Doctoral Thesis

Ring-opening Copolymerization of 1,6-Anhydro Galactomannose and Mannose Monomers into Galactomannans with Potent Anti-HIV Activity

開環重合による高い抗ウイルス性を持つ合成ガラクトマンナン

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ABBREVIATIONS and ACRONYM

CC_{50}	50% Cytotoxic concentration on MT4 cell
CH_2Cl_2	Dichloromethane
$CHCl_3$	Chloroform
COSY	Correlated spectroscopy
c1	Concentration 1%
EC_{50}	50% Effective concentration on HIV
EtOAc	Ethyl acetate
Et_3N	Trimethylamine
FT-IR	Fourier transform infrared spectroscopy
DLS	Dynamic light scattering
DMF	Dimethylformaldehyde
DMSO	Dimethyl sulfoxide
DP	Degree of polymerization
DS	Degree of sulfation
GPC	Gel permeation chromatography
Hex	Hexane
HIV	Human immunodeficience virus
HMQC	Heteronuclear multiple quantum correlation
KBr	Potassium bromide
LGTBE	1,6-anhydro-2, 3, 4-tri-*O*-benzyl-β-D-glucopyranose
LMTBE	1,6-anhydro-2, 3, 4-tri-*O*-benzyl-β-D-mannopyranose

LMGABE	1,6-anhydro-2, 3-di-*O*-benzyl-4-*O*-(2', 3', 4', 6'-tetra-*O*-benzyl-α-D-galactopyranosyl)-α-D-mannopyranose
MeOH	Methanol
Na	Sodium, lump in kerosene
NaH	Sodium hydride
NaOH	Sodium hydroxide
Na_2SO_3	Sodium sulfite
Na_2SO_4	Sodium sulfate
NH_3	Ammonia
NMR	Nuclear magnetic resonance
PSA	Piperidine N-sulfonic acid
PF_5	Phosphorus pentafluoride
SPR	Surface plasmon resonance
TMAOTf	Trimethylsilyl trifluoromethanesulfonate
$[\alpha]_D^{25}$	Specific rotation
$\overline{M}_n$	Molecule weight
^1H	Proton
^{13}C	Carbon

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CHAPTER 1

General introduction

1.1. Antiviral and biological activities of sulfated polysaccharides

Polysaccharides are rich sources in the earth and some polysaccharides have of microelements, amino group and bioactive peptide, which are originated potent special biological activities such as antioxidant, anticoagulant, antitumor and antiviral activities etc. Polysaccharides are generally long linier and branched polymers consisted of monosaccharides derivatives, in which are xanthan, cellulose, chitin, starch, glycogen, carrageenans and galactomannans have high molecules weights and are used for pharmaceuticals, cosmetics and food industries by ingredient of products.

Galactomannans mixed with other polysaccharides, xanthan and carrageenans, are used as matrix, binder and coating materials in tablet drug (Joana Lea 2011) and as thickener for milk, cream dessert, ice cream, in processed cheese etc. In addition, polysaccharides are cured meat in food and biomarker for fungal diagnostic test in United States, microbiological diagnostic criteria proposed by European organization for research and treatment cancer (Zaw Min 2012).

Galactomannan in seeds having mainly (1→4)-β-D-mannopyranose backbone with single D-galactopyranose branches linked by (1→6)-α, which determined by methylation analysis and NMR (Tamaki, 2010).

In general, since natural galactomannans have high molecular weights and are insoluble, so galactomannans are hydrolyzed by acid to obtain lower molecular weight galactomannans. The structural analysis and identification of four natural occurring galactomannans were established by comparison with the specific rotations, shape of the GPC profiles, and high resolution NMR spectroscopies (Tegshi and Yoshida, 2011).

Furthermore, it was found that sulfated natural galactomannans, which were obtained by sulfation, had potent anti-HIV activity (Tegshi and Yoshida, 2012).

Naturally occurring polysaccharides have complex structures of homo-, hetero- and branched of different monosaccharide units, it is difficult to elucidate the relationship between structures and biological activity and important new instrumental analyses into polysaccharide research have been developed for more sensitivies (Yoshida T, 2001). In particular, polysaccharide research used by NMR spectrometer, FT-IR, elemental analysis, the specific rotation by polarimeter, GPC, LC-MS, SPR, particle size and zeta potential analyzer etc.

In 1916, Jay McLean discovered Heparin for the first time but it's used in clinical practice in 20 years later and Dinish reported potent anticoagulant activity of naturally occurring sulfated polysaccharides (Dinish da Gama A, 2008).

Researchers found many sulfated polysaccharides extracted from sea algae. Gerber and Sherman found that sulfated polysaccharides extracted from *Gelidium cartilagenium* were protected infection of influenza B and mump virus to embryonic eggs (Gerber 1958). Nakashima and Yamamoto found anti-HIV activity of naturally sulfated polysaccharides from red algae *Schizymenia pacifica* in 1987 *(*Nakashima 1987*)*.

Yoshida has reported the synthesis of branched polysaccharides by the ring-opening polymerization of anhydro disaccharide and trisaccharide monomers and has investigated the relationship between the structure and biological activies, such as the anticoagulation and anti-HIV activities of sulfated polysaccharides after sulfation of synthetic and naturally polysaccharides by piperidine-N-sulfonic acid and sulfur trioxide pyridine complex (Yoshida 2001).

1.1.1. Naturally occurring sulfated polysaccharides

A wide range of naturally occurring sulfated polysaccharides are attained from marine algae, seeds, tubers, plant cell wall, yeast, bacteria, fungi and animals. This polysaccharides is very interesting subject because of having potential health, antiviral and biological activities for nontoxic in body.

Polysaccharides are exudates galactomannans, glucomannans, celluloses, β-glucans, pectins, starches, xyloglucans, and gum arabic etc from tissue wounding, carrageenans, agarose, alginates and furcellaran from seaweeds, chitosan and hyaluronan from animal resources, xanthan, dextran, curdlan and gellan from bacterial fermentations, scleroglucan / schizophyllan from fungi (S.Thomas 2013).

Naturally occurring polysaccharides in the plant have high molecular weights and solubility in water is very low. Bo reported hydrolysis by diluted sulfuric acid was carried out to decrease to molecule weights and sulfation with piperidine-N-sulfonic acid and SO_3-pyridine complex gave sulfated glucomannan have potent anti-HIV and anticoagulant activities (Surina Bo and Yoshida, 2012).

The cell walls of marine algae are rich in sulfated polysaccharides such as fucoidans in brown algae, carrageenans in red algae and ulvans in green algae (Wijesekara *et al*, 2010).

Chemically anionic sulfated polysaccharides are widespread not only in plants but also occur in aimals such as mammals and invertebrates (Mourao and Pereira, 1999, Mourao 2007)

They occur in great variety of animals, including the sulfated glycosaminoglucans found in vertebrates and invertebrates animals (Nader et al., 2004).

Recently, naturally occurring sulfated polysaccharides and synthetic sulfated polysaccharides are attracted an increasing of much attention because of their wide variety of special biological and antiviral activities as shown table 1 and 2.

Table 1: The structures of naturally occurring polysaccharides in origin (Heinze, 2006)

Polysaccharides type	Source	Structure	Reference
Cellulose	Plants	β-(1→4)-D-glucose	Klemn *et al*, 2002
Curdlan	Bacteria	β-(1→3)-D-glucose	Nakata *et al*, 1998
Scleroglucan	Fungi	β-(1→3)-D-glucose main chain, β-(1→6)-D-glucose branches	Giavasis *et al*, 2002
Schizophyllan	Fungi	β-(1→3)-D-glucose main chain, D-glucose branches	Rau 2002, Misaki *et al*, 1993
Dextran	Bacteria	α-(1→6)-D-glucose main chain	Huynh *et al*, 1998
Pullulan	Fungi	α-(1→6) linked maltotriosyl units	Shingle KI, 2004
Starch	Plants		Shogren RL, 1998
Amylose		α-(1→4)-D-glucose	
Amylopectin		α-(1→4) and α-(1→6)-D-glucose	
Xylan	Plants	β-(1→4)-D-xylose main chain	Ebringerova *et al*, 2000
Guar	Plants	β-(1→4)-D-mannose main chain, (1→6)-D-galactose branches	Maier h *et al*, 1993
Inulin	Plants	β-(1→2)-fructofuranose	Franck *et al*, 2002
Chitin	Animals	β-(1→4)-D-(N-acetyl)-glucosamin	Roberts GAF, 1992
Chitosan		β-(1→4)-D-glucosamin	
Alginate	Algae	α-(1→4)-L-guluronic acid, β-(1→4)-mannuronic acid	Sabra *et al*, 2005
Carrageenans	Red algae	Sulphated galactans (6 type)	Stanley et al, 1987
Ulvans	Green algae	Sulphated disaccharides [→4)-β–D-*Glcp*A- (1→4)- α-L-Rha*p*(1-]	Marc Lahaye *et al*, 2007
fucoidans	Brown algae	sulfated homo and heteropolysaccharides	Vishchuk *et al,* 2012

1.1.2. Synthetic sulfated polysaccharides

Recently, branched polysaccharides are attached attention because branched structures are biological activities.

In 1966, Ruckel and Scheurch were synthesized (1→6)-α-D-glucopyranan by ring-opening polymerization of 1, 6-anhydro-2, 3, 4-tri-*O*-benzyl-β-D-glucopyranose monomer (Scheurch 1966). Scheurch reported two 1, 6-anhydro disaccharide monomers were polymerized dextran with α and β linked glucoses by ring-opening copolymerization of 1, 6-anhydro maltose (Veruovice and Scheurch, 1970) and 1, 6-anhydro-cellobiose (Masura and Scheurch, 1970), respectively.

Synthetic and natural sulfated polysaccharides were synthesized relationship between structure and biological activities such as anti-HIV and antiviral activities were reported.

Sulfated (1→6)-α-lactopyranans were found to have blood anticoagulant and high anti-HIV activities by ring-opening copolymerization between 1,6-anhydro-2,3-di-*O*-benzyl-4-*O*-(2',3',4',6'-tetra-*O*-benzyl–β-D-galactopyranosyl)-β-D-glucopyranose and 1,6-anhydro-2',3',4'-tri-*O*-benzyl-β-D-glucopyranose, debenzylation and sulfation (Han and Yoshida, 2009). Synthesis of oligosaccharide-branched ribofuranan (Yoshida and Yoshida, 2009), block copolysaccharide by polymerization of anhydro sugar derivatives (Choi and Uryu, 1997) and sulfated alkyl (1→6)-α-glucopyranans with potent anti-HIV activity (Bai and Yoshida, 2015) were reported.

Table 2: The structures of synthetic polysaccharides

Polysaccharides type	Structure	Reference
Levoglucosan	α-(1→6)-D-glucose	Rukel *et al*, 1966
Arabinans	α-(1→4)-L-arabinopyranoses	Yoshida *et al*, 1986
Branched dextran	α-(1→6)-D-glucose main chain	Hatanaka *et al*, 1989
Maltose derivative	α-(1→6)-D-glucose main chain, α-(1→4)-D-glucose branches	Veruovic and Schuerch, 1970
Cellobiose derivative	α-(1→6)-D-glucose main chain, β-(1→4)-D-glucose branches	Masura and Schuerch, 1970
Octadecylated glucopyranans	α-(1→6)-D-glucose main chain, 3–*O*–octadecylated	Kobayashi *et al*, 1985
Mannopyranan	α-(1→6)-D-mannopyranosyl main chain, α-(1→4)-D-mannopyranosyl branches	Kobayashi *et al*, 1992
Glucopyranan	α-(1→4)-D-glucopyranose derivatives	Yoshida and Uryu, 1997
Ribofuranan	α-(1→4)-D-ribofuranose, α-(1→5)-D-ribofuranose, , α-(1→5)-D-xylofuranose	Choi and Uryu, 1997
Astragalus	α-(1→4)-D-glucan	Liu and Uryu, 2003
Lactopyranans	α-(1→6)-D-glucopyranose main chain, β-(1→3)-D-galactopyranose branched	Han and Yoshida, 2008
Glucan	Methylated (1→6)-α-D-glucopyranose	Yoshida and Yoshida, 2008
Ribofuranan	3-*O*-(β-D-lactosyl)-α-D-ribofuranan	Yoshida and Yoshida, 2009
Poly-mannosamine	α-(1→6)-D-mannosamine derivatives	Hattori and Yoshida, 2012
Alkyl glucopyranan	α-(1→6)-D-glucopyranose, 3-*O*-long alkyl groups	Bai and Yoshida, 2015
Galactomannan	α-(1→6)-D-mannopyranose, α-(1→4)-D-galactopyranose	Budragchaa and Yoshida, 2015

1.2 Ring-opening polymerization

The ring-opening polymerization is a good method for stereoregular polysaccharides by polymerized of anhydro-sugar monomer derivatives to high molecule weight as catalyst and which is two type such as random and block copolymerization. Therefore, ring-opening polymerization of anhydro sugars is an important for preparing controllable polysaccharides, for agricultural, medicinal and pharmaceutical industries (Nuyken and Pask, 2013).

Several types of stereoregular polysaccharides such as liner, branched, amino and deoxy polysaccharides were synthesized by ring-opening polymerization of anhydro-sugar derivatives to reveal the relationship between structure and biological activities (Yoshida 2009).

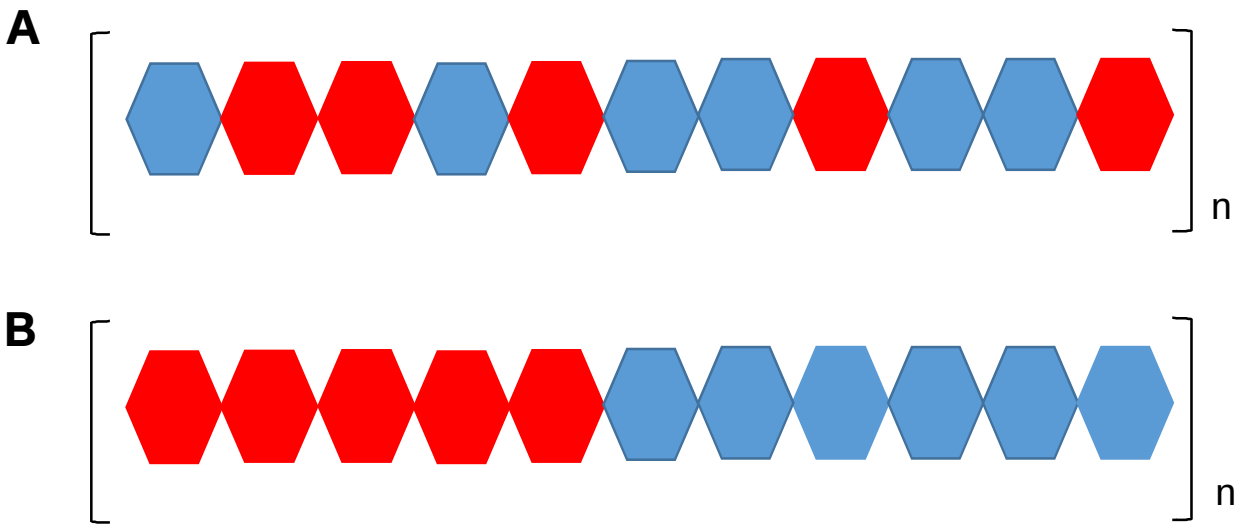

Figure 1: Random (**A**) and Block copolymerization (**B**) of strereoregular polysaccharides.

1.3 HIV

Recently, the human immunodeficiency virus (HIV) is quickly increasing on world and is one of many infection disease in human to causes the acquired immune deficiency syndrome (AIDS). The HIV has two types such as HIV-1 and HIV-2 as shows people living with HIV in figure 1 and structure of HIV in figure 2.

The clinical symptoms of two different types are very slowly in HIV-2 infection than HIV-1. In West Africa and Portugal in Europe, HIV-2 is mainly restricted and HIV-1 is occurs worldwide (Nyamweya *et al,* 2013).

Sulfated polysaccharides are worked by blocking the binding of gp120 glycoprotein of HIV to cell surface receptor, CD4.

Anti-HIV and antiviral activities of sulfated polysaccharides were reported exactly by our laboratory. Nakashima and Yamamoto found the potent anti-HIV activity of natural sulfated polysaccharides lambda-carrageenan that were extracted from sea algae (Nakashima and Yamamoto, 1987). Sulfated polysaccharides extracted from *Erylus discophorus* were avoided the infection of HIV by 90 to 95% inhibition (Esteves and Goncalves, 2011). Mucopolysaccharide and artificial sulfated polysaccharide prepared

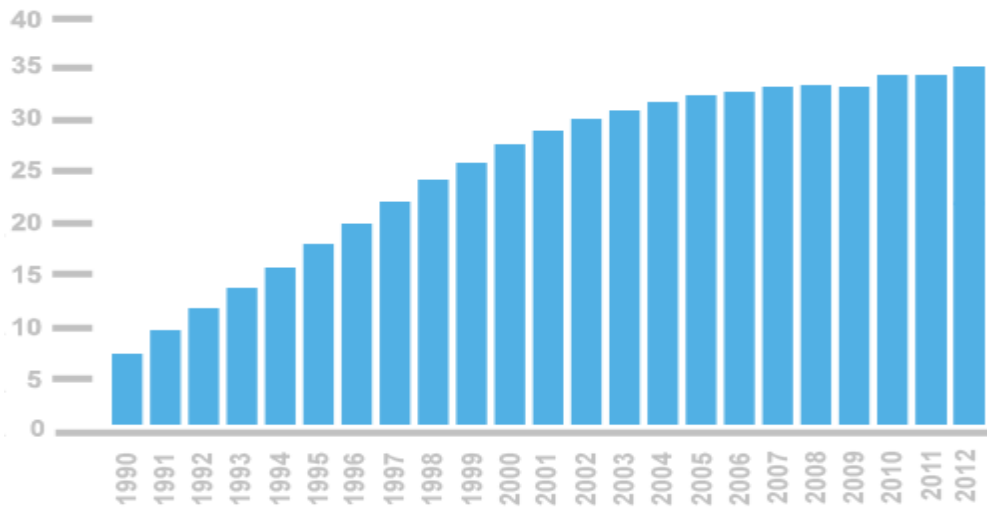

Figure 2: The number of people living with HIV rose (million)
(http://www.avert.org/worldwide-hiv-aids-statistics.htm)

from a marine were also effective (Hashimoto and Shigeta, 1996). Synthetic sulfated polysaccharides: sulfated alkyl glupyranans (Bai and Yoshida, 2015), sulfated lactopyranans (Han and Yoshida, 2008), sulfated polyvinyl alcohol and sulfated copolymers of acrylic acid with vinyl alcohol (Baba and Gorog, 1990) and sulfated alkyl oligosaccharides (Nakashima and Schinazi, 1995) were reported.

Natural polysaccharides such astragalus (Liu and Uryu, 2003), konjac glucomannan (Bo and Yoshida, 2013) and galactomannans (Tegshi and Yoshida, 2012), after sulfation gave anti-HIV activity.

Recently, much attention to the search for potential drug candidates containing higher inhibitory activity against various HIV strains is for prevention and therapy drugs, increasing in pharmaceutical industry.

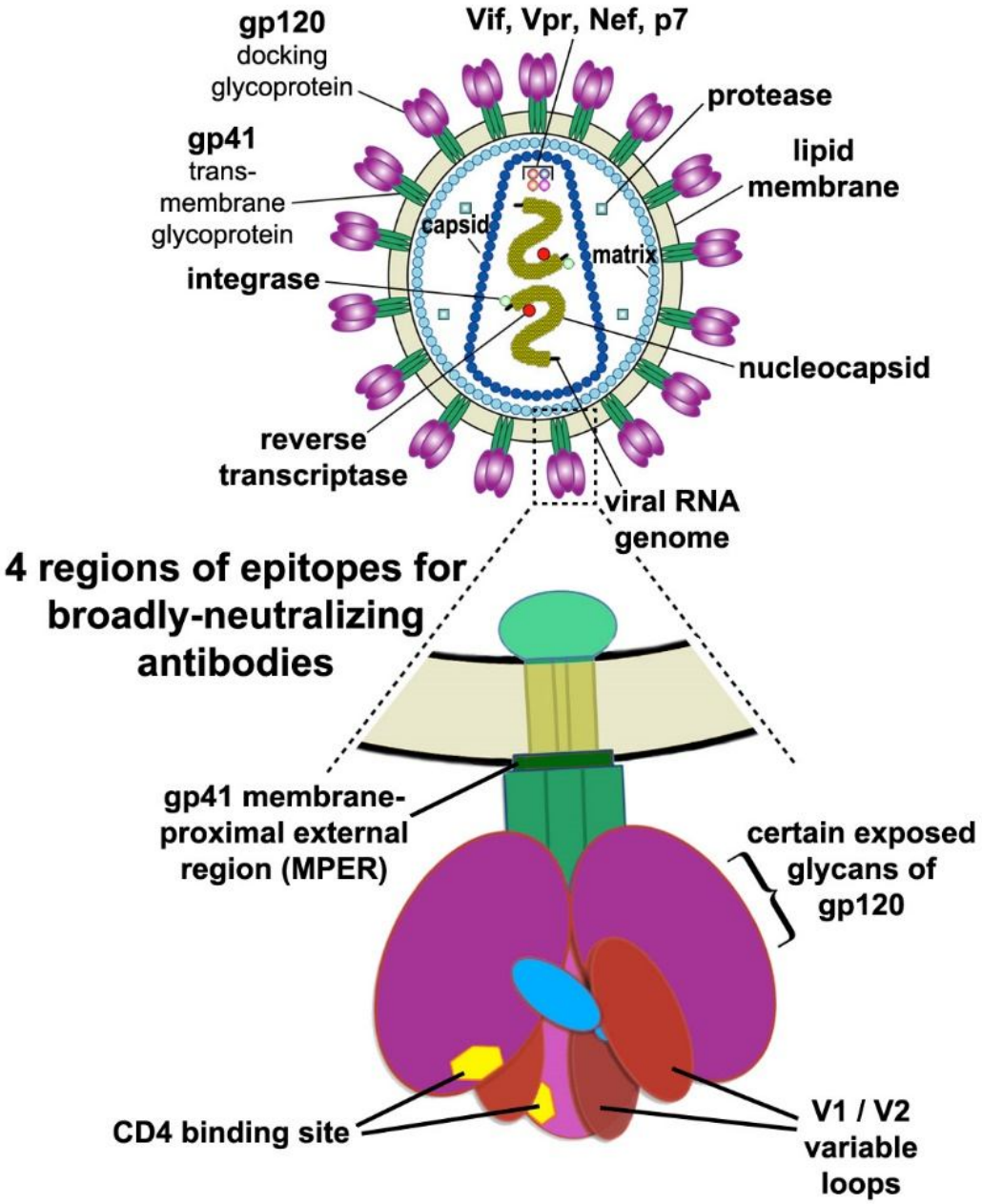

Figure 3: The structure of HIV-1
Haynes *et al*. (2012. Nature Biotechnology 30 (423-433)

1.5 Surface plasmon resonance and Dynamic light scattering

Surface plasmon resonance (SPR) and dynamic light scattering (DLS) are useful to analyze electrostatic interaction between sulfated polysaccharides and model peptid of HIV, gp120. The anti-HIV active is interaction of negatively charged sulfated groups of sulfated polysaccharides and positively charged amino groups as gp120 peptide of HIV. The last years, mostly used search for sulfated polysaccharides and their biological mechanism by SPR and DLS measurements.

Surface plasmon resonance (SPR):

SPR method is a label-free technique, on sensor ship with immobilized EDC / NHS and biopolymers. Many types of sensor chip appeared as CM5 (small organic moleculses to protein, nucleic acid and carbohydrates), CM4 (lower degree that CM5), HPA (long-chain alkanethiol molecules) and L1 (lipophilic residues) etc.

SPR was found in the protein interaction with heparin (Osmond and Coombe, 2002). Interaction between sulfated laquer polysaccharides and poly-L-lysin (Bai and

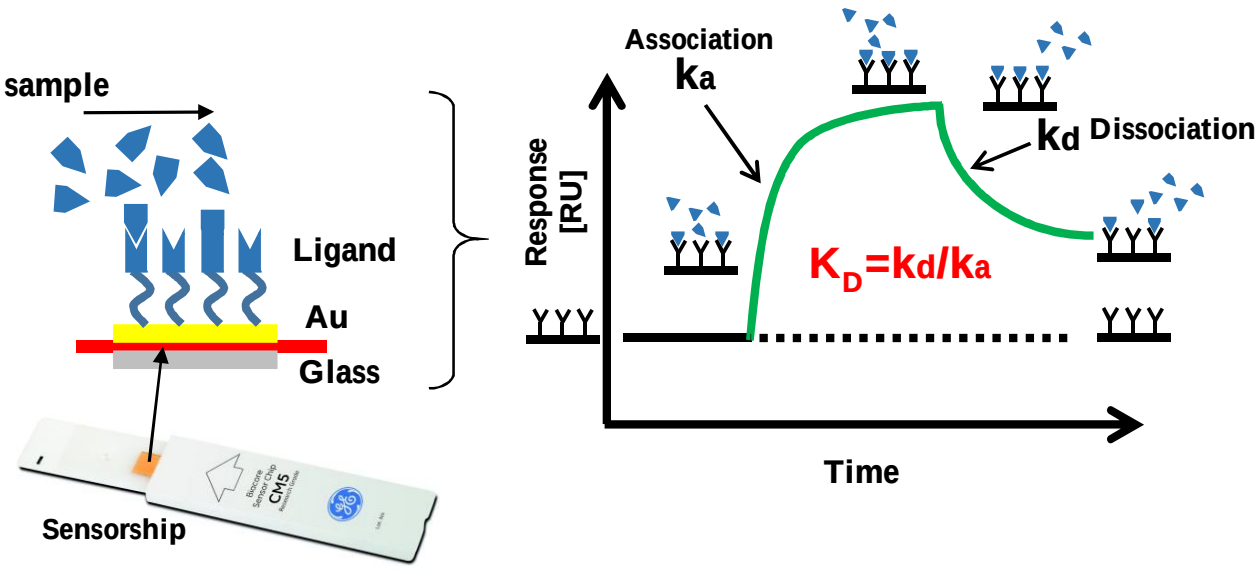

Figure 4. Illustrated scheme of Sulfate plasmon resonance (SPR)

Yoshida, 2013) were reported by our laboratory and carbohydrate-carbohydrate interaction between gangliosides and Gg3-carrying polystyrene (Matsuura and Kobayashi, 2004) and protein-protein interaction (Kim and Chung, 2006) were examined by SPR.

On-off map is summarize diagram of mapping the relationship between sulfated polysaccharides and model peptid by both axes of association rate constant (k_a) and dissociation rate constant (k_d) of SPR measurement.

Dynamic light scattering (**DLS**):

DLS is one of the mostly popular methods for determining a particle size of biopolymers such as proteins-polysaccharides, glucoproteins-polysaccharides, and protein-nucleic acid.

Bai and Yoshida (2015) were examined the particle size and zeta potential of sulfated alkyl glucopyranans with strongly interaction to poly-L-lysin in buffer solution by Otsuka Electronics ELSZ-1000ZS particle size and zeta potential analyzer.

The effect of electrostatic interaction between chitosan and gellan gum was determined by dynamic light scattering measurements (Picone and Cunha, 2013).

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CHAPTER 2.

Synthetic Galactomannans

ABSTRACT

Ring-opening polymerization of a new 1,6-anhydro disaccharide monomer, 1, 6-anhydro-2, 3-di-O-benzyl-4-O-(2', 3', 4', 6'-tetra-O-benzyl-α-D-galactopyranosyl)-β-D-mannopyranose, was carried out using PF_5 as a catalyst under high vacuum at -60°C to give galactose branched mannopyranan (synthetic galactomannan), 4-O-α-D-galactopyranosyl-(1→6)- α-D-mannopyranan, after debenzylation with Na in liquid NH_3. The ring-opening copolymerization with 1, 6-anhydro-tri-O-benzyl-β-D-mannopyranose in various feeds was also performed to give synthetic galactomannans with various proportions of galactose branches. The synthetic galactomannans are different structures of naturally occurring galactomannans.

2.1. INTRODUCTION

We have investigated the synthesis of sulfated polysaccharides with high molecular weights and defined structures by ring-opening polymerization of anhydrosugar monomers and subsequent sulfation to elucidate the relationship between polysaccharide structures and biological activities such as antiviral activities (Yoshida T, 2001). In addition, naturally occurring polysaccharides were also sulfated to give sulfated polysaccharides with antiviral activities (Yoshida T, 2001). Although naturally occurring polysaccharides are the most abundant organic molecules on the earth, such polysaccharides have complex structures so that it is difficult to predict the relationship between structure and biological activity. The ring-opening polymerization of anhydro sugar monomers with Lewis acid catalysts is a good method for a stereo-controlled

synthesis of polysaccharides with defined structures and high molecular weights (Uryu T, 1990, Scheurch C, 1970). Anhydro disaccharide monomers give monosaccharide-branched polysaccharides, which are one of the common structures of natural polysaccharides. Synthesis of polysaccharides with defined structures is important to investigate the relationship between the structure and biological activities.

Six reports have appeared so far on the ring-opening polymerization of anhydro di- and trisaccharide monomers. In the early works, two 1, 6-anhydro disaccharide monomers, benzylated 1, 6-anhydro maltose (Veruovice and Scheurch, 1970) and 1, 6-anhydro-cellobiose (Masura and Scheurch, 1970), were polymerized, to afford dextrans with α- and β-linked glucoses, respectively, in every glucose residue in the main chain. Kobayashi reported the polymerization of a 1, 6-anhydro mannobiose monomer[6] that was synthesized by glycosylation of 1, 6-anhydro mannose with imidated mannose to produce 4-O-α-D-mannopyranosyl-(1→6)-α-D-mannopyranan, which became a model compound for a cell-specific biomedical material using α-D-mannose as a recognition signal. We also reported the polymerization of 1, 5-anhydro ribo-disaccharide (Yoshida and Endo, 2001), ribo-trisaccharide (Yoshida and Yoshida, 2009), and 1, 6-anhydro lactose (Han and Yoshida, 2009) monomers to give the corresponding mono and disaccharide branched polysaccharides.

In particular, sulfated (1→6)-α-D-lactopyranans were found to have potent anti-HIV and blood anticoagulant activities, which depended on the proportion of branches, suggesting that the distance between branches on the main chain polysaccharide plays an important role in the biological activities.

Naturally occurring galactomannans having a (1→4)-β-D-mannopyranosidic main chain structure attached to (1→6)-α-D-galactopyranose branches are found in plant seeds and widely used in food industries (Srivastava and Kapoor, 2005). Interaction with other polysaccharides in water increased viscosity and production of hydrogels (Yalpani, 1998, Tanaka and Hatakeyama, 1998, Pinheiro, and vicente, 2011). The gelation is enhanced by

decreasing the galactose concentration; however, the detailed mechanisms are still unclear.

In general, natural galactomannans have high molecular weights and are insoluble, so galactomannans are hydrolyzed by acid to obtain lower molecular weight galactomannans. The structural analysis and identification of four natural occurring galactomannans were established by comparison with the specific rotations, shape of the GPC profiles, and high resolution NMR spectroscopies (Tegshi and Yoshida, 2011). Furthermore, we found that sulfated natural galactomannans, which were obtained by sulfation, had potent anti-HIV activity (Tegshi and Yoshida, 2012).

In the first part of this paper, we report the synthesis and ring-opening polymerization of a new disaccharide monomer, 1, 6-anhydro-2, 3-di-O-benzyl-4-*O*-(2', 3', 4', 6'-tetra-*O*-benzyl-α-D-galactopyranosyl)-β-D-mannopyranose (LMGABE), to give the corresponding synthetic galactomannan, (1→6)-α-D-mannopyranans with a (1→4)-β-D-galactose branch at position 4 of the mannose residue in the main chain, after deprotection of benzyl protective groups to recover hydroxyl groups. The copolymerization of LMGABE with 1, 6-anhydro-2, 3, 4-tri-O-benzyl-β-D-mannopyranose (LMTBE) was also performed to give (1→6)-α-D-mannopyranans with different proportions of galactose branches.

2.2. EXPERIMENTAL

2.2.1. Materials

1, 6-Anhydro-2, 3, 4-tri-O-benzyl-β-D-mannopyranose (LMTBE) was synthesized by the modified method described in the literature (Sondheimer and Schuerch, 1978).

Dry methylene chloride was distilled under a vacuum below 10^{-5} mmHg using a high vacuum line and stored in a glass ampule under the same high vacuum. The amount of phosphorus pentachloride needed was divided in an ampule under the high vacuum and used directly under pressure. Poly-L-lysine with molecular weight of 1000-5000 was purchased from Wako Pure Chemical Industries, Ltd., Japan. Commercially available anhydrous dimethyl sulfoxide (Wako Pure Chemical Industries, Ltd), piperidine (Sigma-Aldrich, USA), and other chemicals were used without further purification.

2.2.2. Synthesis of 1, 6-anhydro-2, 3-di-O-benzyl-4-O-(2', 3', 4', 6'-tetra-O-benzyl-α -D-galactopyranosyl)-β -D-mannopyranose (LMGABE)

1, 6-Anhydro-2, 3-*O*-benzylidene-β-D-mannopyranose (1.2 g, 4.8 mmol) and 2, 3, 4, 6-tetra-*O*-benzyl-1-*O*-trichloroacetimidoyl-β-D-galactopyranose (1.0 g, 1.5 mmol) were dissolved in anhydrous CH_2Cl_2 (30 ml) under N_2 atmosphere at room temperature and then trimethylsilyl trifluoromethanesulfonate (TMAOTf) (1 ml, 5.5 mmol) was added to the mixture. After stirring for 24 h at room temperature, the mixture was neutralized with trimethylamine (Et_3N), and the precipitates that appeared were filtered out. The methylene chloride layer was washed with water several times, dried with anhydrous sodium sulfate, and then evaporated under reduced pressure. The residue was purified by silica gel column chromatography eluted with EtOAc-hexane in the proportion of 1:2 to give 1, 6-anhydro-2, 3-O-benzylidene-4-O-(2', 3', 4', 6'-tetra-O-benzyl-α-D-galactopyranosyl)-β-D-mannopyranose (1.02 g) in 43.8% yield.

1, 6-Anhydro-2, 3-O-benzylidene-4-O-(2', 3', 4', 6'-tetra-O-benzyl-α-D-galactopyranosyl)-β-D-mannopyranose (1.0 g, 1.3 mmol) was dissolved in MeOH (40 ml)

and then acetyl chloride (0.4 g, 5.2 mmol) was added. After stirring for 30 min at room temperature, the reaction mixture was neutralized with Et_3N and then evaporated to dryness. The residue was extracted with $CHCl_3$. The $CHCl_3$ was washed with water several times, dried with anhydrous Na_2SO_3, and then evaporated. The residue was subjected to silica gel column chromatography eluted with EtOAc-hexane (1:2 v/v) to give pure 1, 6-anhydro-4-O-(2', 3', 4', 6'-tetra-O-benzyl-α-D-galactopyranosyl)-β-D-mannopyranose (0.7 g) in 79% yield after crystallization in the same mixed solvent. To a dry DMF solution (30 ml) of NaH, which was obtained by washing NaH in a 60% oil dispersion (0.24 g, 6.0 mmol) with dry hexane, 1, 6-anhydro-4-O-(2', 3', 4', 6'-tetra-*O*-benzyl-α-D-galactopyranosyl)-β-D-mannopyranose (1.4 g, 2.0 mol) in dry DMF (10 ml) was added at room temperature. After stirring for 30 min, the mixture was cooled by using an ice bath, and then benzyl bromide (0.71 ml, 6.0 mmol) in dry DMF (5 ml) was added dropwise. The mixture was stirred for 18 h at room temperature and then an excess of MeOH was added. The mixture was concentrated by using a rotary evaporator and then the benzylated product was extracted with $CHCl_3$.

The $CHCl_3$ layer was washed with water several times, dried with Na_2SO_4, and evaporated. The residue was purified by silica gel column chromatography eluted with EtOAc-hexane (1:2) to give the disaccharide monomer, 1, 6-anhydro-2, 3-di-*O*-benzyl-4-O-(2', 3', 4', 6'-tetra-O-benzyl-α-D-galactopyranosyl)-β-D-mannopyranose (LMGABE), in 85% (1.5 g) yield after crystallization with $CHCl_3$-hexane solution. The coupling constant between H_1 and H_2 protons in the galactose branch was $J_{1,2}$=4.03 Hz. The specific rotation was =+11.4° (c1, $CHCl_3$) and melting point (mp) =104.8°C. Elementary analysis: observed for C: 74.98%, H: 6.53%, O: 18.50%. Calculated for $C_{54}H_{56}O_{10}$, C: 74.78%, H: 6.54%, O: 18.40%.

2.2.3. Ring-opening polymerization of disaccharide monomer

The ring-opening polymerization of LMGABE and copolymerization with LMTBE were performed with a PF5 catalyst under a high vacuum condition (below 10-5 mmHg) at -60°C by the procedure of the previous paper (Schuerch T, 1972). The results are shown in Table 3.

2.2.3. Debenzylation into synthetic galactomannan

Typical procedures for debenzylation and sulfation of synthetic galactomannans are as follows (no. 2 in Table 4 and no. 2 in Table 5). Copoly(LMGABE) (0.46g) was dissolved in dimethoxyethane (6 ml) and the solution was added dropwise to a Na (0.5 g)-liquid ammonia solution (50 ml) at -78°C. After stirring for 1 h, ammonium chloride and then a small amount of MeOH were added. After the ammonia in the white solution was evaporated at room temperature, deionized water (50 ml) was added. The aqueous layer was extracted with chloroform to remove oily substances and then dialyzed against deionized water for 2 d to give (1→6)-α-D-galactomannnopyranan (0.21 g) in 82.4% yield after freeze-drying from water.

2.2.4. Measurement

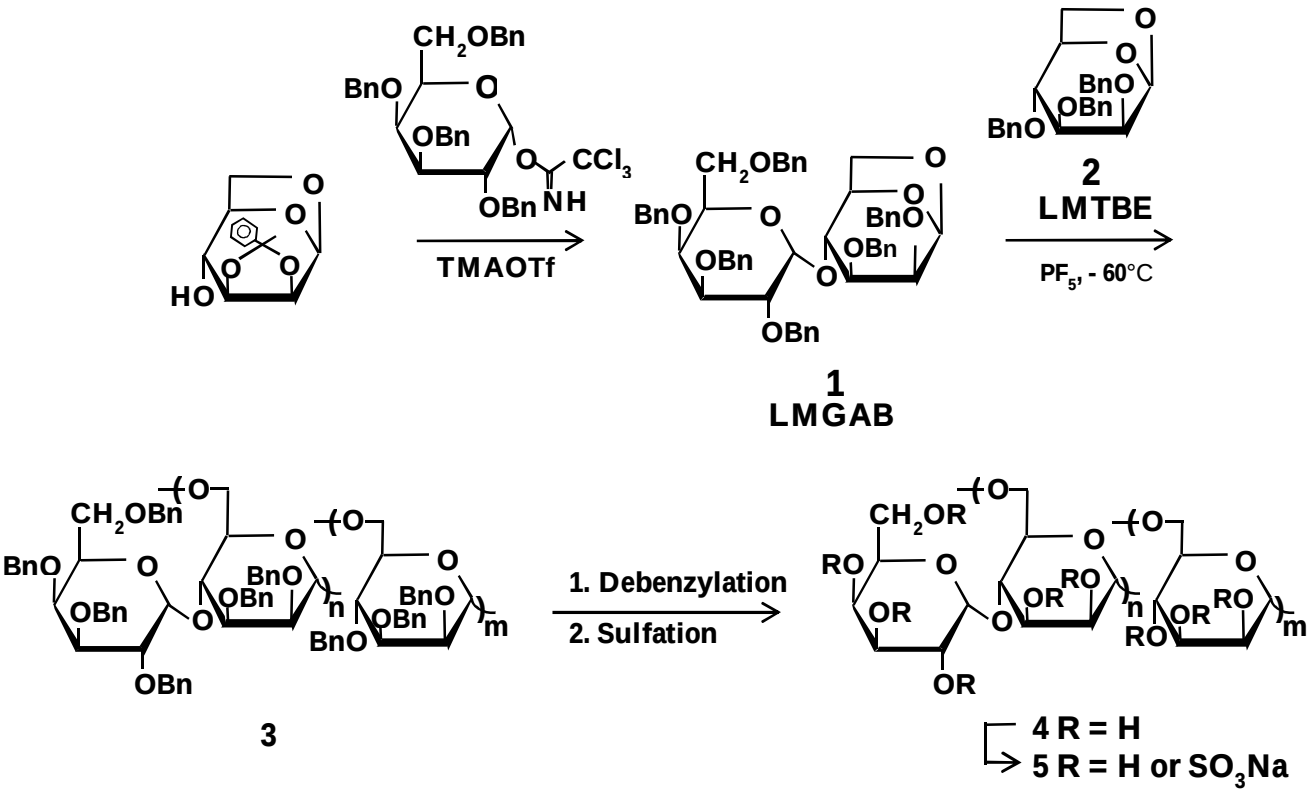
^{1}H and ^{13}C NMR spectra were measured by JEOL JMN AEC-600 and ECM-400 spectrometers at 25°C or 40°C in CDCl3, DMSO-d_6, or D_2O solvent. Two-dimensional spectra were recorded by using JEOL provided programs. Molecular weights were measured by an organic or aqueous phase GPC system using organic phase TSK-gel columns (G2500PWXL, G4000PWXL, and G5000PWXL, 7.6 mm x 300 mm x 3 eluted with chloroform) or aqueous phase columns (G2500HXL, G4000HXL, and G5000HXL 7.6 mm x 300 mm x 3 eluted with 66.7 mmol of phosphate buffer at pH=6.68) at 40°C.

Polystyrene (Shodex standard SM-105) or dextran (Shodex standard P-82) was used as a molecular weight reference. The measurement of specific rotations was carried out with a JASCO DIP-140 digital polarimeter in chloroform or water at 25°C.A Perkin Elmer Spectrum One FT-IR spectrometer was used for measuring IR spectra by a KBr pellet method.

2.3. RESULTS and DISCUSSION

2.3.1. The synthesis and polymerization scheme of the new disaccharide monomer (LMGABE)

Scheme 1 shows the synthesis and polymerization scheme of the new disaccharide monomer, LMGABE. The disaccharide monomer was prepared by glycosylation of 1, 6-anhydro-2, 3-*O*-benzylidene-β-D-mannopyranose with 2, 3, 4, 6-tetra-*O*-benzyl-1-*O*-trichloroacetimidoyl-β-D-galactopyranose with TMAOTf and followed by debenzylidenation to recover hydroxyl groups in the mannose residue and benzylation of the hydroxyl groups in 29.4% yield from the 1, 6-anhydro benzylidenated mannose derivative.



Scheme 1. Synthesis and ring-opening copolymerization of 1,6-anhydro-2, 3-di-*O*-benzyl-4-*O*-(2, 3, 4, 6-tetra-*O*-benzyl-α-D-galactopyranosyl)-β-D-mannopyranose (LMGABE) **1** with 1, 6-anhydro-2, 3, 4-tri-*O*-benzyl-b-D-mannopyranose (LMTBE) **2** into stereoregular sulfated synthetic galactomannan **5**.

2.3.2. Synthesis of disaccharide monomer, *LMGABE*

Figure 7 shows the ^{1}H and ^{13}C NMR spectra, respectively, of the disaccharide monomer, LMGABE, in which all signals were assigned by the 2D NMR measurements of H-H COSY in figure 17 and HMQC in figure 18. In the ^{1}H NMR spectrum (7A), the H1 signals due to 1, 6-anhydro mannose and branched galactose appeared at 5.43 ppm and 4.82 ppm, respectively. The two singlet signals of LMGABE monomer appeared at 101 ppm and 98.5 ppm due to the anhydro mannose and branched galactose (7B), respectively. The specific rotation was $[\alpha]_D^{25}$ =+11.4° (c1, CHCl3) and the coupling constant between H1 and H2 protons ($J_{1,2}$) in the galactose branch was $J_{1,2}$=4.03 Hz. These results suggest that galactose was attached by an α-linkage. The elementary analysis agreed with the calculated one.

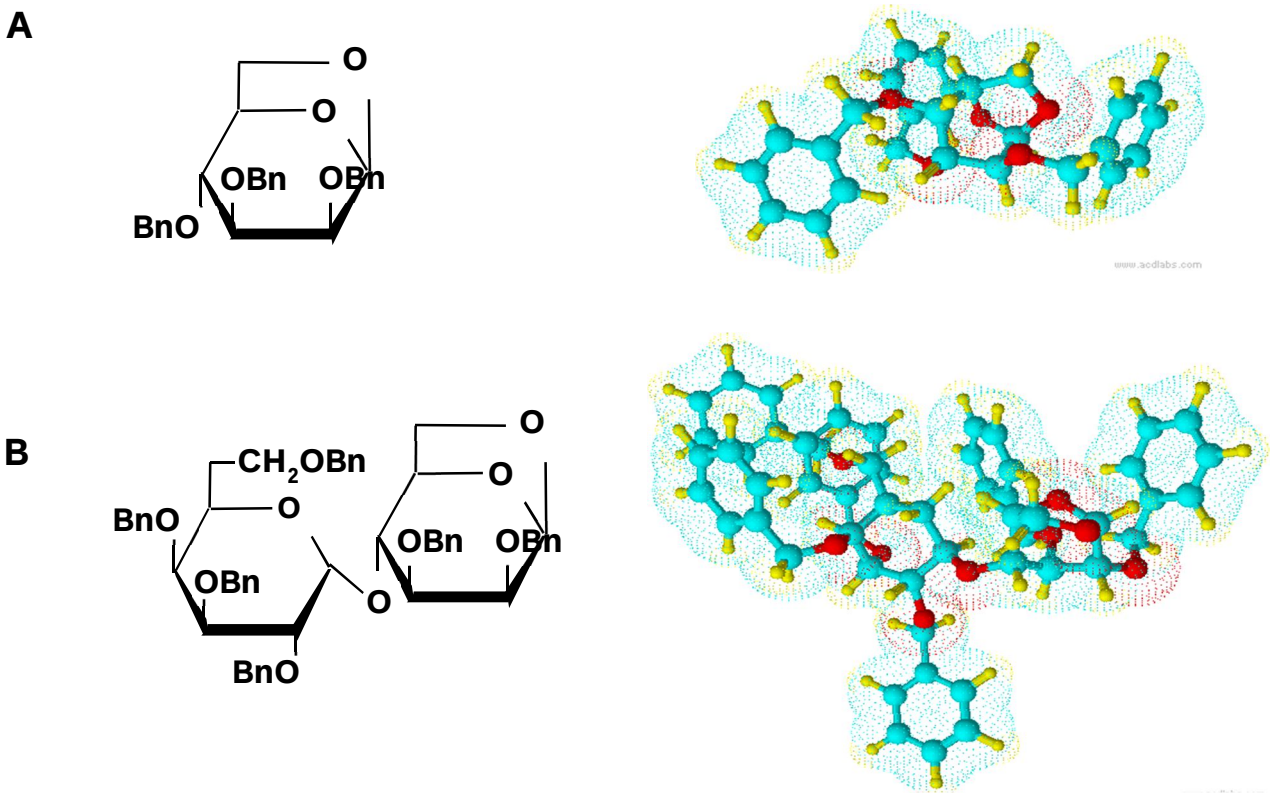


Figure 5: 3D chemical structure of LMTBE (**A**) and LMGABE (**B**)

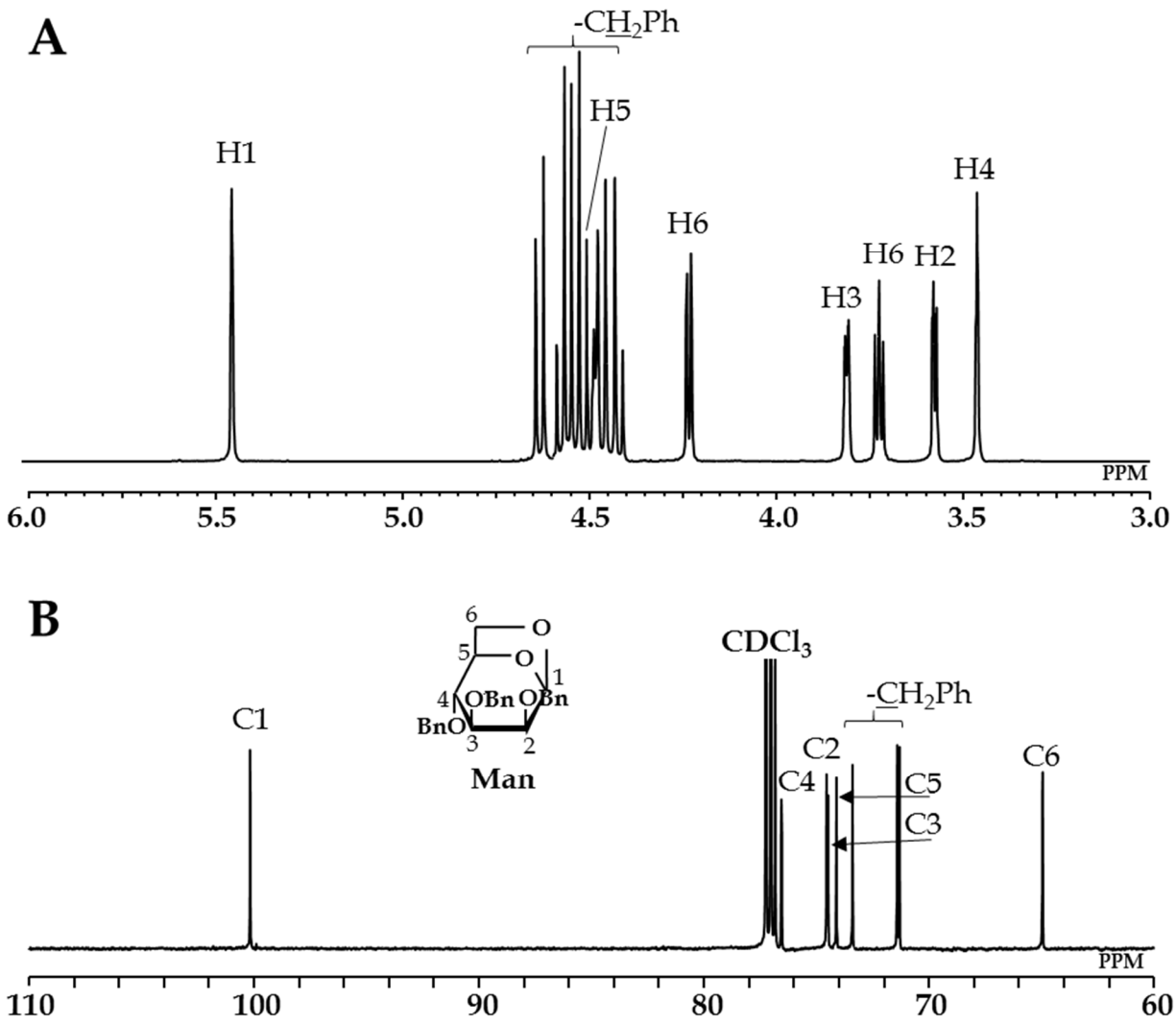


Figure 6: 600 MHz ^{1}H (**A**) and 150 MHz ^{13}C (**B**) NMR spectra of 1, 6-anhydro-2, 3, 4-tri-*O*-benzyl-β-D-mannopyranose (LMTBE) in $CDCl_3$ at 25° C. The specific rotation was $[\alpha]_D^{25}$ =-33.9° (c1, $CHCl_3$). All signals were assigned by H-H COSY and HMQC spectra.

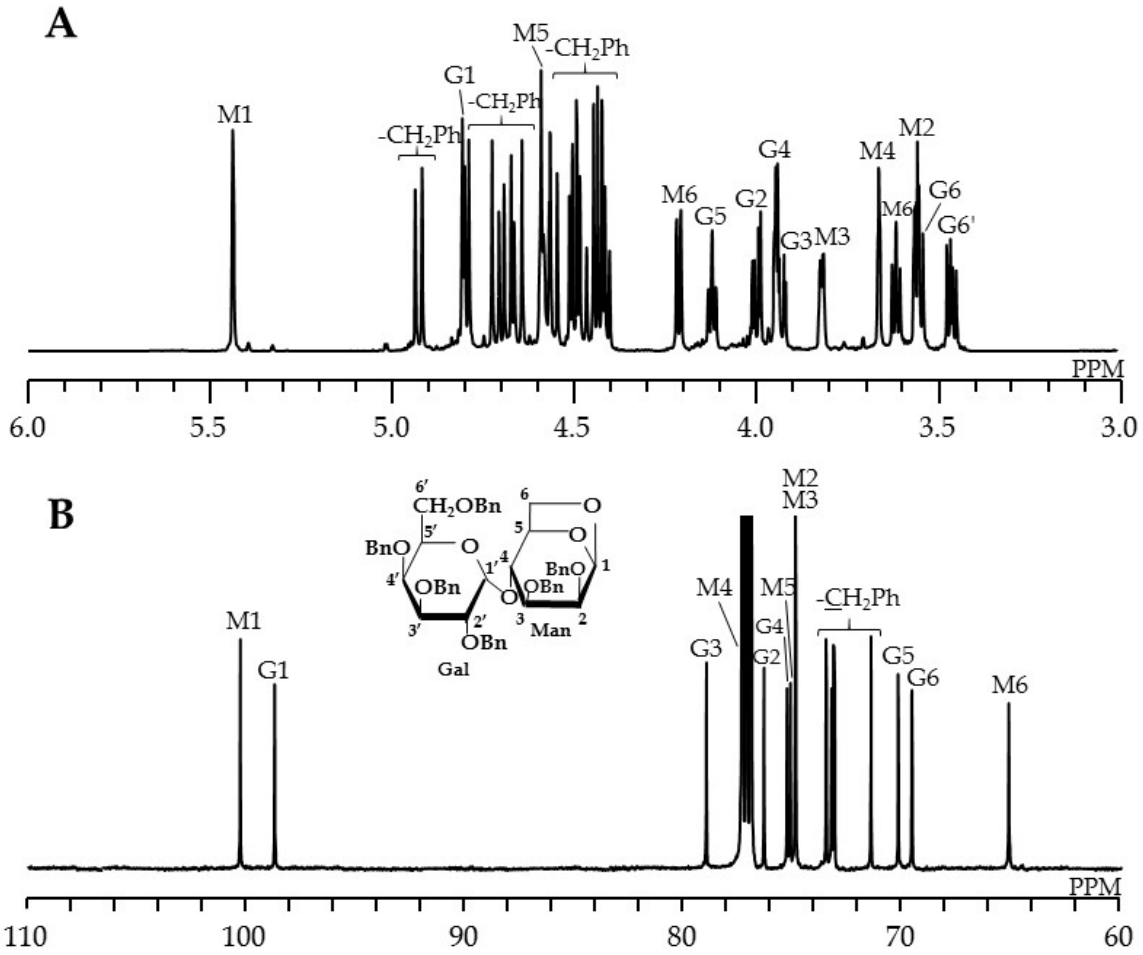


Figure 7: 600 MHz ^{1}H and 150 MHz ^{13}C NMR spectra of 1, 6-anhydro-2, 3-di-*O*-benzyl-4-*O*-(2', 3', 4', 6'-tetra-*O*-benzyl-b-D-galactopyranosyl)-b-D-mannopyranose (LMGABE) in $CDCl_3$ at 25° C. The specific rotation was =+11.4° (c1, $CHCl_3$). All signals were assigned by H-H COSY and HMQC spectra

2.3.3. Polymerization of disaccharide monomer (LMGABE)

The ring-opening polymerizations of the anhydro disaccharide monomer, LMGABE, and copolymerization with benzylated 1, 6-anhydro mannose monomer, LMTBE, were carried out with PF_5 catalyst under high vacuum at -60°C to give the corresponding homo and copolymers with various proportions of galactose branches as shown in Scheme 1. Table 3 shows the results of the homo- and copolymerizations. Catalyzed by large amounts of PF_5 was needed for polymerization of the disaccharide monomer compared to that of monosaccharide monomers because the catalyst was consumed by many protective benzyl oxygens of hydroxyl groups. When the homopolymerization of the disaccharide monomer, LMGABE, was carried out with 20 mol% of PF_5 for 26 h, the disaccharide monomer was found to be polymerized to give the corresponding galactose-branched polymer, poly(LMGABE). However, neither yield nor molecular weight was very high, 46.7% and $\overline{M}_n$ = 7.6x10^3, respectively.

The low reactivity of the disaccharide monomers is probably due to the larger molecular weights and the steric hindrance of the crowed branched structure in the resulted polymers (Veruovice and Schuerch, 1970, Yoshida and Endo, 2001). Therefore, copolymerization with the tribenzylated 1, 6- anhydro mannose monomer, LMTBE, was performed to form the copolysaccharides with different proportions of galactose branches, with high molecular weights, and in high yields. The copolymerization with LMTBE monomers in the proportion of 25 mol% afforded the copolymer in the proportion of LMGABE unit of 86.5 mol% (no. 2). The yield and molecular weight increased to 72.5% and $\overline{M}_n$=22.8x10^3, respectively. Providing a higher proportion of LMTBE monomer for the copolymerization increased the yields and molecular weights of the corresponding copolymers, probably due to the high polymerizability of LMTBE. With LMTBE in a proportion of more than 50 mol%, copolymers with higher molecular weights were obtained in high yields as shown in nos. 3 and 4 of Table 3.

Table 3: Ring-opening copolymerization of LMGABE and LMTBE[a]

No	Monomer				Time	Polymer			
	LMGABE		LMTBE			Yield	$\overline{M}_n$ [b]	$[\alpha]_D^{25}$	LMGABE unit[d]
	g	mol%	g	mol%	h	%	x10^3	deg[c]	mol%
1	0.50	100	0	0	26	46.7	7.6	+47.6	100
2	0.43	75	0.07	25	26	72.5	22.8	+60.1	86.5
3	0.33	50	0.17	50	24	93.5	75.4	+53.6	52.2
4	0.20	25	0.30	75	24	92.6	102.2	+59.4	18.1
5	0	0	0.50	100	5	93.7	113.5	+60.6	0

a) Total monomer weight: 0.5g, Solvent: CH_2Cl_2; 1 ml, Catalyst: PF_5; 20 mol%, temperature: -60°C.
b) Determined by chloroform GPC.
c) Measured in $CHCl_3$ (c1)
d) Calculated from ^{13}C NMR spectrum.

Figure 8 shows the ^{13}C NMR spectra of homo- and copolymers of LMGABE and LMTBE monomers. The signals of homopoly(LMTBE) in Figure 2E were assigned by 2D H-H COSY and HMQC measurements. The spectrum of homopoly(LMTBE) gave single signals corresponding to each carbon and a high and positive specific rotation, +60.6° (c1, $CHCl_3$), suggesting that the homopolymer had complete (1→6)-α stereoregularity.

In spectrum 8A of homopoly(LMGABE), the galactose branches were attached at position 4 of every mannose residue in the main chain, and the two C1 signals appeared at 97.5 ppm and 100.5 ppm due to mannose in the main chain and branched galactose, respectively. The specific rotation was also high and positive at $[\alpha]_D^{25}$= +47.6° (c1, $CHCl_3$), suggesting that the homopoly(LMGABE) had a (1→6)-α-D-pyranosidic structure.

Copolymers in Figures 8B-8D gave complex and overlapped signals in the ^{13}C NMR spectra because of random copolymerization of the two monomers. The C1 signal

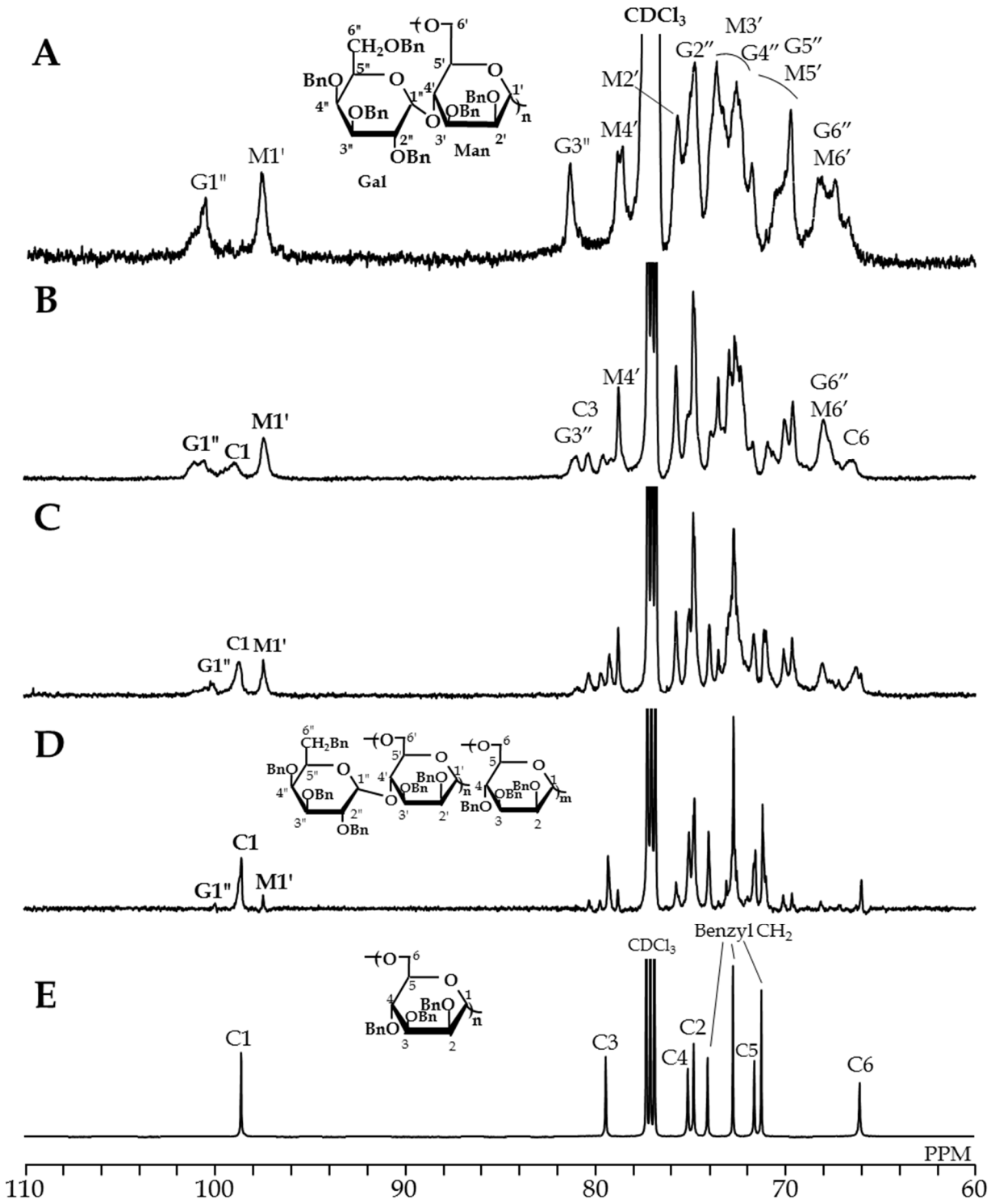


Figure 8: 150 MHz and 125 MHz (E) ^{13}C NMR spectra of (A) poly(LMGABE), (B)-(D) copoy(LMTBE-LMGABE)s, and (E) poly(LMTBE) in $CDCl_3$ at 25 C. The proportions of LMGABE and LMTBE units in the copolymers were (B) 81.9:18.1, (C) 47.8:42.4, and (D)13.5:86.5, respectively. The specific rotations were =+47.6° , +60.1° , +53.6° , +59.4° , and +60.6° (c1, $CHCl_3$), respectively. Signals were assigned by H-H COSY and HMQC spectra. Abbreviations: M, mannose; G, galactose.

at 97.5 ppm due to the mannose main chain decreased with increasing LMTBE units, and a new C1 signal at 98.5 ppm appeared due to the C1 signal of the mannose main chain with out attached galactose branches. The polymerization of 1, 6-anhydro hexose monomers proceeded by the trialkyloxonium ion mechanism. The copolymers also had (1→6)-α stereoregularity, because both homopolymers had a (1→6)-α-pyranosidic structure and the specific rotations of the copolymers had high and positive values.

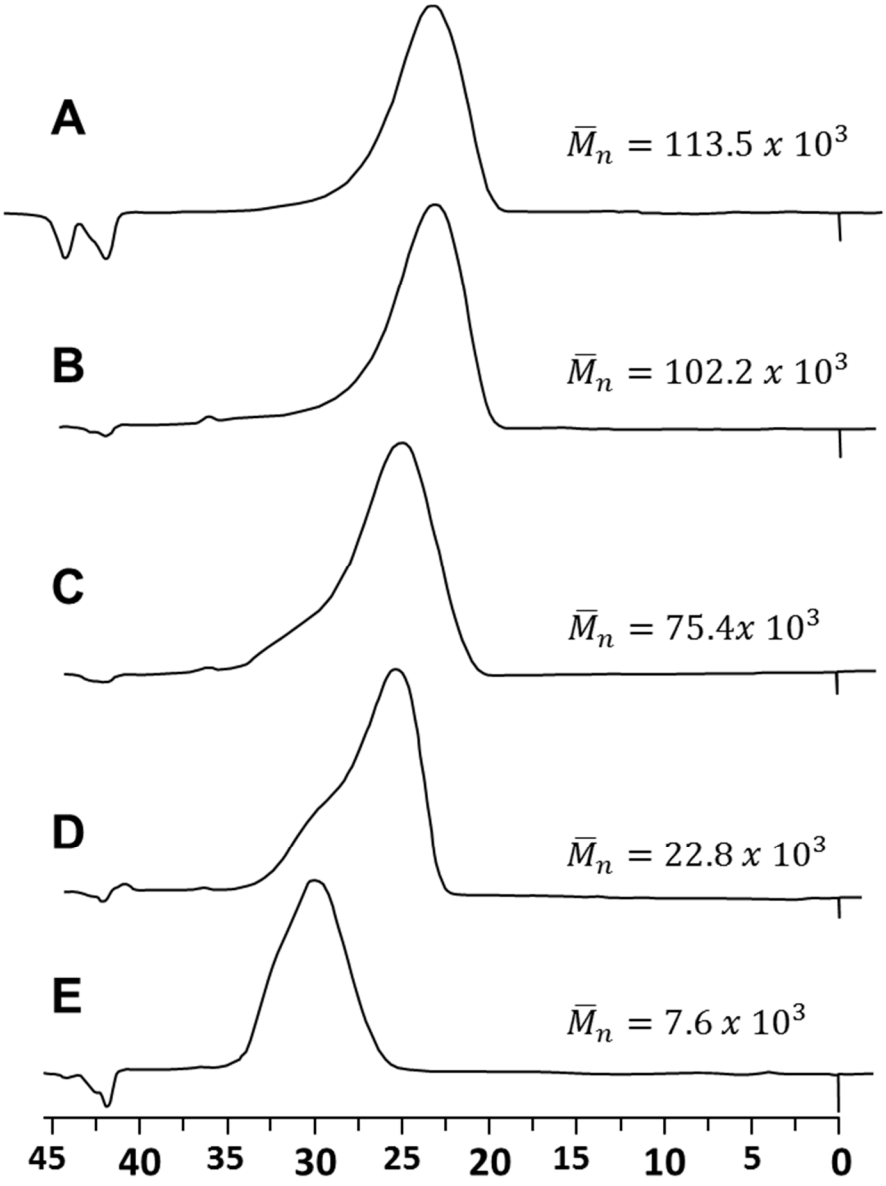


Figure 9: Chloroform GPC profiles of benzylated galactomannans. **A**. (1→6)-α-D-mannopyranan, galactomannans in copoly(**B-D**) were 81.9-18.1, 47.8:42.4, and 13.5:86.5, **E**. (1→6)-α-D-galactomannopyranan at $CHCl_3$ as solvent, 40° C

2.3.4. Debenzylation of benzyl Galactomannans

The benzyl groups in homopoly(LMGABE) and copoly(LMGABE-LMTBE)s were successfully removed by a Birch reduction using Na in liquid ammonia to give the corresponding galactose-branched mannopyranans (synthetic galactomannans) with free hydroxyl groups. The results are presented in Table 4. The yields were relatively high, and the specific rotations decreased with decreasing the proportion of disaccharide residues in the copolysaccharides. Homopoly(LMGABE) gave a synthetic galactomannan with galactose-branches in every mannose residue in the main chain and the molecular weight was $\overline{M}_n$ =4.2x10^3. The copolysaccharides had molecular weights between $\overline{M}_n$ = 12.6x10^3 and 19.1x10^3. By debenzylation, synthetic galactomannans with different proportions of galactose branches from 78.2 mol% to 28.6 mol% were obtained with newly structures.

Table 4. Debenzylation of copoly(LMGABE and LMTBE)s[a]

No	Benzylated polymer					Free galactomannan					
	LMGABE	LMTBE	wt	$\overline{M}_n$	$[\alpha]_D^{25}$	wt	Yield	$\overline{M}_n$[b]	$\overline{M}_w/\overline{M}_n$	Disaccharide unit	$[\alpha]_D^{25}$[d]
	mol%		g	x10^3	deg	g	%	x10^3		mol%[c]	deg
1	100	0	0.20	7.6	+47.6	0.08	86.0	4.2	1.7	100	+135.7
2	86.5	13.5	0.46	22.8	+60.7	0.21	82.4	12.6	1.6	78.2	+129.7
3	42.2	47.8	0.40	75.4	+53.6	0.11	50.5	13.0	1.3	56.6	+126.4
4	18.1	81.9	0.35	102.2	+59.4	0.13	65.7	19.1	1.1	28.6	+108.6
5	0	100	0.40	113.5	+60.6	0.14	95.3	37.2	1.3	0	+90.7

a) Condition: Na; 0.4 g, solvent: Liq. NH_3; 50 mL, Time: 60 min, Temp: -78°C
b) Calculated by aqueous GPC
c) Calculated from ^{13}C NMR spectrum in D_2O
d) Measured in D_2O (c1)

Figure 10 shows the ^{13}C NMR spectra of homopolysaccharides in Figures 10A and 10E and copolysaccharides in Figures 10B-10D corresponding to those in Figure 8. After debenzylation, the ^{13}C NMR spectra became simple, and the C6 signals due to main chain mannose and branched galactose appeared around 63.5 ppm, and the intensity decreased with decreasing proportion of the disaccharide residues in Figures 10B-10D. The C1 signals appeared as complex peaks in contrast to the C6 signals. However, the homo- and copolysaccharides had high and positive specific rotations of more than $[\alpha]_D^{25} = +100°$ (c1, H_2O), indicating (1→6)-α stereoregularity. In spectrum 10E, after debenzylation, poly(LMTBE) gave complete (1→6)-α stereoregular mannopyranan with $[\alpha]_D^{25} = +90.7°$ (c1, H_2O) because the C1 and other signals appeared as single peaks, respectively.

Naturally occurring galactomannans have a (1→4)-β-D-mannopyranosidic main chain with a single or several (1→6)-α-D-galactopyranose branches. The synthetic galactomannans had a (1→4)-α-D-mannopyranosidic main chain attached with a α-D-galactopyranosidic branch at the C4 position of the mannose residues. Although the structure of the synthetic galactomannans was different from that of natural galactomannans, we investigated the anti-HIV activity and interaction with poly-L-lysine as a model peptide of the surface protein of HIV by SPR, DLS, and zeta potential.

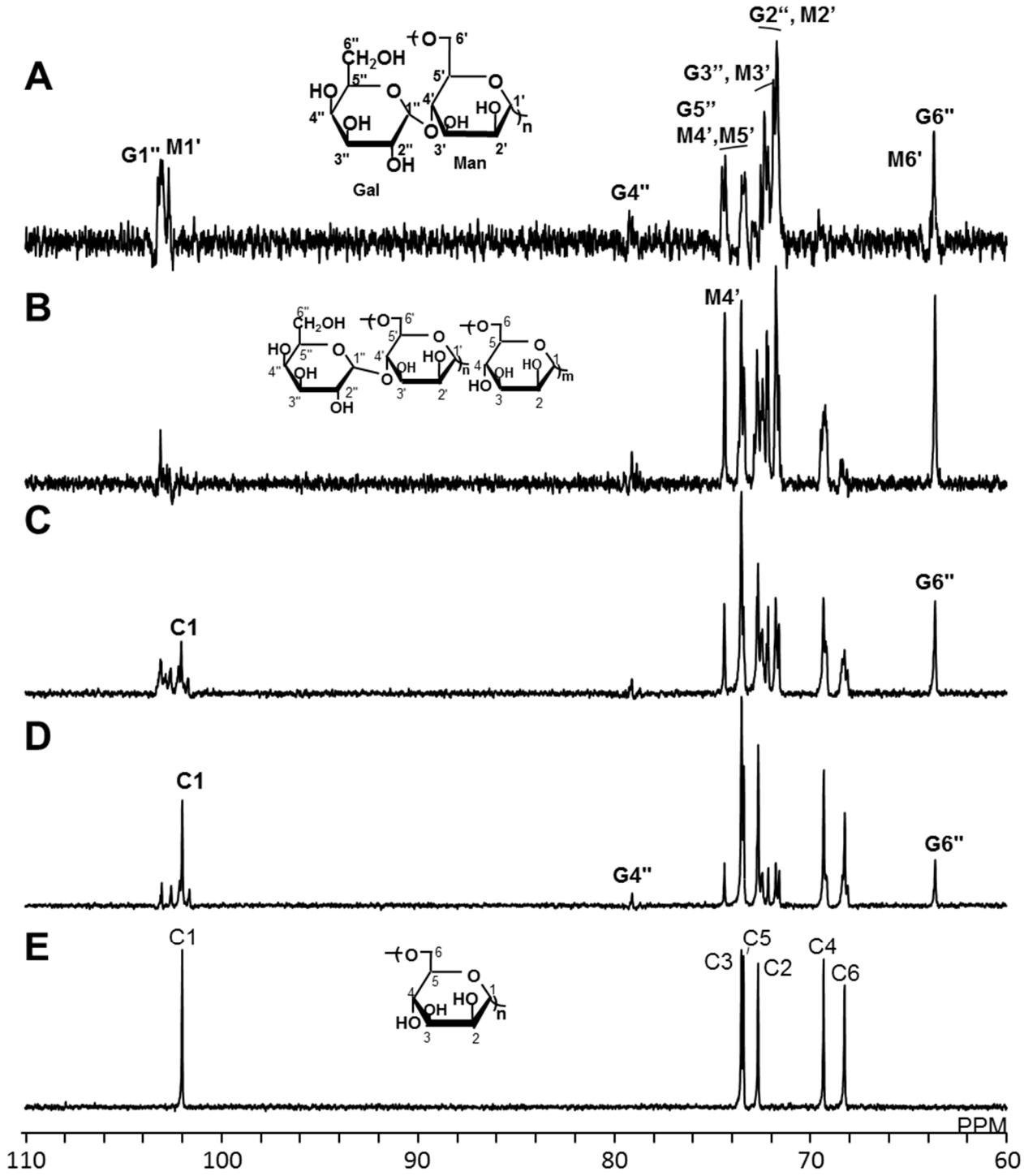


Figure 10. 150 MHz and 125 MHz (E) ^{13}C NMR spectra of (1→6)-α-D-galactomannans in D_2O at 40° C. (A) (1→6)-α-D-galactomannan, (B)-(D) (1→6)-α-D-galactomannans, (E) (1→6)-α-D-mannopyranan. The proportion of Gal and Man units in the copolysaccharides was (B) 71.4:28.6, (C) 43.4:56.6, and (D) 21.8:78.2, respectively. The specific rotation was =+135.7° , +129.7° , +126.4° , +108.6° , and +90.7° , respectively. Signals were assigned by H-H COSY and HMQC spectra. Abbreviations: M, mannose; G, galactose.

2.4 Conclusion

We have synthesized benzyl (1→6)-α-D-galactomannans with branched galactose by ring-opening copolymerization of LMGABE and LMTBE. ^{1}H NMR spectra of benzyl and debenzylated galactomannans were calculated proportion of branched galactose units and all signals were assigned by the 2D NMR measurements of H-H COSY, H-C HMQC and more structures analysis by FT-IR spectroscopy. The molecule weights and specific rotations were examined the synthetic galactomannans.

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CHAPTER 3.

Sulfation and anti-HIV activity of synthetic galactomannans

ABSTRACT

Synthetic sulfated galactomannans were found to have anti-HIV activity and cytotoxicity as high and low as those of standard curdlan and dextran sulfates, respectively, which are potent ant-HIV sulfated polysaccharides with low cytotoxicity.

Sulfation of synthetic galactomannan was carried out by using piperidine-*N*-sulfonic acid solution in dimethylsulfoxide (DMSO) to give sulfated synthetic galactomannans. Degree of sulfation was calculated by elemental analysis.

3.1. INTRODUCTION

We are known to sulfated polysaccharides have special biological activity. In recent time, researchers were extracted of sulfated polysaccharides with therapeutic potential from seaweeds and their discovered newly sulfated oligosaccharides (Seema Patel, 2012). Carrageenans, pentosan polysulfate, dextran sulfate, fucoidan and sulfated some polysaccharides has been demonstrated to have potent inhibitors for human immunodeficiency virus, vesicular stomatitis virus, sindbis virus, herpes simplex virus and human cytomegalovirus (Masanori Baba and Erik de Clerco, 1978). Therefore, much attention for semisynthetic sulfated polysaccharides because their study to development novel drug, cosmetics, pharmaceutical and addition food industries. Sulfated polysaccharides are rich source to never end of most naturally occurring products. Thus, by separate technique and new instrumental have been developed now.

In the latter part, we described the synthesis of sulfated galactomannans by sulfation (Bo and Yoshida, 2013, Han and Yoshida, 2009) of the synthetic galactomannans. Synthetic sulfated galactomannans were found to have potent anti-HIV activity.

3.2.EXPERIMENTAL

3.2.1. Materials

Piperidine-N-sulfonic acid was synthesized by method in literature (Nagasawa and Yoshidome, 1986). Debenzylated (1→6)-α-D-Galactomannnopyranan was synthesized sulfated galactomannans by piperidine-*N*-sulfonic acid. Commercially available anhydrous dimethyl sulfoxide was obtained Wako Pure Chemical Industries, Ltd, Piperidine was purchased from Sigma-Aldrich, USA and other chemicals were used without firthur purification.

The structure analysis of the synthetic sulfated galactomannans was characterized by high resolution NMR spectroscopy, FT-IR spectroscopy and degree of sulfation by elemental analysis.

3.2.2. Sulfation of synthetic Galactomannans

(1→6)-α-D-Galactomannnopyranan (0.135 g) was dissolved in dry DMSO (15 ml), and piperidine-*N*-sulfonic acid (2.0 g) was added at room temperature. The solution was heated at 90°C and stirred for 1.5 h. After cooling to room temperature, 10% NaOH solution was added to the mixture for neutralization. The solution was dialyzed by deionized water for 2 day and the dialysate was concentrated and freeze-dried to give sulfated (1→6)-α-D-galactomannnopyranan (0.225 g).

3.2.3. Anti-HIV activity

Anti-HIV activity was assayed by the MTT method described in the previous paper[18] and calculated by the 50% effective and cytotoxic concentrations (EC_{50} and CC_{50}) of synthetic sulfated galactomannans. We used HIV-$1_{HTLV\text{-}IIIB}$ virus and MT-4 cells, which is an HIV-sensitive cell by in vitro.

3.3. RESULTS and DISCUSSION

3.3.1. Sulfation and anti-HIV activity of synthetic Galactomannans

The synthetic galactomannans (80 mg, no3 in table 5) were sulfated with piperidine-*N*-sulfonic acid in DMSO to give sulfated galactomannans (148 mg) in relatively good conversions as shown in Table 5.

Figure 11 shows the ^{13}C NMR spectra of (A) sulfated synthetic galactomannan with a galactose branch in every mannose residue, (B) with 56.6 mol% of galactose branches in mannose main chain, and (C) sulfated (1→6)-α-D-mannopyranan, respectively. The spectra were broadened and complex due to the sulfation.

Table 5. Sulfation and antiviral activity of (1→6)-α-D-galactomannans[a]

	Free galactomannan							Sulfated galactomannan								
No	Disaccharide Proportion mol%	Mannose wt mg	PSA g	Temp °C	Time h	Yield mg	$\overline{M}_n$ x10^3	DP[b] deg	$[\alpha]_D^{25}$	Elemental analysis C %	H %	S %	DS[c]	EC_{50}[d] µg/ml	CC_{50}[e] µg/ml
1	100	0	46	0.5	90	1.5	78	6.5	20.1	+88.7	21.66	3.64	11.1	1.16	0.23	>200
2	78.2	21.8	135	2.0	90	1.5	225	4.0	15.1	+62.3	26.73	4.38	13.2	1.11	2.14	>200
3	56.6	43.4	80	1.0	90	1.5	148	4.6	20.5	+61.2	26.01	4.34	12.9	1.12	1.93	>200
4	28.6	71.4	90	1.0	90	1.5	172	5.2	27.7	+54.8	25.65	4.32	13.2	1.15	0.44	>200
5	0	100	100	1.0	90	1.5	189	7.5	46.5	+45.6	20.49	3.68	13.9	1.52	0.18	>200
	Dextran sulfate							8.5					18.4	2.1	0.06	>391
	Curdlan sulfate							79.0					14.1	1.4	0.14	>760

a) Sulfation was carried out with piperidine-*N*-sulfonic acid in DMSO.
b) Degree of polymerization
c) Degree of sulfation.
d) 50% Effective concentration on HIV.
e) 50% Cytotoxic concentration on MT4 cell.

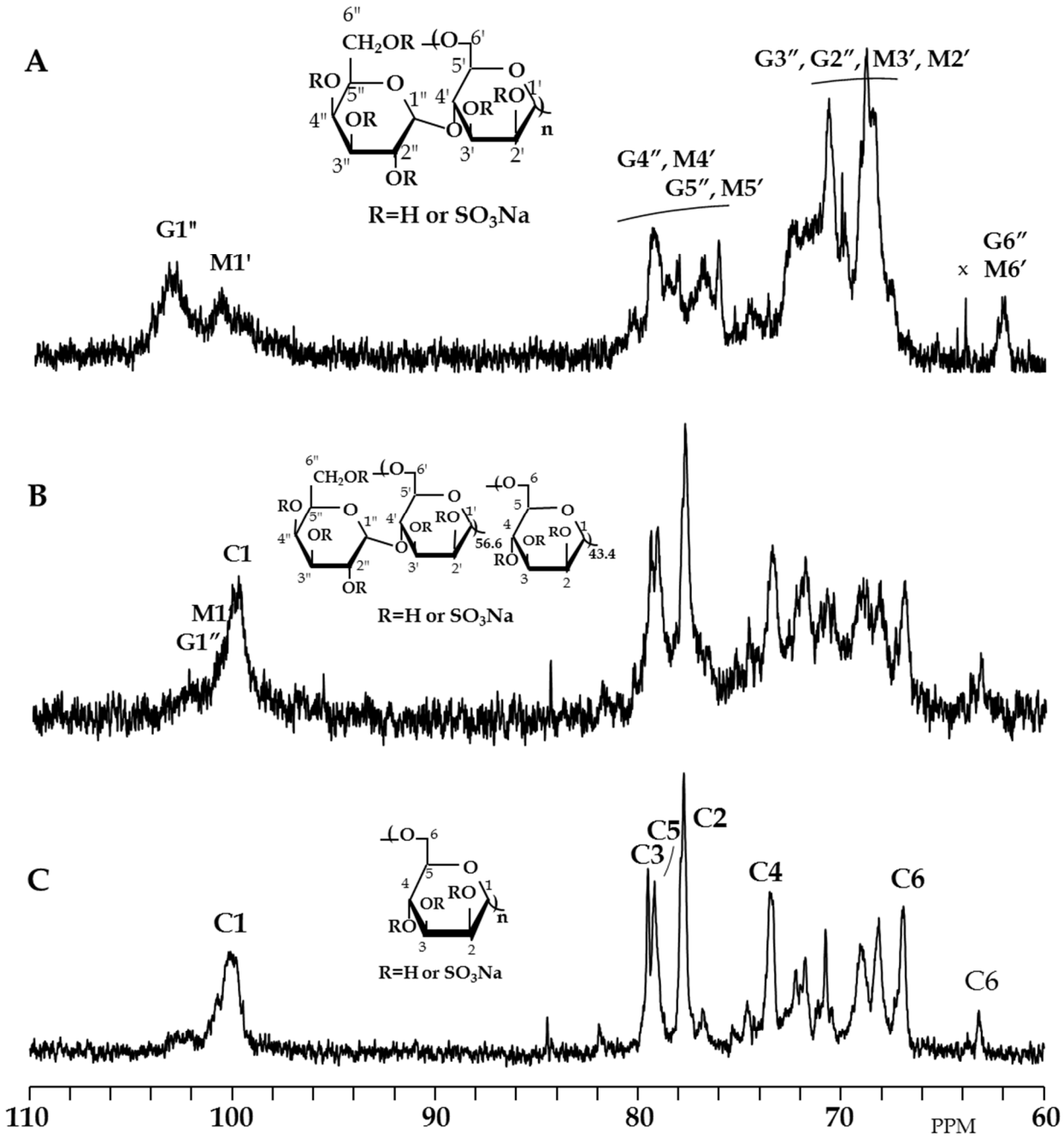

Figure 11: 600 MHz ^{13}C NMR spectra of (A) sulfated (1→6)-a-D-galactomannan, (B) sulfated (1→6)-a-D-galactomannan with 56.6 mol% of galactose branch in the mannose main chain, and (C) sulfated (1→6)-a-D-mannopyranan at D_2O at 40°C.

In Figure 11A, two C1 signals appeared due to the galactose branch at 103 ppm and mannose at 100 ppm in the main chain, respectively, and the intensity of the C1 signal due to the galactose branch decreased with increasing mannose residue. Further assignments were difficult because of the complex and overlapping signals by the sulfation. The molecular weights were around $\overline{M}_n = 5x10^3$ and decreased in comparison with those before sulfation, probably due to acidic conditions during sulfation. The specific rotations decreased gradually from $[\alpha]_D^{25}$ = +88.7° to +45.6° (c1, H_2O) with the decreasing proportion of galactose branches, indicating that the galactose branch was attached by an α-linkage to mannose in the main chain. The degree of sulfation was calculated to be around 1.1 by the results of elementary analysis.

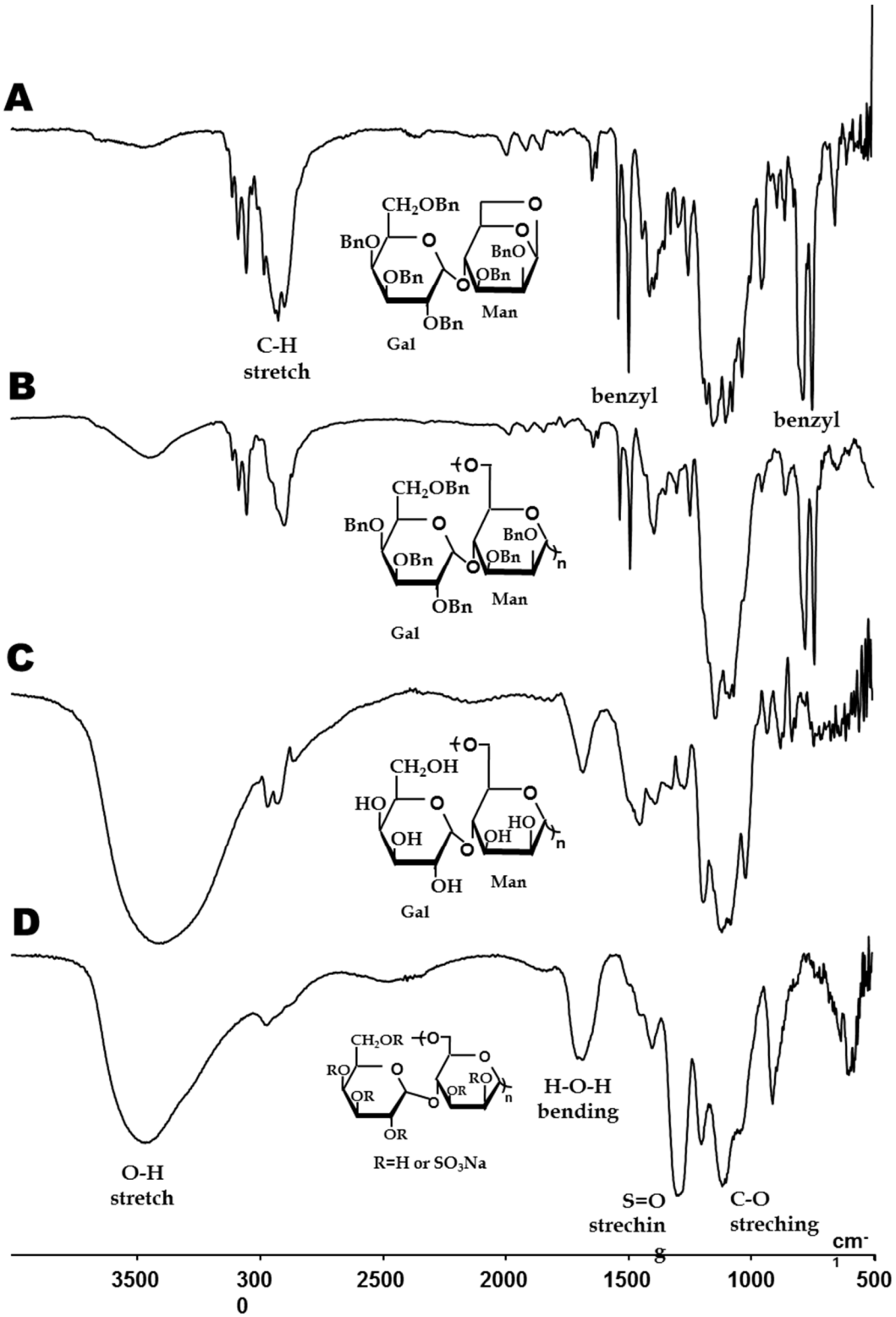

Figure 12. FT-IR spectra of (A) LMGABE monomer, (B) poly(LMGABE), (C) (1→6)-α-D-galactomannan, and (D) sulfated (1→6)-α-D-galactomannan by a KBr pellet method.

Figure 12 shows the FT-IR spectra of (A) LMGABE monomer, (B) homopoly(LMGABE), (C) 4-*O*-α-D-galactopyranosyl-(1→6)-α-D-mannopyranan, and (D) sulfated 4-*O*-α-D- galactopyranosyl-(1→6)-α-D-mannopyranan, respectively. In spectra 5A and 5B, characteristic signals of benzyl groups appeared around 2890 cm^{-1}, 1605 cm^{-1}, 1460 cm^{-1}, and 700 cm^{-1}, respectively, due to C-H stretching and bending vibrations. After debenzylation followed by sulfation, a large absorption due to an OH stretching vibration appeared around 3450 cm^{-1} and a S=O stretching vibration due to an $-OSO_3Na$ group appeared around 1250 cm^{-1} in spectra 5C and 5D. On the other hand, the large absorption around 1650 cm^{-1} was assigned to H-O-H bending vibrations due to water in debenzylated and sulfated galactomannans.

The anti-HIV activity was determined by the MTT method according to the reported procedure (Pauwels and DeCercq, 1988) and evaluated by the 50% effective concentration (EC_{50}) of synthetic sulfated galactomannans for prevention of the multiplication of HIV as presented in Table 5. The cytotoxicity denotes the 50% cytotoxic concentration (CC_{50}) on MT-4 cells. We found that the anti-HIV activity of sulfated synthetic galactomannans was as high as that of standard dextran and curdlan sulfates, which are the most effective sulfated polysaccharides with low cytotoxicity, even though the sulfated synthetic galactomannans synthesized here had lower molecular weights than those of the standards. The cytotoxicity was low, more than 200 μg / ml. These results suggest that the high anti-HIV activity originates from the branched structure of the synthetic galactomannans.

3.4. Conclusion

We synthesized benzyl galactomannans by the ring-opening polymerization of a newly 1, 6-anhydro disaccharide monomer with 3-*O*-galactose branched and after debenzylation to recover hydroxyl groups. Therefore, Synthetic sulfated galactomannans, which is obtained sulfation of synthetic galactomannans by piperidine-*N*-sulfonic acid, having potent inhibited infection of MT-4 cells with HIV.

This synthesis procedure is checked, and the structures of debenzylated and sulfated galactomannans was determined by high resolution NMR spectroscopy, their molecular weights by gel permeation chromatography (GPC) and degree of sulfation was calculated elemental analysis.

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CHAPTER 4.

Electrostatic interaction of synthetic sulfated galactomannans with poly-L-lysin

ABSTRACT

The biological mechanism of synthetic sulfated galactomannans were estimated by using SPR, DSL, and zeta potential measurements with poly-L-lysine as a model peptide of the HIV surface glycoprotein and which is suggesting that the electrostatic interaction between negatively charged sulfate groups of synthetic sulfated galactomannans and positively charged amino groups of poly-L-lysine was an important role in the biological mechanism.

4.1. INTRODUCTION

Naturally occurring sulfated polysaccharides have special biological activities such as antiviral, anti-HIV, anticoagulant, antitumor, anticancer, anti-oxidant and immunomodulatory activities.

Optical technique of surface plasmon resonance (SPR) is used to study molecular interaction of two different molecules in which one (Drescher and Drescher, 2009).

Many investigations of the relationship between protein-protein, nucleic acid-protein, carbohydrate-protein, and carbohydrate-carbohydrate interaction using SPR have been reported (Tegshi and Yoshida, 2011). Therefore, a naturally occurring laquer polysaccharides (Bai and Yoshida, 2013) and konjac glucomannans (Bo and Yoshida, 2013) to decreased molecular weights were found interaction between sulfated laquar polysaccharides and poly-L-lysin by SPR and anti-HIV active, after sulfation.

In this chapter, we report electrostatic interaction between the synthetic sulfated galactomannans and the interaction with poly-L-lysine as a model compound of HIV

surface glycoproteins was examined by surface plasmon resonance (SPR), dynamic light scattering (DLS), and zeta potential measurements to elucidate the biological mechanism.

4.2. EXPERIMENTAL

4.2.1. Materials

Synthetic sulfated galactomannans was synthesized by our laboratory. Poly-L-lysin with molecular weight of 1000-5000 was purchased from Wako pure Chemical Industries, Ltd., Japan. Techniques of A Biacore X100 instrument and Otsuka Electronics ELSZ-1000ZS particle size and zeta potential analyzer were used to study of electrostatic interaction and biological mechanism.

4.2.2. Measurement

A Biacore X100 instrument (GE healthcare UK) was used for the measurement of SPR spectrum at 25°C using a CM5 sensor chip. The dynamic light scattering (DLS) and zeta (ζ) potential were measured at 25°C in phosphate buffer solution (pH=7.4) by an Otsuka Electronics ELSZ-1000ZS particle size and zeta potential analyzer. The ultrasonicated clear buffer solution (4 ml) of sulfated galactomannan (1.0 mg / ml) in no. 3 of Table 6, for example, and poly-L-lysine (1.0 mg / ml) was used for the measurements.

4.3. RESULTS and DISCUSSION

4.3.1. SPR, DLS and zeta potential

To evaluate the interaction of sulfated synthetic galactomannans with HIV, we measured SPR, particle size using DSL, and zeta potential. The results are summarized in Table 4. Poly-L-lysine was used as a model peptide of HIV surface glycoprotein, gp120. For measuring SPR, poly-L-lysine was immobilized on a CM5 sensor chip by an amine coupling method according to the manufacturer's instructions to get 1604 response units (RU).

The apparent kinetic constants, association-rate (k_a), dissociation-rate (k_d), and dissociation (K_D) constants, of sulfated synthetic galactomannans were compared to those of dextran sulfate, which is a potent anti-HIV active polysaccharide. The sulfated synthetic galactomannans had almost the same kinetic constants as those of dextran sulfate, revealing that the sulfated synthetic galactomannans had a higher binding affinity to poly-L-lysine. As shown in no. 1, the sulfated synthetic galactomannan with a galactose branch at every mannose residue had a higher k_a and smaller particle size, suggesting that the compact branched structure plays a role in the interaction with poly-L-lysine.

On the other hand, a sulfated synthetic galactomannan (no. 3) with 56.6 mol% of galactose branches showed the largest particle size, 50 nm, suggesting that the galactomannan molecule might be enlarged by the repulsion of sulfated galactose branches with suitable distances in the main chain. After addition of poly-L-lysine, the particle size of sulfated synthetic galactomannans increased in comparison with that before addition. These results indicated that an interaction occurred between sulfated synthetic galactomannans with negatively charged sulfated groups and poly-L-lysine with positively charged amino groups and increased the particle size.

The zeta potential for sulfated synthetic galactomannans was determined in phosphate buffer, which is the same solvent for measuring SPR. The zeta potential of

sulfated synthetic galactomannans had negative values. After addition of poly-L-lysine, the zeta potential was still negative but increased in accordance to the interaction with poly-L-lysine having positive zeta potential.

The SPR, DSL, and zeta potential measurements revealed that sulfated synthetic galactomannans interacted with poly-L-lysine.

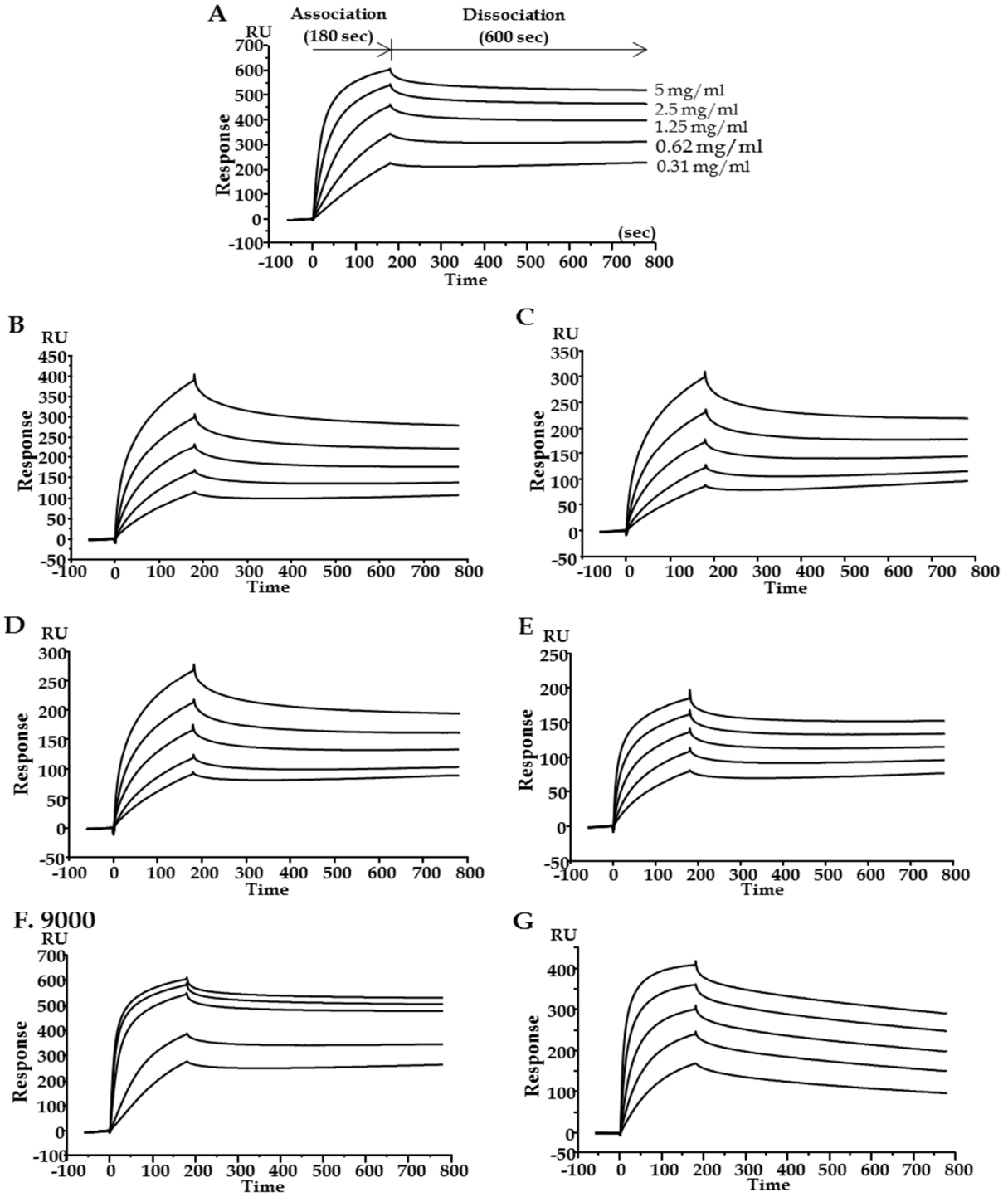

Figure 13: Binding curves of sulfated galactomannans (A), Sulfated galactomannan with 78.2 mol% of galactose branched (B), Sulfated galactomannan with 56.6 mol% of galactose branched (C), Sulfated galactomannan with 28.6 mol% of galactose branched (B) and sulfated mannan (E) were compared to standard dextran sulfate with 9000 (F) and 6500 (G) of molecule weights to immobilized poly-L-lysine..

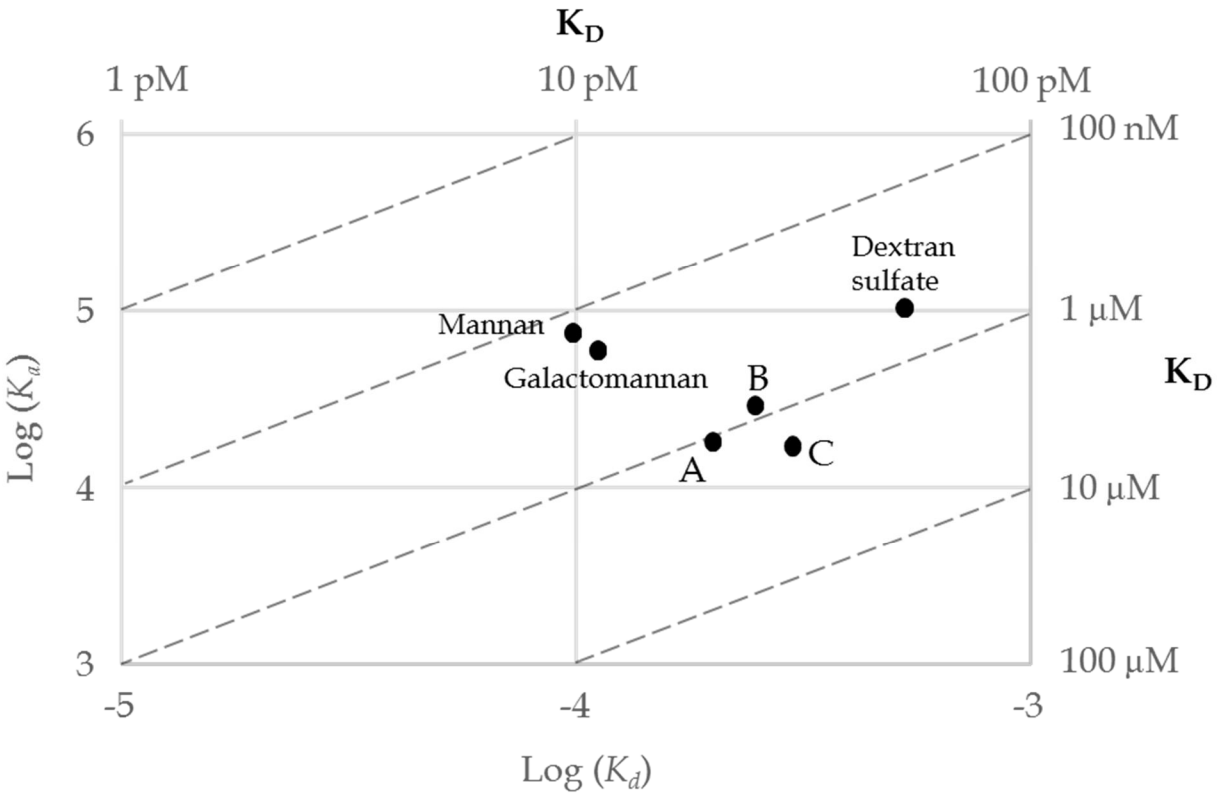

Figure 14: On-off map of synthetic sulfated galactomannans were compared Dextran sulfate with poly-L-lysine. Relationship between association (k_a) and dissociation (k_d) rate constant of sulfatedgalactomannans with 78.2 mol% of galactose branched (A), 56.6 mol% (B) and 28.6 mol% (C). The dotted line shows the dissociation constant, K_D.

Table 6. Apparent kinetic results, DLS measurements, and zeta potential of sulfated synthetic galactomannans[a]

No	Proportion		$\overline{M}_n$	DS[d]	Kinetic result			Poly-L-lysine[c]			
								Absent		Present	
	Disaccharide	Mannose			k_a	k_d	K_D	Particle size	ζ	Particle size	ζ
	mol%		x10³		1/Ms	1/s	M	nm	mV	nm	mV
1	100	0	6.5	1.16	5.9×10^{4}	1.12×10^{-4}	1.90×10^{-9}	27.8±5.1	-13.12	108.8±11.3	-12.20
2	78.2	21.8	4.0	1.11	1.7×10^{4}	3.00×10^{-4}	1.75×10^{-8}	31.3±17.3	-18.55	104.6±11.8	-10.34
3	56.6	43.4	4.6	1.12	1.8×10^{4}	2.01×10^{-4}	1.11×10^{-8}	50.0±8.2	-20.17	111.9±21.0	-11.37
4	28.6	71.4	5.2	1.15	2.9×10^{4}	2.49×10^{-4}	8.54×10^{-9}	41.0±7.2	-23.99	129.5±24.1	-11.00
5	0	100	7.5	1.52	7.4×10^{4}	0.99×10^{-4}	1.34×10^{-9}	17.5±3.4	-17.36	90.7±12.1	-10.93
	Dextran sulfate[e]		6.5	2.1	10.3×10^{4}	5.29×10^{-4}	5.14×10^{-9}	11.4±2.9	-14.12	60.2±8.2	-9.17

a) Sulfated galactomannans were injected 90 µl for 180 s at a flow rate of a HBS-EP running buffer (pH=7.4) at 25°C and then the running buffer was further flowed for 600 s. Concentrations of galactomannans were 500, 250, 125, 62.5, and 31.3 µg/ml, respectively. CM5 sensor chip immobilized poly-L-lysine(response bound: 1604) was used.

b) The particle size (nm) and zeta potential (mV) of the sulfated galactomannans in the presence or absence of poly-L-lysine were determined at 25°C by a dynamic light scattering measurement in phosphate buffer solution (pH=7.4) at a concentration of 1 mg/ml. Before measuring, all samples were subjected to ultrasonication for 5 min.

c) Commercially available poly-L-lysine (1 mg/ml or 0.5 mg/ml) with the molecular weight of 1000-5000 was used. The particle size and zeta potential were 26.5±4.5 nm and +0.41 mV, respectively.

d) Degree of sulfation was calculated from the results of elemental analysis.

e) Standard dextran sulfate with $\overline{M}_n=6.5\times10^{3}$ was used.

4.4. Conclusion

Synthetic sulfated galactomannans was determined electrostatic interaction with poly-L-lysin to biological mechanism by SPR and DLS measurements. It is tested in laboratory to evaluate interaction. Results of SPR presented graphics and on-off map of interaction between association rate and dissociation rate were compared to dextran sulfate, which is potent anti-HIV activity. Sulfated galactomannans was mixed in phosphate buffer solution with poly-L-lysine. Their increased the particle size than only sulfated galactomannans and which are interaction of negatively charged sulfated groups and positively charged amino groups.

Summarized of results SPR and DLS, sulfated galactomannans has demonstrated with strongly interaction of carbohydrate and oligo-protein.

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APPENDIX

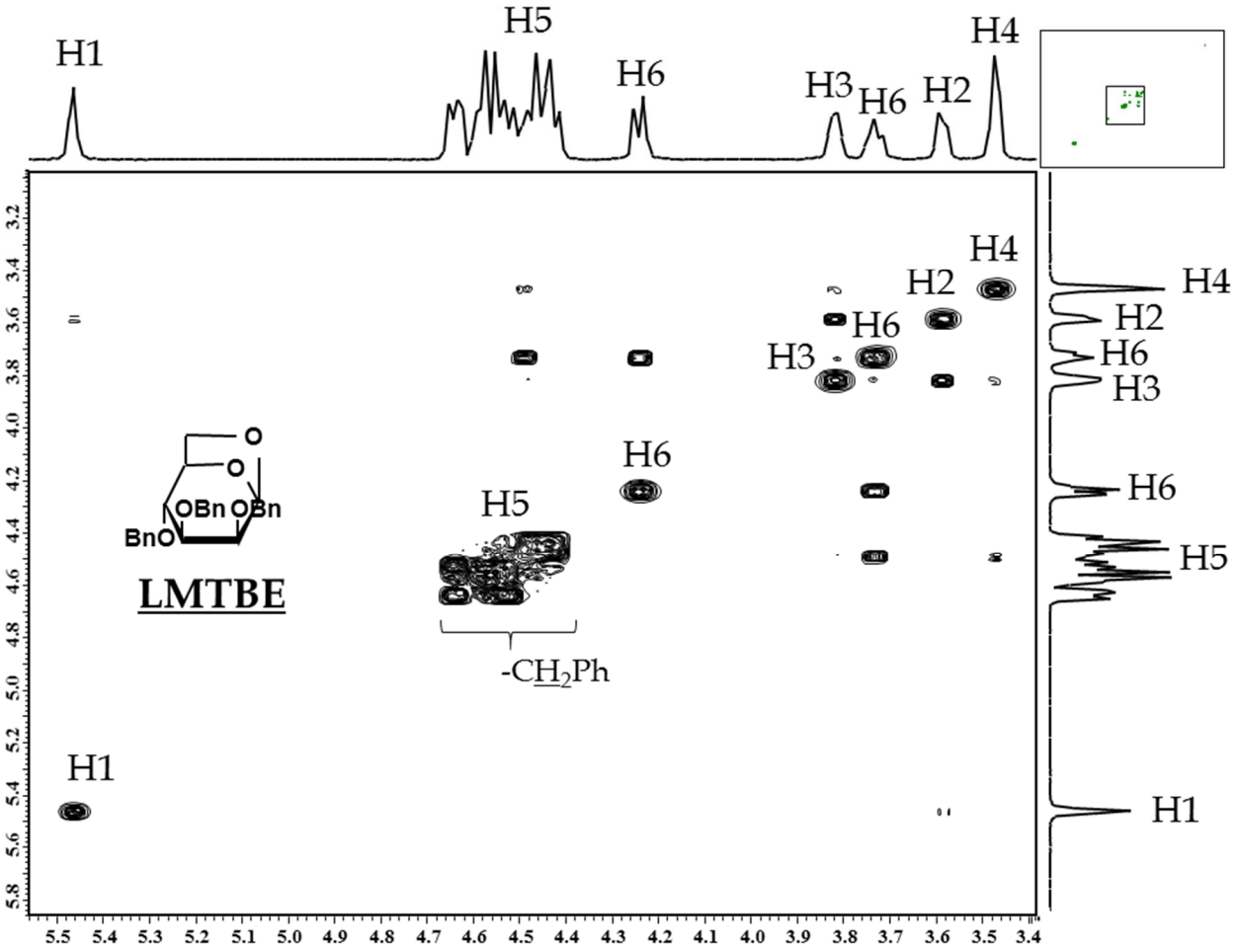

Figure 15: H-H COSY NMR spectrum of LMTBE with in $CDCl_3$ at 25°C. The specific rotation was =-33.9° (c1, $CHCl_3$).

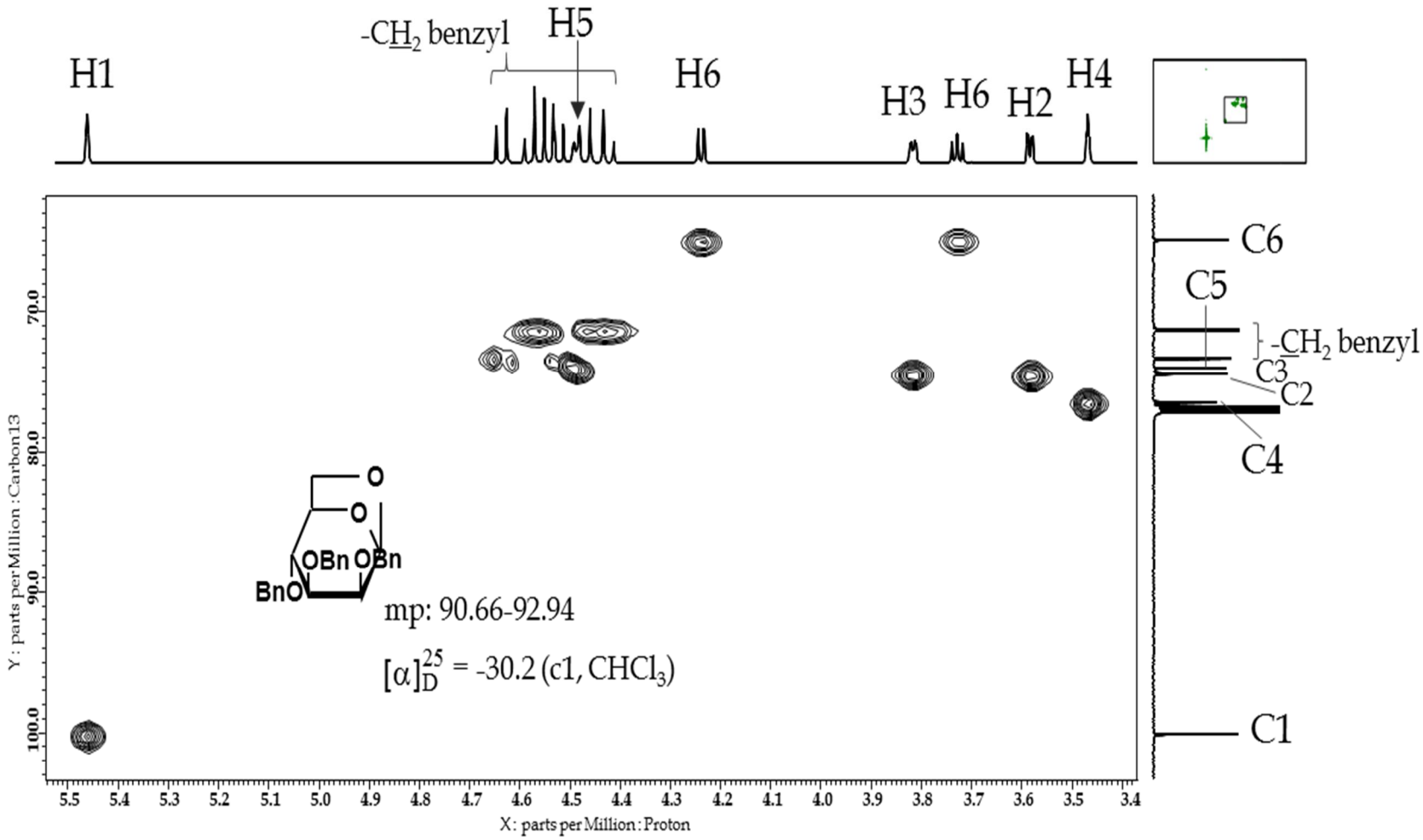

Figure 16: H-C HMQC NMR spectrum of LMTBE with in $CDCl_3$ at 25°C.

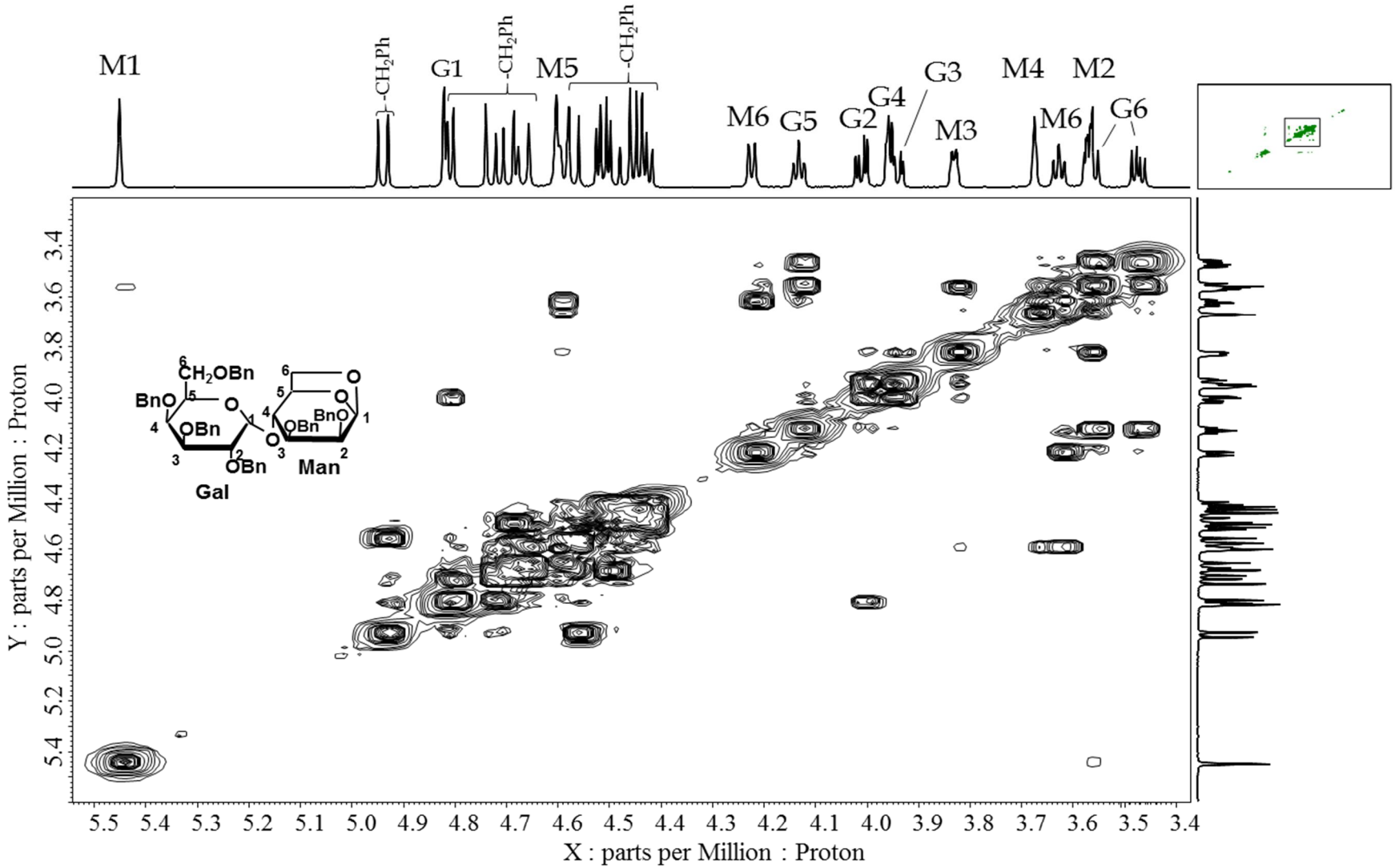

Figure 17: H-H COSY NMR spectrum of disaccharide, LMGABE with in $CDCl_3$ at 25°C. The specific rotation was =+11.4° (c1, $CHCl_3$).

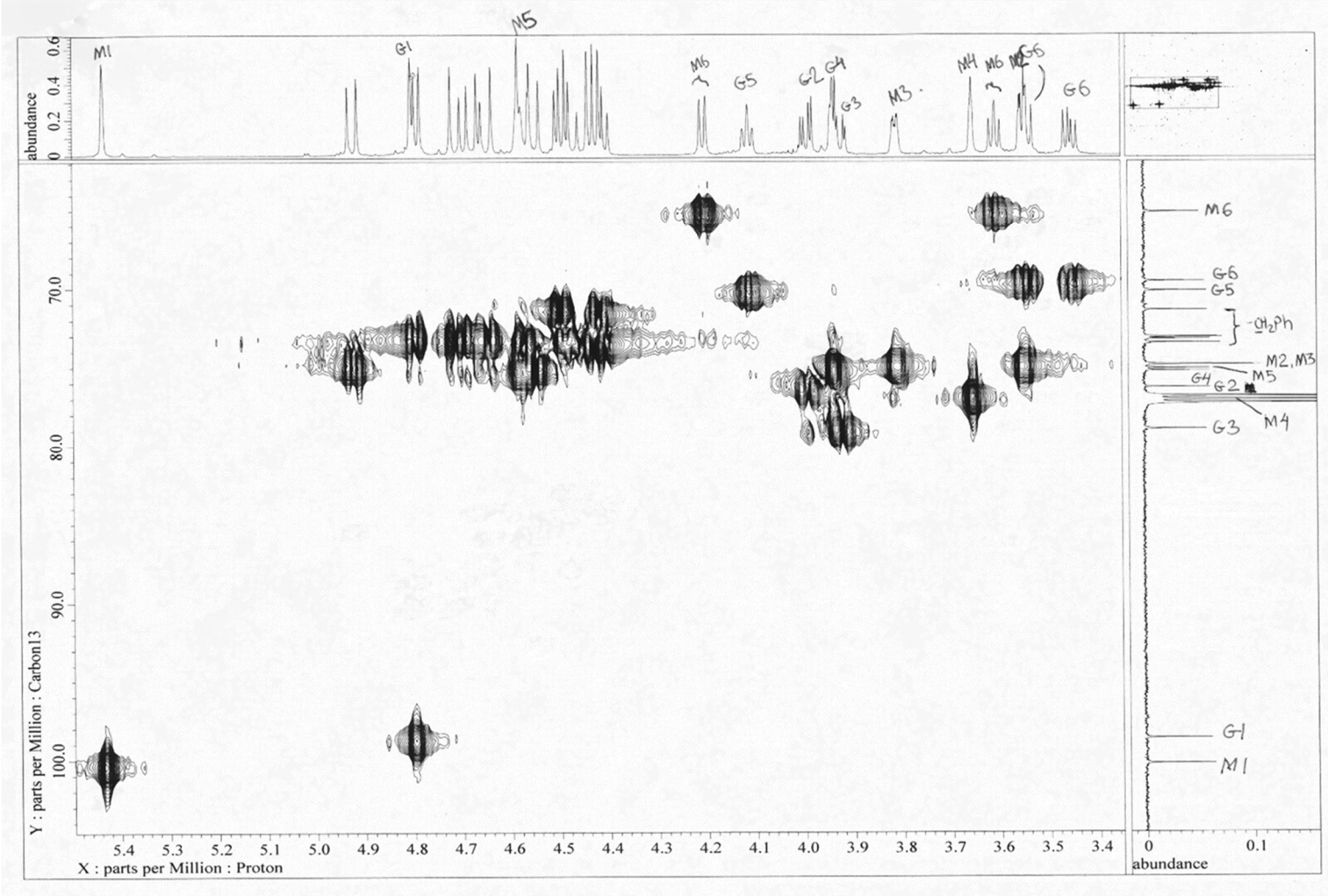

Figure 18: H-C HMQC NMR spectrum of disaccharide, LMGABE

SUMMARY

Homopolymerization of benzylated anhydro disaccharide (LMGABE) and copolymerization with 1, 6-anhydro-2, 3, 4- tri-O-benzyl-β-D-mannopyranose (LMTBE) were carried out by ring-opening polymerization with PF_5 catalyst under high vacuum at -60°C. The homopoly(LMGABE) and copoly(LMGABE and LMTBE) were debenzylated with sodium in liquid ammonia to give synthetic (1→6)-α-D-galactomannans. The sulfation of synthetic (1→6)-α-D-galactomannans with piperidine-*N*-sulfonic acid in DMSO was carried out synthetic sulfated galactomannans and their degree of sulfation was calculated between 1.11 and 1.52 by elemental analyzer.

We found that the synthetic sulfated galactomannans had potent anti-HIV activity. The biological mechanism was elucidated by measuring SPR, DSL, and zeta potential with poly-L-lysine as a model peptide of the HIV surface protein, and the sulfated synthetic galactomannans had strong interactions with poly-L-lysine, suggesting pthat the anti-HIV activity of sulfated synthetic galactomannans makes it hypothetical enough due to the interaction of the negatively charged sulfated groups with the positively charged surface of HIV. The precise interactions between sulfated polysaccharides and virus proteins were further investigated by the synthesis of structurally defined sulfated polysaccharides.

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Davaanyam BUDRAGCHAA

1st September, 2015

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