

THE SYNTHESIS, IMMUNOLOGICAL CHARACTERIZATION
AND NMR ANALYSIS OF CELL-WALL OLIGOSACCHARIDES
OF BACTERIAL ORIGIN

by

Kerry Bruce Reimer

M.Sc. Simon Fraser University, 1987

B.Sc. University of British Columbia, 1985

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
in the Department
of
Chemistry

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SIMON FRASER UNIVERSITY

March 1991

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APPROVAL

Name: Kerry B. Reimer
Degree: Ph.D. Chemistry
Title of Thesis: The Synthesis, Immunological Characterization
and NMR Analysis of Cell-Wall Oligosaccharides
of Bacterial Origin

Examining Committee:

Chairperson: P. Percival, Professor

B. M. Pinto, Senior Supervisor,
Associate Professor

K. N. Slessor, Professor

~~R. K. Pomeroy, Professor~~

T. J. Borgford, ~~Assistant~~ Professor,
Internal Examiner

~~O. Hindsgaul, Associate~~ Professor,
External Examiner,
Department of Chemistry,
University of Alberta

Date Approved:

March 11, 1991

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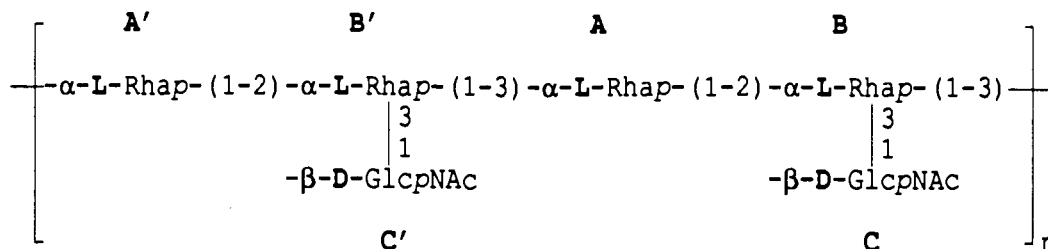
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ABSTRACT

Trisaccharide [B(C)A], pentasaccharide [B(C)AB'C'] and hexasaccharide [B(C)AB'(C')A'] segments of the cell-wall polysaccharide of the β -hemolytic *Streptococci* Group A (shown below) have been prepared



by means of a series of Königs-Knorr glycosylations. The synthesis of a fully functionalized branched trisaccharide sequence, is also described; this unit has served as a key intermediate in an efficient, convergent block synthesis of a hexasaccharide portion of the polysaccharide. The trisaccharide and pentasaccharide moieties were prepared as both propyl and 8-(methoxycarbonyl)octyl glycosides, the former for use as haptens in antigen/antibody binding studies, and the latter for use in the preparation of synthetic antigens. All synthetic intermediates and final products were fully characterized by a full complement of 2-dimensional NMR experiments. Glycoconjugates of [AB'C'], [B(C)A] and [B(C)AB'C'] segments of the polysaccharide with the proteins bovine serum albumin (BSA) and horse hemoglobin (horse-Hb) were prepared from the corresponding 8-(methoxycarbonyl)octyl glycosides. Polyclonal antisera against the BSA glycoconjugates were raised in rabbits, and a panel of disaccharide through pentasaccharide haptens were used in a series of indirect inhibition ELISAs to characterize the binding profiles of the antisera. A panel of monoclonal antibodies was generated by using a culture of heat-killed *Streptococci* Group A bacteria as an immunogen.

The BSA and Horse-Hb glycoconjugates were used as screening reagents in the monoclonal antibody protocol to identify carbohydrate-directed antibodies. The binding profiles of the chosen monoclonal antibodies were characterized by a series of indirect inhibition ELISAs, incorporating the glycoconjugates as solid phase antigens and a panel of disaccharide through pentasaccharide sequences of the polysaccharide as inhibitors. A monoclonal antibody (SA-2C) with greater affinity for the pentasaccharide sequence than the smaller hapten sequences was identified for use as an immunodiagnostic reagent. In a separate study, a heptasaccharide sequence of the *Shigella flexneri* variant Y lipopolysaccharide antigenic determinant was fully characterized by 2-dimensional NMR techniques. Transient nOe effects in the rotating frame were used to infer a model of hapten conformation.

DEDICATION

To my wife Carol, who knows what a "marathon" session is really like.
Her continuing support and understanding has helped make this marathon
seem shorter.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Mario Pinto, for providing a rewarding and stimulating research environment. Our many discussions and the hours he has spent on my behalf are very much appreciated. I am grateful to Dr. D. R. Bundle of the National Research Council of Canada, Ottawa, for providing the resources necessary for me to carry out the immunochemical aspects of this thesis; his guidance throughout my stay at his laboratory was appreciated. I am indebted to the staff of the Immunochemistry Section at the National Research Council of Canada, particularly Marg Gidney and Barb Sinnot, for their patience and invaluable assistance throughout my stay there. I also thank Marcey Tracey for her efficient and friendly service in the course of obtaining several NMR spectra, and for being particularly accommodating in allowing me access to hours of spectrometer time. I thank Noah Wandu and Shannon Harris for providing technical assistance, M. Yang for providing the microanalyses, and Dr. Mehrshid Alai-Safar for recording the PD-MS spectra. The social and scientific exchanges with the many graduate students and Faculty of the Chemistry Department were enjoyed and will be remembered fondly. I also thank the members of my examining committee for the time they have taken in appraisal of this thesis.

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LIST OF ABBREVIATIONS

Ac	Acetyl
All	Allyl
BIRD	Bilinear rotation decoupling
BSA	Bovine serum albumin
Bz	Benzoyl
Bzl	Benzyl
COSY	Correlated spectroscopy
CW	Continuous wave
DMF	<i>N,N</i> -Dimethylformamide
ELISA	Enzyme linked immunosorbent assay
EIA	Enzyme immunoassay
FID	Free induction decay
β -D-GlcpNAc	2-Acetamido-2-deoxy- β -D-glucopyranosyl
Hb	Hemoglobin
HSEA	Hard sphere exo-anomeric effect
IgG	Immunoglobulin class G
IgM	Immunoglobulin class M
LPS	Lipopolysaccharide
LTMP	Lithium tetramethylpiperidide
MLEV	Malcom Levitt
NHAc	Acetamido
NMR	Nuclear magnetic resonance
$^{13}\text{C}\{^1\text{H}\}$ -NMR	Proton-decoupled carbon-13 NMR
nOe	nuclear Overhauser enhancement

NOESY	nuclear Overhauser enhancement spectroscopy
PBS	Phosphate buffered saline
Phth	Phthalimido
p-NPP	para-Nitrophenol phosphate
PTA	Phosphate buffered saline containing Tween-20 and NaN_3
α -L-Rhap	α -L-Rhamnopyranosyl
RIA	Radioimmunoassay
ROESY	Rotating frame NOE spectroscopy
silver triflate	Silver trifluoromethanesulphonate
SEM	β -(trimethylsilyl)ethoxymethyl
Tfl	Triflate
TLC	Thin layer chromatography
TMU	Tetramethyl urea
TOCSY	Total correlation spectroscopy
Tr	Trityl

CHAPTER 1

I: INTRODUCTION

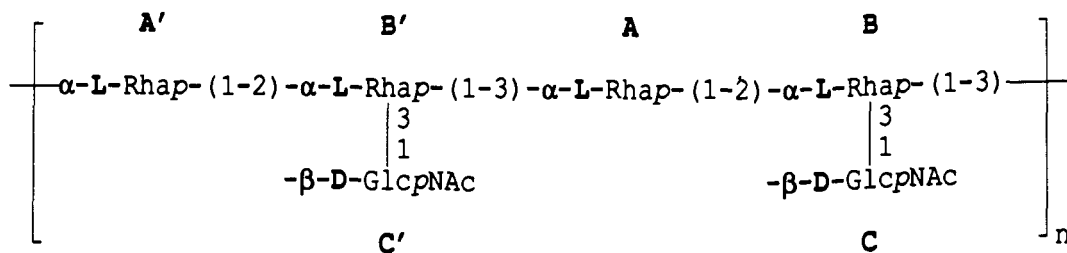
The field of carbohydrate chemistry is a rapidly expanding and exciting branch of chemistry today. Much of the interest is a result of the increased understanding of the role of carbohydrates in biological systems.¹ Carbohydrates are commonly known to have important roles as structural elements (cellulose and chitin) and energy storage compounds (starch and glycogen). In addition to these functions, carbohydrates, in the form of glycoproteins and glycolipids, are known to be involved in many important inter-cellular, and inter-molecular interactions. These interactions include cell-cell recognition, hormone interactions, cell-differentiation, and immune recognition.

Many of the studies of these processes carried out today have been made possible due to advances in synthetic carbohydrate chemistry.² The techniques which have been developed, particularly those for stereospecific glycosylation reactions, have made possible the synthesis of many biologically important oligosaccharides. The present study describes such synthetic efforts and falls under the category of antigen-antibody interactions; specifically, the interaction of *Streptococci* Group A cell-wall polysaccharides with complementary antibodies. The long-term objective of the present work is to develop an antibody-based immunodiagnostic reagent for use in the rapid detection of streptococcal infections and also for study of the interactions of anti-streptococcal antibodies with various human tissues.

A: Streptococci Group A Infections

The Gram positive β -hemolytic *Streptococci* Group A is one of the primary infective agents in humans, causing streptococcal pharyngitis, commonly known as strep throat.³ In a small number of cases the initial streptococcal infection can develop into the more serious condition of rheumatic fever.⁴ Streptococcal infections are also implicated in the development of other disease conditions such as heart-valve disease, glomerulonephritis, rheumatoid arthritis, and other rheumatic disorders.⁵ The detection of streptococcal diseases and their rapid treatment is therefore important. The initial impetus for this project was the development of reagents for use in rapid detection methods.

The cell-wall of *Streptococci* bacteria is composed of protein, carbohydrate, and mucopeptide. The cell-wall carbohydrates are the structures responsible for the serological classification of *Streptococci* into the various groups. The cell-wall carbohydrates of the Group A *Streptococci* have been found to be comprised of a rhamnose backbone consisting of alternating α -L-(1-2) and α -L-(1-3) linkages, with β -D-N-acetylglucosamine residues attached to the 3-positions of the rhamnose backbone⁶. These cell-wall polysaccharides can serve as markers for the detection of *Streptococci* Group A infections.



The diagnosis of streptococcal infections are usually carried out using bacterial culture methods. This involves obtaining a throat swab

from the patient and growing bacterial cultures from the swab. Microbiological and immunological techniques are then used to identify the resulting colonies. These methods usually require overnight or longer to obtain the results. This delay in time often means that treatment is postponed, or is initiated without proper confirmation of infection; a rapid diagnostic reagent is therefore desirable. Several rapid detection methods have been developed⁷ which can detect streptococcal antigen directly from a throat swab. For the most part, these tests rely on the extraction of bacterial antigen from a patient's throat swab, using enzymatic or nitrous acid extraction, and detection of the extracted polysaccharide antigen by macroscopic agglutination of antibody-coated latex particles. Clinical evaluation⁸⁻¹⁰ of these methods has indicated good specificity as compared with the culture method.¹¹ The sensitivity of some of these tests^{9,10} was, however, significantly lower than that obtained using the standard culture techniques, requiring the confirmation of negative results obtained with the kits by the culture method.

One of the goals of this research was to synthesize a panel of oligosaccharides corresponding to the cell-wall polysaccharide of *Streptococci* Group A, as well as to prepare glycoconjugates of these sequences. These compounds would be used in inhibition binding studies with anti-*Streptococci* Group A antibodies to identify an oligosaccharide sequence corresponding to a highly defined *Streptococci* Group A antigen. Once such an oligosaccharide sequence was identified, the corresponding glycoconjugate (i.e. artificial antigen) could be used in an ELISA protocol to directly detect anti-*Streptococci* Group A antibodies in a patient's serum sample (see Figure 1-1).

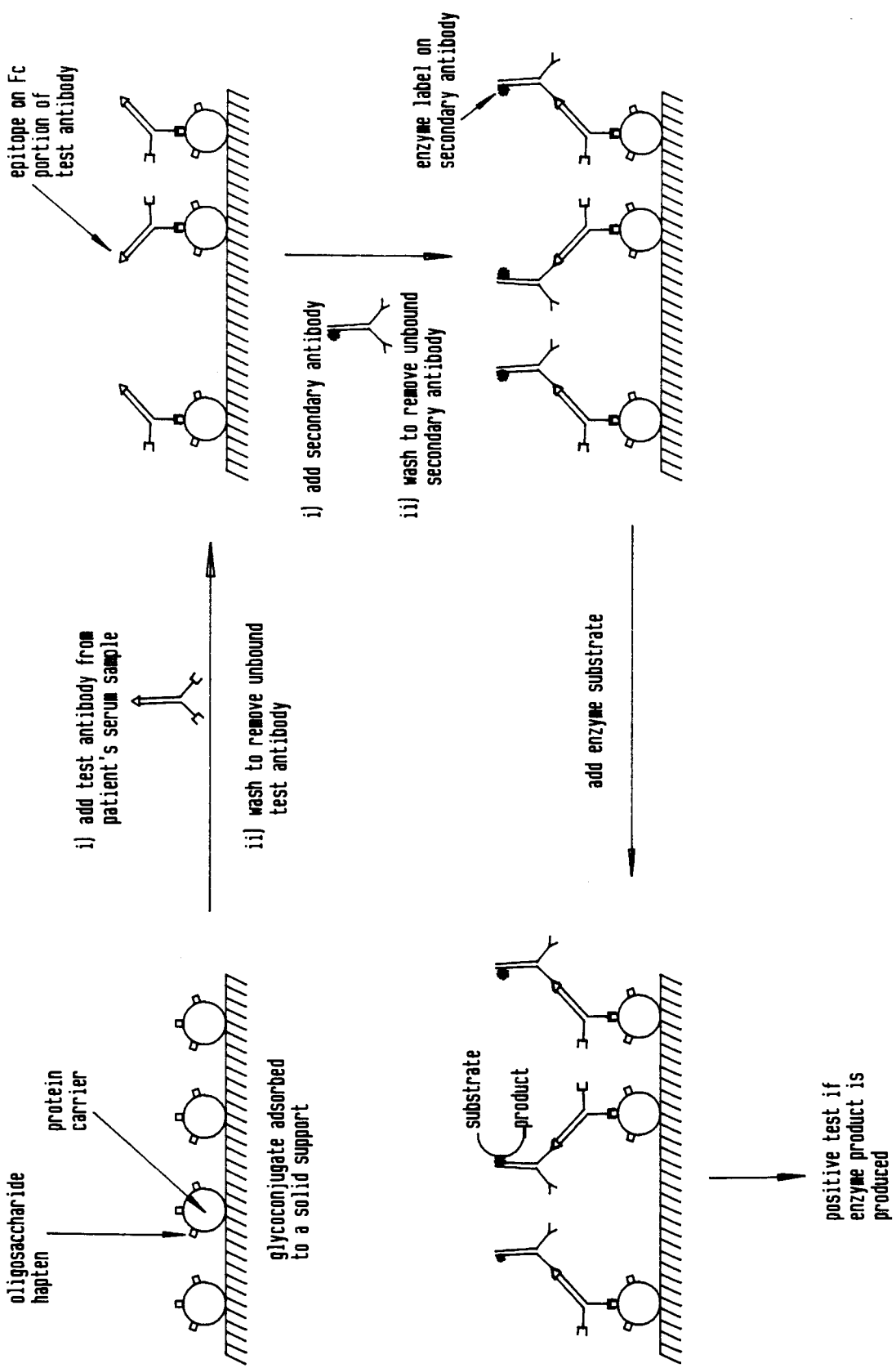


Figure 1-1: Use of a glycoconjugate in an ELISA to detect anti-Streptococci Group A antibodies.

Monoclonal antibodies directed against a *Streptococci* Group A antigen were to be generated, using the synthetic oligosaccharides and glycoconjugates in the hybridoma-monoclonal antibody protocol¹² to select a suitable monoclonal antibody. The selected monoclonal antibody could then be incorporated into an ELISA protocol to detect extracted *Streptococci* Group A antigen from a patient's throat swab (see Figure 1-2). It was envisaged that a detection method based on a monoclonal antibody, directed against a highly defined carbohydrate epitope, would be both more specific and more sensitive than those currently in use which are based on polyclonal antisera. The above artificial antigens and monoclonal antibodies could be used to improve or replace existing reagents in currently available diagnostic test-kits.

Another important use of a monoclonal antibody directed against a *Streptococci* Group A epitope would be the investigation of the relationship between an initial *Streptococci* Group A infection and the development of rheumatic heart disease. Patients with rheumatic diseases show an abnormal immune response to cell-wall antigens of *Streptococci* Group A. The antigens are known to be cross reactive with heart and other muscle tissue antigens. Components of the *Streptococci* Group A cell-wall which have been implicated in immunological cross reactions include the type-specific M proteins¹³ and the group-specific carbohydrates.¹⁴⁻¹⁶ The group-specific carbohydrates have also been shown to be immunologically cross reactive with the structural glycoproteins of bovine and human heart valve tissues.^{14,15} A monoclonal antibody with a highly defined binding specificity would provide a valuable probe in the search for common epitopes in human

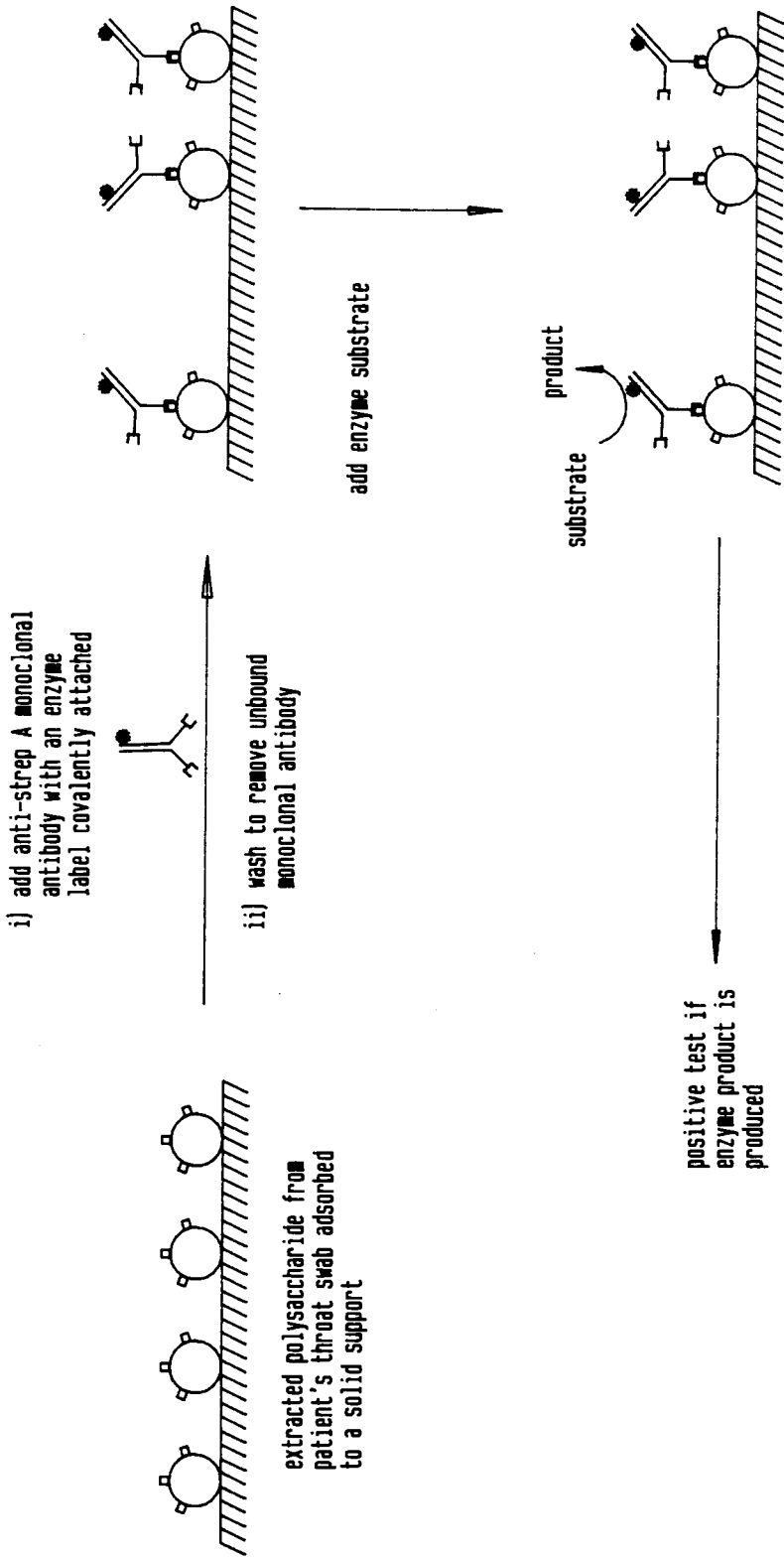


Figure 1-2: Use of a monoclonal antibody in an ELISA to detect extracted Streptococci Group A polysaccharide from a throat swab.

tissues and *Streptococci* Group A cell-wall components.

B: Thesis Overview

In Chapter 2 of this thesis, the synthesis of trisaccharide, pentasaccharide, and hexasaccharide sequences of the *Streptococci* Group A cell-wall polysaccharide structure are detailed. A highly efficient, convergent synthesis of a fully functionalized hexasaccharide from a key trisaccharide intermediate is described. The syntheses made use of the allyl group as a protecting group for the anomeric centres, permitting the ready preparation of glycosyl chlorides for use as glycosyl donors. Benzyl ethers and benzoate esters were used as persistent hydroxyl blocking groups, and the acetate and β -(trimethylsilyl)ethoxymethyl groups were used as temporary hydroxyl protecting groups. NMR analysis of the synthetic intermediates and the final compounds was performed using a variety of one- and two-dimensional NMR methods. Glycoconjugates of oligosaccharide portions of the polysaccharide structure were prepared from two different trisaccharides, and from a pentasaccharide sequence. The latter syntheses afforded the necessary reagents to initiate immunochemical studies of the *Streptococci* Group A group-specific cell-wall carbohydrates.

In Chapter 3 of this thesis, the immunochemical studies of both polyclonal and monoclonal antibodies are described. The glycoconjugates, prepared in Chapter 2, were used to immunize a series of rabbits. The binding specificities of the polyclonal antibodies thus obtained were characterized by inhibition ELISA studies, using a panel of synthetic oligosaccharide inhibitors. These studies served to demonstrate that the glycoconjugates could be used as synthetic

antigens, and that antibodies specific to the carbohydrate portions of the glycoconjugates could be generated. Following this, a monoclonal antibody production protocol was initiated. A series of monoclonal antibodies was generated using a *Streptococci* Group A bacterial vaccine; the synthetic inhibitor oligosaccharides, in conjunction with the glycoconjugates, were used to select a monoclonal antibody with high binding specificity for a pentasaccharide sequence. The selected monoclonal antibody fulfills the requirements for an immunochemical diagnostic reagent. Studies will be ongoing in this laboratory which make use of this antibody in this capacity.

Chapter 4 of this thesis describes the full characterization by NMR spectroscopy of a heptasaccharide portion of the *Shigella flexneri* Variant Y lipopolysaccharide (LPS) antigenic determinant. This, and related compounds, have been synthesized in our laboratory as part of a study of the interactions between the *Shigella flexneri* Variant Y LPS antigen and complementary monoclonal antibodies. With the synthesis of ever increasingly complex carbohydrate structures, characterization by NMR methods of the synthetic intermediates and final products is an expanding analytical technique. In addition to the confirmation of structure, more examples of the use of NMR methods for the conformational analysis of carbohydrates are appearing in the literature.¹⁷ The heptasaccharide was submitted to a detailed analysis using several recently developed 2-dimensional NMR techniques, and a model of hapten conformation has been inferred. This study serves as an example of the nature of analyses which are possible using modern NMR methods.

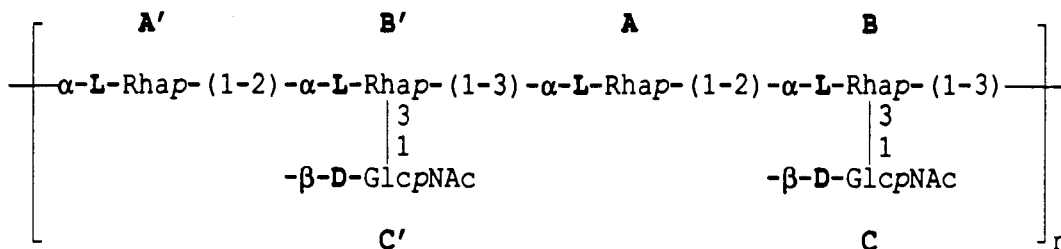
Many problems currently being addressed by chemists require

collaboration with workers in other fields of science. The synthetic compounds prepared in the chemist's laboratory often provide the key elements in some other study in another field of endeavor, such as biology, biochemistry or immunology. This thesis is an example of the interdisciplinary approach which is required if one wishes to investigate many of the interesting biological interactions of carbohydrates.

CHAPTER 2

I: INTRODUCTION

The *Streptococci* Group A cell-wall polysaccharide is composed of a rhamnose backbone consisting of α -L-(1-2) and α -L-(1-3) linkages with β -D-N-acetylglucosamine units at the 3-positions of every other rhamnose residue.⁶ Thus far, workers in our laboratory have synthesized



disaccharide (BC)¹⁸, trisaccharide (AB'C')¹⁸, and tetrasaccharide [AB'(C')A']¹⁹ segments of this polysaccharide structure as their propyl glycosides. In addition, the synthesis of the disaccharide (BC) as its 8-(methoxycarbonyl)octyl glycoside has been reported.²⁰ This chapter describes the synthesis of trisaccharide [B(C)A], pentasaccharide [B(C)AB'C'] and hexasaccharide [B(C)AB'(C')A'] segments of this polysaccharide structure. Of particular significance is the synthesis of a branched trisaccharide which is suitably functionalized to serve as a common intermediate in the synthesis of higher order oligosaccharides of the *Streptococci* Group A polysaccharide. The preparation of protein glycoconjugates of the trisaccharide and the pentasaccharide sequences is also described.

A: Glycoside Synthesis

The synthetic problems encountered in glycoside synthesis are those

of selective protection and deprotection of hydroxyl functional groups at specific positions, activation of specific centres, and the control of the stereochemical outcome of the coupling reactions. The protection of functional groups²¹ follows from other fields of organic synthesis; however, the specific activation of the anomeric centres, and the coupling of two sugar residues are synthetic methods which are unique to carbohydrate chemistry. The techniques used in the syntheses described in this thesis are common to modern synthetic carbohydrate chemistry, and these methods are well described in several review articles;²² however, a brief description of some of the salient features of glycoside synthesis is given below.

The acetal linkage between two sugar residues is termed a glycosidic linkage. The bond is formed between the C-1 (the anomeric carbon) of one residue, and a hydroxyl group of the other residue (the aglycone). Monosaccharides are polyhydroxyl compounds; therefore, the selective addition of another monosaccharide unit usually requires that all but one hydroxyl group be protected by blocking groups. In general, the chemical synthesis of a glycosidic bond involves the nucleophilic attack of a hydroxyl group of one residue on the activated anomeric carbon of another residue. In the nomenclature of carbohydrate chemistry, the protected sugar unit with the activated anomeric centre is called the glycosyl donor, and the sugar unit with one hydroxyl group free is termed the glycosyl acceptor (see Figure 2-1). Three main problems must be overcome in the formation of a glycosidic bond; firstly, the protection of the various hydroxyl groups of the two reacting species, with selective deprotection of the desired hydroxyl group of the glycosyl acceptor, secondly, the activation of the anomeric centre of the glycosyl donor,

and thirdly, control of the stereochemistry of the coupling reaction.

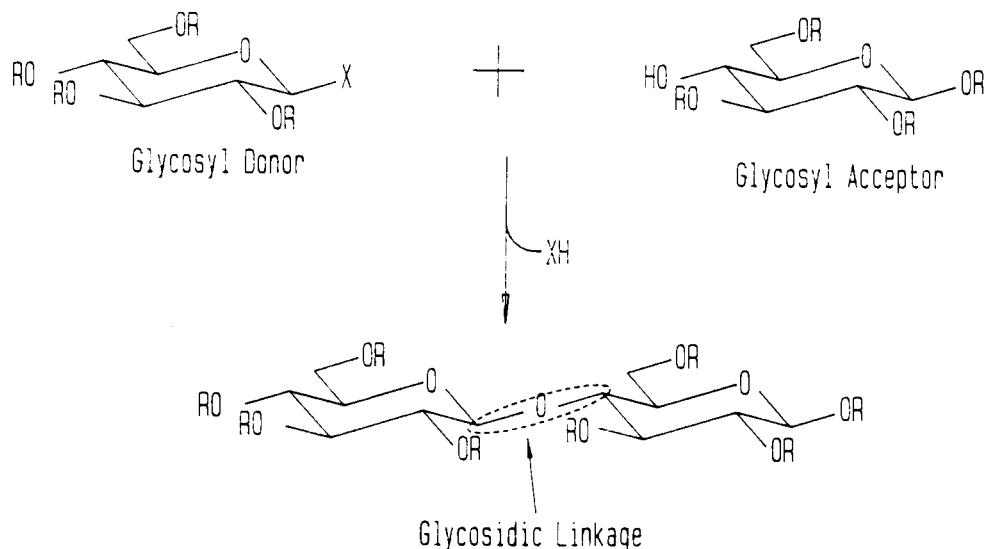


Figure 2-1: A generalized scheme for a glycoside synthesis.

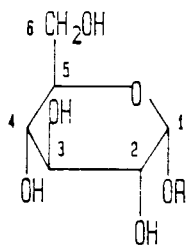
B: Protecting Groups in Carbohydrate Chemistry

The hydroxyl groups of the sugar residues are the primary functional groups requiring protection and selective deprotection. The blocking groups used, are for the most part, in common usage in the various fields of synthetic organic chemistry. Since several hydroxyl groups usually need to be protected, with one position remaining free, a variety of hydroxyl protecting groups are required. Different protecting groups are stable to different reaction conditions, and may be removed by use of a range of conditions. By careful selection of the combination of protecting groups, specific hydroxyl groups within a molecule may be selectively deprotected during a synthetic sequence. Several different classes of hydroxyl protecting groups are commonly used, including various acyl groups, ethers, acetal groups, and orthoesters. A

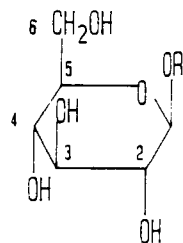
comprehensive list of protecting groups for various functional groups, and descriptions of their use may be found in reference 21. In many sugars, one of the hydroxyl groups is substituted with an amino group, and this group requires protection as well. Various protecting groups have been used for this purpose including the acetyl group and the phthalimido group. In addition, an azide group may be reduced in the final stages of the synthesis to yield the amino sugar.

C: Stereochemical Control of Glycosylation Reactions

There are two general types of glycosidic linkages, 1,2-*cis* and 1,2-*trans*, where the hydroxyl group at the two position of the sugar unit is either *cis* or *trans*, respectively to the exocyclic oxygen atom at the 1-position (see Figure 2-2). Different strategies are used for the



1,2-*cis* linkage



1,2-*trans* linkage

R= another sugar residue

Figure 2-2: Two main types of glycosidic linkages.

preparation of these two types of linkages; in general, the 1,2-*trans* linkages are easier to form than the 1,2-*cis* linkages. The usual method for the formation of 1,2-*trans* linkages is to have an activated glycosyl

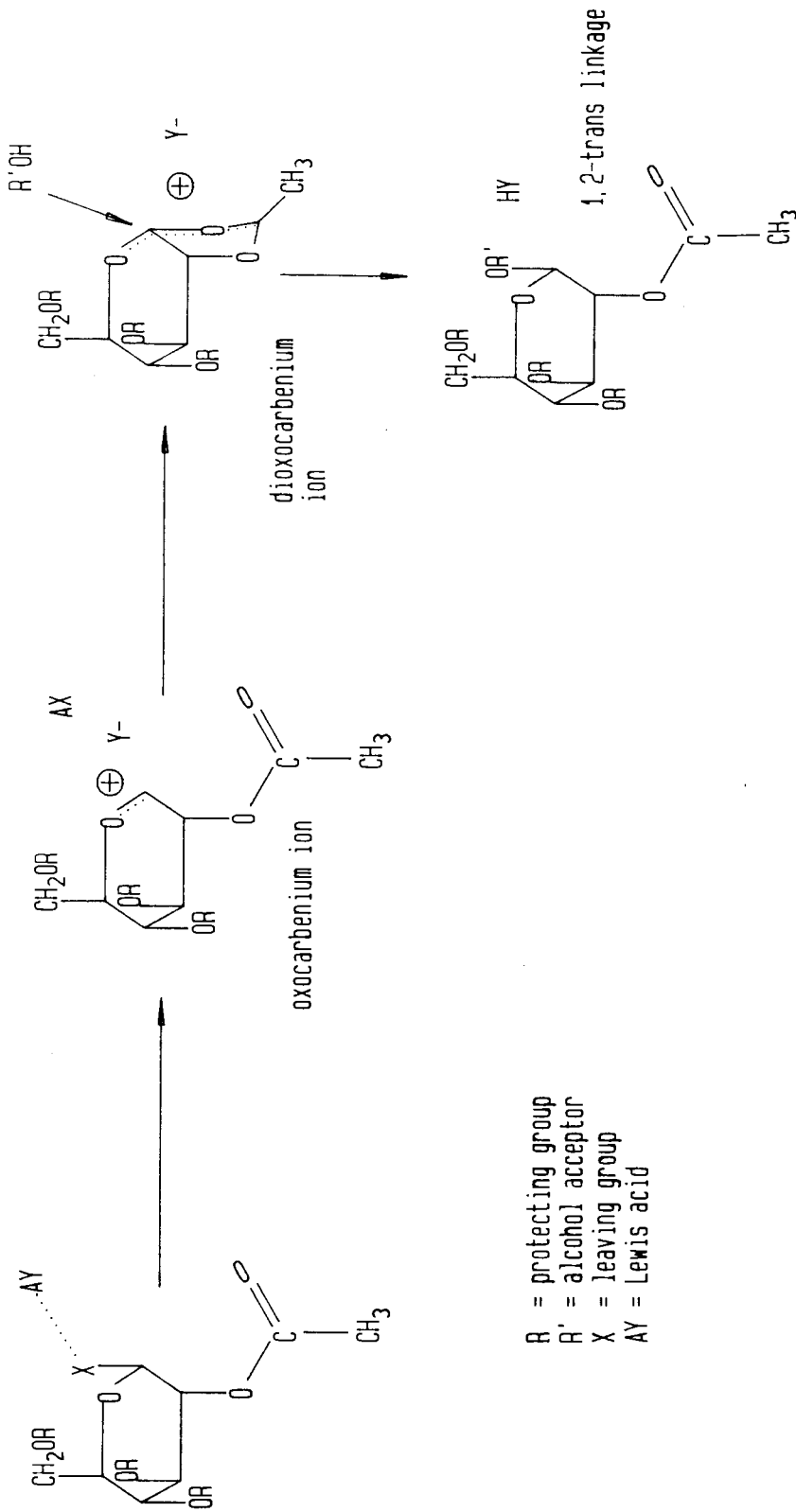


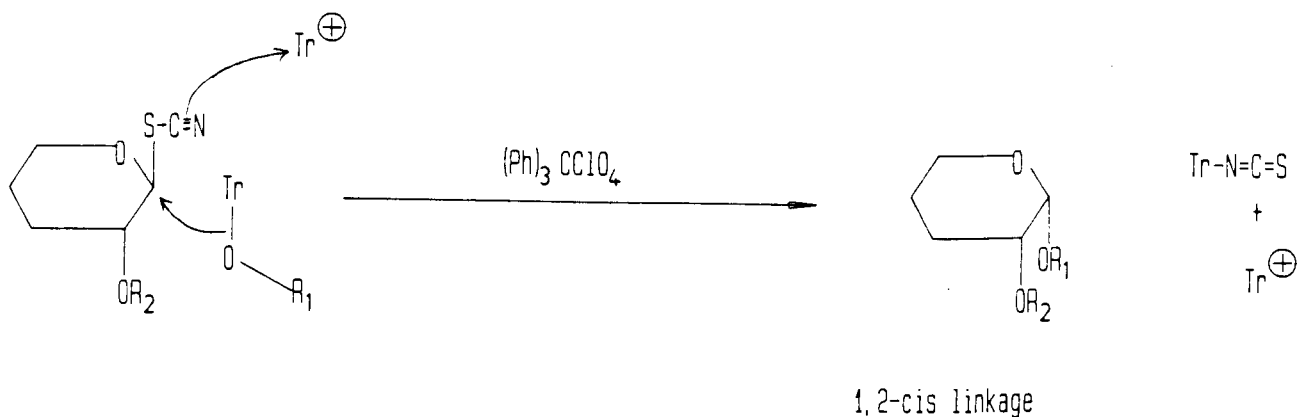
Figure 2-3: Formation of a 1,2-trans glycosidic linkage.

donor which has at the 2-position, a participating group such as an acetate or a benzoate group. Under Lewis acid catalysis the leaving group at the anomeric position departs and results in the formation of an oxocarbenium ion. This oxocarbenium ion is stabilized by the participation of the carbonyl oxygen of the substituent at the 2-position to give a dioxocarbenium ion. The alcohol component of the acceptor substrate then opens the dioxocarbenium ring in an S_N2 fashion, with the neighbouring-group protecting the *cis* face of the ring from nucleophilic attack²³ (see Figure 2-3). A particularly effective protecting group for the production of 1,2-*trans* linkages in 2-amino sugars is the phthalimido group.²⁴

Formation of 1,2-*cis* glycosidic linkages can be achieved by use of blocking groups at the two position which are inactive in neighbouring group participation. Blocking groups such as benzyl ethers are often used for this purpose, or in the case of 2-amino sugars the azido group may be used. The glycosylation reaction is then carried out such that the hydroxyl group of the glycosyl acceptor displaces the leaving group (which is *trans* to the group at the 2-position) of the glycosyl donor in an S_N2 fashion and results in inversion of configuration at the anomeric centre. If glycosyl halides are being used as donors, then the very reactive β -anomers are required for the formation of 1,2-*cis* linkages in the gluco- and galacto-series of sugars. The use of the reactive β -halides may be problematic; a method which circumvents this problem is the use of the "halide-ion catalyzed glycosylation" reaction of Lemieux et al.²⁵ By use of this approach one can form an α -glycosidic linkage starting from the more stable α -halide. The isomerization of the α -halide to the more reactive β -halide is catalyzed, *in situ*, by halide

ions and silver salts. The alcohol acceptor then reacts with the more reactive β -halide, and proceeds with inversion to give an α -glycoside.

Another method for the generation of 1,2-*cis* linkages has been developed by Kochetkov et al.²⁶ In this method a *trans* glycosyl thiocyanate is prepared, and is reacted together with an acceptor which has its alcohol function protected as a trityl ether; the reaction is catalyzed by triphenylmethylm perchlorate (see Figure 2-4). The



R₁ = another sugar unit

R₂ = ether protecting group

Tr = -C(Ph)₃, (Tr[⊕] is catalytic)

Figure 2-4: Formation of 1,2-*cis* glycosidic linkages, by use of glycosyl thiocyanates.

mechanism is thought to involve simultaneous attack of the triphenylmethylm ion on the nitrogen atom of the thiocyanate group, and backside attack of the nucleophilic oxygen of the *O*-trityl group on the anomeric carbon, and results in an S_N2 displacement of the thiocyanate group and inversion of configuration at the anomeric centre, to yield the 1,2-*cis* glycoside.

D: Activation of The Anomeric Centre

Many strategies have been developed for the activation of the anomeric centre; some of the most common methods are the use of glycosyl halides, the imidate method^{22b}, and activation by use of thioglycosides.²⁷

1: Glycosyl Halides

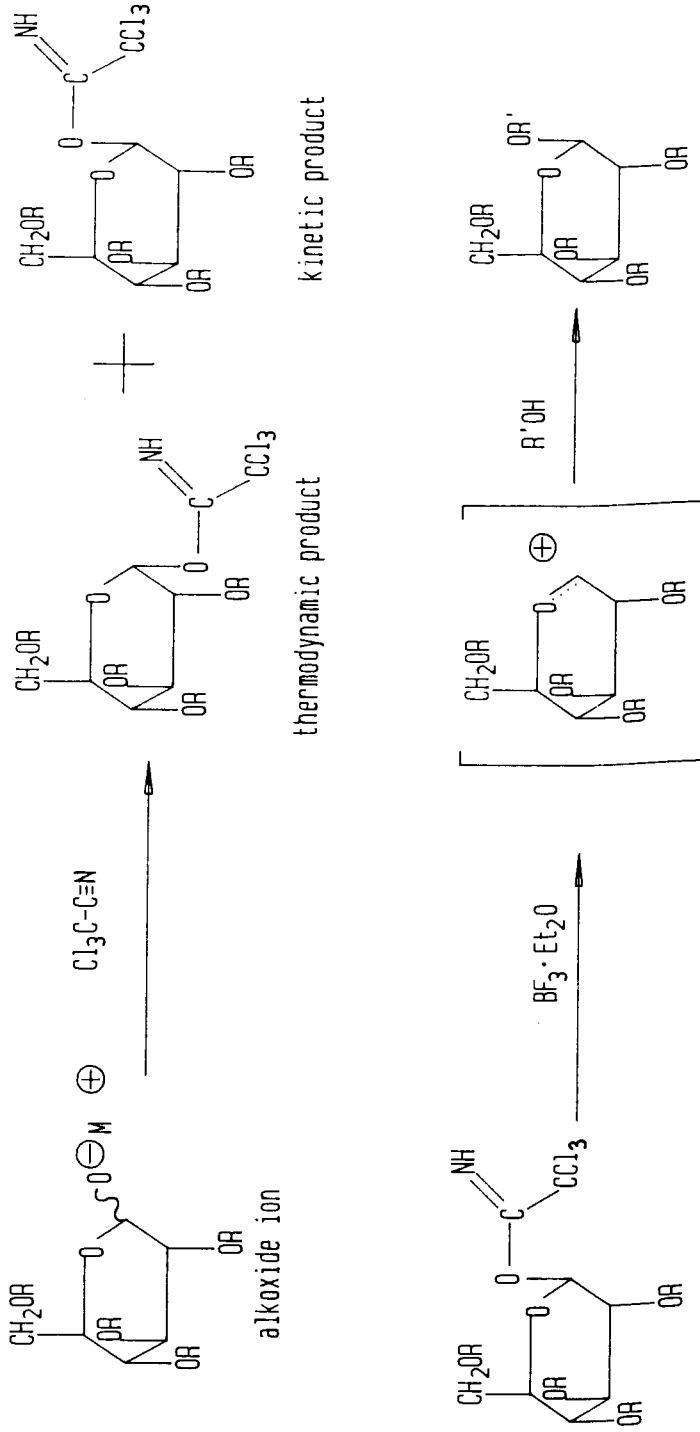
The use of glycosyl halides to activate the anomeric centre was one of the first methods used to prepare oligosaccharides,²⁸ and is still successfully used in a wide variety of applications. Glycosyl halides may be generated in a variety of ways; one of the most common is the reaction of a protected 1-O-acetate with a strong acid such as HCl or HBr. The subsequent glycosylation reaction is promoted with a variety of Lewis acids such as silver salts or mercury salts. If silver salts are used as glycosylation promoters the reaction is termed a Königs Knorr reaction,^{28a} whereas Helferich conditions²⁹ utilize mercury salts as glycosylation promoters. The choice of glycosylation promoter influences the reactivity of the glycosyl donor, and therefore the stereochemical outcome of the reaction is also often affected. In general, with more reactive glycosyl donor species, it is more difficult to control the stereochemistry of the glycoside synthesis. The order of reactivities of the various glycosyl halides is $F < Cl < Br$, with the glycosyl chlorides and bromides the most commonly used species. One of the disadvantages of the glycosyl halides is that they are relatively unstable, and generally cannot be stored for long periods of time. The strength of the various glycosylation promoters are $AgTfI > AgClO_4 > HgBr_2 > Hg(CN)_2$.

2: Glycosyl Imidates

As an alternative to the glycosyl halide method, activation of the anomeric centre by use of glycosyl imidates has been developed.³⁰ Glycosyl imidates may be prepared by treating a blocked hemiacetal with trichloroacetonitrile under mild basic conditions, to give an *O*-glycosyl trichloroacetimidate^{30a} (see Figure 2-5). Both the α - and the β -anomers of the glycosyl imidate may be prepared; the α -anomer is the thermodynamic product and the β -anomer the kinetic product. The glycosyl imidates may be used as glycosyl donors by use of $\text{BF}_3 \cdot \text{Et}_2\text{O}$ or Me_3SiCF_3 as glycosylation catalysts. The general rules for the formation of 1,2-*trans* and 1,2-*cis* glycosides apply to the use of glycosyl trichloroacetimidates as glycosyl donors. One of the advantages of glycosyl imidates, over glycosyl halides, is that they are more stable, allowing for the isolation and storage of either of the α - or β -anomers. It is noteworthy that activation of a tetrasaccharide, by means of a glycosyl imidate, has led to the successful synthesis of a bisected undecasaccharide structure.³¹

3: Thioglycosides

The use of thioglycosides as glycosyl donor substrates is becoming one of the main glycosylation methods currently in use.²⁷ Thioglycosides may be prepared from the corresponding 1-*O*-acyl sugars, by treating the acylated sugar with the appropriate thiol (usually methanethiol or ethanethiol) under Lewis acid catalysis.³² The resulting thioglycosides are stable and can be purified by chromatography, and in some cases recrystallization. The activation of the thioglycoside is achieved through the generation of a sulfonium ion and results in a good leaving



A = acyl protecting group

A' = alcohol acceptor

1,2-trans linkage

Figure 2-5: Trichloroacetimidate method for the formation of glycosidic linkages.

group. A variety of reagents have been used to activate the sulfur atom of the thioglycoside including methyl triflate^{27c,33}, dimethyl(methylthio)sulfonium triflate^{27b,27c,34} and methylsulphenyl triflate³⁵, benzeneselenenyl triflate³⁶ and nitrosyl tetrafluoroborate.³⁷ Good results are obtained with these thiophilic glycosylation promoters however, their use is somewhat hazardous due to the toxicity of some of these reagents. A novel method for the *in situ* activation of thioglycosides with bromine has been developed.³⁸ In this method a glycosyl bromide is generated from the thioglycoside by treatment with bromine.^{27c,39} The resulting glycosyl bromide is then activated by the presence of silver triflate. The reaction is performed in one pot, containing the thioglycoside, the alcohol acceptor, bromine and an acid acceptor such as tetramethyl urea. This method promises to be an efficient alternative to the use of toxic thiophilic promoters.

4: Emerging Techniques

One new method of glycoside synthesis is the oxidative coupling of glycols recently reported by Danishefsky *et al.*⁴⁰ The addition of alcohols to the double bonds of glycols was first carried out by Lemieux⁴¹ and Thiem⁴² by use of iodonium perchlorate or *N*-iodosuccinimide as promoters. Danishefsky has used this method to couple two glycols together, where one glycol is protected with ether protecting groups, and the acceptor glycol is protected with acyl groups. Since the glycol with the ether protecting groups is activated, relative to the glycol with ester groups, glycoside formation results with no self condensation of the acceptor glycol. The resulting disaccharide is itself a glycol, and replacement of the ester blocking groups with ether groups again

activates the glycal allowing for further rounds of glycal addition (see Figure 2-6). This manipulation of protecting groups of the newly formed product is one of the disadvantages of this method. Glycoside formation results in α -stereoselectivity with a 1,2-*trans* iodine atom at the

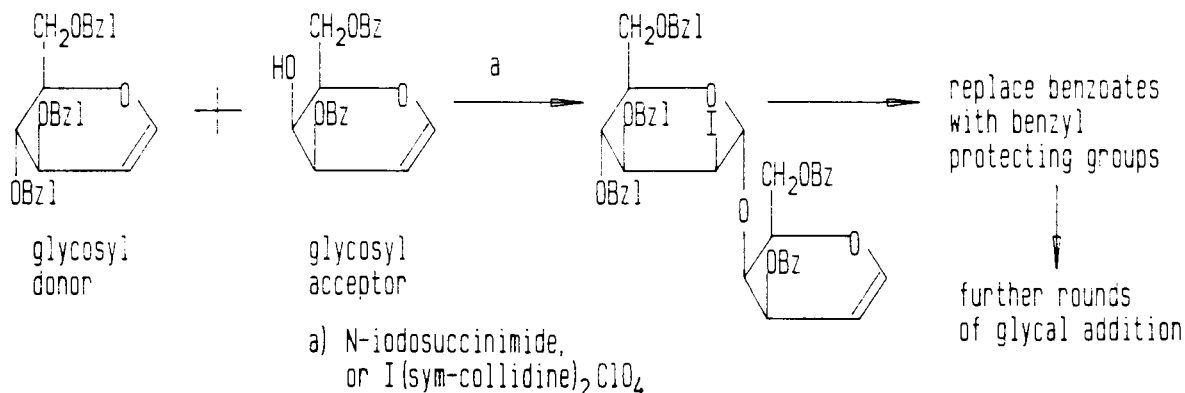
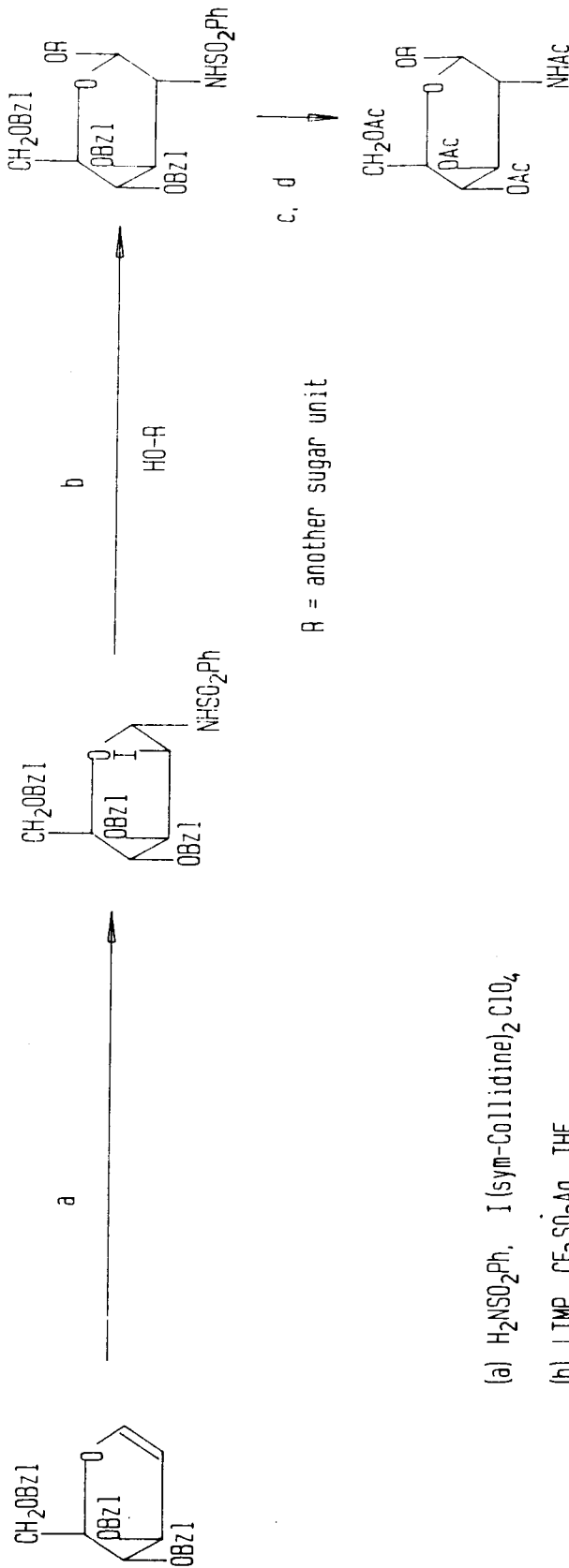


Figure 2-6: Haloetherification synthesis of glycosides.

2-position of the former donor moiety. There is some difficulty in replacing the 2-iodo groups of the growing oligosaccharide product with oxygen and nitrogen heteroatoms⁴⁰, requiring up to four additional steps to incorporate these functionalities.

A more direct route to 2-amino β -linked glycosides starting from glycals has also been developed by Danishefsky et al (see Figure 2-7).⁴³ The glycal is first treated with benzenesulfonamide in the presence of iodonium di-*sym*-collidine perchlorate to give the 2- β -iodo-1- α -sulfonamido-hexoses. When this species was treated with an excess of base in the presence of an acceptor saccharide, 2- α -sulfonamido-1- β -linked glycosides were formed. The N-acetylated products could be formed by



(a) H₂NSO₂Ph, I (sym-Collidine)₂ClO₄

(b) LTMP, CF₃SO₃Ag, THF

(c) Na/NH₃

(d) Ac₂O/py

Figure 2-7: Synthesis of glycosides via sulfonamidoglycosylation of glycals.

reduction of the sulfonamide to the free amine by use of sodium in ammonia, followed by acetylation.

Another method being developed by Danishefsky is glycoside synthesis via epoxide opening⁴⁴ (see Figure 2-8). The epoxide may be prepared from

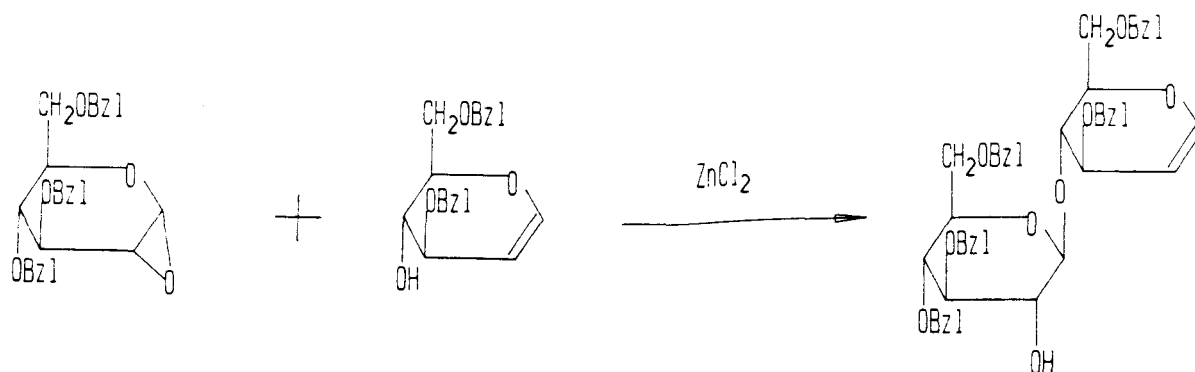


Figure 2-8: Epoxide opening to give glycosides.

the corresponding glycal by treatment of the glycal with dimethyldioxirane.^{44a, 45} Addition of a glycosyl acceptor to the α -epoxide results in formation of a β -glycosidic linkage with a hydroxyl group *trans* at the 2-position of the former epoxide species. If the acceptor substrate is a glycal, then the resulting disaccharide may be converted into an epoxide donor for further rounds of addition, or alternatively, if an α -linkage is required, then the newly formed glycal may be used directly as a glycosyl donor.

Work recently carried out by Fraser-Reid *et al* is complementary to the methods developed by Danishefsky. Fraser-Reid has developed the use of pent-4-enyl glycosides as glycosyl donors.⁴⁶ The pent-4-enyl glycosides may be hydrolyzed under mild conditions⁴⁷ and the resulting

oxocarbenium ion intermediates may be trapped with the hydroxyl group of an acceptor alcohol, and results in glycoside formation.⁴⁸ The use of ether protecting groups on the donor residue, and ester protecting groups on the acceptor residue, gives the so called⁴⁹ "armed" and "disarmed" pent-4-enyl glycosides. Addition of the "armed" residue to the "disarmed" residue gives a "disarmed" disaccharide. In an analogous fashion to Danishefsky's work with the glycols, replacement of the ester blocking groups with ether groups, then gives the "armed" disaccharide ready for further rounds of glycosylation. The same idea of "armed" donors and "disarmed" acceptors has been extended to the use of thioglycosides by van Boom et al,⁵⁰ where the donor thioglycoside is blocked with ether groups and the acceptor thioglycoside is blocked with ester groups.

E: Use of Enzymes in Glycoside Synthesis

Chemical synthesis is still the most common method used when specific oligosaccharide sequences are required. Recently, the use of enzymes has been coupled with chemical synthesis for the preparation of carbohydrate structures.⁵¹ There are two types of enzymes which have been used in the synthesis of carbohydrates; the glycosyltransferases,^{52,53} and the glycosidases.^{53,54}

1: Glycosyltransferases

The glycosyltransferases make up a group of enzymes that are responsible for the synthesis of specific glycosidic linkages in biological systems⁵². The reaction consists of the transfer of an activated monosaccharide unit to an acceptor substrate (see Figure 2-9).

The activated monosaccharide is usually in the form of a nucleotide sugar with the nucleotide moiety a diphosphonucleoside; uridine and guanosine diphosphosugars are the most common donor substrates. The acceptor group may be a protein or a lipid, or in a growing polysaccharide chain, a

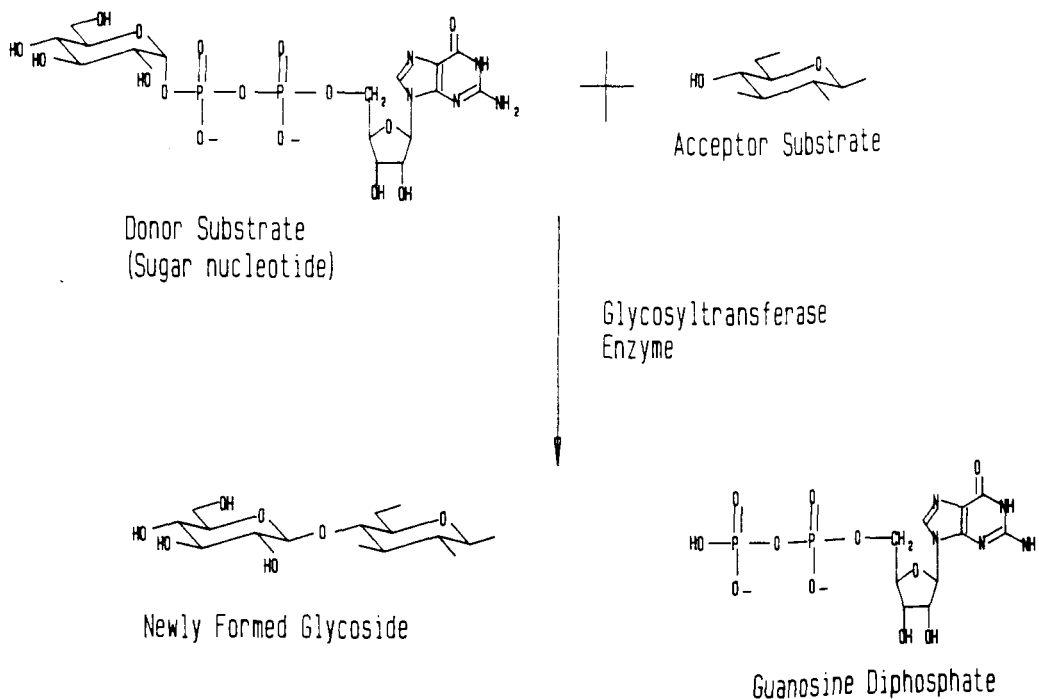


Figure 2-9 A generalized scheme for the glycosyltransferase-catalyzed transfer of a glucosyl unit to a glycosyl acceptor.

hydroxyl group of the non-reducing end in the polysaccharide chain. Glycosyltransferases may be used in the *in vitro* preparation of oligosaccharide structures. This requires that the donor monosaccharide unit be available in the activated diphosphonucleoside form. The glycosyltransferase specific for the desired glycosidic linkage may then be used to catalyze the transfer to a suitable acceptor molecule. The glycosyltransferases show a high degree of specificity for both the donor molecule and the acceptor molecule, yielding both regio- and

and stereospecific glycosidic linkages. These enzymes have, however, been shown to accept modified donors⁵⁵ and acceptors^{56,57} where the modifications are sterically conservative (eg. deoxygenated substrates).

2: Glycosidases

The glycosidases are a group of enzymes that catalyze the cleavage of glycosidic bonds *in vivo*⁵⁴. The endo-glycosidases cleave bonds within a polysaccharide segment, and the exo-glycosidases cleave the glycosidic bonds of terminal monosaccharide units. The mechanism of cleavage of the glycosidic bond is thought to occur in the same fashion as acid catalyzed

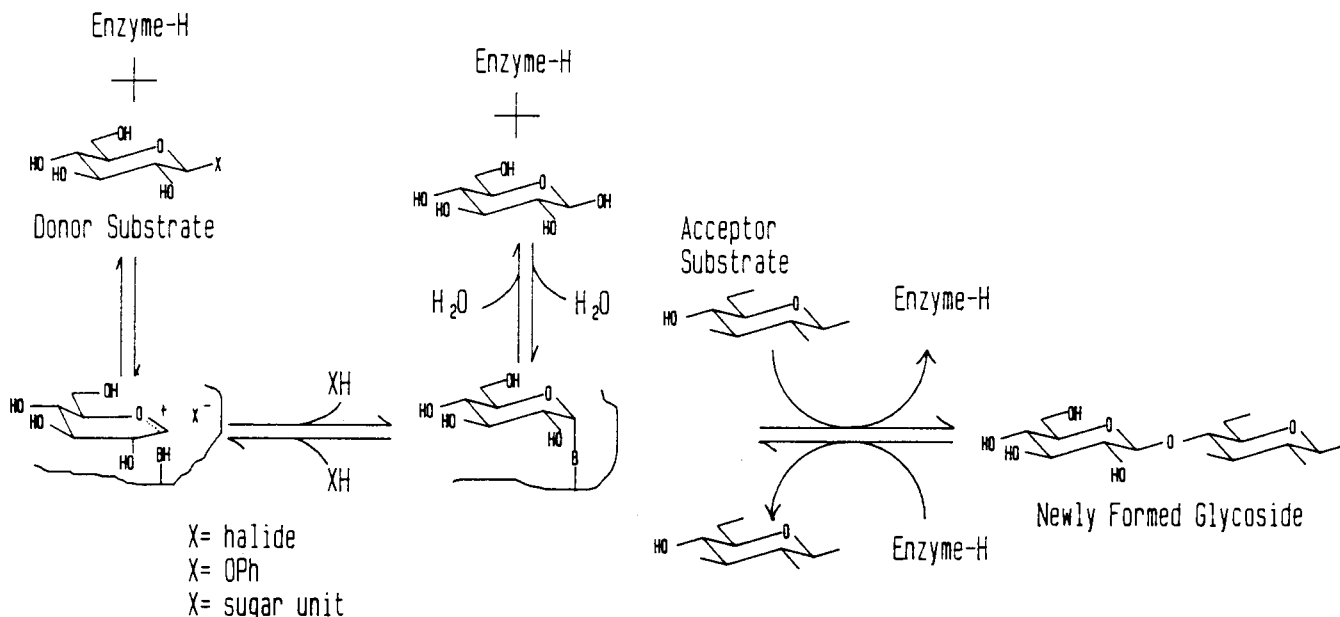
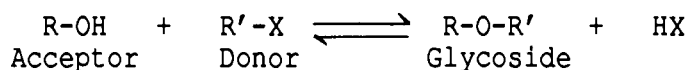


Figure 2-10 A generalized scheme for a glycosidase-catalyzed transfer of a glucosyl donor to a glucosyl acceptor.

cleavage, going through an oxocarbenium ion intermediate. The hydrolysis reaction generally proceeds with net retention of configuration meaning that either the reaction is a double displacement reaction going through an enzyme bound intermediate⁵⁸ or that the carbocation intermediate is

stabilized and shielded by the enzyme such that only one face of the intermediate is available for attack. In either of these mechanisms the intermediate could be trapped by a nucleophile other than water, leading to the transfer of the glycosyl moiety to the intervening nucleophile. If the nucleophile is another carbohydrate moiety, a transglycosylation reaction occurs⁵⁹ (see Figure 2-10). Glycosidases may be made to catalyze the reverse reaction (the formation of glycosides) by adjusting the reaction conditions so that the equilibrium is driven towards the formation, rather than the hydrolysis, of glycosidic bonds.



In general the glycosidases show high specificity for the glycosyl moiety and for the glycosidic linkage, but show little specificity for the aglycone moiety.^{51,54} Glycosidases have been shown to accept a variety of glycosyl donors other than the free monosaccharides.⁵⁹ These include aryl glycosides, glycosyl fluorides, and disaccharides. Because of the low specificity of the glycosidases for the aglycone, regiochemical isomers may be formed when glycosidases are used synthetically.

The prospects for the use of enzymes in oligosaccharide synthesis are increasing as more enzymes become available. The combined use of chemical synthesis, in conjunction with stereospecific enzymatic coupling of the synthetic intermediates, is becoming a viable alternative to traditional total chemical synthesis.⁵⁷ The developments occurring in both chemical glycosylation methods, and in enzymatic glycosylation synthesis, give the chemist valuable new tools with which to prepare complex carbohydrate structures for biological or physical studies.

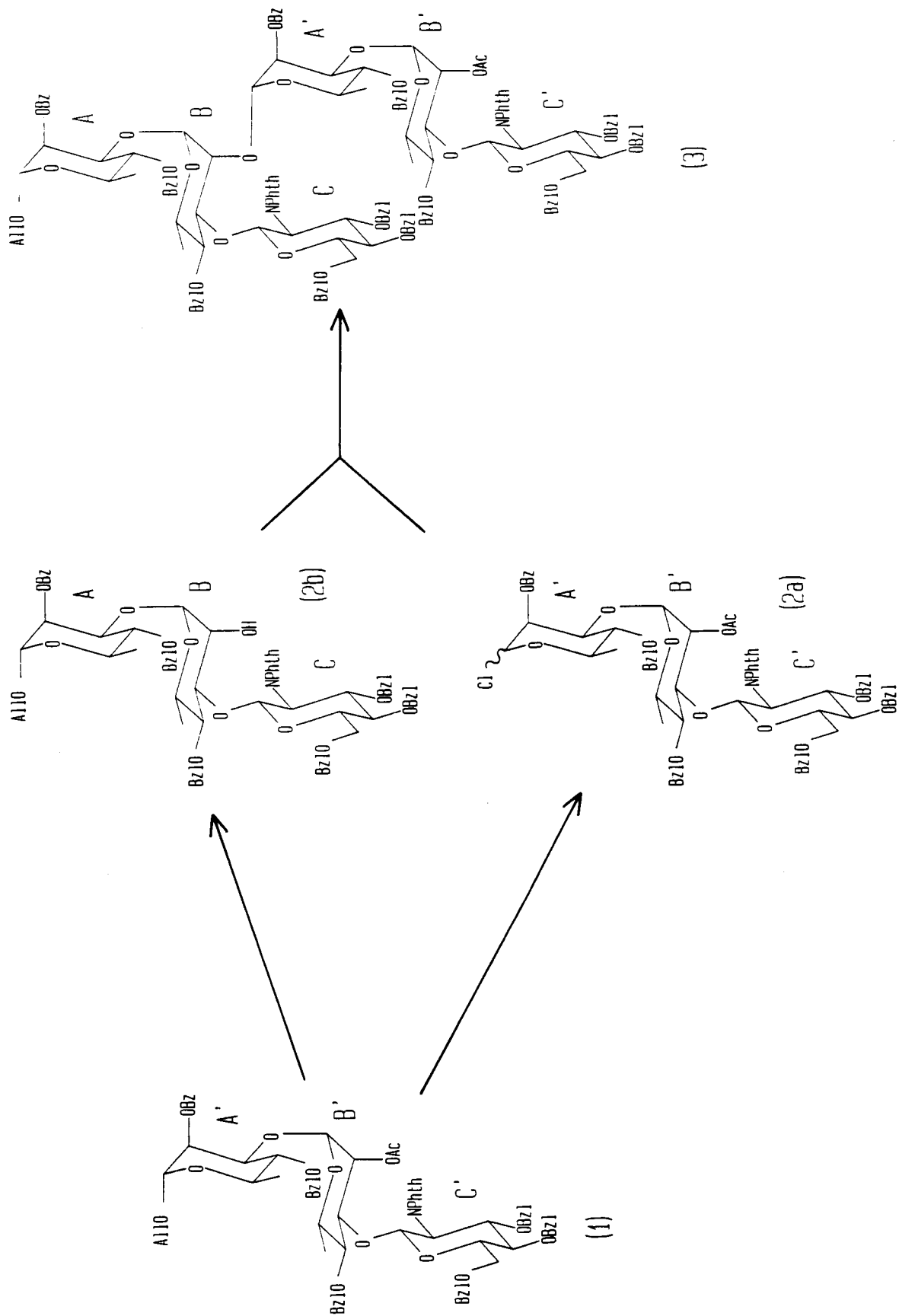
II: RESULTS AND DISCUSSION

A: Synthesis

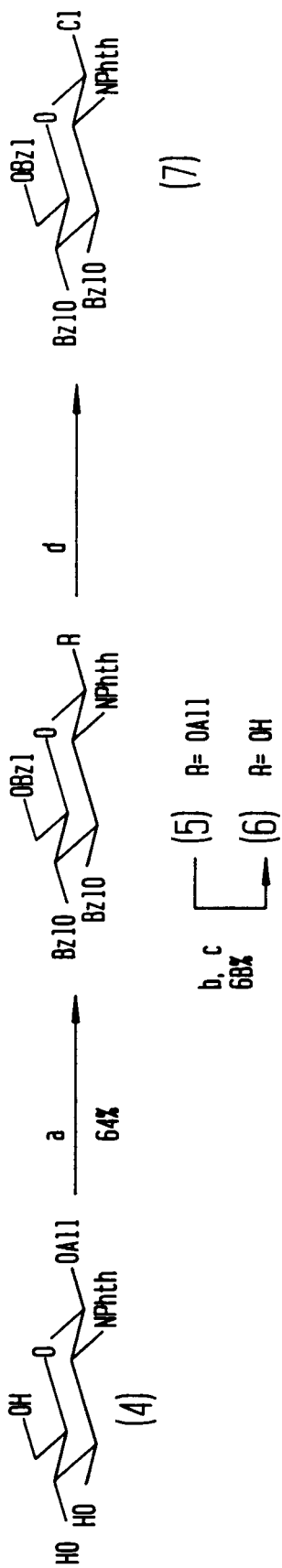
Retrosynthetic analysis of the cell-wall polysaccharide of *Streptococci* Group A bacteria indicated that a suitably functionalized trisaccharide could be used to prepare higher order sequences of the antigenic determinant. A trisaccharide unit such as (1) would be suitable (Scheme 1). This trisaccharide features different participating groups at the 2- and the 2'-positions, with persistent blocking groups at all remaining positions. The synthesis of this unit would rely on conventional blocking group chemistry, as well as some of the monosaccharide blocks utilized in previous syntheses carried out in our laboratory on the *Shigella flexneri* variant Y O-antigen.⁶⁰ The trisaccharide (1) could be made to function as either a glycosyl donor or a glycosyl acceptor in future glycosylation reactions. Removal of the 1-O-allyl group by isomerization to the prop-1-enyl group,⁶¹ followed by hydrolysis to the hemiacetals,⁶² and conversion of the hemiacetals to the glycosyl halide (2a) by use of Vilsmeier-Haack reagents,⁶³ would allow the trisaccharide to function as a glycosyl donor in glycosylation reactions. Alternatively, selective removal of the 2'-O-acetyl group to give the free hydroxyl, would afford the trisaccharide acceptor (2b). Glycosylation of the trisaccharide donor (2a) with the trisaccharide acceptor (2b) would yield a hexasaccharide (3) which could be manipulated in the same fashion as the parent trisaccharide (1) (see Scheme 1); hence oligosaccharides of increasing size could be synthesized.

The required trisaccharide (1) would have to contain persistent blocking groups at the 2,3,4, and 6-positions of the β -D-GlcpNAc unit and

Scheme 1



Scheme 2



(a) NaH, $C_6H_5CH_2Br$, THF, 36h;

(b) Rh($PPPh_3$) $_3Cl$, EtOH-H $_2$ O (9:1), reflux 18h;

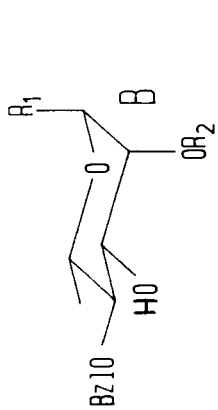
(c) HgO/HgCl $_2$, $(CH_3)_2CO-H_2O$ (10:1), 18h;

(d) $C_2O_2Cl_2$, DMF, CH_2Cl_2 , 2h.

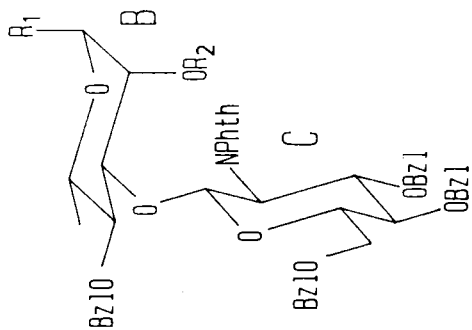
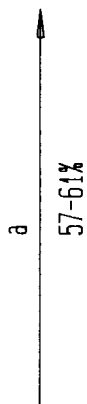
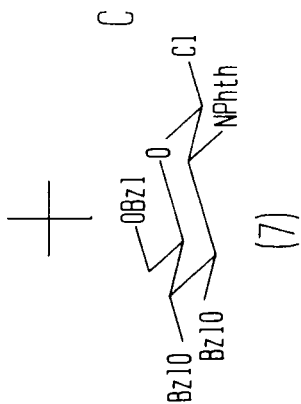
at the 4-position of the rhamnose units, leaving the 2'-position of the rhamnose unit accessible for glycosylation. We envisaged the use of benzyl ethers as the persistent blocking groups for the hydroxyl functions,⁶⁴ a phthalimido group for protection of the amino function, and an acetate as the latent blocking group on the 2'-position of rhamnose.

Accordingly, allyl 2-deoxy-2-phthalimido- β -D-glucopyranoside (4)^{60b} was converted to its tri-O-benzyl derivative (5) with sodium hydride and benzyl bromide in 64% yield (Scheme 2). Conversion of the allyl group in (5) to the prop-1-enyl group by use of Wilkinson's catalyst⁶¹ and subsequent hydrolysis⁶² of the vinyl ether then afforded the hemiacetals (6). Treatment of the hemiacetals (6) with *N,N*-dimethyl(chloromethylene)ammonium chloride⁶³ then gave the glycosyl chloride (7) [68% yield from (5)]. To the best of our knowledge, compound (7) has never been used as a glycosyl donor; although a related donor, namely 4-O-acetyl-3,6-di-O-benzyl-2-deoxy-2-phthalimido- α,β -D-glucopyranosyl chloride, has been used in glycosylation reactions.⁶⁵ Glycosylation of allyl 2-O-acetyl-4-O-benzyl- α -L-rhamnopyranoside (8) with the donor (7) under silver trifluoromethanesulfonate promotion in the presence of collidine²⁴ afforded the disaccharide (9) in 61% yield (Scheme 3). Removal of the allyl group of (9), as with (6) above, gave the disaccharide hemiacetals which could be converted into the disaccharide donor (10), as with (7) above. Glycosylation of the rhamnosyl monosaccharide acceptor (11),^{60a} would then give the key trisaccharide (1). This reaction, however, did not proceed with the desired stereochemistry, yielding a preponderance of the β -anomer rather than the desired α -anomer. The stereochemistry was assigned by

Scheme 3

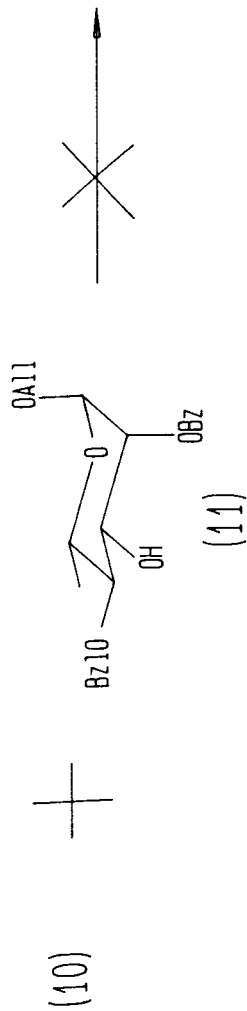


- (8) $R_1 = \text{OAlI}$; $R_2 = \text{AC}$
 (12) $R_1 = \text{O}(\text{CH}_2)_8\text{CO}_2\text{CH}_3$; $R_2 = \text{Bz}$



- (9) $R_1 = \text{OAlI}$; $R_2 = \text{AC}$
 (10) $R_1 = \text{Cl}$; $R_2 = \text{AC}$
 (13) $R_1 = \text{O}(\text{CH}_2)_8\text{CO}_2\text{CH}_3$; $R_2 = \text{Bz}$

(a) $\text{CF}_3\text{SO}_3\text{Ag}$, collidine, CH_2Cl_2 , -78 to 25°C .



(1)

comparison of the NMR spectral data with those of the authentic α -anomer. The authenticity of the α -anomer was confirmed, in turn, by examination of the $^1J_{13C-1H}$ values (see experimental) of the completely deblocked compound. In addition to the β -anomer being formed, a significant amount of the 1-2 β -elimination product was isolated, this species is presumably the result of abstraction of the proton at the 2-position of the disaccharide donor during the glycosylation reaction. This result led us to conclude that the disaccharide donor was too reactive a species. An analogous disaccharide donor was prepared with the 2-O-acetyl group replaced by a benzoate ester. This disaccharide donor was then added to the same monosaccharide acceptor (**11**) as before. This reaction was carried out to determine the effectiveness of the acetyl group as a participating group, relative to the benzoate group. The glycosylation went with very good stereoselectivity, and results in exclusive production of the desired α -anomer. However, the trisaccharide produced in this manner could not be elaborated into larger structures since it contained a benzoate group at both the 2- and the 2'-positions, but the result did show that the benzoate group was a better participating group than the acetate group. At this point, it was decided to abandon the route to the key trisaccharide (**1**) and instead, utilize the disaccharide (**9**) as the precursor to a disaccharide glycosyl acceptor, by removal of the 2-O-acetyl group.

Since it was also desirable to prepare the oligosaccharides in a form suitable for the coupling to carrier proteins (preparation of synthetic antigens), an analogous disaccharide to (**9**) was prepared. This disaccharide incorporated the 8-(methoxycarbonyl)octyl chain⁶⁶ for the covalent attachment to protein⁶⁷ in the subsequent preparation of the

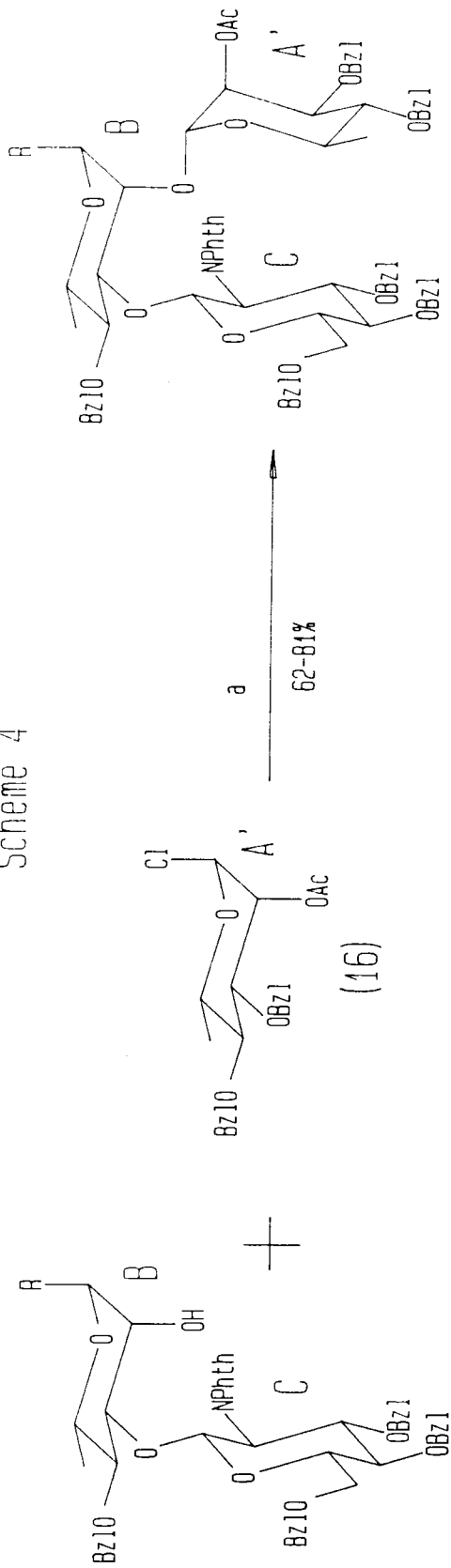
glycoconjugates. Glycosylation of 8-(methoxycarbonyl)octyl 2-O-benzoyl-4-O-benzyl- α -L-rhamnopyranoside (12) with the donor (7) under silver trifluoromethanesulfonate promotion in the presence of collidine²⁴ afforded the disaccharide (13) in 57% yield (see Scheme 3). Transesterification of (9) and (13) then yielded the required disaccharide acceptors, (14) and (15), respectively.

Treatment of the glycosyl acceptors (14) or (15) with 2-O-acetyl-3,4-di-O-benzyl α -L-rhamnopyranosyl chloride (16)⁶⁸ under silver trifluoromethanesulfonate promotion in the presence of 1,1,3,3-tetramethylurea⁶⁹ afforded the desired branched trisaccharides (17) and (18) in 81 and 62% yield, respectively (Scheme 4). Elaboration of the pentasaccharide unit now required a suitable trisaccharide donor. Thus, deallylation of trisaccharide (19),¹⁸ and conversion of the resultant hemiacetals (20) to the glycosyl chloride (21), as described above, furnished the desired compound (Scheme 5). Glycosylation of the acceptor (15) with the trisaccharide chloride (21) under analogous conditions to those described for the preparation of (17) and (18) then gave the pentasaccharide (22) in 43% yield.

Deprotection of compounds (17), (18), and (22) was accomplished by 1) base-catalyzed methanolysis of the ester functions, 2) hydrogenolysis of the benzyl ethers [and hydrogenation of the allyl ether in the case of (17)], 3) hydrazinolysis of the phthalimido group, and 4) selective N-acetylation of the resultant amine. Thus, the deblocked trisaccharides (23) and (24), and the pentasaccharide (25) were obtained.⁷⁰

Another attempt at the synthesis of a key trisaccharide intermediate, such as (1) was made where the benzyl groups on the glucosamine moiety

Scheme 4



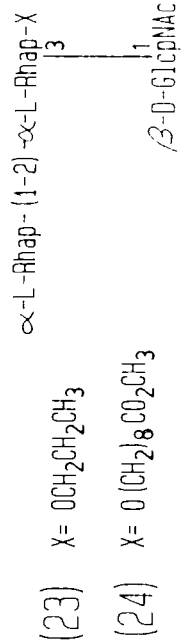
(a) CF₃SO₃Ag, TMU, CH₂Cl₂, -78 to 25°C;

(b) NaOMe, MeOH, 10h;

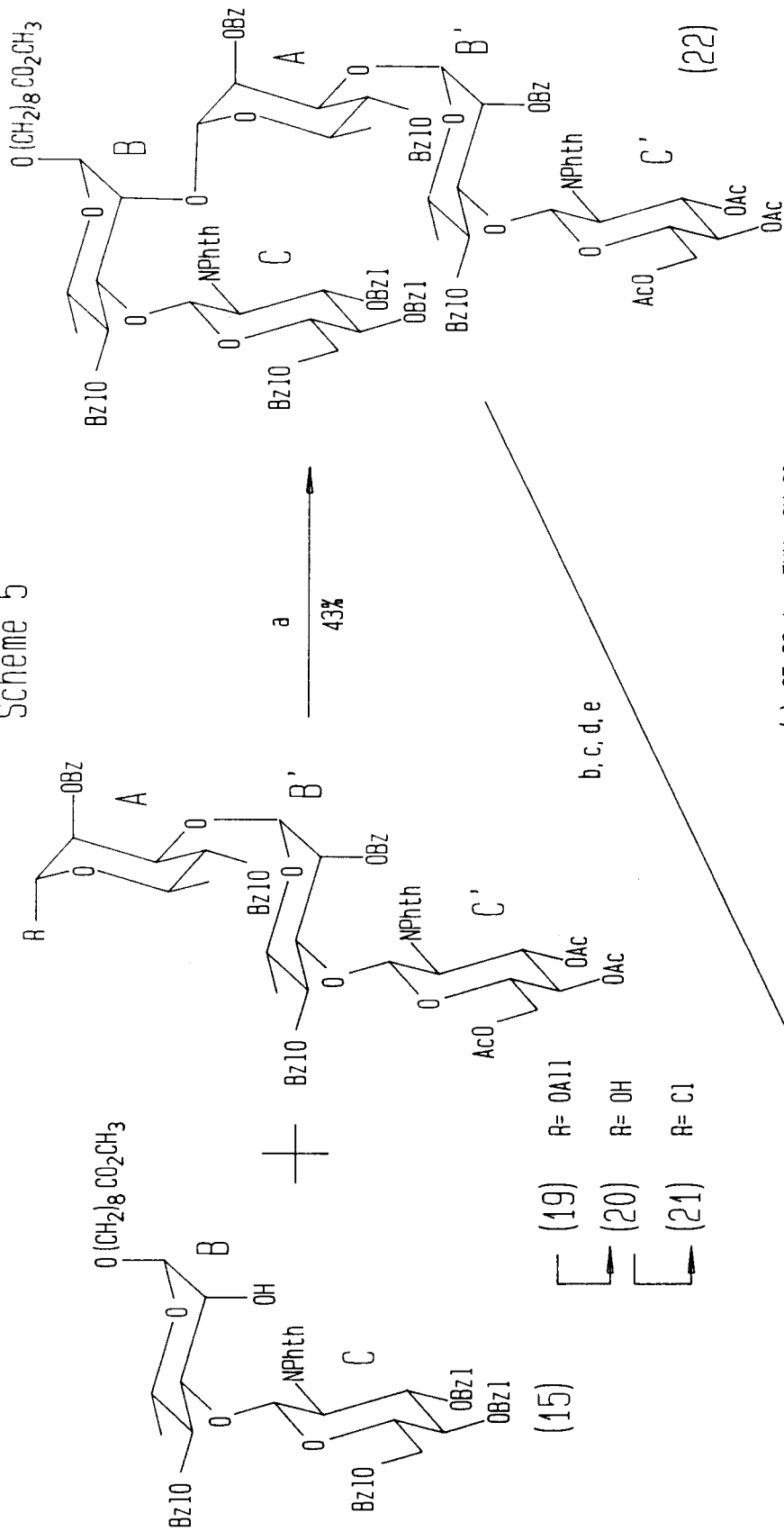
(c) Pd/C, H₂, HOAc-H₂O (4:1);

(d) N₂H₄·H₂O, EtOH, reflux;

(e) Ag₂O/MeOH.



Scheme 5



(a) CF₃SO₃Ag, TMU, CH₂Cl₂.

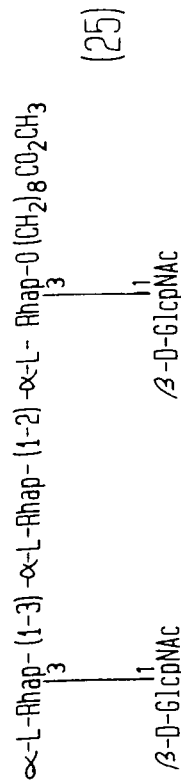
-78 to 25°C;

(b) NaOMe, MeOH, 10h;

(c) Pd/C, H₂, HOAc-H₂O (4:1);

(d) N₂H₄·H₂O, EtOH, reflux

(e) Ac₂O/MeOH.



(15) R = OAlI

(19) R = OH

(20) R = Cl

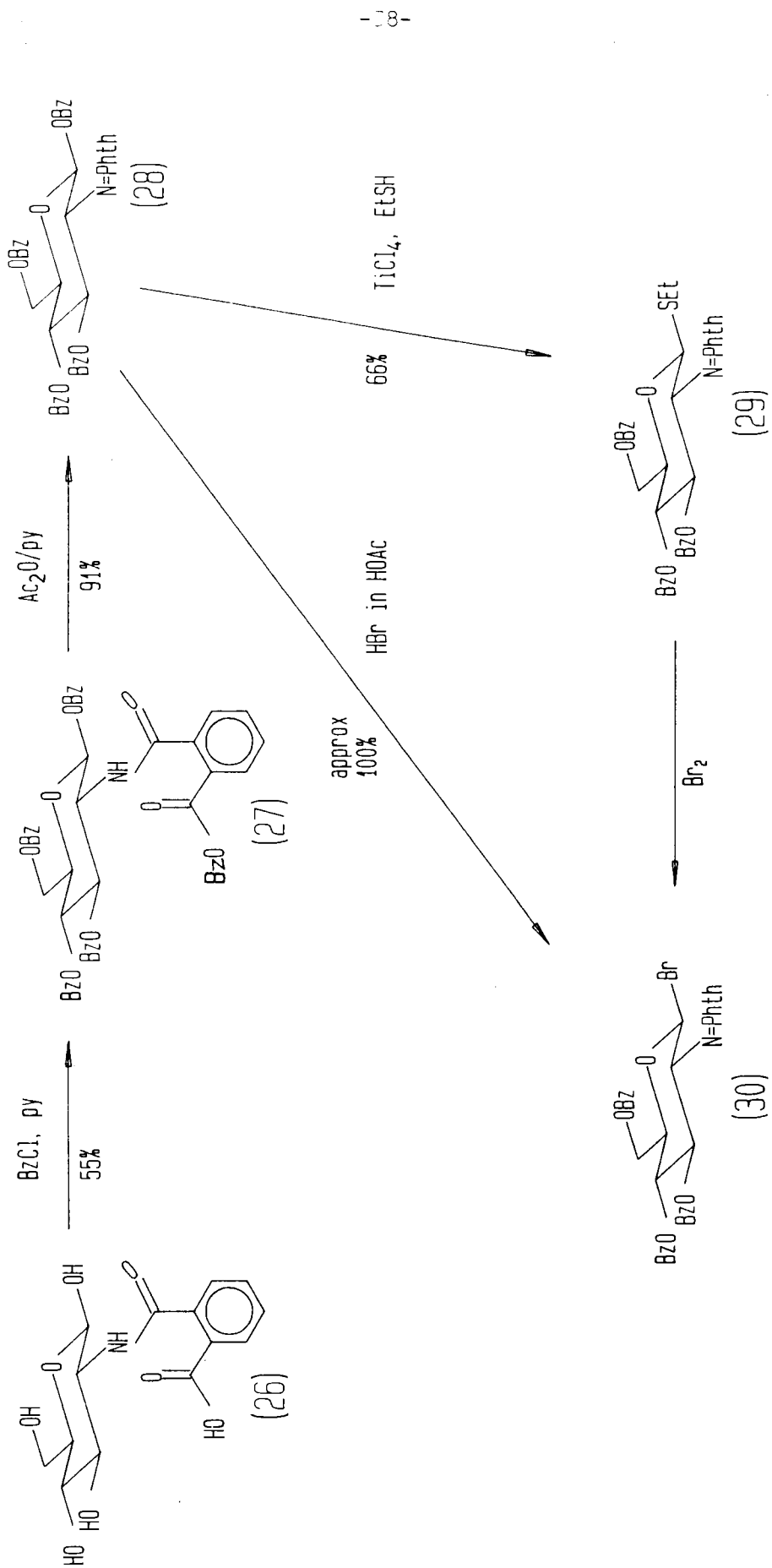
b, c, d, e

were replaced with benzoate esters. It was envisaged that the 2-*O*-acetyl group of the proposed trisaccharide could be selectively removed in the presence of the benzoate esters. It was thought that the replacement of the benzyl groups with benzoates might render the disaccharide donor less reactive than the disaccharide donor (10). The first step then was the preparation of a suitably blocked glucosamine moiety.

The glucosamine derivative (26) was benzoylated by treatment with benzoyl chloride in pyridine (55% yield), in an analogous fashion to the preparation of the well known tetra-*O*-acetyl-2-deoxy-2-phthalimido- β -*D*-glucopyranoside²⁴ (Scheme 6). It was discovered that the carboxybenzamido group of (26), had failed to condense to the phthalimido group as was expected. The mixed anhydride group of (27) was converted to the phthalimido group by refluxing with acetic anhydride in a solution of pyridine; the mixed anhydride readily condensed to give the tetra-*O*-benzoyl-2-deoxy-2-phthalimido- β -*D*-glucopyranoside (28) in 91% yield. The synthesis of this block is considerably more straightforward than that of the corresponding perbenzylated derivative (5); it requires a three step synthesis by use of relatively inexpensive starting materials, and all synthetic intermediates are solid compounds, eliminating the need for chromatography.

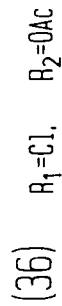
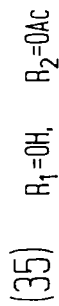
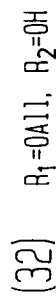
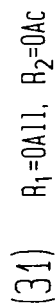
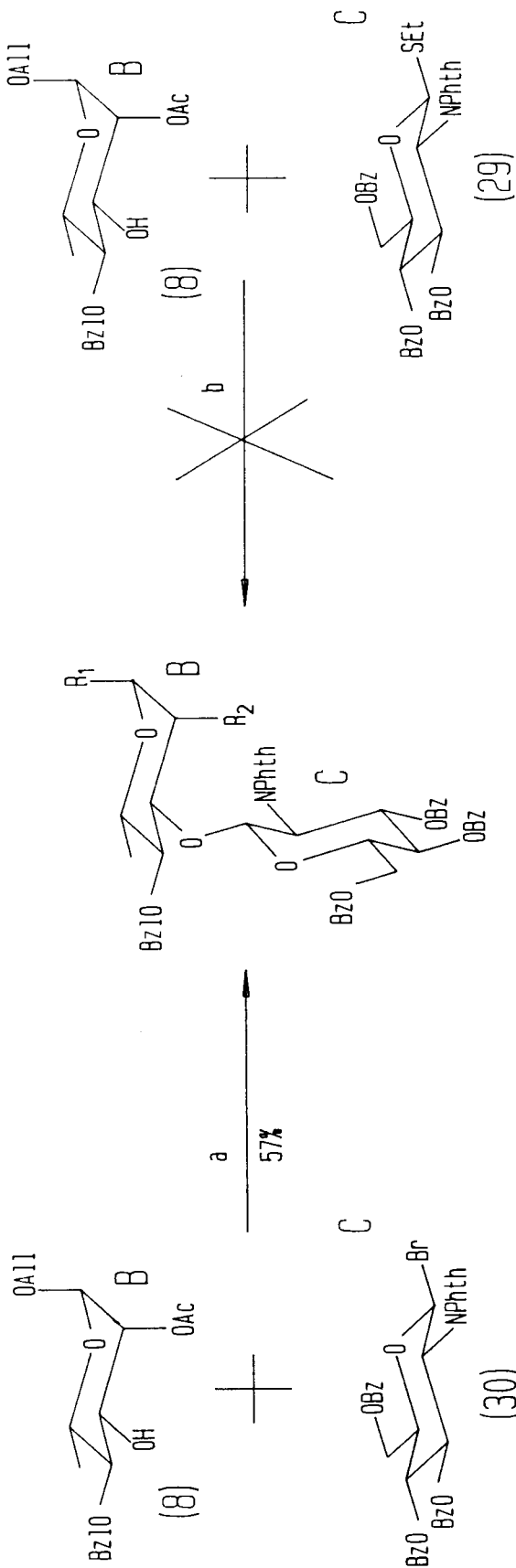
The initial plan was to use the glucosyl donor as its thioglycoside; therefore, the thioglycoside (29) was prepared by treating a solution of the tetra-*O*-benzoyl compound (28) and ethanethiol in dichloromethane with titanium tetrachloride.^{27a} The reaction proceeded to give the crystalline compound (29) in 66% yield (Scheme 6). Attempts to use the thioglycoside (29) as a glycosyl donor, with the rhamnosyl acceptor (8), proved to be unsuccessful. Activation of the thioglycoside, by use of

Scheme 6



methyl triflate^{27c,33} as a thiophilic promoter gave some of the desired product, but a larger amount of a second material was isolated which was found to be the allyl glycoside of the glycosyl donor; namely allyl 3,4,6-tri-*O*-benzoyl-2-deoxy-2-phthalimido- β -D-glucopyranoside. Kihlberg and Bundle's method of *in situ* generation of glycosyl bromides from thioglycosides was also attempted.³⁸ In this procedure a dichloromethane solution of the thioglycoside (29), the acceptor alcohol (8), and silver trifluoromethanesulfonate were stirred together. This mixture was then treated with bromine, and results in the *in situ* generation of the glycosyl bromide, and then the subsequent activation of the glycosyl bromide by the silver trifluoromethanesulfonate in the usual fashion. This reaction also proved to be unsuccessful, with only a small amount of the desired disaccharide being produced. This was thought to be due to the addition of bromine to the double bond in the allyl group of (8), and results in degradation of the alcohol acceptor. With these results in hand, it was decided to generate the glycosyl bromide separately, and then react the activated glucosamine donor with the alcohol acceptor (8), in the usual fashion by use of silver trifluoromethanesulfonate as glycosylation promoter. Initially, the glycosyl bromide (30) was generated from the thioglycoside (29) by treating a dichloromethane solution of the thioglycoside with bromine.^{27c,39} A more direct route to the glycosyl bromide was provided by treatment of a dichloromethane solution of the tetra-*O*-benzoyl derivative (28) with a solution of hydrogen bromide in glacial acetic acid (48%); the reaction proceeded quantitatively and the 3,4,6-tri-*O*-benzoyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl bromide (30) thus generated was used directly in the subsequent glycosylation reaction without further purification.

Scheme 7



80%
76%

(a) CF_3SO_3Ag , collidine, CH_2Cl_2 .

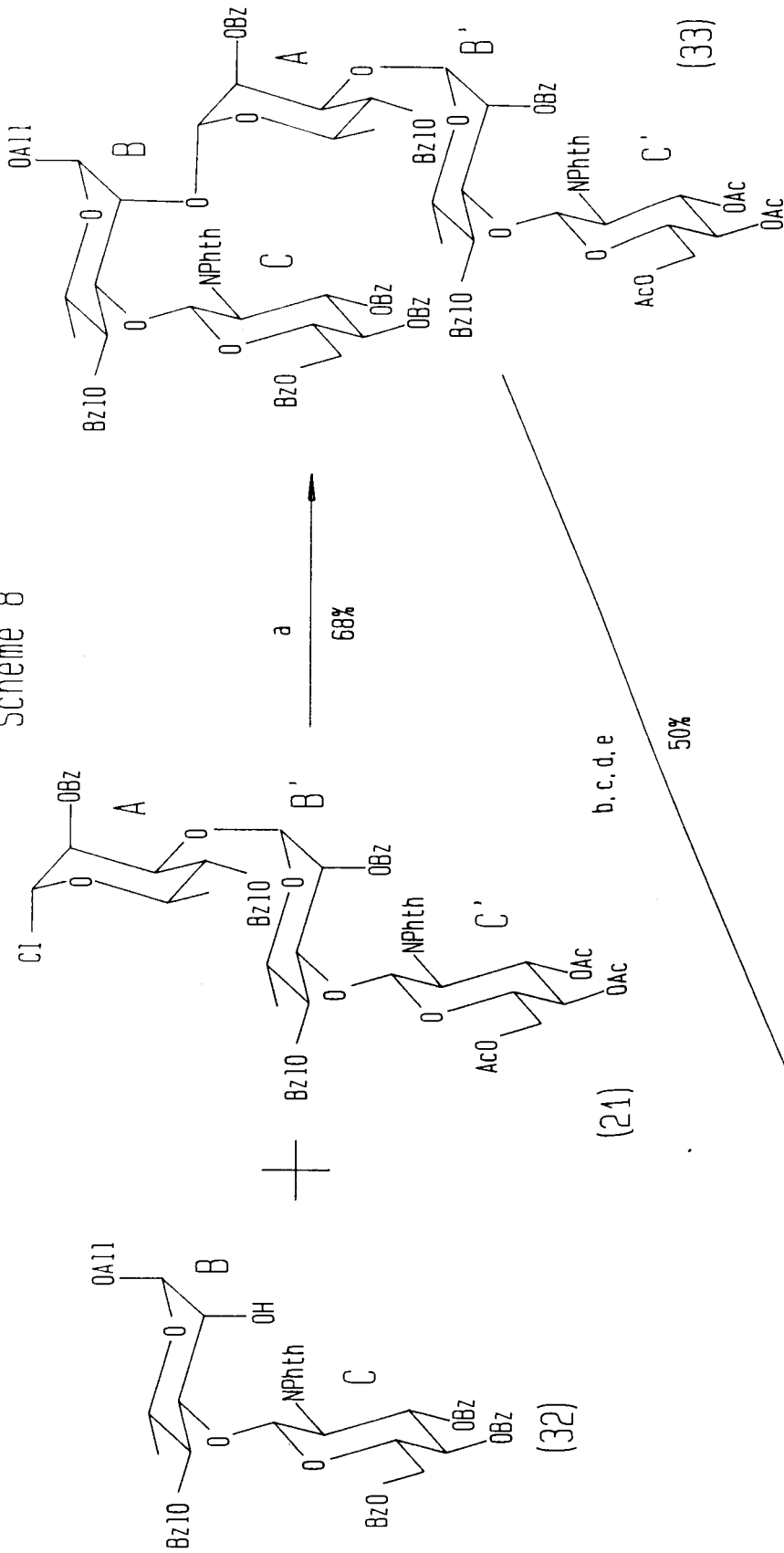
-30 to 25°C;

(b) methyl triflate, CH_2Cl_2 .

Glycosylation of allyl 2-O-acetyl-4-O-benzyl- α -L-rhamnopyranoside (8), prepared in analogous fashion to the 2-O-benzoyl derivative,^{60a} with the donor (30) under silver trifluoromethanesulfonate promotion in the presence of collidine²⁴ afforded the disaccharide (31) in 57% yield (Scheme 7). The crucial test of this synthetic sequence was whether one could successfully remove the 2-O-acetyl group of the disaccharide (31) without removing any of the benzoyl groups on the glucosyl moiety. There was precedent for this type of reaction in which a secondary acetate was removed in the presence of other secondary benzoates,⁷¹ but no precedent for a reaction in which a secondary acetate was removed in the presence of a primary benzoate, as would be required with the removal of the acetate in (31). This selective deprotection was achieved by mild transesterification conditions. The disaccharide (31) was treated with 3% methanolic HCl to give the disaccharide (32) with the free 2-hydroxyl, in 76% yield.

A pentasaccharide sequence was required for use as a hapten in inhibition binding studies; for these studies it was preferable to have the pentasaccharide as its propyl glycoside, (in its deblocked form) rather than as its 8-(methoxycarbonyl)octyl glycoside. In an analogous fashion to the preparation of pentasaccharide (22), glycosylation of the disaccharide acceptor (32) with the trisaccharide chloride (21) afforded the pentasaccharide (33) in 68% yield (Scheme 8). Deprotection of pentasaccharide (33) was accomplished by sodium methoxide catalyzed transesterification of all the acyl groups, followed by catalytic hydrogenolysis of the benzyl ethers, as well as reduction of the allyl group to the propyl group. The phthalimido group was removed by treatment with hydrazine, and the resulting free amine was selectively

Scheme 8



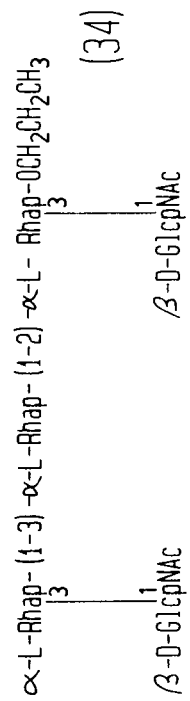
(a) $\text{CF}_3\text{SO}_3\text{Ag}$, TMU, CH_2Cl_2 ,
-78 to 25°C.

(b) NaOMe, MeOH, 10h;

(c) Pd/C, H_2 , HOAc- H_2O (4:1);

(d) $\text{N}_2\text{H}_4\cdot\text{H}_2\text{O}$, EtOH, reflux;

(e) $\text{Ac}_2\text{O}/\text{MeOH}$.



(21)

b, c, d, e

50%

68%

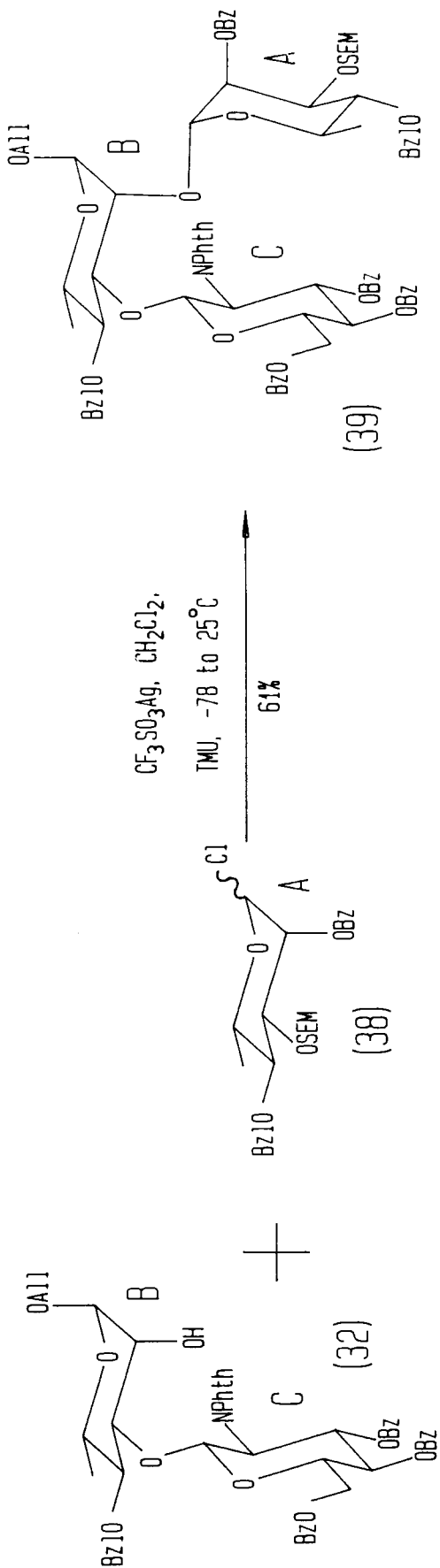
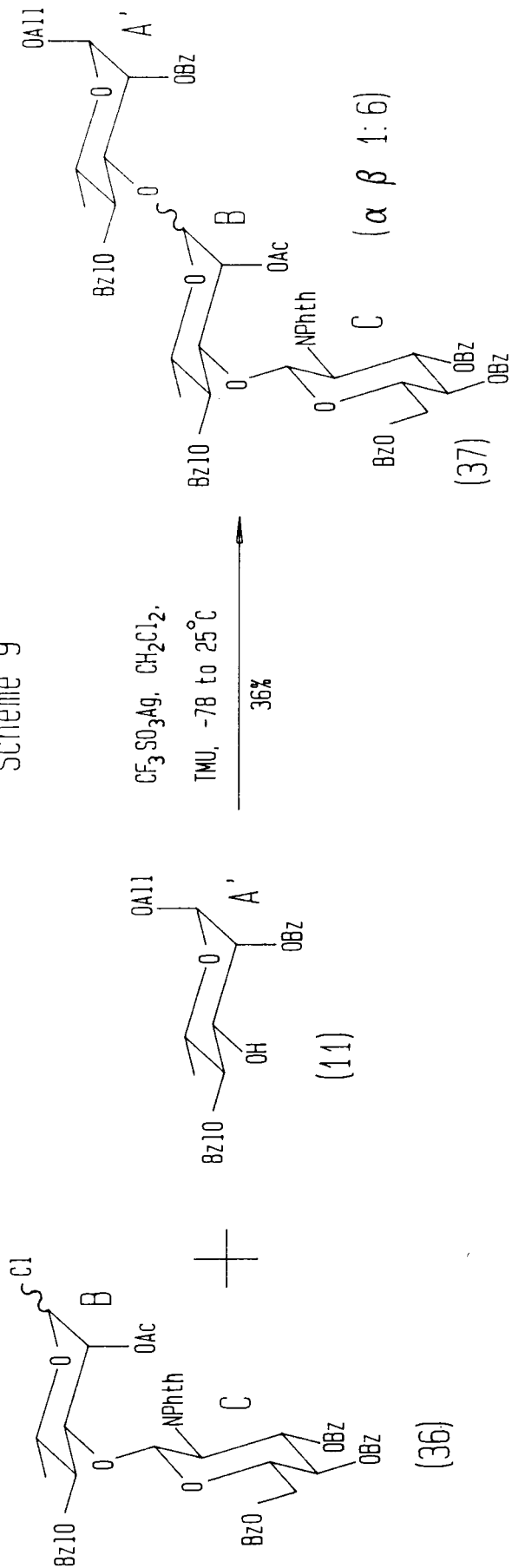
(33)

acetylated by treatment with acetic anhydride in methanol to give the fully deblocked pentasaccharide as its propyl glycoside (34) in 50% yield.

The question still remained as to whether the disaccharide (31) could be used to prepare a linear trisaccharide such as (1). To determine this, the allyl group of (31) was removed [as described above for (6)] to give the hemiacetals (35) in 80% yield from (31) (Scheme 7). Treatment of the hemiacetals (35) with *N,N*-dimethyl(chloromethylene)ammonium chloride⁶³ then gave the glycosyl chloride (36) in quantitative yield which was not isolated but was used immediately in the subsequent glycosylation reaction without further purification. Glycosylation of the rhamnosyl monosaccharide acceptor (11)^{60a} with the disaccharide (36), by use of silver trifluoromethanesulfonate as promoter, in the presence of tetramethyl urea, gave a mixture of products, with the major product being the unwanted β -anomer (36% yield; α/β 1:6) (Scheme 9). The stereochemistry was confirmed as described earlier (see page 33). This result was identical to the result obtained earlier by use of the disaccharide (10) as the glycosyl donor. The failure to prepare the linear trisaccharide (37) with α -stereoselectivity led us to abandon the route to a key **linear** trisaccharide.

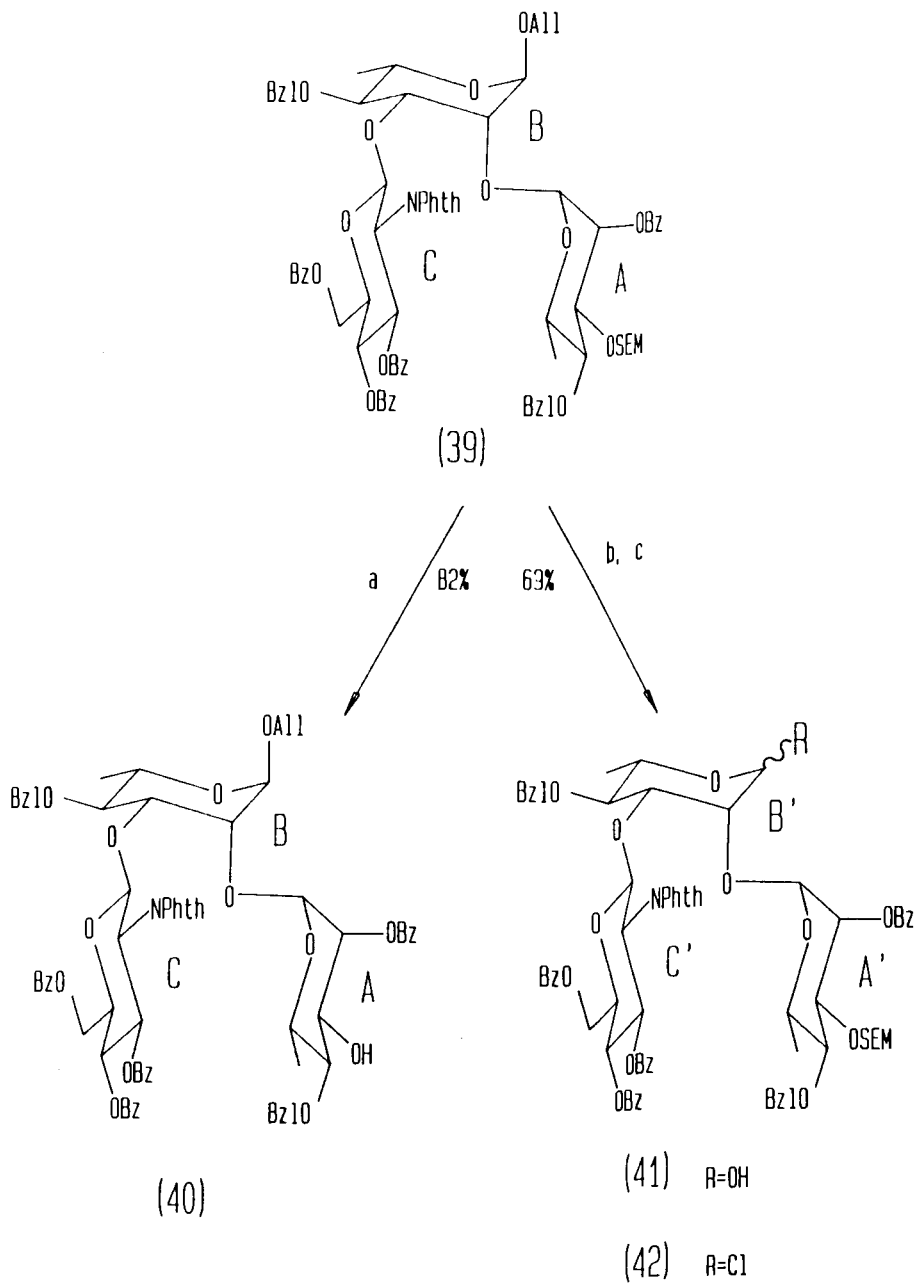
One more possibility existed for the synthesis of a common trisaccharide building block for use in the synthesis of higher order structures. This was the preparation of a [B-(C)-A] **branched** trisaccharide synthetic intermediate. Accordingly, the disaccharide acceptor (32) was glycosylated with the monosaccharide glycosyl chloride (38)⁷² (Scheme 9) in an analogous fashion to the synthesis of

Scheme 9



trisaccharides (17) and (18). The reaction proceeded with α -stereoselectivity to give trisaccharide (39) in a 61% yield. This trisaccharide could serve, in principle, as both a glycosyl donor and acceptor in future glycosylation reactions. Previous work in our laboratory had indicated that the SEM acetal could be selectively removed in the presence of benzoate esters. Indeed, the 3'-position of (39) was deblocked by treatment with methanolic HCl,⁷² to give the trisaccharide acceptor (40) in 82% yield (Scheme 10). The key question remaining was whether the glycosyl chloride derived from the trisaccharide (39) would function adequately as a glycosyl donor. The concern was the absence of a participating group at the 2-position of the proposed glycosyl donor; this being replaced by a rhamnosyl unit. There was a precedent in the literature for this sort of a reaction. Bundle et al⁷³ had prepared several homo-polymers of 1-2 α -linked purosamine (4,6-dideoxy-4-formamido-D-mannose) units. In these glycosylation reactions, there were no participating acyl groups such as benzoate or acetate esters, instead there were glycosyl residues at the 2-positions of the glycosyl donors. Good α -stereoselectivity was observed in these reactions, the stereoselectivity being due presumably to the steric bulk of the purosamine residues at the 2-positions of the glycosyl donors. Other examples of the synthesis of α -linkages in the mannose series, utilizing mannosyl donors without neighbouring group participation, can be found. Ogawa et al have used both α -D-mannosyl bromides,⁷⁴ and α -D-mannosyl trichloroacetimidates³¹ to prepare oligosaccharides with α -stereoselectivity; in both cases the mannosyl donors had other sugar residues at the 2-positions. The reaction we planned was analogous to the ones described. Accordingly, the trisaccharide (39) was converted to the

Scheme 10



(a) 3% HCl in MeOH, CH₂Cl₂

(b) Rh(PPh₃)₃Cl, EtOH-H₂O (9:1), reflux

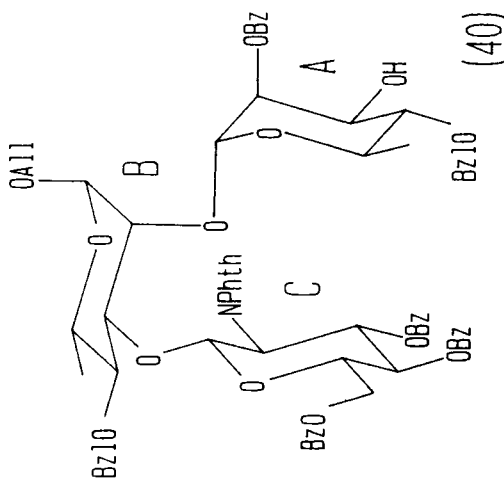
(c) HgO/HgCl₂, acetone-H₂O (10:1)

hemiacetals (41) [69% yield from (39)] and then to the glycosyl chloride (42), in an analogous fashion to (6) above.

Glycosylation of the trisaccharide acceptor (40) with the trisaccharide donor (42) was attempted to determine whether the branched trisaccharide (39) would serve as a common synthetic intermediate for the preparation of higher-order structures. The glycosylation reaction (Scheme 11) was performed in an analogous fashion to the synthesis of pentasaccharide (33), and proceeded with exclusive α -stereoselectivity to give (43) in 50% yield. The hexasaccharide (43) thus prepared had the same features as the parent trisaccharide (39); namely an allyl group as the aglycone which could be removed to generate a glycosyl donor, and the SEM group which could be selectively removed to generate a glycosyl acceptor. This result confirms the usefulness of the key trisaccharide intermediate (39) as a common synthetic intermediate in the synthesis of higher-order structures of the *Streptococci* Group A cell-wall polysaccharides and defines an efficient convergent synthetic route. The hexasaccharide (43) results from only three glycosylation reactions and a nonasaccharide could, in principle, be derived from a fourth glycosylation reaction [i.e. removal of the SEM group of hexasaccharide (43) and glycosylation with the trisaccharide (42)].

Analytically pure samples of the deprotected compounds were generally obtained following successive chromatography on silica gel and sephadex LH20. Owing to the hygroscopic nature of the compound, satisfactory combustion microanalysis results were not obtained for compounds (24) and (34) despite several attempts. Therefore, plasma desorption mass spectra⁷⁵ were obtained as a confirmation of composition. The peaks appearing at m/e 707 and 927 in the spectra were assigned to

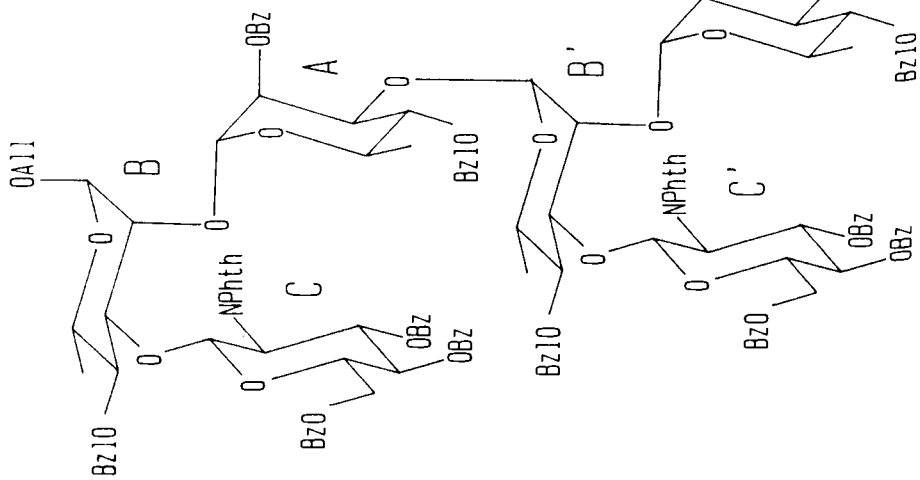
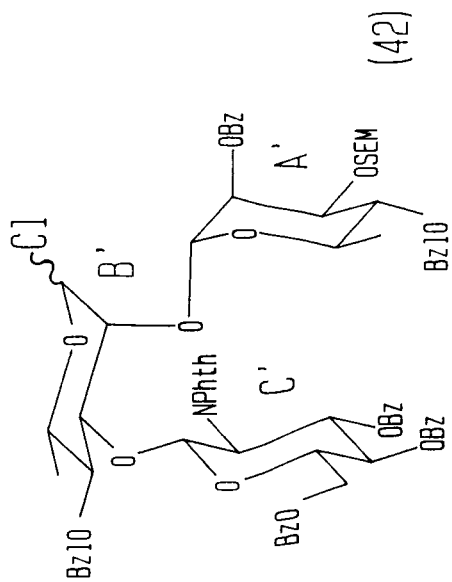
Scheme 11



$\text{CF}_3\text{SO}_3\text{Ag}$, collidine, CH_2Cl_2

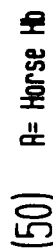
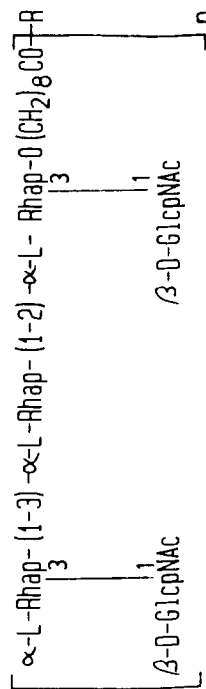
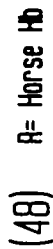
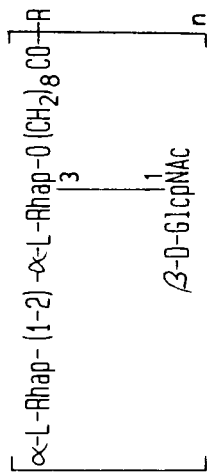
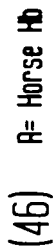
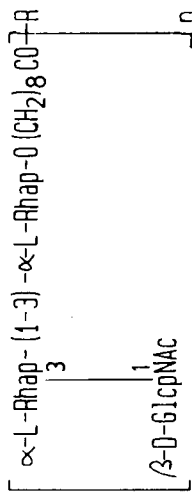
-78 to 25°C, 24h

50%



(43)

Scheme 12



(a) $\text{N}_2\text{H}_4 \cdot \text{H}_2\text{O}$, EtOH, 20 °C, 12h;

(b) $\text{N}_2\text{O}_4/\text{CH}_2\text{Cl}_2$, DMF;

(c) BSA (buffer solution).

the M^+ ions of the sodium salts of compounds (24) and (34), respectively. $M+Na^+$ ions are commonly observed in plasma desorption mass spectra, particularly of compounds containing labile hydrogens or anionic moieties.⁷⁶

B: Preparation of synthetic antigens

The glycoconjugates of the trisaccharides (24), (44),¹⁸ and pentasaccharide (25) with bovine serum albumin (BSA) and with horse hemoglobin (Horse-Hb), were prepared by the modified acyl azide methodology of Pinto and Bundle.⁶⁷ Thus, the esters (24), (44) and (25) were converted into their hydrazides, and the latter were then treated with dinitrogen tetroxide as the nitrosating agent. The resultant acyl azides were then treated immediately with BSA (or horse Hb) in buffer solution to provide the corresponding glycoconjugates, (45)-(50) (see Scheme 12). Levels of hapten incorporation ranging from 13 to 30%, were achieved, and afforded glycoconjugates possessing between 8 and 18 haptens per molecule of protein.⁷⁷ These synthetic antigens were used in the hybrid-myeloma protocol as immunizing antigens and also as screening agents for monoclonal antibodies (see Chapter 3).

C: NMR Spectroscopic Results

1: General

The assigned structures were in accord with their 1H and ^{13}C NMR spectral data. Compounds were characterized by use of routine 1H , ^{13}C and $^{13}C\{^1H\}$ NMR spectra. 1H -Homonuclear chemical-shift correlated (COSY) experiments⁷⁸ were performed on compounds (13), (18), (22), (23), (24),

(25), (33), (34) and (43) in order to facilitate assignments, and ^{13}C - ^1H chemical-shift correlated experiments⁷⁹ were performed on compounds (18), (22), (25), (33), (34) and (43). The ^{13}C - ^1H chemical-shift correlated experiments performed on compounds (22), (25), (33), (34) and (43) were carried out in the inverse mode,⁸⁰ thereby taking advantage of the sensitivity of the ^1H nucleus. The spectra were measured on samples of approximately 10mg. The proton chemical-shifts were displayed along the F_2 -axis and the carbon chemical-shifts were displayed along the F_1 -axis. The experiments were carried out without carbon-decoupling during acquisition, permitting the measurement of the one-bond ^{13}C - ^1H coupling constants ($^1J_{13\text{C}-1\text{H}}$) for the anomeric carbons.

The stereochemical integrity of the trisaccharides (23) and (24) and the pentasaccharides (22), (25) and (34) and the hexasaccharide (43) was confirmed by examination of the one-bond ^{13}C - ^1H coupling constants, $^1J_{13\text{C}-1\text{H}}$, for the anomeric carbons.⁸¹

The vicinal coupling constants of the ring-protons in the monosaccharide units within oligosaccharides were consistent with a $^4\text{C}_1$ (D) conformation for the N-acetylglucosamine ring and with a $^1\text{C}_4$ (L) conformation for the rhamnopyranosyl units.

Full characterization of the NMR spectra of the various compounds was crucial in confirming their structure. The analysis of the NMR spectra for the monosaccharide up to the trisaccharide structures was, for the most part, straightforward and could be carried out by use of conventional 1-dimensional NMR techniques. Assignment of the spectra of the monosaccharides aided in the assignment of the spectra of the disaccharides, which in turn, allowed for the assignment of the trisaccharide spectra. In this fashion, trends in chemical shifts could

be noted and used for the assignment of the more complicated spectra. For the pentasaccharides and hexasaccharide, as well as all the deblocked structures, the overlap of ^1H -NMR signals within the spectra made complete assignment of signals impossible from the analysis of only 1-dimensional spectra. To facilitate the assignment of these crowded spectra several 2-dimensional NMR spectra were recorded.

2: Assignment of ^1H - and ^{13}C -NMR Spectra of Pentasaccharide (34)

The complete assignment of the ^1H - and ^{13}C -NMR spectra of the deblocked pentasaccharide (34) is described below as an example of the analyses which have been carried out on the various synthetic intermediates, and final deblocked compounds.

The first experiment carried out was the COSY experiment; the spectrum is shown in Figure 2-11a, with an expanded region shown in Figure 2-11b. The anomeric proton signals are seen between 4.5 ppm and 5.2 ppm, with one signal mostly obscured by the residual H₂O peak. The rhamnosyl H-2's, seen between 4.0 and 4.3 ppm, as well as the rhamnosyl methyl signals, are all relatively well resolved. The remaining ring proton signals of the various rings fall into a crowded envelope of signals between 3.4 and 3.9 ppm. The analysis of a COSY spectrum usually starts with the identification of a clearly resolved signal which can be assigned to a particular proton within the molecule. By following the pattern of off-diagonal cross-peaks, the complete spin system to which the isolated signal belongs may be identified. The spectrum of the pentasaccharide should contain five separate spin-systems (not counting the aglycone), each corresponding to a particular rhamnose or *N*-acetyl glucosamine ring; the first step in the analysis was then to identify the

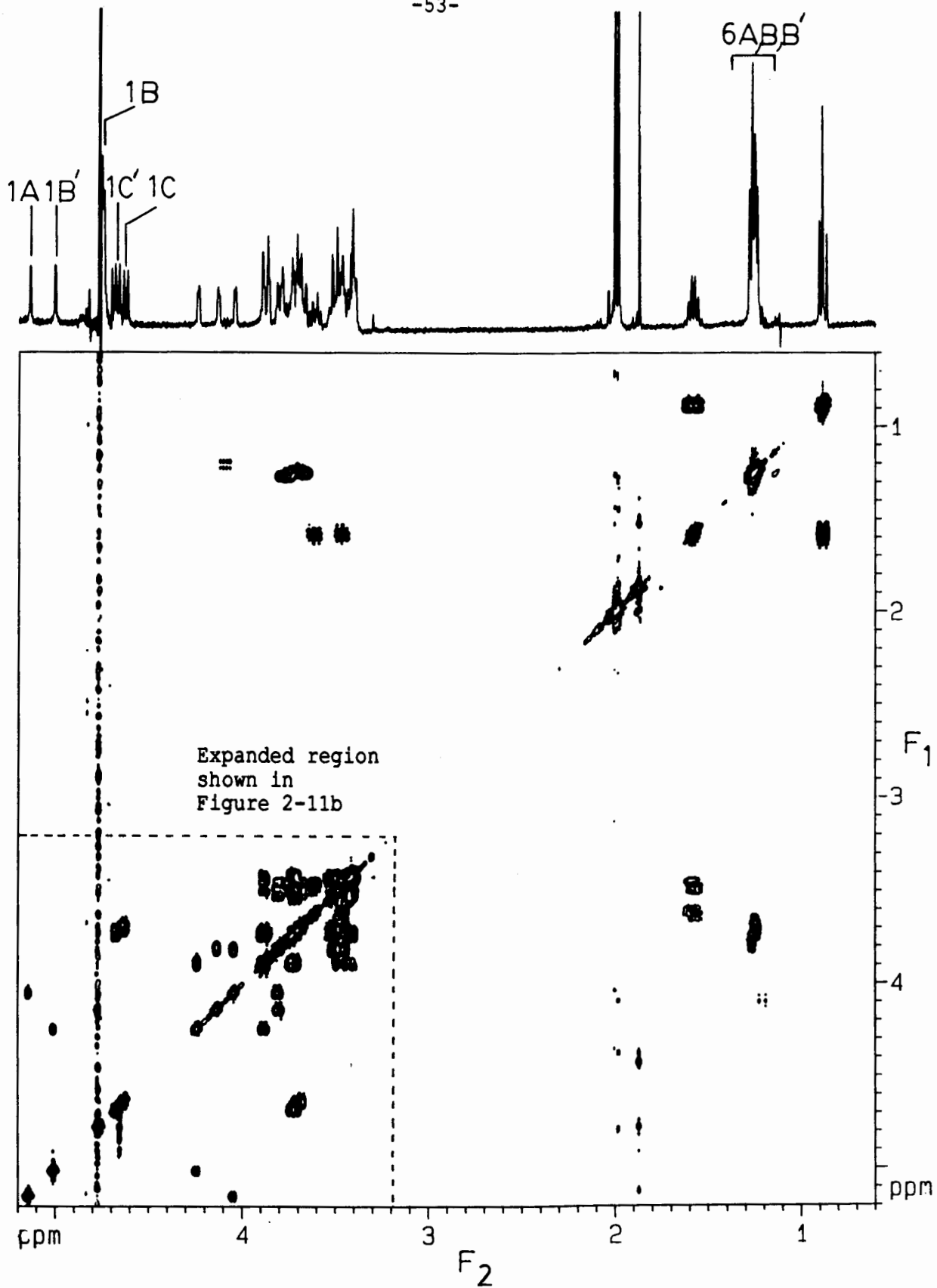


Figure 2-11a: 400 MHz $2\text{D-}^1\text{H-NMR}$ COSY spectrum of pentasaccharide (34).

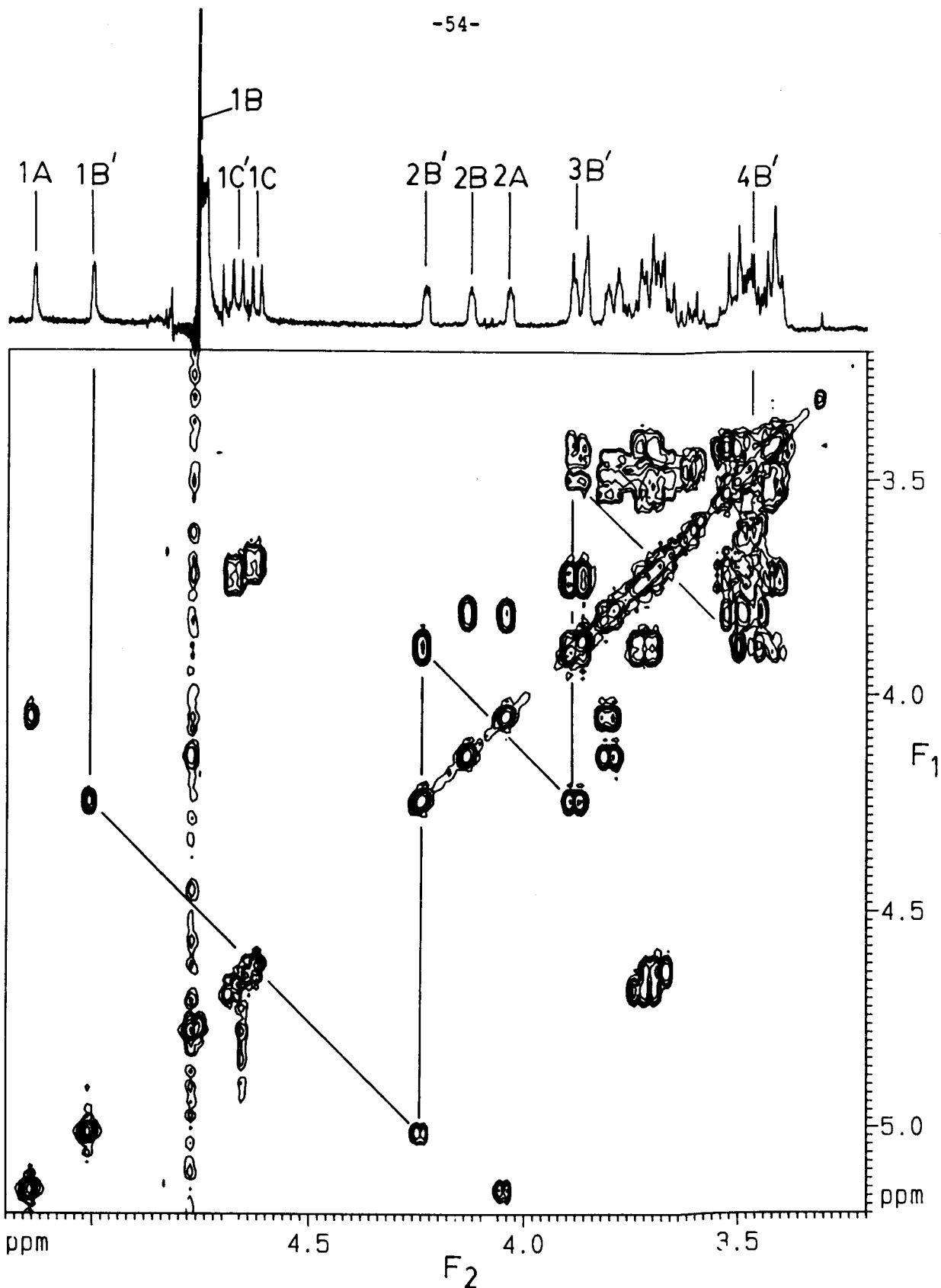


Figure 2-11b: Partial 400 MHz $2\text{D-}^1\text{H-NMR}$ COSY spectrum of pentasaccharide (34)

five spin systems.

One cross-peak pattern for one of the rhamnosyl spin systems is shown in Figure 2-11b. The rhamnosyl H-1 at 5.0 ppm shows a cross-peak at 4.23 ppm indicating the chemical shift of the H-2 of the same spin system. A vertical line drawn at this chemical shift, perpendicular to the F_2 -axis identifies the cross-peak between the H-2 and the H-3, to give the chemical shift of the H-3. In similar fashion the chemical shift of the H-4 of the same spin system could be determined. The cross-peak between the H-4 and the H-5 could not be unambiguously assigned due to the high degree of overlap in that region of the spectrum, meaning that the assignment of the H-5 and the H-6 methyl signals could not be made. The situation is the same for the other two sets of rhamnosyl signals; in addition, the signals for the two *N*-acetyl glucosamine rings very nearly overlap one another. To make the analysis easier it was decided to acquire a TOCSY spectrum⁸², TOCSY standing for "Total Correlation Spectroscopy".

In a TOCSY spectrum, by use of long mixing times, each proton signal shows correlation cross-peaks with every other signal within the same spin system. For example an anomeric signal (H-1) will have a cross-peak for the adjacent H-2, as well as for the H-3 which is J-coupled to the H-2 but not directly to the anomeric H-1; in addition, cross-peaks for the H-4 and the remaining signals of the spin system may be seen. An expanded region of the TOCSY spectrum of the pentasaccharide is shown in Figure 2-12. Correlation cross-peaks between the rhamnosyl H-2's and the H-3's, H-4's and H-5's can clearly be seen. In fact a slice of the 2-dimensional spectrum, parallel to the F_2 -axis, at the chemical shifts of the rhamnosyl H-2's, gives three 1-dimensional spectra showing the

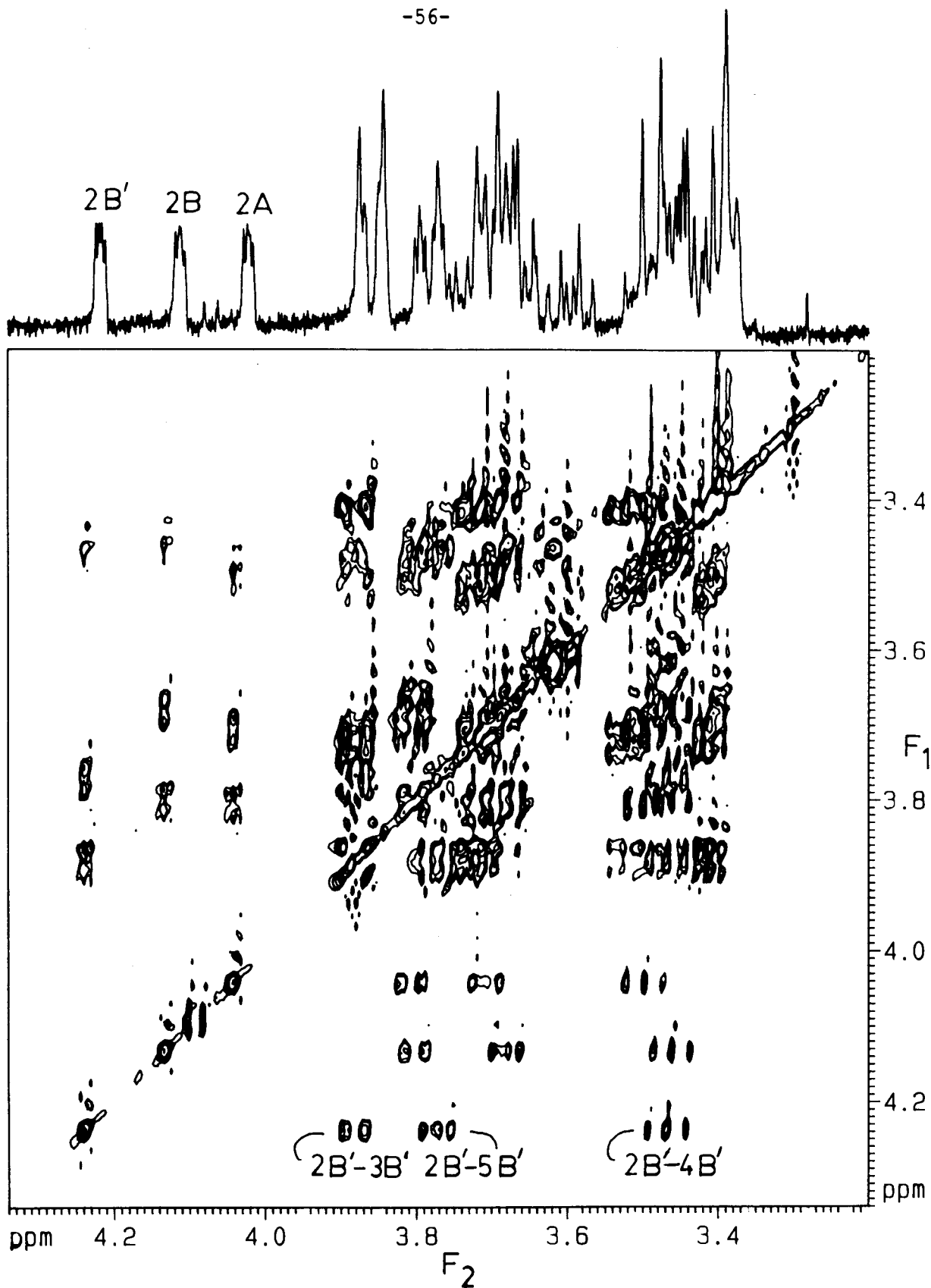


Figure 2-12: Partial 400 MHz $2D-^1\text{H}$ -NMR TOCSY spectrum of pentasaccharide (34)

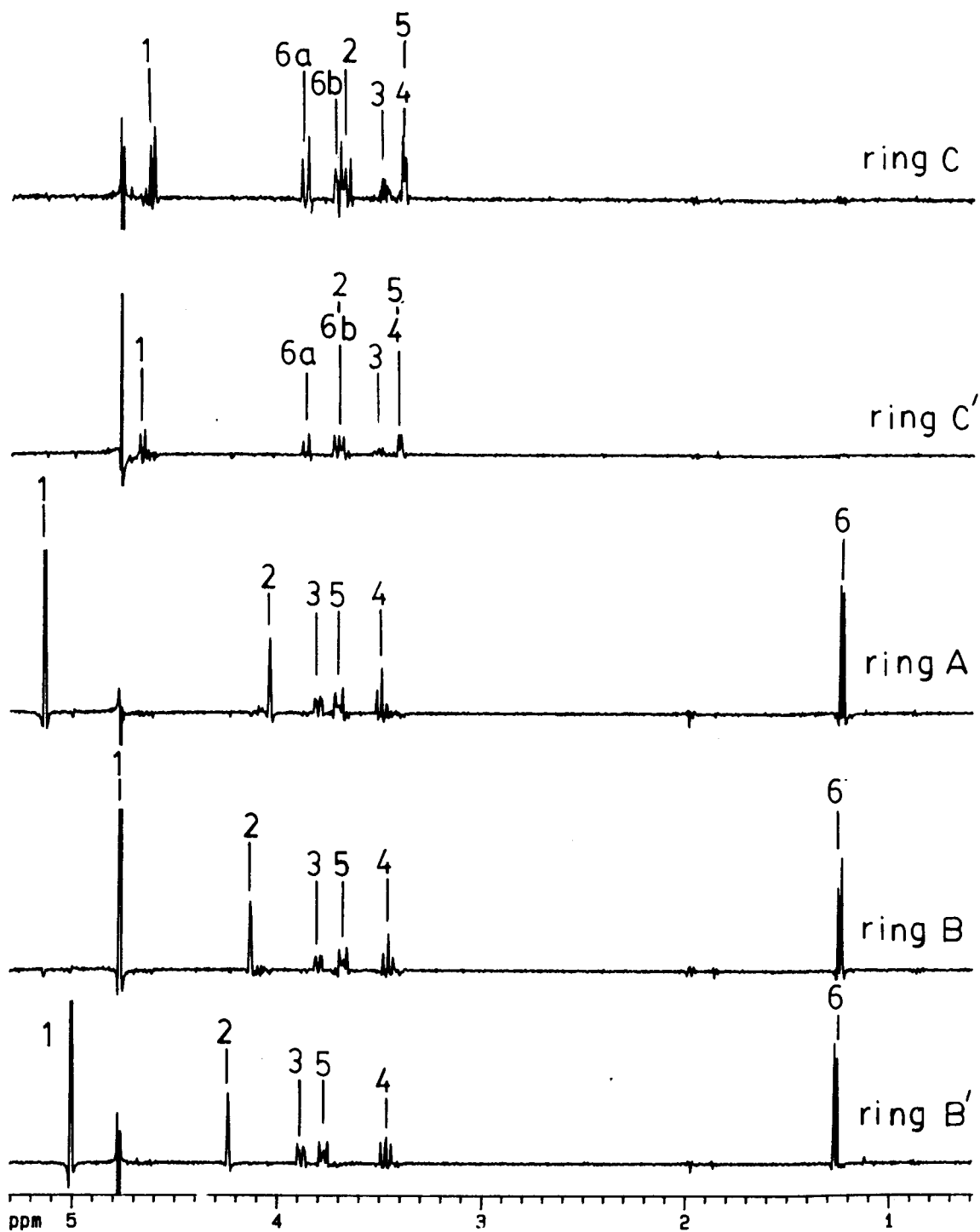


Figure 2-13: One-dimensional "slices" of the TOCSY spectrum of pentasaccharide (34).

complete spin systems of the three rhamnosyl rings. These 1-dimensional slices of the TOCSY spectrum are shown in Figure 2-13. Slices were also taken along the F_2 -axis at the chemical shifts of the two *N*-acetyl glucosamine H-1's to give the spin systems for these two rings. In this fashion, the five separate spin systems could be identified in a straightforward and unambiguous fashion.

Completion of the assignment of the ^1H -NMR spectrum now required the assignment of the individual spin systems to particular rings within the molecule. The sets of signals due to the *N*-acetyl glucosamine rings were clearly distinguished from the sets of signals due to the rhamnosyl rings on the basis of their characteristic coupling constants; in particular, the large coupling constant (approx. 8.5 Hz) of the anomeric protons. The assignment of the sets of *N*-acetyl glucosaminyl and rhamnosyl signals to specific rings was not as straightforward. To accomplish this, a ROESY⁸³ spectrum was obtained; the term ROESY standing for rotating frame n.o.e. spectroscopy. In a ROESY spectrum, proton signals which share a dipolar coupling, rather than a scalar coupling are correlated. In oligosaccharide systems it is commonly observed that the anomeric protons show dipolar coupling to the protons which are across the glycosidic linkage, in addition to the expected intra-ring dipolar contacts. This information may be used to locate spin systems relative to one another, hence permitting sequence assignment.

The ROESY spectrum of the pentasaccharide is shown in Figure 2-14. Five sets of cross-peaks, each lying on a line parallel to the F_2 -axis, can be seen. These sets of cross-peaks are due to dipolar coupling between the anomeric protons and other protons close in space. The five sets of cross-peaks lie on lines which give the chemical shifts of the

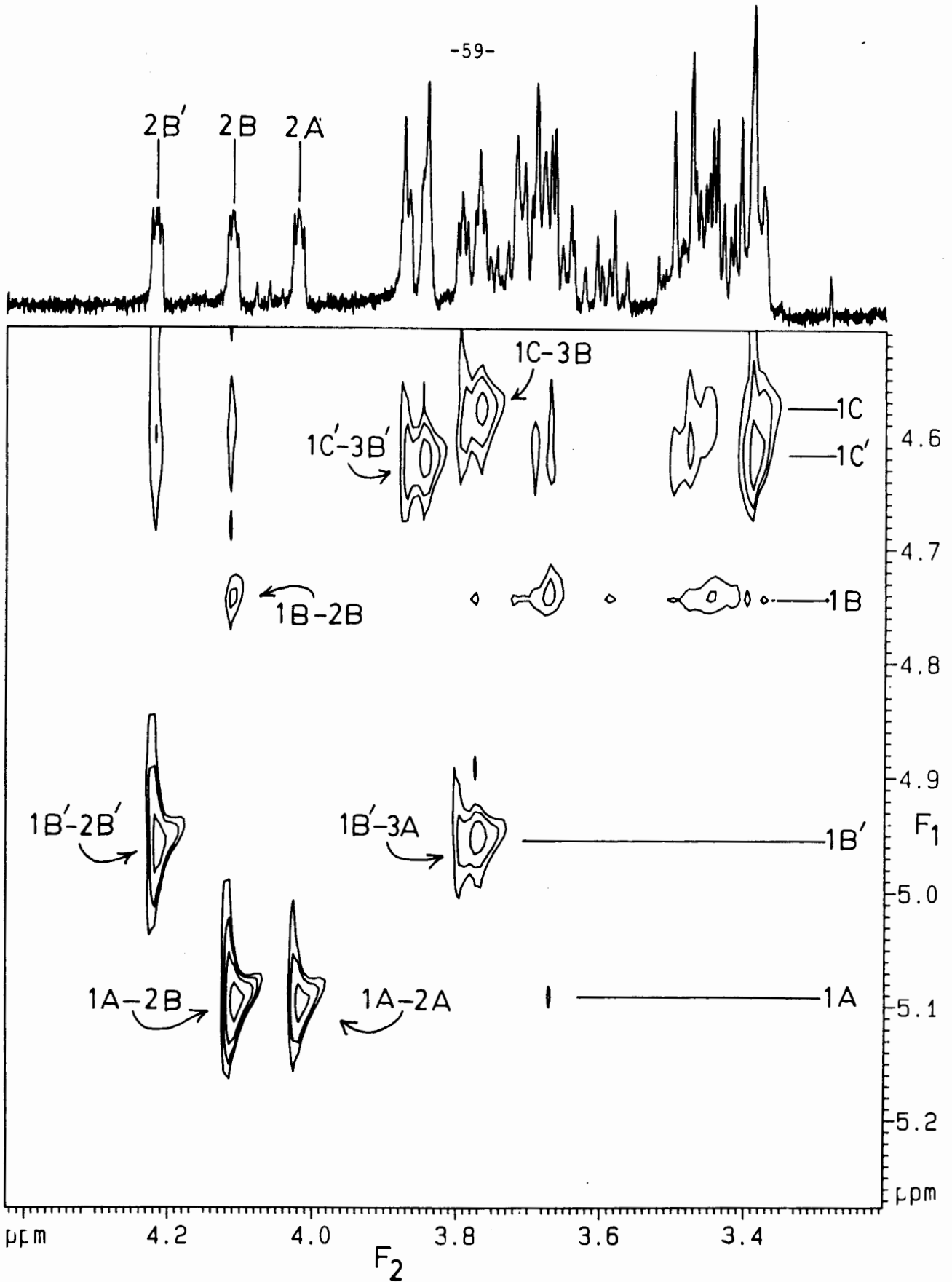


Figure 2-14: Partial 400 MHz 2D-¹H-NMR ROESY spectrum of pentasaccharide (34).

corresponding anomeric signals in the F_1 -direction. The anomeric signal due to the B-ring can be identified as the signal at 4.74 ppm, since the cross-peaks between this anomeric signal and the proton signals at 3.68 and 3.45 ppm are due to a dipolar coupling to the diastereotopic protons of the propyl aglycone. The rhamnosyl H-2 at 4.10 ppm may then be assigned to the B-ring as well, based on the expected intra-ring dipolar coupling between rhamnosyl H-1's and H-2's. The rhamnosyl H-2 at 4.10 ppm has an additional cross-peak, this one due to an interglycosidic contact with the anomeric signal of the A-ring. The anomeric signal of the A-ring is thus identified as being the signal at 5.10 ppm. The remaining rhamnosyl anomeric signal may then be assigned to the B'-ring; the expected inter- and intra-ring cross-peaks are observed.

The two sets of signals due to the *N*-acetyl glucosamine rings may be assigned in a similar fashion. The more downfield H-1 signal of the two shows an interglycosidic cross-peak to the H-3 of the B'-ring, allowing for the assignment of this H-1 signal to the C'-ring. The remaining set of *N*-acetyl glucosamine signals may then be assigned to the C-ring. This then completes the assignment of all the ring proton signals in the ^1H -NMR spectrum of pentasaccharide (34).

The assignment of the ^{13}C -NMR signals followed directly from a ^{13}C - ^1H correlation spectrum. Furthermore, an inverse detected ^{13}C -NMR spectrum, without ^1H decoupling during acquisition, was used to confirm the stereochemical integrity of the pentasaccharide (Figure 2-15). Thus, the one-bond ^{13}C - ^1H coupling constants ($^1J_{13\text{C}-1\text{H}}$), measured in the F_2 -direction of the Fourier transformed spectrum, were used to confirm the configuration about the anomeric centres.⁸¹ The anomeric signals attributed to the rhamnose units had coupling constants of 172-175 Hz,

consistent with an α -L-configuration. The coupling constants for the anomeric carbons of the *N*-acetyl glucosamine units were found to be 163Hz, which were consistent with a β -D-configuration about these centres.

3: Assignment of NMR Spectra of the Remaining Compounds

The NMR experiments described in the preceding section for the case of the pentasaccharide (34), were used, as necessary, to assign the ^1H - and ^{13}C -NMR spectra of the synthetic intermediates and the other final products. The data for the ring protons and carbons are summarized in Tables 2-I through 2-VIII. The remaining signals are recorded in the Experimental section.

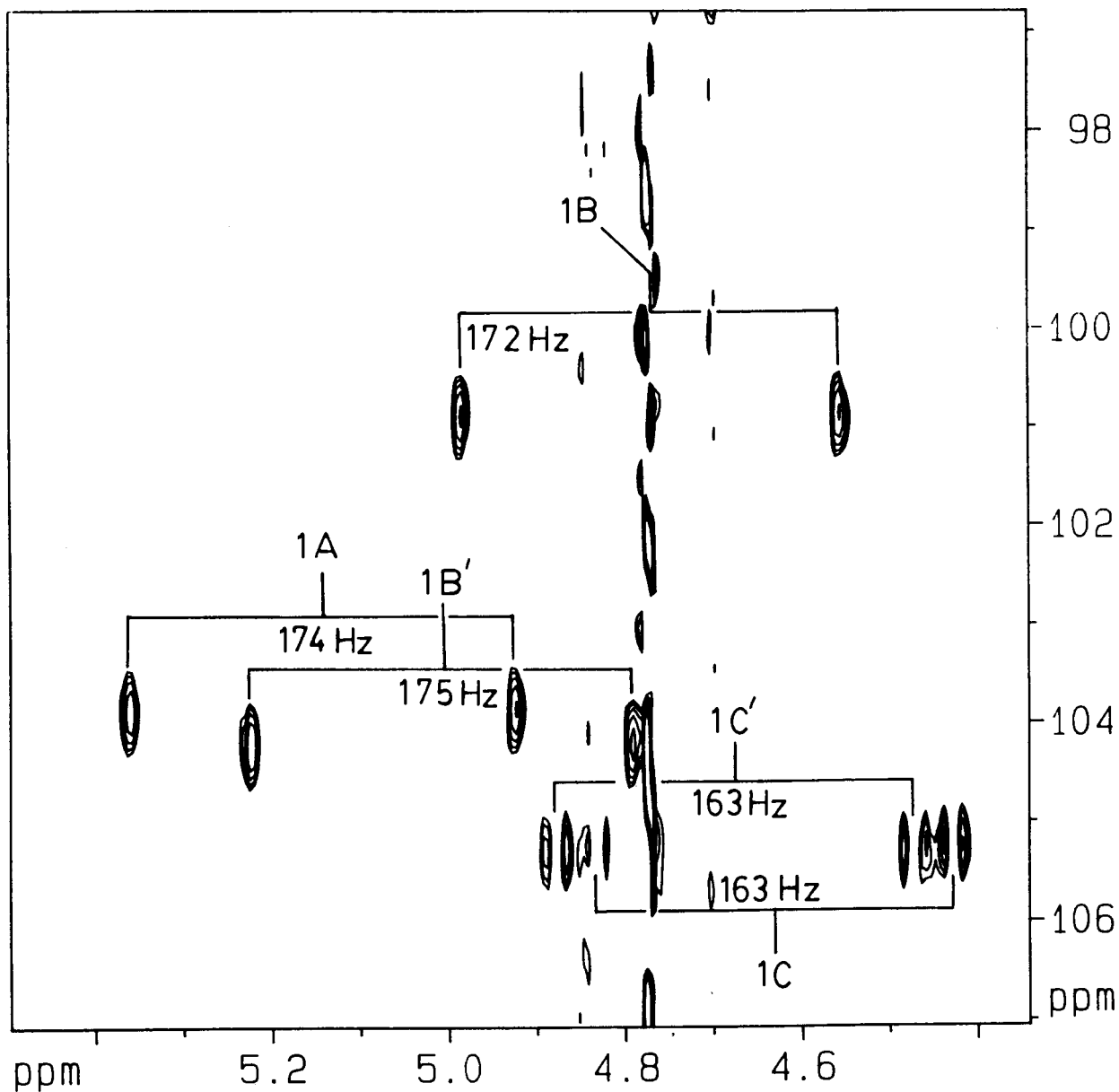


Figure 2-15: Inverse ^{13}C - ^1H correlation spectrum of pentasaccharide (34), indicating the correlations between the anomeric ^{13}C - and ^1H -signals.

Table 2-I ¹H-NMR Data^a for the Ring Protons for (9), (13), (14), (15), (17), (18), (23), and (24)

Ring Protons	(9)	(13)	(14)	(15)	(17)	(18)	(23)	(24)
1B ^b	4.81 (1.75)	4.84 (1.8)	4.75	4.69 (1.7)	4.64 (1.2)	4.58	4.78 (1.8)	4.78
2B	5.29 (1.75, 3.75)	5.43 (1.8, 3.5)	4.08 (1.5, 3.5)	4.03	4.23 (1.2, 3.5)	4.19 (1.2, 3.5)	4.10 (1.8, 3.0)	4.11
3B	4.05 (3.75, 9.25)	4.11 (3.5, 9.5)	3.84 (3.5, 9.0)	3.81 (3.2, 9.0)	3.96 (3.5, 9.0)	3.93 (3.5, 9.5)	3.80 (3.0, 9.5)	3.80 (3.2, 9.5)
4B	3.37 (19.0) ^c	3.44 (19.0) ^c	3.47 (18.0) ^c	3.33 (19.0) ^c	3.15 (18.0) ^c	3.13 (19.0) ^c	3.46	3.47
5B	3.67	3.64	3.56 (6.0, 9.0)	3.53	3.53 (9.0, 6.2)	3.50	3.67	3.67 (6.2)
6B	1.12 (6.0)	1.08 (6.2)	1.08 (6.0)	1.07 (6.2)	1.01 (6.2)	0.99 (6.2)	1.22 (6.1)	1.16
1C	5.44 (8.1)	5.44 (8.0)	5.39 (8.0)	5.41 (8.0)	5.37 (8.0)	5.375 (8.0)	4.64 (8.3)	4.66 (8.5)
2C	4.30 (8.1, 10.5)	4.23	4.26 ↑ multiplet ↓ 4.37	4.37 ↑ multiplet ↓ 4.28	4.45 (8.0, 10.5)	4.456 (8.0, 10.9)	3.69	3.70
3C	4.36 (8.5, 10.5)	4.28	4.37	4.28	4.38 (8.1, 10.5)	4.388 (8.0, 10.9)	3.50	3.52
4C	3.97 (8.0, 8.5)	3.80 (18.2) ^c	3.81	3.78	3.80	3.77 (17.5) ^c	3.41	3.43
5C	3.67	3.62	3.64	3.64	3.60	3.64	3.40	3.41
6C	3.91 (3.5, 11.0)	3.82 (3.5, 11.0)	3.82	3.82	3.87 multiplet ↓ 3.80	3.87 multiplet ↓ 3.80	3.87 (11.5)	3.88 (12.0)
6'C	3.82 (1.5, 11.0)	3.68	3.64	3.64	3.60	3.80	3.71	3.73

(Table 2-I continued)

Ring Protons	(17)	(18)	(23)	(24)
1A'	5.26 (1.8)	5.27 (1.8)	5.11 (1.8)	5.13
2A'	5.61 (1.8, 3.5)	5.625 (1.8, 3.2)	3.99 (1.8, 3.2)	4.01 (1.6, 3.2)
3A'	3.94 (3.5, 9.0)	3.96 (3.1, 9.5)	3.73 (3.2, 9.5)	3.74 (3.2, 9.5)
4A'	3.45 (18.0) ^c	3.46 (19.0) ^c	3.39	3.40
5A'	3.83 (9.0, 6.2)	3.86 (6.2, 9.5)	3.67	3.68
6A'	1.29 (6.2)	1.30 (6.2)	1.22 (6.1)	1.17 (6.2)

^a In CDCl₃ for 9, 13, 14, 15, 17, 18, and in D₂O for 23, and 24. The numbers in parentheses denote coupling constants, in Hz.

^b Indicates the ring to which the aglycone is attached.

^c These values are the sums of the individual coupling constants $J_{AX} + J_{BX}$.

Table 2-II $^{13}\text{C-NMR}$ Data^a for the Ring Carbons in (9), (13), (14), (15), (17), (18), (23) and (24)

Ring Carbons (9)	(13)	(14)	(15)	(17)	(18)	(23)	(24)
1B ^b	96.4	97.0	98.3	98.2	98.1	98.7	101.2 (171)
2B	72.0	72.9	67.0	66.8	78.4	78.3	
3B	79.43	79.7	82.8	82.9	80.5	80.4	79.6
4B	79.37	79.33	79.6	79.6	79.2	79.0	74.8
5B	68.2	68.1	68.9	68.9	67.7	67.3	71.7
6B	17.6	17.8	17.5	17.7	17.7	17.6	19.4

1C	99.2	99.2	98.4	99.1	99.7	99.5	105.3 (162)
2C	56.2	56.2	55.9	55.9	56.13	55.9	58.7
3C	79.17	79.1	78.9	78.9	79.1	78.8	76.6
4C	79.24	79.28	79.3	79.2	79.6	79.4	
5C	74.8	74.7	74.7	74.7	74.8	74.6	78.6
6C	68.7	68.8	69.6	69.6	69.3	69.1	63.7

(Table 2-II continued)

Ring Carbons	(17)	(18)	(23)	(24)
1A'	99.6	99.4	104.4 (169)	104.3 (175)
2A'	68.8	68.6		
3A'	77.6	77.4	82.7	82.7
4A'	80.3	80.1	73.9	73.9
5A'	67.9	67.7	71.9	
6A'	17.9	17.9		19.4

^a In CDCl₃ for 9, 13, 14, 15, 17, 18, and in D₂O for 23, and 24. The numbers in parentheses denote the one-bond ¹³C-¹H coupling constants (¹J_{13C-1H}) in Hz.

^b Indicates the ring to which the aglycone is attached.

Table 2-III ^1H and ^{13}C NMR Data^a for (20), (22) and (25)

Ring	$^1\text{H-NMR}$			$^{13}\text{C-NMR}$		
	(20)	(22)	(25)	(20)	(22)	(25)
1B ^b		4.58	4.76		98.8 (170) ^d	100.9 (171) ^d
2B		4.21	4.12		78.7	79.2
3B		3.85 (3.2, 9.5)	3.78		81.3	82.7
4B		3.22 (19.0) ^c	3.45		78.7	73.6
5B		3.47	3.66		67.2	71.4
6B		.095 (6.2)	1.24		17.7	19.0

1C		5.37 (8.4)	4.62 (8.5)		99.6 (163) ^d	105.2 (162) ^d
2C		4.49 (8.4, 10.5)	3.68		55.9	58.3
3C		4.30	3.55		79.0	76.3
4C		3.70	multiplet ↑ ↓		79.7	72.4
5C		3.66		3.40		74.2
6C		3.59 (10.9)	3.88		69.1	63.1
6'C		3.50	3.72			

1A	5.27 (1.6)	5.43	5.13 (1.8)	91.9 (171) ^d	99.1 (177) ^d	103.8 (173) ^d
2A	5.39	5.62	4.04 (1.8, 3.0)	72.9	72.7	72.4
3A	4.34	4.31	3.80	77.5	77.1	79.6
4A	3.65 (19.2) ^c	3.68	3.49	80.1	80.7	73.9
5A	4.07	3.93	3.69	67.8	67.9	71.7
6A	1.35	1.31	1.23	18.1	18.1	18.2

(Table 2-III Continued)

Ring	¹ H-NMR			¹³ C-NMR		
	(20)	(22)	(25)	(20)	(22)	(25)
1B'	5.18 (1.8)	5.25 (1.8)	5.00 (1.8)	98.7 (173) ^d	98.8 (173) ^d	104.2 (172) ^d
2B'	5.39	5.48 (1.8, 3.2)	4.23 (1.8, 3.1)	72.1	72.3	72.5
3B'	4.06 (3.4, 9.5)	4.17 (3.2, 9.5)	3.88	79.7	79.6	82.5
4B'	3.38 (18.0) ^c	3.49	3.46	78.7	78.7	73.3
5B'	3.70	3.94	3.77	68.3	68.2	71.7
6B'	0.92 (6.2)	1.18 (6.2)	1.26	17.6	17.7	19.2

1C'	5.36 (8.5)	5.46 (8.5)	4.68 (8.5)	98.5 (173) ^d	98.5 (166) ^d	105.3 (164) ^d
2C'	4.26 (8.5, 10.5)	4.30	3.72	54.7	54.7	58.3
3C'	5.54 (9.0, 10.5)	5.60 (9.3, 10.7)	3.55 ↑ multiplet ↓ 3.40	70.8	70.8	76.3
4C'	4.97 (9.0, 10.0)	5.01 (19.0) ^c		68.2	68.2	72.4
5C'	3.06	3.17		71.2	71.2	78.3
6C'	3.88 (3.4, 12.5)	3.92	3.88	61.1	61.1	63.1
6'C'	3.75 (2.0, 12.5)	3.79 (2.0, 10.5)	3.72			

^a In CDCl₃ for **20** and **22**, and in D₂O for **25**. The numbers in parentheses denote coupling constants in Hz.

^b Indicates the ring to which the aglycone is attached.

^c The values are the sums of the individual coupling constants, $J_{AX} + J_{BX}$.

^d These values are the one-bond ¹³C-¹H coupling constants (J_{13C-1H}) in Hz.

Table 2-IV ^1H and ^{13}C NMR Data^a for Compounds (27) (28) (29) and (30)

Ring	$^1\text{H-NMR}$ Data				$^{13}\text{C-NMR}$ Data			
	(27)	(28)	(29)	(30)	(27)	(28)	(29)	(30)
1C	6.33 (8.2)	6.94 (8.9)	5.70 (10.5)	6.61 (9.5)	94.1	90.5	81.4	
2C	4.91 (8.2, 10.0)	4.95 (8.9, 10.6)	4.66 ^b (21.0) ^b	4.90 (9.5, 10.5)	62.0	53.9	54.1	
3C	6.00 ^b (19.5) ^b	6.48 (9.5, 10.6)	6.33 (9.4, 10.5)	6.25 (10.5, 9.5)	73.3	69.6	72.1	
4C	5.80 ^b (19.5) ^b	5.84 (19.0) ^b	5.705 (9.4, 10.3)	5.81 ^b (19.0) ^b	69.4	70.9	70.3	
5C	4.43 (3.0, 4.9, 10.0)	4.50 (2.0, 4.5, 9.50)	4.29 (3.0, 4.5, 10.3)	4.35 (3.0, 5.0, 10.4)	74.1	72.9	76.3	
6C	4.65 (3.0, 12.2)	4.66 (2.0, 9.0)	4.63 (3.0, 12.2)	4.66 (3.0, 12.4)				
6'C	4.50 (4.9, 12.2)	4.52 (4.5, 9.0)	4.51 (4.5, 12.2)	4.52 (5.0, 12.4)	63.0	62.7	63.5	

^a In CDCl_3 . The numbers in parentheses denote coupling constants, in Hz.

^b These values are the sums of the individual coupling constants $J_{\text{Ax}} + J_{\text{Bx}}$.

Table 2-v ^1H and ^{13}C NMR Data^a for Compounds (31) (35) and (32)

Ring	^1H -NMR Data			^{13}C -NMR Data		
	(31)	(35) ^d	(32)	(31)	(35) ^d	(32)
1B ^b	4.71 (1.8)	5.07	4.55 (1.7)	96.3	91.7	98.2
2B	5.35 (1.8, 3.5)	5.46 (1.8, 3.5)	4.06	74.6	72.0	70.0
3B	4.15 (3.5, 9.5)	4.27 (3.5, 9.5)	3.95 (3.2, 9.2)	79.3	79.1	78.7
4B	3.38 (19.0) ^c		3.39 (18.7) ^c	79.8	79.2	83.6
5B	3.65 (6.4, 9.5)	3.87	3.58 (6.2, 9.5)	67.4	67.4	67.1
6B	1.11 (6.4)	1.09	1.10 (6.2)	17.7	17.7	17.6
1C	5.85 (8.5)	5.95 (8.5)	5.82 (8.5)	99.0	98.9	98.5
2C	4.67 (8.5, 10.6)		4.70 (8.5, 10.7)	55.2	55.1	54.6
3C	6.22 (9.5, 10.6)	6.26 (9.5, 10.6)	6.25 (9.2, 10.7)	71.4	71.3	71.1
4C	5.75 (19.5) ^c	5.77 (19.0) ^c	5.64 (9.2, 10.0)	69.7	69.5	69.6
5C	4.26	4.35	4.34 (2.7, 7.0, 10.7)	71.4	72.0	72.6
6C	4.59 (2.8, 12.2)	4.65 (2.8, 12.2)	4.74 (2.7, 12.2)	62.8	62.6	62.8
6'C	4.45 (4.0, 12.2)	4.46 (4.0, 12.2)	4.49 (7.0, 12.2)			

^a In CDCl_3 . The numbers in parentheses denote coupling constants in Hz.

^b Indicates the ring to which the aglycone is attached.

^c The values are the sums of the individual coupling constants, $J_{\text{AX}} + J_{\text{BX}}$.

^d The data is given for the α -anomer only.

Table 2-VI ^1H and ^{13}C NMR Data^a for Compounds (39) (40) and (41)

Ring	^1H -NMR Data			^{13}C -NMR Data	
	(39)	(40)	(41)	(39)	(40)
1B ^b	4.65 (1.5)	4.66 (1.5)	5.01	97.9	97.8
2B	4.33	4.23	4.31	72.2	72.2
3B	4.06 (3.2, 9.5)	4.08 (3.2, 9.5)	4.12 (3.2, 9.3)	81.6	81.7
4B	3.36 (19.0) ^c	3.28 (19.0) ^c	3.34 (19.0) ^c	78.9	79.3
5B	3.59	3.59 (6.2, 9.5)	3.81	67.5	67.5
6B	1.06 (6.3)	1.07 (6.2)	1.03 (6.2)	17.8	17.8
1C	5.85 (8.5)	5.82 (8.5)	5.85 (8.5)	99.9	99.8
2C	5.11 (8.5, 10.6)	5.08 (8.5, 10.8)	5.13 (8.5, 10.6)	54.9	55.0
3C	6.31 (9.4, 10.6)	6.33 (9.4, 10.8)	6.32 (9.4, 10.6)	71.4	71.1
4C	5.79 (19.0) ^c	5.85 (19.0) ^c	5.81 (19.0) ^c	70.2	70.1
5C	4.25	4.32	4.31	75.4	75.1
6C	4.64	4.65 (2.5, 12.0)	4.61 (2.5, 11.5)	63.5	63.4
6'C	4.35	4.43 (6.8, 12.0)	4.36 (7.0, 11.5)		
1A	5.45 (1.8)	5.34 (1.8)	5.42 (1.8)	99.7	99.7
2A	5.71 (1.8, 3.2)	5.66 (1.8, 3.2)	5.68 (1.8, 3.2)	71.6	70.4
3A	4.26	4.34	4.25	79.4	79.6
4A	3.60 (19.0) ^c	3.53 (19.0) ^c	3.58 (19.0) ^c	80.2	80.9
5A	3.94 (6.2, 9.5)	3.94 (6.2, 9.5)	3.92 (6.2, 9.5)	67.8	67.9
6A	1.24 (6.2)	1.30 (6.2)	1.22 (6.2)	18.1	18.2

Footnotes for Table 2-VI

- ^a In CDCl₃. The numbers in parentheses denote coupling constants in Hz.
- ^b Indicates the ring to which the aglycone is attached.
- ^c The values are the sums of the individual coupling constants, $J_{AX} + J_{BX}$.

Table 2-VII ^1H and ^{13}C NMR Data^a for Compounds (33) and (34)

Ring	^1H -NMR Data		^{13}C -NMR Data	
	(33)	(34)	(33)	(34)
1B ^b	4.59 (1.8)	4.77	97.7	101.2 (172)
2B	4.23 (1.8, 3.2)	4.13	79.3	79.3
3B	4.00 (3.2, 9.5)	3.80	81.6	82.8
4B	3.28 (19.0) ^c	3.46	78.5	73.6
5B	3.53 (6.2, 9.5)	3.68	67.4	72.0 ^d
6B	1.02 (6.2)	1.25	17.7	19.35
1C	5.80 (8.4)	4.63	99.8	105.3 (163)
2C	5.03	3.69	54.9	58.6
3C	6.26 (9.0, 10.5)	3.50	71.4	76.6
4C	5.78 (19.5) ^c	3.44 ↑ multiplet	70.0	72.6 ^e
5C	4.30	3.38 ↓	72.0	78.5 ^d
6C	4.62 (2.3, 11.8)	3.88	63.4	63.6 ^d
6'C	4.39 (7.3, 11.8)	3.72		
1A	5.46	5.14	99.2	104.1 (174)
2A	5.59 (1.8, 3.2)	4.04	72.9	72.6 ^e
3A	4.30	3.81	78.3	79.7
4A	3.67 (19.0) ^c	3.50	80.0	74.3
5A	3.89	3.71	67.6	74.2 ^d
6A	1.22 (6.2)	1.24	17.9	19.5

Table 2-VII Continued

Ring	¹ H-NMR Data		¹³ C-NMR Data	
	(33)	(34)	(33)	(34)
1B'	5.29 (1.8)	5.00	98.7	104.4 (175)
2B'	5.51 (1.8, 3.2)	4.24	72.0	72.6 ^e
3B'	4.18 (3.2, 9.5)	3.88	79.9	82.7
4B'	3.46 (19.0) ^c	3.47	78.9	74.0
5B'	3.88	3.77	68.1	72.1 ^d
6B'	1.09 (6.2)	1.26	17.5	19.30
1C'	5.47 (8.5)	4.68	98.4	105.4 (163)
2C'	4.31 (8.5, 11.0)	3.72	54.6	58.5
3C'	5.58 (9.0, 11.0)	3.52	70.8	76.5
4C'	5.01 (9.0, 10.0)	3.44 ↑ multiplet ↓ 3.38	68.3	72.6 ^e
5C'	3.18 (2.0, 3.0, 10.0)		71.2	78.4 ^d
6C'	3.94 (3.0, 11.2)	3.88	61.1	63.4 ^d
6'C'	3.83 (2.0, 11.2)	3.72		

^a In CDCl₃ for 33 and in D₂O for 34. The numbers in parentheses denote coupling constants in Hz.

^b Indicates the ring to which the aglycone is attached.

^c The values are the sums of the individual coupling constants, $J_{AX} + J_{BX}$.

^d These assignments may be interchanged between like carbons within like rings.

^e These assignments may be interchanged.

Table 2-VIII ^1H and ^{13}C NMR Data^a for Compound (43)

Ring	^1H -NMR Data	^{13}C -NMR Data
	(43)	(43)
1B ^b	4.61	97.9 (171) ^d
2B	4.29	78.3
3B	4.06 (3.2, 9.5)	81.7
4B	3.35 (19.0) ^c	78.9
5B	3.59	67.7
6B	1.06 (6.2)	17.6
1C	5.69 (8.5)	100.1 (165) ^d
2C	5.14	54.7
3C	6.22 (9.4, 10.5)	71.4
4C	5.77 (19.5) ^c	70.1
5C	3.68	71.8
6C	4.25	62.5
6'C	4.18	
1A	5.58	98.9 (175) ^d
2A	5.58	73.2
3A	4.23	77.6
4A	3.61 (19.0) ^c	80.3
5A	3.83	67.8
6A	1.10 (6.2)	17.8

Table 2-VIII Continued

Ring	¹ H-NMR Data	¹³ C-NMR Data
	(43)	(43)
1B'	5.14	100.9 (171) ^d
2B'	4.34	80.1
3B'	4.10 (3.2, 9.5)	81.3
4B'	3.36 (19.0) ^c	78.8
5B'	3.87 (6.2, 9.5)	68.7
6B'	1.06 (6.2)	17.9
1C'	5.84 (8.5)	99.9 (168) ^d
2C'	5.11	55.0
3C'	6.28 (9.0, 10.5)	71.5
4C'	5.78 (19.0) ^c	69.7
5C'	4.32	72.1
6C'	4.61	63.4
6'C'	4.38	
1A'	5.40 (1.6)	99.6 (174) ^d
2A'	5.64	71.5
3A'	4.25	75.2
4A'	3.54 (19.0) ^c	80.1
5A'	4.00	68.0
6A'	1.12 (6.2)	18.1

Footnotes for Table 2-VIII

- ^a In CDCl₃. The numbers in parentheses denote coupling constants in Hz.
- ^b Indicates the ring to which the aglycone is attached.
- ^c The values are the sums of the individual coupling constants, $J_{AX} + J_{BX}$.
- ^d These values are the one-bond ¹³C-¹H coupling constants (J_{13C-1H}) in Hz.

III: EXPERIMENTAL SECTION

A: General

Mp's were determined on a Fisher-John's melting point apparatus and are uncorrected. ^1H NMR and ^{13}C NMR spectra were recorded on either a Bruker WM-400 or a Bruker AMX-400 NMR spectrometer at 400.13 and 100.6 MHz, for proton and carbon respectively. All spectra were recorded in deuteriochloroform unless otherwise stated, and chemical shifts are given in ppm downfield from TMS. For those spectra measured in deuterium oxide, chemical shifts are given in ppm downfield from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Chemical shifts and coupling constants were obtained from a first-order analysis of the spectra.

The ^1H -homonuclear chemical-shift correlated (COSY) spectra were acquired by use of the pulse sequence $d1-90^\circ-d0-45^\circ$ -FID with quadrature detection in both dimensions. The initial data sets of 512x2048 data points were zero-filled once in the F_1 direction to give a final data set of 1024x1024 real data points. A non-shifted sine bell function was applied prior to Fourier transformation. The magnitude spectra were symmetrized about the diagonal before analysis.

The TOCSY spectrum was recorded by use of the pulse sequence $d1-90^\circ-d0$ -[MLEV spinlock]-FID, with a solvent presaturation pulse of 2 s during $d1$. The power level used for the spinlock gave a 25 μs 90° pulse. The spinlock (MLEV-17) was applied for a period of 250 ms. 512 experiments of 24 scans each were acquired to give an initial data set of 512x2048 data points. The initial data set was zero-filled once in the F_1 -direction to give a final data set of 1024x1024 real data points. A \cos^2 function was applied to the phase sensitive data set prior to Fourier transformation, the spectrum was then phase corrected and a base-

line correction routine was applied prior to analysis, by use of Bruker UXNMR software.

The ROESY spectrum was acquired by use of the pulse sequence d1-90-d0-spin lock-FID, with a presaturation pulse of 2 s during the relaxation delay d1. The CW spin-lock was applied for 250 msec at 0.5 watts of power at the frequency of the H₂O peak. 512 experiments of 24 scans each were recorded by use of phase sensitive detection. The data set was phase corrected, and a baseline correction was applied prior to analysis by use of the standard Bruker UXNMR software routines.

For the inverse detection experiments a 4-pulse sequence incorporating a BIRD pulse in the preparation period, with phase sensitive detection was used. The data set of 512x2048 data points was zero-filled once in both the F₁- and the F₂-directions, to give a final data set of 1024x2048 real data points, with a digital resolution of 9.3Hz/pt and 1.5Hz/pt in the F₁- and the F₂-directions, respectively. The data set was phase corrected, and a base-line correction applied prior to analysis.

The CF-252 plasma desorption mass spectra were obtained on a BIN-10K instrument from BIO-ION Nordic (Uppsala, Sweden). The samples were prepared in solutions of methanol/water and electrosprayed onto aluminum foils. The spectrum was acquired and the mass was assigned by use of the BIO-ION data system, based upon the PDP 11/73 processor. The experimental masses were obtained by determination of the time centroid of each peak above the baseline and by comparison of these with the times of flight of H⁺ and Na⁺ peaks appearing in the spectrum. Mass accuracy is approximately ±1 amu in the mass range of 500-1000 amu.

Analytical thin-layer chromatography (TLC) was performed on pre-

aluminum plates precoated with Merck silica gel 60F-254 as the adsorbent. The developed plates were air-dried, exposed to UV light and/or sprayed with 5% sulfuric acid in ethanol, and heated at 150°. All compounds were purified by medium pressure column chromatography on Kieselgel 60 (230-400 mesh) according to a published procedure.⁸⁴ Purification at each stage was crucial to the success of subsequent glycosylation reactions.

Solvents were distilled before use and were dried, as necessary, by literature procedures. Solvents were evaporated under reduced pressure and below 40°C.

Reactions performed under nitrogen were also carried out in deoxygenated solvents. Transfers under nitrogen were effected by means of standard Schlenk-tube techniques.

B: Experimental Procedures

Allyl 3,4,6-tri-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (5).--
-A solution of allyl 2-deoxy-2-phthalimido-β-D-glucopyranoside (4)^{60b} (4.80g, 13.7mmol) in anhydrous tetrahydrofuran (30mL) was added by means of a cannula under nitrogen to a cold suspension of NaH (2.0g, 42mmol) in tetrahydrofuran (20mL). The flask was rinsed with additional portions of tetrahydrofuran (2x3mL) and the contents were transferred as before. The mixture was stirred for 15min and then benzyl bromide (5.1mL, 42mmol) was added dropwise during 45min to the cold solution. The mixture was stirred under nitrogen for 24h at room temperature after which time an additional portion of NaH (0.20g, 4.1mmol) was added followed by the dropwise addition of benzyl bromide (0.51mL, 4.6mmol). After 12h the mixture was poured into a cold solution of 2N HCl and extracted with

ethyl acetate. The organic layer was washed with water until neutral pH and dried (Na_2SO_4). The filtrate was concentrated and the resulting syrup was chromatographed by use of hexane-ethyl acetate (2:1) as eluant. Compound (5) was obtained as a clear colorless syrup (5.49g, 64%); $[\alpha]_{\text{D}}^{25} +1.13^\circ$ (c 1.9 in CH_2Cl_2); $^1\text{H-NMR}$ (400.13MHz): δ 7.8-6.85 (19H, m, ArH); 5.18 (1H, d, $J_{1,2}=8.5\text{Hz}$, 1-H), 4.37 (1H, dd, $J_{2,3}=10.2\text{Hz}$, $J_{3,4}=9\text{Hz}$, 3-H), 4.22 (1H, dd, $J_{1,2}=8.5$, $J_{2,3}=10.2\text{Hz}$, 2-H), 3.78 (3H, m, 4-H, 6- H_a and 6- H_b), and 3.65 (1H, dt, $J_{5,6a}=3.0$, $J_{5,6b}=2.7\text{Hz}$, 5-H); $^{13}\text{C}\{^1\text{H}\}\text{-NMR}$ (100.6MHz): δ 133.9 ($\text{CH}_2\text{CH}=\text{CH}_2$), 117.1 ($\text{CH}_2\text{CH}=\text{CH}_2$), 97.5 (C-1), 79.8, 79.4 and 75.1 (PhCH_2), 74.8 and 74.7 (C-5 and C-4), 73.5 (C-3), 69.6 ($\text{CH}_2\text{CH}=\text{CH}_2$), 68.8 (C-6), and 55.9 (C-2). Anal. Calcd. for $\text{C}_{38}\text{H}_{38}\text{NO}_7$: C, 73.64; H, 6.18; N, 2.26. Found: C, 73.76; H, 6.15; N, 2.10.

3,4,6-Tri-O-benzyl-2-deoxy-2-phthalimido- β -D-glucofuranose (6). ---

Tris(triphenylphosphine)rhodium(I) chloride (0.80g, 0.87mmol) was added to a solution of the allyl glycoside (5) (5.4g, 8.7mmol) in ethanol-water (9:1) (300mL) and the mixture was heated at reflux for 18h under nitrogen. Removal of the solvent left a light brown residue which was taken up in ethyl acetate and filtered through a column of silica gel. Evaporation of the solvent gave a syrup which was dissolved in acetone-water (10:1) (250mL) containing yellow mercury (II) oxide (1.88g, 8.68mmol). To this solution mercuric (II) chloride (2.36g, 8.69mmol) in acetone-water (10:1) (50mL) was added dropwise, followed by dropwise addition of acetone-water (10:1) (20mL). The mixture was stirred for 18h following which the solvent was evaporated, and the resulting syrup was taken up in ethyl acetate. The solution was filtered through Celite, and the filtrate was washed successively with saturated aqueous potassium

iodide (2x), aqueous sodium thiosulfate (2x), and water (2x). The organic layer was dried (Na_2SO_4), and the solvent was removed by evaporation. The resulting syrup was purified by chromatography by use of hexane-ethyl acetate (2:1) as eluant to give *Compound (6)* as a clear colorless syrup (3.53g, 70%); $^1\text{H-NMR}$ (400.13MHz): δ 7.67-6.84 (19H, m, ArH), 5.36 (1H, br t, $J_{1,2}+J_{1,\text{OH}}=16\text{Hz}$, 1-H), 4.42 (1H, dd, $J_{1,2}=8\text{Hz}$, $J_{2,3}=11\text{Hz}$, 2-H), 4.11 (1H, dd, $J_{2,3}=11\text{Hz}$, $J_{3,4}=8.5\text{Hz}$, 3-H), 3.56 (4H, m, 4-H, 5-H, 6- H_a and 6- H_b), and 3.46 (1H, br d, $J_{1,\text{OH}}=8\text{Hz}$, D_2O exchangeable, OH); $^{13}\text{C}\{^1\text{H}\}\text{-NMR}$ (100.6MHz): δ 168.2 (carbonyl), 92.9 (C-1), 79.6, 79.1 and 75.0 (PhCH_2), 74.8 and 74.6 (C-4 and C-5), 73.4 (C-3), 68.7 (C-6), and 57.6 (C-2).

3,4,6-Tri-O-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl chloride (7).---Oxalyl chloride (2.1mL, 24mmol) was added to a stirred solution of DMF (1.7mL, 24mmol) in anhydrous dichloromethane (15mL) and the mixture was kept under nitrogen for 5min. The solvent was evaporated under reduced pressure and the white salt was dried *in vacuo* for 0.75h. The *NN*-dimethyl(chloromethylene)ammonium chloride was then dissolved in anhydrous dichloromethane and the solution was cooled to 0°C. A solution of the hemiacetals (**6**) (3.5g, 6.0mmol) in anhydrous dichloromethane (15mL) was transferred by means of a double tipped needle. The flask was rinsed with an additional portion (5mL) of solvent and the contents were transferred as before. The mixture was stirred under nitrogen for 2h after which time the reaction was quenched by the addition of cold aqueous sodium hydrogen carbonate (15mL). The organic layer was diluted with dichloromethane, then washed with sodium hydrogen carbonate and water, and dried (Na_2SO_4). Evaporation of the solvent gave the glycosyl

chloride (7) as a yellow syrup (3.48g, 97%). The syrup was dried in vacuo and used directly in subsequent glycosylation reactions; $^1\text{H-NMR}$ (400.13MHz): δ 6.25 (0.4H, d, $J_{1,2}=3\text{Hz}$, 1_{α}-H), and 5.98 (0.6H, d, $J_{1,2}=8\text{Hz}$, 1_{β}-H).

Allyl 2-O-acetyl-4-O-benzyl- α -L-rhamnopyranoside (8).---*Allyl 4-O-benzyl- α -L-rhamnopyranoside*^{60a} (5.18g, 17.6mmol) was dissolved in acetonitrile (25mL) containing methylorthoacetate (3mL). *p*-Toluenesulphonic acid (0.125g) was added, the mixture was partially concentrated under vacuum at 50°C on a rotatory evaporator, and then stirred at room temperature for 12h. Triethylamine (2.2mL) was added and the mixture was concentrated to give a syrup. The syrup was dissolved in 80% aqueous acetic acid (20mL) and after 5min the solution was evaporated to dryness. The residue was chromatographed on silica gel by use of hexane-ethyl acetate (3:1) as eluant to yield the title compound (8) as a yellow syrup (5.02g, 85%); $[\alpha]_{\text{D}}^{25} +5.5^{\circ}$ (c 2.8 in CH_2Cl_2); $^1\text{H-NMR}$ (400.13MHz): δ 7.39-7.27 (5H, m, ArH), 5.11 (1H, dd, $J_{1,2}=1.75\text{Hz}$, $J_{2,3}=3.8\text{Hz}$, 2-H), 4.77 (1H, d, $J_{1,2}=1.75\text{Hz}$, 1-H), 4.12 (1H, dd, $J_{2,3}=3.8$, $J_{3,4}=9.5\text{Hz}$, 3-H), 3.75 (1H, m, 5-H), 3.36 (1H, t, $J_{3,4}+J_{4,5}=19\text{Hz}$, 4-H), 2.15 (3H, s, OCOCH_3), and 1.36 (3H, d, $J_{5,6}=6.5\text{Hz}$, 6- H_3); $^{13}\text{C}\{^1\text{H}\}\text{-NMR}$ (100.6MHz): δ 133.5 ($\text{CH}=\text{CH}_2$), 117.4 ($\text{CH}=\text{CH}_2$), 96.6 (C-1), 81.7 (C-4), 75.1 (CH_2Ph), 72.9 (C-2), 70.2 (C-3), 68.1 ($\text{CH}_2\text{CH}=\text{CH}_2$), 67.5 (C-5), 20.9 (OCOCH_3), and 17.9 (C-6).
Anal. Calcd for $\text{C}_{18}\text{H}_{24}\text{O}_6$: C, 64.27; H, 7.19. Found: C, 64.33; H, 7.11.

Allyl 2-O-acetyl-4-O-benzyl-3-O-(3,4,6-tri-O-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl)- α -L-rhamnopyranoside (9).---A mixture of allyl 2-O-acetyl-4-O-benzyl- α -L-rhamnopyranoside (8) (1.27g, 3.79mmol),

silver trifluoromethanesulfonate (1.46g, 5.68mmol), collidine (0.75mL, 5.7mmol) and 4Å molecular sieves in anhydrous dichloromethane (5mL) was stirred under nitrogen and cooled to -78°C. A solution of 3,4,6-tri-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl chloride (7) (3.40g, 5.68mmol) in anhydrous dichloromethane (8mL), previously stirred with 4Å molecular sieves for 0.5h under nitrogen and cooled to -78°C, was added dropwise over 0.5h. The flask was rinsed with additional portions of dichloromethane (2x2mL) and the contents were transferred as before. The mixture was allowed to warm to room temperature and was stirred for 36h. The solids were removed by filtration and the filtrate was washed successively with aqueous sodium hydrogen carbonate and aqueous sodium chloride. The organic layer was dried (Na₂SO₄) and concentrated to give a syrup which was purified by chromatography by use of hexane-ethyl acetate (2:1) as eluant. *Compound (9)* was obtained as a clear colorless syrup (2.07g, 61%). An analytically pure sample was obtained as white prisms from methanol, m.p. 106-107°C; $[\alpha]_D^{25}$ -4.04° (c 1.09 in CH₂Cl₂); ¹H-NMR(400.13MHz): δ7.52-6.85 (24H, m, ArH), and 2.13 (3H, s, OCH₃), see Table 2-I also; ¹³C{¹H}-NMR(100.6MHz): δ170.7 (carbonyl), 117.6 (CH=CH₂), 75.0, 74.8, 74.7 and 74.6 (PhCH₂), 67.2 (CH₂-CH=CH₂), and 20.9 (OCOCH₃) see Table 2-II also. Anal. Calcd. for C₅₃H₅₅NO₁₂: C, 70.88; H, 6.17; N, 1.56. Found: C, 70.65; H, 6.15; N, 1.48.

8-(Methoxycarbonyl)octyl 2-O-benzoyl-4-O-benzyl-α-L-rhamnopyranoside (12).-- 8-(Methoxycarbonyl)octyl-α-L-rhamnopyranoside⁶⁸ (10.0g; 0.030 mmol) in acetone (200mL), *p*-toluenesulphonic acid (0.050g), and 2,2-dimethoxypropane were stirred at room temperature for 1h. Triethylamine (4.0mL) was added and the mixture was concentrated to a syrup. The syrup

was dried over P_2O_5 for 1h. A solution of the syrup in THF (50mL) was added to a suspension of sodium hydride (1.33g, 60.0mmol). After stirring for 10min., benzyl bromide (5.0g; 30.0mmol) was added dropwise and the stirring was continued at 20°C overnight. Additional sodium hydride (0.8g; 8mmol) was added to the stirred solution. The reaction mixture was added rapidly to a stirred solution of HCl (0.1N) at 0°C. Ethyl acetate (150mL) was added and the organic layer was removed. The aqueous phase was extracted with ethyl acetate (3X150mL). The combined organic layer was washed with sodium bicarbonate, dried ($MgSO_4$) and was then concentrated to a syrup. Chromatography on silica gel, with hexane-ethyl acetate (6:1) as eluant, gave a colorless syrup (11.2g; 80.6%). A portion of this syrup (10.0g; 21.5mmol) was taken up in HCl (0.5N, 100mL), and methanol (100mL) and refluxed for 1h. TLC, hexane-ethyl acetate (3:1), indicated that the reaction was essentially quantitative. The cooled reaction mixture was quenched with solid K_2CO_3 , filtered, and the filtrate was extracted with dichloromethane (3X50mL). The combined organic layer was dried ($MgSO_4$), and concentrated to a residue. The residue was purified on a short column of silica gel column by use of hexane-ethyl acetate (3:1) as eluant. The solvent was removed and part of the syrup obtained (3.0g; 7.0mmol), was dissolved in acetonitrile (80mL) containing trimethylorthobenzoate (1.5g; 8.0mmol). *p*-Toluenesulfonic acid (0.25g) was added and the mixture was partially concentrated at 50°C on a rotary evaporator and then stirred at 20°C for 18h. Triethylamine was added and the reaction mixture was concentrated to give a syrup. The syrup was dissolved in acetic acid (80%, 50mL), and after 5min., the solution was evaporated to dryness. The residue was chromatographed on silica gel with hexane-ethyl acetate (3:1) as eluant.

Pure (12) was obtained as a light yellow syrup (2.57g; 70%); $[\alpha]_D^{25}$ - 13.6° (c 3.4 in CH_2Cl_2); $^1\text{H-NMR}$ (400.13MHz): δ 5.17 (1H, dd, $J_{1,2}=1.8\text{Hz}$, $J_{2,3}=3.5\text{Hz}$, H-2), 4.83 (1H, d, $J_{1,2}=1.8\text{Hz}$, H-1), 4.86 and 4.78 (2H, ABq, $J_{A,B}=11.0\text{Hz}$, OCH_2Ph), 4.23 (1H, dd, $J_{2,3}=3.5\text{Hz}$, $J_{3,4}=9.5\text{Hz}$, H-3), 3.65 (1H, dq, $J_{4,5}=9.5\text{Hz}$, H-5), 3.45 (1H, t, $J_{3,4}+J_{4,5}=19.0\text{Hz}$, H-4), 3.24 [2H, m, $\text{OCH}_2(\text{CH}_2)_8\text{CO}_2\text{CH}_3$], 2.22 (1H, d, OH), 1.58 [4H, m, $\text{OCH}_2\text{CH}_2(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{CO}_2\text{CH}_3$], 1.38 (3H, d, $J=6.0\text{Hz}$, H-6), and 1.3 [8H, m, $\text{OCH}_2\text{CH}_2(\text{CH}_2)_4\text{CH}_2\text{CH}_2\text{CO}_2\text{CH}_3$]; $^{13}\text{C}\{^1\text{H}\}$ -NMR(100.6MHz): δ 174 and 165 (carbonyl), 97.4 (C-1), 81.4 (C-4), 76.6 (OCH_2Ph), 75.2 (C-3), 70.6 (C-2), 67.9 [$\text{OCH}_2(\text{CH}_2)_7\text{CO}_2\text{CH}_3$], 67.4 (C-5), 51.4 [$\text{OCH}_2(\text{CH}_2)_7\text{CO}_2\text{CH}_3$], 34.0, 29.4, 29.1, 28.98, 28.9, 26.1 and 24.9 [$\text{OCH}_2(\text{CH}_2)_7\text{CO}_2\text{CH}_3$], and 18.2 (C-6). Anal. Calcd. for $\text{C}_{30}\text{H}_{40}\text{O}_8$: C, 68.16; H, 7.63. Found: C, 67.98; H, 7.48.

8-(Methoxycarbonyl)octyl 2-O-benzoyl-4-O-benzyl-3-O-(3,4,6-tri-O-benzyl-2-deoxy-2-phthalimido- β -D-glucofuranosyl)- α -L-rhamnopyranoside (13).---A mixture of the monosaccharide (12) (0.703g; 1.33mmol), silver trifluoromethanesulfonate 0.606g; 2.36mmol), and collidine (0.32mL; 2.4mmol) in dichloromethane (3mL) was stirred under nitrogen with 4Å molecular sieves and cooled to -78°C . To this mixture was added dropwise a cooled solution (-78°C) of the glycosyl chloride (7) (1.41g; 2.36mmol) in dichloromethane (3mL), previously stirred with 4Å molecular sieves. The reaction mixture was stirred under nitrogen and in the dark and allowed to warm to room temperature. After 72h the reaction mixture was worked up, as with (9), and the resulting syrup was chromatographed on silica gel by use of hexane-ethyl acetate (3:1) as eluant. The title compound (13) (R_f 0.38) was obtained as a syrup (0.826g; 57%); $[\alpha]_D^{22}$ -

12.8' (c 0.73 in CHCl_3); $^1\text{H-NMR}$ (400.13MHz): δ 4.72 and 4.61 (2x1H, AB_q , $J_{\text{AB}}=10.8\text{Hz}$, OCH_2Ph), 4.73 and 4.38 (2x1H, AB_q , $J_{\text{AB}}=12.0\text{Hz}$, OCH_2Ph), 4.36 and 4.26 (2x1H, AB_q , $J_{\text{AB}}=11.5\text{Hz}$, OCH_2Ph), 4.39 and 4.24 (2x1H, AB_q , $J_{\text{AB}}=12.0\text{Hz}$, OCH_2Ph), 3.67 (3H, s, $\text{O}(\text{CH}_2)_8\text{CO}_2\text{CH}_3$), 3.57 and 3.39 (2x1H, overlapped m, $\text{OCH}_2(\text{CH}_2)_7\text{CO}_2\text{CH}_3$), see Table 2-I also; $^{13}\text{C}\{^1\text{H}\}$ -NMR(100.6MHz): δ 174.2 ($\text{O}(\text{CH}_2)_8\text{CO}_2\text{CH}_3$), 171.0 (OCOCH_3), 75.2, 74.6, 74.5 and 73.4 (OCH_2Ph), 67.2 ($\text{OCH}_2(\text{CH}_2)_7\text{CO}_2\text{CH}_3$), 51.3 ($\text{O}(\text{CH}_2)_8\text{CH}_2\text{CH}_3$), 34.1, 29.4, 29.3, 29.1 (2 carbons), 26.0 and 24.9 ($\text{OCH}_2(\text{CH}_2)_7\text{CO}_2\text{CH}_3$), see Table 2-II also. Anal. Calcd. for $\text{C}_{65}\text{H}_{71}\text{O}_{14}\text{N}$: C, 71.61; H, 6.56; N, 1.28. Found: C, 71.54; H, 6.76; N, 1.11.

Allyl 4-O-benzyl-3-O-(3,4,6-tri-O-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl)- α -L-rhamnopyranoside (14)---A solution of the disaccharide (9) (1.00g, 1.11mmol) in methanolic HCl (25mL) [prepared by treating anhydrous methanol (25mL) with acetyl chloride (1.45mL)] was kept at room temperature for 4h. The mixture was neutralized by the addition of triethylamine. The solvent was removed and the residue dissolved in ethyl acetate. The resulting precipitate was removed by filtration. Evaporation of the filtrate gave a syrup which was chromatographed by use of hexane-ethyl acetate (1:1) as eluant. *Compound (14)* was obtained as a clear colorless syrup (0.86g, 90%); $^1\text{H-NMR}$ (400.13MHz): δ 7.51-6.81 (24H, m, ArH), and 3.18 (1H, D_2O exchangeable, OH), see Table 2-I also; $^{13}\text{C}\{^1\text{H}\}$ -NMR(100.6MHz): δ 167.9 (carbonyl), 117.3 ($\text{CH}=\text{CH}_2$), 74.80, 74.78, 74.4 and 73.5 (PhCH_2), and 67.7 ($\text{CH}_2-\text{CH}=\text{CH}_2$) see Table 2-II also. Anal. Calcd. for $\text{C}_{51}\text{H}_{53}\text{O}_{11}\text{N}$: C, 71.56; H, 6.24; N, 1.64. Found: C, 71.40; H, 6.24; N, 1.52.

8-(Methoxycarbonyl)octyl 4-O-benzyl-3-O-(3,4,6-tri-O-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl)- α -L-rhamnopyranoside (15).---A sample of the disaccharide (13) (0.051g; 0.047mmol) was taken up in sodium methoxide in methanol (5.0mL; 0.1M). The solution was let stand at room temperature under nitrogen for 36h. The reaction mixture was poured into HCl (0.15M), and the solution was extracted with dichloromethane (5x10mL). The combined organic fractions were washed with aqueous sodium hydrogen carbonate followed by aqueous sodium chloride. The organic layer was then dried (Na_2SO_4), and filtered. Evaporation of the filtrate gave a syrup which was chromatographed by use of hexane-ethyl acetate (2:1) as eluant. The title compound (15) (R_f 0.36) was obtained as a clear colorless syrup (27mg; 58%); $[\alpha]_D^{22}$ -12.8 (c 0.39 in CHCl_3); $^1\text{H-NMR}$ (400.13MHz): δ 4.84 and 4.64 (2x1H, AB_q , $J_{\text{AB}}=11.0\text{Hz}$, OCH_2Ph), 4.77 and 4.41 (2x1H, AB_q , $J_{\text{AB}}=11.9\text{Hz}$, OCH_2Ph), 4.59 and 4.52 (2x1H, AB_q , $J_{\text{AB}}=12.0\text{Hz}$, OCH_2Ph), 4.33 and 4.21 (2x1H, AB_q , $J_{\text{AB}}=11.7\text{Hz}$, OCH_2Ph), 3.66 (3H, s, $\text{O}(\text{CH}_2)_8\text{CO}_2\text{CH}_3$), 3.53 and 3.30 (2x1H, dt, J' s=7.1 and 10.0Hz, $\text{OCH}_2(\text{CH}_2)_7\text{CO}_2\text{CH}_3$), and 3.18 (1H, br s, exchangeable OH) see Table 2-I also; $^{13}\text{C}\{^1\text{H}\}$ -NMR(100.6MHz): δ 174.2 ($\text{O}(\text{CH}_2)_8\text{CO}_2\text{CH}_3$), 173.6 (OCOCH_3), 74.9, 74.8, 74.63 and 74.59 (OCH_2Ph), and 67.5 ($\text{OCH}_2(\text{CH}_2)_7\text{CO}_2\text{CH}_3$), see Table 2-II also. Anal. Calcd. for $\text{C}_{58}\text{H}_{67}\text{O}_{13}\text{N}$: C, 70.64; H, 6.85; N, 1.42. Found: C, 70.40; H, 7.10; N, 1.22.

Allyl 2-O-(2-O-acetyl-3,4-di-O-benzyl- α -L-rhamnopyranosyl)-3-O-(3,4,6-tri-O-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl)-4-O-benzyl- α -L-rhamnopyranoside (17)---A mixture of the disaccharide (14) (0.85g, 0.99mmol), silver trifluoromethanesulfonate (0.763g, 2.97mmol), 1,1,3,3-tetramethylurea (0.35mL, 3.0mmol) and 4Å molecular sieves in anhydrous

dichloromethane (5mL) was stirred under nitrogen for 0.5h. The reaction mixture was cooled to -78° and a solution of 2-O-acetyl-3,4-di-O-benzyl- α -L-rhamnopyranosyl chloride (**16**)⁶⁸ (1.20g, 2.97mmol) in anhydrous dichloromethane (3mL), previously stirred with 4Å molecular sieves for 0.5h under nitrogen and cooled to -78°C, was added by means of a cannula under nitrogen. The flask was rinsed with additional portions of dichloromethane (2x2mL) and the contents were transferred as before. The mixture was allowed to warm gradually to room temperature and was stirred for 36h. The solids were removed by filtration and the filtrate was washed successively with sodium hydrogen carbonate solution and sodium chloride solution. The organic layer was dried (Na₂SO₄) and concentrated to give a syrup that was chromatographed by use of hexane-ethyl acetate (3:1) as eluant. Compound (**17**) was obtained as a white foam (0.957g, 81%); $[\alpha]_D^{24}$ -3.4° (c 0.95 in CH₂Cl₂); ¹H-NMR(400.13MHz): δ 7.44-6.71 (34H, m, ArH), and 2.12 (3H, s, OCOCH₃), see Table 2-I also; ¹³C{¹H}-NMR(100.6MHz): δ 169.9 (carbonyl), 117.1 (CH₂CH=CH₂), 74.7, 74.5, 74.2, 73.6 and 71.3 (OCH₂Ph), 67.6 (CH₂-CH=CH₂), and 21.0 (OCOCH₃), see Table 2-II also. Anal. Calcd. for C₇₃H₇₇O₁₆N: C, 71.60; H, 6.34; N, 1.14. Found: C, 71.63; H, 6.40; N, 1.04.

8-(Methoxycarbonyl)octyl 2-O-(2-O-acetyl-3,4-di-O-benzyl- α -L-rhamnopyranosyl)-3-O-(3,4,6-tri-O-benzyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl)-4-O-benzyl- α -L-rhamnopyranoside (**18**).---A mixture of the disaccharide (**15**) (0.109g; 0.110mmol), silver trifluoromethanesulfonate (0.16g; 0.62mmol), 1,1,3,3-tetramethylurea (80 μ L; 0.67mmol), and 4Å molecular sieves in anhydrous dichloromethane (2.0mL) was stirred under nitrogen for 30min. and cooled to -78°C. A cooled (-78°C) solution of

the rhamnopyranosyl chloride (**16**)⁶⁸ (0.177g; 0.436mmol) in anhydrous dichloromethane (2.0mL), previously stirred with 4Å molecular sieves, was added dropwise to the alcohol solution. The reaction mixture was stirred in the dark and under nitrogen. The temperature of the solution was allowed to reach room temperature. After 34h the reaction mixture was filtered, and the filtrate was evaporated to dryness to give a syrup which was chromatographed by use of hexane-ethyl acetate (3:1). The title compound (**18**) (R_f 0.18) was obtained as a clear colorless syrup (0.93g; 62%); ¹H-NMR(400.13MHz): δ4.95 and 4.61 (2x1H, AB_q, J_{AB} =11.0Hz, OCH₂Ph), 4.87 and 4.60 (2x1H, AB_q, J_{AB} =11.5Hz, OCH₂Ph), 4.85 and 4.65 (2x1H, AB_q, J_{AB} =11.0Hz, OCH₂Ph), 4.78 and 4.45 (2x1H, AB_q, J_{AB} =12.2Hz, OCH₂Ph), 4.59 and 4.52 (2x1H, AB_q, J_{AB} =11.5Hz, OCH₂Ph), 4.16 and 4.05 (2x1H, AB_q, J_{AB} =11.9Hz, OCH₂Ph), 3.66 (3H, s, O(CH₂)₈CO₂CH₃), 3.50 and 3.26 (2x1H, dt, J 's=7.1Hz and 10.0Hz, OCH₂(CH₂)₇CO₂CH₃), and 2.11 (3H, s, OCOCH₃), see Table 2-I also; ¹³C{¹H}-NMR(100.6MHz): δ174.0 (O(CH₂)₈CO₂CH₃), 169.6 (OCOCH₃), 75.0, 74.5, 74.3, 74.1, 73.4 and 71.1 (OCH₂Ph), 67.2 (OCH₂(CH₂)₇CO₂CH₃), 51.1 (O(CH₂)₈CO₂CH₃), 33.8, 29.1, 28.95, 28.88 (2 carbons), 25.8 and 24.7 (OCH₂(CH₂)₇CO₂CH₃), and 20.9 (OCOCH₃), see Table 2-II also. Anal. Calcd. for C₈₀H₉₁O₁₈N: C, 70.93; H, 6.77; N, 1.03. Found: C, 71.51; H, 6.87; N, 0.72.

3-O-(3-O-(3,4,6-Tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-2-O-benzoyl-4-O-benzyl-α-L-rhamnopyranosyl)-2-O-benzoyl-4-O-benzyl-α-L-rhamnopyranose (**20**).---A sample of trisaccharide (**19**)¹⁸ (1.96g;1.70mmol) was taken up in ethanol-water (9:1; 55mL).

Tris(triphenylphosphine)rhodium (I) chloride (0.085g; 0.092mmol) was added and the solution was stirred at reflux under nitrogen for 12h. The

solvent was removed by evaporation and the resulting dark brown syrup was taken up in ethyl acetate and filtered through a short column of silica gel. The filtrate was evaporated to dryness and the resulting foam was dissolved in 90% aqueous acetone (70mL). To the solution was added yellow mercury(II) oxide (0.543g; 2.51mmol), followed by the dropwise addition of a solution of mercury(II) chloride (0.676g; 2.49mmol) in 90% aqueous acetone (10mL). The reaction mixture was stirred for 12h, the solvent was evaporated, and the residue was taken up in ethyl acetate and filtered through celite. The filtrate was washed successively with saturated aqueous potassium iodide (2x), aqueous sodium thiosulfate (2x), and water (2x). The organic layer was dried (Na_2SO_4), the solvent was evaporated and the yellow syrup was chromatographed by use of hexane-ethyl acetate (1:1) as eluant. The title compound (**20**) R_f 0.36 was obtained as a clear light yellow syrup (1.37g; 72%); $^1\text{H-NMR}$ (400.13MHz): δ 5.11 and 4.78 (2x1H, AB_q, $J_{\text{AB}}=11.2\text{Hz}$, OCH_2Ph), 4.34 and 4.21 (2x1H, AB_q, $J_{\text{AB}}=12.5\text{Hz}$, OCH_2Ph), 2.79 (1H, s, exchangeable OH), 1.95, 1.76 and 1.71 (3x3H, s's, OCOCH_3), see Table 2-III also; $^{13}\text{C}\{^1\text{H}\}\text{-NMR}$ (100.6MHz): δ 170.7, 170.1, 169.2 and 165.7 (2 carbons) (carbonyl's), 75.1 and 73.8 (OCH_2Ph), 20.5 and 20.3 (2 carbons) (OCOCH_3), see Table 2-III also. Anal. Calcd. for $\text{C}_{60}\text{H}_{61}\text{O}_{20}\text{N}$: C, 64.57; H, 5.51; N, 1.25. Found: C, 64.54; H, 5.52; N, 1.26.

3-O-(-3-O-(3,4,6-Tri-O-acetyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl)-2-O-benzoyl-4-O-benzyl- α -L-rhamnopyranosyl)-2-O-benzoyl-4-O-benzyl- α -L-rhamnopyranosyl chloride (**21**).---Compound (**21**) was prepared in a similar fashion as described above for (**7**). Oxalyl chloride (0.10mL, 1.1mmol) was added to a stirred solution of DMF (0.090mL; 1.1mmol) in anhydrous

dichloromethane (3.0mL), and the mixture was stirred under nitrogen for 5min. The solvent was removed by evaporation and the white salt was dried *in vacuo* for 1.5h. The *N,N*-dimethyl(chloromethylene)ammonium chloride was then taken up in dichloromethane (1.5mL) and a solution of (20) (0.248g; 0.223mmol in 2.0mL dichloromethane) was transferred to the salt solution; the flask was rinsed with additional portions of dichloromethane (3x0.75mL) and the washings added to the reaction mixture. The mixture was stirred under nitrogen for 2h, and then quenched by the addition of cold aqueous sodium hydrogen carbonate. The organic layer was diluted with dichloromethane and was washed successively with aqueous sodium hydrogen carbonate and aqueous sodium chloride. The organic layer was dried over potassium bicarbonate and the solvent was evaporated to give a clear light yellow syrup (0.248g; 98%) which was dried *in vacuo*, and used directly, without chromatography, in subsequent glycosylations.

8-(Methoxycarbonyl)octyl 2-*O*-(3-*O*-(3-*O*-(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido- β -*D*-glucopyranosyl)-2-*O*-benzoyl-4-*O*-benzyl- α -*L*-rhamnopyranosyl)-2-*O*-benzoyl-4-*O*-benzyl- α -*L*-rhamnopyranosyl)-3-*O*-(3,4,6-tri-*O*-benzyl-2-deoxy-2-phthalimido- β -*D*-glucopyranosyl)-4-*O*-benzyl- α -*L*-rhamnopyranoside (22).---A sample of trisaccharide chloride (21) (0.248g; 0.218mmol) and 1,1,3,3-tetramethylurea (0.060mL; 0.50mmol) in anhydrous dichloromethane (1.5mL) was stirred with 4Å molecular sieves for 30min, and then cooled to -78°C. This solution was added dropwise, under nitrogen, to a cooled mixture (-78°C) of disaccharide alcohol (15) (0.135g; 0.137mmol), silver trifluoromethanesulfonate and 4Å molecular sieves in dichloromethane (2.0mL). The flask was rinsed with additional

portions of dichloromethane (3x1.0mL) and transferred as before. The reaction mixture was stirred in the dark under nitrogen and allowed to warm to room temperature. After 48h the mixture was filtered and the filtrate evaporated to dryness. The resulting syrup was purified by chromatography by use of hexane-ethyl acetate (1:1) followed by chromatography by use of toluene-ethyl acetate (3:1) as eluants. The title compound (**22**) was obtained as a clear colorless syrup (0.124g; 43%); $[\alpha]_D^{22}$ -20.8° (c 0.83 in CHCl_3); $^1\text{H-NMR}$ (400.13 MHz) see Table 2-III; $^{13}\text{C}\{^1\text{H}\}\text{-NMR}$ (100.6 MHz) see Table 2-III. Anal. Calcd. for $\text{C}_{118}\text{H}_{126}\text{O}_{32}\text{N}_2$: C, 68.00; H, 6.09; N, 1.34. Found: C, 67.85; H, 6.16; N, 1.39.

Propyl 2-O-(α -L-rhamnopyranosyl)-3-O-(2-acetamido-2-deoxy- β -D-glucopyranosyl)- α -L-rhamnopyranoside (23).---The fully blocked trisaccharide (**17**) (0.180g; 0.147mmol) was dissolved in methanolic HCl (6mL) [prepared by treating anhydrous methanol (100mL) with acetyl chloride (5.7mL)] and the solution was let stand under N_2 for 16h. Triethylamine was added dropwise to the stirred solution until neutral pH. The solution was concentrated and the precipitated salt was removed by filtration in ethyl acetate. The filtrate was evaporated to dryness and the resulting syrup was purified by column chromatography by use of hexane-ethyl acetate (2.5:1) as eluant; R_f 0.30. The syrup was then taken up in 80% aqueous acetic acid (14mL) and hydrogenolyzed over 10% palladium-carbon (0.120g) at a hydrogen pressure of 52 p.s.i. for 5 days. The solids were removed by filtration through celite and the filtrate was evaporated to dryness. The residue was evaporated with ethanol (3x75mL) to remove excess acetic acid and then dried *in vacuo* for 16h. The

resulting light brown syrup was taken up in ethanol (20mL) to which was added hydrazine hydrate (100%) (0.3mL). The solution was heated at reflux under N_2 for 24h. Filtration of a fine grey precipitate, followed by evaporation of the filtrate, gave a clear colorless syrup which was taken up in methanol (16mL) and then treated with acetic anhydride (4mL). The solution was let stand under N_2 at RT for 12h after which time TLC indicated that the reaction was complete. Removal of the solvent, followed by silica gel chromatography of the residue, by use of ethyl acetate-methanol-water (7:2:1) as eluant (R_f 0.34), gave a white solid which was passed through a column of Sephadex LH20 by use of methanol as eluant. Compound (23) was obtained as a white amorphous powder (0.040g; 49%); $[\alpha]_D^{25}$ 8.1° (c 1.78 in H_2O); 1H -NMR(400.13MHz, D_2O): δ 3.60 (1H, $J_{a,b}=10.0Hz$, $J_{a,CH_2}=6.3Hz$, $OCH_aH_bCH_2CH_3$), 3.46 ($OCH_aH_bCH_2CH_3$), 1.98 (3H, s, $NHCOCH_3$), 1.56 (2H, m, $OCH_2CH_2CH_3$), and 0.87 (3H, t, $J=7.5Hz$, $OCH_2CH_2CH_3$), see Table 2-I also; $^{13}C\{^1H\}$ -NMR(100.6MHz, D_2O): δ 177.5 ($NHCOCH_3$), 72.9, 72.8, 72.7 and 72.5 ($OCH_2CH_2CH_3$, C-4_C, C-2_A, and C-2_B), 25.0 ($NHCOCH_3$), 24.7 ($OCH_2CH_2CH_3$), 19.4 (C-6_B and C-6_A), and 12.6 ($OCH_2CH_2CH_3$), see Table 2-II also. Anal. Calcd. for $C_{23}H_{41}O_{14}N$: C, 49.72; H, 7.43; N, 2.52. Found: C, 49.44; H, 7.20; N, 2.37.

8-(Methoxycarbonyl)octyl 2-O-(α -L-rhamnopyranosyl)-3-O-(2-acetamido-2-deoxy- β -D-glucopyranosyl)- α -L-rhamnopyranoside (24).---A sample of the blocked trisaccharide (18) (0.121g, 0.0893mmol) was taken up in sodium methoxide (14mL; 0.13M). The reaction mixture was stirred at room temperature under nitrogen for 10h and was then neutralized by stirring with Amberlyst 15 (H^+) resin beads. The resin was removed by filtration and the filtrate was evaporated to dryness. The resulting syrup was

chromatographed by use of hexane-ethyl acetate (2:1) as eluant (R_f 0.45). The purified syrup (0.974g) was then taken up in 80% aqueous acetic acid (15mL); ethanol (2mL) was added to completely dissolve the sample. To this solution was added 10% palladium on carbon (0.125g). The reaction mixture was stirred under hydrogen (52p.s.i.) at room temperature for 14h. The reaction mixture was filtered through a pad of Celite, the filtrate was concentrated, and the syrup co-evaporated several times with ethanol to remove traces of acetic acid. The dried syrup was then taken up in ethanol (20mL) and hydrazine hydrate (100%; 0.15mL) was added. The clear light brown solution was heated at reflux under nitrogen for 18h. The reaction mixture was then filtered to remove a fine grey precipitate and the filtrate evaporated to dryness. The resulting syrup was then dried *in vacuo* for 2h to remove traces of hydrazine. The dried syrup was then taken up in methanol (12mL) and acetic anhydride was added (3.0mL). The solution was stirred under nitrogen for 24h. The solvent was reduced to half volume, additional methanol was added and the volume again reduced to half. This was repeated several times in order to remove the acetic anhydride without allowing the solution to get concentrated, thus avoiding the formation of the corresponding hydrazide from the methyl ester of the aglycone. Finally, the solvent was evaporated to dryness and the residue was chromatographed by use of ethyl acetate-methanol-water (7:2:1) as eluant (R_f 0.54). Further purification was carried out by passing the sample through a column of Sephadex LH20 by use of methanol as eluant. The title compound (**24**) was obtained as a white amorphous powder (20mg; 33%); $^1\text{H-NMR}$ (400.13MHz, D_2O): δ 3.62 ($\text{O}(\text{CH}_2)_8\text{CO}_2\text{CH}_3$), and 2.00 (NHCOCH_3), see Table 2-I also; $^{13}\text{C}\{^1\text{H}\}$ -NMR(100.6MHz, D_2O): δ 180.6 ($\text{O}(\text{CH}_2)_8\text{CO}_2\text{CH}_3$), 177.5 (NHCOCH_3), 72.8, 72.7

and 72.6 (C-2_B, C-2_A, and C-4_C), 71.9 and 71.7 (C-5_B and C-5_A), 70.8 (OCH₂(CH₂)₇CO₂CH₃), 54.8 (O(CH₂)₈CO₂CH₃), 36.5, 31.1, 30.94, 30.88 (2 carbons), 28.0 and 27.0 (OCH₂(CH₂)₇CO₂CH₃), and 25.0 (NHCOCH₃), see Table 2-II also. PD-MS Calcd. for C₃₀H₅₃NO₁₆Na: m/e 707. Found: m/e 708 (MNa)⁺.

8-(Methoxycarbonyl)octyl 2-O-(3-O-(3-O-2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl)-3-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-L-rhamnopyranoside (25).---A sample of the fully blocked pentasaccharide (22) (0.141g; 0.0677mmol) was deblocked as described above for (24). Following chromatography by use of ethyl acetate-methanol-water (7:2:1) eluant R_f 0.18 the title compound (25) was obtained as a white amorphous powder (18.2mg; 26%); [α]_D²² - 52.2° (c 0.21 in H₂O); ¹H-NMR (400.13 MHz, D₂O) see Table 2-III; ¹³C{¹H}-NMR (100.6 MHz, D₂O) see Table 2-III. Anal. Calcd. for C₄₄H₇₆O₂₅N₂: C, 51.16; H, 7.42; N, 2.71. Found: C, 50.74; H, 7.39; N, 2.80.

Benzoyl 3,4,6-tri-O-benzoyl-2-deoxy-2-(2-benzoyloxycarbonylbenzamido)-β-D-glucopyranoside (27).---To a sample of (26) (9.69g, 29.6mmol), dissolved in pyridine (80mL), was added benzoyl chloride (22mL) over a period of 10min. Upon addition of the benzoyl chloride, the yellow reaction mixture changed to a semi-solid yellow-red suspension. The reaction mixture was stirred at room temperature for a further 3h. The semi-solid mixture was then poured into ice (400mL), and the ice mixture extracted with dichloromethane (3x150mL). The combined extracts were dried (Na₂SO₄), and evaporated to dryness to give a pink solid. The solid was taken up in dichloromethane (150mL), and washed with

hydrochloric acid (1N), until neutral, and then washed with water. The organic layer was dried (Na_2SO_4), and evaporated to dryness to give a solid compound. The solid was suspended in diethyl ether, and collected by filtration. The title compound (27) was obtained as a white amorphous solid (12.1g, 55%); m.p. 218-220°C; $[\alpha]_D^{23}$ -44.4° (c 2.4 in CHCl_3); $^1\text{H-NMR}$ (400.13MHz): see Table 2-IV; $^{13}\text{C}\{^1\text{H}\}$ -NMR(100.6MHz): δ 166.3, 165.5, 165.4, 164 (benzoyl carbonyl), see Table 2-IV also. Anal. Calcd. for $\text{C}_{49}\text{H}_{37}\text{NO}_{13}$: C, 69.42; H, 4.40; N, 1.65. Found: C, 69.73; H, 4.28; N, 1.77.

1,3,4,6-tetra-O-Benzoyl-2-deoxy-2-phthalimido- β -D-glucoopyranoside (28) .--

- To a sample of (27) (6.58g, 8.85mmol) in pyridine (112mL) was added acetic anhydride (56mL). The solution was refluxed under nitrogen for 25h. Following reflux, the solution was poured into ice (250mL), and the resulting mixture extracted with dichloromethane (2x75mL). The organic layer was dried (Na_2SO_4) and evaporated to dryness. The resulting syrup was taken up in dichloromethane, and washed successively with hydrochloric acid (2N), and aqueous sodium hydrogen carbonate. The organic layer was dried (Na_2SO_4) and the solvent was evaporated to give a yellow foam. The foam was then triturated with ethanol (100%), affording a cream colored solid (28) (5.85g, 91%); m.p. 170-172°C; $[\alpha]_D^{23}$ 24.8° (c 5.2 in CHCl_3); $^1\text{H-NMR}$ (400.13MHz) see Table 2-IV; $^{13}\text{C}\{^1\text{H}\}$ -NMR(100.6MHz): δ 167.5 (2 C's, phthalimido carbonyl) 166.0, 165.5, 165.1, 164.1 (benzoyl carbonyl) see Table 2-IV also. Anal. Calcd. for $\text{C}_{42}\text{H}_{31}\text{NO}_{11}$: C, 69.51; H, 4.31; N, 1.93. Found: C, 69.34; H, 4.41; N, 1.96.

Ethylthio 3,4,6-tri-O-benzoyl-2-deoxy-2-phthalimido-β-D-glucopyranoside (29).---To a sample of (28) (6.35g, 8.75mmol) in anhydrous dichloromethane with 4Å molecular sieves, was added ethanethiol (1.3mL, 17.5mmol). The solution was maintained under an atmosphere of nitrogen and cooled to 0°C. Titanium tetrachloride (1.3mL, 11.8mmol) was added, and the solution removed from the ice-bath and allowed to warm to room temperature. Following 2h of reaction time, the reaction mixture was filtered through a pad of Celite and the filtrate washed with ice-cold sulfuric acid (1N). The organic layer was then washed successively with aqueous sodium hydrogen carbonate, and water. The organic layer was dried (Na₂SO₄), and the solvent was removed by evaporation. The resulting syrup was crystallized from ethanol (100%) to give the *title compound (29)* (3.86g, 66%); m.p. 142-143°C; [α]_D²³ 57.4° (c 2.3 in CHCl₃); ¹H-NMR(400.13MHz): δ2.76 and 2.68 (2x1H, ABdq, J=7.5Hz and J=12.7Hz, SCH₂CH₃), 1.23 (3H, t, J=7.5Hz, SCH₂CH₃), see Table 2-IV also; ¹³C[¹H]-NMR(100.6MHz): δ166.1, 165.7, 165.2 (benzoyl carbonyl), 24.4 (SCH₂CH₃), 15.0 (SCH₂CH₃), see Table 2-IV. Anal. Calcd. for C₃₇H₃₁NO₉S: C, 66.76; H, 4.69; N, 2.10. Found: C, 66.61; H, 4.72; N, 2.19.

3,4,6-Tri-O-benzoyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl bromide (30).---To a sample of compound (28) (7.31g, 10.1mmol) in anhydrous dichloromethane (50mL), was added a solution of 48% hydrogen bromide in acetic acid (15mL). The reaction mixture was stirred under nitrogen at room temperature for 2h. The reaction mixture was worked up by washing successively (by use of ice-cold solutions) with distilled water, and aqueous sodium hydrogen carbonate. The dichloromethane fraction was dried (Na₂SO₄), and the solvent was removed by evaporation to give a

white foam. The reaction proceeded quantitatively, and the product was used in the subsequent glycosylation reaction without further purification; $^1\text{H-NMR}$ (400.13MHz): see Table 2-IV.

Allyl 3-O-(3,4,6-tri-O-benzoyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl)-2-O-acetyl-4-O-benzyl- α -L-rhamnopyranoside (**31**).---A sample of 3,4,6-tri-O-benzoyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl bromide (**30**) (6.84g, 10.0mmol) in anhydrous dichloromethane (12.0mL) was stirred with 4Å molecular sieves, under nitrogen at room temperature for 30min. to this solution was added silver trifluoromethanesulfonate (2.60g, 10.1mmol). The solution was stirred for a further 10min, then collidine (1.34mL, 10.2mmol) was added. The solution of (**30**) was then cooled to -30°C , and a solution of allyl 2-O-acetyl-4-O-benzyl- α -L-rhamnopyranoside (**8**) (2.26g, 6.72mmol) in anhydrous dichloromethane (11.0mL), previously stirred with 4Å molecular sieves, was added dropwise under nitrogen. The dropping funnel was rinsed with additional portions of anhydrous dichloromethane and the washings added to the reaction mixture. The reaction mixture was removed from the cooling bath, and stirred in the dark under nitrogen for 8h. The reaction mixture was then filtered to remove the solids, and the filtrate was washed successively with hydrochloric acid (1N), aqueous sodium hydrogen carbonate, and distilled water. The organic layer was dried (Na_2SO_4), and the solvent removed by evaporation. The resulting syrup was purified by chromatography by use of toluene-ethyl acetate (15:1) as eluant. Compound (**31**) was obtained as a clear colorless syrup (3.82g, 57%); $[\alpha]_{\text{D}}^{23}$ 16.3° (c 2.6 in CHCl_3); $^1\text{H-NMR}$ (400.13MHz): δ 5.82 (1H, m, $\text{CH}_2\text{CH}=\text{CH}_2$), 5.25 (1H, m, $J_{\text{trans}}=15.5\text{Hz}$, $\text{CH}_2\text{CH}=\text{CHH trans}$), 5.17 (1H, m, $J_{\text{cis}}=10.5\text{Hz}$, $\text{CH}_2\text{CH}=\text{CHH cis}$), 4.41 and 4.26

(2x1H, ABq, $J_{A,B}=12.0\text{Hz}$, OCH_2Ph), 4.07 and 3.90 (2x1H, ABq, $J_{A,B}=12.5\text{Hz}$, $\text{CH}_2\text{CH}=\text{CH}_2$), 2.01 (3H, s, OCOCH_3), see Table 2-V also; $^{13}\text{C}\{^1\text{H}\}$ -NMR(100.6MHz): δ 170.1 (acetyl carbonyl), 166.1 154.7, 165.1 (benzoyl carbonyl) 117.7 ($\text{OCH}_2\text{CH}=\text{CH}_2$), 72.1 (OCH_2Ph), 68.3 ($\text{OCH}_2\text{CH}=\text{CH}_2$), 20.9 (OCOCH_3), see Table 2-V also. Anal. Calcd. for $\text{C}_{53}\text{H}_{49}\text{NO}_{15}$: C, 67.72; H, 5.25; N, 1.49. Found: C, 67.94; H, 5.33; N, 1.42.

Allyl 3-(3,4,6-tri-O-benzoyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl)-4-O-benzyl- α -L-rhamnopyranoside (32).---To the dissaccharide (31) (3.59g, 3.82mmol) was added methanolic HCl (35mL) [prepared by treating anhydrous methanol (100mL) with acetyl chloride (4mL)]. Anhydrous dichloromethane (25mL) was added to the solution to completely dissolve the sample. The reaction mixture was stirred under nitrogen at room temperature for 72h. Some starting material still remained, however, some breakdown products were forming, so the reaction was worked up at this point. The reaction mixture was washed with aqueous sodium hydrogen carbonate until neutral. The organic layer was dried (Na_2SO_4), and then evaporated to dryness. The resulting syrup was purified by chromatography by use of hexane-ethyl acetate (3:1) as eluant. *Compound (32)* was obtained as a clear colorless syrup which was dried *in vacuo* to give a white foam (2.61g, 76%); $[\alpha]_D^{23}$ 1.43° (c 2.1 in CHCl_3); ^1H -NMR(400.13MHz): δ 5.77 (1H, m, $\text{CH}_2\text{CH}=\text{CH}_2$), 5.21 (1H, m, $J_{\text{trans}}=15.5\text{Hz}$, $\text{CH}_2\text{CH}=\text{CHH trans}$), 5.15 (1H, m, $J_{\text{cis}}=10.5\text{Hz}$, $\text{CH}_2\text{CH}=\text{CHH cis}$), 4.39 and 4.26 (2x1h, ABq, $J_{A,B}=12.0\text{Hz}$, OCH_2Ph), 4.02 and 3.79 (2x1H, ABq, $J_{A,B}=12.5\text{Hz}$, $\text{CH}_2\text{CH}=\text{CH}_2$), see Table 2-V also; $^{13}\text{C}\{^1\text{H}\}$ -NMR(100.6MHz): δ 166.1, 165.5 165.2 (benzoyl carbonyl), 117.4 ($\text{OCH}_2\text{CH}=\text{CH}_2$), 74.5 (OCH_2Ph), 67.8 ($\text{OCH}_2\text{CH}=\text{CH}_2$), see Table 2-V also.

Anal. Calcd. for $C_{51}H_{47}NO_{14}$: C, 68.22; H, 5.27; N, 1.56. Found: C, 68.04; H, 5.40; 1.47.

Allyl 2-O-(3-O-(3-O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-2-O-benzoyl-4-O-benzyl-α-L-rhamnopyranosyl)-2-O-benzoyl-4-O-benzyl-α-L-rhamnopyranosyl)-3-O-(3,4,6-tri-O-benzoyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-4-O-benzyl-α-L-rhamnopyranoside (33). ---A sample of the trisaccharide chloride (**21**) (0.442g, 0.390mmol) in anhydrous dichloromethane (3.0mL) was stirred with 4Å molecular sieves for 30min, then transferred to a dropping funnel and cooled to -78°C. This solution was added dropwise to a cooled (-78°C) solution of the disaccharide alcohol (**32**), in anhydrous dichloromethane (1.5mL), containing silver trifluoromethanesulfonate (0.15g, 0.408mmol), and 1,1,3,3-tetramethylurea (0.050mL, 0.42mmol). The dropping funnel was rinsed with additional portions of dichloromethane (3x1.0mL) and the washings were added to the reaction mixture. The reaction mixture was stirred in the dark, under a nitrogen atmosphere, and allowed to warm to room temperature. After 60h and 100h reaction time, additional portions of silver trifluoromethanesulphonate (0.10g, 0.39mmol) and 1,1,3,3-tetramethylurea (0.05mL, 0.42mmol) were added to the reaction mixture. After 110h reaction time the reaction mixture was filtered and the filtrate was washed with hydrochloric acid (1N). The organic layer was dried (Na_2SO_4), and the solvent evaporated to give a syrup which was purified by chromatography by use of toluene-ethyl acetate (5:1) as eluant. The *title compound* (**33**) was obtained as a clear, light-yellow syrup which was dried *in vacuo* to give a foam (0.301g, 68%); $[\alpha]_D^{23}$ -6.7° (c 4.3 in $CHCl_3$); 1H -NMR(400.13MHz): 85.65 (1H, m, $CH_2CH=CH_2$), 5.16 and

4.81 (2x1H, ABq, $J_{A,B}=11.2\text{Hz}$, OCH_2Ph), 5.5 (1H, m, $J_{\text{trans}}=15.5\text{Hz}$, $\text{CH}_2\text{CH}=\text{CHH}$ trans), 5.08 (1H, m, $J_{\text{cis}}=10.5\text{Hz}$, $\text{CH}_2\text{CH}=\text{CHH}$ cis), 4.42 and 4.27 (2x1H, ABq, $J_{A,B}=12.5\text{Hz}$, OCH_2Ph), 4.18 and 4.13 (2x1H, ABq, $J_{A,B}=12.0\text{Hz}$, OCH_2Ph), 3.96 and 3.75 (2x1H, ABq, $J_{A,B}=12.5\text{Hz}$, $\text{OCH}_2\text{CH}=\text{CH}_2$) see Table 2-VII also; $^{13}\text{C}\{^1\text{H}\}$ -NMR(100.6MHz): δ 170.6, 169.9 and 169.1 (acetyl carbonyls), 165.9, 165.6 (2 carbons), 165.5 and 165.1 (benzoyl carbonyls), 117.1 ($\text{OCH}_2\text{CH}=\text{CH}_2$) 75.1, 74.3 and 73.7 (OCH_2Ph), 67.6 ($\text{OCH}_2\text{CH}=\text{CH}_2$), 20.4 and 20.2 (OCOCH_3) see Table 2-VII also. Anal. Calcd. for $\text{C}_{111}\text{H}_{106}\text{N}_2\text{O}_{33}$: C, 66.79; H, 5.35; N, 1.40. Found: C, 66.87; H, 5.58; N, 1.41.

Propyl 2-O-(3-O-(3-O-(2-acetamido-2-deoxy- β -D-glucopyransyl)- α -L-rhamnopyranosyl)- α -L-rhamnopyranosyl)-3-O-(2-acetamido-2-deoxy- β -D-glucopyranosyl)- α -L-rhamnopyranoside (34).---A sample of the fully protected pentasaccharide (33) (0.075g, 0.0383mmol) was dissolved in sodium methoxide in methanol (1.0M, 3.0mL). The solution was stirred at room temperature under nitrogen for 40h, and then neutralized by stirring with Amberlyst 15 (H^+) resin beads. The resin was removed by filtration, and the filtrate evaporated to dryness. The resulting syrup was taken up in 80% aqueous acetic acid (10mL) and was stirred at room temperature with 10% palladium on carbon (0.10g) under a hydrogen atmosphere (52p.s.i.) for 20h. The reaction mixture was then filtered through a pad of Celite with ethanol (100%). The filtrate was evaporated to dryness, and the resulting syrup evaporated several times with ethanol (100%) to remove traces of acetic acid. The dried syrup was then taken up in ethanol (100%) (15mL) to which was added hydrazine hydrate (100%) (0.10mL). The solution was refluxed under nitrogen for 10h. Following

reflux, the solution was filtered to remove a fine grey precipitate, and the filtrate was evaporated several times with methanol to remove traces of hydrazine hydrate. The resulting syrup was then taken up in methanol (8.0mL) to which was added acetic anhydride (1.5mL). After 10min TLC (EtOAc-MeOH-H₂O 6:3:1) indicated that the reaction was complete. The reaction mixture was evaporated to dryness and the residue was evaporated with ethanol (100%) to remove traces of acetic anhydride. The resulting syrup was purified by chromatography by use of ethyl acetate-methanol-water (6:3:1) as eluant. The purified sample was then taken up in water (1.0mL) and passed through a syringe filter (0.22 μ m) to remove traces of dissolved silica gel. The filtrate was then lyophilized, and the *title compound* (**34**) (0.0175g, 50%) was obtained as a white amorphous solid; ¹H-NMR(400.13MHz): see Table 2-VII; ¹³C(¹H)-NMR(100.6MHz): see Table 2-VII. PD-MS Calcd. for C₃₇H₆₄N₂O₂₃Na: m/e 928. Found: m/e 927 (MNa)⁺.

3-O-(3,4,6-tri-O-benzoyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl)-2-O-acetyl-4-O-benzyl- α -L-rhamnopyranose (**35**).---To a sample of the disaccharide (**31**) (0.249g, 0.265mmol) in ethanol-water (9:1) (35mL), was added tris(triphenylphosphine)rhodium(I) chloride (60mg). The reaction mixture was refluxed under nitrogen for 24h. Following reflux the reaction mixture was evaporated to dryness and the resulting syrup was taken up in ethyl acetate and filtered through a short column of silica gel. Evaporation of the filtrate gave a syrup which was taken up in acetone-water (10:1) (9.0mL) containing yellow mercury(II) oxide (0.086g, 0.397mmol). To this solution was added mercury(II) chloride (0.108g, 0.397mmol). The reaction mixture was stirred for 40h and the solvent was then removed by evaporation. The yellow syrup was taken up in ethyl

acetate and filtered through a pad of Celite. The filtrate was washed successively with saturated aqueous potassium iodide (2x), aqueous sodium thiosulfate (2x), and water (2x). The ethyl acetate layer was dried (Na_2SO_4), and concentrated to a syrup, which was purified by chromatography by use of hexane-ethyl acetate (2:1) as eluant. Compound (35) was obtained as a clear light yellow foam (0.192g, 80%); ^1H -NMR(400.13MHz): see Table 2-V; $^{13}\text{C}\{^1\text{H}\}$ -NMR(100.6MHz): δ 170.2 (acetyl carbonyl), 166.1, 165.6, 165.0 (benzoyl carbonyl), 74.5 (OCH_2Ph), 20.8 (OCOCH_3), see Table 2-V also. Anal. Calcd. for $\text{C}_{50}\text{H}_{45}\text{NO}_{15}$: C, 66.74; H, 5.04; N, 1.56. Found: C, 66.52; H, 5.26; N, 1.47.

3-O-(3,4,6-Tri-O-benzoyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl)-2-O-acetyl-4-O-benzyl- α -L-rhamnopyranosyl chloride (36).---To a solution of DMF (0.08mL, 1.0mmol) in anhydrous dichloromethane (1.0mL) was added oxalyl chloride (0.10mL, 1.1mmol). The mixture was stirred under nitrogen for 5min, then the solvent was evaporated under reduced pressure, and the resulting white salt was dried *in vacuo* for 1h. To the *NN*-dimethyl(chloromethylene)ammonium chloride salt was added a solution of the disaccharide hemiacetal (35) (0.164g, 0.182mmol) in anhydrous dichloromethane (1.0mL); the flask was rinsed with additional portions of dichloromethane (3x0.75mL) and the washings added to the bulk reaction mixture. The reaction mixture was stirred under nitrogen for 2.5h, after which time the reaction was quenched by the addition of ice-cold aqueous sodium hydrogen carbonate. The organic layer was then washed with saturated aqueous sodium chloride solution, and dried (K_2CO_3), and the solvent was removed by evaporation. The reaction proceeded nearly quantitatively (0.158g, 95%), and the product (36) was used immediately,

without further purification, in the subsequent glycosylation reaction.

Allyl 3-O-(3-O-(3,4,6-tri-O-benzoyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-2-O-acetyl-4-O-benzyl-L-rhamnopyranosyl)-2-O-benzoyl-4-O-benzyl-α-L-rhamnopyranoside (37).---A sample of the disaccharide chloride (36) (0.158g, 0.173mmol) in anhydrous dichloromethane (3.5mL) was stirred with 4Å molecular sieves for 30min. The solution was transferred to a dropping funnel and cooled to -78°C, and added dropwise to a cooled mixture (-78°C) of the alcohol acceptor (11)^{60a} (0.0800g, 0.200mmol), silver trifluoromethanesulfonate (0.160g, 0.623mmol) and 1,1,3,3-tetramethylurea (0.70mL, 0.58mmol). The dropping funnel was rinsed with additional portions of dichloromethane (3x0.75mL) and the washings were added to the reaction mixture. The reaction mixture was stirred in the dark, under nitrogen, and allowed to warm to room temperature. After 36h, the reaction mixture was filtered to remove the solids, and the filtrate was evaporated to dryness. The resulting syrup was purified by chromatography, by use of hexane-ethyl acetate (3:1) as eluant. A co-eluting mixture of products was obtained, consisting of a mixture of the α- and β-anomers of the desired trisaccharide; with the β-anomer, the major product (α:β= 1:6). Compound (37) was obtained as a white foam (0.796g, 36% α/β combined); ¹H-NMR(400.13MHz) (β-anomer unless otherwise noted): δ5.94 (1H, dd, $J_{2,3}=10.6\text{Hz}$, $J_{3,4}=9.5\text{Hz}$, H-3_C), 5.74 (1H, dd, $J_{1,2}=2.0\text{Hz}$, $J_{2,3}=3.4\text{Hz}$, H-2_A), 5.65 (1H, t, $J_{3,4}+J_{4,5}=19.0\text{Hz}$, H-4_C), 5.64 (1H, d, $J_{1,2}=8.5\text{Hz}$, H-1_C), 5.43 (1H, dd, $J_{1,2}=2.5\text{Hz}$, H-1_B), 4.80 (1H, d, $J_{1,2}=2.0\text{Hz}$, H-1_A), 4.76 and 4.57 (2x1H, ABq, $J_{A,B}=11.3\text{Hz}$, OCH₂Ph), 4.73 (1H, dd, $J_{1,2}=8.5\text{Hz}$, $J_{2,3}=10.6\text{Hz}$, H-2_C), 4.58 (1H, dd, $J_{1,2}=2.5\text{Hz}$, $J_{2,3}=4.0\text{Hz}$, H-2_B), 4.45 and 4.27 (2x1H, ABq, $J_{A,B}=12.2\text{Hz}$, OCH₂Ph), 4.28

(1H, dd, $J_{2,3}=3.4\text{Hz}$, $J_{3,4}=9.5\text{Hz}$, H-3_A), 3.92 (1H, dd, $J_{2,3}=4.0\text{Hz}$, $J_{3,4}=9.5\text{Hz}$, H-3_B), 3.90 (1H, dq, $J_{4,5}=9.5\text{Hz}$, $J_{5,6}=6.2\text{Hz}$, H-5_A), 3.57 (1H, t, $J_{3,4}+J_{4,5}=19.0\text{Hz}$, H-4_A), 3.43 (1H, t, $J_{3,4}+J_{4,5}=18.5\text{Hz}$, H-4_B), 3.32 (1H, dq, $J_{4,5}=9.0\text{Hz}$, $J_{5,6}=6.2\text{Hz}$, H-5_B), 2.86 (1H, dt, $J_{4,5}=10.0\text{Hz}$, H-5_C), 1.44 (3H, d, $J_{5,6}=6.2\text{Hz}$, H₃-6_A), 1.10 (3H, d, $J_{5,6}=6.2\text{Hz}$, H₃-6_B). Anal. Calcd. for C₇₃H₆₉NO₂₀: C, 68.48; H, 5.43; N, 1.09. Found: C, 68.37; H, 5.59; N, 1.18.

Allyl 2-O-(2-O-benzoyl-4-O-benzyl-3-O-((β-(trimethylsilyl)ethoxy)methyl)-α-L-rhamnopyranosyl)-3-O-(3,4,6-tri-O-benzoyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-4-O-benzyl-α-L-rhamnopyranoside (39).---A sample of the disaccharide alcohol (32) (2.61g, 2.91mmol) in anhydrous dichloromethane (15mL) with silver trifluoromethanesulfonate (2.24g, 8.72mmol) and 1,1,3,3-tetramethylurea (1.0mL, 8.36mmol), was stirred with 4Å molecular sieves for 30min, and then cooled to -78°C. A solution of the monosaccharide chloride (38)⁷² (2.21g, 4.36mmol) in anhydrous dichloromethane (7.0mL), previously stirred with 4Å molecular sieves for 30min and then cooled to -78°C, was added dropwise to the stirred solution of (32). The dropping funnel containing (38) was rinsed with additional portions of dichloromethane (3x3.0mL) and the washings were added to the reaction mixture. The reaction mixture was stirred under nitrogen, in the dark, and allowed to warm to room temperature. After 17h of reaction, the mixture was filtered through a pad of Celite, and the filtrate was washed successively with hydrochloric acid (1N), and saturated aqueous sodium chloride solution. The organic phase was dried (Na₂SO₄) and the solvent was evaporated. The remaining syrup was purified by chromatography, by use of hexane-ethyl acetate (3:1) as

eluant. The title compound (**39**) was obtained as a clear, light-yellow syrup which was dried *in vacuo* (2.24g, 61%); $[\alpha]_D^{23}$ -19.8° (c 4.8 in CHCl_3); $^1\text{H-NMR}$ (400.13MHz): 85.68 (1H, m, $\text{OCH}_2\text{CH}=\text{CH}_2$), 5.17 (1H, m, $J_{\text{trans}}=15.5\text{Hz}$, $\text{OCH}_2\text{CH}=\text{CHH}$ trans), 5.10 (1H, m, $J_{\text{cis}}=10.5\text{Hz}$, $\text{OCH}_2\text{CH}=\text{CHH}$ cis), 5.03 and 4.88 (2x1H, ABq, $J_{\text{A,B}}=7.0\text{Hz}$, $\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$), 4.97 and 4.71 (2x1H, ABq, $J_{\text{A,B}}=11.0\text{Hz}$, OCH_2Ph), 4.38 and 4.21 (2x1H, ABq, $J_{\text{A,B}}=12.5\text{Hz}$, OCH_2Ph), 4.00 and 3.80 (2x1H, ABq, $J_{\text{A,B}}=12.5\text{Hz}$, $\text{OCH}_2\text{CH}=\text{CH}_2$), 3.71 (2H, m, $\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$), 1.00 (2H, m, $\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$), -0.075 (9H, s, $\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$) see Table 2-VI also; $^{13}\text{C}\{^1\text{H}\}$ -NMR(100.6MHz): 8168.3, 166.9, 166.0, 165.7, 165.6 and 165.2 (carbonyls), 117.3 ($\text{OCH}_2\text{CH}=\text{CH}_2$), 94.1 ($\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$), 75.1 and 74.4 (OCH_2Ph), 67.7 ($\text{OCH}_2\text{CH}=\text{CH}_2$), 65.6 ($\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$), 19.9 ($\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$), -1.4 ($\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$), see Table 2-VI also. Anal. Calcd. for $\text{C}_{77}\text{H}_{81}\text{NO}_{20}$: C, 67.58; H, 5.96; N, 1.02. Found: C, 67.81; H, 6.01; N, 0.98.

Allyl 2-O-(2-O-benzoyl-4-O-benzyl- α -L-rhamnopyranosyl)-3-O-(3,4,6-tri-O-benzoyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl)-4-O-benzyl- α -L-rhamnopyranoside (40).---A sample of the trisaccharide (**39**) (0.906g, 0.662mmol) was dissolved in methanolic HCl (20mL) [prepared by treating anhydrous methanol (100mL) with acetyl chloride (4.0mL)]. This solution was diluted by the addition of anhydrous methanol (20mL), and anhydrous dichloromethane (5.0mL). The reaction mixture was stirred at room temperature, under nitrogen, for 3h. The reaction was quenched by adding aqueous sodium hydrogen carbonate. This solution was extracted with dichloromethane, and the organic layer dried (Na_2SO_4), and the solvent removed by evaporation. The remaining syrup was purified by

chromatography by use of hexane-ethyl acetate (2:1) as eluant. The title compound was obtained as a clear colorless syrup which was dried *in vacuo* to give a white foam (0.675g, 82%); $[\alpha]_D^{23}$ -22.7° (c 2.7 in CHCl_3); $^1\text{H-NMR}$ (400.13MHz): δ 5.68 (1H, m, $\text{OCH}_2\text{CH}=\text{CH}_2$), 5.18 (1H, m, $J_{\text{trans}}=15.5\text{Hz}$, $\text{OCH}_2\text{CH}=\text{CHH}$ trans), 5.09 (1H, m, $J_{\text{cis}}=10.05\text{Hz}$, $\text{OCH}_2\text{CH}=\text{CHH}$ cis), 4.94 and 4.77 (2x1H, ABq, $J_{\text{A,B}}=11.0\text{Hz}$, OCH_2Ph), 4.24 and 4.14 (1H, ABq, $J_{\text{A,B}}=12.0\text{Hz}$, OCH_2Ph), 3.99 and 3.80 (2x1H, ABq, $J_{\text{A,B}}=12.5\text{Hz}$, $\text{OCH}_2\text{CH}=\text{CH}_2$), 2.62 (1H, d, $J_{\text{OH,3A}}=5.3\text{Hz}$, OH) see Table 2-VI also; $^{13}\text{C}\{^1\text{H}\}\text{NMR}$ (100.6MHz): δ 166.2, 166.0, 165.7 and 165.1 (benzoyl carbonyls), 117.3 ($\text{OCH}_2\text{CH}=\text{CH}_2$), 74.5 and 73.8 (OCH_2Ph), 67.8 ($\text{OCH}_2\text{CH}=\text{CH}_2$), see Table 2-VI also. Anal. Calcd. for $\text{C}_{71}\text{H}_{67}\text{NO}_{19}$: C, 68.87; H, 5.45; N, 1.13. Found: C, 68.64; H, 5.45; N, 1.13.

2-O-(2-O-Benzoyl-4-O-benzyl-3-O-(β -trimethylsilyl)ethoxy)methyl)- α -L-rhamnopyranosyl)-3-O-(3,4,6-tri-O-benzoyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl)-4-O-benzyl- α -L-rhamnopyranose (41).---To a sample of the trisaccharide (39) (1.36g, 0.994mmol) in ethanol-water (9:1) (60mL) was added tris(triphenylphosphine)rhodium(I) chloride (0.265g). The reaction mixture was refluxed under nitrogen for 14h. Following reflux, the solvent was removed by evaporation, and the residue was taken up in ethyl-acetate and filtered through a short column of silica gel. The filtrate was evaporated to dryness, and the resulting syrup was dissolved in acetone-water (10:1) (40mL). To this solution was added yellow mercury(II) oxide (0.323g, 1.49mmol), followed by the addition of mercury(II) chloride (0.405g, 1.49mmol). The reaction mixture was stirred at room temperature for 40h. The solvent was then removed by evaporation and the residue was taken up in ethyl-acetate and filtered

through a pad of Celite. The filtrate was washed successively with saturated aqueous potassium iodide (2x), aqueous sodium thiosulfate (2x), and water (2x). The organic layer was dried (Na_2SO_4) and the solvent was removed by evaporation. The remaining syrup was purified by chromatography by use of hexane-ethyl acetate (2:1) as eluant. The title compound was obtained as a clear, light yellow syrup, which was dried *in vacuo* to give a foam (0.909g, 0.685mmol); $^1\text{H-NMR}$ (400.13MHz): δ 5.01 and 4.87 (2x1H, ABq, $J_{\text{A,B}}=7.0\text{Hz}$, $\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$), 4.96 and 4.70 (2x1H, ABq, $J_{\text{A,B}}=11.2\text{Hz}$, OCH_2Ph), 4.25 and 4.18 (2x1H, ABq, $J_{\text{A,B}}=12.0\text{Hz}$, OCH_2Ph), 3.70 (2H, m, $\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$), 2.75 (1H, d, $J_{\text{OH,1B}}=3.3\text{Hz}$, OH), 0.98 (2H, m, $\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$), -0.10 (9h, s, $\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$) see Table VI for other signals; $^{13}\text{C}\{^1\text{H}\}$ -NMR(100.6MHz): see Table 2-VI also. Anal. Calcd. for $\text{C}_{74}\text{H}_{77}\text{NO}_{20}\text{Si}$: C, 66.90; H, 5.84; N, 1.05. Found: C, 66.94; H, 6.03; N, 1.01.

2-O-(2-O-Benzoyl-4-O-benzyl-(β -trimethylsilyl)ethoxy)methyl)- α -L-rhamnopyranosyl)-3-O-(3,4,6-tri-benzoyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl)-4-O-benzyl-L-rhamnopyranosyl chloride (42).---To a solution of DMF (0.23mL, 2.97mmol) in anhydrous dichloromethane (5.0mL) was added oxalyl chloride (0.26mL, 2.98mmol). The mixture was stirred under nitrogen for 5min, then the solvent was evaporated under reduced pressure, and the resulting white salt dried *in vacuo* with gentle heating for 25min. To the *NN*-dimethyl(chloromethylene)ammonium chloride salt was added a solution of the trisaccharide hemiacetal (41) (0.774g, 0.582mmol) in anhydrous dichloromethane (5.0mL); the flask was rinsed with additional portions of dichloromethane (3x1.0mL). The reaction mixture was stirred under nitrogen, at room temperature for 30min, after which

time the reaction was quenched by the addition of ice-cold aqueous sodium hydrogen carbonate. This mixture was diluted with dichloromethane and the organic layer was then washed with saturated aqueous sodium chloride solution, and dried (K_2CO_3), and the solvent was removed by evaporation. The reaction proceeded nearly quantitatively as determined by TLC (0.775g, 98%), and the product (42) was used immediately, without further purification, in the subsequent glycosylation reaction.

Allyl 2-O-(3-O-(2-O-(2-O-benzoyl-4-O-benzyl-3-O-(β -trimethylsilyl)ethoxy)methyl)- α -L-rhamnopyranosyl)-3-O-(3,4,6-tri-O-benzoyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl)-4-O-benzyl- α -L-rhamnopyranosyl)-2-O-benzoyl-4-O-benzyl- α -L-rhamnopyranosyl)-3-O-(3,4,6-tri-O-benzoyl-2-deoxy-2-phthalimido- β -D-glucopyranosyl)-4-O-benzyl- α -L-rhamnopyranoside (43).---A sample of the trisaccharide alcohol (40) (0.198g, 0.149mmol) in anhydrous dichloromethane (3.0mL) with silver trifluoromethanesulfonate (0.102g, 0.397mmol) and collidine (0.6mL), was stirred with 4Å molecular sieves for 30min, and then cooled to $-78^\circ C$. A solution of the trisaccharide chloride (42) (0.345g, 0.256mmol) in anhydrous dichloromethane (4.0mL), previously stirred with 4Å molecular sieves for 30min and then cooled to $-78^\circ C$, was added dropwise to the stirred solution of (40). The dropping funnel containing (42) was rinsed with additional portions of dichloromethane (3x0.75mL) which were added to the reaction mixture. The reaction mixture was stirred under nitrogen, in the dark, and allowed to warm to room temperature. After 24h of reaction, the mixture was filtered, and the filtrate was washed successively with hydrochloric acid (1N), aqueous sodium hydrogen carbonate, and water. The organic phase was dried (Na_2SO_4) and the

solvent was evaporated to dryness. The remaining syrup was purified by chromatography, by use of hexane-ethyl acetate (2:1) as eluant. The title compound (43) was obtained as a clear light yellow syrup which was dried in vacuo (0.190g, 50%); $[\alpha]_D^{23}$ -14.6° (c 2.8 in CHCl_3); ^1H -NMR(400.13MHz): δ 5.92 (1H, m, $\text{OCH}_2\text{CH}=\text{CH}_2$), 5.18 (1H, m, $J_{\text{trans}}=15.5\text{Hz}$, $\text{OCH}_2\text{CH}=\text{CHH}$ trans), 5.10 (1H, m, $J_{\text{cis}}=10.5\text{Hz}$, $\text{OCH}_2\text{CH}=\text{CHH}$ cis), 4.99 and 4.62 (2x1H, ABq, $J_{\text{A,B}}=11.0\text{Hz}$, OCH_2Ph), 4.97 and 4.81 (2x1H, ABq, $J_{\text{A,B}}=7.0\text{Hz}$, $\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$), 4.94 and 4.69 (2x1H, ABq, $J_{\text{A,B}}=11.5\text{Hz}$, OCH_2Ph), 0.94 and 0.91 (2H, m, $\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$), -0.13 (9H, s, $\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$) see Table 2-VIII also; $^{13}\text{C}\{^1\text{H}\}$ -NMR(100.6MHz): δ 168.3, 166.0, 165.7 (2 carbonyls), 165.5, 165.4, 165.1 and 164.9 (benzoyl carbonyls), 117.2 ($\text{OCH}_2\text{CH}=\text{CH}_2$), 93.8 ($\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$), 75.2, 74.8, 74.4 and 73.7 (OCH_2Ph), 67.7 ($\text{OCH}_2\text{CH}=\text{CH}_2$), 65.5 ($\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$), 17.9 ($\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$), -1.5 ($\text{OCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{CH}_3)_3$), see Table 2-VIII also. Anal. Calcd. for $\text{C}_{145}\text{H}_{142}\text{N}_2\text{O}_{38}\text{Si}$: C, 68.33; H, 5.61; N, 1.09. Found: C, 68.12; H, 5.58; N, 1.10.

Preparation of BSA-glycoconjugate (45).---The 8-(methoxycarbonyl)octyl glycoside (44)¹⁸ (8.9mg, 13.0 μ mol) was dissolved in ethanol (200 μ L), and hydrazine hydrate (100 μ L) was added. The reaction mixture was let stand at room temperature, and after 12h, TLC by use of ethyl acetate-methanol-water (7:2:1) indicated that all of the starting ester had been consumed, to yield a more-polar component. The solvent was removed by evaporation, the residue was evaporated with water (2x1mL), and then taken up in water and lyophilized. The white powder was used directly in the subsequent reaction. The lyophilized hydrazide was taken up in freshly distilled *N,N*-dimethylformamide (400 μ L) and the solution was cooled to -40°C. A solution of N₂O₄ in CH₂Cl₂ (54 μ L, 19.5 μ mol, 0.36M) was added by means of a pre-cooled syringe. The solution was stirred for 15min. This solution was then added dropwise to a stirred solution of bovine serum albumin (BSA) (3.64mL, 10mg mL⁻¹) in buffer (0.08M in Na₂B₄O₇ and 0.35M in KHCO₃) at 0°C. The BSA solution was stirred at 0°C for 12h and then dialyzed against distilled water (6x6mL) by use of an Amicon ultrafiltration cell equipped with a PM-10 membrane. The residue was taken up in water and lyophilized to give (45) as a white powder (39mg) with a level of hapten incorporation of 13% (with the assumption of 60 lysine residues per BSA molecule), or 8 haptens per molecule of BSA. The incorporation level was established on the basis of carbohydrate content, as determined by the method of Dubois *et al*,⁷⁷ by use of rhamnose as the standard sugar.

Preparation of Horse Hb-glycoconjugate (46).---The 8-(methoxycarbonyl)octyl glycoside (44) (19.44mg, 28.4 μ mol) was dissolved in ethanol (400 μ L), and hydrazine hydrate (200 μ L) was added. The

reaction mixture was let stand at room temperature, and after 16h, TLC by use of ethyl acetate-methanol-water (7:2:1) indicated that all of the starting ester had been consumed, to give a more-polar component. The solvent was removed by evaporation, the residue was evaporated with water (3x2mL), and then taken up in water and lyophilized. The white powder was divided into two portions, part of which (9.94mg, 14.5mmol) was used directly in the subsequent reaction. The lyophilized hydrazide was taken up in freshly distilled *N,N*-dimethylformamide (400 μ L) and the solution was cooled to -40°C. A solution of N_2O_4 in CH_2Cl_2 (0.51M) was added by means of a pre-cooled syringe. Additional portions of N_2O_4 in CH_2Cl_2 were added until the reaction was complete (total volume added: 500 μ L, 255 μ mol). The solution was stirred for approximately 60min. This solution was then added dropwise to a stirred solution of Horse Hemoglobin (Hb) (3.8mL, 10mg mL⁻¹) in buffer (0.08M in $Na_2B_4O_7$ and 0.35M in $KHCO_3$) at 0°C. The Horse-Hb solution was stirred at 0°C for 12h. After this step it was noticed that some of the proteins had precipitated out of solution. The solution was then dialyzed against distilled water (2x200mL), after which the precipitated proteins were once again resuspended, to give a clear red solution. The solution was removed from the dialysis tubing while rinsing with distilled water; lyophilization afforded a red solid (46), with a level of hapten incorporation of 23% (with the assumption of 44 lysine residues per molecule of Horse-Hb), or 10 haptens per molecule of Horse-Hb. The incorporation level was established on the basis of carbohydrate content, as determined by the method of Dubois et al⁷⁷, by use of rhamnose as the standard sugar.

Preparation of BSA-glycoconjugate (47).---The 8-(methoxycarbonyl)octyl glycoside (**24**) (11.7mg, 17.1 μ mol) was dissolved in ethanol (200 μ L), and hydrazine hydrate (100 μ L) was added. The reaction mixture was let stand at room temperature, and after 12h, TLC by use of ethyl acetate-methanol-water (7:2:1) indicated that all of the starting ester had been consumed, to give a more-polar component. The solvent was removed by evaporation, the residue was evaporated with water (3x1mL), and then taken up in water and lyophilized. The white powder was used directly in the subsequent reaction. The lyophilized hydrazide was taken up in freshly distilled *N,N*-dimethylformamide (0.4mL) and the solution was cooled to -40°C. A solution of N_2O_4 in CH_2Cl_2 (95 μ L, 34 μ mol, 0.36M) was added by means of a pre-cooled syringe. The solution was stirred for 15min and an additional portion of N_2O_4 in CH_2Cl_2 (45 μ L, 0.36M) was added. After a further 20min the reaction mixture was poured into a stirred solution of bovine serum albumin (BSA) (4.7mL, 10mg mL⁻¹) in buffer (0.08M in $Na_2B_4O_7$ and 0.35M in $KHCO_3$) at 0°C. The BSA solution was stirred at 0°C for 12h and then dialyzed against distilled water (6x6mL) by use of an Amicon ultrafiltration cell equipped with a PM-10 membrane. The residue was taken up in water and lyophilized to give (**47**) as a white powder (36.2mg) with a level of hapten incorporation of 15% (with the assumption of 60 lysine residues per BSA molecule), or 9 haptens per molecule of BSA. The incorporation level was established on the basis of carbohydrate content, as determined by the method of Dubois *et al.*⁷⁷, by use of rhamnose as the standard sugar.

Preparation of Horse Hb-glycoconjugate (48).---The 8-(methoxycarbonyl)octyl glycoside (**24**) (5.65mg, 8.26 μ mol) was dissolved

in ethanol (150 μ L), and hydrazine hydrate (75 μ L) was added. The reaction mixture was let stand at room temperature, and after 16h, TLC by use of ethyl acetate-methanol-water (7:2:1) indicated that all of the starting ester had been consumed, to give a more-polar component. The solvent was removed by evaporation, the residue was evaporated with water (3x2mL), and then taken up in water and lyophilized. The white powder was divided into two portions, part of which (2.95mg, 4.31 μ mol) was used directly in the subsequent reaction. The lyophilized hydrazide was taken up in freshly distilled *N,N*-dimethylformamide (200 μ L) and the solution was cooled to -40°C. A solution of N₂O₄ in CH₂Cl₂ (0.51M) was added by means of a pre-cooled syringe. Additional portions of N₂O₄ in CH₂Cl₂ were added until the reaction was complete (total volume added: 200 μ L, 102 μ mol). The solution was stirred for approx. 60min. This solution was then added dropwise to a stirred solution of Horse Hemoglobin (Hb) (1.12mL, 10mg mL⁻¹) in buffer (0.08M in Na₂B₄O₇ and 0.35M in KHCO₃) at 0°C. The Horse-Hb solution was stirred at 0°C for 12h. The solution was then dialyzed against distilled water (2x6mL), by use of an Amicon ultrafiltration cell equipped with a PM-10 membrane. During this procedure, it was noticed that there was some damage to the membrane by the solvents contained in the original solution, and resulted in some leakage through the membrane. As a result, extensive dialysis was not carried out, which resulted in the possibility of some unbound hapten remaining in the sample; the carbohydrate content of this sample was therefore not determined. The solution was removed from the filtration cell, the cell was rinsed with distilled water, and the combined solution was lyophilized to give a red solid (48).

Preparation of BSA-glycoconjugate (49).---Pentasaccharide (25) (7.0mg, 6.8 μ mol) was dissolved in ethanol (200 μ L) and hydrazine hydrate (100 μ L). After 12h the reaction mixture was worked up as with (48) and the lyophilized powder was dissolved in freshly distilled *N,N*-dimethylformamide (0.4mL). The solution was cooled to -40°C and a solution of N₂O₄ in CH₂Cl₂ (70 μ L, 0.36M) was added. After 30min this mixture was poured into a stirred solution of BSA (1.8mL, 10mg mL⁻¹) in buffer (0.08M in Na₂B₄O₇ and 0.35M in KHCO₃) at 0°C. After 12h of stirring at 0°C the reaction mixture was dialyzed against distilled water (6x6mL), by use of an Amicon ultrafiltration cell equipped with a PM-10 membrane, to remove any unbound hapten. The residue was taken up in distilled water and lyophilized to give a white powder (18.7mg) with a hapten incorporation level of 30%, or 18 haptens per molecule of BSA.

Preparation of Horse Hb-glycoconjugate (50).---The 8-(methoxycarbonyloctyl glycoside (25) (8.85mg, 8.57 μ mol) was dissolved in ethanol (200 μ L), and hydrazine hydrate (100 μ L) was added. The reaction mixture was let stand at room temperature, and after 16h, TLC by use of ethyl acetate-methanol-water (7:2:1) indicated that all of the starting ester had been consumed, to give a more-polar component. The solvent was removed by evaporation, the residue was evaporated with water (3x2mL), and then taken up in water and lyophilized. The white powder was divided into two portions, part of which (4.41mg, 4.27 μ mol) was used directly in the subsequent reaction. The lyophilized hydrazide was taken up in freshly distilled *N,N*-dimethylformamide (200 μ L) and the solution was cooled to -40°C. A solution of N₂O₄ in CH₂Cl₂ (0.51M) was added by means of a pre-cooled syringe. Additional portions of N₂O₄ in CH₂Cl₂

were added until the reaction was complete (total volume added: 250 μ L, 127 μ mol). The solution was stirred for approximately 60min. This solution was then added dropwise to a stirred solution of Horse Hemoglobin (Hb) (1.16mL, 10mg mL⁻¹) in buffer (0.08M in Na₂B₄O₇ and 0.35M in KHCO₃) at 0°C. The Horse-Hb solution was stirred at 0°C for 12h. The solution was then dialyzed against distilled water (2x6mL), by use of an Amicon ultrafiltration cell equipped with a PM-10 membrane. During this procedure, as with compound (48), it was noticed that there was some damage to the membrane. Extensive dialysis was not carried out, and resulted in the possibility of some unbound hapten remaining in the sample; therefore, the carbohydrate content of this sample was not determined. The solution was removed from the filtration cell, the cell was rinsed with distilled water, and the combined solution was lyophilized to give a red solid (50).

CHAPTER 3

I: INTRODUCTION

The Gram positive β -hemolytic *Streptococci* Group A are widespread bacteria, which are pathogenic in humans. Infection with this organism can result in the commonly known condition of streptococcal pharyngitis, or strep throat.³ The initial streptococcal infection has been implicated in the development of the more serious condition of rheumatic fever.⁴ In addition, there appears to be a connection between the initial *Streptococci* infection and the development of other diseases such as acute glomerulonephritis, and rheumatoid arthritis.⁵ One of the goals of the present work was to generate an antibody directed against the *Streptococci* Group A cell-wall polysaccharide, which could be used in a diagnostic test kit for the rapid detection of streptococcal infections,¹⁰ by use of synthetic, chemically defined haptens to select and/or characterize its binding properties.

A: Background

1: Antibody Production

When a foreign antigen is injected into a suitable recipient (i.e. immunization with an antigen), an immune response is initiated in the recipient against the antigen. The antigen may be a complex biomolecule with several unique molecular features on its surface. Each of these unique features represents a distinct antigenic determinant, or epitope. The antibody producing cells (B- and T-lymphocytes) of the immunized individual will be stimulated by the various epitopes present on the antigen, with each epitope causing the stimulation of a particular clone of antibody producing cell. Thus, a multivalent antigen will cause the stimulation of a variety of

B- and T-lymphocytes, and results in the production of several different clones of the antibody. If a serum sample is obtained from the immunized individual, then the serum will be found to contain a variety of antibodies with specificities for the various epitopes on the original antigen; such a serum sample is termed "polyclonal serum" since it results from several different lymphocyte clones.

In recent years, the production of antibodies has been revolutionized by the development of methods for the production of antibodies resulting from a single antibody producing clone of lymphocytes.¹² These antibodies are termed monoclonal antibodies, and since they all result from the same parent lymphocyte, they have identical specificities and affinities. A schematic diagram illustrating monoclonal antibody production is shown in Figure 3-1. The first step is the immunization of a mouse with the antigen of interest. After a series of immunizations designed to stimulate the B-lymphocytes, the antibody producing cells are extracted from the spleen of the immunized mouse. The B-lymphocytes from the mouse spleen are fused with malignant B-lymphocytes called myeloma cells. The fusion product, termed a hybridoma, will grow in tissue culture like a tumor cell, but will now produce the immunoglobulin determined by the activated B-lymphocyte. Under selective tissue culture conditions, the hybridoma continues to grow and divide, whereas the unfused spleen cells and myeloma cells die out. The hybridoma colonies are diluted and plated out such that new colonies which result are descended from a single hybridoma fusion product, hence each new colony produces a particular immunoglobulin. The hybridoma colonies are then screened, generally in an ELISA (see below), with a purified antigen to identify colonies which are producing the antibodies with the desired binding specificities. Larger amounts of the antibody may then be produced by

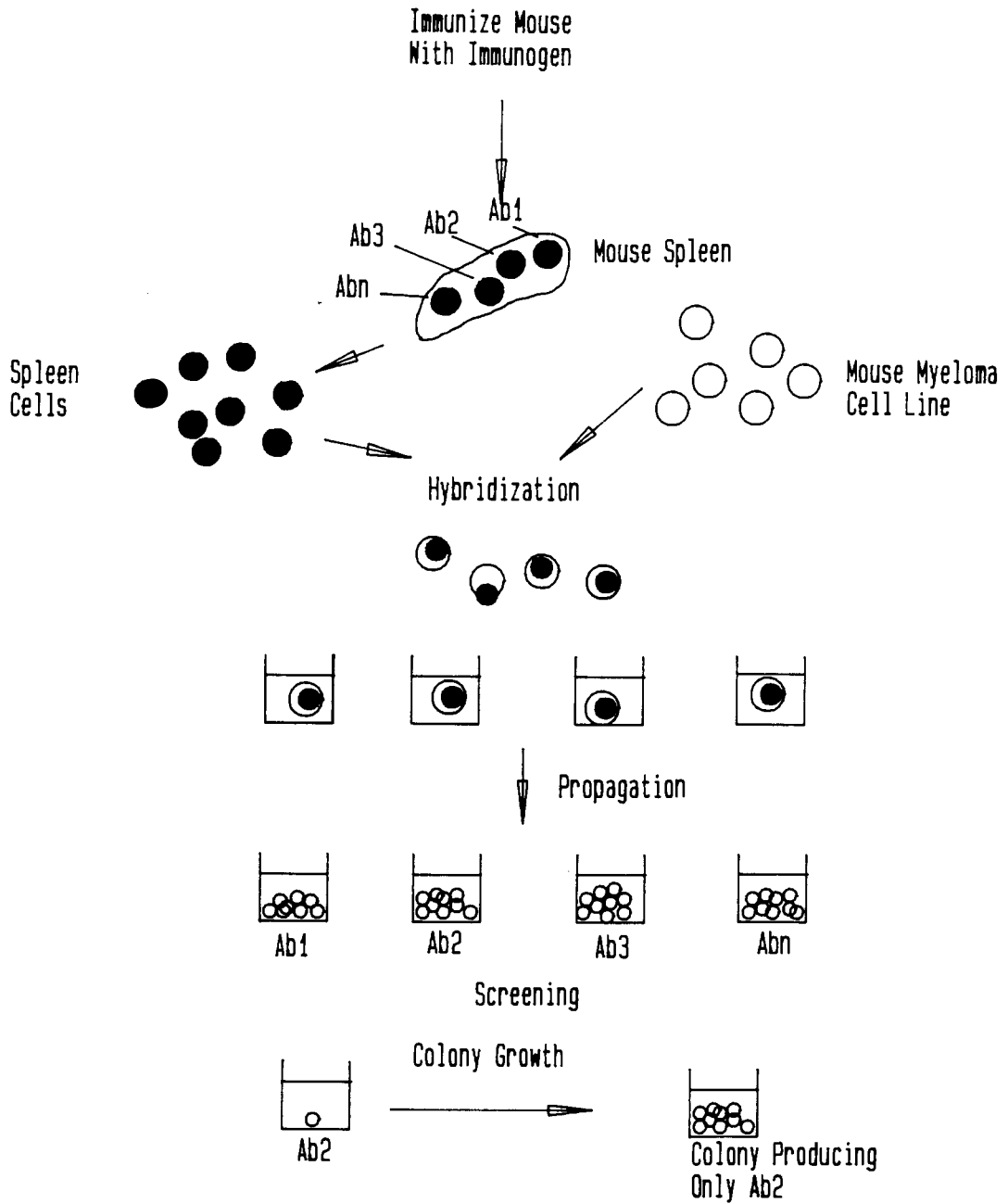


Figure 3-1: Production of a monoclonal antibody by use of the hybrid myeloma protocol.

propagating the hybridoma by use of fermentation technologies, or by raising ascites fluid in suitable animals.

2: *Immunoassays*

Several methods exist for the characterization of antigen-antibody binding interactions. When a soluble multivalent antigen is mixed with a solution of antibody, at an optimum concentration of both, insoluble immune complexes consisting of cross-linked antigens and antibodies will precipitate out of solution; this reaction is known as the precipitin reaction.⁸⁵ The immune complex is the result of more than one antibody molecule binding to the same multivalent antigen molecule. If the antigen is present in sufficient excess, then only a few of the epitopes of the antigen will be bound, and no cross-linking will occur. Conversely, if the antibody is in excess, then most antibody molecules will have only one of their binding sites occupied, and again little cross-linking will take place. At an optimum concentration of antibody and antigen, the so called equivalence point, the number of epitopes is roughly equivalent to the number of binding sites and cross-linking, or lattice formation, can occur. The amount of precipitate formed may be equated to the amount of antigen in a test solution. This reaction may be carried out in solution or semi-solid supports such as agar gels, the latter are used in immunodiffusion and immunoelectrophoresis techniques.

Other immunoassays may involve the labelling of either antibody or antigen with a covalently linked radioactive label, or enzyme label. In radioimmunoassays (RIA) either the antibody or the antigen is radioactively labelled; in Figure 3-2, one such assay is illustrated. In the first step, the antibody is adsorbed to a solid support. The solid support is then

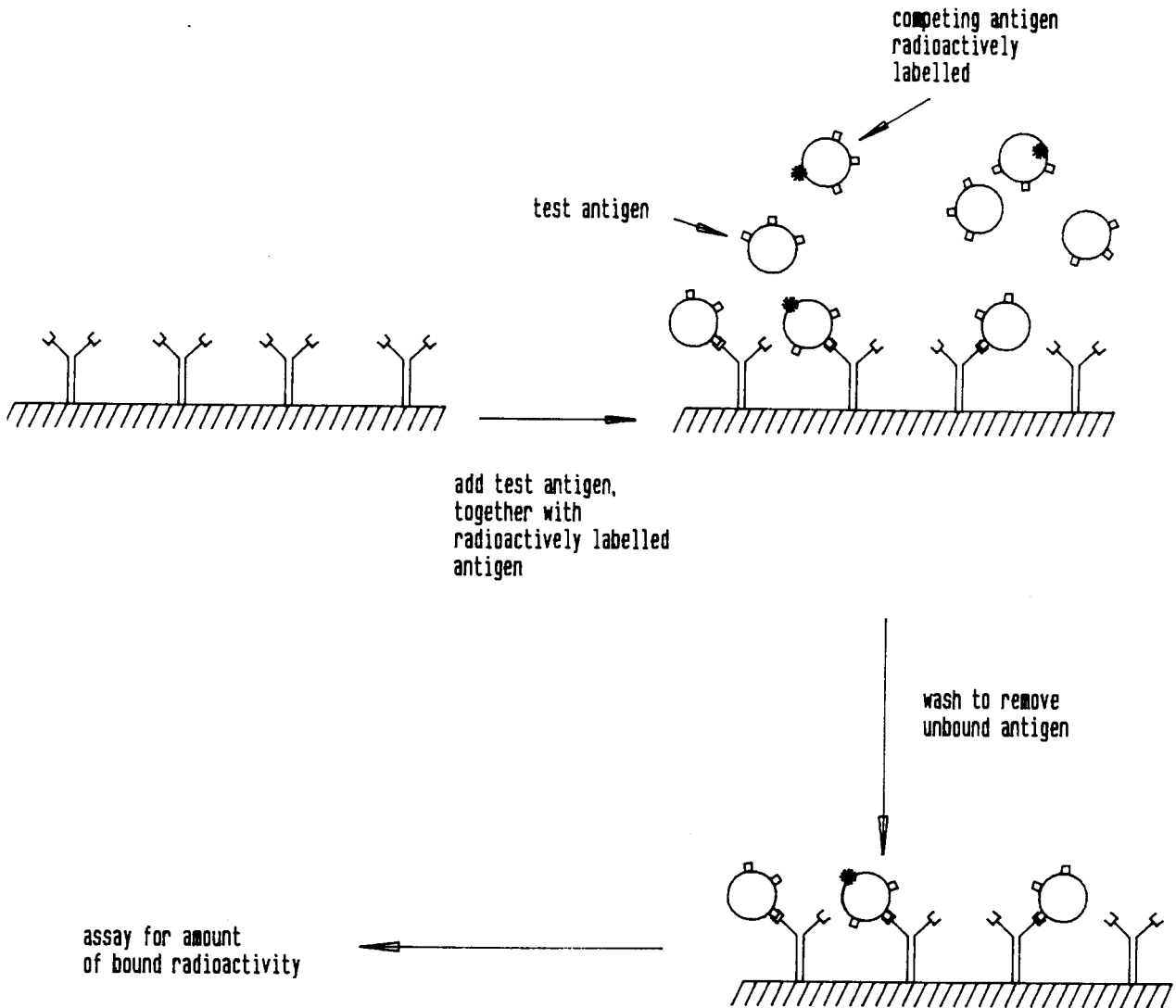


Figure 3-2: Competitive radioimmunoassay (RIA) by use of radioactively labelled antigen.

washed to remove any unbound antibody, followed by the addition of a solution containing the antigen of interest, and a radioactively labelled antigen with the same specificity. The labelled and the unlabelled antigen compete for the binding sites on the antigen; a second wash then removes any unbound antigen. The amount of radioactivity remaining bound to the plate may then be measured, with the radioactivity level inversely proportional to the amount of antigen in the test sample.

In enzyme linked immunosorbant assays (ELISA),⁸⁶ the radioactive label is replaced with an enzyme label. Although not as sensitive as the RIA the ELISA utilizes non-radioactive reagents which also have a longer shelf life than the radioactively labelled reagents. The RIA and the ELISA may both be carried out in an indirect fashion. One such indirect ELISA is illustrated in Figure 3-3. An antigen is adsorbed to a solid support, followed by the addition of a test solution containing antibody. The presence of the antibody is then detected by the addition of a second antibody which has a binding specificity for an epitope of the antibody in the test sample. This secondary antibody is labelled with a covalently attached enzyme. Addition of the enzyme substrate results in a color change in the solution, with the intensity of the color change which is proportional to the amount of antibody in the test solution.

Information regarding the specificity of an antibody binding site may be obtained if the immunoassay is carried out in a competitive fashion by use of a variety of different inhibitor antigens. An inhibitor antigen which has a high degree of complementarity for the antigen binding site of the antibody will bind tightly to the antibody, thus competing effectively with the test antigen. The indirect ELISA performed in a competitive fashion is illustrated in Figure 3-4. The differences in binding energies (G) of the

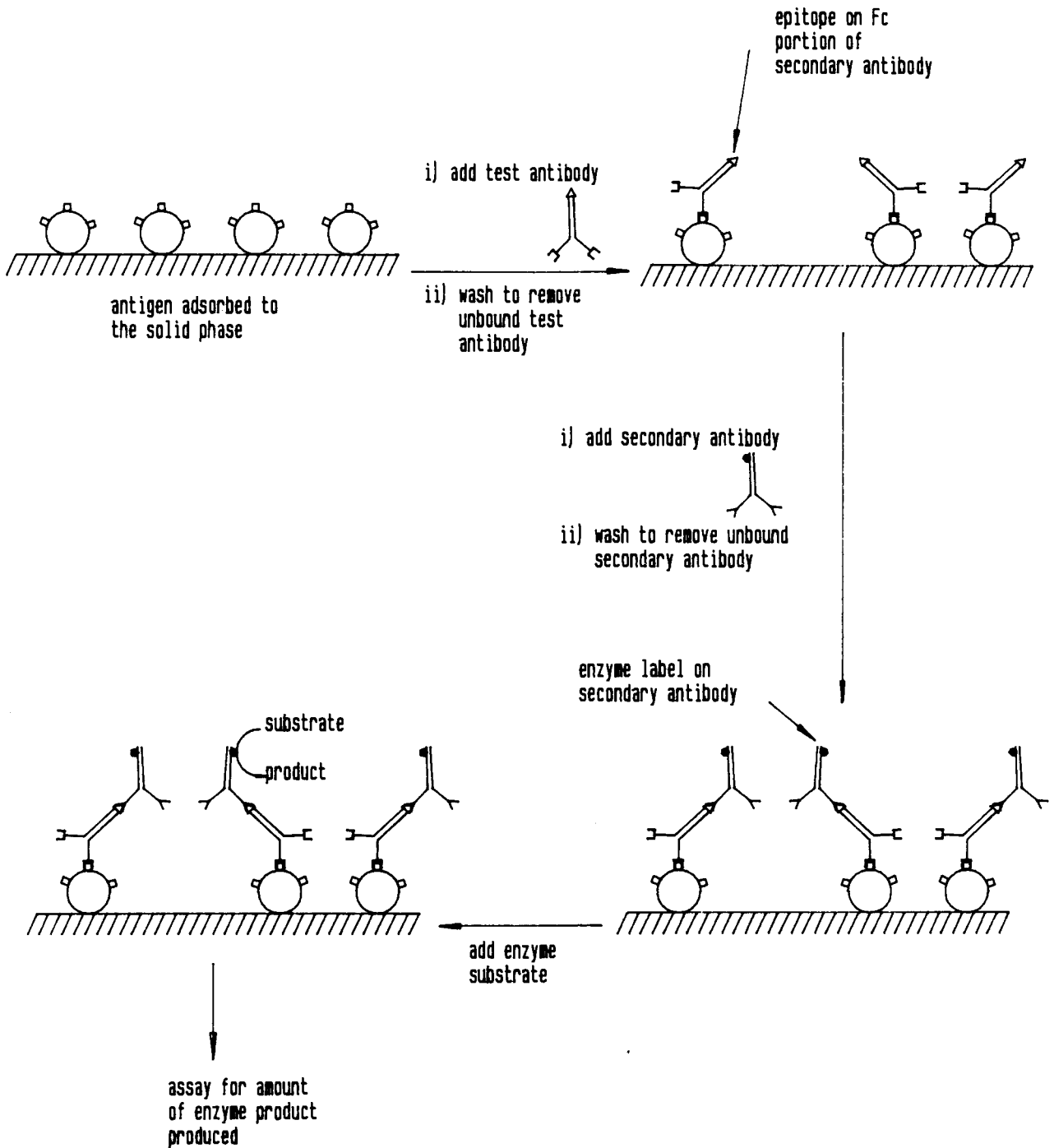


Figure 3-3: Indirect ELISA by use of solid phase antigen and enzyme labelled secondary antibody.

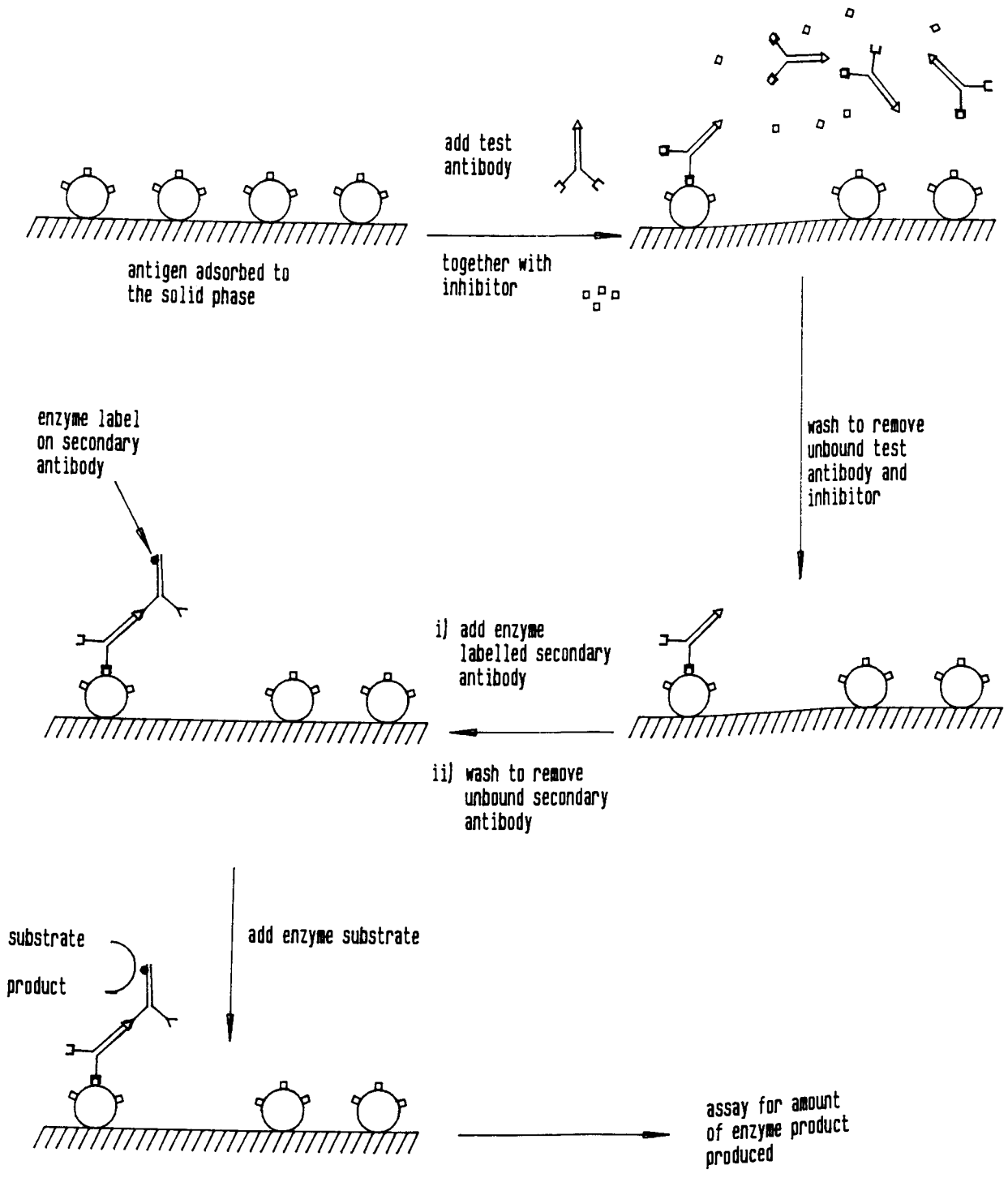
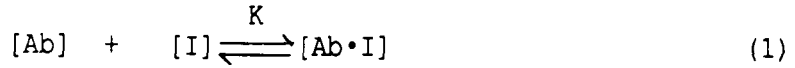


Figure 3-4: Competitive indirect ELISA.

various inhibitors may be related to the molar amounts of inhibitor required to achieve 50% inhibition of the binding.⁸⁷ The equilibrium between the antibody binding site and an inhibitor hapten is given in equation (1).



The equilibrium constant (K) is then given by (2)

$$K = \frac{[Ab \cdot I]}{[Ab][I]} \quad (2)$$

The difference in free energies of binding for two different inhibitor molecules is then given by (3).

$$\Delta\Delta G = \Delta G_1 - \Delta G_2 \quad (3)$$

This difference in free energies may be expressed in terms of equilibrium constants, by use of the Gibb's free energy relationship ($\Delta G = -RT \ln K$):

$$\Delta\Delta G = -RT \ln K_1 - (-RT \ln K_2) \quad (4)$$

$$\Delta\Delta G = RT (\ln K_2 - \ln K_1)$$

$$\Delta\Delta G = RT \ln (K_2 / K_1) \quad (5)$$

Substituting the concentrations for K_1 and K_2 , equation (6) is derived.

$$\Delta\Delta G = RT \ln \left[\frac{\frac{[Ab \cdot I_2]}{[Ab][I_2]}}{\frac{[Ab \cdot I_1]}{[Ab][I_1]}} \right] \quad (6)$$

At 50% inhibition $[Ab] = [Ab \cdot I]$, and equation (6) simplifies to (7).

$$\Delta\Delta G = RT \ln ([I_1] / [I_2]) \quad (7)$$

or

$$\Delta\Delta G = RT \ln (K_{rel})$$

where the relative association constant (K_{rel}) is given by the ratio of concentrations of a particular inhibitor to a reference inhibitor at 50%

inhibition ($K_{rel} = [I_1]_{50\%} / [I_2]_{50\%}$). If I_2 is chosen as a reference inhibitor,

then positive values for $\Delta\Delta G$ indicates poorer binding for I_1 .

3: *The Binding of Carbohydrate Antigens By Proteins*

The interactions of carbohydrate structures with antibody combining sites is a field of increasing interest, as this study also has implications for the interactions of carbohydrates with other classes of proteins. The nature of this interaction was first suggested by Emil Fisher when he proposed his now famous lock and key hypothesis of the binding of small molecules by proteins.⁸⁸ The general features of the nature of the interactions of antibodies and carbohydrates have been elucidated by Kabat.⁸⁹

Early work with human antisera raised against linear dextrans (homopolymers of glucose with predominantly α -1-6 linkages) gave some general information on the size of a binding site. Disaccharide through heptasaccharide portions of the dextran polymer were available, and could be used to competitively displace the dextran polymer from its binding site on the anti-dextran antibody. The relative inhibitory strength of various sized inhibitors was determined on a molar basis. The inhibitor strength was seen to increase with increasing size of the inhibitor, approaching a plateau with a heptasaccharide inhibitor. If the antisera were fractionated, subpopulations of antibodies were found with varying sizes of combining sites; the upper limit had a binding site able to accommodate a hexasaccharide or heptasaccharide.

Work on the binding of blood group antigens to lectins and monoclonal antibodies permitted the identification of some of the forces involved, and a model for the mechanism of the binding between a carbohydrate antigen and a protein binding site has been advanced by Lemieux.⁹⁰ Inhibition studies were carried out with modified blood group antigens and the binding proteins. By

systematically altering specific residues on the antigen molecules, and by use of these modified structures in inhibition binding studies, the contribution of individual residues to the overall binding energy was elucidated. The following model (termed the "hydrated polar-group gate effect")⁹¹ of antigen antibody binding emerged. Two or three hydroxyl groups on the carbohydrate antigen, usually from different sugar residues, comprise a cluster of polar groups termed the "key polar grouping". The rest of the antigen consists of essentially a non-polar surface. Several of the hydroxyl groups of the antigen are thought to be involved in intramolecular hydrogen bonds, rendering polar regions of the oligosaccharide more hydrophobic. It is thought that the key polar group is responsible for the initial highly specific interaction of the antigen with a complementary polar grouping in the combining site of the antibody or lectin. The polar grouping in the combining site was termed the "hydrated gate". This hydrated gate was thought to occupy a position near the edge of the binding site, with the remainder of the binding site comprised mainly of a lipophilic region. The initial binding interaction is thought to be between the key polar group of the oligosaccharide and the polar grouping of the binding site, hence the term hydrated polar-group gate effect. On binding of the antigen, the waters of hydration of both the polar "gate" in the binding site, and the polar grouping of antigen, are released into the bulk solvent, and results in intermolecular hydrogen bonding between the protein and the antigen. This intermolecular hydrogen bonding is postulated to be responsible for the specificity of the interaction. The stability of the interaction, or the affinity of the binding site for the ligand, is thought to be due mainly to non-polar interactions between lipophilic regions on the protein and the antigen. Complementarity between the two hydrophobic regions on the antigen

and the protein binding site is required for this hydrophobic binding to be effective.

Recently, the crystal structure of the lectin *Griffonia simplicifolia*, and that of its complex with the Lewis b tetrasaccharide, was determined.⁹² The contacts observed between the protein, and the Lewis b antigen, support the conclusions drawn from binding studies of the same lectin with several modified Lewis b tetrasaccharides. Other examples of support for the main tenets of the "hydrated polar-group gate effect" model for the binding of carbohydrate antigens, come from the results of inhibition studies of monoclonal antibodies raised against the *Shigella flexneri* variant Y lipopolysaccharide antigen with various oligosaccharide inhibitors.⁹³ Key polar hydroxyl groups are seen on the surface of the antigen, replacement of which results in a marked decrease in inhibitor potency; as well, extended hydrophobic regions can be identified on the surface of the oligosaccharide.

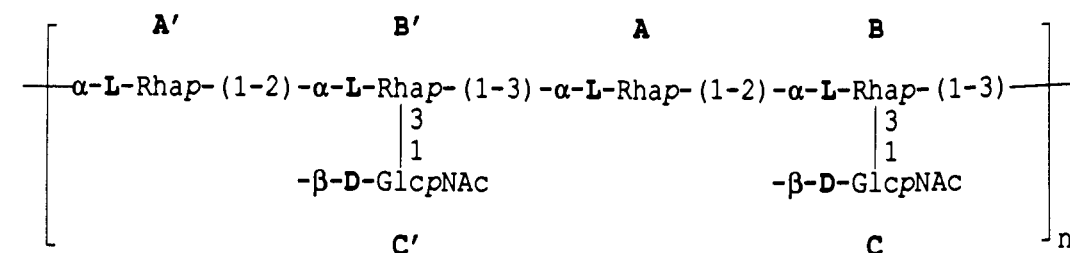
The above studies suggest that the binding of carbohydrate antigens by proteins is a result of a combination of specific polar interactions between regions of the antigen and its binding site, and the complementary fit of hydrophobic regions of the antigen and the binding site.

In contrast to the view that hydrophobic binding is the main stabilizing factor in carbohydrate-protein interactions, is the view that hydrogen bonding is the dominant stabilizing factor. Study of the high resolution crystal structure of the arabinose-binding protein-sugar complex by Quijcho et al⁹⁴ revealed an extensive network of hydrogen bonds between the protein and the bound sugar. On binding of the sugar to the protein, the solvation shell of both the protein and the sugar is released into the bulk solvent and new hydrogen bonds are formed between the protein and the sugar. It is argued that the hydrogen bonds formed between the sugar and the protein are

stronger than those between the bulk solvent and the protein and the unbound sugar; this is due to the stable arrangement of hydrogen bonds within the binding site. Other examples of the involvement of hydrogen bonding in complex formation can be found in the studies of the glycogen phosphorylase-glucose complex.⁹⁵ Clearly several factors are important in carbohydrate protein interactions, including hydrogen bonding, hydrophobic bonding, and van der Waals forces. The relative contributions of the individual factors may vary depending on the system which is under investigation.

B: Production of Antibodies to a Streptococci Group A Antigen

The Streptococci Group A cell-polysaccharide consists of a poly-L-rhamnopyranosyl backbone composed of alternating α -L-(1-2), and α -L-(1-3) linkages. The 3-positions of the poly rhamnopyranosyl backbone have branching β -D-N-acetylglucosamine residues.⁶ It was hoped that a variety of monoclonal antibodies could be generated to various epitopes present within this structure. Towards this end, oligosaccharide portions of this structure were prepared by chemical synthesis (see Chapter 2). Two trisaccharide sequences, and a pentasaccharide sequence were prepared with the 8-(methoxycarbonyl)octyl linking arm as the aglycone.⁶⁶ This linking arm allowed for the attachment of the oligosaccharides to a protein carrier⁶⁷ rendering the oligosaccharide fragments immunogenic, and gave



the artificial antigens shown in Figure 3-5. The carrier proteins chosen

were bovine serum albumin (BSA) and horse hemoglobin. The first question to be answered was whether antibodies could be generated against the carbohydrate portions of the artificial antigens. This question was addressed by use of the glycoconjugates as immunizing agents to generate polyclonal rabbit antisera. Previous work with artificial blood group antigens,^{66b, 96} and more recently, with an artificial antigen prepared from plant cell-wall components,⁹⁷ have shown that polyclonal sera as well as monoclonal antibodies can be successfully generated by immunizing with artificial antigens.

The BSA glycoconjugates (45), (47) and (49), were used to immunize three groups of rabbits; each group of rabbits were immunized with a different glycoconjugate. The purpose of the immunizations was to determine whether the synthetic antigens would elicit an immune response specific for the bound oligosaccharides, and to determine the binding specificities of the resulting antisera. The binding specificities of the polyclonal antisera were determined by a series of inhibition ELISA studies in which oligosaccharide portions (disaccharide through pentasaccharide) of the cell-wall polysaccharide were used as inhibitors of the antibody-glycoconjugate binding.

The next goal of this work was to generate a monoclonal antibody with high specificity for the *Streptococci* Group A cell-wall polysaccharide. It was hoped that such an antibody could be used in a variety of diagnostic schemes to either detect streptococcal infections, or to locate cross-reacting antigens in human tissues. These cross-reacting antigens may be important in autoimmune disorders such as rheumatoid arthritis and other rheumatoid disorders. A monoclonal antibody is ideal for these sorts of studies since the binding specificities may be well characterized and

determined in a quantitative fashion. Towards this end, monoclonal antibodies were prepared against a streptococcal group A vaccine.

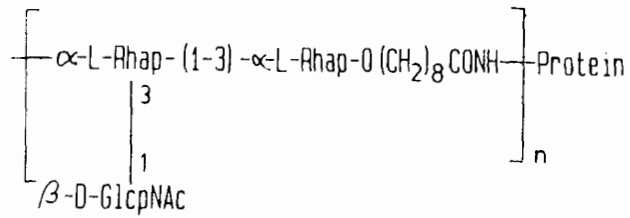
Mice were immunized with a vaccine prepared from a heat-killed, pepsin treated culture of *Streptococci* Group A bacteria. Following immunization, a series of monoclonal antibody fusion experiments¹² were performed. The glycoconjugate structures proved to be vital elements in the screening for a monoclonal antibody. In order to ensure the isolation of an individual monoclonal antibody, a highly purified antigen is required in the screening protocol; the synthetic antigens provided such reagents. Several antibody producing hybridomas were isolated, and four of these were selected for production of ascites fluid. The binding specificities of the monoclonal antibodies thus produced were investigated by a series of inhibition studies by use of various oligosaccharide inhibitors in an indirect inhibition ELISA protocol.

II: RESULTS AND DISCUSSION

A: Polyclonal Antisera to Oligosaccharide Glycoconjugates

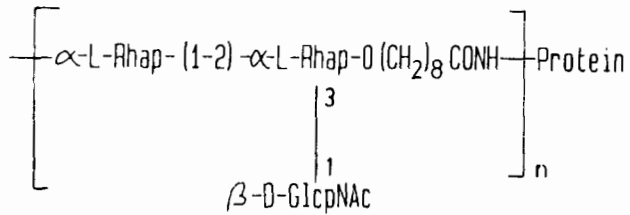
1: Immunizations of Rabbits With Glycoconjugates

Three groups of four New Zealand White rabbits were immunized with the glycoconjugates, (45), (47) and (49) shown in Figure 3-5. Prior to immunization, serum samples were obtained from each of the rabbits. The sera were screened by use of an indirect ELISA, by use of the three BSA-glycoconjugates as coating antigens. The pre-immunization screen was carried out to determine if there was any background antibody activity to the glycoconjugates prior to the immunizations. All of the sera tested showed no activity toward the three glycoconjugates, therefore the immunization procedures were continued. After a series of subcutaneous injections of the glycoconjugates of two times per week for three weeks, trial bleed sera were obtained from each of the rabbits. The trial bleed sera were screened by use of an ELISA. Since it was expected that there would be a substantial immune response to the BSA carrier protein moiety of the glycoconjugates, the three oligosaccharide haptens were coupled to a different carrier protein, namely Horse Hemoglobin. The hemoglobin glycoconjugates, (46), (48) and (50), were used as the coating antigen in the ELISA screening of the trial bleed sera. All of the trial bleed sera showed activity toward the complementary hemoglobin glycoconjugates, so the rabbits were exsanguinated, and the sera collected. The binding of the antisera to the hemoglobin glycoconjugates indicated that there were antibodies present in the antisera which recognized the hapten portions of the glycoconjugates and not just the protein carrier. The rabbit sera were pooled, with each pool from the immunization of a group



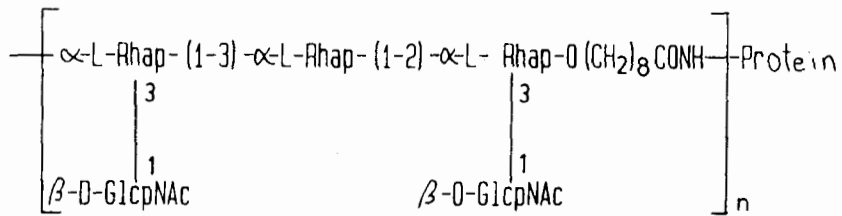
45: Protein= Bovine Serum Albumin

46: Protein= Horse Hemoglobin



47: Protein= Bovine Serum Albumin

48: Protein= Horse Hemoglobin



49: Protein= Bovine Serum Albumin

50: Protein= Horse Hemoglobin

Figure 3-5: Glycoconjugates incorporating synthetic oligosaccharide haptens.

of four rabbits with a given glycoconjugate. The antisera pools "A", "B" and "C" resulted from the immunization with BSA-glycoconjugates (45), (47) and (49), respectively.

Before further studies of the binding characteristics of the three pools of antisera were carried out, the antisera were purified. The first step in the purification was to isolate the IgG class of immunoglobulins from the other serum components. This was achieved by passing the sera through a protein A affinity column. Protein A is a protein which will, under appropriate salt and pH conditions, bind the heavy chain portion of IgG molecules.⁹⁸ The bound immunoglobulins can then be separated from the other serum constituents. The bound IgG molecules were then eluted from the Protein A affinity column by adjusting the salt and pH balance of the eluting buffer, to give the purified IgG fraction of the sera. A major component of the purified fractions contained antibodies directed towards the BSA portion of the artificial antigens used in the immunizations. To remove these antibodies, the protein A purified anti-sera was then passed through a BSA affinity column. This column consisted of a Sepharose gel to which BSA molecules were covalently attached. Anti-BSA IgG molecules bound to the column, whereas antibodies directed at the hapten portions of the glycoconjugates passed on through the column. The antibody containing fractions from the column were pooled, and dialyzed against a Tris-HCl buffer by ultrafiltration to give a solution of IgG enriched in antibodies directed against the oligosaccharide haptens. The protein concentrations of the pooled antisera were determined by measuring the absorbances at 280 nm.

2: Titration of Rabbit Polyclonal Antisera

The pooled antisera were titrated by means of an ELISA, by use of the

three hemoglobin glycoconjugates (46), (48) and (50) as coating antigens on the solid support, (see Figures 3-6 through 3-8). The concentration of antibody required to give an absorbance reading of 0.1 or greater (titration end-point) in the ELISA was compared for the various pools of antibodies. The titration end-point is an approximate indication of the affinity of the antibodies which were tested, for the antigen used in the ELISA assay. The titrations of the three antisera pools with the linear trisaccharide glycoconjugate (46) (Figure 3-6), gave almost identical titration curves for pool "A" and pool "C" antisera. Antiserum "B" titrated out at a greater concentration than the other two pools of antisera. The titrations of the three antisera with the branched trisaccharide glycoconjugate (48) (Figure 3-7), gave similar titration curves for "B" and "C" antisera, but the "A" antiserum required a much greater concentration to achieve the same endpoint. Titrations which used the pentasaccharide glycoconjugate (50) (Figure 3-8), gave similar curves for "A" and "B" antisera, with antiserum "C" titrating out at a lower concentration.

The titration results show that the antibodies raised against a linear trisaccharide hapten (45) had a stronger cross-reaction with the pentasaccharide hemoglobin glycoconjugate (50) than they did with the branched trisaccharide hemoglobin glycoconjugate (48). In a similar fashion, the antibodies raised against a branched trisaccharide hapten (47), showed less cross-reactivity with a linear trisaccharide hemoglobin glycoconjugate (46), than with a pentasaccharide hemoglobin glycoconjugate (50). The antiserum raised against a pentasaccharide hapten (49) cross-reacted strongly with both trisaccharide glycoconjugates (46) and (48).

These results indicate that the antibodies of the individual pools of antisera have a greater affinity for the hemoglobin glycoconjugates which

Figure 3-6
Titration of Rabbit Polyclonal Antisera
By Use Of Glycoconjugate (46)

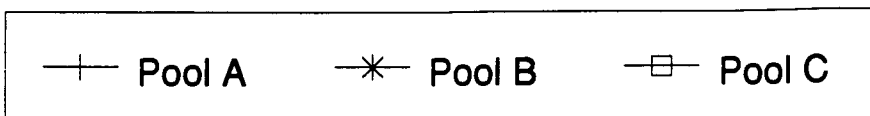
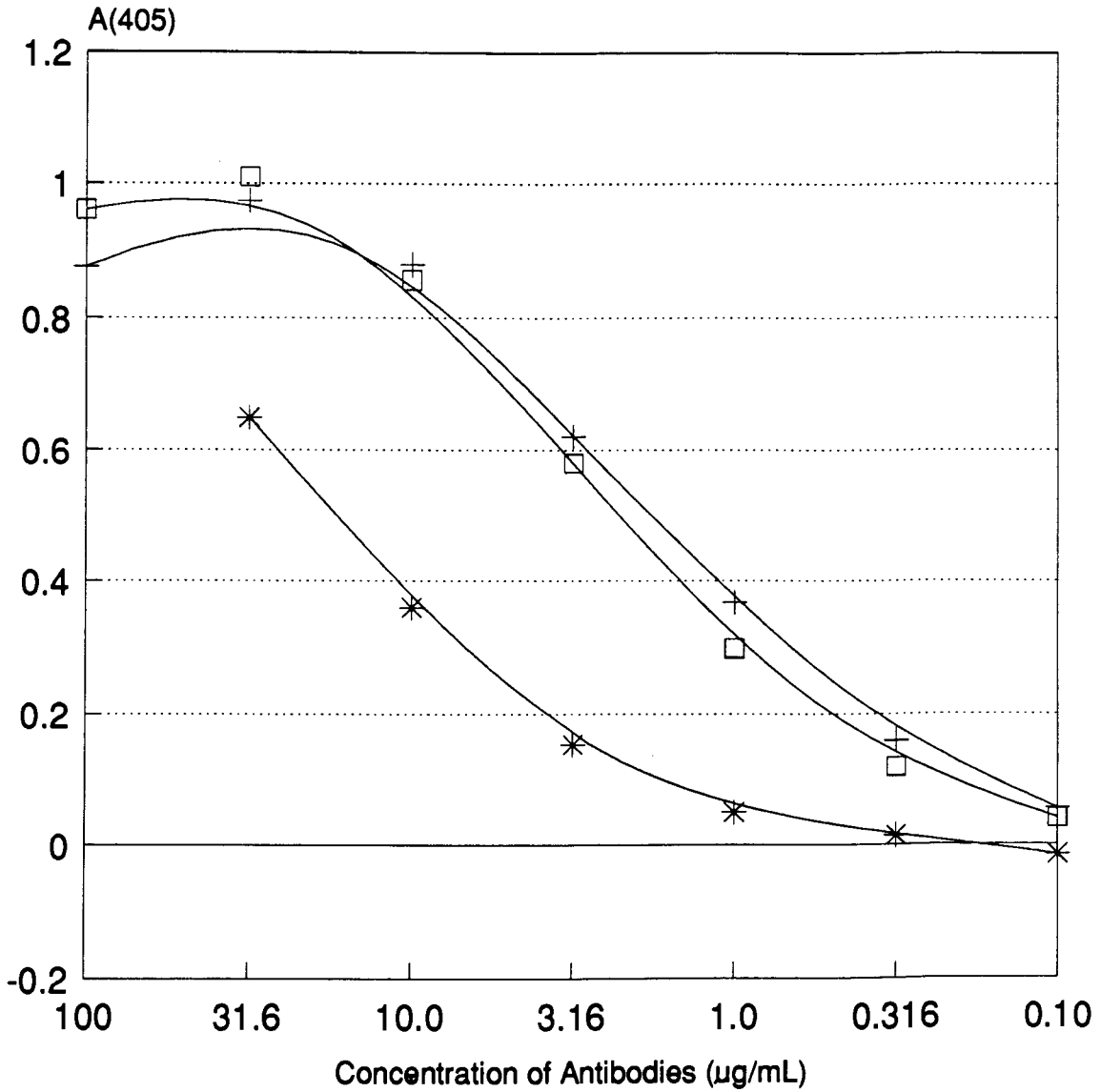


Figure 3-7
Titration of Rabbit Polyclonal Antisera
By Use Of Glycoconjugate (48)

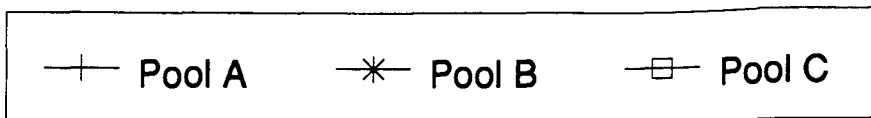
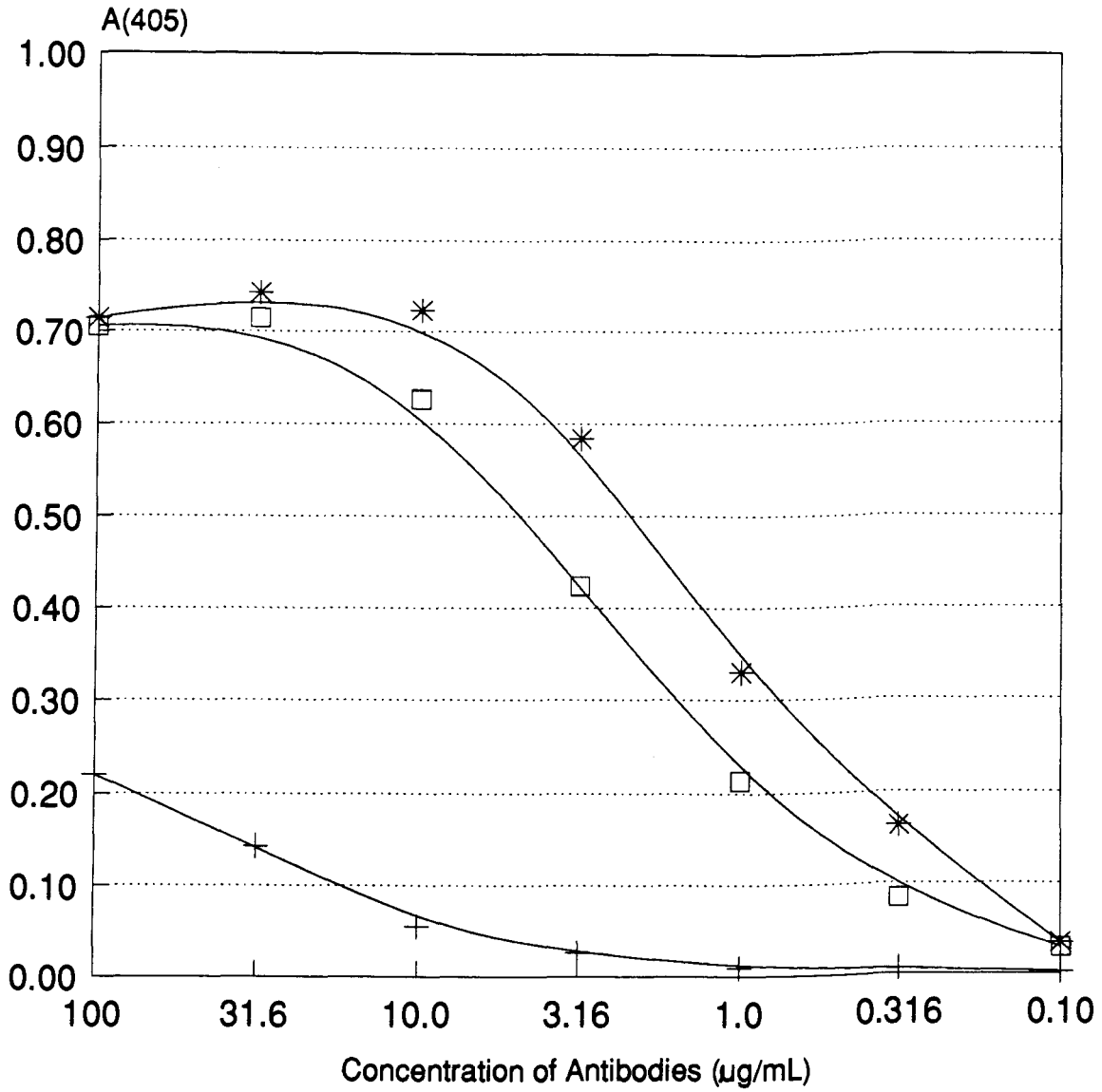
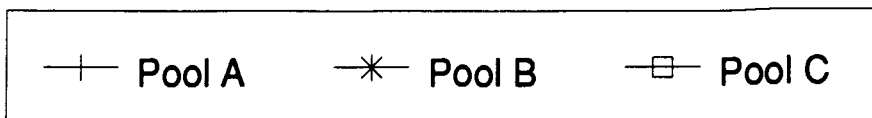
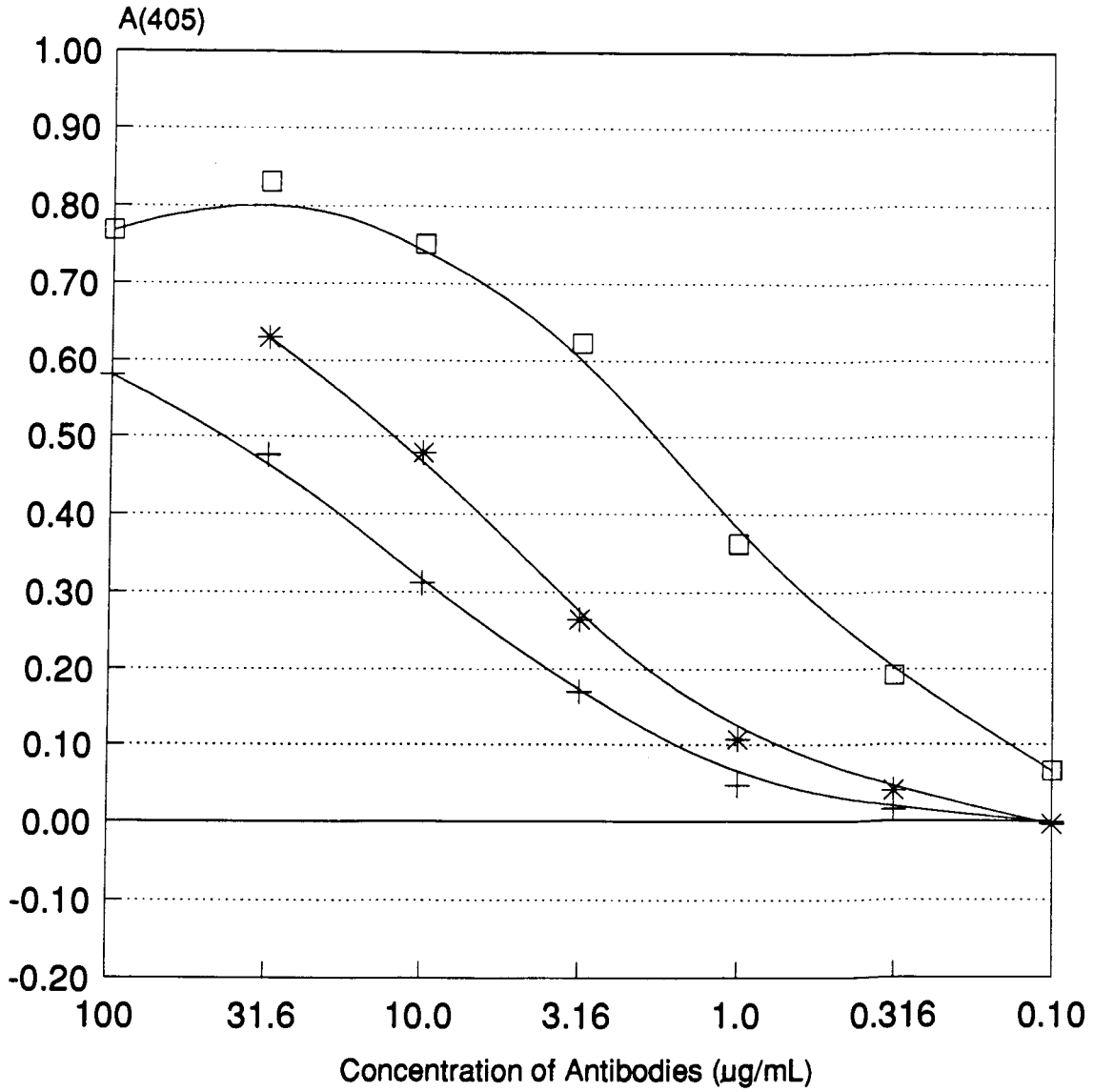


Figure 3-8
Titration of Rabbit Polyclonal Antisera
By Use Of Glycoconjugate (50)



have the homologous oligosaccharide sequences attached. There are also some clear cross-reactivities between the different antisera and the various glycoconjugates, indicating that there are some similarities in the binding surfaces of the various haptens. If one looks at just the chemical structures of the hapten portions of the three pairs of glycoconjugates, (see Figure 3-5), it can be seen that the pentasaccharide hapten incorporates both the branched trisaccharide sequence, as well as the linear trisaccharide sequence. It might then be expected that antiserum to a pentasaccharide hapten would cross-react with both the branched trisaccharide and the linear trisaccharide glycoconjugates. Similarly, antisera to either of the trisaccharide glycoconjugates might be expected to cross-react with the pentasaccharide glycoconjugate. However, the branched and linear trisaccharides have less overlapping structure, and therefore one might expect the corresponding antisera to show less cross-reactivity towards non-complementary trisaccharide haptens. The titration results, shown in Figures 3-6 through 3-8, appear to follow the expected trends.

3: *Inhibition ELISA's of Rabbit Polyclonal Antisera*

To investigate the binding of the antisera further, a series of inhibition ELISA experiments were performed. For each series of inhibition studies, the complementary hemoglobin glycoconjugate for a particular antiserum was used as the coating antigen in the ELISA. A typical assay consisted of the following. If, for example, the antiserum "A" which was tested [this antiserum was raised against the linear trisaccharide BSA-glycoconjugate (45)], a 96-well enzyme immunoassay (EIA) plate was coated with the linear trisaccharide hemoglobin glycoconjugate (46) at a suitable concentration (see Figure 3-9 for a typical EIA plate arrangement). The

plate was then washed to remove unbound glycoconjugate, and the test

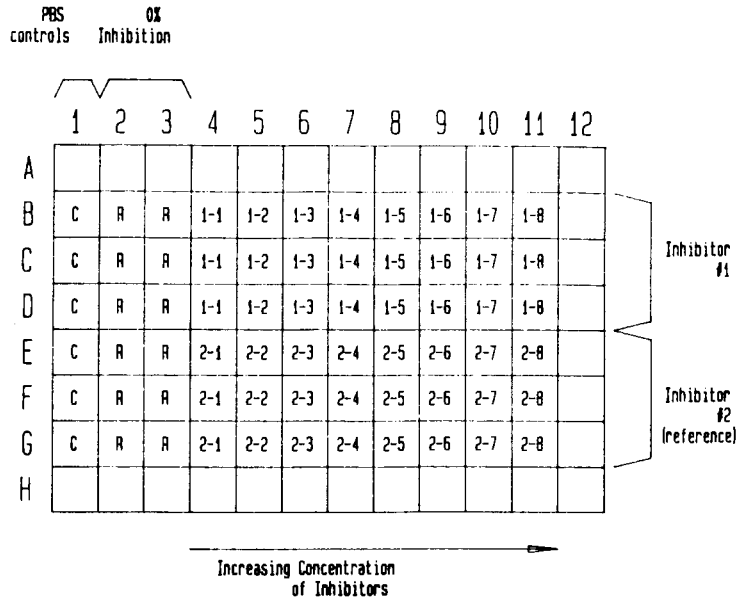


Figure 3-9: Arrangement of inhibitor and control wells on a 96-well microtitre EIA plate.

antiserum was then added at a fixed concentration together with a given concentration of an oligosaccharide inhibitor (see Figure 3-10 for structure of the inhibitors used). The concentrations of the inhibitors were serially diluted, with concentrations typically ranging from 1-1000µg/mL. After a period of incubation, the plate was again washed, and the bound antibody was detected by the addition of a secondary antibody, in this case a goat anti-rabbit IgG which was labelled with an alkaline phosphatase enzyme. The plate was again washed to remove unbound detection antibody, followed by the addition of the enzyme substrate *p*-nitrophenol phosphate. The development of color indicated the level of inhibition at any particular inhibitor concentration. The results of the inhibition assays are displayed in Figures 3-11 through 3-13 and summarized in Table 3-I.

In all cases, the inhibition was the greatest when the inhibitor was

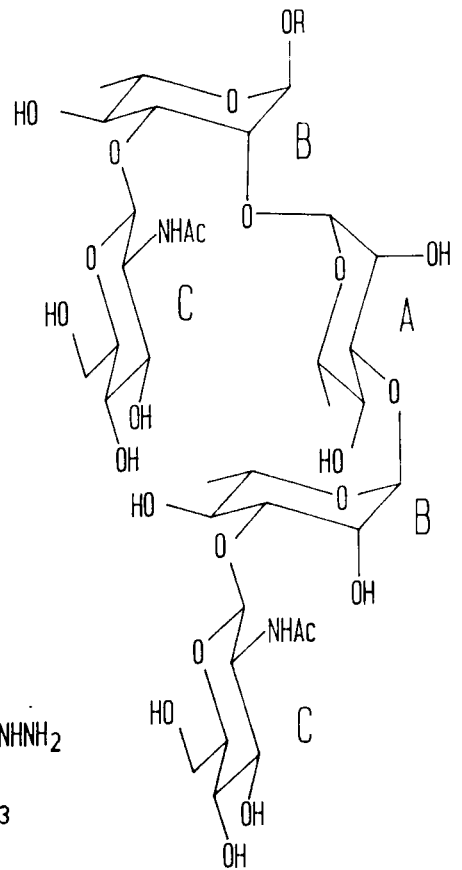
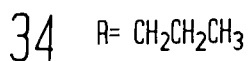
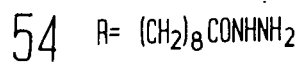
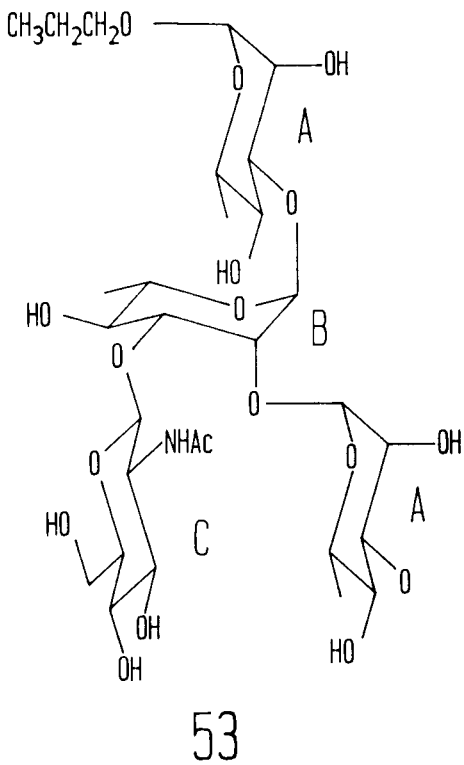
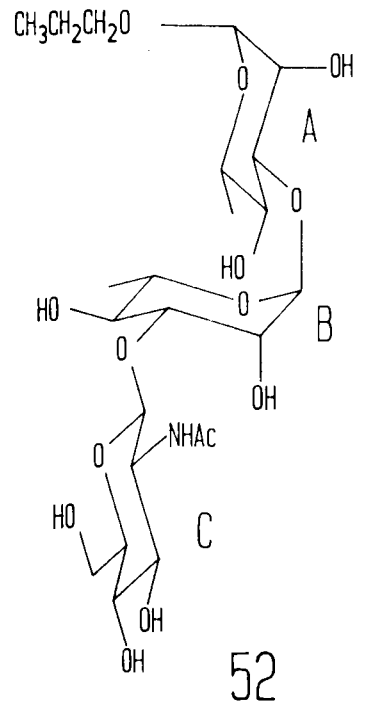
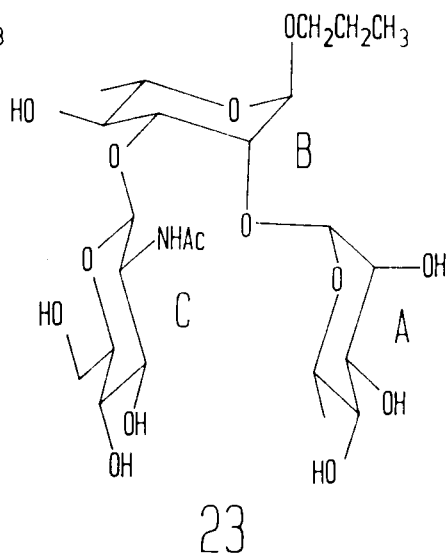
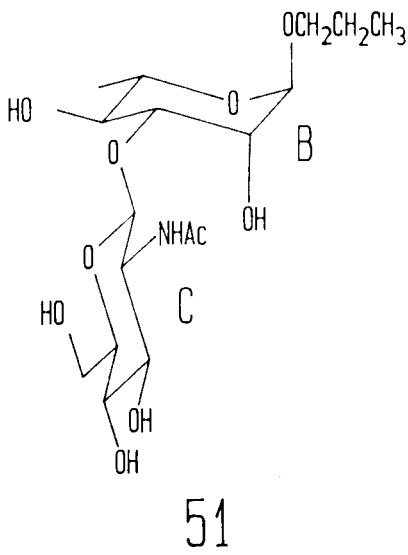


Figure 3-10: Panel of inhibitors⁹⁹ used in binding studies with rabbit polyclonal antisera and monoclonal antibodies.

complementary to the oligosaccharide hapten on the BSA glycoconjugate which was used to generate a given pool of antiserum. In the inhibition studies with antiserum "A" (see Figure 3-11), the order of the potency of the

Table 3-I: Inhibition Data For Rabbit Polyclonal Antisera

Antisera	Inhibitors									
	51		52		23		53		54	
	μg/mL	μM	μg/mL	μM	μg/mL	μM	μg/mL	μM	μg/mL	μM
Pool A	>10 ⁴	>24400	22.5	40.5 ^c (0.0) ^d	a	a	2500 ^b	3500 ^b (2.6)	2500 ^b	2400 ^b (2.4)
Pool B	a	a	a	a	21.1	37.8 ^c (0.0)	1400 ^b	2000 ^b (2.3)	900	870 (1.8)
Pool C	700	1700 (3.3)	>10 ⁴	>18000	>10 ⁴	>18000	2500 ^b	3500 ^b (3.72)	6.2	6.0 ^c (0.0)

^a The inhibitor showed no activity with the antiserum indicated.

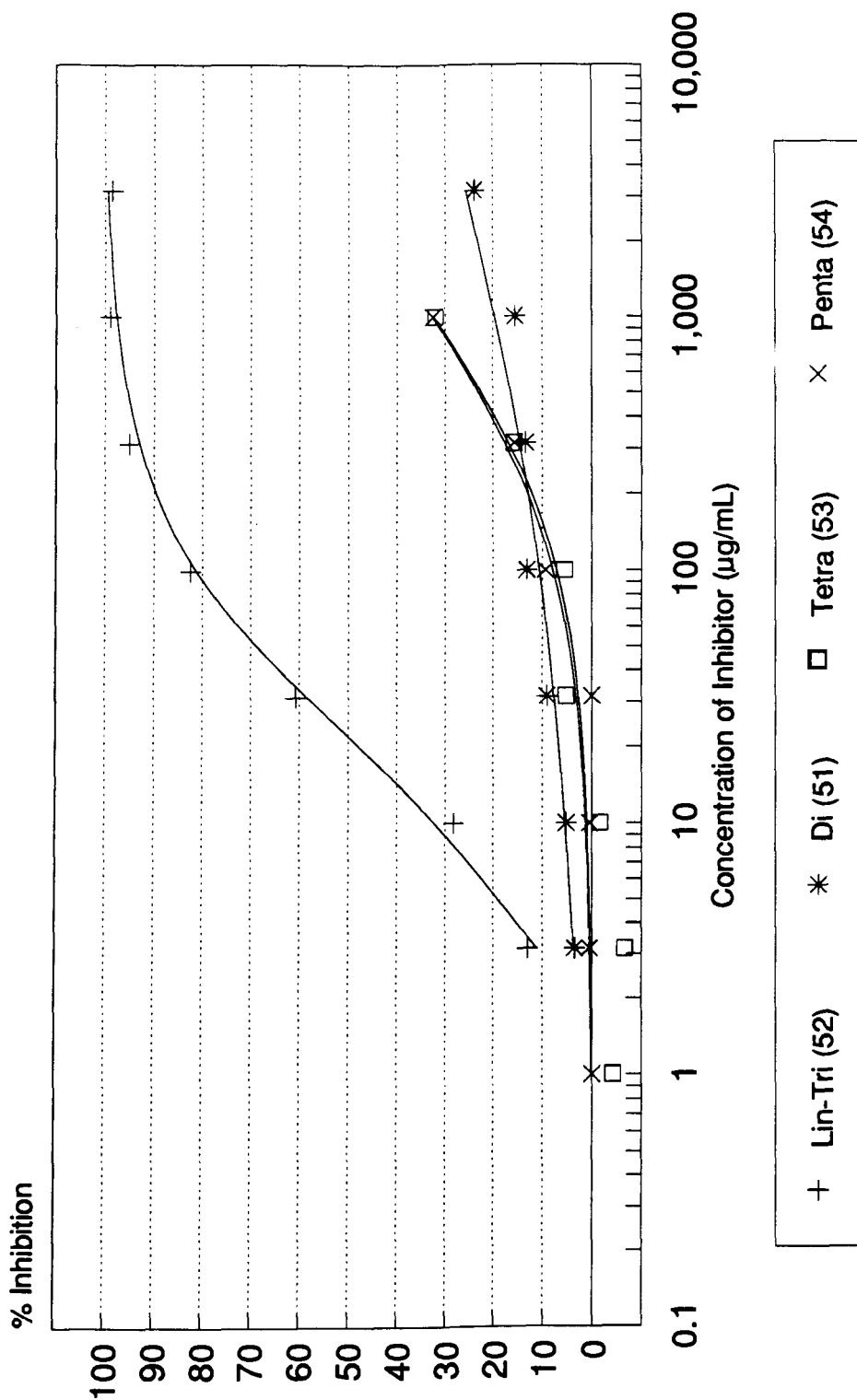
^b These values are based on extrapolated curves.

^c This inhibitor was used as the reference for the indicated series.

^d The values in parentheses are for $\Delta\Delta G$ in kcal/mol, determined from the expression $\Delta\Delta G = RT \ln([I_1]/[I_2])$, where $[I_2]$ is the concentration of the reference inhibitor for a particular series, $[I_1]$ is the concentration of the other inhibitors, $R = 1.98 \text{ calK}^{-1} \text{ mol}^{-1}$, and $T = 295 \text{ K}$; positive values for $\Delta\Delta G$ indicate poorer binding.

inhibitors was linear trisaccharide (52) >> pentasaccharide (54) ~ tetrasaccharide (53) > disaccharide (51). The branched trisaccharide inhibitor (23) was not active. Antiserum "A" bound the tetrasaccharide (53), and pentasaccharide (54) inhibitors to a certain degree, but to a lesser degree than the complementary linear trisaccharide sequence (52). The disaccharide inhibitor (51) seemed to be bound very slightly, whereas the branched trisaccharide sequence (23) failed to inhibit the binding at all. This inhibition pattern might be explained by the fact that both the

Figure 3-11
Inhibition of Polyclonal Antiserum "A"
By Use Of (46) as Solid Phase Antigen



pentasaccharide and the tetrasaccharide sequences contain the linear trisaccharide sequence, and hence a certain degree of inhibition is seen with these compounds. The additional residues which are present possibly inhibit the binding relative to the inhibition seen with the homologous linear trisaccharide. The antibodies in antiserum "A" bind to the linear trisaccharide sequence best, and any additional residues seem to introduce unfavorable contacts. This suggests a small binding site which does not allow for the possibility of a larger sequence fitting in and making extra favorable contacts.

The binding between antiserum "B" and the homologous glycoconjugate (48) (see Figure 3-12), was inhibited most strongly by the complementary branched trisaccharide sequence (23). The tetrasaccharide (53) and pentasaccharide (54) sequences were poor inhibitors, while the linear trisaccharide (52), and disaccharide (51) sequences were not active at all. It is possible that the branching rhamnosyl "A" residue is required for binding with these antibodies, since both the pentasaccharide and the tetrasaccharide sequences have this residue in common with the branched trisaccharide, whereas the linear trisaccharide and the disaccharide sequences both lack this residue.

With antiserum "C" (see Figure 3-13) the most potent inhibitor, other than the complementary pentasaccharide hapten, was the disaccharide sequence. This result seems somewhat puzzling, since all of the oligosaccharide sequences are represented in the pentasaccharide sequence. One might expect that the inhibition of the oligosaccharides would increase with increasing size. This pattern might be expected, since as their size increased, greater homology in structure to the pentasaccharide might be expected. It is possible that the *N*-acetylglucosamine residue is important in binding with these antibodies. The pentasaccharide sequence has two *N*-acetylglucosamine

Figure 3-12
Inhibition of Polyclonal Antiserum "B"
By Use Of (48) as Solid Phase Antigen

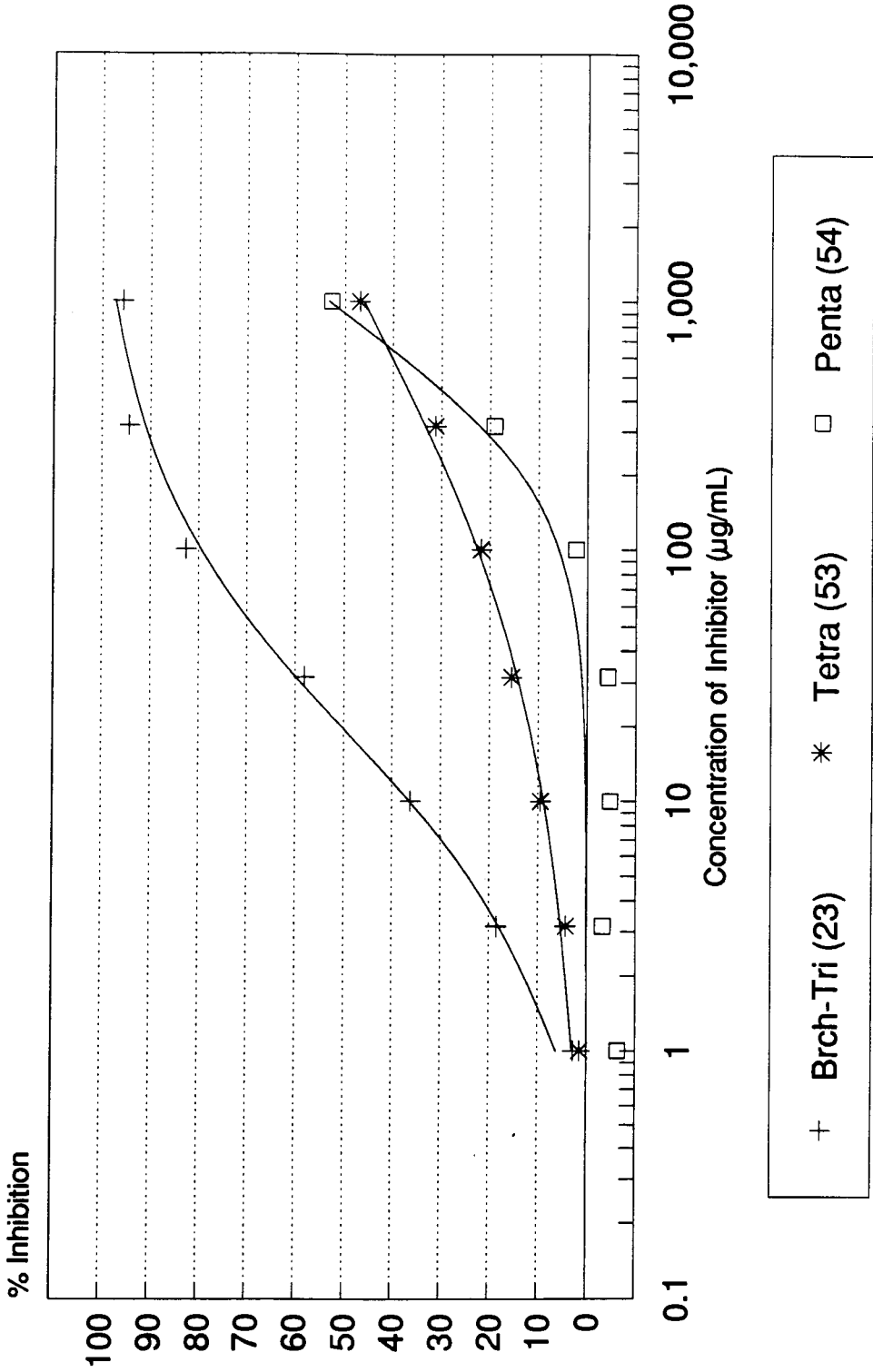
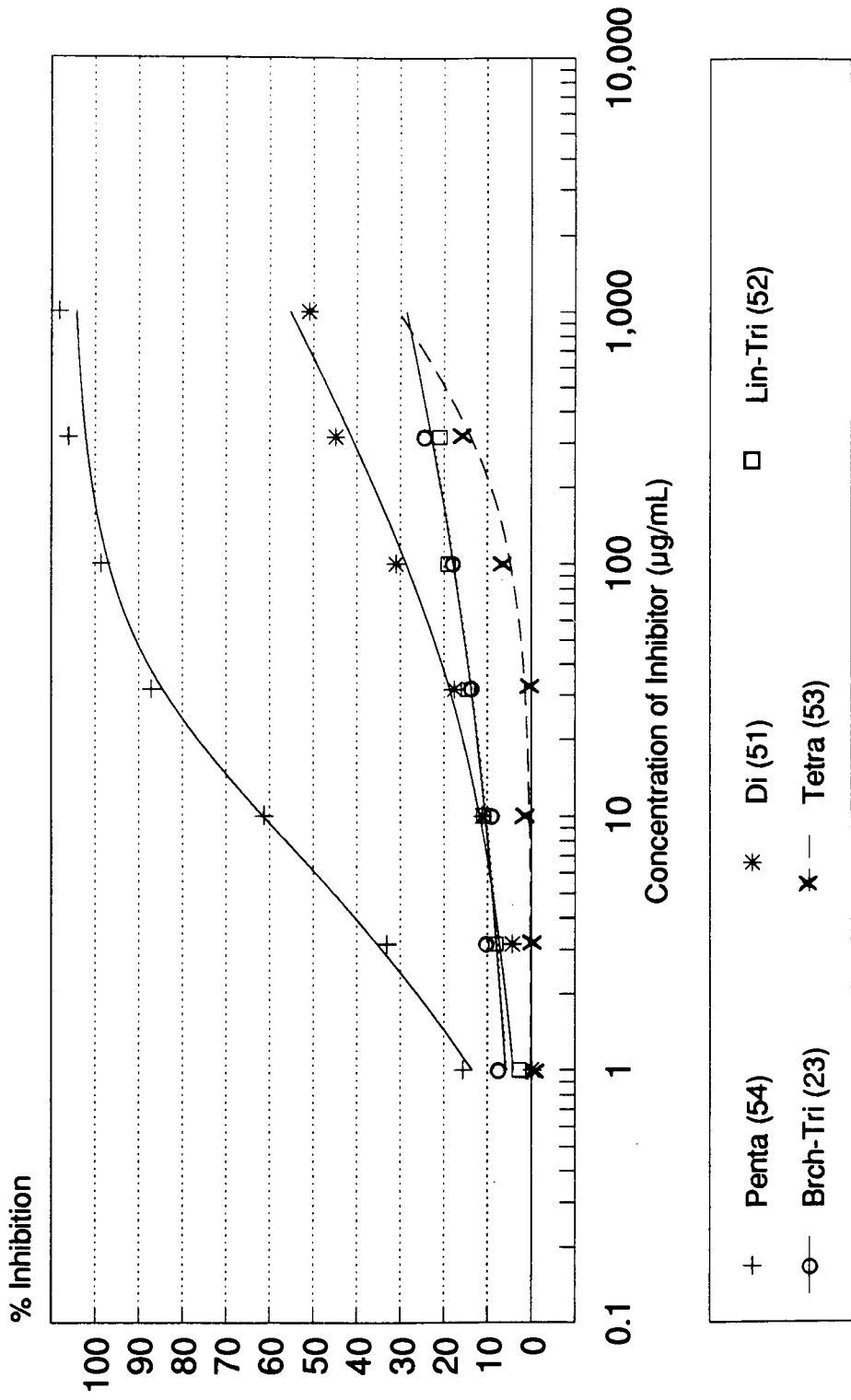


Figure 3-13
Inhibition of Polyclonal Antiserum "C"
By Use Of (50) as Solid Phase Antigen



residues, whereas the other inhibitor sequences have only one. Of the other inhibitors with one *N*-acetylglucosamine residue, one could speculate that the *N*-acetylglucosamine residue in the disaccharide makes a large relative contribution to binding than in the trisaccharide and the tetrasaccharide sequences. The disaccharide sequence may lack some of the residues present in the trisaccharide and tetrasaccharide sequences which may otherwise be involved in unfavourable interactions. This might explain the fact that of the four non-complementary inhibitors, the disaccharide sequence exhibits the greatest inhibition with the Pool "C" antiserum.

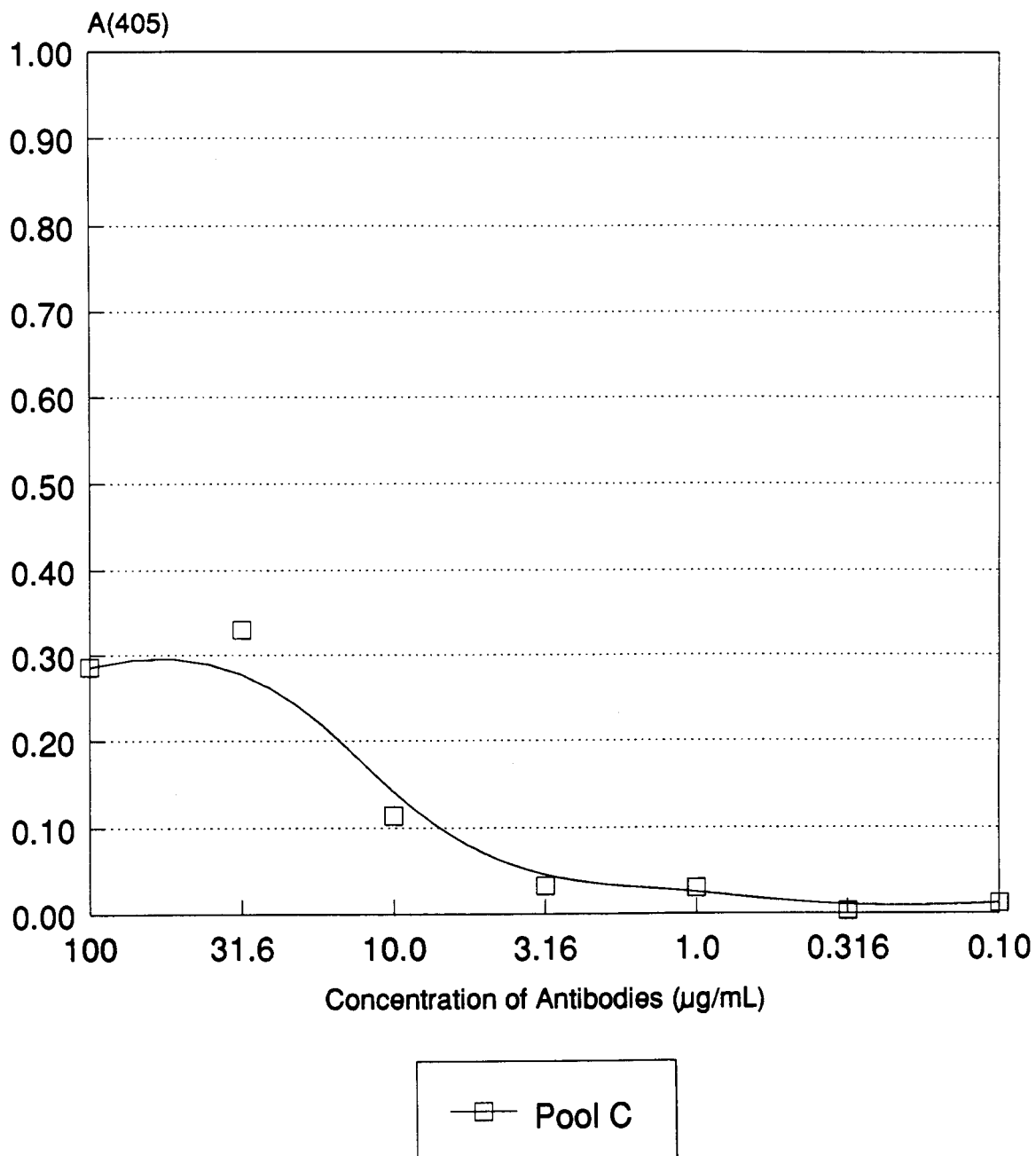
The results from the titration of the antisera with the various glycoconjugates seem to indicate a fair degree of cross-reactivity between the antisera and non-homologous glycoconjugates. From the inhibition data, however, it is apparent that the antibodies bind very specifically to the oligosaccharide sequences which were used in the corresponding immunizations. The question then arises as to whether the three antisera would bind to the natural polysaccharide. This question is important, since it was thought that the glycoconjugates themselves could be used as immunizing agents in a monoclonal antibody production protocol. One of the rationales for immunizing with a synthetic glycoconjugate with bound oligosaccharides as haptens, is that the antigen is presented to the B-lymphocytes in a highly defined form, and thus the immune response is more specific for the antigen of choice, rather than for spurious epitopes that might also be present in a heterogeneous polysaccharide preparation, or a whole cell vaccine.

One of the tests of the legitimacy of this approach for the production of an antibody to a *Streptococcal* Group A antigen, would be to see if the antisera which were raised against the BSA-glycoconjugates, would also bind to the native polysaccharide. To test this, all the purified antisera were

titrated in an ELISA, by use of *Streptococcal* Group A polysaccharide conjugated to BSA¹⁰⁰ as the coating antigen. Since the polysaccharide was conjugated to BSA, and not to horse hemoglobin, there was the possibility of residual anti-BSA antibodies in the rabbit antisera binding to the BSA moiety of the polysaccharide glycoconjugate. To account for this, control wells in the ELISA were set up in which the wells were coated with BSA alone, in place of the polysaccharide glycoconjugate. The activity observed in the control wells was subtracted from the test wells to give the true binding of the rabbit antisera to the polysaccharide structure. The surprising result was that none of the antisera bound very strongly to the natural polysaccharide (see Figure 3-14). Of the three groups of antisera, only antiserum "C", which was raised against a pentasaccharide hapten, showed any sign of binding to the polysaccharide structure. This suggests that the antibodies raised against the synthetic antigens are very specific for the oligosaccharide sequences to which they were raised.

Based on the binding studies, one can speculate on the shape of the antigen binding sites of the antibodies raised against the glycoconjugates. The antigen binding sites are most likely pocket shaped, rather than groove shaped. A pocket shaped binding site would require a very specific match with the antigen; this sort of specificity was observed in the inhibition studies. A small binding pocket would be unlikely to bind an extended polysaccharide structure; in fact the antisera raised against the two trisaccharide glycoconjugates did not bind to the polysaccharide glycoconjugate. The larger the binding pocket, the more likely it would also bind to regions of an extended polysaccharide; the observation that only the antiserum raised against the pentasaccharide sequence showed any binding activity towards the polysaccharide would support this, since of the

Figure 3-14
Titration of Rabbit Polyclonal Antisera
By Use Of Polysaccharide-BSA



inhibitor sequences, the pentasaccharide sequence is the one which would be expected to best model the structure of the polysaccharide.

The above observations have implications for the generation of antibodies which are to be used for diagnostic purposes. If one wishes to obtain antibodies which recognize the streptococcal group A polysaccharide, then the natural polysaccharide, or a polysaccharide glycoconjugate, should be used as the immunogen. If on the other hand, oligosaccharide segments are to be used then they should be larger than a pentasaccharide segment.

B: Monoclonal Antibodies to a Streptococci Group A Polysaccharide

1: Production¹⁰¹ and Purification of Monoclonal Antibodies

The antigen used in the monoclonal antibody protocol was derived from a heat-killed bacterial sample of the Streptococci Group A. The surface protein antigens were removed by a pepsin digest to expose the cell-wall carbohydrate antigen. Two groups of mice were injected with the heat-killed bacteria: six BALB/c mice and six A/J mice. Two fusion experiments were carried out. Indirect ELISA with the Streptococci Group A polysaccharide conjugated to BSA was performed on the hybridomas to select colonies which were secreting antibody specific for the polysaccharide. Six hybridomas from the fusion of the A/J mice, and one from the fusion of the BALB/c mice were recloned. These seven hybridomas were then screened with disaccharide and trisaccharide BSA-glycoconjugates. The class and subclass of the monoclonal antibodies produced by the seven hybridomas were determined by use of an Amersham isotyping kit. Of the six hybridomas produced from the fusion of the A/J mice, six of them were found to be of the class IgM_K, and one of the class IgG3_K. The hybridoma produced from the fusion of the BALB/c mice, was

found to be an IgM λ . Of the seven hybridomas four colonies produced strong titres to both the polysaccharide and the oligosaccharide glycoconjugates. These four hybridomas [(SA-1A (IgM κ), SA-1BC (IgM λ), SA-2B (IgM κ), SA-2C (IgM κ)] were selected for further study. Larger amounts of the antibodies were generated by raising ascites fluid for each of the clones. The monoclonal antibodies were purified from the ascites fluid by performing an ammonium sulfate precipitation. The protein concentrations of the purified monoclonal antibody preparations were determined by measuring the absorbance at 280nm.

The monoclonal antibodies were titrated against linear trisaccharide, and polysaccharide BSA-glycoconjugates in an indirect ELISA, to get an initial indication of their binding characteristics (see Figures 3-15 and 3-16). The antibodies bound to both the polysaccharide and the trisaccharide glycoconjugates. Of the four monoclonal antibodies the one which gave the highest titre was SA-2C.

2: *Inhibition ELISA's of Monoclonal Antibodies*

A series of inhibition ELISA studies were performed in order to determine the binding specificities of the four monoclonal antibodies. A panel of inhibitors was used, ranging in size from a disaccharide to a pentasaccharide sequence (see Figure 3-10). In the inhibition studies it was decided to use the linear trisaccharide sequence as the coating antigen in the indirect ELISA. By use of the trisaccharide glycoconjugate, rather than the polysaccharide glycoconjugate, the binding would be strong enough to detect, but not so strong that the binding could not be inhibited at a reasonable concentration with the panel of inhibitors.

Prior to the inhibition assays, an ELISA was carried out in which the

Figure 3-15
Titration of Monoclonal Antibodies
By Use Of Glycoconjugate (46)

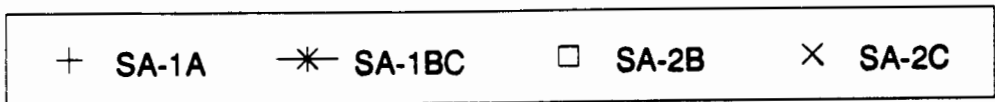
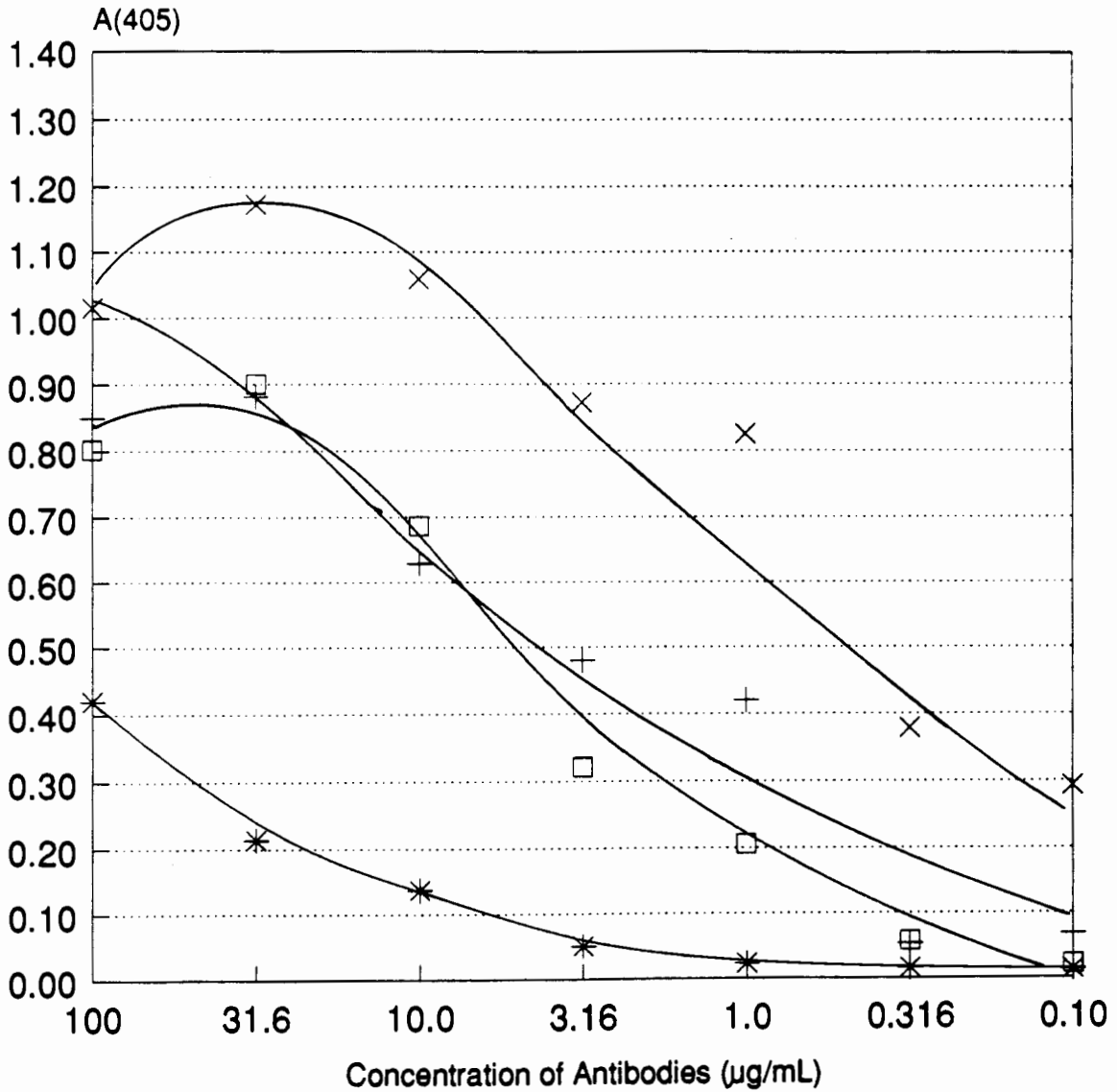
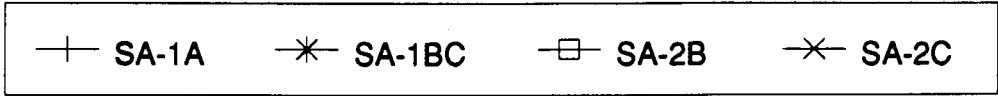
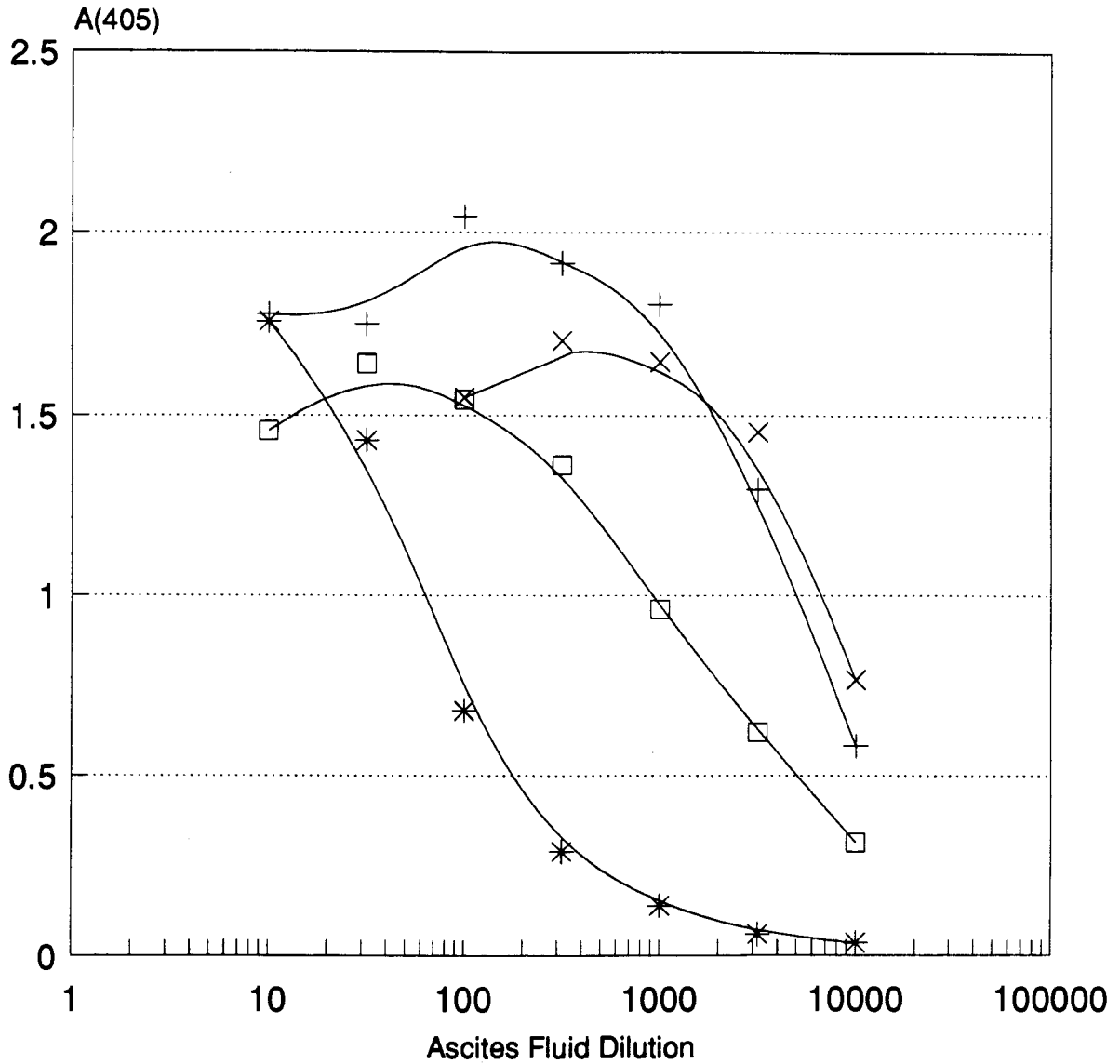


Figure 3-16

Titration of Monoclonal Antibodies
By Use of Polysaccharide-BSA

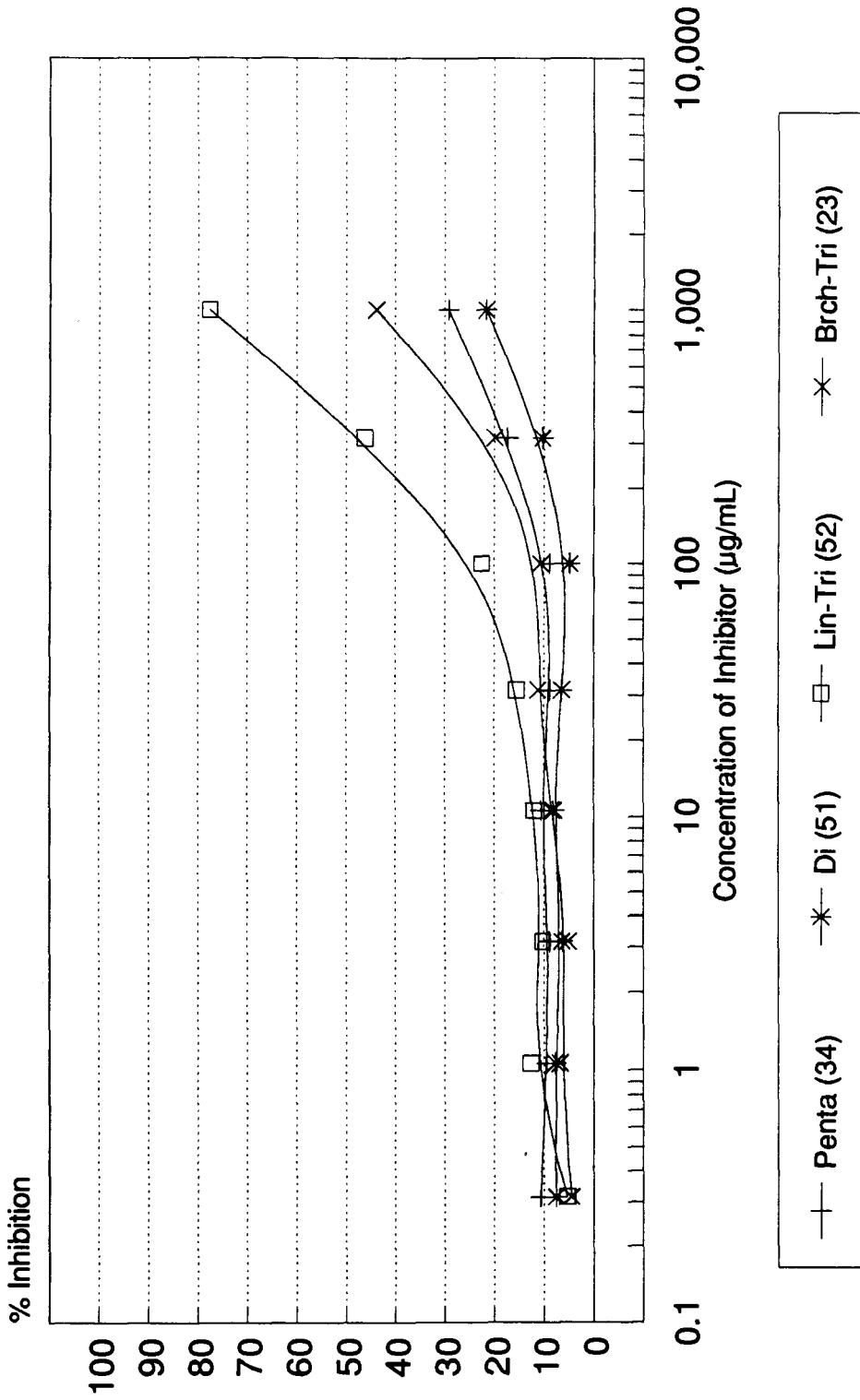


concentrations of both the coating antigen, and the monoclonal antibody were varied. For the inhibition assays, a combination of glycoconjugate coating and antibody concentration was chosen which gave an absorbance value of 0.8-1.0 units. The lowest possible coating level of the glycoconjugate was chosen, so as to increase the effectiveness of the inhibitors at lower concentration levels.

The first set of inhibition studies was carried out with the monoclonal antibody SA-1A. The results of these studies are shown in Figure 3-17. None of the inhibitors was found to give 100% inhibition of the binding, even at the highest level of inhibitor used. The coating of the glycoconjugate in the inhibition assay was reduced to a minimum; however, the binding could still not be effectively inhibited. Of the 4 inhibitors tested, the one which showed the greatest level of inhibition was the linear trisaccharide structure (52), followed by the pentasaccharide (34), the branched trisaccharide (23) and disaccharide (51). The disaccharide and the branched trisaccharide had approximately the same inhibitory strength. Initially, the pentasaccharide (34) was expected to show the greatest inhibition due to its greater homology with the polysaccharide structure, so the greater potency of the linear trisaccharide structure was not expected.

The possibility existed that the inhibition observed with the linear trisaccharide was an artifact because the glycoconjugate which was used as the coating antigen in the ELISA bore the same linear trisaccharide sequence. To test this possibility, the experiment was repeated by use of a branched trisaccharide glycoconjugate (47) in place of the linear trisaccharide glycoconjugate (45) as the coating antigen in the ELISA. The results obtained were the same as when the linear trisaccharide glycoconjugate (45) was used as the solid phase antigen. One possible explanation for the binding pattern

Figure 3-17
Inhibition ELISA of McAb SA-1A
By Use of (45) as Solid Phase Antigen



of SA-1A could be that the binding is due to non-specific sorts of interactions such as interactions with the BSA moiety of the glycoconjugate, or adhesion to the surface of the EIA plate. This last possibility was unlikely since the antibody and inhibitor solutions were made up in a 1% BSA solution; the BSA was intended to block any uncoated surfaces on the EIA plate. If non-specific interactions were responsible for the observed binding, then the oligosaccharide inhibitors would have little effect on the binding; but, some inhibition was observed. Another possibility could be that the antibody is a high affinity antibody with a relatively small binding pocket which binds an epitope on the polysaccharide structure which the linear trisaccharide most closely resembles. Due to the poor inhibitory strength of all the inhibitors, a clear picture of the binding pattern of this monoclonal antibody was not obtained. The amount of inhibitor substance available was limited so it was decided not to carry out the assays at increased inhibitor concentrations.

The aim of this work was to identify a monoclonal antibody which would bind to a streptococcal Group A polysaccharide structure with high specificity. The most likely possibility would be an antibody with an extended binding site, which would interact with a larger surface of the polysaccharide exhibiting unique features. An antibody with a relatively small binding site would be more likely to cross-react with other polysaccharides of similar structure to the streptococcal Group A polysaccharide, thereby reducing the usefulness of such an antibody for any diagnostic purpose. These considerations argue against the use of SA-1A for the detection of streptococcal group A polysaccharide.

An analogous series of indirect inhibition ELISA's were performed by use of the monoclonal antibody SA-2C. Of the four antibodies this was the

antibody which gave the highest titre. All of the five inhibitors tested were able to inhibit the binding up to 100%, by use of an inhibitor concentration of less than 1000 μ g/mL. The inhibition curves obtained are shown in Figures 3-18 and 3-19, with the results displayed in Table 3-II. The relative strength of the

Table 3-II: Inhibition Data For Monoclonal Antibody SA-2C

Mono-clonal Ab	Inhibitors									
	51		52		23		53		34	
	μ g/mL	μ M	μ g/mL	μ M	μ g/mL	μ M	μ g/mL	μ M	μ g/mL	μ M
SA-2C	40.1	97.8 (1.0)	69.2	126 (1.1)	62.9	113 (1.1)	47.4	67.6 (0.8)	16.1	17.8 ^a (0.0)

^a This inhibitor used as the reference for the indicated series.

^b The values in parentheses are for $\Delta\Delta G$ in kcal/mol, determined from the expression $\Delta\Delta G = RT \ln([I_1]/[I_2])$, where $[I_2]$ is the concentration of the reference inhibitor, $[I_1]$ is the concentration of the other inhibitors, $R=1.98 \text{ calK}^{-1}\text{mol}^{-1}$, and $T=295\text{K}$; positive values for $\Delta\Delta G$ indicate poorer binding.

inhibitors was found to be: pentasaccharide > tetrasaccharide > disaccharide \approx branched trisaccharide \approx linear trisaccharide. The differences in the concentrations of inhibitors required to give 50% inhibition for the two trisaccharides, and for the disaccharide, were not significantly different from one another. The trend observed is the one expected for an antibody with an extended binding site; the level of inhibition of a hapten should increase as the size of the inhibitor increases, thereby filling the binding site more completely. A perfectly linear increase in inhibition with increase in size is not necessarily expected. The binding of a ligand with an antibody is the sum of attractive and repulsive forces. The addition of an extra residue may bring into play more repulsive forces than attractive ones, thereby decreasing the affinity of the antibody for the ligand. This

Figure 3-18
Inhibition ELISA of McAb SA-2C
By Use Of (45) as Solid Phase Antigen

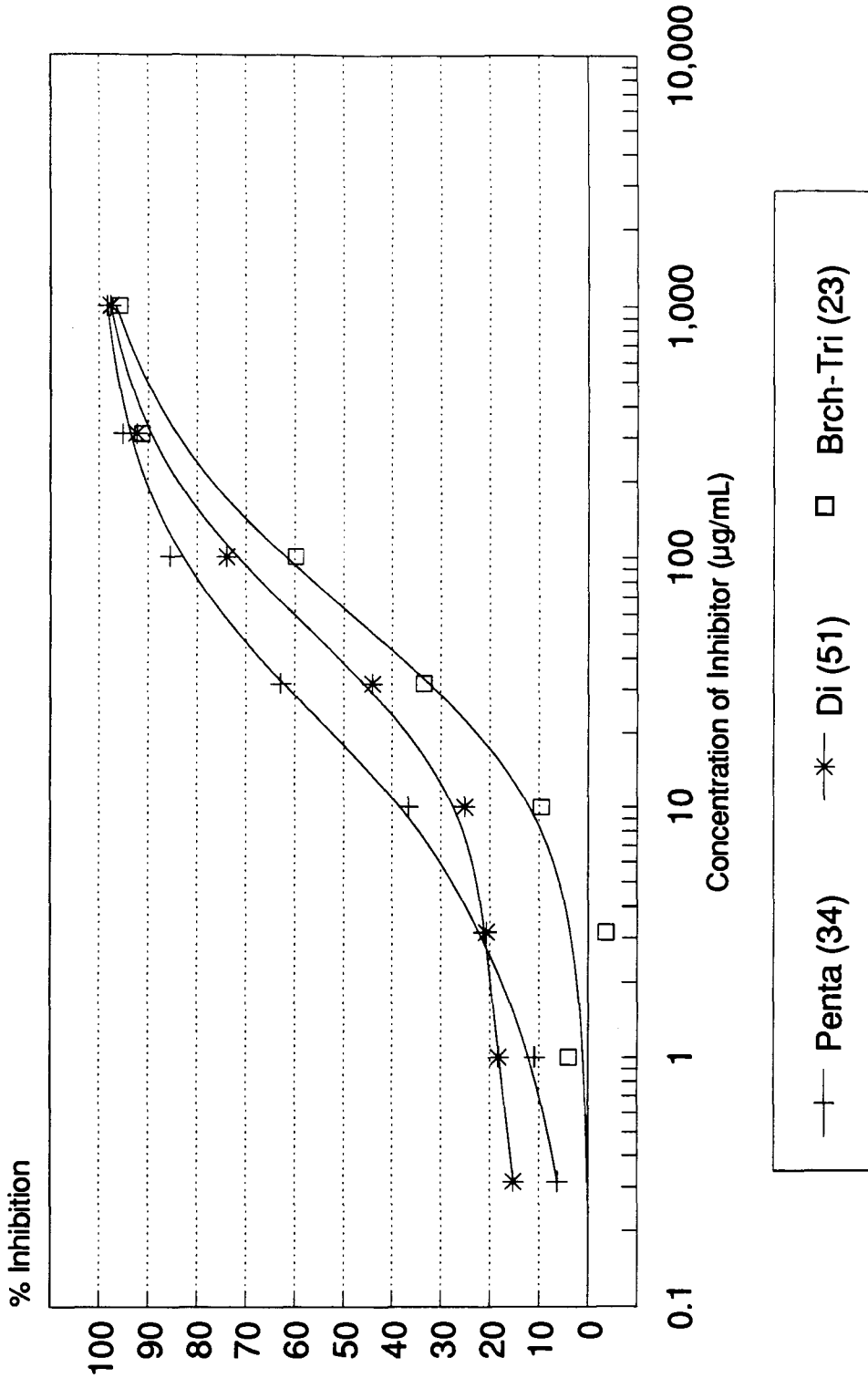
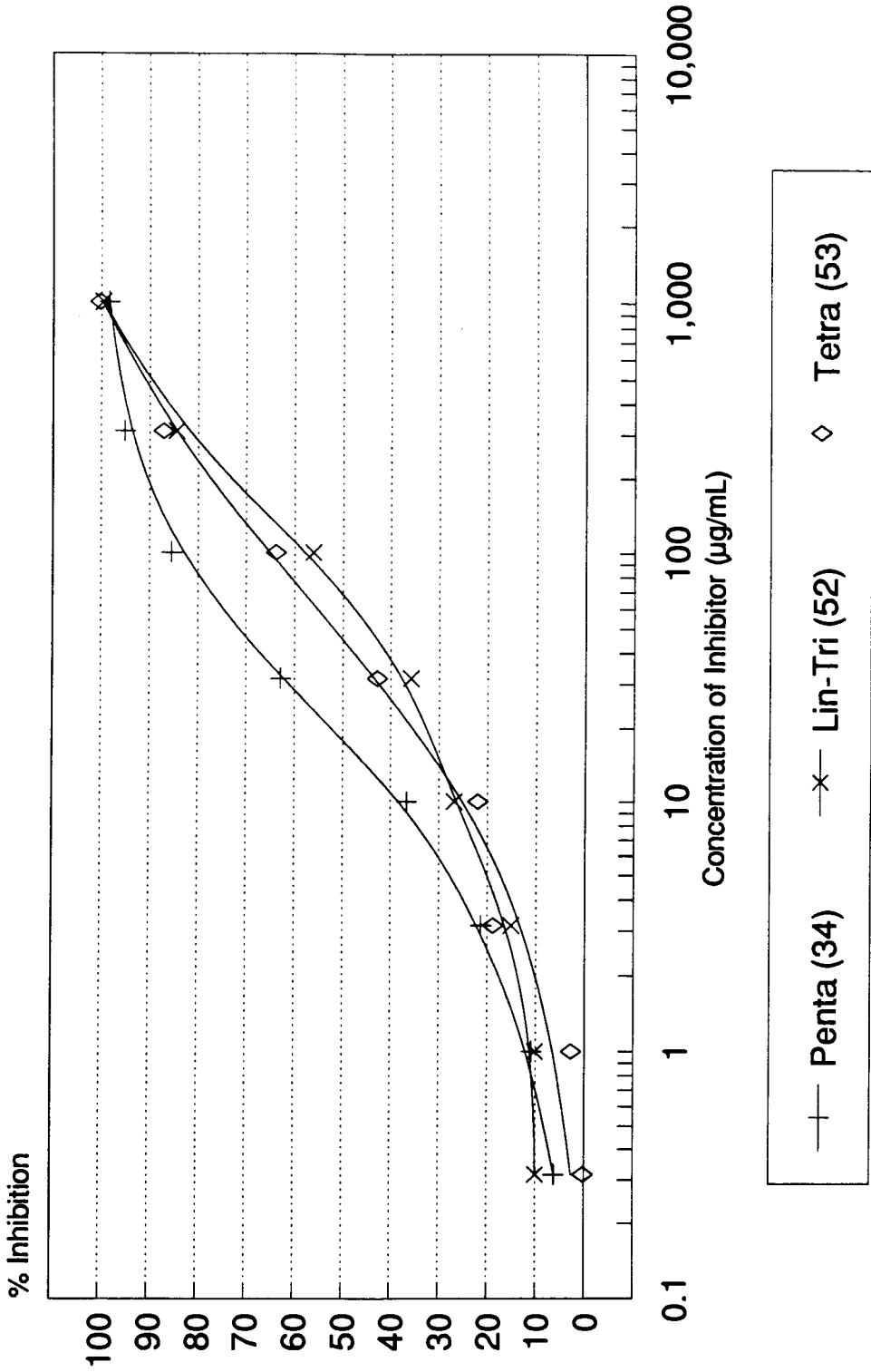


Figure 3-19
Inhibition ELISA of McAb SA-2C
By Use Of (45) as Solid Phase Antigen



may be the explanation why the inhibition of the disaccharide was nominally greater than that for the branched and linear trisaccharides.

The inhibition studies with the antibodies SA-2B and SA-1BC proved to be inconclusive. None of the inhibitors were effective in inhibiting the binding of either antibody. The level of glycoconjugate coating was decreased to a minimum in order to increase the effectiveness of the inhibitors, but to no avail. The binding profiles of these antibodies remain uncharacterized.

Previous workers¹⁰² have produced monoclonal antibodies to a streptococcal group A vaccine. They concluded that there were two basic types of antibody produced. One type was a high affinity antibody directed at an extended portion of the polysaccharide chain, with the binding site probably resembling a shallow groove. The other type of antibody they found was a low affinity antibody with a small binding site likely directed at the tips of the polysaccharide chain, and able to accommodate only single sugar residues. Extensive inhibition studies were not carried out with these antibodies; however, both types were shown to bind *N*-acetylglucosamine. The SA-2C antibody would appear to have similar binding characteristics to the high-affinity antibodies directed at internal sequences which were identified by Braun.¹⁰²

The maximum size of oligosaccharide which is bound by the monoclonal antibody SA-2C remains undetermined. The inhibition with the pentasaccharide hapten (34) was greater than with any other inhibitor, and it is not known if the inhibition would continue to increase with increasing size of the inhibitor, or if a maximum amount of inhibition would be reached with a hexasaccharide structure. As discussed earlier, the size of an antibody binding site for a carbohydrate binding antibody, is generally thought to be

large enough to accommodate a maximum of six sugar residues. There are, however, examples of antibodies binding many more than six residues.¹⁰³ One example of such behaviour is found with antibodies directed against a group B meningococcal polysaccharide derivative (a homopolymer of α -(2-8)-linked sialic acid residues).¹⁰⁴ In this study it was seen that inhibitor strength increases with increasing number of sialic acid residues. Oligosaccharides of up to eleven monosaccharide units were used as inhibitors, and no levelling off of inhibitor strength was observed. This is thought to be due to the conformational properties of the oligosaccharides; specifically, the finding that terminal regions of these types of oligosaccharides have different conformations than the internal regions, with the internal regions forming the epitope which is bound by the antibody. It is possible that the binding of the streptococcal group A polysaccharide by the monoclonal antibody SA-2C is of a similar nature. To fully characterize the binding site, specifically the number of residues which are recognized, further inhibition studies are required by use of hexasaccharide and larger oligosaccharides.

From the inhibition studies of the four monoclonal antibodies, the antibody SA-2C, was singled out as the candidate which showed the most promise for use as a diagnostic reagent. It appears that the binding site of the antibody is extended, binding at least five residues, and probably more. On the basis of the binding studies presented here the monoclonal antibody SA-2C will be used in further studies designed to develop an antibody-based immunodiagnostic reagent.

III: EXPERIMENTAL

A: Immunization of Rabbits With BSA Glycoconjugates

Twelve milligrams of each of the 3 glycoconjugates (45) (47) and (49) (shown in Figure 3-10) were dissolved in 0.01 M phosphate buffered saline (PBS) (12.0mL, pH 7.0). To each of these solutions was added Freund's Complete Adjuvant (15.0mL) (Difco Laboratories, Detroit Michigan). Three groups of 4 New Zealand White rabbits were given a subcutaneous injection of 1.0mL/rabbit 2 times per week for a period of 3 weeks, each group of four rabbits being immunized with a separate glycoconjugate solution. Following this immunization protocol, the rabbits were exsanguinated and the blood samples allowed to incubate at 37°C for three hours and then the blood clots allowed to contract and separate from the blood serum by cooling to 4°C for 8h. The blood clots were separated from the serum, and the serum centrifuged and the supernatant collected and frozen at -20°C until further use.

B: Preparation of BSA Affinity Gel

To a stirred slurry of Sepharose 6B gel (100mL) (Pharmacia, Uppsala, Sweden) in Na_2CO_3 2M (100mL) at 5°C, was added a $\text{CH}_3\text{CN}/\text{CNBr}$ solution (10.0mL, 2.0g/mL). The slurry was stirred at 5°C for 3min, and then poured into a sintered glass funnel and rinsed with NaHCO_3 0.1M (1000mL), followed by a rinse with distilled water (1000mL). The activated gel was then transferred to a beaker, by use of $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ 0.2M, pH 9.5 (100mL). To this suspension was added a solution of Bovine Serum Albumin (BSA), crystallized 99%, (400mg in 5.0mL $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$, 0.1M, pH9.5) (ICN Biochemicals, Cleveland, Ohio). The solution was let stand at room temperature, with occasional stirring, for 2.5h. The BSA-gel was poured into a sintered glass

funnel and filtered. The protein content of the filtrate was determined by measuring the A_{280} , and it was estimated that 98% of the BSA was bound. The gel was then taken up in $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ 0.2M, pH 9.5, (200mL) containing glycine 0.2M to block any unbound activated sites. The solution was let stand at room temperature for 3h with occasional stirring. The gel was then filtered on a sintered glass funnel, and washed with NaHCO_3 0.1M, pH 8.2 (200mL), followed by NaOAc 0.1M, pH 4.0 (200mL). The cycle of washings was repeated 4 times, and then the gel was taken up in PBS, containing NaN_3 (0.05%), and stored at 4°C till needed.

C: Purification of Rabbit Sera

Samples of the antisera from each of the 4 rabbits in each group were pooled to give three separate samples of serum, one from each glycoconjugate immunization. Serum samples (5.0mL) were applied to a protein A-Sepharose CL-4B column system (Pharmacia Canada Inc., Dorval, Quebec, Canada) which was maintained at 4°C. The IgG fraction was bound by use of glycine 1.5M, containing NaCl 3M, adjusted to pH 8.9 by use of 5M NaOH . The bound antibodies were eluted by use of citric acid (100mM), adjusted to pH 4.0 by use of 5M NaOH . The antibody containing fractions were pooled and dialyzed against Tris/HCl, 0.1M, pH 8.5, containing NaCl , 0.5M, and concentrated to the original 5mL volume, by use of an Amicon ultra filtration apparatus. The sample was then loaded onto a BSA-Sepharose 6B affinity column (prepared as described above) in the same buffer, and the eluted fractions were combined and dialyzed against Tris/HCl, 50mM, pH 8.0, containing NaCl , 150mM, and concentrated, by use of an Amicon ultra filtration apparatus. The protein concentrations for the purified antibody preparations were determined by measuring the A_{280} ($\epsilon=1.35 \text{ mLcm}^{-1}\text{mg}^{-1}$ for IgG immunoglobulin).

D: Indirect Enzyme-Linked Immunosorbent Assay

In a typical indirect ELISA, 96 well microtitre plates (Linbro; Flow Laboratories, Mississauga, Ontario, Canada) were coated with a glycoconjugate solution, 10µg/mL in PBS, 100µL/well. The glycoconjugate solution was allowed to bind at room temperature for 3h, and then washed three times (SKATRON AS Microwash 2) with PBS, containing 0.05% Tween 20 and 0.02% NaN_3 (PTA). Alternatively, the plates could be stored at 4°C until needed. To the washed plates, an antibody solution was then added, 100µL/well. All antibody dilutions were made in PBS. The plates were left at room temperature for 3h and then washed three times with PTA. An alkaline phosphatase labelled goat anti-rabbit IgG antibody (Miles-Yeda Ltd., 61-275) was added, 100µL/well, diluted 1:1000 in PBS containing 1% BSA. The labelled antibody was allowed to react for 1h at room temperature and then the plates were washed three times with PTA. The enzyme substrate para-nitrophenol phosphate (p-NPP) was added, 100µL/well, 1.0mg/mL in $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ 0.05M, pH 9.8, containing 0.001M MgCl_2 . The plates were developed for 1h at room temperature, and the absorbance of the wells was read at 405nm (Titertek Multiscan MC plate reader).

E: Indirect Inhibition ELISA By use of Rabbit Polyclonal Antibodies

Prior to the inhibition assay the appropriate concentrations to use for each glycoconjugate/antibody combination were determined in an ELISA assay in which the concentrations of the coating glycoconjugate and the antibody to be tested, were varied simultaneously in a checker board fashion. Following this determination, the EIA plates were coated with an appropriate glycoconjugate solution concentration, 1-10µg/mL in PBS, 100µL/well. The glycoconjugate solution was allowed to bind at room temperature for 3h, and

the plates were then washed three times with PTA. To the washed plates, solutions of inhibitors were added at 2x the final concentration, 50 μ L/well, serially diluted in PBS containing 0.1% BSA (dilution factor used was $\sqrt{10}$). An antibody solution was then added, 1.5-10 μ g/mL, 50 μ L/well in PBS containing 0.1% BSA. All inhibitor concentrations were run in triplicate on the same plate. Several wells were used as reference wells, where in place of an inhibitor solution, 50 μ L of PBS containing 1% BSA was added. These wells were used to calculate the 0% inhibition value. The plates were left at room temperature for 3h and then washed three times with PTA. An alkaline phosphatase labelled goat anti-rabbit IgG antibody was added, 100 μ L/well, diluted 1:1000 in PBS containing 1% BSA. The labelled antibody was allowed to react for 1h at room temperature and then the plates were washed three times with PTA. The p-NPP substrate was then added, 100 μ L/well, 1.0mg/mL in NaHCO₃/Na₂CO₃ 0.05M, pH 9.8, containing 0.001M MgCl₂. The plates were developed for 1h at room temperature, and the absorbance of the wells was read at 405nm.

F: Immunizations of Mice

A *Streptococci* Group A vaccine was prepared as described previously.¹⁰⁵ The vaccine was prepared from a heat-killed, pepsin treated culture of *Streptococcus pyrogens* Group A type 4, strain J17A4. The vaccine was determined to contain 300 μ g/mL rhamnose by use of a phenol-sulphuric acid test.⁷⁷ A/J mice and BALB/c mice were given a series of 6 and 7 intraperitoneal injections, respectively, over periods of 53 and 67 days. The final injections were given 3 days prior to fusion. The injections consisted of 0.1mL of vaccine in PBS; the equivalent to 30 μ g rhamnose.

G: Fusions, ELISA Screening, and Cloning

Two separate fusion experiments were carried out, where in each fusion experiment spleen cells from two immunized mice were fused with the nonimmunoglobulin-producing Sp2/0 plasmacytoma cell line¹⁰⁶ (Institute for Medical Research, Camden, N. J.), as previously described.¹⁰⁷ The cell colonies were screened initially with a *Streptococci* Group A polysaccharide BSA glycoconjugate, and further screenings were carried out with disaccharide and trisaccharide glycoconjugates as well. The screenings were carried out as described above for indirect ELISA. Bound antibody was detected by use of an alkaline phosphatase labelled goat anti-mouse immunoglobulin M (IgM) and IgG antibody. Hybridomas judged to be positive in the ELISA screen were cloned by limited dilution, by use of mouse spleen cells as feeders. All hybrids were cloned twice to ensure clonality and stability before cell samples were frozen and ascites fluid was raised. The fusion experiment with the A/J mice produced 6 stable hybridomas, and the fusion experiment with the BALB/c mice produce 1 stable hybridoma.

H: Ascites Fluid

BALB/c mice were primed by intraperitoneal injection with 0.5mL of 2,6,10,14-tetramethylpentadecane (pristane). After 14 days, the mice were injected (intraperitoneal) with 10⁶ hybridoma cells, and tapped for ascites fluid after a further 1-10 days. The ascites fluid was centrifuged and filtered, and stored at -20°C until further use.

I: Purification of Monoclonal Antibodies by (NH₄)₂SO₄ Precipitation

The ascites fluid samples were thawed and centrifuged at 4°C for 30min (10,000g); the pellet was discarded. The supernatant was filtered through a

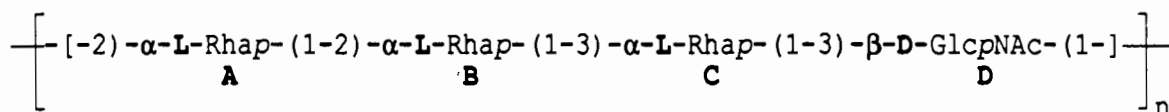
0.22 μ m filter, and to the filtrate was added dropwise with stirring, an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$. The solution was left stirring at 4°C for 16h, and then centrifuged at 4°C for 30min (10,000g). The supernatant was discarded, and the pellet resuspended in PBS (0.5 times the original volume of ascites fluid). The resuspended antibody solutions were then dialyzed against PBS containing 0.02% NaN_3 . The dialyzed solutions were centrifuged at 4°C for 20min (10,000g), and the supernatants were filtered through a 0.8 μ m filter. The protein concentrations of the antibody solutions were determined by measuring the A_{280} ($\epsilon=1.2 \text{ mLcm}^{-1}\text{mg}^{-1}$ for IgM immunoglobulin).

J: Inhibition Studies of Monoclonal Antibodies

The inhibition studies were carried out by use of an indirect inhibition ELISA, as described above. Concentration levels for each glycoconjugate/antibody combination were determined in an ELISA assay where the concentrations of the coating glycoconjugate and the antibody to be tested, were varied simultaneously in a checker board fashion. Bound antibody was detected by use of an alkaline phosphatase labelled goat anti-mouse immunoglobulin M (IgM) antibody, diluted 1:1000 in PBS containing 1.0% BSA.

CHAPTER 4**I: INTRODUCTION**

Our laboratory has been active in the synthesis of oligosaccharides for use in investigations of the interactions of antigens with antibodies at a molecular level. One of the systems under investigation is the interaction of the *Shigella flexneri* Variant Y lipopolysaccharide (LPS) O-antigen with corresponding monoclonal antibodies.⁹³ The *Shigella flexneri* variant Y LPS O-antigen¹⁰⁸ is comprised of a four sugar biological repeating unit¹⁰⁹ containing α -L-rhamnose and β -D-N-acetylglucosamine residues. The approach used is to synthesize portions of the polysaccharide, and to test these sequences in inhibition binding



studies with complementary monoclonal antibodies.¹⁰⁹ From these studies inferences may be made as to the nature of the surfaces of the antigen which are important in recognition and binding with antibodies. Work in our laboratory has furnished pentasaccharide^{60a, 60b} hexasaccharide^{60b} and heptasaccharide^{60c} fragments of the *Shigella flexneri* Variant Y polysaccharide for use in these studies (see Figure 4-1).

During the chemical synthesis of these compounds, NMR spectroscopy played a vital role in confirming the structures of the synthetic intermediates. For the final deblocked compounds, in addition to confirming structural integrity, ¹H- and ¹³C-NMR spectroscopy can give information on the three-dimensional conformation of the compounds in solution. The information obtained from NMR experiments can be used in conjunction with theoretical calculations¹¹⁰ to model the three-dimensional shape of the antigenic surface.

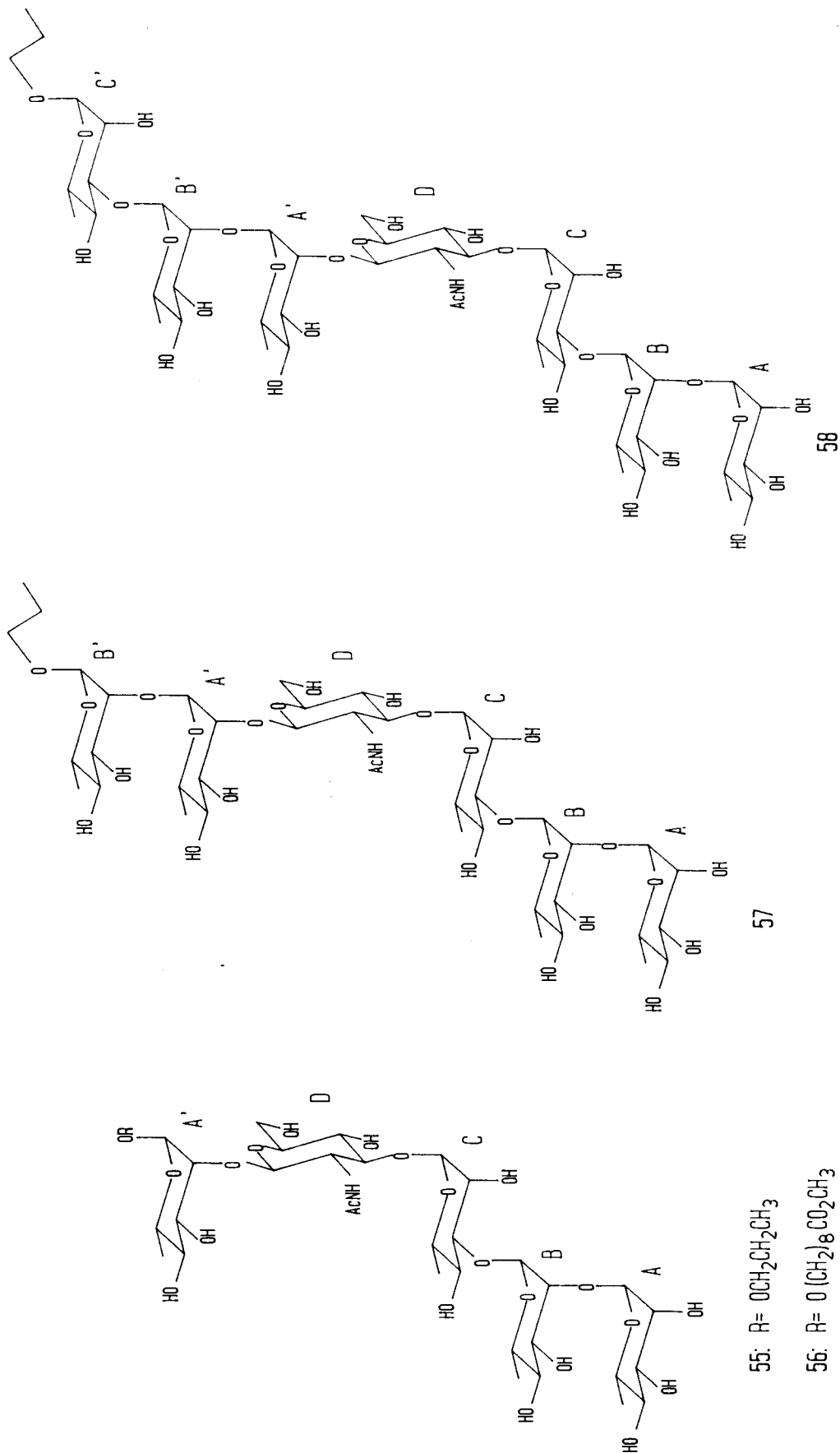


Figure 4-1: Synthetic oligosaccharide sequences of the *Shigella flexneri* Variant Y lipopolysaccharide.

Prior to ^1H -NMR analysis of the conformational properties of the oligosaccharides, a complete assignment of the ^1H -NMR spectrum is required. The ^1H -NMR spectra for these compounds are not easily analyzed due to the extensive overlap of signals, particularly in the region 3.0-4.5 ppm. These problems may be overcome by utilizing two-dimensional NMR techniques which allow for the assignment of signals within highly overlapped regions of the spectrum. To illustrate the nature of the analyses which have been performed, the NMR analysis of the heptasaccharide (58) will be outlined in this chapter. These results provide the basis for a more detailed conformational analysis of the heptasaccharide structure by use of computational methods; these studies are currently ongoing.¹¹¹ Similar analyses have been performed for the penta- and hexasaccharides (55)-(57), and also for oligosaccharides corresponding to the cell-wall polysaccharide of the β -hemolytic *Streptococci* Group A; the synthesis of the latter compounds are described in Chapter 2 of this thesis.

A: Background

In recent years, new NMR techniques have been developed at a rapid pace. The introduction of two-dimensional NMR methods have opened up whole new areas of research. Several reviews in this area have been published,¹¹² and only a brief discussion of a few of the methods used in the present research will be given.

In the familiar one-dimensional NMR experiment, a 90° pulse of an appropriate radio frequency is applied to a sample. The magnetization vectors of the individual nuclei are rotated into the xy plane, perpendicular to the externally applied magnetic field along the z-axis.

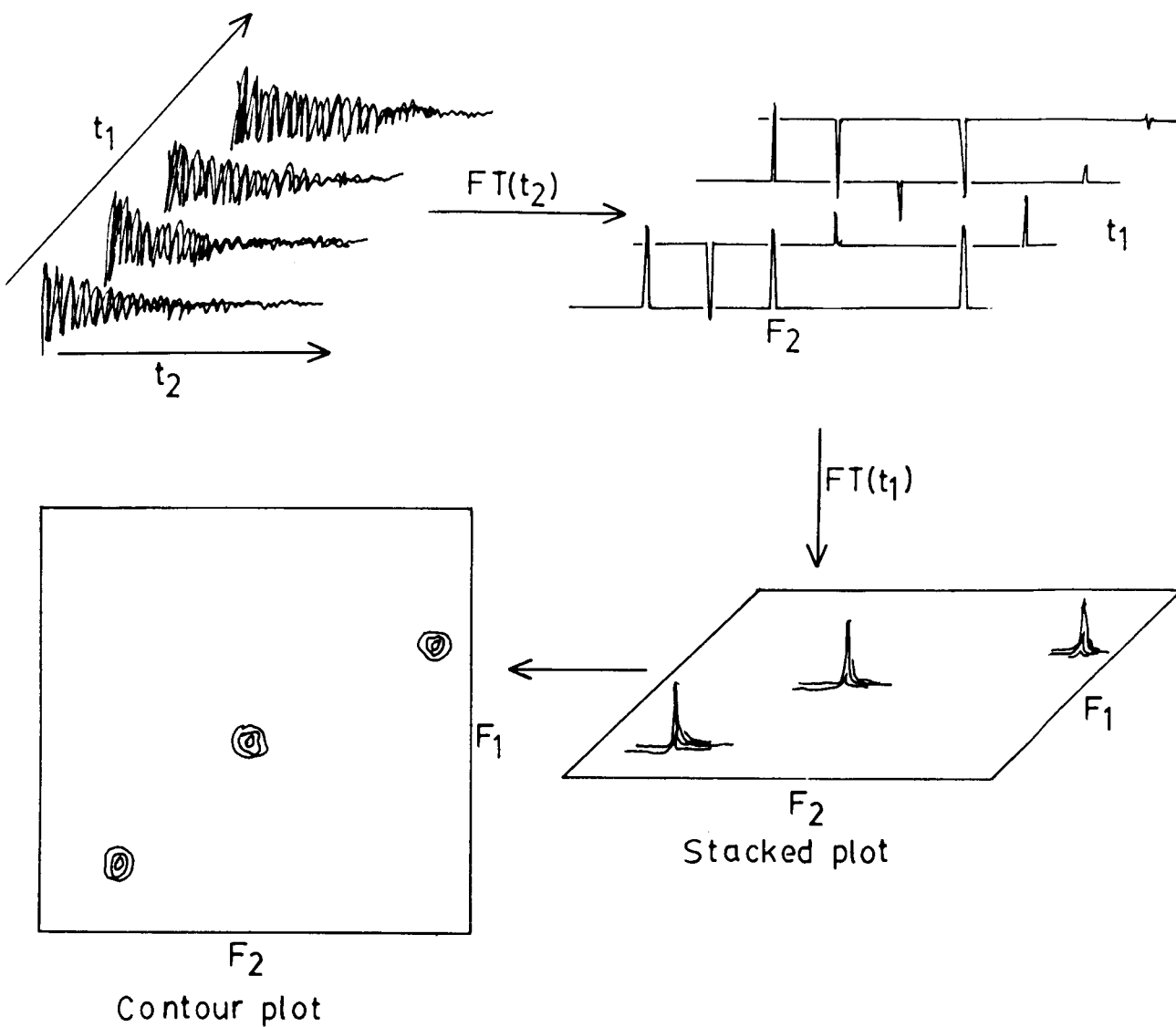
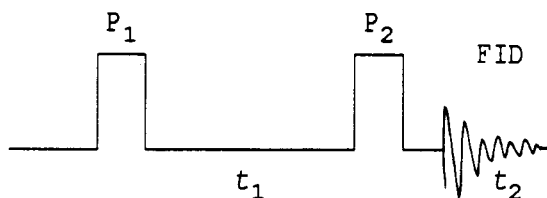


Figure 4-2: General scheme for the acquisition and display of a two-dimensional NMR spectrum.

The nuclei precess about the z-axis, decaying with time back to alignment with the z-axis. This precession about the z-axis in the xy-plane induces a signal in the receiver coil. This signal decays as the magnetization vectors decay back to the z-axis, and is termed a free induction decay (FID). The FID is composed of oscillating signals, the frequencies of which may be extracted by a Fourier transformation. Initially, the NMR signal (FID) is obtained as a function of time $S(t_2)$, which when it is Fourier transformed gives an NMR signal as a function of frequency $S(F_2)$.

In a two-dimensional NMR experiment an additional time variable is introduced into the pulse sequence. The generalized pulse sequence for a two-dimensional NMR experiment is shown below.



The pulses P_1 and P_2 may be single pulses, or more complex series of pulses and fixed delay times. A general scheme for the acquisition of a two-dimensional NMR spectrum is illustrated in Figure 4-2. The acquisition of the FID during t_2 corresponds to the detection period in a conventional one-dimensional experiment. If the newly introduced time variable t_1 is regularly incremented, and a one-dimensional NMR spectrum is obtained for each value of t_1 , then an NMR signal as a function of two time variables will be obtained $S(t_1, t_2)$. If the series of FID's are Fourier transformed with respect to t_2 , a series of one-dimensional spectra will be obtained where the individual signals are modulated in amplitude and phase as a function of t_1 , $S(t_1, F_2)$. A second Fourier

transformation of these signal modulations, perpendicular to the F_2 -axis, then gives a two dimensional NMR spectrum as a function of two frequencies, $S(F_1, F_2)$. These frequency axes (often termed the F_1 and the F_2 axes) may correspond to various NMR frequencies.

In ^{13}C - ^1H correlation spectroscopy⁷⁹ one frequency axis (F_2) corresponds to the ^{13}C -chemical shift and the other (F_1) to the ^1H -chemical shift. In these experiments the chemical shifts of carbon signals may be correlated to the chemical shifts of proton signals to which they are coupled. In homonuclear chemical-shift correlation spectroscopy, referred to as COSY⁷⁸ (the simplest, and one of the most useful 2-D NMR experiments), both frequency axes correspond to proton chemical shift. In a COSY spectrum the chemical shifts of proton signals which share a through-bond coupling are correlated. Many variations of the COSY sequence exist, including the relayed-COSY experiment,¹¹³ and the TOCSY experiment.⁸² In these experiments, proton signals which are not directly coupled, but which are coupled to a common intervening proton, are correlated. The experiments are very useful when there is overlap of cross-peaks in the COSY spectrum.

Another variation of the COSY experiment is a two-dimensional nOe experiment, referred to as a NOESY experiment.¹¹⁴ Again, both frequency axes correspond to proton chemical-shift; however, only signals which are dipolar coupled, rather than scalar coupled, will show a cross-peak. This experiment is very useful for small molecules where the product of the spectrometer angular frequency (ω_0) and the molecular rotational correlation time (τ_c) is much less than unity, and results in positive nOe's, and for large molecules with $\omega_0\tau_c > 1$ (negative nOe). For molecules of intermediate size the correlation time of the molecule may be such

that $\omega_0\tau_c \approx 1$, and the nOe vanishes. An alternate form of the experiment is the CAMELSPIN experiment,¹¹⁵ now commonly called rotating-frame Overhauser enhancement spectroscopy, or ROESY.⁸³ In this experiment, unlike with the nOe, the ROE is positive for molecules where $\omega_0\tau_c < 1$, and increases with increasing correlation time. The effect of the correlation time of a molecule on the magnitude of both the nOe and the ROE is shown in Figure 4-3.^{115,116} The ROE does not pass through zero, so for molecules which cannot be studied with the NOESY experiment, the ROESY experiment is a viable alternative.

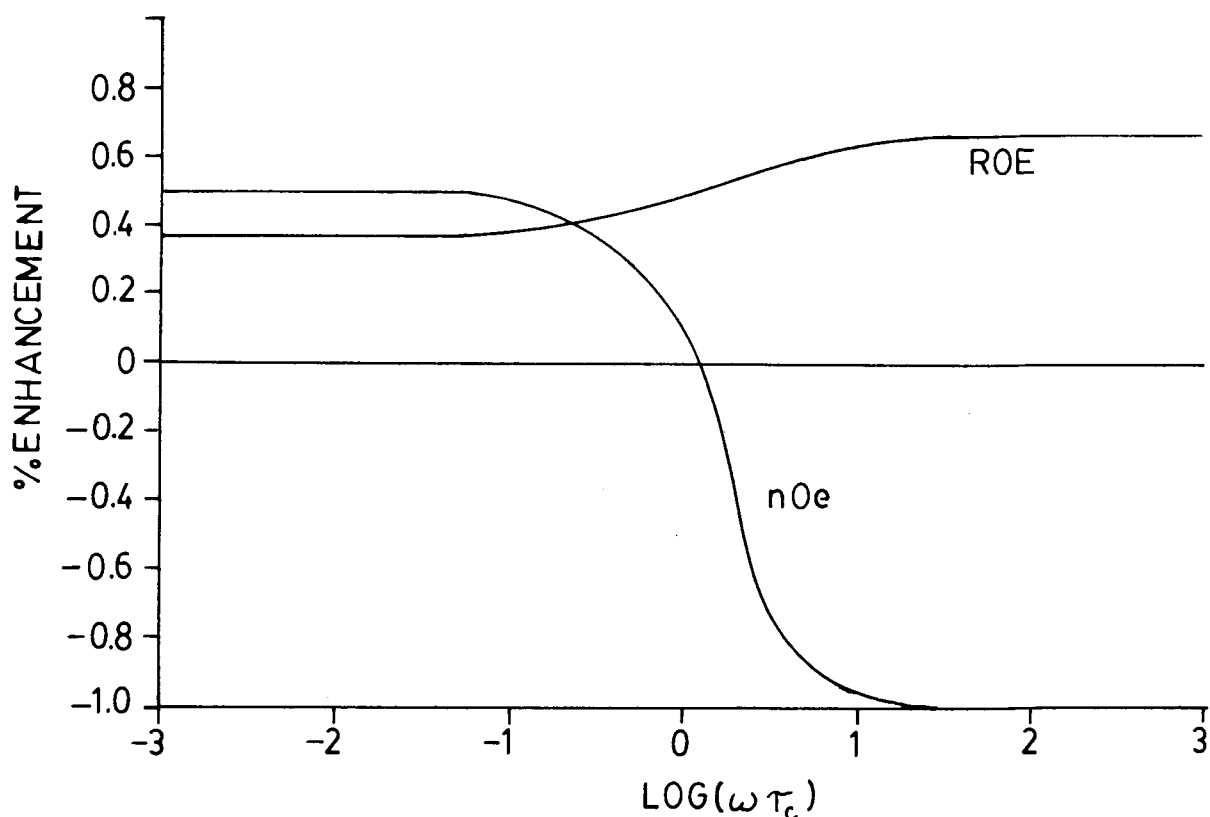


Figure 4-3: Dependence of the nOe, and the nOe in the rotating frame (ROE), on $\omega_0\tau_c$.

II: RESULTS AND DISCUSSION

The one-dimensional ^1H -NMR spectrum of compound (58) is shown in Figure 4-4. The seven anomeric protons signals are seen between 4.60 and 5.20 ppm. The signals due to the methyl groups of the rhamnose residues appear at 1.10-1.30 ppm. The remaining ring proton signals all appear within an envelope of signals only 1.0 ppm wide, between 3.2 and 4.2 ppm. There are 32 proton signals within this envelope, making assignment of all the individual signals by use of one-dimensional NMR techniques impossible.

A: COSY Spectrum of Heptasaccharide (58)

The COSY spectrum of compound (58) is shown in Figures 4-5a through 4-5d. All of the ring protons of a given ring comprise separate J-coupled spin systems. The complete assignment of the proton spectrum requires that first, the complete spin systems be identified, and then the spin systems be assigned to particular regions of the molecule. The COSY cross-peak pattern is interpreted in the following way. Normally, there will be a particular signal in the NMR spectrum which is readily assignable and is clearly resolved. Such a signal serves as a good starting point when interpreting a COSY spectrum. The anomeric signals of the sugar residues, seen between 4.60 and 5.20 ppm, make good starting points for the analysis.

The off-diagonal cross-peaks in the COSY spectrum indicate proton signals which are J-coupled. Thus, for example, the signal at 5.11 ppm, due to a rhamnosyl H-1, shows a cross-peak at 4.12 ppm, indicating the chemical shift of H-2 of the same sugar ring. Since the anomeric proton

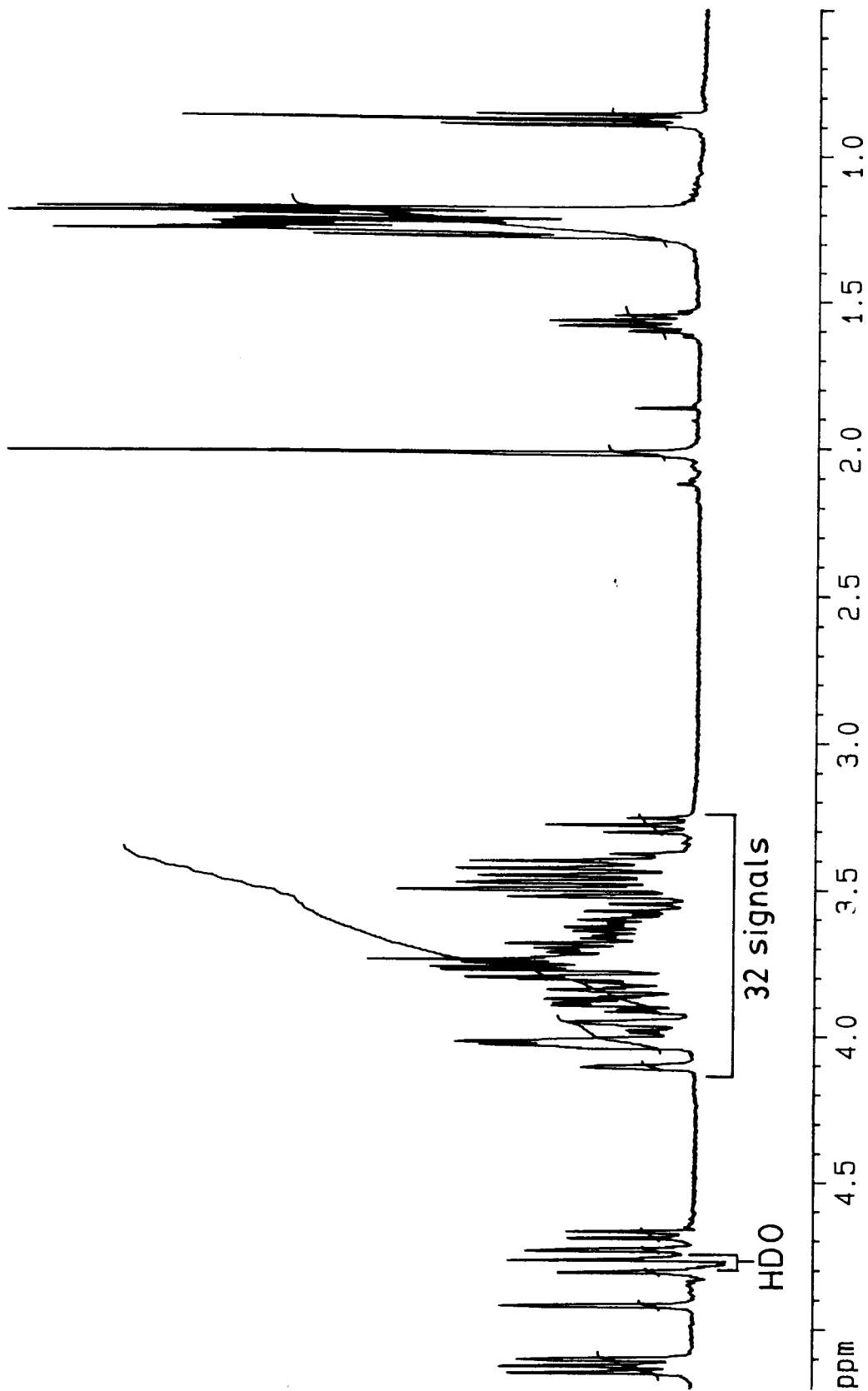


Figure 4-4: 400 MHz $^1\text{H-NMR}$ spectrum of heptasaccharide (58).

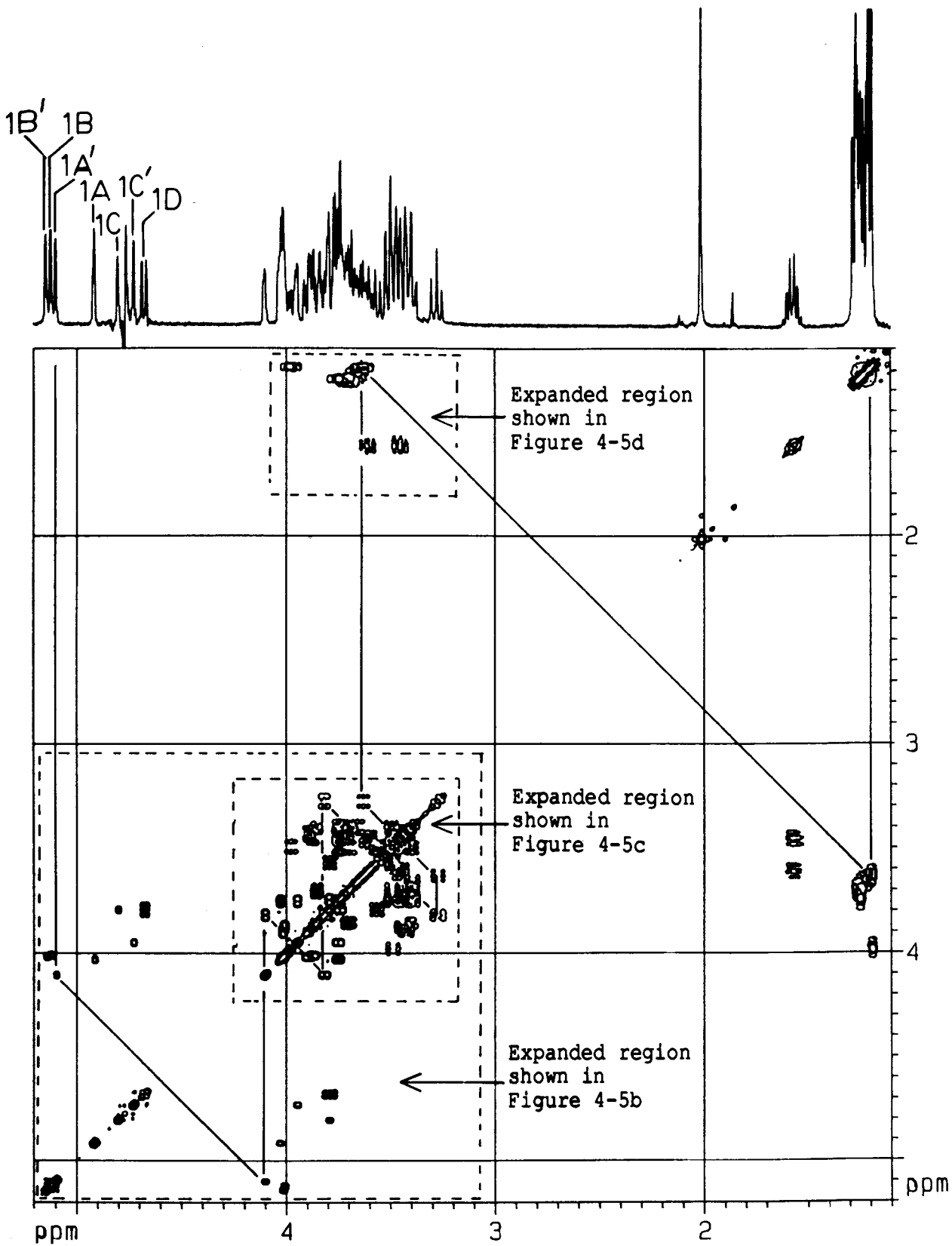


Figure 4-5a: 400 MHz COSY spectrum of heptasaccharide (58).

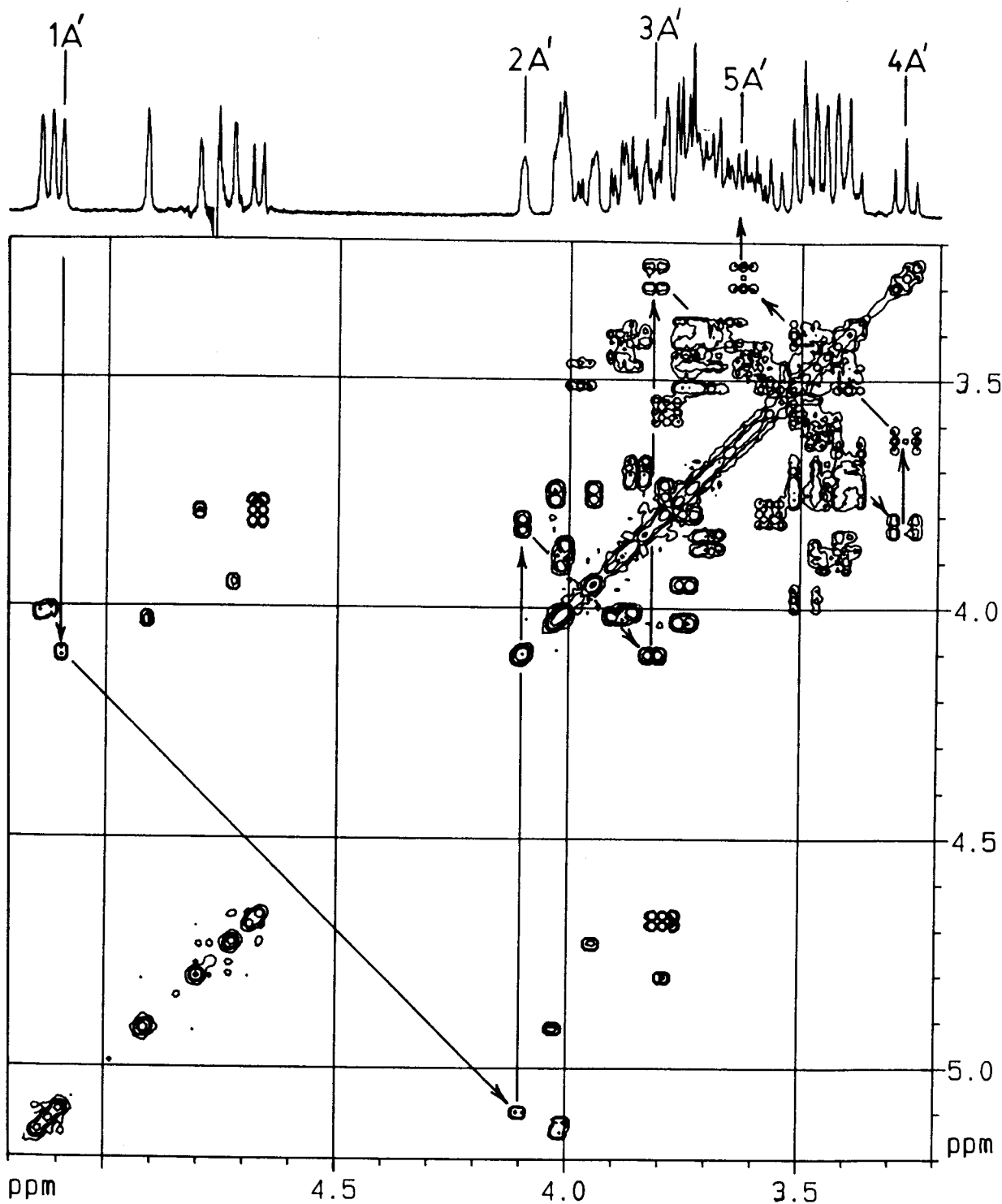


Figure 4-5b: Partial 400 MHz COSY spectrum of heptasaccharide (58). The cross-peak connectivity pattern is shown for one rhamnosyl ring.

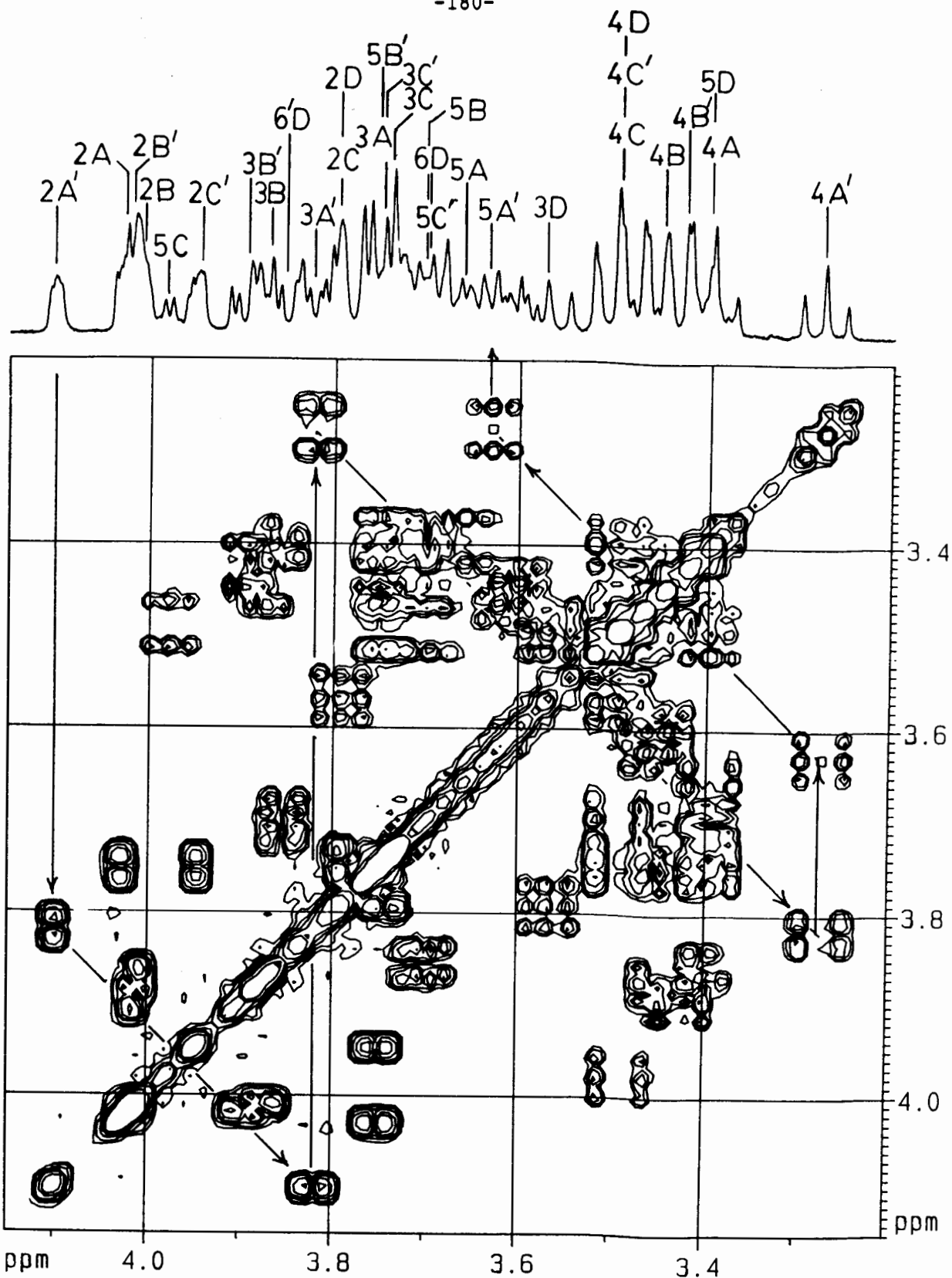


Figure 4-5c: Expanded region of the 400 MHz COSY spectrum of heptasaccharide (58).

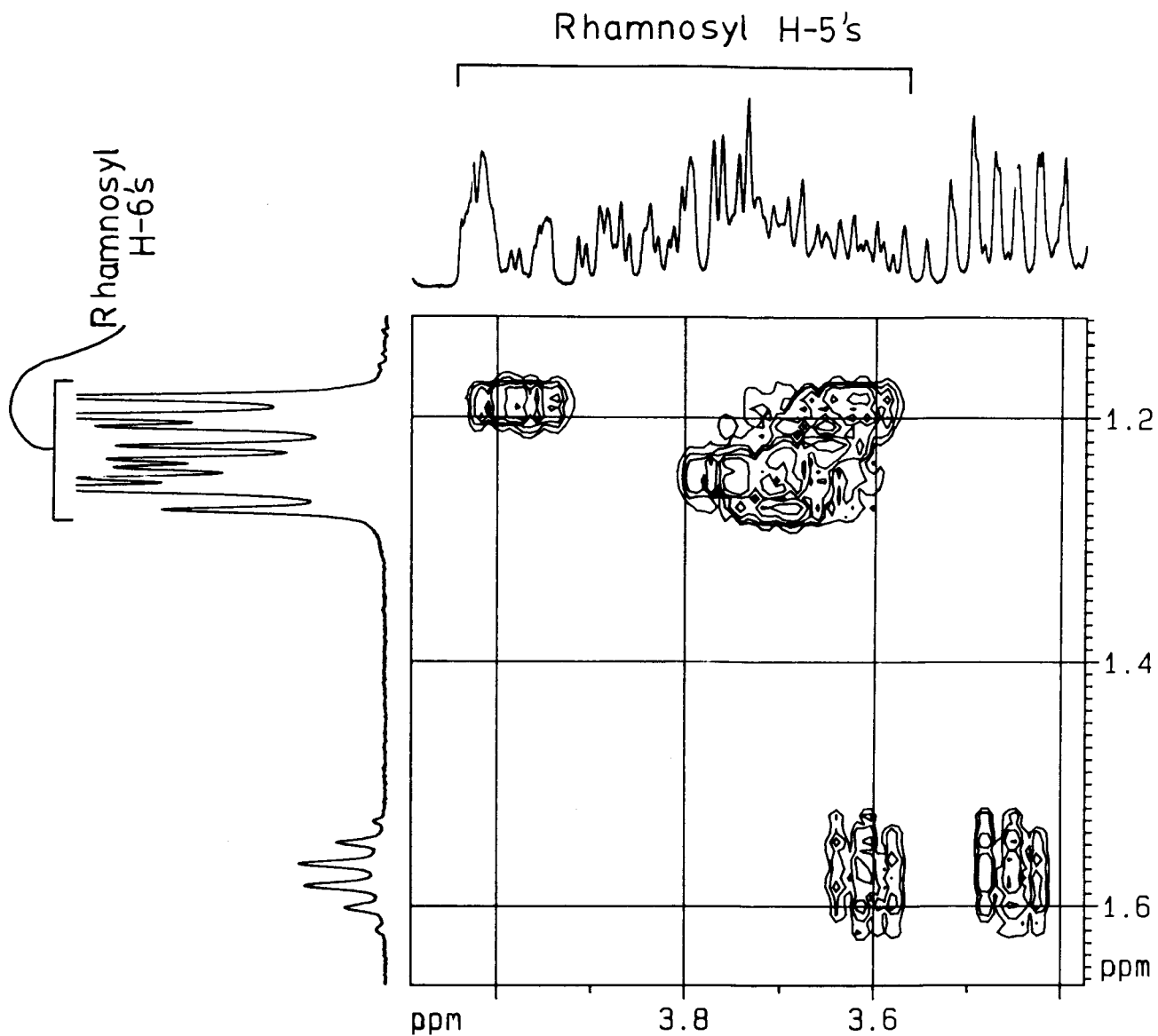


Figure 4-5d: Expanded region of the 400 MHz COSY spectrum of heptasaccharide (58). Correlation cross-peaks between the rhamnosyl H-5's and H-6's are shown.

is only coupled to one other proton, namely H-2, there is only one cross peak seen on the vertical line drawn at 5.11 ppm. The H-2, however, is coupled to two protons, the H-1 and the H-3, hence there are two cross-peaks on the vertical line drawn at the chemical shift of H-2. The cross-peak at 5.11 ppm, indicates the position of the H-1 to which it is coupled, and the cross-peak at 3.83 ppm indicates the chemical shift of the H-3 to which it is coupled. In a similar fashion the H-4, H-5 and H-6₃ of the same ring may be assigned. If any of the signals in the spin system may be unambiguously assigned to a particular proton within the molecule, then the remaining signals within the spin system may be assigned as well; however, this is not possible for this set of signals simply from chemical shift information alone.

The spin system containing the anomeric signals at 4.69, 5.13, and 5.15 ppm can also be identified by following the cross-peak patterns starting with the anomeric signals. The anomeric signal at 4.69 may be easily assigned to the H-1 of the *N*-acetylglucosamine residue on the basis of the vicinal coupling constant. The observed value of 8.6 Hz is consistent with a β -linkage, the remaining coupling constants of the same ring are consistent with a 4C_1 -chair conformation of the pyranose ring.

The identification of the spin systems containing the anomeric proton signals at 4.74, 4.82 and 4.93 is not as straightforward. The chemical shifts of the H-1's and the H-2's for these spin systems may be easily identified; however, the H-3's all have overlapping chemical shifts at about 3.76 ppm (see Figure 4-5c). This overlap means that the coupling patterns cannot be traced through to the corresponding H-4's and the rest of the spin systems. This problem of overlapping signals in the COSY spectrum, can be solved by acquiring a relayed-COSY spectrum.

B: Relayed-COSY Spectrum of Heptasaccharide (4)

If one considers an AMX spin system, in a relayed-COSY experiment, a cross-peak will appear at the chemical shifts of the A and the M signals; however, in addition to this cross-peak an additional cross-peak is also seen at the chemical shifts of the A and the X signals. The chemical shift of the A signal is correlated with the chemical shifts of the M and the X signals; thus, the coupling information of the A signal is relayed to the X signal as well.

The relayed COSY spectrum of (58) is shown in Figure 4-6a, with expanded regions shown in Figures 4-6b through 4-6e. A typical coupling pattern is illustrated by tracing out the spin system which contains the rhamnosyl H-1 signal at 5.11 ppm (see Figure 4-6b). A vertical line drawn at 5.11 ppm shows two cross-peaks at this position. The peak at 4.12 ppm is the same peak seen in the COSY spectrum, this cross-peak indicates the chemical shift of the H-2 of the same ring. The cross-peak at 3.83 ppm is the relay cross-peak, which gives the chemical shift of H-3. Similarly, a vertical line drawn at the chemical shift of H-2 shows three cross-peaks, indicating the chemical shifts of H-1 (5.11 ppm), H-3 (3.83 ppm) and H-4 (3.28 ppm). A vertical line drawn at the chemical shift of H-3 (see Figures 4-6b and 4-6c) shows four cross-peaks corresponding to the chemical shifts of H-1 (5.11 ppm), H-2 (4.12 ppm), H-5 (3.64 ppm) and H-4 (3.28 ppm). Notice that at the chemical shift of H-4, one would expect to see four cross-peaks in an analogous fashion to H-3. The H-4/H-5 cross-peak, normally a relatively weak cross-peak in the COSY spectrum, however, appears as a very weak signal in the relayed COSY spectrum, making the identification of these particular cross-peaks very difficult.

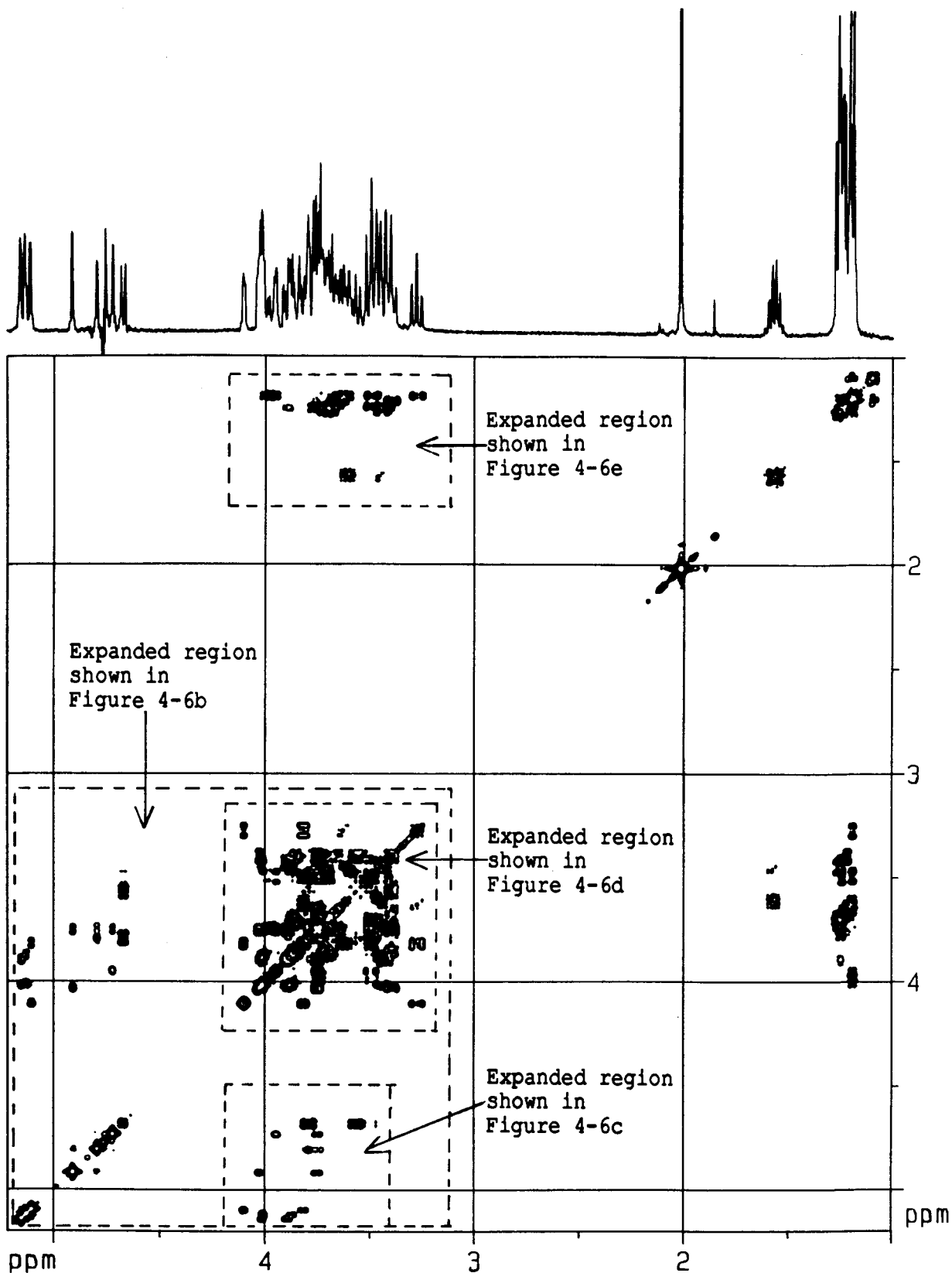


Figure 4-6a: 400 MHz Relayed-COSY spectrum of heptasaccharide (58).

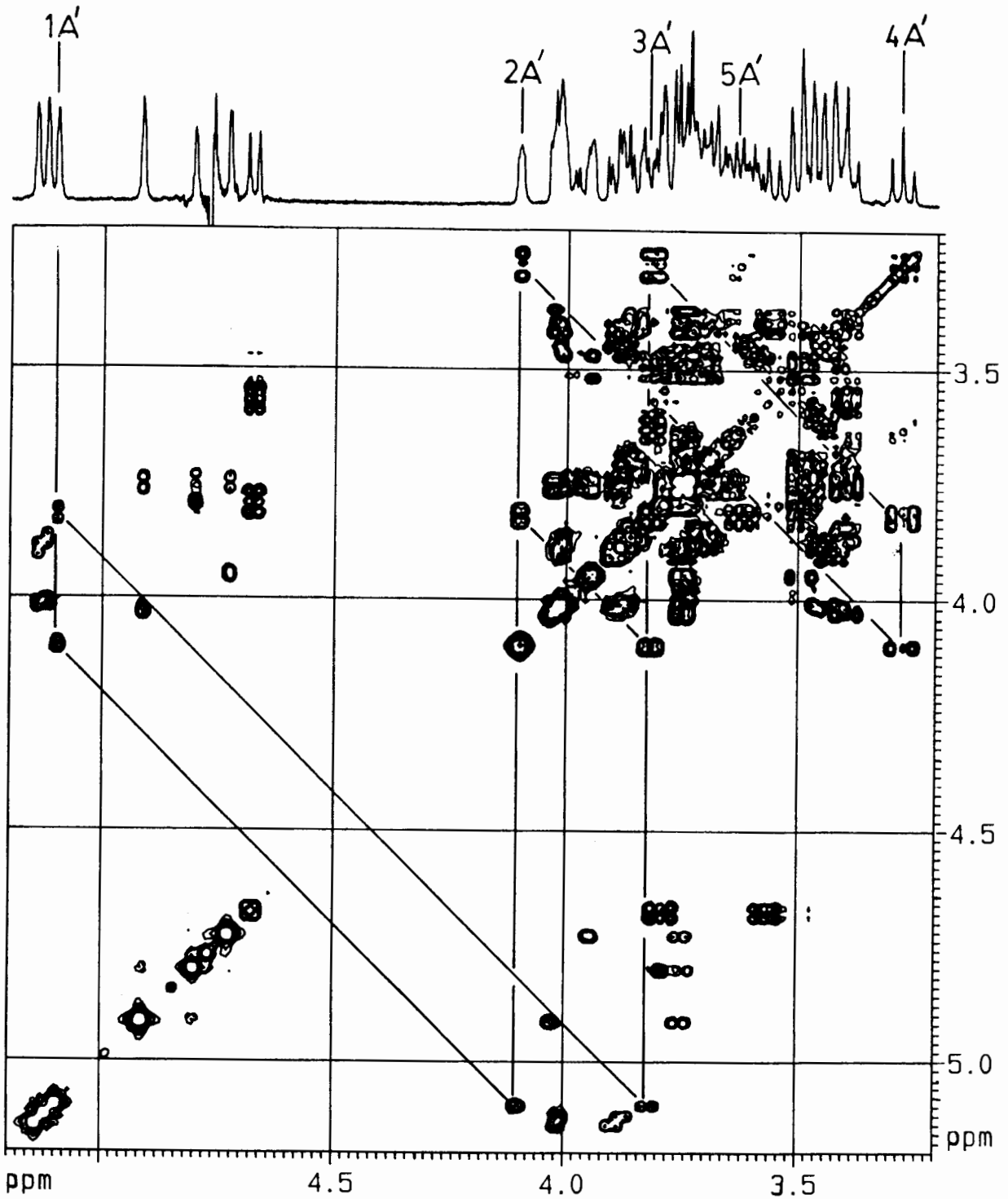


Figure 4-6b: Partial 400 MHz Relayed-COSY spectrum of heptasaccharide (58) . .

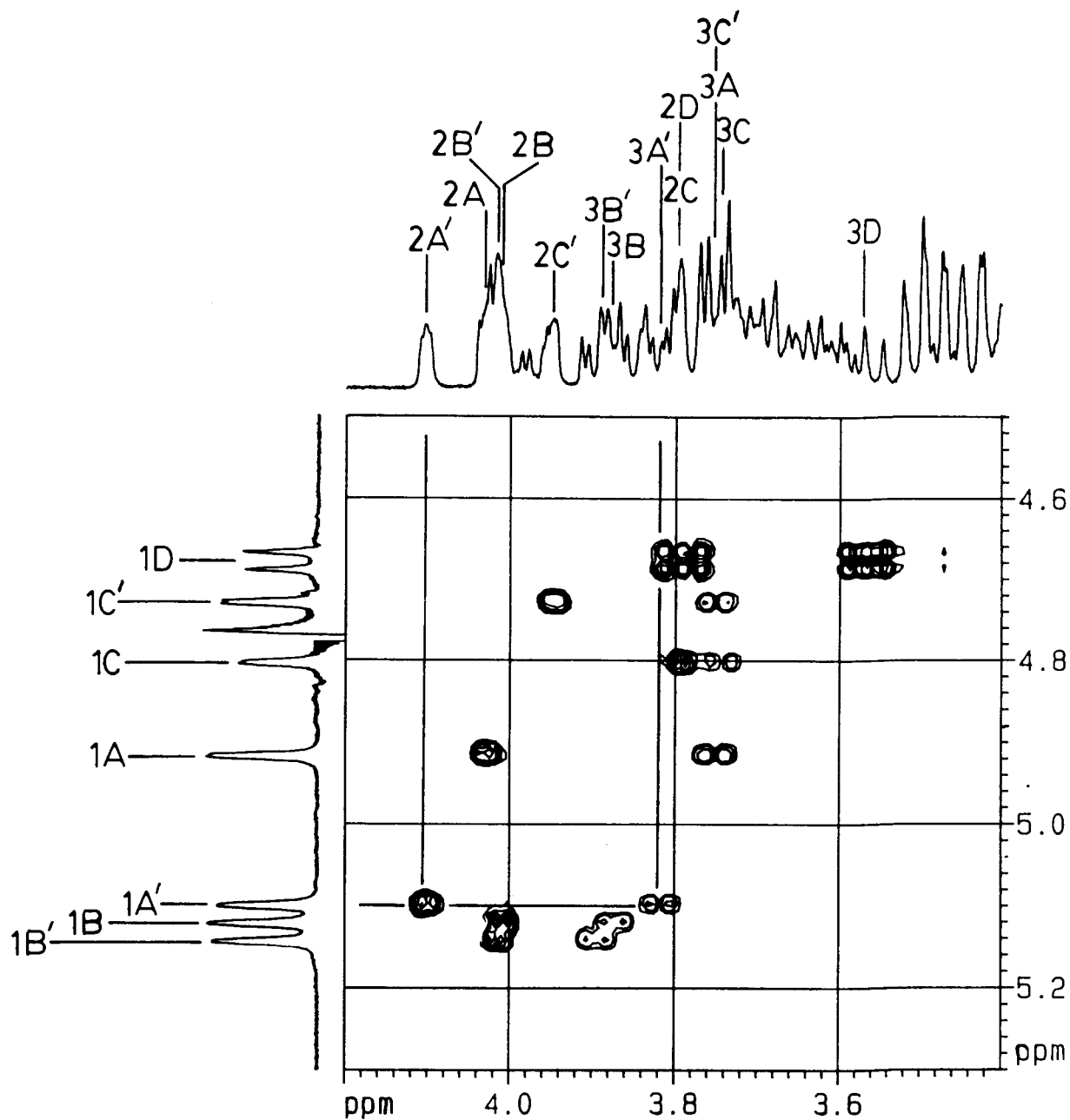


Figure 4-6c: Partial 400 MHz Relayed-COSY spectrum of heptasaccharide (58). Correlation cross-peaks between the anomeric signals and the H-2's and H-3's are shown.

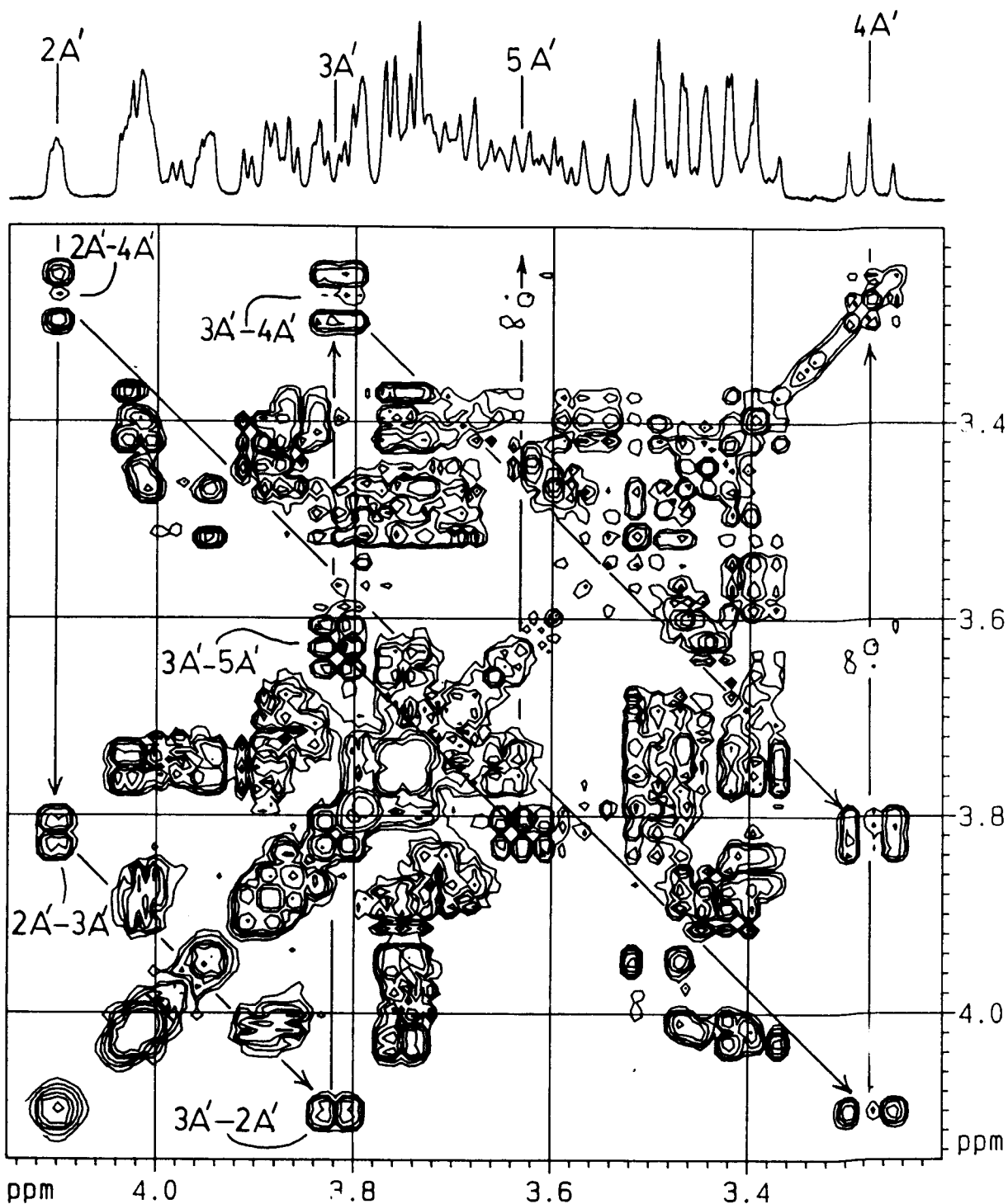


Figure 4-6d: Partial 400 MHz Relayed-COSY spectrum of heptasaccharide (58). Relay cross-peaks between the rhamnosyl H-2's and H-4's can be seen in the upper left corner of the spectrum.

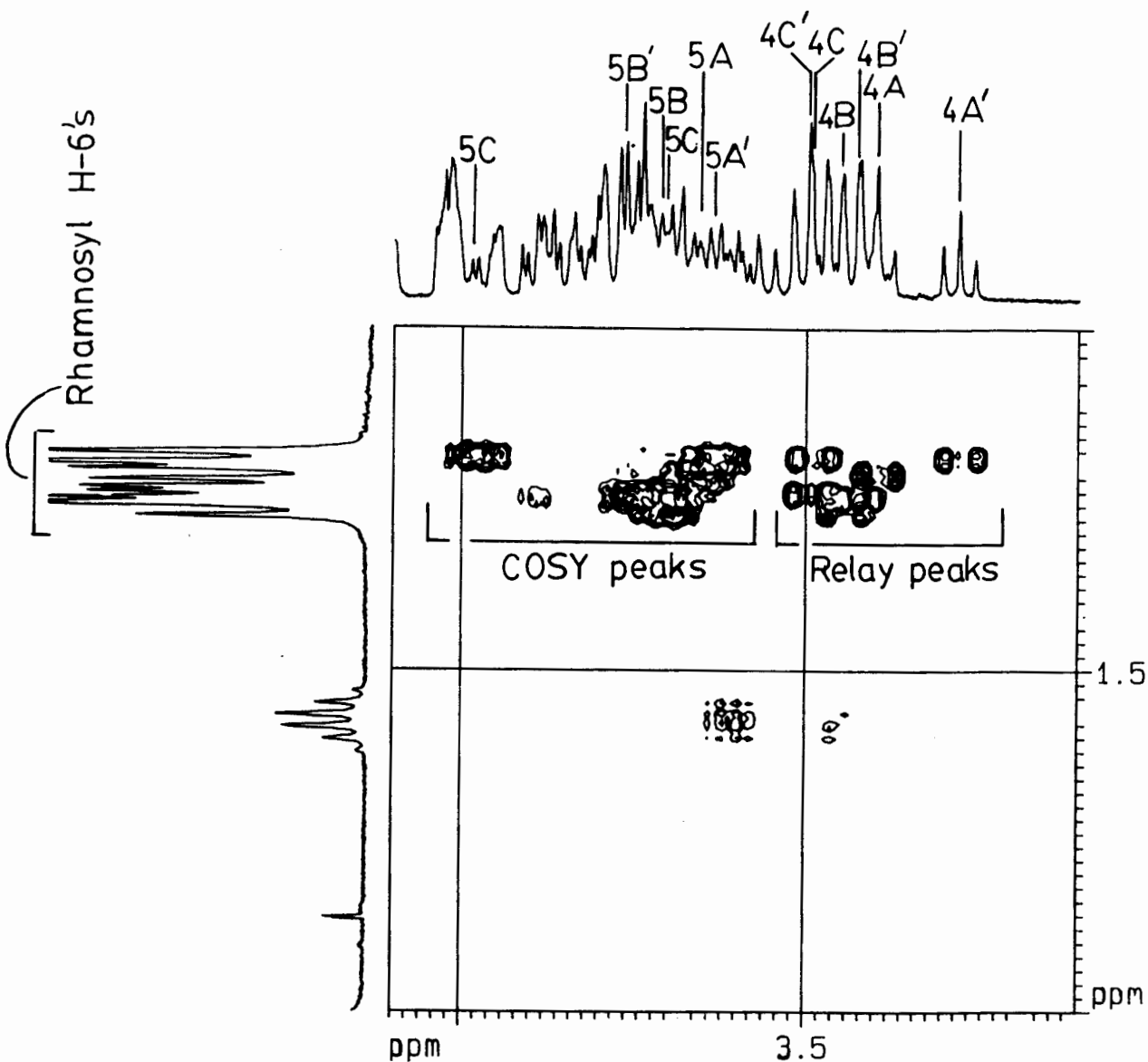


Figure 4-6e: Partial 400 MHz Relayed-COSY spectrum of heptasaccharide (58). Correlation cross-peaks between the rhamnosyl H-6's and the H-5's and H-4's are shown.

An expansion of the region containing the anomeric proton cross-peaks is shown in Figure 4-6c. The correlations between the anomeric signals and the corresponding H-2's and H-3's can be clearly seen in this region. In Figure 4-6d, the relay cross-peaks between the rhamnosyl H-2's and H-4's can be seen in the upper left quadrant of the expanded spectrum. These cross-peaks are important in that they allow for the identification of the corresponding H-4's in spite of the overlap of the three rhamnosyl H-3's between 3.75-3.76 ppm

The expansion shown in Figure 4-6e shows the correlation peaks between the H₃-6 methyl protons, and the rhamnosyl H-5's and H-4's (relay cross-peaks). The chemical shifts of the rhamnosyl H-4's are indicated clearly by the positions of the H₃-6 methyl/H-4 relay cross-peaks. These cross-peaks also show more clearly the chemical shifts of the H₃-6 methyl groups (compare Figures 4-5d and 4-6e). The individual signals of the various spin systems are most easily identified by examination of both the COSY and the relayed COSY spectrum together. In some cases the COSY spectrum will show the coupling patterns more clearly, whereas in other cases the pattern may be more easily seen in the relayed COSY spectrum.

C: TOCSY Spectrum of Heptasaccharide (4)

A TOCSY (Total Correlation Spectroscopy) experiment affords similar information to the relayed-COSY experiment. In a TOCSY experiment the coupling information of each spin can be relayed to all the other spins within the same spin-system, whereas in a single step relayed-COSY experiment, the relay is only to the next non-coupled spin. This is very useful for situations in which there is considerable overlap in the COSY spectrum. Since all the spins of a spin system contain the coupling and

chemical shift information of all the other spins in the same spin system, in principle, all that is required to determine the chemical shift of each ^1H -signal is that at least one of the signals be clearly resolved. The clearly resolved signal will have cross-peaks for all the other spins in the same spin system.

The TOCSY spectrum of heptasaccharide (58) is shown in Figure 4-7a, with expansions shown in Figures 4-7b and 4-7c. The cross-peaks of the anomeric signals of the individual rings (Figure 4-7b) provide clearly resolved signals which can be used to determine the chemical shifts of all the signals within the various spin systems. The cross-peaks of the methyl signals of the rhamnose residues (Figure 4-7c) are also generally well resolved, although not as clearly as the anomeric signals.

The disadvantage of a TOCSY experiment is that if there is no prior knowledge of the coupling constants or relative chemical shifts of the individual signals within the spin system, then assignment of the signals may be difficult, since all spins are coupled to one another. The TOCSY experiment is most useful in these cases following examination of the COSY spectrum, the COSY spectrum permitting the sequential assignment of at least parts of the spin systems. Alternatively, a TOCSY experiment employing short mixing times may be used to relay magnetization to only the next nearest neighbor.

Examination of the COSY, relayed-COSY, and TOCSY spectra made possible the assignment of all the ring protons to a given spin system. The next step was to make the assignment of a given set of signals to a particular sugar ring within the molecule. All the information that is necessary to assign all the signals of a particular spin system to a given ring is the unambiguous assignment of at least one of the signals

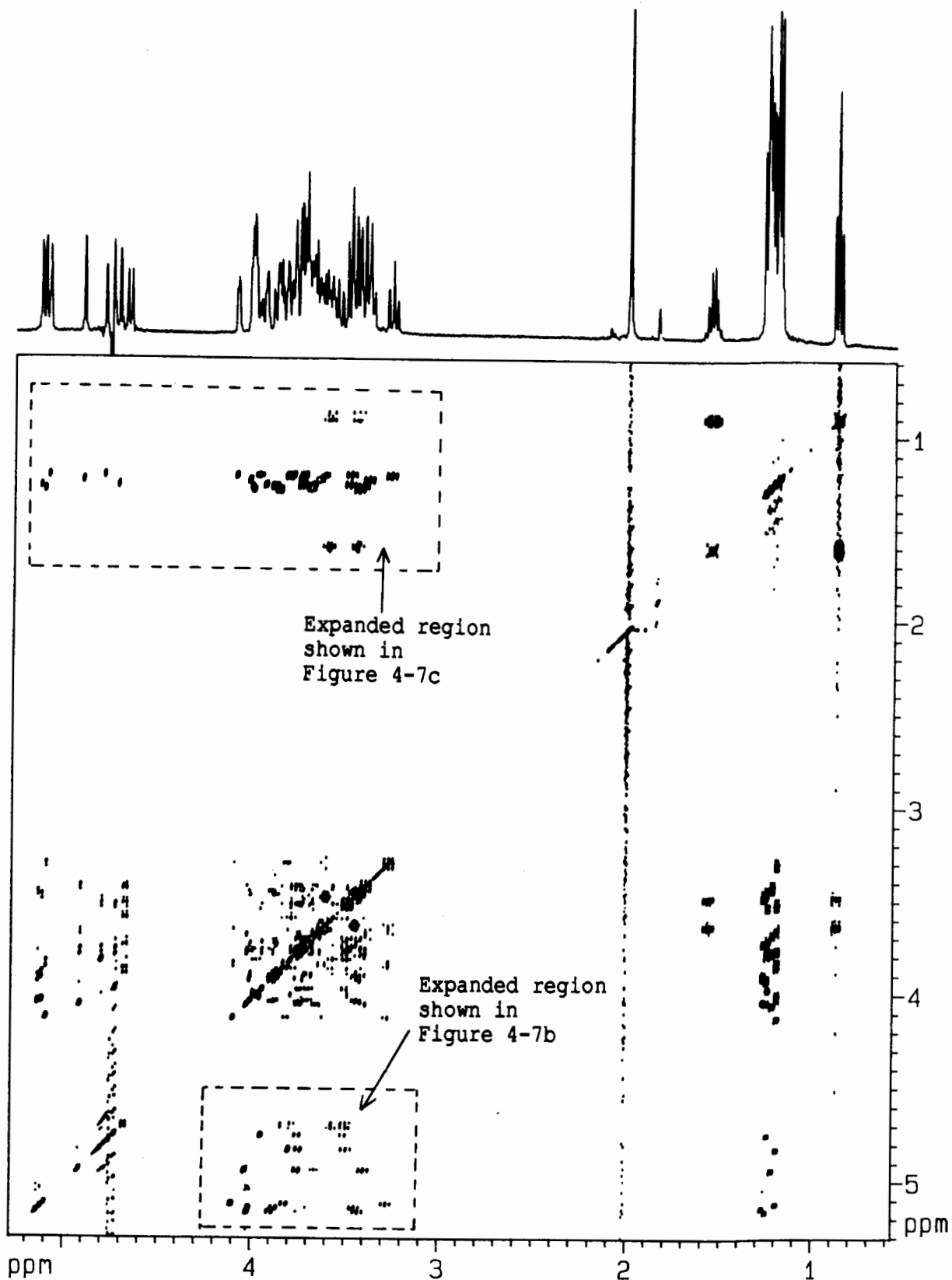


Figure 4-7a: 400 MHz TOCSY spectrum of heptasaccharide (58).

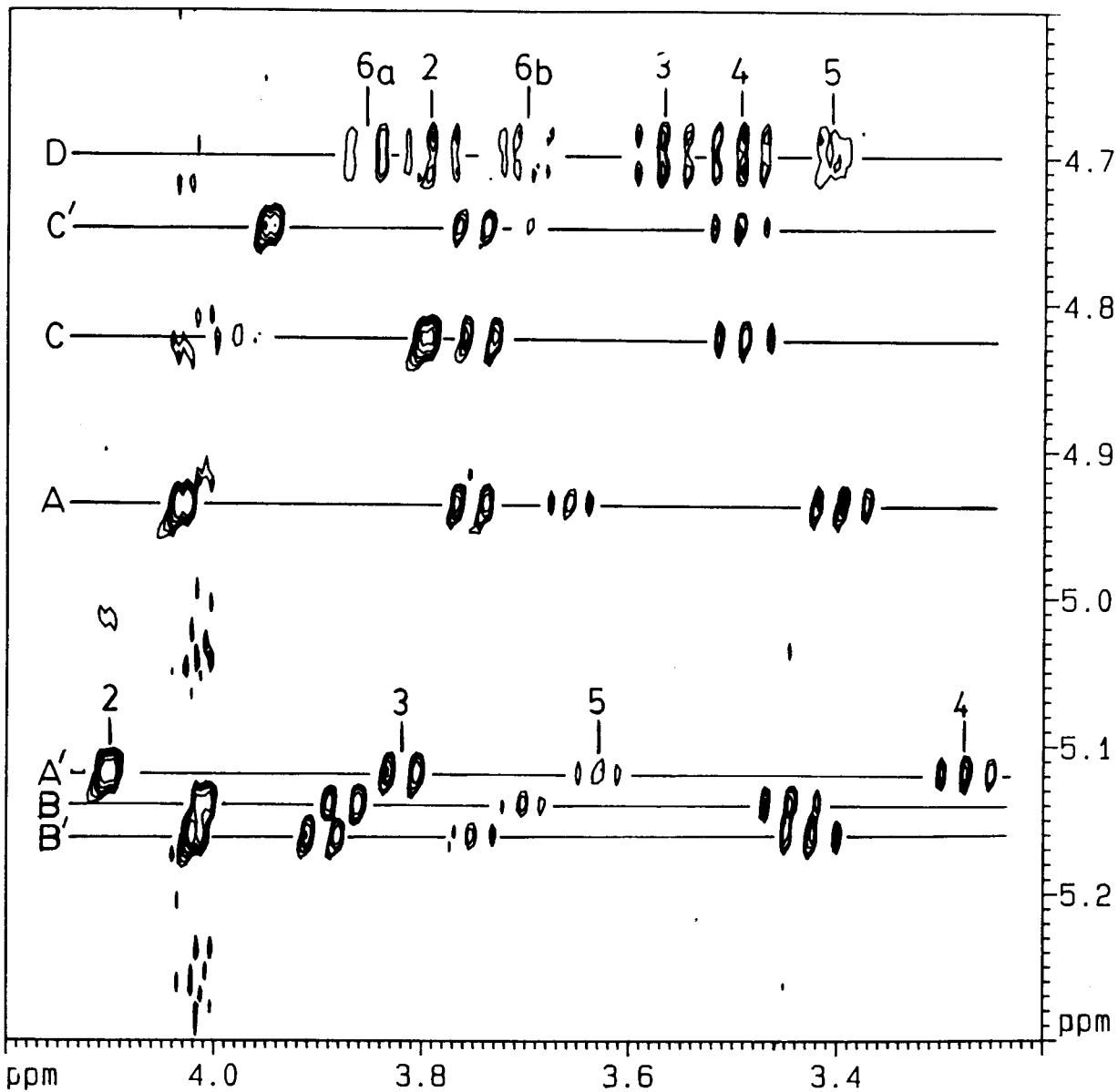
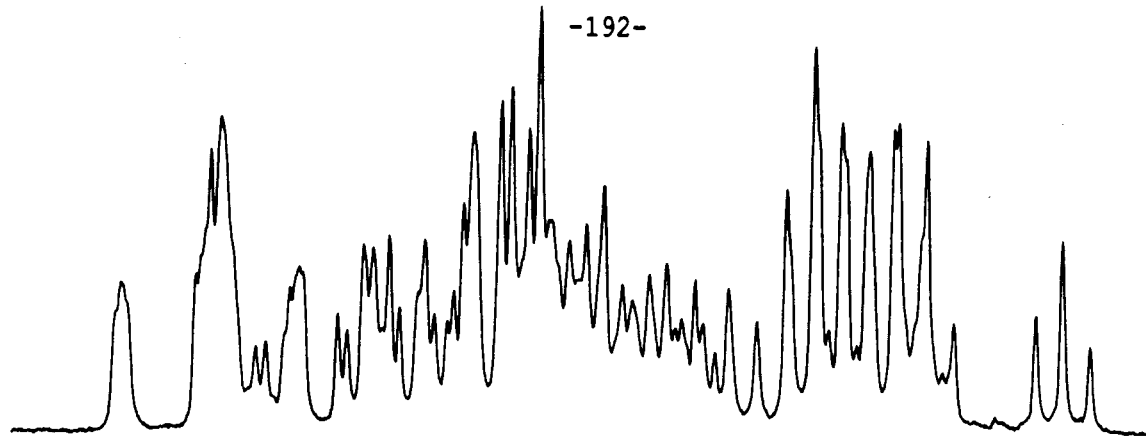


Figure 4-7b: Partial 400 MHz TOCSY spectrum of heptasaccharide (58). Correlation cross-peaks are between the anomeric signals and the remaining ring proton signals.

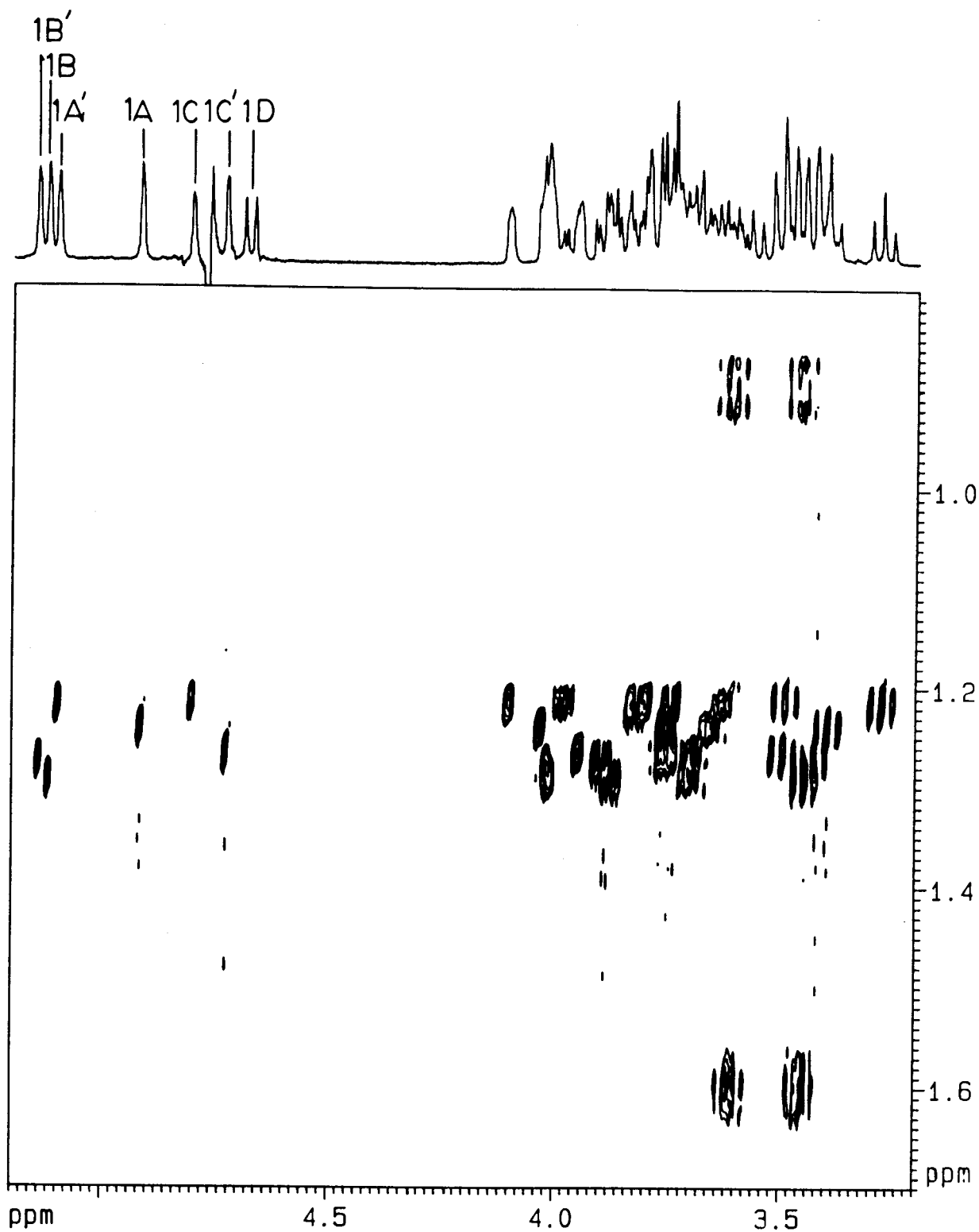


Figure 4-7c: Partial 400 MHz TOCSY spectrum of heptasaccharide (58). Correlation cross-peaks are between the rhamnosyl H-6's the remaining ring proton signals.

within the group to particular ring; this was possible for at least two of the spin systems. The signals due to the *N*-acetylglucosamine ring were easily distinguished from the sets of rhamnosyl signals by their characteristic coupling constants; the H-1 of the *N*-acetylglucosamine ring has a $J_{1,2}=8.6$ Hz, compared with a $J_{1,2}=1.8$ Hz for the rhamnosyl H-1's. Assignment of the various sets of rhamnosyl signals to particular rings was not as straightforward.

The spin system containing the rhamnosyl H-5 at 3.99 ppm was assigned to the C-ring. This assignment was based on a specific deshielding observed for the H-5 of a rhamnose ring which is α -(1-3) linked to a β -D-*N*-acetylglucosamine residue.^{110a} A tentative assignment of the most upfield rhamnosyl H-1 to the C'-ring was made. This assignment was based on the common observation that the anomeric proton is deshielded when the aglycone is another sugar residue. The C'-ring has an aglycone that is not another sugar residue but rather a propyl group, and thus, the glycosylation deshielding would not be expected.

D: ROESY Spectrum of Heptasaccharide (4)

Discrimination between the sets of signals due to the A-, A'-, B- and the B'-rings could not be made unambiguously, based on chemical shift arguments alone. Assignment of the various spin systems to these rings was made by examination of the ROESY spectrum (see Figure 4-8). The ROESY spectrum is a form of 2-dimensional NOE spectroscopy, and as such, correlates spins which share a dipolar coupling relationship. It is commonly observed that anomeric protons show significant dipolar coupling to the ring protons across the glycosidic linkage.¹¹⁰ This interaction may be used to determine the arrangement of the sugar residues relative

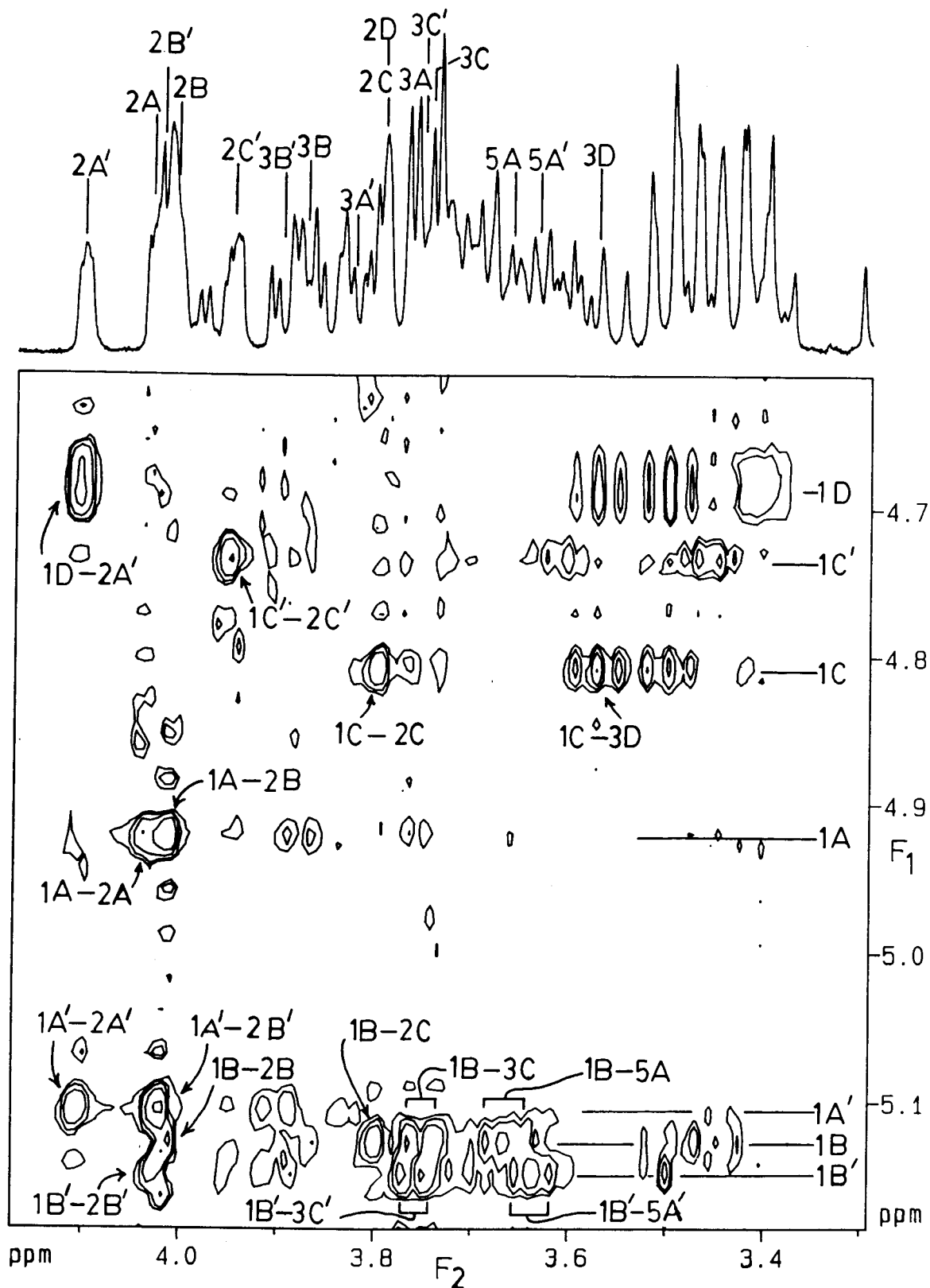


Figure 4-8: Partial 400 MHz ROESY spectrum of heptasaccharide (58). The chemical shifts of the corresponding anomeric proton signals are indicated along the F₁-axis.

to one another.

In conventional one-dimensional nOe spectroscopy, selective saturation of an anomeric proton signal results in an nOe in neighboring proton signals. The interglycosidic proton due to its proximity in space will show an nOe, thereby identifying the adjacent ring. The ROESY spectrum is a two-dimensional version of this one-dimensional experiment. The analysis consists of identifying the anomeric proton signals and the associated cross-peaks. The cross-peaks indicate protons close in space. For example, the H-1 signal of the D-ring (4.69 ppm) showed, in addition to intra-ring cross-peaks, a strong cross-peak with a rhamnosyl H-2 signal (4.12 p.p.m). This suggested the assignment of this signal to the rhamnosyl H-2 of the A'-ring. By referring back to the spin system containing this H-2, the H-1 signal at 5.11 ppm, and the remaining signals in the spin system of the A' ring could then assigned. The H-1_A, showed a cross-peak to another rhamnosyl H-2 signal at 4.03 ppm Based on this observation, this signal, and the other signals within the same spin system, were then assigned to the B'-ring. In a similar fashion, the previous assignment of the rhamnosyl H-1 signal at 4.74 ppm to the C'-ring (based on the expected relative shielding of the anomeric signal at the reducing terminus) was confirmed by the cross-peak between H-1_B, and the rhamnosyl H-3 at 3.76 ppm; this H-3 is part of the same spin system which contains the H-1 signal (4.74 ppm). The assignment of the H-1 signal at 4.82 ppm to the C-ring was similarly confirmed by the cross-peak between this signal and the H-3 signal of the N-acetylglucosamine ring. All but two sets of signals were assigned at this point. Working backwards from the C-ring, the cross-peak between the H-3_C at 3.75 ppm and the anomeric proton signal at 5.13 ppm, identified the set of signals

due to the B-ring. The remaining set of signals was assigned to the A-ring; this assignment was once again confirmed by the cross-peak between the H-1_A and the H-2_B. All of the expected intra-ring cross-peaks were observed; for example, between H-1 and H-2 of the rhamnosyl rings, and between H₃-6 and H-4 of the rhamnosyl rings.

By careful analysis of the COSY, relayed COSY, TOCSY and ROESY spectra, all of the ring proton signals were assigned in an unambiguous fashion to a specific proton within the molecule. Of all the sets of signals, those due to the B- and the B'-rings most closely overlap. This is not surprising since they are both in very similar chemical environments, both being bounded by an A- and a B-ring. The chemical environments of the pairs of A- and C-rings differ more than for the pair of B-rings, and accordingly, it is seen that these sets of signals do not overlap as closely.

E: ¹³C-¹H Correlation Spectrum of Heptasaccharide (4)

Complete assignment of the ¹H-NMR spectrum allowed for the straightforward assignment of all the ¹³C signals following analysis of the ¹³C-¹H NMR chemical shift correlation spectrum. The acquisition of the spectrum was carried out in the inverse mode,⁸⁰ meaning that ¹H-spectra rather than ¹³C-spectra were acquired for the two-dimensional data set, thus taking advantage of the greater sensitivity of the ¹H nucleus. The ¹³C-chemical shift correlations were obtained by Fourier transformation of the two-dimensional data set in the F₁-direction. The experiment was carried out without ¹³C-decoupling during acquisition, meaning that the one-bond ¹³C-¹H coupling constants (¹J_{13C-1H}) could be measured in the F₂-direction of the Fourier transformed spectrum

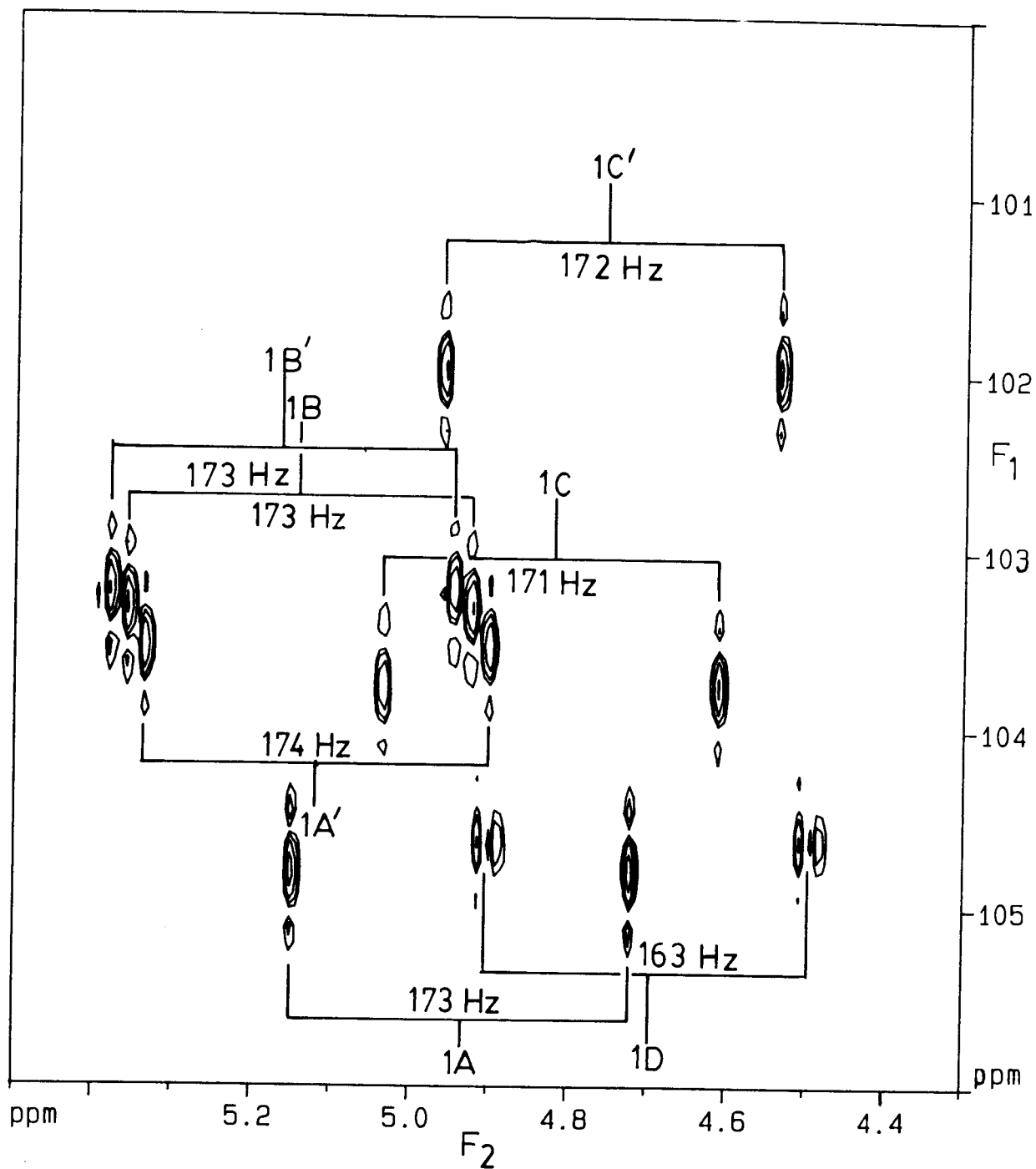


Figure 4-9: Inverse ^{13}C - ^1H chemical shift correlation spectrum of heptasaccharide (58).

(see Figure 4-9). These values for the anomeric signals confirmed the configurations about the anomeric centres. Values of 171-174 Hz for the rhamnosyl C-1's are consistent with an α -L-configuration, whereas the 163 Hz observed for the N-acetylglucosamine unit is consistent with a β -D-linked residue.⁸¹ It was attempted to measure these one-bond coupling constants ($^1J_{13C-1H}$), by use of the traditional method of acquiring a 1H -coupled ^{13}C -NMR spectrum. Since there was only a small amount of sample available it required approximately 16 hours to acquire a suitable spectrum. Due to the overlap of signals in the ^{13}C -spectrum, extraction of the coupling constants was not possible for all the anomeric signals. These coupling constants were easily extracted from the inverse detected ^{13}C -coupled ^{13}C - 1H correlation spectrum (see Figure 4-9); the time required to record the spectrum was approximately 8 hours.

F: Solution Conformation of Heptasaccharide (4)

The ROESY spectrum, apart from providing the information required to assign the sets of proton signals to specific sugar residues within the structure, also provides information on the solution conformation of the molecule. The individual sugar units within an oligosaccharide structure remain fixed in their chair conformations; the conformation of an oligosaccharide is therefore determined by the rotations about the phi (ϕ) and psi (ψ) angles of the glycosidic linkages, and further possible rotations about the (ω) angle in 1-6 linked structures (see Figure 4-10). As mentioned earlier, significant nOe effects are observed between the anomeric proton signals and the proton on the aglycone across the glycosidic linkage. A single nOe contact is insufficient to determine the conformation of the glycosidic linkage; however, two or more nOe contacts between two adjacent rings will fix the conformation about the

glycosidic linkage if the nOe contacts can be related to distances. These sorts of interactions were observed in the ROESY spectrum, and although exact distances between protons were not determined, a semi-quantitative description of the conformation of the heptasaccharide was possible.

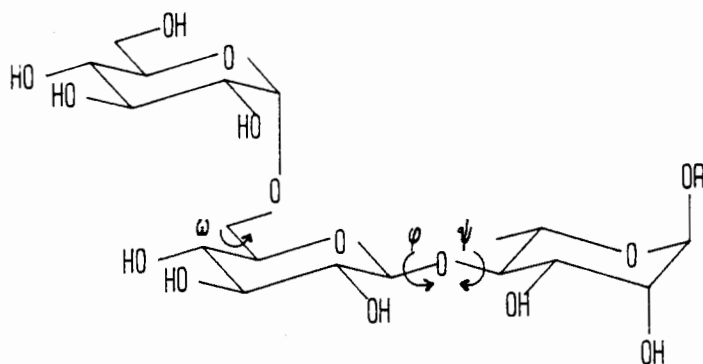


Figure 4-10: Possible rotations about the glycosidic linkages in an oligosaccharide.

Bock et al have carried out conformational studies on disaccharide through tetrasaccharide segments of the *Shigella flexneri* variant Y polysaccharide, as well as the polysaccharide structure itself.^{110a} They measured several NMR parameters including ^{13}C -chemical shifts, ^{13}C - ^1H one- and three-bond coupling constants, ^1H -chemical shifts, ^1H -coupling constants, as well as ^1H - ^1H T_1 and nOe measurements. The conformation of the tetrasaccharide repeating unit was also calculated by use of hard-sphere *exo*-anomeric (HSEA) calculations.¹¹⁷ Distances determined from the calculated structure were used to calculate specific nOe and T_1 interactions. These calculated values were compared to the experimentally determined values for the same interactions. Various H••O

interactions which were seen in the calculated structure, were used to rationalize various specific deshieldings and nOe results observed in the $^1\text{H-NMR}$ spectra. The NMR parameters compared favorably with interactions predicted by the calculated structure. The HSEA calculations predicted values of approximately $45\text{-}50^\circ$ for the phi (ϕ) angles, and $10\text{-}15^\circ$ for the psi (ψ). This structure was in good agreement with the various interproton distances as determined from nOe and T_1 measurements. The calculated structure also correctly predicted several of the specific

Table 4-I: Observed Cross-Peaks in the ROESY spectrum of (58)

<u>Glycosidic Linkages</u>					
A-B	B-C	C-D	D-A'	A'-B'	B'-C'
1A-2A	1B-2B	1C-2C	1D-3D	1A'-2A'	1B'-2B'
2A-3A	2B-3B	2C-3C	1D-5D	2A'-3A'	2B'-3B'
4A-6A	4B-6B	4C-6C	1D-2A'	4A'-6A'	4B'-6B'
5A-6A	5B-6B	5C-6C		5A'-6A'	5B'-6B'
1A-2B	1B-3C	1C-3D		1A'-2B'	1B'-3C'
5A-1B	1B-2C			5A'-1B'	

Cross-peaks in italics represent inter-ring contacts.

deshieldings, seen in the $^1\text{H-NMR}$ spectra, which would result from the proximity (less than 2.7 \AA) of hydroxyl groups to particular protons.

The ROESY spectrum of heptasaccharide (58) indicated several through-space proton interactions. The through space interactions between the various proton signals, as indicated by the cross-peaks in the ROESY spectrum, are given in Table 4-III. As described above, the contact between the anomeric proton and the proton on the aglycone across the glycosidic linkage, served to establish the sequence of sugar residues. In addition to these interglycosidic contacts, the anomeric protons showed contacts with other protons on the adjacent rings. For these residues, a particular conformation about the glycosidic linkages could be inferred. A model of the heptasaccharide structure was then constructed in which the conformations about the glycosidic linkages were consistent with the observed inter-proton contacts. This model is shown in Figure 4-11, with double-headed arrows indicating the various inter-ring contacts observed in the ROESY spectrum.

The inter-residue cross-peaks are described below. The H-1_A signal showed an ROE cross peak to the H-2_B signal, but no other inter-ring contacts are observed. This was insufficient to fix the conformation about the A-B linkage; however, an additional contact observed between H-1_B and H-5_A suggested a particular conformation about this linkage. A phi (ϕ) angle of 50-60°, with a psi (ψ) angle of anywhere from -30° to 30°, would bring H-1_B into close contact with H-5_A. The inter-residue contacts seen in the ROESY spectrum are the same NOE contacts seen previously by Bock et al in smaller oligosaccharide sequences.^{110a} It is likely that the conformation about this linkage is that predicted by the HSEA calculations carried out previously by Bock. From the ROESY spectrum it appears that the contacts which are seen between the A-ring and the B-ring are also seen between the A'-ring and the B'-ring,

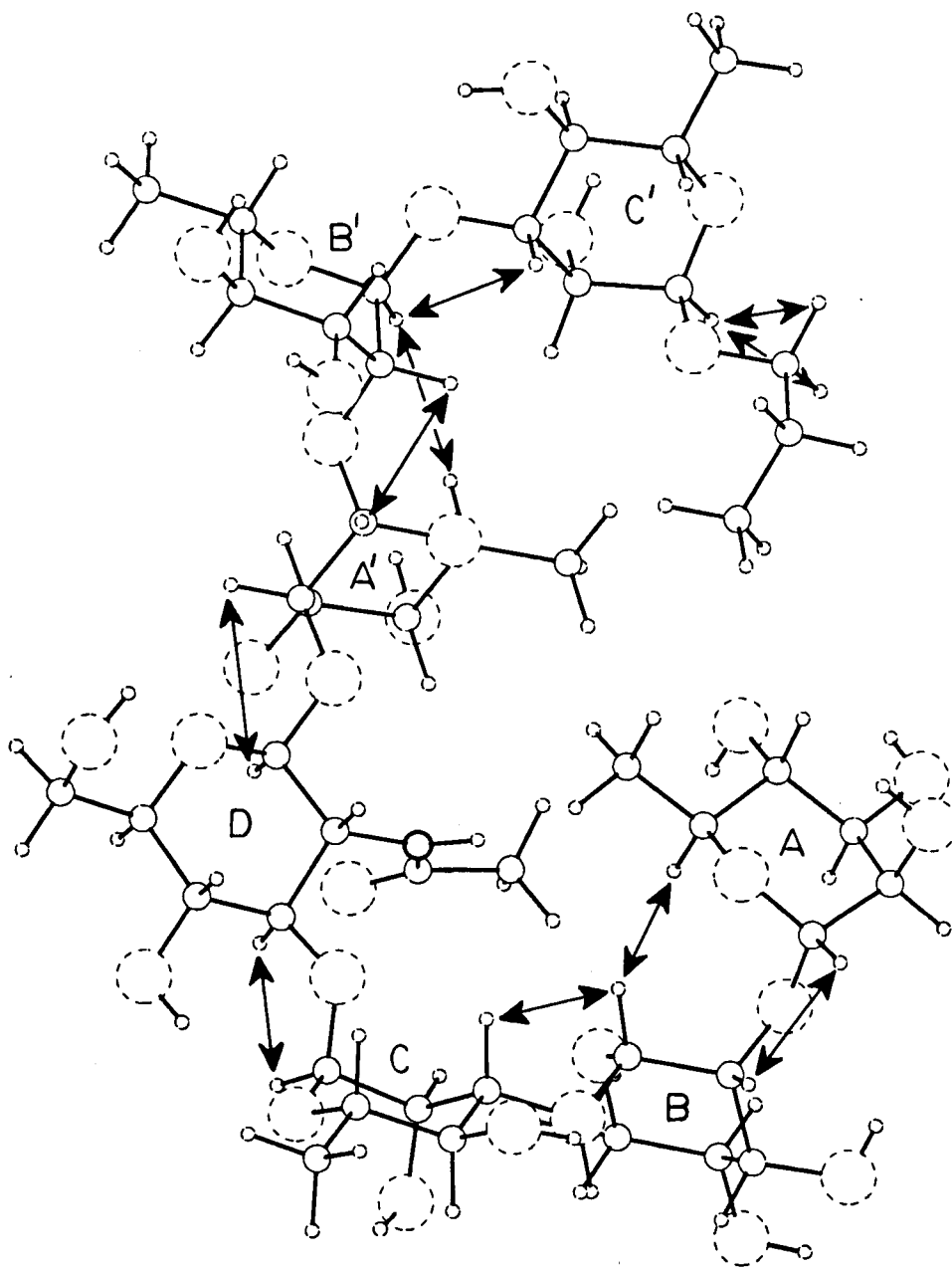


Figure 4-11: Solution conformation of heptasaccharide (58) inferred from the inter-proton contacts observed in the ROESY spectrum. Double-headed arrows indicate the observed inter-ring contacts.

suggesting a similar conformation about these linkages.

The H-1_B showed an interglycosidic contact, H-1_B/H-3_C, as well as a contact with H-2_C; this suggests that the psi (ψ) angle is such that H-1_B is "straddled" by H-3_C and H-2_C. This particular conformation would require negative phi (ϕ) and psi (ψ) angles ($\phi \approx -60$ and $\psi \approx -45$), in contrast to the angles predicted by Bock et al ($\phi \approx 50^\circ$, $\psi \approx 15^\circ$). It should be noted that only the H-1_B/H-3_C contact was observed by Bock et al.^{110a} The conformation about this particular linkage suggested by Bock predicted a specific deshielding of H-3_C due to a close contact between this proton and the ring oxygen of the B-ring; however, this specific deshielding was not observed in the ¹H-NMR spectrum. In the present model (negative phi and psi angles), no such deshielding is predicted.

In contrast to the above results, the contacts observed between H-1_B and the C'-ring are somewhat different from those observed between H-1_B and the C-ring. The analogous cross-peak to the one seen between H-1_B/H-2_C (ie. H-1_B/H-2_{C'}) was not seen, or was very weak. In addition, H-2_{C'} is deshielded, relative to H-2_C by about 0.15 ppm. Both of these effects may be explained by a rotation about the phi (ϕ) angle of the B'-C' linkage from a negative value to a positive value, with small changes in the psi (ψ) angle. This rotation would increase the H-1_B/H-2_{C'} distance, while at the same time decreasing the distance between H-2_{C'} and the ring oxygen of the B'-ring. The increase in the H-1_B/H-2_{C'} distance is consistent with the observed reduction in the ROE cross-peak for this contact, while the decrease in the distance between H-2_{C'} and the ring oxygen of the B-ring is consistent with the specific deshielding of H-2_{C'}. This difference between the B-C and the B'-C' linkages is somewhat unexpected, since both of the B-C linkages are in

seemingly similar chemical environments. It must, however, be noted the C'-ring is not bounded by a D-ring, as is the case in the B-C linkage. This is a very interesting point and brings up the question as to which of the two linkages is representative of the B-C linkage in the polymeric structure.

The contacts observed in the ROESY spectrum between the C-ring and the D-ring are consistent with those observed previously by Bock.^{110a} Only one ROE cross-peak was seen between these rings. In addition to this cross-peak, there was a specific deshielding of H-5_C of about 0.25 ppm relative to H-5 in the A/A' and B/B' rings. This same deshielding has been observed previously in the ¹H-NMR spectra of smaller oligosaccharide fragments, and has been attributed to a close contact between H-5_C and the hydroxyl group at the four position of the D-ring.^{110a} A close contact between H-1_C and H-3_D, as well as a specific deshielding of H-5_C, dictates a specific conformation about the C-D linkage. The conformation about this linkage, previously calculated by Bock using HSEA calculations, predicts a phi (Ø) angle of 50°, and a psi (ψ) angle of 10°. The results of the present study are consistent with this conformation about the C-D linkage.

There was also only one contact observed between the D-ring and the A'-ring, namely between H-1_D and H-2_{A'}. The deshielding of H-1_D by about 0.1 ppm when linked to an α-L-Rhap unit, has been attributed to a close approach of the hydroxyl at the 2-position of the A'-ring.^{110a} The ROE cross-peak, together with the small deshielding of H-1_D, require the C-D linkage to have positive values for both the phi (Ø) and the psi (ψ) angles. This conformation is consistent with the conformation previously calculated by Bock, using HSEA calculations (phi=45°, psi=15°).

A rigorous molecular mechanics calculation of the minimum energy conformation of the heptasaccharide (58), falls beyond the scope of this thesis. These types of calculations form part of an ongoing study of the conformational analysis of complex carbohydrate structures, currently being carried out in our laboratory.¹¹¹ Of particular interest will be the result obtained from calculation on the conformation of the B-C type linkages; specifically whether the experimental finding that the B-C and the B'-C' linkages take up different conformations can be successfully matched through calculation. The assignment of the complete ¹H-NMR spectrum, together with the contacts obtained from the ROESY spectrum will form the starting point for the more detailed analysis of the solution conformation of the heptasaccharide (58). In particular, the experimentally observed contacts can be used as constraints in the modelling studies to select an initial local-minimum energy structure.

III: EXPERIMENTAL

The NMR spectra were recorded on a Bruker AMX-400 spectrometer in deuterium oxide, with chemical shifts given in ppm downfield from 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). Before the ROESY spectrum was recorded, the sample was degassed by subjecting it to a freeze-thaw cycle, repeating the cycle 6 times and sealing the sample *in vacuo*. All spectra were acquired by use of quadrature detection in both directions.

The COSY spectrum was acquired by use of the pulse sequence $d1-90^{\circ}-d0-45^{\circ}-FID$, incorporating a solvent presaturation pulse of 2s during the relaxation delay ($d1$). 512 experiments of 8 scans each gave an initial data set of 512×2048 data points which was recorded over a sweep width of 3030Hz in both F_1 and F_2 . The initial data set was zero-filled once in the F_1 direction to give a final data set of 1024×1024 real data points. The same data set size and sweep widths were used for the relayed-COSY and the ROESY spectra. A non-shifted sine bell function was applied prior to Fourier transformation. The magnitude spectrum was symmetrized about the diagonal before analysis.

The relayed-COSY spectrum was recorded by use of the pulse sequence $d1-90^{\circ}-d0-d2-180^{\circ}-d2-90^{\circ}-FID$. A fixed delay time ($d2$) of 28msec was used with a solvent presaturation pulse of 2s during $d1$. 512 experiments of 16 scans each were recorded. A non-shifted sine bell function was applied to the data set prior to Fourier transformation. The magnitude spectrum was calculated and symmetrized about the diagonal before analysis.

The TOCSY spectrum was recorded by use of the pulse sequence $d1-90^{\circ}-d0-[MLEV \text{ spinlock}]-FID$, with a solvent presaturation pulse of 2s during $d1$. The power level used for the spinlock gave a $25 \mu s$ 90° pulse. The spinlock (MLEV-17) was applied for a period of 250ms. 512

experiments of 24 scans each were acquired to give an initial data set of 512x2048 data points. The spectrum was recorded over a sweep width of 1886Hz in both F_1 and F_2 . The initial data set was zero-filled once in the F_1 -direction to give a final data set of 1024x1024 real data points. A \cos^2 function was applied to the phase sensitive data set prior to Fourier transformation; the spectrum was then phase corrected and a baseline correction routine applied prior to analysis, by use of Bruker UXNMR software.

The ROESY spectrum was acquired by use of the pulse sequence d1-90-d0-[spin lock]-FID, with a presaturation pulse of 2s during the relaxation delay d1. The CW spin-lock was applied for 250ms at 0.5 watts of power at the frequency of the HOD peak. 512 experiments of 24 scans each were recorded by use of phase sensitive detection. The data set was phase corrected, and a baseline correction applied prior to analysis by use of the standard Bruker UXNMR software routines.

The ^{13}C - ^1H correlation spectrum was recorded in the inverse mode, by use of a 4-pulse sequence incorporating a BIRD pulse in the preparation period, by use of phase sensitive detection. The data set of 512x2048 data points was recorded over a sweep width of 3030Hz in the F_2 direction and 9615Hz in the F_1 direction which, following zero-filling once in the F_1 -direction, gave a final data set of 1024x1024 real data points with digital resolutions of 2.9Hz/Pt and 9.4Hz/Pt in F_2 and F_1 , respectively. The data set was phase corrected, and a baseline correction applied prior to analysis by use of the standard Bruker UXNMR software routines.

Tables 4-I and 4-II contain the ^1H -NMR and ^{13}C -NMR data, respectively for heptasaccharide (58). The assignments follow from the analysis of the spectra described above.

Table 4-II. ^1H NMR data^a for the ring protons in the heptasaccharide (58).

(Ring)	H-1	H-2	H-3	H-4	H-5	H ₃ -6
C' ^b	4.74 (1.8)	3.96	3.76	3.50	3.71	1.257 (6.2)
B'	5.15 (1.8)	4.03	3.91	3.43	3.77	1.263 (6.2)
A'	5.11 (1.8)	4.12 (4.8) ^c	3.83	3.28 (19.5) ^c	3.64	1.20 (6.2)
D	4.69 (8.6)	3.81	3.58	3.50	3.40	3.71 3.86
C	4.82 (1.8)	3.81	3.75	3.50	3.99	1.20 (6.2)
B	5.13 (1.8)	4.02	3.88	3.45	3.72	1.29 (6.2)
A	4.93 (1.8)	4.05	3.76	3.40	3.67	1.23 (6.2)

^aChemical shifts (+/- 0.01ppm) in D₂O. The numbers in brackets denote coupling constants in Hz (+/- 0.1Hz).

^bIndicates the ring to which the aglycone is attached.

^cThese values are the sums of the individual coupling constants, $J_{\text{AX}} + J_{\text{BX}}$.

Table 4-III. ^{13}C NMR data^a for the ring carbons in the heptasaccharide (58).

(Ring)	C-1	C-2	C-3	C-4	C-5	C-6
C' ^b	102.2 (172)	71.7	80.2	74.5	71.0	19.3
B'	103.4 (173)	80.9	72.6	74.8	71.3	19.3
A'	103.7 (174)	81.4	72.3	75.0	71.8	19.2
D	104.8 (163)	58.4	84.2	71.7	78.6	60.1
C	103.9 (171)	72.7	79.9	74.3	71.8	19.1
B	103.5 (173)	80.7	72.6	74.8	71.3	19.3
A	105.0 (173)	72.7	73.3	74.7	71.8	19.5

^aChemical shifts (+/- 0.1ppm) in D_2O . The numbers in brackets denote coupling constants in Hz (+/- 2.9Hz).

^bIndicates the ring to which the aglycone is attached.

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