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Synthesis of characteristic *Mycobacterium* peptidoglycan (PGN) fragments utilizing with chemoenzymatic preparation of *meso*-diaminopimelic acid (DAP), and their modulation of innate immune responses†

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Peptidoglycan (PGN) is a major component of bacterial cell wall and is recognized as a potent immunostimulant. The PGN in the cell envelope of *Mycobacterium Tuberculosis* has been shown to possess several unique characteristics including the presence of *N*-glycolyl groups (in addition to *N*-acetyl groups) in the muramic acid residues, and amidation of the free carboxylic acid of D-Glu or of *meso*-DAP in the peptide chains. Using a newly developed, highly stereoselective, chemoenzymatic approach for the synthesis of *meso*-DAP in peptide stems, we successfully synthesized for the first time, a series of *Mycobacterium* PGN fragments that include both mono- and disaccharides of MurNGlyc or 1,6-anhydro-MurNGlyc, as well as peptide-amidated variants. The ability of these PGN fragments to stimulate the immune system through activation of human Nod1 and Nod2 was examined. The PGN fragments were found to modulate immune stimulation, specifically, amidation at the D-Glu and *meso*-DAP in the peptide stem strongly reduced hNod1 activation. This effect was dependent on modification position. Additionally, *N*-glycolyl (instead of acetyl) of muramic acid was associated with slightly reduced human Nod1 and Nod2 stimulatory capabilities.

Introduction

Mycobacterium tuberculosis (TB) is a major global health issue, ranked as the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus (HIV).[1] *Mycobacteria*, including *Mycobacterium tuberculosis* (*M. tuberculosis*), are intracellular pathogens that evade both innate and adaptive host immunity.[2] There is a clear and urgent need for a deeper understanding of *Mycobacteria* biology and the epidemiology of infection.

Innate immune responses are the first line of defense against foreign invaders, and host immune cells employ pattern recognition receptors (PRRs) to recognize evolutionarily conserved microbial components called pathogen-associated molecular patterns (PAMPs). PRRs include membrane-bound Toll-like receptors (TLRs) and cytosolic Nod-like receptors (NLRs).[3,4] Peptidoglycan (PGN), as a major component of bacterial cell walls in both Gram-positive and Gram-negative bacteria,[5] is known as a potent immunostimulator. Nod1 and Nod2, founding members of the NLR protein family, have been shown to recognize bacterial PGN fragments (muropeptides) and regulate innate and adaptive immune responses by activating transcription factors including NF-κB.[6] Nod1 recognizes *meso*-DAP-containing muropeptides, with the basic motif required for ligand binding being iE-DAP (γ-D-glutamyl diaminopimelic acid).[7] The minimum ligand for Nod2, on the other hand, is MDP (muramyl dipeptide: MurNAc-L-Ala-D-isoGln).[8,9]

PGN is comprised of long glycan chains with repeating β(1–4) disaccharide, generally *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), which are linked *via* peptide bridges. The branched position of the peptide is usually a diaminocarboxylic acid, such as L-Lys (in many Gram-positive bacteria) or *meso*-diaminopimelic acid (*meso*-DAP, in Gram-negative bacteria and some Gram-positive

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bacteria, *e.g.*, *Mycobacteria* and *Bacillus* species). The highly cross-linked PGN layer in *M. tuberculosis* displays unique chemical features:[10] amidated carboxylic acid of D-Glu and *meso*-DAP in the peptide stem, and *N*-glycolylmuramic acid (MurNGlyc), in addition to the conventional *N*-acetylmuramic acid (MurNAc), in the glycan chain (Fig. 1A). This amidation may prevent the bacteria from being recognized by host Nod1—a finding also observed in *Bacillus subtilis*.[11] The glycolylated PGN exhibited increased stability in the presence of β-lactam antibiotics and lysozyme and was also hypothesized to stabilize the cell wall through hydrogen bonding.[12] These modifications were thought to be closely related to the intracellular survival and consequent pathogenesis of *M. tuberculosis*, but no direct evidence had been obtained so far.

With the exception of *N*-glycolyl MDP,[13–15] the immunostimulatory activity of PGN partial structures specific to *M. tuberculosis* has not been well characterized. Especially, the immunostimulatory capability of *meso*-DAP-containing *Mycobacterium* PGN fragments has been rarely studied. In this study, we synthesized various PGN partial structures, including MurNGlyc and *meso*-DAP containing tri- or tetrapeptide sequences with various amidation patterns, in order to investigate their Nod1 and Nod2 stimulatory potentials. We have shown that smaller PGN fragments, such as monosaccharide and disaccharide fragments, exhibited higher Nod2-stimulating activity than the corresponding longer glycan fragments.[16,17] It was also demonstrated that monosaccharide and disaccharide fragments with DAP-containing tripeptides exhibited potent Nod1-stimulating activity.[18] Hence, we chose to focus on monosaccharide and disaccharide fragments as synthetic targets in this study.

The (2*S*,6*R*)-*meso*-DAP has been identified as the most potent Nod1 stimulator compared with other three isomers in our previous study.[19] Although several synthetic methods for generation of *meso*-DAP, the key moiety for Nod1 recognition, have been reported,[18] we developed a chemoenzymatic method for the synthesis of the orthogonally protected *meso*-DAP. This newly developed method was advantageous for large-scale synthesis over the reported methods due to short reaction steps, mild conditions, and high enantioselectivity. Tripeptides (L-Ala-D-isoGln-*meso*-DAP) and tetrapeptides (L-Ala-D-isoGln-*meso*-DAP-D-Ala) were prepared and then coupled to mono- and disaccharides to construct a series of *Mycobacterium* PGN fragments with glycolyl- and amide-modifications (Fig. 1B). We then examined their modulation of human innate immune system *via* the interaction with cytosolic receptor proteins Nod1 and Nod2. This is the first demonstration that *Mycobacterium* PGN fragments exhibited very weak hNod1 and hNod2 activation, which provides direct proof for the cytosolic immune evasion of *M. tuberculosis*.

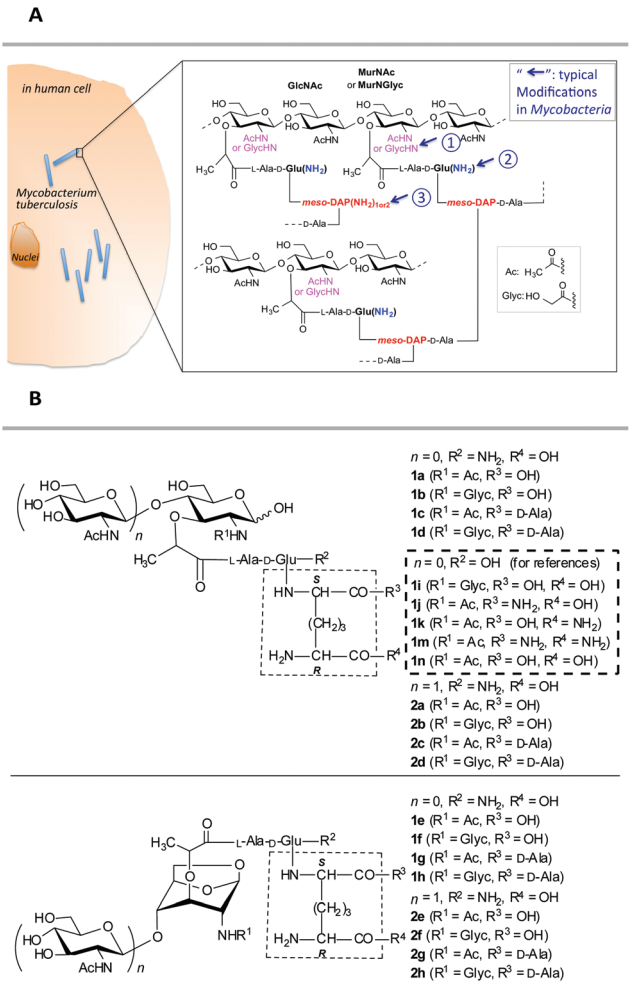


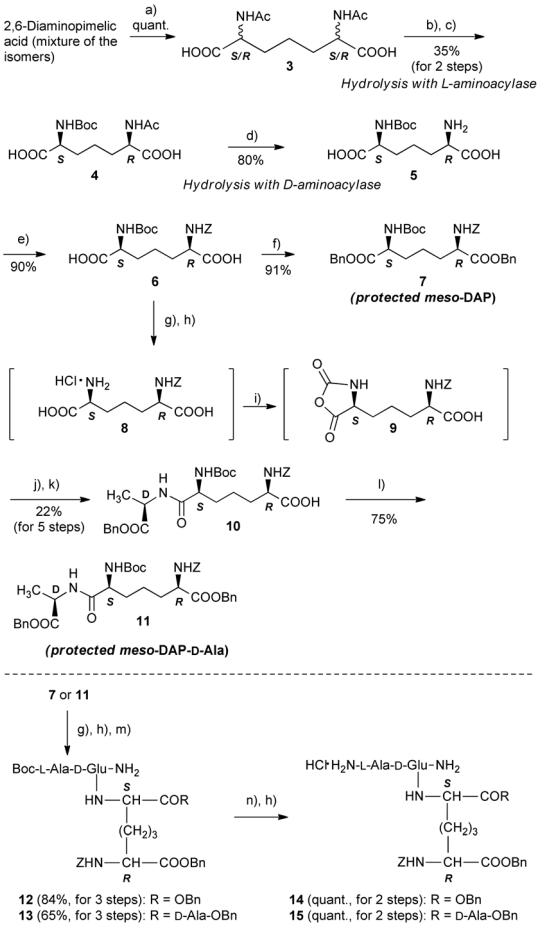
Fig. 1 (A) Schematic structure of *M. Tuberculosis* peptidoglycan. (B) Chemically synthesized *M. Tuberculosis* peptidoglycan fragments.

Results and discussion

An efficient chemoenzymatic method for the large-scale synthesis of *meso*-DAP derivatives

We initially developed a chemoenzymatic method for the large-scale synthesis of *meso*-DAP (Scheme 1). L-Aminoacylase and D-aminoacylase can specifically hydrolyze *N*-acetyl groups with L- and D-configurations, respectively, under mild conditions (40 °C in 0.05 M phosphate buffer). The commercially available racemic 2,6-diaminopimelic acid was first acetylated. Then, the L-*N*-acetyl group of **3** was hydrolyzed by L-aminoacylase (Acylase Amano) and the free amino group was protected with a *tert*-butoxycarbonyl (Boc) group to generate **4** with *S*- and *R*-chiral centers. Herein, a mixture of three compounds: (2*R*,6*R*)-di-*N*-acetyl DAP, (2*S*,6*S*)-di-*N*-Boc DAP, and compound **4**, was obtained, which was completely separated by extraction with ethyl acetate at different pH values. At a pH range of 5–6, all of the (2*S*,6*S*)-di-*N*-Boc DAP and a small amount of compound **4** were extracted to the organic layer. After acidification at a pH range of 1–2, compound **4** was transferred to the organic layer while (2*R*,6*R*)-di-*N*-acetyl DAP remained in the water layer. Compound **4** displayed high purity as revealed by its ^{1}H-NMR spectrum.

In order to facilitate the deprotection of acetyl group and also improve optical purity, *N*-acetyl in D-configuration of **4** was



Scheme 1 Synthesis of *meso*-DAP derivatives **14** and **15**: (a) Ac_2O, pyridine, H_2O, overnight; (b) L-aminoacylase, $CoCl_2{\cdot}6H_2O$, phosphate buffer, 40 °C, 5 d; (c) Boc_2O, TEA, H_2O/1,4-dioxane = 2 : 1, overnight; (d) D-aminoacylase, $CoCl_2{\cdot}6H_2O$, phosphate buffer, 40 °C, 7 d; (e) ZCl, Na_2CO_3, H_2O/1,4-dioxane = 2 : 1, 6 h; (f) BnBr, Na_2CO_3, DMF, 7 h; (g) 20% TFA/DCM, 30 min; (h) 1 M HCl in Et_2O; (i) triphosgene, THF, 50 °C, 6 h; (j) HCl·NH_2-D-Ala-OBn, Et_3N, DMF, 0 °C – r.t., 1 h; (k) Boc_2O, TEA, H_2O/1,4-dioxane = 1 : 1, overnight; l) BnBr, Cs_2CO_3, DMF, 6 h; (m) Boc-L-Ala-D-isoGln-COOH, WSCD·HCl, HOBt, Et_3N, THF, overnight; (n) TFA, 30 min.

cleaved by D-aminoacylase to yield chiral compound **5** in a manner similar to L-aminoacylase.

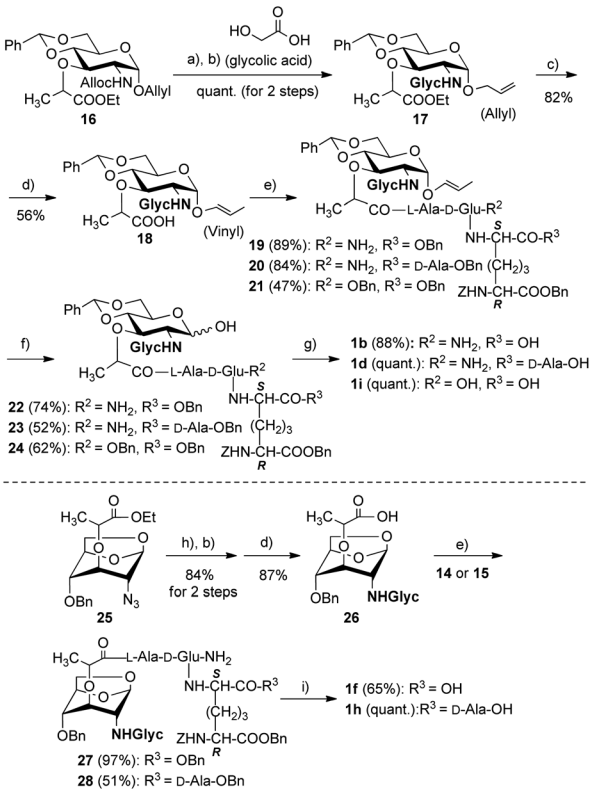
In the enzyme reactions, the addition of proper concentration of cobalt(II) ions effectively accelerated the reaction. We optimized the concentration of cobalt(II) (Tables S1 and S2†). For the L-aminoacylase reaction generating compound **S1** from compound **3**, addition of 1 mM $CoCl_2$ led to higher yield (60% *meso*-DAP in 4 d) than reaction conducted in the absence of Co(II) ions (32% in 4 d). However, 10 mM $CoCl_2$ produced almost the same yield as 1 mM. For D-aminoacylase reaction generating compound **5** from compound **4**, yield of compound **5** was increased when the concentration of Co(II) was increased; Specifically, 10% (no $CoCl_2$ in 4 d), 32% (1 mM $CoCl_2$ in 4 d), 67% (10 mM $CoCl_2$ in 4 d), and 83% (10 mM $CoCl_2$ in 8 d).

The liberated amino group of compound **5** was protected with a benzyloxycarbonyl (Z) group to obtain compound **6**. The two carboxylic acids were protected with benzyl groups yielding the orthogonally protected *meso*-DAP **7**. The Boc group of compound **7** was deprotected with trifluoroacetic acid (TFA) and 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 in THF, to give fully protected tripeptide **12**. The optical purity of **12** was confirmed by chiral HPLC analysis (CHIRALPAK AD-H, Daicel Chemical), showing none of the other enantiomer and diastereomers were contained. The Boc group of **12** was then removed to obtain the key intermediate, tripeptide **14** (H-L-Ala-D-isoGln-*meso*-DAP).

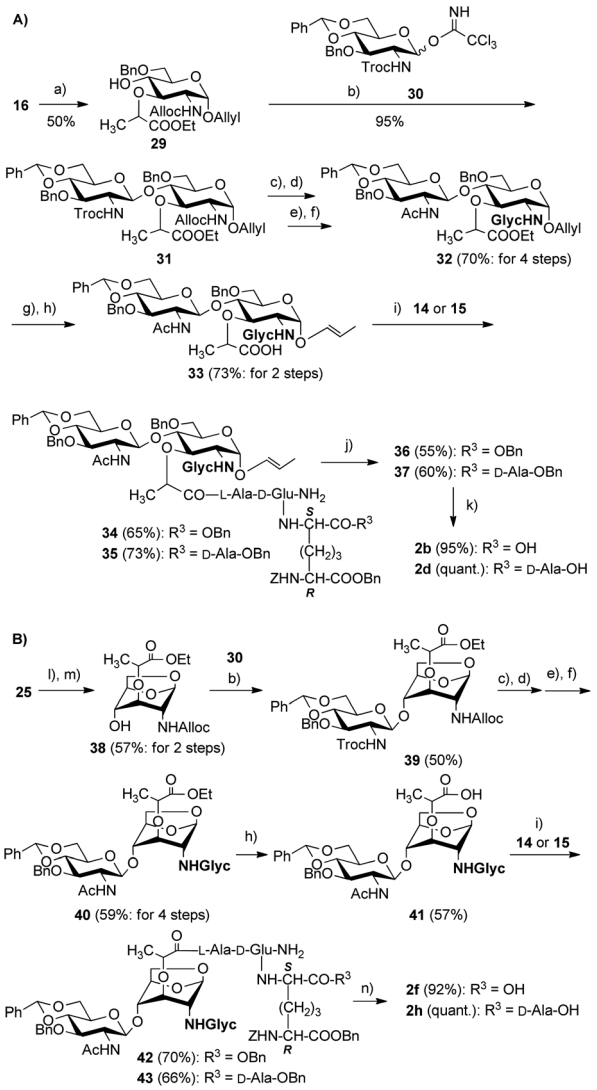
To prepare tetrapeptide, the Boc group of compound **6** was cleaved with TFA, and deprotected product **8** was reacted with triphosgene to form a *N*-carboxyanhydride (NCA) ring in compound **9**.[20] The ring-opening polymerization of α-amino acid *N*-carboxyanhydrides (NCAs) has been reported to be the most economical and expedient process for the synthesis of polypeptide-containing block copolymers.[21] Herein, the ring-opening reaction was conducted by coupling with HCl·H-D-Ala-OBn to generate the dipeptide with a free amino group, which was protected with Boc group in **10**. Tetrapeptide **15** (H-L-Ala-D-isoGln-*meso*-DAP-D-Ala) was synthesized from **10** in a similar manner to the synthesis of **14**. With the peptides in hand, we continued the synthesis of *meso*-DAP containing PGN fragments.

Synthesis of *meso*-DAP containing *Mycobacterium* PGN fragments

The synthetic procedures for the generation of monosaccharide compounds containing *N*-glycolyl are depicted in Schemes 2 and S1.† (The synthesis of MurNAc-containing structures is described in Scheme S2.†) For MurNGlyc-containing fragments, the Alloc protection group in compound **16**[16] was cleaved and then glycolylated using 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium Chloride (DMT-MM)[22] to prevent esterification of the liberated hydroxy group of glycolic acid. The resulting compound **17** was obtained in quantitative yield. The allyl group in compound **17** was isomerized using H_2-activated $[Ir(cod)(MePh_2P)_2]PF_6$ (cod = 1,5-cyclooctadiene), followed by the deprotection of ethyl ester with LiOH to give the product **18**. Compound **18** was subsequently condensed with tripeptide **14**, tetrapeptide **15**, or HCl·NH_2-L-Ala-D-isoGlu-OBn-*meso*-DAP$(OBn)_2$[18] to yield compounds **19**, **20**, or **21**, respectively. The removal of vinyl group with iodine and water and final hydrogenation afforded the final compounds **1b**, **1d**, and **1i** in high yields. The 1,6-anhydro-muramic acid-containing compounds were synthesized from an azide sugar **25**.[18] The azide group of compound **25** was reduced to a free amino group which was glycolylated to generate compound **S4**. Compound **S4** underwent ethyl-ester removal, peptide-coupling, and deprotection to generate the final compounds, **1f** and **1h**.

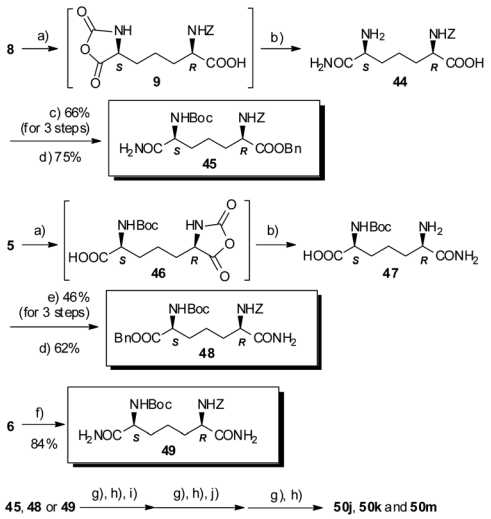


Scheme 2 Synthesis of *Mycobacterium* PGN fragments with monosaccharides. (a) $Pd(PPh_3)_4$, TMSDMA, $CHCl_3$, 2 h; (b) glycolic acid, DMT-MM, THF, 2.5 h; (c) $[Ir(cod)(H)(MePh_2P)_2]PF_6$, THF, 2 h; (d) LiOH, THF/1,4-dioxane/H_2O = 4 : 2 : 1, 1 h; (e) **14**, **15** or HCl·NH_2-L-Ala-D-isoGlu-OBn-*meso*-DAP(OBn)$_2$, DMT-MM, Et_3N, CH_3OH/H_2O = 2 : 1, overnight; (f) I_2, THF/H_2O = 1 : 1, 2 h; (g) H_2 (2.0 MPa), $Pd(OH)_2$, AcOH; (h) Zn, THF/AcOH = 1 : 1, 30 min; (i) H_2 (2.0 MPa), $Pd(OH)_2$, THF/H_2O/AcOH = 1 : 1 : 0.1.



Scheme 3 Synthesis of *Mycobacterium* PGN fragments with disaccharides. (A) having a MurNGlyc moiety and (B) a MurNGlyc(anh) moiety. (a) $Me_3N{\cdot}BH_3/BF_3{\cdot}Et_2O$, CH_3CN, 1 h; (b) TMSOTf, MS(4 Å), CH_2Cl_2, −15 °C, 15 min; (c) Zn/Cu, AcOH/THF = 1 : 1, 1 h; (d) Ac_2O, pyridine, overnight; (e) $Pd(PPh_3)_4$, TMSDMA, $CHCl_3$, 2 h; (f) glycolic acid, DMT-MM, THF, 2 h; (g) $[Ir(cod)(H)(MePh_2P)_2]PF_6$, THF, 2 h; (h) LiOH, THF/1,4-dioxane/H_2O = 4 : 2 : 1, 1 h; (i) DMT-MM, Et_3N, CH_3OH/H_2O = 2 : 1, overnight; (j) I_2, THF/H_2O = 1 : 1, 2 h; (k) H_2 (2.0 MPa), $Pd(OH)_2$, AcOH, 24 h; (l) H_2 (1.5 MPa), $Pd(OH)_2$, THF, 36 h; (m) AllocCl, Et_3N, CH_2Cl_2, 1.5 h; (n) H_2 (2.0 MPa), $Pd(OH)_2$, THF/H_2O/AcOH = 1 : 1 : 0.1, 24 h.

The synthesis of *Mycobacterium* PGN fragments with disaccharides was also succeeded following the routes in Schemes 3 and S3† (MurNGlyc-containing structure) and Scheme S4† (MurNAc-containing structure). For GlcNAc-MurNGlyc-containing structures (Scheme 3A), the glycosyl acceptor **29** was prepared from the regioselective ring opening of 4,6-*O*-benzylidene of **16** to form 6-*O*-benzyl derivative by using $BH_3{\cdot}Me_3N/BF_3{\cdot}Et_2O$. Glycosylation of compound **29** with *N*-Troc glucosaminyl trichloroacetimidate **30** in the presence of TMSOTf afforded the desired disaccharide **31**. Compound **30** was chosen as the glycosyl donor because of its excellent β-selective glycosylation ability due to the neighboring participation of Troc group. The Troc group in **31** was cleaved with Zn/Cu and the liberated amino group was acetylated to give compound **S14**. The Alloc group in compound **S14** was then replaced either by a glycolyl group (as shown in Scheme 3A) to generate the key intermediate **32** for the synthesis of glycolyl-containing compounds, or an acetyl group to generate the intermediate for synthesis of acetyl-containing compounds (Scheme S4†). After isomerization of the allyl group of compound **32** to a vinyl group with an Ir-complex, the cleavage of ethyl ester generated compound **33** in 73% yield in 2 steps. Coupling of compound **33** with peptides and subsequent deprotection of the vinyl group (with I_2) and benzyl-type groups (*via* hydrogenation) generated the *N*-glycolyl disaccharide fragments **2b** and **2d**. The GlcNAc-MurNGlyc(anh)-containing structures **2f** and **2h** (Scheme 3B) were synthesized similarly to the synthesis of compounds **2b** and **2d** from compound **38**. Compound **38** was prepared in 57% yield from azide sugar **25** *via* hydrogenation and the introduction of an Alloc group in 2 steps. The synthesis of *Mycobacterium* PGN fragments with



Scheme 4 Synthesis of amidated derivatives of *meso*-DAP-containing peptides: (a) triphosgene, THF, 50 °C, 5 h; (b) 0.5 M NH_3 in 1,4-dioxane, 0 °C – r.t., 1.5 h; (c) Boc_2O, TEA, H_2O/DMF = 1 : 2, overnight; (d) BnBr, Cs_2CO_3, DMF, overnight; (e) ZCl, Na_2CO_3, H_2O/1,4-dioxane = 1 : 1, 2 h; (f) 28% ammonia, WSCD·HCl, HOBt, Et_3N, THF, overnight; (g) TFA, 30 min; (h) 1 M HCl in Et_2O; (i) Boc-D-Glu-OBn, WSCD·HCl, HOBt, Et_3N, THF, overnight; (j) Boc-L-Ala-OH, WSCD·HCl, HOBt, Et_3N, THF, overnight.

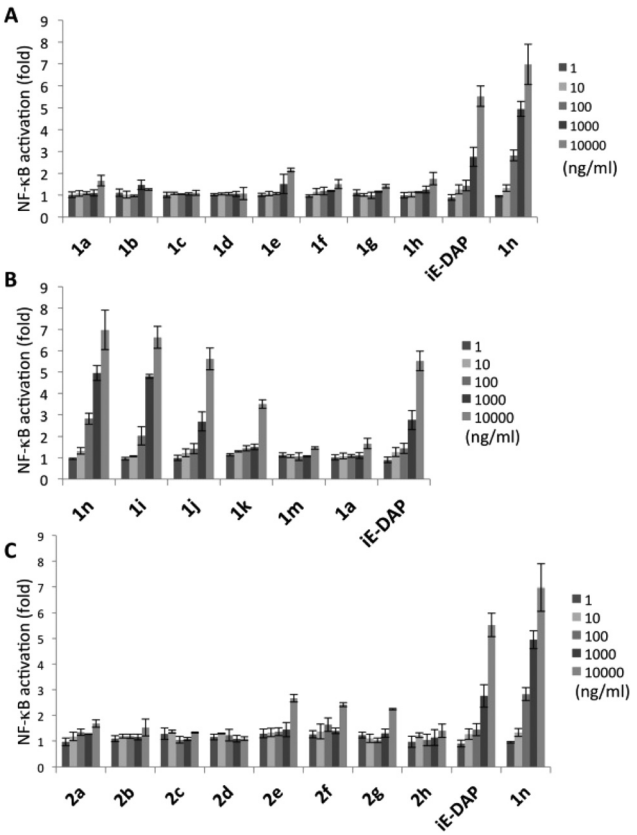


Fig. 2 Stimulation of human Nod1 with synthetic PGN fragments. (A) Monosaccharide-containing fragments (**1a–1h**) of *M. tuberculosis* PGN, and iE-DAP and **1n** (MS-$3P_{A\text{-}iE\text{-}DAP}$; non-amidated compound) were used as references. (B) Monosaccharide-containing fragments with or without amidation. (C) Disaccharide-containing fragments (**2a–2h**) of *M. tuberculosis* PGN.

monosaccharides (compounds **1a–1h**) and disaccharides (compounds **2a–2h**) were thus achieved.

In order to comprehensively compare the effect of amidation modification on the host immune stimulation, PGN fragments **1j**, **1k** and **1m** with peptides, **50j**, **50k** and **50m**, were also synthesized (Schemes 4 and S5†). For the synthesis of L-amide-*meso*-DAP **45**, NCA in **9** was reacted with anhydrous NH_3 (5 equiv.) in 1,4-dioxane to give the L-amidated **44**. After protection of the free amino and carboxyl groups, **45** was obtained successfully. The D-amide-*meso*-DAP **48** was also prepared from **5** in a similar way. The two –COOH groups in **6** were amidated by 28% ammonia aqueous solution with WSCD·HCl, HOBt and triethylamine to give L,D-di-amide-*meso*-DAP **49**. 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 **50j**, **50k** and **50m**, were successfully synthesized, which were used for the synthesis of compounds **1j**, **1k**, and **1m** in Scheme S5.†

Innate immunomodulation of *Mycobacterium* PGN fragments

The immunomodulating abilities of the synthetic *Mycobacterium* PGN fragments were evaluated *via* hNod1 and hNod2 activation with cell assays utilizing HEK293T based conditions; hNod1 assay was done with stably human *NOD1* transfected cells (HEG1G) (Fig. 2), whereas hNod2 assay was conducted with transiently human *NOD2* transfected cells (Fig. 3), to find out the structural motifs that affect the innate immune modulation.

In the human Nod1 assay (Fig. 2), iE-DAP and **1n** (MS-$3P_{A\text{-}iE\text{-}DAP}$), were used as the references. Compound **1n** had been shown to have more potent activity than **iE-DAP** in our previous study.[18] Analysis of structure–activity relationships systematically revealed the effects of amidation patterns at distinct peptides (Glu or *meso*-DAP). Amidation at the α-carboxylic acid of D-Glu dramatically reduced hNod1 activation (**1a–1h**) compared to **1n** (Fig. 2A), which was consistant with our finding from the corresponding peptides: 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).[23] In Fig. 2B, different amidation patterns were compared based on their hNod1 activation potentials. Amidation of one of the two carboxyl groups of *meso*-DAP (**1j** and **1k**) displayed a milder influence on hNod1 activation in comparison with the D-Glu modification (**1a**). Amidation of the carboxylic acid at the D-position of *meso*-DAP (**1k**) led to a greater reduction in hNod1 activation potential than that at the L-position (**1j**), implying that the D-COOH was more important than the L-COOH for hNod1 sensing. The glycolyl modification had little effect on hNod1 activation as demonstrated by **1n** and **1i**.

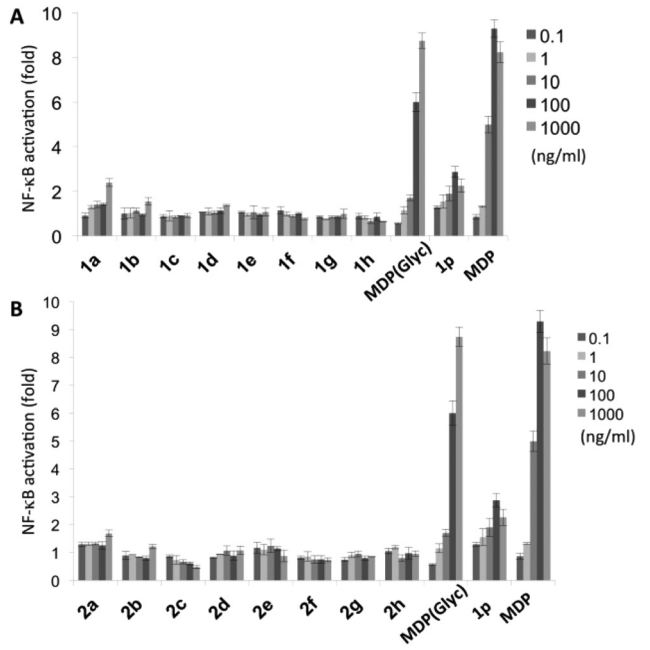


Fig. 3 Stimulation of human Nod2 with synthetic PGN fragments. (A) Monosaccharide-containing fragments (**1a–1h**) of *M. tuberculosis* PGN, and MDP and **1p** (MurNAc-L-Ala-D-isoGln-L-Lys) were used as references. (B) Disaccharide-containing fragments (**2a–2h**) of *M. tuberculosis* PGN.

We also found that anhydro-MurN-containing structures were slightly more potent activators of hNod1 than regular MurN structures (Fig. 2A and C). Disaccharide fragments (**2a–2h**) showed similar hNod1 activation tendencies as their corresponding monosaccharides varieties. The above results demonstrate that amidation at D-Glu as well as *meso*-DAP in *Mycobacterium* PGN fragments reduce their recognition by hNod1, and this may provide advantage to the bacteria by allowing them to escape from the detection by the host's innate immune system.

In the human Nod2 assay (Fig. 3), **MDP** and **1p** (MurNAc-L-Ala-D-isoGln-L-Lys)[16] were used as the references. The *N*-glycolyl MDP [**MDP(Glyc)**] displayed strong hNod2 activation; however, it was less potent than MDP. Comparing *N*-acetylated tripeptide monosaccharide **1a** and disaccharide **2a** with *N*-glycolylated tripeptide monosaccharide **1b** and disaccharide **2b**, *N*-acetylated **1a** and **2a** induced slightly greater activation of hNod2 than the corresponding **1b** and **2b**, respectively. Thus, *N*-glycolyl modification led to reduced hNod2 activation, although the effect was not so significant. The effect of DAP residues on activation of hNod2 was also investigated for the first time in detail. Tripeptide-containing fragments, **1a**, **1b**, **2a**, and **2b**, displayed very weak hNod2 stimulatory capabilities, while Lys-containing tripeptide, **1p** (MurNAc-L-Ala-D-isoGln-L-Lys), showed modest activity. These results clearly demonstrate that the *meso*-DAP moiety dramatically decreases hNod2 activation. Almost no hNod2-activating function was observed for DAP-containing tetrapeptides, **1c**, **1d**, **2c**, and **2d**. As expected, fragments with (anh)MurN (**1e–1h** and **2e–2h**) were unable to activate Nod2 due to modification of the MurN backbone.

Conclusions

The effective chemoenzymatic synthesis of *meso*-DAP derivatives greatly facilitated the preparation of various *meso*-DAP-containing PGN fragments whose innate immunity modulating efficacy was comprehensively explored. Glycolylation and amidation are the major structural modification characteristics of *M. tuberculosis* peptidoglycans. This study initially manifests that *Mycobacterium* PGN fragments exhibit very weak hNod1 and hNod2 activation, providing a potential mechanism for *M. tuberculosis* cytosolic immune evasion. Amidation is responsible for the reduced hNod1 responses, which is consistent with previously reported studies using peptides and regular monosaccharide-containing structures.[7,10] In addition, this study systematically evaluated the effect of the *meso*-DAP residue on hNod2 activation for the first time. We previously reported that MDP, the minimal ligand required for hNod2 activation, elicited the strongest hNod2 activation and Lys-containing tri- or tetrapeptides also possessed significant Nod2-stimulatory activity, although their potency decreased as the length of the peptide chain increased.[16,17] We demonstrate here that the *meso*-DAP residue considerably reduces the hNod2-stimulatory activity of PGN fragments.

On the other hand, the glycolylation modification had no significant effect on either hNod1 or hNod2 stimulation; *N*-glycolylated compounds showed slightly weaker activities than the corresponding acetylated ones. The glycolyl group may, rather, contribute to maintenance of cell wall integrity and improve resistance to lysozyme—another important element for bacterial survival.

Experimental section

General

^{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 compounds were 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.

(2*S*,6*R*)-6-acetamido-2-((*tert*-butyloxycarbonyl)amino)heptanedioic acid (4). Compound **3** (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 solution was stirred at 40 °C for 5 d. The solution was cooled to room temperature and acidified to pH 2–3 before being added activated carbon, then warmed to 70 °C for 10 min. The black suspension was filtrated, and the filtrate was evaporated to give a crude compound.

Without any purification, the crude compound was dissolved in H_2O (200 mL) and triethylamine (60 mL). The solution of Boc_2O (70 mL) in 1,4-dioxane (100 mL) was added to the water solution dropwise at 0 °C. The reaction was stirred at room temperature and monitored by TLC until complete reaction. The mixture was adjusted to pH 5–6 with 2 M aq. HCl and extracted with ethyl acetate to remove di-butoxycarbonated compound. Further adjusted to pH 2–3 and extracted with ethyl acetate several times to get pure **4**. 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 aq. NH_3, then the solution was lyophilized to give compound **4** as a 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<u>H</u>(NHAc)–), 3.82 (1H, br s; –C<u>H</u>(NHBoc)–), 1.94 (3H, s; –NHCOC<u>H</u>$_3$), 1.78–1.48 (4H, m; 3-C<u>H</u>$_2$, 5-C<u>H</u>$_2$), 1.37–1.29 (11H, m; –OC(C<u>H</u>$_3$)$_3$, 4-C<u>H</u>$_2$); ^{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 (5). D-aminoacylase (5.65 g) was dissolved in phosphate buffer (pH = 8.2, 0.05 M), heated to 40 °C. $CoCl_2{\cdot}6H_2O$ (1.62 g, 6.81 mmol) was added to the solution followed by the addition of **4** (11.3 g). Without stirring, the mixture was reacted at 40 °C for 7 d. The solution was cooled to room temperature and acidified to pH 2–3 before being added activated carbon, then warmed to 70 °C for 10 min. The black suspension was filtrated, and the filtrate was evaporated to give a crude compound. The residue was purified with HP20 to give compound **5** as a purple solid (7.90 g, 80%).

^{1}H NMR (500 MHz, D_2O): δ 4.18 (0.2H, br s; –C<u>H</u>(NHAc)–), 3.93 (1H, br s; –C<u>H</u>(NHBoc)–), 3.78 (0.8H, m; –C<u>H</u>(NH$_2$)–), 2.02–1.67 (4H, m; 3-C<u>H</u>$_2$, 5-C<u>H</u>$_2$), 1.52–1.49 (11H, m, –OC(C<u>H</u>$_3$)$_3$, 4-C<u>H</u>$_2$); 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 (6). To the solution of compound **5** (7.5 g, 26 mmol) and Na_2CO_3 (10.95 g) in water (250 mL) was added benzyl chloroformate (5.57 mL, 39 mmol) in 1,4-dioxane (125 mL) dropwise at 0 °C, stirred at 0 °C for 1 h, then stirred at room temperature for 5 h. The solution was washed with diethylether, and the water layer was acidified to pH 2–3 and extracted with ethyl acetate three times. The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. The crude compound was purified by silica-gel flash column chromatography ($CHCl_3$:CH_3OH : CH_3COOH = 12 : 1 : 0.1) to give compound **6** as a white solid (9.18 g, 90%).

^{1}H NMR (400 MHz, CD_3OD): δ 7.36–7.25 (5H, m; Ar<u>H</u>), 5.08 (2H, s; –C<u>H</u>$_2$Ph–), 4.15 (1H, dd, J = 4.6 Hz, 8.4 Hz; –C<u>H</u>(NHBoc)–), 4.07 (1H, dd, J = 4.6 Hz, 8.2 Hz; –C<u>H</u>(NHZ)–), 1.93–1.60 (4H, m; 3-C<u>H</u>$_2$, 5-C<u>H</u>$_2$), 1.54–1.42 (11H, m; –OC(C<u>H</u>$_3$)$_3$, 4-C<u>H</u>$_2$); ^{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.

Dibenzyl (2*S*,6*R*)-6-[{(benzyloxy)carbonyl}amino]-2-{(*tert*-butyloxycarbonyl) amino}heptanedioate (7). Compound **6** (4.0 g, 9.42 mmol) and Na_2CO_3 (2.0 g, 18.8 mmol) were dissolved into dry DMF (25 mL). The resulting suspension was stirred at room temperature for 30 min before BnBr (4.5 mL, 37.7 mmol) were added dropwise. The reaction was reacted for 7 h before quenching with H_2O and extracted with ethyl acetate twice. The combined organic layers were washed with brine, dried over Na_2SO_4 and concentrated *in vacuo*. The crude compound was purified by silica-gel flash column chromatography (toluene : AcOEt = 8 : 1) to give compound **7** as a white solid (5.18 g, 91%).

(2*R*,6*S*)-7-[{(*R*)-1-(benzyloxy)-1-oxopropan-2-yl}amino]-2-[{(benzyloxy)carbonyl}amino]-6-{(*tert*-butoxycarbonyl)amino}-7-oxoheptanoic acid (10). Compound **6** (2.0 g, 4.71 mmol) was dissolved in 50% TFA in CH_2Cl_2 (30 mL) and the mixture was stirred for 30 min at room temperature. The solution was then concentrated and co-evaporated with toluene to remove TFA. Addition of 1 M HCl diethylether solution gave a white precipitate. The supernatant was removed to give Boc-deprotected intermediate **8**, which was lyophilized in 1,4-dioxane. The crude **8** was dissolved in dry THF at 50 °C, followed by addition of triphosgene (1.54 g, 5.18 mmol) in dry THF. The solution was stirred at 50 °C for 6 h. After cooling to room temperature, the solvent was evaporated quickly, and the oily solid **9** was washed by dry hexane twice. Without purification, the next step was proceeded immediately. To the solution of the crude compound **9** in dry DMF (60 mL) was added HCl·H-D-Ala-OBn (3.05 g, 14.13 mmol) and triethylamine (2.6 mL) in dry DMF (30 mL) at 0 °C. The mixture was stirred at 0 °C for 0.5 h, and then was warmed to room temperature for 0.5 h. After removing DMF, the next step was carried out directly. The crude compound was dissolved into H_2O/1,4-dioxane (1 : 1, 80 mL) followed by addition of triethylamine (4.8 mL) at 0 °C. Boc_2O (13 mL) was then added dropwise to the solution, and the solution was stirred at room temperature overnight. The solution was acidified to pH 2–3 and extracted with ethyl acetate five times. The combined organic layers were dried over Na_2SO_4 and concentrated *in vacuo*. 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 **10** (606.8 mg, 22% for 5 steps).

^{1}H NMR (400 MHz, CD_3OD): δ 7.36–7.24 (10H, m; ArH), 5.17–5.08 (4H, m; –O–CH_2-Ph × 2), 4.41 (1H, q, J = 7.3 Hz; D-Ala-αH), 4.12 (1H, dd, J = 4.8 Hz, 9.0 Hz; –CH(NHBoc)–), 4.03 (1H, t, J = 4.8 Hz; –CH(NHZ)–), 1.88–1.42 (15H, m; DAP 3-CH_2, 4-CH_2, 5-CH_2, –OC(CH_3)$_3$), 1.37 (3H, d, J = 7.3 Hz; D-Ala-βCH_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.

Protected *meso*-DAP-D-Ala (11). Compound **10** (600 mg, 1.02 mmol) and Cs_2CO_3 (199 mg, 0.61 mmol) were dissolved into dry DMF (5 mL). The resulting suspension was stirred at room temperature for 30 min before BnBr (242 μL, 2.4 mmol) were added dropwise. The reaction was reacted for 6 h before quenching with H_2O and extracted with ethyl acetate three times. The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The crude compound was purified by silica-gel flash column chromatography ($CHCl_3$: acetone = 20 : 1) to give compound **11** as a white solid (5.18 g, 75%).

Fully-protected L-Ala-D-isoGln-*meso*-DAP (12). Compound **7** (1.0 g, 1.65 mmol) was dissolved in 50% TFA in CH_2Cl_2 (20 mL) and the mixture was stirred for 30 min at room temperature. The reaction mixture was then concentrated and co-evaporated with toluene. Addition of 1 M HCl diethylether solution gave a white precipitate. The supernatant was removed to give Boc-deprotected intermediate. To the residue was added anhydrous THF, 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. Triethylamine (689 μL, 4.95 mmol) was then added to the solution at 0 °C. The mixture was stirred overnight at room temperature. 10% citric acid solution was added to the mixture and extracted with ethyl acetate three times. The combined organic layers were washed with saturated aq. $NaHCO_3$, brine and dried over Na_2SO_4, and concentrated *in vacuo*. The crude compound was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 20 : 1) to give **12** as a white solid (1.11 g, 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.

Protected H-L-Ala-D-isoGln-*meso*-DAP (14). Compound **12** (800 mg, 1 mmol) was dissolved in TFA (3 mL) and the resulting mixture was stirred for 30 min at room temperature. The reaction mixture was then concentrated and co-evaporated with toluene. Addition of 1 M HCl diethylether solution gave a white solid. The supernatant was removed with decantation to give Boc-deprotected intermediate **14**. The compound was used without further purification.

Protected L-Ala-D-isoGln-*meso*-DAP-D-Ala (13). Compound **13** was synthesized from compound **11** with similar method to the synthesis of compound **12**. The crude compound was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 30 : 1) to give **13** as a white solid (450 mg, 65%).

^{1}H NMR (500 MHz, CD_3OD): δ 7.34–7.26 (15H, m; ArH), 5.18–5.04 (6H, m; –CH_2-Ph × 3), 4.43 (1H, q, J = 7.3 Hz; D-Ala-αH), 4.33 (1H, dd, J = 3.2 Hz, 10.9 Hz; Gln-αH), 4.26 (1H, dd, J = 5.5 Hz, 7.6 Hz; DAP 2-H), 4.17 (1H, m; DAP 6-H), 4.00 (1H, q, J = 7.2 Hz; L-Ala-αH), 2.32–2.22 (3H, m; Gln-βCH, γCH_2), 1.84–1.42 (16H, m; Gln-βCH, DAP 3-CH_2, 5-CH_2, 4-CH_2, –OC(CH_3)$_3$), 1.37 (3H, d, J = 7.3 Hz; D-Ala-βCH_3), 1.28 (3H, d, J = 7.2 Hz; L-Ala-βCH_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.

Protected H-L-Ala-D-isoGln-*meso*-DAP-D-Ala (15). Compound **15** was synthesized from compound **13** with similar method to the synthesis of compound **14**. The compound was used without further purification.

The synthetic procedures of **1b**, **1d** and **1i** in Scheme 2 are shown in ESI.†

Compound 1b. ^{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-αH, Gln-αH, Lac-αH, DAP 2-H), 4.04–3.93 (3H, m; –NHCOCH_2OH, H-2), 3.86–3.66 (4H, m; DAP 6-H, H-3, H-5, H-6), 3.58–3.41 (2H, m; H-4, H-6′), 2.35–2.32 (2H, m; Gln-γCH_2), 2.14–2.07 (1H, m; Gln-βCH), 1.97–1.91 (1H, m; Gln-βCH), 1.89–1.63 (4H, m; DAP 3-CH_2, 5-CH_2), 1.43–1.29 (8H, DAP 4-CH_2, Lac-βCH_3, Ala-βCH_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.

Compound 1d. ^{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-αH, Ala-αH, Gln-αH, Lac-αH, DAP 2-H), 4.03–3.92 (3H, m; –NHCOCH_2OH, H-2), 3.85–3.65 (4H, m; DAP 6-H, H-3, H-5, H-6), 3.57–3.42 (2H, m; H-4, H-6′), 2.32 (2H,br s; Gln-γCH_2), 2.14–2.06 (1H, m; Gln-βCH), 1.92–1.66 (5H, m; Gln-βCH, 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 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.

Compound 1i. ^{1}H NMR (400 MHz, D_2O): δ 5.07 (1H, d, J = 2.5 Hz; H-1β), 4.23–4.16 (4H, m; Ala-αH, Glu-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; Glu-γCH$_2$), 2.11–2.02 (1H, m; Glu-βCH), 1.91–1.62 (5H, m; Glu-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_2O): 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.

2-Glycolylamino-1,6-anhydro-2-deoxy-4-*O*-benzyl-3-*O*-[(1*R*)-1-(carbonyl)ethyl]-β-D-glucopyranose (26). To the solution of compound **25** (100.0 mg, 0.265 mmol) in AcOH/THF 1 : 1 was added zinc (172.2 mg, 2.65 mmol) at 0 °C. After stirring for 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 a 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 being added DMT-MM (132 mg, 0.477 mmol). The mixture was stirred at room temperature for further 2.5 h. 10% citric acid solution was added to the mixture and extracted with $CHCl_3$ twice. The combined organic layers were washed with saturated aq. $NaHCO_3$, brine and dried over Na_2SO_4, and then concentrated *in vacuo*. The crude compound was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 25 : 1) to give compound, *1,6-anhydro-2-deoxy-2-glycolylamino-4-O-benzyl-3-O-[(1R)-1-(carbo-nyl) ethyl]-β-D-glucopyranose* as a white solid (81 mg, 84%).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.39–7.32 (5H, m; ArH), 6.97 (1H, d, J = 9.6 Hz; NH), 5.37 (1H, s; H-1), 4.70 (1H, d, J = 11.8 Hz; PhCH–O–), 4.61 (1H, d, J = 5.3 Hz; H-5), 4.58 (1H, d, J = 11.7 Hz; PhCH–O–), 4.24–4.15 (5H, m; Lac-αH, –COOCH$_2$CH$_3$, H-2, H-6), 4.05 (1H, s; –NHCOCH$_2$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-βCH$_3$), 1.29 (3H, t, J = 7 Hz; –COOCH$_2$CH$_3$); ^{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.

The obtained compound, *1,6-anhydro-2-deoxy-2-glycolyl-amino-4-O-benzyl-3-O-[(1R)-1-(carbonyl)ethyl]-β-D-glucopyranose* (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 H_2O, then by methanol to give compound **26** as a white solid (62.3 mg, 87%). 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 (27). Compound **26** (22 mg, 0.058 mmol) and **14** (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 $CHCl_3$ three times. The combined organic layers were washed with 10% citric acid solution, saturated aq. $NaHCO_3$, brine and dried over Na_2SO_4, and then concentrated *in vacuo*. The crude compound was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 20 : 1) to give compound **27** as a 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$_2$Ph × 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$_2$OH), 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$_2$OH), 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 (28). Compound **28** was synthesized from **26** and **15** with similar method to the synthesis of **27**. The crude compound was purified by silica-gel flash column chromatography ($CHCl_3$/MeOH 16 : 1) to give compound **28** as a 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; NH), 5.41 (1H, s; H-1), 5.15–5.05 (6H, m; –COOCH$_2$Ph × 3), 4.69–4.65 (2H, m; PhCH–O–, H-5), 4.58–4.47 (3H, m; PhCH–O–, Gln-αH, DAP 2-H), 4.37–4.30 (3H, m; D-Ala-αH, DAP 6-H, Ala-αH), 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; –NHCOCH$_2$OH), 3.76 (1H, m; H-6′), 3.48 (1H, s; H-3), 3.45 (1H, s; H-4), 2.61 (1H, s; –NHCOCH$_2$OH), 2.27 (2H, br s; Gln-γCH$_2$), 2.14 (1H, br s; Gln-βCH), 1.85–1.63 (5H, m; Gln-βCH, DAP 3-CH$_2$, 5-CH$_2$), 1.39–1.31 (11H, DAP 4-CH$_2$, Lac-βCH$_3$, Ala-βCH$_3$, D-Ala-βCH$_3$); ^{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 **27** (50 mg, 0.0469) and

$Pd(OH)_2$ (60 mg, 0.427 mmol) were mixed well in THF/H_2O/AcOH 1 : 1 : 0.1 (2.1 mL), and reacted under H_2 atmosphere (2.0 MPa) 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 **1f** as a white solid (20 mg, 65%).

^{1}H NMR (500 MHz, D_2O): δ 5.42 (1H, s; H-1), 4.63 (1H, d, J = 4.6 Hz; H-5), 4.30–4.20 (4H, m; Ala-αH, Gln-αH, H-6, DAP 2-H), 4.11 (1H, q, J = 6.8 Hz; Lac-αH), 4.01 (2H, d, J = 2.9 Hz; –NHCOCH_2OH), 3.95 (1H, s; H-2), 3.84 (1H, s; H-4), 3.77–3.74 (2H, m; DAP 6-H, H-6′), 3.35 (1H, s; H-3), 2.35–2.32 (2H, m; Gln-γCH_2), 2.14–2.07 (1H, m; Gln-βCH), 1.95–1.66 (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), 1.30 (3H, d, J = 6.8 Hz; Lac-βCH_3); ^{13}C NMR (125 MHz, D_2O): 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 **28** 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-αH, Ala-αH, Gln-αH, H-6, DAP 2-H), 4.25 (1H, q, J = 6.8 Hz; Lac-αH), 4.15 (2H, d, J = 3.3 Hz; –NHCOCH_2OH), 4.09 (1H, s; H-2), 4.01–3.97 (2H, m; DAP 6-H, 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-γCH_2), 2.28–2.21 (1H, m; Gln-βCH), 2.06–1.79 (5H, m; Gln-βCH, DAP 3-CH_2, 5-CH_2), 1.59–1.52 (2H, m; DAP 4-CH_2), 1.51–1.43 (9H, m; Ala-βCH_3, Lac-βCH_3, D-Ala-βCH_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.

The synthetic procedures of **2b**, **2d**, **2f** and **2h** in Scheme 3 were shown in ESI.†

Compound 2b. ^{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 2d. ^{1}H NMR (600 MHz, D_2O): δ 5.21 (0.57 H, d, J = 3.5 Hz; H-1β), 4.57 (1H, q, J = 6.5 Hz; Lac-αH), 4.49–4.46 (1H, m; H-1′), 4.28–4.20 (3H, m; Ala-αH, Gln-αH, DAP 2-H), 4.09 (1H, q, J = 7.6 Hz; D-Ala-αH), 4.01–3.97 (2H, m; –COCH_2OH), 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-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-γCH_2), 2.12–2.06 (1H, m; Gln-βCH), 1.97 (3H, s; –NHCOCH_3), 1.94–1.64 (5H, m; Gln-βCH, DAP 3-CH_2, 5-CH_2), 1.41–1.24 (11H, m; DAP 4-CH_2, Ala-βCH_3, Lac-βCH_3, D-Ala-βCH_3); ^{13}C NMR (150 MHz, D_2O): 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.

Compound 2f. ^{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-αH), 4.25–4.22 (2H, m; Gln-αH, DAP 2-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; –NHCOCH_2OH), 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-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-γCH_2), 2.15–2.08 (1H, m; Gln-βCH), 1.99 (3H, s; –NHCOCH_3), 1.96–1.64 (5H, m; Gln-βCH, DAP 3-CH_2, 5-CH_2), 1.44–1.36 (5H, m; DAP 4-CH_2, Ala-βCH_3), 1.31 (3H, d, J = 6.5 Hz; Lac-βCH_3); ^{13}C NMR (125 MHz, D_2O): 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 2h. ^{1}H NMR (500 MHz, D_2O): δ 5.41 (1H, s; H_{anh}-1), 4.65 (2H, br s; H_{anh}-5, H-1), 4.31–4.26 (2H, m; D-Ala-αH, Ala-αH), 4.24–4.21 (3H, m; Gln-αH, DAP 2-H, H_{anh}-6), 4.13 (1H, q, J = 6.9 Hz; Lac-αH), 4.05 (2H, s; –NHCOCH_2OH), 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-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-γCH_2), 2.15–2.08 (1H, m; Gln-βCH), 1.99 (3H, s; –NHCOCH_3), 1.93–1.65 (5H, m; Gln-βCH, DAP 3-CH_2, 5-CH_2), 1.44–1.30 (11H, m; DAP 4-CH_2, Lac-βCH_3, Ala-βCH_3, D-Ala-βCH_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 per mL penicillin and 100 μg mL^{-1} streptomycin (all culture reagents were purchased from Gibco). The assay procedure was as follows: 5×10^5 cells per 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) 293 T was cultured in Dullbecco's modified Eagle Media (purchased from Sigma), containing 10% heat-inactivated fetal calf serum with 100 units per mL penicillin and 100 μg mL^{-1} streptomycin (all culture reagents were purchased 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 per mL HEK293T cells were seeded to transparent 24-well plate and incubated overnight before transfection; transfected plasmids Nod2 (20 ng per well pMX2-HA-Nod2) and reporter plasmid: NF-κB-dependent pBxIV-luc (5 ng per well) and control pEF1BOS-β-gal (20 ng per well) were transfected into cells by calcium phosphate method; 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.

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