



HOKKAIDO UNIVERSITY

Title	Syntheses and Biochemical Evaluation of Novel Glycoligands
Author(s)	Matsuoka, Koji
Degree Grantor	北海道大学
Degree Name	博士(理学)
Dissertation Number	甲第3550号
Issue Date	1995-03-24
DOI	https://doi.org/10.11501/3082564
Doc URL	https://hdl.handle.net/2115/51227
Type	doctoral thesis
File Information	000000285014.pdf



**Syntheses and Biological Evaluation of
Novel Glycoligands**

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Syntheses and Biochemical Evaluation of Novel Glycoligands

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Submitted for the degree of Doctor of Philosophy
in the Graduate School of Science

HOKKAIDO UNIVERSITY
1995

Abstract

Synthetic glycoconjugates as a novel class of useful tools for basic glycoscience studies were systematically designed and synthesized. The main purpose of this work was focused on creation of a new glycochemical field by hybridizing the conventional sugar-chemistry, polymer chemistry, and biochemistry. The thesis was composed of five chapters. The first chapter provided a general introduction to the roles of glycoconjugates in nature and the significance of molecular recognition based on the interaction of carbohydrates and proteins such as lectins and enzymes.

In order to investigate the structures and functions of a variety of glycoconjugates, it should be noted that we must consider some new strategy and methodology for the preparation of well characterized glycoligands and glycosubstrates in conjunction with glycobiology and glycotechnology. In the second chapter, facile and efficient syntheses of activated cyclic aldose derivatives were explained on the basis of a new combination of mercuric (II) bromide and 2,4,6-trimethylpyridine.¹ Reactive intermediates such as orthoester and oxazoline derivatives synthesized here can be useful and versatile precursors for glycoconjugates syntheses and the application of these procedures will be discussed in following chapters.

The third chapter under the title of "Glycoprotein Models: Polymeric Sugar Ligands Available for Determining The Binding Specificity of Lectins" gave a new strategy for the investigation of binding specificity of lectins with sugars using polymeric glycoligands derived from a variety of polymerizable glycosides of *N*-acetyllactosamine and the related isomers. It was clearly suggested that a lectin isolated from *Erythrina collarodendron* showed a specific affinity for $\beta(1\rightarrow4)$ type disaccharides and the precise evaluation of the interaction of carbohydrates and lectins can be firstly performed by means of polymeric sugar-cluster effect.²

Chapter 4 was devoted to the syntheses and biochemical evaluation of novel bi-fluorescence-labeled lactoside as a high performance substrate for ceramide glycanase isolated from leech (*Macrobdella decora*). This unique substrate prepared from *n*-pentenyl lactoside involved two kind of fluorophores as a donor and an acceptor of fluorescence energy transfer in the molecule and showed two emission maximums at around 335 and 540 nm when the substrate was excited at 260 or 290 nm. Using a bi-fluorescence-labeled substrate for kinetic analysis in the enzyme reaction, the K_M of the leech ceramide glycanase against the substrate was found to be 7.7 μM and the V_{max} was determined to be 0.16 nmol/min.^{3, 4}

Concluding remarks will be described in Chapter 5.

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Abbreviations

Ac	Acetyl
APS	Ammonium persulfate
Asn	<u>L</u> -Asparagine
Bz	Benzoyl
CGase	Ceramide glycanase
CHCl ₃	Chloroform
CDCl ₃	Chloroform- <i>d</i>
CRM	Carbohydrate recognition molecules
CSA	10-Camphorsulfonic acid
DABSYL	4-{[4'-(dimethylamino)phenyl]azo} benzoyl
Dansyl	5-Dimethylamino-1-naphthalenesulfonamido
DMF	Dimethylformamide
DNPH	2,4-dinitrophenylhydrazine
D ₂ O	Deuterium oxide
DSS	Sodium 2,2-dimethyl-2-silapentane-5-sulfonate
ECorL	<i>Erythrina corallodendron</i> lectin
EDANS	5-[(2'-aminoethyl)amino]naphthalenesulfonyl
Et	Ethyl
Et ₄ NBr	Tetraethylammonium bromide
Fuc	<u>L</u> -Fucose
Gal	<u>D</u> -galactose
GalNAc	<i>N</i> -Acetyl- <u>D</u> -galactosamine
GalT	Galactosyl transferase
Glc	<u>D</u> -Glucose
GlcNAc	<i>N</i> -Acetyl- <u>D</u> -glucosamine
GPC	Gel permeation chromatography
GSL	Glycosphingolipid
HEPES	<i>N</i> -2-Hydroxyethylpiperazine- <i>N</i> '-ethanesulfonic acid
HgBr ₂	Mercuric (II) bromide
Hg(CN) ₂	Mercuric (II) cyanide
Lac	Lactose: Galβ(1→4)Glc
LacNAc	<i>N</i> -Acetylactosamine: Galβ(1→4)GlcNAc
LacNAc-13	Galβ(1→3)GlcNAc
LacNAc-16	Galβ(1→6)GlcNAc
Le ^x	Lewis X: Galβ(1→4)[Fucα(1→3)]GlcNAc
Mal	Maltose: Glcα(1→4)Glc
Man	<u>D</u> -Mannose

Me	Methyl
MS	Molecular sieves
Neu5Ac	<i>N</i> -Acetylneuraminic acid
Neu5Gc	<i>N</i> -Glycolylneuraminic acid
NLD	Naphthyl-Lac-Dansyl
NLP	Naphthyl-Lac-Pent
NMR	Nuclear magnetic resonance
PAD	Pulsed amperometric detector
PBS	Phosphate buffered saline
Pent	<i>n</i> -Pentenyl
Ser	<u>L</u> -Serine
SnCl ₄	Stannic (IV) chloride
TEMED	<i>N,N,N',N'</i> -Tetramethylethylenediamine
THF	Tetrahydrofuran
Thr	<u>L</u> -Threonine
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TMSOTf	Trimethylsilyltrifluoromethanesulfonate
Tr	Triphenylmethyl
Typ	<u>L</u> -Tryptophan
Tyr	<u>L</u> -Tyrosine
UDP	Uridine 5'-diphosphate
WGA	Weat germ agglutinine

Acknowledgment

I wish to express my gratitude to my thesis advisor, Professor Shin-Ichiro Nishimura, for his suggestions, supports, and discussions. The work described in this dissertation would not have been successful without him.

I am grateful to Professors Norio Nishi, Akihiko Yamagishi, and Takashi Yoshida of Hokkaido University for a critical reading and suggestions of the dissertation manuscript. I would like to gratitude to Professor Seiich Tokura of Hokkaido University for his encouragement to me.

I would also like to thank Professor Yuan Chuan Lee of The Johns Hopkins University for assistance and discussions, especially for research in Chapter 2 and 4, during the two years in USA.

I am indebted to Professor Keisuke Kurita of Seikei University and Mr. Tetsuya Furuike of Hokkaido University for valuable discussion and suggestions. I would also like to acknowledge Mr. Hiroyuki Shiota and my colleague of both Professor Nishimura' lab of Hokkaido University and Professor Lee's lab of The Johns Hopkins University for support and encouragement.

Finally, I would like to acknowledge Miss Yasuko Sado for her encouragement.

Dedication

I would like to dedicate this dissertation to my parents, Shinji and Kazuko Matsuoka.

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Seikei University

Prof. Keisuke Kurita
Dr. Shigeru Ishii
Mr. Sadafumi Aibe
Mr. Osamu Kohgo
Mr. Hitosi Yoshino

Chapter 1

1.1 General Introduction

Recently, it has been recognized that carbohydrate chains of glycoconjugates represent an important group of informational biomolecules.¹ Although the term “glycoconjugates” usually includes glycoproteins, glycolipids, and proteoglycans, activity in all carbohydrate field is constantly escalating and this situation is documented by the large number of manuscripts submitted to an increasing number of scientific journals devoted solely to sugars, their derivatives, and their biological functions. Carbohydrate chains of the glycoconjugates show indispensable roles in nature through interactions with proteins,² nucleic acid,³ and oligosaccharides.⁴ Figure 1 shows, for example, a typical illustration of glycoconjugates located on the surface of erythrocytes. Glycoproteins and glycolipids are existing as important components of biomembranes together with phospholipids and antenna of oligosaccharides are directed to outside of the cell owing to its hydrophilicity. Sphingoglycolipids are known as major components serve important information for cell-cell interactions.⁵ When the oligosaccharide chains have sialic acid residues such as *N*-acetylneuraminic acid (Neu5Ac) or *N*-glycolylneuraminic acid (Neu5Gc) at non-reducing end or internal sugar sequences, these are classified as

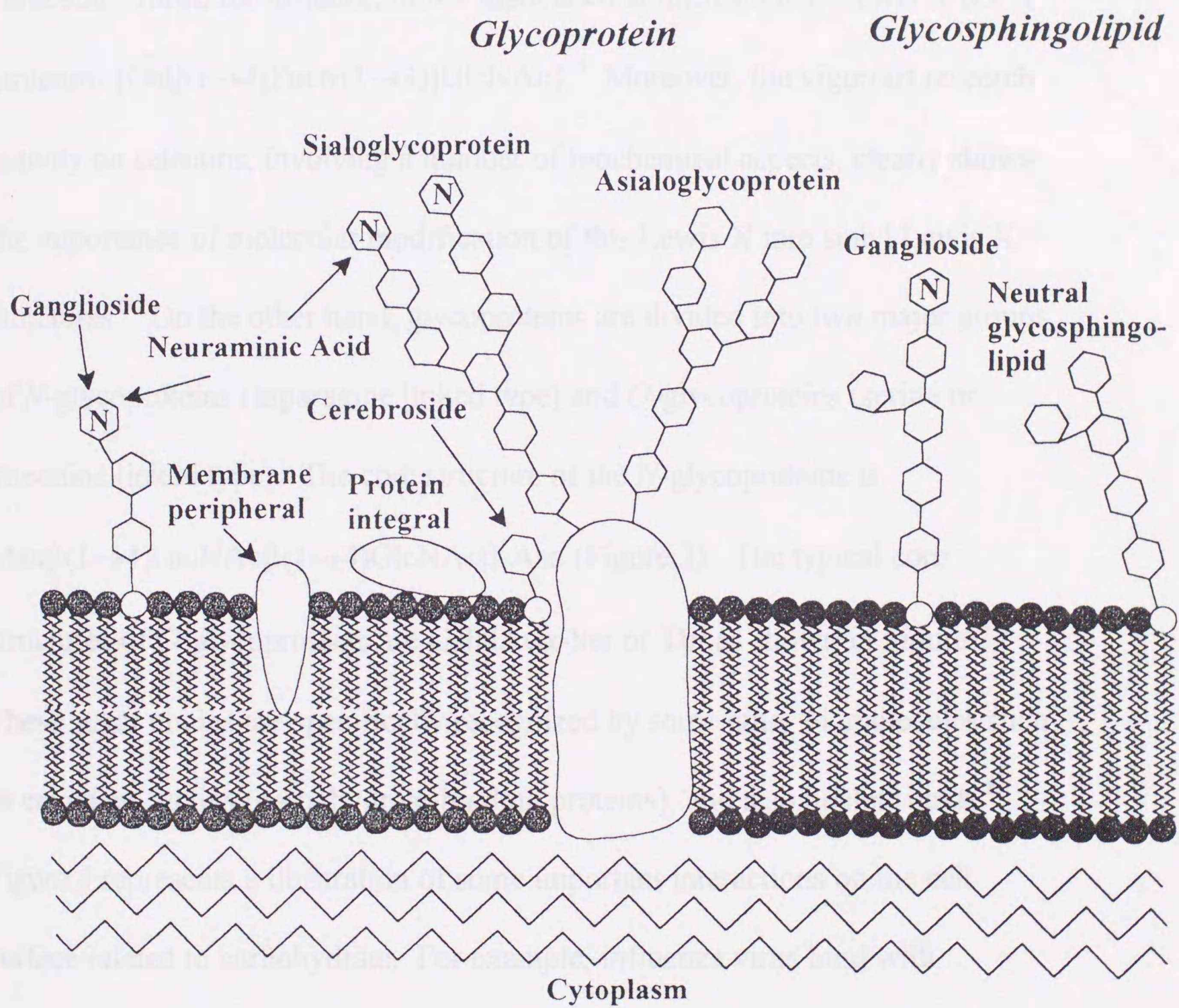


Figure 1. Glycoconjugates on the cellular surface.

gangliosides. Major monosaccharides found in oligosaccharide chains are generally D-glucose (Glc), D-mannose (Man) D-galactose (Gal), L-fucose (Fuc), *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-galactosamine (GalNAc), and sialic acid (Neu5Ac) as shown in Figure 2. These carbohydrate chains sometimes form, for instance, tumor associated antigen such as Lewis X (Le^{X}) structure $\{\text{Gal}\beta 1\rightarrow 4[\text{Fuc}\alpha(1\rightarrow 3)]\text{GlcNAc}\}$.⁶ Moreover, the vigorous research activity on selectins, involving a number of biochemical aspects, clearly shows the importance of molecular modification of this Lewis X into sialyl Lewis X structures.⁷ On the other hand, glycoproteins are divided into two major groups of *N*-glycoproteins (asparagine linked type) and *O*-glycoproteins (serine or threonine linked type). The core structure of the *N*-glycoproteins is $\text{Man}\beta(1\rightarrow 4)\text{GlcNAc}\beta(1\rightarrow 4)\text{GlcNAc}\beta\text{-Asn}$ (Figure 3). The typical core structures of *O*-glycoproteins are $\text{GalNAc}\alpha\text{-Ser or Thr}$ as shown in Figure 3. These sugar chains are specifically recognized by some other biomolecules such as enzymes, lectins (carbohydrate-binding proteins), and also nucleic acids.⁸ Figure 4 represents a illustration of some important interactions on the cell surface related to carbohydrate. For example, influenza virus bind with oligosaccharide chains bearing sialic acids of glycolipids and this step is supposed to be initial process of the infection to an erythrocyte.⁹ Bacteria have been reported to contain several carbohydrate binding tentacles and bind to the

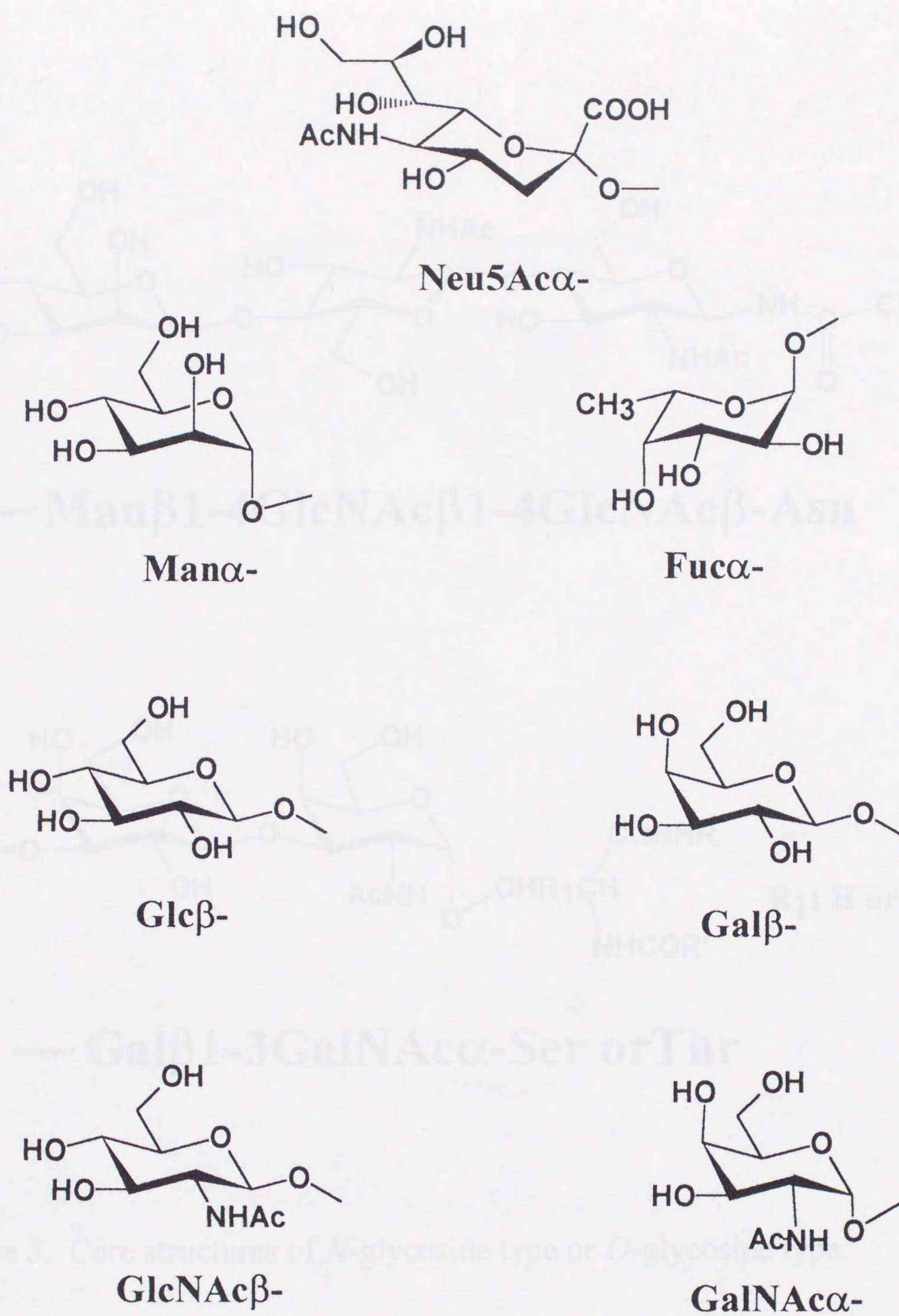
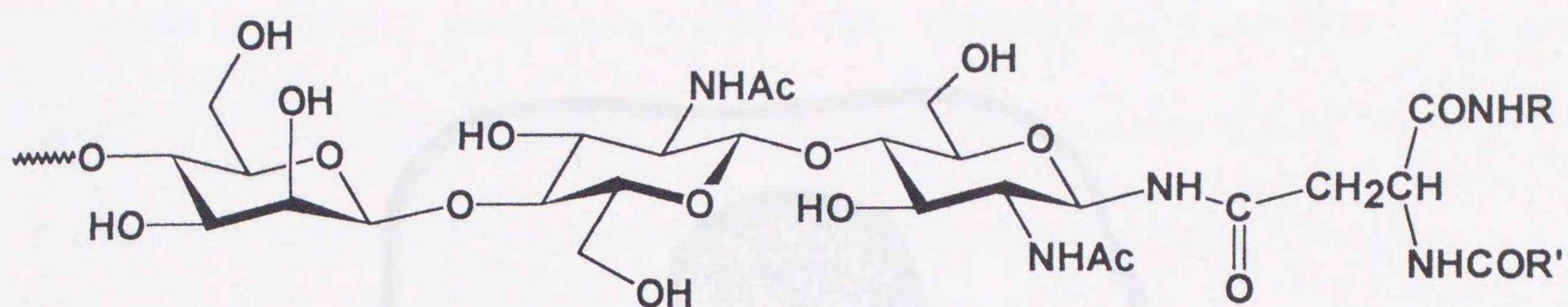
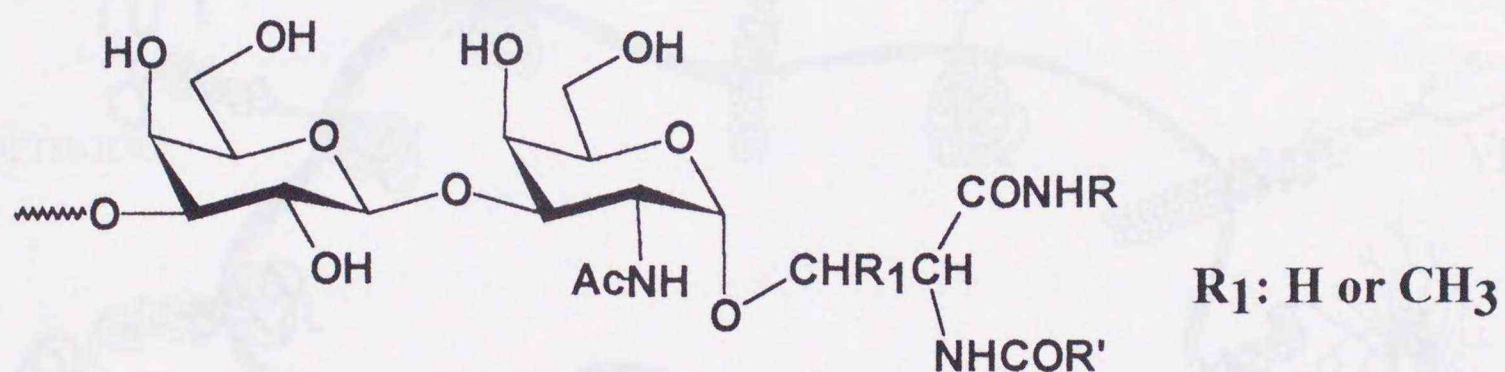


Figure 2. Chemical structures of monosaccharides formed of major glycoproteins and glycolipids.



~~~~~ **Man $\beta$ 1-4GlcNAc $\beta$ 1-4GlcNAc $\beta$ -Asn**



~~~~~ **Gal $\beta$ 1-3GalNAc $\alpha$ -Ser or Thr**

Figure 3. Core structures of *N*-glycoside type or *O*-glycoside type.

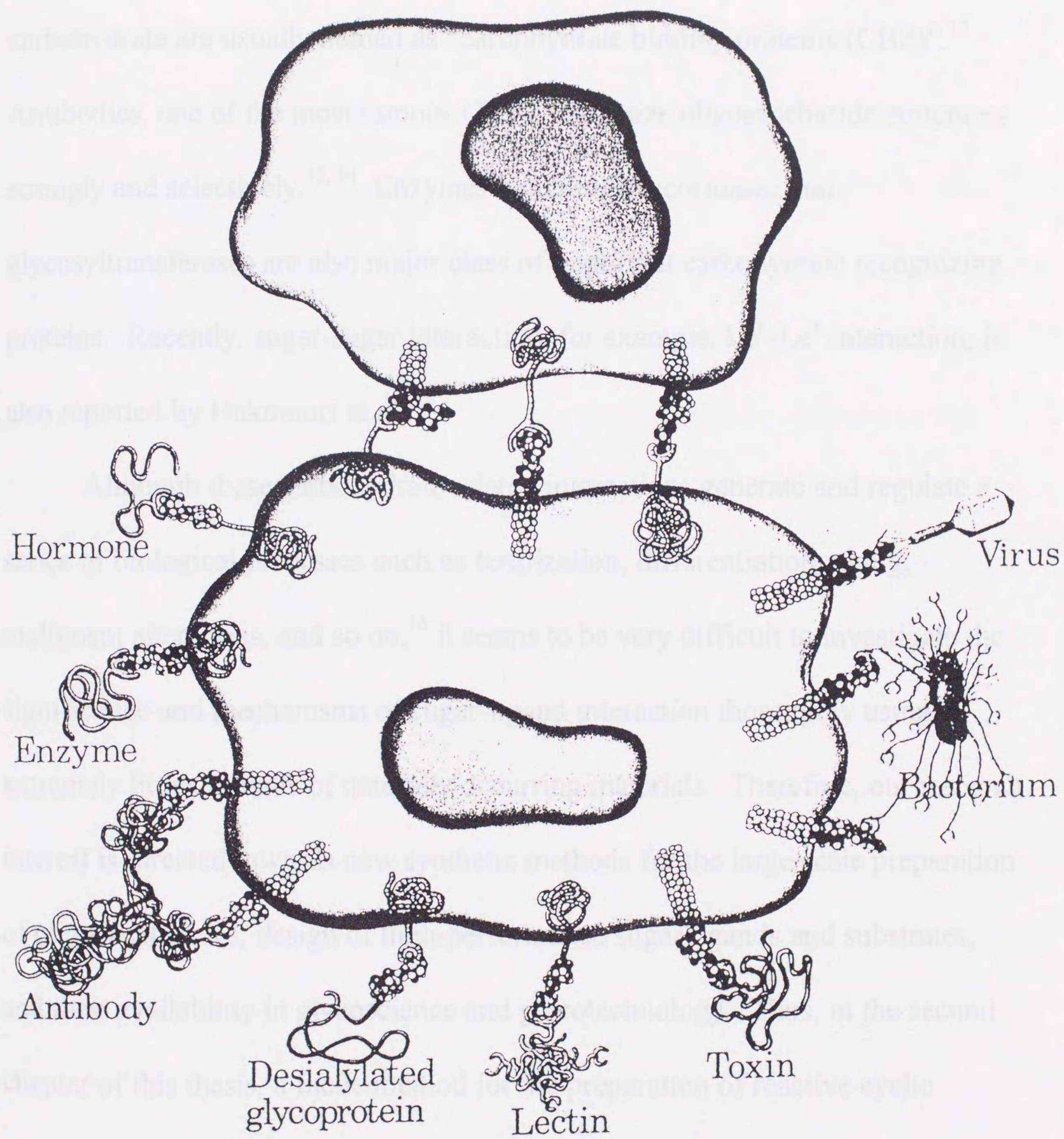


Figure 4. Cell surface carbohydrate interactions.

oligosaccharide chains on a cell surface through the tentacles.¹⁰ Lectins are defined as a group of carbohydrate binding proteins and have at least two binding sites in the molecule.¹¹ Proteins which have only one binding site against a carbohydrate are usually named as “carbohydrate binding proteins (CBP)”.¹² Antibodies, one of the most famous CBPs, recognize oligosaccharide structures strongly and selectively.^{13, 14} Enzymes including glycosidases and glycosyltransferases are also major class of important carbohydrate recognizing proteins. Recently, sugar-sugar interaction, for example, Le^x-Le^x interaction, is also reported by Hakomori et al..¹⁵

Although these carbohydrate related interactions generate and regulate a series of biological processes such as fertilization, differentiation, aging, malignant alterations, and so on,¹⁶ it seems to be very difficult to investigate the significance and mechanisms of sugar-ligand interaction thoroughly using extremely little amounts of naturally occurring materials. Therefore, our research interest is directed towards new synthetic methods for the large scale preparation of oligosaccharides, design of high-performance sugar ligands and substrates, and their availability in glycoscience and glycotechnology. Thus, in the second chapter of this thesis, a facile method for the preparation of reactive cyclic intermediates from Gal, GlcNAc, maltose (Mal), and lactose (Lac) will be described. This simple method can be utilized for making potential glycosides as

precursors of the target ligands for the characterization of the sugar-lectin or sugar-enzyme interactions.

In order to explain an enhanced affinity constant observed in the case of interaction between a lectin and a multivalent sugar ligand, Lee et al.¹⁷ has proposed so called "sugar cluster effects". Figure 5 exhibits a typical cluster effect demonstrated by means of isolated some multivalent ligands which have mono-, bi-, and triantennary *N*-acetyllactosamine [LacNAc; Gal β -(1 $\rightarrow$ 4)-GlcNAc] structures respectively. The binding constant of the monovalent ligand has been dramatically enhanced by converting the biantennary ligand, and this phenomenon has been confirmed as a much higher affinity observed by employing a triantennary ligand. From the point of view of assembling and enhancement of the binding affinity of sugar ligands, polymeric clusters derived from some polymerizable glycosides¹⁸ are expected as one of the most valuable ligands which will exhibit amplified binding specificity in sugar-lectin interactions. Thus, in the third chapter, syntheses and versatility of the polymeric sugar-ligands as a new class of "glycoprotein models" will be discussed in relation to the sugar-binding specificity of *Erythrina corallodendron* lectin (ECorL).

Carbohydrate substrate having some photosensitive groups are useful tools to probe and investigate the nature of enzymatic biosynthesis and metabolism of

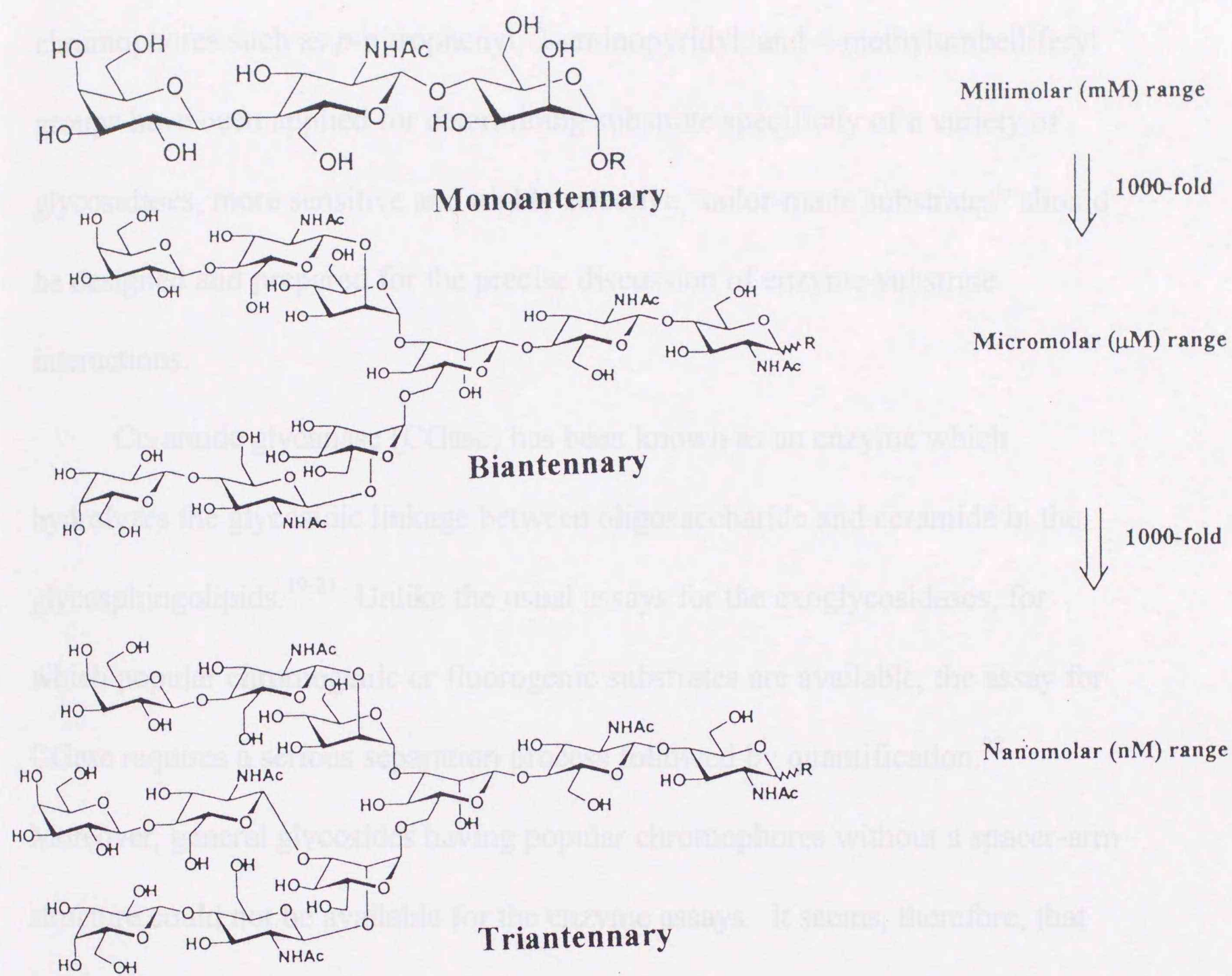


Figure 5. "Cluster effects" by Oligosaccharides, Lee et al..¹⁷

“glycoconjugates”. Actually, although some simple synthetic substrates bearing chromophores such as *p*-nitrophenyl, 2-aminopyridyl, and 4-methylumbelliferyl groups have been applied for determining substrate specificity of a variety of glycosidases, more sensitive and highly selective “tailor-made substrates” should be designed and prepared for the precise discussion of enzyme-substrate interactions.

Ceramide glycanase (CGase) has been known as an enzyme which hydrolyzes the glycosidic linkage between oligosaccharide and ceramide in the glycosphingolipids.¹⁹⁻²¹ Unlike the usual assays for the exoglycosidases, for which popular chromogenic or fluorogenic substrates are available, the assay for CGase requires a serious separation process followed by quantification.²² Moreover, general glycosides having popular chromophores without a spacer-arm structure could not be available for the enzyme assays. It seems, therefore, that CGase obviously recognize not only the stereostructure of glycosidic bond between sugar moiety and ceramide but the hydrophobic ceramide portion in addition to the oligosaccharide chains. As a new “glycolipid mimetic”, it is demonstrated in Chapter 4 that a novel and sophisticated bi-fluorescence-labeled substrate can be easily preparable from *n*-pentenyl lactoside as a key starting material. Energy transfer theory proposed by Stryer²³ is proved to be applicable to materialize high-sensitive and specific CGase substrates.

In the series of this study, novel synthetic method, assembling as polymeric cluster ligands, bi-fluorescence-labeled substrate, and their biochemical evaluation will be systematically discussed.

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Chapter 2

New Glycosidation Strategy: A Facile and Quantitative Preparation of Activated Cyclic Sugar Derivatives Using HgBr₂ and 2,4,6-Collidine

An easy and convenient catalyzing system of a combination of mercuric bromide (HgBr₂) and 2,4,6-collidine was found to promote the formation of activated cyclic sugar derivatives such as 1,2-orthoesters and an oxazoline derivative in quantitative yields at room temperature. Slightly hindered skew like conformation of the lactose orthoester derivative was revealed by ¹H-NMR analysis. It was also suggested that the reaction of a lactosyl 1,2-orthoester with trimethylsilyl azide proceeded smoothly and gave stereoselectively β-lactosyl azide which is available intermediate for constructing glycopeptides and neoglycoconjugates in high yield.

2.1. Introduction

Reactive intermediates of aldoses are much interested in the chemical syntheses of a variety of glycosides which linked by *O*-, *N*-, *S*-, and *C*- substituent atoms. Cyclic aldose 1,2-orthoesters have been also regarded as one of the useful precursors not only for syntheses of simple 1,2-*trans*-glycoside¹⁻³ but for temporary protection of 1,2-di-hydroxyl groups.⁴ Although these orthoesters have been prepared in moderate yields from acetohalo sugars using conventional promoters such as Et₄NBr–2,4,6-collidine,⁵ *N,N*-dimethylformamide dialkylacetals–silver triflate,⁶ silver triflate–2,4,6-collidine,⁷ trialkylstanyl methoxide–Et₄NBr,⁸ and silver nitrate–2,4,6-collidine,⁹ these procedures sometimes involve difficulties in handling, purification processes, lower yields, and require expensive silver salts. Therefore, a simpler and more efficient procedure might be expected to achieve easier-handling, higher-yields, versatility, and lower costs of the reactions. In this paper we introduce a convenient and versatile method for the preparation of reactive cyclic aldoses such as orthoesters and oxazoline derivatives from acetohalo sugars using an economical and efficient catalyzing system of HgBr₂ and 2,4,6-collidine.

2.2. Results and Discussion

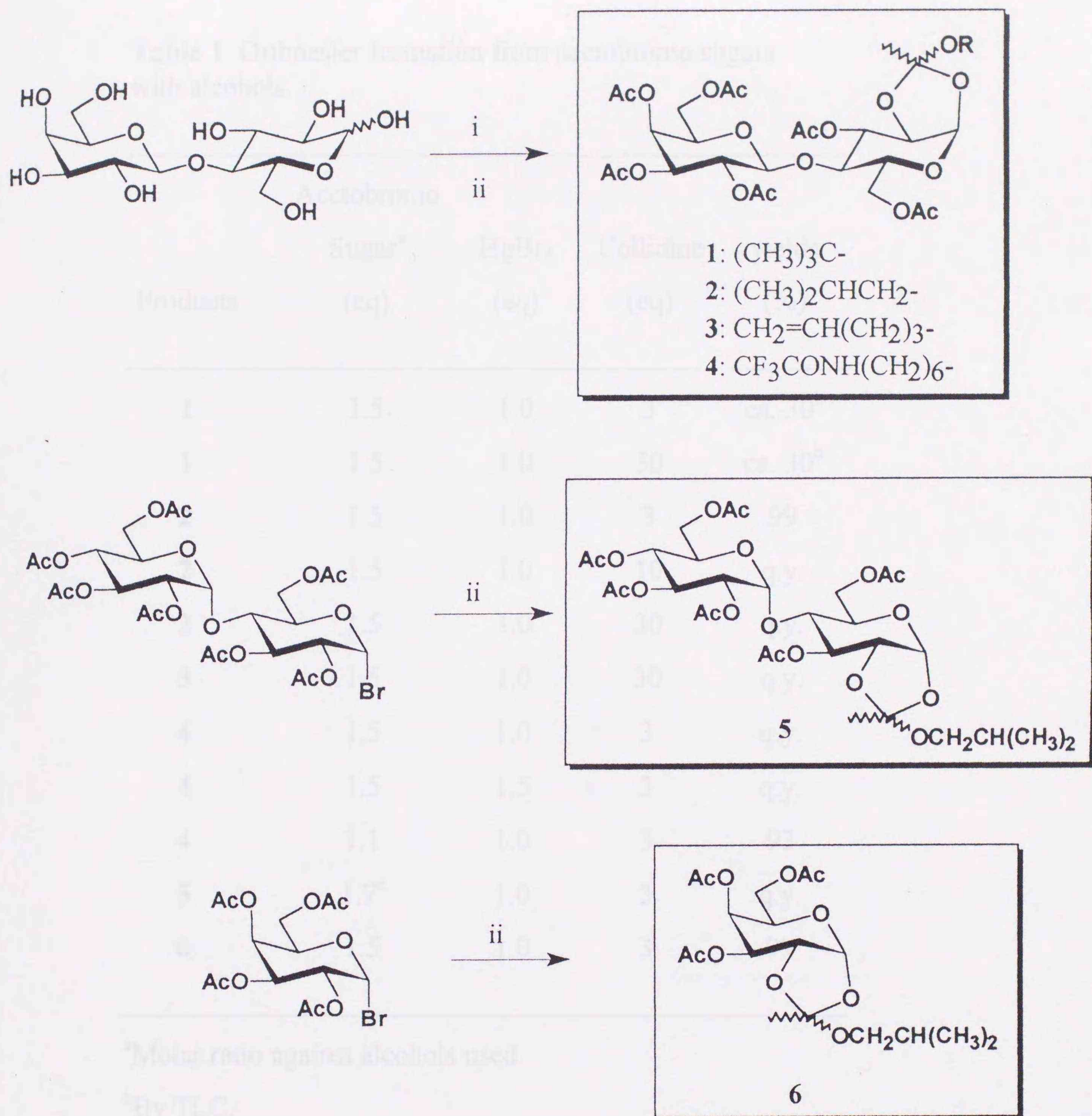
Synthesis of 1,2-orthoesters. Although orthoesters and oxazoline derivatives have been known as major by-product or intermediates of glycosidation reactions from acetohalo sugars and highly labile compounds,¹⁰ glycosidation on the basis of these activated cyclic aldoses are still expected to be effective and economical procedures in order to produce simple but available glycosides for the preparation of neoglycoconjugates.¹¹

In the course of our synthetic studies on neoglycoconjugates, we have previously reported the preparation of a number of *N*-protected ω -aminohexyl glycosides by the reaction of acetohalo sugars with alcohols using mercuric cyanide $[\text{Hg}(\text{CN})_2]$ as catalyst.^{12,13} As is observed in common Königs–Knorr type glycoside bond formations, we had also observed that acetoxonium cation is presumably formed initially, and the alcoholic oxygen atom either attacks the anomeric carbon under slightly acidic condition or the methyl linked carbon of the acetoxonium cation under weakly basic conditions such as pyridine⁴ and 2,4,6-collidine.⁵ Therefore, when the alcoholic oxygen attacks the carbon of latter case, the main product can be 1,2-orthoester.

Our interest therefore was directed toward a simpler combination of mercuric salts and weak bases to achieve high yield formation of orthoesters. After the

careful examination of the reagents, a combined use of HgBr_2 and 2,4,6-collidine was the most convenient method as a novel promoter for the preparation of orthoesters and oxazolines as shown in Scheme 1. Equivalent molar of HgBr_2 and excess of 2,4,6-collidine promoted efficiently the formation of a variety of orthoester. Table 1 shows versatility of this procedure for the preparation of lactose, maltose, and galactose orthoesters and yields were almost quantitative.

Synthesis of an oxazoline derivative by HgBr_2 -2,4,6-collidine. It was also suggested that the known 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy- α -D-glucopyranosyl chloride¹⁴ was efficiently converted to the oxazoline derivative under the same condition employed for the preparation of orthoesters. Firstly, bromo anion derived from HgBr_2 seemed to induce *in situ* anomerization of glycosyl chloride, and then cyclization proceeded smoothly to afford an oxazoline derivative as indicated in Scheme 2. Although similar methods based on the same mechanism were reported and used for the preparation of oxazoline derivatives,^{15,16} the present method is also regarded as one of the practically available strategies because of high yield of the reaction and easy handling.



Scheme 1. Reagents and conditions: i) $\text{Ac}_2\text{O}-\text{HBr}/\text{AcOH}$, then HBr/AcOH ,²⁰ ii) $\text{R}-\text{OH}$, powdered $\text{MS4\AA}$, HgBr_2 , and 2,4,6-collidine in 1:1:1 (v/v/v) of benzene–nitromethane–chloroform at ambient temperature.

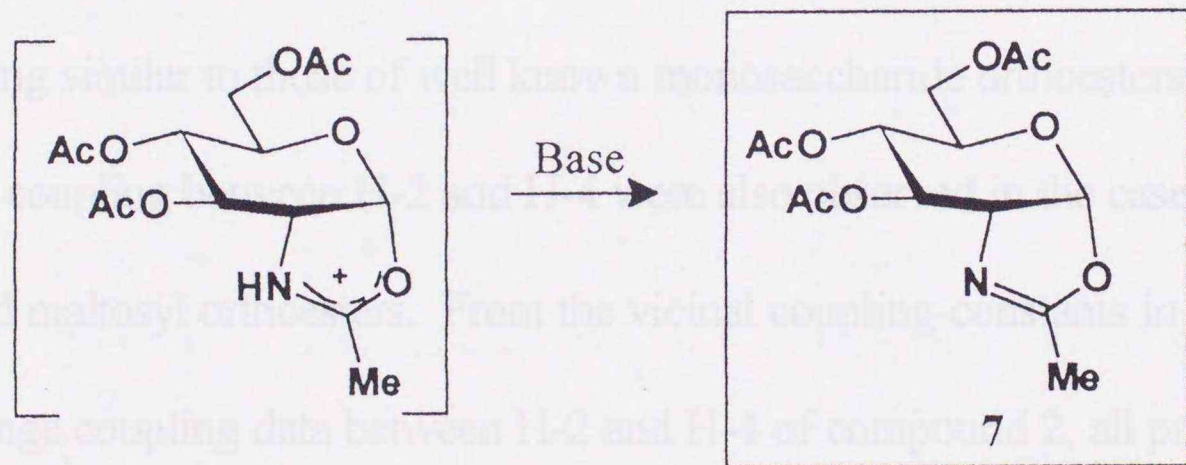
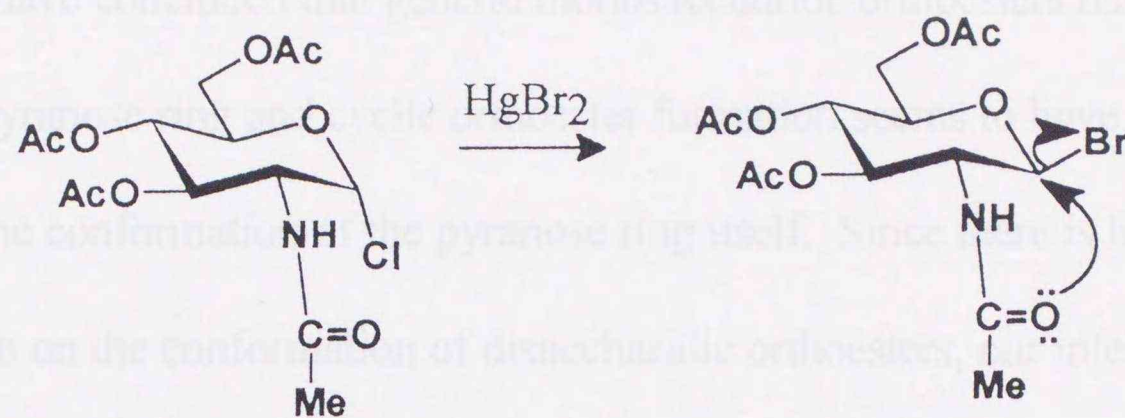
Table 1 Orthoester formation from acetobromo sugars with alcohols.

| Products | Acetobromo | | | yields
(%) |
|----------|----------------------------|---------------------------|-------------------|---------------------|
| | Sugar ^a
(eq) | HgBr ₂
(eq) | Collidine
(eq) | |
| 1 | 1.5 | 1.0 | 3 | ca. 30 ^b |
| 1 | 1.5 | 1.0 | 30 | ca. 30 ^b |
| 2 | 1.5 | 1.0 | 3 | 99 |
| 2 | 1.5 | 1.0 | 10 | q.y. |
| 2 | 1.5 | 1.0 | 30 | q.y. |
| 3 | 1.5 | 1.0 | 30 | q.y. |
| 4 | 1.5 | 1.0 | 3 | q.y. |
| 4 | 1.5 | 1.5 | 3 | q.y. |
| 4 | 1.1 | 1.0 | 3 | 93 |
| 5 | 1.7 ^c | 1.0 | 3 | q.y. |
| 6 | 1.5 | 1.0 | 3 | 99 |

^aMolar ratio against alcohols used.

^bBy TLC.

^cThe bromide was not pure by TLC.



Scheme 2. Proposed mechanism of the formation of oxazoline derivative 7.

Conformation of a 1,2-orthoester of a disaccharide. Conformation of 1,2-orthoesters derived from monosaccharides was precisely discussed by Lemieux et al.⁵ They have concluded that general monosaccharide orthoesters have slightly distorted pyranose ring and cyclic orthoester formation seems to have no significant effect on the conformation of the pyranose ring itself. Since there is little information on the conformation of disaccharidic orthoesters, our interest is now focused on the analysis of the conformation of C-4 substituted orthoester derivatives compared with those of monosaccharide orthoester. ¹H-NMR data of the orthoesters prepared here are listed in Table 2 and 3. Although it is evident in view of the magnitudes of coupling constants that disaccharide orthoesters show distorted pyranose ring similar to those of well known monosaccharide orthoesters, specific long-range coupling between H-2 and H-4 were also observed in the cases for lactosyl and maltosyl orthoesters. From the vicinal coupling-constants in addition to the long-range coupling data between H-2 and H-4 of compound **2**, all proton dihedral angles were estimated by employing modified Karplus equation¹⁷ and a proposed conformation is shown as an inset of Figure 1. In comparison with the monosaccharide orthoester which exhibit a slightly distorted pyranose ring, disaccharide orthoesters seem to have further hindered skew-like conformation. This data suggests that the bulky substituents at C-4 position of the reducing glucose

Table 2 ^1H -NMR data for synthesized compounds.^a

| Hydr.
Atm. | Chemical shifts (δ) and multiplicity | | | | |
|---------------|---|------------------|------------------|-----------|------------------|
| | 2 | 3 | 4 | 5 | 6 |
| H-1 | 5.65, d | 5.65, d | 5.65, d | 5.71, d | 5.81, d |
| H-2 | 4.31, ddd | 4.31, ddd | 4.29, ddd | 4.34, ddd | 4.31, dd |
| H-3 | 5.54, dd | 5.54, dd | 5.52, dd | 5.05, dd | 5.06, dd |
| H-4 | 3.65, d | 3.65, dd | 3.66, d | 3.64, d | 5.43, dd |
| H-5 | 3.87, ddd | 3.86, ddd | 3.85, ddd | 3.90, ddd | 4.3 ^b |
| H-6a | 4.1 ^b | 4.1 ^b | 4.1 ^b | 4.21, dd | 4.17, dd |
| H-6b | 4.25, dd | 4.25, dd | 4.26, dd | 4.31, dd | 4.12, dd |
| H-1' | 4.63, d | 4.62, d | 4.61, d | 5.53, d | |
| H-2' | 5.19, dd | 5.19, dd | 5.19, dd | 4.87, dd | |
| H-3' | 5.01, dd | 5.01, dd | 5.00, dd | 5.06, t | |
| H-4' | 5.38, dd | 5.38, dd | 5.38, dd | 5.41, t | |
| H-5' | 3.95, dt | 3.95, dt | 3.94, dt | 4.04, ddd | |
| H-6'a | 4.1 ^b | 4.1 ^b | 4.1 ^b | 4.08, dd | |
| H-6'b | 4.1 ^b | 4.1 ^b | 4.1 ^b | 4.25, dd | |

^a ^1H -NMR data were measured in CDCl_3 with tetramethylsilane as an internal standard at 20°C .

^bOverlapped and could not be determined.

Table 3 ^1H - ^1H coupling constants for synthesized compounds^a

| Coupled
Protons | <i>J</i> -values (Hz) | | | | |
|--------------------|-----------------------|------|------|------|------|
| | 2 | 3 | 4 | 5 | 6 |
| $J_{1,2}$ | 5.1 | 5.1 | 5.1 | 5.3 | 4.8 |
| $J_{2,3}$ | 2.7 | 2.7 | 2.6 | 2.8 | 6.8 |
| $J_{2,4}$ | 0.9 | 1.1 | 1.0 | 1.3 | ND |
| $J_{3,4}$ | 1.5 | 1.3 | 1.3 | 0.6 | 3.4 |
| $J_{4,5}$ | 9.6 | 9.7 | 9.6 | 8.8 | 2.5 |
| $J_{5,6a}$ | 5.4 | 5.4 | 5.4 | 5.8 | 6.6 |
| $J_{5,6b}$ | 2.3 | 2.3 | 2.2 | 2.2 | 6.6 |
| $J_{6a,6b}$ | 12.0 | 12.0 | 12.1 | 12.2 | 11.4 |
| $J_{1',2'}$ | 7.9 | 7.9 | 7.9 | 4.0 | |
| $J_{2',3'}$ | 10.4 | 10.4 | 10.4 | 10.3 | |
| $J_{3',4'}$ | 3.5 | 3.5 | 3.5 | 9.5 | |
| $J_{4',5'}$ | 0.9 | 1.0 | 1.0 | 10.1 | |
| $J_{5',6'a}$ | 6.6 | 6.6 | 6.8 | 2.2 | |
| $J_{5',6'b}$ | 6.6 | 6.6 | 6.8 | 4.4 | |
| $J_{6'a,6'b}$ | b | b | b | 12.7 | |

^aCondition was described in Table 2.^bOverlapped and could not be determined.

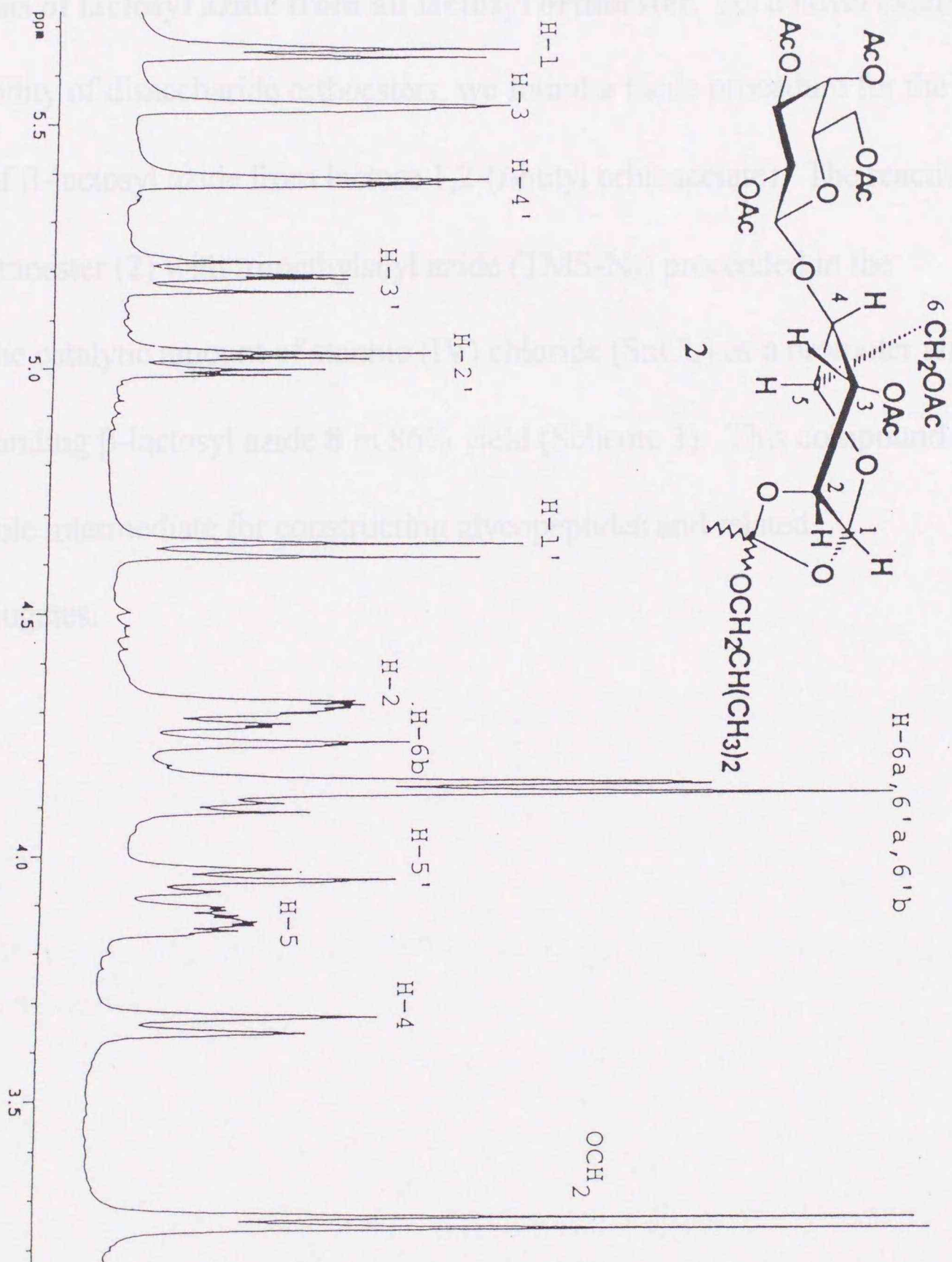
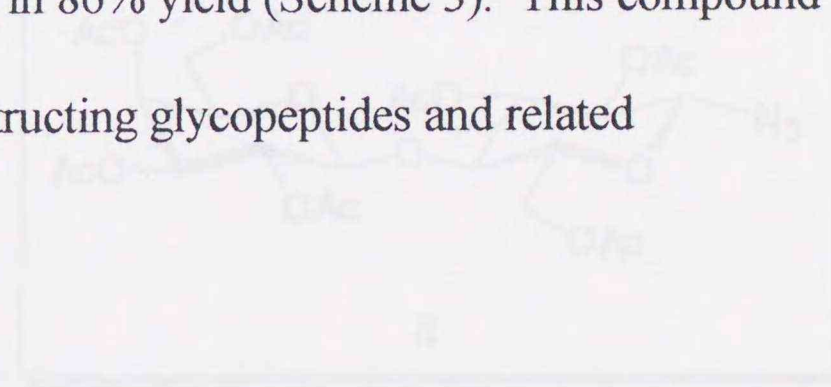


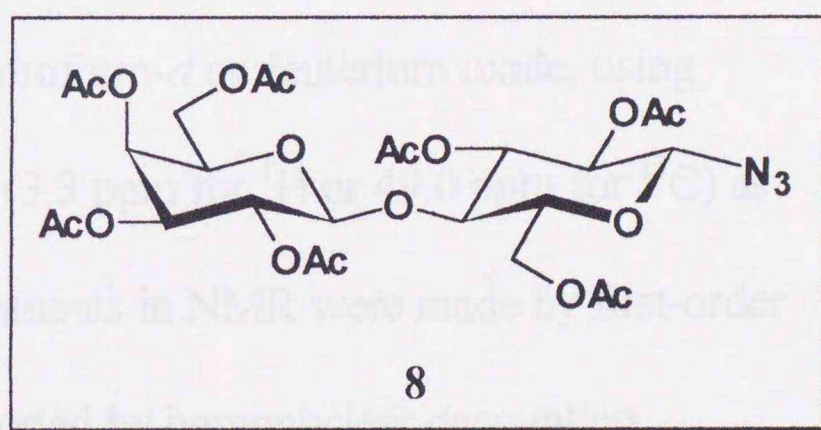
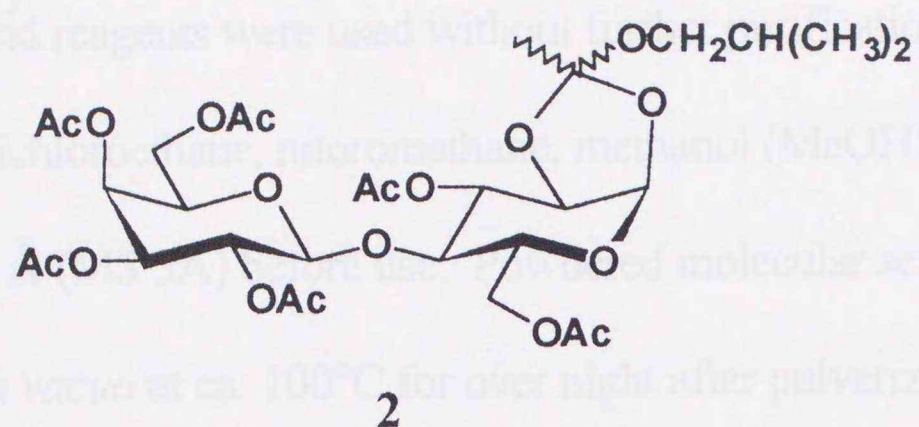
Figure 6. ^1H -NMR spectrum of compound 2.

residue greatly influence the conformation of the orthoester-bearing sugar moiety as indicated in the case for oxazoline derivatives.^{18,19}

Synthesis of lactosyl azide from an lactosyl orthoester. As a novel example of the availability of disaccharide orthoesters, we found a facile procedure for the preparation of β -lactosyl azide from lactose 1,2-(*i*-butyl orthoacetate). The reaction of lactosyl orthoester (**2**) with trimethylsilyl azide (TMS-N₃) proceeded in the presence of the catalytic amount of stannic (IV) chloride (SnCl₄) as a promoter and gave corresponding β -lactosyl azide **8** in 86% yield (Scheme 3). This compound will be valuable intermediate for constructing glycopeptides and related neoglycoconjugates.



Scheme 3. Reagents and conditions: (i) TMS-N₃, SnCl₄, in dichloromethane at ambient temperature.



Scheme 3. Reagents and conditions: i) TMS-N₃, SnCl₄, in dichloromethane at ambient temperature.

2.3. Exeperimental Section

General Procedures. Unless otherwise stated, all commercially available solvents and reagents were used without further purification. Benzene, Chloroform (CHCl_3), dichloroethane, nitoromethane, methanol (MeOH) were stored over molecular sieves 3 Å (MS 3Å) before use. Powdered molecular seives 4Å (MS 4Å) was dried over *in vacuo* at ca. 100°C for over night after pulverizing. ^1H and proton decoupled carbon NMR spectra were recorded at 300 and 75.47 MHz respectively, with a Bruker AMX-300 spectrometer in chloroform-*d* or deuterium oxide, using tetramethylsilane (TMS), or methanol (3.3 ppm for ^1H or 49.0 ppm for ^{13}C) as internal standards. Ring-proton assignments in NMR were made by first-order analysis of the spectra, and were supported by homonuclear decoupling experiments. Reactions were monitored by thin layer chromatography (TLC) on a precoated plate of silica gel 60 F₂₅₄ (layer thickness, 0.25 mm; E. Merck, Darmstadt, Germany). The solvent systems used were (A) 2:1, (B) 1:1 (C) 1:2 (v/v) toluene–ethyl acetate, and (D) 65:25:4 (v/v/v) chloroform–methanol–water. For detection of the sugar components, TLC sheets were sprayed with (a) a solution of 85:10:5 (v/v/v) methanol–concd sulfuric acid–*p*-anisaldehyde, and heated for a few minutes (for carbohydrate) or (b) an aqueous solution of 5wt% potassium permanganate and heated similarly (for double bond). Column chromatography was

performed on silica gel (Silica Gel 60; 0.015-0.040 mm, E. Merck) or Sephadex LH-20 (Pharmacia Fine Chemicals). All extractions were concentrated below 45°C under diminished pressure.

***O*-(2,3,4,6-Tetra-*O*-acetyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-3,6-di-*O*-acetyl- α -D-glucopyranose 1,2-[(Trifluoroacetamido)-1-hexyl orthoacetate] (4).** A mixture of powdered MS 4Å (5 g), mercuric bromide (HgBr₂) (1.91 g, 5.2 mmol), 2,4,6-collidine (2.00 mL, 15.0 mmol) and 6-(trifluoroacetamido)-1-hexanol (1.07 g, 5.0 mmol) in 1:1 (v/v) of benzene-nitromethane (20 mL) was stirred for 20 min at room temperature. A solution of *O*-(2,3,4,6-tetra-*O*-acetyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-2,3,6-tri-*O*-acetyl-D-glucopyranosyl bromide²⁰ (5.25 g, 7.5 mmol) in 10 mL of dry chloroform was dropwisely added to the mixture. The reaction mixture was continuously kept stirring for 22 h at room temperature. The mixture was filtered and the filtrate was evaporated under diminished pressure. The residue was diluted by chloroform, then the chloroform solution was successively washed with 1 N aq. sulfuric acid, saturated sodium hydrogenbicarbonate, and 1.5 M sodium chloride, dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo*. The syrupy residue was once purified by passing through Sephadex LH-20 column (ϕ 5 cm x 200 cm) with 95% ethanol as eluent. The fractions containing title compound (t.l.c. for sugar assay and phenol-sulfuric acid assay) was combined and concentrated.

The syrup was subsequently chromatographed on silica gel with 3:1 to 1:1 (v/v) of toluene–ethyl acetate as the eluent. The yield of **4** was 4.25 g (q.y.) as clear syrup: R_F 0.47 (solvent C), $^1\text{H-NMR}$ δ (CDCl_3) 1.37 (m, 4 H, 2 CH_2), 1.58, (m, 4 H, 2 CH_2), 1.72 (s, 3 H, CH_3), 1.98, 2.04, 2.07, 2.12, 2.13, 2.17 (each s, 18 H, 6 COCH_3), 3.36 (dd, 2 H, J 6.8 Hz, NHCH_2), 3.48 (t, 2 H, J 6.4 Hz, OCH_2), 6.64 (brs, 1 H, NH), and the data of the ring protons are given in Table 1 and 2.

***O*-(2,3,4,6-Tetra-*O*-acetyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-3,6-di-*O*-acetyl- α -D-glucopyranose 1,2-(*i*-Butyl orthoacetate) (2).** A *O*-(2,3,4,6-tetra-*O*-acetyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-2,3,6-tri-*O*-acetyl-D-glucopyranosyl bromide (1.05 g, 1.50 mmol) was allowed to react with *i*-butyl alcohol (92.3 mL, 1.0 mmol) by the same manner as described above for the preparation of **4**. The compound **2** had R_F 0.49 (solvent B), $^1\text{H-NMR}$ δ (CDCl_3) 0.90 (d, 6 H, J 6.7 Hz, 2 CH_3), 1.72 (s, 3 H, CH_3), 1.78 (m, 1 H, CH), 1.98, 2.04, 2.07, 2.12, 2.13, 2.17 (each s, 18 H, 6 COCH_3), 3.26 (d, 2 H, J 6.6 Hz, OCH_2), and the data of the ring protons are given in Table 1 and 2.

***O*-(2,3,4,6-Tetra-*O*-acetyl- β -D-galactopyranosyl)- 3,6-di-*O*-acetyl- α -D-glucopyranose 1,2-(*n*-Pentenyl orthoacetate) (3).** A title compound was prepared from *O*-(2,3,4,6-tetra-*O*-acetyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-2,3,6-tri-*O*-acetyl-D-

glucopyranosyl bromide (1.05 g, 1.50 mmol) with *n*-pentenyl alcohol (0.104 mL, 1.0 mmol) by same condition as described for **4**. The compound **3** had R_F 0.50 (solvent B), 0.66 (solvent C), $^1\text{H-NMR}$ δ (CDCl_3) 1.72 (s, 3 H, CH_3), 1.81 (m, 2 H, OCH_2CH_2), 1.98, 2.04, 2.07, 2.12, 2.12, 2.17 (each s, 18 H, 6 COCH_3), 2.15 (m, 2 H, $\text{CH}_2\text{CH=}$), 3.49 (t, 2 H, J 6.5 Hz, OCH_2), 5.00 (m, 2 H, CH=CH_2), 5.80 (m, 1 H, CH=CH_2), and the data of the ring protons are given in Table 1 and 2.

***O*-(2,3,4,6-tetra-*O*-acetyl- α -D-glucopyranosyl)-(1 $\rightarrow$ 4)-3,6-di-*O*-acetyl- α -D-glucopyranose 1,2-(*i*-Butyl orthoacetate) (**5**).** A title compound was prepared from crude *O*-(2,3,4,6-tetra-*O*-acetyl- α -D-glucopyranosyl)-(1 $\rightarrow$ 4)-2,3,6-tri-*O*-acetyl-D-glucopyranosyl bromide (2.01 g, 2.87 mmol) with *i*-butyl alcohol (0.156 mL, 1.69 mmol) by the same condition as described for **4**. The compound **5** had R_F 0.52 (solvent B), $^1\text{H-NMR}$ δ (CDCl_3) 0.90 (dd, 6 H, J 6.7 Hz, 2 CH_3), 1.73 (s, 3 H, CH_3), 1.80 (m, 1 H, OCH_2CH), 2.03, 2.04, 2.09, 2.10, 2.11, 2.13 (each s, 18 H, 6 COCH_3), 3.23 (m, 2 H, OCH_2), and the data of the ring protons are given in Table 1 and 2.

3,4,6-Tri-*O*-acetyl- α -D-galactopyranose 1,2-(*i*-Butyl orthoacetate) (6**).** A title compound was prepared from 2,3,4,6-tetra-*O*-acetyl-D-galactopyranosyl bromide (0.62 g, 1.5 mmol) with *i*-butyl alcohol (92.3 mL, 1.0 mmol) by the same

manner for the preparation of **4**. The compound **6** had R_F 0.60 (solvent A), ^1H -NMR δ (CDCl_3) 0.90 (dd, 6 H, J 6.7 Hz, 2 CH_3), 1.59 (s, 3 H, CH_3), 1.82 (m, 1 H, OCH_2CH), 2.07, 2.07, 2.12 (each s, 9 H, 3 COCH_3), 3.27 (dd, 2 H, J 6.7 and <1 Hz, OCH_2), and the data of the ring protons are given in Table 1 and 2.

2-Methyl-(3,4,6-tri-*O*-acetyl-1,2-dideoxy- α -D-glucopyrano)-[2,1-d]-2-oxazoline (7**).¹⁸** A mixture of powdered MS 4Å (1 g), HgBr_2 (1.02 g, 2.84 mmol), 2,4,6-collidine (1.09 mL, 8.20 mmol) in 1:1 (v/v) of benzene–nitromethane (4 mL) was stirred for 40 min at room temperature. To the solution was slowly added a solution of crude 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy- α -D-glucopyranosyl chloride¹⁴ (1.00 g, 2.73 mmol) in 2 mL of dry chloroform, then the mixture was continuously stirred for 3 h at 50°C. The reaction mixture was evaporated *in vacuo*, diluted with chloroform, and filtered. The filtrate was washed with saturated sodium hydrogen bicarbonate solution and brine, dried over sodium sulfate, filtered, and evaporated. The residual syrup was subjected to a column of silica gel with 100:200:1 (v/v/v) of toluene–ethyl acetate–triethylamine as the eluent to give pure oxazoline derivative **7** (0.76 g, 84.4%); R_F 0.60 (solvent A), ^1H -NMR δ (CDCl_3), 2.09, 2.16, 2.12 (each s, 9 H, 3 COCH_3), 2.10 (s, 3 H, CH_3 -oxazoline), 3.61 (dt, 1 H, $J_{5,6}$ 4.5 Hz, H-5), 4.14 (m, 1 H, H-2), 4.18 (d, 2 H, H-6a and -6b), 4.93 (dt, 1 H,

$J_{2,4} \sim 1$ Hz, $J_{4,5}$ 9.3 Hz, H-4), 5.27 (t, 1 H, $J_{3,4}$ 2.4 Hz, H-3), and 5.98 (d, 1 H, $J_{1,2}$ 7.4 Hz, H-1).

***O*-(2,3,4,6-Tetra-*O*-acetyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-2,3,6-tri-*O*-acetyl- β -D-glucopyranosyl Azide (**8**).²¹** A solution of an orthoester **2** (0.67 g, 0.97 mmol) in dry dichloroethane (6 mL) was treated with trimethylsilyl azide (TMS-N₃) (428 μ L, 2.90 mmol) and 1.0 M stannic (IV) chloride in dichloromethane (97 μ L, 97 μ mol) as a promoter for 11 h at room temperature. The reaction mixture was poured into cold saturated sodium hydrogen bicarbonate solution, extracted by chloroform, washed with brine, dried over anhydrous Na₂SO₄, filtered, and concentrated *in vacuo*. The resulting syrup was purified by column of silica gel with 2:1 (v/v) of toluene–ethyl acetate as eluent, to give clear syrup of **8** (0.55 g, 85.9 %); R_F 0.54 (solvent C), $\nu_{\max}$ 2121 cm⁻¹ (N₃), 1755 cm⁻¹ (C=O), 1370 cm⁻¹, 1220 cm⁻¹, 1062 cm⁻¹, ¹H-NMR δ (CDCl₃), 1.96-2.15 (each s, 21 H, 7 COCH₃), 3.70 (ddd, 1 H, $J_{5,6a}$ 5.0 Hz and $J_{5,6b}$ 2.0 Hz, H-5), 3.82 (t, 1 H, $J_{4,5}$ 9.9 Hz, H-4), 3.88 (t, 1 H, $J_{5',6'a}$ 7.1 Hz $J_{5',6'b}$ 6.6 Hz, H-5'), 4.08 (dd, 1 H, $J_{6'a,6'b}$ 11.1 Hz, H-6'a), 4.12 (dd, 1 H, H-6a), 4.13 (dd, 1 H, H-6'b), 4.49 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1'), 4.51 (dd, 1 H, $J_{6a,6b}$ 12.1 Hz, H-6b), 4.63 (d, 1 H, $J_{1,2}$ 8.8 Hz, H-1), 4.86 (dd, 1 H, $J_{2,3}$ 9.5 Hz H-2), 4.96 (dd, 1 H, $J_{3',4'}$ 3.4 Hz, H-3'), 5.10 (dd, 1 H, $J_{2',3'}$ 10.4 Hz, H-2'), 5.21 (t, 1 H, $J_{3,4}$ 9.0 Hz, H-3), 5.35 (dd, 1 H, $J_{4',5'}$ 1.0 Hz, H-4').

2.4. Conclusion

We found a facile and an efficient method for the preparation of aldose 1,2-orthoester and oxazoline derivatives using inexpensive HgBr_2 -2,4,6-collidine system. These reactive intermediates prepared here can be useful as synthons for variety of glycoconjugate syntheses. Indeed, the oxazoline derived from GlcNAc was essentially converted into its functional glycosides with alcohols in the presence of acid catalyst.²²

2.5. References

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Chapter 3

Glycoprotein Models: Polymeric Sugar Ligands Available for Determining the Binding Specificity of Lectins

Systematic syntheses of polymerizable *N*-acetylglucosamine and related disaccharide derivatives have been accomplished by introducing an *n*-pentenyl group at each reducing end as a simple and versatile polymerizable aglycon. Radical copolymerizations of these sugar monomers with acrylamide proceeded smoothly in water by means of ammonium persulfate and *N,N,N',N'*-tetramethylethylenediamine as initiators and to give water soluble glycopolymers in good yields. In addition to chemical syntheses of glycopolymers, chemoenzymic galactosylation of the polymeric GlcNAc ligand was also completely performed by means of bovine galactosyl transferase activity in the presence of uridine 5'-diphospho-galactose as a sugar donor. These glycopolymers were demonstrated to exhibit enhanced binding capacity with lectins based on polymeric sugar-cluster effect. It was suggested that the polymeric LacNAc ligand showed much higher affinity to *Erythrina corallodendron* lectin than other polymers having positional LacNAc isomers from the inhibitory assay of hemagglutination by *Erythrina corallodendron*. The order of the inhibitory effects of these polymers on hemagglutination by *Erythrina*

corallodendron was $\text{Gal}\beta(1\rightarrow4)\text{GlcNAc}(\text{LacNAc}) \gg \text{Gal}\beta(1\rightarrow4)\text{Glc}(\text{Lac}) \gg$

$\text{Gal}\beta(1\rightarrow3)\text{GlcNAc} > \text{Gal}\beta(1\rightarrow6)\text{GlcNAc} \cong \text{Gal}.$

3.1. Introduction

Although sugar chains of the glycoconjugates could play important roles as biologically active structures on the cell surface, themselves usually bound to the peptide chains or the ceramides. This suggests that sugar chains usually act as partial structures of macromolecules or conjugated molecules. As it is known that the synthetic trisaccharide or small sugar derivatives exhibited only weak affinity to the hemagglutinin molecules,² it seems to be important to investigate how such weak carbohydrate-protein interactions are eventually amplified in the successful recognition processes, but with extreme carbohydrate specificity. In order to thoroughly understand these interaction on molecular level, our attention has been focused on glycoprotein models as the synthetic glycoconjugates which may be available for the investigation of the recognition processes related to sugar-protein interactions. Actually, synthetic polymers including well-defined sugar residues are used for biochemical purposes such as affinity chromatography of lectins, carbohydrate antigens, and cell culture systems.³⁻⁵

Recently we have reported a convenient preparation of water-soluble carbohydrate monomers which have *n*-pentenyl group (PentO-; $\text{CH}_2=\text{CHCH}_2\text{CH}_2\text{CH}_2\text{O}-$) at the reducing end as a polymerizable functional group, and copolymers from the corresponding monomers with acrylamide.^{6,7} This method

has been applicable for the syntheses of a variety of sugar monomers containing *N*-acetyl-D-glucosamine (GlcNAc),⁷ *N,N'*-diacetylchitobiose [GlcNAc β (1 $\rightarrow$ 4)GlcNAc],⁷ *N*-acetyllactosamine [Gal β (1 $\rightarrow$ 4)GlcNAc],⁶ and Le^x-type trisaccharide {Gal β (1 $\rightarrow$ 4)[Fuc α (1 $\rightarrow$ 3)]GlcNAc} of tumor-associated carbohydrate antigen.⁸

N-Acetyllactosamine [*O*-(β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-2-acetamido-2-deoxy-D-glucopyranose; Gal β (1 $\rightarrow$ 4)GlcNAc; LacNAc] is well known as a biologically important disaccharide core structure of lactosaminoglycans, tumor-associated antigenic carbohydrates, and many carbohydrate receptors in glycoproteins and glycolipids.⁹⁻¹¹ We have demonstrated a highly selective adhesion of rat hepatocyte on a polyacrylamide gel including LacNAc residues which clearly showed much higher affinity than another type of gel including *N,N'*-diacetylchitobiose,⁶ since hepatic lectins have been reported to exhibit specific affinity to LacNAc containing sugar chains of glycoproteins.¹² It was also demonstrated for determining the sugar binding specificity of wheat germ agglutinin (WGA) that density of GlcNAc residues on the glycoprotein models significantly affected a successful combining to the subsites of WGA using high-density GlcNAc polymer prepared from ω -acryloyl amido type sugar monomer.¹

We describe herein the syntheses and binding properties with lectins of new polymers having sequences related to LacNAc such as Gal, Lac,

Gal β (1 $\rightarrow$ 3)GlcNAc (LacNAc-13), and Gal β (1 $\rightarrow$ 6)GlcNAc (LacNAc-16), respectively. The specific interaction of the glycopolymers with *Erythrina corallodendron* lectin will be discussed by means of inhibitory effects of glycopolymer ligands on hemagglutination induced by the lectin.

3.2. Results and Discussion

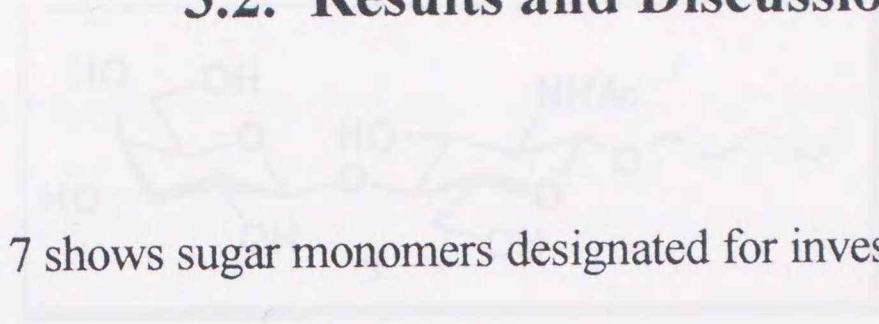


Figure 7 shows sugar monomers designated for investigating structural accessibility in interaction between ECorL and lactosamine oligosaccharides. Compounds **9** and **11** were prepared according to the method reported previously through the simple reactions of oxazoline derivatives with *n*-pentenyl alcohol. Galactose derivative **10** and lactose derivative **12** were selected as sugar monomers to prepare glycopolymer ligands lacking the reducing GlcNAc residue. In order to evaluate the importance of the inter-glycosidic bond between Gal and GlcNAc residues, two positional isomers LacNAc-13 (**13**) and LacNAc-16 (**14**) were regarded as synthetic targets of this study. Since we have already demonstrated the versatility of *n*-pentenyl group for creating glycopolymers having branching oligosaccharide structure such as Le^x trisaccharide,⁸ all sugar monomers used here were expected to show good polymerizability to give polymers with high molecular weights.

Figure 7. Glycosyl monomers related to LacNAc.

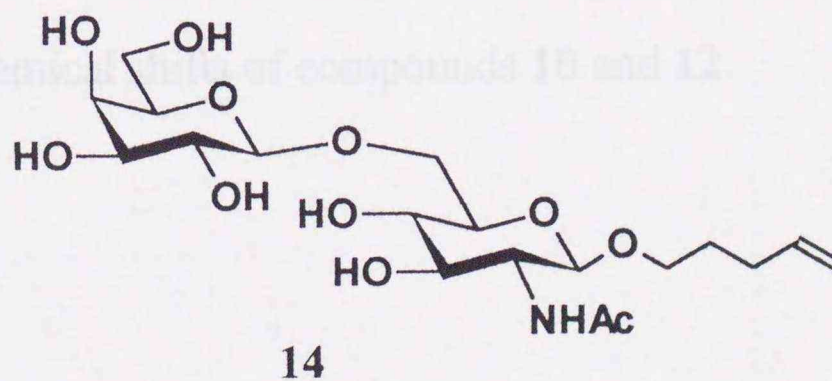
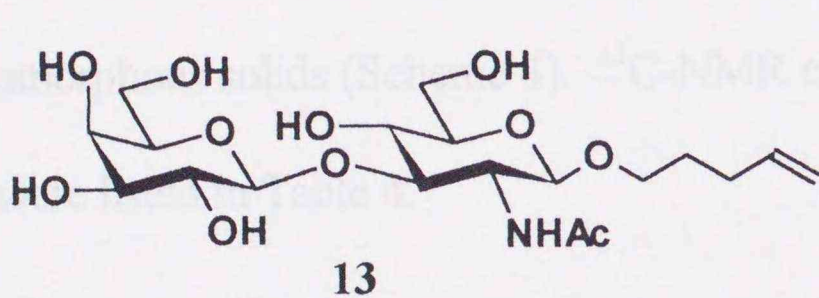
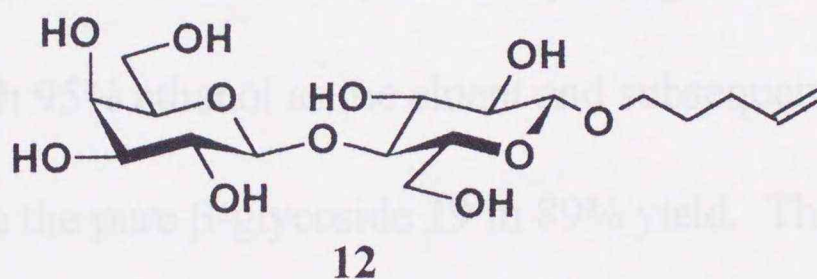
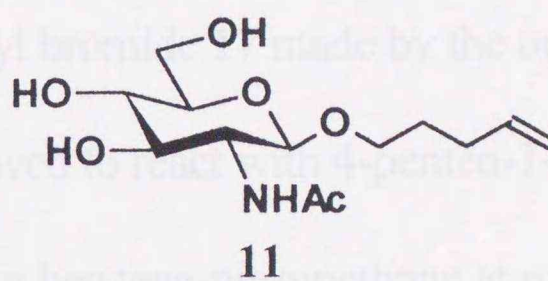
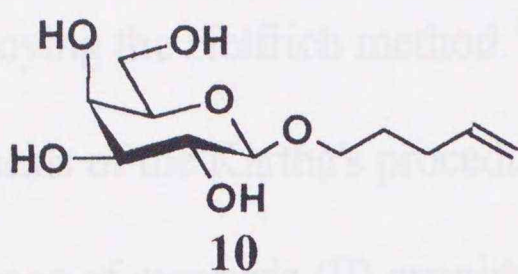
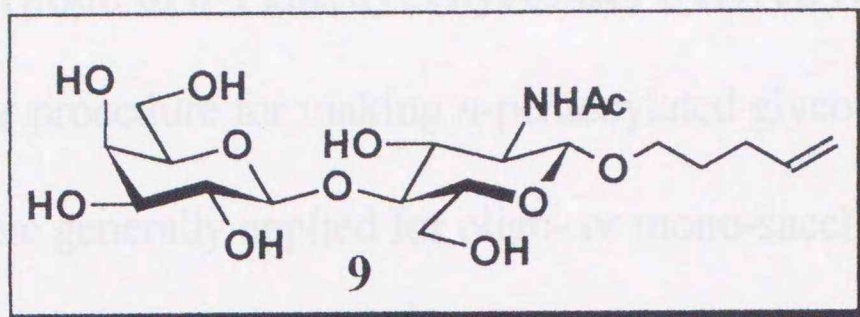
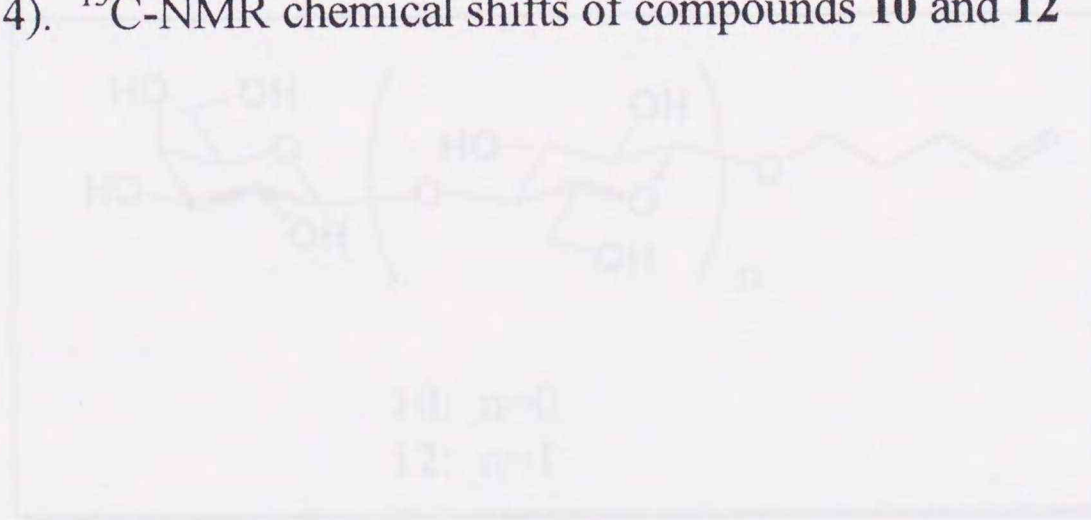
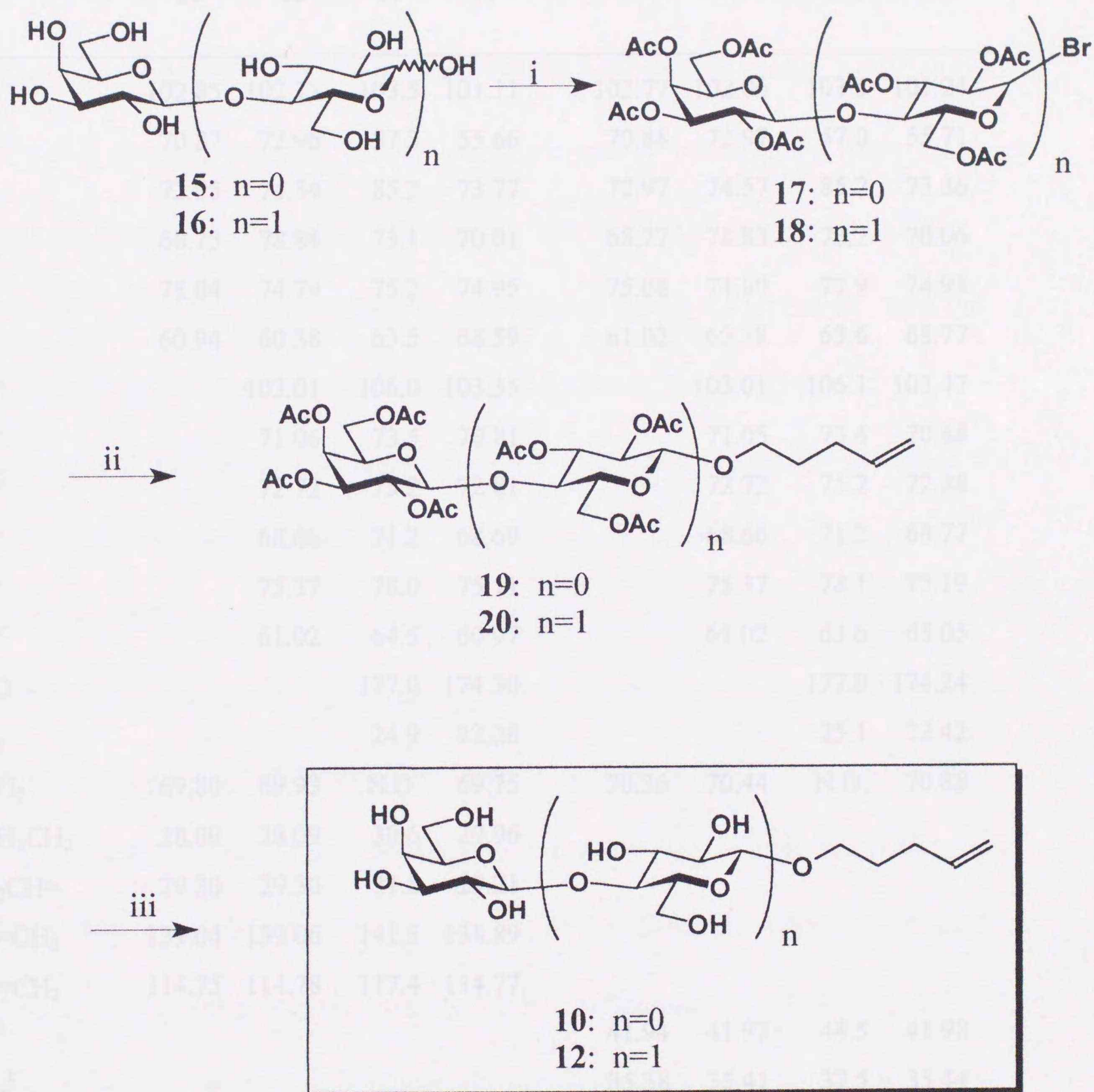


Figure 7. Glycosyl monomers related to LacNAc.

Preparation of *n*-Pentenyl Glycosides Derived from Galactose and Lactose.

Lactose. Our procedure for making *n*-pentenylated glycosides *via* an oxazoline derivative were generally applied for oligo- or mono-saccharides in which have a GlcNAc residue at the reducing end. Thus, Gal and Lac which are lacking the 2-acetamido group at reducing end were converted to *n*-pentenyl glycosides by employing the Helfrich method.¹³ A galactosyl bromide **17** made by the one pot synthesis of the Kartha's procedure¹⁴ was allowed to react with 4-penten-1-ol in the presence of mercuric (II) cyanide [Hg(CN)₂] in benzene-nitromethane at room temperature. The reaction mixture was purified by using chromatography on Sephadex LH-20 with 95% ethanol as the eluent and subsequent silica gel chromatography gave the pure β -glycoside **19** in 89% yield. The per-*O*-acetate **19** was treated with sodium methoxide in methanol to give water soluble **10** as an amorphous solids (Scheme 4). ¹³C-NMR chemical shifts of compounds **10** and **12** were listed in Table 4.





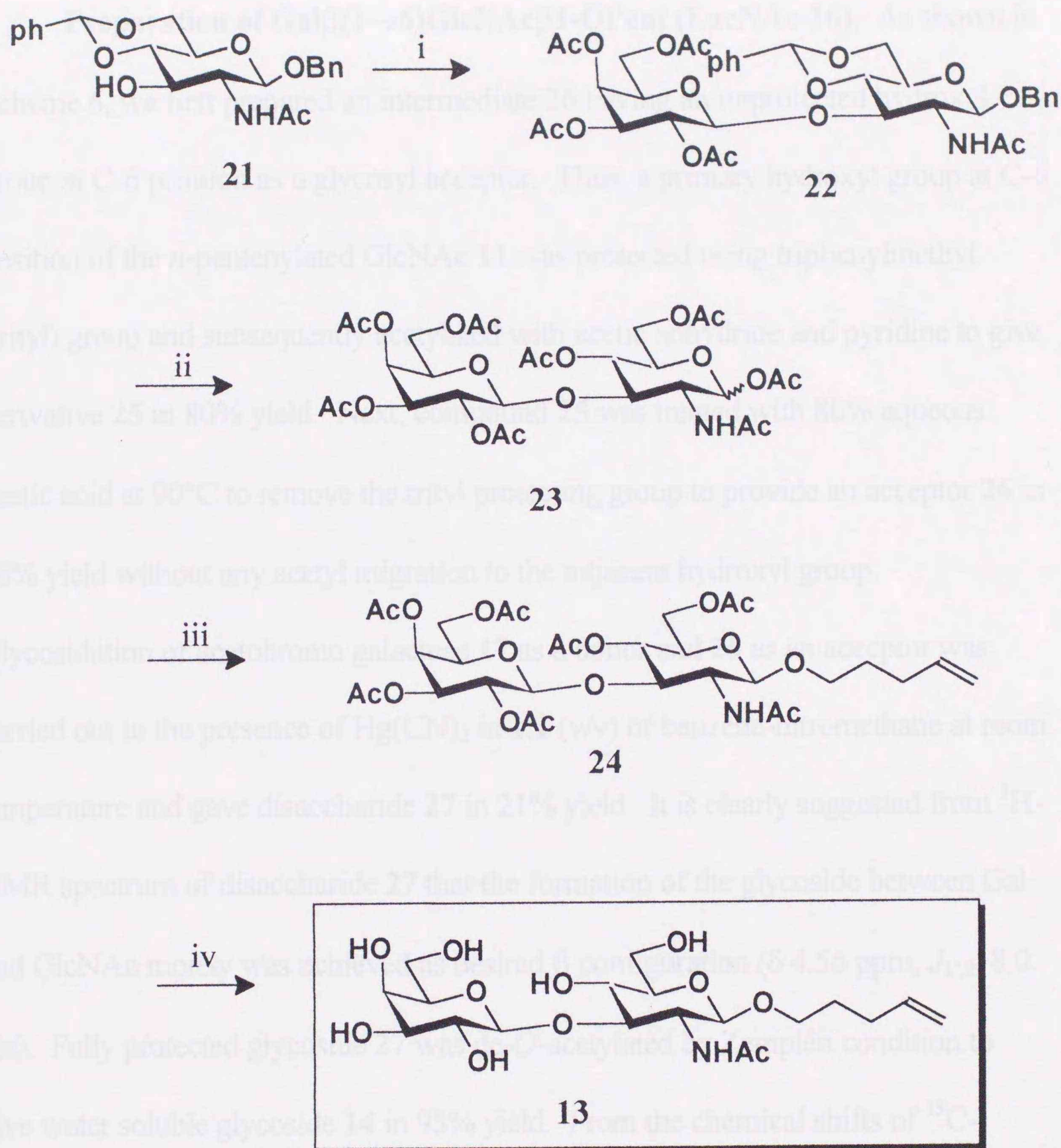
Scheme 4. Reagents and conditions: i) Ac_2O , HBr/AcOH , r.t., then further HBr/AcOH r.t.; ii) PentOH, MS 4Å, $\text{Hg}(\text{CN})_2$, Benzene-Nitromethane, r.t.; iii) NaOMe/MeOH , r.t..

Table 4. ^{13}C -NMR chemical shifts^a of carbohydrate monomers and copolymers.

| | Monomers | | | | Copolymers | | | |
|----------------------------------|----------|--------|-----------------|--------|------------|--------|-----------------|--------|
| | 10 | 12 | 13 ^c | 14 | 10 | 12 | 13 ^c | 14 |
| C-1 | 102.85 | 102.13 | 103.5 | 101.11 | 102.77 | 102.06 | 103.5 | 101.24 |
| C-2 | 70.87 | 72.96 | 57.3 | 55.66 | 70.88 | 72.97 | 57.0 | 55.71 |
| C-3 | 72.95 | 74.54 | 85.2 | 73.77 | 72.97 | 74.57 | 85.2 | 73.86 |
| C-4 | 68.73 | 78.84 | 73.1 | 70.01 | 68.77 | 78.83 | 73.2 | 70.06 |
| C-5 | 75.04 | 74.79 | 75.2 | 74.95 | 75.08 | 74.80 | 77.9 | 74.98 |
| C-6 | 60.94 | 60.38 | 63.5 | 68.59 | 61.02 | 60.38 | 63.6 | 68.77 |
| C-1' | | 103.01 | 106.0 | 103.35 | | 103.01 | 106.1 | 103.47 |
| C-2' | | 71.06 | 73.5 | 70.81 | | 71.05 | 73.4 | 70.88 |
| C-3' | | 72.72 | 75.2 | 72.81 | | 72.72 | 75.2 | 72.88 |
| C-4' | | 68.66 | 71.2 | 68.69 | | 68.66 | 71.2 | 68.77 |
| C-5' | | 75.37 | 78.0 | 75.11 | | 75.37 | 78.1 | 75.19 |
| C-6' | | 61.02 | 64.5 | 60.97 | | 61.02 | 63.6 | 65.05 |
| C=O | | | 177.0 | 174.30 | | | 177.0 | 174.24 |
| CH ₃ | | | 24.9 | 22.26 | | | 25.1 | 22.42 |
| OCH ₂ | 69.80 | 69.93 | N.D. | 69.75 | 70.36 | 70.44 | N.D. | 70.88 |
| OCH ₂ CH ₂ | 28.09 | 28.09 | 30.6 | 27.96 | | | | |
| CH ₂ CH= | 29.30 | 29.30 | 31.8 | 29.21 | | | | |
| CH=CH ₂ | 139.04 | 139.06 | 141.5 | 138.89 | | | | |
| CH=CH ₂ | 114.75 | 114.78 | 117.4 | 114.77 | | | | |
| CH ^b | | | | | 41.94 | 41.97 | 44.5 | 41.98 |
| CH ₂ ^b | | | | | 35.38 | 35.41 | 37.5 | 35.44 |
| C=O ^b | | | | | 179.40 | 179.42 | 182.1 | 179.42 |

^aChemical shifts were determined from the signal of methanol (49.0 ppm) as the internal standard. ^bSignals were observed as multiplets owing to the polymer main chain structure.

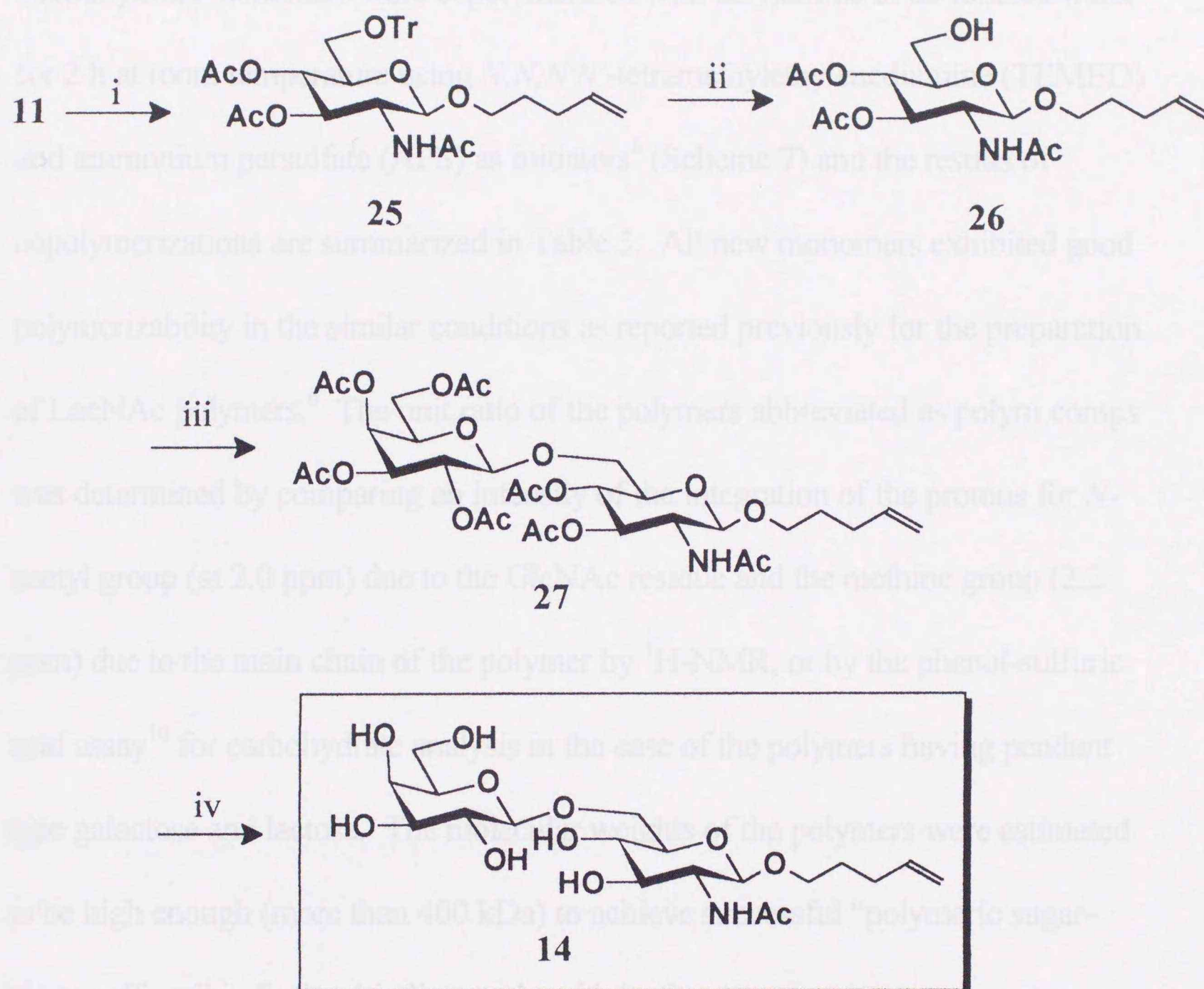
Preparation of Gal β (1 $\rightarrow$ 3)GlcNAc β 1-OPent (LacNAc-13). Scheme 5 indicates a synthetic route of LacNAc-13. Because of a poor solubility of a benzylidene derivative of *n*-pentenyl GlcNAc **11** as a glycosyl acceptor, the key intermediate had to be changed to a benzylidene derivative of benzyl 2-acetamido-2-deoxy- β -D-glucopyranoside **21**.¹⁵ Glycosyl acceptor **21** was coupled with galactosyl bromide **17** by means of Hg(CN)₂ as a catalyst to provide **22** in 68% yield. Disaccharide derivative **22** was converted to the per-*O*-acetate **23**, then the trimethylsilyltrifluoromethanesulfonate (TMSOTf) was employed for the preparation of a reactive oxazoline derivative which was a convenient precursor for the glycoside formation. The oxazoline derivative was allowed to react with 4-penten-1-ol in the presence of catalytic amount of camphorsulfonic acid (CSA) which is usually added until pH 3, and finally the *n*-pentenyl glycoside **24** was de-*O*-acetylated by the Zemplén method to give pure water soluble **13** in good yield. The purity and structure of glycoside **13** was confirmed by ¹H and ¹³C-NMR spectra. The ¹H -NMR spectrum of compound **13** revealed the signal assignable to H-1 at δ 4.45 ppm as a doublet with $J_{1,2}$ 7.8 Hz, indicating that the glycosidic bond newly formed had the β configuration. Chemical shifts of the fully assigned ¹³C-NMR spectrum for **13** is listed in Table 4.



Scheme 5. Reagents and conditions: i) 17, $\text{Hg}(\text{CN})_2$, Benzene-Nitromethane, 60°C , 8 h; ii) $\text{H}_2/\text{Pd-c}$, $\text{EtOH-AcOH-H}_2\text{O}$, 60°C , 9 h, then Ac_2O , Pyridine; iii) TMSOTf , $\text{ClCH}_2\text{CH}_2\text{Cl}$, 50°C , 5 h, then Et_3N , r.t., and PentOH , CSA , $\text{ClCH}_2\text{CH}_2\text{Cl}$, 90°C , 5 h; iv) NaOMe/MeOH , r.t., 3 h.

Preparation of Gal β (1 $\rightarrow$ 6)GlcNAc β 1-OPent (LacNAc-16). As shown in Scheme 6, we first prepared an intermediate **26** having an unprotected hydroxyl group at C-6 position as a glycosyl acceptor. Thus, a primary hydroxyl group at C-6 position of the *n*-pentenylated GlcNAc **11** was protected using triphenylmethyl (trityl) group and subsequently acetylated with acetic anhydride and pyridine to give derivative **25** in 80% yield. Next, compound **25** was treated with 80% aqueous acetic acid at 90°C to remove the trityl protecting group to provide an acceptor **26** in 76% yield without any acetyl migration to the adjacent hydroxyl group.

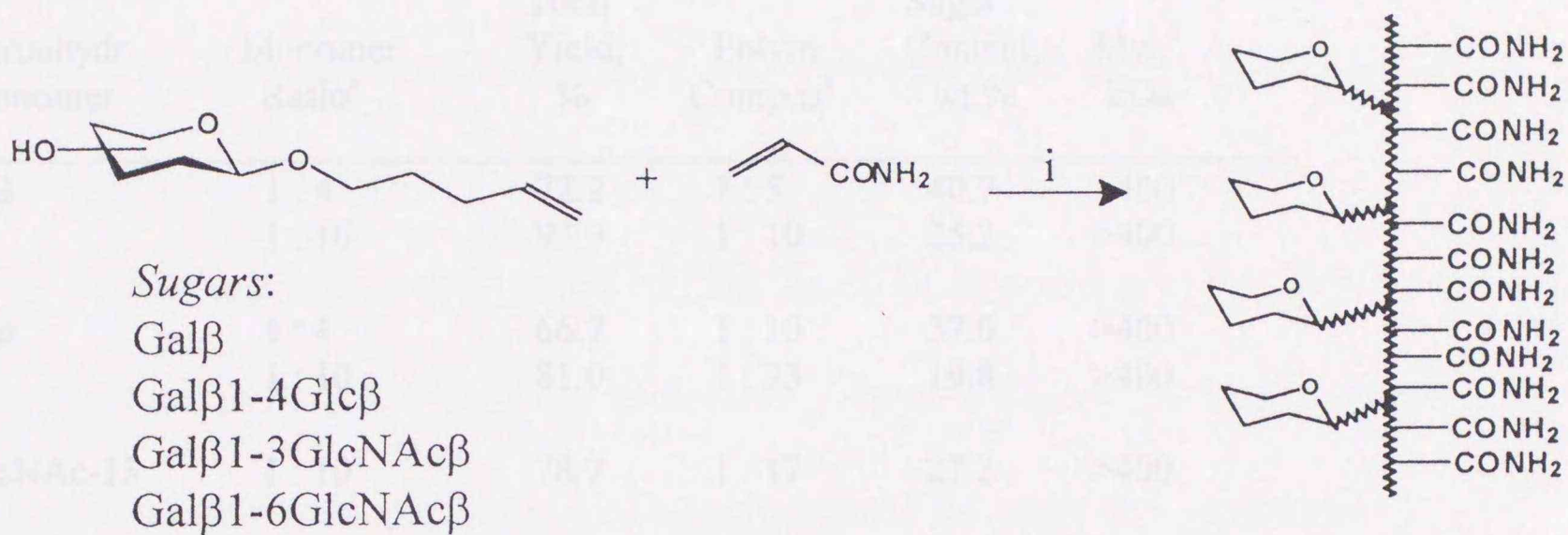
Glycosidation of acetobromo galactose **17** as a donor and **26** as an acceptor was carried out in the presence of Hg(CN)₂ in 1:1 (v/v) of benzene-nitromethane at room temperature and gave disaccharide **27** in 21% yield. It is clearly suggested from ¹H-NMR spectrum of disaccharide **27** that the formation of the glycoside between Gal and GlcNAc moiety was achieved as desired β configuration (δ 4.56 ppm, $J_{1,2}$ 8.0 Hz). Fully protected glycoside **27** was de-*O*-acetylated by Zemplén condition to give water soluble glycoside **14** in 95% yield. From the chemical shifts of ¹³C-NMR spectrum of compound **14**, it was shown that only C-6 signal of GlcNAc residue distinctively deshielded and shifted to 68.6 ppm (Table 4).



Scheme 6. Reagents and conditions: i) TrCl, Pyridine, 90°C, 40min, then Ac₂O, r.t., 3 h; ii) 80% aqueous AcOH, 90°C, 1 h; iii) 17, MS 4Å, Hg(CN)₂, Benzene-Nitromethane, r.t., 3 d; iv) NaOMe/MeOH, r.t., 4.5 h.

Radical copolymerization of water soluble glycosides with acrylamide.

Carbohydrate monomers were copolymerized with acrylamide in de-ionized water for 2 h at room temperature using *N,N,N',N'*-tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) as initiators⁶ (Scheme 7) and the results of copolymerizations are summarized in Table 5. All new monomers exhibited good polymerizability in the similar conditions as reported previously for the preparation of LacNAc polymers.⁶ The unit ratio of the polymers abbreviated as polym comps was determined by comparing an intensity of the integration of the protons for *N*-acetyl group (at 2.0 ppm) due to the GlcNAc residue and the methine group (2.2 ppm) due to the main chain of the polymer by ¹H-NMR, or by the phenol-sulfuric acid assay¹⁶ for carbohydrate analysis in the case of the polymers having pendant type galactose and lactose. The molecular weights of the polymers were estimated to be high enough (more than 400 kDa) to achieve successful “polymeric sugar-cluster effects” in further binding study with lectins.



Scheme 7. Reagents and condition: i) TEMED, APS, H₂O, r.t., 2 h.

Table 5. Results of copolymerization of carbohydrate monomers with acrylamide.

| Carbohydr. Monomer | Monomer Ratio ^a | Total Yield, % | Polym Compos ^a | Sugar Content, wt. % | Mw, ^b kDa |
|--------------------|----------------------------|----------------|---------------------------|----------------------|----------------------|
| Gal | 1 : 4 | 72.2 | 1 : 5 | 40.7 | >400 |
| | 1 : 10 | 93.9 | 1 : 10 | 25.2 | >400 |
| Lac | 1 : 4 | 66.7 | 1 : 10 | 37.0 | >400 |
| | 1 : 10 | 81.0 | 1 : 23 | 19.8 | >400 |
| LacNAc-13 | 1 : 10 | 78.7 | 1 : 17 | 27.2 | >400 |
| LacNAc-16 | 1 : 4 | 62.7 | 1 : 8 | 44.0 | >400 |
| | 1 : 10 | 77.4 | 1 : 23 | 23.0 | >400 |

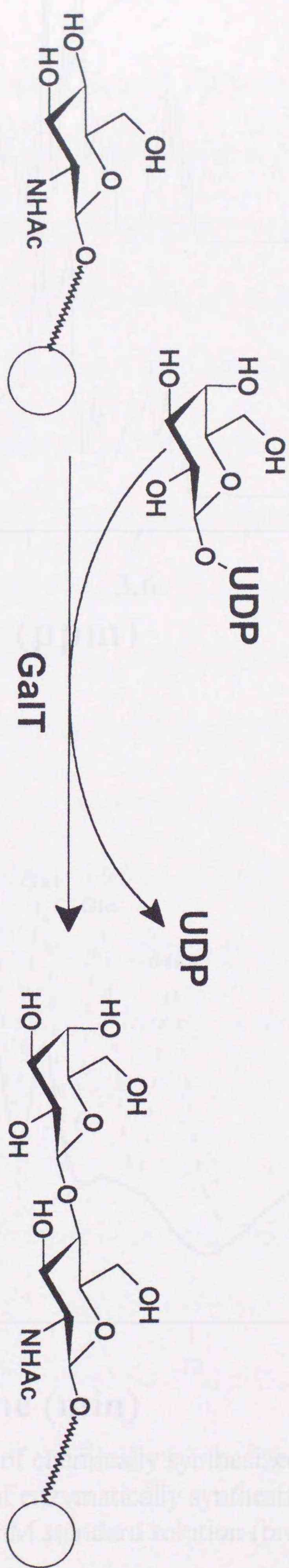
^aRatio of carbohydrate monomer to acrylamide. ^bMWs were estimated by the GPC method with asahipak G-510 column [pullulans (5.8, 12.2, 23.7, 48.0, 100, 186, and 380 kDa, Shodex Standard P-82) were used as standards].

Enzymatic Preparation of Glycopolymer Having LacNAc Side Chains.¹⁷

Chemoenzymatic syntheses of carbohydrates have been regarded as an important strategy for the preparation of structurally complex oligosaccharides owing to their stereo- and regioselectivity in the formation of glycoside linkages.¹⁸ The galactosyl transferase (GalT) is a readily available and a well evaluated enzyme.¹⁹ Therefore, our interests were paid towards chemical and enzymatic syntheses of glycopolymers in order to establish more convenient and facile procedures for this purpose.

It has been known that galactosylation into a 4-OH position of the GlcNAc in oligosaccharides by β -1,4-GalT has succeeded to provide *N*-acetyllactosamine type oligosaccharide chains even in case of introduction of galactose to a sepharose-GlcNAc conjugate in approximately 20% yield.²⁰ Since Zehavi et al. also demonstrated the enzymatic elongation approach by using a water soluble polymer substrate in which polymer had pendant type glucose residues,²¹ galactosylation yield was found to be 36% owing to a poor accessibility of rigid polymer structures against enzymatic reactions. In order to enhance the transfer yield on the polymer chains, *n*-pentenyl group was expected to provide the acceptor sugar side chains with an adequate spacer arm functions. Actually, the distance between the main chain of the polymer and sugar moiety was estimated to be 7.48 Å. As anticipated, elongation of Gal into the GlcNAc polymer with GalT proceeded smoothly to give LacNAc-type polymer in quantitative yield (Scheme 8). Integration ratio of H-1 and

H-1' of the enzymatically synthesized polymer having LacNAc measured in ^1H -NMR spectrum revealed this quantitative galactosylation (Figure 8B). And the spectrum was completely identical with that of synthetic LacNAc polymer shown in Figure 8A. Moreover, high performance anion exchange chromatography (HPAEC) analysis of acid hydrolysate of LacNAc polymer also supported this result as shown in Figure 8C. Calculated area of Gal and GlcNAc were found to be completely the same based on elution profiles of standard samples.²²



Scheme 8. Reagents and conditions: α -Lactalbumin, MnCl_2 (10 mM), 50 mM HEPES, pH 6.0, 37°C, 24 h.

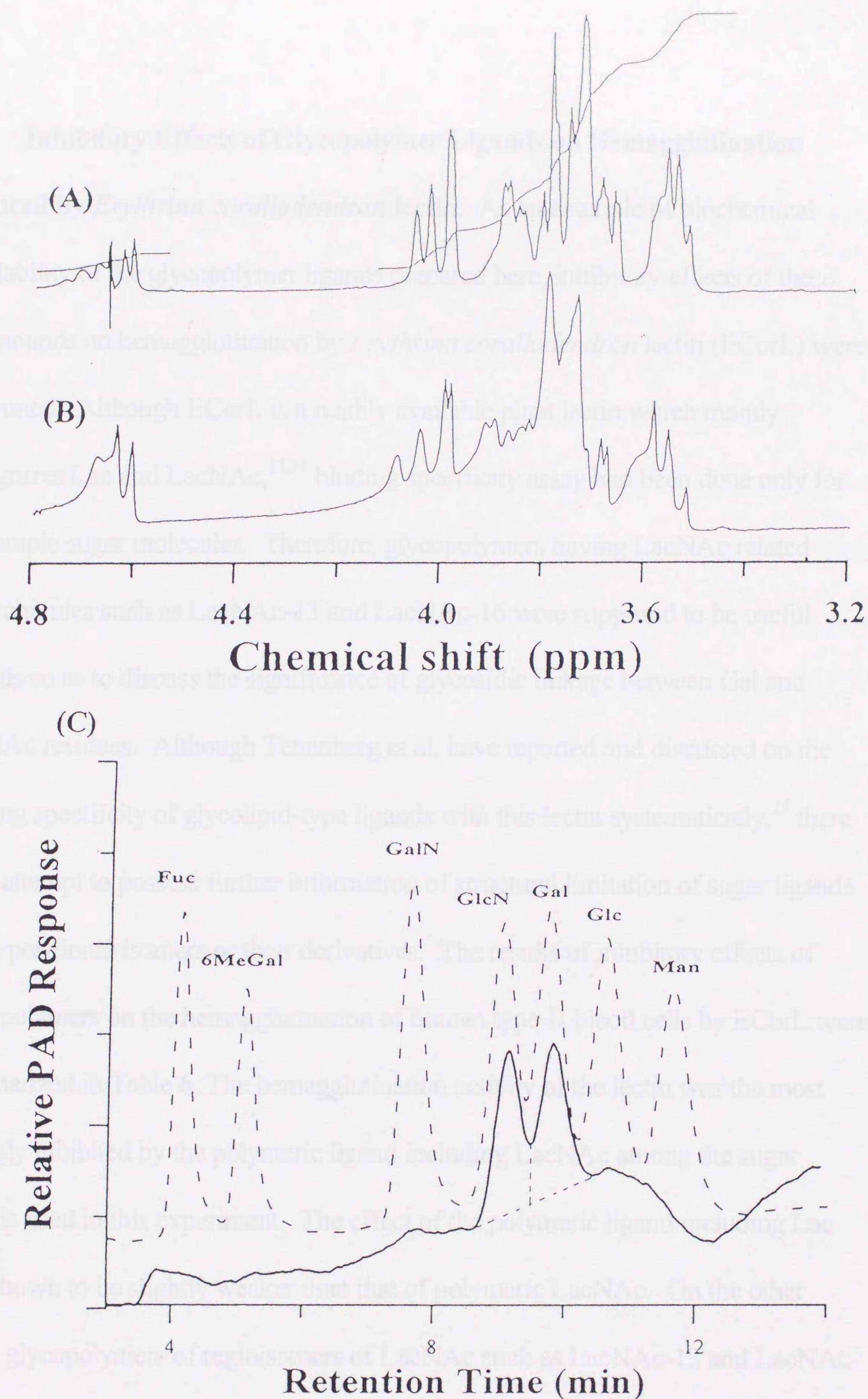


Figure 8. (A) 500 MHz of ^1H -NMR spectrum of chemically synthesized LacNAc polymer. (B) 300 MHz of ^1H -NMR spectrum of enzymatically synthesized LacNAc polymer. (C) HPAEC patterns of 50 mM standard solution (broken line) and hydrolysate of LacNAc polymer (solid line).

Inhibitory Effects of Glycopolymer Ligands on Hemagglutination

Induced by *Erythrina corallodendron* lectin. As an example of biochemical availability of the glycopolymer ligands prepared here, inhibitory effects of these compounds on hemagglutination by *Erythrina corallodendron* lectin (ECorL) were examined. Although ECorL is a readily available plant lectin which mainly recognizes Lac and LacNAc,^{23,24} binding specificity assay had been done only for the simple sugar molecules. Therefore, glycopolymers having LacNAc related disaccharides such as LacNAc-13 and LacNAc-16 were supposed to be useful ligands so as to discuss the significance of glycosidic linkage between Gal and GlcNAc residues. Although Tenenberg et al. have reported and discussed on the binding specificity of glycolipid-type ligands with this lectin systematically,²⁵ there is no attempt to possess further information of structural limitation of sugar ligands using positional isomers or their derivatives. The results of inhibitory effects of glycopolymers on the hemagglutination of human type-B blood cells by ECorL were summarized in Table 6. The hemagglutination activity of the lectin was the most strongly inhibited by the polymeric ligand including LacNAc among the sugar ligands used in this experiment. The effect of the polymeric ligand including Lac was shown to be slightly weaker than that of polymeric LacNAc. On the other hand, glycopolymers of regioisomers of LacNAc such as LacNAc-13 and LacNAc-16 showed only a faint or weak inhibitory effect on the hemagglutination by lectin.

It was apparently suggested that lectin recognizes specifically inter-glycosidic linkage between Gal and GlcNAc residues. This suggests that the binding pocket of the lectin probably is tight and strictly designated for the combining with the structures of Gal β (1 $\rightarrow$ 4)Glc or Gal β (1 $\rightarrow$ 4)GlcNAc. Use of the polymers having sugar residues is proved to be quite efficient methodology for determining the binding specificity of a lectin. When the polymeric ligand is applied for such experiments, the affinity constant will be observed as exponentially amplified values than that of monomeric ligand at the same concentrations based on polymeric sugar-cluster effects. Usually, the binding constants of polymeric ligands are estimated to be 10^3 times as much as that of monomeric sugar itself, thus we could compare exactly the differences in the binding constants of sugar ligands with lectins. As shown in Table 3, it was clearly demonstrated that the order of the inhibitory effects of glycopolymers on hemagglutination by lectin is LacNAc polymer $\gg$ Lac polymer, LacNAc monomer $\gg$ LacNAc-13 polymer $>$ LacNAc-16 polymer, Gal polymer $\gg$ GlcNAc polymer.

Table 6. Inhibitory effects of glycopolymers^a on *Erythrina corallodendron* lectin hemagglutination.^b

| Ratio ^c | Control | Monomer | 1:10 | 1:10 | 1:10 | 1:10 | 1:10 | 1:10 |
|----------------------|---------|---------|--------|--------|--------|-------|--------|------|
| | LacNAc | Gal | GlcNAc | LacNAc | LacNAc | Lac | LacNAc | |
| MC ^d (μM) | >>1000 | >25 | >1000 | >>1000 | >500 | >1000 | >25 | >1 |
| | | | | | -13 | -16 | | |

^aGlycopolymers were derived from those *n*-pentenyl glycosides with acrylamide. ^bFresh human type-B red blood cells were treated with heparin and used as the concentration estimated to be 1% (v/v) in 0.05 M PBS (pH 7.3). The lectin concentration was estimated to be 2.0 μM by using the extinction coefficient A_{280} . ^cMonomer ratio of sugar to acrylamide. ^dMinimum concentration of inhibitory potency in the hemagglutination were estimated by the concentration of the sugar residue.

3.3. Experimental Section

General Procedures. Unless otherwise stated, all commercially available solvents and reagents were used without further purification. Benzene, chloroform (CHCl_3), dimethylformamide (DMF), nitromethane, methanol (MeOH) were stored over molecular sieves ($3 \text{ \AA}$) and, pyridine and triethylamine were stored with NaOH pellets before use. Powdered molecular sieves $4 \text{ \AA}$ ($\text{MS4 \AA}$) were dried over *in vacuo* at ca. 100°C for over night after pulverizing. Acrylamide was recrystallized from benzene. Melting points were determined with a Laboratory Devices melting point apparatus or Fisher-Johns melting point apparatus and were not corrected. ^1H -NMR spectra were recorded at 270, 300, or 500 MHz with a JEOL JNM-GX270, Bruker AMX-300, or JEOL EX-500 spectrometer in chloroform-*d* or deuterium oxide. ^{13}C -NMR spectra were recorded at 67.8 or 75 MHz with the same instruments. Tetramethylsilane (TMS), methanol (3.3 ppm for ^1H or 49.0 ppm for ^{13}C), or 3-(trimethylsilyl)propanesulfonic acid sodium salt (DSS) were used as internal standards. Ring-proton assignments in NMR were made by first-order analysis of the spectra, and were supported by homonuclear decoupling experiments. Elemental analyses were performed with a Yanaco MT-3 CHNcorder or done by Galbraith Laboratories, Inc. on the samples extensively dried ca. 24 h *in vacuo*

(50°C, 0.1 Torr) over phosphorus pentoxide. Average molecular weights of the polymers were estimated by the gel permeation chromatography (GPC) method with an Asahipak GS-510 column, and pullulans (5.8, 12.2, 23.7, 48.0, 100, 186, and 300 kDa, Shodex Standard P-82) were used as standards. Reactions were monitored by thin-layer chromatography (TLC) on a precoated plate of silica gel 60F₂₅₄ (layer thickness, 0.25 mm; E. Merck, Darmstadt, Germany). The solvent systems used are (A) 2:1, (B) 1:1, (C) 1:4 (v/v) toluene-ethyl acetate, (D) 5:4:1 (v/v/v) chloroform-ethyl acetate-methanol, and (E) 65:25:4 (v/v/v) chloroform-methanol-water. For detection of the intermediates, TLC sheets were sprayed with (a) a solution of 85:10:5 (v/v/v) methanol-concd sulfuric acid-*p*-anisaldehyde, and heated for a few minutes (for carbohydrate) or (b) an aqueous solution of 5wt% potassium permanganate and heated similarly (for double bond). Column chromatography was performed on silica gel (Wakogel c-200; 100-200 mesh, Wako Pure Chemical Industries, Osaka, Japan) or (Silica Gel 60; 0.015-0.040 mm, E. Merck). Dialyses were performed against deionized water using a dialysis tubing (molecular cut off, 12,000-14,000 kDa). All extractions were concentrated below 45°C under diminished pressure. Galactosyl transferase from bovine milk (GalT; EC 2.4.1.90), uridine 5'-diphosphate galactose (UDP-Gal), and *Erythrina corallodendron* lectin (ECorL) were obtained from Sigma Co., ST Louis, MO.

***n*-Pentenyl 2,3,4,6-Tetra-*O*-acetyl- β -D-galactopyranoside (19).** A mixture of powdered MS4Å (1 g), mercuric (II) cyanide [Hg(CN)₂] (2.48 g, 9.73 mmol), and 4-penten-1-ol (1.01 mL, 9.73 mmol) in 1:1 (v/v) of benzene-nitromethane (5 mL) was stirred for 40 min. To the mixture was added **17**¹⁴ (2.0 g, 4.86 mmol) and the reaction mixture was stirred for 24 h at room temperature. The mixture was filtered and the filtrate was evaporated under diminished pressure. The residue was dissolved in chloroform, and the suspension was filtered to remove precipitates of mercuric salts. The organic layer was washed with 1.5 M sodium chloride, dried over anhydrous sodium sulfate, filtered, and evaporated *in vacuo*. The residual syrup was subjected to the Sephadex LH-20 column (ϕ 5 x 200 cm) with 95% ethanol as eluent. The fractions containing the glycoside **19** determined by TLC were combined and concentrated. The syrup was chromatographed on silica gel with 4:1 (v/v) toluene-ethyl acetate as the eluent to give pure **19** (1.80 g, 88.7%): *R*_f 0.57 (solvent A); ¹H-NMR δ (CDCl₃) 1.70 (m, 2 H, OCH₂CH₂), 1.99, 2.05, 2.06, 2.15 (each s, 12 H, 4 COCH₃), 2.11 (m, 2 H, CH₂CH=), 3.71 (m, 2 H, OCH₂), 3.9 (m, 1 H, H-5), 4.12 (dd, 1 H, *J*_{5,6a} 6.9 Hz and *J*_{6a,6b} 11.2 Hz, H-6a), 4.19 (dd, 1 H, *J*_{5,6b} 6.6 Hz, H-6b), 4.46 (d, 1 H, *J*_{1,2} 7.9 Hz, H-1), 4.96-5.05 (m, 2 H, CH=CH₂), 5.02 (dd, 1 H, *J*_{3,4} 3.3 Hz, H-3),

5.21 (dd, 1 H, $J_{2,3}$ 10.5 Hz, H-2), 5.39 (dd, 1 H, $J_{4,5}$ 0.8 Hz, H-4), and 5.72-5.86 (m, 1 H, $\text{CH}=\text{CH}_2$).

***n*-Pentenyl β -D-Galactopyranoside (10).** To a solution of **19** (1.80 g, 4.32 mmol) in methanol (20 mL) was added sodium methoxide (93.4 mg, 1.73 mmol), and the mixture was stirred for 15 h at room temperature. Dowex 50W X-8 (H^+) resin was added to neutralize the solution and the suspension was filtered, and evaporated to give a quantitative yield of carbohydrate monomer **10** (1.07 g): R_f 0.40 (solvent E); $^1\text{H-NMR}$ δ (D_2O) 1.68 (m, 2 H, OCH_2CH_2), 2.10 (m, 2 H, $\text{CH}_2\text{CH}=\text{}$), 3.8 (m, 2 H, OCH_2), 3.45 (dd, 1 H, $J_{2,3}$ 9.9 Hz, H-2), 3.59 (dd, 1 H, $J_{3,4}$ 3.4 Hz, H-3), 3.87 (m, 1 H, H-4), 4.33 (d, 1 H, $J_{1,2}$ 7.9 Hz, H-1), 4.95-5.08 (m, 2 H, $\text{CH}=\text{CH}_2$), and 5.79-5.93 (m, 1 H, $\text{CH}=\text{CH}_2$); $^{13}\text{C-NMR}$ δ (D_2O) was listed in Table 4.

Anal. Calcd. for $\text{C}_{11}\text{H}_{20}\text{O}_6 \cdot 0.9\text{H}_2\text{O}$: C, 49.95; H, 8.30. Found: C, 50.18; H, 8.59.

***n*-Pentenyl *O*-(2,3,4,6-Tetra-*O*-acetyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 4)-2,3,6-tri-*O*-acetyl- β -D-glucopyranoside (20).** A mixture of MS4Å powder (3 g), $\text{Hg}(\text{CN})_2$ (2.55 g, 10.0 mmol), and 4-penten-1-ol (1.56 mL, 15.0 mmol) in 1:1 (v/v) of benzene-nitromethane (10 mL) was stirred for 40 min. To the

suspension was added **18**¹⁴ (3.50 g, 5.00 mmol) at room temperature as described in the case for the preparation of **19**. The reaction mixture was worked up by the same manner for **19**, and the residue was purified by chromatography on silica gel with 2:1 (v/v) toluene-ethyl acetate as the eluent to afford **20** (1.42 g, 40.3%): R_f 0.51 (solvent B); $^1\text{H-NMR}$ δ (CDCl_3) 1.65 (m, 2 H, OCH_2CH_2), 1.97, 2.04, 2.05, 2.05, 2.07, 2.12, 2.16 (each s, 21 H, 7 COCH_3), 2.09 (m, 2 H, $\text{CH}_2\text{CH=}$), 3.60 (ddd, 1 H, $J_{5,6b}$ 5.0 Hz and $J_{5,6b}$ 2.0 Hz, H-5), 3.66 (m, 2 H, OCH_2), 3.80 (t, 1 H, $J_{4,5}$ 9.8 Hz, H-4), 3.88 (t, 1 H, $J_{5',6'}$ and $J_{5',6'b}$ 6 Hz, H-5'), 4.11 (m, 3 H, H-6a, 6'a, 6'b), 4.45 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.49 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1'), 4.47 (m, 1 H, H-6b), 4.89 (dd, $J_{2,3}$ 9.6 Hz, H-2), 4.95 (dd, 1 H, $J_{3',4'}$ 3.4 Hz, H-3'), 4.95-5.04 (m, 2 H, CH=CH_2), 5.11 (dd, 1 H, $J_{2',3'}$ 10.4 Hz, H-2'), 5.20 (t, 1 H, $J_{3,4}$ 9.3 Hz, H-3), 5.35 (d, $J_{4',5'}$ $\sim$ 0 Hz, H-4'), and 5.78 (m, 1 H, CH=CH_2).

***n*-Pentenyl *O*-(β -**D**-Galactopyranosyl)-(1 $\rightarrow$ 4)- β -**D**-glucopyranoside**

(12). Compound **12** was prepared from **12** (1.42 g, 2.02 mmol) by the procedure for the preparation of **10** (0.82 g, 98.9%): mp 162.5-165.5°C (from ethanol); R_f 0.38 (solvent E); $^1\text{H-NMR}$ δ (D_2O) 1.68 (m, 2 H, OCH_2CH_2), 2.09 (m, 2 H, $\text{CH}_2\text{CH=}$), 3.26 (dd, 1 H, $J_{2,3}$ 9.3 Hz, H-2), 3.49 (dd, 1 H, $J_{2',3'}$ 9.9 Hz, H-2'), 4.33 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.40 (d, 1 H, $J_{1',2'}$ 7.7 Hz, H-1'), 4.82-5.08 (m, 2

H, CH=CH₂), and 5.79-5.93 (m, 1 H, CH=CH₂); ¹³C-NMR δ (D₂O) was listed in Table 4.

Anal. Calcd. for C₁₇H₃₀O₁₁•0.8H₂O: C, 48.06; H, 7.49. Found: C, 48.24; H, 7.71.

Benzyl *O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→3)-2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (22). A solution of **21**¹⁵ (3.0 g, 7.51 mmol) in 1:1 (v/v) of benzene-nitromethane (300 mL) was evaporated to ca. 50 mL. Hg(CN)₂ (1.71 g, 6.76 mmol) and a solution of **17** (3.24 g, 7.89 mmol) in benzene (30 mL) were added to the mixture, and the reaction mixture was stirred for 8 h at 60°C. The solution was diluted by chloroform, washed with aqueous saturated sodium hydrogen bicarbonate and brine, dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. The syrup was chromatographed on silica gel eluted with 5:1~1:1 (v/v) toluene-ethyl acetate to give pure **22** (3.7 g, 67.5%): ¹H-NMR δ (CDCl₃) 1.92-2.10 (5 s, 15 H, 5 COCH₃), 4.57 and 4.74 (each d, 2 H, CH₂-Ph), 4.66 (d, 1 H, *J*_{1,2} 9.3 Hz, H-1'), 5.24 (d, 1 H, *J*_{1,2} 8.3 Hz, H-1), 5.52 (s, 1 H, benzylidene), 5.79 (d, 1 H, *J* 7.1 Hz, NH), and 7.26-7.47 (m, 10 H, aromatic).

Anal. Calcd. for C₃₆H₄₃O₁₅N₁•1H₂O: C, 57.82; H, 5.80; N, 1.87. Found: C, 58.08; H, 5.92; N, 2.01.

***O*-(2,3,4,6-Tetra-*O*-acetyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 3)-2-acetamido-1,4,6-tri-*O*-acetyl-2-deoxy-D-glucopyranose (**23**).** A mixture of **22** (300 mg, 0.411 mmol), 10% palladium-carbon (300 mg) in 2:1:1 (v/v/v) of ethanol-acetic acid-water (20 mL) was continuously stirred under hydrogen atmosphere for 9 h at 60°C. The mixture was neutralized with triethylamine until pH 7, filtered, and concentrated. The residual syrup was dissolved in pyridine (10 mL) and acetic anhydride (10 mL) at 0°C. The mixture was stirred for 12 h at room temperature. The reaction mixture was evaporated, poured into ice-water, and extracted with chloroform. The chloroform layer was successively washed with 1 N sulfuric acid, saturated aqueous sodium hydrogen bicarbonate, and brine. The solution was dried over magnesium sulfate, filtered, and evaporated. The residue was purified by silica gel chromatography with 20:1~1:0 (v/v) chloroform-methanol to give pure **23** (200 mg, 71.8%): R_f 0.50 (solvent D); $^1\text{H-NMR}$ δ (CDCl_3) 1.97-2.20 (8 s, 24 H, 8 COCH_3), 4.62 (d, 1 H, $J_{1',2'}$ 7.3 Hz, H-1'), 5.36 (d, 1 H, $J_{3'4'}$ 2.9 Hz, H-4'), 5.61 (d, 1 H, $J_{1,2}$ 9.8 Hz, H-1), and 6.07 (d, 1 H, J 3.7 Hz, NH).

Anal. Calcd. for $\text{C}_{28}\text{H}_{39}\text{O}_{18}\text{N}_1 \cdot 1\text{H}_2\text{O}$: C, 48.34; H, 5.94; N, 2.01. Found: C, 48.28; H, 5.73; N, 2.04.

***n*-Pentenyl *O*-(2,3,4,6-Tetra-*O*-acetyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 3)-2-acetamido-4,6-di-*O*-acetyl-2-deoxy- β -D-glucopyranoside (24).** A solution of **23** (440 mg, 0.649 mmol) in dichloroethane (10 mL) was treated with trimethylsilyl-trifluoromethanesulfonate (132 mL, 0.681 mmol) under nitrogen atmosphere for 5 h at 50°C. Triethylamine (272 mL, 1.95 mmol) was added to the mixture, and the solution was directly subjected to a column of silica gel with 100:200:1 (v/v/v) toluene-ethyl acetate-triethylamine as an eluent to give an oxazoline derivative (290 mg, 70.3%): R_f 0.56 (solvent D).

A solution of the freshly prepared oxazoline derivative (290 mg, 0.456 mmol) in dichloroethane (5 mL) was allowed to react with 4-penten-1-ol (141 μ L, 1.37 mmol) in the presence of 10-camphorsulfonic acid-*d* (30 mg) under a nitrogen atmosphere for 5 h at 90°C. The solution was cooled and diluted with chloroform, and the organic solution was washed with saturated aqueous hydrogen bicarbonate and brine, dried over anhydrous magnesium sulfate, filtered, and evaporated *in vacuo*. The residue was purified by silica gel chromatography with 100:1~1:0 (v/v) chloroform-methanol to give **24** (337 mg, 73.8%): $^1\text{H-NMR}$ δ (CDCl_3) 1.67 (m, 2 H, OCH_2CH_2), 1.97-2.15 (7 s, 21 H, 7 COCH_3), 4.52 (d, 1 H, $J_{1',2'}$ 7.3 Hz, H-1'), 4.58 (d, 1 H, $J_{1,2}$ 7.6 Hz, H-1).

Anal. Calcd. for $\text{C}_{31}\text{H}_{45}\text{O}_{17}\text{N}_1 \cdot 1\text{H}_2\text{O}$: C, 51.59; H, 6.56; N, 1.98. Found: C, 50.94; H, 6.22; N, 1.98.

***n*-Pentenyl *O*-(β -D-Galactopyranosyl)-(1 $\rightarrow$ 3)-2-acetamido-2-deoxy- β -D-glucopyranoside (13).** White solid of **13** was prepared from **24** (240 mg, 0.341 mmol) by the procedure for the preparation of **10** (110 mg, 71.4%): mp 238°C (Dec.) (from ethanol); ^{13}C -NMR δ (D_2O) was listed in Table 4.

Anal. Calcd. for $\text{C}_{19}\text{H}_{33}\text{O}_{11}\text{N}_1 \cdot 1\text{H}_2\text{O}$: C, 48.61; H, 7.51; N, 2.98. Found: C, 48.54; H, 7.36; N, 2.92.

***n*-Pentenyl 2-Acetamido-3,4-di-*O*-acetyl-2-deoxy-6-*O*-triphenylmethyl- β -D-glucopyranoside (25).** A solution of **11** (4.9 g, 16.9 mmol) in pyridine (50 mL) was added with triphenylmethyl chloride (7.08 g, 25.4 mmol) and the mixture was stirred under nitrogen atmosphere for 40 min at 90°C. The mixture was cooled to room temperature, and added with acetic anhydride (16.5 mL, 0.17 mol). After 3 h, the reaction mixture was evaporated and poured into ice-water. The mixture was extracted with chloroform, and worked up as the same manner described for **23**. The residue was applied to a silica gel column with 1:0~20:1 (v/v) chloroform-ethanol as eluent to give white powdery **25** (8.2 g, 80.4%) which was recrystallized from ethanol: mp 178-179°C; ^1H -NMR δ (CDCl_3) 1.72 (m, 2 H, OCH_2CH_2), 1.69, 1.94, 2.00 (each s, 9 H, 3 COCH_3), 2.16 (m, 2 H, $\text{CH}_2\text{CH=}$), 3.59 (m, 2 H, H-6a, 6b), 3.93 (m, 2 H,

OCH₂), 4.64 (d, 1 H, $J_{1,2}$ 8.3 Hz, H-1), 4.97 (t, 1 H $J_{4,5}$ 11 Hz, H-4), 5.14 (m, 2 H, CH=CH₂), 5.17 (t, 1 H, $J_{3,4}$ 9.0 Hz, H-3), 5.84 (m, 2 H, NH and CH=CH₂), and 7.17-7.46 (m, 15 H, 3 Ph).

Anal. Calcd. for C₃₅H₄₁O₈N₁: C, 69.63; H, 6.84; N, 2.32. Found: C, 69.48; H, 6.71; N, 2.24.

***n*-Pentenyl 2-Acetamido-3,4-di-*O*-acetyl-2-deoxy- β -D-glucopyranoside (26).** Compound **25** (1.7 g, 2.82 mmol) was treated with a solution of 80% aqueous acetic acid (25 mL) for 1 h at 90°C. The solution was evaporated, and the residue was directly purified by silica gel chromatography with 1:2 (v/v) toluene-ethyl acetate as eluent to give **26** (0.8 g, 76.2%): mp 155-156°C (from ethyl acetate-hexane); ¹H-NMR δ (CDCl₃) 1.67 (m, 2 H, OCH₂CH₂), 1.96, 2.04, 2.05 (each s, 9 H, 3 COCH₃), 2.10 (m, 2 H, CH₂CH=), 2.38 (br s, 1 H, OH-6), 3.52 (m, 1 H, H-5), 3.60 (br dd, 1 H, $J_{5,6a}$ <1, H-6a), 3.70 (m, 2 H, OCH₂), 3.74 (br d, 1 H, $J_{5,6b}$ 13 Hz, H-6b), 3.88 (m, 1 H, H-2), 4.68 (d, 1 H, $J_{1,2}$ 8.4 Hz, H-1), 5.0 (m, 3 H, H-4, CH=CH₂), 5.33 (t, 1 H, $J_{2,3}$ 10.6 Hz and $J_{3,4}$ 9.4 Hz, H-3), 5.79 (m, 1H, CH=CH₂), and 5.68 (d, 1 H, J 8.7 Hz, NH).

Anal. Calcd. for C₁₇H₂₇O₈N₁: C, 54.68; H, 7.29; N, 3.75. Found: C, 54.46; H, 7.29; N, 3.71.

***n*-Pentenyl *O*-(2,3,4,6-Tetra-*O*-acetyl- β -D-galactopyranosyl)-(1 $\rightarrow$ 6)-2-acetamido-3,4-di-*O*-acetyl-2-deoxy- β -D-glucopyranoside (27).** A mixture of Hg(CN)₂ (1.89 g, 7.39 mmol), powdered MS4Å (3 g), and **26** (1.38 g, 3.70 mmol) in 10 mL of 1:1 (v/v) benzene-nitromethane was stirred for 40 min at room temperature. Glycosyl donor **17** (1.01 g, 2.46 mmol) was added to the mixture, and the mixture was stirred for 3 days at room temperature. The reaction mixture was filtered, evaporated, and the residue was dissolved in chloroform, then filtered again to remove mercuric salts. The work-up procedure was similar as described for **19**. The product was purified by column chromatography on silica gel with 1:3 (v/v) toluene-ethyl acetate as eluent to give **27** (0.37 g, 21.4%): *R*_f 0.34 (solvent C); mp 122-123°C (from ethyl acetate-hexane); ¹H-NMR δ (CDCl₃) 1.69 (m, 2 H, OCH₂CH₂), 1.95, 1.98, 2.03, 2.03, 2.06, 2.06, 2.15 (each s, 21 H, 7 COCH₃), 2.13 (m, 2 H, CH₂CH=), 3.61 (dd, 1 H, *J*_{6a,6b} 10.5 Hz, H-6a), 3.65 (m, 2 H, OCH₂), 3.69 (m, 1 H, *J*_{5,6b} 7.4 Hz and *J*_{5,6b} 2 Hz, H-5), 3.79-3.92 (m, 2 H, H-6b, 5'), 3.82 (m, 1 H, *J*_{2,3} 10.6 Hz, H-2), 4.21 (m, 2 H, H-6'a, 6'b), 4.56 (d, 1 H, *J*_{1',2'} 8.0 Hz, H-1'), 4.60 (d, 1 H, *J*_{1,2} 8.3 Hz, H-1), 4.87 (t, *J*_{4,5} 9.5 Hz, H-4), 4.99 (dd, 1 H, *J*_{3',4'} 3.4 Hz, H-3'), 4.95-5.05 (m, 2 H, CH=CH₂), 5.21 (dd, 1 H, *J*_{2',3'} 10.5 Hz, H-2'), 5.25 (dd, 1 H, *J*_{3,4} 9.3 Hz, H-3), 5.39 (d, 1 H, *J*_{4',5'} 0.9 Hz, H-4'), 5.53 (d, 1 H, *J* 8.8 Hz, NH), and 5.80 (m, 1 H, CH=CH₂).

Anal. Calcd. for $C_{31}H_{45}O_{17}N_1 \cdot 0.5H_2O$: C, 52.25; H, 6.50; N, 1.97.

Found: C, 52.31; H, 6.54; N, 1.82.

***n*-Pentenyl *O*-(β -D-galactopyranosyl)-(1 $\rightarrow$ 6)-2-acetamido-2-deoxy- β -D-glucopyranoside (14).** Compound **14** as white solids was prepared from **27** (133.3 mg, 0.189 mmol) by the method for preparation of **10** (81.5 mg, 95.3%): R_f 0.17 (solvent E); mp 205-208°C (from methanol); 1H -NMR δ (D_2O) 1.60 (m, 2 H, OCH_2CH_2), 1.99 (s, 3 H, $COCH_3$), 2.02 (m, 2 H, $CH_2CH=$), 4.18 (d, 1 H, J 11.4 Hz, H-6b), 4.40 (d, 1 H, $J_{1,2}$ 7.6 Hz, H-1), 4.47 (d, 1 H, $J_{1',2'}$ 8.3 Hz, H-1'), 4.99 (m, 2 H, $CH=CH_2$), and 5.83 (m, 1 H, $CH=CH_2$); ^{13}C -NMR δ (D_2O) was listed in Table 4.

Anal. Calcd. for $C_{19}H_{33}O_{11}N_1 \cdot 1.5H_2O$: C, 47.70; H, 7.58; N, 2.93.

Found: C, 47.89; H, 7.35; N, 2.88.

Copolymerization of Carbohydrate Monomers with Acrylamide. A solution of a carbohydrate monomer (100 mg) and 4 or 10 equivalent molar of acrylamide in 1.0 mL of deionized water was degassed for a while using water pump, to which were added TEMED (0.1 equivalent molar of the carbohydrate monomer) and APS (0.04 equivalent molar of the carbohydrate monomer). The reaction mixture was continuously stirred for 2 h at room temperature, diluted

with 0.1 M acetic acid-pyridine buffer (pH 4.9), dialyzed against deionized water, and lyophilized to give water soluble copolymer as white soft powder. The ^{13}C -NMR data were shown in Table 4, and the physical data are shown in Table 5.

Enzymatic Galactosylation of A Polymer Having *N*-Acetyl-D-glucosamine.¹⁵ Acceptor glycopolymer derived from GlcNAc **11** (10.0 mg, 12 μmol of GlcNAc), UDP-Gal (9.63 mg, 17 μmol), α -lactalbumine (100 μg), and GalT (1.0 unit) were incubated in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (HEPES) (pH 6.0) for 24 h at 37°C. The mixture was directly applied to a column of the Sephadex G-50 (ϕ 2.5 x 100 cm) with 50 mM ammonium acetate as the eluent. The fractions containing glycopolymer were collected and freeze-dried to give white powdery polymer having *N*-acetylglucosamine (11.8 mg) in quantitative yield: ^1H -NMR δ (D_2O) 1.6 and 1.7 (br m, CH_2), 2.01 (s, 3 H, COCH_3), 2.2 and 2.3 (br m, CH), 3.52 (m, 1 H, H-2'), 3.55 (m, 1 H, H-5), 3.64 (d, 1H, H-3), 3.7 (m, 8 H, OCH_2 , H-2, -3, -4, -5', -6'a, and -6'b), 3.83 (m, 1H, H-6a), 3.91 (d, 1 H, H-4'), 3.97 (br d, 1 H, H-6b), 4.43 (d, 1 H, H-1), 4.48 (d, 1 H, H-1').

High Performance Anion Exchange Chromatography with Pulsed Amperometry Detection (HPAEC-PAD) analysis. Acid hydrolysis of the polymer was carried out under 2 M trifluoroacetic acid at 100°C for 2 h.²⁶ After the hydrolysis reaction, the solution was filtered to remove the main polymer chain, and the filtrate was successively subjected to the HPAEC-PAD analysis. HPAEC was carried out using Dionex BioLC (Sunnyvale, CA) equipped with a Carbopak PA-1 column and a pulsed amperometric detector (PAD-II). The quantitative analysis was carried out using a standard solution of monosaccharides of 50 mM solution respectively. The resulting pattern is showing in Figure 8C.

Effects of Glycopolymers on Hemagglutination by ECorL. The inhibitory activity of the synthetic polymers was tested in 0.05 M phosphate buffered saline (PBS; pH 7.3) using a fresh suspension [ca. 4% (v/v)] of human type-B red blood cells. The human blood cells were treated by heparin to avoid aggregation by themselves and used without further purification. The concentration was estimated to be 2.0 μ M from Abs. at 280 nm, using the extinction coefficient $A_{1\text{ cm}}$ 1.53. The procedure was as follows: Various concentrations of the test polymeric inhibitors (10 μ L) were added to a suspension of the human type-B red blood cells (10 μ L), PBS (30 μ L), and the ECorL solution (10 μ L). The mixture was incubated for a few hours at room temperature before visual examination of

hemagglutination activity. The minimum concentration of inhibitor required to inhibit the aggregation could be estimated by testing a dilution series of each test ligand.

3.4. Conclusion

We have systematically synthesized LacNAc-related oligosaccharides having *n*-pentenyl group as a polymerizable aglycon, and these glycosides were smoothly converted to the water soluble copolymers as the simple and convenient polymeric sugar ligands related to lactosaminoglycan models. The enzymatic elongation of sugar chain in the GlcNAc-branching type polymer has been also demonstrated using GalT and UDP-Gal to give a quantitative yield of LacNAc-branching type polymer. It was suggested that these glycopolymer ligands are new type of sugar cluster ligands for lectins to discuss the binding specificity between sugar and sugar-binding proteins. This method will be applied for creating further complex glycoprotein mimetics and ligands such as sialic acid containing structures and sulfated oligosaccharides.

3.5. References

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Chapter 4

Glycolipid Models: A Novel Bi-Fluorescence-Labeled Substrate for Ceramide Glycanase Based on Fluorescence Energy Transfer

An alkyl lactoside containing two different fluorescence probes as an energy donor and an energy acceptor was synthesized as a substrate for ceramide glycanase. An *n*-pentenyl lactoside was converted to its 4',6'-(2-naphthylmethylidene)-derivative, followed by benzylation of all remaining OH groups. The fully protected lactoside was treated with borane-trimethylamine complex and aluminum chloride in tetrahydrofuran [Garegg, P. J. (1984) *Pure & Appl. Chem.* 56, 845-858] for selective opening of the 4',6'-acetal group to give the 6'-(2-naphthylmethyl) derivative in high yield. After de-*O*-benzylation, the aglycon chain was extended by Michael addition with HS(CH₂)₂NH₂·HCl. The amino group of the lactoside was dansylated to give the target lactoside, which has two different fluorescent probes at each end. Excitation at 260 nm (of 2-naphthyl group) of the bi-fluorescence-labeled lactoside showed emissions at 335 nm (2-naphthyl) and at 540 nm (dansyl). The distance between the naphthyl group and the dansyl group was estimated to be 12 Å by the Förster relationship.

Digestion of this lactoside with leech (*Macrobdella decora*) ceramide glycanase resulted in an increase in the naphthyl emission with a concomitant decrease in the dansyl emission. These changes can be used for continuous monitoring of the ceramide glycanase activity. The K_M and V_{max} of the leech ceramide glycanase was estimated to be 7.7×10^{-6} M and 0.16 nmol/min, respectively, using this lactoside as a substrate.

4.1. Introduction

The value of resonance energy transfer using a suitable pair of fluorescent probes for measurement of distances in biomolecules has been demonstrated by Stryer and Haugland.¹ When the donor fluorophore is excited, the emitted light is absorbed by the second fluorophore, which in turn fluoresces at a different wavelength. The efficiency of intermolecular resonance energy transfer is strongly dependent on both the distance between donor and acceptor, and the spectral overlap of the wavelength of the fluorescent emission of the donor with the absorption of the acceptor. Fluorescence energy transfer, most suitable for the distance range of 10–60 Å, has been applied to proteins, oligonucleotides, and lipids.^{2,3} Recently Rice et al.⁴⁻⁶ have utilized fluorescence energy transfer to determine solution conformations of the complex type glycopeptides.

Fluorescently labeled substrates for proteases have been used to study the kinetics of rennin by Matayoshi et al.⁷⁻⁹ The substrates used were octa to decapeptide labeled, for example, with 5-[(2'-aminoethyl)amino]naphthalenesulfonyl group (EDANS) at *C*-terminus and with 4-{[4'-(dimethylamino)phenyl]azo}benzoyl group (DABCYL) at *N*-terminus. When these groups co-exist on a peptide, the DABCYL group effectively quenches the fluorescence emission by the EDANS group. As the enzyme cleaves the

substrate, this quenching is removed and the emission by the EDANS group is restored. These substrates have proved to be very useful for the assay of endopeptidases.

Ceramide glycanase (CGase) is an enzyme which cleaves the glycosidic linkage between oligosaccharide and ceramide in the ceramide glycosides (glycosphingolipids). A number of ceramide glycanases have been isolated from the European leech *Hirudo medicinalis*,¹⁰ the American leech *Macrobdella decora*,^{11, 12} earthworm *Lumbricus terrestris*,¹³ *Rhodococcus* sp.,^{14, 15} and *Corynebacterium* sp..¹⁶ The leech CGase can cleave a wide variety of glycosphingolipids (GSL) as well as a number of synthetic β -lactosides.¹¹ Zhou et al. found that CGase require the presence of a detergent such as sodium cholate for effective hydrolysis of GSL.¹¹ The enzyme is quite stable between pH 4.5 and 8.5, and the optimum pH of the enzyme was 5.0 when G_{M1} was used as substrate, for which the K_M was estimated to be 15.4 μ M. Unlike the assays for the exoglycosidases, for which chromogenic or fluorogenic substrates are available, the assay for CGases (and other endo-type carbohydrates) is by means of separation of the products followed by quantification.^{11, 15} According to an assay by Zhou et al., for example, the typical assay requires 0.2 unit of the enzyme and 30 nmol of GSL, and is not convenient for a large number of samples nor is it amenable to continuous monitoring of the enzyme activity.¹¹

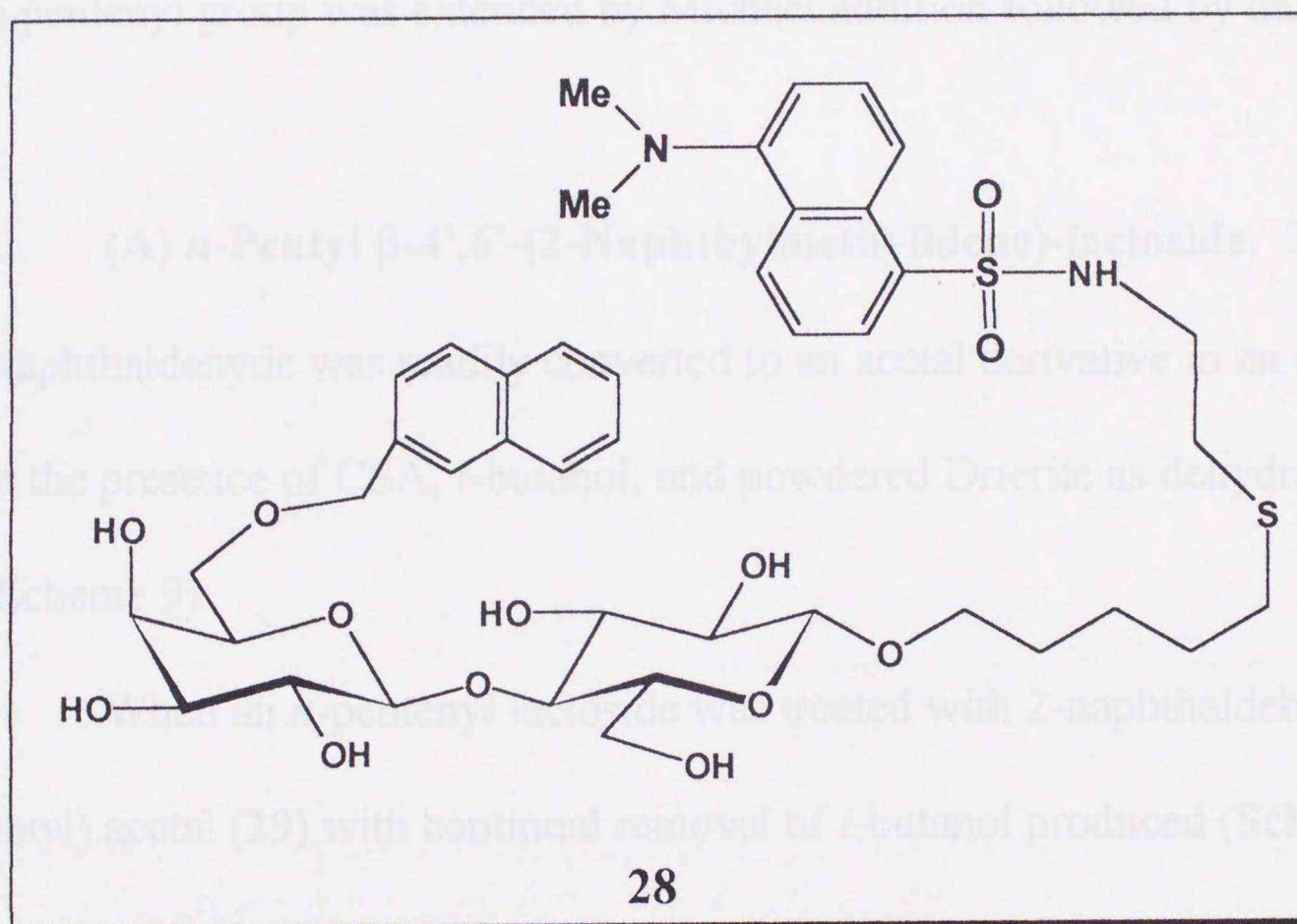


Figure 9. Structure of NLD 28.

4.2. Results and Discussion

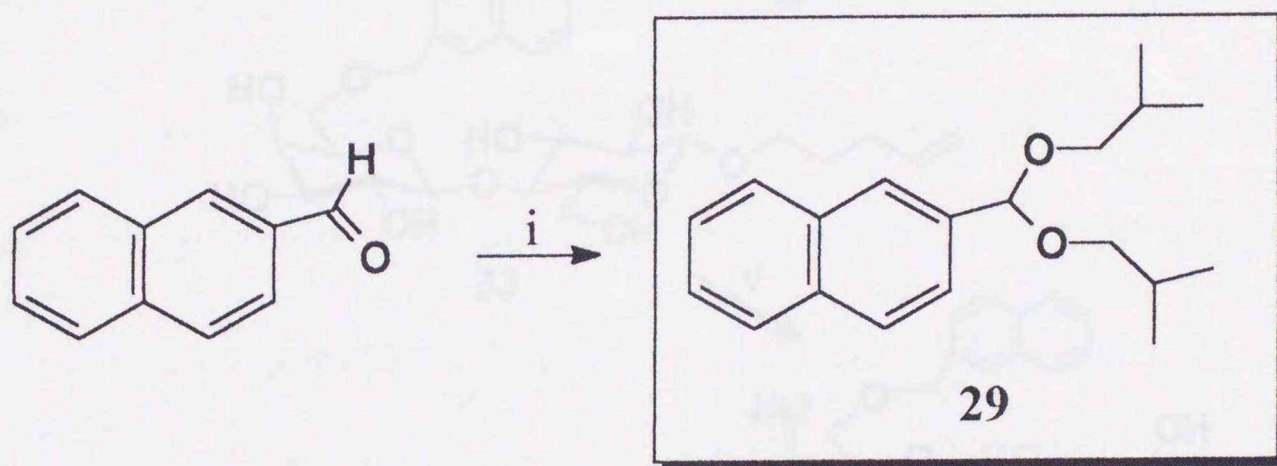
Synthesis of Bi-fluorescence-Labeled Lactoside. *n*-Pentenyl β -lactoside¹⁷ was modified with 2-naphthaldehyde at the galactosyl residue, and the *n*-pentenyl group was extended by Michael addition followed by dansylation.

(A) *n*-Pentyl β -4',6'-(2-Naphthylmethylidene)-lactoside. 2-

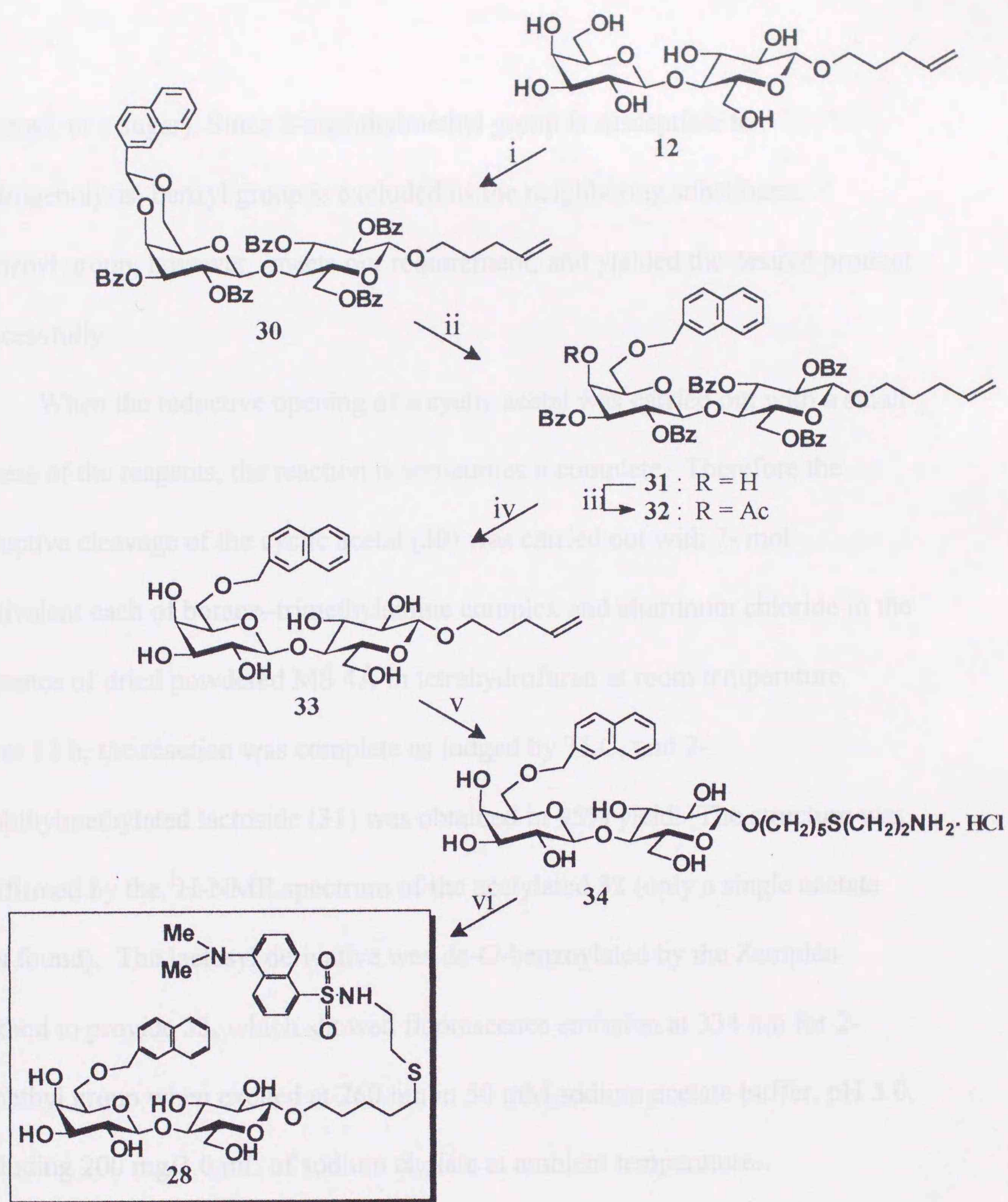
Naphthaldehyde was readily converted to an acetal derivative in an excellent yield in the presence of CSA, *i*-butanol, and powdered Drierite as dehydrating agent (Scheme 9).

When an *n*-pentenyl lactoside was treated with 2-naphthaldehyde di-(*i*-butyl) acetal (**29**) with continual removal of *i*-butanol produced (Scheme 10) in the presence of CSA as an acid catalyst, the acetal exchange reaction proceeded smoothly. The cyclic acetal derived from the lactoside was purified with a Sephadex LH-20 column, and the acetal was successively benzoylated to give **30** in 55% yield.

(B) *n*-Pentenyl β -6'-(2-naphthylmethyl)-lactoside. The regioselective ring opening reaction of a cyclic acetal reported by Garegg¹⁸ requires that the substituent adjacent to the cyclic acetal must be a bulky group (e.g., benzyl,



Scheme 9. Reagents and condition: i) *i*-Buthanol, powdered Drierite, 10-camphorsulfonic acid (CSA), r.t., 2 days.



Scheme 10. Reagents and conditions: ia) **29**, CSA, DMF, reduced pressure, 55°C, 2 h; ib) Bz-Cl, pyridine, 0°C to r.t., 3 h; ii) borane-triethylamine complex, MS 4Å, AlCl₃, THF, r.t., 11 h; iii) Ac₂O, pyridine, r.t.; iv) NaOMe, MeOH; v) HSCH₂CH₂NH₂·HCl, MeOH, uv irradiation, r.t., 3 days; vi) Et₃N, dansyl-Cl, MeOH, r.t., 30 min.

benzoyl, or a sugar). Since 2-naphthylmethyl group is susceptible to hydrogenolysis, benzyl group is excluded as the neighboring substituent.

Benzoyl group, however, meets our requirement, and yielded the desired product successfully.

When the reductive opening of a cyclic acetal was carried out with a small excess of the reagents, the reaction is sometimes incomplete. Therefore the reductive cleavage of the cyclic acetal (**30**) was carried out with 7- mol equivalent each of borane-trimethylamine complex and aluminum chloride in the presence of dried powdered MS 4Å in tetrahydrofuran at room temperature.

After 11 h, the reaction was complete as judged by TLC, and 2-naphthylmethylated lactoside (**31**) was obtained in 95% yield. The structure was confirmed by the ¹H-NMR spectrum of the acetylated **32** (only a single acetate was found). The lactosyl derivative was de-*O*-benzoylated by the Zemplén method to provide **33**, which showed fluorescence emission at 334 nm for 2-naphthyl group when excited at 260 nm in 50 mM sodium acetate buffer, pH 5.0, including 200 mg/3.0 mL of sodium cholate at ambient temperature.

(C) The Synthesis of Bi-Fluorescence Labeled Lactoside. *n*-Pentenyl glycosides are versatile intermediates in carbohydrate chemistry.¹⁹ They can be used as glycosylating agents in different ways, and the ω-alkene group in their

aglycon is amenable to modification such as oxidation (producing an aldehyde), gem-hydroxylation (for improved aq. solubility), and Michael addition (for extension of the aglycon chain). The Michael addition of cysteamine thiol to a C=C has been accomplished,^{20, 21} providing a thioether product in an anti-Markovnikov manner. Using an analogous reaction, the lactosyl derivative **33** was extended with cysteamine by the Michael addition reaction to the ω -alkene group to afford **34** in 66% yield. Dansylation of the amino group was carried out in dry methanol at room temperature to give **28** in 91% yield.

Physical Properties of NLD. Uv-absorption spectrum of NLD showed the presence of peaks at 260 nm and 335 nm, as expected from the presence of the 2-naphthyl group and the dansyl group. The NLD having bi-fluorescence probes at the each terminal was tested as a function of excitation wavelength from 260 to 300 nm and the emission spectra of 5 μ M solution of NLD are shown in Figure 10. These emission spectra included two peaks due to 2-naphthyl group at around 335 nm and dansyl group at 540 nm because the fluorescence energy transfer was observed from 2-naphthyl as the energy donor to dansyl as the acceptor. The relative fluorescence intensity of emission peaks excited at 260 nm were found to be the most effectively enhanced among all wavelengths tested. Therefore, we selected this excitation wavelength for further enzyme reaction.

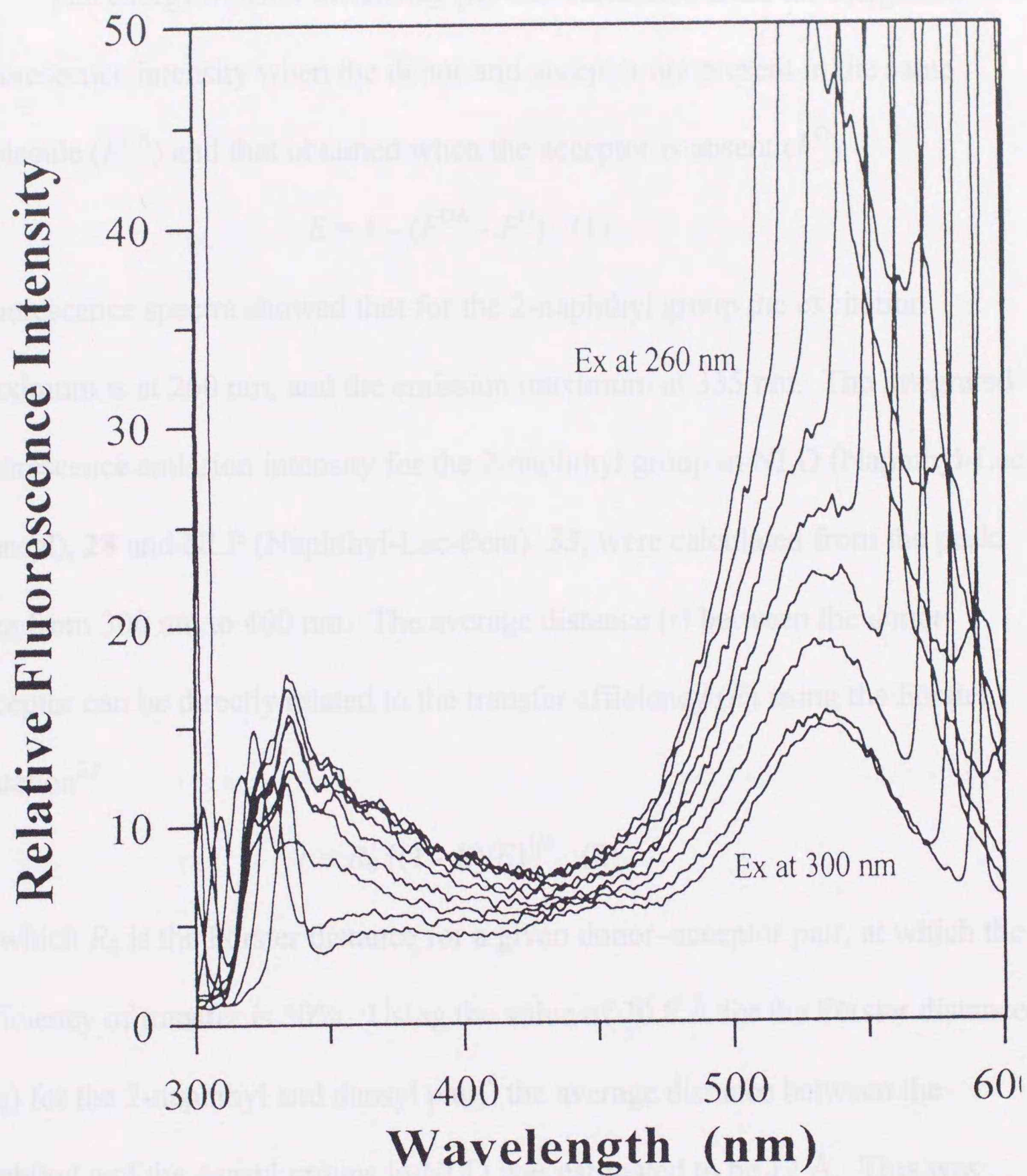


Figure 10. Dependence of excitation wavelength of NLD on the emission spectra. The conditions were as follows: ambient temperature, 3.0 mL of 50 mM sodium acetate, pH 5.0, containing 200 μ g of sodium cholate. Slit size of 5.0 nm was used for both the excitation and emission. The spectra were scanned at 500 nm/min.

The energy transfer efficiency (E) was calculated from the integrated fluorescence intensity when the donor and acceptor are present in the same molecule (F^{DA}) and that obtained when the acceptor is absent (F^D):

$$E = 1 - (F^{DA} - F^D) \quad (1)$$

Fluorescence spectra showed that for the 2-naphthyl group the excitation maximum is at 260 nm, and the emission maximum at 335 nm. The integrated fluorescence emission intensity for the 2-naphthyl group in NLD (Naphthyl-Lac-Dansyl), **28** and NLP (Naphthyl-Lac-Pent), **33**, were calculated from the peak area from 300 nm to 400 nm. The average distance (r) between the donor-acceptor can be directly related to the transfer efficiency (E) using the Förster equation²²

$$r = R_0 [(1 - E)/E]^{1/6} \quad (2)$$

in which R_0 is the Förster distance for a given donor-acceptor pair, at which the efficiency of transfer is 50%. Using the value of 20.8 Å for the Förster distance (R_0) for the 2-naphthyl and dansyl pair,⁴ the average distance between the naphthyl and the dansyl groups in NLD was estimated to be 12 Å. This was shorter than the value calculated when the glycoside is fully extended.

Digestion of NLD by The CGase. NLD and G_{M1} were separately incubated with 10 µL of CGase (0.29 U) in 200 µL of 50 mM sodium acetate

buffer, pH 5.0, at 37°C for 17 has performed by Zhou et al.¹¹ The TLC showed that both G_{M1} and NLD were completely hydrolyzed. The released 2-naphthylmethylated lactose (NL) had R_F 0.44, and the other product 5-(2-dansylamido-ethanethio)-pentanol had R_F 0.94 in the solvent (c). Zhou et al. (Zhou et al., 1989) tested many ceramide glycoside substrates, including *Al*-Lac-Cer (the lactosyl-ceramide in which the primary alcohol (6'-OH) of the galactose residue was converted to an aldehyde group). Hydrolysis of *Al*-Lac-Cer was only 5% of Lac-Cer, and they suggested that the galactose residue in the Lac-Cer was necessary to interact with the enzyme. Our results showed that NLD in which the 6' position of the galactose is substituted by a hydrophobic and bulky 2-naphthylmethyl group could be cleaved by ceramide glycanase as readily as G_{M1} , showing the unimportance of the free 6'-OH group.

Time Course of Enzymatic Reaction using NLD as substrate.

Digestion of NLD was carried out in the cuvette at ambient temperature using the following condition: A 3.0 mL solution of 10 μ M in NLD containing 200 μ g of sodium cholate was made fresh. To the solution (3.0 mL) placed in the cuvette was added CGase (40 μ L, 1.14 U) and mixed quickly. An emission spectrum was taken immediately after the enzyme addition and this was regarded

as the 0 min spectrum. The spectra taken at proper intervals (up to 240 min.) are shown in Figure 11.

The emission spectra of the reaction mixture shown in Figure 11 were taken by excitation at 260 nm, which also showed a double scattered peak at around 520 nm. As expected, the enzymic digestion caused the decrease in the dansyl emission by diminishing the energy transfer from the 2-naphthyl group. It also caused an increase in the 2-naphthyl emission by removing the quenching by dansyl group. The 2-naphthyl emission at 334.5 nm gradually increased up to 90 min, after which, the reaction tapered off until it had almost stopped at 210 min. The decreases in the dansyl emission at 540.3 nm as the reaction progressed were also measured. The inset in Figure 11 shows the time course of the formation of the product (○), 2-naphthymethylated lactose (NL), determined from the increase of the 2-naphthyl emission at 334.5 nm, and that of hydrolysis of NLD (●) determined by the dansyl emission at 540.3 nm. The concentrations of the NL were calibrated from a standard curve of the emission of **33** (2-Naphthyl-Lac-Pent or NLP) under the same spectrometric conditions.

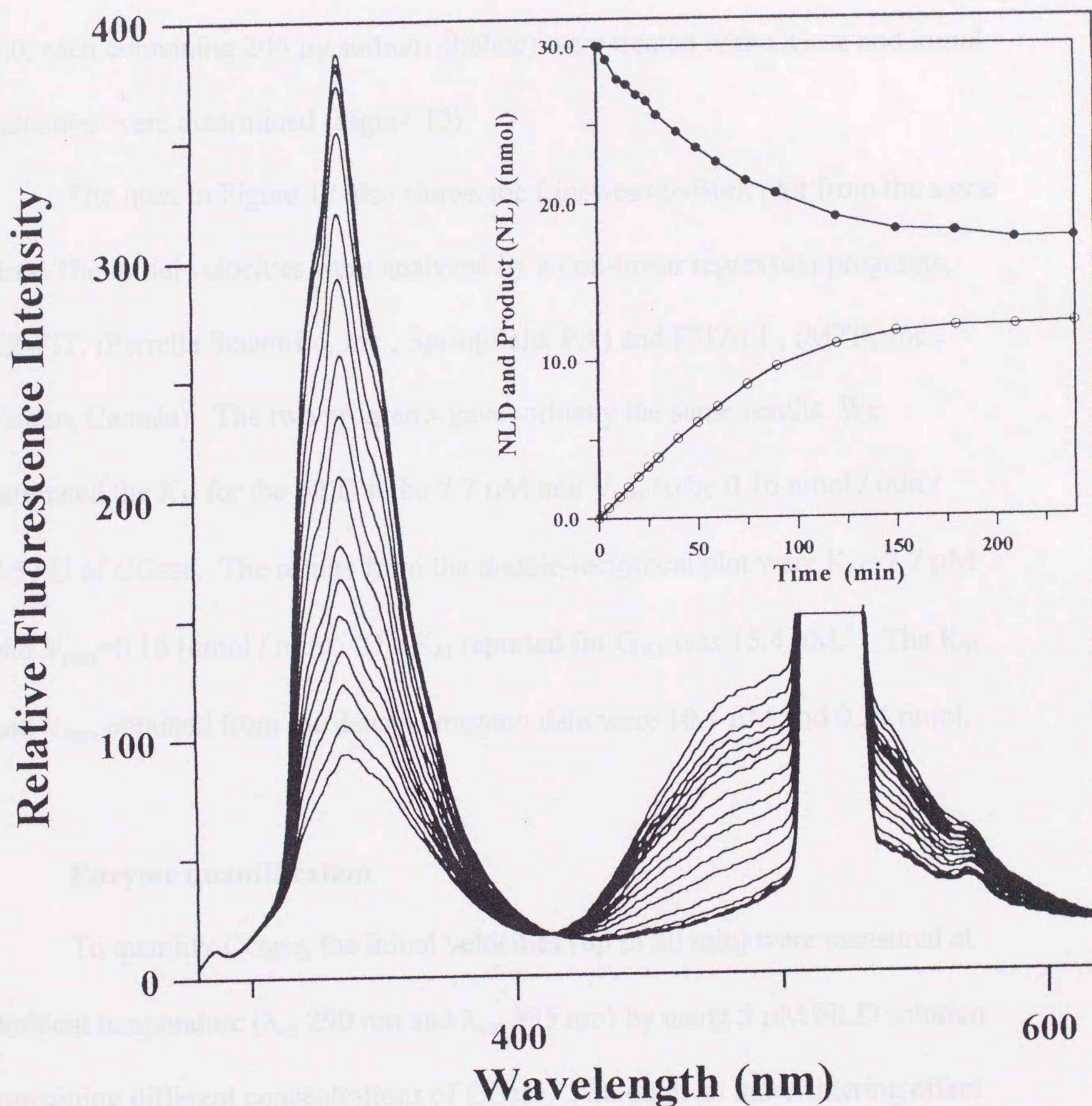


Figure 11. The time course (1.5, 5, 10.5, 15, 20, 25, 30, 40, 50, 60, 75, 90, 120, 150, 180, 210, and 240 min) of the relative fluorescence emission of NLD during hydrolysis with CGase. The reaction conditions were as follows: ambient temperature, 3.0 mL of 50 mM sodium acetate, pH 5.0, containing 200 μ g of sodium cholate, 10 μ M of the NLD (30 nmol/3.0 mL), 1.14 U of the CGase. Slit size of 5.0 nm was used for both the excitation and emission. The spectra were scanned at 500 nm/min exciting at 260 nm for the 2-naphthyl group. The inset shows the result of the time course expressed as the concentrations of the product (NL) and the remaining NLD.

Enzyme Kinetics. A series of freshly prepared solutions (3.0 mL) containing different concentrations of NLD in 50 mM sodium acetate buffer (pH 5.0, each containing 200 μ g sodium cholate) were treated with CGase and initial velocities were determined (Figure 12).

The inset in Figure 12 also shows the Lineweaver-Burk plot from the same data. The initial velocities were analyzed by a non-linear regression programs, EZ-FIT, (Perrella Scientific, Inc., Springfield, PA) and FITALL, (MTR, Inc., Tronto, Canada). The two programs gave virtually the same results. We estimated the K_M for the NLD to be 7.7 μ M and V_{max} to be 0.16 nmol / min / 0.57 U of CGase. The results from the double-reciprocal plot were $K_M=7.7 \mu$ M and $V_{max}=0.16$ (nmol / min). The K_M reported for G_{M1} was 15.4 μ M.¹¹ The K_M and V_{max} obtained from the dansyl emission data were 10.1 μ M and 0.24 nmol.

Enzyme quantification

To quantify CGase, the initial velocities (up to 20 min) were measured at ambient temperature (λ_{ex} 290 nm and λ_{em} 335 nm) by using 5 μ M NLD solution containing different concentrations of CGase. Although an inner filtering effect was negligible at this substrate concentration, measurements were carried out at the non-maximal excitation wavelength to avoid overlapping of the second scattering peak with the dansyl emission peak. As shown in Figure 13, the initial

velocities were proportional to the amount of CGase added.

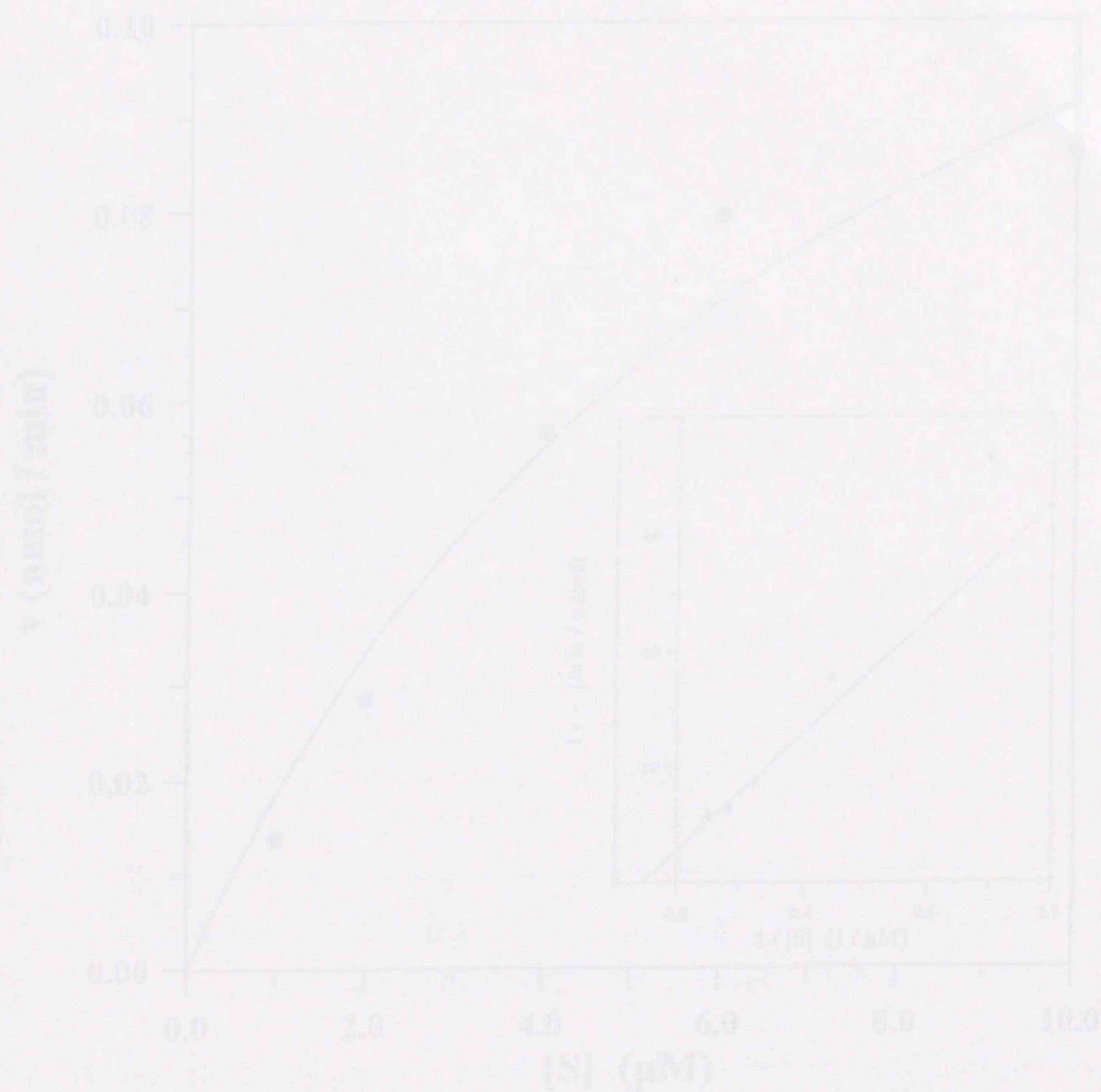


Figure 12. The initial rates of hydrolysis by CGase as a function of NLD concentration. The reaction conditions were as follows: ambient temperature, 3.0 mL of 50 mM sodium acetate, pH 5.0, containing 200 μg of sodium cholate, 0.57 U of CGase, 500 μL of 5.0 mM NLD, and 500 μL of 0.1 M sodium acetate. The spectra were recorded at 500 nm, and the excitation wavelength was 280 nm. The inset figure shows the Lineweaver-Burk plot.

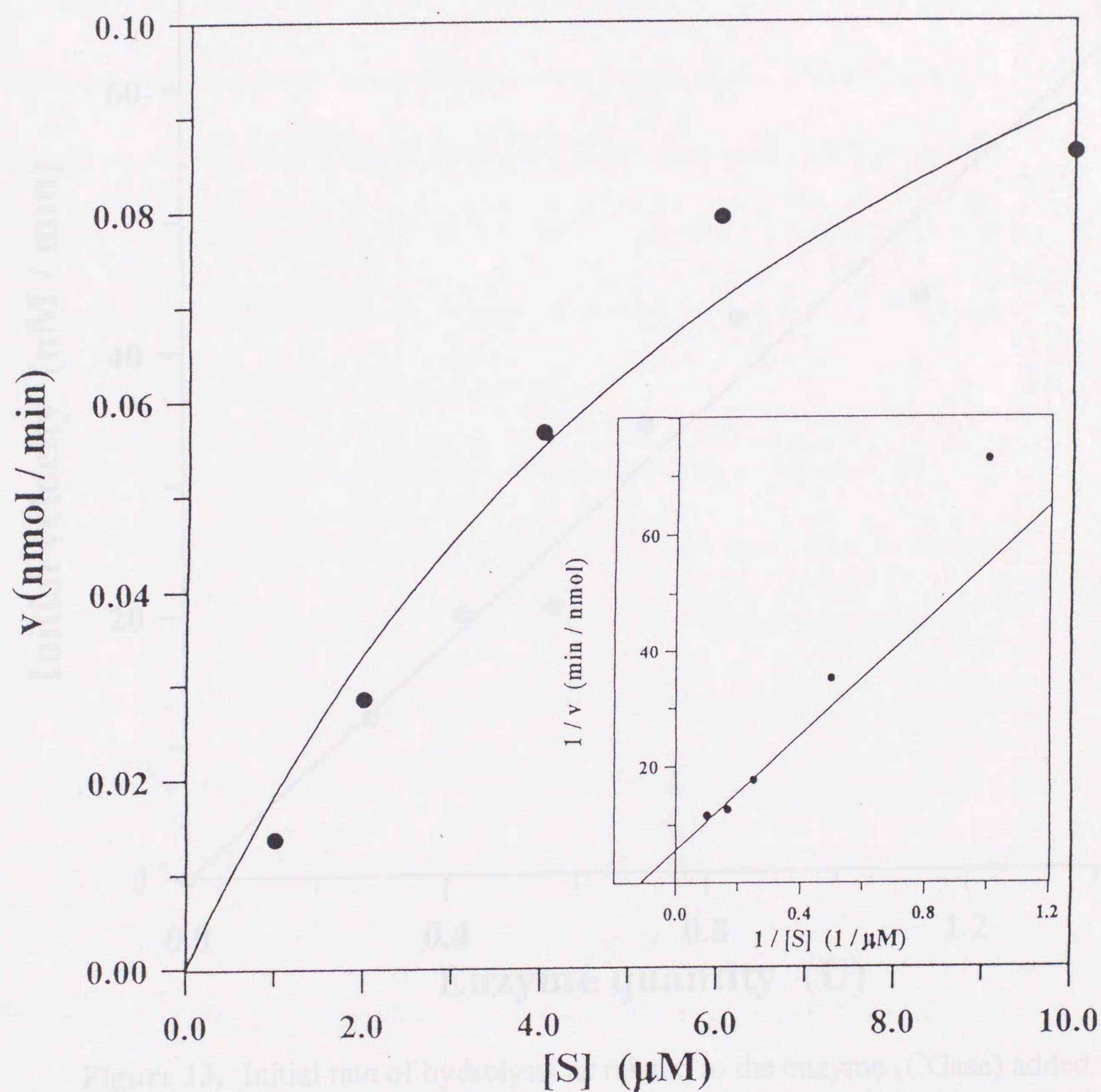


Figure 12. The initial rates of hydrolysis by CGase as a function of NLD concentration. The reaction conditions were as follows: ambient temperature, 3.0 mL of 50 mM sodium acetate, pH 5.0, containing 200 μg of sodium cholate, appropriate amount of NLD, 0.57 U of CGase. Slit size of 5.0 nm was used for both the excitation and emission. The spectra was scanned at 500 nm/min exciting at 260 nm for the 2-naphthyl group. The inset figure shows the Lineweaver-Burk plot.

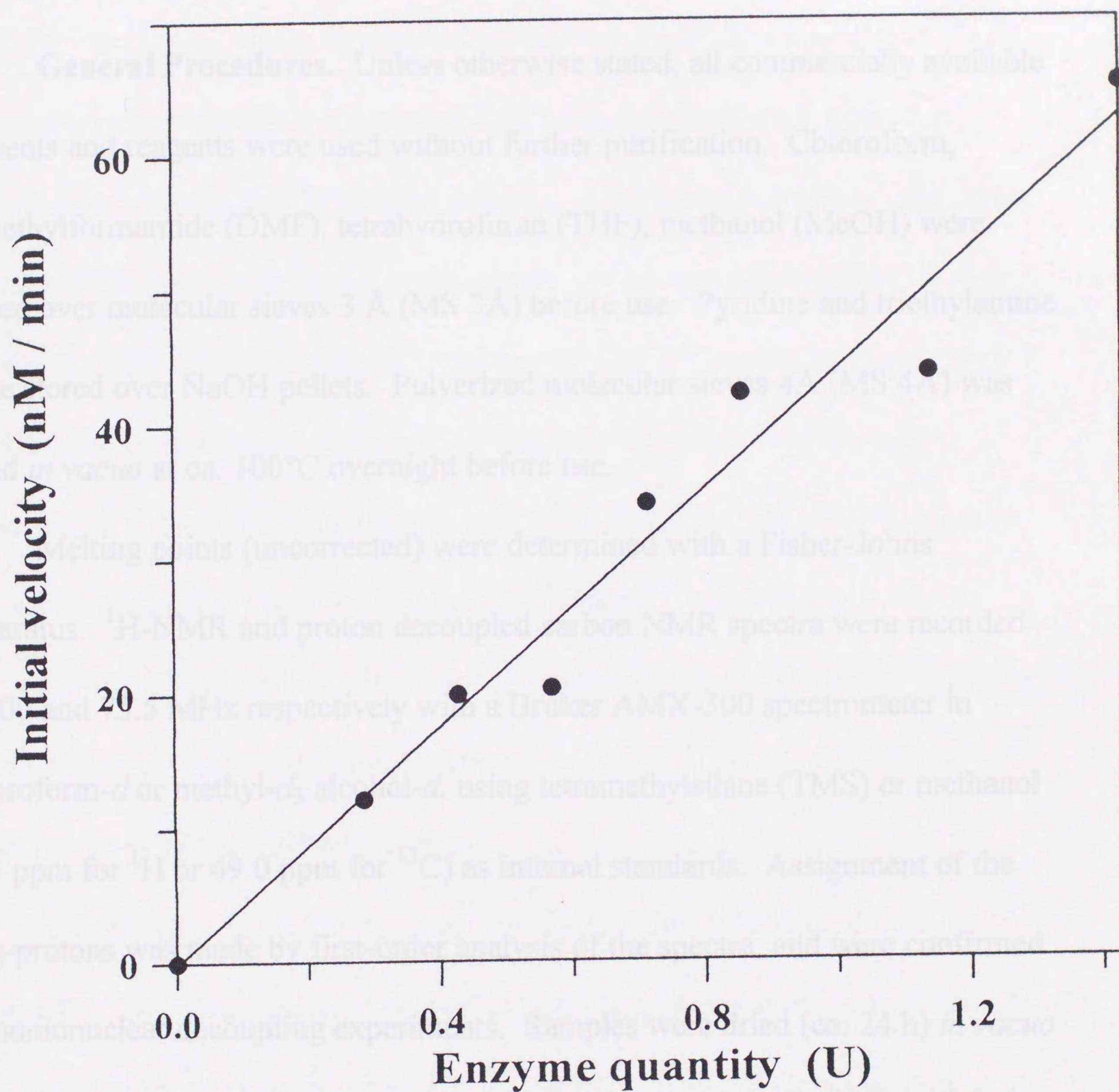


Figure 13. Initial rate of hydrolysis as related to the enzyme (CGase) added. The calculated data of the velocity were obtained from the emission spectra measured at 335 nm, and excited at 290 nm at ambient temperature. 5 μ M solution of NLD was used for the enzymatic reactions.

4.3. Experimental Section

General Procedures. Unless otherwise stated, all commercially available solvents and reagents were used without further purification. Chloroform, dimethylformamide (DMF), tetrahydrofuran (THF), methanol (MeOH) were stored over molecular sieves 3 Å (MS 3Å) before use. Pyridine and triethylamine were stored over NaOH pellets. Pulverized molecular sieves 4Å (MS 4Å) was dried *in vacuo* at ca. 100°C overnight before use.

Melting points (uncorrected) were determined with a Fisher-Johns apparatus. ¹H-NMR and proton decoupled carbon NMR spectra were recorded at 300 and 75.5 MHz respectively with a Bruker AMX-300 spectrometer in chloroform-*d* or methyl-*d*₃ alcohol-*d*, using tetramethylsilane (TMS) or methanol (3.3 ppm for ¹H or 49.0 ppm for ¹³C) as internal standards. Assignment of the ring-protons was made by first-order analysis of the spectra, and were confirmed by homonuclear decoupling experiments. Samples were dried (ca. 24 h) *in vacuo* (50°C, 0.1 Torr) over NaOH pellets before elemental analyses by Galbraith Lab., Inc. (Knoxville, TN).

Synthetic and hydrolytic reactions were monitored by thin-layer chromatography (TLC) on precoated plates of silica gel 60 F₂₅₄ (layer thickness, 0.25 mm; E. Merck, Darmstadt, Germany). The solvent systems used were (A)

5:1 (v/v) toluene–ethyl acetate, (B) 9:1, (C) 5:1 (v/v) chloroform–methanol, (D) 65:25:4 (v/v/v) chloroform–methanol–water, (E) 60:35:8 (v/v/v) chloroform–methanol–0.02% aq. calcium chloride. For detection of the components, TLC plates were sprayed with a solution of 85:10:5 (v/v/v) methanol–conc. sulfuric acid–*p*-anisaldehyde, and heated for a few min (for carbohydrate) or an aqueous solution of 5% (wt) potassium permanganate and heated similarly (for double bond). Visualization of aldehydic components was by heating the plate sprayed with a 2,4-dinitrophenylhydrazine (DNPH) solution which was made by dissolving 0.4 g of DNPH in 100 mL of 2 M HCl and 100 mL of 95% ethanol. An amino group was visualized by heating the plates sprayed with 0.2% ninhydrin in 95% ethanol. Column chromatography was performed on silica gel (Silica Gel 60; 0.015–0.040 mm, E. Merck). A 4W F4T5/CW, Cool White uv lamp was used in the Michael reaction.

n-Pentenyl β -lactoside, **12**, was prepared by the method previously reported.¹⁷ 2-Naphthaldehyde, borane-trimethylamine complex, sodium cholate and dansyl chloride were purchased from Aldrich Chemical Co. (Milwaukee WI). G_{M1} as a standard solution of 5 mg / mL in 2:1 (v/v) chloroform–methanol was purchased from Matreya Inc. (Pleasant Gap, PA). Ceramide glycanase (CGase) from leech (*Macrobdella decora*) was purchased from V-Labs, Inc. (Covington, LA). The CGase as supplied usually contains 0.29 units/10 mL and

21.8 mg protein/10 mL. One unit of CGase is defined as the amount of the enzyme which hydrolyses 1 nmol of G_{M1} per min at pH 5.0, 37°C. The stock sodium cholate solution (1.0 g/10.0 mL) was prepared in deionized water. A 1 mM methanolic solution of the 2-naphthylmethylated lactoside with ω -dansyl aglycon (NLD) was prepared and stored at -15°C in the dark. Fluorescence measurement was carried out with a Perkin-Elmer luminescence spectrometer LS50B (Perkin-Elmer Corp., Rockvill, MD), and the data were processed with an OBEY fluorescence data manager software (Perkin-Elmer).

2-Naphthylaldehyde di-(*i*-Butyl)acetal (29). To a solution of 2-naphthaldehyde (2.00 g, 12.8 mmol) in *i*-butanol (20 mL) containing powdered Drierite (3.0 g) was added 10-camphorsulfonic acid (3.0 mg, 12.8 μ mol) with stirring, and the mixture was continuously stirred for 2 d at room temperature. The reaction mixture was diluted with chloroform (50 mL) and filtered. The filtrate was washed with cold saturated sodium hydrogen bicarbonate solution and brine, then dried over anhydrous sodium sulfate. After filtration, the chloroform solution was concentrated *in vacuo* to give syrupy **29** (3.29 g, 89.6%) containing a trace of starting material: R_F 0.81 (solvent toluene), and the compound was used for next step without further purification.

n-Pentenyl *O*-[2,3-Di-*O*-benzoyl-4,6-*O*-(2-naphthylmethyldene)- β -D-galactopyranosyl]-(1 $\rightarrow$ 4)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranoside (**30**). To a solution of *n*-pentenyl *O*-(β -D-galactopyranosyl)-(1 $\rightarrow$ 4)- β -D-glucopyranoside (**12**) (2.04 g, 4.97 mmol) and **29** (2.85 g, 9.94 mmol) in dry dimethylformamide (20 mL) at 0°C was added 10-camphorsulfonic acid (0.93 g, 4.00 mmol), and the mixture was heated at 55°C for 2 h while removing the liberated *i*-butanol with a water aspirator. The reaction mixture was neutralized with triethylamine (0.56 mL, 4.02 mmol) and vacuum-evaporated to remove DMF. The residual syrup was fractionated on a column of Sephadex LH-20 (ϕ 5 x 200 cm) using 95% ethanol as eluant. The fractions containing both carbohydrate and aldehyde when analyzed by TLC were concentrated. The syrup was dissolved in pyridine (30 mL), to which benzoyl chloride (5.83 mL, 49.7 mmol) was added with stirring at 0°C. After 3 h at room temperature, methanol (0.5 mL) was added dropwise to the mixture, and the reaction mixture was diluted with chloroform, and the solution was successively washed with cold 0.5 M sulfuric acid, cold saturated sodium hydrogen bicarbonate, and brine. The extract was dried over anhydrous sodium sulfate, filtered, and evaporated. The residue was chromatographed on a column of silica gel eluted first with 20:1 then with 10:1 (v/v) toluene–ethyl acetate to give pure **30** (2.90 g, 54.6%): R_F 0.45 (solvent A); m.p. 243–245°C (from methanol); $^1\text{H-NMR}$ δ (CDCl_3) 1.53 (m, 2 H, $\text{CH}_2\text{CH=}$), 2.35 (m, 2 H,

OCH₂CH₂), 3.02 (s, 1 H, H-5'), 3.6 (m, 2 H, OCH₂), 3.63 (dd, 1 H, $J_{6'a, 6'b}$ 10.7 Hz, H-6'a), 3.8 (m, 2 H, H-5, 6'b), 4.23 (t, 1 H, $J_{4, 5}$ 9.0 Hz, H-4), 4.37 (d, 1 H, $J_{4', 5'} \sim 0$ Hz, H-4'), 4.44 (dd, 1 H, $J_{5, 6a}$ 4.2 Hz and $J_{6a, 6b}$ 12.6 Hz, H-6a), 4.62 (dd, 1 H, $J_{5, 6b}$ 2.0 Hz, H-6b), 4.67 (d, 1 H, $J_{1, 2}$ 7.8 Hz, H-1), 4.76 (m, 2 H, CH₂=CH), 4.86 (d, 1 H, $J_{1', 2'}$ 7.9 Hz, H-1'), 5.20 (dd, 1 H, $J_{3', 4'}$ 3.6 Hz, H-3'), 5.33 (dd, 1 H, $J_{2, 3}$ 9.5 Hz, H-2), 5.44 [s, 1 H, CH-(2-naphthyl)], 5.57 (m, 1 H, CH=CH₂), 5.83 (dd, 1 H, $J_{2', 3'}$ 10.4 Hz, H-2'), 5.86 (t, 1H, $J_{3, 4}$ 9.5 Hz, H-3), 7.59 (m, 32 H, 5 Bz, 2-naphthyl).

Anal. Calcd. for C₆₃H₅₆O₁₆·H₂O: C, 69.61; H, 5.37. Found C, 69.74; H, 5.55.

A small amount of perbenzoylated *n*-pentenyl lactoside was also obtained as a byproduct: R_F 0.59 (solvent A); ¹H-NMR δ (CDCl₃) 1.57 (m, 2 H, CH₂CH=), 1.93 (m, 2 H, OCH₂CH₂), 3.64 (m, 2 H, OCH₂), 3.69 (dd, 1 H, $J_{6'a, 6'b}$ 11.3 Hz, H-6'a), 3.75 (dd, 1 H, 6'b), 3.90 (t, 1 H, $J_{5', 6'a}$ 6.9 Hz and $J_{5', 6'b}$ 6.5 Hz, H-5'), 3.9 (m, 1 H, H-5), 4.27 (t, 1 H, $J_{4, 5}$ 9.6 Hz, H-4), 4.50 (dd, 1 H, $J_{5, 6a}$ 4.2 Hz and $J_{6a, 6b}$ 12.1 Hz, H-6a), 4.61 (dd, 1 H, $J_{5, 6b} < 1$ Hz, H-6b), 4.69 (d, 1 H, $J_{1, 2}$ 7.9 Hz, H-1), 4.77 (m, 2 H, CH₂=CH), 4.89 (d, 1 H, $J_{1', 2'}$ 7.9 Hz, H-1'), 5.38 (dd, 1 H, $J_{3', 4'}$ 3.4 Hz, H-3'), 5.48 (dd, 1 H, $J_{2, 3}$ 9.8 Hz, H-2), 5.60 (m, 1 H, CH=CH₂), 5.74 (dd, 1 H, $J_{2', 3'}$ 10.3 Hz, H-2'), 5.74 (m, 1 H, H-4'), 5.82 (t, 1H, $J_{3, 4}$ 9.3 Hz, H-3), 7.61 (m, 35 H, 7 Bz).

***n*-Pentenyl *O*-[2,3-Di-*O*-benzoyl-6-*O*-(2-naphthylmethyl)- β -D-galactopyranosyl]-(1 $\rightarrow$ 4)-2,3,6-tri-*O*-benzoyl- β -D-glucopyranoside (31).** A mixture of **30** (703 mg, 0.66 mmol), powdered MS 4Å (1.5 g), and borane-trimethylamine complex (346 mg, 4.61 mmol) in 10 mL of dry THF was stirred for 40 min at room temperature, to which was added anhydrous aluminum chloride (614 mg, 4.61 mmol) with stirring, and the stirring was continued for 11 h at room temperature. When TLC showed complete disappearance of the acetal group, the mixture was filtered, and the powdered MS 4Å was thoroughly washed with chloroform. The filtrate was successively washed with cold 0.5 M sulfuric acid, cold saturated sodium hydrogen bicarbonate, and brine, dried over anhydrous sodium sulfate, filtered, and concentrated. The syrup was separated on a column of silica gel eluting with 10:1 (v/v) toluene-ethyl acetate to give pure **31** (668 mg, 94.7%): R_F 0.42 (solvent A); m.p. 171–173°C (from methanol); $^1\text{H-NMR}$ δ (CDCl_3) 1.52 (m, 2 H, $\text{CH}_2\text{CH=}$), 1.87 (m, 2 H, OCH_2CH_2), 2.26 (d, 1 H, OH-4'), 3.05 (m, 2 H, H-6'a, 6'b), 3.46 (t, 1 H, $J_{5,6}$ 5.9 and 6.2 Hz, H-5'), 3.58 (m, 2 H, OCH_2), 3.78 (m, 1 H, H-5), 4.16 (t, 1 H, $J_{4,5}$ 9.2 Hz, H-4), 4.19 (br t, 1 H, $J_{4',\text{OH}}$ 2.3 Hz, H-4'), 4.38 [m, 2 H, OCH_2 -(2-naphthyl)], 4.39 (dd, 1 H, $J_{5,6a}$ 4.6 Hz and $J_{6a,6b}$ 11.8 Hz, H-6a), 4.57 (dd, 1 H, $J_{5,6b}$ $\sim$ 1 Hz, H-6b), 4.62 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.74 (m, 2 H, $\text{CH}_2=\text{CH}$),

4.77 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1'), 5.11 (dd, 1 H, $J_{3',4'}$ 3.1 Hz, H-3'), 5.37 (dd, 1 H, $J_{2,3}$ 9.5 Hz, H-2), 5.56 (m, 1 H, CH=CH₂), 5.69 (dd, 1 H, $J_{2',3'}$ 10.3 Hz, H-2'), 5.71 (t, 1H, $J_{3,4}$ 9.0 Hz, H-3), 7.58 (m, 32 H, 5 Bz, 2-naphthyl).

Anal. Calcd. for C₆₃H₅₈O₁₆: C, 70.65; H, 5.45. Found C, 70.76; H, 5.65.

A small amount of **31** (22 mg, 0.2 mmol) was *O*-acetylated by the usual method of pyridine–acetic anhydride to give pure mono-acetate **32** : R_F 0.57 (solvent A); ¹H-NMR δ (CDCl₃) 1.52 (m, 2 H, CH₂CH=), 1.78 (s, 3 H, Ac), 1.87 (m, 2 H, OCH₂CH₂), 2.78 (t, 1 H, $J_{5',6'}$ 8.1 Hz and $J_{6'a,6'b}$ 9.1 Hz, H-6'a), 2.97 (dd, 1 H, $J_{5',6'b}$ 5.2 Hz, H-6'b), 3.51 (m, 2 H, OCH₂), 3.61 (dd, 1 H, H-5'), 3.77 (m, 1 H, H-5), 4.18 (t, 1 H, $J_{4,5}$ 9.6 Hz, H-4), 4.36 [m, 2 H, OCH₂-(2-naphthyl)], 4.38 (dd, 1 H, $J_{5,6a}$ 4.4 Hz and $J_{6a,6b}$ 12.1 Hz, H-6a), 4.56 (dd, 1 H, $J_{5,6b}$ <1 Hz, H-6b), 4.62 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 4.76 (d, 1 H, $J_{1',2'}$ 7.8 Hz, H-1'), 4.77 (m, 2 H, CH₂=CH), 5.22 (dd, 1 H, $J_{3',4'}$ 3.3 Hz, H-3'), 5.38 (dd, 1 H, $J_{2,3}$ 9.6 Hz, H-2), 5.55 (m, 1 H, CH=CH₂), 5.53 (d, 1 H, $J_{4',5'}$ ~0 Hz, H-4'), 5.54 (dd, 1 H, $J_{2',3'}$ 10.4 Hz, H-2'), 5.69 (t, 1H, $J_{3,4}$ 9.0 Hz, H-3), 7.56 (m, 32 H, 5 Bz, 2-naphthyl).

***n*-Pentenyl *O*-[6-*O*-(2-Naphthylmethyl)- β -D-galactopyranosyl]-(1→4)- β -D-glucopyranoside (**33**).** To a solution of **31** (667.6 mg, 0.623 mmol) in dry methanol (5 mL) and dry THF (5 mL) was added sodium methoxide (20.2 mg, 0.374 mmol), and the solution was stirred for 64 h at room temperature. The

reaction mixture was neutralized by adding Dowex 50W X-8 (H^+) resin. The suspension was filtered, and the filtrate was evaporated *in vacuo*. The residue was purified by passing through a column of a Sephadex LH-20 ($\phi 2.5 \times 50$ cm), eluting with 95% ethanol, to give homogeneous **33** (260.8 mg, 76.0%): R_F 0.18 (solvent B); $^1\text{H-NMR}$ δ (CD_3OD) 1.66 (m, 2 H, $\text{CH}_2\text{CH=}$), 2.10 (m, 2 H, OCH_2CH_2), 3.25 (m, 1 H, H-2'), 3.51 (m, 1 H, H-3'), 3.53 (m, 1 H, H-2), 4.24 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1'), 4.32 (d, 1 H, $J_{1,2}$ 7.4 Hz, H-1), 4.68 [m, 2 H, CH_2 -(2-naphthyl)], 4.95 (m, 2 H, $\text{CH}_2=\text{CH}$), 5.79 (m, 1 H, $\text{CH}=\text{CH}_2$), 7.62 (m, 10 H, 2-naphthyl); $^{13}\text{C-NMR}$ δ (CD_3OD) 30.06 (OCH_2CH_2), 31.21 ($\text{CH}_2\text{CH=}$), 62.05 (C-6), 70.24, 70.37, 70.55, 72.38, 74.48, 74.68, 74.79, 75.30, 76.27, 76.45, 81.53 [C-2, 3, 4, 5, 2', 3', 4', 5', 6', OCH_2CH_2 , CH_2 -(2-naphthyl)], 104.22 (C-1), 105.19 (C-1'), 115.19 ($\text{CH}_2=\text{CH}$), 126.92, 127.06, 127.10, 127.73, 128.64, 128.98, 129.11, 134.49, 134.74, 136.95 (10 C, attributable to 2-naphthyl), 139.47 ($\text{CH}=\text{CH}_2$).

5-(2-Aminoethanethio)-pentyl *O*-[6-*O*-(2-Naphthylmethyl)- β -D-galactopyranosyl]-(1 $\rightarrow$ 4)- β -D-glucopyranoside Hydrochloride Salt (34**).** A solution of **33** (40.4 mg, 73.4 μmol) in dry methanol (0.5 mL) was allowed to react with 2-aminoethanethiol hydrochloride (85.1 mg, 0.734 mmol) under the uv lamp for 3 d at room temperature. The reaction mixture was evaporated *in*

vacuo, and the residue was dissolved in a small amount of water. The aqueous solution was fractionated on a column of Sephadex G-10 ($\phi 1.5 \times 14$ cm), first eluted with water then with 0.5 M aqueous acetic acid, to give homogeneous **34** (32.1 mg, 65.9%): R_F 0.13 (solvent D).

5-[2-(5-Dimethylamino-1-naphthalenesulfonamido)-ethanethio]-pentyl O-[6-O-(2-Naphthylmethyl)- β -D-galactopyranosyl]-(1 $\rightarrow$ 4)- β -D-glucopyranoside (28). To a solution of **34** (32.1 mg, 48.3 μ mol) and dry triethylamine (13.4 μ L, 96.7 μ mol) in dry methanol (2.0 mL) was added dansyl chloride (52.1 mg, 193 μ mol) at room temperature. After 30 min, the mixture was directly applied to a column of Sephadex LH-20 ($\phi 2.5 \times 50$ cm) eluted with 95% ethanol to give crude **28** (37.8 mg, 90.9%). An analytical sample was chromatographed on silica gel with 10:1 (v/v) chloroform–methanol as eluent to give pure **28**: R_F 0.33 (solvent C); $^1\text{H-NMR}$ δ (CD_3OD) 1.36 (m, 4 H, 2 CH_2), 1.55 (m, 2 H, OCH_2CH_2), 2.26 (m, 2 H, $\text{CH}_2\text{SCH}_2\text{CH}_2\text{NH}$), 2.39 (m, 2 H, $\text{SCH}_2\text{CH}_2\text{NH}$), 2.98 (m, 2 H, CH_2NH), 4.28 (d, 1 H, $J_{1',2'} 7.8$ Hz, H-1'), 4.37 (d, 1 H, $J_{1,2} 7.3$ Hz, H-1), 4.73 [m, 2 H, OCH_2 -(2-naphthyl)], 7.25–8.57 (m, 13 H, dansyl, 2-naphthyl).

Hydrolysis of NLD with Ceramide Glycanase. The enzymatic reaction conditions were based on Zhou et al.¹¹: A solution of NLD (30 nmol) and a solution of sodium cholate (2.0 μ L, 200 mg) was placed in a 1.5 mL screw-capped vial (USA/Scientific Plastics, Ocala, FL), and the solution was evaporated to dryness *in vacuo* with a SpeedVac (Savant Instruments Inc., NY). To the dried residue, 200 μ L of 50 mM sodium acetate buffer, pH 5.0, was added, and the mixture was ultrasonicated for 1 min. The enzyme (10 μ L, 0.29 U) was added to the vial, and the vial was shaken in a water bath at 37°C in the dark. After incubation at 37°C for 17 h, a portion of the reaction mixture was directly analyzed by TLC using solvents (C), (D), or (E). The components were detected either by uv illumination or by charring.

Continuous Assay of Ceramide Glycanase with NLD by Intramolecular Fluorescence Quenching. Fluorescence measurement of emission spectra and excitation spectra was carried out at ambient temperature, in a Teflon-stoppered cuvette (12.5 mm wide x 45 mm high) containing a 3.0 mL sample. The slit width of the excitation and the emission was 5.0 nm, and the scan speed was 500 nm/min.

A typical enzymatic reaction is carried out at room temperature as follows: Appropriate amounts of 1 mM NLD solution and the sodium cholate solution in

a screw-capped vial was evaporated to dryness. To the vial was added 4.0 mL of 50 mM sodium acetate buffer, pH 5.0, and the vial was sonicated for 1 min. A 3.0 mL portion was pipeted into a Teflon-stoppered cuvette, and an appropriate amount of the enzyme (10–40 μ L, 0.29–1.14 U) was added and quickly mixed by inversion. The initial spectrum at 0 min was taken immediately after the enzyme was added. The quantification of NLD by measurement of dansyl emission was estimated by subtracting the dansyl emission (540.3 nm) at 240 min from the value at 0 min. The amount of NLD hydrolysed during this period was regarded to be equal to the 6'-(2-naphthylmethyl)-lactose (NL) formed in the same reaction period. Linear proportionation of the decrease of the dansyl emission yielded the quantity of the hydrolysed NLD. These emission spectra showed an isoemissive point at 414 nm.

4.4. Conclusion

We have successfully synthesized a bi-fluorescence-labeled lactoside (NLD) regio and stereoselectively. This substrate showed clear emission spectrum in which emission peaks were at around 335 and 540 nm due to 2-naphthyl and dansyl emissions, respectively, when the substrate was excited at 260 to 300 nm. The synthetic substrate was demonstrated its usefulness as a substrate for Cgase from leech based on fluorescence quenching and energy transfer. Cgase cleaved the substrate and gave K_m 7.7 μ M and V_{max} 0.16 nmol / min measured by 2-naphthyl emission at 335 nm. The NLD provides a number of advantages such as sensitivity, speed, and feasibility of continuous assay.

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Chapter 5

Concluding Remarks

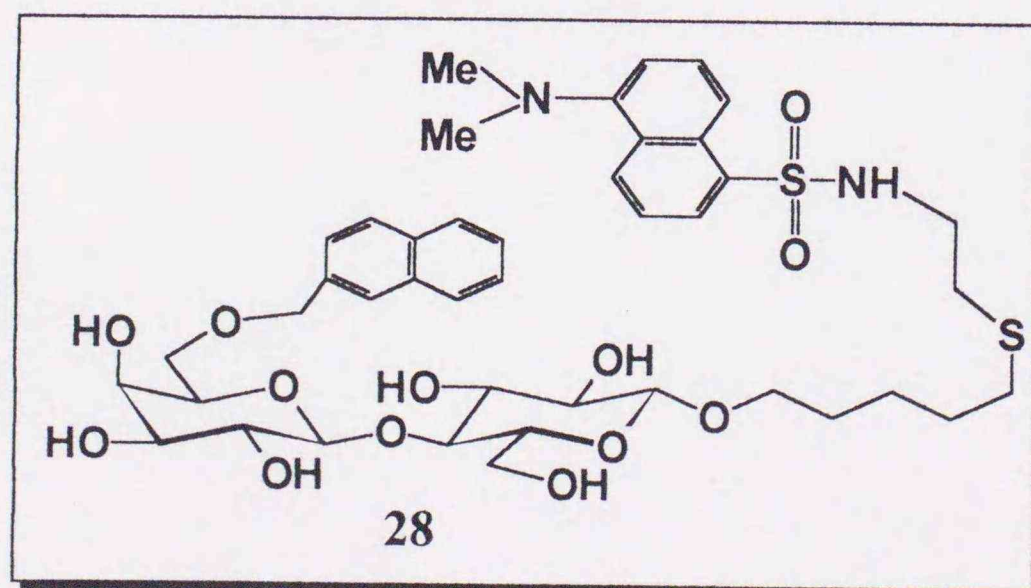
It has been described as major subjects through this study that 1) syntheses of novel glycoligands have been systematically accomplished, 2) their biochemical evaluation has been effectively investigated. The contents of the findings and conclusions are summarized as follows:

- ☞ An easy and convenient catalyzing system of a combination of mercuric bromide (HgBr_2) and 2,4,6-collidine was found to promote the formation of activated cyclic sugar derivatives such as 1,2-orthoesters and an oxazoline derivative in quantitative yields at room temperature.
- ☞ It was also suggested that the reaction of a lactosyl 1,2-orthoester with trimethylsilyl azide proceeded smoothly and gave stereoselectively β -lactosyl azide which is available intermediate for constructing glycopeptides and neoglycoconjugates in high yield.
- ☞ Systematic syntheses of polymerizable *N*-acetylglucosamine and related disaccharide derivatives have been accomplished by introducing an *n*-pentenyl group at each reducing end as a simple and versatile polymerizable

aglycon. Radical copolymerizations of these sugar monomers with acrylamide proceeded smoothly in water by means of ammonium persulfate and *N,N,N',N'*-tetramethyl-ethylenediamine as initiators and to give water soluble glycopolymers in good yields.

☞ Using novel glycopolymers, The order of the inhibitory effects of glycopolymers as structures below on hemagglutination by *Erythrina corallodendron* lectin was proved to be $\text{Gal}\beta(1\rightarrow4)\text{GlcNAc}(\text{LacNAc}) \gg \text{Gal}\beta(1\rightarrow4)\text{Glc}(\text{Lac}) \gg \text{Gal}\beta(1\rightarrow3)\text{GlcNAc} > \text{Gal}\beta(1\rightarrow6)\text{GlcNAc} \cong \text{Gal}$.

☞ An alkyl lactoside containing two different fluorescence probes as an energy donor and an energy acceptor as shown in below was synthesized to create a substrate for ceramide glycanase from *n*-pentenyl lactoside in good yield.



☞ Digestion of the bi-fluorescence-labeled lactoside with leech (*Macrobdella decora*) ceramide glycanase resulted in an increase in the naphthyl emission (λ_{em} 335 nm) with a concomitant decrease in the dansyl emission (λ_{em} 540 nm). These changes can be used for continuous monitoring of the ceramide glycanase activity. The K_M and V_{max} of the leech ceramide glycanase was estimated to be 7.7×10^{-6} M and 0.16 nmol/min, respectively, using this lactoside as a substrate.

I hope that unique synthetic strategies obtained here will open new trends of biomimetic chemistry for investigating glycoscience and glycotechnology.



