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Synthesis of crosslinked peptidoglycan fragments for investigation of their immunobiological functions

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ABSTRACT

The synthesis of crosslinked peptidoglycan (PGN) fragments from *Streptococcus pneumoniae* cell wall was achieved. The immunostimulatory activities, including the induction of IL-6 and the stimulation of the intracellular receptor Nod2, were also determined. The crosslinked PGN fragments were not major human Nod2 ligands.

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The innate immunity is an evolutionary ancient defense system, which is activated by microbial molecules with various sensor proteins, called pathogen-recognizing receptors (PRRs).[1] The bacterial cell wall peptidoglycan (PGN) is one of the components, which activates the immune system. PGN consists of polysaccharide chains, which are crosslinked with peptides, to form a three-dimensional mesh-like structure (Fig. 1). Polysaccharide is a β(1→4) glycan composed of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), and the carboxyl group of the MurNAc is linked to the peptide. At the branched position of the peptide, 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), is typically present.

To investigate the recognition of PGN with PRRs and the function of PGN fragments, we have previously synthesized several PGN fragments, including Lys-type,[2,3] and DAP-type fragments including tracheal cytotoxin (TCT).[4] We and French group have independently shown that the intracellular Nod1 and Nod2, which are founding members of the Nod-like receptors (NLRs), are PGN receptors; Nod1 recognizes γ-D-glutamyl diaminopimelic acid (iE-DAP)[5] and the muropeptides containing iE-DAP,[6] whereas Nod2 recognizes *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide: MDP) and muropeptides containing MDP.[7,8,3] PGN is also considered to be recognized by Toll-like receptor 2 (TLR2) that is a member of membrane-bound Toll-like receptors (TLRs). However, our previous studies using the synthetic PGN partial structures indicated that TLR2 does not sense muropeptides,[2,3] although it is still debated.[9] PGN is also a target of other types of proteins, which include peptidoglycan recognition proteins (PGRPs) and lectins. PGRP is a major protein family responsible for the defense against microorganisms in insects, and in animals

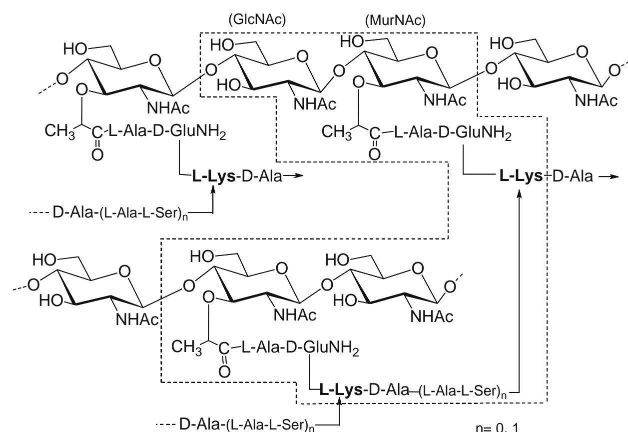

Figure 1. Schematic structure of peptidoglycan (Lys-type) from *Streptococcus pneumoniae* ($n = 0$: major structure). Segment enclosed by the dashed line is one unit of the linked structure.

it also plays important roles together with other immune receptors (Fig. 2).[10,11]

Because natural PGN is a heterogeneous polymer often contaminated with other immunostimulating substances, detailed bio-

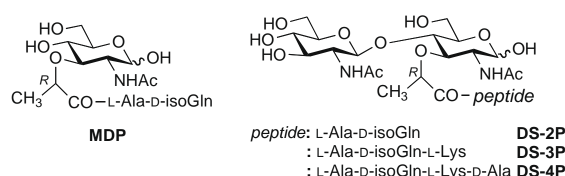

Figure 2. Structures of MDP, DS-2P, DS-3P, and DS-4P.

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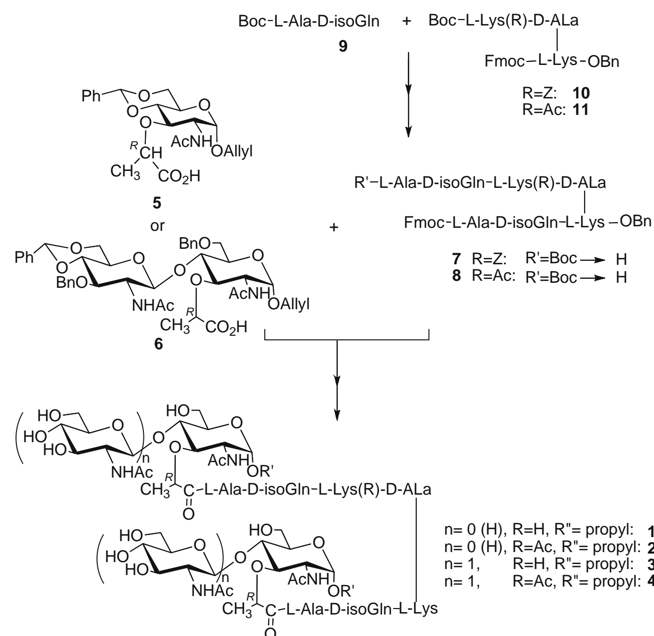
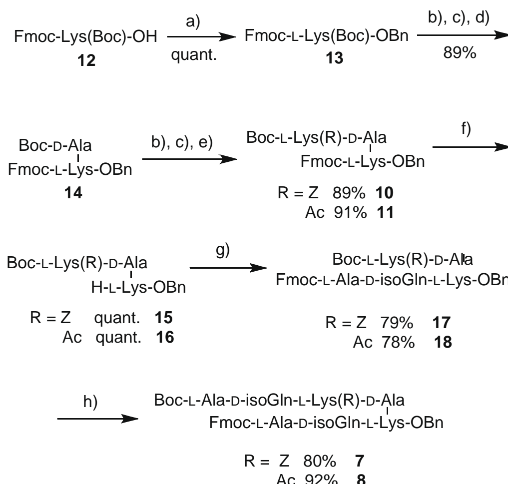
functional studies of PGN have been investigated using synthetic specimens. However, the biological activity of the crosslinked fragments has not been well elucidated, due to the synthetic difficulties. Previously, Boons and Mariuzza's group has reported the synthesis of a crosslinked PGN structure from *Staphylococcus aureus* by solid-phase method for analysis of PGRPs recognition, the structure only contained a monosaccharide moiety.[12] Herein, we report the successful synthesis of crosslinked PGN structures with a repeating disaccharide (GlcNAc-MurNAc) unit using a solution-phase synthesis. Fragments with monosaccharides and/or shorter peptide chains were also synthesized. The synthesized structures are the major partial structures of *Streptococcus pneumoniae* PGN, which has the simplest peptide chain containing L-alanine, D-isoglutamine, L-lysine, and D-alanine.[13] The established synthetic approach should be applicable to the synthesis of crosslinked PGN structures with longer glycan chains. We also evaluated their immunostimulatory activities and compared these activities to the previously synthesized PGN fragments with shorter peptide structures.[3]

Scheme 1 shows the synthetic strategy for the linked compounds. Initially, we prepared the peptide parts **7** and **8** by the condensation of **9** and **10**/**11**; and then peptides **7**′ and **8**′ were connected to the monosaccharide **5** or disaccharide **6**, to obtain crosslinked PGN partial structures **1**–**4**.

Branched peptides **7** and **8** were prepared as shown in Scheme 2. We initially attempted to introduce two molecules of dipeptide Boc-L-Ala-D-isoGln **9** to the two lysines of the branched L-Lys-[ε-(L-Lys-D-Ala)], but were unsuccessful. Hence, we introduced the dipeptide **9** to compounds **10** or **11** in a stepwise manner. Because of the relative reactivities of lysines, Fmoc protected α-amino group of lysine was first reacted. Starting from protected Lys **12**, D-Ala and L-Lys residues were introduced to the side chain of **12** to give **10** and **11**. In the preparation, Boc group was cleaved with trifluoroacetic acid (TFA), and the obtained compound was precipitated with Et_2O/HCl as a HCl salt, and then coupled with another amino acid. The Fmoc group of **10** or **11** was then cleaved to give **15** or **16**. Dipeptides Fmoc-L-Ala-D-isoGln and Boc-L-Ala-D-isoGln **9** were then introduced to afford **7** and **8**, respectively. After deprotection of **10** and **11**, the peptides were insoluble in DMF; thus, the peptides were solubilized with DMF/DMSO to carry out the reactions.



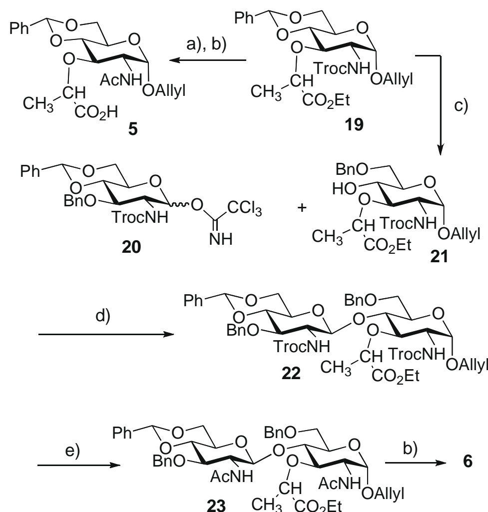
Scheme 1. Synthetic strategy for the crosslinked peptidoglycan partial structures.



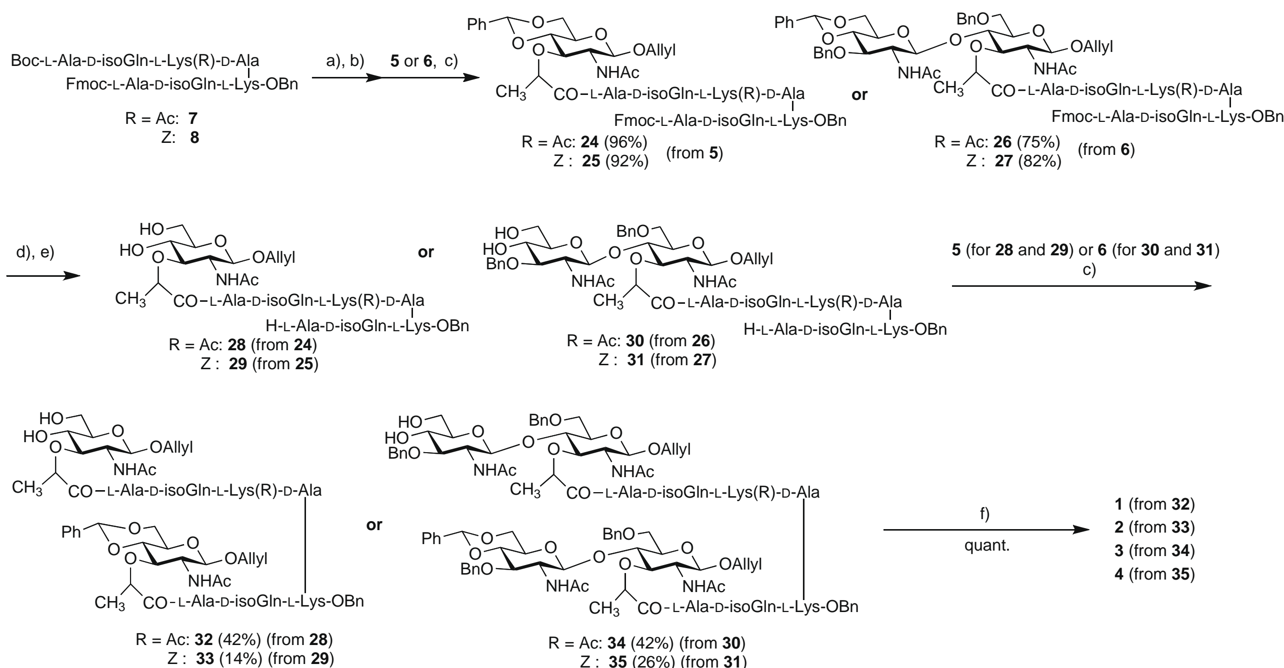
Scheme 2. Preparation of peptides **7** and **8**. (a) BnBr, Cs_2CO_3, DMF, overnight; (b) TFA; (c) HCl·Et_2O; (d) Boc-D-Ala, WSCD·HCl, HOBt, Et_3N, DMF, overnight; (e) Boc-L-Lys(*R*), WSCD·HCl, Et_3N, HOBt, DMF, overnight; (f) 20% piperidine, DMF; (g) Fmoc-L-Ala-D-isoGln, WSCD·HCl, HOBt, Et_3N, DMF/DMSO (2:1), overnight; (h) Boc-L-Ala-D-isoGln, HATU, DMF/DMSO (1:1), overnight. WSCD = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, HOBt = 1-Hydroxy-1*H*-benzotriazole, HATU = 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate methanaminium.

Glycan parts **5** and **6** were prepared from **19** and **20**, which were derived from D-glucosamine as described (Scheme 3).[2–4] The Troc group of **19** was replaced to acetyl group, and then the ethyl ester at the lactate was cleaved to give monosaccharide unit **5**. To prepare disaccharide unit **6**, selective cleavage of the benzylidene acetal of compound **19** yielded **21**.[14] Synthesis of disaccharide **22** with a β(1→4) linkage was achieved by means of neighboring group participation of the *N*-Troc (Troc = 2,2,2-trichloroethoxycarbonyl) group and the appropriate reactivity of *N*-Troc-glucosaminyl trichloroacetimidate of **20** and **21**. The Troc group of **21** was then converted to an acetyl group to obtain **22**, and the ethyl ester was cleaved with LiOH for the further coupling reaction with the peptides, to give **6**.

Peptides **7**/**8** and glycans **5**/**6** were then linked to give PGN fragments **1**–**4** (Scheme 4). We initially attempted to introduce two

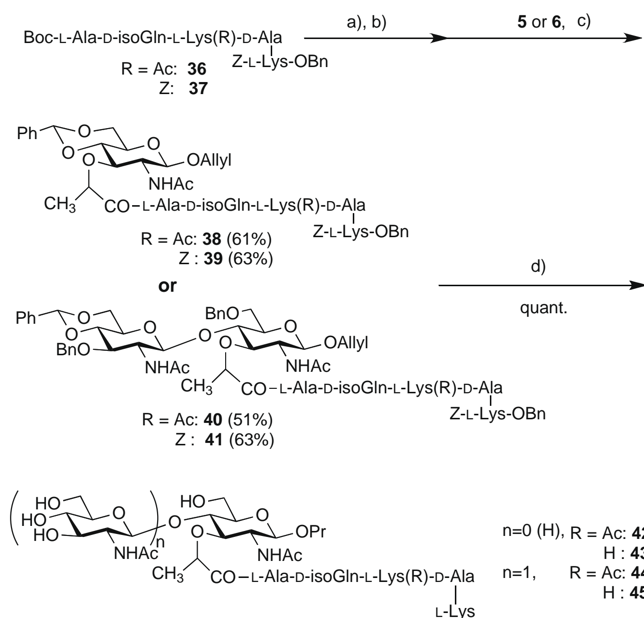


Scheme 3. Synthesis of glycans 5 and 6. (a) Zn–Cu, AcOH then Ac_2O, Py., 69%; (b) LiOH, THF/1,4-dioxane/H_2O = 4/2/1; (c) $Me_3N·BH_3$, $BF_3·Et_2O$, CH_3CN; (d) TMSOTf (0.1 equiv), CH_2Cl_2, −15 °C, 10 min, 88%; (e) Zn/Cu, THF, AcOH/Ac_2O = 1/1/1, 46%. TMSOTf = trimethylsilyl trifluoromethanesulfonate.



Scheme 4. Synthesis of crosslinked PGN fragments **1**–**4**. (a) TFA; (b) $HCl{\cdot}Et_2O$; (c) HATU, Et_3N, DMF/DMSO = 1:1; (d) 10% TFA/CH_2Cl_2; (e) 20% piperidine/DMSO; (f) H_2, $Pd(OH)_2$, AcOH.

glycan chains to the two L-Ala of the linked structure, but the reactivity of amino groups was insufficient to obtain the desired compounds. Hence, the *N*-Boc-protection of one of the L-Ala residues was cleaved and glycan moiety **5** or **6** was subsequently introduced using HATU and Et_3N in DMF/DMSO (1:1) to give **24**–**27**. We then tried the deprotection of the *N*-Fmoc group of another L-Ala in order to introduce a second glycan to **24**–**27**. However, Fmoc deprotection was difficult due to the very low solubility of compounds **24**–**27**. Hence, we cleaved the benzylidene acetal at the 4- and 6-positions of glucosamine with 10% trifluoroacetic acid (TFA) before Fmoc cleavage. Another unit of glycan **5** or **6** was introduced in a manner similar to that using HATU. All benzyl, benzyloxycarbonyl



Scheme 5. Synthesis of PGN fragments **42**–**45**. (a) TFA; (b) $HCl{\cdot}Et_2O$; (c) HATU, Et_3N, DMF/DMSO (1:1); (d) H_2, $Pd(OH)_2$, AcOH.

and benzylidene groups of **32**–**35** were removed by catalytic hydrogenation with $Pd(OH)_2$ and H_2 in acetic acid to give **1**–**4**, respectively. Moreover, we synthesized the PGN fragments with pentapeptides **42**–**45,** which contain the branched part of the peptides, L-Ala-D-isoGln-L-Lys-D-Ala-(ε)-L-Lys, using a method similar to that used to prepare compounds **1**–**4**[15] (Scheme 5).

The biological activities of synthetic PGN fragments, that is, linked structures **3** (DS_2-link(Ac)) and **4** (DS_2-link)), and the monosaccharides (**42** (MS-B5P(Ac)) and **43** (MS-B5P)) and disaccharides (**44** (DS-B5P(Ac)) and **45** (DS-B5P)) with branched pentapeptide, were evaluated along with the previously synthesized monosaccharides and disaccharides with a series of the peptides (MDP, DS-2P, DS-3P, and DS-4P). The ability of synthesized PGN fragments to induce cytokine (IL-6) secretion from human peripheral blood mononuclear cells (PMNs) was determined (Fig. 3).[3] The samples were incubated with human blood mononuclear cells (PMNs) for 12 h, and the cytokine production was determined by ELISA. The PGN fragments, that is, MDP, DS-2P, DS-3P, and DS-4P showed potent IL-6 inducing activity. The potency was decreased in ascending order of peptide chain length. The compounds having

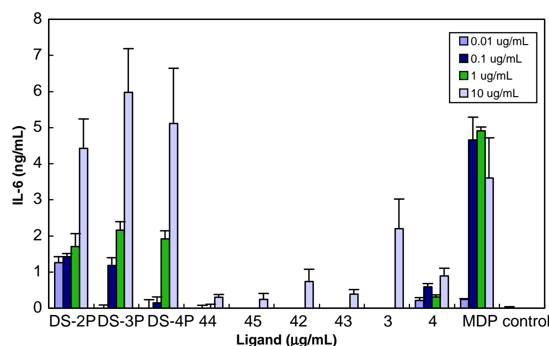


Figure 3. IL-6 induction of the PGN fragments; DS-2P, DS-3P, DS-4P, DS-B5P(Ac) **44**, DS-B5P **45**, MS-B5P(Ac) **42**, MS-B5P **43**, $(DS)_2$-Link(Ac) **3**, $(DS)_2$-Link **4**, and MDP. Samples were incubated with human peripheral blood mononuclear cells (PMNs)[5] for 12 h, and cytokine production was determined by ELISA.

branched pentapeptide (MS-B5P(Ac), MS-B5P, DS-B5P(Ac), and DS-B5P) scarcely showed the activity. As for the linked compounds, $(DS)_2$-Link(Ac) **3** showed low activation, whereas $(DS)_2$-Link **4** showed slightly higher activity. Similar tendency was observed in IL-8 induction (see Supplementary data).

The human Nod2 stimulating activity of each synthetic PGN fragment was then evaluated by the HEK293T bioassay as previously described[16] (Fig. 4). MDP and DS-2P exhibited similar Nod2 stimulatory activities, while DS-3P showed a 10-fold lower activity than MDP. Notably, DS-4P showed only very weak Nod2 stimulatory activity, and DS-B5P, MS-B5P, $(DS)_2$-Link, and $(DS)_2$-Link(Ac) did not show the Nod2 stimulatory activities less than 1 μg/ml. These results clearly indicated that crosslinked PGN fragments with peptides are not major Nod2 ligands. These results along with previous studies[3] suggest that Nod2 should recognize PGN fragments containing MDP, which are digested by enzymes from both bacteria and host animals. In fact, our recent studies revealed that many bacteria excrete soluble Nod1 and Nod2 ligands, which should be PGN fragments produced by bacterial enzymes.[16] These PGN fragments might be transported into the cells through phagocytosis, molecular adhesion, or specific transport system.

DS-4P, $(DS)_2$-Link, and $(DS)_2$-LinkAc showed weak but significant activities in IL-6 and IL-8 induction from human blood mononuclear cells, suggesting the posibility of enzyme digestion of the PGN fragments in mononuclear cells during the incubation period, or facilitated up-take of these molecules in mononuclear cells. The former mechanism was also reported recently; the intracellular degradation of bacteria in the phago-lysosome proved to be important for activation of the macrophage through Nod2.[17]

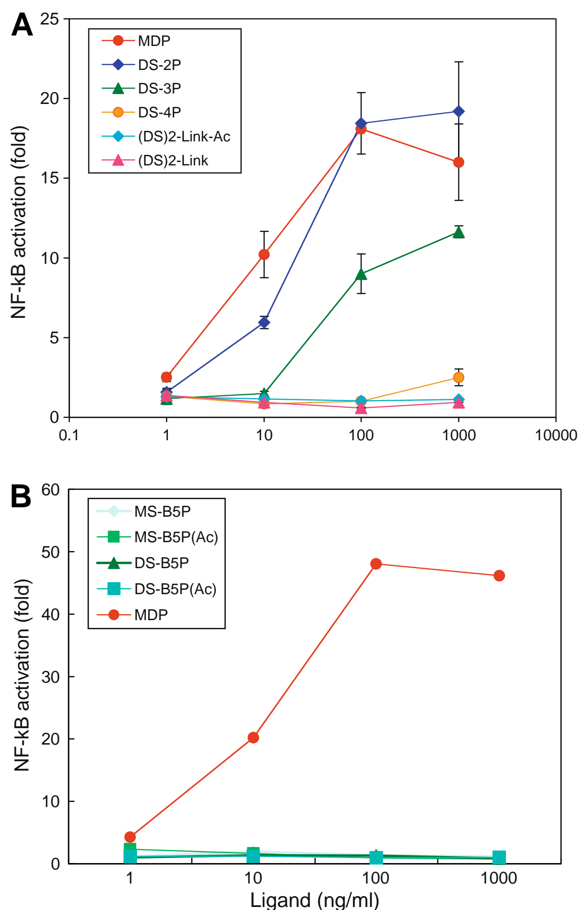


Figure 4. Stimulation of Nod2 by the PGN fragments: (A) MDP, DS-2P, DS-3P, DS-4P, $(DS)_2$-Link **4,** and $(DS)_2$-LinkAc **3**. (B) MS-B5P **43**, MS-B5P(Ac) **42**, DS-B5P **45**, and DS-B5P(Ac) **44**. HEK293T cells were transfected with human-Nod2, and the indicated amount of each compound was added to the cells. Ability of each compound to activate NF-κB was determined by luciferase reporter assay.[16]

In the host defence system against bacterial infection, along with the NLRs and TLRs, some other recognizing proteins are also known; one of major families is peptidoglycan recognition protein (PGRP) family. PGRP seems to recognize a relatively longer glycan with a peptide moiety, but the recognizing structures and its functions are only partially known. The compounds synthesized in the present research would also be useful to investigate these structural bases.[18,19]

In conclusion, we successfully synthesized glycan linked PGN fragments, and observed their biological activities. The established synthesis in this Letter offers possibilities to construct longer PGN structures, which are useful for the investigation in innate immunostimulation of PGN. Based on these PGN fragments library, exploring of other biological activities, and determination of the detailed recognition structures of other types of PGN recognition molecules including PGRPs and PGN recognizing lectins would be possible.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2009.03.081.

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15. *Compound* **3**: ESI-MS (positive) m/z = 1849.76 $[M+H]^+$; ^{1}H NMR (500 MHz, CD_3OD): δ = 4.44–4.15 (m, 9H), 3.86–3.30 (m, 11H), 3.08–3.20 (m, 4H), 2.06–2.40 (m, 8H), 1.91 (s, 6H, NHC(O)CH_3), 1.85 (s, 6H, NHC(O)CH_3), 1.83 (m, 3H, Lys-ε-NHC(O)CH_3), 1.42–1.50 (m, 4H), 1.26–1.34 (m, 22H), 0.85–0.80 (m, 6H, propyl CH_3).
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