A STRUCTURAL STUDY OF THE CAPSULAR ANTIGENS OF *ESCHERICHIA COLI* K36 AND *KLEBSIELLA* K68

THESIS

Submitted in Fulfilment of the

Requirements for the Degree of

MASTER OF SCIENCE

of RHODES UNIVERSITY

by

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SEPTEMBER 1986

ACKNOWLEDGEMENTS

The author wishes to express his sincere gratitude to Professor H.Parolis, for his invaluable advice, unending encouragement and dedicated supervision throughout this study.

The author also expresses grateful appreciation to:
Dr. L.A.S.Parolis for help and advice during the time the research was being carried out;
Mr D.Morley for valuable technical assistance;
Miss J.V.Humphrey for time spent proof reading this manuscript;
Mr A.N.Anderson for help in culturing the E.coli K36 bacteria and extracting the capsular polysaccharide;
the C.S.I.R., Pretoria, for financial support and the use of their n.m.r. facilities;
Rhodes University for financial support;
the mass spectrometry unit of the University of Cape Town for the use of their m.s.

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1 THE ANATOMY OF THE BACTERIAL CELL SURFACE

Bacterial cells all have a cytoplasmic membrane (see Figure 1) which regulates the movement of ions and molecules into and out of the bacterium. Enclosing this membrane is a cell wall of which there are

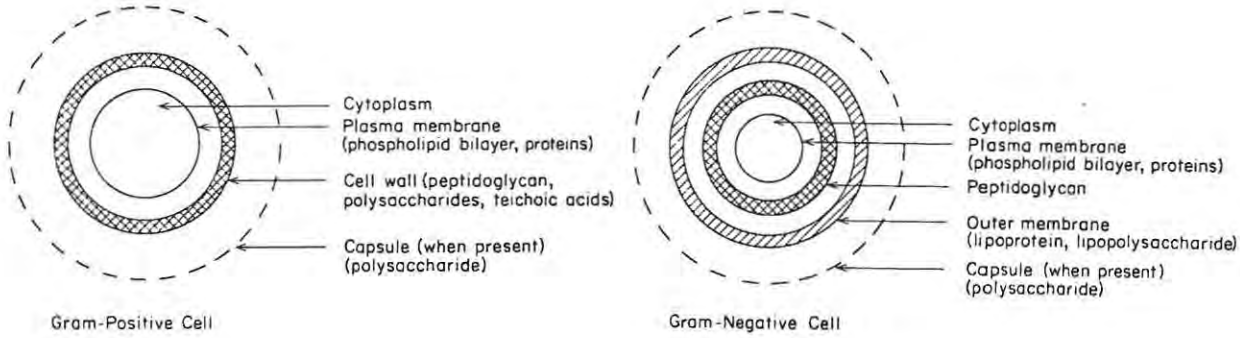


Figure 1. THE BACTERIAL CELL.(01)

two general types, which are differentiated by the Gram stain(02) as being either gram positive or gram negative (depending upon whether they hold the gram stain after washing with ethanol). The cell wall provides the cell with shape and rigidity and is composed, in the case of gram positive types, of peptidoglycan, and in the case of gram negative bacteria, of a peptidoglycan and an outer membrane (see Figure 2). The peptidoglycan layer, common to both cell wall types, consists of a backbone of alternating units of N-acetylglucosamine and N-acetylmuramic acid to which peptides are attached by amide

links. This heteropolymer is a highly cross linked mosaic and this

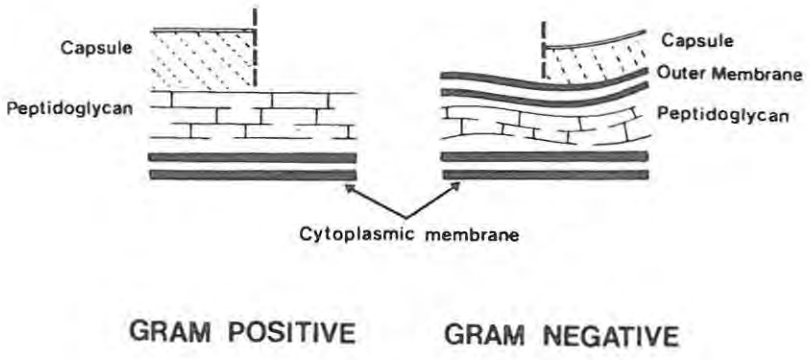


Figure 2. DIAGRAMATIC REPRESENTATION OF THE CELL SURFACE OF GRAM POSITIVE AND GRAM NEGATIVE BACTERIAL CELLS.(03)

gives it strength and rigidity. In gram positive bacteria, this layer also contains two carbohydrate antigens, a simple polysaccharide and a teichoic acid; these are usually the type specific or major group antigens of the bacterium.

Many of the bacteria also produce exopolysaccharides (see Figure 3) either as discrete capsules (for example, the Enterobacteriaceae K antigens) or unattached slime layers (for example, the Enterobacteriaceae M antigens). The vast majority of these polysaccharides are heteroglycans(03) composed of contiguous oligosaccharide repeating units. Their monosaccharide components are largely neutral hexoses, 6-deoxy hexoses and also amino sugars.(03) Pentose units are rare.(03) The capsular polysaccharides usually have a high content of

acidic constituents such as uronic acids, phosphate groups, or pyruvate ketals.(01)

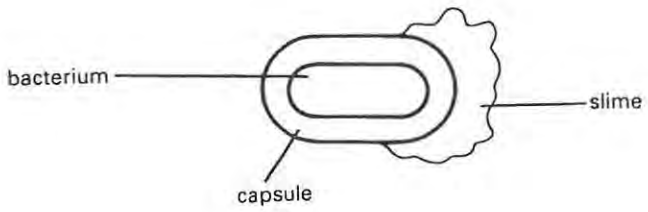


Figure 3. DIAGRAMATIC REPRESENTATION OF THE EXOPOLYSACCHARIDES SURROUNDING THE BACTERIAL CELL.(03)

2 CHEMISTRY AND SEROLOGY OF THE *E.COLI*

2 CHEMISTRY AND SEROLOGY OF THE ESCHERICHIA COLI

Escherichia coli fall within the family Enterobacteriaceae and are a group of mostly motile saprophytic gram negative bacilli.(04) They are also the predominant constituent of the colonic flora, where they are important for intraintestinal physiological functions; colonisation occurs shortly after birth. Generally they remain safely confined to the intestinal lumen, however, should the host be debilitated, immunosuppressed or have critical anatomical barriers disrupted, E.coli can become pathogenic.(04) Some of the E.coli strains, because of their ability to overcome the host's defence mechanism in a healthy individual, are inherently pathogenic; such strains are mainly implicated in three disease states, namely, urinary tract infections, neonatal meningitis, and diarrhoeal disease.(04)

Kauffmann subdivided the E.coli into a number of O-groups and as such was the first person to successfully classify the E.coli by serological methods.(05) In conjunction with Vahlne, he also classifed the envelope or capsule (K) antigen; when present these mask the O-somatic antigen. The criterion for the presence of the K antigen was the inagglutinability of a non-heated bacterial culture in O-antiserum.(05) Three different types of K antigen were described and designated A,B, and L. This classical division has been reviewed by Ørskov et al(05) who recommended that it be discontinued because of the difficulties associated with it; they suggested that the nomenclature of K antigens be restricted to polysaccharides (acidic) and

protein (fimbrial) K antigens only.(05) At present the E.coli are serotyped, not only on the basis of the lipopolysaccharide O-somatic antigen and the capsular K antigen, but also by the flagella, or so called H antigen. Thus, they are categorised into O:H:K serotypes.

The O-somatic antigen, a lipopolysaccharide (LPS) surface antigen that is thermostable, is found in all smooth (S) Enterobacteriaceae. Mutants without the O-specific polysaccharide (see Figure 4), known as the rough (R form), can arise spontaneously from the S forms.(05)

Figure 4. General structure of the bacterial LPS.(05)

The chemical basis for the specificity of the O-antigen of gram negative bacteria arises from the structure of the O-specific polysaccharide. At present, 164 structurally different O antigens have been identified and, with the exception of the mannans of O8 and O9,(06) they are heteropolysaccharides.(05)

The K antigens, with the exception of K88 and K99 which are proteins, are all thought to be polysaccharides. These capsules or K antigens, if present, can easily be detected by immunoelectrophoresis. This is a more reliable method for detection than the inagglutination

reaction in O-antisera. It was previously thought that over one hundred of the K antigens existed, however, further study has shown some strains have no surface antigen other than the O-antigen, while others share the same K antigen. At present seventy four different K antigens have been identified.(05) Ørskov et al noted that the polysaccharide K antigens associated with O8, O9, O101 and probably O20 differ from those found in association with all other O-antigen combinations.(05) The K antigens of this group can be subdivided into those having polysaccharides with or without amino sugars. Those devoid of the amino sugars have molecular weights in the range 3 x 10^5 to 10^6, are physically heterogeneous, but can be rendered homogeneous by treatment with dilute alkali.(05) They have a strong similarity to the K antigens of Klebsiella. Polysaccharides with amino sugars are homogeneous with a M_W between 10^5 and 3 x 10^5.(05)

Certain conditions, such as low temperatures and a high osmotic pressure, cause many of the Enterobacteriaceae to produce a non-specific slime antigen; this is loosely associated with the cell surface and is distinct from the K antigens. This ubiquitous slime capsule, commonly termed the M-antigen, is represented by a group of five acidic polysaccharides all very similar in structure.(05)

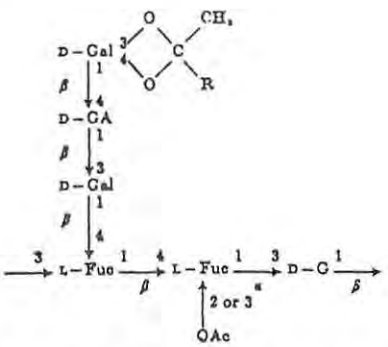


Figure 5. THE STRUCTURE OF THE M ANTIGEN.(05)

At present, approximately a third of the known K antigens have been structurally elucidated and reported in the literature (see Section 6). Glucuronic acid, galactose, glucose, KDO, galacturonic acid and mannose commonly occur as components of the repeating unit. Although the size of the repeat unit can vary from two to six monosaccharides, many of the units are disaccharides. Several of the structures, viz. those from K2$^{(07)}$, K12$^{(08)}$, K52$^{(09)}$, and K100$^{(119)}$ also have phosphate units attached. In many respects, some of the polysaccharides from the E.coli do not differ greatly from the bacterial polysaccharides found in other species of bacteria; for example, the Klebsiella.

3 STRUCTURAL ELUCIDATION OF BACTERIAL POLYSACCHARIDES

3 STRUCTURAL ELUCIDATION OF BACTERIAL POLYSACCHARIDES

A CHEMICALLY BASED ANALYTICAL PROCEDURES

i) INTRODUCTION

Chemical procedures, either alone or in combination with instrumental techniques, are the mainstay of bacterial polysaccharide primary structure elucidation. The procedures employed should define the total monosaccharide composition, absolute configuration of the sugars, inter-monosaccharide covalent bonds, molecular size, and the glycosyl sequence. Unfortunately, chemical procedures in general are destructive and, especially in cases where the amount of polysaccharide is limited, this tends to be a drawback. To a certain extent this has been overcome by using smaller samples (i.e. in the milligram range) as the analytical procedures have become more sensitive, and in some cases, a single milligram may be sufficient for all the chemical analyses that have to be performed on the polysaccharide.

A detailed discussion of all the chemical techniques available for polysaccharide structural elucidation is not within the scope of this review. Only those techniques that are widely used (for example, partial acid hydrolysis for glycosyl sequencing) and those techniques which have been used in the experimental part of this thesis will be mentioned.

ii) COMPOSITION AND MOLECULAR SIZE

Monosaccharide composition and molecular weight, which give the size of the repeat unit and the number of repeat units per chain respectively, are both required for the overall characterization of the bacterial polysaccharide under study. While the uniform nature of the repeat unit allows the composition to be quoted (within limits) as an exact value, the same is not true of the molecular weight determination; this value, because the polysaccharides consist of chains of varying length, is usually quoted as an average.[10]

(a) Composition analysis

The identification of sugar components, as well as their quantification, gives essential information on the primary structure of the polysaccharide. Composition analysis is predominantly by chemical means, although certain instrumental methods are also used in the analysis of carbohydrates, such as nuclear magnetic resonance and infra red spectroscopy. Often these prove to be useful adjuncts to the chemical methods as a number of acid labile constituents can be readily identified by these techniques, helping to devise a suitable, chemically based, analytical procedure.[10]

In order to determine the sugar ratio of the polysaccharide, the glycosidic linkages between the component monosaccharides must be cleaved. A number of methods have been described for achieving this, including: hydrolysis, acetolysis, methanolysis, and

mercaptolysis.[10] The method and specific reaction conditions needed to cleave the polymer vary according to the monosaccharide composition, configuration of the glycosidic linkages, and the ring form of the component sugars. To obtain maximum depolymerisation and minimum degradation, the following factors are taken into account: furanosidic are more labile than pyranosidic linkages and beta linkages are generally more stable than alpha. In addition, the presence of either a hexuronic acid or an amino sugar stabilises a linkage to acid hydrolysis.[11]

Degradation of labile sugars can be overcome by the protecting the anomeric hydroxyl group as soon as the glycosidic linkages are broken; e.g. methanolysis of sialic acids. Chemical modification of the polysaccharide prior to acid hydrolysis can also increase the yield of the free sugar; for example, milder conditions are needed to cleave the linkages after conversion of a glycosyl uronic acid or an amino sugar to the parent sugar. The modification of the hexuronic acid to the neutral hexose can be achieved by treatment with water soluble carbodiimide and sodium borohydride, or with lithium aluminium hydride in tetrahydrofuran (THF).[10] An additional benefit of this is that the uronic acid can then be estimated by gas liquid chromatography (g.l.c.); uronic acids, due to their low volatility, even after conversion to alditol acetates or peracetylated aldononitriles (PAANs), can not be analysed directly by g.l.c. techniques.

Methods for complete acid hydrolysis have been described[11,12]

using mineral (especially sulfuric, nitric, and hydrochloric acids), formic, acetic, and trifluoroacetic acids (TFA). TFA is recommended for use, because (a) the yields are at least equal to those from hydolysis with a mineral acid, and (b) it may be readily removed by evaporation.[12] Glycoside cleavage with HCl in dry methanol, methanolysis, is also recommended as the sugars are released as their methyl-glycosides, thereby minimising the possibilities of side reactions.[13,14] Formic acid is recommended as it has the advantage of being able to solubilise less hydrophilic polymers that are not fully soluble in the other acids available.

Analytical methods for the hydrolysate fall into two broad categories; those based on liquid chromatographic separation: high performance liquid chromatography (h.p.l.c.), thin layer chromatography (t.l.c.), paper chromatography and exchange chromatography- where the analysis is usually of the reducing sugars themselves; and those employing g.l.c. of suitable derivatives. Analysis may also be done spectrophotometrically where the sugars are reacted with specific reagents to give various coloured products. Aspinall[10] has listed the commonly used colorimetric methods.

In this laboratory, preliminary sugar identification is carried out by paper chromatography of the hydrolysate. This method can separate and identify components of the hydrolysate quickly, simply, and accurately with the expenditure of less than a milligram of material;[15] also a high degree of sample purity is not a prerequisite. The paper chromatograms are run in several different solvent

developers and then visualisation, using selective colour reagents, allows sugars to be identified on the basis of their position on the chromatogram relative to that of a standard run concurrently with the sample. The standard consist either of glucose, in which case the sugars are identified relative to glucose (R_{gl})[19], or of all the commonly occurring sugars, where the identification is by finding the corresponding sugar in the standard. Churms lists a value, relative to glucose and 2,3,4,6-tetra-O-methyl-glucose, for all of the commonly encountered sugars when run in several developers.[16]

There are four distinct types of colour reagents used which take advantage of one of the following: (a) the reducing power of the sugars, (b) the action of acids upon the sugar to produce furfural derivatives which give coloured products after treatment with aromatic amines and phenols, (c) the cleavage reactions of periodate and lead tetra-acetate followed by tests for fragments, and (d) reactants specific for structural features.[25] In this laboratory, we commonly use three detection reagents, namely:

(i) akaline silver nitrate-sodium thiosulphate, specifically for reducing sugars,[17]

(ii) periodate-benzidine reagent, which can be used for both non-reducing and reducing sugars,[18] and

(iii) p-anisidine reagent, which differentiates between hexoses and pentoses.[19]

The solvent developers used to separate the hydrolysate are

selected depending upon the nature of the substance being analysed. Solvent combinations commonly used in our laboratory are:

	EthAc	Pyr	HAc	1-But	FormA	EtOH	H_2O	Common use:	pH
1.	8	:2					:1	monomers	basic
2.	18		:3		:1		:4	momomers	acidic
3.	5	:5	:1				:3	uronic and amino monomers/oligomers	slightly basic
4.				40		:11	:9	monomers	neutral
5.			1	:2			:1	oligomers	acidic

KEY:
EthAc*Ethyl acetate
Pyr *Pyridine
HAc *Acetic acid
1-But*1-Butanol
FormA*Formic acid
EtOH *Ethanol

The solvent mixture is chosen on the basis of suitability for the separation being carried out, reactivity towards the sample, and its stability as a mixture.[15] The solvent developers have a number of disadvantages; they are highly volatile and, thus, liable to changes in the solvent ratio; they are sensitive to temperature change; ethyl acetate has a tendency to undergo hydrolysis; and the composition of the mixture may be altered as a result of esterification (if the mixture contains an acid). In addition, if pyridine is present, any heat applied in the removal of the solvent may cause epimerisation of the sugars.[15]

There are a number of methods for quantitative estimation of sugars from the hydrolysate using paper chromatography, the most accurate of these involve dissection of the paper chromatogram after

developing in the solvent, extraction of the monomers from each section, followed by microanalysis of the extract.[15] In this laboratory the use of paper chromatography to determine the total sugar ratio has been superceded by g.l.c. analysis of derivatised sugars from the hydrolysate.

Gas-liquid chromatographic methods of carbohydrate analysis are restricted to material that is volatile, stable to the operating conditions of the instrument, and does not bind irreversibly to the chromatographic column. It is necessary, as a result of their low volatility, that the free reducing sugars released by glycosidic cleavage be derivatised. Some of the derivatives of neutral sugars used for g.l.c., e.g. trimethylsilyl, acetyl and trifluoroacetyl, trimethylsilylated and acetylated methyl-glycosides, give rise to multiple peaks for each sugar, producing complex chromatograms which make quantitative analysis difficult.[20] The peracetylated aldononitrile acetates (PAAN) and trimethylsilylated and acetylated alditol acetates, which give single peaks as well as having good chromatographic properties are, as a result, the derivatives of choice.[20]

Preparation of PAANs from the free sugars is done, by us, using the method described by McGinnis[21] which utilises N-methylimidazole as both solvent and catalyst; this derivatisation has also been found, by others, to be both simple and rapid.[22] The alditol acetates are not used to derivatise xylose, owing to the symmetry of xylitol, or arabinose and lyxose as they yield the same alditol acetate.[20] The method for producing alditol acetates that we use is essentially

that reported by Sloneker;[23] however, Blakeney et al [24] have developed this method further, so that the need to transfer or evaporate the material during derivatisation is avoided. The latter method should thus give better percentage yields of the alditol acetates.

(b) Molecular size determination

A wide variety of methods are available for determining the macromolecular size of polysaccharides and the most useful of these give either number average values M_n, weight average values M_w, or the relative molecular weight M_r.[10] The M_n is the arithmetic mean weight of molecules, and is given by methods that count the number of particles in solution, while M_w is an average of the weight fractions; consequently the result depends on the method used.[26] In addition, the difference between these two values gives an indication of the polydispersity of the sample.[27] Lastly, the M_r is a value relative to some standard (for example, dextrans of known weight) and is used when the determination is via a secondary method, i.e. the weight is not determined directly.

Of the methods available, we find gel filtration, a simple procedure employing commonly available apparatus, to be the most applicable to our polysaccharides. This method, in order to separate molecules of differing size, relies upon the ability of some molecules to enter pores in the gel, which hold the stationary phase, more readily than other molecules.[28] Very large molecules never enter the stationary phase and, thus, move directly through the chromatographic bed, while smaller ones, which can enter the pores and consequently spend a portion of time there, move more slowly. Molecules are, therefore, eluted in order of decreasing molecular size.[28] A linear relationship exists between V_e- the elution volume- and the log molecular

weight of the solute eluted and it is on this relationship that the molecular weight is determined.[28]

A drawback of this method is that it is a secondary technique and, therefore, needs calibration with structurally related compounds of known molecular weight. The first step, thus, in determining the M_r of a sample is to plot the calibration curve for the column (V_e against log molecular weight). This requires establishing V_e for the standard, which is taken as the volume at which its peak concentration elutes. This can be ascertained colorimetrically after treatment of the eluate with phenol-sulphuric[29] reagent. The next step is to determine V_e for the sample by the same procedure. The molecular weight can then be determined graphically from the calibration curve. Finally, the elution profile and thus the molecular weight distribution, is plotted.

The eluants used in gel filtration are aqueous solutions of weak acids, weak bases, or electrolytes. These are used in preference to distilled water because of their ability to retard adsorption or acceleration of charged solutes as a result of interactions with the gel.[29]

There are several limitations to this method, the most important of these being that the polysaccharides take up different conformations in solution and this can affect the partitioning between the pores and mobile phase, distorting results.[30]

iii) CONFIGURATIONAL ANALYSIS OF MONOSACCHARIDES

Monosaccharides consist of a number of contiguous assymetric centers and to assign the absolute configuration the full stereochemistry must be specified; for example, β-D-Glucose specifies the configuration at all the centers. Once the sugar has been identified and, therefore, the assignment of the stereochemistry at most of the centers specified, only the D/L and α and β configurations remain unassigned. Commonly, resolution of the anomeric linkages as either α or β is, in most cases, by n.m.r. spectroscopy, where a signal downfield of 5 ppm is assigned as a and those upfield of 5 ppm are assigned as β. In general, the procedures used to assign the absolute configuration of the component sugars involve either, polarimetry, specific enzymes or, more recently, g.l.c. analysis of stereospecific monosaccharide derivatives.

Of these, the usual method of assigning either the D or the L configuration to a specific sugar in our laboratory was by the measurement of the specific optical rotation of the sugars, but, this technique has several drawbacks. These include: (a) it requires a substantial amount of material; (b) the sugars have to be separated after hydrolysis (c) the sample must be highly purified; and (d) the specific rotation varies with temperature and solvent used.[31] For these reasons, we use g.l.c. as an alternative method to polarimetry when assigning the D or L configuration to the component monosaccharides of a polymer.

Gas-liquid chromatography can be used to differentiate enantiomers either by the application of an achiral stationary phase, or the conversion of the enantiomers into diasteriomers with a chiral reagent and subsequent separation on a non-chiral phase.[32] We have chosen the latter alternative since it was the most convenient,(an OV-225 column can be used as the non chiral phase; this was also used to separate alditol acetates and aldononitriles). Chiral reagents that are recommended in the literature for producing diasteriomers from sugar enantiomers include: (-)-2-butanol,[32] (+)-1-phenyl ethanethiol,[33] and (+)-2-octanol;[34] of these we elected to use octanol and separate the diastereomers on an OV-225 column. This method has successfully assigned configurations of sugars in a hydrolysate containing three different sugars (described in this thesis). Uronic acids require conversion to the neutral parent sugar prior to reaction with the chiral reagent if they too are to be detected during g.l.c. analysis. This method does not seem to be applicable to some amino sugars;[35] in these cases specific enzymes for the D or L configuration of the amino sugar are combined with paper chromatographic analysis to determine if the sugar is enzymatically degraded by either the enzyme specific for the D or the L configuration.

iv) LINKAGE PATTERN

After identification and quantification of the individual monosaccharide components, as well as assignment of their absolute configuration, the linkage pattern has to be determined. Most frequently this is effected by chemical procedures, e.g. methylation, although it is possible that in the future, physical methods, e.g. double quantum coherence n.m.r. spectroscopy,[36,95] may become paramount in this field. Chemical linkage pattern analyses utilise both methylation and oxidative methods.

(a) Methylation analysis

The most common way of resolving the manner in which the sugars are linked involves the conversion of all the free hydroxy groups to methyl ethers; these O-methyl bonds are resistant to acid cleavage and, therefore, any free hydroxyls on the sugar residue after hydrolysis of the methylated polymer indicate a position of attachment. The partially methylated monosaccharides can then be separated from each other, usually as suitably volatile derivatives, by g.l.c. (we have found experimentally that the alditol acetates are superior to PAANs). Identification, hence, is by either g.l.c., where the retention times, compared to a standard, are collated with reference data[37] and the permethylated sugar identified, or g.l.c.-m.s. where identification of the peaks is on the basis of the fragmentation pattern of the eluting compound.[38,39] More recently, mass spectrometry alone has

been sufficient, with all the different methylated monosaccharides in a hydrolysate being successfully identified by mass spectrometry *per se*.[40] Etherification of the free hydroxyl groups, prior to the introduction of the Hakomori method in 1964,[41] was by treatment of the polymer with dimethyl sulphate and sodium hydroxide[42,43] or the modified Purdie and Irvine method put forward by Kuhn,[44] which employed silver oxide and methyl iodide in N,N-dimethylformamide. These methods required repeated treatments of the polymer to effect complete methylation. The Kuhn method is still used by us, primarily for polymers that have only been partially methylated by the Hakomori method and also for uronic acid containing polymers which are methylated by the Hakomori method, carboxyl group reduced and then remethylated by the Kuhn method. (This methylates the 6 position on what was previously the uronic acid sugar). The Hakomori method by which, it is claimed, complete methylation is ordinarily achieved in a single step, involves alkoxide formation from the free hydroxyl groups of the polysaccharide catalysed by methylsulfinyl carbanion in dimethyl sulphoxide (DMSO). Methylation occurs at these alkoxide groups after treatment with methyl iodide.[41]

A number of modifications to the original procedure have been suggested, including (i) the addition of 1,1,3,3,-tetramethylurea to restrict intra- and inter-molecular hydrogen bonding of the polymer and, thus, lower resistance to O-methylation;[45] (ii) the replacement of sodium hydride with one of the following:butyl lithium,[46] potassium hydride,[47] or potassium *tert* butoxide,[48] for the

generation of the methyl sulfinyl carbanion from DMSO (these are supposed to give a more stable reagent and, therefore, chromatograms with fewer artifacts) and (iii) the optimisation of the steps, to improve both recovery and reduce the time required to successfully methylate the polymer.[49] However, not all of these modifications have been found to be successful, for example, potassium tert butoxide was found, in a study, to be unsuitable for methylation.[49] In addition to modifications of the Hakomori method, several alternative techniques have been put forward which claim to give better methylation results,[50,51] and protect material that would be degraded under the alkaline conditions of a Hakomori methylation.[52,53]

Methylation analysis is a valuable technique for resolving the linkage position of the intermonosaccharide bond between the sugars, but, in order to determine the actual monosaccharide sequence, other methods are used.

(b) Periodate/lead tetra-acetate oxidation of polysaccharides

Treatment of the intact polymer with periodate or lead tetra-acetate is a useful supplement to methylation analysis and, in fact, at times the techniques are used sequentially. Selective oxidation with either of the compounds mentioned results in a 1,2 glycol scission[54] of the 1,2 diol and 1,2,3 triol groups found in some of the sugar residues. If a sugar is substituted in a manner that leaves no hydroxy groups adjacent to each other, it will be resistant to oxidation by these compounds, for example, a 3-O-linked hexopyranosyl residue will, thus, not be oxidised under these conditions. The choice of oxidant, because of the difficulties in finding a suitable solvent for both the lead tetra-acetate and the carbohydrate, is, in most cases, restricted to periodate.[55]

Several approaches to structural elucidation using periodate exist and the specific method used depends upon the nature of the polysaccharide and the information to be derived. One of the procedures, monitoring the uptake of periodate per mole of repeat unit, gives the number of susceptible linkages within the repeat unit, while the rate of reaction[56] and/or the end products of the reaction gives information on the type of structure being cleaved by oxidation.[55] Similarly, modifications of oxidation to include steps to remove all oxidised residues (sometimes, using less periodate than required to cleave all the oxidatively labile linkages) coupled with recovery of the unoxidised portion of the polysaccharide, also

reveals the nature of the sample more clearly. In comparison to the unmodified method, this procedure also furnishes information on the monosaccharide sequence. Selective removal of the oxidised polysaccharide is accomplished using either the Barry[57] or Smith[58] method. Of the two, the latter has been found by others[56] to be the more satisfactory. We have also found this to be suited to our polysaccharides.

The quantitative nature of this reaction and the specific products from some of the structural features, makes the selective oxidation of vic-diols a useful analytical tool, especially when accompanied by methylation analysis of the unoxidised portions of the polymer. Unfortunately, as with methylation analysis, selective oxidation gives little information on the monosaccharide sequence and this must be obtained by other methods.

v) MONOSACCHARIDE SEQUENCING

Several techniques are available for determining the glycosyl sequence in a polysaccharide, including: β-elimination of residues attached to C-4 of glycopyranosyluronic acid residues, selective acetolysis, keto degradation, acid-catalysed partial hydrolysis and also enzymic hydrolysis.[77] Most of these methods are of limited value in monosaccharide sequencing and are used, more often, just as supplementary techniques to either partial acid hydrolysis or selective enzymic hydrolysis methods. For this reason, the discussion of the procedures used in sequencing will only include these last two methods. Acid-catalysed partial hydrolysis, the classical method of glycosyl sequencing, has been employed frequently and consequently there are numerous examples where sequencing has been by this technique;[e.g.63,70,75] however, the emphasis is changing, several papers have been published where enzymes alone were used for sequencing (notably those that are bacteriophage-borne endoglycosidases).[64-69] In this thesis, the acidic capsular polysaccharide from E.coli K36 was sequenced using a bacteriophage-borne endo-galactosidase.

(a) Partial acid hydrolysis

Acid hydrolysis of a polysaccharide, stopped prior to the reaction going to completion, gives fragments of varying molecular weight.

These oligosaccharides, which consist of two or more glycosyl residues, can then be separated by one of the following procedures: gel permeation, ion-exchange, partition, or high performance liquid chromatography. Characterization of these fragments after isolation provides complementary and overlapping data from which the glycosyl sequence can be pieced together.

In heteropolysaccharides, where a number of different glycosyl linkages exist, the glycosidic bonds are hydrolysed at different rates dependant upon the hydrolytic conditions and sugar residues involved. For example, during aqueous acidic hydrolysis, furanosidic linkages are hydrolysed 10-10^3 times faster than corresponding pyranosidic linkages.[40] This differential lability of the polysaccharide glycosidic linkages is essential for the success of the experiment. If all the linkages have an equivalent probablity of being cleaved, the number of different oligosaccharides would be large, yields low, and separation difficult. In order to get the best yield of the desired oligosaccharides the following are optimised:

i) the procedure, which, in most cases, entails heating a solution of 0.5%, or less, of the polysaccharide in acid, after which the acid is removed and the oligosaccharides are isolated. There are several modifications[60,61] to this approach e.g. the continuous removal (by dialysis) of the smaller fragments from the acid medium, as they are formed preserves the acid labile oligosaccharides from degradation,

ii) the reaction conditions, for example, liquid

hydrogen fluoride used at -40° gives optimum yield of the larger oligosaccharides and at -23° of the smaller oligosaccharides,[63]

iii) the hydrolytic reagent- often this is an acid in an aqueous solution, although, occasionally, non-aqueous reagents are used if, (a) alternate splitting patterns to the aqueous solutions can be achieved (and these are of use in sequencing the polymer) or, (b) the sugars are less liable to be degraded under non-aqueous conditions or, (c) the polysaccharide is insoluble in water.[10] Several of the reagents have a specific application, for example, the glycosyl linkage of a 2-amino-2-deoxy- residue and (1->6) linkages, both resistant under normal hydrolytic conditions, can be easily cleaved by acetolysis.[10]

Partial acid hydrolysis is a widely applied technique for glycosyl sequencing, but, when dealing with polymers with acid labile components, alternative methods must be employed.

(b) Enzymic hydrolysis

Hydrolytic enzymes, in particular those which selectively cleave glycosyl bonds, are increasingly applied in bacterial polysaccharide structural elucidation and are, in some cases, the only method of preparing oligosaccharides that correspond exactly to the integral repeat unit. This is especially valid in those polysaccharides that contain acid labile constituents, e.g. acetal[64] or pyruvate.[65] The enzymes used, either purified isolates or those that are bacteriophage-borne, are highly specific (they are often of the endo series, which are specific for both linkage type and the monosaccharide residue[73]). They give controlled hydrolysis of a single bond type in the polymer; enzymic cleavage, therefore, produces oligosaccharides that consist of either one, or more, integral repeat units. Of the two types of enzymes used for sequencing, bacteriophage enzymes have found wider application and a number of structures have been published where sequencing has been by bacteriophage depolymerisation.[59,62,64-69,79] For this reason, much of the discussion will be confined to those procedures involving bacteriophages.

There are several sources of the enzymes used, as bacteria, fungi and bacteriophages all produce enzymes that hydrolyse polysaccharides in their host organism. Some of the glycoside hydrolyases are well characterized and commercially available, but, in certain instances, the required enzyme must be isolated by the researcher; this involves either (1) enzyme induction, brought about by growing a suitable orga-

nism on a sole carbon source similar to the polysaccharide to be cleaved and then extraction of the induced enzyme,[72] or, (2) the isolation of a suitable bacteriophage (sewage provides a rich source of bacteriophages). Some of the bacteriophages that interact with the E.coli are designated K bacteriophages, this is because the K antigen, generally the acidic polysaccharide that surrounds the bacterium, acts as a receptor for the bacteriophage.[73] These phages carry, associated with their tail spikes, endo-glycosidases[74] which depolymerise the capsular polysaccharide of the host strain. We have found the phages to be highly host specific, the majority will adsorb to and lyse only their host strain.[71] Similarly, they will not adsorb to acapsular mutants of the host strain,[74] thus, the polysaccharide is essential for the phage/bacterium interaction. The specificity of the phage for the host proves to be highly advantageous as bacteriophage hydrolysis of the polysaccharide yields oligosaccharides that correspond to either a single repeat unit (designated P1) or multiples thereof (e.g. P2 etc.).

Incubation of polysaccharide with the homologous phage (2mg of polysaccharide per ml of solution containing ca. 1×10^{10} phage particles) at 37° for several days is usually sufficient to hydrolyse the polysaccharide. Thereafter, separation and purification of the bacteriophage depolymerised polysaccharide is by chromatography. In this laboratory, we have found paper and gel permeation chromatography (using Biogel® P2 or P4) to be efficient methods of isolating pure samples of P1 and P2. Following isolation, characterization of P1

and P2 is by the same methods as those applied to the polysaccharide and, in fact, a great deal of information is derived from comparing and contrasting the information from the oligo- and poly-saccharide. Methylation of P1 reveals, when the results are compared to those from the native polysaccharide, (i) which sugars are the reducing and non-reducing termini of the oligosaccharide; (ii) the position of attachment of the terminal sugar when it is in the polysaccharide chain and the sugar to which it is linked; and (iii) for trisaccharides and branched chain tetrasaccharides, the complete glycosyl sequence. If, as happened in the bacteriophage depolymerisation of E.coli K36, the phage enzyme cleaves at a branch point, and there are less than three residues attached, the linkage position of the various residues can also be resolved by methylation. Supplementary sequencing information is provided by an n.m.r. study of the P1 oligosaccharide, which, because it is released by the enzyme with a terminal reducing sugar, gives rise to "twinned" signals.[78] There is a mutarotational equilibrium between the α and β orientation of the terminal sugar anomeric hydroxyl and two different molecules coexist. The situation is further complicated by the pyranose/furanose equilibrium for the terminal sugar that can occur under these conditions, and in fact, four different molecules may coexist in the solution.[67] The reducing terminus is, therefore, readily identifiable as it has a signal for both the α and β anomers. The mutarotational effect is transmitted along the oligosaccharide chain and those sugars in closest proximity to the reducing sugar are the more profoundly affected, for example,

in the spectrum of P1 from E.coli K36 and from Klebsiella K68 (both included in this thesis) the signal from the 2-O- linked mannosyl residue linked to the terminal reducing galactose shows the biggest chemical shift difference between its "twinned signals". The chemical shift difference falls off considerably with increasing distance of the sugar from the reducing terminus. Methylation and n.m.r. study of P1 are, therefore, complimentary techniques as any structural hypothesis made from the methylation results can be tested by examination of the n.m.r. spectra. Other methods are available for characterizing P1, for example, the method of Morrison[76] identifies the reducing terminus, but, these are seldom required if methylation and n.m.r. results are unambiguous.

Enzyme applications are not limited to generating oligosaccharides, some, usually well characterized purified enzyme isolates, are used to determine whether a structural feature is present or absent in a molecule; for example, the anomeric configuration of the glucuronic acid residue in E.coli K27 capsular polysaccharide was assigned when a β-D-glucuronidase was applied to the polymer and the enzyme did not split the molecule, therefore, assuming the enzyme was active the structure had to be α-D-glucuronic acid.[75]

Enzymatic procedures are rapid and can be adapted to a microscale. Optimal reaction conditions at pH 7, or thereabouts, and temperatures near room temperature, ensure only selected bonds react and the sugar or quarternary structures are not altered by extremes of pH or temperature. However, should the enzyme be contaminated with

other glycoside hydrolyases, due to inadequate purification, more than one oligomer will result from the hydrolysis, giving an ambiguous result. Selective enzymic hydrolysis, when a suitable enzyme (especially a bacteriophage-borne endo-glycanase) is available, is a convenient method of preparing an oligosaccharide that corresponds to one, or more, intact repeat units, particularly for those polymers containing components not stable to the conditions required for partial acid-catalysed hydrolysis.

B INSTRUMENTAL ANALYTICAL TECHNIQUES

i) INTRODUCTION

This section deals with those methods which involve instruments for the identification and determination of carbohydrate structures. The procedures/instruments that we use include:

(1) ultra-violet spectrophotometry (u.v.), a useful technique for monitoring the uptake of sodium metaperiodate during periodate oxidation,

(2) polarimetry, although the use of this instrument is dwindling owing to the inherent requirement of the method, namely, the high degree of sample purity, it is still used for the determination of the overall optical rotation of the poly- or oligosaccharides,

(3) mass spectrometry (m.s.), (either alone or in tandem with g.l.c. known as g.l.c.-m.s.) is used to identify compounds derived from methylation analysis. Moreover, this technique can also be used to determine the molecular weight, sequence, and position of the glycosidic linkages in small oligosaccharides (i.e. those with up to five monosaccharide units),[40]

(4) nuclear magnetic resonance (n.m.r.), a method that gives a great deal of usable information, for example, the proton attached to the anomeric carbon in monosaccharides have larger chemical shifts when in the equatorial position compared to the axial posi-

tion,

(5) gas-liquid chromatography (g.l.c.) is used to analyse a hydrolysate for component monosaccharides and more importantly, is used for analyses of the permethylated sugar mixtures produced during methylation analysis. It is also possible to separate and identify the various anomers and establish the ring size of sugars by this method.

The latter techniques- mass spectrometry, g.l.c., g.l.c.-m.s., and n.m.r.- because of their applicabilty to structural studies of carbohydrates, warrant a more detailed review.

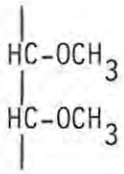
ii) GAS-LIQUID CHROMATOGRAPHY AND G.L.C.-M.S

Gas liquid chromatography is a process in which the components of the sample are volatilised and distributed between a moving inert gas phase (He,H_2 or N_2) and a liquid phase held stationary on a support. Those compounds that are most compatible with the liquid phase will spend a correspondingly larger portion of their time held in the stationary phase than those compounds with less affinity for the liquid, therefore, the net result is that components are eluted progressively with increasing affinity for the stationary phase.

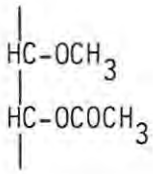
The sample, usually a solution of volatile derivatives of the saccharide components (e.g. acetylated aldononitriles) in a volatile solvent, is injected into a heated chamber whereupon it is volatilised and carried along the column by the gas stream. The column is maintained uniformly at a temperature sufficient to keep the components volatile and afford a suitable partition ratio of the sample between the mobile and stationary phases.[80] Compounds eluting from the column are monitored with an appropriate detector, for example, a flame ionisation detector (FID).

Identification of the compounds is by comparing the retention time (time taken to travel the full length of the column) with that of a standard. In some cases, the eluate is fed directly into a mass spectrometer (known as g.l.c.-m.s.) and this allows the identities of the compounds, which are already tentatively assigned by their retention times, to be confirmed by their mass spectra. Absolute iden-

tification of methylated sugars by mass-spectral analysis alone is not possible. Firstly, different isomers having the same substitution pattern give identical spectra, thus, no distinction can be made between them; secondly, alditols derived from 2,4- and 3,5-di-O-methyl hexoses will give identical mass spectra.[82] To a large extent, the latter problem can be overcome by reducing the permethylated sugars with borodeuteride instead of borohydride, thus ensuring a unique spectrum from each molecule.[83] The fragmentation of permethylated alditol acetates gives rise to primary fragments that arise by fission between two carbon atoms in the alditol chain, with either of the carbon fragments carrying the positive charge, and secondary fragments that are formed from the primary fragments by elimination of acetic acid and/or ketene.[82] The intensities of the fragment peaks in the analysis of alditol acetates are influenced by the structures present. Fission occurs preferentially between certain chemical groups on the molecule, for example, fission occurs between the carbon atoms in partial structure 1 and 2 in preference to partial structure 3.[82]



1.



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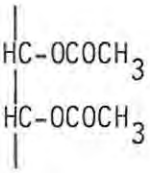


3.

In addition, fission also occurs more frequently between the carbons in partial structure 1 than in partial structure 2.[82] A discrete fragmentation pattern will, therefore, be obtained for most of the

substitution patterns on the monosaccharide. This means that, combined with the identification by relative retention time and a knowledge of the polysaccharide composition, g.l.c.-m.s. should give unambiguous identification of partially methylated monosaccharides.

Quantitation of the various components eluted during the g.l.c. run requires either determination of the molar response factor (m.r.f.) of individual compounds analysed by the detector, or an assumption of these values. In some cases, where accuracy is not of paramount importance or the compounds are similar, it is satisfactory, as a comparison, to consider that the molar response is equal to the weight response. The effective carbon response factor is also used to calculate individual sugar responses. This theory assumes that each type of carbon (e.g. carbonyl or ether moiety) contributes to the same extent in all molecules regardless of the identity of the the compound, therefore, the total response will be the summation of all the individual carbon responses.[84] However, for accurate analyses the m.r.f. should be determined for each compound.[81]

The choice of an appropriate liquid phase is important in obtaining meaningful results in g.l.c. analysis; both the ability of the liquid to effect separation and its stability under the operating conditions are considered. The stationary phase can be either non-polar or polar. The non-polar phases are most efficient when separating components of differing carbon number, while those that are polar afford better separation of molecules that fall within a narrow and low molecular weight range and are therefore, par-

ticularly well suited to separating monosaccharides. Those liquids that have found application in separating sugar derivatives include: ECNSS-M, OV-17, OV-225, and apiezon greases.[37] The column type is selected to suit the application. There are two types of column available, those filled with a solid support phase to which the liquid phase adheres, and those known as a wall coated or a capillary column. The latter have the liquid phase bound to the wall of a small diameter column and are the most suited to analytical work because they give better peak separation[37] than the more conventional packed columns. They are of little use, however, in preparative separation as the sample size is limited to 1µg or less.[80]

For the majority of our research using the g.l.c., we use a bonded fused silica OV-225 column as this, in most cases, successfully separates volatile derivatives (PAANs or alditol acetates) of monosaccharides, octyl glycosides, and partially methylated alditol acetates. It also has greater stability under the operating conditions of the instrument compared to some of the other liquid phases available e.g. ECNSS-M.[80] Occassionally OV-17 is used in preference to the OV-225 as the liquid phase as the order in which the partially methylated alditol acetates are eluted is different and the quantitative determination properties are superior to OV-225.[37] In addition, some of the partially methylated sugars co-elute on a particular column, e.g. 3,4,6- and 2,4,6-tri-O-methylhexose derivatives, which emerge as a single peak on OV-225, but, are separated on OV-17. The reverse is true when dealing with 2,3,6- and 2,3,4-tri-O-methyl hexose

derivatives. However, it is feasible to resolve all the different methylated sugars by the correct use of both OV-17 and OV-225.

Gas-liquid chromatography and g.l.c.-m.s. have been limited to a few fields because of the inherent restrictions of the method, in particular, the components to be separated must be stable and volatile under the operating conditions. At present, when used in carbohydrate research, they are used only in the analysis of the component monosaccharides after hydrolysis of the polysaccharide, the assignment of the absolute configuration by separation of diasteriomers produced by chiral reagents and, more importantly, for the analysis of partially methylated sugars produced by hydrolysis of the permethylated polymer.

iii) NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY OF CARBOHYDRATES

(a) Introduction

Nuclear magnetic resonance (n.m.r.) is a powerful tool for obtaining structural information on poly-, oligo- and di-saccharides. It has an advantage over other methods as it is non-destructive, that is, the sample is generally neither destroyed nor modified during analysis. This ability to examine and then recover the sample intact is especially appealing when the saccharide is available in limited quantities. In addition, methods and instrumentation have been evolved that make it possible to analyse a sample in a solid form, thus, ensuring the true physical and chemical nature of a solid carbohydrate is preserved.

Most n.m.r. spectra can be fully described by five sets of parameters,[96] namely:

(1) the integral, which is the area of the signal and is proportional to the number of nuclei resonating at that particular frequency,

(2) the chemical shift, which is defined with respect to an arbitrarily selected standard, e.g. tetramethylsilane (TMS), these values are quoted in dimensionless units of parts per million or the σ scale, where

$$\sigma = \frac{\text{frequency separation (Hz) between the resonance and TMS} \times 10^6}{\text{spectrometer radiofrequency (Hz)}} ;$$

(3) the spin coupling, which causes signal splitting, is reported in Hz,

(4) spin-lattice relaxation time, or t_1 value, is a time constant for the transfer of absorbed energy from the resonating nucleus to the environment, and

(5) the spin-spin relaxation time, or t_2 value (the time constant for the loss of phase coherence of the resonating nuclei).

The resolution of these parameters has improved vastly compared to earlier instruments and advances in a number of areas are responsible for this. The introduction of superconducting solenoids (which provide a stronger magnetic field than the electromagnets used previously) allow the operational frequency of the instruments to be increased from less than 100 MHz to over 500 MHz. The benefits operating at higher frequency are two-fold, firstly, there is an appreciable increase in the sensitivity of the machine when used at higher magnetic field and secondly, the chemical shifts of the nuclei are magnified while the couplings remain invariant. Clearly, therefore, the spectrum is more spread out and interpretation is easier, which allows more detailed information to be obtained and smaller samples to be analysed. Similarly, the use of pulsed Fourier transform (FT) techniques, instead of continuous wave methods, has increased the sensitivity of n.m.r. analysis thus allowing nuclei, other than protons, to be used as the probe. Dedicated computers (and in some cases array processors) have become necessary additions to the machines used

since the advent of FT methods; these have, by improved data manipulation, increased the number of parameters that can be studied on a routine basis. For example, spin-lattice (t_1) and spin-spin (t_2) relaxation times, which furnish detailed information on the nature of the polysaccharide, can now be determined. Unfortunately, despite these advances, no single method allows all the parameters to be accurately determined in a single experiment and, as a result, it is necessary to choose the technique that will give the majority of the information required (see Table 2).

Problem	Experiment	Results
Carbon assignments are known, but what are the proton assignments?	Two-dimensional heteronuclear correlated	The proton spectrum will be along one axis, the carbon along the other. In contour format, the spots will show the correlations.
Which protons are coupled to which?	Two-dimensional J-correlated (COSY)	The one-dimensional spectrum will be along the diagonal. The off-diagonal peaks will show the coupling correlations.
Which groups are nearby each other in space?	Two-dimensional NOE (NOESY) or one-dimensional difference NOE	In two dimensions, the one-dimensional spectrum will be along the diagonal. Those protons nearb each other can be correlated by the off-diagona peaks. In one dimension, the magnitude of the NOE is related to the distance between nuclei.
What are the proton-proton coupling constants in a heavily overlapping region of the spectrum?	Two-dimensional J-resolved	The proton-decoupled proton spectrum will be along one axis and the proton-proton coupling constants will be spread out along the other.
CH and CH_2 regions of the carbon spectrum are heavily overlapped. Which are which?	Insensitive nuclei enhanced by polarization transfer (INEPT), distortionless enhancement by polarization transfer (DEPT)	Intensity enhancement is obtained and proper choice of the delay times in these pulse sequences allows selective inversion of the different types of carbons.
How can carbon signals from the deuterated solvent be suppressed?	INEPT, DEPT	These pulse sequences rely upon transfer of polarization from protons to the insensitive nucleus, and thus the deuterated solvent is not observed.
Which carbons are connected to which?	Two-dimensional ^{13}C-correlated	Correlations between the carbons that are connected to each other can be made immediately.

Table 2. NUCLEAR MAGNETIC RESONANACE EXPERIMENTAL OPTIONS.[85]

One aspect of determining the five n.m.r. parameters that has lead to

confusion is the large number of standards used, for example, external carbon disulphide, internal sodium 2,2,3,3,-tetra-deuterio-4,4, -dimethyl-4-silapentanoate (TSP), sodium 4,4-dimethyl-4-silapentane -1-sulphonate (DSS), as well as external TMS, have all been used as standards.[92] The net result is the introduction of an experimental variation in the chemical shift of substituents.

At present most of the n.m.r. studies of carbohydrates utilise either proton or carbon substituents as the nuclear probe. These are used to furnish information on the total sugar ratio, sugar and chain conformations, nature of the anomeric linkage, additional substituents, and solution conformations.[85-90,94]

(b) Proton magnetic resonance spectroscopy

^{1}H-n.m.r. data from polysaccharides and oligosaccharides are affected, principally, in two ways. The first is by exchangeable protons (O-H,N-H) in the molecule. These give rise to a strong residual water peak known as the HOD signal and this has a chemical shift at ambient temperature, such that it interferes with signals in the important anomeric region. (This signal can be reduced, but not removed,by exchanging the protons with deuterons using quality deuterium oxide >99.98%). Thus, some of the anomeric signals are obscured by the HOD signal and its spinning sidebands. The second effect is from both signal broadening and the low sensitivity of the ^{1}H nuclear chemical shift to changes in chemical and stereochemical

environment. The signal broadening is due to the short spin-spin relaxation times of the polysaccharide protons as a result of the high viscosity of the polymer solutions.[89] These result in a considerable overlapping of resonances, especially where the molecule is large and complex. Clearly, to obtain easily decipherable spectra from ^{1}H-n.m.r. experiments these problems known as "hidden resonance"[90] must be overcome. Several methods have been put forward. These can be subdivided into (1) chemical and (2) physical/instrumental methods, although, in some cases, a combination approach can be adopted with greater success.

The chemical methods used include:

(a) solvent induced shifts; in practice these are seldom used as the induced shift is small (<0.5 p.p.m.) and polysaccharide solubility is usually limited to water and solvents of similar nature,

(b) functional derivatisation of hydroxyl groups, as this often results in a downfield shift of protons on adjacent carbon atoms,

(c) specific deuteration; specific protons are replaced with deuterons and, thus, no longer give rise to a interfering resonance,

(d) deploymerisation; the viscosity is reduced by either producing oligosaccharides that correspond to the intact repeat unit or cleaving a few intersaccharide linkages, and this gives smaller linewidths than in the spectra of the polymer,

(e) paramagnetic shift reagents (e.g. Lanthanide) when added to the sample induce large shifts (up to 40 ppm) in some of the resonances,[90] thereby shifting interfering resonances to different parts of the spectrum.

Unfortunately, all of these methods to some extent alter the sample; so under conditions where it is important to recover the sample unchanged, instrumental solutions to these problems are preferable. As mentioned previously, chemical shifts are dependant upon the external magnetic field and, therefore, the transmitter radiofrequency. Thus, a facile method of circumventing resonance overlap is to perform experiments at higher frequency and instruments are available that operate at fields up to 600MHz. Unfortunately, even at the highest field available, not all of the resonances are discrete (especially those in the non-anomeric region[88]) and this has lead to the development of several experiments to overcome this. Included here are spin decoupling, spin tickling, internuclear double resonance (INDOR), partially relaxed Fourier transform, and two dimensional techniques (see page 49) to improve the resolution in these areas.[89]

The effect of the HOD signal is minimised by either raising the temperature of the sample during analysis, whereupon, the HOD signal is displaced upfield (approximately 4.2 ppm at 95°) and that part of the spectrum previously hidden will be exposed or, by using one of the pulsed FT techniques. For example, saturation decoupling[89] effectively removes the HOD signal by using an appropriate pulse

sequence.

The anomeric protons of the polysaccharide give the most useful information and of the five parameters determined for these protons during a ^{1}H-n.m.r. experiment, the chemical shift (σ) and the spin-spin couplings (J) are the most useful. The shift value is indicative of the environment of the proton and the coupling constant correlates with the size of the dihedral angle (θ) between the anomeric proton and H-2. This value is at its greatest when the angle is 180° (the protons are transdiaxial), falling to a minimum when the angle is 90° (the protons are gauche). When applied to those signals where the difference in the angle between H-1 and H-2 of the α and β form is large, the J value is characteristic of the anomeric linkage. For example, the value for a β galactose is 8 Hz (both protons are axial) and 3.5 Hz for α galactose (H-1 equatorial,H-2 axial). However, where the difference is small (as in mannosyl residues) the coupling constant is not a reliable method of assigning anomeric configuration.

Although the "hidden resonance" problems associated with complex carbohydrates threaten to curtail the use of ^{1}H-n.m.r. experiments in favour of other nuclei as probes, there is a wealth of information to be derived by using this technique. Indeed, the full potential of proton magnetic resonance has yet to be achieved.

(c) Nuclei other than protons as nuclear probes

The majority of n.m.r investigations of carbohydrates have utilised protons as the nuclear probe, but there are several other nuclei suitable for this type of study- ^{15}N, ^{19}F, ^{31}P, and especially ^{13}C. It only became possible to study ^{13}C nuclei after the introduction of FT instrumentation which provided a substantial increase in the sensitivity of n.m.r. analyses. This was because the low natural abundance (about 1.1%) and the limited intrinsic sensitivity (approximately 1.6% of the ^{1}H nucleus sensitivity) prevented meaningful analysis using the pre-FT instruments and methods.(91) These drawbacks are, fortunately, compensated by the enhanced sensitivity (nearly 30 fold) of the ^{13}C chemical shifts to changes in chemical and stereochemical environment compared to an equivalent proton substituent. Similarly, the line broadening as a result of high viscosity that occurs in ^{1}H-n.m.r. spectra does affect ^{13}C spectra. Hence, the resonances are sharp and well spread out, which is an advantage when dealing with polysaccharides where the "signal density" is high. The radiofrequency of the instrument does not have to be as high as in ^{1}H-n.m.r. experiments, a frequency of 100MHz is quite adequate to ensure the separation of the resonances even in complex spectra.

The parameters that can be determined in a ^{13}C n.m.r. experiment include the chemical shift, spin-spin coupling constants between the carbon and other resonating nuclei, nuclear Overhauser enhancement (nOe) and the nuclear relaxation times t_1 and t_2.(93) The chemical

shift, characteristic of the environment of the nucleus, is used to identify various substituents and the configurations of the anomeric carbons in the molecule. Anomeric configuration can be assigned on the basis of the $^{1}J_{CH}$ coupling constant for an anomeric linkage. This is because coupling constants give information on the angles of the bonds connecting the coupled nuclei.(93) It was shown(93) that the $^{1}J_{CH}$ coupling constants for the α and β anomer are 160Hz and 169Hz respectively. Other couplings of interest include the $^{3}J_{C-1',H}$ value which is used to determine the torsion angles of the glycosidic linkages in the molecule. Three ^{13}C parameters studied nOe, t_1 and t_2 are of use in evaluating internuclear distances and polymer substituent mobility, but, due to the absence of a comprehensive data bank of values for reference compounds, their application to carbohydrate structural studies is limited.

Experimentally, there are two ways of obtaining ^{13}C n.m.r spectra. The first, using broad band decoupling of protons, produces spectra that are simple as each carbon in a different environment produces a single sharp resonance. This is because at natural abundance levels ^{13}C-^{13}C couplings are not observed. While using the second method, where the protons remain coupled, the signals from those carbons covalently bonded to one, or several, protons are split into two or more resonance peaks. Furthermore, in this method spectra are complicated by longer range couplings which cause signal broadening and splitting. The negative aspects are, however, offset by the increase in the usable information obtained from this experiment.

Experimental options for analysis of ^{13}C resonances and ^{13}C coupling constants include: ^{1}H decoupling, selective ^{13}C saturation combined with gated ^{1}H decoupling, off resonance decoupling, FT difference spectroscopy, as well as two dimensional techniques.(90) Therefore, it is often possible to select a technique that is well suited to the task at hand.

(d) Two dimensional nuclear magnetic resonance spectroscopy

The techniques discussed previously have been mainly one dimensional (1D) in which the chemical shift data are displayed along a single horizontal axis, while in two dimensional (2D) n.m.r. a second axis is introduced on which either shift or coupling values are displayed. Experimentally the techniques differ in the pulse sequence used; the 1D pulse sequence (see Figure 6) is altered so that the evolution period t_1 becomes variable within a sequence of pulse cycles.

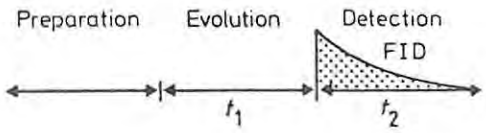


Figure 6. TIME SEQUENCE OF MODERN PULSE EXPERIMENTS.(36)

If t_1 is increased (from t_1 =0) by a constant time increment Δt_1, the receiver signal and therefore the resonance amplitude, will become dependant upon t_1. Therefore, the data can be transformed with

respect to t_2 and also t_1 to obtain frequency variables F_2 and F_1, respectively. F_2 yields the chemical shifts of the nuclei, as in 1D experiments, while F_1 reveals the modulation, i.e. the coupling constants.

Two dimensional n.m.r. experiments can be subdivided into correlated and J-resolved 2D spectra. The latter, characterized by one axis for the coupling information and one for the chemical shift data, resolves both the coupling constants and the chemical shift, while the former, where both of the axes contain chemical shift data, correlates nuclei based on interactions such as the nOe or coupling constant.[36] There is a slight experimental difference between the two as correlation spectra require an additional time interval, called the mixing time, between the evolution and detection periods.[36] The connection between F_1 and F_2 axis is established through scalar coupling or dipolar coupling with measurements of coupling constants made from a contour plot of the J-resolved spectra. Hence, the 2D-n.m.r. experiment chosen depends upon the information to be derived. For example, ^{1}H COSY (correlated spectroscopy) is used to determine the homonuclear (proton) couplings within a molecule, while ^{1}H 2D-J-resolved experiments are used for determining the size of the homonuclear coupling.

Two dimensional n.m.r., despite its ability to improve upon the poor resolution found in some of the 1D experiments and its unsurpassed ability to pinpoint couplings, is unlikely to entirely replace 1D experiments. This is because 2D experiments require a longer acquisi-

tion time and more sophisticated pulse transmitters than 1D. Moreover, 1D data reduces the minimum 2D acquisition times and simplifies 2D spectral interpretation. The techniques are, therefore, complementary.

(e) Conclusion

Clearly, n.m.r. has already proven itself a useful method of determining carbohydrate structures. It can identify component monosaccharides, anomeric configuration, position of linkages and the conformation of both monosaccharides and glycosidic linkages, without alteration or destruction of the sample. Yet, all of the techniques discussed 1D and 2D, (^{13}C and ^{1}H n.m.r.), have the potential to be developed further as technology advances, whereupon, the information will be available in greater detail than at the present. Examples of these developments are already becoming evident; the introduction of array processors, for example, has reduced the time required for data manipulation, an important advance in a technique where the computation is often time consuming. These advances hold exciting implications for the carbohydrate chemist involved in the field of structural elucidation.

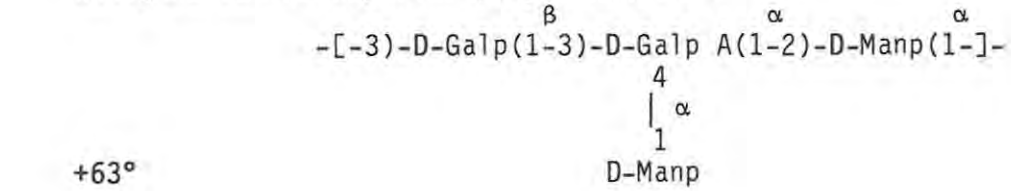
4 THE STRUCTURAL ELUCIDATION OF THE *E.COLI* K36 CAPSULAR ANTIGEN

4 THE STRUCTURAL ELUCIDATION OF THE E.COLI K36 CAPSULAR ANTIGEN

i) ABSTRACT

The structure of the acidic capsular polysaccharide isolated from E.coli strain K36 has been established, primarily, by spectroscopic and chemical analyses of the bacteriophage-depolymerised polysaccharide. In addition, a few complimentary experiments were performed upon the intact polysaccharide.

In concurrence with the results, the following structure is assigned to the capsular polysaccharide:



ii) INTRODUCTION

The E.coli are invariably encapsulated when examined fresh from an established infection(03) and it is the polysaccharides that envelop the bacterial cell that are often important in the pathogenesis of the infection. Unfortunately, information on the role of the capsule is relatively scarce, however, it is thought that they reduce the susceptabilty of the bacterium to phagocytosis and that they restrict the access of opsonins to deeper immunogenic structures, thus, interfering with complement fixation.(03) They are also poorly immunogenic.(03)

It is noteworthy that the E.coli capsular polysaccharides also have diversity of structure and this reduces the likelihood that the host has had previous exposure to the antigen, limiting the possibility that there will be a specific antigen present.(03) Similarly, some of the E.coli, for example the strain K5,(97) have capsules that closely resemble the body's polysaccharides and, as a result, are non-immunogenic. The E.coli are, thus, able to mediate the host's defence mechanism to some extent.

The study of the E.coli strain K36 capsular polysaccharide, which we report on now, forms a part of a study to identify the structure of all the K antigens.

iii) RESULTS AND DISCUSSION

The E.coli K36 was cultured on Mueller-Hinton agar (see Annexure 2) with 2% sodium chloride added and the polysaccharide was extracted from the cells with 2% aqueous phenol. The isolation and purification involved ethanol and cetyltrimethylammonium bromide precipitation steps as well as dialysis. The native polysaccharide, examined by gel permeation chromatography on a Sepharose® 4B Cl column, was found to be monodisperse (M_r of 2.7×10^7 relative to dextran) with respect to its molecular weight distribution (see Profile 1)

The enzymatic depolymerisation of the capsular polysaccharide with a polymer specific bacteriophage-borne endoglycanase gave a 78% yield of material with a M_w below 6 000; from this material an oligo-

saccharide (P1), that corresponds to a single intact polysaccharide repeat unit, was isolated by paper chromatography in a 20% yield. The optical rotation of P1 (+89°) when compared with the optical rotation of the polysaccharide (+63°), suggests that a β linkage had been cleaved during the depolymerisation.

Total sugar analysis of both the polysaccharide and oligosaccharide, with and without reduction of any carboxylic acid groups present, gave an overall molar ratio (see Table D) of 2:1:1 for mannose, galactose and galacturonic acid. The monosaccharides were all shown to be of the **D** configuration by g.l.c. analysis of the sugars as their derived[34] acetylated 2(-)-octyl glycosides.

The K36 oligoalditol P1 (produced by borohydride reduction of oligosaccharide P1) was methylated, carboxyl reduced with $LiAlH_4$ and then treated with trifluoroacetic acid (TFA) to release the permethylated monosaccharides. G.l.c analysis of these sugars as the derived alditol acetates revealed (see Table C,column(c)) that the oligoalditol P1 is a linear tetrasaccharide with a 3-O- linked galactitol and a non-reducing mannose as the termini. A 4-O- galacturonic acid and a 2-O- linked mannose comprise the rest of the chain. There are, thus, only two possible structures for this P1 oligoalditol:

A. Manp(1-4)Galp A(1-2)Manp(1-3)Galactitol

B. Manp(1-2)Manp(1-4)Galp A(1-3)Galactitol

The ^{1}H-n.m.r. spectrum of P1 recorded at both ambient temperature (30°) and 95° (see Table A and Spectrum 1 and 2) is characterized by several fractional signals. These arise due to the mutarotation of

the terminal sugar unit and the pyranose/furanose equilibrium of the terminal sugar that occurs in solution.[67] In the spectrum of P1 recorded at 95° the resonances at 5.28 ppm (0.55 H) and 4.62 ppm (0.45 H) which together integrate for a single proton, represent H-1 of the α and β pyranosyl configuration of the terminal residue. These may be confidently assigned as the α and β galactose signals on the basis of the methylation and Morrison[76] analyses, both of which identified the galactose as the reducing terminus. The presence of the fractional signals in the spectrum of P1 at 30° and 95° allows other assignments to be made. This is because the mutarotational equilibrium measurably affects the resonances of those residues in close proximity to the reducing terminal. Thus, four signals arise from one of the mannose residues, of which the signals at 5.23 ppm and 5.22 ppm (at 95°) represent H-1 of a mannosyl residue when the oligosaccharide has an α and a β galactopyranosyl reducing terminus respectively, while the other resonances at 5.16 and 5.13 ppm arise when the molecule has an α and β galactofuranosyl terminal residue. The different forms of the galactose, thus, have a strong influence upon the chemical shift of the mannose residue causing its anomeric proton signal to be "twinned".[67] It may thus be concluded that the mannose residue is linked to the terminal galactose in P1. The "twinning" further enables the assignment of the more downfield of the mannosyl signals to the mannose linked to the galactose, i.e. the 2-O-linked mannose.

The correctness of the spectral assignments made from the P1 spectrum were confirmed by the spectrum (see Table A and Spectrum 3)

of P1 oligoalditol (P1 reduced with $NaBH_4$). The terminal galactose residue has been reduced to galactitol (whereupon the residue no longer has a signal in the anomeric region) which causes the "twinned" H-1 signal of the 2-O- linked mannosyl sugar to collapse into a single signal (5.20 ppm). The signal for the anomeric proton of the second mannosyl residue could not be conclusively assigned as either α or β from the ^{1}H-n.m.r. data. A generalisation, when dealing with anomeric resonances of individual monosaccharides, is that H-1 signals downfield of 5 ppm arise from sugars with α anomeric configurations (that is the protons are in the equatorial plane), whilst those upfield of 5 ppm arise from sugars of the β anomeric configuration. The second mannosyl signal falls almost upon 5 ppm and therefore no anomeric assignment can be made for this mannose, from the ^{1}H spectrum. It was necessary, in order to ascertain the nature of this last anomeric configuration, to perform a ^{13}C proton coupled experiment. The value for the C-1,H-1 coupling for this mannose was 169.83 Hz, which is consistent for a coupling where the proton linked to the anomeric carbon is in the equatorial position;[93] the anomeric configuration can thus be confidently assigned as α.

The polysaccharide was examined by proton and ^{13}C-n.m.r. spectroscopy. It was, however, necessary to partially autohydrolyse (PA) the native polysaccharide prior to n.m.r. analysis due to the high viscosity of the polysaccharide solution. The ^{1}H-n.m.r. spectrum of the PA polysaccharide showed six well resolved signals in the anomeric region (see Spectrum 4). From the chemical shift and coupling

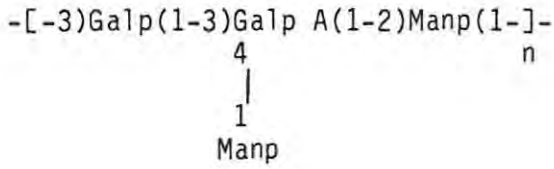
constant (4.63 ppm,7.5 Hz) of the galactose residue[70,99] in the polysaccharide its anomeric configuration was assigned as ß.

Finally, after assigning the resonances of the four anomeric protons from the chemical shift and coupling data (^{1}H and ^{13}C) of the P1 and polysaccharide spectra (see Tables A and B) there are a further two signals in the ^{1}H-n.m.r. anomeric region without assignments. These assignments were made from a ^{1}H-two dimensional correlated n.m.r. spectroscopic (2D COSY) spectrum of a structural analogue of the E.coli K36 P1 oligosaccharide (see Spectrum 13). (This analogue was derived from the deploymerisation of Klebsiella K68 capsular polysaccharide by a homologous bacteriophage). In the 2D COSY (^{1}H) experiment the chemical shifts of the individual protons are displayed along a 45° diagonal and each pair of coupled protons give rise to two symmetrically displaced off diagonal responses. The resonances are correlated to their off diagonal responses by a connectivity plot, illustrating those protons that are coupled to each other (see Figure I,Annexure 1) Using this technique we were able to assign these signals as the H-4 (4.63 ppm) and H-5 (4.49 ppm) resonances of the galacturonic acid residue.

Analyses were conducted on the neutral sugars of methylated native polysaccharide and methylated native polysaccharide reduced with lithium aluminium hydride (see Table C,columns (a) and (b)). These data showed that the galacturonic acid along with the 2-O- linked mannose and 3-O- linked galactose constitute a trisaccharide backbone to which the last mannosyl residue is linked to the galacturonic acid

as a lateral non-reducing terminal sugar. Thus, if the mannose is linked in this manner, the structure A for the oligoalditol is the correct one.

Furthermore, by comparing and contrasting the two sets of methylation data (Table C), the assumption can be made that the galactose is, in the native polysaccharide, linked to the galacturonic acid at the 3-O-position. The monosaccharide sequence is, therefore, as follows:



These results were confirmed by a periodate oxidation of the polysaccharide; it was found that there were three accessible vic diols per repeat unit in the molecule. Ebisu et al found that the side group of Pneumococcus S-14 polysaccharide could be selectively removed by a Smith degradation[58] after a limited periodate oxidation, despite the presence of susceptible linkages in the main chain.[98] With the same purpose in mind, the E.coli K36 polysaccharide was subjected to a limited oxidation (the reaction was halted after a single mole of periodate per mole of repeat unit was consumed). However, both mannose residues were equally affected by the periodate oxidation step and, hence, a stepwise oxidation was not feasible (see Spectrum 5).

iv) CONCLUSION

On the basis of these experimental results the structure of the E.coli K36 capsular antigen was established as shown in the abstract.

The use of a bacteriophage-borne endogalactosidase circumvented the need for a number of other experiments which are normally required in order to sequence the polysaccharide. Had a more traditional approach been adopted it would have been necessary to perform a partial hydrolysis as well as other complementary experiments in order to fully elucidate the polysaccharide structure. When available, a bacteriophage provides a convenient alternative to the other methods of structural elucidation and this technique may, in certain circumstances, be the only method of locating the position of attached labile constituents; for example, O-acetyl groups.[100]

The viral endogalactosidase cleaved the D-Galp($1\xrightarrow{\beta}3$)-D-Galp A-bond to produce the following oligomer:

D-Manp($1\xrightarrow{\alpha}4$)-D-Galp A($1\xrightarrow{\alpha}2$)-D-Manp ($1\xrightarrow{\alpha}3$)-D-Gal-$\overset{\alpha/\beta}{O}$H ..... P1 +89°

A survey of the literature shows that the primary structure of the E.coli K36 capsular polysaccharide is identical to that from Klebsiella K57[101] and very closely related to the structure of Klebsiella K68[70] capsular antigen. The latter differs only in the linkage of the galactose to the galacturonic acid (O-2 instead of O-3) and the presence of pyruvate on the pendant mannose. It is of interest to note that these differences are sufficient to preclude the adsorption of the heterologous (φK36,K68) bacteriophages by either strain.

Table A. ^{1}H-n.m.r. data[a] for E.coli K36 polysaccharide, derived oligo- and polysaccharides (500 MHz).

I. OLIGOSACCHARIDE P1: derived from the depolymerisation of the capsular polysaccharide with a phage-borne endogalactosidase.

Chemical shift[b] (ppm)		$J_{1,2}$ (Hz)	Integral (H)		Assignment
Ambient temperature[d]	95°C		Ambient temperature	95°C	
4.47	4.49	-	1	1	H-4 of ^{4}-Gal A$^{\alpha}$
4.62	4.62	7.5	0.3	0.45	^{3}-Gal$^{\beta}$OH
	4.72	-		0.4	H-5 of ^{4}-Gal A$^{\alpha}$
	4.74	-		0.6	H-5 of ^{4}-Gal A$^{\alpha}$
4.91	4.93	-	1	0.9	Man$^{\alpha}$
	5.13	-		0.2	^{2}-Man$^{\alpha}$Galf^{β}OH
	5.16	-		0.2	^{2}-Man$^{\alpha}$Galf^{α}OH
5.22 5.24	5.22 5.23	-	0.95	0.6	^{c2}-Man$^{\alpha}$
5.26 5.27	5.26 5.27	-	0.9	1	^{4}-Gal A$^{\alpha}$
5.28 5.29	5.28 5.29	-	0.5	0.55	^{3}-Gal$^{\alpha}$OH

II. OLIGOALDITOL P1: oligosaccharide PI reduced with $NaBD_4$

Chemical shift (ppm)	$J_{1,2}$ (Hz)	Integral (H)	Assignment
4.47	-	1	H-4 of ^{4}-Gal A$^{\alpha}$
4.72	-	0.6	H-5 of ^{4}-Gal A$^{\alpha}$
4.94	-	0.5	Man$^{\alpha}$
5.22	-	0.7	^{2}-Man$^{\alpha}$
5.29	-	0.8	^{4}-Gal A$^{\alpha}$

III. PARTIALLY AUTOHYDROLYSED NATIVE POLYMER.

Chemical shift (ppm)	$J_{1,2}$ (Hz)	Integral (H)	Assignment
4.43	-	1	H-4 of ^{3,4}-Gal A$^{\alpha}$
4.63	-	1	H-5 of ^{3,4}-Gal A$^{\alpha}$
4.68	7.5	1	^{3}-Gal$^{\beta}$
5.05	-	1	Man$^{\alpha}$ / ^{2}-Man$^{\alpha}$ / ^{3,4}-Gal A$^{\alpha}$
5.23	-	2	

IV. SMITH DEGRADED POLYSACCHARIDE: produced from native polysaccharide subjected to aqueous sodium metaperiodate for two hours prior to treatment with 0.5M trifluoroacetic acid.

Chemical shift (ppm)	$J_{1,2}$ (Hz)	Integral	Assignment
4.59	-	1	H-4 of ^{3,4}-Gal A$^{\alpha}$
4.64	7.5	0.6	^{3}-Gal$^{\beta}$
4.78	-	0.45	H-5 of ^{3,4}-Gal A$^{\alpha}$
4.98	-	0.2	Man$^{\alpha}$
5.11	-	0.4	^{2}-Man$^{\alpha}$
5.27	-	0.9	^{3,4}-Gal$^{\alpha}$

[a] Signals tabulated and assigned are those of protons with a chemical shift between 4.3 ppm and 5.5 ppm.
[b] Chemical shift downfield from 4,4-dimethyl-4-silapentane-1-sulphonate(DSS), measured from acetone (2.23 ppm) as the internal standard.
[c] The symbol ^{2}-Man$^{\alpha}$, refers to the anomeric proton signal of a 2-O-linked mannosyl residue in the α anomeric configuration. The absence of a numerical prefix, as in Man-, indicates a non-reducing terminal group.
[d] Ambient temperature is 30°

Table B. ^{13}C-n.m.r. data[a] for E.coli K36 polysaccharide, derived oligo- and polysaccharides (100MHz).

I. OLIGOSACCHARIDE P1: derived from the depolymerisation of the capsular polysaccharide with a phage-borne endogalactosidase.

Chemical shift[b] (ppm)	Assignment
93.05	^{3}-Gal$^{\alpha}$-OH
95.1	^{2}-Man$^{\alpha}$-Gal$^{\alpha}$-OH
95.41	^{2}-Man$^{\alpha}$-Gal$^{\beta}$-OH
97.04	^{3}-Gal$^{\beta}$-OH[c]
101.75	Man$^{\alpha}$
102.49	^{4}-Gal A$^{\alpha}$
173.14	COOH of ^{4}-Gal A$^{\alpha}$

II. OLIGOALDITOL P1: oligosaccharide PI reduced with $NaBD_4$.

Chemical shift (ppm)	$J_{C_1-H_1}$(Hz)	Assignment
(A.) Proton decoupled spectrum		
100.29		^{2}-Man$^{\alpha}$
101.58		Man$^{\alpha}$
102.51		^{4}-Gal A$^{\alpha}$
173.15		COOH of ^{4}-Gal A$^{\alpha}$
(B.) Proton coupled spectrum		
	169.83	Man$^{\alpha}$
	169.83	^{2}-Man$^{\alpha}$
	168.57	^{4}-Gal A$^{\alpha}$

III. NATIVE POLYMER: after partial autolysis.

Chemical shift (ppm)	Assignment
95.47	^{2}-Man$^{\alpha}$
100.79	Man$^{\alpha}$
101.65	^{3,4}-Gal A$^{\alpha}$
105.16	^{3}-Gal$^{\beta}$
173.1	COOH of ^{3,4}-Gal A$^{\alpha}$

[a] Signals tabulated and assigned are of those carbons with a chemical shift between 90 ppm and 180 ppm.
[b] Chemical shift downfield from DSS, measured from acetone as the internal standard (37.07 ppm).
[c] The symbols ^{3}-Gal$^{\beta}$ refer to the signal of the anomeric carbon of a 3-O-linked galactosyl residue in the β-configuration.

Table C. METHYLATION ANALYSIS OF K36 NATIVE POLYSACCHARIDE, CARBOXYL-REDUCED POLYSACCHARIDE AND OLIGOALDITOL P1.

STARTING MATERIAL:	METHYLATED SUGARS:					
	1,2,4,5,6-Gal[b]	2,3,4,6-Man	3,4,6-Man	2,4,6-Gal	2,3-Gal	2-Gal[d]
	T[a] 0.75	0.98	1.55	1.70	3.21	4.03
(a).NATIVE POLYSACCHARIDE[I]		1.0[c]	1.13	1.3		
(b).NATIVE POLYSACCHARIDE[II] (COOH reduced)		1.0	1.28	1.2		1.6
(c).OLIGOALDITOL P1[III] (COOH reduced)	1.29	1.0	1.55		0.79	

I, Methylated and hydrolysed.

II, Methylated, reduced with $LiAlH_4$ then hydolysed.

III, Methylated and methanolysed.

[a] Retention time relative to tetra-O-methyl-glucitol-di-O-acetate on an OV-225 column isothermally at 205°

[b] 1,2,4,5,6-Gal refers to a 1,2,4,5,6-penta-O-methyl-galactitol-3-O-acetate.

[c] Values shown are the molar ratios of the corresponding derived alditol acetates, quantification is by the use of the ECR(ref. 84) values.

[d] All assignments were confirmed by g.l.c.-m.s.

Table D. OVERALL MONOSACCHARIDE RATIOS OF K36 POLYSACCHARIDE AND DERIVED OLIGOSACCHARIDES.

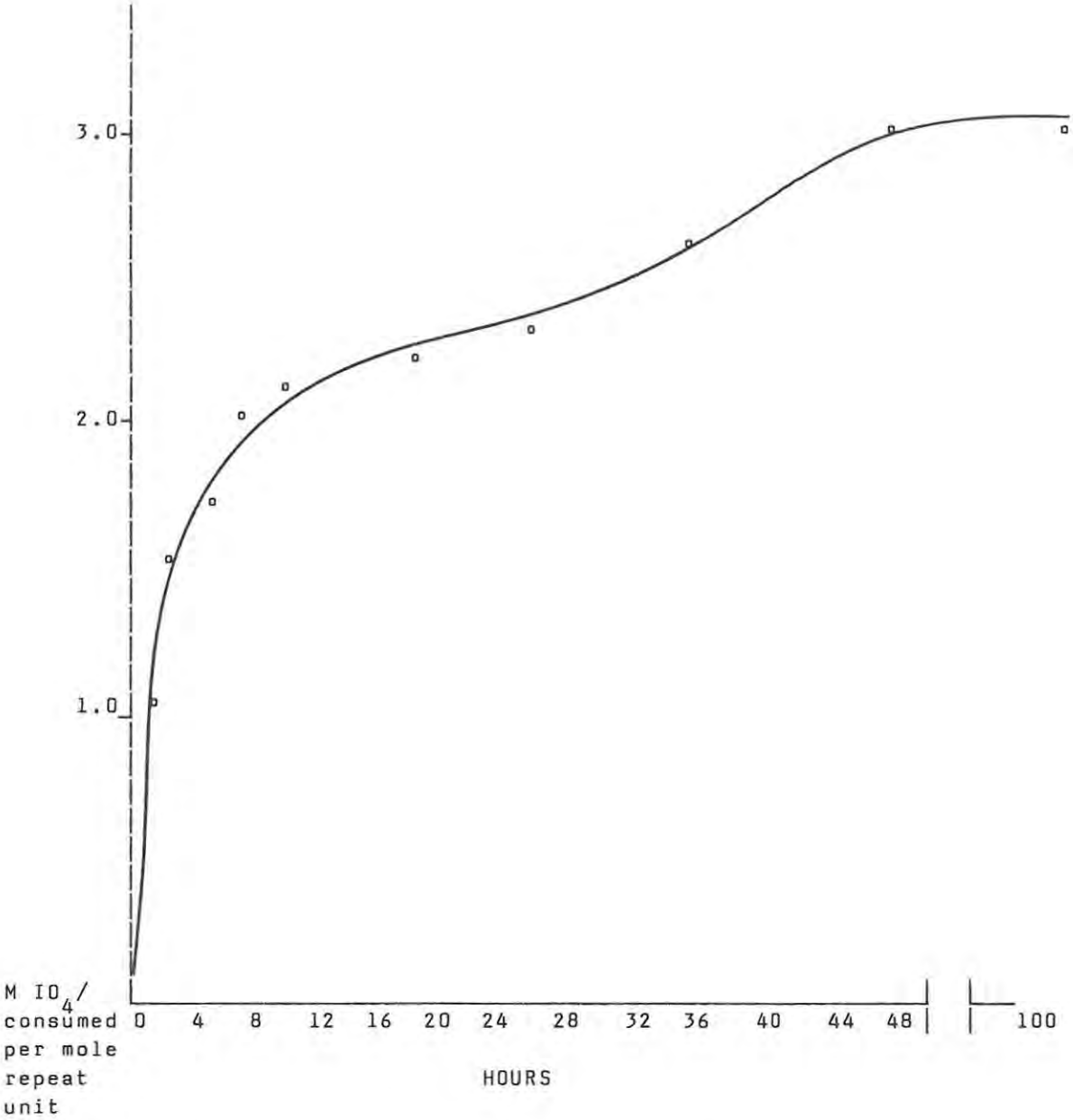
		Galactose[a]	Mannose (MOLAR RATIO).
	T[c]	13.8	17.4
1.Hydrolysate: polysaccharide (First growth).		1.95[b]	1.0
2.Hydrolysate: polysaccharide (Second growth).		2.05	1.0
3.Methanolysate: polysaccharide (First growth).		0.82	1.0
4.Methanolysate: polysaccharide (Second growth).		1.13	1.0
5.Hydrolysate: oligosaccharide		2.05	1.0
6.Methanolysate: oligosaccharide		1.22	1.0

[a] Identification of the monosaccharide PAAN derivatives was on the basis of their actual retention times, compared to those of authentic standards.
[b] Molar ratio of the monosaccharide as the peracetylated aldononitrile derivative, determined on an OV-225 Durabond® fused silica column at 250°(isothermally).
[c] Approximate retention times of the derivatives at 250°.

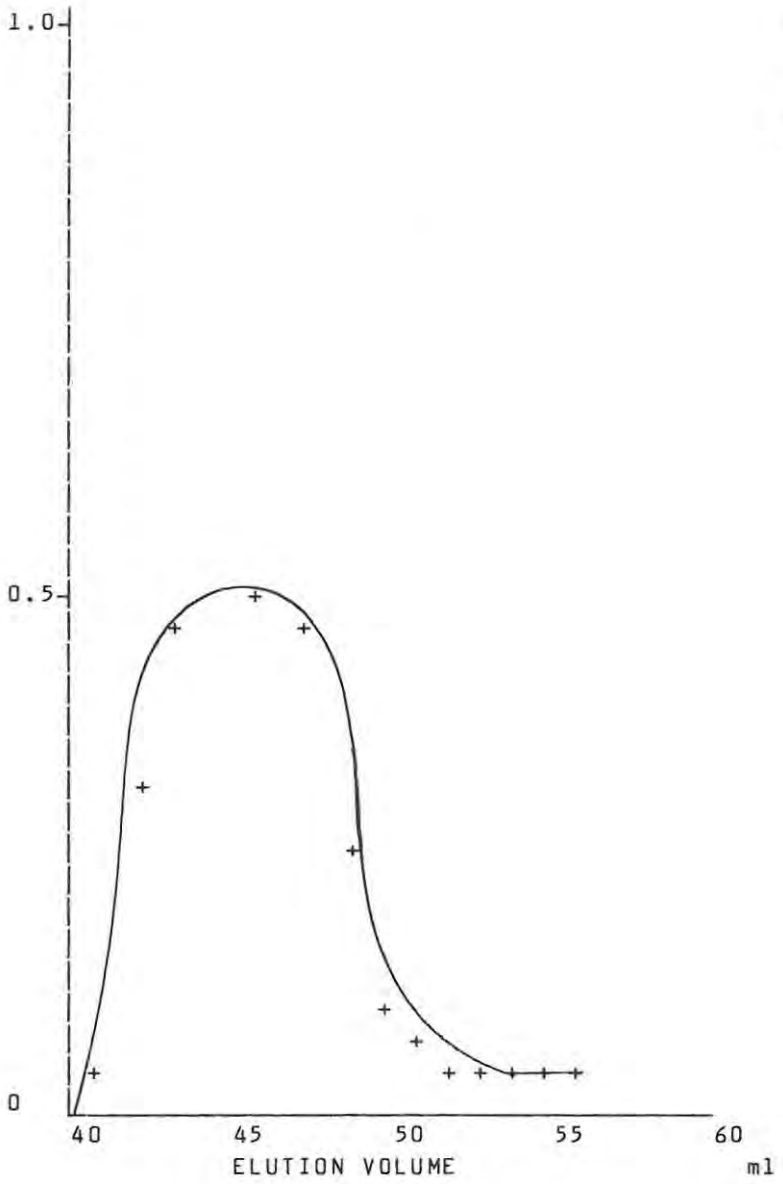
Table E. RELATIVE RETENTION TIMES OF DERIVED MANNOSE AND GALACTOSE ACETYLATED 2-(-)-OCTYL GLYCOSIDES.

D	Peak number	1	2	3	4
Mannose	T[a]	1.207(L)	1.255(S)	1.436(S)	1.546(S)
Galactose	T	1.277(L)	1.496(M)	1.539(M)	1.625(S)
L					
Mannose	T	1.170(L)	1.340(M)	1.492(S)	1.571(S)
Galactose	T	1.299(M)	1.338(L)	1.365(M)	1.905(S)

[a] Retention times relative to mannitol acetate on an OV-225 column at 230°
L-Large M-Medium S-Small peaks.



Graph 1. PERIODATE OXIDATION OF THE E.COLI K36 CAPSULAR POLYSACCHARIDE.



+NATIVE POLYMER in the acidic form.

Profile 1.ELUTION PROFILE OF THE POLYSACCHARIDE IN THE ACIDIC FORM.

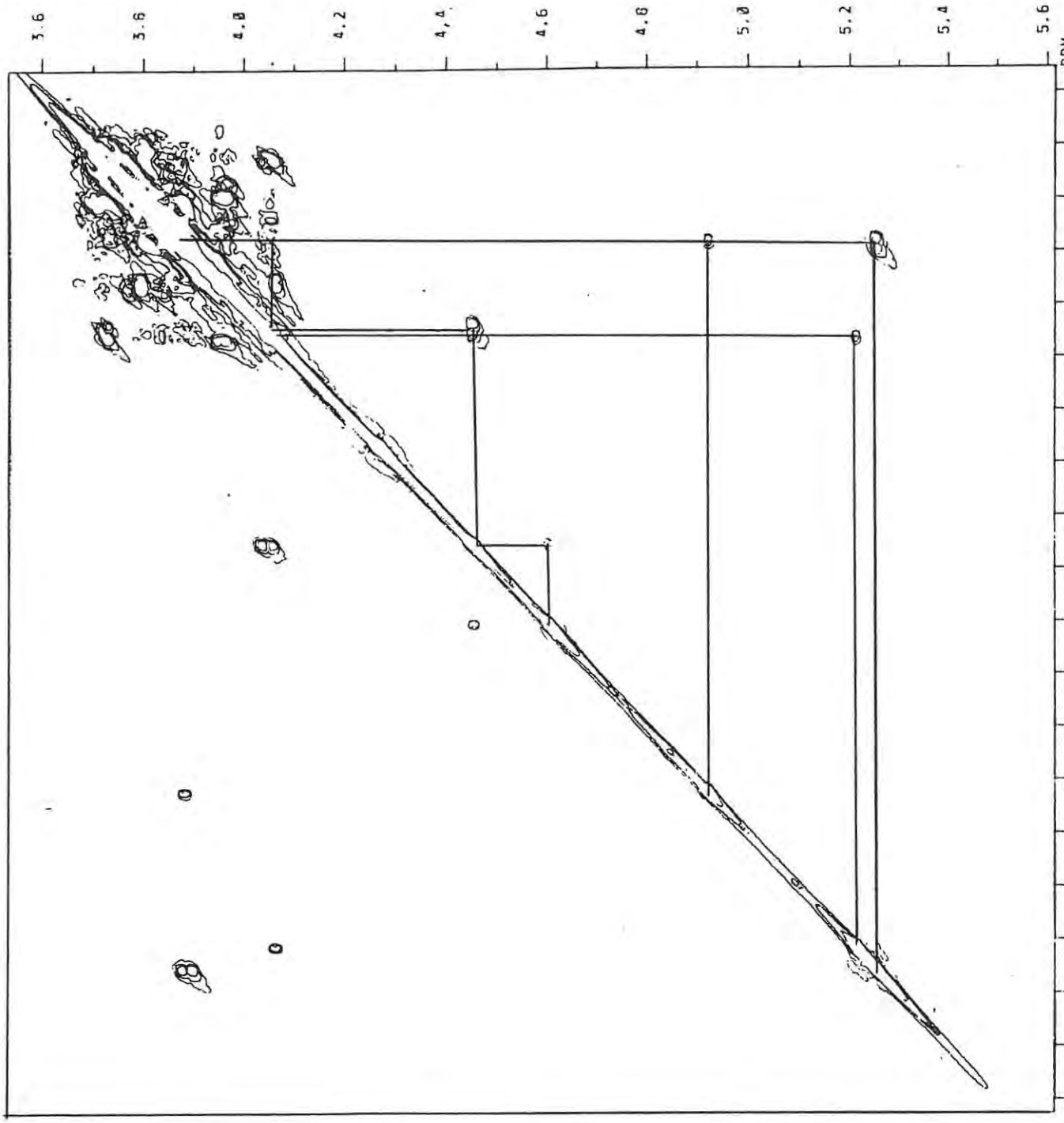


Figure I COSY correlation plot (K68) P1

v) EXPERIMENTAL

(a) General methods

Solutions were concentrated under reduced pressure at temperatures not exceeding 40°, unless specifically stated.

(b) Chromatography

For preparative and analytical paper chromatography, both by the descending method, Whatman number 1 paper was used with one of the following solvent systems as the eluant; viz.

(a). 8:2:1 v/v ethyl acetate, pyridine, water;

(b). 2:1:1 v/v 1-butanol, acetic acid, water;

(c). 5:1:5:3 v/v ethyl acetate, acetic acid, pyridine, water;

(d). 40:11:9 v/v 1-butanol, ethanol, water.

Chromatograms were either developed with silver nitrate[17] or periodate-benzidine[18] reagents. A Sepharose® 4B-CL gel column with 1M NaCl in water as the mobile phase was used for the molecular weight determining gel permeation chromatography. A Hewlett-Packard 5890 A gas-liquid chromatograph fitted with a flame ionisation detector was used for analytical g.l.c. separations. A Durabond® fused silica capillary column (30m x.25mm, wall coated with either .25μm OV-225 or OV-17) was fitted. Integration of the signal output was by a Hewlett-Packard 3392 A recording integrator. G.l.c.-m.s. was performed with a V.G. Micromass 16F spectrophotometer; spectra were

recorded at 40eV with an ion source temperature of 170°.

(c) Nuclear magnetic resonance spectroscopy

Proton magnetic resonance experiments on the samples (20mg±) were recorded at 95° and, in some cases, at ambient temperature (30°) using a Bruker WM 500 MHz FT spectrometer. Preparation was by dissolution of the sample in 99.8% deuterium oxide followed by lyophilisation. This was repeated three times after which single exchanges with 99.96% and 99.996% D_2O followed. It was then run in 99.996% D_2O with acetone (2.23 ppm-^1H, 31.07ppm-^{13}C) as the internal standard. (This was measured downfield from aqueous sodium 4,4-dimethyl-4-silapentane for ^1H-n.m.r. spectra and from DSS for ^{13}C n.m.r. spectra). In all cases except where stated, all the ^{13}C determinations were proton-decoupled experiments.

(d) Optical rotation

The optical rotation of a filtered solution (8.0μm millipore LCWP filter) of the sample was determined in a 0.1dm cell at 23°(±2°) with a Perkin Elmer model 141 polarimeter (sodium lamp).

(e) Polysaccharide isolation

A culture of E.coli O9:H19:K36 (culture no. A 198a) was obtained

from Drs. F. and I. Orskov and grown on Mueller-Hinton agar (see Annexure 2) with 2% NaCl added. Five trays were used (1.5l of medium, surface area: 1650cm^2). The slime was harvested, diluted with an equal volume of 2% aqueous phenol and the extraction was allowed to continue for 20 hours (4°), whereafter the cells and debris were removed by ultacentrifugation at 35K (5°) for 180 minutes. The supernatant was added to a large volume of ethanol (6x that of the supernatant) and cooled to 4° prior to the precipitate being removed. The precipitate was then dissolved in water and precipitated a second time using 5% cetyltrimethylammonium bromide to form an insoluble complex with the acidic polysaccharide. After recovery, this complex was broken by dissolving the precipitate in 3M NaCl, whereupon ethanol precipitation and several dialyses were performed to purify the polysaccharide further. The polysaccharide (1.1743g) was obtained finally as the solid sodium salt after freeze-drying the contents of the dialysis bag.

(f) Bacteriophage depolymerisation

The polysaccharide (508mg) as the sodium salt was dissolved in a 250ml suspension containing 2.8 x 10^{13} plaque forming units (PFU) of the homologous K36 bacteriophage which had been isolated by methods described previously.[102] (see page 94, this thesis). This suspension had been partially purified by dialysis (M_w cutoff 12 000 -14 000, for 2 days) against tap water. The phage/polymer mixture was stirred

at 29° for three days with chloroform added to discourage bacterial growth, after which time the mixture was freeze dried and the lyophilisate (743mg) dissolved in 50ml of distilled water and dialysed exhaustively (M_w cutoff <8 000) against distilled water (five changes of 50ml each). The dialysates were combined, freeze dried and oligosaccharide P1 was isolated by paper chromatography in solvent C.

Oligosaccharide P1 (20mg/10ml water) was reduced to the oligoalditol with $NaBH_4$ (200mg). Excess borohydride was removed by passage down an Amberlite® IR-120 $(H)^+$ column, followed by additions of methanol (2ml) and evaporation to dryness under reduced pressure repeated several times.

(g) Analysis of the total sugar ratio

Hydrolysis of a sample (5mg) was effected with 2M TFA (2ml)at 100° for approximately 16 hours, after which the TFA was removed and the sample concentrated under reduced pressure. The liberated sugars were transformed into peracetylated aldononitriles (PAANs) using the method described previously by Mc Ginnis.[21] Another sample (6mg) was methanolysed with 3% anhydrous HCl (2ml) at 100° for approximately 16 hours under dry refluxing conditions and the acid neutralised with $PbCO_3$ (100mg). Insoluble material was removed by centrifugation, whereafter the solution was treated with $NaBH_4$ (200mg). Finally the solution was passed down an Amberlite® IR-120 $(H)^+$ column, methanol added to the eluate (2ml x 4) which was concentrated under reduced

pressure. The methyl glycosides were cleaved with 2M TFA (2ml,16h,100°) to release the free reducing sugars which were converted to PAANs. Analysis of the hydrolysate and methanolysate PAAN mixtures was by g.l.c. on an OV-225 column at 225° (see Table D).

In addition, some of the underivatised hydrolysate and methanolysate was also separated by paper chromatography using solvent systems A,B,C,and D. In both cases, silver nitrate-sodium thiosulphate was used to develop the chromatograms; monosaccharides were identified by comparison with a standard containing all the commonly encountered sugars, run concurrently with the sample.

(h) Determination of the absolute configuration of the sugar components

The D-/L- configuration of the monosaccharide units present was determined by the g.l.c. analysis of the derived acetylated octyl glycosides on an OV-225 column. These were derived by reacting[34] the free reducing monosaccharides from a hydrolysate (10mg) and a methanolysate (12mg) with 2(-)octanol (0.5ml,140°,18hr). Removal of the octanol was at 100° under reduced pressure and was followed by acetylation (pyridine,acetic anhydride 1:1,100°,1hr.) of the derived octyl glycosides. Analysis was on an OV 225 column at 230°. Assignments were made by comparing the results with those in Table E.

(i) Methylation analysis

The polysaccharide (48mg, as the acid) was methylated under

Hakomori conditions,[41] followed by the Kuhn methylation procedure.[44] Hydrolysis of the permethylated polymer (26mg) with 2M TFA (2ml,100°,12hr) was followed by reduction with $NaBH_4$ (200mg in 5ml H_2O,1hr). The solution was passed down an Amberlite® IR-120 $(H)^+$ column, whereafter, methanol (2ml) was added to the eluate which was evaporated to dryness (repeated four times). Acetylation (pyridine,acetic anhydride 1:1,100°,1hr) of the product afforded a mixture of partially methylated alditol acetates.

Methylated polymer(23mg) was reduced with $LiAlH_4$ (200mg) in anhydrous tetrahydrofuran (4ml) at 23° (±2°) for 12 hours. The sample was then heated to 65° for 16 hours under reflux conditions. Excess $LiAlH_4$ was removed by the addition of ethyl acetate (5ml) then water (10ml). After acidification with tartaric acid (100mg), the methylated and carboxyl reduced polysaccharide was extracted with $CHCl_3$ (4X20ml). The extract was passed down an Sephadex® LH 20 column, concentrated, hydrolysed, and derivatised. Analysis was on an OV-225 column at 205° (see Table C).

The oligosaccharide P1 (20mg) was treated under Hakomori conditions[41] after being treated with $NaBH_4$ (200mg) for an hour, the permethylated oligoalditol was methanolysed using the same procedure as detailed in section g. The free permethylated monosaccharides from the oligosaccharide were converted into alditol acetate derivatives.[23] All permethylated alditol acetates were separated and analysed by g.l.c on an OV-225 and OV-17 columns (205°) (see Table C). Confirmation of these results was by g.l.c.-m.s. analysis.

(j) Periodate Oxidation

A solution of the polysaccharide (1.5mg/ml) was mixed with an equal volume of an 0.03M $NaIO_4$ solution. The reaction which was allowed to proceed at 24°(±2°) in the absence of light, was monitored spectrophotometrically for the $NaIO_4$ concentration, using a Beckman DB u.v. spectrophotometer (214nm); by reference to a calibration curve the moles of $NaIO_4$ consumed during the reaction were determined (see Graph 1).

To 0.03M sodium metaperiodate solution (20ml) was added polysaccharide (30mg in 20ml of water). The oxidation was monitored spectrophotometrically and the reaction was terminated by the addition ethylene glycol (5ml) after a single mole of periodate per mole of repeating unit had been consumed. After dialysis for two days (M_w cutoff 4 000), the solution was freeze dried and the material recovered was made up to 50ml with distilled water and reduced with $NaBH_4$ (100mg) for 12 hours, excess borohydride was neutralised with 20% acetic acid and the solution dialysed (3d,tap water,M_w cutoff 4 000) and freeze-dried. The freeze dried product (23mg) was D_2O exchanged and analysed by ^{1}H-n.m.r. spectroscopy (see Table A,IV and Spectrum 4).

(k) Partial autohydrolysis (PA) of the polysaccharide

The polymer was converted to the acidic form by passage down an Amberlite® IR-120 $(H)^+$ column, the eluate was heated to 60° for 10 minutes, dialysed (M_w cutoff 12 000) for 24 hours against tap water and then the contents of the dialysis bag were freeze-dried.

5 THE PREPARATION AND N.M.R. STUDY OF THE REPEATING UNIT OF *KLEBSIELLA* K68 POLYSACCHARIDE

5 THE PREPARATION AND N.M.R. STUDY OF THE REPEATING UNIT OF KLEBSIELLA K68 POLYSACCHARIDE

i) ABSTRACT

The Klebsiella K68 acidic capsular polysaccharide was treated with the homologous Klebsiella K68 bacteriophage which was isolated from sewage. This bacteriophage, which possesses an endoglycanase that cleaves Galp(1-2)Galp A bonds, produced several oligosaccharides. The oligosaccharides correspond to the repeat unit of the intact polysaccharide and multiples thereof. The structure of the smallest oligosaccharide, designated P1, which corresponds to a single intact repeat unit, was found to be as follows:

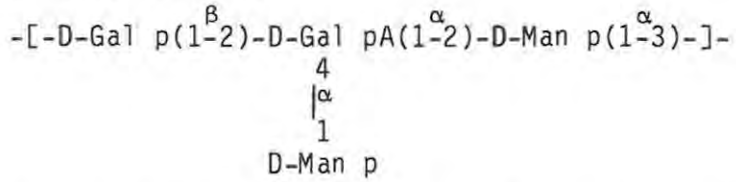
(Pyruvalated)-Manp($1\overset{\alpha}{-}3$)Galp A($1\overset{\alpha}{-}2$)Manp($1\overset{\alpha}{-}3$)Gal$\overset{\alpha/\beta}{-}$OH P1

This tetrasaccharide, without pyruvate, was isolated during the structural elucidation of the E.coli K36 acidic capsular polysaccharide.

ii) INTRODUCTION

Stirm demonstrated[74] that there are bacteriophages which are specific for Enterobacteriaceae K strains and these often possess glycosidases that depolymerise the acidic capsular polysaccharides. Each of these enzymes is specific for one or a few capsular polysaccharides. The products of the depolymerisation are oligosaccharides that consist of either one or more repeating units of the polysaccharide and these can be valuable in the study of the capsular polysaccharide.

The capsular polysaccharide of Klebsiella K68 has been reported[70] to have a pyruvalated tetrasaccharide repeating unit, which is composed of D-galacturonic acid and D-mannose and D-galactose in the ratio of 1:2:1. The polysaccharide structure, which was established[70] by methylation analysis, Smith degradation, and partial hydrolysis studies, is as follows:



During the partial hydrolysis study only non-pyruvalated oligosaccharides were isolated. We now report on the production of the intact pyruvalated tetrasaccharide repeating unit (P1) of K68 by bacteriophage depolymerisation, its isolation by gel permeation chromatography and on the n.m.r. spectroscopic study of P1.

iii) RESULTS AND DISCUSSION

The propagation of the Klebsiella K68 bacteriophage on its host proved difficult as the titre of the phage suspensions from the flask lyses (at 37°, dialysable P medium[102] (see Annexure 2)) was approximately 1 x 10^8 plaque forming units(PFU)/ml. (In general, for a selective enzymic hydrolysis approximately 250ml of a 4 x 10^{10} PFU/ml suspension is required to depolymerise one gram of polysaccharide). It appeared that the copious quantities of capsule produced by the Klebsiella K68 bacteria (capsular polysaccharide was harvested from a culture in high yield- 560mg per litre of solid medium[35]) and the slow growth characteristic of this strain adversely affected the ability of the

phage to adhere to the bacteria and to multiply. Several attempts were made to improve the yield of the phage. The lysis was carried out at 29° as it was noticed in this laboratory, that K68 grew faster at this temperature. However, there was no appreciable improvement in the titre of the product. The host was grown in a carbon restricted medium (see Annexure 2) at 29° and, again, the result was the same (titre: 1×10^8 PFU/ml). It was unlikely that we were going to get a sufficiently concentrated phage solution from a flask lysis. Consequently, all the phage suspensions were pooled (2 800ml, 9×10^7 PFU/ml) and concentrated to a volume of 250ml and a titre of 1×10^9 PFU/ml, by dialysis against a saturated solution of polyethlene glycol 6 000. Although there were only 2.5×10^{11} PFU this suspension was used to depolymerise the polysaccharide (500mg), for, even though the yield of P1 was low, about 40%, this was outweighed by the advantage of obtaining other useful oligosaccharides (P2 etc.).

After depolymerisation (three days,29°) the saccharide/phage mixture was freeze dried. The crude lyophilisate, when examined by analytical gel permeation chromatography on a Biogel® P4 column at 50° and at ambient temperature (24°) showed four and three distinct peaks respectively (see Profiles 2 and 3). This material was dialysed against distilled water and low molecular weight material (less than 4 000) from the dialysate, corresponding to the first, second and third days of dialysis and the contents of the dialysis bag, were separated by preparative gel permeation chromatography on a Biogel® P4 column

(see Profiles 3-6). The eluate corresponding to the slowest eluting peak was collected and lyophilised; carbohydrate material from this fraction was shown by n.m.r. analysis to be oligosaccharide P1. Higher oligosaccharides were isolated, but were not investigated further. Oligosaccharide P1 was subjected to a series of n.m.r. experiments (see Tables F and G).

The spectra obtained from ^{1}H- and ^{13}C-n.m.r. analysis of P1 were clear and the information obtained, particularly when considered in conjunction with the data from K68 polysaccharide, was unambiguous. Previous analyses[70] of the K68 polysaccharide had shown a β galactose as well as galacturonic acid and two mannose residues which were all of the alpha anomeric configuration. Comparison of the ^{1}H-n.m.r. spectra of K68 polysaccharide (Table F,I) and of K68 P1 (Table F,II) shows that a β linkage has been cleaved by the phage enzyme and this establishes the galactose as the reducing terminus of P1. From a methylation analysis,[70] we know that, with the exception of the attached pyruvate group and the β galactose linkage at O-2 instead of O-3 of the galacturonic acid residue, the linkage, sequence and composition of the Klebsiella K68 and E.coli K36 polysaccharide are the same. Since the β galactose linkage has been cleaved, P1 has the same linkage and sugar sequence as P1 from K36 polysaccharide. Comparing the ^{1}H-n.m.r. data from both oligosaccharides (Tables A and F,Spectra 2 and 10) a few differences were apparent. These can be ascribed to the influence of the pyruvate moiety, since this is the only difference between the two oligosaccharides.

Although the majority of the non-anomeric signals are contained

in a region approximately 1 ppm wide, a few are shifted downfield into the anomeric region. For example, it has been shown that the H-5 glucuronic acid signal resonates in the anomeric region, especially when the anomeric configuration is α-D-.[103] The presence of these signals has led to a degree of confusion over signal assignments. In the structural elucidation of Klebsiella K17 Dutton and Folkman found that the spectrum of the polysaccharide was difficult to interpret as there were six signals present between 4.5 and 5.5 ppm one of which was a non-anomeric signal. They assigned the signal at 4.60 ppm to the β-D-glucose residue.[103] However, as mentioned by the authors,[103] the coupling constant of 10 Hz is larger than that normally associated with β-D-glucose. Thus, the possibilty exists that this signal and the H-5 glucuronic acid signal have been incorrectly assigned. To confidently assign the non-anomeric signals in the oligosaccharide ^{1}H-n.m.r. spectra, a two dimensional correlation (2D COSY) experiment was performed upon the Oligosaccharide P1 (depyruvalated). The 2D COSY experiment, by way of a connectivity plot linking all coupled protons with their symmetrically displaced off-diagonal responses, identifies coupled protons and it is possible to positively assign signals on the basis of their couplings. The P1 spectrum has two non-anomeric signals in the region 4.4 to 5.5 ppm, that is, there are a total of seven signals in the anomeric region. From the 2D COSY (see Spectrum 13) it was evident that both non-anomeric resonances arose from the α-D-galacturonic acid. The one (4.73 ppm), identified from the correlation plot as the H-5 proton of the uronic acid, has been

assigned as such before,[70] the second signal was assigned as H-4 of the acid. These assignments were confirmed by the expanded scale plot of the 1D spectrum; H-4 (double doublet) and H-5 (doublet) both have a coupling of 1.5 Hz, values which are consistent for a galacturonic acid H-4 and a H-5 signal.[104] The H-2 resonance signals of both mannose residues could be unambiguously identified and it is noteworthy that a 2-O- linkage has a downfield effect of .17 ppm on this signal, but does not shift it into the anomeric region.

iv) CONCLUSION

When a suitable molecular weight range gel is available, gel permeation is an appropriate means of separating the oligosaccharides produced by bacteriophage enzyme hydrolysis, allowing not only the recovery of the P1 oligosaccharide but also the recovery of P2 and P3 oligosaccharides. These can be used during a structural analysis to provide complementary data to that obtained from the P1 molecule.

In concurrence with the n.m.r. results, the structure of the oligosaccharide P1, produced by the action of the viral endogalactosidase on the Klebsiella K68 capsular polysaccharide, is as follows:

$$\begin{array}{l} \text{Manp}(1\overset{\alpha}{-}4)\text{Galp A}(1\overset{\alpha}{-}2)\text{Manp}(1\overset{\alpha}{-}3)\text{Gal}\overset{\alpha/\beta}{-}\text{OH} \\ \quad\ \text{X} \\ \quad\ \text{Pyr} \end{array}$$

This is in agreement with the structure assigned to the Klebsiella K68 capsular antigen by Dutton et al.[70]

Table F. ^{1}H-n.m.r. data[a] for Klebsiella K68 polysaccharide, derived oligo- and polysaccharides (500 MHz).

I. OLIGOSACCHARIDE P1: derived from the depolymerisation of the capsular polysaccharide with a phage-borne endogalactosidase.

Chemical shift (ppm)	$J_{1,2}$ (Hz)	Integral (H)	Assignment
1.58	-	2.2	CH_3 of Pyruvate
4.44	-	0.9	H-4 of ^{4}-Gal A$^{\alpha}$ .....Pyr[d]
4.48	-	0.3	H-4 of ^{4}-Gal A$^{\alpha}$
4.63	7.5	0.4	^{3}-Gal$^{\beta}$OH
4.71	-	0.8	H-5 of ^{4}-Gal A$^{\alpha}$ .....Pyr
4.73	-	0.2	H-5 of ^{4}-Gal A$^{\alpha}$
4.90	-	0.7	Man$^{\alpha}$ .....Pyr
4.94	-	0.3	Man$^{\alpha}$
5.13	-	0.2	^{2}-Man$^{\alpha}$Galf^{β}OH
5.16	-		^{2}-Man$^{\alpha}$Galf^{α}OH
5.23	-	0.5	^{2}-Man$^{\alpha}$[c] .....Pyr
5.27	-	1.1	^{2}-Man$^{\alpha}$
5.28	-		^{4}-Gal A$^{\alpha}$
5.29	-		^{3}-Gal$^{\alpha}$OH

II. OLIGOALDITOL P1: oligosaccharide P1 reduced with $NaBH_4$.

Chemical shift (ppm)		$J_{1,2}$ (Hz)	Integral (H)		Assignment
Ambient temperature	95°C		Ambient temperature	95°C	
1.58	1.58	-	2.9	1.3	CH_3 of Pyruvate
4.43	4.43	-	1.1	0.7	H-4 of ^{4}-Gal A-$^{\alpha}$ .....Pyr
	4.47	-		0.5	H-4 of ^{4}-Gal A-$^{\alpha}$
	4.72	-		0.4	H-5 of ^{4}-Gal A-$^{\alpha}$ .....Pyr
	4.74	-		0.6	H-5 of ^{4}-Gal A-$^{\alpha}$
4.86	4.89	-	1	0.7	Man-$^{\alpha}$ .....Pyr
	4.93	-		0.4	Man-$^{\alpha}$
5.24	5.21	-	1.0	1.1	^{2}-Man-$^{\alpha}$
5.28	5.27	-	0.9	0.4	^{4}-Gal A-$^{\alpha}$
	5.29	-		0.6	^{4}-Gal A-$^{\alpha}$

III. PARTIALLY AUTOHYDROLYSED NATIVE POLYMER.[e]

Chemical shift (ppm)	$J_{1,2}$ (Hz)	Integral (H)	Assignment
1.68	-	0.3	CH_3 of pyruvate
4.52	-	1	H-4 of ^{3,4}-Gal A$^{\alpha}$
4.65	7.5	1	^{3}-Gal$^{\beta}$
4.72	-	1	H-5 of ^{3,4}-Gal A$^{\alpha}$
4.95	-	1	Man$^{\alpha}$
5.21	-	1	^{2}-Man$^{\alpha}$
5.46	-	1	^{3,4}-Gal A$^{\alpha}$

[a] Signals tabulated and assigned are those of protons with a chemical shift between 4.3 ppm. and 5.5 ppm.
[b] Chemical shift downfield from 4,4-dimethyl-4-silapentane-1-sulphonate(DSS), measured from acetone (2.23 ppm.) as the internal standard.
[c] The symbols ^{2}-Man$^{\alpha}$, refer to the anomeric proton signal of a 2-O- linked mannosyl residue in the α anomeric configuration. The absence of a numerical prefix, as in Man-, indicates a non-reducing terminal group.
[d] Resonances from protons in the oligosaccharide when a pyruvate is attached to the molecule.
[e] Reference 70.

Table G. ^{13}C-n.m.r. data[a] for Klebsiella K68 polysaccharide, derived oligo- and polysaccharides (100MHz).

I. OLIGOSACCHARIDE P1: derived from the depolymerisation of the capsular polyasaccharide with a phage-borne endogalactosidase.

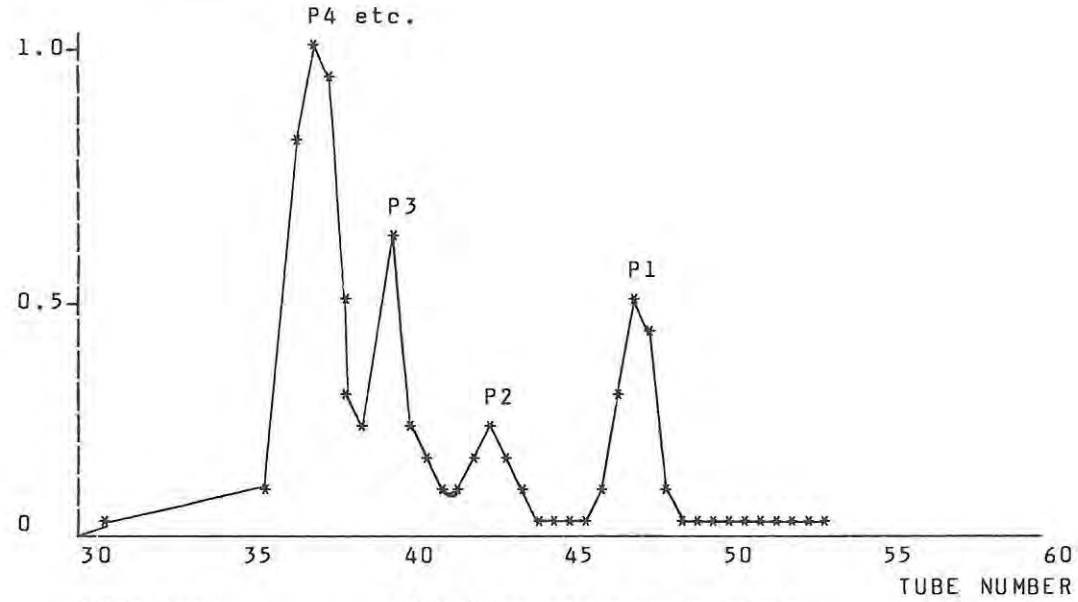
Chemical shift[b] (ppm)	Assignment
25.45	CH_3 of Pyruvate
93.01	^{3}-Gal$^{\alpha}$OH
95.01	^{2}-Man$^{\alpha}$-Gal$^{\alpha}$OH
95.39	^{2}-Man$^{\alpha}$-Gal$^{\beta}$OH
97.14	^{3}-Gal$^{\beta}$OH[c]
100.76	Man$^{\alpha}$
101.76	Man$^{\alpha}$ .....Pyr
102.51	^{4}-Gal A$^{\alpha}$
103.06	^{4}-Gal A$^{\alpha}$ .....Pyr
173.16	COOH of ^{4}-Gal A$^{\alpha}$
174.01	COOH of ^{4}-Gal A$^{\alpha}$ ....Pyr

II. OLIGOALDITOL P1: derived by reducing oligosaccharide PI with $NaBH_4$.

Chemical shift (ppm)	Assignment
25.27	CH_3 of Pyruvate
99.97	^{2}-Man$^{\alpha}$
101.24	Man$^{\alpha}$
102.70	^{4}-Gal A$^{\alpha}$
172.54	COOH of ^{4}-Gal A$^{\alpha}$

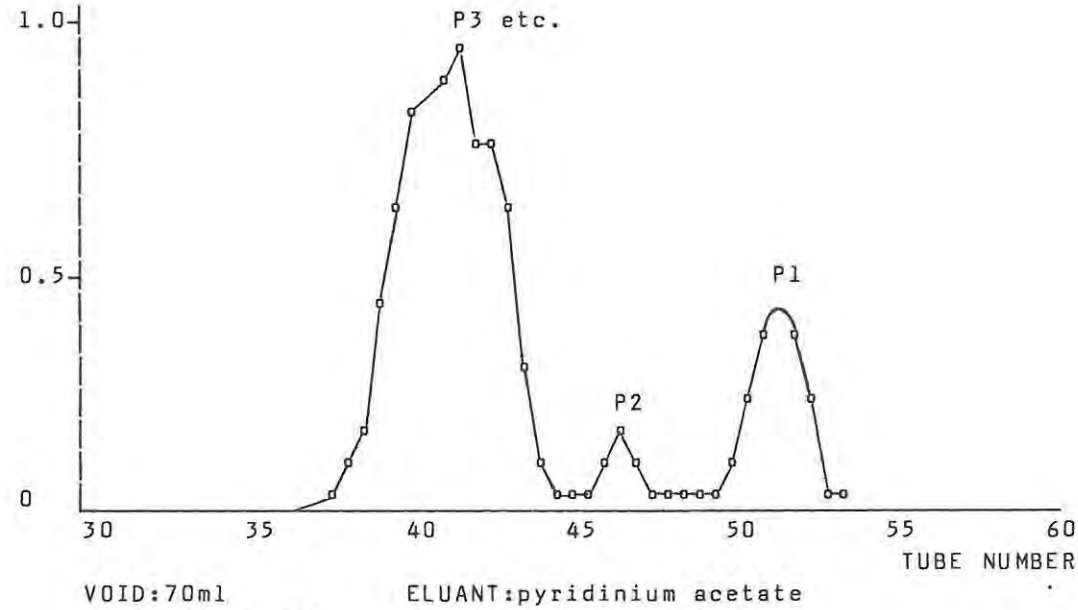
[a] Signals tabulated and assigned are of those carbons with a chemical shift between 90 ppm and 180 ppm.
[b] Chemical shift downfield from DSS, measured from acetone as the internal standard (37.07 ppm).
[c] The symbols ^{3}-Gal$^{\beta}$ refer to the signal of the anomeric carbon of a 3-O- linked galactosyl residue in the β-configuration.
[d] These are the resonances from carbons in oligosaccharides where a pyruvate group is attached.

iv). COLUMN 1.6 X 40cm.



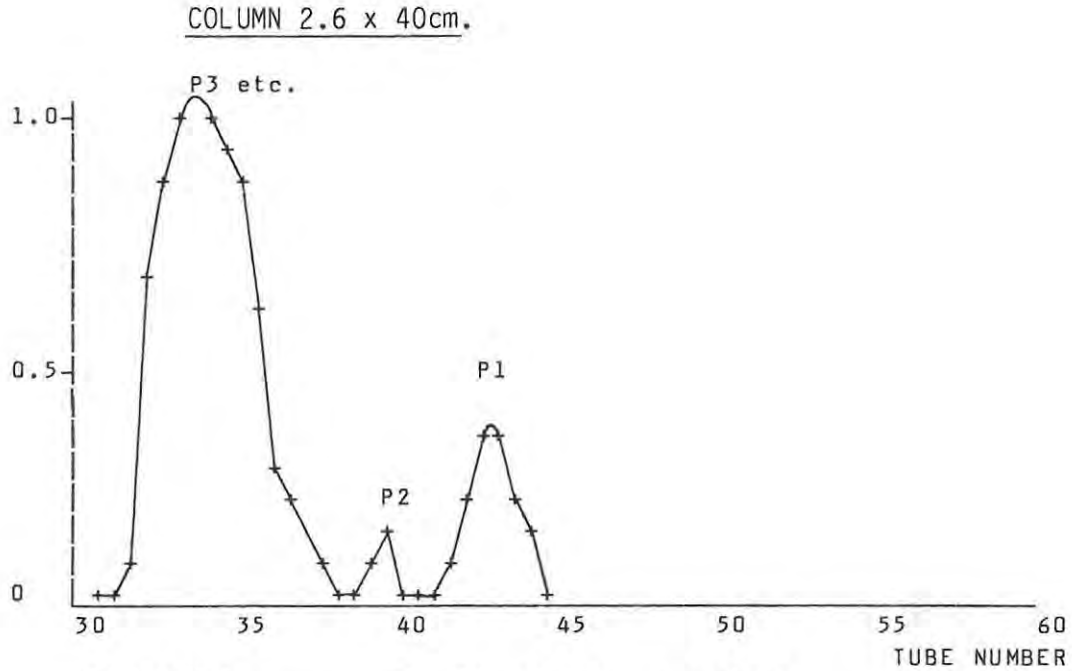
VOID:50ml ELUANT:pyridinium acetate
TUBE VOLUME:5ml TEMP:50°

Profile 2. ANALYSIS AT 50° OF THE CRUDE PRODUCT FROM K68 ENZYMIC HYDROLYSIS.

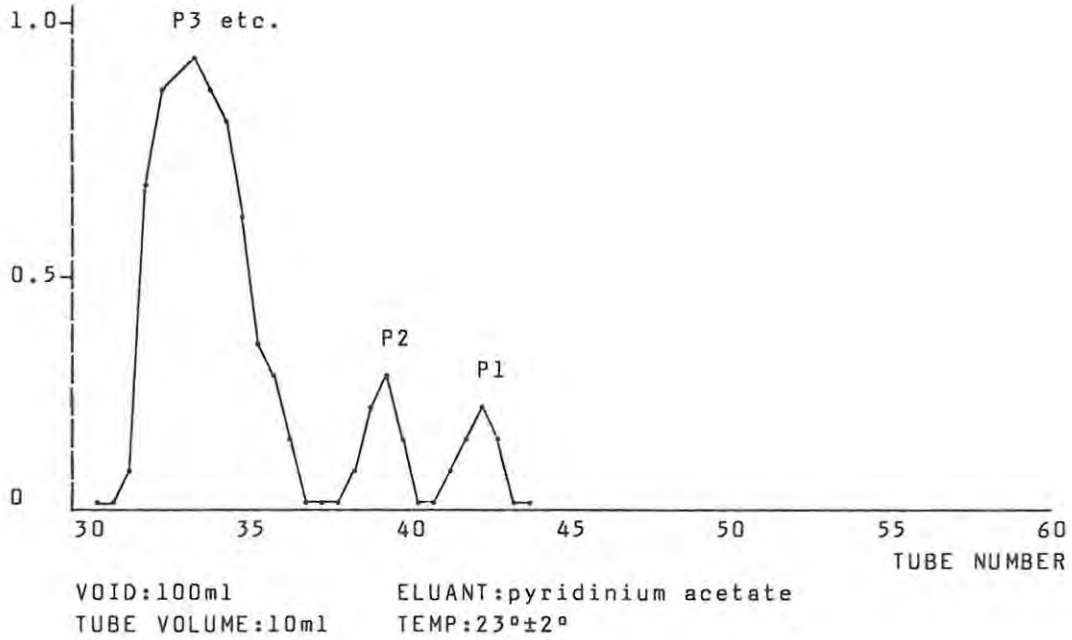


VOID:70ml ELUANT:pyridinium acetate
TUBE VOLUME:5ml TEMP:24°

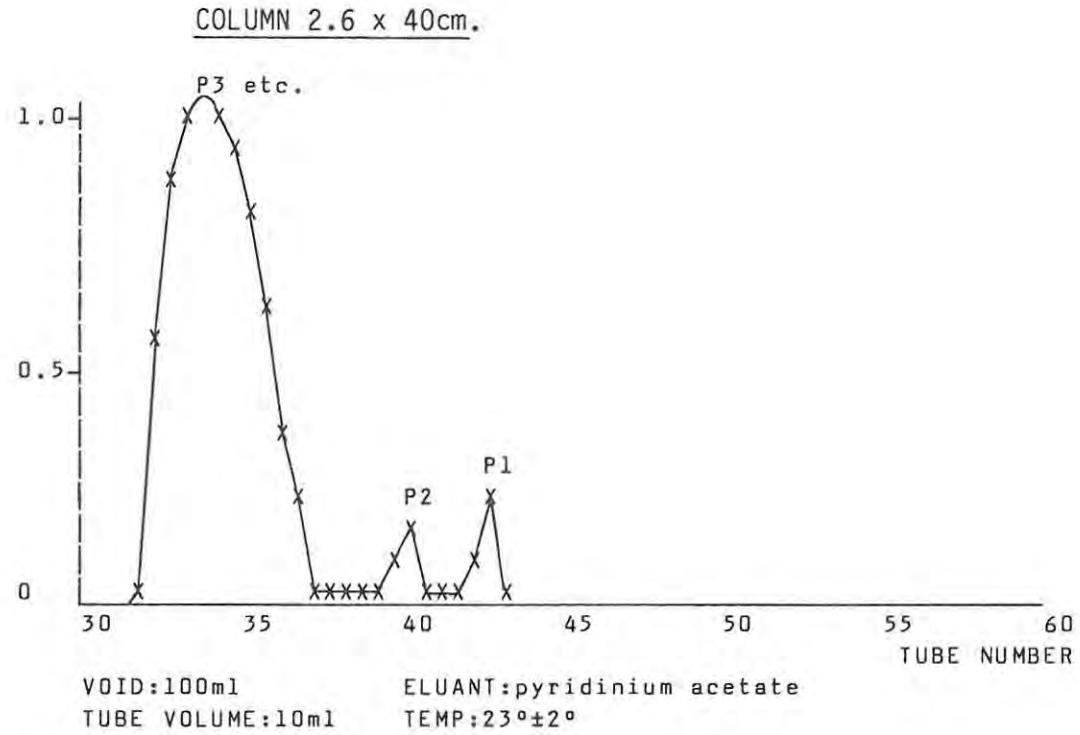
Profile 3. ANALYSIS AT 24° OF THE CRUDE PRODUCT FROM THE K68 ENZYMIC HYDROLYSIS.



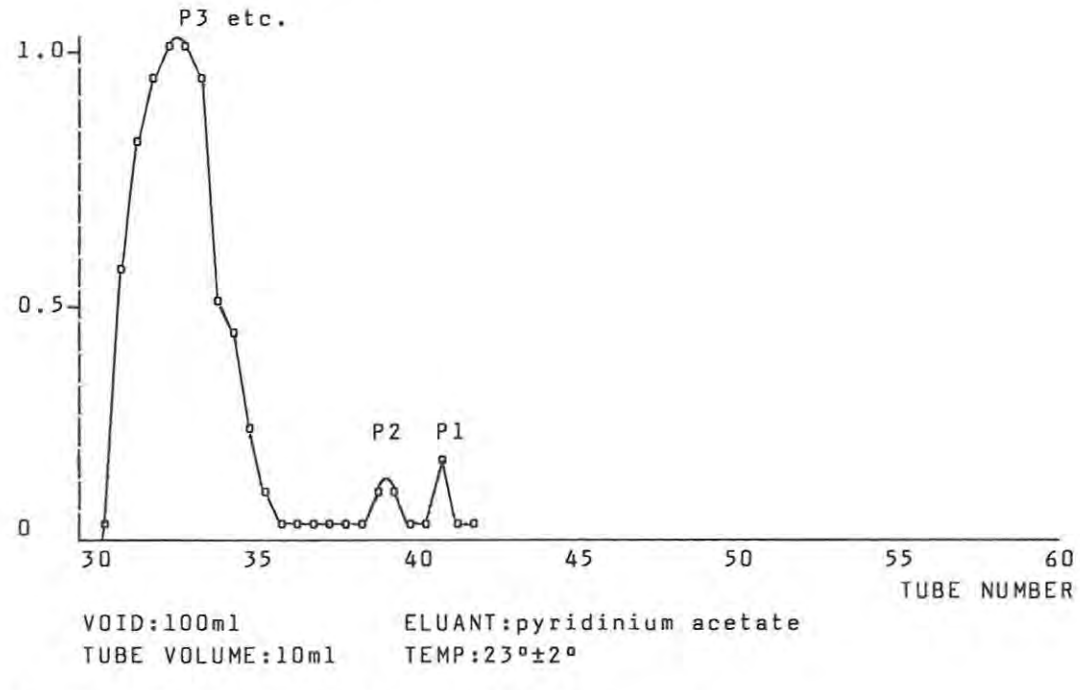
Profile 3. MATERIAL FROM FIRST DAY OF DIALYSIS.



Profile 4. MATERIAL FROM THE SECOND DAY OF DIALYSIS.



Profile 6. MATERIAL FROM THIRD OF DIALYSIS.



Profile 7. CONTENTS OF THE DIALYSIS BAG.

vi) EXPERIMENTAL

The procedures used in the structural study of Klebsiella K68 capsular polysaccharide were essentially the same as those used in the elucidation of the E.coli K36 antigen; and therefore, only those methods which differ will be discussed.

(a) Bacteriophage isolation

To isolate a suitable bacteriophage a 10ml culture (6hr. old) of Klebsiella K68 (obtained from Drs. F. and I.Ørskov,Copenhagen) was added to 10ml of sewage (from Port Elizabeth) and 50ml of nutrient broth (see Annexure 2). This was shaken at 37° for six hours, after which a small amount (5ml) of chloroform was introduced. The bacterial cells and other debris were then removed by centrifugation (15K x 10min.), leaving a clear supernatant containing the bacteriophages. Bacteriophage activity was tested for by applying a drop (approximately 0.006ml) of the supernatant (10^{-2} to 10^{-8} serial dilution of the suspension in saline) to a fresh lawn of the host bacterium (Mueller-Hinton agar plates seeded with Klebsiella K68). As a control a drop of pure saline was applied to a lawn and incubated at 29°. After 12 hours at 29° the plates were inspected for clear areas i.e. plaques. Whereafter, in order to purify the bacteriophages found, a single plaque with a large halo was picked from the the 10^{-6} dilution plate and added to a nutrient broth tube (5ml), freshly innoculated with the host strain. This bacteriophage/host

mixture was centrifuged (with 1ml chloroform added) after incubation at 37° for six hours. A pure line of the phage was established by successive single plaque isolations. Assays of the phage containing suspensions were done by applying a known volume of a serial dilution to a fresh lawn of the host, which was incubated at 29° for 12 hours. Plates with between 30 and 300 plaques, had the number of plaques counted. After the dilution was corrected for, the titre of the suspension was quoted from these numbers as plaque forming units (PFU) per ml of suspension.

The titre and volume of the bacteriophage were increased first by a tube lysis and then several flask lyses. Tube lysis involved inoculating six 10ml nutrient broth tubes with 1ml of a six hour old bacterial cell culture after which at 0, 15, 30, 45, 60 and 75 minutes successive tubes were innoculated with 0.1ml of the purified phage suspension. Those tubes that cleared after several hours were treated with $CHCl_3$ to kill any remaining bacterial cells and centrifuged (15K x 10 min.) leaving pure phage suspension in nutrient broth. A flask lysis represents the same procedure on a larger scale. All volumes used were scaled up five times, thus 5ml of the bacterial cell culture is added to 50ml of medium and 0.5ml of the phage solution is added after the various time intervals. The medium used for the flask lyses was dialysable P-medium (see Annexure 2).

The flask lyses were repeated until there were approximately 2.5×10^{11} plaque forming units (PFU), in 2 800ml. However, the

volume of this suspension was too large and was reduced by removing the water by osmosis. The method applied in the case of Klebsiella K68 homologous bacteriophage was to put the suspension into a dialysis tube (M_w cutoff <4 000) and this was then suspended in a saturated solution of polyethylene glycol 6 000. The final suspension was further dialysed against tap water in an attempt to purify the phage solution before it was added to the polysaccharide.

(b) Bacteriophage deploymerisation of the polysaccharide

A sample (500mg) of the pure Klebsiella K68 capsular antigen was treated with 250ml (10^9PFU/ml) of the bacteriophage suspension. After incubation with stirring at 37° for three days with $CHCl_3$ included to act as a bactericide, the mixture was freeze dried. The lyophilisate (770mg) was made up to 50ml with distilled water and then dialysed (M_w cutoff <4 000) against 50ml of distilled water for 24 hours. This was repeated twice. In each case the dialysate was lyophilised and retained separate from the the other fractions.

Four fractions, viz. the contents of the dialysis bag (153mg) and dialysates 1 (161mg),2 (120mg) and 3 (155mg), were separated on a Biogel® P4 column (2.6 x 40cm, 20ml/hr) using pyridinium acetate (water,acetic acid,pyridine 500:2:5) as the eluant. A better separation of the oligosaccharides was effected by elevating the column temperature to 50° with a water jacket. After the elution profile was

determined by spectrophotometric analysis (at 490nm) of samples treated with phenol-sulphuric acid reagent, those fractions identified as containing carbohydrate material were lyophilised and retained.

6 THE STRUCTURES AND COMPONENTS OF THE *E.COLI* CAPSULAR ANTIGENS REPORTED IN THE LITERATURE

A. PUBLISHED E.COLI K ANTIGEN STRUCTURES.

Where more than one structure has been reported for a single K antigen, the one most recently reported is shown.

K1.(106)

-[-8)N acetylneuraminic acid(2-]-

K2.(07)

-[-P-4-Galp(1-2)Glyc(1-3)-]-....-[-P-4-Galf(1-2)Glyc(1-3)-]-

K5.(97)

-[-4)Glc A($1\overset{\beta}{-}4$)Glc N Ac($1\overset{\alpha}{-}$]-

K6.(107)

-[-2)D-Ribf($1\overset{\beta}{-}2$)-D-Ribf($1\overset{\beta}{-}7$)-D-KDO($1\overset{\alpha}{-}$]-

K7.(108)

-[-3)D-Man N Ac A($1\overset{\beta}{-}4$)-D-Glc($1\overset{\beta}{-}$]-
6
|
OAc

K12.(08)

Rha($1\overset{\alpha}{-}2$)Rha($1\overset{\alpha}{-}5$)KDO(2-[$\overset{\beta}{-}3$)Rha($1\overset{\alpha}{-}2$)Rha($1\overset{\alpha}{-}5$)KDO$\overset{\beta}{-}$]-phosphodiester-phosphatidic acid
7(8) 7(8)
| |
OAc OAc

K13.(110)

-[-3)Ribf($1\overset{\beta}{-}7$)KDO($2\overset{\beta}{-}$]-
4
|
OAc

K14.(109)

-[-6)-2-acetamido-2-deoxy-D-Galp(1$^{\beta}_{-}$5)KDO(2$^{\beta}_{-}$]-

K20.(110)

-[-3)Rib$f$(1$^{\beta}_{-}$7)KDO(2$^{\beta}_{-}$]-
5
|
OAc

K23.(110)

-[-3)Rib$f$(1$^{\beta}_{-}$7)KDO(2$^{\beta}_{-}$]-

K27.(75)

-[-4)D-Glc(1$^{\alpha}_{-}$3)-D-Glc A(1$^{\alpha}_{-}$3)-D-Fuc(1$^{\alpha}_{-}$]-
3
|α
1
D-Gal

K28.(100)

-[-3)-D-Glc(1$^{\alpha}_{-}$4)-D-Glc A(1$^{\beta}_{-}$4)-D-Fuc(1$^{\alpha}_{-}$]-
4 3
|β |
1 OAc
D-Gal

K29.(69)

-[-2)-D-Manp(1$^{\alpha}_{-}$3)-D-Glcp(1$^{\beta}_{-}$3)-D-Glcp A(1$^{\beta}_{-}$3)-D-Galp(1$^{\alpha}_{-}$]-
4
|α
1
D-Manp
2
|β
1
D-Glcp
X
4 6
Pyr

K30.(113)

-[-2)-D-Manp(1$\overset{\alpha}{-}$3)-D-Galp(1$\overset{\beta}{-}$]-
3
|α
1
D-Galp
3
|β
1
D-Glcp A

K32(A).(114)

-[-3)Glc(1$\overset{\alpha}{-}$4)Rha(1$\overset{\alpha}{-}$3)Gal(1$\overset{\alpha}{-}$]-
3
|β + OAc
1
Glc A

K40.(112)

-[-4)Glcp A(1$\overset{\beta}{-}$4)-2-acetamido-2-deoxy-D-Glcp(1$\overset{\alpha}{-}$6)-2-acetamido-2-deoxy-D-Glcp(1$\overset{\alpha}{-}$]-
|
L-Serine

K42.(115)

-[-3)Gal(1-3)Gal A(1-2)Fuc(1-]-

K52.(09)

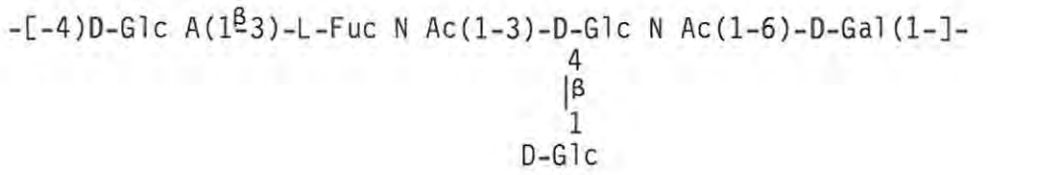
-[-3)Galp(1$\overset{\alpha}{-}$]-O-P=O
2 |
| |
1 (O^-)
Fruc*f*.....+ OAc or O propionate

K54.(116)

-[-3)-D-Glc A(1$\overset{\beta}{-}$3)-L-Rha(1$\overset{\alpha}{-}$]-
6
|
Threonine(90%) or Serine(10%)

K62.(07).....(see K2)

K87.(117)



K92.(118)

K95.(111)

K100.(119)

B. COMPONENTS OF THE E.COLI CAPSULAR (polysaccharide) ANTIGENS.

K antigen	acidic/amino sugars	neutral sugars						other
		Gal	Glc	Man	Fuc	Rha	Rib	Fruc	
K1	NANA[e]								
K2		+							glycine
K4[a]	Gal A,[b] Gal N[c]		+						
K5	Glc A, Glc N Ac								
K6	KDO[d]						+		
K7	Man N Ac A	+							OAc[f]
K8[a]	Glc A, Gal N, Glc N	+							
K9[a]	NANA, Gal N	+							
K12	KDO					+			phosphatidic acid and OAc
K13	KDO						+		OAc
K14	KDO,	+							acetamido
K17[a]	Glc A, Gal N	+							
K20	KDO						+		OAc
K23	KDO						+		
K25[a]	Glc A, Gal N				+				
K26[a]	Glc A	+				+			
K27	Glc A	+	+		+				
K28	Glc A	+	+		+				OAc
K29	Glc A	+	+	+					pyruvate
K30[a]	Glc A	+		+					
K31[a]	Glc A	+	+						
K32	Glc A	+	+			+			OAc
K34[a]	Glc A, Gal A	+	+	+					
K40	Glc A		+						acetamido/serine
K42	Gal A	+			+				
K52		+						+	OAc/propionate
K54[a]	Glc A					+			Threonine/serine
K57[a]	Gal A, Gal N	+							
K62		+							glycine/OAc
K85	Glc A, Glc N								
K87	Glc A, Glc N Ac, Fuc N Ac	+	+						
K92	NANA								
K95	KDO						+		
K100							+		Phosphate

[a]-Reference 05.
[b]-Galacturonic acid.
[c]-Galactosamine.
[d]-3-deoxy-D-manno-octulosonic acid.
[e]-N-acetylneuraminic acid.
f-O acetyl group.

7 ANNEXURE 1

ANNEXURE 1

A NUCLEAR MAGNETIC RESONANCE SPECTRA

i) E.coli K36 proton magnetic resonance spectra

(1)-Oligosaccharide P1 run at ambient temperature.

(2)-Oligosaccharide P1 run at 95°.

(3)-Oligoalditol P1 run at 95°.

(4)-Partially autohydrolysed native polysaccharide run at 95°.

(5)-Periodate oxidised polysaccharide run at 95°.

ii) E.coli K36 ^{13}C resonance spectra

(6)-Oligosaccharide P1.[a]

(7)-Oligoalditol P1.

(8)-Oligoalditol P1 proton coupled spectrum.

(9)-Partially autohydrolysed native polysaccharide.

iii) Klebsiella K68 proton magnetic resonance spectra

(10)-Oligosaccharide P1 run at 95°.

(11)-Oligoalditol P1 run at ambient temperature.

(12)-Oligoalditol P1 run at 95°.

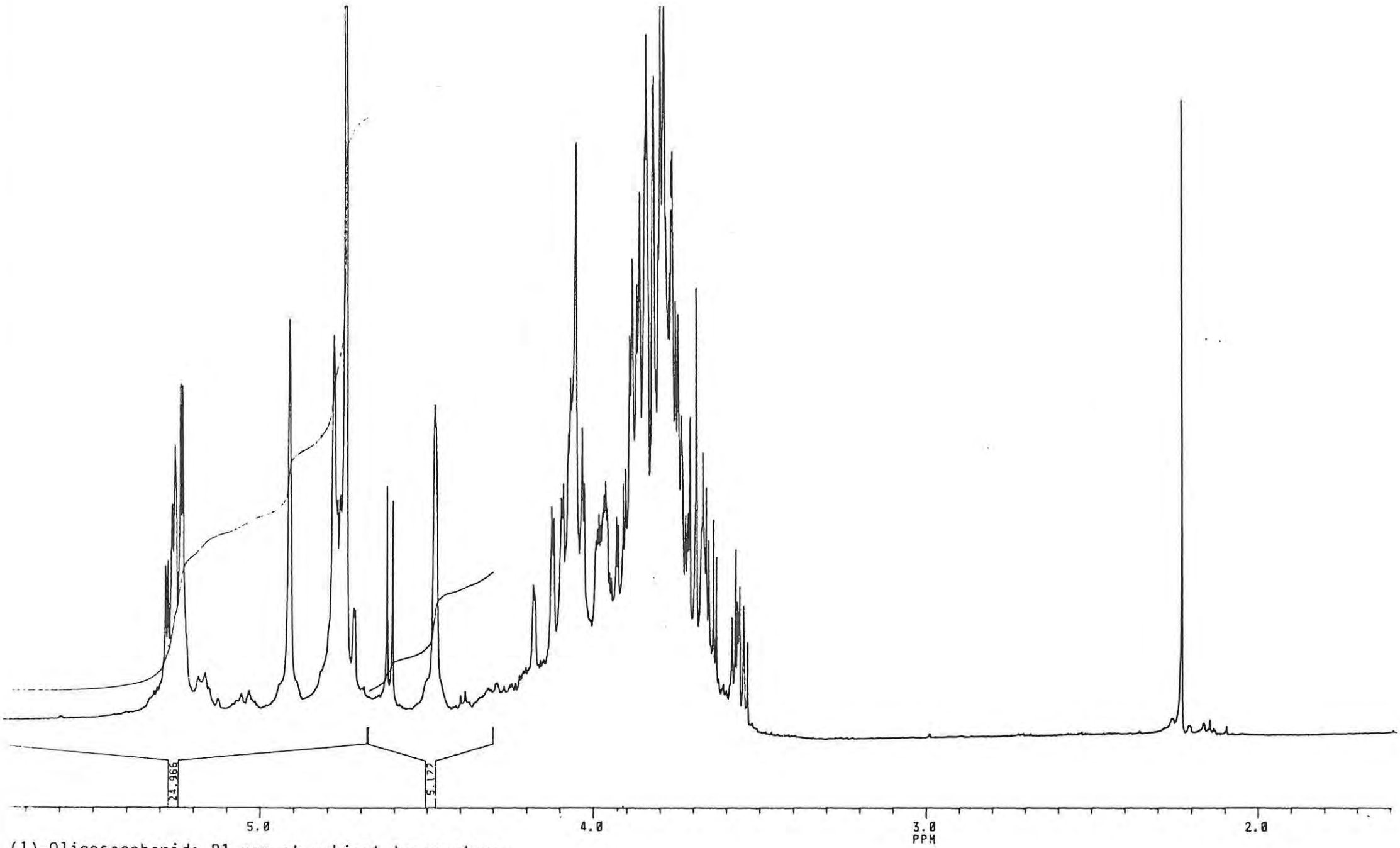
(13)-Two dimensional COSY plot for the oligosaccharide P1. (Figure I)

iv) Klebsiella K68 ^{13}C resonance spectra

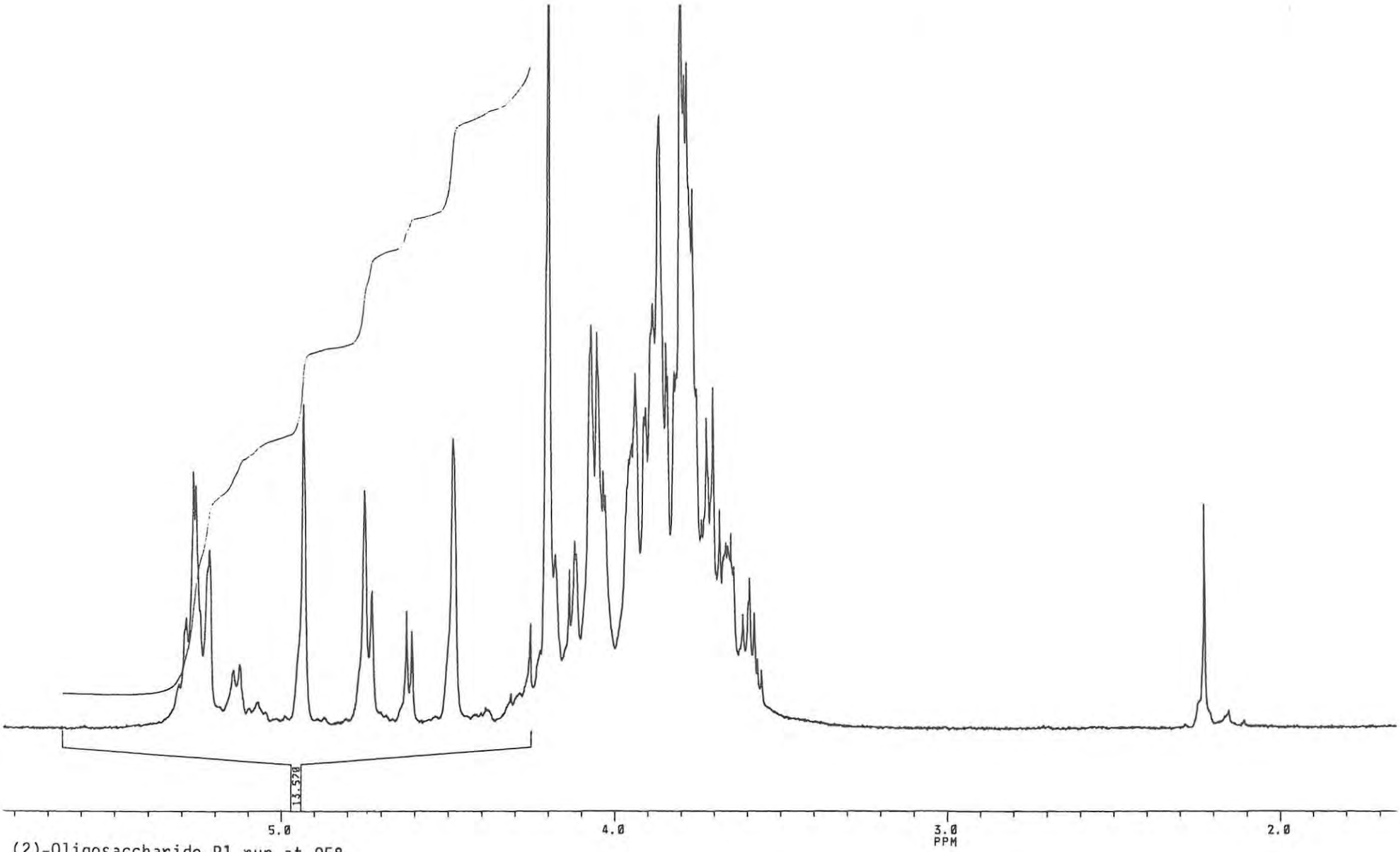
(14)-Oligosaccharide P1.

(15)-Oligoalditol P1.

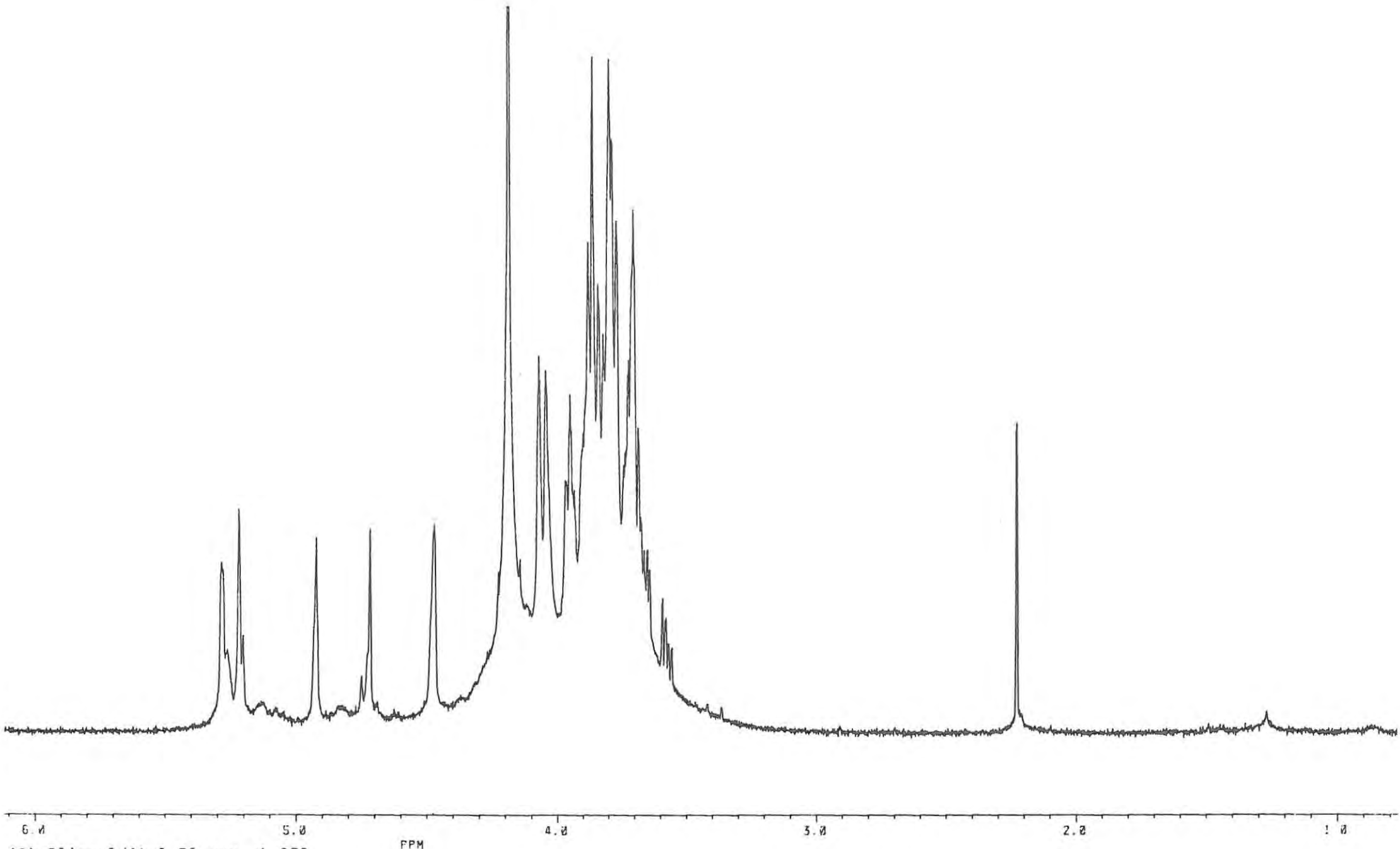
[a] Except where specifically stated, all ^{13}C spectra were run as proton-decoupled experiments.



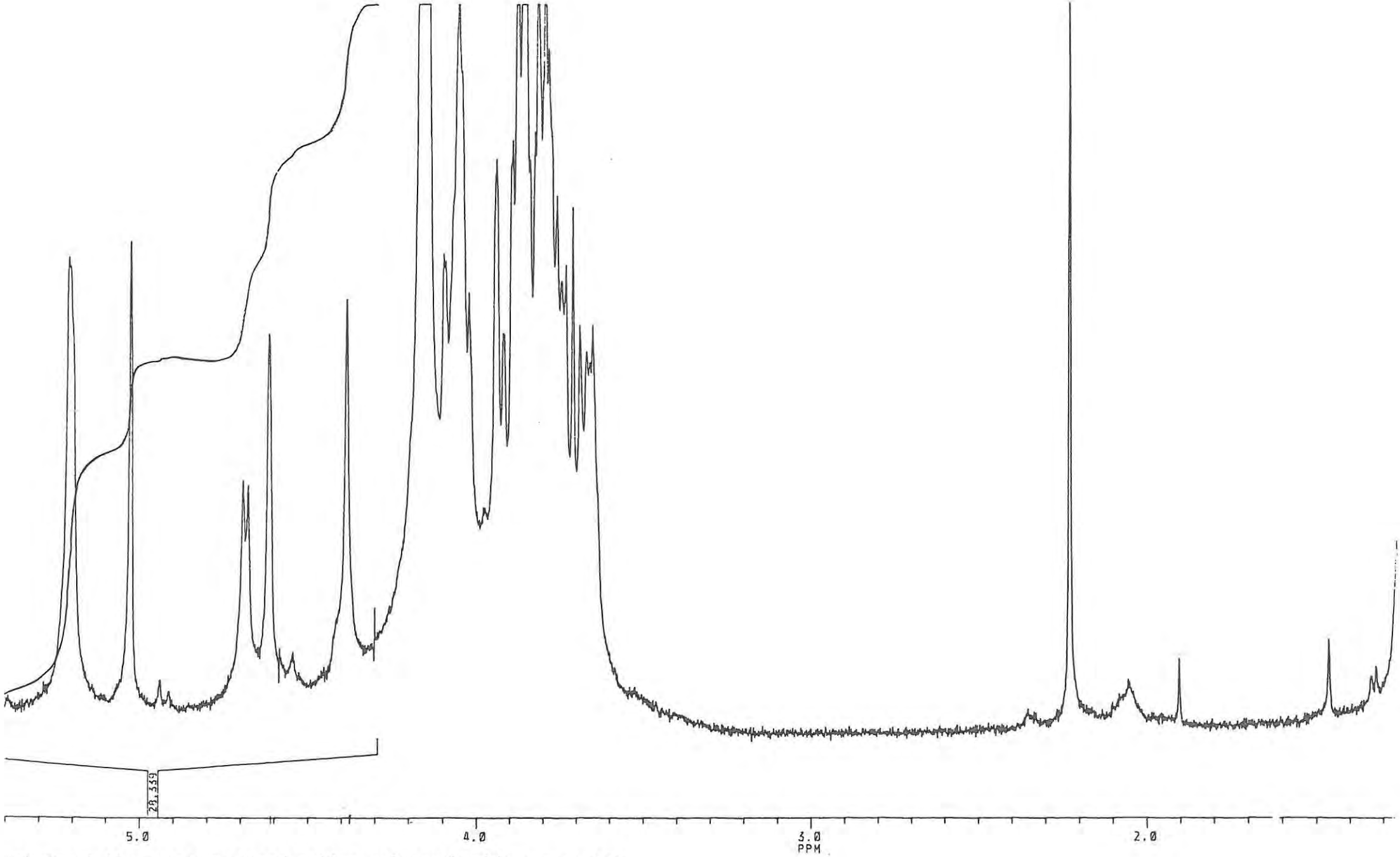
(1)-Oligosaccharide P1 run at ambient temperature.



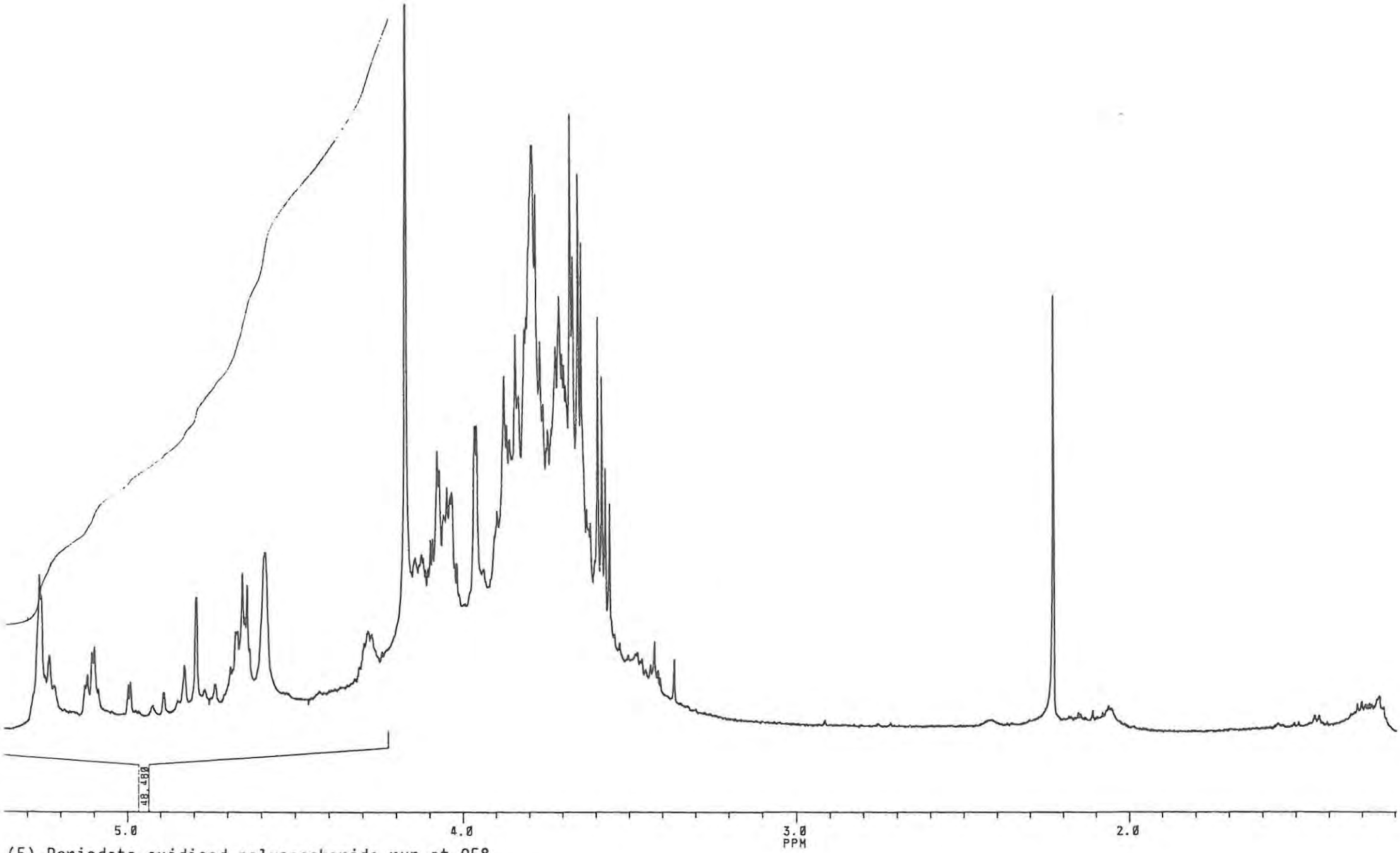
(2)-Oligosaccharide P1 run at 95°.



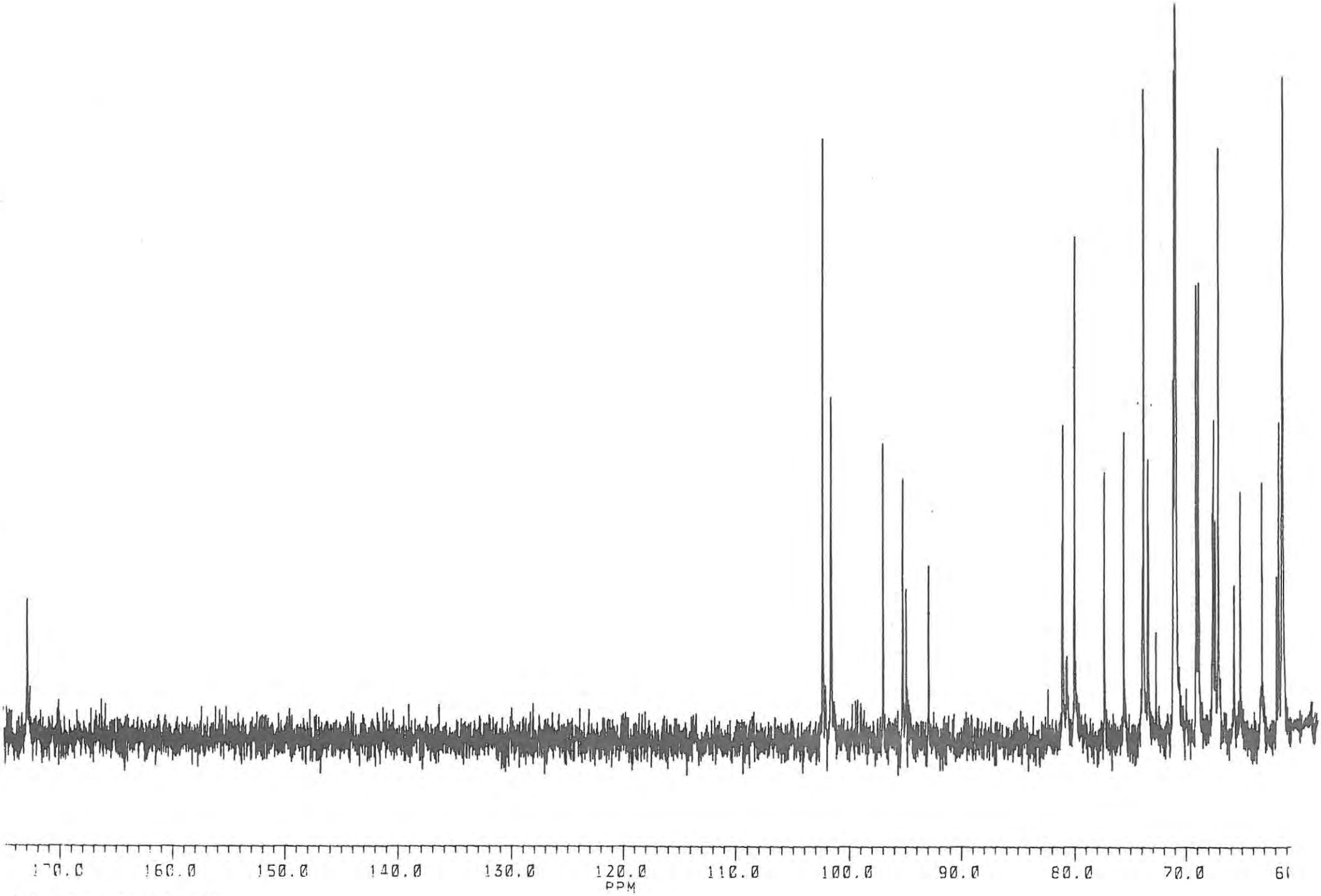
(3)-Oligoalditol P1 run at 95°.



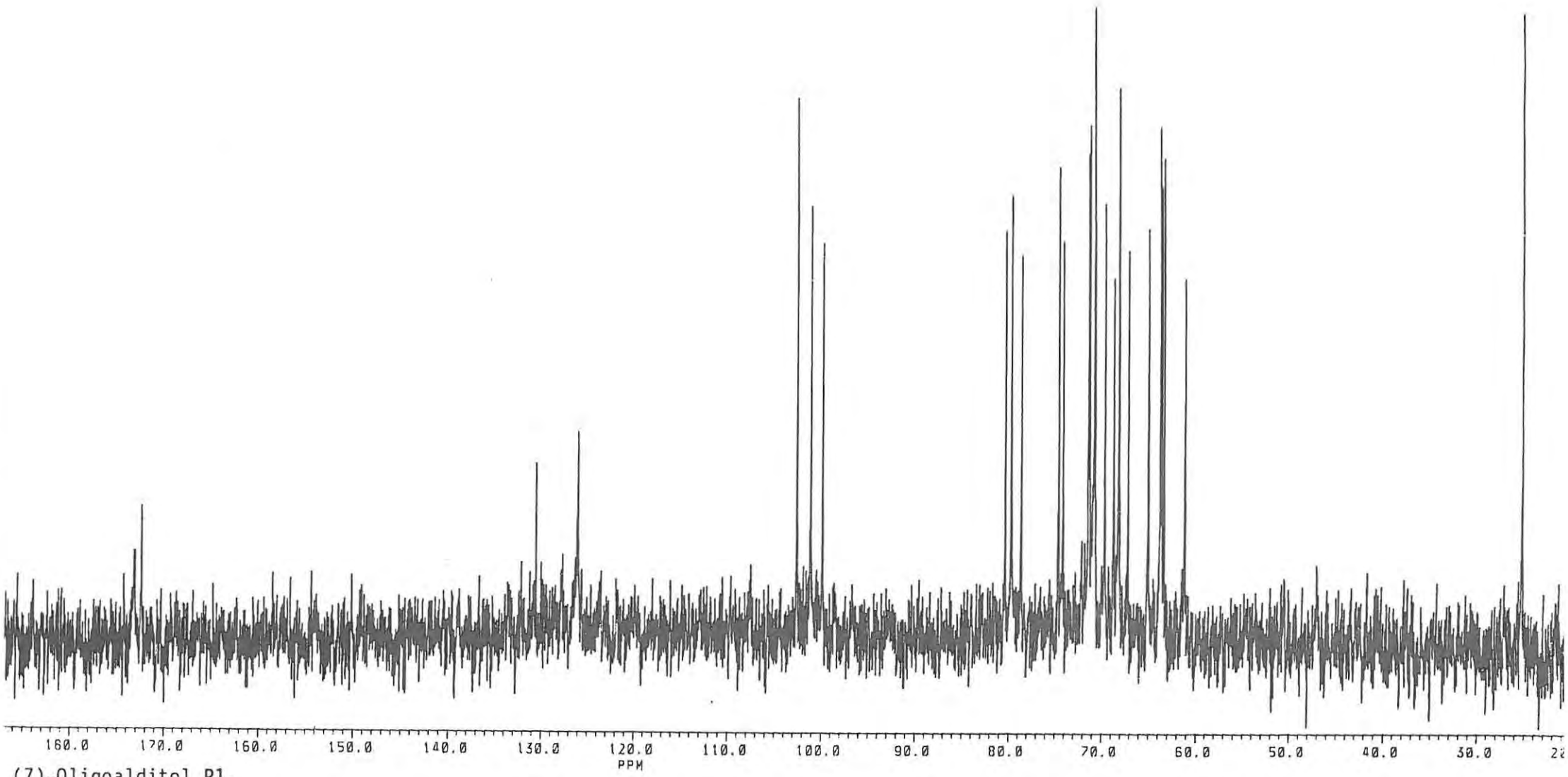
(4)-Partially autohydrolysed native polysaccharide run at 95°.



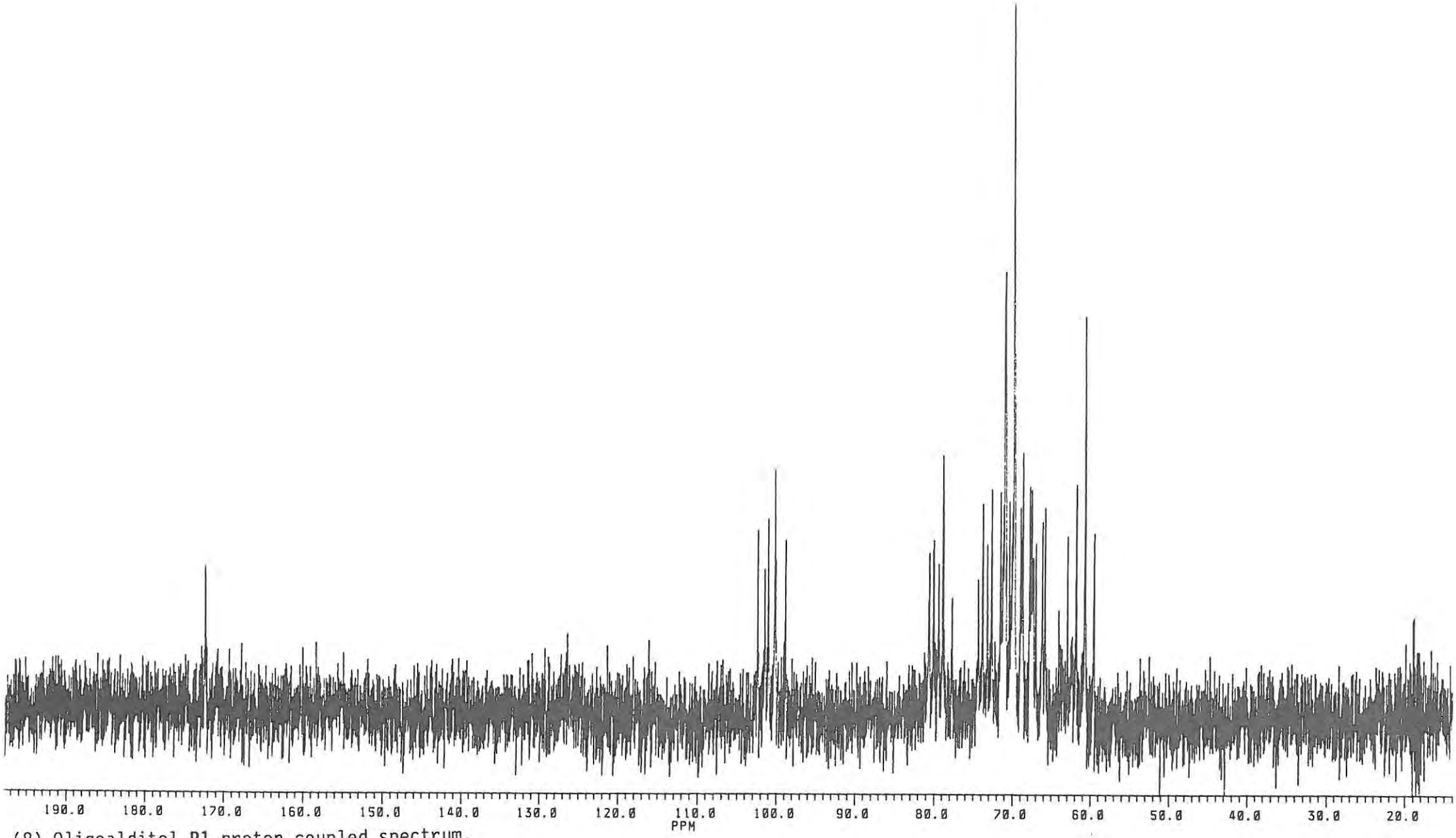
(5)-Periodate oxidised polysaccharide run at 95°.



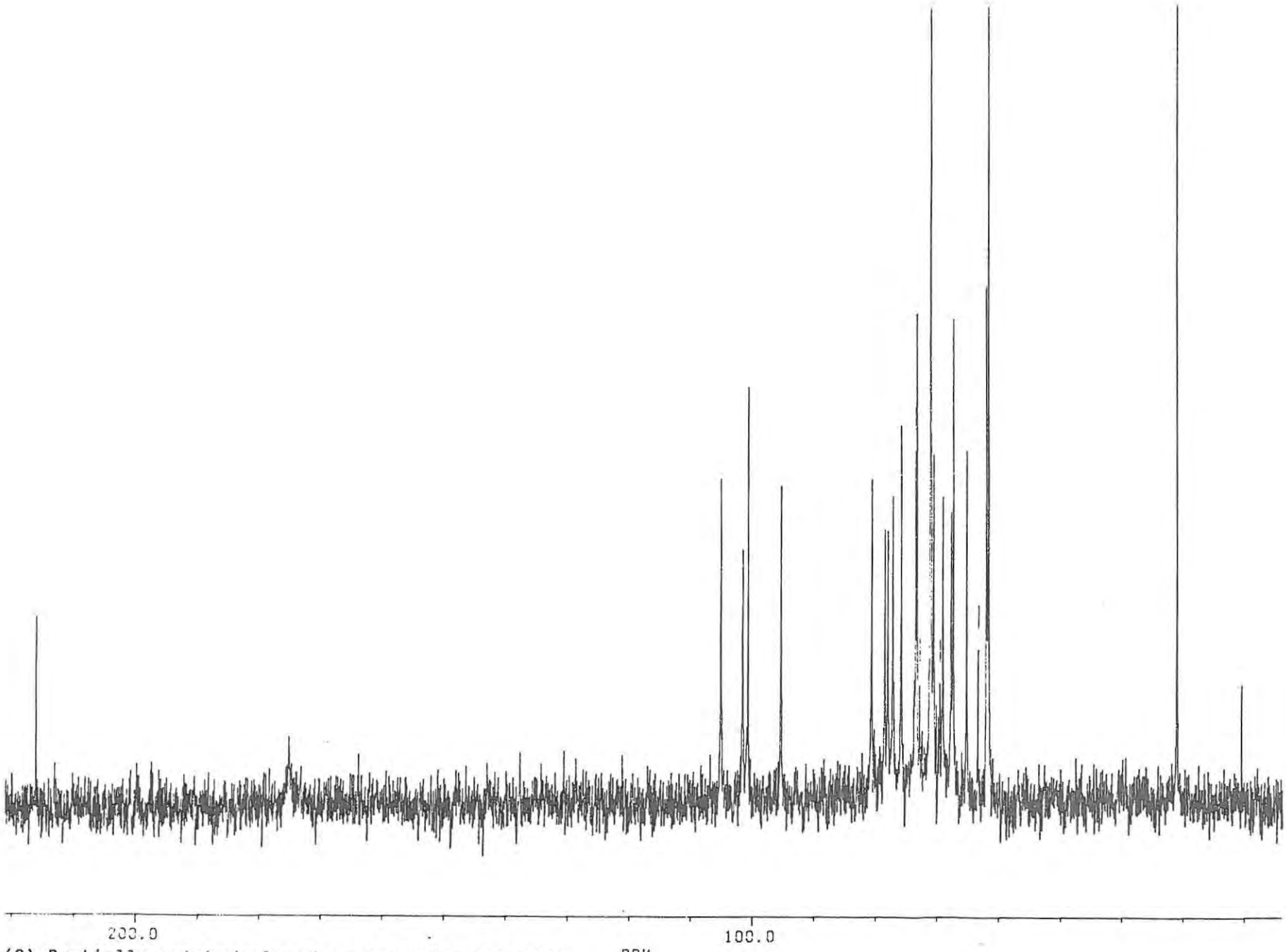
(6)-Oligosaccharide P1.



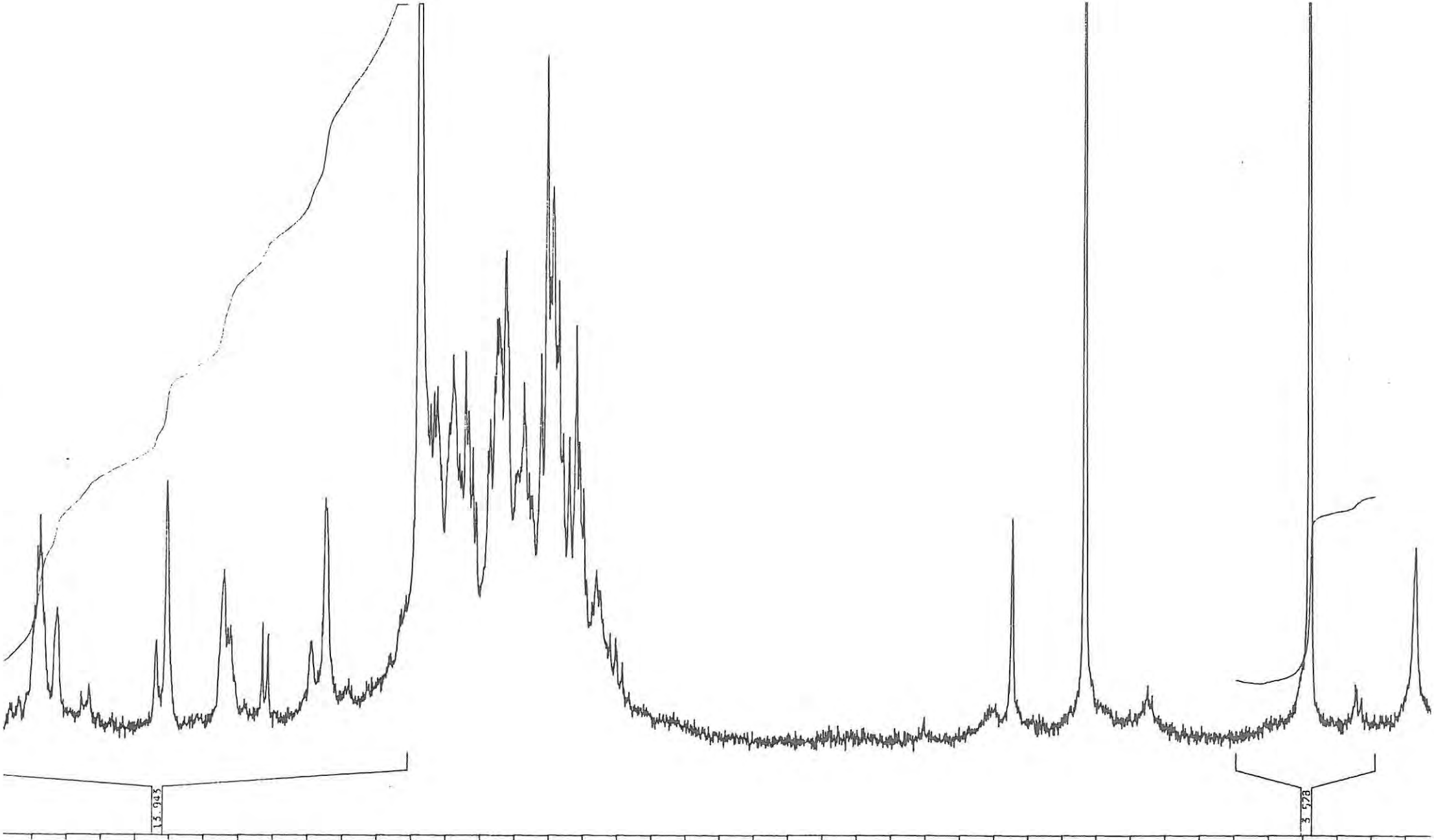
(7)-Oligoalditol P1.



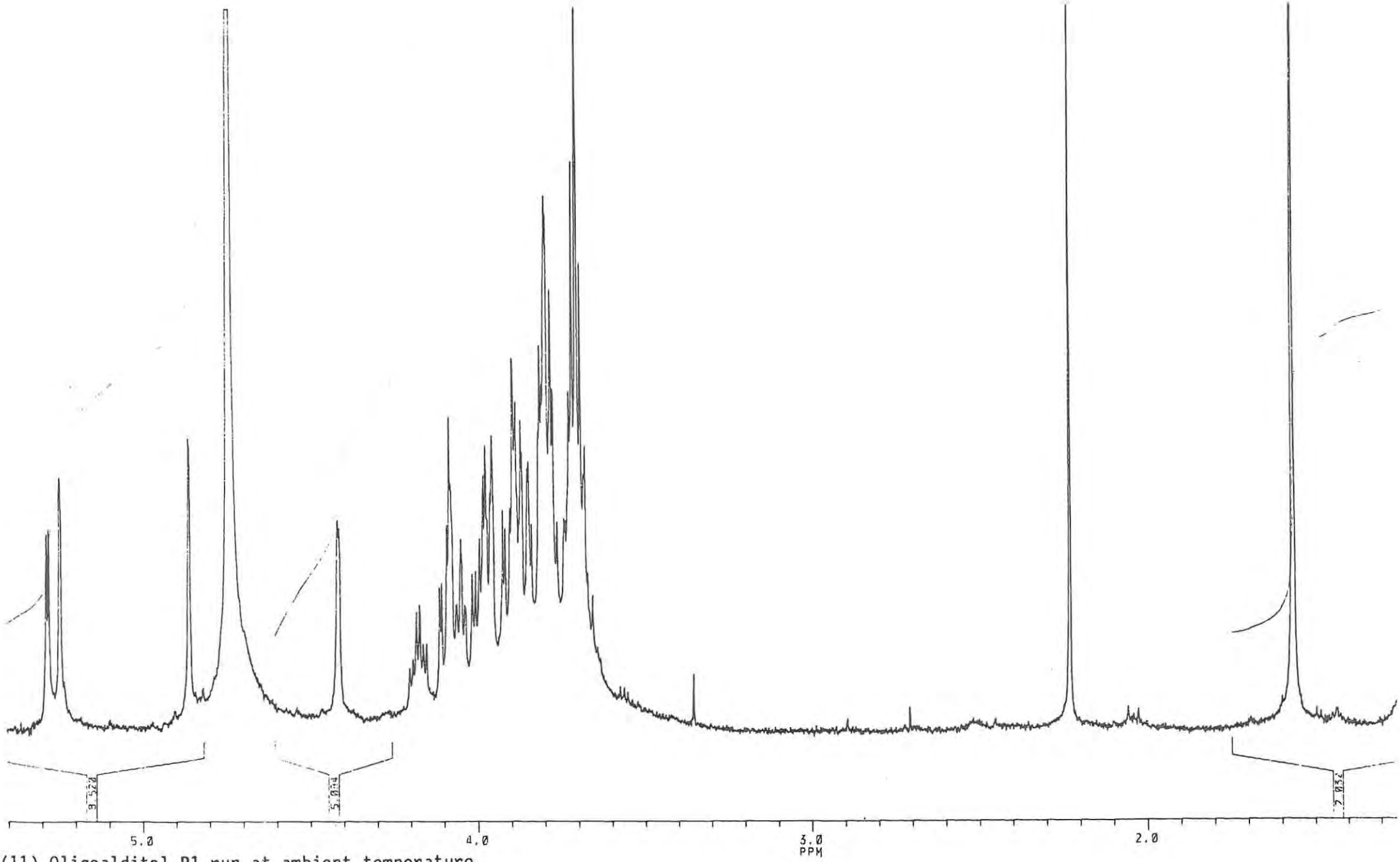
(8)-Oligoalditol P1 proton coupled spectrum.



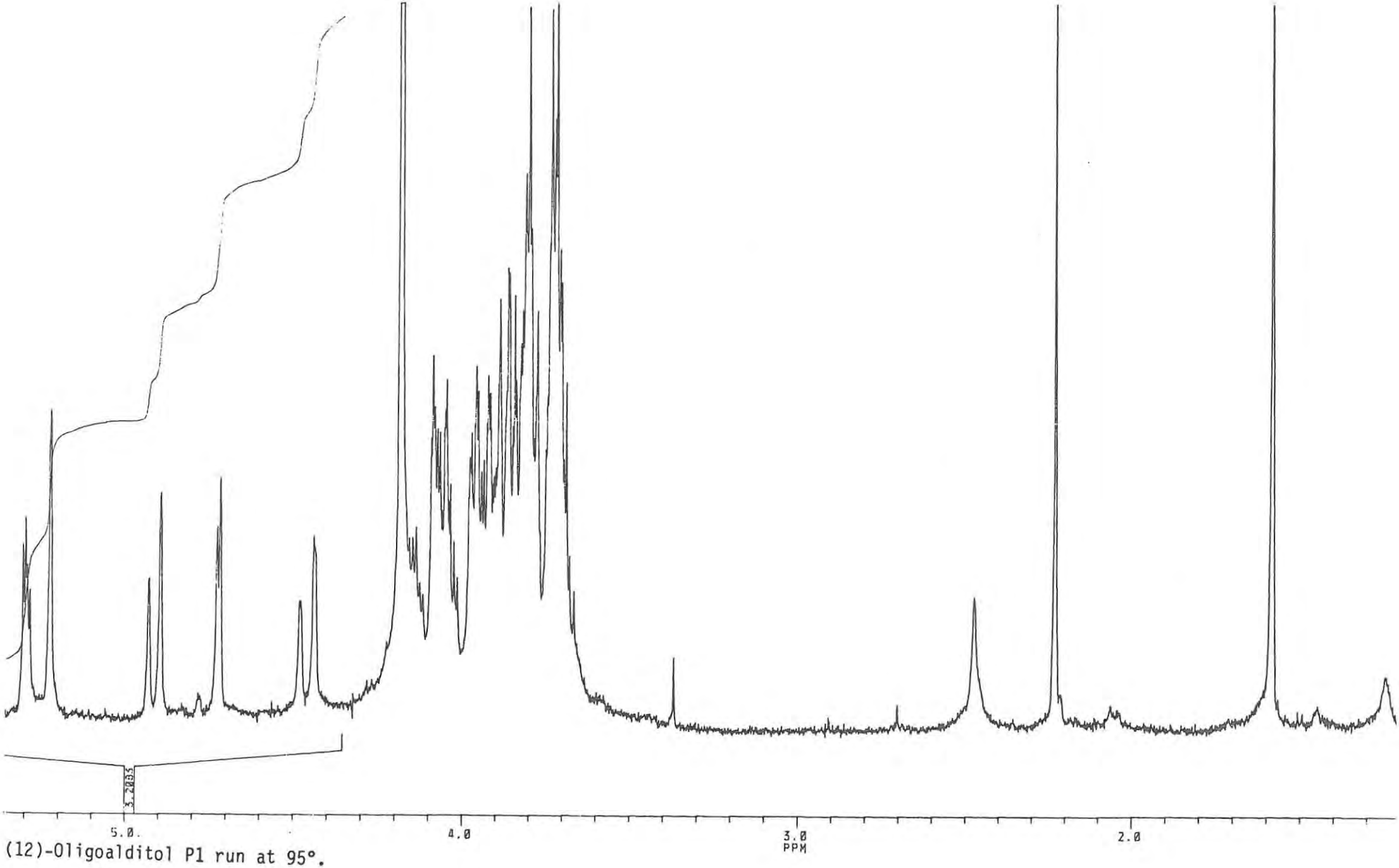
(9)-Partially autohydrolysed native polysaccharide.



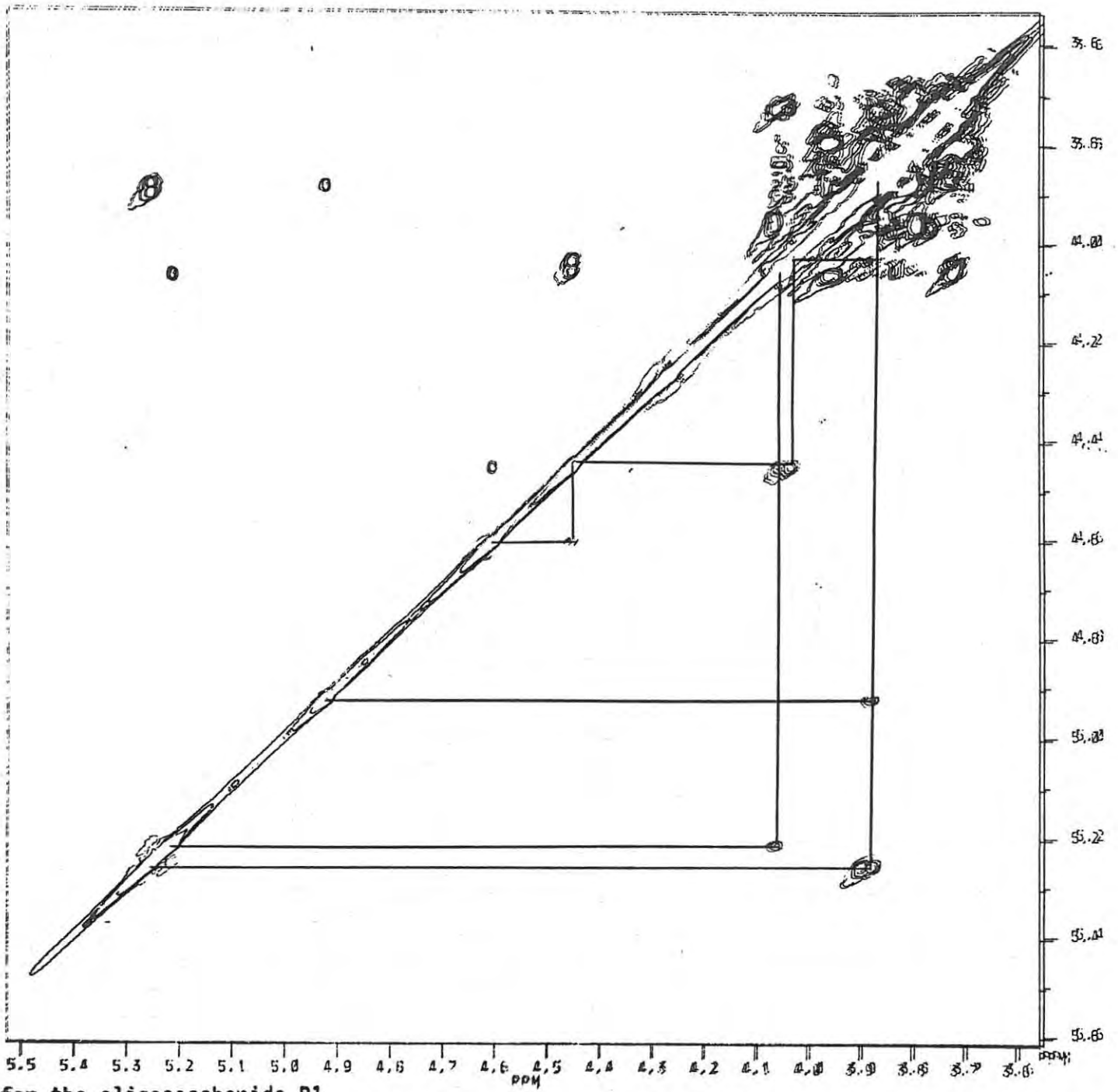
(10)-Oligosaccharide P1 run at 95°.



(11)-Oligoalditol P1 run at ambient temperature .

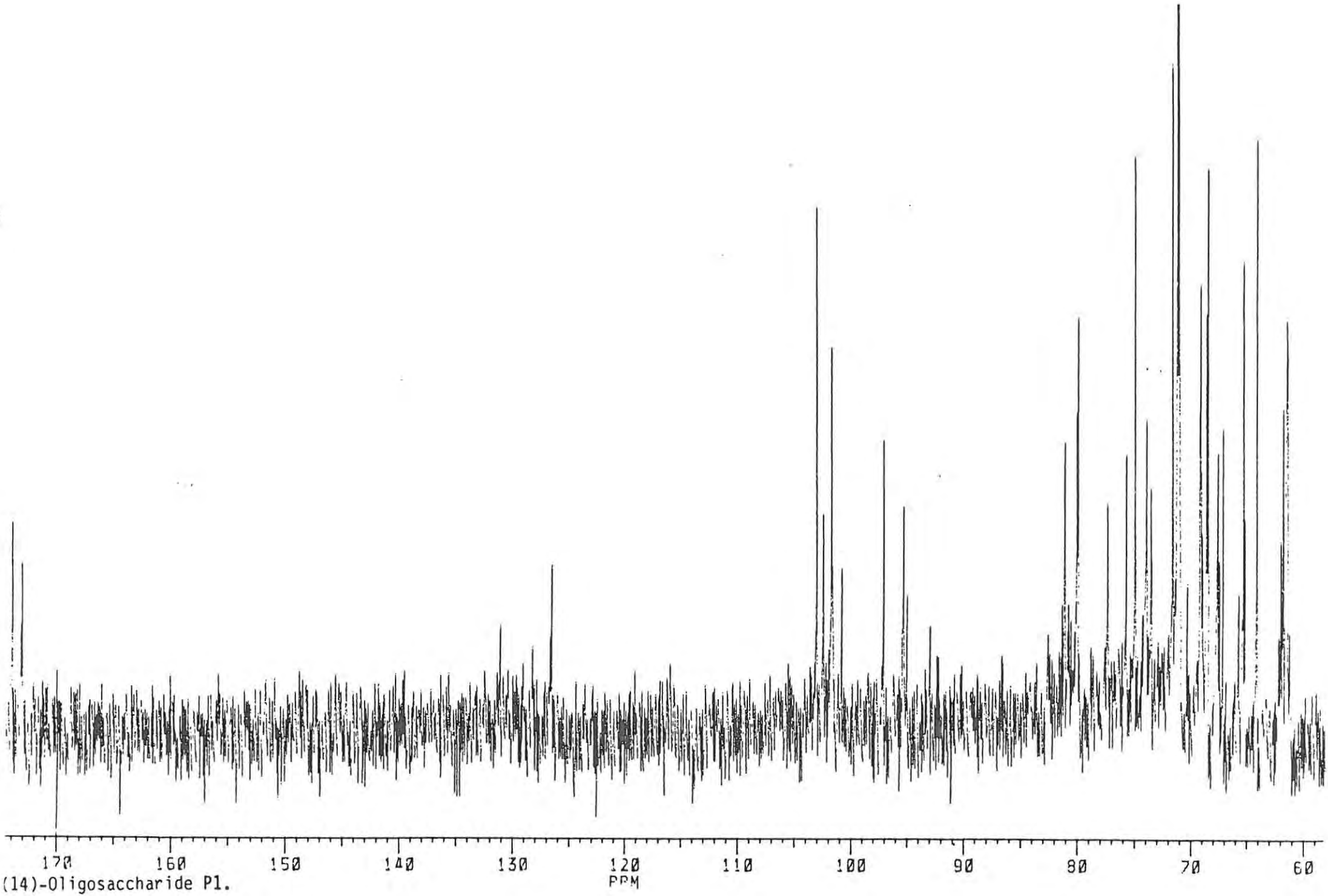


(12)-Oligoalditol P1 run at 95°.

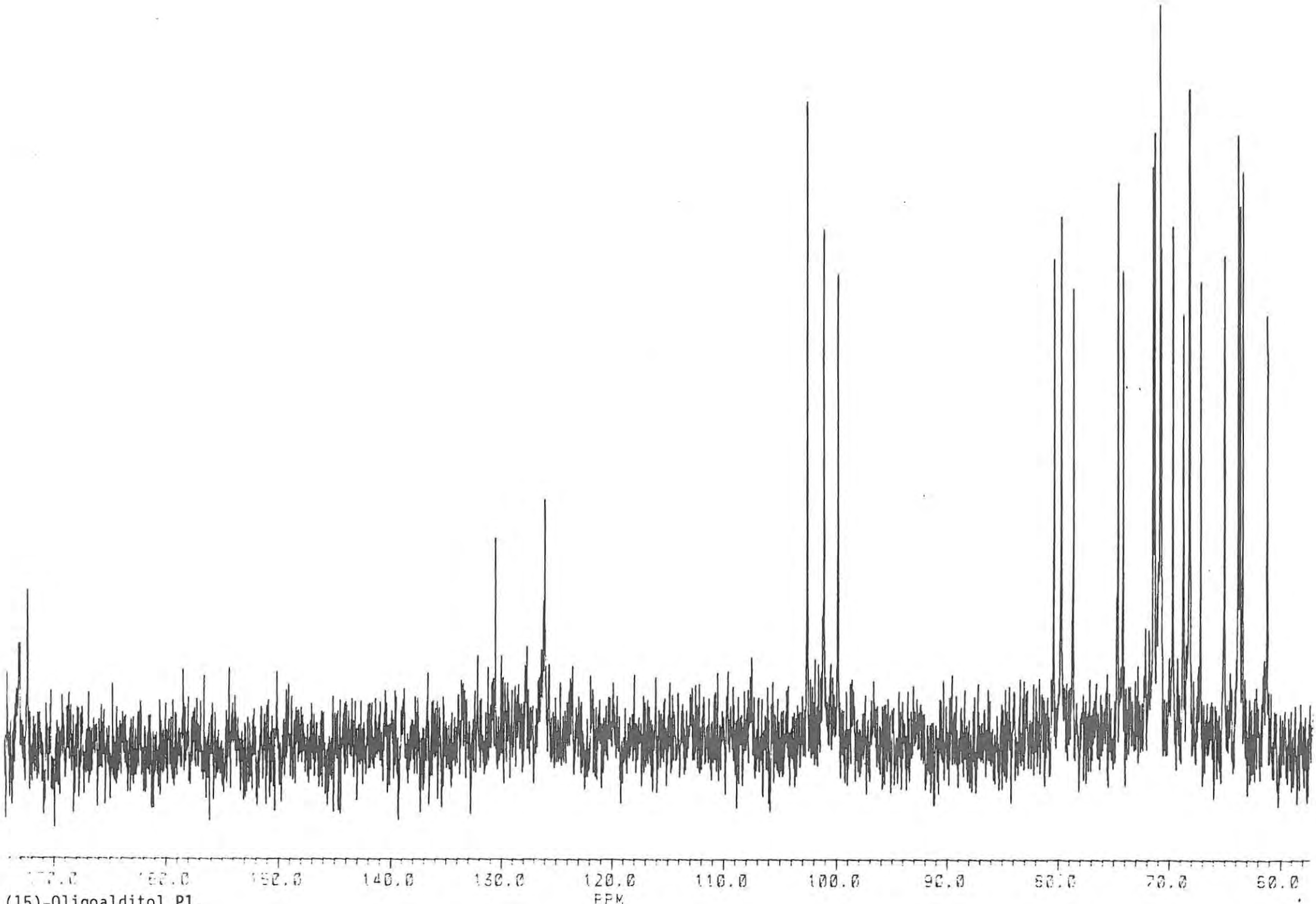


(Figure I)

(13)-Two dimensional COSY plot for the oligosaccharide P1.



(14)-Oligosaccharide P1.



(15)-Oligoalditol P1.

8 ANNEXURE 2

MEDIA.

A. Mueller Hinton Agar.

	g/l
Meat infusion	5.0
Casein hydrolysate	17.5
Starch	1.5
Agar	14.0

B. Mueller Hinton Broth.

	g/l
Meat infusion	5.0
Casein hydrolysate	17.5
Starch	1.5

C. DIALYSABLE P MEDIUM.[105]

	g/l
Glucose	5.0
Casamino acids (Difco® B230)	5.0
L-tryptophan	0.32
L-cysteine	0.24
KH_2PO_4	2.0
$Na_2HPO_4.12H_2O$	12.48
NH_4Cl	1.04
Gelatin	0.008
$MgSO_4.7H_2O$	0.5
$FeSO_4$	0.001
$CaCl_2$	0.1

D DIALYSABLE P-MEDIUM (carbohydrate restricted)

	g/l
Glucose	1.0
Casamino acids(Difco® B230)	5.0
L-trptophan	0.32
L-Cysteine	0.24
KH_2PO_4	2.0
$Na_2HPO_4.12H_2O$	12.48
NH_4Cl	1.04
Gelatin	0.008
$MgSO_4.7H_2O$	0.5
$FeSO_4$	0.001
$CaCl_2$	0.1
Glycerol	2.0

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