SCHOOL OF POSTGRADUATE STUDIES
UNIVERSITY OF LAGOS

CERTIFICATION

THIS IS TO CERTIFY THAT THE THESIS -

STRUCTURAL STUDIES OF SOME BACTERIAL POLYSACCHARIDES AND SYNTHESIZED MODEL TRISACCHARIDES

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STRUCTURAL STUDIES OF SOME BACTERIAL POLYSACCHARIDES AND SYNTHESIZED MODEL TRISACCHARIDES.

BY

ADENRELE ADENIKE ADEYEYE (MISS)

December 1988

STRUCTURAL STUDIES OF SOME BACTERIAL POLYSACCHARIDES AND SYNTHESIZED MODEL TRISACCHARIDES.

A Thesis submitted in partial fulfilment of the award of
the Doctor of Philosophy Degree in Chemistry at the Department of
Chemistry, University of Lagos,
Akoka, Yaba, Lagos.

By

Adenrele Adenike Adeyeye (Miss)

December 1988

Dedication

This work is dedicated

To

The Glory of God,

My Ever Loving Parents,

and My Sons 'Tayo and 'Gbenga.

School of Postgraduate Studies.

University of Lagos.

CERTIFICATION

This is to certify that the Thesis titled "STRUCTURAL STUDIES OF SOME BACTERIAL POLYSACCHARIDES AND SYNTHESIZED MODEL TRISACCHARIDES" submitted to the School of Postgraduate Studies, University of Lagos for the award of the degree of Doctor of Philosophy (Ph.D) in Chemistry is a record of original research carried out by Miss A.A. Adeyeye in the Department of Chemistry.

Author

Supervisor

Internal Examiner

Internal Examiner

External Examiner

DECLARATION

"STRUCTURAL STUDIES OF SOME BACTERIAL POLYSACCHARIDES AND SYNTHESIZED MODEL TRISACCHARIDES"

I declare that the above named thesis is my own the work, done by me and it has not been submitted in any other form in a previous application for a higher degree. Information derived from the published or unpublished work of others has been acknowledged in the text.

Candidate

Supervisor.

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I wish to express my profound gratitude to my supervisor Dr.(Mrs) M.Ogunlesi for introducing me to and training me in the field of Carbohydrate Chemistry, and for her unflinching academic and moral support as well as making it possible to make this dream come true. Thank you and God Bless Ma.

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I also wish to thank all the members of my family and my friends who have given me their kindness, moral and financial support throughout the "Acada" period.

ABSTRACT

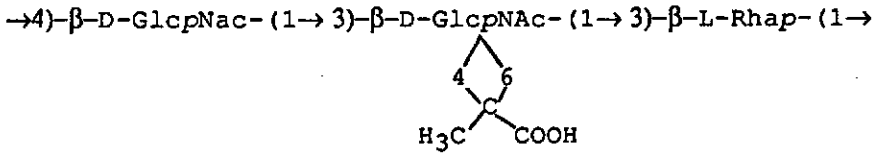
This thesis reports the structures of five bacterial antigens as well as the syntheses of four model oligosaccharides. The bacterial antigens investigated are the lipopolysaccharides (LPS) isolated from *Brucella abortus* strain 7 and strain Mustapha, *Campylobacter coli* Labet 227, *Escherichia coli* O149 and the capsular polysaccharide from *Streptococcus pneumonia* type 37.

The techniques of investigation were sugar analysis, methylation analysis, specific degradation studies and n.m.r.spectroscopy.

The polysaccharide from *Brucella abortus* (*B.abortus*) was found to be homopolymers of 1,2-linked 4-formamido-4,6-dideoxy-α-D-mannopyranosyl units.

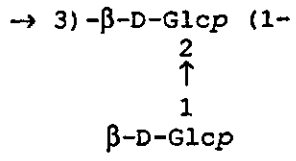
Campylobacter coli Labet 227 was found to synthesize only the core. Two oligosaccharides labelled OS (I) and (OS II) were isolated. OS(I) was found to contain glucose, galactose, 2-acetamido-2-deoxyglucose, 2-acetamido-2-deoxy-galactose and heptose, while OS(II) was found to contain glucose, 2-acetamido-2-deoxy-galactose and very small quantities of heptose.

The O-antigen polysaccharide from *Escherichia coli* O149 was composed of trisaccharide repeating-units having the structure **A.1**.



A.1.

The capsular polysaccharide elaborated by *Streptococcus pneumonia* type 37 was found to consist of disaccharide repeating units having the structure **A.2**.



A.2.

Four model trisaccharides were synthesized for structural and conformational studies and are listed (a) to (d).

a. α-D-Glucose-(1→2)-α-D-Glucose-(1→3)-α-D-Glucose^{1}→OMe

b. α-D-Glucose-(1→2)-β-D-Glucose-(1→3)-α-D-Glucose^{1}→OMe

c. β-D-Glucose-(1→2)-β-D-Glucose-(1→3)-α-D-Glucose^{1}→OMe

d. β-D-Glucose-(1→2)-α-D-Glucose-(1→3)-α-D-Glucose^{1}→OMe

Extensive conformational analysis studies on the trisaccharides are reported using the HSEA calculation techniques. The n.m.r. and conformational studies showed that the changes in the glycosidation shifts from the n.m.r.spectra of disaccharides can be used for those of related trisaccharides with the additivity of the glycosylation shifts of the 1→2 and 1→3 disaccharide units taken into consideration.

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INTRODUCTION

CHAPTER 1.

1.0. THE NEED FOR BACTERIAL POLYSACCHARIDE STUDIES.

The correlation of the chemical structure of a biosynthetic product with its physical and chemical properties as well as its activity and role is a very important study. The first procedure when such a correlation is to be made is structural characterisation and organic chemistry has been applied extensively.

The elucidation of the structure of bacterial polysaccharides is important because it gives a better understanding of the structure/ activity relationship, the cross-reactivity and the classification of bacteria thereby aiding the development of diagnostic tools and methods.

Some bacterial antigens such as the lipopolysaccharides in gram-negative bacteria form part of the cell wall and other antigens form a capsule that surrounds the bacteria. Most of these antigens have immunological properties. In addition, since the immunological characteristics of bacteria are determined to a great extent by the polysaccharides on their surface, the elucidation of the structure of these polysaccharides will offer the chemical explanation for some of their immunogenic properties and immunological reactions.

As a defence against bacteria, human beings and animals develop antibodies which react with antigens on the bacterial surface. Artificial immunity can be induced through vaccination. Early vaccines were based on whole organisms preparation but research in this area later showed that purified cell wall and capsular polysaccharides could be used. Although, the introduction of antibiotics in treating bacterial diseases overshadowed the early promise of polysaccharide vaccines, it has been repeatedly observed that bacterial strains can become resistant to antibiotics. These observations have given impetus to the development of vaccines using

bacterial polysaccharides. The final and long term objective of the structural determination of bacterial polysaccharides is the development of artificial vaccines. Many artificial antigens[1] have been synthesized recently by linking oligosaccharides through a p-aminophenyl arm to an innocuous immunogenic carriers such as protein. These oligosaccharides which have been found to be the repeating units in the cell wall or capsular polysaccharides through the structural studies are produced either by synthesis or through phage enzyme mediated partial hydrolysis. The covalent linkage of the oligosaccharide to the immunogenic carrier molecules has resulted in non-toxic O-antigen specific glycoconjugates. When these glycoconjugates are used as vaccines (either for eliciting antibodies for passive protection studies or for eliciting active immunity in mice as the experimental animal model), specific and efficient immunity was achieved[1].

CHEMICAL NOMENCLATURE

The chemical nomenclature used in this thesis conforms to those that have been adopted in contemporary reports in Carbohydrate Chemistry and is in conformity with the IUPAC rules for Carbohydrate nomenclature[1a].

1.1. MICROBIOLOGICAL BACKGROUND TO THE STRUCTURAL STUDIES OF BACTERIAL POLYSACCHARIDES.

1.1.1. Nomenclature and Classification of Bacteria.

A variety of formal classifications of bacteria have been prepared over the years but the three main ones are the Bergey's manual[2], Skerman[3] key based on Bergey classification, and the Cowan[4] keys. The latter is a clear and concise practical manual for the identification of medically important bacteria in the diagnostic microbiological laboratory.

Nomenclature

The Bergey's manual is divided into nineteen main groups on the basis of of structural elucidation of bacterial polysaccharides.

The part describes the nature of the bacteria i.e. whether gram-positive or negative, aerobic or anaerobic, bacillus i.e.rod-like, or cocci i.e.spherical, spiral or involuted forms.

The family name is derived from either the habitat e.g. Enterobacteriaceae for the enteric bacteria or from the shape e.g. Micrococcus or a combination of both e.g. Lactobacillus indicating rod-like organisms from milk. Also the group name is taken into consideration in some instances. However, there are others of uncertain affiliations and they are named rather arbitrarily.

The genus of the bacteria is the group from which the individual bacterial strains take their names. The names are usually derived from those of the men who first isolated these bacteria. Some examples are Salmonella from Salmon, Brucella from Bruce and Klebsiella from Kleb[5].

The actual species are commonly named after the type of diseases they cause or the source from which they were first isolated.e.g. Salmonella typhi causes typhoid fever, Brucella abortus is the causative agent of contagious abortion in cattle while Vibrio cholerae is responsible for causing cholera.

Mutation by some bacterial strains may lead to an incomplete elaboration of the O-specific polysaccharide or its total absence of the O-specific side chain in the LPS. Such bacteria are referred to as the rough forms (R strains) while those that synthesize the complete O-specific polysaccharide are referred to as the smooth strains. Bacteria strains could be classified as rough (R) or smooth (S)[5a].

Generally, bacterial classification is complicated and is frequently changed, as is the naming of bacteria but for simplicity, bacteria have been divided into three groups, gram-negative bacteria, gram-positive

bacteria, and other bacteria, as was done essentially by Stacey and Barker[6].

1.1.1.1. Classification according to the reaction to gram-stain. Gram-positive and Gram-negative Bacteria.

Bacteria have been classified as being either gram-positive or gram-negative in accordance to their reaction with the Gram stain[5]. The Gram stain was developed by Christian Gram, a histologist, as a method of staining bacteria in tissue. It is an arbitrary procedure consisting of four steps which are as follows:

(a) Primary staining with triphenylmethane dye such as crystal violet and usually containing a mordant such as ammonium oxalate.

(b) Application of dilute Lugol's iodine (1:15 w/v) in ethanol.

(c) Decolourisation most commonly with 95% ethanol.

(d) Counter staining with a dye of contrasting colour such as safranin which gives a pink colour.

The gram-positive are those which retain the primary stain and are deep violet in colour while the gram-negative bacteria are those which are decolourised and stained by the counterstain to pink as in the case of safranin. The basis of the empirical distinction between gram-positve and gram-negative is the types of cell envelopes in these two different classes of bacteria.

Examples of gram-positive bacteria are the *Micrococcus flavus* which is of commercial importance in the flavouring and ripening of cheese, *Staphylococcus aureus* which is notorious as the cause of suppurative (pyogenic or pus forming) conditions e.g.mastitis in women and in cows, boils, carbuncles,infantile impetigo and food poisoning. Some others are *Streptococcus pneumonia* which is responsible for pneumonia, and *Corynebacterium diphtheria* which causes diphtheria.

Examples of gram-negative bacteria are the genera *Escherichia*, *Shigella*, *Salmonella*, *Klebsiella*, *Enterobacter*, and *Yersinia*[5] which belong to the

Enterobactericeae many of which are found in the intestinal tract of man. They comprise of a large number of species many of which are human pathogens. They can cause enteric diseases (typhoid fever, paratyphoid fever, dysentery, infantile diarrhoea and infections of the urinary tract and other organs of the body).

Other common examples of gram-negative bacteria are of the genus Brucella which is capable of producing brucellosis in a wide range of mammals including man, the genus Pasteurella which causes heamorrhagic septiceamia, the genus Heamophilus influenzae which causes acute bacterial meningitis which is common in children but rare in adults and the genus Rhizobium to which the symbiotic nitrogen fixation microorganisms of the leguminous plants belong.

Classification based on antigens.

Bacteria are also classified according to the antigens they possess. Among the Enterobacteriaceae for example, the designation of genus and in many cases species, is based primarily on biochemical parameters. Further differentiation to specific rank in some instances and to serogroups and serotypes and varieties is dependent upon serological reactions which detect surface antigens. Most Enterobacteriaceae have three chemically distinct antigenic determinants[5]. They are the somatic antigens or cell wall O-antigens also called lipopolysaccharides, the flagellar or H antigens and the capsular or K-antigens i.e.capsular polysaccharides. In addition, many species also possess pili also called fimbriae[5].
A schematic representation of a bacterial cell showing these components is given in Figure 1.1 on page 6.

O-antigens: The somatic O-antigens are heat stable lipopolysaccharides (LPS) common to all smooth gram-negative bacteria. They are present in the outer membrane and are responsible for the endotoxic activity allied with

gram negative bacteria. The antigenic specificity of these O-antigens resides in the O-specific side chain of the LPS and is dependent upon the kinds of sugar residues and their arrangements in the side chain.

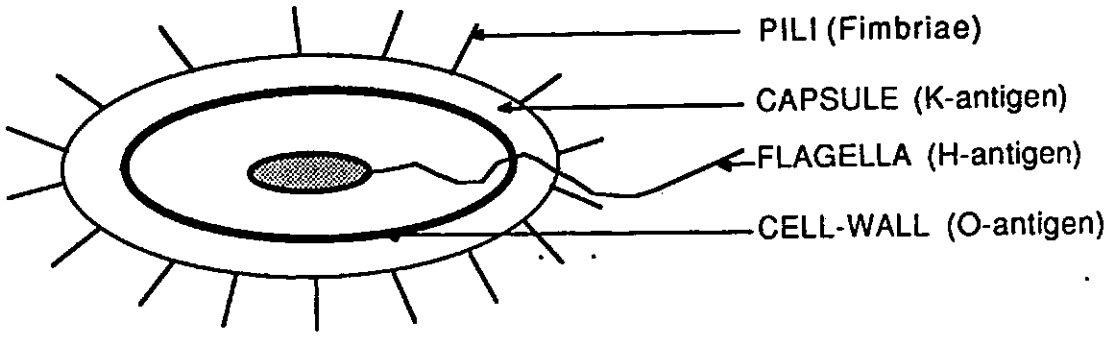


FIGURE 1.1 :A schematic representation of a bacteria cell showing the various antigens.

H-antigens: The flagella of motile bacteria contain serologically specific proteins. These flagellins unlike the O-antigens are heat labile and are also destroyed by ethanol.

K-antigens: This third class of antigens appear in Enterobacteriaceae as capsules or as envelope antigens which overlie the surface O-antigens. Antigens of these class are polysaccharides and their reactivity is usually altered by heating.

Fimbriae : Many species of Enterobacteriaceae possess fimbriae also called pili. These are different from flagella. Pili are extremely fine filamentous appendages extending outwards from the surfaces of the bacteria. Chemical studies show that pili are composed entirely of protein. The entire function of the pili is not well understood but it is known that they are involved in the adhesion of bacteria to host tissues and the ability of microorgarnism to adhere to host tissues is thought to be an important factor in pathogenesis.

1.1.2. The structure of the bacterial cell envelope.

Most bacteria have two structures that surround their cytoplasm, the cytoplasmic membrane and the cell wall while some may have a third structure: the capsule as in figure 1.1. These layers collectively are often referred to as the cell envelope. The bacterial polysaccharides are found both in the cell wall and the capsule or slime layer.

1.1.2.1. The Capsule[7]

Many bacteria can develop an envelope layer of considerable thickness which is termed the capsule. Capsules are produced only by certain species of bacteria and often only when these are grown under certain nutritional conditions. The capacity to form capsules may be lost on prolonged cultivation.

Capsules vary in their chemical composition but many of them consist of polysaccharides charged with a high proportion of acidic constituents such as hexuronic acids, neuraminic acid, or pyruvate substitution[6]. Other capsules are composed of only one or two amino-acids. These amino acids are generally of the unnatural D-configuration as opposed to the natural L-configuration always found in proteins. Capsules are frequently found with bacteria obtained from pathogenic material.

1.1.2.2. The Cell wall[5].

Bacteria are surrounded by cell walls which have the following properties and characteristics[4].

a) They give mechanical support and distinctive shape.

b) They withstand the high osmotic pressure inside the cells which can be as high as 20 atmospheres.

c) They are of considerable medical importance because these cell walls and associated substances are largely responsible for the virulence of the bacteria.

d) When injected into experimental animals, they can produce the symptoms of numerous bacterial diseases because the characteristic antigens of the bacteria are located on the cell walls. The antigen is what is recognised by the body as a foreign substance that has to be destroyed.

e) When extracted and injected into animals they make the animals acquire immunity.

f) When their synthesis is disturbed or prevented, the bacteria are killed.This is the mechanism of the action of penicillin and other antibiotics.

1.2.1. The Chemical Structure of Bacterial Cell Walls[6,8].

The chemical stucture of bacterial cell walls is responsible for its rigid nature. The backbone of the cell wall is the macromolecule known as the peptidoglycan consisting of two major subunits which are amino sugars and amino acids. The amino-sugars in peptidoglycan are N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAM) are chemically related to glucose. These two sub-units alternate to form a high molecular weight polymer. Although this polymer is found in the procaryotic*[1] cell walls, it bears a chemical relationship to the cellulose found in plants cell walls and to the chitin found in insect and crustacean exoskeletons and in fungal cell walls.

In a peptidoglycan, a chain of several amino acids is attached to each N-acetyl muramic acid molecule. Only a few of the 20 amino-acids usually found in proteins occur in the peptidoglycan and its exact composition varies in different bacterial species. Many of the amino acids in the cell wall are of D-configuration, whereas those in proteins are of the L-configuration. The cell walls of both the gram-positive and gram negative bacteria are further discussed as follows.

---

[1] a primitive bacteria characterised by lack of nuclear membrane.

1.2.1. The Cell Wall of Gram-positive Bacteria[6,8]

In the Gram-positive bacteria, the polysaccharide is found in the thick cell wall of about 250Å wide which surrounds the plasma membrane. This cell wall is composed of peptidoglycan and teichoic acids. The cross section of the cell wall of the gram-positive bacteria is shown in Figure 1.2.

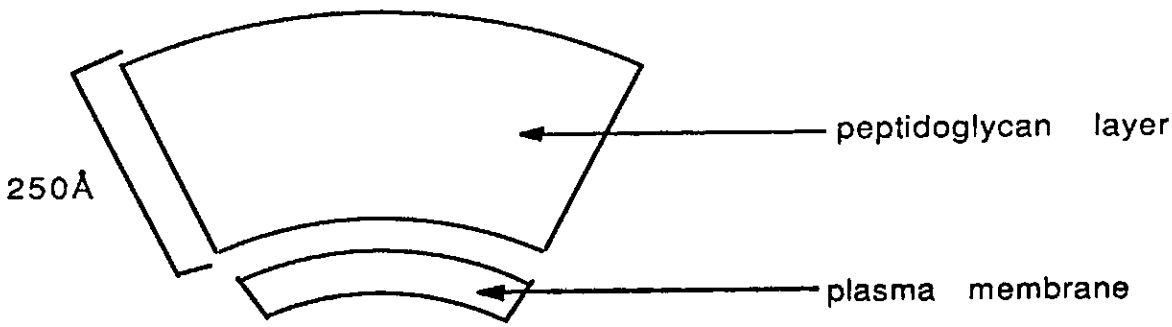


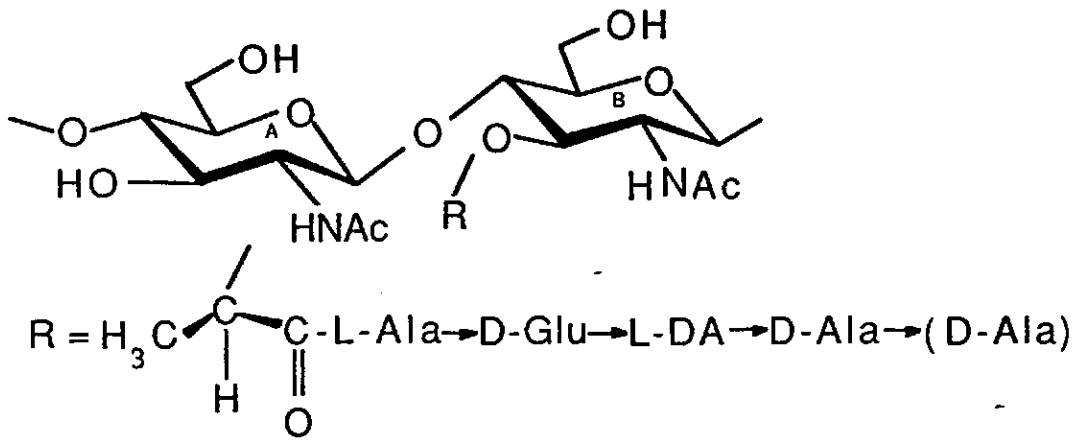
FIGURE 1.2. The cross section of the cell wall of gram-positive bacteria.

The Peptidoglycan[8]

The chemistry of the peptidoglycan has been adequately summarised by Scheleifer and Kander[8]. The peptidoglycan is made up of a polysaccharide which consists of a disaccharide repeating unit of muramic acid (A) and glucosamine (B) illustrated in [I.1] . The muramic acid or 2-amino-3-O-[(R)-1-carboxyethyl]-2-deoxy-D-glucose, is unique as it has not been reported present in the cell wall of other organisms. The polysaccharide chain displays some variation. For example, the amino group in a muramic acid residue is generally acetylated but may also be free, acylated with glycolic acid or form an internal amide with the adjacent carboxyl group. The muramic acid may be replaced by the isomer which has the D-mannose configuration. The muramic acid residues may be further O-acetylated. Finally, some 2-amino-2-deoxy-D-glucopyranosyl residues may lack the N-acetyl group.

The protein moiety of the peptidoglycan present as a tetrapeptide or pentapeptide is linked to the carboxyl group of the muramic acid moiety.

There is considerable variation in this structure, the greatest being observed for the third amino acid residue (L-DA), which may be L-lysine, L-ornithine, L-hydroxylysine, *meso*-diamino-pimelic acid or some other less common amino acid.



[I.1]

The peptide moiety is unusual in two respects. It contains D-amino acids which are never found in proteins. Moreover the D-glutamine residue forms a peptide linkage at its side chain γ-carboxyl group. In the intact peptidoglycan, the N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) alternate in sequence to form a linear polysacchride chain. The cross-linking between the peptidoglycan chains is effected by the formation of amide linkages between different peptides. The common mode of cross-linking in gram-negative bacteria is the formation of amide linkages between the carboxyl groups of D-alanine residue belonging to one chain and the amino group of the ω-amino-acids of another chain.
In gram-positive bacteria in which cross-linking is more extensive the same groups are connected via a short peptide which may be a pentaglycine as found in Staphylococcus aureus[9].

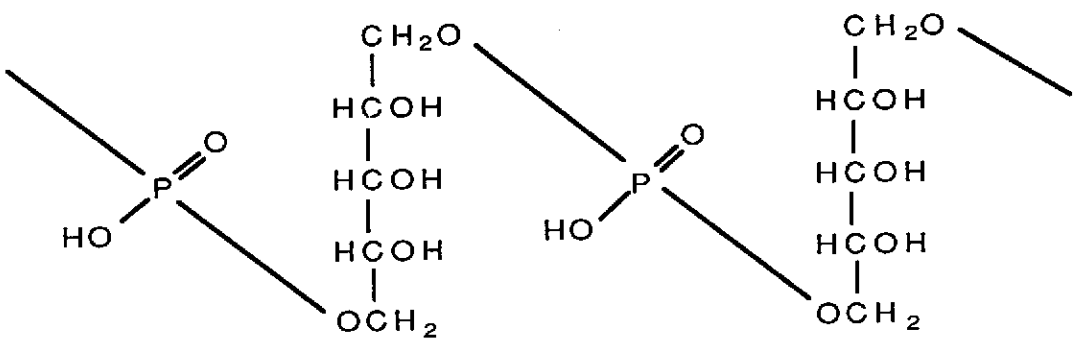
In the individual glycan strands the chain length may vary between 10 and 64 disaccharide units. This complex heteropolymer is further modified particularly in gram-positive bacteria by the attachment of oligo- and poly- saccharides and of teichoic acids. The peptidoglycan is therefore a highly cross-linked mosaic, and this structure undoubtedly accounts for the

properties of strength and rigidity that are necessary for its function of containing and protecting the bacterial cell.

Teichoic acid[9-11].

Teichoic acids cover the peptidoglycan in gram-positive bacteria. Teichoic acids are polymers of oligosaccharides joined together by phosphoric ester linkages. There are two main types of teichoic acids. These are the ribitol teichoic acids and glycerol teichoic acids which are found in the cell-wall or the membrane.

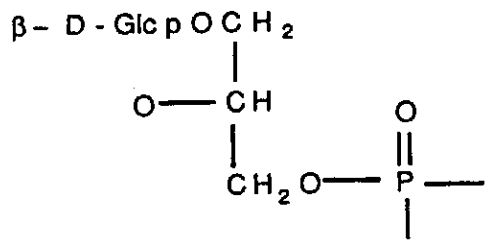
In the ribitol teichoic acids, the phosphoric diester linkages are between O-1 and O-5 of adjacent ribitol residues[I.2]. These residues are generally mono-O-acylated with D-alanine. Different glycosyl groups e.g.α or β-linked D-glucopyranosyl and 2-acetamido-2-deoxy-D-glucopyranosyl groups may be further linked to the ribitol residues.



[I.2]

In the glycerol teichoic acids, the glycerol residues are connected through phosphoric diester linkages between O-1 and O-3. The 2-position may be acylated with D-alanine or may be glycosylated. There are exceptions however, where the glycerol residues are linked between O-1 and O-2.Examples are the teichioc acid from Bacillus subtilis [12] and shown in [I.3] Streptomyces antibioticus[13] .

Two types of glycerol teichoic acids in which the glycosyl residues are part of the main chain have been observed. In one of these, as in the cases of teichoic acids isolated from the Staphylococcus and Micrococcus



[I.3]

species[14], the repeating unit contains two phosphoric diester groupings. In the other type, the sugar is glycosidically linked to the glycerol moiety. The cell wall teichoic acids are covalently linked to the peptidoglycan i.e. they are attached to the NAM-NAG backbone of the peptidoglycan by a phosphodiester bond. The linkage region has been found to be the same or similar for the different types of the cell wall teichoic acids[11-13].

The membrane or glycerol teichoic acids also referred to as the lipoteichoic acids contain a polyglycerol phosphate chain linked to a glycobiosylglycerol in which the glycosyl moiety is further acylated by fatty acids. Different disaccharides namely kojibiose, lactose and gentiobiose have been observed to be present. The poly-glycerol phosphate is partially glycosylated or O-acylated with D-alanine.

Teichoic acids do not contribute to the rigidity of the cell wall and their function is somewhat uncertain. However, they are believed to contribute to the magnesium binding and thus maintain proper ionic condition for cationic-dependent enzymes in the cell envelope. Although not usually immunogenic in the isolated state, they are immunogenic when combined with the other cell wall components in the whole cell and thus constitute an important surface antigenic component in many gram-positive bacteria[15].

1.2.2. The Cell Wall of Gram-negative Bacteria[5].

The cell walls of gram-negative bacteria are more complex but much thinner than the cell wall of gram-positive bacteria and consequently the wall of gram-negative cells are more easily broken by mechanical forces than those of the gram-positive. The diagram of the cross section of the cell wall of the gram-negative bacteria is as shown in Figure 1.3.

The peptidoglycan layer of gram-negative cells such as the *E. coli* or *Salmonella typhi* which is about 30-80Å thick surrounds the plasma membrane. The peptidoglycan layer is in turn covered by an outer layer of 60-100Å thick. This outer membrane is a mosaic of phospholipid, proteins and lipopolysaccharide (LPS) and it has a bilayer arrangement like the plasma membrane.

Between the plasma membrane and the peptidoglycan layer is the periplasmic space which serves to transport fluids and enzymes to and from the plasma membrane and the cell wall. The periplasmic space also serves as a distiguishing factor between the gram-positive and gram-negative bacteria, as it has not been found in the gram-positive bacteria.

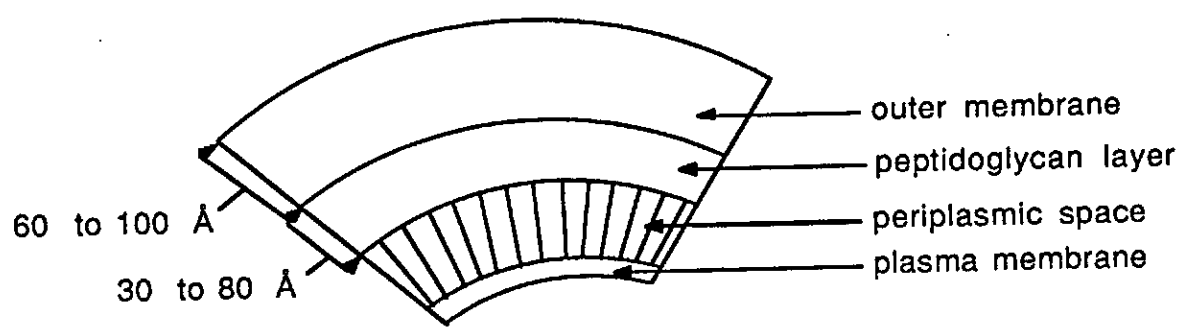


Figure 1.3. The cross section of the cell wall of the gram-negative bacteria.

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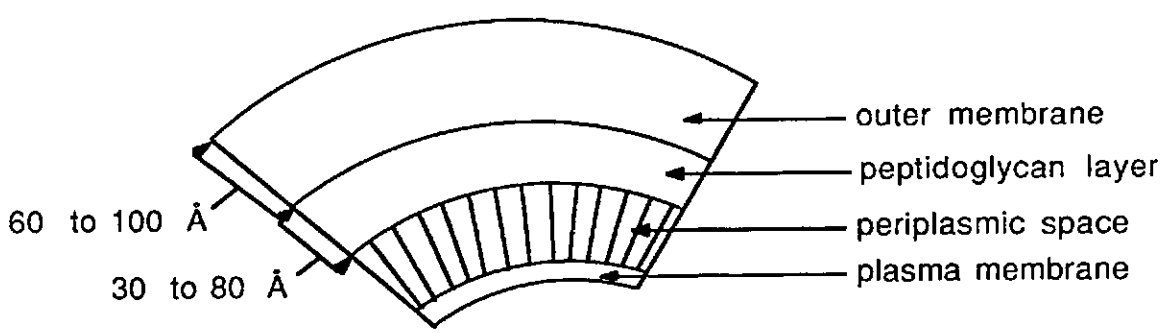


Figure 1.3. The cross section of the cell wall of the gram-negative bacteria.

The protein, phospholipid and the lipopolysaccharide are held together by weak non-covalent bonds as evident by the ease of dissociation of this huge complex during the hot phenol-water extraction of the lipopolysaccharide of the various Enterobacteriaceae[15].

1.2.2.1. The Lipopolysaccharides (LPS)

The LPS form a large unique class of macromolecules representing a characteristic attribute of the gram-negative bacteria. Occuring as part of the outer membrane and associated with proteins and phospho- lipids, they are located in the outer leaflet of the outer membrane of the bacterial cell[16]. In this exposed position on the cell surface, LPS are involved in the interaction of the cell with the environment. Thus the contact of the bacterium with the immune system leads to the stimulation of specific antibodies directed predominantly against the determinant structures of the LPS. Hence the LPS represent the major surface antigenic components and they have for many years been known as the O-antigens[17] .

Another term used synonymously for the LPS is endotoxin. This is because a wide spectrum of endotoxic reactions are observed when purified LPS or bacteria which contain LPS i.e. gram negative bacteria are injected into experimental animals. These effects, in contrast to the specific immune delayed response[18-20], are in general nonspecific and acute, and would include such phenomena as fever, changes in white cell counts, shock and death after high doses of LPS.

The Biosynthesis of LPS.

The biosynthesis of the enterobacterial LPS is very complex but more

main feature of this synthesis is the independent synthesis of O-specific side chain and the core oligosacharide. The latter is probably synthesized on the lipid A moiety as the acceptor. It is itself, the acceptor for the O-specific polysaccharide which is assembled from the oligosaccharides repeating unit on an intermediary acceptor and then transferred to the completed core oligosaccharide[21].

However it is known that the LPS are synthezised in the inner cytoplasmic membrane of the bacterial cell and, immediately after completion are exported to the outer membrane, where with the protein they form the external face of the bilayer. The inner face contains proteins and phospholipids.

The enzymes responsible for the biosynthesis of LPS are determined by a number of of genes or gene clusters on the bacterial chromosome[22]. Briefly the main gene loci and their functions are *rfa* (closely linked to *xyl*;the locus determining xylose utilization), which gives the code for the synthesis of the core oligosaccharide; *rfb* (closely linked to *his* the locus determining the synthesis of histidine) which gives the code for the synthesis of the oligosaccharide repeating units of the O-specific side chain; and *rfc* (closely linked to PMI, the locus determining the enzyme phosphomannose isomerase) which gives the code for a polymerase joining the O-specific oligosaccharide repeating units to give the O-specific polysaccharide. The understanding of this mechanism is important for the understanding for the appreciation of the enterobaterial R mutants and the SR mutants[22].

Mutant LPS[22].

Bacteria which synthesize the complete LPS are referred to as being the smooth strains and these LPS are reffered to as the smooth LPS i.e S-LPS. Mutations affecting the gene loci *rfb* or *rfa* lead to bacterial forms which are termed R mutants. Correspondingly, their LPS are termed R-LPS and the strains of bacteria which elaborate this type of LPS are termed rough strains. Mutations in the *rfb* locus block the synthesis of the O-specific side chain and such R(*rfb*) mutants also referred to as Ra mutants have LPS which consists only of core oligosaccharide and lipid A. Mutations in the *rfa* locus interfere with the synthesis of the core oligosaccharide which remains more or less incomplete. Thus the O-specific polysaccharide cannot be transfered and the resulting *(rfa)* mutants are referred to as Rb to Re mutants. These mutants have an LPS which, like those of *rfb* mutants, lack the O-specific side chain and in addition have a defective coreoligosaccharide. Both types of mutation result in the loss O-specificity and in the appearance of a new specificity, the R-specificity which is cryptic in the wild-type S forms.

1.2.2.2. The Structural Features of the LPS.

The complete LPS elaborated by the smooth strains of the gram-negative bacteria, and especially those of the Enterobacteria are composed of three structural regions namely the lipid region, which is termed Lipid A, the core oligosaccharide and the O-specific side chain both of which form a long, covalently linked heteropolysaccharide as shown schematically in Figure 1.4[23]. The lipid A, the core, and the O-chain are interlinked by covalent linkages. It is generally accepted that, certainly in Enterobacteriaceae, but also in more remote bacterial families, the

general architecture of the LPS is as shown in Figure 1.4. This probably implies that there exists related pathways of evolution of these molecules as well as common mechanisms of biosynthesis and principle of their genetic determination.

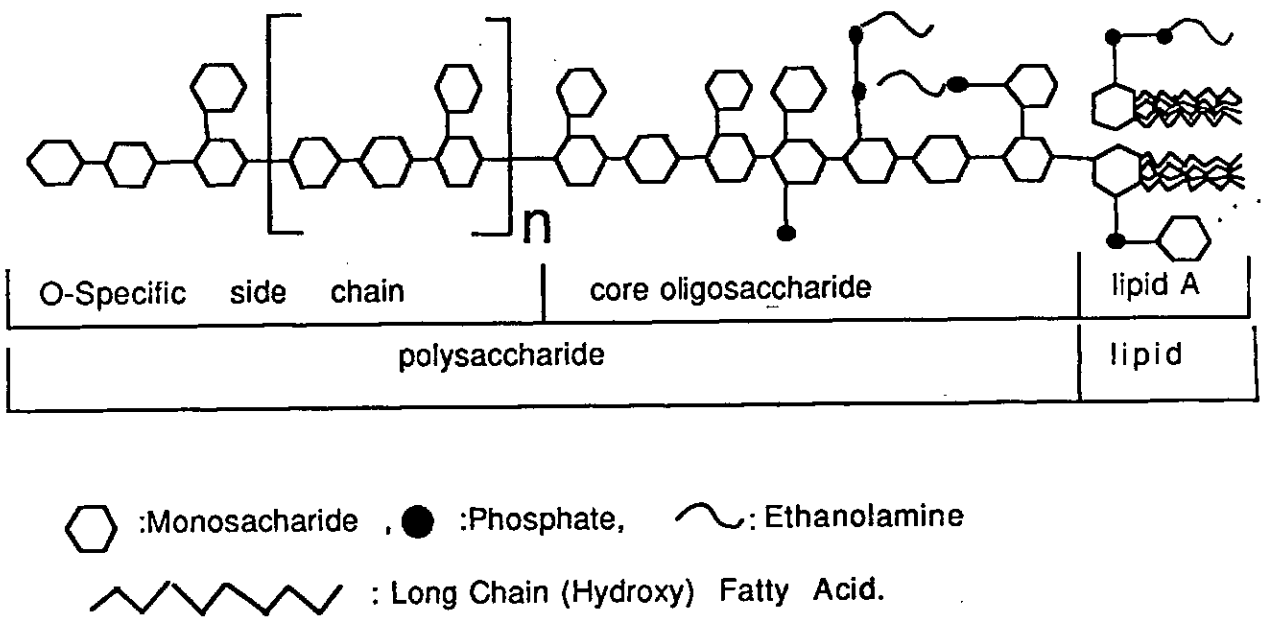


Figure 1.4. The schematic diagram of the Lipopolysaccharide[23]

In spite of great variations in sugar composition, the functions and biological properties of the LPS are similar. The three regions of the LPS are not only distinct in their chemical structure but also in their biological and functional properties.

The lipid A, is the most conservative part, and has a similar structure in most of the LPS investigated. The LPS are anchored in the outer membrane via their lipid component which is the lipid A . Lipid A is a unique glycophospholipid, containing glucosamine, fatty acids, and phosphates.

The core was originally thought to have the same structure in large groups of bacteria, e.g. in Salmonella but it was found later that a number

of different core oligosaccharides exist which are structurally closely related. These are few and there is a great similarity in their structure when compared to the O-specific side chain.

The O-specific side chain determines the O-antigenic specificity of the bacteria and there is an enormous amount of structural variation. The O-specific side chain consists of oligosaccharide repeating units which is a structural feature that seems to be common for most bacterial polysaccharides. There may be many sugar residues present in many combinations involving different glycosidic linkages.

There is evidence that several LPS are linked together through pyrophosphodiester bridges in the Lipid A moiety. It was reported that three LPS units would form one large molecule on the bacterial surface. By virtue of their amphiphilic character, LPS once isolated, tend to form large micelles in aqueous solutions[23]. Figure 1.5 on page 19 shows the schematic representation of the S (smooth) form (a), Ra (rough) form(b), and the core defective (c) lipopolysaccharides.

1.1.2.3. LIPID A.

Lipid A is an amphiphatic molecule with both hydrophobic (a disaccharide acylated with long chain fatty acid) and hydrophilic (phosphorylated sugars) regions. Moreover it is an amphoteric molecule, carrying both acidic (phosphate) and basic (ethanolamine) residues.

Lipid A is bound to the polysaccharide portion through a ketosidic linkage of the 2-keto-3-deoxy-D-mannooctulosonic acid (KDO). This ketosidic linkage is highly susceptible to mild acid hydrolysis and therefore the free Lipid A is obtained from the LPS by mild acid

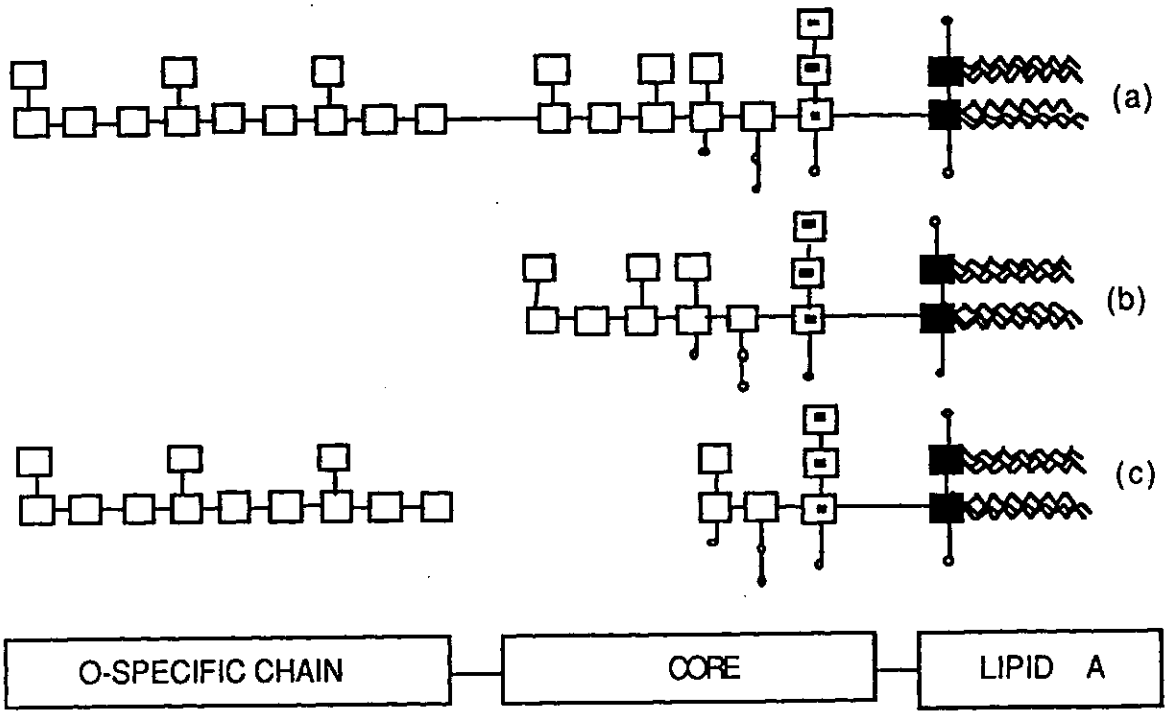


Fig.1.5. The schematic representation of the Smooth (S) form (a), Rough (Ra) form (b), and the core defective (c) lipopolysaccharides[23].

hydrolysis. This is the only means of obtaining lipid A since there are no known enzymes which cleave this linkage and there are no bacterial mutants which synthezise only lipid A.

The three main characteristic constituents of the Lipid A are glucosamine, phosphate and long chain fatty acids.

The glucosamine phosphate represents the hydrophilic backbone of lipid A, to which are attached long chain fatty acids which impart the hydrophobic properties to the molecule. The glucosamine residues which usually have the D-configuration are the main saccharide component of lipid A and is thus a characteristic constituent. The glucosamine is present as a disaccharide and is referred to as the lipid A backbone. There are

exceptions however. For example in the lipid A of Rhodopseudomonas viridis and Rhodopseudomonas palustris, no glucosamine has been found, instead, 2,3-diamino-2,3-dideoxy-D-glucose is present[24,25].

In some lipid A structures other sugars have been detected beside glucosamine. Galactosamine has been found in the lipid A preparations of Neisseria catarrhalis, Moraxella duplex, and Micrococcus calcoeticus[26], Chromatium vinosum[27] has mannose in addition to glucosamine in its lipid A.

Phosphoethanolamine and ethanolamine are frequently encountered in lipid A. Phosphate is identified in most lipid A preparations but there are also some exceptions as well.

Long chain nonhydroxylated as well as hydroxylated fatty acids (C10-C18) represent the major components of lipid A. The fatty acids are responsible for the hydrophobic properties of lipid A and seem to play a role in the endotoxic activity since their removal leads to preparations of reduced endotoxic activities. The fatty acids are bound to the lipid A backbone through ester and amide linkages. Depending upon the source of lipid A, various kinds of fatty acids are present. In Enterobacteriaceae, unsubstituted and 3-hydroxy fatty acids predominate, both types of acids being saturated, even numbered, and straight chained. Unsaturated and notably cyclopropane fatty acids have not been encountered in significant quantities. The lipid A in Salmonella[28,29] has been studied in some detail but that of E. coli[29-31] which has been recently studied by chemical and physical means is known in greater detail. Figure 1.6. shows the chemical structure of E. coli lipid A as it is presently known[29-30].

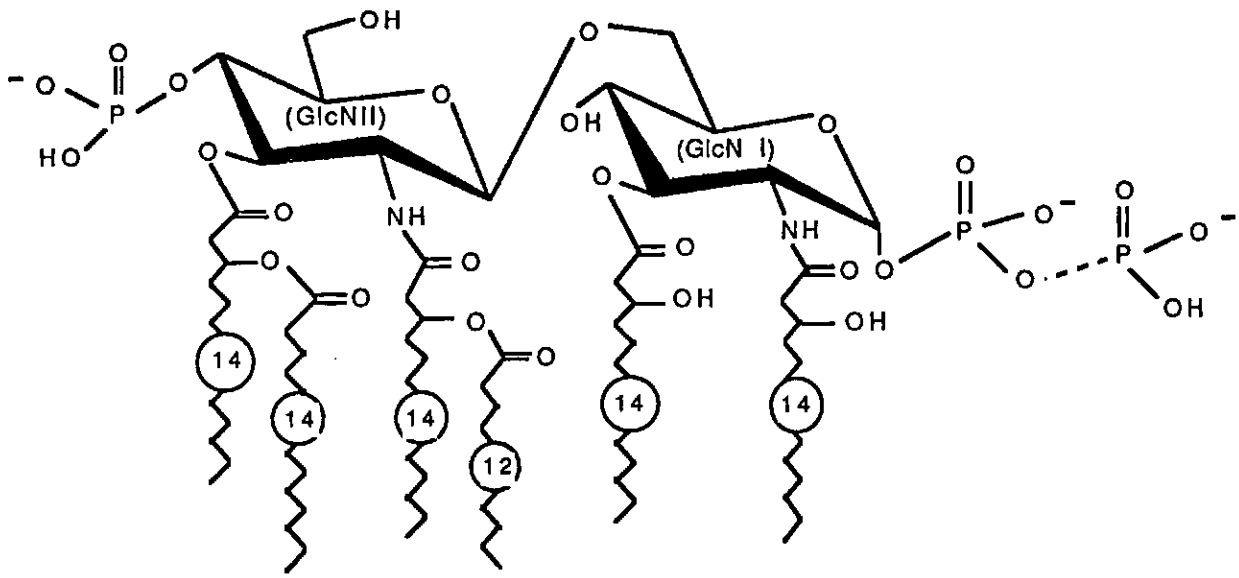


Figure 1.6. Structure of lipid A from E coli[29,30].

(Numbers in circles indicate the number of carbon atoms in the acyl chains)

The lipid A of E. coli like other enterobacterial lipid A structures contains D-glucosamine, phosphate and long chain fatty acids among which (R)-3-hydroxytetradecanoic [$CH_3(CH_2)_{10}CH(OH)CH_2COOH$] acid predominates. There are two 2-deoxy-2-amino-D-glucopyranose residues interlinked by a β-(1→6) glycosidic bond. This disaccharide carries phosphate groups in position 4'of the non-reducing glucosaminyl residue (GlcN II) and in position 1 of the reducing glucosaminyl residue (GlcN I), the latter being α-linked.

Presently it is known that the hydrophilic backbone is substituted by 2-keto-3-deoxy-octulosonic acid (KDO), another phosphoryl group and a characteristic spectrum of long chain fatty acids comprising of (R)-3-hydroxytetradecanoic acid(C14) (about 2 mol), (R)-3-dodecanoloxytetradecanoic acid (C14→C12),(about 1 mol), (R)-3-tetradecanoloxytetradecanoic acid (C14→C14), (about 1 mol). The KDO is attached to the primary hydroxyl group of GlcN II the additional phosphate group is linked to the glycosidic phosphoryl residue on GlcN I. The amino group in position 2 of the two

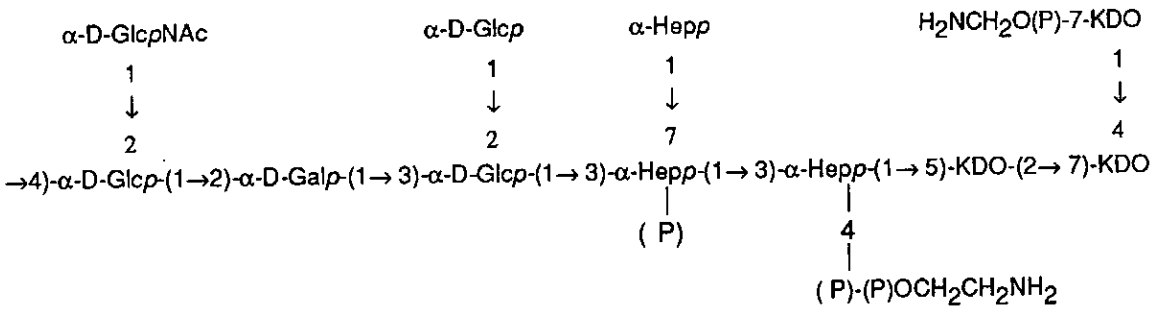
backbone sugars are acylated by one mole of both (R)-3-dodecanoloxytetra-decanoic acid(C12→C14) and (R)-3-hydroxytetra-decanoic acid C14:30H, the former being linked to GlcNII and the latter being linked to GlcNI.

The hydroxyl group in position 3'(GlcN II) carries (R)-3-tetradecano-loxytetradecanoic acid, and that in position 3, nonacylated (R)-3-hydroxy-tetradecanoic acid. The hydroxyl group in position 4 of GlcN I in a fraction of free Lipid A, was shown to be free. It is presently under investigation whether this hydroxyl group is also free in the bound Lipid A. For most LPS, lipid A seems to have a closely related structure, although, the fatty acid composition may vary.

1.2.2.4. The Core Structure.

The glycosidic linkage of the 2-keto-3-deoxy-D-manno-octulosonic acid (KDO) residues which link the core structure to the lipid A are extremely labile to acid hydrolysis and it is generally possible to cleave this linkages without affecting other glycosidic linkages in the LPS. By such treatment, the core and the O-specific side chain are separated from the lipid A.

The LPS from the rough mutants therefore on mild acid hydrolysis yield fractions consisting of the complete or incomplete core and these provide materials for studying the core. The best studied core structure is that from Salmonella LPS, the Ra core [I.4][32,33]. In this structure Hep stands for L-glycero-D-manno-heptose. The KDO is pyranosidic and as of now there is no information concerning its anomeric nature. The heptose and KDO regions have not been investigated in the same detail for other LPS but seem to have similar structures in Enterobacteriaceae.
Other core structures that have been studied are those of Niesseria[34].



[I.4] The Core structure of the Salmonella LPS [33]

1.2.2.5. Structure of the O-Specific Side Chain.

The O-specific side chains of LPS are made up of identical repeating units of oligosaccharides[32]. These units usually contain different constituents, thus the O-chain represents a heteropolysaccharide. In some cases the repeating units may contain an oligomer of a single type though in a distinct linkage sequence, hence repeating units can also be recognised. In these cases the O-chain represents a homopolysaccharide, for instance, a mannan in *E. coli* O9[35] and a galactan in *Klebsiella* O8[36]. Some O-chains which are also homopolysaccharides have also been identified with only one type of linkage such as the β-(1→2)-linked poly-6-deoxy-L-altropyranose isolated from *Yersinia enterocolitica*[37] and α-(1→2) linked 4-amino-4,6-deoxy-D-mannopyranose isolated from *B.abortus* .

The average number of repeating units (n) in the O-specific side chain appears to be in the order of n = 20 to 35 but, also larger (up to n = 40) and smaller (n = 5) repeating units have also been found.

The O-chains contain the immunodeterminant structures against which the anti-O antibodies formed during infection or on immunisation are directed[22,38]. Each bacterial serotype synthesises a unique LPS, characterised by a specific composition of the O-chain and by an individual O-antigenicity. There consequently exists in nature as many distinct LPS as there exists bacterial serotypes.

This number is certainly very high.

There is a great diversity of constituent sugars, linkage types and structural peculiarities present in O-specific side chains. These constituents include neutral sugars (hexoses,pentoses, deoxy- and O-methyl derivatives) and charged sugars (amino-hexoses and pentoses, hexuronic acid,hexoaminuronic acid) which may carry a variety of substituents such as amino acids, phosphoryl-, glyceryl-, lactyl-,pyruvyl- and acetyl groups.

However,the nature, sequence and the type of linkage (and substitution) of the individual monosaccharide residue within a repeating unit is characteristic and unique for a given LPS and the parental bacterial strain.

The O-specific side chain generally contains three or more sugar constituents. The best studied are the Salmonella antigens and those from other groups of the Enterobacteriaceae like the Escherichia, Arizona, Shigella while others have been analyzed less systematically and only occassionally. It is noteworthy that only some sugars are very commonly encountered. These are as discussed below.

More than forty combinations made up of different sugars i.e. chemotypes, have been found in specific polysaccharides[39]. Several combinations which have never been found or encountered together include two 3,6-dideoxy-hexoses or a deoxy-hexose together with a dideoxyhexose. On the other hand,two heptoses or three hexoses may well occur in one antigen. For most O-antigens, only the "chemical" repeating unit has been determined. For some of them, however, the non-reducing terminal or the sugar linking the side chain to the core has been identified and thereby "biological" repeating units can been determined.

The gram-negative bacteria have been classified in chemotypes according to the sugar components in their O-specific side chains and tables giving chemotypes and serotypes have been compiled[39].

1.3. The Sugar Constituents of Bacterial Polysaccharides.

1.3.1. Sugar Constituents of the O-Specific Side Chains.

More than 20 constituent sugars both common and rare types have been found in the O-antigens of gram-negative bacteria. This is because bacteria find many ways of converting usual to unusual derivatives which suit their demands for special surface properties. The structures of some rare sugars are shown on the next page.

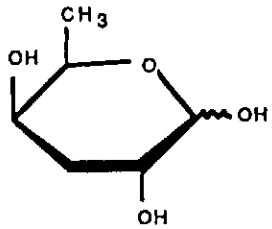
a) HEXOSES: The most commonly found hexoses are glucose and galactose. In *Salmonella* for example, these two sugars are constituents of the specific polysaccharides and therefore present in all O-antigens. Mannose is also frequently found. The hexoses usually have D-configuration. The occurrence of gulose has been reported but not confirmed[40] in some strains of Klebsiella. D-fructose has been reported as being present as levans in the *Vibrio cholerae* LPS[41].

b) 6-DEOXYHEXOSES: L-Fucose (6-deoxygalactose) and L-rhamnose[42] (6-deoxymannose) are frequently found in *Salmonella* and some other Enterobacterial species while D-rhamnose has been found in *Xanthomonas campestris*[43]. 6-deoxy-L-talose has been identified in the LPS of *E.coli* O45[44] and 6-deoxytalose (D or L) was identified in other *E. coli* serotypes[44], 6-deoxy-arabinohexose (6-deoxyaltrose) has also been found in *Citrobacter* LPS[45].

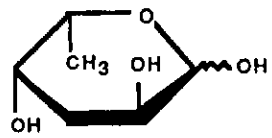
c) 3,6-DIDEOXYHEXOSES : Five representatives of this class of sugars have so far been found in Enterobacteriaceae[39,43].

i) 3,6-dideoxy-D-xylohexose (abequose) in *Salmonella* groups B,C_2 and C_3, *Citrobacter* 4 and 5 as well as *Pasteurella pseudotuberculosis*[46,47] (*P. pseudotuberculosis*) type II.

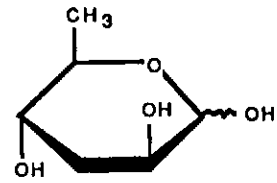
Some Rare Sugars from Bacterial Polysaccharides



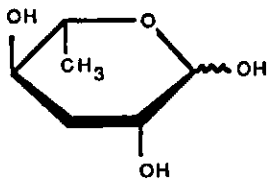
3,6-dideoxy-D-xylohexose
(Abequose)



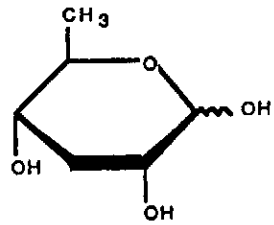
3,6-dideoxy-L-xylohexose
(Colitose)



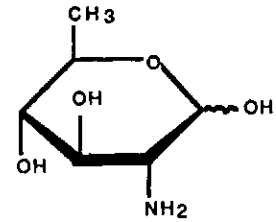
3,6-dideoxy-D-arabinohexose
(Tyvelose)



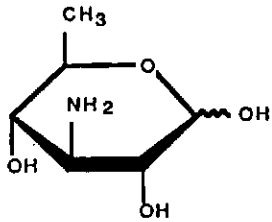
3,6-dideoxy-L-arabinohexose
(Ascarylose)



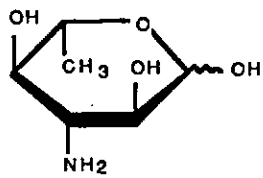
3,6-dideoxy-D-ribohexose
(Paratose)



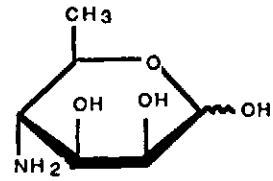
2-amino-2,6-dideoxy-D-glucose



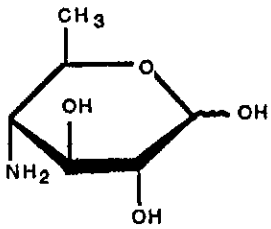
3-amino-3,6-dideoxy-D-glucose



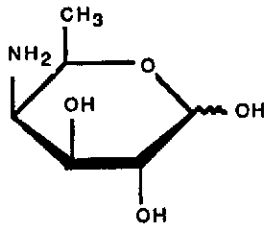
3-amino-3,6-dideoxy-L-glucose



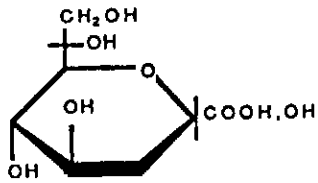
4-amino-4,6-dideoxy-D-mannose
(Perosamine)



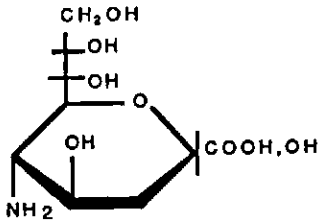
4-amino-4,6-dideoxy-D-glucose
(Viosamine)



4-amino-4,6-dideoxy-D-galactose
(Thomosamine)



2-keto-3-deoxymanno-octulosonic acid
(KDO)



5-Amino-3,5-dideoxy-D-galacto-D-glycero-nonulosonic acid
(Neuraminic acid)

ii) 3,6-dideoxy-L-xylohexose (colitose) occurs in some strains of Salmonella e.g Strains 35 and 50, E. coli O111 and O55, and Arizona strains 9 and 20.

iii) 3,6-dideoxy-D-arabinohexose (tyvelose) occurs in Salmonella groups D and P.pseudotuberculosis type IV.

iv) 3.6-dideoxy-L-arabinohexose (ascarylose) occurs in some strains of P. pseudotuberculosis type IV and Yersinia. Ascarylose is also a constituent of the ascaris egg cell wall glycolipid where this sugar was first discovered[48].

(v) 3,6-dideoxy-D-ribohexose (paratose) has been found in Salmonella groups A and B and P.pseudomonas types I and II.

d) AMINOSUGARS[39]: There is a wide variety of aminosugars. Glucosamine and galactosamine occur quite frequently in many O-specific side chains. Mannosamine has been found in some species of Escherichia, Salmonella, and Arizona. D-fucosamine and rhamnosamine have also been encountered in the polysaccharides of C. violaceum and E. coli. Some unusual amino sugars which have been found are: 2-amino-2,6-dideoxy-D-glucose (D-quinovosamine) as a constituent of an unidentified gram-negative bacterium, 3-amino-3,6-dideoxy-D-galactose in the plant pathogen Xanthomonas campestris. 3-amino-3,6-di-deoxyglucose (D and L) which have been found in E.coli, Salmonella, Arizona and Citrobacter strains, 4-amino-4,6-dideoxyglucose (viosamine) which has been isolated from C.violaceum and 4-amino,4,6-dideoxy-D-galactose (thomosamine) which has also been identified to be present in some E.coli strains. Also, 4-amino-4,6-dideoxy-D-mannose (perosamine) has been found in the different serotypes of Vibrio cholerae, Inaba and Ogawa.

e) URONIC ACIDS: Some uronic acids have been found and they include hexuronic acids such as

i) glucuronic acid in *Shigella boydii*[49] and some *E. coli* and *Klebsiella* groups[39].

ii) galacturonic acid in *Xanthomonas*[50] and some other *E. coli*[51] and *Klebsiella* strains.

iii) galactosamine uronic acid in *Citrobacter intermedium*, *Rhodospirillaceae* and *Pseudomonas aeruginosa*[52].

Other sugar acids that have been found are a pentulosonic acid such as 2-keto-3-deoxypentonic acid which has been found in *Klebsiella*, while an hexulosonic acid 2-keto-3-deoxygalactonic acid has been found in an *Azotobacter vinelandii* strain[53]. Others are 2-keto-3-deoxyoctulosonic acid i.e. KDO (2-keto-3-deoxy-D-manno-octulosonic acid) which has been found as a common constituent of the O-antigens[39]. Neuraminic acid which is a derivative of nonulose sugars has been isolated from *E. coli* O24 and O56[39].

Generally, it is believed that the presence of uronic acids in the LPS is an indication of contamination by the capsular (K) antigen of the bacteria. In some cases, however, such as those above, the uronic acids were shown to be non-separable and were therefore covalently linked constituents of the polysaccharide.

f) PENTOSES: D-ribose is a constituent sugar found in some *Salmonella* groups[54-55]. Its presence in other O-antigens is most frequently an indication of nucleic acids as contaminant. However, xylose has been found in *Citrobacter freundii* O8[56], *Nisseria* and *Pseudomonas*. L-arabinose has been reported in photosynthetic procaryotes, while D-threo-pentulose has been found in *Pseudomonas*.

g) O-METHYL SUGARS[57]: These are frequently found in the family Rhodospirillaceae They include mono- and di-O-methyl ethers of pentoses, hexoses, and aminohexoses. 3-O-methyl xylose has been found in Rhodospirillaceae viridis F (R.viridis F) and R. palustris I while 3-O-methylmannose has been found in R. viridis F as well as E.coli O8 and Klebsiella O5. In R. capsulata 37b4 and Klebsiella O10, 3-O-methyl-L-rhamnose and in R. palustris III.

i) NON-SUGAR SUBSTITUENTS: These include the O-acetyl group and the O-phosphate group. The latter was found linked to glycerol in E. coli 100[58] and to ribitol in Proteus mirabilis[59]. Ammonia and lysine have been found linked via the α-amino group[60] to the carboxyl group of galacturonic acid in Xanthomonas sinensis[60] and P. mirabilis[59] respectively. Alanine has been found linked to the amino group of galactosamine in Pseudomonas aeruginosa[61].Lactic acid has been found linked as an ether to glucose in Shigella dysenteriae type 3 and in the "Shigella like" E. coli O124[62,63]. Pyruvic acid has also been found acetalically linked to N-acetyl glucosamine in E. coli O149[64], and to galactose in Shigella dysenteriae type 9 [65] via its carbonyl group. Ethanolamine has also been identified in P. aeruginosa[66].

1.3.2. SUGAR CONSTITUENTS OF THE CAPSULAR POLYSACCHARIDES[67].

Several types of sugar residues have been detected in the capsular polysaccharides but the commonest monosaccharides are the following:

a) HEXOSES: D-glucose, D-galactose, and D-mannose are the commonest sugar constituents occuring virtually in almost all the capsules studied to date[67]. D-allose has also been reported in P.viscogena. Fructose has once been found in H.infuenzae type e capsular polysaccharide.

b) 6-DEOXYHEXOSES[67]: L-fucose and L-rhamnose have been frequently reported. They provide an interesting example of L-isomers of sugars found in nature. There is a high incidence of L-rhamnose in the pneumococcal polysaccharides[67].

c) 3,6-DIDEOXYHEXOSES: These sugars appear to be confined to the LPS of gram-negative bacteria.This is because these sugars have not so far been reported as being constituents of capsular polysaccharides.

d) PENTOSES: These have also been less commonly found and they include ribose, arabinose, and xylose. The ribose is usually found as ribitol phosphate in *Heamophilus influenzae* and *Streptococcus pneumoniae*. In *Heamophilus influenzae Type b* capsular polysaccharide, the antigen has been shown to consist of double strands in which ribosyl-ribose disaccharides of the trehalose type are doubly joined through phosphate diester bridges. Ribose has been found in the capsular polysaccharides of several strains of *E.coli*[67] and the *Rhizobium* family[67], occuring in the furanose form and as ribitol. However, arabinose and xylose have been less commonly encountered.

e) HEPTOSES: This class of sugars as well as the octoses have not been commonly found in capsular polysaccharides although, D-glycero-D-manno-heptose has been reported found as being present in the extracellular polysaccharides of *Beijeincka mobilis*[67].

f) AMINO-SUGARS: The variability in amino sugar components is suprisingly great and they are usually N-acetylated. The commonly found aminosugars are D-glucosamine, D-galactosamine, and

D-mannosamine. Fucosamine has also been encountered and an unusual

D-mannosamine. Fucosamine has also been encountered and an unusual amino sugar which is 2-acetamido-4-amino-2,4,6-trideoxy hexose has been found in the species-specific polysaccharide termed C polysaccharide. It is suprising however, that so far amino sugars have been found lacking in Klebsiella capsular polysaccharides.

g) URONIC ACIDS[67]: Apart from the neutral hexoses mentioned above, uronic acids are the commonest constituents of the capsular polysaccharides. They seem to be present in many strains of the pneumococcal polysaccharides and also in all Klebsiella[68] strains. Both species produce large capsules of acidic polysaccharides. D-glucuronic acid has been most frequently noted while D-galacturonic acid, D-mannuronic acid and L-guluronic acid have also been found. Alginic acid found in the brown algae has also been found in P. aeruginosa, and Azotobacter vinelandii. Other sugar acids such as colominic acid which are homopolysaccharides of sialic acid elaborated by several strains of E. coli have been found in the analysis of their capsules. KDO has also been found in the many strains of E. coli.

h) NON-SUGAR CONSTITUENTS[67,69]: Two types of non-sugar components are found in bacterial capsules, namely organic and inorganic.

Organic substituents

The following are commonly encountered organic substituents:

i) O-acetate groups

Acetate groups have long been known as a component of capsular polysaccharides, and O-acetyl residues are probably among the most widespread non-carbohydrate modification found on neutral or acidic sugars. They are found in polysaccharides from diverse organisms such as Streptococcus pneumoniae, Rhizobium radicicolum,

vinelandii and K. aerogenes. In those polymers containing amino sugars, N-acetyl groups are consistently found.

ii) Pyruvate residues[69]

The first capsular polysaccharides from which pryruvate residues was detected was that of Xanthomonas campestris and has been found in many more capsular polysaccharides of Klebsiella, E. coli, Rhizobium and Achromobacter families. The pyruvate in these polymers is linked acetalically as pyruvic acid to a sugar unit. The carboxylic acid is thus free and contributes to the overall charge on the polymer as well as being available for the possible binding of salts. Pyruvate residues have been found in many capsular polysaccharides in which acetates are present. Pyruvylgalactose appears to be the most frequently encountered residue. Pyruvic acid has also been found linked to glucose, rhamnose, mannose in a number of cases and also to glucuronic acid as well. Pyruvic acid residues are relatively stable to acid or alkaline hydrolysis unlike O-acetyl groups which are extremely labile to alkali and to acid treatment. It is thus simpler to obtain pyruvylated sugars from oligosaccharides obtained by partial acid hydrolysis of the polysaccharides.

iii) Succinate residues[69].

Another non-carbohydrate organic substituent that has been detected was the succinate residue. This has been found in capsular polysaccharide produced by a strain of Alcaligenes feacalis or (A.myxogenes). The polymer contained glucose as the principal sugar along with smaller amounts of galactose. Uronic acids and amino sugars were absent, so the polymer differed from most others in which acyl sugars have been found and which contain various charged sugars. The succinate was probably linked as an ester to free hydroxyl groups

of glucose moieties. The succinyl groups are similar to O-acetyl groups in their lability to alkaki and mild acid treatment.

i) INORGANIC CONSTITUENTS[67,69].

Crude preparations of capsular polysaccharides almost always contain a considerable amount of salts. It is sometimes difficult to determine whether these salts form an essential part of the polymer or are non-specifically adsorbed to it. Phosphate is certainly a component of one group of capsular polysaccharides from *S. pneumoniae* and *H.influenzae*. This is exemplified by the specific polysaccharides of the types 6, 8A and 34 *pneumococcus*[69]. The phosphate occurs sometimes as ribitol phosphate or as glycerol phosphate which is reminiscent of certain teichoic acids in the cell wall of gram-positive bacteria in the *S.pneumonia*[67,69]. The phosphate occurs as phosphate diester bridges in the *Heamophilus influenzae*[69] making the intricate structure of the polysaccharide to bear some resemblance to ribonucleic acids. This polysaccharide is in fact hydrolysed by ribonuclease. In *H. parasuis*, disaccharide units of the trehalose-type consisting of N-acetylglucosamine and a hexose (probably galactose) are joined by phosphodiester bridges to form single strands. Phosphate esters have been found lacking in the *Klebsiella*[67] types found so far. The presence of other inorganic compounds remains unproven. It does seem suprising that sulphate group, which is found in a number of polysaccharides derived from the higher algae and from mammalian tissues[70] has not so far been found in bacterial capsular polysaccharides.

1.4. ISOLATION AND PURIFICATION OF BACTERIAL POLYSACCHARIDES.

1.4.1. Isolation and Purification of Cell wall polysaccharides.

Isolation of Cell wall polysaccharides

The methods used for the isolation of cell wall polysaccharides are:

a) Extraction with chloral hydrate[71]. This procedure is used mainly for the extraction of amylose and amylopectin from starch but can also be used for the extraction of some bacterial polysaccharides of the amylopectin type.

b) Extraction with anhydrous diethylene glycol[72]. Diethylene glycol is used to extract the washed and dried organism. The advantages of this procedure are that it is neutral in reaction, it is miscible with water, ethanol and acetone in all proportions and it is free from nitrogen. It is also readily eliminated by dialysis and can be used at low temperatures. The use of the anhydrous reagent minimizes the risk of degradation or modification of extracted polysaccharides being brought about by bacterial enzymes.

c) Extraction with trichloroacetic acid[73]. This is the simplest method but it is old and not as efficient as the other methods because it might lead to the denaturation of the polysaccharides.

d) The hot phenol-water extraction[74]. Aqueous 45% (w/v) phenol in used for the extraction at 65-68°C. This is by far the most efficient method for the extraction of LPS. This is because after exhaustive pretreatment with other LPS-protein extracting agents, such as those mentioned in (a) to (c) above, the preextracted bacteria will still release significant amounts of LPS during the hot phenol-water extraction. The reason for this is that phenol is an excellent solvent for many proteins. Polysaccharides, mucopolysaccharides and lipopolysaccharides are water soluble but phenol insoluble and are thus dissolved in the aqueous phase. The partition coefficients of

proteins in biphasic phenol-water mixtures very often allows an almost complete extraction of these substance from aqueous solutions under controlled pH and ionic strength. Also phenol is a weak acid having a dissocciation constant of 1.1 to 1.2 $x10^{-10}$ at 18-19°C.
The mixture of phenol and water has a high dielectric constant which aids the solubility of the proteins in the mixture. The partition coefficients of proteins in biphasic phenol-water mixtures very often allows an almost complete extraction of these substances from aqueous solutions under controlled pH and ionic strength.

A modified procedure of the phenol-water extractions, the phenol-chloroform-petroleum ether (PCP) procedure is however used to selectively extract R-LPS[74a] in the presence of S-LPS. This procedure has proved to be highly efficient and specific. Water-soluble preparations of high purity of the R-LPS are thus obtained. These facts thus form the basis of the method of partition for a combination of proteins, polysaccharides and nucleic acids between phenol and water. Therefore various polysaccharides can be precipitated from aqueous solutions by the addition of liquid phenol. The phenol-water procedure yields a water soluble extract that is subsequently purified by high speed centrifugation.

It has occassionally been observed that LPS, which are more lipophilic in nature, partition mainly into the phenol phase during phenol-water extraction[74a]. The LPS from the smooth strains of Brucella abortus (B.abortus) species, Citrobacter freundii and the Xanthomonas are examples. Some investigators[74a] have shown that the crude endotoxin (S-LPS) from the smooth B.abortus strains and X.campestris are isolated into the phenol phase along with proteins and other contaminants from the cell, when the cell walls are extracted during the hot phenol-water procedure. This is because of the apparently strong association between the S-LPS and the proteins by strong non-covalent bonds. The LPS from rough strains i.e.R-LPS are however isolated in the aqueous phase like

most other bacterial species with some exceptions such as the R-LPS of the "heptoless" mutant of Salmonella minnesota[74a].

Purification of the LPS[74].

For the LPS extracted by the methods (a) to (d) above, the preparation in general contains small amount of contaminants such as protein (about 1%). The contaminants such as ribonucleic and (RNA) and other proteins have to be removed in order to obtain the purified LPS. The nucleic acids and the proteins are removed by treatment with proteases or ribonuclease and cationic detergents such as cetyltrimethyl ammonium bromide (cetavlon) or cetyl trimethyl pyridinium chloride or sodium dodecyl (lauryl) sulphate (SDS). Other methods of purification are fractional ethanol precipitation and repeated high speed centrifugation.

Isolation and Purification of Capsular polysaccharides[7].

There are various procedures for the isolation and purification of capsular polysaccharides.

Isolation of Capsular polysaccharides[7].

The different methods of isolation of capsular polysaccharides are stated below.

a) Simple water or saline extractions.

The extraction is effected by vigorously shaking or mixing the capsule with water in a Waring blender.

b) Physical Treatment.

Removal of the capsule from cell surface by physical treatment such as forcing the suspension of encapsulated bacteria through a hypodermic needle or chromatogram sprayer.

c) Centrifugation, concentration and dialysis.

When abundant and excreted into the medium, the slime layer or capsule is isolated by removing the bacteria by centrifugation or

concentrating the culture medium under reduced pressure. It can also be isolated by precipitating the antigens with ethanol or acetone. Another method is to dialyse the capsular material to remove low molecular weight materials.

d) Chemical Extraction.

As some capsules are not readily removed from some strains, more drastic procedures such as boiling or treatment with dilute alkali are sometimes used. However, these methods inevitably lead to the production of a polymer containing various contaminants and some degradations.

Purification of Capsular polysaccharides

The removal of extraneous matter from capsular polysaccharides presents several problems some of which are due to the high viscosity of the polymers in aqueous solutions.

There are however several methods of purification which include

a) Fractional precipitation with organic solvents such as ethanol, acetone, chloroform and butanol. This has been of only limited value and gives poor separation from other cell material contaminants which may be present. This may include nucleic acids if the cells have undergone lysis. The deproteinization technique by repeated treatment with chloroform and butanol has widely been applied and it can be successful provided that dilute(< 0.5%,w/v) solutions of the polysaccharides are used. Dimethyl sulphoxide, a dipolar aprotic solvent, which shows selective solubility for polysaccharides has also been used.

b) Removal of either nucleic acids or proteins is probably best accomplished by enzymic digestion. Successive treatments with deoxyribonuclease, ribonuclease, trypsin and pronase may be required.

After mild heating to destroy the enzymes, the polysaccharides are recovered from the supernatant fluids after centrifugation.

c) Treatment with cationic detergents: the ability of acidic polysaccharides to combine with quaternary ammonium salts which are usually cationic detergents e.g. cetyltrimethyl ammonium bromide (cetavlon) or cetyl trimethyl pyridinium chloride to form complexes is made use of in the purification procedure. The complexes formed are precipitated and can be separated from soluble neutral material by centrifugation. The polysaccharides are recovered from the complexes in strong salt solutions.

d) By using the phenol-water extraction procedure developed for the extraction of lipopolysaccharides from the cell wall of gram-negative bacteria.

e) On a micro scale, precipitation with specific antisera provides an elegant method for recovering the purified capsular polysaccharide present by precipitation.

CHAPTER 2.

2.0. Methods for Structural Studies of Bacterial Polysaccharides.

The primary structure of a bacterial polysaccharide can be studied by the following procedures:

a) the analysis of the glycosyl residue composition commonly referred to as sugar analysis[75,75a] This involves the determination of the constituent monosaccharides and their proportions.

b) the determination of the glycosidic linkages by methylation analysis[76,77].

c) the determination of the sequence of the glycosyl residues.

d) the determination of the anomeric configuration.

e) the determination of the absolute configuration.

f) the detection and location of non-carbohydrate substituents.

2.1. Determination of Glycosyl Residue Composition: Sugar Analysis.

The identification of the constituent monosaccharides of polysaccharides and their respective proportions can be determined by acid hydrolysis followed by the characterisation of the sugars obtained on G.L.C. Sugar analysis is the first step in the structural determination of a polysaccharide.

Acid hydrolysis.

The standard conditions for hydrolysis are as follows:

a) 0.5M 2,2,2-trifluoroethanoic acid (trifluoroacetic acid- TFA) for 16 hours or 2M TFA for 2 hours at 120°C

b) 2N sulphuric acid at 100°C for 3 hours.

c) 4M hydrochloric acid for 4 hours at 100°C.

d) 1N formic acid for one hour, then 0.5N sulphuric acid for 16 hours.

e) Liquefied gaseous hydrogen fluoride for 3hours at room temperature.

f) 48% hydrofluoric acid at -4°C for 120 hours.

g) 1% oxalic acid at 80°C for 3 to 5 hours. This is required for furanosidic sugars e.g. fructose which are very acid labile.

Low concentration of acids are employed in the hydrolysis of the polysaccharides containing acid labile sugars. This usually takes place during partial acid hydrolysis. A full discussion of partial acid hydrolysis is in Section 2.3.1.

The problems posed by the resistance of O-methyl glycosides of uronic acids and 2-amino-2-deoxyhexosides to hydrolysis are the same as in the parent polysaccharide. Polysaccharides containing uronic acid and N-acetyl hexosamines are generally more difficult to hydrolyse after treatment with acids. The major portion of the products may consist of resistant oligosaccharides. In the case of uronic acid-containing polysaccharide, complete hydrolysis is not possible without accompanying decomposition hence complete depolymerisation is usually possible only after reduction to the corresponding hexose residues. The reduction may be performed with lithium aluminium hydride or deuteride in tetrahydrofuran or similar solvents either on the permethylated polysaccharides or on the products of partial depolymerisation with the protection of the reducing sugars if necessary. Reduction with a labelled reagent provides a convenient means of distinguishing those hexose derivatives formed from uronic acids with the incorporation of two deuterium atoms ($COOH \rightarrow CD_2OH$) when mass spectroscopy is used for identification. The problem of incomplete depolymerisation of

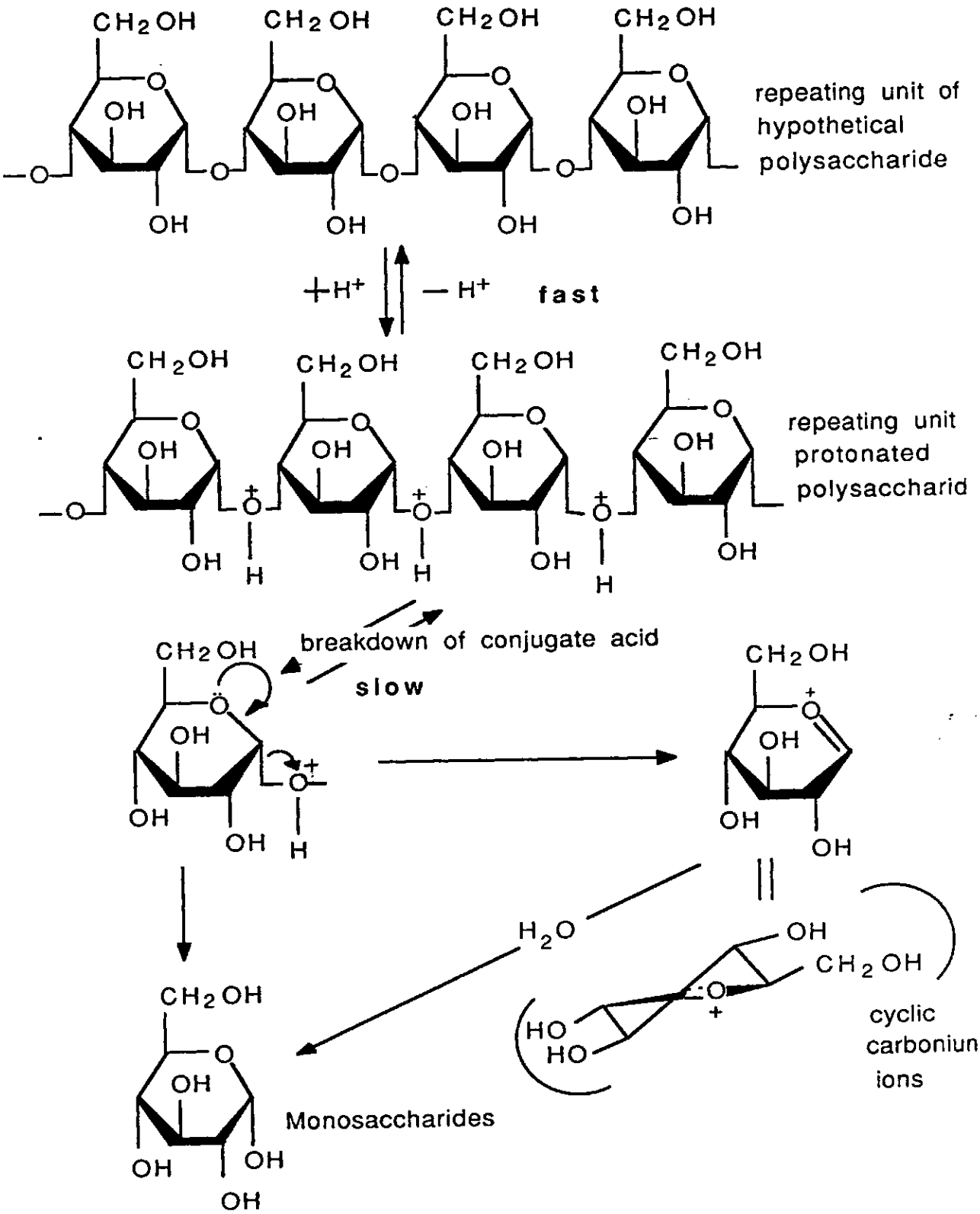
amino-deoxy glycosides can be largely averted by using acetolysis and thus minimizing N-deacetylation.

The methods (a) to (d) are normally used for the hydrolysis of the polysaccharides containing N-acetyl hexosamines but the most successful method is the treatment of these polysaccharides with liquefied gaseous hydrogen fluoride for 3hours at room temperature which cleaves the N-acetyl hexosamine glycosidic linkages preferentially.

Mechanism of Acid Hydrolysis.

Hydrolysis in polysaccharides has been shown to occur mainly with the fission of each respective glycosyl-oxygen bond with the glycosyl carbonium ions being the more stable intermediates. The mechanism involves protonation of the glycosyl-oxygen atom, followed by the slow breakdown of the conjugate acid to the cyclic carbonium ion which is then rapidly attacked by water to give the free sugar. The carbonium ion is stabilised by mesomeric electron release from the ring oxygen and for this reason the cyclic ion adopts a half chair conformation and the C-1 atom becomes sp^2 hybridized. See Scheme.2.1.on the following page.

During the hydrolysis of the N-acetyl hexosamine-containing polysaccharides, the N-acetyl residue is removed to produce free amino group on the C-2. The amino group is very effective in hindering the approach of the proton to the glycosidic bond because of electrostatic shielding by the quaternary ammonium ion formed under the acidic conditions of hydrolysis.



Scheme 2.1. : Acid Hydrolysis of Polysaccharides

2.1.1. Identification of the constituent monosaccharides.

The constituent monosaccharides can be identified by

a. paper chromatographic analysis

b. thin layer chromatography

c. gas liquid chromatography after conversion of each monosaccharide residue to suitable volatile derivative such as alditol acetate[75,76]. This is the commonest procedure in use. The sugar is reduced to the corresponding alditol and then converted to the acetate. Comparison of the retention times of the alditol acetates of the constituent monosaccharides with those of the standards sugars after injection on the gas chromatograph usually gives an indication of what the constituent monosaccharides are.

Other types of derivatives which have been used for the G.L.C. determination of the constituent sugars are methyl glycosides[78], acetates[79], aldononitrile acetates[80], trimethylsilyl esters[81], trifluoroacetates[81]. A serious disadvantage of derivatives preserving the anomeric centre e.g. methyl glycoside is that a single sugar may give rise to at least two or four peaks on g.l.c. i.e.α- and β- pyranosides and α- and β- furanosides. One advantage of using alditol acetates is that each aldose derivative will give only one peak on the g.l.c. Ketose derivatives will however give two peaks. Another advantage of using alditol acetates is that quantitation can be performed without the use of response factors[82] except for the analysis of mixtures containing N-acetamido sugars. The symmetry introduced by converting sugars into alditols is avoided by using sodium borodeuteride as the reducing agent.

Although most polysaccharides are composed of common sugar constituents, unusal sugars are sometimes encountered especially as

constituents of microbial polysaccharides. These may include deoxy- sugars, those of uncommon configurational type, and those with stable ether substituents. Such sugars may be detected first as "unknown peaks"in the chromatographic separation, and the mass spectrum from g.l.c.-m.s. may give a clue to the structural features. Thereafter, the suspected unknown or rarely encountered sugar is isolated for more complete structural characterisation by n.m.r. and if necessary by degradative methods. Confirmatory synthesis may be desirable to ensure correct configurational assignments.

2.2. The Determination of Linkage Position.

The glycosidic linkages can be determined by methylation analysis[83,84]. This procedure is used in determining the linkage point of one glycosyl residue to the other in the polysaccharide.

Methylation analysis is the oldest but still by far the most widely used procedure for the glycosidic linkage determination in polysaccharides and glycoconjugates. Basically the method involves the complete etherification of all the free hydroxyls and hydrolysis to obtain the methylated monosaccharides, borohydride reduction, acetylation followed by analysis on the G.L.C. The free hydroxyl group are those not involved in ring formation, intersugar glycosidic linkages or those carrying substituents stable to conditions used for methylation analysis.

The most widely used method for the permethylation of carbohydrates is that described by Hakomori[83]. Other methylation methods formerly used but which find application from time to time in special circumstances are mainly those of Haworth[84], Purdie[85], and Kuhn[86].

Scheme 2.2. on the following page depicts methylation analysis .

The following are the various procedures of methylation analysis :

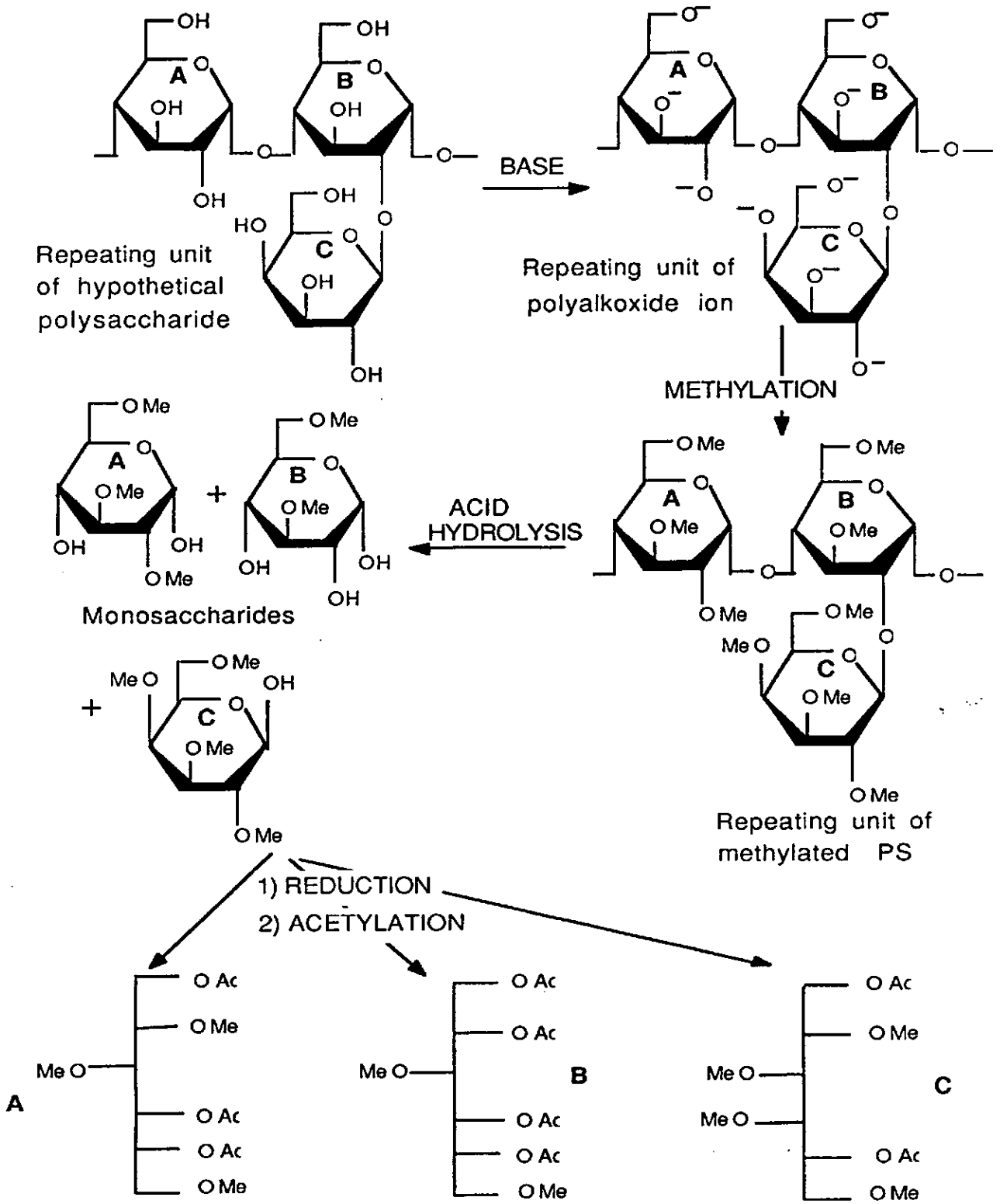
a) The method of Haworth[84] employs dimethyl sulphate and aqueous 30% sodium hydroxide at 40-60°C for 1.5 to 2 hours. Now it is mainly used in the permethylation of slightly soluble materials in dimethyl sulphoxide (DMSO) in order to improve their solubility in this solvent

b) The Purdie methylation method[85] uses methyl iodide as both the solvent and alkylating agent with silver oxide as the base. It is generally less effective and its use is restricted to materials already partially methylated and therefore soluble in methyl iodide.

c) The method developed by Kuhn et al[86] involves the use of N,N-dimethyl formamide or DMSO as a dipolar aprotic solvent with methyl iodide or dimethyl sulphate as alkylating agent and silver oxide or barium oxide (sometimes with added barium hydroxide) as base . In the Hakomori's procedure[83], the polyalkoxide ion of the substrate in anhydrous DMSO is first prepared by reaction with sodium methylsulphinylmethanide (Dimsyl sodium). Methyl iodide is subsequently added to effect methylation. This procedure has largely replaced the method of Harworth and Purdie.

With important exceptions such as base-catalysed β-eliminations, glycosidic linkages are stable to the strongly basic conditions often required for alkylation.

Scheme 2.2. : Methylation Analysis of Polysaccharides.



Partially methylated alditol acetates (analysis by g.l.c. and m.s.)

2.2.1. Methylation Analysis Procedures.

The etherification of the polysaccharide is dependent on a sufficient degree of ionisation of hydroxyl groups to achieve alkoxide formation with enhanced nucleophilicity towards alkylating agents usually methyl iodide or methyl sulphate. Effective reaction is also dependent on the polysaccharide being soluble in a convenient polar solvent. For most methylations, the method of choice is that of Hakomori in which the polysaccharide dispersed in dimethyl sulfoxide (DMSO) is allowed to react with methyl iodide. The strong base which is the conjugate base of the solvent pKa 35 ensures complete alkoxide formation and efficient etherification is achieved in one step. If the polysaccharides contain uronic acids, these are simultaneously transfered into methyl esters[87,88]. For acidic polysaccharides it is important that complete methylation be achieved in a single operation, since uronic esters are very susceptible to base catalysed β-elimination[88]. In practice, the alkoxides are so much strongly nucleophilic towards the alkylating agent than carboxylate ions that the net base concentration is markedly decreased before esterification occurs and substantial degradation during methylation is averted. The N-acyl amino sugars are recovered as N-acyl, N-methyl derivatives[89] because the presence of dimsyl ion ensures that the N-acyl groups are ionised. The O-acyl groups present in many polysaccharides are cleaved in strongly alkaline conditions but acetal and ketal functions e.g. pyruvic acid are stable.

A prerequisite for the methylation reaction is that the substrate is soluble in DMSO. Solubilisation is often facilitated by ultrasonic treatment or warming up to 70°C. Although some methylations have been achieved with partially or completely insoluble substrates,e.g. mixtures

of polysaccharides in plant cell wall preparations, most undermethylations are due to incomplete dissolution. In such cases, a portion of the material may be completely methylated while the remaining insoluble part is unmethylated. For individual polysaccharides, different methods to achieve solubility can be adopted if necessary. Low solubilities can be circumvented by acetylation with acetic anhydride and pyridine in dimethyl formamide[90] (the O-acyl groups introduced will be split off during the subsequent base treatment) or by making a partial methylation using the Hakomori, Haworth or Purdie conditions. N-methyl morpholine N-oxide (MMNO) has been shown to dissolve non-degradatively some polysaccharides that are insoluble in DMSO alone, so that methylation can be effected in the MMNO-DMSO mixture[91]. For very high molecular weight polysaccharides with certain linkages which render them insoluble, reducing the molecular weight[92-94] leads to the polysaccharide becoming completely soluble in DMSO and then methylation can be fully effected .For example, the solubilities of (1→3)-β-D glucans are known to be related to their molecular weights[92,93].

A recent alternative approach to methylation, which has been successfully applied to LPS uses the highly reactive methyltrifluoromethane sulfonate (methyl triflate) as alkylating reagent in the presence of 2,6-di-tertbutylpyridine and trimethyl phosphate as solvent[95].

The completeness of methylation can be ascertained by:

a) methoxyl determination if a large enough quantity of the methylated derivative is available for microanalysis.

b) i.r.spectroscopy: the absence of O-H stretching vibrations in the infra red spectrum simply confirms full methylation.

c) by spotting on TLC plates and comparing with the unmethylated material.

Analysis of the methylated sugars formed on hydrolysis of a fully methylated polysaccharide should, by its internal consistency, give confirmation of full etherification since there should be equimolar proportion of non-reducing end groups and branch points. The significance of minor products formed on hydrolysis of a methylated polysaccharide should be assesed with caution in the light of the structural evidence obtained by independent means. In particular, care should be taken not to assign branch points to products of incomplete methylation, which may arise either from undermethylation of the polysaccharide or from limited demethylation during hydrolysis.

2.2.2. Recovery of methylated sugars.

The methylated material is recovered by the following methods which are

a) dialysis for polymeric material.

b) reverse phase chromatography for oligomeric and polymeric materials using the Sep-pak C_{18} catridges[96] supplied by Waters Associates.

c) by partitioning between water and chloroform (for monomeric, oligomeric and polymeric materials[77].

Subsequent hydrolysis is then performed with any of the following

a) 0.5M 2,2,2-trifluoroethanoic acid (trifluoroacetic acid) for 16 hours at 100°C.

b) 2M 2,2,2-trifluoroethanoic acid for 1 hour at 120°C.

c) 4M hydrochloric acid for 4hours at 100°C.

d) 1M formic acid for 1 hour, evaporating and adding 0.5M Sulphuric acid at 100°C for 16 hours.

e) Liquified anhydrous hydrogen fluoride at room temperature for 3 hours. Most of these conditions are suitable for most polysaccharides but in certain cases stronger or weaker conditions may be necessary depending on the structural feature of the material.

Since methyl ethers are stable to hot dilute acids, the methyl groups introduced during methylation are unaffected. Hence characterisation of the hydrolysis products will then identify those hydroxyl groups since they are methylated. The hydroxyl groups formerly involved in inter-sugar linkages can then be identified since they are not methylated. For the methylated sugars the presence of a methoxyl group at C-4 or C-5 defines the ring size as pyranose or furanose.

2.2.3. Characterisation and analysis of the methylated sugars.

The complete characterisation of a permethylated polysaccharide requires the identification and quantitative analysis of all the methylated sugars formed on depolymerisation.

Separation and identification of methylated sugars.

Before the analysis of methylated sugars by g.c.-m.s became widely adopted, the mixtures of methylated sugars formed in preparative quantities were separated by

a) partition chromatography on filter sheets or column of cellulose[97].

b) adsorbtion chromatography on columns of charcoal or charcoal-celite mixture.The individual methyl derivatives were identified by

i) their characteristic physical constants e.g. melting points, optical rotations, and X-ray powder diagrams[98],

ii) demethylation by boron trichloride or tribromide and identification of the parent sugar formed[99] and by examination of the products of periodate oxidation[100].

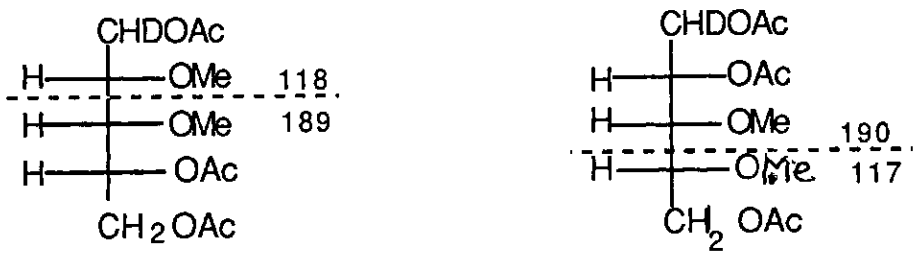
The resolving power of the gas-liquid chromatography columns coupled with the use of mass spectrometry to characterize substitution patterns in sugar derivatives, preparative-scale isolation is now rarely necessary.

Methyl esters of all the commonly occuring sugars as their suitable alternative volatile derivatives may usually be separated by using a variety of different liquid phases of the columns of the G.L.C. and the G.C-M.S. Those commonly used are the partially methylated derivatives of alditol acetates[75,76], methyl glycosides[78], acetates[79], aldononitrile acetates[80], trimethyl silyl (TMS) esters[81], and trifluoroacetates[81]. Acyclic derivatives of the methylated sugars are usually preferred because reducing sugars give rise to the formation of multiple derivatives from the same sugar. Reducing sugars, on acetylation or on conversion to TMS esters form α- and β-anomers and may, if unsubstituted on O-4 and O-5 give rise to pyranose and furanose forms. Also for this reason, methanolysis of methylated polysaccharide, with the formation of equilibrium mixture of methyl glycosides is now less frequently performed. However, exceptional situations may be encountered in which it is necessary to protect sugar derivatives, which would otherwise undergo decomposition. Examples are methylated polysaccharides containing residues of sialic acids[101] or 3,6-anhydrohexoses[102].

Partially methylated alditol acetates, formed on reduction with sodium borohydride followed by acetylation, are the most widely used derivatives for the characterisation of methylated sugars. The mass spectra of these compounds are normally simple to interprete with fragmentaton patterns

characteristic of constitution, and especially of substitution pattern. However, mass spectra is rather insensitive to stereochemical differences[77]. Molecular ions are not seen in electron inpact spectra taken at 70eV, but molecular weights can be obtained by extrapolation from fragment ions coupled with an intelligent use of g.l.c. retention time data. Direct observation of molecular ions is frequently possible in chemical ionization mass spectra using isobutane as reagent gas[103] .

The main limitation of the use of partially methylated alditol acetates for the characterisation of methylated sugars lies in the structural symmetry that may exist when the primary hydroxyl group (O-5 in pentoses and O-6 in hexoses) is not etherified. This difficulty, however can be overcome by introducing deuterium at C-1 by reduction of the sugar with sodium borodeuteride.



[I.5] **Figure 2.1.** [I.6]

Thus, in the example shown in figure 2.1, the partially methylated alditol acetate from 2,3- **[I.5]** and 3,4-di-O-methylpentitol triacetate **[I.6]** can be differentiated by the observation of the relevant isotopic shifts in primary fragments ions. Two identical m/e values of 117 and 189 would have been obtained if **[I.5]** and **[I.6]** were undeuterated and it would have been difficult to distinguish them.

Partially methylated acetylated aldononitriles are acyclic derivatives

readily formed from reducing sugars by reaction with hydroxylamine in pyridine, followed by the addition of acetic anhydride to effect elimination of acetic acid from oxime acetates and acetylation of the unsubstituted hydroxyl groups. These derivatives, although less extensively used, appear to give good g.l.c. separations, and their mass spectra can be readily interpreted without the problem of structural symmetry.

2.2.4. Other Applications of methylation analysis.

Apart from being used generally to determine the linkage positions of the constituent monosaccharides in a polysaccharide methylation analysis can also be applied in the following:

a) the presence and the position of the methylated sugars in polysaccharides which contain naturally methylated sugars. These units can be readily identified by the g.l.c.-m.s. of the derived alditol acetate obtained on hydrolysis of the polysaccharide and the subsequent reduction-acetylation. This technique has been used in the identification of the 3-O -methyl-L-rhamnose in *Klebsiella* O group 10 LPS[104] and *Rhodopseudomonas capsulata*[105] as well as for 3-O-methyl and L-xylose in the LPS from *R. viridis* and *Myxococcus fulvus*[106], respectively. Also, the location of the 3-O-methyl-L-rhamnose in the *Klebsiella* O group 10 was determined by this technique. The permethylation was performed with deuteriomethyl iodide in order to distinguish between the natural methoxy groups and those introduced in the analytical procedure. In the subsequent G.C.-M.S. analysis of the alditol acetates from the hydrolysate of the trideuteriomethylated polysaccharide it could be established that the naturally methylated sugars occupy only the terminal positions in the polysaccharide chains

since the O-methyl group was found only in the 3-O-methyl-2,4-di-O-trideuteriomethyl-L-rhamnose derivative.

b) Methylation analysis is valuable for monitoring and evaluating various specific and non-specific degradations of polysaccharides performed in the sequence determination of their sugar residues[77,107]. Partial acid hydrolysis, followed by methylation analysis, of polysaccharides containing acid labile sugar residues when compared with the methylation analysis of the intact material gives information on the attachment sites of these residues. Likewise methylated polysaccharides may be partially hydrolysed, followed by borohydride reduction and remethylation using trideuteriomethyl iodide. This method yields similar but more detailed information on the position of acid labile sugars[107]. The Smith degradation may conveniently be evaluated in this way. In particular, the polyalcohol obtained on periodate oxidation-borohydride reduction may be methylated before the mild acid hydrolysis step. Trideuteriomethylation after this treatment will give detailed information on the positions to which the oxidised residues were linked[107].

c) Methylation analysis has also been used to investigate the products obtained on uronic acid degradation of polysaccharides and on oxidative-alkaline elimination of partially methylated polysaccharide[107].

d) Methylation analysis is also an important technique for the location of O-acyl groups in natural carbohydrates. In the method devised by Norrman and de Belder[108], the substrate is first acetalated by treatment with vinyl ether and then investigated by methylation analysis. The position of the O-methyl groups in the monomeric sugars reflect

the substitution of O-acyl groups in the native material[109,110].

e) The use of methylation analysis to monitor enzymic degradations of polysaccharides and glycoconjugates has as yet only found limited application but offers obvious possibilities. In such experiments, information could be obtained on the penultimate residue in the degraded material.

2.3. Determination of the Glycosyl Sequence.

Various specific degradation methods are used in the sequence analysis of a polysaccharide. There are no standard procedures since each polysaccharide presents its own problems.

There are a range of conceivable methods for the specific degradation of polysacharides. However, a summary of the more common general procedures including those used to determine the sequence of *E. coli* O149 and the *Streptococcus pneumoniae* type 37 is discussed below. They are:

a) Partial acid hydrolysis.

b) Alkaline degradation which causes β-elimination mainly for the uronic acid components.

c) Oxidative hydrolysis.

d) N-deacetylation and deamination.

In principle any reaction that can be applied at the monomer level should be applicable to polysaccharide. Applications of consecutive degradations each of which should be ideally quantitative should facilitate determination of complicated structures with moderate efforts. Liquid chromatography is an established method of separation of the low molecular weight products formed and high-pressure liquid chromatography is becoming increasingly important. Mass-spectroscopy and nuclear magnetic resonance

spectroscopy, especially when combined with pulse Fourier transformation are very important for the characterisation of the degradation products.

2.3.1. Partial Acid Hydrolysis.

An often-used technique for sequence determination is partial acid hydrolysis followed by isolation and characterisation of the oligosaccharides formed. The isolated degraded poly- and oligosaccharides then give more detailed information and often unambiguous information on the sequence in addition to providing confirmation of linkage types.

There are several polysaccharides in which some of the glycosidic linkages differ in lability. Alternatively it is sometimes possible to create such linkages by chemical modification of the polysaccharide. Under such conditions fragmentation can be performed with a high degree of selectivity. Weak glycosidic linkages are most frequenty associated with furanose and deoxy-sugar residues[109]. Fructose, ribose, apiose occur as furanosides in nature while arabinose and galactose may be either furanosidic or pyranosidic. Furanosides are hydrolysed faster than corresponding pyranosides by a factor of 10 to 10^3 but 6-deoxyhexosidic linkages e.g. those of fucose, rhamnose are hydrolysed only 5 times faster than those of the corresponding hexoses. Deoxy-sugars especially when the deoxygenated carbon atoms are in the ring, are also readily liberated from terminal sites even when in pyranosidic linkage.

3,6-dideoxy-hexopyranosidic linkages which occur in certain bacteria e.g. abequose[110] (3,6-dideoxy-D-galactose) are hydrolysed more readily. Glycosides having deoxy functions in the position vicinal to the glycosidic carbon atom are very sensitive to acid hydrolysis.
Representatives of this class are the 3-deoxy-glyculosonic acids the most common being 2-keto-3-deoxy-D-manno-octulosonic (KDO) and neuraminic acid

(5-amino,3,5- dideoxy-D-glycero-D-galacto-nonulosonic acid). These linkages will be selectively hydrolysed in polysaccharides under mild acid conditions producing monosaccharides or oligosaccharides or both. The investigation of these products will therefore produce information of structural significance by sugar analysis and methylation analysis.

Conversely, glycosiduronic acids are much more resistant to hydrolysis than the corresponding neutral glycosides and complete hydrolysis is not possible without accompanying decomposition[111] involving extensive epimerisation and degradation. Therefore partial acid hydrolysis of a glycusiduronic acid-containing polysaccharide conveniently leads to the isolation of acidic disaccharides (aldobiuronic acids) and higher oligosaccharides. However, the reduction of the hexuronic acids to hexose residues may be achieved before hydrolysis by successive treatment of the polysaccharide with a water soluble carbodiimide and sodium hydroxide[111a].

Polysaccharides containing 2-amino-2-deoxyglycosidic linkages are resistant to acid hydrolysis, because of the electrostatic shielding by the quartenary ammonium ion present under acidic conditions[112]. Oligosaccharides are therefore obtained on hydrolysis of polysaccharides containing such bonds. In order to take advantage of the acid resistance of the 2-amino-2-deoxy aldosidic linkages,the polysaccharides can be N-deacetylated[113-115]. The resulting 2-amino-2-deoxy-glycosidic linkages are then almost completely resistant to acid hydrolysis and amino disaccharides can be readily isolated as products of partial hydrolysis.

Despite the fact that complex mixtures of oligosaccharides may be obtained and that the yields of individual oligosaccharides may be low, partial acid hydrolysis is still useful when combined with G.C.- M.S. or high performance liquid chromatography.

2.3.2. Partial Depolymerisation under Non-Aqueous Conditions.

The non-aqueous conditions for partial depolymerisation are

a) Methanolysis

b) Acetolysis

c) Trifluoroacetolysis

The two possible advantages which may result from the use of non-aqueous conditions for partial depolymerisation are that

i) When depolymerization is carried out under non-aqueous conditions, the reducing sugar is converted to the glycoside which is a more stable form of the sugar. On hydrolysis of the low molecular weight material obtained from the depolymerisation the end sugar remains stable because of the protection of C-1 by the alkyl group in the reaction medium. This is an advantage of the procedure in non-aqueous solvent. Certain sugars e.g. 3,6-anhydrohexoses are readily destroyed under normal conditions of hydrolysis but may be trapped by derivative formation.

ii) Another advantage is that during acetolysis, the relative susceptibilities of the different glycosidic linkage to cleavage may be altered to an extent that the "cracking pattern" may lead to a significantly different collection of oligosaccharide as products of partial depolymerisation and thus shed more light on the structure of the polysaccharide.

a) Methanolysis[107].

This is the most commonly used of all these procedures because of the first advantage stated. The reagent used is 2M methanolic hydrochloric acid at 80°C for 16 hours leading to the methylation of the free OH group on C-1 of the reducing sugar generated.

b) Acetolysis[107].

Partial acetolysis is generally performed with a mixture of acetic

anhydride, acetic acid and 2-4% sulphuric acid on a polysaccharide or preferably its acetylated derivative. The resulting mixture of the sugars are then de-O-acetylated with sodium or barium methoxide to give mixture of mono- and oligosaccharides. Acetolysis is complementary to acid hydrolysis as the relative rates of cleavage of the glycosides in the two reactions are sufficently different and are sometimes reversed, e.g. the (1→6) linkages between hexose residues which are more resistant to partial acid hydrolysis are prefentially split during the procedure. It is also a convenient procedure when the parent polysaccharide is insoluble in most aqueous solution but soluble in acetic anhydride-acetic acid mixture. For example cellulose is depolymerised by this method. Although acetolysis has been used mostly for several structural determinations involving polysaccharides from different sources e.g. yeast and fungi[116] algae[117], glycopeptides[118], it has been also used for the isolation of neuraminic acid (sialic acid)-containing oligosaccharide obtained from a bacterial polysaccharide. Neuraminic acid linkages are extermely labile to aqueous acid often with decomposition of the liberated sugar, but may remain intact to permit the isolation of neuraminic acid-containing oligosaccharides.

b) Trifluoroacetolysis.

The potential applications of trifluoroacetolysis using mixtures of trifluoroacetic anhydride and trifluoroacetic acid have been employed primarily for glycoconjugates[119] and oligosaccharides containing N-acetylhexosamine[120]. However, it is a procedure used mainly for the N-deacetylation of polysaccharides[121].

2.3.3. Periodate oxidation of Polysaccharides.

The commonly used procedure of oxidative hydrolysis in the sequence determination of bacterial polysaccharides is the periodate oxidation. Although periodate oxidation is not an absolute method for structural analysis of polysaccharide, it does provide invaluable information when taken together with the results of identification of the components of hydrolysis of either the polysaccharide itself or of its methyl ether while particular attention must be paid to the methods used for the determination of the products and of the consumption of the periodate[122].

The oxidation can be carried out with periodic acid, sodium metaperiodate, or potassium metaperiodate. The concentration of periodate is usually in the range of 0.01 to 0.1M and always in excess. The reaction is carried out in the absence of light since some of the products such as formaldehyde are oxidised in bright sunlight whereas they are not at all oxidised in the dark[123].

Vicinal hydroxyl groups are oxidised by the periodate and are hydrolysed faster in a slightly acidic medium pH 3 to 5. The reaction is generally carried out in distilled water and care should be taken to prevent the medium becoming alkaline since the aldehydic products of cleavage are very sensitive to alkali. There is also the possibility of "overoxidation" or "underoxidation"[124-126]. Overoxidation is most frequently encountered when the oxidation results in the formation of tartronic acid half-aldehyde products or tartronaldehyde derivatives from hexofuranosides or heptopyranosides[124,125]. The incomplete oxidation of vulnerable sugar residues may arise when hemiacetal formation between aldehyde fragments in oxidatively cleaved residues and hydroxyl groups in adjacent but not yet oxidised residues protects the latter from oxidation.

Incomplete oxidation may also occur in 4-linked polysaccharides whose sugar residues do not carry primary hydroxyl groups at C-6 also due to the formation of hemiacetal. The hemiacetal linkages are disrupted on reduction with sodium borohydride, and the protected groups but formerly vulnerable diol groups are reexposed for oxidation. Depending on the polysaccharide, the oxidation-reduction sequence may have to be repeated twice before full oxidation is achieved. Another cause of protection against peroxidate oxidation arises from hydrogen bonding between one pair of hydroxyl groups, normally susceptible to oxidation, and a suitably disposed acetamido group on a neighbouring sugar residue.

Formic acid is liberated from either the non-reducing end groups or units linked in the (1→6) fashion in polysaccharides containing hexopyranose units. Formaldehyde is produced from polysaccharides containing hexofuranose units if both 5 and 6 positions are not involved in glycosidic linkages. Formaldehyde is also produced from polysaccharides containing heptose units if positions 6 and 7 are not involved in glycosidic linkages. Hexopyranose units linked 1→3 or engaged in branching at position 1→3→6 or 1→2→4 positions and 1→3→4 are resistant to periodate attack because of the absence of vicinal hydroxyl groups. Owing to the possibility of overoxidation, considerable caution should be exercised in interpreting the data of polysaccharides containing uronic acids particularly when they are present as non-reducing terminal units or where they are linked through the 2-position.

The parts of a polysaccharide that are not oxidisable can be obtained separately from the oxidised residues as mono-, oligo- or poly- saccharides and characterisation of these products then give significant structural information. It is essential that no overoxidation occurs. The latter is excluded by performing the reaction with the exclusion of light at low

temperature 5°C and at pH 3 to 3.5. The use of oxygen-free water and the addition of ethylene glycol as a radical scavenger also lessens radical induced depolymerisation during oxidation.

The two major methods now available for the controlled degradation of the periodate-oxidised polysaccharides are:

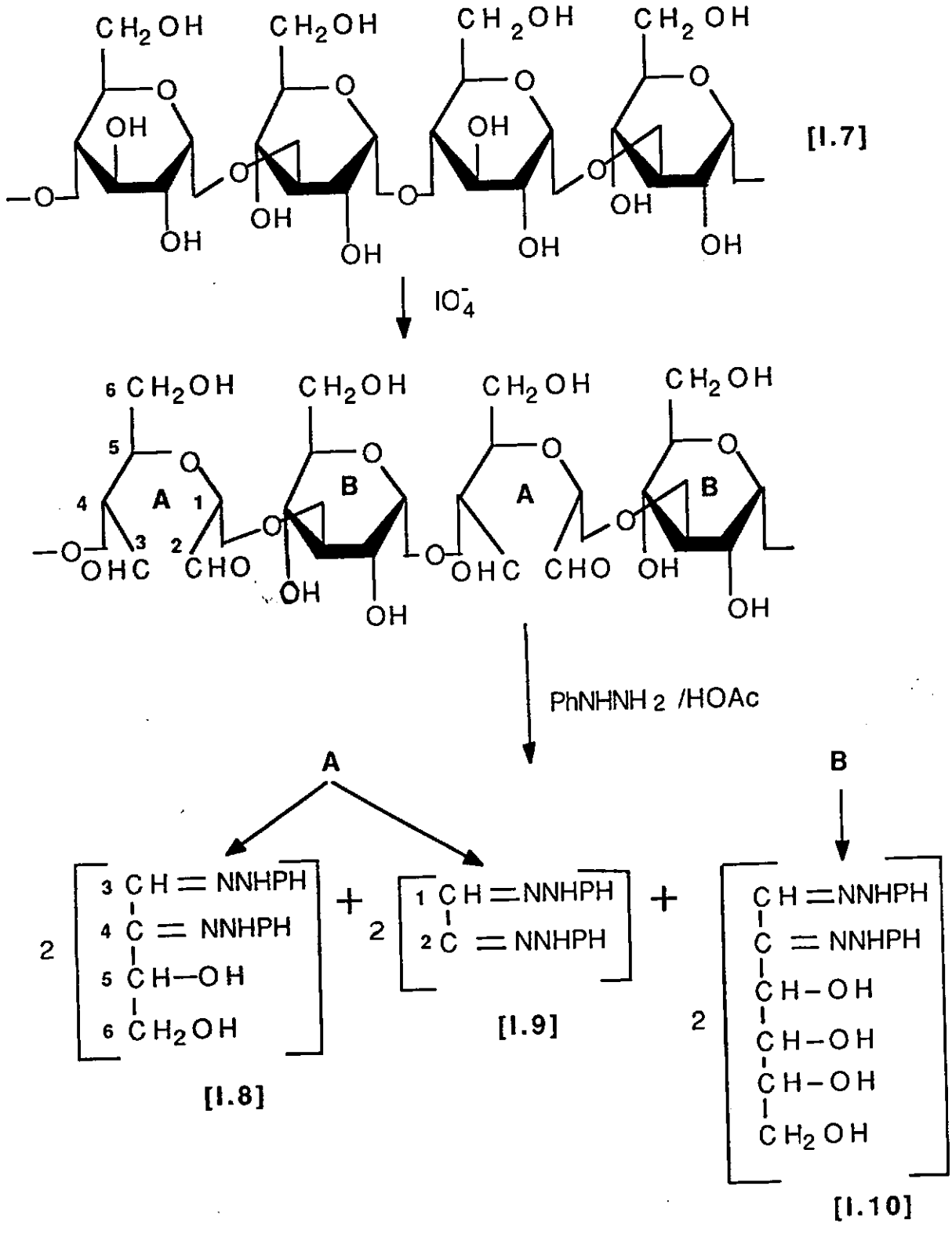
a) Barry degradation[127].

b) Smith degradation[128].

a) Barry degradation

This procedure involves the treatment of the periodate-oxidised polysaccharide with phenyl hydrazine in dilute acetic acid. Such a degradation is based on the fact that phenyl hydrazine in dilute acetic acid converts an acetate of glyoxal $OCH.CH(OR)_2$ into glyoxalosazone with the liberation of the alcohol which is ROH. An example is the degradation of periodate oxidised nigeran[129] [I.7], see Scheme 1.3. In the Barry degradation, as exemplified by nigeran [I.7], the oxidised residues are split off as bis phenyl hydrazones of a triulose, tetrulose [I.8] or glyoxal [I.9]. Depending on whether they are adjacent or not, the non-oxidised residues give phenyl osazones of mono-, oligo- or poly-saccharides [I.10]. Such a degradation is valuable in structural determination whether or not there are long sequences of periodate resistant residues which would yield oligo- or poly- saccharide osazones or whether are isolated units as in the case sited. The polysaccharide remaining after the degradation is analysed and can be subjected to consecutive degradation. However the Barry degradation has lost some of its importance because similar results are obtained by the Smith degradation which also gives similar but cleaner products.

Scheme 1.3. : Barry Degradation of Nigeran.



b) Smith degradation[128].

This method uses the sequence

i) Periodate oxidation of the vicinal hydroxyl groups.

ii) Subsequent borohydride reduction of the aldehyde functional groups at room temperature.

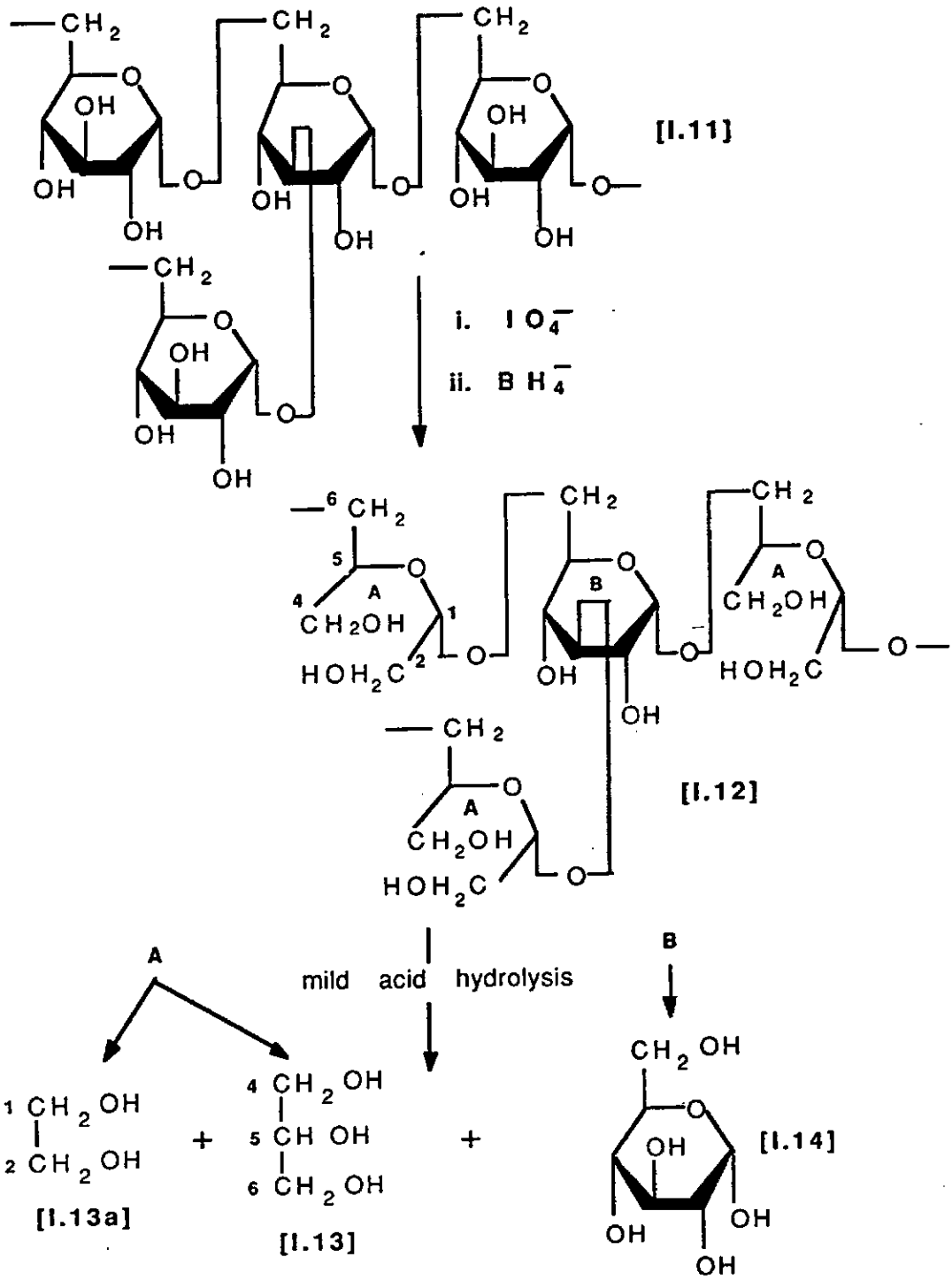
iii) Mild acid hydrolysis with dilute aqueous acid.

When a sugar residue of a polysaccharide is cleaved by periodate and reduced, the resulting alcohol derivative being a true acetal is sensitive to acid, whereas, when a sugar unit which survives cleavage is joined to a unit which is cleaved, the surviving unit appears as a glycoside which is relatively stable to acid. Because of the marked difference in the stability between acetals and glycosides, it is now possible to obtain a wide variety of polysaccharides, glycosides of mono-,di-, and oligosaccharide structures which throw light on the fine structure of the parent polysaccharide. After the oxidation, the modified sugar residues contain acyclic acetal residues which are hydrolysed much faster than glycosidic linkages. The acid hydrolysis used are very mild so that normal glycosidic linkages are generally not broken. The product therefore contains small fragments of C-2, C-3, C-4 polyalcohols and other cleavage fragments derived from oxidised residues. They can then be separated by chromatography and identified. Characterisation of the degradation products often affords considerable structural information. Complete hydrolysis of the polyalcohols and characterisation of the products may also give results of structural significance.

The reduction of the periodate oxidised material can be performed catalytically by hydrogen over platinum or a Raney nickel catalyst under pressure (700 to 1500 psi) at 85 to 90°C for 7 hours, but the use of sodium

borohydride is more convenient. Concentrated sodium borohydride (10mg/ml) is used to lessen competing β-elimination reactions. The aldehyde groups in the oxidised polysaccharide form cyclic hemiacetals with suitably situated groups. The rate of hydrolysis of these cyclic structures is then of the same order of magnitude as that of the intact glycosidic linkages but on the reduction to the polyalcohols however they become part of the non-cyclic structures and are hydrolysed at a much higher rate than the glycosidic linkages.

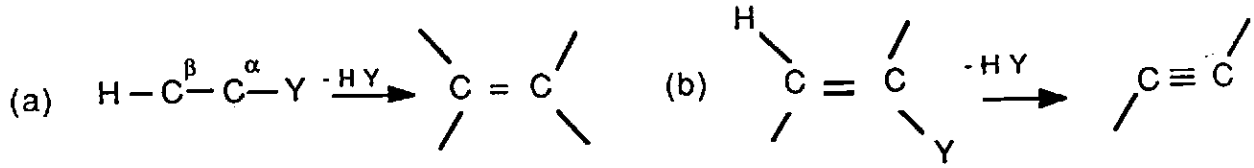
One advantage of the Smith degradation is that the aglycone of the low molecular weight glycoside formed provides structural information concerning the sugar residue from which it was derived e.g. a 2-O-substituted tetritol would generally be derived from a 4-substituted hexopyranose residue, while a 2-O-substituted glycerol will be derived from a 4-O-substituted pentopyranose. A 1-O-substituted glycerol will be derived from a 6-linked hexopyranose residue. Smith degradation is the specific degradation technique most frequently used in identifying which of the sugar moieties are resistant to periodate attack e.g. 3-linked units or 3-blocked units generally. An example of the procedure is its application to dextran the partial structure which is **[I.11]** in Scheme 2.4. Acid hydrolysis of **[I.12]** yields a mixture of glycerol **[I.13]** and glucose **[I.14]**.



Scheme 2.4 : Smith Degradation of Dextran

2.3.4. Degradation based on β-Elimination.

Elimination reactions involve the removal of two atoms or a group from a molecule of without their being replaced by other atoms or groups. In the great majority of such reactions, the atoms are lost from adjacent carbon atoms, one of them very often being a proton and the other a nucleophile Y: or Y^- resulting in the formation of multiple bond. This is the 1,2 or α, β-elimination and it is often referred to as β-elimination as shown in (a) and (b) of Scheme 2.5.



Scheme 2.5.

Examples of β-elimination are the base induced elimination of hydrogen halide from alkyl halide and the acid catalysed dehydration of alcohols There are other types of elimination reactions in which

i) both atoms are lost from the same carbon atom i.e. 1,1-eliminations

ii) atoms further apart than the 1,2- i.e.the reversal of the 1,4-additions.

iii) carbon atoms 1 and 5,or 1 and 6 i.e.1,5 and 1,6-elimination leading to cyclisation. However β-elimination is by far the most common.

β-elimination in polysaccharides[130,131]

Several kinds of groups e.g. alkoxyl and hydroxyl groups in the β-position to an electron withdrawing group, such as carbonyl, carboxylic ester, amide, sulphone are eliminated on treatment with base. The presence of a hydrogen atom in the α-position to these groups is essential and alkoxyl groups are eliminated more readily than hydroxyl groups.

β-elimination at the reducing sugar residue has been useful in structural studies of oligosaccharides[130,131] but it has been found to be only of limited value when applied directly to polysaccharides. This is because side reactions may completely obscure the results after the first three or four residues have been eliminated from a polysaccharide and in some instances, the degradation may stop after only a few residues have been eliminated. For polysaccharides of irrregular structure, the structural composition in the vicinity of the reducing terminal may not be the same in the different molecules. Also as a result of biosynthesis or the isolation procedure, there may not even be any reducing terminal in the polysaccharide molecules or at most only in some of them.

In the β-elimination reactions involving polysaccharides, the electron withdrawing groups are first introduced chemically into the polysaccharide; this may be done by esterification of a carboxylic acid group, by oxidation of an alcoholic group to a carbonyl group, or by neucleophilic displacement reactions.

β-elimination reactions made use of in glycosyl sequence determination in polysaccharides are

a) Degradation preceeded by oxidation

b) Degradation by using sulphone derivatives

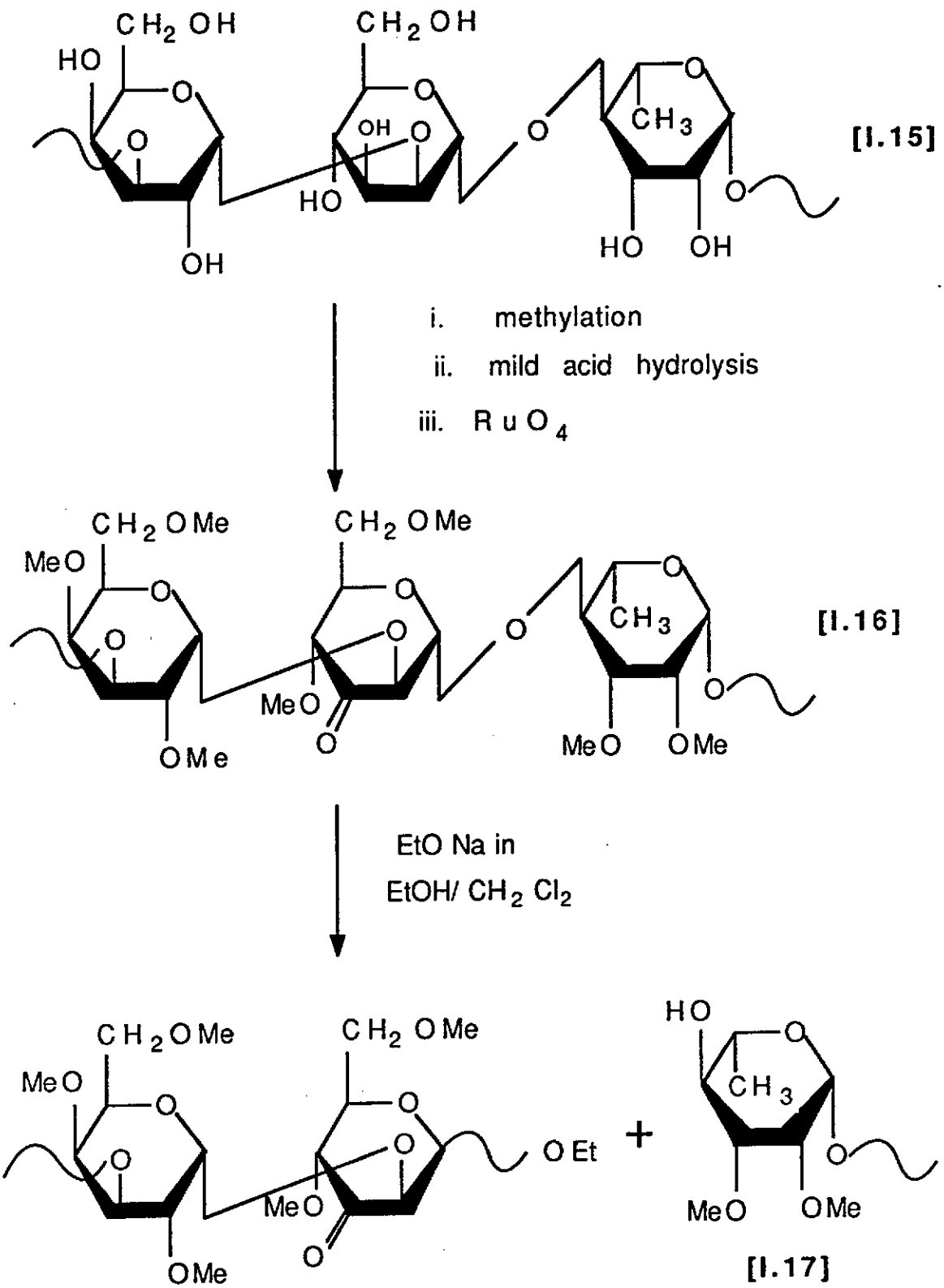
c) Degradation of polysaccharides containing uronic acid residues.

a) Degradation preceeded by oxidation.

By applying suitable selective procedures, methylated polysaccharides having limited number of free hydroxyl groups can be prepared. A method has been developed by which such modified polysaccharides may be specifically degraded by oxidation of the alcohol groups to carbonyl groups followed by alkaline β-elimination and mild acid hydrolysis.

Although, there are only a few published examples of this degradative method in literature, its applicability in structural polysaccharide chemistry looks quite favourable.

As an example, a polysaccharide of a known structure which has been subjected to this degradation is that from the LPS of Salmonella typhimurium LT 2[131]. The fully methylated LPS composed of oligosaccharide repeating units **[I.15]** in **Scheme 2.6.**, was first subjected to mild acid hydrolysis whereby the abequose residues linked to the O-3 of the D-mannopyranose residues were hydrolysed off together with the lipid components. The partially methylated polysaccharide resulting was oxidised with ruthenium tetraoxide and the products, composed of the same trisaccharide repeating units **[I.16]** but with a keto function at the C-3 of the mannopyranosyl residue was treated with sodium ethoxide in ethanol-dichloromethane. Some of the D-galactopyranose residue carry a terminal D-glucopyranose residue at C-4, which in the present treatment is disregarded. From the results of the model experiment, it was predicted that the 2,3-di-O-methyl-L-rhamnose residue **[I.17]** should be released by this treatment. Methylation of the degraded product with trideuteriomethyl iodide, followed by hydrolysis yielded 2,3-di-O-methyl-4-O-trideuterio- methyl-L-rhamnose. All the sugar residues except D-mannose in the original polysaccharide were accounted for and from the results of the degradation, the sequence →3)-Man-(1→4)-Rha-(1- was established as in shown in Scheme 2.6.



Scheme 2.6. : β - elimination Degradation of Salmonella typhi LT 2 PS

b) Degradation by way of sulphone derivatives.

It is possible to replace the primary hydroxyl group at C-6 in aldohexoses and related polysaccharides by an electron attracting group such as para-toluene sulfonate followed by displacement with iodine. In a subsequent nucleophilic substitution, this group is replaced with iodine. For example when methyl β-D-glucopyranoside is subjected to this treament, the resulting product is 6-deoxy-6-iodo-β-D-glucopyranoside. On treatment with base, the glycosidic linkage is cleaved. By applying this sequence of reactions certain linkages in the polysaccharide may be specifically cleaved. This degradation has been applied to the capsular polysaccharide elaborated by Pneumococcus type II[133] after methylation analysis and uronic acid elimination and the results obtained were in accordance with the results obtained from another procedure. However, the sulphone degradation is laborious because of the several steps involved. There is also the difficulty in obtaining good yields hence it has not been used frequently in the structural studies of polysaccharides. There is also the difficulty in obtaining good yields hence it has not been used frequently in the structural studies of polysaccharides.

c) Degradation of polysaccharides containing uronic acid.

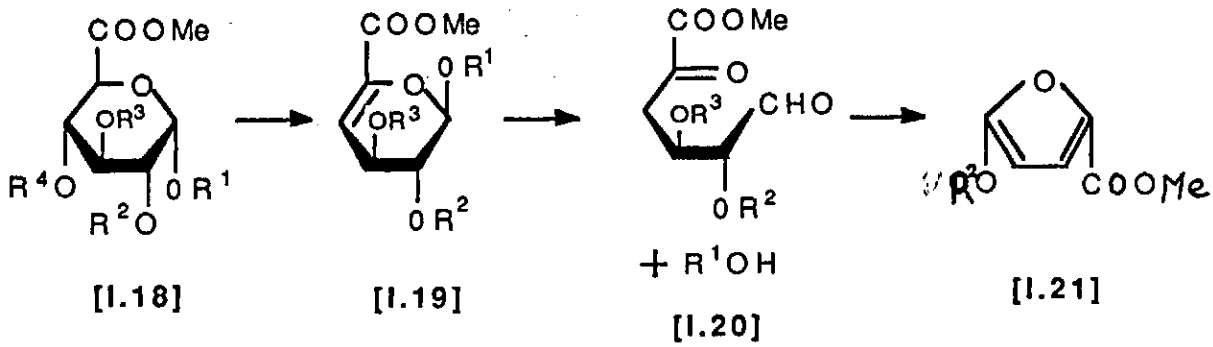
On esterification, each carboxyl group in the uronic acid residues becomes a carboxylate and on subsequent treatment with base, β-elimination occurs.

This degradation when applied to polysaccharides, is subject to certain practical limitations. The esterification of the polysaccharide by use of ethylene oxide or propylene may take several weeks, and the yield in the degradation is moderate (about 50%). A further restriction is that the uronic acid residue should be substituted at position 4. To overcome these limitations, the polysaccharide is first methylated by the Hakomori

limitations, the polysaccharide is first methylated by the Hakomori procedure[83,134] with sodium methyl sulfinyl carbanion (dimsyl sodium) and methyl iodide in DMSO whereby all the hydroxyl groups are etherified and the carboxyl groups are esterified simultaneously.

Elimination does not occur during the methylation presumably because the strong base is rapidly decomposed by reaction with methyl iodide which is present in excess. On treatment of the methylated product with base, either sodium methoxide in methanol or dimsyl sodium in DMSO, the 4-substituent on the uronate residue [I.18] in Scheme 2.7.[135] is eliminated. Anhydrous conditions are essential to avoid saponification and may be maintained by adding 2,2-dimethoxypropane and a catalytic amount of para-toluene sulfonic acid to the system before the addition of the base. The sodium methyl sulfinyl carbanion reagent also adds to the ester group, with the formation of a methylsulfinyl ketone but this occurs at a rate considerably lower than that of the β-elimination. The unsaturated product [I.19] which is an ester is labile to acids and on mild acid hydrolysis releases the aglycone R_1OH.

The intermediate [I.20] should by analogy with the 3-deoxy-glyculosonic acids[135] react further and yield furan [I.21] with the simultaneous release of the substituents at O-2 and O-3. The reaction sequence is shown in Scheme 2.7. However the reactions are not as simple as shown in the scheme. This is because some cleavage of the glycosiduronic linkage has also been observed during the alkaline treatment of the fully methylated polysaccharide containing uronic acid. Elimination of a terminal uronic acid residue by successive treatment with base and mild acid hydrolysis at the site of linkage produces a hydroxyl group. The hydroxyl group may be located by etherification of the product preferably with trideuteriomethyl or ethyl iodide. This is followed by acid hydrolysis and the analysis of the resulting mixture of sugars by G.L.C.-M.S.

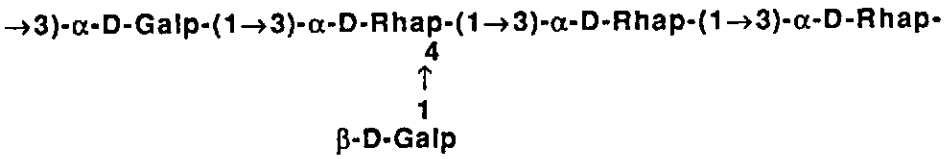


R^1 = Sugar residue , R^2, R^3 & R^4 = alkyl group or sugar residue.

Scheme 2.7.

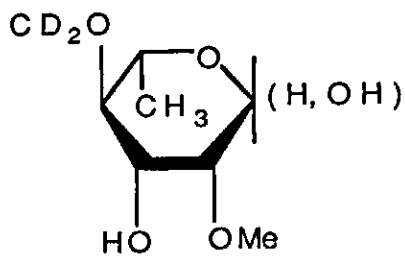
The freshly generated hydroxyl groups in the fully methylated polysaccharide, may be used as starting points for a new degradation.

This method has been tested on the Pneumococcus type II[133] and Klebsiella type 9 capsular polysaccharides composed of pentasaccharide repeating units [I.22].



[I.22]

The expected sugar [I.23] representing the sugar residues to which the uronic acid had originally been linked was obtained in the analysis of the polymeric products. The yield in these degradations were 90%[136].



[I.23]

Other specifc degradation reactions of Uronic acid.

Other specific degradation reactions involving the selective cleavages of the glycosiduronic acid linkages that have been applied in sequence determination but are not of widespread use are:

i) Hofmannn-Weerman Rearrangement[137].

ii) Curtius Rearrangement[138].

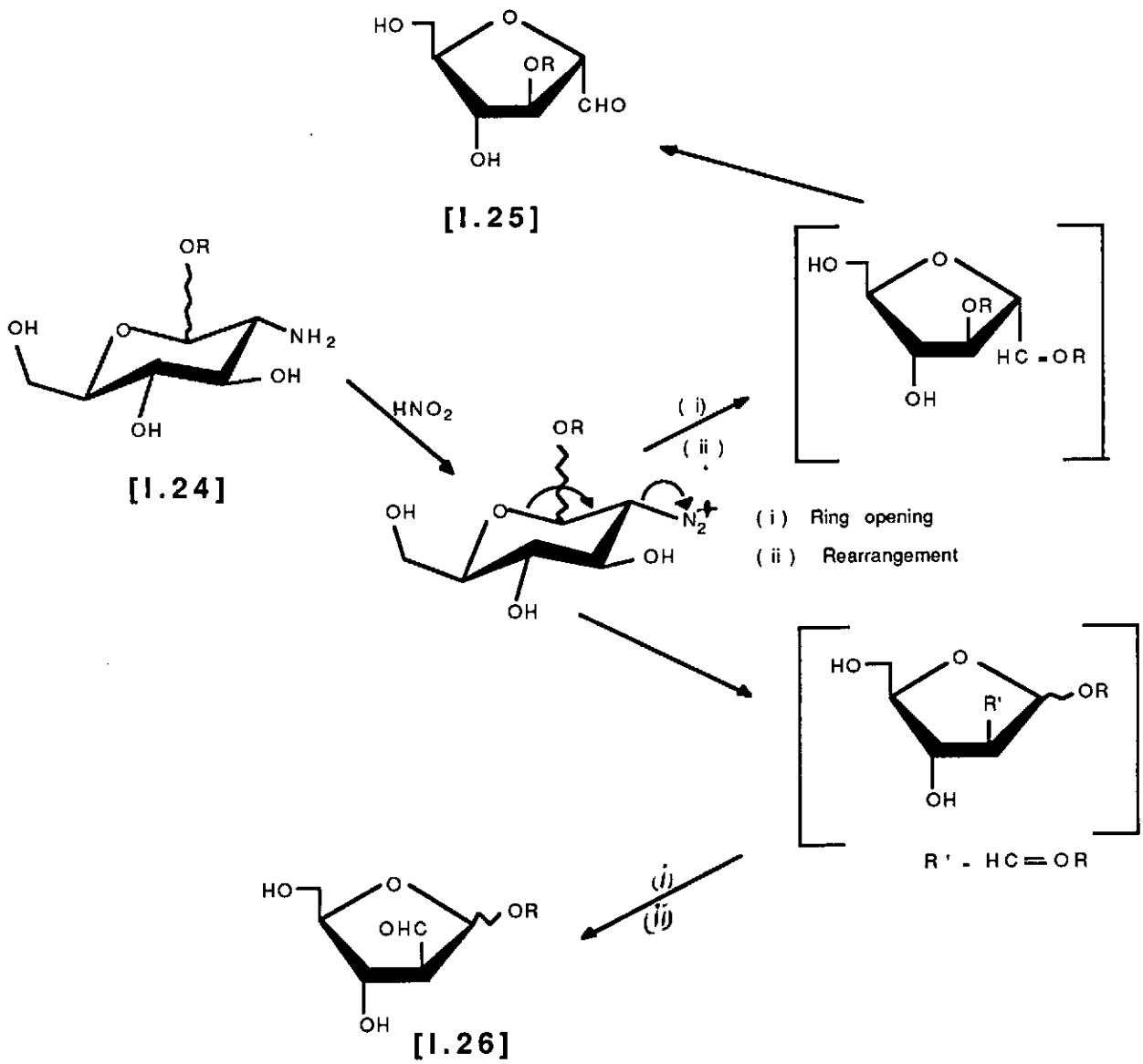
iii) Lossen Rearrangement[139].

2.3.5. N-Deacetylation and Deamination

This is applicable to 2-amino-2-deoxyglycosidic bonds. Degradation by deamination is the selective cleavage of the equatorially oriented 2-amino-2-deoxyglycosidic bonds by nitrous acid with the formation of 2,5-anhydro sugars. The deamination proceeds by rearrangement resulting from the attack upon the intermediate diazonium ion by the ring oxygen atom which is in a trans and parallel disposition. The glycosidic linkage (OR^1 at position 1) is simultaneously cleaved rendering the reaction useful in structural studies[140-142].

Most frequently, the amino sugars occur as N-acetyl derivatives and have to be N-deacetylated[113-115] before deamination[143,144]. This reaction is best accomplished either by hydrazinolysis with anhydrous agent and a catalytic quantity of hydrazine sulfate[113] or by treatment with sodium hydroxide in DMSO[114] or by trifluoroacetolysis[115]. For the deamination, the nitrous acid is prepared in situ and the reaction is allowed to proceed for not more than one hour so that the amount of unwanted by-products is minimized in the mixture.

The diazonium ion from a 2-amino-2-deoxy-D-glucoside [I.24] yields 2,5-anhydro-D-mannose [I.25] as shown in scheme 2.8. while the ion from the 2-amino-2-deoxy-D-galactoside yields 2,5-anhydro-D-talose. Amino sugars



Scheme 2.8. The degradative pathways of nitrous acid deamination

incorporated in a polysaccharide chain react similarly. Studies have revealed that the deamination of 2-amino-2-deoxy-D-glucosides is more complicated than previously assumed. It has been reported[143] that in addition to the 2,5-anhydro-D-mannose, 2-C-formyl pentofuranosides [I.26] are formed. This reaction occurs with epimerization at C-2 with the liberation of 3-O-glycosyl substituents when present. The two degradative pathways of the nitrous acid deamination are shown in Scheme 2.8. above.

Deamination of aminodeoxyglycosides gives a variety of products

depending on location and stereochemical orientation of the amino groups in relation to the neighbouring groups; rarely is a single type of product formed[143]. However the 2,5-anhydro sugars are usually formed in addition to other side products. One of the first polysaccharides to be studied was chitin which was N-deacetylated with hydrazine and the product deaminated to give 2,5-anhydromannose[145].

2.4. Determination of Anomeric Configuration.

The anomeric configuration can be determined by three methods namely

a) Chromium trioxide oxidation[146].

b) Enzyme catalysed oxidation[147-149]

c) ^{1}H and ^{13}C n.m.r. nuclear magnetic resonance spectroscopy[150].

a) Chromium trioxide oxidation.

Fully acetylated aldopyranosides in which the aglycon is equatorially oriented in the most stable chair form generally the β-anomers are oxidised by chromium trioxide in acetic acid with a high degree of selectivity. An aldohexoside yields a 5-hexulosonate in this reaction. The anomer with the axially attached aglycone group . i.e.the α-anomer is oxidised only slowly[146]. However, the validity of this procedure is dependent on the conformational stability of the commonly occuring aldopyranosides and may be unreliable for conformationally flexible sugars for which the energy differences between the two chair forms is small e.g. glycosides of 3,6-dideoxy-D-arabinohexose. Another problem that may be encountered when this method is applied is the difficulty of preparing fully acetylated polysaccharide. Sugar residues containing free hydroxyl groups will be oxidised with chromium trioxide independent of their anomeric configuration and this will obscure results. For this reason, the method has failed

completely in some instances. Furthermore it is not possible to protect the the hydroxyl groups by methylation, as methoxyl groups are oxidised to formyl groups at a rate comparable to that at which β-glycosides are oxidised.

However, in the instances when this procedure has been applied successfully to some bacterial polysaccharides[151-153], the ester linkages (acetates) formed during the oxidation are cleaved during the subsequent methylation analysis. By comparing the original methylation analysis with that of the oxidised sample, the sequence of the sugar residues may also be determined. An example is the oxidation of the acetylated LPS from Salmonella kentucky[154] revealed that the L-rhamnose and the D-galactose are β-linked and the D-mannose residues are α-linked as shown by the structure of the repeating unit [I.27].

```
→ 4)-β-L— Rhap-(1→ 2)-α-D-Manp-(1→ 2)-α-D-Manp-(1→ 3)-β-D-Galp-(1
          3                                  4
          ↑                                  ↑
          1                                  1
     α-L-Abep                           2AcO--D-Glcp
```

[I.27]

The sugar analysis of [I.27] before oxidation shows

i) An Abequose unit

ii) A Rhamnose unit

iii) Two Mannose units

iv) A Galactose unit

v) A glucose unit

After the chromium trioxide oxidation, rhamnose and galactose were not recovered. On the methylation analysis of the oxidised product, 2,3,4,6-tetra-O-methyl-D-mannose (terminal) and 3,4,6-tri-O-methyl-D-mannose were

obtained. As the two main residues are the only α-D-linked sugars in the chain, then they are adjacent as shown in the structure of the oligosaccharide repeating unit.

b) Enzyme catalysed hydrolysis.

Various types of enzymes have been used for the determination of anomeric configuration and this usually leads to partial hydrolysis of the bacterial polysaccharides producing oligosaccharides and thus can give information on the sequence. All glycosidases and glycanases (glycan hydrolases) are specific for the sugar unit undergoing hydrolysis and for its anomeric configuration. Exoglycosidases whether used individually or generated in a stepwise manner by sequential induction, act on the polysaccharide substrate by removal of the non-reducing terminal units usually without regard to the type of linkage to the next innermost residue.

Exoglycanases also act from non-reducing termini but are highly specific as to linkage type and may give rise to products of greater complexity than simple monosaccharides. The specificity of these enzymes require that they can only approach a limited distance of other structural features, e.g., a different linkage type and a branch point.

In contrast, endoenzymes are not limited by action pattern. They can cleave unbranched regions of both internal and external chains of the requisite type, but they are similarly restricted to branch points or other different structural features.

A great advantage of enzymatic hydrolysis in the determination of polysaccharide structure is that only the more acid-resistant glycosidic linkages are cleaved in the presence of acid-sensitive linkages e.g. L-arabinofuranosyl linkages are left intact in the presence of xylopyranose residues[147].

Highly specific endoglycanases are derived from bacteriophages[148]. These enzymes act on capsular polysaccharides, giving oligosaccharides that represent the chemical repeating unit of the corresponding polysaccharide, although not necessarily the biosynthetic repeating unit. An example is provided by the isolation of a trisaccharide [I.28] from the capsular polysaccharide of Klebsiella type O5[149] with the repeating unit [I.29] by the action of the bacteriophage Ω 8. The phage enzyme therefore cleaves only the α-(1→3) linkages specifically.

β-D-Manp-(1→2)-α-D-Manp-(1→2)-D-Man

[I.28]

→2)-α-D-Manp-(1→2)-α-D-Manp-(1→3)-β-D-Manp

[I.29]

Determination of anomeric configuration by N.M.R. spectroscopy.

This is usually determined by ^{1}H and ^{13}C n.m.r. With polysaccharides made up of several kinds of sugar residues, n.m.r. spectroscopy can be of great advantage despite the high degree of complexity inherent in their spectra. The use of high field spectrometer is especially beneficial for ^{1}H applications in this area. In both ^{1}H and ^{13}C n.m.r., anomeric resonances are well separated from signals produced by most of the other nuclei. This fact helps greatly in determining the number of different residues in oligo- and polysaccharides and in estimating their relative proportions. It also makes it easier to obtain from these signals the chemical shifts and coupling parameters necessary for assigning anomeric configurations[150].

Proton magnetic resonance spectroscopy (p.m.r.).

The absolute value of the chemical shift for an anomeric hydrogen atom is in itself of little value for the determinaton of the configuration of the anomeric centre. This situation arises because such shifts are not only

highly dependent on the configuration of the sugar but also on the conformational properties and substitutional changes.

In the case of unsubstituted and saturated glycosyl groups, the anomeric hydrogen atom generally produces its signal to the lowest field. Care has to be exercised in the assignment of signals to anomeric hydrogen atoms in complex structures because strong specific electrostatic shielding may occur as a result of close proximity[155]. However, the determination of the anomeric configuration by p.m.r. has become largely a routine matter following well established rules[155-158] for coupling of the anomeric hydrogen atom with the hydogen atom(s) at C-2. This applies to the vast majority of anomeric structures of known sugars which usually occur as aldopyranoses. However exceptions to these rules have been observed arising from the existence of unexpected conformational equilibria or when a polar substituent is in axial position[157,158].

The simple rule[159] that an axially oriented hydrogen atom resonates at a higher field than a chemically similar but equatorially disposed hydrogen atom is normally valid, but it also has many exceptions. The relationship may be reversed in the case of 2-ulosides[160] or by substitutional changes, especially at C-2[161].

In the absence of long range coupling, compounds which do not have a hydrogen atom at C-2 produce a singlet signal for H-1. The determination of the anomeric configuration must then be based on the interpretation of the chemical shift. Since there is no hydrogen atom present at the anomeric centre of ketoses, pmr measurements cannot provide direct evidence on anomeric configuration. Convincing evidence may however be achieved by the use of proton magnetic resonance to establish the relative disposition of the ring atoms and then to predict which anomeric configuration best accomodates the conformation adopted by the ring.

Also the measurement of the relaxation time for the anomeric proton can provide information on its orientation, since the axial hydrogen atom of the β-D anomers have shorter relaxation times than the equatorial hydrogen atoms of the α-D anomers[162].

Carbon-13 Magnetic resonance spectroscopy.

The chemical shifts of carbon-13 atoms are very sensitive to changes in chemical bonding and steric interactions arising from changes in configuration[163,164]. The magnitudes of these shift differences are readily measured by high field-Fourier Transform spectrometers. Normally all the spin-decoupled signals for a monosaccharide are resolved, and indeed this situation is often nearly achieved for complex oligosaccharide structures[165-166]. The method has proved to be useful for examination of repeating units of a great number of polysaccharides.

The signal for the anomeric carbon atom of a glycopyranoside which has the aglycone in the equatorial orientation i.e. the β-orientation tends to occur at lower field than the corresponding signal for the α anomer[163,164] and this feature can be very useful for the assignment of anomeric configuration. This is especially so since these signals occur in a region of the spectrum usually devoid of signals from other carbon atoms. Exceptions have been noted to this rule and hence elucidation of the anomeric configuration may not be possible from ^{13}C-chemical shifts alone.

By selective decoupling of hydrogen atoms, anomeric configuration is determined from the one-bond coupling constant for the anomeric carbon atom $^{1}J_{C-1,H-1}$[167,168]. The coupling constant for aldopyranoses with H-1 in equatorial orientation is 170Hz; this value is 10Hz larger than that of the corresponding β-D anomer which is usually around 160 Hz.

^{13}C-nmr has been particularly useful for the study of anomeric

^{13}C -n.m.r. in the delineation of the structures of antigens based on sialic acid- a homopolymer of neuraminic acid, and allowing the complete assignment of the structures for colominic acid (another homopolymer of neuraminic acid although in a different linkage sequence), including the determination of anomeric configuration and purity[166].

2.5. Determination of Absolute Configuration.

The absolute configuration of the constituent monosaccharide residues of the polysaccharide can be determined by three methods :

a) Optical rotation measurements which requires highly purified monosaccharides and substantial amount of material. This method is not convenient for those natural carbohydrates which are available only in small quantities[98].

b) Enzymic methods which requires specific enzymes which may not always be available[147,148].

c) High resolution capillary gas-liquid chromatography (g.l.c.)[169,170]. This is a rapid and reliable method for determining the absolute configurations of sugars using only small amounts of materials. The D and L monosaccharides which are enantiomers are converted into diastereoisomers by an optically active secondary alcohol such as 2(-) or (+)- butanol or 2(-) or(+)-octanol. Suitable derivatives of these diastereomers are separated on a non-chiral phase of a capillary column of a g.l.c. In the procedure by Gerwig et al[170], the polysaccharides are hydrolysed and from the monosaccharides (-)-2-butyl glycosides are prepared from the (-)-2-butyl alcohol. The diastereo-isomers thus formed are then converted to the trimethylsilyl ethers which are then injected into the G.L.C. to separate and identify them as D or L sugars. The identification is achieved by comparing with the retention

times of the derivatives with those of the standards. For further unambiguous identification of these diastereoisomers, mass spectrometry is also employed by the use of combined G.C.-M.S.

2.6. Conformational analysis

The conformational analysis of polysaccharides has become important in order to understand the interaction of a polysaccharide with protein receptors[171,172] such as enzymes, antibodies and lectins.

The conformation of polysacharides can be determined by :

a) X-ray or neutron diffraction studies[172].

b) Chiroptical methods[173].

c) Nuclear magnetic resonance spectroscopic data[174-178].

a) X-ray or neutron diffraction studies.

The diffraction studies require the polysaccharide molecule to be crystalline, which is a limitation because many polysaccharides do not crystallize very readily. In addition, lattice forces may cause deviation from the conformation which is predominating in solution because the interaction of the polysaccharides and proteins takes place in solution.

b) Chiroptical methods[173].

Chiroptical methods have been used extensively and yield information about polysaccharides in solution. The use of these methods has been limited to simple polysaccharides having small oligosaccharide repeating units e.g. amylose.This is because the results obtained for complex oligosaccharides are more difficult to interprete.

c) N.M.R.Spectroscopy[174-178].

Detailed conformational analysis of complex polysaccharides at present is only possible using n.m.r. data. High resolution n.m.r spectrometers

operating at 300MHz or higher fields have made it possible to obtain experimental n.m.r. parameters which contain detailed conformational information about synthetic oligosaccharides and polysaccharides.

In order to make complete interpretation of the n.m.r. data and draw relevant conclusions from the results, it is necessary to support these experimentally-acquired data with a model which allows a simple theoretical evaluation of the preferred conformation of the oligo- or polysaccharides in solution.

There are several theoretical approaches used in the study of carbohydrate conformations but the most frequently applied are

a) Ab initio calculations

b) Force field calculations

c) Hard sphere calculations e.g.Hard Sphere Exo Anomeric calculations(HSEA) or Geometry of Saccharides (GESA).

Conformational Analysis by N.M.R. spectroscopy[174-178].

^{1}H and ^{13}C-n.m.r. spectroscopy are the most direct methods by which information about the preferred conformation in solution can be obtained.

^{1}H-n.m.r. parameters

The conformation of a polysaccharide can be obtained after a complete assignment of the resonance signals in the two-dimensional (2-D) ^{1}H-n.m.r. spectrum. The major problem in the analysis of spectra of oligo- or polysaccharides is the assignment of the signals from the ring protons which resonate between δ 3.2 and 4.3.

Difference homo-decoupling experiments[174,175] are useful in assigning the chemical shifts of protons outside the region between δ3.2 and 4.3. Partially relaxed spectra[176,177] allow the signals from the fast relaxing protons of the hydroxymethyl groups to be distinguished from the slowly relaxing protons[174]. This experiment can be combined with a double-

resonance experiment[178], which increases the possibility of assigning the "hidden resonances"[179].

The most powerful technique for the assignment of complex spectra are two-dimensional n.m.r. experiments. 2-D-J-resolved spectroscopy[180] results in spectra in which the chemical shifts and coupling constants are separated along two axis. Other experiments such as proton-proton (COSY) and proton-carbon shift correlation spectroscopy also allows the complete assignment of the resonance signals.

The proton n.m.r.data from which conformation can be determined are

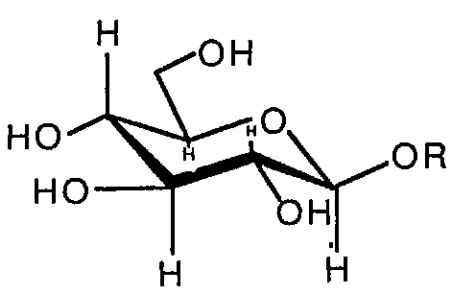
i) Chemical shifts

ii) Coupling constants

iii) Spin lattice relaxation rates

iv) Nuclear Overhauser enhancements

The proton chemical shifts can be used in the analysis of the interglycosidic conformation because protons will be shifted downfield if they are geometrically close to oxygen atoms (>2.7 Å) from neighbouring units[176,181] as shown in the example [I.30] and [I.31]. A downfield shift is observed for the H-3 and the H-5 protons in pyranoses when the anomeric configuration is changed from β- to α- as in β-D-glucopyranose [I.30] and α-D-glucopyranose [I.31].



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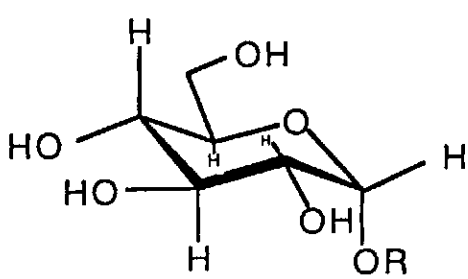
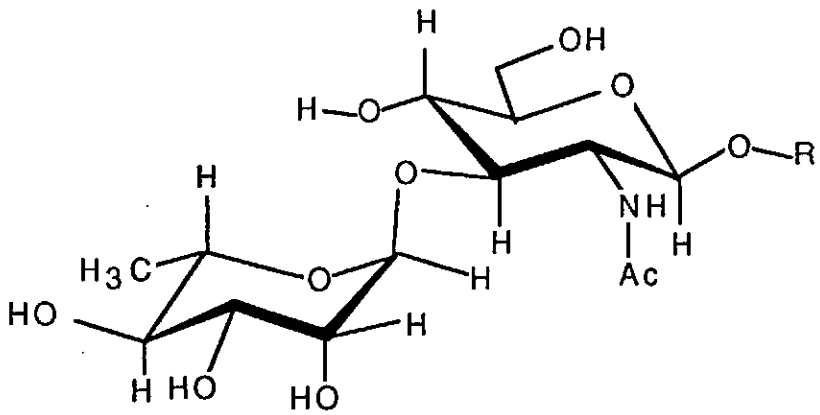


[I.31]

The chemical shifts of the H-3 and the H-5 are respectively 3.37 and 3.35ppm in [I.30] while they are 3.61 and 3.72 ppm respectively in [I.31].

If the molecules contain functional groups which exhibit strong anisotropy e.g.C=O groups in N-acetyl derivatives, upfield shifts may be similarly observed. Thus an upfield shift is observed for the anomeric proton of the α-L-rhamnose unit in the α-L-rhamnopranosyl-(1→3)-β-D-N-acetylglucopyranoside disaccharide [I.32].



[I.32]

The spin-spin coupling constants can be used to confirm that the chair conformations of the individual units are similar to those found in the parent monosaccharides.

The application of proton spin lattice relaxation rates in the study of interglycosidic conformations rely on the strong dependence of the intramolecular proton-proton distances[182,183]. Generally, relaxation contributions from another monosacharide unit can be observed if the proton-proton distances are below 3.00Å. The problem with the application of proton-relaxation rates in conformational analysis is that it is generally difficult to determine the individual relaxation contributions. A quantitative measure for the proton-proton interactions in the neighbouring pyranose rings is therefore difficult to obtain. The problem can be solved by the measurement of the nuclear Overhauser enhancements[184]. In this experiment, the individual proton-proton relaxation contribution can be determined between the proton saturated and the protons receiving relaxation contribution. The experiment is performed in the difference mode

and because it gives very reliable results, it is possible to measure enhancements as small as 2% with confidence[176,177,181]. This experiment is one of the most powerful tools in the conformational analysis of polysaccharides.

^{13}C-n.m.r. Parameters.

Even though the carbon atoms are not located in the plane of the ring, unlike the protons which are axially and equatorially disposed in space, the ^{13}C-n.m.r.data contain some conformational information.

The ^{13}C-n.m.r.parameters which are of importance in a conformational analysis are as follows:

i) Chemical shifts

ii) Long range coupling constants

iii) Spin-lattice relaxation rates.

Before these parameters can be used, it is necessary to assign all the carbon resonances. The most frequently used techniques are correlation with model compounds and correlation with proton spectra. The ^{13}C-^{13}C satellite spectra either in the 1-dimensional[185] or 2-dimensional[186] mode are also increasingly being used . Generally correlation with proton spectra either through a selective proton-decoupling experiment[187] or through heteronuclear 2-dimensional correlated spectroscopy[188] (HECTOR), is the most reliable method to assign the carbon signals in a given spectrum.

The ^{13}C-chemical shifts do not yield much information about the glycosidic conformation as the corresponding ^{1}H-n.m.r. chemical shifts do. In cases where crowding occurs as for example in branched trisaccharides, small valence and/or bond angle deformations may take place. This will result in a different hybridisation of the ^{13}C-nucleus and thus in a different ^{13}C-chemical shift. This effect causes upfield shifts to be observed in the resultant spectra[174,176].

Due to the angular dependence of the three bond C-O-C-H long-range coupling constants[187-189], it is possible to obtain information about the interglycosidic torsion angles from high resolution proton coupled spectra of the polysaccharides[190], or from the proton spectra of the isotopically labelled molecules [191]. These values have been used successfully in several cases in the conformational analysis of oligosaccharides[176,177,192-193]. The only limitation to the use of these long-range coupling constants is that the line width is to some extent dependent on the molecular size which implies that it is not generally possible to use this approach for polysaccharides having large groups of oligosaccharide repeating units.

Finally relaxation rates can be used in conformational analysis of polysaccharides, but due to the dominating dipole-dipole relaxation mechanism from the protons directly bonded to the carbon atoms, these values are mainly used in the study of the molecular motion of the individual monosacharide unit in the polysaccharides[194].

Theoretical calculations.

i) Ab initio calculations[195-196].

This method has not been much used to calculate the preferred conformation of oligosaccharides. This is because these calculations are very expensive and in general not practically possible on large molecules. Most ab initio calculations have been performed on model compounds which simulate the atoms involved in the glycosidic linkages.

ii) Force fields calculations.

The calculations include both bond and angle deformations, torsional terms, non-bonded interactions, coloumbic terms, and hydrogen bonding. They have been carried out on several monosaccharides and some disaccharides[197-199]. These methods are simpler but rather expensive and time consuming to carry out on molecules larger than disaccharides.

iii) Hard sphere calculations method

This is a simple and inexpensive method, and it gives reliable results even on larger oligosaccharides despite the fact that simple functions are used to describe the non-bonded interactions between the component monosaccharides of the oligosaccharides[200]. The hard sphere calculations method which take into consideration the importance of the exoanomeric effect is referred to as the hard sphere exoanomeric calculations (HSEA). These calculations give results which are in excellent accord with evidence from n.m.r. spectra.

The primary assumption for the hard sphere calculations is that the monosaccharide units exist as rigid bodies, i.e. they exist in their regular unstrained chair conformations. The validity of the rigid body assumption has been investigated for 161 X-ray structures of compounds containing pyranose rings[201] and from the results obtained it has been possible to establish coordinates for an average pyranose ring. The assumption which appears to be reasonable from the results has been discussed by other workers[202].

The coordinates are taken from a good neutron or X-ray diffraction experiments in order to obtain as accurate results as possible. The proton coordinates from the neutron diffraction studies can only be used because the preferred conformation of the oligosaccharides is to a large extent determined by the atoms, particularly the atoms located around the glycosidic linkage. However if only X-ray diffraction data are available, only the experimentally determined coordinates from the heavy atoms are used. The proton coordinates are generated by modifying the data using a computer programme.

This programme positions the protons at a distance of 1.1 Å from the carbon atom along a vector defined by the remaining carbon-carbon and carbon-oxygen bond vectors. Hydrogen atoms of hydroxyl groups are not included in the calculations. These coordinates are stored in a library

which can be used directly in the conformational analysis of a specific oligosaccharide. If the coordinates have not been determined for a particular monosaccharide unit, it is possible to perform bond modifications on related structures and thus calculate the appropriate coordinates.

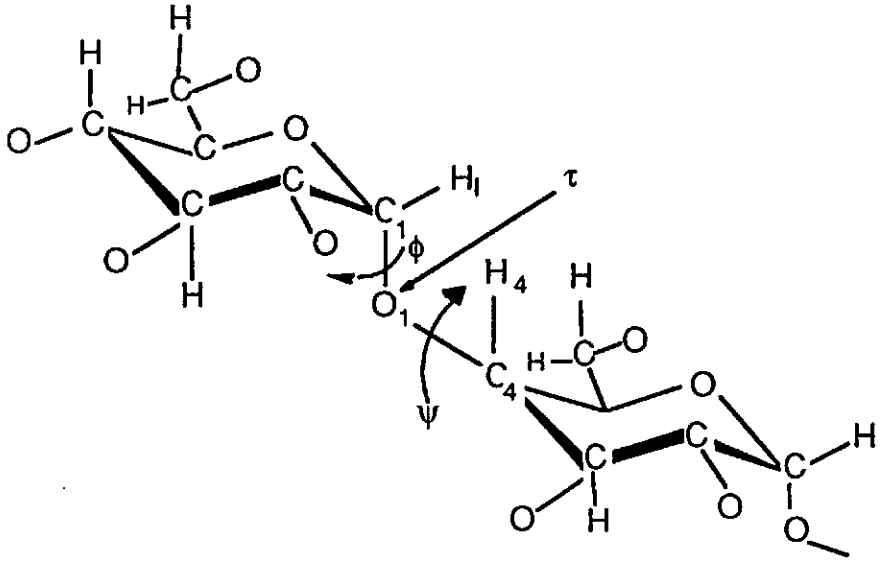
If maltose **[I.33]** is taken as an example, there are three degrees of freedom which remain to be investigated, and they are :

i) the rotations around the C_1- O_1 bond ϕ_H rotation for the H_1-C_1-O_1-C_4 fragment **[I.34]**. This is defined as positive according to IUPAC recommendations[203].

ii) rotations around the O_1-C_4 bond ψ_H rotation for the C_1-O_1-C_4-H_4 fragment **[I.35]**).

iii) the size of the glycosidic bond angle (τ). Experimental data from X-ray or neutron diffraction studies suggest the τ-angle in most oligosaccharides can be considered constant (=117°)[203], but the value can be included as a variable in the calculations.

Hard sphere calculations primarily take into account the non-bonded interactions between atoms.Some workers[204] have calculated the potential functions for the interaction between the atoms but the values published by Kitaygorodsky[205] are more commonly used.



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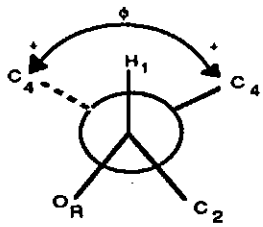
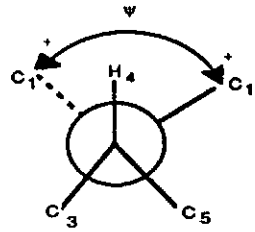


[I.34]



[I.35]

The data have been used to estimate the non-bonded interaction between two atoms by the equation (1)

$v_{pot} = 3.5[-0.04/z^6 + 8.5x10^3 (e^{-13z})]$ kcal/ mole ----------(1)

in which $z = r_{ij}/r_o$ and r_o and r_{ij} are the equilibrium distance and the actual distance respectively between the two interacting atoms.

The value of r_o has been set arbitrarily at $1.11(r_i+r_j)$ and r_i and r_j are the van der Waal radii between the two interacting atoms[204].

With known values of the van der Waal radii for the atoms or groups of atoms obtained from literature (see Table 2.1.), potential energy curves for the interactions between them, calculated from equation (1) can be drawn as shown in figure 2.2 for H-H **(A)** and H-O **(B)** .

TABLE 2.1.

van der Waal radii for atoms(in Å) used in HSEA calculations

r_H	= 1.20	$r_{o,H-H}$	= 2.66
r_N	= 1.55	$r_{o,H-O}$	= 3.00
r_C	= 1.70	$r_{o,H-C}$	= 3.22
r_O	= 1.50	$r_{o,C-C}$	= 3.78
r_{CH_3}	= 1.85		

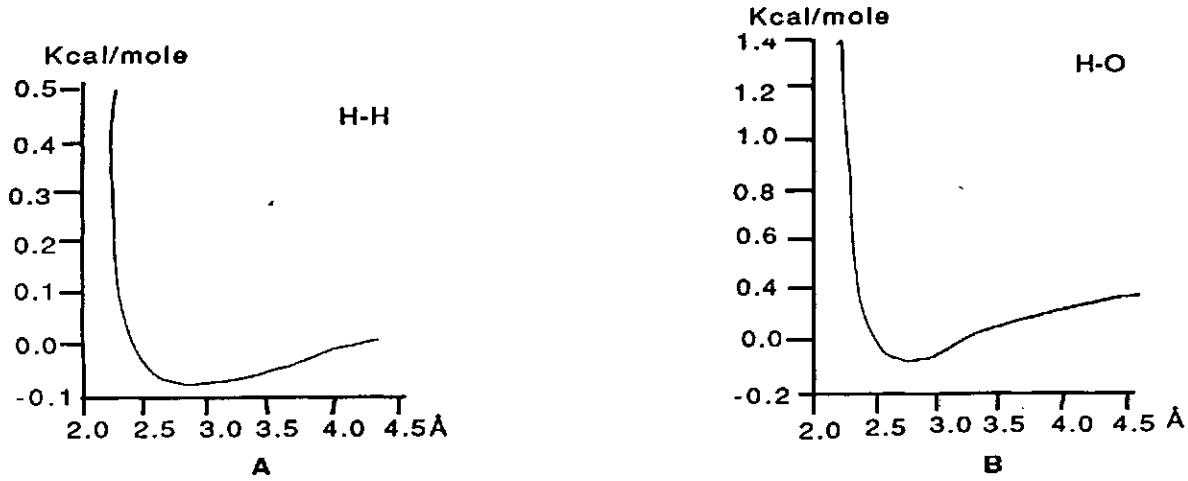


Figure 2.2.

The interaction energies for a given set of values for ϕ_H , ψ_H and τ are then summed up for all interactions between two monosaccharides units and the sum represents the pure non-bonded interaction energy for that conformation. ϕ_H , ψ_H is then varied through 360° in given intervals e.g.5° and this leads to a series of interaction energies which are functions of ϕ_H and ψ_H. The values obtained can be used to describe the energy surface in a three dimensional picture as a function of ϕ_H and ψ_H.

A plot of isoenergy contours around the minimum obtained from such values is also frequently used to visualise this problem. Figure 2.3. shows an example of an isocontour map for a 1→3 linked disaccharide[206].

However, experimental[177,181] and theoretical evidence has shown that the exoanomeric effect makes an important contribution to the preferred conformation of the glycosidic linkage. The consequence of the exoanomeric effect is that the aglyconic carbon prefers an orientation in which the ϕ-angle is ≈ +60° in the β-D-glycosides and ≈ -60° in the α-D-glycosides respectively.

For the hard sphere exoanomeric calculations, Lemieux et al[181], have proposed a torsional potential based on the ab-initio calculations of

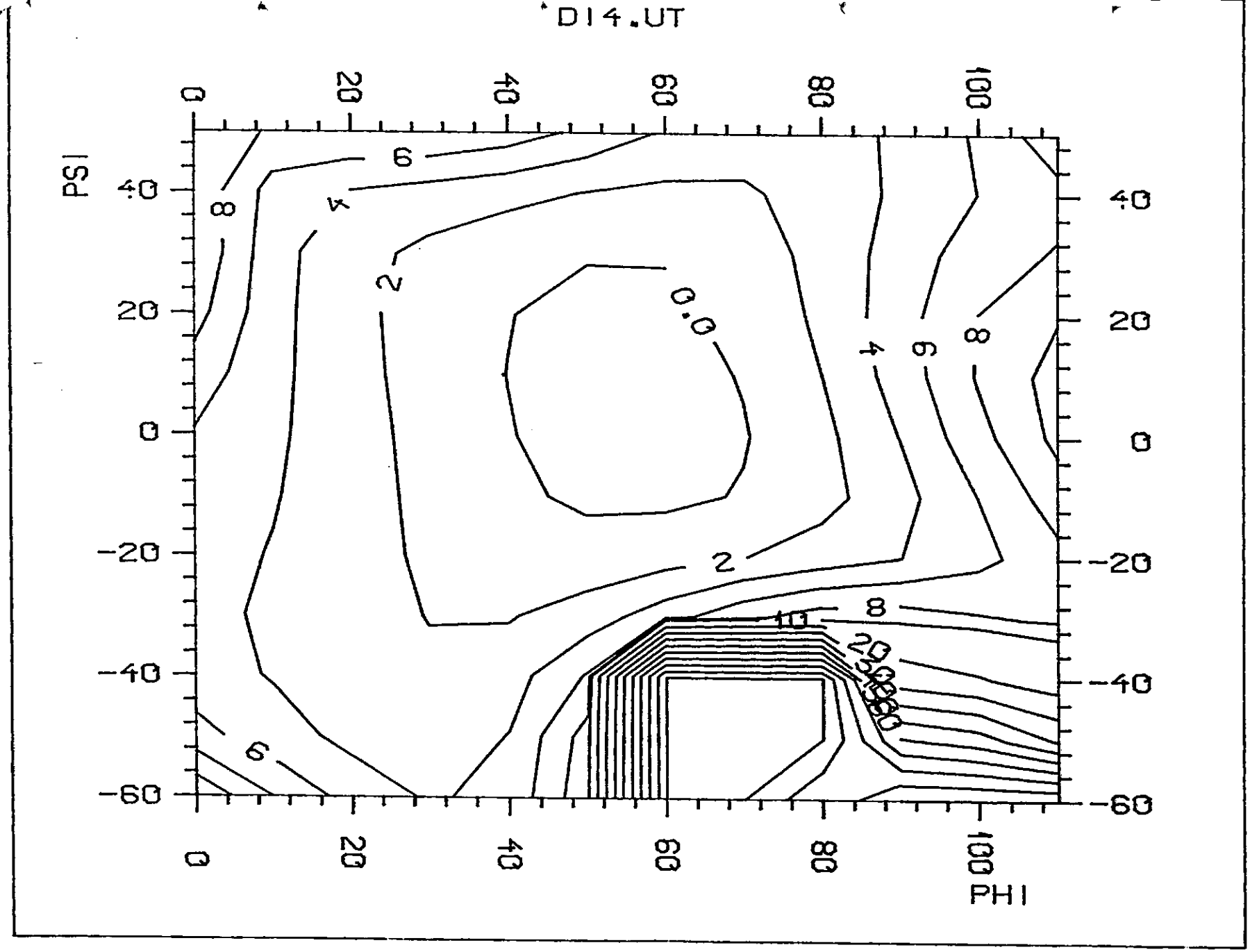


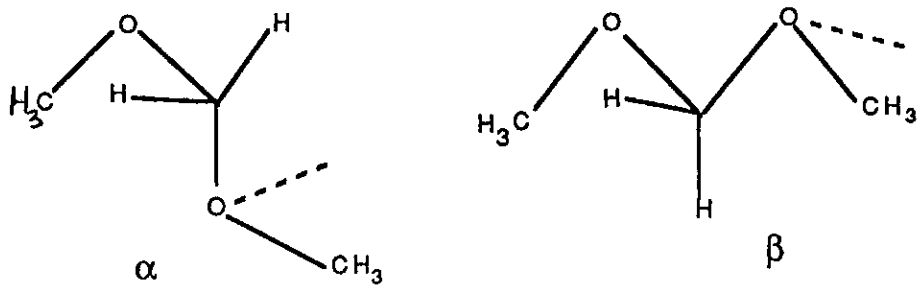
Figure 2.3. Iso-contour Energy Map of a 1,3- linked Disaccharide.

dimethoxymethane [I.36] which was used as a model in describing the exoanomeric effect for the α- and β-glycosides.

$v_{exo-\alpha} = 1.58(1-\cos\phi)-0.74(1-\cos2\phi)-0.70(1-\cos3\phi)+ 1.72$ kcal/mole -----(2)

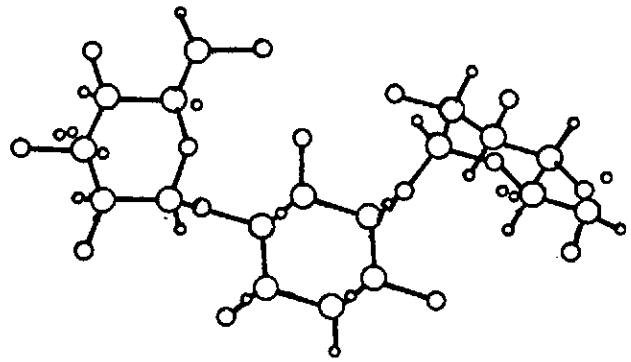
$v_{exo-\beta} = 2.61\ (1-\cos\phi)-1.21\ (1-\cos2\phi)-1.18\ (1-\cos3\phi)+2.86$kcal/mole -----(3)

This energy contribution is calculated for each ϕ-value and added to the non-bonded interaction energies according to equation (1) to give the total energy which in these calculations[171,181] are called the hard sphere exoanomeric calculations (HSEA).

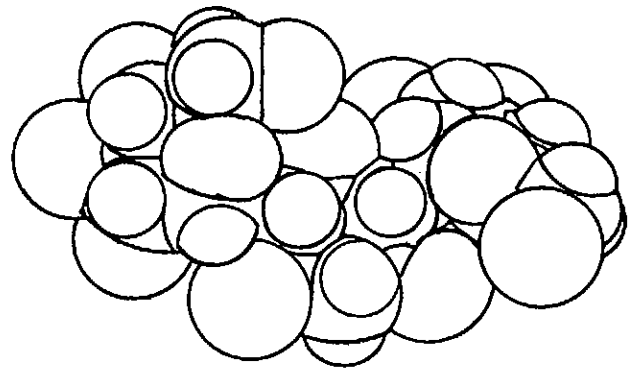


[I.36]

Finally, an analysis of the minimum energy conformation obtained with respect to short proton-proton or proton-oxygen distances or hard interactions is made and related to the experimentally determined n.m.r parameters to support the calculated results. Furthermore, the coordinates determined for this minimum energy conformation can be used in standard molecular plot computer programs. In these programmes, the molecules can be plotted either as ball and stick models [C] or as space filling models [D] as shown in Figure 2.4. which can then be inspected from different angles to identify interactions between the the individual monosaccharides[208]. When colour plot programs are used, the identification of the interactions are much easier[207, 208].



C



D

Figure 2.4. The Ball and Stick Model (C) and the Space Filling Model (D) in their minimum energy conformation.

2.7. The Aim of the Project.

The projects reported in this thesis consists of two parts. The first part deals with the Structural Studies of Bacterial Polysaccharides referred to as "Analytical" while the second part deals with the Synthesis of Oligosaccharides and is referred to as "Synthesis".

The broad aim of the structural studies was to investigate the cell wall and capsular polysaccharides from bacteria of current interest. The bacterial polysaccharides studied were those isolated from

a) *Brucella abortus* Biotype 1, strains Mustapha and 7

b) *Campylobacter coli* Labet 227.

c) *Escherichia coli* O149

d) *Streptococcus pneumonia* Type 37

The reason for choosing each microorganism is discussed below.

a) *Brucella abortus* Biotype 1, Strains Mustapha and 7[5,209].

Both types belong to the genus *Brucella* and are responsible for Malta fever or undulant fever which is also known as brucellosis in man. *Brucella abortus* is also responsible for contagious abortion in cattle, sheep and goats. Brucellosis is a disease common in most parts of the world but it was first reported in Nigeria in 1928. In both man and animals the disease is infectious as well as invasive. Bovine brucellosis is still the most important zoonosis from the economic point of view on a world wide basis since it seriously affects livestock productivity. In Nigeria, the majority of the brucellosis outbreak is caused by biotypes 1,2, and 3. Brucellosis is responsible for staggering economic losses[210] to the Nigerian livestock producers and also constitutes a health hazard because the infective agent are pathogenic to man. The economic importance of brucellosis calls for an extensive study of the causative organism.

Earlier literature[210a] contains some reports on the constituent sugars and lipids in the *Brucella abortus* species. The sugars identified were

glucose, galactose, and mannose. However Carroff et al[210b] with the aid of modern techniques were able to carry out extensive study on the antigenic S-type LPS of B.abortus 1119-3. Their investigation by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy, periodate oxidation and methylation analysis led to the identification of the O-chain polysaccharide of B.abortus S-type lipopolysaccharide as a linear homopolymer of 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl units. Kreutzer et al[210c] determined the fatty acid content and determined that hexadecanoic acid and octadecanoic acid were the major constituents and also noted the absence of 3-hydroxytetradecanoic acid, a common marker of enteric LPS. However Caroff et al[210b] reported the presence of 3-hydroxy tetradecanoic acid in the LPS of Brucella abortus.

In this thesis, we present the results of the chemical studies on the LPS from the cell wall of B.abortus biotype 1, strains 7 and Mustapha. These microorganisms are among those that have been implicated in brucellosis in Nigeria. As mentioned previously, human beings and animals develop antibodies which react with antigens on the bacterial surface as a defence against bacteria. Artificial immunity can be induced through vaccination. Early vaccines were based on whole organisms preparation but research in this area later showed that purified cell wall and capsular polysaccharides could be used. Diseases can thus be controlled by vaccination and the O-antigen has been used as vaccines. It is therefore important that the structures of the O-antigens from as many strains as possible are known if control by vaccination is to be fully effective.

It is because of this need that the cell wall of the two strains were studied.

b) *Campylobacter coli* Labet 227.

Campylobacter coli is one of the newly recognised enteric pathogens of the

genus Campylobacter[211]. It causes diarrhoea, accompanied by abdominal pain, fever, nausea and sometimes vomiting[212]. The organisms are excreted in the feaces of healthy domestic animals such as chicken, turkeys, cattle and pigs. While this microorganism have been found in patients with diarrhoea, non-ill carriers have also been encountered.

C. coli has been recognised to be responsible for this disease worldwide but the statistics in the United states of America estimates that as many as 2 million cases of Campylobacter infections occur there each year. This number is equivalent to the estimated number of salmonellosis and exceeds the number of shigellosis cases. Studies in Canada and England indicate that the C. coli accounts for 3 to 5% of human cases of Campylobacter infections[213].

However, in Nigeria, there is no definite total statistics of the number and group of people infected annually but it has been isolated quite often in a number of diarrhoeal cases from the Lagos University Teaching Hospital, Idi Araba in Lagos. A lot of microbiological as well as immunuological work has been done on many Campylobacter strains in this hospital[213a].

However, there are rather few reports on the cell wall carbohydrates of the Campylobacter strains because they are a newly recognised group of pathogens and also because of some problems which are encountered during resuscitation and culturing.

Two major reports on the isolation and chemical composition of the LPS from C. coli and C. jejuni are in literature[213b,213c]. In the earlier article[213b] the LPS from three strains of Campylobacter jejuni was extracted by aqueous phenol. The sugar components present in all strains were glucose, galactose, L-glyceromannoheptose and glucosamine. One strain contained in addition, galactosamine. The fatty acids present were mainly 3-hydroxy-tetradecanoic acid and n-hexadecanoic acid. The polysaccharide

fraction behaved like core structure in gel filtration experiments.

A notable observation in their report is the non-integral values of the molar ratios of some monosaccharides. For example in Strain E8035, the ratios of L-Heptose:Galactose:Glucose:Glucosamine were 1: 0.5 : 0.8 : 0.9. which is suggestive of a heterogeneous core. In the later report[213c], the LPS from seven type strains of Campylobacter jejuni and one type strain of Campylobacter coli extracted with 45% aqueous phenol was studied. The sugar components present in all strains were glucose, galactose, L-glyceromanno-heptose, 2-keto-3-deoxy-octulosonic acid and glucosamine. All but one strain contained galactosamine and two strains contained in addition mannose. The fatty acids present were mainly 3-hydroxy-tetradecanoic acid and n-hexadecanoic acid and trace amounts of of n-tetradecanoic acid.

On the basis of the sugar components detected, the LPS isolated from the eleven strains of the Campylobacter jejuni and Campylobacter coli can be divided into three main groups. One group comprises the LPS of the Campylobacter coli strains (E8035, 11101) which contains glucose, galactose, L-glycero-D-manno-heptose, and glucosamine. The second group is formed by C. jejuni biotype 1 LPS which contains glucose, galactose, L-glycero-D-manno-heptose, glucosamine and galactosamine.

The LPS of C. jejuni biotype 2 make up the third group by containing mannose as well. The number of sugar molecules relative to fatty acids is low, indicating that if present, O-antigenic side chain must be of a short chain length. The LPS of several strains were also examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and their weight were found to be low. The result from the molecular weight determination confirms the oligosaccharide nature of the LPS, which should have been called lipooligosaccharide.

Because of the paucity of reports on the structural studies of Campylobacter strains in Nigeria and due to its implication in diarrhoea in

Nigeria, it was decided to study the cell wall LPS of this microorganism. Among the strains of the C.coli and C.jejuni picked for study only C.coli Labet 227 was relatively easy to resuscitate and culture.

C.coli strain Labet 227 was isolated from a diarrhoea patient below the age of six years at Ohaozara Local Government area of Imo State, Nigeria in a study supported by World Health Organisation (W.H.O.).

The bacteria was found to be a rough strain hence the lipooligosaccharide from its cell wall was characterised.

c) *Escherichia coli* O149.

E. coli O149 belongs to the genus Escherichia coli which is one of the most extensively studied member of Enterobacteriaceae. Many reviews which deals with a great number of reports have been written on the study of the cell wall of this bacteria[39,63,67,69]. E. coli is found in the intestine of humans and other animals as part of the normal flora. There are over one hundred of different strains[214] within the species that vary in many ways, including their potential to induce diseases such as diarrhoea, meningitis, and urinary tract infections such as phylonephritis and infantile diarrhoea.

Neonatal piglet diarrhoea is often caused by E. coli O149[215] and strains belonging to this O-group also colonise the the intestine of adult man[215]. These strains also elaborate the K-88 capsular polysaccharide. The structure of the O-antigen polysaccharide from Escherichia coli O149 was studied.

The result of this work will be useful in the formulation of vaccines to control the diseases arising from infection by the microorganism.

d) *Streptococcus pneumonia* Type 37.

The structure of the capsular polysaccharide elaborated by Streptococcus

pneumonia type 37 was investigated. Streptococcus pneumonia is a gram-positive bacterium that has been divided into 82 different types one of which is S. pneumonia type 37. Several of these types have been combined into groups on the basis of serological cross reactivity but each type produces its own type specific capsular polysacharide antigen. S. pneumonia causes a variety of diseases[216] such as pneumonia, otitis media, and some other life threatening diseases such as septicemia and meningitis. The morbidity of pneumonia caused by S. pneumonia is high in both adults and children. In children, one of the most common diseases with S.pneumonia as etiological agent is otitis media.

Although, S. pneumonia Type 37 was isolated from the sputum of a pneumonia patient, it has been observed to possess relatively lower virulence. The incidence of this organism causing the disease is very low in man[217]. There is also extensive literature[31,39,67,69,216a] on the studies of the capsular polysaccharide elaborated by this bacteria.

In fact the first capsular polysaccharide vaccine arose from early work on the Streptococcus pneumoniae when a multivalent vaccine containing polysaccharides of six types showed 100% efficacy in preventing pneumonia caused by those organisms when tested on U.S. Army recruits. In 1977, a 14-valent pneumococcal polysaccharide vaccine was developed[216a]. Since then over 130 million individuals have been immunized with capsular polysaccharides with no adverse effects from the vaccination. Further research is continuing on this vaccine to improve its efficacy since the immunity generated by vaccines appear to be long lasting in contrast to antibiotics which bacterial strains may become resistant to.

The capsular polysaccharide from this bacteria was studied so that the knowledge gained would be of further assistance in improving the formulation of the existing vaccine.

CHAPTER 3. OLIGOSACCHARIDE SYNTHESIS.

3.0. The need for oligosaccharide synthesis.

Biological information is carried in oligo- and polysaccharides either free or covalently linked to other substances such as lipids or proteins to form glycolipids, lipopolysaccharides and glycoproteins. These substances play fundamental roles in normal biological processes, in diseases and a host of other life processes[218]. The collaboration of structural carbohydrate chemistry with biological studies has resulted in the identification of a great number of oligosaccharide fragments involved in biological interactions.

In order to be able to exploit this knowledge for the understanding of health and the prevention, diagnosis and therapy of diseases, it is necessary to isolate or synthesize these fragments.

Oligosaccharides are not only fragments of polysaccharides but their simplified models as well. Various delicate questions of the chemical behaviour of polysaccharides, specific cleavage of their chains, spectral and other physico-chemical characterisations in relation to the structural features, were and will continue to be solved by means of model oligosaccharides with exactly known structures. In the study of macromolecule-macromolecule interactions, exemplified by antigen-antibody or lectin-polysaccharide reactions, the interactions are largely determined by the terminal sequences of the oligosaccharide fragment of the biopolymer particle. The behaviour of such binding sites referred to as "determinants" or "antennae", is well modelled by an oligosaccharide of corresponding structure.

N.M.R. spectroscopy, including one- and two-dimensional ^{1}H and ^{13}C spectroscopy has become one of the the most versatile techniques for

structural studies of polysaccharides which are built of oligosaccharide repeating units. Information on residues, substituents and mode of linkages is obtained without destruction of the material. It is also possible to obtain information on the absolute configuration of the different sugars. In order to understand all the information given in the n.m.r.spectra, it is necessary to understand the origin of the glycosylation shifts i.e. change in chemical shift upon glycosylation for different types of linkages. In the present computer age, it is also of interest to develop a computer-assisted analysis of polysaccharides based on n.m.r. data. A computer program[219] called CASPER has been developed for the determination of polysaccharides with regular repeating units. The program utilizes the ^{13}C n.m.r. spectrum of the polysaccharide and the information from sugar and methylation analysis. It is therefore compulsory to have a wide knowledge of the n.m.r. characteristics of model oligosaccharide compounds and an extensive data base of these compounds. This is because of the vast variability of oligosaccharides that have been and yet more to be encountered in the process of structural studies of polysaccharides.
The extent of this variablity can be shown for three different hexoses which can combine to form 1056 different trisaccharides[220].

For the reasons given above, these oligosaccharides must then be available for n.m.r. studies to be carried out on them while it is sometimes possible to obtain some oligosaccharides from natural sources by specific enzymatic degradation techniques. (An example of this being the use of bacteriophages for the isolation of oligosaccharide fragments of the repeating units constituting the outer part of the LPS and capsules of the bacterial cell envelopes). However, it is not always a possible to obtain some desired oligosaccharides from natural sources and even when available, it is usually not in sufficient quantities. Hence it is commonly

necessary to synthesize actual or closely related tailor-made oligosaccharides for the required studies. In addition, the synthetic route usually opens the way to obtain a number of substances analogous to the one identified as having a biological activity. This may be of fundamental importance for elucidating structure/function relationships.

3.1. Different Methods of Oligosaccharide Synthesis.

There are no universal reaction conditions for oligosaccharide synthesis, because each oligosaccharide synthesis remains an independent problem posing a new challenge, requiring a knowledge of methods, considerable systematic research, experience and experimental dexterity.

In order to be able to synthesize an oligosaccharide, there are three points to be considered. They are

a) Glycosidic linkages of given monosaccharides of the desired configurations and ring size that have to be made.

b) Glycosylations of the specific hydroxyl groups of the monosaccharide for which particular glycosidic linkage has to be provided.

c) At higher levels of order i.e. trisaccharides and higher oligosaccharides, the desired sequence of units and branches has to be created. Structures of higher regularity are obvious exceptions.

Hydroxyl groups of sugars and their derivatives undergoing glycosylations have various reactivities in these reactions. These differences are not high enough to provide a routine selective mono-glycosylations of the few hydroxl groups in a molecule. Owing to these differences, however, the results of glycosylations by a particular reagent differ considerably, depending on the nature and the reactivity of the aglyconic component. The main reaction leading to glycosides are accompanied

by side processes which may not involve the aglycone. The role of these reactions can be negligible in glycosylations of highly reactive compounds and can become the determining factor if the reactivity of an aglyconic component is too low for effective competition with the side processes. Therefore the reactivity of an aglycone not only causes a higher or lower reaction rate but sometimes determines whether it is possible to carry out the particular synthetic scheme.

From the viewpoint of reactivity towards electrophilic reagents, in particular to glycosylation, hydroxyl groups of typical carbohydrate derivatives can be distinguished as primary and secondary in spite of the great range of reactivity within each group.

From the above, it is obvious that in an oligosaccharide synthesis, two polyfunctional but complementary reaction partners must be coupled. Both must be able to be selectively blocked and deblocked. In the glycosyl component it must be possible to expose and functionalize the anomeric center: in the aglycone component, only the hydroxyl group intended for the coupling step must be selectively deblocked. The reactivities at the anomeric centre of the glycosyl component and of the hydroxyl group of the reaction partner depend very strongly on the type of blocking pattern of the two compounds. A variation of the blocking pattern can exert a decisive influence on the coupling step. Conformational and steric influences, and the molecular sizes of the two partners are also significant. The coupling step should proceed as stereoselectively as possible and result in only one of two anomers because of the difficulties of separating anomeric mixtures of oligosaccharides particularly in the case of higher oligosaccharides.

The most frequently used method is that of Koenigs-Knorr[221] although several efficient variants have been subsequently[222-225] developed.

In general, the procedures involve the treatment of a glycosyl halide

with an alcohol eitherin the presence of heavy-metal salts preferentially silver salts or an organic base as the acid acceptor; the latter enhances the rate of reaction and also prevents side reactions e.g. deacylation. For the glycopyranosyl halides, the anomer having the halogen substituent in the axial orientation is thermodynamically more stable than the corresponding anomer having the halogen substituent equatorially attached because of the anomeric effect[226]. Under the usual preparative conditions, the more stable halide is obtained, and the less stable anomer can be prepared only by a kinetically controlled reaction.

When synthesizing oligosaccharides by the Koenigs-Knorr reaction, or by any of its modifications, the following points form a general guideline[227-230].

a) The reactivity of the glycosyl donor i.e.glycosyl halide can be varied over relatively wide ranges by the choice of the halogen, the catalyst (promotor) and the protecting group pattern.

b) Diastereoselectivity in the coupling is attained by

   i) participation of neighbouring groups for β-glycosides of D-glucose, D-glucosamine, D-galactose, and D-galactosamine and for α-glycosides of D-mannose, L-fucose and L-rhamnose.

   ii) <u>in situ</u> anomerization of the α-glycosyl halide to give the more reactive β-glycosyl halide[231] which reacts preferentially to give the more stable α-glycosides of D-glucose, D-glucosamine, D-galactose, and D-galactosamine due to the exploitation of the anomeric effect.

   iii) heterogeneous catalysis which gives good result in the difficult formation of the β-mannose linkage[232].

c) The reactivity of the glycosyl acceptor, which for D-glucopyranose, gives the approximate order

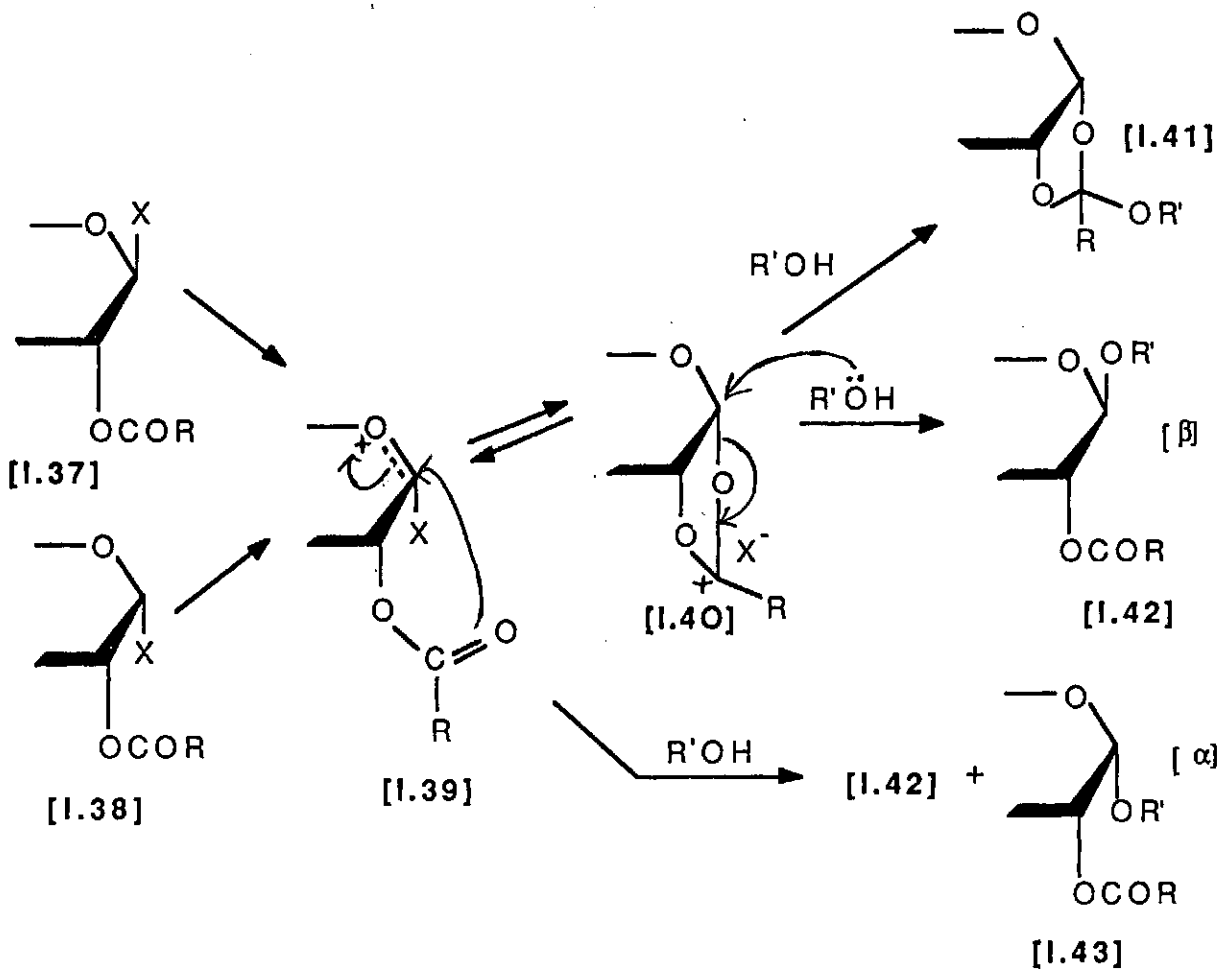
water > methanol > ethanol > 6-OH >>> 3-OH > 2-OH > 4-OH groups of glucose, is controlled by the choice of the protecting groups having steric and electronic effects.Also the modification of the structure e.g. formation of 1,6-anhydro structures affects the reactivity.

The applications of these generalisations has led to excellent results.

The mechanism of the Keonigs Knorr reaction[227].

The general course of glycosidation from glycosyl halides having a participating group at C-2 may be depicted as shown in Scheme 3.1. When a 1,2-cis-glycopyranosyl halide [I.38] is treated with a large excess of alcohol in the presence of a catalyst with strong affinity for the halogen e.g. a silver or mercury salt, the corresponding 1,2-trans-glycopyranoside [I.42] is the favoured product.

This can be explained by an S_N2 type of mechanism. However, under the usual type of Koenigs-Knorr reaction, in which a limited amount of alcohol is used, the reaction proceeds via the intimate ion-pair or the oxocarbenium ion [I.39] to give the dioxocarbenium ion (acetoxonium ion)[I.40] which then reacts with the alcohol with nucleophilic ring opening at C-1 from the upper side to give the 1,2-trans- i.e. β-glycoside [I.42] because of steric reasons.

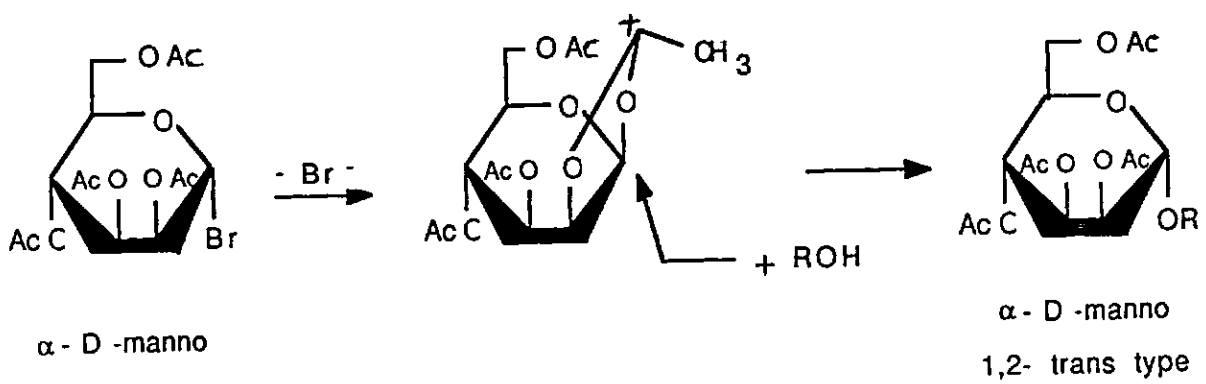


Scheme 3.1

In reactions of moderately reactive alcohols, [I.39] is also capable of reacting resulting in the formation of an anomeric mixture of both [I.42] and 1,2-cis-glycopyranosyl i.e. the α-glycoside [I.43]. In some instances, depending on either the reaction conditions, the nature of the catalyst and alcohol used, the orthoester [I.41] could also be formed. The 1,2-cis-glycopyranoside [1.43] is the major product from [I.38] when mercury (II) salt is used as the

catalyst but the mechanism remains unclear. When the 1,2-transglycopyranosyl halide [I.37] is used as the starting material, the trans-glycopyranoside

orthoester [I.41] are the major products. The reaction sequence [I.38]→ [I.39]→ [I.40]→ [I.42] is a well established procedure which is made use of to bring about stereoselective β-glucopyranoside synthesis. The stereochemistry of the 2-OH in the D-manno series is just opposite to that of the D-gluco, so that at C-1 during a corresponding reaction, an inverted cyclic dioxocarbenium ion is formed as an intermediate. In this case, the nucleophile can open the ring only from below, and the α-D-manno-pyranoside is formed as shown in **Scheme 3.2**.



Scheme 3.2.: The synthesis of β-mannopyranoside.

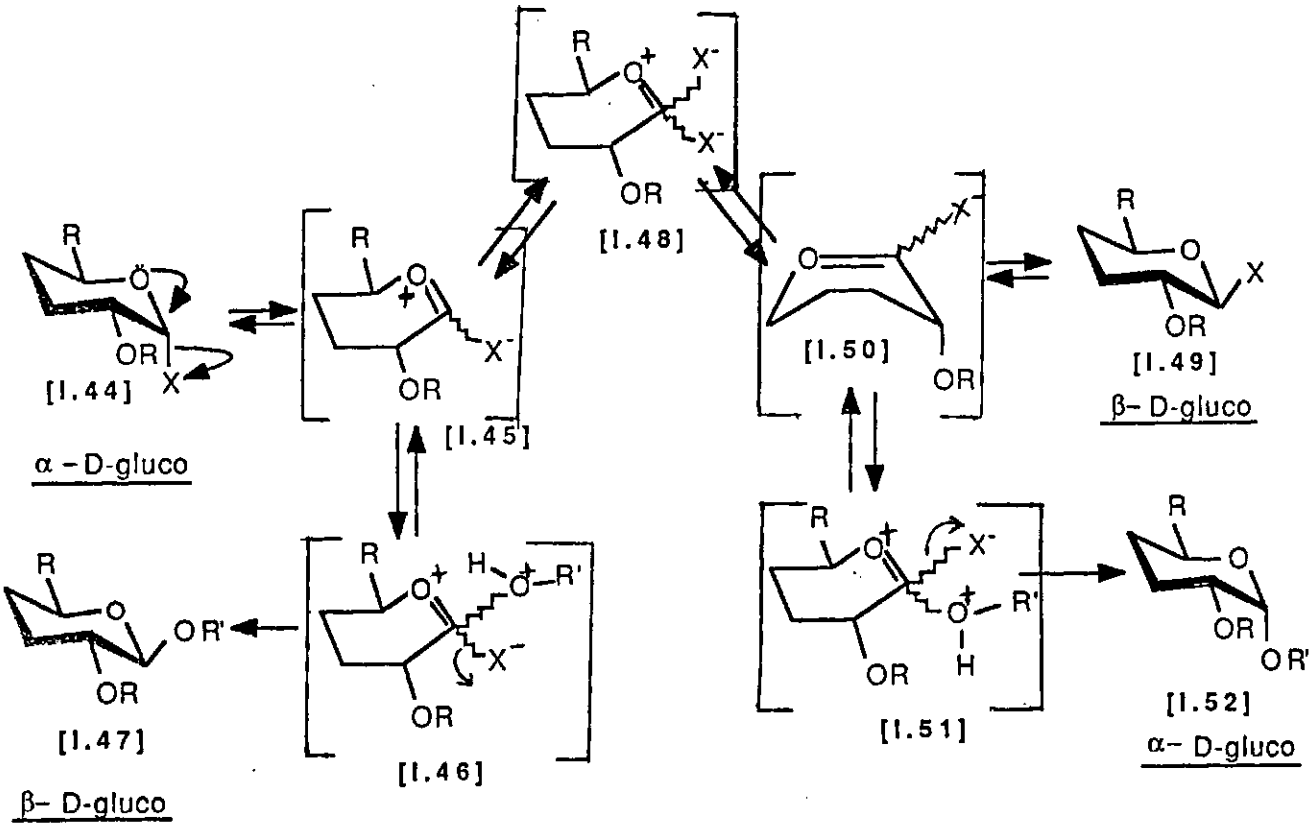
The synthesis of the α-glycoside is much more difficult. It is important that the starting compound has a non-neighbouring group active substituent at C-2, such as the benzyl group which also gives good reactivity of the halide, so that the the reaction product similar to [1.42] in Scheme 3.1. is avoided. Another group is the trichloroacetyl group, the β-halide containing this group is more stable but its reactivity is lower.

In order to achieve an α-glycoside synthesis, the less stable β-halide must be taken as the starting material. The β-halides could be applied and will react under inversion in the sense of an S_N2 reaction. This can be done in a solvent of low polarity such as dichloromethane or ether in the presence of an active catalyst such as silver carbonate and/or silver

the presence of an active catalyst such as silver carbonate and/or silver perchlorate or silver triflate. However this process is possible with relatively stable isolable β-halides. In the majority of cases, the labile β-halides can only be used with difficulty or not at all because they anomerise very easily to the more stable α-halides and the reaction would result in a mixture of the two anomeric glycosides.

Therefore, the α-glycoside synthesis must proceed directly from the more accessible and more stable α-glycosyl halide by *in situ* anomerisation with a non-neighbouring group-active substituent.

As shown in **Scheme 3.3.** the presence of a phase transfer catalyst like tetralkylammonium halide or a metal salt catalyst causes an equilibration between the α-D-glycopyranosyl **[I.44]** and the β-D-glycopyranosyl halides **[I.49]**[231] especially when a solvent of low polarity is used. This anomerisation proceeds via different ion-pairs which are in equilibrium via the ion triplet **[I.48]**. The β-D-glycopyranosyl halide **[I.49]** is destabilised by the anomeric effect (which is very powerful in halide substituents) so that within the equilibrum a much higher amount of the α-D-glycopyranosyl halide is present. Apart from this thermodynamic view of the equilibrium reaction, kinetically, it can be observed that the reaction of the more unstable β-D-halide **[I.49]** to the α-D-glycopyranoside **[I.52]** is a faster reaction compared with the one of the α-D-halide **[I.44]** which occurs under inversion to the β-D-glycopyranoside **[I.47]**.



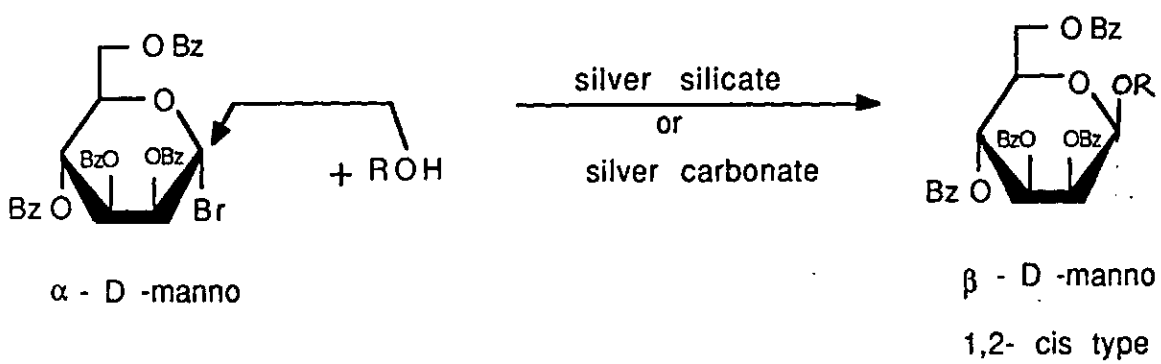
Synthesis of α- D-gluco 1,2-cis type

Scheme 3.3.

This is because the energy barrier for the nucleophilic reactions of the glycosyl halides [I.44] and [I.49] with alcohol R'OH with inversion via [I.44]→[I.45]→[I.46]→[I.47] is higher than that for the corresponding reaction [I.49]→[I.50]→[I.51]→[I.52] so that the formation of the α-glycoside [I.52] is faster than that of [I.47].

Utilizing this difference in reaction rates, the reaction can be made to proceed so that virtually only the α-D-glycoside is produced. This indicates that the reaction must proceed under kinetic control and sufficient difference between both reaction rates must be maintained with a careful coordination of the reactants and catalysts.

The anomeric effect at D-mannose is effective in the same way, and the substituent at C-2 is not involved in the in situ anomerisation procedure for D-mannose, this method will yield the α-D-mannopyranoside which can also be prepared by the neighbouring group assisted procedure. For the preparation of the β-D mannosidic linkage therefore, a solid state catalyst must be applied for the reaction in the heterogeneous phase. Silver carbonate and silver silicate are active catalysts for this reaction[232]. The α-D-manno-pyranosyl halide preferably the bromide can then be transferred under inversion to the β-D-mannopyranoside following the heterogeneous catalyst procedure as shown in **Scheme 3.4.**

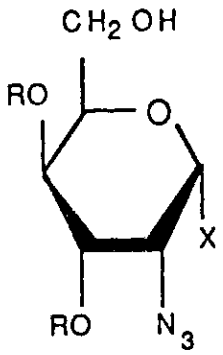
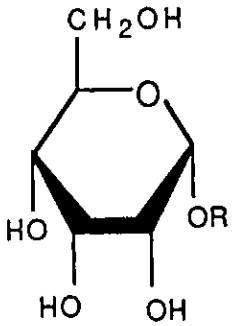


Scheme 3.4.

This heterogeneous catalyst procedure can also be used for other glycosyl halides where the reaction proceeds under inversion, without neighbouring group assistance[233].

The three most important parameters which determine the selectivity and the yield of the glycosidation reaction as shown in Table 3.1. are the reactivity of the halide, of the catalyst and of the hydroxy component[227]. The reactivity of the halide can be varied to a considerable extent, by the choice of the blocking system or the substituents.

Table 3.1.

HALOGEN	CATALYST	ALCOHOL
	Et_4NBr/ molecular sieve 4 Å	
	$Hg(CN)_2$	
	$Hg(CN)_2$ / $HgBr_2$	
	$HgBr_2$ / molecular sieve 4 Å	
	$AgClO_4$, Ag_2CO_3	
R = Bzl > Bz > Ac X = I > Br > Cl	Ag triflate , Ag_2CO_3	HO-CH >> HO-CH R > 6-OH 6-OH >> 3-OH > 2-OH > 4-OH

Acyl substituents like acetyl groups increase the stability and reduce reactivity whereas ether protecting groups, like benzyl ether reduce the stability

and increase the reactivity of the halide. The number of the ether groups is more important than their positions in the molecule hence there is a wide range of possibilities of varying the reactivity for a given halide. There is a range of catalysts of different reactivities which can be utilized for the neighbouring group participation procedure as well as for in situ anomerisation procedure but they are not suitable for the heterogeneous catalyst procedure. However the most difficult problem is to vary the reactivity of the hydroxyl group because generally the glycosyl acceptor has been already predetermined.

Usually primary hydroxyl groups are more reactive than the secondary hydroxyl groups where the reactivity considerably depends on the other substituents in the molecule so that the reactivity can only be determined

with difficulties. For the *in situ* anomerisation procedure, secondary hydroxyl groups with medium reactivity are especially suitable and lead in the desired selectivity to the α-D-glycosides. With primary hydroxyl groups which are much more reactive, the selectivity is reduced in the *in situ* anomerisation because the difference between the two reaction rates of the hydroxy groups to both glycosides is not sufficient and a mixture of the two anomers are obtained.

The solvent is also an important parameter. In the neighbouring group procedure a highly polar aprotic solvent such as nitromethane is usually more suitable while for the in situ anomerisation and the heterogeneous catalyst procedure, a medium polar aprotic solvent e.g. dichloromethane would be more suitable.

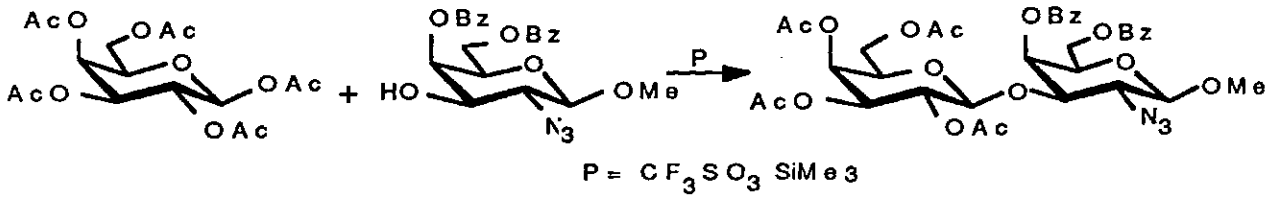
Leaving groups at the anomeric position.

The halides are the classical leaving groups and the order is I > Br > Cl > F. Glycosyl fluoride compounds are not normally employed in oligosaccharide synthesis because the fluoride ion is a relatively weak nucleophile and hence a poor leaving group. It is a relatively stronger base compared to the other halide anions and hence cannot be readily displaced.

There are other leaving groups besides halides in glycosyl compounds which can be used for oligosaccharide synthesis depending on the particular synthesis. They are the following:

i) O-acetyl group. This is a leaving group with a rather low reactivity which can be eliminated by a Lewis acid catalyst under neighbouring group participation. This method is also suitable for a neighbouring group procedure[234] as exemplified by the reaction of β-D-galactose pentaacetate [I.53] and methyl 2-azido 4,6-dibenzyl-galactoside [I.54] shown in Scheme 3.5. The fundamental sequence of

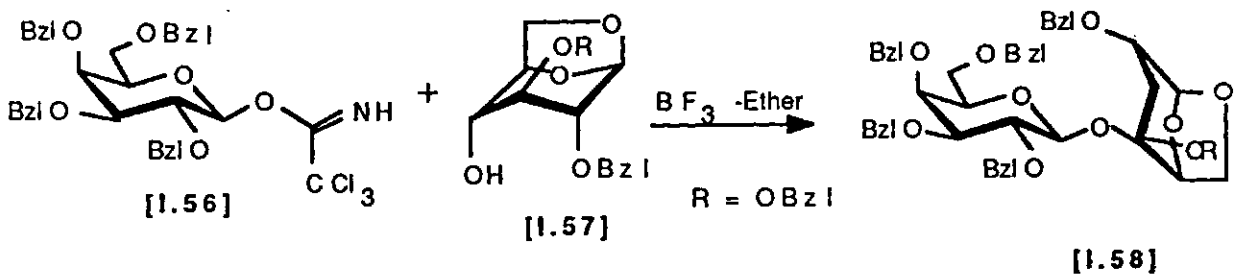
the O-glycoproteins has been achieved by using this procedure[235] with the reaction in **Scheme 3.5.** as the starting material.



Scheme 3.5.

ii) Trichloro-imidate method[236, 237]. The trichloroaceto imidate group is less reactive than a halide, but much more reactive than the acetyl group. When the trichloroaceto imidate group is used as a leaving group, it should be used in the neighbouring group assisted procedure in order to obtain good selectivities to the β-D-glycosides. The trichloroaceto imidates can also be used for the reaction without neighbouring group assistance but the inversion leads from the imidates to the corresponding α-glycosides. It is however not easy to prepare the β-imidate and the reaction of the β-imidates often yields anomeric mixtures.

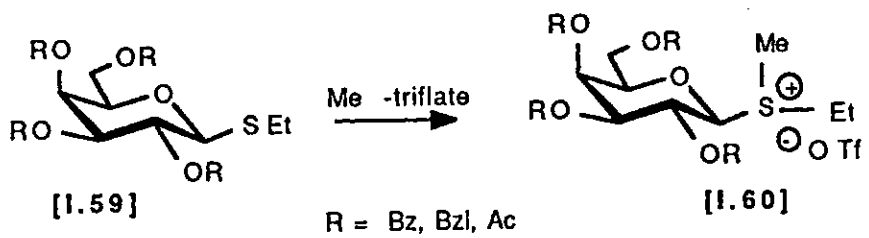
An example of this method is the synthesis of a 1→2 linked disaccharide **[I.58]** using the trichloroaceto-imidate method which is shown in **Scheme 3.6.** The reaction is that of the trichloro-acetoimido



Scheme 3.6.

2,3,4,6-tetrabenzyl glucopyranoside [I.56] and 1,2-dibenzyl-4,6-anhydrogalactopyranoside [I.57].

iii) The thioglycosides method[238,239]. Using methyl triflate, the thioglycosides e.g. [I.59] can be converted by methylation to the unstable intermediate sulfonium compounds e.g. [I.60], the methylated sulfonium aglycone groups of which are effective leaving group.



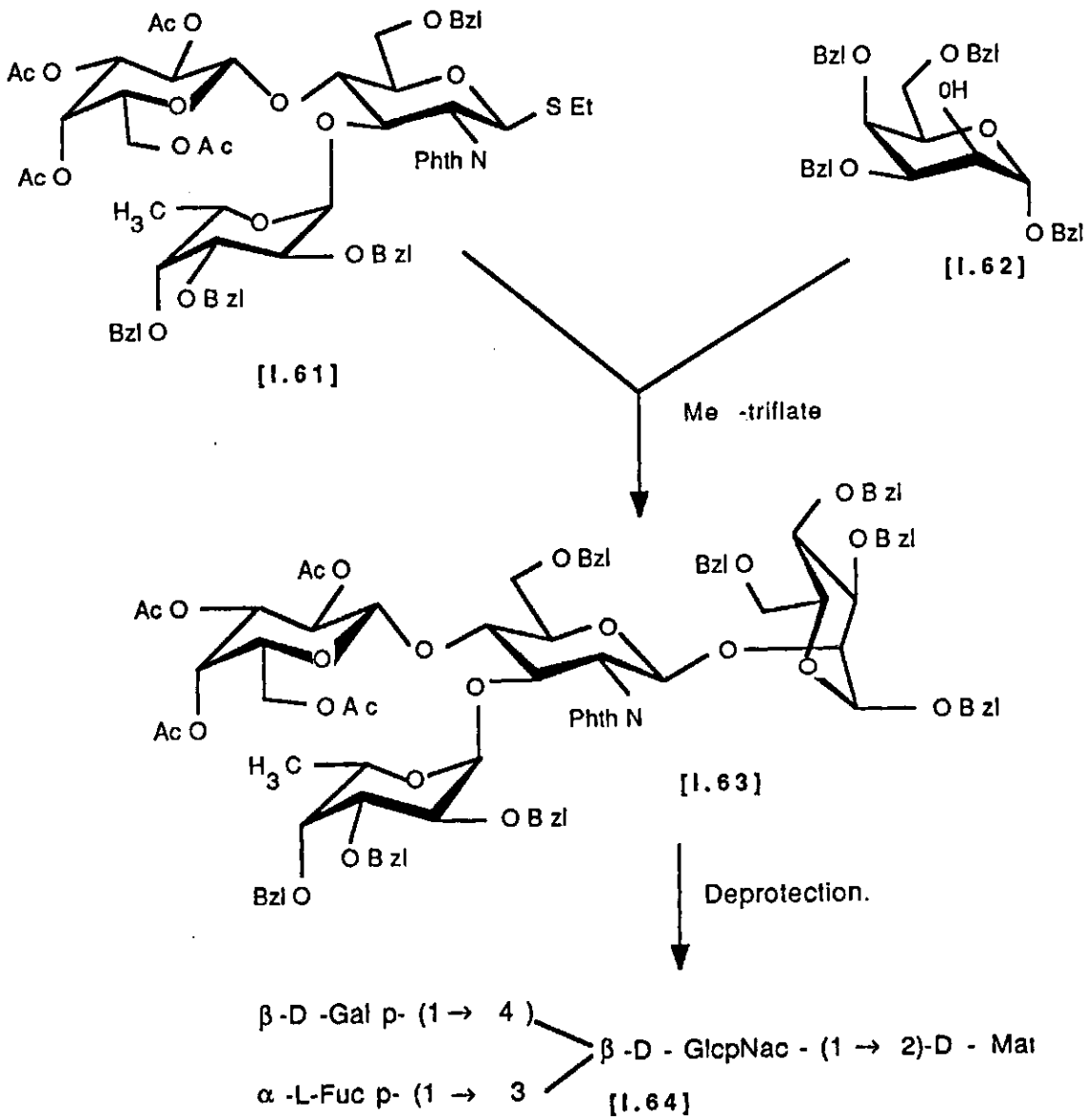
Using thioglycosides with a neighbouring group assistance, this method yields oligosaccharides with good selectivity to the β-D-glycosides but the corresponding reaction with a non-neighbouring active group yields a mixture of α and β glycosides, the proportion of which depends to a large extent on the solvent. The application of this method in its neighbouring group assisted form has been used in the synthesis of large oligosaccharide units such as a heptasaccharide and a nonasccharide[239].

As seen in the example in Scheme 3.7., the tetrasaccharide [I.64] can be obtained by the deprotection of [I.63] after the reaction of the trisaccharide [I.61] with the D-manno derivative [I.62] forming the β-D-glycosidically linked product. Instead of the highly toxic methyl triflate, the trimethyl disulfonium triflate can be used as the promotor (catalyst)[239]. With neighbouring group assistance, this method only affords the β-glycosidically linked product in high yields but under

the non-neighbouring group assistance the reaction leads to a mixture of anomers as the selectivity is decreased due to the formation of a more reactive intermediate. Also, the proportion of the anomers formed depends strongly on the solvent used. It is also possible to convert the thioglycoside to the glycosyl chloride or bromide, to activate the anomeric centre, by reacting with chlorine or bromine or a copper(II)bromide/tetra-butyl ammonium bromide complex. The glycosylation reaction of the resulting halide then proceeds under the glycosyl halide reaction conditions with or without the neighbouring group assistance to yield selectively the α or β oligosaccharide or a mixture of both depending on the reaction conditions.

More recently glycoyl fluoride has been generated from the thioglycoside by N-bromosuccinimide/diethylaminosulfur trifluoride N-bromosuccinimide /hydrogen fluoride-pyridine complex. It has been observed that when using thioglycosides directly in the glycosylation reactions, the thioglycoside group causes the deactivation of the other hydroxyl groups in the molecule and this may raise some difficulties with this method.

Furthermore the building up glycoside synthesis with the methyl triflate or metal salt catalysts can also be applied to a limited extent. Despite these facts, the thioglycosides have their advantages over the glycosyl halides which are the classical donors in glycosylation reactions. This is because problems may arise in the conversion of many oligosaccharide derivatives (such as glycosides glycosyl esters) into glycosyl halides resulting in the low yielding steps quite far into the synthetic sequence. Thioglycosides have therefore attracted considerable attention for use in glycosydation reactions.



Scheme 3.7: The synthesis of a tetrasaccharide using the thioglycoside method.

3.2. Synthetic Studies

The n.m.r. study of methyl 1→2 and 1→3 linked glucose disaccharides has been previously described[240]. It is the aim of the project to carry out the n.m.r. studies of trisaccharides i.e. the next higher oligosaccharide to determine if known changes in the glycosidation shifts from the n.m.r.spectrum of the disaccharides can be used for those of related trisaccharides after the additivity of the 1→2 and 1→3 disaccharide units are taken into consideration. In addition, the purpose of this study was to provide part of the data for a computer-based analysis of n.m.r. spectra from polysaccharides and larger oligosaccharides[219]. Another aim of the study is to determine the effect of temperature on the glycosylation shifts of the constituent monosaccharides in their n.m.r. spectra.

Four trisaccharides made up of glucose monomers with the middle glucose unit vicinally disubstituted were synthesized with the variation of the anomeric configuration at the linkage position. These were to serve as models to check if the results from the n.m.r. and conformational studies would answer the questions raised above.

The four trisaccharides that were synthesized can be represented as **i- iv**.

i. α-D-Glucose(1→2)-α-D-Glucose(1→3)-α-D-Glucose(1→OMe

ii α-D-Glucose(1→2)-β-D-Glucose(1→3)-α-D-Glucose(1→OMe.

iii. β-D-Glucose(1→2)-β-D-Glucose(1→3)-α-D-Glucose(1→OMe.

iv. β-D-Glucose(1→2)-α-D-Glucose(1→3)-α-D-Glucose(1→OMe.

EXPERIMENTAL

ER 4

. CHEMICALS AND REAGENTS FOR THE STRUCTURAL AND SYNTHETIC STUDIES.

The following chemicals and reagents used for both the structural and synthetic studies are listed in alphabetial order under the headings of suppliers (written in **bold type**) and are of analytical grade except when indicated otherwise.

E.Merck, Darmstadt W.Germany.

Acetic acid (glacial)

Acetic anhydride

Acetonitrile

Benzaldehyde

Benzyl bromide

Benzyl chloride

Celite

Chloroform

Deuterochloroform

Deuterium oxide 99% and 99.9%

Dichloromethane

2,4-Dinitrophenyl hydrazine

Ethanethiol

Ethanol (Absolute and 95%)

Ethyl acetate

Hexane p.a.

Hydrochloric acid (37%)

Hydrogen fluoride (liquified)

Magnesium sulphate.

Methanol

Methyl iodide

E.Merck, Darmstadt W.Germany.

Palladium on Carbon (10%)

Petroleum Ether 40-60°C

Petroleum Ether 60-80°C

Phosphorous pentachloride

Phosphorous pentoxide

Phosphorous tribromide

Silica gel 60

Sodium (in oil)

Sodium bisulphite

Sodium chloride

Sodium iodide

Sodium metaperiodate.

Sodium 3-trimethylsilylpropionate-d_4 (TSP)

Sulphuric acid 98%

Tetrahydrofuran

Tetrabutyl ammonium bromide

Tetramethyl silane

Toluene p.a.

p-Toluene sulphonic acid

2,4,6-trimethylpyridine.

Aldrich-Chemie, Stenheim, West Germany.

S(+)-2-butanol 99%.

2,2,2-Trifluoroethanoic acid (Trifluoroacetic acid, TFA) 99%.

Riedel de Haen AG Seelze-Hanover.

Methyl-α-D-glucopyranose

Pyridine p.a.

Sodium acetate.

Fluka AG Buchs,Switzerland.

Acetyl chloride

Silver nitrate

Silver trifluoromethane sulphonate

Sodium borodeuteride

Sodium borohydride

Sodium hydride (60% suspension in oil)

BDH, England

Copper(II) bromide

Ethanol (Absolute and 95%)

Dimethylformamide (g.p.r.)

D-Glucose

Methanol

Raffinose

Stachyose

Sodium hydroxide

Sodium hydrogen carbonate

Sodium sulphate

Sucrose

Zinc chloride

Fischer Scientific Company, USA.

Bromine

Dimethyl sulphoxide (DMSO)

J.T.Baker, USA.

Ammonium Hydroxide 25%.

Kebo Lab, Sweden

Molecular Sieve 3Å and 4Å

Biorad Laboratories, Sweden.

Biogel P-2, P-4 and P-10 column packing.

Pharmacia Fine Chemicals, Sweden

Dextran 10.

RNA-ase

Sodium dodecyl sulphate.

Sigma Chemical Company, USA.

Charcoal

Xylose

Sigma Chemical Company, USA

Sil A (Trimethylsilylating reagent)

Atlanta Heidelberg,Georgia,USA.

Fatty acid methyl esters:

N-decanoic acid C-10:0; n-dodecanoic acid C-12:0; anti iso tridecanoic acid a.i.C-13:0; n-tridecanoic acid C-13:0; iso tetradecanoic acid i.C-14:0, n-tetradecanoic acid C-14:0, anti iso pentadecanoic acid a.i.C-15:0; n-pentadecanoic acid C-15:0; iso hexadecanoic acid i.C-16:0, n-hexa decanoic acid C-16:0, anti iso heptadecanoic acidC-17:0; iso heptadecanoic acid; C-17:0 used as internal standard in fatty acid analysis, n-octadecanioc acid C-18:0; iso octadecanioc acid C-18:0; anti iso nonadecanoic acid C-19:0 ; iso nonadecanoic acid C-19:0; n-eicosaanoic acid C-20:0; iso eicosaanoic acid
C-20:0; 3-hydroxydecanoic acid 3-OH-C-10:O, 2-hydroxydodecanoic acid 2-OH C-12:O; 3-hydroxydodecanoic acid 3-OH-C-12:O; 3-hydroxytetradecanioc acid 3-OH-C-14:O, 3-OH 3-hydroxyoctadecanioc acid C-18:O.

EQUIPMENTS

The following equipments were made use of :-

Beckman Refrigerated Centrifuge by Beckman, England.

Bransonic 220 Ultrasonicator by Belos Ingeniofirm Aktiebolag, Sweden.

Custom made columns for Biogel P-4 and P-10 column supplied by Pharmacia

Fine Chemicals, Sweden.

Hetossic CD 52 freeze dryer with an attached Pfeiffer vacuum pump supplied by Heto Lab Equipments, Denmark.

Hydrogen Fluoride (HF) Reaction Apparatus by Peninsula Laboratories, California, USA.

HP 5890-MS 5970 Combined Gas Chromatograph-Mass Spectrometer coupled to a Computer Work station 59970 by Hewlett Packard, USA.

HP 5830A Gas Chromatograph fitted with a Flame Ionisation Detector and an SE-54 fused-silica capillary column by Hewlett Packard

JEOL FX 100, GX-270 and 400 Nuclear Magnetic Resonance Spectrometer by JEOL Limited, Japan.

Parr Hydrogenation Apparatus by Parr Instrument Company, Illinois USA.

Perkin Elmer 241 Polarimeter by Perkin Elmer, USA.

Pye Unicam 204 Gas Chromatograph fitted with a Flame Ionisation Detector from Pye Unicam, England and supplied by Phillips (Nig) Ltd.

6feet Supelco SP 2340 packed column by Supelco Inc. Switzerland.

Peristalsic pump P-3 by Pharmacia Fine Chemicals, Sweden.

Refractometer Optilab Multiref 902 made by LKB Instruments, Sweden.

Rotary Evaporator from Technik AB Switzerland.

Sep-Pak C-18 Catrigdes made by Waters Associates.

Serum vials and Teflon septa obtained from Supelco Inc ,USA

Sorval SS-3 Automatic Superspeed Centrifuge by Sorval Company USA.

Vibromix Vibrator from Heidolph, West Germany.

Wifug Laboratory Centrifuge 10206 by Wifug Limited, England.

PREPARATION OF ANHYDROUS SOLVENTS.

Dimethyl sulphoxide (DMSO)

The DMSO was redistilled under vacuum over calcium hydride and stored over molecular sieve 4Å.

Dichloromethane

The dichloromethane was always freshly distilled over P_2O_5 before use.

Tetrahydrofuran (THF)

The tetrahydrofuran was redistilled over sodium with benzophenone as an indicator. Benzophenone imparted a blue coluration to the THF when the sodium was spent. The THF can then be recharged with more sodium.

Pyridine.

Anhydrous pyridine was obtained by redistilling over phosphorous pentoxide and stored over molecular sieve.

Diethyl ether: Dry diethylether was obtained by storing over sodium drawn out as a wire.

PREPARATION OF STANDARD REAGENTS

0.5 % 2,4-dinitrophenyl hydrazine reagent.

This reagent was prepared by dissolving 2,4-dinitrophenyl hydrazine (500mg) in 2M hydrochloric acid (100ml).

Phenol reagent (aqueous) 90% w/v·

The phenol reagent was always freshly prepared before use by adding distilled water (10mL) to phenol (90gm) and stirring with a glass rod to ensure complete dissolution.

Cold Methanol Reagent

20ml of methanol was saturated with sodium acetate. To make a 100mL of the reagent, 1mL of the saturated solution was added to 99 mL of methanol and mixed thoroughly. The reagent was stored at $4^{o}C$.

2M Dimsyl sodium for methylation analysis.

5g of sodium hydride (50% suspension in oil) was weighed into a 100 ml serum vial and suspended in 25 mL hexane to remove the oil. The hydride was allowed to settle and the liquid was decanted. This procedure was repeated twice and the washed sodium hydride was then dried by flushing

with dry nitrogen. Dried DMSO was added to the vial which was sealed with a rubber septum and flushed with nitrogen via two injection neeedles. The vial was placed in an oil bath and heated to 50°C.

Hydrogen was evolved and vented through an injection needle. After about 5 hours, the hydrogen evolution ceased and the resulting greyish-yellow opalescent solution of dimsyl sodium was ready. The reagent was stored in the freezer as it is stable for about three months at about -18 to -20°C.

4.2. ISOLATION AND ANALYSIS OF THE BACTERIAL POLYSACCHARIDES

4.2.1. *Brucella abortus (B.abortus)* Biotype 1,Strains 7 and Mustapha.

The two strains of B.abortus, biotype 1 strains 7 and Mustapha typed at the Ministry of Agriculture and Veterinary Laboratory, New Haw, Weybridge, Surrey in England, were obtained from the collection of the National Veterinary Research Institute (N.V.R.I.) Vom. Strain 7 was isolated from the South Devon breed 27 at the Livestock Investment Breeding Centre in Kano while strain Mustapha was isolated from Alhaji Mustapha's herd, a local milk collection centre in Maiduguri.

Culturing conditions

The two strains were supplied as lyophilized cultures and grown on potato dextrose agar under carbon dioxide for 48 hours at 37°C. The bacterial growth was harvested with sterile 1% saline. The cells were then centrifuged and killed with acetone to obtain the acetone dried cells.

Extraction of the Crude LPS

The LPS was extracted by the hot water-phenol[74] as follows: The acetone-dried cells of B.abortus (20g) was suspended in distilled

water (680mL) and heated to 66°C in a water bath. The phenol reagent (760mL) was heated to the same temperature and added to the cell suspension giving a phenol-water ratio of 1:1 w/w. The mixture was agitated thoroughly by power stirring for 15 minutes and distributed into six 200mL Beckmann centrifuge tubes and cooled very rapidly to 5°C in an ice bath. This was followed by centrifugation at 13,000 x g for 15 mins at 4°C which separated the content in each tube into four layers: a bottom layer of sedimented cell residue on which floated a water-saturated phenol layer on top of which was an interphase precipitate and an uppermost layer consisting of phenol-saturated water.

The uppermost aqueous layer was sucked off carefully using a Pasteur pipette. It was dispensed into centrifuge tubes, 50mL in each tube and centrifuged. The supernatant from the aqueous phases was pooled, dialysed against several changes of distilled water, concentrated to 50% of its volume by pervaporation at room temperature. To this was added 5 volumes of cold methanol reagent (one part of methanol saturated with sodium acetate to 99 parts of methanol) slowly and with stirring. After standing at 4°C for several hours, some precipitation was observed. This was lyophilized to yield the crude aqueous phase LPS (APLPS) for each of the two strains.

The interface precipitate and the lower water-saturated phenol layer were separated by filtration through a Whatman No.42 paper under gentle suction.

Methanol reagent was added to the phenol layer (3:1 v/v). The solution was mixed for 5minutes and left at 4°C for 1 hour during which precipitation was observed. The material was centrifuged for 15 minutes at 4°C. The supernatant was discarded while the precipitate was suspended in 400mL of distilled water and stirred overnight on a magnetic stirrer. The suspension was centrifuged at 10,000 x g for 30 mins at 4°C.

The supernatant liquid was kept while the precipitate was resuspended in 400mL of distilled water, stirred for 1hour and centrifuged. This step was repeated once more. The supernatant liquids from the three extractions were pooled, dialysed against several changes of distilled water and pervaporated at room temperature to a final volume of 200mL. After centrifugation at 10,000 x g for 15 min, the precipitate that was obtained was dissolved in 80mL of distilled water and lyophilized to yield the crude phenol-phase LPS (PPLPS) for each strain.

Purification of the Crude APLPS and PPLPS[241].

The crude LPS had been found to contain other bacterial components such as proteins, nucleic acids and other polysaccharides apparently associated with the LPS by strong non-covalent bonds. This necessitated the following purification process.

Batches of 100mg each of the crude LPS were dissolved in 20mL of dimethyl sulphoxide and stirred for 20 min at room temperature. 20mL of 4M NaI was added with slow stirring. The NaI served as a chaotropic agent to disrupt the noncovalent association between the LPS and other bacterial components.

The solution was kept at room temperature for a further 20 min after which was added 120mL of the cold methanol reagent with stirring. The resultant mixture was kept at 4°C for 1hour during which the LPS precipitated. The precipitate was redissolved in 20 mL DMSO and 20mL of NaI after which was added 120mL of the cold methanol reagent to effect precipitation. The procedure was repeated four more times. After the last precipitation with the methanol reagent the LPS was redissolved in 80mL of distilled-deionised water at 4°C and lyophilized .

Isolation of the PS and lipid A from the LPS of B.abortus biotype 1, Strains 7 and Mustapha.

Delipidation was carried out on 50mg strain of the PPLPS of each strain and for 3hours at 100°C using 10mL 0.1M acetic acid. After cooling, the solution was centrifuged to remove the precipitated lipid. The solution was next extracted with 5mL n-hexane three times to remove any lipid left in solution. The aqueous solution was lyophilised to obtain the phenol phase polysaccharide (PPPS). The lipid was also lyophilized and the sugars present in both the (PPPS) and the lipid were analysed on the G.L.C. as their alditol acetates.

Sugar Analysis of the PPPS

4M hydrochloric acid (2mL) was added to the PPPS (5mg) in a pear shaped flask and heated at 100°C on a water bath for 4hours. The product was allowed to cool and sodium borohydride (25mg) was added, checked for alkalinity and left for 2 hours at room temperature. The excess sodium borohydride was decomposed by the addition of 50% acetic acid to a pH of 3.5. The product was then evaporated to near dryness on a rotary evaporator at 40°C. To remove the borates generated in the reaction mixture as their methyl derivatives, 5% acetic acid in methanol (v:v) was added and also evaporated to dryness. This step was repeated twice more. 95% aqueous acetic anhydride was added, the flask was tightly stoppered and heated at 100°C for 1hour. After the flask has cooled down, the excess acetic anhydride was removed by co-distillation with toluene (5mL). This step was repeated thrice more until the product was completely evaporated to dryness. Dichloromethane (3mL) was added, and followed by washing twice with water. The dichloromethane layer was withdrawn into a sample tube, concentrated and injected on the G.L.C.

A Pye Unicam 5830A instrument fitted with a flame ionisation

detector and a 6 feet SP-2340 packed column was employed. Both injector and detector temperatures were 250°C while the column temperature was at 210°C held for 10 minutes and increased to 250°C at the rate of 4°/min.

The sugar analysis was repeated using 2M TFA at 120°C for 1hour and the work up done was essentially as above to obtain the alditol acetates.

Preparation of the Alditol Acetate Standards.

The alditol acetates of the following sugars were prepared as standards. They were arabinose, rhamnose, mannose, glucose, galactose, N-acetyl-glucosamine, and N-acetylgalactosamine. A solution of each sugar (5mg/ml) in distilled water was made, sodium borohydride (25mg) was added, and the derivatisation continued as in the above procedure for the PPPS.

^{13}C N.M.R.

To 20mg of each PPPS of the two strains, was added 0.4mL of 99% D_2O. The spectra were recorded on a GX-100 Jeol spectrometer at 70°C with full proton decoupling. Chemical shifts are reported using dioxane (^{13}C δ 67.4 ppm.) as internal reference for strain 7 and acetone as internal reference (^{13}C δ 31.04 ppm.) for strain Mustapha.

Sugar Analysis of Lipid A of B.abortus biotype 1 Strains 7 and Mustapha[242].

1mg of the lipid contained in a 13mm screw-cap tube was hydrolysed at 100°C using 0.5mL of each of the various acidic media under these conditions - 4M HCl for 4hr, 4M HCl for 18hr, and 2M TFA for 18hr. The excess acid was evaporated by flushing with air at room temperature.

The hydrolysate was reduced with aqueous 0.2mL sodium borohydride solution (10mg/mL $NaBH_4$) for 30min at room temperature. The excess borohydride was removed by adding 50% acetic acid to pH 3. The borates generated were removed as the methyl derivatives by carrying out

evaporation to dryness with 10% acetic acid (5ml) in methanol followed by two more evaporations with methanol (5mL). The alditols formed were acetylated with 0.5mL dry acetic anhydride-pyridine mixture (1:1 v/v) at 120°C for 20min. The excess reagents were removed by the addition of toluene (2mL) and flushing with air at room temperature. The evaporation was carried out twice more. The alditol acetate mixture obtained was partitioned between water and dichloromethane. The dicloromethane layer was withdrawn into a sample tube, concentrated and injected on the G.L.C.: a Hewlett Packard 5830A instrument fitted with a flame ionisation detector and an SE 54 fused silica capillary column. Both the injector and detector temperatures were 270°C while the column temperature was 185°C held for 8minutes and increased to 230°C at 5°/min. In order to determine the percentage of the backbone sugars present in the lipid, the procedure was repeated using mannose as internal standard.

Preparation of the Alditol Acetate Standards for Fatty Acid Sugar Analysis.

The alditol acetates of the following sugars were again prepared as standards. They were arabinose, rhamnose, mannose, glucose, galactose, N-acetylglucosamine, and N-acetylgalactosamine. The sugar (2mg) was dissolved in distilled water (0.5 mL) and sodium borohydride solution (10mg/mL) (2mL) was added, and the derivatization continued as in the above procedure for the fatty acid sugar analysis.

Fatty acid analysis of the B.abortus biotype 1 Strains 7 and Mustapha[243]

Each of the the APLPS and the PPLPS (3mg) was methanolysed in 2M methanolic HCl (2ml) for 18hours at 80°C in a 13mm screw cap tube. After cooling, the volume was concentrated to half by evaporation. Half-saturated sodium chloride (2mL) was added and the fatty acid methyl

esters were extracted with hexane. The hexane extract was concentrated and injected on the G.L.C. as carried out for the sugar analysis but using a temperature programme of 180°C, held for 10min and subsequently increased to 220°C at 5°/min. n-Heptanoic acid was used as internal standard. A similar analysis as decribed above was carried out on the lipid A obtained after delipidation of the APLPS and APLPS.

4.2.2. *Campylobacter coli* Labet 227

Isolation and purification of Lipooligosaccahride (LOS).

C.coli Labet 227 was isolated from a diarrhoea patient below the age of six years at Ohaozara Local Government area of Imo State and obtained in the lyophilised form, from the collection of the Department of Medical Microbiology, College of Medicine, University of Lagos.

The freeze dried bacteria cells were extracted twice with 45% aqueous phenol at 66°C as described for isolation of the two strains of the lipopolysaccahride B.abortus biotype 1. The aqueous phase was dialysed and purified by ultracentrifugation and treatment with RNA-ase as done for the B.abortus. The washed LOS was freeze-dried.

Calibration of the Biogel P-10 column[244]

600mL of a mM aqueous formic acid containing 0.5% of 1,1,1-trichloro-butanol as the bacteriostatic agent was added to 250g of the dry Biogel P-10 powder in a Buchner flask. The mixture was heated for 1hour at 90°C on a water bath in order to swell the gel properly. The gel was allowed to cool down and was completely deaerated by connecting to a vacuum pump for a period of 30minutes. After disconnecting from the pump, the gel was poured gently and carefully into a (90 x3 cm) transparent plastic column, the end of which was closed. The pouring was done along the side of the column to eliminate trapping of air bubbles. After the gel has packed to

3cm below the top of the column, it was allowed to run freely and the column left to pack carefully under gravity. The column was then connected to a reservoir containing the eluent which was a mM aqueous formic acid containing 0.5% of 1,1,1-trichlorobutanol. The volume of the eluent equivalent to twice the bed volume was allowed to run through the column continuously.

The column was then calibrated by applying 2ml of the solution of a mixture of 5mg each of glucose, sucrose, raffinose, stachyose and dextran 10 to the top of the column and using the same eluent under controlled conditions such rate of flow of 27mL/hour, chart speed e.t.c. The eluate was monitored using a differential refractometer. The polysaccharide (dextran 10) was eluted in the void volume as a peak and the remaining four sugars were eluted according to their molecular weights as four consecutive peaks.

Preparation of the C.coli Oligosaccharide (OS) samples.

The OS was obtained from the LOS (91mg) by treatment with 0.1M aqueous acetic acid (20ml) at 100°C for 3hours followed by centrifugation at 5000 r.p.m. to remove the insoluble lipid A. The supernatant was extracted with n-hexane (2x25ml) to remove any remaining lipids still present in solution. The OS was freeze-dried and the lyophilised material was redissolved in water (2ml) and applied on a Biogel P-10 column (90x3cm) which had been previously calibrated with a mixture of glucose, sucrose, raffinose, stachyose and dextran 10. The materials were eluted with mM aqueous formic acid and the eluate was monitored using a differential refractometer. Four peaks corresponding to four different fractions were obtained.

Sugar analysis of the C.coli OS[242].

The oligosaccharide sample (1mg) in a 13x100mm screw cap tube was hydrolyzed with 0.5ml of 2M trifluoroethanoic acid (TFA) at 120°C for 1hr. The excess acid was evaporated by flushing with air at room temperature.

The hydrolysate was reduced with sodium borohydride solution (0.2ml of 10mg/ml $NaBH_4$) for 30 min at room temperature. Excess borohydride was removed by adding 50% acetic acid to pH 3. The borates generated were removed as the methyl derivatives by successive evaporations with 10% acetic acid in methanol (2x5ml) and methanol (2x5ml).

The alditols formed were acetylated with dry acetic anhydride-pyridine mixture (1:1 v:v) at 120°C for 20min. Excess reagents were removed by addition of toluene (3x1mL) and flushing with air at room temperature. The alditol acetate mixture obtained was partitioned between water and dichloromethane. The dichloromethane layer was withdrawn into a sample tube, concentrated and injected on the G.L.C.

A Hewlett-Packard 5830A instrument fitted with a flame ionisation detector and an SE-54 fused silica capillary column was employed. Both injector and detector temperatures were 270°C while the column temperature was at 210°C held for 3 minutes and increased to 250°C at the rate of 2°/min. The alditol acetates were further confirmed by a G.C.-M.S.on a Hewlett-Packard 5970 instrument fitted with an SE-54 fused silica capillary column. The temperature conditions for the characterisation of the alditol acetates were the same for the gas chromatograph unit of the G.C.-M.S. as in the G.L.C.and the spectra were recorded at an ionisation potential of 70eV with the scan of the fragments ranging from 50 to 550m.u.

Methylation analysis of the *C.coli* OS.

The methylation was carried out according to the method of Hakomori[83]

modified by Jansson et al[77]. The freeze-dried OS (3mg) was dissloved in dry DMSO (3mL) contained in a 10mL serum vial which was sealed with a rubber septum. The vial was flushed with dry nitrogen via two injection needles. The LOS was dissolved by stirring using a small magnetic stirrer bar. 2M Dimsyl sodium (1.5mL) in DMSO was added dropwise via an injection needle using a syringe. The vial was agitated in an ultrasonic bath for one hour and then left at room temperature overnight. The vial was cooled in an ice-water bath and 1.5mL methyl iodide was added dropwise using a syringe. Excess pressure was released via a second needle. The vial was again ultrasonicated for 30minutes at room temperature and stirred for 1 hour after which it was opened and the excess methyl iodide removed by flushing with nitrogen.

The methylated products were recovered by reverse phase chromatography on Sep-pak C-18 catridges[96]. After dilution with an equal volume of water, the methylated material was applied to the catridge which had previously been washed successively with ethanol (40ml) and water (10ml). The methylated material was retained on the column and was washed with water (2x4ml) and a 15% aqueous acetonitrile solution (2x4ml). The sample was subsequently eluted with (2x2ml) 100% acetonitrile.

Conversion to partially methylated alditol acetates followed by analysis on GLC were carried out as described under sugar analysis of the C.coli OS.

The temperature program was 150°C held for 2min and increased to 220°C at the rate of 2°C/min. The partially methylated alditol acetates were further analysed on the G.C.-M.S. and from the m/e values obtained, the substitution patterns of the sugar derivatives were identified.

^{1}H N.M.R.Spectra:Sample preparation of the C.coli OS.

The C. coli OS (5mg) was treated twice with 99% deuterium oxide (D_2O)

(0.4ml) and freeze dried after each treatment. It was then dissolved in 99.9% D_2O(0.4ml) and the ^1H n.m.r. spectrum was recorded at 85°C with a JEOL GX-400 instrument. Chemical shifts were recorded in ppm relative to internal sodium 3-trimethylsilylpropionate-d_4 (TSP).

Fatty acids analysis of the LPS of the C.coli:

LOS (3mg) was methanolysed with 0.1M HCl in methanol(2mL) at 85°C for 18hours[243]. The methyl esters were extracted with hexane and the volume was reduced under a stream of nitrogen. Analysis was on G.L.C. as for sugar analysis using a temperature programme of 180°C, held for 10min and subsequently increased to 220°C at 5°C/min. n-Heptadecanoic acid methyl ester was used as internal standard.

4.2.3. *Escherichia coli*(*E. coli*)O149.

E. coli O149 strains 853-76 and 512-N were isolated from neonatal piglets and the lyophilised purified LPS was obtained from the National Bacteriological Laboratory, Stockholm, Sweden.

Preparation of the polysaccharide samples

The LPS was isolated from the aqueous phase during the hot phenol-water procedure and purified as previously described under the *B.abortus* experimental.

Fully delipidated polysaccharide (PS 1) and (PS 2).

The fully delipidated polysaccharide was isolated from the lipopolysaccharide by mild acid hydrolysis with aqueous 0.1N acetic acid for 1.5 hours and 3 hours at 100°C to obtain the partially pyruvylated polysaccharide (PS 1) and completely depyruvylated polysaccharide respectively (PS 2). The subsequent work-up was performed as described

previously under C.coli delipidation procedure. .

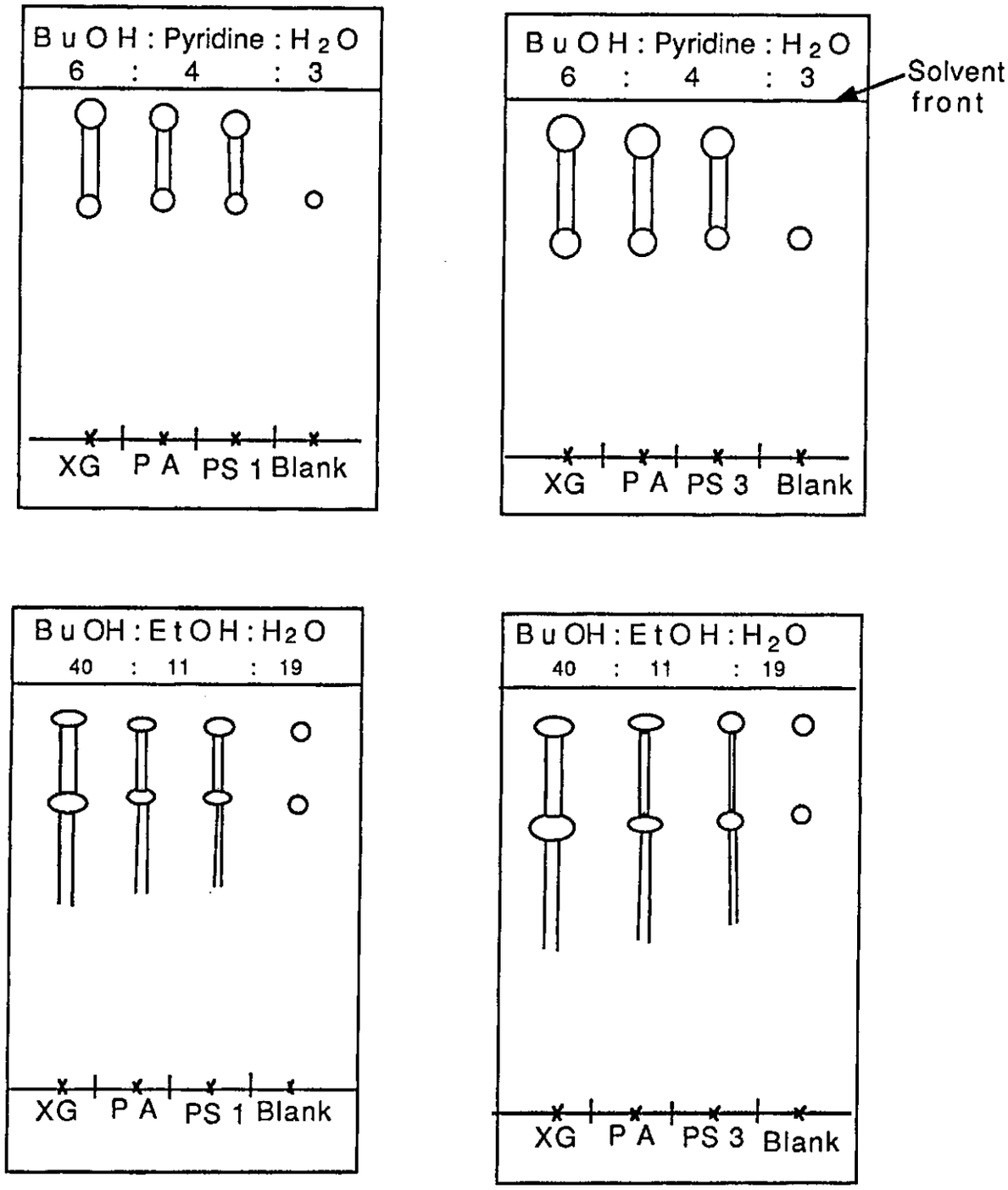
Partially delipidated polysaccharide (PS 3).

The partially delipidated and fully pyruvylated polysaccharide (PS 3) was prepared by treating the native lipopolysaccharide with 0.1N aqueous sodium hydroxide for 16 hours with vigorous stirring at room temperature. The excess sodium hydroxide was neutralised with 0.1N aqeuous acetic acid and the product was dialysed against distilled water for 48 hours. The dialysed solution was freeze-dried. The recovered material was purified by chromatography on a previously calibrated column (90 x 3cm) of Bio-Gel P-10 column eluted with mM aqueous formic acid. The polysaccharide (PS 3) was eluted in the void volume.

The pyruvic acid test

The polysaccharide (PS 3) was tested for pyruvic acid according to the procedure of Nimmich[245]. As standards, pure pyruvic acid and xanthan gum (a model polysaccharide known to contain pyruvic acid) were used. A blank was also run. The partially (PS 2) and fully pyruvylated polysaccharides (PS 3)(2mg) each were hydrolysed for 3 hours at 100°C with 1mL of 1M hydrochloric acid. To each of the hydrolysates, 2,4-dinitrophenylhydrazine reagent (0.5mL) was added, mixed thoroughly and allowed to stand at room temperature for 10 minutes. On adding the 2,4-dinitrophenyl hydrazine, the hydrolysates of both the E.coli polysaccharides and Xanthan gum became very cloudy and yellow, while a yellow precipitate was obtained from the pure pyruvic acid sample due to the formation of the phenyl hydrazone derivative of the pyruvic acid.

The reaction mixture was extracted with ethylacetate (2mL). On adding ethyl acetate, the ethylacetate phase of the samples changed to deep golden yellow. A clear yellow solution was obtained from the blank.



Marked out areas are yellow and faint when developed on the TLC plates but are reddish purple when observed under the U V light.

P A = Pyruvic acid P S 1 = E. coli O 149 PS 1 ▯ = TLC plates

X G = Xanthan Gum P S 3 = E. coli O 149 PS 3

Fig. 4.1. The Thin Layer Chromatogram of the Pyruvic Acid Test.[245]

The ethylacetate phase was reextracted by shaking vigorously with 10% sodium hydroxide (1mL). The golden yellow colour of the ethyl acetate phases became deep red wine in colour on the addition of sodium hydroxide, but the blank retained its yellow colour. The sodium hydroxide solution was treated with 2M hydrochloric acid to pH 3.0 and after acidification, the solution was again extracted with ethylacetate (0.5mL). Fine yellow precipitates of the dinitrophenyl hydrazones were now formed in the tubes containing the three samples except that of the blank. The precipitates were all soluble in the ethyl acetate which was used to reextract them.

The final ethylacetate extracts of the four materials were spotted on a TLC plate and the chromatogram was run with a mixture of n-butanol-pyridine-water (ratio 6:4:3) as the eluent. Another run was made using the n-butanol-ethanol-water mixture in a ratio of (40:11:19) for another TLC spotted plate. The chromatograms are shown in Figre 4.1.

Sugar analysis of the E.coli polysaccharides.

Hydrogen fluoride (HF) solvolysis[246]

Before carrying out the sugar analysis, hydrogen fluoride solvolysis was carried out in order to completely cleave off the glucosamine linkages present in the polysaccharides.

The freeze-dried polysaccharides (PS 1)and a stirring bar were placed in the reaction tube and connected to the hydrogen fluoride reaction apparatus(PS 3). All the components of the apparatus are made of polytetrafluoroethane (PTFE) sold under the trademark of Teflon. See Figure 4.2. for the schematic diagram of the HF apparatus. The whole assembly was checked for leaks and made airtight. The HF apparatus reservoir containing cobalt chloride (as the drying agent) was evacuated with the aid of the vacuum pump that was connected to the whole assembly.

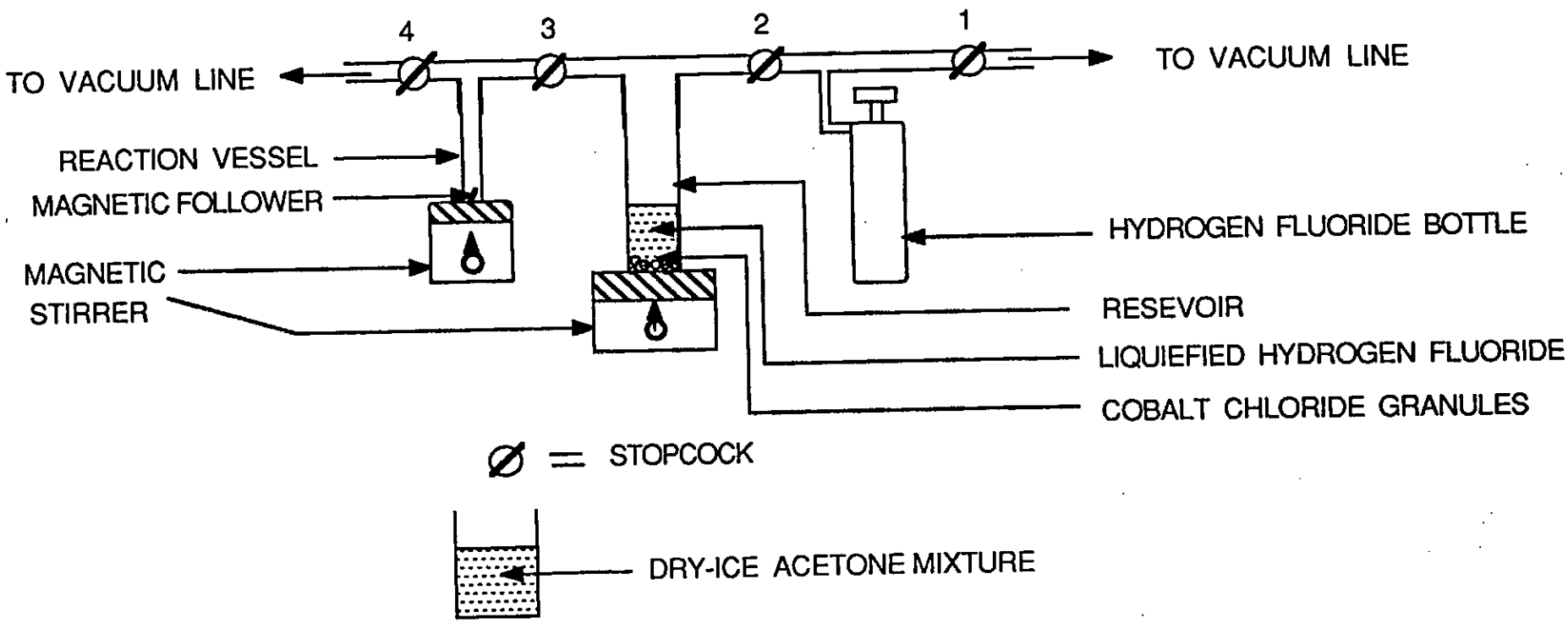


FIGURE 4.2. A SCHEMATIC DIAGRAM OF THE HYDROGEN FLUORIDE SOLVOLYSIS APPARATUS

The stopcock 1 connecting liquid HF bottle to the vacuum pump was then closed to the vacuum pump. The reservoir was immersed in liquid nitrogen, and on slowly opening stopcock 2 connecting the reservoir and the HF bottle, 50mL of the HF was distilled from the HF tank into the reservoir. After the HF has been collected, stopcock 2 was closed and the rest of the HF solvolysis apparatus was opened to the vacuum line for about 30 minutes.

The entire apparatus except for the reservoir was thereby evacuated. After all the other stopcocks in the apparatus except that opening to the reaction vessel were closed, HF (5mL) was distilled into the reaction vessel by cooling the reaction vessel in a dry ice-acetone bath and stirring its contents and slowly opening the the stopcock 3 connecting the reservoir and the reaction vessel. After the HF (5mL) had distilled over, the reservoir was completely shut off from the rest of the system. The timing of the reaction was started after removing the dry ice-acetone bath and the reaction was allowed to continue for three hours at room temperature.

After the completion of the reaction, the stopcock 4 leading to the vacuum pump was opened and the excess HF was evacuated completely over a period of 30 minutes. The reaction product was transferred with the aid of 2M TFA used for further hydrolysis during sugar analysis as described earlier. Precautions: Liquid HF is very corrosive to the skin, eyes and the mucous membranes causing severe burns hence extra care must be taken when handling the reagent.

Sugar analysis as described essentially for *C.coli* was carried out immediately on each of the partially (PS 2) and the depyruvylated polysaccharides (PS 3) after the hydrogen fluoride solvolysis. However, the conditions of the hydrolysis was for 2 hours at 100°C with 2M TFA.

^{1}H N.M.R. Spectra: Sample preparation

The depyruvylated (PS 2) and pyruvylated polysaccharide (PS 2) (5mg) each was treated twice with 99% deuterium oxide (D_2O) (0.4ml) and freeze dried after each treatment. It was then dissolved in 99.9% D_2O(0.4ml) and the ^{1}H n.m.r. spectrum was recorded at 85°C with a JEOL GX-270 and 400 instruments. Chemical shifts were recorded in ppm relative to internal sodium 3-trimethylsilylpropionate-d_4 (TSP).

^{13}C N.M.R.

To the PS 2 and PS 3 (20mg) each was added 0.4mL of 99% D_2O. The spectra were recorded on a GX-270 and 400 Jeol specrometer at 70°C with full proton decoupling. Chemical shifts are reported using dioxane (^{13}C δ 67.4 ppm.) as internal reference.

COSY Experiment

The COSY experiment was performed on the sample prepared as for ^{1}H n.m.r. according to the basic pulse sequences of $\pi/2 - \tau - \pi/2$ - Acquire where τ is an incremented delay.

Partial acid hydrolysis[107].

The depyruvylated polysaccharide (PS 2)(15mg) was hydrolysed with 0.5M trifluoroacetic acid (5mL) for 2 hours at 100°C. The excess acid was evaporated on the rotary eavaporator and the PS was freeze-dried. The residue was applied on a (90 x3cm) Bio-Gel P-4 column calibrated with Dextran 10, raffinose, stachyose, sucrose and glucose and eluted with mM aqueous formic acid. The fractionation was monitored by using a differential refractometer and the main product was eluted in the disaccharide region. This product was reduced with aqueous sodium borodeuteride solution (0.2ml of 10mg/ml $NaBH_4$) for 30 min at room

to pH 3. The borates generated were removed as the methyl derivatives by successive evaporations with 10% acetic acid in methanol (2x5ml) and methanol (2x5ml), after which the reduced material was methylated as described above for C.coli Labet 227. The methylated product was characterised on the G.L.C. and the combined G.C.- M.S. using a 12m long, methyl silicone column operated at a temperature of 200°C for 5minutes and increased at the rate of 8°C per minute to 300°C and held for 30minutes. The retention times of the products were obtained relative to permethylated lactitol acetate.

Methylation analysis

This was performed as previously described in the procedure for C.coli Labet 227 on the both the pyruvylated (PS 2)and the depyruvylated polysaccharides (PS 3).

^{1}H N.M.R.

The ^{1}H N.M.R. spectrum of the product of the partial acid hydrolysis was also obtained. The sample was prepared as described earlier.

Absolute Configuration Determination.

Absolute configurations of the sugars were determined according to the procedure of Gerwig et al[170].

The depyruvylated polysaccharide (PS 3)(1mg) in a 13mm screw cap tube was subjected to partial acid hydrolysis for 1hour with 2M TFA (500mg) at 100°C. The product was evaporated to dryness by flushing with air. The residue was allowed to dry overnight under vacuum over phosphorous pentoxide in a dessicator.

To the dry residue, (+)-2-butanol (250µL) and acetyl chloride (20µL) were added and heated at 80°C for 16hours. The product was allowed to cool

to room temperature and was reacetylated with acetic anhydride (200µL) for 2 hours at room temparature.

It was then evaporated to dryness and Sil-A (a commercial silylating agent made up of a mixture of trimethylchlorosilane, hexamethylenedisilazane and pyridine as catalyst) (100µL) was added and allowed to stand for 1 hour at room temperature. The excess Sil-A was removed by flushing with air to dryness and the residue was extracted with n-hexane. The hexane extract was injected on the G.L.C and the combined G.C.-M.S. to determine the absolute configuration of the sugars by comparison with the retention times of that of the standards.

Preparation of Standards for Absolute Configuration Determination

The sugar standards for this determination were N-acetylglucosamine and rhamnose. To each of the anhydrous sugar standard (1mg) (+)-2-butanol (250µL) and acetyl chloride (20µL) were added and heated at 80°C for 16hours. To another sample of each of the standard (1mg) in different tubes, racemic (+/-)-2-butanol (250µL) and acetyl chloride (20µL) were added and also heated at 80°C for 16hours. The derivatization of the standards continued as in the procedure of the *E.coli* polysaccharide above.

Determnination of Anomeric Configuration.

The anomeric configuration was determined solely from the chemical shift and coupling constant values obtained from the ^{1}H n.m.r. and the COSY spectra using JEOL standard pulse sequence.

4.2.4. Streptococcus Pneumoniae Type 37 (PN 37 or S-37)

Preparation of purified native polysaccharide.

The already lyophilized crude capsular polysaccharide was obtained

The already lyophilized crude capsular polysaccharide was obtained from the State Serum Institute DK-2300 Copenhagen S, Denmark. The crude capsular polysaccharides was found to be contaminated with protein and was purified by the hot water-phenol procedure. The crude capsular polysaccharide (200mg) in water (100mL) was extracted by the hot water-phenol[74] as follows. The crude capsular polysaccharide of S-37 (200mg) dry weight was suspended in distilled water (100mL) and heated to 66°C in a water bath. The phenol reagent (90%w/v) (110mL) was heated to the same temperature and added to the crude capsular polysaccharide solution to give a phenol-water ratio of 1:1 (w/w).

The mixture was agitated thoroughly by power stirring for 15 minutes and distributed into the Wifug centrifuge tubes. This was followed by centrifugation at 5,000 x g for 15 mins at room temperature which separated the content in each tube into three layers: a water-saturated phenol layer on top of which was an interphase precipitate and an uppermost layer consisting of phenol-saturated water.

The uppermost aqueous layer was carefully removed by using a Pasteur pipette, and the extraction procedure repeated twice more. The three aqueous phases were pooled, dispensed into centrifuge tubes, recentrifuged once more to remove any precipitate that was still present. The clear aqueous phases were pooled, dialysed against several changes of distilled water over a period of 48hours, concentrated to 50% of its volume by pervaporation at room temperature and freeze dried.

The freeze dried purified capsular polysaccharide was subsequently dissolved in distilled water (10ml) and applied to a column of DEAE-Trisacryl (1.6 x 20 cm) which was irrigated first with water (150mL) and then with a linear gradient of aqueous sodium chloride (0-0.5 M). The only fraction that was obtained was in the aqueous region and was dialysed and freeze-dried to give the pure S-37 polysaccharide.

Partial depolymerisation of S-37 polysaccharide

Pure S-37 (50mg) was dissolved in 80% formic acid (20mL) and the solution kept at 85°C for 20minutes. The excess formic acid was removed under reduced pressure on the rotary evaporator and distilled water (10mL x 2) was added and evaporated twice more to remove the formic acid completely. The residue obtained after the removal of the acid was dissolved in water (10mL)and the solution was dialysed against several changes of distilled water over a period of 48hours, and freeze-dried. The product was dissolved in water (2mL) and fractionated on a precalibrated column of (90 x3 cm) of Biogel P10 that was irrigated with mM aqueous formic acid as earlier described. The polysaccharide eluted in the void volume was freeze-dried to give the pure partially depolymerised polysaccharide S-37 (I).

Methylation analysis.

Methylation analysis was carried out on the pure S-37, the partially depolymerised sample S-37 (I) as described previously for C.coli Labet 227.

Sugar Analysis

The sugar analysis was carried out as for that of C.coli Labet 227 as described earlier.

N.m.r. spectroscopy.

For the n.m.r. spectroscopy, the solutions of both pure S-37 and the depolymerised S-37(1) were prepared as earlier described for the E.coli 0149. The n.m.r. spectra of solutions in deuterium oxide were recorded at 70° (^{13}C) and 85° (^{1}H) with a JEOL GX- 400 instument. Chemical shifts were reported in p.p.m. using internal dioxane (δ 67.4) and internal sodium

3-trimethylsilylpropanoate-d_4 (TSP) for ^{13}C and ^{1}H n.m.r. spectroscopy, respectively. COSY and the C-H correlation spectroscopy experiments were performed according to JEOL standard pulse sequences. For the correlation spectroscopy a 90° mixing pulse was used. Relaxation times (T_1) were determined using the inversion recovery method.

Smith degradation[128].

The partially depolymerised polysaccharide S-37(I) (60mg) was oxidised with 0.2M sodium metaperiodate (5mL) in sodium acetate buffer of pH 6 (15mL) and kept in the dark for 120hours at $4^{o}C$. The excess periodate was reduced with ethylene glycol (3mL) and the solution was dialysed against tap water and freeze-dried. The product obtained was dissolved in water and sodium borohydride (200mg) was added and the mixture was kept for 16 hours. The excess the sodium borohydride was decomposed with aqueous acetic acid and the solution was again dialysed and freeze-dried to give the polyalcohol. Part of the polyalcohol (10 mg) was dissolved in 0.5M trifluoroacetic acid and kept at room temperature for 16 hours. The product was freeze-dried and fractionated on a standardised column (90x3cm) of Bio-Gel P-10 column that was irrigated with mM formic acid. The main product was a polysaccharide (3mg) S-37 (II) which was eluted in the void volume.

Polysaccharide obtained from Smith degradation S-37 (II).

i) This polysaccharide S-37 (II) was subjected to both sugar and methylation analysis as described earlier.

ii) The ^{1}H n.m.r. spectrum of the S-37 (II) polysaccharide was also obtained and the sample preparation was as described for the samples of the other bacterial polysaccharides discussed earlier.

Absolute Configuration Determination.

Absolute configurations of the constituent sugars of the S-37 was determined as described under the procedure for absolute configuration determination for E.coli O149.

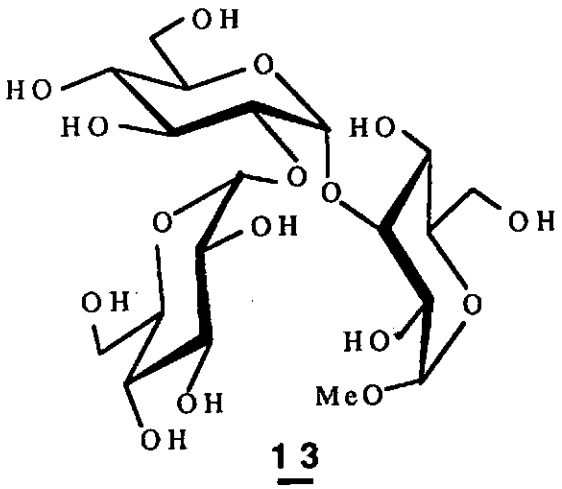
Anomeric Configuration Determnination

The anomeric configuration was determined solely from the chemical shift and coupling constant values obtained from the ^{1}H n.m.r., COSY, and the C-H correlation spectroscopy spectra using the JEOL standard pulse sequence.

4.3. SYNTHESIS OF TRISACCHARIDES

EXPERIMENTAL PROCEDURES: MATERIALS

The trisaccharides methyl *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranoside (**13**), methyl *O*-α-D-glucopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranoside (**21**),



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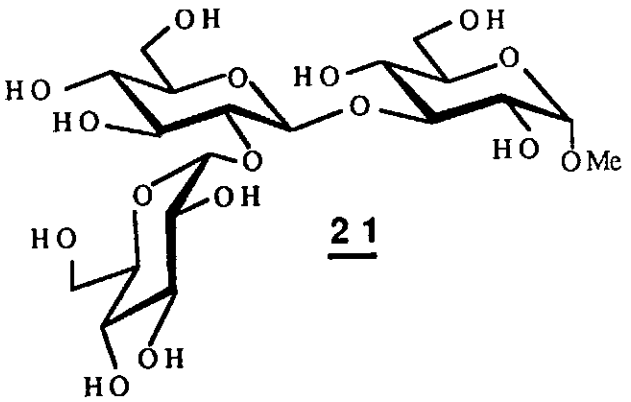

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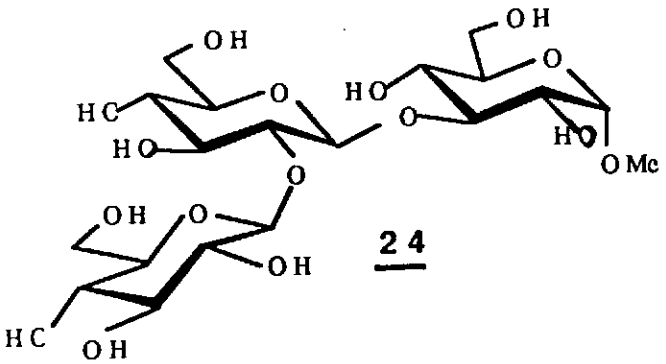
methyl *O*-β-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranoside (**24**) and methyl *O*-β-D-glucopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranoside (**30**) made only of glucose



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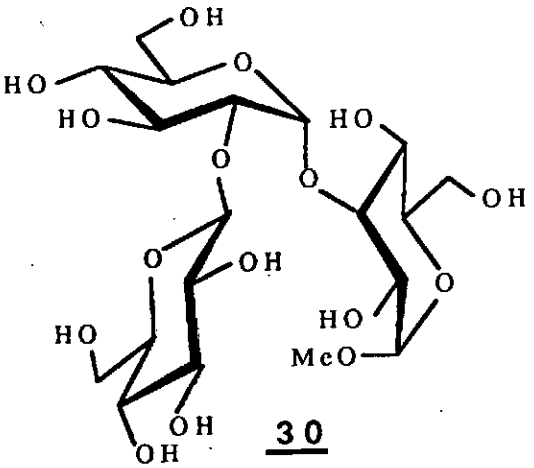

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as the monosaccharide with different anomeric configuration but the same linkage sequences were obtained under the conditions of silver trifluoromethane sulphonate (silver triflate) promotion[247]. The glycosidation products were deprotected by classical methods and were purified on a Biogel P-2 column before the n.m.r. spectra were run.

GENERAL METHODS

Concentrations were performed under diminished pressure at a bath temperature less than 40°C. Optical rotations were measured at 22°C in chloroform or water where applicable for 1.04-1.74% solutions with a Perkin-Elmer 241 polarimeter.

Thin layer chromatography was performed on Silica Gel F_{254}(Merck) with detection by u.v.light when applicable or by charring with sulphuric acid. Column chromatography was performed on Silica Gel 60(0.035-0.07mm, Amicon.) with loading in the range 1/25-1/100 and elution with toluene-ethyl acetate mixtures unless otherwise stated. Organic solutions were dried over $MgSO_4$. Molecular sieves 3Å or 4Å were desiccated in a vaccum at 300° overnight and ground immediately before use.

Products were generally purified by column chromatography and characterised by n.m.r.spectrocopy. The n.m.r spectra were recorded for solutions in $CDCl_3$ or D_2O using JEOL JMN FX 100 , GX 270 or 400. Chemical shifts were recorded in p.p.m. from that of internal tetramethylsilane for solutions in $CDCl_3$ at 25°C. For solutions in deuterium oxide the chemical shifts were recorded at 70° with dioxane δ_C 67.4 p.p.m. for ^{13}C n.m.r. and sodium 3-(trimethylsilyl)-$^2H_4$-propionate (TSP) δ_H 0.00p.p.m.for ^1H n.mr. as internal references. N.m.r. spectra recorded for all new compounds were in agreement with the postulated structures and only selected data are reported.

For the assignment of signals proton-proton and carbon-proton shift correlation spectroscopy (COSY) were used. Chemical shifts of overlapping signals were obtained from the center of the cross-peaks in the proton-proton shift correlation spectra. The HSEA program[171,181] was used to estimate minimum energy conformations and rotational freedom. This program accounts for non-bonded interactions as expressed by the Kitaygorodsky algorithim, together with a term for the exoanomeric effect.

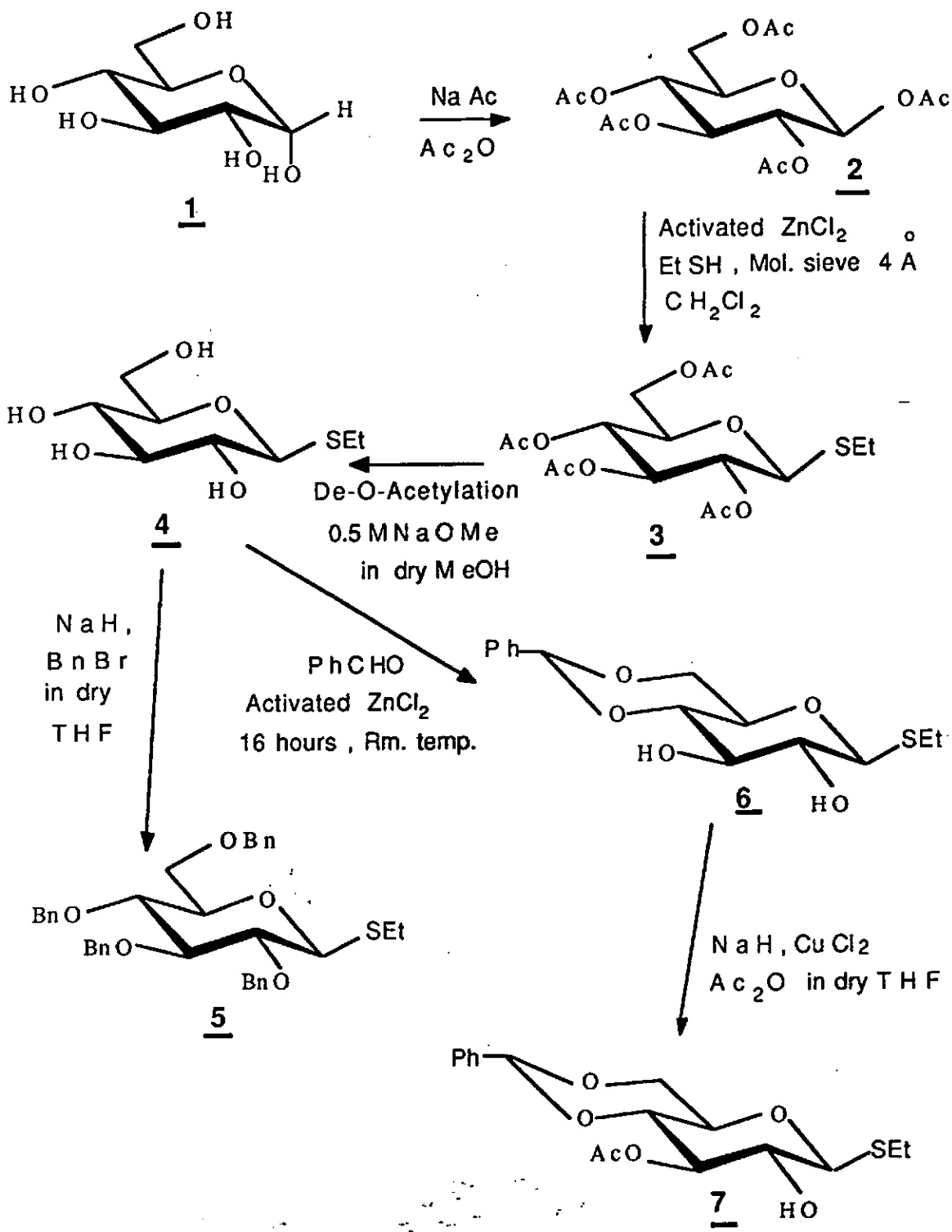
Synthesis of Ethyl 3-O-Acetyl-4,6-O-Benzylidene-β-D-thioglucopyranoside (7)

The sequence of the reactions leading to ethyl 3-O-Acetyl-4,6-O-Benzylidene-β-D-thioglucopyranoside(7) through the intermediate monosaccharide derivatives β-D-glucopyranose pentaacetate (2) to ethyl 4,6-O-benzylidene-β-D-thioglucopyranoside (6) is shown in scheme 4.1. on the following page. The reaction procedures of these derivatives are described as follows.

β-D-glucopyranose pentacetate (2)

β-D-glucopyranose pentacetate was synthezised by the procedure of Wolfrom and Thompson[248a] with D-glucose (1) as the starting material. (2) was synthezised as follows:-

A suspension of anhydrous sodium acetate (10g) of anhydrous sodium acetate in acetic anhydride (140ml) in 500mL round botttoned flask was heated over a flame to the boiling point in an efficient fume hood. About 0.5g of anhydrous D-glucose from 20g supply was added, and the flask without shaking was heated carefully at the point nearest to the sugar laying on the bottom. Initiation of the reaction was indicated by continued boiling after the removal of the flame, the flask was placed on a cork ring and the flame extinguished.



Scheme 4.1.

The remainder of the sugar was added in small portions at a rate which maintained the boiling temperature of the mixture. The flask was gently

maintained the boiling temperature of the mixture. The flask was gently shaken occassionally to prevent the accumulation of the solid sugar at the bottom of the flask. When the reaction stopped, it was started again by heating before more sugar was added to the flask.

After the addition of all the sugar, and when the reaction has subsided, the solution was brought to a full boil. It was then cooled and poured with stirring onto 500mL of cracked ice. After standing for 3hours with occasional stirring, the crystalline material was filtered with suction and washed with cold water. The crude product was purified by crystallization from 200mL of hot 95% ethanol followed by filtration with decolourising charcoal as soon as the temperature cooled to room temperature. The recrystallization was repeated once more to give the pure 19g of β-D-glucose pentaacetate (2) in 59% yield.

^{13}C n.m.r.data: (2) δ 20.52 to 20.76 (Five Acetyl CH_3), 91.67 (C-1).

Ethyl 2,3,4,6-tetra-O-acetate β-D-thioglucopyranoside (3) and Ethyl-β-D-1-thioglucopyranoside (4)

Ethyl-β-D-1-thioglucopyranoside (4) was synthezised according to the method of Pascu and Wilson[249] via ethyl 2,3,4,6-tetra-O-acetate β-D-thioglucopyranoside (3) using β-D-glucopyranose pentacetate as the starting material. The procedures of the syntheses of (3) and (4) are descibed below.

Ethyl 2,3,4,6-tetra-O-acetate β-D-thioglucopyranoside (3)

β-D-glucose pentaacetate (10g, 25mmole) was dissolved in 50ml of freshly distilled dichloromethane. 4.5ml(50mmoles) ethanethiol (ethyl mercarptan) and crushed molecular sieves 4Å (8g) was added. The mixture was cooled to 0°C on an ice bath and crushed activated zinc chloride 12.5g (75mmoles) was added slowly. The reaction mixture was stirred for 16 hours whilst

shaken occassionally to prevent the accumulation of the solid sugar at the bottom of the flask. When the reaction stopped, it was started again by heating before more sugar was added to the flask.

After the addition of all the sugar, and when the reaction has subsided, the solution was brought to a full boil. It was then cooled and poured with stirring onto 500mL of cracked ice. After standing for 3hours with occasional stirring, the crystalline material was filtered with suction and washed with cold water. The crude product was purified by crystallization from 200mL of hot 95% ethanol followed by filtration with decolourising charcoal as soon as the temperature cooled to room temperature. The recrystallization was repeated once more to give the pure 19g of β-D-glucose pentaacetate (2) in 59% yield.

^{13}C n.m.r.data: (2) δ 20.52 to 20.76 (Five Acetyl CH_3), 91.67 (C-1).

Ethyl 2,3,4,6-tetra-O-acetate β-D-thioglucopyranoside (3) and Ethyl-β-D-1-thioglucopyranoside (4)

Ethyl-β-D-1-thioglucopyranoside (4) was synthezised according to the method of Pascu and Wilson[249] via ethyl 2,3,4,6-tetra-O-acetate β-D-thioglucopyranoside (3) using β-D-glucopyranose pentacetate as the starting material. The procedures of the syntheses of (3) and (4) are descibed below.

Ethyl 2,3,4,6-tetra-O-acetate β-D-thioglucopyranoside (3)

β-D-glucose pentaacetate (10g, 25mmole) was dissolved in 50ml of freshly distilled dichloromethane. 4.5ml(50mmoles) ethanethiol (ethyl mercarptan) and crushed molecular sieves 4Å (8g) was added. The mixture was cooled to 0°C on an ice bath and crushed activated zinc chloride 12.5g (75mmoles) was added slowly. The reaction mixture was stirred for 16 hours whilst

monitoring the reaction with t.l.c. When the reaction was complete, mixture was filtered through celite and rinsed with 2x25ml dichloromethane. The solution was now washed succesively with 50ml each of 1M sulphuric acid, saturated sodium hydrogen carbonate solution, and water. The product obtained, ethyl 2,3,4,6-tetra-O-acetate β-D-thioglucopyranoside (3) was dried with sodium sulphate, filtered and evaporated. It was codistilled three times with toluene to distill off any excess ethanethiol.

Ethyl-β-D-1-thioglucopyranoside. (4)

Compound (3) was de-O-acetylated[248b] by dissolving in 20 ml of methanol and adding a catalytic amount of 0.5M methanolic sodium methoxide (1mL) and stirring for two hours at room temperature. The reaction mixture was neutralised with Dowex 50(H^+) resin (about 3 spatula full) and concentrated. The remaining traces of methanol was removed by leaving the sample on the vacuum pump for about two hours to give the dry sample of (4).The yield was 7.3g (89%) of (4).

^{13}C n.m.r.data: (4) δ 15.2 (CH_3CH_2S), 24.8 (CH_3CH_2) 86.8 (C-1).

Ethyl-2,3,4,6-tetrabenzyl-β-D-1-thioglucopyranoside (5)

Compound (5) was synthezised essentially according to the procedure of Lichti et al[250]. Sodium hydride (4.8g) (50% suspension in oil) was weighed into a 500ml round bottomed flask and washed with 2x150ml of petroleum ether (b.pt.40- 60°). After decanting the second portion of the petroleum, the sodium hydride was carefully dried with nitrogen. (2.4g) of (4) was dissolved in dry dimethylformamide (125 mL) and mixed with benzyl bromide (9.3ml). Both were transferred into a dropping funnel attached to the round-bottomed flask containing the sodium hydride which was cooled to 0°C in an

ice bath. The mixture was added to the sodium hydride dropwise with stirring. The reaction was monitored with t.l.c. using petroleum ether-ethylacetate mixture 5:1 until the reaction was complete. When the reaction was complete, methanol (25mL) was carefully added to quench the excess sodium hydride and the contents of the flask was transferred to a 1-litre separating funnel. Water (200mL) and toluene (400mL) were added and shaken. The water phase was washed once more with toluene (200mL) and the two toluene phases were pooled and washed once again with water (200mL). The toluene phase was dried with sodium sulphate, concentrated and the crude product was purified on a short column using petroleum ether:ethylacetate mixture 5:1. The yield of (5) that was obtained was 4.3g (71%).

Ethyl-4,6-O-Benzylidene-β-D-1-thioglucopyranoside (6)

Activated zinc chloride (10gm) was finely ground and added to a solution of ethyl-β-D-1-thioglucopyranoside (4) (2.23gm) in benzaldehyde (10 mL) and was allowed to stir overnight at room temperature, after which the reaction mixture was poured with stirring to 50ml petroleum ether (60-70°), saturated aqueous sodium hydrogen carbonate (50ml) and ice. It was stirred for 30 minutes and filtered off. The product was washed well with petroleum ether and water and recrytallised from water containing enough sodium hydrogen carbonate to make it alkaline. The crystals of (6) were dried over phosphorous pentoxide under vacuum. A yield of compound 6 (2.03g, 66%) was obtained.

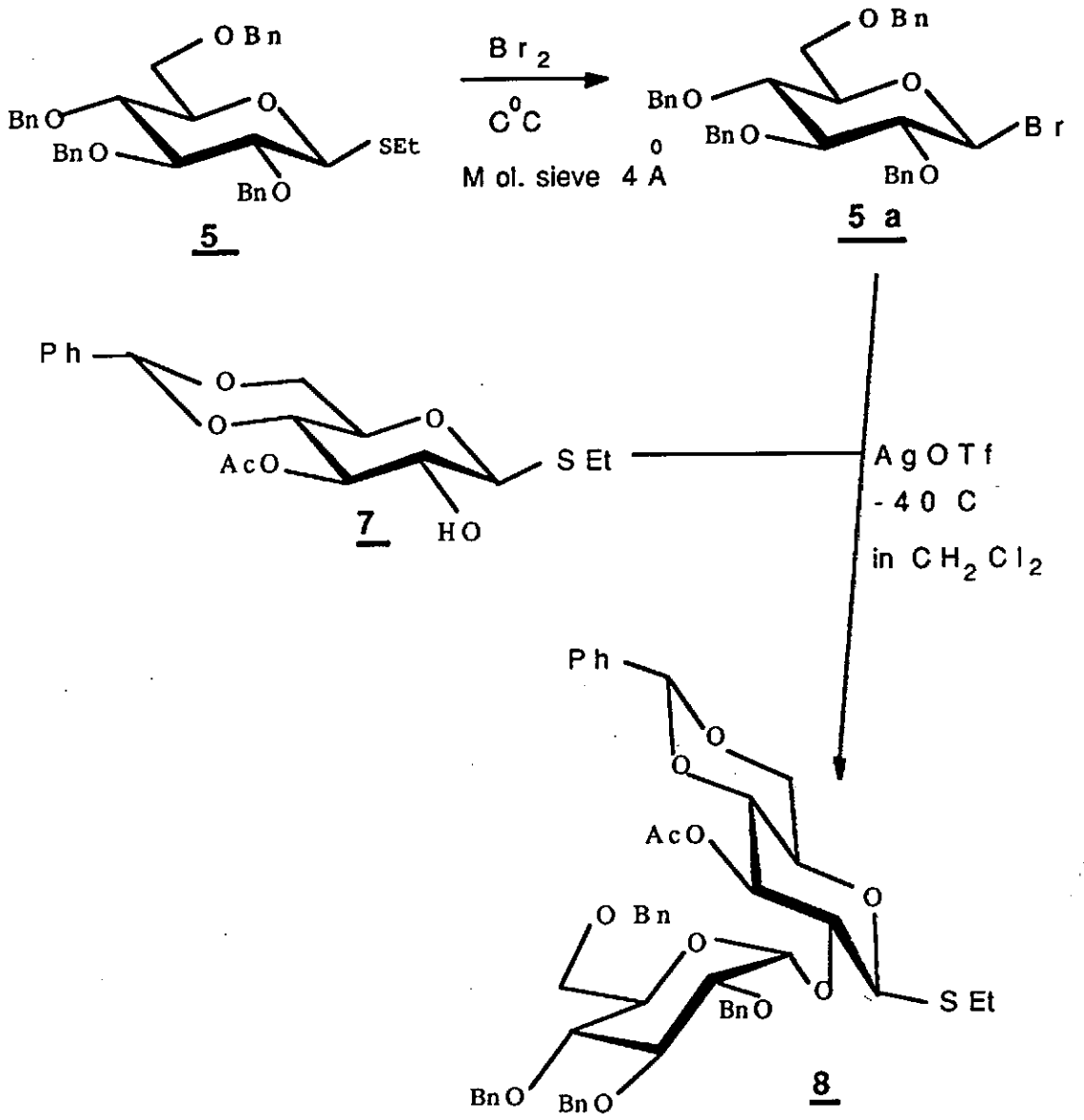
^{13}C n.m.r.data of (6): δ15.2 (CH_3CH_2S), 24.5 (CH_3CH_2), 86.3 (C-1), 101.8 (PhCH).

selective acetylation in position 3 of compound (4). Dry ethyl-4,6-O-benzylidene-β-D-1-thioglucopyranoside (6)(3.1g,10mmoles) was stirred with sodium hydride (9.6g,20 mmoles) (50% in mineral oil) in dry tetrahydrofuran (150mls). After about 6 hours when the evolution of the hydrogen ceased, copper (II) chloride (1.35g,10mmoles) was added. After about 10 minutes, a green solution of the copper chelate resulted. The solution was cooled to 0°C and acetic anhydride (1.5ml,16mmoles) was added. The temperature was kept at 0°C for 30 minutes and the reaction was finished after another 1.5 hours at room temperature, during which it was monitored on the t.l.c. The reaction mixture was concentrated to a solid, dissolved in dichloromethane and washed successively in water, aqueous sodium carbonate and saturated sodium chloride, dried with sodium sulphate and concentrated to a solid. The crude products were separated on a short column using a toluene-ethyl acetate mixture of ratio 5:1as the eluent giving (7) in 80% yield (3.12g, 0.80mmoles).

^{13}C n.m.r. data : δ 15.1 (C H_3CH_2S), 20.9 (Acetyl Me), 24.6 (CH_3C H_2), 78.3 (C-3) , 86.8 (C-1) , 101.2 (PhC), 170.8 (C=O Acetyl).

Synthesis of the Disaccharide Ethyl 3-O-Acetyl-4-6-O-Benzylidene-2-O-(2,3,4,6-tetra-O-benzyl-α-D-glucopyranosyl)-1-thio-β-D-glucopyranoside (8).

The reaction steps between bromo-2,3,4,6-tetrabenzyl-β-D-glucopyranoside(5a) and ethyl-4,6-O-Benzylidene-3-O-Acetyl-β-D-1-thioglucopyranoside(7) leading to the formation of (8) is shown in Scheme 4.2.



Scheme 4.2.

Ethyl 3-O-Acetyl-4-6-O-Benzylidene-2-O-(2,3,4,6-tetra-O-benzyl-α-D-glucopyranosyl)-1-thio-β-D-glucopyranoside (8).

A solution of bromine(72ul,1.4 mmol) in dry dichloromethane (5ml) was added to a cooled mixture of (5) (850mg, 1.4 mmol) in dichloromethane (10ml) and molecular sieves 4Å. with stirring. After 1 hour, the mixture was filtered, concentrated and co-concentrated three times with toluene. The residue (5a) was dissolved in dichloromethane (5ml) and added to a stirred mixture of

(7) (354mg,1.0mmol) and molecular sieves in dichlomethane (5ml) to which 4Å molecular sieves has been added. The mixture was cooled to -40°C and stirred whilst a solution of silver triflate 590mg(2.3mmol) and 2,4,6-trimethyl-pyridine (0.3ml,2mmoles) in dichloromethane (5ml) was added. The mixture was allowed to warm up to room temperature and left for 16 hours, diluted with dichloromethane, and filtered through celite. The filtrate was successively washed with 10% aqueous $Na_2S_2O_3$, water, aqueous 1M H_2SO_4, and saturated $NaHCO_3$, dried and concentrated to dryness to obtain a syrup. The syrupy crude disaccharide (8) was purified by column chromatographyto obtain a yield of 543mg (62 %) of (8).

$[\alpha]_{578}$: 17°

^{13}C n.m.r. data : δ 15.6 (C H_3CH_2S), 21.0 (Acetyl Me), 24.7 (CH_3C H_2S), 85.2 (C-1), 95.9 (C'-1), 101.2 (Ph C) ,169.5(C=O Acetyl).

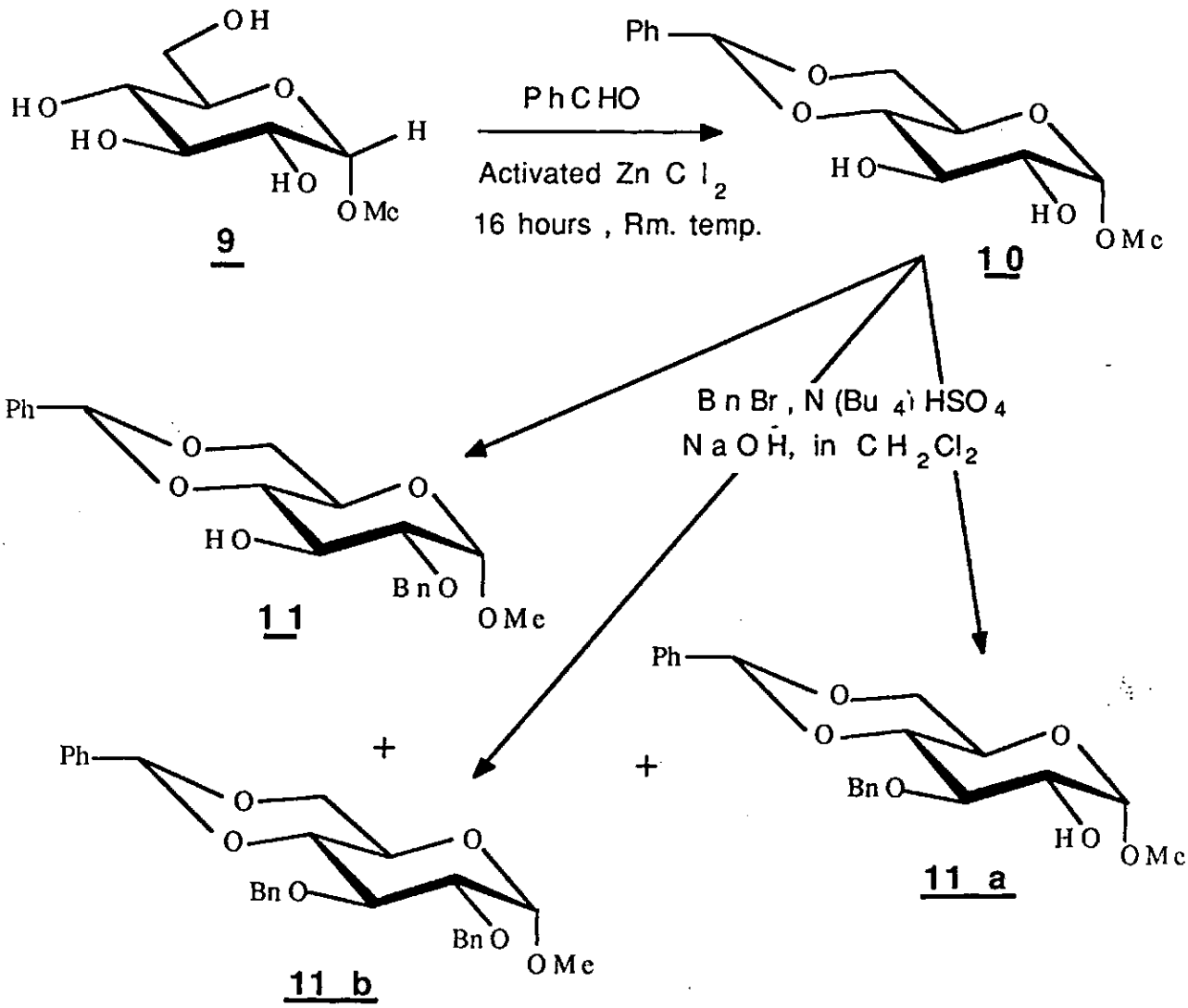
Synthesis of methyl O-2-benzyl-4-6-O-benzylidene-α-D-glucopyranoside (11)

The sequence of reactions leading to the formation of (11) from (9) as the starting material through methyl 4,6-O-benzylidene-α-D-glucopyranoside (10) is shown in Scheme 4.3.

Methyl 4,6-O-benzylidene-α-D-glucopyranoside (10)

Activated zinc chloride (20gm) was finely ground and added to a solution of methyl-α-D-glucopyranoside (9) (3.88gm) in benzaldehyde (10 mL) and was allowed to stir overnight at room temperature, after which the reaction mixture was poured with stirring to 50ml petroleum ether (60-70°), saturated aqueous sodium hydrogen carbonate (50ml) and ice. It was stirred for 30 minutes and filtered off. The product was washed well with petroleum ether and water and recrytallised from water containing enough sodium hydrogen carbonate

to make it alkaline to obtain pure (10). A yield of 3.58g (61%) of compound (10) was obtained.



Scheme 4.3

Methyl O-2-benzyl-4,6-O-benzylidene-α-D-glucopyranoside (11).

The preparation of (11) is essentially as described by Garegg et al[252]. Methyl 4,6-O-benzylidene-α-D-glucopyranoside (10) (3.00g,10.5mmol) tetrabutyl- ammonium hydrogen sulphate (0.72g, 2.1mmol) were dissolved in dichloromethane (180ml). Aqueous sodium hydroxide (5ml of a 5% solution) was

added and the mixture boiled under reflux for 48 hours. The reaction was monitored by thin layer chromatography. The mixture was shaken with water, dried with sodium sulphate, and concentrated. Column chromatography of the residue on silica gel yielded 53% of **(11)** (6.4g,15.9mmol), methyl O-3-benzyl-4,6-O-benzylidene-α-D-glucopyranoside **(11a)** and methyl O-2,3-dibenzyl-4,6-O-benzylidene-α-D-glucopyranoside **(11b)** were also formed and recovered in 21% yield (2.53g,6.3mmol) and 5% yield (7.4g, 1.5mmol) respectively.

Synthesis of Methyl *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranoside (13)

The reaction of ethyl 3-O-Acetyl-4-6-*O*-Benzylidene-2-*O*-(2,3,4,6-tetra-O-benzyl-α-D-glucopyranosyl)-1-thio-β-D-glucopyranoside **(8)** and methyl O-2-benzyl-4,6-O-benzylidene-α-D-glucopyranoside **(11)** leading to the formation of the blocked trisaccharide methyl *O*-(2,3,4,6-tetra-benzyl-α-D-glucopyranosyl)-(1→2)-*O*-(3-O- Acetyl-4,6-O-benzylidene-α-D-glucopyranosyl)-(1→3)-*O*-(2-O-benzyl-4,6-O-benzylidene)-α-D-glucopyranoside **(12)** and subsequently methyl *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→3)-*O*-α- D-glucopyranoside **(13)** after deprotection of the former is as shown in Scheme 4.4. The various reactions procedures are described below.

Methyl *O*-(2,3,4,6-tetra-benzyl-α-D-glucopyranosyl)-(1→2)-*O*-(3-O- Acetyl-4,6-O-Benzylidene-α-D-glucopyranosyl)-(1→3)-*O*-(2-O-benzyl-4,6-O-Benzylidene)-α-D-glucopyranoside **(12)**.

A solution of **(8)** (1.23g ,1.4mmol) in dichloromethane (5ml) was treated with bromine 0.30ml(1.4mmol) in dichloromethane (5ml) as described in the preparation of **(5a)** to yield **(8a)** . The residue **(8a)** was mixed with **(7)**

(0.387g 1mmol) and molecular sieves 4Å in dichloromethane (10ml). The mixture was cooled to -25°. Silver triflate 770mg (3mmol) and 2,4,6-trimethylpyridine 0.3ml(2mmol) were dissolved in 5ml dichloromethane and was added to the cooled mixture of (7) and (8a) whilst stirring. After 30 minutes of stirring , the reaction was complete and the mixture processed as described in the preparation of (8). The yield after column chromatography was 406mg (33%) of (12) .

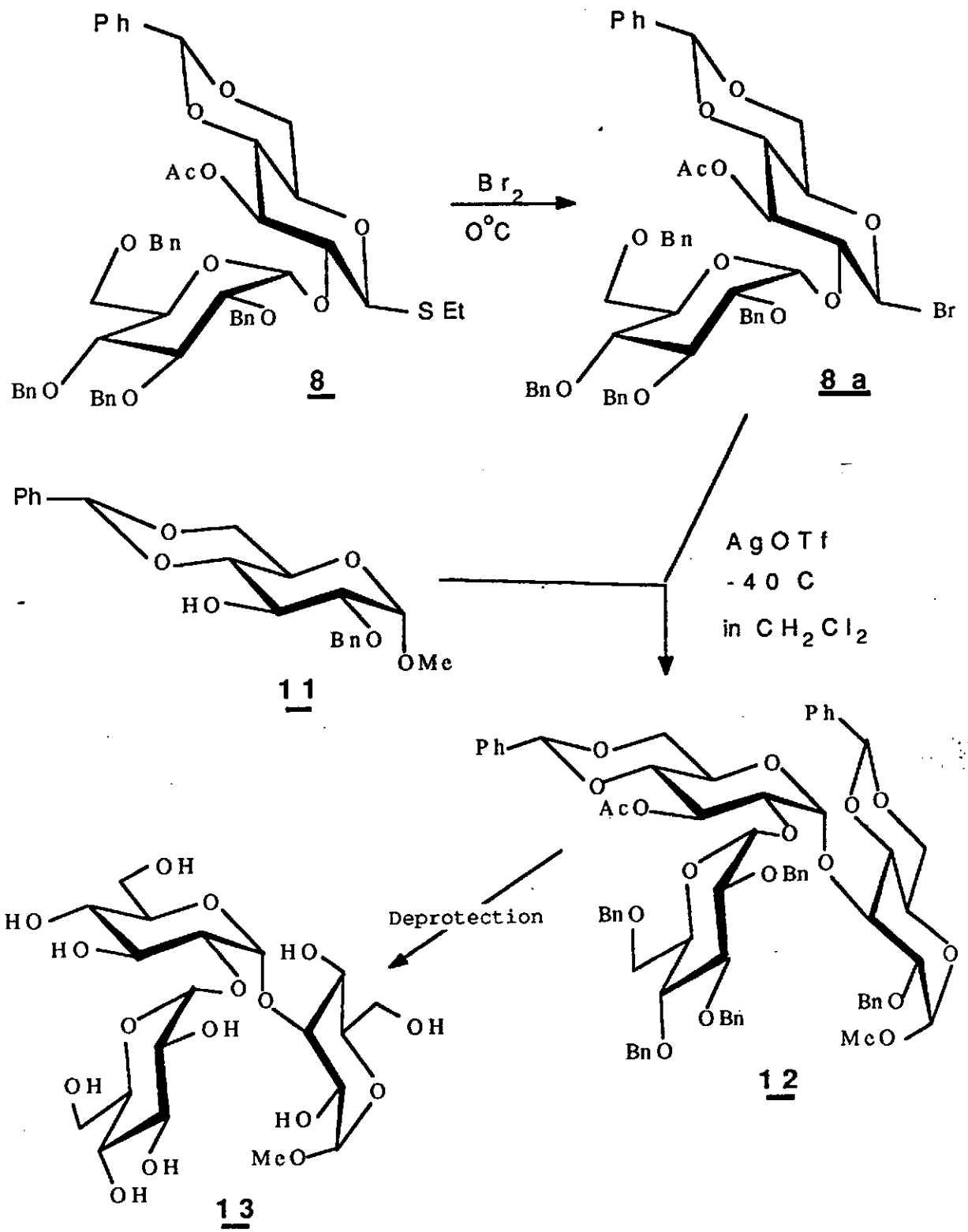
$[\alpha]_{578}$: 73°

^{13}C N.m.r. data : δ 21.1 (Acetyl Me) , 55.2 (Me O), 61.6 (C-6) , 94.5 (C-1'), 95.6 (C-1"), 101.2, 101.6 (Ph C), 169.8 (C=O Acetyl).

Methyl O-α-D-glucopyranosyl-(1→2)-O-α-D-glucopyranosyl-(1→3)-O -α- D-glucopyranoside (13)

350mg of (12) was de-O-acetylated as described for (3). The de-O-acetylated (12) was fully deprotected by catalytic hydrogenation with 10% Palladium on Carbon in 80% aqueous acetic acid at 400kP for 16 hours. The product was filtered and concentrated to evaporate off the acetic acid. The concentrated residue was dissolved in water and purified by chromatography on a short silica gel column using using a mixture of EtOAc:HOAc:MeOH:H_2O in ratio 12:3:3:2 as eluent followed by gel filtration on a Biogel P-2 column with water as the eluent. The resulting pure product was lyophilised to obtain 138mg (90%) of (13). See Scheme 4.4 on the following page.

$[\alpha]_{578}$: 191°


Ph
AcO
O Bn
BnO
BnO
BnO
S Et
8
Br2
0°C
Br
8 a
AgOTf
-40 C
in CH2Cl2
HO
OMe
11
OBn
McO
12
Deprotection
OH
13

Scheme 4.4.

Synthesis of Methyl O-(2-hydroxy-3,4,6-tri-O-benzyl-β-D-glucopyranose)-(1→3)-O-(2-O-benzyl-4,6-O-benzylidene)-α-D-glucopyranoside (19).

For the synthesis of (19), the common disaccharide from which methyl α-D-glucopyranosyl-(1→2)-O-β-D-glucopyranosyl-(1→3)-O-α-D-glucopyranoside (21) and Methyl β-D-glucopyranosyl-(1→2)-O-β-D-glucopyranosyl-(1→3)-O-α-D-glucopyranoside (24) were formed, the raction steps are shown in Scheme 4.5. on page 161 . For this reaction the starting compound was 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (14) was converted through intermediate compounds 3,4,6-Tri-O-acetyl-1,2-O-(1-ethoxylidene)-α-glucopyranose (15) and 3,4,6-Tribenzyl-1,2-O-(1-ethoxyethylidene)-α-D-glucopyranose (16) to produce 2-O-Acetyl-3,4,6-tri-O-benzyl-D-glucopyranosyl bromide (17) in Scheme 4.5. (17) as the glycosyl donor was allowed to react with methyl O-2-benzyl-4,6-O-benzylidene-α-D-glucopyranoside(11) as the aglycone to give fully blocked disaccharide methyl O-(2-Acetyl-3,4,6-tri-O-benzyl-β-D-glucopyranose)-(1→3)-O-(2-O-benzyl-4,6-O-benzylidene)-α-D-glucopyranoside(18). (18) was de-O-acetylated to produce the disaccharide (19). The procedures for these reactions are described below.

2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl bromide (14)[248c].

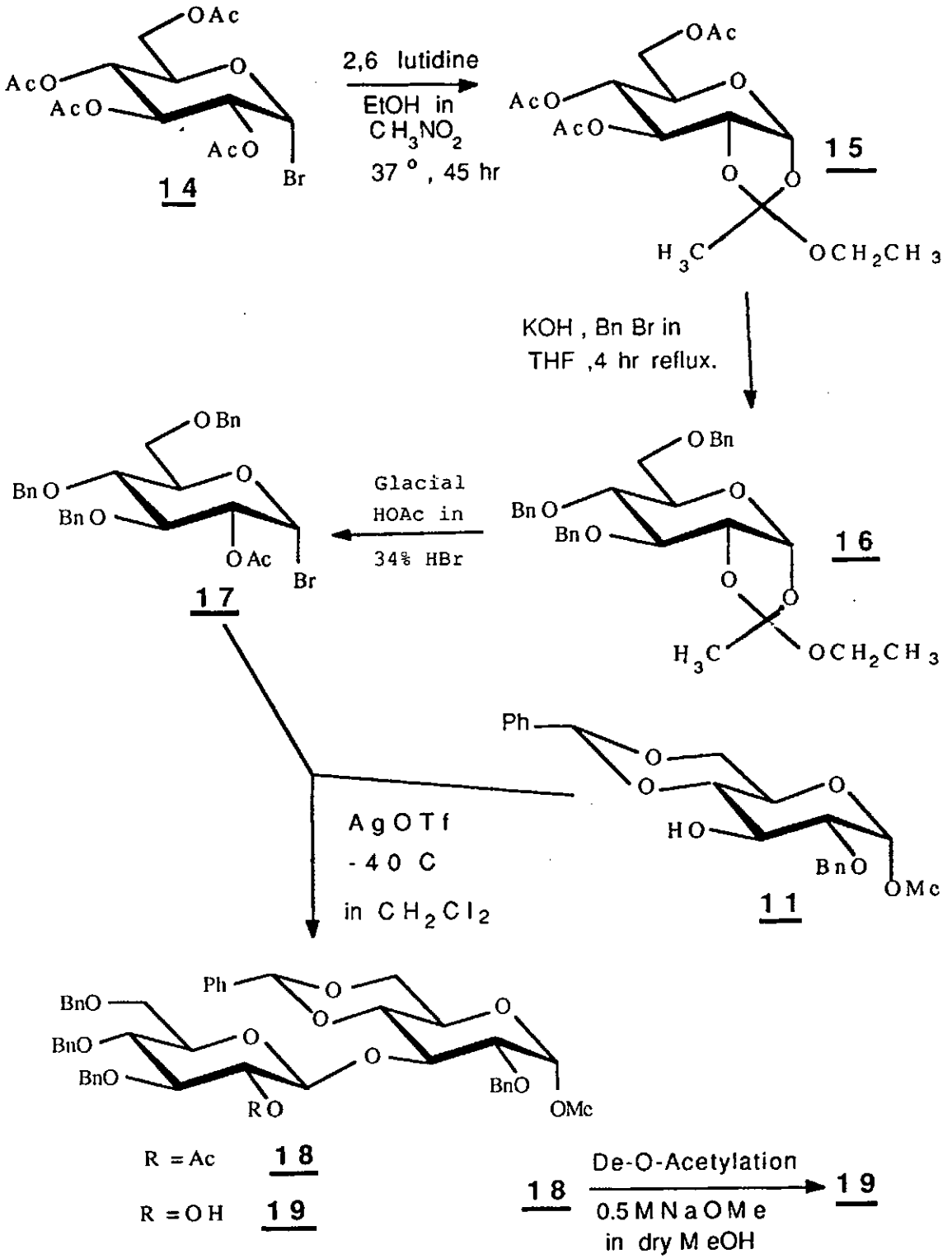
40mL of acetic anhydride in a three-necked flask equipped with an efficient stirrer and a thermometer and cooled with an ice and water mixture, 0.24ml of 70% perchloric acid was added dropwise. The solution was allowed to warm to room temperature and anhydrous D-glucose (10g) of (1) was added at such a rate, over a period of about 10 minutes to keep the reaction temperature between 30-40°C.

6.5mL of phosphorus bromide was also carefully added whilst keeping

the temperature below 20°C. 3.6mL of water was added dropwise to the continuously stirred and cooled mixture over about 15 minutes period to prevent the temperature from rising above 20°C. The reaction was then kept for 2 hours at room temperature. 30ml of chloroform was added and the mixture was filtered through a bed of fine glass wool. The reaction flask and the filter funnel were washed with 5ml of chloroform and the washing added to the filtrate. The filtrate was poured into 80ml of water (near 0°) contained in a 250ml separatory funnel.

After washing, the chloroform layer was drawn off into a 100ml separatory funnel which contains 30ml of 0° water. The operation is repeated by adding chloroform (5mL) to the original aqueous mixture and combining the chloroform extracts. After vigorous shaking, the chloroform layer was poured into stirred aqueous solution of sodium hydrogen carbonate (50ml) in a beaker. The mixture was transferred to another 250mL separatory funnel with the aid of a little chloroform and shaken vigorously. The chloroform layer was dried by stirring with sodium sulphate for 10 minutes to dry it. The mixture was filtered and the faintly yellow solution was evaporated under reduced pressure below 60° in a rotary evaporator to a hard crystalline mass.

The solid was transferred to a mortar with the aid of 2:1 (v:v) mixture of petroleum ether and diethylether (50mL) and ground in the solvent. The crude tetra-O-acetyl-α-D-glucopyranosyl bromide (**14**) was dried under reduced pressure over sodium hydroxide. The yield of pure (**14**) obtained by crystallization of the crude product from diethylether was 18.93g (83%).


OAc
AcO
AcO
AcO
Br
14
2,6 lutidine
EtOH in
CH3NO2
37 °, 45 hr
OAc
AcO
AcO
15
H3C
OCH2CH3
KOH, Bn Br in
THF, 4 hr reflux.
OBn
BnO
BnO
16
H3C
OCH2CH3
Glacial
HOAc in
34% HBr
OBn
BnO
BnO
OAc
Br
17
Ph
HO
BnO
OMe
11
AgOTf
-40 C
in CH2Cl2
Ph
BnO
BnO
BnO
RO
BnO
OMe
R = Ac 18
R = OH 19
18
De-O-Acetylation
0.5 M NaOMe
in dry MeOH
19

Scheme 4.5.

3,4,6-Tri-O-acetyl-1,2-O-(1-ethoxylidene)-α-glucopyranose (15)[253].

8.22g (20mmoles) of compound (14) was dissolved in a mixture of nitromethane (20mL), 2,6-lutidine (4.65ml,40mmoles) and absolute ethanol (5.8ml,100mmoles). The solution was allowed to stir continuously at 37° for hours after which 2M silver nitrate (15ml,30mmoles), water (25mL) and acetone (50mL) were added. The solution was filtered, and the filtrate was diluted with chloroform (100mL) and hexane (250mL) . The organic layer separated and washed twice with water. Chromatographically pure (15) was obtained on evaporation and was crystallized from ethanol and ether-hexane mixture yielding 4.37g (57%).

^{13}C n.m.r. data. δ 15.3 ($C H_3$), 22.0 ($C H_3CH_2O$), 58.5 ($CH_3C H_2O$), 97.8 (C-1), 121.0 (Orthoester C).

3,4,6-Tribenzyl-1,2-O-(1-Ethoxyethylidene)-α-D-glucopyranose (16)[253,254].

A solution of (15) 2.5g and benzyl bromide (2.6mL) in dry tetrahydrofuran (10mL) was treated with powdered potassium hydroxide (5g) and boiled under reflux for 4 hours with stirring. After being cooled, the mixture was diluted with dichloromethane (60mL), and was successively washed with water (5 x 20mL), saturated sodium hydrogen carbonate (2 x 10ml) and water (2 x 10mL), dried with potassium carbonate and evaporated to give a yellow oil that was chromatographed on a column of silica gel with 4:1 toluene-diethylether containing 0.1% triethylamine affording 5.26g (77%) of (16) as a pale yellow oil.

2-O-acetyl-3,4,6-tri-O-benzyl-D-glucopyranose was also obtained as the slow moving fraction was and crystalllized from ether to give a yield of 3% (40.7mg).

2-O-Acetyl-3,4,6-tri-O-benzyl-D-glucopyranosyl bromide (17)[254].

A solution of (16) (2g) in dichloromethane (50mL) was cooled to 0°, treated with a 34% solution of hydrogen bromide in glacial acetic (5mL) and stirred for 30min. The mixture was diluted with dichloromethane (250mL), successively washed with ice-water (2 x 100ml), saturated sodium hydrogen carbonate solution (2 x 100ml), dried with magnesium sulphate, filtered and evaporated. 1.86g of compound (17) was obtained as a syrup in 85% yield and was used without further purification because it is unstable. Scheme 4.5 shows the various reaction steps.

Methyl *O* -(2-Acetyl-3,4,6-tri-O-benzyl-β-D-glucopyranose)-(1→3)-*O*-(2-O-benzyl-4,6-O-benzylidene)-α-D-glucopyranoside (18).

(17) 1.00g (2mmol) , (11) 470mg (1mmol) were dissolved in dichloromethane (10ml) and ground molecular sieves 4Å were added. 590mg (2.3mmol) silver triflate and 0.16ml (1.2mmol) 2,4,6-trimethylpyridine were dissolved in dichloromethane (5ml) and was added to the cooled solution at -25°C of (14) and (11) whilst stirring continuously. After 15 minutes, the reaction was complete and the reaction mixture was worked up as done for (8). The crude product was chromatographed to give a yield of 583mg (73%) of (18) as in Scheme 4.5.

$[\alpha]_{578}$: 4°

^{13}C N.m.r. data: δ 21.0 (Acetyl Me), 55.3 (MeO), 62.0 (C-6), 98.9 (C-1), 100.8 (C-1'), 101.5 (PhC).

Methyl *O*-(2-hydroxy-3,4,6-tri-O-benzyl-β-D-glucopyranose)-(1→3)-*O*-(2-O-benzyl-4,6-O-benzylidene)-α-D-glucopyranoside (19)[254].

860mg (1mmol) of (18) was dissolved in a mixture dichloromethane and methanol (ratio 1:1)(16mL). 1M Sodium methoxide (1mL) was added and the

solution was stirred overnight. Dichloromethane (100ml) was added, and this mixture was washed with water, dried, and concentrated to dryness giving a yield of 707mg (86%) of (19) of Scheme 4.5.

^{13}C N.m.r. data of (19): δ 55.2 (MeO), 62.4 (C-6) , 98.3 (C-1), 101.2 (PhC) , 104.5 (C-1') .

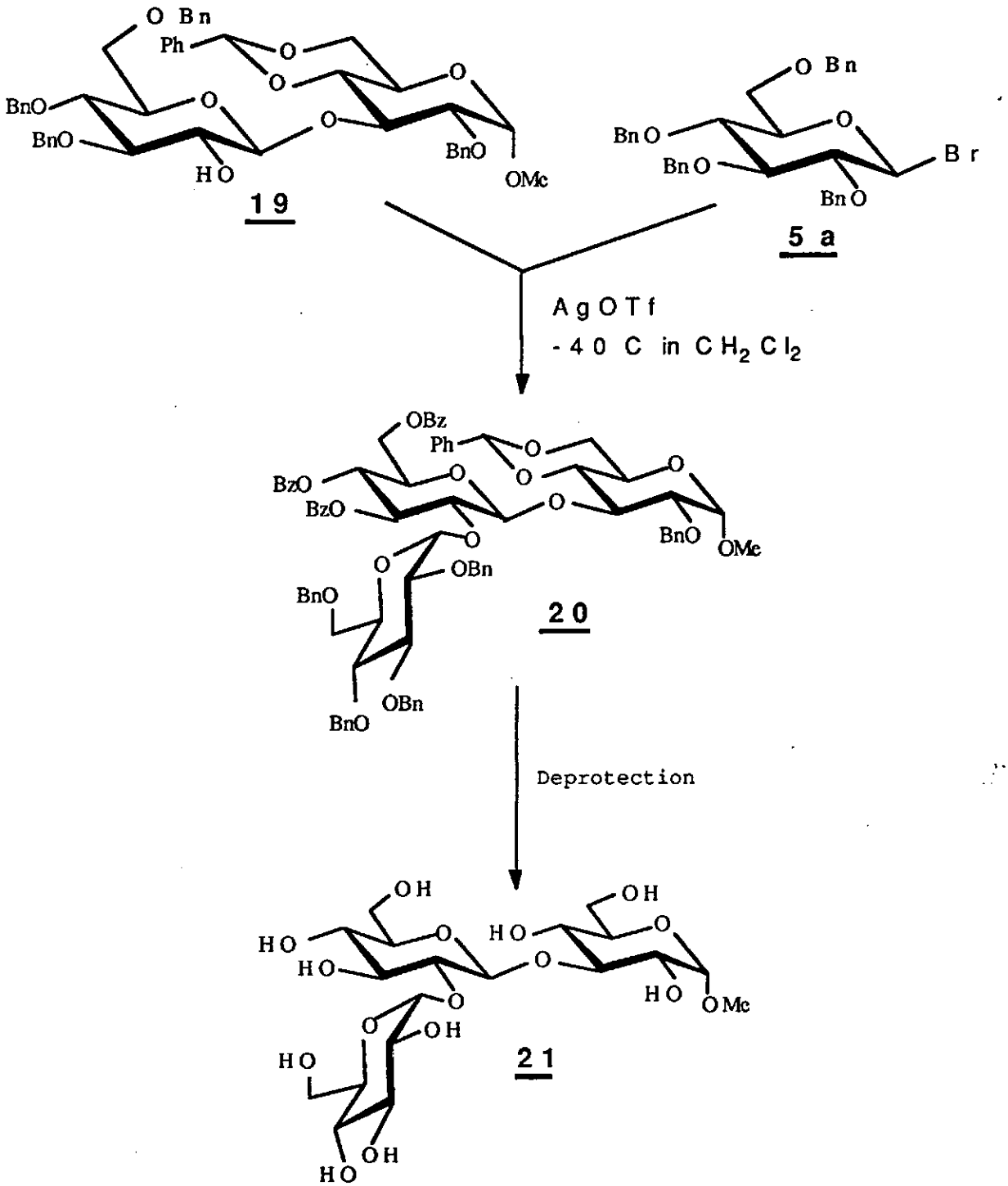
Synthesis of the Trisaccharide methyl α-D-glucopyranosyl-(1→2)-O-β-D-glucopyranosyl-(1→ 3)-O-α-D-glucopyranoside (21)

The synthesis of the blocked trisaccharide methyl O-(2,3,4,6-tetrabenzyl-α-D-glucopyranosyl)-(1→2)-O-(3,4,6-tri-benzyl-β-D-glucopyranosyl)-(1→3)-O-2-O-benzyl-4,6-O-benzylidene-α-D-glucopyranoside (20) by the reaction between (19) and (5a) is as shown in scheme 4.6. on page 46. (21) was obtained from methyl O-(2,3,4,6-tetrabenzyl-α-D-glucopyranosyl)-(1→2)-O-(3,4,6-tri-benzyl-β-D-glucopyranosyl)-(1→3)-O-2-O-benzyl-4,6-O-benzylidene-α-D-glucopyranoside (20) after deprotection.

All the syntheses are described below.

Methyl O-(2,3,4,6-tetrabenzyl-α-D-glucopyranosyl)-(1→2)-O-(3,4,6-tri-benzyl-β-D-glucopyranosyl)-(1→3)-O-2-O-benzyl-4,6-O-benzylidene-α-D-glucopyranoside (20)

1.17g (2mmol) of (5)in 10ml of dichloromethane was converted to the bromo-sugar (5a)by reading it with bromine as described for the preparation of (8) using 0.12ml of bromine. The resulting (5a) and 0.980g (1.2mmol) of (19) were dissolved in dichloromethane (10ml), molecular sieves 4Å was added and the mixture was cooled to -25°. 0.771g (3mmol) of silver triflate and 0.27ml (2mmol) of 2,4,6-trimethyl-pyridine dissolved in dichloromethane were added to the cooled mixture of (5a) and (19) whilst stirring continuosly. After 15 minutes, the reaction was complete the mixture was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Ph
O
O
O
BnO
BnO
O
O
BnO
HO
OMe
19
O Bn
BnO
O
BnO
Br
BnO
5 a
AgOTf
-40 C in CH2Cl2
OBz
Ph
O
O
O
BzO
BzO
O
O
BnO
OMe
O
OBn
BnO
20
OBn
BnO
Deprotection
OH
HO
O
HO
HO
O
O
OH
HO
OMe
O
OH
HO
21
OH
HO

Scheme 4.6.

processed as described for (8) to give the pure (20). Column chromatography yielded 493mg (30%) of (20).

$[\alpha]_{578}$: 52°

^{13}C N.m.r. data: δ 55.2 (MeO), 61.9 (C-6) , 94.8 (C-1"), 98.2 (C-1)
101.6 (Ph *C*), 101.4 (C-1').

Methyl α-D-glucopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranoside (21).

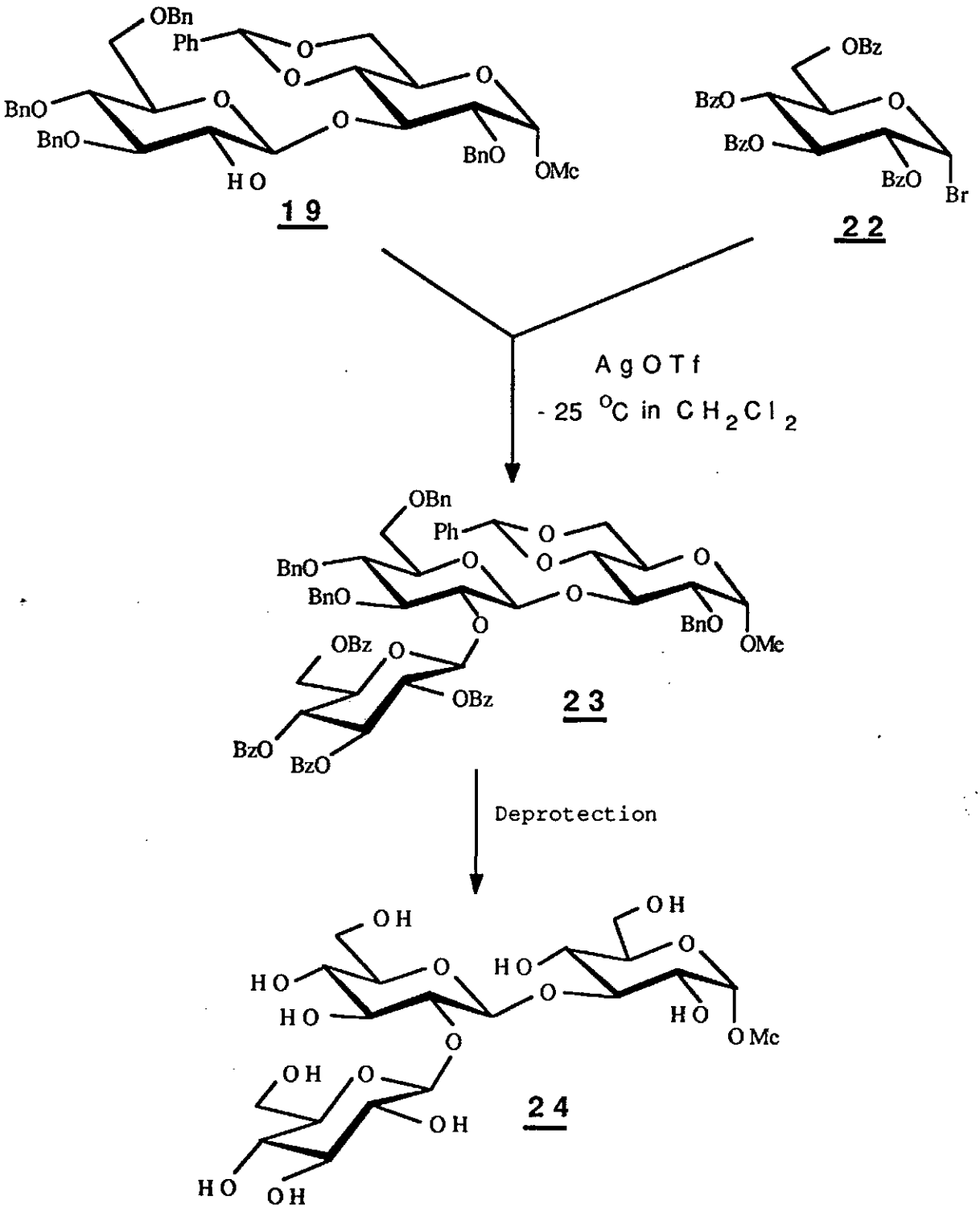
The full deprotection of 200mg of (20) was achieved by hydrogenation over 10% palladium on carbon.(200mg) in 80% aqueous acetic acid at 400kPA for 16hours as described for (13). The resulting crude (21) was purified as described for (13) yielding pure 68.4mg (93%) of (21) after lyophilisation.

$[a]_{578}$: 52°

^{13}C N.m.r. data: d 55.2 (MeO), 61.9 (C-6) , 94.8 (C-1"), 98.2 (C-1)
101.6 (Ph *C*), 101.4 (C-1').

Synthesis of Trisaccharide Methyl *O*-β-D-glucopyranosyl-(1→2)-O-β-D-glucopyranosyl)-(1→3)-O-α-D-glucopyranoside(24)

The blocked trisaccharide methyl *O*-(2,3,4,6-tetrabenzoyl-β-D-glucopyranosyl)-(1→2-*O*-(3,4,6-tribenzyl-β-D-glucopyranosyl)-(1→3)-*O*- 2-O-benzyl-4,6-O-benzylidene-α-D-glucopyranoside (23) was obtained by reacting methyl *O*-(2-hydroxy-3,4,6-tri-O-benzyl-β-D-glucopyranose)-(1→3)-*O*-(2-O-benzyl-4,6-O-benzylidene)-α-D-glucopyranoside (19) and tetra-O-benzoyl-α-D-glucopyranosyl bromide(22). (23) was then deprotected as described earlier for (12) to give (24). These reaction steps are shown in Scheme 4.7 on the next page.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O
O
BnO
O
BnO
O
BnO
OMe
HO
19
OBz
BzO
O
BzO
BzO
Br
22
AgOTf
-25 °C in CH2Cl2
OBn
Ph
O
O
O
BnO
O
BnO
O
BnO
OMe
O
OBz
O
OBz
23
BzO
BzO
Deprotection
OH
OH
HO
O
HO
O
HO
HO
OMe
O
OH
O
OH
24
HO
OH

Scheme 4.7.

Tetra-O-benzoyl-α-D-glucopyranosyl bromide (22)[248d].

D-glucopyranose pentabenzoate (5.65g) was dissolved in 6ml of 1,2-dichloroethane, and the solution was treated with 34% (w/w) hydrogen bromide (5.8mL) in glacial acetic acid. The container was tightly stoppered and kept at 2 hours at room temperature. Toluene (140mL) was then added and the solvent was evaporated under reduced pressure.Another batch of toluene (40mL) was again evaporated from the syrup and then 20ml of dry ether. The semi-crystalline mass was dissolved in 50mL of dry ether; and the solution was diluted with 75ml of dry pentane and left at 0°. A yield of 4.75g (90%) of needle shaped crystals of (22) was obtained and recrystallization of the compound from a mixture of 27 parts of dry ether and 74 parts of pentane gave pure (22) (4.73g).

Methyl *O*-(2,3,4,6-tetrabenzoyl-β-D-glucopyranosyl)-(1→2-*O*-(3,4,6-tribenzyl-β-D-glucopyranosyl)-(1→3)-*O*-2-O-benzyl-4,6-O-benzylidene-α-D-glucopyranoside (23)

1.19g (2mmol) of (19) was added to 0.980g (1.2mmol) of (22) and the glycosidation and processing of the reaction mixture was as described for (20). The reaction product was purified by column chromatography to yield (23) as described earlier. The reaction product was purified by column chromatography to yield 1.05g (62%) (23).

$[\alpha]_{578}$: 15°

^{13}C N.m.r. data : δ 55.2 (MeO), 62.3 , 62.9 (C-6',C-6"), 98.1 (C-1), 100.6,100.7 (C-6', C-6"), 101.6 (Ph *C*)

Methyl *O*-β-D-glucopyranosyl-(1→2)-O-β-D-glucopyranosyl)-(1→3)-O-α-D-glucopyranoside (24).

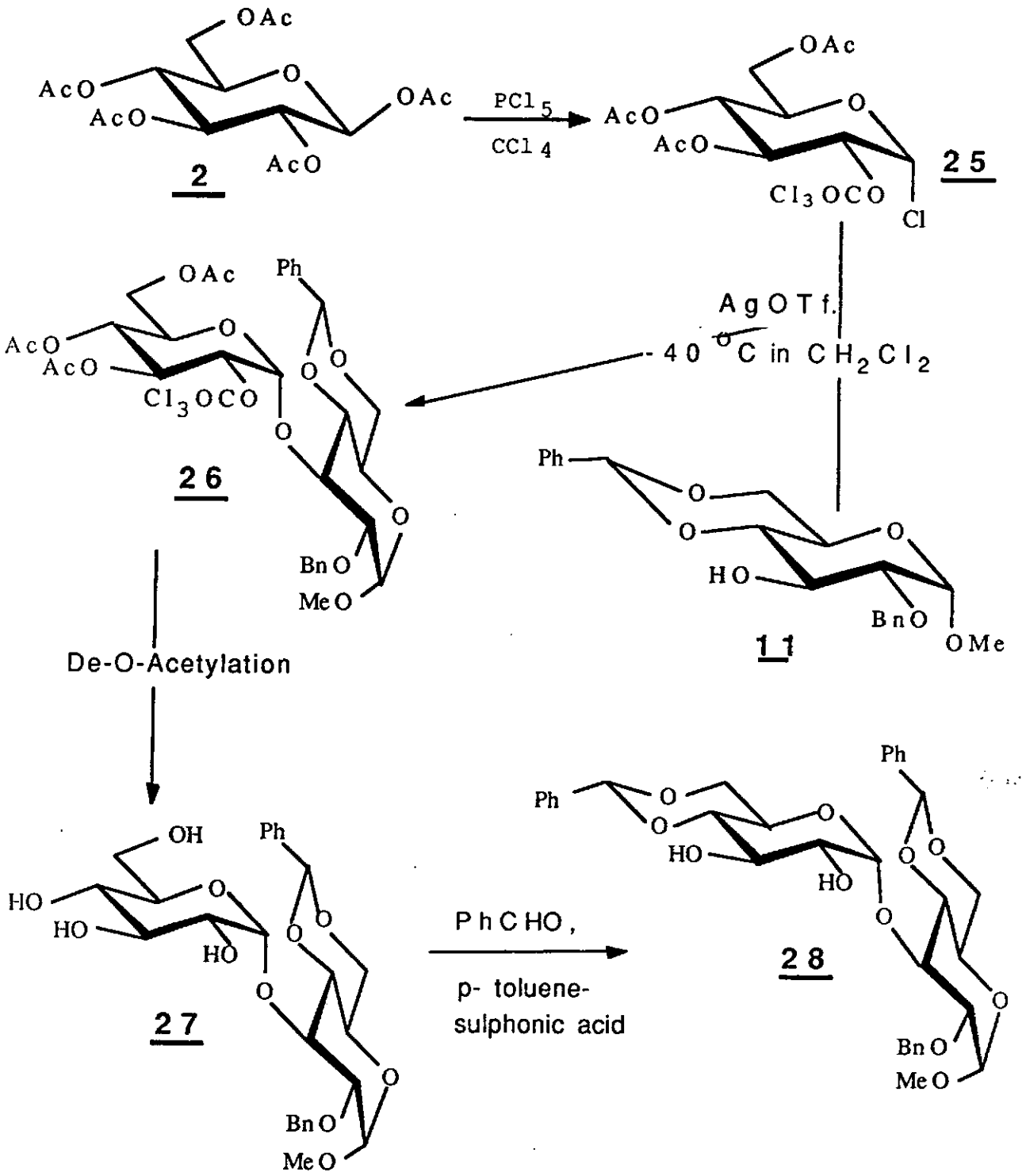
The full deprotection of (23) was achieved by de-O-benzoylation using

0.5M methanolic sodium methoxide. 300mg of (23) was dissolved in 10mL of amixture of dichloromethane:methanol (1:1v/v). 1mL of the methanolic sodium methopxide was added and was allowed to stir for 16hours. The work-up was done as described earlier for (12) and a yield of 101mg (90%) of (24) was obtained.

Synthesis of the intermediate disaccharide methyl O-(4,6-O-benzylidene-α-D-glucopyranosyl)-(1→3)-*O*-2-O- benzyl-α-D-4,6-O-benzylidene-α-D-glucopranoside(28)

Scheme 4.8. shows the reaction steps leading to the disaccharide (28). The starting compound β-D-glycopyranose pentacetate (2) was derivatised to 3,4,6-tri-O-acetyl-2-trichloroacetyl-α-D-glucopranosylchloride (25) which reacted with methyl O-2-benzyl-4,6-O-benzylidene-α-D-glucopyranoside(11) to produce methyl *O*-(2-trichloroacetyl-3,4,6-tri-O-acetyl-α-D-glucopranosyl)-(1→3)-*O*-2-O-benzyl-4,6-benzylidene-α-D-glucopyranoside (26). (26) was de-O- trichloroacetylated to give methyl *O*-α-D-glucopranosyl)- (1→3)-*O*-2-O-benzyl-4,6-benzylidene-α-D-gluco- pyranoside(27). (28) was obtained by O-benzylidenation of (27). The various reaction procedures are described below.

3,4,6-tri-O-acetyl-2-trichloroacetyl-α-D-glucopranosylchloride (25)[248e].
Pure, finely powdered, dry β-D-glucopyranose pentaacetate (2)(7.8g)was thoroughly mixed with 17.7g of powdered phosphorus pentachloride in a 100ml round-bottomed flask equipped with a reflux condenser. Carbon tetrachloride (10mL) was added, and the reaction mixture was protected from atmospheric moisture with a calcium chloride tube. The mixture was heated for 5 hours in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AcO
AcO
O
OAc
AcO
2
PCl5
CCl4
OAc
AcO
AcO
O
Cl3OCO
Cl
25
AgOTf.
-40 °C in CH2Cl2
OAc
Ph
AcO
AcO
O
Cl3OCO
O
O
O
O
BnO
MeO
26
Ph
O
O
O
HO
BnO
OMe
11
De-O-Acetylation
OH
Ph
HO
HO
HO
O
O
O
O
BnO
MeO
27
PhCHO,
p- toluene-
sulphonic acid
Ph
Ph
O
O
O
HO
HO
O
O
O
O
BnO
MeO
28

Scheme 4.8.

a fumehood in an oil bath at 120°C. After about 30minutes, the mixture became partially fluid and volatile components begin to reflux with the evolution of hydrogen chloride. At the end of the reaction period, a clear yellowish solution was obtained. The solution was evaporated under reduced pressure until the bath temperature rose to about 85°. The thick syrup was dissolved in 16ml of dry ether and cooled overnight at -10°C. The crystalline precipitate was collected on a Buchner funnel, pressed to a firm cake and washed first with 2ml each of cold methanol and dry ether to yield the crude product. The yield was 3.82g (40%) of the crude product. The crude (25) was recrystallized from ether twice to obtain the pure product with a final yield of 2g (19%).

Methyl O -(2-trichloroacetyl-3,4,6-tri-O-acetyl-α-D-glucopranosyl)-(1→3)-O -2-O-benzyl-4,6-benzylidene-α-D-glucopyranoside (26).

(25) 640mg (1.4mmol) and 390mg (1.0mmol) of (11) were dissolved in dichloromethane (10ml), molecular sieves 4Å (1g) was added and cooled to -40°. A mixture of 1.54g (6mmol) of silver triflate and 2,4,6-trimethylpyridine 0.5ml (3mmol) dissolved in 5ml of dichloromethane was added to the mixture (11) and (25). After the reaction was complete, it was worked up as described for the other glycosylations. Column chromatography using petroleum ether (40°-60°C)-ethyl acetate 4:1 mixture as eluent yielded 305mg (38%) of (26).

$[a]_{578}$: 85°

^{13}C N.m.r. data : δ 55.4 (MeO), 61.2 ,61.9 (C-6,C-6'), 101.6 (PhC), 160.7 ($C{=}OCl_3$), 169.5, 169. 8, 170.6 (C=O Acetyl)

Methyl O-α-D-glucopranosyl)-(1→3)-O-2-O-benzyl-4,6-benzylidene-α-D-glucopyranoside (27).

(26) (810mg,1mmol) was de-O-acylated with 0.5M methanolic sodium methoxide (0.66ml) in 15ml of methanol for 2hours at room temperature. The solution was neutralised with Dowex 50 (H^+) resin and concentrated to give 396mg of compound (27) in (82%) yield.

^{13}C N.m.r. data of (27) : ($CDCl_3$ δ 77.17, CD_3OD) δ 54.3 (MeO), 60.7, 60.8 (C-6,C-6'), 97.6, 100.3 (C-1,C-1") 102.7 (Ph *C*).

Methyl O-(4,6-O-benzylidene-α-D-glucopyranosyl)-(1→3)-O-2-O-benzyl-4,6-O-benzylidene-α-D-glucopranoside (28).500mg (1.06mmol) of (27) was dissolved in benzaldehyde (1mL) and 50mg of para-toluene sulphonic acid was added and was allowed to stir for 16hours at room temperature. The reaction mixture was then poured with stirring into 25ml petroleum ether (60-70°), saturated aqueous sodium hydrogen carbonate (25ml) and ice.

It was stirred for 30 minutes and filtered off. The crude product was washed well with petroleum ether and water and recrytallised from water containing enough sodium hydrogen carbonate to make it alkaline. A yield of 358mg (54%) of pure (28) was obtained.

^{13}C N.m.r. data : (CD_3Cl δ 77.17 , CD_3OD) δ 55.1 (MeO), 61.7, 62.5 (C-6, C-6'), 98.4, 99.3 (C-1,C-1") 101.2, 101.7 (Ph*C*)

Synthesis of the trisaccharide Methyl β-D-glucopyranosyl-(1→2)-α-D-glucopyranosyl)-(1→3)-α-D-glucopyranoside (30)

Scheme 4.9 on the next page shows the reaction steps between (28) and 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (14) leading to trisaccharide methyl O-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl)-(1→2)-O-(4,6-O-benzylidene-α-D-glucopyranosyl)-(1→3)-O-2-O-benzyl-4,6-O-

(4,6-O-benzylidene-α-D-glucopyranosyl)-(1→3)-*O*-2-O-benzyl-α-D-4,6-O-benzylidene-α-D-glucopranoside (29) which was deprotected to give (30). The reaction steps are described further below.

Methyl O-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl)-(1→2)-O-(4,6-O-benzylidene-α-D-glucopyranosyl)-(1→3)-*O*-2-O-benzyl-α-D-4,6-O-benzylidene-α-D-glucopranoside (29)

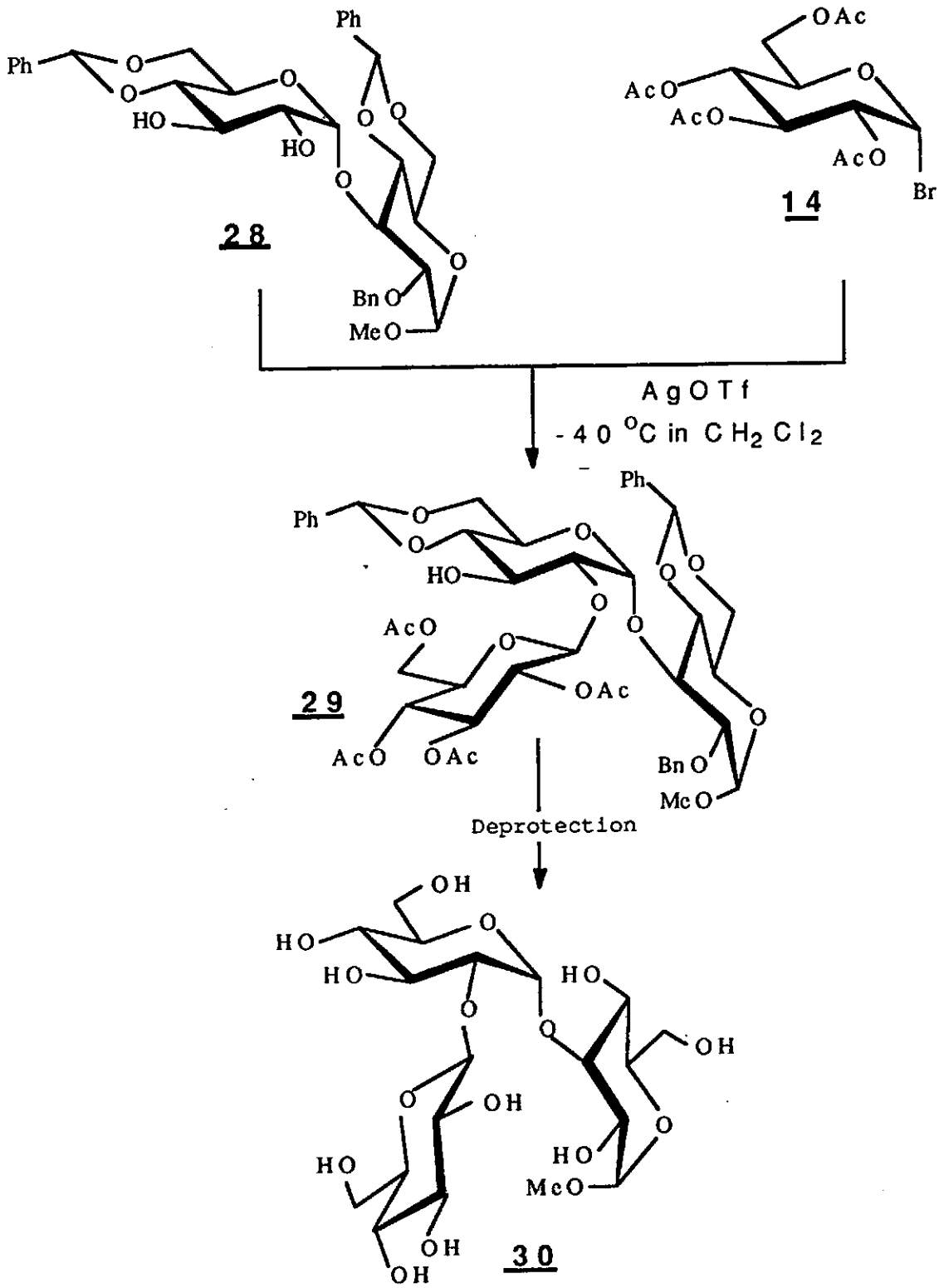
(28) (590mg, 1mmol) and (14) (822mg, 2mmol) were dissolved in dichloromethane (10ml) and molecular sieves 4Å were added . The mixture was cooled to -40°C and silver triflate 590mg (2.3mmol) dissolved in a toluene-dichloromethane mixture (ratio 1:1) was added whilst stirring continuously. After 30 minutes the reaction was complete and the reaction mixture was processed as for the earlier glycosylations to give (29) having 85 % anomeric purity.

^{13}C N.m.r. data of (29) : δ 20.3, 20.6, 20.7, 20.8 (Acetyl Me), 55.2 (MeO), 98.2,98.4,100.1 (C-1,C-1', C-1"), 101.1,102.1 (PhC).

About 15% yield of the 3-linked product was formed and was seperated from (29) during column chromatography.

Methyl *O*-β-D-glucopyranosyl-(1→2)-α-D-glucopyranosyl)-(1→3)-α-D-glucopyranoside (30).

55mg of (29) in 5ml of methanol was de-O-acetylated with 0.15mL of 0.5M methanolic sodium methoxide for one hour at room temperature. The crude product was fully deprotected and worked up as for (12) and 25mg (93%) of (30) (85% pure as determined from ^{1}H n.m.r.) was obtained after lyophilisation. Scheme 4.9. shows the sequence of reactions.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O
O
HO
HO
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Ph
O
O
O
Bn O
Me O
28
OAc
AcO
AcO
O
AcO
Br
14
AgOTf
-40 °C in CH2Cl2
Ph
O
O
HO
O
O
AcO
O
OAc
29
AcO
OAc
Ph
O
O
O
Bn O
Me O
Deprotection
OH
HO
HO
O
HO
O
OH
O
OH
HO
HO
Me O
OH
OH
30

Scheme 4.9.

INDEX I.

INDEX of ^{13}C N.M.R.SPECTRA of SYNTHESIZED COMPOUNDS (2) to (30) .

Figures 4.3 to 4.28.

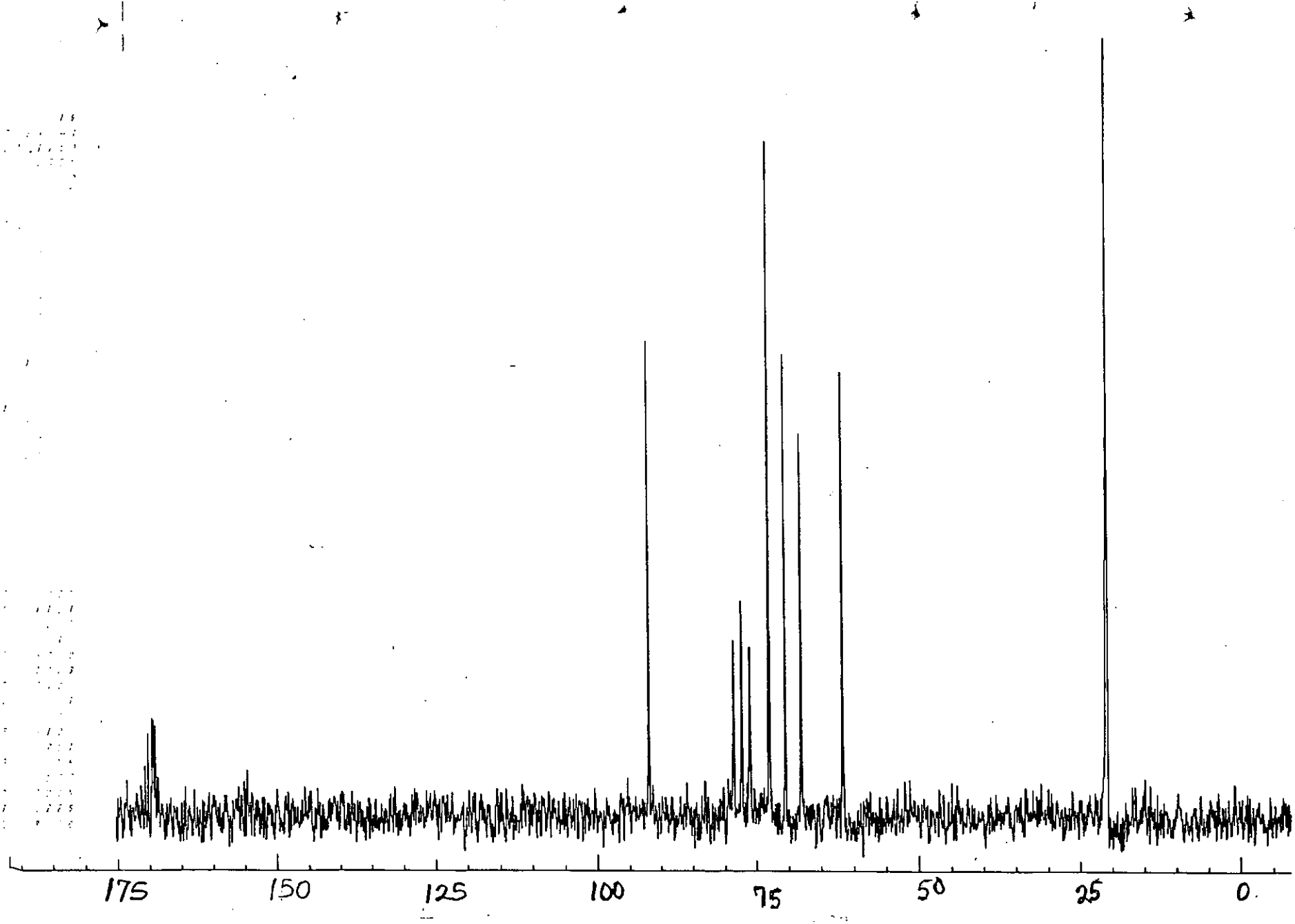


Figure 4.3. ^{13}C n.m.r. spectrum of β-D-glucopyranose pentaacetate. Compound 2.

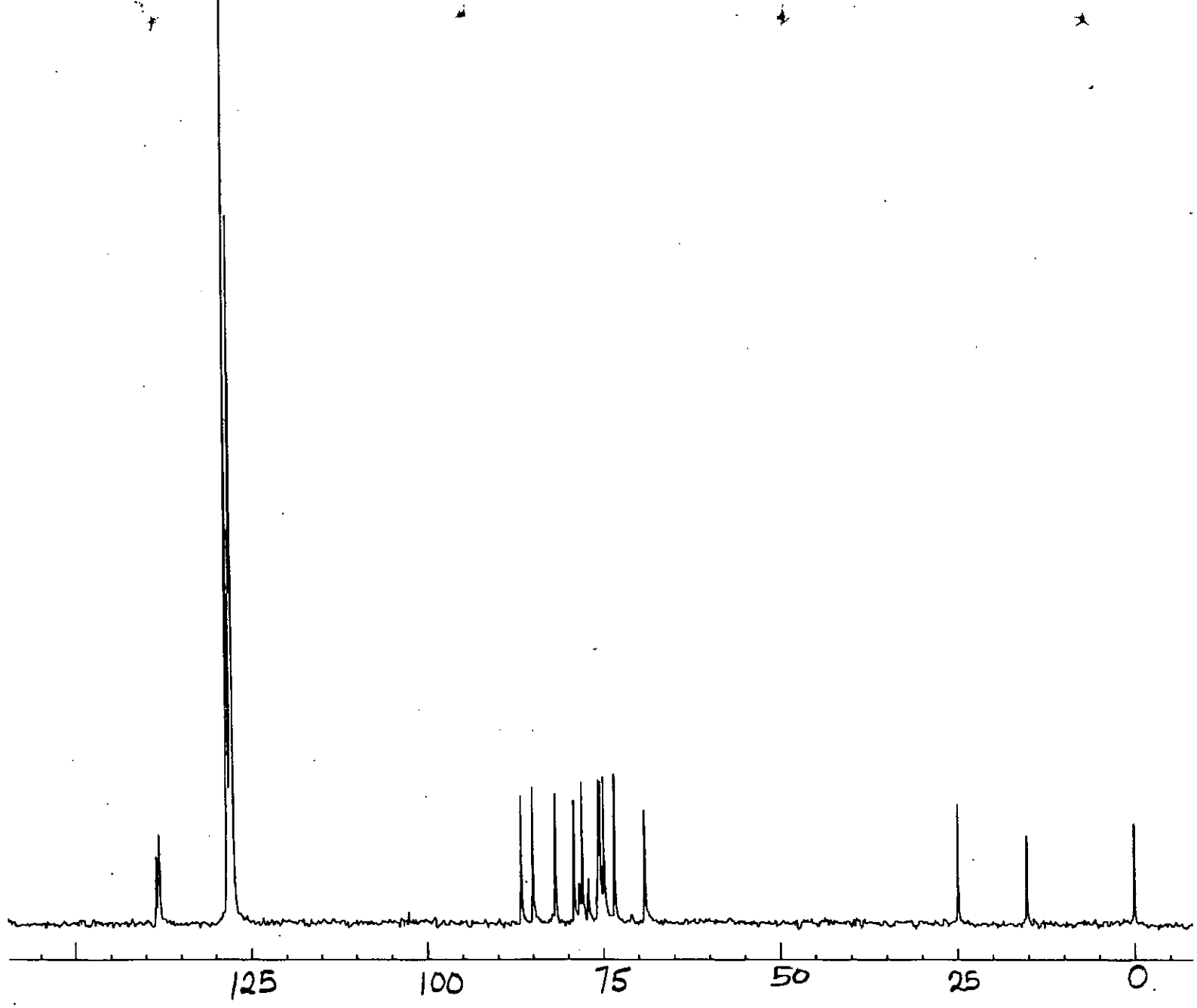


Figure 4.5. ^{13}C n.m.r. spectrum of Ethyl-2,3,4,6-tetrabenzyl-β-D-1-thioglucopyranoside. Compound 5.

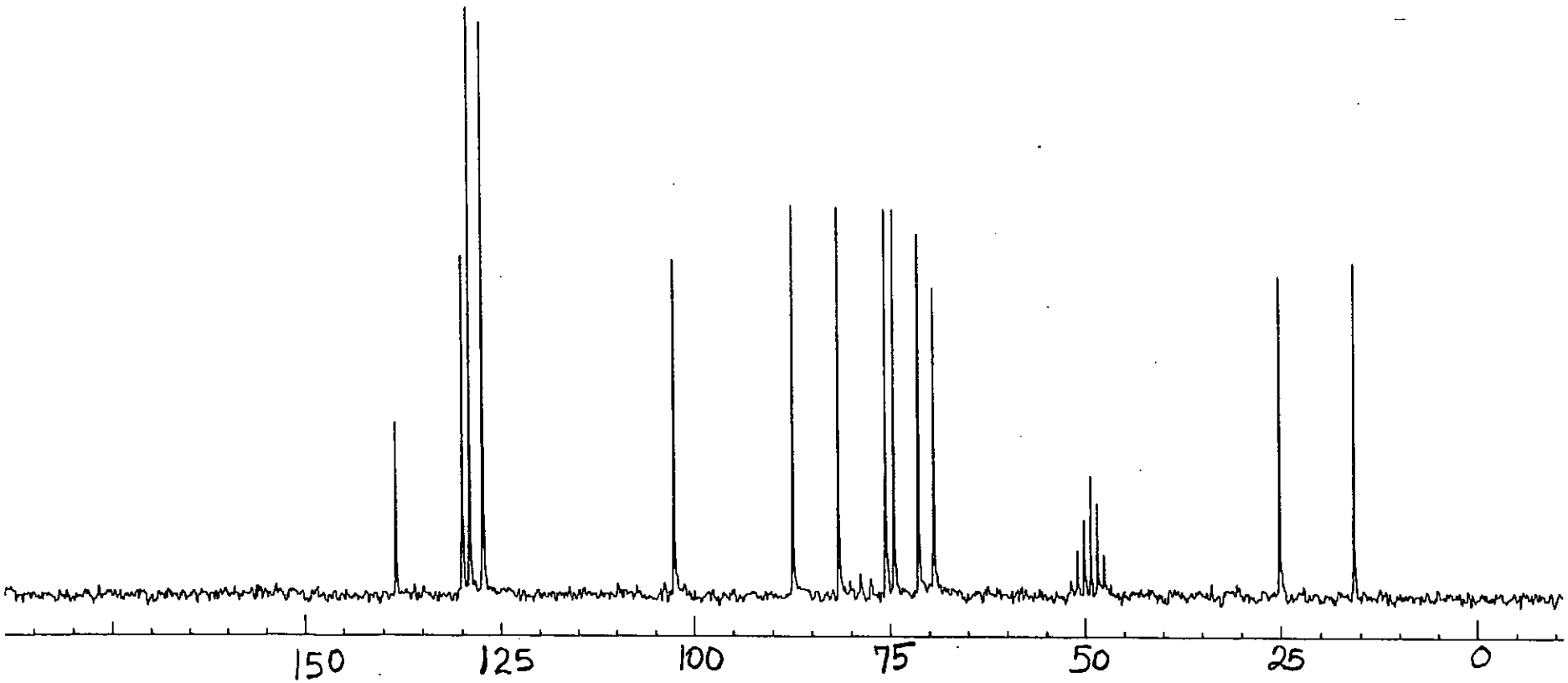


Figure 4.6. ^{13}C n.m.r. spectrum of Ethyl 4,6-O-benzylidene-β-D-thioglucopyranoside. Compound 6.

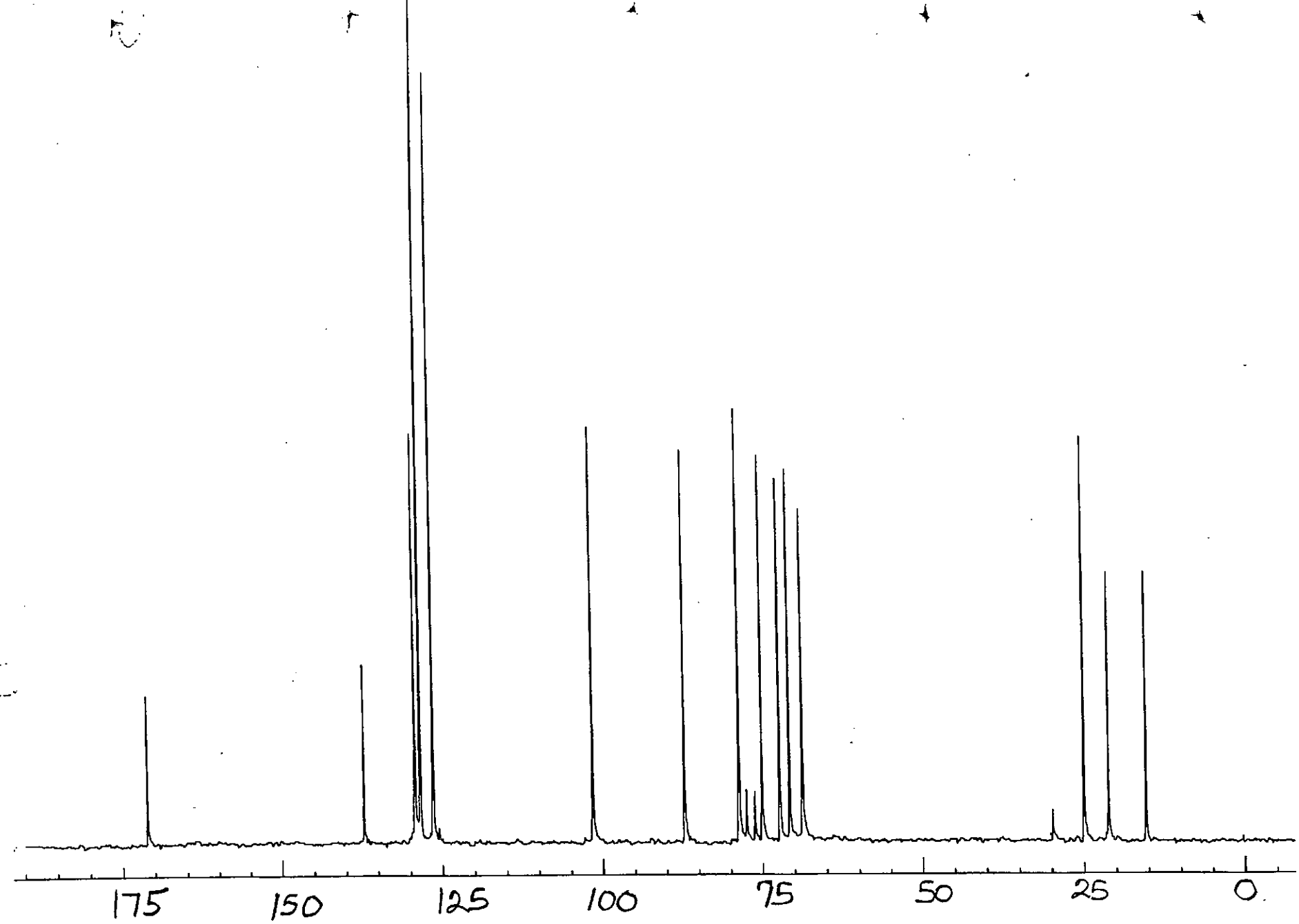


Figure 4.7. ^{13}C n.m.r. spectrum of Ethyl 3-O-Acetyl-4,6-O-Benzylidene-β-D-thioglucopyranoside.Compound 7.

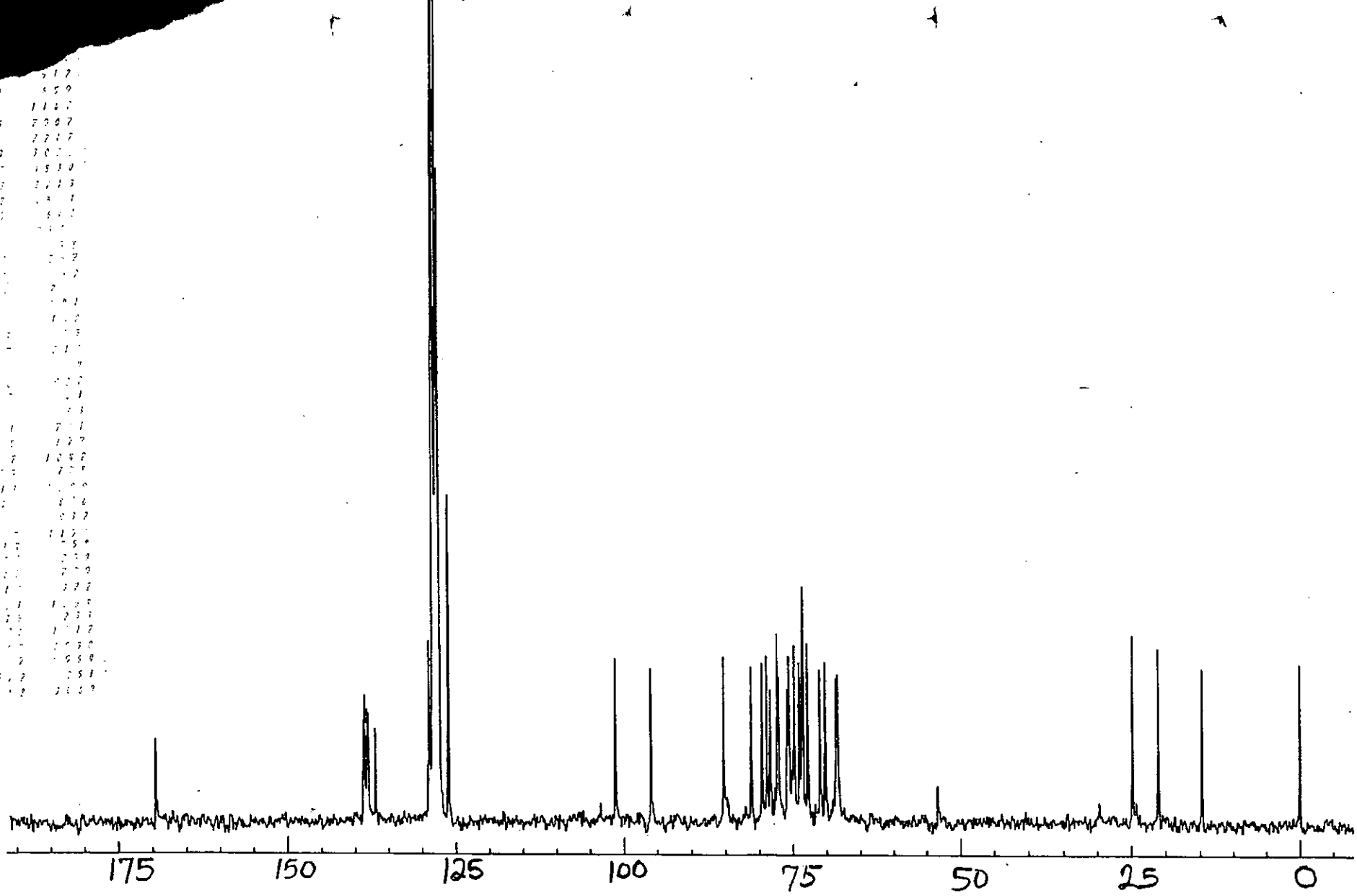
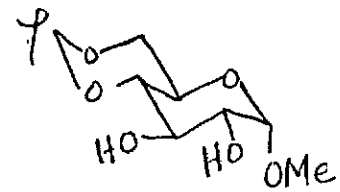


Figure 4.8. ^{13}C n.m.r. spectrum of Ethyl 3-O-Acetyl-4-6-*O*-Benzylidene-2-*O*-(2,3,4,6-tetra-O-benzyl-α-D-glucopyranosyl)-1-thio-β-D-glucopyranoside. Compound 8.



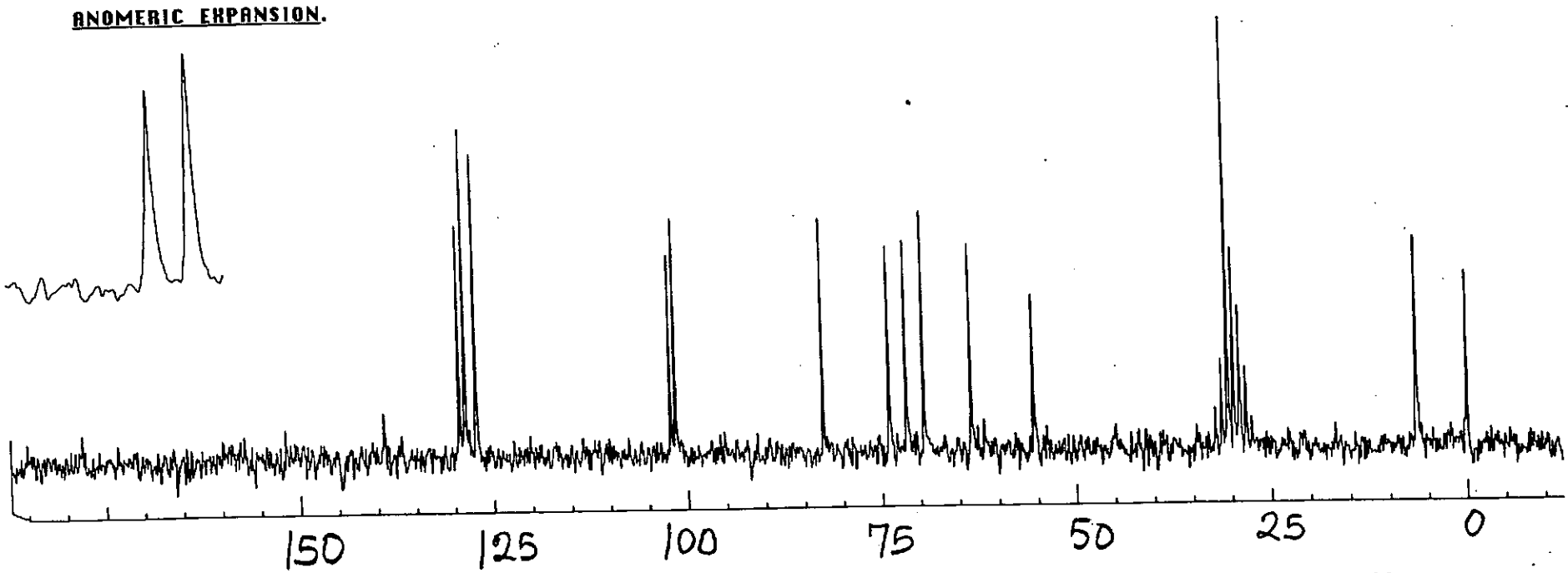


Figure 4.9. ^{13}C n.m.r. spectrum of Methyl 4,6-O-benzylidene-α-D-glucopyranoside. Compound 10.

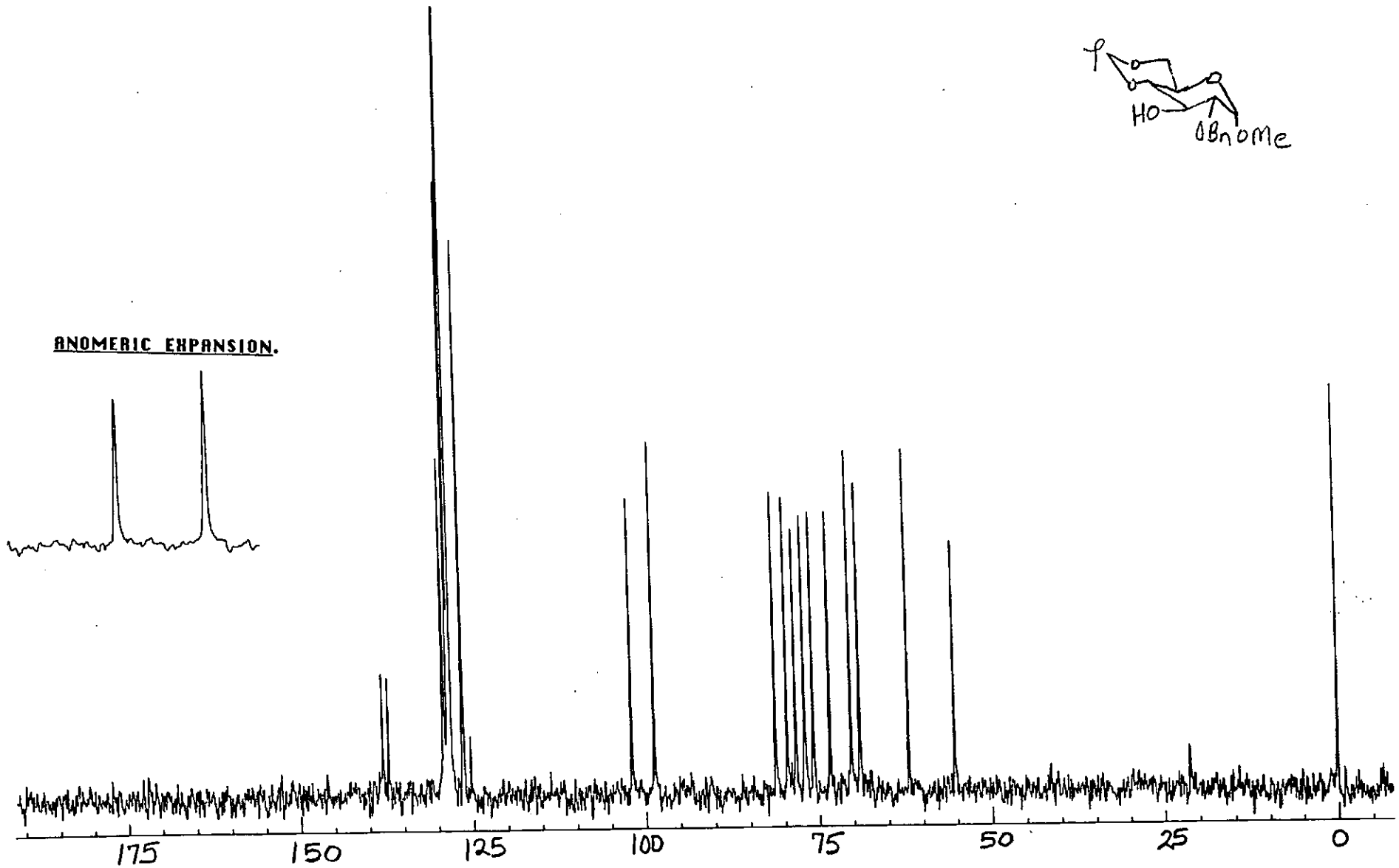


Figure 4.10. ^{13}C n.m.r. spectrum of Methyl O-2-benzyl-4-6-O-benzylidene-α-D-glucopyranoside. Compound 11

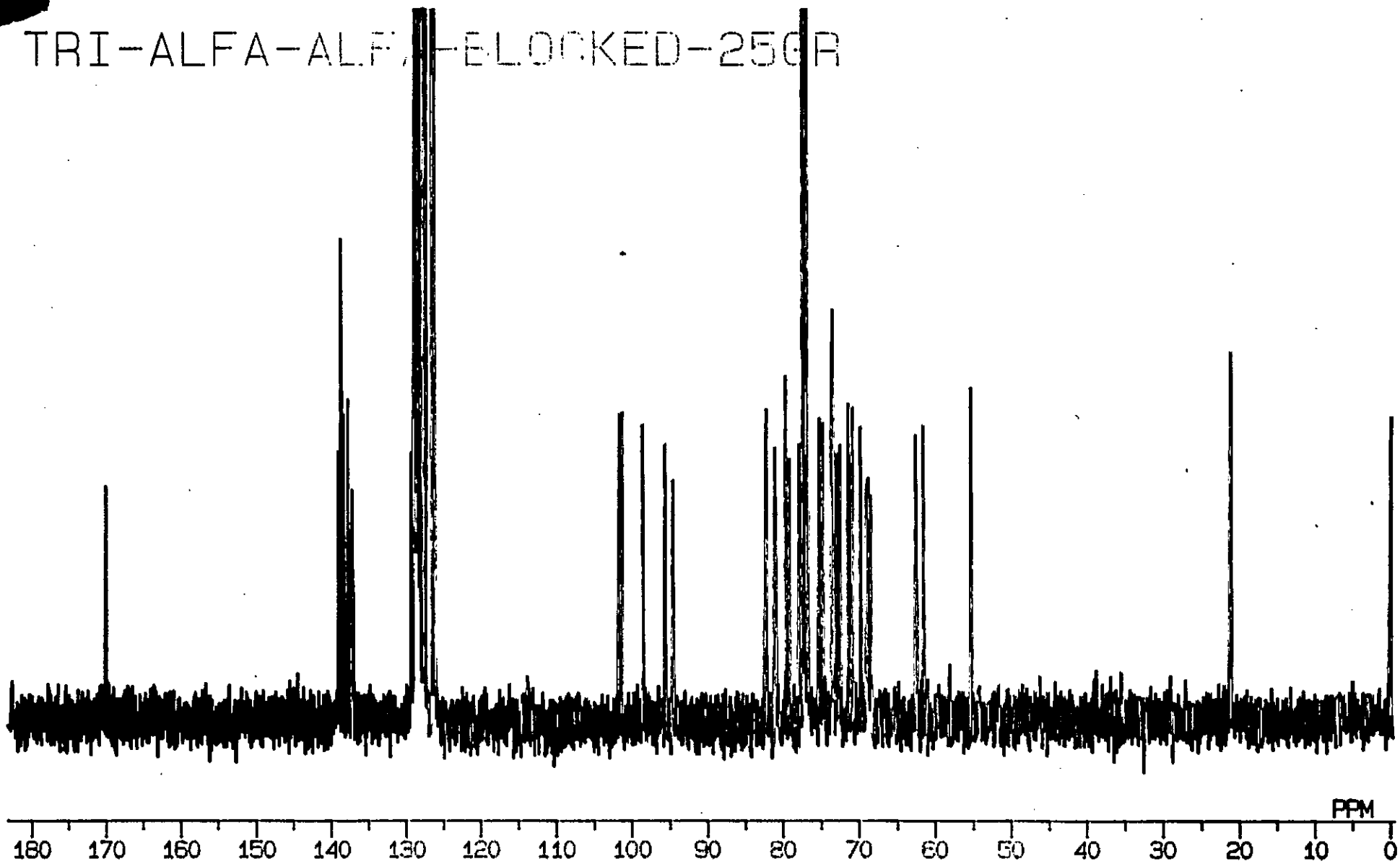


Figure 4.11. ^{13}C n.m.r. spectrum of Methyl *O*-(2,3,4,6-tetra-benzyl-α-D-glucopyranosyl)-(1→2)-*O*-(3-O-Acetyl-4,6-O-benzylidene-α-D-glucopyranosyl)-(1→3)-*O*-(2-O-benzyl-4,6-O-benzylidene)-α-D-glucopyranoside. Trisaccharide 12.

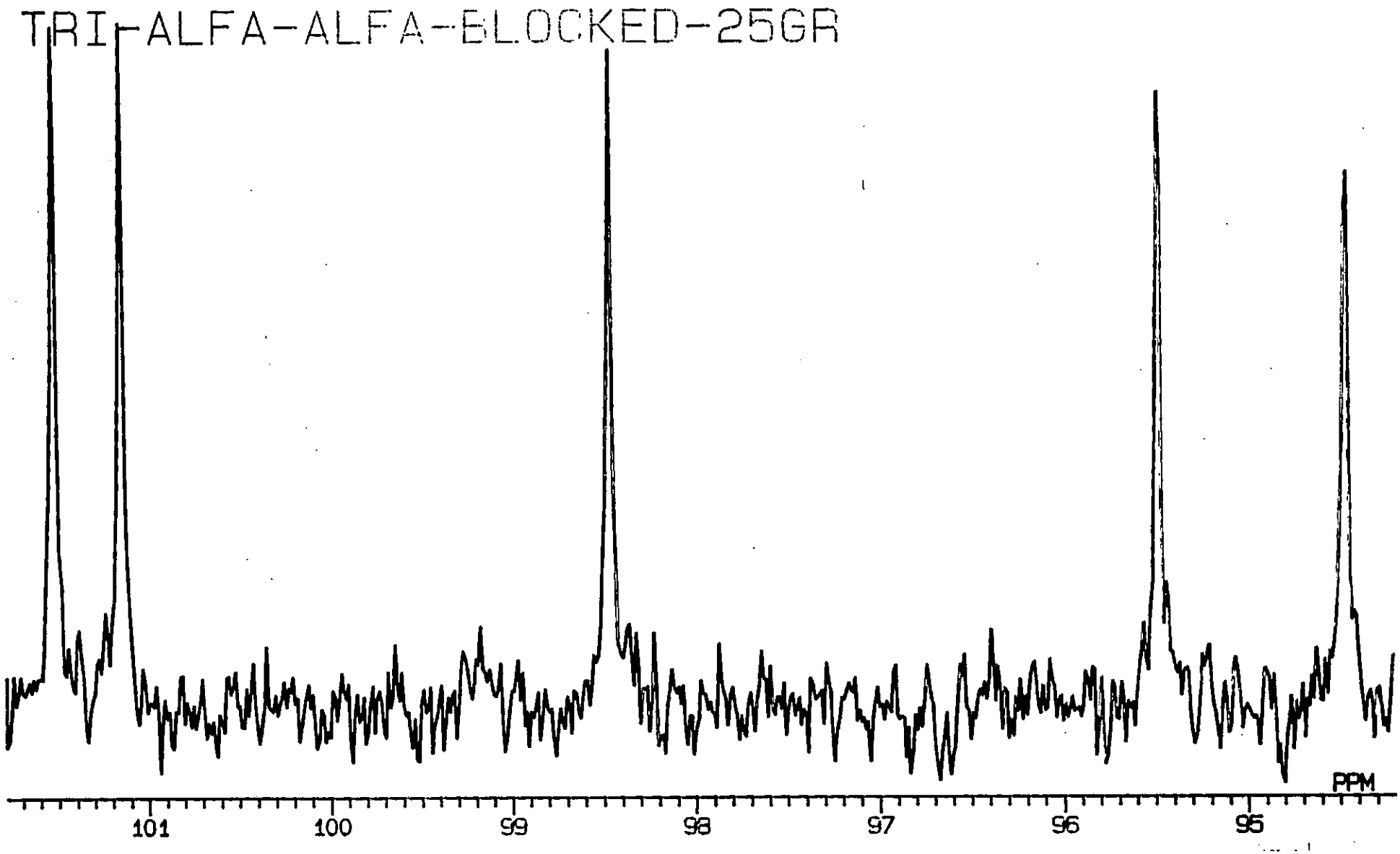


Figure 4.11a. ^{13}C n.m.r. spectrum of Methyl *O*-(2,3,4,6-tetra-benzyl-α-D-glucopyranosyl)-(1→2)-*O*-(3-O-Acetyl-4,6-O-benzylidene-α-D-glucopyranosyl)-(1→3)-*O*-(2-O-benzyl-4,6-O-benzylidene)-α-D-glucopyranoside. Trisaccharide 12. ANOMERIC EXPANSION.

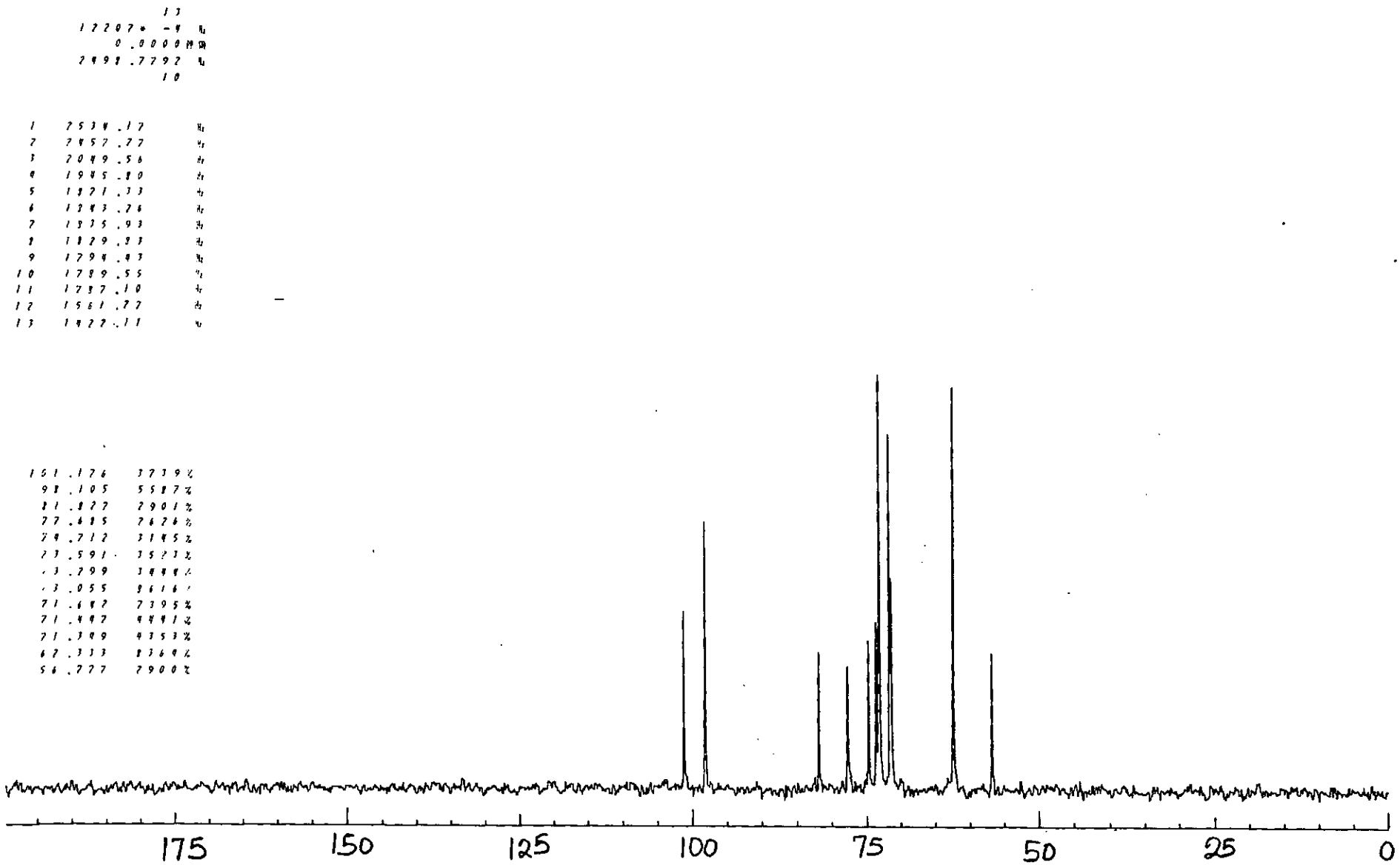


Figure 4.12. ^{13}C n.m.r. spectrum of Methyl *O*-α-D-glucopyranosyl-(1→ 2)-*O*-α-D-glucopyranosyl-(1→ 3)-*O*-α-D-glucopyranoside. Trisaccharide <u>13</u>.

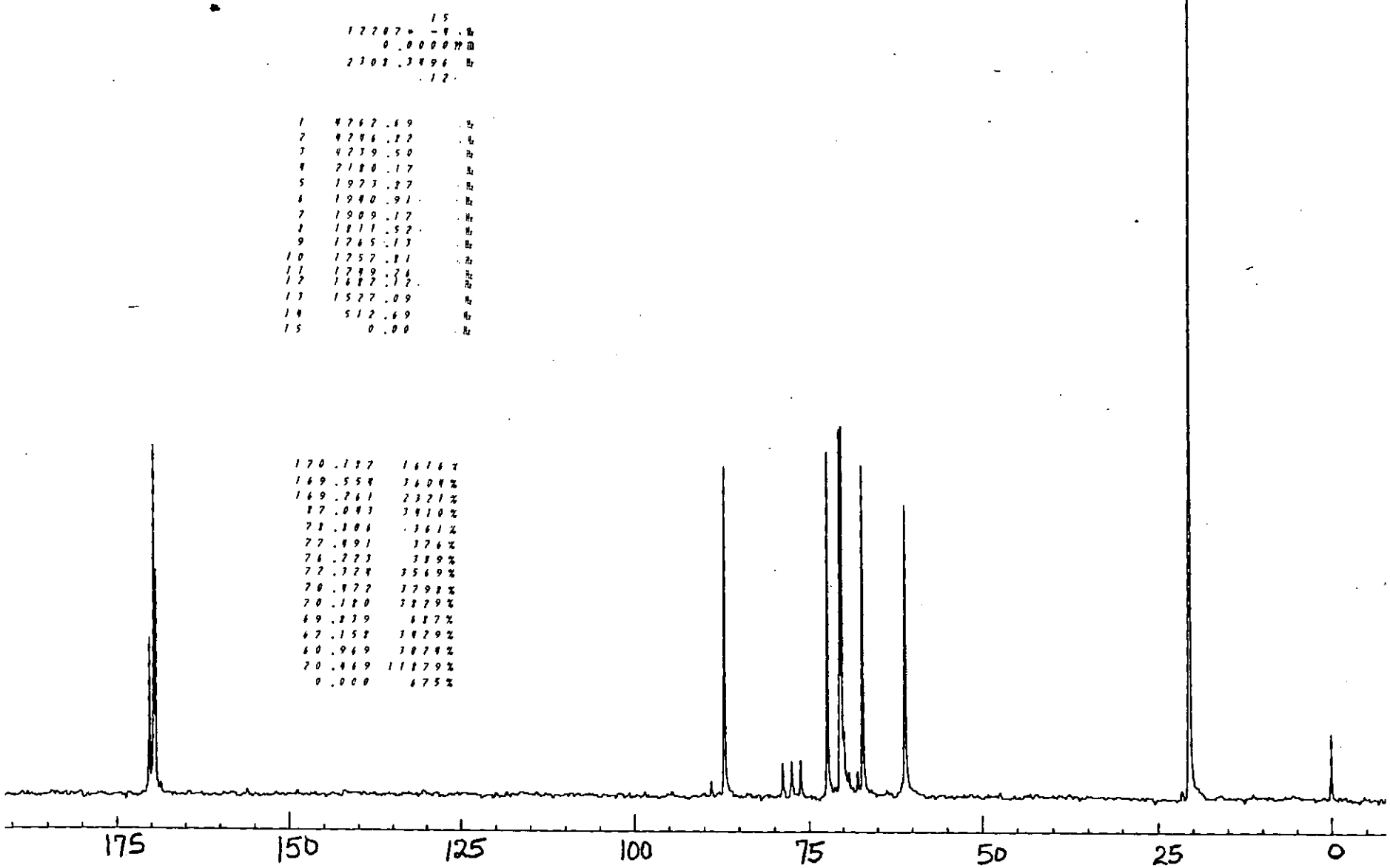


Figure 4.13. ^{13}C **n.m.r. spectrum of 2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl bromide. Compound 14**

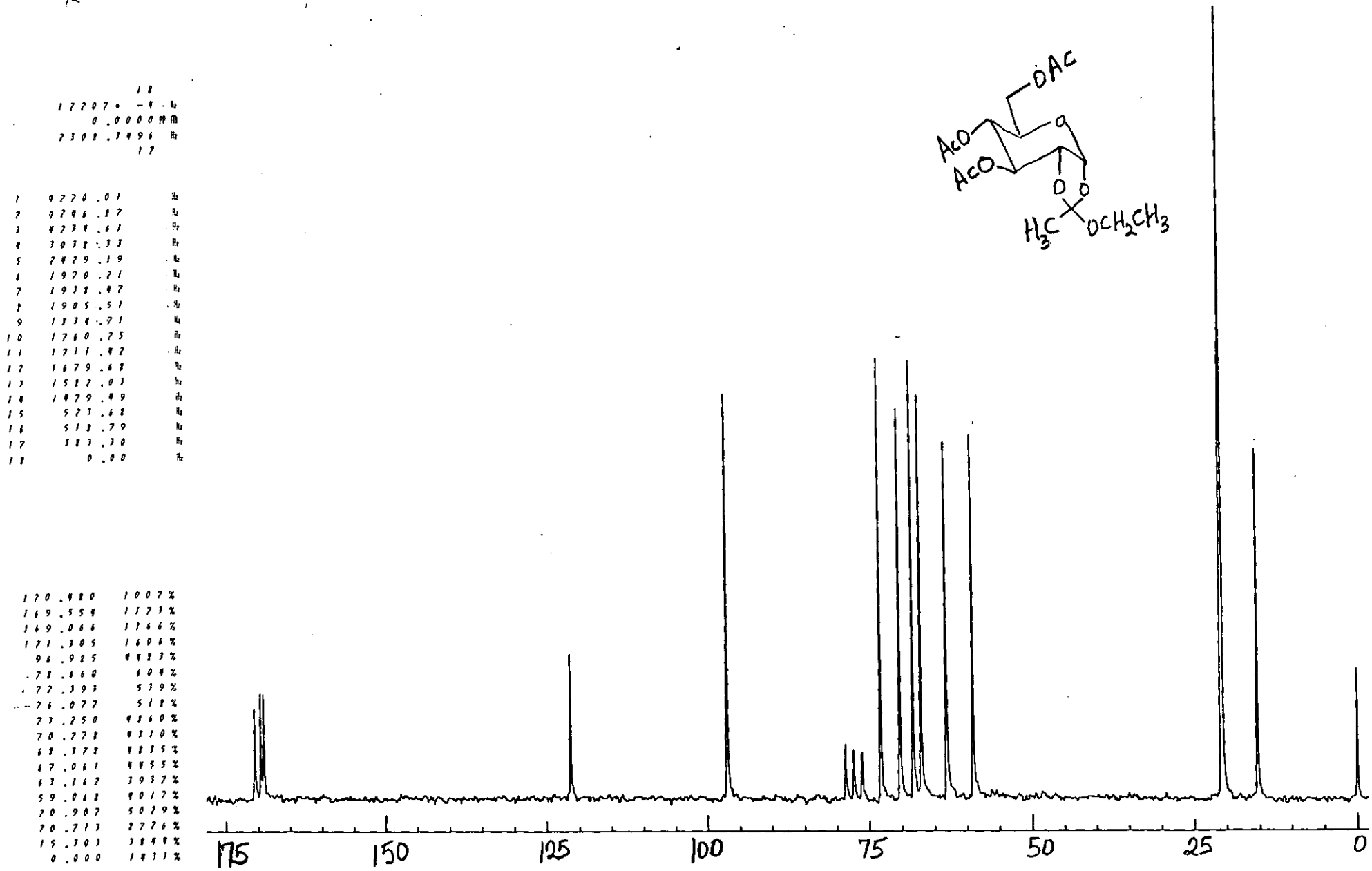


Figure 4.14. ^{13}C n.m.r. spectrum of 3,4,6-Tri-O-acetyl-1,2-O-(1-ethoxylidene)-α-D-glucopyranose. Compound 15.

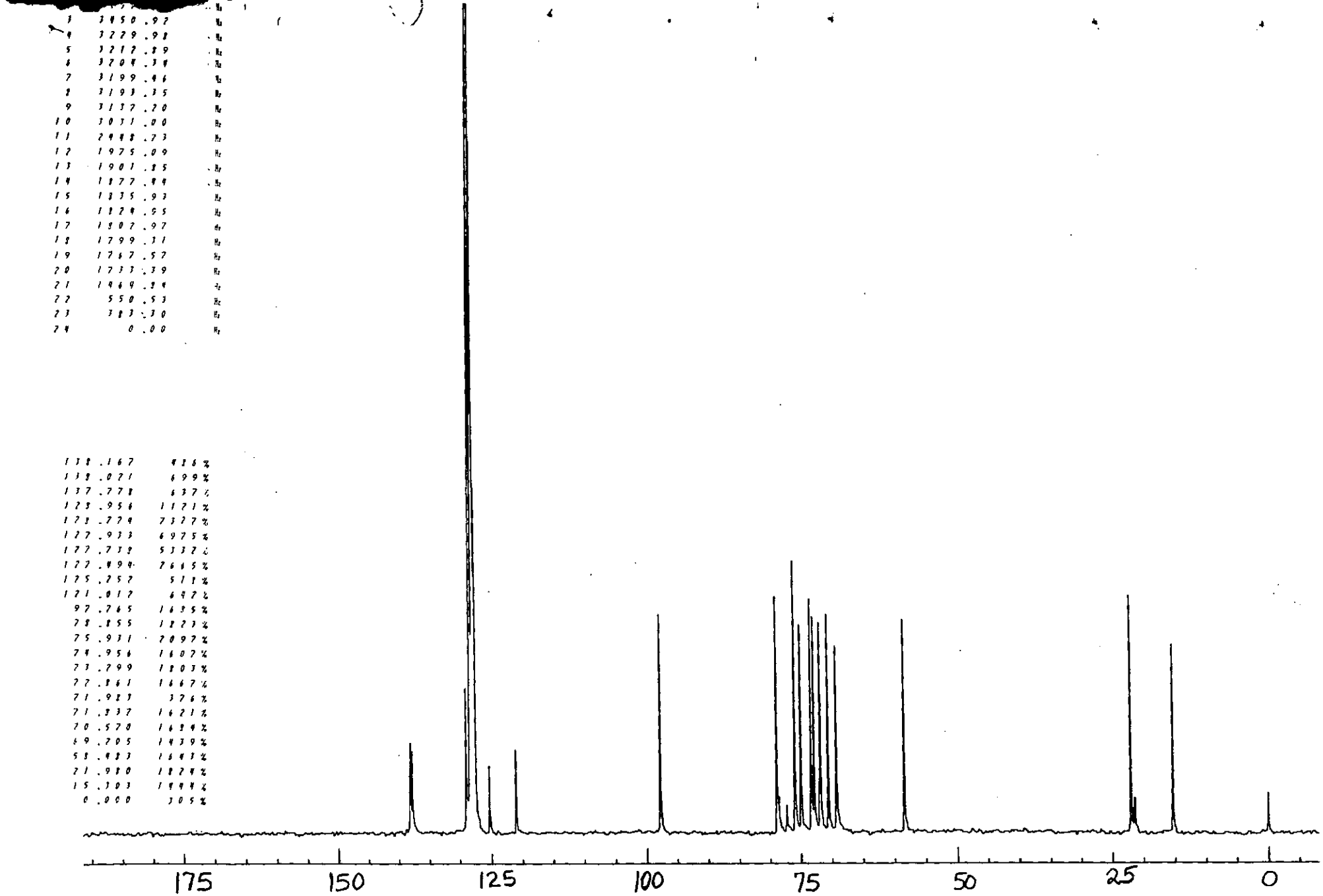


Figure 4.15. ^{13}C **n.m.r. spectrum of 3,4,6-Tribenzyl-1,2-O-(1-Ethoxyethylidene)-α-D-glucopyranose. Compound 16.**

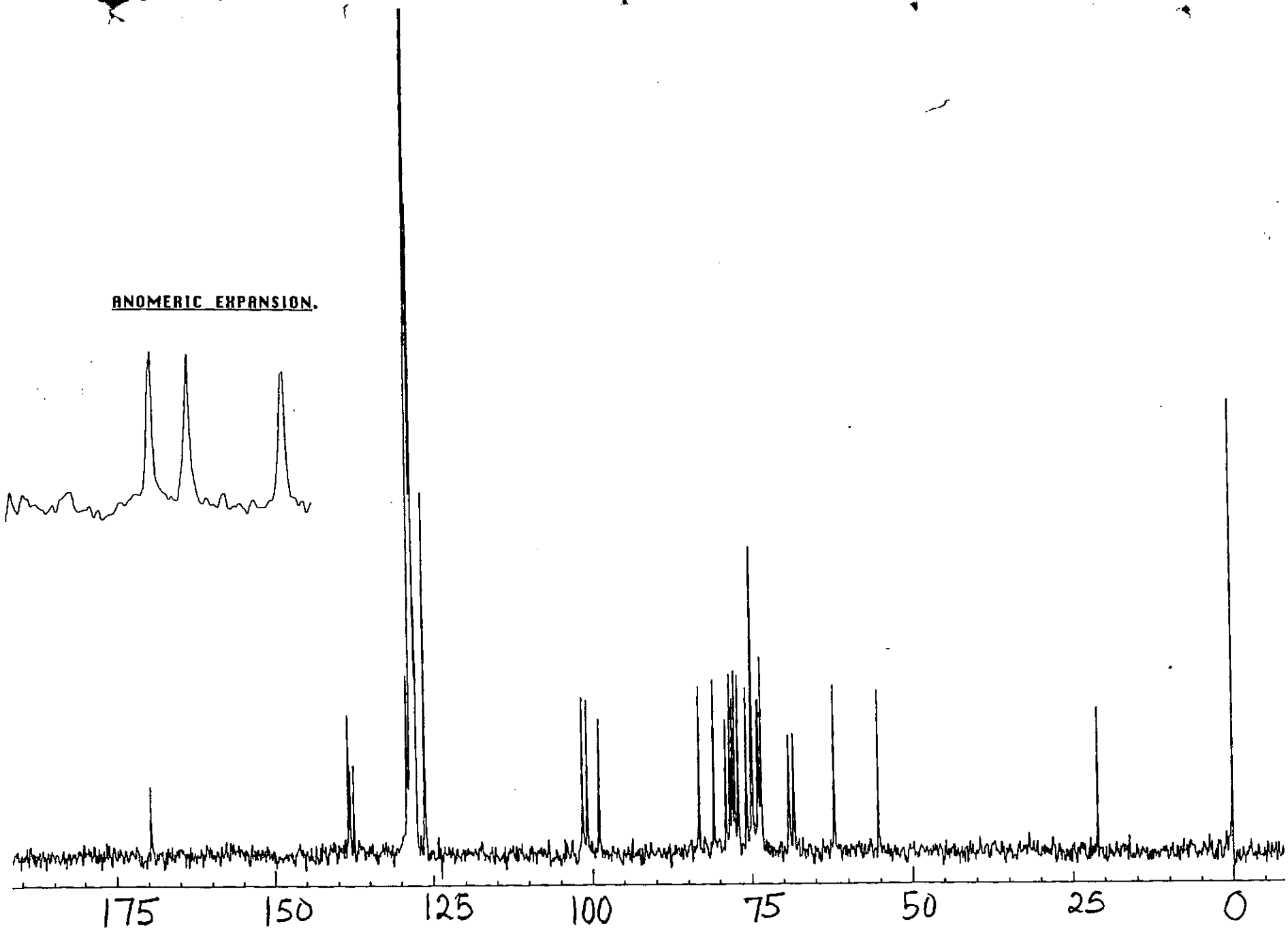


Figure 4.16. ^{13}C n.m.r. spectrum of Methyl *O*-(2-Acetyl-3,4,6-tri-O-benzyl-β-D-glucopyranose)-(1→3)-*O*-(2-O-benzyl-4,6-O-benzylidene)-α-D-glucopyranoside. Compound 18.

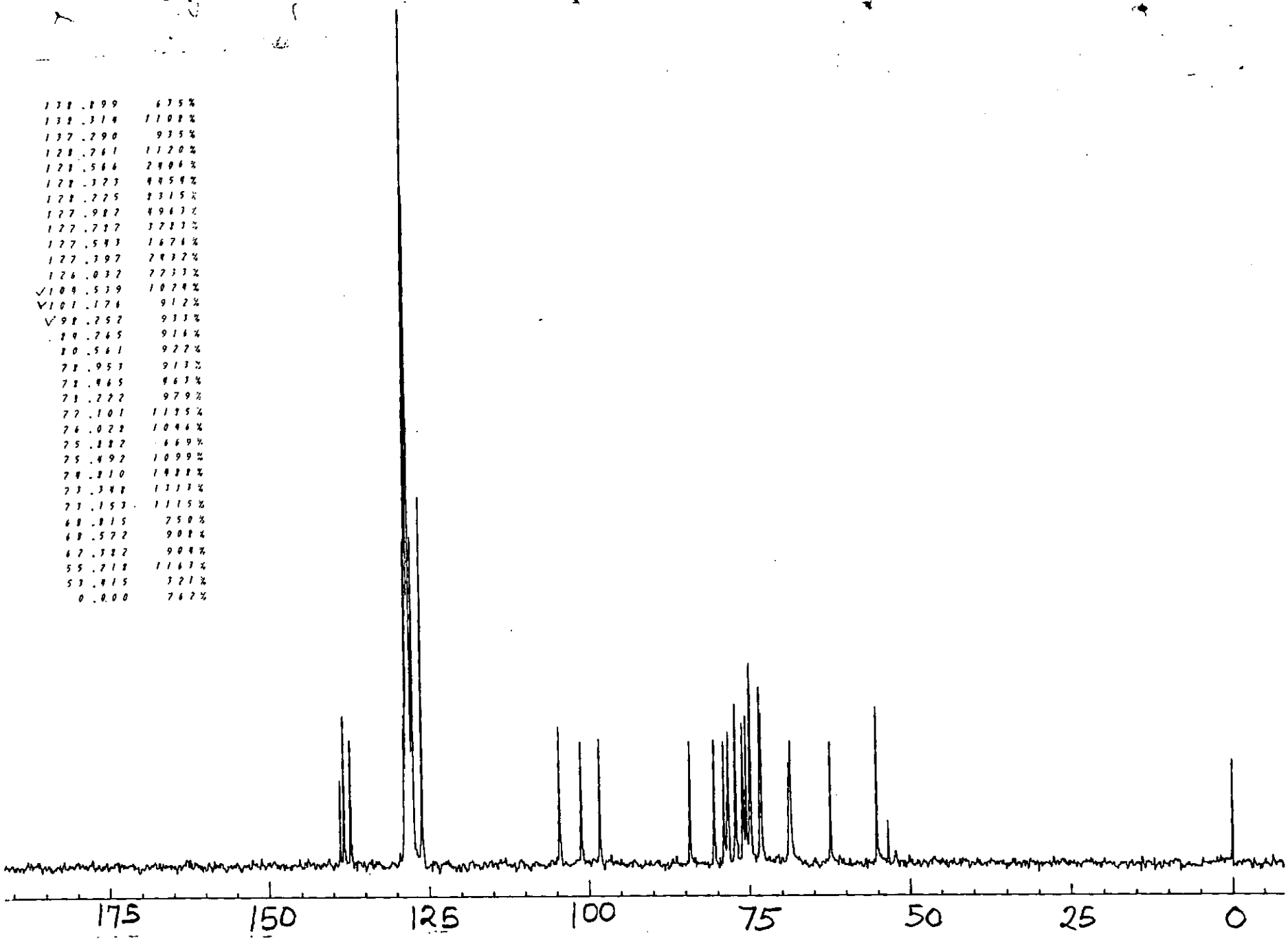


Figure 4.17. ^{13}C n.m.r. spectrum of Methyl *O*-(2-hydroxy-3,4,6-tri-O-benzyl-β-D-glucopyranose)-(1→3)-*O*-(2-O-benzyl-4,6-O-benzylidene)-α-D-glucopyranoside. Compound 19.

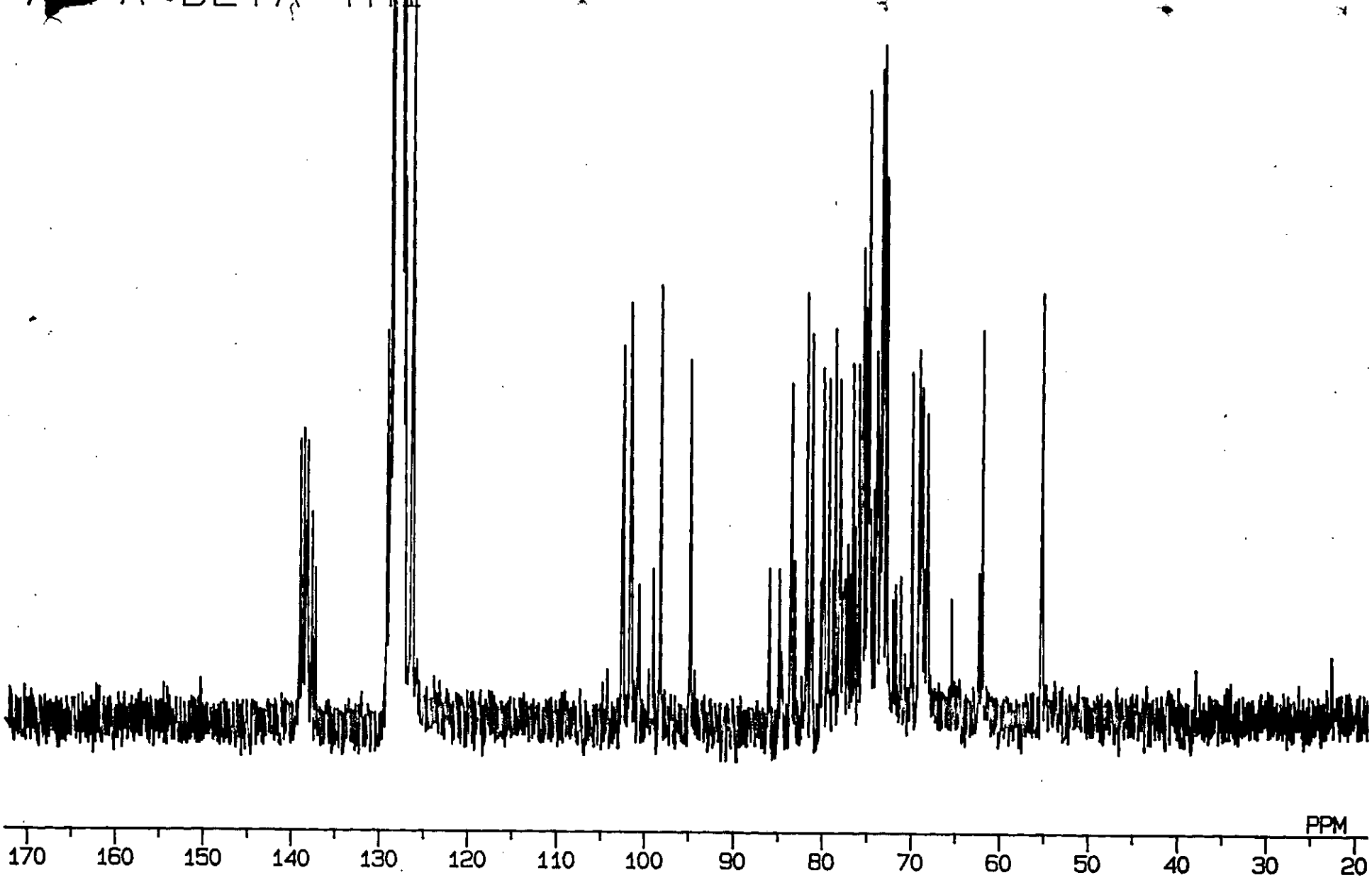


Figure 4.18. ^{13}C n.m.r. spectrum of Methyl *O*-(2,3,4,6-tetrabenzyl-α-D-glucopyranosyl)-(1→2)-*O*-(3,4,6-tri-benzyl-β-D-glucopyranosyl)-(1→3)-*O*-2-O-benzyl-4,6-O-benzylidene-α-D-glucopyranoside. Trisaccharide 20.

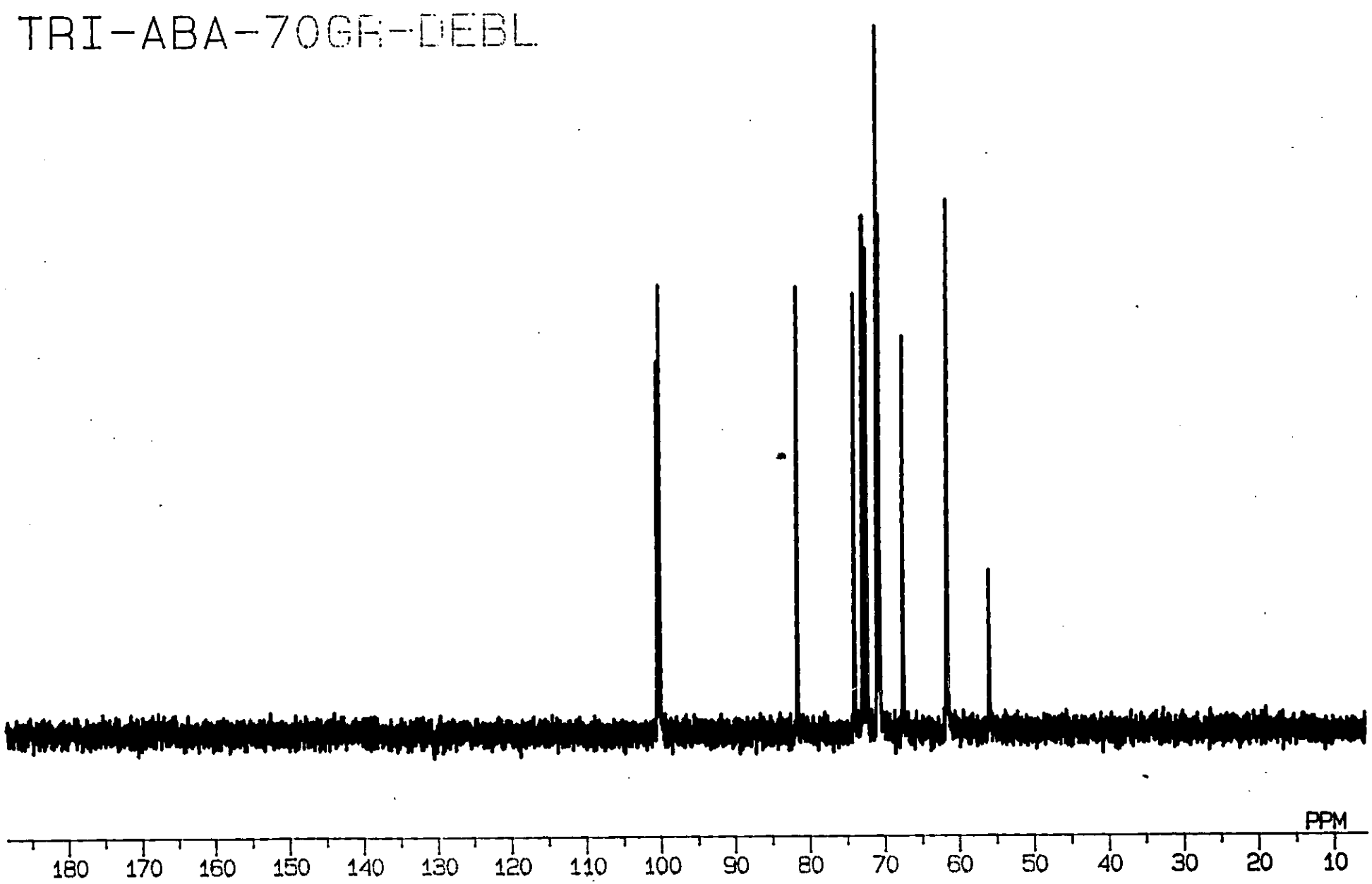


Figure 4.19. ^{13}C n.m.r. spectrum of Methyl *O*-α-D-glucopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranoside. Trisaccharide 21

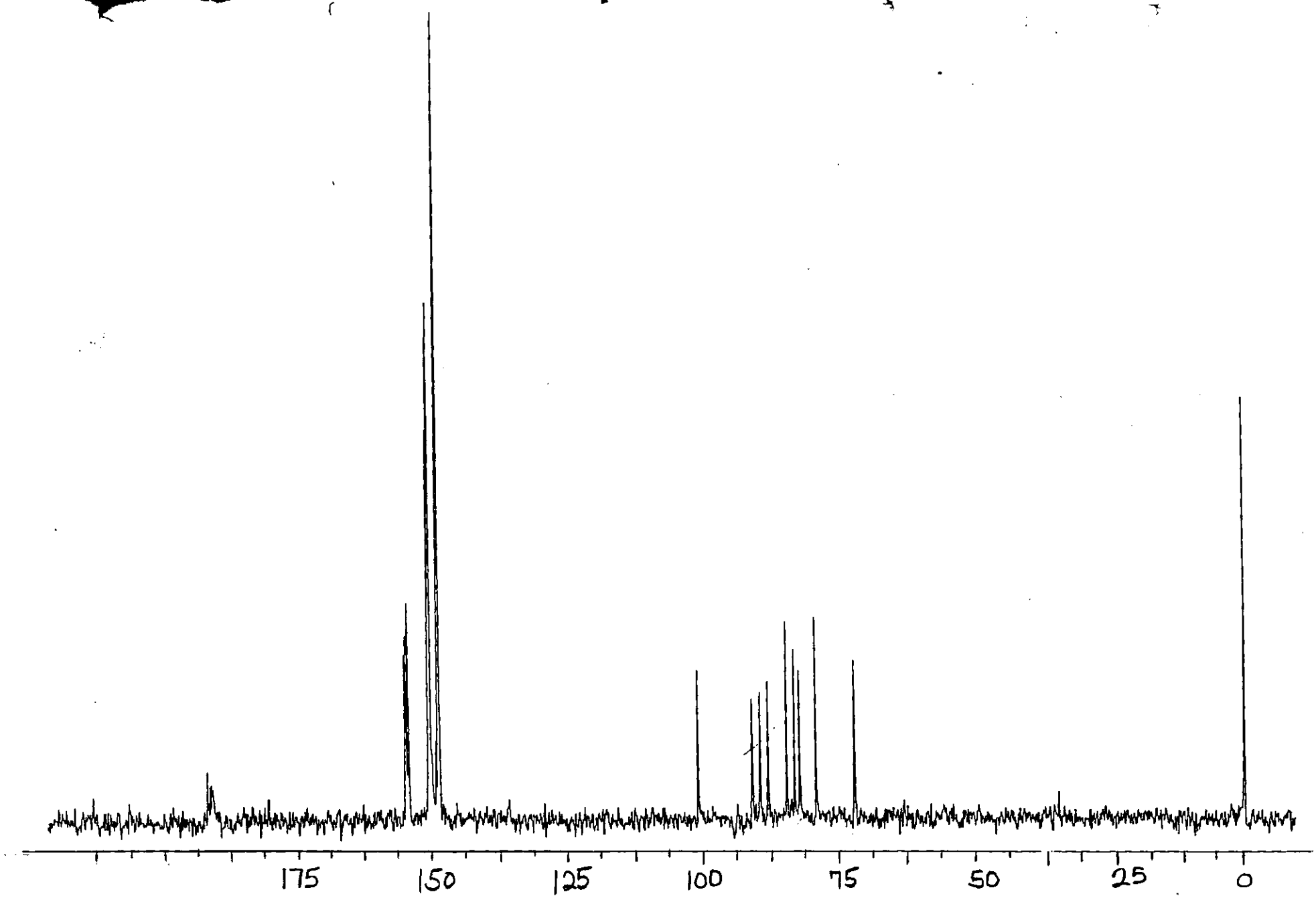


Figure 4.20. ^{13}C n.m.r. spectrum of Tetra-O-benzoyl-α-D-glucopyranosyl bromide. Compound 22.

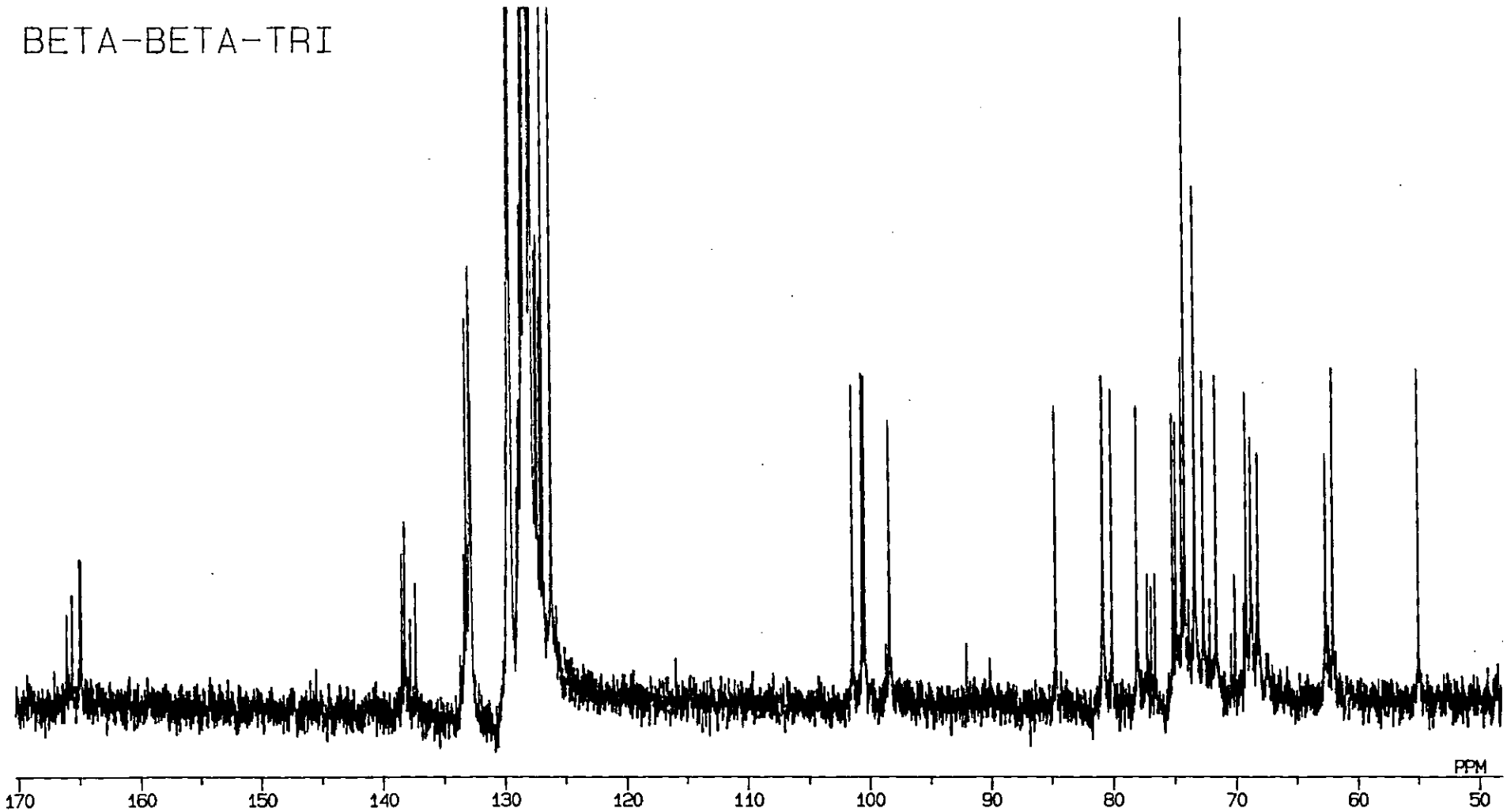


Figure 4.21. ^{13}C n.m.r. spectrum of Methyl *O*-(2,3,4,6-tetrabenzoyl-α-D-glucopyranosyl)-(1→2)-*O*-(3,4,6-tribenzyl-β-D-glucopyranosyl)-(1→3)-*O*- 2-O-benzyl-4,6-O-benzylidene-α-D-glucopyranoside. Trisaccharide 23.

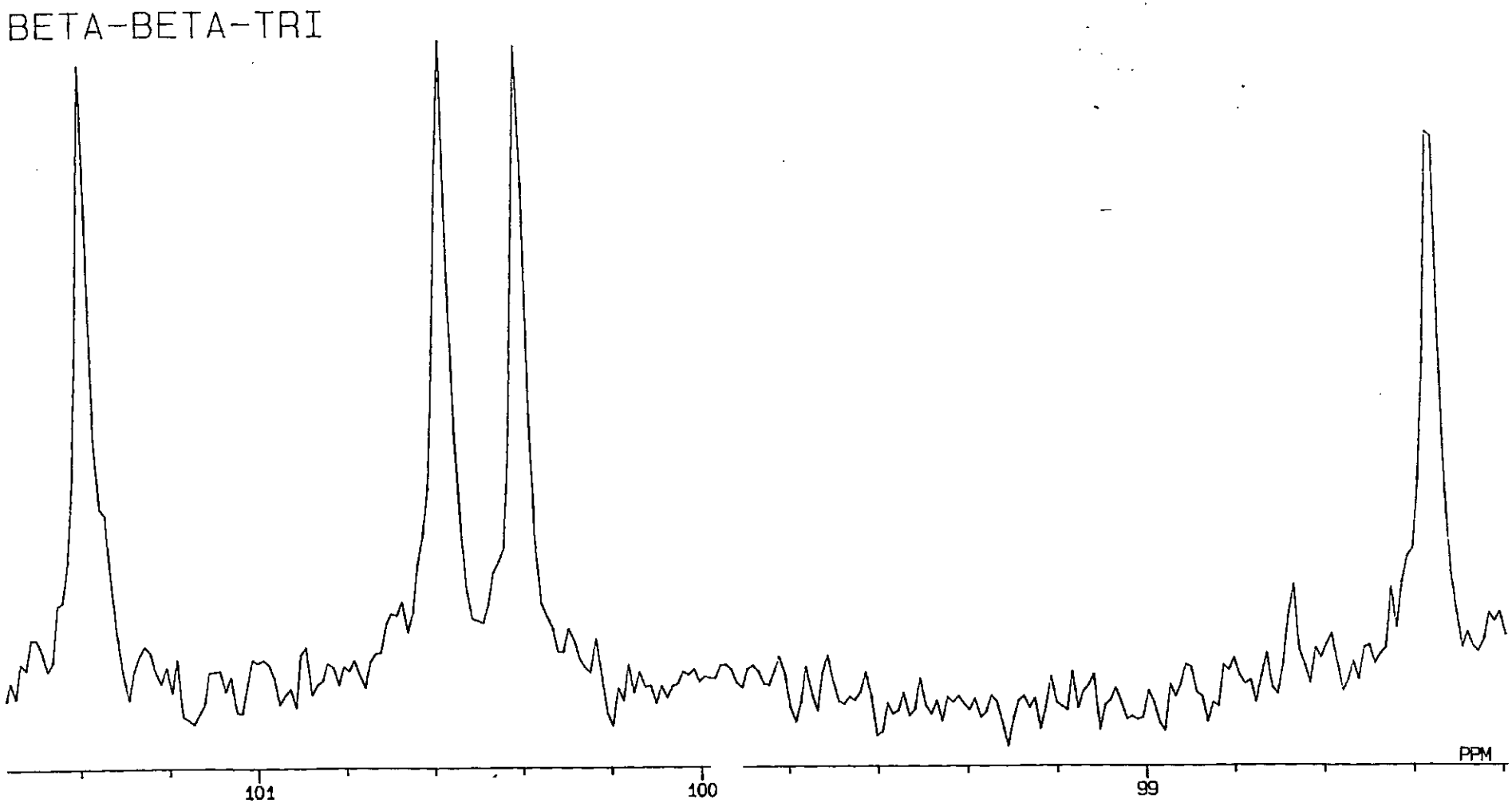


Figure 4.21a. ^{13}C n.m.r. spectrum of Methyl *O*-(2,3,4,6-tetrabenzoyl-β-D-glucopyranosyl)-(1→2)-*O*-(3,4,6-tribenzyl-β-D-glucopyranosyl)-(1→3)-*O*-2-O-benzyl-4,6-O-benzylidene-α-D-glucopyranoside. Trisaccharide 23. ANOMERIC EXPANSION.

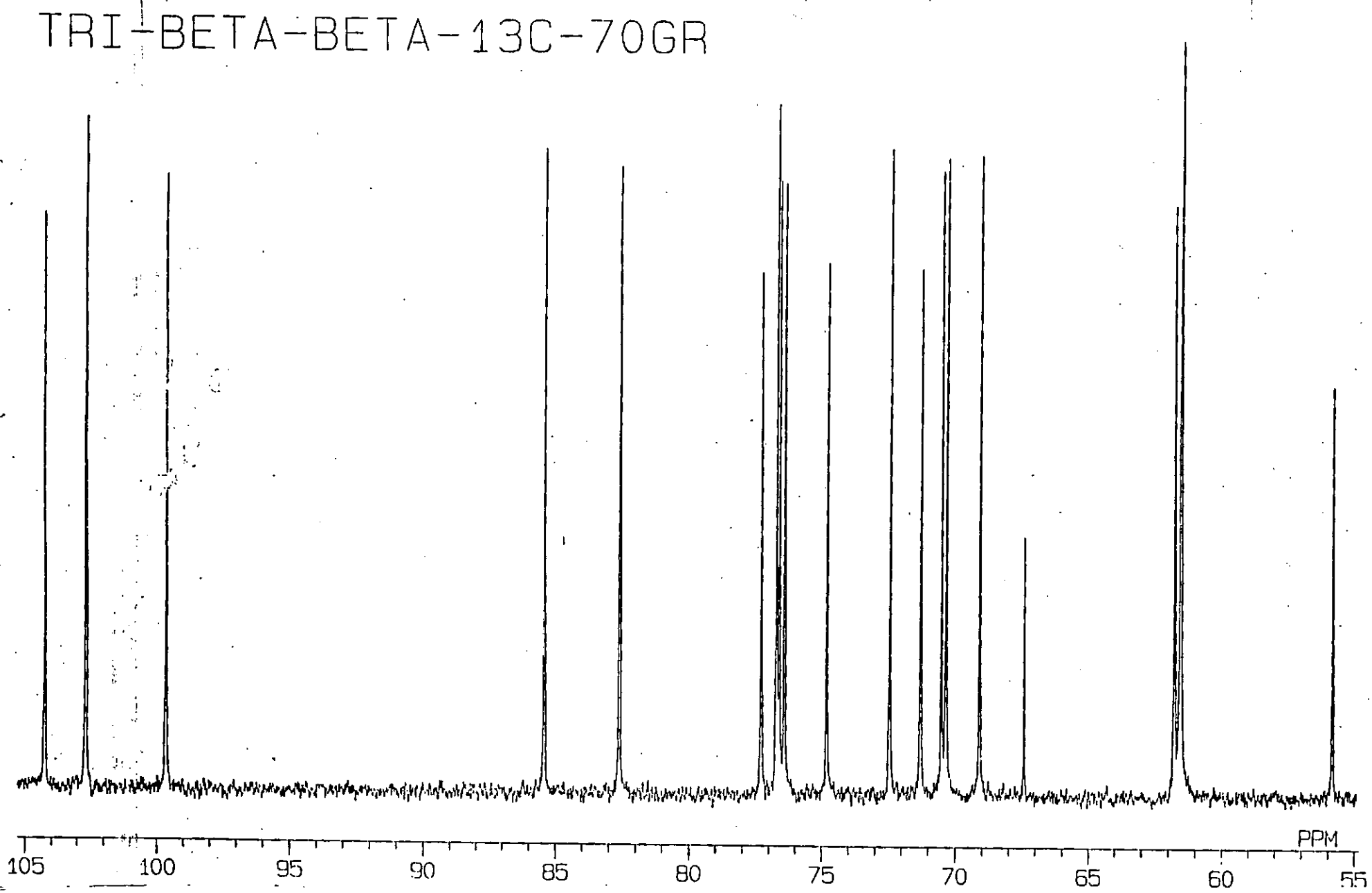


Figure 4.22. **^{13}C n.m.r. spectrum of Methyl *O*-β-D-glucopyranosyl-(1→ 2)-*O*-β-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranoside. Trisaccharide 24.**

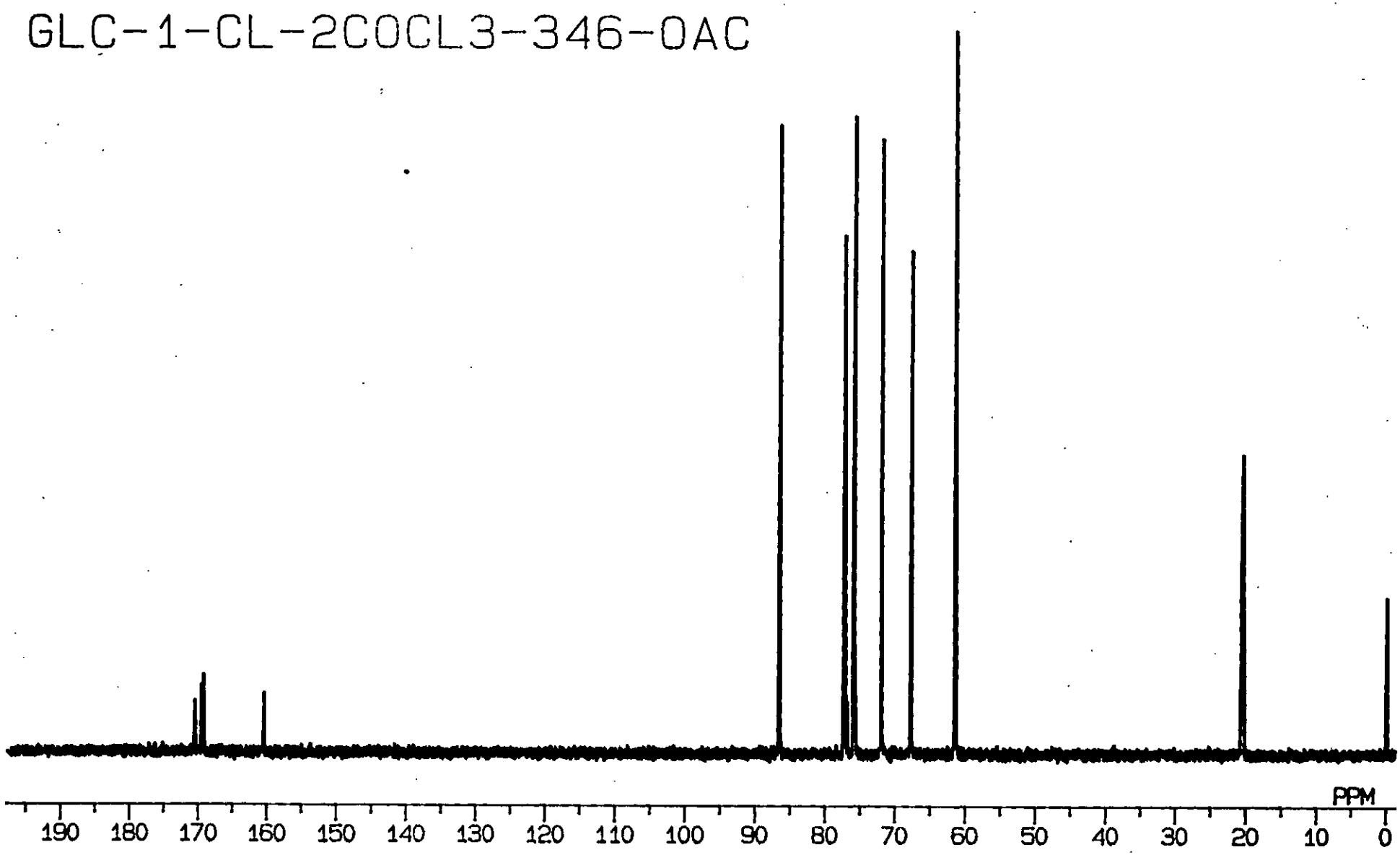


Figure 4.23. ^{13}C n.m.r. spectrum of 3,4,6-tri-O-acetyl-2-trichloroacetyl-α-D-glucopranosyl chloride. Compound 25.

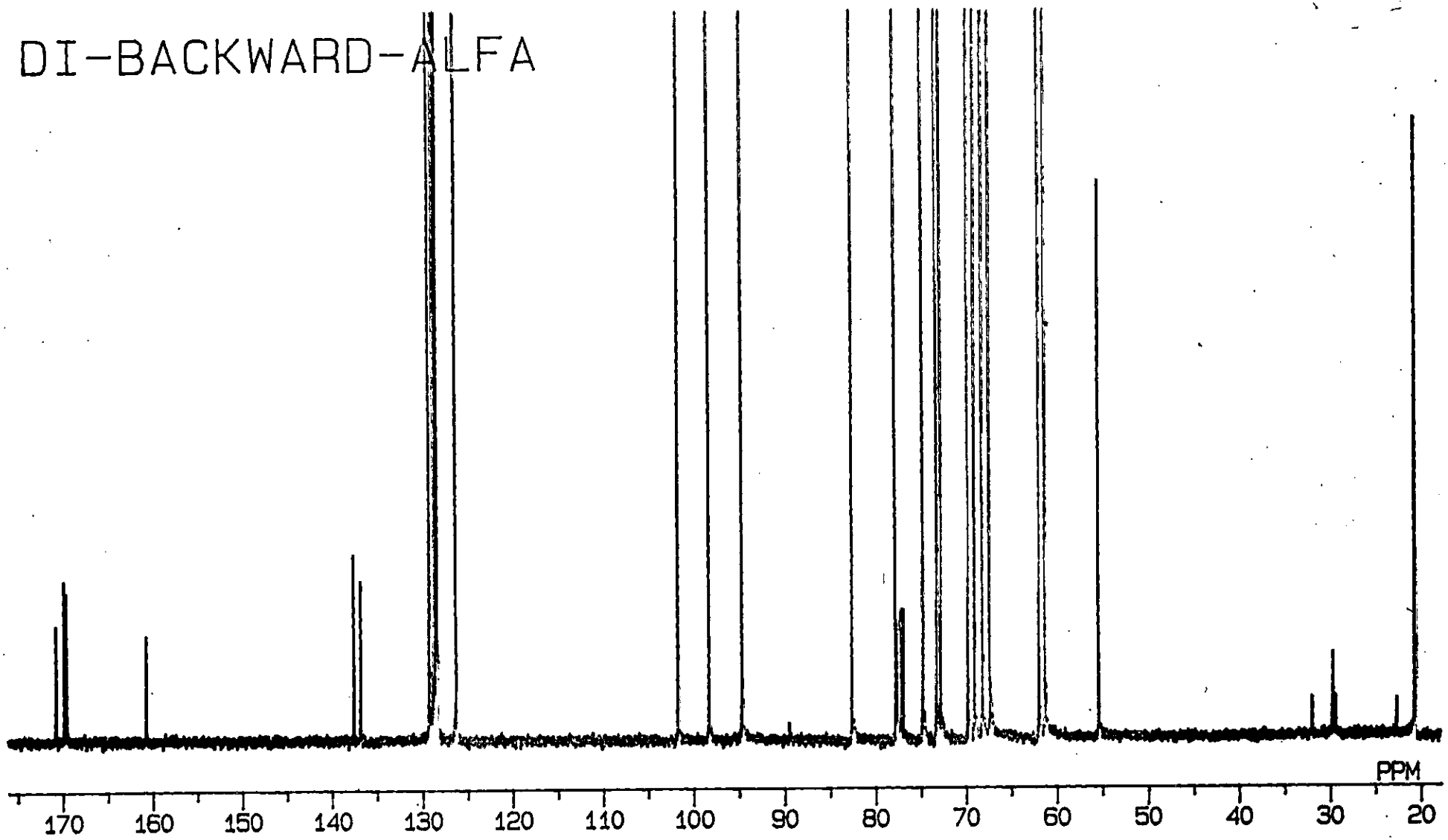


Figure 4.24. **^{13}C n.m.r. spectrum of Methyl *O*-(2-trichloroacetyl-3,4,6-tri-O-acetyl-α-D-glucopranosyl)-(1→ 3)-*O*-2-O-benzyl-4,6-benzylidene-α-D-glucopyranoside. Compound 26.**

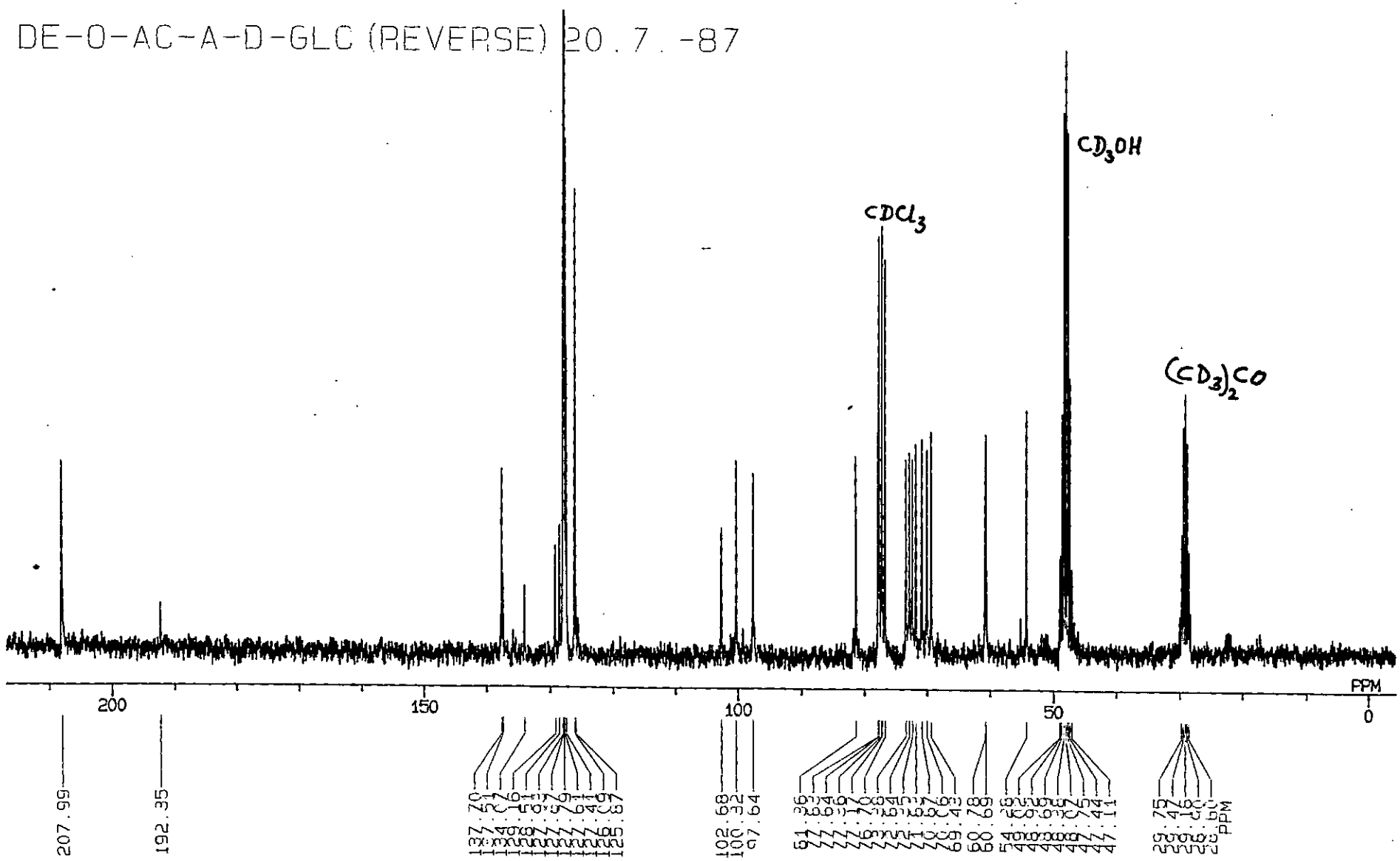


Figure 4.25. ^{13}C n.m.r. spectrum of Methyl *O*-α-D-glucopranosyl)-(1→ 3)-*O*-2-*O*-benzyl-4,6-benzylidene-α-D-glucopyranoside. Compound 27.

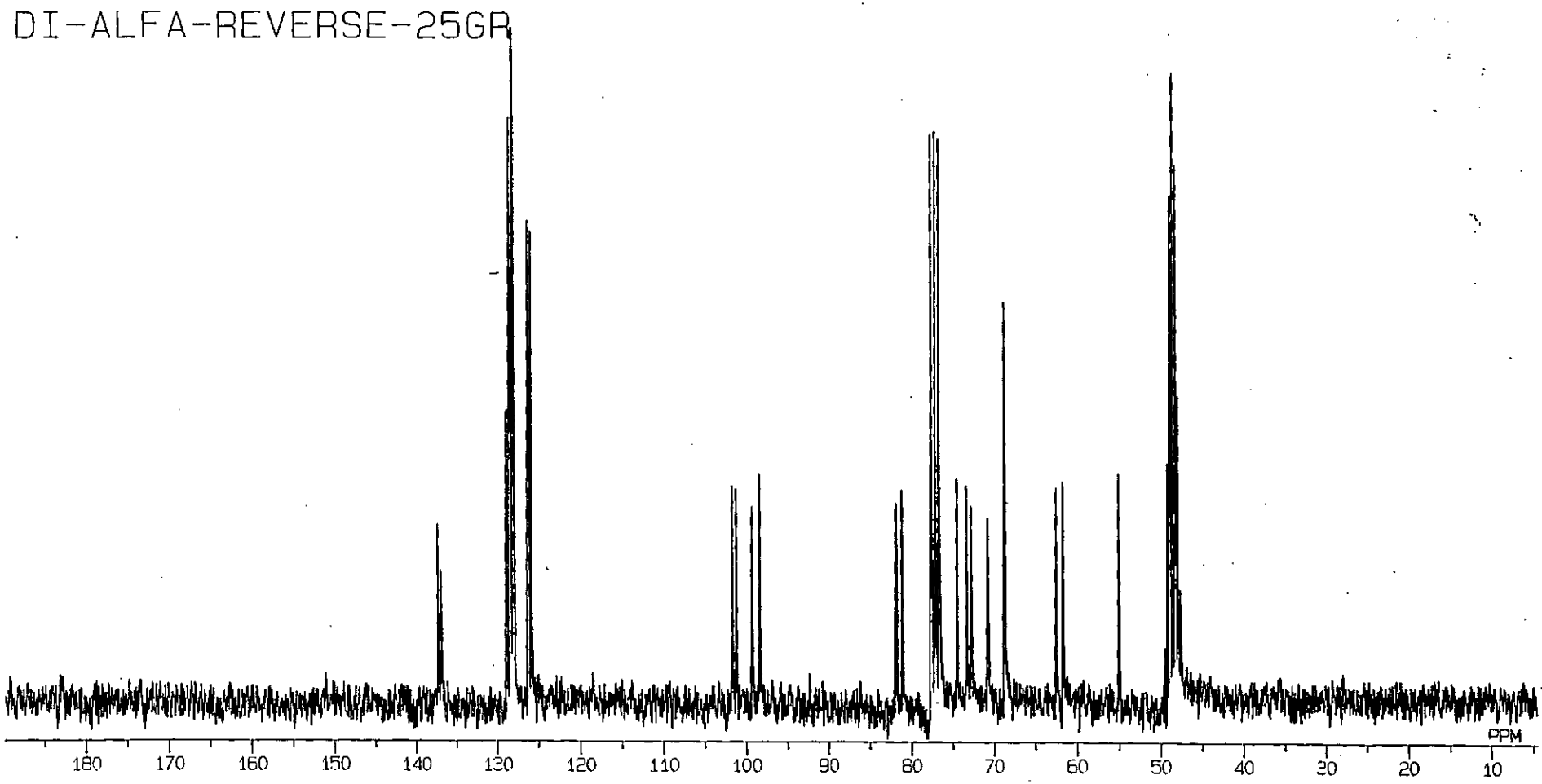


Figure 4.26. ^{13}C n.m.r. spectrum of Methyl O-(4,6-O-benzylidene-α-D-glucopyranosyl)-(1→3)-*O*-2-O-benzyl-4,6-O-benzylidene-α-D-glucopranoside. Compound **28**.

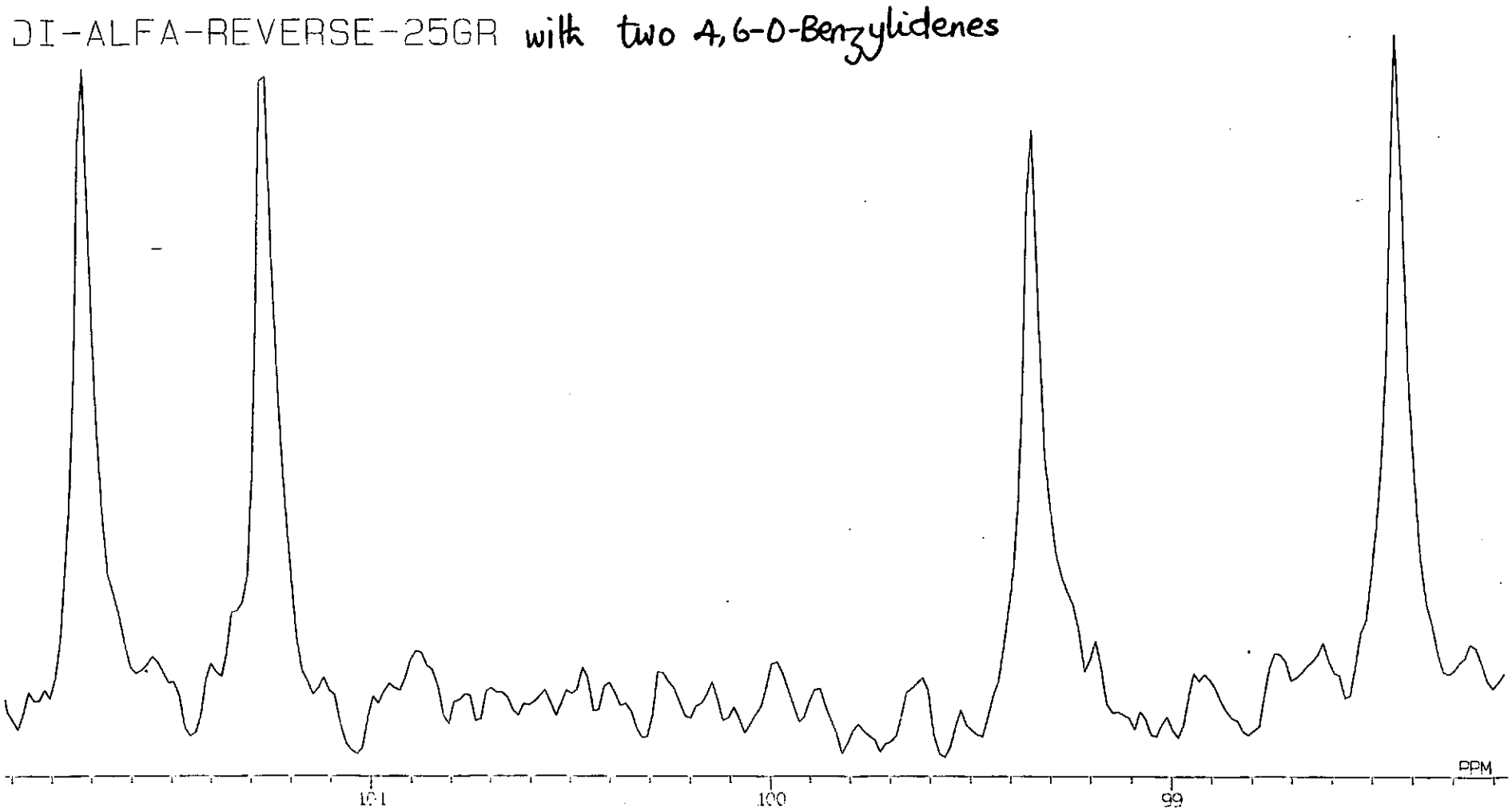


Figure 4.26a. ^{13}C n.m.r. spectrum of Methyl O-(4,6-O-benzylidene-α-D-glucopyranosyl)-(1→3)-*O*-2-O-benzyl-4,6-O-benzylidene-α-D-glucopranoside. Compound 28. ANOMERIC EXPANSION.

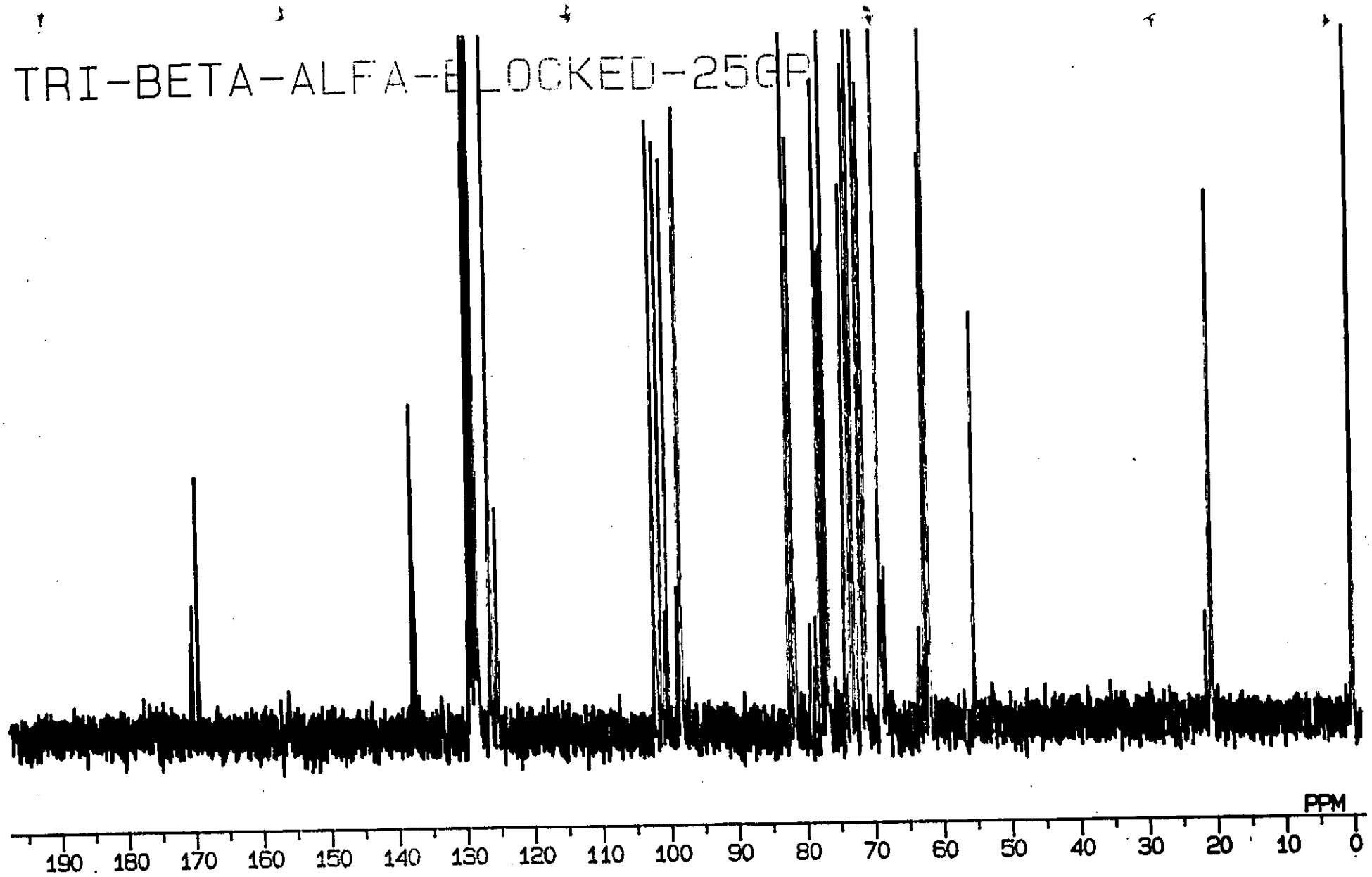


Figure 4.27. ^{13}C n.m.r. spectrum of Methyl O-(2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl)-(1→2)-O-(4,6-O-benzylidene-α-D-glucopyranosyl)-(1→ 3)-*O*-2-O-benzyl-4,6-O-benzylidene-α-D-glucopranoside. Trisaccharide 29.

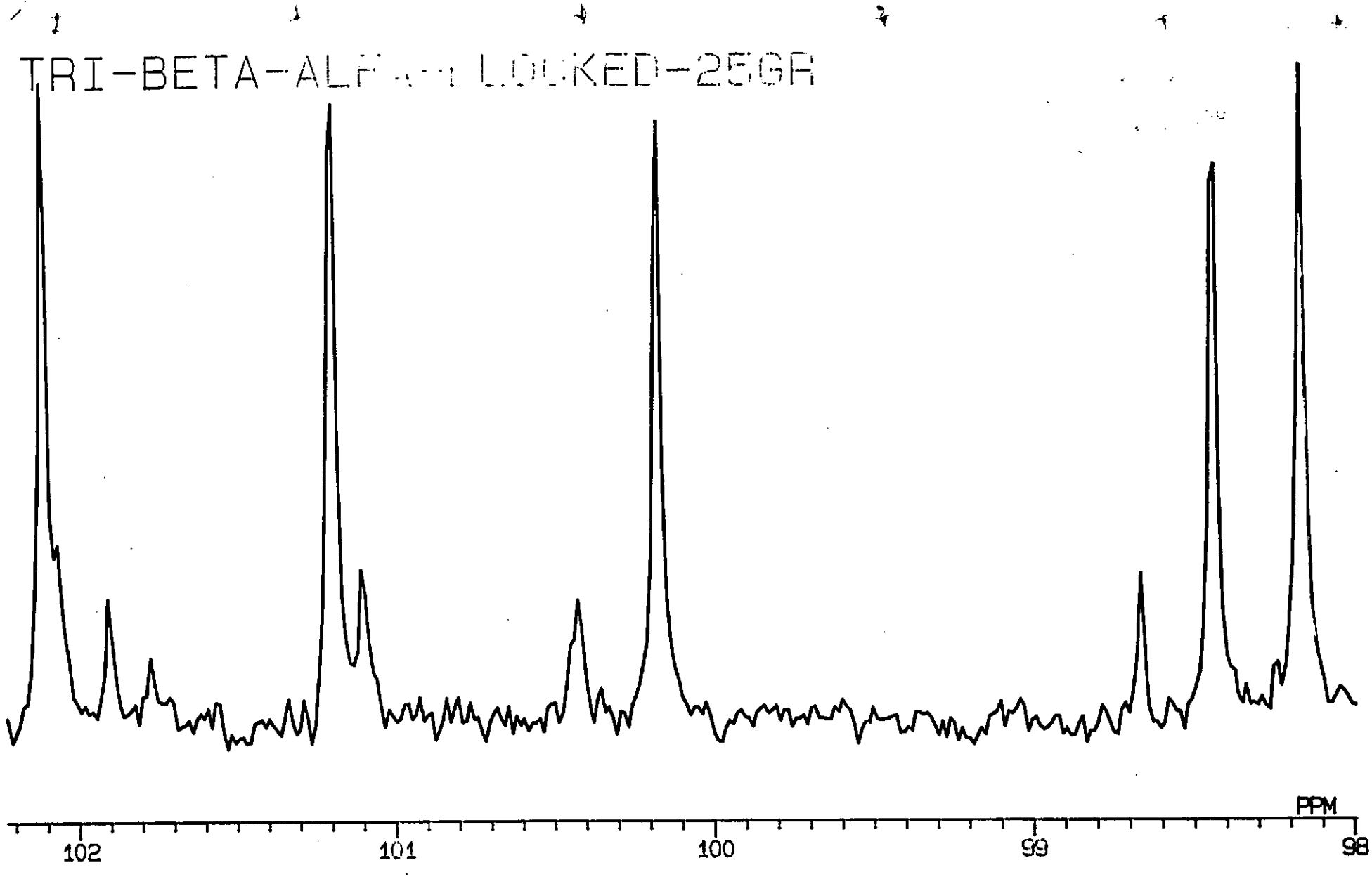


Figure 4.27a. **^{13}C n.m.r. spectrum of Methyl O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)-(1→ 2)-O-(4,6-O-benzylidene-α-D-glucopyranosyl)-(1→ 3)-*O*-2-O-benzyl-4,6-O-benzylidene-α-D-glucopranoside. Trisaccharide 29. ANOMERIC EXPANSION.**

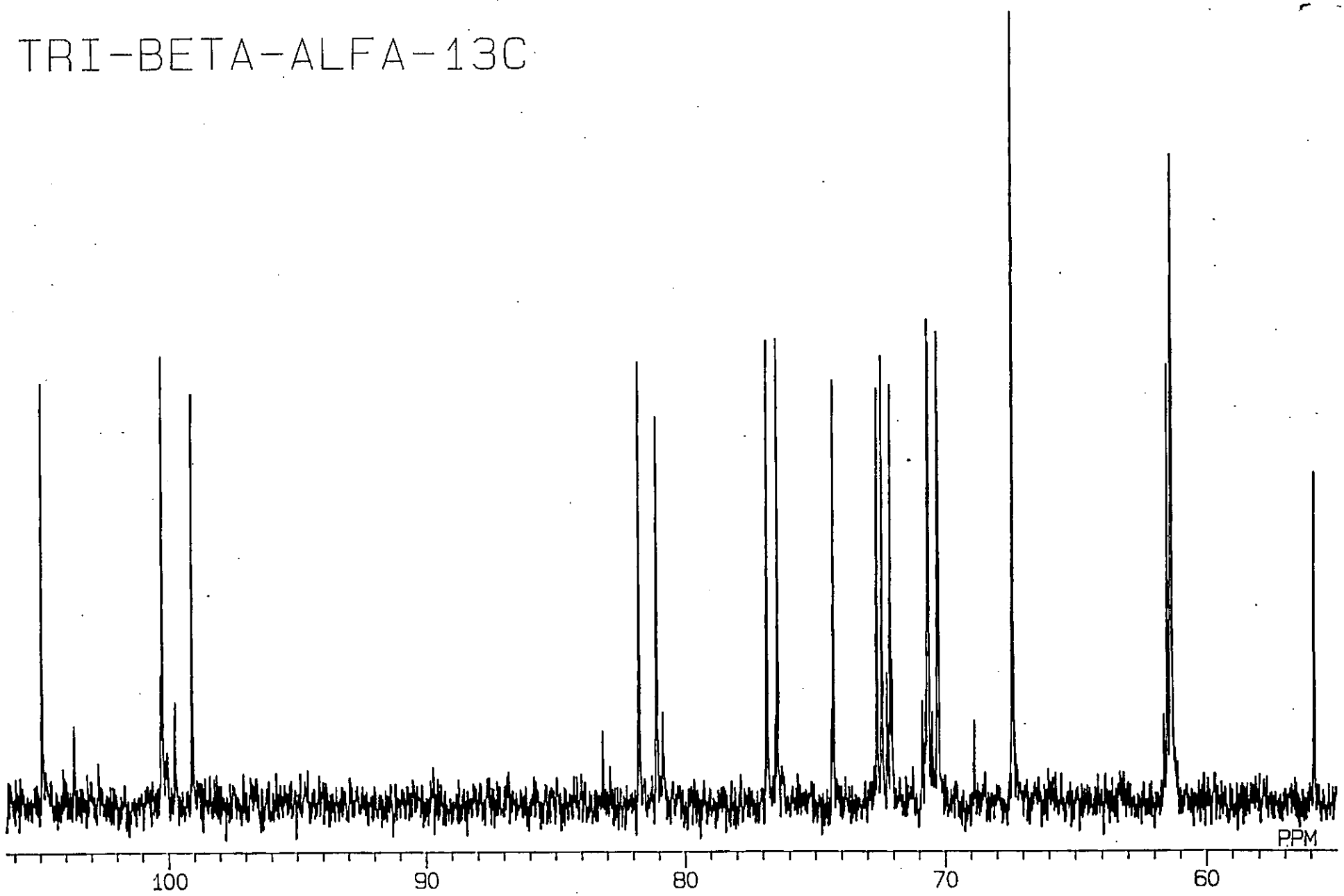


Figure 4.28. ^{13}C n.m.r. spectrum of Methyl *O*-β-D-glucopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→3)-*O*-α-D-glucopyranoside. Trisaccharide **30**.

RESULTS AND DISCUSSION

Chapter 5

* All the ^{1}H and ^{13}C n.m.r. and mass spectra of the bacterial polysaccharides are attached as Index II after page 216 at the end of "Results and Discussion" involving the structural studies of these polysaccharides.

5.1. *Brucella abortus* biotype 1, strains 7 and Mustapha.

The sterile saline-harvested bacteria killed and dried with acetone[241] gave an average yield of 1.7g of the acetone-dried cells from 10g of harvested wet cells. Extraction of the LPS of the B.abortus biotype 1, strains 7 and Mustapha from the acetone dried cells was carried out by the hot phenol-water method[74] followed by ultracentrifugation of the concentrated dialysed aqueous and phenol phases to obtain the precipitated LPS. This afforded 2 distinct LPS : the S-type LPS that was found exclusively in the phenol layer and the R-type LPS that was found in the aqueous phase of the phenol-water extract.

Our main attention was directed to the phenol phase LPS because it had already been demonstrated[255] that it contained the main antigenic determinants of the bacterium and it was responsible for the serological cross reactions with Yersinia enterocolitica and Vibrio cholerae antigens. The crude phenol-phase LPS was obtained in an average yield of 8.5% for the two strains i.e. 1.732g for strain Mustapha and 1.698g for strain 7 from 20g of the acetone-dried cells. This average yield of crude aqueous phase LPS from the acetone dried cells is within the limits of the yields usually obtained i.e. 5 to 10% from the acetone dried cells of other bacteria especially the Enterobacteriaceae[256].

The crude aqueous-phase LPS obtained from 20g of the two strains were 2.003g and 1.979g for strains Mustapha and 7 respectively. Also, this yield also falls within the experimental limits that is usually obtained for other bacteria as described above[256].

The crude phenol-phase LPS (PPLPS) purified in batches of 100mg gave a yield of 32mg of the purified phenol-phase LPS. From the sugar analysis carried out on the LPS, it was considered free of contaminants such as RNA since ribose a sugar which is the marker for the presence of RNA was absent. Delipidation of the PPLPS (50mg) by mild acid hydrolysis of strains Mustapha and 7 each gave an aqueous solution and an oily insoluble lipid. The lipid fraction was lyophilized to yield 15mg and 17mg respectively for strains Mustapha and 7. The Biogel P-10 gel-filtration of the concentrated water soluble products and lyophilisation of the material obtained from the void-volume gave 22mg of the O-chain polysaccharide (PPPS) for strain 7 and 23mg for strain Mustapha. No peaks were found in the elution range between the void volume and the monosaccharide region indicating the absence of material charcteristic of the core oligosaccharides. The O-chain fraction was a readily soluble white powder. In order to determine the types of sugar(s) present in the PPPS of the two strains, sugar analysis was done. However during hydrolysis, on separate treatment of the polysaccharides with 2M 2,2,2-trifluoroethanoic acid (TFA) for 1hour at 120°C and 4M HCl for 4hours at 100°C, an extensive decomposition or degradation of the polysaccharide took place as evident by the black coloration of the solution obtained under the two different conditions. On the completion of the derivatisation of the sugars that were supposed to be present in the polysaccharides, and the analysis of the products on the gas liquid chromatograph (G.L.C.) as well as the combined gas chromatograph-mass spectrometer (G.C.-M.C.) as their alditol

acetates, no sugar could be identified. The sugars present in the PPPS could not be detected by the particular methods of acid hydrolysis because no peaks corresponding to those of known sugar standards were obtained from the G.L.C. The results from the G.C.-M.S. further indicated that no sugars were present as the fragmentation patterns of the peaks obtained from the mass spectra showed the absence of alditol acetates. It may be inferred from these results that the sugar residue(s) which make up the repeating unit of the polysaccharide must be acid labile. Because of the difficulty usually encountered during the hydrolysis of labile sugars, it was decided to determine the structure of the repeating unit of the PPPS of the two strains from mainly n.m.r. studies.

However, the ^{13}C n.m.r. data of the phenol phase O-polysaccharides of the two strains are recorded in Table 5.1. below and the spectra are shown in Figures. 5.1. and 5.2. of Index 1. The decoupled spectra of the strain 7 showed signals at 101.32, 77.93, 69.01, 52.93, 68.56, 17.79 and 165.60 ppm and are assigned to the carbon atoms of 1, 2-linked 4,6-dideoxy-4-acyl-α-hexopyranosyl residue according to the earlier assignment of Caroff et al[255]. The decoupled spectra of strain Mustapha showed signals at 101.43, 77.98, 69.11, 52.98, 68.53, 17.79 and 166.76 ppm and are assigned as for strain 7. Table 5.1. also showed the values of (δ^a-δ) where δ^a are the chemical shift values of the carbon atoms of methyl-α-L-rhamnopyranoside[257] and the δ are the values reported above for the PS of two strains under investigation. Also shown are the values (δ^c-δ) reported where δ^c are the chemical shift values reported for the 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl residue present in the LPS of the B.abortus 1119-3[255]. The presence of only one signal in the anomeric region from the spectra of both strains indicates that the LPS is a homopolymer.

The data of Nunez et al[258] indicate that the anomeric carbon atoms of methyl-α-hexopyranoside are shifted upfield relative to the corresponding atoms of the β-glycosides.

It will be observed from Table 5.1. that the chemical shift values for C-1 of the LPS of strain 7 and strain Mustapha are shifted upfield by about 0.5ppm relative to methyl-α-rhamnopyranoside thus suggesting that the anomeric configuration in each case is α like that of the sugar residue in the LPS of the B.abortus strain 1119-3 earlier studied.

Table 5.1.

^{13}C N.M.R. shifts of the native O-chain from the phenol-phase LPS of the B.abortus strain 7(S-7) and strain Mustapha(S-M).

	Shifts O-PS (ppm)		$(\delta^a-\delta)$ ppm			$(\delta^c-\delta)$ ppm	
Carbon atom	S-7	S-M	S-7	S-M	1119-3	S-7	S-M
C-1	101.32	101.43	+0.58	+0.47	+0.19	+0.49	+0.38
C-2	77.93	77.98	6.93	-6.98	-7.26	+0.36	+0.28
C-3	69.01	69.11	+2.29	+2.19	+1.78	+ 0.41	+ 0.41
C-4	52.93	52.93	+20.1	+20.1	+19.95	+0.22	+0.17
C-5	68.56	68.53	+0.8 4	+0.87	+0.56	+0.28	+0.31
C-6	17.79	17.79	-0.0 9	-0.09	-0.37	+0.28	+0.28
NHCHO	165.60	166.76				+0.56	-0.60

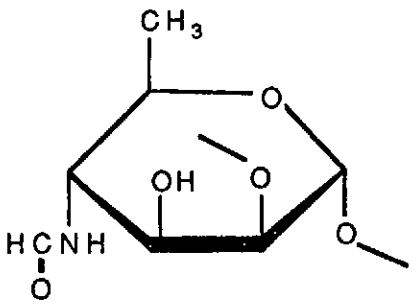
Two significant observations in the values of $(\delta^a-\delta)$ in Table 1 are a downfield shift of about 7ppm at C-2 and an upfield shift of about 20ppm at C-4 relative to the respective secondary carbinol in methyl-α-L-rhamnopyranoside. The results of the N.M.R. studies of the methyl glycosides of the 1,2-linked[240] and the 1,4-linked[240] disaccharides

and some methyl hexopyranosides[258,259] can readily be used to determine the glycosylated carbon atom and the carbon atom carrying the N-acyl substituent.

In an N.M.R. study of the 1,2-linked disaccharides[240] at 70°C, a downfield shift of 4-9 ppm at C-2 of the methyl glycoside residue relative to the carbinol carbon atom was observed. Although the monosaccharide constituents of the model sugars used in the latter study were glucose and fucose the downfield shifts of about 7ppm in the N.M.R. spectra of the strains 7 and Mustapha can be said to be indicative of a 1,2-α-linked homopolymer. In the study of the methyl glycosides of the 1,4-linked disaccharides[257], a downfield shift of 7-9ppm at C-4 of the methyl glycoside residue was observed. This suggests that the polysaccharide in the LPS of the B. abortus are not 4-linked. According to Bundle et al[260] an upfield shift of about 15-20ppm upfield of a secondary carbinol is indicative of an aminodeoxy derivative at C-2. This observation coupled with the preceeding conclusions can be used to locate the N-acyl group on C-4 of the hexopyranose unit present in the O-chain of the polysaccharide.

There are seven signals in the proton decoupled ^{13}C- n.m.r. spectra. Six of these are assigned to the ring carbon atoms and the seventh to the substituent on C-4. The signal being about 166ppm indicates this seventh carbon atom to be a carbonyl carbon atom. The substituent cannot be an acetamido group because this would give rise to an additional signal due to the methyl group of the acetamido group. This then suggests that the substituent on C-4 is a formamido group and not an acetamido group. The N.M.R. data strongly indicate the homopolymer of the LPS of strains 7 and Mustapha to be 1,2 linked-4,6-dideoxy-4-formamido-α-linked hexopyranosyl units **R.1**. The very small values in the (δ^{C}-δ) columns lead us to conclude

that the polysaccharide in the LPS of the strains 7 and Mustapha are identical with the corresponding one in the B.abortus 1119-3.



R.1

An attempt to hydrolyse the O-PS of the two strains by 4M HCl and 2M TFA resulted in a dark yellow coloration and a black precipitate indicating degradation of the polysaccharide as suspected earlier. Even after the attempted complete preparation of the expected alditol acetates was carried out, no sugar was identified on the G.L.C. This degradation can be attributed to the formamido group present on the carbon-4.

The backbone sugars analysed from the lipid A of the PPLPS from the two strains were glucose, glucosamine and galactosamine. Using a known amount of an internal standard of mannose, 1mg of the lipid A of strain Mustapha was found to contain ~5µg of glucose, ~23µg of glucosamine and ~7µg of galactosamine, while 1mg of the lipid A strain 7 gave a yield of only ~10µg of glucose and ~23µg of glucosamine. The presence of the sugars were confirmed by G.C.-M.S. However, the quantity of the sugars indicated by the results of the sugar analysis of the lipid A was rather small to account for being the only backbone sugars for the fatty acid molecules present. The various hydrolytic conditions of 2M TFA for 1hour at 120°C, 4M HCl for 4hours at 100°C and 4M HCl for 16hours at 100°C stated under

experimental were employed to find out if a higher recovery of the backbone sugars could be obtained. However essentially the same results were obtained. No worker[30] so far has been able to determine what the backbone sugar of the lipid A of the LPS of B.abortus is. However, there is reason to believe that these sugars in addition to a possible labile component that can be easily degraded form the backbone sugars because the procedure was repeated several times with essentially the same results being obtained. The difficulty of obtaining a good n.m.r.spectrum of the lipid (because of the poor solubility of the lipid in most n.m.r. solvents that are employed in n.m.r. spectroscopy) also did not aid the investigation of the backbone sugars of the precipitated lipid A. In the intact LPS, it is even more difficult because of the presence of other sugar residues present in the O-specific side chain. These make the interpretation of the spectra very difficult.

The amount of individual fatty acids present in the phenol phase LPS and the isolated lipid A from strains 7 and Mustapha was calculated from gas-liquid chromatograms using n-heptadecanoic acid as internal standard. As shown in Table 5.2. on page 183, the major fatty acid of the phenol phase LPS was n-hexadecanoic acid. n-Octadecanoic acid was also found present and the presence of these two fatty acids was confirmed by G.C.-M.S. 3-hydroxydodecanoic acid was found present in the lipid A but not in the LPS of the two strains. 3-hydroxy- tetradecanoic acid was identified in both the LPS and lipid A of the two strains. It was found present in large proportions in strain Mustapha: 34% in the LPS and 25 % in the lipid A. It is present to a much smaller extent in the LPS and lipid A of strain 7. The presence of 3-hydroxy tetradecanoic acid [$CH_3(CH_2)_{10}CHOHCH_2COOH$] which is a marker for enteric bacteria deserves some comments because it had earlier

been reported absent in the LPS of Brucella[261-263] except for a contrary report by Caroff et al[255]. In view of these earlier reports, a minimum of about 10 determinations was carried out on the LPS of each strain and this fatty acid was found present in all the determinations. Since the final proof of its presence rested on mass spectral analysis, the samples were injected on the G.C.-M.S. However, at the operating conditions of the instrument, the signal corresponding to 3-hydroxy-tetradecanoic acid on the GLC was very weak on the G.C.-M.S. Because of this poor response, it was not possible to obtain the mass spectra of the fatty acid. The identification therefore rested on the results from the GLC and a typical chromatogram is shown in figure 5.3 of Index 1. It is quite possible that the use of capillary GLC in this analysis has made this identification possible. It has been suggested that these hydroxy acids could be trimethylsilylated to improve the chromatography and sensitivity.

The aqueous phase LPS was not investigated at this time since it did not appear to be of direct immmunological importance. However, it of note that on mild acid hydrolysis the LPS gave a lipid A indistinguishable in composition from the phenol phase LPS lipid A and the PS also was degraded by mild acid hydrolysis during delipidation. It is however remarkable that the two forms of the LPS are quantitatively separated into the water and phenol phases.

Table 5.2

Characterization of the fatty acids present in the phenol phase LPS and the isolated lipid A of B.abortus strains 7 (S-7) and Mustapha (S-M).

Fatty acid methyl ester	GLC relative retention time (t_R)[a]	% of fatty acids				Mass Spectrometry	
		S-7		S-M		Base Peak	Characteristic ion
		LPS	Lipid A	LPS	Lipid A		
14:0	1.00	-	-	3	4	(b)	
16:0	1.41	64	73	44	52	74	270(M), 227(M-43)
18:0	1.78	23	18	19	12	74	298(M), 255(M-43)
3-OH-12:0	0.88	-	3	-	7	(c)	
3-OH-14:0	1.33	13	6	34	25	(c)	

[a]Relative retention times are based on tetradecanoic acid methyl ester (14:0, t_R = 1.00).

(b) Signals were very weak on G.C-M.S.

(c) Although strong signals were observed on the GLC, the response under the operating conditions of the G.C.-M.S. was rather weak.

Campylobacter coli Labet 227

The lipooligosaccharide LOS was isolated from the bacteria Campylobacter coli Labet 227 (C.coli) by the hot water-phenol method[74] as previously described for B.abortus. The oligosaccharide (OS) was prepared by treatment with aqueous acetic acid followed by the conventional work-up. The product obtained was freeze-dried and a yield of 17mg of the OS was obtained from 91mg of the LOS. The freeze-dried OS was redissolved in water (2ml) and applied on a Biogel P-10 column (90x3cm) which had been previously calibrated with a mixture of glucose, sucrose, raffinose, stachyose and dextran 10. Four peaks corresponding to four fractions were obtained. The two earlier peaks were eluted in the oligosaccharide region and the last two in the monosaccharide and salt region respectively. After freeze-drying, a yield 7mg of the higher molecular weight oligosaccharide material OS(I) was obtained while a yield of 5mg of the lower molecular weight oligosaccharide OS(II) material was obtained. From the plot of the elution volume against the logarithm of molecular weight of the known sugars in Fig 5.3a which was used to estimate the size of the eluted oligosaccharides, the OS(I) was estimated to contain between six and seven sugar units and OS(II) four sugar units.

OS(I).

The sugar analysis of this oligosaccharide indicated the presence of glucose 36%, galactose 17%, 2-acetamido-2-deoxy glucose 15%, 2-acetamido-2-deoxygalactose 7% and heptose 25%. These results are shown in Table 5.3 on page 192. The chromatogram of the sugar analysis is shown in Figure 5.4a in Index I. The m/e values corresponding to the additol acetates are reported in Table 5.4 on page 192. The molar ratio of these consituent

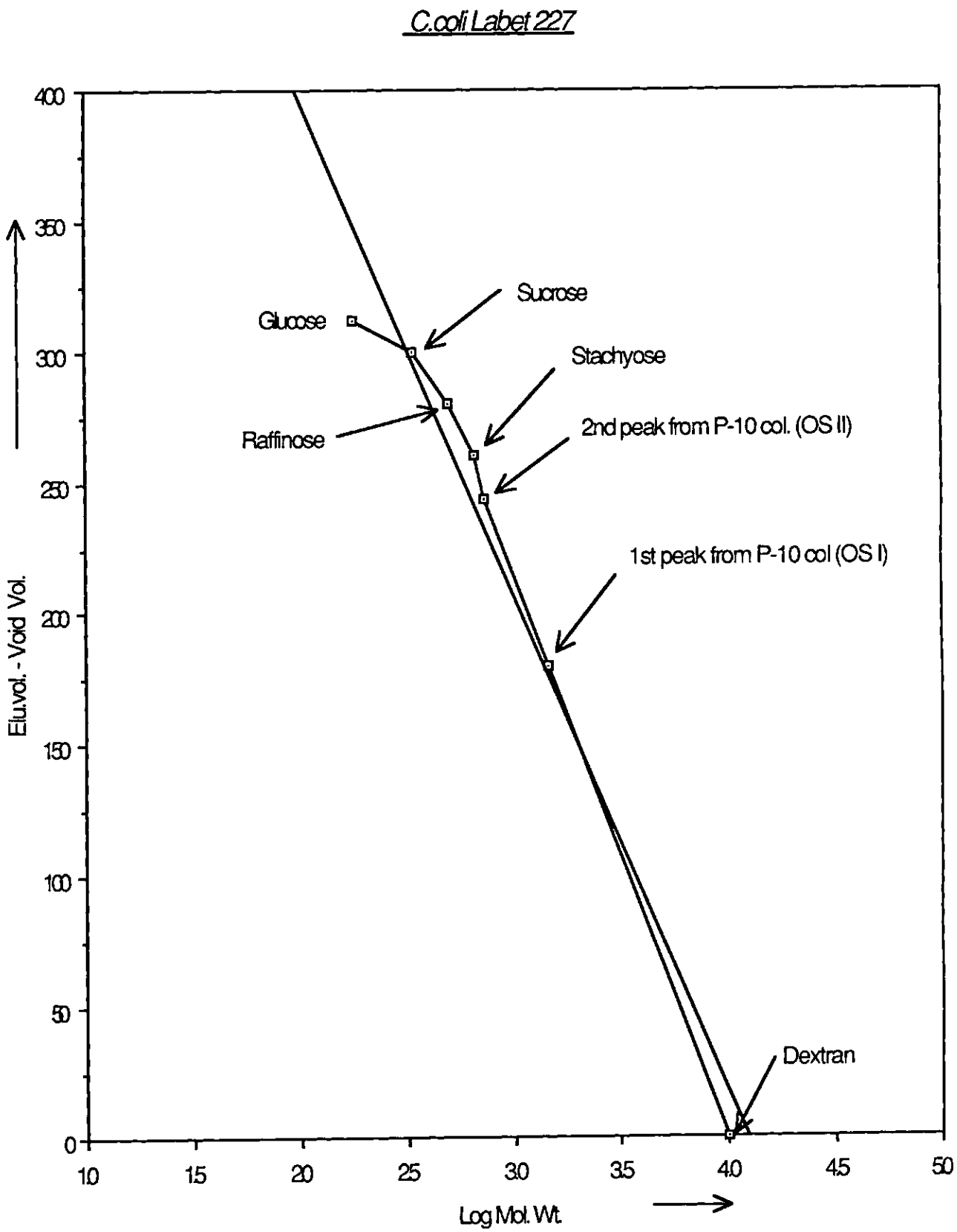


Fig. 5.3a : E. vol. vs Log. Mol.wt of C.coli OS

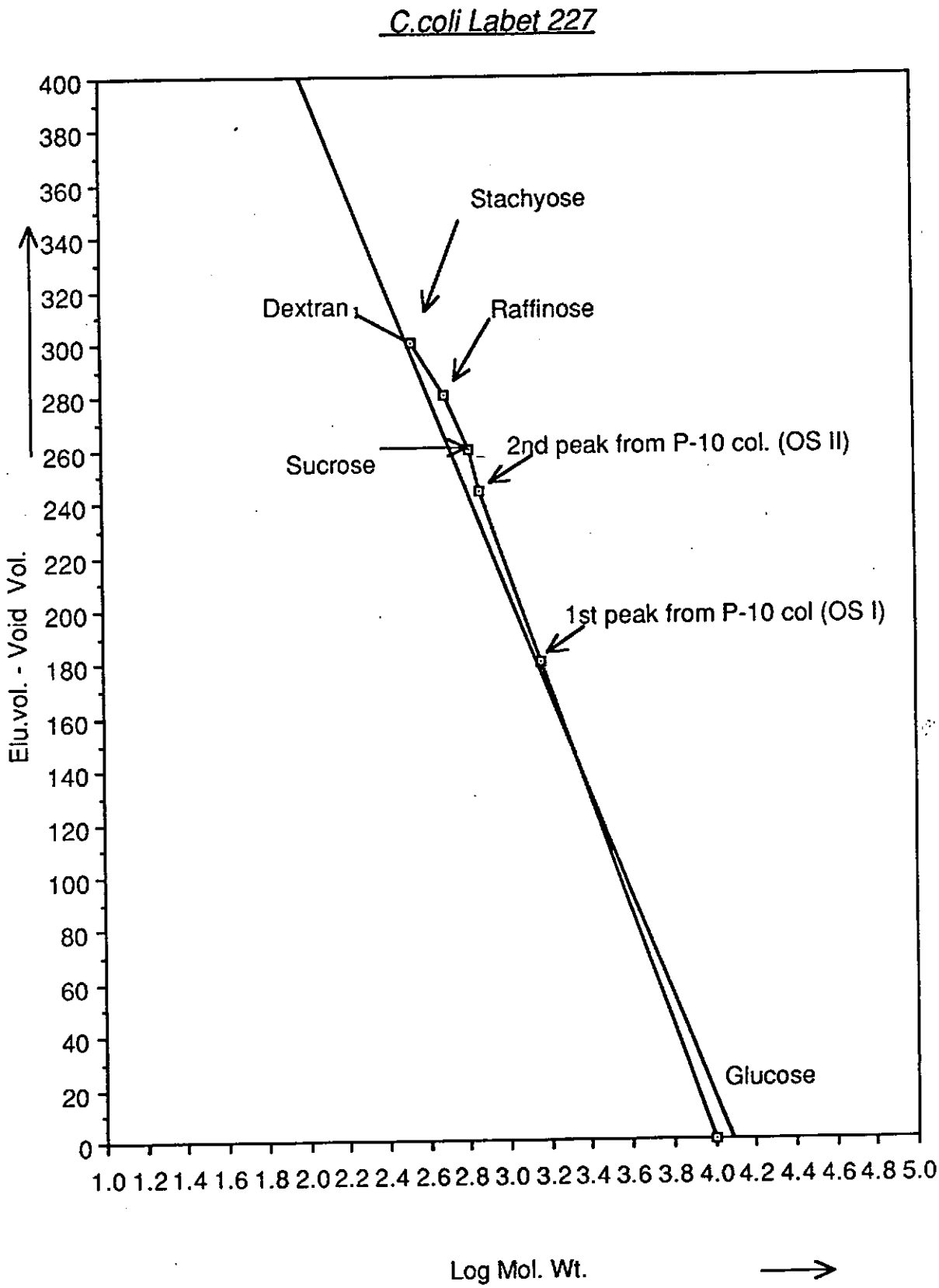


Figure 5.3a : E. vol. vs Log Mol.wt of C.coli OS

monosaccharides Glc:Gal:GlcNAc:GalNAc:Hep is 2:1:1:0.5:2 thus implying 6.5 sugar residues per mole of the oligosaccharide. A 0.5 molar ratio has been assigned to 2-acetamido-2-deoxygalactose because the results from the sugar analysis of OS(II) show that the recovery of the alditol acetate from this acetamido sugar is quantitative when compared to that of glucose. However such non-integral values for sugar residues have been reported for the LPS of some Campylobacter species[213a,213b]. The non-integral values suggest that in OS(I), 2-acetamido-2-deoxygalactose is sometimes present, the value of 0.5 being an average. Thus OS(1) may be a mixture of oligosaccharides of similar molecular sizes but differing in composition by the presence or absence of 2-acetamido-2-deoxygalactose. Thus OS(1) may be a mixture of a hexasaccharide and a heptasaccaharide. However the chromatogram of the separation on the Biogel column does not indicate a co-elution of oligosaccharides of different sizes at the elution volume of OS(I). It is however possible for the proposed hexa- and hepta- saccharides to be of similar spatial arrangement and hence co-elute. The other option is that the 2-acetamido-2-deoxygalactose is a contaminant but this seems unlikely since if it was present as a monosaccharide, it would elute separately in a region of much higher elution volume.

A plot of the logarithm of the molecular weight against the elution volume of the standard sugars stated earlier was used to estimate the size of the eluted oligosaccharides. OS(I) was eluted in the region corresponding to a molecular weight of 1415. The hexasaccharide proposed for OS(I) would have a molecular weight of about 1230 while the heptasaccharide would have molecular weight of about 1582, if a mean value of 360 is used as the 'chromatographic' molecular weight of a 2-acetamido-2-deoxyhexose[264] and the number of molecules of water eliminated is taken

into consideration. The mean of the molecular weight of the hexa- and hepta- saccharide is about 1406.

Analysis of the partially methylated alditol acetates derived from OS(I) on the G. C.-M.S. indicated the presence of five neutral sugars which were 2,3,4,6-hexitol (4%) where the numbers indicate the carbon atoms carrying the O-methyl groups, 2,3,4,6-hexitol (32%); 2,4,6-hexitol (16%); 2,3,4,6,7-heptitol (20%); 2,6,7- heptitol (8%) and 4,6,7-heptitol (20%). The relative proportions and the linkages of these sugars are as shown in Table 5.5 on page 193. These have been arranged in the order of elution from the gas chromatograph column. The characteristic m/e values for the major fragments from the partially methylated alditol acetates identified in OS(I) are shown in Table 5.6 on page 194. The mass spectra of the partially methylated heptoses are shown in figure 5.4b to 5.4d of Index I and the fragmentation patterns showing the origin of the peaks are shown as R.2, R.3 and R.4.of scheme 5.1 on pages 195 and 196.

The first peak corresponding to 2,3,4,6-hexitol is rather small and was not integrated in some of the analyses. It is therefore suggested that it is not taken into account in arriving at the constituent monosaccharides in OS(I).

The 2,3,4,6-hexitols arising from glucose and galactose residues are well separated on the SE-54 capillary column, glucose being eluted before galactose, thus a peak corresponding to a 2,3,4,6-hexitol is the derivative from a definite hexose and is not a mixture. The 2,3,4,6-hexitol has the same retention time as 2,3,4,6-glucitol and was eluted before the 2,4,6-hexitol on this column under same operating conditions. Thus the 2,3,4,6-hexitol (32%) is derived from the glucose and the 2,4,6-hexitol is derived from the galactose present in the oligosaccharide.

The sugar analysis indicates the presence of two heptoses and these are recovered as 2,3,4,6,7-heptitol (20%) and 4,6,7-heptitol (20%) in the methylation analysis. The molar ratio of the partially methylated alditol acetates recorded in quantitative yields i.e. 2,3,4,6-hexitol: 2,4,6-hexitol: 2,3,4,6,7-heptitol: 4,6,7-heptitol is 2:1:1:1. Thus the two glucose units are present as terminal sugars $Glc^{1}\rightarrow$, the galactose as a 1,3-linked sugar $-^{3}Gal^{1}\rightarrow$, one heptose as terminal $Hep^{1}\rightarrow$, and the other heptose as branch point, linked at positions 1, 2 and 3, i.e.

$$\begin{array}{l} __^{3/2}Hep^{1}\rightarrow \\ \;\;^{2/3}\,| \end{array}$$

The five neutral sugars which were recovered after methylation and acetylation in addition to the acetamido sugar(s) not recovered suggest a total of six or seven monosaccharides in OS (I). The 2-acetamido-2-deoxyglucose and 2-acetamido-2-deoxygalactose were not recovered during the methylation analysis on the g.c.-m.s. probably because of the poor response factor of the acetamido sugars, their thermal lability and the comparatively low volatility leading to very weak signals to be obtained. Another reason could be due to the heterogeniety of the LOS, the OS taken up for the methylation analysis does not have stoichiometric proportions of the 2-acetamido-2-deoxy-glucose and 2-acetamido-2-deoxy-galactose present in it. If a hexasaccharide structure is proposed for OS(I) i.e two glucose, one galactose, one 2-acetamido-2-deoxyglucose and two heptoses, then the result of methylation would imply that there are three terminal sugars and only one branch point. It is of course possible that the acetamido sugar is a branch point. This however still leaves one branch point of one terminal sugar unaccounted for. If a heptasacchride structure is present, then all the terminal sugars could then be accounted for, if the 2-acetamido sugars

are branch points. Unfortunately, the acetamido sugars were not recovered in the methylation analysis, so this matter cannot be resolved now. The 2,6,7-heptitol (8%) is a branch point i.e $\text{—}^{4/3}\text{Hep}^{1}\rightarrow$ (3/4 |) to which a terminal sugar can be glycosidically linked either at position 3 or 4, but the sugar analysis does not indicate the presence of a third heptose. Its origin cannot be satisfactorily explained except if it is said to arise from an undermethylated terminal heptose.

The ^{1}H n.m.r. spectra of OS(1) is shown in Figure 5.5 of Index I . The anomeric hydrogen resonances are found between δ4.6 and δ5.5, the ring proton resonances between δ3.4 and δ4.0, the N-acetyl and O-acetyl proton resonances between δ1.9 and δ2.1 and the deoxy proton resonances at δ1.1. The signals in the anomeric region between δ5.08 and δ5.44 are integrated as 2.64. If the upfield signal at δ4.65 which is also in anomeric region is added on to this, the integrated value would be about 2.92. Since a hexa- or heptasaccharide structure has been proposed for OS (1) it seems that the number of anomeric protons would be double this value thus giving a total of about six anomeric protons. Thus the ^{1}H n.m.r. would seem to favour the hexasaccharide structure proposed for OS (I).

The ratio of the integrations of anomeric:acetyl:deoxy protons is 2.92: 5.59: 1.00. Since the numbers anomeric protons has been equated to six, then there would be eleven acetyl, protons and two deoxy protons. The eleven acetyl protons are present in N-acetyl and O-acetyl groups. Usually the resonances about δ2.0 arise from N-acetyl protons. The signal at δ2.01 are integrated to arise from 4.25 protons which can be assigned to N-acetyl protons thus suggestive of an average of 1.5 acetamido sugars in OS (1) as found in sugar analysis. It should be pointed out that is is not possible to entirely distinguish the signals corresponding to the N-acetyl protons

from those corresponding to O-acetyl protons because of the possibility of the superposition of the signals from the two sets of protons. However, the large excess from the eleven protons compared to the three or six N-acetyl protons which are expected from one or two acetamido sugars shows that some of the neutral sugar residues in the native oligosaccharide are acetylated.

The two deoxy protons which resonate at δ 1.1 are probably present in some labile side chains present on a constituent monosaccharide since no deoxy sugars were identified and there is no signal in the ^{1}H n.m.r. corresponding to a 3,6-dideoxy sugar.

OS(II)

The sugar analysis on this oligosaccharide indicated the presence of glucose 52%, 2-acetamido-2-deoxygalactose 44%, and heptose 4% as shown in Table 5.3. on page 192. The chromatogram of the sugar analysis of OS II is shown in Figure 5.6a of Index I. The heptose being present in such minor quantities can be ignored. This then means that the glucose and the 2-acetamido-2-deoxyglalactose are present in equimolar amounts.

The molecular weight of OS(II) as estimated from Biogel chromatography was 750. This value when analysed as done for OS(I) is in fairly good agreement with an oligosaccharide containing two glucose and two 2-acetamido-2-deoxygalactose units, thus suggesting a 'chromatographic' molecular weight of about 220 for each acetamido sugar[264]. OS(II) was eluted before stachyose on the Biogel column. This confirms that the proposed tetrasaccharide structure for OS(II) is of higher molecular weight than a tetrasaccharide composed of neutral sugars, the difference in molecular weight value arising from the higher value for the acetamido sugars.

The ^{1}H n.m.r. spectra of OS(II) is shown in figure 5.6 of Index 1. The signals from the anomeric protons were at δ 5.23, δ 4.65 and δ 4.55. The integral of the signal at δ 5.23 and the sum of the integrals at δ 4.65 and δ 4.55 were in the ratio of 1:1.14. This confirms the conclusion arrived at from the results of the sugar analysis of OS(II) that the two constituent monosaccharides namely glucose and 2-acetamido-2-deoxy-galactose are present in equimolar quantities. Since it is estimated that OS(II) is a tetrasaccharide, then the integral of 2.14 in the anomeric region should correspond to four protons. Thus the signal at δ 5.23 corresponds to two protons, while the signals at δ 4.65 and δ 4.55 each correspond to one proton.

The ratio of the integration of the anomeric:acetyl:deoxy protons is 2.14:8.27:0.33. Since the integral of 2.14 in the anomeric region has been equated to four protons, then OS(II) should contain a total of sixteen protons arising from N-acetyl and O-acetyl groups and resonating between δ 1.9 and δ 2.1 as well as 0.7 proton in the deoxy region of OS (II). Six of these protons are present in the two units of 2-acetamido-2-deoxygalactose while the remaining ten protons would arise from O-acetyl groups. Thus some of the neutral sugar residues in OS(II) are acetylated as in OS(I).

The presence of two oligosaccharides in the core of this microorganism can be interpreted in two ways. One is that there is only a single core oligosaccharide consisting of OS(I) and OS(II) adding up to a total of ten or eleven sugar units depending on whether OS(I) is a hexasaccharide or a heptasaccharide. This is in line with the usual number of the sugar residues which varies from 9 to 11 that are present in the core oligosaccharide of the many lipopolysaccharides that has been investigated. This proposal however suggests that there is an acid-labile sugar which is

This proposal however suggests that there is an acid-labile sugar which is responsible for the cleavage of the oligosaccharide during delipidation. Such a sugar might actually be present and the alditol acetate formed during sugar analysis might be volatile thus not making its detection possible on the GLC. However the ^{1}H n.m.r. spectra of both OS (I) and OS (II) do not indicate the presence of such a sugar. Both the OS(I) and the OS(II) add up to between ten to eleven sugar residues and probably represents the the core oligosacccharide as elaborated by this microorganism.

Another proposal is that the core is heterogeneous and two separate lipooligosaccharides are elaborated by the microorganism. The heterogeneity proposed for OS(I) and the non-integral values of sugars e.g 0.5 unit of 2-acetamido-2-deoxygalactose in OS(I) as well as non-integral values of protons e.g. 0.7 proton in the deoxy region seem to be in favour of this model.

On comparing these results with those previously reported for the LPS from C.coli and C.jejuni[213a,213b], it is observed that the sugars identified in the oligosaccharide are the same types as in those reports and this is one of the very few strains reported to contain both 2-acetamido- 2-deoxyglucose and 2-acetamido-2-deoxygalactose. This further confirms that the 2-acetamido- 2-deoxyglucose and 2-acetamido-2-deoxygalactose are present in the LPS of some C.coli strains.

The application of the ^{1}H n.m.r.spectroscopy has been useful in the clarification of the form of the amino sugars present in the oligosaccharide. The signals indicating the methyl protons on the acetyl groups ~(δ 2.0) confirm that these amino sugars are actually acetamido sugars.

TABLE 5.3

Sugars	Percentage Composition	
	OS (I)	OS (II)
Glucose	36	52
Galactose	17	-
2-acetamido-2-deoxyglucose	15	-
2-acetamido-2-deoxygalactose	7	44
Heptose	25	4

TABLE 5.4
m/e values for the major fragments from the alditol acetates identified in OS(I) and OS(II).

Alditol acetate	R_T	m/e(%)
Glucose	1.00	43(100),85(4), 115(12), 139(7), 145(7),187(7), 217(4), 259(3), 289(4).
Galactose	1.02	43(100), 85(4), 115(12),139(8), 145(5), 187(8), 217(5), 259(5). 289(2).
2-acetamido-2-deoxyglucose	1.43	43(100), 84(44), 102(18), 139(10), 144(15), 259(6), 318(9), 360(1).
2-acetamido-2-deoxygalactose	1.51	43(100), 84(30), 102(14), 139(9), 144(10), 259(3), 318(3), 360(2).
Heptose	1.61	43(100), 85(3), 115(10), 139(7), 157(6), 187(7), 217(3), 259(5), 289(4), 331(3), 361(4), 433(2).

R_T = Relative to Glucitol Hexaacetate.

TABLE 5.5.

The relative proportions and the linkages of the partially methylated alditol acetates in OS(1)

Sugar derivative[a]	%	Remarks	Linkage positions
2,3,4,6-Hexitol	4	Terminal hexose linked at position 1	$Hex^{1}\rightarrow$
2,3,4,6-Hexitol	32	Terminal hexose linked at position 1	$Glc^{1}\rightarrow$
2,4,6-Hexitol	16	3-linked hexose	$\rightarrow{}^{3}Gal^{1}\rightarrow$
2,3,4,6,7-Heptitol	20	Terminal heptose linked at position 1	$Hep^{1}\rightarrow$
2,6,7-Heptitol	8	Heptose linked at positions 3 and 4	$\rightarrow{}^{4/3}Hep^{1}\rightarrow$ 3/4 |
4,6,7-Heptitol	20	Heptose linked at positions 2 and 3	$\rightarrow{}^{3/2}Hep^{1}\rightarrow$ 2/3 |

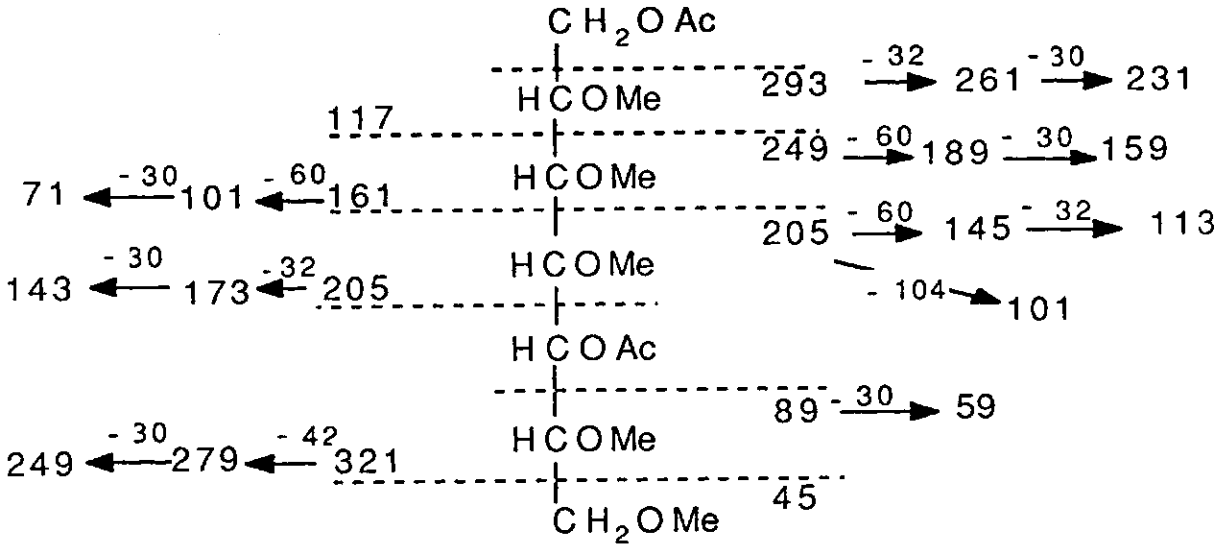
a. The numbers indicate the positions of the methyl groups i.e. 2,3,4,6-hexitol is 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylhexitol.

Table 5.6 m/e values for the major fragments from the partially methylated alditol acetates identified in OS(I).

Sugar derivative	RT	m/e
2,3,4,6-Hexitol	1.00	43(100), 45(37), 71(14), 87(20), 101(69) 117(33), 129(35), 101(32), 145(25), 161(24), 205(5), 233(9).
2,4,6-Hexitol	1.28	43(100), 45(32) 117(63), 129(53), 101(32), 161(20), 87(17), 201(9), 233(9).
2,3,4,6,7-Heptitol	1.55	43(100), 89(44),101(70),161(15),189(2), 205(14), 249(4),321(1)
2,6,7-Heptitol	2.23	43(100), 45(15), 59(2), 89(17), 117(58), 215(3), 303(2), 349(1) 377(9).
4,6,7-Heptitol	2.44	43(100), 45(18), 59(17), 85(20), 89(23), 99(19), 101(12), 127(19), 145(4), 159(4), 169(13), 201(7), 215(3), 243(2), 261(13), 303(2), 345(1), 377(8).

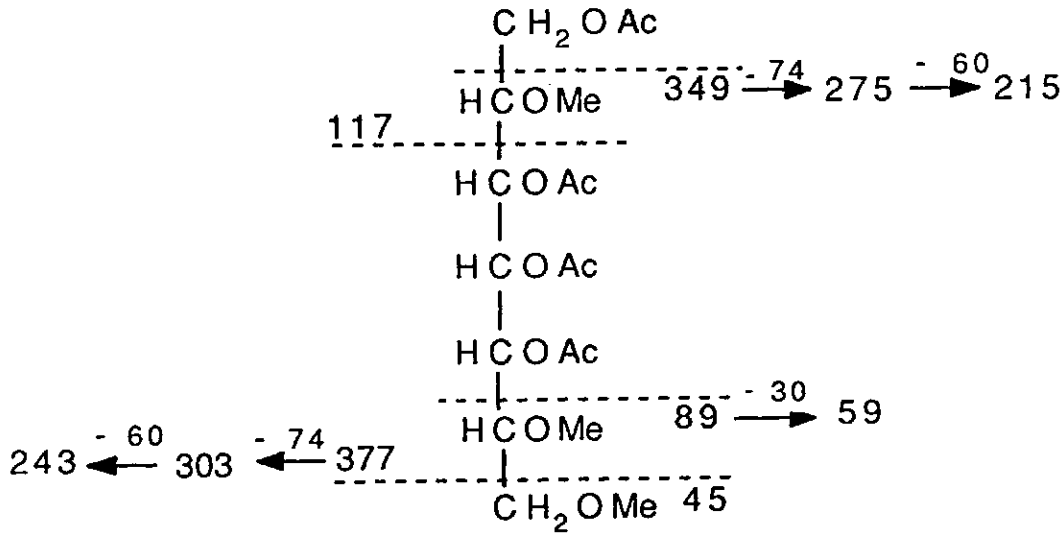
R_T = relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylglucitol on an HP-SE 54 column.

Scheme 5.1 The fragmentation pattern of the partially methylated heptitol acetates showing the origin of the peaks in the mass spectra .



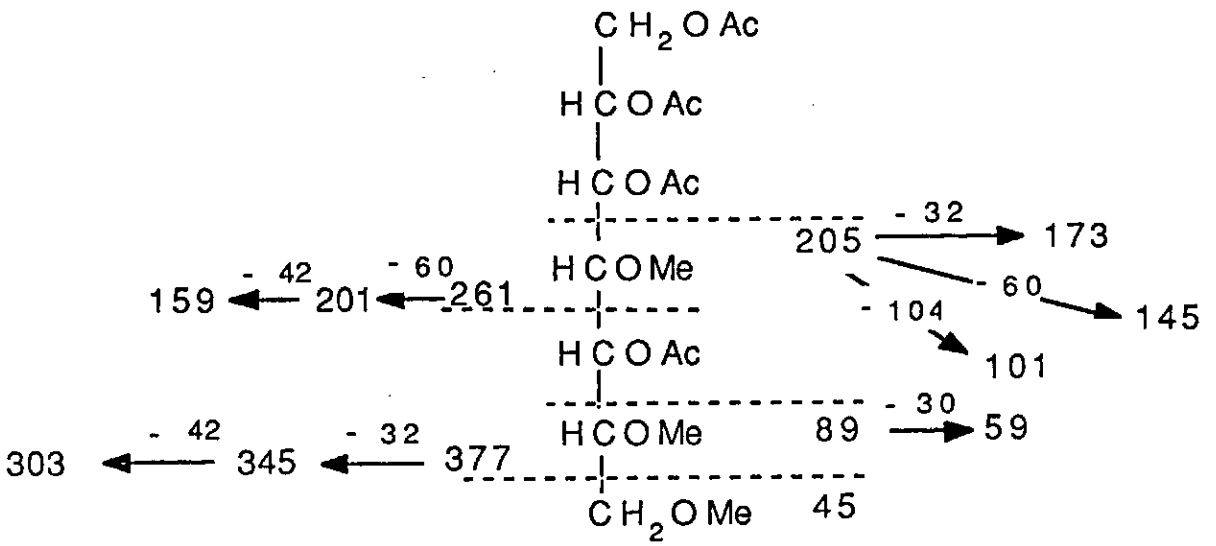
1,5-di-O-acetyl-2,3,4,6,7-penta-O-methyl heptitol.

R.2



1,3,4,5-tetra-O-acetyl-2,6,7-tri-O-methyl heptitol.

R.3



1,2,3,5-tetra-O-acetyl-4,6,7-tri-O-methyl heptitol.

R. 4

Fatty acid composition of the Lipid A of *C.coli* Labet 227.

The fatty acids in the Lipid A of this bacteria were also determined by methanolysis.The acids identified were n-tetradecanoic acid 14:0 ,3-hydroxy n-tetradecanoic acid 3-OH 14:0, n-hexadecanoic acid 16:0 and octadecanoic acid 18:0 and their relative proportions are as shown in Table 5.7 on the following page. The results obtained from the fatty acid analysis indicate that the Lipid A is composed of predominantly of 3-hydroxy n-tetradecanoic acid and n-hexadecanoic acid in almost equal proportions having a molar ratio (1:1). The same type of fatty acids have been found present in the LPS of most other enteric bacteria and the presence of the 3-hydroxy- tetradecanoic acid which is a marker in enteric bacteria further shows that this baceria is an enteric

type. The other fatty acids that have been identified are those commonly found in the Lipid A of other bacteria.

Table 5.7. Fatty acid composition and relative abundance in Lipid A of C.coli

Fatty acid	Mole(%)
n-tetradecanoic acid	4
3-hydroxy n-tetradecanoic acid	49
n-hexadecanoic acid	41
octadecanoic acid	6

E. coli 0149

The partially pyruvylated PS 1 and fully depyruvylated PS 2 polysaccharides were prepared from the LPS by mild acid hydrolysis with aqueous acetic acid at 100°C for 1.5 and 3hours respectively followed by conventional work-up as described under "Experimental". The average yield of the PS1 and PS2 from 100mg of the LPS was generally about 53% from both the 1.5hour and 3hour hydrolysis. After gel filtration and lyophilisation, the final yield of the purified PS was 17 to 21% of the dry weight of the LPS. The fully pyruvylated polysaccharide (PS 3) was obtained from the partial delipidation of the LPS by treatment with 0.1M sodium hydroxide at room temperature for 16 hours. The yield of the PS 3 from the LPS was 57% and the final yield of the purified PS 3 was 23% of the LPS after gel filtration and lyophilisation.

The polysaccharide PS 1 obtained from the 1.5 hour hydrolysis of the LPS gave a complicated n.m.r. spectra containing inter alia signals at δ_H 1.5 and δ_C 25.4. The intensities of these signals, relative to those given by N-acetyl groups and methyl groups of the 6-deoxyhexose residue, decreased with increasing the time of hydrolysis. The signals were almost non-existent in the ^{13}C n.m.r.as well as the ^{1}H n.m.r. of the polysaccharide obtained from the 3hour hydrolysis. The signals at δ_H 1.5 and δ_C 25.4 are typical for pyruvic acid residues of 4,6-O-carboxy-ethylidene glycopyranosides in which the methyl group occupies an equatorial position. The presence of the pyruvic acid in the LPS was further demonstrated by the procedure of Nimmich[245].

The polysaccharide obtained from the sodium hydroxide delipidation i.e. the partially delipidated polysaccharide (PS 3) tested for pyruvic acid according to the procedure of Nimmich as described earlier

confirmed the presence of pyruvic acid. When The final ethylacetate extracts of the four materials were spotted on TLC plates and the chromatogram was run with a mixture of n-butanol-pyridine-water (6:4:3) as the eluent, The PS1, PS 3, pure pyruvic acid, and the Xanthan gum samples showed identical characteristic shapes with yellow coloration on the TLC plates and had the same reddish purple colour when observed under the ultra violet light. The blank showed a faint yellow circular shape and was not visible under the u.v. light. From another TLC plate using n-butanol-ethanol-water mixture (40:11:19) as eluent the chromatogram obtained was similar to the previous one that was obatained using a mixture of n-butanol-pyridine-water (6:4:3). This test confirms the presence of pyruvic acid residues in the polysaccharide of the E. coli. The polysaccharide (PS 2) that was obtained by extending the acid hydrolysis time from 1.5hrs to 3hours was free from the pyruvic acid as evident from the absence of the signal for pyruvic acid in the n.m.r. spectrum and the negative result obtained from the Nimmich test on the PS 2. The reaction scheme is shown in figure 5.8 below.

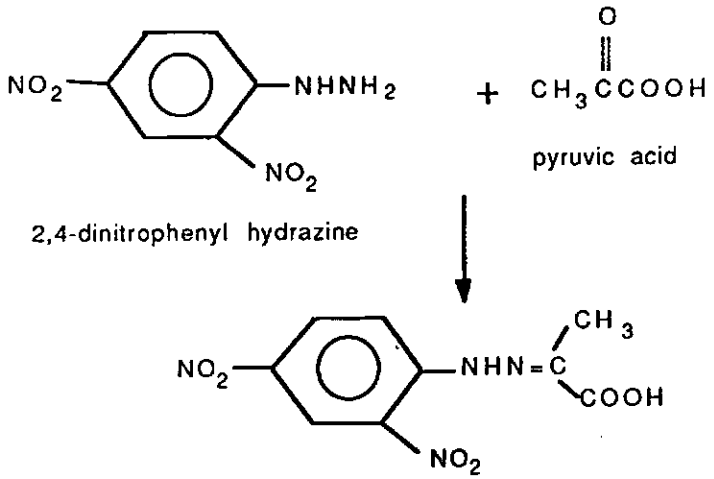


Figure 5.8. Reaction of pyruvic acid and the 2,4-dinitrophenyl hydrazine

The sugar analysis of the PS 2 and PS 3 gave L-rhamnose, glucose, galactose, a heptose and 2-amino-2-deoxy-glucose in the relative proportions shown below in Table 5.8. In this analysis, hydrolysis with acid was preceeded by solvolysis with liquid hydrogen fluoride, in order to ensure the complete cleavage of the 2-acetamido-2-deoxy-D-glucosidic linkages. The very small amounts of glucose, galactose, and the trace amount of the heptose are from the core oligosaccharide sugars which were part of the O-side chain in the LPS. These were recovered in small amounts because the size of the core is very small compared to the polysaccharides.

Table 5.8. Sugar analysis of the E.coli O149 Polysaccharides.

Sugar	Mole %
N-acetylglucosamine	50
Rhamnose	37
Glucose	9
Galactose	4
Heptose	trace

N.m.r.evidence discussed below demonstrate that the amino sugars were N-acetylated. The ^{1}H n.m.r. spectrum in figure 5.8 of Index I. of the fully depyruvylated polysaccharide (PS 2) of Index I showed, inter alia, signals for the anomeric protons at δ 4.85 (n.r., H), 4.72 ($J_{1,2}$ ~7Hz, H)and δ 4.62 ($J_{1,2}$ ~7Hz, H), for N-acetyl groups at δ 2.02 (s, 6H) and for the H-6 of L-rhamnopyranoside residues at δ 1.30 ($J_{1,2}$ ~5Hz, 3H).In fig 5.9, the corresponding signals in the ^{1}H.n.m r. spectra of the fully pyruvylated and partially delipidated polysaccharide (PS 3) occured at δ 4.82(2 H), 4.75 (H), δ 2.02 (6H), δ 1.30 (9H) and a signal for the methyl

group of the pyruvic acid residue at δ 1.50 (s, 3H). This material also showed a number of weak extra resonances in the anomeric region which on prolonged treatment, increased in intensity. The agreement between the two spectra is good except for the large signal at δ 1.30 in the spectrum of the partially delipidated LPS. The reason for this discrepancy is not understood. Fig 5.7 shows ^{1}H n. m. r. of PS 1 while Fig.5.8. shows the ^{13}C n.m.r. of PS 1. ^{13}C n.m.r. spectrum of the fully depyruvylated and delipidated polysaccharide and PS 2 showed inter alia, signals for anomeric carbons at δ103.2, 101.8 and 101.2, for carbons linked to nitrogen at δ 56.8 and 55.6, for methyl of N-acetyl groups at 23.3 and 23.2 and for C-6 of L-rhamnose residues at δ17.5. The corresponding signals in the partially delipidated LPS occurred at δ103.2, 102.2 and 101.3, 56.8, 55.6, 23.3, 23.2 and 17.5, together with signals given by the pyruvic acid residue at δ103.2 and 25.4. Figures 5.11 and 5.12 of Index 1 show the ^{13}C n.m.r. spectra of PS 2 and PS 3.

The methylation analysis of the PS 1, PS 2 and PS 3 gave the sugars listed in Table 5.9 on page 202. The results of the methylation analysis of the three polysaccharides- PS 1, PS 2 and PS 3 showed the presence of three sugar residues in each of these polysaccharides. These PS 2 from table 5.9 showed that the three partially methylated alditol acetates present are 1,3,5-tri-O-acetyl-2,4-di-O-methyl-6-deoxy-hexitol (33%) 1,4,5-tri-O-acetyl-2-acetamido-2-deoxy-2N-methyl-3-O-methyl-hexitol(39%), 1,3,5-tri-O-acetyl-2-acetamido-2-deoxy-2N-methyl-4-O-methyl-hexitol (28%) which showed that the three sugars residues are present in a molar ratios of 1:1:1. From the combined results of the sugar analysis and methylation analysis, it can be inferred that the rhamnose residue is linked in positions 1 and 3 while one of the 2-acetamido-2-deoxy-glucoses is linked

in positions 1,3 and the other one is linked in positions 1 and 4.

The results of the methylation analysis of the partially pyruvylated PS 1 and the fully pyruvlated PS 3 also showed the presence of a 2-acetamido-2-deoxy-glucose which is linked at positions 1, 3, 4 and 6. This showed that the pyruvyic acid is present on the 1,3 linked 2-acetamido-2-deoxy-glucose and is linked to this sugar in positions 4 and 6. The relative ratios of the 1,3,4,6-linked 2-acetamido-2-deoxy-glucose in the PS (1) and PS (3) in table 5.9 further showed that the PS 1 is partially pyruvylated while PS 3 is fully pyruvlated. The complete absence of the 1,3,5-tri-O-acetyl-2-acetamido-2-deoxy-2N-methyl-4-O-methyl-hexitol in PS 3 further confirms that the 1,3-linked 2-acetamido-2-deoxy-glucose is further substituted by the pyruvic acid residues in positions 4 and 6.

Table 5.9.

Methylation analysis of the polysaccharides of E.coli 0149

Sugar[a]	T[b]	Mole %		
		A	B	C
2,4-Rha	0.93	43	33	51
2,3,6-GlcNAc	2.38	17	39	26
2,4,6-GlcNAc	2.56	30	28	-
2-GlcNAc	3.18	9	-	23

A = PS 1; B = PS 2; C = PS 3.

[a]2,4-Rha = 2,4-di-O-methyl-L-rhamnose,e.t.c.

[b]Retention time of the corresponding alditol acetate on an HP-54 column, relative to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol.

The absolute configuration determinations according to the procedure of Gerwig et al[170] described under "Experimental" whereby the retention times of the trimethylsilyl derivatives of the component sugars of the PS 2 were compared with those of the L-rhamnose and 2-acetamido-2-deoxy-D-glucose which were the known standards. From the results obtained from the determinations of the absolute configuration of the constituent sugars, the rhamnose residue was identified as having the L-configuration while the 2-acetamido-2-deoxy-glucose residues has the D-configuration.

From the chemical shifts of the anomeric signals in both the ^{1}H and the ^{13}C n.m.r. of PS 2 and PS 3, it can be easily concluded that the two 2-acetamido-2-deoxy-glucose present in the polysaccharide have the β-configuration.

However, the chemical shifts of the anomeric signals which are δ 4.85 in the ^{1}H n.m.r. and δ 101.2 in the ^{13}C n.m.r. spectra corresponding to the rhamnose residue are in the borderline region which are not definite for either the the α- or the β-configuration. Also β-L-rhamnopyranose is a rare sugar. However, to be able to determine the actual anomeric configuration of the rhamnose residue, the COSY experiment was carried out on the PS 2. From the COSY spectrum, shown in Fig 5.13 in Index I, the values of the chemical shifts of the protons of the rhamnose residue were determined. Table 5.10 on the next page shows the chemical shifts of the protons present in the rhamnopyranose residues and those of α and β-L-rhamnopyranose[265].

Table 5.10. Chemical Shifts of the Rhamnopyranose residue in the COSY spectrum and the α and β-L-Rhamnopyranoses[265].

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6(3 equivalent protons)
L-Rhap residue	4.85	3.92	4.09	3.51	3.39	1.30
α-L-Rhap	5.12	3.92	3.81	3.45	3.86	1.28
β-L-Rhap	4.85	3.93	3.59	3.38	3.39	1.30

For a conclusive identification of the anomeric nature of the L-rhamnopyranosyl residue, comparison of the chemical shift of the H-5 of this residue with the chemical shift of only the H-5 of an α-L-rhamnopyranose or β-L-rhamnopyranose can be used. This is because H-1 of this residue might have been shifted upfield or downfield because of its anomeric position which is involved in the glycosidic linkage to the 2-acetamido-2-deoxy-glucose residue. The glycosylation or substituent effect would have affected the chemical shift of H-3 of the rhamopyranosyl residue being the proton at the substitution position by the N-acetyl-glucosamine. The chemical shifts of H-2 and H-4 which are vicinal protons to H-1 would also have been similarly affected. However the chemical shift of H-2 in α- and β-L-rhamnopyranose have almost the same values and cannot be used as a distinguishing factor. The chemical shift of H-5 of this residue in the PS 2 was found to be δ3.39. The corresponding values for the α and the β-L-rhamnopyranose are δ3.86 and δ3.39. H-5 resonances in the α-L-rhamnopyranosyl residues may appear considerably upfield from δ3.86 for the α-anomer when the H-5 is close to a proton in the opposing ring[257,265]. Assuming that the L-rhamnopyranosyl residue is α-linked, no such interactions are indicated from the ball and stick model of the trisaccharide, independent of whether it is linked to O-3 or to O-4 of a

2-acetamido-2-deoxy-D-glucopyranosyl residue. From the values in the table 5.10 above, it can be seen that the chemical shift value that distinguishes the α from the β-rhamnopyranose is that of H-5. It is thus concluded that the L-rhamnopyranosyl residue in the O-poly- saccharide of the E. coli O149 has the β-configuration.

The results of the sugar analysis, methylation analysis and the absolute configuration determinations in conjunction with the n.m.r.results discussed above demonstrate that the E.coli O149 O-polysaccharide is composed of trisaccharide repeating units containing two residues of 2-acetamido-2-deoxy-β-D-glucopranose, linked through O-3 and O-4 respectively and one residue of β-L-rhamnopyranose linked through O-3. Pyruvic acid is linked as an acetal with the S configuration[266] to the 4 and 6-positions of the 1,3-linked 2-acetamido-2-deoxy-D-glucopyranosyl residue.

In order to distinguish between the two possible trisaccharides repeating units **R.6** and **R.7**, the depyruvylated polysaccharide PS 2 was subjected to partial acid hydrolysis.

→3)-β-D-GlcNacp-(1→4)-β-D-GlcpNAc-(1→3)-β-L-Rhap-(1-

R.6

→4)-β-D-GlcNacp-(1→3)-β-D-GlcpNAc-(1→3)-β-L-Rhap-(1-

R.7

The partial acid hydrolysis of the depyruvylated polysaccharide PS 2 after gel filtration on the Biogel P-4 column and lyophilisation gave a main product eluted in the disaccharide region. 15 mg of the PS 2 which was partially hydrolysed with 2,2,2 trifluoroethanoic acid (TFA) gave a yield

of 3.7 mg (24.6%). This product was reduced with sodium borodeuteride.

The ^{1}H n.m.r. spectrum of the sodium borodeuteride reduced product was complicated and the information that could be gathered from the spectrum did not shed further light on the sequence of the monosaccharide residues in the trisaccharide repating unit of the E. coli polysaccharide. Figure. 5.14 of Index I shows the ^{1}H n. m. r. of partial acid hydrolysis product. This product was methylated and characterised on the both the G.L.C.and G.C.-M.C. using a 12m long, 100% methyl silicone column indicated a mixture of 3 different methylated disaccharide alditols. The retention times of these products were obtained relative to permethylated lactitol acetate. These three products showed that the partial acid hydrolysis product was a mixture of 3 different disaccharides arising originally from the cleavage of the trisaccharide during the partial acid hydrolysis. After reduction and methylation, these disaccharides gave rise to the methylated disaccharide alditols of

i. N-acetyl-glucosamine linked in position 1 to position 3 of rhamnose **R.8** arising from the disaccharide ($GlcNAc^{1}\rightarrow^{3}Rha$).

ii. rhamnose linked in position 1 to position 4 of N-acetylglucosamine **R.9**, arising from ($Rha^{1}\rightarrow^{4}GlcNAc$).

iii. N-acetylglucosamine linked in position 1 to position 3 of N-acetylglucosamine **R.10**, arising from ($GlcNAc^{1}\rightarrow^{3}GlcNAc$).

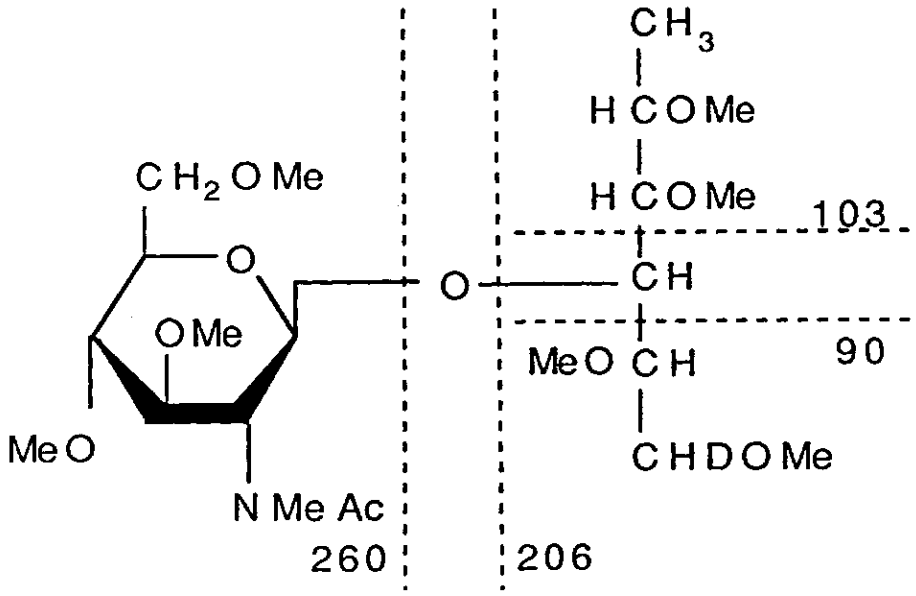
R.8, **R.9**, and **R.10**. are shown in scheme 5.2 on pages 207 and 208. The relative molar proportions of **R.8**, **R.9**, and **R.10** and their m/e values of the characteristic peaks from the G.C.-M.C. for the three products are shown in table 5.11 below. The mass spectra of **R.8.**, **R.9** and **R.10** are shown in Figure 5.15 of Index I.

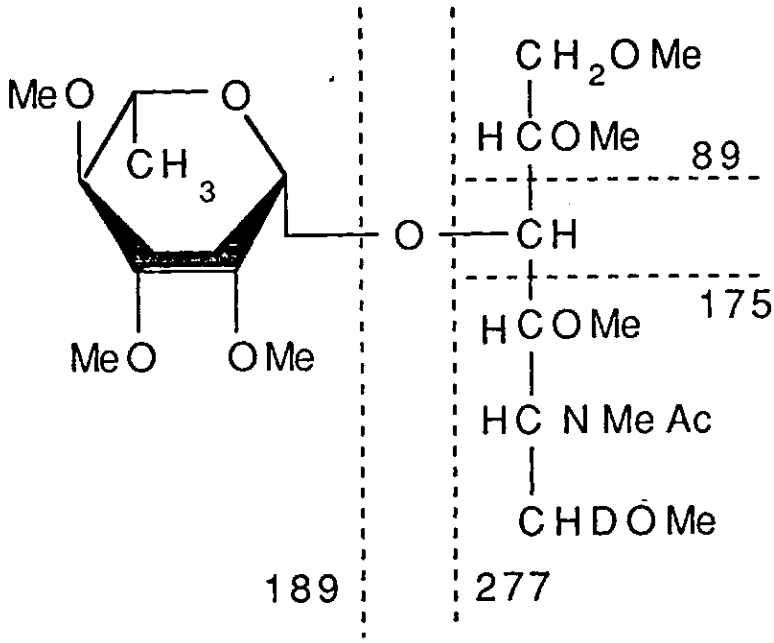
Table 5.11. m/e values for the diagnostic peaks obtained for products obtained from partial acid hydrolysis.

Permethylated Disaccharide Alditol	R_T	Mole %	m/e
GlcNAc$^1\rightarrow{}^3$Rha	1.24	45	90(23), 101(100), 106(26) 206(9), 260(21), 266 (23) 276(39)
Rha$^1\rightarrow{}^4$GlcNAc	1.25	34	88(100), 89(64), 101(54), 175(21), 189(22), 277(63)
GlcNAc$^1\rightarrow{}^3$GlcNAc	1.70	21	89(60), 131(23), 133(7), 260(93), 277(100).

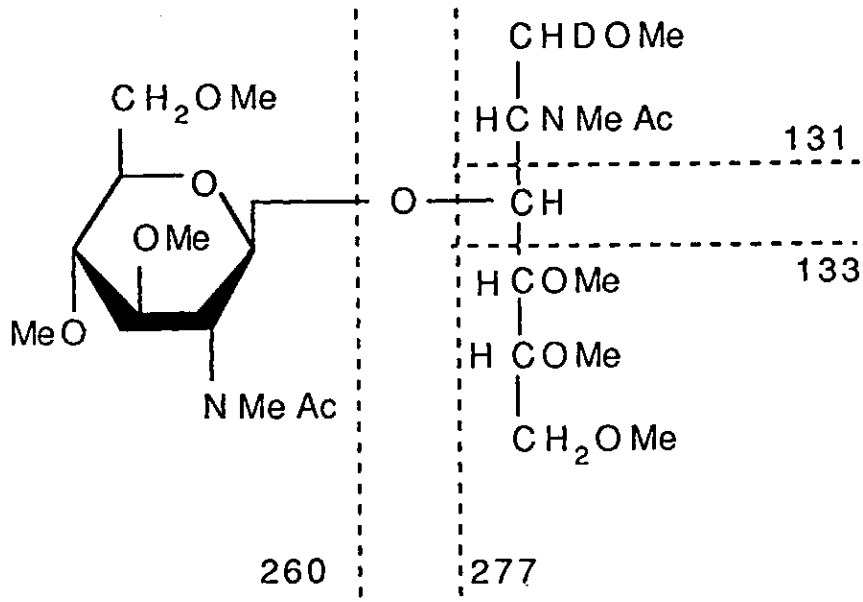
R_T = The retention time relative to permethylated lactitol acetate.

Scheme 5.2: The fragmentation pattern of the methylated disaccharide alditols showing the origin of peaks in the mass spectra.





R.9

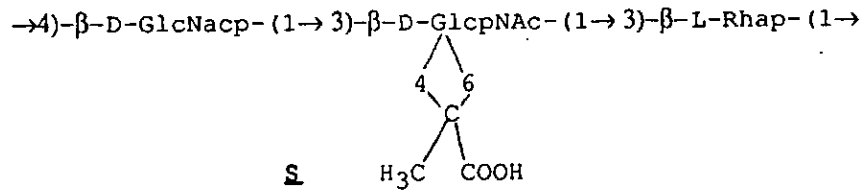


R.10

From the combined results obtained above, it is concluded that the E.coli O149 O-polysacchaaride is composed of trisaccharide repeating units

having the structure **R.11**.

Pyruvic acetals are common in capsular polysaccharides from gram-positive and gram-negative bacteria but have only been found once before in a polysaccharide namely that elaborated by *Shigella dysenteriae* type[65] .



R.11

Streptococcus pneumonia Type 37.

The crude S-37 which was contaminated by protein as indicated by the ^{1}H n.m.r was purified by partition between water and phenol by the hot water-phenol procedure at 66°C. In this procedure used for the purification of bacterial LPS, the PS accumulates in the aqueous phase and the protein in the phenol phase. The only fraction obtained was from the aqueous phase and was dialysed and freeze-dried to give a yield of 88mg (44%) from 200mg from the crude S-37. The solutions of the purified S-37 was not monodisperse as indicated by the high viscosity of the aqueous solutions of this polysaccharide. The high viscosity probably arose from the very high molecular weight of the polysaccharide and the poor solubility is probably due to the linkages present in the high molecular weight of the PS. This is because it has been demonstrated that the solubilities of some (1→3)-β-D-glucans are known to be related to their molecular weights[92,93]. From the sugar analysis, it was shown that glucose is the only constituent sugar in the S-37 capsular polysaccharide.

Due to the high viscosity,and the resultant poor dispersity, the ^{1}H- and ^{13}C.n.m.r. spectra were of poor quality. There was also the failure to achieve complete methylation of the purified S-37 by the Hakomori procedure and therefore no partially methylated alditol acetates of the constituent sugars of the polysaccharide were obtained. Only a mixture alditol acetates were obtained.

The ^{1}H and ^{13}C.n.m.r spectra of the native purified PS showed that S-37 did not contain non-carbohydrate substituents such as acetyl, pyruvyl or lactate groups[39,67,69] as there were no signals in the high frequency field or the deoxy region of the n.m.r.specrtum where the methyl protons belonging to these groups resonate. This is an uunusual feature for a

capsular PS because most capsular PS encountered so far usually possess one or more of acetyl, pyruvyl or lactate groups.

Also the absence of acidic sugars i.e.uronic acid residues was noticable as capsular PS usually possess acidic sugars which are responsible for the negative changes present on the surface of the capsules. ^{1}H and ^{13}C n.m.r. spectra of the native S-37 polysaccharide are shown in Figures 5.16 and 5.17.

Partial depolymerisation of S-37 obtained by the treatment of this polysaccharide with 80% aqueous formic acid at 85°C for 20 minutes gave a yield of 54% of a more soluble product S-37 I and an improved n.m.r spectra. Comparison of the ^{1}H- and ^{13}C-n.m.r. spectra of this material S-37 I and purified native S-37 showed that structural changes introduced by the partial depolymerisation were negligible because the ^{13}C n.m.r. spectrum of the S-37 was almost identical to that of the S-37 I. The two signals in the anomeric region that were obtained from the ^{13}C n.m.r. and the ^{1}H n.m.r. were at δ 102.9 and δ 100.0, δ 5.12 (doublet 7.7 Hz) and δ4.68 (doublet 8.0Hz) respectively as shown in Table 5.12 below. The ^{1}H- and ^{13}C-n.m.r. spectra of S 37 I are shown in Figures 5.18 and 5.19 in Index 1 . The specific rotation $[\alpha]_{578}$ of a 1% aqueous solution of the native S-37 and S 37 I was -2° which gave an indication that the predominant anomeric configuration of the sugar constituents of the polysaccharide may be β in nature. This is because it has generally been accepted that the α-sugars usually give high and positive values of specific rotation while β-sugars often give low and positive value of specific rotation.

^{1}H and ^{13}C N.M.R. spectra of the native S-37 polysaccharide are shown in Figures 5.16 and 5.17. The values for the $J_{1,2}$ coupling constants which

are 7.8 and 7.7 Hz indicate that both the D-glucopyranosyl residues in S-37 are β-linked. This conclusion is further corroborated by the low and negative values of the specific rotation. The NOESY spectrum from which the $^{1}J_{C,H}$ values were obtained is shown in Figure 5.22. The values for the $^{1}J_{C,H}$ coupling constants of 166.5 and 166.5 Hz, however, fall between those generally observed for α- and β-glycopyra- nosides which are 170Hz for the former and 160Hz for the latter[167].

Table 5.12

^{1}H and ^{13}C N.M.R. Anomeric chemical shifts of PN 37.

	A	B
→3)-β-D-Glc-(1- 2↑	5.12 (7.7)	4.68 (8.0)
β-D-Glc-(1-	4.97 (7.8)	--
→3)-β-D-Glc-(1- 2↑	102.9 (166.5)	--
β-D-Glc-(1-	100.0 (166.5)	--

A, Depolymerised polysaccharde; B, Smith degraded polysaccharide.

(For the anomeric protons and carbons, one bond coupling constants are given in brackets.)

Methylation analysis of S-37 I gave comparable amounts 2,3,4,6-tetra-O-methyl-D-glucose (52%) which is a terminal sugar and 4,6-di-O-methyl-D-glucose (44%), which is a branch point but no other products, indicating a highly branched structure. The results are shown in Table 5.13 below. The G.C.-M.S. spectra of these partially methylated sugars are shown in Figure 5.19 of Index 1.

All the signals in the ^{1}H-and ^{13}C-n.m.r. spectra of partially depolymerised polysaccharide S-37 I could be identified as shown in Tables 5.12 and 5.13 by means of COSY and C-H correlation spectroscopy. The COSY and C-H correlation spectra of S 37 I are shown in Fig 5.20 and 5.21.

These results confirm that S-37 is composed of disaccharide repeating units. In agreement with the previous results, the Smith degradation of the depolymerised S-37 gave a linear 1,3-linked glucan, as indicated by methylation analysis, which gave 2,4,6-tri-O-methyl-D-glucitol as the sole product as shown in Table 5.13.

Table 5.13

Methylation analysis of PN 37

Sugar[a]	T[b]	Mole %	
		A	B
2,3,4,6-Glc	1.00	58	--
4,6-Glc	1.30	42	--
2,4,6-Glc	1.21	--	100

[a] 4,6-Glc = 4,6-di-O-methyl-D-glucose, e.t.c..

[b] Retention time of the corresponding alditol acetate on an HP-54 column, relative to that of 1,5-di-O-acetyl-2,3,4,6-tetra-methyl-D-glucitol.

A, PN37 Polysaccharide; B, Smith degraded polysaccharide.

The ^{1}H. n. m. r. of the Smith degraded polysaccharide is shown in Figure 5.23 and the G.C.-M.S.of the product of Smith Degradation on S 37 I is shown in Figure 5.24.

From the combined evidence it is concluded that S-37 is composed of disaccharide repeating units with the structure **R.12**.

→ 3)-β-D-Glcp (1-
2
↑
1
β-D-Glcp-

R.12

The chemical shift of an anomeric proton of a chain residue on S-37 should, as a first approximation, be equal to the chemical shifts of the H-1 resonance in a β-1,3-glucan δ 4.71, plus the shift of the H-1 resonance when going from methyl β-D-glucopyranose to methyl-β-sophoroside, namely 0.11 p.p.m.[240]. The calculated value, δ 4.82, is considerably lower than that actually observed δ 5.12.

Table 5.14

The chemical shifts of the sugar residues in partially depolymerised S-37.

	H/C						
Sugar residue	1	2	3	4	5	6	6'
→3)-β-D-Glc-(1- 2↑	5.12 (0.48)	3.92 (0.67)	4.09 (0.59)	3.59 (0.17)	3.51 (0.05)	3.75 (0.03)	3.93 (0.03)
	100.04 (3.2)	80.38 (5.63)	82.49 (5.73)	68.86 (-1.85)	86.51 (-0.26)	61.77 (0.07)	
β-D-Glc-(1-	4.97 (0.33)	3.40 (0.15)	3.54 (0.14)	3.40 (-0.02)	3.45 (-0.01)	3.74 (0.02)	3.93 (0.03)
	102.94 (6.10)	74.89 (-0.31)	76.87 (0.11)	71.12 (0.41)	77.12 (0.36)	62.29 (0.45)	

[Chemical shifts displacements (relative to β-D-glucopyranose) are given in brackets.]

Data for protons 2-6' were assigned from COSY and for carbons 2-6 from the CH correlation experiment.

A possible explanation may be that S-37, because of its crowded, comb-like structure assumes a conformation in which O-2 of a neighbouring chain residue is in close contact with this anomeric proton causing the proton to resonate at a lower field. The signal of the anomeric proton in the terminal β-D-glucopyranosyl group, at δ 4.97 also occurs at much lower field that the corresponding proton in methyl β-sophoroside, at δ4.76. In an energy minimised model containing six sugar residues, i.e. three repeating units obtained, using the HSEA program[176, 181], the middle β-D-glucopyranosyl group comes close to the anomeric proton of the adjacent group, which may account for the downfield shift. It is evident from the modelling that the molecule is severely crowded and thus other ways of escaping repulsion, like ring deformation, may be at hand, not accounted for in the HSEA program. This however, would probably not change the position of the ring oxygen in terminal sugar residues very much. The chemical shifts for the carbon atom resonances in S-37 and β-1,3-glucan[240] are given in Table 5.15 below.

The shifts caused by substitution of the β-1,3-glucan with β-D-glucopyranosyl groups in 2-positions are -3.4, 6.4 and -3.2 p.p.m. for C-1, C-2, and C-3 resonances, respectively showing a marked downfield shift for C-2 and upfield shifts for C-1 and C-3 which are the two neighbouring carbon atoms to the linkage atom. The corresponding values in methyl β-sophoroside[240] are -1.4, 7.3, and 0.0 p.p.m., respectively. The signal for the C-1 response of the β-D-glucopyranosyl group in S-37 is δ 102.9. These deviations indicates that there are inter-residue atomic contacts in S-37 which are caused by the steric crowding and which are not present in the disaccharide model.

Table 5.15

Chemical Shifts in the ^{13}C-n.m.r. spectra of S-37 I, 1,3-glucan, methyl β-sophoroside and the pertinent monosaccharides.

Sugar Residue	Chemical Shift δ					
	C-1	C-2	C-3	C-4	C-5	C-6
→3)-β-D-Glc-(1* 2↑	100.0 (-3.4)	80.4 (6.4)	82.5 (3.2)	68.9 (-0.3)	86.5 (1.1)	61.8 (0.1)
β-D-Glc-(1-*	102.9	74.9	76.9	71.1	77.1	62.3
→3)-β-D-Glc-(1-	103.4	74.0	85.7	69.2	76.6	61.7
β-D-Glc-(1→ 3)-β-D-Glc-OMe	102.7 (-1.4)	83.1 (7.3)	76.8 (0.00)	70.5	76.6	61.7
β-D-Glc-OMe	104.1	74.0	76.8	70.7	76.8	61.8
β-D-Glc	96.9	75.2	76.8	70.7	76.8	61.8

* Repeating units present in S-37.

INDEX II.

^{13}C AND ^{1}H N.M.R. AND MASS SPECTRA OF THE POLYSACCHARIDES.

FIGURES 5.1 to 5.3 for
Brucella abortus Biotype 1, strains Mustapha and 7

FIGURES 5.4 to 5.7 for
Campylobacter coli Labet 227

FIGURES 5.8 to 5.15 for
Escherichia coli O149

FIGURES 5.16 to 5.24 for
Streptococcus pneumonia Type 37

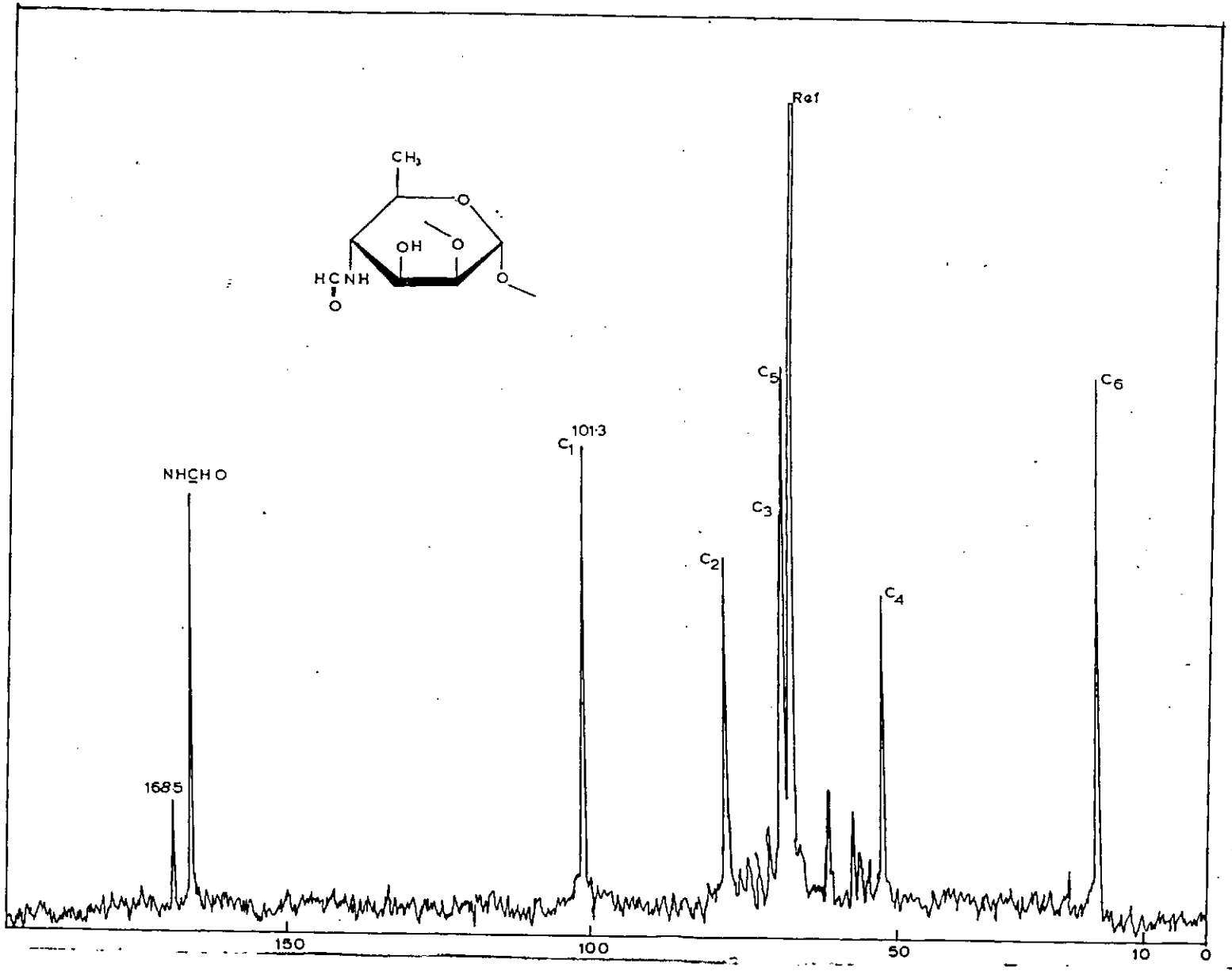


Figure 5.1. ^{13}C n.m.r. spectrum of *Brucella abortus* Strain 7.

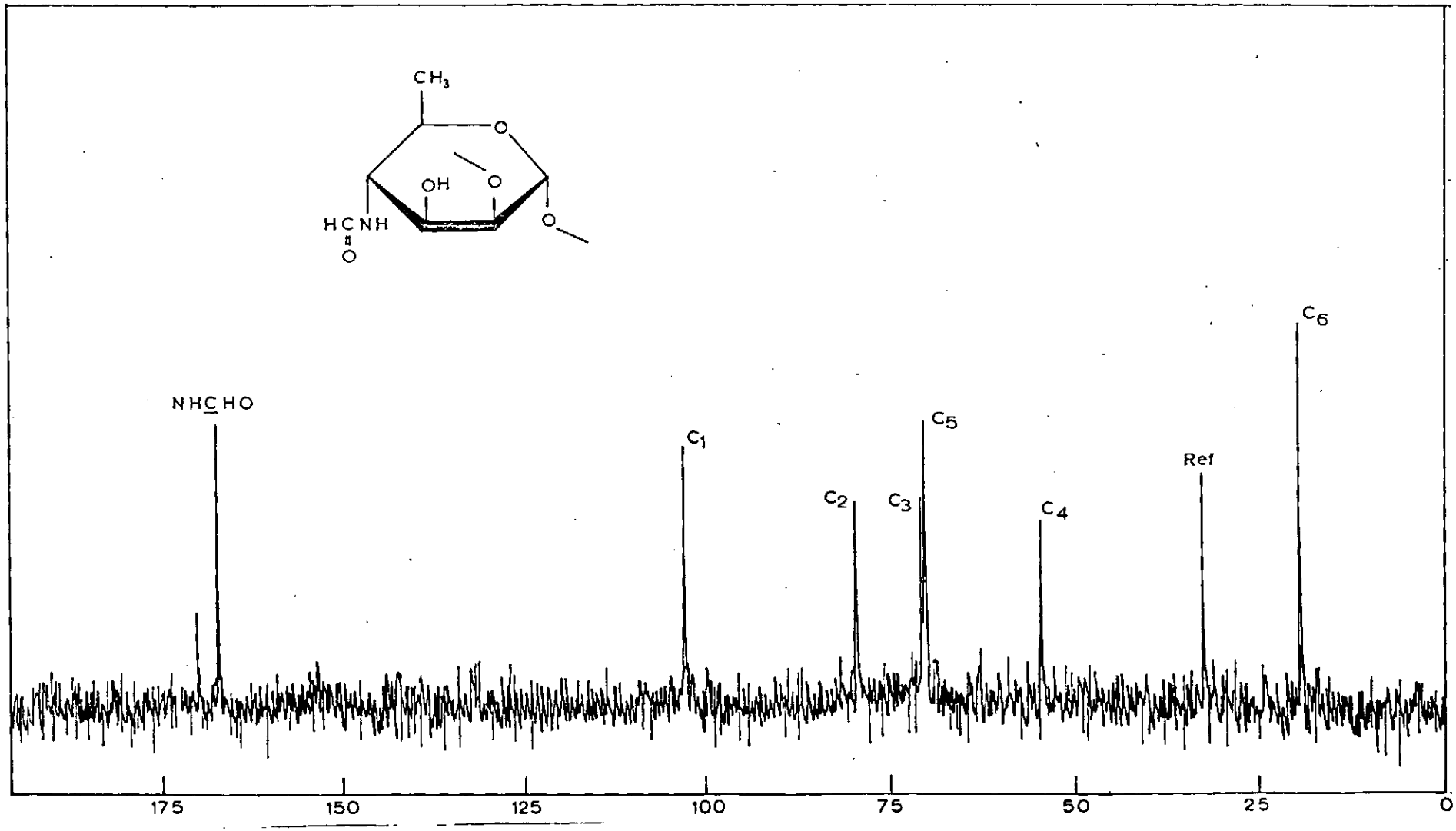


Figure 5.2. ^{13}C n.m.r. spectrum of Brucella abortus Strain Mustapha.

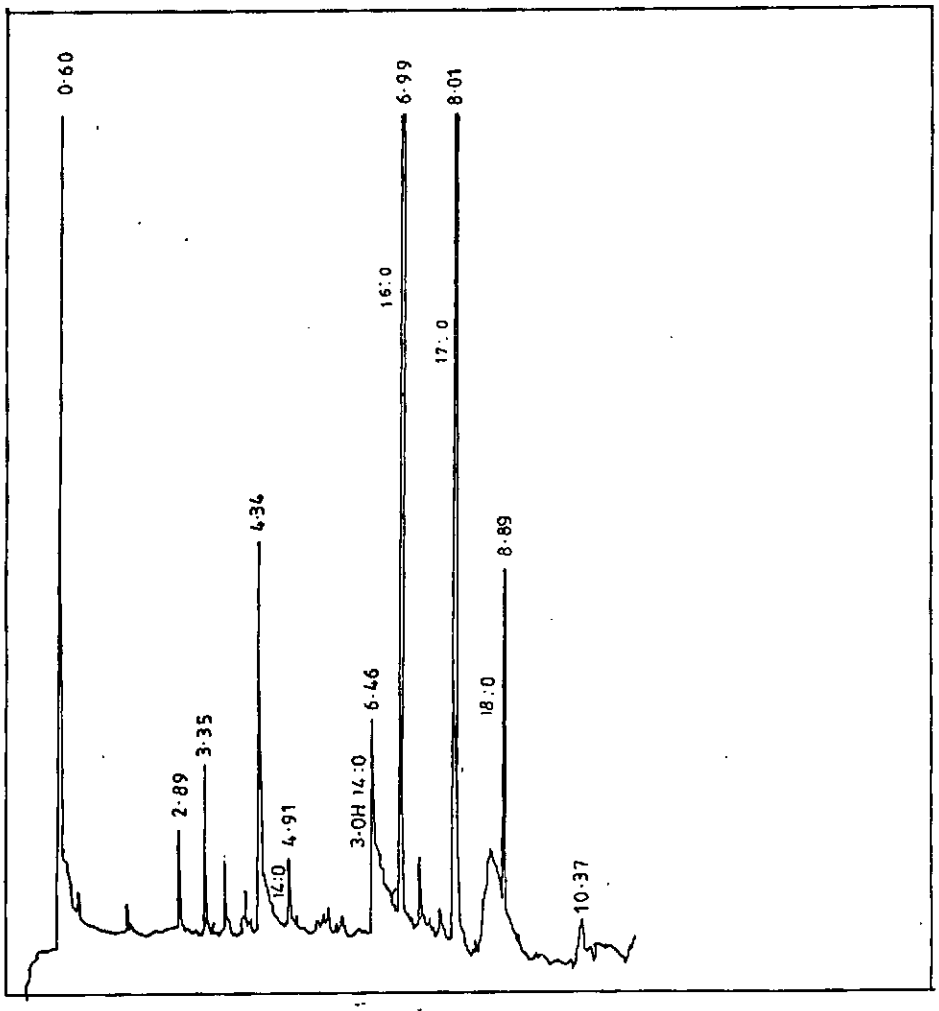


Figure 5.3. **Chromatogram of Fatty Acid Analysis in Lipid A of <u>Brucella</u> <u>abortus</u> strain 7.**

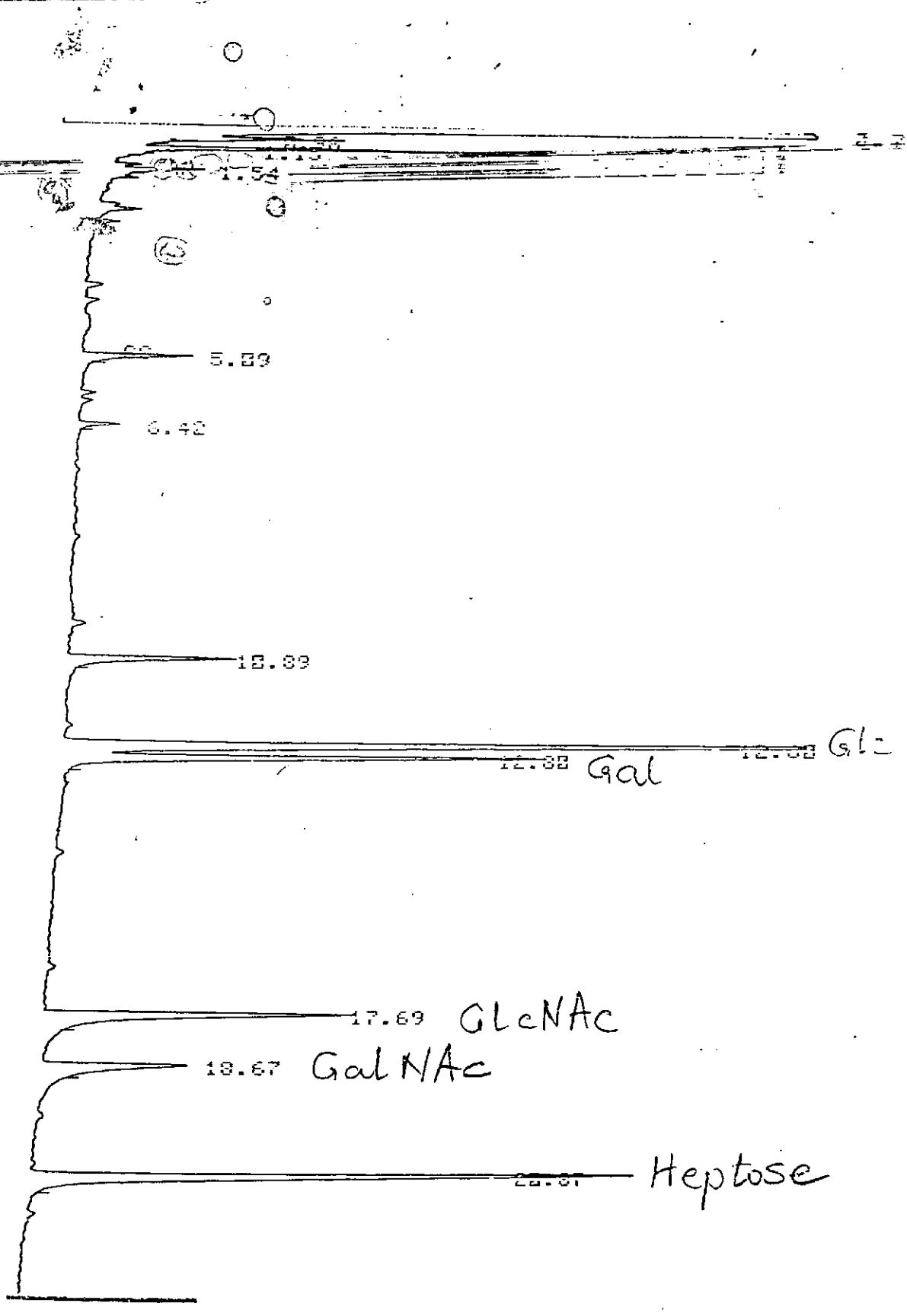


Figure 5.4a

Sugar Analysis of OS I of <u>Campylobacter</u> <u>coli</u> Labet 227.

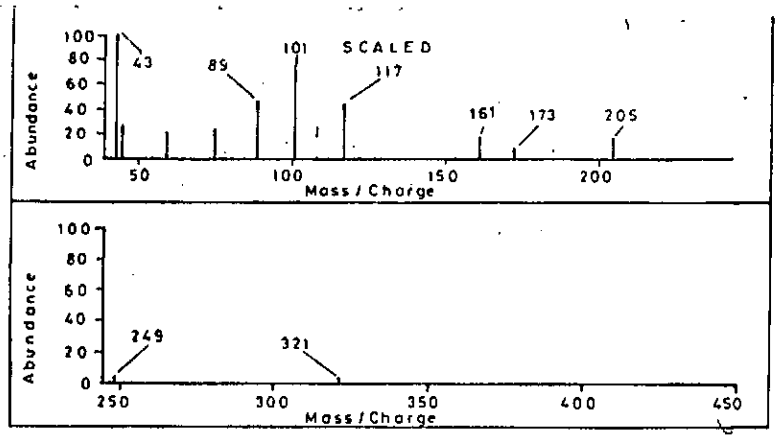


Figure 5.4b. Mass Spectra of R .2. of <u>Campylobacter</u> <u>coli</u> Labet 227 (OS I).

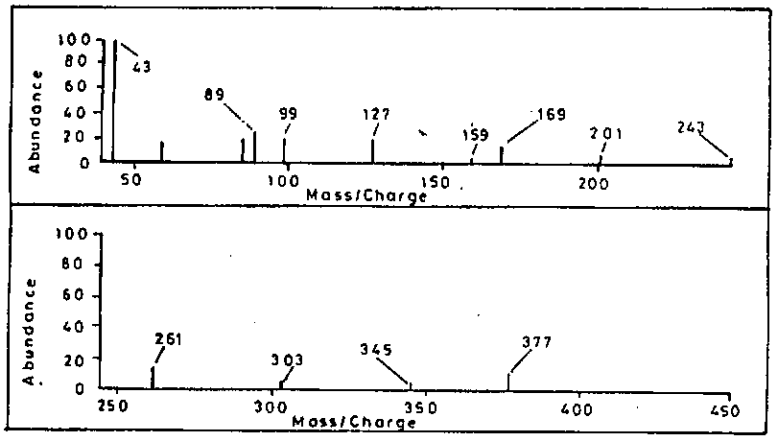


Figure 5.4c. Mass Spectra of R .3. of <u>Campylobacter</u> <u>coli</u> Labet 227 (OS I).

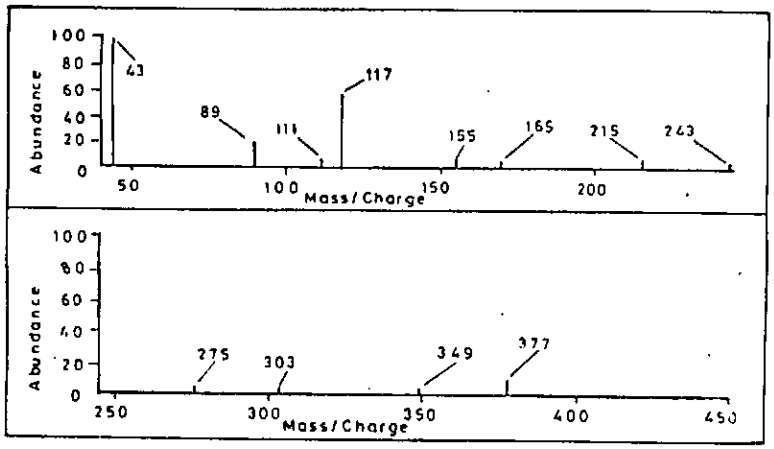


Figure 5.4d. Mass Spectra of R .4. of <u>Campylobacter</u> <u>coli</u> Labet 227 (OS I).

Campylobacter coli Labet 227

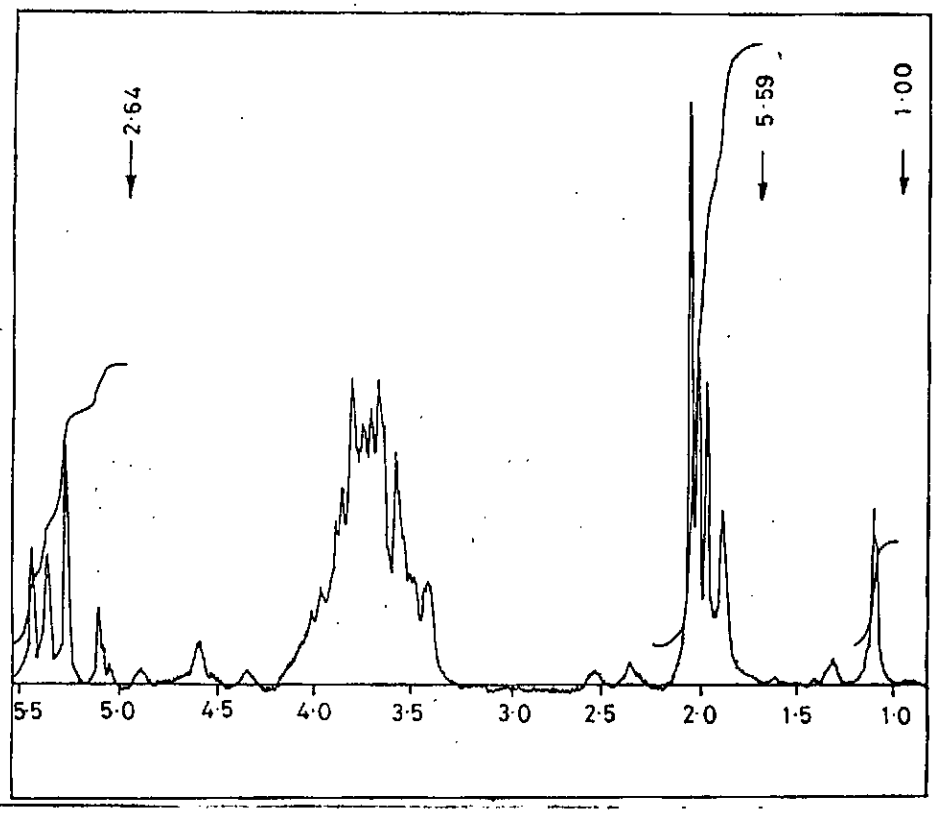


Figure 5.5. ^{1}H n.m.r. spectrum of Campylobacter coli Labet 227 (OS I).

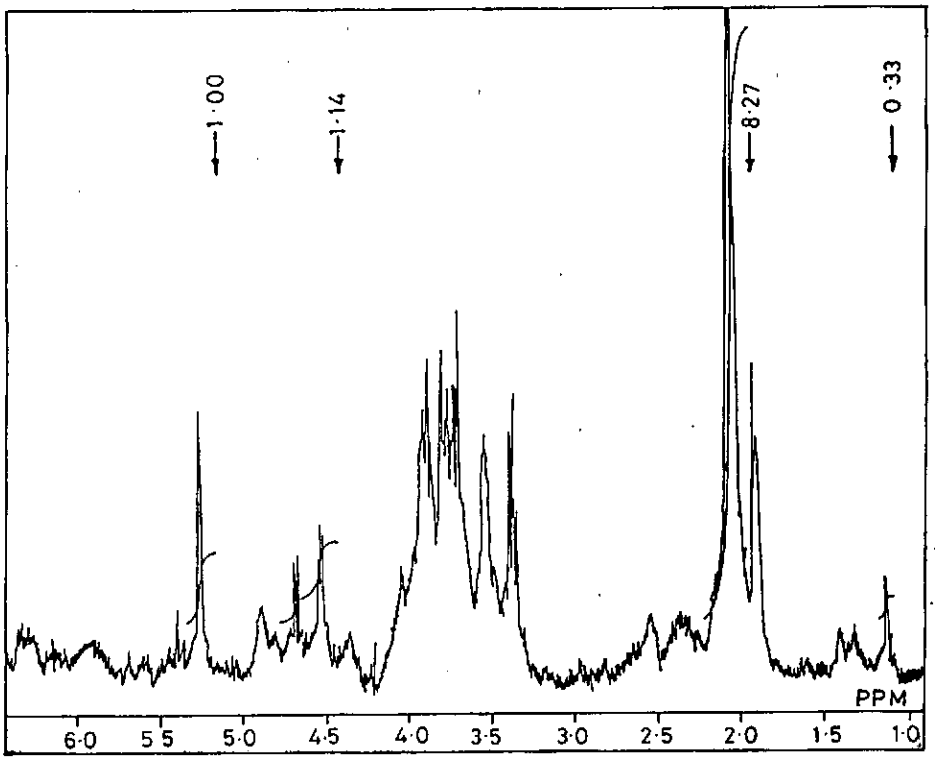


Figure 5.6. ^{1}H n.m.r. spectrum of Campylobacter coli Labet 227 (OS II).

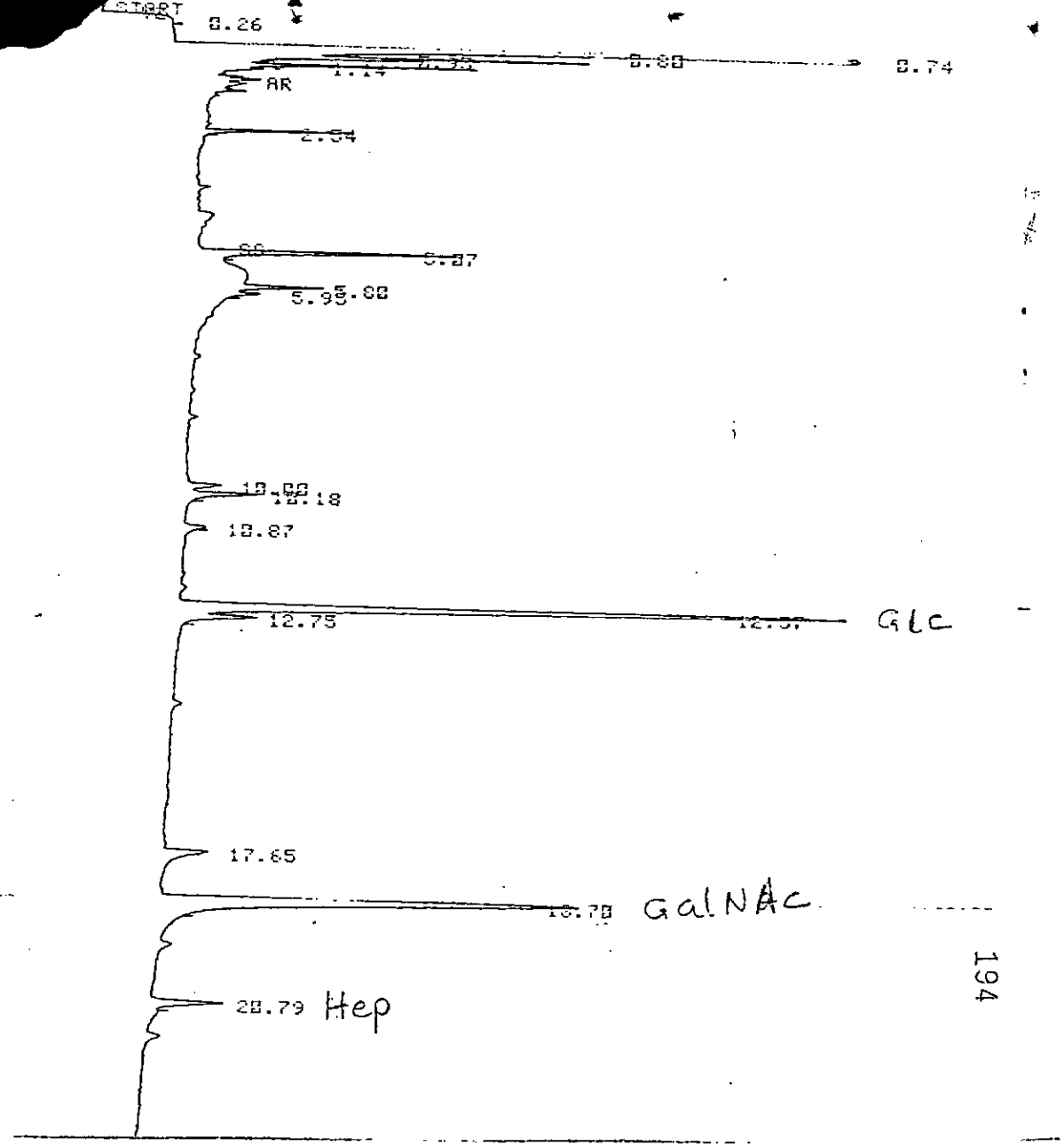


Figure 5.6a. Sugar Analysis of Campylobacter coli Labet 227 (OS 11).

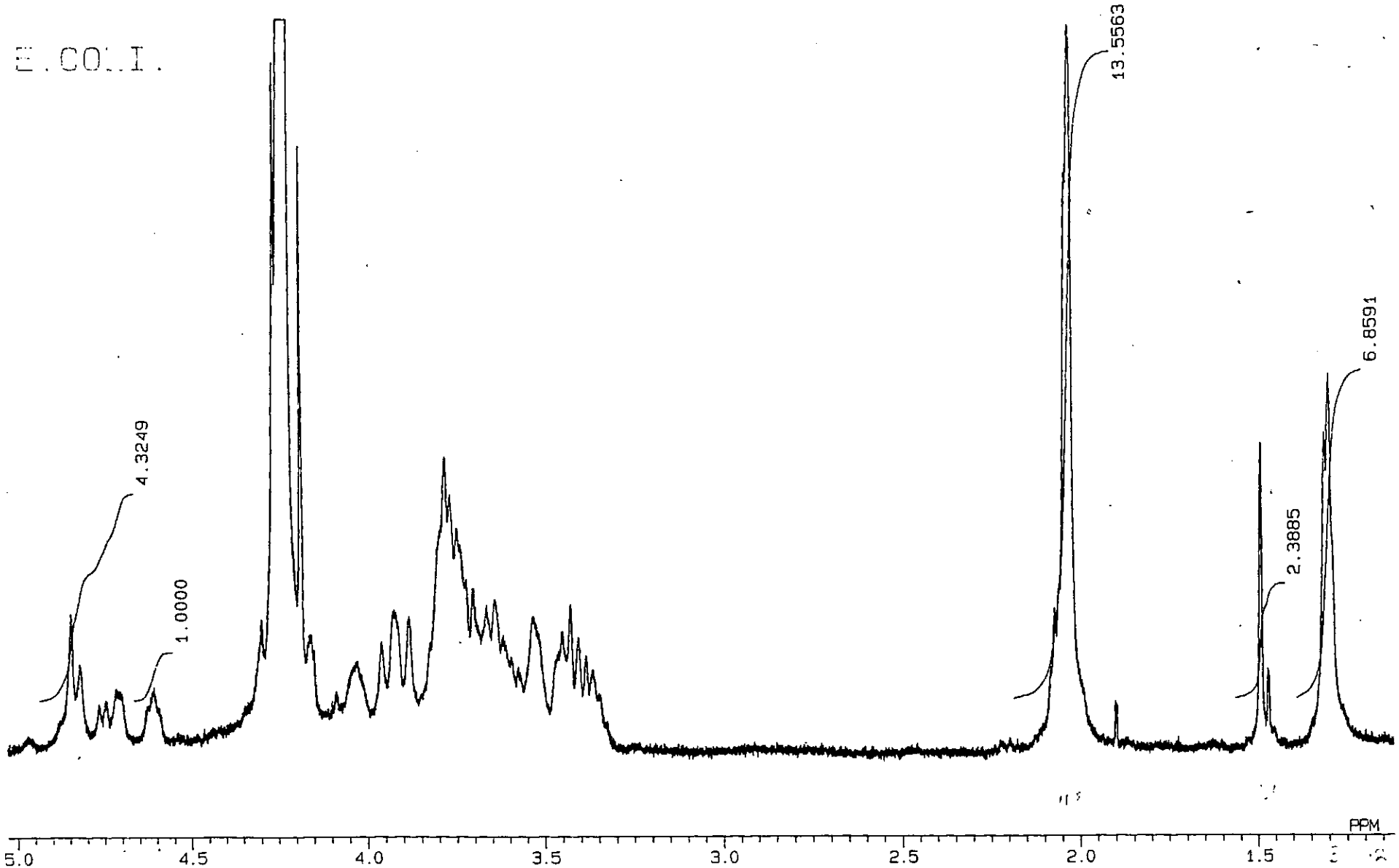


Figure 5.7. ^{1}H n.m.r. spectrum of <u>Escherichia coli</u> 0149 PS I.

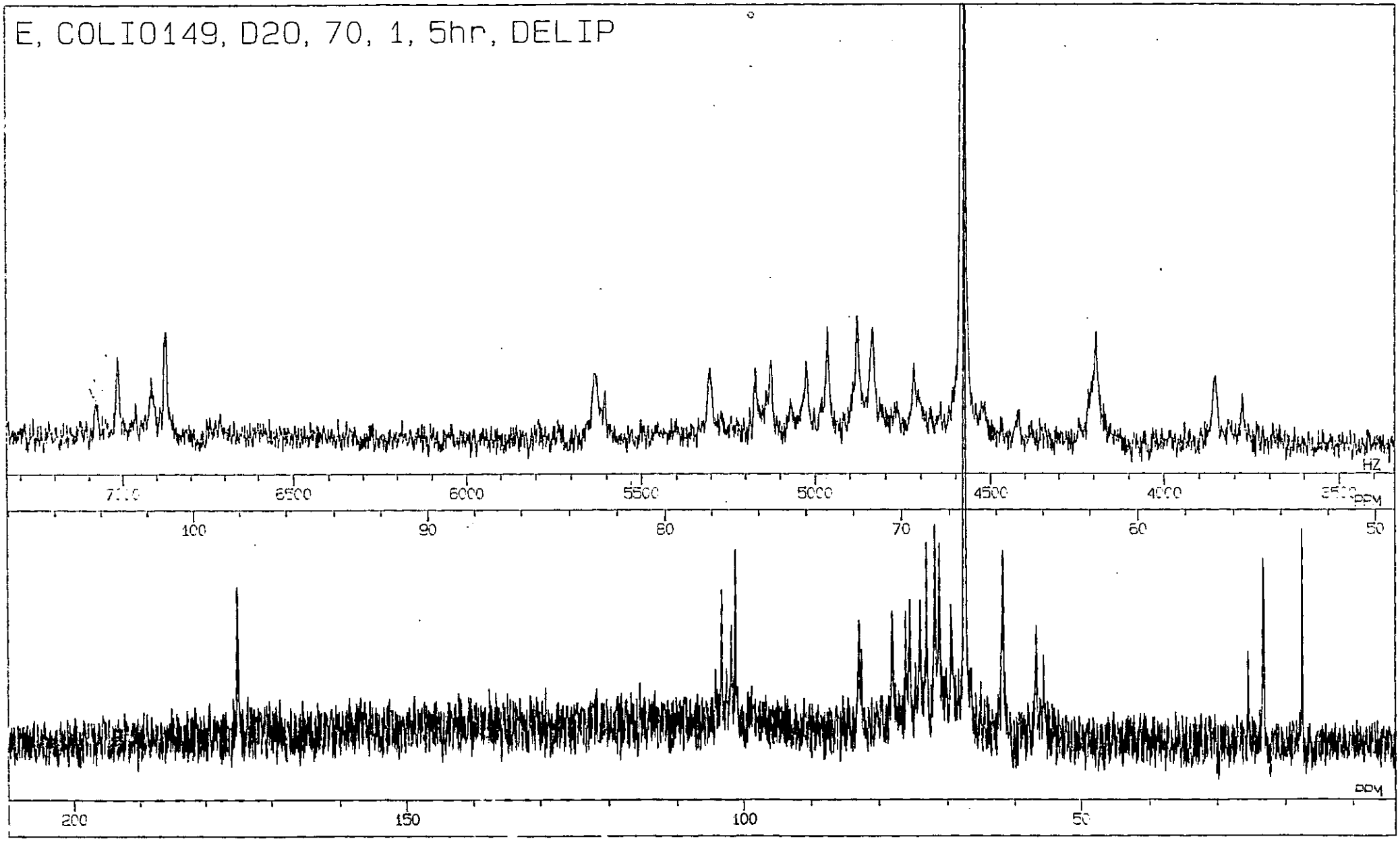


Figure 5.8. ^{13}C n.m.r. spectrum of <u>Escherichia coli</u> 0149 PS I.

E coli 0149-PS2 - 3hr delip.

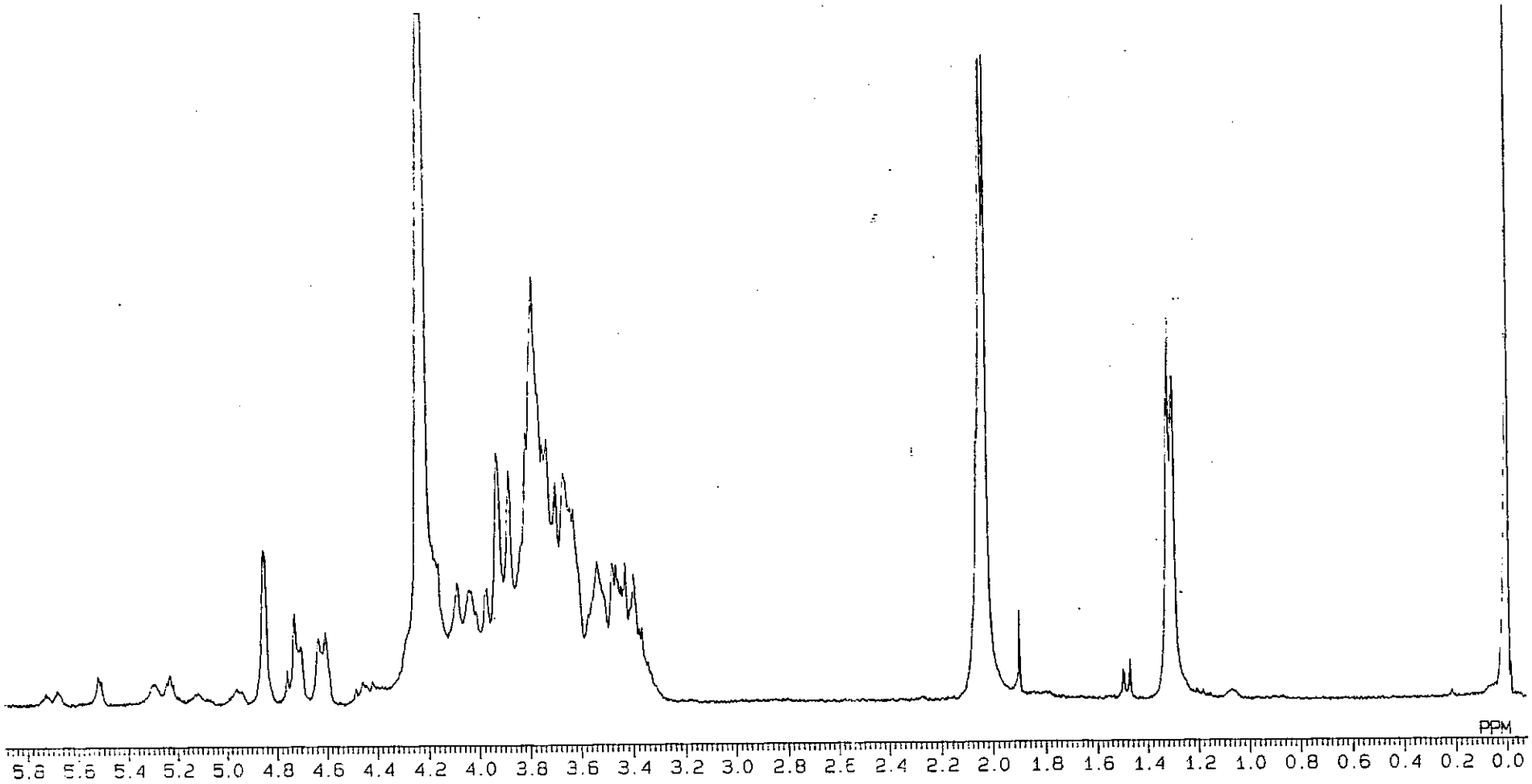


Figure 5.9. ^{1}H **n.m.r. spectrum of** ***Escherichia coli*** **0149 PS 2.**

PS2

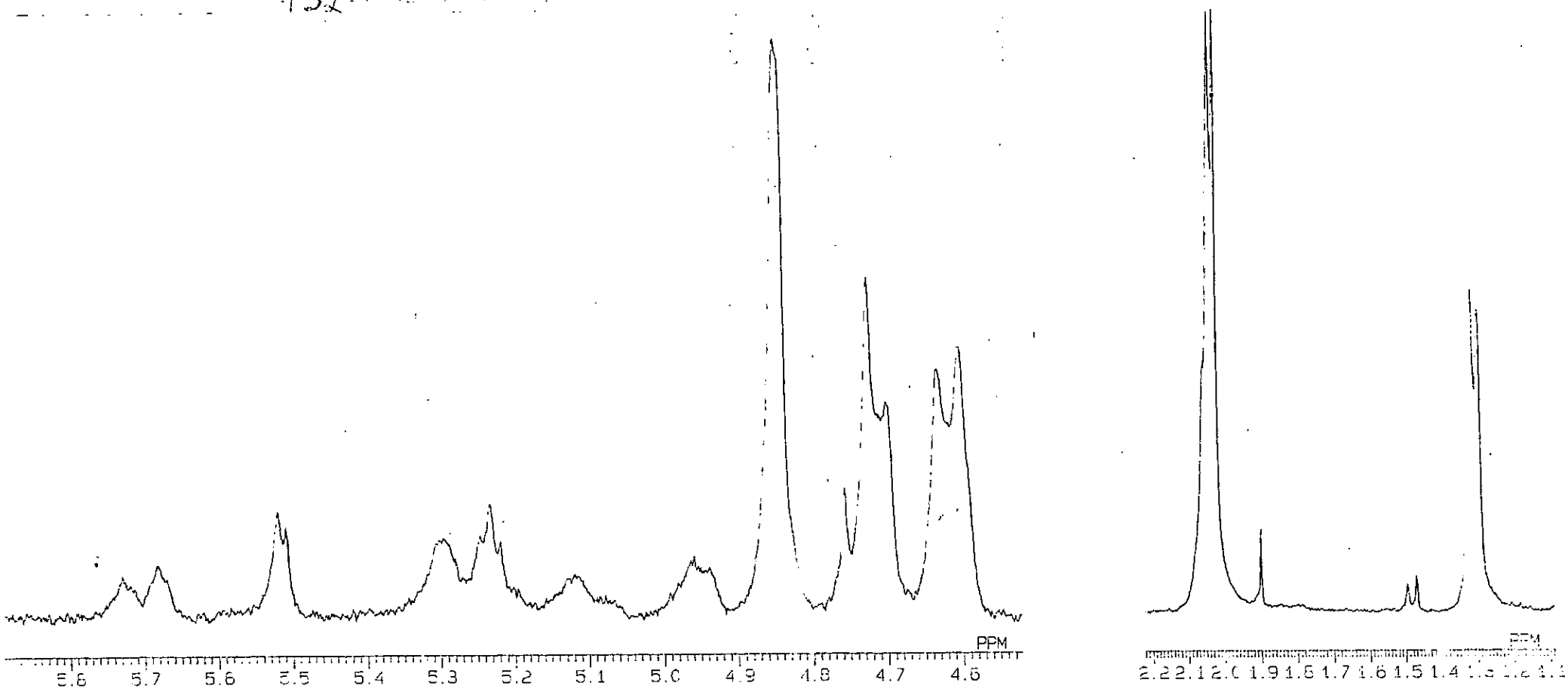


Figure 5.9. ^{1}H n.m.r. spectrum of *Escherichia coli* 0149 PS 2. Anomeric Expansion.

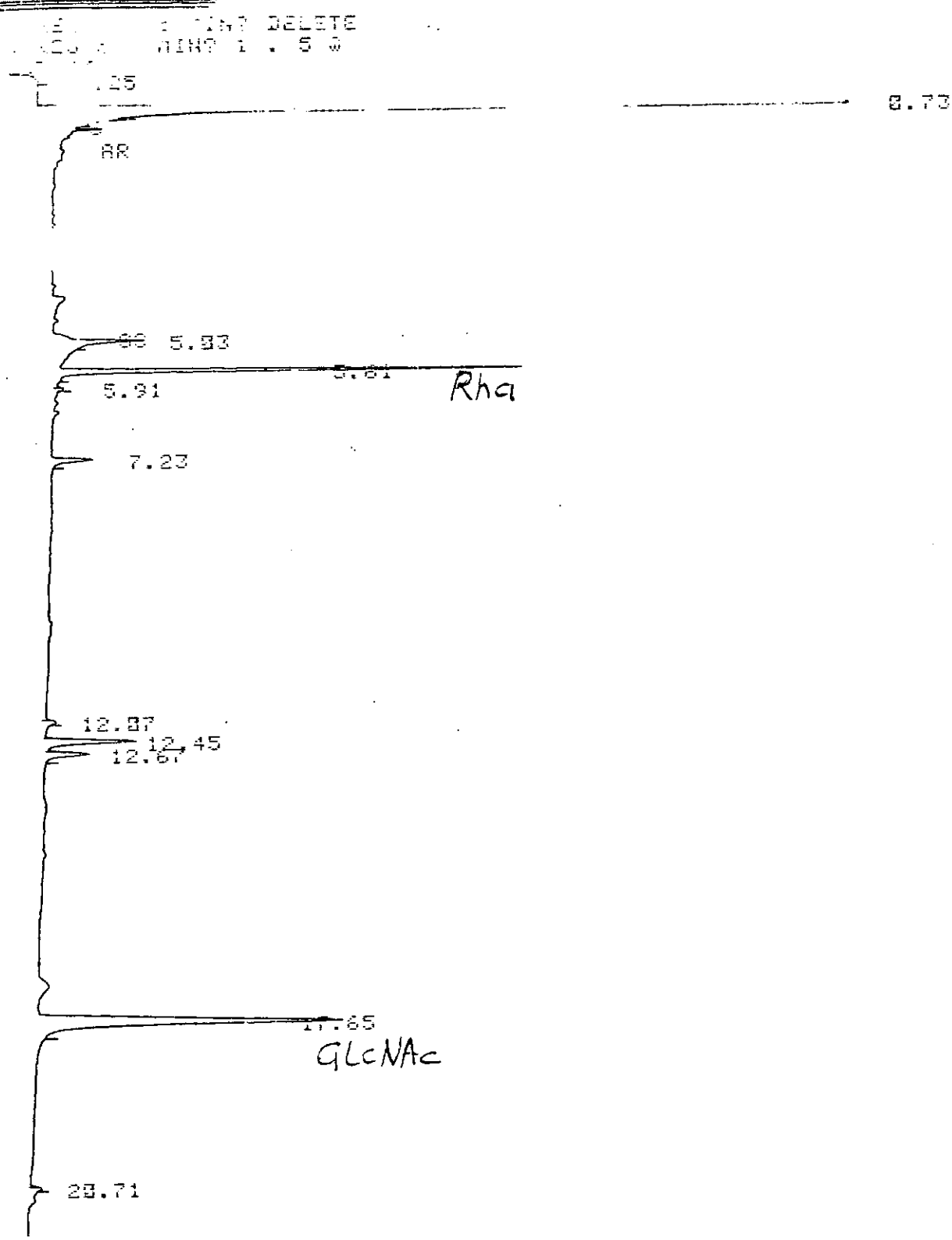


Figure 5.9a. Sugar Analysis of *Escherichia coli* 0149 PS 2.

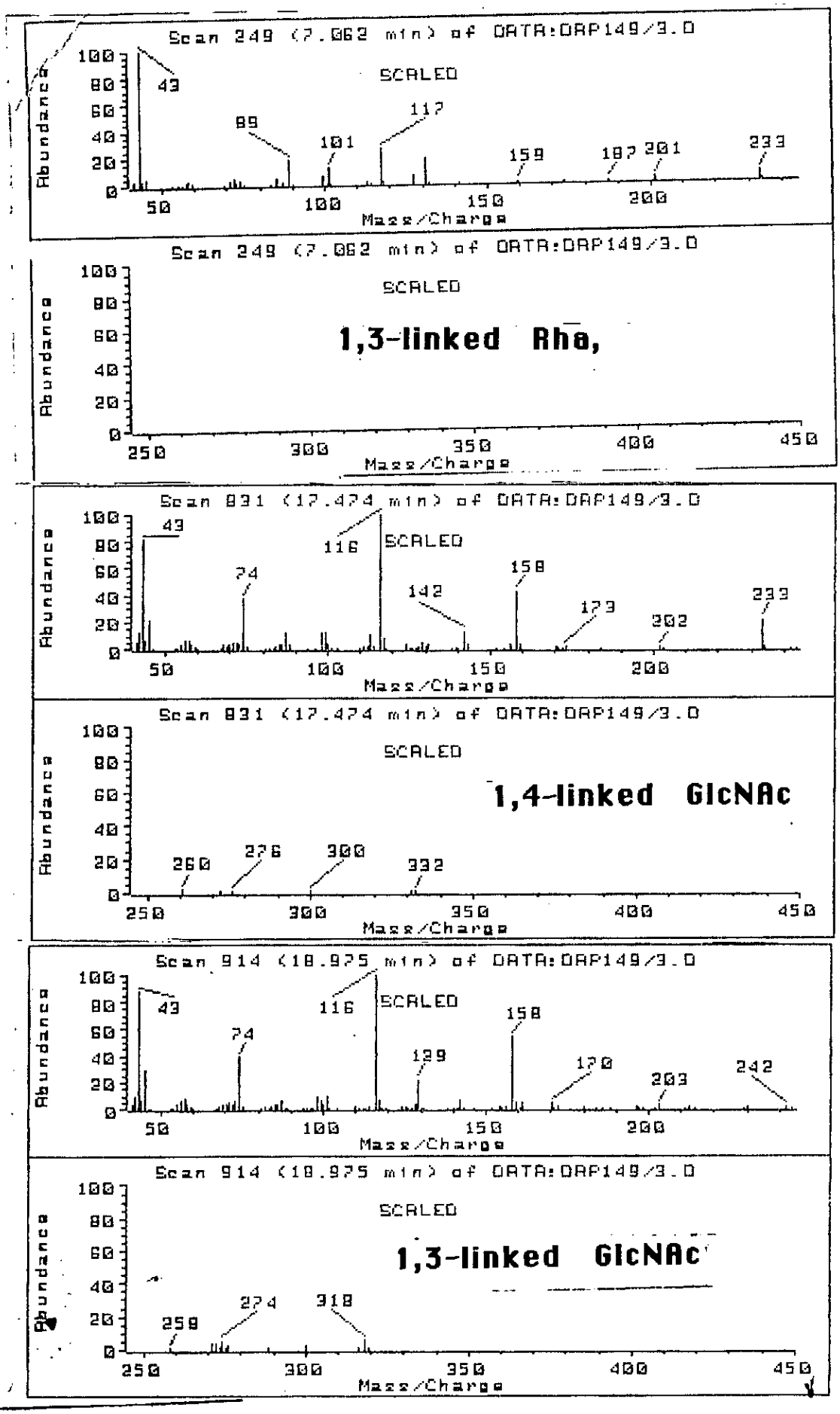


Figure 5.9b.

Mass Spectra of PMAA of *Escherichia coli* 0149 PS 2.

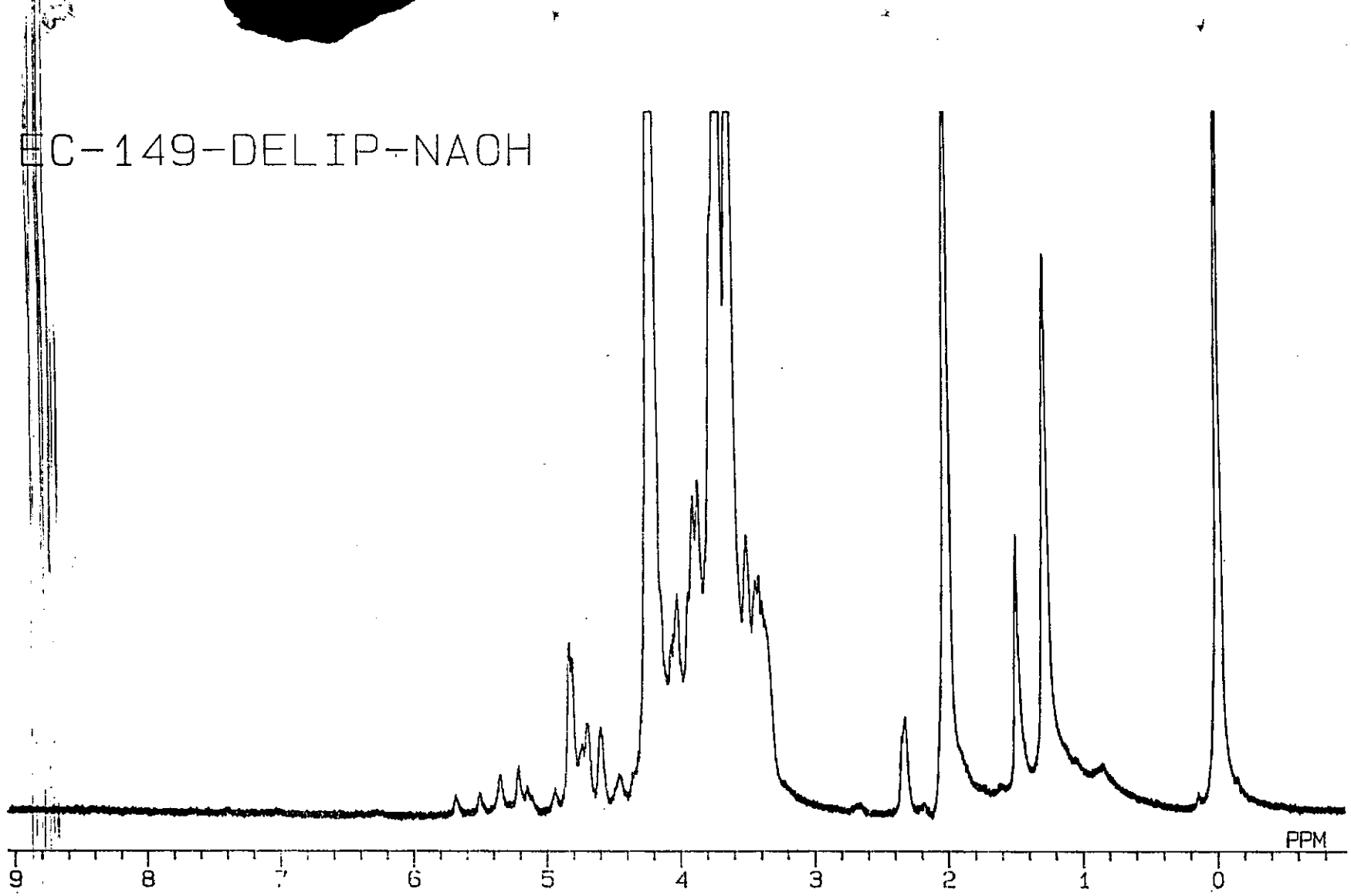


Figure 5.10. ^{1}H n.m.r. spectrum of *Escherichia coli* 0149 PS 3.

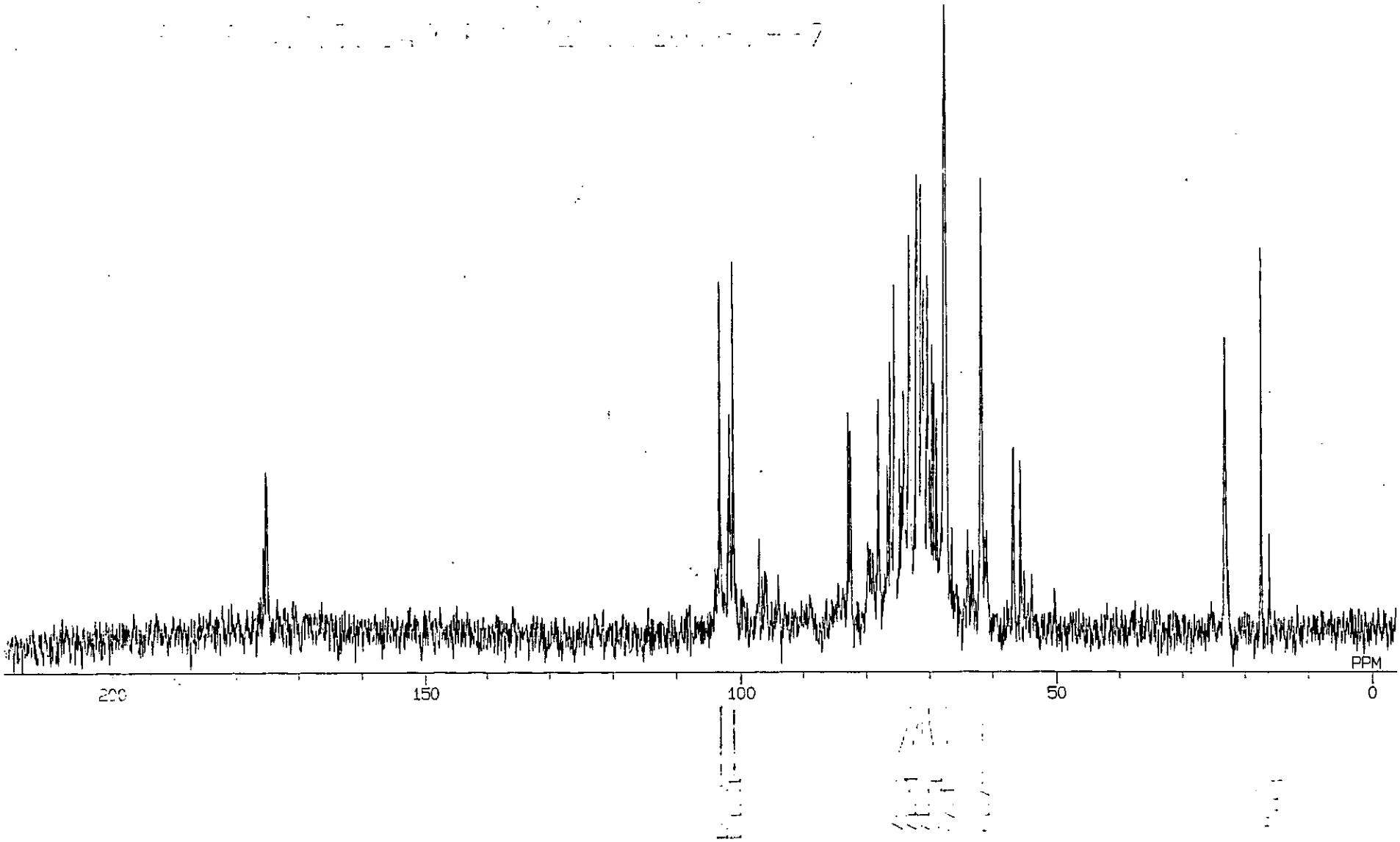


Figure 5.11. ^{13}C n.m.r. spectrum of *Escherichia coli* 0149 PS 2.

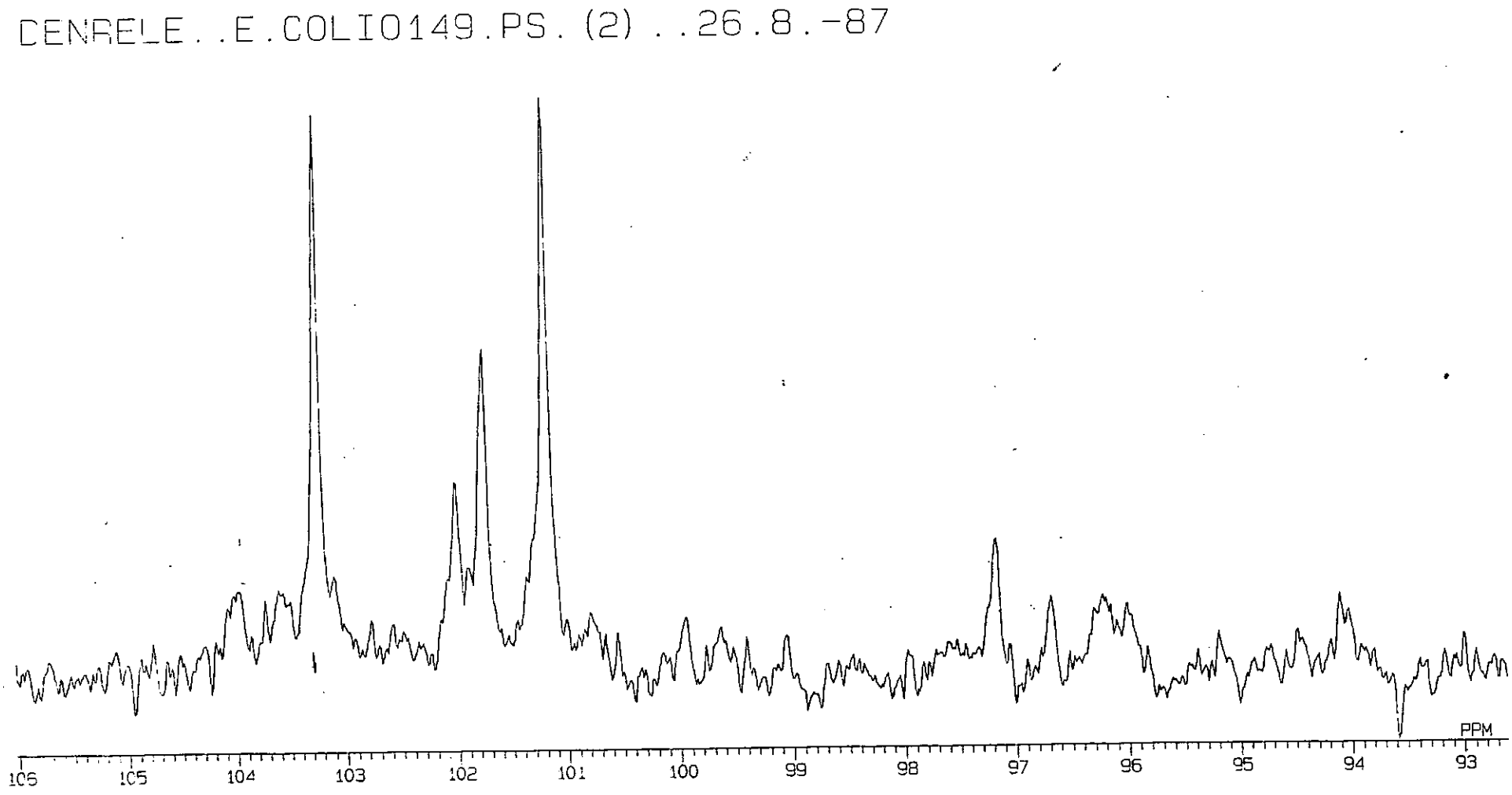


Figure 5.11. ^{13}C n.m.r. spectrum of *Escherichia coli* 0149 PS 2. Anomeric Expansion.

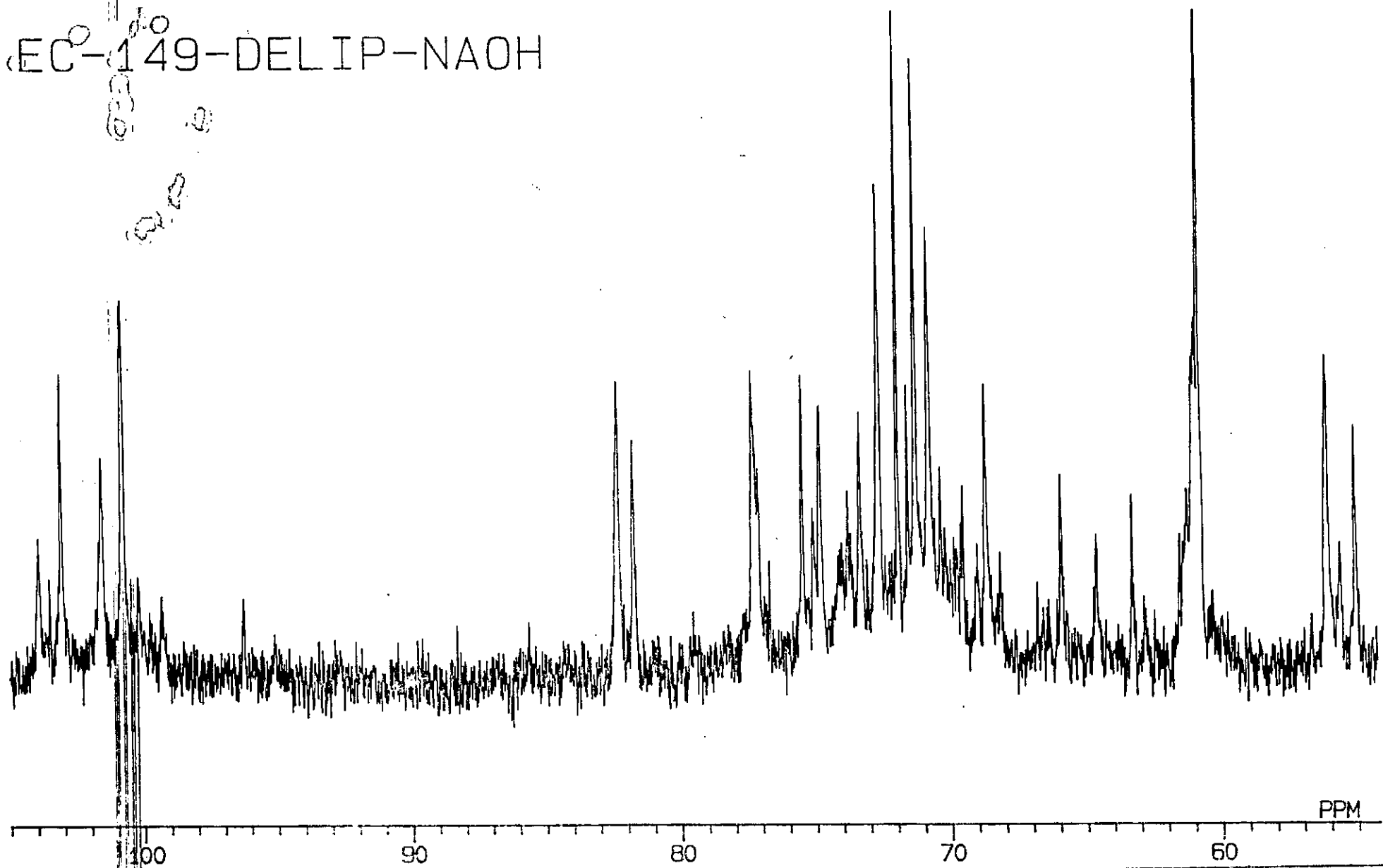
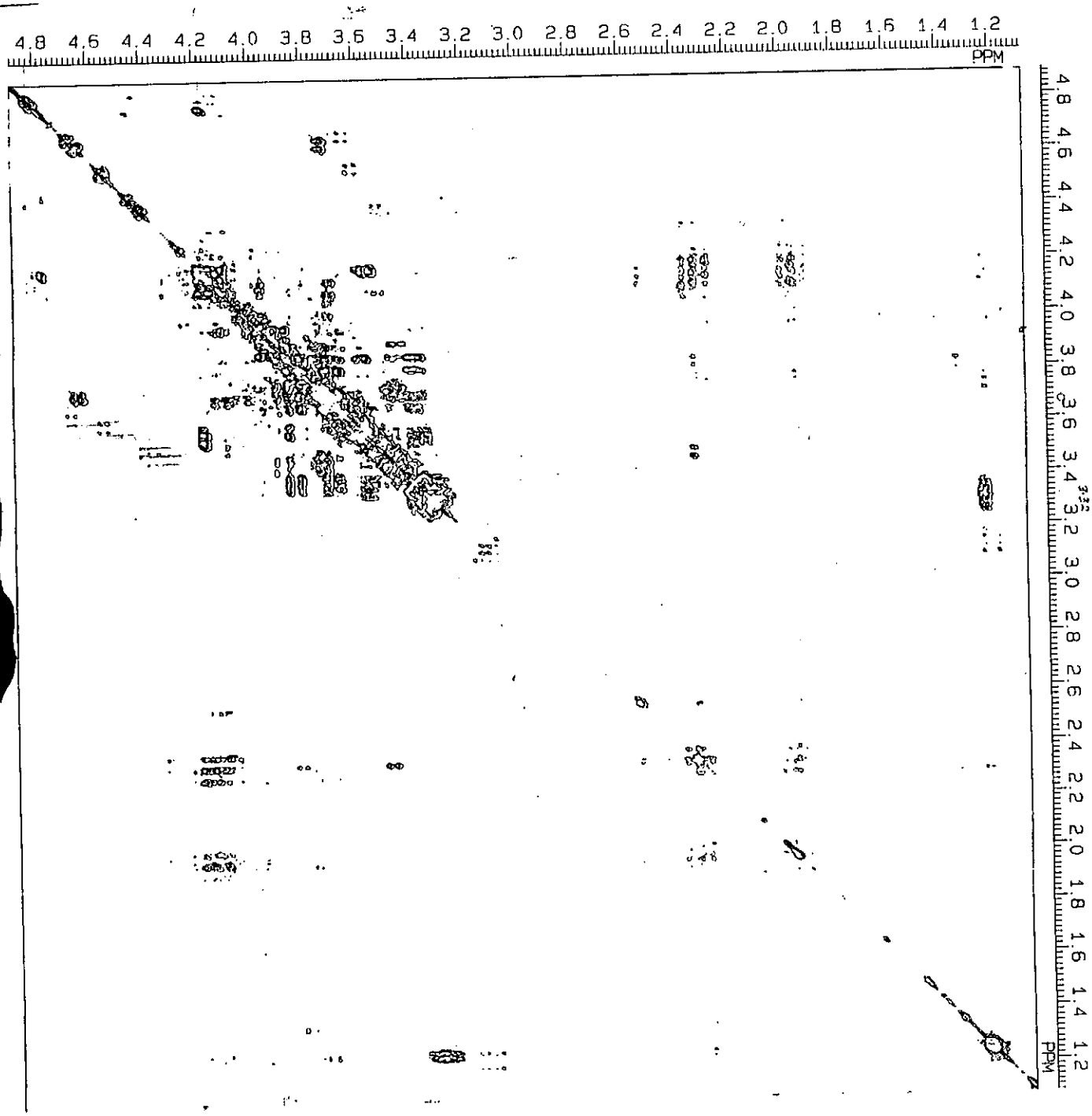


Figure 5.12. ^{13}C n.m.r. spectrum of *Escherichia coli* 0149 PS 3. Anomeric Expansion.

Figure 5.13. COSY spectrum of *Escherichia coli* 0149 PS 2.



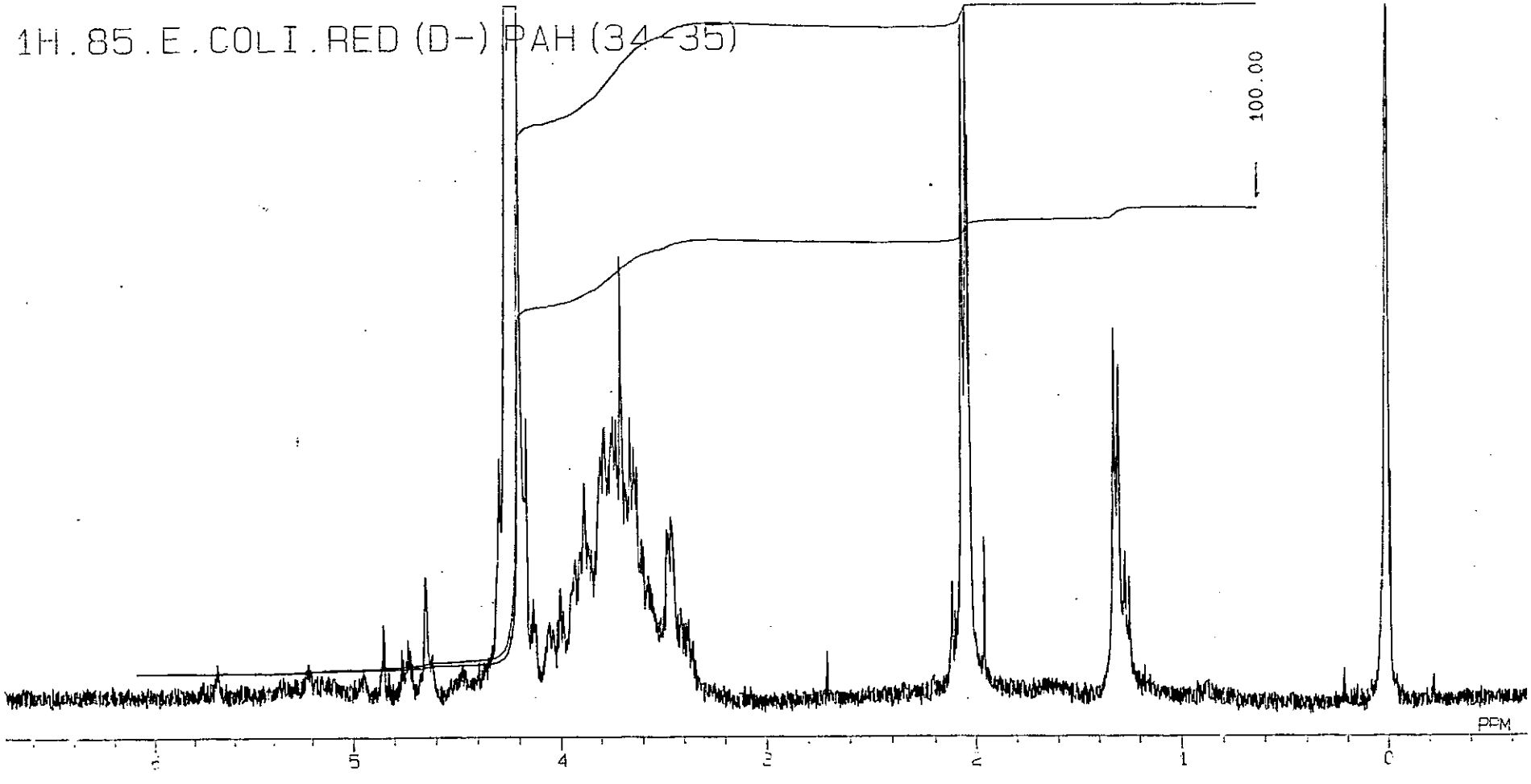


Figure 5.14. ^{1}H n.m.r. spectrum of Partial Acid Hydrolysis of *Escherichia coli* 0149 PS 2.

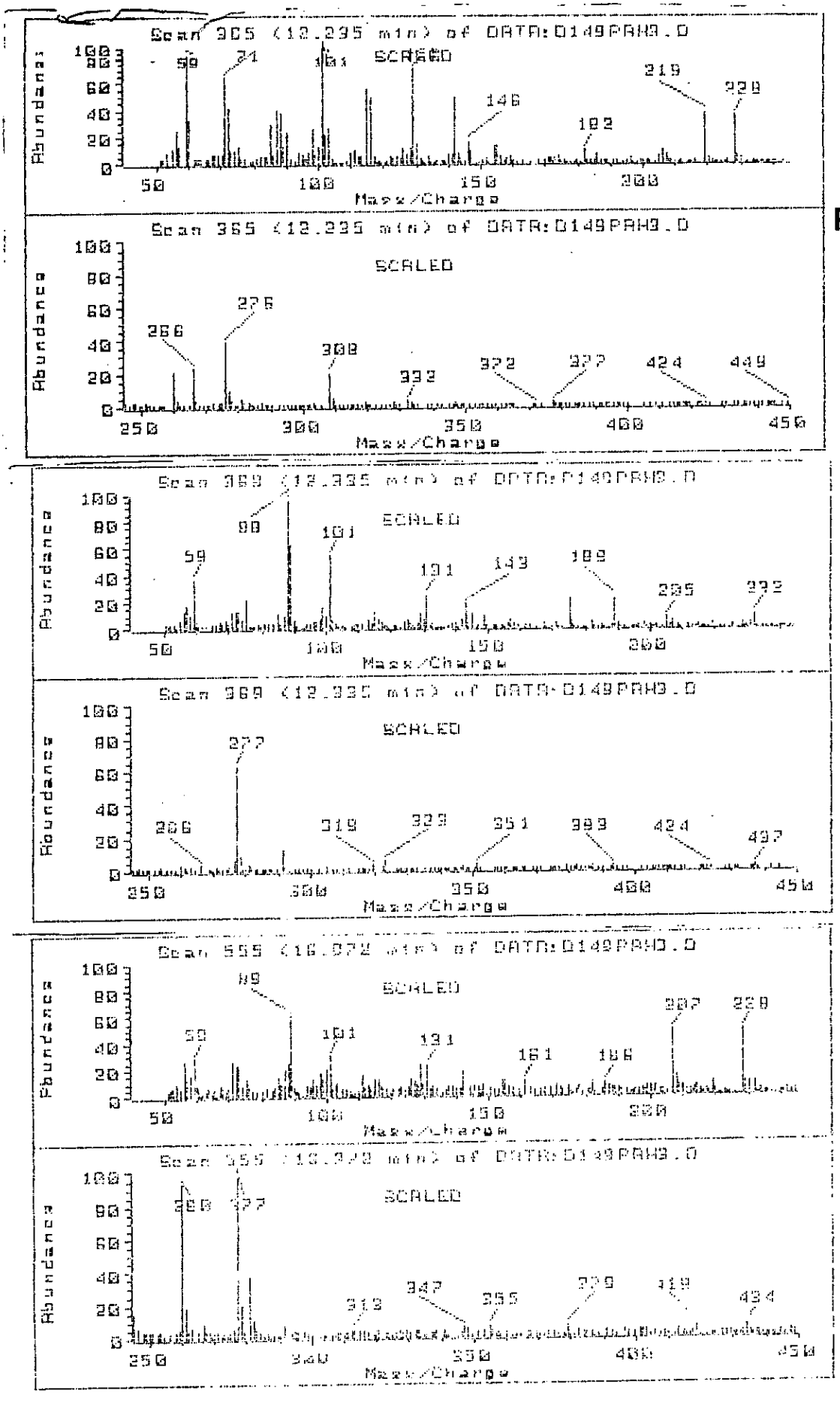


Figure 5.15.

Mass Spectra of R.8, R.9, R.10 from PAH of *Escherichia coli* 0149 PS 2.

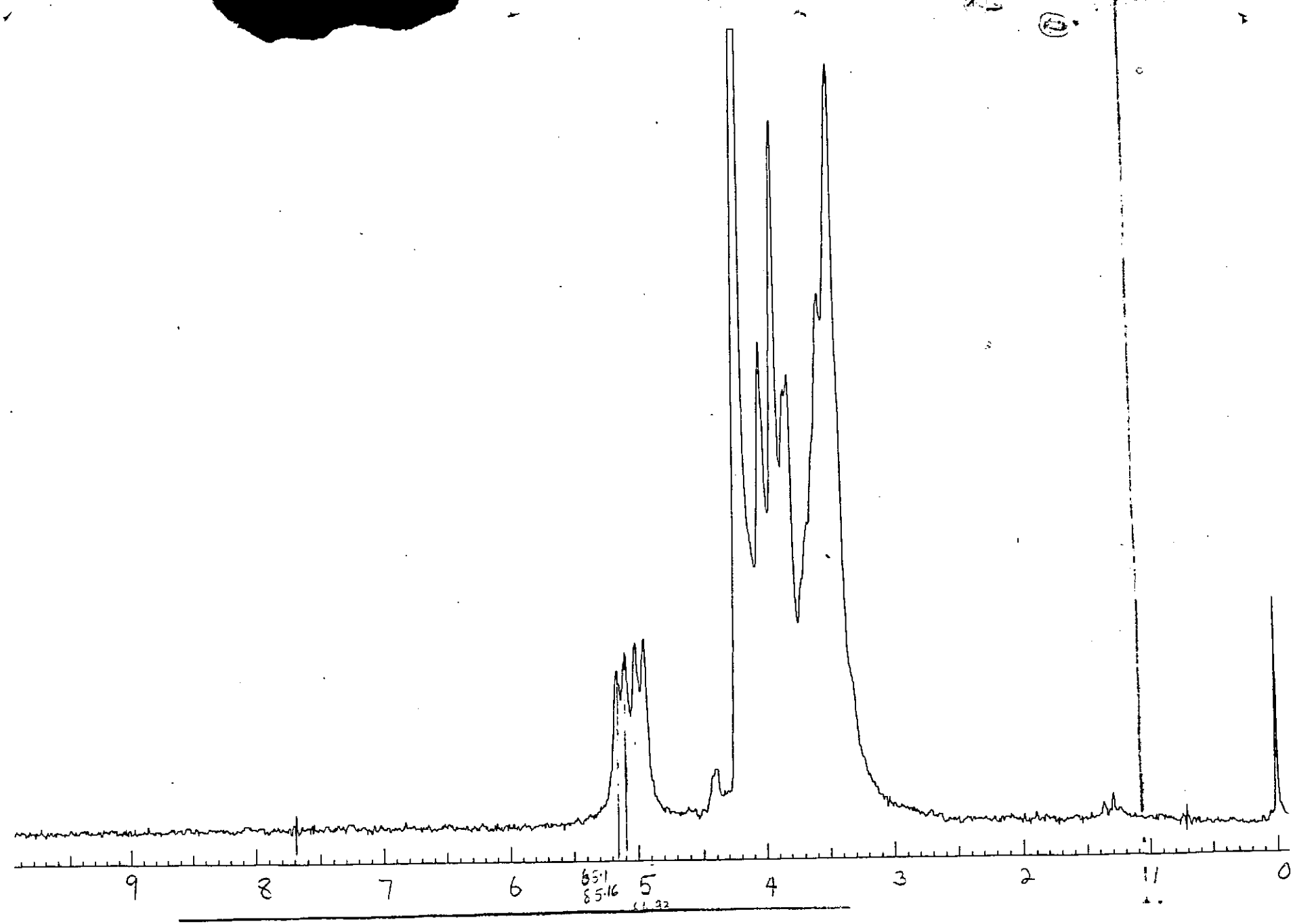


Figure 5.16. ^{1}H n.m.r. spectrum of <u>Streptococcus Pneumoniae</u> Type 37 Native PS.

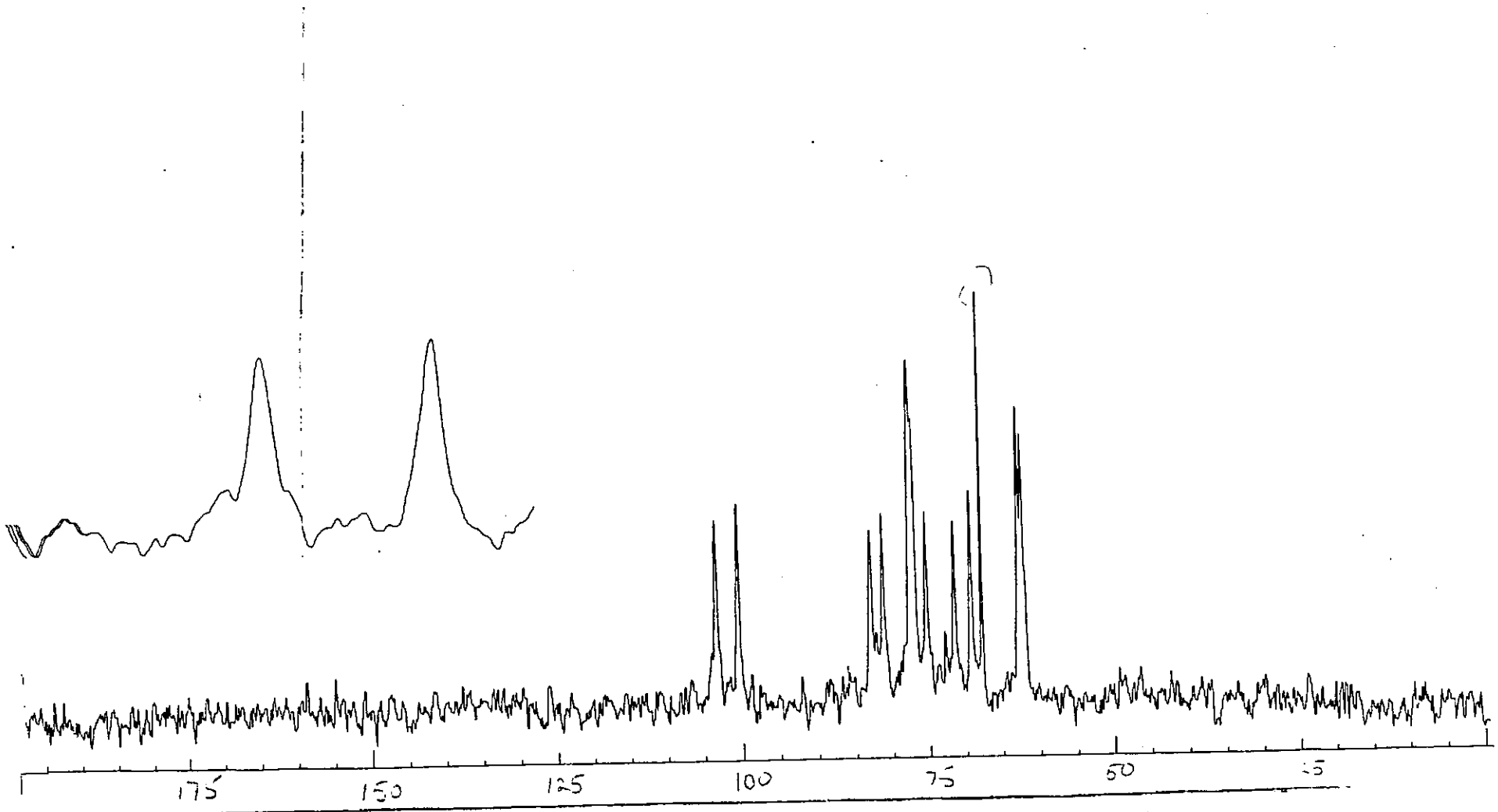


Figure 5.17. ^{13}C n.m.r. spectrum of Streptococcus Pneumoniae Type 37 Native PS

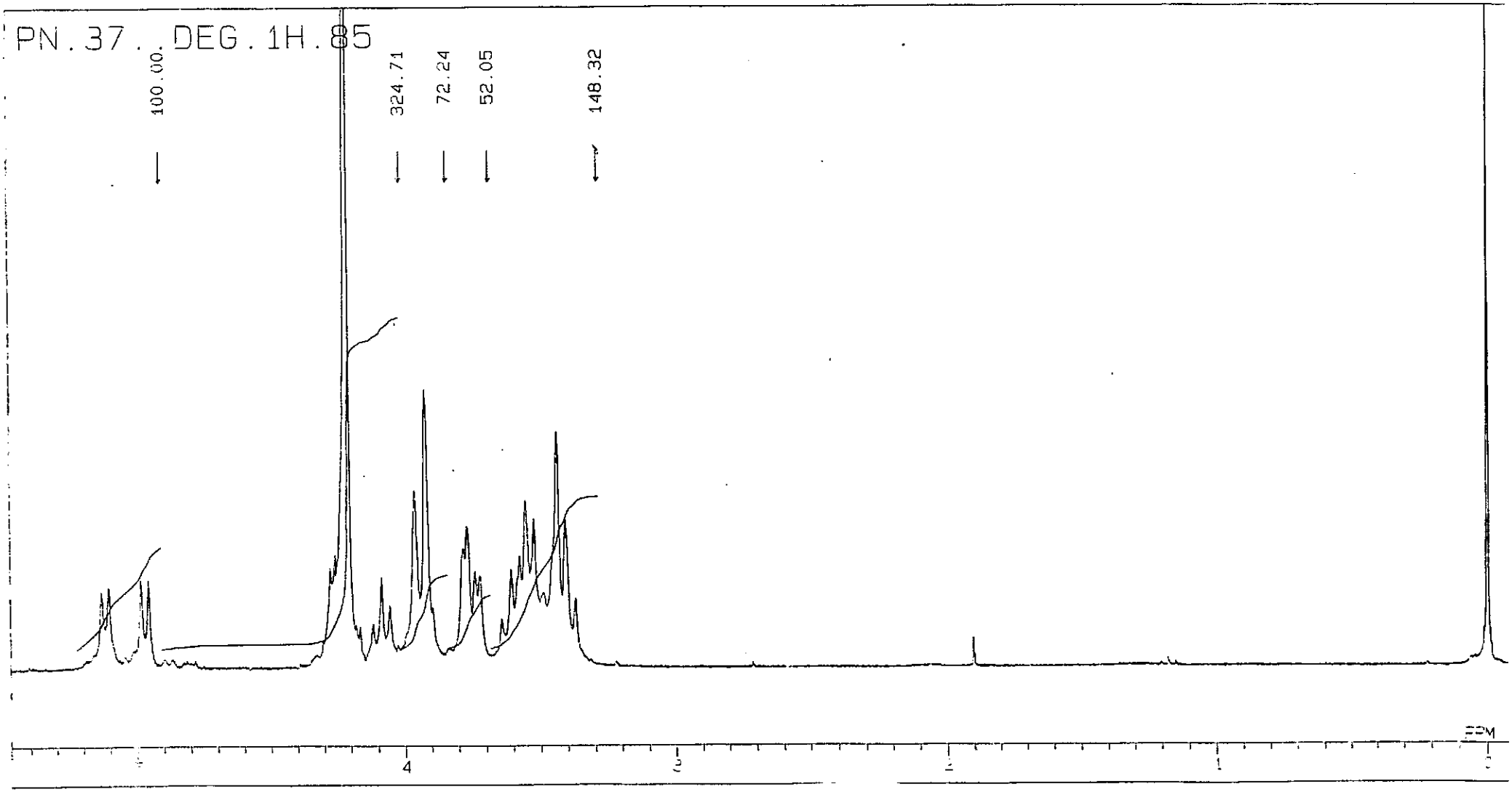
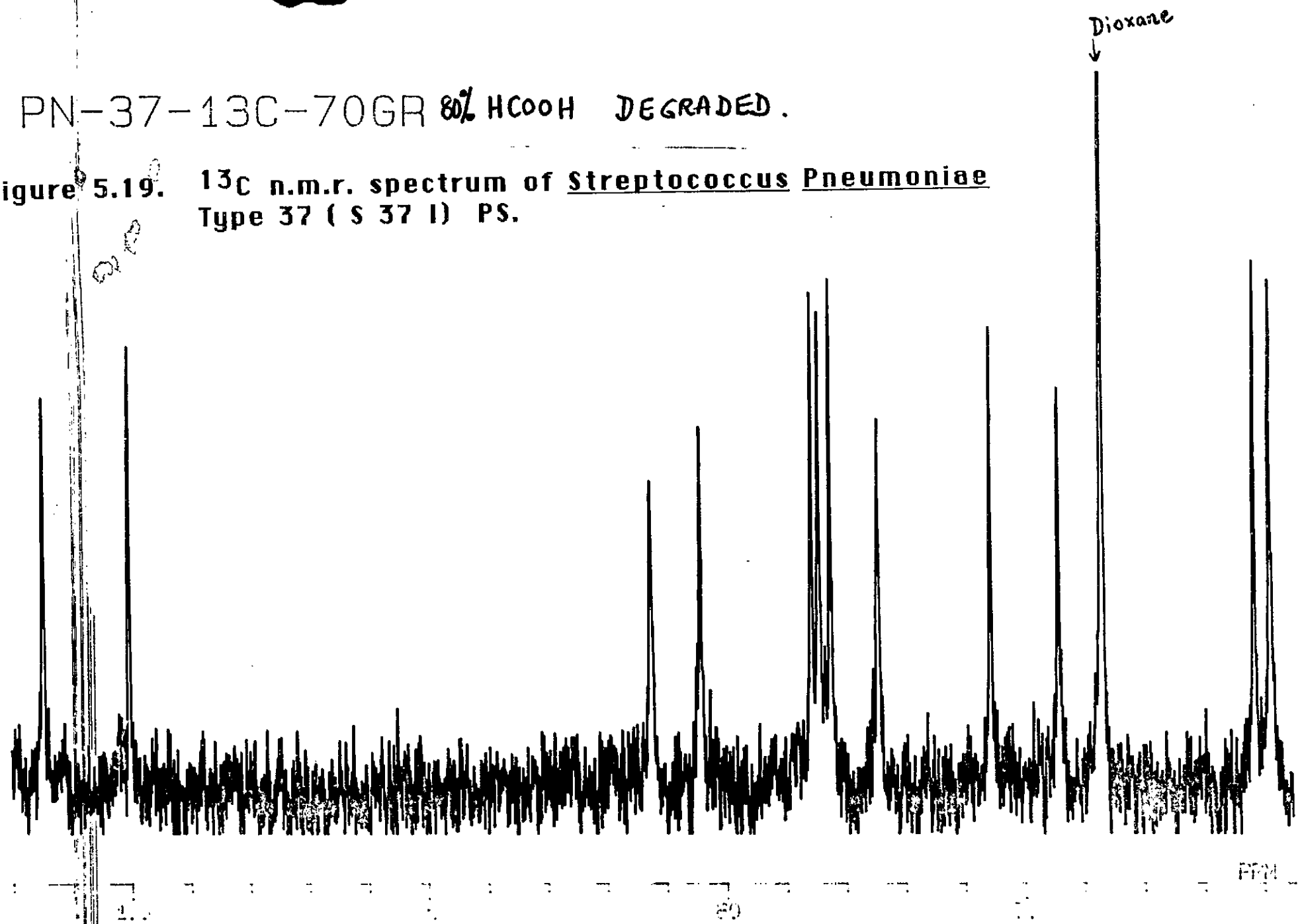


Figure 5.18. ^{1}H n.m.r. spectrum of Streptococcus Pneumoniae Type 37 (S 37 I) PS

PN-37-13C-70GR 80% HCOOH DEGRADED.

Figure 5.19. ^{13}C n.m.r. spectrum of Streptococcus Pneumoniae Type 37 (S 37 I) PS.



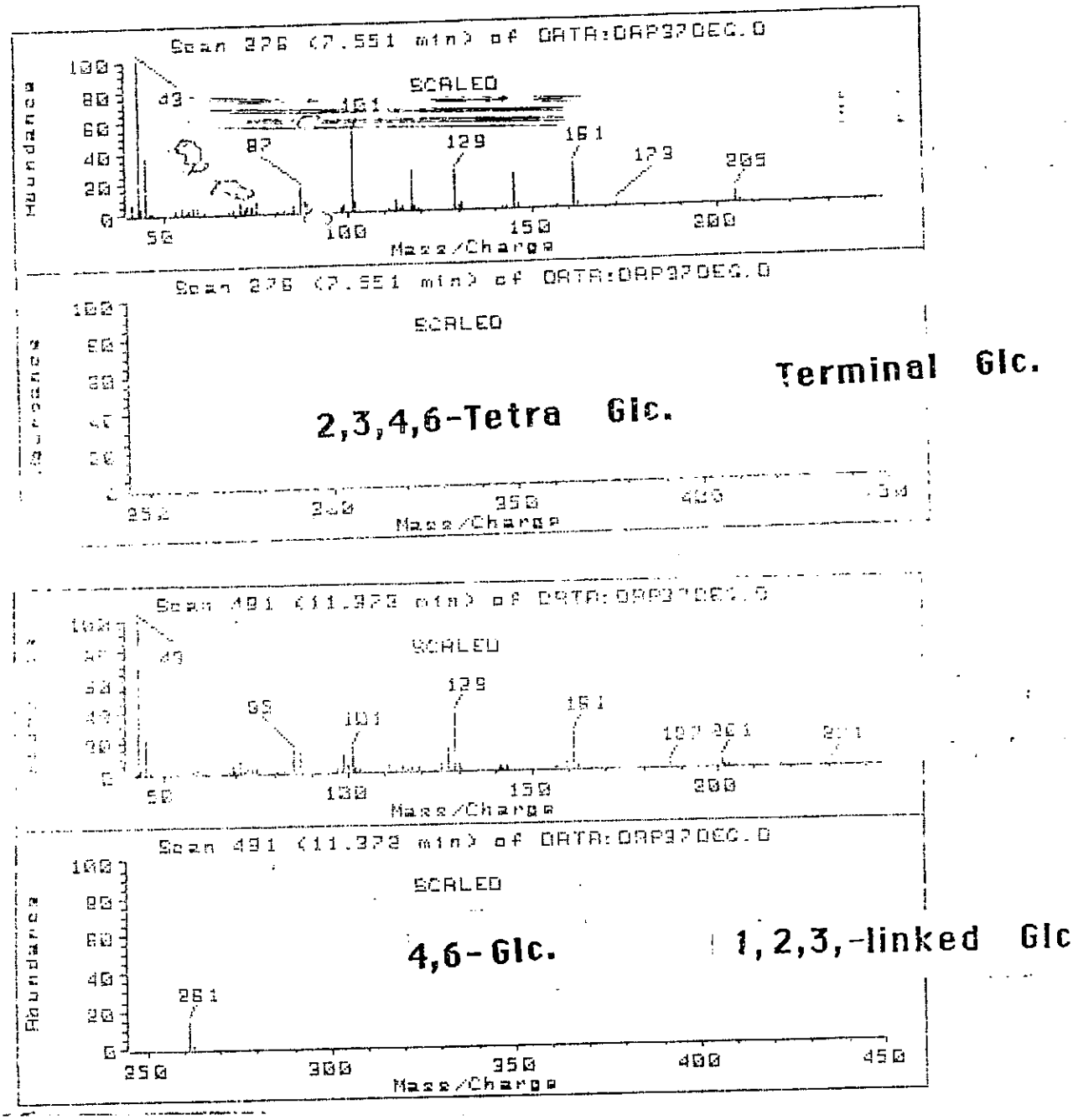


Figure 5.19a.

Mass Spectra of PMAA of <u>Streptococcus</u> <u>Pneumoniae</u> Type 37 (S 37 I) PS.

Figure 5.20. COSY spectrum of <u>Streptococcus</u> <u>Pneumoniae</u> Type 37 (S 37 I) PS

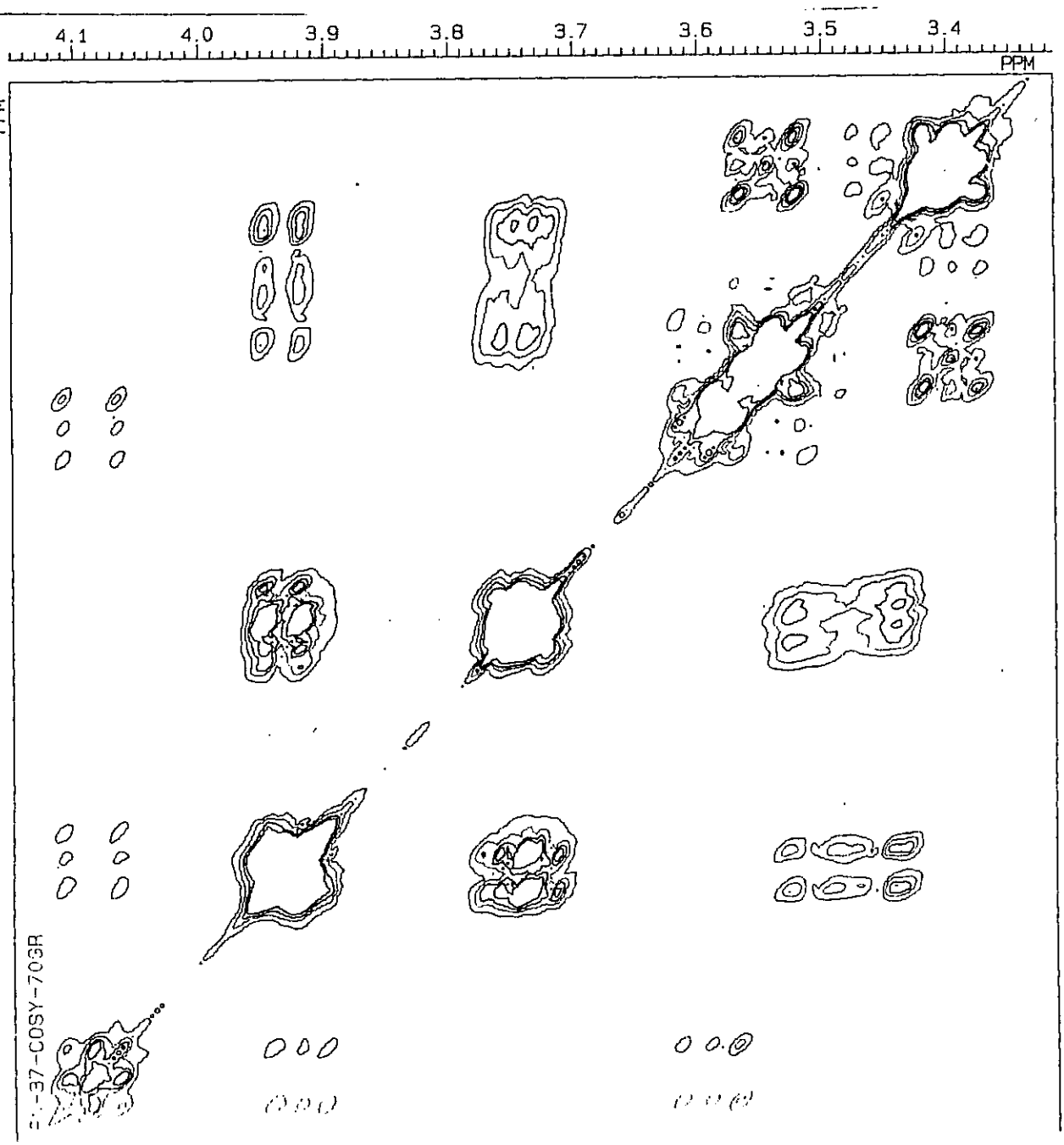
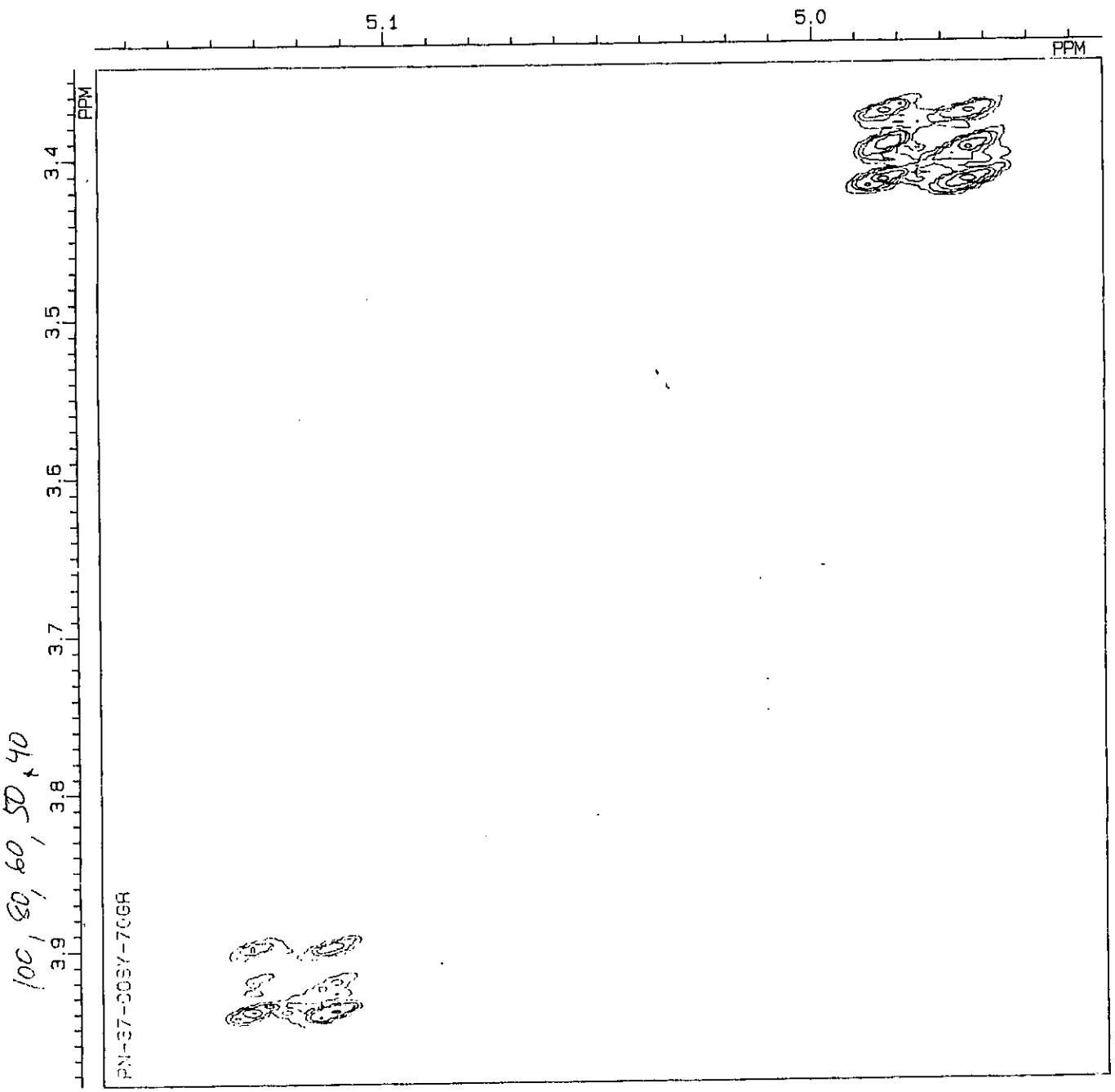


Figure 5.20. COSY spectrum of <u>Streptococcus</u> <u>Pneumoniae</u> Type 37 (S 37 I) PS continued.



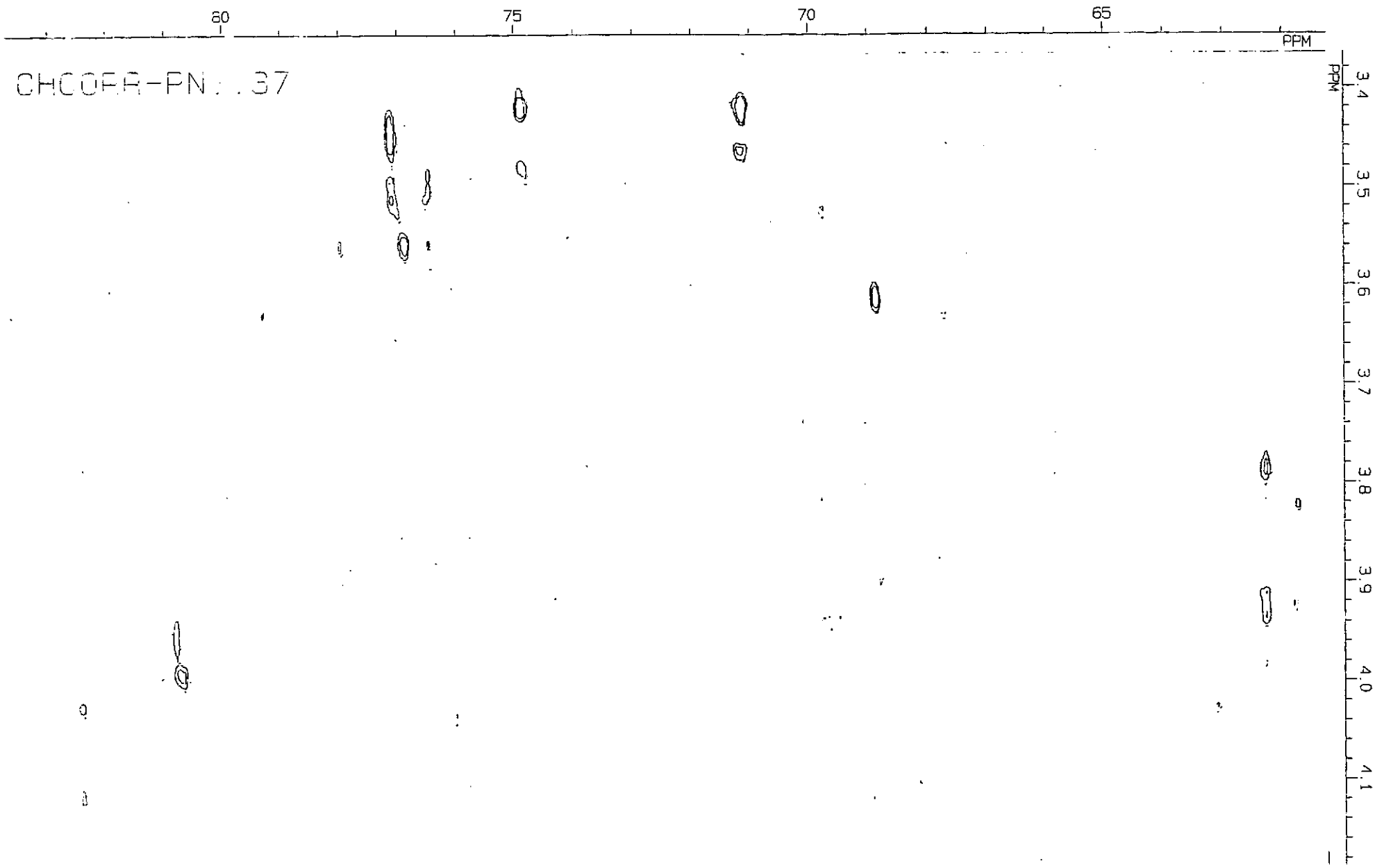


Figure 5.21. CH Correlated spectrum of <u>Streptococcus</u> <u>Pneumoniae</u> Type 37(S37I) PS.
Type 37 (S 37 I) PS

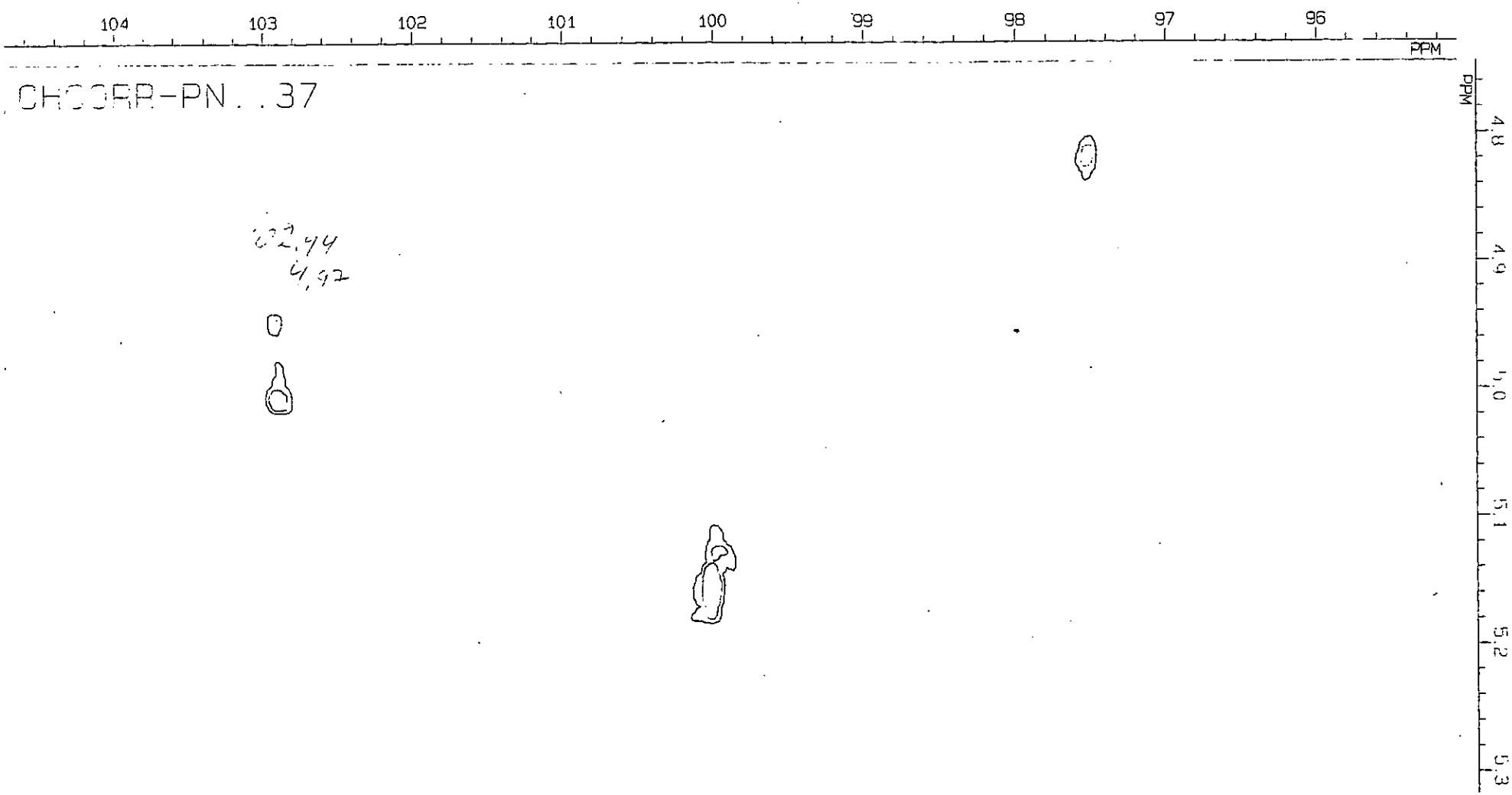


Figure 5.21. CH Correlated spectrum of Streptococcus Pneumoniae Type 37 (S 37 I) PS continued.

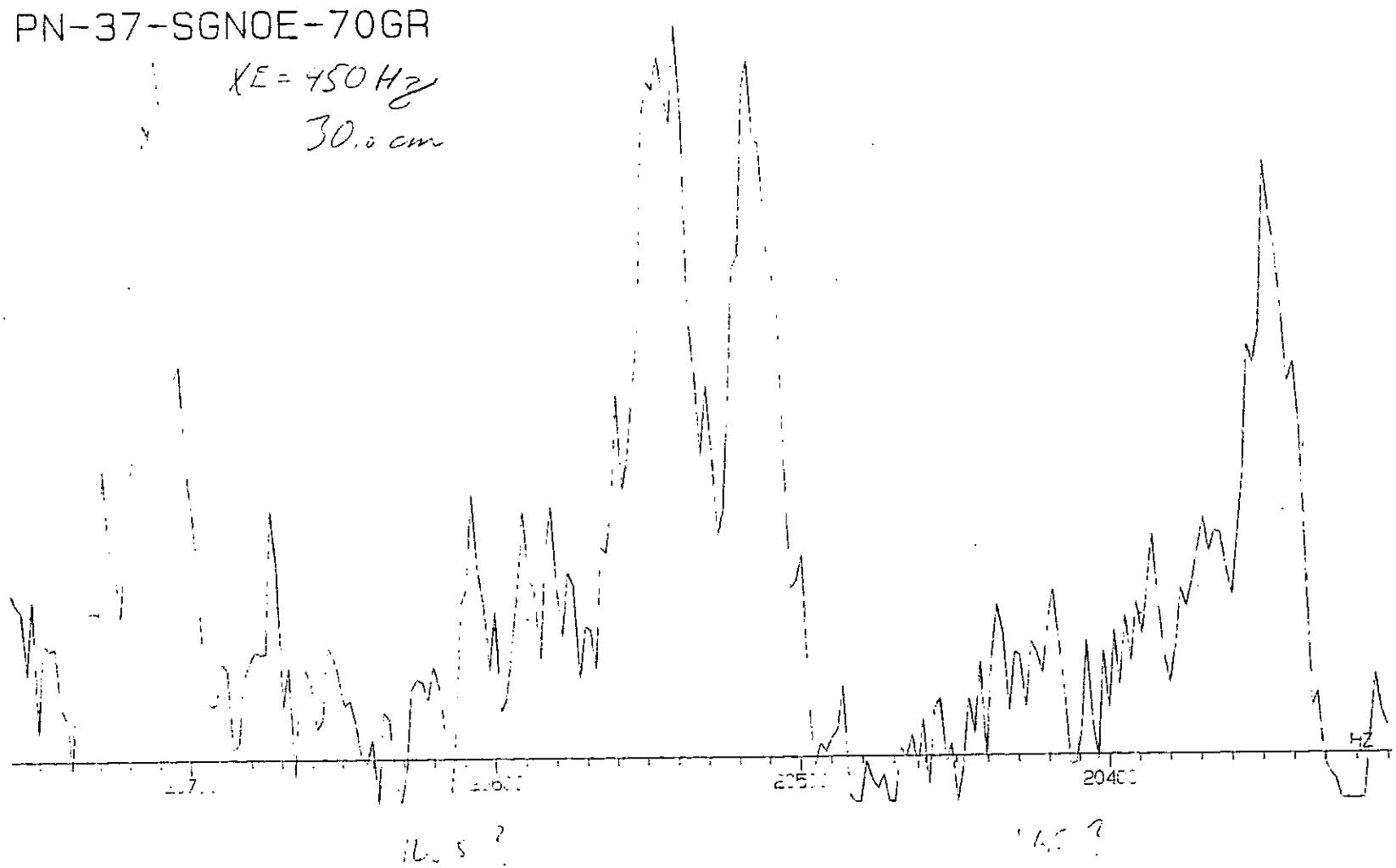


Figure 5.22. **NOESY spectrum of <u>Streptococcus</u> <u>Pneumoniae</u> Type 37 (S 37 I) PS.**

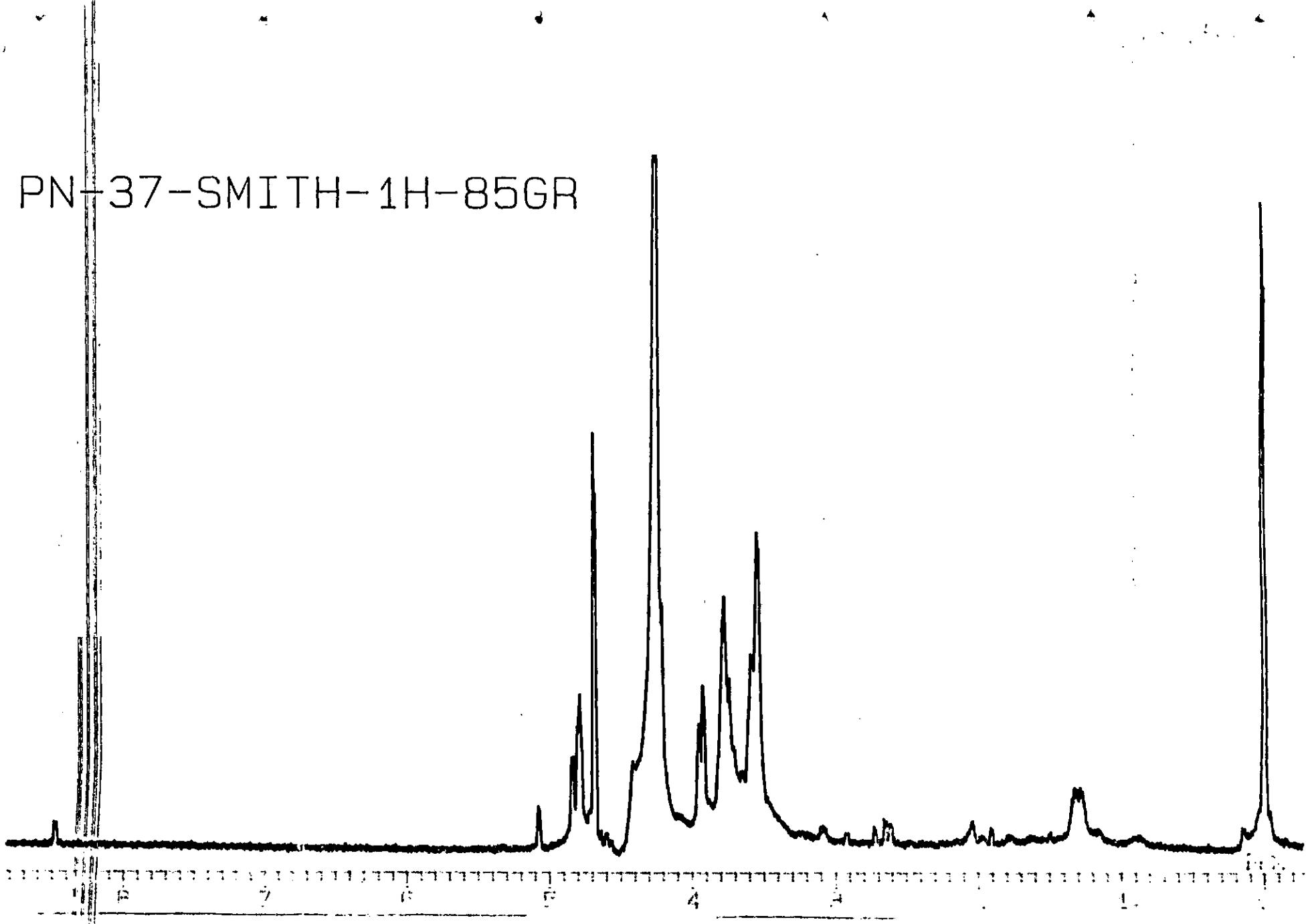
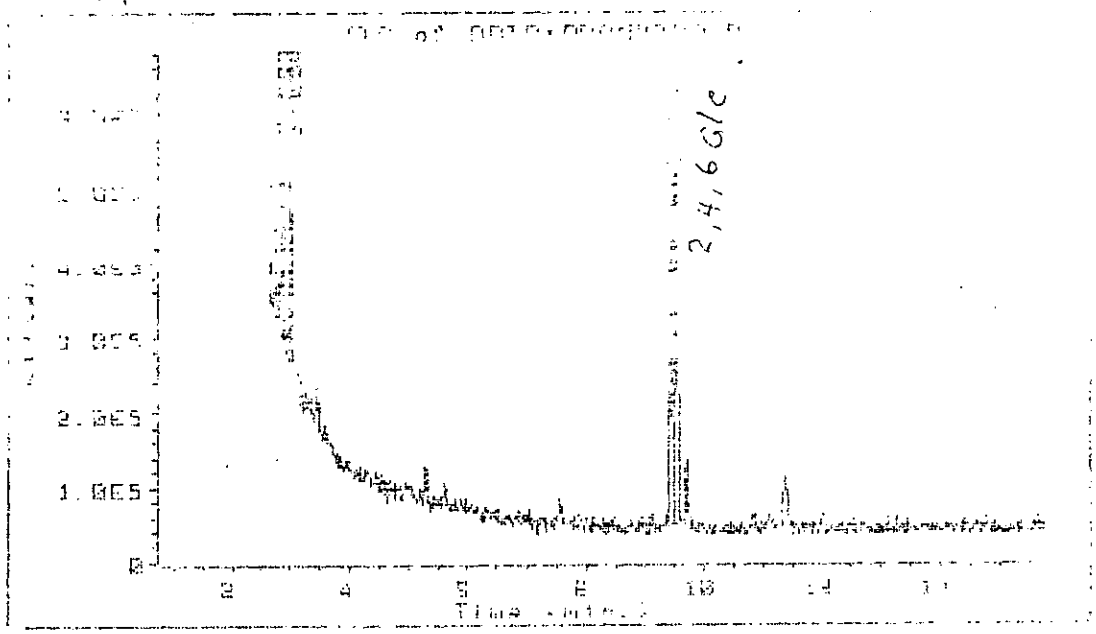


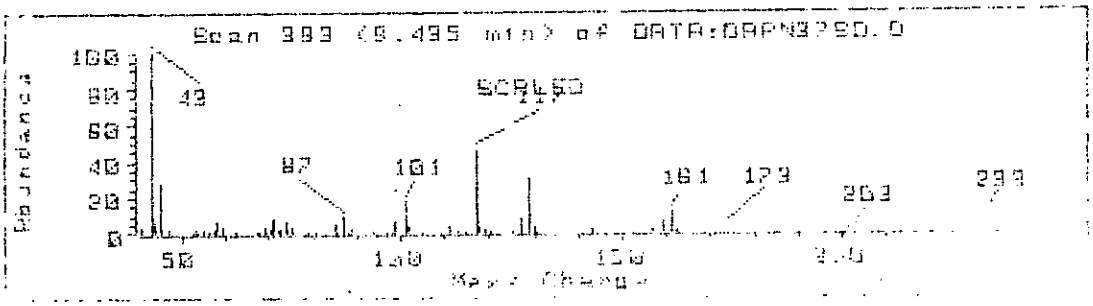
Figure 5.23. ^{1}H n.m.r. spectrum of <u>Streptococcus Pneumoniae</u> Type 37 (S 37 I) PS Smith Degradation Product.

Name Info: PN 37 HCOOH degraded Smith degradation MA
Misc Info: 190/250C,3min,HP SE-54 c 1,2Mpa,[illegible]
Operator : DENGLE

Date: [illegible] May 87 5:37 pm
Instrument: MS 5970
Inlet : GC

Sequence index : 0
Ms bottle num : 0
Replicate num : 1





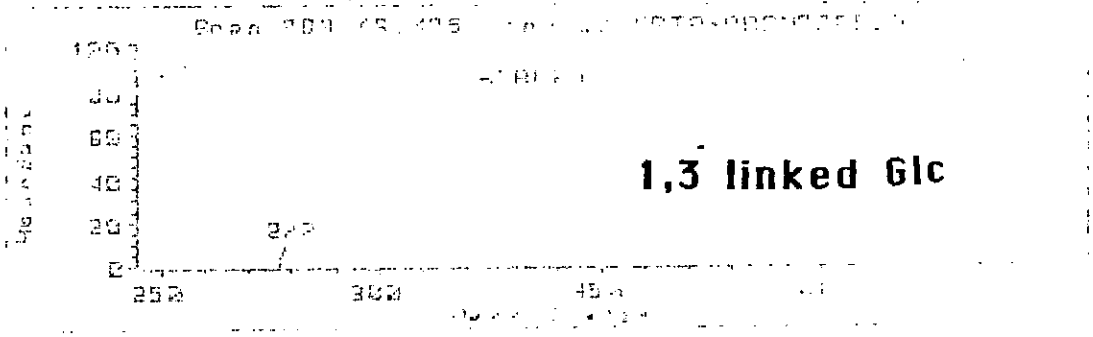
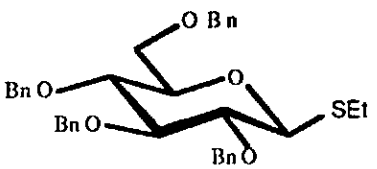


Figure 5.24.

Mass Spectra of PMAA of (S 37 I) PS Smith Degradation Product.

5.5. Synthesis, N.M.R. and Conformational studies of the Model Trisaccharides.

For the preparation of the four combinations of the linear trisaccharides D-Glc-(1→2)-D-Glc-(1→3)-α-D-Glc-(1→OMe, two routes were attempted. One route working towards the reducing end and another route working towards the non-reducing end were employed. Silver trifluoromethanesulfonate mediated glycosidations[247,267] were used in all the reaction steps. The first route was used for synthesis of α-D-Glc-(1→2)-α-D-Glc-(1→3)-α-D-Glc-1→OMe (**13**). Ethyl 2,3,4,6-tetra-O-benzyl-1-thio-β-D-glucopyranoside (**5**) was treated with bromine to yield 2,3,4,6-tetra-O-benzyl-α-D-glucopyranosyl bromide (**5a**) which was condensed with ethyl 3-O-acetyl-4,6-O-benzylidene-1-thio-β-D-glucopyranoside (**7**) to yield the disaccharide (**8**) in 63% yield. Treatment of (**8**) with bromine gave a disaccharide bromide (**8a**) which was used in the glycosidation of methyl-2-O-benzyl-4,6-O-benzylidene-α-D-glucopyranoside (**11**) to yield the blocked trisaccharide (**12**) in 34% yield. The protecting groups were removed from (**12**) by treatment with methanolic sodium methoxide followed by catalytic hydrogenolysis with palladium on carbon, to give after gel-filtration, α-D-Glc-(1→2)-α-D-Glc-(1→3)-α-D-Glc-(1→OMe in 95% yield based on the weight of the blocked trisaccharide. For the synthesis of α-D-Glc-(1→2)-β-D-Glc-(1→3)-α-D-Glc-(1→OMe (**21**) and β-D-Glc-(1→2)-β-D-Glc-(1→3)-α-D-Glc(1→OMe (**24**), the second route with a common disaccharide intermediate (**19**) using (**16**) as the starting material. 3,4,6-tri-O-benzyl-1,2-O-(1-ethoxylidene)-α-D-glucopyranoside (**16**) was treated with hydrogen bromide in glacial acetic acid[253, 254] to give the 2-O-acetyl-3,4,6-tri-O-benzyl-α-D-glucopyranosyl bromide (**17**) which was used in the glycosidation of (**11**) to give the disaccharide (**18**) in 73% yield.



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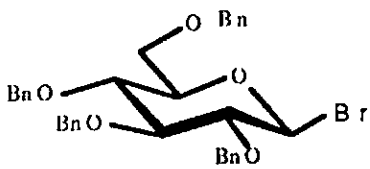

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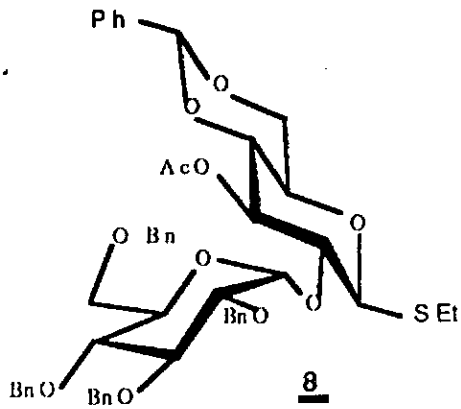

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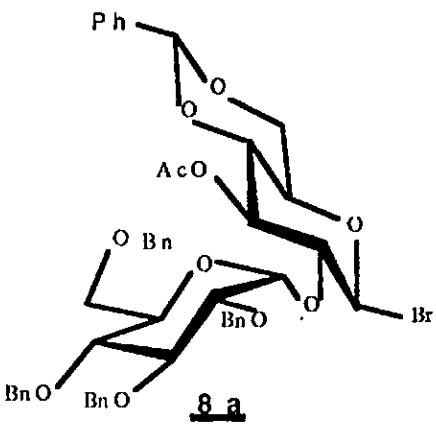

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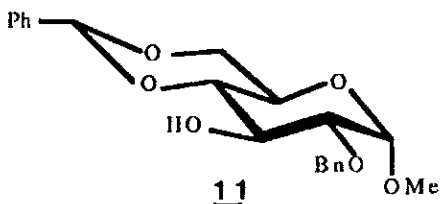

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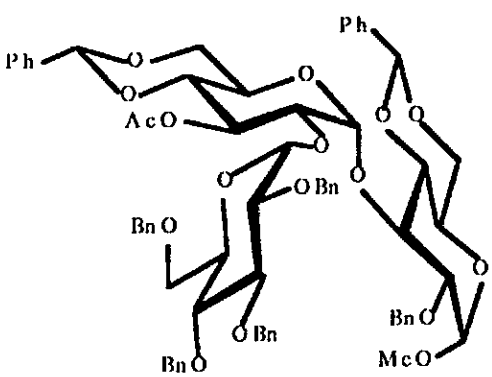
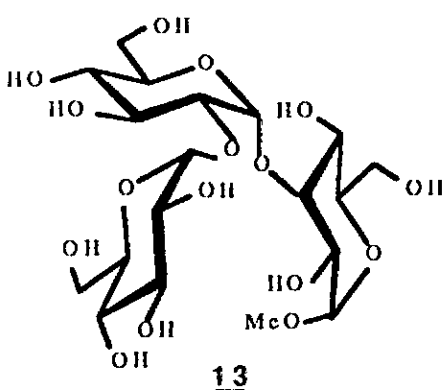


12

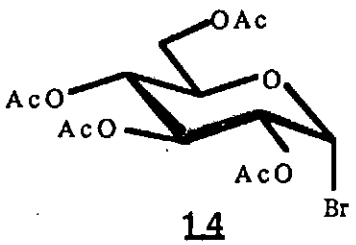


13

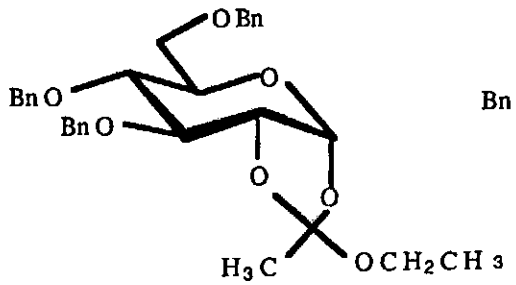
O-deacetylation of (18) in methanolic sodium methoxide gave (19) in 87% yield. This disaccharide was condensed with (5a)to give (20) (in 36% yield) which was deblocked as described for (13) to give α-D-Glc-(1→2)-β-D-Glc-(1→3)-α-D-Glc-(1→OMe (21) in 95% yield.

For the synthesis of (24), disaccharide (19) was condensed with tetra-O-benzoyl-α-D-glucopyranosyl bromide (22) in a glycosidation to yield (23) which was deblocked as earlier described for (α-D-Glc-(1→2)-β-D-Glc-(1→3)-α-D-Glc-(1→OMe (21) to give β-D-Glc-(1→2)-β-D-Glc-(1→3)-α-D-Glc-(1→OMe (24) in 76% yield. The preparation of the last trisaccharide β-D-Glc-(1→2)-α-D-Glc-(1→3)-α-D-Glc-(1→OMe (30) also employed a second route in which 2-O-trichloroacetyl-3,4,6-tri-O-acetyl-α-D-glucopyranosyl chloride[248e] (25) was condensed with (11) to give (26) in 38% yield. O-deacetylation of (26) with methanolic sodium methoxide yielded (27) which was treated with benzaldehyde and zinc chloride to give (28) in 60% yield. This disaccharide was condensed with 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (14) to yield (29) which was delocked as described earlier to give β-D-Glc-(1→2)-α-D-Glc-(1→3)-α-D-Glc-(1→OMe (30) with an anomeric purity of 85%.

The reason for choosing the reverse route in order to make (30) can be found in the following discussion. Our first approach to synthesize (30) was by trying to glycosylate (19) with (5) but this did not give the expected β-1-2-linked disaccharide after work-up. Using another procedure to synthesize the trisaccharide, the β-1-2-linked disaccharide (8) was subjected to glycosylation with (11) using methyl triflate. The expected β-1,2-α-1,3-linked trisaccharide was not formed but a transglycosylation product of 1,3 linked disaccharide was obtained.



14



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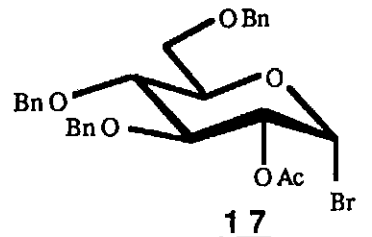

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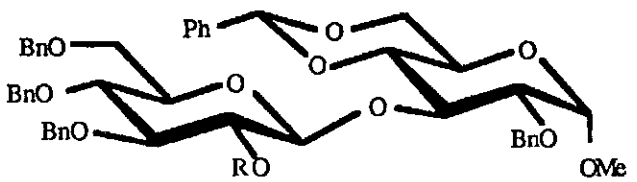
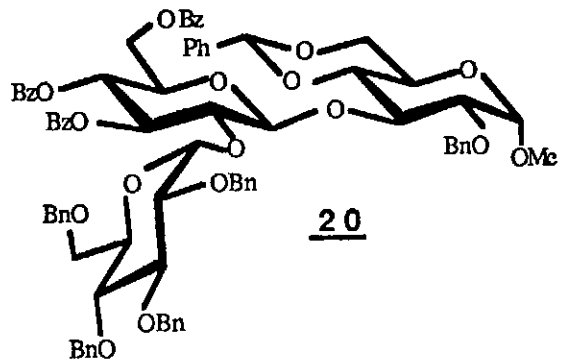


R = Ac 18

R = OH 19



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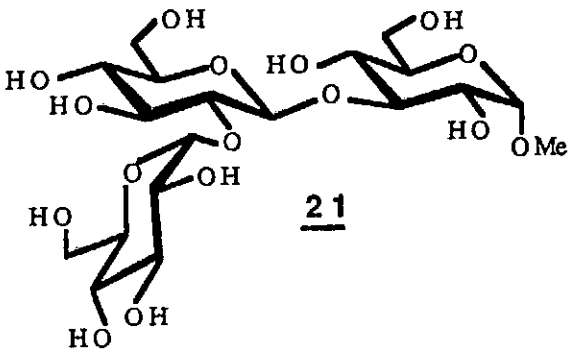

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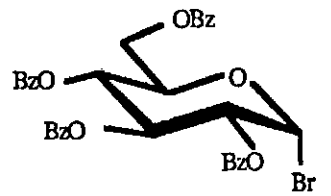

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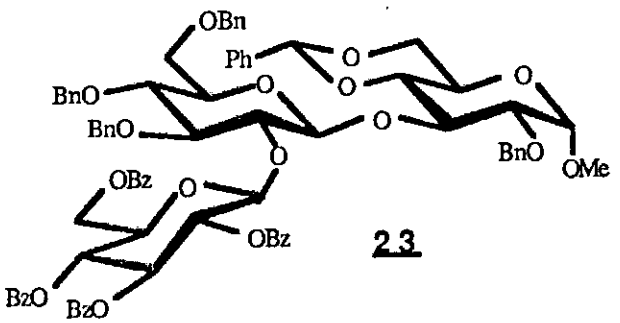

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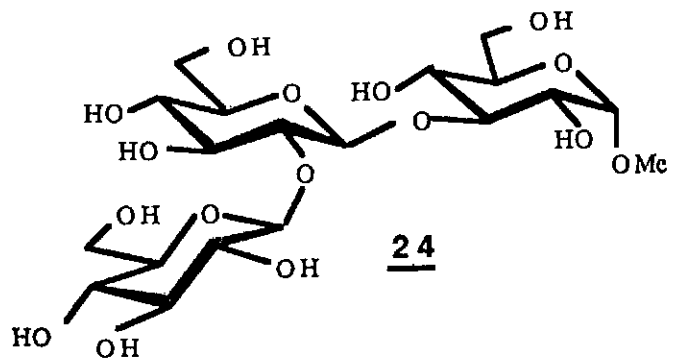

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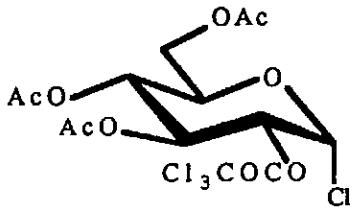

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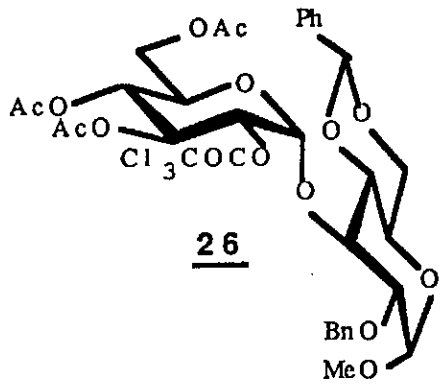
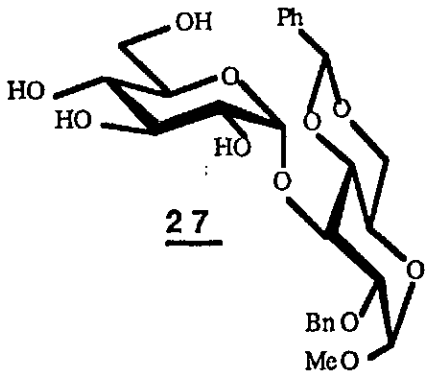
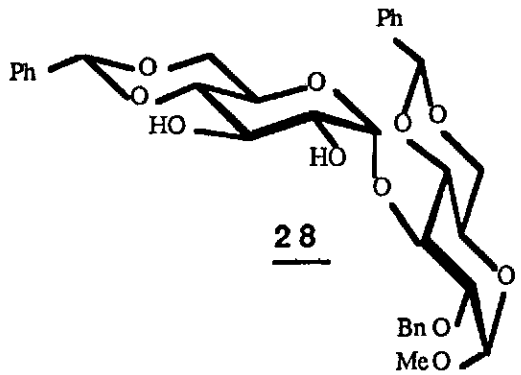


26



27



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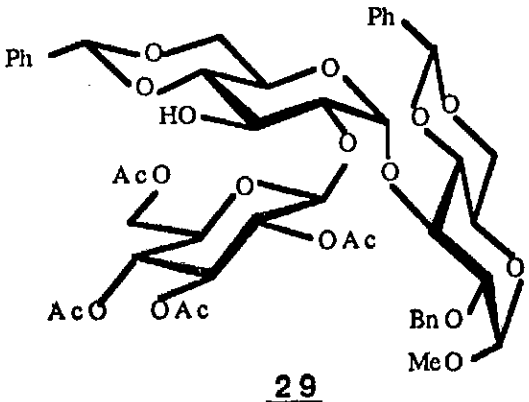

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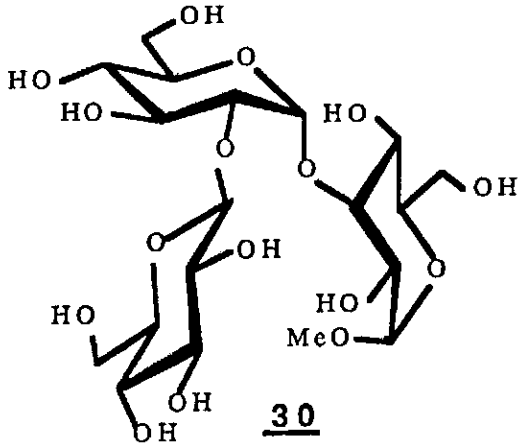


30

The reverse approach was then attempted. Compound (**26**) was selectively O-de(trichloro)acetylated using ammonia in diethylether[248e] but attempting to glycosylate the resulting disaccharide with (**14**) failed due to the unreactivity of the disaccharide and the expected trisaccharide was again not obtained.

The ^{1}H and ^{13}C n.m.r. spectra of **13**, **21**, **24**, and **30** as well as some important intermediate disaccharides and trisaccharides are shown in Index III.

N. M. R. Assignments.

^{1}H n.m.r. spectra

The ^{1}H n.m.r spectra of **13**, **21**, **24** and **30** are shown in Figures 5.25 to 5.28 in Index III. The chemical shifts and chemical shift differences for all protons relative to those of parent monomers are given in Table 5.17. The signals for the anomeric protons are usually separated from the "bulk" regions and thus are easily observable in the n.m.r. spectra. In the glycosyl groups downfield shifts for signals of anomeric protons are obseved for the H' and H". For the protons of residues which are involved in the linkages, most of the chemical shifts displacements are downfield and they vary between 0.07 and 0.38ppm. α-glucosyl 5'-H appears for **13**, **21**, **24** and **30** at between δ3.97 and δ4.05 which is a downfield displacement of ~0.12 to 0.20ppm downfield of the chemical shift in α-glucopyranose. A possible explanation for this displacement and those of other protons in similar arrangements has been given by Lemieux et al[176]. Downfield shifts may be obtained when the proton is close to one or two oxygen atoms in the neighbouring sugar residues. This effect is borne out by oxygen-hydrogen or hydrogen-hydrogen contacts arising from the HSEA calculations of the minimum energy conformations shown in in Table 5.16.

The displacements for the signals of the neighbouring β-protons of the middle glucopyranosyl residue are appreciable. All shifts are positive i.e. downfield and the largest 0.38ppm is that for H'2 in **30**. In the α-glucopyranosyl groups small downfield shifts are obtained for most protons.

The smaller shift of the anomeric protons having the α-anomeric configuration is of structural significance since this may be due to the restricted rotational freedom observed from the HSEA calculations. Similar effects on 5'H to 5"-H are observed for the glucopyranosyl residues having α-anomeric linkages as in **13** and **21**. The anomeric proton of the glucopyranosyl non-reducing end of **13** is shifted upfield of that in **21** and might be due to the influence of the different anomeric linkage present in the middle glycopyranosyl residue. The same trend is observed in **24** and **30** where the chemical shift of the anomeric proton of the non-reducing glucopyranosyl of **24** is displaced upfield relative to the corresponding residue in **30**. This might probably be a short range effect where the anomeric configuration of one residue affects the chemical shift of a residue linked next to it.

The same effect is observed for the chemical shifts of the anomeric proton of the β-linked residues where the middle glucopyranosyl in **21** is shifted downfield to the largest extent because it is linked to an α-anomeric residue.

Similar shifts are observed for those of 1,2 and 1,3 disaccharides for which HSEA calculated conformations indicate additional proton interactions to 1'-H [240]. For the disaccharide residues Di's. 1a, 1b, 2a, 3b and 4b in the trisaccharides both proton-proton and proton-oxygen contacts for 1'-H are indicated and the anomeric signals have only a minor

shift.

For the trisaccharides in which the anomeric proton is shifted downfield an interaction with O-3 is indicated in the HSEA calculations. In addition to the effect observed for the anomeric protons, downfield shifts for 2'-H of the β-glucosides are observed.

Signals for 5'-H are shifted downfield in the α-D-glucopyranosyl unit but are almost unaffected or shifted upfield in the β-linked glycopyranosyl residues. The calculated contacts from 5' and 5"-H with oxygen or hydrogen are probably responsible for the downfield or upfield shifts respectively.

For all the units in which 1'-H has a calculated contact to an oxygen in the methyl glycoside residue e.g to O-3, the corresponding proton (H-3) has the largest downfield shift of the two protons adjacent to the linkage. The substituent shifts for the **13**, **21**, **24** and **30** do not have the same values and can be explained that the anomeric nature and thus the disposition of the glycosyl units in the preferred conformation have a noticeable effect on the chemical shifts.

From the comparison between substituent shifts for signals of protons on the linkage carbon and the adjacent carbon atoms, it may be possible to determine the linkage positions from the magnitude of the chemical shift displacement in this set of trisaccharides. The magnitude of these displacement shifts is comparable to those reported for some disaccharides[206,240,259]. The same trend has been observed in the ^{13}C.n.m.r. spectra of the four trisaccharides.

^{13}C N.m.r spectra.

The ^{13}C n.m.r spectra of **13**, **21**, **24** and **30** are shown in Figures

5.29 to 5.32 in Index II .

The ^{13}C n.m.r. chemical shifts and chemical shift differences for Tri's 1 to 4 are given in Table 5.18. The chemical shift differences were obtained for the glycosyl group by comparison with the corresponding reducing sugar and for the methyl glycoside residue by comparison with the corresponding methyl glycoside. Chemical shifts of the relevant monomers are also given in Table 5.18. The non-reducing glucose monosaccharides are used in the comparison of the chemical shifts of the glucopyranosyl groups because these are unaffected by the methyl group of the methyl glucoside. In the following discussion, the convention of designating substituted carbons α-carbons and their vicinal carbons β-carbons is used.

For the glycosyl residues at the non-reducing end, downfield changes in the chemical shifts of the C"-1 are observed in all the four trisaccharides. The changes in C"-1 vary between 4.2 and 8.00 ppm with the lower shifts being recorded for the C"-1 atoms with the α-anomeric linkages.

In general, significant chemical shift changes (> 4 p.p.m.) are obtained only for the anomeric carbon of the glycosyl residues (C-1'and C-1") as well as the substituted carbons C-2' and C-3. However the most prominent shifts were recorded for C'-1, C'-2 and C'-3 (β-carbon) for the middle glycosyl unit substituted in position 2 and linked to the methyl glycoside residue. Also the values of the displacements of the chemical shifts for the α and the β-anomeric linkage carbons were consistent with the values of the downfield shifts of the former being lower than that of the latter.

In the middle glucopyranosyl residues of the trisaccharides, the substituted C-2 and their β-carbon atoms were remarkably shifted downfield

with the highest downfield shift being recorded for the C-3. The highest downfield chemical shift difference of 11.3 ppm is that obtained in the C-3 of the methyl glucoside residue of the **24**. All the β-atoms to the substituted atoms were shifted upfield and this is characteristic of all the four trisaccharides with varying values of -0.0 to -1.7 ppm, the highest chemical shift difference for the β-atoms being recorded for **13**. On the glycosylation of C-3 in methyl α-D-glucopyranoside, by the middle glucopyranosyl residue, downfield displacements or small upfield displacement in the chemical shifts are observed: for C-1 a value of 4.2 ppm to 6.7 ppm. in the glycosyl group and for C-3 a value of 6.8 to 11.3 ppm in the methyl glucoside residue. Smaller upfield shifts of 0.7 to 1.5 ppm are observed for C-2.

For the C-4 in the methyl-glucoside residue i.e. the β-carbon atom in **21** and **30** to which the middle glucopyranosyl residue is linked by β-anomeric linkage, there is an upfield shift of 1.7 and -1.6 ppm respectively. This chemical shift displacement for the C-4 in the methyl-glucoside residue is noticeably low or even absent in **13** and **24** respectively. The signal for C-5" of the glucopyranosyl residue of the non-reducing end of the four trisaccharides are shifted downfield, the chemical shifts displacements varying from 0.2 to 0.5ppm.

The difference in the ^{13}C n.m.r spectra of **13** and **30** gives an indication of the substituent effects for glycosyl groups with an axial or equatorially disposed linkages. In the spectra of **13**, **21**, **24** and **30**, the substituents shifts for all the β-carbons are consistent becausealmost all are shifted upfield with just one exception. Since all the residues have similar absolute configuration, but different anomeric

configurations, the variation of the chemical shift displacement must derive from steric interactions between the monosaccharide residues over their respective glycosidic bonds.

As no major changes in conformation were observed from the HSEA calculations, the chemical shift displacement must derive from interactions between different atoms which are in van der Waals contact. The chemical shift displacement for carbon atoms with similar α- anomeric linkages in the two glycopyranosyls and the methyl glucopyranoside residues are somewhat similar and differ from those having β-configurations which have also their similar values.
This is in line with the fact that the α-anomers in ^{13}C n.m.r. spectra have their shifts upfield of the β-anomers which are normally displaced downfield from the rest of the other signals.

Calculation of ^{13}C n.m.r and ^{1}H n.m.r spectra of Trisaccharides using substituent shift of Related Disaccharides.

One of the aims of this project was to determine if from the chemical shift of a disaccharide having the same anomeric configuration and linkage could be used to compute the chemical shifts of a similar trisaccharide taking the additivity of the glycosidation or substituent shifts taken into consideration. Starting from the chemical shifts of α or β-D-glcucopyranose from ^{13}C n.m.r spectra, and firstly to account for glucosyl in the 2-position, the substituent shifts for the 1,2-linked disaccharide relative to the parent monosaccharide are added. To account for the glucosyl residue which is linked to position 3, the substituent shifts for 1,2 and 1,3-linked disaccharide[240] are added relative to the parent monosaccharide to

the corresponding chemical shift of α or β-D-glucopyranose. For the calculated chemical shifts of the methyl glucoside residue in the trisaccharides the substituent shift from the 1,3-linked disaccharide are added to the corresponding chemical shift of methyl α-D-glucopyranose. These sets of values give the calculated chemical shifts for the trisaccharides. These calculations are carried out for the ^{13}C n.m.r. chemical shifts and the results obtained for the four trisaccharides are shown in Tables 5.19 to 5.22. These calculated chemical shifts are compared to the observed values and the differences noted.

For example in Table 5.19, 92.99 ppm is the chemical shift for the C-1 of the α-D-glucopyranose and the substituent shift relative to the α-1,2 linked disaccharide is 4.58ppm[240], for C"-1, the sum of these two values is 97.57ppm. The difference between the experimentally observed chemical shift 97.18ppm and the calculated value is -0.039 ppm.

For C-1', the sum of the chemical shift for the C-1 of the α-D-glucopyranose which is 92.99 ppm, the substituent shift relative to the α-1,2 linked disaccharide -2.76ppm and the substituent shift relative to the α-1,3 linked disaccharide 6.96ppm is 97.19 ppm. The observed chemical shift is 97.25ppm, and the difference between the observed and the calculated value is 0.06ppm.

For C-1 of the methyl glycoside residue, chemical shift of methyl glycoside is 100.19ppm and the the substituent shift relative to the α-1,3 linked disaccharide 0.11ppm. The sum of these two values is 100.32 ppm and this gives the calculated chemical shift for C-1 of the trisaccharide. The difference between the observed which is 100.30ppm and the calculated value is 0.02ppm. This procedure is repeated for all the carbon atoms of

the trisaccharide. These calculations are shown in Tables 5.19 to 5.22 for the ^{13}C n.m.r. spectra.

The same procedure is repeated for the chemical shifts of the protons obtained from the ^{1}H n.m.r spectra of the four trisaccharides. Tables 5.23 to 5.26 show the calculations for the ^{1}H n.m.r. data for these trisaccharides.

For example in Table 5.23, 5.23 ppm is the chemical shift for the H-1 of the α-D-glucopyranose and the substituent shift relative to the α-1,2 linked disaccharide is -0.16ppm, for H-1", the sum of these two values is 5.07ppm. The difference between the experimentally observed chemical shift of 5.16ppm and the calculated value is 0.09 ppm.

For H-1', the sum of the chemical shift for the H-1 of the α-D-glucopyranose which is 5.23 ppm, the substituent shift relative to the α-1,2 linked disaccharide 0.20ppm and the substituent shift relative to the α-1,3 linked disaccharide 0.09ppm is 5.52 ppm. The experimentally observed chemical shift is 5.51ppm and the difference between the observed and the calculated value is -0.01ppm.

For H-1 of the methyl glycoside residue, chemical shift of methyl glycoside is 4.81ppm and the the substituent shift relative to the α-1,3 linked disaccharide 0.01ppm. The sum of these two values is 4.82 ppm and this gives the calculated chemical shift for H-1 of the trisaccharide. The difference between the observed chemical shift which is 4.82ppm and the calculated value is 0.00ppm.

A summary of Tables 5.19 to 5.22 is shown in Tables 5.27 while that of Tables 5.23 to 5.26 is shown in Table 5.28.

The differences between the observed and calculated chemical shifts

although not uniform are consistent for both the ^{1}H n.m.r.spectra and the ^{13}C n.m.r. Small differences between the observed and calculated chemical shifts are obtained in the ^{1}H n.m.r.spectra and the majority of the values are in a range of 0.01ppm and 0.02ppm. Only ten relatively high values between 0.05 and 0.16ppm are found in a total of 84 calculations. The same observation is made when the observed and the calculated ^{13}C n.m.r. chemical shifts are compared.

The agreement between the observed and the calculated values appears to be good indicating that data from 1,2- and 1,3- linked disaccharides may be used with the addition of the substituent shifts for the estimation of the chemical shifts of similar trisaccharides which contain 1,2- and 1,3- linkages and have the same anomeric configuration.

However there is need to carry out n.m.r. studies on more disaccharides and trisaccharides made up of similar sugar residues having the same type of linkages and anomeric configuration. This may assist in establishing if a definite pattern or relationship will emerge between the chemical shifts of many similar disaccharides and trisaccharides which contain the same types of linkages and anomeric configuration.

Tables 5.16 to 5.33 are shown in Index of Tables at the end of "Results and Discussion".

Temperature dependence of ^{13}C n. m. r. chemical shifts

The temperature shifts of the ^{13}C n..m.r. spectra of the trisaccharides obtained on changing the temperature from 30°C to 70°C are shown in Tables 5.29 to 5.32. and summarised in Table 5.33.

The ^{13}C n.m.r. spectra of the four trisaccharides at 70° are shown in Figures 5.29 to 5.32 while the ^{13}C n.m.r. spectra at 30° are shown in

Figures 5.33 to 5.36.

The values are relative to internal dioxane which has the chemical shift of 67.40ppm at both temperatures. The spectra of model substances are normally recorded at ambient temperatures but those of oligo- and polysaccharides should preferably be run at elevated temperatutes in order to improve resolution. Chemical shifts change with temperature, so it is necessary to have the spectra of model compounds and polysaccharides recorded at the same temperature using the same internal reference. It has been observed that most signals are shifted to lower fields on heating. In very few cases even upfield chemical shifts displacements have been observed such as that of C"-1 of trisaccharides **13** and **21**.

Significant changes are observed mainly for the linkage carbons C-2 and C-3. Large temperature shifts are more marked for the linkage carbons which contain α-glycopyranosyl linked units than the β-linked glucopyranosyl units. This may also be due to the restricted rotation of the α-linked disaccharide units found in the HSEA calculations. It is evident from the substituent shifts of C"1 and C'-2 and C-3 of the four trisaccharides, that the anomeric centre plays an important role by possibly affecting interactions across the glycosidic linkage.
The downfield shift of C'1 signal in **13** is smaller than the other C'1 signals which may be due to the 1'-H vs 1-H α-guache interaction as indicated by HSEA calculations. The same effect has been observed for the 1'H of some α-linked disaccharides[240,258,259].

The chemical shift diferences range from -0.01 to 0.91 which is the highest with the majority of the values being around 0.2ppm.
The differences appear pronounced for the methyl glucoside residues with

that in **24** having the highest value. The chemical shift displacements increase upward from **13, 21, 24 and 30** and the general trend of the downfield shifts relative to that of internal dioxane upon increment in temperature is far from uniform. However from Table 5.33, a wide variation is observed in the chemical shift displacement values of the trisaccharides **13, 21, 24,** and **30,** due to a change in temperature. Since the displacement of chemical shifts are not uniform on changing the temperature at which the n.m.r. is obtained, a general statement cannot be made about changes in ^{13}C n.m.r. chemical shift with temperature.

Homonuclear shift correlated spectroscopy (COSY) and Homonuclear shift correlated spectroscopy (HECTOR).

The information from an n.m.r. experiment can be plotted in two dimensions. This presentation in two dimensions (i.e two frequency axes at 90° to one another) allows much more information to be assembled and correlated than would be concievable in a normal 'one' dimensional plot. COSY and HECTOR have recently become the more routine two-dimensional n.m.r.experiments and establishes which proton couples with which protons or carbons respectively. The presentation contains information which completely unambiguously establishes the coupling relationships among all of the protons or between protons and carbons.

The whole map is to be reflected about a diagonal to ensure that all spurious peaks are eliminated. The signals located off the diagonal correlate those protons which are coupling to each other or to carbons. This way all the protons in the molecule can be identified in COSY and the protons as well as carbons in HECTOR.

COSY and HECTOR Spectra of Trisaccharides 13, 21, 24 and 30

The signal for the protons of the methyl group in the O-methyl glucose residues was taken as the unambiguous starting point in the assignment of the chemical shifts for all the ^{1}H n.m.r. signals COSY-spectra. The chemical shifts for all the ^{13}C n.m.r. signals were obtained from the HECTOR spectra. The COSY spectra of trisaccharides **13**, **21**, **24** and **30** are shown in Figures 5.37 to 5.40 while their HECTOR spectra are shown in Figures 5.41 to 5.44.

Hard Sphere Exoanomeric Calculations (HSEA) calculations[171,181]

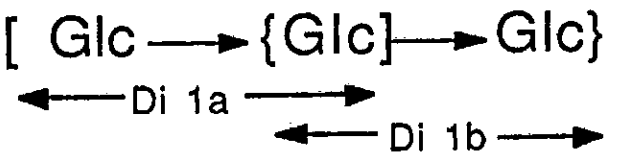
This discussion will mainly be concerned with various chemical shift displacements deriving from stereochemistry around the glycosidic bond.

The energy plots ϕ/ψ and the minimum energy conformations for the trisaccharides **13, 21, 24 and 30** were obtained as described in the "Experimental" section using the HSEA-program. In this approach only van der Waals interactions together with the exoanomeric effect were evaluated and the rigid-body assumption was made, i.e. an atom in a monomer is fixed relative to the other atoms in the monomer. The HSEA calculations were performed as described earlier using crystal coordinates sets for α-D-glucose, methyl α-D-glucose, β-D-glucose and methyl β-D-glucose which were obtained from the library [201,202] A value of 117° was used for the glycosidic bond angle τ, and ϕ/ψ-values had 5° increments in the calculations. The plots were prepared by using CHEM-X[208]. Iso-contour levels were set at 0.1, 0.5, 1, 2, 3 and 4 Kcal above the energy of the minimum energy molecule.

The energy plots ϕ (psi) vs ψ (phi) together with contacts deriving from changes in the ϕ and ψ angles of the four trisaccharides are shown in Figures 5.45 to 5.48. of Index III. The values of the ϕ and ψ angles of the minimum energy molecules and inter-residual atomic distances <3.0 Å in **13, 21, 24 and 30** are shown in Table 5.16 of the index of Tables. Lemieux and co-workers[176,181] have found that the conformations derived by ^{1}H n.m.r. are well anticipated by theoretical calculations using HSEA-program. This takes into account non-bonded interactions (van der Waals interaction) corrected for the influence of the exo-anomeric effect. The HSEA calculations do not include oxygen lone-pairs or hydrogens linked to oxygens. In many cases, HSEA-calculations have provided conformations in

which strongly deshielded protons are found close to oxygen atoms in other sugar units[181] which explains chemical shifts of signals at otherwise unexpected fields.

For the ϕ/ψ energy plots made from the HSEA calculations, of the four synthesized trisaccaharides **13**, **21**, **24**, and **30** have been named Tri 1, Tri 2, Tri 3 and Tri 4 respectively for the purpose of this discussion. Also, each trisaccharide is considered as two overlapping disaccharide units and the minimum energy calculations for each of the two overlapping disaccharide units are made separately. As an example, Tri 1 is considered as being made up of the Di.1a and Di.1b. Di.1a is the α-1, 2 linked disaccharide residue while Di.1b is the α-1,3 linked disaccharide residue with the glucoside residue of Di.1a overlapping the glucosyl residue of Di.1b. A representation of an hypothetical trisaccharide is shown in R.13 below.



R.13

Therefore in the following discussion the four trisaccaharides, Tri 1 to 4 will be regarded as being made up of Di.1a and Di.1b, Di.2a and Di.2b, Di.3a and Di.3b, Di.4a and Di.4b respectively.

The overall flexibility of the trisaccharides is rather large at a level of 8.2 KJ (2.0 kcal) above that of the minimum energy molecule and the absolute value of ϕ in the minimum energy conformations of Tri 1 to Tri 4 is 50° (±10°) whereas the values for ψ vary with in a range of -25° to 20°. A prerequisite for taking the minimum energy conformation as a

weighted average is that the energy well is symmetrical. The general shape of the energy wells shown in Figures 5.25 to 5.28, is ellipsoid rather than circular and showing a somewhat larger degree of freedom for ϕ than for ψ.

From the energy maps, Di.1a, Di.1b, Di.2a and Di.4b have similar shapes while Di.2b, Di.3a, Di.3b and Di.4aaa have similar shapes which are quite distinct and different from those of the former set. As observed from these energy maps, restricted rotational freedom for smaller ϕ/ψ values for the α-D glycosides units are present due to contacts from 5'-H while greater freedom were observed from the β-D units. It is thus observed that there is a higher degree of rotation for the residues containing the β-anomeric linkages. Also rotational freedom is function of τ, the glycosidic bond angle which has been set to 117°, a relatively large value compared to those found in crystal structures. On decrease of τ the calculated energy increases but the values of ϕ and ψ for the minimum energy molecule remain constant. However it is observed for the α-D disaccharide unit linked by a β-D disaccharide unit greater rotational freedom is also observed in the four pairs of making up Tri 1 to Tri 4 which may be due to a short range effect of the β-D unit on the α-D unit. This is in conformity with the pattern observed in 1,2 and 1,3 disaccharides[240].

On comparison of the maps for Di.1b and Di.3b, the effect of an axial hydroxyl group on a β-carbon in the methyl glycoside residue can be observed as a shift of the position of high energy conformers. Thus for Di.1b and Di.3b, such conformers are obtained on going to high ϕ, low ψ. In Di.2b, Di.3a, Di.4a and Di.4b, more of this space is accessible.

An explanation for this is that the equatorial hydroxyl group OH-2 in these four units has more interaction with the linkage oxygen than the β-residues. This effect is not observed when an α-D-glucosyl group is substituted into the α-D-glucoside residues of Di.1a, Di.1b, Di.2a, and Di.3b. In Tri's 1 to 4, there are several different stereochemical arrangements around the glycosidic linkages and the interacting atoms differ. The 1,3-linked pairs have the anomeric centre of the methyl glycoside residue in common but this has very little effect on the shapes of energy maps of Di 1b to Di 4b. However, Tri 2 and Tri 3 have similar internuclear interaction due to the similar anomeric linkages although they are in opposite directions.

The basic difference between Di's 3b and 2a is that Di 3b has an axial substituents in position 1 of the glycosyl group in addition to two neighbouring equatorial substituents. In the latter, the substituent in position 1 occupies an equatorial position as well as two neighbouring equatorial substituents.

An important observation is that the presence of the methyl group on O-1 has no significant effect on the result of the calculations. The disaccharide units linked by the α anomeric linkage of the Tris have a long-range interaction from 5'-H to the opposing equatorial substituent next to the linkage in the other ring. This is in contrast to those linked by the β-anomeric configuration.

The anomeric protons 1'-H and 1"-H as well as the ring oxygen O-5' and 0.5" are always in contact with the proton on the linkage carbon and the anomeric proton in most examples is also in contact with one of the neighbouring substituents.

Conformational dependence of ^{13}C n.m.r. chemical shifts has been observed[268,269]. Bock et al[270] have shown that there is some correlation between the induced chemical shift differences of C-1 for α-D-gluco-and α-D-galactopyranosyl residues in oligosaccharides and the ψ-angle. For low values of ψ, a low value of the chemical shift is obtained. There is an increase observed going from 97.2 p.p.m. at -35° to 99.0 p.p.m.. at -5°. Such a correlation was also observed for the aglycone carbon. It was suggested that a similar dependence should be valid for other glycosides. In agreement with the assumption, the ψ -values for Di.1b, Di.2b, Di.3b, are -25°, -5° and 10° respectively and C-1 chemical shifts at 97.3, 99.0 and 99.2 ppm.

Recently Lipkind and Kochetkov[271] presented conformational analyses of some 1,3-linked disaccharides using theoretical calculations and measurements of the nuclear Overhauser effect obtained by saturation of the anomeric proton. A large population of conformers of low energy and short distances between 1'-H and 4-H or 1'-H and 2-H was observed for disaccharides with the same stereochemistry around the glycosidic bond as that in Di.1a, Di.1b, Di.2b, and Di.4b respectively. This observation suggested that the steric γ-gauche effect was the reason for the strong upfield shift of signals for C-4 and C-2, respectively, and for the corresponding small glycosidation shift of C-1 in these disaccharides. Similar short distances could be observed in the minimum energy conformation of Di.1b, Di.2b, Di.3b, and Di.4b shown in Table 5.16 and in conformers with low energy shown in Figure 5.25 and 5.28.

Recent studies[257,269] have shown that the signal of an anomeric proton which is close to an oxygen is shifted downfield. We have also observed that proximity with protons causes an upfield shift[257].

The anomeric protons present are in proximity with oxygen and therefore are shifted downfield. Although, the shifts vary between -4.19 and 8.00 ppm. The upfield shifts of 1-H may be caused by the short distance to 3-H.

In conclusion, the calculations do not show any significant differences between the four trisaccharides because the monosaccharide residues do not interact in space due to lack of steric crowding.

Conclusion

From the investigation of the trisaccharide glycosides **13**, **21**, **24**, and **30**, it is concluded that a typical set of substituent shifts in ^{1}H and ^{13}C.n.n.r. spectroscopy is obtained on glycosylation at the 2 and 3-positions in a sugar residue. These shifts depend upon the stereochemistry around the glycosidic linkage. It should therefore be possible to calculate chemical shift differences for trisaccharide residues which are similar in these respects.

The agreement between the observed and the calculated chemical shifts appear to be good indicating that data from 1,2- and 1,3- linked disaccharides may be used (with the addition of the substituent shifts or a factor) for the estimation of the chemical shifts of similar trisaccharides containing 1,2- and 1,3- linkages and having the same anomeric configuration.

The results obtained make up part of the material being used for the expansion of the library data of the computer program, CASPER[219] from which the spectra of oligosaccharides and polysaccharides containing oligosaccharide repeating units may be simulated. With an extensive database, sequence information for oligo-and polysaccharides could be obtained.

INDEX III.

^{13}C AND ^{1}H N.M.R. OF THE SYNTHESIZED TRISACCHARIDES **13,21,24,30.**

Figures 5.25 to 5.28 for ^{1}H N.M.R.Spectra of Trisaccharides **13,21,24,30.**

Figures 5.29 to 5.32 for ^{13}C N.M.R.Spectra of Trisaccharides **13,21,24,30** at 70°C

Figures 5.33 to 5.36 for ^{13}C N.M.R.Spectra of Trisaccharides **13,21,24,30.**at 30°C.

Figures 5.37 to 5.40 for COSY Spectra of Trisaccharides **13,21,24,30.**

Figures 5.41 to 5.44 for CH correlation Spectra of Trisaccharides **13,21,24,30.**

Figures 5.45 to 5.48 for Isocontour Energy Maps of Trisaccharides **13,21,24,30.**

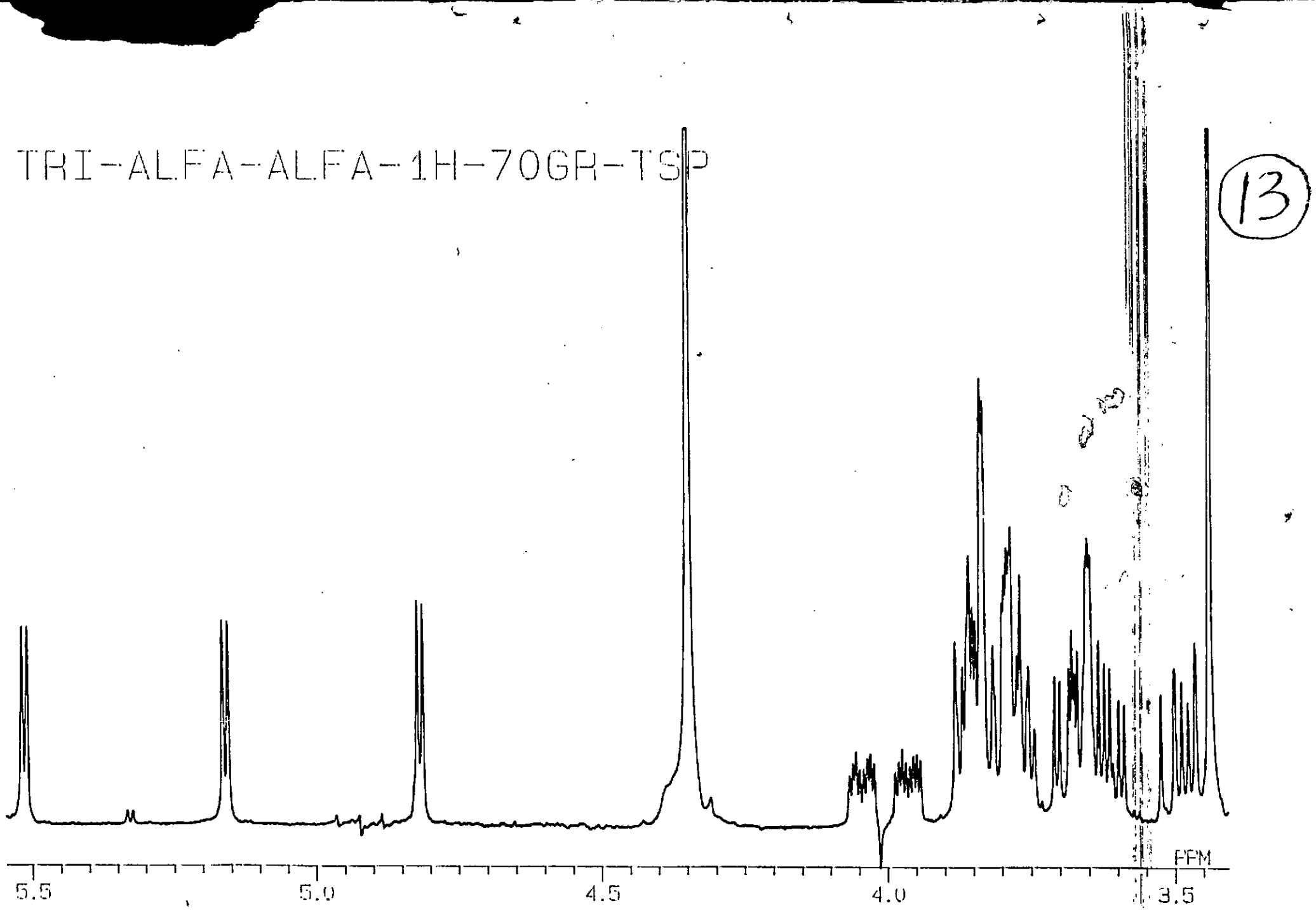


Figure 5.25. ^{1}H n.m.r. spectrum of Trisaccharide 13.

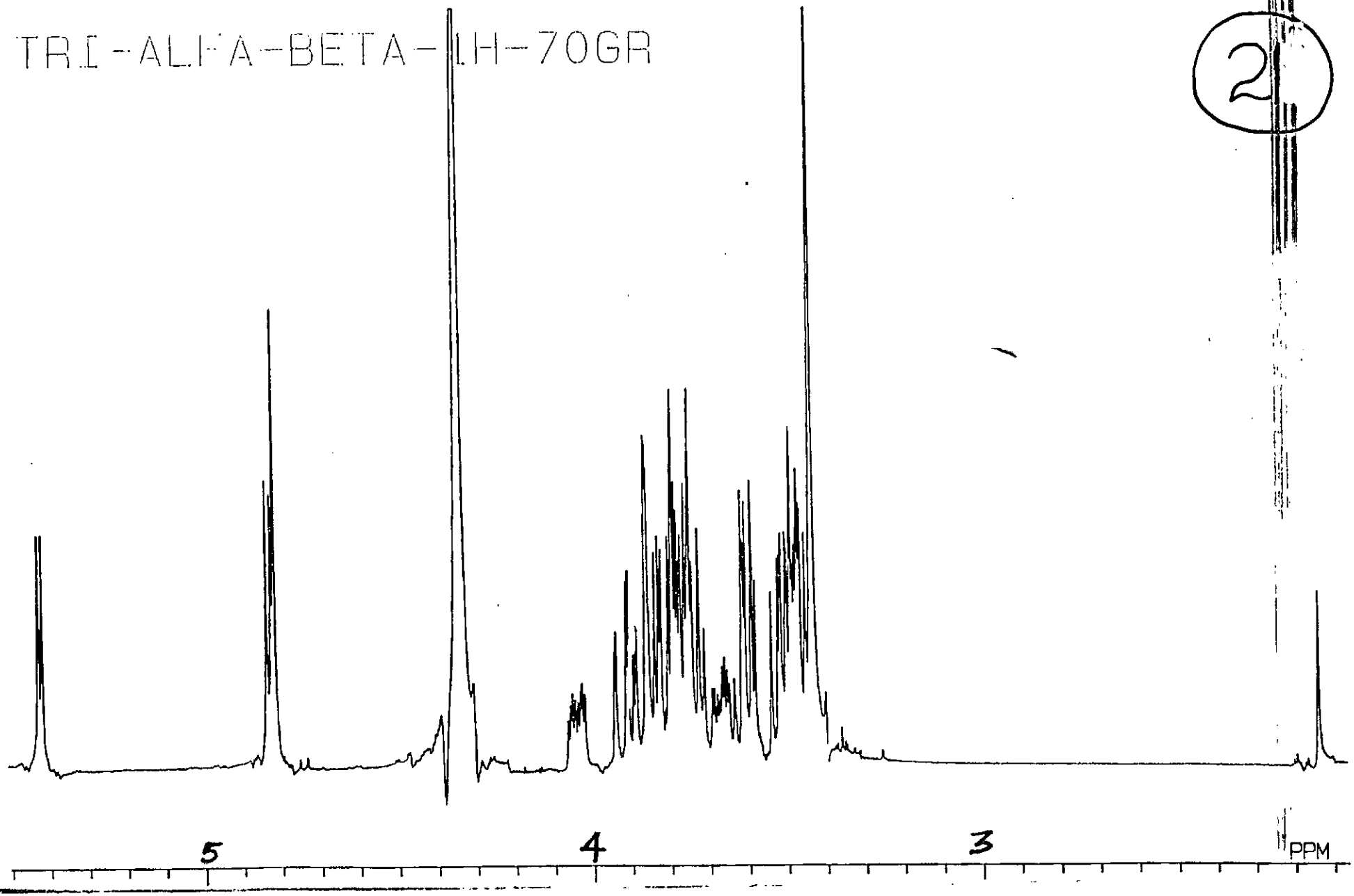


Figure 5.26. ^{1}H n.m.r. spectrum of Trisaccharide 21.

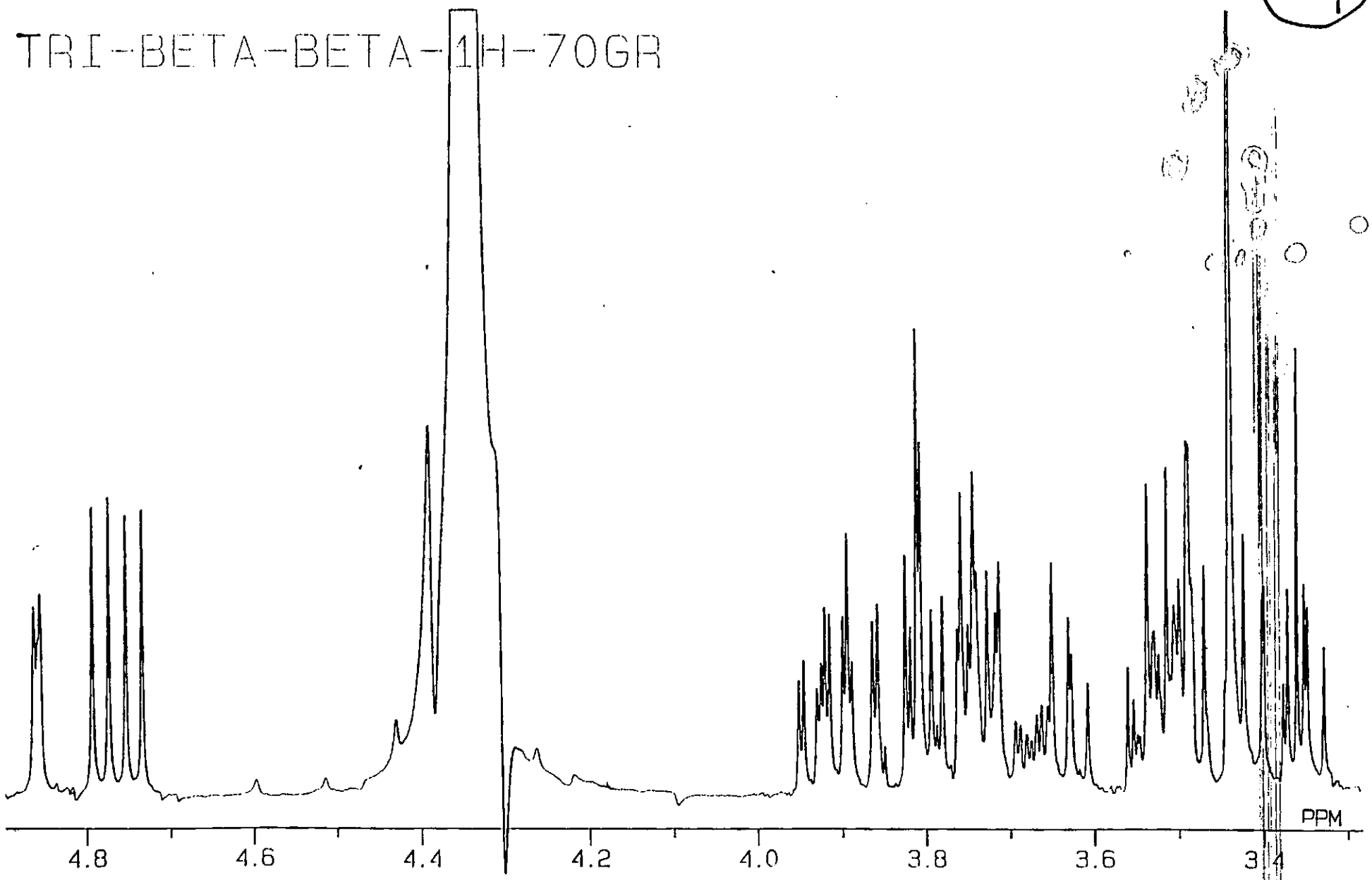


Figure 5.27. ^{1}H **n.m.r. spectrum of Trisaccharide 24.**

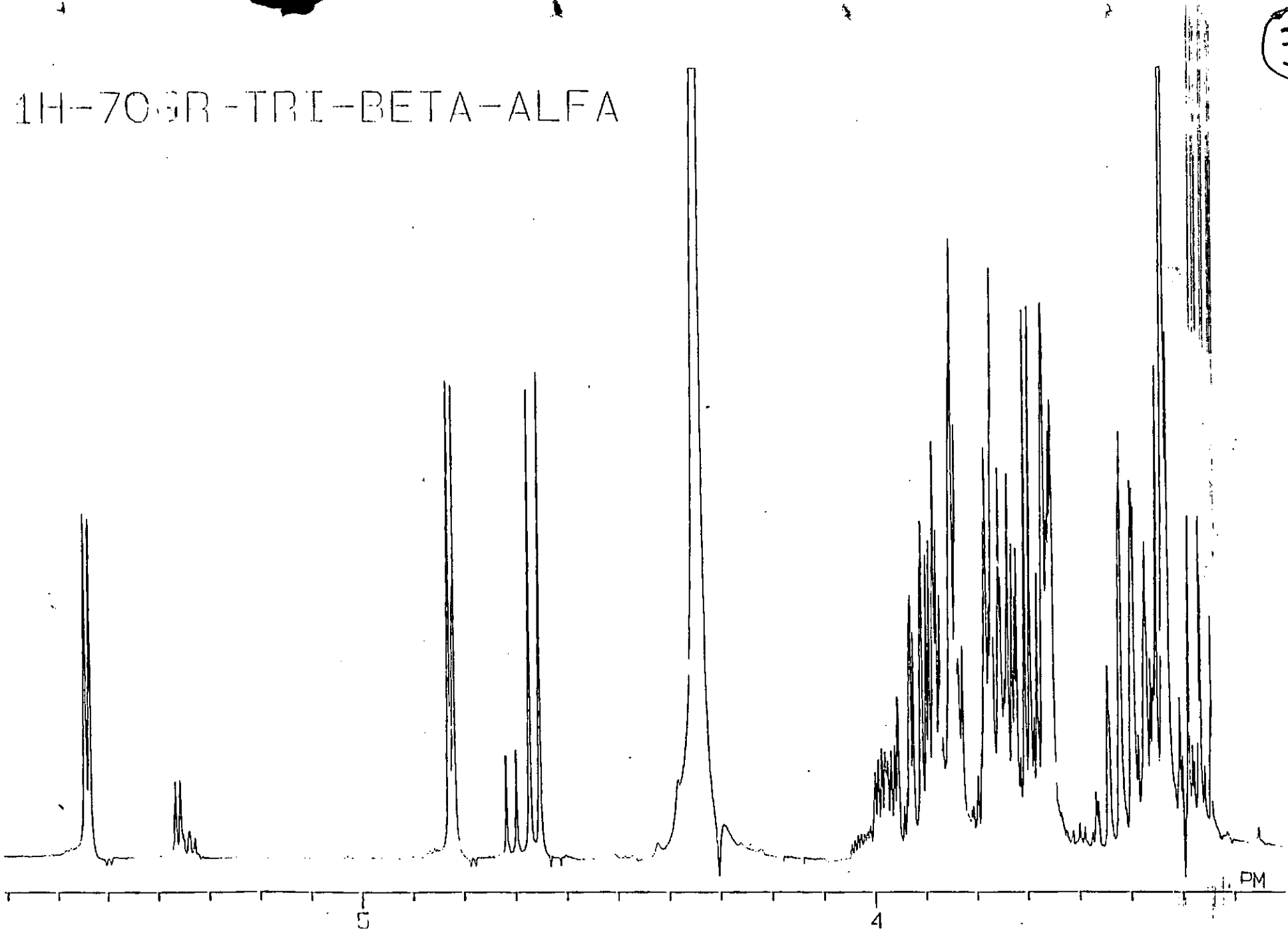


Figure 5.28. ^{1}H n.m.r. spectrum of Trisaccharide 30.

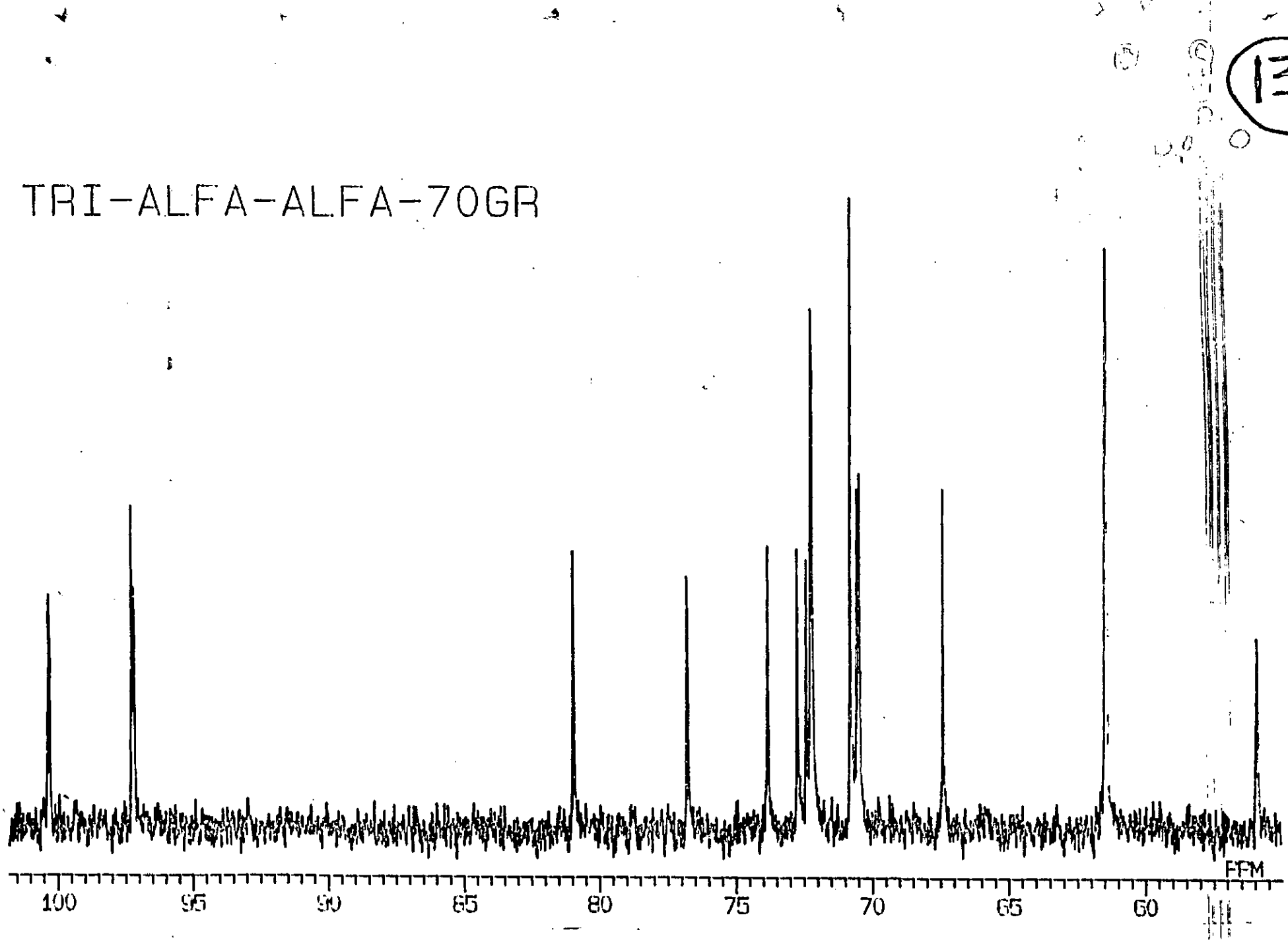


Figure 5.29. ^{13}C n.m.r. spectrum of Trisaccharide 13 at 70°C.

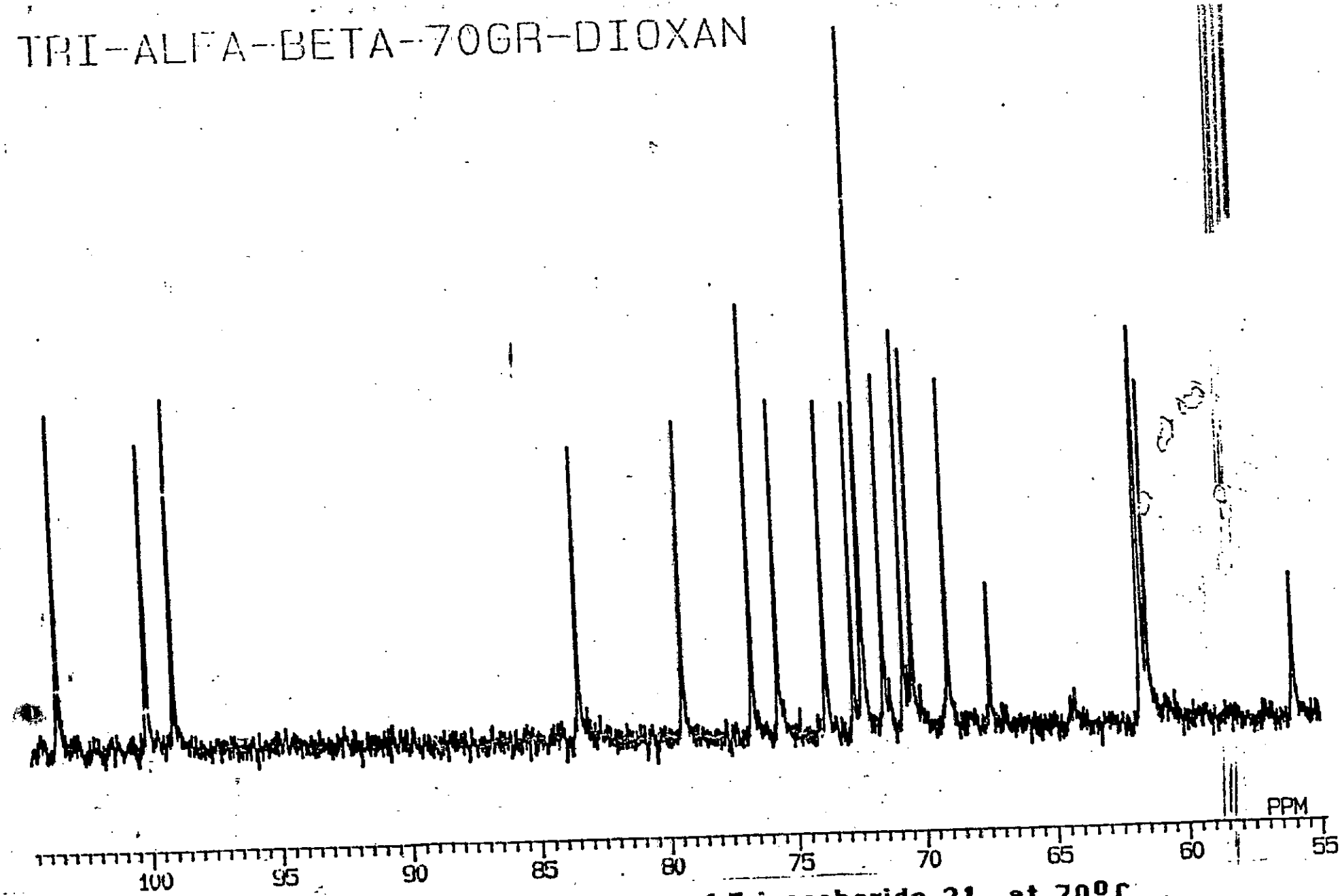


Figure 5.30. ^{13}C n.m.r. spectrum of Trisaccharide 21 at 70°C.

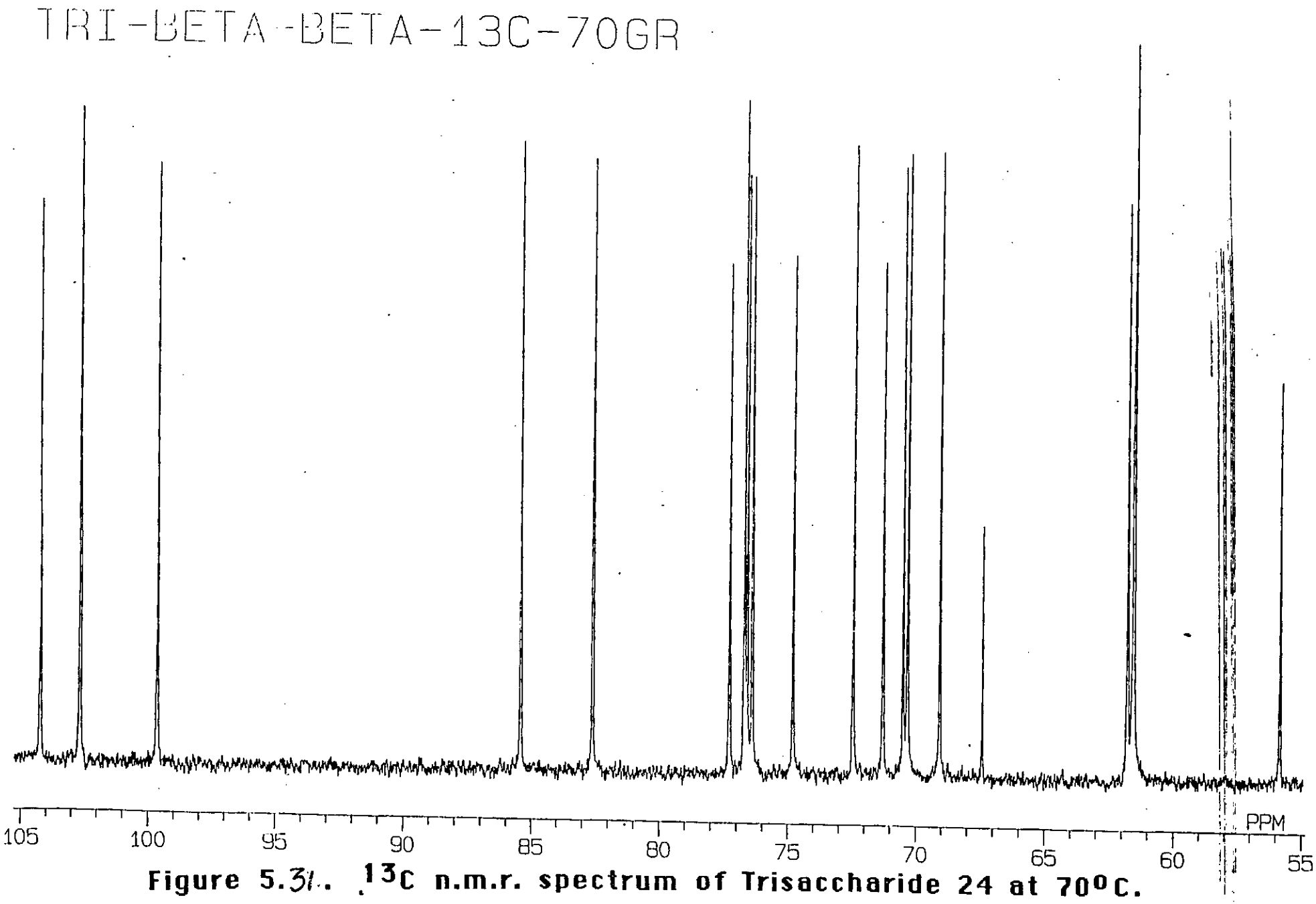


Figure 5.31. ^{13}C n.m.r. spectrum of Trisaccharide 24 at 70°C.

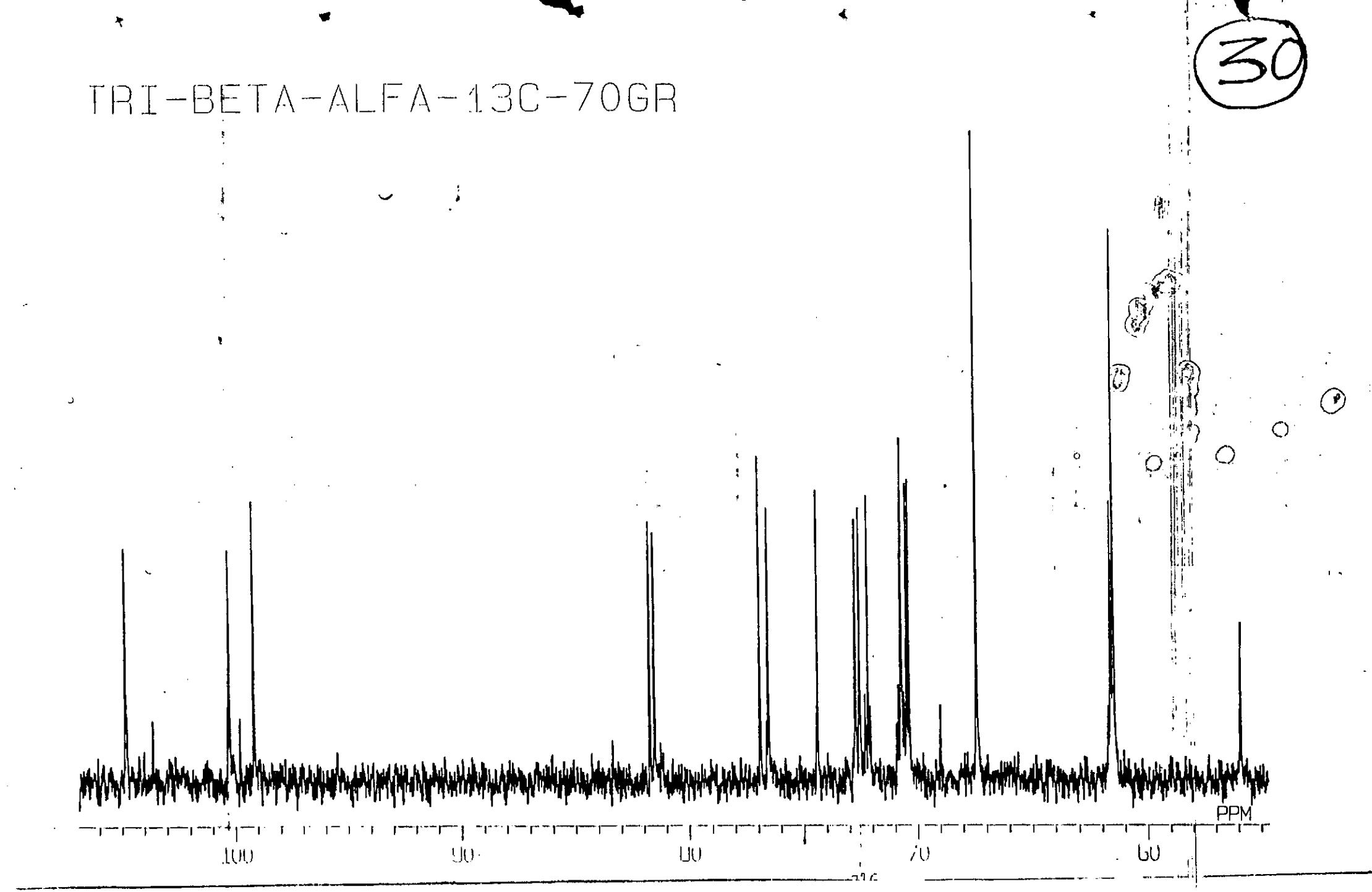


Figure 5.32. ^{13}C n.m.r. spectrum of Trisaccharide 30 at 70°C.

13

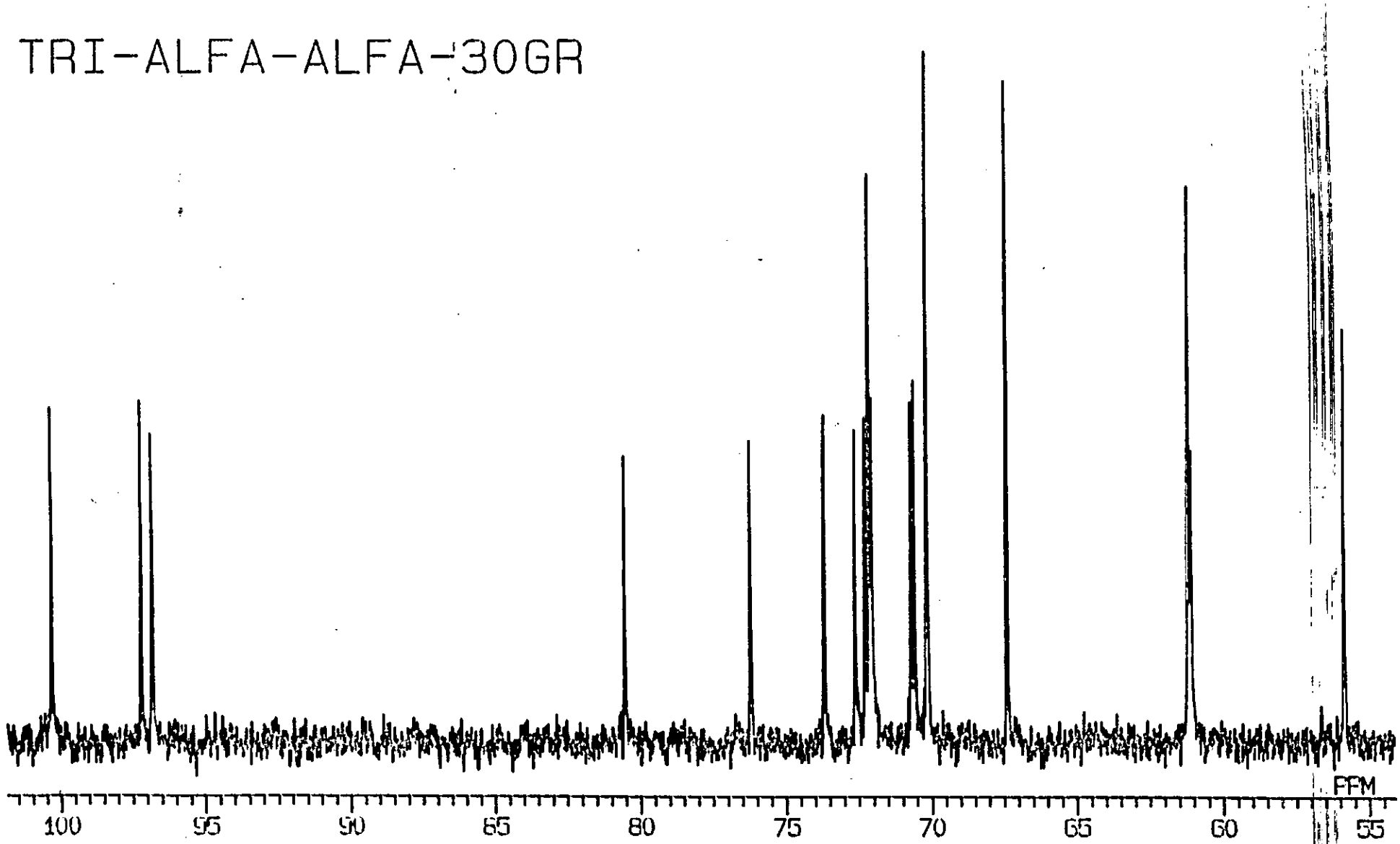


Figure 5.33. ^{13}C n.m.r. spectrum of Trisaccharide 13 at 30°C.

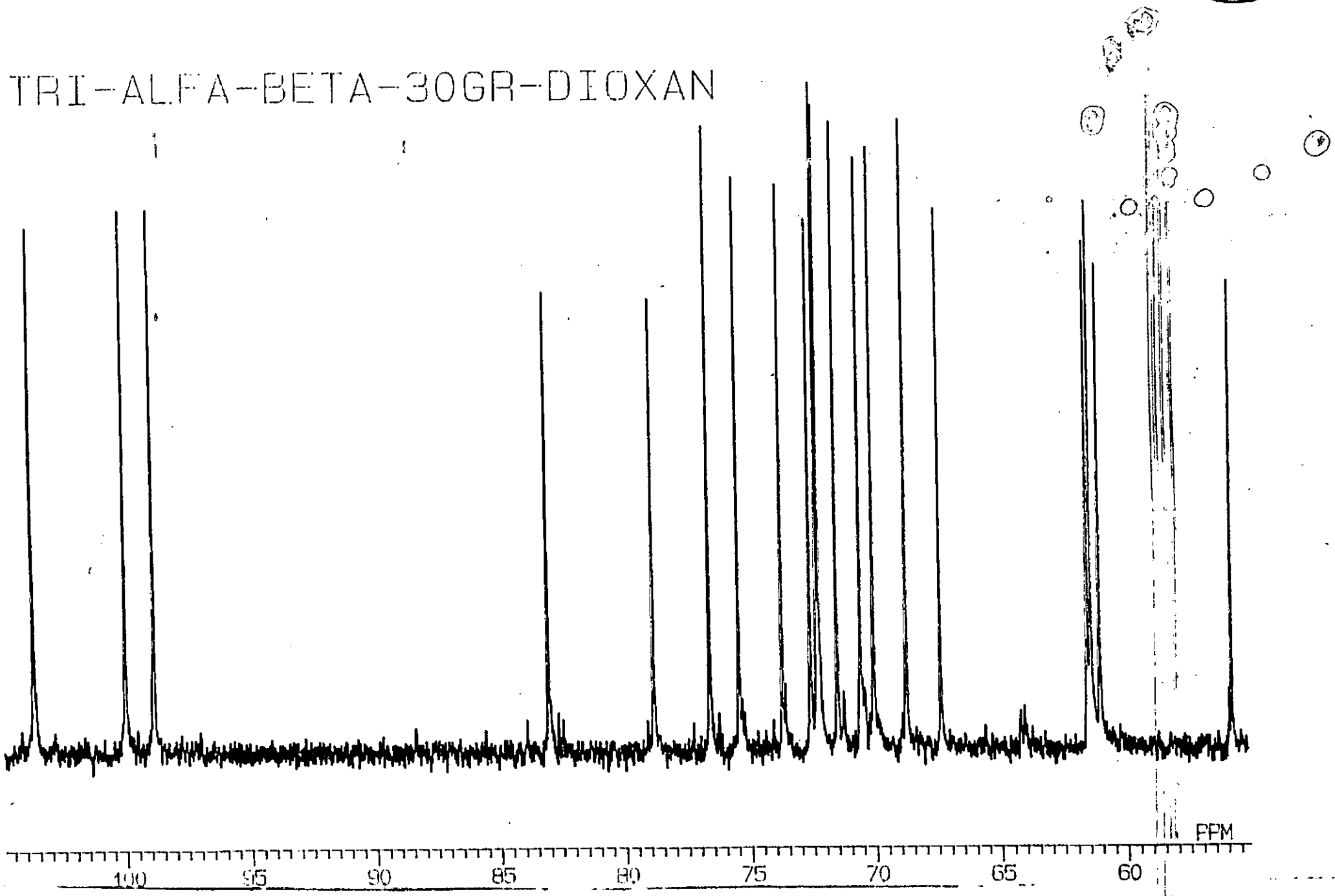


Figure 5.34. ^{13}C n.m.r. spectrum of Trisaccharide 21 at 30°C.

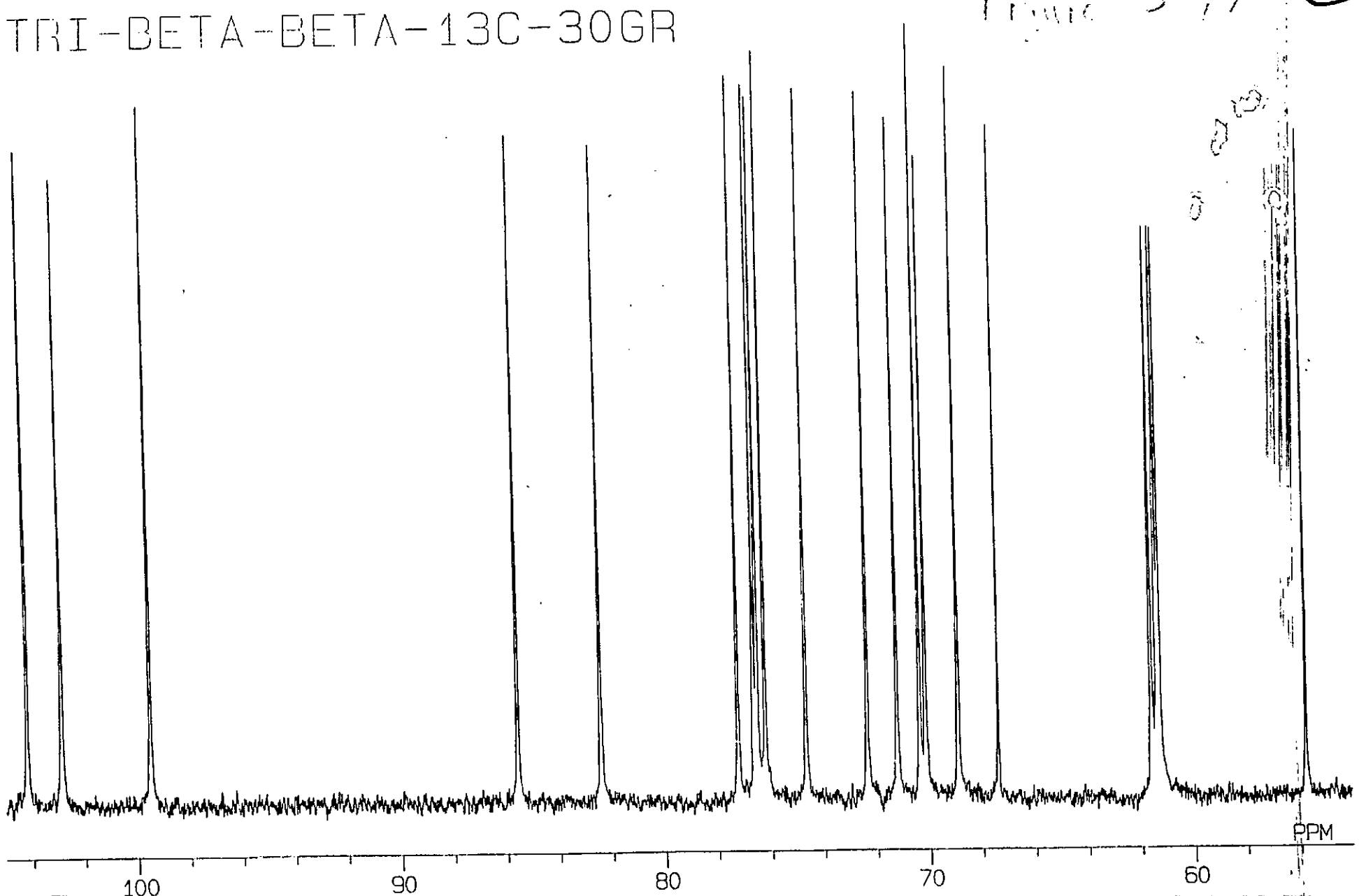


Figure 5.35. ^{13}C n.m.r. spectrum of Trisaccharide 24 at 30°C.

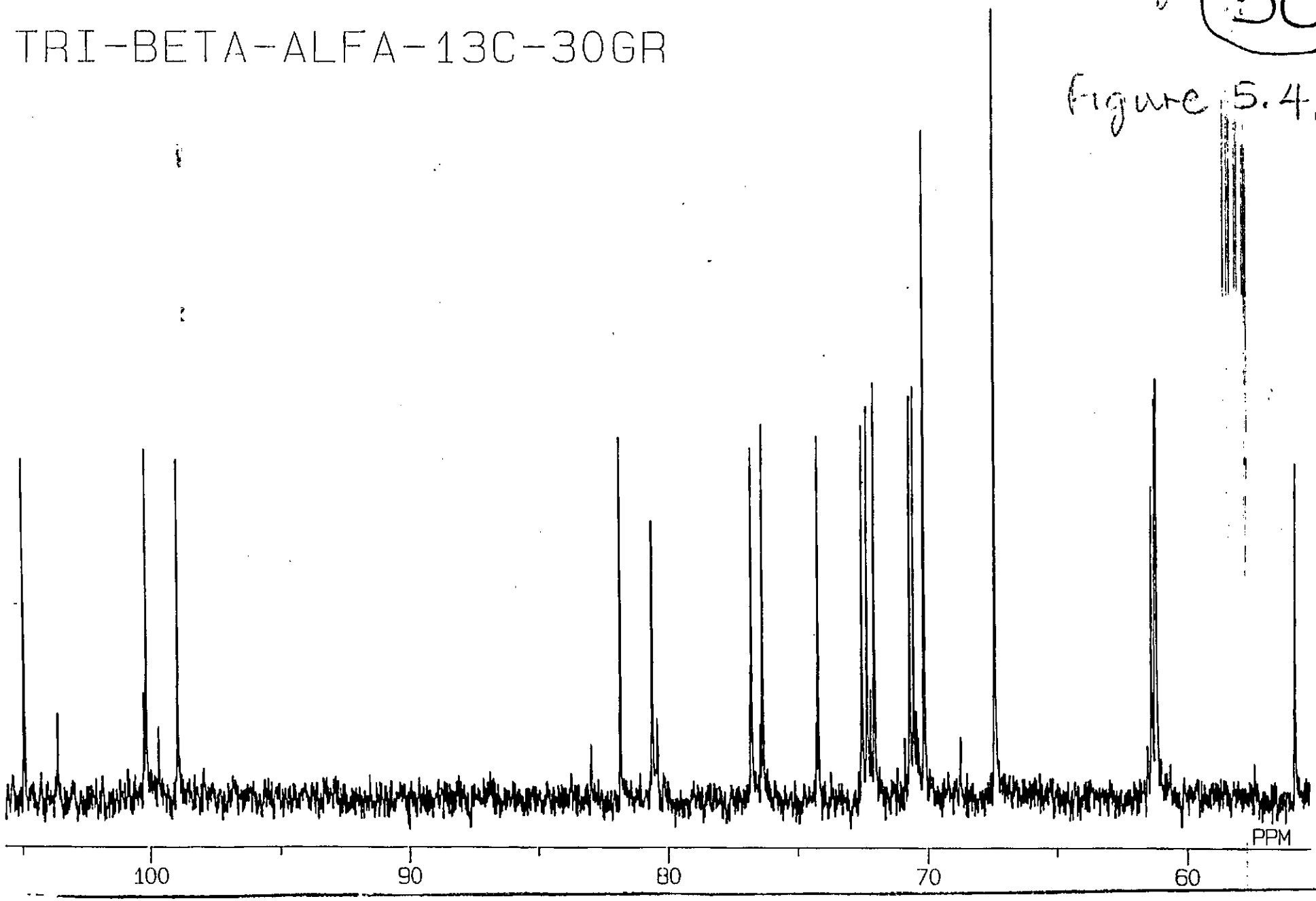


Figure 5.36. ^{13}C n.m.r. spectrum of Trisaccharide 30 at 30°C.

TRI-ALFA-ALFA-COSY

Figure 5.37. COSY spectrum of of Trisaccharide 13.

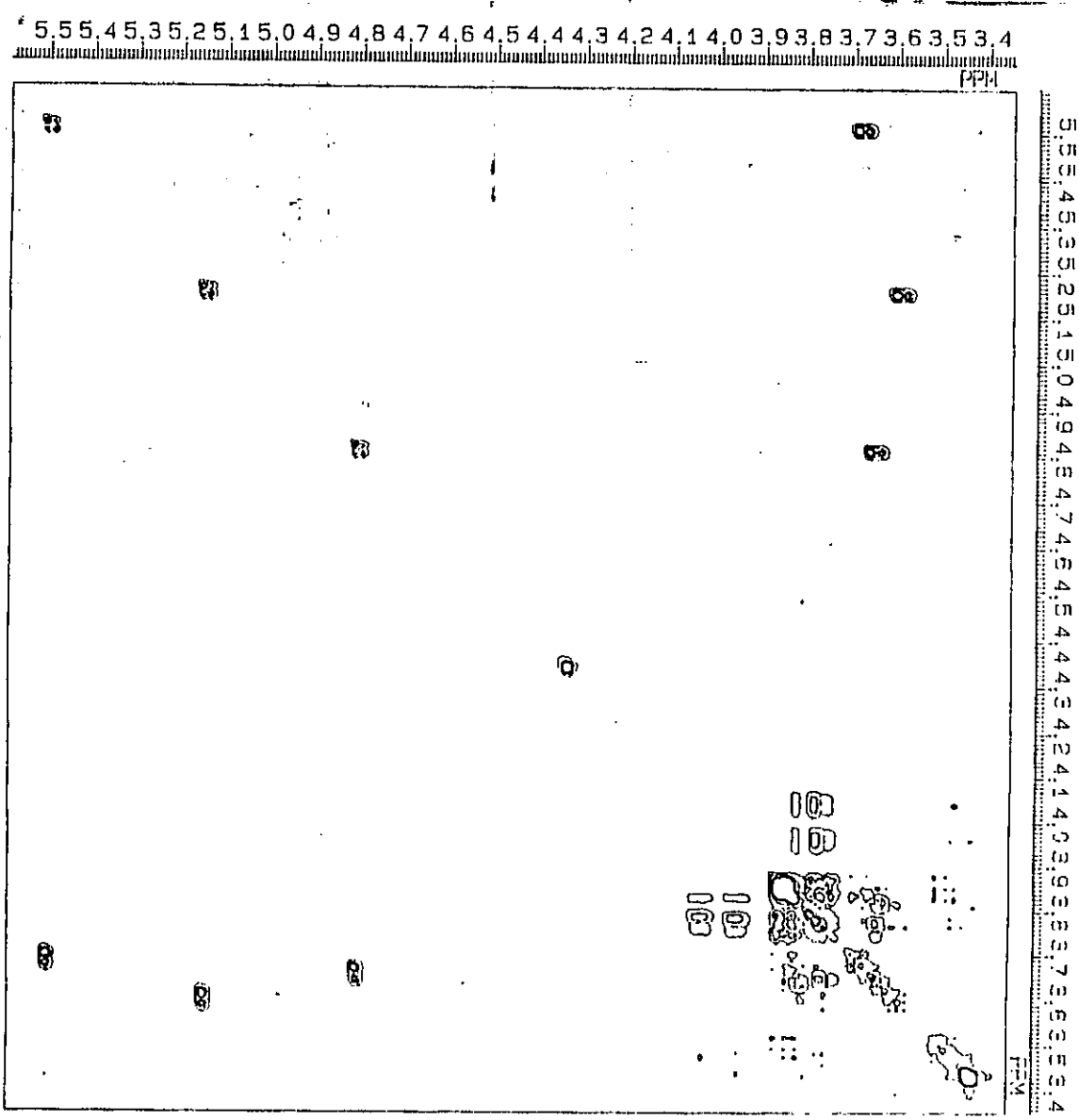
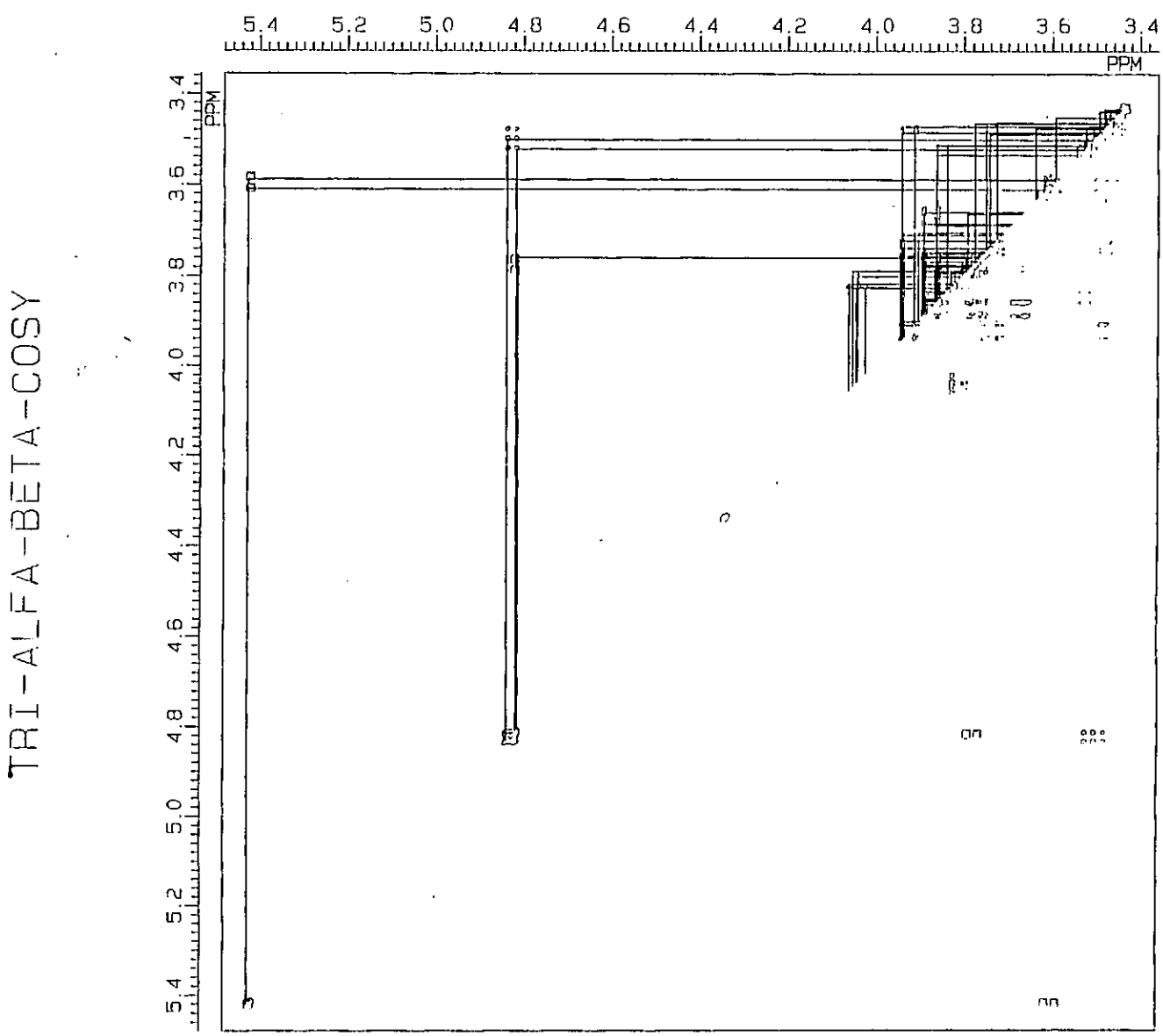


Figure 5.38. COSY spectrum of of Trisaccharide 21.



TRI-BETA-BETA-COSY

Figure 5.39. COSY spectrum of of Trisaccharide 24.

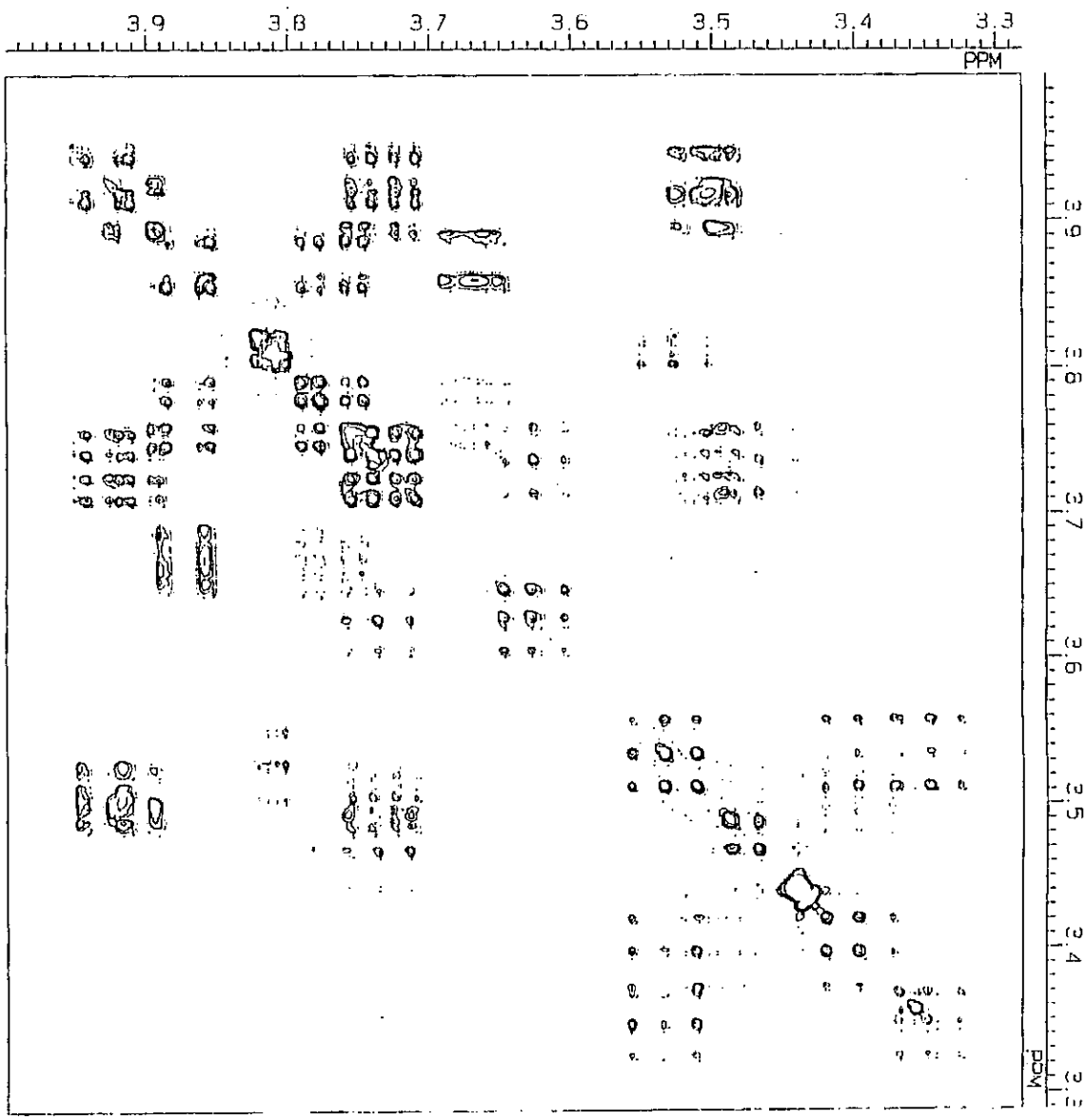
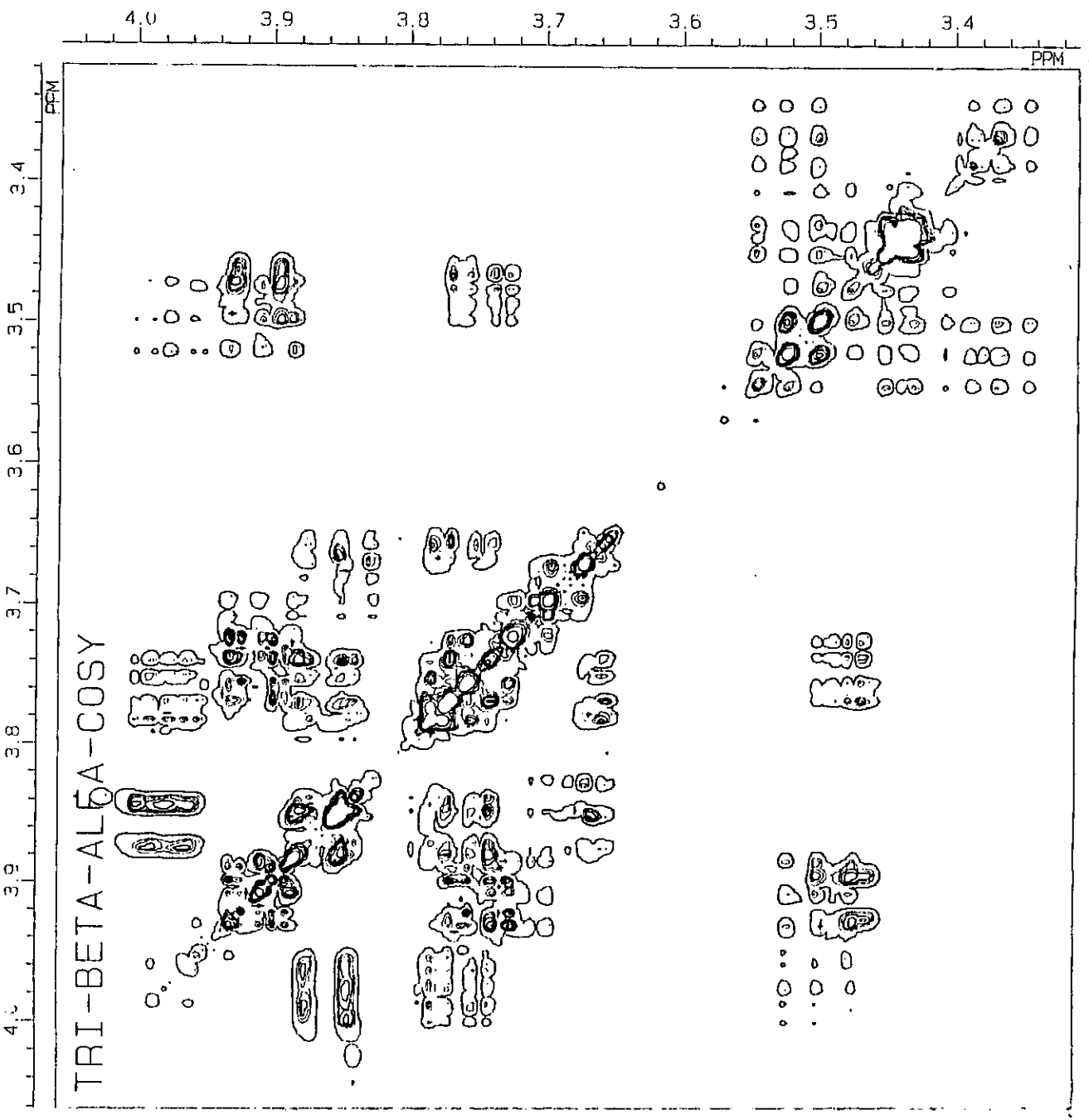


Figure 5.40. COSY spectrum of of Trisaccharide 30.



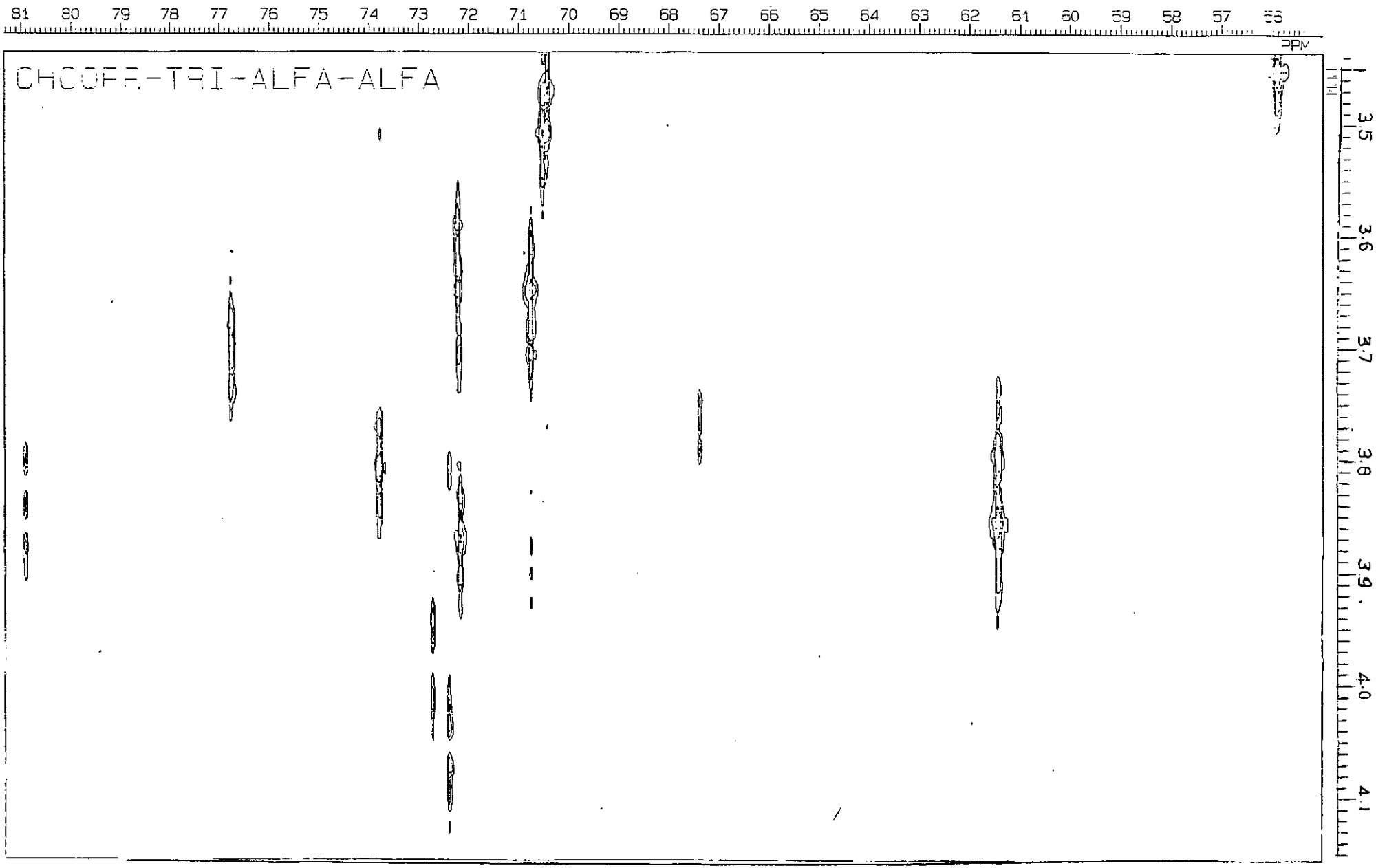


Figure 5.41. CH Correlated spectrum of Trisaccharide 13.

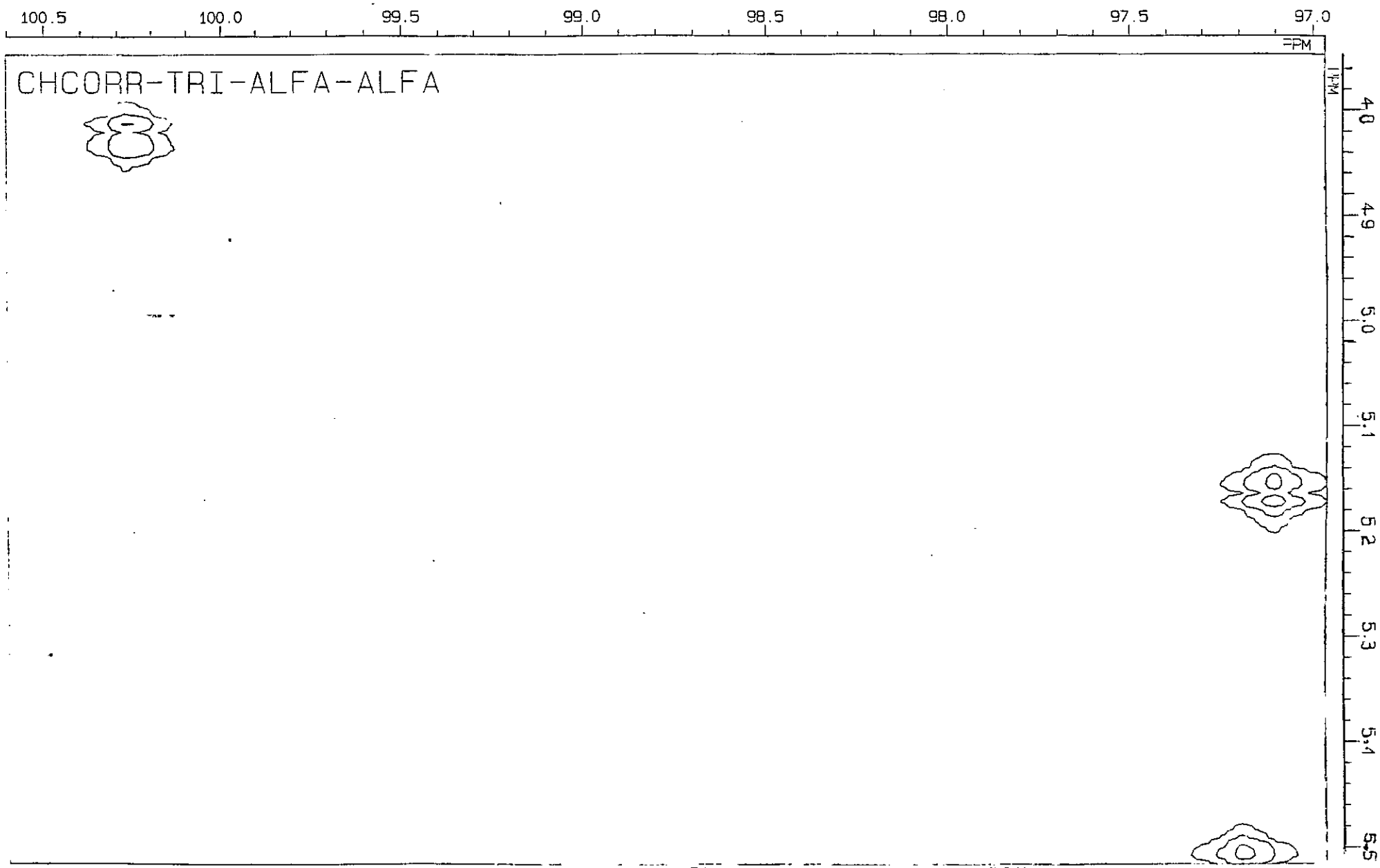


Figure 5.41a. CH Correlated spectrum of Trisaccharide 13 continued.

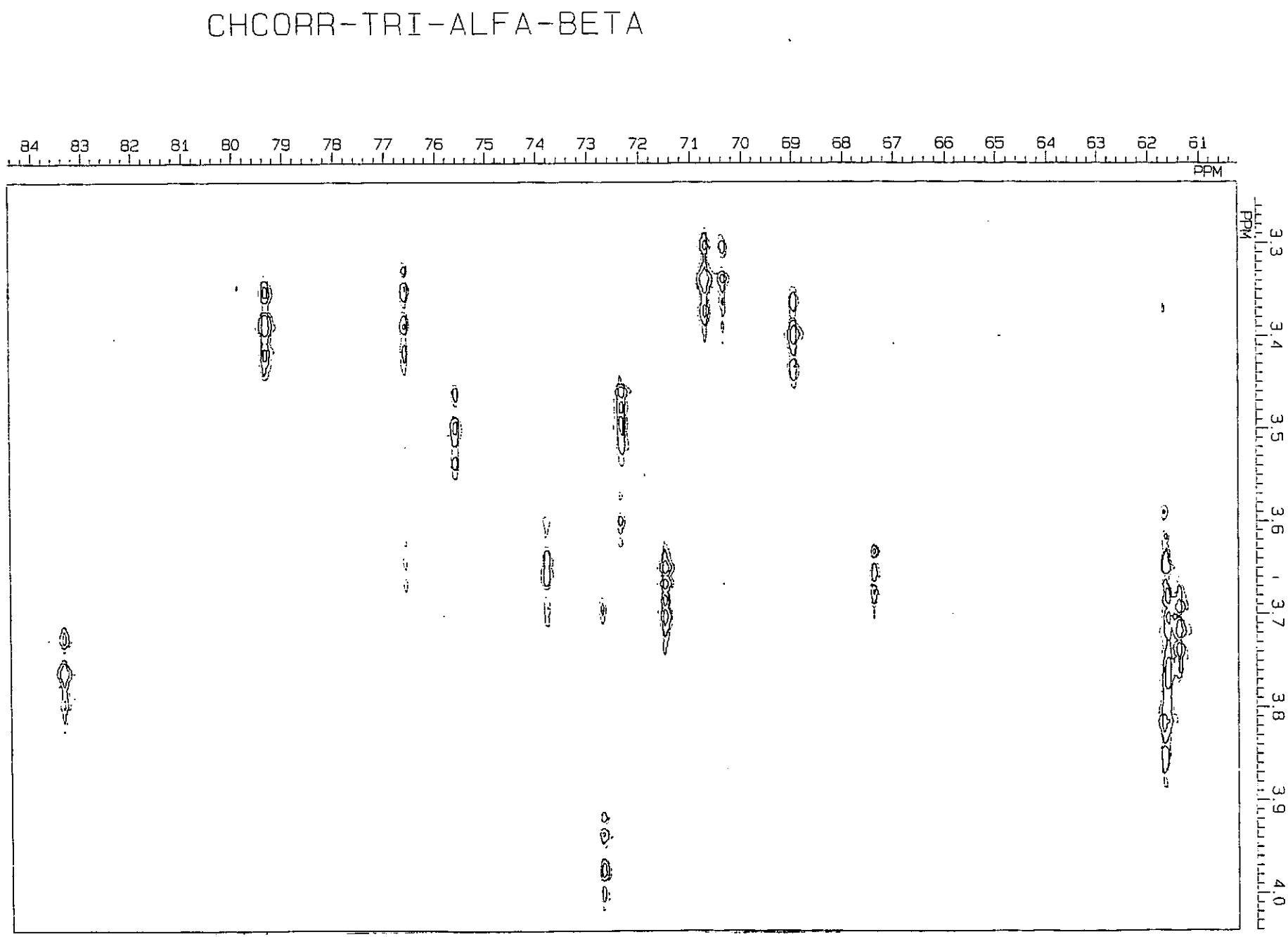


Figure 5.42. CH Correlated spectrum of Trisaccharide 21.

CHCORR-TRI-ALFA-BETA

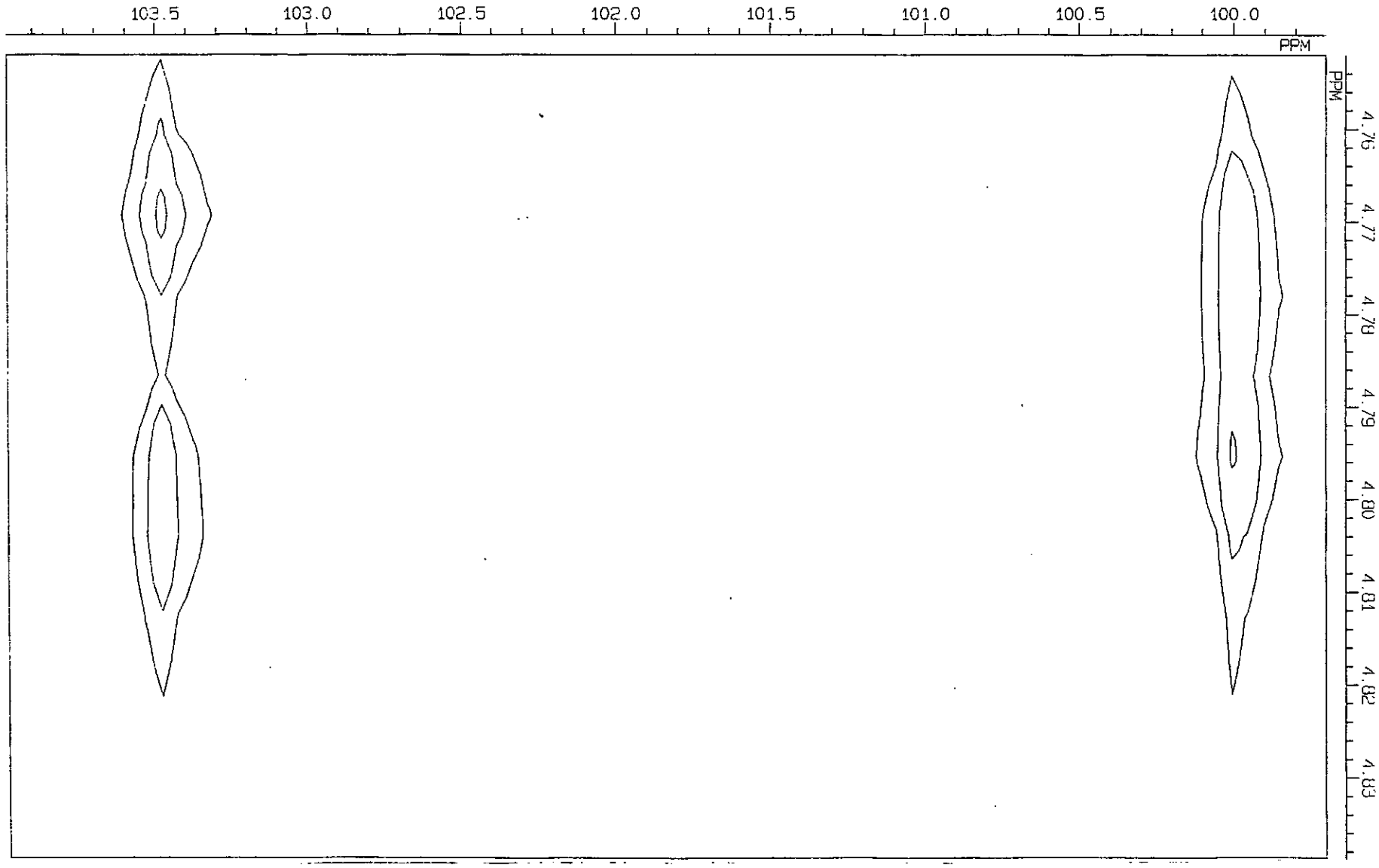


Figure 5.42a. CH Correlated spectrum of Trisaccharide 21 continued.

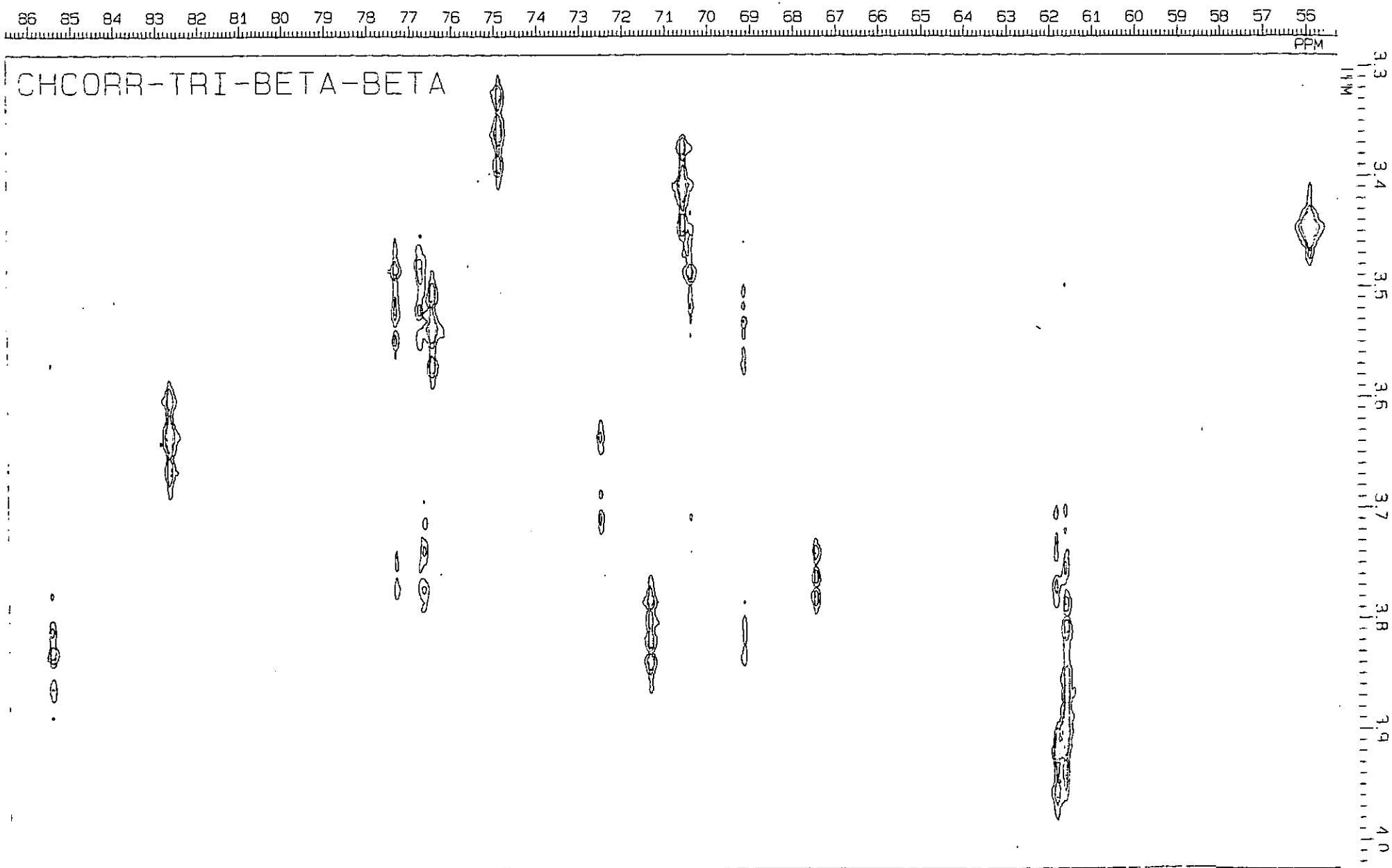


Figure 5.43. CH Correlated spectrum of Trisaccharide 24.

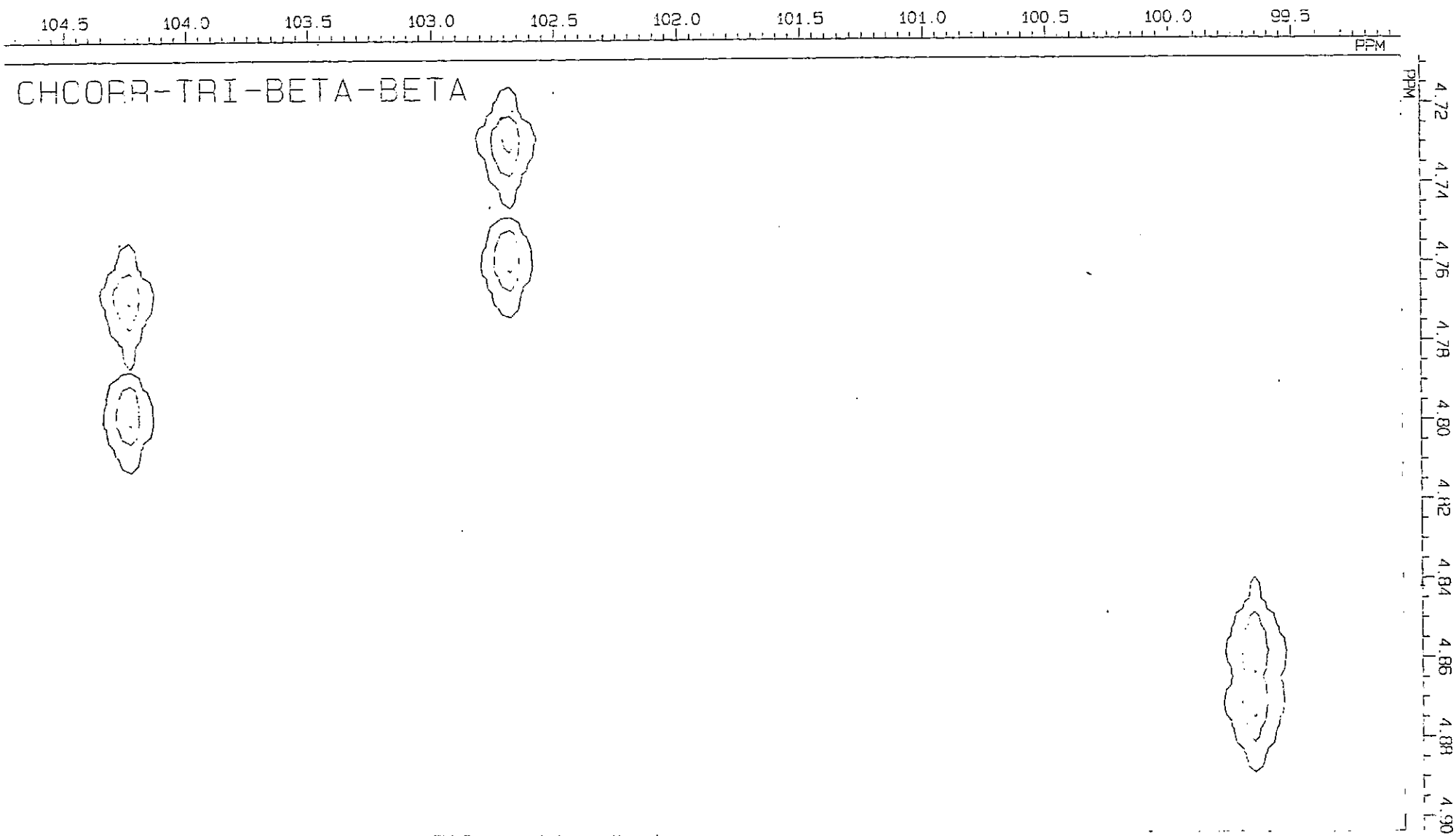


Figure 5.43a. CH Correlated spectrum of Trisaccharide 24 continued.

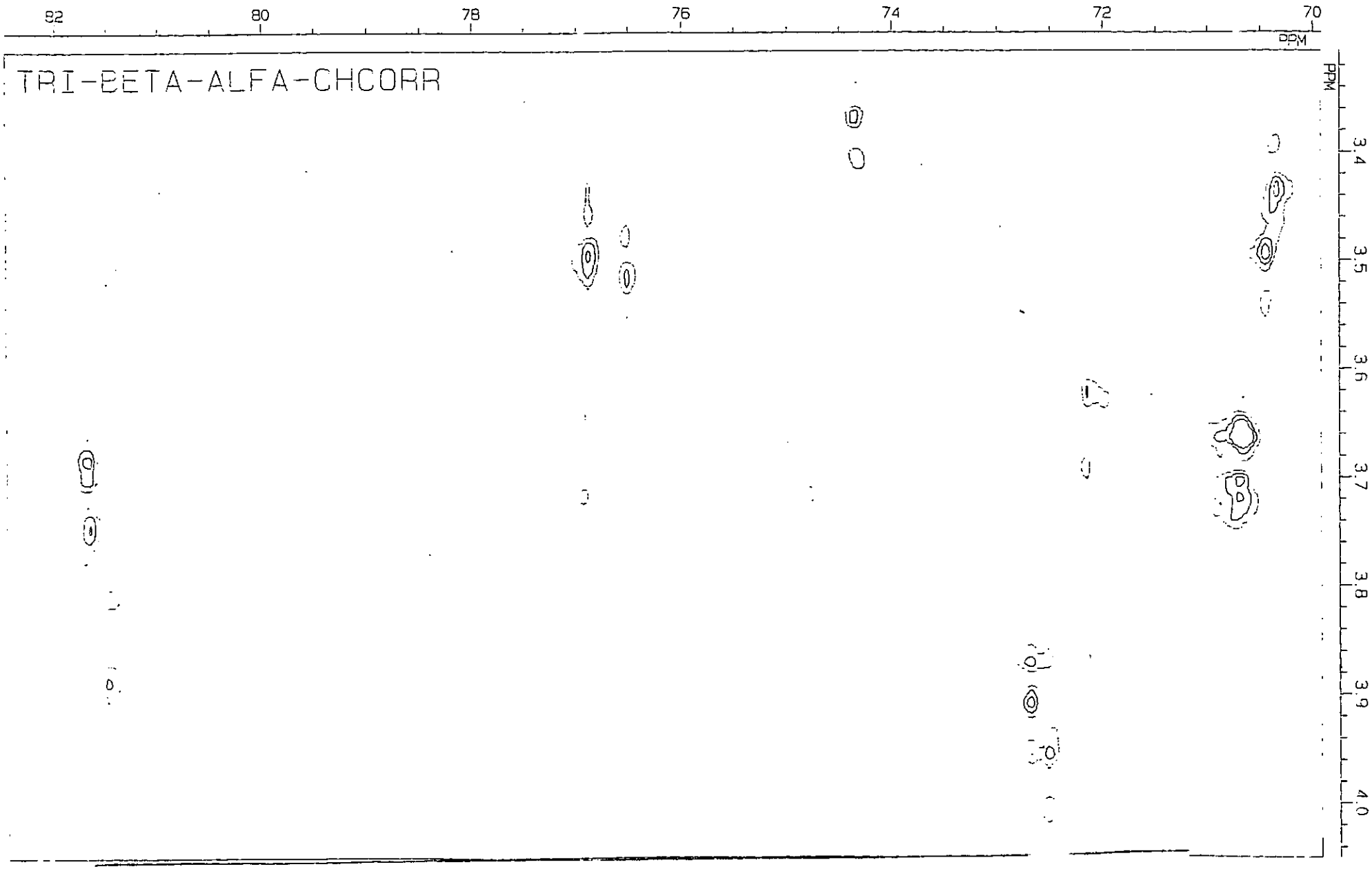


Figure 5.44. CH Correlated spectrum of Trisaccharide 30.

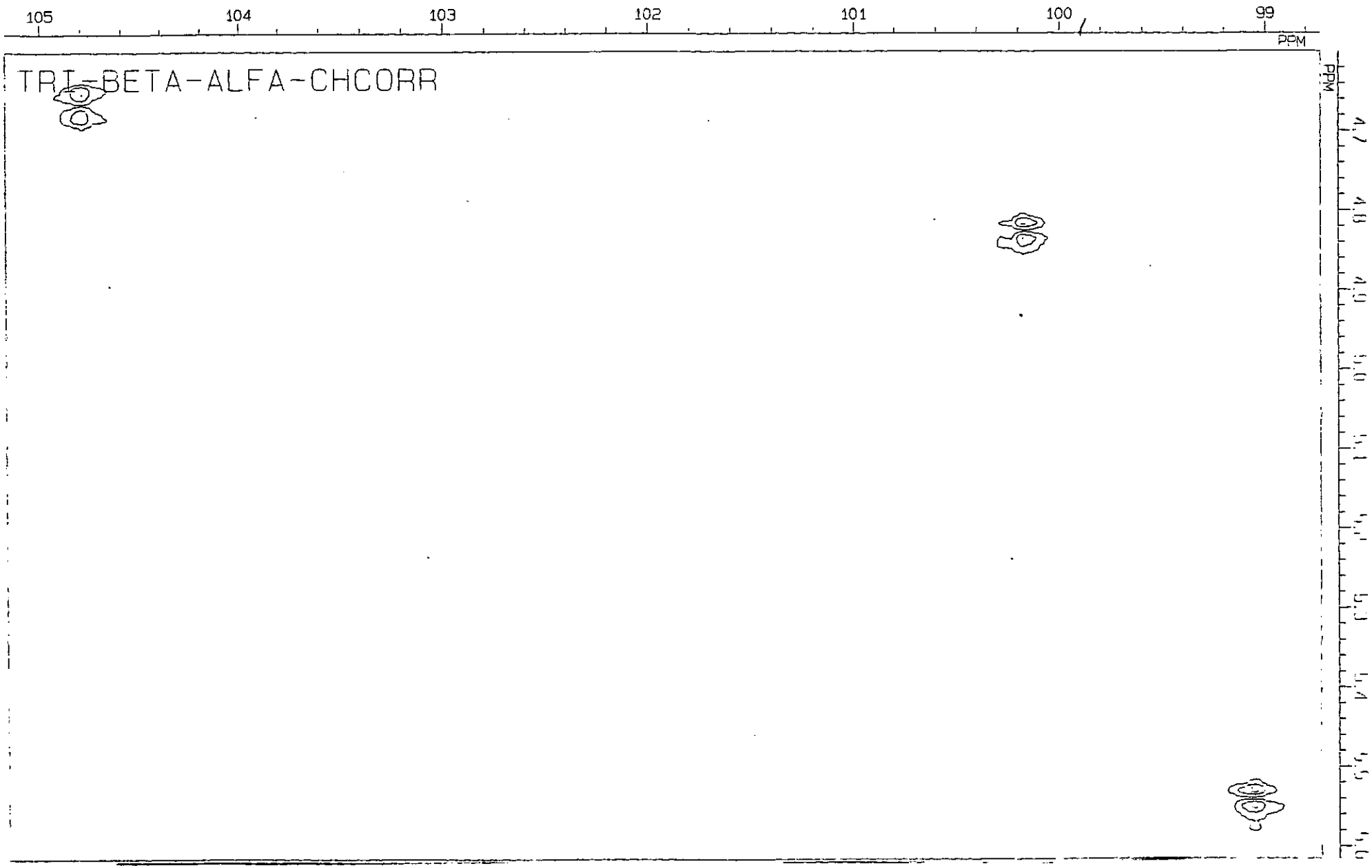


Figure 5.44a. CH Correlated spectrum of Trisaccharide 30 continued.

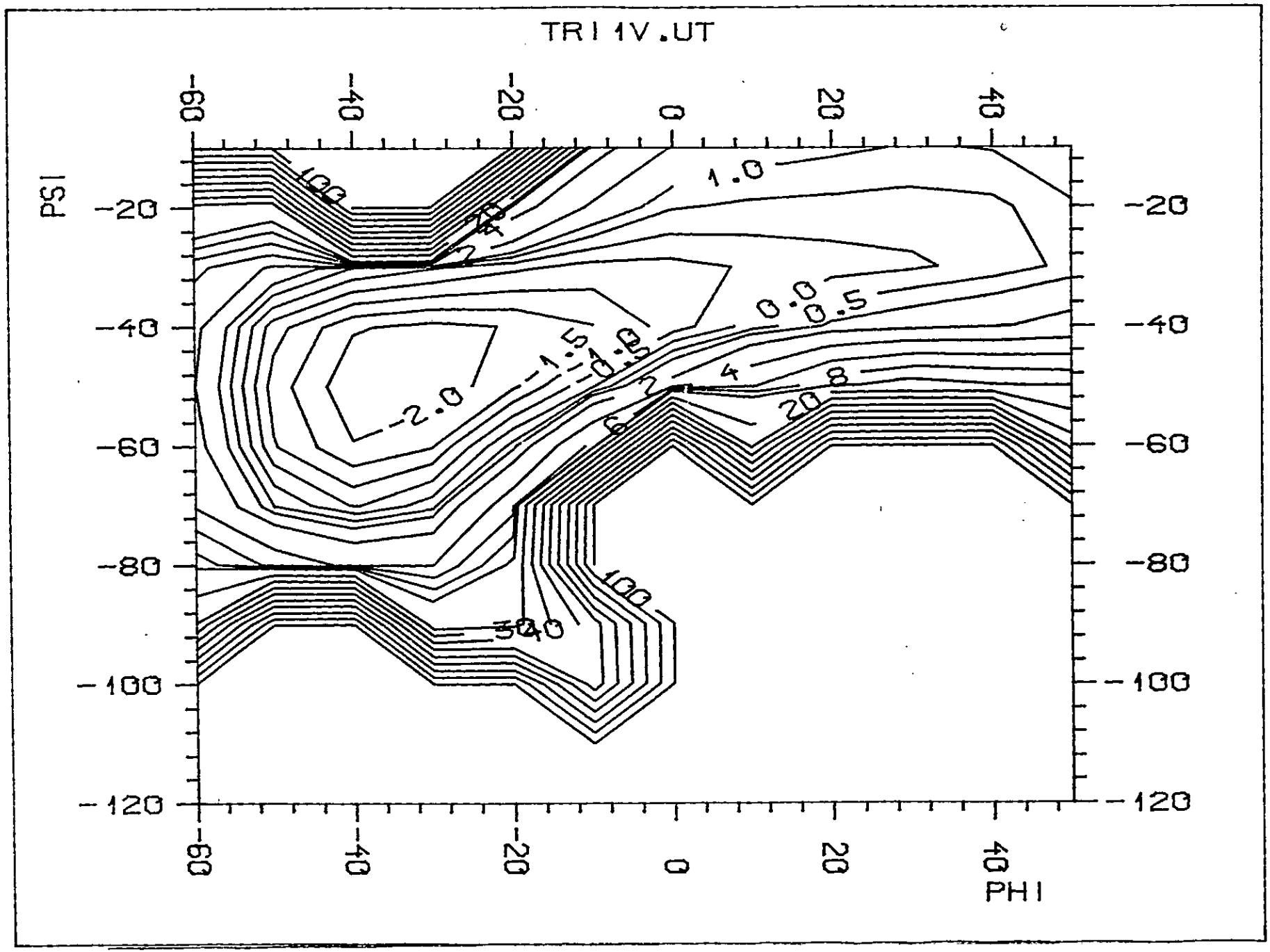


Figure 5.45. Iso-contour Energy Map of Di.1a residue of Trisaccharide 13.

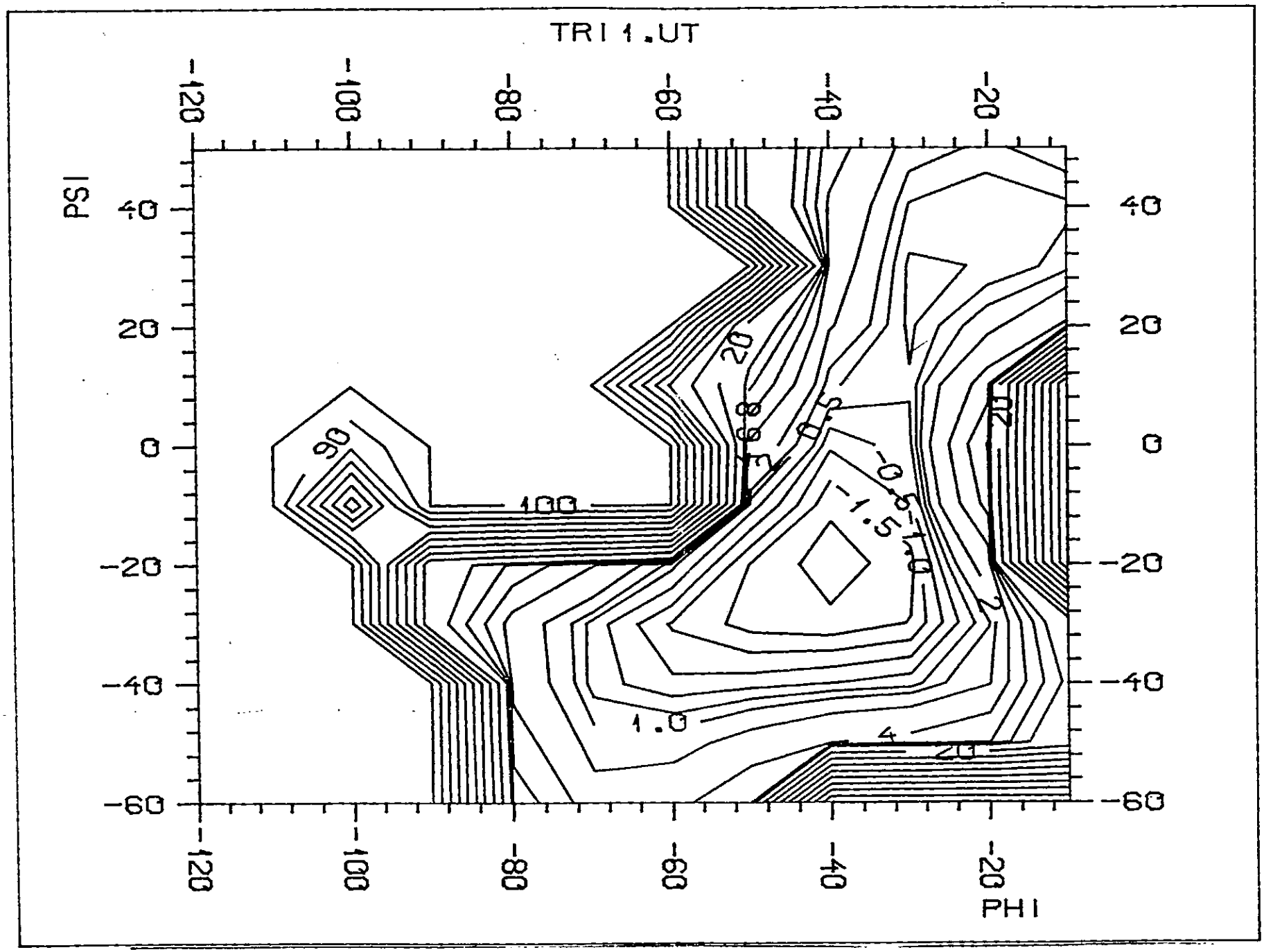


Figure 5.45a. Iso-contour Energy Map of Di.1b residue of Trisaccharide 13.

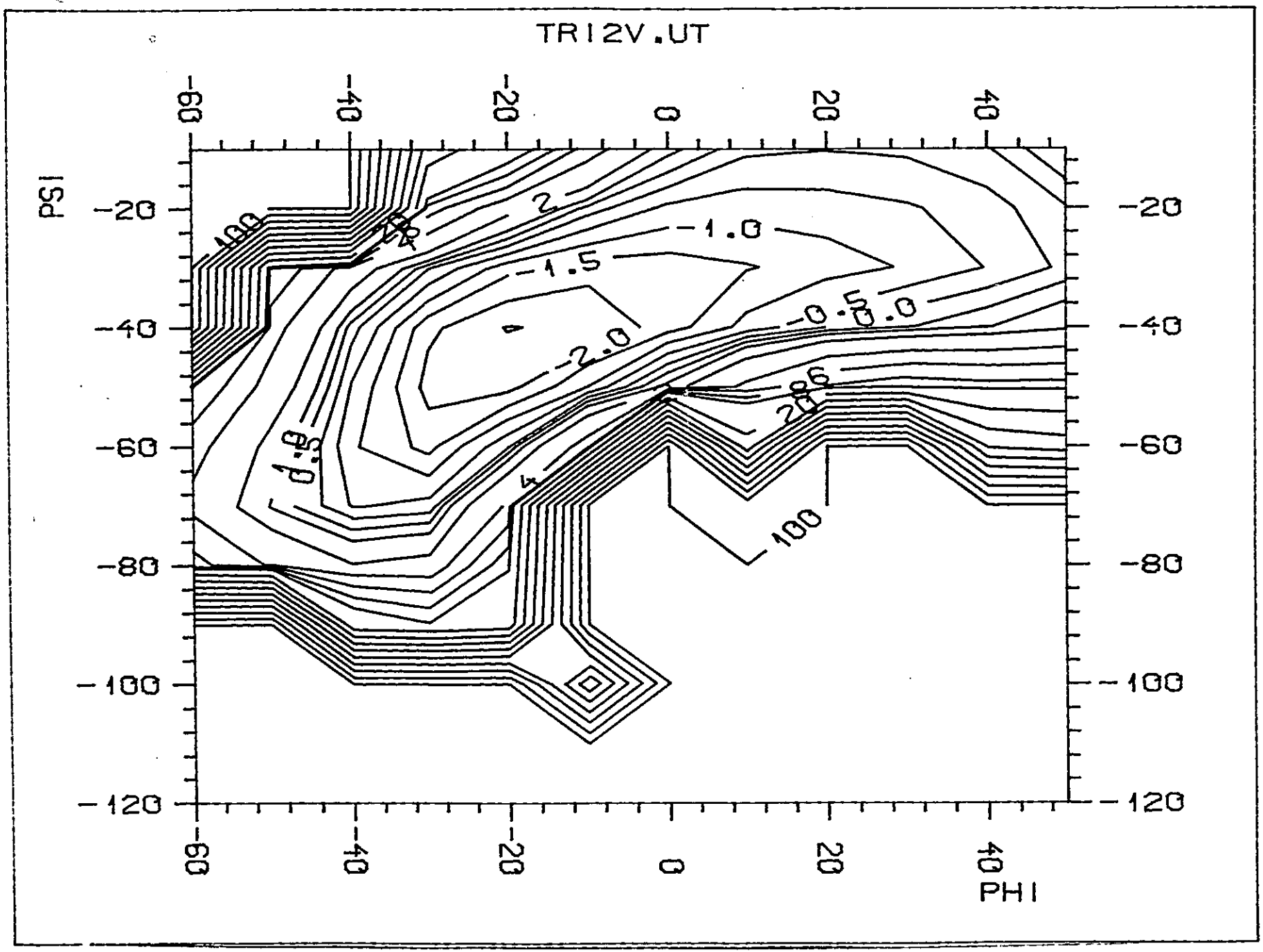


Figure 5.46. Iso-contour Energy Map of Di.2a residue of Trisaccharide 21.

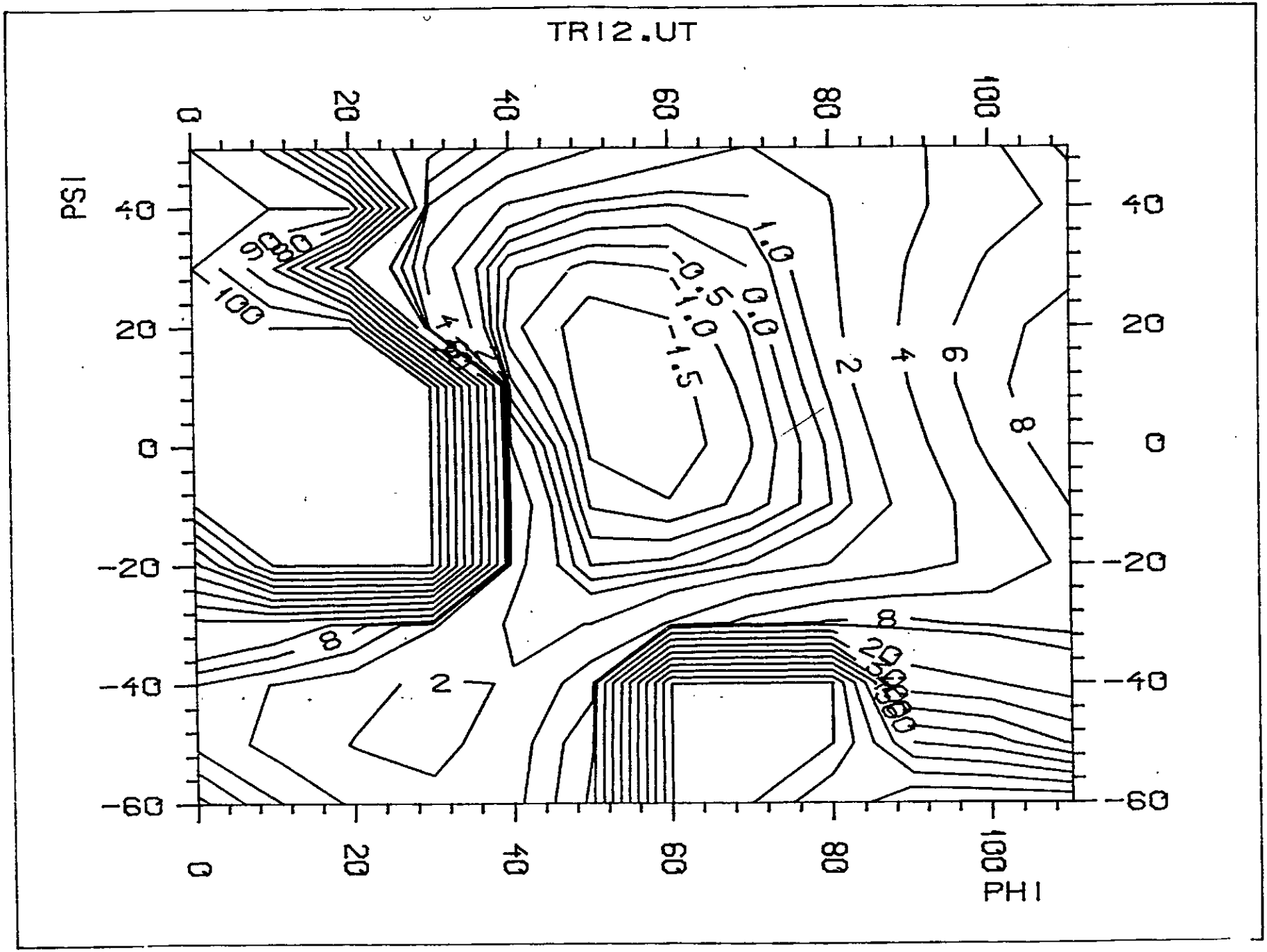


Figure 5.46a. Iso-contour Energy Map of Di.2b residue of Trisaccharide 21.

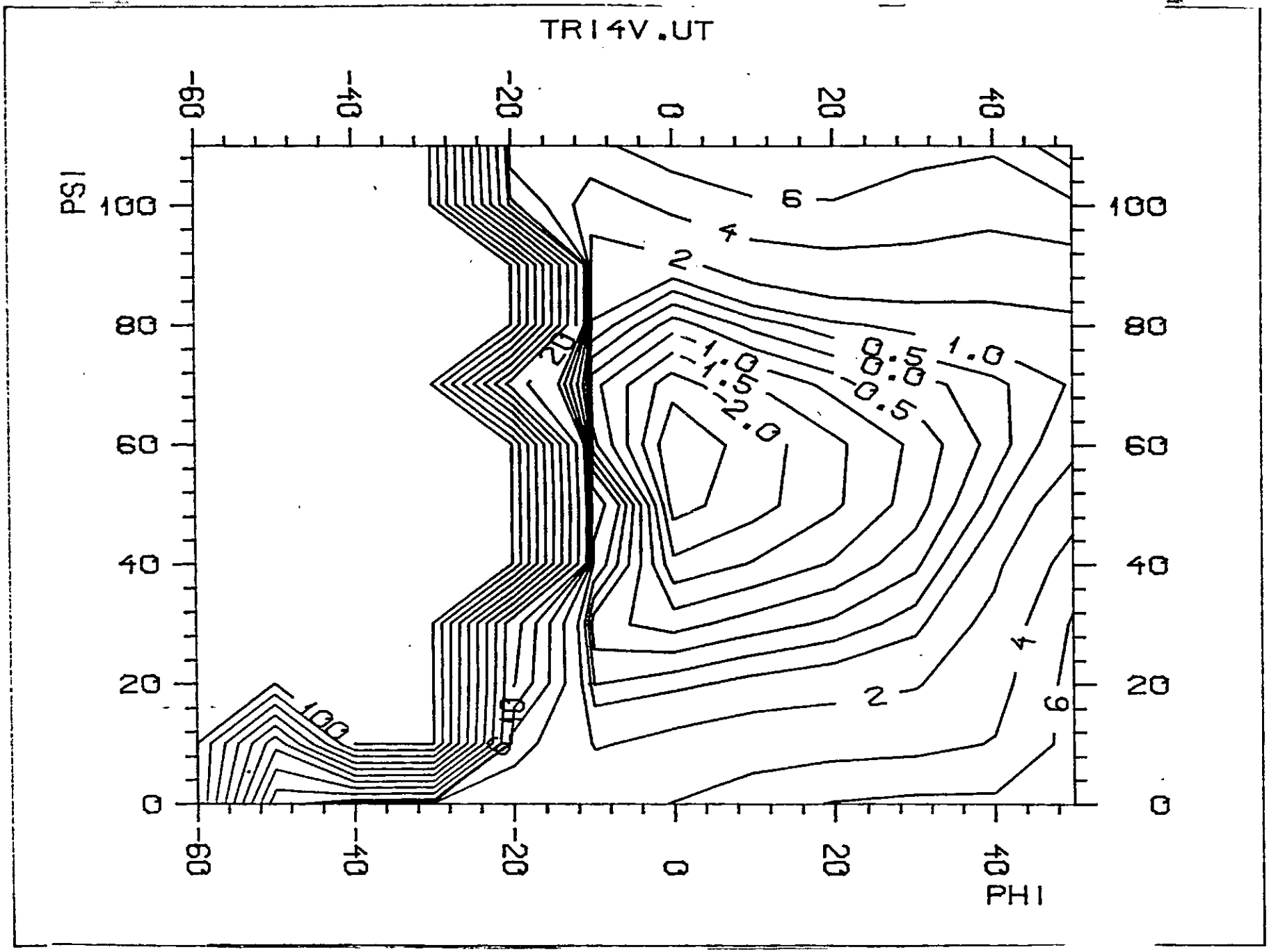


Figure 5.47. Iso-contour Energy Map of Di.3a residue of Trisaccharide 24.

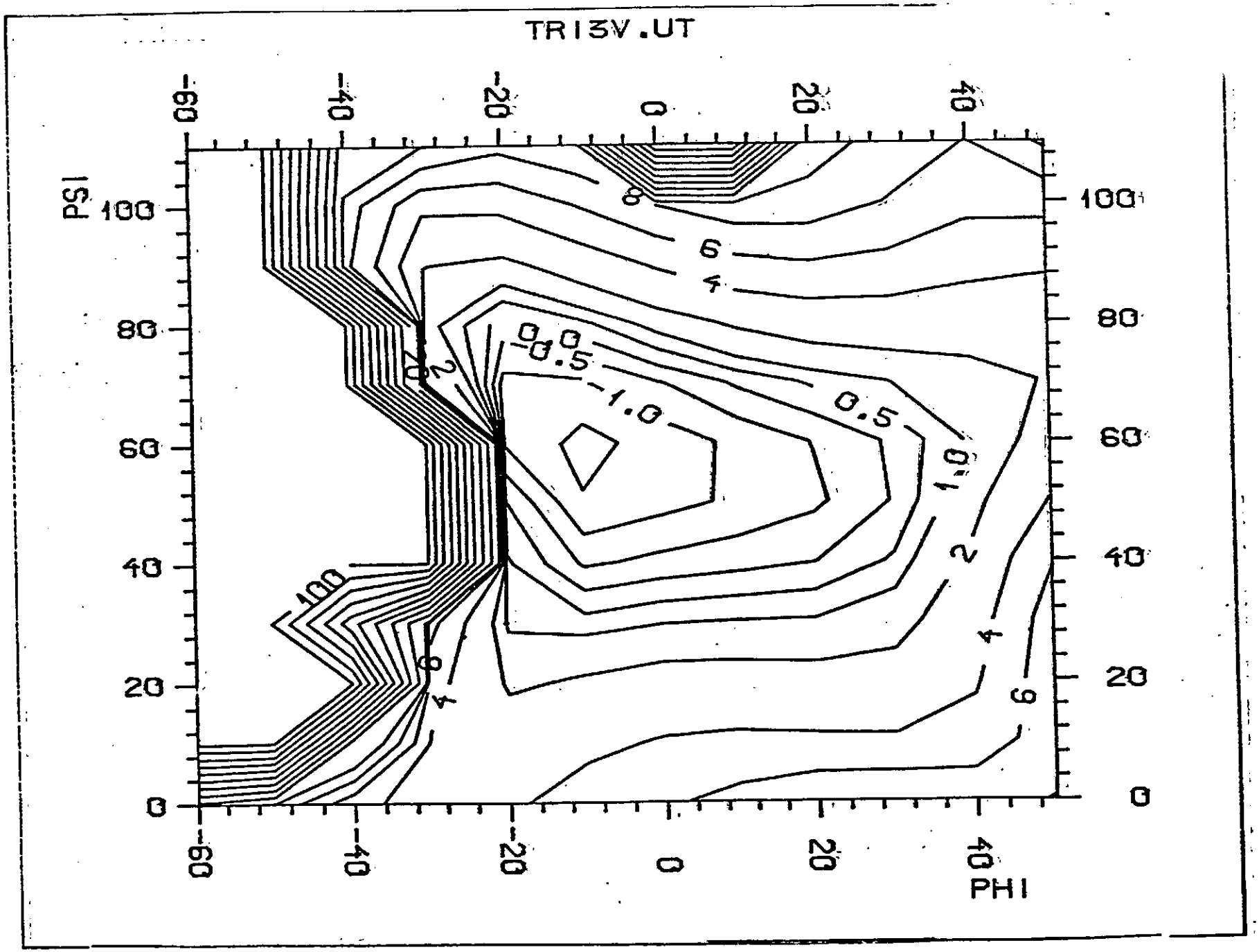


Figure 5.48. Iso-contour Energy Map of Di.4a residue of Trisaccharide 30.

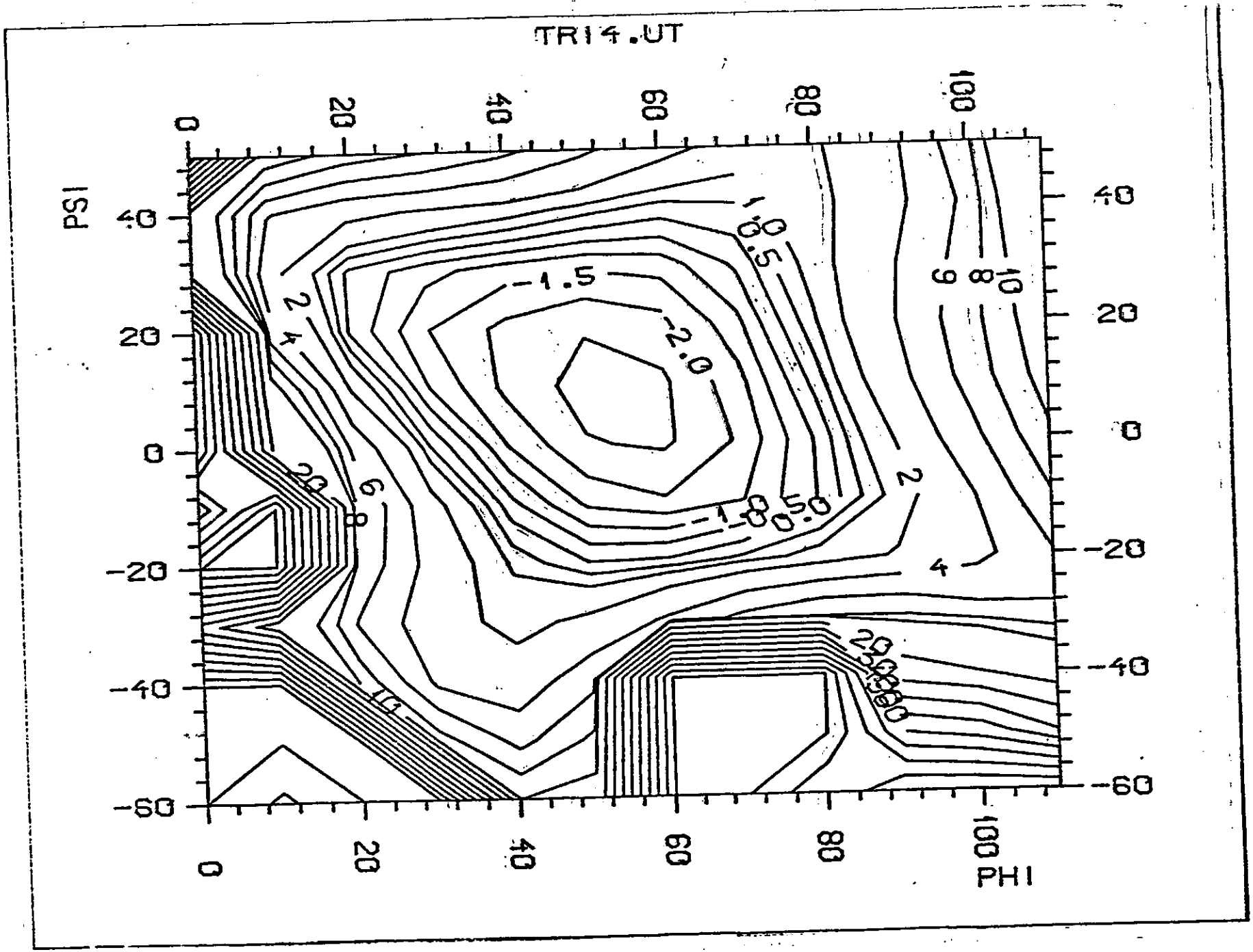


Figure 5.47a. Iso-contour Energy Map of Bi.3b residue of Trisaccharide 24.

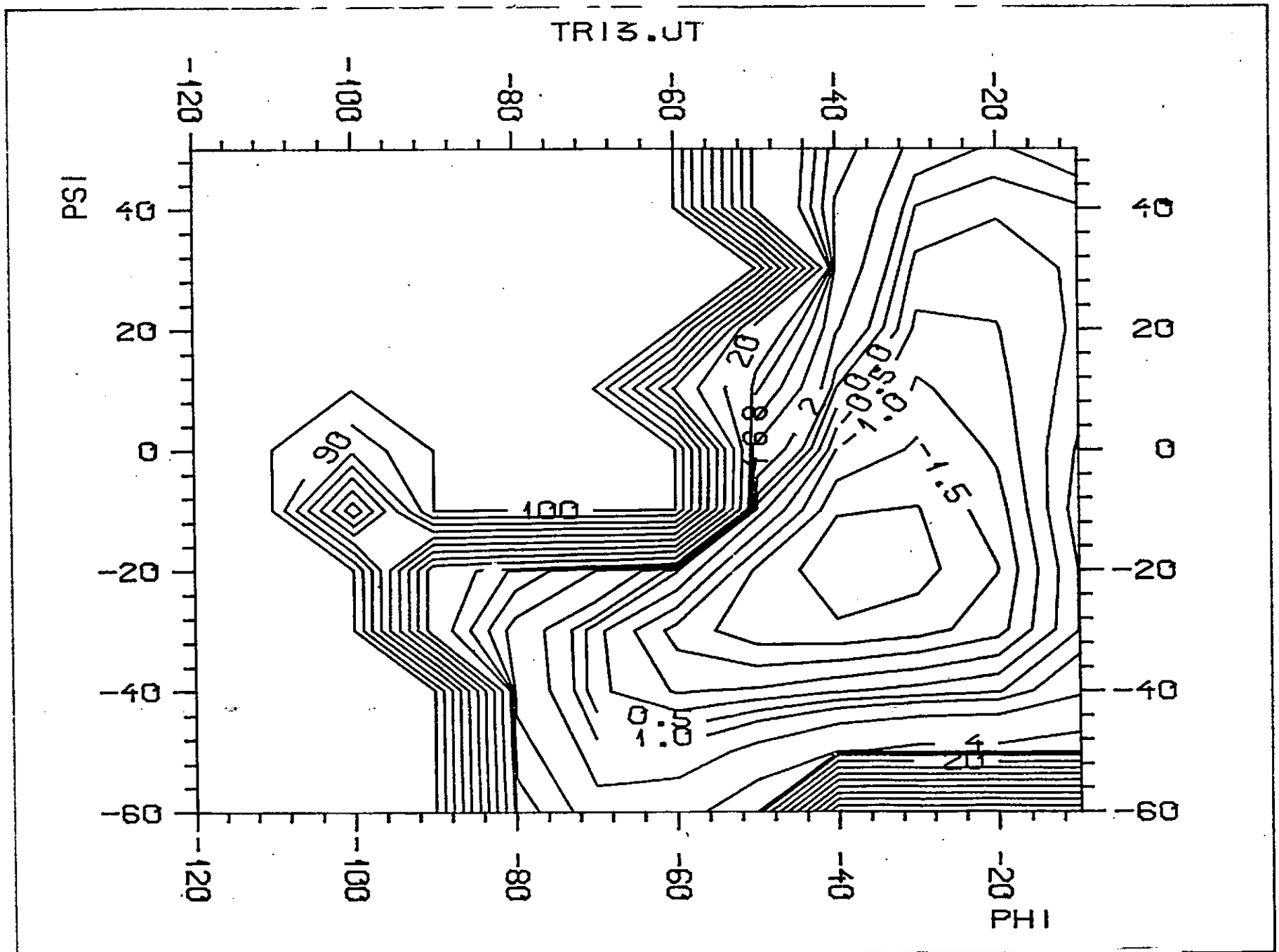


Figure 5.48a. Iso-contour Energy Map of Di.4b residue of Trisaccharide 30.

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TABLES 5.16 TO 5.33
Chemical shift data for Trisaccharides **13,21,24,30 .**

Table 5.16. Values for the ϕ and ψ angles in degrees together with inter-residual internuclear distance <3 A in the minimum energy conformations of **13, 21, 24 and 30**.

Disaccharide residue	ϕ_1/ψ_1	ϕ_2/ψ_2	1"H	5"H	O-5"	1'H	5'H	O-5'
Di.1a	-50/-35	-	2.26(1'H)	2.60(0-3')	2.48(2-H') 2.70(3'-H)	- 2.88(2-H')	- -	-
Di.1b	-	-40/-25	-	-	-	2.26(2-H)	2.48(0-3)	2.70(2-H)
Di.2a	-55/-5	-	1.91(0-1')	2.64(2'H')	2.58(2'-H')			
Di.2b	-	55/5	-	-	-	1.91(0-1')	2.64(2'-H)	2.58(3-H)
Di.3a	60/20		2.34(2'-H)		2.39(1'-H)			
Di.3b	-	-40/-25	-	-	-	2.34(2'-H) 2.70(3-H)	-	2.39(1-H)
Di.4a	60/0	-	2.43(3'-H)	2.59(3-H')				
Di.4b	-	50/10	-	-	-	2.49(2'-H)	-	2.58(3-H)

Table 5.17. ^{1}H n.m.r.chemical shifts of the trisaccharides 13, 21, 24, 30 and the pertinent monosaccharides.

(Chemical shift differences are given in parenthesis.)

*Tris.	1".H	2".H	3".H	4".H	5".H	6".Ha	6'.Hb	1'.H	2.H	3'.H	4'.H	5'.H	6'.Ha	6.Hb	1.H	2.H	3.H	4.H	5.H	6.Ha	6.Hb	OMe
13	5.16	3.60	3.79	3.46	3.97	3.85	3.78	5.51	3.69	3.86	3.50	4.05	3.85	3.78	4.82	3.66	3.83	3.66	3.64	3.87	3.78	3.44
	(- 0.07)	(0.06)	(0.07)	(0.04)	(0.13)	(0.01)	(0.02)	(0.28)	(0.15)	(0.14)	(0.08)	(0.21)	(0.01)	(0.02)	(0.01)	(0.10)	(0.15)	(0.25)	(0.00)	(0.00)	(0.00)	(0.00)
21	5.42	3.60	3.75	3.47	4.04	3.84	3.78	4.83	3.50	3.61	3.45	3.49	3.93	3.73	4.82	3.77	3.86	3.51	3.67	3.87	3.77	3.44
	(- 0.19)	(0.12)	(0.03)	(0.04)	(0.20)	(0.00)	(0.02)	(0.19)	(0.25)	(0.11)	(0.03)	(0.03)	(0.03)	(0.01)	(0.01)	(0.09)	(0.18)	(0.10)	(0.00)	(0.00)	(0.10)	(0.00)
24	4.78	3.35	3.54	3.40	3.51	3.91	3.73	4.74	3.63	3.74	3.47	3.51	3.93	3.73	4.86	3.81	3.82	3.53	3.67	3.87	3.77	3.44
	(0.14)	(0.01)	(0.04)	(-0.02)	(0.05)	(0.01)	(0.01)	(0.01)	(0.38)	(0.24)	(0.05)	(0.05)	(0.03)	(0.01)	(0.05)	(0.13)	(0.14)	(0.12)	(0.03)	(0.00)	(0.10)	(0.00)
30	4.66	3.37	3.52	3.48	3.91	3.75	3.78	5.54	3.71	3.91	3.50	3.98	3.86	3.76	4.82	3.69	3.85	3.69	3.66	3.86	3.76	3.44
	(0.02)	(0.12)	(0.02)	(-0.01)	(0.01)	(0.03)	(0.03)	(0.31)	(0.17)	(0.19)	(0.08)	(0.10)	(0.02)	(0.00)	(0.01)	(0.01)	(0.17)	(0.28)	(-0.02)	(0.01)	(-0.11)	(0.00)
α–D–Glucopyranose	5.23	3.54	3.72	3.42	3.84	3.84	3.76															
β–D–Glucopyranose	4.64	3.25	3.50	3.42	3.46	3.90	3.72															
Methyl-α–D-Glucopyranose															4.81	3.56	3.68	3.41	3.64	3.87	3.76	3.44

*Tris = Trisaccharides

Table 5.18. ^{13}C n.m.r. chemical shift of Trisaccharides 13, 21, 24, 30 and the pertinent monosaccharides

(Chemical shift differences are given in parentheses)

Trisaccharide	C1"	C2"	C3"	C4"	C5"	C6"	C1'	C2'	C3'	C4'	C5'	C6'	C1	C2	C3	C4	C5	C6	OMe
αG-2αG-3αG-OMe (13)	97.18 (4.19)	72.22 (-0.25)	73.81 (0.03)	70.44 (-0.27)	72.74' (-0.37)	61.45 (-0.39)	97.25 (4.26)	76.78 (4.31)	72.18 (-1.60)	70.53 (-0.18)	72.40 (0.03)	61.45 (-0.39)	100.32 (0.13)	70.76 (-1.47)	80.94 (6.84)	70.76 (0.08)	72.22 (-0.30)	61.45 (-0.22)	55.92 (0.01)
αG-2βG-3αG-OMe (21)	99.00 (6.01)	72.37 (-0.10)	73.61 (-0.03)	70.39 (-0.32)	72.73 (0.36)	61.41 (-0.37)	103.54 (6.70)	79.36 (4.16)	75.62 (-1.14)	70.63 (0.02)	96.63 (- 0.13)	61.63 (0.21)	100.05 (- 0.14)	71.52 (-0.71)	83.40 (9.30)	68.98 (-1.70)	72.37 (-0.15)	61.64 (0.02)	55.90 (-0.03)
βG-2βG-3αG-OMe (24)	104.22 (7.38)	74.79 (-0.41)	76.40 (-0.36)	70.50 (-0.21)	77.28 (0.52)	61.79 (-0.05)	102.66 (5.82)	82.89 (10.12)	76.60 (-0.16)	70.33 (-0.38)	76.70 (-0.16)	61.54 (- 0.03)	99.63 (- 0.56)	71.23 (- 1.00)	85.41 (11.31)	69.07 (-1.61)	72.43 (- 0.09)	61.57 (0.10)	55.84 (- 0.09)
βG-2αG-3αG-OMe (30)	104.84 (8.00)	74.37 (-0.83)	76.52 (-0.24)	70.35 (-0.36)	76.90 (0.14)	61.60 (- 0.24)	99.16 (6.17)	81.70 (9.23)	72.71 (-1.07)	70.45 (- 0.26)	72.54 (0.17)	61.47 (- 0.37)	100.25 (- 0.06)	70.71 (-1.52)	81.50 (7.40)	70.68 (0.00)	72.16 (-0.36)	61.47 (0.26)	55.90 (0.03)
α-D-Glucopyranose	92.99	72.47	73.78	70.71	72.37	61.84													
β-D-Glucopyranose	96.84	72.20	76.76	70.71	76.76	61.84													
Methyl-α-D-Glucopyranose													100.19	72.73	74.10	70.68	72.52	61.67	55.93

Table 5.19. Calculation of the simulated chemical shifts from the ^{13}C n.m.r. spectra and the difference between the simulated and the observed chemical shifts for α-D-Glc(1→2)-α-D-Glc(1→3)-α-Glc-OMe (13).

	(a)	(b)	(c)			
	Mns.	Δ2	Δ3	Σ	Obs	Obs-calc.
α	92.99	4.58		97.57	97.18	-0.39
	72.47	-0.21		72.26	72.22	-0.04
	73.78	-0.02		73.76	73.81	-0.05
	70.71	-0.17		70.54	70.44	-0.10
	72.37	0.48		72.85	72.74	-0.11
	61.84	-0.33		61.51	61.45	-0.06
α	92.99	-2.76	6.96	97.19	97.25	0.06
	72.47	4.63	0.19	77.29	76.78	-0.51
	73.47	-1.63	0.13	72.28	70.53	-0.01
	70.71	-0.02	0.15	70.54	70.53	-0.01
	72.37	-0.14	0.44	72.67	72.40	-0.27
	61.84	-0.04	-0.36	61.44	61.45	0.01
α	100.19		0.11	100.30	100.32	0.02
	72.23		-1.40	70.83	70.76	-0.07
	74.10		7.41	81.51	80.94	-0.57
	70.68		0.15	70.83	70.76	-0.07
	72.52		-0.23	72.29	72.22	-0.07
	61.67		-0.12	61.55	61.45	-0.10
OMe	55.93		-0.01	55.92	55.92	0.00

Mns = chemical shift of α- or β-linked glucopyranose

$\Delta 2^{ref}$ = the chemical shift difference between the 1,2 linked disaccharides and the corresponding α- or β-D-glucopyranose.

Δ3 = the chemical shift difference between the 1,3 linked disaccharides and the corresponding α- or β-D-glucopyranose.

Σ = Total sum of the added chemical shifts from columns a, b, and c.

Obs = The observed chemical shifts from the n.m.r.spectra.

Calc = The calculated chemical shift from columns 1,2 and 3.

Table 5.20. Calculation of the simulated chemical shifts from the ^{13}C n.m.r. spectra and the difference between the simulated and the observed chemical shifts for α-D-Glc(1→2)-β-D-Glc(1→3)-α-Glc-OMe (21).

	(a) Mns.	(b) Δ2	(c) Δ3	Σ	Obs	Obs-calc
α	92.99	5.65		98.64	99.00	0.36
	72.47	-0.15		72.32	72.37	0.05
	73.78	-0.01		73.77	73.81	0.04
	70.71	-0.21		70.50	70.39	-0.11
	72.37	-0.38		72.62	72.73	0.11
	61.84	-0.38		61.46	61.41	-0.05
β	96.84	0.51	6.83	104.18	103.54	-0.64
	75.20	4.68	-0.78	79.10	79.36	0.26
	76.76	-1.37	-0.16	75.23	75.62	0.39
	70.71	0.17	-0.13	70.75	76.73	-0.02
	76.76	-0.21	0.13	76.68	76.63	-0.05
	61.84	0.02	-0.20	61.66	61.63	-0.03
α	100.19	-0.15		100.04	100.05	0.01
	72.23	-0.62		71.61	71.52	-0.09
	74.10	9.54		83.74	83.40	-0.34
	70.68	-1.55		69.13	68.98	-0.15
	72.52	-0.17		72.35	72.37	0.02
	61.67	0.04		61.71	61.69	-0.02
OMe	55.93	-0.01		55.92	55.90	-0.02

Table 5.21. Calculation of the simulated chemical shifts from the ^{13}C n.m.r. spectra and the difference between the simulated and the observed chemical shifts for β-D-Glc(1→2)-β-D-Glc(1→3)-α-Glc-OMe (24).

	(a) Mns.	(b) Δ2	(c) Δ3	Σ	Obs	Obs-calc
β	96.84	6.54		103.38	104.22	0.84
	72.47	-0.62		74.58	74.79	0.21
	73.78	-0.13		76.63	76.40	-0.23
	70.71	-0.17		70.54	70.50	-0.04
	72.37	0.17		76.93	77.28	0.35
	61.84	-0.10		61.74	61.79	-0.05
β	96.84	-1.40	6.83	102.27	102.66	0.39
	75.20	7.29	-0.78	81.71	82.59	0.88
	76.76	0.01	-0.16	76.61	76.52	-0.01
	70.71	-0.15	-0.13	70.43	70.33	-0.01
	76.76	-0.15	0.13	76.74	76.70	-0.04
	61.84	-0.08	-0.20	61.56	61.54	-0.02
α	100.19		-0.15	100.04	99.63	0.41
	72.23		-0.62	71.61	71.23	-0.38
	74.10		9.54	83.74	85.41	1.67
	70.68		-1.55	69.13	68.98	-0.06
	72.52		-0.17	72.35	72.37	0.08
	61.67		0.04	61.71	61.69	-0.14
OMe	55.93		-0.01	55.92	55.84	-0.08

Table 5.22. Calculation of the simulated chemical shifts from the ^{13}C n.m.r. spectra and the difference between the simulated and the observed chemical shifts for β-D-Glc(1→2)-α-D-Glc(1→3)-α-Glc-OMe (30).

	(a) Mns.	(b) Δ2	(c) Δ3	Σ	Obs	Obs-calc.
β	96.84	7.75		104.59	104.84	-0.25
	75.20	-0.95		74.25	74.37	-0.12
	76.76	-0.11		76.65	76.52	-0.13
	70.71	-0.14		70.57	70.35	-0.22
	76.76	0.00		76.76	76.90	-0.14
	61.84	-0.01		61.83	61.60	-0.23
α	92.99	-0.38	6.96	99.57	99.16	0.41
	72.47	8.87	-0.19	81.53	81.70	0.17
	73.78	-1.10	0.13	72.81	72.71	-0.10
	70.71	-0.11	-0.15	70.45	70.45	0.00
	72.37	-0.29	0.44	72.52	72.54	0.02
	61.84	-0.06	-0.36	61.42	61.47	0.05
α	100.19		0.11	100.04	100.25	-0.05
	72.23		-1.40	71.61	70.71	-0.12
	74.10		7.41	81.51	81.50	-0.01
	70.68		-1.55	70.83	70.68	-0.15
	72.52		-0.17	72.29	72.16	-0.13
	61.67		-0.12	61.55	61.47	-0.08
OMe	55.93		-0.01	55.92	55.90	-0.02

Table 5.23. Calculation of the simulated chemical shifts from the ^{1}H n.m.r. spectra and the difference between the simulated and the observed chemical shifts for α-D-Glc(1→2)-α-D-Glc(1→3)-α-Glc-OMe (13).

	(a) Mns.	(b) Δ2	(c) Δ3	Σ	Obs	Obs-calc.
α	5.23	-0.16		5.07	5.16	0.09
	3.54	0.02		3.56	3.60	0.04
	3.72	0.06		3.78	3.79	0.01
	3.42	0.03		3.45	3.46	0.01
	3.84	0.08		3.92	3.97	0.05
	3.76	0.01		3.85	3.85	0.00
				3.77	3.78	0.01
α	5.23	0.20	0.09	5.52	5.51	-0.01
	3.54	0.10	0.04	3.68	3.69	0.01
	3.72	0.10	0.04	3.86	3.86	0.00
	3.42	0.06	0.02	3.50	3.50	0.00
	3.84	0.02	0.13	3.99	4.05	0.06
	3.84	0.01	0.03	3.88	3.85	-0.03
	3.76	0.01	0.02	3.79	3.78	-0.01
α	4.81		0.01	4.82	4.82	0.00
	3.56		0.10	3.66	3.66	0.00
	3.68		0.14	3.83	3.83	0.01
	3.41		0.25	3.66	3.66	0.00
	3.64		0.02	3.64	3.64	-0.02
	3.87		-0.02	3.87	3.87	-0.02
	3.76		0.00	3.78	3.78	0.02
OMe	3.43		0.01	3.44	3.44	0.00

Table 5.24. Calculation of the simulated chemical shifts from the ^{1}H n.m.r. spectra and the difference between the simulated and the observed chemical shifts for α-D-Glc(1→2)-β-D-Glc(1→3)-α-Glc-OMe (21).

	(a) Mns	(b) Δ2	(c) Δ3	Σ	Obs	Obs-calc.
α	5.23	-0.05		5.28	5.42	0.16
	3.54	0.00		3.54	3.60	0.04
	3.72	0.03		3.75	3.79	0.00
	3.42	0.02		3.44	3.47	0.03
	3.84	0.16		4.00	4.04	0.04
	3.84	-0.01		3.83	3.84	0.01
	3.76	0.01		3.77	3.78	0.01
β	4.64	0.14	0.04	4.82	4.83	0.01
	3.25	0.12	0.12	3.49	3.50	0.01
	3.50	0.10	0.04	3.64	3.61	0.03
	3.42	0.02	0.01	3.45	3.45	0.00
	3.46	-0.01	0.04	3.49	3.49	0.00
	3.90	0.01	0.02	3.93	3.93	0.00
	3.72	0.00	0.02	3.74	3.73	-0.01
α	4.81		0.02	4.83	4.82	-0.00
	3.56		0.19	3.75	3.77	0.02
	3.68		0.19	3.87	3.86	-0.01
	3.41		0.11	3.52	3.51	-0.00
	3.64		0.03	3.67	3.67	0.00
	3.87		0.01	3.87	3.87	-0.01
	3.76		0.01	3.77	3.77	0.00
OMe	3.43		0.01	3.44	3.44	0.00

Table 5.25. Calculation of the simulated chemical shifts from the ^{1}H n.m.r. spectra and the difference between the simulated and the observed chemical shifts for β-D-Glc(1→2)-β-D-Glc(1→3)-α-Glc-OMe (24).

	(a) Mns.	(b) Δ2	(c) Δ3	Σ	Obs	Obs-calc.
β	4.64	0.12		4.72	4.78	0.02
	3.25	0.09		3.34	3.35	0.01
	3.50	0.02		3.52	3.54	0.02
	3.42	0.01		3.43	3.40	-0.03
	3.46	-0.05		3.41	3.51	0.10
	3.90	-0.02		3.92	3.91	-0.01
	3.72	0.02		3.74	3.93	-0.01
β	4.64	0.11	0.04	4.79	4.74	-0.05
	3.25	0.28	0.12	3.65	3.63	-0.01
	3.50	0.20	0.04	3.74	3.74	0.00
	3.42	0.03	0.01	3.47	3.47	0.01
	3.46	-0.02	0.04	3.51	3.51	0.03
	3.90	0.01	0.02	3.93	3.93	0.02
	3.72	0.01	0.02	3.73	3.73	-0.02
α	4.81		0.02	4.83	4.86	-0.03
	3.56		0.19	3.75	3.81	0.02
	3.68		0.19	3.87	3.82	-0.05
	3.41		0.11	3.52	3.53	-0.01
	3.64		0.03	3.67	3.67	0.00
	3.87		0.01	3.88	3.87	-0.01
	3.76		0.01	3.77	3.77	0.00
OMe	3.43		0.01	3.44	3.44	0.00

Table 5.26. Calculation of the simulated chemical shifts from the ^{1}H n.m.r. spectra and the difference between the simulated and the observed chemical shifts for β-D-Glc(1→2)-α-D-Glc(1→3)-α-Glc-OMe (30).

	(a) Mns.	(b) Δ2	(c) Δ3	Σ	Obs	Obs-calc.
β	4.64	0.02		4.62	4.66	0.04
	3.25	0.12		3.37	3.37	0.00
	3.50	0.02		3.52	3.52	0.00
	3.42	0.01		3.43	3.43	-0.00
	3.46	-0.03		3.43	3.48	0.05
	3.90	0.01		3.91	3.91	-0.00
	3.72	0.02		3.75	3.75	-0.01
α	5.23	0.18	0.09	5.50	5.54	-0.04
	3.54	0.11	0.04	3.69	3.71	-0.02
	3.72	0.16	0.04	3.92	3.91	-0.01
	3.42	0.04	0.02	3.48	3.50	0.02
	3.84	-0.01	0.13	3.98	3.98	0.00
	3.84	0.01	0.03	3.88	3.86	-0.02
	3.76	0.01	0.02	3.79	3.76	-0.03
α	4.81		0.01	4.82	4.82	0.00
	3.56		0.10	3.66	3.39	0.03
	3.68		0.14	3.82	3.85	0.03
	3.41		0.25	3.66	3.69	0.00
	3.64		0.02	3.66	3.66	0.00
	3.87		-0.02	3.85	3.86	0.01
	3.76		0.00	3.76	3.76	0.00
OMe	3.43		0.01	3.44	3.44	0.00

Table 5.27. A summary of Tables 5.19 to 5.22 of the ^{13}C n.m.r. chemical shifts showing the differences between the calculated and the observed values.

Trisaccharide		C1"	C2"	C3"	C4"	C5"	C6"	C1'	C2'	C3'	C4'	C5'	C6'	C1	C2	C3	C4	C5	C6	OMe
αG-2αG-3αG-OMe	Obs	97.18	72.22	73.81	70.44	72.74	61.45	97.25	76.78	72.18	70.53	72.40	61.45	100.32	70.76	80.94	70.76	72.22	61.45	55.92
(13)	Calc	97.57	72.26	73.76	70.54	72.85	61.51	97.19	77.29	72..67	70.54	72.67	61.44	100.30	70.83	81.51	70.83	72.29	61.55	55.92
	Obs -Calc	- 0.31	- 0.04	0.05	- 0.10	- 0.11	- 0.06	0.06	- 0.51	- 0.10	- 0.01	- 0.27	0.01	0.02	- 0.07	- 0.57	- 0.07	- 0.07	- 0.10	0.00
αG-2βG-3αG-OMe	Obs	99.00	72.37	73.81	70.39	72.73	61.41	103.54	79.36	75.62	70.73	76.63	61.63	100.05	71.52	83.40	68.98	72.37	61.69	55.90
(21)	Calc	98.64	72.32	73.77	70.50	72.62	61.46	104.18	79.10	75.23	70.75	76.68	61.66	100.04	71.61	83.74	69.13	72.35	61.71	55.92
	Obs-calc	0.36	0.05	0.04	- 0.11	0.11	- 0.05	- 0.64	0.26	0.39	- 0.02	- 0.05	-0.03	0.01	-0.09	-0.34	- 0.15	0.02	- 0.02	- 0.02
βG-2βG-3αG-OMe	Obs	104.22	74.79	76.40	70.50	77.28	61.79	102.66	82.89	76.60	70.33	76.70	61.54	99.63	71.23	85.41	69.07	72.43	61.57	55.84
(24)	Calc	103.38	74.58	76.63	70.54	76.93	61.74	102.27	81.71	76.61	70.43	76.74	61.56	100.04	71.61	83.74	69.13	72.35	61.75	55.92
	Obs-Calc	0.84	0.21	- 0.23	- 0.04	0.35	0.05	0.39	0.88	- 0.01	- 0.10	- 0.04	- 0.02	- 0.41	- 0.38	1.67	- 0.06	0.08	- 0.14	- 0.08
βG-2αG-3αG-OMe	Obs	104.84	74.37	76.52	70.35	76.90	61.60	99.16	81.70	72.71	70.45	72.54	61.47	100.25	70.71	81.50	70.68	72.16	61.47	55.90
(30)	Calc	104.59	74.25	76.65	70.57	76.76	61.83	99.57	81.53	72.81	70.45	72.52	61.42	100.30	70.83	81.51	70.83	72.29	61.55	55.92
	Obs-calc	0.25	0.12	0.13	-0.22	0.14	-0.23	- 0.41	0.17	- 0.10	0.00	0.02	0.05	- 0.05	- 0.12	- 0.01	- 0.15	- 0.13	- 0.08	- 0.02

Table 5.28. Summary of Tables 5.23 to 5.26 showing the differences between the calculated and the observed chemical shift of the ^{1}Hn.m.r. spectra for Trisaccharides 13, 21, 24 and 30.

Trisaccharide		1".H	2".H	3".H	4".H	5".H	6".Ha	6'.Hb	1'.H	2.H	3'.H	4'.H	5'.H	6'.Ha	6.Hb	1.H	2.H	3.H	4.H	5.H	6.Ha	6.Hb	OMe
αG-2αG-3αG-OMe	Obs	5.16	3.60	3.79	3.46	3.97	3.85	3.78	5.51	3.69	3.86	3.50	4.05	3.85	3.78	4.82	3.66	3.83	3.66	3.64	3.87	3.78	3.44
	Calc	5.07	3.56	3.78	3.45	3.92	3.85	3.77	5.52	3.68	3.86	3.50	3.99	3.88	3.79	4.82	3.66	3.82	3.66	3.66	3.85	3.76	3.44
(13)	Obs- Calc	0.09	0.04	0.01	0.01	0.05	0.00	0.01	- 0.01	0.01	0.00	0.00	0.06	0.03	- 0.01	0.00	0.00	0.01	0.00	- 0.02	0.02	0.02	0.00
αG-2βG-3αG-OMe	Obs	5.42	3.60	3.75	3.47	4.04	3.84	3.78	4.83	3.50	3.61	3.45	3.49	3.93	3.73	4.82	3.77	3.86	3.51	3.67	3.87	3.77	3.44
	Calc	5.28	3.54	3.75	3.44	4.00	3.83	3.77	4.82	3.49	3.64	3.45	3.49	3.93	3.74	4.83	3.75	3.87	3.52	3.67	3.88	3.77	3.44
(21)	Obs- Calc	0.14	0.06	0.01	0.03	0.04	0.01	0.01	0.01	0.01	0.00	0.00	0.06	0.03	- 0.01	- 0.01	0.00	- 0.01	0.00	- 0.02	0.02	0.00	0.00
βG-2βG-3αG-OMe	Obs	4.78	3.35	3.54	3.40	3.51	3.91	3.73	4.74	3.63	3.74	3.47	3.51	3.93	3.73	4.86	3.81	3.82	3.53	3.67	3.87	3.77	3.44
	Calc	4.76	3.34	3.52	3.43	3.41	3.92	3.74	4.79	3.65	3.74	3.46	3.49	3.91	3.95	4.83	3.75	3.87	3.52	3.67	3.88	3.77	3.44
(24)	Obs- Calc	0.02	0.01	0.02	- 0.03	0.10	- 0.01	- 0.01	- 0.05	-0.02	0.00	0.00	0.06	0.03	- 0.02	0.03	0.06	- 0.05	0.01	- 0.01	0.02	0.00	0.00
βG-2αG-3αG-OMe	Obs	4.66	3.37	3.52	3.48	3.91	3.75	3.78	5.54	3.71	3.91	3.50	3.98	3.86	3.76	4.82	3.69	3.85	3.69	3.66	3.86	3.76	3.44
	Calc	4.62	3.37	3.52	3.43	3.91	3.76	3.77	5.50	3.69	3.92	3.48	3.98	3.88	3.79	4.82	3.66	3.82	3.66	3.66	3.85	3.76	3.44
(30)	Obs- Calc	0.04	0.00	0.00	0.05	0.00	- 0.00	0.01	0.04	0.02	- 0.00	0.02	0.00	- 0.02	- 0.03	0.00	0.03	0.03	0.03	0.00	0.01	0.02	0.00

Table 5.29. The temperature dependence of the ^{13}C n.m.r. chemical shift for α-D-Glc(1→2)-α-D-Glc(1→3)-α-Glc-OMe (13)

	70°	30°	Δ		70°	30°	Δ		70°	30°	Δ
C1ᵐ	97.18	96.80	0.38	C1'	97.25	97.18	0.07	C1	100.32	100.31	0.01
2	72.22	72.10	0.12	2	76.78	76.19	0.59	2	70.76	70.57	0.19
3	73.81	73.70	0.16	3	72.18	72.01	0.17	3	80.94	80.52	0.42
4	70.44	70.05	0.30	4	70.53	70.14	0.39	4	70.76	70.68	0.08
5	72.74	72.57	0.14	5	72.40	72.24	0.16	5	72.22	72.10	0.12
6	61.45	61.17	0.28	6	61.45	61.09	0.36	6	61.45	61.17	0.28
								OMe	55.92	55.86	0.06

Table 5.30. The temperature dependence of the ^{13}C n.m.r. chemical shift for α-D-Glc(1→2)-β-D-Glc(1→3)-α-Glc-OMe (21)

	70°	30°	Δ		70°	30°	Δ		70°	30°	Δ
C1ᵐ	99.00	98.83	0.17	C1'	103.54	103.68	-0.14	C1	100.05	99.98	0.07
2	72.37	72.21	0.16	2	79.36	78.84	0.52	2	71.52	71.52	0.00
3	73.81	73.70	0.11	3	75.62	75.43	0.19	3	83.40	83.03	0.37
4	70.39	70.05	0.34	4	70.73	70.57	0.16	4	68.98	68.76	0.22
5	72.73	72.57	0.16	5	76.63	76.59	0.04	5	72.37	72.32	0.05
6	61.41	61.06	0.35	6	61.63	61.40	0.23	6	61.69	61.55	0.14
								OMe	55.90	55.82	0.08

Table 5.31. The temperature dependence of the ^{13}C n.m.r. chemical shift for β-D-Glc(1→2)-β-D-Glc(1→3)-α-Glc-OMe (24)

	70°	30°	Δ		70°	30°	Δ		70°	30°	Δ
C1''	104.22	104.19	0.03	C1'	102.66	102.87	-0.21	C1	99.63	99.49	0.14
2	74.79	74.67	0.12	2	82.59	82.42	0.17	2	71.23	71.22	0.01
3	76.40	76.21	0.19	3	76.60	76.51	0.09	3	85.41	85.57	-0.16
4	70.50	70.37	0.13	4	70.33	70.15	0.18	4	69.07	68.91	0.16
5	77.28	77.23	0.05	5	76.70	76.64	0.06	5	72.43	72.35	0.08
6	61.79	61.61	0.18	6	61.54	61.30	0.24	6	61.57	61.40	0.17
								OMe	55.87	55.78	0.06

Table 5.32. The temperature dependence of the ^{13}C n.m.r. chemical shift for β-D-Glc(1→2)-α-D-Glc(1→3)-α-Glc-OMe (30)

	70°	30°	Δ		70°	30°	Δ		70°	30°	Δ
C1''	104.84	104.94	0.10	C1'	99.16	98.94	0.22	C1	100.25	100.1	0.10
2	74.37	74.22	0.15	2	81.70	81.81	0.11	2	70.25	70.53	0.18
3	76.52	76.35	0.18	3	72.71	72.51	0.20	3	81.50	80.59	0.19
4	70.35	70.12	0.23	4	70.45	70.12	0.33	4	70.68	70.67	0.01
5	77.28	77.23	0.05	5	72.54	72.32	0.22	5	72.16	72.05	0.11
6	61.60	61.37	0.23	6	61.47	61.23	0.24	6	61.47	61.17	0.27
								OMe	55.90	55.83	0.07

Table 5.33. The summary of Tables 5.29 to 5.32.

^{13}C nmr Chemical shift differences in ppm on variation of temperature[a].

Trisaccharide	C1"	C2"	C3"	C4"	C5"	C6"	C1'	C2'	C3'	C4'	C5'	C6'	C1	C2	C3	C4	C5	C6	OMe
αG-2αG-3αG-OMe (13)	0.38	0.12	0.16	0.30	0.14	0.28[b]	0.07	0.59	0.17	0.39	0.16	0.36	0.01	0.19	0.42	0.08	0.12	0.28	0.06
αG-2βG-3αG-OMe (21)	0.17	0.16[b]	0.11	0.34	0.16	0.35	-0.14	0.52	0.19	0.16	0.04	0.23	0.07	0.00	0.37	0.22	0.05[b]	0.14	0.08
βG-2βG-3αG-OMe (24)	0.03	0.12	0.19	0.13	0.05	0.18	-0.21	0.17	0.09	0.18	0.06	0.24	0.14	0.01	-0.16	0.16	0.08	0.17[b]	0.06
βG-2αG-3αG-OMe (30)	-0.10	0.15	0.18	0.23	0.12	0.23[b]	0.22	-0.11	0.20	0.33	0.22	0.24[b]	0.10	0.18[c]	0.91	0.01	0.11	0.27[b]	0.07

[a] $\delta = \delta(70^{o}) - (30^{o})$. Dioxane was taken as δ 67.40 ppm for all temperatures.

[b,c] These values could be interchanged.

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