Synthesis of Peptidoglycan Fragment Library for Analysis of Receptor Recognition

(認識タンパク質探索・解析を目指したペプチドグリカン・フラグメントライブラリ構築)

Ning Wang

Graduate School of Science

Osaka University

Contents

List of Abbreviations

Ac	acetyl
Ala	alanine
Alloc	allyloxycarbonyl
Apaf	apoptotic peptidase activating factor
Ar	aryl
Boc	*t*-butoxycarbonyl
Bn	benzyl
BSA	bovine serum albumin
Bu	butyl
CARD	caspase-recruitment domain
CHAP	cysteine and histidine dependent amidokydrolases/peptidases
DCM	dichloromethane
DIPEA	*N,N*-diisopropylethylamine
DMA	dimethylamine
DMAP	*N,N*-dimethyl-4-aminopyridine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
ESI	electrospray ionization
Et	ethyl
Fmoc	9-fluorenylmethyloxycarbony
GlcNAc	*N*-acetyl glucosamine
Gln	glutamine
HATU	*O*-(7-Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
iE-DAP	γ-D-glutamyl meso-diaminopimelic acid
IFN	interferon
IgG	immunoglobulin G
IKK	IκB kinase
IL	interleukin
kDa	kilodaltons
LPS	lipopolysaccharide

LRRs	leucine-rich repeats
Lys	lysine
LysM	lysine motif
MALDI	matrix assisted laser desorption ionization
MAMPs	microbe-associated molecular patterns
MDP	muramyl dipeptide
Me	methyl
MS	mass spectrometry
MS4A	molecular sieves 4A
MurNAc	*N*-acetylmuramic acid
NF-κB	nuclear factor κB
NLR	NOD-like receptor
NMR	nuclear magnetic resonance
NOD	nucleotide-binding oligomerization domain
OTf	trifluoromethansulfonate
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffer solution
PGN	peptidoglycan
PRGPs	peptidoglycan recognition proteins
Ph	phenyl
Pr	propyl
PRRs	pattern recognition receptors
QTOF	quadrupole time-of-flight
quant	quantitative
RICK	recruit receptor-interacting serine/threonine kinase
RIP-1	receptor interacting protein-1
RLRs	RIG-I receptors
TBAF	tetra-butyl ammonium fluoride
Tf	trifluoromethanesulfonyl
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TIR	Toll/IL-1 receptor
TLC	thin-layer chromatgraphy
TLR	Toll-like receptor
TMS	trimethylsilyl
Troc	2,2,2-trichloroethoxycarbonyl
p-Ts	*p*-toluenesulfonyl

UV	ultraviolet
WSCD	water-soluble carbodiimide; 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
Z	benzyloxycarbonyl

Abstract

Peptidoglycan (PGN) is a major component of bacterial cell wall, consisting of glycans and peptide chains that form a three-dimensional mesh-like structure outside the plasma membrane. The glycan chain consists of alternating residues of β(1→4) linked *N*-acetylglucosamine and *N*-acetylmuramic acid and is connected each other with peptide-linkages. Specific microbial molecules including PGN are known as the stimulating components of innate immune system, activating sensor proteins called pathogen-recognition receptors (PRRs). PGN is also known as the target of antibiotics, and also other enzymes and lectins.

Recent studies revealed that several protein families recognize distinct PGN partial structures at various different positions. There was a great deal of effort for the chemical synthesis of PGN fragments in the last decade in order to obtain the pure and discrete species for precise structural and biochemical studies. In the previous work of our group, nucleotide-binding oligomerization domain protein 1 (Nod1) and 2 (Nod2) were identified as the innate immune receptors of PGN. However, the comprehensive analysis of the substrate structures recognized by PGN receptors and other PGN recognition proteins using complex PGN fragments has not been well-conducted. In order to understand the recognition and functions of bacterial peptidoglyan with host multi-cellular organisms and also in the bacteria themselves, the library of PGN fragments was synthesized in this study. The library included two kinds of sequences of alternating glycans (GlcNAc/MurNAc), with various length of glycan chains. The targets covered the compounds having disaccharide,

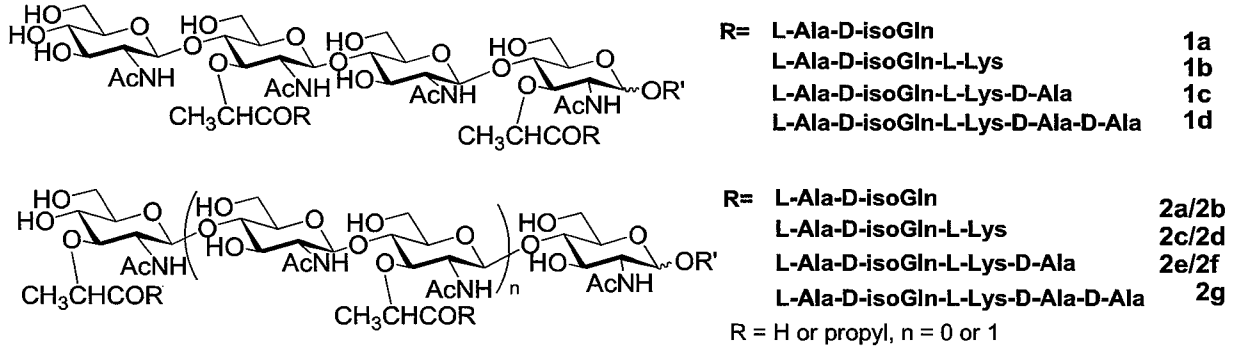


Figure 1. Peptidoglycan fragments structures.

tetrasaccharide and octasaccharide.

The synthetic targets **2b~h** (Figure 1), which are the fragments expected to be produced by bacterial enzymes[9] releasing to the environment, have not been chemically synthesized. The

previously synthesized fragments **1a~d** (in our group), which are considered to be cleaved by the enzymes in animals were also prepared for comparison in this research. The Nod2 stimulating activity of each synthetic peptidoglycan fragment was then evaluated.

Construction of PGN arrays was then performed by attaching synthesized PGN fragments on a solid surface of the array platforms. In order to introduce the PGN fragments to the array, several linkage structures were examined, and finally a linker with terminal amine was successfully attached to the PGN fragments. The arrays could be used to detect the PGN's receptors and recognition proteins (eg. peptidoglycan recognition proteins (PGRP), enzymes and lectins) and measure the binding properties of these proteins.

Many proteins containing PGN recognition domain, such as LysM, recognize relatively longer glycan chains. For the analysis of the detailed recognition of LysM domain, especially with LysM containing enzyme AtlB and AtlC from *Enterococcus faecalis*, the tetrasaccharide and octasaccharide containing PGN structures were synthesized. In order to prepare various fragment structures having longer glycan chains, a new efficient synthetic pathway was investigate. Appropriately protected 4-*O*-Fmoc protected disaccharide was thus used as the key intermediate in this route. Synthesis of tetrasaccharide as well as the octasaccharide with heptapeptide was successfully achieved.

Chapter I Introduction

Innate immunity

The innate immune system comprises the mechanisms that defend the host from infection by microorganisms as the first line of the defense.[1] When microbial pathogens, such as bacteria, viruses, and fungi invade a host organism, the innate immune system detects these microbes with pathogen-recognition receptors (PRRs), which recognize the components specific to microorganisms to induce anti-infectious response and activate immune system. These unique microbial molecules are called microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs). Major MAMPs are lipopolysaccharide (LPS) from Gram-negative bacteria, bacterial lipoproteins, peptidoglycan, DNA and RNA from bacteria and viruses, lipoteichoic acids, β-glucans from fungi, and etc. Various PRRs have been identified as receptors against these MAMPs (Figure I-1).[2] One important class of PRRs are extracellular proteins such as C-reactive protein and mannan-binding lectin (MBL) that bind to native or opsonized pathogens and mediates their elimination through recruitment of antimicrobial factors, such as complement molecules. Another important class of PRRs is involved in microbicidal host responses through intracellular signaling events. The latter group of PRRs includes Toll-like receptors (TLRs),[3] nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs)[4] and retinoic acid-inducible gene-I-like (RIG-I) receptors (RLRs).[5] Each PRR in this group is responsible for the recognition of the respective molecular species from pathogens including bacteria and viruses. Mammalian TLRs comprise a large family consisting of 13 members. TLR1-9 are conserved among most mammals and TLR1-10 have been identified in the human.[3] TLRs are single-spanning transmembrane proteins that contain leucine-rich repeats (LRRs) in their ectodomains for MAMP recognition. TLR signaling is mediated through a conserved cytoplasmic part, called a Toll/interleukin-1 receptor (TIR) domain. The intracellular TIR domain of TLRs plays an essential role in transducing TLR signaling events by interacting with cytosolic factors. The LRRs presenting on the extracellular or extraendosomal domain recognize common constituents of microbes that are conserved during evolution as the particular structural motif to microbes. For example, TLR4 is the receptor for lipopolysaccharide (LPS); diacyl lipopeptides are recognized by TLR2/TLR6 heterodimer, triacyl lipopeptides by TLR1/TLR2 heterodimer, flagellin by TLR5. Another major class of PRR family is cytoplasmic NLRs. The representative NLRs are Nod1[6] and

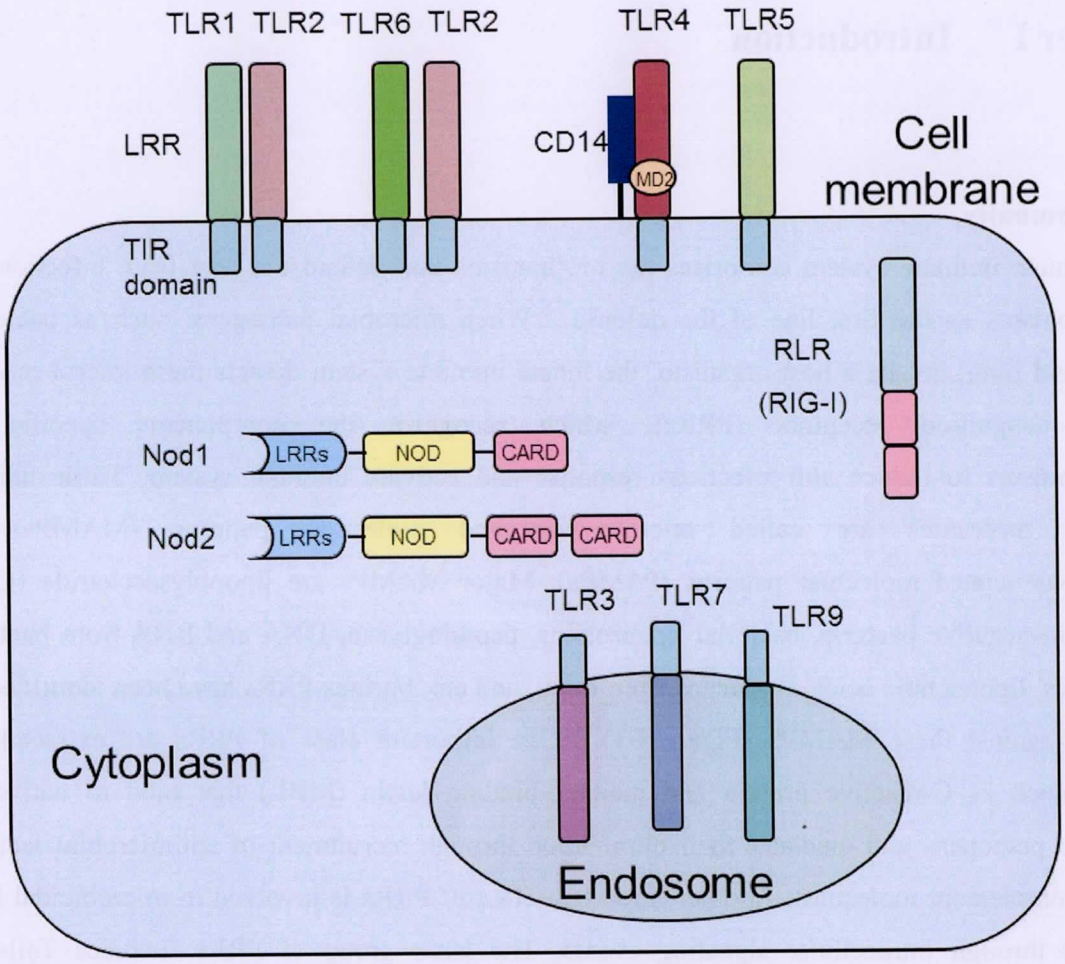


Figure I-1. Representative PRRs (Ref 2).

Nod2,[7] which recognize peptidoglycan and its partial structures (Fig. I-1). The details of NLRs are described later. In addition to TLRs and NLRs, two of the RLRs, RIG-I and MDA5 have been identified as intracellular receptors against viral dsRNA[5]. Recent studies have shown that PRRs also recognize the host-derived molecules from damaged, injured or stressed cells as alarm signals. Innate immune signals from both microbial and host molecules through PRRs are associated with acute as well as chlonic inflamation.

PGN of Bacteria

Bacteria are subdivided into two main categories on the basis of the Gram reaction (crystal violet stain) and morphology. Peptidoglycan (PGN) is a bacterial cell wall component, consisting of glycans and peptide chains that form a three-dimensional mesh-like structure outside the plasma membrane (Figure I-2). The Gram-positive bacteria are characterized by having thick PGN layer while the cell surface of Gram-negative bacteria is composed of the outer membrane, thin cell wall

of peptidoglycan, and inner cytoplasmic membrane. The glycan component of PGN consists of alternating residues of β(1→4) linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues. Glycan chains are connected each other with peptide linkages. The first two residues of the stem peptide are generally L-Ala and D-Glu or D-Glu-NH_2 (D-isoGln), while the last residue is typically D-Ala. 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.[8)]

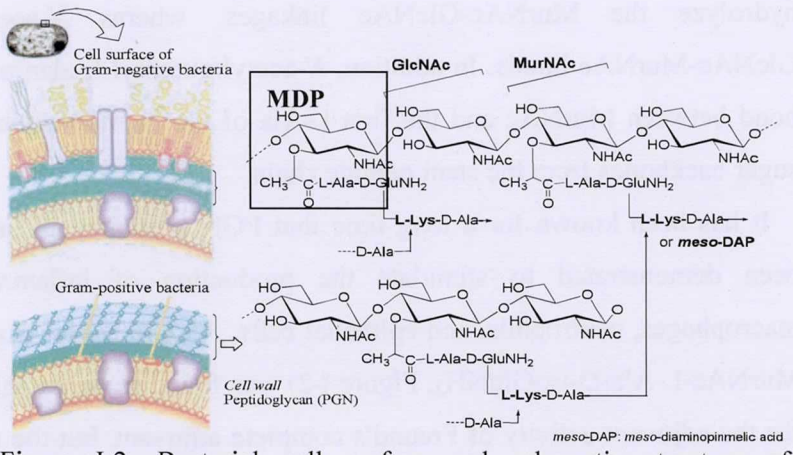


Figure I-2. Bacterial cell surface and schematic structure of peptidoglycan (PGN).

Although, most of the bacteria have conservative common structure especially glycan and the glycan connecting di- and tripeptide part, substitutions and modifications to the basic PGN structure sometimes occur in both the peptide stem and bridge regions and in the *N*-acetylglucosamine and *N*-acetylmuramic acid disaccharide backbone.[8)] The disaccharide backbone can be modified by addition of glycolic acid, via *N*-acylation, to muramic acid residues as occurs in *Mycobacterium tuberculosis*. Modifications to the peptide stem portion of PGN include substitutions of Gly or L-Ser residues for the L-Ala. Also, D-Gln at secondary position in the stem peptide can be substituted.

On the other hand, bacteria employ several classes of PGN-hydrolyzing enzymes that participate in assembly and disassembly of the bacterial cell wall during the processes of bacterial growth and division. PGN-hydrolyzing enzymes that bind to and degrade intact bacterial cells or PGN of the producing organism are classified as autolysins. Several pathogenic bacterial species have hydrolases or autolysins that are required for bacterial pathogenicity in eukaryotic hosts. PGN hydrolases are defined by their catalytic specificities. Two classes of these enzymes function to digest the PGN glycan backbone (Figure I-3): *N*-acetylmuramidases such as lysozymes

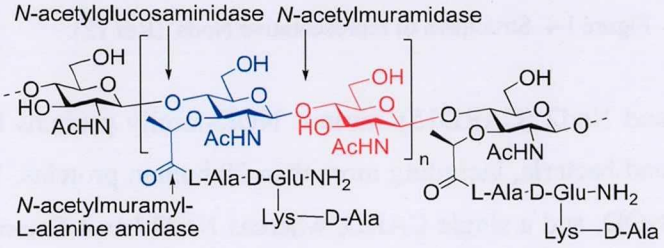


Figure I-3. PGN-hydrolyzing enzymes.

hydrolyze the MurNAc-GlcNAc linkages, wheras *N*-acetylglucosaminidases cleave the GlcNAc-MurNAc bonds. In addition, *N*-acetylmuramyl-L-alanine amidases (Figure I-3) cleave the bond between MurNAc and the first L-Ala of the peptide chain,[9] leading the separation of PGN sugar backbones from the stem peptide chain.

It has been known for a long time that PGN promotes an inflammatory response.[10] PGN has been demonstrated to stimulate the production of inflammatory cytokines in monocytes, macrophages, neutrophils, and epithelial cells. A PGN partial structure, muramyl dipeptide (MDP : MurNAc-L-Ala-D-isoGluNH$_2$, Figure I-2) was found to be the minimal chemical structure required for the adjuvant activity of Freund's complete adjuvant, but the mechanism of the recognition had not been revealed until recently as described in next part (Nod-like receptors). PGN is also known as the target of antibiotics, and also other enzymes and lectins.

Nod-like receptors

The Nod family was originally identified by the homology to the apoptosis regulator Apaf-1 in a Gen-BankTM database search.[11] Nod proteins exist in cytoplasm. They have a similar domain structure (Figure I-4) composed of a central nucleotide-binding oligomerization domain (NOD), a *C*-terminal domain consisting in leucine-rich repeats (LRRs), and *N*-terminal effector binding domain, such as caspase recruitment domain (CARD), pyrin domain (PYD), and Toll-interleukin-1 receptor domain (TIR).[11] LRRs domain has been thought to be responsible for sensing of respective ligands. Since the identification of Nod1 (CARD4) and Nod2 (CARD15), various NOD-family proteins have been found from animals, plants, fungi and bacteria, including more than 20 human proteins. Nod1 consists of a C-terminal LRR, a central NOD, and a single CARD, whereas Nod2 has a C-terminal LRR, a central NOD, and two CARDs (Figure I-4).[12]

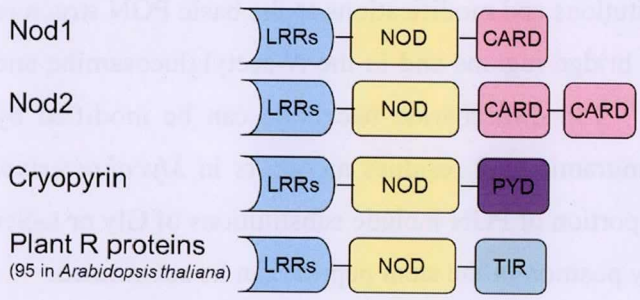


Figure I-4. Structures of representative Nods (Ref 12).

Activation of Nod proteins with their ligands leads to the oligomerization of Nods via interactions of the central NOD to induce intracellular signaling through the clustering of signaling molecules with the effector binding domains.

Our group, i.e., chemistry group in Osaka University and Dr. Inohara and Dr. Nuñez's biology group in University of Michigan, previously identified that Nod1 and Nod2 are the innate immune

receptors against PGN and PGN fragments. Nod1 recognizes peptidoglycan having *meso*-diaminopimelic acid and γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP) as the minimum ligand,[13] whereas the minimum Nod2 ligand is muramyl dipeptide (MDP).[14] At the same period, Dr. Philpott's group reported the identical results.[42), 43)] Because PGN from both Gram-positive and Gram-negative bacteria contains MDP, Nod2 functions as a general sensor of bacteria.

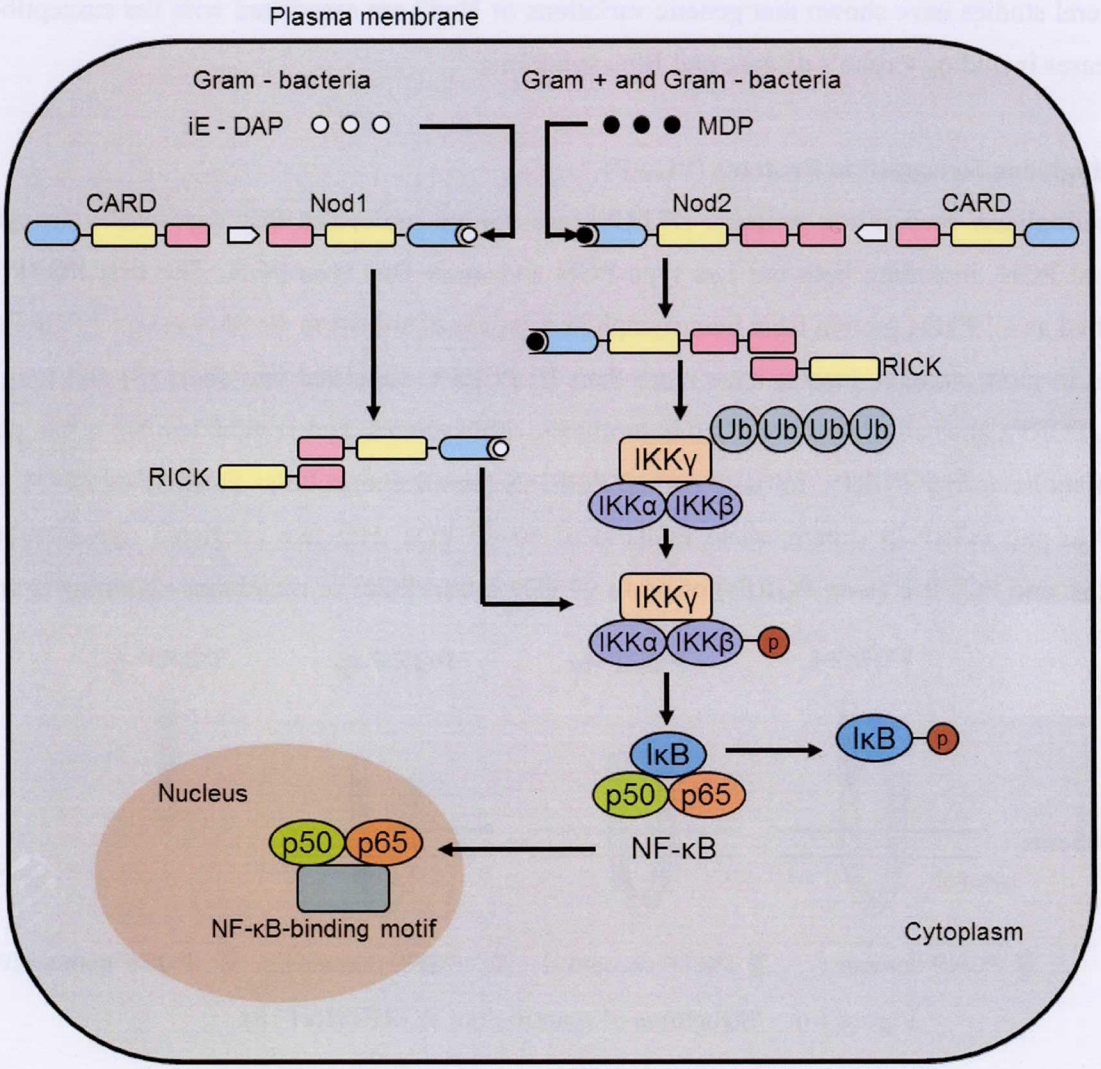


Figure I-5. Signal pathways of Nod1 and Nod2 (Ref 15).

However, recognition process of PGN with Nod1 and Nod2 is unclear at present. Though some amino acid residues in the LRRs of Nod1 and Nod2 are required for ligand sensing, no apparent observations for direct interaction of Nod proteins with their ligands have been reported.

On the other hand, downstream signals of Nod2 have been disclosed as follows (Figure I-5). Upon activation of Nod2 by peptidoglycan and MDP, serine/threonine kinase (RICK) is recruited through CARD–CARD interactions to activate RICK. Activation of RICK leads to K63

(Lys63)-linked polyubiquitylation of IKKγ, the scaffold of the inhibitor of NF-κB (IκB)-kinase complex (IKK complex, which consists of IKKα and IKKβ). The subsequent phosphorylation of IKKβ leads to phosphorylation of IκB. The phosphorylated IκB was then ubiquitinated and then degraded. The released nuclear factor-κB (NF-κB) is translocated to the nucleus to activate the expression of specific genes that concern immunoactivation.[15)]

Several studies have shown that genetic variations of Nod2 are associated with the susceptibility to diseases including Crohn's disease and Blau syndrome.[16)]

Peptidoglycan Recognition Proteins (PGRP)

Peptidoglycan recognition proteins (PGRPs) are unique molecules that specifically recognize bacterial PGN, including both the Lys type PGN and *meso*-Dap type PGN. The first PGRP was identified as a 19 kDa protein from hemolymph and cuticle of silkworm *Bombyx mori*.[17)] PGRPs are present in most animals. Insects have more than 10 PGRPs, classified into short (S) and long (L) forms. PGRPs have also been identified in mollusks, echinoderms, and vertebrates, but not in plants. Mammals have four PGRPs, initially named PGRP-S (small extracellular PGRPs) of 20–25 kDa, PGRP-Iα and PGRP-Iβ (intermediate PGRPs) of 40–45 kDa with two predicted transmembrane domains, and PGRP-L (long PGRPs) of up to 90 kDa intracellular or membrane-spanning proteins

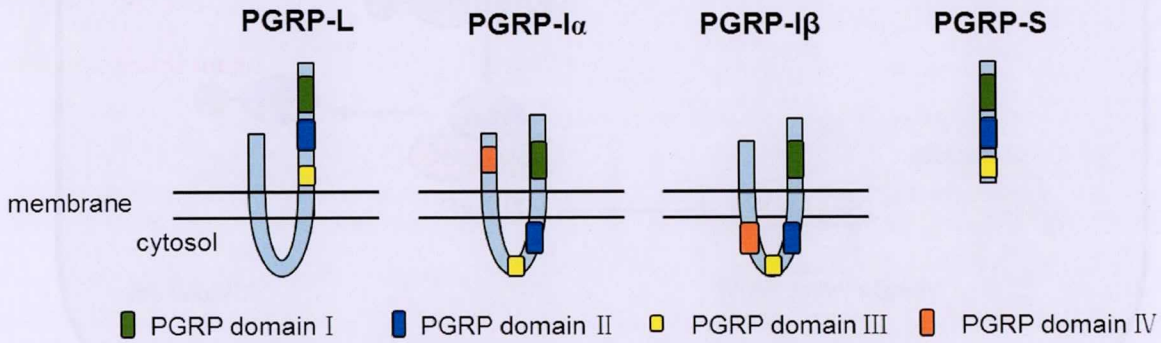


Figure I-6. Structures of mammalian PGRPs (Ref 18).

(Figure I-6), based on the predicted structures.[18)] Subsequently, the changing of names appeared as PGLYRP-1, PGLYRP-2, PGLYRP-3, and PGLYRP-4, respectively.

PGRPs were identified as a set of crucial PRRs in both insects and mammals. Recent studies have shown the diverse functions of respective PGRPs. Generally, their functions are categorized at least three classes. PGRPs in the first category work as PRRs that recognize and bind bacterial PGN, followed by activation or inhibition of downstream immune responses, such as prophenoloxidase system, Toll and IMD pathway, and JNK pathway. In the second category, PGRPs act as

N-acetylmuramyl-L-alanine amidase that cleaves the lactylamide bond between muramic acid and the peptide chain in bacterial PGN, and exhibit the bactericidal activity like lysozyme. In the second category, PGRPs are identified as the opsonin to induce agglutination or phagocytosis.

Lysin Motif (LsyM)

The lysin motif (LysM) is a ubiquitous protein module that binds to various types of peptidoglycan and chitin. The common structure recognized by LysM is *N*-acetylglucosamine moiety. LysM domains are generally composed of 40–60 amino acid residues. LysM has been found in more than 4000 proteins, e.g., bacterial lysins, bacteriophage proteins, and certain proteins of eukaryotes.[19] Various bacterial enzymes such as PGN hydrolases, peptidases, chitinases, esterases, reductases, and nucleotidases contain LysM(s).[20] Bacteriophage lysins are muramidases having

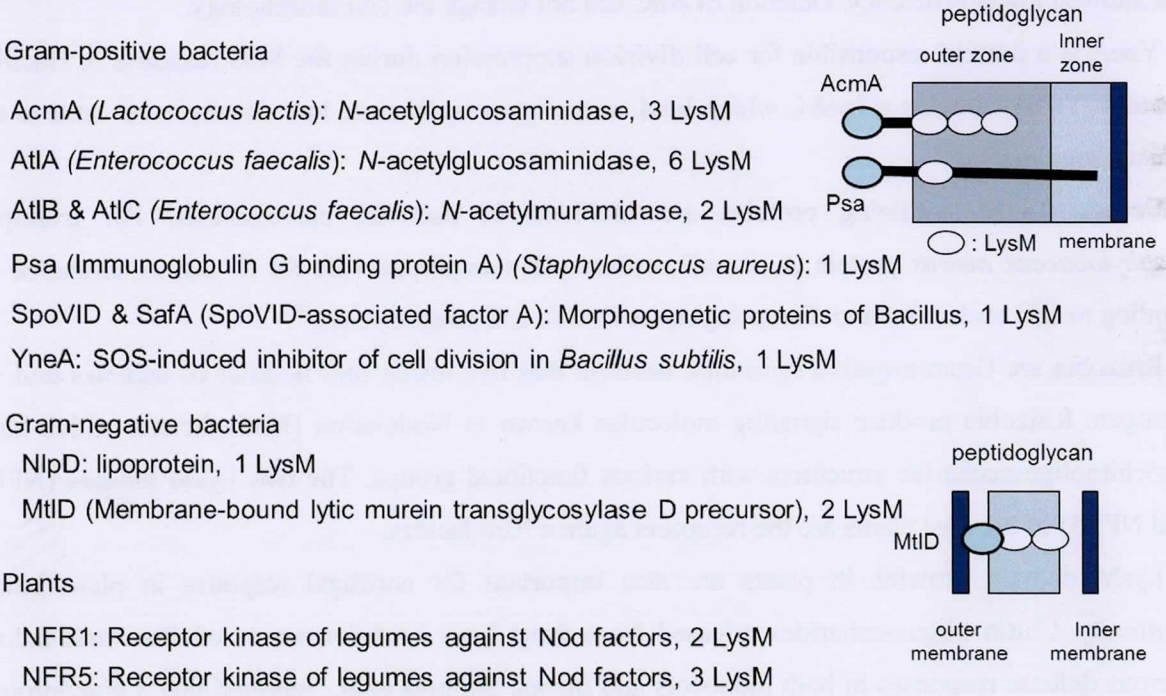


Figure I-7. Presentation of various cellular locations of LysM-containing proteins (Ref 19).

LysM domains. Bacterial proteins possessing LysM(s) retain at the cell surface by the interaction of LysM with peptidoglycan layer (Fig. 1-7).

A major class of LysM-containing proteins is a group of peptidoglycan hydrolases, i.e., glycosylases such as *N*-acetylmuramidases and *N*-acetylglucosaminidases as well as peptidoglycan endopeptidases such as Cysteine and histidine dependent amidohydrolases/peptidases (CHAP) superfamily. LysM domains of the glycosylases are located at the C-terminal part, whereas those in the peptidase are present in the *N*-terminal part.

One of the most well-characterized LysM proteins is AcmA, *N*-acetylglucosaminidase of *Lactococcus lactis*. AcmA binds to the cell wall in a non-covalent manner and is responsible for cell lysis of both autolysis (self-destruction of bacteria) and allolysis (lysis induced by other cells). AcmA has three C-terminal LysMs in the LysM domain, which binds to outer wall zone, high density peptidoglycan–teichoic acid complex layer.[21] LysM domain then proved to be responsible for binding to peptidoglycan.[22]

Mesnage et al. identified AtlA as a major *N*-acetylglucosaminidase of *Enterococcus faecalis*. AtlA contains six C-terminal LysMs that bind peptidoglycan.[23] AtlA digests the septum and is required for cell separation after cell division.[24] They also identified two LysM proteins, AtlB and AtlC, from *Enterococcus faecalis*. Both AtlB and AtlC were found to be *N*-acetylmuramidase.[24] The physiological roles of these two proteins were different; AtlB also digests the septum like AtlA but showed lower efficiency. Deletion of AtlC did not change the cell morphology.

YneA is a protein responsible for cell division suppression during the SOS response in Bacillus subtilis. YneA contains a LysM, which bind to the inner wall zone, low density layer next to the plasma membrane.[25]

Several LysM-containing proteins are involved in bacterial pathogenesis. For example, *Staphylococcus aureus* protein A on cell surface plays important role for *S. aureus* virulence by binding to IgG molecules and disrupting opsonization and phagocytosis.[26]

Rhizobia are Gram-negative symbiotic bacteria that live inside root nodules of legumes and fix nitrogen. Rhizobia produce signaling molecules known as Nodulation (Nod) factors, which have lipochitooligosaccharide structures with various functional groups. The two LysM kinases (NFR1 and NFR5) of the host plants are the receptors against Nod factors.

LysM domain proteins in plants are also important for antifugal response in plant innate immunity. Chitin oligosaccharides released from fungi have been known as elicitors that induce various defense responses in both monocots and dicots. Shibuya et al., reported that a glycoprotein CEBiP (chitin elicitor binding protein) of *Oryza sativa* possessing two LysMs is involved in chitin-induced defence response.[27] They then found that CERK1 (chitin elicitor receptor kinase) (also known as LysM-RLK1 (LYK1)[29]), a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis.[28] They also found that CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in *Oryza sativa*.[30] Wan et al. reported that LYK1 and LYK-4 are important for chitin signaling and plant innate immunity in Arabidopsis.[29), 31]

LysM proteins in plants are also important for antibacterial response. Arabidopsis LysM proteins LYM1, LYM3, and CERK1/LYK1 proved to mediate peptidoglycan sensing and immunity to

bacterial infection.[32)]

Previous studies in structure-activity relationship of PGN

From the middle of last century, several investigators attempted to elucidate the structural entity responsible for immunostimulation by PGN. In the middle of 1970s, the collaborative group of Prof. Shiba and Prof. Kotani in Osaka University[33)] and Prof. Lederer's group in Pasteur Institute[34)] independently demonstrated that the minimum structure required for the immunostimulation is MDP. From then on, many derivatives and analogs of MDP have been synthesized and the relationship between the chemical structures and immunological activities has been studied. Synthetic monosaccharide fragments such as MDP, MurNAc-tri- and tetrapeptide (Figure I-8), which were the representatives of firstly synthesized PGN fragments, enhanced immune responses of guinea pigs against a given protein antigen in manners similar to those of natural peptidoglycan preparations in standard in vivo test systems. In contrast, MurNAc-L-Ala and the tetrapeptide without MurNAc moiety did not.[35)]

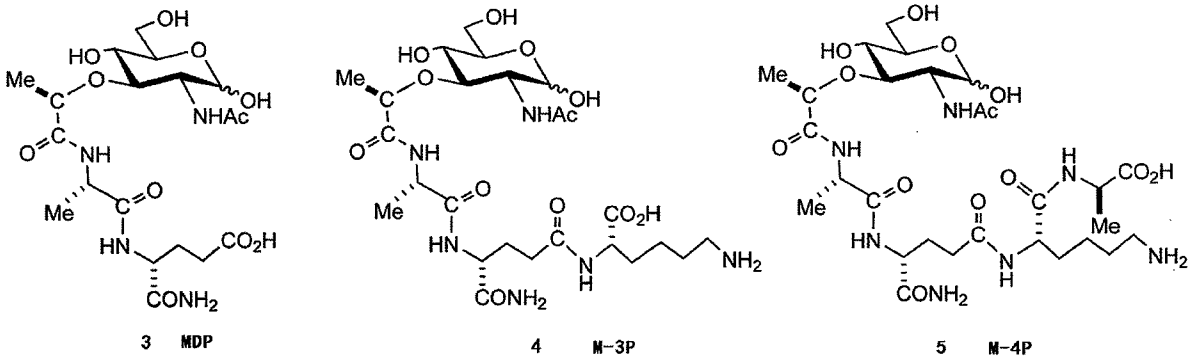


Figure I-8. Muramyl pepetides.

In those days, peptidoglycan digests by endopeptidase, which is capable of hydrolyzing the cross linkages between the neighboring glycan chains, as well as digests by glycosidases, which split the glycan chain, generally were shown to be more active than MDP.[36)] These results suggested that presence of long glycan chain rather than the peptide network is required for efficient manifestation of these biological activities (However, the author's present study revealed longer glycan chain rather decreased the immunostimulating activity).

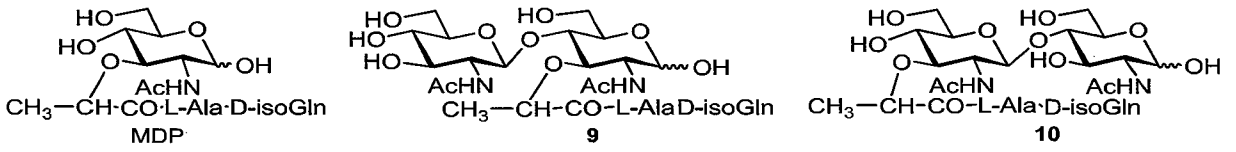
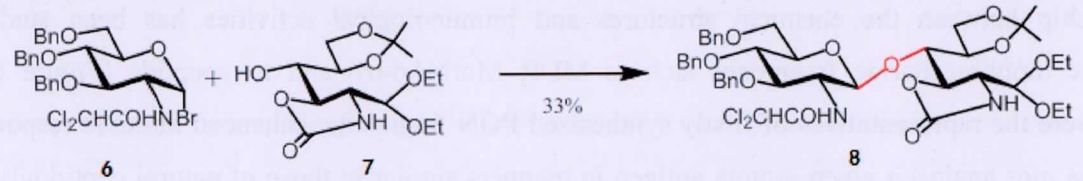


Figure I-9. Synthetic disaccharide with dipeptide fragments.

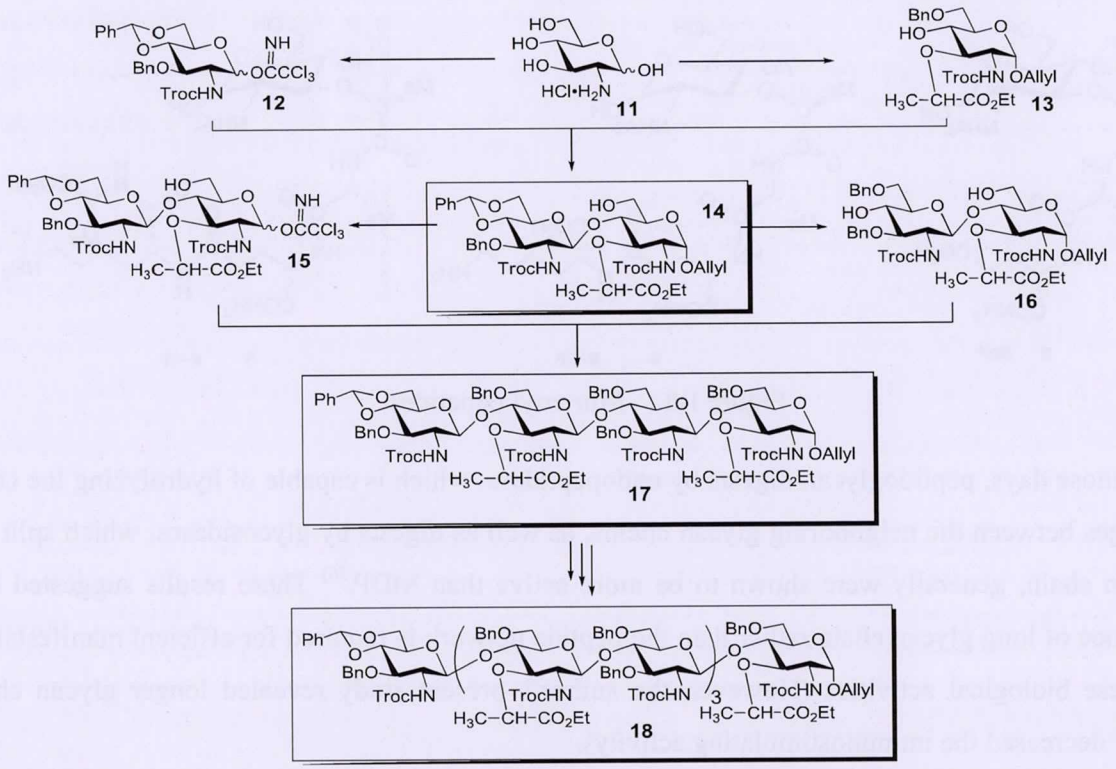
Regarding to the peptide chain, no distinct difference was observed in the biological activity

among MurNAc-di-, -tri-, and-tetrapeptides, indicating MDP residue is sufficient for the full activity.[37] Hence, our group synthesized two disaccharide dipeptides fragments (Figure I-9) with alternative repeating units of PGN while their biological activities were almost identical with MDP.[38]

In order to systematically examine the effect of the glycan chain length on the activity, our group investigated the synthesis of peptidoglycan partial structures having longer repeating β(1→4) glycan chains (Scheme I-1) corresponding to the common backbone structure of bacterial PGN.



Scheme I-1. Previous glycosylation method.



Scheme I-2. Synthetic route for long saccharide.

Inamura *et al.* reported the effective construction of repeating glycans of PGN accomplished by the coupling of a key disaccharide glucosaminyl-β-(1→4)-muramic acid unit (Scheme I-2). Stereoselective glycosylation of disaccharide units was achieved by neighboring group participation of the *N*-Troc (Troc = 2,2,2-trichloroethoxycarbonyl) group and appropriate reactivity of

N-Troc-glucosaminyl trichloroacetimidate. By using this efficient synthetic strategy, mono-, di-, tetra- and octasaccharide fragments of PGN were synthesized.[39)]

After the discovery of Toll-like receptors (TLRs) as innate immune receptors in 1997,[40)] TLR2 was once considered as the PGN receptor. However, Boneca *et al.* revealed that highly purified PGN fragments are not recognized by TLR2.[41b)] Synthetic PGN fragments also did not show TLR2 dependency.[39)] Recently, TLR2 is mainly considered to be the receptor of lipoproteins.[41)] In 2003, Inohara *et al.*[13), 14)] and Philpott *et al.*[42), 43)] independently identified two cytoplasmic proteins named Nod1 and Nod2 which belong to the family of NLR proteins are the peptidoglycan receptors.

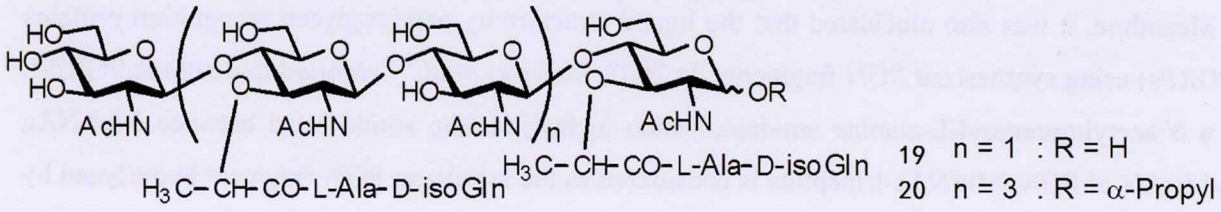


Figure I-10. Tetra- and octasaccharide dipeptide fragments of peptidoglycan.

Therein, Nod2 proved to be receptor to MDP and peptidoglycan fragments,[14)] which has been looked for decades after the discovery of MDP as the minimal adjuvant active structure of PGN. The synthetic tetrasaccharide and octasaccharide having dipeptides linked to each MurNAc residue (Figure I-10) also showed definite Nod2 induced NF-κB activation while glycan chain without peptide did not.[14)] MDP was most active and the potency decreased as the glycan chain length increased from di-, tetra- to octasaccharides (Figure I-11).[14)] The effect of peptide chain length on

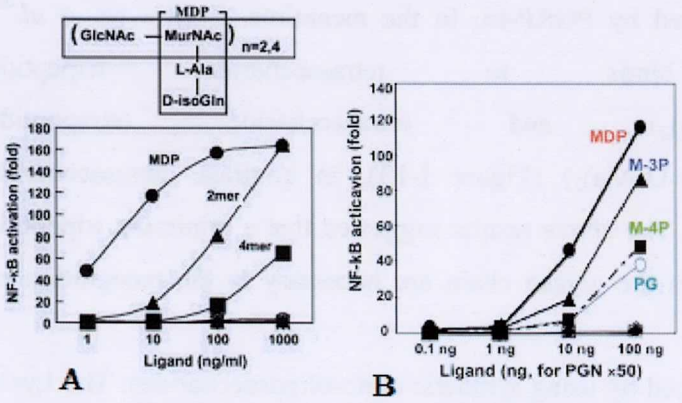


Figure I-11. Stumulation of Nod2 by PGN fragments. A. Saccharide with dipeptide. B. Muramyl peptides

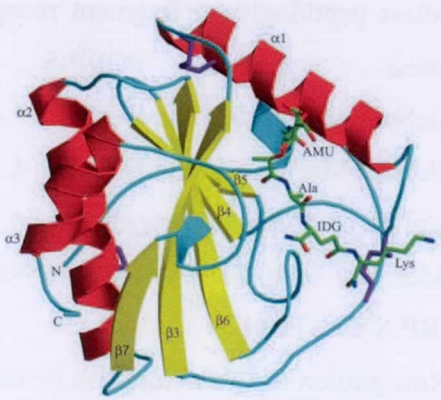


Figure I-12. Structure of the PGRP-Iα–MTP complex (Fig from Ref 33).

Nod2 activation had also been elucidated. MDP showed the most potent activity compared with muramyl tripeptide or tetrapeptide (Figure I-11).[39)] On the other hand, Nod1 proved to be the receptor to *meso*-diaminopimelic acid type PGN and the minimal ligand structure for Nod1 was

found to be γ-D-glutamyl-diaminopimelic acid (iE-DAP).[13)]

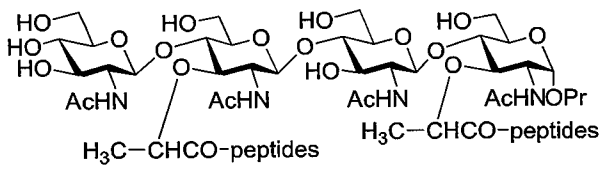


Figure I-13. Synthesized tetrasaccharide with two tri-, tetra-, and pentapeptide units.

peptides :

L-Ala—D-isoGln—L-Lys **1b**
L-Ala—D-isoGln—L-Lys(Ac) **21**
L-Ala—D-isoGln—L-Lys-D-Ala **1c**
L-Ala—D-isoGln—L-Lys(Ac)-D-Ala **22**
L-Ala—D-isoGln—L-Lys-D-Ala-D-Ala **1d**
L-Ala—D-isoGln—L-Lys(Ac)-D-Ala-D-Ala **23**

Meantime, it was also elucidated that the ligand structure by peptidoglycan recognition proteins (PGRPs) using synthesized PGN fragments. In 2003, Dziarski *et al.*[44)] reported that human PGRP-L is a *N*-acetylmuramoyl-L-alanine amidase, which hydrolyzes the amide bond between Mur-NAc and L-Ala of PGN. MurNAc-tripeptide is considered as the minimum PGN fragment hydrolyzed by PGRP-L. Other members of the human PGRP family did not show the amidase activity. In the following a few years, Mariuzza *et al.*[45)] evaluated the interactions of a range of synthetic peptidoglycan derivatives with PGRP-Iα, PGRP-Iβ and PGRP-S. They determined the crystal structure of the C-terminal PGN-binding domain of human PGRP-Iα in complex with the lysine-type PGN muramyl tripeptide in 2004 (Figure I-12). The normalized data for the lysine-type muramyl tetra and pentapeptide showed that these compounds have similar but much lower affinity than muramyl tripeptide. Further study showed that the compounds contain only peptide chain as well as muramyldipeptide exhibited no binding, indicating that the muramyltripeptide is the smallest peptidoglycan fragment recognized by PGRP-Iα. In the meantime, Ezekowitz *et al.*[46)] showed that PGRP-S binds to tetrasaccharide tripeptide ((GlcNAc-MurNAc-L-Ala-D-isoGln-L-Lys)$_2$) and tetrasaccharide tetrapeptide ((GlcNAc-MurNAc-L-Ala-D-isoGln-L-Lys-D-Ala)$_2$) (Figure I-13), in contrast, tetrasaccharide dipeptide or MurNAc tetrapeptide did not. The above results suggested that a minimum tripeptide residue and both GlcNAc and MurNAc in the glycan chain are necessary in the recognition of PGRP-S with PGN.

Recognition of LysM was also investigated by using synthetic chito-oligosaccharides. The LysM domains from bacterial proteins show affinity to various PGN. In contrast, LysMs present in chitinases in some eukaryotes and green algae bind to chitin. Interaction of LysM domain derived from *Pteris ryukyuensis* chitinase-A (PrChi-A) with *N*-acetylglucosamine oligomers ((GlcNAc)$_n$) was investigated using differential scanning calorimetry, isothermal titration calorimetry, and NMR spectroscopy. This LysM has high affinity with (GlcNAc)$_5$ with the stoichiometry of

$(GlcNAc)_5$/LysM domain (1:1).[47]

Synthesis of peptidoglycan fragment library for analysis of receptor recognition in this study

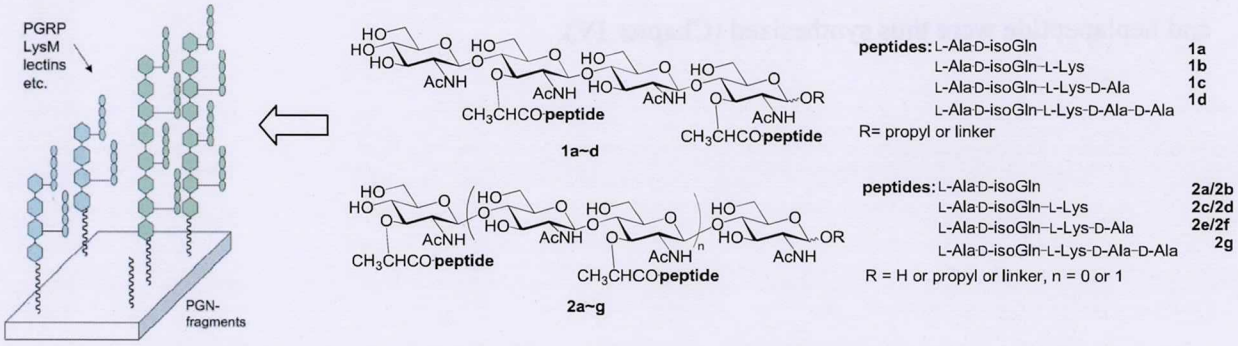


Figure 2. Peptidoglycan fragments structures and their schematic structure fixed on a plate.

Since the PGN hydrolyzing enzymes in host cells are well known as lysozymes which degrade the bacteria as *N*-acetylmuramidases, previous synthetic works mainly focused on PGN with GlcNAc-MurNAc repeating glycan units. In order to understand the recognition and functions PGN in multi-cellular organisms and also in the bacteria themselves, a PGN fragments library including two kinds of glycan sequences (GlcNAc-MurNAc and MurNAc-GlcNAc) with various lengths of glycan chains is necessary. In this thesis, the author designed the synthetic targets **2a~g** (Figure 2), which are the fragments expected to be produced by bacterial enzymes, and synthesized them for the first time as shown in Chapter II. The previously synthesized fragments **1a~c**, which are considered to be produced by the enzymes in animals, were also synthesized for comparison. With these compounds, Nod2 stimulating activity of these synthetic peptidoglycan fragments was evaluated (Chapter II).

The author then studied to construct PGN arrays for the high-throughput elucidation of interactions with a variety of receptors and recognition proteins (lectins, PGRPs, LysM proteins, etc) (Chapter III). PGN fragments having linker for the conjugation with the array were thus synthesized and then successfully attached to the array platforms. The properties the PGN arrays were then assessed by measuring the interaction with wheat germ agglutinin (WGA), which binds *N*-acetylglucosamine, as well as PGRP-S, which proved to recognize the peptidoglycan tetrasaccharide fragments. The assay results clearly indicated high performance of the PGN arrays for studies of interactions of PGN with PGN-recognition proteins.

The author also elucidated the synthesis of peptidoglycan fragments having long peptide chain in order to investigate the effect of peptide chain length to the interaction with LysM domains. The target LysM proteins are *N*-acetylmuramidases AtlB and AtlC from *Enterococcus faecalis* characterized by Mesnage et al.[24] The tetrasaccharide heptapeptide and octasaccharides depeptide and heptapeptide were thus synthesized (Chapter IV).

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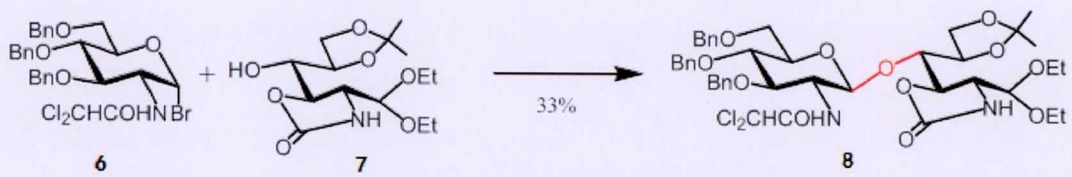
Chapter II Synthesis of Peptidoglycan (PGN) Fragments for Analysis of Glycan Sequence Recognition by Nod2

II-1 Introduction

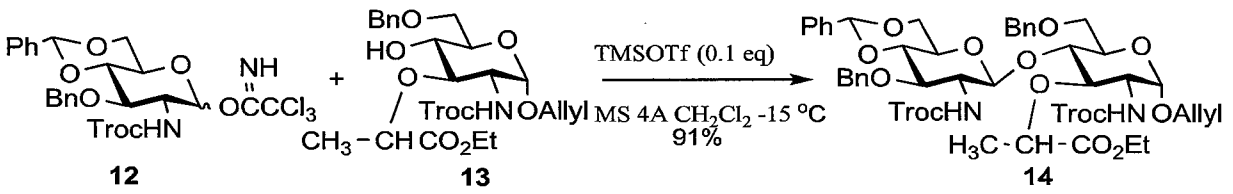
Since chemically synthesized PGN fragments are essential in order to analyze the various ligand-protein interactions, our group have developed the synthetic procedure for PGN partial structures such as tetrasaccharides and octasaccharides.[3] Synthesis of PGN partial structures with longer glycan chain had not been elucidated until 2000 because of the synthetic difficulty (eg. stereoselective construction of β(1→4) glycosidic linkage at hindered 4-hydroxy group of a muramic acid residue). In our laboratory, a special synthetic strategy was developed to construct the disaccharide.[4] In order to circumvent the low reactivity of the 4-OH group in the acceptor, an open-chain derivative of glucosamine was used as a glycosyl acceptor (Scheme II-1).[1]



Scheme II-1. Previous glycosylation method.

Recent progress in glycosylation reactions then changed the situation. β-Selective glycosylation with glucosaminyl donor was established by means of neighboring participation of 2-*N*-trichloroethoxycarbonyl (*N*-Troc) group (Scheme II-2).[2), 3)] Glycosyl trichloroacetimidates[3)] have proved to be highly efficient for β-glucosaminylation. Various compounds, including lipid A and (immunostimulating glycoconjugates of Gram-negative bacteria) their analogs were successfully synthesized by this method. Inamura applied this glycosylation method for the synthesis of GlcN-β(1→4)-MurN structure. The key disaccharide GlcN-β(1→4)-MurN was thus synthesized in high yield. This disaccharide was used as a common synthetic intermediate, which was separately converted to both disaccharide acceptor and donor. A tetrasaccharide was obtained by the glycosylation of these disaccharide blocks. Octasaccharide was synthesized from the tetrasaccharide acceptor and donor in a similar strategy. Introduction of the peptide moieties to

3-*O*-lactyl groups followed by deprotection afforded the peptidoglycan fragments with tetrasaccharide and octasaccharide.[3), 4)]



Scheme II-2. Modified glycosylation method.

Although several PGN fragments were successfully synthesized,[4)] more fragment structures were required to elucidate the biofunctions of PGN in host organisms and in the bacteria themselves. During the Lysis of bacterial cells, the various PGN fragments are released from bacteria. These fragements should contain two glycan sequences that have GlcNAc-MurNAc or MurNAc-GlcNAc repeating units, while the lysozyme, also known as muramidase, of host organisms cleaves 1,4-β-linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues in a peptidoglycan to afford glycans having GlcNAc-MurNAc repeating unit. Since peptidoglycan fragments having more than two MurNAc-GlcNAc repeating units had not been synthesized, we were interested in their biological activities. In the present study, we focused to the syhthesis of tetrasaccharide fragments. The author hence established the synthesis for both sequences of PGN fragments as described below.

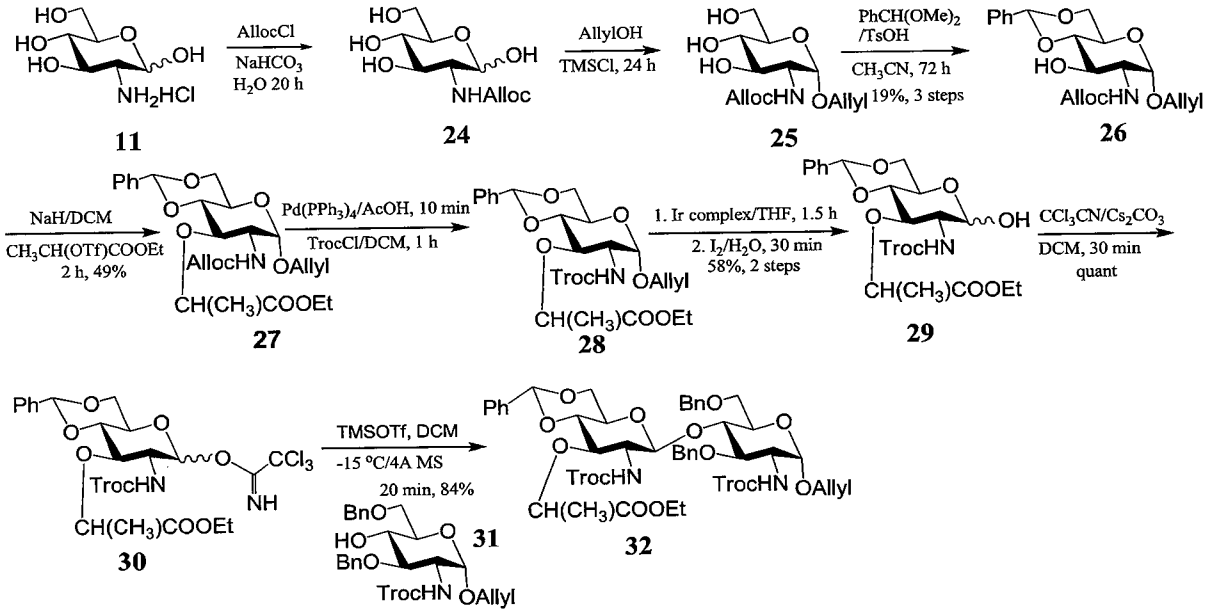
II-2 Synthesis of Disaccharide/Tetrasaccharide Key Intermediate

II-2-a Preparation of Disaccharide

Two disaccharide units **32** and **14** were prepared from D-glucosamine hydrochloride **11** as shown in Scheme II-3 and Scheme II-4. Compound **32** has MurNAc-GlcNAc unit, whereas **14** has GlcNAc-MurNAc unit.

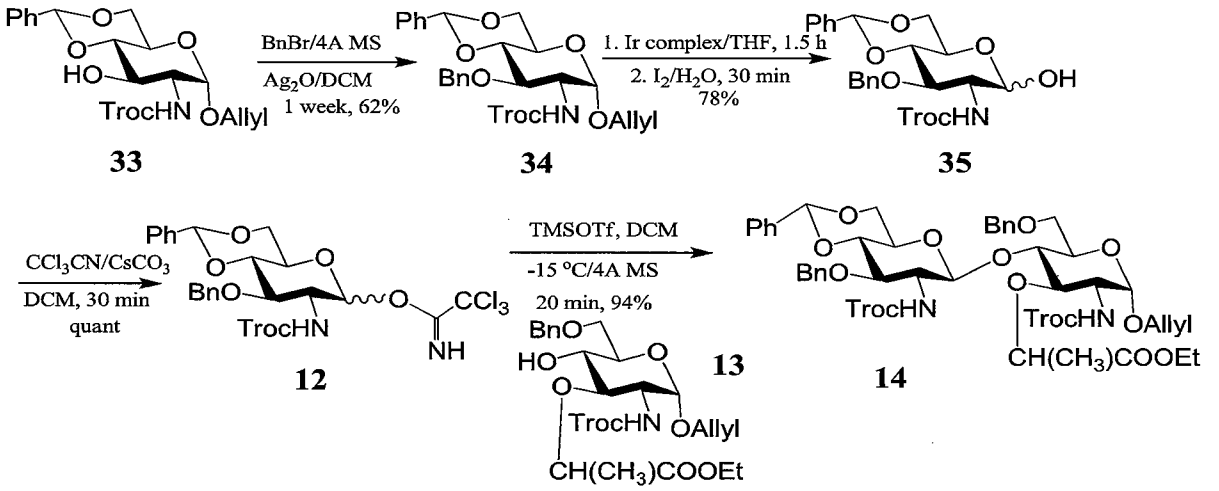
For the synthesis of **32**, D-Glucosamine hydrochloride **11** was first treated with TrocCl in the presence of $NaHCO_3$ in water to give the 2-*N*-Troc product **24**. The α-allyl glycoside **25** was obtained by Fischer glycosidaation[5)] with allyl alcohol and trimethylsilyl (TMS) chloride under reflux. Treatment of **25** with benzaldehyde dimethyl acetal and *p*-toluenesulfonic acid (TsOH) in acetonitrile gave 4,6-*O*-Benzylidene derivative **26**. Treatment of **26** with NaH followed by addition

of trifluoromethanesulfonyl-L-(*S*)-2-propionic acid ethyl ester afforded muramic acid derivative **27**. After the Alloc group of **27** was replaced with the Troc group by treatment with Pd $(PPh_3)_4$ (0.3 equiv) in the presence of acetic acid followed by addition of TrocCl to afford **28**, the 1-*O*-Allyl group of **28** was isomerized to 1-propenyl group using iridium complex $([Ir(cod)(MePh_2P)_2]PF_6$[6] (the complex was activated as follows: a solution in THF was degassed and then the complex was activated with H_2 for 1 min, H_2 in the reaction vessel was then replaced with Ar and the solution was used for the isomerization) followed by cleavage with iodine and water yielding 1-free compound **29**. Then glycosylation donor Schmidt's trichloroacetimidate[7] **30** was prepared using trichloroacetonitrile and cesium carbonate. Glycosylation of *N*-Troc acceptor **31** with the donor **30** proceeded smoothly by using TMSOTf (0.1 equiv) as a catalyst at –15 °C in the presence of MS 4A in CH_2Cl_2 under Ar. The key disaccharide **32** (MurN-GlcN) was thus obtained in 84% yield.



Scheme II-3. Synthesis of disaccharide **32**.

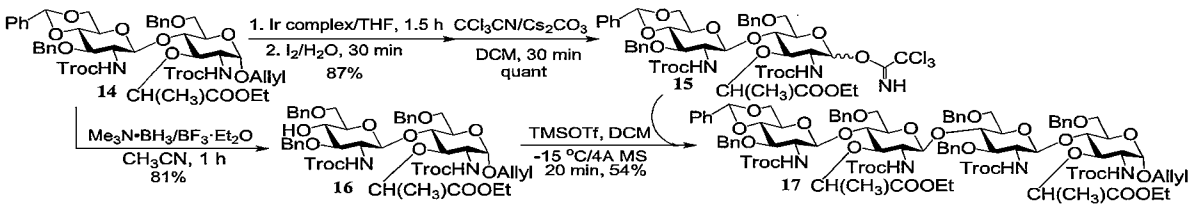
Similarly, glycosylation of the glucosaminyl donor **12** with muramic acid acceptor **13** gave disaccharide **14** (GlcN-MurN) in 94% yield as shown in Scheme II-4.[3]



Scheme II-4. Synthesis of disaccharide **14**.

II-2-b Preparation of Tetrasaccharide

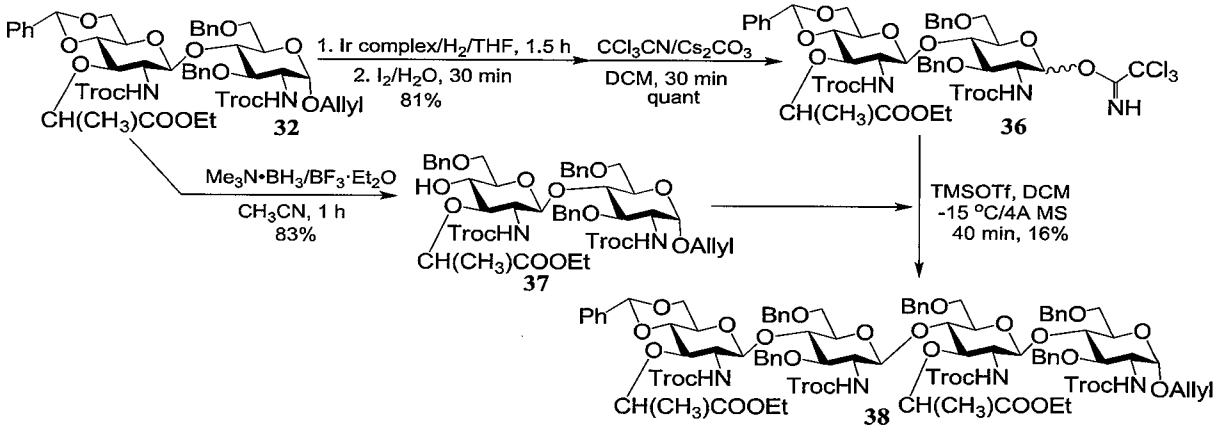
Tetrasaccharide **17** was then synthesized by using **14** as common synthetic intermediate for both glycosyl donor and an acceptor. Disaccharide donor **15** was prepared via cleavage of the allyl glycoside and subsequent conversion to the trichloroacetimidate. Regioselective reductive ring opening of the 4',6'-*O*-benzylidene of **14** was carried out by using $BH_3 \cdot Me_3N$ and $BF_3 \cdot Et_2O$ in CH_3CN[8] to afford the disaccharide glycosyl acceptor **16** with a free 4-hydroxy group in 81 % yield. These disaccharide blocks were then coupled by the same glycosylation conditions mentioned above to afford tetrasaccharide (GlcN-MurN repeating units) **17** in a moderate 54% yield (Scheme II-5).



Scheme II-5. Preparation of tetrasaccharide **17**.

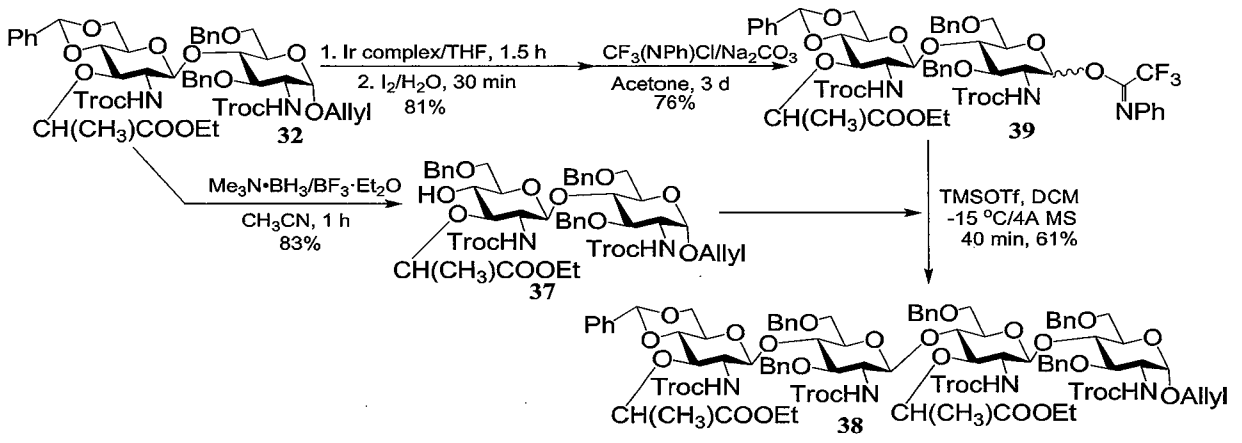
Unfortunately, the same glycosylation strategy was not successful for the synthesis of the

tetrasaccharide composed of $(MurN\text{-}GlcN)_2$. The glycosylation between glycosyl trichloroacetimidate **36** and glycosyl acceptor **37** in the presence of TMSOTf as a Lewis acid and Molecular Sieve 4A (MS4A) gave the desired tetrasaccharide **38** only in 16% yield, accompanied with 62% yield of recovered **37** (Scheme II-6). The increase of the reaction temperature or the equivalents of reactants did not improve the yield. The possible reasons of the low yield are: 1) The 4-OH group of the disccharide acceptor **37** has the considerably low reactivity because of the steric hindrance of 3-*O*-lactyl moiety in muramic acid residue; 2) trichloroacetimidate **36** is highly reactive but the activated cationic intermediate should be decomposed prior to the desired glycosylation since the attack of **37** to the intermediate seemed to be sterically-encumbered.



Scheme II-6. Preparation of tetrasaccharide **38** using trichloroacetimidate **36**.

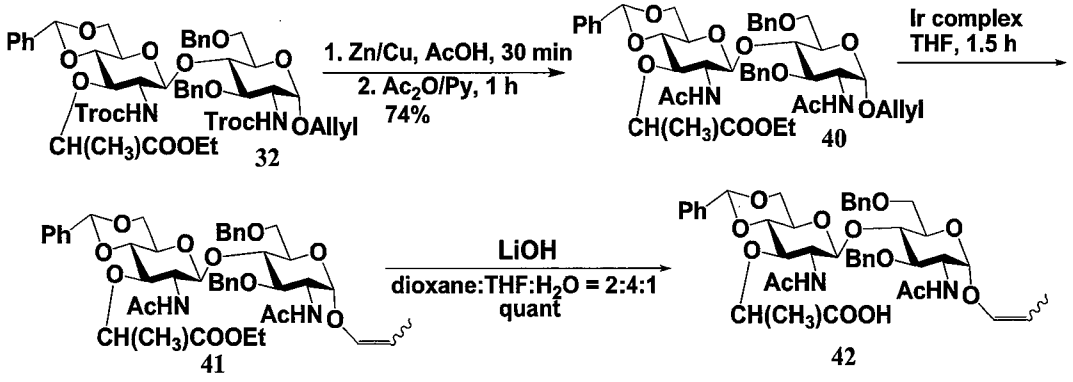
Glycosyl trichloroacetimidates generally afford the corresponding *N*-glycosyl trichloroacetamides as by-products, especially, if the reactivity of acceptor is not high enough. The author was then used *N*-phenyltrifluoroacetimidate[9] **39** as the glycosyl donor. Generally, glycosyl *N*-phenyltrifluoroacetimidates has also high reactivity but improved stability as compared to the corresponding trichloroacetimidates. Formation of the *N*-glycosyl trifluoroacetamides as by-products is considerably reduced since *N*-phenyl group of the eliminated *N*-phenyltrifluoroacetamide should suppress the undesired attack of the amide to the cationic intermediates. In order to promote the glycosylation with *N*-phenyltrifluoroacetimidate **39**, the equivalents of acceptor **37** increased (ratio of donor : acceptor was 1: 1.5). The yield of glycosylation with acceptor **37** was dramatically improved to give tetrasaccharide **38** in 61% yield (Scheme II-7).



Scheme II-7. Preparation of tetrasaccharide **38** using *N*-Phenyltrifluoroacetimidate **39**.[9)]

II-2-c Construction of Key Carboxylic Acid Intermediates

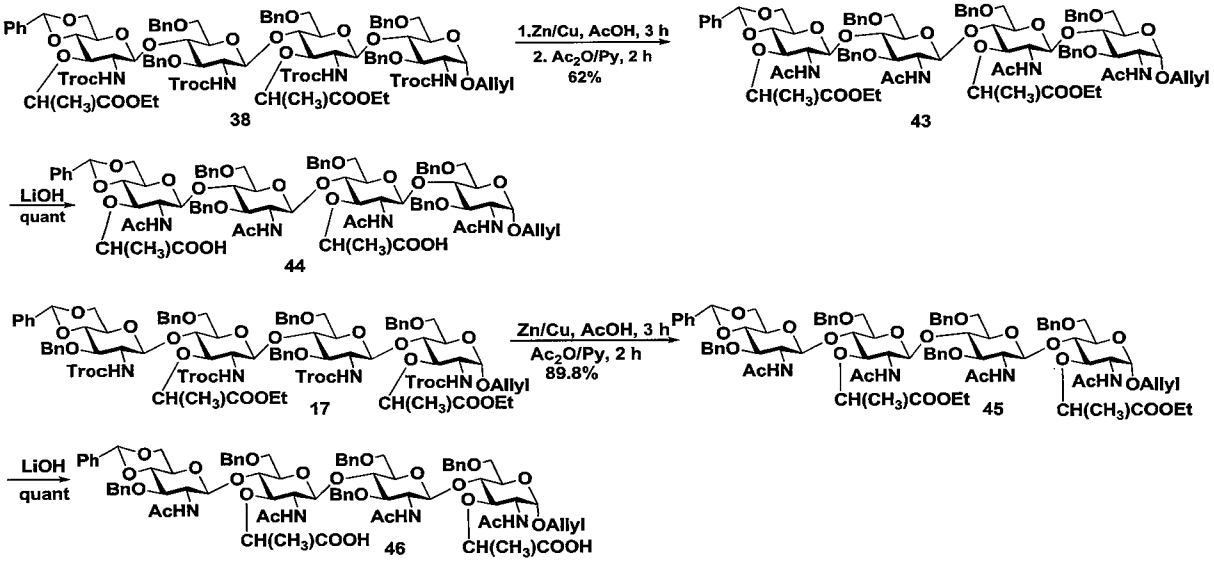
For the later condensation with peptides, *N*-Troc groups in glycan parts were changed to *N*-acetyl groups and the ethyl ester moiety (moieties) was cleaved to give the key carboxylic acid intermediate(s). The synthesis of disaccharide with carboxylic acid **42** is shown in Scheme II-8. Deprotection of *N*-Troc groups in **32** was effected by using Zn-Cu couple in AcOH. Free amino groups were then subsequently acetylated with Ac_2O and pyridine to give **40** in 74% yield. Isomerization of the allyl group to a vinyl group was carried out with H_2-activated $[Ir(cod)(MePh_2P)_2]PF_6$. Although both *E* and *Z* isomers were produced after the isomerization of



Scheme II-8. Preparation of carboxylic acid **42**.

ally group, the mixture was used for the further reactions without separation. Ethyl ester was the cleaved with LiOH and the resulting carboxylic acid **42** was isolated with hydrophobic chromatography using Diaion HP 20 column (eluted with H_2O-methanol) quantitatively.

Tetrasaccharides possessing two carboxylic acid moieties were synthesized in a similar manner. After the cleavage of Troc groups of **38** by using Zn-Cu couple and AcOH, subsequent acetylation gave **43** in 62% yield. Without the procedure of isomerization, saponification of two ethyl ester groups in **43** gave corresponding tetrasaccharide with two carboxylic acids **44** in quantitative yield after purification by HP-20 (Scheme II-9). The carboxylic acid **46** was obtained in a similar manner from **17**.



Scheme II-9. Preparation of carboxylic acid **44** and **46**.

II-2-d Investigation of Troc Deprotection

The Troc (2,2,2-trichloroethoxycarbonyl) group was used as amino protecting group in this work. It has been widely used for protection of amino and hydroxy functions in organic synthesis. As described above, 2-*N*-Troc group has been used for β-selective glycosylation by using the neighboring participation in oligosaccharide synthesis. Troc group is stable under various acidic and oxidative conditions and is readily removed by a reductive elimination process such as Zn or Zn-Cu in AcOH, Zn-Pb in AcOH. However, reductive substitution of a chlorine atom to hydrogen sometimes occurs as a side reaction during the deprotection process. The acidic conditions restrict the application to the acid stable compounds. Therefore, the author's group developed a new method for the removal of Troc group under mild conditions using tetrabutylammonium fluoride (TBAF). The author partially took charge of this study as shown in Table II-1.

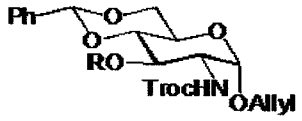
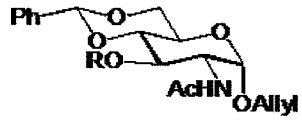
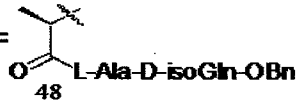
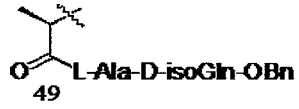
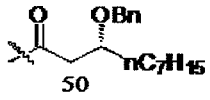
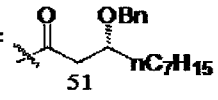
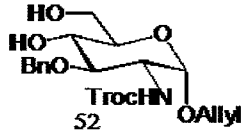
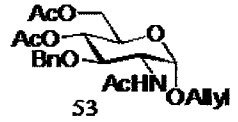
Entry	Substrate	Product	Yield
			
1	R= Bn (34)	R= Bn (47)	97%
2	R=  (48)	R=  (49)	90%
3	R=  (50)	R=  (51)	77%
4	 (52)	 (53)	78%

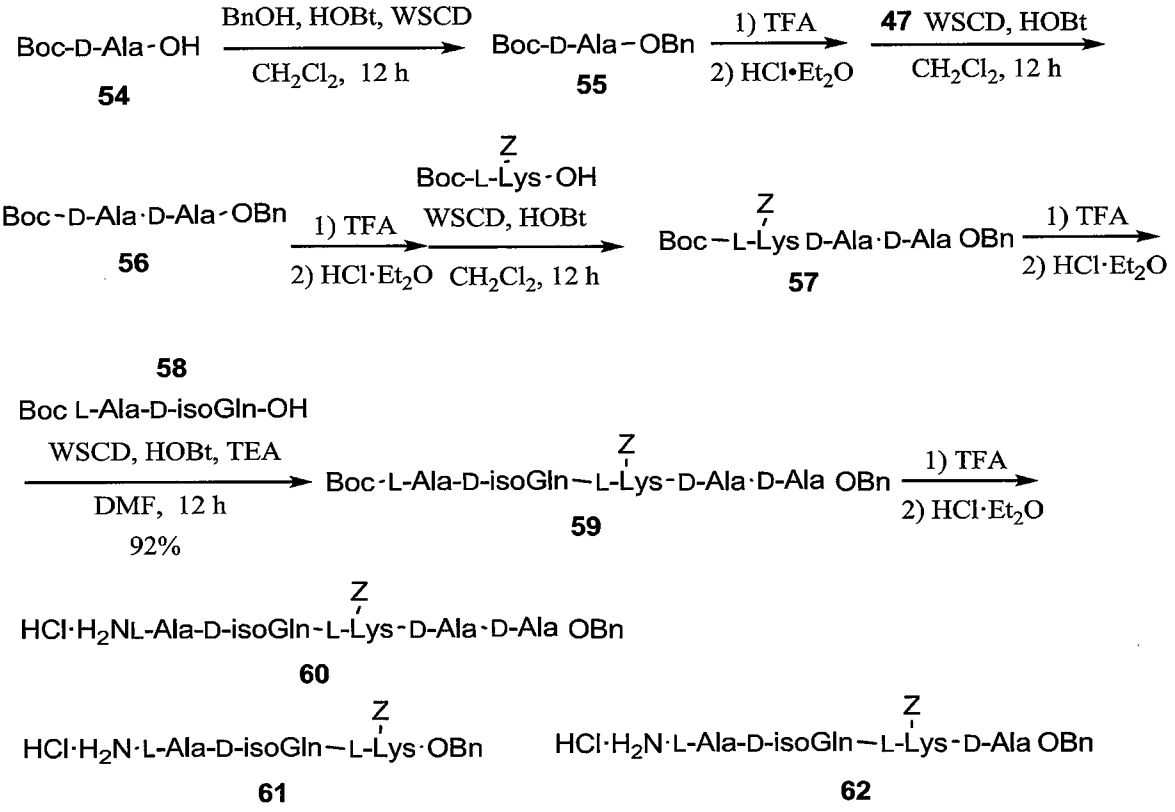
Table II-1. Examples of cleavage of Troc group with TBAF.

Treatment of *N*-Troc glucosamine derivative **34** with 1M TBAF in THF smoothly cleaved Troc group at room temperature. The resulting amino group was then acetylated to give **47** in 97% yield. Other trials on some different glucosamine and muramic acid derivatives, such as **48**, **50** and **52**, also afforded good yields (Table II-1). The present method can be applied to various oligosaccharide synthesis and other organic synthesis.

II-3 Preparation of Peptide Moieties

The peptide moieties **60**, **61**, **62** were synthesized by the coupling of the dipeptide (Boc-L-alanyl-D-isoglutamine) with L-Lys, L-Lys-D-Ala, and L-Lys-D-Ala-D-Ala, respectively. For example, the synthesis of pentapeptide is shown in Scheme II-10. Tripeptide **57** was

synthesized by usual stepwise elongation from C-terminal. Boc group of **57** was the removed by TFA and the resulting TFA salt was changed to the hydrochloride salt. Condensation of the dipeptide **58** with the hydrochloride salt using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide



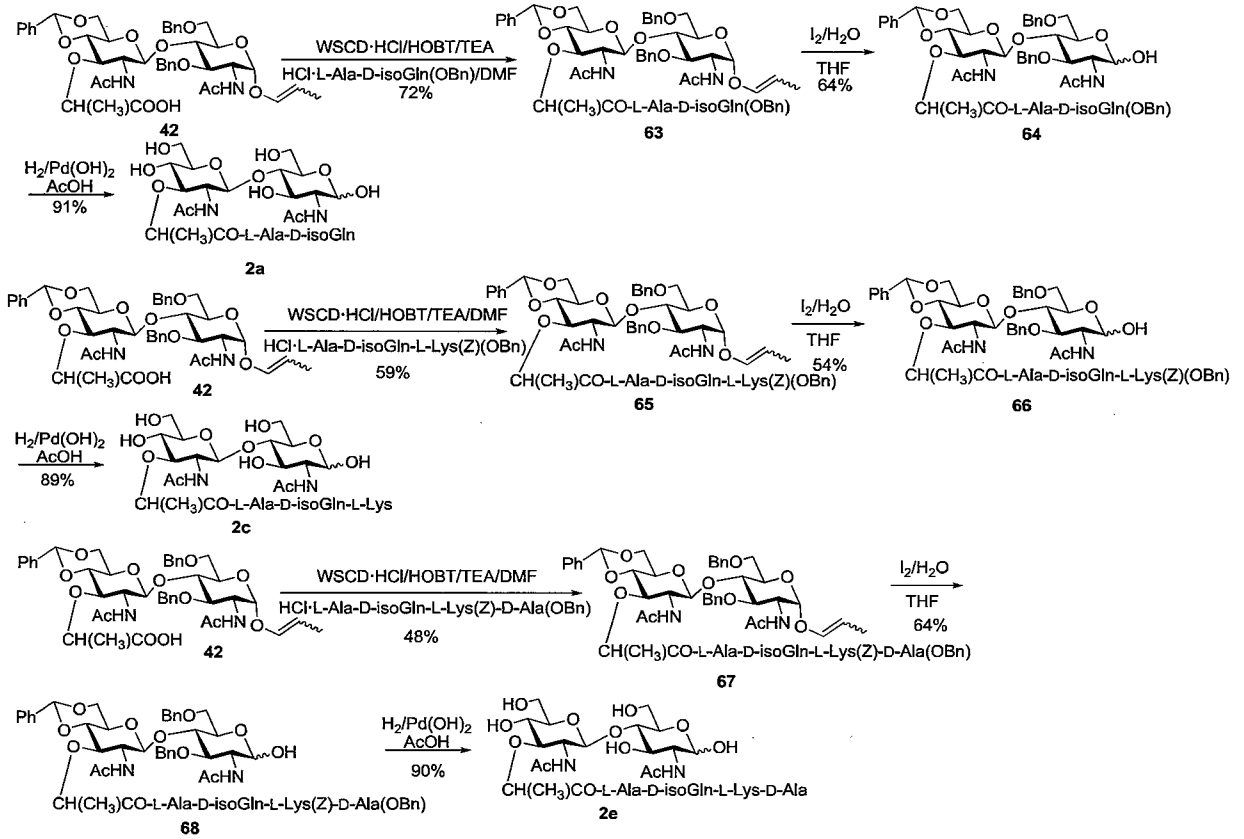
Scheme II-10. Synthesis of peptides for PGN synthesis.

hydrochloride (WSCD·HCl), 1-hydroxybenzotriazole (HOBt) gave pentapeptide **59**. Removal of the Boc group by TFA afforded **60**, which was used for the coupling with glycan chain. Tripeptide **61** and tetrapeptides **62** were also synthesized in a similar manner.

II-4 Synthesis of Disaccharide PGN Fragments

The disaccharide carboxylic acid **42** was then coupled with di-, tri-, tetrapeptide using WSCD·HCl and HOBt in DMF. Condensation reaction between disaccharide and all peptides proceeded smoothly to give glycan peptide conjugates, **63**, **65**, **67**, respectively. Products were purified by silica-gel chromatography. After the cleavage of vinyl group by iodine and H_2O,

deprotection of all benzyl-type protecting groups was performed by catalytic hydrogenolysis (H_2, 2 MPa) in acetic acid using palladium hydroxide on carbon. Lyophilization from water gave the target compounds **2a**, **2c** and **2e** successfully (Scheme II-11).

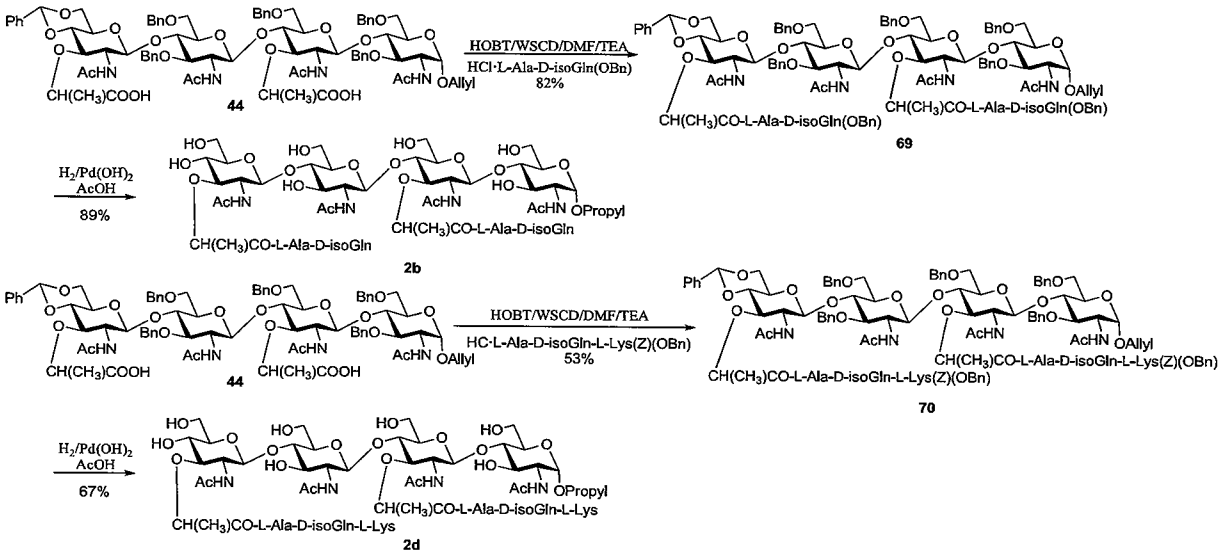


Scheme II-11. Complete synthesis of PGN disaccharide fragments.

II-5 Synthesis of Tetrasaccharide PGN Fragments

II-5-a Preparation of Fragments Possessing MGMG Sequence

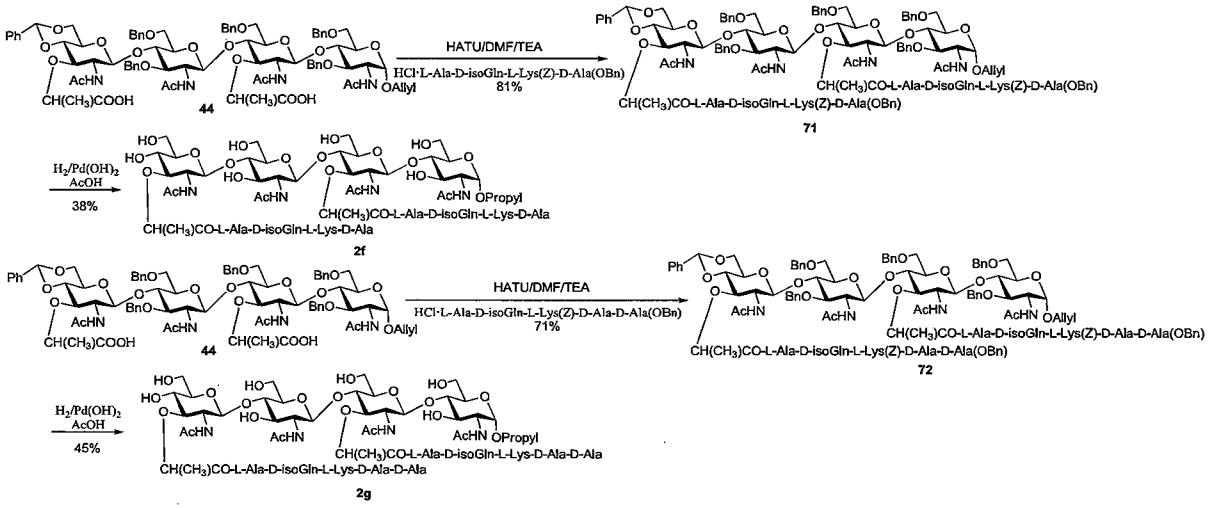
The tetrasaccharide intermediate having two carboxyl groups **44** (MGMG: MurNAc-GlcNAc-MurNAc-GlcNAc) was then coupled with di-, tri-, tetra and pentapeptides using condensation reagents in DMF. Condensation of **44** with HCl·L-Ala-D-isoGln(OBn) or HCl·L-Ala-D-isoGln-L-Lys(Z)(OBn) were effected by using WSCD, HOBt, and triethylamine to give protected tetrasaccharide containing two units of the dipeptide **69** in 82 % yield and tripeptide **70** in 53% yield, respectively (Scheme II-12).



Scheme II-12. Preparation of MGMG Sequence Fragments **2b** and **2d**.

For the condensation with longer peptides **71** and **72**, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HATU) was used as a coupling reagent. Tetrasaccharide with tetrapeptide **71** was obtained in 81% and tetrasaccharide with pentapeptide **72** in 71% yield (Scheme II-13). Isomerization of allyl group and the subsequent removal of the 1-propenyl group with I_2 and H_2O in **71** and **72** seemed to be difficult. In fact, isomerization of allyl group in complex molecules is generally not easy and the cleavage yields of 1-propenyl groups with I_2 and H_2O were moderate even in disaccharides (50-60% yields, Scheme II-11). Fortunately, previous results indicated the presence of 1-*O*-propyl glycoside did not significantly affect the immunostimulating activity (Figure II-2).[4] Cleavage of allyl group was thus

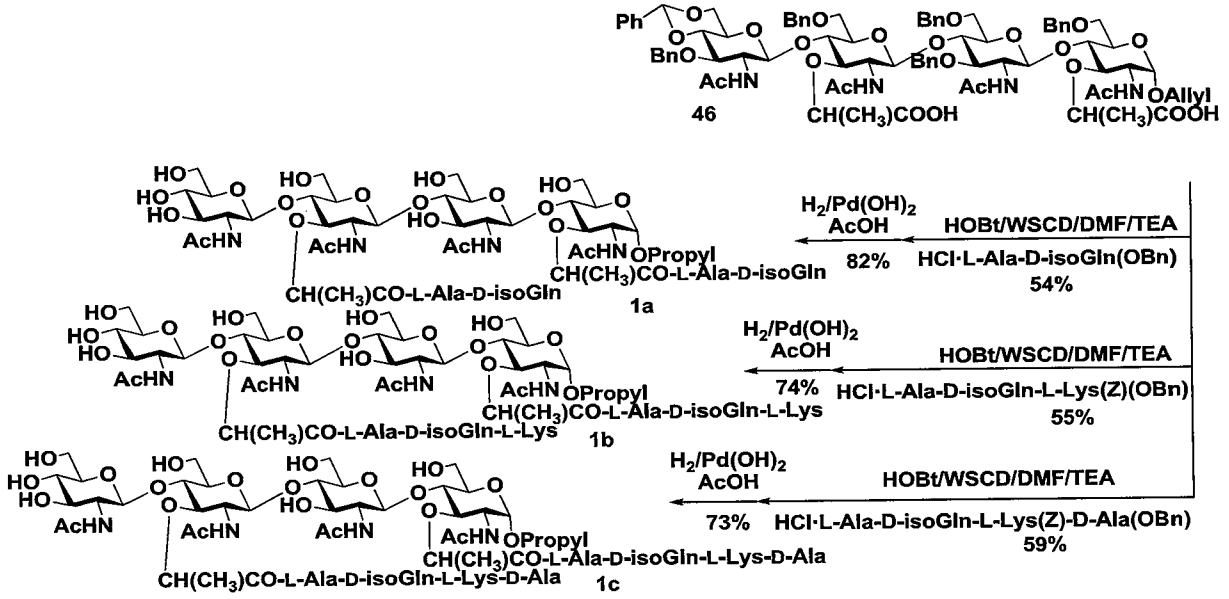
avoided. All aromatic groups were removed by catalytic hydrogenation with $Pd(OH)_2$ and H_2. Reactions were monitored by TLC and MS. Dipeptide fragment **69** and tripeptide fragment **70** were completely deprotected after 12 h, while fragments with tetrapeptide **71** and pentapeptide **72** required longer reaction time for deprotection. Frequently, benzylidene group was remained after 24 h hydrogenation for the deprotection of **71** and **72**. Thus, 10% TFA in water was used to remove the benzylidene after filtration of palladium catalyst and concentration. Purification by reverse phase chromatography using C-18 (elution with H_2O) afforded tetrasaccharide with two peptide units (**2b**, **2d**, **2f** and **2g**).



Scheme II-13. Preparation of MGMG Sequence Fragments **2f** and **2g**.

II-5-b Preparation of GMGM Sequence Fragments

PGN fragments possessing GMGM (GlcNAc-MurNAc-GlcNAc-MurNAc) sequence **1a**, **1b** and **1c** were also prepared by the similar synthetic route in reasonable yields as shown below (Scheme II-14).



Scheme II-14. Preparation of GMGM Sequence Fragments **1a**, **1b** and **1c**.

II-6 Human Nod2 Activation of Synthetic Compounds

In order to investigate the relationship between PGN structure and Nod2 activity, various partial PGN structures synthesized in the present study were tested by Dr. Inohara of the University of Michigan, Medical School. The structures of synthetic PGN fragments and their abbreviations are shown in Figure II-1. The capital letters in the abbreviations represent the monosaccharide units in glycan chain; **M** is for *N*-acetylmuramic acid and **G** is for *N*-acetylglucosamine, whereas the number in the abbreviations indicates the length of peptide. For example, MurNAc-GlcNAc with dipeptide **2a** is abbreviated as **MG2**. The Nod2 activation was assessed by NF-κB activation in HEK293T cells transfected with human Nod2. Bioactivity of 1 ng/mL of MDP was used as the standard. The results of Nod2 activation assay are listed below (Figure II-2).

The previous results were reproduced and new interesting results were also obtained in the present study. All the fragments tested showed Nod2 activation. Disaccharide with dipeptide **MG2** and **GM2** showed potent Nod2 activation equivalent to MDP (Figure II-2-a). In the previous paper, Kusumoto reported that the adjuvant activities of **MG2** and **GM2** were identical with that of MDP.[1)]

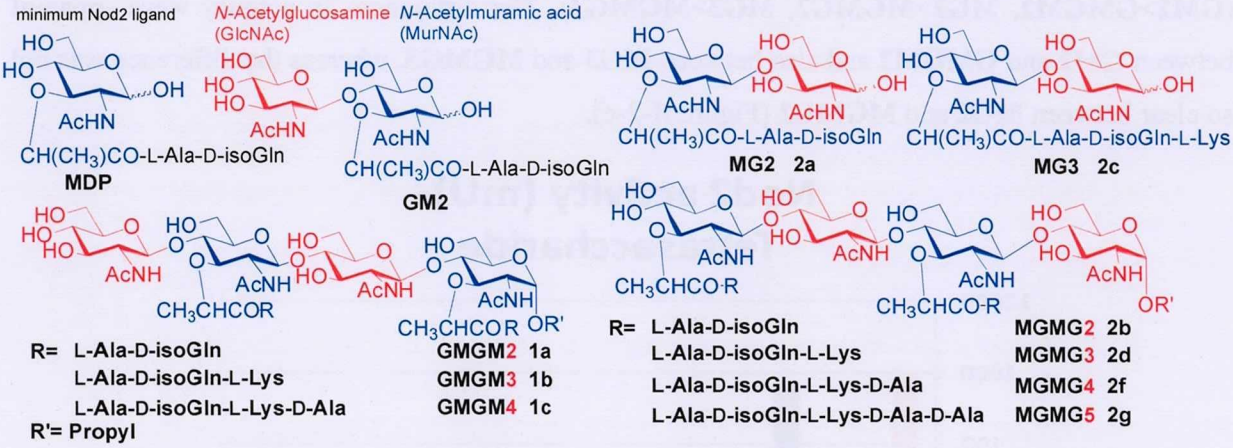


Figure II-1. Structures of all the compounds for Nod2 activition assay.

These results were confirmed at the molecular level in the present study.

Two apparent trends were observed. One trend is that the potency decreased as the molecular size increased. Among the compounds having the same glycan length with different peptides, dipeptides were most potent and the activities decreased as the peptide chain length increased (**MG2>MG3**, **GMGM2>GMGM3>GMGM4**, **MGMG2>MGMG3>MGMG4=MGMG5**) (Figure II-2-a). Our group previously showed that the GMGM sequence PGN fragments with longer peptide chain have

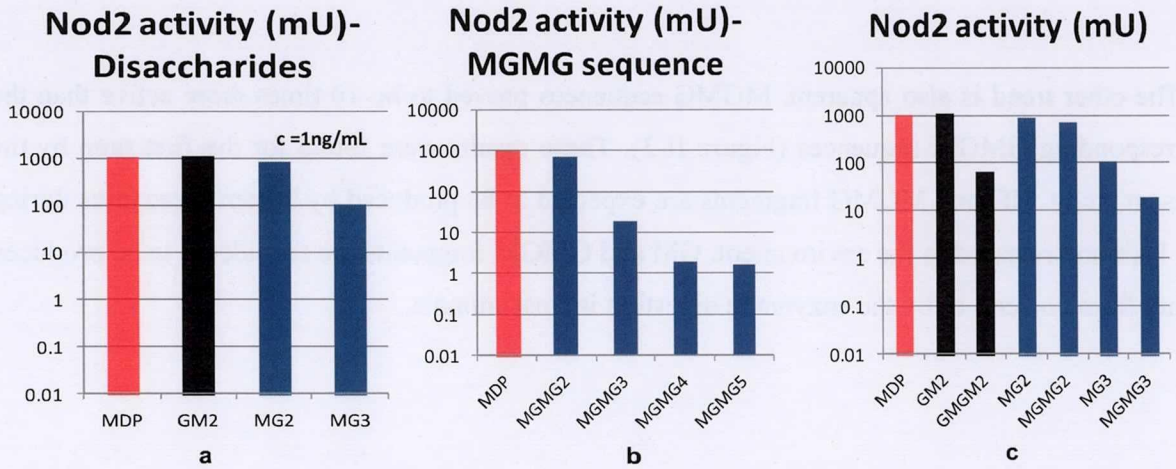


Figure II-2. Human Nod2 Activation-1. 1000 mU = bioactivity of 1ng/mL MDP.

weaker activity (Figure II-2-b). The author confirmed the previous results and also found the similar trends in MG and MGMG sequence PGN fragments.

Glycan chain length also affected the biological activity. Disaccharides with the same peptide sequence generally showed higher Nod2 activation than corresponding tetrasaccharides (**GM2>GMGM2**, **MG2>MGMG2**, **MG3>MGMG3**). The differences in potency were apparent between **GM2** and **GMGM2** and also between **MG3** and **MGMG3**, whereas the difference was not so clear between **MG2** and **MGMG2** (Figure II-2-c).

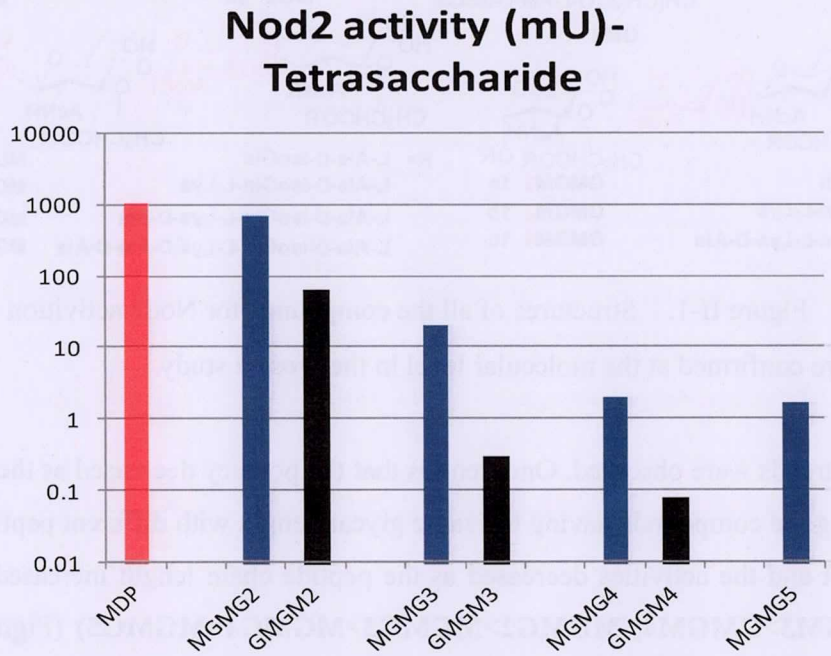


Figure II-3. Human Nod2 Activation-2. 1000 mU = bioactivity of 1ng/mL MDP.

The other trend is also apparent. MGMG sequences proved to be 10 times more active than the corresponding GMGM sequences (Figure II-3). These results were found for the first time by the present study. MG and MGMG fragments are expected to be produced by bacterial enzymes during the lysis and released to the environment. GM and GMGM fragments are considered to be produced either from bacteria or by the enzymatic digestion in host animals.

II-7 Summary

In this Chapter, the author described the synthesis of various PGN fragments, which contain alternating GlcNAc-MurNAc or MurNAc-GlcNAc sequences of disaccharides and tetrasaccharides with a series of peptide chains from di- to pentapeptide. These fragments were prepared via efficient synthetic strategy in good yields. Human Nod2 activation activities with these synthetic fragments were systematically investigated. A notable difference between MGMG and GMGM sequence PGN fragments was observed for the first time. MGMG sequence fragments showed higher activity than the corresponding GMGM fragments having the same peptides. Considering of the origins of these two sequences of PGN fragments, GM repeating units are produced by enzymatic digestion with *N*-acetylmuramidases of both bacteria and mammals, whereas MG repeating units are mainly produced by bacterial lytic enzymes *N*-acetylglucosaminidases. In host animals, bacterial cells and peptidoglycan released from dead bacteria are incorporated into the host immune cells by endocytosis and then digested with lysozyme and peptidases. Therefore, major Nod2 ligands produced by this system seem to be small fragments having GM unit. Bacterial lysis should produce both GM and MG series as well as MDP and release these small PGN fragments to the environment. These small fragments are probably incorporated into the immune cells through transporters and recognized by Nod2.

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Chapter III Synthesis of PGN Fragments with Linkers for Microarray Analysis

III-1 Introduction

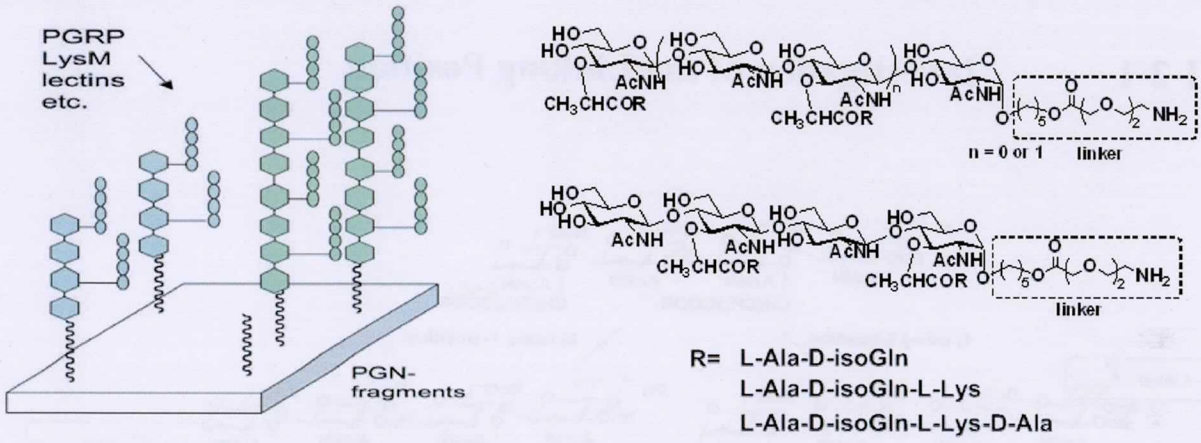


Figure III-1. Designed PGN fragments and microarray.

The author has established the synthesis of PGN fragments and applied them for the precise analysis for the recognition with Nod2 as described above. The author then planned to elucidate the PGN structures recognized by other proteins. In fact, peptidoglycans are multifunctional molecules, which interact with various proteins such as peptidoglycan recognition of proteins (PGRPs),[1] lectins, and also the proteins having LysM domain,[2] in addition to PGN receptors, Nod1 and Nod2. However, precise analysis of PGN structures responsible for the recognition and action mechanisms have not been investigated well. So far, interactions between PGN and recognition proteins have been investigated by bio-assays or SPR with proteins or cells using PGN fragments prepared by chemical synthesis or enzymatic digestion.[3]

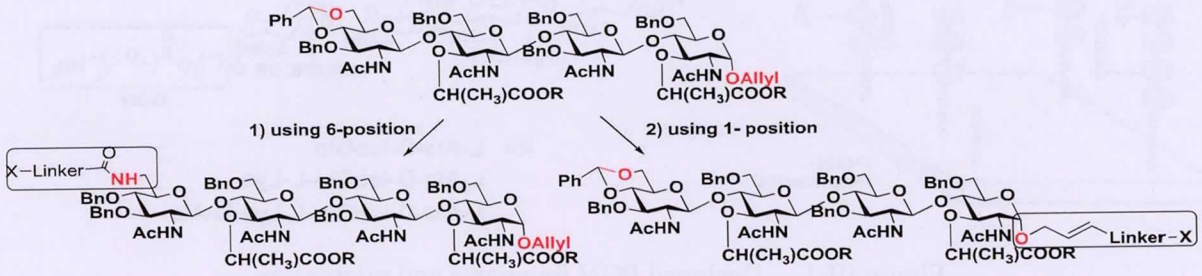
Glycan arrays have proven to be versatile for the high-throughput analysis of interactions of various glycans with a variety of targets such as proteins, antibodies, proteins, viruses and cells.[4] The author therefore developed the peptidoglycan array for the precise and comprehensive elucidation of the interactions of PGN with the recognition proteins (Figure III-1).

In order to introduce the PGN fragments to the array, several linkage structures were examined,

and finally an ester linker having terminal free amino group was successfully attached to the PGN fragments. PGN arrays were then prepared by attaching synthesized PGN fragments on the array platforms (Figure III-1). The binding assays using arrays with wheat germ agglutinin (WGA) and PGRP-S revealed the high performance of PGN arrays for the recognition studies of PGN with PGN recognition proteins.

III-2 Investigation of the Linker for Microarray Preparation

III-2-1 Investigation of the Linking Position

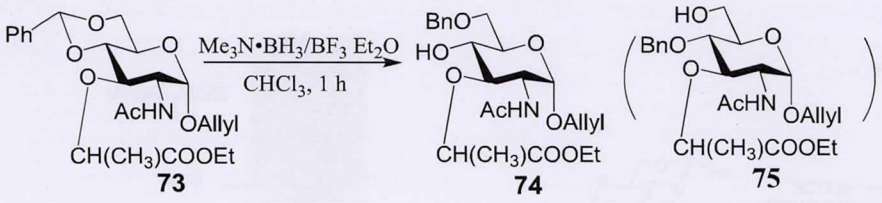


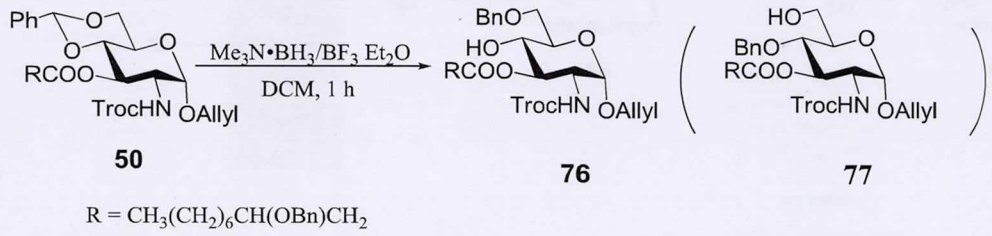
Scheme III-1. Possible synthetic route for introduction of linker to PGN fragments.

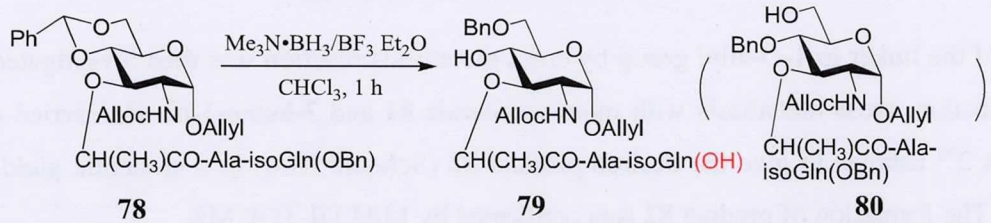
In general, appropriate linkers are necessary to introduce substrates to the arrays. In order to achieve efficient interaction between substrate and protein, selection of the types, the length, and the attachment position of the linker is critically of importance. Two positions seemed to be appropriate as the attachment position. One positions is the 6-OH in the non-reducing end of the glycan chain. The possible method for introduction of the linker is the replacement of 6-OH with amino group followed by introduction of the linker by amide formation. The other position is the glycosidic position. The 1-*O*-allyl group at the reducing end of the glycan can be used for the introduction of the linker by cross metathesis reaction (Scheme III-1). Since the trial of the introduction of amino group to the 6-OH position of the model compound was not successful, the latter method was finally employed for the microarray preparation.

The key reaction for the former route is the conversion of 6-OH to the amino group. The model monosaccharide for this trial was prepared as shown below. Several reductive opening reactions of 4,6-*O*-benzylidene group[5] in both muramic acid and glucosamine derivatives were tested in order to obtain the 4-*O*-benzylated 6-OH compounds, regioselectively. In our laboratory, it was

reported that reductive opening of 4,6-*O*-benzylidene group in 3-*O*-benzylated pyranose derivatives by using $Me_3N{\cdot}BH_3$ and $BF_3{\cdot}OEt_2$ in CH_2Cl_2 afforded 4-*O*-benzylated 6-OH compounds with high regio-selectivity. However, reaction of muramic acid derivative **73** having 3-*O*-alkoxycarbonylate by using $Me_3N{\cdot}BH_3$ and $BF_3{\cdot}OEt_2$ in $CHCl_3$ gave the undesired 4-OH compound **74**. The 3-*O*-acylated glucosamine derivative **50** and MDP derivative **78** gave similar results (Scheme III-2). In addition, benzyl ester part in **78** was reduced to the corresponding alcohol derivative **79**. Structures of all the resulted 4-OH, 6-*O*-benzylated compounds were determined by 1D and 2D NMR analysis.

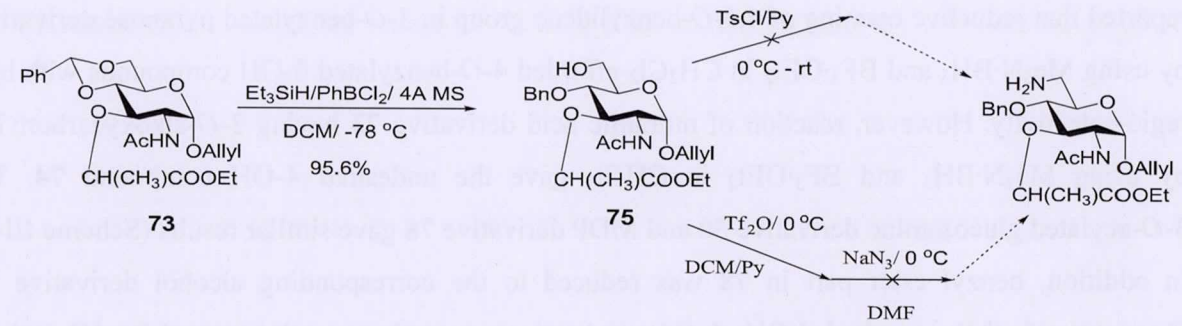




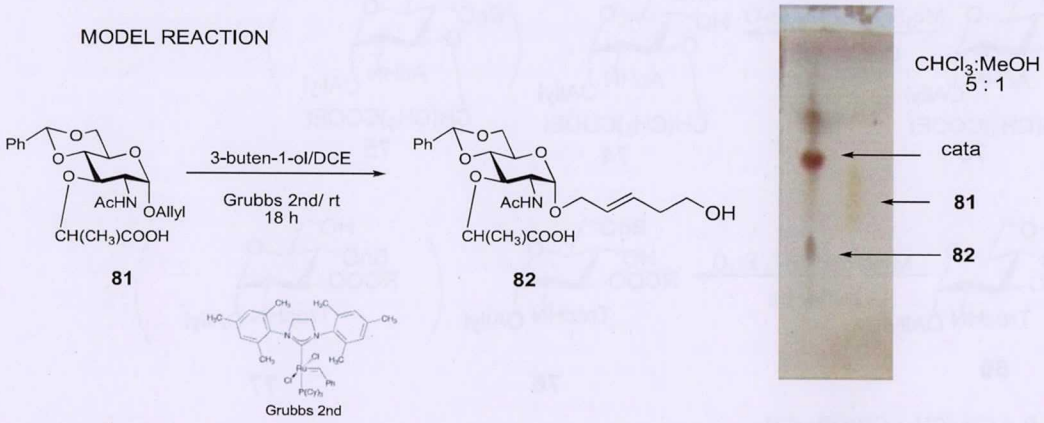


Scheme III-2. Trials of reductive opening with $Me_3N{\cdot}BH_3$ and $BF_3{\cdot}OEt_2$.

The author then found that the reductive opening of **73** with $Et_3SiH/PhBCl_2$ gave the desired 6-OH, 4-*O*-benzylated compound **75** in high yield.[6] However, introduction of Ts group to the 6-OH as well as substitution of 6-*O*-triflate with azide did not succeed (Scheme III-3). Since the latter method using the linker introduced by cross metathesis found to be successful eventually, the replacement from 6-OH to 6-NH_2 was not further elucidated.



Scheme III-3. Reductive opening of monosaccharide **73** and the subsequent trial.



Scheme III-4. Model reaction of cross metathesis.

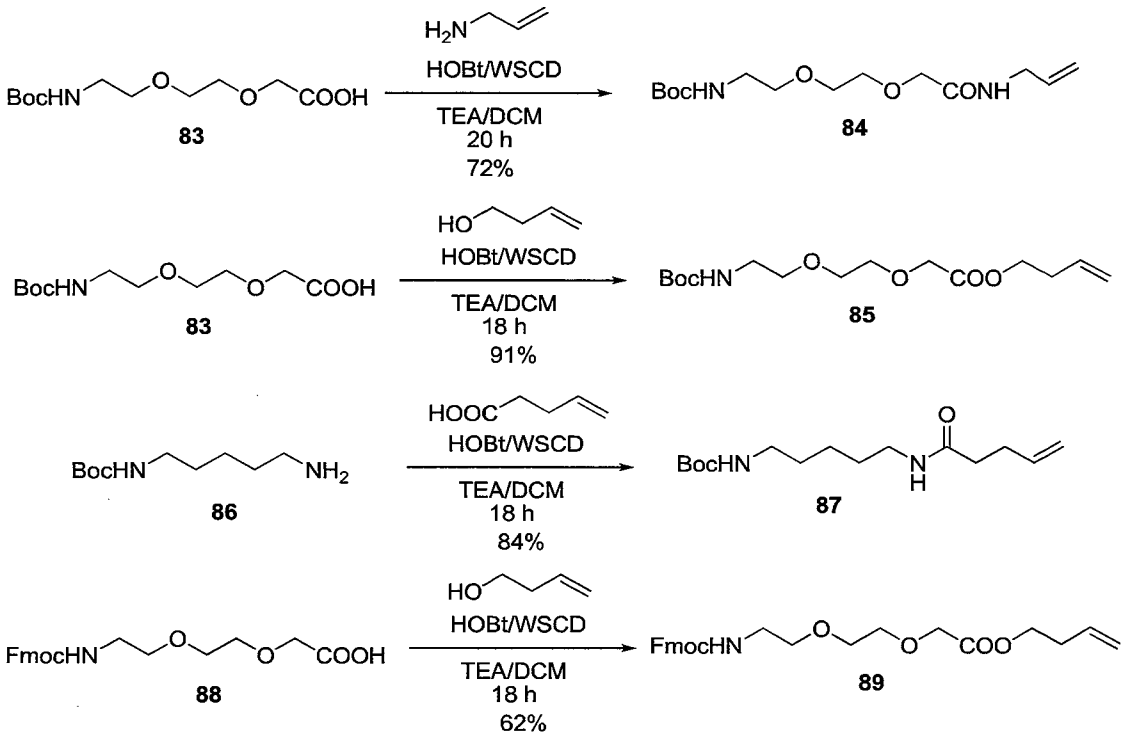
Introduction of the linker to 1-*O*-allyl group by cross metathesis reaction was then investigated.[7] As the model reaction, cross metathesis with monosaccharide **81** and 3-buten-1-ol was carried out by using Grubbs 2nd catalyst to give the desired product **82** (Scheme III-4) in a moderate yield as judged by TLC. The formation of product **82** was confirmed by MALDI-TOF MS.

The author then turned to other substrates containing longer saccharide units and modified the reaction conditons, which are described in the Section 4 in this chapter.

III-2-2 Preparation of the Linkers

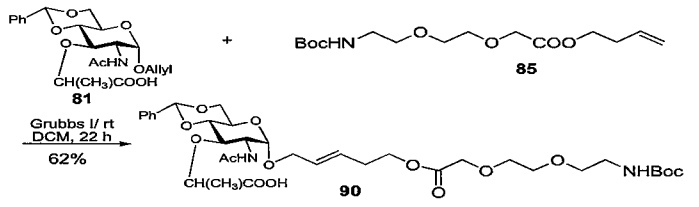
Several linkers tested for the connection of PGN fragments to the array plate were prepared as shown in Scheme III-5. Linkers having ester (**85**) and amide (**84**) linkage were prepared by the condensation of commercial available carboxylic acid **83** possessing ethylene glycol unit with corresponding allylamine and homoallylalcohol, respectively. Linker **87** was prepared from

N-Boc-1,5-pentanediamine **86** and 4-pentenoic acid. Fmoc protected linker **89** was also synthesized in a manner similar to the preparation of **85**.



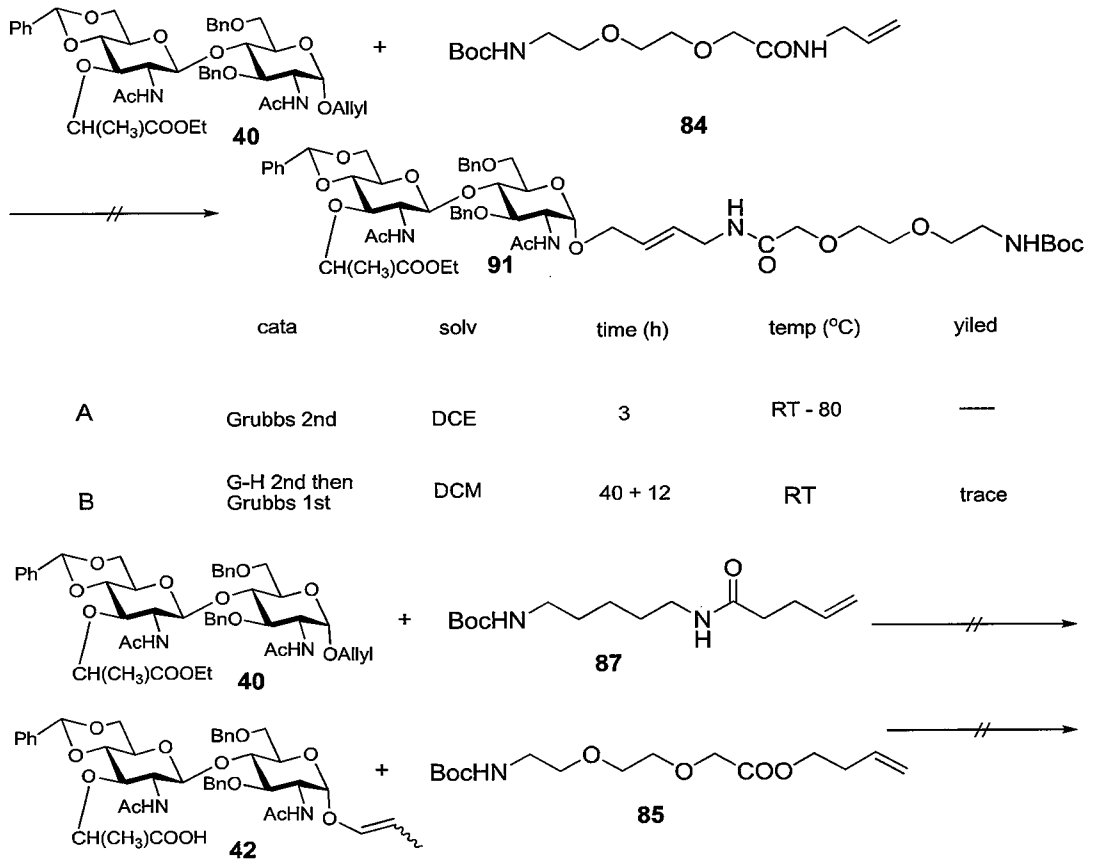
Scheme III-5. Preparation of linkers for connection of PGN fragments to array.

III-2-3 Cross Metathesis of PGN Fragments and Linkers



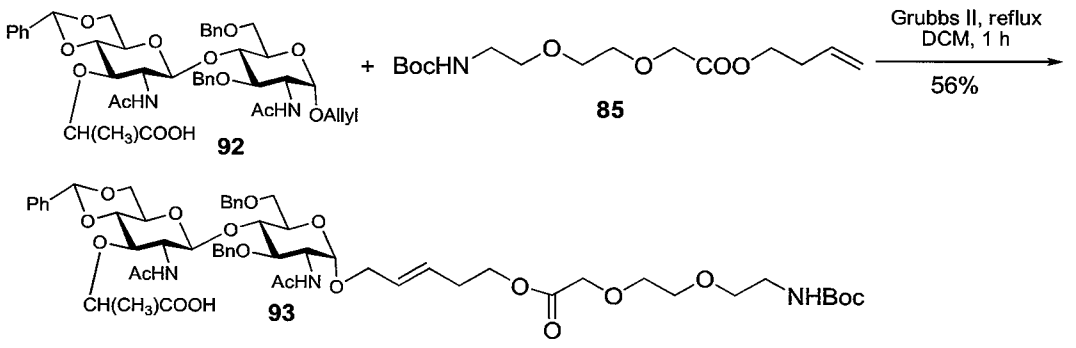
Scheme III-6. Preparation of monosaccharide with linker **90**.

Connection of the linker to PGN fragments was then examined. As shown in Scheme III-6, linker **85** was introduced to MurNAc derivative **81** by cross metathesis using Grubbs 1st generation catalyst. After the reaction, the reaction mixture was concentrated and directly purified by flash silica-chromatography to avoid the regeneration of starting compounds. The desired **90** was thus obtained in 62% yield.



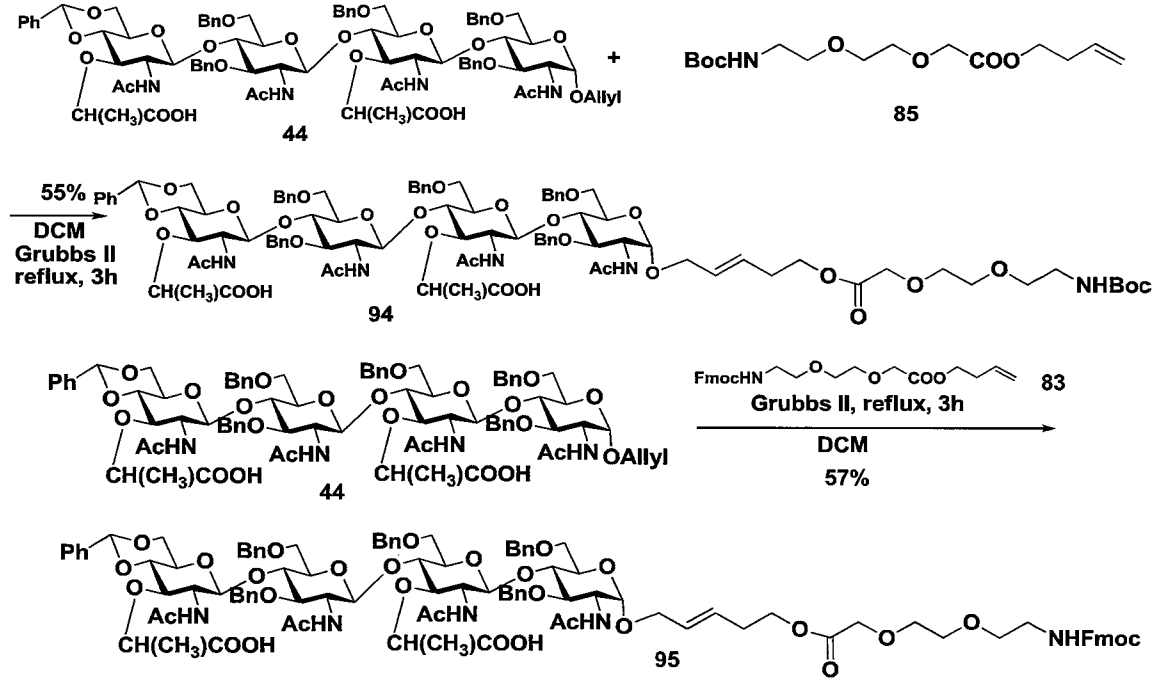
Scheme III-7. Trials of cross metathesis of disaccharides with linkers.

Connection of the linkers to the disaccharides was then investigated (Scheme III-7 and III-8). An intermediate **40** (MurNAc-GlcNAc without peptide) was first tested. Since ethyl ester in **40** should be removed by base for further condensation with peptide part, the base-stable amide linker **84** and **87** were used for the reaction. However, the desired cross metathesis didn't proceed or scarcely proceeded, even after extensive investigations in types and amounts of Grubbs' catalysts, solvents, reaction time, temperature (Scheme III-7). On the other hand, the ester-type linker **85** was also found to be useful for the cross metathesis with the disaccharide **92**. In this case, Grubbs' 2nd generation catalyst gave a good result. The reaction of **92** and **85** using Grubbs' 2nd generation catalyst was carried out in 1 h at rerflux to give the desired coupling product **93** in a moderate yield (Scheme III-8). Cross metathesis of the vinyl intermediate **42** with **85** was failed because of the low reactivity of vinyl group (Scheme III-7).

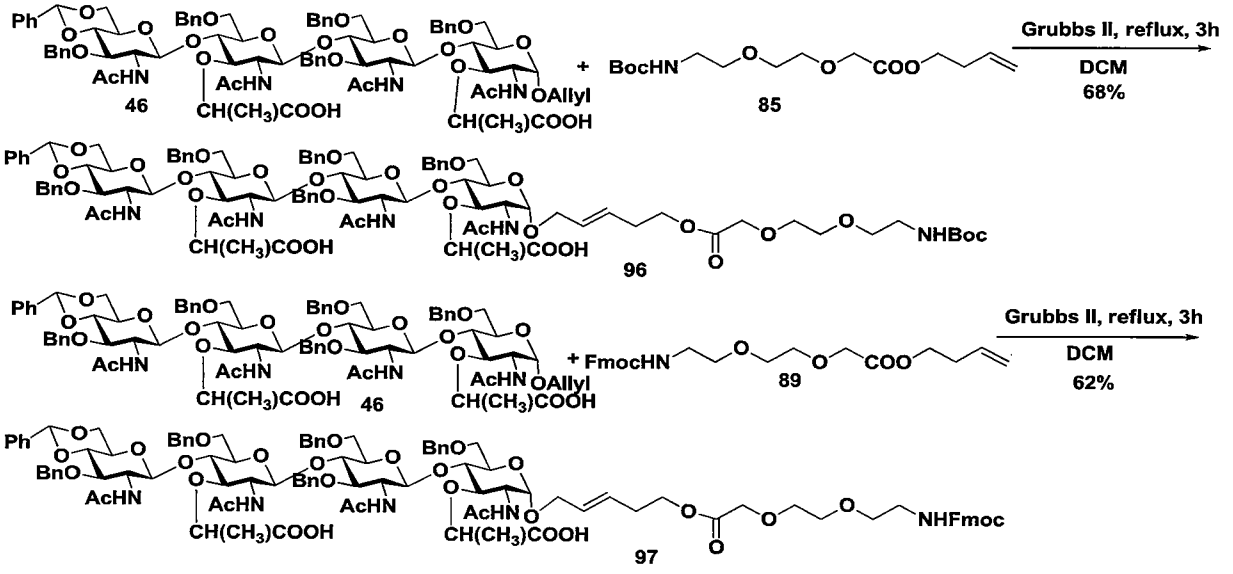


Scheme III-8. Cross metathesis of disaccharide **92** with linker **93**.

The cross metathesis reactions of the ester-type linker **85** (Boc protected) and **93** (Fmoc protected) with the MGMG-type tetrasaccharide **44** and GMGM-type tetrasaccharide **46** were then effected by using Grubbs' 2nd generation catalyst in DCM at reflux for 3 h (Scheme III-9 and Scheme III-10). The reactions proceeded smoothly to give the desired products **94-97** in satisfactory yields. Fmoc-protected linker **93** was used for the preparation of PGN fragments having tri and tetrapeptides, since the side-chain amino group of Lys was protected by Boc group. In order to promote the cross metathesis, 4 equivalents of the linker was used against the allyl glycosides. The procedure of the metathesis was also important; the catalyst was added after all reagents were added and stirred in DCM at reflux under Ar atmosphere to give the desired product in better yields.



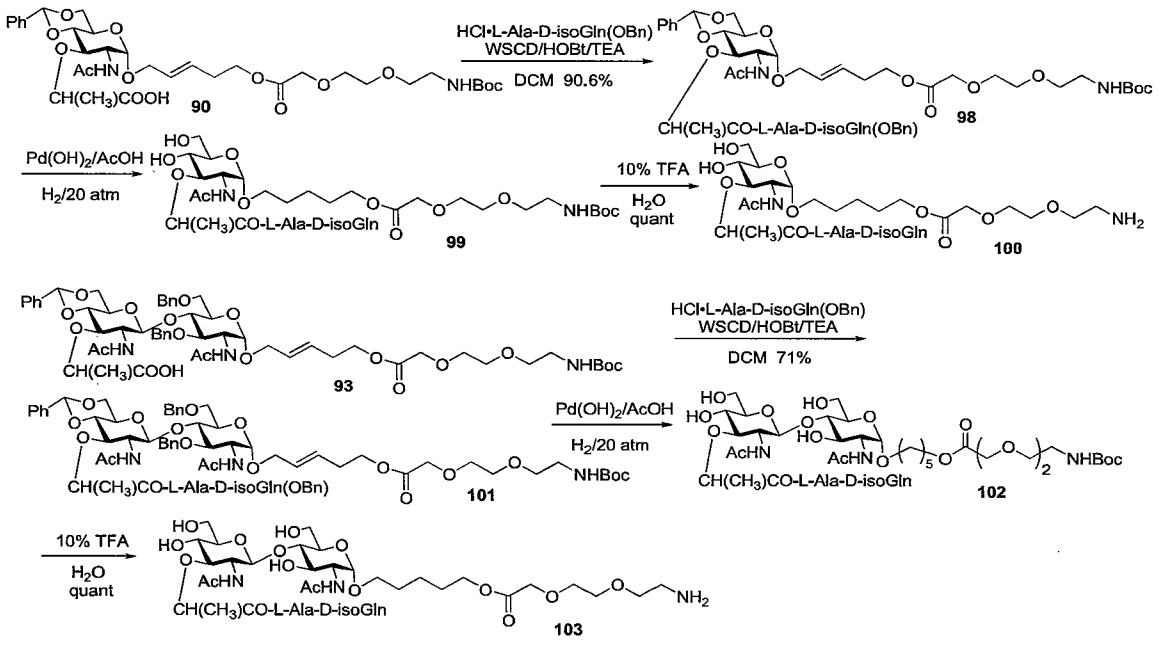
Scheme III-9. Synthesis of MGMG type PGN glycan with Linkers.



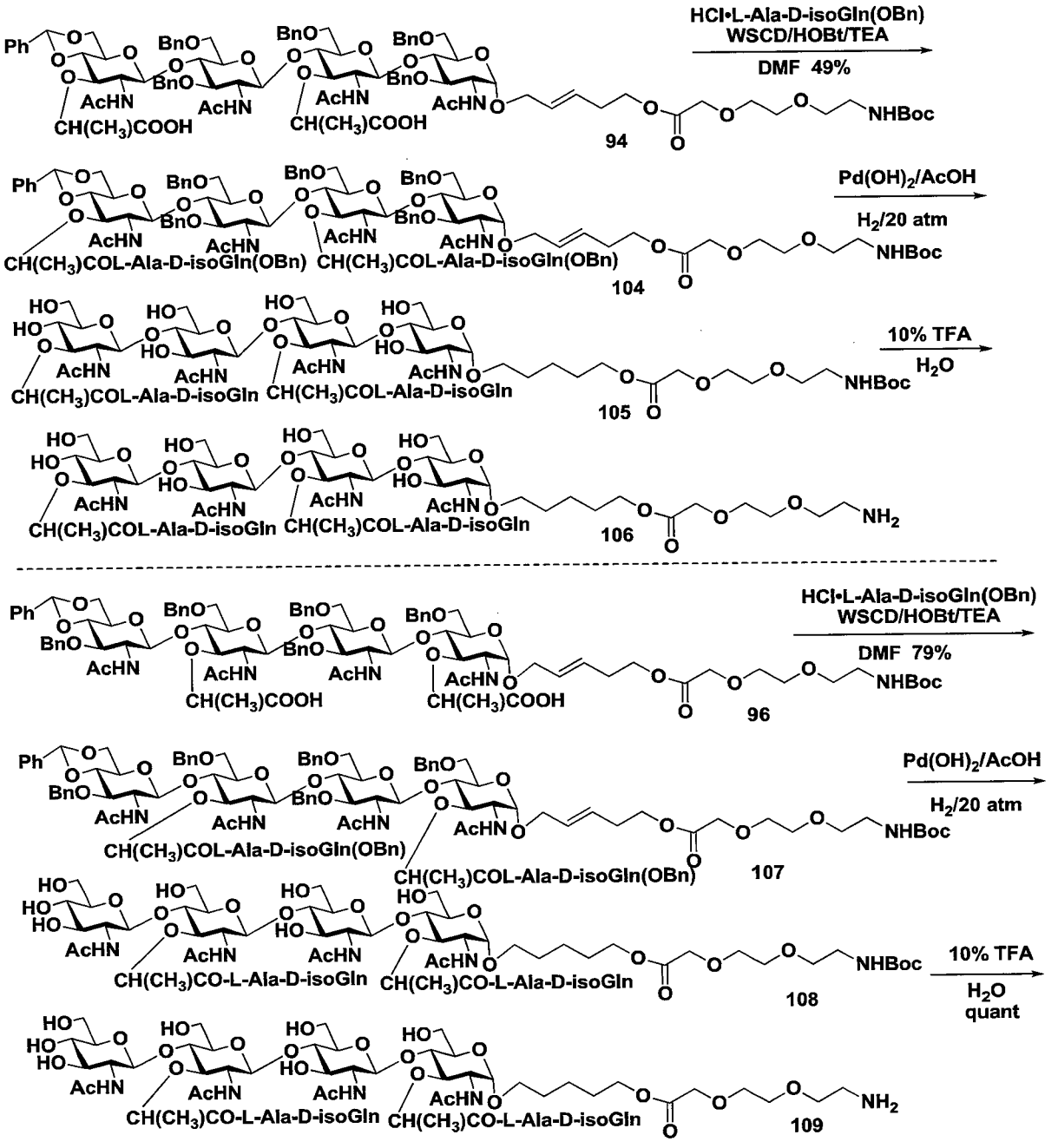
Scheme III-10. Synthesis of GMGM type PGN glycan with Linkers.

III-3 Synthesis of PGN Fragments with Linker

Peptide moieties (di-, tri and tetrapeptides) were then introduced to the carboxyl group(s) at the MurNAc in glycan parts with linker by using WSCD·HCl and HOBt in DCM or DMF. Coupling reactions between the mono, di, and tetrasaccharides with dipeptide proceeded smoothly to give glycan peptide conjugates **98**, **101**, **104** and **107** in satisfactory yields, respectively (Scheme III-11 and Scheme III-12). All the products were purified by silica-gel column chromatography (mobile phase : chloroform/methanol). Catalytic hydrogenolysis was carried out for the deprotection of all the aromatic protecting groups in acetic acid by using palladium hydroxide on carbon. Boc group was then removed with 10% TFA in water to give compound **100**, **103**, **106** and **109** having the free amino group at the terminal of the linker, which were purified by reversed-phase C-18 column and then lyophilized with water. Since Rf values of the Boc-protected compounds with corresponding deprotected compounds **100**, **103**, **106** and **109** in TLC were similar (mobile phase : BuOH : AcOH : H_2O = 2 : 1 : 1, Rf : in the range of 0.3 to 0.6), the reaction was monitored by MALDI-TOF MS.



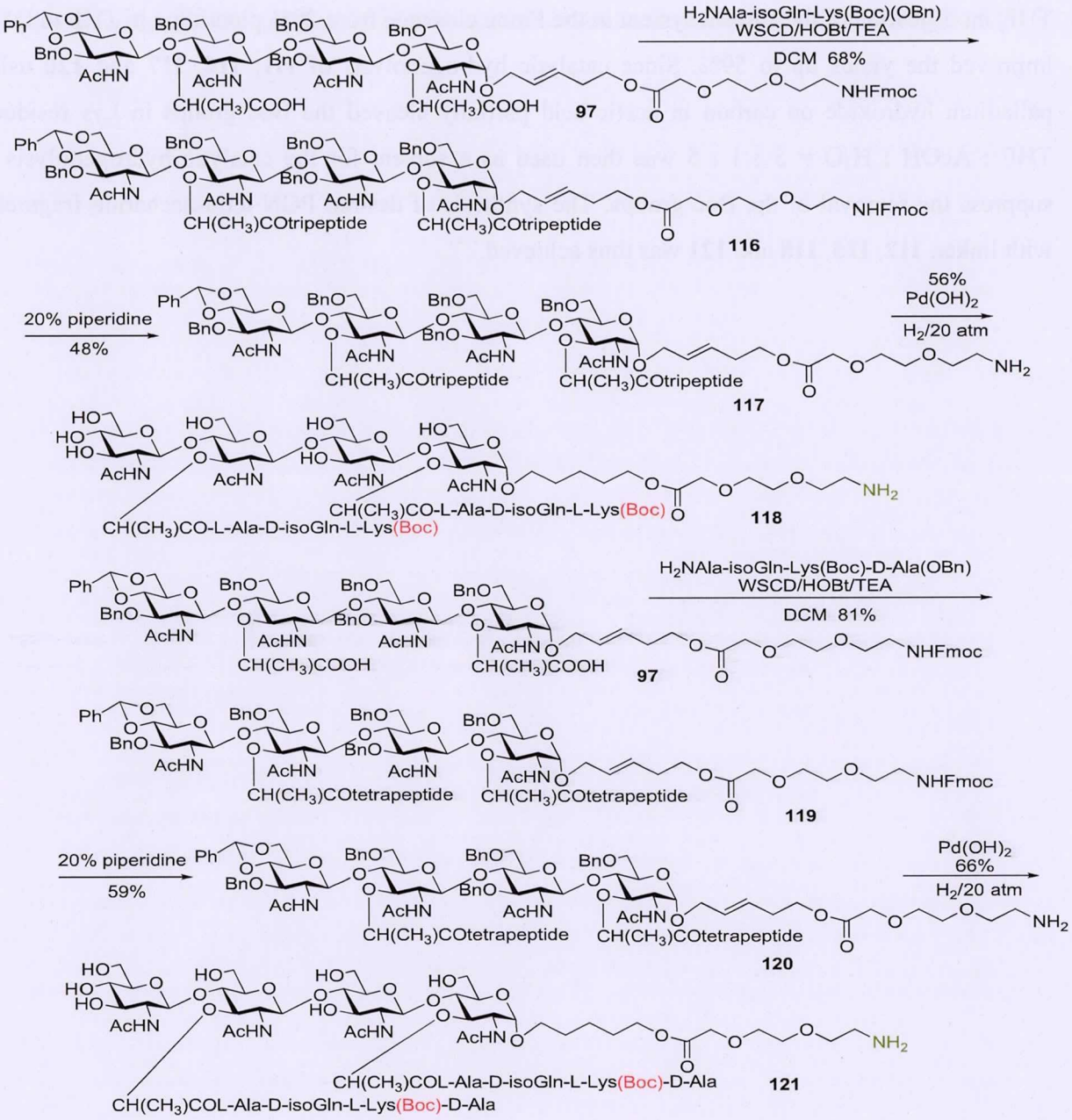
Scheme III-11. Synthesis of mono and disaccharide dipeptide with linker **100** and **103**.



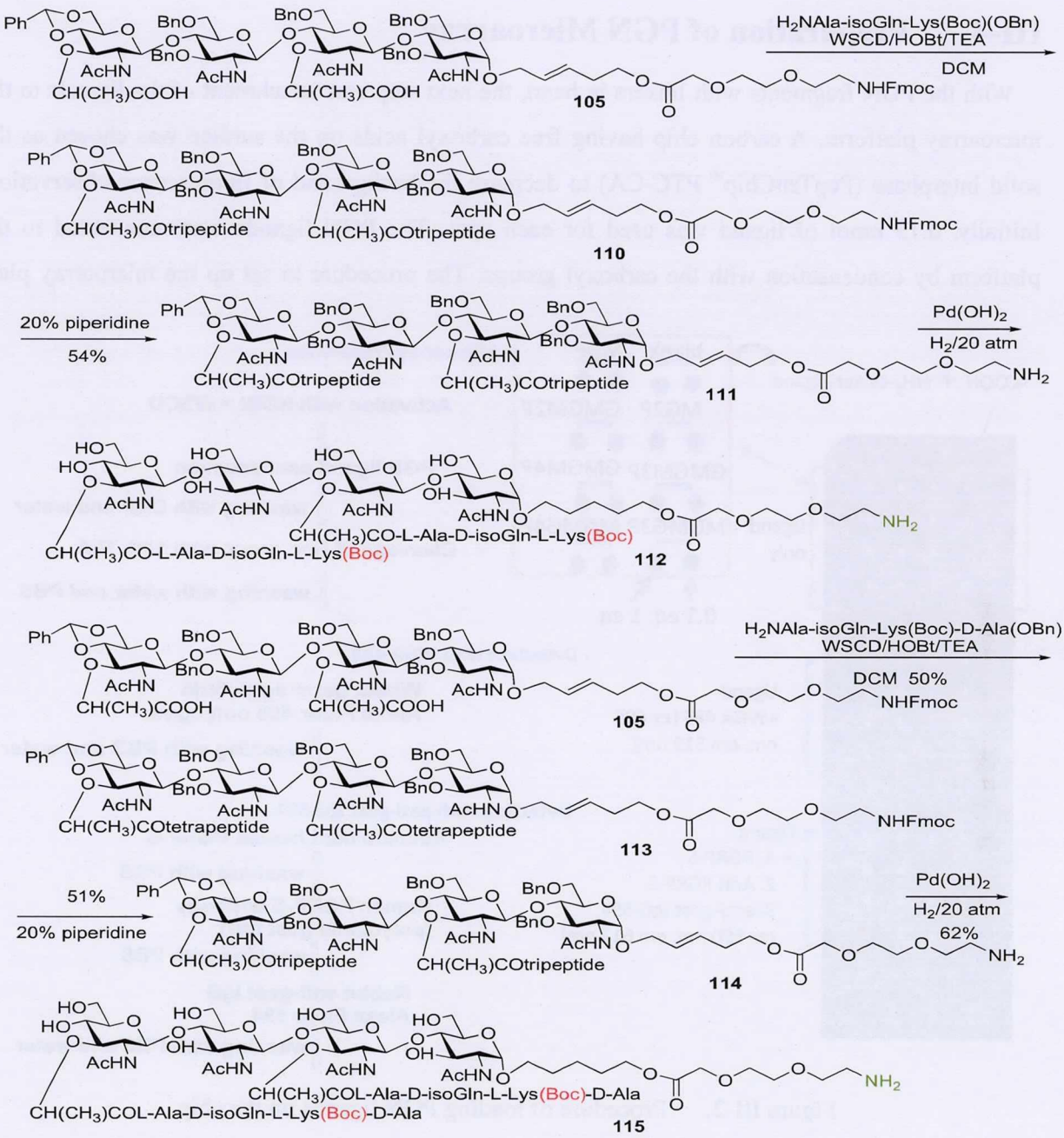
Scheme III-12. Synthesis of tetrasaccharide dipeptide with linker **106** and **109**.

Coupling reactions between the tetrasaccharides with Fmoc linker **95** and **97** and tripeptide or tetrapeptide containing Lys(Boc) residue proceeded smoothly by using WSCD·HCl and HOBt in DCM to give glycan peptide conjugates **110** and **116** respectively (Scheme III-13, Scheme III-14). Removal of the Fmoc group in the linker with 20% piperidine in DMF gave free amines **111** and **117** in moderate yields. Since ester group in the linker was partially cleaved in 20% piperidine in

THF, modification of the solvent system in the Fmoc cleavage from 20% piperidine in THF to DMF improved the yields up to 59%. Since catalytic hydrogenolysis of **111**, **114**, **117** and **120** using palladium hydroxide on carbon in acetic acid partially cleaved the Boc groups in Lys residues, THF : AcOH : H_2O = 5 : 1 : 5 was then used as a solvent for the catalytic hydrogenolysis to suppress the removal of the Boc groups. The synthesis of desired PGN tetrasaccharide fragments with linker, **112**, **115**, **118** and **121** was thus achieved.



Scheme III-14. Synthesis of PGN fragments with GMGM sequence and linker **118** and **121**.



Scheme III-13. Synthesis of PGN fragments with MGMG sequence and linker **112** and **115**.

III-4 Preparation of PGN Microarray

With the PGN fragments with linkers in hand, the next step was attachment of the ligands to the microarray platform. A carbon chip having free carboxyl acids on the surface was chosen as the solid interphase (PepTenChip® PTC-CA) to decrease the background of fluorescence observation. Initially, 0.13 nmol of ligand was used for each spot. The PGN ligands were connected to the platform by condensation with the carboxyl groups. The procedure to set up the microarray plate

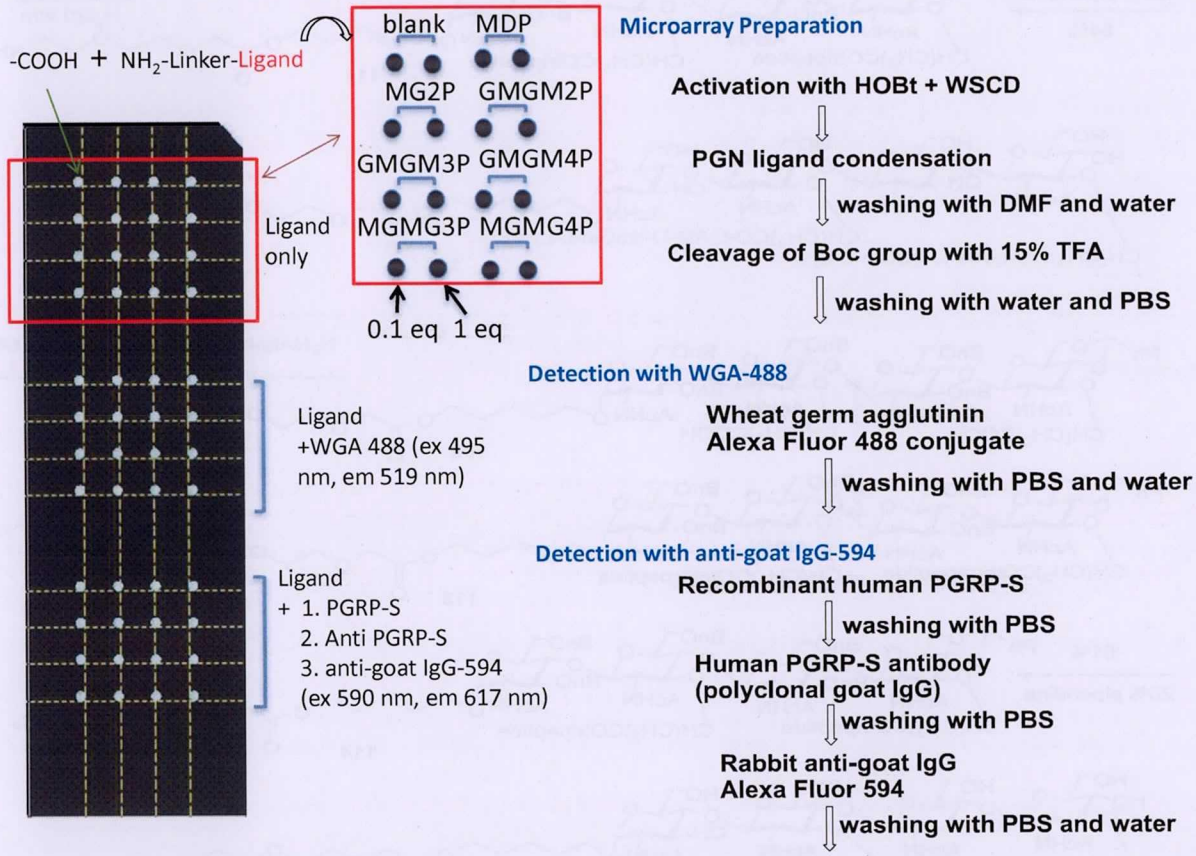


Figure III-2. Procedure of loading PGN ligands on the chip.

was performed as shown in Figure III-2. A solution of condensation reagents HOBt and WSCD in DMF was firstly loaded to the spots on the plate and the plate was shaken for 1 h. After washing by DMF, PGN ligands were then loaded to the spots with different concentrations, respectively. After 1 h, the plate was washed five times with DMF followed by washing with water. Then, 15% TFA solution in water was put on the spots and the plate was shaken for 20 min to cleave the Boc protection on the L-Lys in some of the ligands. TFA was then removed and the plate was soaked in PBS. PGN microarray was thus prepared and used for binding assay with proteins.

III-5 Studies of PGN-Protein Interactions Using PGN Microarray

As the first trial, two series of proteins, wheat germ agglutinin (WGA: lectin, binding with GlcNAc containing glycans) and human PGRP-S (proved to recognize tetrasaccharide with tri and tetrapeptide), were used. PGRP-S was loaded at the bottom area of the plate, followed by anti-PGRP-S (Goat) and anti-Goat IgG-594. Afterward, WGA-488 was loaded in the middle area of the plate. Final wash by PBS and water completed the loading of ligands on the plate. The plate was then kept in dark and used for the fluorescence measurement.

Fluorescence detection was performed using two range of light wavelength. Length from 473 nm to 575 nm was used to observe WGA-488, and length from 532 nm to 585 nm was set for the anti-Goat IgG-594. The result of the scan was shown in Figure III-3. In both cases, the top area contained only ligands did not show obvious fluorescence, which was considered as background. On the other hand, the blank spots where PGN ligands were not loaded showed the weak fluorescence in each area, indicating the existence of non-specific binding between proteins with the

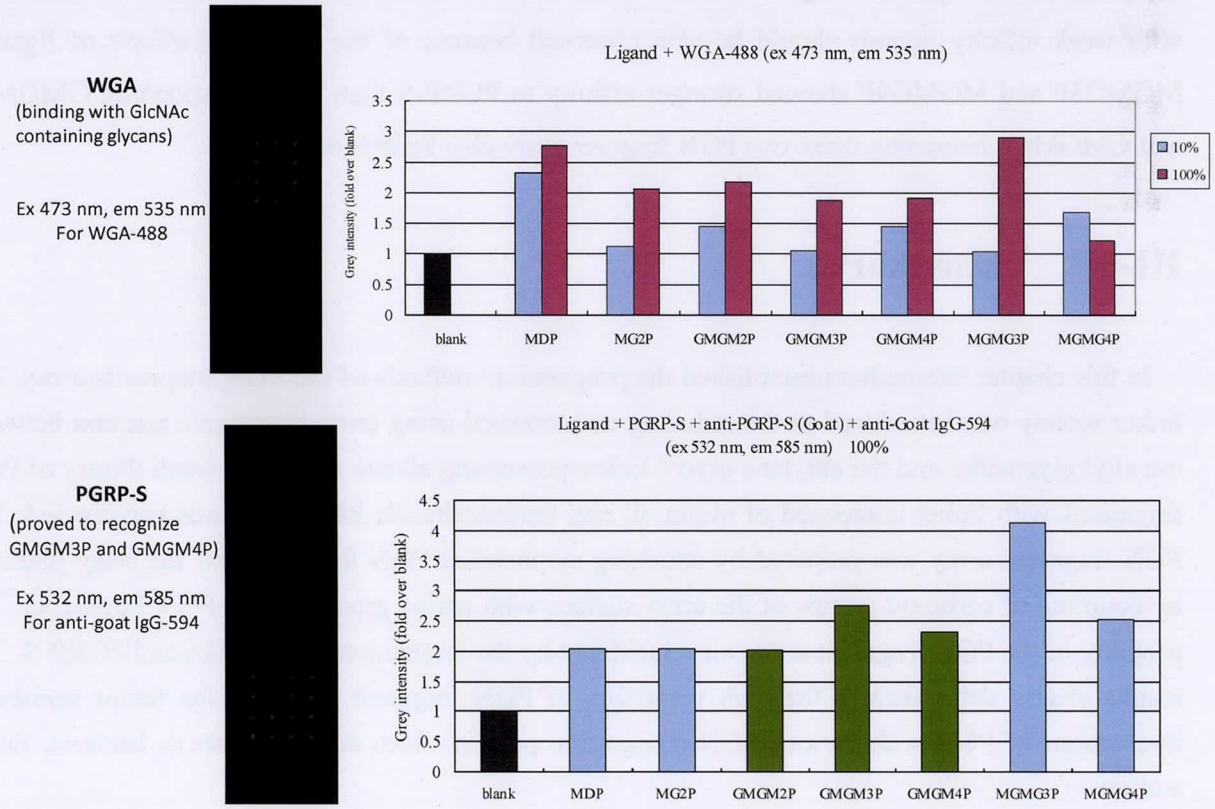


Figure III-3. Fluorescence scan of microarray using two wavelength.

carboxyl groups on the plate surface. The specific bindings between ligands and proteins were still remarkable. WGA bound to all peptidoglycan partial structures and the binding affinity varied depending on the ligands (Fig. III-3). In general, 100% loading spots showed stronger affinity to WGA than corresponding 10% loading spots. Biding of PGRP-S to the array was assessed using 100% loading spots. PGRP-S also bound to all PGN fragments and MGMG3P showed strongest binding with PGRP-S.

Ezekowitz et al. previously reported the biding property of PGRP-S to PGN by using our synthetic GM series PGN partial structures, i.e., GMGM3P, GMGM4P, GMGM2P, GM2P, M3P, and MDP. Their SPR study using PGRP-S loaded chip clearly indicated that PGRP-S bound to GMGM3P and GMGM4P but did not to GMGM2P, GM2P, M3P, and MDP. GMGM3P showed the stronger affinity than GMGM4P.[3] From the microarray analysis in this study, the author observed that PGRP-S bound to both GMGM3P and GMGM4P, and GMGM3P showed stronger affinity. The recognition of PGRP-S to these fragments was thus reproducible in microarray analysis. On the other hand, bindings to MDP and GMGM2P were also observed in microarray analysis with certain affinity. In this study, PGN fragments were introduced to the solid-surface and therefore interactions with weak affinity ligands should be also observed because of the clustering effects of ligands. MGMG3P and MGMG4P showed stronger affinity to PGRP-S than the corresponding GMGM3P and GMGM4P, indicating these two PGN fragments are also ligands of PGRP-S.

III-6 Summary

In this chapter, the authors established the preparation methods of the PGN fragments array. The linker moiety was introduced to the reducing end terminal using cross metathesis reaction between the allyl glycosides and the ethylene glycol linker possessing alkene moiety. A small library of PGN fragments with linker composed of mono, di and tetrasaccharide backbones was constructed. The PGN fragment array was prepared by attaching synthesized PGN fragments on the array platform by coupling of carboxyl groups of the array surface with amine groups of the PGN fragments. The property of the PGN fragment array was elucidated by the interactions with WGA and PGRP-S. The results clearly demonstrated the high versatility of PGN fragment array for the future studies of interactions of PGN with various PGN recognition proteins from animals, insects, bacteria, fungi, and etc.

References

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Chapter IV Synthesis of Tetrasaccharide and Octasaccharide Fragments for Binding Studies with LysM

IV-1 Introduction

LysM domain containing proteins are known as the most conserved carbohydrate binding modules. LysM proteins are found in bacteriophages, bacteria, and eukaryotes such as fungi, plants, and mammals. LysM domains recognize a wide range of polysaccharides containing *N*-acetylglucosamine (GlcNAc) residues such as PGN and chitin.

Enterococcus faecalis produces three LysM proteins, AtlA, AtlB, and AtlC. AtlA is an *N*-acetylglucosaminidase, whereas AtlB and AtlC are *N*-acetylmuramidase.[2] In order to investigate the precise recognition of LysM domains of AtlB and AtlC with PGN, the author studied the synthesis of PGN fragments from *E. faecalis*.

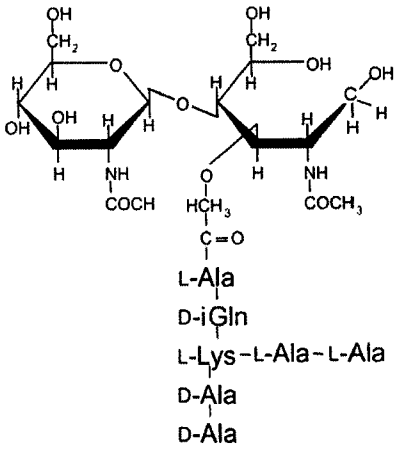
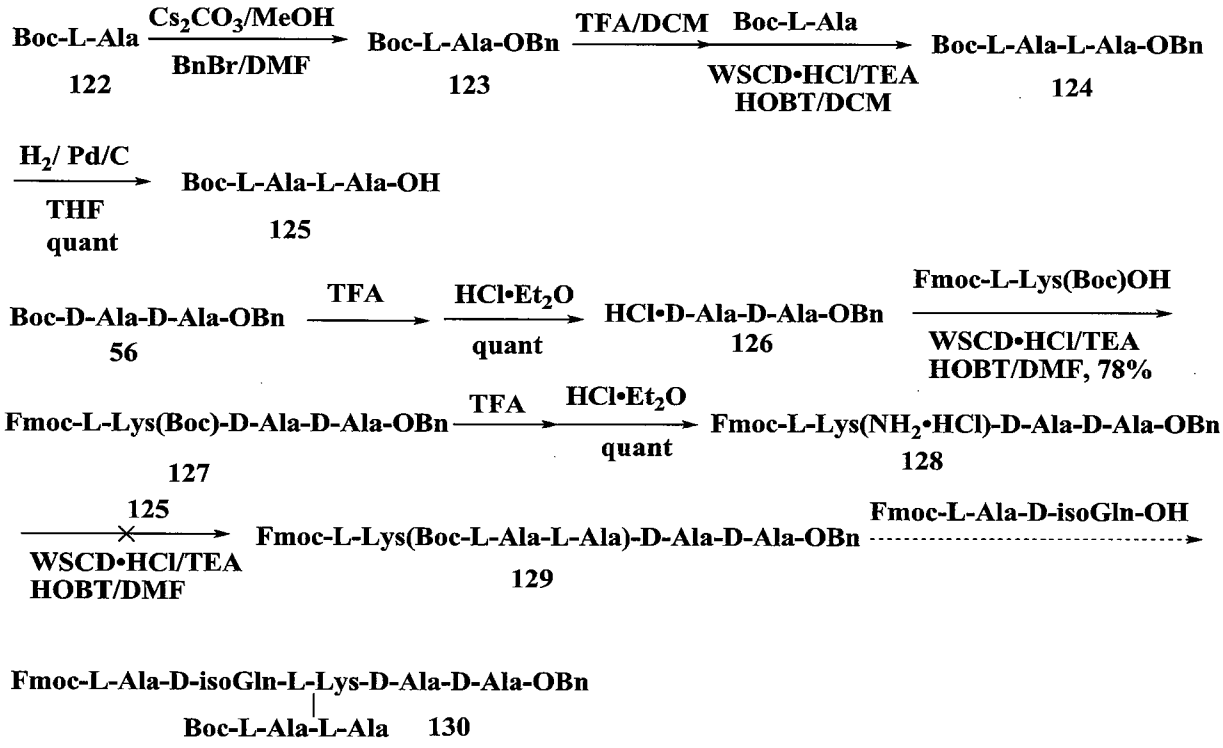


Figure IV-1. Structure of a major fragment obtained from *Enterococcus faecalis* PGN.

Structure of a disaccharide fragment obtained by AtlB digestion of *E. faecalis* PGN followed by reduction with sodium borohydride is shown in Figure IV-1.[1] This fragment has a particular heptapeptide structure, in which L-Ala-L-Ala dipeptide attaches to the amino group of L-Lys in the typical L-Ala-iso-D-Gln-L-Lys-D-Ala-D-Ala pentapeptide stem. In *E. faecalis* PGN, a stem peptide, L-Ala-D-iGln-L-Lys(L-Ala-L-Ala)-D-Ala moiety is linked to the amino group of L-Ala-L-Ala part to form network structure. The author therefore focused on the synthesis of PGN partial structures containing the heptapeptide. Since longer glycan chains might lead to stronger binding, the PGN fragments with tetrasaccharide and octasaccharide backbones were thus selected as the synthetic targets. At the same time, tetrasaccharide and octasaccharide without peptide were also synthesized for comparison.

IV-2 Synthesis of Heptapeptide

The heptapeptide part of the repeating unit structure of *E. faecalis* was firstly synthesized as shown below.

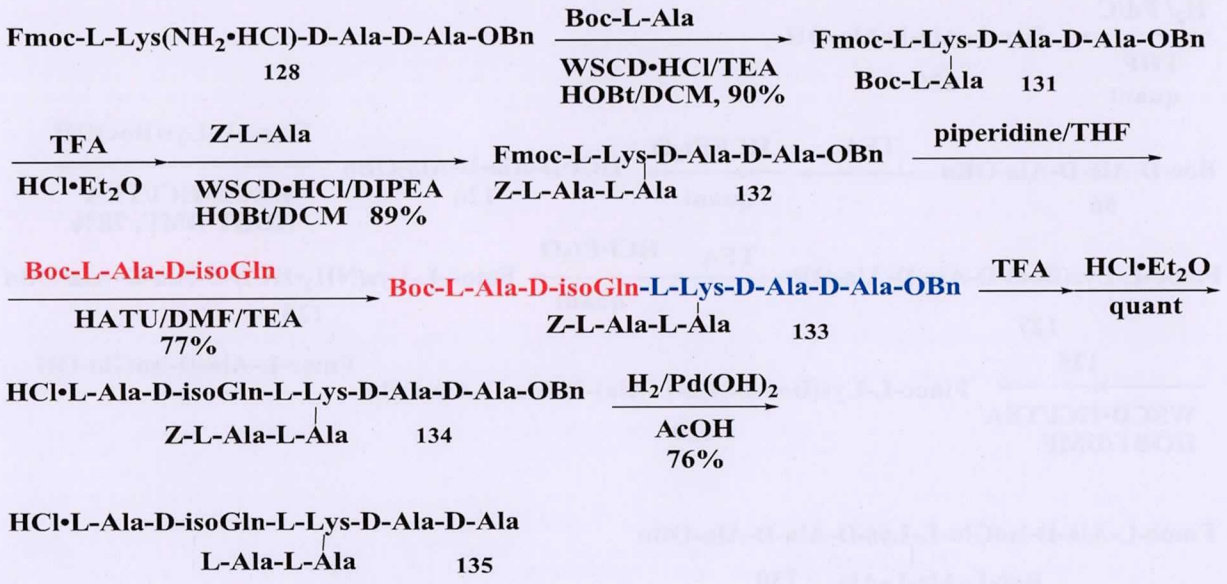


Scheme IV-1. Initial synthetic route for heptapiptide **130**.

Initial plan for the synthesis of the heptapeptide is shown in Scheme IV-1. The branch dipeptide **124** (Boc-L-Ala-L-Ala-OBn) was synthesized by the coupling of Boc-L-Ala-OH **122** with H-L-Ala-OBn. Cleavage of the benzyl ester in **124** gave **125**, which is used as the branch peptide in the heptapeptide for later condensation. Tripeptide **127** was prepared from dipeptide **126** and commercial available Fmoc-L-Lys(Boc). The side chain Boc group in **127** was then removed by TFA and the resulting TFA salt was changed to hydrochloride **128**. However, condensation of tripeptide **128** and dipeptide **125** did not proceed (Scheme IV-1). In addition, C-terminal Ala residue in dipeptide might be racemized during the coupling in this route. The next route was then investigated as shown in Scheme IV-2.

Condensation of Boc-L-Ala-OH with the tripeptide **128** was carried out by using WSCD•HCl and HOBt to give tetrasaccharide **131**. The second L-Ala in the L-Lys branch was introduced by the coupling with Z-L-Ala-OH to form pentapeptide **132**. The resulting **132** was hardly soluble in water and organic solvents, such as DCM, $CHCl_3$, and EtOAc. Fmoc group of **132** was then removed by treatment with piperidine in THF and the resulting pentapeptide amine was coupled with the dipeptide (Boc-L-Ala-D-isoGln-OH) using HATU to give the protected heptapeptide **133** in 77% yield. Although the solubility of **133** in organic solvents was lower than **132**, purification with

silica-gel column chromatography using $CHCl_3$-MeOH as an eluent was possible. Cleavage of Boc group of **133** and treatment with $HCl \cdot Et_2O$ afforded hydrochloride salt of heptapeptide **134**, which was used for further condensation with glycan parts. The fully deprotected heptapeptide **135** was



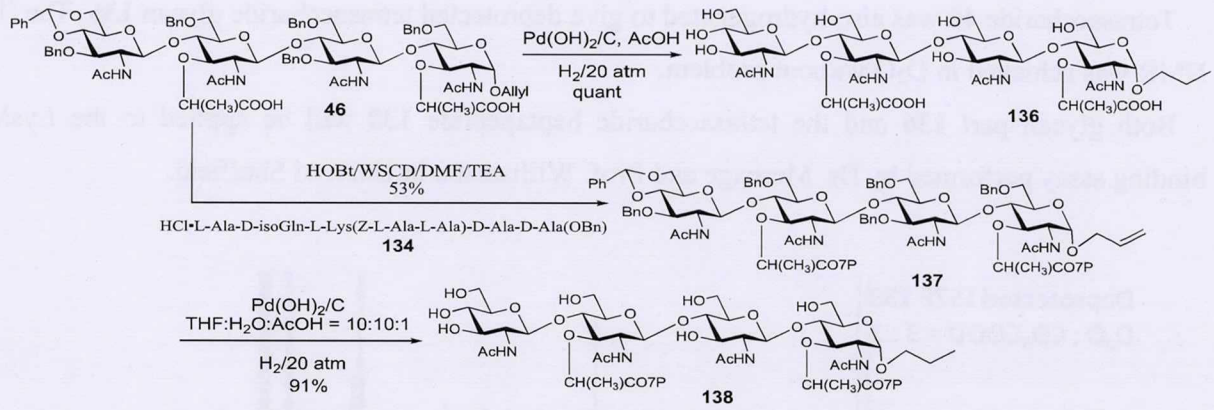
Scheme IV-2. Synthesis of heptapiptide part.

obtained by catalytic hydrogenation of **134**.

IV-3 Synthesis of Tetrasaccharide with Heptapeptide from *E. faecalis*

Synthesis of the tetrasaccharide heptapeptide **138** (TS7P) is shown in (Scheme IV-3). The tetrasaccharide intermediate **46** and a hydrochloride salt of heptapeptide **134** were coupled by using WSCD and HOBt or HATU in DMF to give **137**. Both HOBt/WSCD and HATU used as the condensation reagents gave similar results.

The structure of **137** was confirmed as follows. MALDI-TOF MS of **137** clearly showed the $[M+Na]^+$ peak at 3246.5. ^1H NMR spectrum of **137** in $CDCl_3$ gave the broad signals in the range of 3 to 5.5 ppm corresponding to the carbohydrate signals, whereas relatively sharp signals were observed in 0.7-2.3 ppm as peptide side-chain signals (Figure IV-2). ^1H NMR of **137** in $CD_3OD/CDCl_3$ (1:1) gave the better resolution, though the integral intensity in carbohydrate signals was still smaller than theoretical value probably due to the long T1 and short T2 values caused by the aggregation. The ^{13}C NMR of **137** was measured in DMF-*d*7. Though most of the carbon signals afforded peaks, anomeric carbon signals were not observed probably owing to the same reason.



Scheme IV-3. Synthesis of tetrasaccharide with heptapeptide **138** (TS7P).

Hydrogenation of **137** was then carried out in THF : H_2O : AcOH = 10 : 10 : 1 to give tetrasaccharide heptapeptide **138** (TS7P). MALDI-TOF MS of **138** afforded the $[M+Na]^+$ peak at 2240.0. The MS/MS analysis also showed reasonable fragmentation patterns for the peptide sequence, such as peaks $[(M\text{-Ala})+2Na]^{2+}$ at 1107.0425 and $[(M\text{-2Ala})+2Na]^{2+}$ at 1071.4998. ^1H

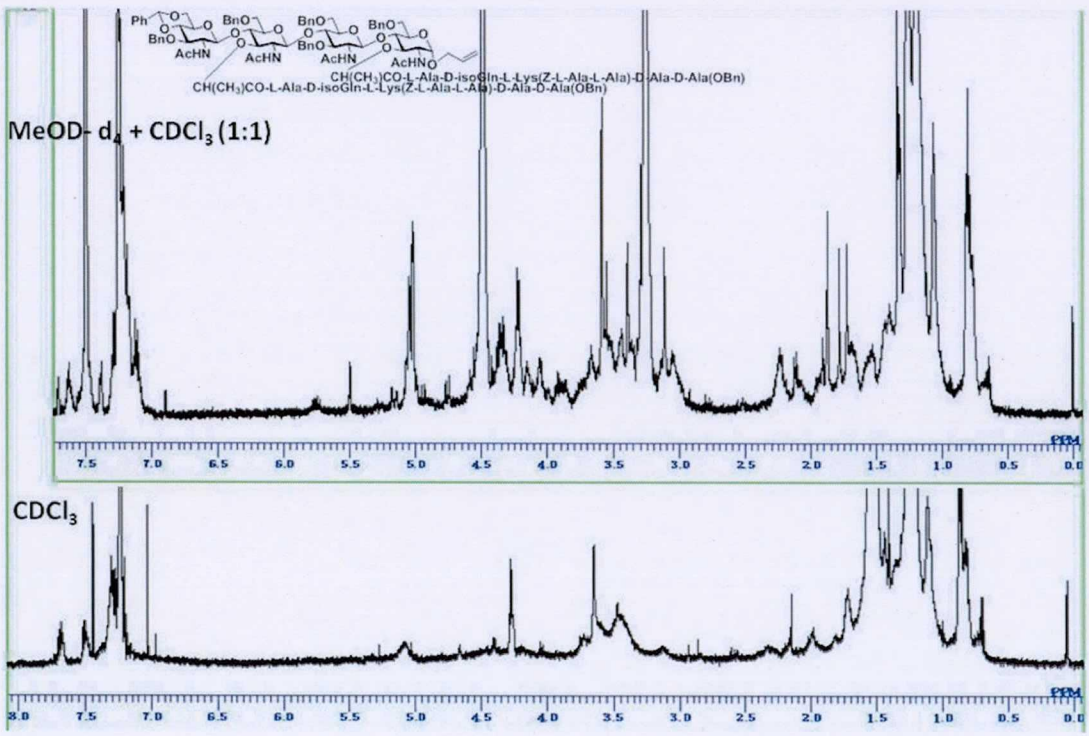


Figure IV-2. ^1H NMR of **137** in different solvent systems.

NMR of **138** was measured in a mixture of D_2O : CD_3COOD = 3 : 2 to give clear signals as shown in Fig. IV-3.

Tetrasaccharide **46** was also hydrogenated to give deprotected tetrasaccharide glycan **136**. The ^1H NMR was achieved in D_2O without problem.

Both glycan part **136** and the tetrasaccharide heptapeptide **138** will be applied to the LysM binding assay performed by Dr. Mesnage and Prof. Williamson in Univ. of Sheffield.

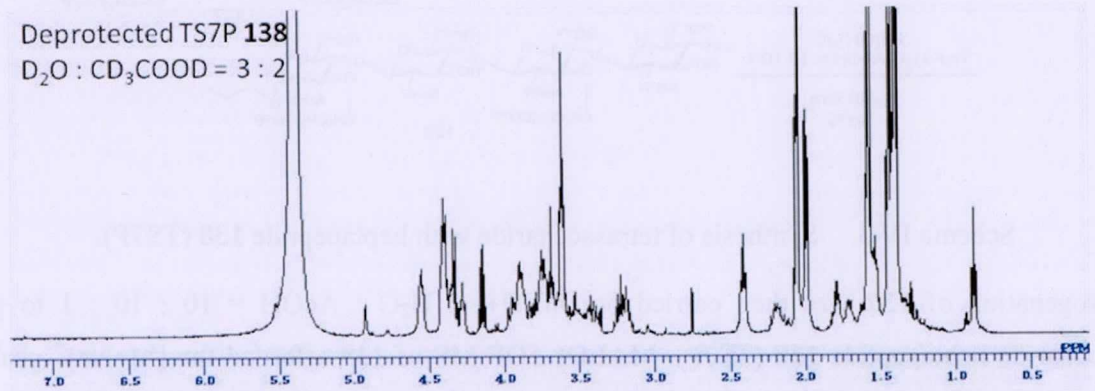
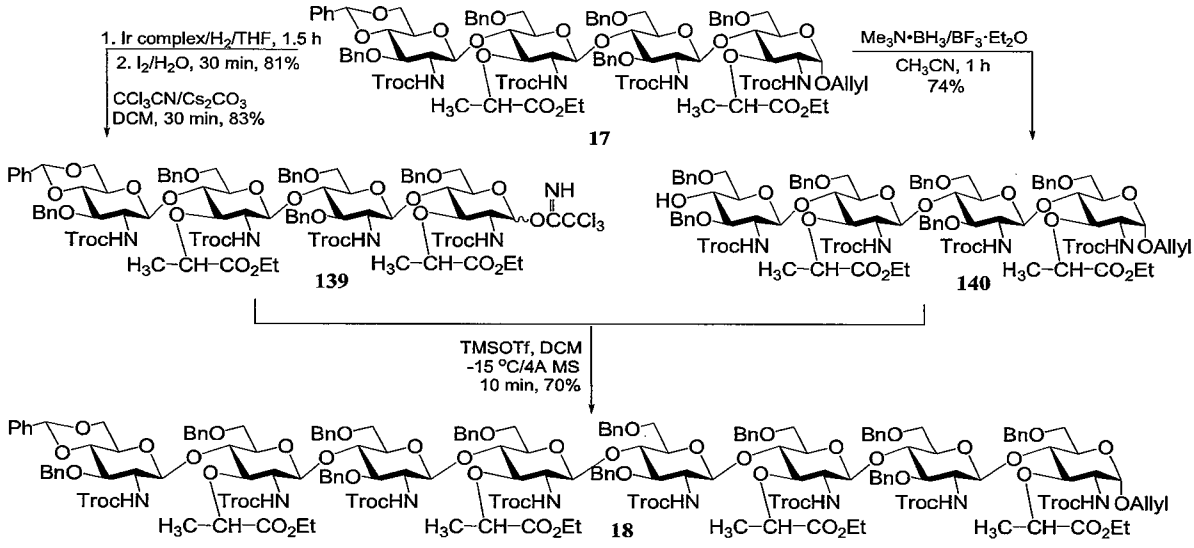


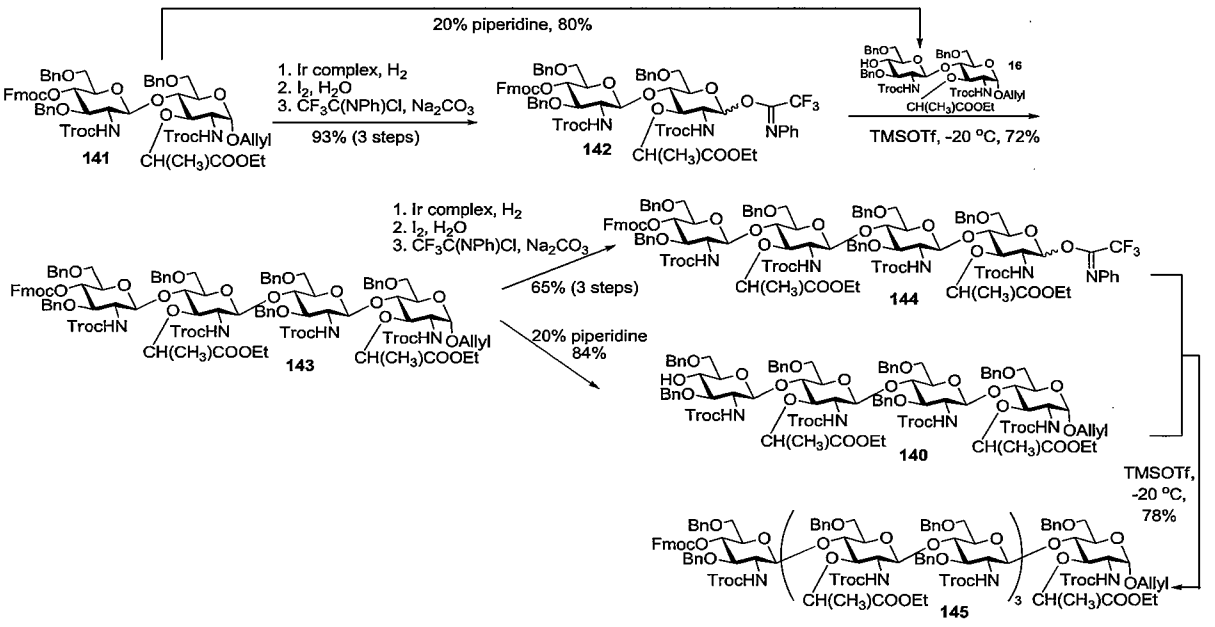
Figure IV-3. ^1H NMR of **138** in D_2O : CD_3COOD = 3 : 2.

IV-4 Improvement of Procedure for Synthesis of Octasaccharide Fragments



Scheme IV-3. Synthesis strategy of Octasaccharide **18**

Because it was suggested that the LysM domain in the *E. faecalis*, AtlB and AtlC recognize longer glycan chains, octasaccharide fragments were also synthesized. Inamura *et al.* previously synthesized octasaccharide containing repeating GlcNAc-MurNAc units with dipeptides and also prepared the protected hexadecasaccharide chains, although the deprotection process of the hexadecasaccharide was not successful.[3] The Troc protected tetrasaccharide **17** was used as the key precursor for longer glycan chains in their study. Tetrasaccharide **17** was converted to glycosyl donor **139** via cleavage of the allyl glycoside and the subsequent trichloroacetimidation in 60% yield (2 steps). Regioselective ring opening of the 4,6-*O*-benzylidene group in **17** with BH_3·Me_3N and BF_3·Et_2O afforded the tetrasaccharide acceptor **140** in 74% yield. Glycosylation of **139** with **140** was carried out by using TMSOTf as a catalyst. The octasaccharide **18** was thus obtained in 70% yield, which was a satisfactorily yield as a glycosylation reaction between tetrasaccharide donor and acceptor (Scheme IV-3). The author re-investigated the same reactions in order to obtain the octasaccharide **18**. However, the yield of **18** was only 30% even under the careful control of the reaction conditions.



Scheme IV-4. Synthesis of octasaccharide **145** using 4-*O*-Fmoc blocks.

To improve the synthetic yield and find an alternative pathway for the synthesis of the longer oligosaccharides, the author then employed another disaccharide intermediate with 4-*O*-Fmoc protection, based on the preliminary investigation by Dr. Huang in our group.[4] Since the *O*-Fmoc group can be cleaved by mild base such as piperidine or triethylamine, it was chosen to obtain the 4-OH acceptor in better yields. In the previous method, the yields of regioselective ring opening of the benzylidene group of longer glycans were lower than those of shorter glycans. The cleavage of Fmoc group in both disaccharide **142** and tetrasaccharide **143** was effected by using 20% piperidine in THF to afford the corresponding acceptors **16** and **140** in ca. 80% yields, respectively.

Since Fmoc group is readily removed under the basic conditions for preparation of trichloacetimidates, *N*-phenyltrifluoroacetimidates, which were proven to be useful in chapter II, were also used as glycosyl donors. Removal of allyl glycoside in **141** followed by *N*-phenyltrifluoroacetimidation[5] gave **142** in 93% yield for 3 steps (Scheme IV-4). Glycosylation of **140** and **144** was then carried out by using TMSOTf as a catalyst at -20 °C to give the tetrasaccharide **143** in good yield. The high stability of glycosyl donor **142** during the storage was another advantage of this synthetic pathway. The tetrasaccharide donor **144** and acceptor **140** were then synthesized from **143**. Glycosylation of **140** with **144** also proceeded successfully to give octasaccharide **145** in 78% yield. In this glycosylation step, byproducts indentified as **146** and **147**

(Figure IV-3) were somehow formed.

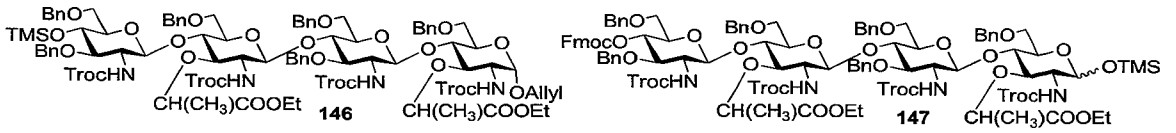
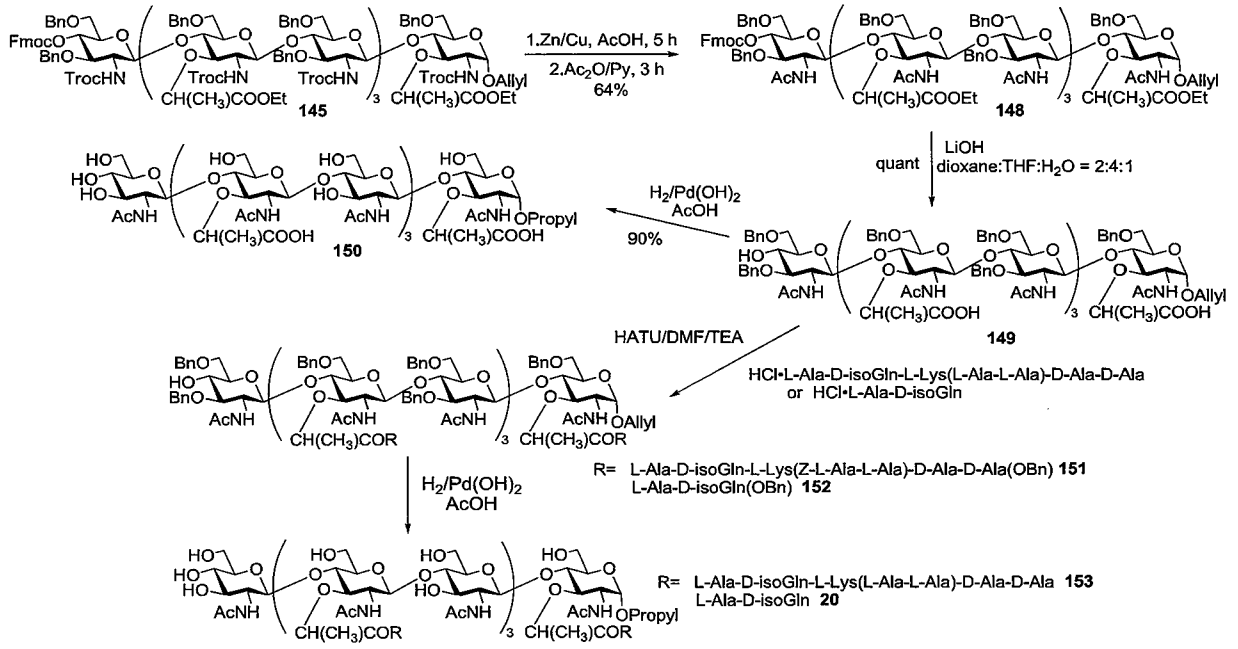


Figure IV-4. Byproducts of glycosylation reaction of **140** and **144**.

IV-5 Synthesis of Octasaccharide with Peptides

As shown in Scheme IV-5, deprotection of *N*-Troc groups using Zn-Cu couple in AcOH followed by acetylation with Ac_2O and Pyridine gave **148** in 64% yield. Removal of ethyl esters with LiOH resulted in carboxylic acid **149**, which was purified by hydrophobic chromatography using Diaion HP 20 column. Deprotection of all of benzyl-type protecting groups was carried out by catalytic hydrogenolysis (H_2, 2 MPa) in acetic acid using palladium hydroxide on carbon to give fully deprotected octasaccharide **150**, which was purified by HPLC and lyophilized. Molecular ion peaks of compounds **148**, **149** and **150** were observed in MALDI-TOF MS. Coupling of **149** with heptapeptide using HATU in DMF proceeded smoothly to give glycan peptide conjugate **151**. After silica-gel chromatography purification, $[M+H]^+$ was observed at 6397.01 in MALDI-TOF MS though the peak intensity was weak. Octasaccharide dipeptide **152** was also synthesized in a similar manner and clear peak $[M+Na]^+$ of **152** was observed at 4232.97 in MALDI-TOF MS. Deprotection of **151** and **152** was then carried out by catalytic hydrogenolysis in acetic acid using palladium hydroxide as a catalyst to give **153** and **20**, respectively. After removal of palladium, the filtrates were concentrated and the residues were lyophilized from H_2O. ESI-QTOF MS and MALDI-TOF MS of **153** and **20**, however, did not afford molecular ion peaks under normal measurement conditions probably owing to the aggregate formation. The structural analysis of octasaccharide fragments **153** and **20** by the spectroscopy is therefore under investigation.



Scheme IV-5. Synthesis of octasaccharide with peptides.

IV-6 Summary

In this Chapter, heptapeptide PGN fragments were designed and synthesized to elucidate the interaction between peptidoglycan and LysM containing proteins. The tetrasaccharide and ocatasaccharide fragments were synthesized via new synthetic routes based on Fmoc protection at 4-OH positions of the disaccharide and the tetrasaccharide unit. NMR and MS spectroscopy of the tetrasaccharide heptapeptide (TS7P) and octasaccharide with peptides revealed their strong tendency to aggregate. This feature made the spectroscopic analysis of these PGN fragments extremely difficult. It is still necessary to confirm the structures of the final deprotected octasaccharide with peptides. Further analysis of PGN interaction with proteins by using NMR is now under investigation by collaborators in Univ. of Scheffild.

References

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[2] S. Mesnage, F. Chau, L. Dubost, M. Arthur, *J. Biol.Chem.* **2008** *283*, 19845–19853.

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[4] C. Huang, Y. Konishi, Y. Fujimoto, K. Fukase, *IKCOC-11*, **2009**, poster.

[5] a) B. Yu, H. Tao, *Tetrahedron Letters* **2001**, *42*, 2405-2407; b) B. Yu, H. Tao, *J. Org. Chem.* **2002**, *67*, 9099-9102; c) M. Adinolfi, G. Barone, A. Iadonisi, M. Schiattarella, *Synlett* **2002**, 269-270; d) S. Cai, B. Yu, *Org. Lett.* **2003**, *5*, 3827-3830.

Chapter V Conclusion

As described above, the author achieved the comprehensive synthesis of PGN partial structures in order to study the precise PGN structures responsible for the immunostimulating activity as well as the interactions between PGN structures and PGN-binding proteins. Various PGN fragments were synthesized according to the strategy previously developed for the synthesis of tetrasaccharide fragments **1a~d**. These fragments having GMGM sequence are considered to be formed by lysozyme digestion of PGN in animals. In this study, the author synthesized the fragments **2a~g** expected to be produced by bacterial glucosaminidase for the first time. Fragments **2a**, **2c**, and **2e** have disaccharide MG sequence, wheras fragments **2b**, **2d**, **2f**, and **2g** have tetrasaccharide MGMG sequence. Compound **1a~c** were also prepared for comparison. The author found that *N*-phenyltrifluoroacetimidate donor was superior to trichloroacetimidate donor in the coupling between the disaccharides MG donor and MG acceptor and hence established the synthesis tetrasaccharide PGN fragments having MGMG sequence.

Nod2 stimulating activity of the PGN fragment library including PGN with disaccharide and tetrasaccharide was then evaluated. Compounds having shorter glycans and shorter peptides showed more potent activity. Dipeptide fragments (both GM and MG) having dipeptide had the equivalent activity as MDP. In conclusion, MGMG sequence PGN fragments showed higher activity than corresponding GMGM sequence PGN fragments.

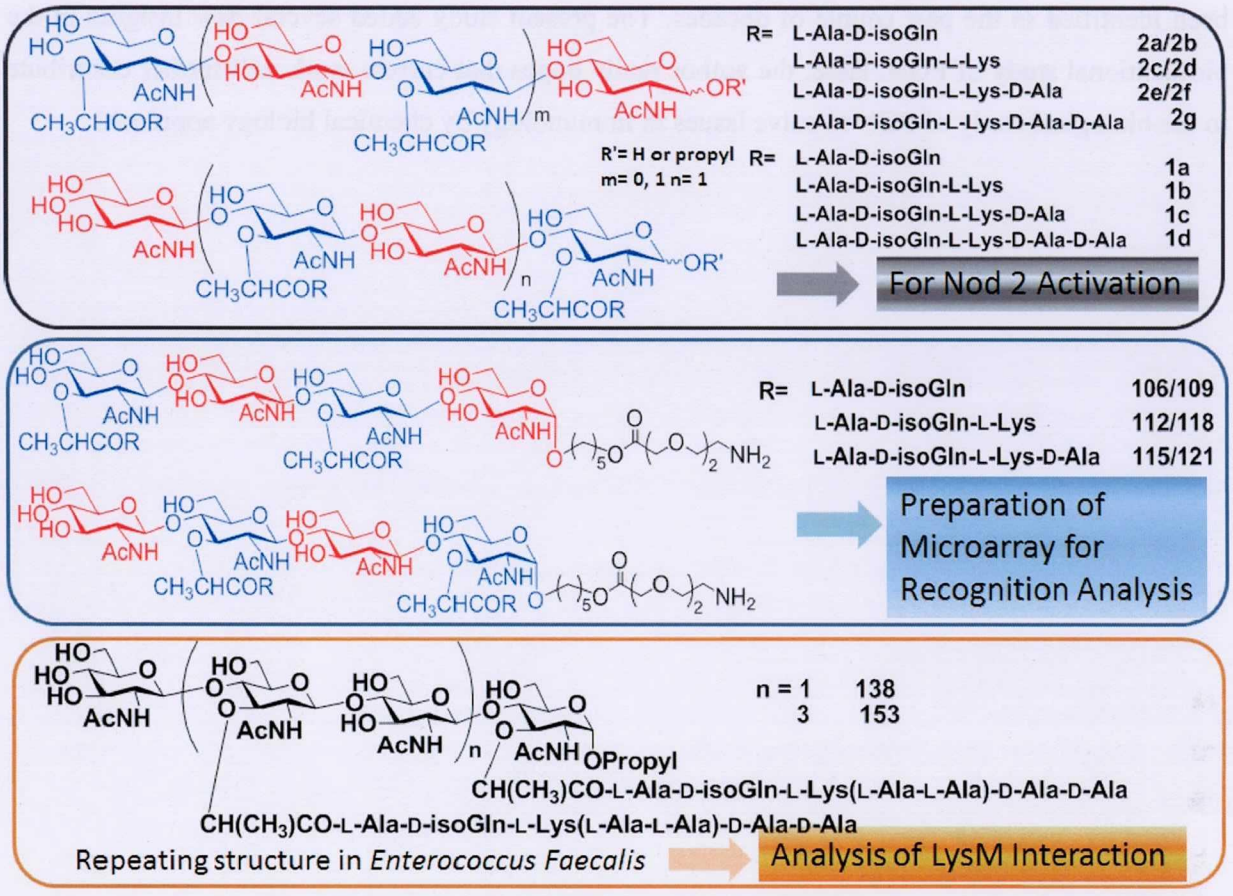


Figure V-1. Peptidoglycan fragment library.

PGN microarrays were constructed by attaching synthesized PGN fragments on the array platforms. The PGN fragment library having mono, di and tetrasaccharide backbones with di, tri, and tetrapeptides were introduced to the array via an appropriate linker. Binding study of WGA lectin and PGRP-S revealed the high efficiency of the array for the study of interactions between OGN with the receptors and recognition proteins.

The tetrasaccharide and octasaccharide fragments containing heptapeptide stem were synthesized to elucidate the interaction between peptidoglycan and LysM domain from AtlB and AtlC. A new synthetic route was established for the synthesis of octasaccharide fragments by using Fmoc protected disaccharide and tetrasaccharide units. Further analysis of interactions of PGN with proteins is now ongoing.

The aim of this research was to synthesize various biofunctional PGN fragments and to elucidate the interaction of PGN with the receptors and recognition proteins, The importance of PGN in host defence system has been revealed and various receptors and recognition proteins against PGN have

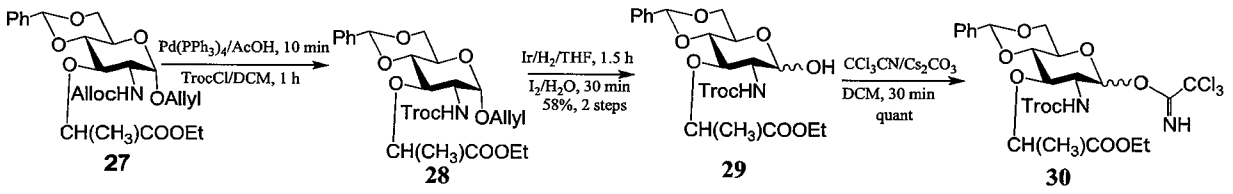
been identified in the past couple of decades. The present study added several new insights in the biofucntional study of PGN. Here, the author really hopes that current work will further contribute to the biological study of PGN to solve issues in immunology by chemical biology approach.

Chapter VI Experimental

General

^{1}H NMR spectra were recorded in indicated solvents by using a JEOL JNM-LA 500, or a JEOL ECA 500, or a Varian INOVA 600 spectrometers. The chemical shifts in $CDCl_3$ are given in *d* values from tetramethylsilane (TMS) as an internal standard. For the measurement in D_2O, HDO signal (4.718 ppm at 30 °C) was used as a reference. ESI-QTOF mass spectrometry were carried out using Applied Biosystem Mariner™ Biospectrometry Workstation. High resolution mass spectrometry was measured by using Micromass Q-Tof *micro*™ (ESI-QTOF-MS). Elemental analyses were performed with Yanaco CHN corder MT-6. Silica-gel column chromatography was carried out using Kieselgel 60 (Merck, 0.040-0.063 mm) or Silica Gel 60 N (Kanto Chemical Co., spherical, neutral, 0.040-0.050 mm) at medium pressure (2-4 kg/cm^2). Gel permeation chromatography (GPC) was carried out using Sephadex LH20 at atmospheric pressure. Precoated Kieselgel 60 F 254 (Merck Co., 0.5 mm) was used for preparative thin layer chromatography. TLC analysis was performed on Silica-gel 60 F_{254} (Merck) and compound visualized by UV (254 nm), phosphomolybdic acid solution (5.0% in EtOH), 0.03% p-methoxybenzaldehyde in EtOH-conc.H_2SO_4-acetic acid or 0.2% ninhydrin in EtOH-collidine-acetic acid. MS4A was activated by heating at 250 °C in vacuo for 3 h before use. Unless otherwise stated all reactions were performed at room temperature. Non-aqueous reactions were carried out under argon atmosphere unless otherwise noted. Anhydrous CH_2Cl_2 was distilled from calcium hydride. Anhydrous THF was purchased from Kanto Chemicals, Tokyo, Japan. Anhydrous DMF was purchased from NACALAI TESQUE INC., Kyoto, Japan. Distilled water purchased from Otsuka (Tokyo, Japan) or prepared by a combination of Arium® 611 UV (Sautorius) or Toray Pure LV-308 (Toray) and GSL-200 (Advantec, Tokyo, Japan). All other reagents and solvents used were also purchased from commercial sources.

Experiments in Chapter II

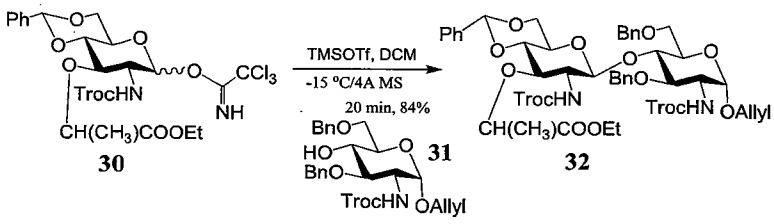


4,6-*O*-Benzylidene-2-deoxy-3-*O*-((*R*)-1-(ethoxycarbonyl)ethyl)-2-(2,2,2-trichloroethoxycarbonylamino)-D-glucopyranosyl Trichloroacetimidate (30)

To a solution of **27** (10.0 g, 20.3 mmol) in dry CH_2Cl_2 (200 mL) were added AcOH (1.8 mL, 30.5 mmol) and $Pd(PPh_3)_4$ (7.0 g, 6.1 mmol) and the mixture was stirred at rt for 10 min. TrocCl (5.7 mL, 40.6 mmol) was then added to the deep green solution. After being stirred at rt for 1 h, the reaction was quenched with sat. $NaHCO_3$ aq (100 mL) and the organic layer were concentrated. The residue was dissolved with EtOAc and insoluble materials were filtered off and then the filtrate was concentrated. This process was repeated until no insoluble materials were observed. The EtOAc solution was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (350 g, toluene : EtOAc = 10 : 1) to give **28** as a yellow solid.

To a degassed solution of **28** (1.0 g, 1.7 mmol) in dry THF (12 mL) was added $[Ir(cod)(MePh_2P)_2]PF_6$ (29 mg, 0.034 mmol) which had been activated with hydrogen for 30 s. The mixture was stirred under nitrogen atmosphere at room temperature for 1.5 h. Iodine (0.87 g, 3.43 mmol) and water (6 mL) were then added and the reaction mixture was stirred for additional 30 min. After excess iodine was quenched with aqueous $Na_2S_2O_3$ (5%, 50 mL), the mixture was extracted with EtOAc (100 mL). The organic layer was washed with aqueous $Na_2S_2O_3$ (5%, 50 mL × 2) and brine (50 mL), and dried over Na_2SO_4. After removal of the solvent in vacuo, the crude product was purified by silica-gel chromatography (100 g, toluene : EtOAc = 25 : 1) to give **29** as a yellow solid (0.54 g, 58%).

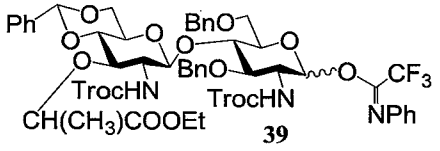
To a solution of **29** (320 mg, 0.59 mmol) in dry CH_2Cl_2 (20 mL) at rt were added Cs_2CO_3 (96 mg, 0.3 mmol) and CCl_3CN (0.6 mL, 5.9 mol). After being stirred for 30 min, insoluble materials were filtered off by celite and the filtrate was concentrated. Residue was lyophilized from benzene gave **30** as a pale yellow solid (410 mg, quant.) without further purification.



Allyl 3,6-di-*O*-benzyl-2-deoxy-4-*O*-[4,6-*O*-benzylidene-2-deoxy-3-*O*-{(*R*)-1-(ethoxycarbonyl)ethyl}-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl]-2-(2,2,2-trichloroethoxycarbonyl

amino)-α-D-glucopyranoside (32)

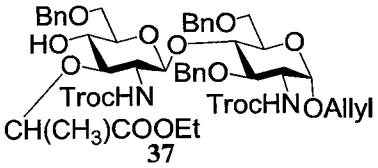
To a mixture of the imidate **30** (411 mg, 0.59 mmol), the acceptor **31** (282 mg, 0.49 mmol), and MS4A in dry CH_2Cl_2 (20 mL) at -15 °C was added TMSOTf (9 μL, 0.05 mmol). After being stirred at the same temperature for 20 min, the reaction was quenched with chilled saturated aqueous $NaHCO_3$ (10 mL), and the mixture was extracted with $CHCl_3$ (50 mL). The organic layer was washed with sat. $NaHCO_3$ aq (20 mL) and brine (20 mL), dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (60 g, toluene : EtOAc = 10 : 1) to give **6** as a white solid (453 mg, 84%). ^1H NMR (500 MHz, $CDCl_3$) δ 7.42-7.24 (m, 15H), 6.61 (brs, 1H), 6.07 (brs, 1H), 5.86 (m, 1H), 5.62 (d, J = 7.5 Hz, 1H), 5.41 (s, 1H), 5.27-5.20 (m, 2H), 5.00 (d, J = 9.0 Hz, 1H), 4.93-4.88 (m, 3H), 4.78 (d, J = 11.5 Hz, 1H), 4.72-4.60 (m, 4H), 4.46-4.42 (m, 2H), 4.24-4.19 (m, 3H), 4.10-4.09 (m, 2H), 4.01-3.94 (m, 3H), 3.87 (d, J = 10.5 Hz, 1H), 3.67 (m, 1H), 3.61-3.49 (m, 4H), 3.40 (m, 1H), 3.26 (t, J = 10.5 Hz, 1H), 3.06 (m, 1H), 1.37 (d, J = 7.0 Hz, 3H), 1.30-1.27 (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.8, 163.4, 155.3, 154.2, 138.9, 137.9, 137.1, 133.3, 129.0, 128.7, 128.5, 128.3, 128.2, 127.4, 127.3, 125.9, 118.2, 102.2, 101.0, 96.6, 95.4, 82.3, 78.1, 78.1, 77.1, 75.1, 74.6, 74.4, 73.5, 70.6, 68.5, 67.6, 65.6, 61.1, 57.4, 54.7, 18.8, 14.2; HRMS (ESI-QTOF) Anal. Calcd for $C_{47}H_{54}Cl_6N_2O_{15}Na$ $[M+Na]^+$: 1119.1553, found: 1119.1559.



39

3,6-Di-*O*-benzyl-2-deoxy-4-*O*-[4,6-*O*-benzylidene-2-deoxy-3-*O*-{(*R*)-1-(ethoxycarbonyl)ethyl}-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl]-2-(2,2,2-trichloroethoxycarbonyl amino)-D-glucopyranosyl (*N*-phenyl)trifluoroacetimidate (39)

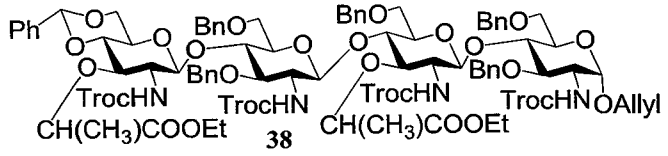
To a solution of **32** (0.87 g, 0.79 mmol), the solution of $[Ir(cod)(MePh_2P)_2]PF_6$ (32 mg, 0.04 mmol) activated with H_2 in dry THF (1 mL) was added. After being stirred at room temperature for 1.5 h, iodine (400 mg, 1.6 mmol) and water (2 mL) were added and the reaction mixture was stirred for additional 30 min. To the reaction mixture was rapidly added $Na_2S_2O_3$ aq (5%, 100 mL). The mixture was then extracted with EtOAc (50 mL). The organic layer was washed with $Na_2S_2O_3$ aq (5%, 50 mL × 2), sat. $NaHCO_3$ aq (100 mL × 2), brine (50 mL), dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica-gel chromatography (80 g, toluene : EtOAc = 5 : 1) to give 1-liberated-disaccharide (677 mg, 81%) as a pale yellow solid, HRMS (ESI-QTOF) Anal. Calcd for $C_{44}H_{50}Cl_6N_2O_{15}K$ $[M+K]^+$: 1095.0979, found: 1095.0933. To a solution of 1-liberated-disaccharide (329 mg, 0.31 mmol) in acetone (10 mL) at 0 °C was added Na_2CO_3 (987 mg, 9.3 mmol) and *N*-PhenyL-2,2,2-Trifluoroacetimidoyl chloride (0.1 mL, 0.47 mmol). After being stirred for 3 d at rt, insoluble materials were filtered off through celite and the filtrate was concentrated. The residue was purified by silica-gel chromatography (30 g, toluene : EtOAc = 12 : 1) to give **39** as a pale yellow solid (290 mg, 76%).



37

Allyl 3,6-di-*O*-benzyl-2-deoxy-4-*O*-[6-*O*-benzyl-2-deoxy-3-*O*-{(*R*)-1-(ethoxycarbonyl)ethyl}-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl]-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranoside (37)

To a solution of **32** (138 mg, 0.125 mmol) and trimethylamine-borane (10 mg, 0.138 mmol) in dry CH_3CN (15 mL) at 0 °C was added boron trifluoride diethyl etherate (53 mg, 0.376 mmol) dropwise and the mixture was stirred at rt for 1 h. The reaction was then quenched with ice and sat. $NaHCO_3$ aq (20 mL) and the mixture was extracted with EtOAc (50 mL × 2). The organic layer was washed with citric acid (1 M, 15 mL × 4), sat. $NaHCO_3$ aq (50 mL), and brine (50 mL), dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (30 g, toluene : AcOEt = 5 : 1) to give **37** as a colorless solid (115 mg, 83%). ^1H NMR (500 MHz, $CDCl_3$) δ 7.39-7.26 (m, 15H), 5.84 (m, 1H), 5.73 (d, J = 6.5 Hz, 1H), 5.26-5.18 (m, 2H), 4.94-4.87 (m, 4H), 4.76 (d, J = 12.0 Hz, 1H), 4.71-4.61 (m, 5H), 4.43 (d, J = 11.9 Hz, 1H), 4.33 (s, 2H), 4.45-4.18 (m, 3H), 4.09-4.07 (m, 1H), 3.96-3.85 (m, 4H), 3.66 (d, J = 10.0 Hz, 1H), 3.59-3.55 (m, 3H), 3.52-3.50 (m, 3H), 3.26 (m, 1H), 3.15 (m, 2H), 1.38 (d, J = 6.9 Hz, 3H), 1.29 (t, J = 7.2 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.6, 155.3, 154.2, 139.1, 137.9, 137.2, 133.3, 128.6, 128.5, 128.9, 127.9, 127.6, 127.4, 127.0, 118.1, 102.0, 96.6, 95.9, 79.7, 78.0, 75.4, 74.5, 74.3, 74.1, 73.7, 73.4, 71.8, 70.5, 68.4, 67.6, 61.2, 56.0, 54.7, 19.0, 14.2; HRMS (ESI-QTOF) Anal. Calcd for $C_{47}H_{56}Cl_6N_2O_{15}Na$ $[M+Na]^+$: 1121.1710, found: 1121.1663.

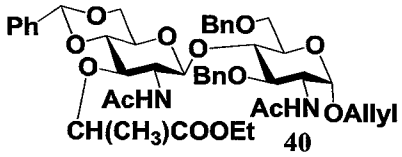


38

Allyl 3,6-di-*O*-benzyl-4-*O*-[[6-*O*-benzyl-4-*O*-[3,6-di-*O*-benzyl-4-*O*-{{4,6-*O*-benzylidene-2-deoxy-3-*O*-{(*R*)-1-(ethoxycarbonyl)-ethyl}-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl}}-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl]-2-deoxy-3-*O*-{(*R*)-1-(ethoxycarbonyl)-ethyl}-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl]]-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranoside (38)

To a mixture of the imidate **39** (135 mg, 0.11 mmol), the acceptor **37** (200 mg, 0.18 mmol), and MS4A in dry CH_2Cl_2 (20 mL) at -15 °C was added TMSOTf (5 μL, 0.03 mmol). After being stirred at the same temperature for 20 min, the reaction was quenched with chilled saturated aqueous $NaHCO_3$ (10 mL), and the mixture was extracted with $CHCl_3$ (50 mL). The organic layer was washed with sat. $NaHCO_3$ aq (20 mL) and brine (20 mL), dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (30 g, toluene : EtOAc = 7 : 1) to

give **38** as a colorless solid (141 mg, 61%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45-7.18 (m, 30H), 5.85-5.82 (m, 2H), 5.41 (s, 1H), 5.24-5.12 (m, 3H), 4.88-4.59 (m, 14H), 4.50-4.10 (m, 13H), 4.02-3.87 (m, 7H), 3.77 (m, 1H), 3.67-3.49 (m, 8H), 3.40-3.38 (m, 3H), 3.25 (t, J = 10.0 Hz, 1H), 3.17 (brs, 1H), 3.06 (d, J = 10.0 Hz, 1H), 2.97-2.94 (m, 2H), 1.38 (d, J = 7.0 Hz, 3H), 1.29-1.24 (m, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.6, 173.9, 155.9, 155.5, 154.3, 154.0, 139.5, 138.9, 138.1, 137.8, 137.6, 137.2, 133.5, 129.5, 129.1, 129.1, 128.6, 128.5, 128.4, 128.4, 128.3, 128.2, 128.1, 127.9, 127.8, 127.6, 127.4, 127.3, 127.1, 125.9, 117.9, 102.8, 101.8, 100.2, 96.6, 96.0, 95.6, 95.5, 82.4, 80.4, 78.2, 77.7, 75.0, 74.8, 74.6, 74.5, 74.3, 74.1, 74.0, 73.4, 73.9, 70.8, 68.4, 68.4, 68.0, 67.5, 65.7, 61.2, 61.1, 57.5, 57.3, 57.0, 54.7, 18.8, 18.4, 14.2, 14.1; HRMS (ESI-QTOF) Anal. Calcd for $C_{91}H_{104}Cl_{12}N_4O_{29}K$ $[M+K]^+$: 2175.2686, found: 2175.2556.

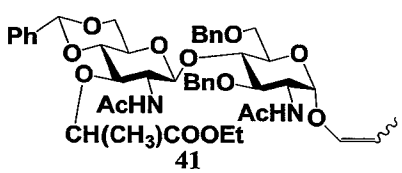


40

Allyl

2-acetylamino-4-*O*-[2-acetylamino-4,6-*O*-benzylidene-2-deoxy-3-*O*-{(*R*)-1-(ethoxycarbonyl)-ethyl}-β-D-glucopyranosyl]-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside (40)

To a solution of **32** (50 mg, 1.9 mmol) in AcOH (2 mL) was added Zn–Cu (prepared from 300 mg of Zn), the mixture was stirred at room temperature for 30 min. The insoluble materials were filtered off and the filtrate was concentrated in vacuo. The residue solvent was removed by coevaporation with toluene (10 mL). The residue was dissolved in pyridine (2 mL) and acetic anhydride (2 mL) and the solution was stirred at room temperature for 1 h. The solution was removed by concentration with toluene (10 mL). The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: acetone = 9 : 1) to give **40** as a white solid (28 mg, 74%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43-7.26 (m, 15H), 6.15 (d, J = 7.5 Hz, 1H), 5.84 (m, 1H), 5.44 (s, 1H), 5.28 (d, J = 9.0 Hz, 1H), 5.23-5.19 (m, 2H), 4.86-4.84 (m, 2H), 4.77 (d, J = 12.0 Hz, 1H), 4.58 (d, J = 12.0 Hz, 1H), 4.48-4.39 (m, 3H), 4.19-4.11 (m, 5H), 3.98-3.72 (m, 5H), 3.62-3.60 (m, 2H), 3.54-3.47 (m, 2H), 3.37 (t, J = 10.0 Hz, 1H), 3.13 (m, 1H), 2.00 (s, 3H), 1.84 (s, 3H), 1.37 (d, J = 7.0 Hz, 3H), 1.27 (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.6, 172.3, 170.3, 148.7, 140.2, 139.2, 138.1, 137.2, 133.6, 129.0, 128.5, 128.2, 128.1, 127.7, 127.3, 125.8, 125.6, 117.8, 102.1, 101.9, 96.2, 82.5, 77.7, 74.9, 74.0, 73.5, 70.9, 68.5, 68.3, 67.7, 75.7, 61.1, 55.7, 52.1, 23.5, 23.3, 18.8, 14.1; HRMS (ESI-QTOF) Anal. Calcd for $C_{45}H_{57}N_2O_{13}$ $[M+H]^+$: 833.3861, found: 833.3839.



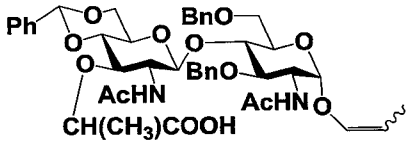
41

Prop-1-enyl

2-acetylamino-4-*O*-[2-acetylamino-4,6-*O*-benzylidene-2-deoxy-3-*O*-{(*R*)-1-(ethoxycarbonyl)-et

hyl}-β-D-glucopyranosyl]-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside (41)

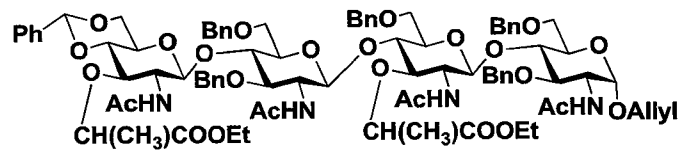
To a solution of **40** (50 mg, 0.06 mmol) in THF (2 mL) was added H_2-activated $[Ir(cod)(MePh_2P)_2]PF_6$ (2.5 mg, 0.003 mmol) in dry THF (1 mL). After being stirred under an argon atmosphere at room temperature for 1.5 h, the reaction mixture was quenched with sat. $NaHCO_3$ aq (10 mL) and the mixture was extracted with AcOEt (20 mL × 2). The organic layer was washed with sat. $NaHCO_3$ aq (20 mL) and brine (20 mL), dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: acetone = 10 : 1) to give **41** as a white solid (34 mg, 68%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44-7.26 (m, 15H), 6.14 (d, J = 7.6 Hz, 1H), 6.09 (dd, J = 12.3, J = 1.6 Hz, 1H), 5.45 (s, 1H), 5.26 (d, J = 8.5 Hz, 1H), 5.12-5.08 (m, 1H), 5.04 (d, J = 3.8 Hz, 1H), 4.88 (d, J = 12.2 Hz, 1H), 4.76 (d, J = 12.0 Hz, 1H), 4.61 (d, J = 12.2 Hz, 1H), 4.48-4.39 (m, 3H), 4.23-4.16 (m, 4H), 4.01 (t, J = 9.0 Hz, 1H), 3.79-3.76 (m, 2H), 3.69 (d, J = 15.0 Hz, 1H), 3.63 (d, J = 3.9 Hz, 1H), 3.59-3.53 (m, 2H), 3.48 (d, J = 5.1 Hz, 1H), 3.39 (t, J = 10.0 Hz, 1H), 3.15 (m, 1H), 1.97 (s, 3H), 1.81 (s, 3H), 1.53 (d, J = 6.4 Hz, 3H), 1.37(d, J = 7.0 Hz, 3H), 1.28 (t, J = 7.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.3, 171.6, 169.9, 142.8, 139.2, 138.2, 137.3, 129.0, 128.6, 128.3, 128.3, 128.2, 128.1, 127.9, 127.4, 125.9, 104.7, 102.2, 101.0, 96.9, 82.6, 77.4, 77.4, 74.9, 74.3, 73.6, 71.2, 68.6, 67.5, 65.7, 61.0, 55.6, 51.9, 23.5, 23.3, 18.9, 14.2, 12.4; HRMS (ESI-QTOF) Anal. Calcd for $C_{45}H_{56}N_2O_{13}Na$ $[M+Na]^+$: 855.3680, found: 855.3613.



42

Prop-1-enyl 2-acetylamino-4-*O*-[2-acetylamino-4,6-*O*-benzylidene-2-deoxy-3-*O*-{(*R*)-1-carboxyethyl}-β-D-glucopyranosyl]-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside (42)

To a solution of **41** (23 mg, 0.03 mmol) in dioxane : THF : H_2O (2 : 4 : 1, 4.0 mL) was added LiOH (4 mg, 0.17 mmol) and stirred at room temperature for 1 h. The solution was neutralized with Dowex H^+ (Dowex 50W × 8, 200–400 mesh H form, DowChemicals) and then applied to an HP-20 column (2 cm × 10 cm). Organic and inorganic salts were removed by elution with H_2O (160 mL), then eluted with MeOH and concentrated in vacuo to give a disaccharide with a free lactic acid moiety **42** as a white solid (22 mg, quant). HRMS (ESI-QTOF) Anal. Calcd for $C_{43}H_{52}N_2O_{13}Na$ $[M+Na]^+$: 827.3367, found: 827.3291.

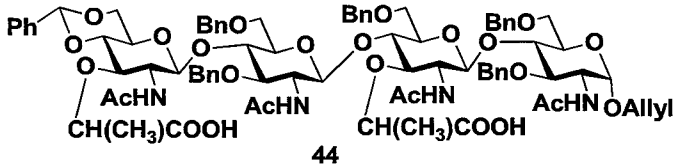


43

Allyl 2-acetylamino-4-*O*-[[2-acetylamino-4-*O*-[2-acetylamino-3,6-di-*O*-benzyl-4-*O*-{{2-acetylamino-

4,6-*O*-benzylidene-3-*O*-{(*R*)-1-(ethoxycarbonyl)ethyl}-2-deoxy-β-D-glucopyranosyl}}-2-deoxy-β-D-glucopyranosyl]-6-*O*-benzyl-2-deoxy-3-*O*-{(*R*)-1-(ethoxycarbonyl)ethyl}-β-D-glucopyranosyl]]-3,6-di-*O*-benzyl-2-deoxy-D-glucopyranoside (43)

To a solution of **38** (200 mg, 0.09 mmol) in AcOH (2 mL) was added Zn–Cu (prepared from 500 mg of Zn), the mixture was stirred at room temperature for 30 min. The insoluble materials were filtered off and the filtrate was concentrated in vacuo. The residue solvent was removed by coevaporation with toluene (10 mL). The residue was dissolved in pyridine (2 mL) and acetic anhydride (2 mL) and the solution was stirred at room temperature for 1 h. The reagents were removed by concentration with toluene (10 mL). The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: acetone = 9 : 1) to give **43** as a white solid (93 mg, 62%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44-7.21 (m, 31H), 6.98 (m, 1H), 6.74 (d, J = 6.8 Hz, 1H), 6.07 (d, J = 8.4 Hz, 1H), 5.81(m, 1H), 5.47 (s, 1H), 5.24-5.14 (m, 3H), 4.95 (d, J = 12.8 Hz, 1H), 4.84 (d, J = 3.6 Hz, 1H), 4.74-4.64 (m, 5H), 4.55-4.45 (m, 5H), 4.36-4.02 (m, 14H), 3.96-3.38 (m, 20H), 3.23 (t, J = 9.6 Hz, 1H), 3.13 (m, 1H), 3.05 (m, 1H), 1.98 (s, 3H), 1.94 (s, 3H), 1.88 (s, 3H), 1.78 (s, 3H), 1.37 (d, J = 6.8 Hz, 3H), 1.31-1.21(m, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.9, 174.7, 172.4, 172.3, 171.8, 169.8, 139.6, 138.8, 138.3, 138.1, 138.1, 138.0, 137.2, 133.7, 129.1, 128.5, 128.3, 128.3, 128.2, 128.2, 128.0, 127.9, 127.8, 127.7, 127.4, 127.3, 127.2, 125.9, 117.5, 102.4, 101.1, 100.8, 100.5, 96.3, 95.5, 82.6, 80.3, 76.4, 75.8, 75.4, 74.9, 74.6, 74.1, 73.8, 73. 4, 73.4, 73.2, 72.8, 71.1, 68.5, 68.5, 68.2, 68.0, 65.9, 61.3, 61.0, 55.4, 55.0, 53.37, 52.1, 29.7, 29.3, 23.9, 23.6, 23.5, 23.4, 23.1, 18.8, 18.6, 14.2, 14.1; HRMS (ESI-QTOF) Anal. Calcd for $C_{87}H_{108}N_4O_{25}Na$ $[M+Na]^+$: 1631.7200, found: 1631.7169.

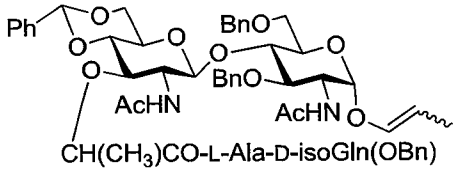


44

Allyl 2-acetylamino-3,6-di-*O*-benzyl-4-*O*-[[2-acetylamino-6-*O*-benzyl-3-*O*-{(*R*)-1-carboxyethyl}-4-*O*-[2-acetylamino-4-*O*-{{2-acetylamino-4,6-*O*-benzylidene-3-*O*-{(*R*)-1-carboxyethyl}-2-deoxy-β-D-glucopyranosyl}}-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranosyl]-2-deoxy-β-D-glucopyranosyl]]-2-deoxy-D-glucopyranoside (44)

To a solution of **43** (93 mg, 0.06 mmol) in dioxane : THF : H_2O (2 : 4 : 1, 4.0 mL) was added LiOH (16 mg, 0.7 mmol) and stirred at room temperature for 1 h. The solution was neutralized with Dowex H^+ (Dowex 50W × 8, 200–400 mesh H form, DowChemicals) and then applied to an HP-20 column (2 cm × 10 cm). Organic and inorganic salts were removed by elution with H_2O (160 mL), then eluted with MeOH and concentrated in vacuo to give a disaccharide with a free lactic acid moiety **44** as a white solid (89 mg, quant). ^{1}H NMR (400 MHz, CD_3OD) δ 7.34-6.99 (m, 30H), 5.83 (m, 1H), 5.40(s, 1H), 5.20 (dd, J = 17.6 Hz, J = 1.6 Hz, 1H), 5.09-5.00 (m, 2H), 4.84 (d, J = 11.2 Hz, 1H), 4.68 (d, J = 3.6 Hz, 1H), 4.60-4.37 (m, 8H), 4,25-4.21 (m, 3H), 4.09-4.00 (m, 2H),

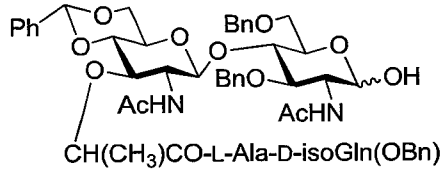
3.95-3.31 (m, 22H), 3.04- 2.95 (m, 2H), 1.89 (m, 6H), 1.77 (s, 6H), 1.31-1.19 (m, 6H); HRMS (ESI-QTOF) Anal. Calcd for $C_{83}H_{100}N_4O_{25}Na$ $[M+Na]^+$: 1575.6574, found: 1575.6508.



63

Prop-1-enyl 2-acetylamino-4-*O*-[[2-acetylamino-4,6-*O*-benzylidene-2-deoxy-3-*O*-[(*R*)-propionyl-{benzyl-(L-alanyl-D-isoglutaminate)}]-β-D-glucopyranosyl]]-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside (63)

To a solution of **42** (20 mg, 0.025 mmol), HCl·L-Ala-D-isoGln(OBn) (25 mg, 0.073 mmol), and HOBt (6 mg, 0.044 mmol) in DMF(3 mL) were added WSCD·HCl (6 mg, 0.037 mmol) and triethylamine (11 μL, 0.079 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 20 mL), H_2O (20 mL), sat. $NaHCO_3$ aq (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 20 : 1) to give **63** as a white solid (20 mg, 72%). ^1H NMR (500 MHz, $CDCl_3$: CD_3OD = 4 : 1) δ 7.84 (m, 1H), 7.72 (m, 1H), 7.38-7.17 (m, 24H), 6.13 (d, J = 12.5 Hz, 1H), 5.45 (s, 1H), 5.09-5.03 (m, 3H), 5.02 (d, J = 3.5 Hz, 1H), 4.87 (d, J = 12.0 Hz, 1H), 4.75 (d, J = 12.0 Hz, 1H), 4.65-4.59 (m, 2H), 4.53-4.39 (m, 4H), 4.25-4.15 (m, 3H), 4.07-4.01 (m, 2H), 3.86-3.80 (m, 2H), 3.72-3.66 (m, 3H), 3.54-3.52 (m, 2H), 2.22-2.17 (m, 2H), 2.00-1.91 (m, 5H), 1.85 (s, 3H), 1.56-1.54 (m, 3H), 1.43-1.40 (m, 3H), 1.34-1.31 (m, 3H); ^{13}C NMR (150 MHz, $CDCl_3$: CD_3OD = 4 : 1) δ 174.2, 173.5, 173.4, 173.0, 172.0, 171.4, 143.0, 139.1, 138.1, 137.2, 135.7, 129.3, 128.8, 128.6, 128.5, 128.5, 128.4, 128.4, 128.2, 127.9, 127.8, 127.7, 126.1, 105.0, 101.5, 101.1, 97.0, 81.5, 79.2, 76.7, 74.3, 73.9, 71.6, 68.7, 68.3, 67.0, 66.8, 65.9, 56.3, 52.5, 52.3, 30.6, 27.0, 23.2, 22.8, 17.6, 17.1, 12.4; HRMS (ESI-QTOF) Anal. Calcd for $C_{58}H_{71}N_5O_{16}Na$ $[M+Na]^+$: 1116.4794, found: 1116.4772.

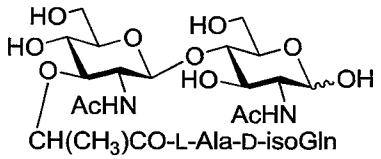


64

2-Acetylamino-4-*O*-[[2-acetylamino-4,6-*O*-benzylidene-2-deoxy-3-*O*-[(*R*)-propionyl-{benzyl-(L-alanyl-D-isoglutaminate)}]-β-D-glucopyranosyl]]-3,6-di-*O*-benzyl-2-deoxy-D-glucopyranoside (64)

To a solution of **63** (20 mg, 0.018 mmol) in THF (5 mL), iodine (10 mg, 0.039 mmol) and water (0.5 mL) were added and the reaction mixture was stirred for 2 h. The reaction was quenched by the

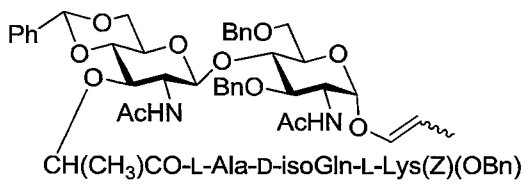
addition of $Na_2S_2O_3$ aq (5%, 10 mL). The mixture was then extracted with $CHCl_3$ (20 mL). The organic layer was washed with $Na_2S_2O_3$ aq (5%, 10 mL×2), sat. $NaHCO_3$ aq (20 mL×2), and brine (20 mL), dried over Na_2SO_4, and then concentrated in vacuo. The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 10 : 1) to give 1-liberated **64** as a white solid (12 mg, 64%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.65 (m, 1H), 7.35-7.24(m, 19H), 5.39 (s, 1H), 5.03 (m, 3H), 4.73 (d, J = 11.5 Hz, 1H), 4.62-4.53 (m, 3H), 4.42 (d, J = 11.5 Hz, 1H), 4.32 (brs, 1H), 4.10-4.01 (m, 4H), 3.93 (m, 1H), 3.83-3.63 (m, 4H), 3.48-3.39 (m, 4H), 2.42-2.36 (m, 2H), 2.11 (m, 1H), 1.88-1.81 (m, 7H), 1.25-1.19 (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.9, 173.7, 173.0, 171.9, 171.2, 138.6, 137.6, 136.9, 135.4, 129.0, 128.4, 128.1, 128.1, 128.0, 128.0, 127.9, 127.5, 127.4, 125.8, 101.1, 100.7, 90.5, 87.8, 81.2, 78.6, 76.4, 73.7, 73.6, 70.9, 68.6, 68.3, 66.5, 65.7, 55.9, 52.2, 51.9, 30.2, 27.0, 22.9, 22.6, 19.0, 16.6; HRMS (ESI-QTOF) Anal. Calcd for $C_{55}H_{67}N_5O_{16}Na$ $[M+Na]^+$: 1076.4481, found: 1076.4492.



2a

2-Acetylamino-4-*O*-[2-acetylamino-2-deoxy-3-*O*-{(*R*)-propionyL-(L-alanyl-D-isoglutamine)}-β-D-glucopyranosyl]-2-deoxy-D-glucopyranoside (2a)

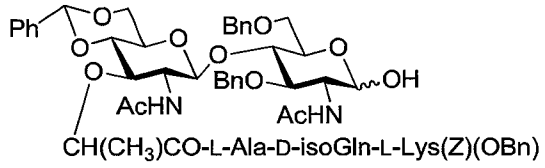
To a solution of **64** (11 mg, 0.01 mmol) in AcOH (4 mL) was added palladium hydroxide (60 mg) in AcOH and stirred under H_2 (2 MPa) for 1 d. The reaction was monitored by TLC analysis and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite, and the filtrate was concentrated. The residue was lyophilized from acetonitrile-H_2O to give **2a** (6.5 mg, 91%) as a white solid. ^{1}H NMR (500 MHz, D_2O) δ 5.11 (d, J = 2.6 Hz, 1H), 4.50 (d, J = 8.6 Hz, 1H), 4.26-4.15 (m, 3H), 3.87-3.44 (m, 12H), 2.26 (dd, J = 7.8 Hz, J = 7.0 Hz, 2H), 2.06-1.87 (m, 8H), 1.36 (d, J = 7.2 Hz, 3H), 1.30 (d, J = 6.7 Hz, 3H); ^{13}C NMR (125 MHz, D_2O) δ 177.0, 176.2, 175.8, 175.4, 175.1, 174.7, 102.0, 95.5, 91.1, 83.1, 79.9, 78.9, 76.3, 75.2, 73.2, 70.7, 69.9, 69.4, 61.2, 60.8, 60.7, 56.8, 55.7, 54.3, 53.9, 50.4, 33.3, 27.9, 22.9, 22.6, 19.4, 17.3; HRMS (ESI-QTOF) Anal. Calcd for $C_{27}H_{44}N_5O_{16}Na_2$ $[M+2Na-H]^+$: 740.2578, found: 740.2542.



65

Prop-1-enyl 2-acetylamino-4-*O*-[[2-acetylamino-4,6-*O*-benzylidene-2-deoxy-3-*O*-[(*R*)-propionyl-{benzyl-(L-alanyl-D-isoglutamyl-ε-N-benzyloxycarbonyl-L-lysinate)}]-β-D-glucopyranosyl]]-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside (65)

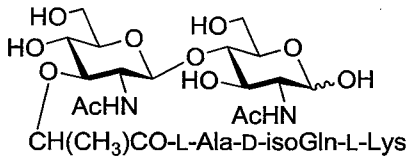
To a solution of **42** (14 mg, 0.017 mmol), HCl·L-Ala-D-isoGln-L-Lys(OBn) (14 mg, 0.023 mmol), and HOBt (6 mg, 0.044 mmol) in DMF (1 mL) were added WSCD·HCl (6 mg, 0.037 mmol) and triethylamine (10 μL, 0.079 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 20 mL), H_2O (20 mL), sat. $NaHCO_3$ aq (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 18 : 1) to give **65** as a white solid (14 mg, 59%). ^{1}H NMR (500 MHz, $CDCl_3$: CD_3OD = 4 : 1) δ 7.79 (m, 1H), 7.44-7.29 (m, 28H), 6.13 (d, J = 13.5 Hz, 1H), 5.58 (m, 1H), 5.45 (s, 1H), 5.20-5.02 (m, 7H), 4.87 (d, J = 11.5 Hz, 1H), 4.73 (m, 1H), 4.62-4.59 (m, 2H), 4.60-4.45 (m, 4H), 4.28-4.00 (m, 5H), 3.88-3.67 (m, 5H), 3.54-3.52 (m, 2H), 3.13-3.11 (m, 2H), 2.31-2.27 (m, 2H), 2.11-2.09 (m, 1H), 1.93 (s, 4H), 1.85 (s, 4H), 1.73 (m, 1H), 1.55-1.54 (m, 3H), 1.48-1.42 (m, 4H), 1.36-1.26 (m, 6H); HRMS (ESI-QTOF) Anal. Calcd for $C_{72}H_{89}N_7O_{19}Na$ $[M+Na]^+$: 1378.6111, found: 1378.6103.



66

2-Acetylamino-4-*O*-[[2-acetylamino-4,6-*O*-benzylidene-2-deoxy-3-*O*-[(*R*)-propionyl-{benzyl-(L-alanyl-D-isoglutamyl-ε-N-benzyloxycarbonyl-L-lysinate)}]-β-D-glucopyranosyl]]-3,6-di-*O*-benzyl-2-deoxy-D-glucopyranoside (66)

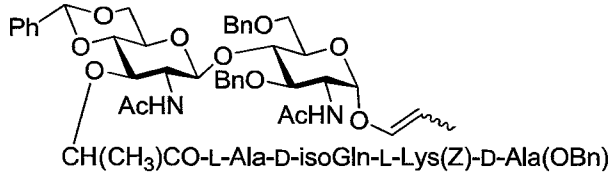
To a solution of **65** (14 mg, 0.010 mmol) in THF (5 mL), iodine (10 mg, 0.039 mmol) and water (0.5 mL) were added and the reaction mixture was stirred for 2 h. The reaction was quenched by the addition of $Na_2S_2O_3$ aq (5%, 10 mL). The mixture was then extracted with $CHCl_3$ (20 mL). The organic layer was washed with $Na_2S_2O_3$ aq (5%, 10 mL×2), sat. $NaHCO_3$ aq (20 mL×2), and brine (20 mL), dried over Na_2SO_4, and then concentrated in vacuo. The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 10 : 1) to give 1-liberated **66** as a white solid (7 mg, 54%). ^{1}H NMR (500 MHz, DMF-*d*7) δ 8.18 (d, J = 8.2 Hz, 2H), 7.87 (m, 1H), 7.68 (m, 1H), 7.54-7.34 (m, 19H), 7.08 (brs, 1H), 6.89 (d, J = 4.4 Hz, 1H), 5.76 (s, 1H), 5.24-4.94 (m, 4H), 4.79-4.70 (m, 3H), 4.44-4.42 (m, 2H), 4.24 (m, 1H), 4.15-3.93 (m, 7H), 3.87-3.83 (m, 2H), 3.76-3.68 (m, 2H), 3.40-3.36 (m, 4H), 2.50-2.40 (m, 2H), 2.19 (m, 1H), 2.03 (s, 3H), 1.97 (s, 4H), 1.54-1.34 (m, 8H), 1.18 (t, J = 7.1 Hz, 2H), 1.08 (t, J = 7.1 Hz, 2H); ^{13}C NMR (125 MHz, DMSO-*d*6) δ 173.0, 172.0, 171.9, 171.8, 171.5, 169.5, 169.1, 156.0, 139.3, 138.4, 137.5, 137.2, 135.9, 128.7, 128.3, 128.3, 128.2, 128.0, 127.9, 127.9, 127.8, 127.7, 127.6, 127.6, 127.4, 127.3, 127.2, 127.1, 127.1, 127.0, 125.8, 100.0, 90.6, 79.9, 78.7, 77.5, 77.3, 76.6, 73.3, 71.9, 69.7, 67.7, 65.7, 65.6, 65.0, 55.6, 52.7, 52.1, 48.0, 31.4, 30.4, 28.9, 27.8, 23.0, 22.6, 22.5, 18.8, 18.2; HRMS (ESI-QTOF) Anal. Calcd for $C_{69}H_{85}N_7O_{19}Na$ $[M+Na]^+$: 1338.5798, found: 1338.5793.



2c

2-Acetylamino-4-*O*-[2-acetylamino-3-*O*-{(*R*)-propionyl-(L-alanyl-D-isoglutamyl-L-lysine)}-2-deoxy-β-D-glucopyranosyl]-2-deoxy-D-glucopyranoside (2c)

To a solution of **66** (5 mg, 0.01 mmol) in AcOH (4 mL) was added palladium hydroxide (17 mg) in AcOH and stirred under H_2 (2 MPa) for 1 d. The reaction was monitored by TLC analysis and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite and the filtrate was concentrated. The residue was lyophilized from acetonitrile-H_2O to give **2c** (3 mg, 89%) as a white solid. ^{1}H NMR (500 MHz, D_2O) δ 5.11 (d, J = 2.6 Hz, 1H), 4.49 (d, J = 8.5 Hz, 1H), 4.27 (dd, J = 9.5 Hz, J = 4.5 Hz, 1H), 4.22-4.13 (m, 2H), 3.87-3.44 (m, 14H), 3.32-3.26 (m, 3H), 2.47-2.32 (m, 2H), 2.15-1.88 (m, 8H), 1.36-1.29 (m, 7H), 1.11-1.00 (m, 5H); ^{13}C NMR (150 MHz, D_2O) δ 178.9, 175.9, 175.6, 175.1, 174.5, 174.2, 174.0, 101.3, 94.8, 90.4, 82.5, 79.7, 78.2, 75.6, 78.2, 75.6, 74.6, 72.5, 70.0, 69.3, 68.7, 61.3, 60.6, 60.1, 54.9, 53.6, 49.7, 39.3, 31.9, 31.1, 27.0, 26.3, 27.0, 26.3, 22.2, 22.1, 21.9, 18.8, 16.6; HRMS (ESI-QTOF) Anal. Calcd for $C_{33}H_{57}N_7O_{17}Na$ $[M+Na]^+$: 846.3709, found: 846.3697.

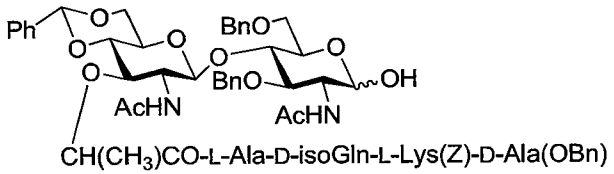


67

Prop-1-enyl 2-acetylamino-4-*O*-[[2-acetylamino-4,6-*O*-benzylidene-2-deoxy-3-*O*-[(*R*)-propionyl-{benzyl-(L-alanyl-D-isoglutamyl-ε-*N*-benzyloxycarbonyl-L-lysyl)-D-alaninate}]-β-D-glucopyranosyl]]-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside (67)

To a solution of **42** (10 mg, 0.012 mmol), HCl·L-Ala-D-isoGln-L-Lys(Z)-D-Ala(OBn) (12 mg, 0.019 mmol), and HOBt (6 mg, 0.044 mmol) in DMF (3 mL) were added WSCD·HCl (6 mg, 0.037 mmol) and triethylamine (6 μL, 0.04 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 20 mL), H_2O (20 mL), sat. $NaHCO_3$ aq (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 20 : 1) to give **67** as a white solid (8.8 mg, 48%). ^{1}H NMR (400 MHz, DMF-*d*7) δ 8.30 (d, J = 7.2 Hz, 1H), 8.17-8.14 (m, 3H), 7.91 (d, J = 7.2 Hz, 1H), 7.59 (m, 1H), 7.49-7.28 (m, 26H), 7.11-7.07 (m, 2H), 6.30 (m, 1H), 5.69 (s, 1H), 5.16-5.02 (m, 6H), 4.89 (m, 1H), 4.74-4.60 (m, 3H), 4.44-4.35 (m, 4H), 4.19-3.54 (m, 14H), 3.31 (m, 1H), 3.08-3.05 (m, 2H), 2.32-2.30 (m, 2H), 2.19 (m, 1H), 1.96-1.75 (m, 7H), 1.61-1.20 (m, 18H); ^{13}C

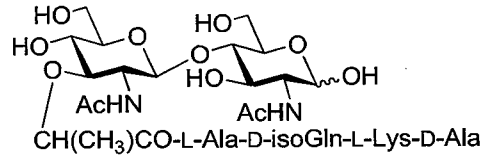
NMR (100 MHz, DMF-*d7*) δ 174.1, 173.2, 172.7, 170.8, 170.2, 157.1, 139.7, 138.7, 138.5, 137.1, 129.4, 129.1, 129.0, 128.9, 128.7, 128.6, 128.4, 128.3, 128.1, 128.1, 128.0, 127.9, 127.9, 127.7, 126.7, 102.1, 101.3, 98.0, 81.5, 80.1, 78.8, 78.7, 74.5, 73.0, 69.3, 66.7, 66.0, 56.9, 53.9, 53.1, 49.8, 48.8, 41.2, 32.7, 32.6, 29.5, 29.0, 23.6, 22.7, 19.4, 18.1, 17.3, 12.4; HRMS (ESI-QTOF) Anal. Calcd for $C_{75}H_{94}N_8O_{20}Na$ $[M+Na]^+$: 1449.6482, found: 1449.6439.



68

2-Acetylamino-4-*O*-[[2-acetylamino-4,6-*O*-benzylidene-2-deoxy-3-*O*-[(*R*)-propionyl-{benzyl-(L-alanyl-D-isoglutamyl-ε-*N*-benzyloxycarbonyl-L-lysyl)-D-alaninate}]-β-D-glucopyranosyl]]-3,6-di-*O*-benzyl-2-deoxy-D-glucopyranoside (68)

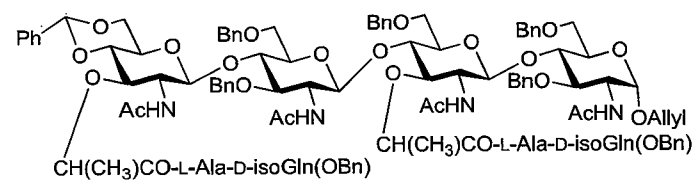
To a solution of **67** (4.5 mg, 0.004 mmol) in THF (2 mL), iodine (5 mg, 0.02 mmol) and water (0.5 mL) were added and the reaction mixture was stirred for 2 h. The reaction was quenched by the addition of $Na_2S_2O_3$ aq (5%, 10 ml). The mixture was then extracted with $CHCl_3$ (20 mL). The organic layer was washed with $Na_2S_2O_3$ aq (5%, 10 mL×2), sat. $NaHCO_3$ aq (20 mL×2), and brine (20 mL), dried over Na_2SO_4, and then concentrated in vacuo. The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 10 : 1) to give 1-liberated **68** as a white solid (2.8 mg, 64%). HRMS (ESI-QTOF) Anal. Calcd for $C_{72}H_{90}N_8O_{20}Na$ $[M+Na]^+$: 1409.6169, found: 1409.6152.



2e

2-Acetylamino-4-*O*-[2-acetylamino-3-*O*-{(*R*)-propionyl-(L-alanyl-D-isoglutamyl-L-lysiyl-D-alanine)}-2-deoxy-β-D-glucopyranosyl]-2-deoxy-D-glucopyranoside (2e)

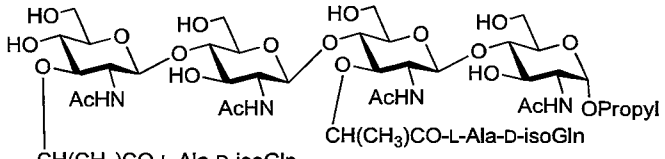
To a solution of **68** (1.6 mg, 0.001 mmol) in AcOH (1 mL) was added palladium hydroxide (10 mg) in AcOH and stirred under H_2 (2 MPa) for 1 d. The reaction was monitored by TLC analysis and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite and the filtrate was concentrated. The residue was lyophilized from acetonitrile-H_2O to give **2e** (0.9 mg, 90%) as a white solid. HRMS (ESI-QTOF) Anal. Calcd for $C_{36}H_{63}N_8O_{18}$ $[M+H]^+$: 895.4260, found: 895.4213.



69

Protected tetrasaccharide (MurNAc-GlcNAc-MurNAc-GlcNAc) with dipeptide (69)

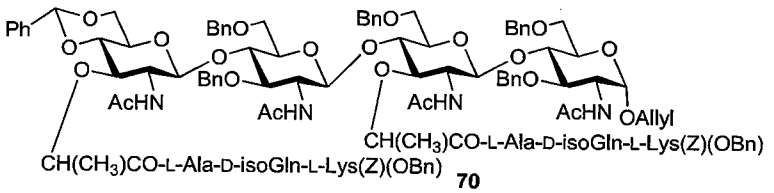
To a solution of **44** (25 mg, 0.016 mmol), HCl·L-Ala-D-isoGln(OBn) (22 mg, 0.064 mmol), and HOBt (7 mg, 0.048 mmol) in DMF (3 mL) were added WSCD·HCl (8 mg, 0.048 mmol) and triethylamine (15 μL, 0.096 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 20 mL), H_2O (20 mL), sat. $NaHCO_3$ aq (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 15 : 1) to give **69** as a white solid (28 mg, 82%). ^1H NMR (400 MHz, DMF-*d*7) δ 8.12-8.04 (m, 3H), 7.85 (d, J = 9.2 Hz, 1H), 7.68-7.00 (m, 48H), 5.90 (m, 1H), 5.70 (s, 1H), 5.14 (dd, J = 17.2 Hz, J = 1.9 Hz, 1H), 5.08-5.17 (m, 6H), 4.95 (d, J = 11.2 Hz, 1H), 4.84 (d, J = 8.4 Hz, 1H), 4.76 (d, J = 3.7 Hz, 1H), 4.64-4.29 (m, 15H), 4.20-3.57 (m, 24H), 3.25-3.15 (m, 3H), 2.49 (t, J = 8.0Hz, 4H), 2.31-2.20 (m, 2H), 1.86-1.77 (m, 14H), 1.29-1.20 (m, 12H); ^{13}C NMR (100 MHz, DMF-*d*7) δ 173.9, 173.9, 173.3, 173.2, 173.1, 173.0, 170.8, 170.8, 170.1, 140.7, 140.4, 139.8, 139.7, 139.6, 138.7, 137.3, 137.3, 135.3, 129.4, 129.1, 128.9, 128.7, 128.6, 138.5, 128.2, 128.2, 128.1, 128.0, 127.9, 127.8, 127.4, 126,7, 116,9, 101.8, 101.3, 97.5, 92.9, 81.6, 80.2, 80.0, 79.8, 79.6, 77.2, 75.3, 74.5, 73.0, 72.9, 71.0, 69.2, 68.8, 68.5, 66.7, 66.3, 53.3, 52.8, 50.0, 49.6, 36.0, 31.0, 28.0, 23.7, 23.6, 23.4, 22.8, 19.4, 19.2, 18.3, 17.7; HRMS (ESI-QTOF) Anal. Calcd for $C_{113}H_{138}N_{10}O_{31}Na_2$ $[M+2Na]^{2+}$: 1088.4662, found: 1088.4655.



2b

Tetrasaccharide (MurNAc-GlcNAc-MurNAc-GlcNAc) with dipeptide (2b)

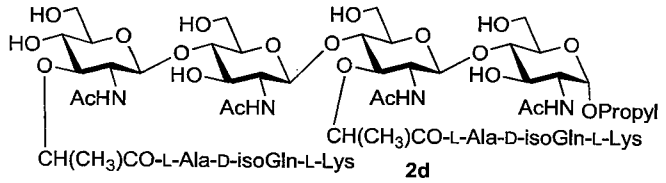
To a solution of **69** (28 mg, 0.013 mmol) in AcOH (1 mL) was added palladium hydroxide (55 mg) in AcOH and stirred under H_2 (2 MPa) for 1 d. The reaction was monitored by TLC analysis and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite and the filtrate was concentrated. The residue was lyophilized from acetonitrile-H_2O to give **2b** (16.6 mg, 89%) as a white solid. ^1H NMR (500 MHz, CD_3OD) δ 4.81-4.25 (m, 9H), 3.87-3.29 (m, 26H), 2.38 (brs, 4H), 2.19 (brs, 2H), 1.97 (m, 14H), 1.60-1.58 (m, 2H), 1.43-1.34 (m, 12H), 0.95-0.91 (dd, J = 12.0 Hz, J = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, CD_3OD) δ 176.3, 175.9, 175.6, 175.2, 175.2, 173.9, 173.9, 173.8, 173.5, 103.3, 103.1, 101.8, 98.2, 83.4, 81.9, 81.6, 80.0, 78.7, 78.4, 78.0, 77.4, 76.8, 76.0, 73.9, 72.2, 71.2, 70.9, 70.8, 62.5, 62.0, 61.6, 61.4, 57.0, 56.4, 55.9, 55.1, 53.9, 53.8, 50.7, 49.6, 31.6, 28.8, 28.4, 23.7, 23.4, 22.5, 19.5, 18.9, 17.7, 17.5, 11.0; HRMS (ESI-QTOF) Anal. Calcd for $C_{57}H_{94}N_{10}O_{31}Na$ $[M+Na]^+$: 1437.5984, found: 1437.6008.



70

Protected tetrasaccharide (MurNAc-GlcNAc-MurNAc-GlcNAc) with tripeptide (70)

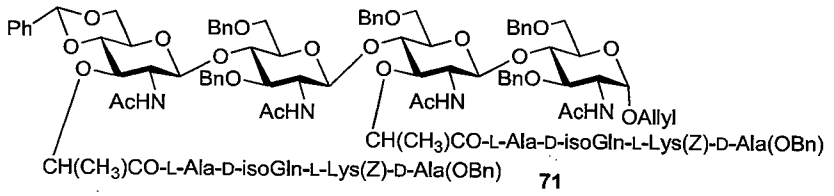
To a solution of **44** (17 mg, 0.011 mmol), HCl·L-Ala-D-isoGln-L-Lys(Z)(OBn) (26 mg, 0.044 mmol), and HOBt (5 mg, 0.033 mmol) in DMF (3 mL) were added WSCD·HCl (6 mg, 0.037 mmol) and triethylamine (10 μL, 0.04 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was concentrated, and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 20 mL), H_2O (20 mL), sat. $NaHCO_3$ aq (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 12 : 1) to give **70** as a white solid (15 mg, 53%). ^1H NMR (500 MHz, $CDCl_3$) δ 7.52-7.26 (m, 50H), 5.83 (m, 1H), 5.45 (s, 1H), 5.15-5.07 (m, 8H), 4.82-3.12 (m, 54H), 2.28-2.17 (m, 4H), 2.04-1.74 (m, 16H), 1.43-1.25 (m, 24H); HRMS (ESI-QTOF) Anal. Calcd for $C_{141}H_{174}N_{14}O_{37}Na_2$ $[M+2Na]^{2+}$: 1350.5980, found: 1350.5985.



2d

Tetrasaccharide (MurNAc-GlcNAc-MurNAc-GlcNAc) with tripeptide (2d)

To a solution of **70** (7 mg, 0.003 mmol) in AcOH (1 mL) was added palladium hydroxide (12 mg) in AcOH and stirred under H_2 (2 MPa) for 1 d. The reaction was monitored by TLC analysis and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite, and the filtrate was concentrated. The residue was lyophilized from acetonitrile-H_2O to give **2d** (3.7 mg, 67%) as a white solid. ^1H NMR (400 MHz, D_2O) δ 4.40-4.35 (m, 2H), 4.26 (m, 1H), 4.10-4.03 (m, 5H), 3.79-3.20 (m, 29H), 2.84 (t, J = 7.0 Hz, 4H), 2.26-2.24 (m, 4H), 2.07-2.03 (m, 2H), 1.90-1.80 (m, 14H), 1.66 (m, 2H), 1.58-1.51 (m, 6H), 1.43-1.39 (m, 2H), 1.31-1.21 (m, 16H), 0.74 (t, J = 7.4 Hz, 3H); HRMS (ESI-QTOF) Anal. Calcd for $C_{69}H_{119}N_{14}O_{33}K$ $[M+H+K]^{2+}$: 855.3851, found: 855.3832.

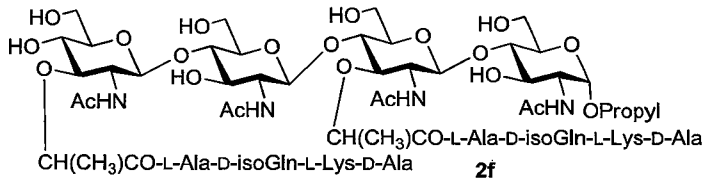


71

Protected tetrasaccharide (MurNAc-GlcNAc-MurNAc-GlcNAc) with tetrapeptide (71)

To a solution of **44** (15 mg, 0.006 mmol) and HCl·L-Ala-D-isoGln-L-Lys(Z)-D-Ala(OBn) (19 mg,

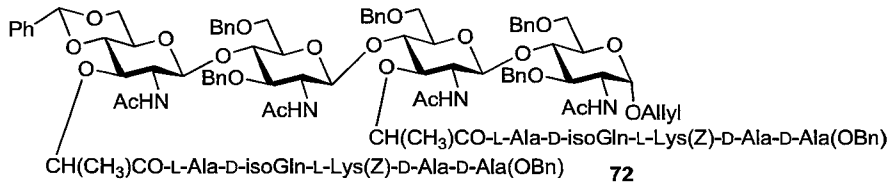
0.019 mmol) in DMF (3 mL) were added HATU (11 mg, 0.019 mmol) and triethylamine (8 μL, 0.039 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was concentrated, and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 20 mL), H_2O (20 mL), sat. $NaHCO_3$ aq (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 15 : 1) to give **71** as a white solid (23 mg, 81%). ^1H NMR (500 MHz, $CDCl_3$: CD_3OD = 4 : 1) δ 7.41-7.21 (m, 50H), 5.85 (m, 1H), 5.43 (s, 1H), 5.25-5.06 (m, 8H), 4.93-4.81 (m, 2H), 4.68-4.25 (m, 16H), 4.13-3.89 (m, 11H), 3.47-3.36 (m, 11H), 3.12 (m, 4H), 2.35-2.18 (m, 6H), 2.02-1.79 (m, 14H), 1.48-1.10 (m, 30H); HRMS (ESI-QTOF) Anal. Calcd for $C_{147}H_{184}N_{16}O_{39}Na_2$ $[M+2Na]^{2+}$: 1421.6351, found: 1421.6357.



2f

Tetrasaccharide (MurNAc-GlcNAc-MurNAc-GlcNAc) with tetrapeptide (2f)

To a solution of **71** (22 mg, 0.008 mmol) in AcOH (1 mL) was added palladium hydroxide (33 mg) in AcOH and stirred under H_2 (2 MPa) for 1 d. The reaction was monitored by TLC analysis and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite and the filtrate was concentrated. The residue was lyophilized from acetonitrile-H_2O to give **2f** (5.4 mg, 38%) as a white solid. ^1H NMR (400 MHz, D_2O) δ 4.39-4.37 (m, 3H), 4.25-4.22 (m, 2H), 4.17-4.10 (m, 8H), 3.76-3.15 (m, 26H), 2.85 (t, J = 7.2 Hz, 4H), 2.28-2.26 (m, 4H), 2.07-2.03 (m, 2H), 1.90-1.80 (m, 14H), 1.63-1.52 (m, 10H), 1.44-1.42 (m, 2H), 1.31-1.17 (m, 20H), 0.74(t, J = 7.4 Hz, 3H); HRMS (ESI-QTOF) Anal. Calcd for $C_{75}H_{129}N_{16}O_{35}Na$ $[M+H+Na]^{2+}$: 918.4352, found: 918.4310.

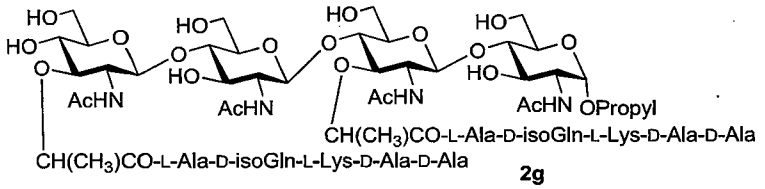


72

Protected tetrasaccharide (MurNAc-GlcNAc-MurNAc-GlcNAc) with pentapeptide (72)

To a solution of **44** (15 mg, 0.006 mmol) and HCl·L-Ala-D-isoGln-L-Lys(Z)-D-Ala-D-Ala(OBn) (22 mg, 0.019 mmol) in DMF (3 mL) were added HATU (11 mg, 0.019 mmol) and triethylamine (8 μL, 0.039 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 20 mL), H_2O (20 mL), sat. $NaHCO_3$ aq (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 15 : 1) to give **72** as a white solid (20 mg, 71%). HRMS (ESI-QTOF) Anal. Calcd

for $C_{153}H_{194}N_{18}O_{41}Na_2$ $[M+2Na]^{2+}$: 1492.6722, found: 1492.6753.

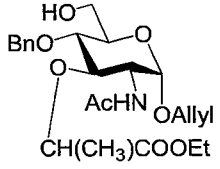


2g

Tetrasaccharide (MurNAc-GlcNAc-MurNAc-GlcNAc) with pentapeptide (2g)

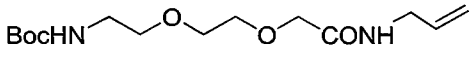
To a solution of **72** (20 mg, 0.007 mmol) in AcOH (1 mL) was added palladium hydroxide (29 mg) in AcOH and stirred under H_2 (2 MPa) for 1 d. The reaction was monitored by TLC analysis and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite and the filtrate was concentrated. The residue was lyophilized from acetonitrile-H_2O to give **2g** (6.2 mg, 45%) as a white solid. ^1H NMR (400 MHz, D_2O) δ 4.43-4.38 (m, 2H), 4.30-4.06 (m, 11H), 3.88-3.25 (m, 28H), 2.87 (t, J = 7.6 Hz, 4H), 2.29-2.26 (m, 4H), 2.09-2.01 (m, 2H), 1.92-1.82 (m, 14H), 1.67-1.53 (m, 8H), 1.48-1.41 (m, 2H), 1.33-1.24 (m, 28H), 0.77 (t, J = 7.6 Hz, 3H); HRMS (ESI-QTOF) Anal. Calcd for $C_{81}H_{140}N_{18}O_{37}$ $[M+2H]^{2+}$: 978.4813, found: 978.4783.

Experiments in Chapter III



75

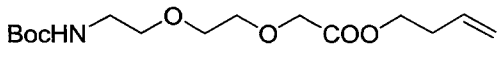
A solution of **73** (63 mg, 0.14 mmol) in DCM (5 mL) and MS4A were stirred for 30 min under Ar at rt. The suspension was cooled to -78 °C and Et_3SiH (70 μL, 0.42 mmol) and $PhBCl_2$ (60 μL, 0.42 mmol) were added. The mixture was stirred for 2 h. Et_3N (1 mL) and MeOH (1 mL) were added and the mixture was stirred for 15 min, then warmed up to rt, diluted with EtOAc (10 mL) and filtered over a pad of Celite. The filtrate was diluted with EtOAc (30 mL) and washed with H_2O (20 mL), sat. $NaHCO_3$ aq (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (10 g, $CHCl_3$: acetone = 8 : 1) to give **84** as a colorless oil (61 mg, 96%). ^1H NMR (500 MHz, $CDCl_3$) δ 7.92-7.91 (m, 1H), 7.40-7.30 (m, 5H), 5.88-5.80 (m, 1H), 5.28(d, J = 3.7 Hz, 1H), 5.26-5.21 (m, 1H), 5.15-5.12 (m, 1H), 4.73 (s, 2H), 7.53 (dd, J = 9.0 Hz, J = 17.5 Hz, 1H), 4.25-4.15 (m, 2H), 4.07 (m, 1H), 3.99-3.94 (m, 1H), 3.84-3.62 (m, 6H), 2.36-2.33 (m, 1H), 2.04 (s, 3H), 1.39 (d, J = 8.5 Hz, 3H), 1.27 (t, J = 9.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.3, 171.0, 137.6, 134.0, 128.6, 128.0, 127.9, 127.7, 127.6, 116.8, 96.0, 79.6, 76.7, 76.6, 75.1, 75.0, 71.6, 68.4, 61.3, 61.2, 54.3, 23.1, 19.1, 14.1.



84

***tert*-Butyl 2-(2-(2-(allylamino)-2-oxoethoxy)ethoxy)ethylcarbamate (84)**

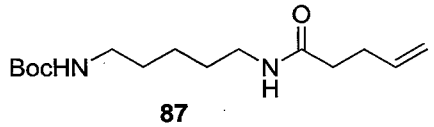
To a solution of Boc-NH-(PEG)-COOHDCHA (50 mg, 0. 11 mmol), allyl amine (34 μL, 0.45 mmol), and HOBt (23 mg, 0.17 mmol) in DCM (3 mL) were added WSCD·HCl (27 mg, 0.17 mmol) and triethylamine (31 μL, 0.22 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 20 mL), H_2O (20 mL), sat. $NaHCO_3$ aq (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 15 : 1) to give **84** as a colorless oil (24 mg, 72%). ^1H NMR (500 MHz, $CDCl_3$) δ 6.99 (brs, 1H), 5.90-5.83 (m, 1H), 5.25-5.15 (m, 2H), 4.88 (brs, 1H), 4.02 (s, 2H), 3.96-3.93 (m, 2H), 3.70-3.67 (m, 2H), 3.65-3.62 (m, 2H), 3.56-3.54 (t, J = 5.0 Hz, 2H), 3.33-3.31 (m, 2H), 1.45(s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.6, 155.9, 134.1, 116.4, 79.4, 71.0, 70.5, 70.4, 70.0, 41.1, 40.3, 28.4; MALDI-MS (positive): m/z = 325.45 $[M+Na]^+$.



85

Boc protected ester linker (85)

To a solution of Boc-NH-(PEG)-COOHDCHA (100 mg, 0.22 mmol), 3-buten-1-ol (78 μL, 0.89 mmol), and HOBt (46 mg, 0.33 mmol) in DCM (3 mL) were added WSCD·HCl (54 mg, 0.33 mmol) and triethylamine (62 μL, 0.45 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 20 mL), H_2O (20 mL), sat. $NaHCO_3$ aq (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (10 g, toluene : EtOAc = 3 : 1) to give **85** as a colorless oil (65 mg, 91%). ^1H NMR (500 MHz, $CDCl_3$) δ 5.82-5.74 (m, 1H), 5.14-5.03 (m, 3H), 4.22 (t, J = 6.5 Hz, 2H), 4.14 (s, 2H), 3.72-3.70 (m, 2H), 3.67-3.64 (m, 2H), 3.56-3.54 (t, J = 5.0 Hz, 2H), 3.32-3.31 (m, 2H), 2.44-2.39 (m, 2H), 1.44 (s, 9H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.4, 156.0, 133.7, 117.5, 79.2, 70.9, 70.4, 70.3, 68.6, 63.8, 40.4, 33.0, 28.4; HRMS (ESI-QTOF) Anal. Calcd for $C_{15}H_{27}NO_6Na$ $[M+Na]^+$: 340.1736, found: 340.1732.

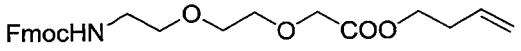


87

***tert*-Butyl 5-pent-4-enamidopentylcarbamate (87)**

To a solution of mono-*t*-butoxycarbonyl 1,5-diaminopentane toluenesulfonic acid salt (100 mg, 0.27 mmol), pent-4-enoic acid (87 μL, 0.8 mmol), and HOBt (156 mg, 1.2 mmol) in DCM (3 mL) were added WSCD·HCl (188 mg, 1.2 mmol) and triethylamine (160 μL, 1.2 mmol) at 0 °C and the

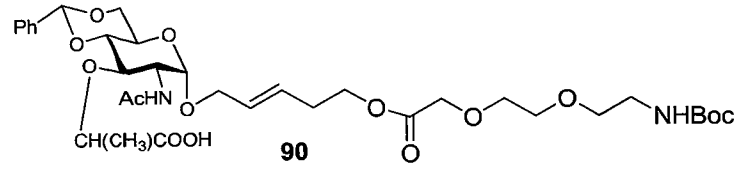
mixture was stirred at rt overnight. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 20 mL), H_2O (20 mL), sat. $NaHCO_3$ aq (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 15 : 1) to give **87** as a white solid (64 mg, 84%). ^1H NMR (400 MHz, $CDCl_3$) δ 5.87-5.77 (m, 2H), 5.09-4.98 (m, 2H), 3.25 (dd, J = 6.8 Hz, J = 12.8 Hz, 2H), 3.11 (dd, J = 6.4 Hz, J = 12.8 Hz, 2H), 2.42-2.36 (m, 2H), 2.28-2.24 (m, 2H), 1.60-1.43 (m, 13H), 1.37-1.25 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.3, 156.1, 137.1, 115.4, 79.0, 40.2, 39.2, 35.8, 29.6, 29.1, 28.4, 23.9; MALDI-MS (positive): m/z = 307.43 $[M+Na]^+$.



89

Fmoc protected ester linker (89)

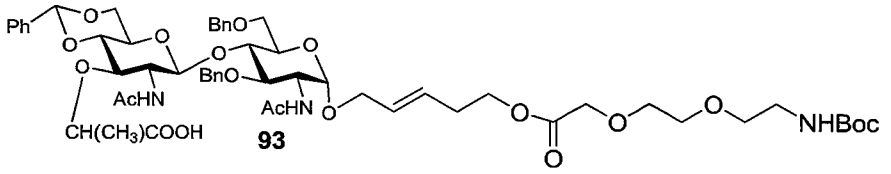
To a solution of Fmoc-NH-(PEG)-COOH (99 mg, 0.26 mmol), 3-buten-1-ol (89 μL, 1.0 mmol), and HOBt (42 mg, 0.31 mmol) in DCM (3 mL) were added WSCD·HCl (50 mg, 0.31 mmol) and triethylamine (39 μL, 0.28 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was concentrated, and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 20 mL), H_2O (20 mL), sat. $NaHCO_3$ aq (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (10 g, toluene : EtOAc = 3 : 1) to give **89** as a colorless oil (70 mg, 62%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, J = 7.6 Hz, 2H), 7.61 (d, J = 7.2 Hz, 2H), 7.41-7.36 (m, 2H), 7.33-7.26 (m, 2H), 5.80-5.70 (m, 1H), 5.40 (brs, 1H), 5.12-5.00 (m, 2H), 4.39 (d, J = 7.2 Hz, 2H), 4.23-4.19 (m, 3H), 3.71-3.66 (m, 4H), 3.59-3.57 (m, 2H), 3.43-3.41 (m, 2H), 2.39 (dd, J = 6.8 Hz, J = 13.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.3, 156.5, 144.0, 141.3, 133.6, 127.6, 127.0, 125.0, 119.9, 117.5, 70.9, 70.3, 70.1, 68.5, 66.6, 63.8, 47.2, 40.9, 33.0. HRMS (ESI-QTOF) Anal. Calcd for $C_{25}H_{29}NO_6Na$ $[M+Na]^+$: 462.1893, found: 462.1888.



90

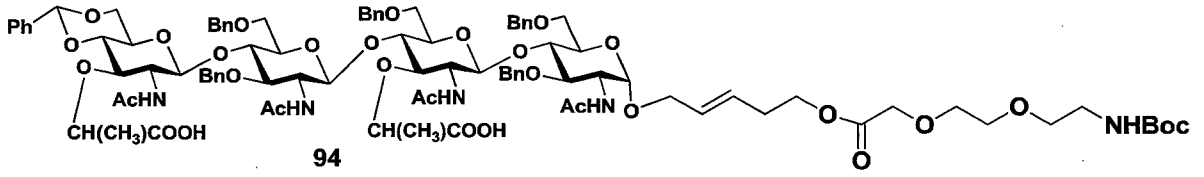
Protected muramic acid with linker (90)

Solution of monosaccharide **81** (3.8 mg, 0.1 mmol) and Boc protected ester linker **85** (8.5 mg, 0.27 mmol) was mixed in dry DCM (0.5 mL) for 10 min. Grubbs catalyst 1st generation (1.5 mg, 0.002 mmol) was added to the system, and the reaction mixture was stirred at rt overnight. The flask was opened to the air for 2 h. The residue was purified by silica-gel chromatography (3 g, $CHCl_3$: MeOH = 12 : 1) to give **90** as a colerless oil (4 mg, 62%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.45 (m, 2H), 7.38-7.37 (m, 3H), 5.65-5.58 (m, 3H), 4.50 (m, 1H), 4.26-4.11 (m, 7H), 3.79-3.64 (m, 10H), 3.56-3.55 (m, 2H), 3.32 (m, 2H), 2.42-2.41 (m, 2H), 2.04 (s, 3H), 1.46-1.43 (m, 9H), 1.25 (m, 3H); MALDI-MS (positive): m/z = 733.40 $[M+Na]^+$.



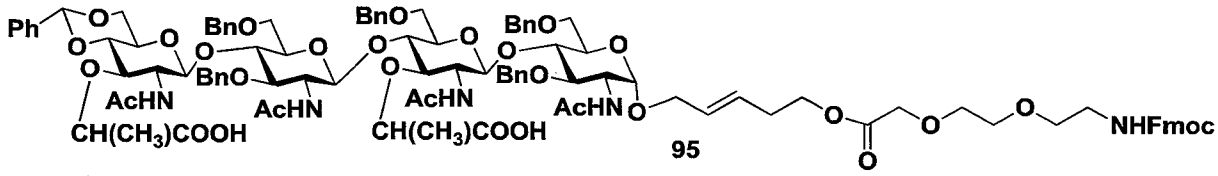
Protected disaccharide with linker (93)

Solution of disaccharide **92** (5 mg, 0.006 mmol) and Boc protected ester linker **85** (4 mg, 0.27 mmol) was refluxed in dry DCM (1 mL) for 20 min under Ar. Grubbs catalyst 2nd generation (1.1 mg, 0.001 mmol) was added to the system, and the reaction mixture was stirred at 50 °C for 1 h. The flask was cooled and opened to the air for 1 h. The residue was purified by silica-gel chromatography (3 g, $CHCl_3$: MeOH = 12 : 1) to give **93** as a colerless solid (4 mg, 62%). HRMS (ESI-QTOF) Anal. Calcd for $C_{56}H_{75}N_3O_{19}Na$ $[M+Na]^+$: 1116.4893, found: 1116.4972.



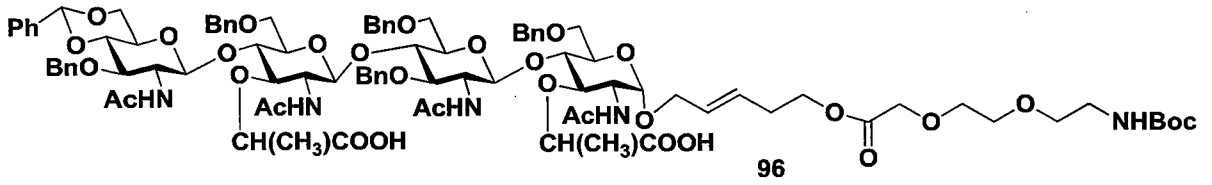
Protected tetrasaccharide (MurNAc-GlcNAc-MurNAc-GlcNAc) with Boc linker (94)

Solution of tetrasaccharide **44** (4 mg, 0.003 mmol) and Boc protected ester linker **85** (3.5 mg, 0.01 mmol) was refluxed in dry DCM (2 mL) for 20 min under Ar. Grubbs catalyst 2nd generation (1.1 mg, 0.001 mmol) was added to the system and the reaction mixture was stirred at 50 °C for 3 h. The flask was cooled and opened to the air for 1 h. The residue was purified by silica-gel chromatography (3 g, $CHCl_3$: MeOH = 10 : 1 → 5 : 1) to give **94** as a colerless solid (2.6 mg, 55%). ^1H NMR (500 MHz, CD_3OD) δ 7.69-7.54 (m, 2H), 7.41-7.06 (m, 28H), 5.67 (m, 1H), 5.47 (s, 1H), 5.08 (d, J = 12.0 Hz, 1H), 4.58-4.47 (m, 12H), 4.30-4.28 (m, 4H), 4.17-4.13 (m, 5H), 3.98-3.89 (m, 6H), 3.73-3.43 (m, 25H), 3.22-3.21 (m, 2H), 3.16-3.15 (m, 1H), 2.03-1.85 (m, 12H), 1.34-1.26 (m, 15H); MALDI-MS (positive): m/z = 1864.47 $[M+Na]^+$. HRMS (ESI-QTOF) Anal. Calcd for $C_{96}H_{123}N_5O_{31}Na_2$ $[M+2Na]^{2+}$: 944.4016, found: 944.4014.



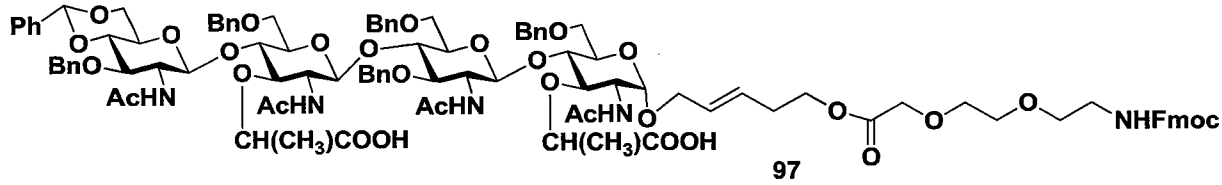
Protected tetrasaccharide (MurNAc-GlcNAc-MurNAc-GlcNAc) with Fmoc linker (95)

Solution of tetrasaccharide **44** (4 mg, 0.003 mmol) and Fmoc protected ester linker **89** (5 mg, 0.01 mmol) was reflux in dry DCM (3 mL) for 20 min under Ar. Grubbs catalyst 2nd generation (1.4 mg, 0.001 mmol) was added to the system, and the reaction mixture was stirred at 50 °C for 3 h. The flask was cooled and opened to the air for 1 h. The residue was purified by silica-gel chromatography (3 g, $CHCl_3$: MeOH = 10 : 1 → 5 : 1) to give **95** as a colerless solid (2.9 mg, 57%). MALDI-MS (positive): m/z = 1986.11 $[M+Na]^+$. HRMS (ESI-QTOF) Anal. Calcd for $C_{106}H_{124}N_5O_{31}Na_3$ $[M+3Na-H]^{2+}$: 1016.4003, found: 1016.4086.



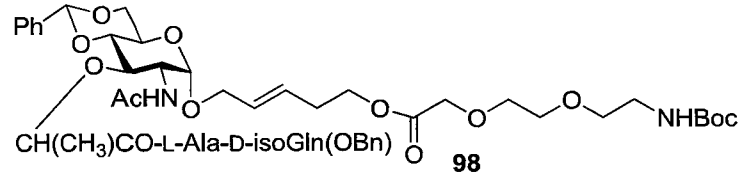
Protected tetrasaccharide (GlcNAc-MurNAc-GlcNAc-MurNAc) with Boc linker (96)

Solution of tetrasaccharide **46** (12 mg, 0.008 mmol) and Boc protected ester linker **85** (9.8 mg, 0.03 mmol) was reflux in dry DCM (3 mL) for 20 min under Ar. Grubbs catalyst 2nd generation (0.6 mg, 0.001 mmol) was added to the system, and the reaction mixture was stirred at 40 °C for 3 h. The flask was cooled and opened to the air for 1 h. The residue was purified by silica-gel chromatography (3 g, $CHCl_3$: MeOH = 10 : 1 → 5 : 1) to give **96** as a colerless solid (10 mg, 68%). MALDI-MS (positive): m/z = 1865.24 $[M+Na]^+$.



Protected tetrasaccharide (GlcNAc-MurNAc-GlcNAc-MurNAc) with Fmoc linker (97)

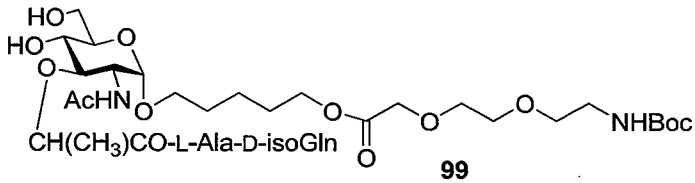
Solution of tetrasaccharide **46** (6.4 mg, 0.004 mmol) and Fmoc protected ester linker **89** (7.2 mg, 0.016 mmol) was reflux in dry DCM (2 mL) for 20 min under Ar. Grubbs catalyst 2nd generation (0.7 mg, 0.001 mmol) was added to the system and the reaction mixture was stirred at 50 °C for 3 h. The flask was cooled and opened to the air for 1 h. The residue was purified by silica-gel chromatography (3 g, $CHCl_3$: MeOH = 10 : 1 → 5 : 1) to give **97** as a colerless solid (5 mg, 62%). HRMS (ESI-QTOF) Anal. Calcd for $C_{106}H_{124}N_5O_{31}Na_3$ $[M+3Na-H]^{2+}$: 1016.4003, found: 1016.4001.



Protected MDP with Boc linker (98)

To a solution of **90** (4 mg, 0.006 mmol), HCl·L-Ala-D-isoGln(OBn) (4 mg, 0.011 mmol), and HOBt (1.2 mg, 0.009 mmol) in DCM (3 mL) were added WSCD·HCl (1.4 mg, 0.009 mmol) and triethylamine (3 μL, 0.019 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 10 mL), H_2O (10 mL), sat. $NaHCO_3$ aq (10 mL), and brine (10 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (3 g, $CHCl_3$: MeOH = 15 : 1) to give **98** as a white solid (5.1 mg, 90.6%). HRMS

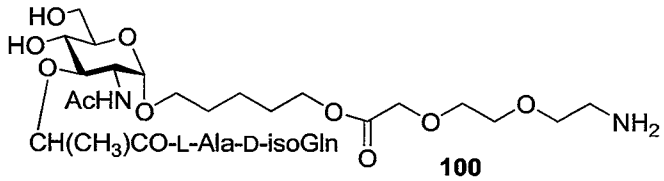
(ESI-QTOF) Anal. Calcd for $C_{49}H_{69}N_5O_{17}Na$ $[M+Na]^+$: 1022.4586, found: 1022.4594.



99

MDP with Boc linker (99)

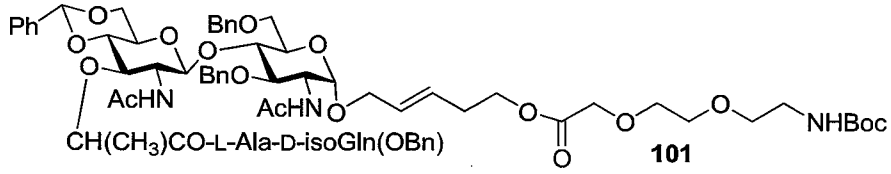
To a solution of **98** (2 mg, 0.002 mmol) in AcOH (1 mL) was added palladium hydroxide (3 mg) in AcOH and stirred under H_2 (2 MPa) for 1 d. The reaction was monitored by TLC analysis and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite, and the filtrate was concentrated. The residue was lyophilized from acetonitrile-H_2O to give **99** (1.4 mg, 90%) as a white solid. HRMS (ESI-QTOF) Anal. Calcd for $C_{35}H_{61}N_5O_{17}Na$ $[M+Na]^+$: 846.3960, found: 846.3967.



100

MDP with linker (100)

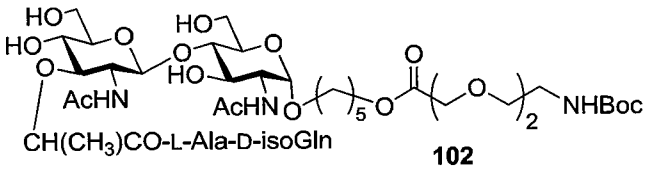
99 (1.4 mg, 0.001 mmol) was dissolved in 10% TFA in H_2O (1 mL) and stirred for 2 h. The reaction was monitored by MS (MALDI-TOF) analysis and continued until deprotection of Boc group was completed. The reaction system was diluted by H_2O and lyophilized to give **100** (1.2 mg, quant) as a white solid. HRMS (ESI-QTOF) Anal. Calcd for $C_{30}H_{54}N_5O_{15}$ $[M+H]^+$: 724.3616, found: 724.3616.



101

Protected disaccharide dipeptide (MG2P) with Boc linker (101)

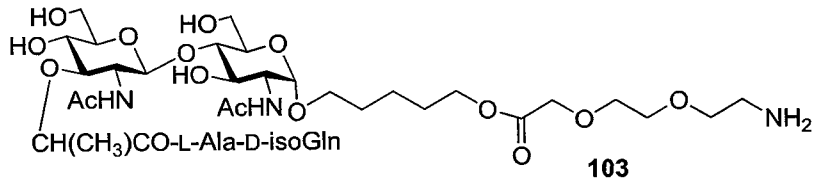
To a solution of **93** (4 mg, 0.004 mmol), HCl·L-Ala-D-isoGln(OBn) (3 mg, 0.008 mmol), and HOBt (1 mg, 0.007 mmol) in DCM (3 mL) were added WSCD·HCl (1 mg, 0.007 mmol) and triethylamine (3 μL, 0.019 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 10 mL), H_2O (10 mL), sat. $NaHCO_3$ aq (10 mL), and brine (10 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (2 g, $CHCl_3$: MeOH = 15 : 1) to give **101** as a white solid (4.3 mg, 71%). HRMS (ESI-QTOF) Anal. Calcd for $C_{71}H_{94}N_6O_{22}Na$ $[M+Na]^+$: 1405.6319, found: 1405.6317.



102

Disaccharide dipeptide (MG2P) with Boc linker (102)

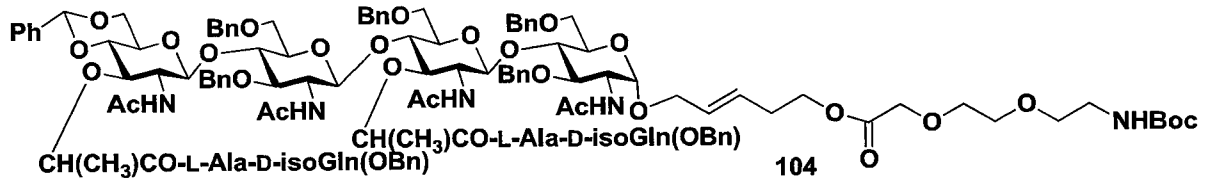
To a solution of **101** (2 mg, 0.001 mmol) in AcOH (1 mL) was added palladium hydroxide (3 mg) in AcOH and stirred under H_2 (2 MPa) for 1 d. The reaction was monitored by TLC analysis and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite and the filtrate was concentrated. The residue was lyophilized from acetonitrile-H_2O to give **102** (1 mg, 73%) as a white solid.



103

Disaccharide dipeptide (MG2P) with linker (103)

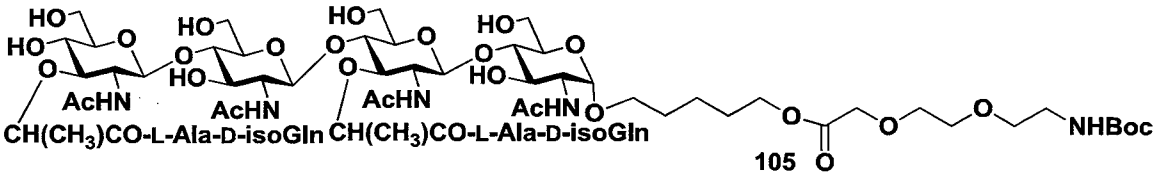
102 (1 mg, 0.001 mmol) was dissolved in 10% TFA in H_2O (1 mL) and stirred for 2 h. The reaction was monitored by MS (MALDI-TOF) analysis and continued until deprotection of Boc group was completed. The reaction system was diluted by H_2O and lyophilized to give **103** (0.9 mg, quant) as a white solid. MALDI-MS (positive): m/z = 949.02 $[M+Na]^+$.



104

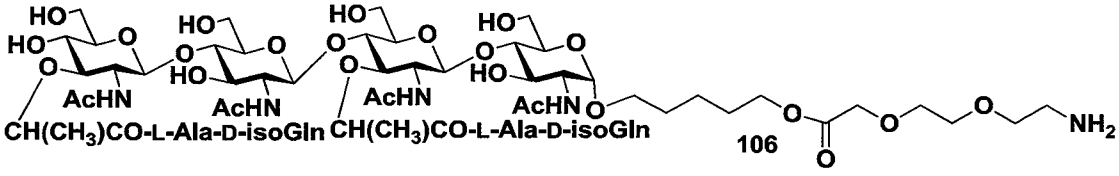
Protected tetrasaccharide dipeptide (MGMG2P) with Boc linker (104)

To a solution of **94** (2.6 mg, 0.001 mmol), HCl·L-Ala-D-isoGln(OBn) (2.2 mg, 0.006 mmol), and HOBt (0.7 mg, 0.04 mmol) in DMF (1 mL) were added WSCD·HCl (0.8 mg, 0.004 mmol) and triethylamine (1.5 μL, 0.008 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 10 mL), H_2O (10 mL), sat. $NaHCO_3$ aq (10 mL), and brine (10 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 10 : 1) to give **104** as a white solid (1.6 mg, 49%). ^1H NMR (500 MHz, $CDCl_3$) δ 7.33-7.09 (m, 40H), 5.51 (m, 2H), 5.37 (s, 1H), 5.05-4.99 (m, 5H), 4.85-4.72 (m, 4H), 4.52-3.84 (m, 26H), 3.57-3.53 (m, 9H), 3.43 (m, 11H), 3.20 (m, 2H), 3.06 (m, 2H), 2.39-2.32 (m, 4H), 2.11 (m, 2H), 1.86-1.52 (m, 14H), 1.36-1.29 (m, 12H), 1.05 (m, 9H); MALDI-MS (positive): m/z = 2443.25 $[M+Na]^+$.



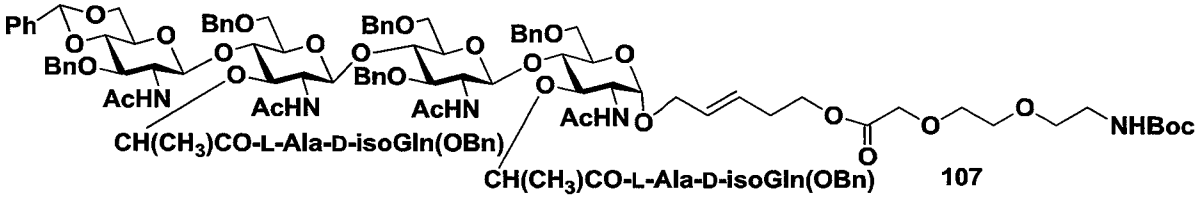
Tetrasaccharide dipeptide (MGMG2P) with Boc linker (105)

To a solution of **104** (1.6 mg, 0.001 mmol) in AcOH (1 mL) was added palladium hydroxide (2 mg) in AcOH and stirred under H_2 (2 MPa) for 1 d. The reaction was monitored by TLC analysis and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite, and the filtrate was concentrated. The residue was lyophilized from acetonitrile-H_2O to give **105** (0.6 mg, 53%) as a white solid. ^{1}H NMR (500 MHz, D_2O) δ 4.73-4.71 (m, 2H), 4.43-4.38 (m, 3H), 4.23-4.21 (m, 2H), 4.16-4.09 (m, 5H), 3.82-3.28 (m, 34H), 3.15 (t, J = 5.5 Hz, 2H), 2.16-2.12 (m, 4H), 1.99 (m, 2H), 1.93-1.82 (m, 14H), 1.49-1.43 (m, 6H), 1.35-1.18 (m, 21H); MALDI-MS (positive): m/z = 1726.24 $[M+Na]^+$.



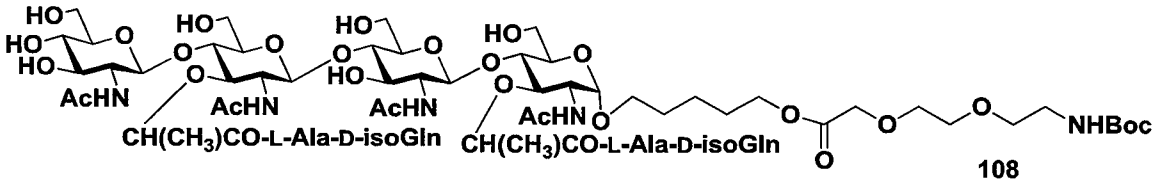
Tetrasaccharide dipeptide (MGMG2P) with linker (106)

105 (0.6 mg, 0.001 mmol) was dissolved in 10% TFA in H_2O (1 mL) and stirred for 2 h. The reaction was monitored by MS (MALDI-TOF) analysis and continued until deprotection of Boc group was completed. The reaction system was diluted by H_2O and lyophilized to give **106** (0.6 mg, quant) as a white solid.



Protected tetrasaccharide dipeptide (GMGM2P) with Boc linker (107)

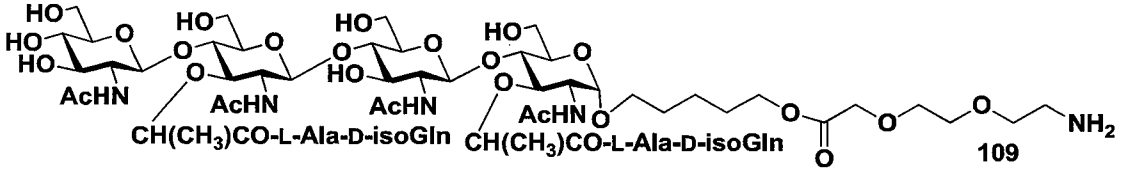
To a solution of **96** (5.6 mg, 0.003 mmol), HCl·L-Ala-D-isoGln(OBn) (4.4 mg, 0.012 mmol), and HOBt (1.2 mg, 0.01 mmol) in DMF (1 mL) were added WSCD·HCl (1.6 mg, 0.009 mmol) and triethylamine (2 μL, 0.018 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 20 mL), H_2O (20 mL), sat. $NaHCO_3$ aq (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 10 : 1) to give **107** as a white solid (5.8 mg, 79%). HRMS (ESI-QTOF) Anal. Calcd for $C_{126}H_{161}N_{11}O_{37}Na_2$ $[M+2Na]^{2+}$: 1233.5442, found: 1233.5437.



108

Tetrasaccharide dipeptide (GMGM2P) with Boc linker (108)

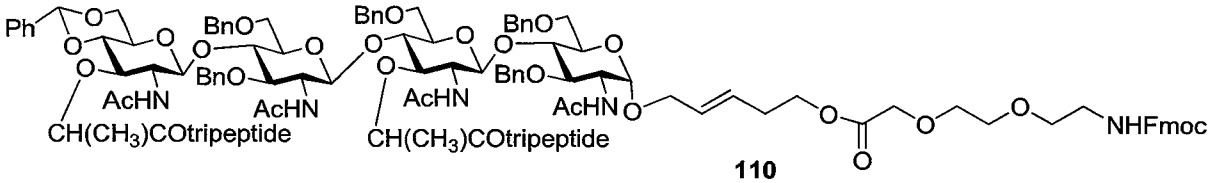
To a solution of **107** (3 mg, 0.001 mmol) in AcOH (1 mL) was added palladium hydroxide (5 mg) in AcOH and stirred under H_2 (2 MPa) for 1 d. The reaction was monitored by TLC analysis and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite and the filtrate was concentrated. The residue was lyophilized from acetonitrile-H_2O to give **108** (1.4 mg, 68%) as a white solid. MALDI-MS (positive): m/z = 1726.51 $[M+Na]^+$.



109

Tetrasaccharide dipeptide (GMGM2P) with linker (109)

108 (1.4 mg, 0.001 mmol) was dissolved in 10% TFA in H_2O (1 mL) and stirred for 2 h. The reaction was monitored by MS (MALDI-TOF) analysis and continued until deprotection of Boc group was completed. The reaction system was diluted by H_2O and lyophilized to give **109** (1.3 mg, quant) as a white solid. ^1H NMR (400 MHz, D_2O) δ 4.41-4.08 (m, 11H), 3.79-3.30 (m, 25H), 2.32 (m, 5H), 2.08-1.83 (m, 15H), 1.54-1.26 (m, 18H); MALDI-MS (positive): m/z = 1626.16 $[M+Na]^+$. HRMS (ESI-QTOF) Anal. Calcd for $C_{65}H_{109}N_{11}O_{35}Na_2$ $[M+2Na]^{2+}$: 824.8441, found: 824.8415.

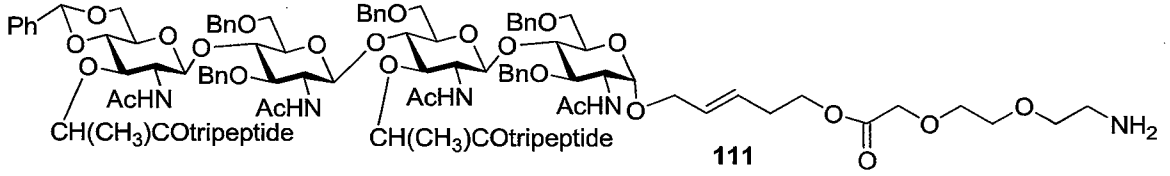


110

Protected tetrasaccharide tripeptide (MGMG3P) with Fmoc linker (110)

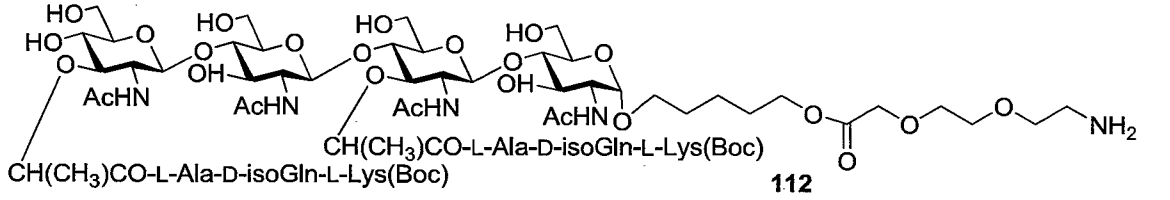
To a solution of **95** (3 mg, 0.001 mmol), HCl·L-Ala-D-isoGln-L-Lys(OBn) (2.4 mg, 0.003 mmol), and HOBt (0.7 mg, 0.004 mmol) in DCM (1 mL) were added WSCD·HCl (0.8 mg, 0.004 mmol) and triethylamine (1 μL, 0.004 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 10 mL), H_2O (10 mL), sat. $NaHCO_3$ aq (10 mL), and brine (10 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (3 g, $CHCl_3$: MeOH = 10 : 1) to give **110** as a white solid (3 mg, 66%). ^1H NMR (500 MHz, DMF-*d*7) δ 8.28-8.25 (m, 2H), 8.21-8.14 (m, 3H), 7.94-7.92 (m, 3H), 7.75-7.69 (m, 4H), 7.55-7.52 (m, 2H), 7.48-7.26 (m, 32H), 7.17-7.15 (m, 7H), 6.69 (brs, 2H), 5.73-5.68 (m, 3H), 5.21-5.09 (m, 6H), 4.96 (d, J = 11.0 Hz, 1H), 4.85 (d, J = 7.5 Hz, 1H), 4.76 (d, J

= 3.5 Hz, 1H), 4.65-4.62 (m, 9H), 4.42-4.30 (m, 12H), 4.18-4.12 (m, 6H), 4.07-3.88 (m, 11H), 3.76-3.51 (m, 16H), 3.31-3.28 (m, 4H), 2.99 (m, 4H), 2.39-2.32 (m, 6H), 2.19-2.17 (m, 2H), 1.96-1.87 (m, 14H), 1.80-1.69 (m, 4H), 1.44-1.37 (m, 26H), 1.32-1.27 (m, 12H); MALDI-MS (positive): m/z = 3022.09 $[M+Na]^+$. HRMS (ESI-QTOF) Anal. Calcd for $C_{158}H_{203}N_{15}O_{43}Na_2$ $[M+2Na]^{2+}$: 1522.6994, found: 1522.6992.



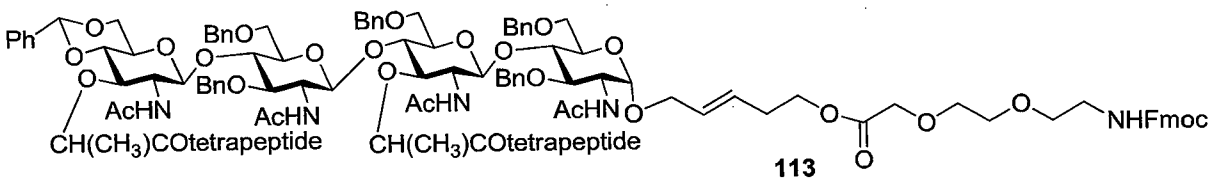
Protected tetrasaccharide tripeptide (MGMG3P) with linker (111)

110 (3 mg, 0.001 mmol) was dissolved in 20% piperidine in DMF (1 mL) and stirred for 2 h. The reaction was monitored by TLC and continued until deprotection of Fmoc group was completed. The mixture was concentrated and purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 10 : 1 → 3 : 1) to give **111** as a white solid (1.5 mg, 54%). ^{1}H NMR (500 MHz, DMF-*d7*) δ 8.30-8.17 (m, 5H), 8.08 (m, 1H), 7.92 (d, J = 9.0 Hz, 1H), 7.79-7.71 (m, 4H), 7.55-7.74 (m, 2H), 7.47-7.12 (m, 24H), 7.05 (brs, 1H), 6.68 (m, 2H), 5.73-5.69 (m, 3H), 5.20-5.09 (m, 4H), 5.10 (d, J = 11.5 Hz, 1H), 4.96 (d, J = 11.0 Hz, 1H), 4.86 (d, J = 8.0 Hz, 1H), 4.76 (d, J = 3.5 Hz, 1H), 4.72-4.54 (m, 9H), 4.45-4.34 (m, 10H), 4.20-4.12 (m, 7H), 4.01-3.91 (m, 10H), 3.73-3.58 (m, 16H), 3.31-3.26 (m, 3H), 3.16(t, J = 5.0 Hz, 1H), 2.99 (m, 4H), 2.41-2.33 (m, 4H), 2.18-2.17 (m, 2H), 1.96-1.88 (m, 14H), 1.80-1.69 (m, 4H), 1.44-1.27(m, 38H); MALDI-MS (positive): m/z = 2799.45 $[M+Na]^+$. HRMS (ESI-QTOF) Anal. Calcd for $C_{143}H_{194}N_{15}O_{41}Na$ $[M+H+Na]^{2+}$: 1400.6744, found: 1400.6743.



Tetrasaccharide tripeptide (MGMG3P) with linker (112)

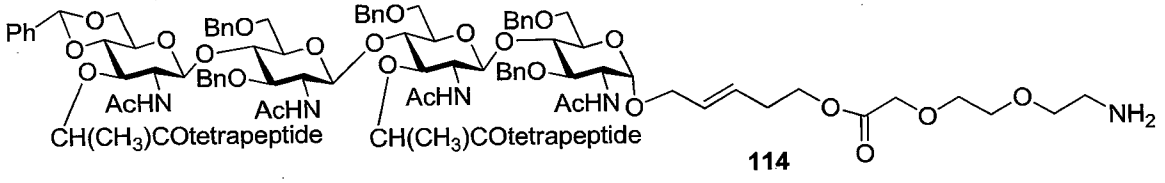
To a solution of **111** (1.5 mg, 0.001 mmol) in THF : H_2O : AcOH (5 : 5 : 1) was added palladium hydroxide (2 mg) and stirred under H_2 (2 MPa) for 1 d. The reaction was monitored by MS analysis (MALDI-TOF) and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite and the filtrate was concentrated. The residue was lyophilized from acetonitrile-H_2O to give **112** (0.8 mg, 72%) as a white solid. ^{1}H NMR (400 MHz, D_2O) δ 4.43-4.38 (m, 4H), 4.30-4.04 (m, 12H), 3.82-3.35 (m, 28H), 3.11 (m, 1H), 3.05-3.02 (m, 5H), 2.95-2.92 (m, 4H), 2.29-2.25 (m, 4H), 2.08-2.00 (m, 2H), 1.92-1.82 (m, 16H), 1.67-1.47 (m, 14H), 1.34-1.03(m, 32H); HRMS (ESI-QTOF) Anal. Calcd for $C_{87}H_{149}N_{15}O_{41}Na_2$ $[M+2Na]^{2+}$: 1052.9915, found: 1052.9880.



113

Protected tetrasaccharide tetrapeptide (MGMG4P) with Fmoc linker (113)

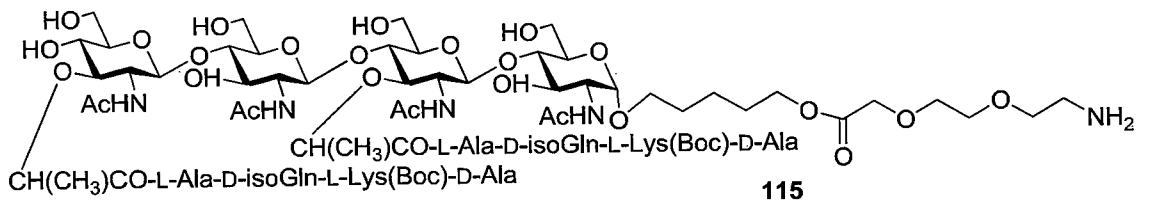
To a solution of **95** (2.9 mg, 0.006 mmol), HCl·L-Ala-D-isoGln-L-Lys(Z)-D-Ala(OBn) (2.7 mg, 0.019 mmol) and HOBt (0.6 mg, 0.044 mmol) in DCM (1 mL) were added WSCD·HCl (0.8 mg, 0.037 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 10 mL), H_2O (10 mL), sat. $NaHCO_3$ aq (10 mL), and brine (10 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 10 : 1) to give **113** as a colorless solid (2.3 mg, 51%). ^1H NMR (500 MHz, DMF-*d*7) δ 8.19-8.17 (m, 2H), 8.05 (m, 3H), 7.81-7.79 (m, 4H), 7.61-7.60 (m, 4H), 7.34-7.02 (m, 37H), 6.53 (m, 2H), 5.59-5.55 (m, 3H), 5.06-4.96 (m, 5H), 4.83 (d, J = 11.0 Hz, 1H), 4.72 (d, J = 8.0 Hz, 1H), 4.62-4.46 (m, 9H), 4.42 (d, J = 12.0 Hz, 1H), 4.33-4.12 (m, 14H), 4.05-3.99 (m, 6H), 3.94-3.40 (m, 26H), 3.17-3.13 (m, 4H), 2.86-2.85 (m, 4H), 2.26-2.18 (m, 4H), 2.10-2.06 (m, 3H), 1.83-1.74 (m, 13H), 1.63 (m, 4H), 1.48-1.43 (m, 3H), 1.28-1.14 (m, 42H); MALDI-MS (positive): m/z = 3163.73 $[M+Na]^+$. HRMS (ESI-QTOF) Anal. Calcd for $C_{164}H_{213}N_{17}O_{45}Na_2$ $[M+2Na]^{2+}$: 1593.7365, found: 1593.7307.



114

Protected tetrasaccharide tetrapeptide (MGMG4P) with linker (114)

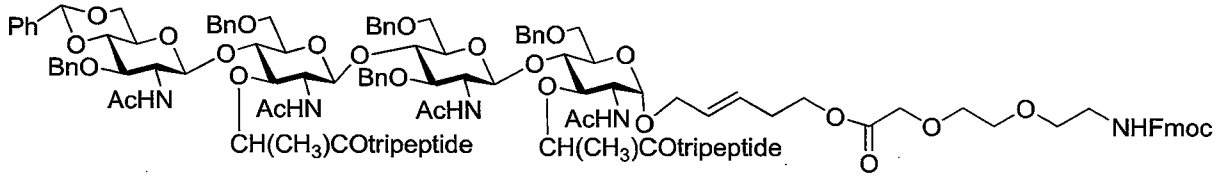
113 (2.3 mg, 0.001 mmol) was dissolved in 20% piperidine in DMF (1 mL) and stirred for 2 h. The mixture was concentrated and purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 10 : 1 → 3 : 1) to give **114** as a white solid (1 mg, 51%). ^1H NMR (400 MHz, CD_3OD) δ 7.41-7.08 (m, 37H), 7.20 (m, 3H), 5.69-5.66 (m, 2H), 5.46 (s, 1H), 5.13-5.10 (m, 5H), 4.96-4.92 (m, 2H), 4.76 (m, 1H), 4.62-4.57 (m, 10H), 4.44-4.26 (m, 11H), 4.15-3.48 (m, 32H), 3.13-2.97 (m, 6H), 2.40-2.38 (m, 2H), 2.31 (m, 4H), 2.19-2.15 (m, 2H), 1.95-1.86 (m, 14H), 1.73-1.60 (m, 6H), 1.41-1.28 (m, 42H); MALDI-MS (positive): m/z = 2940.70 $[M+Na]^+$.



115

Tetrasaccharide tetrapeptide (MGMG4P) with linker (115)

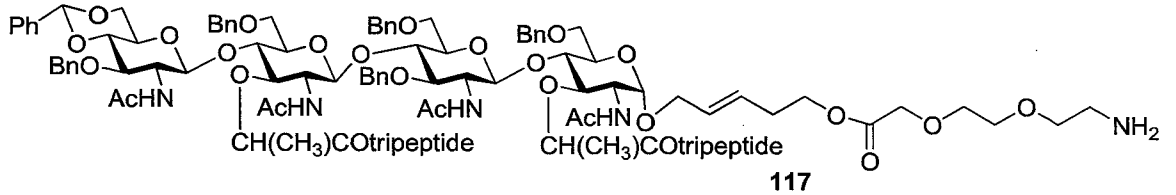
To a solution of **114** (1 mg, 0.001 mmol) in THF : H_2O : AcOH (5 : 5 : 1) was added palladium hydroxide (2 mg) and stirred under H_2 (2 MPa) for 1 d. The reaction was monitored by MS analysis (MALDI-TOF) and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite and the filtrate was concentrated. The residue was lyophilized from acetonitrile-H_2O to give **115** (0.5 mg, 62%) as a white solid. ^{1}H NMR (500 MHz, D_2O) δ 4.78-4.70 (m, 3H), 4.43-4.38 (m, 2H), 4.28 (m, 1H), 4.22-4.03 (m, 9H), 3.76-3.40 (m, 37H), 2.95-2.92 (m, 4H), 2.30-2.27 (m, 4H), 2.11-2.08 (m, 2H), 1.92-1.78 (m, 14H), 1.69-1.63 (m, 8H), 1.58-1.51 (m, 6H), 1.49-1.45 (m, 4H), 1.36-1.19 (m, 36H); HRMS (ESI-QTOF) Anal. Calcd for $C_{93}H_{159}N_{17}O_{43}Na_2$ $[M+2Na]^{2+}$: 1124.5303, found: 1124.5240.



116

Protected tetrasaccharide tripeptide (GMGM3P) with Fmoc linker (116)

To a solution of **97** (2.6 mg, 0.001 mmol), HCl·L-Ala-D-isoGln-L-Lys(OBn) (2.2 mg, 0.004 mmol), and HOBt (0.6 mg, 0.004 mmol) in DCM (1 mL) were added WSCD·HCl (0.7 mg, 0.004 mmol) and triethylamine (1 μL, 0.004 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 10 mL), H_2O (10 mL), sat. $NaHCO_3$ aq (10 mL), and brine (10 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (3 g, $CHCl_3$: MeOH = 10 : 1) to give **116** as a white solid (2.7 mg, 68%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.68 (d, J = 7.5 Hz, 1H), 7.63 (m, 1H), 7.53 (d, J = 7.0 Hz, 1H), 7.47-7.43 (m, 2H), 7.34-7.12 (m, 43H), 5.54-5.48 (m, 2H), 5.38 (m, 1H), 5.16-5.05 (m, 8H), 4.88-3.96 (m, 28H), 3.86-3.33 (m, 25H), 3.14-2.96 (m, 8H), 2.29-2.26 (m, 6H), 2.17-2.10 (m, 2H), 2.01-1.86 (m, 14H), 1.77-1.67 (m, 4H), 1.38-1.35 (m, 26H), 1.21-1.19(m, 12H); HRMS (ESI-QTOF) Anal. Calcd for $C_{158}H_{203}N_{15}O_{43}Na_2$ $[M+2Na]^{2+}$: 1522.6994, found: 1522.6943.

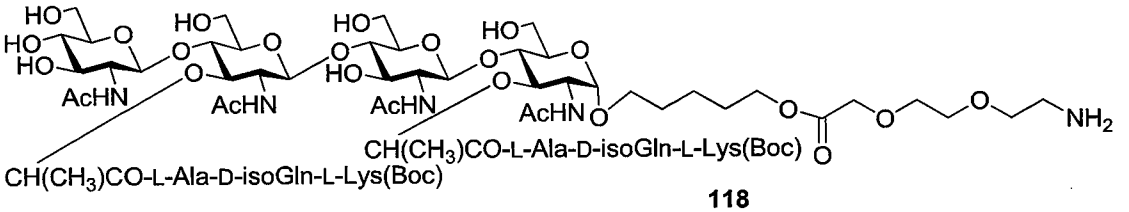


117

Protected tetrasaccharide tripeptide (GMGM3P) with linker (117)

116 (2.7 mg, 0.001 mmol) was dissolved in 20% piperidine in DMF (1 mL) and stirred for 2 h. The reaction was monitored by TLC and continued until deprotection of Fmoc group was completed. The mixture was concentrated and purified by silica-gel chromatography (2 g, $CHCl_3$: MeOH = 10 : 1 → 3 : 1) to give **117** as a white solid (1.2 mg, 48%). HRMS (ESI-QTOF) Anal. Calcd for

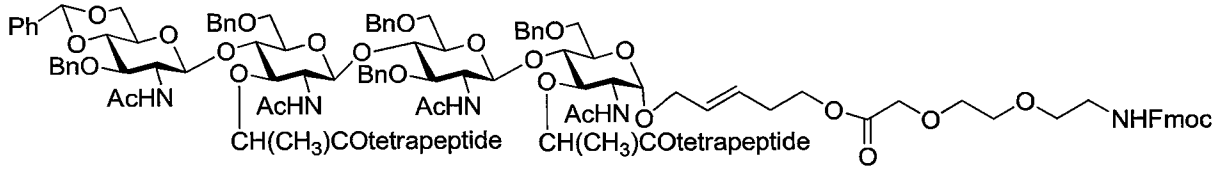
$C_{143}H_{194}N_{15}O_{41}Na$ $[M+H+Na]^{2+}$: 1400.6744, found: 1400.6744.



118

Tetrasaccharide tripeptide (GMGM3P) with linker (118)

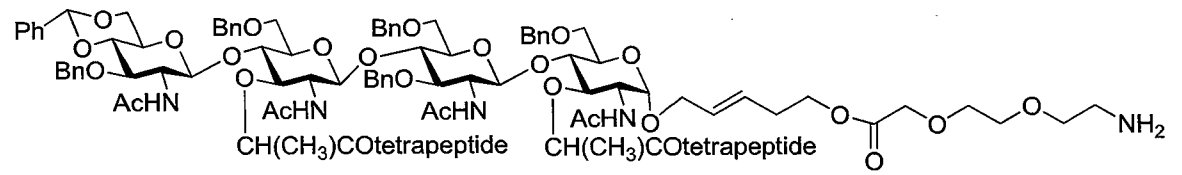
To a solution of **117** (1.2 mg, 0.001 mmol) in THF : H_2O : AcOH (5 : 5 : 1) was added palladium hydroxide (2 mg) and stirred under H_2 (2 MPa) for 1 d. The reaction was monitored by MS analysis (MALDI-TOF) and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite, and the filtrate was concentrated. The residue was lyophilized from acetonitrile-H_2O to give **118** (0.5 mg, 56%) as a white solid. ^1H NMR (400 MHz, D_2O) δ 4.80 (m, 1H), 4.36 (m, 3H), 4.10-3.98 (m, 7H), 3.60-3.20 (m, 37H), 3.07 (m, 2H), 2.91-2.86 (m, 4H), 2.25 (m, 4H), 2.07 (m, 2H), 1.92-1.81 (m, 16H), 1.52-1.42 (m, 10H), 1.31-1.26 (m, 36H); MALDI-MS (positive): m/z = 2083.02 $[M+Na]^+$.



119

Protected tetrasaccharide tetrapeptide (GMGM4P) with Fmoc linker (119)

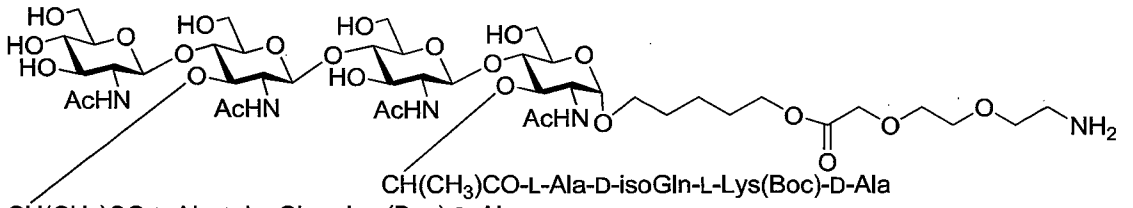
To a solution of **97** (4.7 mg, 0.002 mmol), HCl·L-Ala-D-isoGln-L-Lys(Z)-D-Ala(OBn) (4.3 mg, 0.007 mmol) and HOBt (1 mg, 0.007 mmol) in DCM (1 mL) were added WSCD·HCl (1.2 mg, 0.007 mmol) and triethylamine (1 μL, 0.007 mmol) at 0 °C and the mixture was stirred at rt overnight. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 20 mL), H_2O (20 mL), sat. $NaHCO_3$ aq (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (3 g, $CHCl_3$: MeOH = 10 : 1) to give **119** as a white solid (6.1 mg, 81%). ^1H NMR (500 MHz, $CDCl_3$) δ 7.73 (d, J = 7.5 Hz, 1H), 7.68 (m, 1H), 7.58 (d, J = 7.5 Hz, 1H), 7.51-7.47 (m, 2H), 7.40-7.17 (m, 43H), 5.58-5.50 (m, 3H), 5.12-5.06 (m, 6H), 4.89-4.81 (m, 4H), 4.67-4.11 (m, 30H), 3.86-3.08 (m, 30H), 2.34-2.27 (m, 6H), 2.16-2.15 (m, 2H), 2.04-1.89 (m, 14H), 1.73-1.66 (m, 4H), 1.40-1.35 (m, 26H), 1.28-1.24(m, 18H); MALDI-MS (positive): m/z = 3163.52 $[M+Na]^+$.



120

Protected tetrasaccharide tetrapeptide (GMGM4P) with linker (120)

119 (3 mg, 0.001 mmol) was dissolved in 20% piperidine in DMF (1 mL) and stirred for 2 h. The reaction was monitored by TLC and continued until deprotection of Fmoc group was completed. The mixture was concentrated and purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 10 : 1 → 3 : 1) to give **120** as a white solid (1.6 mg, 59%). HRMS (ESI-QTOF) Anal. Calcd for $C_{149}H_{204}N_{17}O_{43}Na$ $[M+H+Na]^{2+}$: 1471.7115, found: 1471.7106.



121

Tetrasaccharide tetrapeptide (GMGM4P) with linker (121)

To a solution of **120** (0.8 mg, 0.001 mmol) in THF : H_2O : AcOH (5 : 5 : 1) was added palladium hydroxide (1 mg) and stirred under H_2 (2 MPa) for 1 d. The reaction was monitored by MS analysis (MALDI-TOF) and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite and the filtrate was concentrated. The residue was lyophilized from acetonitrile-H_2O to give **121** (0.4 mg, 66%) as a white solid. ^1H NMR (400 MHz, D_2O) δ 4.83 (d, J = 3.6 Hz, 1H), 4.42-4.40 (m, 3H), 4.20-4.06 (m, 8H), 3.80-3.23 (m, 38H), 3.11-3.09 (t, J = 5.0 Hz, 2H), 2.95-2.92 (t, J = 6.4 Hz, 4H), 2.32-2.28 (m, 4H), 2.13-2.04 (m, 2H), 1.95-1.84 (m, 16H), 1.57-1.44 (m, 10H), 1.37-1.21 (m, 42H); HRMS (ESI-QTOF) Anal. Calcd for $C_{93}H_{159}N_{17}O_{43}Na_2$ $[M+2Na]^{2+}$: 1124.5303, found: 1124.5281.

Experiments in Chapter IV

Fmoc-L-Lys(Boc)-D-Ala-D-Ala(OBn)

127

2-((9H-fluoren-9-yl)methoxy)carbonyl-ε-*t*-butoxycarbonyl-L-lysyl-D-alanyl-D-alanine benzyl ester (Fmoc 3P) (127)

Boc-D-Ala-D-Ala-OBn (**56**) (37 mg, 0.10 mmol) was dissolved in TFA (5 mL) and the mixture was stirred for 30 min. After removal of TFA, the residue was dissolved with CH_2Cl_2. 1 M HCl in Et_2O (2 mL) and Et_2O were added. The supernatant was removed by decantation and the precipitate was washed with Et_2O for three times and dried in vacuo to give HCl·NH_2-D-Ala-D-Ala-OBn as white solid (**126**). To a solution of Fmoc-L-Lys(Boc)(OH) (73 mg, 0.16 mmol), **126** and HOBt (28 mg, 0.2 mmol) in DCM (10 mL) was added WSCD·HCl (34 mg, 0.2 mmol) and TEA (58 μL, 0.4 mmol) at 0 °C and the mixture was stirred for 12 h. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 20 mL), H_2O (20 mL), sat. $NaHCO_3$ aq (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (10 g, $CHCl_3$:

MeOH = 25 : 1) to give **127** as a colorless oil (57 mg, 78%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, J = 7.6 Hz, 2H), 7.61 (d, J = 7.4 Hz, 1H), 7.41-7.29 (m, 10H), 5.15 (dd, J = 26.1, J = 12.2 Hz, 3H), 4.46-4.37 (m, 4H), 4.22-4.19 (m, 2H), 3.15-2.85 (m, 2H), 2.29(m, 2H), 2.18 (m, 1H), 1.93-1.90 (m, 1H), 1.81-1.70 (m, 2H), 1.42-1.37 (m, 16H).

Fmoc-L-Lys-D-Ala-D-Ala(OBn)
Boc-L-Ala 131

2-((9H-fluoren-9-yl)methoxy)carbonyl-ε-(*t*-butoxycarbonyl-L-alanyl)-L-lysyl-D-alanyl-D-alanine benzyl ester (Fmoc 4P) (131)

Fmoc-L-Lys(Boc)-D-Ala-D-Ala-OBn (**127**) (176 mg, 0.25 mmol) was dissolved in TFA (10 mL) and the mixture was stirred for 30 min. After removal of TFA, the residue was dissolved with CH_2Cl_2. 1 M HCl in Et_2O (2 mL) and Et_2O were added. The supernatant was removed by decantation and the precipitate was washed with Et_2O for three times and dried in vacuo to give Fmoc-L-Lys(HCl·NH_2)-D-Ala-D-Ala-OBn as white solid (**128**). To a solution of Boc-L-Ala(OH) (62 mg, 0.33 mmol), **128** and HOBt (68 mg, 0.5 mmol) in DCM (20 mL) was added WSCD·HCl (82 mg, 0.5 mmol) and TEA (70 μL, 0.75 mmol) at 0 °C and the mixture was stirred for 12 h. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 30 mL), H_2O (30 mL), sat. $NaHCO_3$ aq (30 mL), and brine (30 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (20 g, $CHCl_3$: MeOH = 20 : 1) to give **131** as a colorless oil (175 mg, 90%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.74 (d, J = 7.2 Hz, 2H), 7.59-7.58 (m, 2H), 7.40-7.37 (m, 2H), 7.34-7.27 (m, 7H), 6.86 (m, 2H), 6.43 (s, 1H), 5.77 (m, 1H), 5.16-5.08 (m, 3H), 4.58-4.55 (m, 1H), 4.48 (t, J = 7.0 Hz, 1H), 4.40-4.39 (m, 2H), 4.19 (t, J = 7.0 Hz, 1H), 4.13-4.11 (m, 2H), 3.23 (m, 2H), 1.83 (m, 1H), 1.52 (m, 2H), 1.42-1.36 (m, 10H), 1.32-1.27 (m, 12H).

Fmoc-L-Lys-D-Ala-D-Ala(OBn)
Z-L-Ala-L-Ala 132

2-((9H-fluoren-9-yl)methoxy)carbonyl-ε-(benzyloxycarbonyl-L-alanyl-L-alanyl)-L-lysyl-D-alanyl-D-alanine benzyl ester (Fmoc 5P) (132)

Fmoc-L-Lys(Boc-L-Ala)-D-Ala-D-Ala-OBn (**131**) (175 mg, 0.23 mmol) was dissolved in TFA (10 mL) and the mixture was stirred for 30 min. After removal of TFA, the residue was dissolved with CH_2Cl_2. 1 M HCl in Et_2O (2 mL) and Et_2O were added. The supernatant was removed by decantation and the precipitate was washed with Et_2O for three times and dried in vacuo to give Fmoc-L-Lys(HCl·L-Ala)-D-Ala-D-Ala-OBn as white solid. To a solution of Z-L-Ala(OH) (75 mg, 0.34 mmol), Fmoc-L-Lys(HCl·L-Ala)-D-Ala-D-Ala-OBn and HOBt (61 mg, 0.45 mmol) in DCM (30 mL) was added WSCD·HCl (73 mg, 0.45 mmol) and TEA (47 μL, 0.67 mmol) at 0 °C and the mixture was stirred for 12 h. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 30 mL), H_2O (30 mL), sat. $NaHCO_3$ aq (30

mL), and brine (30 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (20 g, $CHCl_3$: MeOH = 20 : 1) to give **132** as a colorless solid (176 mg, 89%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76-7.74 (m, 2H), 7.61-7.52 (m, 4H), 7.40-7.18 (m, 13H), 7.17-7.10 (m, 1H), 6.39 (d, J = 2.7 Hz, 1H), 6.07 (brs, 1H), 5.13-5.06 (m, 4H), 4.51 (m, 1H), 4.44-4.30 (m, 4H), 4.21-4.18 (m, 2H), 4.09 (m, 1H), 3.20 (m, 2H), 1.76 (brs, 1H), 1.62 (m, 1H), 1.51 (m, 2H), 1.39-1.26 (m, 14H).

Boc-L-Ala-D-isoGln-L-Lys-D-Ala-D-Ala(OBn)
Z-L-Ala-L-Ala **133**

Protected heptapeptide (133)

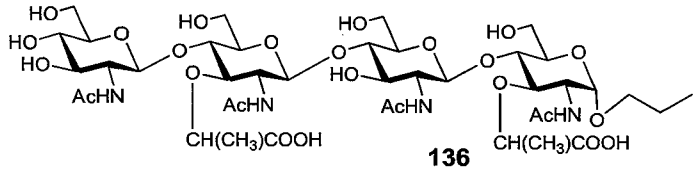
Fmoc-L-Lys(Z-L-Ala-L-Ala)-D-Ala-D-Ala-OBn (**132**) (134 mg, 0.15 mmol) was dissolved in 20% piperidine in THF (20 mL) and the mixture was stirred for 30 min. After removal of solvent, the residue was purified by silica-gel chromatography (5 g, $CHCl_3$: MeOH = 6 : 1) to give NH_2-L-Lys(Z-L-Ala-L-Ala)-D-Ala-D-Ala-OBn as white solid (91 mg, 91%). To a solution of Boc-L-Ala-D-isoGln(OH) (88 mg, 0.27 mmol), NH_2-L-Lys(Z-L-Ala-L-Ala)-D-Ala-D-Ala-OBn and HATU (106 mg, 0.27 mmol) in DMF (10 mL) was added TEA (38 μL, 0.4 mmol) at 0 °C and the mixture was stirred for 12 h. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 20 mL), H_2O (20 mL), sat. $NaHCO_3$ aq (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (20 g, $CHCl_3$: MeOH = 10 : 1) to give **133** as a colorless solid (89 mg, 77%). ^{1}H NMR (500 MHz, DMF-*d*7) δ 8.20 (d, J = 7.0 Hz, 1H), 8.11 (d, J = 8.0 Hz, 1H), 8.02-7.99 (m, 4H), 7.70 (brs, 1H), 7.40-7.33 (m, 10H), 7.25-7.15 (m, 1H), 7.09 (brs, 1H), 6.97 (m, 1H), 5.19-5.06 (m, 4H), 4.46-4.34 (m, 4H), 4.24 (m, 1H), 4.19 (m, 1H), 4.14-4.11 (m, 1H), 3.13 (m, 2H), 2.31 (m, 2H), 2.15 (m, 1H), 1.87 (m, 1H), 1.78 (m, 1H), 1.65 (m, 1H), 1.47-1.28 (m, 28H); ^{13}C NMR (125 MHz, DMF-*d*7) δ 174.1, 173.8, 173.2, 173.0, 172.8, 172.5, 157.0, 156.5, 138.1, 137.1, 129.6, 129.1, 129.0,128.8, 128.6, 128.5, 128.4, 128.3, 125.9, 79.1, 66.7, 66.4, 54.5, 53.0, 51.6, 51.2, 49.4, 48.9, 48.8, 39.3, 32.5, 31.9, (30.6, 30.4, 30.3, 30.1, 29.9, 29.8, 29.7, 29.6,) 28.9, 28.4, 23.6, 221.3, 18.6, 18.3, 18.1, 18.1, 17.2; MALDI-MS (positive): m/z = 977.06 $[M+Na]^+$.

HCl·L-Ala-D-isoGln-L-Lys-D-Ala-D-Ala
L-Ala-L-Ala **135**

Heptapeptide (135)

Boc-L-Ala-D-isoGln-L-Lys(Z-L-Ala-L-Ala)-D-Ala-D-Ala-OBn (**133**) (9 mg, 0.01 mmol) was dissolved in TFA (2 mL) and the mixture was stirred for 30 min. After removal of TFA, the residue was dissolved with CH_2Cl_2. 1 M HCl in Et_2O (2 mL) and Et_2O (2 mL) were added. The supernatant was removed by decantation and the precipitate was washed with Et_2O for three times and dried in vacuo to give HCl·L-Ala-D-isoGln-L-Lys(Z-L-Ala-L-Ala)-D-Ala-D-Ala-OBn (**134**) as a white solid. To a solution of **134** in AcOH (1 mL) was added palladium hydroxide (45 mg) and stirred under H_2

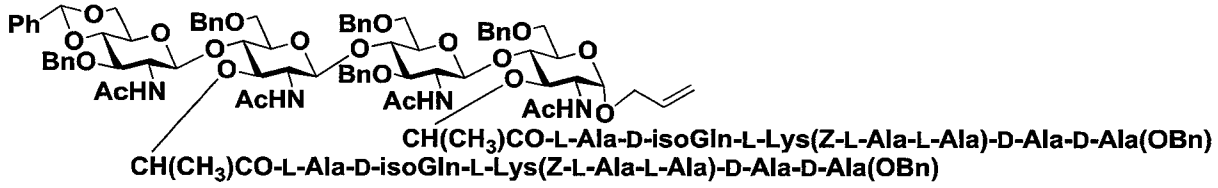
(2 MPa) for 1 d. The reaction was monitored by MS analysis (MALDI-TOF) and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite, and the filtrate was concentrated. The residue was lyophilized from H_2O to give **135** (6 mg, 76%) as a white solid. ^{1}H NMR (500 MHz, D_2O) δ 4.33-4.27 (m, 2H), 4.22-4.17 (m, 2H), 4.09-4.00 (m, 3H), 3.18-3.14 (m, 1H), 3.12-3.08 (m, 1H), 2.37-2.33 (m, 2H), 2.11-2.07 (m, 1H), 1.96-1.90 (m, 1H), 1.72-1.62 (m, 2H), 1.50-1.46 (m, 8H), 1.32-1.26 (m, 11H); ^{13}C NMR (125 MHz, D_2O) δ 180.0, 176.3, 175.5, 175.1, 174.6, 174.3, 171.6, 171.2, 54.7, 53.6, 51.5, 50.7, 49.8, 49.5, 39.6, 31.8, 31.2, 28.5, 27.6, 23.0, 17.9, 17.4, 17.3, 17.3, 17.2; MALDI-MS (positive): m/z = 652.87 $[M+Na]^+$.



136

Propyl
2-acetylamino-3-*O*-((*R*)-1-carboxyethyl)-4-*O*-[[2-acetylamino-4-*O*-[2-acetylamino-3-*O*-((*R*)-1-carboxyethyl)-4-*O*-(2-acetylamino-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranosyl]-2-deoxy-β-D-glucopyranosyl]]-2-deoxy-D-glucopyranoside (136)

To a solution of **46** (2 mg, 0.001 mmol) in AcOH (1 mL) was added palladium hydroxide (5 mg) and stirred under H_2 (2 MPa) for 1 d. The reaction was monitored by MS analysis (MALDI-TOF) and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite, and the filtrate was concentrated. The residue was lyophilized from H_2O to give **136** (1.3 mg, quant) as a white solid.

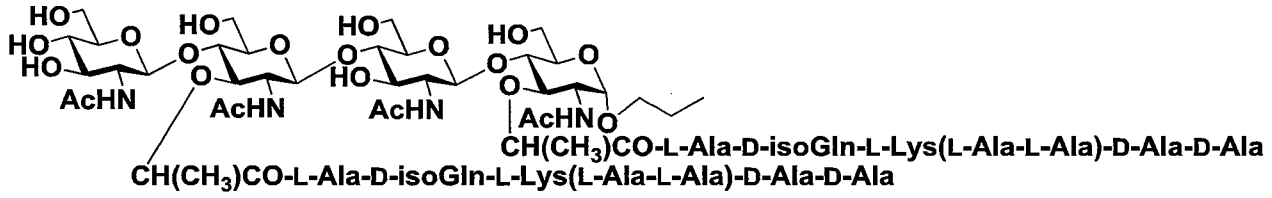


137

Protected tetrasaccharide (GMGM) with heptapeptide (137)

To a solution of **46** (15 mg, 0.01 mmol), HCl·L-Ala-D-isoGln-L-Lys(Z-L-Ala-L-Ala)-D-Ala-D-Ala-OBn (**134**) and HATU (25 mg, 0.03 mmol) in DMF (3 mL) was added TEA (8 μL, 0.07 mmol) at 0 °C and the mixture was stirred for 12 h. The mixture was concentrated and the residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was washed with citric acid (1 M, 20 mL), H_2O (20 mL), sat. $NaHCO_3$ aq (20 mL), and brine (20 mL). The organic layer was dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (10 g, $CHCl_3$: MeOH = 10 : 1) to give **137** as a colorless solid (16 mg, 53%). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.26-8.19 (m, 3H), 8.15-8.13 (m, 3H), 8.00-7.99 (m, 5H), 7.87-7.77 (m, 5H), 7.46-7.44 (m, 2H), 7.38-7.23 (m, 50H), 7.12-7.08 (m, 5H), 6.94 (brs, 1H), 5.82 (m, 1H), 5.66 (s, 1H), 5.24 (d, J = 16.8 Hz, 1H), 5.12-4.88 (m, 12H), 4.70 (d, J = 12.0 Hz, 1H), 4.57-4.01 (m, 29H), 3.90-3.39 (m, 21H), 3.12-2.88 (m, 4H), 2.12-2.11 (m, 4H), 1.90 (m, 2H),

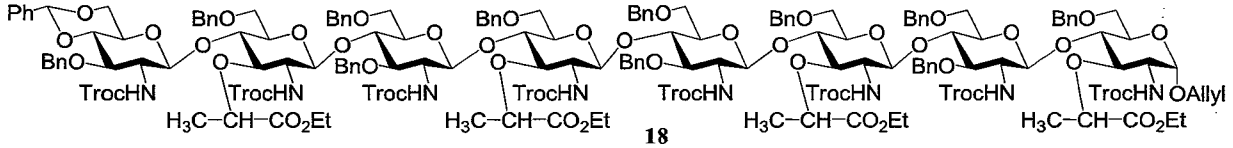
1.81-1.76 (m, 14H), 1.56-1.47 (m, 4H), 1.35-1.13 (m, 44H); MALDI-MS (positive): m/z = 3246.56 $[M+Na]^+$. HRMS (ESI-QTOF) Anal. Calcd for $C_{165}H_{214}N_{22}O_{45}Na_2$ $[M+2Na]^{2+}$: 1635.2481, found: 1635.2391.



138

Tetrasaccharide heptapeptide (TS7P) (138)

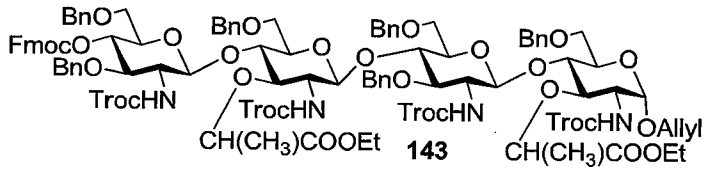
To a solution of **137** (4 mg, 0.001 mmol) in THF : H_2O : AcOH (10 : 10 : 1) was added palladium hydroxide (5 mg) and stirred under H_2 (2 MPa) for 1 d. The reaction was monitored by MS analysis (MALDI-TOF) and the hydrogenolysis was continued until deprotection was completed. The Pd catalyst was filtered off by celite, and the filtrate was concentrated. The residue was lyophilized from H_2O to give **138** (2.4 mg, 91%) as a white solid. ^1H NMR (500 MHz, D_2O : CD_3COOD = 3 : 2) δ 4.95 (d, J = 3.5 Hz, 1H), 4.59 (m, 3H), 4.44-4.30 (m, 12H), 4.20-4.16 (dd, J = 7.0 Hz, J = 14.0 Hz, 2H), 4.00-3.39 (m, 28H), 3.30-3.18 (m, 4H), 2.46-2.43 (t, J = 7.5 Hz, 4H), 2.26-2.22 (m, 2H), 2.11-2.02 (m, 14H), 1.87-1.73 (m, 4H), 1.66-1.41 (m, 46H), 0.93-0.90 (t, J = 7.5 Hz, 3H); MALDI-MS (positive): m/z = 2240.35 $[M+H]^+$. HRMS (ESI-QTOF) Anal. Calcd for $C_{93}H_{158}N_{22}O_{41}Na_2$ $[M+2Na]^{2+}$: 1143.0392, found: 1143.0376.



18

Protected octasaccharide $(GM)_4$ (18)

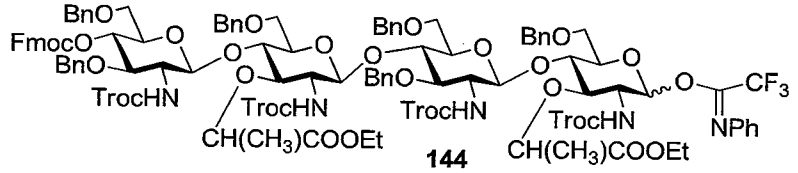
To a mixture of the imidate **139** (135 mg, 0.041 mmol), the acceptor **140** (80 mg, 0.036 mmol), and MS4A in dry CH_2Cl_2 (10 mL) at -15 °C was added TMSOTf (12 μL, 0.008 mmol). After being stirred at the same temperature for 20 min, the reaction was quenched with chilled saturated aqueous $NaHCO_3$ (10 mL), and the mixture was extracted with $CHCl_3$ (20 mL). The organic layer was washed with sat. $NaHCO_3$ aq (20 mL) and brine (20 mL), dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (30 g, toluene : EtOAc = 7 : 1) to give **18** as a white solid (56 mg, 35%). ^1H NMR (500 MHz, $CDCl_3$) δ 7.51-7.13 (m, 60H), 5.73-5.80 (m, 1H), 5.56 (s, 1H), 5.24-3.16 (m, 110H), 1.42-1.22 (m, 24H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.8, 175.7, 175.3, 175.2, 156.0, 156.0, 154.0, 139.4, 139.4, 138.1, 137.9, 137.8, 137.6, 137.6, 137.2, 133.9, 129.5, 129.1, 129.0, 128.5, 128.4, 128.3, 128.2, 127.8, 127.4, 127.2, 126.0, 101.2, 100.7, 96.7, 96.2, 96.1, 96.0, 95.9, 95.6, 95.5, 95.5. 95.4, 82.3, 80.9, 80.8, 79.2, 78.0, 75.2, 75.0, 74.7, 74.5, 74.4, 73.9, 73.8, 73.4, 73.3, 72.9, 70.2, 68.8, 68.3, 67.4, 67.3, 65.7, 61.4, 61.2, 60.6, 57.5, 57.0, 50.2, 50.9, 45.3, 31.9, 29.7, 22.7, 18.8, 18.4, 14.2.



143

4-*O*-Fmoc protected tetrasaccharide (143)

To a mixture of the imidate **142** (374 mg, 0.26 mmol), the acceptor **16** (236 mg, 0.21 mmol), and MS4A in dry CH_2Cl_2 (3 mL) at -20 °C was added TMSOTf (8 μL, 0.03 mmol). After being stirred at the same temperature for 40 min, the reaction was quenched with chilled saturated aqueous $NaHCO_3$ (10 mL), and the mixture was extracted with $CHCl_3$ (50 mL). The organic layer was washed with sat. $NaHCO_3$ aq (20 mL) and brine (20 mL), dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (30 g, toluene : EtOAc = 7 : 1) to give **143** as a white solid (365 mg, 72%). ^1H NMR (400 MHz, $CDCl_3$) δ 7.78-7.74 (m, 2H), 7.58-7.15 (m, 46H), 5.88-5.78 (m, 1H), 5.25-5.12 (m, 4H), 4.88-4.79 (m, 6H), 4.69-4.11 (m, 30H), 3.97-3.58 (m, 10H), 3.43-3.40 (m, 7H), 3.19-2.94 (m, 3H), 1.36-1.26 (m, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.7, 175.5, 156.0, 154.8, 154.2, 135.9, 153.8, 143.3, 143.1, 141.3, 139.4, 137.8, 137.6, 137.4, 133.8, 129.8, 129.3, 129.0, 128.4, 128.4, 128.3, 127.9, 127.8, 127.7, 127.5, 127.5, 127.3, 127.4, 125.0, 124.9, 120.1, 117.4, 99.8, 96.2, 96.0, 95.6, 95.4, 80.7, 76.7, 76.0, 75.2, 75.0, 74.6, 74.4, 74.2, 74.0, 73.7, 73.5, 72.9, 72.3, 70.1, 70.0, 69.4, 61.3, 61.2, 57.4, 57.0, 55.3, 46.7, 18.5, 18.4, 14.1, 14.1.

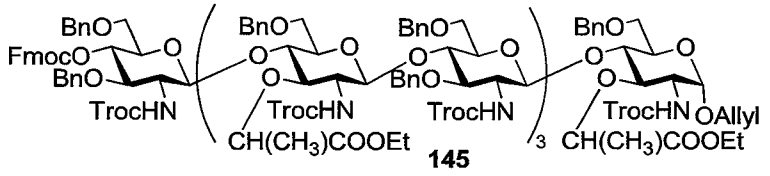


144

4-*O*-Fmoc protected tetrasaccharide donor (144)

To a solution of **143** (410 mg, 0.17 mmol), the solution of $[Ir(cod)(MePh_2P)_2]PF_6$ (2.9 mg, 0.003 mmol) activated with H_2 in dry THF (1 mL) was added. After being stirred at room temperature for 1.5 h, iodine (88 mg, 0.35 mmol) and water (0.5 mL) were added and the reaction mixture was stirred for additional 30 min. To the reaction mixture was rapidly added $Na_2S_2O_3$ aq (5%, 100 mL). The mixture was then extracted with EtOAc (50 mL). The organic layer was washed with $Na_2S_2O_3$ aq (5%, 50 mL × 2), sat. $NaHCO_3$ aq (100 mL × 2), brine (50 mL), dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by silica-gel chromatography (80 g, toluene : EtOAc = 5 : 1) to give 1-liberated-disaccharide as a pale yellow solid. To a solution of 1-liberated-disaccharide in acetone (10 mL) at 0 °C was added Na_2CO_3 (432 mg, 4.1 mmol) and *N*-PhenyL-2,2,2-Trifluoroacetimidoyl chloride (0.04 mL, 0.21 mmol). After being stirred for 3 d at rt, insoluble materials were filtered off through celite and the filtrate was concentrated. The residue was purified by silica-gel chromatography (30 g, toluene : EtOAc = 12 : 1) to give **144** as a pale yellow solid (279 mg, 65% for 3 steps). ^1H NMR (400 MHz, $CDCl_3$) δ 7.52-7.25 (m, 31H), 7.10-7.08 (m, 2H), 6.77 (d, J = 8.6Hz, 2H), 5.58 (s, 1H), 5.14 (d, J = 12.3 Hz, 1H), 4.88-4.79 (m,

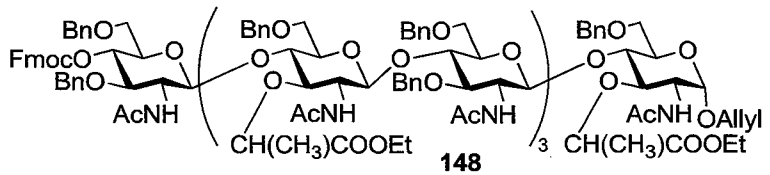
5H), 4.74 (d, J =10.0 Hz, 2H), 4.70-4.68 (m, 2H), 4.63-4.61 (m, 3H), 4.58-4.55 (m, 2H), 4.50-4.47 (d, J = 10.8 Hz, 2H), 4.41-4.38 (m, 2H), 4.32-4.15 (m, 7H), 3.98-3.83 (m, 7H), 3.74-3.46 (m, 12H), 3.33 (d, J = 10.0 Hz, 1H), 3.18 (m, 4H), 2.83 (d, J = 8.8 Hz, 1H), 1.37-1.25 (m, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.6, 175.3, 156.0, 155.2, 154.0, 143.4, 139.4, 138.1, 137.8, 137.6, 137.4, 137.2, 129.6, 129.2, 129.1, 129.1, 128.9, 128.7, 128.5, 128.4, 128.3, 128.2, 128.0, 127,8, 127.4, 127.2, 126.0, 124.2, 119.5, 102.0, 101.2, 100.7, 96.2, 95.8, 95.5, 82.3, 80.6, 78.0, 77.2, 76.7, 76.4, 76.2, 75.4, 74.7, 74.4, 74.3, 74.2, 73.8, 73.5, 73.0, 68.8, 68.3, 67.2, 66.6, 65.7, 61.5, 61.4, 57.5, 57.2, 57.0, 54.9, 29.7, 18.8, 18.4, 14.16, 14.1.



145

4-*O*-Fmoc protected octasaccharide (145)

To a mixture of the imidate **144** (35 mg, 0.013 mmol), the acceptor **140** (20 mg, 0.009 mmol), and MS4A in dry CH_2Cl_2 (2 mL) at -20 °C was added TMSOTf (3 μL, 0.002 mmol). After being stirred at the same temperature for 20 min, the reaction was quenched with chilled saturated aqueous $NaHCO_3$ (10 mL), and the mixture was extracted with $CHCl_3$ (20 mL). The organic layer was washed with sat. $NaHCO_3$ aq (20 mL) and brine (20 mL), dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel chromatography (3 g, toluene : EtOAc = 7 : 1) to give **145** as a colorless solid (10 mg, 24%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.72-7.69 (s, 1H), 7.40-7.18 (m, 67H), 6.52 (brs, 1H), 5.81-5.74 (m, 2H), 5.19 (m, 3H), 5.11-4.96 (m, 5H), 4.82-4.74 (m, 10H), 4.64-4.41 (m, 28H), 4.23-3.84 (m, 34H), 3.68-3.52 (m, 17H), 3.38-3.36 (m, 10H), 3.16-3.02 (m, 5H), 2.94-2.83 (m, 4H), 1.30-1.20 (m, 24H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.7, 156.0, 154.9, 154.1, 154.0, 139.5, 139.4, 138.1, 137.9, 137.9, 137.6, 137.56, 137.5, 137.4, 133.8 129.8, 129. 3, 129.2, 129.0, 128.9, 128.6, 128.5, 128.4, 128.3, 128.2, 127.9, 127.5, 127.4, 127.3, 127.2, 125.0, 120.1, 117.4, 102.2, 102.1, 100.3, 96.2, 96.1, 96.0, 95.6, 95.5, 95.4, 83.2, 83.0, 80.7, 79.2, 77.8, 77.4, 76.9, 76.8, 75.8, 75.2, 75.1, 75.0, 74.8, 74.6, 74.5, 74.4, 74.3, 74.1, 73.9, 73.8, 73.6, 73.4, 73.1, 73.0, 71.4, 70.3, 70.2, 70.0, 69.1, 68.9, 68.4, 68.4, 68.3, 68.2, 67.0, 61.3, 57.8, 57.0, 55.4, 29.7, 22.7, 18.5, 14.1, 14.1; Anal. Calcd for $C_{194}H_{216}Cl_{24}N_8O_{59}$: C, 52.31; H, 4.89; N, 2.52%, found: C, 52.35; H, 4.76; N, 2.63%.

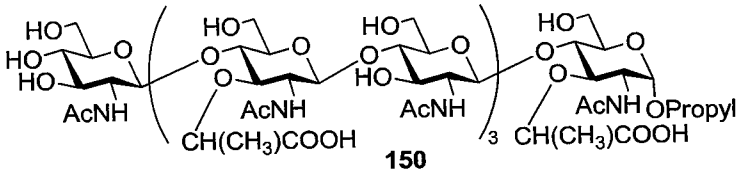


148

4-*O*-Fmoc protected octasaccharide (148)

To a solution of **145** (45 mg, 0.01 mmol) in AcOH (2 mL) was added Zn–Cu (prepared from 30 mg of Zn), the mixture was stirred at room temperature for 5 h. The insoluble materials were filtered off and the filtrate was concentrated in vacuo. The residue solvent was removed by coevaporation with

toluene (10 mL). The residue was dissolved in pyridine (1 mL) and acetic anhydride (1 mL) and the solution was stirred at room temperature for 3 h. The reagents were removed by concentration with toluene (10 mL). The residue was purified by silica-gel chromatography (5 g, $CHCl_3$: acetone = 9 : 1) to give **148** as a white solid (20 mg, 64%). ^1H NMR (500 MHz, $CDCl_3$) δ 7.75-7.73 (m, 4H), 7.57-7.48 (m, 4H), 7.40-7.19 (m, 60H), 5.84 (m, 1H), 5.31-5.11 (m, 4H), 4.79-3.50 (m, 95H), 2.94 (m, 1H), 2.35-1.94 (m, 24H), 1.31-1.19 (m, 24H).



150

Octasaccharide (150)

To a solution of **149** (4 mg, 0.001 mmol) in AcOH was added palladium hydroxide (5 mg) and stirred under H_2 (2 MPa) for 1 d. The Pd catalyst was filtered off by celite, and the filtrate was concentrated. The residue was lyophilized from H_2O to give **138** (2.4 mg, 90%) as a white solid. ^1H NMR (400MHz, D_2O) δ 5.03 (d, J = 3.0 Hz, 1H), 4.45-4.34 (m, 10H), 3.81-3.24 (m, 54H), 1.93-1.84 (m, 24H), 1.42-1.36 (dt, J = 8.0 Hz, J = 14.0 Hz, 2H), 1.26-1.20 (m, 12H), 0.71 (t, J = 7.2 Hz, 3H).

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