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Synthetic study of peptidoglycan partial structures. Synthesis of tetrasaccharide and octasaccharide fragments

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Abstract—Partial structures of peptidoglycan, a potent immunostimulating glycoconjugate of bacteria, were synthesized for precise biological studies. A key disaccharide glucosaminyl-β(1–4)-muramic acid was prepared by stereoselective glycosylation of a *N*-Troc (Troc = 2,2,2-trichloroethoxycarbonyl) muramic acid acceptor with a *N*-Troc-glucosaminyl trichloroacetimidate. The disaccharide was converted to either a disaccharide acceptor or a donor. They were then coupled together by the same glycosylation method to give a tetrasaccharide in a good yield. Octasaccharide was also obtained in a good yield in a similar manner. *N*-Acetylation and coupling with the dipeptide moiety of L-alanyl-D-isoglutamine followed by deprotection afforded the repeating peptidoglycan tetrasaccharide and octasaccahride peptide conjugates for the first time.

Bacterial cell wall peptidoglycan (PGN) has been well-known as a strong immunopotentiator.[1] PGN induces many kinds of mediators such as cytokines, prostaglandins, platelet activating factor, and NO, which stimulate the immune system and also cause clinical manifestations of bacterial infections such as fever, inflammation, and hypotension. Recent studies have revealed the recognition mechanisms of PGN and other bacterial immunopotentiator by immunocompetent cells. Membrane CD14 (*m*CD14), a glycosylphosphatidylinositol-anchored protein expressed on macrophage/monocyte, binds PGN and other microbial products, e.g. lipopolysaccharide (LPS) of gram-negative bacteria.[2,3] TLR4 (toll-like receptor 4) proved to mediate cellular activation by LPS[4,5] whereas TLR2 was shown to be the receptor for PGN,[5–7] lipoproteins,[8] and lipoteichoic acids.[6]

PGN consists of polysaccharide chains linked to a peptide network to form a three-dimensional rigid structure. The former is β(1→4)glycan composed of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurAc) whose carboxy group is the point of linkage to the peptide. Two research groups including ourselves independently demonstrated that the minimum structure required for the immunostimulation is *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide: MDP) (**1**)[9–11] (Fig. 1). We then prepared a number of derivatives and structural analogs of MDP to study their biological activities. Since endopeptidase digests of PGN have been shown to be more active than MDP and digests by glycosidases,[12] we synthesized two alternating disaccharide dipeptides corresponding to the repeating units of PGN but their biological activities were almost identical with MDP.[13] MDP was shown to inhibit the binding of soluble PGN (sPGN) to human monocytes[2] but a recent study indicated MDP does not inhibit the binding potency of soluble CD14 to sPGN–agarose.[3] These results suggest that the binding of MDP to CD14 is considerably weaker than that of PGN. In spite of their biological significance, larger but defined partial structures of PGN were, however, not available even after our above work. We therefore, undertook synthetic approach to PGN fragments in order to clarify the role of chain length in the binding with CD14 and TLR2. In this paper, we describe a new synthetic route of tetrasaccharide and octasaccharide fragments of PGN.

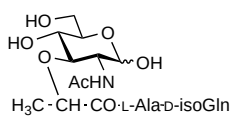

Figure 1. MDP: muramyl dipeptide (**1**).

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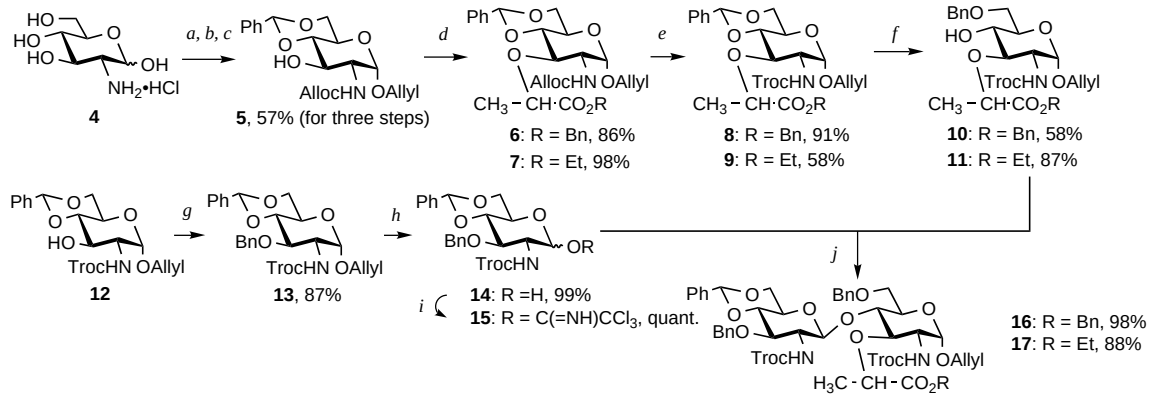
The first key for the synthesis was stereoselective construction of β(1–4) glycosidic linkage, in particular, glycosylation at the hindered 4-hydroxy group of a muramic acid (Mur) residue. In fact, glycosylation at this position did not proceed at all when an oxazoline derivative was used as a glycosyl donor in our previous work.[14] We employed the *N*-Troc-glucosaminyl trichloroacetimidate which proved to be an effective glycosyl donor for the formation of β-glucosaminyl linkages in our synthesis of lipid A derivatives.[15,16] The second key was the regioselective ring opening of 4,6-*O*-benzylidene of GlcN and Mur residues to form 6-*O*-benzyl derivatives by the combined used of $BH_3{\cdot}Me_3N$ with $BF_3{\cdot}Et_2O$.[17] With these two key reactions, we planned a simple strategy to construct the repeating glycan chain as follows. A key disaccharide β(1–4)GlcN-Mur was prepared as a common synthetic intermediate, which was separately converted to either a disaccharide acceptor or a donor. A tetrasaccharide was obtained by the coupling of these disaccharide components. An octasaccharide was synthesized from the tetrasaccharide in a similar manner. Introduction of the dipeptide moiety of L-alanyl-D-isoglutamine followed by deprotection afforded the alternating peptidoglycan tetrasaccharide and octasaccharide with peptide moieties.

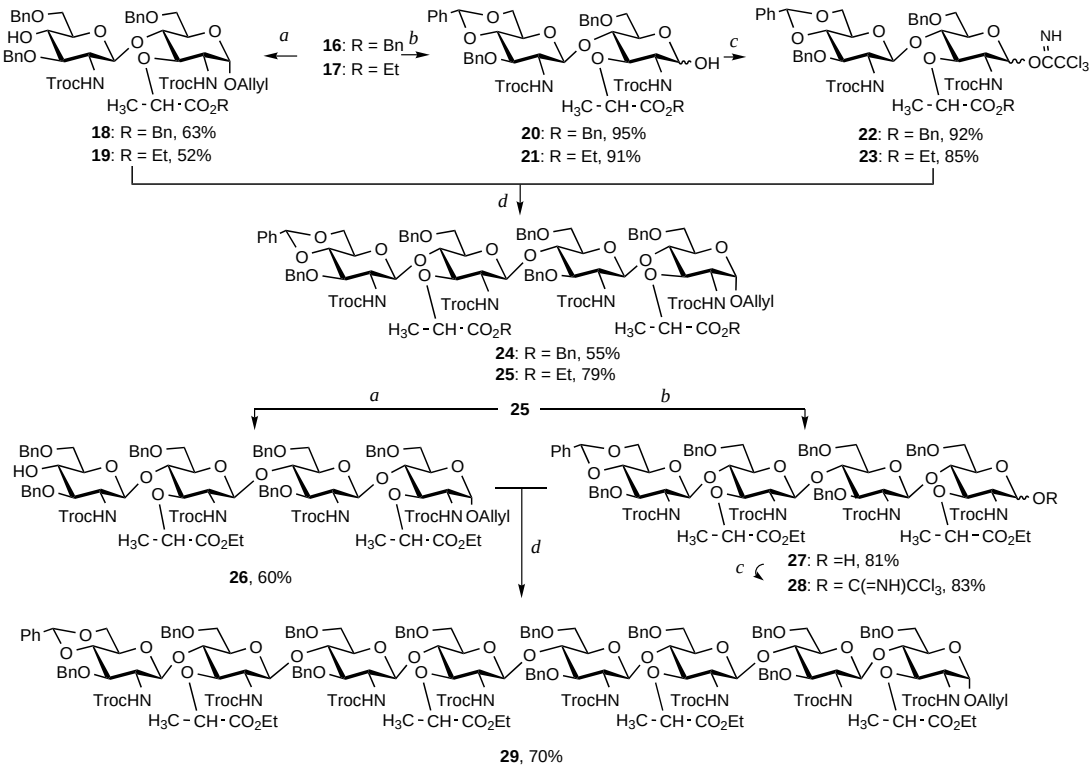
The key disaccharide **16** was synthesized as shown in Scheme 1. A new *N*-Troc-muramic acid acceptor **10** was prepared via a new effective route. *N*-Alloc-GlcN allyl glycoside (alloc = allyloxycarbonyl) **5** was employed as a precursor for the Mur residue, since the alloc group is stable under the basic conditions for introduction of the lactic acid moiety. Treatment of **5** with NaH followed by addition of trifluoromethanesulfonyl-L-(*S*)-2-propionic acid benzyl ester afforded Mur derivative **6** in a good yield.[18] The alloc group of **6** was then replaced with the Troc group, since isomerization of the 1-*O*-allyl group to 1-*O*-2-propenyl group did not proceed at all by an Ir complex[19] in the presence of the *N*-alloc function. Deprotection of the *N*-alloc group was carried out by treatment with Pd $(PPh_3)_4$ (0.3 equiv.) in the presence of acetic acid as an additive. TrocCl was then added to the reaction mixture to give **8**. The undesired cyclic lactam was formed when ammonium formate or dimedone was used as an additive. Regioselective reductive ring opening of the 4,6-*O*-benzylidene of **8** was carried out by using $BH_3{\cdot}Me_3N$ and $BF_3{\cdot}Et_2O$ in CH_3CN to afford the glycosyl acceptor **10** with a free 4-hydroxy group in 67% yield. *N*-Troc-glucosaminyl donor **15** was prepared from 3-*O*-benzyl-4,6-*O*-benzylidene allyl glycoside **13**[20] via cleavage of the allyl glycoside and subsequent conversion to the trichloroacetimidate. Glycosylation reaction of **10** with **15** (1.2 equiv. to **10**) proceeded smoothly by using TMSOTf (0.1 equiv.) as a catalyst at −15°C in the presence of MS 4 Å in CH_2Cl_2 under N_2 to afford the key disaccharide **16** in 98% yield. The corresponding ethyl ester derivatives **7, 9, 11, 17** were also prepared in similar manners (Scheme 1).

Tetrasaccharide **24** and octasaccharide **29** were then synthesized from **16** as shown in Scheme 2. The allyl glycoside in **16** was cleaved and the product with the free 1-hydroxy group was converted to glycosyl trichloroacetimidate **22**. Regioselective ring opening of the 4′,6′-*O*-benzylidene group in **16** with $BH_3{\cdot}Me_3N$ and $BF_3{\cdot}Et_2O$ afforded the disaccharide acceptor **18** in 63% yield. Glycosylation of **18** with **22** (1.5 equiv. to **18**) was carried out in a manner similar to the synthesis of **16** by using TMSOTf as a catalyst. The tetrasaccharide **24** was thus obtained in 55% yield. For the synthesis of octasaccharide **29**, tetrasaccharide ethyl ester **25** was synthesized in a similar manner. Both tetrasaccharide donor **28** and acceptor **26** were derived from **25** in a manner similar to the synthesis of **22** and **18**. Glycosyl coupling of **26** with **28** was then attempted. In this case, 1.5 equiv. of donor **28** was used against acceptor **26**. The desired glycosylation was proceeded smoothly at −15°C to give octasaccharide **29** in 70% yield, which was a satisfactorily high yield for a glycosylation reaction between large segments at a hindered 4-hydroxy group.

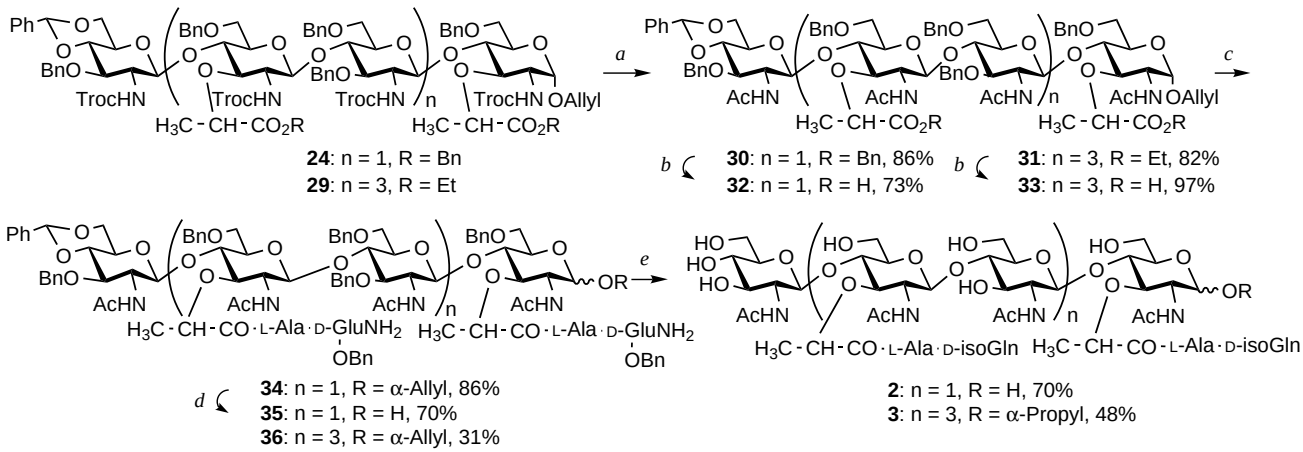
Condensation of the dipeptide with the glycan part **24** and **29** was then investigated (Scheme 3). Using Zn–Cu



Scheme 1. Synthesis of a key disaccharide: (a) allocCl, $NaHCO_3$, H_2O; (b) AllylOH, DOWEX 50 W X8 200–400 mesh, 80°C; (c) $PhCH(OMe)_2$, *p*-TsOH; (d) NaH then trifluoromethanesulfonyl-L-(*S*)-2-propionic acid benzyl ester; (e) $Pd(PPh_3)_4$, AcOH, CH_2Cl_2, then TrocCl; (f) $Me_3N{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, CH_3CN; (g) BnBr, AgO, CH_2Cl_2; (h) Ir complex, H_2, THF, then I_2, H_2O; (i) CCl_3CN, Cs_2CO_3, CH_2Cl_2; (j) TMSOTf (0.1 equiv.), MS 4 Å, CH_2Cl_2, −15°C.



Scheme 2. Synthesis of tetra-, octasaccharide: (a) $Me_3N{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, CH_3CN; (b) Ir complex, H_2, THF, then I_2, H_2O; (c) CCl_3CN, Cs_2CO_3, CH_2Cl_2; (d) TMSOTf (0.1 equiv.), MS 4 Å, CH_2Cl_2, –15°C.



Scheme 3. Synthesis of tetra-, octasaccharide dipeptide: (a) Zn–Cu, AcOH, then Ac_2O, Py; (b) for **32**: 1 M NaOMe, THF, H_2O; for **33**: LiOH, dioxane, THF, H_2O; (c) HCl·H-L-Ala-D-Glu(OBn)-NH_2, WSCI·HCl, HOBt, TEA, CH_2Cl_2; (d) Ir complex, H_2, THF, then I_2, H_2O; (e) $Pd(OH)_2$, AcOH, H_2 (10 atm).

couple in AcOH effected deprotection of the four *N*-Troc groups in **24**. Free amino groups were then acetylated with Ac_2O and pyridine. Saponification of the two benzyl esters in the resulting tetrasaccharide **30** gave **32**, which was isolated in 73% yield by adsorption chromatography using a Diaion HP-20 column followed by purification by silica-gel column chromatography. Condensation of **32** with H-L-Ala-D-Glu(OBn)-NH_2 was effected by using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (WSCI), 1-hydroxybenzotriazole (HOBt), and triethylamine to give protected tetrasaccharide dipeptide conjugate **34** in 86% yield. After deprotection of the 1-*O*-allyl group of **34** by treatment with an Ir complex and I_2, all the benzyl and benzylidene groups were removed by catalytic hydrogenation with $Pd(OH)_2$ and H_2. Purification with a HP 20 column (elution with H_2O) followed by gel-permeation chromatography using Sephadex LH-20 (elution with H_2O) afforded tetrasaccharide dipeptide **2** in 70% yield. ESI-MS (negative mode, $m/z = 685.3$ $[M-2H]^{2-}$, 1371.8 $[M-H]^-$) and NMR provided evidences for the structure **2**. The correct anomeric configurations of the protected precursor **34** were confirmed by the chemical shift values of the ^1H NMR signals of each anomeric protons (H-1 at

$\delta = 4.83$ and H-1′, 1″, 1‴ at 4.45–4.30), which correspond to α and β anomers, respectively.

Protected octasaccharide dipeptide **36** was prepared in a similar manner. Since the anomeric configuration at the terminal 1-position in long glycan chain was not expected to influence the biological activity, catalytic hydrogenation of **36** was carried out without cleavage of the 1-*O*-allyl group to give octasaccharide dipeptide conjugate **3** in the form of α-propyl glycoside in 48% yield (MALDI-MS, positive mode, $m/z = 2792.13$[M+Na]$^+$).

As described tetra- and octasaccharide peptide conjugates were synthesized corresponding to the dimer and tetramer of the basic structural unit of peptidoglycan, i.e. GlcNAc-MurNAc disaccharide linked to the dipeptide of L-alanyl-D-isoglutamine. The present study was the first success for the synthesis of peptidoglycan fragments composed of more than three saccharide residues. In view of the sufficiently high yield of each synthetic transformation, the present work provided synthetic routes to more complex various partial structures of peptidoglycan.

A biological study of peptidoglycan fragments **2** and **3** will be reported in due course.

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