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Synthesis of peptidoglycan fragments from *Enterococcus faecalis* with Fmoc-strategy for glycan elongation

Ning Wang,[a] Hiroki Hasegawa,[a] Cheng-yuan Huang,[a] Koichi Fukase,*[a] and Yukari Fujimoto*[a],[b]

Abstract: Peptidoglycan (PGN) is an essential structural component of the bacterial cell wall conferring cell shape, which can be recognized by host recognition proteins and receptors as well as bacterial surface proteins. In this work, the PGN partial structures from *Enterococcus faecalis* that contain tetrasaccharide and octasaccharide with a unique heptapeptide were synthesized with Fmoc-strategy for elongation of the glycan chains. Namely, 4-*O*-Fmoc-protected disaccharide was utilized as the key intermediate in this efficient synthetic pathway for preparing various PGN fragments. Both tetrasaccharide and octasaccharide with heptapeptide were successfully synthesized for the first time.

Bacterial cell wall peptidoglycan (PGN) is a huge polymer composed of glycan chains and peptide chains that form three dimensional mesh-like structures. Each glycan consists of alternating *N*-acetylglucosamine (GlcNAc) and *N*-muramic acid (MurNAc) linked with β(1→4) bond (Figure 1A). The glycans in PGN are connected each other with peptide linkage at the carboxylic acid of MurNAc. The glycan and the first three amino acids connected to the glycan are quite common in bacteria.

LysM domain is a PGN recognition motif,[1] found in various proteins from bacteriophages, bacteria, eukaryotes, plants, and mammals.[2-9] In *Enterococcus faecalis*, there are three LysM-containing proteins, named AtlA, AtlB and AtlC.[10,11] AtlA is an *N*-acetylglucosaminidase, whereas AtlB and AtlC are *N*-acetylmuramidase. Structure of a disaccharide fragment was confirmed by AtlB digestion of *E. faecalis* PGN (Figure 1A) followed by reduction with sodium borohydride.[12] Additionally, *E. faecalis* PGN has a unique heptapeptide structure as the peptide linkage; L-Ala-D-isoGln-L-Lys(L-Ala-L-Ala)-D-Ala-D-Ala, in which the branched L-Ala-L-Ala dipeptide attaches to the amino group of L-Lys in the typical L-Ala-D-isoGln-L-Lys-D-Ala-D-Ala pentapeptide stem.[10]

In the present study, the PGN fragments from *E. faecalis* were synthesized in order to investigate the precise recognition of LysM domains of AtlB and AtlC with PGN. It was demonstrated recently that longer glycan chains lead to stronger binding with LysM domains. For example, LysM domain derived from *Pteris ryukyuensis* chitinase-A bound to GlcNAc oligomers, $(GlcNAc)_n$, with 1:1 stoichiometry and $(GlcNAc)_5$ showed higher affinity than the shorter glycans.[13] The effect of the peptide chain length for LysM recognition, however, has not been elucidated. Thus, the *E. faecalis* PGN fragments, i.e., $(GlcNAc\text{-}MurNAc)_4$ octasaccharide with the unique heptapeptide moiety were synthesized for the first time by fragment condensation strategy using Fmoc-protected glycans for elongation of the glycan (Figure 1B), and the detailed scheme is shown in Scheme 1.

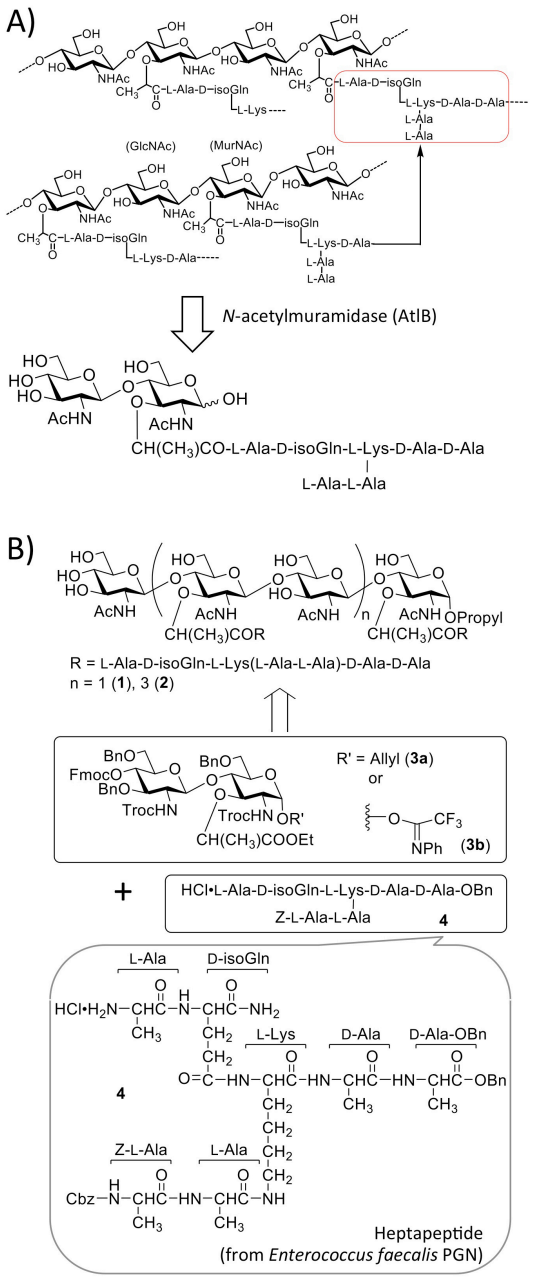

Figure 1. A) Structure of peptidoglycan from *Enterococcus faecalis*, and the structure of a disaccharide fragment obtained by AtlB digestion of *E. faecalis* PGN followed by reduction with sodium borohydride. B) Synthetic targets **1** and **2** as the substrates of the AtlB.

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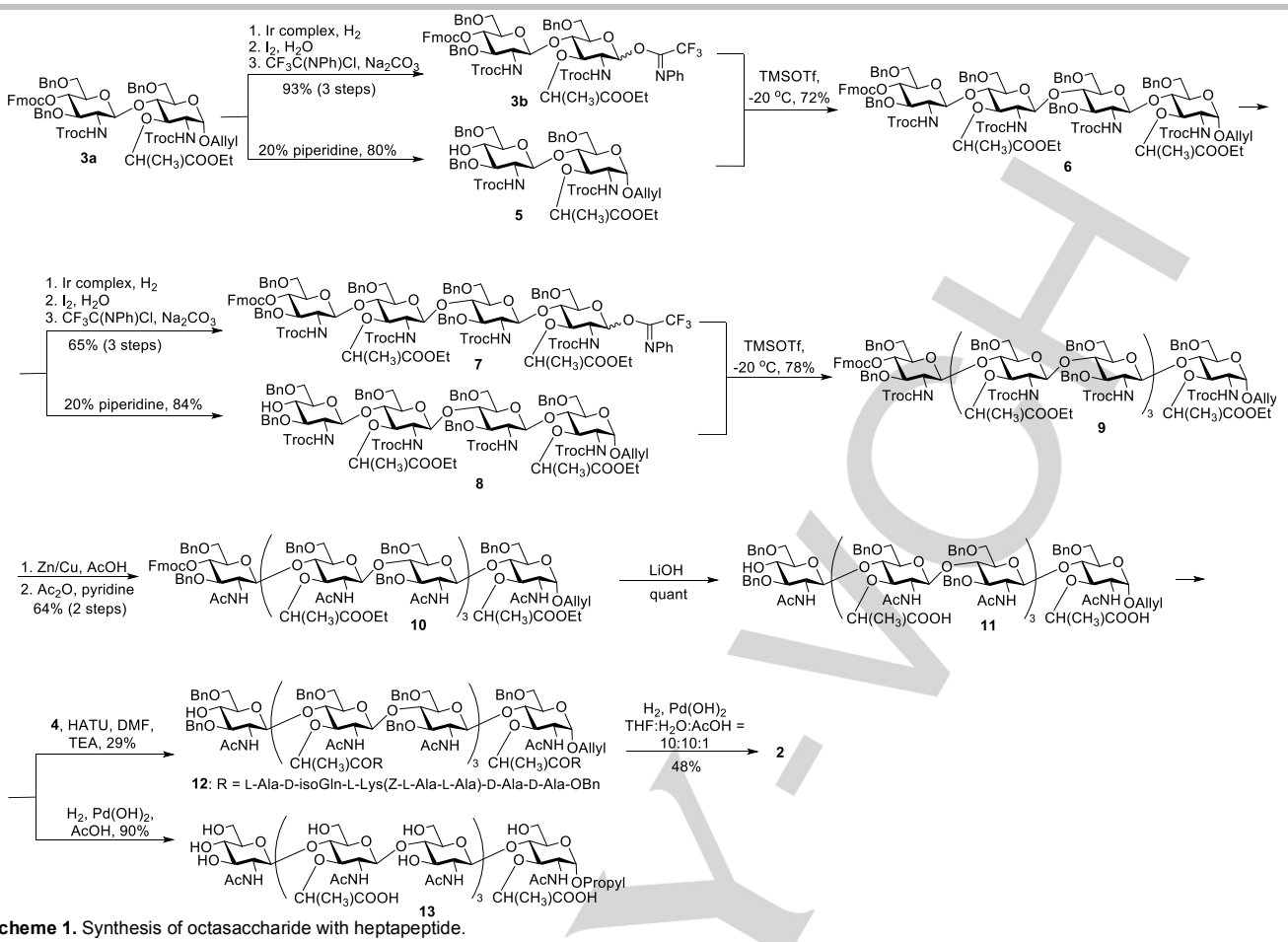
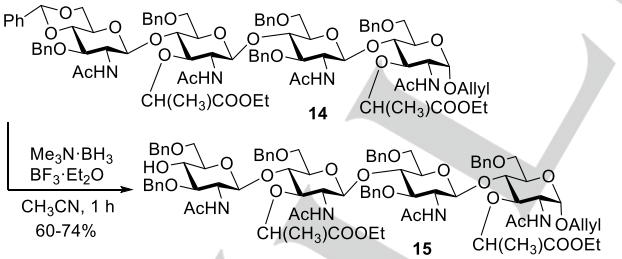


Scheme 1. Synthesis of octasaccharide with heptapeptide.

We previously synthesized the octasaccharide dipeptide containing four GlcNAc-MurNAc units.[14,15] One of the key reactions was the regioselective ring opening reduction of the benzylidene group in long glycans (such as **14** to **15** in Scheme 2). However, in the case of longer glycan, the yields and reproducibility of the regioselective ring opening reaction for the oligosaccharide intermediates were not sometimes satisfactory.

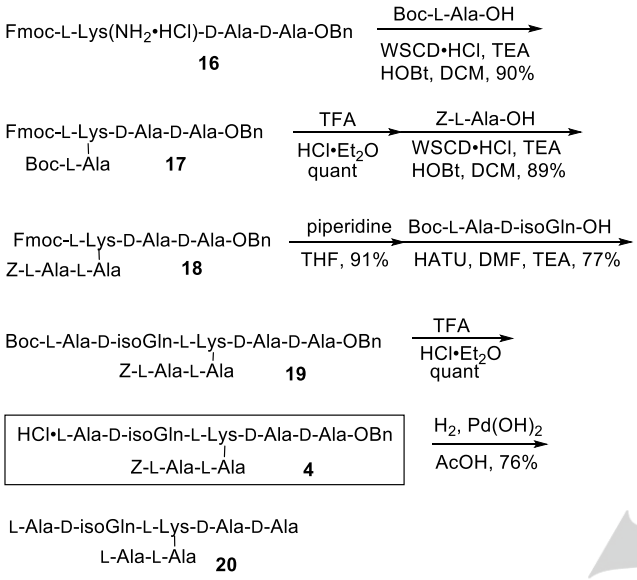


Scheme 2. Preparation of glycosyl acceptor via benzylidene protected intermediate.

To improve the approach to the synthesis of the octasaccharide, we employed a disaccharide with 4'-*O*-Fmoc protection (**3a**) as the key intermediate, since the *O*-Fmoc group can be cleaved by a mild base, such as piperidine or trimethylamine, to give the 4-OH glycosyl acceptor in better yields. The Fmoc-method is also suitable for solid-phase synthesis of PGN glycans.[16] The other improvement of the synthesis was the use of the *N*-phenyltrifluoroacetimidates as glycosyl donors.[17-20] In our previous synthetic study for the tetrasaccharide containing two MurNAc-GlcNAc repeating units, we found that the typical trichloacetimidate donors were more readily decomposed in glycosylation with low reactive hydroxy groups than the corresponding *N*-phenyltrifluoroacetimidates.[21] The improved stability of *N*-phenyltrifluoroacetimidates also showed advantage to the glycosylation between large tetrasaccharide fragments in present study. In addition, the basic condition for the preparation of the trichloacetimidate was not tolerated by the glycosylation donor, leading to the removal of Fmoc group. We thus used the *N*-phenyltrifluoroacetimidates as the suitable glycosyl donors in this study.

The efficient construction of heptapeptide **4** was performed using sequential coupling method as shown in Scheme 3. Since the solubility of the protected linear pentapeptide in organic solvents was very low, we designed the synthetic route where the side-chain L-Ala-L-Ala was introduced to the tripeptide **16** which was prepared in lab (see SI). Since the direct coupling of Boc-L-Ala-L-Ala-OH and **16** did not proceed, the two branched L-Ala moieties were attached to **16** step by step. Condensation of Boc-L-Ala-OH with the tripeptide **16** was carried out by using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (water-soluble carbodiimide; WSCD·HCl), 1-hydroxybenzotriazole (HOBt) and TEA to give tetrapeptide **17**. The second L-Ala in the L-Lys branch was introduced by the coupling with *Z*-L-Ala-OH to form pentapeptide **18**. Fmoc group of **18** was then removed by treatment with piperidine in THF and the resulted pentapeptide amine was coupled with the dipeptide (Boc-L-Ala-D-isoGln-OH) using *O*-(7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HATU) to

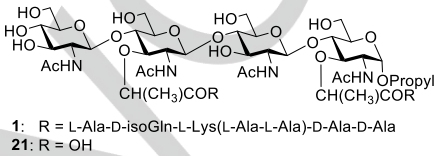
give the protected heptapeptide **19** in 77% yield. Although the solubility in organic solvents was low, purification of **19** with silica-gel column chromatography was achieved by using $CHCl_3$-MeOH as an eluent. Cleavage of Boc group of **19** and treatment with HCl in Et_2O afforded hydrochloride salt of heptapeptide **4**, which was used for further condensation with glycan fragments (Scheme 1). The fully deprotected heptapeptide **20** was also obtained by catalytic hydrogenation of **4** with $Pd(OH)_2$ and H_2 in acetic acid.



Scheme 3. Synthesis of heptapeptide.

Synthesis of the tetrasaccharide and octasaccharide fragments with the heptapeptide was then carried out. In our strategy, **3a** and **6** served as the precursors for glycosylation donors (**3b** and **7**) as well as acceptors. Specifically, cleavage of Fmoc group in disaccharide **3a** and tetrasaccharide **6** was achieved by using 20% piperidine in THF to afford the corresponding acceptors **5** and **8** in 80% or 84% yield, respectively. Removal of allyl glycoside in **3a** followed by *N*-phenyltrifluoroacetimidation gave **3b** in 93% yield for 3 steps (Scheme 1). Glycosylation of **3b** and **5** was then carried out by using TMSOTf as a catalyst at -20 ˚C to give the tetrasaccharide **6** in good yield. Trace amount of the byproducts, such as the OTMS replacement of OH group in **5** induced by TMSOTf, could be observed in the reaction system. The high stability of glycosyl donor **3b** during the storage was another advantage of this synthetic pathway (stable for 3 months under -20°C). The tetrasaccharide donor **7** and acceptor **8** were then synthesized from **6**. Glycosylation of **7** with **8** also proceeded successfully to give octasaccharide **9** in 78% yield. The yield was slightly higher than the glycosylation by using the corresponding benzylidene protected tetrasaccharide trichloacetimidate (70%).[15] Considering the stability during the storage and reproducibility of the glycosylation, the present results showed the versatility of *N*-phenyltrifluoroacetimidates for fragment condensation of longer glycans.

After cleavage of the Troc group with Zn–Cu in AcOH and subsequent acetylation with Ac_2O, the cleavage of ethyl ester with LiOH was carried out to give **11**. Introduction of heptapeptide **4** to the liberated four carboxyl groups was also the key reaction for the synthesis. We avoided the usage of carbodiimides as condensation reagents since they sometimes afford *N*-acylurea by-products. Hence, condensation of **11** with **4** by using *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) and triethylamine (TEA) in DMF successfully afforded the glycan peptide conjugate **12**. Deprotection of all of benzyl-type protecting groups by catalytic hydrogenolysis (H_2, 2 MPa) in acetic acid (AcOH) using palladium hydroxide on carbon successfully gave fully deprotected octasaccharide with heptapeptide **2**. The liberated octasaccharde **13** was also prepared from **11** with the catalytic hydrogenolysis in 90% yield.



1: R = L-Ala-D-isoGln-L-Lys(L-Ala-L-Ala)-D-Ala-D-Ala
21: R = OH

Figure 2. Structure of tetrasaccharide fragments **1** and **21**.

The tetrasaccharide fragments **1** and **21** (Figure 2) were also synthesized for the analysis of the binding with LysM domain of the AtlB (The details of the synthesis are in the Supporting Information, Scheme SI-2). The synthesized PGN fragments was used for the elucidation of the interaction between PGN and LysM domains from *E. faecalis*.[22] The results of the binding with a single LysM module was as flollows; the tetrasaccharide $(GlcNAc\text{-}MurNAc)_2$ **21** (K_D values 650 μM) showed higher affinitiy than the $(GlcNAc\text{-}MurNAc\text{-}dipeptide(L\text{-}Ala\text{-}D\text{-}isoGln))_2$ or $(GlcNAc\text{-}MurNAc\text{-}heptapeptide)_2$ **1** (K_D values 1.8±0.5 mM or >1 mM, respectively), while the binding affinity increased with the length of the glycan chains (K_D = 80 μM for $(GlcNAc\text{-}MurNAc)_4$ **2**).[22]

In conclusion, we have developed an Fmoc-strategy for the synthesis of bacterial PGN with utilizing 4-*O*-Fmoc protected *N*-phenyltrifluoroacetimidates as the glycosyl donors. The PGN fragments containing heptapeptide stem was synthesized for the first time. The octasaccharide with heptapeptide was the largest PGN fragment so far synthesized. The synthetic PGN fragments have been used for the elucidation of the interaction between PGN and LysM domains from *E. faecalis*[22] and further analysis will be reported in elsewhere.

Experimental Section

The synthetic procedures and characterization of the compounds are described in the Supporting Information.

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Keywords: peptidoglycan • glycosylation • carbohydrates • peptides • bioorganic chemistry

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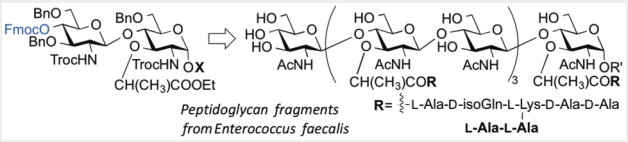

The peptidoglycan fragments, octasaccahride heptapeptide, from *Enterococcus faecalis* were synthesized with using the Fmoc-strategy for long glycan elongation.

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