1, 156.2, 142.4, 137.1, 135.5, 135.4, 129.2, 128.7, 128.6, 128.6, 128.5, 128.4, 128.3, 128.0, 127.8, 126.1, 106.2, 101.7, 97.5, 81.2, 78.1, 77.8, 68.8, 67.3, 67.1, 63.4, 62.0, 54.0, 53.6, 52.4, 48.4, 31.8, 31.5, 31.0, 29.3, 21.7, 19.3, 17.2, 17.1, 12.3.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{61}H_{75}N_7O_{18}Na$: 1216.5061 $[M+Na]^+$; found: 1216.5076.

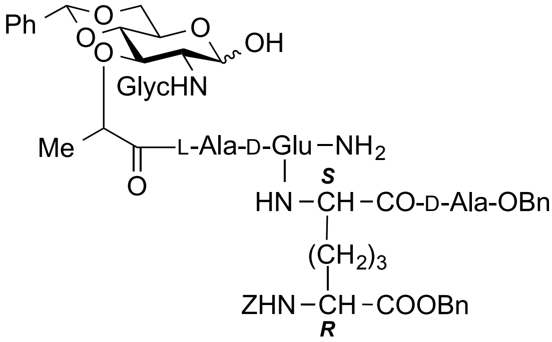


1-*O*-Deprotected monosaccharide containing *N*-glycolylmuramyl group and tripeptide (59) Compound **57** (50 mg, 0.045 mmol) was dissolved in THF/H_2O 2:1 (7.5 mL). I_2 (23 mg, 0.089 mmol) was added to the solution, the reaction was stirred at room temperature for 2 h. The dark brown solution was quenched by 10% aqueous $Na_2S_2O_3$ and the mixture was extracted with $CHCl_3$. The organic layer was washed with 10% aqueous $Na_2S_2O_3$, saturated aqueous $NaHCO_3$, and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 15:1) to give compound **59** as white solid (23 mg, 74%).

^{1}H NMR (400 MHz, DMSO-d_6): δ 8.22 (1H, d, J = 6.8 Hz; NH), 8.14 (1H, d, J = 8.2 Hz; NH), 7.72 (1H, d, J = 7.8 Hz; NH), 7.66 (1H, d, J = 8.5 Hz; NH), 7.42-7.33 (22 H, m; ArH, NH × 2), 7.03-7.00 (2H, m; NH, 1-OH), 5.69 (1H, s; Ph-CH=O_2), 5.47 (1H, t, J = 5.3 Hz; -NHCOCH$_2$OH), 5.10-5.00 (7H, m; H-1, -CH$_2$Ph ×3), 4.29-3.57 (13H, m; Gln-αH, DAP 2-H, H-2, H-6, Lac-αH, Ala-αH, DAP 6-H, -NHCOCH$_2$OH, H-3, H-4, H-5, H-6'), 2.15-2.11 (2H, m; Gln-γCH$_2$), 1.91 (1H, br s; Gln-βCH), 1.67-1.58 (5H, m; Gln-βCH, DAP 3-CH$_2$, 5-CH$_2$), 1.34 (2H, br s; DAP 4-CH$_2$), 1.22-1.18 (6H, m; Lac-βCH$_3$, Ala-βCH$_3$).

^{13}C NMR (125 MHz, DMSO-d_6): δ 173.0, 172.1, 172.1, 172.0, 171.9, 171.9, 171.8, 156.1, 137.6, 136.8, 135.9, 135.9, 128.7, 128.3, 128.3, 128.0, 128.0, 127.9, 127.8, 127.7, 127.6, 125.9, 125.8, 100.3, 91.4, 81.2, 76.6, 75.9, 68.1, 65.9, 65.8, 65.5, 62.1, 61.3, 53.8, 53.0, 52.1, 51.9, 48.0, 31.5, 30.2, 30.2, 27.7, 21.9, 18.9, 18.2.

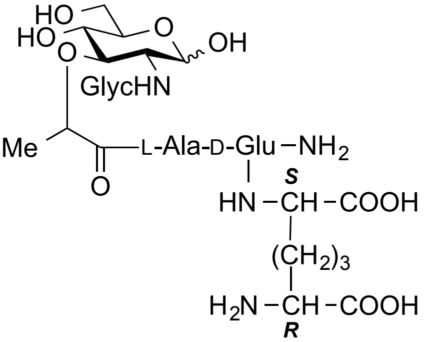
HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{55}H_{66}N_6O_{17}Na$: 1105.4377 $[M+Na]^+$; found: 1105.4381.



1-*O*-Deprotected monosaccharide containing *N*-glycolylmuramyl group and tetrapeptide (60) Compound **60** was synthesized from **58** with similar method to the synthesis of **59**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 15:1) to give compound **60** as white solid (14 mg, 52%).

^{1}H NMR (500 MHz, DMSO-d_6): δ 8.33 (1H, d, J = 7.3 Hz; NH), 8.13 (1H, d, J = 8.2 Hz; NH), 7.87 (1H, d, J = 8.0 Hz; NH), 7.70 (1H, d, J = 7.6 Hz; NH), 7.66 (1H, d, J = 8.9 Hz; NH), 7.44-7.26 (21 H, m; ArH, NH × 2), 7.02 (1H, d, J = 3.5 Hz; 1-OH), 6.99 (1H, s; NH), 5.68 (1H, s; Ph-CH=O_2), 5.46 (1H, t, J = 5.8 Hz; -NHCOCH$_2$OH), 5.12-4.98 (7H, m; H-1, -C$\underline{H}_2$Ph ×3), 4.31-3.66 (14H, m; D-Ala-α$\underline{H}$, Gln-αH, DAP 2-$\underline{H}$, H-2, H-6, Lac-α$\underline{H}$, Ala-α$\underline{H}$, DAP 6-$\underline{H}$, -NHCOC$\underline{H}_2$OH, H-3, H-4, H-5, H-6'), 2.15-2.08 (2H, m; Gln-γC$\underline{H}_2$), 1.98-1.92 (1H, m; Gln-βC$\underline{H}$), 1.69-1.27 (7H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$, 4-C$\underline{H}_2$), 1.26-1.18 (9H, m; Lac-βC$\underline{H}_3$, Ala-βC$\underline{H}_3$, D-Ala-βC$\underline{H}_3$).

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{58}H_{71}N_7O_{18}Na$: 1176.4748 $[M+Na]^+$; found: 1176.4761.

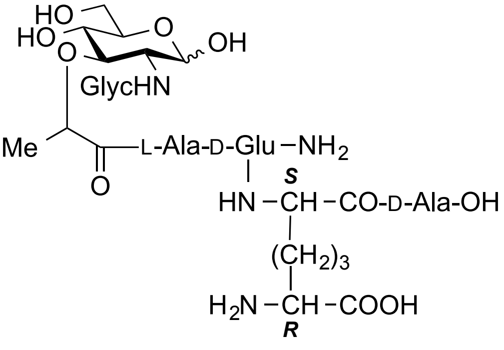


Monosaccharide containing *N*-glycolylmuramyl group and tripeptide (1b) Compound **59** (20 mg, 0.0185) and $Pd(OH)_2$ (40 mg, 0.285 mmol) were mixed well in AcOH (2 mL), and reacted under H_2 atmosphere (20 kg cm^{-2}) for 24 h. The $Pd(OH)_2$ was removed by membrane filtration, the filtrate was concentrated in vacuo. The residue was resuspended with ultrapure H_2O and lyophilized to give **1b** as white solid (11 mg, 88%).

^{1}H NMR (500 MHz, D_2O): δ 5.13 (1H, d, J = 3.5 Hz; H-1β), 4.30-4.15 (4H, m; Ala-α$\underline{H}$, Gln-α$\underline{H}$, Lac-α$\underline{H}$, DAP 2-$\underline{H}$), 4.04-3.93 (3H, m; -NHCOC$\underline{H}_2$OH, H-2), 3.86-3.66 (4H, m; DAP 6-$\underline{H}$, H-3, H-5, H-6), 3.58-3.41 (2H, m; H-4, H-6'), 2.35-2.32 (2H, m; Gln-γC$\underline{H}_2$), 2.14-2.07 (1H, m; Gln-βC$\underline{H}$), 1.97-1.91 (1H, m; Gln-βC$\underline{H}$), 1.89-1.63 (4H, m; DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.43-1.29 (8H, DAP 4-C$\underline{H}_2$, Lac-βC$\underline{H}_3$, Ala-βC$\underline{H}_3$).

^{13}C NMR (125 MHz, D_2O): δ 176.6, 176.4, 176.0, 175.8, 175.5, 175.3, 175.1, 91.5, 79.6, 76.4, 72.3, 69.8, 61.6, 61.4, 55.2, 54.7, 53.9, 53.7, 50.4, 32.4, 31.4, 30.7, 27.4, 21.8, 19.3, 17.2.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{26}H_{44}N_6O_{15}Na$: 703.2757 $[M+Na]^+$; found: 703.2767.



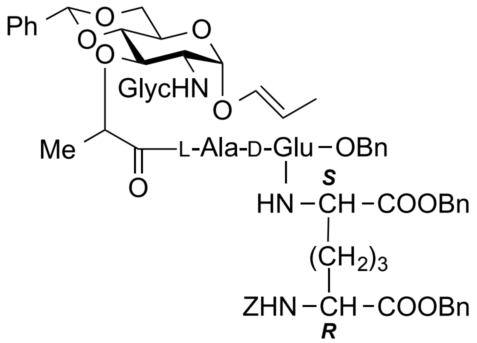
Monosaccharide containing *N*-glycolylmuramyl group and tetrapeptide (1d)

Compound **1d** was synthesized from **60** with similar method to the synthesis of **1b** (quant.).

^{1}H NMR (500 MHz, D_2O): δ 5.12 (1H, d, J = 3.3 Hz; H-1β), 4.29-4.14 (5H, m; D-Ala-α$\underline{H}$, Ala-α$\underline{H}$, Gln-α$\underline{H}$, Lac-α$\underline{H}$, DAP 2-$\underline{H}$), 4.03-3.92 (3H, m; -NHCOC$\underline{H}_2$OH, H-2), 3.85-3.65 (4H, m; DAP 6-$\underline{H}$, H-3, H-5, H-6), 3.57-3.42 (2H, m; H-4, H-6’), 2.32 (2H,br s; Gln-γC$\underline{H}_2$), 2.14-2.06 (1H, m; Gln-βC$\underline{H}$), 1.92-1.66 (5H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.41-1.28 (11H, DAP 4-C$\underline{H}_2$, Lac-βC$\underline{H}_3$, Ala-βC$\underline{H}_3$, D-Ala-βC$\underline{H}_3$).

^{13}C NMR (125 MHz, D_2O): δ 176.5, 176.4, 176.3, 176.0, 175.7, 175.5, 175.1, 173.7, 91.5, 79.6, 76.4, 72.3, 69.8, 61.6, 61.4, 55.2, 54.5, 54.0, 53.5, 51.2, 50.4, 32.2, 31.5, 30.7, 27.5, 21.7, 19.3, 18.0, 17.2.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{29}H_{49}N_7O_{16}Na$: 774.3128 [M+Na]$^+$; found: 774.3136.



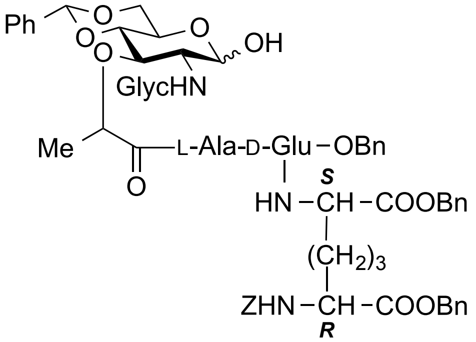
Protected monosaccharide containing *N*-glycolylmuramyl group and tripeptide (62)

Compound **62** was synthesized from **56** and **61** (HCl• NH_2-L-Ala-D-isoGlu-*meso*-DAP) with similar method to the synthesis of **57**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 50:1) to give compound **62** as white solid (25 mg, 47%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.47-7.45 (2H, m; N$\underline{H}$ × 3), 7.35-7.11 (25H, m; Ar$\underline{H}$), 6.71 (1H, d, J = 8.0 Hz; N$\underline{H}$), 6.10 (1H, d, J = 11.7 Hz; -O-C$\underline{H}$=CH-), 5.57 (1H, s; Ph-C$\underline{H}$=O_2), 5.46 (1H, d, J = 8.2 Hz; O$\underline{H}$), 5.19-5.01 (10H, m; -O-CH=C$\underline{H}$-, H-1, -C$\underline{H}_2$Ph × 4), 4.51-4.38 (2H, m; Gln-αH, DAP 2-$\underline{H}$), 4.29-4.25 (5H, m; H-2, H-6, Lac-α$\underline{H}$, Ala-α$\underline{H}$, DAP 6-$\underline{H}$), 4.11-3.96 (2H, m; -NHCOC$\underline{H}_2$OH), 3.88-3.67 (4H, m; H-3, H-4, H-5, H-6’), 2.27-2.06 (4H, m; Gln-γC$\underline{H}_2$, Gln-βC$\underline{H}_2$), 1.77-1.59 (4H, m; DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.53 (3H, d, J = 6.7 Hz; -CH=CH-C$\underline{H}_3$), 1.42-1.26 (8H, m; Lac-βC$\underline{H}_3$, Ala-βC$\underline{H}_3$, DAP 4-C$\underline{H}_2$).

^{13}C NMR (100 MHz, $CDCl_3$): δ 174.1, 173.9, 172.5, 172.3, 172.1, 171.1, 156.2, 142.3, 137.1, 135.2, 135.2, 129.1, 128.7, 128.3, 128.2, 128.1, 126.0, 106.1, 101.6, 97.5, 82.1, 68.8, 67.4, 67.3, 63.2, 62.4, 53.9, 53.8, 52.1, 52.0, 51.9, 49.7, 32.0, 31.6, 31.0, 26.9, 21.2, 19.6, 17.8, 12.3.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{65}H_{75}N_5O_{18}Na$: 1236.4999 $[M+Na]^+$; found: 1236.5015.

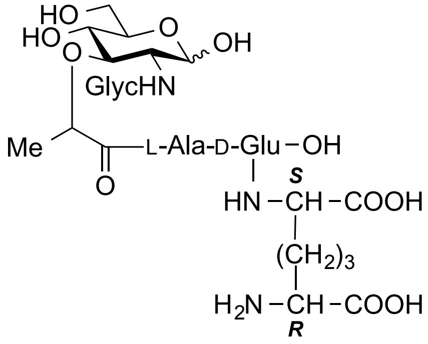


1-*O*-Deprotected monosaccharide containing *N*-glycolylmuramyl group and tripeptide (63) Compound **63** was synthesized from **62** with similar method to the synthesis of **59**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 25:1) to give compound **63** as white solid (12 mg, 62%).

^{1}H NMR (400 MHz, DMSO-d_6): δ 8.43 (1H, d, J = 7.6 Hz; NH), 7.70 (1H, d, J = 7.8 Hz; NH), 7.64 (1H, d, J = 9.2 Hz; NH), 7.40-7.24 (26 H, m; ArH, NH), 5.66 (1H, s; Ph-CH=O_2), 5.07-4.97 (9H, m; H-1, -CH_2Ph ×3), 4.35-4.12 (5H, m; Gln-αH, DAP 2-H, Lac-αH, Ala-αH, H-4), 4.03-3.98 (4H, m; -NHCOCH_2OH, DAP 6-H, H-2), 3.90-3.64 (4H, m; H-6, H-3, H-4, H-5, H-6'), 2.18-2.14 (2H, m; Gln-γCH_2), 1.96 (1H, br s; Gln-βCH), 1.81-1.53 (5H, m; Gln-βCH, DAP 3-CH_2, 5-CH_2), 1.32 (2H, br s; DAP 4-CH_2), 1.22-1.18 (6H, m; Lac-βCH_3, Ala-βCH_3).

^{13}C NMR (100 MHz, DMSO-d_6): δ 172.6, 172.4, 172.3, 172.0, 171.9, 171.7, 171.6, 170.7, 156.4, 137.8, 137.0, 136.0, 135.7, 128.9, 128.6, 128.3, 128.2, 128.0, 127.8, 126.1, 126.0, 100.5, 91.6, 81.3, 76.9, 76.1, 68.3, 66.5, 66.2, 66.0, 66.7, 62.4, 56.8, 54.0, 53.2, 52.2, 52.0, 31.3, 30.8, 30.4, 28.8, 26.9, 22.0, 19.0, 19.0.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{62}H_{71}N_5O_{18}Na$: 1196.4686 $[M+Na]^+$; found: 1196.4702.



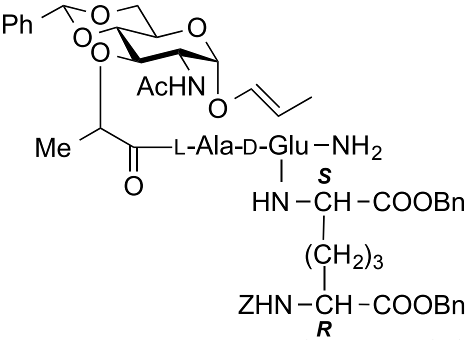
Monosaccharide containing *N*-glycolylmuramyl group and tripeptide (1k)

Compound **1k** was synthesized from **63** with similar method to the synthesis of **1b** (quant.).

^{1}H NMR (400 MHz, D$_2$O): *δ* 5.07 (1H, d, *J* = 2.5 Hz; H-1β), 4.23-4.16 (4H, m; Ala-αH, Gln-αH, Lac-αH, DAP 2-H), 3.99-3.87 (3H, m; -NHCOCH$_2$OH, H-2), 3.77-3.63 (4H, m; DAP 6-H, H-3, H-5, H-6), 3.58-3.39 (2H, m; H-4, H-6’), 2.27-2.23 (2H, m; Gln-γCH$_2$), 2.11-2.02 (1H, m; Gln-βCH), 1.91-1.62 (5H, m; Gln-βCH, DAP 3-CH$_2$, 5-CH$_2$), 1.37-1.25 (8H, DAP 4-CH$_2$, Lac-βCH$_3$, Ala-βCH$_3$).

^{13}C NMR (100 MHz, D$_2$O): *δ* 176.2, 176.0, 176.0, 175.7, 175.4, 175.3, 174.8, 91.5, 79.6, 76.4, 72.2, 69.7, 61.6, 61.4, 56.5, 54.9, 53.9, 53.4, 50.2, 32.2, 30.9, 30.5, 27.5, 21.6, 19.2, 17.4.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{26}H_{44}N_5O_{16}$: 682.2778 [M+H]$^+$; found: 682.2780.



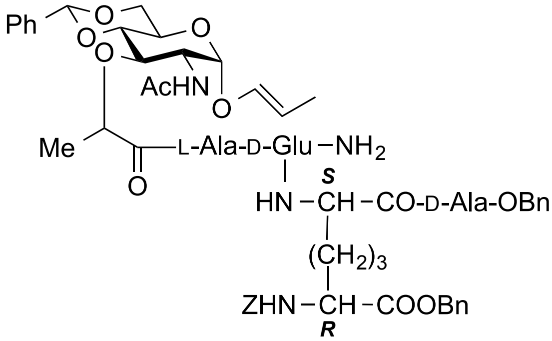
Protected monosaccharide containing *N*-acetylmuramyl group and tripeptide (70)

Compound **69** (45 mg, 0.107 mmol), **34** (50 mg, 0.071 mmol), WSCD•HCl (20.5 mg, 0.107 mmol), and HOBt (14.4 mg, 0.107 mmol) were dissolved in dry DMF under argon atmosphere, triethylamine (30 μL, 0.214 mmol) was syringed to the solution at 0 °C. The mixture was reacted overnight at room temperature. Then 10% citric acid solution was added to the mixture and extracted with ethyl acetate, the resulting organic layer was washed with saturated aqueous NaHCO$_3$ and brine, dried over Na$_2$SO$_4$, and concentrated in vacuo. The residue was purified by silica-gel flash column chromatography (CHCl$_3$/MeOH 20:1) to give compound **70** as white solid (40 mg, 54%).

^{1}H NMR (400 MHz, DMSO-d$_6$): *δ* 8.22 (1H, d, *J* = 7.1 Hz; NH), 8.18 (1H, d, *J* = 8.0 Hz; NH), 8.13 (1H, d, *J* = 8.0 Hz; NH), 7.72 (1H, d, *J* = 7.6 Hz; NH), 7.56 (1H, d, *J* = 7.3 Hz; NH), 7.44-7.29 (21 H, m; ArH, NH), 7.00 (1H, s; NH), 6.22 (1H, dd, *J* = 1.6 Hz, 12.4 Hz ; -O-CH=CH-), 5.70 (1H, s; Ph-CH=O$_2$), 5.12-4.98 (8H, m; -O-CH=CH-, H-1, -CH$_2$Ph × 3), 4.33-4.00 (7H, m; Ala-αH, Gln-αH, DAP 2-H, 6-H, Lac-αH, H-2, H-5), 3.79-3.62 (4H, m; H-4, H-3, H-6, H-6’), 2.14-2.10 (2H, m; Gln-γCH$_2$), 1.96-1.89 (1H, m; Gln-βC$\underline{\text{H}}$), 1.81 (3H, s; -NHCOC$\underline{\text{H}}_3$), 1.75-1.56 (5H, m; Gln-βC$\underline{\text{H}}$, DAP 3-C$\underline{\text{H}}_2$, 5-C$\underline{\text{H}}_2$), 1.51 (3H, dd, J = 1.4 Hz, 6.9 Hz; -CH=CH-C$\underline{\text{H}}_3$), 1.35 (2H, br s; DAP 4-C$\underline{\text{H}}_2$), 1.22-1.19 (6H, m; Lac-βC$\underline{\text{H}}_3$, Ala-βC$\underline{\text{H}}_3$).

^{13}C NMR (125 MHz, $CDCl_3$:CD_3OD 10:1): δ 174.9, 174.2, 173.8, 173.3, 172.4, 171.8, 156.6, 142.7, 141.8, 137.2, 136.3, 135.4, 129.2, 128.7, 128.6, 128.6, 128.4, 128.3, 128.1, 126.1, 105.8, 101.7, 97.4, 81.7, 78.3, 68.9, 67.3, 67.1, 63.4, 53.9, 53.4, 52.6, 52.2, 32.2, 31.7, 31.0, 28.7, 22.8, 21.7, 19.5, 17.2, 12.3.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{58}H_{70}N_6O_{16}Na$: 1129.4741 [M+Na]$^+$; found: 1129.4751.

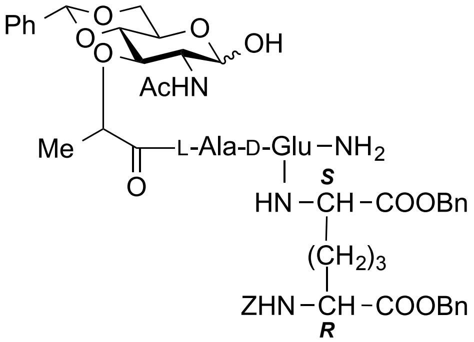


Protected monosaccharide containing *N*-acetylmuramyl group and tetrapeptide (71) Compound **71** was synthesized from **69** and **35** with similar method to the synthesis of **70**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 20:1) to give compound **71** as white solid (21 mg, 62%).

^{1}H NMR (500 MHz, $CDCl_3$:CD_3OD 8:1): δ 7.46-7.30 (20 H, m; Ar$\underline{\text{H}}$), 6.11 (1H, d, J = 12.1 Hz ; -O-C$\underline{\text{H}}$=CH-), 5.56 (1H, s; Ph-C$\underline{\text{H}}$=O_2), 5.15-5.08 (8H, m; -O-CH=C$\underline{\text{H}}$-, H-1, -C$\underline{\text{H}}_2$Ph × 3), 4.53-4.12 (8H, m; D-Ala-α$\underline{\text{H}}$, Ala-α$\underline{\text{H}}$, Gln-α$\underline{\text{H}}$, DAP 2-$\underline{\text{H}}$, 6-$\underline{\text{H}}$, Lac-α$\underline{\text{H}}$, H-2, H-5), 3.82-3.68 (4H, m; H-4, H-3, H-6, H-6’), 2.24 (3H, br s; Gln-γC$\underline{\text{H}}_2$, Gln-βC$\underline{\text{H}}$), 1.94 (3H, s; -NHCOC$\underline{\text{H}}_3$), 1.86-1.62 (5H, m; Gln-βC$\underline{\text{H}}$, DAP 3-C$\underline{\text{H}}_2$, 5-C$\underline{\text{H}}_2$), 1.56 (3H, d, J = 6.0 Hz; -CH=CH-C$\underline{\text{H}}_3$), 1.40-1.35 (11H, m; Lac-βC$\underline{\text{H}}_3$, Ala-βC$\underline{\text{H}}_3$, D-Ala-βC$\underline{\text{H}}_3$, DAP 4-C$\underline{\text{H}}_2$).

^{13}C NMR (125 MHz, $CDCl_3$:CD_3OD 8:1): δ 173.8,173.7, 173.1, 172.6, 172.5, 172.1, 171.4, 156.4, 142.5, 137.2, 136.3, 135.5, 135.4, 129.1, 128.7, 128.6, 128.6, 128.5, 128.3, 128.3, 128.2, 128.1, 128.0, 127.8, 126.0, 105.8, 101.6, 97.5, 81.1, 78.3, 68.8, 67.2, 671, 67.0, 67.0, 63.4, 54.0, 53.8, 53.0, 50.1, 31.8, 31.6, 31.0, 29.9, 23.0, 21.7, 19.4, 17.3, 17.0, 12.3.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{61}H_{75}N_7O_{17}Na$: 1200.5112 [M+Na]$^+$; found: 1200.5122.

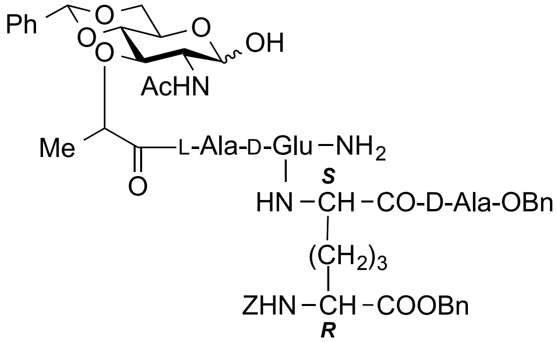


1-*O*-Deprotected monosaccharide containing *N*-acetylmuramyl group and tripeptide (72) Compound **72** was synthesized from **70** in the similar method to the synthesis of **59**. The crude product was purified by silica-gel flash column chromatography (CHCl$_3$/MeOH 15:1) to give compound **72** as white solid (20 mg, 56%).

^{1}H NMR (500 MHz, DMSO-d$_6$): δ 8.22 (1H, d, J = 7.5 Hz; NH), 8.13 (1H, d, J = 8.5 Hz; NH), 8.04 (1H, d, J = 7.8 Hz; NH), 7.72 (1H, d, J = 7.3 Hz; NH), 7.55 (1H, d, J = 7.0 Hz; NH), 7.44-7.26 (21 H, m; ArH, NH), 7.00 (1H, s; NH), 6.90 (1H, d, J = 4.6 Hz; 1-OH), 5.68 (1H, s; Ph-CH=O$_2$), 5.10-4.99 (7H, m; H-1, -CH$_2$Ph × 3), 4.30-3.65 (11H, m; Ala-αH, Gln-αH, DAP 2-H, 6-H, Lac-αH, H-2, H-3, H-4, H-5, H-6, H-6’), 2.14-2.11 (2H, m; Gln-γCH$_2$), 1.91 (1H, br s; Gln-βCH), 1.81 (3H, s; -NHCOCH$_3$), 1.72-1.56 (5H, m; Gln-βCH, DAP 3-CH$_2$, 5-CH$_2$), 1.34 (2H, br s; DAP 4-CH$_2$), 1.24-1.19 (6H, m; Lac-βCH$_3$, Ala-βCH$_3$).

^{13}C NMR (125 MHz, DMSO-d$_6$): δ 173.0, 172.1, 172.1, 171.9, 171.8, 169.5, 156.1, 137.7, 136.8, 135.9, 128.7, 128.3, 128.0, 128.0, 127.9, 127.8, 127.7, 127.6, 125.9, 125.8, 100.3, 91.4, 81.2, 76.7, 75.8, 68.1, 65.9, 65.7, 65.5, 62.1, 53.8, 53.7, 52.1, 51.9, 48.1, 31.5, 30.2, 30.1, 27.8, 22.6, 21.9, 18.9, 18.3.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{55}H_{66}N_6O_{16}Na$: 1089.4428 $[M+Na]^+$; found: 1089.4435.



1-*O*-Deprotected monosaccharide containing *N*-acetylmuramyl group and

tetrapeptide (73) Compound **73** was synthesized from **71** with similar method to the synthesis of **59**. The crude product was purified by silica-gel flash column chromatography (CHCl$_3$/MeOH 12:1) to give compound **73** as white solid (10.2 mg, 66%).

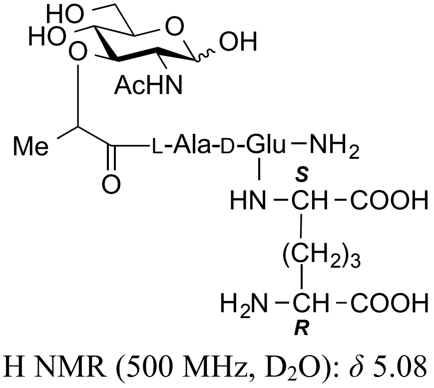
^{1}H NMR (500 MHz, DMSO-d$_6$): δ 8.32 (1H, d, J = 7.1 Hz; NH), 8.13 (1H, d, J = 8.0 Hz; NH), 8.05 (1H, d, J = 8.0 Hz; NH), 7.87 (1H, d, J = 8.0 Hz; NH), 7.70 (1H, d, J = 7.3 Hz; NH), 7.54 (1H, d, J = 7.0 Hz; NH), 7.44-7.26 (21 H, m; ArH, NH), 6.99 (1H, s; NH), 6.91 (1H, d, J = 4.2 Hz; -OH), 5.68 (1H, s; Ph-CH=O$_2$), 5.11-4.98 (7H, m; H-1, -CH$_2$Ph × 3), 4.29-3.60 (12H, m; D-Ala-αH, Ala-αH, Gln-αH, DAP 2-H, 6-H, Lac-αH, H-2, H-3, H-4, H-5, H-6, H-6’), 2.14-2.10 (2H, m; Gln-γCH$_2$), 1.93 (1H, br s; Gln-βCH), 1.81 (3H, s; -NHCOCH$_3$), 1.71-1.33 (7H, m; Gln-βCH, DAP 3-CH$_2$, 5-CH$_2$, DAP 4-CH$_2$), 1.26-1.19 (9H, m; D-Ala-βCH$_3$, Lac-βCH$_3$, Ala-βCH$_3$).

^{13}C NMR (125 MHz, DMSO-d$_6$): δ 173.1, 172.2, 172.2, 172.1, 172.0, 171.5, 171.3, 169.5, 156.1, 137.7, 136.8, 135.9, 135.9, 128.7, 128.4, 128.3, 128.3, 128.0, 127.9, 127.9, 127.8, 127.6, 127.6, 125.9, 125.8, 123.7, 123.1, 100.3, 91.4, 81.2, 76.7, 75.9, 68.1, 65.8, 65.8, 65.5, 62.1, 54.0, 53.7, 52.1, 51.9, 48.1, 47.6, 31.8, 31.7, 30.2, 27.9, 22.7, 21.8, 18.9, 18.2, 16.9.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{58}H_{71}N_7O_{17}Na$: 1160.4799 [M+Na]$^+$; found: 1160.4807.

Monosaccharide containing *N*-acetylmuramyl group and tripeptide (1a) and monosaccharide containing *N*-acetylmuramyl group and tetrapeptide (1c) Compounds **1a** and **1c** were synthesized from **72** and **73**, respectively, with the similar method to the synthesis of **1b**.

Compound **1a** (76%)

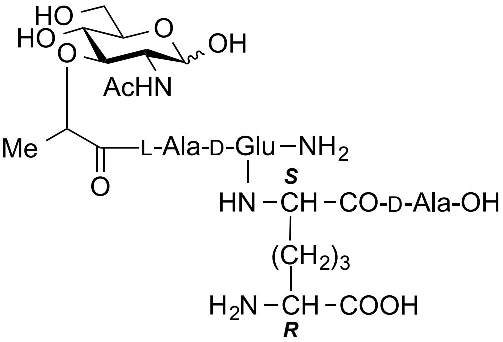


^{1}H NMR (500 MHz, D$_2$O): δ 5.08 (1H, d, J = 3.6 Hz; H-1β), 4.25-4.13 (4H, m; Ala-αH, Gln-αH, Lac-αH, DAP 2-H), 3.89-3.60 (5H, m; H-2, H-3, H-5, H-6, DAP 6-H), 3.51-3.38 (2H, m; H-4, H-6’), 2.33-2.30 (2H, m; Gln-γCH$_2$), 2.13-2.05 (1H, m; Gln-βCH), 1.96-1.89 (4H, m; Gln-βCH, -NHCOCH$_3$), 1.86-1.61 (4H, m; DAP 3-CH$_2$, 5-CH$_2$), 1.42-1.28 (8H, DAP 4-CH$_2$, Lac-βCH$_3$, Ala-βCH$_3$).

^{13}C NMR (125 MHz, D_2O): δ 176.5, 176.5, 175.7, 175.2, 175.1, 174.8, 174.5, 91.5, 80.2, 76.2, 72.0, 69.5, 61.0, 55.0, 54.7, 54.2, 53.5, 50.3, 32.2, 31.3, 30.6, 27.4, 22.5, 21.7, 19.2, 17.1.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{26}H_{44}N_6O_{14}Na$: 687.2808 $[M+Na]^+$; found: 687.2812.

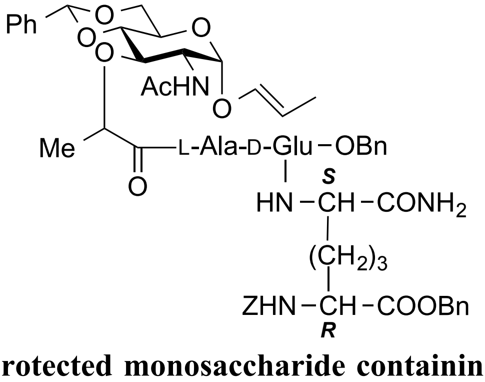
Compound **1c** (93%)



^{1}H NMR (500 MHz, D_2O): δ 5.08 (1H, d, J = 3.5 Hz; H-1β), 4.24-4.18 (5H, m; D-Ala-αH, Ala-αH, Gln-αH, Lac-αH, DAP 2-H), 3.90-3.60 (5H, m; H-2, H-3, H-5, H-6, DAP 6-H), 3.51-3.38 (2H, m; H-4, H-6'), 2.34-2.31 (2H, m; Gln-γCH_2), 2.14-2.05 (1H, m; Gln-βCH), 1.93-1.62 (8H, m; Gln-βCH, -NHCOCH_3, DAP 3-CH_2, 5-CH_2), 1.41-1.28 (11H, DAP 4-CH_2, Lac-βCH_3, Ala-βCH_3, D-Ala-βCH_3).

^{13}C NMR (125 MHz, D_2O): δ 180.4, 176.6, 176.5, 175.7, 175.5, 175.2, 174.7, 173.5, 91.6, 80.2, 76.4, 72.2, 69.7, 61.2, 55.2, 54.5, 54.3, 53.5, 51.6, 50.4, 32.2, 31.5, 30.7, 27.6, 22.7, 21.7, 19.3, 18.2, 17.3.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{29}H_{49}N_7O_{15}Na$: 758.3179 $[M+Na]^+$; found: 758.3191.



Protected monosaccharide containing *N*-acetylmuramyl group and tripeptide (78)

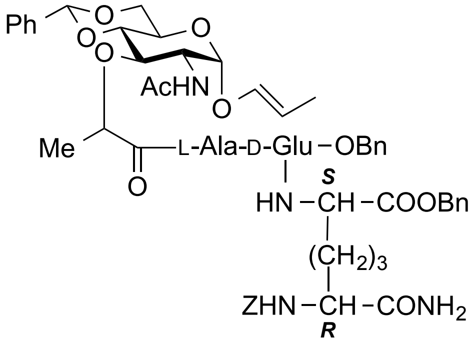
Compound **78** was synthesized from **69** and **50** with similar method to the synthesis of **70**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 30:1) to give compound **78** as white solid (46 mg, 51%).

^{1}H NMR (400 MHz, DMSO-d_6): δ 8.39 (1H, d, J = 7.3 Hz; NH), 8.16 (1H, d, J = 7.8 Hz; NH), 7.75 (1H, d, J = 8.0 Hz; NH), 7.66 (1H, d, J = 7.8 Hz; NH), 7.50 (1H, d, J = 7.8

Hz; NH), 7.36-7.22 (20 H, m; ArH), 6.88 (1H, s; NH), 6.14 (1H, d, *J* = 12.4 Hz; -O-CH=CH-), 5.63 (1H, s; Ph-CH=O_2), 5.03-4.91 (8H, m; -O-CH=CH-, H-1, -CH_2Ph × 3), 4.31-4.11 (3H, m; Ala-αH, Gln-αH, Lac-αH), 4.08-3.91 (4H, m; DAP 2-H, 6-H, H-2, H-4), 3.73-3.58 (4H, m; H-5, H-3, H-6, H-6'), 2.13-2.09 (2H, m; Gln-γCH_2), 1.97-1.91 (1H, m; Gln-βCH), 1.74 (3H, s; -NHCOCH_3), 1.75-1.50 (5H, m; Gln-βCH, DAP 3-CH_2, 5-CH_2), 1.43 (3H, d, *J* = 6.0 Hz; -CH=CH-CH_3), 1.26-1.14 (8H, m; DAP 4-CH_2, Lac-βCH_3, Ala-βCH_3).

^{13}C NMR (100 MHz, DMSO-d_6): δ 173.5, 172.2, 171.7, 171.4, 170.9, 169.7, 156.1, 143.5, 137.4, 136.8, 135.9, 135.8, 128.7, 128.4, 128.3, 128.1, 127.9, 127.8, 127.6, 125.9, 104.1, 100.3, 97.0, 80.6, 76.7, 75.6, 67.8, 65.9, 65.8, 65.5, 63.1, 54.0, 52.8, 52.0, 51.7, 47.7, 31.4, 31.2, 30.3, 26.8, 22.5, 21.9, 18.9, 18.9, 12.1.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{58}H_{71}N_6O_{16}$: 1107.4921 $[M+H]^+$; found: 1107.4941.

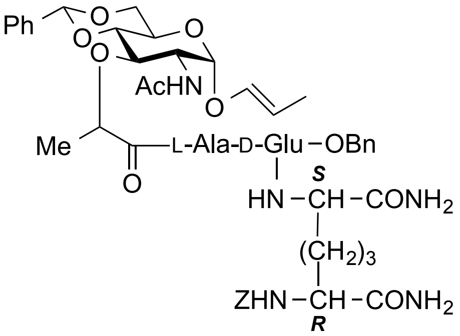


Protected monosaccharide containing *N*-acetylmuramyl group and tripeptide (79)

Compound **79** was synthesized from **69** and **51** with similar method to the synthesis of **70**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 30:1) to give compound **79** as white solid (19 mg, 55%).

^{1}H NMR (400 MHz, DMSO-d_6): δ 8.45 (1H, d, *J* = 7.6 Hz; NH), 8.22 (1H, d, *J* = 7.6 Hz; NH), 7.56 (1H, d, *J* = 8.0 Hz; NH), 7.44-7.19 (20 H, m; ArH), 7.20 (1H, d, *J* = 8.2 Hz; NH), 6.94 (1H, s; NH), 6.21 (1H, dd, *J* = 1.6 Hz, 12.4 Hz; -O-CH=CH-), 5.70 (1H, s; Ph-CH=O_2), 5.11-5.00 (8H, m; -O-CH=CH-, H-1, -CH_2Ph × 3), 4.38-4.11 (5H, m; Ala-αH, Gln-αH, Lac-αH, DAP 2-H, 6-H,), 4.02-3.64 (6H, m; H-2, H-4, H-5, H-3, H-6, H-6'), 2.20-2.16 (2H, m; Gln-γCH_2), 2.01-1.93 (1H, m; Gln-βCH), 1.80 (3H, s; -NHCOCH_3), 1.83-1.55 (5H, m; Gln-βCH, DAP 3-CH_2, 5-CH_2), 1.49 (3H, dd, *J* = 1.6 Hz, 6.9 Hz; -CH=CH-CH_3), 1.37-1.20 (8H, m; DAP 4-CH_2, Lac-βCH_3, Ala-βCH_3).

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{58}H_{71}N_6O_{16}$: 1107.4921 $[M+H]^+$; found: 1107.4941.

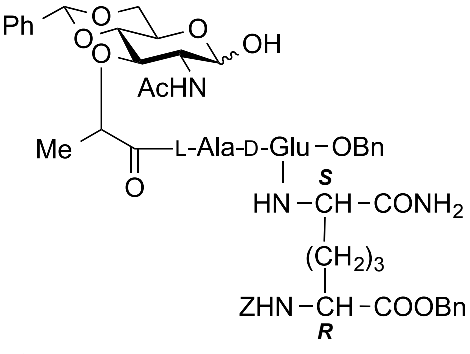


Protected monosaccharide containing *N*-acetylmuramyl group and tripeptide (80) Compound **80** was synthesized from **69** and **52** with similar method to the synthesis of **70**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 30:1) to give compound **80** as white solid (26 mg, 47%).

^{1}H NMR (400 MHz, DMSO-d_6): δ 8.45 (1H, d, J = 7.6 Hz; NH), 8.22 (1H, d, J = 7.8 Hz; NH), 7.82 (1H, d, J = 8.0 Hz; NH), 7.56 (1H, d, J = 7.8 Hz; NH), 7.44-7.26 (18 H, m; ArH, NH × 3), 7.20 (1H, d, J = 8.2 Hz; NH), 6.92 (1H, s; NH), 6.21 (1H, dd, J = 1.6 Hz, 12.4 Hz; -O-CH=CH-), 5.71 (1H, s; Ph-CH=O_2), 5.11-4.96 (6H, m; -O-CH=CH-, H-1, -CH_2Ph × 2), 4.39-4.09 (5H, m; Ala-αH, Gln-αH, Lac-αH, DAP 2-H, 6-H,), 4.02-3.62 (6H, m; H-2, H-4, H-5, H-3, H-6, H-6'), 2.20-2.16 (2H, m; Gln-γCH_2), 2.04-1.96 (1H, m; Gln-βCH), 1.81 (3H, s; -NHCOCH_3), 1.77-1.56 (5H, m; Gln-βCH, DAP 3-CH_2, 5-CH_2), 1.50 (3H, dd, J = 1.4 Hz, 6.9 Hz; -CH=CH-CH_3), 1.35-1.20 (8H, m; DAP 4-CH_2, Lac-βCH_3, Ala-βCH_3).

^{13}C NMR (100 MHz, DMSO-d_6): δ 173.8, 173.6, 172.2, 171.7, 171.4, 170.9, 169.7, 155.9, 143.5, 137.4, 137.0, 135.8, 128.7, 128.3, 128.2, 128.0, 127.9, 127.6, 127.6, 125.9, 104.1, 100.3, 97.0, 80.5, 76.7, 75.6, 67.7, 65.9, 65.3, 63.1, 54.4, 52.8, 52.2, 51.7, 47.6, 31.7, 31.2, 26.8, 22.5, 21.9, 18.9, 18.9, 12.0.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{51}H_{66}N_7O_{15}$: 1016.4611 $[M+H]^+$; found: 1016.4623.

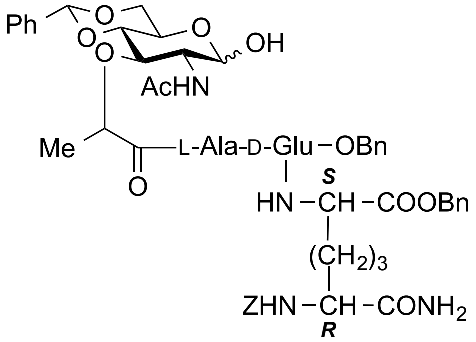


1-*O*-Deprotected monosaccharide containing *N*-acetylmuramyl group and tripeptide (81) Compound **81** was synthesized from **78** with similar method to the synthesis of **59**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 15:1) to give compound **81** as white solid (24 mg, 77%).

^{1}H NMR (400 MHz, DMSO-d$_6$): δ 8.44 (1H, d, J = 8.0 Hz; NH), 8.06 (1H, d, J = 8.3 Hz; NH), 7.82 (1H, d, J = 7.8 Hz; NH), 7.72 (1H, d, J = 7.6 Hz; NH), 7.51 (1H, d, J = 7.8 Hz; NH), 7.44-7.28 (21 H, m; ArH, NH), 6.94 (1H, s; NH), 6.90 (1H, d, J = 4.6 Hz; 1-OH), 5.68 (1H, s; Ph-CH=O$_2$), 5.12-4.98 (7H, m; H-1, -CH$_2$Ph × 3), 4.38-3.96 (6H, m; Ala-αH, Gln-αH, DAP 2-H, 6-H, Lac-αH, H-6), 3.90-3.60 (5H, m; H-2, H-3, H-4, H-5, H-6), 2.20-2.16 (2H, m; Gln-γCH$_2$), 2.04-1.93 (1H, br s; Gln-βCH), 1.79 (3H, s; -NHCOCH$_3$), 1.77-1.57 (5H, m; Gln-βCH, DAP 3-CH$_2$, 5-CH$_2$), 1.37-1.19 (8H, m; Lac-βCH$_3$, Ala-βCH$_3$, DAP 4-CH$_2$).

^{13}C NMR (100 MHz, DMSO-d$_6$): δ 173.5, 172.2, 171.7, 171.5, 170.9, 169.5, 156.1, 137.7, 136.8, 135.9, 135.8, 128.7, 128.4, 128.3, 128.0, 127.9, 127.8, 127.7, 127.6, 125.9, 125.8, 100.3, 91.4, 81.2, 76.7, 75.8, 68.1, 65.9, 65.8, 65.5, 62.1, 54.0, 53.7, 52.0, 51.6, 47.6, 31.4, 31.2, 30.3, 26.8, 22.6, 21.9, 18.9, 18.9.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{55}H_{66}N_6O_{16}Na$: 1089.4428 [M+Na]$^+$; found: 1089.4437.



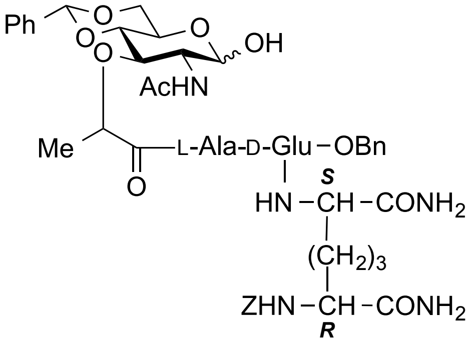
1-*O*-Deprotected monosaccharide containing *N*-acetylmuramyl group and tripeptide (82) Compound **82** was synthesized from **79** with similar method to the synthesis of **59**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 20:1) to give compound **82** as white solid (14 mg, 86%).

^{1}H NMR (400 MHz, DMSO-d$_6$): δ 8.44 (1H, d, J = 7.6 Hz; NH), 8.23 (1H, d, J = 7.4 Hz; NH), 8.06 (1H, d, J = 8.0 Hz; NH), 7.51 (1H, d, J = 8.0 Hz; NH), 7.44-7.27 (21 H, m; ArH, NH), 6.95 (1H, s; NH), 6.90 (1H, d, J = 3.9 Hz; 1-OH), 5.68 (1H, s; Ph-CH=O$_2$), 5.09-5.00 (7H, m; H-1, -CH$_2$Ph × 3), 4.39-4.11 (5H, m; Ala-αH, Gln-αH, DAP 2-H, Lac-αH, H-6), 3.89-3.85 (3H, m; DAP 6-H, H-2, H-5), 3.76-3.61 (3H, m; H-3, H-4, H-6’), 2.20-2.16 (2H, m; Gln-γCH$_2$), 2.00-1.95 (1H, br s; Gln-βCH), 1.79 (3H, s; -NHCOCH$_3$), 1.84-1.47 (5H, m; Gln-βCH, DAP 3-CH$_2$, 5-CH$_2$), 1.37-1.19 (8H, m;

Lac-βCH$_3$, Ala-βCH$_3$, DAP 4-CH$_2$).

^{13}C NMR (100 MHz, DMSO-d$_6$): *δ* 173.7, 172.2, 171.9, 171.7, 171.4, 169.5, 155.9, 137.7, 137.0, 135.9, 135.8, 128.7, 128.3, 128.2, 128.0, 127.9, 127.7, 127.6, 125.9, 100.3, 91.4, 81.2, 76.7, 75.8, 68.1, 65.9, 65.7, 65.3, 62.1, 54.1, 53.7, 52.1, 51.7, 47.6, 31.4, 31.1, 30.4, 26.8, 22.6, 21.9, 18.9, 18.9.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{55}H_{66}N_6O_{16}Na$: 1089.4428 [M+Na]$^+$; found: 1089.4437.



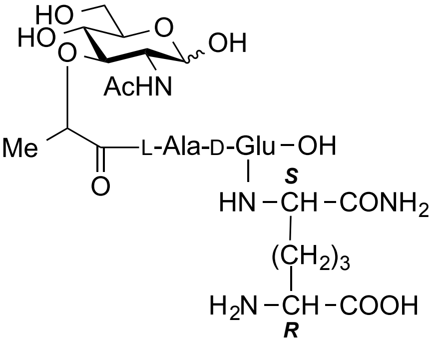
1-*O*-Deprotected monosaccharide containing *N*-acetylmuramyl group and tripeptide (83) Compound **83** was synthesized from **80** with similar method to the synthesis of **59**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 7:1) to give compound **83** as white solid (14 mg, 46%).

^{1}H NMR (500 MHz, DMSO-d$_6$): *δ* 8.45 (1H, d, *J* = 7.7 Hz; NH), 8.06 (1H, d, *J* = 8.2 Hz; NH), 7.83 (1H, d, *J* = 8.2 Hz; NH), 7.51 (1H, d, *J* = 7.9 Hz; NH), 7.44-7.21 (16 H, m; ArH, NH), 6.93 (1H, s; NH), 6.90 (1H, d, *J* = 3.7 Hz; 1-OH), 5.68 (1H, s; Ph-CH=O$_2$), 5.09-5.00 (5H, m; H-1, -CH$_2$Ph × 3), 4.36-4.05 (6H, m; Ala-αH, Gln-αH, DAP 2-H, 6-H, Lac-αH, H-6), 3.91-3.84 (2H, m; H-2, H-5), 3.76-3.59 (3H, m; H-3, H-4, H-6'), 2.19-2.16 (2H, m; Gln-γCH$_2$), 2.03-1.96 (1H, br s; Gln-βCH), 1.79 (3H, s; -NHCOCH$_3$), 1.76-1.42 (5H, m; Gln-βCH, DAP 3-CH$_2$, 5-CH$_2$), 1.37-1.19 (8H, m; Lac-βCH$_3$, Ala-βCH$_3$, DAP 4-CH$_2$).

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{48}H_{61}N_7O_{15}Na$: 998.4118 [M+Na]$^+$; found: 998.4130.

Monosaccharide containing *N*-acetylmuramyl group and tripeptide (1l) and (1m) Compounds **1l** and **1m** were synthesized from **81** and **82,** respectively, with similar method to the synthesis of **1b**.

Compounds **1l** (**quant.**)

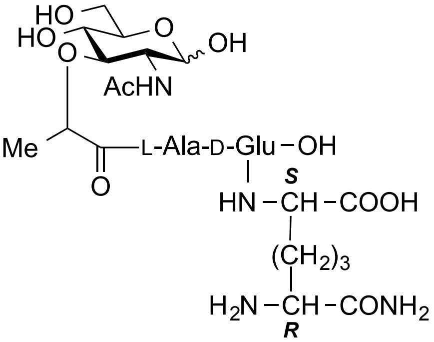


^{1}H NMR (700 MHz, D_2O): δ 5.13 (1H, d, J = 3.6 Hz; H-1β), 4.64 (1H, d, J = 8.4 Hz; H-1α), 4.28-4.20 (4H, m; Ala-α$\underline{H}$, Gln-α$\underline{H}$, Lac-α$\underline{H}$, DAP 2-$\underline{H}$), 3.94-3.64 (5H, m; H-2, H-3, H-5, H-6, DAP 6-$\underline{H}$), 3.62-3.48 (2H, m; H-4, H-6'), 2.34-2.30 (2H, m; Gln-γC$\underline{H}_2$), 2.16-2.12 (1H, m; Gln-βC$\underline{H}$), 1.95 (3H, s; -NHCOC$\underline{H}_3$), 1.94-1.69 (5H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.47-1.35 (8H, DAP 4-C$\underline{H}_2$, Lac-βC$\underline{H}_3$, Ala-βC$\underline{H}_3$).

^{13}C NMR (176 MHz, D_2O): δ 176.9, 176.1, 175.5, 174.4, 174.4, 174.3, 174.0, 91.0, 79.6, 77.8, 77.5, 71.6, 69.9, 61.5, 60.8, 53.5, 53.1, 49.8, 31.5, 30.6, 29.9, 27.2, 22.1, 21.0, 18.7, 16.9.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{26}H_{44}N_6O_{14}Na$: 687.2808 $[M+Na]^+$; found: 687.2817.

Compound **1m** (95%)

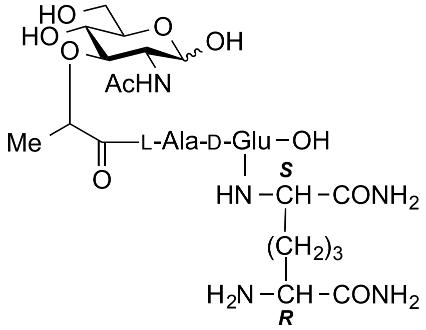


^{1}H NMR (400 MHz, D_2O): δ 5.09 (1H, d, J = 3.2 Hz; H-1β), 4.25-4.15 (4H, m; Ala-α$\underline{H}$, Gln-α$\underline{H}$, Lac-α$\underline{H}$, DAP 2-$\underline{H}$), 3.96-3.41 (7H, m; H-2, H-3, H-5, H-6, DAP 6-$\underline{H}$, H-4, H-6'), 2.27-2.23 (2H, m; Gln-γC$\underline{H}_2$), 2.06 (1H, br s; Gln-βC$\underline{H}$), 1.90 (3H, s; -NHCOC$\underline{H}_3$), 1.86-1.64 (5H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.43-1.30 (8H, DAP 4-C$\underline{H}_2$, Lac-βC$\underline{H}_3$, Ala-βC$\underline{H}_3$).

^{13}C NMR (100 MHz, D_2O): δ 176.7, 176.4, 175.6, 174.9, 174.9, 174.7, 172.3, 91.6, 80.2, 78.4, 76.4, 72.2, 69.7, 61.5, 61.2, 54.4, 53.5, 50.3, 32.6, 31.4, 31.0, 28.3, 22.7, 21.5, 19.3, 17.6.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{26}H_{44}N_6O_{14}Na$: 687.2808 $[M+Na]^+$; found:

687.2814.

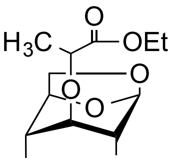


Monosaccharide containing *N*-acetylmuramyl group and tripeptide (1n)

Under argon atmosphere, *m*-cresol (8.6 μL, 0.082 mmol) and anisole (2.7 μL, 0.025 mmol) were added to compound **83** (0.8 mg, 0.00082 mmol) at room temperature. Then TMSOTf (4.5 μL, 0.025 mmol), thioanisole (2.9 μL, 0.025 mmol) and TFA (25μL) were added sequentially at 0 ^{o}C, and the mixture was allowed to react at 0 ^{o}C for 2 h. After that time, the mixture was washed by hexane and ether before adding water. The water solution was neutralized with pyridine and concentrated in vacuo. The residue was resuspended with ultrapure H_2O and lyophilized to give **1n** as pale white solid (0.5 mg, 92%).

^{1}H NMR (500 MHz, D_2O): δ 5.08 (1H, d, J = 3.3 Hz; H-1β), 4.23-4.11 (4H, m; Ala-α$\underline{H}$, Gln-α$\underline{H}$, Lac-α$\underline{H}$, DAP 2-$\underline{H}$), 3.92-3.37 (7H, m; H-2, H-3, H-5, H-6, DAP 6-$\underline{H}$, H-4, H-6’), 2.25-2.22 (2H, m; Gln-γC$\underline{H}_2$), 2.08-2.00 (1H, br s; Gln-βC$\underline{H}$), 1.90 (3H, s; -NHCOC$\underline{H}_3$), 1.86-1.64 (5H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.45-1.31 (8H, DAP 4-C$\underline{H}_2$, Lac-βC$\underline{H}_3$, Ala-βC$\underline{H}_3$).

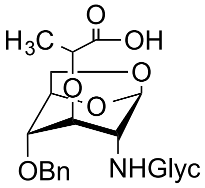
HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{26}H_{45}N_7O_{13}Na$: 686.2968 $[M+Na]^+$; found: 686.2976.



2-Glycolylamino-1,6-anhydro-2-deoxy-4-*O*-benzyl-3-*O*-[(1*R*)-1-(ethyoxycarbonyl)ethyl]-β-D-glucopyranose (65) Compound **64** (100.0 mg, 0.265 mmol) was dissolved in AcOH/THF 1:1, and zinc (172.2 mg, 2.65 mmol) was added to the above solution at 0 ^{o}C. After 30 min, zinc was removed by membrane filtration, and the filtrate was concentrated in vacuo. The residue was purified directly by silica-gel flash column chromatography ($CHCl_3$/MeOH 20:1) to give the reduced intermediate as white solid (83.8 mg, 90%). Glycolic acid (27.2 mg, 0.358 mmol) and the above intermediate were mixed in THF for 10 min before adding DMT-MM (132 mg, 0.477 mmol). The mixture was stirred at room temperature for further 4 h. Without evaporating THF, 10% citric acid solution was added to the mixture and extracted with $CHCl_3$. The organic layer was washed with saturated aqueous $NaHCO_3$, brine and dried over Na_2SO_4, and then concentrated in vacuo. The residue was purified directly by silica-gel flash column chromatography ($CHCl_3$/MeOH 25:1) to give compound **65** as white solid (81 mg, 84%).

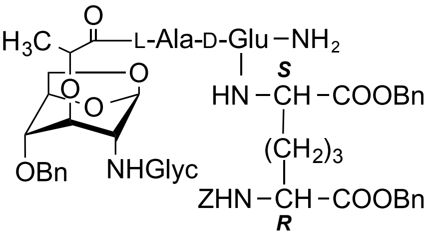
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.39-7.32 (5H, m; Ar$\underline{H}$), 6.97 (1H, d, J = 9.6 Hz; N$\underline{H}$), 5.37 (1H, s; H-1), 4.70 (1H, d, J = 11.8 Hz; PhC$\underline{H}$-O-), 4.61 (1H, d, J = 5.3 Hz; H-5), 4.58 (1H, d, J = 11.7 Hz; PhC$\underline{H}$-O-), 4.24–4.15 (5H, m; Lac-α$\underline{H}$, -COOC$\underline{H_2}$CH$_3$, H-2, H-6), 4.05 (1H, s; -NHCOC$\underline{H_2}$OH), 3.75 (1H, m; H-6'), 3.48 (1H, s; H-3), 3.44 (1H, s; H-4), 1.39 (3H, d, J = 6.9 Hz; Lac-βC$\underline{H_3}$), 1.29 (3H, t, J = 7 Hz; -COOCH$_2$C$\underline{H_3}$). ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.7, 170.9, 137.3, 128.7, 128.3, 127.9, 100.8, 76.6, 75.8, 74.3, 74.0, 71.5, 65.4, 62.0, 61.3, 49.7, 18.1, 14.1.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{20}H_{27}NO_8Na$: 432.1629 $[M+Na]^+$; found: 432.1631.



2-Glycolylamino-1,6-anhydro-2-deoxy-4-*O*-benzyl-3-*O*-[(1*R*)-1-(carbonyl)ethyl]-β-D-glucopyranose (66) Compound **65** (77 mg, 0.188 mmol) and LiOH·H_2O (8.68 mg, 0.207 mmol) were dissolved in THF/1,4-dioxane/H_2O 4:2:1 (3 mL) and stirred at room temperature for 1 h. Then the solution was neutralized with Dowex H^+ and filtrated to get clear solution. After evaporation, the residue was passed through HP-20 column, first washed by ultrapure H_2O to remove organic and inorganic salts, then by methanol to give compound **66** as white solid (71.7 mg, quant.).

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{18}H_{23}NO_8Na$: 404.1316 $[M+Na]^+$; found: 404.1328.

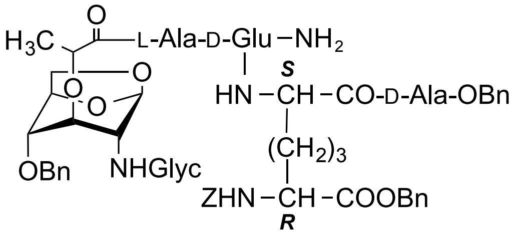


Protected monosaccharide (anh) containing *N*-glycolylmuramyl group and tripeptide (67) Compound **66** (22 mg, 0.058 mmol) and **34** (64 mg, 0.087 mmol) were dissolved to MeOH/H_2O 2:1 (12 mL), then triethylamine (16 μL, 0.116 mmol) was added. The mixture was stirred at room temperature for 10 min before adding DMT-MM, then allowed to react overnight. After dissolving the white solid to H_2O, the mixture was extracted with $CHCl_3$. The organic layer was washed with 10% citric acid solution, saturated aqueous $NaHCO_3$, brine and dried over Na_2SO_4, and then concentrated in vacuo. The residue was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 20:1) to give compound **67** as white solid (60 mg, 97%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.84 (1H, d, J = 6.6 Hz; NH), 7.55 (1H, d, J = 7.6 Hz; NH), 7.39-7.30 (21H, m; ArH, NH), 6.99 (1H, d, J = 9.5 Hz; NH), 6.73 (1H, s; NH), 5.70 (1H, d, J = 8.0 Hz; NH), 5.42 (1H, s; H-1), 5.33 (1H, s; NH), 5.17-5.05 (6H, m; -$COOCH_2Ph$ × 3), 4.71-4.66 (3H, m; PhCH-O-, H-5, Gln-αH), 4.56 (1H, d, J = 11.9 Hz; PhCH-O-), 4.48 (1H, m; DAP 2-H), 4.39-4.31 (2H, m; DAP 6-H, Ala-αH), 4.26 (1H, d, J = 7.9 Hz; H-6), 4.08-4.04 (2H, m; H-2, Lac-αH), 4.02 (2H, d, J = 5.2 Hz; -$NHCOCH_2OH$), 3.77 (1H, dd, J = 5.7 Hz, 7.5 Hz; H-6'), 3.48 (1H, s; H-3), 3.47 (1H, s; H-4), 2.46 (1H, s; -$NHCOCH_2OH$), 2.35-2.26 (2H, m; Gln-γCH_2), 2.08 (1H, br s; Gln-βCH), 1.96 (1H, br s; Gln-βCH), 1.87-1.69 (4H, m; DAP 3-CH_2, 5-CH_2), 1.47-1.33 (8H, DAP 4-CH_2, Lac-βCH_3, Ala-βCH_3).

^{13}C NMR (125 MHz, $CDCl_3$): δ 173.5, 173.5, 173.2, 173.0, 172.1, 171.2, 156.1, 137.1, 136.2, 135.3, 135.2, 128.7, 128.7, 128.6, 128.6, 128.5, 128.5, 128.4, 128.3, 128.2, 100.5, 76.5, 76.0, 75.8, 74.0, 71.7, 67.3, 67.2, 67.1, 65.1, 62.2, 53.7, 52.7, 51.8, 49.6, 47.1, 32.5, 31.2, 30.6, 30.2, 21.3, 17.8, 17.1.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{55}H_{66}N_6O_{16}Na$: 1089.4428 $[M+Na]^+$; found: 1089.4413.

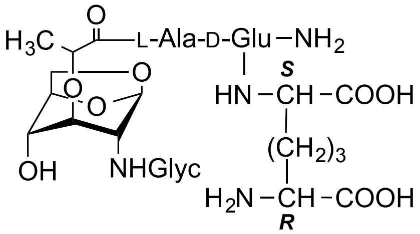


Protected monosaccharide (anh) containing *N*-glycolylmuramyl group and tetrapeptide (68) Compound **68** was synthesized from **66** and **35** with similar method to the synthesis of **67**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 16:1) to give compound **68** as white solid (18 mg, 51%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.88 (1H, d, J = 5.2 Hz; NH), 7.65 (1H, d, J = 7.3 Hz; NH), 7.56 (1H, d, J = 7.0 Hz; NH), 7.44 (1H, d, J = 7.7 Hz; NH),7.36-7.30 (20H, m; ArH), 7.18 (1H, s; NH), 7.09 (1H, d, J = 8.8 Hz; NH), 5.78 (1H, d, J = 7.2 Hz; NH), 5.49 (1H, s; N$\underline{H}$) , 5.41 (1H, s; H-1), 5.15-5.05 (6H, m; -COOC$\underline{H}_2$Ph × 3), 4.69-4.65 (2H, m; PhC$\underline{H}$-O-, H-5), 4.58-4.47 (3H, m; PhC$\underline{H}$-O-, Gln-α$\underline{H}$, DAP 2-$\underline{H}$), 4.37-4.30 (3H, m; D-Ala-α$\underline{H}$, DAP 6-$\underline{H}$, Ala-α$\underline{H}$), 4.23 (1H, d, *J* = 7.7 Hz; H-6), 4.10-4.05 (2H, m; H-2, Lac-αH), 4.02 (2H, br s; -NHCOC$\underline{H}_2$OH), 3.76 (1H,m; H-6’), 3.48 (1H, s; H-3), 3.45 (1H, s; H-4), 2.61 (1H, s; -NHCOCH$_2$O$\underline{H}$), 2.27 (2H, br s; Gln-γC$\underline{H}_2$), 2.14 (1H, br s; Gln-βC$\underline{H}$), 1.85-1.63 (5H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.39-1.31 (11H, DAP 4-C$\underline{H}_2$, Lac-βC$\underline{H}_3$, Ala-βC$\underline{H}_3$, D-Ala-βC$\underline{H}_3$).

^{13}C NMR (125 MHz, CDCl$_3$): δ 173.6, 173.5, 173.1, 172.8, 172.3, 171.9, 171.5, 156.2, 137.1, 135.3, 128.7, 128.6, 128.5, 128.4, 128.2, 128.1, 127.9, 127.8, 100.5, 75.9, 75.7, 73.9, 71.7, 67.1, 65.1, 62.2, 53.8, 53.2, 51.6, 49.7, 48.3, 47.3, 32.1, 30.8, 29.9, 21.6, 17.8, 17.5, 17.3.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{58}H_{71}N_7O_{17}Na$: 1160.4799 [M+Na]$^+$; found: 1160.4813.



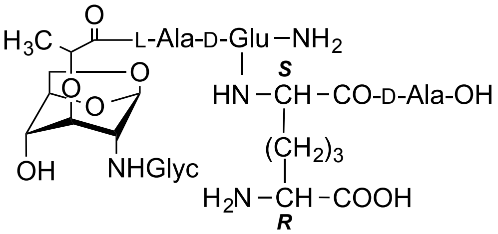
Monosaccharide (anh) containing *N*-glycolylmuramyl group and tripeptide (1f)

Compound **67** (50 mg, 0.0469) and Pd(OH)$_2$ (60 mg, 0.427 mmol) were mixed well in THF/H$_2$O/AcOH 1:1:0.1 (2.1 mL), and reacted under H$_2$ atmosphere (20 kg cm^{-2}) for 24 h. The Pd(OH)$_2$ was removed by membrane filtration, the filtrate was concentrated in vacuo. The residue was resuspended with ultrapure H$_2$O and lyophilized to give **1f** as white solid (20 mg, 65%).

^{1}H NMR (500 MHz, D$_2$O): δ 5.42 (1H, s; H-1), 4.63 (1H, d, *J* = 4.6 Hz; H-5), 4.30-4.20 (4H, m; Ala-α$\underline{H}$, Gln-α$\underline{H}$, H-6, DAP 2-$\underline{H}$), 4.11 (1H, q, *J* = 6.8 Hz; Lac-α$\underline{H}$), 4.01 (2H, d, *J* = 2.9 Hz; -NHCOC$\underline{H}_2$OH), 3.95 (1H, s; H-2), 3.84 (1H, s; H-4), 3.77-3.74 (2H, m; DAP 6-$\underline{H}$, H-6’), 3.35 (1H, s; H-3), 2.35-2.32 (2H, m; Gln-γC$\underline{H}_2$), 2.14-2.07 (1H, m; Gln-βC$\underline{H}$), 1.95-1.66 (5H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.45-1.40 (2H, m; DAP 4-C$\underline{H}_2$), 1.36 (3H, d, *J* = 7.2 Hz; Ala-βC$\underline{H}_3$), 1.30 (3H, d, *J* = 6.8 Hz; Lac-βC$\underline{H}_3$).

^{13}C NMR (125 MHz, D$_2$O): δ 176.3, 175.5, 175.4, 175.2, 175.1, 174.5, 101.7, 80.7, 77.5, 77.1, 70.3, 66.3, 62.5, 54.7, 54.1, 53.5, 50.7, 33.0, 32.1, 31.3, 30.1, 28.8, 22.6, 18.5, 17.9.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{26}H_{42}N_6O_{14}Na$: 685.2651 [M+Na]$^+$; found: 685.2661.

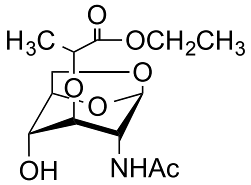


Monosaccharide (anh) containing *N*-glycolylmuramyl group and tetrapeptide (1h) Compound **1h** was synthesized from **68** with similar method to the synthesis of **1f** (quant.).

^{1}H NMR (500 MHz, D_2O): δ 5.56 (1H, s; H-1), 4.77 (1H, br s; H-5), 4.47-4.34 (5H, m; D-Ala-α<u>H</u>, Ala-α<u>H</u>, Gln-α<u>H</u>, H-6, DAP 2-<u>H</u>), 4.25 (1H, q, J = 6.8 Hz; Lac-α<u>H</u>), 4.15 (2H, d, J = 3.3 Hz; -NHCOC<u>H</u>$_2$OH), 4.09 (1H, s; H-2), 4.01-3.97 (2H, m; DAP 6-<u>H</u>, H-4), 3.89 (1H, dd, J = 5.7 Hz, 7.9 Hz; H-6'), 3.49 (1H, dd, J = 1.6 Hz, 1.6 Hz; H-3), 2.48-2.44 (2H, m; Gln-γC<u>H</u>$_2$), 2.28-2.21 (1H, m; Gln-βC<u>H</u>), 2.06-1.79 (5H, m; Gln-βC<u>H</u> , DAP 3-C<u>H</u>$_2$, 5-C<u>H</u>$_2$), 1.59-1.52 (2H, m; DAP 4-C<u>H</u>$_2$), 1.51-1.43 (9H, m; Ala-βC<u>H</u>$_3$, Lac-βC<u>H</u>$_3$, D-Ala-βC<u>H</u>$_3$).

^{13}C NMR (125 MHz, D_2O): δ 177.6, 176.9, 176.7, 176.1, 175.1, 174.7, 174.7, 100.9, 79.4, 77.0, 77.0, 68.8, 66.3, 61.9, 55.0, 54.7, 53.9, 50.8, 49.9, 49.6, 32.5, 31.7, 30.9, 27.9, 22.0, 19.1, 17.7, 17.3.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{29}H_{48}N_7O_{15}$: 734.3203 $[M+H]^+$; found: 734.3216.



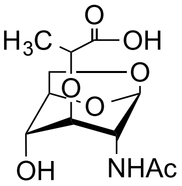
2-Acetylamino-1,6-anhydro-2-deoxy-3-*O*-[(1*R*)-1-(ethyoxycarbonyl)ethyl]-β-D-glucopyranose (74) $Pd(OH)_2$ was added to the THF solution (2 mL) of compound **64** (100 mg, 0.265 mmol) and the mixture as stirred for 36 h under H_2 atmosphere (15 kg cm^{-2}). After that time, $Pd(OH)_2$ was removed by membrane filtration, and the filtrate was concentrated in vacuo. Acetic anhydride (1 mL) and pyridine (1 mL) were added to the above residue and reacted for 20 min. The solution was concentrated in vacuo and the residue was purified directly by silica-gel flash column chromatography (toluene/AcOEt 15:1) to give compound **74** as white solid (61 mg, 76%).

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.42 (1H, d, J = 8.3 Hz; N<u>H</u>), 5.37 (1H, s; H-1), 4.50 (1H, d, J = 5.5 Hz; H-5), 4.29 (1H, d, J = 6.9 Hz; Lac-α<u>H</u>), 4.25–4.17 (3H, m;, -COOC<u>H</u>$_2$CH$_3$, H-6), 4.08 (1H, d, J = 8.7 Hz; H-2), 3.77-3.70 (2H, m; H-4, H-6'), 3.42

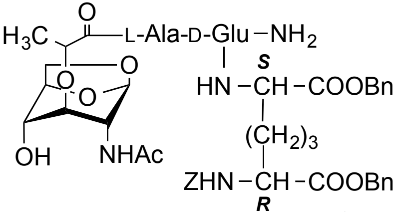
(1H, s; H-3), 2.00 (3H, s; -NHCOCH$_3$), 1.42 (3H, d, *J* = 6.9 Hz; Lac-βCH$_3$), 1.29 (3H, t, *J* = 7.1 Hz; -COOCH$_2$CH$_3$).

^{13}C NMR (100 MHz, CDCl$_3$) *δ* 172.9, 169.8, 101.1, 77.9, 74.1, 70.5, 65.3, 61.2, 50.3, 23.2, 18.1, 14.1.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{13}H_{21}NO_7Na$: 326.1210 [M+Na]$^+$; found: 326.1212.



2-Acetylamino-1,6-anhydro-2-deoxy-3-*O*-[(1*R*)-1-(carbonyl)ethyl]-β-D-glucopyranose (75) Compound **75** was synthesized from **74** with similar method to the synthesis of **56**. Compound **75** was used without further purification.



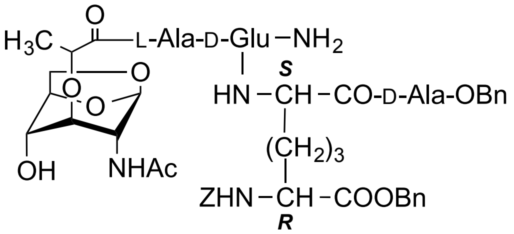
Protected monosaccharide (anh) containing *N*-acetylmuramyl group and tripeptide (76) Compound **76** was synthesized from **75** and **34** with similar method to the synthesis of **70**. The crude product was purified by silica-gel flash column chromatography (CHCl$_3$/MeOH 15:1) to give compound **76** as white solid (52.4 mg, 84%).

^{1}H NMR (500 MHz, CDCl$_3$:CD$_3$OD 20:1): *δ* 7.93 (1H, d, *J* = 6.2 Hz; NH), 7.73 (1H, d, *J* = 7.5 Hz; NH), 7.69 (1H, d, *J* = 7.5 Hz; NH), 7.36-7.25 (20H, m; ArH, NH), 6.97 (1H, d, *J* = 9.0 Hz; NH), 5.99 (1H, d, *J* = 8.5 Hz; NH), 5.9 (1H, s; NH), 5.39 (1H, s; H-1), 5.17-5.06 (6H, m; -CH$_2$Ph × 3), 4.54-4.43 (3H, m; DAP 2-H, H-5, Gln-αH), 4.33-4.28 (2H, m; DAP 6-H, Ala-αH), 4.25 (1H, d, *J* = 7.5 Hz; H-6), 4.11 (1H, d, *J* = 6.0 Hz; Lac-αH), 3.96 (1H, s; H-2), 3.75 (1H, dd, *J* = 6.0 Hz, 7.5 Hz; H-6’), 3.67 (1H, s; H-4), 3.38 (1H, s; H-3), 2.34-2.23 (2H, m; Gln-γCH$_2$), 2.11 (1H, br s; Gln-βCH), 1.98 (3H, s; -NHCOCH$_3$), 1.95-1.91(1H, m; Gln-βCH), 1.84-1.64 (4H, m; DAP 3-CH$_2$, 5-CH$_2$), 1.41-1.34 (8H, m; DAP 4-CH$_2$, Lac-βCH$_3$, Ala-βCH$_3$).

^{13}C NMR (125 MHz, CDCl$_3$:CD$_3$OD 20:1): *δ* 174.2, 173.9, 173.7, 173.4, 172.5, 172.4, 170.8, 136.3, 135.4, 128.7, 128.6, 128.6, 128.4, 128.3, 128.3, 128.1, 100.7, 78.9, 76.3, 75.4, 69.2, 67.3, 67.3, 67.1, 65.2, 53.9, 52.6, 52.0, 48.1, 32.2, 31.7, 30.9, 29.0, 22.8, 22.8, 21.6, 17.7, 17.0.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{48}H_{60}N_6O_{15}Na$: 983.4009 [M+Na]$^+$; found:

983.4005.

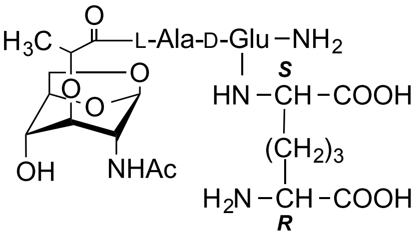


Protected monosaccharide (anh) containing *N*-acetylmuramyl group and tetrapeptide (77) Compound **77** was synthesized from **75** and **35** with similar method to the synthesis of **70**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 20:1) to give compound **77** as white solid (19 mg, 60%).

^{1}H NMR (500 MHz, $CDCl_3$:CD_3OD 20:1): δ 7.95 (1H, d, J = 6.5 Hz; N$\underline{H}$), 7.68 (1H, br s; N$\underline{H}$), 7.36-7.25 (20H, m; Ar$\underline{H}$), 6.90 (1H, d, J = 9.0 Hz; N$\underline{H}$), 5.99 (1H, d, J = 8.3 Hz; N$\underline{H}$), 5.70 (1H, s; N$\underline{H}$), 5.39 (1H, s; H-1), 5.16-5.01 (6H, m; -C$\underline{H}_2$Ph × 3), 4.56-4.52 (2H, m; D-Ala-α$\underline{H}$, Gln-α$\underline{H}$), 4.38 (1H, dd, J = 2.9 Hz, 10.8 Hz; H-5), 4.34-4.31 (2H, m; DAP 2-$\underline{H}$, 6-$\underline{H}$), 4.28-4.22 (2H, m; Ala-α$\underline{H}$, H-6), 4.11 (1H, q, J = 6.6 Hz; Lac-α$\underline{H}$), 3.98 (1H, s; H-2), 3.75 (1H, dd, J = 6.2 Hz, 6.2 Hz; H-6'), 3.67 (1H, s; H-4), 3.40 (1H, s; H-3), 2.29-2.20 (3H, m; Gln-γC$\underline{H}_2$, βC$\underline{H}$), 1.97 (3H, s; -NHCOC$\underline{H}_3$), 1.90-1.58 (5H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.44-1.35 (11H, m; DAP 4-C$\underline{H}_2$, D-Ala-βC$\underline{H}_3$, Lac-βC$\underline{H}_3$, Ala-βC$\underline{H}_3$).

^{13}C NMR (125 MHz, $CDCl_3$:CD_3OD 20:1): δ 174.0, 173.7, 173.0, 172.6, 172.5, 172.2, 170.8, 170.7, 135.6, 135.4, 128.7, 128.6, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 100.7, 78.9, 76.2, 75.4, 68.9, 67.2, 67.0, 65.2, 54.0, 53.5, 51.0, 48.4, 31,8, 31.5, 31.1, 29.8, 22.8, 21.7, 17.9, 17.0, 17.0.

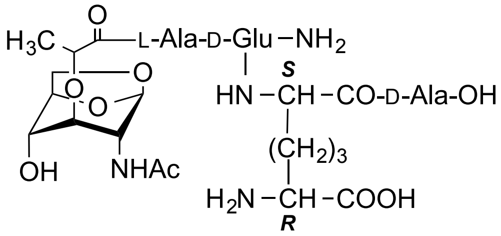
HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{51}H_{65}N_7O_{16}Na$: 1054.4380 [M+Na]$^+$; found: 1054.4386.



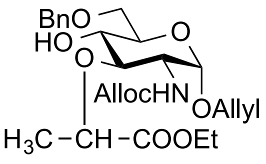
Monosaccharide (anh) containing *N*-acetylmuramyl group and tripeptide (1e) Compound **1e** was synthesized from **76** with similar method to the synthesis of **1f** (74%).

^{1}H NMR (500 MHz, D_2O): δ 5.41 (1H, s; H-1), 4.61 (1H, d, J = 5.6 Hz; H-5), 4.29-4.18 (4H, m; Ala-α$\underline{H}$, Gln-α$\underline{H}$, H-6, DAP 2-$\underline{H}$), 4.10 (1H, q, J = 6.9 Hz; Lac-α$\underline{H}$), 3.85 (1H, s;

H-2), 3.83-3.79 (2H, m; DAP 6-H, H-4), 3.73 (1H, dd, *J* = 5.9 Hz, 7.7 Hz; H-6'), 3.33 (1H, s; H-3), 2.35-2.32 (2H, m; Gln-γCH_2), 2.14-2.06 (1H, m; Gln-βCH), 1.94 (3H, s; -NHCOCH_3), 1.91-1.67 (5H, m; Gln-βCH, DAP 3-CH_2, 5-CH_2), 1.45-1.40 (2H, m; DAP 4-CH_2), 1.36 (3H, d, *J* = 7.2 Hz; Ala-βCH_3), 1.29 (3H, d, *J* = 6.9 Hz;, Lac-βCH_3).
^{13}C NMR (125 MHz, D_2O): *δ* 177.1, 177.0, 176.8, 176.2, 176.1, 174.8, 174.7, 101.1, 79.6, 77.0, 77.0, 69.2, 66.3, 55.1, 54.0, 50.8, 50.6, 32.6, 31.3, 30.8, 27.9, 22.9, 22.1, 19.1, 17.8.
HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{26}H_{43}N_6O_{13}$: 647.2883 $[M+H]^+$; found: 647.2905.



Monosaccharide (anh) containing *N*-acetylmuramyl group and tetrapeptide (1g)
Compound **1g** was synthesized from **77** with similar method to the synthesis of **1f** (77%).
^{1}H NMR (500 MHz, D_2O): *δ* 5.41 (1H, s; H-1), 4.61 (1H, d, *J* = 5.7 Hz; H-5), 4.33-4.18 (5H, m; Ala-αH, D-Ala-αH, Gln-αH, H-6, DAP 2-H), 4.10 (1H, q, *J* = 6.9 Hz; Lac-αH), 3.90-3.85 (2H, m; DAP 6-H, H-2), 3.79 (1H, d, *J* = 1.3 Hz; H-4), 3.73 (1H, dd, *J* = 5.8 Hz, 7.8 Hz; H-6'), 3.33 (1H, dd, *J* = 1.6 Hz, 1.6 Hz; H-3), 2.34-2.30 (2H, m; Gln-γCH_2), 2.14-2.06 (1H, m; Gln-βCH), 1.93 (3H, s; -NHCOCH_3), 1.90-1.65 (5H, m; Gln-βCH, DAP 3-CH_2, 5-CH_2), 1.45-1.40 (2H, m; DAP 4-CH_2), 1.36 (3H, d, *J* = 7.3 Hz; Ala-βCH_3), 1.33 (3H, d, *J* = 7.3 Hz; D-Ala-βCH_3), 1.29 (3H, d, *J* = 6.9 Hz;, Lac-βCH_3).
^{13}C NMR (125 MHz, D_2O): *δ* 176.8, 176.4, 176.3, 175.6, 175.6, 174.3, 174.2, 173.4, 100.5, 79.0, 76.4, 68.5, 65.7, 54.1, 53.8, 53.3, 50.3, 50.0, 49.3, 32.0, 31.1, 30.1, 27.4, 22.3, 21.3, 18.6, 17.1, 16.6.
HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{29}H_{48}N_7O_{14}$: 718.3254 $[M+H]^+$; found: 718.3269.

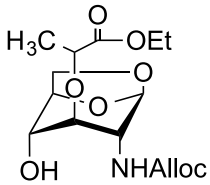


1-Allyl-2-allyloxycarbonylamino-6-benzyloxy-2-deoxy-4-hydroxyl-3-*O*-[(*R*)-1-(ethoxycarbonyl)ethyl]-α-D-glucopyranoside (84) To a solution of **53** (1.0 g, 2.03 mmol) and trimethylamine-borane (297 mg, 4.07 mmol) in dry CH_3CN (6 mL) at 0 °C was added boron trifluoride diethyl etherate (1.54 mL, 12.18 mmol) dropwise and the mixture was stirred at 0 °C for 30 min and then at room temperature for 1 h. The reaction was quenched with saturated aqueous $NaHCO_3$ and extracted with ethyl acetate. The organic layer was washed with aqueous 10% citric acid, saturated aqueous $NaHCO_3$, and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel flash chromatography (toluene/AcOEt 8:1) to give compound **84** as white solid (502 mg, 50%).

^{1}H NMR (500 MHz, DMSO-d_6): δ 7.37-7.26 (5H, m; Ar$\underline{H}$), 7.50 (1H, d, J = 5.9 Hz; N$\underline{H}$), 5.88 (2H, m; -O-CH$_2$-C$\underline{H}$=CH$_2$ × 2), 5.47 (1H, d, J = 7.2 Hz; 4-O$\underline{H}$), 5.27 (2H, dd, J = 1.2 Hz, 17.2 Hz; -O-CH$_2$-CH=C$\underline{H}$H × 2), 5.17 (1H, ddd, J = 1.3 Hz, 3.0 Hz, 10.5 Hz; -O-CH$_2$-CH=CH$\underline{H}$), 5.12 (1H, dd, J = 1.5 Hz, 10.5 Hz; -O-CH$_2$-CH=CH$\underline{H}$), 4.92 (1H, d, J = 3.3 Hz; H-1), 4.61 (1H, q, J = 6.9 Hz; Lac-α$\underline{H}$), 4.51-4.46 (4H, m; PhC$\underline{H}_2$-O-, -O-C$\underline{H}_2$-CH=CH$_2$), 4.16-4.07 (3H, m; -O-C$\underline{H}$H-CH=CH$_2$, -COOC$\underline{H}_2$CH$_3$), 3.93 (1H, dd, J = 5.6 Hz, 13.6 Hz; -O-CH$\underline{H}$-CH=CH$_2$), 3.67 (1H, m; H-6), 3.60-3.56 (2H, m; H-5, H-6’), 3.49 (1H, dd, J = 8.8 Hz, 10.8 Hz; H-3), 3.39-3.32 (2H, m; H-2, H-4), 1.31 (3H, d, J = 6.9 Hz; Lac-βC$\underline{H}_3$), 1.20 (3H, t, J =7.2 Hz; -COOCH$_2$C$\underline{H}_3$).

^{13}C NMR (125 MHz, DMSO-d_6): δ 174.3, 155.6, 138.6, 134.4, 133.6, 128.2, 127.3, 127.3, 116.9, 116.6, 95.6, 77.7, 74.9, 72.3, 71.7, 70.8, 69.1, 67.3, 64.4, 60.7, 54.8, 18.7, 13.9.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{25}H_{35}NO_9Na$: 516.2204 [M+Na]$^+$; found: 516.2205.

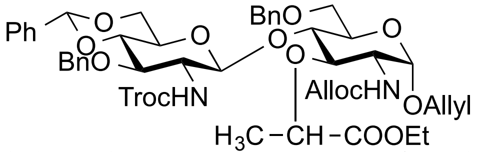


2-Allyloxycarbonylamino-1,6-anhydro-2-deoxy-4-hydroxyl-3-*O*-[(1*R*)-1-(ethyoxycarbonyl)ethyl]-β-D-glucopyranose (85) Compound **64** (580 mg, 1.537 mmol) and $Pd(OH)_2$ (600 mg, 4.27 mmol) were mixed well in THF (6 mL), and reacted under H_2 atmosphere (15 kg cm^{-2}) for 36 h. The $Pd(OH)_2$ was removed by membrane filtration, the filtrate was concentrated in vacuo. Without any further purification, the residue was dissolved in anhydrous DCM (12 mL) under argon protection. Triethylamine (320 µL, 2.31 mmol) was syringed to the solution at 0 °C, followed by allyl chloroformate (196 µL, 1.84 mmol). Then the reaction was allowed to react at room temperature for 1.5 h. The reaction was quenched with saturated aqueous $NaHCO_3$ and extracted with $CHCl_3$. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel flash chromatography ($CHCl_3$/MeOH 40:1) to give compound **85** as white solid (300 mg, 57%).

^{1}H NMR (500 MHz, CDCl$_3$): δ 5.95-5.87 (1H, m; -O-CH$_2$-C$\underline{H}$=CH$_2$), 5.57 (1H, d, J = 8.5 Hz; N$\underline{H}$), 5.41 (1H, s; H-1), 5.31 (1H, d, J = 17.2 Hz; -O-CH$_2$-CH=C$\underline{H}$H), 5.22 (1H, d, J = 10.5 Hz; -O-CH$_2$-CH=CH$\underline{H}$), 4.60-4.55 (2H, m; -O-C$\underline{H}_2$-CH=CH$_2$), 4.52 (1H, d, J = 5.3 Hz; H-5), 4.25-4.19 (4H, m; H-6, -COOC$\underline{H}_2$CH$_3$, Lac-αH), 3.86 (1H, d, J = 9.2 Hz; H-2), 3.76 (1H, dd, J = 5.9 Hz, 7.2Hz; H-6'), 3.73 (1H, s; H-4), 3.47 (1H, s; H-3), 2.82 (1H, s; O$\underline{H}$), 1.42 (3H, d, J = 6.8 Hz; Lac-βC$\underline{H}_3$), 1.28 (3H, t, J = 7.2 Hz; -COOCH$_2$C$\underline{H}_3$).

^{13}C NMR (125 MHz, CDCl$_3$): δ 158.1, 144.3, 126.0, 114.2, 100.8, 80.9, 79.3, 75.8, 72.6, 72.1, 68.9, 61.1, 34.4, 31.2, 19.9.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{15}H_{23}NO_8Na$: 368.1316 [M+Na]$^+$; found: 368.1318.

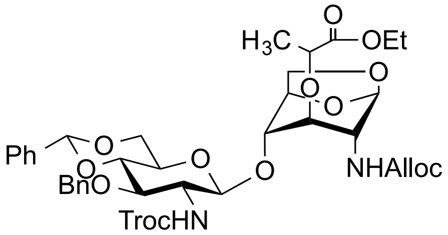


1-Allyl-2-allyloxycarbonylamino-6-*O*-benzyl-4-*O*-(3'-*O*-benzyl-4',6'-*O*-benzylidene -2'-deoxy-2'-(2,2,2-trichlorethoxycarbonylamino)-β-D-glucopyranosyl)-2-deoxy-3-*O*-[(*R*)-1-(ethoxycarbonyl)ethyl]-α-D-glucopyranoside (87) TMSOTf (6 μL, 0.0608 mmol) was added to a mixture of the imidate **86** (309 mg, 0.456 mmol), the acceptor **84** (150 mg, 0.304 mmol), and MS4A 4 Å molecular sieves in dry CH$_2$Cl$_2$ (8 mL) at -15 ^{o}C. After the mixture had been stirred at the same temperature for 10 min, the reaction was quenched with chilled saturated aqueous NaHCO$_3$, filtrated to remove the solid. The filtrate was extracted with CH$_2$Cl$_2$, and the organic layer was washed with brine, dried over Na$_2$SO$_4$, and concentrated in vacuo. The residue was purified by silica-gel flash chromatography (toluene/AcOEt 10:1) to give compound **87** as white solid (390 mg, 95%).

^{1}H NMR (500 MHz, CDCl$_3$): δ 7.53-7.12 (15H, m; Ar$\underline{H}$), 7.08 (1H, s; N$\underline{H}$), 6.00-5.80 (2H, m; -O-CH$_2$-C$\underline{H}$=CH$_2$ × 2), 5.57 (1H, s, Ph-C$\underline{H}$=O$_2$), 5.31-5.13 (5H, m; -O-CH$_2$-CH=C$\underline{H}_2$ ×2, H-1), 4.88 (1H, d, J = 12.2 Hz; Ph-C$\underline{H}$-O-), 4.87 (1H, d, J = 11.9 Hz; Ph-C$\underline{H}$-O-), 4.65 (1H, d, J = 12.2 Hz; Ph-C$\underline{H}$-O-), 4.84 (1H, d, J = 12.5 Hz; -O-C$\underline{H}$-CCl$_3$), 4.61-4.52 (4H, m; -O-C$\underline{H}$-CCl$_3$, -O-C$\underline{H}_2$-CH=CH$_2$, Lac-α$\underline{H}$), 4.41 (1H, dd, J = 5.1 Hz, 10.5 Hz; H-6'), 4.29-4.11 (4H, m; Ph-C$\underline{H}$-O-, -O-C$\underline{H}_2$-CH$_3$, H-1'), 4.06 (1H, ddt, J = 1.3 Hz, 5.5 Hz, 13.1 Hz; -O-C$\underline{H}$-CH=CH$_2$), 3.97 (1H, dd, J = 5.5 Hz, 13.1 Hz; -O-C$\underline{H}$-CH=CH$_2$), 3.92 (1H, m; H-3), 3.77-3.56 (7H, m; H-6, H-6, H-6', H-4', H-4, H-2, $\underline{N}$H), 3.49-3.41 (2H, m; H-2', H-5), 3.26-3.19 (2H, m; H-3', H-5'), 1.33 (3H, d, J = 7.1 Hz; Lac-βC$\underline{H}_3$), 1.28 (3H, t, J = 7.2 Hz; -COOCH$_2$C$\underline{H}_3$).

^{13}C NMR (125 MHz, CDCl$_3$): δ 175.4, 156.5, 154.0, 138.2, 137.7, 137.2, 134.0, 129.1, 129.1, 128.7, 128.4, 128.4, 128.3, 128.2, 127.8, 126.0, 117.3, 116.9, 101.2, 100.9, 96.5, 82.3, 77.9, 77.8, 75.2, 74.7, 74.6, 74.2, 73.8, 69.9, 69.0, 68.8, 67.1, 67.0, 65.6, 65.2, 61.3, 57.5, 55.2, 18.9, 14.1.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{48}H_{57}Cl_3N_2O_{15}Na$: 1031.2701 [M+Na]$^+$; found: 1031.2706.

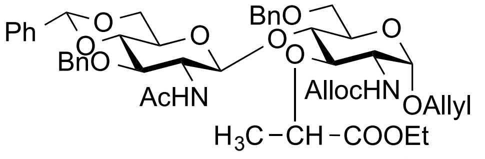


1,6-Anhydro-2-allyloxycarbonylamino-4-*O*-[3’-*O*-benzyl-4’,6’-*O*-benzylidene-2’-deoxy-2’-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl]- -2-deoxy-3-*O*-[(*R*)-1-(ethoxycarbonyl)ethyl]-D-glucopyranoside (88) Compound **88** was synthesized from **86** and **85** with similar method to the systhesis of **87**. The crude product was purified by silica-gel flash chromatography (toluene/AcOEt 5:1) to give compound **88** as white solid (149 mg, 50%).

^{1}H NMR (500 MHz, CDCl$_3$): δ 7.51-7.29 (10H, m; Ar$\underline{H}$), 5.98-5.90 (1H, m; -O-CH$_2$-C$\underline{H}$=CH$_2$), 5.69 (1H, br s; N$\underline{H}$), 5.60 (1H, s, Ph-C$\underline{H}$=O$_2$), 5.36-5.32 (2H, m; -O-CH$_2$-CH=C$\underline{H}$H, H$_{anh}$-1), 5.21 (1H, dd, J = 1.3 Hz, 10.5 Hz; -O-CH$_2$-CH=CH$\underline{H}$), 4.99 (1H, d, J = 5.9 Hz; N$\underline{H}$), 4.91 (1H, d, J = 11.9 Hz; Ph-C$\underline{H}$-O-), 4.70 (1H, d, J = 11.9 Hz; Ph-C$\underline{H}$-O-), 4.83-4.80 (2H, m; -O-C$\underline{H}$-CCl$_3$, H-1), 4.61-4.54 (3H, m; -O-C$\underline{H}$-CCl$_3$, -O-C$\underline{H}_2$-CH=CH$_2$), 4.47 (1H, d, J = 5.8 Hz; H$_{anh}$-5), 4.33 (1H, dd, J = 4.9 Hz, 10.5 Hz; H-6), 4.24-4.16 (4H, m; -O-C$\underline{H}_2$CH$_3$, Lac-α$\underline{H}$, H$_{anh}$-6), 3.91 (1H, d, J = 9.1 Hz; H$_{anh}$-2), 3.86 (1H, dd, J = 9.5 Hz, 9.5 Hz; H-3), 3.81-3.70 (4H, m; H-6’, H-4, H$_{anh}$-6’, H$_{anh}$-4), 3.56 (1H, s; H$_{anh}$-3), 3.50-3.42 (2H, m; H-2, H-5), 1.41 (3H, d, J = 6.9 Hz; Lac-βC$\underline{H}_3$), 1.28 (3H, t, J = 7.2 Hz; -COOCH$_2$C$\underline{H}_3$).

^{13}C NMR (125 MHz, CDCl$_3$): δ 172.5, 155.7, 154.4, 137.8, 137.1, 132.9, 129.1, 128.6, 128.5, 128.4, 128.1, 126.0, 117.6, 101.4, 101.3, 95.4, 82.4, 75.8, 74.9, 74.6, 74.4, 74.3, 68.5, 66.3, 65.6, 64.8, 61.2, 57.7, 51.2, 18.1, 14.1.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{38}H_{45}Cl_3N_2O_{14}Na$: 883.1809 [M+Na]$^+$; found: 883.1816.



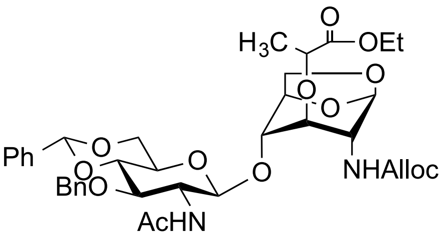
1-Allyl-2-allyloxycarbonylamino-6-*O*-benzyl-4-*O*-(3’-*O*-benzyl-4’,6’-*O*-benzylidene

-2’-deoxy-2’-ethylamino-β-D-glucopyranosyl)-2-deoxy-3-*O*-[(*R*)-1-(ethoxycarbonyl)ethyl]-α-D-glucopyranoside (89) Zn/Cu (prepared from 2 g of Zn) was added to a solution of **87** (380 mg, 0.377 mmol) in AcOH/THF 1:1 (10 mL), and the mixture was stirred at room temperature for 1 h. The insoluble materials were filtered off, and the filtrate was concentrated in vacuo. The residual solvent was removed by coevaporation with toluene. The residue was dissolved in pyridine (5 mL) and acetic anhydride (5 mL), and the solution was stirred at room temperature overnight. The reagents were removed by concentration with toluene. The residue was purified by silica-gel flash chromatography (toluene/AcOEt 3:1) to give compound **89** as white solid (231 mg, 70%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.53-7.14 (15H, m; Ar$\underline{H}$), 7.06 (1H, s; N$\underline{H}$), 5.97-5.81 (2H, m; -O-CH_2-C$\underline{H}$=CH_2 × 2), 5.58 (1H, s, Ph-C$\underline{H}$=O_2), 5.31-5.14 (5H, m; -O-CH_2-CH=C$\underline{H}_2$ × 2, H-1), 4.88 (1H, d, J = 12.2 Hz; Ph-C$\underline{H}$-O-), 4.83 (1H, d, J = 12.2 Hz; Ph-C$\underline{H}$-O-), 4.64 (1H, d, J = 12.2 Hz; Ph-C$\underline{H}$-O-), 4.36 (1H, d, J = 12.2 Hz; Ph-C$\underline{H}$-O-), 4.62-4.56 (3H, m; -O-C$\underline{H}_2$-CH=CH_2, Lac-α$\underline{H}$), 4.48-4.44 (2H, m; H-1’, N$\underline{H}$), 4.40 (1H, dd, J = 5.1 Hz, 10.3 Hz; H-6’), 4.27-4.08 (3H, m; -O-C$\underline{H}_2$$CH_3$, -O-C$\underline{H}$-CH=$CH_2$), 4.00-3.95 (2H, m; -O-C$\underline{H}$-CH=CH_2, H-3), 3.76 (1H, dd, J = 10.5 Hz, 10.5 Hz; H-6’), 3.69-3.57 (7H, m; H-6, H-6, H-4’, H-4, H-2, H-2’, H-3’), 3.47 (1H, m; H-5), 3.29 (1H, m; H-5’), 1.74 (3H, s; -NHCOC$\underline{H}_3$), 1.35 (3H, d, J = 7.1 Hz; Lac-βC$\underline{H}_3$), 1.28 (3H, t, J = 7.3 Hz; -COOCH_2C$\underline{H}_3$).

^{13}C NMR (125 MHz, $CDCl_3$): δ 175.4, 169.8, 156.5, 138.5, 138.0, 137.3, 134.0, 133.5, 129.2, 129.1, 128.8, 128.5, 128.3, 128.1, 127.8, 126.0, 117.1, 116.9, 101.3, 100.3, 96.4, 82.7, 77.6,75.2, 74.8, 74.1, 73.6, 70.2, 68.9, 67.3, 65.7, 65.2, 61.3, 56.1, 55.2, 23.4, 18.8, 14.1.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{47}H_{58}N_2O_{14}Na$: 897.3780 $[M+Na]^+$; found: 897.3791.

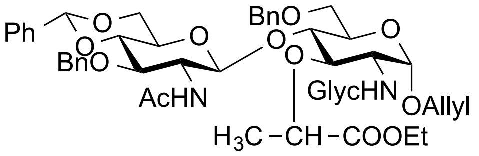


1,6-Anhydro-2-allyloxycarbonylamino-4-*O*-[2’-acetylamino-3’-*O*-benzyl-4’,6’-*O*-benzylidene-2’-deoxy-β-D-glucopyranosyl]-2-deoxy-3-*O*-[(*R*)-1-(ethoxycarbonyl)ethyl]-D-glucopyranoside(90) Compound **90** was synthesized from **88** with similar method to the synthesis of **89**. The crude product was purified by silica-gel flash chromatography (toluene/AcOEt 1:1) to give compound **90** as white solid (83 mg, 76%).

^{1}H NMR (500 MHz, CDCl$_3$): δ 7.50-7.30 (10H, m; ArH), 5.97-5.90 (1H, m; -O-CH$_2$-CH=CH$_2$), 5.61-5.58 (2H, m; NH, Ph-CH=O$_2$), 5.49 (1H, d, J = 7.2 Hz; NH), 5.35-5.31 (2H, m; -O-CH$_2$-CH=CHH, H$_{anh}$-1), 5.21 (1H, dd, J = 1.1 Hz, 10.5 Hz; -O-CH$_2$-CH=CHH), 5.13 (1H, d, J = 8.1 Hz; H-1), 4.90 (1H, d, J = 11.9 Hz; Ph-CH-O-), 4.65 (1H, d, J = 11.9 Hz; Ph-CH-O-), 4.61-4.54 (2H, m; -O-CH$_2$-CH=CH$_2$), 4.45 (1H, d, J = 4.6 Hz; H$_{anh}$-5), 4.33 (1H, dd, J = 4.9 Hz, 10.5 Hz; H-6), 4.23-4.13 (5H, m; -O-CH$_2$CH$_3$, Lac-αH, H$_{anh}$-6, H-3), 3.90 (1H, d, J = 9.4 Hz; H$_{anh}$-2), 3.79-3.67 (4H, m; H-6', H-4, H$_{anh}$-6', H$_{anh}$-4), 3.55-3.49 (2H, m; H$_{anh}$-3, H-5), 3.39-3.34 (1H, m; H-2), 1.89 (3H, s; -NHCOCH$_3$), 1.41 (3H, d, J = 6.9 Hz; Lac-βCH$_3$), 1.28 (3H, t, J = 7.2 Hz; -COOCH$_2$CH$_3$).

^{13}C NMR (125 MHz, CDCl$_3$): δ 172.5, 171.0, 155.6, 138.2, 137.2, 132.9, 129.1, 128.5, 128.4, 128.3, 128.0, 126.0, 117.7, 101.3, 101.2, 99.0, 82.6, 76.0, 75.1, 74.5, 74.3, 72.9, 68.6, 66.2, 65.7, 64.9, 61.2, 57.6, 51.1, 23.6, 18.2, 14.1.

HRMS (ESI-LIT-orbitrap): m/z: calcd for C$_{37}$H$_{46}$N$_2$O$_{13}$Na: 749.2892 [M+Na]$^+$; found: 749.2897.

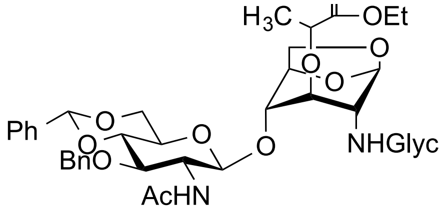


1-Allyl-2-glycolylamino-6-*O*-benzyl-4-*O*-(3'-*O*-benzyl-4',6'-*O*-benzylidene-2'-deoxy-2'-ethylamino-β-D-glucopyranosyl)-2-deoxy-3-*O*-[(*R*)-1-(ethoxycarbonyl)ethyl]-α-D-glucopyranoside (91) Compound **91** was synthesized from **89** with similar method to the synthesis of **54**. The crude product was purified by silica-gel flash column chromatography (AcOEt/toluene 1:1) to give compound **91** as pale yellow solid (116.3 mg, quant.).

^{1}H NMR (500 MHz, CDCl$_3$): δ 7.82 (1H, d, J = 6.1 Hz; NH), 7.53-7.14 (15H, m; ArH), 5.88-5.80 (1H, m; -O-CH$_2$-CH=CH$_2$), 5.58 (1H, s, Ph-CH=O$_2$), 5.25-5.15 (3H, m; -O-CH$_2$-CH=CH$_2$, H-1), 4.88 (1H, d, J = 12.1 Hz; Ph-CH-O-), 4.84 (1H, d, J = 12.1 Hz; Ph-CH-O-), 4.65 (1H, d, J = 12.1 Hz; Ph-CH-O-), 4.36 (1H, d, J = 12.1 Hz; Ph-CH-O-), 4.61(1H, q, J = 7.0 Hz; Lac-αH), 4.46-4.39 (3H, m; H-1', H-6', NH), 4.25-4.06 (5H, m; -O-CH$_2$CH$_3$, -COCH$_2$OH, -O-CH-CH=CH$_2$), 3.99-3.91 (3H, m; -O-CH-CH=CH$_2$, H-2, H-4), 3.75 (1H, dd, J = 10.3 Hz, 10.3 Hz; H-6'), 3.69-3.46 (7H, m; H-2', H-4', H-3', H-5, H-3, H-6, H-6), 3.31-3.26 (1H, m; H-5'), 3.02 (1H, dd, J = 6.2 Hz, 6.2 Hz; -COCH$_2$OH), 1.74 (3H, s; -NHCOCH$_3$), 1.33 (3H, d, J = 7.0 Hz; Lac-βCH$_3$), 1.27 (3H, t, J = 7.2 Hz; -COOCH$_2$CH$_3$).

^{13}C NMR (125 MHz, CDCl$_3$): δ 176.0, 172.3, 169.8, 138.5, 137.9, 137.3, 133.8, 129.2, 129.1, 128.8, 128.5, 128.3, 128.1, 127.9, 126.0, 117.4, 101.3, 100.4, 96.2, 82.7, 77.7, 75.7, 74.8, 74.0, 73.7, 70.2, 68.9, 68.8, 67.2, 65.7, 62.0, 61.4, 56.0, 53.5, 23.4, 18.9, 14.1.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{45}H_{56}N_2O_{14}Na$: 871.3624 $[M+Na]^+$; found: 871.3627.

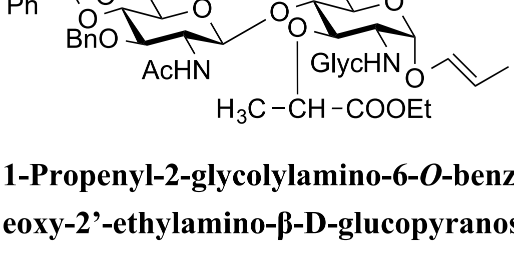


1,6-Anhydro-2-glycolylamino-4-*O*-[2’-acetylamino-3’-*O*-benzyl-4’,6’-*O*-benzylidene-2’-deoxy-β-D-glucopyranosyl]-2-deoxy-3-*O*-[(*R*)-1-(ethoxycarbonyl)ethyl]-D-glucopyranoside (105) Compound **105** was synthesized from **90** with similar method to the synthesis of **54**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 35:1) to give compound **105** as white solid (30 mg, 78 %).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.52-7.31 (10H, m; ArH), 7.01 (1H, d, J = 9.3 Hz; NH), 5.6 (1H, s; Ph-CH=O_2), 5.36-5.33 (2H, m; H_{anh}-1, NH), 4.90 (1H, d, J = 12.2 Hz; Ph-CH-O-), 4.67 (1H, d, J = 12.2 Hz; Ph-CH-O-), 4.58 (1H, br s; -$COCH_2OH$), 4.53 (1H, d, J = 8.5 Hz; H-1), 4.45 (1H, d, J = 5.4 Hz; H_{anh}-5), 4.33 (1H, dd, J = 4.9 Hz, 10.6 Hz; H-6), 4.25-4.08 (7H, m; -O-CH_2CH_3, Lac-αH, H_{anh}-6, H_{anh}-2, -$COCH_2OH$), 3.96-3.91 (1H, m; H-2), 3.84-3.71 (4H, m; H-6’, H-4, H_{anh}-6’, H_{anh}-4), 3.64 (1H, dd, J = 9.7 Hz, 9.7 Hz; H-3), 3.50 (1H, s; H_{anh}-3), 3.42-3.37 (1H, m; H-5), 1.91 (3H, s; -$NHCOCH_3$), 1.40 (3H, d, J = 6.8 Hz; Lac-βCH_3), 1.28 (3H, t, J = 7.2 Hz; -$COOCH_2CH_3$).

^{13}C NMR (125 MHz, $CDCl_3$): δ 172.4, 172.2, 171.9, 138.1, 137.1, 129.1, 128.6, 128.4, 128.2, 126.0, 101.4, 100.9, 99.6, 82.2, 76.2, 74.3, 73.9, 73.3, 72.0, 68.5, 66.5, 64.6, 62.7, 61.3, 55.1, 48.7, 23.5, 18.1, 14.1.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{35}H_{44}N_2O_{13}Na$: 723.2736 $[M+Na]^+$; found: 723.2740.



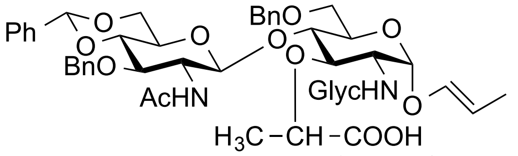
1-Propenyl-2-glycolylamino-6-*O*-benzyl-4-*O*-(3’-*O*-benzyl-4’,6’-*O*-benzylidene-2’-deoxy-2’-ethylamino-β-D-glucopyranosyl)-2-deoxy-3-*O*-[(*R*)-1-(ethoxycarbonyl)ethyl

]-α-D-glucopyranoside (93) Compound **93** was synthesized from **91** with similar method to the synthesis of **55**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/acetone 15:1) to give compound **93** as pale white solid.

^{1}H NMR (500 MHz, $CDCl_3$): *δ* 7.95 (1H, d, *J* = 5.6 Hz; NH), 7.53-7.14 (15H, m; ArH), 6.09 (1H, d, *J* = 1.6 Hz, 12.3 Hz; -O-CH=CH-CH_3), 5.58 (1H, s, Ph-CH=O_2), 5.40 (1H, d, *J* = 3.3 Hz; H-1), 5.12-5.05 (1H, m; -O-CH=CH-CH_3), 4.88 (1H, d, *J* = 12.1 Hz; Ph-CH-O-), 4.83 (1H, d, *J* = 12.1 Hz; Ph-CH-O-), 4.65 (1H, d, *J* = 12.1 Hz; Ph-CH-O-), 4.34 (1H, d, *J* = 12.1 Hz; Ph-CH-O-), 4.62(1H, q, *J* = 7.0 Hz; Lac-αH), 4.45-4.38 (3H, m; H-1', H-6', NH), 4.26-4.05 (4H, m; -O-CH_2CH_3, -COCH_2OH), 3.99 (1H, dd, *J* = 9.6 Hz, 9.6 Hz; H-4), 3.94-3.90 (1H, m; H-2), 3.76 (1H, dd, *J* = 10.3 Hz, 10.3 Hz; H-6'), 3.70-3.44 (7H, m; H-2', H-4', H-3', H-5, H-3, H-6, H-6), 3.31-3.26 (1H, m; H-5'), 2.95 (1H, dd, *J* = 6.2 Hz, 6.2 Hz; -COCH_2OH), 1.73 (3H, s; -NHCH_3), 1.52 (3H, dd, *J* = 1.6 Hz, 6.9 Hz; -O-CH=CH-CH_3), 1.34 (3H, d, *J* = 7.0 Hz; Lac-βCH_3), 1.29 (3H, t, *J* = 7.2 Hz; -COOCH_2CH_3).

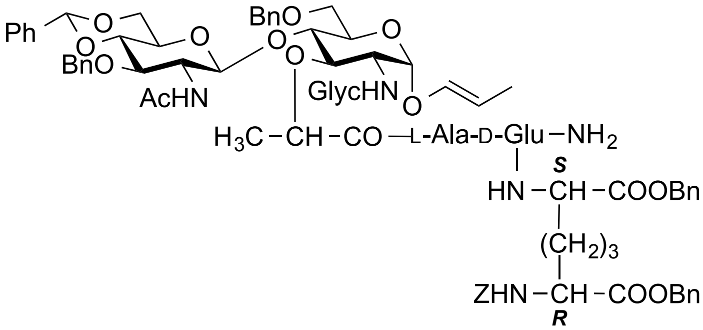
^{13}C NMR (125 MHz, $CDCl_3$): *δ* 175.9, 172.4, 169.7, 143.1, 138.4, 137.8, 137.2, 129.2, 129.1, 128.7, 128.5, 128.4, 128.3, 128.0, 127.8, 126.0, 104.4, 101.2, 100.4, 96.2, 82.6, 77.6, 75.3, 74.8, 74.0, 73.6, 70.5, 68.8, 67.0, 65.7, 61.9, 61.4, 55.8, 53.4,23.4, 18.9, 14.1, 12.3.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{45}H_{56}N_2O_{14}Na$: 871.3624 $[M+Na]^+$; found: 871.3627.



1-Propenyl-2-glycolylamino-6-*O*-benzyl-4-*O*-(3'-*O*-benzyl-4',6'-*O*-benzylidene-2'-deoxy-2'-ethylamino-β-D-glucopyranosyl)-2-deoxy-3-*O*-[(*R*)-1-(carbonyl)ethyl]-α-D-glucopyranoside (95) Compound **93** (110 mg, 0.126 mmol) and LiOH·H_2O (5.8 mg, 0.139 mmol) were dissolved in THF/1,4-dioxane/H_2O 4:2:1 (4 mL) and stirred at room temperature for 2 h. Then the solution was neutralized with Dowex H^+ and filtrated to get clear solution. After evaporation, the residue was passed through HP-20 column, first washed by ultrapure H_2O to remove organic and inorganic salts, then by methanol to give compound **95** as white solid (77.6 mg, 73 % (calculated from **91**)).

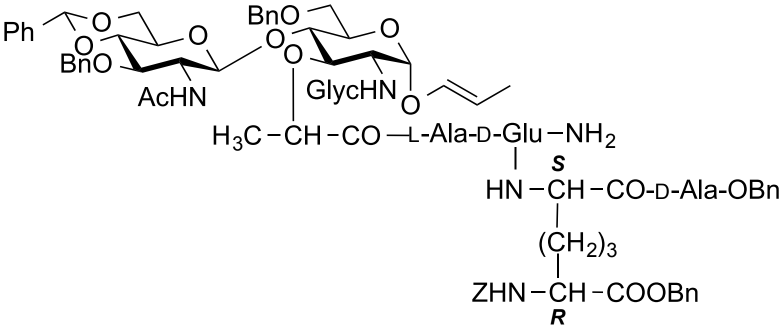
HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{43}H_{52}N_2O_{14}Na$: 843.3311 $[M+Na]^+$; found: 843.3306.



Protected disaccharide containing *N*-glycolylmuramyl group and tripeptide (97)
Compound **97** was synthesized from **95** and **34** with similar method to the synthesis of **57**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 20:1) to give compound **97** as white solid (36 mg, 65 %).

^{1}H NMR (500 MHz, $CDCl_3$:CD_3OD 8:1): δ 7.44-7.20 (30 H, m; Ar$\underline{H}$), 6.09 (1H, d, J = 12.4 Hz; -O-C$\underline{H}$=CH-CH_3), 5.58 (1H, s; Ph-C$\underline{H}$=O_2), 5.26 (1H, d, J = 2.9 Hz; H-1), 5.16-5.06 (7 H, m; -COOC$\underline{H_2}$Ph × 3, -O-CH=C$\underline{H}$-CH_3), 4.86 (1H, d, J = 11.9 Hz ; Ph-C$\underline{H}$-O-), 4.76 (1H, d, J = 11.6 Hz ; Ph-C$\underline{H}$-O-), 4.64 (1H, d, J = 11.9 Hz ; Ph-C$\underline{H}$-O-), 4.56-4.53 (2H, m; Lac-α$\underline{H}$, H-1'), 4.48-4.41 (3H, m; Ph-C$\underline{H}$-O-, Gln-α$\underline{H}$, DAP 2-$\underline{H}$), 4.36-4.31 (2H, m; DAP 6-$\underline{H}$, H-6'), 4.21-4.18 (1H, m; Ala-α$\underline{H}$), 4.06-3.97 (4H, m; H-2, H-5, -COC$\underline{H_2}$OH), 3.75 (1H, dd; J = 10.5 Hz, 10.5 Hz; H-6'), 3.66-3.41 (7H, m; H-6', H-4', H-2, H-3', H-3, H-6, H-6), 3.29-3.25 (1H, m; H-5'), 2.38-1.93 (4H, m; Gln-γC$\underline{H_2}$, Gln-βC$\underline{H_2}$), 1.85-1.63 (4H, m; DAP 3-C$\underline{H_2}$, 5-C$\underline{H_2}$), 1.81 (3H, s; -NHCOCH_3), 1.51 (3H, dd, J = 1.6 Hz, 6.9 Hz; -O-CH=CH-C$\underline{H_3}$), 1.39-1.26 (8H, m; DAP 4-C$\underline{H_2}$, Lac-βC$\underline{H_3}$, Ala-βC$\underline{H_3}$).

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{80}H_{95}N_7O_{22}Na$: 1528.6422 $[M+Na]^+$; found: 1528.6420.

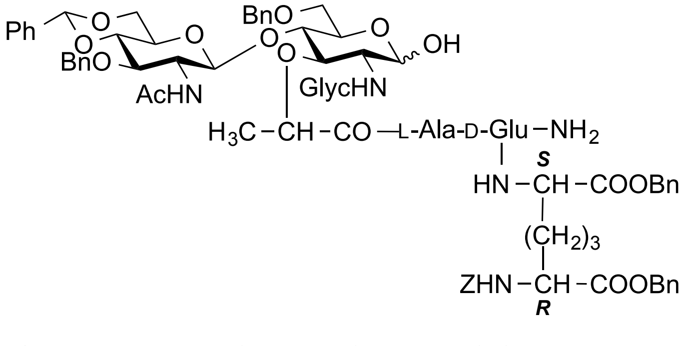


Protected disaccharide containing *N*-glycolylmuramyl group and tetrapeptide (98)
Compound **98** was synthesized from **95** and **35** with similar method to the synthesis of **57**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 20:1) to give compound **98** as white solid (35 mg, 73 %).

^{1}H NMR (500 MHz, DMSO-d$_6$): δ 8.44 (1H, d, J = 5.7 Hz; NH), 8.34 (1H, d, J = 6.9 Hz; NH), 8.20 (2H, d, J = 7.7 Hz; NH ×2), 7.95 (1H, d, J = 7.5 Hz; NH), 7.88 (1H, d, J = 8.3 Hz; NH), 7.70 (1H, d, J = 7.6 Hz; NH), 7.44-7.20 (31H, m; ArH, NH), 6.91 (1H, s; NH), 6.18 (1H, dd, J = 1.4 Hz, 12.2 Hz; -O-CH=CH-CH$_3$), 5.67 (1H, s; Ph-CH=O$_2$), 5.31 (1H, t, J = 5.9 Hz; -COCH$_2$OH), 5.20 (1H, d, J = 3.0 Hz; H-1), 5.12-4.98 (7 H, m; -COOCH$_2$Ph × 3, -O-CH=CH-CH$_3$), 4.78 (1H, d, J = 8.3 Hz; H-1'), 4.73 (1H, d, J = 11.9 Hz ; Ph-CH-O-), 4.50 (1H, d, J = 11.9 Hz ; Ph-CH-O-), 4.60-4.37 (4H, m; Ph-CH-O- × 2, Lac-αH, D-Ala-αH), 4.32-3.98 (6H, m; Ala-αH, H-6', DAP 2-H, 6-H, Gln-αH, H-3'), 3.89-3.50 (11H, m; H-4, H-6', H-4', H-2, H-2', H-6, H-6, H-3, H-5, -COCH$_2$OH), 3.24-3.22 (1H, m; H-5'), 2.18-2.13 (2H, m; Gln-γCH$_2$), 1.98-1.93 (1H, m; Gln-βCH), 1.82 (3H, s; -NHCOCH$_3$), 1.75-1.33 (5H, m; Gln-βCH, DAP 3-CH$_2$, 5-CH$_2$), 1.45 (3H, dd, J = 1.4 Hz, 6.7 Hz; -O-CH=CH-CH$_3$), 1.29-1.22 (11H, m; DAP 4-CH$_2$, Lac-βCH$_3$, Ala-βCH$_3$, D-Ala-βCH$_3$).

^{13}C NMR (125 MHz, DMSO-d$_6$): δ 173.4, 173.1, 172.7, 172.2, 171.9, 171.5, 171.3, 169.7, 156.1, 143.5, 138.7, 138.5, 137.6, 136.8, 135.9, 128.3, 128.3, 128.2, 128.0, 128.0, 128.0, 127.9, 127.8, 127.7, 127.6, 127.4, 127.3, 127.1, 126.0, 103.4, 100.2, 95.7, 81.3, 75.5, 74.5, 73.5, 71.7, 70.7, 68.0, 67.7, 65.9, 65.8, 65.5, 65.4, 61.3, 60.9, 54.0, 53.0, 52.2, 51.8, 48.0, 47.6, 31.8, 31.7, 30.2, 27.9,22.9, 21.8, 19.5, 18.8, 16.9, 12.1.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{83}H_{100}N_8O_{23}Na$: 1599.6794 [M+Na]$^+$; found: 1599.6788.



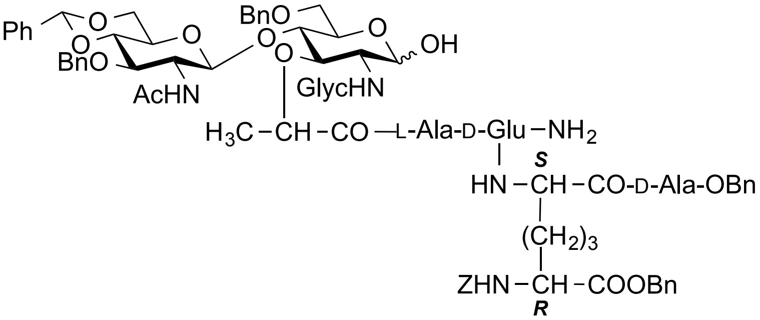
1-*O*-Deprotected disaccharide containing *N*-glycolylmuramyl group and tripeptide (101) Compound **101** was synthesized from **97** with similar method to the synthesis of **59**. The crude product was purified by silica-gel flash column chromatography (CHCl$_3$/MeOH 20:1) to give compound **101** as white solid (17 mg, 55 %).

^{1}H NMR (500 MHz, DMSO-d$_6$): δ 8.27-8.23 (2H, m; NH × 2), 8.20 (1H, d, J = 7.9 Hz; NH), 8.16 (1H, d, J = 7.2 Hz; NH), 7.83 (1H, d, J = 6.6 Hz; NH), 7.76 (1H, d, J = 7.9 Hz; NH), 7.45-7.23 (31 H, m; ArH, NH), 6.97 (1H, s; NH), 6.72 (1H, d, J = 3.9 Hz; 1-OH), 5.69 (1H, s; Ph-CH=O$_2$), 5.33 (1H, t, J = 6.0 Hz; -COCH$_2$OH), 5.11-5.00 (7 H, m; -COOCH$_2$Ph × 3, H-1), 4.76-4.72 (2H, m; H-1', Ph-CH-O-), 4.62-4.52 (3H, m ;

Ph-CH-O- × 3), 4.46 (1H, q, *J* = 6.3 Hz; Lac-αH), 4.41-4.00 (6H, m; Ala-αH, H-6’, DAP 2-H, Gln-αH, DAP 6-H, H-3’), 3.81-3.47 (11H, m; H-4, H-6’, H-4’, H-2, H-2’, H-6, H-6, H-3, H-5, -COCH$_2$OH), 3.25-3.20 (1H, m; H-5’), 2.17-2.14 (2H, m; Gln-γCH$_2$), 1.98-1.89 (1H, m; Gln-βCH), 1.84 (3H, s; -NHCOCH$_3$), 1.76-1.57 (5H, m; Gln-βCH, DAP 3-CH$_2$, 5-CH$_2$), 1.35 (2H, br s; DAP 4-CH$_2$), 1.25-1.22 (6H, m; Lac-βCH$_3$, Ala-βCH$_3$).

^{13}C NMR (125 MHz, DMSO-d$_6$): δ 173.4, 173.1, 172.3, 172.1, 172.0, 172.0, 171.8, 169.6, 156.2, 138.7, 138.6, 137.6, 136.8, 135.9, 135.9, 128.7, 128.4, 128.3, 128.2, 128.1, 128.1, 128.0, 128.0, 127.8, 127.7, 127.7, 127.4, 127.3, 127.1, 126.0, 100.1, 89.9, 81.3, 75.9, 75.8, 75.0, 73.5, 71.7, 69.8, 68.2, 68.0, 65.9, 65.8, 65.5, 65.4, 61.3, 53.8, 53.7, 52.2, 52.0, 48.1, 31.5, 30.2, 30.2, 27.8, 22.9, 21.9, 19.4, 18.6.

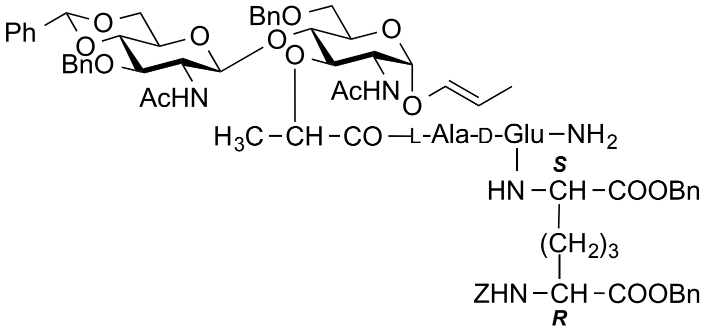
HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{77}H_{91}N_7O_{22}Na$: 1488.6109 $[M+Na]^+$; found: 1488.6106.



1-*O*-Deprotected disaccharide containing *N*-glycolylmuramyl group and tetrapeptide (102) Compound **102** was synthesized from **98** with similar method to the synthesis of **59**. The crude product was purified by silica-gel flash column chromatography (CHCl$_3$/MeOH 25:1) to give compound **102** as white solid (19 mg, 60 %).

^{1}H NMR (400 MHz, DMSO-d$_6$): δ 8.34 (1H, d, *J* = 7.7 Hz; NH), 8.20-8.16 (2H, m; NH ×2), 8.10 (1H, d, *J* = 7.4 Hz; NH), 7.88 (1H, d, *J* = 8.0 Hz; NH), 7.79 (1H, d, *J* = 6.9 Hz; NH), 7.70 (1H, d, *J* = 6.9 Hz; NH), 7.44-7.23 (31 H, m; ArH, NH), 6.92 (1H, s; NH), 6.69 (1H, d, *J* = 3.9 Hz; 1-OH), 5.68 (1H, s; Ph-CH=O$_2$), 5.30 (1H, t, *J* = 5.7 Hz; -COCH$_2$OH), 5.12-4.98 (7 H, m; -COOCH$_2$Ph × 3, H-1), 4.76-4.71 (2H, m; H-1’, Ph-CH-O-), 4.60-4.51 (3H, m; Ph-CH-O- × 3), 4.46 (1H, q, *J* = 6.4 Hz; Lac-αH), 4.39-3.97 (7H, m; D-Ala-αH, Ala-αH, H-6’, DAP 2-H, Gln-αH, DAP 6-H, H-3’), 3.80-3.50 (11H, m; H-4, H-6’, H-4’, H-2, H-2’, H-6, H-6, H-3, H-5, -COCH$_2$OH), 3.24-3.18 (1H, m; H-5’), 2.17-2.12 (2H, m; Gln-γCH$_2$), 2.00-1.93 (1H, m; Gln-βCH), 1.83 (3H, s; -NHCOCH$_3$), 1.75-1.34 (5H, m; Gln-βCH, DAP 3-CH$_2$, 5-CH$_2$), 1.26-1.21 (11H, m; DAP 4-CH$_2$, Lac-βCH$_3$, Ala-βCH$_3$, D-Ala-βCH$_3$).

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{80}H_{96}N_8O_{23}Na$: 1559.6481 [M+Na]$^+$; found: 1559.6486.



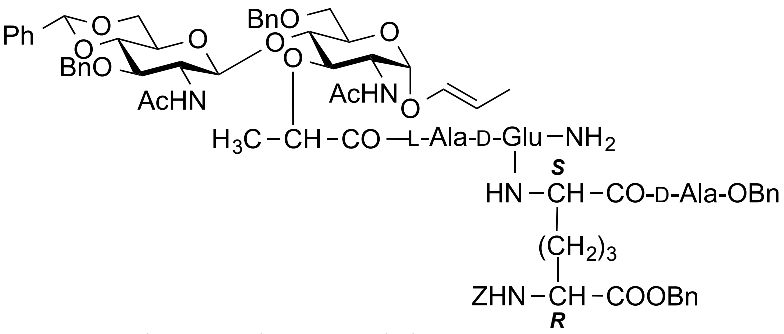
Protected disaccharide containing *N*-acetylmuramyl group and tripeptide (99)

Compound **99** was synthesized from **96** and **34** with similar to the synthesis of **70**. The crude product was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 25:1) to give compound **99** as white solid (32 mg, 65%).

^{1}H NMR (500 MHz, DMSO-d_6): δ 8.27 (1H, d, J = 7.2 Hz; NH), 8.28-8.07 (4H, m; 4NH), 7.75 (1H, d, J = 7.7 Hz; NH), 7.44-7.24 (31H, m; ArH, NH), 6.97 (1H, s; NH), 6.17 (1H, dd, J = 1.3 Hz, 12.2 Hz; -O-CH=CH-CH$_3$), 5.68 (1H, s; Ph-CH=O$_2$), 5.20 (1H, d, J = 3.0 Hz; H-1), 5.10-4.98 (7 H, m; -COOCH$_2$Ph × 3, -O-CH=CH-CH$_3$), 4.81-4.72 (2H, m; Ph-CH-O-, H-1'), 4.60-4.43 (5H, m; Ph-CH-O- × 3, Lac-αH, Ala-αH), 4.26 (1H, dd, J = 4.9 Hz, 10.2 Hz; H-6'), 4.21-4.17 (2H, m; Gln-αH, DAP 2-H), 4.06-4.00 (2H, m; H-3', DAP 6-H), 3.86-3.20 (10H, m; H-4, H-6', H-4', H-2', H-2, H-5, H-6, H-6, H-3, H-5'), 2.16-2.11 (2H, m; Gln-γCH$_2$), 1.96-1.89 (1H, m; Gln-βCH), 1.82 (6H, s; -NHCOCH$_3$ × 2), 1.74-1.56 (5H, m; Gln-βCH, DAP 3-CH$_2$, 5-CH$_2$), 1.45 (3H, dd, J = 1.3 Hz, 6.9 Hz; -O-CH=CH-CH$_3$), 1.35 (2H, br s; DAP 4-CH$_2$), 1.26-1.20 (6H, m; Lac-βCH$_3$, Ala-βCH$_3$).

^{13}C NMR (125 MHz, DMSO-d_6): δ 174.0, 173.1, 173.0, 172.1, 172.0, 171.9, 171.8, 169.7, 156.1, 143.6, 138.7, 138.5, 137.6, 136.8, 135.9, 135.9, 128.4, 128.3, 128.2, 128.1, 128.1, 128.0, 128.0, 127.8, 127.7, 127.7, 127.4, 127.3, 127.2, 127.1, 126.0, 103.3, 103.3, 100.2, 95.7, 81.3, 77.5, 75.3, 75.0, 73.5, 71.7, 67.7, 65.9, 65.8, 65.5, 65.4, 53.8, 53.4, 52.0, 48.0, 31.4, 30.2, 30.2, 27.9, 22.9, 22.7, 21.9, 19.6, 18.9, 12.2.

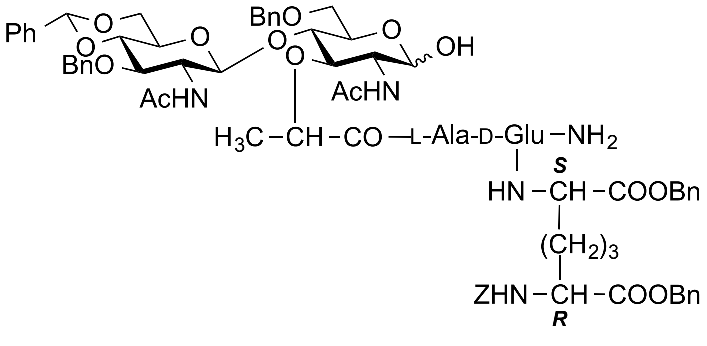
HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{80}H_{95}N_7O_{21}Na$: 1512.6473 [M+Na]$^+$; found: 1512.6466.



Protected disaccharide containing *N*-acetylmuramyl group and tetrapeptide (100) Compound **100** was synthesized from **96** and **35** in a manner similar to the synthesis of **70**. The crude product was purified by silica-gel flash column chromatography (CHCl$_3$/MeOH 25:1) to give compound **100** as white solid (31 mg, 80%).

^{1}H NMR (500 MHz, DMSO-d$_6$): δ 8.54 (1H, d, J = 5.0 Hz; N$\underline{H}$), 8.33 (4H, d, J = 7.1 Hz; N$\underline{H}$), 8.24-8.17 (2H, m; 2N$\underline{H}$), 8.09 (1H, d, J = 6.5 Hz; N$\underline{H}$), 7.88 (1H, d, J = 8.0 Hz; N$\underline{H}$), 7.70 (1H, d, J = 7.9 Hz; N$\underline{H}$), 7.44-7.24 (31H, m; Ar$\underline{H}$, N$\underline{H}$), 6.93 (1H, s; N$\underline{H}$), 6.17 (1H, d, J = 12.5 Hz; -O-C$\underline{H}$=CH-CH$_3$), 5.68 (1H, s; Ph-C$\underline{H}$=O$_2$), 5.20 (1H, s; H-1), 5.10-4.98 (7 H, m; -COOC$\underline{H}_2$Ph × 3, -O-CH=C$\underline{H}$-CH$_3$), 4.81-4.71 (2H, m; Ph-C$\underline{H}$-O-, H-1’), 4.60-4.41 (5H, m; Ph-C$\underline{H}$-O- × 3, Lac-α$\underline{H}$, D-Ala-α$\underline{H}$), 4.31-3.98 (6H, m; H-6’, Gln-α$\underline{H}$, Ala-α$\underline{H}$, DAP 2-$\underline{H}$, H-3’, DAP 6-$\underline{H}$), 3.86-3.20 (10H, m; H-4, H-6’, H-4’, H-2’, H-2, H-5, H-6, H-6, H-3, H-5’), 2.17-2.10 (2H, m; Gln-γC$\underline{H}_2$), 1.99-1.92 (1H, m; Gln-βC$\underline{H}$), 1.82 (6H, s; -NHCOCH$_3$ × 2), 1.74-1.45 (5H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.45 (3H, d, J = 6.8 Hz; -O-CH=CH-C$\underline{H}_3$), 1.37-1.22 (11H, m; DAP 4-C$\underline{H}_2$, Lac-βC$\underline{H}_3$, Ala-βC$\underline{H}_3$, D-Ala-βC$\underline{H}_3$).

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{83}H_{100}N_8O_{22}Na$: 1583.6844 [M+Na]$^+$; found: 1583.6852.

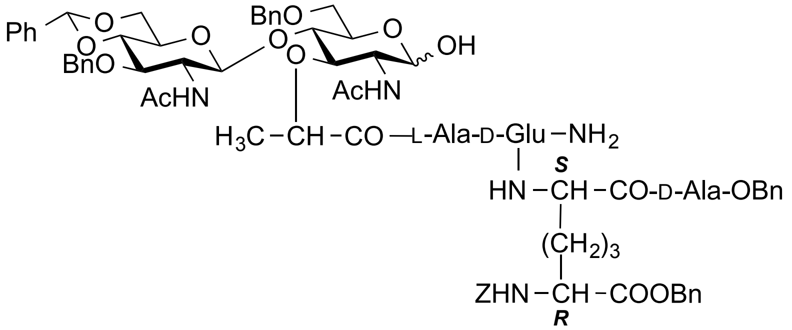


1-*O*-Deprotected disaccharide containing *N*-acetylmuramyl group and tripeptide (103) Compound **103** was synthesized from **99** with similar method to the synthesis of **59**. The crude product was purified by silica-gel flash column chromatography (CHCl$_3$/MeOH 25:1) to give compound **103** as white solid (20 mg, 74 %).

^{1}H NMR (500 MHz, DMSO-d$_6$): δ 8.41 (1 H, d, J = 5.3 Hz; NH), 8.30-8.25 (2 H, m; 2NH), 8.19 (1 H, d, J = 8.0 Hz; NH), 8.04 (1 H, d, J = 6.4 Hz; NH), 7.75 (1 H, d, J = 7.5 Hz; NH), 7.44-7.23 (31 H, m; ArH, NH), 6.96 (1 H, s; NH), 6.62 (1 H, d, J = 3.8 Hz; 1-OH), 5.68 (1H, s; Ph-CH=O$_2$), 5.13-4.98 (7 H, m; -COOCH$_2$Ph × 3, H-1), 4.74-4.72 (2H, m; Ph-CH-O-, H-1'), 4.61-4.38 (5H, m; Ph-CH-O- × 3, Lac-αH, Ala-αH), 4.26 (1H, dd, J = 4.5 Hz, 9.6 Hz; H-6'), 4.21-4.14 (2H, m; Gln-αH, DAP 2-H), 4.04-4.00 (2H, m; H-3', DAP 6-H), 3.76-3.20 (10H, m; H-4, H-6', H-4', H-2', H-2, H-5, H-6, H-6, H-3, H-5'), 2.16-2.13 (2H, m; Gln-γCH$_2$), 1.95-1.89 (1H, m; Gln-βCH), 1.83 (3H, s; -NHCOCH$_3$), 1.80 (3H, s; -NHCOCH$_3$), 1.76-1.56 (5H, m; Gln-βCH, DAP 3-CH$_2$, 5-CH$_2$), 1.35 (2H, br s; DAP 4-CH$_2$), 1.25-1.21 (6H, m; Lac-βCH$_3$, Ala-βCH$_3$).

^{13}C NMR (125 MHz, DMSO-d$_6$): δ 174.1, 173.1, 172.1, 172.0, 172.0, 171.8, 169.4, 156.2, 138.8, 138.6, 137.6, 136.8, 135.9, 135.9, 128.8, 128.4, 128.3, 128.2, 128.1, 128.1, 128.0, 128.0, 127.9, 127.8, 127.7, 127.7, 127.4, 127.3, 127.2, 126.0, 100.2, 89.7, 81.3, 76.0, 75.8, 75.2, 73.5, 71.7, 69.7, 68.2, 68.0, 66.3, 65.9, 65.8, 65.5, 65.4, 54.3, 53.8, 52.2, 52.0, 48.2, 31.5, 30.2, 30.2, 27.8, 22.9, 22.8, 22.0, 19.6, 18.6.

HRMS (ESI-LIT-orbitrap): m/z: calcd for C$_{77}$H$_{91}$N$_7$O$_{21}$Na: 1472.6160 [M+Na]$^+$; found: 1472.6172.



1-*O*-Deprotected disaccharide containing *N*-acetylmuramyl group and tetrapeptide (104) Compound **104** was synthesized from **100** with similar method to the synthesis of **59**. The crude product was purified by silica-gel flash column chromatography (CHCl$_3$/MeOH 25:1) to give compound **104** as white solid (13 mg, 56 %).

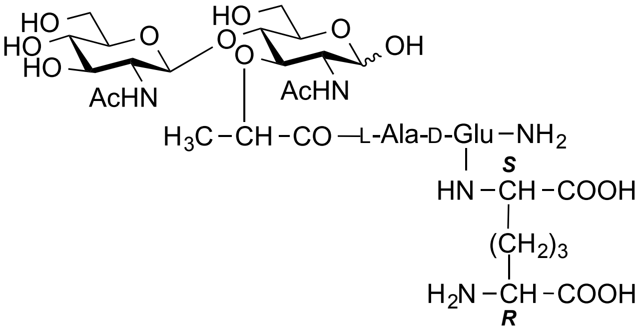
^{1}H NMR (500 MHz, DMSO-d$_6$): δ 8.38 (1H, d, J = 5.6 Hz; NH), 8.33 (1H, d, J = 7.1 Hz; NH), 8.23 (1 H, d, J = 8.1 Hz; NH), 8.16 (1H, d, J = 8.7 Hz; NH), 8.00 (1H, d, J = 7.3 Hz; NH), 7.88 (1 H, d, J = 8.3 Hz; NH), 7.70 (1H, d, J = 7.4 Hz; NH),7.44-7.22 (31 H, m; ArH, NH), 6.91 (1 H, s; NH), 6.59 (1H, d, J = 4.3 Hz; 1-OH), 5.68 (1H, s; Ph-CH=O$_2$), 5.14-4.98 (7 H, m; -COOCH$_2$Ph × 3, H-1), 4.75-4.72 (2H, m; Ph-CH-O-, H-1'), 4.61-4.38 (5H, m; Ph-CH-O- × 3, Lac-αH, D-Ala-αH), 4.30-3.98(6H, m; Ala-αH, H-6', Gln-αH, DAP 2-H, H-3', DAP 6-H), 3.76-3.20 (10H, m; H-4, H-6', H-4', H-2', H-2, H-5, H-6, H-6, H-3, H-5'), 2.21-2.09 (2H, m; Gln-γCH$_2$), 1.99-1.93 (1H, m;

Gln-βC$\underline{H}$), 1.83 (3H, s; -NHCOC$\underline{H}_3$), 1.80 (3H, s; -NHCOC$\underline{H}_3$), 1.74-1.56 (5H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.45-1.23 (11H, m; DAP 4-C$\underline{H}_2$, D-Ala-βC$\underline{H}_3$, Lac-βC$\underline{H}_3$, Ala-βC$\underline{H}_3$).

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{80}H_{96}N_8O_{22}Na$: 1543.6531 $[M+Na]^+$; found: 1543.6533.

Compounds **2a**, **2b**, **2c** and **2d** were synthesized from **103**, **101**, **104** and **102**, respectively, with similar method to the synthesis of **1b**.

Compound **2a** (96%)

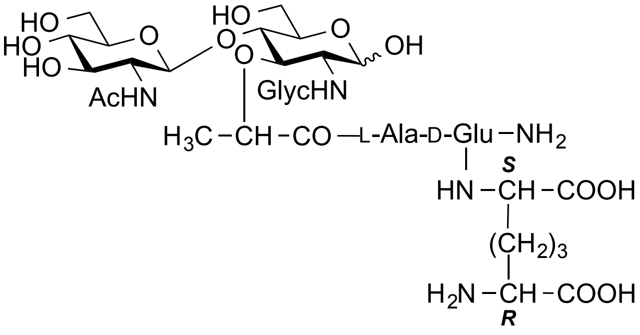


^{1}H NMR (500 MHz, D_2O): δ 5.17 (0.74 H, d, J = 5.7 Hz; H-1β), 4.57 (0.26 H, d, J = 8.3 Hz; H-1α), 4.50-4.45 (2H, m; H-1', Lac-α$\underline{H}$), 4.31-4.19 (3H, m; Ala-α$\underline{H}$, DAP 2-$\underline{H}$, Gln-α$\underline{H}$), 3.88-3.61 (9H, m; H-6, H-6', H-2, H-6, H-2', H-5', H-6', H-3, DAP 6-$\underline{H}$), 3.54-3.33 (4H, m; H-4', H-4, H-5, H-3'), 2.35-2.32 (2H, m; Gln-γC$\underline{H}_2$), 2.15-2.08 (1H, m; Gln-βC$\underline{H}$), 1.98 (3H, s; -NHCOC$\underline{H}_3$), 1.90 (3H, s; -NHCOC$\underline{H}_3$), 2.01-1.65 (5H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.45-1.36 (5H, m; DAP 4-C$\underline{H}_2$, Ala-βC$\underline{H}_3$), 1.33 (3H, d, J = 6.8 Hz; Lac-βC$\underline{H}_3$).

^{13}C NMR (125 MHz, D_2O): δ 178.6, 176.9, 176.5, 175.9, 175.6, 175.5, 175.4, 175.0, 101.3, 91.2, 80.3, 78.9, 78.4, 77.0, 76.4, 74.5, 72.0, 71.2, 62.0, 60.7, 56.9, 55.4, 54.5, 53.8, 50.8, 32.6, 31.7, 30.9, 28.0, 23.0, 23.0, 22.0, 19.1, 17.6 ppm.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{34}H_{58}N7O_{19}$: 868.3782 $[M+H]^+$; found: 868.3796.

Compound **2b** (95%)



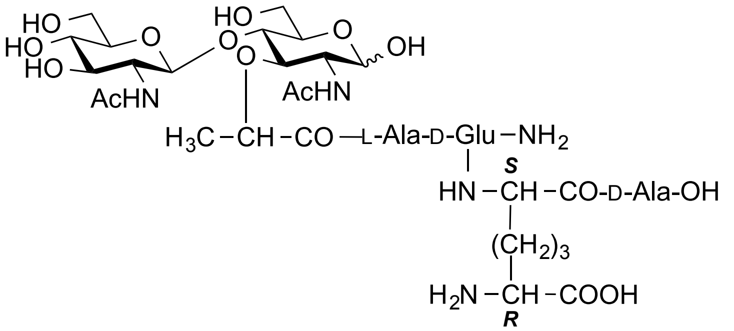
^{1}H NMR (700 MHz, D_2O): δ 5.26 (0.62 H, d, J = 3.6 Hz; H-1β), 4.62 (1H, q, J = 6.7 Hz;

Lac-αH), 4.52 (1H, dd, *J* = 7.9 Hz, 7.9 Hz; H-1’), 4.23-4.27 (2H, m; Ala-αH, Gln-αH), 4.21-4.19 (1H, m; DAP 2-H), 4.06-4.02 (2H, m; -COCH_2OH), 3.93-3.83 (3H, m; H-2, H-6, H-4), 3.80-3.64 (7H, m; H-2’, H-5, H-5’, H-6’, H-3, H-6, DAP 6-H), 3.57-3.51 (1H, m; H-3’), 3.41-3.36 (2H, m; H-6’, H-4’), 2.39-2.37 (2H, m; Gln-γCH_2), 2.16-2.13 (1H, m; Gln-βCH), 2.02 (3H, s; -NHCOCH_3), 2.00-1.68 (5H, m; Gln-βCH, DAP 3-CH_2, 5-CH_2), 1.46-1.33 (8H, m; DAP 4-CH_2, Ala-βCH_3, Lac-βCH_3).

^{13}C NMR (176 MHz, D_2O): *δ* 177.6, 176.0, 175.7, 175.3, 175.0, 174.7, 174.7, 174.5, 100.5, 90.2, 79.2, 77.1, 76.1, 75.6, 73.6, 71.2, 70.3, 61.2, 61.0, 59.8, 56.1, 54.5, 54.1, 53.5, 53.1, 49.9, 31.8, 30.8, 30.0, 26.9, 22.2, 21.2, 18.4, 16.6.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{34}H_{57}N_7O_{20}Na$: 906.3551 $[M+Na]^+$; found: 906.3566.

Compound **2c** (88%)

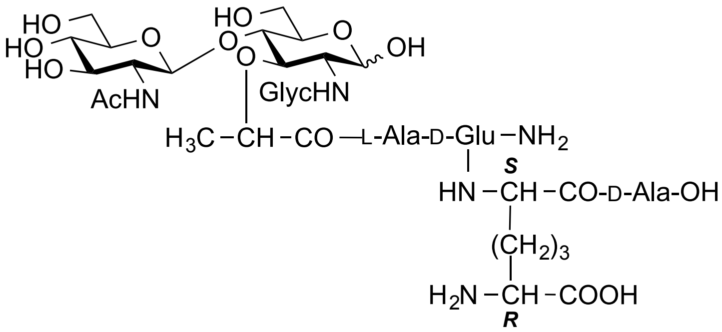


^{1}H NMR (700 MHz, D_2O): *δ* 5.15 (0.56 H, d, *J* = 3.5 Hz; H-1β), 4.49-4.44 (2H, m; H-1’, Lac-αH), 4.30-4.20 (3H, m; D-Ala-αH, Ala-αH, Gln-αH), 4.08-4.05 (1H, m; DAP 2-H), 3.86 (1H, dd, *J* = 4.2 Hz, 11.2 Hz; H-6), 3.81-3.60 (8H, m; H-6’, H-2, H-6, H-2’, H-5’, H-6’, H-3, DAP 6-H), 3.53-3.46 (2H, m; H-4’, H-4), 3.35-3.32 (2H, m; H-5, H-3’), 2.35-2.32 (2H, m; Gln-γCH_2), 2.11-2.06 (1H, m; Gln-βCH), 1.97 (3H, s; -NHCOCH_3), 1.89 (3H, s; -NHCOCH_3), 1.99-1.64 (5H, m; Gln-βCH, DAP 3-CH_2, 5-CH_2), 1.38-1.23 (11H, m; DAP 4-CH_2, D-Ala-βCH_3, Ala-βCH_3, Lac-βCH_3).

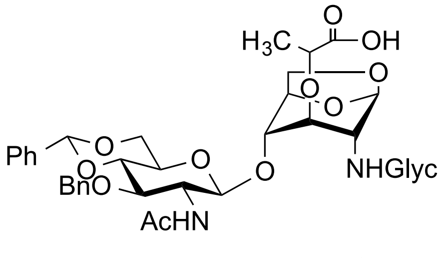
^{13}C NMR (176 MHz, D_2O): *δ* 179.7, 175.9, 175.7, 175.0, 175.0, 174.7, 174.2, 172.9, 100.4, 90.3, 79.4, 78.0, 77.5, 76.1, 75.5, 73.6, 71.1, 70.3, 61.2, 59.8, 56.1, 54.5, 53.9, 52.9, 51.0, 50.0, 31.6, 30.9, 30.1, 27.1, 22.2, 22.1, 21.1, 18.2, 17.6, 16.7.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{37}H_{62}N_8O_{20}Na$: 961.3973 $[M+Na]^+$; found: 961.3978.

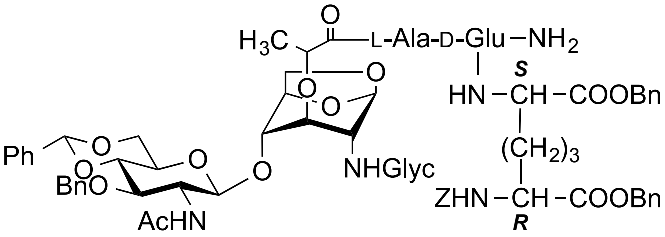
Compound **2d** (quant.)



^{1}H NMR (600 MHz, D$_2$O): δ 5.21 (0.57 H, d, J = 3.5 Hz; H-1β), 4.57 (1H, q, J = 6.5 Hz; Lac-α$\underline{\text{H}}$), 4.49-4.46 (1H, m; H-1’), 4.28-4.20 (3H, m; Ala-α$\underline{\text{H}}$, Gln-α$\underline{\text{H}}$, DAP 2-$\underline{\text{H}}$), 4.09 (1H, q, J = 7.6 Hz; D-Ala-α$\underline{\text{H}}$), 4.01-3.97 (2H, m; -COC$\underline{\text{H}}_2$OH), 3.88-3.78 (3H, m; H-2, H-6, H-4), 3.75-3.60 (7H, m; H-2’, H-5, H-5’, H-6’, H-3, H-6, DAP 6-$\underline{\text{H}}$), 3.51-3.46 (1H, m; H-3’), 3.36-3.33 (2H, m; H-6’, H-4’), 2.36-2.33 (2H, m; Gln-γC$\underline{\text{H}}_2$), 2.12-2.06 (1H, m; Gln-βC$\underline{\text{H}}$), 1.97 (3H, s; -NHCOC$\underline{\text{H}}_3$), 1.94-1.64 (5H, m; Gln-βC$\underline{\text{H}}$, DAP 3-C$\underline{\text{H}}_2$, 5-C$\underline{\text{H}}_2$), 1.41-1.24 (11H, m; DAP 4-C$\underline{\text{H}}_2$, Ala-βC$\underline{\text{H}}_3$, Lac-βC$\underline{\text{H}}_3$, D-Ala-βC$\underline{\text{H}}_3$).
^{13}C NMR (150 MHz, D$_2$O): δ 179.5, 175.9, 175.7, 175.2, 175.0, 175.0, 174.7, 174.5, 172.9, 100.4, 90.2, 79.2, 77.1, 76.1, 75.6, 73.6, 71.2, 70.3, 61.2, 61.0, 59.8, 56.1, 54.5, 53.8, 53.5, 52.9, 50.8, 49.9, 31.6, 30.9, 30.0, 26.9, 22.1, 21.0, 18.4, 17.5, 16.6.
HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{37}H_{62}N_8O_{21}Na$: 977.3922 [M+Na]$^+$; found: 977.3939.



1,6-Anhydro-2-glycolylamino-4-*O*-[2’-acetylamino-3’-*O*-benzyl-4’,6’-*O*-benzylidene-2’-deoxy-β-D-glucopyranosyl]-2-deoxy-3-*O*-[(*R*)-1-(carbonyl)ethyl]-D-glucopyranoside (107) Compound **107** was synthesized from **105** with similar method to the synthesis of **56**. Compound **107** was used without further purification.
HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{15}H_{23}NO_8Na$: 368.1316 [M+Na]$^+$; found: 368.1318.

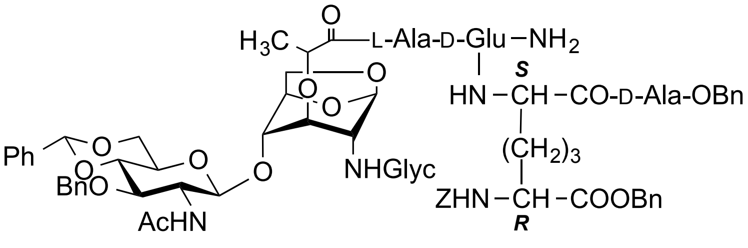


Protected disaccharide (anh) containing *N*-glycolylmuramyl group and tripeptide (109) Compound **109** was synthesized from **107** and **34** with similar method to the synthesis of **57**. The crude product was purified by silica-gel flash column chromatography (CHCl$_3$/MeOH 20:1) to give compound **109** as white solid (19 mg, 70 %).

^{1}H NMR (500 MHz, CDCl$_3$): δ 7.83 (1H, J = 6.6 Hz; NH), 7.61 (1H, J = 6.9 Hz; NH), 7.52-7.31 (26 H, m; ArH, NH), 7.08 (1H, J = 9.2 Hz; NH), 6.73 (1H, s; NH), 5.74 (1H, d, J = 8.4 Hz; NH), 5.61 (1H, s; Ph-CH=O$_2$), 5.40 (1H, s; H$_{anh}$-1), 5.37 (1H, d, J = 8.8 Hz; NH), 5.17-5.05 (6H, m; -COOCH$_2$Ph × 3), 4.91 (1H, d, J = 12.2 Hz; Ph-CH-O-), 4.87 (1H, t, J = 7.2 Hz; -NHCOCH$_2$OH), 4.71-4.66 (2H, m; Ph-CH-O-, Gln-αH), 4.48-4.44 (3H, m; DAP 2-H, H-1, H$_{anh}$-5), 4.37-4.26 (4H, m; DAP 6-H, Ala-αH, H-6, H$_{anh}$-6), 4.21-4.08 (3H, m; Lac-αH, -NHCOCH$_2$OH), 4.00-3.96 (2H, m; H-2, H$_{anh}$-2), 3.84-3.72 (4H, m; H-6’, H-4, H$_{anh}$-4, H$_{anh}$-6’), 3.59 (1H, dd, J = 9.3 Hz, 9.3 Hz; H-3), 3.49 (1H, s; H$_{anh}$-3), 3.41-3.36 (1H, m; H-5), 2.34-2.25 (2H, m; Gln-γCH$_2$), 2.10-2.05 (1H, m; Gln-βCH), 1.92 (3H, s; -NHCOCH$_3$), 1.86-1.64 (5H, m; Gln-βCH, DAP 3-CH$_2$, 5-CH$_2$), 1.45-1.33 (8H, m; DAP 4-CH$_2$, Lac-βCH$_3$, Ala-βCH$_3$).

^{13}C NMR (125 MHz, CDCl$_3$): δ 173.6, 173.5, 173.4, 173.0, 173.0, 172.9, 172.1, 171.9, 156.2, 138.0, 137.0, 136.2, 135.2, 135.2, 129.2, 128.7, 128.7, 128.6, 128.5, 128.5, 128.4, 128.2, 128.2, 126.0, 101.3, 100.8, 99.6, 82.1, 76.7, 73.8, 73.4, 72.4, 68.5, 67.4, 67.2, 67.1, 67.1, 66.5, 64.7, 62.7, 54.8, 53.7, 52.7, 51.7, 49.5, 47.1, 32.4, 31.8, 30.3, 29.7, 23.5, 21.3, 17.5, 17.2.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{70}H_{83}N_7O_{21}Na$: 1380.5534 [M+Na]$^+$; found: 1380.5540.

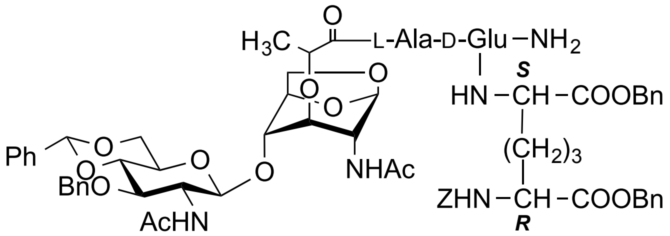


Protected disaccharide (anh) containing *N*-glycolylmuramyl group and tetrapeptide (110) Compound **110** was synthesized from **107** and **35** with similar method to the synthesis of **57**. The crude product was purified by silica-gel flash column chromatography (CHCl$_3$/MeOH 20:1) to give compound **110** as white solid (19 mg, 66 %).

^{1}H NMR (500 MHz, CDCl$_3$): δ 7.94 (1H, s; NH), 7.54-7.28 (28 H, m; ArH, NH × 3), 7.10 (1H, J = 9.0 Hz; NH), 7.02 (1H, s; NH), 5.75 (1H, d, J = 7.3 Hz; NH), 5.59 (1H, s; Ph-CH=O$_2$), 5.40 (1H, s; H$_{anh}$-1), 5.14-5.03 (6H, m; -COOCH$_2$Ph × 3), 4.97 (1H, s; -NHCOCH$_2$OH), 4.90 (1H, d, J = 12.2 Hz; Ph-CH-O-), 4.68 (1H, d, J = 12.2 Hz; Ph-CH-O-), 4.54-4.44 (4H, m; Gln-αH, H$_{anh}$-5, D-Ala-αH, H-1), 4.33-4.26 (5H, m; Ala-αH, H-6, H$_{anh}$-6, DAP 2-H, 6-H), 4.19-4.07 (3H, m; Lac-αH, -NHCOCH$_2$OH), 4.00-3.95 (2H, m; H-2, H$_{anh}$-2), 3.82-3.62 (5H, m; H-6’, H-4, H$_{anh}$-4, H$_{anh}$-6’), 3.53 (1H, s; H$_{anh}$-3), 3.41-3.36 (1H, m; H-5), 2.23-2.10 (3H, br m; Gln-γCH$_2$, Gln-βCH), 1.92 (3H, s; -NHCOCH$_3$), 1.83-1.63 (5H, m; Gln-βCH, DAP 3-CH$_2$, 5-CH$_2$), 1.45-1.32 (11H, m; DAP 4-CH$_2$, Lac-βCH$_3$, Ala-βCH$_3$, D-Ala-βCH$_3$).

^{13}C NMR (125 MHz, CDCl$_3$): δ 173.4, 173.3, 173.1, 173.0, 172.9, 172.2, 172.1, 156.2, 138.1, 137.1, 136.3, 135.3, 129.1, 128.7, 128.5, 128.5, 128.3, 128.2, 128.1, 128.0, 126.0, 101.3, 100.8, 99.5, 82.1, 75.5, 73.9, 73.5, 72.4, 68.5, 67.1, 67.0, 66.5, 64.8, 62.7, 54.9, 53.8, 51.5, 48.4, 47.1, 32.1, 32.0, 30.7, 29.7, 23.4, 21.5, 17.5, 17.5, 17.4.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{73}H_{88}N_8O_{22}Na$: 1451.5905 [M+Na]$^+$; found: 1451.5916.



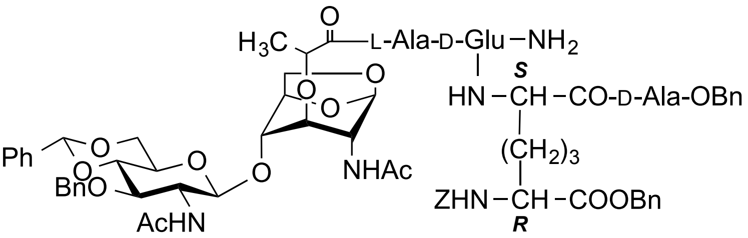
Protected disaccharide (anh) containing *N*-acetylmuramyl group and tripeptide (111) Compound **111** was synthesized from **108** and **34** with similar method to the synthesis of **70**. The crude product was purified by silica-gel flash column chromatography (CHCl$_3$/MeOH 15:1) to give compound **111** as white solid (22 mg, 90 %).

^{1}H NMR (400 MHz, CDCl$_3$): δ 7.85 (1H, d, J = 5.7 Hz; NH), 7.59 (1H, d, J = 7.3 Hz; NH), 7.52-7.29 (26 H, m; ArH, NH), 6.93 (1H, d, J = 9.8 Hz; NH), 6.76 (1H, s; NH), 5.73 (1H, d, J = 8.2 Hz; NH), 5.60 (1H, s; Ph-CH=O$_2$), 5.47 (1H, s; NH), 5.31 (1H, s; H$_{anh}$-1), 5.21 (1H, d, J = 8.5 Hz; NH), 5.17-5.04 (6H, m; -COOCH$_2$Ph × 3), 4.91 (1H, d, J = 12.4 Hz; Ph-CH-O-), 4.69-4.66 (2H, m; Ph-CH-O-, Gln-αH), 4.50-4.25 (7H, m; DAP 2-H, 6-H, H$_{anh}$-5, H-1, H-6, H$_{anh}$-6, Ala-αH), 4.12-3.97 (3H, m; Lac-αH, H-2, H$_{anh}$-2), 3.84-3.78 (2H, m; H-6’, H-4), 3.76 (1H, d, J = 2.1 Hz; H$_{anh}$-4), 3.70 (1H, dd, J

= 5.7 Hz, 7.3 Hz; H$_{anh}$-6’), 3.55 (1H, dd, *J* = 9.6 Hz, 9.6 Hz; H-3), 3.47 (1H, s; H$_{anh}$-3), 3.41-3.35 (1H, m; H-5), 2.35-2.25 (2H, m; Gln-γC$\underline{H}_2$), 2.11-2.05 (4H, m; NHCOC$\underline{H}_3$, Gln-βC$\underline{H}$), 1.91 (3H, s; -NHCOC$\underline{H}_3$), 1.97-1.69 (5H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.46-1.39 (2H, m; DAP 4-C$\underline{H}_2$), 1.35-1.33 (6H, m; Lac-βC$\underline{H}_3$, Ala-βC$\underline{H}_3$).

^{13}C NMR (125 MHz, CDCl$_3$): *δ* 173.8, 173.5, 173.1, 173.0, 172.2, 171.6, 171.2, 156.2, 138.1, 137.1, 136.2, 135.3, 135.2, 129.1, 128.7, 128.6, 128.6, 128.5, 128.5, 128.3, 128.2, 126.0, 101.3, 101.3, 100.2, 82.0, 77.7, 76.4, 75.5, 73.8, 72.4, 68.5, 67.3, 67.1, 67.1, 67.0, 66.4, 64.4, 54.7, 53.7, 52.7, 51.8, 49.6, 46.9, 32.4, 31.7, 30.6, 29.7, 23.7, 22.8, 21.4, 17.4, 17.2.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{70}H_{84}N_7O_{20}$: 1342.5766 [M+H]$^+$; found: 1342.5790.



Protected disaccharide (anh) containing *N*-acetylmuramyl group and tetrapeptide (112) Compound **112** was synthesized from **108** and **35** with similar method to the synthesis of **70**. The crude product was purified by silica-gel flash column chromatography (CHCl$_3$/MeOH 20:1) to give compound **112** as white solid (16 mg, 62 %).

^{1}H NMR (500 MHz, CDCl$_3$): *δ* 7.88 (1H, br s; N$\underline{H}$), 7.62 (1H,br s; N$\underline{H}$), 7.54-7.28 (26 H, m; Ar$\underline{H}$, N$\underline{H}$), 7.03 (1H, br s; N$\underline{H}$), 6.96 (1H, s; N$\underline{H}$), 6.93 (1H, s; N$\underline{H}$), 5.73 (1H, d, *J* = 7.2 Hz; N$\underline{H}$), 5.60 (1H, s; Ph-C$\underline{H}$=O$_2$), 5.31 (1H, s; H$_{anh}$-1), 5.14-5.04 (6H, m; -COOC$\underline{H}_2$Ph × 3), 4.91 (1H, d, *J* = 12.3 Hz; Ph-C$\underline{H}$-O-), 4.68 (1H, d, *J* = 12.3 Hz; Ph-C$\underline{H}$-O-), 4.53-4.25 (9H, m; Gln-α$\underline{H}$, DAP 2-$\underline{H}$, 6-$\underline{H}$, H$_{anh}$-5, H-1, H-6, H$_{anh}$-6, Ala-α$\underline{H}$, D-Ala-α$\underline{H}$), 4.11-3.99 (3H, m; Lac-α$\underline{H}$, H-2, H$_{anh}$-2), 3.84-3.75 (3H, m; H-6’, H-4, H$_{anh}$-4), 3.71 (1H, dd, *J* = 7.1 Hz, 7.1 Hz; H$_{anh}$-6’), 3.55 (1H, dd, *J* = 9.2 Hz, 9.2 Hz; H-3), 3.47 (1H, s; H$_{anh}$-3), 3.40-3.35 (1H, m; H-5), 2.25 (2H, br s; Gln-γC$\underline{H}_2$), 2.11-2.06 (4H, m; -NHCOC$\underline{H}_3$, Gln-βC$\underline{H}$), 1.91 (3H, s; -NHCOC$\underline{H}_3$), 1.87-1.62 (5H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.46-1.32 (11H, m; DAP 4-C$\underline{H}_2$, Lac-βC$\underline{H}_3$, Ala-βC$\underline{H}_3$, D-Ala-βC$\underline{H}_3$).

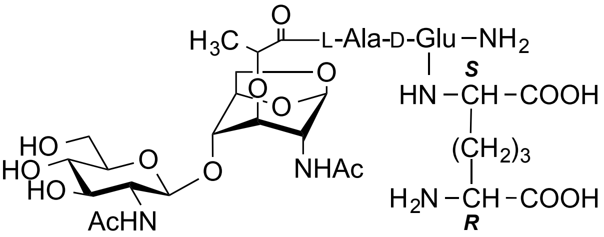
^{13}C NMR (125 MHz, CDCl$_3$): *δ* 173.8, 173.6, 173.5, 173.3, 172.2, 171.7, 171.2, 156.2, 138.1, 137.2, 136.3, 135.3, 129.1, 128.7, 128.6, 128.5, 128.4, 128.3, 128.2, 128.2, 128.1, 128.1, 128.0, 126.0, 101.3, 101.3, 100.2, 82.1, 75.4, 73.9, 72.4, 68.5, 67.2, 67.0, 66.4, 64.5, 54.7, 53.8, 51.6, 49.7, 48.4, 47.0, 32.0, 30.7, 29.7, 23.7, 22.8, 21.5, 17.4, 17.4,

17.3.

HRMS (ESI-LIT-orbitrap): m/z: calcd for $C_{73}H_{88}N_8O_{21}Na$: 1435.5956 $[M+Na]^+$; found: 1435.5973.

Compounds **2e**, **2f**, **2g** and **2h** were synthesized from **109**, **110**, **111** and **112**, respectively, with the similar method to the synthesis of **1f**.

Compound **2e** (quant.)

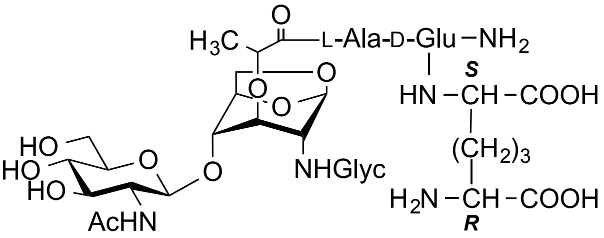


^{1}H NMR (500 MHz, CD_3OD): δ 5.32 (1H, s; H_{anh}-1), 4.61 (1H, d, *J* = 4.5 Hz; H_{anh}-5), 4.49 (1H, d, *J* = 8.4 Hz; H-1), 4.40-4.28 (4H, m; Ala-α$\underline{H}$, Gln-α$\underline{H}$, DAP 2-$\underline{H}$, H_{anh}-6), 4.11 (1H, q, *J* = 6.7 Hz; Lac-α$\underline{H}$), 4.04 (1H, s; H_{anh}-2), 3.89 (1H, dd, *J* = 1.9 Hz, 11.9 Hz; H-6), 3.85 (1H, s; H_{anh}-4), 3.78-3.67 (4H, m; H-2, H-5, DAP 6-$\underline{H}$, H_{anh}-6'), 3.53 (1H, s; H_{anh}-3), 3.46-3.42 (1H, m; H-3), 3.34-3.27 (2H, m; H-6', H-4), 2.37-2.34 (2H, m; Gln-γC$\underline{H}_2$), 2.21-2.15 (1H, m; Gln-βC$\underline{H}$), 2.06 (3H, s; -NHCOC$\underline{H}_3$), 2.02 (3H, s; -NHCOC$\underline{H}_3$), 1.99-1.70 (5H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.56-1.51 (2H, m; DAP 4-C$\underline{H}_2$), 1.41 (3H, d, *J* = 7.0 Hz; Ala-βC$\underline{H}_3$), 1.36 (3H, d, *J* = 6.7 Hz; Lac-βC$\underline{H}_3$).

^{13}C NMR (125 MHz, D_2O): δ 176.9, 176.9, 176.7, 176.4, 176.1, 176.0, 174.6, 174.6, 101.6, 100.9, 78.2, 77.0, 75.3, 74.6, 74.3, 70.8, 65.8, 62.5, 61.6, 56.5, 54.8, 53.8, 53.8, 50.6, 49.9, 32.4, 31.1, 30.7, 27.7, 23.3, 22.9, 21.9, 19.0, 17.6.

HRMS (LTQ-orbitrap MS): m/z: calcd for $C_{34}H_{55}N_7O_{18}Na$: 872.3496 $[M+Na]^+$; found: 872.3507.

Compound **2f** (92%)

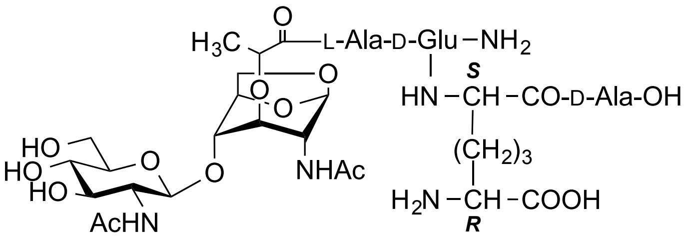


^{1}H NMR (500 MHz, D_2O): δ 5.41 (1H, s; H_{anh}-1), 4.75-4.65 (2H, m; H_{anh}-5, H-1), 4.30 (1H, q, *J* = 7.5 Hz; Ala-α$\underline{H}$), 4.25-4.22 (2H, m; Gln-α$\underline{H}$, DAP 2-$\underline{H}$), 4.19 (1H, dd, *J* = 5.0 Hz, 8.5 Hz; H_{anh}-6), 4.13 (1H, q, *J* = 6.5 Hz; Lac-αH), 4.06 (2H, s; -NHCOC$\underline{H}_2$OH), 3.98 (1H, s; H_{anh}-2), 3.92 (1H, s; H_{anh}-4), 3.83-3.80 (1H, m; H-6), 3.76 (1H, dd, *J* = 6.0 Hz, 8.5 Hz; H_{anh}-6'), 3.71-3.64 (3H, m; DAP 6-$\underline{H}$, H-5, H-2), 3.59 (1H, s; H_{anh}-3), 3.54-3.49 (1H, m; H-3), 3.40-3.39 (2H, m; H-6, H-4), 2.35-2.32 (2H, m; Gln-γC$\underline{H}_2$), 2.15-2.08 (1H, m; Gln-βC$\underline{H}$), 1.99 (3H, s; -NHCOC$\underline{H}_3$), 1.96-1.64 (5H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.44-1.36 (5H, m; DAP 4-C$\underline{H}_2$, Ala-βC$\underline{H}_3$), 1.31 (3H, d, J = 6.5 Hz; Lac-βC$\underline{H}_3$).

^{13}C NMR (125 MHz, D$_2$O): δ 178.1, 176.9, 176.5, 176.0, 175.9, 175.7, 175.4, 175.0, 101.9, 100.7, 77.7, 77.1, 77.0, 75.9, 75.1, 74.5, 70.7, 65.9, 61.9, 61.6, 56.7, 55.4, 54.7, 54.0, 50.6, 49.5, 32.6, 31.6, 31.0, 27.8, 23.2, 22.1, 19.0, 17.7.

HRMS (LTQ-orbitrap MS): m/z: calcd for $C_{34}H_{56}N_7O_{19}$: 866.3625 [M+H]$^+$; found: 866.3633.

Compound **2g** (90%)

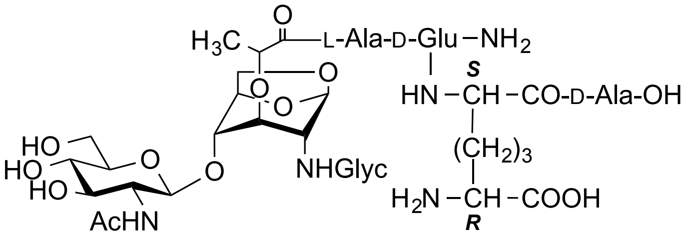


^{1}H NMR (500 MHz, D$_2$O): δ 5.38 (1H, s; H$_{\text{anh}}$-1), 4.65-4.59 (2H, m; H$_{\text{anh}}$-5, H-1), 4.29 (1H, q, J = 7.5 Hz; D-Ala-α$\underline{H}$), 4.24-4.15 (4H, m; Ala-α$\underline{H}$, Gln-α$\underline{H}$, DAP 2-$\underline{H}$, H$_{\text{anh}}$-6), 4.11 (1H, q, J = 7.5 Hz; Lac-α$\underline{H}$), 3.94 (1H, s; H$_{\text{anh}}$-2), 3.91 (1H, s; H$_{\text{anh}}$-4), 3.85-3.82 (1H, m; H-6), 3.76-3.65 (4H,m; H$_{\text{anh}}$-6’, DAP 6-$\underline{H}$, H-5, H-2), 3.52-3.49 (2H, m; H$_{\text{anh}}$-3, H-3), 3.40-3.38 (2H, m; H-6, H-4), 2.36-2.32 (2H, m; Gln-γC$\underline{H}_2$), 2.17-2.10 (1H, m; Gln-βC$\underline{H}$), 2.00 (3H, s; -NHCOC$\underline{H}_3$), 1.99 (3H, s; -NHCOC$\underline{H}_3$), 1.95-1.66 (5H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.42-1.36 (5H, m; DAP 4-C$\underline{H}_2$, D-Ala-βC$\underline{H}_3$), 1.31 (6H, m; Ala-βC$\underline{H}_3$, Lac-βC$\underline{H}_3$).

^{13}C NMR (125 MHz, D$_2$O): δ 179.3, 176.8, 176.6, 176.3, 176.0, 176.0, 175.4, 174.6, 174.2, 101.6, 100.9, 78.2, 77.0, 75.4, 74.6, 74.3, 70.8, 65.8, 61.6, 56.5, 55.4, 54.8, 53.8, 51.0, 50.6, 49.9, 32.5, 31.8, 31.0, 27.8, 23.3, 22.9, 22.0, 19.0, 18.0, 17.7.

HRMS (LTQ-orbitrap MS): m/z: calcd for $C_{37}H_{60}N_8O_{19}Na$: 943.3867 [M+Na]$^+$; found: 943.3876.

Compound **2h** (quant.)



^{1}H NMR (500 MHz, D$_2$O): δ 5.41 (1H, s; H$_{\text{anh}}$-1), 4.65 (2H, br s; H$_{\text{anh}}$-5, H-1), 4.31-4.26 (2H, m; D-Ala-α$\underline{H}$, Ala-α$\underline{H}$), 4.24-4.21 (3H, m; Gln-α$\underline{H}$, DAP 2-$\underline{H}$, H$_{\text{anh}}$-6),

4.13 (1H, q, $J = 6.9$ Hz; Lac-α$\underline{H}$), 4.05 (2H, s; -NHCOC$\underline{H}_2$OH), 3.98 (1H, s; H_{anh}-2), 3.92 (1H, s; H_{anh}-4), 3.83-3.80 (1H, m; H-6), 3.77-3.64 (4H, m; H_{anh}-6’, DAP 6-$\underline{H}$, H-5, H-2), 3.59 (1H, s; H_{anh}-3), 3.54-3.49 (1H, m; H-3), 3.40-3.39 (2H, m; H-6, H-4), 2.39-2.28 (2H, m; Gln-γC$\underline{H}_2$), 2.15-2.08 (1H, m; Gln-βC$\underline{H}$), 1.99 (3H, s; -NHCOC$\underline{H}_3$), 1.93-1.65 (5H, m; Gln-βC$\underline{H}$, DAP 3-C$\underline{H}_2$, 5-C$\underline{H}_2$), 1.44-1.30 (11H, m; DAP 4-C$\underline{H}_2$, Lac-βC$\underline{H}_3$, Ala-βC$\underline{H}_3$, D-Ala-βC$\underline{H}_3$).
^{13}C NMR (125 MHz, D_2O): δ 177.3, 176.5, 176.3, 175.7, 175.6, 175.6, 174.7, 174.5, 174.3, 101.5, 100.3, 77.5, 76.8, 76.7, 75.5, 74.7, 74.2, 70.4, 65.6, 61.6, 61.3, 56.3, 54.8, 54.3, 53.4, 50.3, 49.6, 49.2, 32.1, 31.3, 30.5, 27.5, 22.9, 21.6, 18.7, 17.4, 17.0.
HRMS (LTQ-orbitrap MS): m/z: calcd for $C_{37}H_{60}N_8O_{20}Na$: 959.3816 $[M+Na]^+$; found: 959.3821.

HEG1G cells bioassay for Nod1 activation

HEG1G cells were cultured in Dullbecco’s modified Eagle Media (purchased from Sigma), containing 10% heat-inactivated fetal calf serum with 100 units/mL penicillin and 100 μg/mL streptomycin (all culture reagents from Gibco). The assay procedure was as follows:

- 5×10^5 cells/mL HEG1G cells were seeded to black flat-bottom 96-well plates, incubated overnight before being treated with appropriate ligands for 24 h. NF-κB activation was detected by measuring the GFP fluorescence at 535 nm With Infinite M200 pro.

HEK293T cells bioassay for Nod2 activation

Human embryonic kidney (HEK) 293T was cultured in Dullbecco’s modified Eagle Media (purchased from Sigma), containing 10% heat-inactivated fetal calf serum with 100 units/mL penicillin and 100 μg/mL streptomycin (all culture reagents from Gibco). pMX2-HA-Nod2, pBxIV-luc and pEF1BOS-β-gal plasmids were gifts from N. Inohara (University of Michigan Medical School, Michigan, US). The assay procedure was as follows:

- 0.5×10^5 cells/mL HEK293T cells were seeded to transparent 24-well plate and incubated overnight before transfection. Then plasmids Nod2 (20 ng/well pMX2-HA-Nod2) and reporter plasmids: NF-κB-dependent pBxIV-luc (5 ng/well) and control pEF1BOS-β-gal (20 ng/well) were transfected into cells. Briefly, HEK293T cells were transfected by calcium phosphate method and 16 h post-transfection, cells were treated with various ligands. 24 h later, ligand-dependent NF-κB activation was determined by the luciferase reporter assay with FDSS.

Expression and Purification of ED-PknB

Namely, the nucleotide sequence encoding PknB from Gly_{354} to Gln_{626} (*ED-pknb*) was first inserted to vector pGEX-4T-3 (GE Healthcare) for expression as a glutathione-S-transferase (GST) fusion protein in BL21 cells. The GST tagged ED-PknB was purified by immobilized glutathione agarose (Pierce). The GST tag was cleaved by the thrombin CleanCleave kit (Sigma). Generally, 1 mL GST tagged ED-PknB at concentration of 1.5 mg/mL in cleave buffer was incubated with 200 μL 50% (v/v) suspension of thrombin agarose for 2 h at room temperature. After removing the agarose by centrifugation, the supernatant containing ED-PknB and GST was incubated with 300 mL of 50% (v/v) suspension of Glutathione agarose for 30 min, with 3 times repetition. GST was absorbed by the Glutathione agarose and ED-PknB was dialyzed against phosphate buffered saline (PBS) pH 7.4 before biotinylation reaction.

Protein binding assay and kinetic analysis with BLI

PBST (10 mM PBS, 0.02% Tween20, pH 7.4) was used as the buffer in all steps. The ForteBio 384TW microplate was chosen to save samples (40 μL/well) and increase the response of small molecular analytes. The instrument worked at 30 ^{o}C. Each assay included five typical steps: 1) Initial Baseline measurement; 2) Protein Loading; 3) Baseline (washing process); 4) Association with PGN fragments; 5) Dissociation. In detail, the biotinylated ED-PknB was loaded on high density Super Streptavidin (SSA) biosensor until saturation. The biosensors were dipped to buffer to give the baseline before dipping to the wells with different concentrations of PGN analytes. Six concentrations with two-fold serial dilutions were prepared for each compound, from 6.80×10^3 μM to 212 μM. Then the biosensors were dipped to the same wells as baseline to collect the dissociation signals. Double references were performed to eliminate non-specific binding and other system drift. The binding constants were calculated by globally fitting the data with 2:1 (Heterogenous ligand) Model on the Octet Data Analysis software 8.1.

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