STRUCTURAL STUDIES ON KLEBSIELLA CAPSULAR POLYSACCHARIDES

by

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to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
April 1980

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TO MY WEST COAST PARENTS

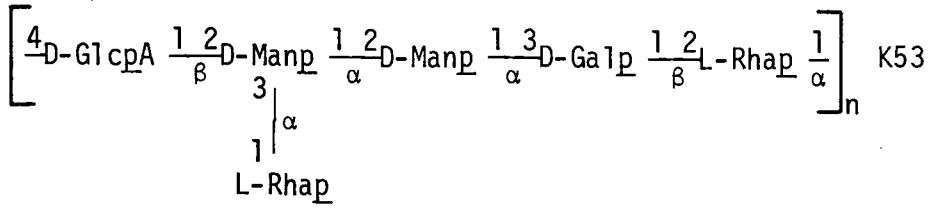
Mr. & Mrs. Allen Cooke

ABSTRACT

The genus Klebsiella is divisible into approximately eighty different strains characterized on the basis of immunochemical tests. These Gram-negative bacteria produce large capsular polysaccharides which are their antigenic determinants. In order to understand the chemical basis of serological differentiation, the structural investigation of all 80 strains is taking place. Until now about fifty structures have been elucidated.

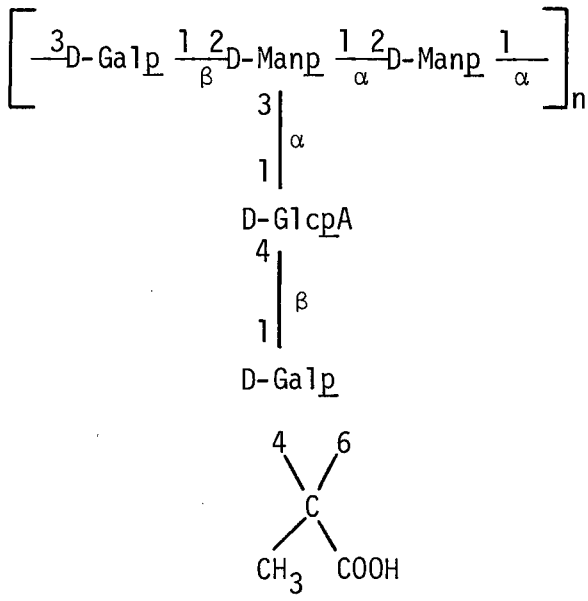
The capsular antigens isolated from Klebsiella serotypes K53 and K74 are presented here and were determined using many different techniques. Methylation analysis, partial hydrolysis, and uronic acid degradation were used to study the sequential arrangement of the sugar constituents in the polysaccharide. Extensive use was made of ^{1}H-and ^{13}C-nuclear magnetic resonance spectroscopy to determine anomeric linkages within isolated oligosaccharides and the native polysaccharide. Methods such as gas liquid chromatography, gas liquid chromatography - mass spectrometry, paper chromatography, and polarimetry have been used to isolate and characterize products obtained from different degradative techniques.

The capsular polysaccharides from Klebsiella serotypes K53 and K74 are found to consist of repeating units of the following structures:



K53

K74


3D-Galp 1 2D-Manp 1 2D-Manp 1
β α α
n
3
1
α
D-GlcpA
4
1
β
D-Galp
4 6
C
CH3 COOH

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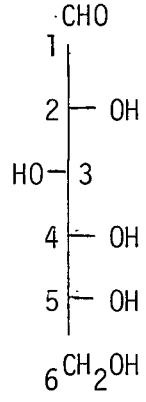
A special mention goes to the members of the department who have helped indirectly during the course of this work, viz. the members of the N.m.r. and Mass-spectrometry Services and Dr. L. D. Hayward and his technician Robert Ng.

Finally, I want to thank Celine Gunawardene for typing this thesis.

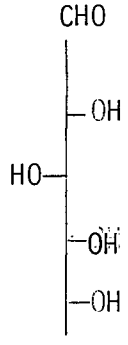
PREFACE*

In an effort to familiarize readers who do not work in the particular area of organic chemistry to which this thesis refers, the following explanation of terms used is offered.

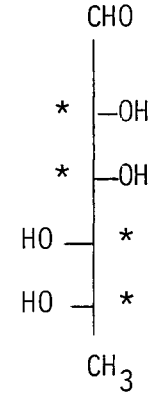
Fischer projection formulae are used to represent the acyclic modificaton of sugars. Some examples are shown below. Numbering commences from the carbonyl group at the top of the chain (I). Note that D-glucuronic acid (II) differs from D-glucose (I) only



D-glucose
(I)



D-glucuronic acid
(II)

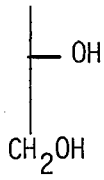


L-rhamnose
(III)

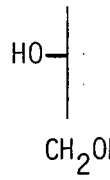
in that C-6 is oxidized to a carboxylic acid group. The C-6 of L-rhamnose (III) is part of a methyl group and is referred to also by another common name, 6-deoxy-L-mannose.

There are four chiral centers in these six-carbon chains (marked with asterisks in structure III) making it important to appreciate the spatial arrangement of atoms (configuration) that is implied by these Fischer representations. To simplify the nomenclature ofall the possible isomers (16 for each of I, II, III),

all those having the hydroxyl group at the highest-numbered chiral center (C-5) projecting to the right in the Fischer projection formulae belong to the D-series, and the others to the L-series.

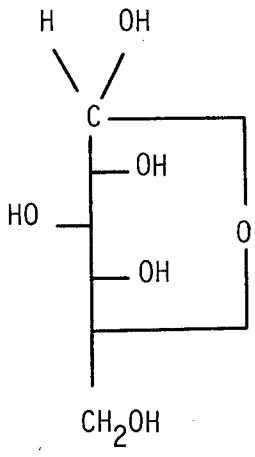


D-series

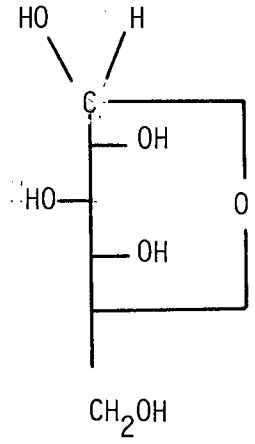


L-series

Physical and chemical evidence indicates that, in fact, these six-carbon polyhydroxyaldehydes exist in a cyclic form. The ring closure occurs by nucleophilic attack of the oxygen atom at C-5 on the aldehydic carbon atom, generating a new chiral (anomeric) center at C-1. This results in two anomers, represented below



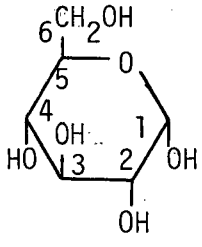
α-D-glucose
(IV)



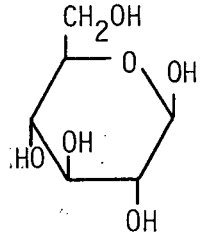
β-D-glucose
(V)

in the Tollens formulae. It should be noted that C-1 is unique in having two attached oxygen atoms, formally making it a hemiacetal carbon.

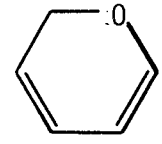
Since the Tollens formulae have obvious limitations with their unequal bond lengths, Haworth developed a perspective method of looking at the six-membered ring (VI and VII). This improvement recognizes that the ring oxygen atom lies behind the carbon chain and that bond lengths are approximately equal. Often in practise regular hexagons are used in Haworth projections,



α-D-glucopyranose
(VI)

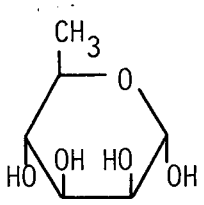


β-D-glucopyranose
(VII)

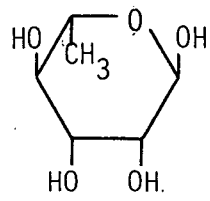


pyran
(VIII)

which he related to such rings at the heterocyclic compound pyran (VIII) and named them pyranoses. Note that hydroxyl groups not involved in ring formation on the right in Fischer and Tollens formulae point down in the Haworth projections and those on the left point up. Similarly, for aldopyranoses, the group on C-5 points up for D (IX) and down for the L enantiomer (X). It follows, then, that when sugar residues are attached there are two possible configurations, an α- or a β-pyranoside, for each linkage.

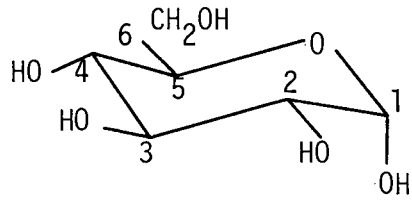


α-D-rhamnopyranose
(IX)



α-L-rhamnopyranose
(X)

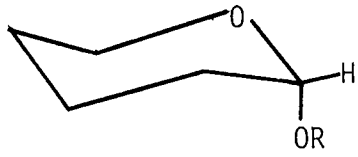
The true conformation of pyranoid carbohydrates is related to the chair form of cyclohexane. X-ray diffraction analysis has shown that a hexose, such as α-D-glucose (XI), consists of a puckered, six-membered, oxygen-containing carbon ring, with hydroxyl substituents at C-1 through C-4, and a hydroxymethyl group at C-5. All substituents on the ring, except for that at C-1, are equatorial.



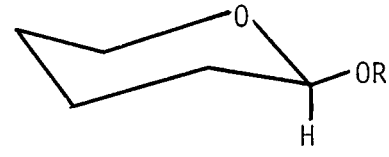
(XI)

Two isomers (anomers) are possible in relation to the anomeric center (C-1), depending on whether a substituent is axial (α-anomer; XII)

or equatorial (β-anomer; XIII), where R = hydrogen, for monosaccharides, and R = another sugar residue, for di-, oligo-, and polysaccharides. Since H-1 is in a different chemical environment for the two anomers, nuclear magnetic resonance spectroscopy can easily distinguish between them and, thereby, provides invaluable assistance in assigning anomeric configurations.



(XII)



(XIII)

Haworth projections are most useful and will be used in this thesis, even though they give no indication of three-dimensional molecular shape. There seems to be little justification for the use of formulae which depict states of molecules as well as structures, when the true states are often unknown or variable.

* Reproduced with the kind permission of T.E. Folkman from his M.Sc. thesis entitled "Structural Studies on Klebsiella Capsular Polysaccharides", University of British Columbia, April 1979.

I. INTRODUCTION

Occurrence and Nature of *Klebsiella* Bacteria

The genus *Klebsiella* is composed of Gram-negative, nonmotile bacteria that conform to the definitions of the family *Enterobacteriaceae* and the tribe *Klebsielleae.* Approximately eighty strains of *Klebsiella* are known grouped in three species: *K. pneumoniae, K. ozaenae,* and *K. rhinoschleromatis.*[1,2] Many of these microorganisms are normally found in healthy carriers in the upper respiratory, intestinal, and genito-urinary tracts. Their pathogenicity to man is well known although some strains are not toxic. They are present in the lungs of patients with respiratory diseases and in the suppurative infections in other parts of the body. *K.pneumoniae* is the most important member of the group. It is the primary cause of pneumonia in 3 per cent of all bacterial pneumonias and has been isolated from patients with pleurisy, appendicitis, cystitis, and pyelonephritis.[3] This species is notable for its destructive action on the tissues, producing abscesses and cavities. The two other members of the group have been isolated from disease conditions of the upper respiratory track of man. *K.rhinoschleromatis* was isolated from the granulomatous nasal lesions of patients with rhinoschleroma, and *K.ozaenae* has been cultured from nasal secretions of individuals with ozena, a fetid, catarrhal condition of the nose. Other sources of *Klebsiella* included feces, pus, blood, abscesses, bones and joints.

Klebsiella strains are resistant to penicillin in standard doses but may be sensitive to high concentrations.[2] Sulfadiazine and

streptomycin have proved to be of value in therapy as well as chloramphenicol, tetracycline, neomycin, kanamycin, etc... It seems that proportion of resistant strains is increasing steadily, which may be due to mutations.

Functions and Composition of Klebsiella Capsular Polysaccharides

Much attention has been given to the morphology, composition and structure of the bacterial cell wall.[4] The plasma membrane of the cell is surrounded by a cell wall composed of murein which in turn is enveloped by a lipophilic complex of lipopolysaccharide (LPS), phospholipid, and protein. The whole cellular structure of some organisms is surrounded by a capsule which is sometimes a protein but more often a polysaccharide. Finally, some bacteria have flagella which are proteins passing through the cell wall. In Figure I.1 a diagrammatic representation of a bacterial cell wall is shown.

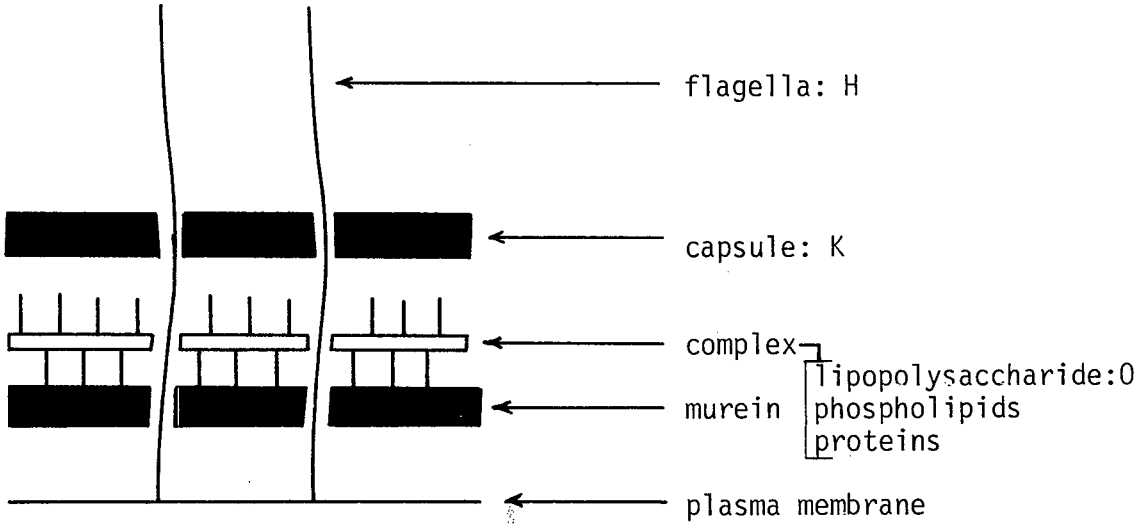


Figure I. 1 Diagrammatic representation of a bacterial cell wall.

According to the general structure of the bacterial cell wall just described many types of antigens have been found:

- antigens H (German; Hauch = fog) corresponding to the flagella - protein
- antigens O (German; ohne Hauch = without fog) also called somatic - lipopolysaccharide
- antigens K (German; Kapsel) corresponding to the presence of a capsule - protein or polysaccharide

It should be noted that as nonmotile bacteria, Klebsiella do not have flagella or H antigens.

A common feature of Gram-negative organisms is the occurrence of polysaccharides on the cell surface. These are either lipopolysaccharides, capsular polysaccharides or both. It has been known for some time that Klebsiella produce large capsules and slime. The polysaccharide nature of both was established by Emmerling,[5] Schardinger,[6] Toenniessen[7] and Kramár.[8] Dudman and Wilkinson found that capsular and slime polysaccharides are identical in chemical composition.[9] Thus, slime can be considered as excreted capsular polysaccharide.

For non-capsulated (K^-) Gram - negative bacteria, serological classification is based on specific reactions of somatic O - antigens with respective O - antibodies. In the case of the genus Klebsiella most bacteria are heavily encapsulated (K^+) and non - capsulated (K^-) variants are difficult to obtain. This heavy capsule shields completely the O - antigen. Consequently, serological classification

of Klebsiella strains is solely based on their capsular K-antigens. To date, approximately 80 strains have been characterized on the basis of immunochemical tests.[10,11] Recently, Ørskov and Fife-Asbury[12] have added K82, and deleted K73, 75, 76, 77, and 78.

The exact role of capsular antigens is not well understood. Among the various functions it has been suggested that they may act as storage material in case of need,[13] be a protection against desiccation,[14] against phagocytosis[15] or against bacteriophages.[16] The latter hypothesis is not always true. As it is shown by works taking place in this laboratory, capsular polysaccharides are excellent substrates of phage enzymes.[17]

Polysaccharides as antigens induce, though weakly, the formation of antibodies in man and animals and react serologically with these antibodies. In other terms they possess immunogenicity (capacity to induce formation of antibodies in mammals) and antigenicity (reactivity with antibodies). Heidelberger et al [18,19] have used anti-K sera to study cross - reactions with other bacterial species. The basis of these immunochemical phenomena is the recognition of partial structures of the polysaccharide antigens by antibody molecules or immune cell receptors. These partial structures (antigenic determinants) may have the size of oligosaccharides or monosaccharide constituents and may be of a carbohydrate or non-carbohydrate nature.[20] For a quantitative consideration of the antigen-antibody reaction knowledge of the structure of the polysaccharide antigen is necessary. For this reason structural investigation of all Klebsiella strains

has been undertaken and will soon be completed. The investigation of K53 and K74 is reported in this thesis.

One of the most outstanding features of bacterial polysaccharides is their ordered structure composed of oligosaccharide repeating units. This is demonstrated in the capsular antigens of Klebsiella. Nimmich[10,11,21] has reported the qualitative composition of all K-types. He found that the majority contained only a charged monosaccharide constituent either D-glucuronic acid or D-galacturonic acid and hexoses like D-mannose, D-glucose and D-galactose; in some strains 6-deoxyhexoses were also present such as L-rhamnose and L-fucose. In addition, acetyl and formyl groups, ketal-linked pyruvate and, very seldom, keto acid were found. These non-carbohydrate constituents may function as determinant groups and may be the cause of cross - reactivities of some Klebsiella polysaccharides.

Capsular polysaccharide antigens of Klebsiella have been studied in many laboratories and different structural patterns (see Appendix I) have emerged from the proposed structures. Some are linear (K6, K70) [22,23] and many are branched (K11, K28)[24,25] so much so that an intricate comb - like structure is obtained. The number of sugars per repeating unit varies from three (K1, K63)[26,27] to seven (K41).[28] Most of the monosaccharide constituents exist in the pyranose form even though some furanose forms have been found in a few polysaccharides (K12, K41).[28,29] According to their composition the different strains of Klebsiella have been classified in chemotypes (see Appendix II) by Heidelberger and Nimmich.[18] Even if they are of

the same chemotype, two polysaccharides can have different structural patterns as illustrated by K31[30] and K33.[31] These two polysaccharides have a pentasaccharide repeating unit and contain D-glucuronic acid, D-galactose, D-glucose, and D-mannose with pyruvate substitution. In K31 the glucuronic acid is the branching sugar, while in K33 a mannose residue is the branching sugar and bears two side-chains. In this laboratory an unusual structure for K60 has been found.[32] The repeating unit of K60 is an heptasaccharide having three side-chains linked to three different sugar residues. The diversity of structures presented by the different K-type strains probably justifies their serological differentiation.

II. METHODOLOGY OF STRUCTURAL ANALYSIS OF POLYSACCHARIDES

To understand what makes a polysaccharide immunogenic and what is the chemical basis of its antigenicity it is desirable to know its structure as precisely as possible. Structural analysis of the polysaccharide has to clarify its sugar composition, the sequence of sugar components, and the nature of the linkages (positions of substitution and anomeric configurations). In the following the most widely used methods are mentioned.

II. 1. Isolation and Purification

Strains of Klebsiella serotypes K53 and K74 were obtained as agar stab cultures from Dr. I. Ørskov in Copenhagen. Using strong growing colonies the bacteria were plated out twice on agar plates. Each strain was grown in a sucrose-yeast extract medium for 4 hours at $37^{o}C$ and then incubated for three days on large trays of sucrose-yeast extract-agar.

Slime was harvested, diluted with aqueous phenol to kill the bacteria and centrifuged. The viscous supernatant contained neutral and acidic polysaccharides. The supernatant was first precipitated in ethanol. The precipitate was dissolved in water and reprecipitated using 10 per cent hexadecyltrimethylammonium bromide (Cetavlon). Addition of an aqueous solution of cationic detergents to solutions of crude polysaccharides in water results in the precipitation of Cetavlon salt of the acidic polysaccharide.[33] Neutral polysaccharides are not precipitated by Cetavlon and can thus easily be separated from acidic

ones. This precipitate was dissolved in 4M sodium chloride and precipitated again in ethanol. The polysaccharide was dissolved in water, dialysed for three days against running tap water and freeze - dried.

II. 2. Nuclear Magnetic Resonance Spectroscopy

II.2.1. ^{1}H-n.m.r. Spectroscopy

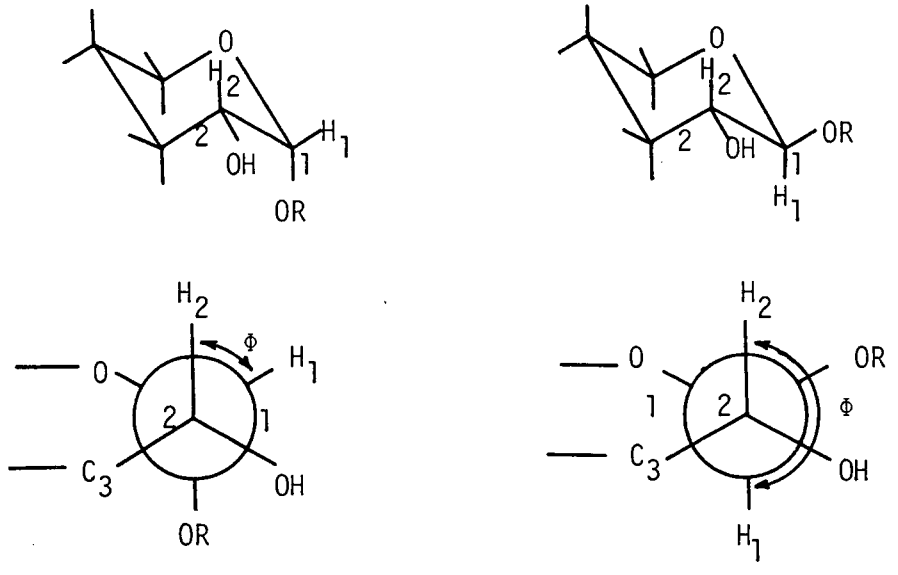
N.m.r. spectroscopy has been used almost exclusively for simple molecules because spectra obtained for polymers were too complex. Lately, this technique has been applied successfully in the structural analysis of Klebsiella polysaccharides.[34,35] Because of their regular repeating units spectra of these polymers are very close to those obtained for their oligomers. Several problems are encountered in performing a p.m.r. experiment on a polysaccharide. Deuteration of all labile hydrogen atoms by repeated exchange with deuterium oxide before measurement of the spectrum is necessary and prevents interference from the large HOD resonance. This exchange is performed by dissolving the polysaccharide in D_2O followed by freeze-drying. The sample is then dried under vacuum for a few hours and the process is repeated two or three times. Even after careful deuteration, a HOD peak is usually observed. This peak appears in the region of the spectrum associated with the anomeric protons (δ 4.5 - 5.5). In such cases it is advantageous to induce a change in the chemical shift of the HOD peak. This can be done by altering either the pH (addition of trifluoroacetic acid) or by recording the spectrum at high temperature which has the effect to shift the HOD peak upfield.

Recording the spectrum at high temperature helps to eliminate another problem related to the viscosity of the sample. To record a p.m.r. spectrum 1-2 per cent solutions of the polysaccharide are necessary but these solutions are so viscous that there is a loss of resolution when the ^{1}H-n.m.r. experiment is performed. Using high temperature reduces the viscosity and homogenizes the sample. Less viscous samples can also be obtained by performing a mild acid hydrolysis on the native polysaccharide. Hydrolysis conditions have to be selected so that acid labile groups, such as pyruvate and acetate, are not completely removed. This technique, in some cases, greatly improves the resolution of the spectrum.

Nowadays anomeric linkages are assigned on the basis of n.m.r. data. In ^{1}H-n.m.r. spectroscopy the anomeric region covers a range between δ 4.5 to δ 5.5. It is well accepted that signals appearing downfield of δ 5.0 are assigned to α - linkages and those upfield to β - linkages. The borderline at δ 5.0 is arbitrary but has been found to be valid in most cases.

To α and β configurations correspond spin - spin coupling constants J between the protons linked to carbons C1 and C2. Karplus has demonstrated that the coupling constant is affected by the dihedral angle ϕ formed by the two bonds involved in the coupling.[36] As illustrated in Figure II.1, for the β anomer of D - glucose the H1 and H2 protons are trans-diaxial ($\phi = 180^o$), the coupling constant is maximum, $J_{1,2}$ = 7 - 9 Hz. On the other hand, for the α - anomer protons H1 and H2 are in a gauche conformation (ϕ=60^o) and the

coupling constant is smaller, $J_{1,2}$ = 2-3 Hz. However, for L-rhamnose the situation is reversed with the α-L-anomeric proton having $J_{1,2} \simeq 2$ Hz and the β-L-anomeric proton $J_{1,2} \simeq 1$ Hz. For this 6-deoxyhexose, because of an axial OH at C2, protons H1 and H2 are in a gauche conformation for both the β(a,e) and α(e,e) forms. This is another feature which aids identifying component sugars though less important than chemical shifts.



Configuration α Configuration β

Figure II.1 Variation of the dihedral angle Φ relative to the anomeric carbon configuration of D-glucopyranose.

Other information obtainable by ^{1}H-n.m.r. include the presence or absence of pyruvate acetals, 6-deoxy sugars, acetate groups, and the number of sugar constituents per repeating unit.

Figure II.2 shows typical spectra of Klebsiella K53 polysaccharide. Spectrum A has been recorded on the native polysaccharide without an internal standard. The spectrum exhibits a doublet at δ1.29 due to the methyl groups of L-rhamnose units. In the anomeric region (δ 4.5-5.5) the signals are not very well resolved. A broad peak appears upfield of δ 5.0 p.p.m. and can be assigned to β-linkages while the three large signals downfield of δ 5.0 p.p.m. can be assigned to α - linkages. In comparing the integration of the different peaks it is possible to estimate that the methyl protons of the rhamnose units and the anomeric protons are in a 1:1 ratio. Also, the ratio of the α-protons to the β-protons seems to be 2:1. Absence of peaks at δ 2.20 and δ 1.50 shows that the polysaccharide does not contain any acetate group or pyruvate acetal. Spectrum B has been obtained after the polysaccharide underwent a mild hydrolysis. As can be seen this treatment did not improve very much the spectrum even if the product was more soluble and the solution less viscous.

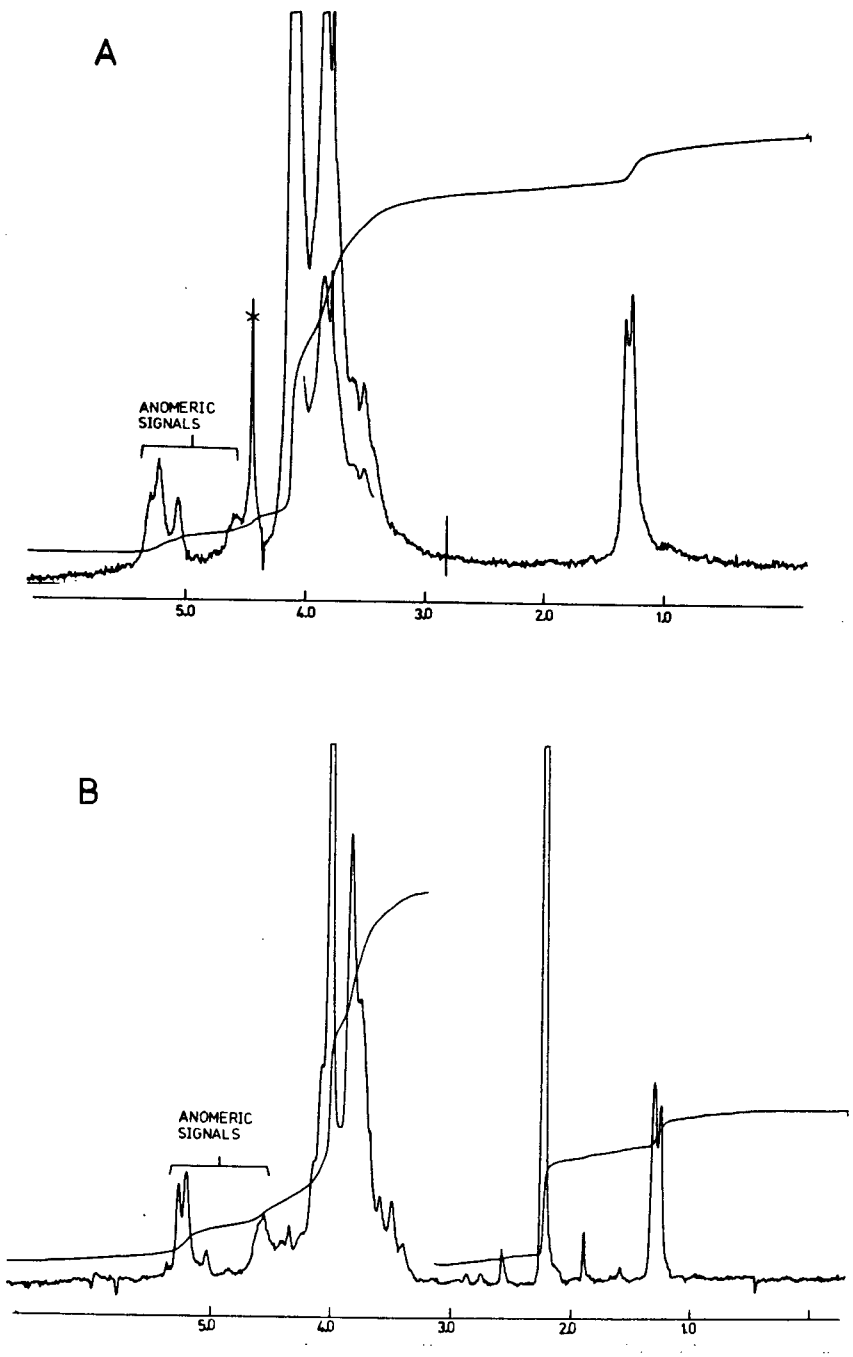


Figure II.2 ^{1}H-n.m.r. spectra of K53 polysaccharide.

A. Native polysaccharide, recorded without internal standard.

B. Partially depolymerized K53, 0.5M TFA, 30 min at 95°C.

II. 2.2. ^{13}C-n.m.r. Spectroscopy

One of the most important physical methods being used in organic chemistry is without any doubt ^{13}C-n.m.r. spectroscopy. The main difficulty in ^{13}C-n.m.r. is the low natural abundance of the carbon - 13 nucleus (1.1%) which makes the technique much less sensitive (1.6%) than ^{1}H-n.m.r. (100%). However, improvements in instrumentation and technique have enabled ^{13}C-n.m.r. spectra of molecules of considerable complexity and high molecular weight to be determined.

Many methods have been developed to increase the sensitivity of ^{13}C-n.m.r. Outstanding in importance is the use of pulse Fourier - transform n.m.r. spectroscopy which is rapid and gives improved signals. The method consists essentially of irradiating the sample with a short radiofrequency pulse which excites simultaneously all the ^{13}C nuclei. Depending on the pulse width this irradiation tips the vector of magnetization by a finite angle (30^{o}, 90^{o} or 180^{o}). When the nuclei relax to their original state the energy absorbed is emitted; this is called a free induction decay (F.I.D.). Accumulation of the F.I.D. in a computer and their Fourier transformation give rise to the ^{13}C spectrum of the product. This method has also the advantage to improve the signal to noise ratio. This ratio improves as the square root of the number of total transients. Because of their low solubility and high viscosity, Klebsiella polysaccharide solutions necessitate a large number of transients (> 100,000) to obtain a reasonable signal to noise ratio.

Another major breakthrough in ^{13}C-n.m.r. spectroscopy was

the discovery of proton broad band decoupling in 1966[37]. Spin decoupling or nuclear magnetic double resonance is achieved by irradiating an ensemble of nuclei not only with a radio-frequency at resonance with the nuclei to be observed but additionally with a second alterning field at resonance with the nuclei to be decoupled. This has the effect of collapsing the spin multiplets into singlets making the spectrum easier to interpret and increasing the sensitivity of n.m.r. measurements. Additional sensitivity enhancement is produced as a side effect of proton decoupling. This phenomenon arises from an intramolecular dipole-dipole relaxation mechanism and is known as the nuclear Overhauser effect.[38]

The ^{13}C-n.m.r. spectrum of a polysaccharide contains useful information related to the fine structure of the molecule. In the light of the broad generalisation recognized in earlier studies of monosaccharides, resonances near 91-105 p.p.m. could be attributable to anomeric carbon atoms. Contrary to ^{1}H-n.m.r. the α-anomeric carbons appear upfield of the β-anomeric carbons due to a shielding effect. It has been found that increased shielding of a ^{13}C nucleus is accompanied by a decrease in the shielding of the appended proton, i.e., ^{13}C and ^{1}H shifts are affected inversely.[39] Usually chemical shifts appearing downfield of 101 p.p.m. represent β-linked sugars and those upfield α-linked sugars. This rule is not always valid as it has been reported,[40] and observed in the course of this work in the investigation of K74. Caution must be exercised when applying

this general rule especially when the molecule contains rhamnose or mannose constituents. In addition to information about anomeric linkages, the presence of deoxy sugars, pyruvate acetal and acetate group may be detected from a ^{13}C-n.m.r. spectrum.

Quite recently ^{13}C-n.m.r. spectroscopy has been applied to carbohydrates in the study of monosaccharides,[41,42] oligosaccharides,[43-45] and polysaccharides.[46-49] This physical method has been used successfully for the structural analysis of the polysaccharides K53 and K74 and their oligosaccharides. The spectra were recorded at 20 MHz and were proton decoupled. Deuterium oxide was used as solvent with acetone as internal standard giving a reference peak at 31.07 p.p.m.

Figure II.3 shows the ^{13}C-n.m.r. proton decoupled spectrum of a sample of K53 having undergone a mild hydrolysis. At approximately 100 p.p.m. seven signals arising from anomeric carbons are apparent but two (102.23 and 103.44 p.p.m.) have been assigned to the same residue. In the region usually associated with ^{13}C nuclei bearing a primary alcohol (60-62 p.p.m.) only one strong peak is observed. This signal probably arises from C6's of the galactose and the two mannose moieties in K53. Upfield of the acetone peak one signal appears at 17 p.p.m. attributable to methyl ^{13}C nuclei from rhamnose residues. This spectrum is in good agreement with the hexasaccharide repeating unit of K53.

II. 3. Total Sugar Ratio

The next step in the investigation of a polysaccharide is total hydrolysis followed by analysis of the hydrolysate qualitatively

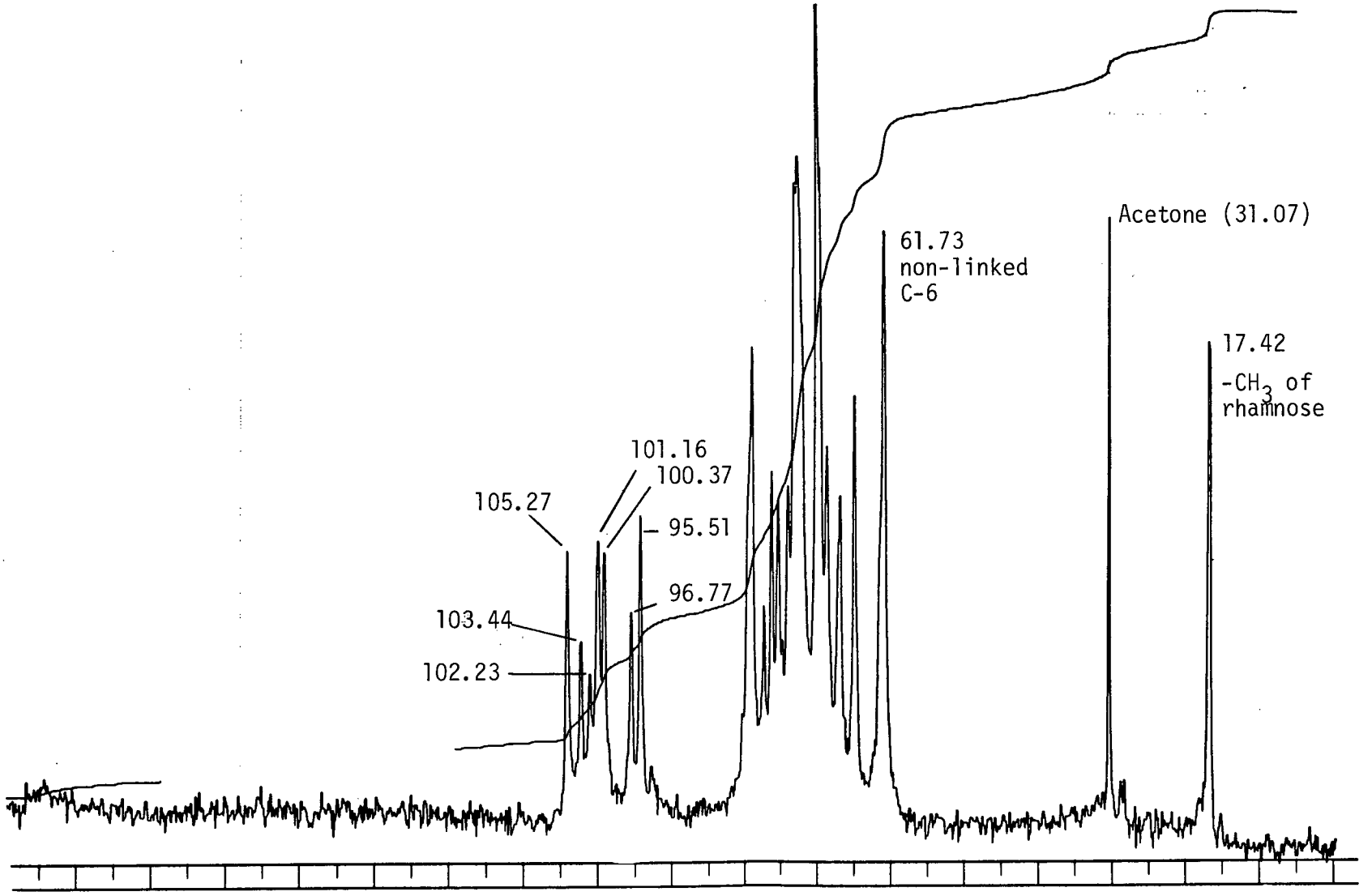


Figure II.3 ^{13}C-n.m.r. spectrum of Klebsiella K53 capsular polysaccharide

and quantitatively. Acids commonly used for this purpose are sulfuric, hydrochloric, formic, and trifluoroacetic. Advantages and disadvantages of the different methods have been discussed extensively.[50,51] Trifluoroacetic acid has the advantage of being easily removed under diminished pressure following the hydrolysis. This acid will normally hydrolyse a neutral polysaccharide within a few hours with a minimum of degradation.

Polysaccharides containing uronic acid residues are more resistant to acid hydrolysis. The presence of electron acceptor, carboxyl groups stabilize uronosyl linkages through the heterocyclic oxygen. To hydrolyse acidic Klebsiella polysaccharides a method has been developed in this laboratory involving the use of methanolysis.[52] Treatment with methanolic hydrogen chloride cleaves most glycosidic bonds but not all uronosyl linkages. At the same time the methyl ester of the uronic acid is formed and can be reduced using sodium borohydride in anhydrous methanol. Reduction is performed in methanol since in water the ester would be saponified. Following the reduction the product is hydrolysed with 2M trifluoroacetic acid. The monosaccharides are then reduced and acetylated to their alditol peracetates for gas-liquid chromatography (g.l.c.) analysis.

The use of alditol acetate derivatives throughout this study is because they are much more volatile than their native sugar counterparts. Also, they give rise to only one peak in g.l.c. analysis, making quantitation easier. Theoretically, for a free sugar five forms are possible (α- and β- pyranosides, α- and β-

furanosides and the linear form) but in practice only four seem to be formed. Trimethylsilylation is fast enough to preserve the equilibrium between the different forms and produces a multiple peak chromatogram for each sugar residue.

II. 4. Structural Analysis

II. 4. 1. Methylation Analysis

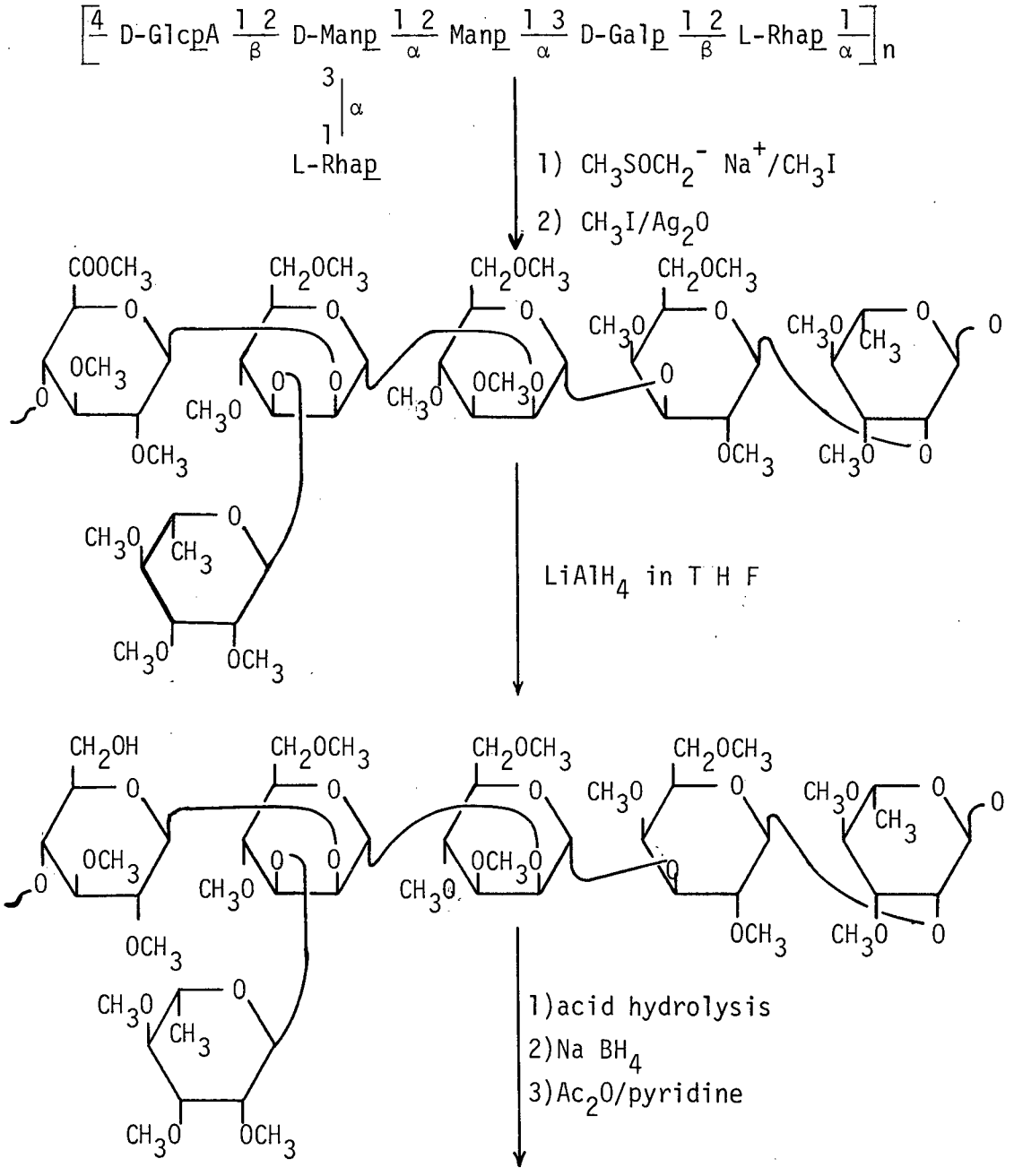
Methylation analysis is a facile method for determining the substitution of monosaccharide units constituting oligo - and polysaccharides. The method gives details of the structural units in the polymer, the presence of branching and terminal residues, but no information on their sequence or the anomeric nature of their linkages. The analysis relies upon the methoxyl substitution of all free hydroxyl groups in the native polysaccharide. This can be achieved by using several well known procedures, e.g., Purdie methylation[53], Kuhn methylation[54], and Hakomori methylation.[55,56]

The most widely used method is that described by Hakomori because it involves milder conditions and gives good results. In this procedure the polysaccharide or oligosaccharide substrate, dissolved in anhydrous dimethyl sulfoxide, is first treated with sodium dimethylsulfinylmethanide (dimsyl sodium) and methyl iodide is subsequently added to effect methylation. Usually the Hakomori procedure gives complete etherification in one step but in this study a subsequent Purdie methylation was necessary for the native polysaccharide. It should be noted that Hakomori methylation

cannot be repeated on a polysaccharide containing uronic acid because the methyl ester of the uronic acid may be degraded by β-elimination during base treatment[57] (see Section II.5.2.).

Following methylation, the material is recovered by dialysis in the case of a polysaccharide or by extraction with chloroform for oligosaccharides. The methylated product is reduced with lithium aluminum hydride in refluxing tetrahydrofuran. The aluminum hydroxide produced after the lithium aluminum hydride has been destroyed tends to adsorb materials having free hydroxyl groups giving rise to losses. This problem can be alleviated by extracting quantitatively the product by the method of Dutton and Smith.[58] The reduced polymer or oligomer is then hydrolysed, reduced to alditols and acetylated. Figure II.4 illustrates these steps in a reaction scheme.

The unmethylated positions of the alditol represent sites of linkage, except in the cases of a uronic acid residue and a residue with pyruvate substitution. For these residues the reduced carboxyl function and the sites of pyruvate substitution will be acetylated too. The derivatives of such sugars can easily be identified by repeating the same sequence of reactions on the carboxyl reduced and/or depyruvylated polysaccharide. The results will be the disappearance of certain peaks and the appearance of new ones in the g.l.c. tracing.



1,5-di-O-acetyl-2,3,4-tri-O-methyl-L-rhamnitol
1,2,5-tri-O-acetyl-3,4-di-O-methyl-L-rhamnitol
1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-D-mannitol
1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-galactitol
1,2,3,5-tetra-O-acetyl-4,6-di-O-methyl-D-mannitol
1,4,5,6-tetra-O-acetyl-2,3-di-O-methyl-D-glucitol

Figure II.4 Methylation analysis scheme for K53 polysaccharide

II. 4.2. G.l.c. Analysis of Partially Methylated Alditol Acetates

Gas-liquid chromatography has been modified repeatedly and is now a simple and reliable method for the separation and quantitation of partially methylated alditol acetates. In general it can be stated that the technique today has nearly reached perfection. The field has been reviewed extensively.[51,59,60]

Various liquid phases are available for separation of mixtures of partially methylated alditol acetates. Best separations are obtained on medium - polar columns such as:

-ECNSS-M: a ethylenesuccinate-cyanoethylsilicone copolymer

-OV-17 : a phenyl, methyl silicone polymer

-OV-225 : a phenyl, cyanopropyl, methyl silicone polymer

-HI-EFF-1B: diethylene glycol succinate

Depending on the difficulties of separation one must use different columns for optimum separation. Good separation of the three di-O-methyl-L-rhamnose isomers can be achieved using HI-EFF-1B column but 2,3,4,6-tetra-O-methyl-D-glucose cannot be separated from the 2,4-di-O-methyl-L-rhamnose. To separate the latter two compounds column OV-17 can be used.[48] In the investigation of Klebsiella K53 and K74 column ECNSS-M was used almost exclusively because it gave good separations. (see Figure II.5.).

Publications by Lindberg et al[60] and Albersheim et al[61] provide relative retention times for numerous partially methylated alditol acetates. In the present work identification of various g.l.c. derivatives was made by using these retention times data which was

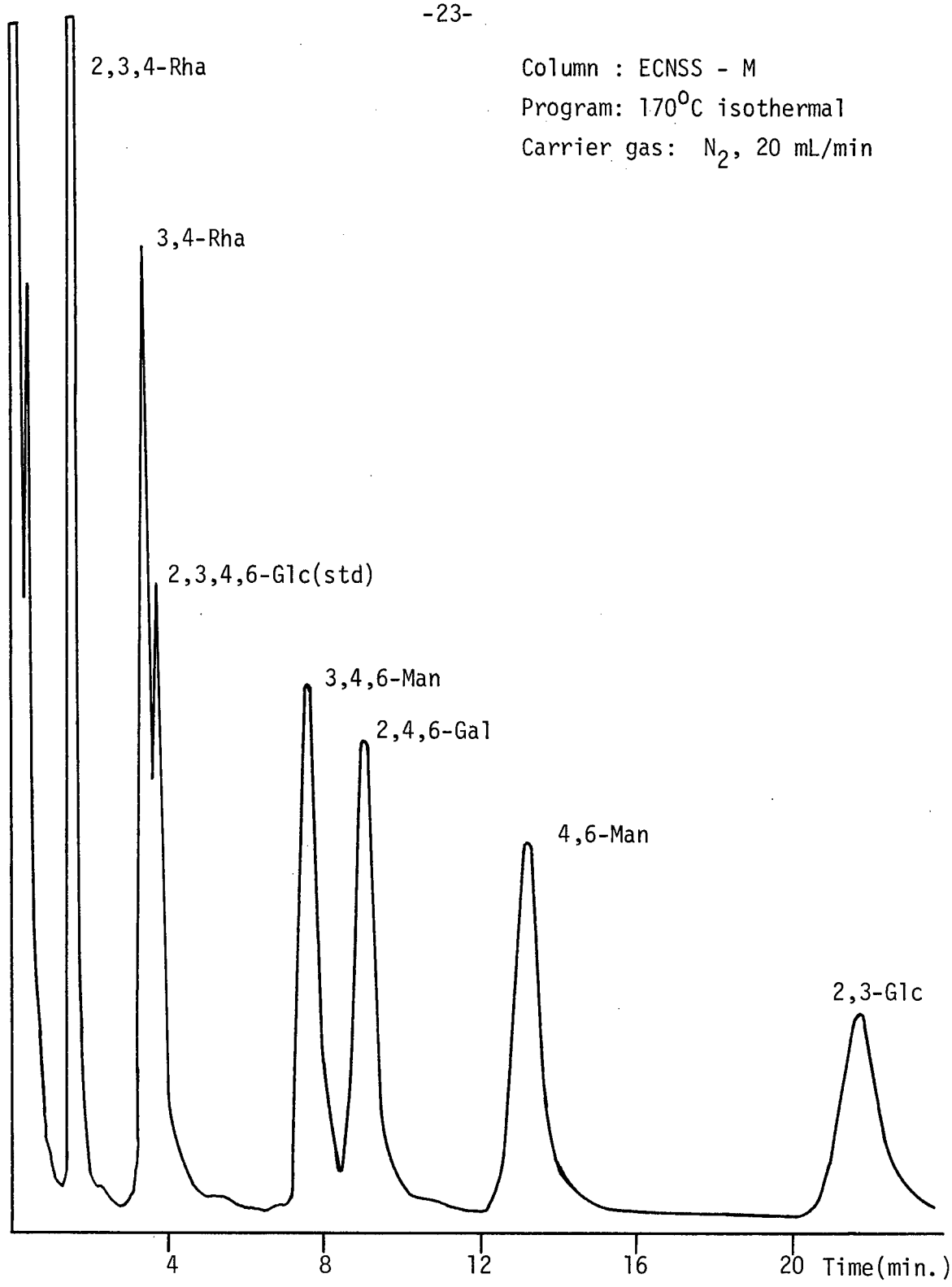


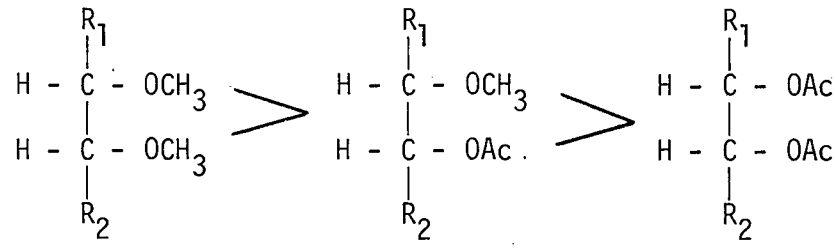
Figure II.5 G.l.c. separation of a mixture of partially methylated alditol acetates obtained from Klebsiella K53 polysaccharide.

confirmed by mass spectrometry (see section II.4.3). Quantitation as determined by peak integration was corrected using molar response factors[61], though Lindberg et al [60] suggested that this correction is not necessary for partially methylated alditol acetates.

II.4.3. Mass Spectrometry of Partially Methylated Alditol Acetates

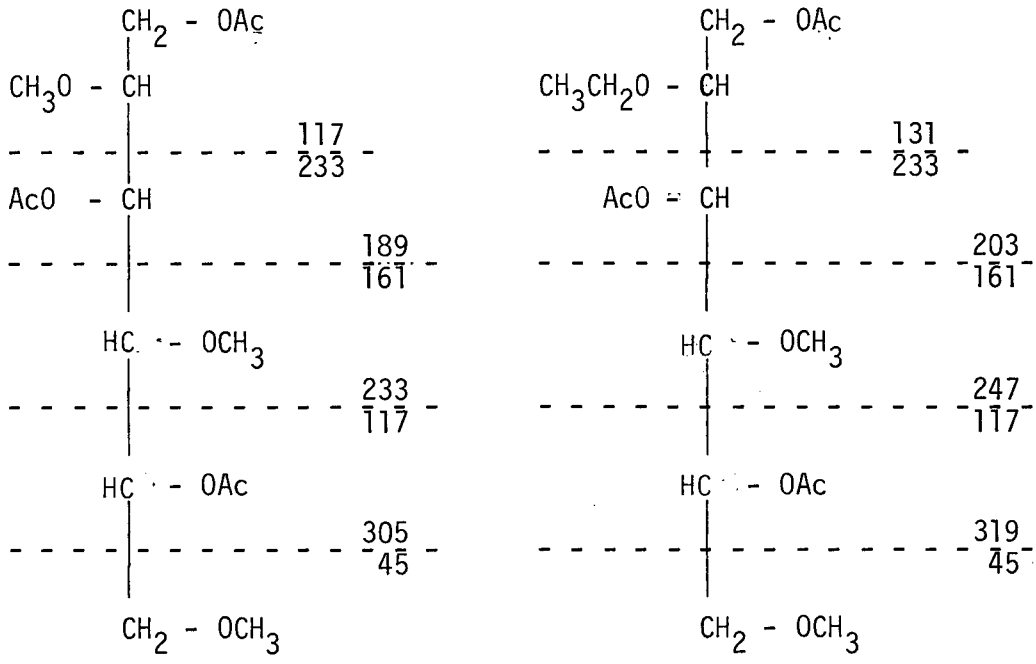
Data obtained from gas-liquid chromatography analysis are informative but do not allow unambiguous identification of the components. However, combined gas-liquid chromatography - mass spectrometry (g.l.c. - m.s.) has become increasingly important to confirm the identification of the components of a mixture of partially methylated alditol acetates. The fragmentation pathways of these carbohydrate derivatives on electron impact have been studied in great detail by some Swedish workers[62] and considerable data are now available.[60]

On electron impact of partially methylated alditol acetates no molecular ion is found in the spectra, and derivatives having the same substitution patterns give similar mass spectra. For example, it is not possible to differentiate stereoisomeric derivatives of glucose, galactose, and mannose. Primary fragments are formed by fission between carbon atoms in the alditol chain. Fission between two methoxylated carbons is more abundant than fission between one methoxylated and one acetoxylated carbon which in turn is preferred to fission between two acetoxylated carbons, as shown below.



Primary fragments give rise to secondary fragments. These are formed by loss of one or more of the following: acetic acid (M.W.60), methanol (M.W.32), ketene (M.W.42), or formaldehyde (M.W.30).

The previous information is useful to identify labelled compounds for which standard spectra are not available. In uronic acid degradation studies the site of linkage on the sugar residue bearing the uronic acid component is ethylated after degradation. This produce an ethylated partially methylated alditol acetate. In the uronic acid degradation of Klebsiella K53 capsular polysaccharide, 1,3,5-tri-O-acetyl-2-O-ethyl-4,6-di-O-methyl-D-mannitol was obtained. By comparing the mass spectrum of the 2-O-ethyl isomer to that of 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-mannitol a characteristic shift of 14 mass units was observed. The spectrum of the tri-O-methyl isomer exhibits a strong peak from the primary fragment m/e 117, while in the spectrum of the 2-O-ethyl isomer this peak is shifted to m/e 131. Primary fragmentation of these two isomers are shown below and their mass spectra are presented in Figure II.6



II.5 Sugar Sequence Determination by Degradation Methods

II.5.1. Partial Hydrolysis

Isolation of oligosaccharides from partial hydrolysis of a polysaccharide is the key to the determination of the sequential arrangement of the constituent monosaccharides in that polymer. These oligosaccharides or block units permit the reconstruction of the repeating unit in an additive manner.

There is no standard method of partial hydrolysis giving optimum yields. Depending on the type of monosaccharide residues, the anomeric configuration of the glycosidic linkages, the position of linkage, etc..., one has to determine the conditions of hydrolysis in order to get the maximum amount of oligosaccharides. Capon has reviewed the rate constant for the acid catalyzed hydrolysis of a

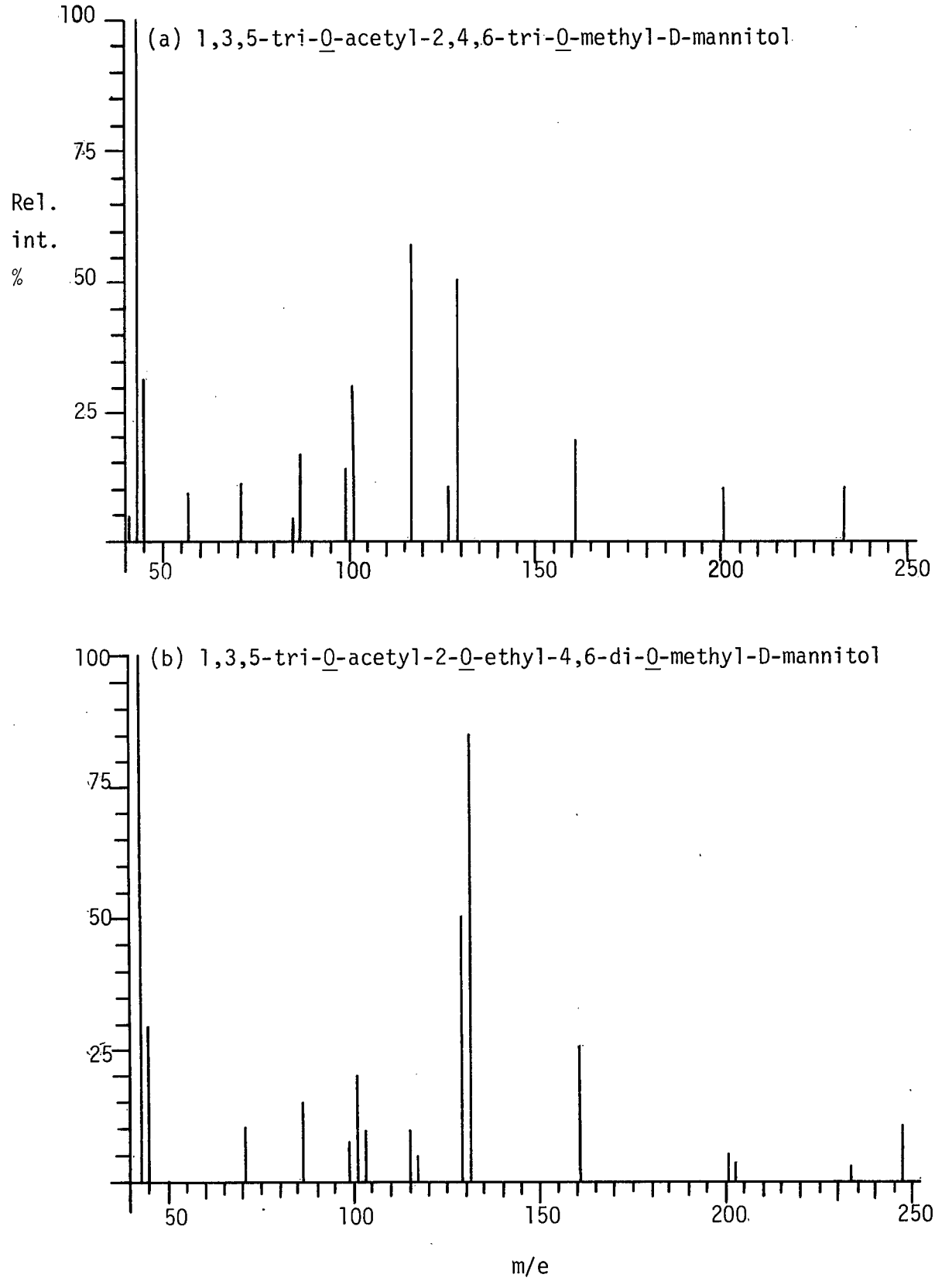
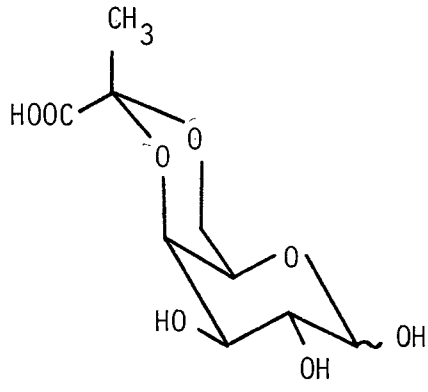


Figure II.6 Mass spectrum of a uronic acid degradation derivative from K53(b) compared to the spectrum of a standard derivative (a).

large variety of glycosides.[63] He found that furanosides are more labile than pyranosides, deoxy sugars are more easily hydrolysed than hexoses, α - glycosidic bonds are cleaved more easily than β - glycosidic bonds and that uronic acids are resistant to hydrolysis. The resistance of the uronosyl bond is an advantage and permits the isolation of large proportions of aldobiouronic acid, and to a lesser extent aldotriouronic acid. Residues present in the side chain are hydrolysed more quickly than residues in the chain. This fact was used in the analysis of K53 where the single unit L-rhamnose side chain was removed to produce a linear polysaccharide.

Pyruvate acetals spanning carbons four and six of an hexose are moderately stable as little steric strain is involved. In some cases, they have been found to survive during partial hydrolysis.[64] During partial hydrolysis studies on K74 no oligomers retaining the pyruvate acetal were isolated but an interesting observation was made. In K74 the pyruvate acetal is linked to the terminal galactose of the side chain as 4,6-O-(1-carboxyethylidene-)-D-galactose, as shown below. A sample of the polysaccharide treated with 0.5M trifluoroacetic acid at 100°C for 1 hour was not completely depyruvylated. On the other hand, the acetal was completely removed by hydrolysing another sample with 0.01M trifluoroacetic acid at 100°C for 6 hours. This could possibly suggest that removal of the acetal is more dependent on the time of hydrolysis than on the acid strength.

In order to improve yields, Galanos et al have developed an apparatus whose basic principle is the continuous removal of



4,6-O-(1-carboxyethylidene-)-D-galactose

oligosaccharides from solution to prevent total hydrolysis to monomers.[65] Although this apparatus is available in this laboratory it was not used in the course of this work because of the problems associated with it. The use of this apparatus is tedious, time-consuming, requires large amounts of starting material, and does not guarantee good yields of oligomers. In the present study one-pot partial hydrolyses were performed on K53 and K74. Before proceeding on a large scale, partial hydrolyses monitered by paper chromatography were made on small samples to determine the best hydrolysis conditions. This batch hydrolysis procedure was successful for both polysaccharides. For K53, di-, tri-, tetra-, and pentasaccharides were isolated; for K74, di-, tri-, and tetrasaccharides were collected.

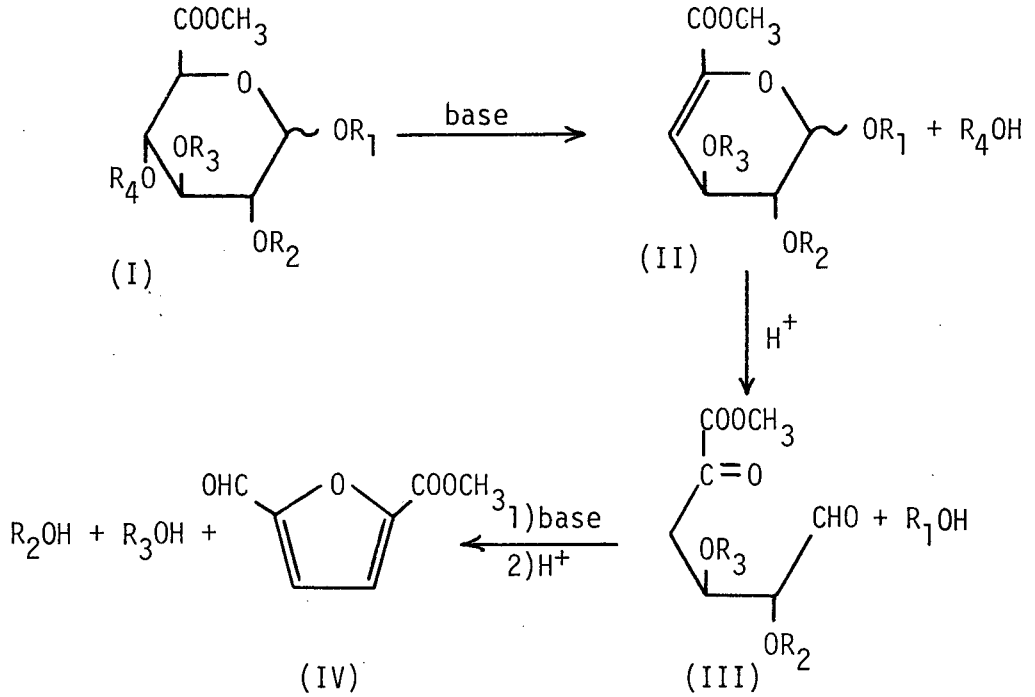
Oligosaccharides obtained from partial hydrolysis were purified by standard chromatographic techniques. The hydrolysate was first separated into acidic and neutral fractions by ion-exchange

chromatography. The acidic oligomers were separated according to molecular size by gel filtration chromatography and purified by descending paper chromatography.

II. 5.2. Uronic Acid Degradation (β - elimination)

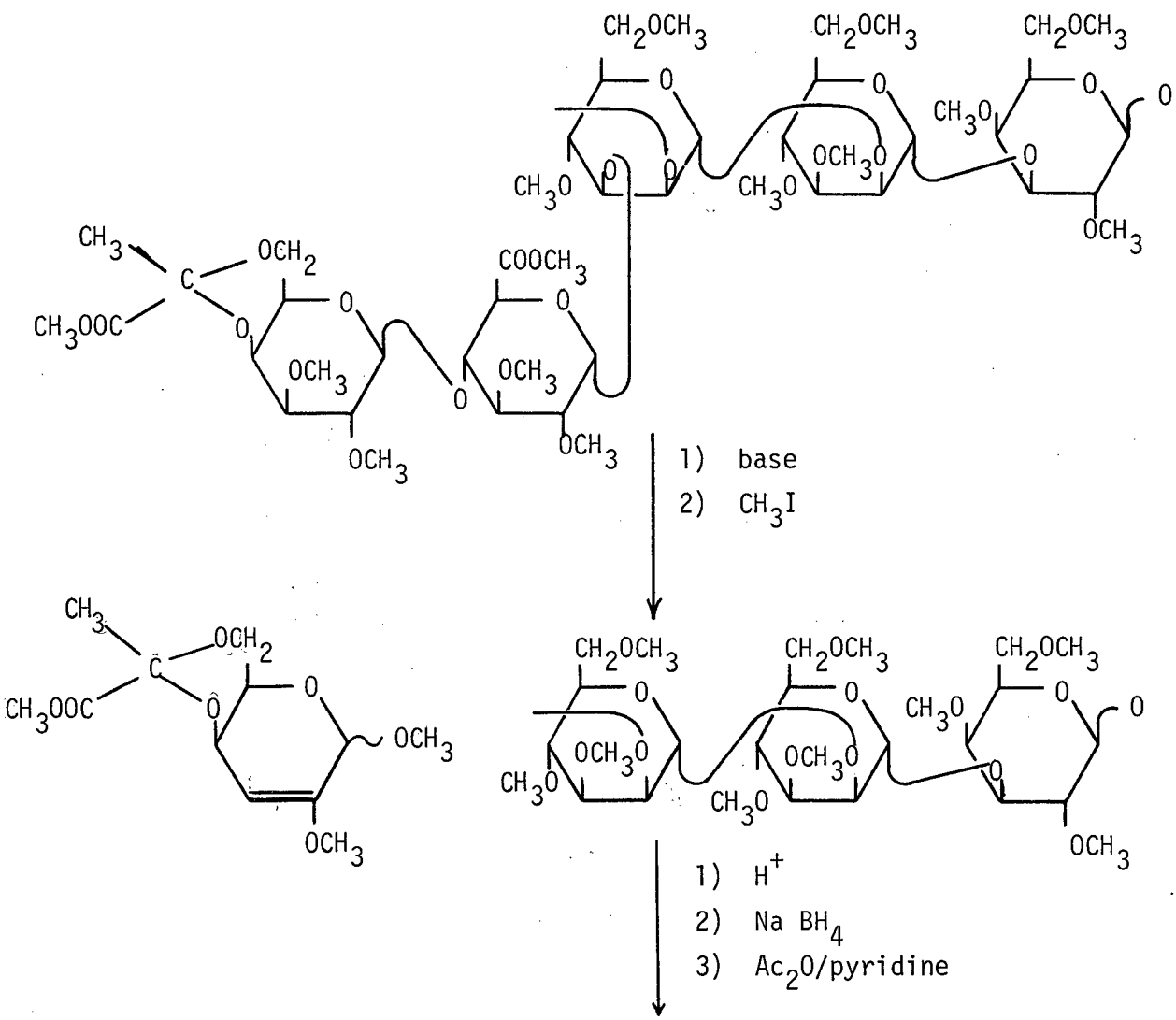
The β-elimination reaction for uronic acids and their derivatives has been reviewed by Kiss.[66] Recently, the technique has been applied with success to the structural investigation of acidic polysaccharides.[67]

The main steps of this degradation are outlined as follows:



On methylation, the esterified carboxyl group in uronic acid residues becomes electron — withdrawing and increases the acidity of the ring proton at C5. On subsequent treatment with base the proton at C5 is removed and the substituent in the β-position (R_4O^-) is eliminated. The hex-4-eno-pyranosyluronic residue (II) is acid sensitive. Treatment under mild acid conditions will degrade this unsaturated residue without affecting intact glycosidic bonds. Aspinall and Rosell have reported that treatment with base results in complete loss of the unsaturated residue (II) and acid hydrolysis is unnecessary.[68] They suggested that after treatment with base, the product is directly alkylated with trideuteriomethyl or ethyl iodide to label the site(s) to which uronic acids were attached. The procedure avoids intermediate isolation of degraded polysaccharide and possible loss of acid labile glycosyl substituents.

The degradation becomes more complex, but also more informative, when the substituent on C4 of the uronic acids is another sugar. On treatment with base, a sugar residue R_4OH is released, and this, having a good leaving group at C3, will further react by a second β-elimination. If this leaving group at C3 happens to be another sugar residue, degradation will continue giving rise to the so-called peeling of the polysaccharide. This can be prevented by protecting reducing sugars by acetylation when elimination is performed in the presence of acetic anhydride.[69] The nature of residues released during the degradation is revealed by analysis of the remaining sugars, as partially methylated alditol acetates, by gas-liquid chromatography - mass spectrometry. Figure II.7 illustrates



1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-D-mannitol
1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl-D-galactitol

Figure II.7 Uronic acid degradation of Klebsiella K74 polysaccharide

uronic acid degradation of Klebsiella K74 polysaccharide.

II. 6. Determination of D - or L - Configuration of Component Sugars

The D or L configuration of individual sugar residues can be determined by the sign of their circular dichroism curves measured on suitable derivatives.[70] These derivatives which can be alditol acetates or partially methylated alditol acetates are collected by preparative gas-liquid chromatography. Measurements are made in acetonitrile at 213 nm and spectra are compared with those obtained from authentic samples.

III. Structural Investigation of *Klebsiella* Serotype K53 Capsular Polysaccharide

III. 1 Abstract

The structure of the Klebsiella serotype K53 capsular polysaccharide has been elucidated by methylation analysis, uronic acid degradation, and characterization of oligosaccharides obtained by partial hydrolysis. Anomeric configurations of the sugar residues were determined by ^{1}H-and ^{13}C-n.m.r. spectroscopy on the original polysaccharide and oligosaccharides. The polymer consists of hexasaccharide repeating units having the following structure.

$$\left[\underline{4}\text{ D-Glc}\underline{p}\text{A }\frac{1\ 2}{\beta}\text{ D-Man}\underline{p}\ \frac{1\ 2}{\alpha}\text{ D-Man}\underline{p}\ \frac{1\ 3}{\alpha}\text{ D-Gal}\underline{p}\ \frac{1\ 2}{\beta}\text{ L-Rha}\underline{p}\ \frac{1}{\alpha}\right]_n$$

with an α-L-Rha$p$ branch at position 3 of the D-Man$p$ residue.

III. 2 Introduction

Nimmich[10,11] has investigated the qualitative composition of the capsular polysaccharide from Klebsiella serotype K53 and found that it contained mannose, galactose, rhamnose, and glucuronic acid. Two other Klebsiella capsular antigens contain these sugars, namely K40 and K80, but their structures are not yet known. In an effort to correlate chemical structure with immunological specificity we now report structural studies of K53.

III. 3. Results and Discussion

Composition and n.m.r. Spectra

Klebsiella K53 bacteria were grown on an agar medium, and the capsular polysaccharide was purified by one precipitation with Cetavlon. The product moved as one band during electrophoresis and had $[\alpha]_D$ -12^o, which compares well to the calculated value of $[\alpha]_D$ $+2.3^o$ using Hudson's rules of isorotation.[71] The molecular weight of the polysaccharide was determined by gel chromatography to be 1.2×10^6.

Paper chromatography of an acid hydrolysate of the polysaccharide showed the presence of rhamnose, galactose, mannose, and glucuronic acid. Methanolysis of K53[52], reduction of the hydrolysate, and analysis of the derived alditol acetates by gas-liquid chromatography confirmed the presence of rhamnose, mannose, galactose, and glucose in the approximate molar ratio of 2.3:1.9:1:1 respectively.

The ^{1}H-n.m.r. spectrum of the native polysaccharide (P1) was recorded in D_2O at 90^oC without acetone as internal standard (see Appendix III, spectrum No. 1). Chemical shift assignments were made relative to that of the methyl protons of the rhamnose residues. The spectrum exhibits a strong doublet at δ1.28, $J_{5,6}$ 6 Hz, and four broad signals in the anomeric region at δ4.55, δ5.05, δ5.23, and δ5.29. The signal at δ1.28 was assigned to methyl protons of the rhamnose sugars, and that upfield of δ5.0 to β-linkages and those downfield to α-linkages. The integral ratio indicates that the repeating unit contains six monosaccharide constituents, two of which are rhamnose, and that two are β-linked and four α-linked. Absence of signals at δ1.5 and δ2.2 shows

that the polysaccharide does not contain any pyruvate ketal or O-acetyl group.[34,35]

The ^{13}C-n.m.r. spectrum of K53 (see Appendix III, spectrum No.2) corroborates the results obtained in the ^{1}H-n.m.r. experiment. Six signals appear in the anomeric region at 96.14, 97.31, 100.44, 101.28, 103.35, and 105.32 p.p.m. The signal at 61.91 p.p.m., attributable to C6's of mannose and galactose, indicates that these residues are not linked to other sugar residues at position 6.[28] The signal at 17.42 p.p.m. was assigned to C6's of rhamnose residues.

^{1}H-and ^{13}C-n.m.r. spectra were also recorded on a sample (Pla) of K53 having undergone a mild hydrolysis, 0.5M trifluoroacetic acid at 95°C for 30 min. (see Appendix III, Spectra No. 3 and No.4). The treatment did not improve very much the ^{1}H-n.m.r. spectrum. The ^{13}C spectrum is quite similar to that of the native polysaccharide except that seven signals appear in the anomeric region intead of six. However, two signals, 102.23 and 103.44 p.p.m., were assigned to the glucuronic acid residue.

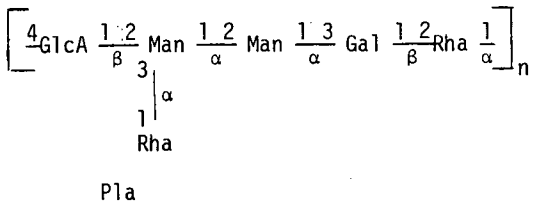
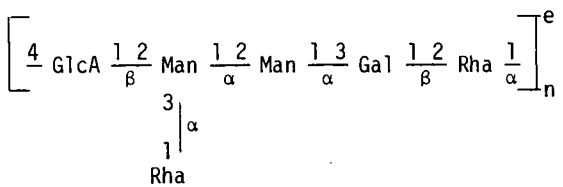
Precise assignment of the signals was achieved after studying ^{1}H-and ^{13}C-n.m.r. spectra of oligosaccharides obtained from partial hydrolysis; see Table III.1.

Methylation Analysis

Methylation [55,56] of K53 polysaccharide and subsequent conversion to alditol acetates and g.l.c.-m.s. analysis [60,62] indicated that K53 is composed of a hexasaccharide repeating unit

TABLE III. I N.m.r. Data of *Klebsiella* K53 Capsular Polysaccharide and Derived Poly - and Oligosaccharides

COMPOUND	δ[a]	$J_{1,2}$ (Hz)[b]	^{1}H-n.m.r. data Integral (H)	Assignment[c]	^{13}C-n.m.r. data p.p.m.[a]	Assignment[d]
GlcA $\frac{1\ 2}{\beta}$ Man-OH	5.30	2	0.7	α-Man-OH	102.49	β-GlcA
	4.99	S	0.3	β-Man-OH	92.91	α,β-Man-OH
A1	4.57	7	1	β-GlcA	61.40	C6 of Man
GlcA $\frac{1\ 2}{\beta}$ Man $\frac{1\ 2}{\alpha}$ Man-OH	5.37	S	0.7	α-Man-OH	102.36	β-GlcA
	5.25	S	0.3	unknown origin	100.84	α-Man-Man
	5.16	1	1	α-Man-Man	93.40	α,β-Man-OH
A2	4.93	S	0.3	β-Man-OH	61.84 }	C6's of Man
	4.58	6	1	β-GlcA	61.38 }	
GlcA $\frac{1\ 2}{\beta}$ Man $\frac{1\ 2}{\alpha}$ Man $\frac{1\ 3}{\alpha}$ Gal-OH	5.29	S	1.2	α-Man-Gal	102.32	β-GlcA
	5.18	S	1	α-Man-Man	101.02	α-Man-Man
	4.65	7 }	1.8	β-Gal-OH	97.21	β-Gal-OH
A3	4.61	7 }		β-GlcA	95.41 }	α-Man-Gal
	1.29	S	0.7	unknown origin	95.12 }	
					93.12	α-Gal-OH
					61.71	C6's of Man,Gal
GlcA $\frac{1\ 2}{\beta}$ Man $\frac{1\ 2}{\alpha}$ Man $\frac{1\ 3}{\alpha}$ Gal $\frac{1\ 2}{\beta}$ Rha-OH	5.35	S	0.5	α-Rha-OH	105.44	β-Gal-Rha
	5.26	S	1	α-Man-Gal	102.31	β-GlcA
	5.19	S	1	α-Man-Man	101.11	α-Man-Man
	4.84	S	0.5	β-Rha-OH	95.53	α-Man-Gal
A4	4.63	7 }	2	β-Gal	93.91	α-Rha-OH
	4.59	7 }		β-GlcA	93.68	β-Rha-OH
	signal for H6's of Rha(δ1.28) was not recorded				61.79	C6's of Man, Gal
					17.63	C6 of Rha-OH
$\left[\frac{4}{}\text{GlcA}\ \frac{1\ 2}{\beta}\ \text{Man}\ \frac{1\ 2}{\alpha}\ \text{Man}\ \frac{1\ 3}{\alpha}\text{Gal}\ \frac{1\ 2}{\beta}\text{Rha}\ \frac{1}{\alpha}\right]_n$	^{1}Hn.m.r. spectrum was not recorded				105.27	β-Gal-Rha
					102.20	β-GlcA
					101.12	α-Man-Man
					100.43	α-Rha-GlcA
P2					95.50	α-Man-Gal
					61.72	C6's of Man, Gal
					17.28	C6 of Rha

COMPOUND	δ[a]	$J_{1,2}$ (Hz)[b]	^{1}H-n.m.r. data Integral (H)	Assignment[c]	^{13}C-n.m.r. data p.p.m.[a]	Assignment[d]
	5.27	S			105.27	β-Gal-Rha
	5.20	S	3.2	α-anomeric H	103.44	β-GlcA
	5.03	S			102.23	
	4.55	b	2	β-anomeric H	101.16	α-Man-Man
	1.28	6	4	H6's of Rha	100.37	α-Rha-GlcA
					96.77	α-Rha terminal
					95.51	α-Man-Gal
					61.73	C6's of Man, Gal
					17.42	C6's of Rha
P1a						
	5.29	b			105.32	β-Gal-Rha
	5.23	b	4	α-anomeric H	103.35	β-GlcA
	5.05	b			101.28	α-Man-Man
	4.55	b	2	β-anomeric H	100.44	α-Rha-GlcA
	1.28	6	6	H6's of Rha	97.31	α-Rha terminal
					96.14	α-Man-Gal
					61.91	C6's of Man, Gal
					17.42	C6's of Rha
P1						

[a]Chemical shift relative to internal acetone; δ2.23 for ^{1}H-n.m.r. and 31.07 p.p.m. for ^{13}C-n.m.r. downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.).

[b]b=broad; s=singlet. [c] For example, α-Man-OH=protonon C1 of α-linked Man residue.

[d]For example, β-Gal-Rha=^{13}C-1 of β-linked non-reducing Gal residue.

[e]Spectra recorded without internal standard; chemical shifts assigned relative to the chemical shifts of H6's and C6 of rhamnose residue, δ1.28 for ^{1}H-n.m.r. and 17.42 p.p.m. for ^{13}C-n.m.r.

TABLE III.2 Methylation Analyses of K53 Capsular Polysaccharide and Derived Poly-and Oligosaccharides

Methylated Sugars[a] (as alditol acetates)	T^b Column (ECNSS-M)	Mole % [c]							
		I[d]	II	III	IV	V	VI	VII	VIII
2,3,4-Rha	0.44	18.6	19.5						30.1
3,4-Rha	0.89	19.0	18.4	18.8				16.2	
2-Et-4,6-Man	1.82								20.5
3,4,6-Man	1.94	17.6	18.9	45.1	50.8	68.3	54.0	45.3	26.1
2,4,6-Gal	2.27	17.2	15.4	30.5			21.4	20.5	23.3
2,3,4-Glc	2.48				49.2	31.7	24.6	18.0	
2,3,6-Glc	2.50		11.6						
4,6-Man	3.31	16.0	14.1						
2,3-Glc	5.32	11.6	2.0	5.6					

[a] 2,3,4-Rha = 1,5-di-O-acetyl-2,3,4-tri-O-methyl-L-rhamnitol, etc.

[b] Retention time relative to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol on an ECNSS-M column, isothermal at 170°C.

[c] Values are corrected by use of effective, carbon response factors given by Albersheim et al. [61]

[d] I, methylated original polysaccharide P1; II, methylated polysaccharide, reduced, remethylated; III, straight chain methylated polysaccharide P2; IV, aldobiouronic acide A1; V, aldotriouronic acid A2; IV, aldotetraouronic acid A3; VII, aldopentaouronic acid A4; VIII, ethylated product from uronic acid degradation.

(Table III. 2, column I). The presence of a dimethyl mannose residue is attributable to a branch point and that of a trimethyl rhamnose to the terminal sugar of a side-chain. Absence of any alditol methylated at the 5 position is an indication of the pyranosidic nature of the sugar constituents. The 2,3-di-O-methyl glucose is the only derivative which could have arisen from the glucuronic acid. This has been shown by the appearance in the g.l.c. tracing of a peak corresponding to the acetate derivative of 2,3,6-tri-O-methyl glucitol and the almost complete disappearance of the 2,3-di-O-methyl glucitol acetate peak, after the permethylated polysaccharide was reduced and remethylated (Table III.2, column II). A small amount of the dimethyl glucose residue in this second analysis was because of incomplete methylation.

Partial Hydrolysis

Partial acid hydrolysis of K53 polysaccharide was followed by separation of acidic and neutral fractions by ion-exchange chromatography. The acidic components were separated by gel filtration chromatography. Figure III. 1 illustrates the chromatogram produced from the weight of fractions collected. Some fractions were pooled and purified by descending paper chromatography. Four oligomers were thus collected.
i) Compound A1, $[\alpha]_D$ -38.9^0 (c 1.97, water), 18mg. Refer to Table III. 1 and Table III. 2, column IV, Appendix III, spectra No. 5 and No. 6. The ^{1}H-n.m.r. spectrum (D_2O, ambient temperature) shows anomeric signals at δ5.30 (0.7 H, $J_{1,2}$ 2 Hz), δ4.99 (0.3 H, singlet), and 4.57 (1 H, $J_{1,2}$ 7 Hz). In the ^{13}C-n.m.r. spectrum, two signals occur in the anomeric region

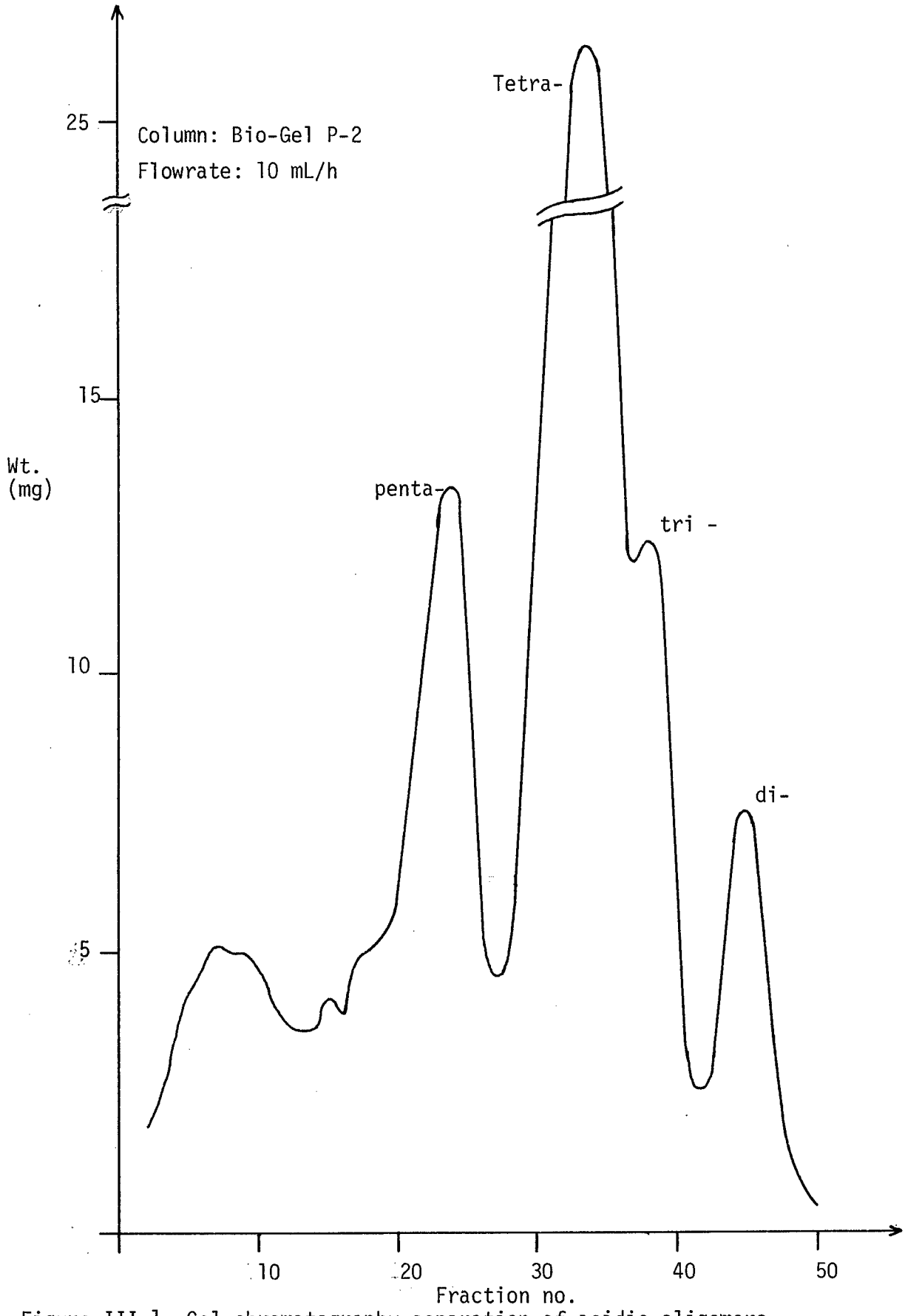


Figure III.1 Gel chromatography separation of acidic oligomers obtained from partial hydrolysis of K53 polysaccharide

at 102.49 and 92.91 p.p.m., and one in the region associated with $-CH_2OH$ groups[28] at 61.40 p.p.m. These results are in good agreement with a disaccharide having a reducing hexose and a non-reducing β-linked glycoside. Methylation, reduction, hydrolysis of A1, and subsequent g.l.c.-m.s. analysis of its alditol acetates[60,62] gave 3,4,6-tri-O-methylmannose and 2,3,4-tri-O-methylglucose in a molar ratio of 1:1. Compound A1 is, therefore, established as being

GlcpA $\frac{1\ 2}{\beta}$ Manp A1

ii) Compound A2, $[\alpha]_D$ -5.8° (c 1.17, water), 35mg. Refer to Table III.1 and Table III.2, column V, Appendix III, spectra No. 7 and No.8. The ^1H-n.m.r. spectrum (D_2O, ambient temperature) exhibits anomeric signals at δ5.37 (0.7 H, singlet), δ5.25 (0.3 H, unknown origin), δ5.16 (1 H, singlet), δ4.93 (0.3 H, singlet) and δ4.58 (1 H, $J_{1,2}$ 6 Hz). In the ^{13}C-n.m.r. spectrum, three anomeric signals occur at 102.36, 100.84 and 93.40 p.p.m., and two in the region associated with non-linked C6[28] at 61.84 and 61.38 p.p.m. Methylation of A2 and subsequent g.l.c. - m.s. analysis [60,62] of its alditol acetates gave 3,4,6-tri-O-methylmannose and 2,3,4-tri-O-methylglucose in a molar ratio of 2:1. The structure of A2 is thus established as

GlcpA $\frac{1\ 2}{\beta}$ Manp $\frac{1\ 2}{\alpha}$ Manp A2

iii) Compound A3, $[\alpha]_D$ +47.1° (c 4.27, water), 91mg. Refer to Table III.1 and Table III.2, column VI, Appendix III, spectra No. 9 and No. 10. A3 was shown by n.m.r. spectroscopy to consist of one reducing hexose and three non-reducing glycosyl units, two α-linked and one

β-linked. The ^{1}H-n.m.r. spectrum shows four anomeric signals at δ5.29 (1.2 H, singlet), δ5.18 (1 H, singlet), two overlapping doublets corresponding to two protons at δ4.65 ($J_{1,2}$ 7 Hz) and δ4.61 ($J_{1,2}$ 7 Hz). One more signal of unknown origin occurs upfield of the acetone peak at δ1.29 (0.7 H, singlet). This peak is characteristic of the chemical shift of methyl protons of a 6-deoxyhexose, such as rhamnose. However no rhamnose was detected either in the ^{13}C-n.m.r. spectrum or in the methylation analysis of the oligomer.

The ^{13}C-n.m.r. spectrum presents some interesting aspects. In this spectrum only five signals were expected in the anomeric region but six appear at 102.32, 101.02, 97.21, 95.41, 95.12, and 93.12 p.p.m. The chemical shifts at 97.21 and 93.12 p.p.m. were assigned respectively to the β and α forms of the reducing galactose and those at 95.41 and 95.12 p.p.m. to the α-mannopyranosyl residue linked to the C3 of the galactose. The initial reaction to these results is that the sample is a mixture of oligosaccharides. This possibility was eliminated by checking the purity of the sample by paper chromatography, only one spot was detected, and by determining the number of reducing end in the sample by the method of Morrison.[72] This method measures the ratio of acetylated aldononitrile to acetylated alditol and gives the degree of polymerisation of the oligomer; only one reducing end was found. Colson and King[73] have reported for some β-D-glycopyranosyl-L-rhamnose disaccharides that the anomeric signal of the glycopyranosyl residue, when linked to C2 of the rhamnose, is split by the passage from the equatorial (β) to axial (α) position of the OH linked to the

anomeric carbon of the reducing rhamnose. Such a phenomenon could explain the splitting in A3 of the C1 signal of the mannopyranosyl residue linked to the C3 of the reducing galactose. Similar observations were made for another tetrasaccharide isolated in the structural investigation of K74 and having the same terminal disaccharide.

Methylation of A3 and subsequent reduction, hydrolysis and g.l.c. - m.s. analysis of its alditol acetates[60,62] gave 3,4,6-tri-O-methylmannose, 2,4,6-tri-O-methylgalactose, and 2,3,4-tri-O-methylglucose in the approximate molar ratio of 2.3:0.9:1.0. Compound A3 is, therefore, established as being

$$\text{GlcpA}\ \overset{1\ 2}{\beta}\ \text{Manp}\ \overset{1\ 2}{\alpha}\ \text{Manp}\ \overset{1\ 3}{\alpha}\ \text{Galp} \qquad \text{A3}$$

iv) Compound A4, $[\alpha]_D$ + 28.5° (c 0.74, water), 29 mg. Refer to Table III.1 and Table III.2, column VII, Appendix III, spectra No. 11 and No. 12.

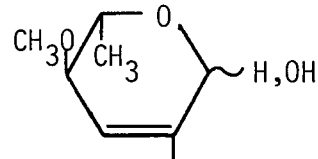
This oligosaccharide was shown by n.m.r. spectroscopy to contain one reducing 6-deoxyhexose and four non-reducing glycosyl constituents, two of which are α-linked and two β-linked. The ^{1}H-n.m.r. spectrum exhibits six peaks in the anomeric region at δ5.35 (0.5H, singlet), δ5.26(1H, singlet), δ5.19 (1H, singlet), δ4.84 (0.5 H, singlet) and two overlapping doublets corresponding to two protons at δ4.63 ($J_{1,2}$ 7 Hz) and δ4.59 ($J_{1,2}$ 7 Hz). The signal due to the methyl protons of the rhamnose residue does not appear because the spectrum was not recorded upfield of the acetone peak. In the ^{13}C-n.m.r. spectrum, six anomeric signals occur at 105.44, 102.31, 101.11, 95.53, 93.91, and 93.68 p.p.m., and one in the region associated with non-linked C6 at 61.79 p.p.m.[28] Finally, the peak

at 17.63 p.p.m. was assigned to the C6 of the rhamnose residue. Methylation of A4 and subsequent reduction, hydrolysis and g.l.c.-m.s. analysis of its alditol acetates[60,62] gave 3,4,6-tri-O-methylmannose, 2,4,6-tri-O-methylgalactose, 2,3,4-tri-O-methyglucose, and 3-4-di-O-methylrhamnose in the approximate molar ratio of 2.3:1.0:0.9:0.8. The structure of A4 is established as

$$\text{GlcpA}\ \overset{1\ 2}{\underset{\beta}{}}\ \text{Manp}\ \overset{1\ 2}{\underset{\alpha}{}}\ \text{Manp}\ \overset{1\ 3}{\underset{\alpha}{}}\ \text{Galp}\ \overset{1\ 2}{\underset{\beta}{}}\ \text{Rhap} \qquad \text{A4}$$

Uronic Acid Degradation

The permethylated polysaccharide K53 was subjected to a base-catalyzed uronic acid degradation.[68,74] In a single operation, the polymer was treated with sodium methylsulfinylmethanide in methyl sulfoxide and directly alkylated with ethyl iodide. Hydrolysis, reduction of the degraded product and subsequent g.l.c.-m.s. analysis of its alditol acetates[60,62] gave 2,3,4-tri-O-methylrhamnose, 2-O-ethyl-4-6-di-O-methylmannose, 3,4,6-tri-O-methylmannose, and 2,4,6-tri-O-methylgalactose (see Table III.2, column VIII). Loss of glucuronic acid residues was accompanied by further degradation of exposed reducing groups as witnessed by complete loss of the 3,4-di-O-methylrhamnose residue. Further degradation was halted at this point because of formation of base-stable 3,6-dideoxy-hex-2-enopyranose units as shown below.



$$\text{4,6-Man}\ \overset{1\ 2}{\underset{}{}}\ \text{3,4,6-Man}\ \overset{1\ 3}{\underset{}{}}\ \text{2,4,6-Gal} - \text{O}$$

$$\begin{array}{l} 3| \\ 1| \\ \text{2,3,4-Rha} \end{array}$$

Degradation of the glucuronic acid and dimethylrhamnose units and the presence of 2-O-ethyl-4,6-dimethylmannose indicate that glucuronic acid is linked to C2 of the branching mannose and to O1 of the in-the-chain rhamnose residue. These results also prove that the terminal non-reducing rhamnose is the only sugar in the side chain linked to C3 of the branching mannose.

The mass spectrum of the 2-O-ethyl isomer exhibits primary fragments at m/e 45, 131, 161, and 247. By comparing this spectrum with that of 1,3,5-tri-O-acetyl-2,4,6-tri-O-methylmannitol a characteristic shift of 14 mass units is observed (see Figure II.6).

Study of Partially Depolymerized K53 Polysaccharide

A mild hydrolysis, 2M TFA at 95°C, 30 min., was performed on a sample of K53 capsular polysaccharide. Methylation of the non-dialyzable product,[55,56] reduction, hydrolysis, and subsequent g.l.c.-m.s. analysis of its alditol acetates[60.62] gave the results shown in Table III.2, column III. Complete loss of 2,3,4-tri-O-methylrhamnose and absence of any other terminal non-reducing residue indicated that the depolymerized K53 was a straight chain polysaccharide, P2 $[\alpha]_D$ +14.2° (c 0.84, water). The specific rotation is in good agreement with the calculated specific rotation, $[\alpha]_D$ +15.2°, using Hudson's rules of isorotation.[71] The low proportion of 2,3-dimethylglucose was because of incomplete reduction of the glucuronic acid methyl ester.

In the ^{13}C-n.m.r. spectrum of P2 (see Appendix III, spectrum No.13) five peaks appear in the anomeric range at 105.27, 102.20, 101.12, 100.43, and 95.50 p.p.m., and another one at 17.28 p.p.m. owing to the C6 of the

rhamnose residue. Comparing this spectrum to that of the native K53 polysaccharide P1, one can see that the peak at 97.31 p.p.m. disappeared and was thus assigned to the α-rhamnose of the side-chain. Removal of the side-chain moved the chemical shift of the glucuronic acid by about 1 p.p.m. upfield, from 103.35 to 102.20 p.p.m. Finally, on the basis of previous n.m.r. data the chemical shift appearing at 100.44 p.p.m. in the spectrum of the native K53 could be assigned to the α-rhamnose linked in the chain.

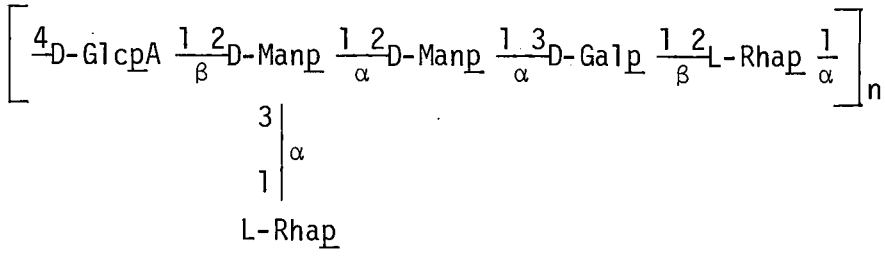
D or L configuration of the component sugars

The D or L configuration of the component sugars was determined by comparing the sign of the circular dichroism (c.d.) curves measured on the corresponding alditol acetates of those obtained from authentic samples.[70] Since galactitol is a meso-alditol the configuration of galactose was determined relative to the peracetyl derivative of 2,4,6-tri-O-methylgalactitol. These derivatives were isolated by preparative gas-liquid chromatography from samples obtained from total sugar ratio and methylation analyses of K53. Measurements were made in acetonitrile at 215-250 nm. Rhamnose was confirmed to be of the L configuration, and glucose, mannose, and galactose were of the D configuration.

Measurements were also made on partially methylated alditol acetates of rhamnose, mannose, and glucose. It showed that 3,4-di-O-methyl-L-rhamnose and 2,3,4-tri-O-methyl-L-rhamnose have a negative c.d. curve, while 3,4,6-tri-O-methyl-D-mannose, 4,6-di-O-methyl-D-mannose, and 2,3-di-O-methyl-D-glucose have a positive c.d. curve.

Proposed Structure

Data obtained in the present structural investigation demonstrate that the capsular polysaccharide from Klebsiella serotype K53 is composed of hexasaccharide repeating units having the following structure.



Of the Klebsiella capsular polysaccharide reported to date, only K52[75] has the same structural pattern but is not of the same chemotype (see Appendices I and II).

Capsular polysaccharides K53 and K52 cross-react heavily in anti-K47 serum.[19] Both antigens, as well as the capsular polysaccharide K47,[76] contain a common disaccharide; i.e. α-L-Rhap-(1→4)-β-D-GlcpA-. In K47, this disaccharide constitutes the lateral side-chain while in K52 and K53, it is part of the backbone. This feature suggests that this dimer is an antigenic determinant. Cross-reactivity of K53 with anti-PnXXIII also suggests that the non-reducing α-L-rhamnose lateral end group could be an immunodominant sugar because the capsular polysaccharide PnSXXIII has the same side-chain.[19]

III. 4. Experimental

General Methods

Descending paper chromatography was carried out using Whatman No. 1 paper for analytical purposes and Whatman 3MM for preparative paper chromatography. The following solvent systems (v/v) were used: (A) ethyl acetate - acetic acid - formic acid - water (18:3:1:4); (B) ethyl acetate - pyridine - water (8:2:1); (C) 1-butanol - acetic acid - water (2:1:1). Chromatograms were developed using alkaline silver nitrate reagent.[77]

Gel filtration chromatography was conducted on a column (100 x 3 cm) of Bio-Gel P-2(100-200 mesh). The column was irrigated with water - pyridine - acetic acid (500:5:2) at a flow rate ∿ 10mL/h. Fractions of 2.0 - 2.5 mL were collected with a Gibson FC-80K micro fractionator or a LKB Radi Rac 3403 B fraction collector. Fractions were freeze-dried, weighed in tared tubes and the results plotted on graph paper to produce a chromatogram.

Analytical g.l.c. separations were performed using a Hewlett Packard model 5710A gas chromatograph fitted with dual flame ionization detectors. A stainless steel column (1.8 m x 3 mm) of 5% ECNSS-M on Gas Chrom Q (100 - 120 mesh) was exclusively used, operated at 170^{o}C isothermal, except as otherwise stated. Preparative g.l.c. was performed on an F & M model 720 gas chromatograph with dual thermal conductivity detectors. A column (1.8 m x 6.3 mm) of 5% Silar 10C on Gas Chrom Q (100-120 mesh) was used for preparative separations. An Infotronics CRS-100 electronic integrator was used to measure peak areas.

G.l.c. - m.s. was performed using a Micromass 12 instrument fitted with a Watson-Biemann separator. Spectra were recorded at 70 eV with an ionization current of 100 μA and an ion source temperature of 200°C.

^{1}H-n.m.r. spectra were recorded on a Varian XL-100 spectrometer at approximately 90°C and, in some cases, at ambient temperature (for water peak suppression). Samples run in D_2O were exchanged and freeze-dried three or four times in 99.7% D_2O. ^{13}C-n.m.r. spectra were recorded on a Varian CFT-20 instrument at ambient temperature in 50 per cent D_2O. In all cases acetone (2.23 for ^{1}H-n.m.r. and 31.07 p.p.m. for ^{13}C-n.m.r. measured against aqueous sodium 4,4-dimethyl-4-silapentane-1-sulfonate, D.S.S.) was used as internal standard. In addition, a ^{13}C-n.m.r. spectrum of the native polysaccharide (P1), recorded at 95°C, was obtained by courtesy of Dr. Michel Vignon, CERMAV/CNRS, Grenoble, France on a Cameca 250 MHz instrument.

Circular dichroism measurements were made on a Jasco J-20 automatic recording spectropolarimeter with a quartz cell of path length 0.01 cm. Optical rotations of previously dried samples were measured at room temperature on a Perkin-Elmer model 141 polarimeter using a 10 cm cell. Infrared spectra were recorded on a Perkin-Elmer 457 spectrophotometer.

Solutions were concentrated under reduced pressure at temperatures not exceeding 40°C.

Isolation and Purification of Klebsiella K53 Polysaccharide

A culture of Klebsiella K53 (1756/51) was obtained from Dr. Ida Ørskov, Copenhagen, and was grown on a 3% sucrose - yeast extract - agar medium composed of 5g NaCl, 2.5g K_2HPO_4, 0.62g $MgSO_4 \cdot 7H_2O$, 0.5g $CaCO_3$, 75g sucrose, 5g Bacto yeast extract, and 37.5g agar in 2.5L of water. The cells were harvested after 3 days, 1L of very viscous slime was collected. Slime was diluted to 3L with 1% aqueous phenol, and centrifuged in batches for 8-12h at 30,000 r.p.m. in a Beckman model L3-50 ultracentrifuge with rotor type 35. The clear, supernatant liquids were decanted, combined (∿2L), and precipitated by pouring into 10L of Solvent 1K (ethanol-methanol, 95:5). Crude polysaccharide was dissolved in 1L of water and precipitated with a 10% Cetavlon solution. The precipitate was isolated by centrifugation, redissolved in 1L of 4M NaCl, and reprecipitated by pouring into Solvent 1K. The purified polysaccharide was collected, dissolved in water, and dialyzed against running tap-water for three days. Freeze-drying of this solution yielded 13g of the sodium salt of the capsular polysaccharide, $[\alpha]_D$ -12° (c 0.24, water). Purity of the polysaccharide was checked by electrophoresis using a 1% solution on a cellulose acetate strip (Sepraphore III; 15 x 2.5 cm) in Veronal buffer pH 8.6 (LKB-Produkter AB,Stockholm 12, Sweden) at 300V for 60 min and then development in alcian blue. Homogeneity was also confirmed by gel chromatography by courtesy of Dr. S.C. Churms, University of Cape Town South Africa, and the molecular weight of K53 polysaccharide was determined to be 1.2×10^6.

Some spectroscopic analyses were performed on K53 polysaccharide that had been partially depolymerized to reduce viscosity. This was achieved by mild hydrolysis in 0.5M trifluoroacetic acid for 30 min at 95°C, and then concentration under reduced pressure and evaporation several times with water to eliminate excess of acid. The hydrolysate was dissolved in water, dialyzed overnight against running tap-water and freeze-dried.

Analysis of Component Sugars

Polysaccharide was hydrolysed overnight with 2M trifluoroacetic acid at 95°C. After evaporation, the hydrolysate was found, by paper chromatography in solvents A and B, to contain rhamnose, galactose, mannose and glucuronic acid.

Methanolysis of 30mg of Klebsiella K53 capsular polysaccharide with 3% methanolic hydrochloric acid and subsequent treatment with sodium borohydride in anhydrous methanol depolymerized the substrate and reduced uronic acid residues.[52] Total hydrolysis, reduction of the free sugars to alditols with sodium borohydride, and acetylation with acetic anhydride-pyridine (1:1) at room temperature overnight yielded rhamnitol pentaacetate, and the hexaacetates of mannitol, galactitol, and glucitol in the g.l.c. tracing using a column of 5% ECNSS-M; programmed at 170°C for 8 min and then at 4°/min to 190°C.

Methylation Analysis

a) Native Polysaccharide P1

About 500 mg of dried K53 capsular polysaccharide was dissolved in 50 mL anhydrous dimethyl sulfoxide with ultrasonic agitation and methylated by treatment with 10mL dimethylsulfinyl anion for 6h, and

then 8mL methyl iodide for 1h.[55,56] After removal of excess reagents by dialysis against running tap-water for three days, the methylated polysaccharide was recovered by freeze-drying, yield 352mg. Subsequent Purdie[78] treatment with silver oxide and methyl iodide gave a permethylated product that showed no hydroxyl group absorption at 3600 cm^{-1} in the infrared spectrum, yield 373 mg.

A sample of 55mg of this dried material was reduced with lithium aluminum hydride in refluxing tetrahydrofuran, 15mL, for 3h and then at room temperature overnight. The white precipitate of aluminum hydroxide was dissolved with 4% hydrochloric acid and the reduced product was extracted with chloroform (5x10mL). The combined extracts were washed with water (3x10mL) and evaporated to dryness under reduced pressure. The reduced polysaccharide, 45mg, was separated into two equal fractions. Fraction one was hydrolysed with 2M trifluoroacetic acid overnight at 95°C, reduced with sodium borohydride, and acetylated with acetic anhydride:pyridine (1:1) at room temperature overnight. The second fraction was remethylated by one Purdie treatment[78] and converted to the corresponding alditol acetates as for fraction one. G.l.c. and g.l.c. - m.s. analyses of both mixtures of partially methylated alditol acetates allowed the assignments given in Table III.2, columns I and II.

b) Partially Depolymerized K53 Polysaccharide, P2

A sample (215mg) of K53 polysaccharide was partially depolymerized with 2M trifluoroacetic acid at 95°C for 30 min. The hydrolysate was concentrated under reduced pressure, evaporated several times with water to eliminate excess of acid, and then dissolved in

water and dialyzed overnight against running tap-water. Freeze-drying of the solution yielded 165 mg of polymeric material, P2, $[\alpha]_D$ + 14.2° (c 0.82, water). Methylation of 16 mg of P2 by Hakomori's procedure,[55,56] derivatisation to the corresponding alditol acetates as for the original polysaccharide, and g.l.c. analysis gave the results listed in Table III.2, column III.

Partial hydrolysis

A batch hydrolysis was performed on 640 mg of K53 polysaccharide with 0.5M trifluoroacetic acid at 95°C for 5 hours. After removal of the acid by evaporation with several portions of water, paper chromatography (solvent A) of the hydrolysate showed the presence of several oligosaccharides. The total amount of material was applied to the top of a column (2x20cm) of Bio-Rad AG1-X2 resin in the formate form. The column was first eluted with 700 mL of water, and then with 500 mL of 10% formic acid. Paper chromatography (solvent B) of the neutral fraction, 288 mg, showed that it contained mainly monosaccharides and very little oligosaccharide.

The acidic fraction, 322 mg, was separated by gel filtration chromatography on a column of Bio-Gel P-2, 100x3 cm. The column was irrigated with a buffer composed of water: pyridine: acetic acid (500:5:2). Fractions of 2.0 - 2.5 mL were collected, freeze-dried, and weighed in tared tubes. The results were plotted on graph paper to produce the chromatogram shown in Figure III.I, page 42. Fractions 20-26 were pooled as well as fractions 29-34, 35-40, 43-46, and purified by descending paper chromatography on Whatman 3 MM paper using

solvent C for three to six days. Four pure oligosaccharides were thus collected, i.e. A1, A2, A3, and A4.

Methylation analysis of these oligosaccharides was performed as follow. Dried samples of 7-10 mg were dissolved in 5 mL anhydrous dimethyl sulfoxide, treated with 3 mL dimethylsulfinyl anion for 6h and 4 mL of methyl iodide for 1 hour.[55,56] The mixtures were diluted with water, neutralized with 10% acetic acid, transferred to separatory funnels and extracted with chloroform (5 x 10 mL). The combined extracts were back extracted with water (3 x 10 mL) and evaporated to dryness under reduced pressure. Permethylated oligosaccharides were reduced with lithium aluminum hydride and converted to the corresponding alditol acetates as previously described for the methylation analysis of the original polysaccharide. G.l.c. analysis of the mixtures of partially methylated alditol acetates allowed the assignments shown in Table III.2, columns IV-VII; results confirmed by g.l.c. - m.s. analyses.

Uronic Acid Degradation[68,74]

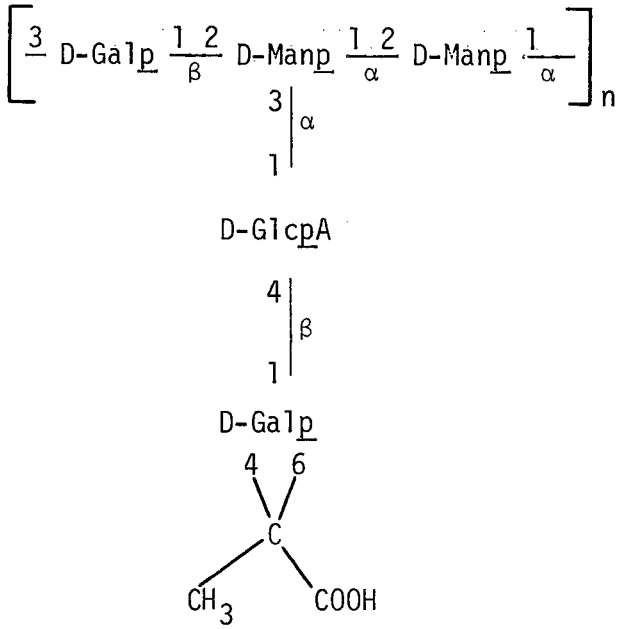
A sample (100 mg) of dried, methylated polysaccharide and 1 mg of p - toluenesulfonic acid were dissolved in 20 mL of a 19:1 (v/v) mixture of dimethyl sulfoxide—2,2 - dimethoxypropane in a flask sealed with a rubber cap. The flask was flushed with nitrogen and 10 mL of dimethylsulfinyl anion was added with a syringe and the mixture was stirred overnight at room temperature. The substrate was then directly re-alkylated by adding 7 mL of ethyl iodide with external cooling and the reaction mixture was stirred one more hour. The mixture

was neutralized with 10% acetic acid and extracted with chloroform (4 x 10 mL). The combined extracts were washed with water (3 x 10 mL) and evaporated to dryness. Hydrolysis of the degraded product with 2 M trifluoroacetic acid, reduction with sodium borohydride, acetylation with a 1:1 (v/v) mixture of acetic anhydride: pyridine overnight at room temperature, and g.l.c. analysis of the corresponding alditol acetates gave the results listed in Table III.2, column VIII; results confirmed by g.l.c. - m.s. analysis.[60,62]

IV Structural Investigation of *Klebsiella* Serotype K74 Capsular Polysaccharide

IV. 1. Abstract

By using the techniques of methylation analysis, partial hydrolysis, and uronic acid degradation, the structure of the capsular polysaccharide from Klebsiella serotype K74 has been investigated. The anomeric natures of glycosidic linkages were determined by ^{1}H-and ^{13}C-n.m.r. spectroscopy on the original polysaccharide and on oligosaccharides obtained from partial hydrolysis. The polymer was shown to consist of pentasaccharide repeating units as shown below



IV. 2. Introduction

In a qualitative analysis of the capsular polysaccharide from Klebsiella type K74, Nimmich[11] reported the presence of glucuronic acid, galactose, mannose, and pyruvate acetal. Six other Klebsiella polysaccharides contain these sugars,[10] and structural investigation of two of these, type K20[79] and type K21,[80] have been published. We now report the structural investigation of K74.

IV. 3 Results and Discussion

Composition and n.m.r. spectra

Isolation and purification of the polysaccharide, as previously described in Section III.4, provided a homogeneous polymer as indicated by electrophoresis. The product had $[\alpha]_D$ + 66° (c 0.21, water), which compares very well with the calculated specific rotation (+65°) using Hudson's rules of isorotation[71], and a molecular weight of 4.7 x 10^6 as determined by gel chromatography.

The ^{1}H-n.m.r. spectrum[34,35] of the original polysaccharide exhibits a sharp singlet at δ1.51, characteristic of the methyl protons of the pyruvate acetal. Absence of signal at δ 2.20 indicates that the polymer does not contain any acetate group. The anomeric region (δ4.5 - 5.5), though poorly resolved, shows one broad signal at δ4.55 assigned to β-linkages and two broad signals at δ5.26 and δ5.37 attributable to α-linkages. By integration the ratio of the α-anomeric protons to the β-anomeric ones seems to be 3:2.

Comparison of the integrals also indicates that there is one pyruvate acetal per pentasaccharide repeating unit. This result has been confirmed by courtesy of Dr. S.C. Churms, University of Cape Town, South Africa, who found that the polysaccharide contains 11.9% of pyruvic acid (calculated, 11.7%). However, Nimmich has reported that K74 contains only 4% of pyruvate acetal.[81]

The ^{1}H-n.m.r. analysis was confirmed by ^{13}C-n.m.r. spectroscopy of the polysaccharide. The spectrum shows five peaks in the anomeric region at 103.94, 103.26, 100.96, 100.50, and

96.16 p.p.m., and three in the region associated with $-CH_2OH$ groups at 62.19, 62.00, and 61.85 p.p.m. Another peak, characteristic of the methyl group of the pyruvate acetal, appears upfield of the acetone signal at 26.12 p.p.m. According to observations reported by Garegg et al, the acetal carbon atom of the pyruvate group is thus assigned to be of the R configuration.[82] For n.m.r. data refer to Table IV.1 and Appendix III, spectra No.20 and No.21.

Total hydrolysis of Klebsiella K74 capsular polysaccharide and subsequent g.l.c. analysis of the corresponding alditol acetates confirmed the presence of mannose, galactose, and glucose in the approximate molar ratio of 2:2:1. Glucose and mannose were confirmed to be of the D configuration by circular dichroism (c.d.) measurements of their alditol acetates.[70] Galactose was also shown to be of the D configuration based on the c.d. of the acetate derivatives of 2,3-di-O-methylgalactitol and 2,4,6-tri-O-methylgalactitol. The derivatives were isolated by preparative gas liquid chromatography from samples prepared for the total sugar ratio and methylation analyses of K74. Measurements were made in acetonitrile at 215-250 nm.

Methylation Analysis

Complete methylation of K74 polysaccharide in its sodium salt form, as well as its free acid form, proved to be difficult owing to the low solubility of the polymer in dimethyl sulfoxide. One Hakomori methylation [55,56] and one Purdie treatment[78] were necessary in order to obtain a product that showed no hydroxyl

Table IV. 1 N.m.r. data of Klebsiella K74 capsular polysaccharide and derived oligosaccharides

Compound	δ[a]	$J_{1,2}$[b] (Hz)	^{1}H-n.m.r. data Integral(H)	Assignment[c]	^{13}C-n.m.r. data p.p.m.[a]	Assignment[d]
GlcA $\frac{1\ 3}{\alpha}$ Man-OH	5.34	3	1	α-GlcA	101.42	α-GlcA
	5.18	S	0.6	α-Man-OH	94.82	α-Man-OH
B1	4.94	S	0.4	β-Man-OH	94.34	β-Man-OH
					61.73	C6 of Man
GlcA $\frac{1\ 3}{\alpha}$ Man $\frac{1\ 2}{\alpha}$ Man-OH	5.37	2	2.1	α-Man-OH	102.85	α-Man-Man
	5.34	3		α-GlcA	101.37	α-GlcA
	5.17	2	0.3	unknown origin	93.38	α,β-Man-OH
B2	5.08	2	1.0	α-Man-Man	61.94	C6's of Man
	4.94	1	0.3	β-Man-OH		
GlcA $\frac{1\ 3}{\alpha}$ Man $\frac{1\ 2}{\alpha}$ Man $\frac{1\ 3}{\alpha}$ Gal-OH	5.34	3	2	α-GlcA	103.07	α-Man-Man
	5.29	4		α-Man-Gal	101.33	α-GlcA-OH
	5.17	S	0.3	α-Gal-OH	97.21	β-Gal-OH
B3	5.07	2	1	α-Man-Man	95.33	α-Man-Gal
	4.64	7	0.9	β-Gal-OH	95.08	
					93.09	α-Gal-OH
					61.92	C6's of Man, Gal
$\left[\overset{3}{\underset{}{}}\text{Gal}\ \frac{1\ 2}{\beta}\ \text{Man}\ \frac{1\ 2}{\alpha}\ \text{Man}\ \frac{1}{\alpha}\right]_n^e$	5.37	b	3	α-anomeric H	103.94	β-Gal
	5.26	b			103.26	
	4.55	b	2	β-anomeric H	100.96	α-GlcA
	1.51	S	3	$-CH_3$ of puruvate	100.50	β-Man-Man
					96.16	α-Man-Gal
					62.19	C6's Man, Gal
					62.00	
					61.85	
					26.12	$-CH_3$ of pyruvate

3 | α
1
GlcA

4 | β
1
Gal

4 6
C
CH_3 COOH

K74 polysaccharide

Footnotes: [a]Chemical shift relative to internal acetone; δ2.23 for ^{1}H-n.m.r. and 31.07 p.p.m. for ^{13}C-n.m.r. downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.).

[b]b=broad, s=singlet. [c]For example, α-GlcA = proton on C1 of α-linked GlcA; α-Man-Man = proton on C1 of the non-reducing α-linked Man [d]As for [c], but for anomeric, ^{13}C nuclei. [e] ^{1}H-n.m.r. spectrum of the original polysaccharide recorded without internal standard; chemical shifts assigned relative to -CH_3 of pyruvate acetal, δ1.51.

absorption in the infrared spectrum. Reduction of the permethylated sample with lithium aluminum hydride, total hydrolysis with 2M trifluoroacetic acid, and subsequent g.l.c. analysis of the corresponding alditol acetates gave the results listed in Table IV.2, column I. These results seem to corroborate those obtained from n.m.r. experiments despite the low molar percentages of the dimethylglucose and the dimethylgalactose units. The low abundance of these two compounds could be due to the fact that they have large retention times and show not well separated, broad peaks in the g.l.c. tracing. Consequently, integration of their peak areas is not very good. When the experiment is repeated using a programme the peaks of these two isomers are sharper and almost as large as the peaks of the other three partially methylated alditol acetates (see Figure IV.1). Integration is then better and the molar percentage ratio is improved (see Table IV.2, column I, values in parentheses). The still low proportion of 2,3-dimethylglucose was probably because of incomplete reduction of the glucuronic acid methyl esters.

As demonstrated by further studies (see below), the presence of 4,6-di-O-methylmannose is due to a branch point and the 1-carboxyethylidene group is present as an acetal spanning O4 and O6 of the terminal 2,3-di-O-methylgalactose.

Partial Hydrolysis

Partial, acidic hydrolysis of the native K74 polysaccharide was followed by separation of the acidic and neutral fractions by ion-exchange chromatography. The neutral fraction was not studied

TABLE IV. 2 Methylation Analysis of Native, and Degraded K74 Capsular Polysaccharide and Derived Oligosaccharides

Methylated sugars[a] (as alditol acetates)	T[b] ECNSS - M	Mole %[c] I[d]	II	III-1	III-2	IV	V
3,4,6 - Man	1.93 (1.98)[e]	23.3(21.2)[e]		16.8	41.5	23.6	58.6
2,4,6 - Man	2.08		51.5	49.2	39.8	32.5	
2,4,6 - Gal	2.25 (2.34)	25.9(23.2)				20.2	35.0
2,3,4 - Glc	2.45		48.5	34.3	18.7	23.7	
4,6 - Man	3.24 (3.34)	22.0(22.0)					6.4
2,3 - Glc	5.27 (4.53)	13.4(13.9)					
2,3 - Gal	5.71 (4.72)	15.4(19.7)					

[a] 3,4,6 - Man = 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-D-mannitol, etc.

[b] Retention time relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol on an ECNSS-M column operated at 170°C, isothermal.

[c] Values corrected by use of effective, carbon response factors given by Albersheim et al. [61]

[d] I, methylated original polysaccharide; II, aldobiouronic acid, B1; III-1 and III-2, aldotriouronic acid, B2; IV, aldotetraouronic acid, B3; V, uronic acid degradation product.

[e] Numbers in parentheses refer to data obtained when temperature programming was used; 160°C for 13 min and then 2°/min to 190°C.

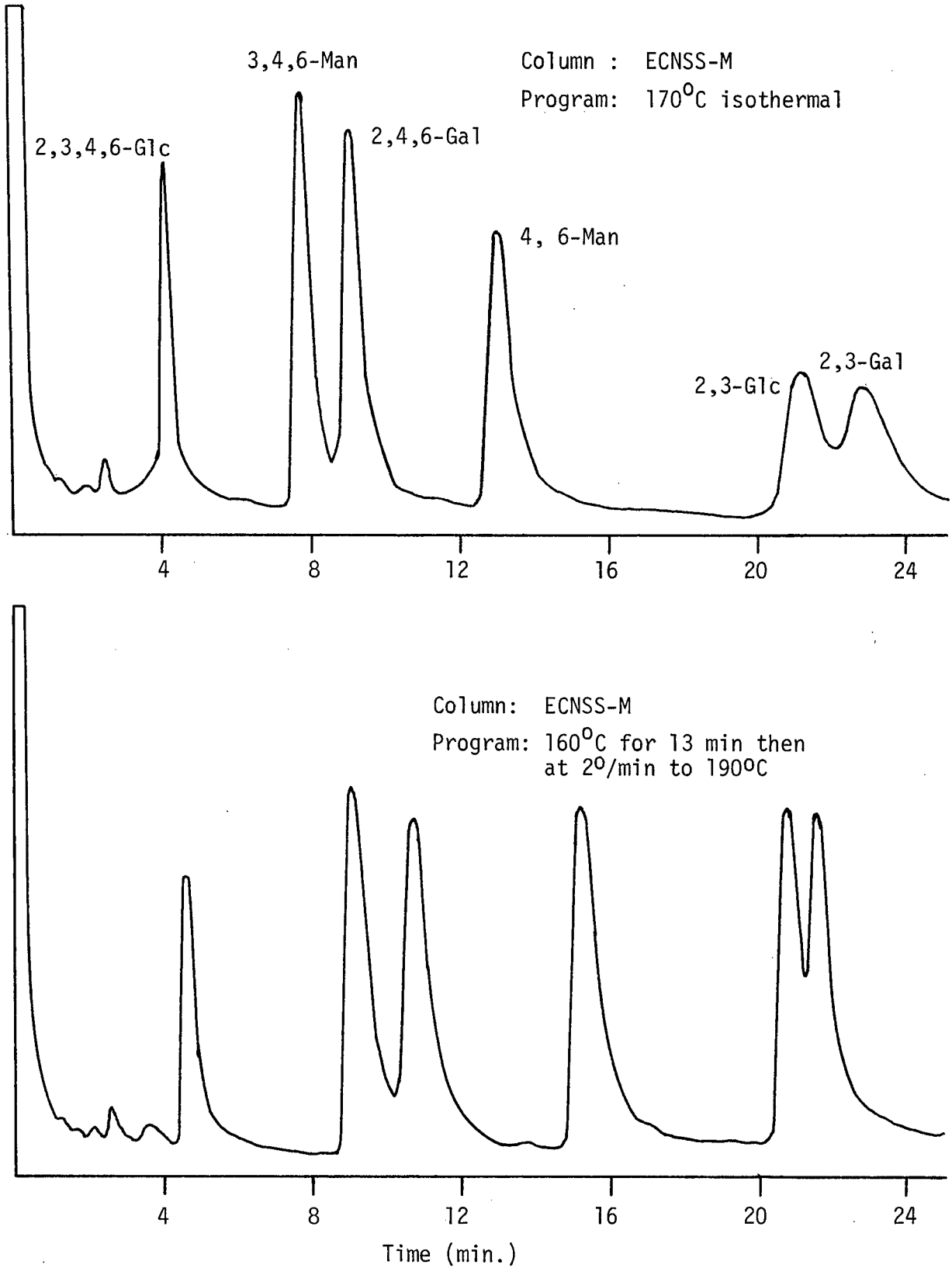


Figure IV.1 G.l.c. separation of a mixture of partially methylated alditol acetates obtained from Klebsiella K74 polysaccharide.

further, while the acidic one was separated by gel filtration chromatography. The chromatogram produced from the weight of the fractions collected is shown in Figure IV.2. Some fractions were pooled and purified by descending paper chromatography. Three pure oligosaccharides were thus collected.

i) Compound B1, $[\alpha]_D$ + 80.7° (c 2.41, water), 52 mg.
Refer to Table IV.1 and Table IV.2, column II, and Appendix III, spectra No. 14 and No. 15. The ^{1}H-n.m.r. spectrum shows signals at δ5.34 (1 H, $J_{1,2}$ 3 Hz), δ5.18 (0.6 H, singlet), and δ4.94 (0.4 H, singlet) in the anomeric region. In the ^{13}C-n.m.r. spectrum three signals occur in the anomeric region at 101.42, 94.82, and 94.34 p.p.m., and one in the region associated with non-linked C6 at 61.73 p.p.m. These data correspond to a compound having one reducing hexose and one α-linked, non-reducing glycosyl residue. Methylation[55,56] of B1 yielded a product that, on hydrolysis and conversion of the products into alditol acetates, gave components corresponding to 2,4,6-tri-O-methylmannose and 2,3,4-tri-O-methylglucose in the molar ratio of 1:1; results confirmed by g.l.c.-m.s.[60,62] The structure of B1 is thus established as

$$\text{D-Glcp}A \xrightarrow[\alpha]{1\ 3} \text{D-Man}p \qquad \text{B1}$$

ii) Compound B2, $[\alpha]_D$ + 89.4° (c 1.61, water), 60 mg. Refer to Table IV.1 and Table IV.2, columns III-1 and III,2, and Appendix III, spectra No. 16 and No.17. The ^{1}H-n.m.r. spectrum exhibits two overlapping doublets corresponding to 2.1 H at δ5.37 ($J_{1,2}$ 2 Hz) and δ5.34 ($J_{1,2}$ 3 Hz), and three more peaks at δ5.17 (0.3 H, $J_{1,2}$ 2 Hz),

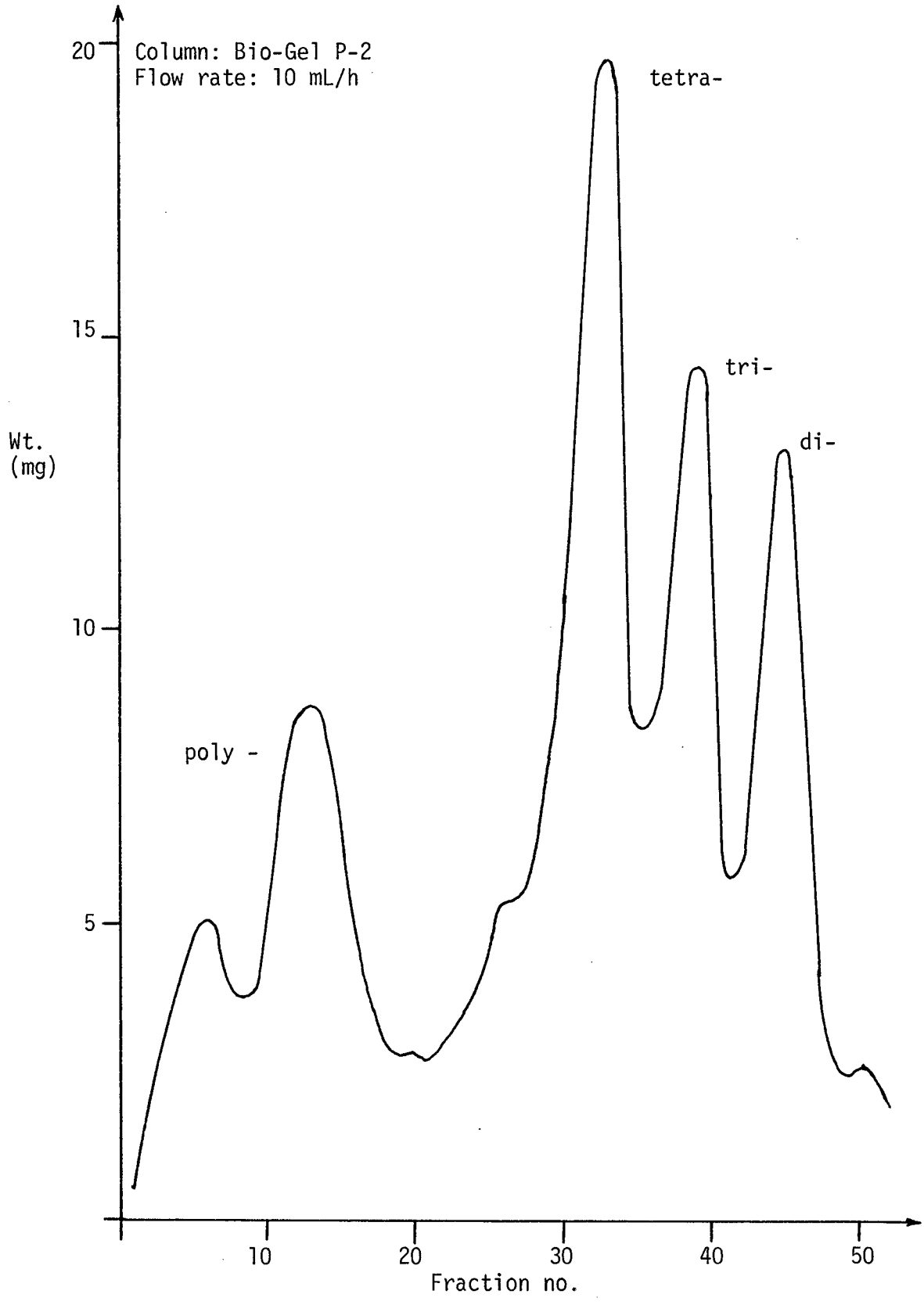


Figure IV. 2 Gel chromatography separation of acidic oligomers obtained from partial hydrolysis of K74 polysaccharide

δ5.08 (1 H, $J_{1,2}$ 2 Hz), and δ 4.94 (0.3 H, $J_{1,2}$ 1 Hz). In the ^{13}C-n.m.r. spectrum three signals appear in the anomeric region at 102.85, 101.37, and 93.38 p.p.m., and one at 61.94 p.p.m. due to non-linked C6's of the mannose residues. Methylation[55,56] of B2 and subsequent g.l.c.-m.s. analysis of the corresponding alditol acetates [60,62] was performed twice. In the first case the analysis gave 3,4,6-tri-O-methylmannose, 2,4,6-tri-O-methylmannose, and 2,3,4-tri-O-methylglucose in the approximate molar ratio of 1:3:2. In the second analysis the molar ratio was approximately 1:1:0.5. These inconsistent results could possibly be attributable to some degradation during the methylation analysis and to incomplete reduction of the glucuronic acid methyl ester. However, the structure of B2 is established as being

D-GlcpA $\frac{1\ 3}{\alpha}$ D-Manp $\frac{1\ 2}{\alpha}$ D-Manp B2

iii) Compound B3, $[\alpha]_D$ + 130° (c 2.10, water), 72 mg. Refer to Table IV.1 and Table IV.2, column IV, and Appendix III, spectra No. 18 and No. 19. In the ^{1}H-n.m.r. spectrum two overlapping doublets corresponding to two protons occur at δ5.34 ($J_{1,2}$ 3 Hz) and δ5.29 ($J_{1,2}$ 4 Hz), and three additional signals at δ5.17 (0.3 H, singlet), δ5.07 (1 H, $J_{1,2}$ 2 Hz), and δ4.64 (0.9 H, $J_{1,2}$ 7 Hz). The ^{13}C-n.m.r. spectrum exhibits six peaks in the anomeric region at 103.07, 101.33, 97.21, 95.33, 95.08, and 93.09 p.p.m., and one signal in the region associated with -CH_2OH groups at 61.92 p.p.m. The spectrum presents the same interesting feature observed in the ^{13}C-n.m.r. spectrum of the tetraouronic acid isolated from K53 (see Section III.3, p 43);

i.e. splitting of the anomeric signal of the mannopyranosyl residue linked to C3 of the reducing galactose. Chemical shifts at 95.33 and 95.08 p.p.m. have been assigned to this mannose residue. As reported for some 1 → 2 linked disaccharides[73], such a splitting could possibly be due to the passage from the equatorial to axial position of the OH linked to C1 of the reducing galactose.

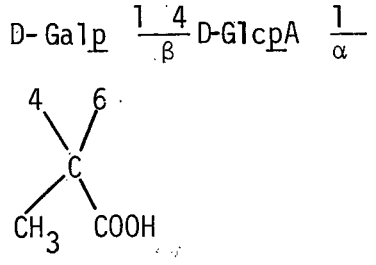
The tetrasaccharide B3 was shown not to be a mixture of oligosaccharides by the method of Morrison.[72] The sample was reduced with sodium borohydride, hydrolysed, and converted to the corresponding alditol acetate and aldononitrile acetate. G.l.c. analysis of the mixture gave mannononitrile pentaacetate and galactitol hexaacetate in the ratio of 2:1. Glucononitrile acetate did not appear in the g.l.c. tracing because the glucuronic acid was not reduced before conversion to the aldononitriles. These results confirmed that galactose is the reducing sugar in the tetrasaccharide. Knowing the structure of the aldobiouronic acid B1, it is also possible to conclude that the two mannose residues are linked together.

Methylation of B3, [55,56] followed by reduction, hydrolysis, and subsequent g.l.c. analysis of the derived alditol acetates gave 3,4,6-tri-O-methylmannose, 2,4,6-tri-O-methylmannose, 2,4,6-tri-O-methylgalactose, and 2,3,4-tri-O-methylglucose in a molar ratio of 1:1.5:1:1; results confirmed by g.l.c. - m.s.[60,62] Compound B3 is therefore established as

D-GlcpA $\frac{1\ 3}{\alpha}$ D-Manp $\frac{1\ 2}{\alpha}$ D-Manp $\frac{1\ 3}{\alpha}$ D-Galp B3

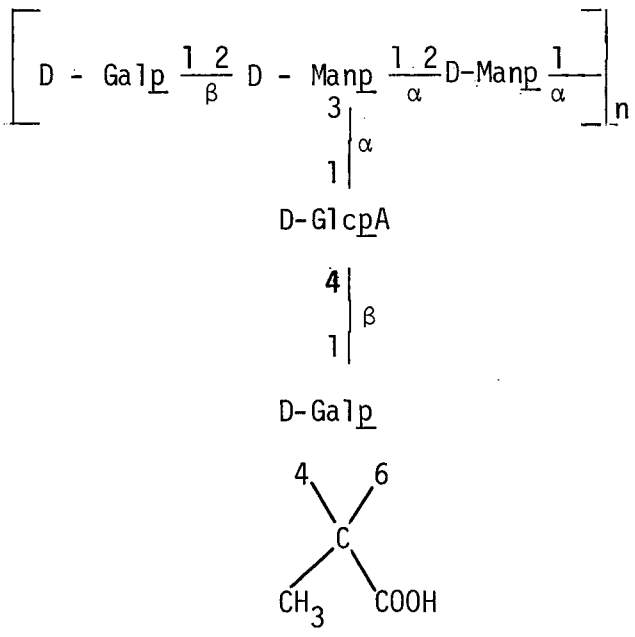
Uronic Acid Degradation

In a single operation, the permethylated polysaccharide was subjected to a base-catalysed uronic acid degradation, using dimethylsulfinyl anion, and directly alkylated with methyl iodide.[68,74] Hydrolysis of the remethylated, degraded material and analysis of the products by g.l.c. -m.s.[60,62] of the alditol acetates gave the results listed in Table IV.2, column V. The presence of some 4,6-di-O-methylmannose is due to incomplete methylation of the degraded polymer. Loss of glucuronic acid residues was accompanied by further degradation of exposed, reducing groups as witnessed by the complete disappearance of 2,3-di-O-methylgalactose. These results indicate that the side-chain of K74 polysaccharide is a pseudo-aldobiouronic acid linked to C3 of the branching mannose and whose structure is established as



From the structure of the tetrasaccharide B3, it has been established that the glucuronic acid is linked to a neutral trisaccharide in the following sequence: GlcA $\frac{1\ 3}{}$ Man $\frac{1\ 2}{}$ Man $\frac{1\ 3}{}$ Gal. Since the glucuronic acid is part of the side-chain, this neutral trimer constitutes the backbone of the polymer. Now connecting the side-chain to the backbone, the structure of the capsular polysaccharide

from Klebsiella serotype K74 is established as



Immunology

The capsular antigen from Klebsiella K74 cross-reacts weakly in antipneumococcal sera Pn II, PnVIII, and PnXX[19]. However, no useful information can be obtained from these reactions since there is no common structural feature between K74 and the pneumococcal capsular polysaccharides PnS II, PnS VIII, and PnS XX.

IV. 4 Experimental

General Methods

Instrumentation used has been described previously (see Section III.4, page 50). For descending paper chromatography, the following solvent systems (v/v) were used: (A) ethyl acetate - acetic acid - formic acid - water (18:3:1:4); (B) ethyl acetate - pyridine - water (8:2:1); (C) freshly prepared 1-butanol-acetic acid - water (2:1:1). Analytical g.l.c. separations were performed using the following stainless steel columns (1.8 m x 3 mm): (A) 3% of SP - 2340 on Supelcoport (100-130 mesh); (B) 5% of ECNSS - M on Gas Chrom Q (100 - 120 mesh); (C) 3% of OV - 225 on the same support. Preparative g.l.c. separations were performed using a column (D) (1.8 m x 6.3 mm) of 5% Silar 10C on Gas Chrom Q (100 - 120 mesh).

The ^{13}C-n.m.r. spectrum of the native polysaccharide was obtained by courtesy of Dr. Michel Vignon, CERMAV/CNRS, Grenoble, France on a Bruker Spectrospin instrument.

Isolation and Purification of Klebsiella K74 Capsular Polysaccharide

A culture of *Klebsiella* serotype K74 (371), obtained by courtesy of Dr. Ida Ørskov, was grown as described previously for *Klebsiella* K53 (see Section III.4, page 52). The isolated polysaccharide (10 g), in the sodium salt form, had $[\alpha]_D$ + 66° (*c* 0.21, water). Purity of the polysaccharide was checked by electrophoresis using a 1% solution on cellulose acetate strips (Sepraphore III; 15 x 2.5 cm) in Veronal buffer pH 8.6 (LKB - Produkter AB, Stockholm 12, Sweden)

at 300 V for 60 min and then developed in alcian blue in citrate buffered ethanol. Homogeneity was confirmed by gel chromatography by courtesy of Dr. S.C. Churms, University of Cape Town, South Africa, and the molecular weight of K74 polysaccharide determined to be 4.7×10^6.

Analysis of Sugar Constituents

Sugar analysis was performed as described in Section III.4, page 53. The alditol acetates of mannose, galactose, and glucose were identified by g.l.c. (column A; programmed at 195°C for 4 min and then 2°/min to 260°C) by comparison with authentic samples and found to be present in the approximate molar ratio of 2:2:1. Paper chromatography (solvents A and B) run on an hydrolysate of the polysaccharide confirmed the presence of mannose, galactose, glucuronic acid, and pyruvic acid.

The D or L configuration of the constituent sugars was determined by measurement of the circular dichroism curve of the alditol acetates of mannose and glucose, and of the partially methylated alditol acetates of galactose. Samples were isolated by preparative g.l.c. (column D; 240°C isothermal). Comparison with authentic standards confirmed the D configuration of all the sugar constituents.

Methylation Analysis

A dried sample (127 mg) of K 74 polysaccharide was dissolved in 100 mL anhydrous dimethyl sulfoxide using ultrasonic agitation and methylated by treatment with 12 mL dimethyl sulfinyl anion overnight, and then 8 mL methyl iodide for 1½ h. The reaction mixture

was dialyzed against running tap-water for three days and freeze-dried. The product was dissolved in chloroform, filtered, and subsequent Purdie treatment[78] of the soluble fraction (153 mg) with methyl iodide and silver oxide gave a product that showed no hydroxyl absorption in the infrared spectrum.

The permethylated sample was reduced with lithium aluminum hydride in refluxing tetrahydrofuran for 5 hours, and the reaction was continued at room temperature overnight. Excess of hydride was destroyed with water and the product was recovered by the method of Dutton and Smith.[58] Hydrolysis of the reduced product with 2M trifluoroacetic acid at 95°C overnight, reduction with sodium borohydride, and g.l.c. - m.s.[60,62] analysis of the partially methylated alditol acetates gave the results listed in Table IV.2, column I.

Partial Hydrolysis

About 540 mg of K74 polysaccharide was partially hydrolysed with 2M trifluoroacetic acid at 95°C for 2.5 h. The solution was concentrated to dryness under reduced pressure and evaporated several times with water to eliminate excess of acid. The product was applied to a column (30 x 1.5 cm) of Bio - Rad AG 1 - X2 resin in the formate form. The neutral fraction was eluted with 600 mL of water and freeze - dried, yield 254 mg. The acidic fraction was eluted with 500 mL of 10% formic acid, evaporated to dryness under reduced pressure several times with water, and freeze - dried, yield 293 mg. Paper chromatography (solvent C) run on the

acidic fraction showed that it contained a disaccharide and higher oligosaccharides.

The acidic oligomers were separated by gel filtration chromatography on a Bio - Gel P-2 column (100 x 3 cm) using a buffer composed of water: pyridine: acetic acid (500:5:2) for irrigation at a flow rate of 10 mL/h. Fractions of 2.0 - 2.5 mL were collected in tared tubes, freeze - dried, and weighed. The chromatogram produced by the weight of the fractions collected is shown in Figure IV.2. Fractions 41-47 were pooled as well as fractions 35-40 and 29-34, and purified by descending paper chromatography for three days using solvent C. Three pure oligosaccharides were thus collected, B1, B2, and B3.

Methylation analysis of these oligosaccharides was performed as described for the oligomers isolated from K53 (see Section III.4, page 55). Results of the analyses are shown in Table IV.2, columns II-IV.

The purity of the tetrasaccharide B3 was checked by g.l.c. (column C; 210°C isothermal) according to the method of Morrison.[72]

Uronic Acid Degradation[68,74]

A dried sample (15 mg) of methylated K74 polysaccharide and 1 mg of p - toluenesulfonic acid were dissolved in 10 mL of a 19:1 (v/v) mixture of dimethyl sulfoxide - 2,2-dimethoxypropane, and treated with 5 mL dimethylsulfinyl anion overnight. An excess of methyl iodide (7 mL) was added with external cooling, the mixture was stirred one hour, and dialyzed against running tap - water for

two days. The polymeric material was extracted with chloroform (5 x 10 mL) and the combined extracts were evaporated to dryness under reduced pressure. Hydrolysis of the product with 2M trifluoroacetic acid, reduction with sodium borohydride, acetylation with a 1:1 (v/v) mixture of acetic anhydride : pyridine overnight at room temperature, and g.l.c. analysis of the alditol acetates gave the results listed in Table IV.2, column V.

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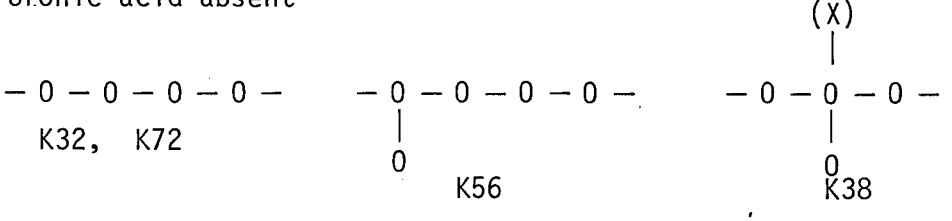
Appendix I

Structural Patterns of Klebsiella Capsular Polysaccharides

Key : X = Uronic Acid O = Neutral sugar; pyruvate and acetate omitted

A. Uronic acid absent



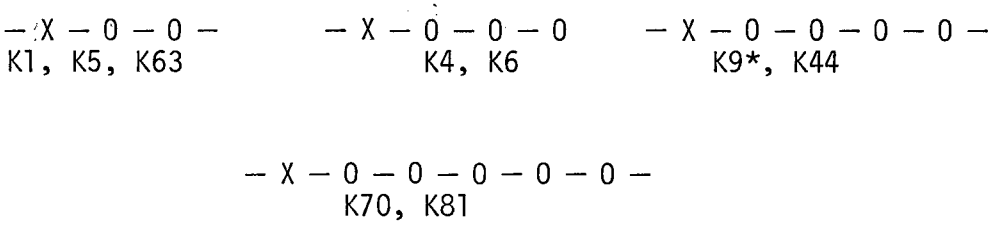
(X) = 3-deoxy-L-glycero-pentulosonic acid

[X] = 4-O [(s)-1-carboxyethyl]-D-glucuronic acid

⟨X⟩ = 2R,3R-hex-4-enopyranosyluronic acid

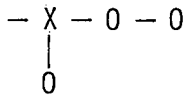
B. Uronic acid in chain

a) linear

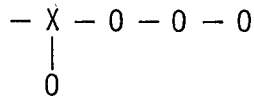


b) branch point on uronic acid

i) single unit side chain

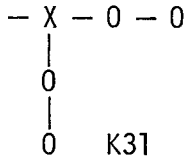


K11, K51, K57



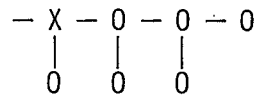
K21, K24

ii) two unit side chain



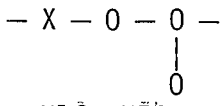
K31

iii) plus branch points on neutral sugars

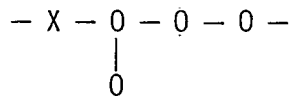


K60

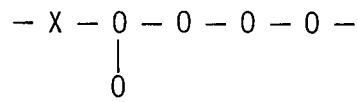
c) branch not on uronic acid



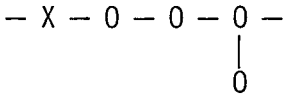
K16, K54



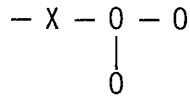
K7, K61, K62



K52, K53

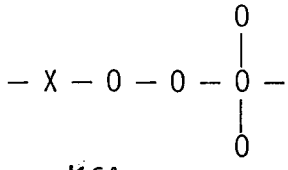


K17



K58

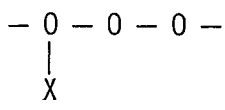
d) double branch not on uronic acid



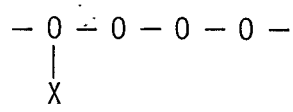
K64

C. Uronic acid in side chain

a) single unit side chain

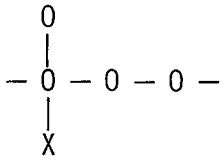


K2, K8, K51

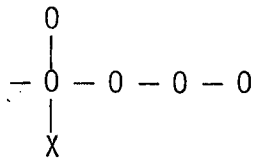


K9, K59

b) two single unit side chains forming a double branch



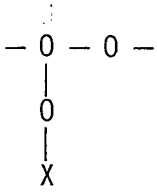
K30, K33



K27

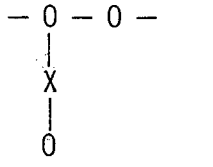
c) two unit side chain

i) uronic acid terminal

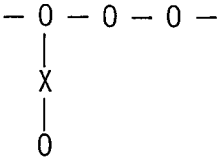


K20, K23, K55, K83

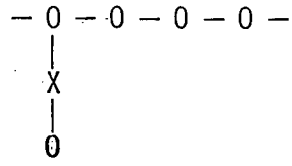
ii) uronic acid non-terminal



K25, K47



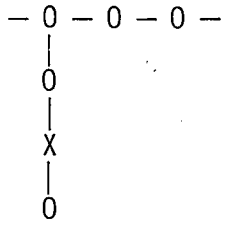
K13, K74



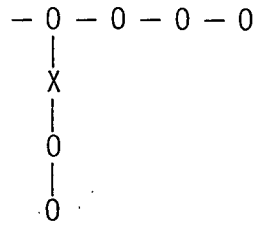
K12, K28, K36

D. Three unit side chain

i) uronic acid non-terminal



K18



K41

Note: K9 and K9* are supposed to be from the same strain of Klebsiella. This strain has been investigated in two laboratories but two different structures have been proposed.

APPENDIX II KLEBSIELLA CAPSULAR POLYSACCHARIDES (K1 - K83) GROUPED ACCORDING TO CHEMOTYPE

Glucuronic Acid, Galactose, Glucose	8^P, 11^P, 15, 51, 25, 27^P
Glucuronic Acid, Galactose, Mannose	20, 21^P, 29^P, 42^P, 43, 66, 74^P
Glucuronic Acid, Galactose, Rhamnose	9, 47, 52, 9*, 81, 83
Glucuronic Acid, Glucose, Mannose	2, 4, 5^P, 24
Glucuronic Acid, Glucose, Rhamnose	17, 44, 71
Glucuronic Acid, Glucose, Fucose	1, 54
Glucuronic Acid, Galactose, Glucose, Mannose	10, 28, 39, 50, 59, 61, 62, 7^P, 13^P, 26^P, 30^P, 31^P, 33^P, 35^P, 46^P, 69^P, 60
Glucuronic Acid, Galactose, Glucose, Fucose	16, 58^P
Glucuronic Acid, Galactose, Glucose, Rhamnose	18, 19, 23, 41, 79, 12^P, 36^P, 45^P, 55^P, 70^P
Glucuronic Acid, Galactose, Mannose, Rhamnose	53, 40, 80^P
Glucuronic Acid, Glucose, Mannose, Fucose	6^P
Glucuronic Acid, Glucose, Mannose, Rhamnose	64^P, 65^P
Glucuronic Acid, Galactose, Glucose, Mannose, Fucose	68^P
Glucuronic Acid, Galactose, Glucose, Mannose, Rhamnose	14^P, 67
Galacturonic Acid, Galactose, Mannose	3^P, 49, 57
Galacturonic Acid, Glucose, Rhamnose	34, 48
Galacturonic Acid, Galactose, Fucose, Rhamnose	63
Pyruvic Acid, Glucose, Rhamnose	72
Pyruvic Acid, Galactose, Rhamnose	32
Pyruvic Acid, Galactose, Glucose, Rhamnose	56
Keto Acid, Galactose, Glucose	22, 37, 38

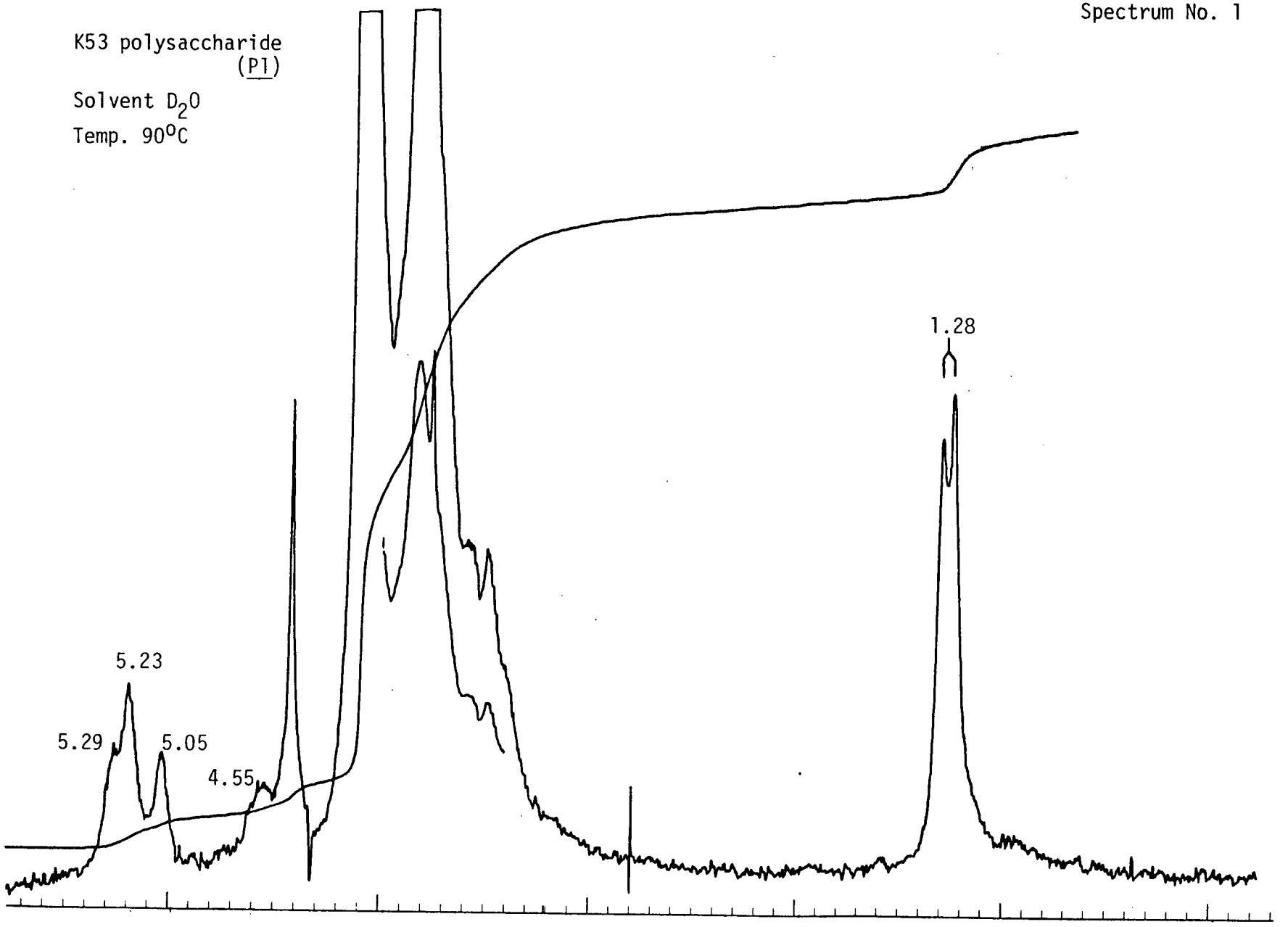
K82 has been added but its qualitative composition is not yet known.[12]

^{P} Pyruvic acid present in addition

Note: K9 and K9*, see Appendix I, p.87.

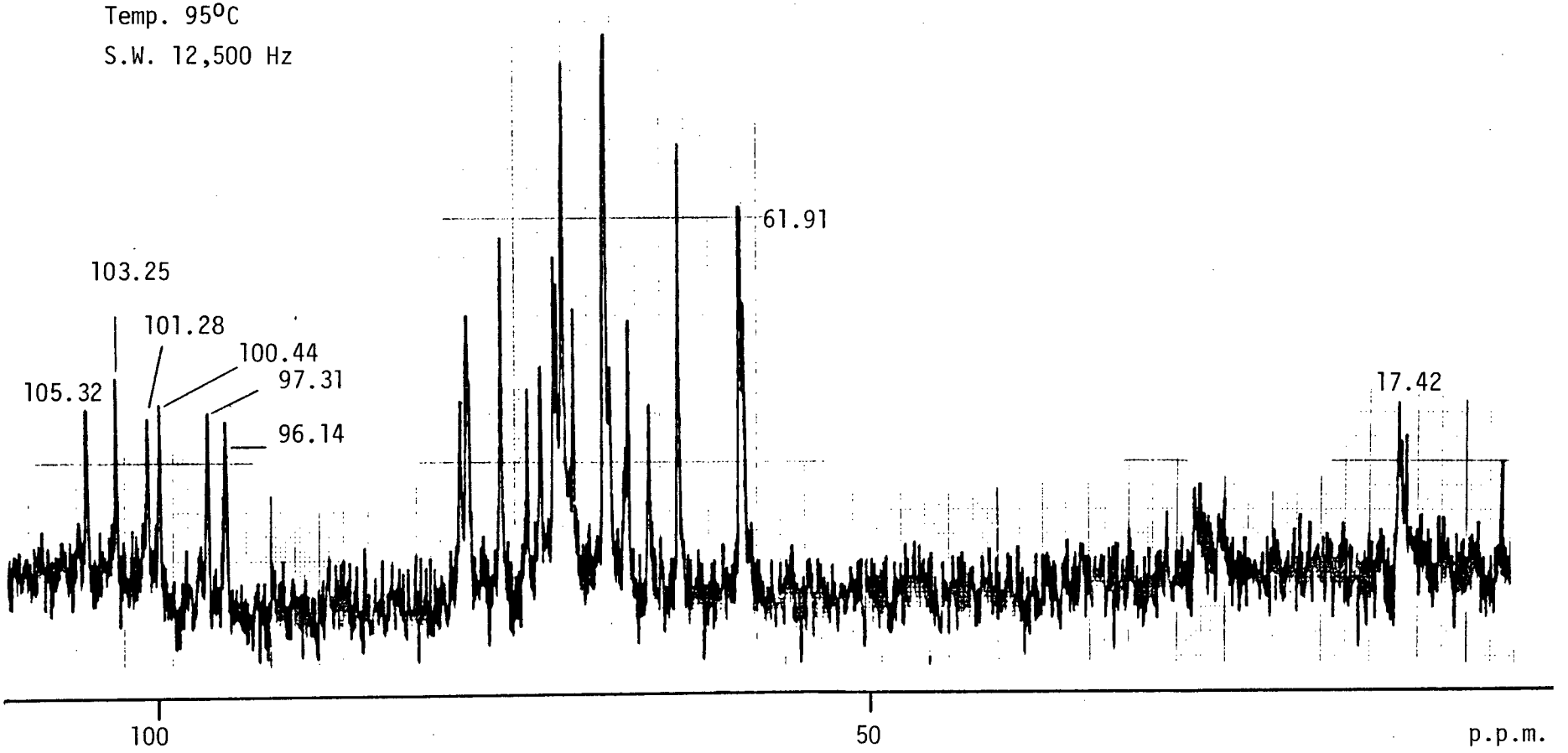
Appendix III

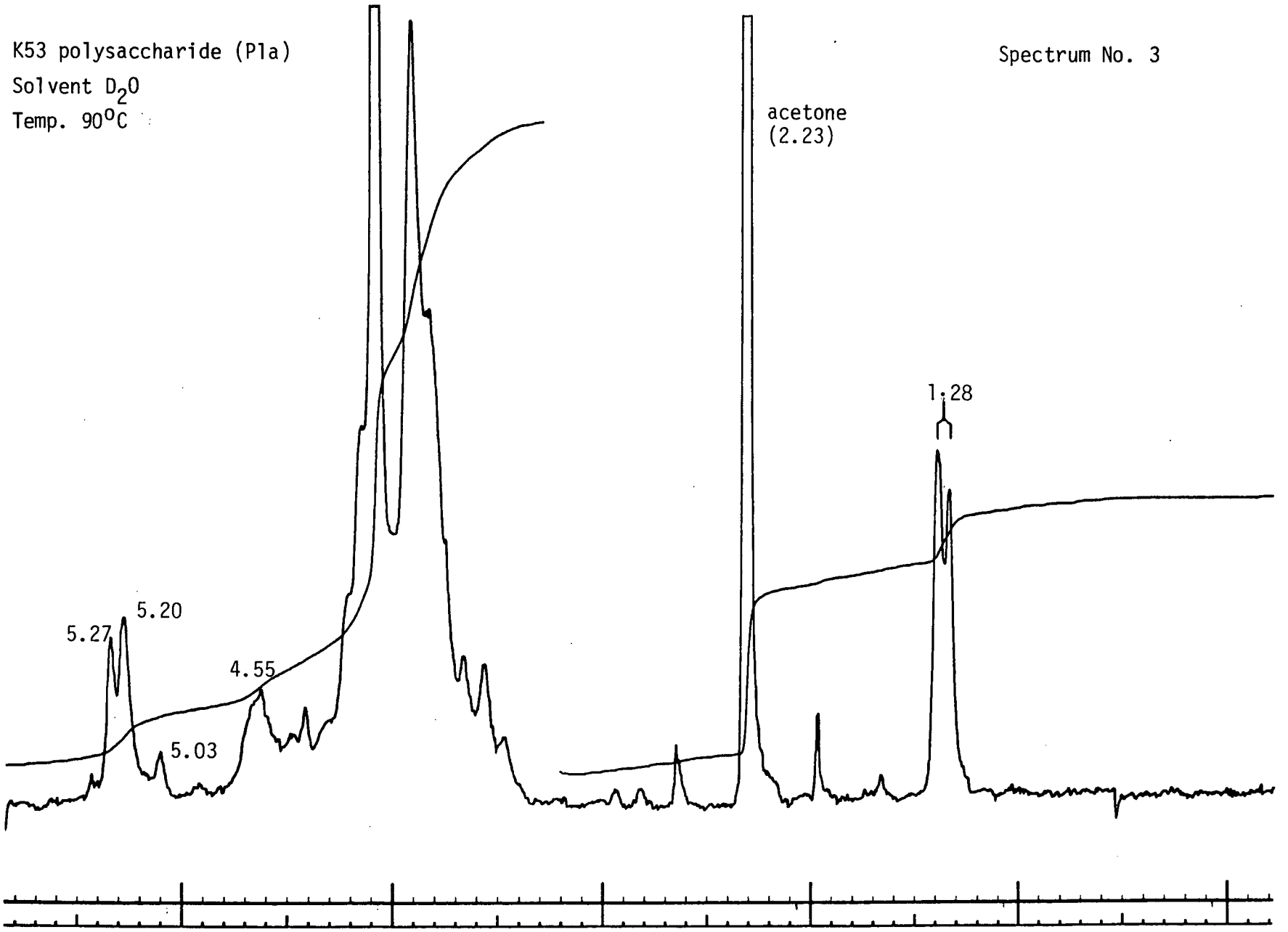
N.m.r. Spectra


Spectrum No. 1
K53 polysaccharide
(P1)
Solvent D_2O
Temp. 90°C
1.28
5.23
5.29
5.05
4.55

K53 polysaccharide (P1)
Solvent D_2O
Temp. 95°C
S.W. 12,500 Hz

Spectrum No. 2




K53 polysaccharide (Pla)
Solvent D_2O
Temp. 90°C
Spectrum No. 3
acetone
(2.23)
1.28
5.27
5.20
4.55
5.03

K53 polysaccharide (P1a)

Solvent D_2O

S.W. 8000 Hz

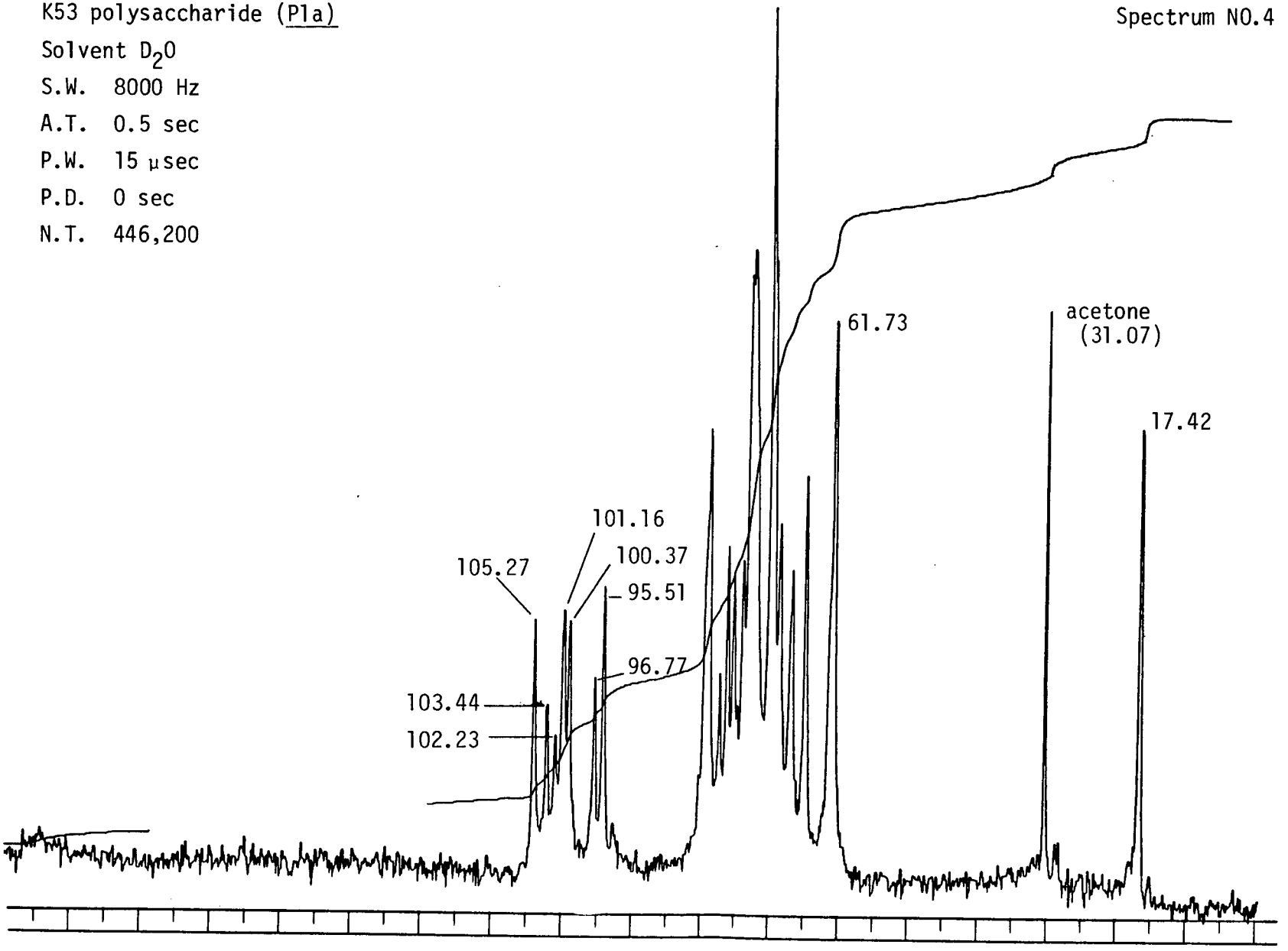
A.T. 0.5 sec

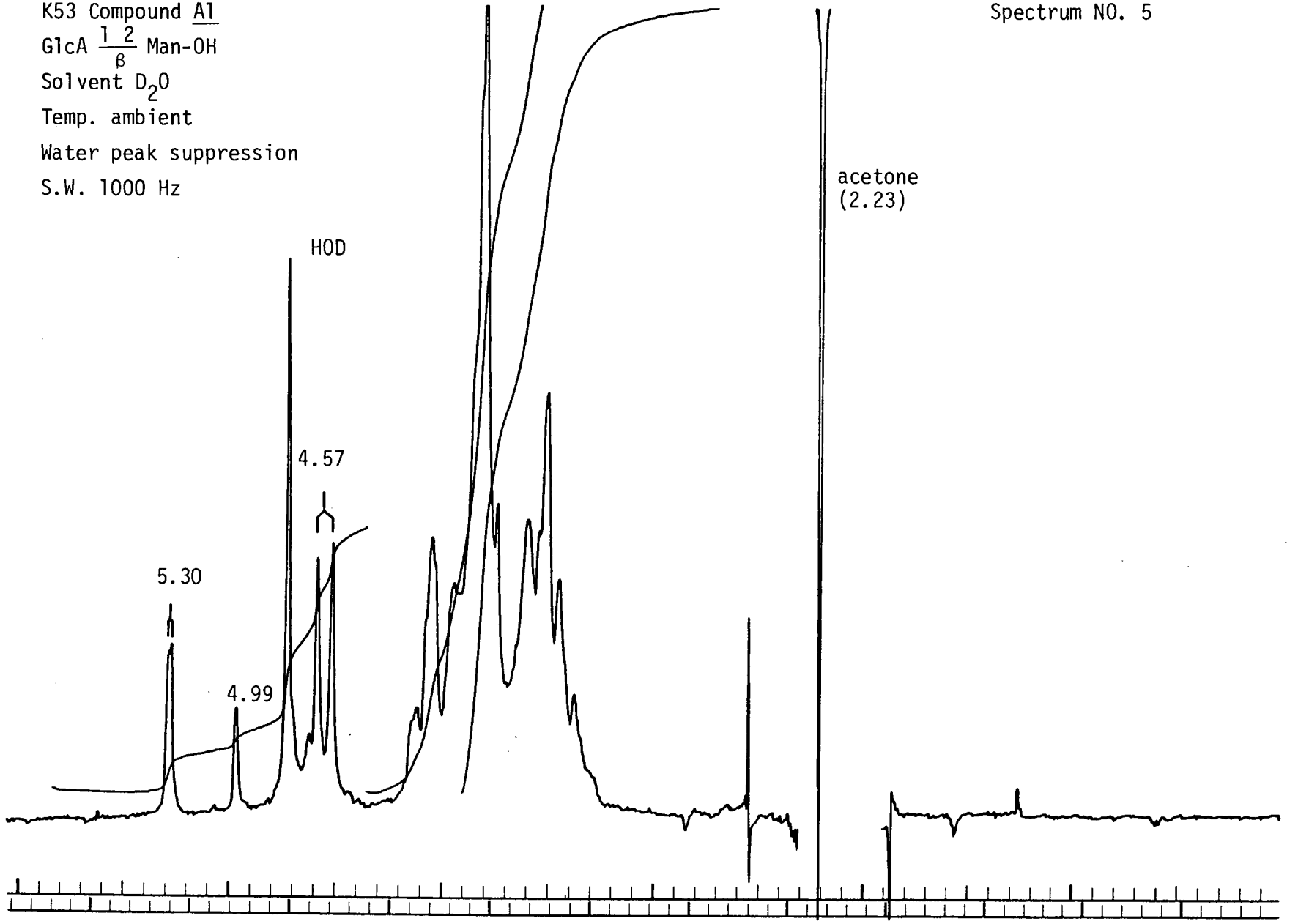
P.W. 15 μsec

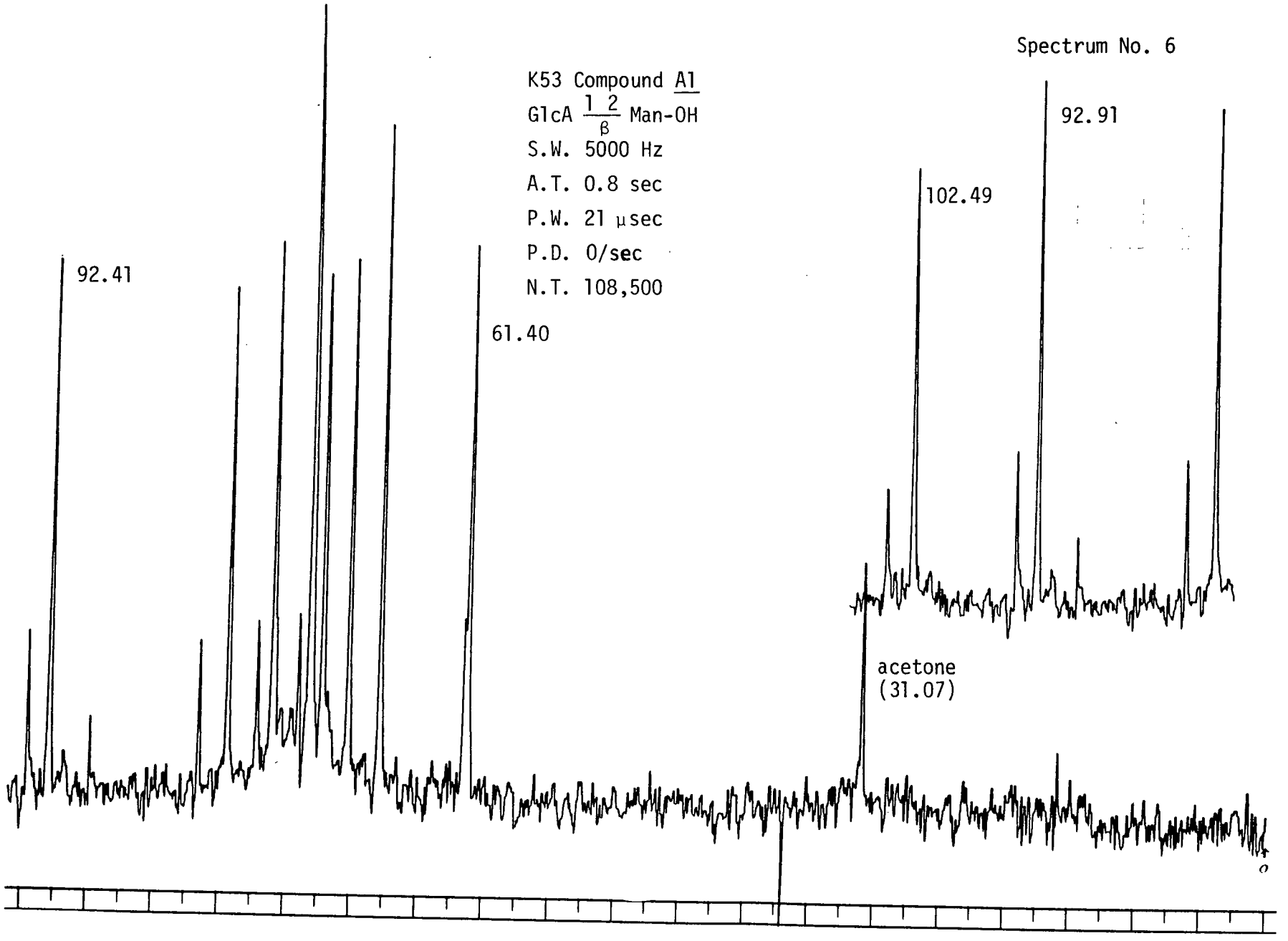
P.D. 0 sec

N.T. 446,200

Spectrum NO.4




K53 Compound A1
GlcA 1 2 β Man-OH
Solvent D_2O
Temp. ambient
Water peak suppression
S.W. 1000 Hz
Spectrum NO. 5
HOD
4.57
5.30
4.99
acetone
(2.23)


Spectrum No. 6
K53 Compound A1
GlcA 1 2 β Man-OH
S.W. 5000 Hz
A.T. 0.8 sec
P.W. 21 μsec
P.D. 0/sec
N.T. 108,500
92.41
61.40
102.49
92.91
acetone
(31.07)

K53 Compound A2

GlcA $\frac{1\ 2}{\beta}$ Man $\frac{1\ 2}{\alpha}$ Man-OH

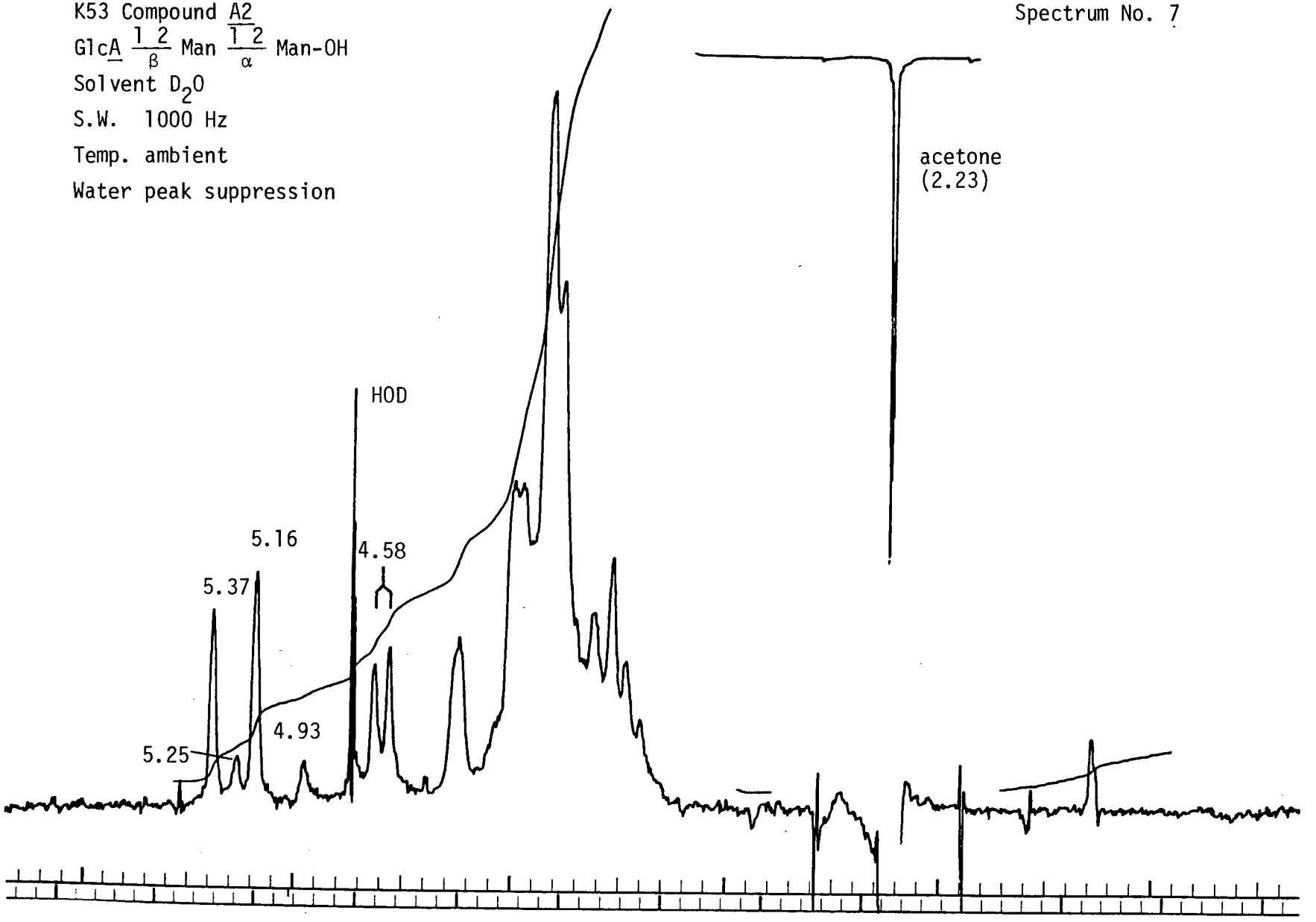
Solvent D_2O

S.W. 1000 Hz

Temp. ambient

Water peak suppression

Spectrum No. 7



K53 Compound A2

GlcA $\frac{1\ 2}{\beta}$ Man $\frac{1\ 2}{\alpha}$ Man-OH

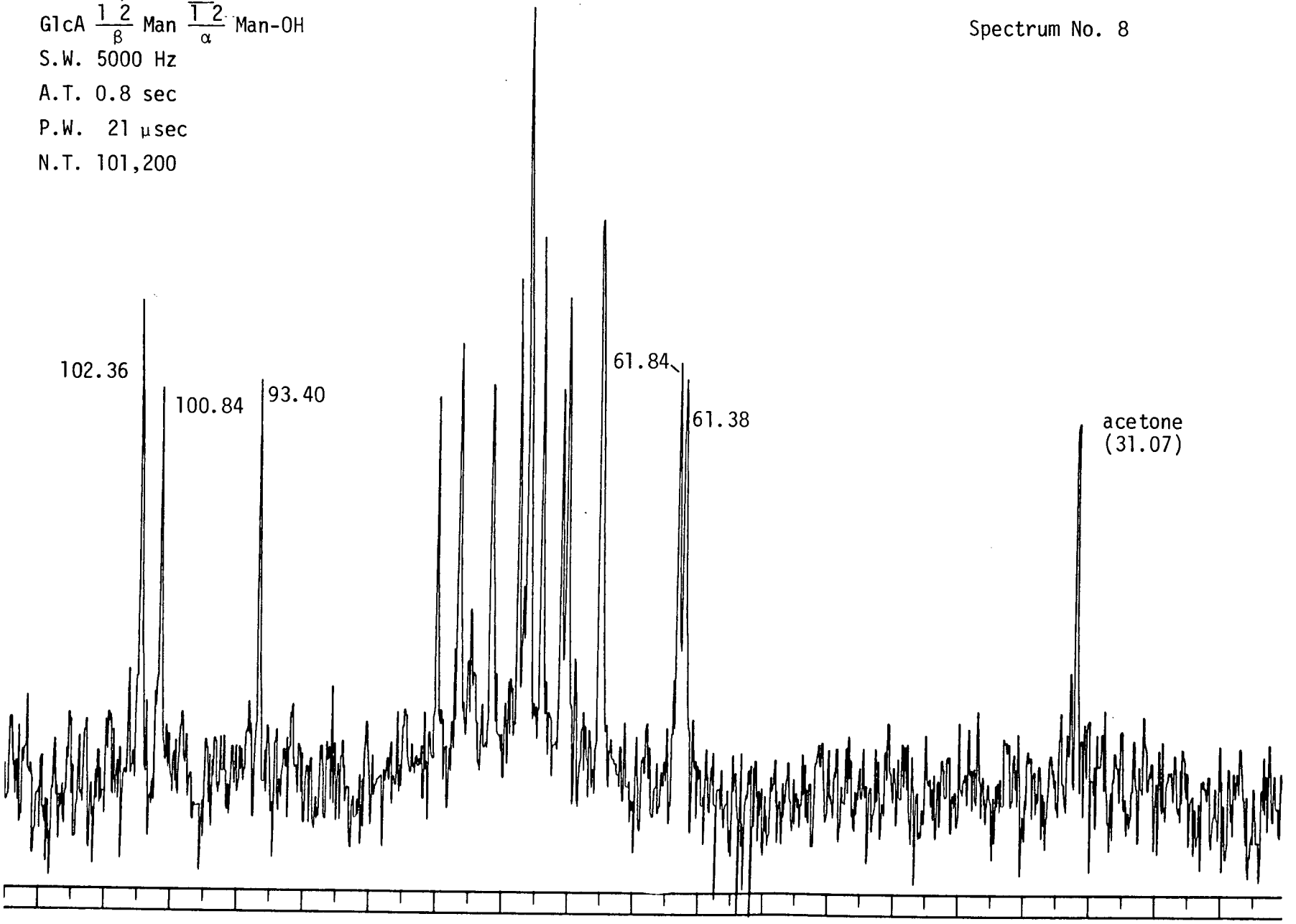
S.W. 5000 Hz

A.T. 0.8 sec

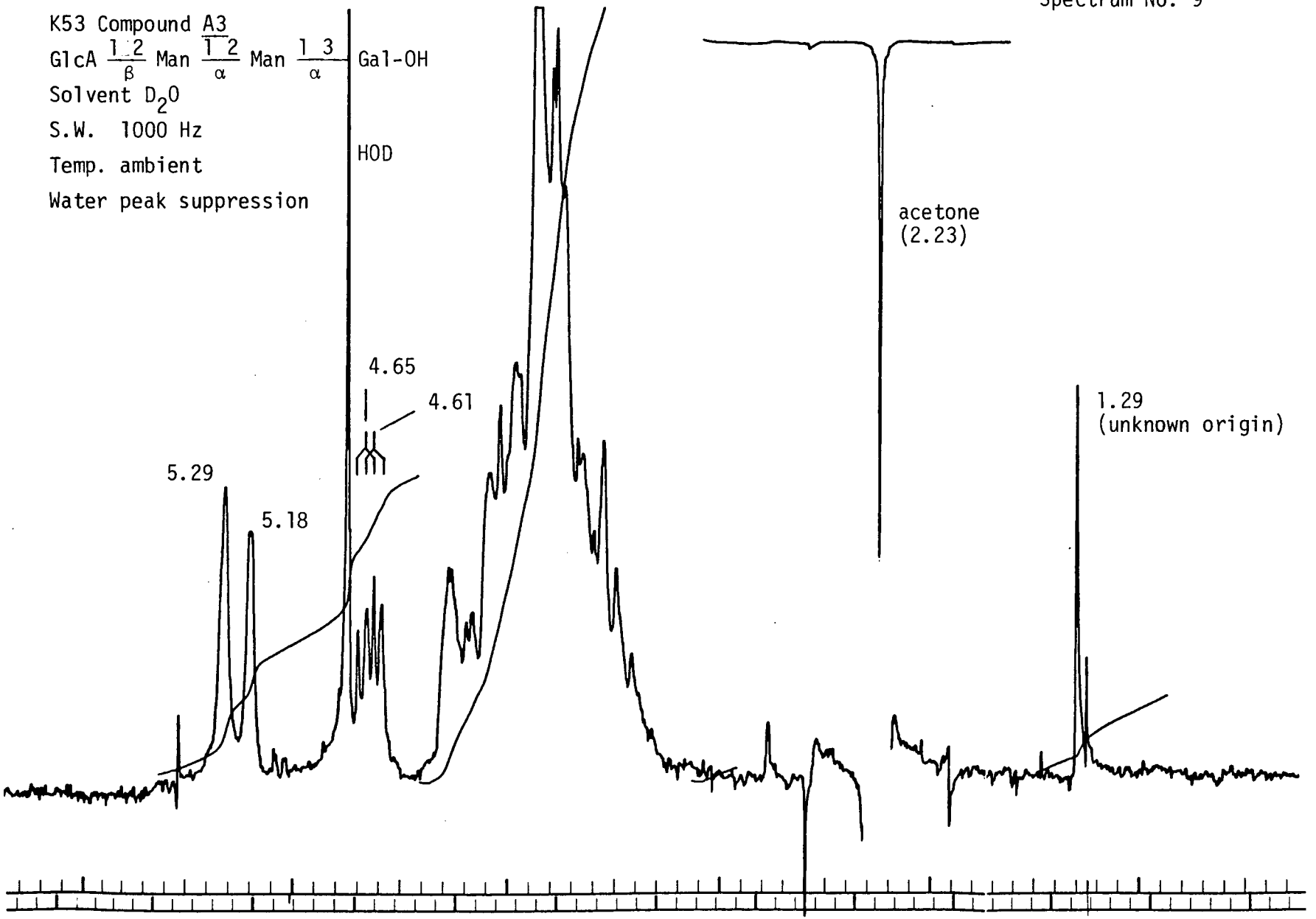
P.W. 21 μsec

N.T. 101,200

Spectrum No. 8



Spectrum No. 9



Spectrum No. 10

K53 Compound A3

GlcA $\frac{1\ 2}{\beta}$ Man $\frac{1\ 2}{\alpha}$ Man $\frac{1\ 3}{\alpha}$ Gal-OH

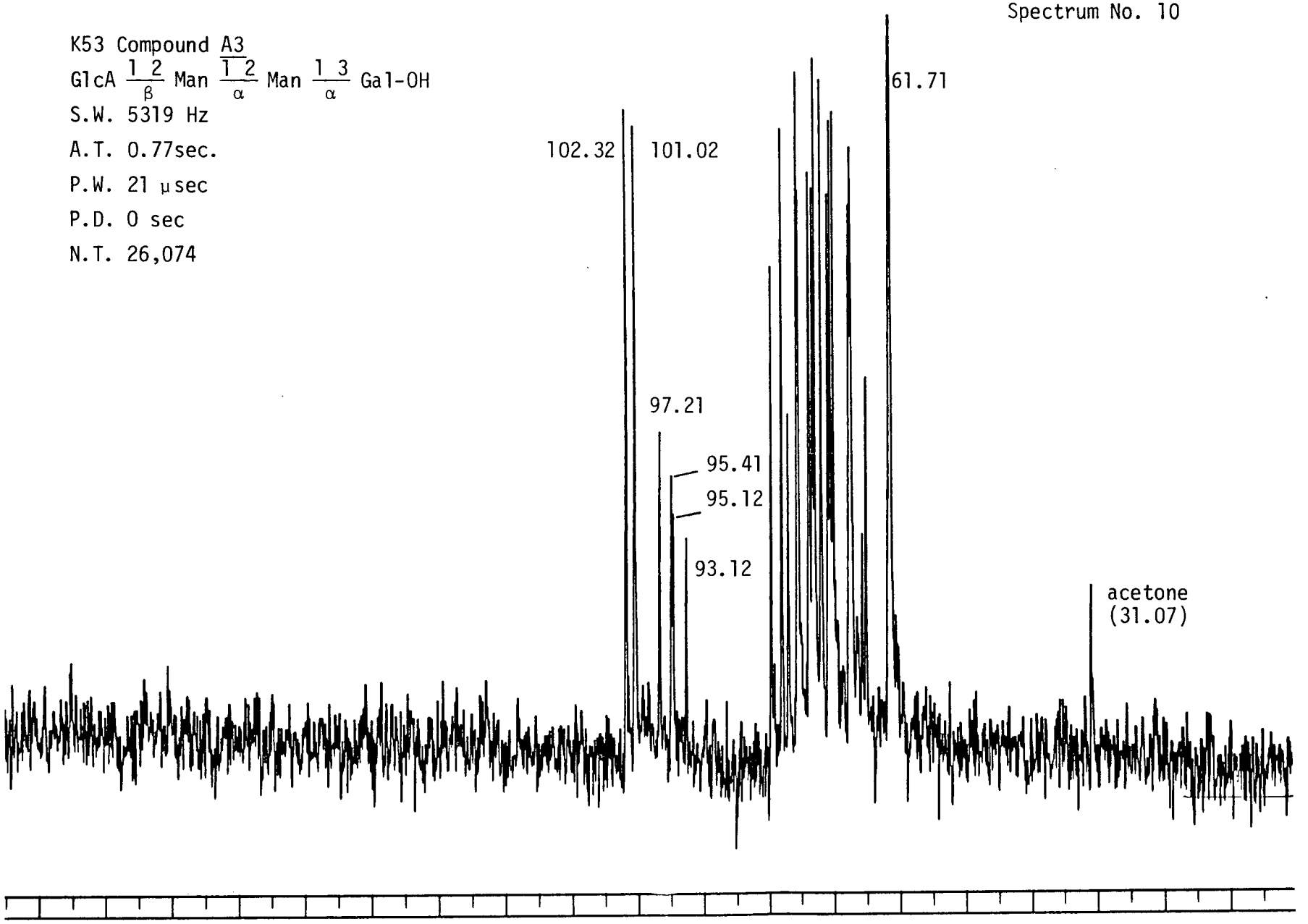
S.W. 5319 Hz

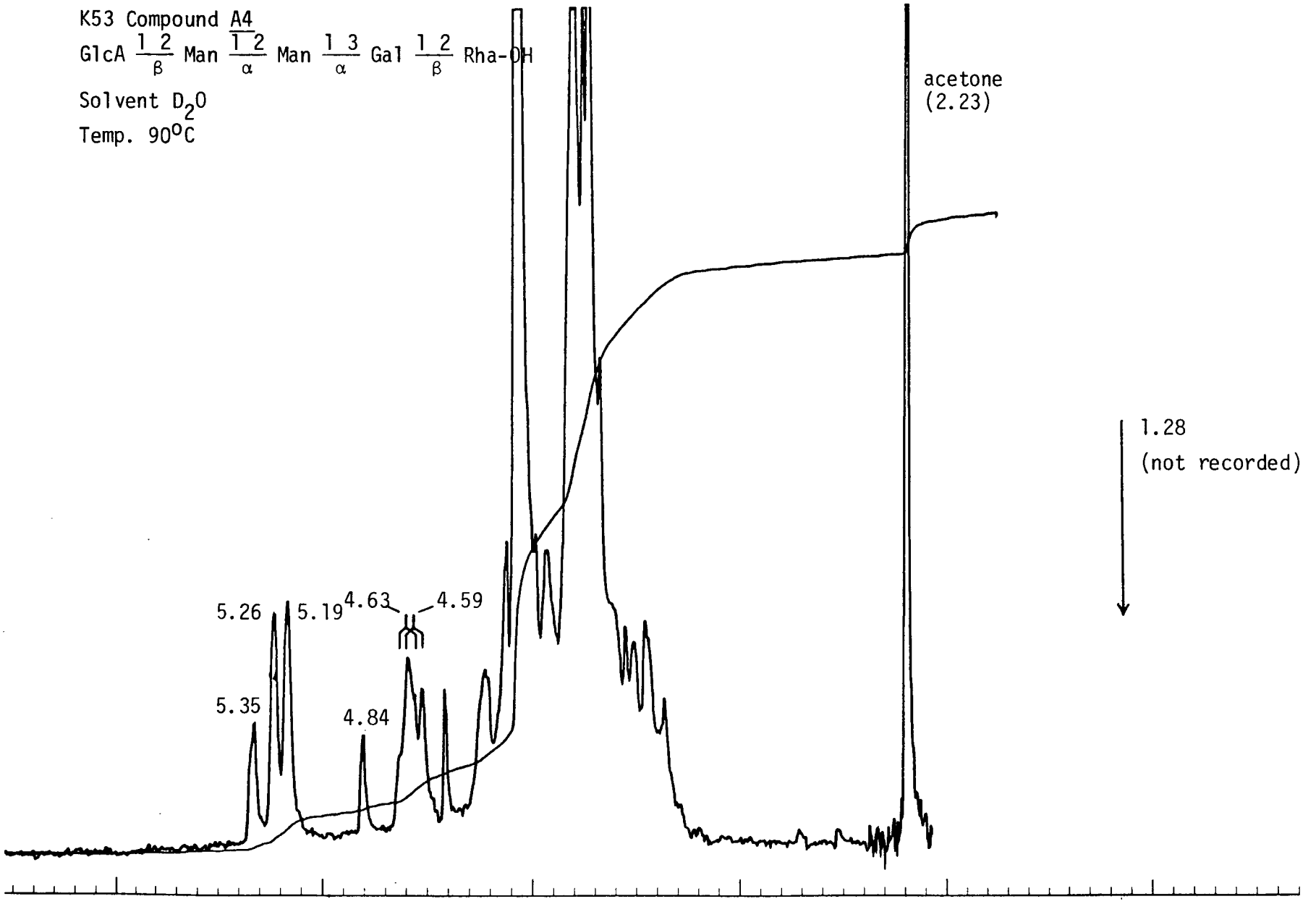
A.T. 0.77sec.

P.W. 21 μsec

P.D. 0 sec

N.T. 26,074




K53 Compound A4
GlcA 1 2 β Man 1 2 α Man 1 3 α Gal 1 2 β Rha-OH
Solvent D_2O
Temp. 90°C
acetone
(2.23)
1.28
(not recorded)
5.26
5.19
4.63
4.59
5.35
4.84

K53 Compound A4

GlcA $\frac{1\ 2}{\beta}$ Man $\frac{1\ 2}{\alpha}$ Man $\frac{1\ 3}{\alpha}$ Gal $\frac{1\ 2}{\beta}$ Rha-OH

S.W. 5000 Hz

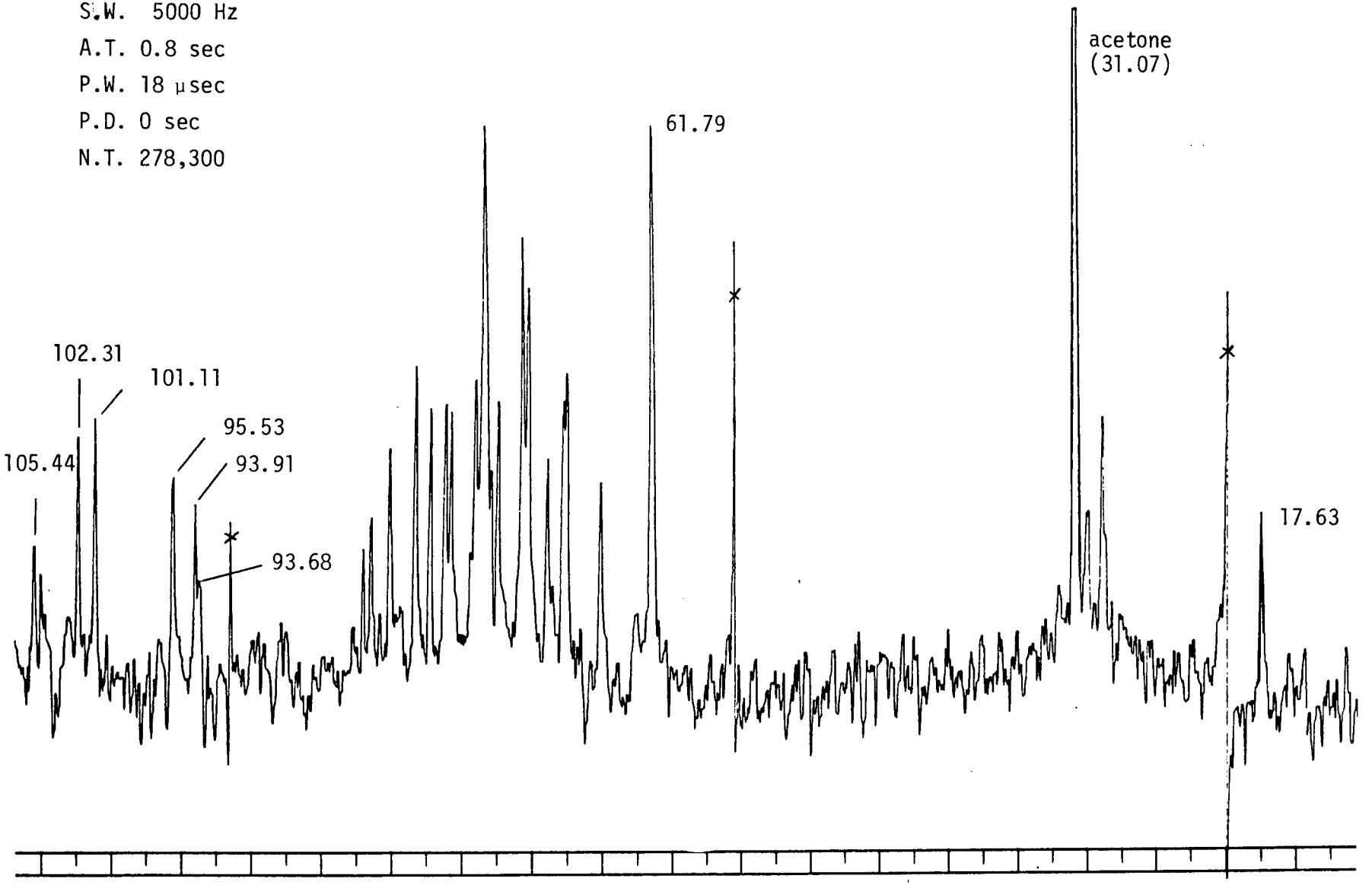
A.T. 0.8 sec

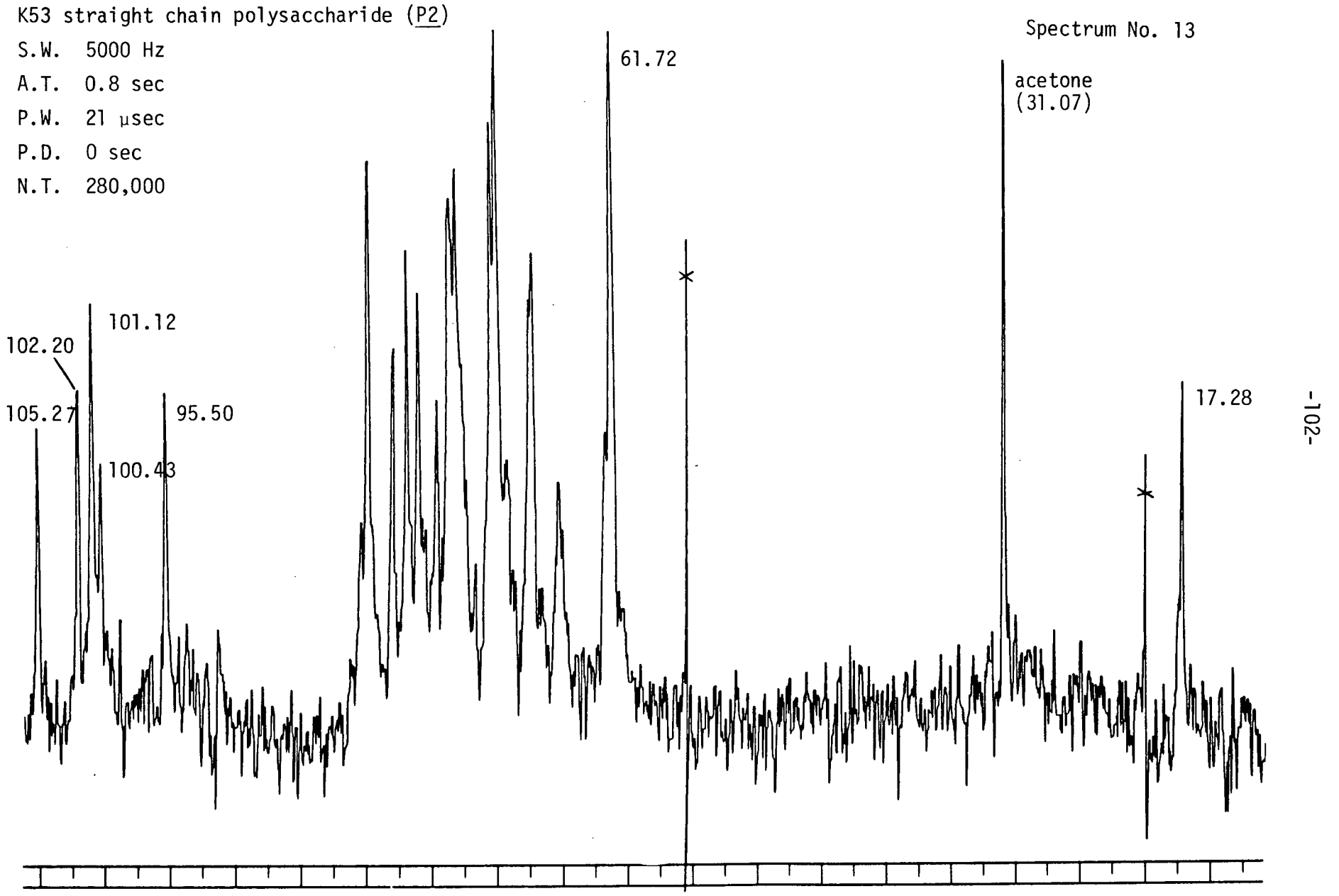
P.W. 18 μsec

P.D. 0 sec

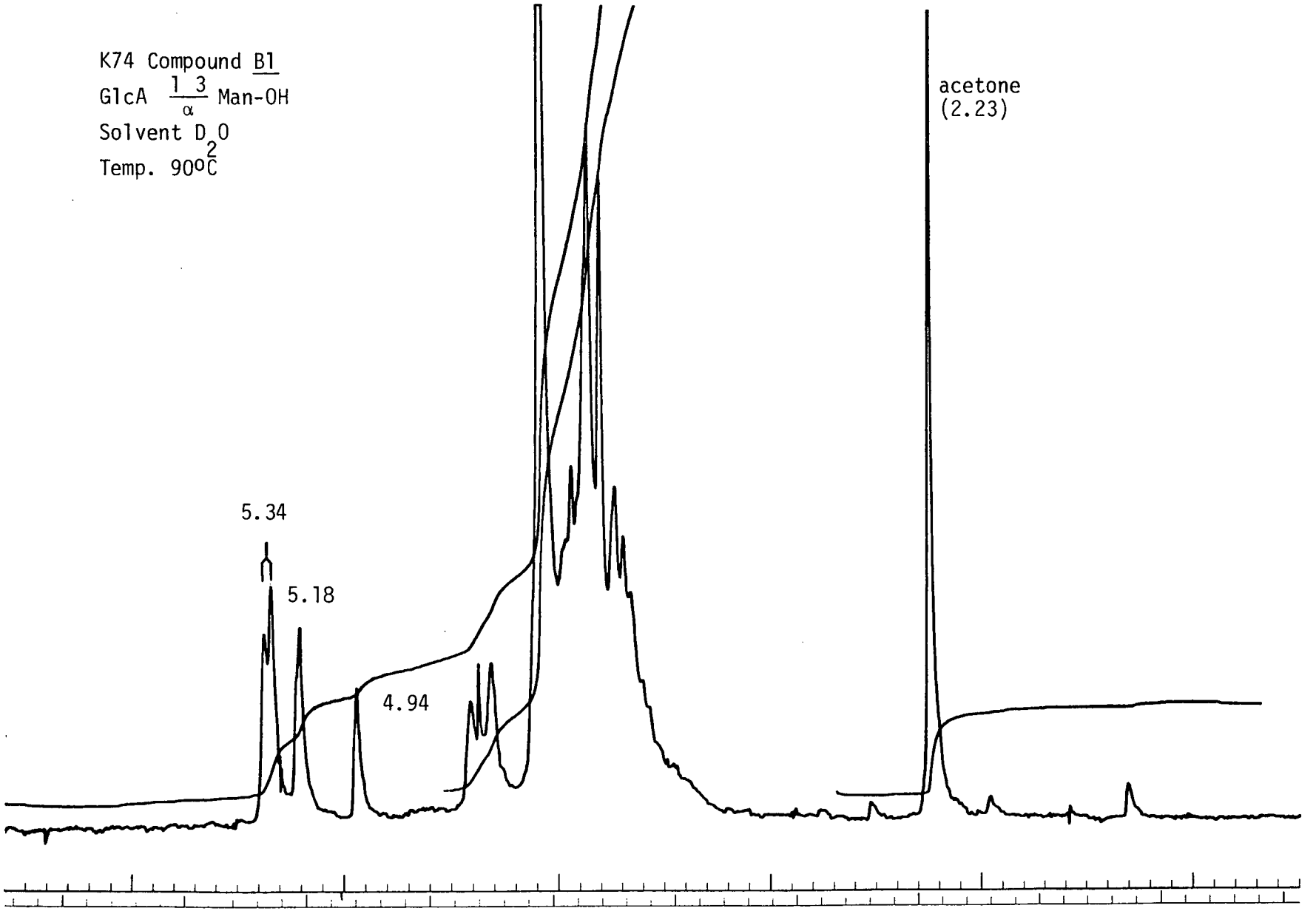
N.T. 278,300

Spectrum No. 12




K53 straight chain polysaccharide (P2)
S.W. 5000 Hz
A.T. 0.8 sec
P.W. 21 µsec
P.D. 0 sec
N.T. 280,000
Spectrum No. 13
61.72
acetone
(31.07)
102.20
101.12
105.27
95.50
100.43
17.28

Spectrum No. 14



K74 Compound B1

GlcA $\frac{1\ 3}{\alpha}$ Man-OH

S.W. 5000 Hz

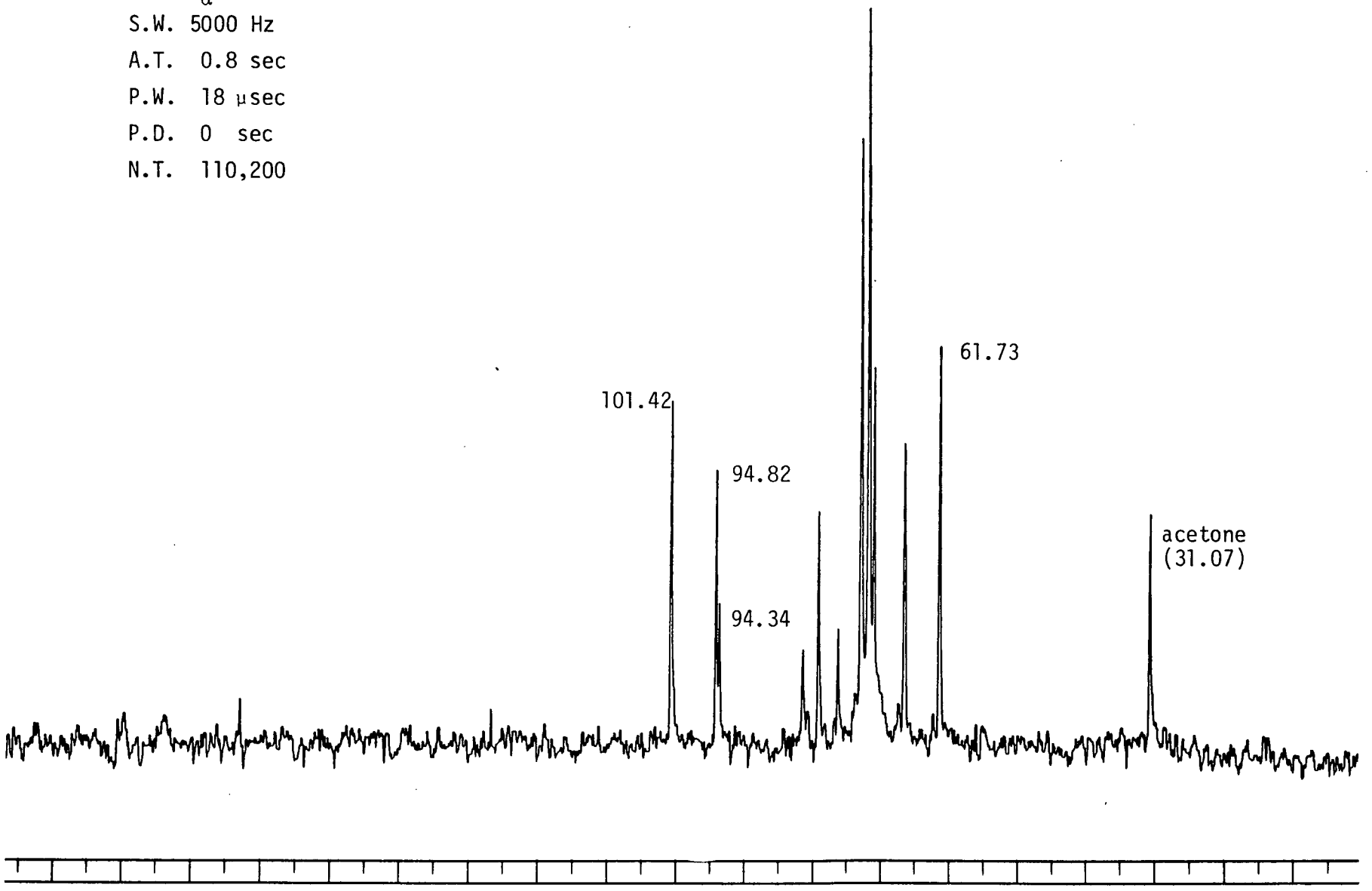
A.T. 0.8 sec

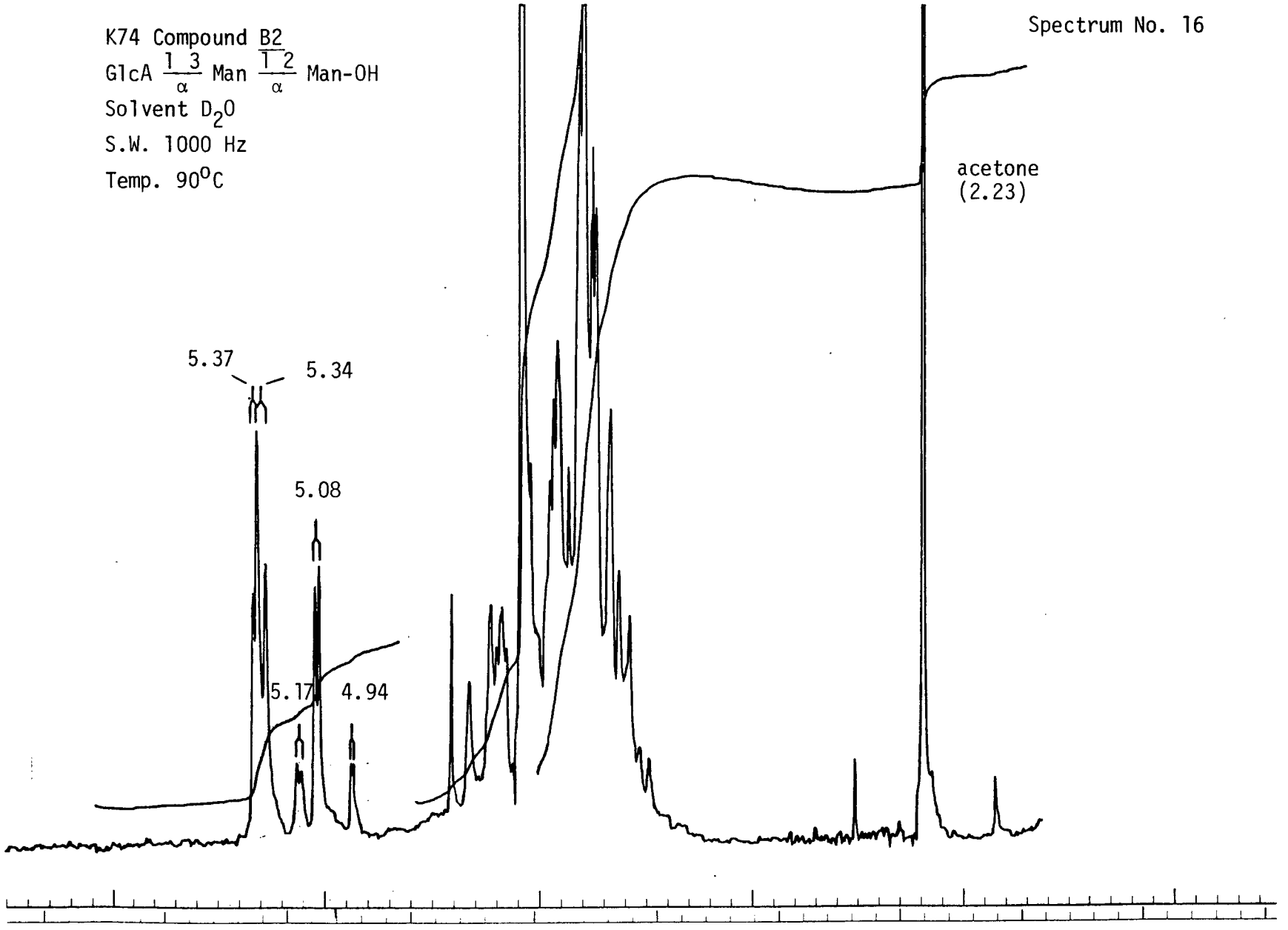
P.W. 18 μsec

P.D. 0 sec

N.T. 110,200

Spectrum No. 15




K74 Compound B2
GlcA 1 3 α Man 1 2 α Man-OH
Solvent D2O
S.W. 1000 Hz
Temp. 90°C
Spectrum No. 16
acetone
(2.23)
5.37
5.34
5.08
5.17
4.94

Spectrum No. 17

K74 Compound B2

GlcA $\frac{1\ 3}{\alpha}$ Man $\frac{1\ 2}{\alpha}$ Man-OH

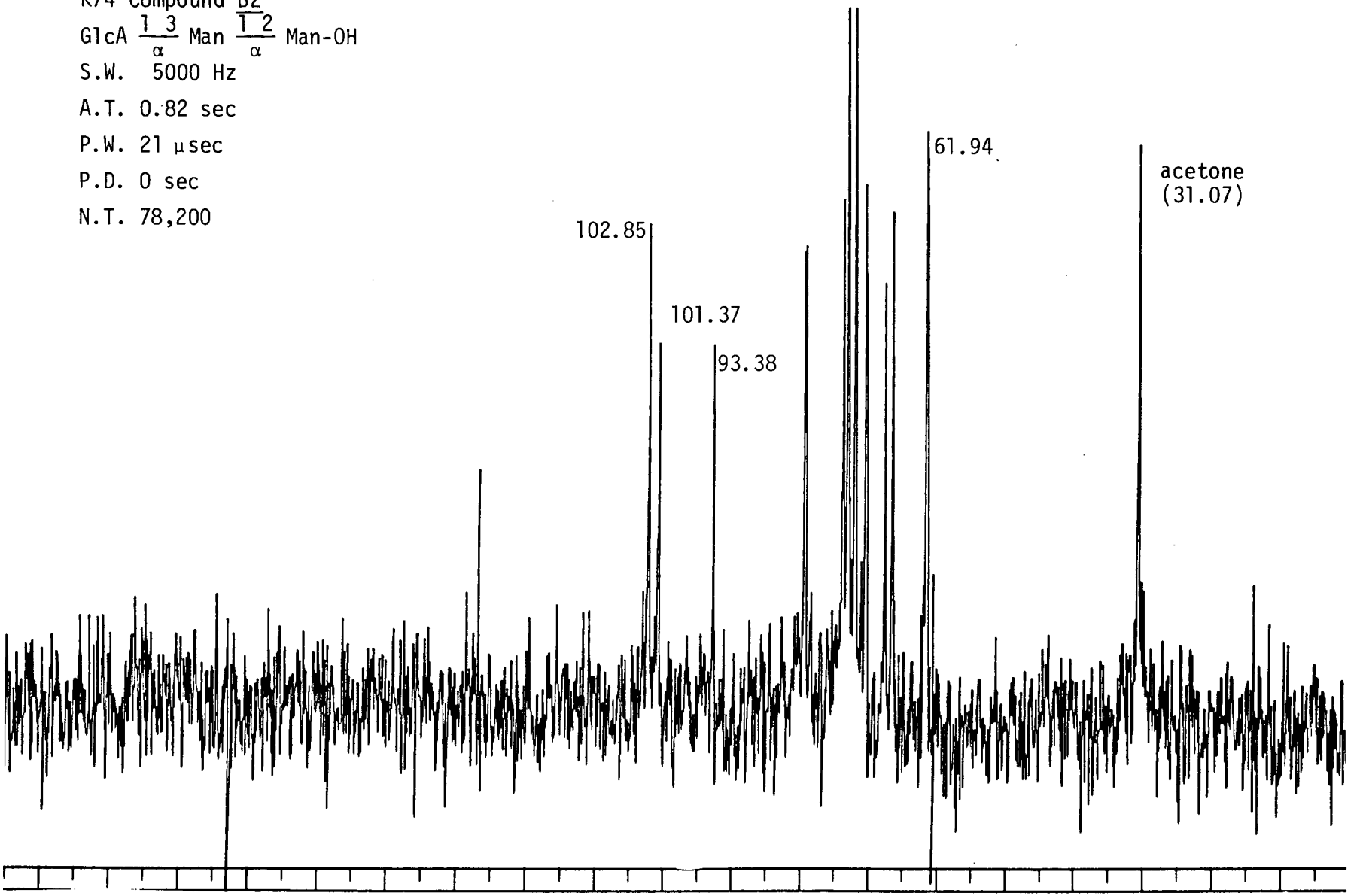
S.W. 5000 Hz

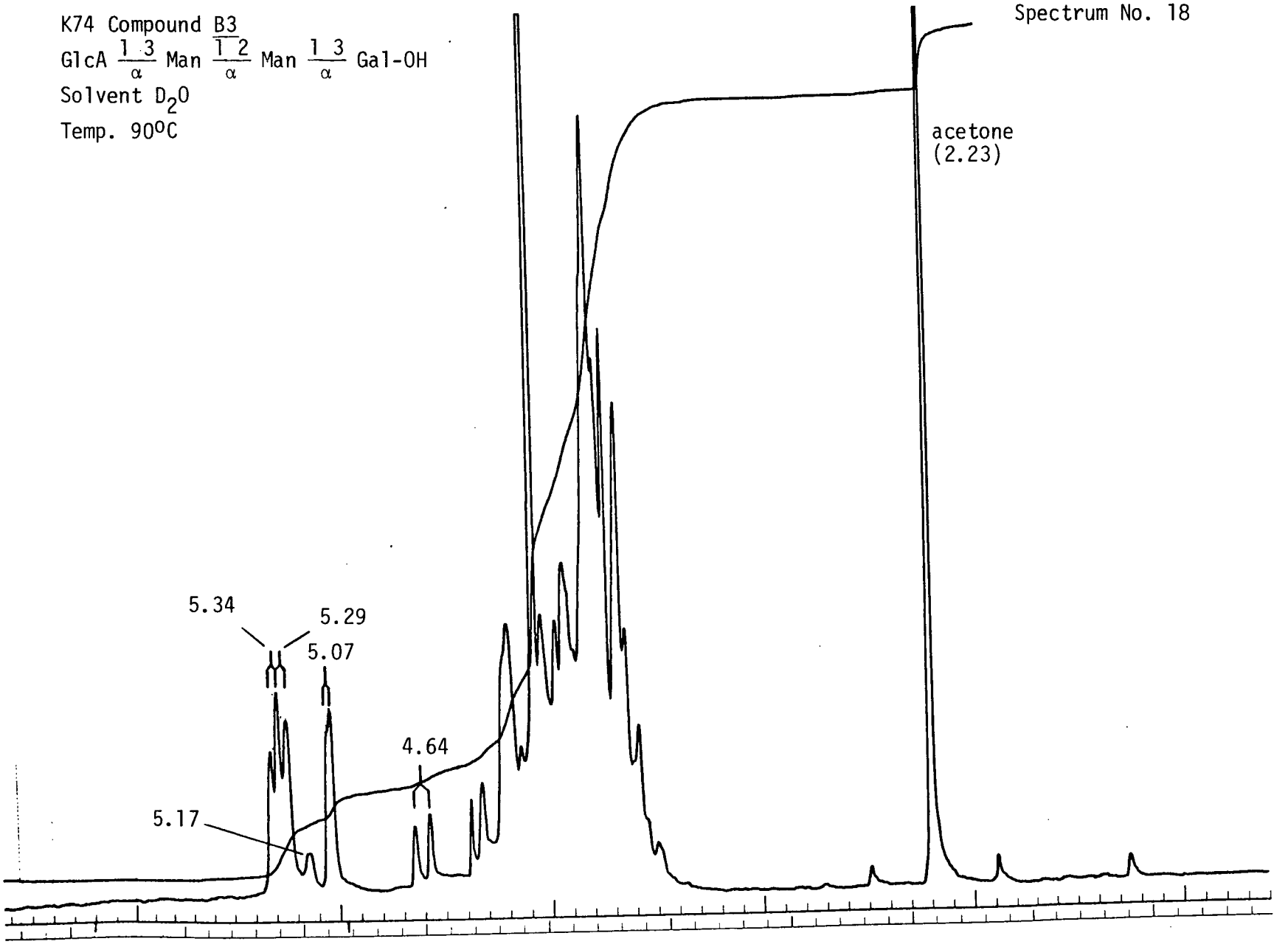
A.T. 0.82 sec

P.W. 21 μsec

P.D. 0 sec

N.T. 78,200




K74 Compound B3
GlcA $\frac{1\ 3}{\alpha}$ Man $\frac{1\ 2}{\alpha}$ Man $\frac{1\ 3}{\alpha}$ Gal-OH
Solvent D_2O
Temp. 90°C
Spectrum No. 18
acetone
(2.23)
5.34
5.29
5.07
4.64
5.17

Spectrum No. 19

K74 Compound B3

GlcA $\frac{1\ 3}{\alpha}$ Man $\frac{1\ 2}{\alpha}$ Man $\frac{1\ 3}{\alpha}$ Gal-OH

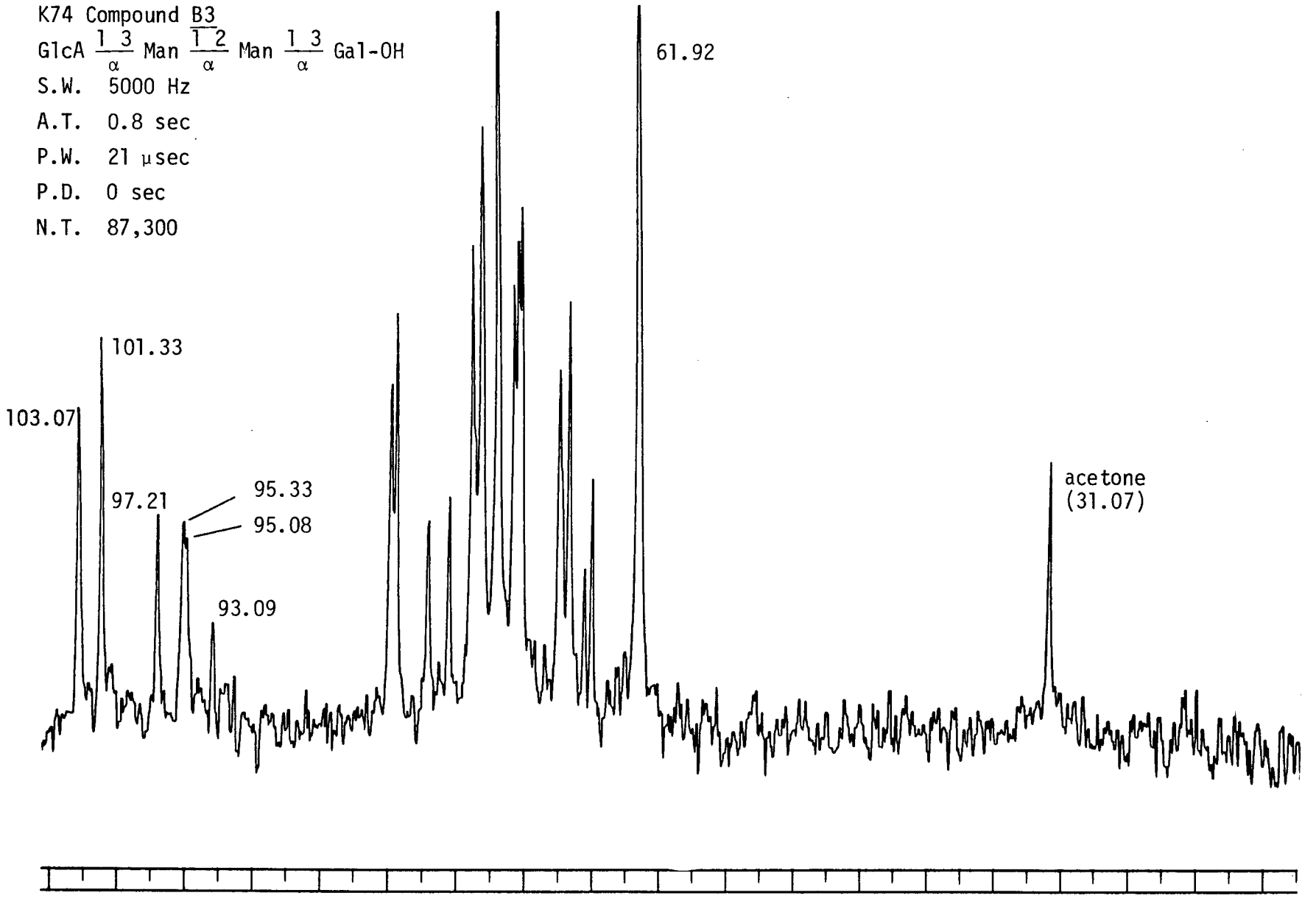
S.W. 5000 Hz

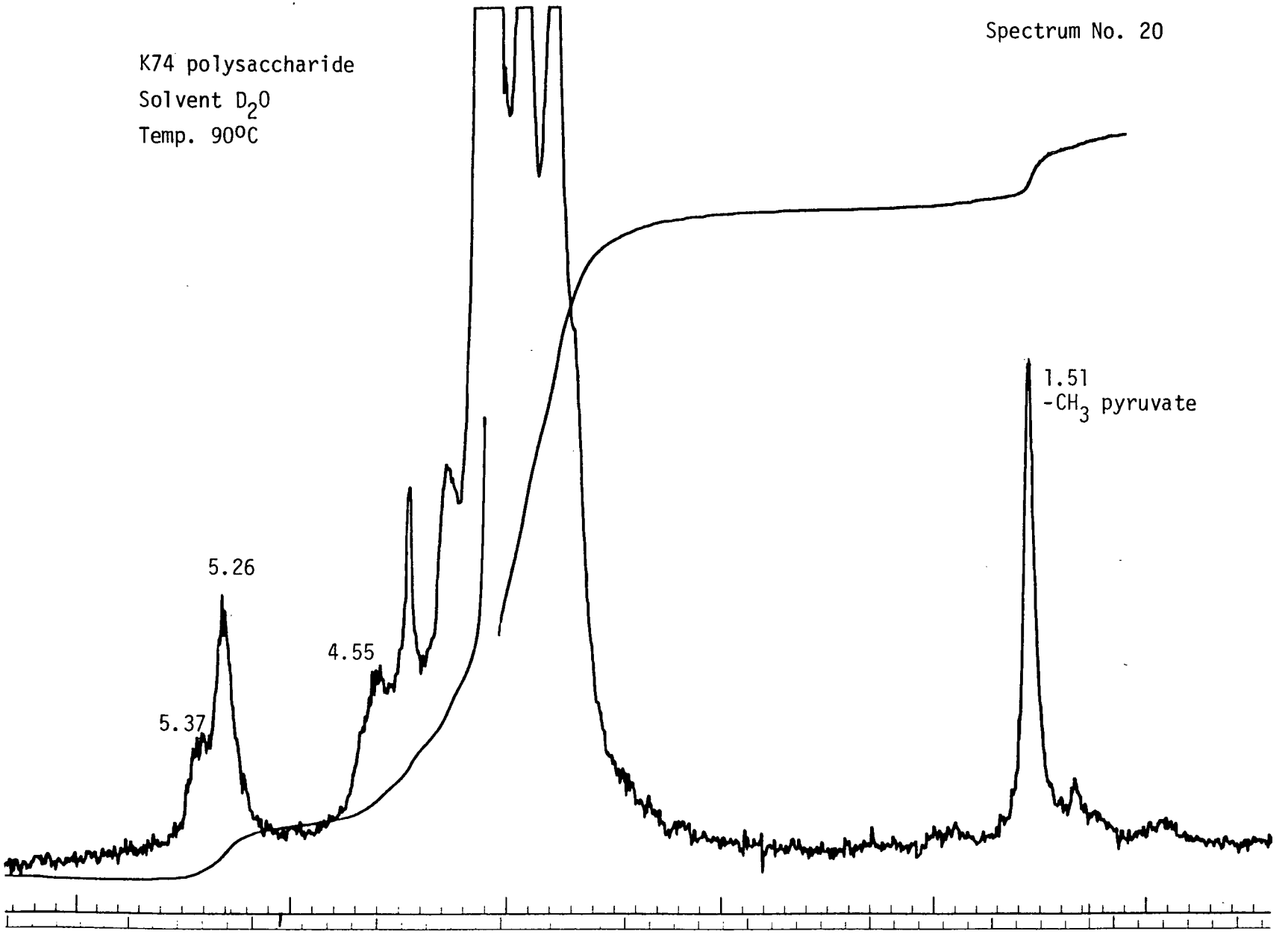
A.T. 0.8 sec

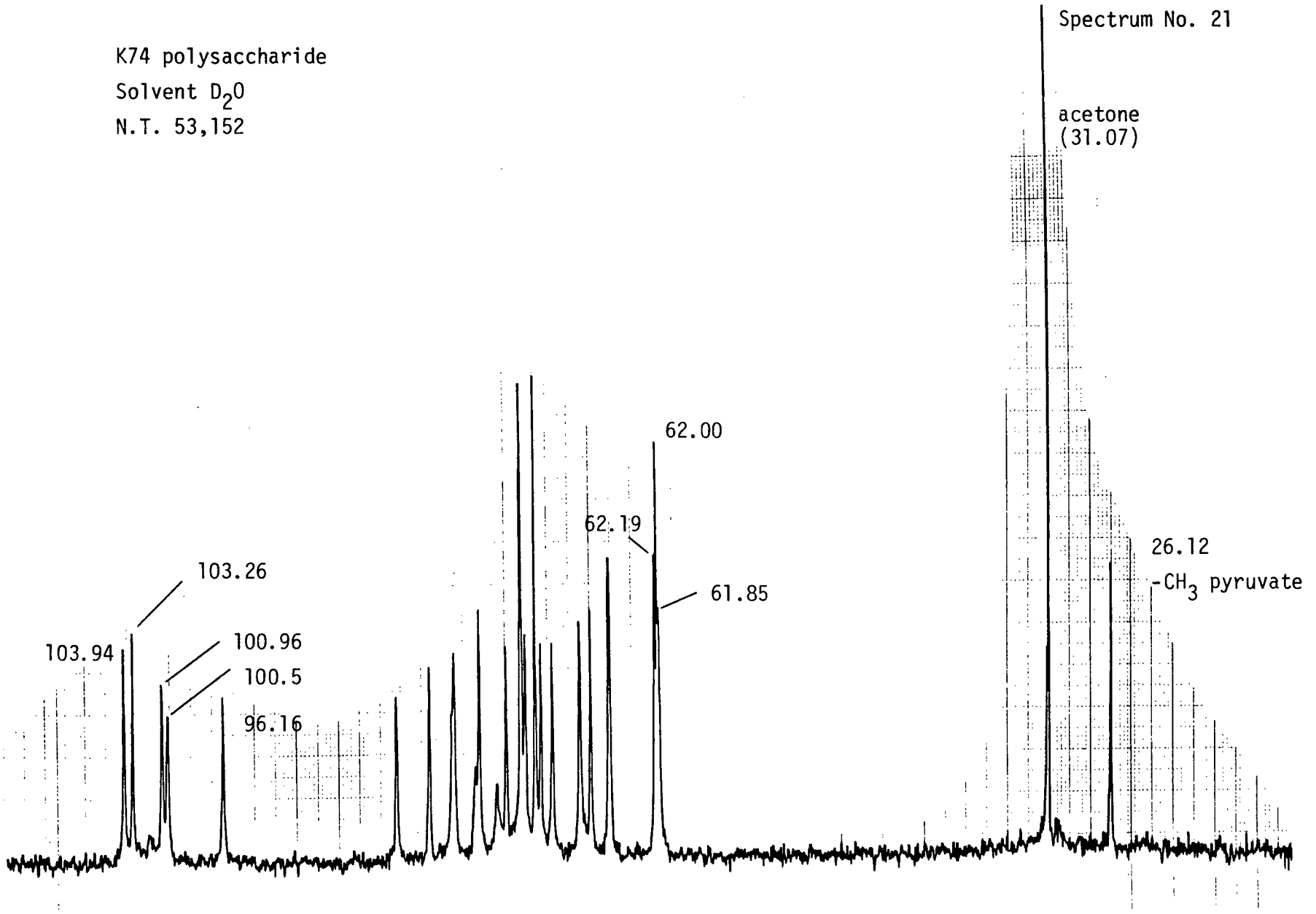
P.W. 21 μsec

P.D. 0 sec

N.T. 87,300




Spectrum No. 20
K74 polysaccharide
Solvent D_2O
Temp. 90°C
1.51
$-CH_3$ pyruvate
5.26
4.55
5.37


K74 polysaccharide
Solvent D_2O
N.T. 53,152
Spectrum No. 21
acetone
(31.07)
62.00
62.19
61.85
26.12
-CH_3 pyruvate
103.26
103.94
100.96
100.5
96.16