CHITOSAN MODIFICATION: TOWARD THE RATIONAL TAILORING OF PROPERTIES

By

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ABSTRACT

The main objective of this work was to develop a method whereby chitosan could be modified to give synthetic analogues of branched polysaccharides. To this end, a variety of allyl glycosides were prepared (99-102, 105, 108,109 and 111) and reductively ozonolyzed, to give the acetaldehydo glycosides 112-119. These aldehydes were then reductively alkylated to chitosan (1), to give branched chitosan derivatives (120-127) of the general structure 157. Pendant residues of α and β-glucopyranose, α and β-D-galactopyranose, 2-acetamido-2-deoxy-D- α and β-glucopyranose, β-D-glucopyranuronic acid and β-D-lactose were incorporated by this method, at various levels of substitution.

Rheological evaluations of these derivatives by steady-shear viscometry demonstrated a relationship between the degree of substitution and rheological properties, as well as the effect of branch size and functionality on aqueous solution properties. Importantly, many of the trends seen in this study are similar to established explanations for the aqueous solution properties of seed galactomannans. It was also shown that intrinsic viscosities of the derivatives were supportive of observations based on concentrated solution properties. Also, it was demonstrated that these water soluble chitosan derivatives interacted, sometimes in a synergistic manner, with xanthan gum solutions.

A similar route, involving the synthesis of 10-undece-

nyl-β-D-glycopyranosides (134-136), reductive ozonolysis and reductive amination to chitosan, provided combined hydrophobic/hydrophilic branched chitosan derivatives (140-142) of the general structure 158. This methodology was demonstrated with the 10 -undecenyl β-D-glycosides of glucopyranose, galactopyranose and lactose. Compounds 140a and 141a, bearing glucose and galactose pendant residues, showed uncommon thermally induced gelation properties in dilute aqueous acid solution. This property was studied by ^{1}H-nmr relaxation measurements and ^{13}C-nmr spectroscopy. It was found that a high degree of substitution was necessary for gel formation, and that the pendant sugar was required, but excess hydrophilicity (such as the disaccharide branch, lactose) precluded gelation.

In addition, a derivative (151) was prepared, which contained a metal-chelating moiety and a hydrophilic spacer group. This compound had substantial copper (II) binding capacity, and useful ion-exchange ability. Finally, a chitosan derivative (156) was synthesized, bearing a pendant 1-thio-β-D-glucopyranose moiety, and was shown to be useful for the affinity chromatographic purification of the enzyme β-glucosidase.

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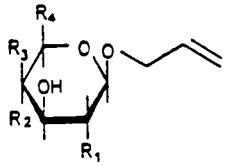
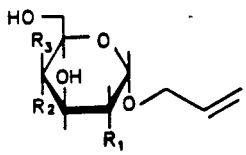
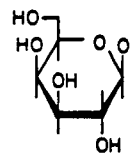
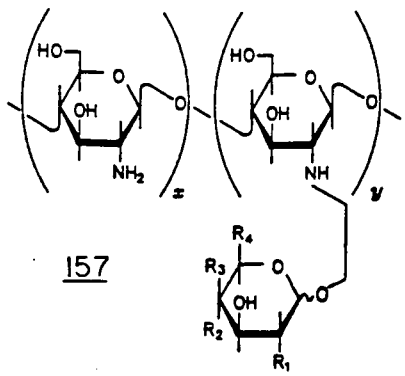


99-102,105



108,109,111

	R_1	R_2	R_3	R_4
99,108	OH	OH	H	CH_2OH
100,109	OH	H	OH	CH_2OH
101	OH	OH	H	CO_2H
105,111	NHAc	OH	H	CH_2OH
102			H	CH_2OH



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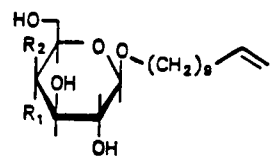
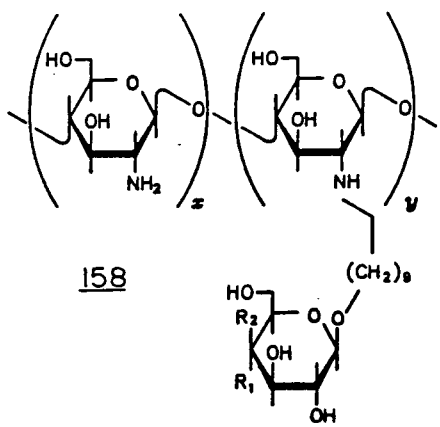
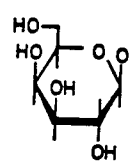
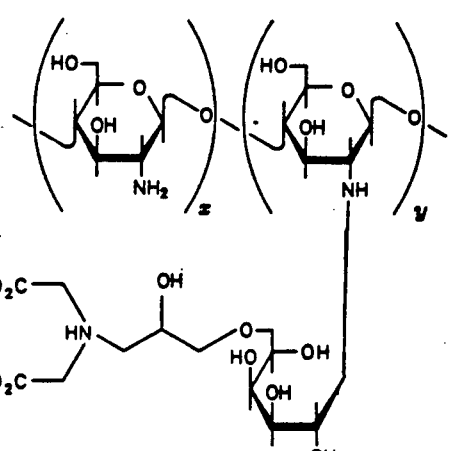


134-136



158

	R_1	R_2
134	OH	H
135	H	OH
136		H



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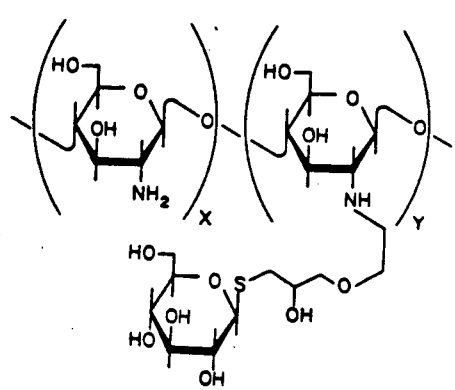

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ABBREVIATIONS

Ac = acetyl

Ac_2O = acetic anhydide

b = broad

bz = benzene

DCC = dicyclohexylcarbodiimide

DMF = dimethylformamide

DMSO = dimethylsulfoxide

EDC = 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide

FT = Fourier transform

Gal = D-galactose

Glc = D-glucose

glc = gas liquid chromatography

GlcA = D-glucuronic acid

GlcNAc = 2-acetamido-2-deoxy-D-glucose

$GlcNH_2$ = 2-amino-2-deoxy-D-glucose

HEC = hydroxyethylcellulose

HOAc = acetic acid

ir = infrared

Lact = D-lactose

m = multiplet

Me = methyl

ms = mass spectroscopy

Ms = methanesulfonyl (mesyl)

NaALG = sodium alginate

nmr = nuclear magnetic resonance

Pc = phenylcarbamoyl

PF = *para*-formaldehyde

Ph = phenyl

i-PrOH = iso-propanol

pyr = pyridine

q = quartet

RaNi = Raney nickel

s = singlet

t = triplet

THF = tetrahydrofuran

tlc = thin layer chromatography

TMS = tetramethylsilane

Ts = *p*-toluenesulfonyl (tosyl)

Tr = trityl

Xan = xanthan gum

CHAPTER 1

INTRODUCTION

1.1 OVERVIEW

Polysaccharides are ubiquitous in nature and participate in many vital biological systems and processes.[1,2] Their roles fall into two general classifications: (1) Structural, as exemplified by cellulose, the main structural polysaccharide in the plant world, and by chitin, which serves as the main structural material in crustacean shells and insect exoskeletons; and (2) functional, as with starch, the main energy storage material in plants, and glycogen, which serves analogously in mammals.

These are but a few of the better known polysaccharides which occur in nature. Myriad others exist,[3,4] whether they be plant exudates (gums), substances in seaweeds (algal polysaccharides), or the exocellular polysaccharides of bacteria. It has long been of interest to learn more about the various roles of polysaccharides in nature. However in recent years a separate, albeit related aspect in the study of carbohydrate polymers has attracted increasing attention from both the academic and industrial sectors, and has led to a diversity of industrial applications.

Polysaccharides, being extremely abundant and often possessing desirable properties,[5] are ideal candidates for

use in a variety of applied areas.[6,7] Some of the major industrial uses of these materials occur in food and food processing, agricultural products, cosmetics, pharmaceuticals, paints, adhesives, paper making, mining aids, waste water treatment, and enhanced oil recovery. They serve as thickening or gelling agents, suspending agents, lubricants and metal sequestrants, amongst other purposes.[6] As well, polysaccharides have found growing biochemical application in the form of solid phase polymeric support materials[1,8,9] for ion exhange and affinity chromatography, and enzyme and cell immobilizations.

While many polysaccharides are isolated from natural sources in a readily utilizable form, many are insoluble, intractable materials unsuitable for many applications. The fact that the very abundant and inexpensive substances, cellulose, starch and chitin fall into the latter category has stimulated research into methods for their modification.[10,11] Since the turn of the century industrial polysaccharide derivatives of cellulose and starch have been known. Various methods for the chemical modification of polysaccharides have been employed,[12,13] typically involving derivatization of the polysaccharide hydroxyl groups under strongly alkaline conditions with reactive organic compounds. While control of the overall degree of substitution (d.s., number of substituents per monosaccharide residue) is possible, these reactions usually afford a random or "statistical" distribution of derivatized sites on the monosaccharide

residue.[10,11,14] In other words, such reactions display little site-selectivity, which can be a major drawback for some applications. As a result, over the last 10-15 years there have been greater efforts to develop new derivatization methods which offer more control over all aspects of the reaction. This requires that accessibility and reactivity difficulties of these largely intractable polymers be overcome or circumvented.

The current interest in the modification of polysaccharides for the preparation of "tailored derivatives" has given this field great impetus, and indeed, novel methodologies[15] are now emerging which have significant potential. However, there must be a concurrent effort directed toward the characterization and evaluation of these complex materials, both structurally and functionally, leading to an understanding of the inter-relationship of structure and function.

A large number of applications of polysaccharides make use of their properties in aqueous solution (i.e. thickeners, gelling agents, suspending agents and lubricants), and not surprisingly these properties have been subject to intensive study.[5,16] Such studies require consideration of the diversity of possible sequences, of the multitude of conformational states available to a particular polymer sequence, as well as the various inter- and intra-molecular associations which may occur.[17] In light of other factors such as structural irregularities, in the form of minor

differences in sequences or substitution pattern (polydispersity) or molecular weight inhomogeneity (polymolecularity), it is clear that such investigations are inherently complex.

In recent years there have been significant advances made in determining the primary structure of complex polysaccharide systems, largely as a result of new chemical and physical methodologies.[1,18,19] More importantly, modern techniques are providing a means to gain insight into the hydrated and condensed states of these hydrocolloid polymers.[17] However, the relationship between structure and properties, from a predictive standpoint, remains elusive. To date, it has been possible only to correlate the properties of polysaccharides that are related through either sequence or conformation, both of which may produce intrinsic physical properties.[17] The main obstacles to understanding these relationships have been the lack of analytical methodology, the unavailability of systematically varied substrates for examination, and the overall complexity of the systems of interest.

This second point provides a locus of interest for chemists working in polysaccharide modification, who could develop methods for preparing an array of related derivatives for structure/property investigations. The "statistical" methods available for polysaccharide modification resulting in complex, random substituent distributions are unsuitable for this purpose. The development of specific modifications

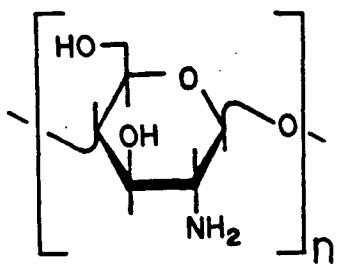
to control product composition is necessary for studies systematically relating structure to macroscopic properties.

Some requirements for site selective control of the modification of polysaccharides are: (1) the presence of a site of unique reactivity; and (2) mild reaction conditions suitable for both polysaccharide and ligand.

The primary objective of this work was to incorporate chemical modification, spectroscopic characterisation and rheological evaluation into a concerted study of branched polysaccharides. As will be seen, excellent examples of studies in each of these areas exist in the current literature; however, remarkably little work has been addressed toward combined studies in these areas. The first task was therefore to develop methodology whereby polysaccharides could be chemically modified to produce materials with specific, desired properties. We required that some degree of selectivity be inherent in this methodology, such that a systematically varied "family" of branched polysaccharide derivatives could be obtained. We wished to prepare derivatives similar in composition and structure to naturally occurring branched polysaccharides, which are already known to have interesting rheological properties.[5,17] And finally, we wished to investigate the relationship between the structural features of the derivatives prepared and their macroscopic solution properties. In addition, while the potential immunological applications[1,20,21] stemming from this methodology will not be directly addressed in this work, it

was intended that the strategy used would be sufficiently general that it could find application in that area.

With these goals in mind, we have chosen the amino polysaccharide chitosan (1), as an exemplar. It is a linear (1→4)-linked homopolymer of 2-amino-2-deoxy-β-D-glucopyranose, and it is derived from chitin. Chitin (2), a (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose polymer, is available in large quantities from crustacea shell wastes, and has found use in numerous industrial[22,23] and biomedical[24-26] applications. The free amine functionality on chitosan renders it an ideal substrate for high yielding site-selective reactions under mild, aqueous, alkylation and acylation conditions.[27] This work will describe the use of the reductive amination reaction to transform chitosan into metal chelating derivatives, affinity chromatography supports, viscosity modifiers and gelling agents. It was intended that the preparation of a series of synthetic branched polysaccharides would provide some insight into the effects of branching and branch composition on rheological properties.



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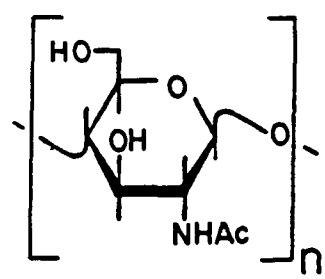

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1.2 BACKGROUND

1.2.1 Introduction to Polysaccharide Modification

The modification of polysaccharides has been employed for over a century and numerous monographs and review articles[7,10-14,28-31] have appeared on this topic. Traditionally the well-known and abundant polysaccharides cellulose[7,10-14,28] and starch,[7,13,29,30] were the substrates of interest. Typical modification reactions have involved relatively harsh treatment of the polymer with reagents, often under heterogeneous conditions, resulting in derivatives with a "statistical" substituent distribution.[10,11,14]

Some of the well-known modifications for celluose and starch include etherification, esterification and oxidation. These methods have been used to prepare polysaccharides for various industrial applications, and they have been described in detail in numerous other articles.[7,10-14,28-31] Some of the more common etherification products include the hydroxyethyl, hydroxypropyl, methyl and carboxymethyl ethers. These are produced by heterogeneous reaction of the polysaccharide (celluose or starch) under alkaline conditions with the respective alkyl epoxides and alkyl halides. These treatments give products with statistical substituent distributions, as dictated by the relative reactivities and accessibilities of the polymer hydroxyl groups.[10,11,14] A large amount of work has been done with cellulose to determine substitution

patterns obtained with various reagents and solvents,[10,11,14] and to explain these in terms of its solid state structure.[32] Much of the difficulty in achieving selectivity with cellulose modifications has stemmed from the necessity of heterogeneous reaction conditions. While this is not the case for all polysaccharides, hydroxyl group accessibility, of both primary and secondary positions, is affected by the molecular associations of the polymer in solution, often masking the inherent reactivity differences of the individual sites. Hence, the result is limited and unpredictable reactivity of the hydroxyl groups, similar to the more extreme case of cellulose.

Well-known esterification products are cellulose acetate, nitrate and xanthate. These cellulose esters are used in variety of industries. The former two are used in textile manufacture, and cellulose acetate is also an important film and packaging material. Cellulose nitrate is a commonly used explosive material. Preparing highly and fully substituted esters is less difficult than for cellulose ethers, but the substitution pattern for partially esterified derivatives remains statistical.

The major oxidative treatments which have been applied to cellulose[12] and/or starch[13] include hypochlorite, hypobromite, nitrogen dioxide, chromic acid, oxygen and periodate oxidations. These conditions/treatments give a variety of oxy-cellulose or oxy-starch derivatives respectively, containing aldehyde, ketone and carboxylate

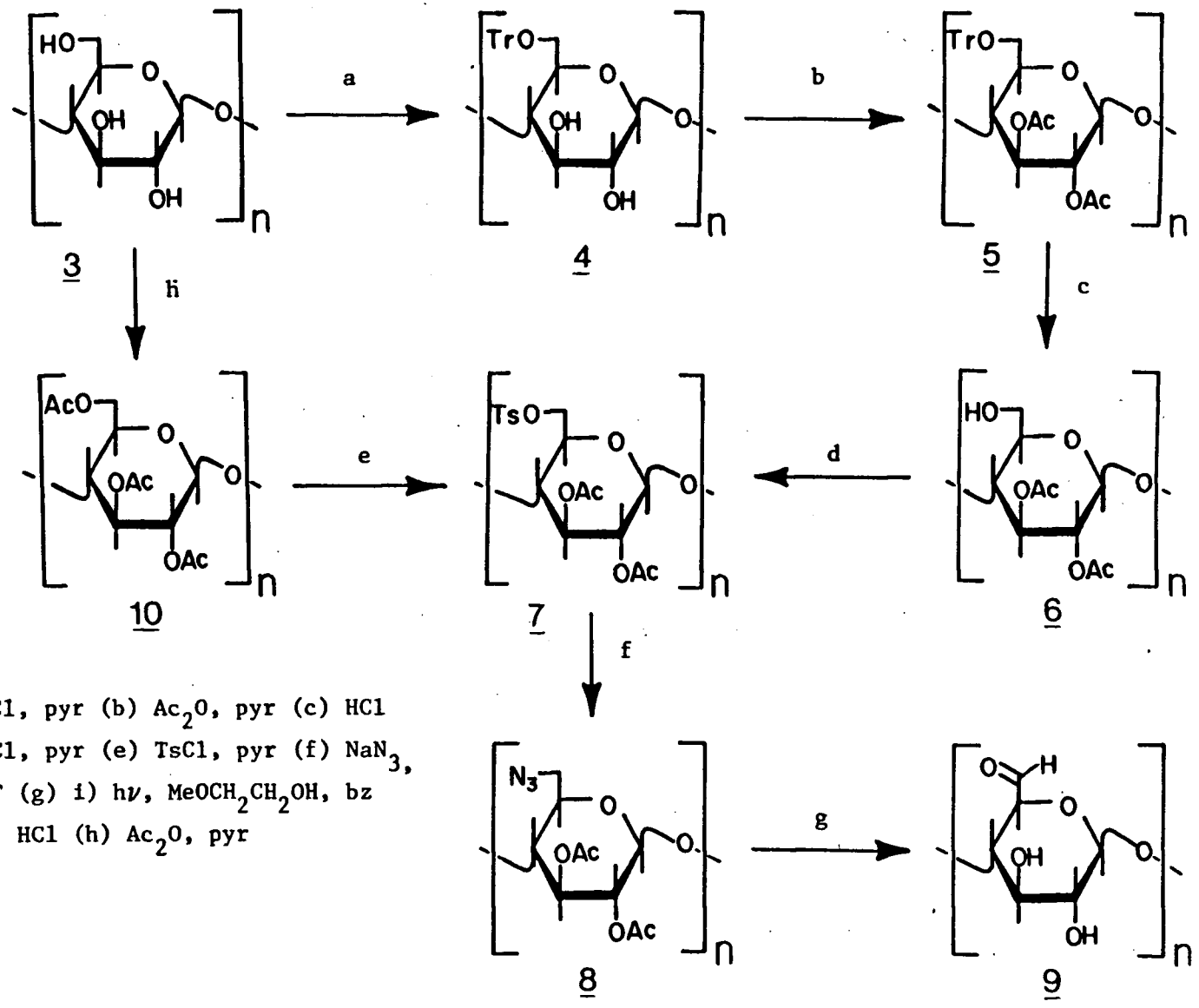
groups. It is not within the scope of this discussion to go into the historical development of these derivatization methods, which have already been well reviewed in the literature.[12,13,31]

The advancements in polysaccharide modification are attributed to several factors. The availability of new reagents, catalysts and solvents have had a profound influence. Also, the greater variety of natural polysaccharides employed as substrates has increased the array of reactions and possible products. However, the introduction of new strategies, which generally involve some degree of selectivity, has created a much greater potential for the generation of "tailored" derivatives.[15] Also, the many new areas in which polysaccharides have found application (i.e. biochemical purifications, solid phase chromatography media, and biomolecule immobilizations), have provided a new range of target materials and increased motivation.[1,8,9,28,33,34] Site-specific modification of polysaccharide carboxyl groups are well-known.[15] However, until the recent renaissance in polysaccharide modification research, relatively few methods for site-selective chemical derivatization of polysaccharides existed. In the last 15 years, considerable attention has been directed toward amine-containing polysaccharides such as chitosan, a (1→4)-linked 2-amino-2-deoxy-β-D-glucopyranose homopolymer, as substrates for selective modifications.[23] Such interest has led to the development of new approaches for the modifi-

cation of amine functions, and perhaps of greater importance, methods for site-selective preparations of synthetic amino polysaccharides.[15] A much larger choice of substrates bearing amino groups is thus available for application in "tailored" modification strategies. In the following section, a review of the existing methods for the preparation and modification of amino polysaccharides will be undertaken.

1.2.2 Synthesis and Modification of Amino Polysaccharides

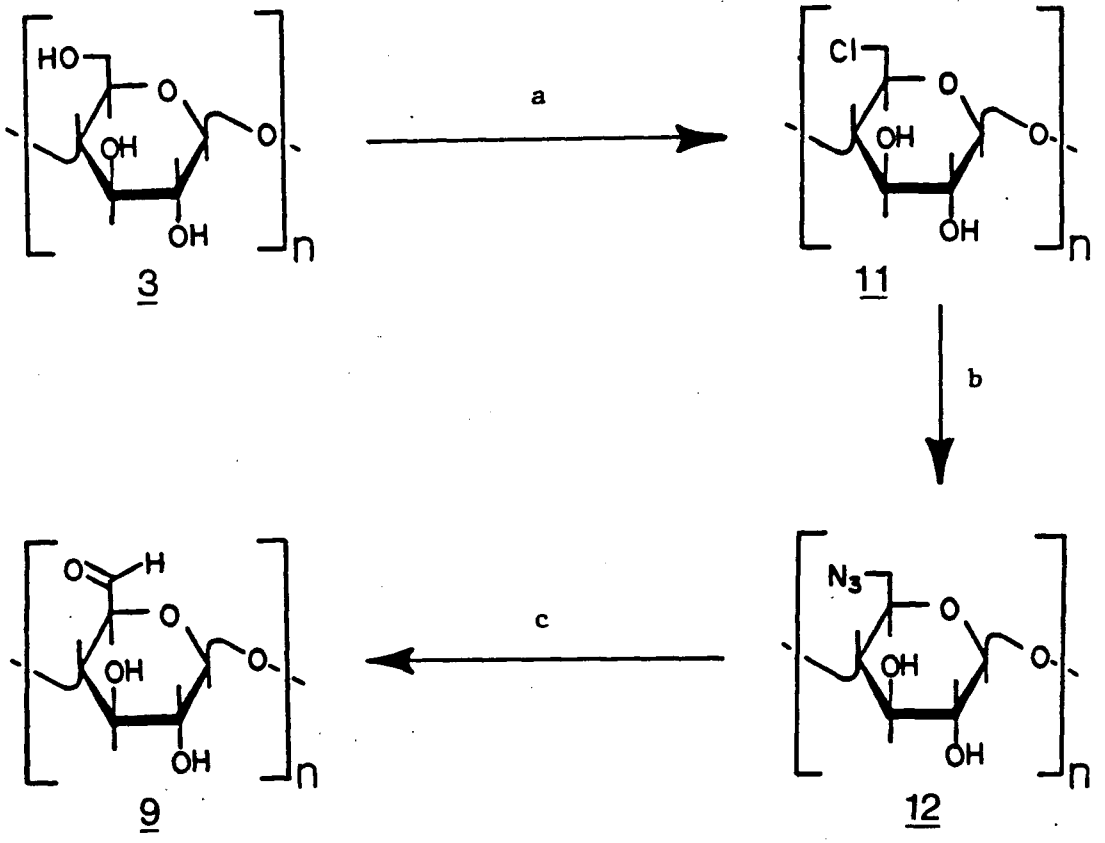
One of the most popular methods of introducing an amine function involves the intermediary formation of a polysaccharide bearing carbonyl functions through oxidation. Hence, methods for the selective oxidation of polysaccharides are important in amino polysaccharide synthesis, as well as for isotope labelling, epimerizations, and potentially for reaction with Grignard and Wittig reagents. Normally, keto groups are introduced at secondary positions while aldehydes or carboxylates result at primary centers; however, over-oxidation can cause cleavage of the monosaccharide unit, and the formation of dialdehydes or dicarboxylates.[35] In most instances this is undesirable as the integrity of the polysaccharide has been decreased. Nevertheless, products of this sort have found application as textile and paper sizes.[13] Fortunately, while many oxidations work in a non-selective or over-oxidative manner, mild reagents and strategies involving protecting groups have provided ways for overcoming some of the problems of polysaccharide oxidations.



(a) TrCl, pyr (b) Ac_2O, pyr (c) HCl
(d) TsCl, pyr (e) TsCl, pyr (f) NaN_3, DMF (g) i) $h\nu$, $MeOCH_2CH_2OH$, bz
ii) HCl (h) Ac_2O, pyr

Scheme 1

The oxidation of primary C-6 positions to an aldehyde occurs under many oxidative conditions. However, the aldehyde usually rapidly oxidizes to the carboxylate under most aqueous conditions. Selective methods, typically involving multistep procedures, have been developed for preparing 6-aldehydo-cellulose 9.[36,37] For example, as shown by Scheme 1, the 6-O-p-tolylsulfonyl derivative 7 was converted into the azide 8, which was subsequently photolyzed to the aldehyde 9.[36] Similarly, a "one-pot" preparation of a

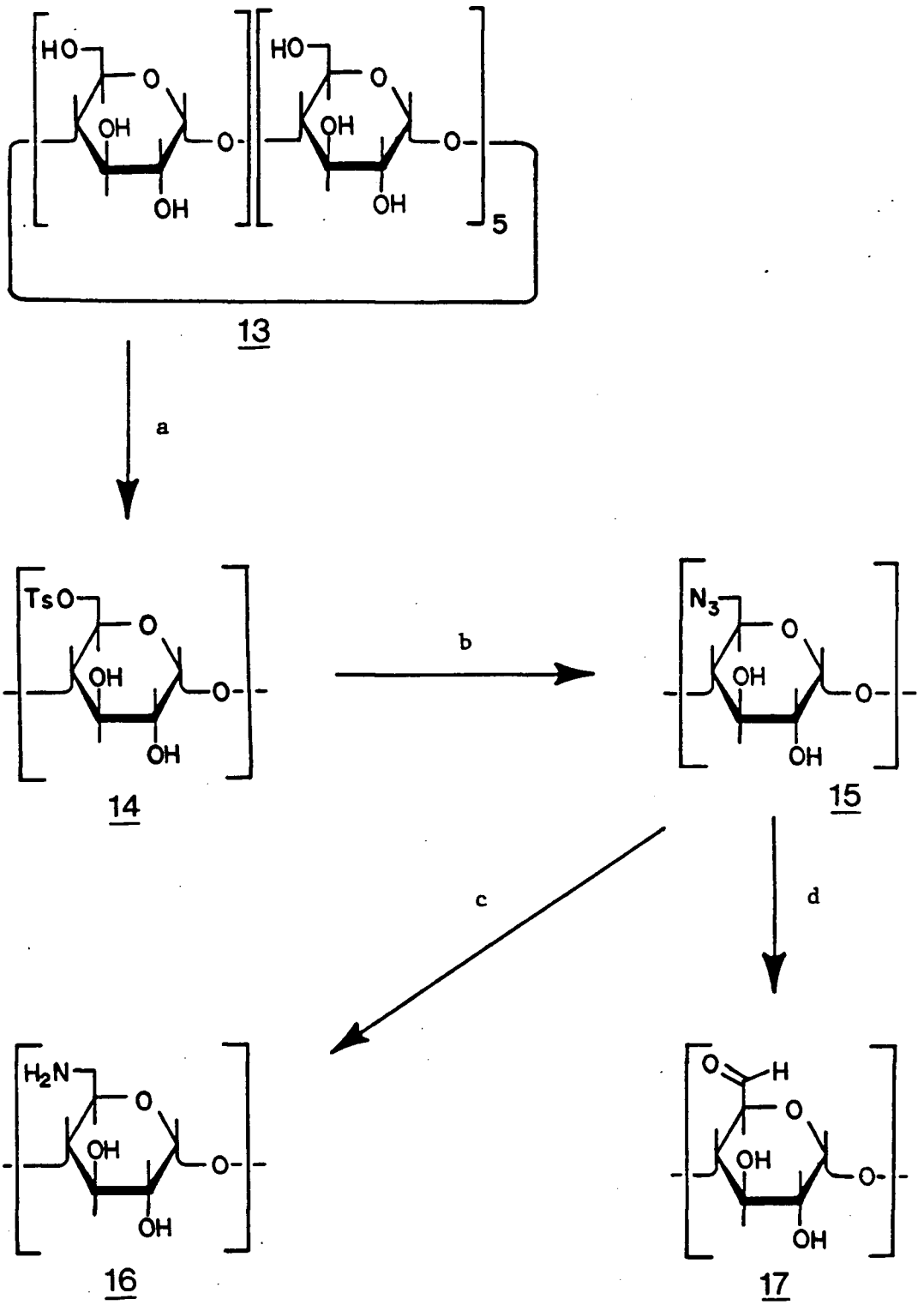


(a) i) MsCl, DMF ii) Na_2CO_3, H_2O (b) NaN_3 (c) $h\nu$

Scheme 2

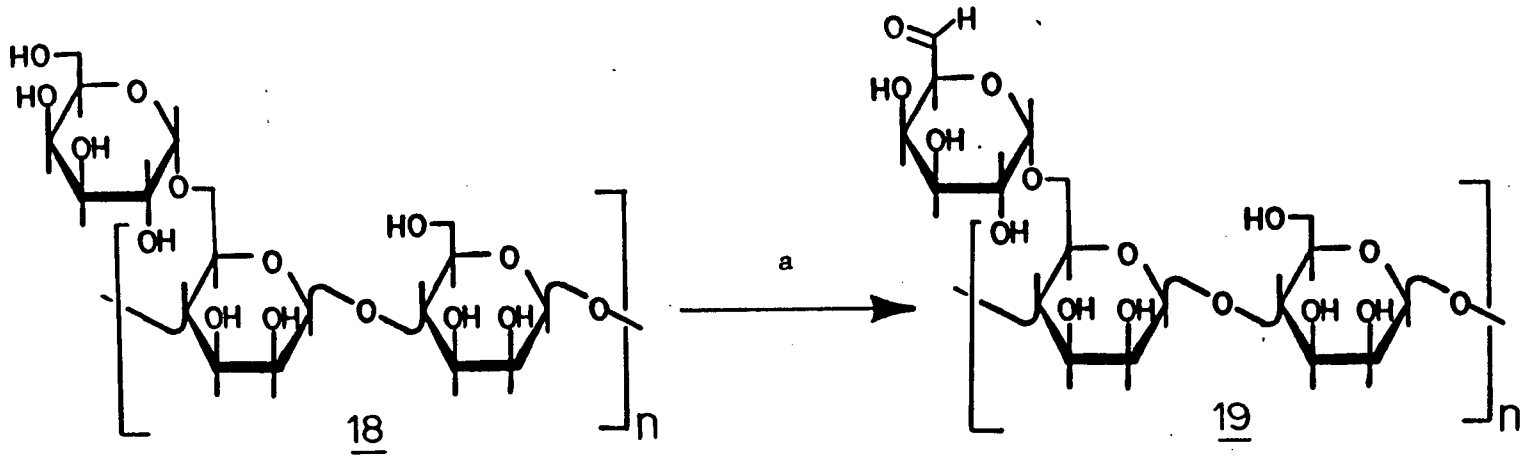
6-chloro-6-deoxy-cellulose derivative 11, followed by nucleophilic substitution by azide ion and photolysis led to the 6-aldehydo product (Scheme 2) having d.s. values of 0.03-0.45, with greater selectivity.[37] While none of the 6-aldehydo-cellulose derivatives have been converted to the corresponding 6-amino-celluloses, there is certainly potential for this transformation. Scheme 3 outlines the analogous preparation of the 6-amino- and 6-aldehydo-cyclcodextrin compounds 16 and 17 from the selectively tosylated cyclcodextrin 14.[38,39] Similar oxidation procedures have been applied to amylose.[40] Unfortunately, these procedures are invariably accompanied by mild to appreciable polymer hydrolysis.

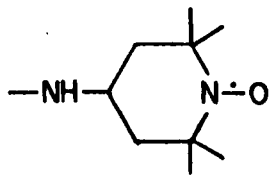
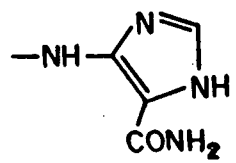
The enzyme galactose oxidase has been employed to generate specifically the C-6 aldehyde moiety in galactose residues of polysaccharides.[41,42] This enzymatic oxidation has been applied to agarose,[43,44] locust bean gum[45] and guar gum.[41,45-47] In the latter case, the 6-aldehydo-guar 19,was reductively aminated to provide the amine-containing derivatives 20-25 (Scheme 4)[45] bearing a variety of functional groups. The enzyme appears to be inhibited by 4-O- and 3-O-substituted galactose residues, and by 2-amino-2-deoxy-D-galactose.[41,46] The oxidative efficiency is typically quite high (60-90%). Treatment of the aldehyde with

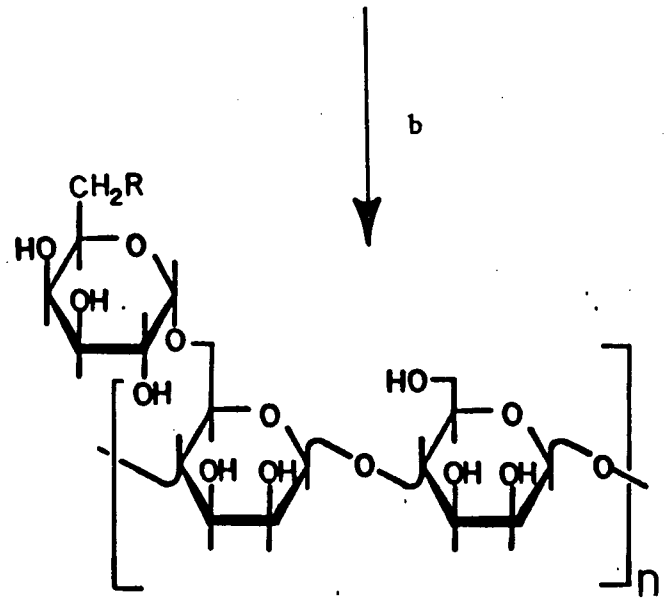


(à) TsCl, pyr (b) NaN_3 (c) $LiAlH_4$ (d) $h\nu$

Scheme 3



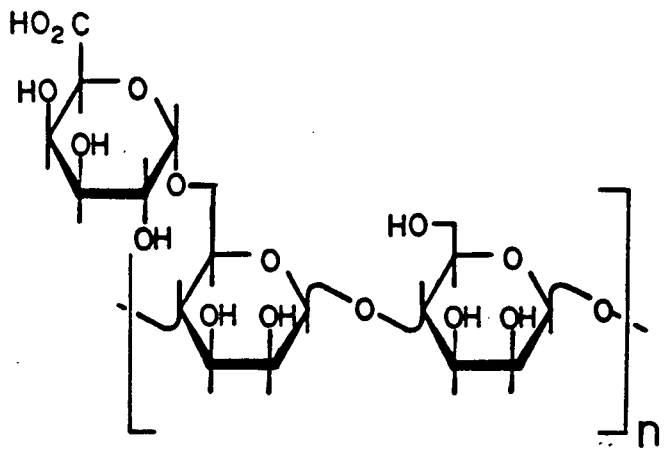
	R
20	$-NH_2$
21	$-NHCH_2CO_2H$
22	—NH—BSA
23	$-NH(CH_2)_3\,OH$
24	
25	



(a) Gal. Oxidase b) $NaCNBH_3$, amine

Scheme 4

aqueous bromine has provided 26,with a D-galacturonic acid branch residue on the mannan backbone .[45]



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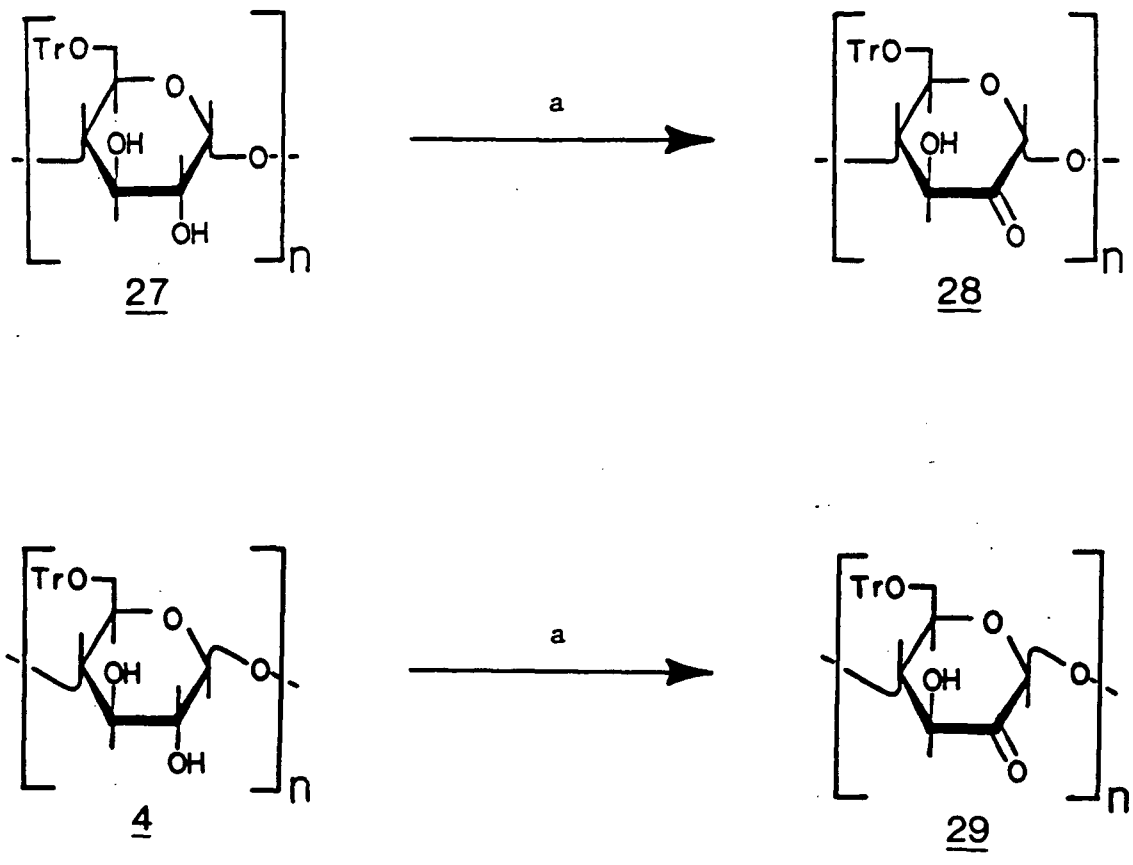
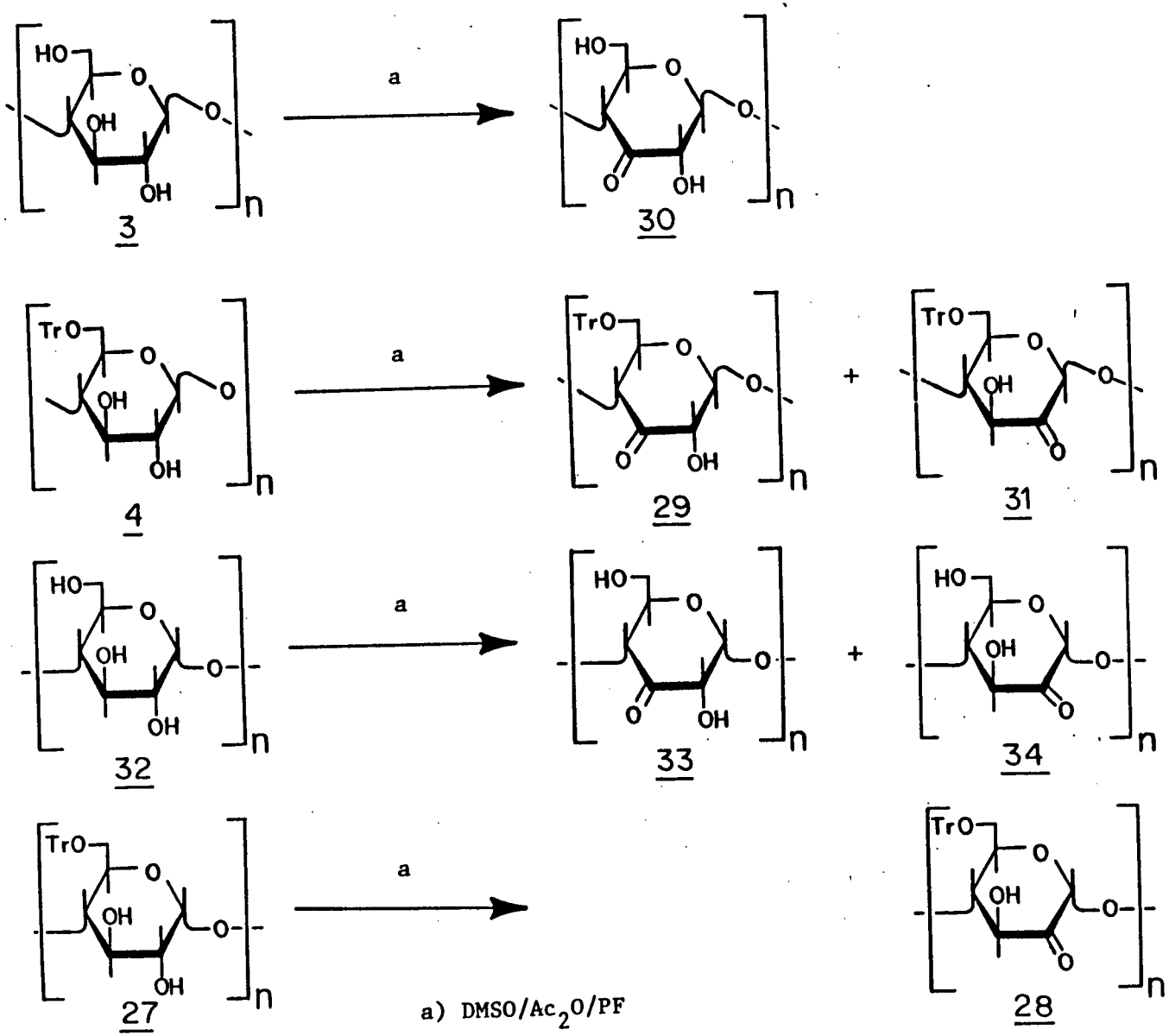
The oxidation of secondary hydroxyl groups of polysaccharides, to keto- or glycosylulose residues, has been accomplished using the dimethyl sulfoxide/acetic anhydride (DMSO/Ac_2O) reagent.[48] The trityl ether is usually employed as a C-6 protecting group, to prevent C-6 oxidation. For example, both 6-O-trityl-amylose 27, and 6-O-trityl-cellulose 4, gave predominantly the respective 2-keto products upon treatment with DMSO/Ac_2O (Scheme 5).[49-51] Native cellulose 3, under the same conditions gave mixtures of 2-oxy, 3-oxy and 2,3-dioxy residues.[52] The 3-oxy product 30 was obtained exclusively in 60-70% yield when the reaction was performed using dimethyl sulfoxide/paraformaldehyde (DMSO/PF) as the solvent system (Scheme 6). [53,54] Similar oxidations of amylose (32) gave predominantly the 3-oxy residue 33, with 10% of the 2-keto product 34. Interestingly, it was found that 6-O-trityl-amylose gave exclusively the C-2 oxidized



(a) DMSO/Ac_2O

Scheme 5

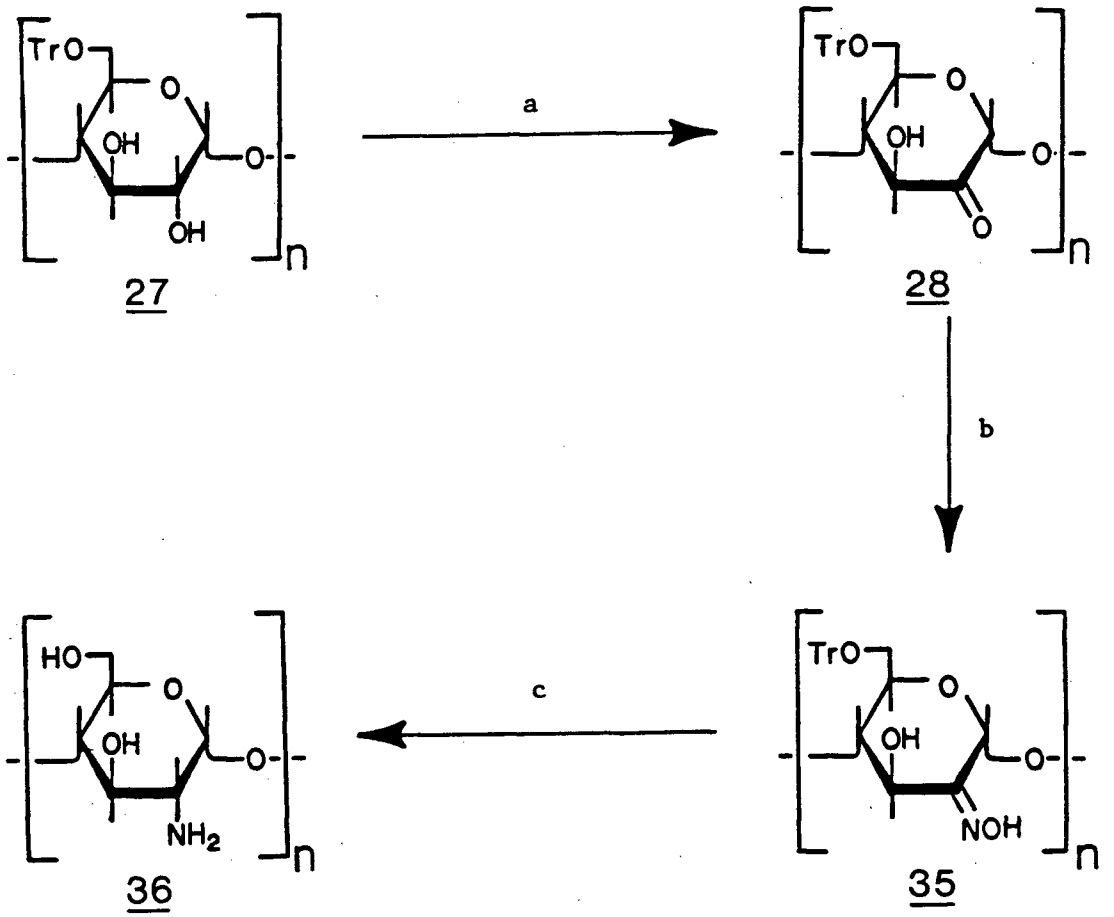
product 28 in the DMSO/Ac_2O/PF system, while 6-O-trityl-cellulose yielded a mixture of 2-oxy 29 (56%) and 3-oxy 31 (36%) residues.[54] Analogously, 6-O-acetyl-amylose gave 56% and 30% of the 2- and 3-oxy monosaccharide residues respectively. Apparently, the bulkiness of the 6-O substitutent influences the site of oxidation, tending to promote C-2 oxidation. Selective oxidation of the C-3 position of cellulose and amylose with DMSO/Ac_2O/PF, is considered to be due



Scheme 6

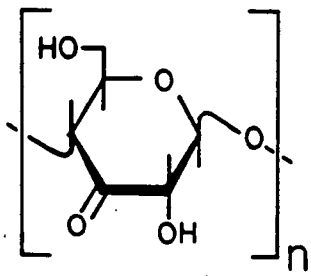
to reversible formation of covalent hydroxymethyl and poly(oxymethylene)ol groups at O-2 and O-6.[53] Thus, when 6-O-trityl derivatives are treated with this reagent, *in situ* protection of O-2 occurs, thereby shifting selectivity from O-2 to O-3. The Pfitzner-Moffat reagent[48] has been reported to achieve similar oxidations and has been applied to cellulose.[52]

The 2-oxy-amylose derivative 28 has been further elaborated, *via* oximation, reduction and detritylation (Scheme 7) to give 2-amino-amylose 36 with d.s. 0.8, and predominantly the D-gluco configuration.[49] Other workers have

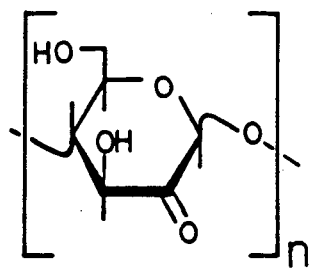


(a) $DMSO/Ac_2O$ (b) $HONH_2Cl$ (c) $LiAlH_4$, THF

Scheme 7



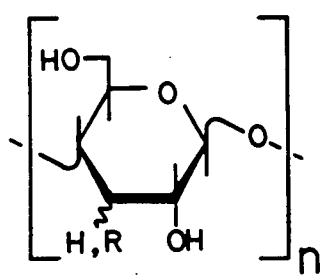
37

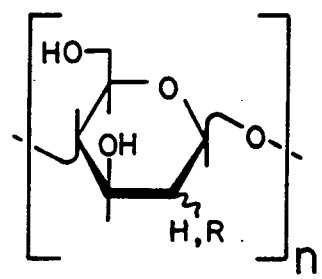


30

a

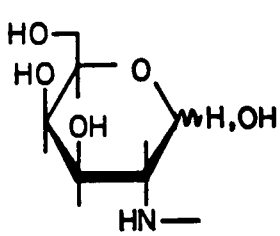
a





R

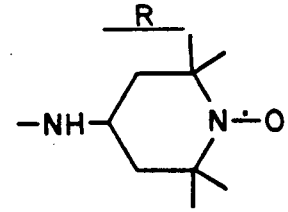
40 $-NH_2$

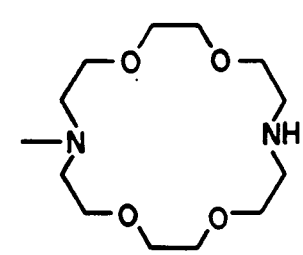


41

R

38





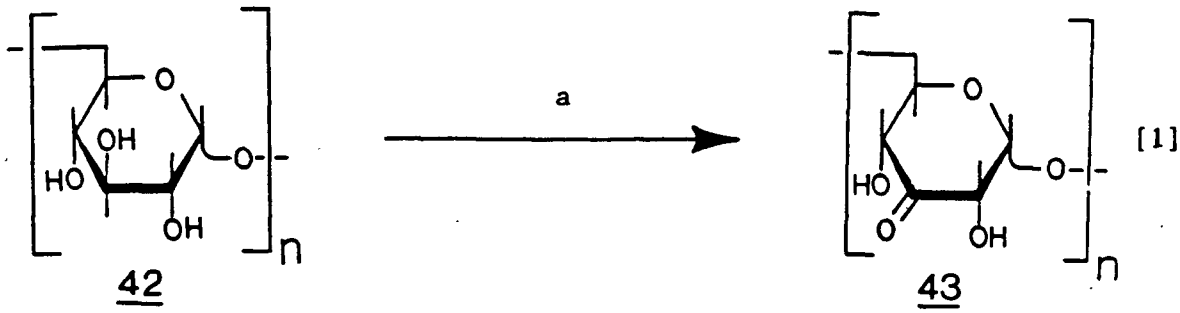
39

(a) $NaCNBH_3$, amine

Scheme 8

also applied this oximation/reduction sequence in 2-amino-polysaccharide syntheses.[55,56] The 2- and 3-keto-cellulose derivatives 30 and 37, have been reductively aminated to provide the 2-amino- and 3-amino-cellulose derivatives 38-41, seen seen in Scheme 8.[57,58] The reaction of glucosamine with 37 gave the (3→2')-amine linked 41, a unique branched polysaccharide.

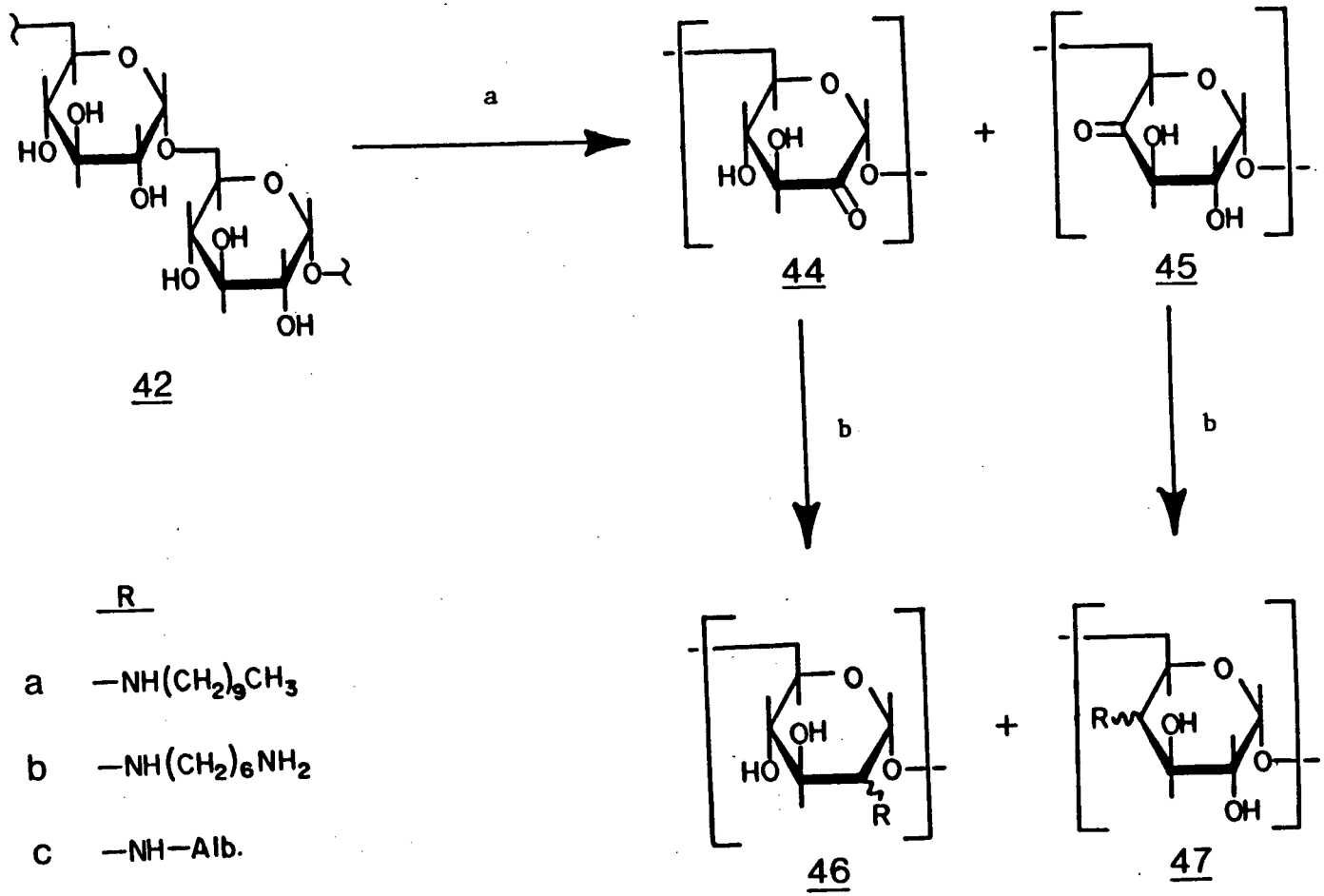
Dextran, a branched polysaccharide with a (1→6)-α-D-glucopyranose main chain with the O-6 position blocked, is an ideal candidate for selective oxidation of secondary centers. The DMSO/Ac_2O reagent, when applied to dextran (Eq. 1), gave mainly the 3-keto derivative 43.[59]



[1]

(a) DMSO/Ac_2O

Aqueous bromine[60] is known to oxidize polysaccharides, and has been used to prepare dextran derivatives having 2-oxy (44) and 4-oxy (45) functionalities in a 0.85:1.00 ratio (Scheme 9).[61,62] These partially oxidized dextrans have been reductively aminated with alkylamines and albumin . Unfortunately, acidic products arising from ring cleavage made up 11% of the product mixture when 1.0 equivalent of oxidant per

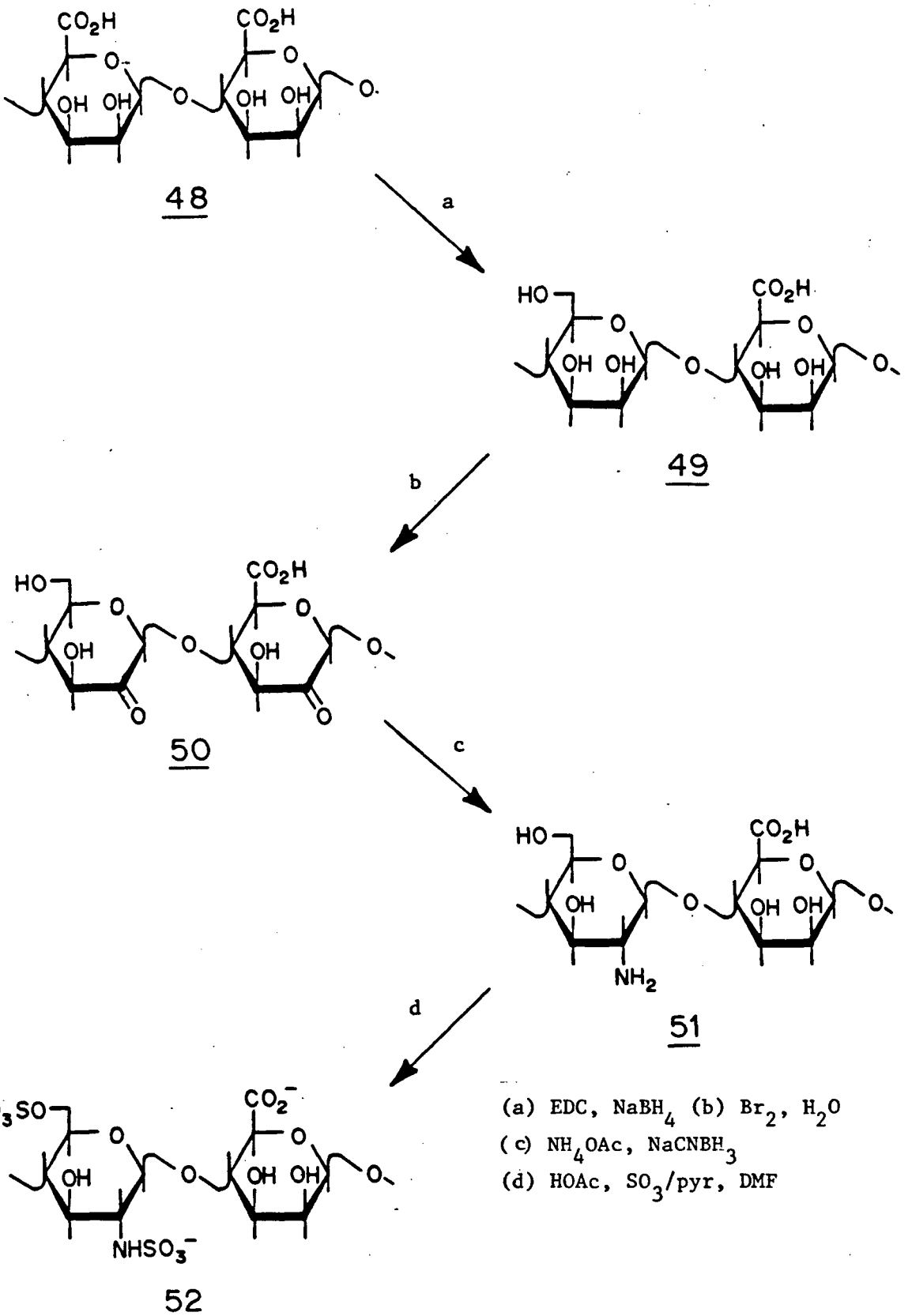


(a) Br_2/H_2O (b) $NaCNBH_3$, amine

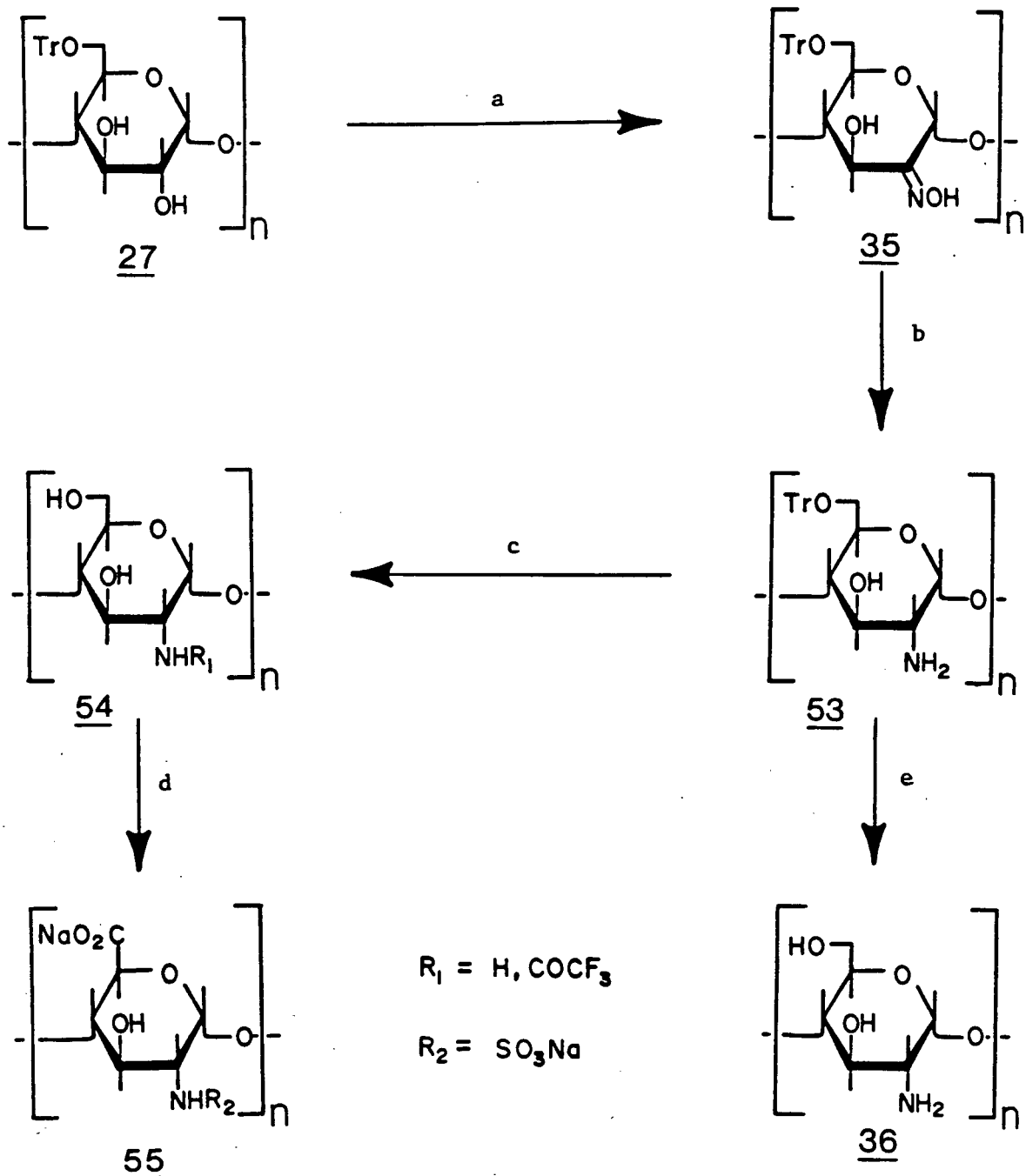
Scheme 9

residue was used. The use of borate, a cis-diol complexing agent,[63] in conjunction with aqueous bromine oxidation has been employed in the oxidation of T-40 dextran.[64] It was found that in the presence of borate the degree of oxidation increased to 65%, from 43% without borate, but with no change in distribution of ketone groups. Also the extent of over-oxidation was reduced from 11% to 3%. Similarly, Sepharose™ (cross-linked agarose) has been treated with aqueous bromine to give residues having mainly the 4-keto functionality. This was then reductively aminated with 1-aminodecane, 1,6-diaminohexane and albumin.[61,65] Cellulose treated under similar conditions showed only a low degree of oxidation at the C-2 and C-3 positions.[65,66] The heparin analogue 52 has been prepared from partially reduced (50%) alginic acid 49, as outlined in Scheme 10, by selective aqueous bromine oxidation, reductive amination with ammonium acetate, and sulfation.[67] Substantial depolymerization of the product was noted.

Oxidations have been performed on amine-containing polysaccharides in order to generate carboxylates at C-6. For example, as depicted in Scheme 11, 2-amino-6-O-trityl-amylose (53), was converted into the heparin analogue 55 having 46% carboxylates at C-6 .[56] Similarly, chitosan (1) has been treated with perchloric acid, chromium trioxide and subsequently chlorosulfonic acid, to give the heparin analogue 57 (Scheme 12).[68] Interestingly, the perchlorate salt acted as a bulky group, sterically limiting oxidation at C-3.

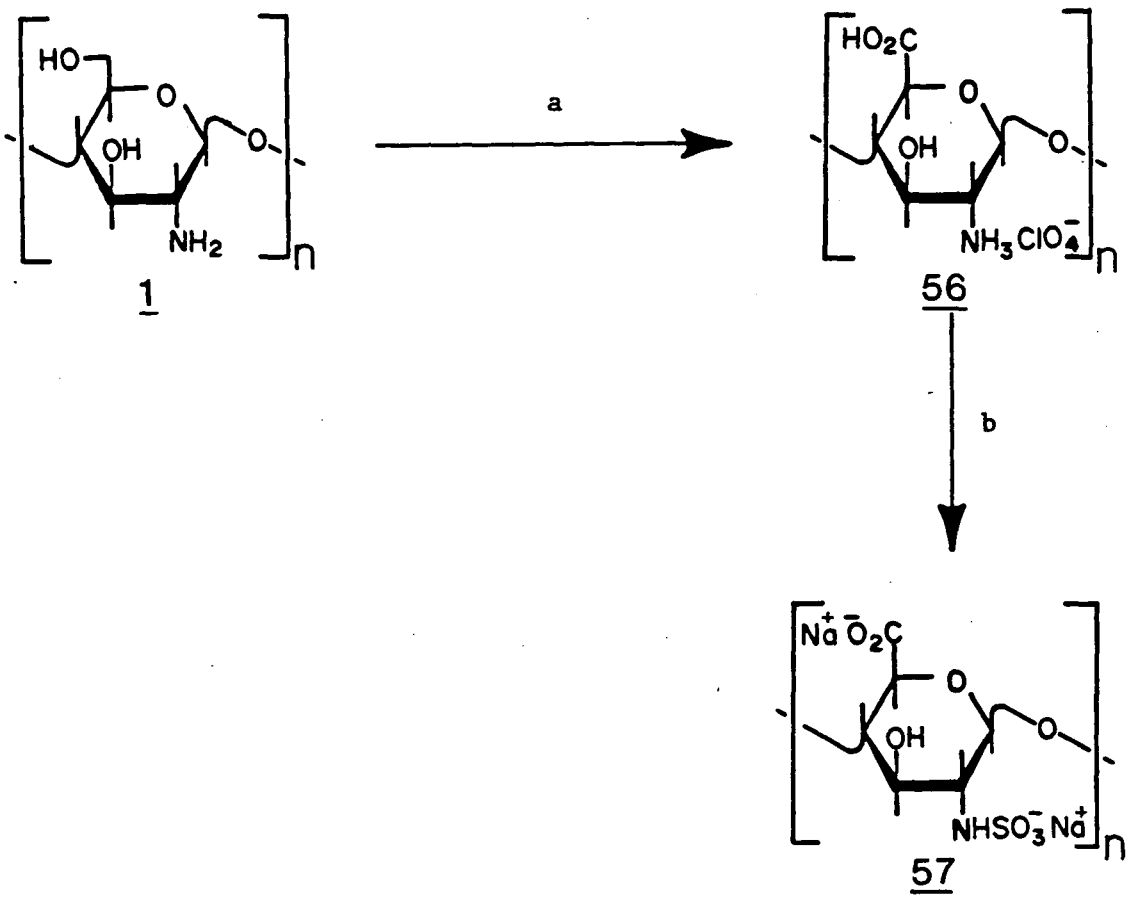

48
a
49
b
50
c
51
d
52
(a) EDC, $NaBH_4$ (b) Br_2, H_2O
(c) NH_4OAc, $NaCNBH_3$
(d) HOAc, SO_3/pyr, DMF

Scheme 10



(a) i) Ac_2O, pyr ii) $HONH_2$, pyr (b) $LiAlH_4$ (c) i) $(CF_3CO)_2O$, pyr ii) HCl, $CHCl_3$ (d) i) O_2/Pt ii) H_3O^+ iii) OH^- iv) SO_3, pyr v) $ClSO_3H$, pyr (e) HCl

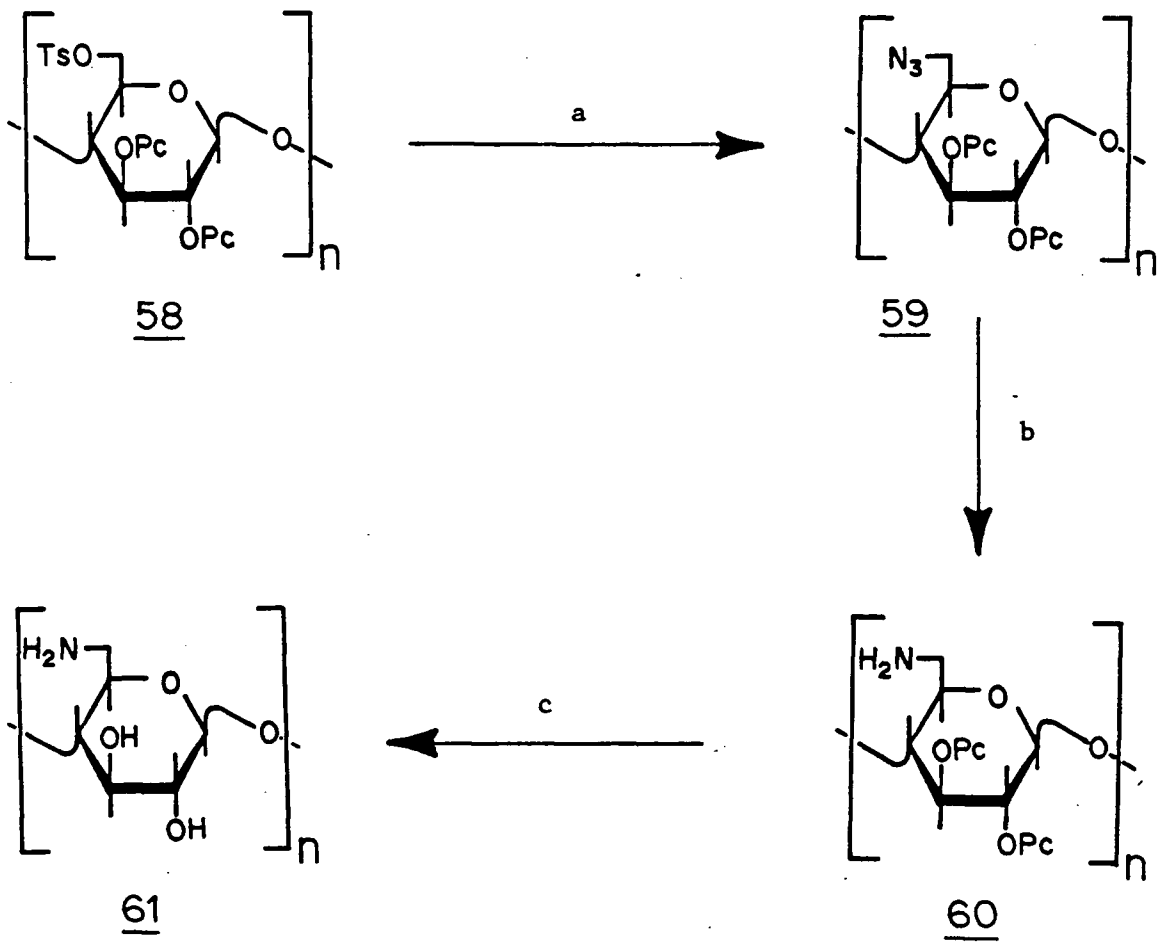
Scheme 11



(a) i) $HClO_4$ ii) CrO_3 (b) i) $ClSO_3H$, pyr ii) NaOH

Scheme 12

Cellulose and amylose derivatives bearing 6-amino moieties have been prepared in low yields by routes similar to those for 6-aldehydo-cellulose. Scheme 13 outlines a route that has been applied to both cellulose[69] and amylose,[70] in which the 2,3-di-O-phenylcarbamoyl-6-O-p-tolylsulfonyl derivative 58 was converted into the amine 61 via azide formation. Amino groups have been introduced at C-6 of

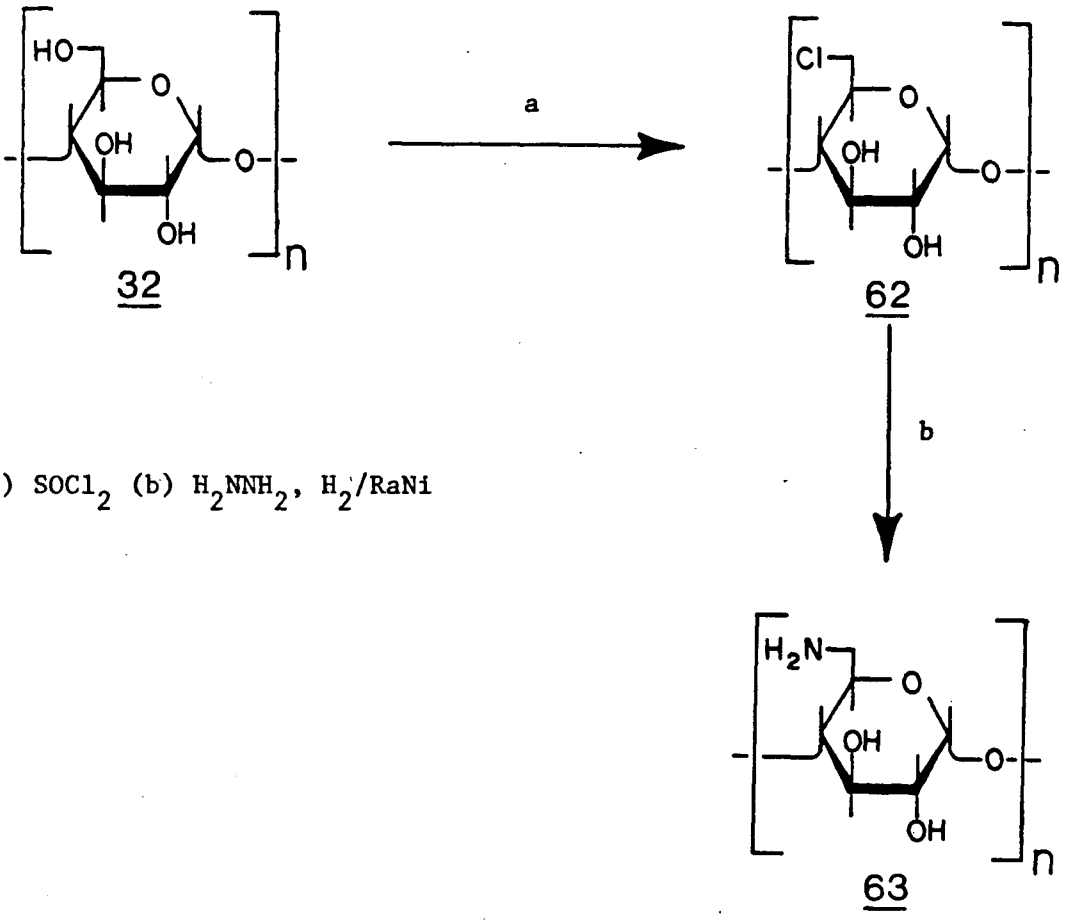


(a) NaN_3, DMSO (b) $LiAlH_4$, THF (c) NaOMe/MeOH

Scheme 13

amylose by treatment with sulfuryl chloride to give the 6-chloro derivative 62, followed by hydrazinolysis and reduction to give 63 (Scheme 14). [71]

The previous section illustrated the many approaches that have been employed to prepare a diversity of synthetic amino polysaccharide derivatives. One of the main drawbacks to many of the procedures was concomitant depolymerization. The use of naturally occuring amino polysaccharides as



(a) $SOCl_2$ (b) H_2NNH_2, H_2/RaNi

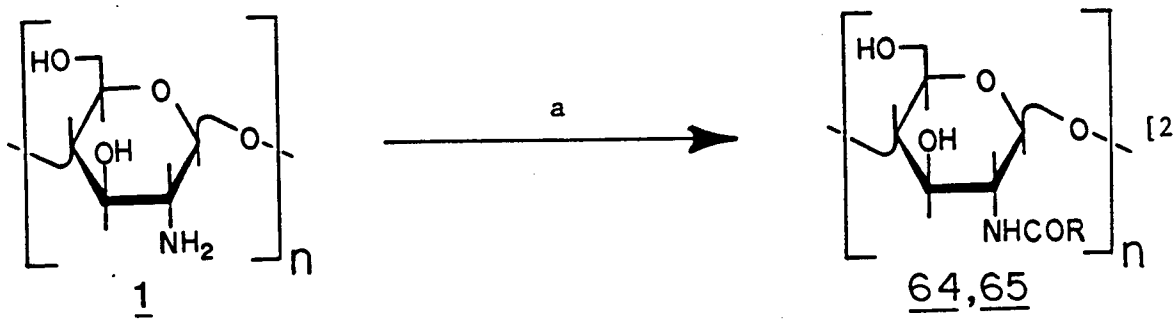
Scheme 14

substrates for mild chemical modification has thus become a favored method for preparing functionalized amino polysaccharides without compromising molecular weight. Modified amino-polysaccharides have been prepared by N-alkylation and N-acylation of natural amine-containing polysaccharides.

Chitin (2), a (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose polymer (with 15% 2-amino-2-deoxy groups), is the most abundant amino polysaccharide , and the second most abundant organic polymer, in nature.[22] It is easily N-deacetylated to provide the 2-amino-2-deoxy-β-(1→4)-D-glucopyranose homopolymer chitosan (1), an ideal substrate for chemical modification. Both of these materials have attracted substantial interest in the form of review articles and monographs which have focussed on their diverse and commercially useful properties.[22-26] The added advantage of facile chemical modification has stimulated researchers to delve into "tailoring" the properties of these relatively intractable materials and expand the potential industrial utility of these polysaccharides.

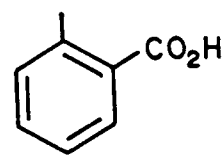
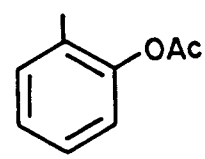
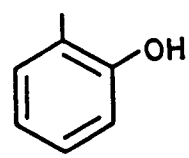
As such, chitosan has been employed extensively as a substrate for N-acylation and N-alkylation. Insoluble in water and most organic solvents, chitosan is readily solubilized in dilute aqueous organic acids (e.g. acetic, formic, oxalic, etc.), in which most of its reactions are conducted. These solvent systems give clear, viscous chitosan solutions suitable for homogeneous chemical modification.

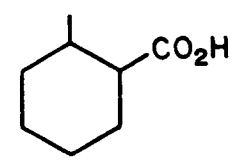
Selective N-acylations have been performed on chitosan with a variety of aryl and alkyl carboxylic anhydrides.[72-80] These reactions were done (in 2-10% aqueous acetic acid) with 2-3 molar equivalents of anhydride to give products 64a-c and 65a-n, with high d.s. (0.8-1.0) in good yields (77-96%), as shown in Eq.2. Succinylation of chitosan provided products



1 64,65

(a) HOAc/MeOH/H_2O, $(RCO)_2O$

	R
64a	
b	
c	

	R
65a	H
b	CH_3
c-k	$(CH_2)_n CH_3$ n = 1,2,4,6,8,10,12,14,16
l	CH_2Cl
m	$CH_2CH_2CO_2H$
n	

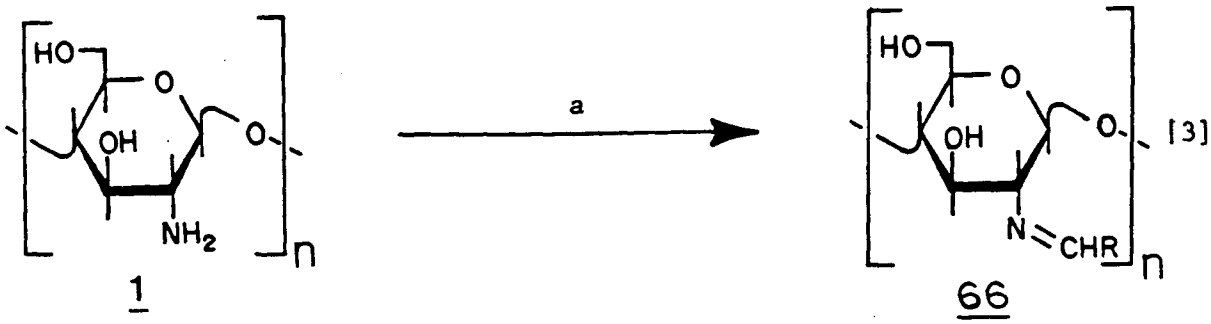
with d.s. 0.2-0.60, with the higher d.s. samples being water-soluble.[74,81] Cross-linking (0.5-1.0%) of these derivatives produced transparent gels suitable for enzyme immobilization matrices. An N-2'-acetoxybenzoyl (aspirin) derivative 64b (d.s. 0.65), obtained by N-acylation of chitosan with 2-acetoxybenzoic anhydride has been evaluated as a potential drug delivery and controlled release system.[82]

The N-alkylation of chitosan has received much attention over the last 10 years. These studies are generally categorized in two ways: (1) involving reaction with alkyl or aryl halides, and (2) by reductive amination with aldehydes or ketones. The former has been used to prepare quaternary alkylammonium derivatives from regenerated chitosan and alkyl iodides in the presence of pyridine or triethylamine.[83] The tri-N-alkylammonium iodide salts of chitosan are water-soluble, with d.s. values ranging from 0.52-0.78. A N,N-dimethyl-N-hydroxyethyl-chitosan salt was obtained by reaction of chitosan with methyl iodide and ethylene oxide.[84]

The reaction of amines with carbonyls is well known.[85] The initial equilibrium yielding the imine or Schiff base, is driven to completion upon addition of borohydride[76] or other reducing agents, which transform the imine into the corresponding substituted amine. This reaction has found extensive application to chitosan, affording derivatives which vary in functionality and properties.

In some cases, it is of interest to isolate the Schiff base derivative rather than the reduced amine. N-Alkylidene

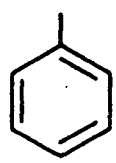
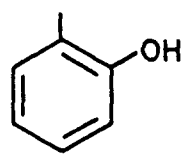
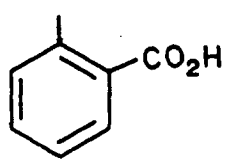
and N-arylidene derivatives of chitosan, 66a-g, have been reportedly derived from formaldehyde and a variety of alkyl[87] and aryl [87-89] aldehydes, some examples of which are given in Eq. 3. These derivatives have served as the protected forms of the chitosan amine moiety during O-alkylations and O-acetylations. Typically, these N-alkylidene and N-arylidene adducts are obtained as gels from reaction in methanolic acetic acid. These gels are insoluble in water and organic



1 66 [3]

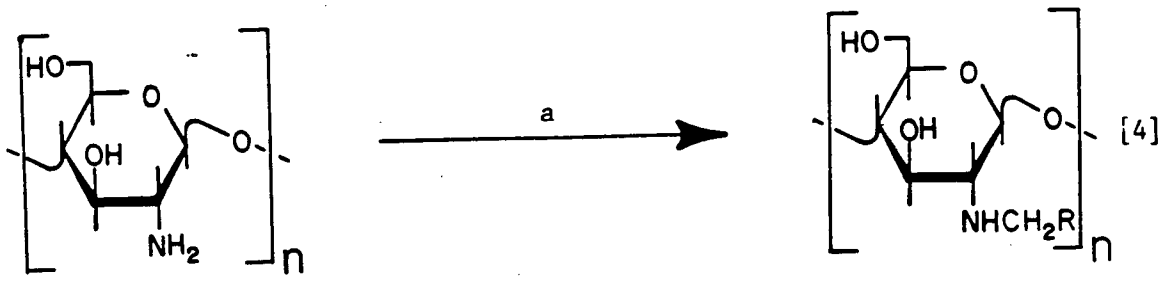
(a) HOAc/MeOH/H_2O, RCHO

	R
66a	H
b	CH_3
c	CH_2CH_3
d	CO_2H

	R
e	
f	
g	

solvents, although in the latter, some N-arylidene products do swell.[78] These materials are of interest for their membrane-forming ability and for their porous ultrastructure, a desirable attribute for gel filtration applications.[90,91]

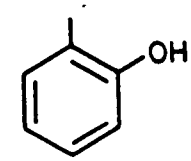
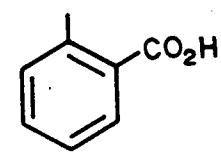
Reductive alkylations have the advantage of affording products of greater hydrolytic stability than the corresponding imine, and are thus of greater applicability for many end uses. Typically, sodium cyanoborohydride is the reducing agent of choice, due to its low reactivity toward aldehydes and ketones, and stability in aqueous reaction media.[86] However, hydrogen/Raney nickel (H_2/RaNi) treatment is also suitable[85] and reacts more quickly. Sodium cyanoborohydride has been used to prepare a wide range of N-alkyl chitosan compounds, with virtually no depolymerization.[15] A variety of alkyl and aryl aldehydes have been reacted using this procedure. Particular attention has been centered on the attachment of functionalized molecules to chitosan in order to generate or enhance specific properties. For example, Eq. 4 shows that salicylaldehyde,[92] o-phthalaldehyde,[93] glyoxylic acid[94] and ascorbic acid[95] have been reductively alkylated to chitosan to produce, respectively, compounds 67-70, all of which demonstrated substantial metal-chelating capacity . A variety of carbohydrate molecules have been attached to chitosan,[96,97] giving the branched chain derivatives 71-76 (d.s. 0.10-0.97) amongst others. In the case of 76, interesting rheological properties were observed.[98] It was demonstrated by N-acetylation of 76 to give 77, and by alkylation

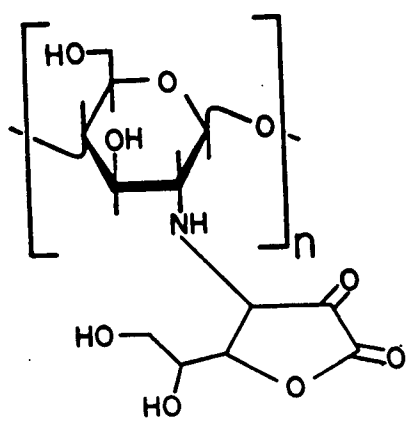


1

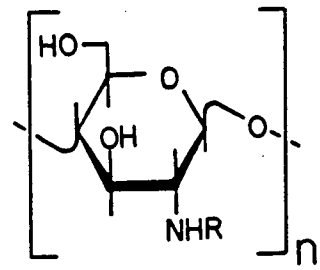
67-69

(a) aldehyde/ketone, $NaCNBH_3$, $HOAc/H_2O$

	R
67	
68	
69	CO_2H

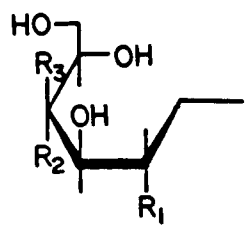


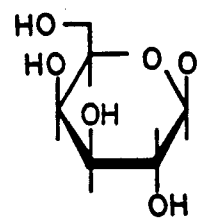
70



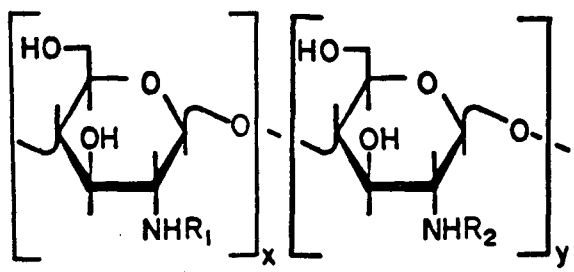
71-76

R

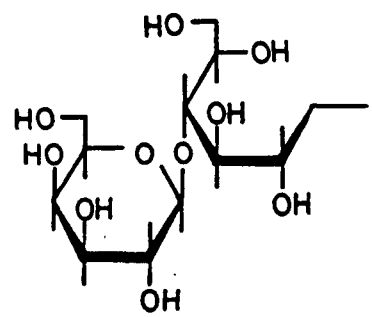


	R_1	R_2	R_3
71	OH	OH	H
72	NHAc	OH	H
73	NH	OH	H
74	OH	H	OH
75	NH	H	OH
76	OH		H

of 76 with propanal to yield 78, that properties such as solubility and hydrophobicity could be manipulated by preparing "mixed" derivatives.[96] In the same report, streptomycin sulfate was similarly coupled to chitosan as was a selectively oxidized cyclodextrin, affording conjugates with potential use in drug delivery and drug carriers. Branch copolymers of chitosan formed by reductive amination with T-10 dextran (d.s. 0.15)[96] and an aldehyde derivative of polyethylene glycol[93] (MW 8,000) have also been described.



76-78

	R_1	R_2
76	H	
77	$COCH_3$	
78	$CH_2CH_2CH_3$	

1.2.3 Nmr Spectroscopy of Polysaccharides

A current trend associated with structural studies of polysaccharides revolves around the use of spectroscopic methods to probe both structure and molecular dynamics.[18] Spectroscopic methods can be used on both intact species or in conjugation with wet chemical and chromatographic techniques. Ultimately, one would like to determine the structure, de novo, of intact polymers with minimal chemical manipulation. Some of the various spectroscopic techniques that have been used in polysaccharide studies are ir, ORD/CD, ^{1}H-nmr and ^{13}C-nmr spectroscopy. The latter two, proton and carbon-13 nuclear magnetic resonance spectroscopy, have become extremely valuable in the last ten years, as evidenced by the numerous recent reviews of their application to oligo[99-101] and polysaccharides,[18,102-104] in both structural and physicochemical investigations. Because these two spectroscopic tools have become invaluable in the area of polysaccharide studies, a brief overview is in order.

Nuclear magnetic resonance spectroscopy has a number of inherent features which make it particularly indispensable in polysaccharide investigations. The parameters of chemical shift (δ), coupling constants (J), and relaxation times (T_1 and T_2) impart information about the chemical structure and identity of the carbohydrate residues present, as well as conformational and dynamic aspects of the system. There is a large volume of literature dealing specifically with these parameters as they apply to the study of carbohy-

drates.[18,99-105] Nmr spectra are usually obtained on samples in solution; however, in the last decade it has become possible to perform routine nmr experiments on samples in the solid state[105] using the ^{13}C-cross polarization/magic angle spinning (^{13}C CP/MAS) experiment. This advancement has greatly enhanced the potential of nmr in the study of intractable materials. These factors, coupled with technological advances in the design of nmr instrumentation and computerization make nmr an invaluable spectroscopic tool in the study of complex carbohydrate polymers. The discussion presented herein will deal solely with solution state nmr spectroscopy.

Before a discussion of ^{1}H and ^{13}C-nmr spectroscopic application to polysaccharides is undertaken, certain factors which affect the acquisition of high resolution spectra (for ^{1}H in particular) must be addressed. These factors are line-broadening of the signals and interference of exchangeable protons (N-H, O-H). Typically, solutions of polysaccharides are prepared in deuterium oxide, as opposed to water, thereby removing the signal from O-H and N-H protons. However, an HOD peak (δ~4.8 ppm) arises from exchange, and may interfere with proton signals of the sample. Also, polysaccharides often contain water of hydration, which further increases the HOD signal. To minimize this interference it is prudent to subject the sample to deuterium exchange, i.e. dissolution in D_2O and lyophilization, repeatedly. Generally, this procedure diminishes the HOD signal sufficiently to give

an unobscured spectrum of the sample of interest. Other methods exist for minimizing the interference of the HOD signal in FT-nmr experiments.[107] For example, saturation decoupling takes advantage of the differential relaxation of solvent (T_1 of H_2O > 2 s) and polymer (T_1 < 0.5 s) protons. Thus a suitable pulse sequence can largely remove the interfering resonance, although spurious peaks may result.

Line-broadening is a manifestation of the short spin-spin relaxation times (T_2) of the polymer protons ($\Delta\nu_{1/2} \propto 1/T_2$). This problem can be partially overcome by acquiring spectra at elevated temperatures. The use of high-field spectrometers by which greater signal dispersion is obtained, helps to counteract line-broadening effects. Another way to obtain spectra with better resolution is to use computer lineshape manipulation techniques, such as convolution difference processing.[103] This problem is not as critical in FT ^{13}C-nmr because of the much greater signal dispersion, as will be discussed shortly, which makes ^{13}C-nmr extremely attractive for studying polysaccharides.

Carbon-13 nmr spectroscopy suffers from a drawback which does not apply to the ^{1}H-nmr experiment. The inherent difficulty in detection of ^{13}C lies in the low natural abundance (1.1%) of this nuclei, and the low relative sensitivity (1.59×10^{-2}), giving an overall sensitivity decrease of $\sim 10^{-4}$, compared to that for ^{1}H. Thus, longer time requirements become a major factor in FT ^{13}C-nmr experiments. The design of higher field instruments (300-500 MHz) has

helped to reduce these requirements, as have the technological advancements in modern FT nmr spectrometers and probes. However, the fact remains that ^{13}C-nmr experiments require relatively large amounts of machine time.

^{1}H-nmr Spectroscopy

^{1}H-nmr spectroscopy can provide specific information about a number of aspects of polysaccharides. The chemical shift of the glycosidic proton can give some indication of the identities of constitutent monosaccharides. However, due to limited dispersion and broad resonances, absolute assignment on this basis is often difficult. The chemical shifts of anomeric protons does distinguish between the α- and β-anomeric configurations, particularly for gluco and galacto residues with H-1 of β-glycosides resonating at 4.5-5.0 ppm and that of the α-anomer occurring slightly downfield at 5.0-5.5 ppm. This shift difference results from the respective axial and equatorial orientations of the C_1-H_1 bond.[109]

The splitting or coupling constant of the anomeric signal can give a clue as to the relative configuration at C-2, and hence of sugar identity. However, broad resonances often preclude visualization of ^{1}H-^{1}H coupling (Table 1). One of the most important contributions of ^{1}H-nmr is the ease of quantitating the respective anomeric resonances, thereby revealing relative proportions of constituent monosaccharides. It may be possible to determine the positions of

^1H	δ (ppm)	^{13}C	δ (ppm)
CH_3C	~1.5	$\underline{C}H_3C$	~15
CH_3CON	1.8–2.1	$\underline{C}H_3COH$	20–23
CH_3CO_2	2.0–2.2	$\underline{C}H_3CO_2$	
$C\underline{H}(NH)$	3.0–3.2	$\underline{C}H_2C$	38
CH_3O	3.3–3.5	CH_3O	55–61
H-2 to H-6′	3.5–4.5	CH(NH)	58–61
H-5	4.5–4.6	CH_2OH	60–65
H-1 (ax)	4.5–4.8	C-2 to C-5	65–75
$\underline{H}—C(OH)_2$	5.2	C—X[b]	80–87
HO	5.0–5.4	C-1 (ax-O, red)	90–95
H-1 (eq)	5.3–5.8	C-1 (eq-O, red)	95–98
HCO_2	5.9	C-1 (ax-O, glyc)	98–103
		C-1 (eq-O, glyc)	103–106
		C-1 (fur)	106–109
		COOH	174–175
		C=O	175–180

Substituent effects on α-^1H and α-^{13}C (ppm)[c]

	O-Alkyl	*O*-Acyl	*O*-Sulfate	*O*-Phosphate
^1H	−0.2–0.3	+0.3–0.5	+0.3–0.6	+0.3–0.5
^{13}C	+7–10	+<3	+6–10	+2–3

Table 1. Representative chemical shifts for ^1H and ^{13}C nuclei of common functional groups found on polysaccharides.[a]

a. Abbreviations: ax, axial; eq, equatorial; red, reducing; glyc, glycosidic; fur, furanosyl.

b. Non-anomeric ^{13}C involved in glycosidic linkage.

c. Downfield, +; Upfield, −.

glycosidic attachment, at which the protons exhibit resonances shifted downfield by ~0.2 ppm relative to the free hydroxyl equivalent. While it is usually difficult to assign other ring proton signals because of overlap, at high fields isolated peaks can provide some indication of residue composition. Proton nmr spectroscopy is particularly useful for establishing the presence of substitutents which are often hydrolyzed or undetected by other analytical methods. Typical substituents are acetates, methyl esters, methyl ethers and carboxyethylidene (pyruvate acetal) groups. The position of substitution can often be assigned as well, due to downfield shifts in ring proton signals. For example, acetates can

shift the corresponding ring protons into the anomeric chemical shift region.[110]

The relaxation parameters, T_1 and T_2 for the various proton resonances can supply information about the motional dynamics of the polymer.[102,111,112] These parameters are especially useful for distinguishing between freely rotating and rigid groups (e.g. H-6 vs ring protons) and between less motion-restricted monosaccharide residues (e.g. branch residues vs backbone residues). The T_2 effects are apparent in signal linewidth ($\Delta\nu_{1/2} \propto 1/T_2$), and can be quantitated using spin echo pulse sequences.[113] T_1 can be measured in a variety of ways[113,114] including inversion recovery, saturation recovery, progressive saturation as well as various modifications of these pulse sequences. The nuclear Overhauser enhancement (n.O.e)[115] effect can be used to give conformational information which has been particularly useful for determining glycosidic linkage and geometry in oligosaccharides.[115,116]

The spectra of polysaccharides in deuterated DMSO solution contain the hydroxyl proton resonances, the relaxation characteristics of which can provide insight into hydrogen-bonding interactions. Similarly, hydrogen-bonding is often reflected in the chemical shift of the hydroxyl group protons involved, again reflecting molecular conformation.

^{13}C-Nmr Spectroscopy

^{13}C-nmr spectroscopy is undoubtedly a preferred method

for the study of polysaccharides, and indeed polymers in general. The problem of solvent interference is eliminated, concomitant with greater dispersion of resonances over a 200 ppm chemical shift range as opposed to 10 ppm for that of ^{1}H. While ^{1}H-^{13}C coupling constants can be obtained, the fully coupled spectra are often difficult to interpret and require longer acquisition times. Usually it suffices to obtain fully decoupled spectra, which give a single resonance per nonequivalent carbon. This gives increased signal amplitude due to collapse of the multiplets and a proton-carbon n.O.e. effect which provides up to 3 times signal enhancement. Partially decoupled spectra from SSFORD (single frequency off resonance decoupled) experiments[113] may help in determining the multiplicity of the carbon resonances. The multiplets are split by a much reduced amount over fully coupled spectra, which have coupling constants of up to 200 Hz, thus reducing the difficulty in visualizing the multiplet. This does not however, yield the heteronuclear coupling constants. Complementary techniques[118] are used to aid in assignments, as are the commonly used spectral comparisons to previously characterized model compounds such as methyl glycosides and oligosaccharides. The information content of a proton-decoupled ^{13}C-nmr spectrum of a polysaccharide is usually greater than that of a ^{1}H spectrum. The chemical shift of the glycosidic carbon is located downfield from those of the ring carbons,[119-121] and reflects to a greater degree than ^{1}H-nmr, the identity of the respective

sugar. The glycosidic configuration can often be inferred from ^{13}C chemical shift data, with the α anomer typically resonating at 98-103 ppm and the β at 103-106 ppm. The signals of ring carbons of most monosaccharides are resolved in ^{13}C spectra at high field (50-100 MHz for ^{13}C nuclei), although linebroadening can cause overlap particularly in complex multiresidue polysaccharides. However, resonance separation in spectra recorded at elevated temperatures is usually sufficient to make assignment possible. The carbon involved in glycosidic attachment generally resonates 6-9 ppm downfield from the signals of the corresponding hydroxylic carbon,[119-121] and is usually easily discernible. If the linkage is at a primary center, this downfield shift will position the resonance of the methylene carbon (C-6) in the ring carbon region. The absence of a signal at 60-65 ppm is an indication of a (1→6)-linked or 6-O-substituted polysaccharide. Partially decoupled spectra may also aid in this assignment as the multiplicity of the primary carbon (triplet) amongst the ring carbon doublets will be diagnostic. It is possible to distinguish C-6 from other carbons using relaxation measurements, since the former has less restricted motion and will undergo relaxation at a faster rate.[102,111,112] There are methods, also based on relaxation phenomena, to distinguish between primary, secondary, tertiary and quaternary centers.[99,118]

The presence of substitutents is readily established by ^{13}C chemical shifts (see Table 1). To obtain meaningful

quantitative data from ^{13}C-nmr spectra, it is crucial to take care in setting aquisition parameters[113] and in sample preparation.[18] One must forego the nuclear Overhauser enhancement, and allow long relaxation delays (3-5 T_1's), thereby increasing the time requirement for the experiment. This is also true for T_1 and T_2 relaxation time determinations in which various relaxation delays and pulse widths must be determined.[113] However, despite the time requirement, ^{13}C-nmr relaxation measurements may provide useful information about the motional dynamics of the polymer.[27] The ^{13}C-nmr spectral linewidths give a qualitative indication of T_2 relaxation, which in turn reflects the motional correlation times.[102,111]

While most of this discussion has been directed to proton-decoupled spectra, useful information can be obtained from the fully coupled spectrum. In particular, the $^3J_{CH}$ value can provide information about the angles ϕ and ψ (Fig. 1), representing the geometry about the glycosidic bond, as formulated by the Karplus relationship.[104]

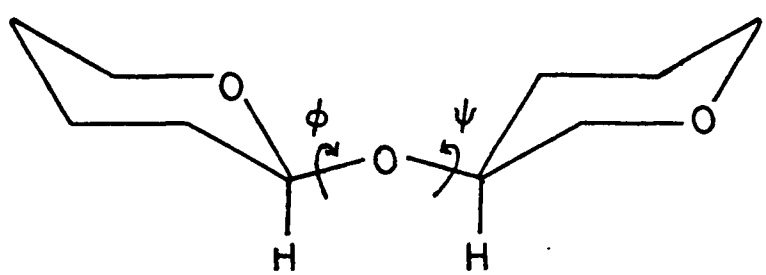


Figure 1. Diagram showing glycosidic conformation, and the angles ϕ, and ψ which define it.

2D-NMR Spectroscopy

To date, two-dimensional nuclear magnetic resonance spectroscopy experiments[122] have found relatively limited application to polysaccharides,[123-125] mainly due to complex signal overlap as well as the added problem of line-broadening. However, it is definite that 2D nmr methods will find increased application in polysaccharide structural studies of both intact species and particularly in conjunction with partially hydrolyzed products, such as bacteriophage-degraded oligosaccharides. The 2D-J experiment has been applied to some polysaccharide systems with success,[125] as a method to overcome the hidden resonance problems. This experiment provides a "homo-nuclear decoupled" (i.e. a single line per resonance) ^{1}H -nmr spectrum in one dimension, as well as coupling information in the other dimension. The COSY and SECSY methods[126,127] have found extensive application to oligosaccharides,[128-130] and are being applied more frequently to polysaccharides having well dispersed ^{1}H-nmr spectra.[123,124] These methods establish connectivity between coupled ^{1}H nuclei. There are ^{13}C 2D-nmr techniques which have been applied to polysaccharides. For example, proton-carbon correlation spectroscopy, which establishes the connectivity between carbon resonances and attached or coupled protons, has proven potential in oligosaccharide studies and limited but expanding utility in polysaccharide structure investigations.[123,124] These can be used to assign anomeric protons, based on the carbon resonance assignments which are often

easier. Another ^{13}C 2D-nmr experiment which has been used in polysaccharide structure determination is 2D ^{13}C-nmr spectroscopy,[131] in which carbon chemical shifts are along one axis and ^{13}C-^{1}H coupling constants are along the other.

At this point, it is appropriate to illustrate with literature examples, the use of nmr spectroscopy in the study of polysaccharides as outlined in the previous section. Quite detailed reviews[18,102-104] on this subject have appeared in the last five years, so an exhaustive survey of this material will not be presented. Instead a few examples illustrating he the important aspects of ^{1}H and ^{13}C-nmr spectroscopy in the study of carbohydrate polymers, will be briefly discussed.

Literature Studies

One family of polysaccharides extensively studied by nmr spectroscopy are the dextrans,[132-143] polymers of (1→6)-α-D-glucopyranose. The ^{1}H-nmr spectrum of dextran[133] (B-742) has resonances at δ 4.9 and 5.2 ppm, due to the H-1 of α-(1→6)-linked glucopyranose backbone and to those bearing a (1→3) or (1→4) branch, respectively. The ratio of these peaks provides a means for determining the degree of branching, which is verified also by chemical methods. Comparison of C-1 intensities in ^{13}C-nmr spectra of dextran B-742 (Fig. 2) affords a reasonable estimate of relative proportions of α-(1→6) (57%), α-(1→4) (9%) and α-(1→3) (34%) linkages.[103,139-143] It was also found that ring resonances in the 70-85 ppm region were diagnostic for α-(1→2), α-(1→3) and

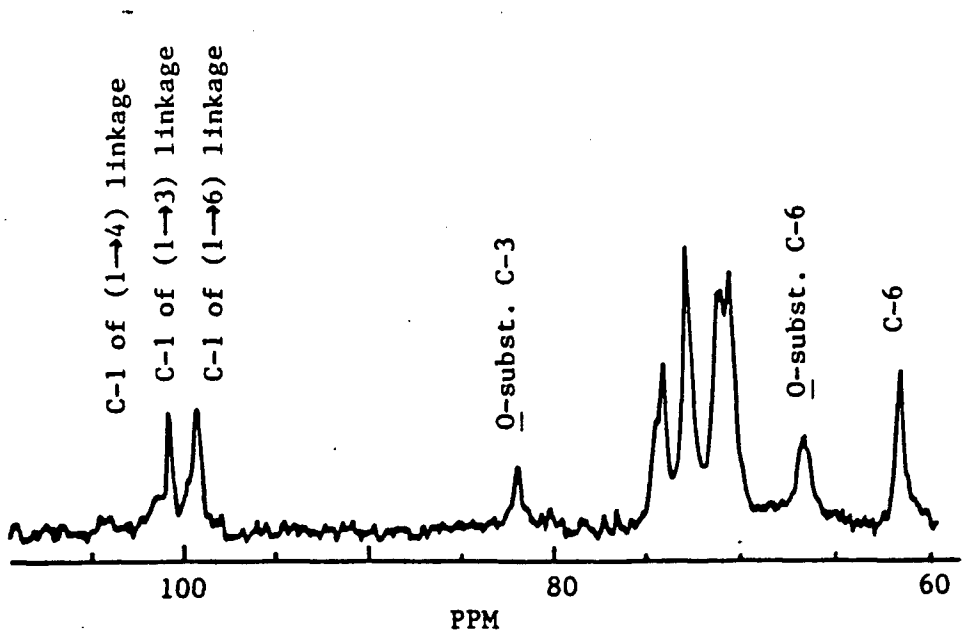


Figure 2. ^{13}C-nmr spectrum of Dextran B-742 in D_2O, at 32°C, showing the signals characteristic of ^{2}branching (ref. external TMS).

α-(1→4) branches. Relative estimates from ^{13}C spectra agree to within 10% with methylation results, and are thus considered useful for analyses of branching in dextrans.

Yeast mannans have undergone similar detailed ^{1}H and ^{13}C-nmr investigations by Gorin and coworkers.[144-151] These polysaccharides possess a (1→6)-α-D-mannopyranose backbone with mainly (1→2)-α- and some (1→3)-α-branches of varying length . ^{1}H-Nmr spectra of these polysaccharides[144-146] have well resolved anomeric regions (Fig. 3a). Chemical shifts of H-1 vary with substitution at O-2, and with location of the monosaccharide in the branch , giving rise to a number of anomeric proton signals. The anomeric

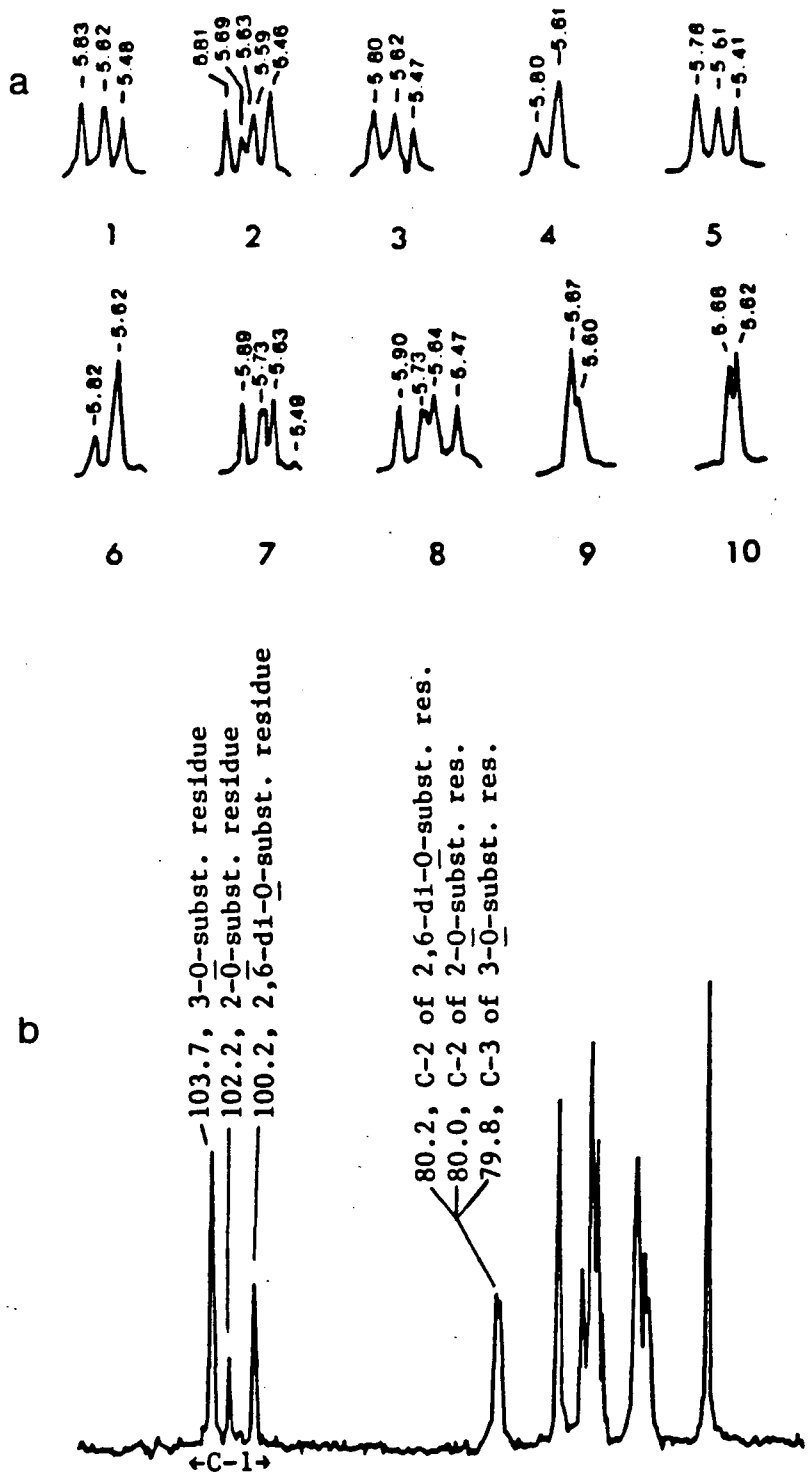


Figure 3. a) The anomeric proton signals from a variety of yeast mannans at 100 MHz in D_2O, showing the different chemical shifts and relative intensities; and b) the ^{13}C-nmr spectrum of branched mannan from bakers yeast in D_2O, at 70°C (ref. external TMS).

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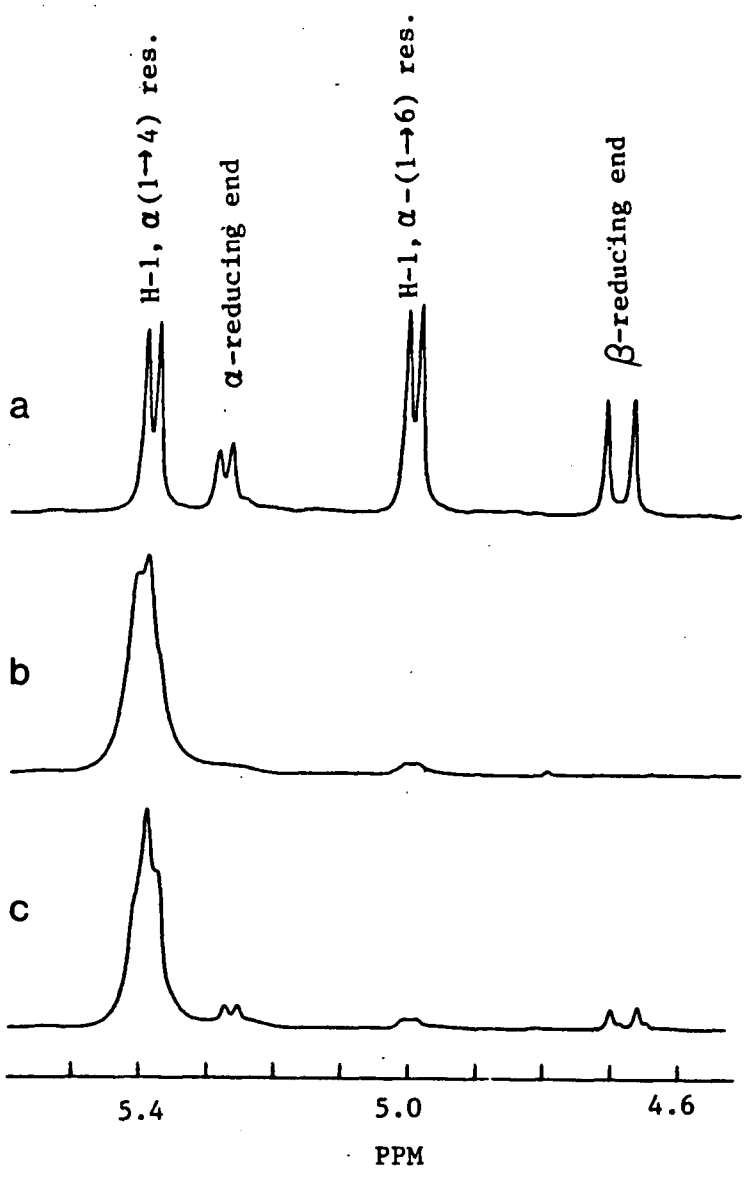
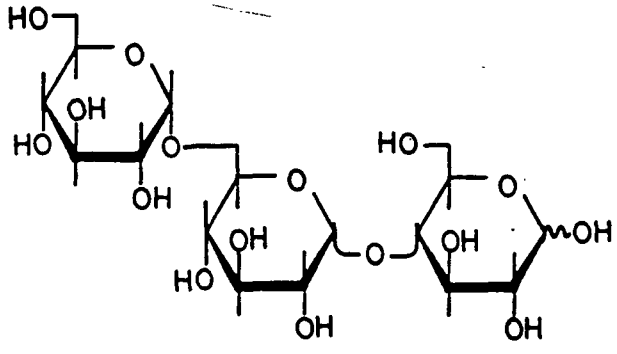


Figure 4. An expanded region of the ^{1}H-nmr spectra of a) panose, b) waxy-maize starch, and c) a degraded starch sample obtained in D_2O solution at 90°C.



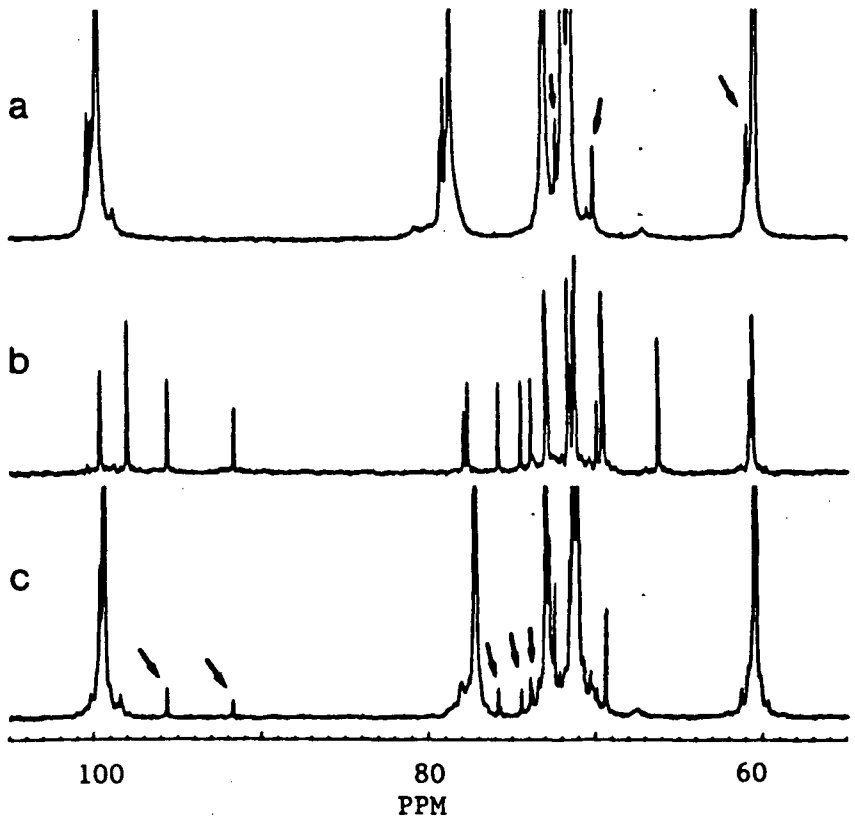
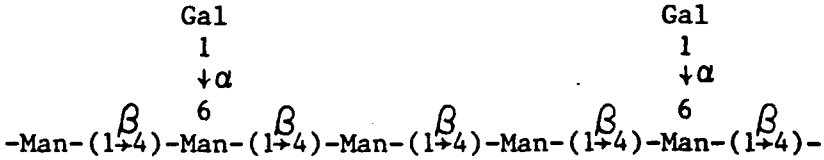


Figure 5. ^{13}C-nmr spectra of a) waxy-maize starch, b) panose, and c) degraded starch in D_2O at 90°C. Arrows in a), and c) arise from non-reducing terminal residues, and reducing terminal residues respectively.

The galactomannans of legume seeds, containing a β-(1→4) mannan backbone with single residue side chains of (1→6)-linked-α-D-galactopyranose (81), differ from previous examples of branched polysaccharides in the monomeric compo-



81

sition of the branch.[155] Both ^{1}H and ^{13}C-nmr spectroscopy gave manno/galacto ratios which agreed with chemical determinations.[156,157] An interesting feature was the splitting of resonances in the anomeric region of the ^{13}C-spectrum (Fig. 6), reflecting the identity of the nearest neighbor. For example, three signals for the C-4 of mannose residues appeared, arising from any two adjacent residues (diads) in which (1) both were substituted, (2) one was substituted and (3) both were unsubstituted. Intensities of these resonances gave relative amounts of the diad sequences.

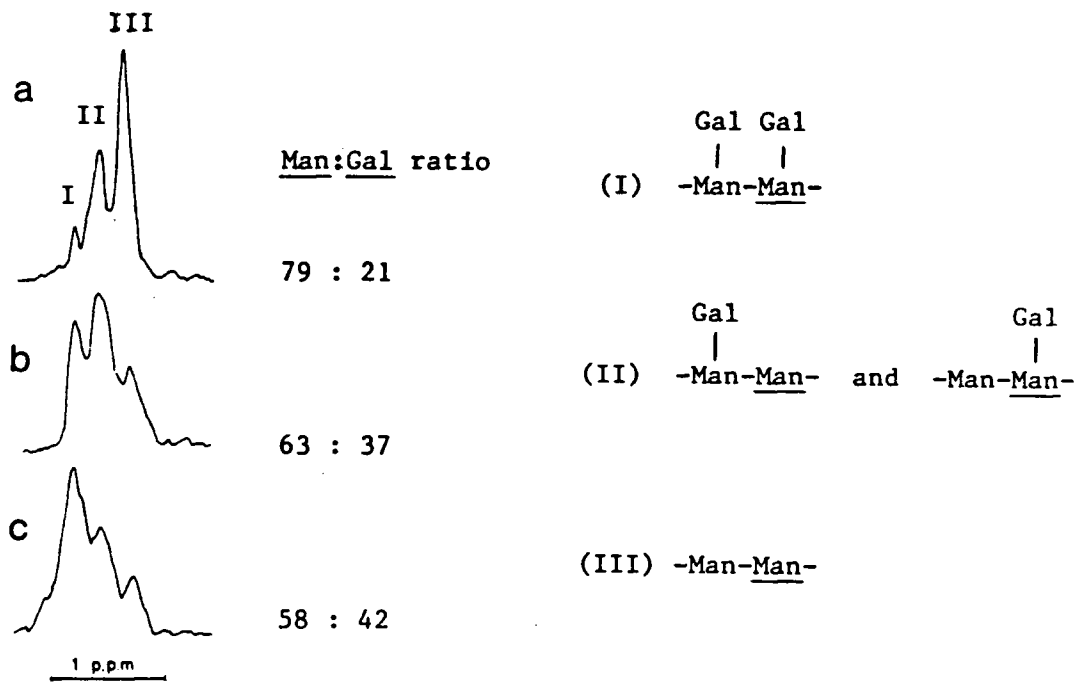


Figure 6. ^{13}C-nmr spectral region, at 25 MHz, showing C-4 of the D-mannose residues in a) locust bean gum; b) guaran; and c) the galactomannan from clover seeds. The diad sequences corresponding to each peak are shown at the right, with the unit involved underlined.

^{13}C-nmr was applied fruitfully to determine the structure of some synthetically branched amylose derivatives.[158] Addition of glucopyranosyl monomers to amylose produced a polysaccharide with unknown branch linkages. While a β linkage was expected based on coupling methods, the ^{13}C spectral data was necessary to confirm this. The ^{13}C spectrum of the synthetic amylose was compared to those of methyl β-D-glucopyranoside and the (1→6)-α branched (1→4)-α-linked polysaccharide glycogen,[103,143] as seen in Fig. 7. The resonance at δ 104.4 ppm confirmed a β-linkage for the branch, and a resonance at δ 80.3 ppm indicated the predominance of branching at the O-6 position of amylose. Otherwise, the spectrum of the synthetic compound looks like a composite of the methyl β-D-glucopyranoside and glycogen spectra. This example demonstrates the importance of nmr spectroscopy in the study of branched polysaccharides, particularly in conjunction with polysaccharide modifications.

The utility of ^{1}H and ^{13}C-nmr spectroscopy is borne out by its increasing application to studies on complex and regular heteropolysaccharides. An excellent example is provided by the work of Dutton and coworkers in which nmr spectroscopy was employed in conjunction with chemical methods in structural investigations of the capsular polysaccharides of *Klebsiella*.[159-167] Nmr spectral analyses are especially applicable to oligosaccharides isolated from the chemical degradation of these complex polysaccharides, as

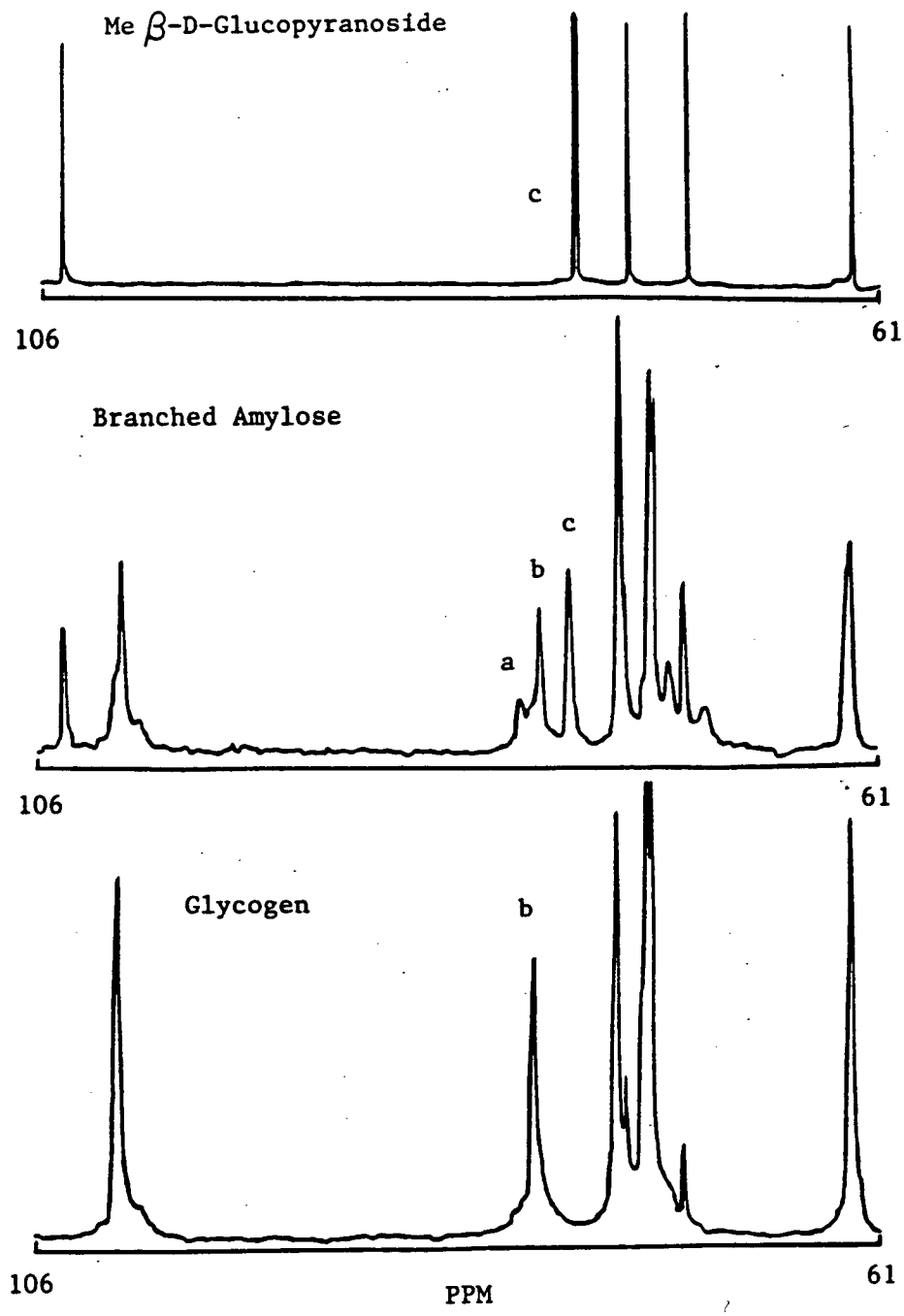


Figure 7. ^{13}C-nmr spectra of methyl β-D-glucopyranoside, a branched amylose derivative, and glycogen at 90°C.

well as to repeating unit oligosaccharides obtained from bacteriophage degradations.[166-169]

Both ^{1}H and ^{13}C-nmr spectroscopy have found application in the analysis of industrially utilized cellulose derivatives. Comprehensive articles on 2-O-hydroxypropyl,[170-172] 2-O-hydroxyethyl,[173,174] methyl[173] and carboxymethyl[173,175] celluloses have appeared. These studies demonstrate the power of nmr spectroscopy as used for the detection of substituents, since the derivatives of interest are complex and highly substituted. Nmr methods enable one to distinguish the relative degree of substitution at each position due to the downfield chemical shifts of substituted sites.

Besides its demonstrated utility in probing structural aspects of polysaccharides, nmr spectroscopy has also been used for conformational studies. The ^{1}H-nmr spectrum of amylose in DMSO solution exhibited signals for OH-2 and OH-3 at lower field ($\delta>5$) than anticipated, a phenomenon attributed to intramolecular hydrogen bonding association,[176,177] as illustrated in Fig. 8. More recently ^{13}C-nmr spectroscopy

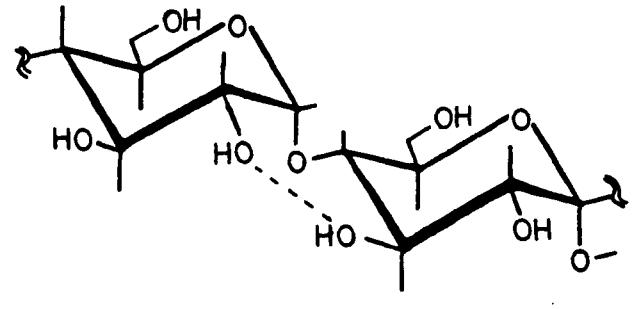


Figure 8. A disaccharide unit of amylose showing the inter-residue interaction between the 2-OH and 3-OH groups.

has been used to investigate the conformation of helical complexes of amylose and amylopectin in solution.[154] In this study, the random coil-to-helix transition was studied by adding DMSO, triiodide or alcohols, to induce helix formation. In general, C-1 and C-4 showed marked downfield shifts, attributed to rotation of the C-O bonds of the glycosidic linkage upon helix formation.[154]

Molecular mobility of polysaccharides (as indicated by the correlation time constant, τ_c) is easily probed by nmr spectroscopy. Line broadening of ^{13}C resonances in (1→3)-β-D-glucans (curdlan) was observed upon gelation.[178-181] Inferences regarding conformation can be made, depending on linewidths of both solutions and gels, as some helical associations allow a greater degree of mobility. Downfield chemical shifts of C-1 and C-3 of curdlan resonances, relative to those of degraded fractions, were explained as an effect of restricted rotation about the glycosidic bond in the helix conformation.[132,179]

Nmr spectroscopic studies on (1→3)-β-D-glucans having (1→6)-β-linked sidechains (lentinan) illustrate the mobility differences which can exist between the main chain and branch residues. In the gel state, the (1→3)-β-linked main chain exhibits no resonances due to an ordered structure and concomitant low mobility, while resonances for branch residues are observed (Fig. 9).[179-181] The studies on this family of gel-forming fungal glucans showed that, at 25.2 MHz, linewidths for main chain residue carbons were as large as

1000 Hz, corresponding to a motional correlation time of $>10^{-6}$ s^{-1}, while side chain ^{13}C resonances had correlation times of 10^{-8}-10^{-9} s, as determined from linewidths, T_1 values and nuclear Overhauser enhancements.[182] Similar

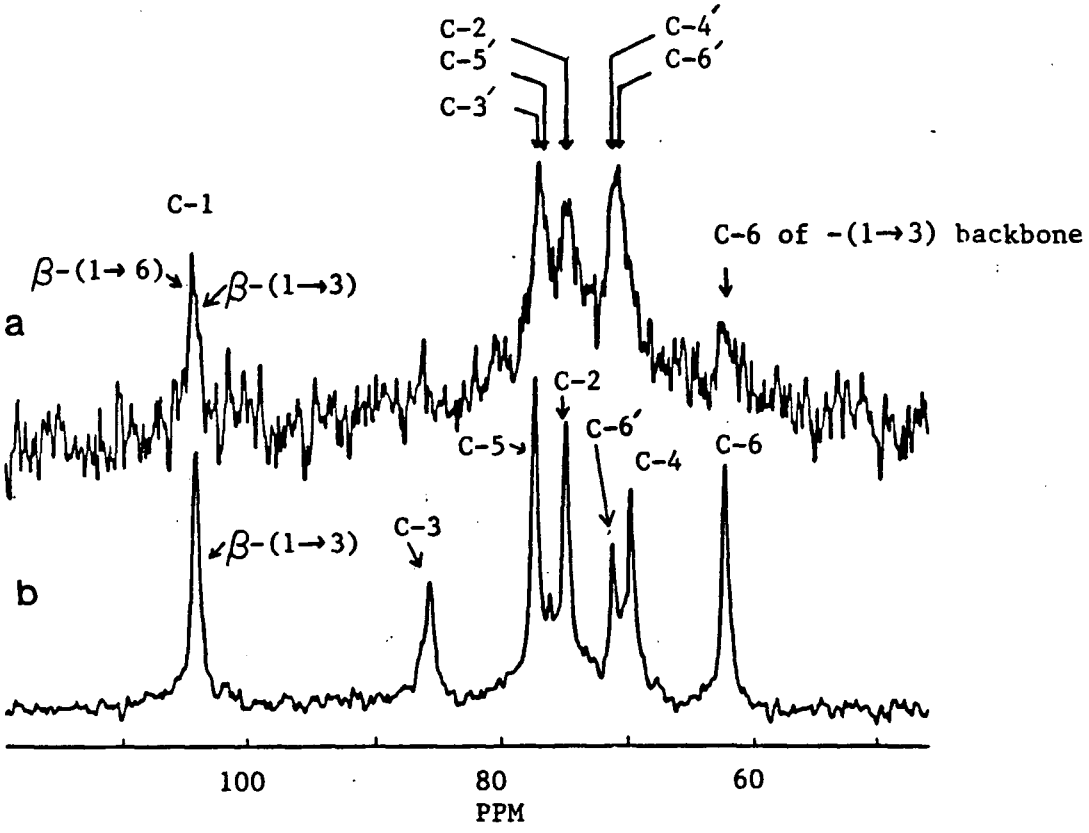


Figure 9. ^{13}C-nmr spectra of a) lentinan gel, and b) a lower molecular weight fraction, in D_2O. The disappearance of signals from the β-(1→3) linked main chain in the gel state spectrum can be seen.

although smaller differences in mobility of backbone and side chain residues were observed in some mannans, as indicated by ^{13}C-T_1 measurements.[183] For example, T_1 values for C-1 of the terminal side chain residue, an adjacent sidechain residue, and a main chain residue in mannans were 0.20, 0.13 and 0.09 s, respectively. Dextran studies gave analogous results,

showing that mobility differences in branched polysaccharides appear to be a general trend[143] and that the relationship between mobility, T_1 values and linewidths can be used as a tool to distinguish branch residues from those of the main chain.

1.2.4 Polysaccharides in Solution

Much of the industrial interest in polysaccharides and their derivatives arises from the properties exhibited by aqueous solutions or dispersions of these materials.[6] While some of the properties follow understandable and predictable trends, such as limited solubility, thickening or gelation and hydrophilicity, the specific interactions at the molecular level are more difficult to comprehend. Although it is known that these molecules are both polymeric and hydrophilic in nature, a complete understanding of the relationship between primary polysaccharide structure and the physicochemical properties of the aqueous solutions has yet to be established. The molecular interactions of the polymer, including both inter- and intra-molecular associations as well as interactions with other solutes and solvent molecules, are wide ranging and complex. Some systems offer examples of specific interactions which can be correlated with the respective resultant properties, allowing some insight to be gained. Unfortunately, the diversity in polysaccharide primary structure and accompanying interactions[17,183] serves to isolate these examples without

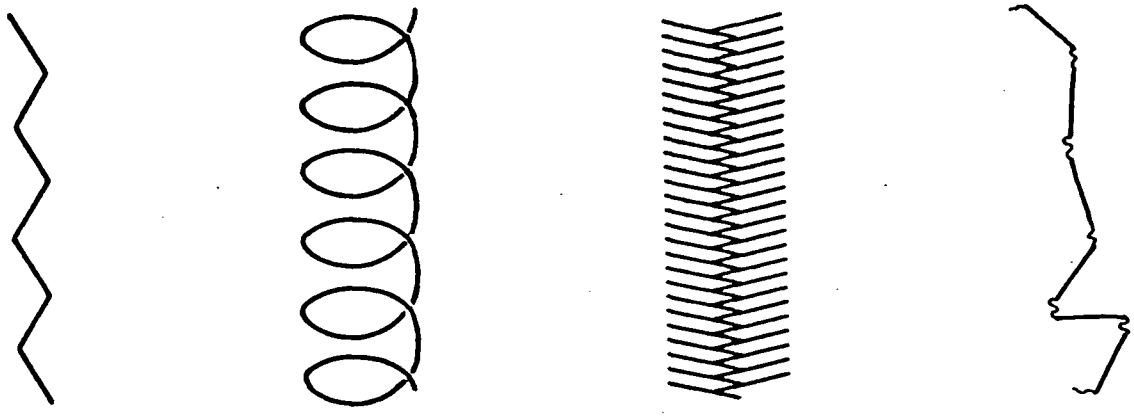
permitting predictable trends to be established. And yet, it is this general understanding which would be invaluable for the "tailored" preparation of polysaccharide derivatives. The following discussion will provide an overview of polysaccharides in solution, focussing on both the causes and evaluation of physicochemical properties.

Solubilization or hydration of polysaccharides is thought to proceed initially at amorphous regions where intermolecular interactions are limited by the disorganized spatial arrangement of the chain residues.[3,184] Further hydration subsequently replaces the intermolecular hydrogen-bonding to an extent which determines solubility. Little or no hydration leaves the polymer undissolved, while limited hydration results in a gel-state solution and extensive hydration yields apparent total dissolution.

The aqueous solubility of natural polysaccharides is dependent on structural and conformational features. For example, linear polysaccharides such as cellulose and chitin are known to adopt highly ordered ribbon-like structures, with substantial crystalline character, resulting in difficult solubilization.[3,17,184] Branched polysaccharides on the other hand, have a less ordered structure and typically solubilize readily. Some of the other factors which often enhance solubility are glycosidic arrangement, charged functionalities, and structural irregularities.

The glycosidic linkages in polysaccharides influence solubility and molecular associations in solution.[17,183] As

mentioned, linear homopolysaccharides such as cellulose, chitin and some mannans, having (1→4)-β-linkages, form flat ribbon sequences of semi-crystalline nature. Similar polysaccharides having the less linear (1→4)-α-linkage (e.g. amylose) form less rigid coiled springs or helices, and are generally more water-soluble. In fact, an examination of the geometry of the various linkages, i.e. (1→6), (1→4), (1→3) and (1→2) in both α and β forms, shows that steric effects will dictate the order and helical parameters (pitch) of the polymer.[1,17,183] Fig. 10 depicts in stick diagram form the effect of linkage position and configuration on conformation.



Type A

e.g. (1→3)-α-D-galactan
(1→4)-α-D-galactan
(1→3)-β-D-glucan
(1→4)-β-D-glucan
(1→3)-α-D-mannan
(1→4)-β-D-mannan
(1→3)-α-D-xylan
(1→3)-β-D-xylan

Type B

e.g. (1→4)-β-D-galactan
(1→3)-β-D-galactan
(1→4)-α-D-glucan
(1→3)-β-D-glucan
(1→2)-α-D-mannan
(1→4)-α-D-mannan
(1→3)-β-D-mannan
(1→4)-α-D-xylan
(1→3)-β-D-xylan

Type C

e.g. (1→2)-α-D-galactan
(1→2)-β-D-galactan
(1→2)-α-D-glucan
(1→2)-β-D-glucan
(1→2)-β-D-mannan
(1→2)-α-D-xylan
(1→2)-β-D-xylan

Type D

e.g. all 1,6-disubstituted homoglycans

Figure 10. Depictions of the relationship between tertiary structure and linkage. Type A, extended ribbon; Type B, flexible helix; Type C, crumpled ribbon; and Type D, flexible coil.

A (1→6)-linkage for example, having a greater degree of freedom, does not exist in a single preferred conformation and is thus usually disordered in solution. This situation is exemplified by (1→6)-α-linked D-glucopyranose polysaccharides of the dextran family,[17] and evidenced by the relatively low viscosity displayed by these polymers.

Charged or ionizable functionalities on a polysaccharide can influence solubility and solution properties by providing loci for hydration, perhaps upon a pH change, or by providing sites for ionic interaction with other charged solutes. An example of the former would be the use of aqueous organic acid solutions to dissolve water insoluble chitosan, likely by disruption of intermolecular hydrogen bonding involving the amine upon formation of the ammonium ion. Interaction of an ionizable polysaccharide with charged solutes is illustrated by the action of Ca^{+2} and other divalent metal ions on sodium alginate solutions.[185-189] According to the "egg-box" model (Fig. 11), the calcium is sequestered between chain segments of L-guluronate residues by means of ionic crosslinking interactions.[190-195] Noncrosslinked D-mannuronate and mixed L-guluronate/D-mannuronate chain segments provide sufficient areas of hydration such that the calcium chelate does not precipitate, but forms a stable gel.[193]

Other polysaccharides with charged functionalities include glycosaminoglycans such as hyaluronate and chondroitin, which have uronic acid and acetamido groups; derma-

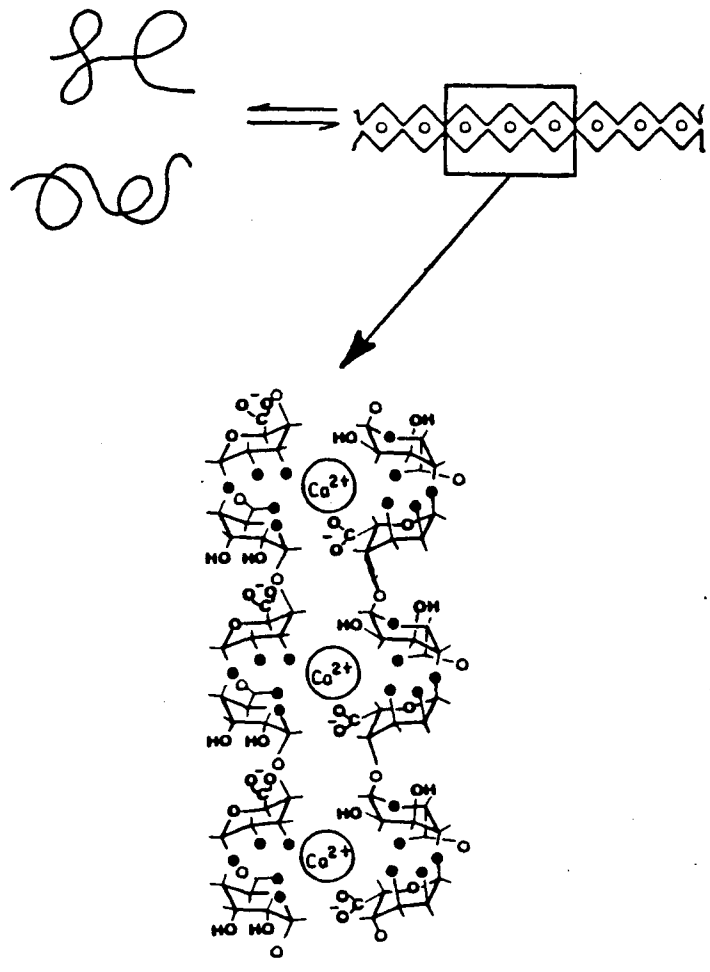


Figure 11. The "Egg box" model for calcium ion induced interaction of poly(L-guluronate) chains.

tan sulfate with sulfate, uronate and acetamido containing residues; and heparins having sulfate, uronate, N-sulfate and acetamido moieties. These functionalities give rise to ionic interactions which determine the solution properties of these polysaccharides, and numerous solid state and solution studies have been done to delineate important structural interactions and the solution conformation of these com-

pounds.[196]

As mentioned earlier, the branches on a linear polysaccharide can have a marked effect on the solution properties. There are relatively few families of polysaccharides which are based on a linear backbone with varying degree of branching, but one such series are the seed galactomannans.[155] The parent (1→4)-β-linked linear mannan (ivory nut mannan), having a ribbon-like structure similar to cellulose, is insoluble in water, and is a very resilient solid material. However, when ~20% of the residues are substituted with (1→6)-α-D-galactopyranose (locust bean gum), the material is soluble in hot water and gels upon cooling. Guar gum, having @ 55% substitution, is largely soluble in water, giving viscous solutions. It is clear that distribution of substituents along the backbone is an important factor in the interactions of galactomannan polysaccharides. The self-association of galactomannans, which were reported to contain substituted and unsubstituted blocks,[197] has been attributed to intermolecular interactions between unsubstituted "smooth" regions, while branched "hairy" regions remain hydrated (Fig. 12a). Proponents of a more random alternating substitution pattern, as supported by recent studies on some galactomannans,[198-201] postulate a two-fold conformation of the mannan backbone, [202,203] resulting in "smooth" and "hairy" faces which could interact[201,204] as shown in Fig. 12b.

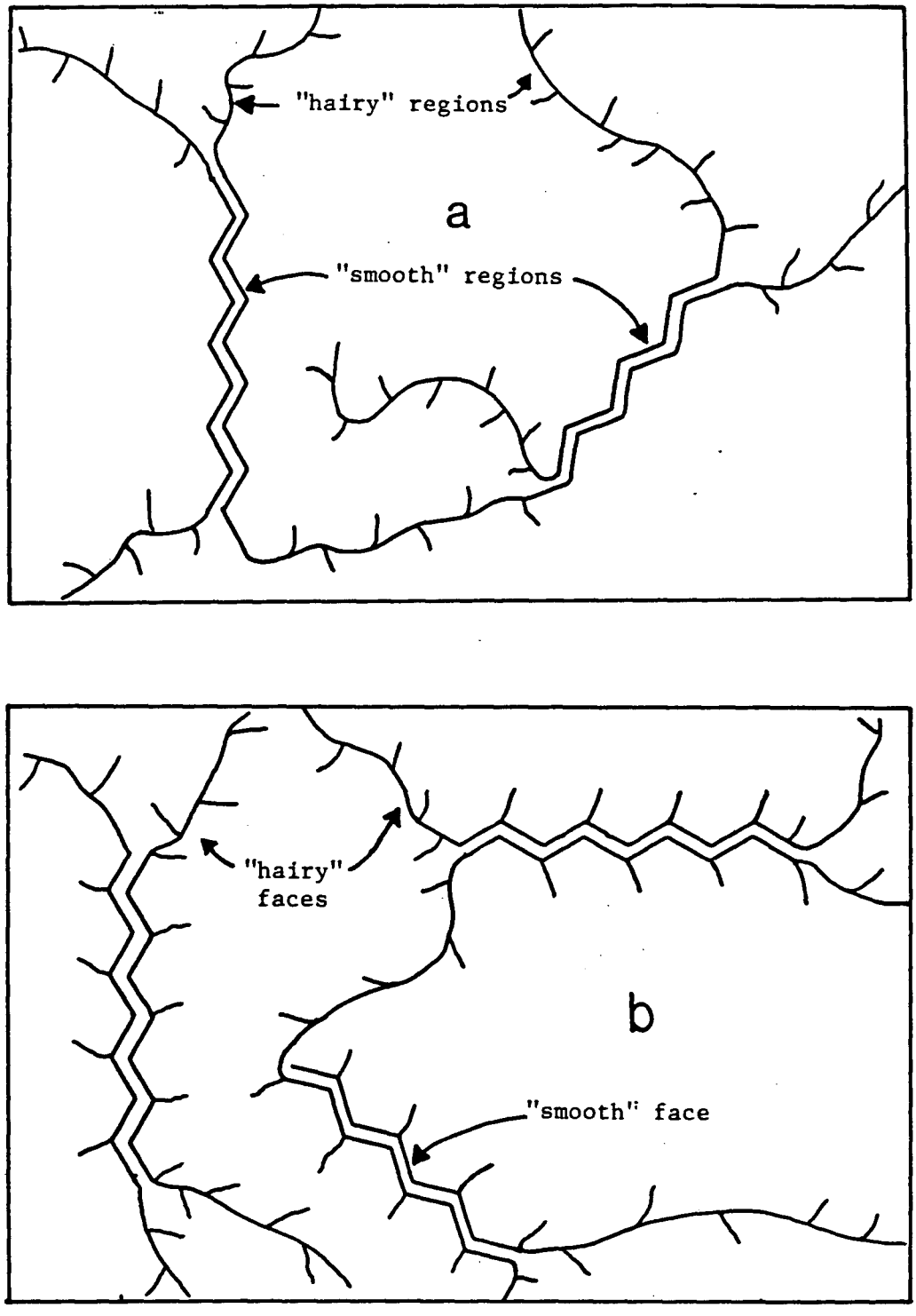


Figure 12. Illustration of galactomannan interactions in which a) block substitution forms "hairy" solvated regions and "smooth" regions which interact; or b) randomly substituted chains have conformationally induced "hairy" faces and self associating "smooth" faces.

Structural irregularity is another feature known to affect hydration and solution properties of polysaccharides. The irregularity can involve a substituent positioned on selected residues of the polysaccharide, or conformational aberrations caused by the primary structure of the polymer. An excellent example of these effects is found in pectins, a poly-(1→4)-α-D-galacturonate polymer having intermittent (1→2)-linked L-rhamnose units. This polymer is found in nature in partially esterified forms,[3] and is known to bind calcium and form gels in a manner analogous to alginates.[19,194,205,206] It has been established that sequences of ~15 residues of unesterified galacturonate are required for calcium-induced chain association,[207,208] and that the L-rhamnose units occur at uniform intervals of ~ 25 galacturonate residues.[194,208] Fully esterified chains found in nature have been subjected to blockwise enzymatic and random chemical de-esterification to give a range of samples useful for probing the effect of esterification pattern on calcium-induced gelation. The calcium-binding capacities of block and randomly esterified chains, as monitored by circular dichroism are compared in Fig. 13.[191,208] The randomly de-esterified samples show little binding capacity until ~50% of the residues are liberated. Upon further de-esterification a sharp increase in binding is observed, while chelation ability increases almost linearly upon blockwise removal of ester functions. These observations are rationalized by the requirement of long chain segments for cooperative calcium-

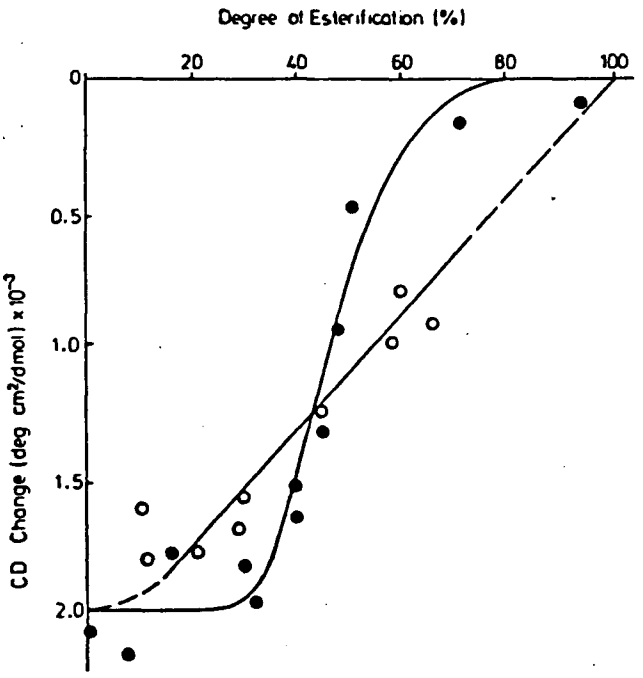


Figure 13. Calcium binding capacity, as monitored by CD, for partially esterified pectin samples having random (●) and block (○) substitution patterns.

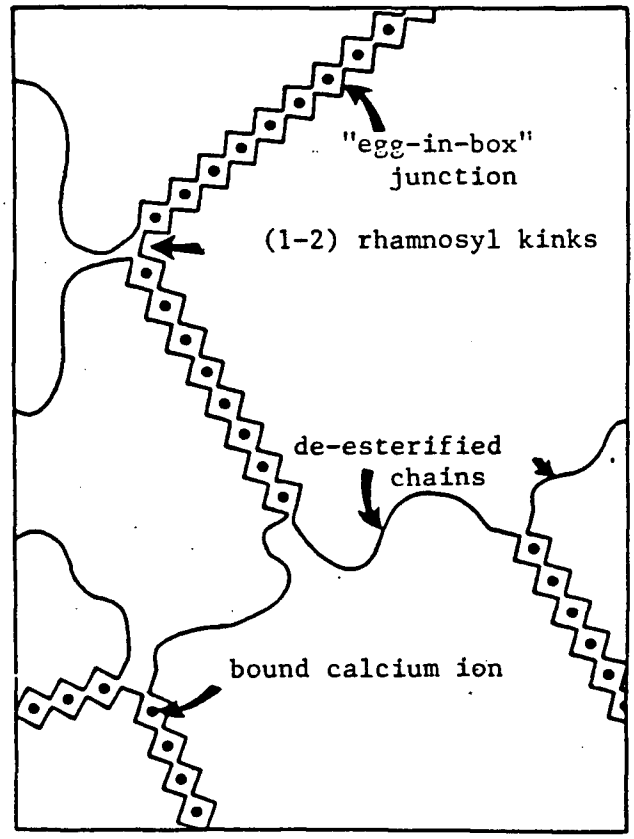


Figure 14. The interactions present in calcium pectate gels.

binding. The (1→2)-L-rhamnose units serve to disrupt the highly ordered chelate by causing kinks, thereby providing sufficient regions of hydration to maintain a stable gel rather than a precipitate (Fig. 14). This study clearly illustrates the importance of a systematic strategy of preparing modified polysaccharides, over and above those naturally available, in order to probe structure/function interactions and ultimately, to allow one to tailor polysaccharide solution properties.

Synergistic interactions, another interesting phenomena exhibited by polysaccharides in solutions, have received considerable attention in the recent literature. These involve quaternary association of unlike polysaccharides, with concomitant enhancement or alteration of solution properties. The interaction of seed galactomannans with xanthan gum typifies the synergistic effect,[155,204,209-214] where viscous solutions or gels can be obtained at concentrations considerably lower than those required for either single component. The galactomannan polysaccharides also participate in mixed associations with carrageenan and agar.[155,215-217] Generally, less substituted galactomannans (e.g. locust bean gum, d.s.~0.2) exhibit more extensive interaction than those with a higher degree of branching[209,218] (e.g. guar gum, d.s.~0.5). Also, galactomannans having a regular alternating branch pattern show substantially stronger interaction with xanthan than do other galactomannans with equivalent overall galactose con-

tent.[201,209] These interactions are of substantial industrial interest for producing viscous solutions and stable gels at low material cost.

Introduction to Rheology

Numerous experimental methods exist for probing the solution interactions of polysaccharides, including chiroptical techniques (ORD/CD), disorder-order transition kinetics, light-scattering measurements, differential scanning calorimetry, nuclear magnetic resonance spectroscopy and viscometry.[17] The last of these offers the additional feature of characterizing the flow behaviour or rheological properties of the system, as well as providing information about molecular interactions. Since the potential applicability is often based on the rheological properties of the material, rheometry is an important method for characterizing polysaccharides in solution. In fact, commercial polysaccharides are often identified by the solution viscosity at a defined concentration. Certainly, viscometry is indispensable for purposes of correlating the interactions and properties of polysaccharides. However, some caution is required in designing and interpreting rheometric measurements because various experimental factors will influence the molecular interactions that are probed. It is intended that the following discussion will provide the reader with some background on the types of viscometry and rheometry commonly employed, and the information that can be extracted from these measure-

ments. Generally, the information procured from studies on dilute and concentrated solutions is different, and thus these concentration regimes are treated separately.

Dilute Solutions

The ability of many polysaccharides to form viscous solutions at relatively low concentrations is well-known and has importance in many industrial and biological applications. This behaviour arises largely from the coil dimensions of the hydrated polymer; however, depending on solvent conditions, contributions from interchain interactions can be appreciable. An index which reflects polymer coil dimensions in solution is intrinsic viscosity,[219] ($[\eta]$), the fractional increase in viscosity per unit concentration (c) for isolated chains (i.e. $c \xrightarrow{\lim} 0$). Intrinsic viscosity increases with coil dimensions according to the Flory-Fox relationship (Eq. 5):

$$[\eta] = \frac{\Phi L^3}{M_r} \qquad [5]$$

where Φ is a constant, L is the average end-to-end chain length and M_r is polymer molecular weight. The molecular weight dependence of intrinsic viscosity is given by the Mark-Houwink equation (Eq. 6):

$$[\eta] = KM_r^{\alpha} \qquad [6]$$

where K is a constant and α is a parameter relating to coil dimensions.

Experimentally, intrinsic viscosities are obtained using

the Kraemer relationship:

$$\frac{Ln(\eta_{rel})}{c} = [\eta] + k'[\eta]^2 c \qquad [7]$$

or the Huggins equation:

$$\frac{\eta_{sp}}{c} = [\eta] + k''[\eta]^2 c \qquad [8]$$

where k' and k'' are constants and relative viscosity (η_{rel}) is the ratio of solution viscosity (η) to solvent viscosity (η_s), as given in Eq. 9:

$$\eta_{rel} = \frac{\eta}{\eta_s} \qquad [9]$$

and specific viscosity (n_{sp})) is obtained from relative viscosity according to Eq. 10:

$$\eta_{sp} = \eta_{rel} - 1 \qquad [10]$$

Plots of η_{sp}/c against c, or of ln η_{rel}/c against c, for a series of dilute solutions, extrapolated to zero concentration, give intrinsic viscosities.[219] By determining intrinsic viscosities for a series of related samples varying in molecular weight it is possible to determine the constant K, and α, of the Mark-Houwink relationship (Eq. 6). These parameters in turn relate to the shape and conformation of the polymer. It is important to point out that because intrinsic viscosity represents the effect of the material on the solution behavior at infinite dilution, they are virtually independent of contribution from inter-chain interactions. Thus intermolecular interactions are not probed by

intrinsic viscosity.

Concentrated Solutions

As one considers more concentrated polymer solutions, a critical concentration, c*, is reached, at which the volume occupied by the polymer equals the solution volume, and the presence of more polymer can be accommodated only by entanglement or interaction of chains. Above this concentration polysaccharide solutions typically have non-Newtonian flow behaviour.[220] That is, the apparent viscosity (η) is dependent on the shear rate ($\dot{\gamma}$). Newtonian fluids on the other hand, have the same viscosity at all shear rates. For polysaccharides, shear thinning or pseudoplastic behaviour is most common, with regions of Newtonian flow behaviour at low shear rates (represented by zero shear viscosity, η_0) and at high shear rates (called infinite shear viscosity, η_∞) (Fig. 15). A measure of shear thinning of a polysaccharide solution is obtained by expressing viscosity as a fraction of zero shear viscosity. Another parameter is $\dot{\gamma}_{0.1}$, the shear rate at which the apparent viscosity is one tenth the magnitude of the infinite shear rate viscosity[220] ($\eta=\eta_0/10$). Empirical models have been developed to represent flow behaviour curves from steady shear viscometric determinations over relatively large shear rate ranges.[221] For example, the power-law model:

$$\eta = m\dot{\gamma}^{1-n} \qquad \text{or} \qquad [11]$$

$$\sigma = m\dot{\gamma}^{n} \qquad [12]$$

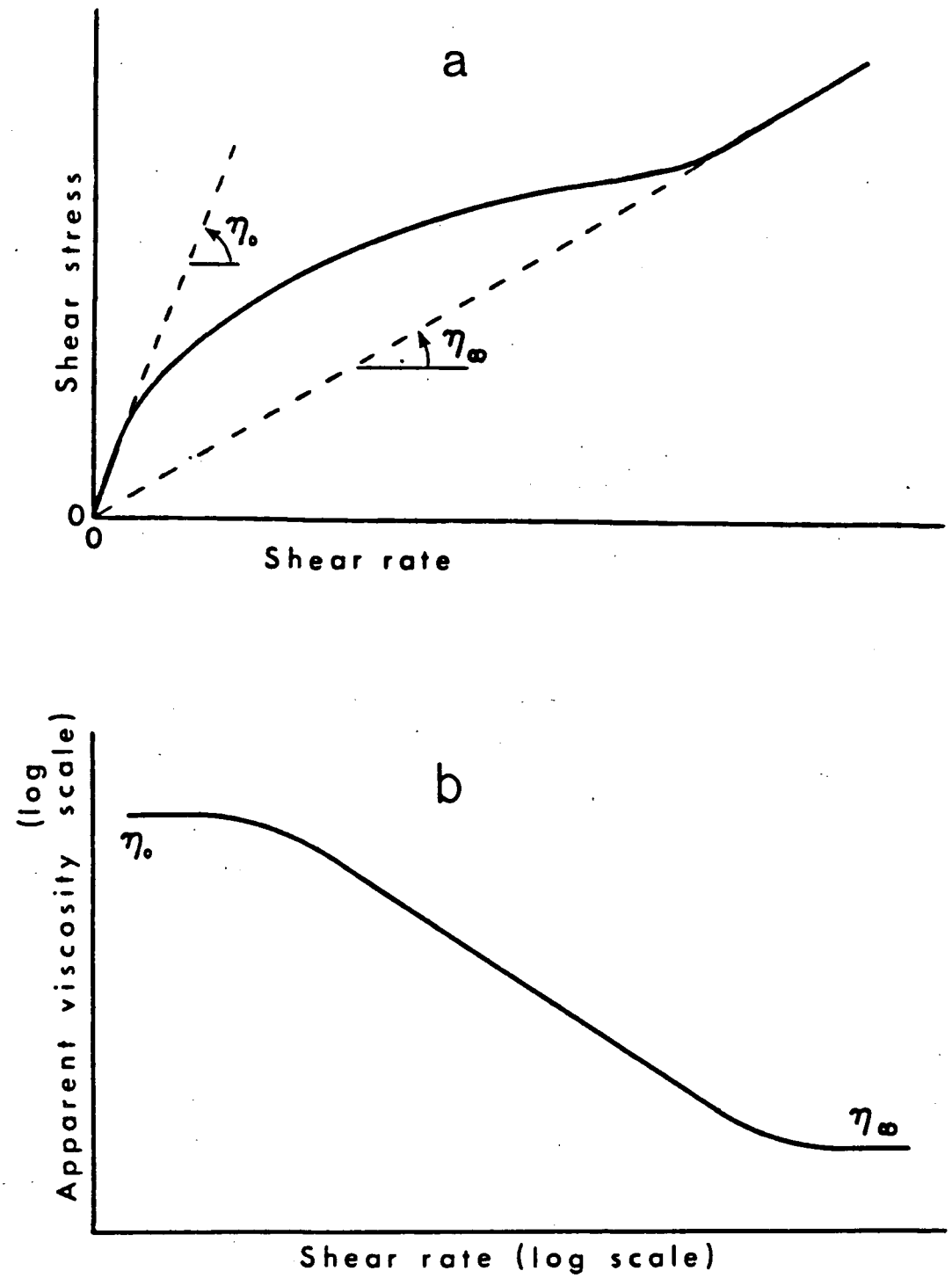


Figure 15. Idealized rheograms of pseudoplastic flow plotted as a) shear stress vs shear rate on arithmetic coordinates; and b) apparent viscosity vs shear rate on logarithmic coordinates.

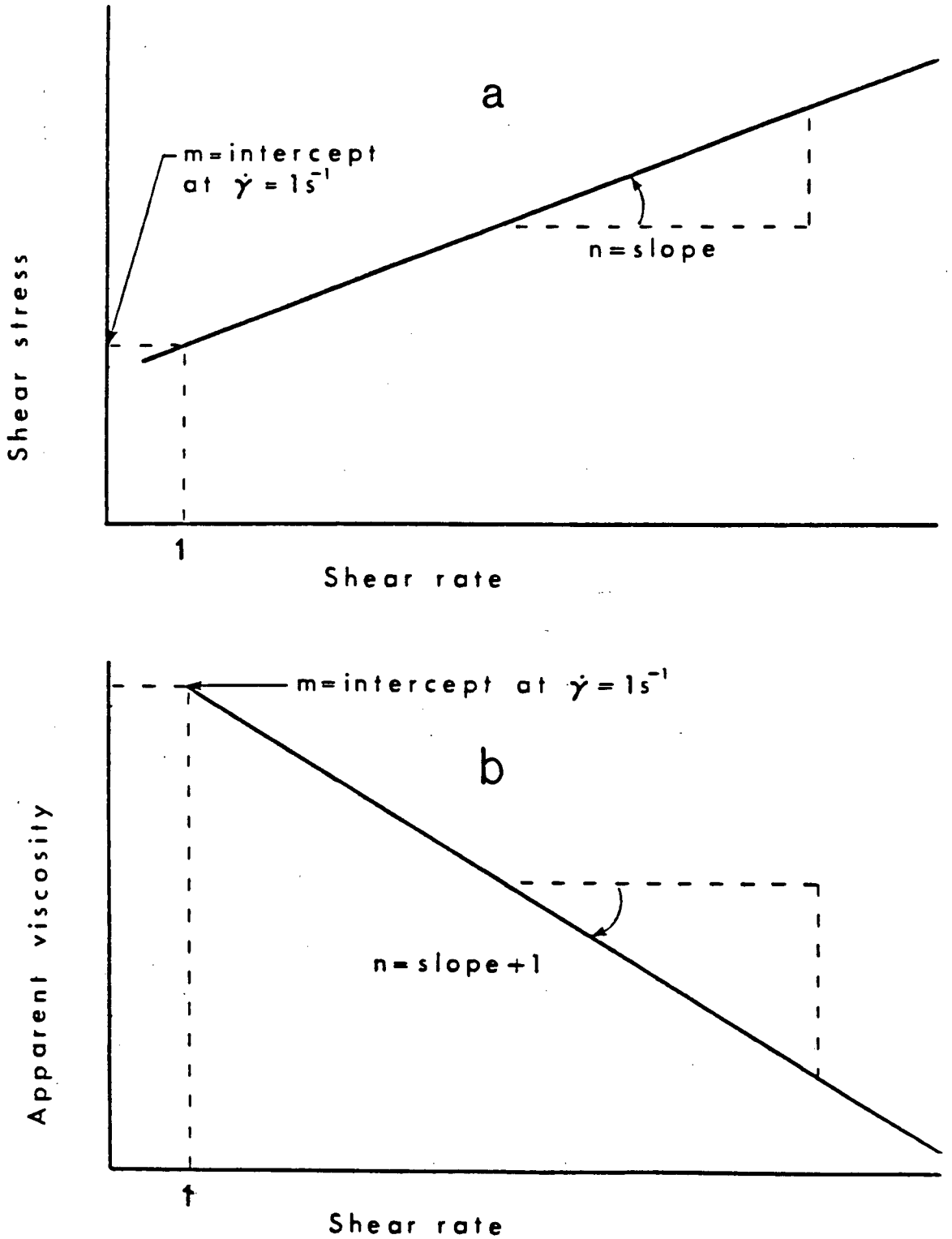


Figure 16. Idealized power-law model rheograms on logarithmic coordinates, plotted as a) shear stress vs shear rate; and b) apparent viscosity vs shear rate.

where m is the consistency coefficient and n is the flow behaviour index, has found extensive use in characterizing non-Newtonian flow behaviour over intermediate shear rate ranges (Fig. 16).[222-224] A variety of constitutive equations exist which can be used to model pseudoplastic flow over shear rate ranges including zero shear or zero and infinite shear behaviour. Parameters obtained from empirical modelling are particularly useful for comparison of flow properties of different polysaccharide solutions. Steady shear viscometric measurements are useful for examining the effects of parameters such as concentration, temperature, pH, and ionic strength on the flow properties of polysaccharide solutions. The cause of shear thinning in polysaccharide solutions is usually specific to the system; however, in general it occurs as the rate of externally imposed movement exceeds the rate of re-entanglement of the polymer chains. Thus, the "crosslink" network is reduced relative to the entanglement-disentanglement equilibrium existing under static or low shear rate conditions.

Some highly ordered polymer solutions and gels have a component of solid-like or plastic behaviour when undisturbed. These materials are often characterized by a yield stress value, or a minimum shear stress value, above which flow will occur. The power-law plastic model [Eq. 13] incorporates the yield stress parameter into the familiar power-law model:

$$\sigma = \sigma_y + m\dot{\gamma}^n \qquad [13]$$

where σ_y is the yield stress.

In considering the rheology of polysaccharide solutions, we have not yet attempted to distinguish between time independent (which has been assumed to this point) and time dependent flow behaviour. While this feature will not be addressed in any depth here, discussions on this aspect of rheology are available in monographs on the subject.[219,221] Time dependent flow describes fluids in which decreasing or increasing effects on apparent viscosity (or shear stress) are evident, at a constant shear rate. Usually this effect is reversible. Samples exhibiting decreasing apparent viscosity are termed thixotropic, while those with increasing viscosity are rheopectic. If an irreversible loss in apparent viscosity occurs, the fluid is said to be rheodestructive. Fig. 17 illustrates some of these features.

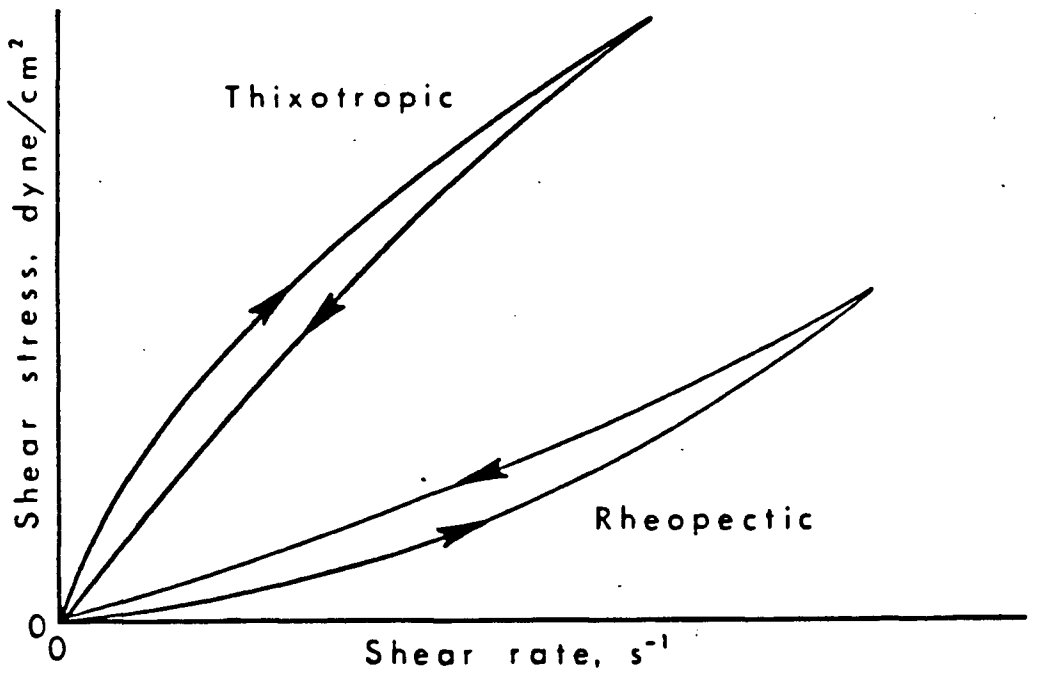


Figure 17. Idealized rheograms of time dependent flow in continuous upcurve and downcurve experiments.

The organization and intermolecular networks of hydrogels can be probed by oscillatory shear or dynamic viscometry.[225] This involves the application of a small oscillatory, sinusoidal, deformation (Eq. 14), and measurement of the sample's

$$\gamma = \gamma_0 \sin(\omega t) \qquad [14]$$

resistance to deformation. For true solids the greatest deformation (strain, γ) occurs when the applied stress (σ) is at a maximum (Eq. 15). That is to say, stress and strain

$$\sigma = k\gamma_0 \sin(\omega t) \qquad [15]$$

are in phase. Liquids, on the other hand, show greatest resistance to flow (stress) when the rate of deformation (Eq. 16) is greatest, and is thus 90° out of phase with strain (Eq. 17) with the applied strain wave. Fig. 18 shows the

$$\frac{d\gamma}{dt} = \dot{\gamma} = \omega\gamma_0 \cos(\omega t) \qquad [16]$$

$$\sigma = \eta\omega\gamma_0 \cos(\omega t) \qquad [17]$$

respective stress and strain waves for a solid, a viscous fluid and a viscoelastic material. Viscoelastic materials will have a phase shift of between 0 and 90° depending on the relative contributions of viscous and elastic behaviour. The stress function for a viscoelastic material is given in Eq. 18;

$$\sigma = \gamma[G'\sin(\omega t) + G''\cos(\omega t)] \qquad [18]$$

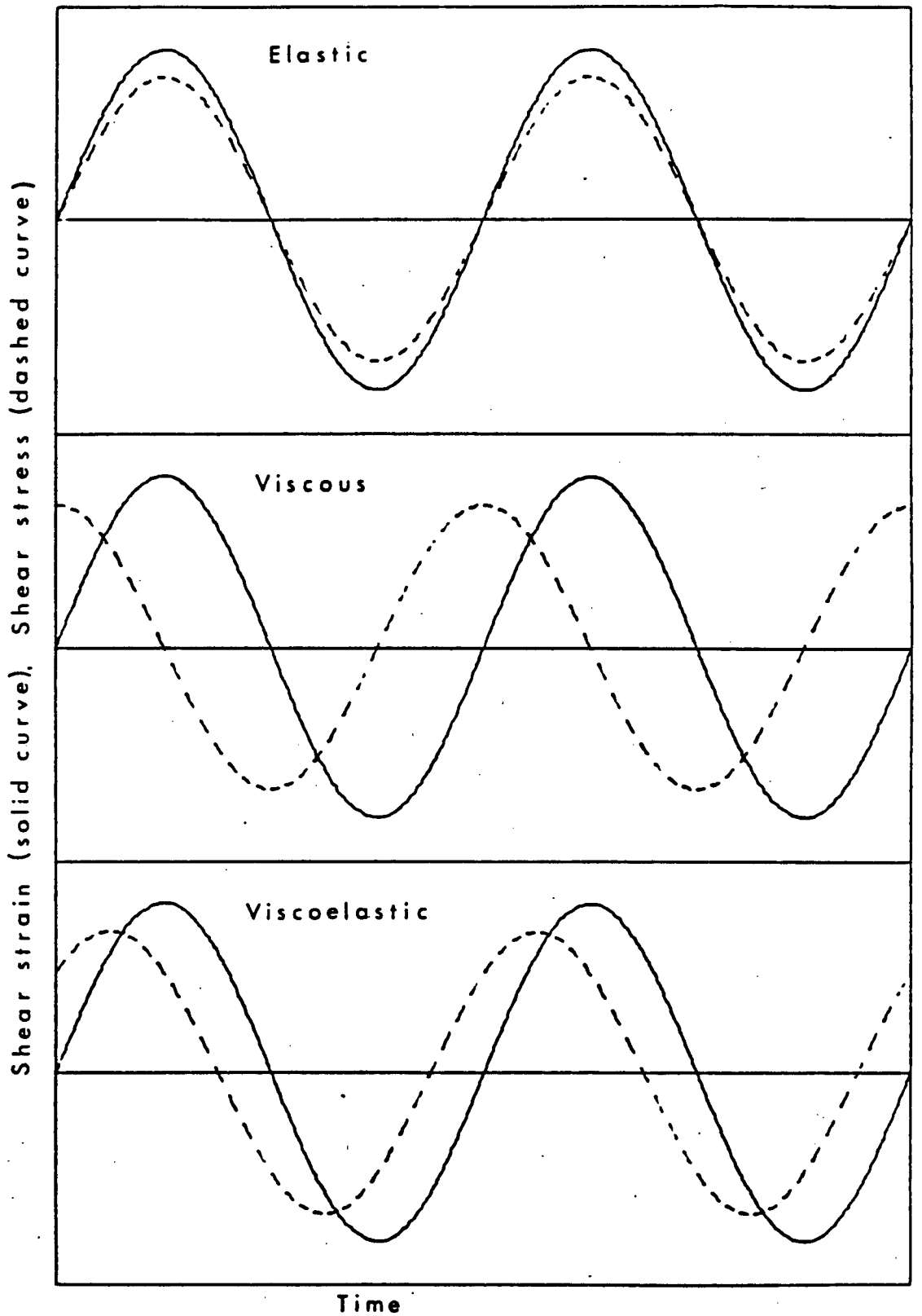


Figure 18. Idealized dynamic response of elastic, viscous and viscoelastic systems to sinusoidal oscillatory shear.

having both viscous and elastic components and the respective constants G', the dynamic storage modulus, and G" the loss modulus. The former is a measure of the energy recovered per cycle of deformation, while the latter is a measure of the energy lost as heat for a deformation cycle. A ratio of the loss and storage moduli (Eq. 19) gives the tangent of the phase shift (δ), and is called the loss tangent. This parameter is sensitive to changes in viscoelastic behaviour

$$\text{Tan } \delta = \frac{G''}{G'} \qquad [19]$$

for a material. The dynamic viscosity, as seen in Eq. 20,

$$\eta^* = \frac{G''}{\omega} \qquad [20]$$

describes the energy loss resulting from oscillatory strain. The dependence of η^*, or of the loss and storage modulus, on angular frequency (ω), can provide information about molecular assocations, particularly the relative extent of crosslinking interactions and their strength. Figure 19 illustrates the dependence of the storage and loss moduli, and dynamic viscosity on angular frequency, for hydrated systems corresponding to the gel state, concentrated solution and dilute solution.[17]

1.3 SUMMARY

In the previous sections an attempt has been made to introduce the Reader to three areas of major importance in polysaccharide chemistry. These were, (1) polysaccharide

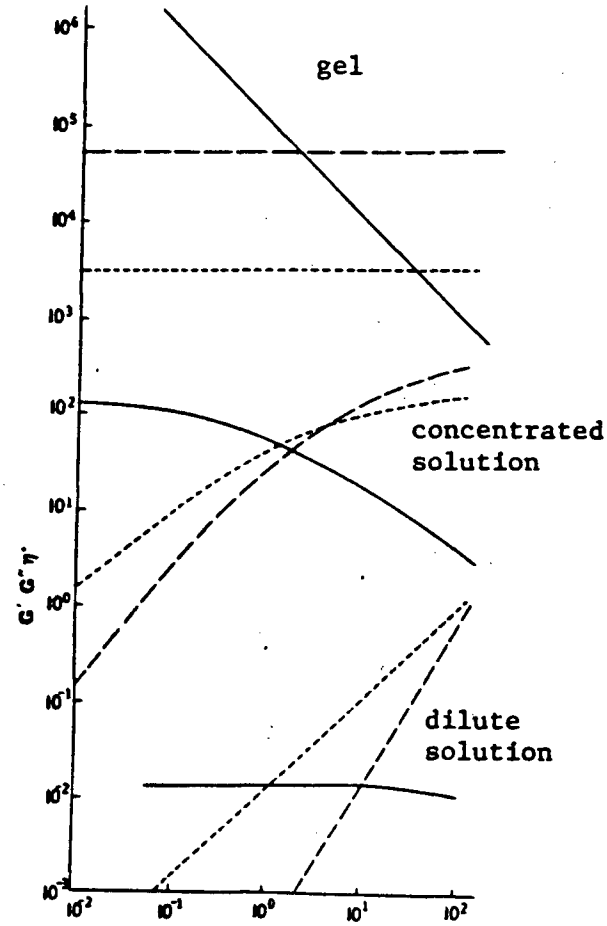


Figure 19. Typical rheograms from oscillatory rheometry showing the solid-like and liquid-like behaviour as characterised by the storage(— —) and loss(----) moduli G' and G" respectively, and by dynamic viscosity η^*. The samples shown are 2% agar, 5% κ-carrageenan, and 5% dextran.

modification (with emphasis on amino polysaccharides), (2) nmr spectroscopy of polysaccharides, and (3) solution properties of polysaccharides. It was intended at the outset of this work that attention would not be directed at a single facet of polysaccharide chemistry, but rather, that the studies should reflect the diverse interest in polysaccharides. This philosophy is reflected in the introduction, and will be equally apparent throughout the discussion.

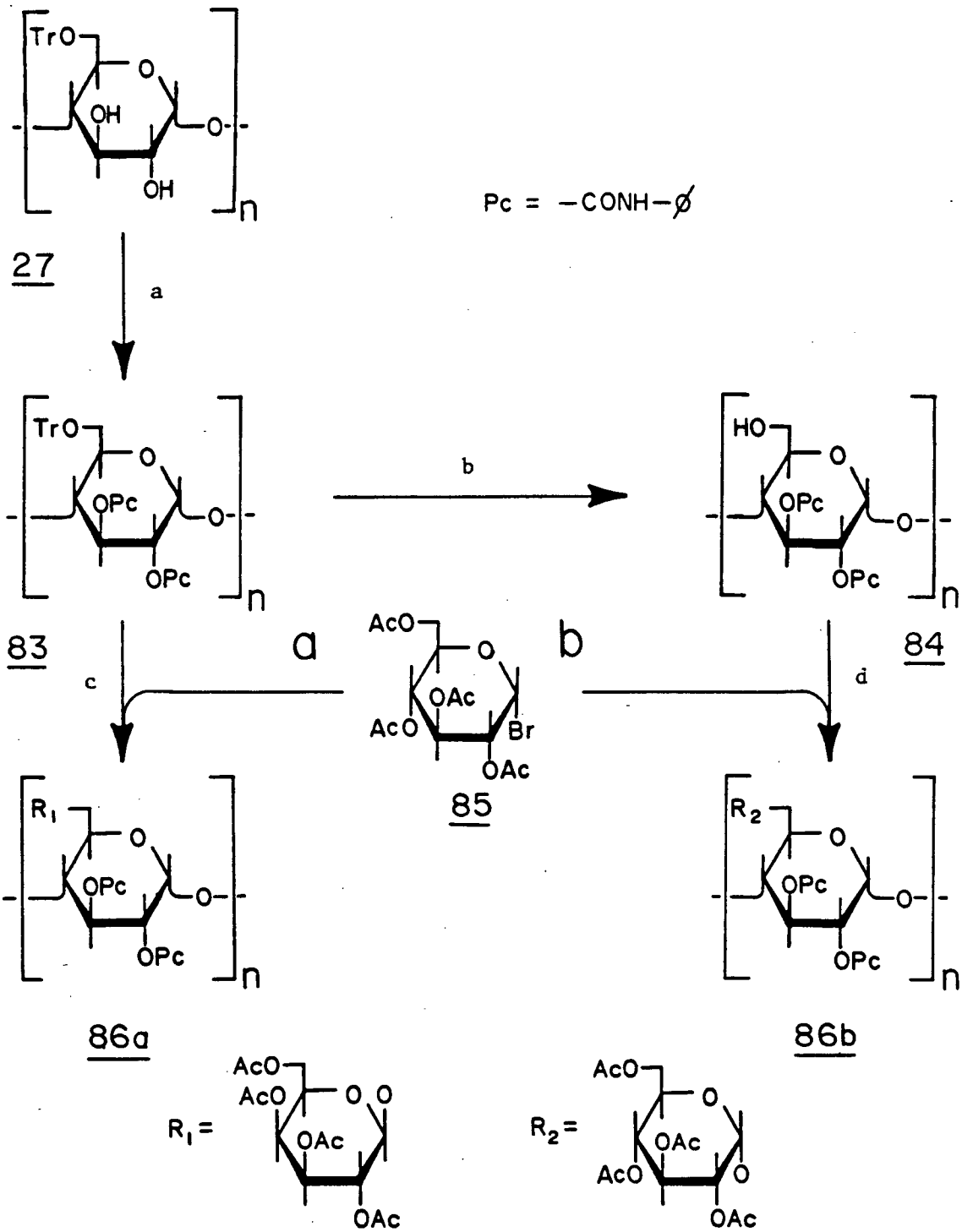
CHAPTER 2

BRANCHED CHITOSAN DERIVATIVES

2.1 INTRODUCTION

Some natural branched polysaccharides, such as xanthan[226] and guar gum,[155,227] are known to possess unique aqueous solution properties.[16,155,226] It has also been established that many branched exocellular polysaccharides have immunogenic activity,[1,2,20,21] a fact which renders them potentially useful in biomedical and pharmacological applications.[228] These two factors have helped to direct attention toward the synthesis of polysaccharides bearing pendant carbohydrate moieties.

The synthesis of branched derivatives of polysaccharides has been accomplished using a variety of different approaches.[15,229] One notable method involves the reaction of acetobromo sugars under glycosidation conditions with amylose and cellulose to produce branched derivatives.[158,230-234] Pfannemuller *et al.*, have coupled the acetobromo derivatives of glucose (**85**), maltose and maltodextrins (up to heptasaccharides) to 2,3-di-*O*-phenylcarbamoyl-6-*O*-trityl derivative (**83**) of the polysaccharides[158,230] (Scheme 15a), or to the 2,3-di-*O*-phenylcarbamoyl derivative[230] **84** (Scheme 15b) . The latter produced (1→6)-α-linkages preferentially with little or no depolymerization, while the former gave (1→6)-β-lin-



(a) phenylisocyanate, pyr (b) HCl, MeOH (c) $AgClO_4$, CH_3NO_2-dioxane

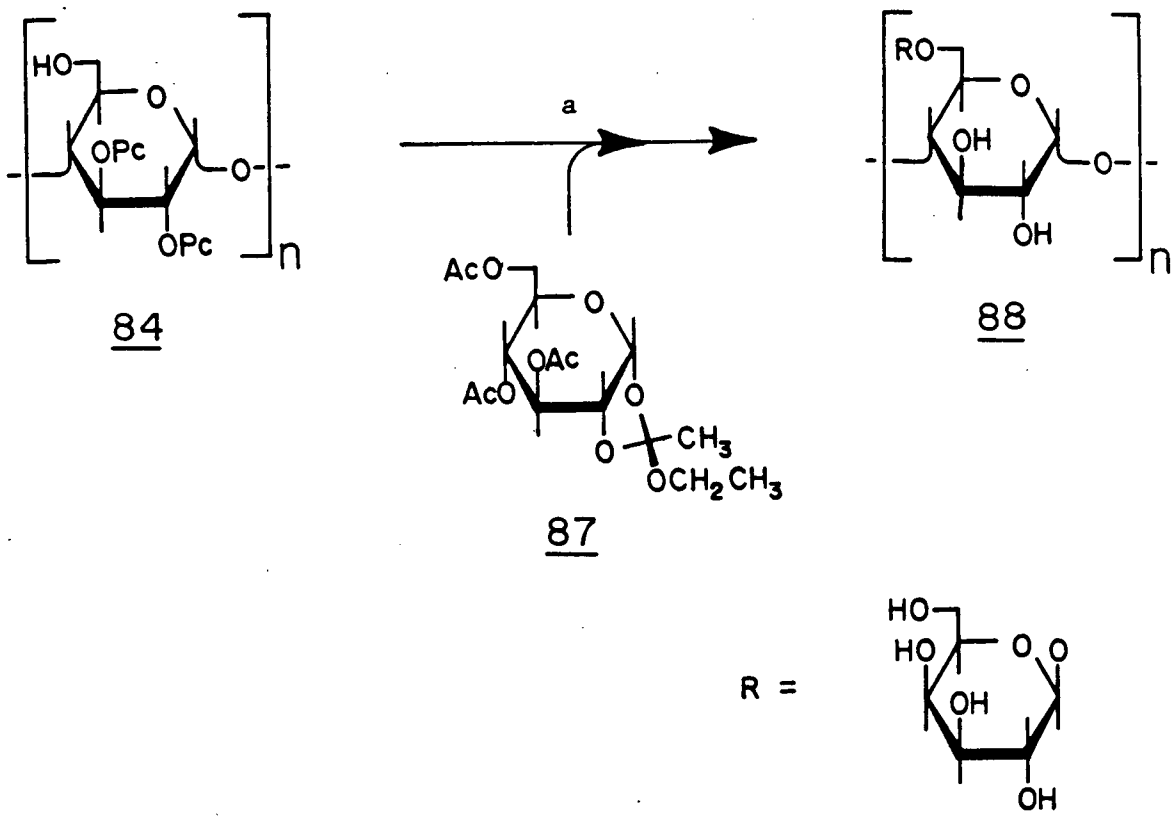
(d) $Hg(CN)_2$, CH_3CN-dioxane

Scheme 15

kages and extensive depolymerization. When acetobromoglucose was employed, the branched amylose was produced in 50-85% yields having degrees of substitution ranging from 0.21-0.44, while the maltodextrin derivative of amylose had d.s. 0.01-0.04.[234] When cellulose was treated in a similar fashion with acetobromoglucose, lower d.s. values of 0.09-0.14 were obtained.

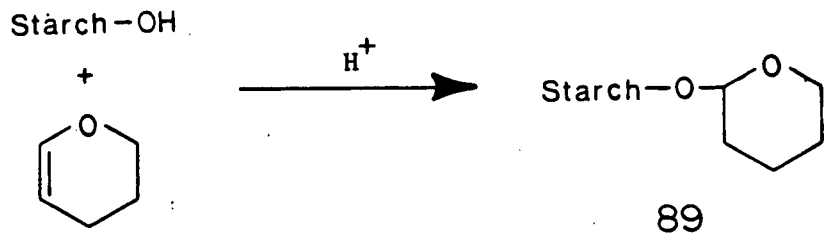
Carbohydrate 1,2-orthoesters have found application in coupling to amylose and cellulose derivatives *via* the cyclic orthoester glycosidation method.[229,231,235] Kochetkov *et al.* have reported the reaction of 3,4,6-tri-*O*-acetyl-α-D-glucopyranose 1,2-(*t*-butyl orthoacetate) with randomly substituted cellulose diacetate, giving a product substituted mainly at primary positions.[235] Pfannemuller *et al.* have used both 1,2-(*t*-butyl orthoacetate) and 1,2-(ethyl orthoacetate) (**87**) derivatives of 3,4,6-tri-*O*-acetyl-α-D-glucopyranose in reactions with 2,3-di-*O*-phenylcarbamoylamylose (**84**) and cellulose derivatives (Scheme 16).[231] The product **88** (d.s. 0.25-0.30) contained largely (1→6)-β-branches with a small amount of (1→6)-α. When the 1,2-(ethyl orthoacetates) of maltose, maltotetrose and maltohexose were similarly treated, branched polysaccharides of d.s. 0.05-0.20 were obtained.

Starch polysaccharides have been reacted with 3,4-dihydro-2H-pyrans, as in Eq. 21, to give derivatives bearing the tetrahydropyran-2-yl acetal. These compounds were water-soluble at low levels of substitution and organic-soluble at high levels.[236] While the pendant group in this



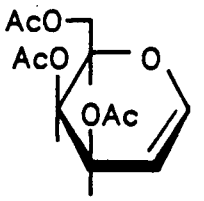
(a) i) lutidinium perchlorate, chlorobenzene ii) NaOMe/MeOH

Scheme 16



[21]

case is not a carbohydrate, it is coupled "glycosidically" and indicates the potential of using known carbohydrate glycals (such as the glucal derivative 90) to prepare derivatives with 2-deoxy-saccharide branches.



90

A variety of linear and branched synthetic polysaccharides have been prepared by polymerization of 1,6-anhydro sugar derivatives.[229,237,238] For example, branched dextran analogues have been prepared from two suitably protected 1,6-anhydro-D-glucose derivatives.[237]

The methods described here have significant potential and reflect the first generation of synthetic methods for making branched polysaccharides. However, various disadvantages are evident in most cases, such as (1) requirement for specific protection, (2) multistep synthetic procedures, (3) activation of the carbohydrate moiety, (4) low coupling efficiencies, (5) poor site-selectivity, and (6) harsh, degradative reaction conditions.

The reductive N-alkylation methods described in the introduction (section 1.2.1) have been used to produce chitosan derivatives, by reaction with reducing mono- and disaccharides, having acyclic carbohydrate branches.[96]

Similarly, enzymatically oxidized guar was extended by reductive amination with aminosugars.[45] In both cases, the branches differ substantially from those on natural polysaccharides.

The work described in this chapter constitutes a new method for controlled solubilization of chitosan via the introduction of hydrophilic groups. Aldehydes obtained from reductive ozonolysis of allyl glycosides have been reductively aminated to the 2-amino group of chitosan to produce pendant glycosidic branches.[239,240] While the linkage is not strictly a glycosidic branch, the saccharide residues are intact pyranosides and variation of the pendant sugar functionality, identity, and linkage configuration are possible, by preparing the appropriate alkenyl glycoside precursors. Viscometric studies on aqueous solutions of this family of branched chitosan derivatives have been undertaken as a means for probing structure/property relationships, which may find more general applicability to other synthetic and natural branched polysaccharides.

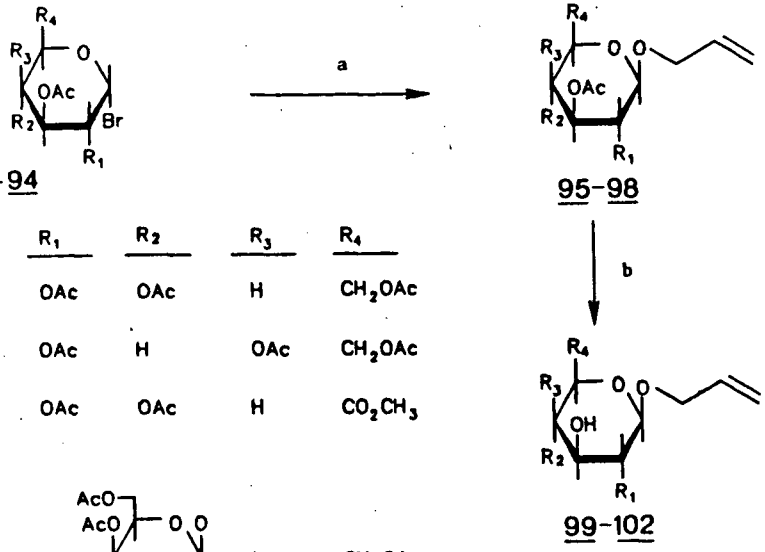
The use of 10-undecenyl β-D-glycosides in analogous derivatizations will demonstrate that variations in the alkyl chain length of these branched chitosans adds another dimension in the tailoring of polysaccharide solution properties. This is an extension of the concept of controlling solubility properties of chitosan by co-reaction with hydrophobic and hydrophilic groups to give mixed branch derivatives[19] (section 1.2.2).

2.2 N-[2'-O-(D-GLYCOPYRANOSYL)ETHYL]CHITOSAN DERIVATIVES

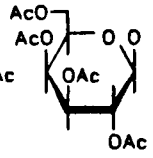
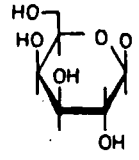
2.2.1 Synthesis and Characterization

A wide variety of allyl glycosides have been reported for use in biochemical studies[241-244] and as intermediates in carbohydrate syntheses.[245,246] The allyl glycosides used in this work were prepared according to methods described by Lee and Lee.[242] The β-D-glycosides were prepared from the respective peracetylated α-D-glycopyranosyl halides.

The acetobromo or acetochloro sugars 91-94 and 103 are well described in the literature, and were prepared using standard methods.[247] Koenigs-Knorr glycosidations[227,248] of the acetobromo sugars 91-94 with allyl alcohol (Scheme 17a), provided the peracetylated allyl β-D-glycosides 95-98. A parallel route to the 2-acetamido-2-deoxy-β-D-glucoside from acetochloroglucose (103), is given in Scheme 17b. Subsequent de-O-acetylation gave the unprotected allyl β-D-glycopyranosides 99-102 and 105. The allyl α-D-glycopyranosides 108 and 109 were prepared by acid-catalyzed glycosidation, as shown in Eq. 22, where the respective free sugars, D-glucose (106) and, D-galactose(107) were refluxed in allyl alcohol with Dowex 50x8, H^+, ion-exchange resin. The reported yields of allyl 2-acetamido-2-deoxy-α-D-gluco pyranoside using this methods were very low (10%),[242] so the preferred method, involving treatment with boron trifluoride-etherate as catalyst, was used to prepare 111 (Eq. 23) from 110 in 40%

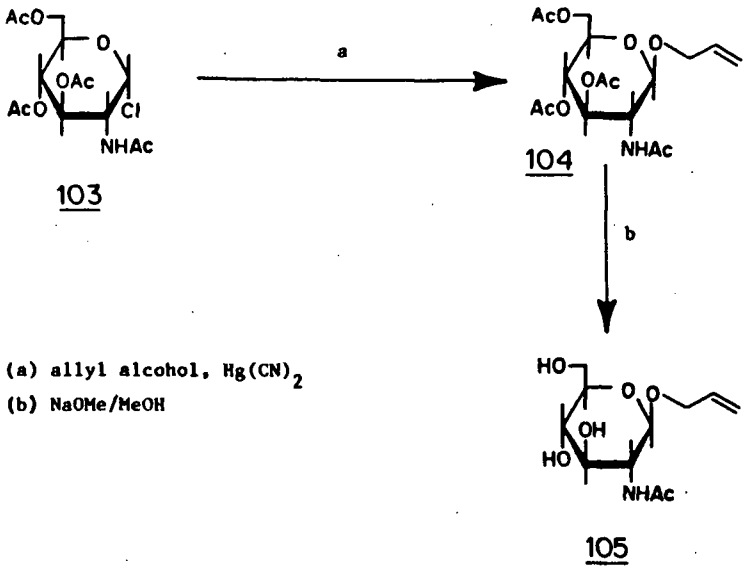


91-94 → (a) → 95-98 → (b) → 99-102

	R_1	R_2	R_3	R_4
91,95	OAc	OAc	H	CH_2OAc
92,96	OAc	H	OAc	CH_2OAc
93,97	OAc	OAc	H	CO_2CH_3
94,98	OAc		H	CH_2OAc
99	OH	OH	H	CH_2OH
100	OH	H	OH	CH_2OH
101	OH	OH	H	CO_2H
102	OH		H	CH_2OH

(a) allyl alcohol, $Hg(CN)_2$, Drierite (b) NaOMe/MeOH

Scheme 17a

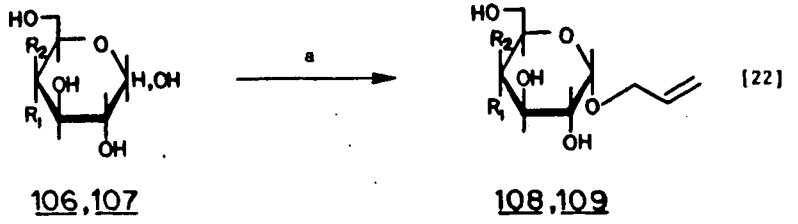


103 → (a) → 104 → (b) → 105

(a) allyl alcohol, $Hg(CN)_2$

(b) NaOMe/MeOH

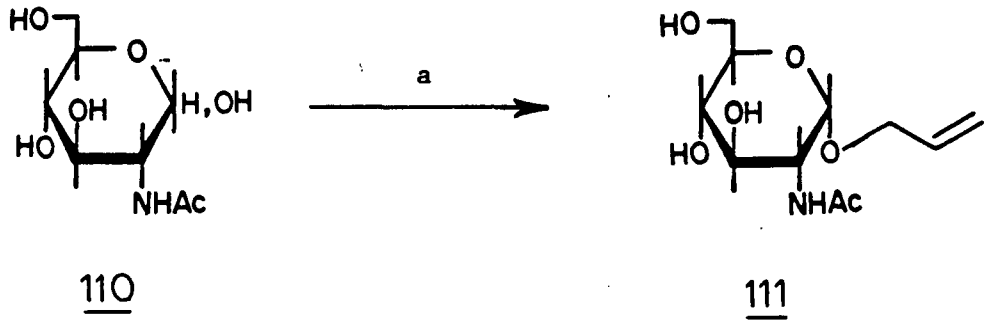
Scheme 17b



106,107 → (a) → 108,109 [22]

(a) allyl alcohol, Dowex 50X8, H^+

	R_1	R_2
106,108	OH	H
107,109	H	OH



[23]

110 111

(a) allyl alcohol, BF_3-etherate

yield. Characterization data, such as melting points and optical rotation values, agreed with those reported in the literature.[242] While published ^1H and ^{13}C-nmr spectroscopic data for allyl glycosides were not available in many cases, the ^1H and ^{13}C-nmr spectra obtained agreed closely with published chemical shift and coupling constant values of the respective methyl glycosides.[99-101]

Previously unreported allyl β-D-glucopyranuronic acid 101, gave a ^{13}C-nmr spectrum comparable to its respective methyl glycoside analogue,[28] and containing the characteristic allyl group carbon resonances. Unfortunately, this compound was not successfully crystallized and an analytically pure sample for specific rotation and melting point determination was not obtained. The crystalline precursor, methyl (allyl 2,3,4-tri-O-acetyl-β-D-glucopyranoside)uronate, 97, was fully characterized. De-O-acetylation of 97 was performed under standard Zemplen conditions, with the tlc analysis of the reaction mixture showing a major component having an R_f value higher than expected for 101. Treatment with aqueous sodium hydroxide converted the component having

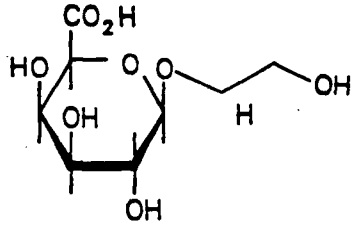
Derivative	Branch	C-1	C-2	C-3	C-4	C-5	C-6	C-1'	C-2'	C-3'
99	β-Glc	99.6	71.5	74.2[a]	68.0[b]	74.2[a]	59.2	68.9[b]	131.8	117.0
100	β-Gal	100.2	69.1	71.1	67.0	73.4	59.3	68.9	131.9	116.9
101	β-GlcA	99.7	71.1	73.7	69.2	72.8	170.4	69.5	131.7	117.1
102	β-Lact									
	(β-Gal)	100.2	69.2	70.9[a]	66.8	73.5	59.3			
	(β-Glc)	99.4	71.1[a]	72.7[b]	76.8	73.0[b]	58.4	68.9	131.7	117.0
105	β-GlcNAc	98.8	54.2	72.5	68.7	74.5	59.5	68.9	132.3	116.5
108	α-Glc	95.7	69.6	71.5	68.0	70.2	59.0	66.8	132.1	116.5
109	α-Gal	96.1	66.9[b]	68.0	67.7	69.3	59.6	66.7[a]	132.2	116.5
111	α-GlcNAc	94.5	52.0	69.4	68.4	70.3	59.0	66.8	132.1	116.2

Table 2. 100.6 MHz ^{13}C-nmr chemical shift data (ppm) for the allyl glycosides in D_2O solution (ref. external TMS).

a. Assignments may be reversed.
b. "

the high R_f into a lower R_f material corresponding to the product. A complete listing of the ^{13}C-chemical shift values for the allyl glycosides prepared is given in Table 2.

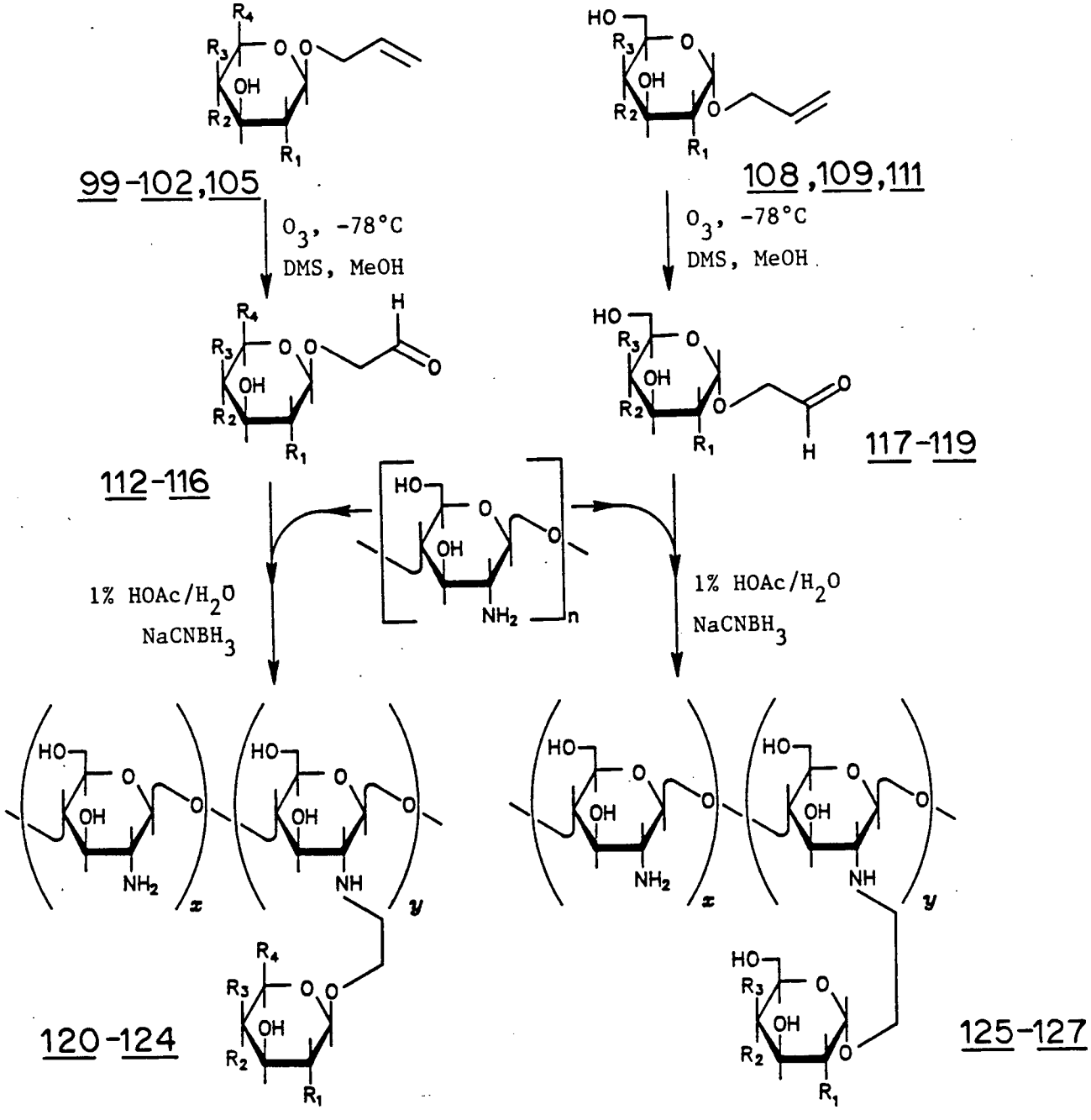
The allyl glycoside precursors 99-102, 105, 108, 109 and 111 were reductively ozonolyzed[249] to provide the respective acetaldehydo glycosides 112-119 (Scheme 18).[240,250] Aldehydes of this type exist in a variety of equilibrium states, including the gem-diol, the intramolecular cyclic hemiacetals and acetal oligomers. Hence direct characterizations were not attempted. Previous work done in this laboratory,[240,250] in which aldehydes of this sort were reduced and acetylated for characterization purposes, established that the ozonolysis of allyl glycosides proceeds in a virtually quantitative manner. Thus in most cases, the aldehyde products were used directly in the subsequent step. The aldehyde 114 was reduced and characterized by ^{13}C-nmr spectroscopy in order to establish the stability of uronosides to ozonolytic conditions. The solution ^{13}C-nmr spectrum of the product 128 established that the carbohydrate portion of the molecule was unaltered, and that the expected 2-hydroxyethyl glycoside was the product.

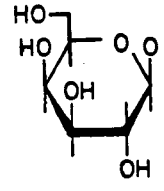


128

Reductive amination[86] of chitosan with the aldehydes 112-119, was performed as outlined in Scheme 18, to yield

Scheme 18



	R_1	R_2	R_3	R_4
99,108,112,117,120,125	OH	OH	H	CH_2OH
100,109,113,118,121,126	OH	H	OH	CH_2OH
101,114,122	OH	OH	H	CO_2H
105,111,116,119,124,127	NHAc	OH	H	CH_2OH
102,115,123	OH		H	CH_2OH

derivatives 120-127. The aldehydes were dissolved in 10-15 mL of the reaction media (5% aqueous acetic acid) and added to a viscous chitosan solution (@ 1 mmol/10 mL). Treatment with excess sodium cyanoborohydride resulted in appreciable foaming which dissipated over time. After the reaction had been stirred for 24 hours, it was filtered to remove insoluble material. The only case where filtration was not possible was in the preparation of 122a, where the product precipitated during the reaction. Varying the molar ratio of aldehyde to chitosan (A/C) gave products with a range of degree of substitution (d.s.) values, as shown in Table 3. For example, a molar ratio of ~ 3 was used to give fully or highly substituted derivatives (e.g. 121a-126a), while a ratio of 0.50 gave products with low d.s. (124d, 125d, 127e). The degree of substitution values were determined from C, H and N elemental microanalyses (see appendix A). Of note is the fact that for the β-D-lactosyl derivative 123a, an A/C ratio of 3.0 resulted in a d.s. of 0.90, indicating that the size of the substituent influenced the coupling efficiency, as would be expected. This is further supported by the results of derivatives 127c and 127d, in which A/C ratios of 1.5 and 0.75 provided d.s. values 0.35 and 0.19 respectively, significantly lower than results for the 121, 123 and 125 series of derivatives. In the case of the lactosyl derivatives, the size effect seemed to be manifested mainly at high d.s. values, hindering complete substitution, while for the acetamido derivatives relatively lower d.s. products were

Derivative	Branch	A/C	d.s. (±.05)	Yield(%)
120a	β-Glc	3.1	1.00	95
121a	β-Gal	2.7	1.00	60
b		1.3	0.70	85
c		0.75	0.38	80
122a	β-GlcA	3.0	1.00	70
b		1.0	0.67	80
123a	β-Lact	3.1	0.90	95
b		1.5	0.76	85
c		0.75	0.35	95
d		0.50	0.32	95
e		0.35	0.24	87
124a	β-GlcNAc	3.0	1.00	85
125a	α-Glc	3.0	1.00	95
b		1.5	0.59	60
c		0.75	0.38	70
d		0.5	0.26	80
126a	α-Gal	3.1	1.00	60
b		2.0	0.86	55
c		1.0	0.48	95
d		0.75	0.32	95
127a	α-GlcNAc	3.1	1.00	90
b		3.1	1.00	85
c		1.5	0.35	95
d		0.75	0.19	95
e		0.5	0.17	95

Table 3. Characteristics of N-[2-O-(D-glycopyranosyl)ethyl]-chitosan derivatives.

obtained at all A/C ratios less than 3.0. This could be an indication that the latter effect was not strictly due to size, but may relate to molecular associations or repulsions involving free amino functionalities on the backbone and

acetamido groups on the branch.

^{13}C-nmr spectra were recorded for all of the highly substituted derivatives for each sugar branch. These derivatives were highly soluble and gave free flowing 5% (w/w) solutions in D_2O. The linewidths of the branch carbon resonances were relatively narrow (5-10 Hz) in comparison to the chitosan main chain resonances (100-200 Hz), as shown in Fig. 20. ^{13}C chemical shift assignments (Table 4) were easily accomplished by comparison to published values for methyl glycosides[99,100] or to values given in Table 2 for the respective allyl glycosides. ^{13}C-nmr spectra of derivatives 121b, 125c and 126c, having lower d.s. values, show substantially broader signals for branch carbons than for the high d.s. analogues 121a, 125a, and 126b as shown in Figs. 20, 21 and 22. This is indicative of interrelationships between the degree of substitution, solution viscosity and branch mobility, as manifested in the correlation time (τ_c) dependence of T_2 and linewidth ($\nu_{1/2}$).[111] It is interesting to note the increased linewidth and reduced intensity of the C-1' carbon of the branch, when compared to C-6 or other ring carbon resonances on the same derivative, illustrating the reduced mobility of positions closer to the main chain. Thus, not only does ^{13}C-nmr spectroscopy provide proof of structural modification, it also allows one to discern resonances on the basis of mobility and it provides a qualitative indication of relative viscosities.

Derivative	Branch	C-1	C-2	C-3	C-4	C-5	C-6	C-2'	C-1'
120a	β-Glc	100.8	71.7	74.2	68.2	74.4	59.4	67.3	45.8
121a	β-Gal	101.4	67.2	71.2	69.3	73.6	59.5	67.5	45.8
122a	β-GlcA	100.7	71.6	74.1	70.4	74.4	174.1	67.4	45.8
123a	β-Lact								
	(β-Gal)	101.4	69.4	71.1	67.0	73.8	59.4		
	(β-Glc)	100.5	71.3	72.8	77.1	73.2	58.7	67.2	45.7
124c	β-GlcNAc	99.5	54.0	72.3	68.4	74.3	59.3	67.2	45.8
125a	α-Glc	97.0	69.9	71.6	68.2	70.4	59.2	65.5	45.6
126b	α-Gal	97.2	66.9	68.1	67.8	69.5	59.7	65.6	45.6
127a	α-GlcNAc	95.6	52.1	69.5	68.5	70.5	59.1	65.4	45.6
129	α-$GlcNH_2$	97.5	53.9	69.1	69.0	71.2	59.8	66.2	45.9
130a	(α-Glc)	96.9	69.8	70.3	68.0	71.5	59.0	65.0	45.5
	(β-GlcNAc)*	99.7	53.8	--	77.5	76.0	60.9		

Table 4. 100.6 MHz ^{13}C-nmr data for the N-ethyl glycoside branched chitosan derivatives, showing chemical shift values (ppm) for pendant sugar resonances (ref. external TMS).

* GlcNAc of backbone, NAc resonances; CH_3 20.5, C=O 173.2.

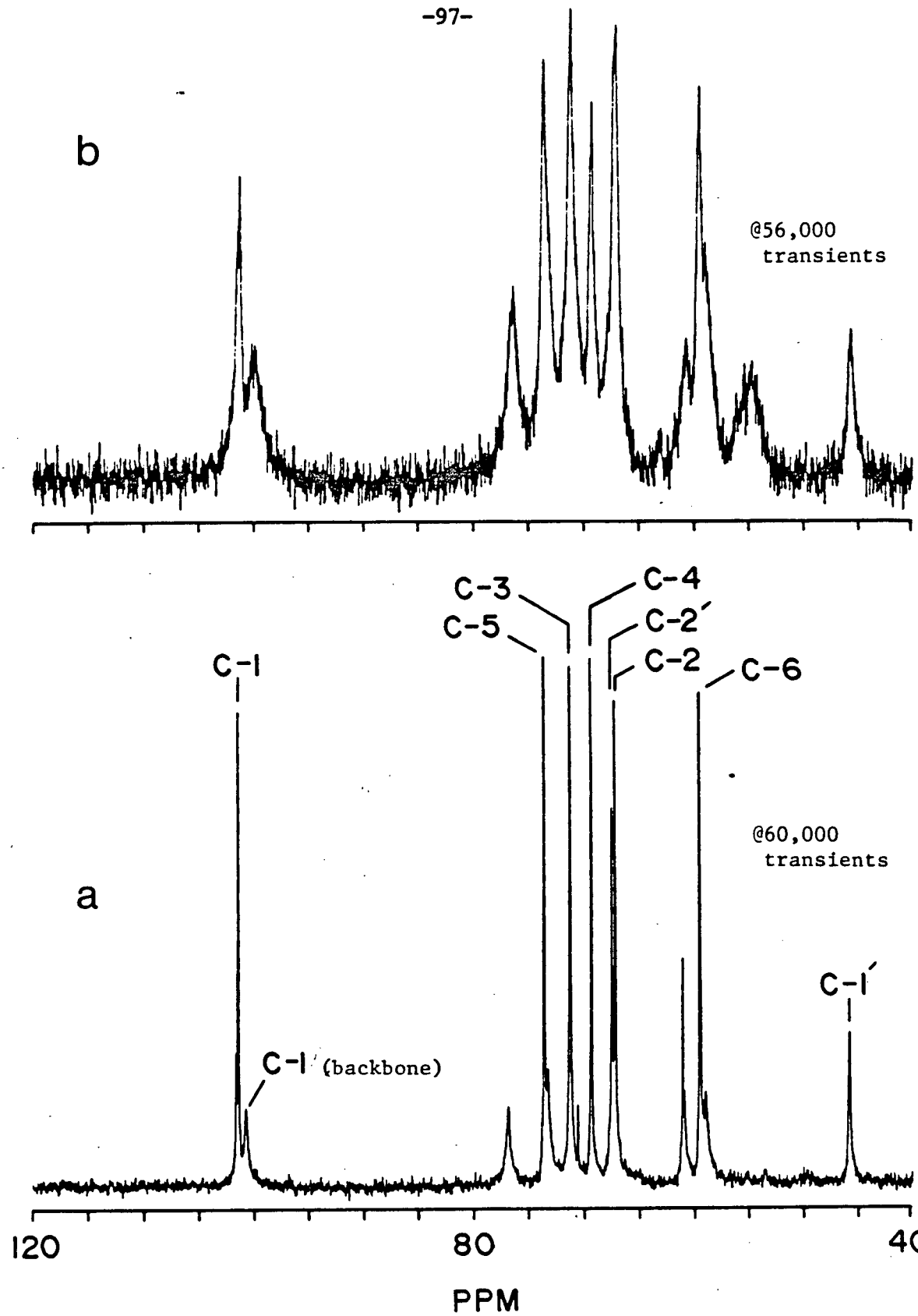


Figure 20. 100.6 MHz ^{13}C-nmr spectral region showing branch residue resonances for a) 121a (d.s. 1.0); and b) 121b (d.s. 0.70), in D_2O (ref. external TMS).

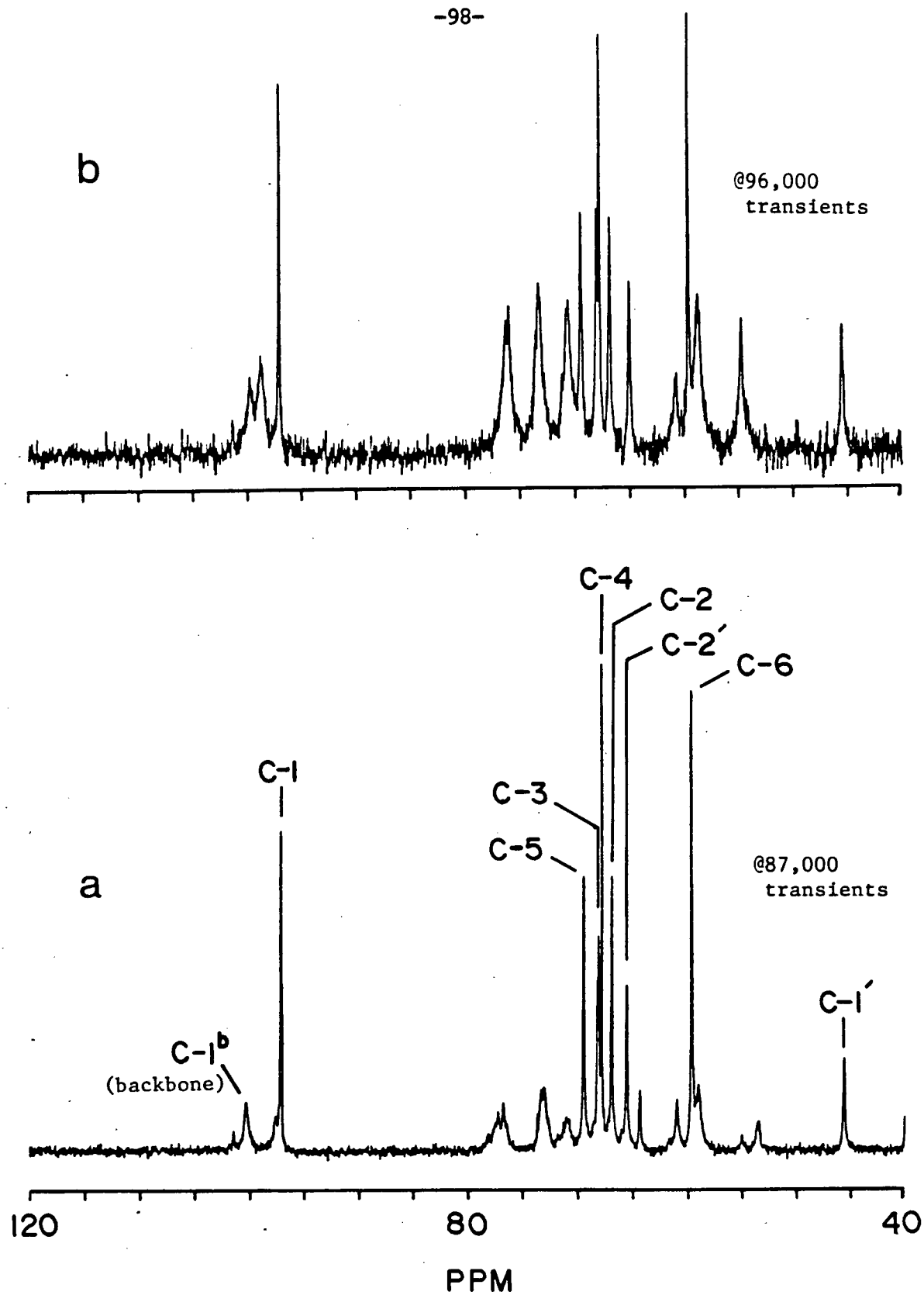


Figure 21. An expanded region of the 100.6 MHz ^{13}C-nmr spectra of a) 126b (d.s. I.00); and b) 126d (d.s. 0.32), in D_2O, showing the branch residue resonances (ref. external TMS).

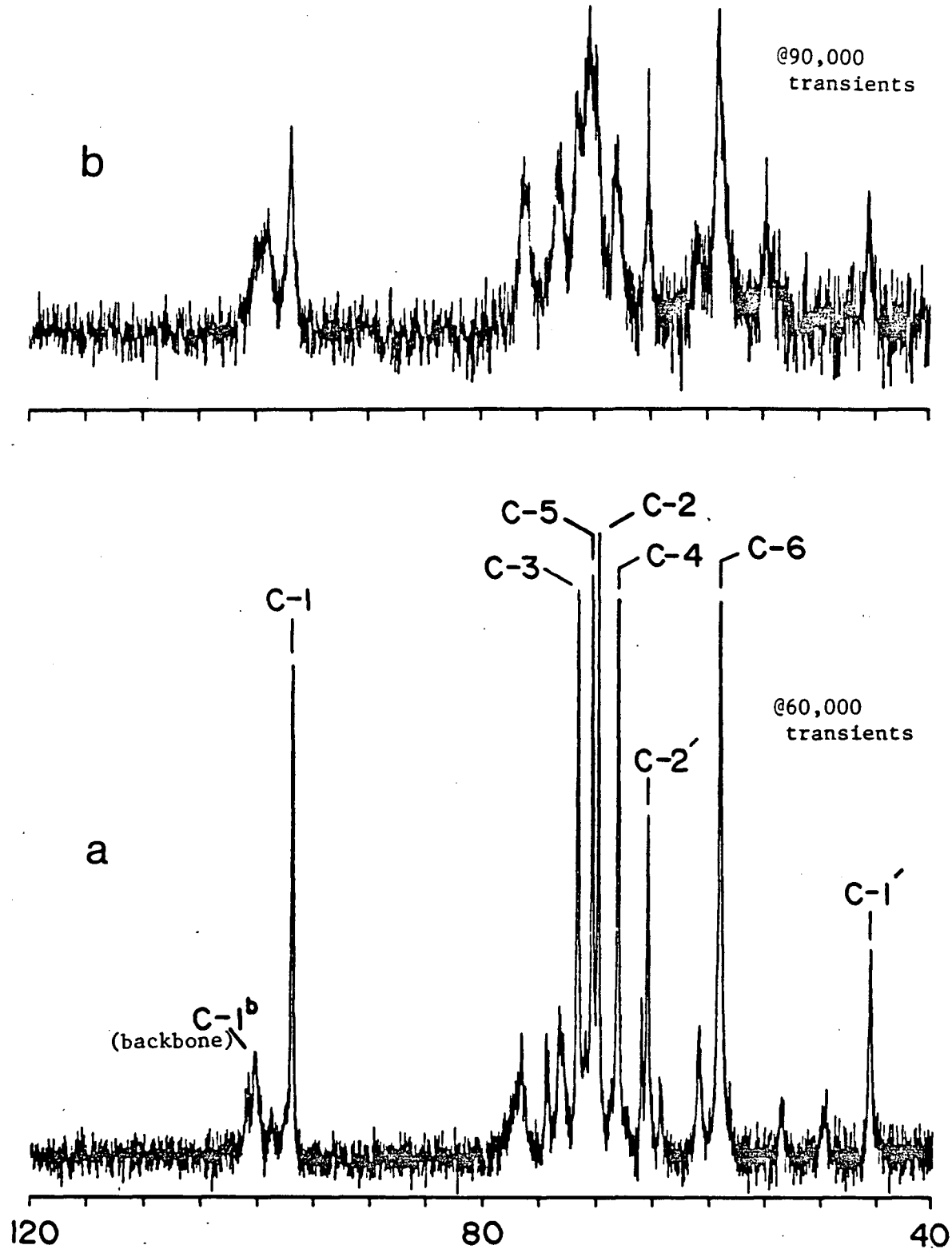
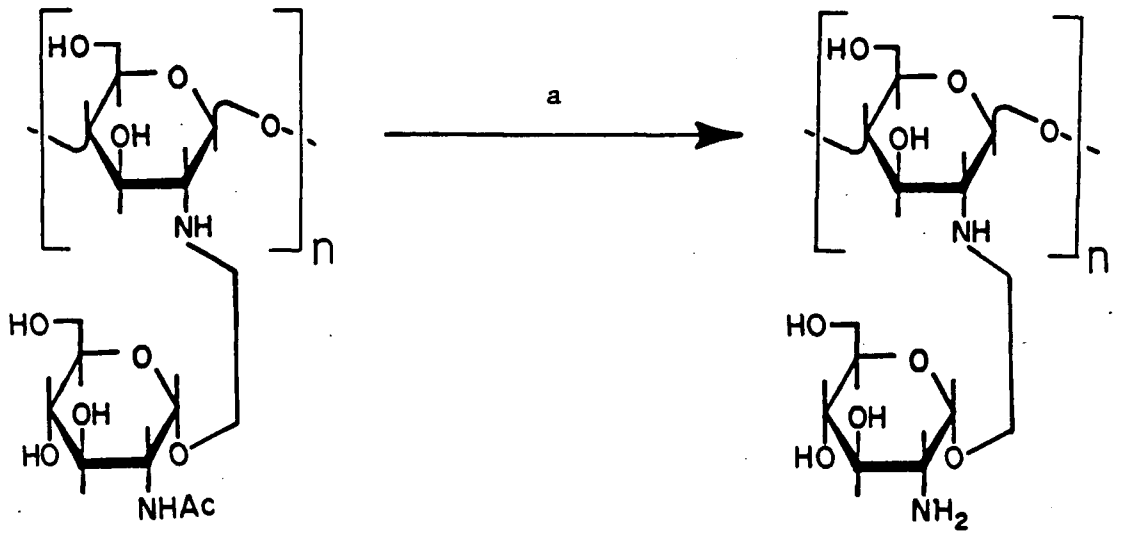


Figure 22. An expanded region of the 100.6 MHz ^{13}C-nmr spectra of a) 125a (d.s. 1.00); and b) 125c (d.s. 0.38), in D_2O, showing the branch residue signals (ref. external TMS).

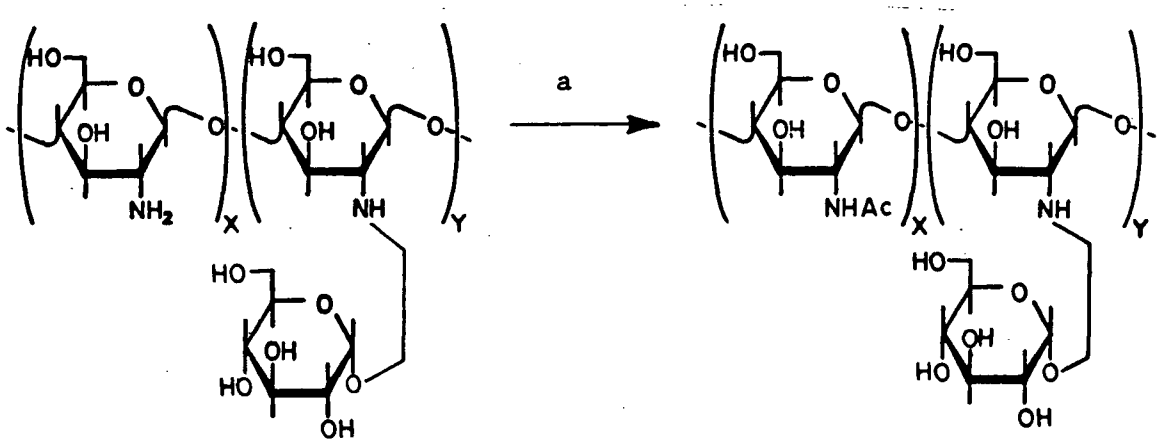
The resonances due to the chitosan backbone are evident to some extent in most of the derivatives' spectra. In general, the resonances of the anomeric carbon (C-1^b) and C-4^b carbon are discernible. No attempt has been made here to assign all the chitosan resonances, particularly in intermediate and low d.s. samples where the splitting of resonances, due to substituted and unsubstituted residues, complicated assignment, and broad lines obscured the peaks. In all cases, it was possible to assign the C-1^b signal to the 98-102 ppm region (depending on substitution) and C-4^b to the 75-80 ppm range.

The utility of secondary modification of chitosan derivatives has been alluded to. The derivatives described here having d.s. <1.0, are suitable candidates for homogeneous chemical reaction in aqueous media. To demonstrate this, three secondary modification sequences were undertaken. Two of these will be mentioned here, and the third will be described in the context of a study to be discussed later in this chapter. One comparison we felt would be of value was to contrast the properties of the 2-acetamido-α-D-glucose derivative (127a) with those of a derivative bearing pendant 2-amino-α-D-glucose units. As such, derivative 127b was subjected to treatment with 40% aqueous NaOH at 100°C (Eq. 24). Both elemental analyses and ^{13}C-nmr spectra verified the absence of N-acetate groups in the desired product 129. Although it remained to be seen whether depolymerization occurred, qualitative observations of derivative 129 were



127 (a) 40% aq NaOH 129 [24]

encouraging. A second procedure, which offers a facile method for controlling or modifying the solubility properties of the N-ethylglycosyl chitosan derivatives is N-acetylation. Treatment of a solution of 125c (d.s. 0.38) in aqueous methanol (1:1) with acetic anhydride provided derivative 130 (Eq. 25), which showed characteristic N-acetate peaks in its



125 (a) $MeOH/H_2O$ (1:1), Ac_2O 130 [25]

^{13}C-nmr spectrum and analyzed for full N-acetylation at all unsubstituted amines (d.s. of HNAc 0.62). Compound 130, having a high degree of N-acetylation, would qualify as a N-[2-O(α-D-glucopyranosyl)ethyl] chitin derivative.

We now have in hand a family of structurally-related water-soluble derivatives bearing pendant carbohydrates with varied functionality, glycosidic configuration, and size at a number of degrees of substitution. This constitutes an ideal array of compounds for use in studies relating solution properties to structural features.

2.2.2 Viscometry

Steady shear rheometric determinations were performed on 2.0% (w/w) solutions (unless otherwise specified) of polysaccharide derivatives and commercial polysaccharides, in distilled water at 20° ±0.5°C. Measurements were done using a rotational viscometer with cone and plate geometry, from which shear stress (σ) values at shear rates ($\dot{\gamma}$) ranging from 1-2500 s^{-1}, were obtained. Apparent viscosities (η) were determined according to Eq. 26.

$$\eta = \frac{\sigma}{\dot{\gamma}} \qquad [26]$$

Rheograms of apparent viscosity against shear rate for solutions of derivatives 120-127 and for commercial samples of xanthan gum, hydroxyethyl cellulose and sodium alginate are presented on linear and logarithmic coordinates in Figs. 23-38. For purposes of comparison, it was decided to employ

the power-law equation:

$$\sigma = m\dot{\gamma}^{n} \qquad [12]$$

where n is the flow behaviour index, and m is the consistency coefficient, to model the flow behaviour of the solutions examined. Thus, regression of logarithm of shear stress against logarithm of shear rate provided the parameter n and the logarithm of m. The parameters, n and m, determined for all solutions, are presented in Table 5. The rheograms on logarithmic axes therefore represent the power-law modelling, and it is immediately obvious that these plots are considerably easier to analyze than those on linear axes. After careful examination of the logarithmic rheograms, it might be concluded that the power-law model is not totally appropriate for all solutions studied. It would be more correct to say that, in some cases, the experimental data extends past the shear rate range where the power-law model is appropriate. There is no doubt, however, that all of the derivatives prepared are adequately modelled by the power-law equation over a substantial portion of the experimental shear rate range. This is supported by the high correlation coefficients (R^2) shown in Table 5. The power-law has been frequently used to model the flow behaviour of polysaccharide solutions and dispersions.[223,224] This method of analysis was felt to be superior to examination of apparent viscosities as a fraction of zero shear viscosity (η_o) or at specific shear rate values representative of the experimental range (e.g. 10

Derivative	Branch	d.s. (±.05)	n ± 3%	m (mPa·s) ± 3%	R^2	#Points
120a	β-Glc	1.00	1.01	22.4	.998	84
121a	β-Gal	1.00	0.879	82.7	.999	36
b		0.70	0.783	434	.997	33
c		0.38	0.588	2300	.995	50
122a	β-GlcA	1.00	0.970	45	.999	36
b		0.67	0.911	109	.997	32
123a	β-Lact	0.90	0.997	24.9	.999	53
b		0.76	0.939	49.4	.999	28
c		0.35	0.841	222	.997	30
d		0.32	0.891	159	.997	34
124a	β-GlcNAc	1.00	1.05	14.3	.999	24
125a	α-Glc	1.00	0.929	65.1	.998	62
b		0.59	0.695	803	.999	34
c		0.38	0.677	1090	.998	33
d[a]		0.26	0.502	3270	.988	41
126a	α-Gal	1.00	1.00	25.3	.999	56
b		0.86	0.931	54.1	.999	59
c		0.48	0.554	5180	.992	43
d		0.32	0.778	525	.998	60
127c	α-GlcNAc	0.35	0.864	196	.997	29
d		0.19	0.822	456	.994	31
e		0.17	0.810	456	.996	36
129	α-$GlcNH_2$	1.00	0.845	110	.999	37
Xan.			0.296	10200	.938	38
HEC			0.426	9230	.985	24
NaALG			0.717	3180	.988	34

Table 5. Power-law parameters for branched chitosan derivative solutions (2.0% w/w in water), xanthan gum (Xan), hydroxyethylcellulose (HEC), and sodium alginate (NaALG).

a. Data given is for a 1% solution.

and 1000 s^{-1}). The parameter n is a direct measure of the shear rate dependence or pseudoplasticity of the solution while m corresponds to viscosity at a shear rate of 1 s^{-1}. For shear thinning fluids, of which polysaccharide solutions are typical examples, n can vary from 1.0, for solutions having Newtonian (no shear rate dependence) to 0.0-0.2, which describe extremely pseudoplastic fluids. Comparison of m values provides an index of relative viscosity (at a shear rate of 1 s^{-1}) for sample solutions. Thus, using these two parameters, n and m, we can begin to examine and contrast the flow behaviour of the solutions characterized in Table 5. Discussion will be limited largely to power-law parameters and the respective logarithmic rheograms. The linear rheograms are included for the readers reference, but they will not be discussed directly.

The first notable trend in the rheological properties of the derivative solutions is that the fully substituted derivatives 120a-122a and 124a-126a and the highly substituted lactosyl compound 123a have Newtonian or close to Newtonian flow behaviour, as indicated by n~1. This is probably indicative of relatively little interchain interaction, and hence the disorder-order equilibria for these derivatives would lie far to the left, and are not further disrupted by shearing forces. Derivatives 121a and 125a are slightly anomalous in that they have n values of ~0.9, and thus have a small shear rate dependence. A possible explanation is that these derivatives are capable of stronger

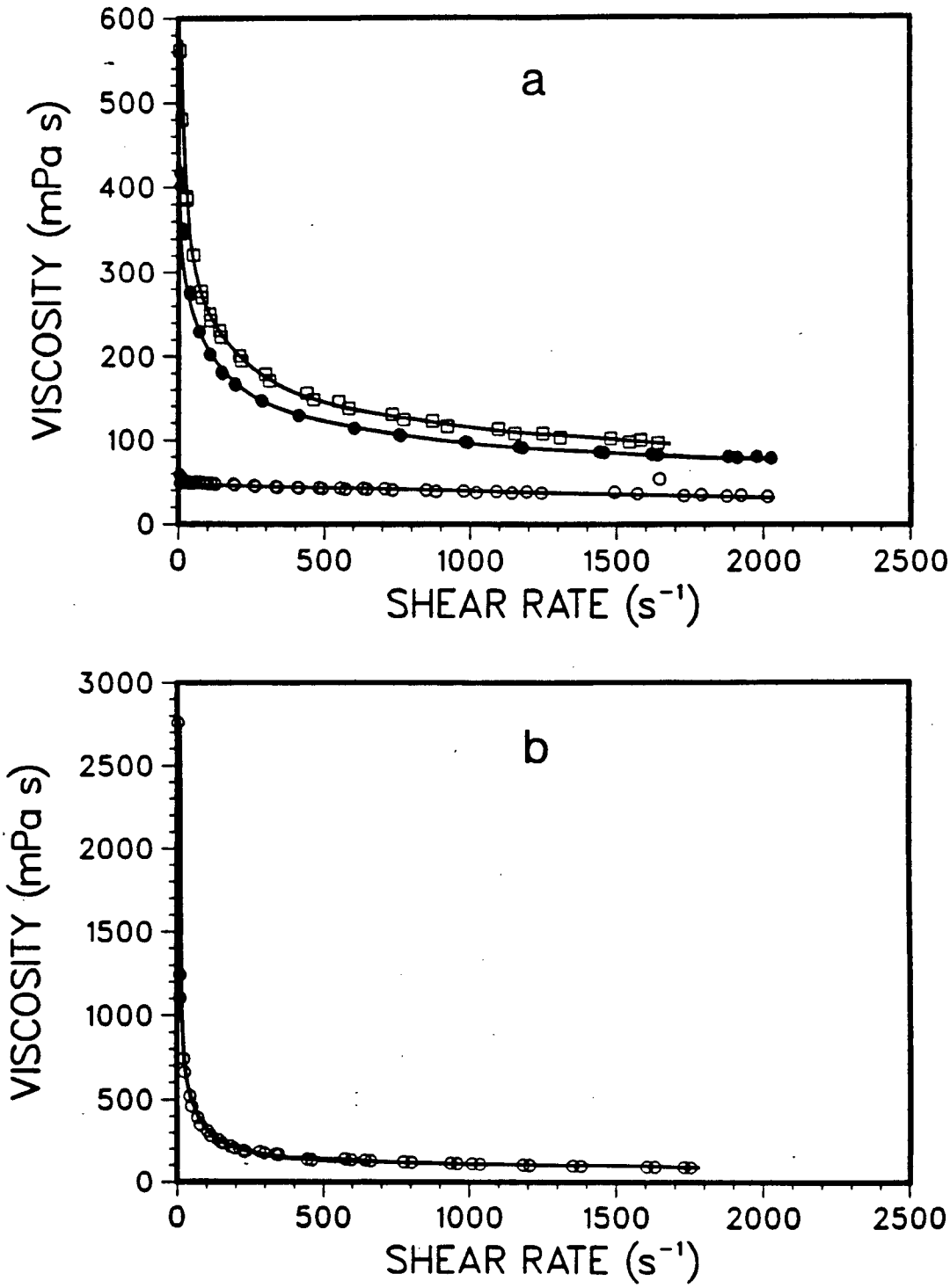


Figure 23. Rheograms for 2.0% aqueous solutions of derivatives a) 125a (○), 125b (●), 125c (□); and b) 125d (○) at 20°C, on linear axes.

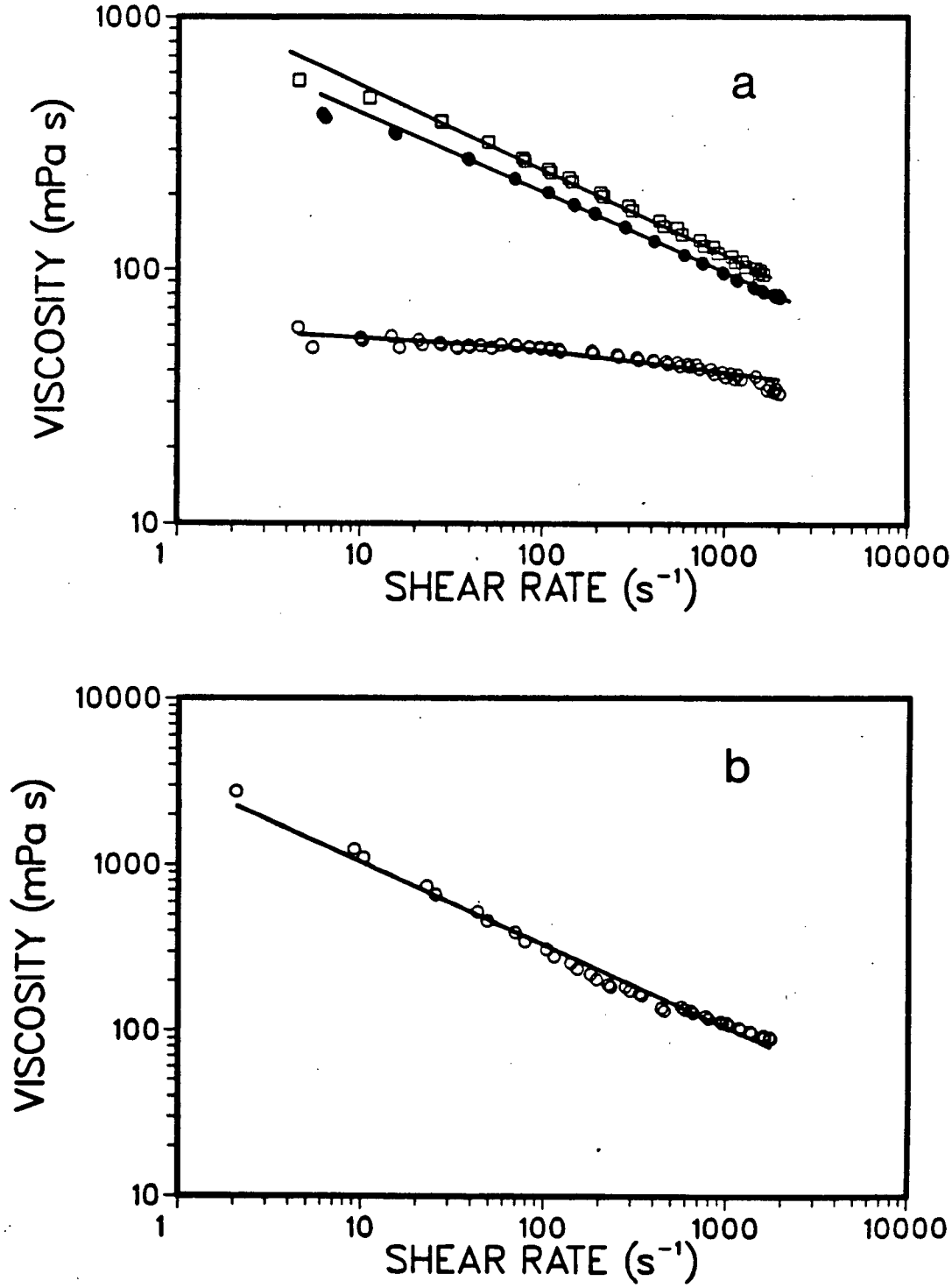


Figure 24. Rheograms for 2.0% aqueous solutions of products a) 125a (○), 125b (●), 125c (□); and b) 125d (○) on logarithmic axes.

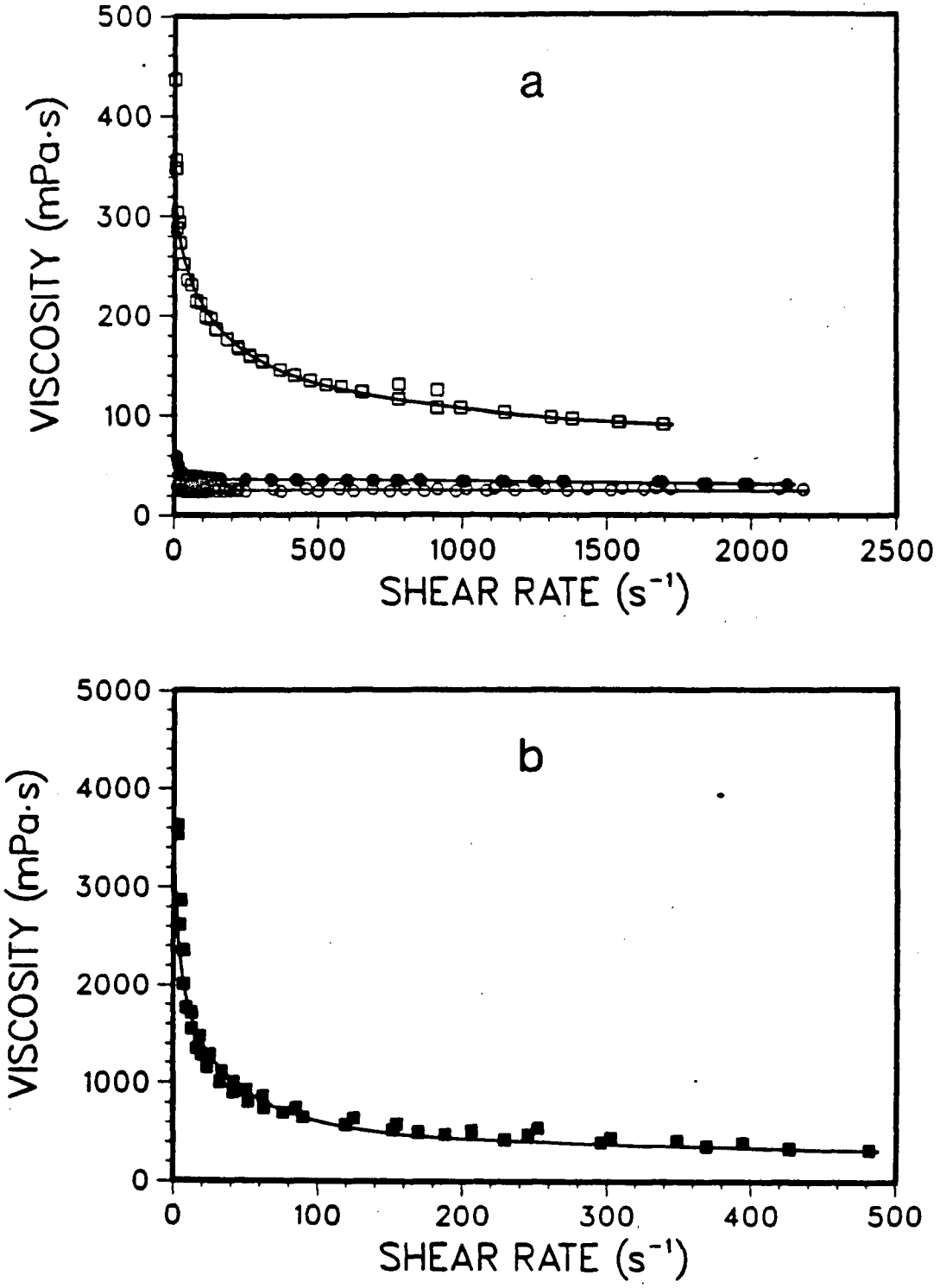


Figure 25. Rheograms for 2.0% aqueous solutions of derivatives a) 126a (○), 126b (●), 126d (□); and b) 126c (■) on linear axes.

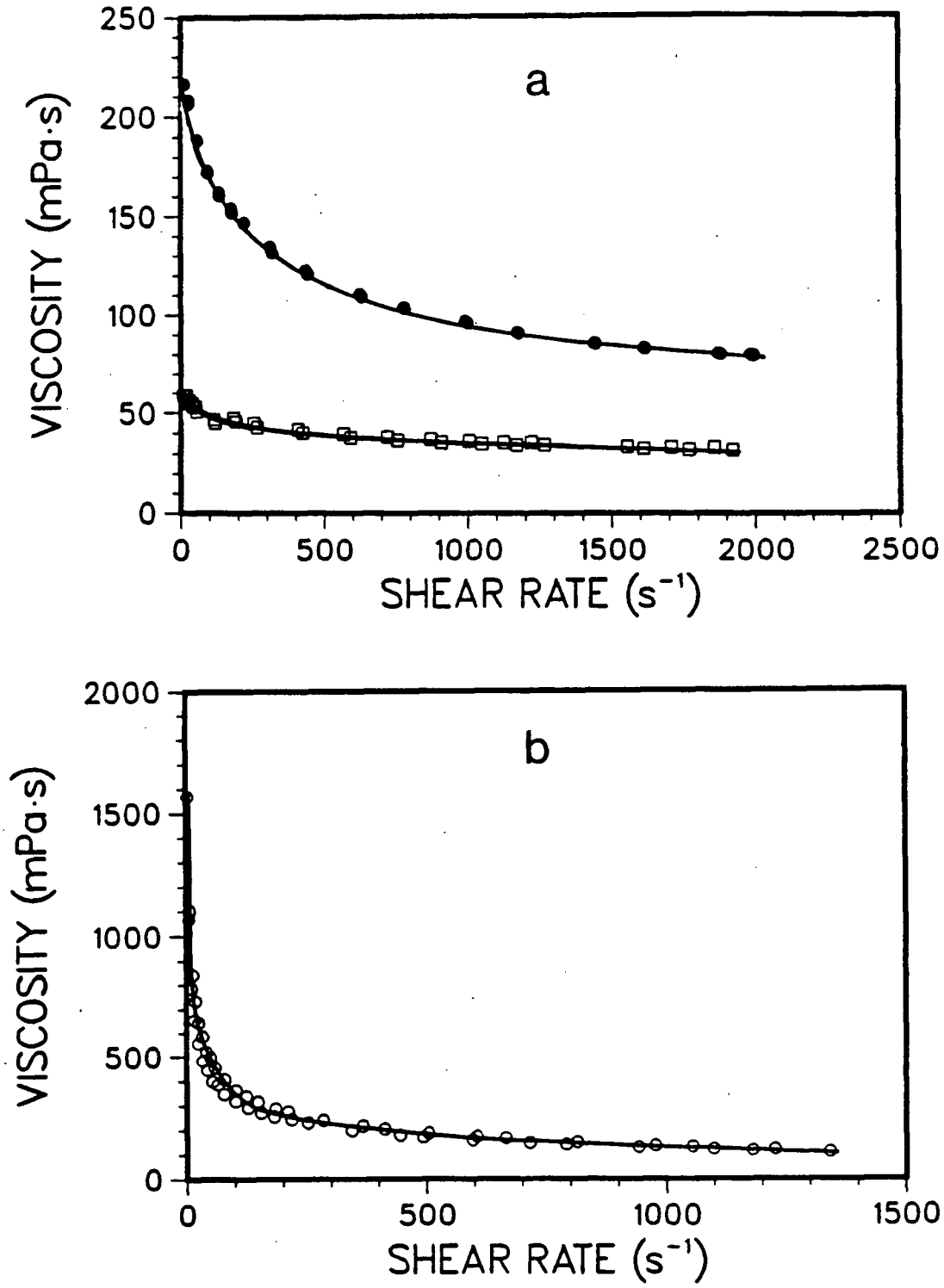


Figure 26. Rheograms for 2.0% aqueous solutions of derivatives a) 121a (□), 121b (●); and b) 121c (○) on linear axes.

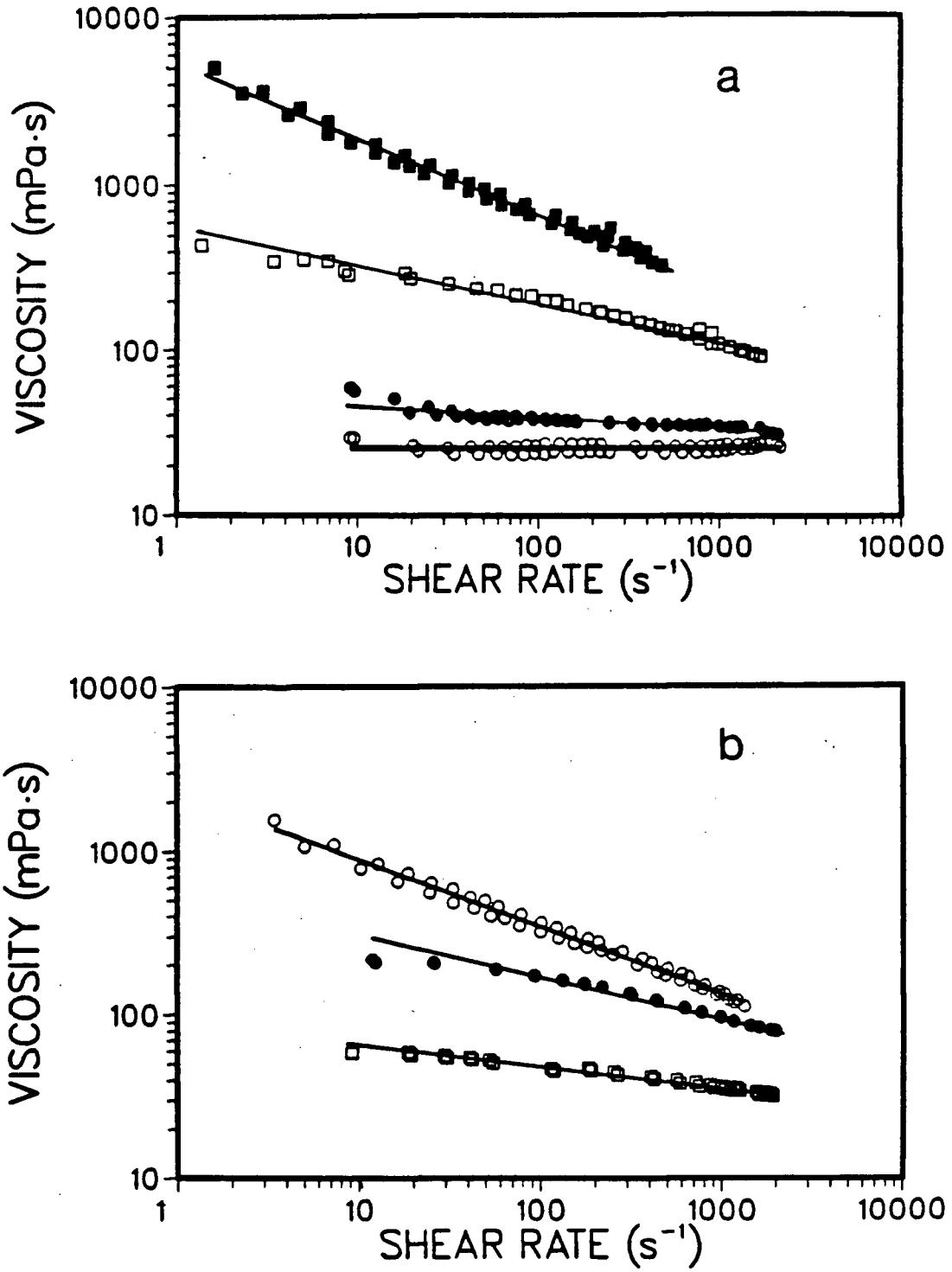


Figure 27. Rheograms for 2.0% aqueous solutions of compounds a) 126a (○), 126b (●), 126c (■) and 126d (□); and b) 121a (□), 121b (●) and 121c (○) on logarithmic coordinates.

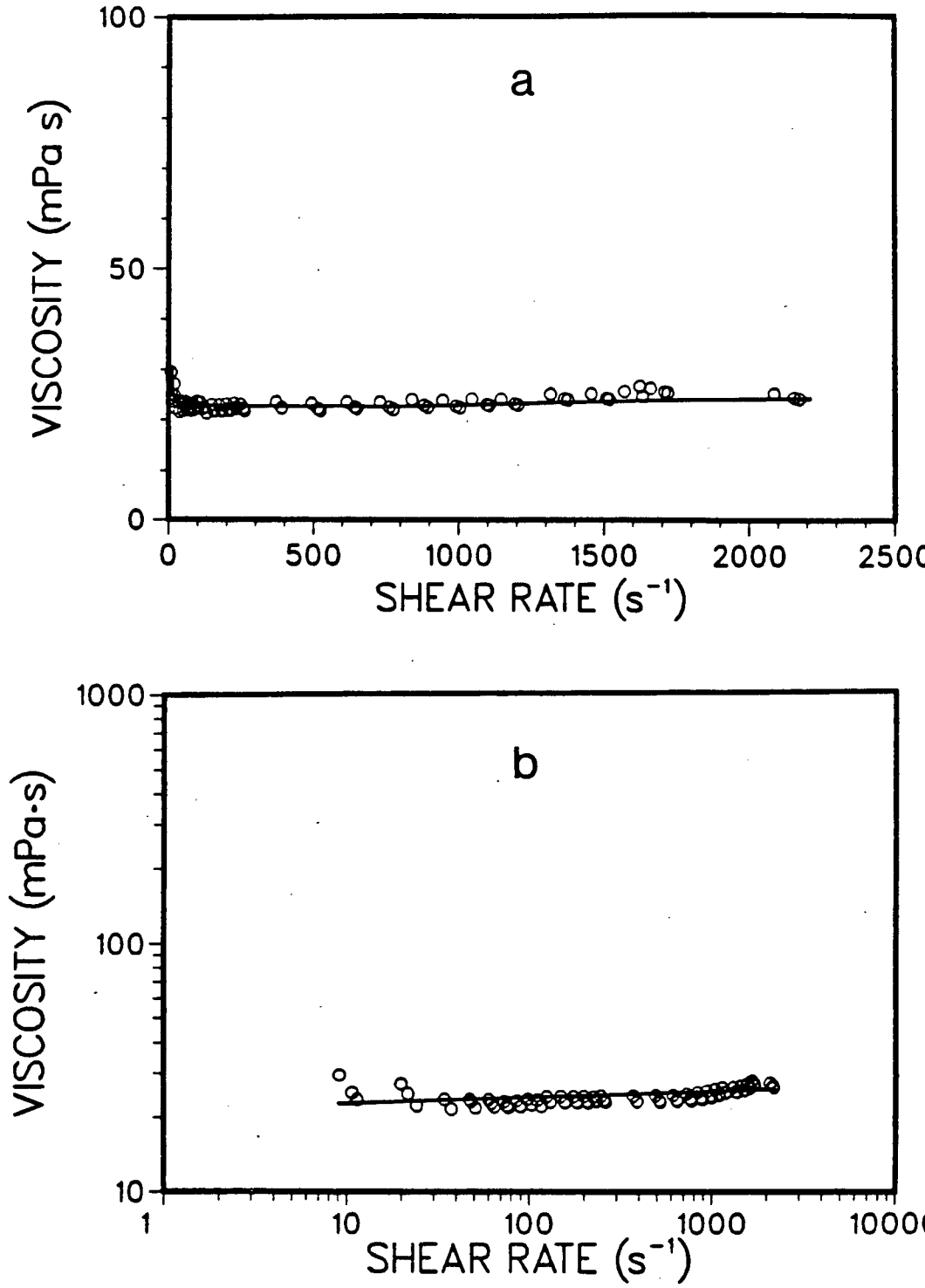


Figure 28. rheograms for 2.0% aqueous solutions of derivatives a) 120a (O) on linear axes; and b) 120a (O) on logarithmic axes.

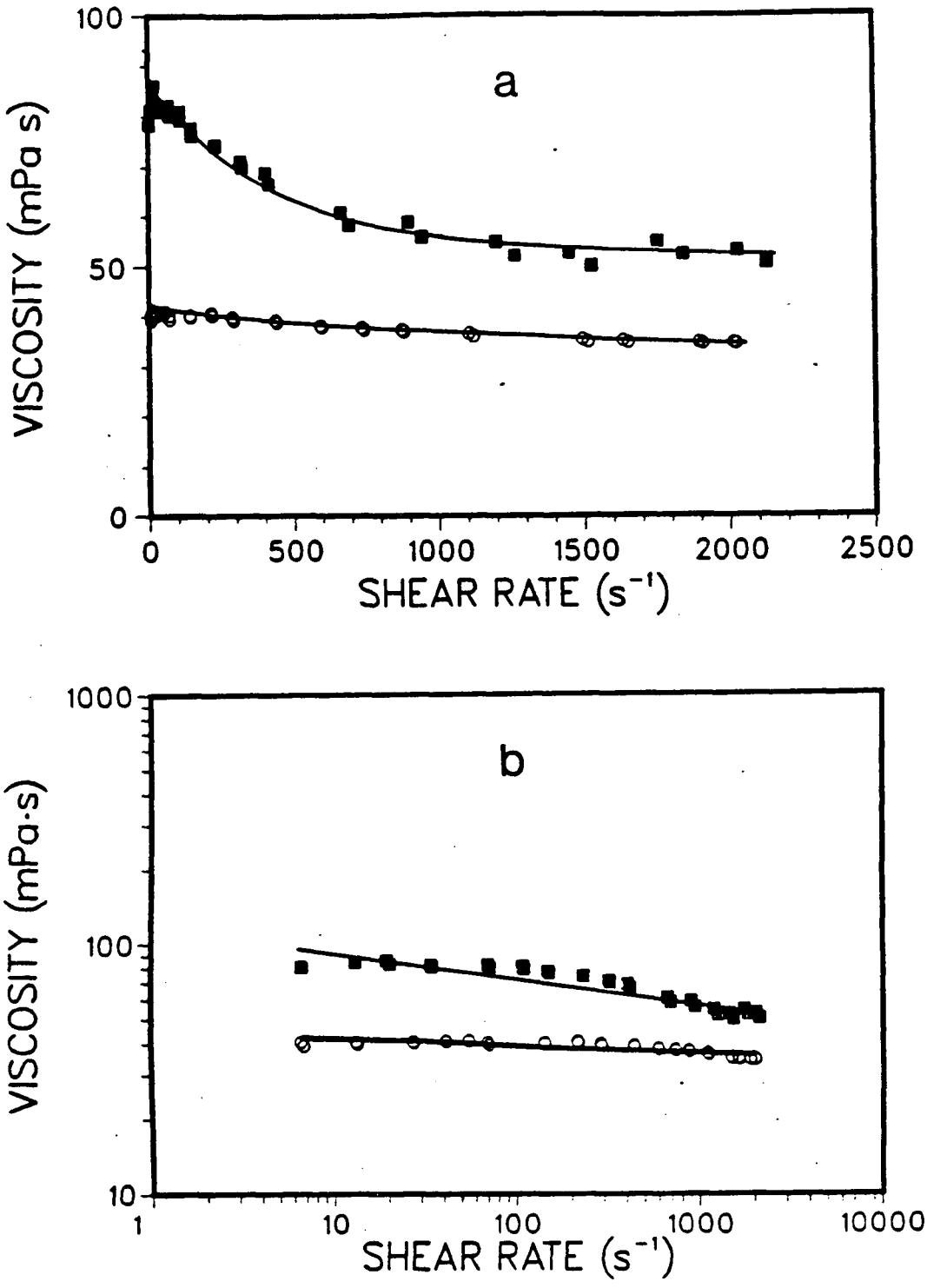


Figure 29. Rheograms for 2.0% aqueous solutions of a) 122a (○), 122b (■) on linear axes; and b) 122a (○) and 122b (■) on logarithmic coordinates.

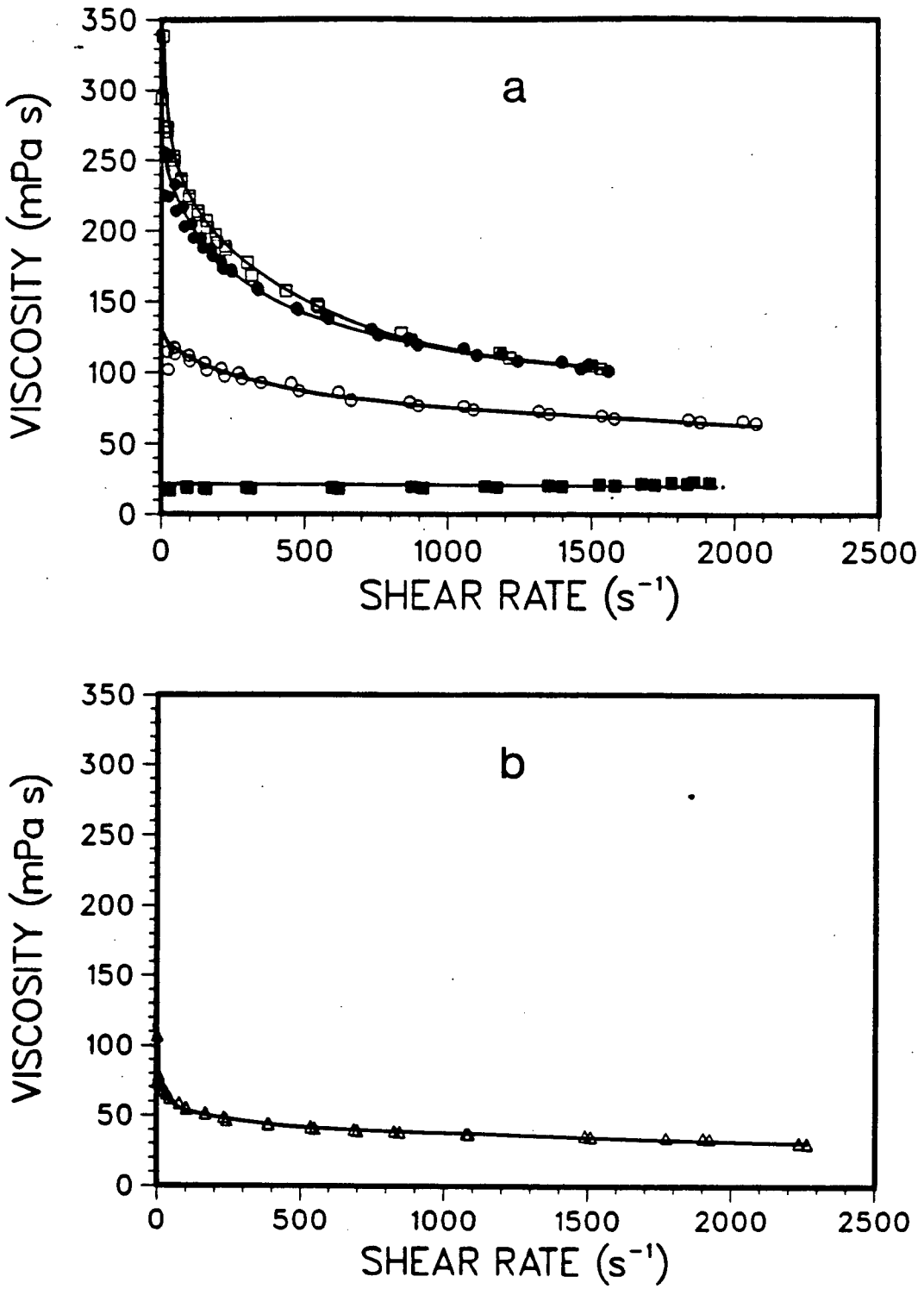


Figure 30. Rheograms for 2.0% aqueous solutions of derivatives a) 127a (■), 127b (○), 127c (□) and 127d (●); and b) 124a (△) at 20 C, on linear axes.

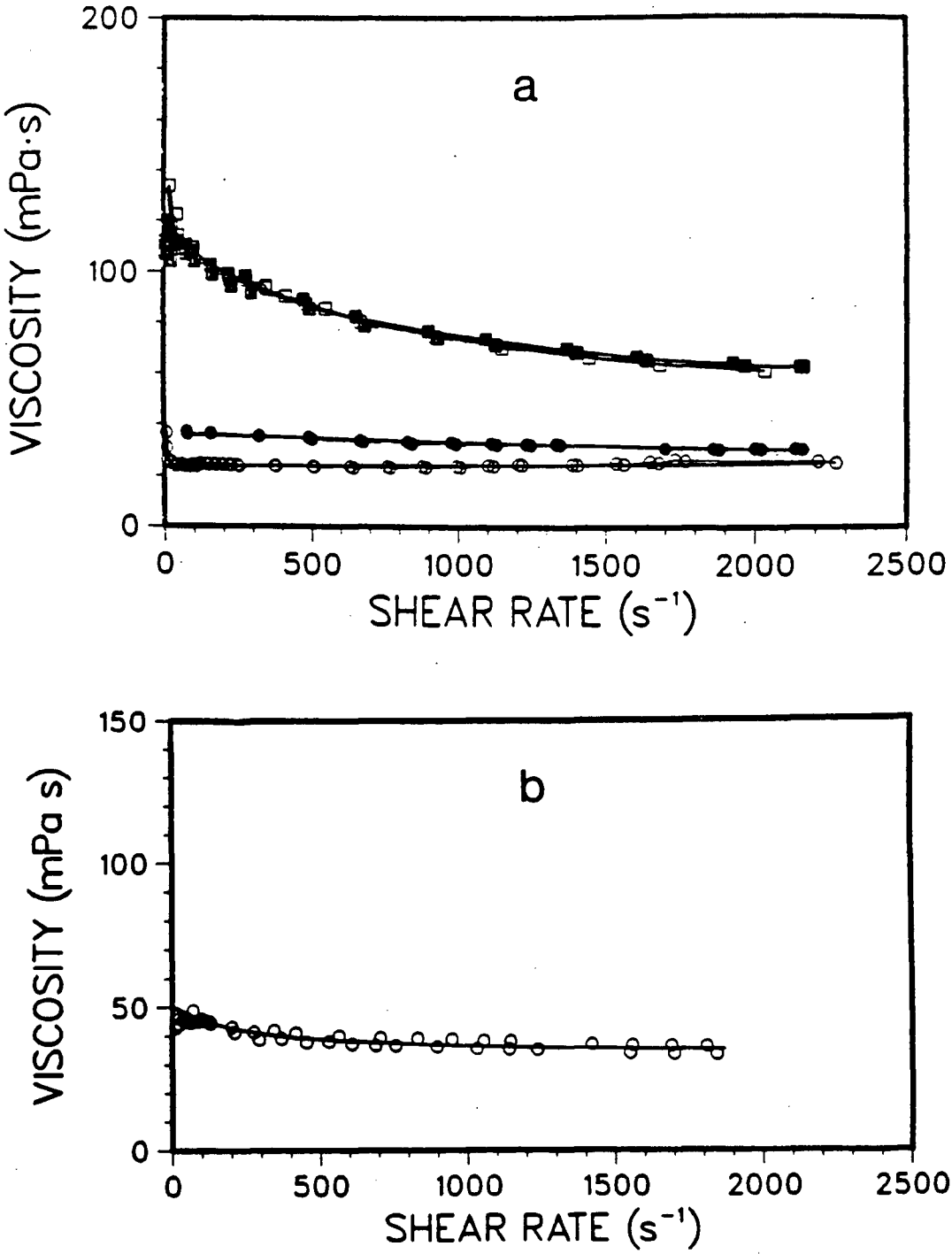


Figure 31. Rheograms for a) 2.0% aqueous solutions of 123a (○), 123b (●), 123c (■) and 123d (□); and b) 2.7% aqueous solution of 123a, at 20 C on linear axes.

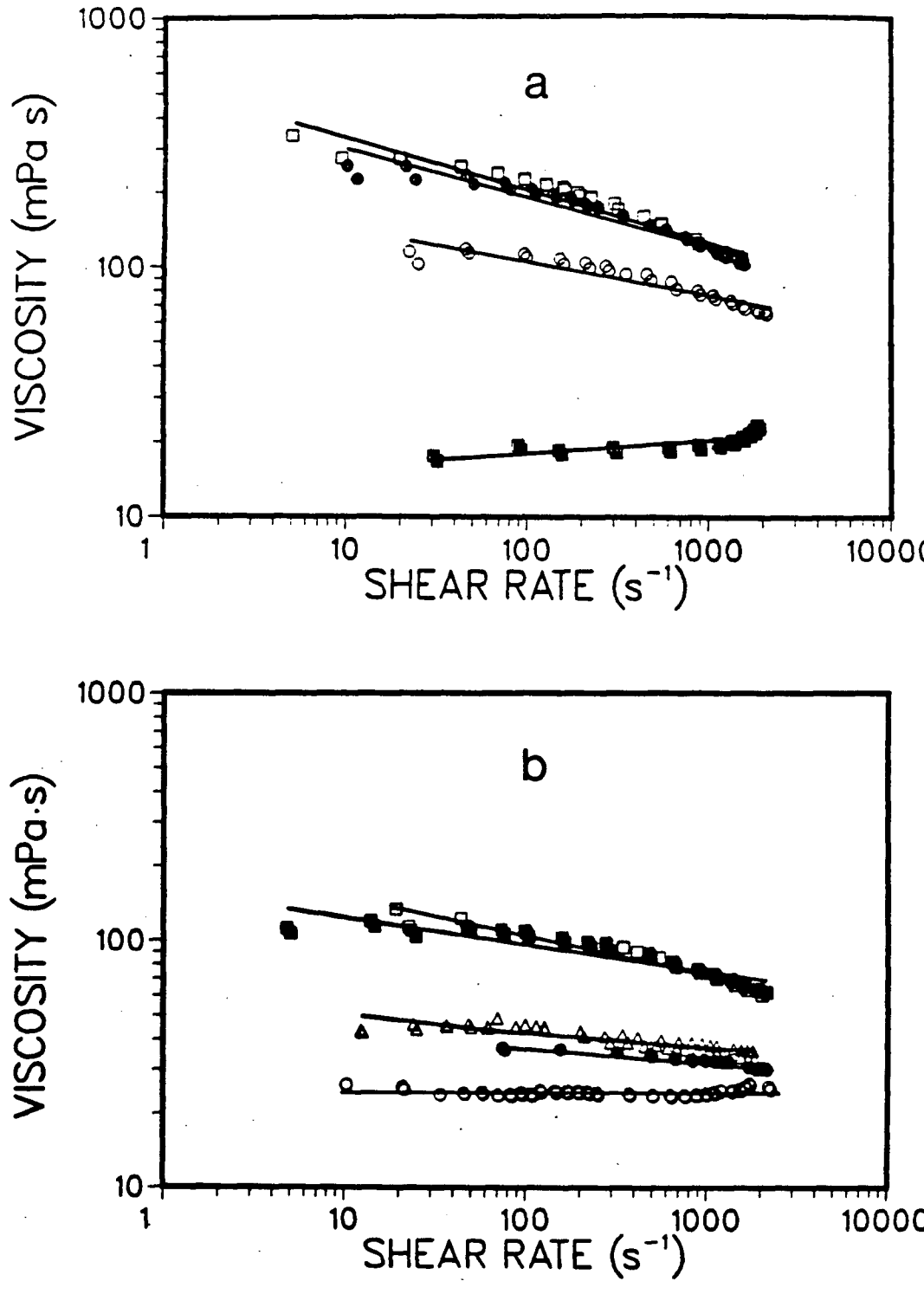


Figure 32. rheograms for 2.0% aqueous solutions of derivatives a) 124a (■), 127a (○), 127b (□), 127c (●); and b) 123a (○), 123b (⊙), 123c (■), 123d (□), and a 2.7% solution of 123a (△) on logarithmic coordinates.

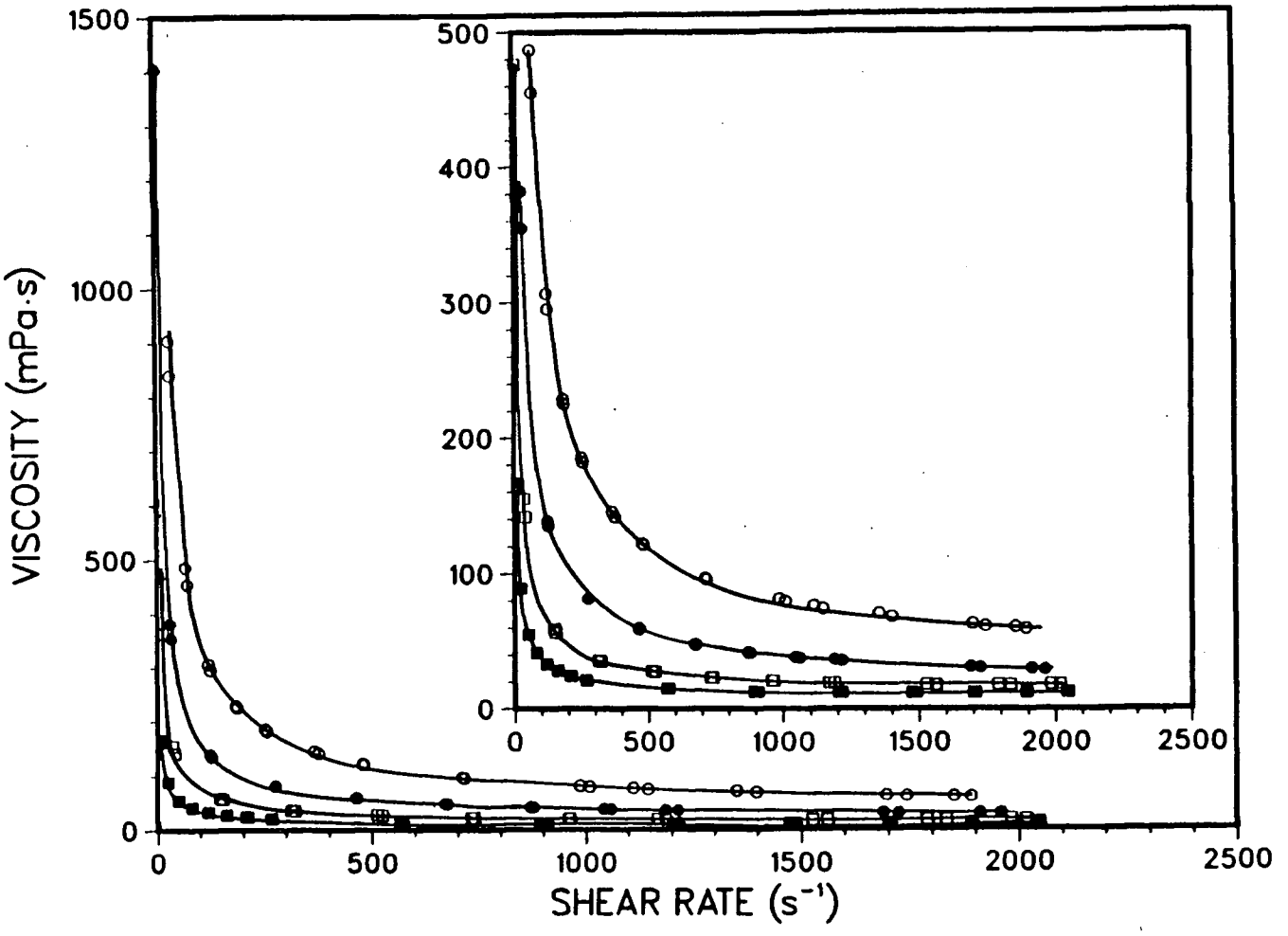


Figure 33. Rheograms for aqueous xanthan dispersions having concentrations of 0.25% (■), 0.5% (□), 1.0% (●), and 2.0% (○) on linear coordinates, at 20°C.

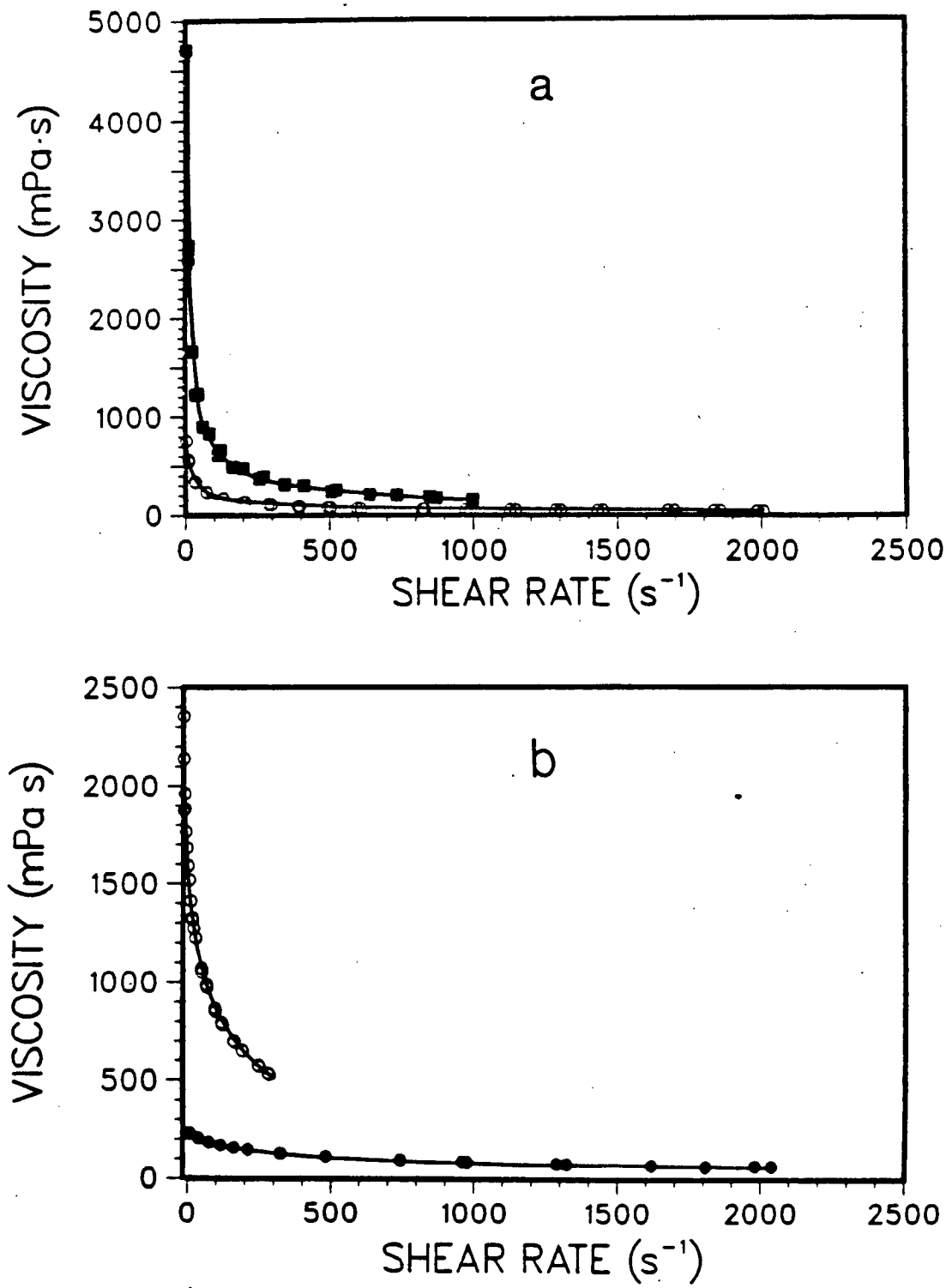


Figure 34. Rheograms for a) hydroxyethyl-cellulose solutions of 1.0% (○) and 2.0% (■) concentration; and b) sodium alginate solutions of 1.0% (●) and 2.0% (○) concentration, on arithmetic axes.

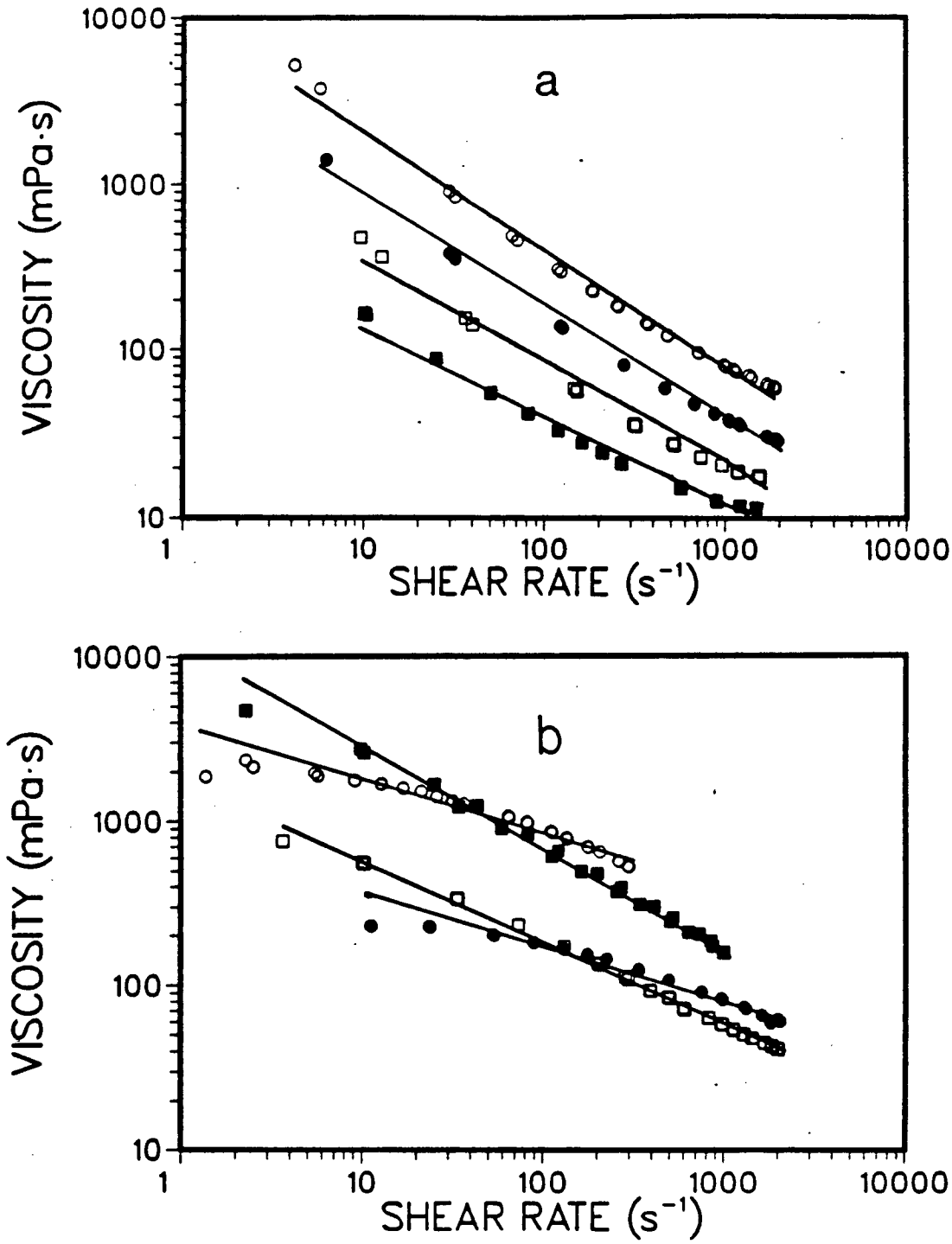


Figure 35. Rheograms on logarithmic axes for a) 0.25% (■), 0.5% (□), 1.0% (●) and 2.0% (○) xanthan gum dispersions; and b) 1.0% (□) and 2.0% (■) hydroxyethyl-cellulose and 1.0% (●) and 2.0% (○) sodium alginate solutions.

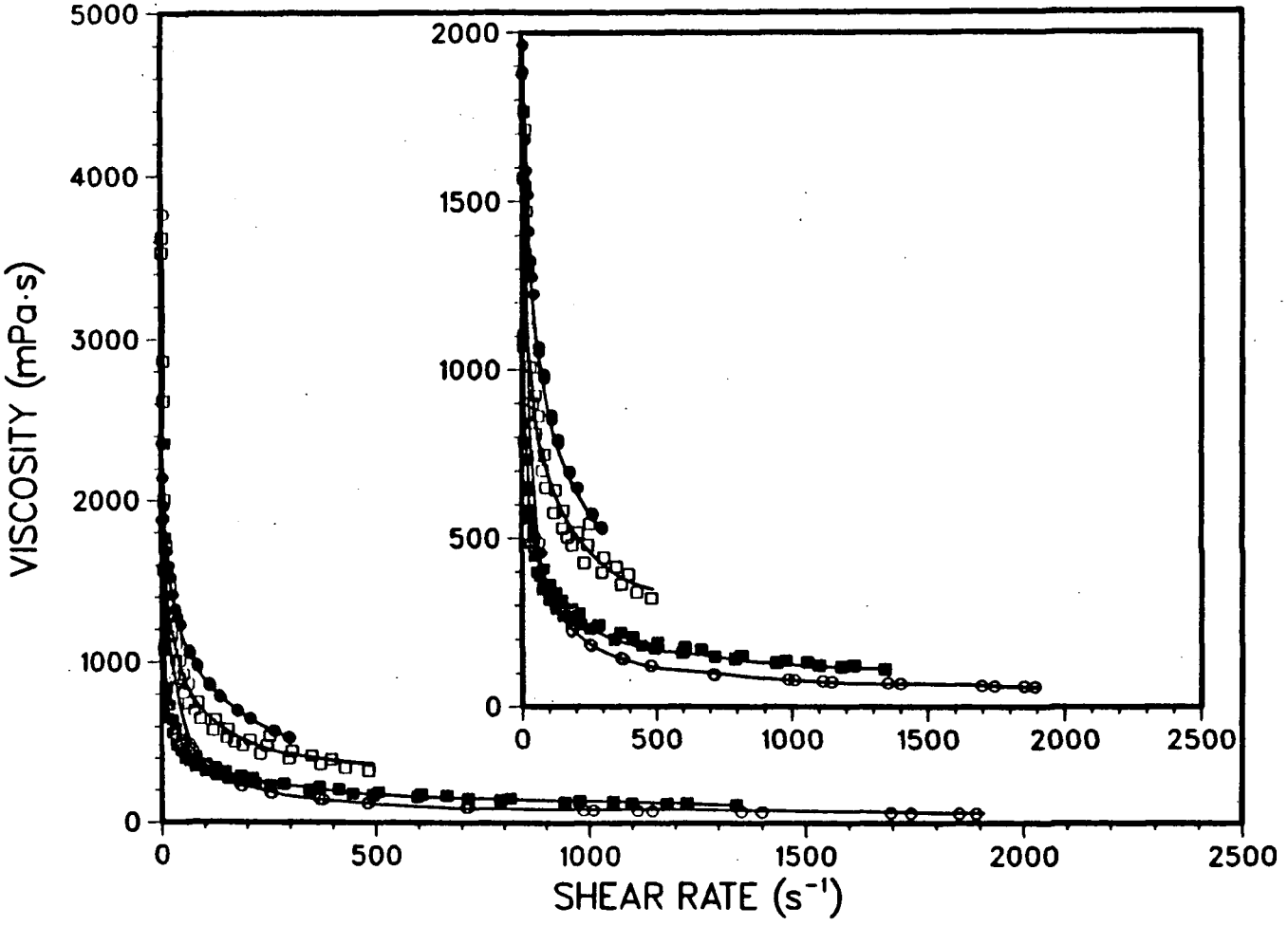


Figure 36. Rheograms comparing 2.0% (w/w) aqueous solutions of xanthan (○), sodium alginate (●), 121c (□), and 126c (■) on linear coordinates.

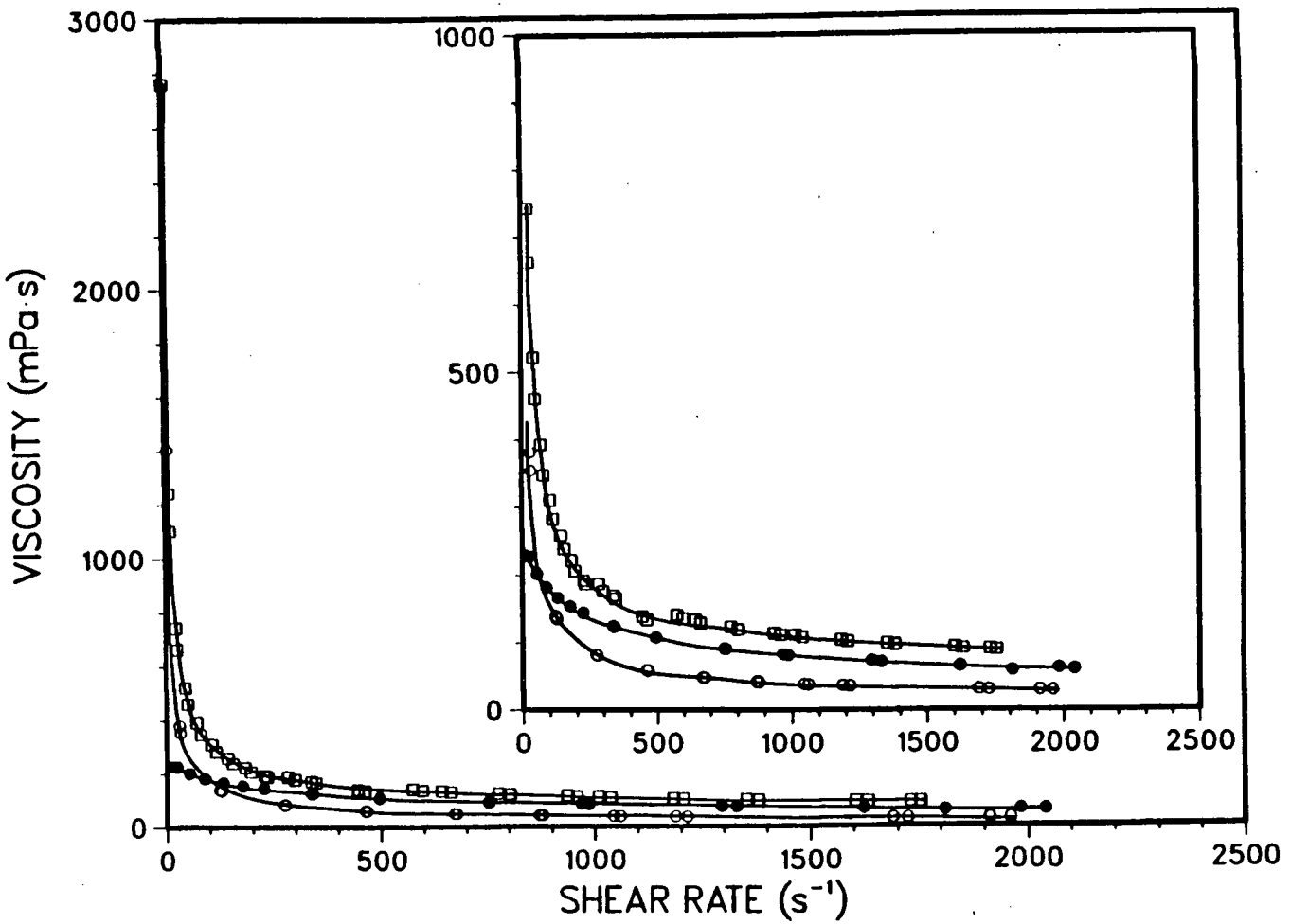


Figure 37. Rheograms for 1.0% aqueous solutions of xanthan (○), sodium alginate (●) and derivative 125d (□), on linear axes.

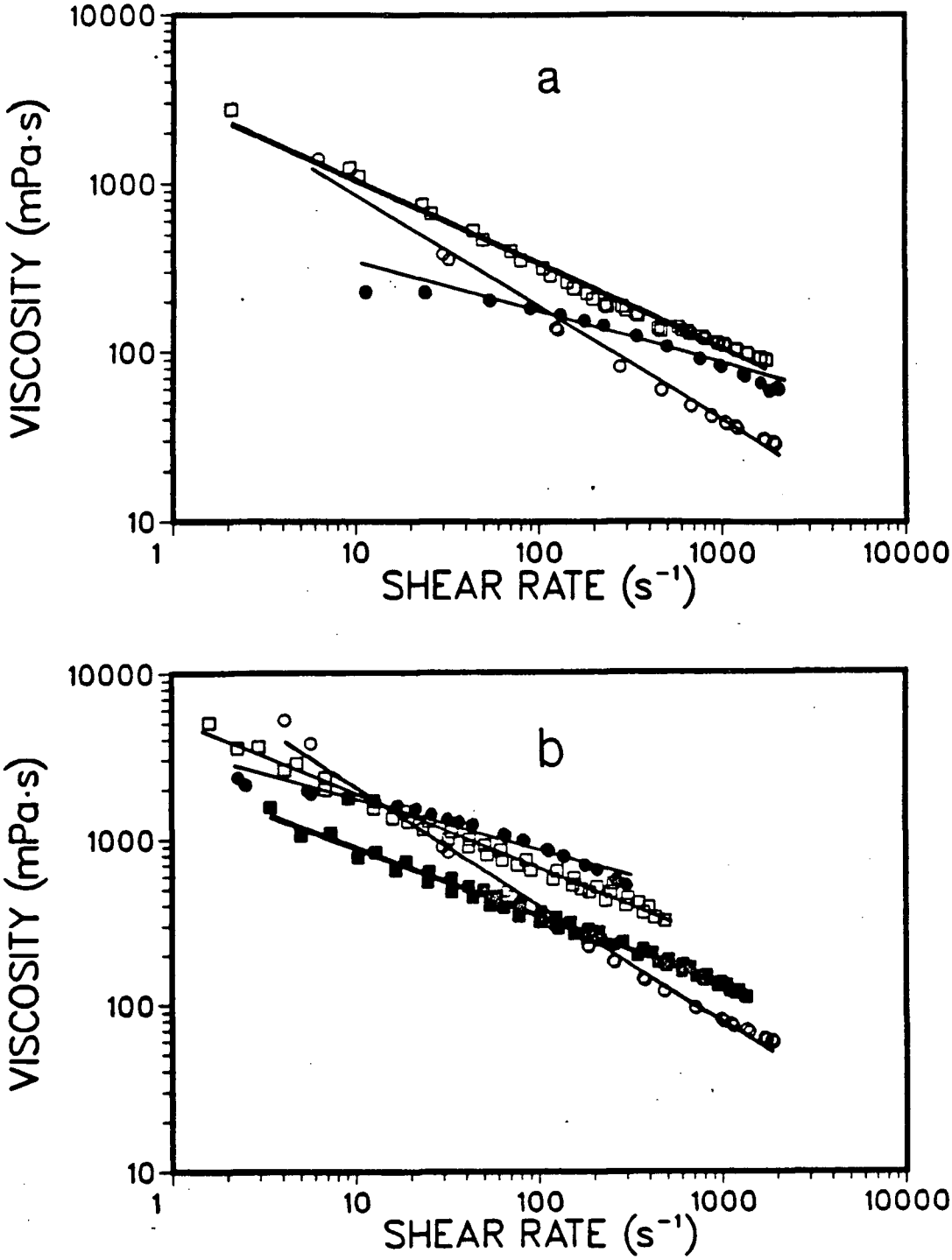


Figure 38. Rheograms on logarithmic coordinates for a) 1.0% aqueous solutions of xanthan gum (○), sodium alginate (●) and derivative 125d (□); and b) 2.0% solutions of xanthan gum (○), sodium alginate (●), 121c (□) and 126c (■).

self-association than the other highly substituted derivatives. However, despite these minor variations it is obvious that all solutions of highly substituted derivatives exhibit very little shear rate dependence. The m parameters for the high d.s. derivatives range from 14-80 mPa·s^n, compared to water with a viscosity of 1.0 mPa·s^1.

A related observation is the increase in m and decrease in n as the d.s. decreases for a series of derivatives containing the same branch. For example, of the β-galactosyl series 121a, 121b, and 121c (Fig. 27b), 121c having a d.s. 0.38 is considerably more viscous (m 2300 mPa·s^n) and pseudoplastic (n 0.588) than 121b (with m 434 mPa·s^n and n 0.783). A similar trend is seen in the α-D-glucosyl (Fig. 24), β-D-lactosyl (Fig. 32b), α-D-2-acetamido-2-deoxy-glucosyl (Fig. 32a) and α-D-galactosyl (Fig. 27a) series. In the lactosyl series, 123a-d, the magnitude of the change is considerably less, both in terms of viscosity and pseudoplasticity. This is undoubtedly a result of the larger subsitutent being unable to form interchain interactions at relatively lower levels of substitution than a monosaccharide branch. All of these observations indicate that samples with low d.s. are more capable of interchain associations. Two possible mechanisms for this are: (1) interdigitization of chains, as depicted in Fig.39, and (2) occurrence of interchain interactions analogous to those present in native chitosan.[23] For the latter case, a particular conformational state might provide "open" and "branched" faces, much like those proposed in seed

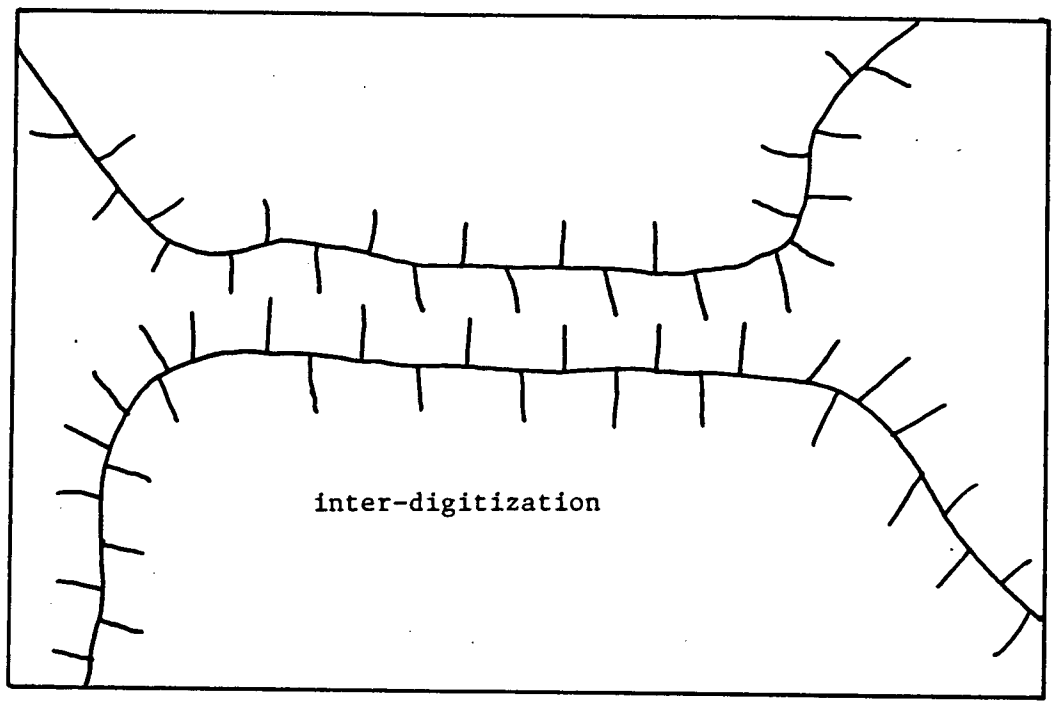


Figure 39. Idealized schematic of the interactions of branched chitosan derivatives in aqueous solution.

galactomannan self-associations.[17,201] The "open" faces could then interact, much as in chitosan, with regions of disruption imparting overall solubility (Fig. 40). The latter model may provide an explanation for the apparently anomalous result for derivative 126c, which has a d.s. of 0.48 and is considerably more viscous than the analogous derivative 126d, which has d.s. 0.32. Assuming that interactions occur as

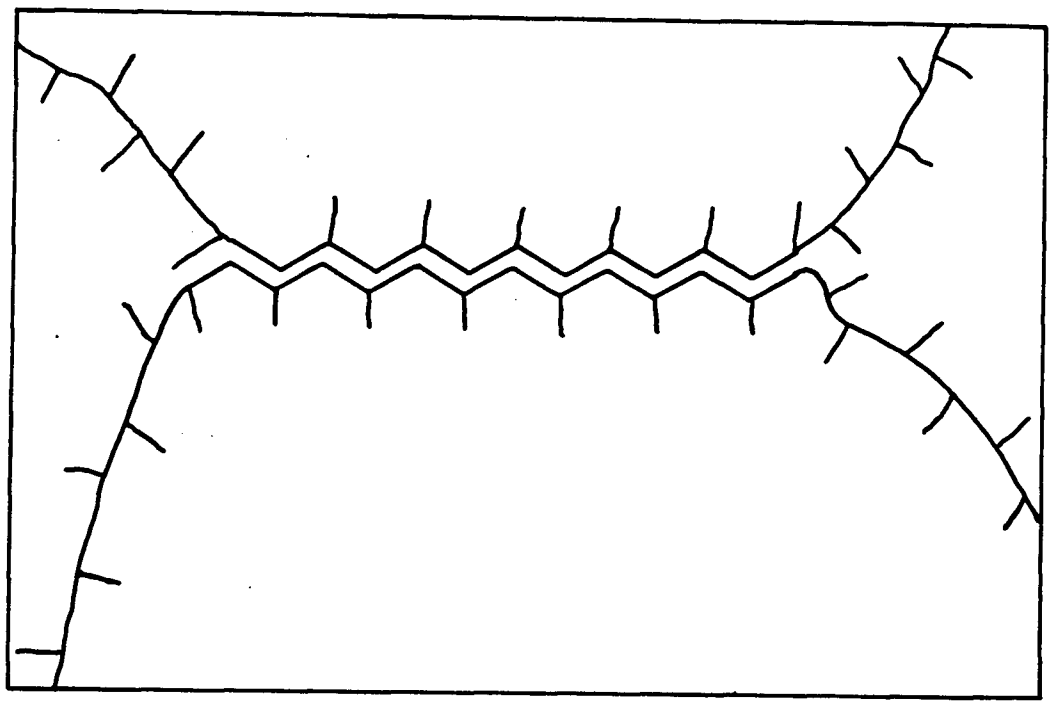


Figure 40. Idealized schematic of interactions in branched chitosan self-association, in which backbone-backbone interaction, between unsubstituted or conformationally accessible chain segment, occurs.

depicted in Fig. 40, it is reasonable that an optimum degree of substitution, and substitution pattern, will exist for maximum interchain association. Indeed, recent studies have shown that both substitution pattern and degree of branching greatly influence galactomannan interactions.[200,209] Thus, perhaps for this system a random substitution of ~50% is near optimum and viscous properties decrease on either side of this value. Of course, it is likely that viscosity and

pseudoplasticity would increase again at quite low d.s. values, as larger blocks of unsubstituted backbone could adopt chitosan-like interactions, eventually resulting in gels and insolubilization. Derivatives 123e and 125d, having d.s. < 0.3 and giving a gel and a very viscous solution respectively, provide some support for this proposal. Fig. 41 shows the experimental data plotted as n vs d.s., for neutral monosaccharide branched derivatives, and the dotted line

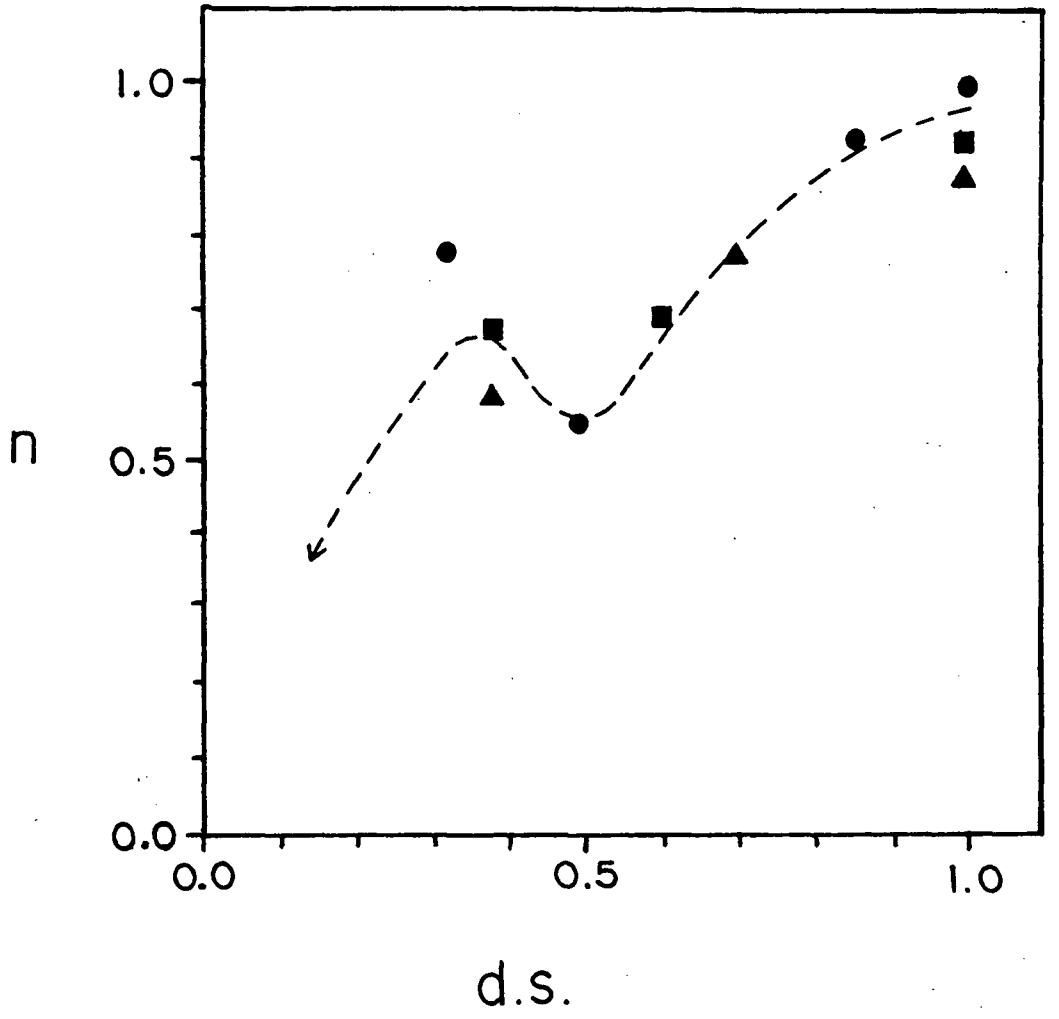


Figure 41. The consistency coefficients (n) of the neutral monosaccharide branch derivatives, 121a-c (▲), 125a-d (■), and 126a-d (●) plotted against degree of substitution (d.s.), and a curve (– –) representing the postulated relationship between n and d.s.

represents an idealized relationship as just put forth. Much of the reasoning behind this postulation has been used in explaining the behaviour of galactomannans such as locust bean and guar gum, and is thus not completely without precedent.[17,200,201,209] Also, it might be expected that derivatives of the sort prepared here would have behaviour most similar to that of natural branched polysaccharides bearing single pendant residues.

It must be remembered that, although 2.0% (w/w) solutions were used for all derivatives throughout these studies, the molar concentration of the solutions varied with the degree of substitution of the sample. Table 6 contains some calculated average molecular weights for hypothetical derivatives bearing neutral monosaccharide branches at a variety of d.s. values. It can be seen that a fully substituted derivative at

d.s.	MW	molar ratio	equimolar conc.(%)
0.2	202.2	1.82	3.64
0.4	243.4	1.51	3.02
0.6	284.6	1.29	2.58
0.8	325.8	1.13	2.26
1.0	367.0	1.00	2.00

Table 6. Variation of molar concentration of 2.0% solutions with d.s., for N-[2-O-(glycopyranosyl)ethyl]chitosan derivatives.

2.0% concentration will have fewer polymer chains (67%) than a derivative having d.s. of 0.4. However, it is believed that

these concentration discrepancies could not account for the changes in rheology that were observed. Tests done on a 2.7% solution of the lactosyl derivative 123a having d.s. 0.9, showed that the 2.7% solution did not vary substantially in rheology from its 2.0% solution, and did not match the 2.0% solution of 123b having a molar concentration ~20% lower (Fig.32b).

In order to investigate the role of functional groups in the interactions of the branched chitosan derivatives described here, polymers bearing pendant α and β-acetamido-D-glucose and β-D-glucuronate residues were prepared. The solution properties of 127c-e having acetamido-α-glucose branches, are somewhat anomalous from the rest of the monosaccharide branched compounds. Even at low d.s. values (0.19 and 0.17 respectively for 127d and 127e) the solutions are not very pseudoplastic. It was earlier mentioned that the coupling efficiencies in the preparation of these compounds were poor, yielding low d.s. at relatively high molar ratios, probably due to steric or electrostatic forces. This may also be the reason for low pseudoplasticity, since as described for the lactosyl derivatives, a bulkier substituent would likely interfere in the interchain association. The derivative 124a, containing the β-D-acetamido-glucose moiety also seems to fit this trend. However, if the sugar size is influencing both the reaction efficiency and resultant solution properties, it seems somewhat surprising that at a mole ratio of 3.0, fully substituted derivatives 124a, 127a

and 127b were easily obtained. Another interesting result was obtained for 127a in the acetamido-glucose series. Surprisingly, this fully substituted derivative did not dissolve in water or aqueous acetic acid, but formed a clear rigid gel. This was anomalous when compared to 124a, the fully substituted β-analogue which was soluble in water. The ^{13}C-nmr spectrum showed however, that 127a actually contained both α and β-2-acetamido-2-deoxy-D-glucose residues in a 7:1 ratio. When derivative 127b, having only α-acetamido-glucose branches was prepared, it was also found to be soluble in water. Thus, in contrast to 127b and 124a containing pure and β-acetamido-glucose substituents respectively, 127a gave a gel, seemingly due to the presence of both the α and β isomers as co-branches.

The N-deacetylation of 127b having 2-acetamido-2-deoxy-α-glucose branches with d.s. 1.0, was undertaken in order to provide the free amino derivative 129 (Eq. 24). It is somewhat inappropriate to directly compare the solution propeties of 129 to the other derivatives because of the extra chemical, and potentially degradative, treatment required for its preparation. Since degradation would result in reduced viscosity, the increased viscosity of 129 in relation to 124a and 127c indicates that the free amino group is involved in stronger interactions than its acetylated analogue. If any depolymerization did occur, it is masked by the increased viscosity due to self-association.

The derivatives 122a and 122b containing pendant

β-D-glucuronate residues, had some interesting features. As mentioned previously the acidic forms of these derivatives were water-insoluble. The sodium salts were readily soluble and gave solutions having relatively low viscosity and little pseudoplastic character. Derivatives of this variety have some potential; it is apparent that, in the acidic form, they have increased solubility at low d.s., while for the sodium salt, viscosity increased with lower d.s. It was believed that derivatives bearing pendant uronic acid residues might be useful as metal chelates. It was found with 122a and 122b that the addition of varying amounts of Ca^{+2} and Cu^{+2} ions had no apparent effect on solution properties. In hindsight however, it seems possible that the analogous derivatives bearing the galacturonate residue, rather than glucuronate, might have more success in this regard. This postulation is based on the proven ability of galacturonate residues (in pectins) to chelate calcium. An attempt to prepare a derivative having galacturonic acid branches was undertaken, based on the galactose oxidase oxidation followed by treatment with aqueous bromine, as described for the synthesis of 26 (section 1.2.2). The oxidation appeared to proceed as expected and afforded a solid precipitate. Unfortunately, after direct treatment with an aqueous bromine solution, and subsequent dialysis, very little material was recovered and a ^{13}C-nmr spectrum was inconclusive. Presumably, hydrolysis occurred due to improper buffering of the bromine reaction and the product dialysed out. Further attempts at this reaction

were not undertaken, although it is still believed that this sequence offers an attractive means for generating galacturonosyl branches.

As a final point, the rheograms of commercial samples of xanthan (Fig. 35a), hydroxyethylcellulose (Fig. 35b) and sodium alginate (Fig. 35b) are provided for contrast. Fig. 38a directly contrasts 2.0% solutions of 121c and 126c with xanthan and sodium alginate, while Fig. 38b compares 1.0% solutions of 125d, xanthan and sodium alginate. It can be seen that the derivatives described here exhibit flow behaviour similar to xanthan and hydroxyethylcellulose, although they are somewhat less viscous and less pseudoplastic. The sodium alginate solutions however can be seen to be quite different in their behaviour, being considerably less pseudoplastic over the shear rate range studied.

Intrinsic viscosities for derivatives 126a, 126b, 126d, 125a, 120a and 122a were determined in order to further probe the behaviour of these derivatives in solution, and the results are presented in Table 7. Looking at the intrinsic viscosities for 126a,b and d, there is again an increasing trend with decreasing d.s. This alludes to a relationship between conformation and d.s. that is consistent with the proposed mechanism for self-association given in Fig. 40. In other words, there is an apparent conformationally related change in the polysaccharide derivatives at lower d.s. values. It then becomes possible, at certain d.s. values, for a maximal inter-chain association to occur, causing increased

Derivative	Branch	d.s. (±.05)	$[\eta]$ ($dL \cdot g^{-1}$)	R^2	#Points
120a	β-Glc	1.00	0.487	.968	8
122a	β-GlcA	1.00	2.63	.998	10
125a	α-Glc	1.00	1.46	.947	8
126a	α-Gal	1.00	0.725	.833	8
b		0.86	2.39	.931	8
d		0.32	5.41	.944	10

Table 7. Intrinsic viscosities for selected derivatives, determined according to the Kraemer relationship (Eq. 7).

viscosity and pseudo-plasticity. The intrinsic viscosities of the fully substituted derivatives 120a and 125a, bearing β and α-glucosyl sugars respectively, are ~.5 and 1.5 ($dL \cdot g^{-1}$). This discrepancy could be an indication of greater molecular order in the case of 125a. The fact that 125a had lower n (0.929) and m values (65.1 $mPa \cdot s^n$) than 120a (n 1.01 and m 22.4) at 2.0% concentration supports this observation. It has already been mentioned that the highly substituted derivatives such as 120a and 125a have little shear rate dependence and are sterically resistant to self-association. Therefore differences in viscosity, even at high (2.0%) concentrations, are probably a reflection of the shape of the polymer molecule itself. The intrinsic viscosity values for 120a and 125a are then the first clue to conformational differences induced by altering the glycosidic

configuration of the branch. The effect on solution properties is not particularly large in magnitude, and the similarity in trends for lower d.s. samples for all series indicate that other associative forces come into play at 2.0% concentration which dominate the resultant properties.

2.2.3 Synergistic Interaction

The increasingly apparent similarity between the N-2'-(glycopyranosyl)ethyl chitosan derivatives and the well studied seed galactomannans, led us to consider using these derivatives to probe synergistic interactions.[213,214] As such, mixtures of xanthan with derivatives 121a, 121b, 121c, 126c, 126d, 125c, and 123b were prepared, such that upon addition of water the resultant solutions contained 0.25% xanthan, and 0.25% of the derivative, with an overall polysaccharide concentration of 0.50%, as described in Table 8.

Derivative	Branch	d.s. (±.05)	Observations
121a	β-Gal	1.00	S,V
b		0.70	S,V
c		0.38	G,B,X
123b	β-Lact	0.76	G,F,X
125c	α-Glc	0.38	G,B,X
126c	α-Gal	0.48	G,B,X
d		0.32	G,B,X

Table 8. Qualitative observations from synergistic mixtures containing 0.25% xanthan and 0.25% of the respective derivative. Code; B, beads; F, fibrous; G, gelatinous; S, solution; V, viscous; X, excluded solvent.

The interactions between xanthan and derivatives 121c, 126c, 126d and 125 resulted in the formation of gelatinous globules which excluded solvent. With 123b, an opaque gelatinous precipitate was obtained while 121a and 121b provided viscous solutions. The rheometric evaluations of these synergistic solutions were compared to those of 0.25% and 0.50% (w/w) xanthan solutions and to a known synergistic mixture containing 0.25% xanthan and 0.25% locust bean gum (Fig. 42 and 43). The power-law parameters obtained for these five solutions are given in Table 9. Interestingly, the mixture of 121b and xanthan was more viscous (m 1780 mPa s) and slightly less pseudoplastic than the 0.50% solution of xanthan (m 1400 mPa s). The interaction of 121a with xanthan produced a less viscous solution, which still had viscosity and pseudoplasticity greater than 0.25% xanthan. Thus, in the first case,

Sample	n ∓ 3%	m (mPa·s) ∓ 3%	R^2	#Points
xan + 121a	0.478	605	.974	30
xan + 121b	0.409	1780	.986	24
xan + LBG	0.402	1670	.965	23
xan(0.5%)	0.397	1400	.946	22
xan(0.25%)	0.518	378	.968	27

Table 9. Power-law parameters obtained from rheological tests on the synergistic mixtures of 0.25% xanthan and 0.25% derivative, at 20°C. Parameters for a xanthan-locust bean gum mixture and 0.50 and 0.25% xanthan solutions are provided for comparison.

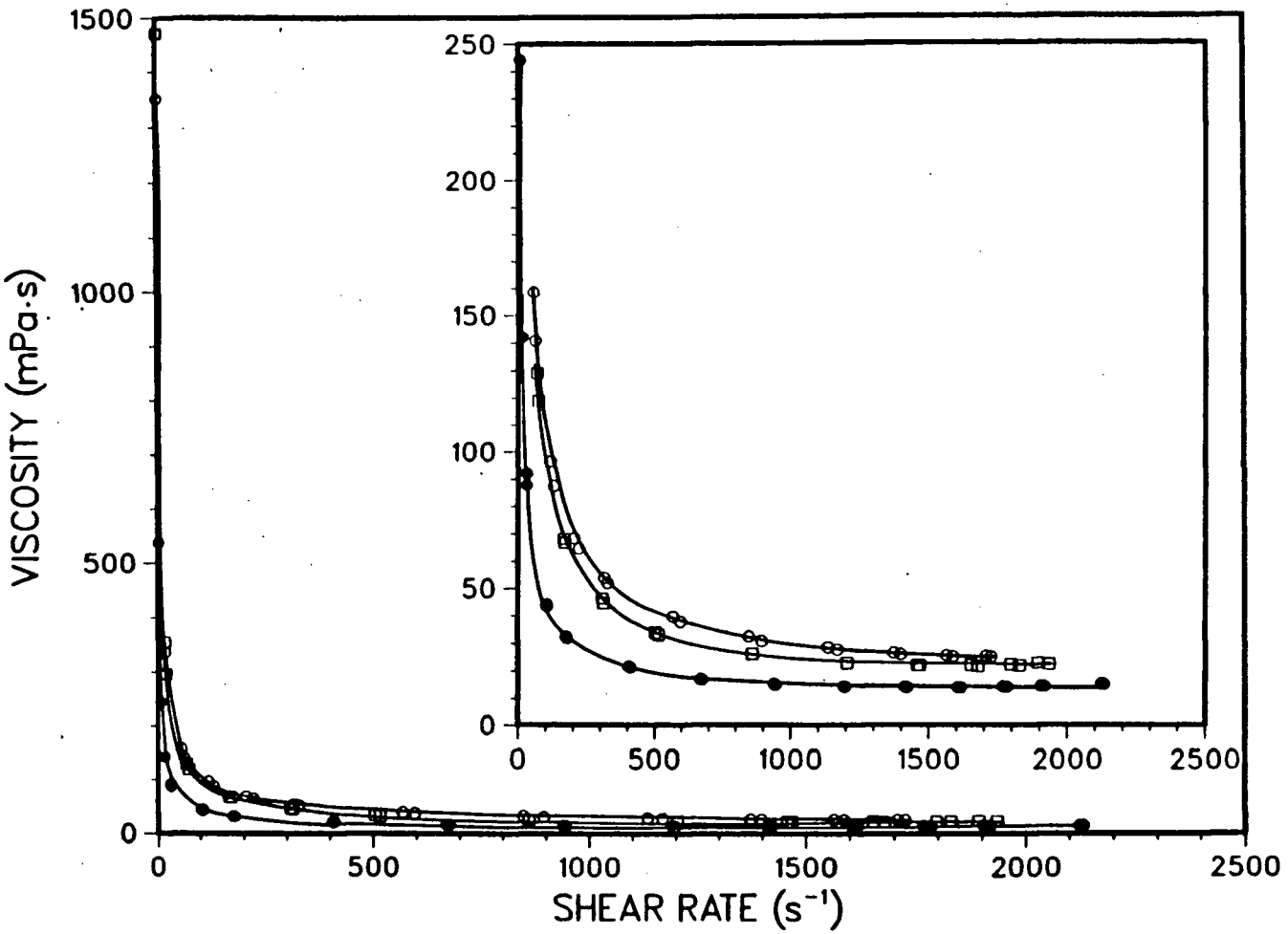


Figure 42. Rheograms for synergistic mixtures containing 0.25% (w/w) xanthan gum and 0.25% 121a (●), 0.25% 121b (○), or 0.25% locust bean gum (□), on linear axes.

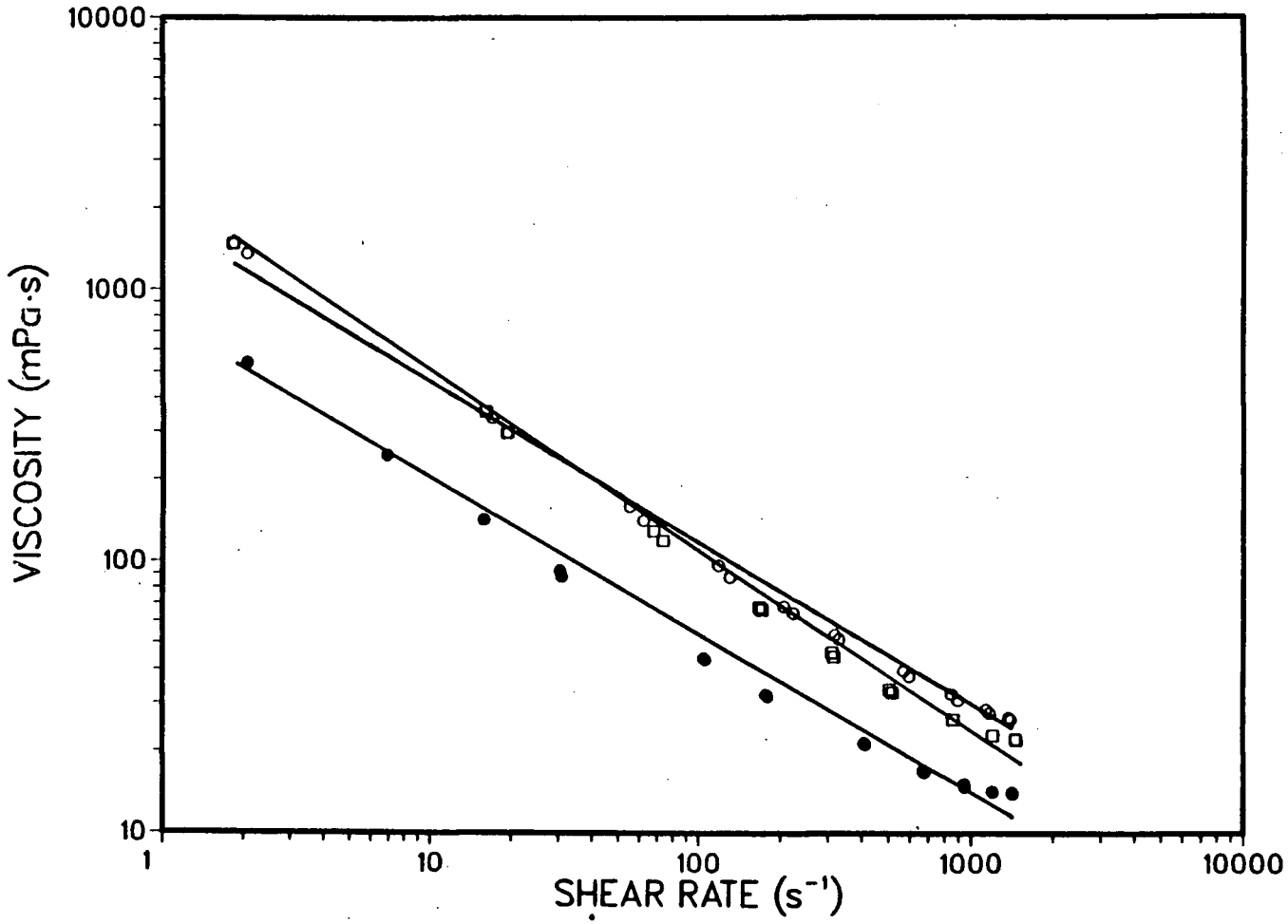


Figure 43. Rheograms for synergistic mixtures containing 0.25% xanthan gum and 0.25% 121a (●), 0.25% 121b (○), or 0.25% locust bean gum (□), on logarithmic coordinates.

an appreciable synergistic effect is seen, giving properties greater than an equivalent concentration of xanthan. The latter case indicates that a small interaction is taking place, resulting in a lower n and larger m, than for the 0.25% xanthan solution. The locust bean gum/xanthan synergistic interaction gave a solution having a similar viscosity and pseudoplasticity to the mixture of 121b and xanthan.

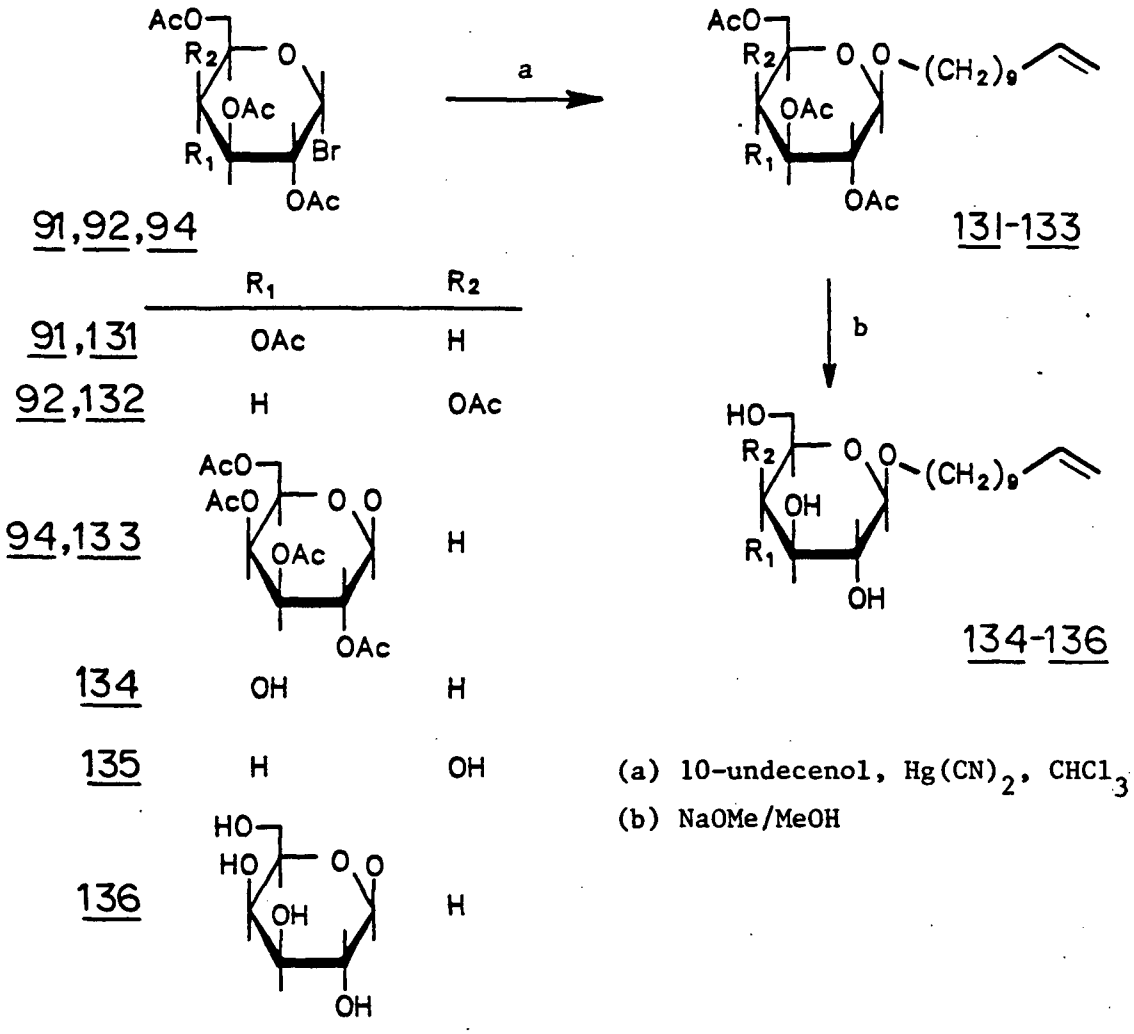
It is important to point out that the interaction of the derivatives prepared in this study with xanthan gum are not necessarily analogous to those between galactomannans and xanthan. Indeed, upon consideration of the primary structure of xanthan, it seems likely that an ionic interaction between the carboxylates of xanthan and the amine groups on the chitosan derivatives would occur. Thus, derivatives having low d.s. permit access of the xanthan branch (bearing the carboxylate) to the free amino groups, giving a salt precipitate. The higher d.s. samples sterically restrict the extent of ionic interaction and yield viscous solutions. Thus, while we have not necessarily probed the synergistic interaction between galactomannans and xanthan, the potential for synergistic interaction between chitosan derivatives and xanthan gum has been established. This observation is exciting, and opens up a whole range of possiblities for viscosity modification. For example, it can be envisioned that chitosan derivatives, such as those presented here, could be further modified in order to limit and control the synergistic response upon mixing with xanthan gum. The simplest way of

doing this is probably by N-acetylation as described in the preparation of the N-ethyl-β-glucosyl chitin derivative 130. Furthermore, there is no reason to limit this effect to xanthan mixtures, as similar and perhaps more interesting observations could result from admixture of chitosan derivatives with other carboxylate-containing polymers, such as proteins. This observation also provides some insight into the behaviour exhibited by the acidic and salt forms of the glucuronoside derivatives 122a and b. It is reasonable to expect that ionic interaction caused precipitation of high d.s. derivatives in the acid form, but at lower d.s. reduced interaction could yield solutions or gels. These ionic crosslinking attractions are removed when the derivatives are in the carboxylate form.

2.3 N-[10'-O-(β-D-GLYCOPYRANOSYL)DECYL]CHITOSAN DERIVATIVES

2.3.1 Synthesis and Characterization

The 10'-undecenyl β-D-glycosides of glucose (134), galactose (135), and lactose (136) were prepared by methods[242] similar to those for the synthesis of allyl β-D-glycosides, as outlined in Scheme 19. The acetobromo sugars were reacted under Koenigs-Knorr glycosidation conditions with two molar equivalents of 10-undecenol in chloroform to give the intermediate peracetylated glycosides 131-133. The crude residue was directly de-O-acetylated to yield the desired 10'-undecenyl β-D-glycopyranosides 134-136.



Scheme 19

It was noted by ^{1}H-nmr that some α-D-glycoside impurity was present in the β-lactoside product. Liquid chromatography of the crude material, using methods reported for long chain alkyl glycosides,[252] afforded the compounds 134, 135 and 136 as waxy solids.

Characterization of the 10'-undecenyl β-D-glycosides was best accomplished using ^{1}H and ^{13}C-nmr. Since similar

molecules are known to behave as non-ionic surfactants and form micelles,[252-254] in aqueous solution, and because their water solubility was limited, the glycosides were dissolved in methanol-d^4. This served to reduce aggregation of the molecules and permit higher resolution spectra to be obtained. It was found that spectra recorded at 50°C were better resolved than those determined at 20°C, which had broader signals. Thus, assignment of ^1H and ^{13}C-nmr spectra recorded at 400 MHz and 100.6 MHz respectively at 50°C was possible. The ^{13}C-nmr chemical shift data for the three glycosides is given in Table 10.

Despite attempts at purification by liquid chromatography, the β-lactoside product contained some α-anomer (~10%) impurity and was carried through as such. This was likely a result of performing the glycosidation at elevated temperatures. Obtaining analytically pure samples for optical rotation determinations and elemental microanalyses was precluded because of the difficulty in crystallization and drying of the glycosides. However, fast atom bombardment (fab) mass spectrometry provided the expected parent peaks as proof of product molecular weights.

Ozonolysis of the alkenyl β-D-glycopyranosides 134 and 135 was performed at -78°C in methanol. Somewhat surprisingly, it was necessary to use a chloroform-methanol mixture (1:5) to solubilize the disaccharide 136. After workup, the aldehydes 137-139 were directly employed in reactions with chitosan. It was found that upon standing for over a day, the

Sample#	Sugar	C-1	C-2	C-3	C-4	C-5	C-6	C-1'	C-9'	C-10'	C-11'
134	β-Gal	102.5	73.3	76.0	70.0	76.3	61.1	69.1	32.9	138.3	112.8
135	β-Glc	103.1	70.8	73.3	69.0	74.7	60.7	68.5	32.9	138.2	112.8
136	β-Lact										
	(β-Gal)	103.0	70.8	72.8[a]	69.7	75.1	60.7				
	(β-Glc)	102.2	72.8[a]	74.5[b]	79.0	74.5[b]	60.2	68.4	32.9	138.3	113.2

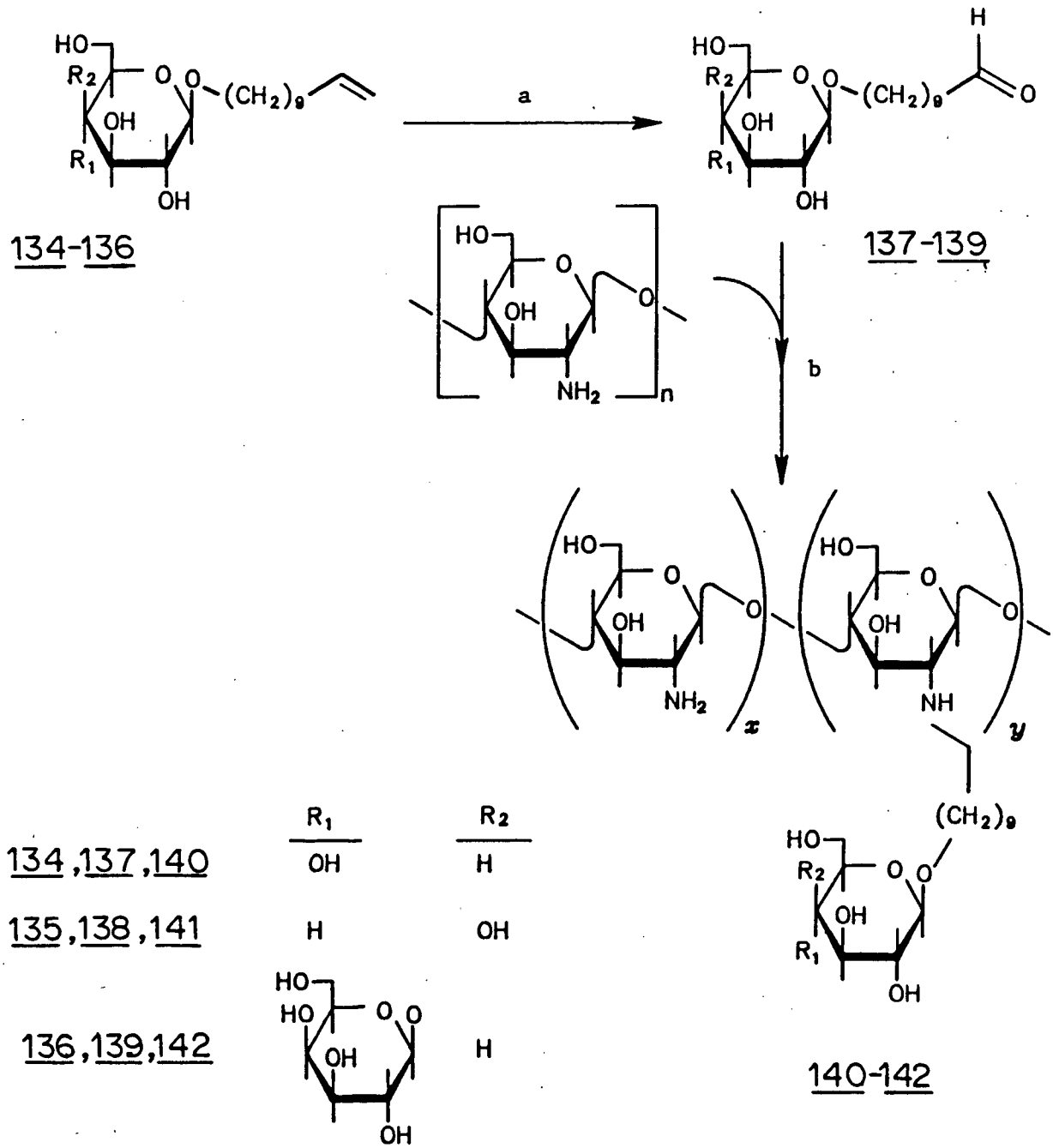
Table 10. 100.6 MHz ^{13}C-nmr chemical shift data (ppm), for saccharide and some aglycon resonances, of the 10'-undecenyl β-D-glycopyranosides in CD_3OD (ref. external TMS).

a. Assignments may be reversed.

b. " " "

aldehydes became insoluble in methanol and water, but dissolved slowly if small amounts of acetic or hydrochloric acid were added. Apparently oligo- and poly-acetal compounds formed, as might be expected for these relatively unhindered decyl-aldehydes.

The reductive amination[86] of the decanalyl β-D-glycopyranosides 137 and 138 to chitosan (Scheme 20) were performed in 5% aqueous acetic acid-methanol (1:1) solvent. The methanol was necessary to solubilize the aldehydes 137 and 138; however, the aldehyde 139 was soluble in the totally aqueous system. Upon addition of sodium cyanoborohydride to the reaction solutions, a marked decrease in viscosity occurred. After the reactions were stirred for 24 hours, they were dialyzed, filtered and lyophilized. The reactions were performed using two different aldehyde-to-chitosan ratios, for each of the aldehydes 137-139, to give the derivatives 140-142a and b, which are listed in Table 11. The degree of substitution values, as determined from elemental microanalysis, immediately told us that the coupling efficiency of the long chain aldehydes was much greater than for those of the allyl glycoside route. Thus in this series, derivatives 140a and 141a had d.s. values of 1.47 and 1.37 when 3 equivalents of aldehyde were employed, while previously a d.s. of 1.0 appeared to be maximal (Table 3). Derivatives with d.s. values lower than 1.0 were prepared by reducing the amount of aldehyde used in the coupling reaction. Obviously, this was a result of the less hindered nature of the decanalyl alde-



	R_1	R_2
134,137,140	OH	H
135,138,141	H	OH
136,139,142		H

(a) O_3, -78 C, DMS, MeOH (b) HOAc/MeOH/H_2O (1:10:10), $NaCNBH_3$

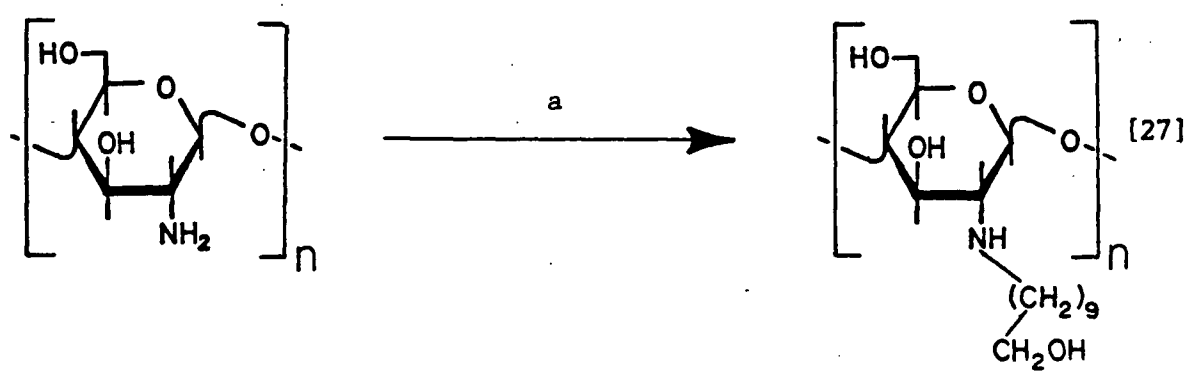
Scheme 20

Derivative	Branch	A/C	d.s. (±.05)	Yield(%)
140a	β-Glc	3.0	1.47	80
b		2.1	0.81	85
141a	β-Gal	3.0	1.37	65
b		1.5	0.22	85
142a	β-Lact	2.9	1.10	70
c		1.6	0.50	70
143a		2.0	1.73	76
b		1.0	1.00	82
144	α-Gal*		0.32	
	β-Gal	2.9	1.04	63

Table 11. Characteristics for the N-[10'-O-(β-D-glycopyranosyl)decyl]chitosan derivatives.

* The N-ethyl-(α-galactosyl) branch was present on 126d prior to its modification to give the mixed derivative 144.

hydes, compared to acetaldehydes, which allowed a substantial amount of N,N-disubstitution. Since this fact complicated molecular formula determinations, it was assumed, for simplification, that if the d.s. was greater than 1.0, no unsubstituted residues remained. For comparison purposes the derivatives 143a and 143b were prepared using standard



1 143

(a) MeOH/i-PrOH/ H_2O (2:1:2), $NaCNBH_3$, 10-hydroxydecanal

conditions involving the reaction of chitosan with 10-hydroxydecanal (Eq. 27) , which was obtained from ozonolysis of 10-undecenol. Derivative 143a precipitated from the reaction solution and was collected by filtration, and 143b was isolated using standard workup procedures. Again high d.s. values were obtained at typical A/C ratios (Table 11).

Disappointingly, none of the derivatives 140-143 were water soluble. They were however, all soluble in dilute organic or mineral acid solutions in water (e.g. 1-2% aqueous acetic acid). The high d.s. samples 140a, 141a and 142a, bearing pendant β-D-glucose, β-D-galactose, and β-D-lactose residues respectively, gave thin, mobile solutions at 5.0% (w/w) polysaccharide concentration in 1% aqueous acetic acid, while the lower d.s. analogues 140b, 141b and 142b, gave slightly more viscous solutions. Solution ^{13}C-nmr spectra of 140a, 141a and 142a had easily discernible resonances for the pendant sugars and alkyl group, but virtually no distinguishable signals from the chitosan backbone. This is indicative of freely rotating pendant sugars, more so even than that seen for the N-ethyl glycosyl series of derivatives. It must however be borne in mind that, due to much larger average residue molecular weights, the decyl samples at 5% concentration have a lower molar concentration than the N-ethyl series at 5% concentration. Despite this factor, however, one would intuitively expect greater mobility in a group having a ten carbon vs a two carbon spacer arm. Total assignment of the branch and alkyl ^{13}C resonances for derivatives 140a, 141a

Derivative	Branch	C-1	C-2	C-3	C-4	C-5	C-6	C-10'
140a	β-Glc	100.8	71.8	74.5	69.0	74.6	59.5	68.4
141a	β-Gal	101.3	67.0	71.4	69.2	73.4	59.2	68.8
142a	β-Lact							
	(β-Gal)	102.5	70.5	72.2	70.0	75.8	60.5	
	(β-Glc)	101.7	72.4	74.1	78.5	74.3	60.0	68.1
144	α-Gal	97.0	66.7	68.0	67.6	69.9	60.4	
	β-Gal	101.2	67.0	71.3	69.1	73.3	59.2	68.8

Table 12. 100.6 MHz ^{13}C-nmr chemical shift (ppm) data for pendant residues of the N-decyl-β-D-glycopyranosides, in 1.0% CD_3COOD/D_2O (ref. external TMS).

and 142a are presented in Table 12, and again they compare well with the methyl glycoside analogues,[99-101] the 10-undecenyl β-D-glycoside precursors (Table 10) and to the respective N-ethyl D-glycopyranosyl chitosans (Table 4). To our chagrin, it was immediately apparent that these solutions had uninteresting rheology at ambient temperatures. Serendipitously, it was noted that upon heating the 5.0% solutions of 140a and 141a to 50°C a stiff opaque gel formed, which dissolved reversibly upon cooling. Solutions of 2.0% concentration did not exhibit this behaviour. This type of reversible temperature induced gelling process was considered to be of some interest and deserving of further investigation.

The non-reversible gelation of some proteins upon heating is known, and is largely attributed to hydrophobic interactions.[255] Long chain (C_8 and longer) alkyl glycosides are non-ionic surfactants, which form micelles at certain critical concentrations.[252-254,256] Such micellar solutions are characterized by a "cloud point", which results from aggregation of micelles upon heating.[256] Interestingly, the gelation of 141a is accompanied by increased solution opacity, and seems to relate to the cloud point phenomena that occurs at the monomeric level. Thus, it appears that the necessary components for temperature dependent behaviour are present on the derivatives described here. That is, the combination of hydrophobic character and polymeric structure appear to be conducive to temperature induced gelling. The reversibility of the interaction indicates that reorientation of the

polymer chains accompanies temperature reduction. For proteins this is not usually the case as irreversible denaturation accompanies gel formation. Another polysaccharide derivative known to have similar behaviour is methylcellulose.[257]

2.3.2 ^{1}H and ^{13}C-NMR Investigations

In order to follow gel formation and to perhaps gain insight into the mechanism, ^{1}H and ^{13}C-nmr experiments were undertaken. It was felt that the mobility of the components of the derivative could be probed by observing the temperature dependence of T_1-relaxation of resonances in the ^{1}H-nmr spectrum of 141a. As such, T_1-relaxation measurements of three resonances, representing the pendant sugar, the alkyl chain, and solvent, in the ^{1}H-nmr spectrum were performed at 20°, 40°, 60° and 80° using the inversion recovery method[113] (Fig. 44). The T_1 values obtained are given in Table 13. The

Temperature	T_1 of Resonances (s)		
(°C)	Sugar	Alkyl	HOD
20	0.34	0.25	3.4
40	0.42	0.30	1.6
60	0.47	0.32	1.4
80	0.80	0.54	1.2

Table 13. T_1 values ,at 20, 40, 60, and 80° C, for the resonances indicated in the 300 MHz ^{1}H-nmr spectrum of 141a (Figure 44), in 1% CD_3COOD/D_2O solution.

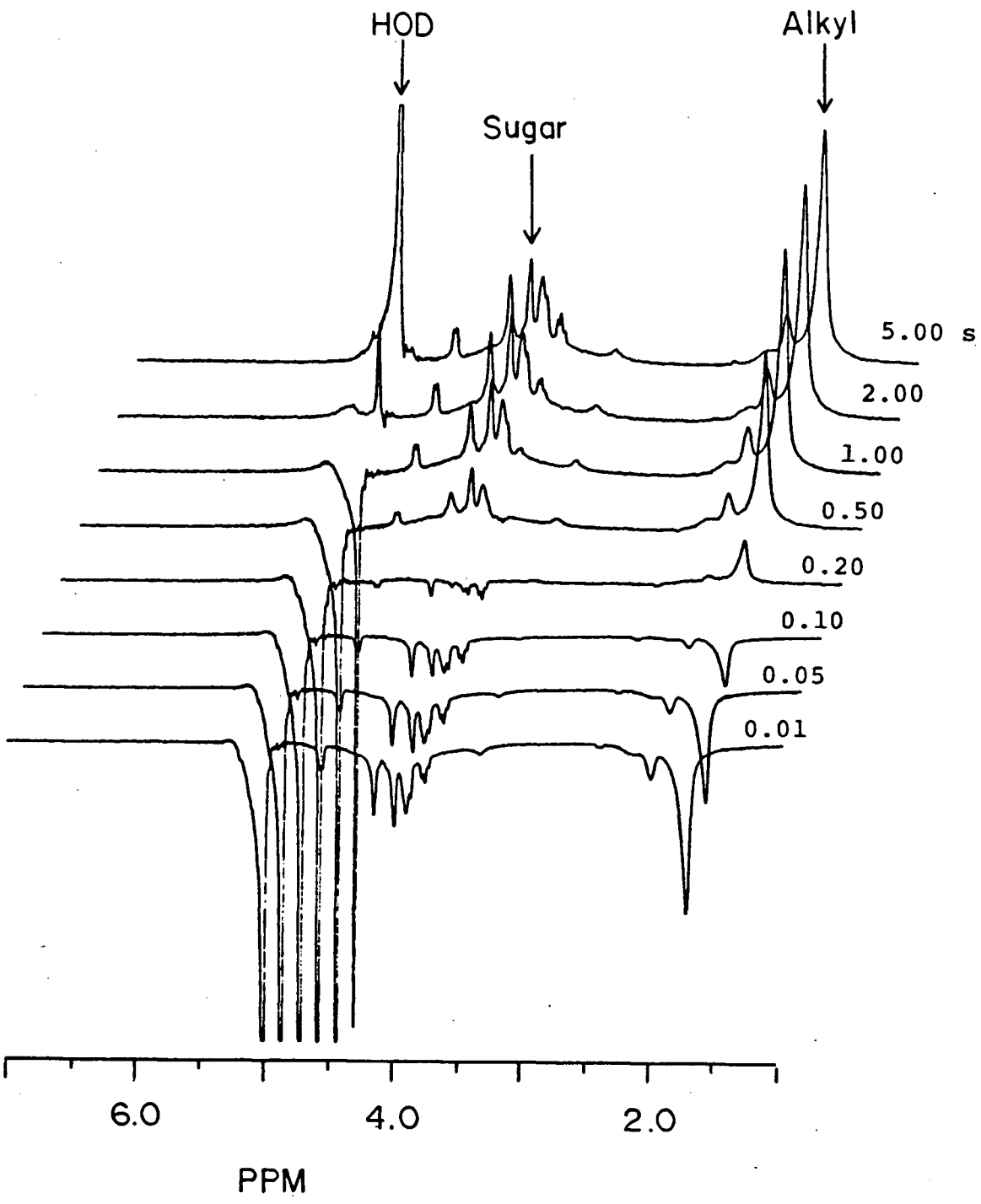


Figure 44. Stacked plots showing the inversion recovery of the resonances in the spectrum of 141a, in 1.0% CD_3CO_2D/D_2O solution at 20°C, at 300 MHz. The peaks for which T_1 values were calculated are indicated.

increase in the T_1-relaxation time of the sugar and alkyl protons indicates that the correlation time (τ_c) of the polymer is sufficiently slow at 20°C that it has passed the minima in the T_1 vs τ_c curve. This is expected since at 300 MHz a τ_c ~$3x10^{-9}$ s^{-1} would result in a T_1 minimum, while chitosan derivatives in solution have been shown to have correlation times of 10^{-9}-10^{-8} s^{-1}.[258] Thus, increased T_1 values at 40°, 60° and 80°C result from reduced mobility of the respective groups in the gel state. The decreasing T_1 value of the solvent or HOD resonance, is supportive of reduced solvent mobility upon gelation. In this case, the water molecules having τ_c~10^{-12}-10^{-11} s^{-1} in solution, are "trapped" in the gel matrix and their reduced mobility causes a reduction in T_1, in the direction of the T_1 minimum at τ_c~$3x10^{-9}$ s^{-1}. Unfortunately, there were no obvious chemical shift changes upon heating that could help illuminate the gelling mechanism. As expected, a general broadening of resonances occurred upon heating, due to the dependence of T_2 and linewidth, on correlation time. As in the ^{13}C-nmr spectrum, no ^{1}H-nmr resonances from the chitosan main chain were discernable. Gelation was also monitored by ^{13}C-nmr spectroscopy. In Fig. 45, the ^{13}C-nmr spectrum of 141a at 30° and 50°C is given. Substantial line-broadening is immediately apparent at 50°C, with linewidths for C-1 being @ 15 and 150 Hz respectively, for the 30° and 50°C spectra, reflecting the substantially reduced mobility of the pendant galactose unit in the gel. Again, no chemical shift changes occur upon

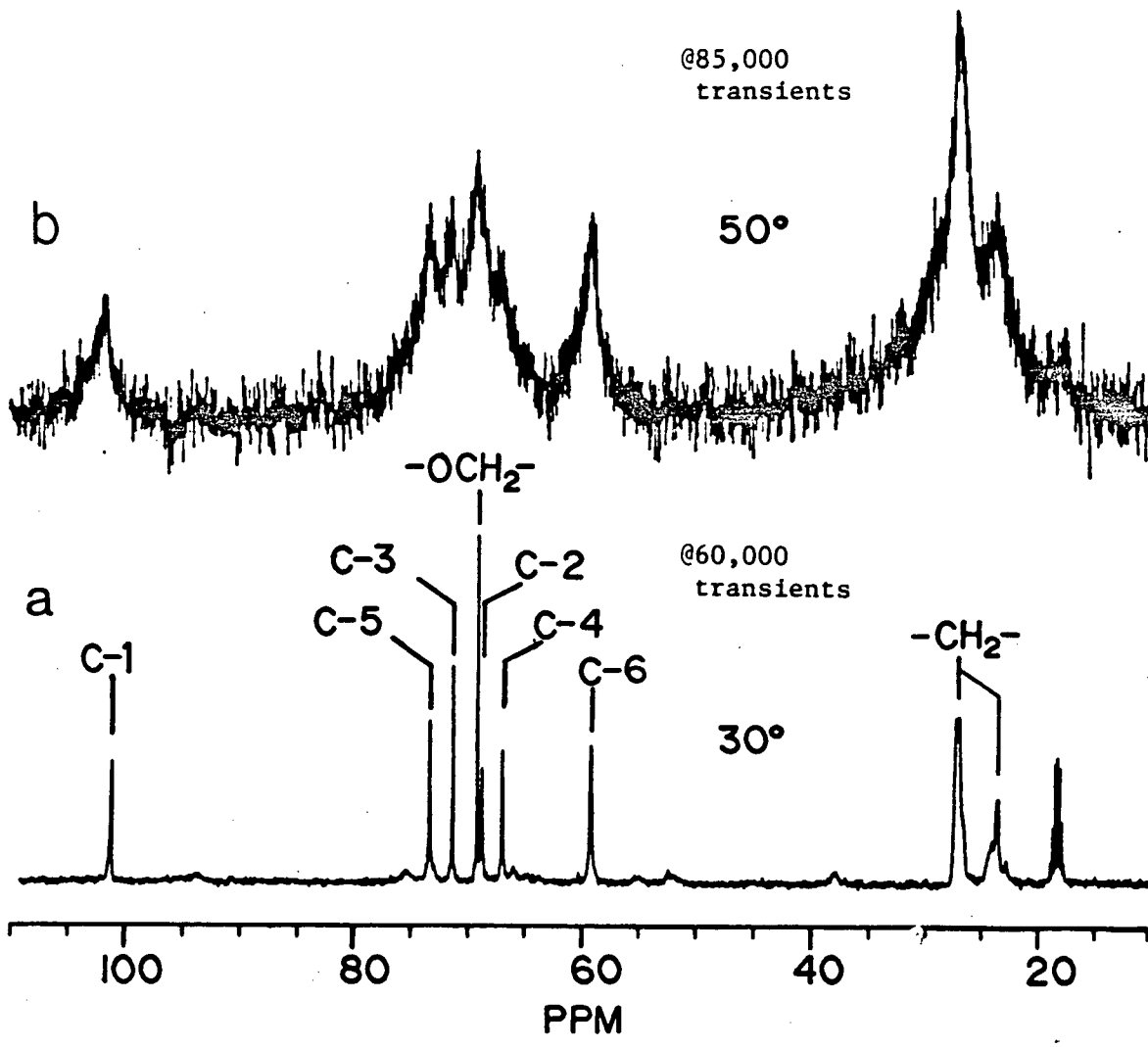


Figure 45. An expanded region of the 100.6 MHz ^{13}C-nmr spectrum of 144 at a) 30°C ; and b) 50°C, in 1.0% CD_3COOD/D_2O (ref. external TMS), showing alkyl and sugar branch resonances.

heating the sample. Thus, while nmr was useful for following the gelation phenomena, the experiments performed were insensitive to the interactions which cause it.

While it is difficult to speculate on the exact mechanism for the observed temperature induced gelation, similar phenomena have been observed in other systems. Phase separa-

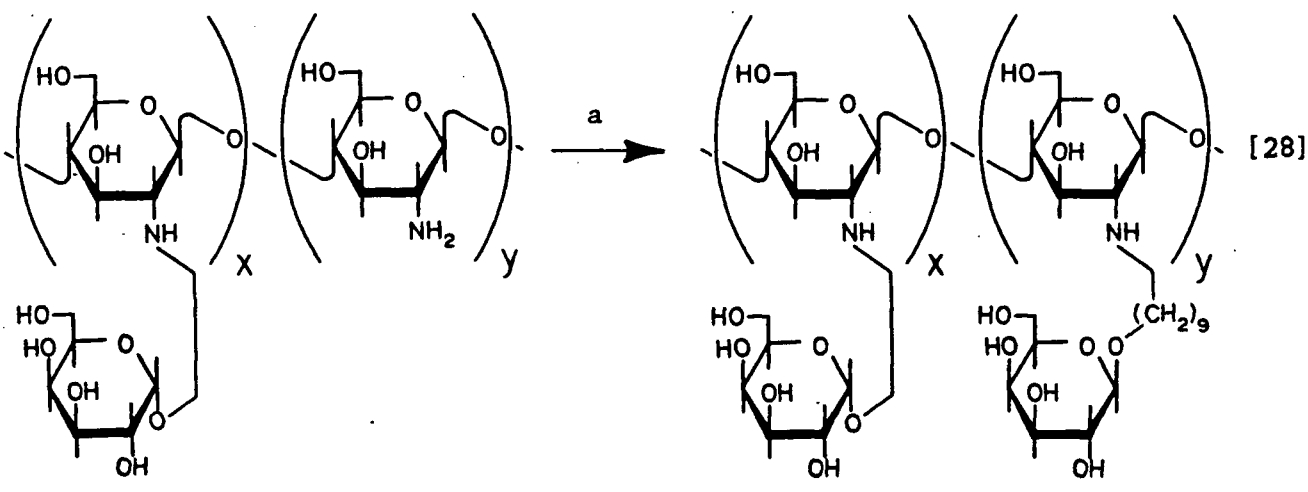
tion is observed in solution of non-ionic surfactants when heated, and is referred to as the "cloud point".[259] Although some structural similarities do exist between the branch units described here, and carbohydrate derived non-ionic surfactants, it is difficult to visualize a micellar like interaction for these compounds. It is likely that, upon heating, the alkyl chains are increasingly repulsed from interaction with water. The behavior seen here for these polysaccharide systems is probably analogous to the temperature dependence observed for polyethylene glycol solutions.[260]

It was interesting to note that the lower d.s. monosaccharide derivatives 140b and 141b, and the lactose derivatives, 142a,b even at 7.5% concentration, did not gel at elevated temperatures. Thus, substantial hydrophobic character appears necessary, and a large pendant group precludes gel formation. The latter is likely a result of the increased hydrophilicity of the disaccharide, counteracting or interfering in the hydrophobic interactions. It has been reported[254] that alkyl lactosides are not as prone to micelle formation, and behave poorly as surfactants. This is likely a further manifestation of that property. Interestingly, of the derivatives 143a and b, which lacked the pendant carbohydrate, 143a was insoluble in aqueous acetic acid and 143b gave a highly viscous solution showing no observable change upon heating. This gratifyingly indicated that gel formation was dependent on the hydrophilic character of the pendant

moiety. Both 143a and b gave clear stiff gels in 1% acetic acid-methanol, while the branched derivatives gave gels in aqueous acetic acid-methanol systems.

2.3.3 Mixed Branch Chitosan Derivatives

The concept of preparing co-branched chitosan derivatives in order to control or enhance solubility properties has been introduced[96] (section 1.2.2). In this study, we felt that N-10'-O-(β-D-glycopyranosyl)decyl branches, and the unique temperature dependence they impart, could be used in conjunction with the controlled solubility properties of the N-2'-O-(D-glycopyranosyl)ethyl branched chitosan derivatives to give products having viscous properties that were stable or enhanced at elevated temperature. To this end, derivative 126c was reductively alkylated with the decanalyl glycoside 138 to give 144 (Eq. 28), as described in Table 11.



126 144 [28]

(a) $MeOH/H_2O$ (1:1), $NaCNBH_3$, 138

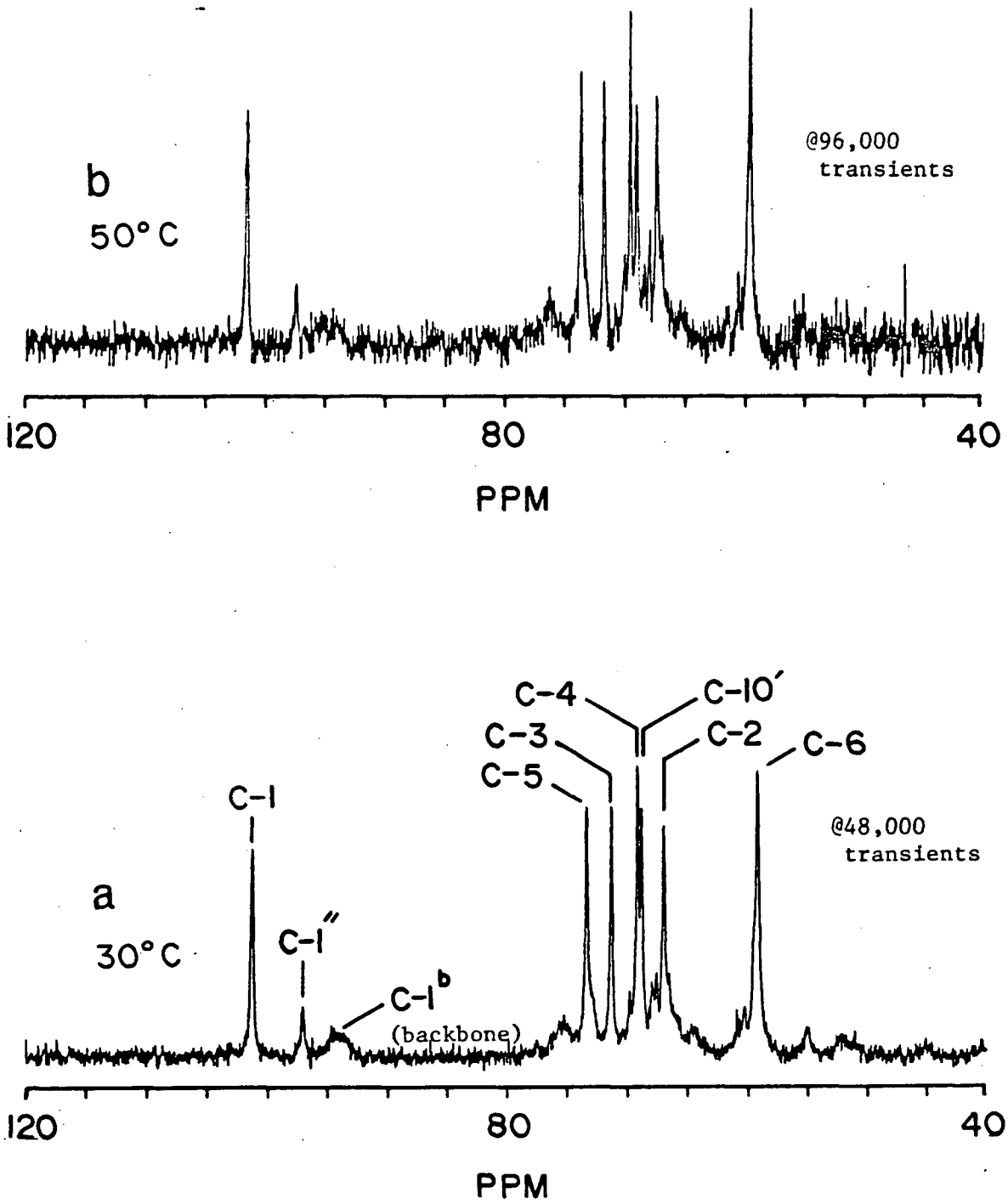


Figure 46. 100.6 MHz ^{13}C-nmr spectral region containing the branch residue signals for derivative 144 at 30° and 50° C, in 1.0% CD_3COOD/D_2O (ref. external TMS).

Disappointingly, it was found that while 144 would swell in water, it would not give a true solution. Again, it was found that a viscous solution was obtained in 2.0% aqueous acetic acid. The ^{13}C-nmr spectrum (Fig. 46) of 144 contains resonances for both branch residues; however, mobility differences result in considerable suppression of the α-galactosyl branch compared with the more extended β-D-galactosyl branch.

Steady shear viscometry on 2.0% (w/w) solutions of 144 and 126c in 2.0% aqueous acetic acid, at 20°C and 50°C provided the data shown in the logarithmic rheograms in Fig. 47. The rheology of the solution of 144 at 20°C was interesting, in that both pseudoplasticity and viscosity were appreciable, and the solution was considerably more viscous than that of 126c. However, the rheograms (and power-law parameters, Table 14) show that the temperature dependence of

Derivative	Temperature (°C)	n ∓ 3%	m (mPa·s) ∓ 3%	R^2	#Points
144	20	0.488	7330	.994	12
	50	0.499	4920	.996	14
126c	20	0.607	1880	.997	13
	50	0.644	1060	.997	11

Table 14. Power-law parameters obtained from rheological evaluation of 126c and 144, at 20°and 50°C, in 1.0% aqueous acetic acid solution.

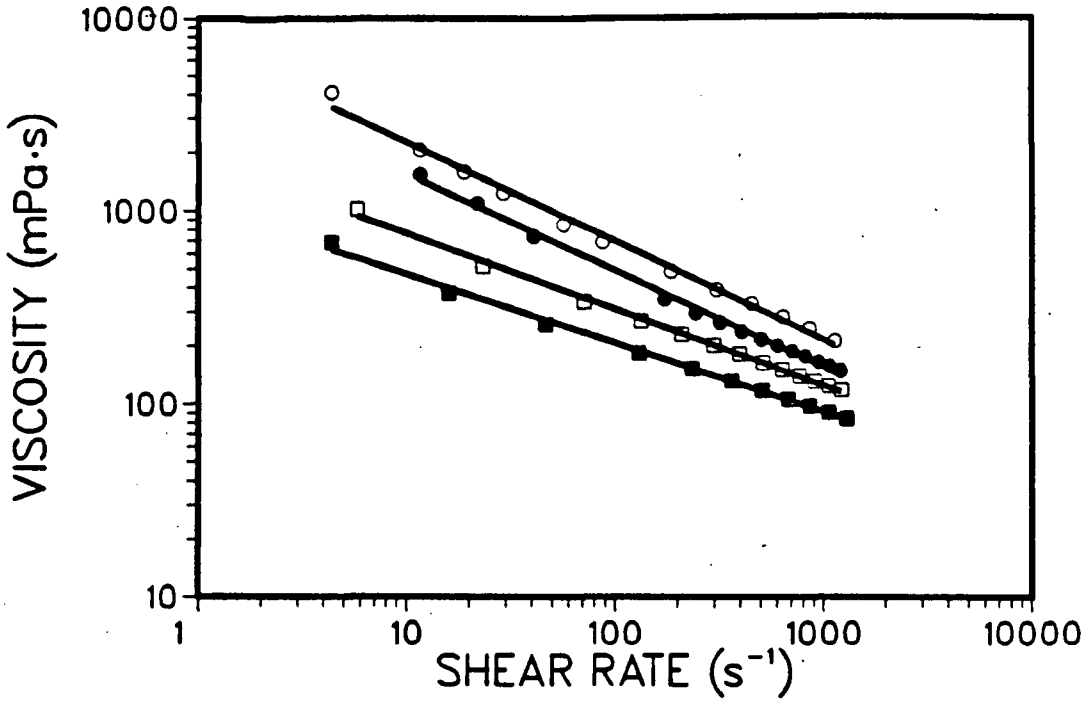


Figure 47. Rheograms of derivatives 144, at 20° (○) and 50°C (●), and 126d, at 20° (□) and 50°C (■) on logarithmic coordinates.

both 144 and 126c are similar and result in a reduction of both viscosity and, to a lesser extent, pseudoplasticity. As an exploratory experiment, these results were rewarding and are certainly indicative of further potential for mixed derivatives.

2.4 CONCLUSION

The work described in this chapter constitutes a significant contribution to existing knowledge on the rational derivatization of chitosan for controlled solubility applications. Analogues of natural branched polysaccharides have been prepared and their similarity to the naturally occurring branched legume seed galactomannans has been discussed. In

addition, potential for modifying and controlling chitosan self-associations as well as synergistic interactions has been demonstrated. The concept of utilizing "mixed" branch derivatives to access unique properties and interactions was further explored, and was extended by incorporating mixed functionality (namely hydrophobicity and hydrophilicity) on a single carbohydrate derived branch, to provide chitosan derivatives having unique gelling properties.

CHAPTER 3

METAL CHELATING AND AFFINITY CONJUGATES OF CHITOSAN

3.1 INTRODUCTION

3.1.1 Metal Chelating Chitosan Derivative

As mentioned in Chapter 1, chitosan has been modified in order to prepare controlled-solubility derivatives, metal sequestrants, drug delivery matrices, drug carriers and immobilization supports.[22-26] Although our main efforts have been directed toward controlled solubility compounds,[96,237] we felt that our experience in chitosan modification could be exploited in the synthesis of metal chelating and affinity ligand conjugates.

Some metal chelating chitosan derivatives have been mentioned (67, 68, 69, and 70).[92-95] Our objective was to incorporate controlled solubility features in conjunction with chelating properties to create a novel chitosan derivative. This was felt to be best accomplished by incorporating a hydrophilic spacer arm between the chitosan backbone and the desired chelating group. Previous reports from this lab in which free sugars were coupled to chitosan to give acyclic hydrophilic branches[96] provided an ideal route to the desired spacer group. Iminodiacetic acid, essentially one half of the well-known chelate, ethylene diamine tetraacetic acid (EDTA), was chosen as an appropriate

chelating functionality. The synthetic route to a precursor having the desired structural features, and its attachment to chitosan will be discussed.

3.1.2 Potential Affinity Chromatography Support

While chitosan has attracted significant attention in biochemical and biomedical applications,[22-26] there has been surprisingly little research into the use of chitosan in affinity chromatography. In collaboration with a group* interested in the enzyme β-glucosidase, we felt the potential of using chitosan conjugates in affinity chromatography could be demonstrated.

A number of well-known concepts have developed in affinity chromatography,[8,32,33] including hydrolytic stability of covalent linkages, choice of a suitable ligand, and the use of a spacer group. In our case, the use of reductive amination procedures[86] eliminated any worry about hydrolysis of the amine linkage to the backbone. As a ligand, a pendant glucose having a glycosidic linkage resistant to enzymatic hydrolysis was desired. 1-Thio-β-D-glycosides, being known inhibitors for glycosidase enzymes and generally immune to enzymatic hydrolysis, provided a logical choice for the ligand.[261]

The spacer group required a degree of bifunctionality such that the thio-β-D-glucose could be attached, and a remaining site was present for reaction with chitosan. The reagent 1-allyloxy-2,3-epoxypropane (allylglycidyl ether)

* Dr. S.G. Withers and coworkers, The Department of Chemistry, The University of British Columbia.

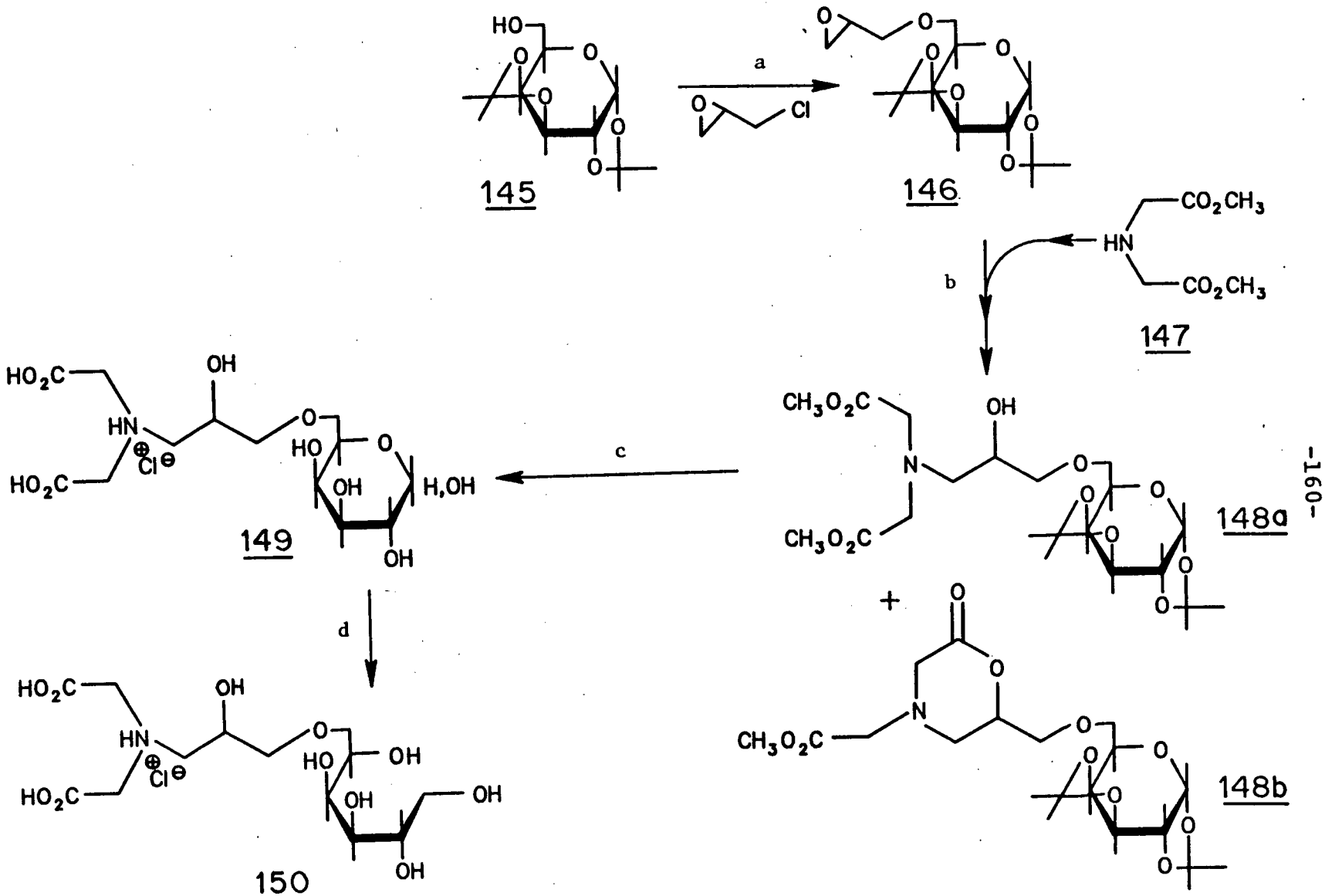
provided both features, with an epoxide amenable to thiolate attack and a double bond which was convertible to an aldehyde. Thus, the synthesis of the appropriate 1-thio-β-D-glucopyranoside and its conjugation with chitosan will be presented, and preliminary evaluations of β-glucosidase binding will illustrate the potential for affinity chromatography applications.

3.2 METAL CHELATING CHITOSAN DERIVATIVE

3.2.1 Synthesis

By design, it was decided to prepare a compound which contained both an iminodiacetic acid functionality for metal chelation, and an aldehyde for coupling to chitosan. We chose to incorporate a carbohydrate-derived, hydrophilic spacer arm to enhance the solubility characteristics of the final product, and to act as a spacer arm. Scheme 21 outlines the synthesis of the coupling precursor 149.

The epoxide 145 was prepared from 1,2:3,4-di-O-iso propylidene galactose (146), by reaction with epichlorohydrin in the presence of sodium hydride. Subsequent reaction of the epoxide with dimethyliminodiacetate (147) gave a 4:1 mixture of the dimethyliminodiacetate 148a, and the morpholone 148b. Although cyclizations similar to that producing 148b have been observed in reactions of epoxides with amino acid esters,[262] it appears to be more favoured for these diester compounds. Total conversion of the mixture to the morpholone



(a) NaH, THF (b) EtOH (c) i) NaOH ii) HCl (d) i) OH^-, $NaBH_4$ ii) HCl .i) HCl

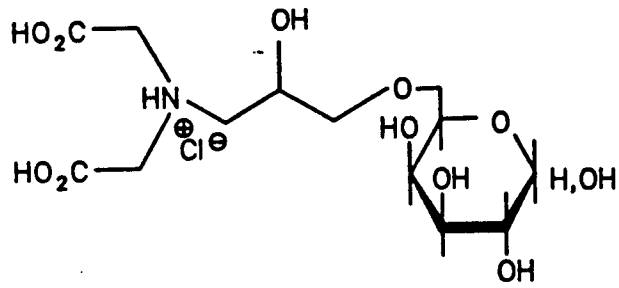
Scheme 21

148b was easily achieved by distillation. Liquid chromatography on silica gel offered some separation of the mixture, but it was determined that further cyclization occurred on the column. Base hydrolysis of the 148 mixture, and subsequent treatment with acid at 50°C, gave the free sugar derivative 149.

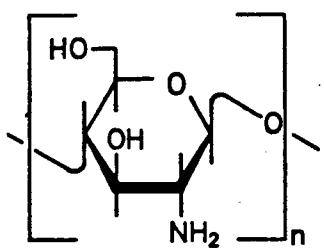
Compound 149 was coupled to chitosan via reductive amination, with sodium cyanoborohydride in 5% aqueous acetic acid, to give the derivatives 151a and 151b (Eq. 29). Control of the degree of substitution was achieved by varying the ratio of aldehyde to chitosan (A/C) as shown in Table 15. Derivative 151a was water-soluble, giving a clear viscous solution at 5.0% (w/w) in distilled water. Compound 151b, on the other hand, gave an opaque gel at 5.0 % (w/w) concentration and at 1.0% retained much of its gelatinous nature.

3.2.2 Characterization

The solution state ^{13}C-nmr spectrum of 151a was obtained and resonances for the hydroxypropyl and iminodiacetate functionalities were easily discernible. Most of the carbohydrate resonances arising from the galactosyl branch were assignable. The chitosan resonances, however, were very broad and assignment was difficult. Interestingly, the linewidth for C-1 of the chitosan backbone is of the order of 90 Hz, while the carbon resonances of C-6' of the galactosyl group had a linewidth of 40 Hz. This reflects the relative mobilities of these portions of the molecule.



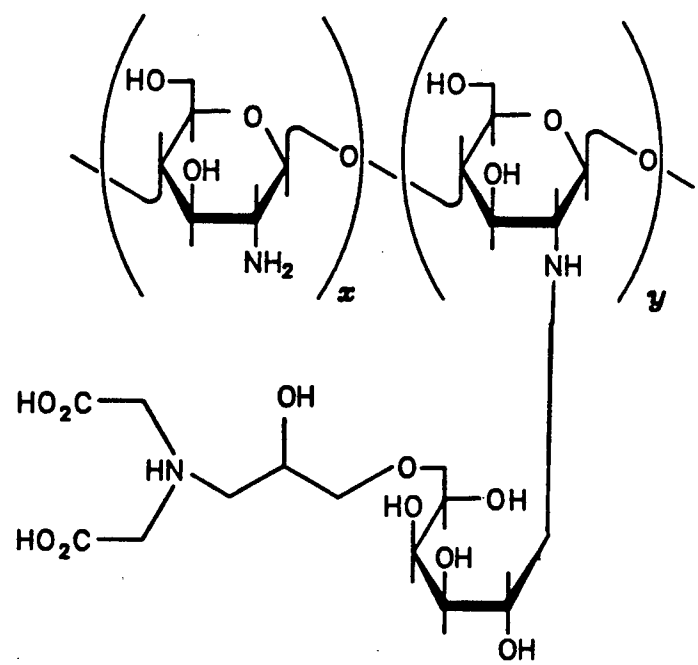
149



1

a

[29]



151

Sample	d.s. (±.05)	Cu(II) Uptake[a]	
		mmol/g	% Theoretical[b]
151a	1.00	2.97	152
151a[c]	1.00	0.33	16.9
151b	0.50	2.45	87.5
Chitosan[d]		2.40	39.0
Chitosan[c,d]		1.15	18.5
Chitosan[e]		0.06	2.0
Chitosan[f]		3.12	51.0

Table 15. Cu(II) chelating capacity for derivatives 151a and 151b contrasted with values obtained in parallel experiments for chitosan, and with literature values for chitosan.

a. A 200 mg sample was dissolved or dispersed in @ 15 mL of distilled water and treated with a 0.5 M Cu(II) acetate solution (15 mL), exhuastively dialysed and freeze-dried.

b. Based on the number of copper ions per GlcN residue.

c. After saturation with Cu(II) these samples were dialysed for 3 d (3x250 mL) against water, 2 d against 0.1 M iminodiacetic acid solution, and finally, exhaustively with water and freeze-dried.

d. Precipitated, lyophilized chitosan.

e. Chitosan flakes, ref. 92

f. Chitosan, ref. 263

3.2.3 pH Titration

The results for pH-titrations of derivatives 151a and 151b gave inflection points corresponding to values of pK_a^1 2.1, pK_a^2 5.9 and p_i 3.9. These values are similar to those reported for N-carboxymethylchitosan,[94] which contains some similar functionalities. Some of the inflections are ill-defined, probably because of the number of titratable groups present, and the insolubilization at pH values of approximately 10 and higher.

3.2.4 Copper(II) Chelation

Table 15 summarizes the evaluation of copper(II) uptake of the derivatives 151a and 151b, and contrasts these with native lyophilized chitosan. The differential uptake of 151a and 151b reflects the higher d.s. value of 151a. The chelating capacity of native chitosan itself, is 2.4 mmol/g and the percent uptake, in terms of copper ions per residue, corresponds to an average of 0.39. For the derivatives 151a and b, more than one ion binds per derivatized residue (i.e. uptake>100%), which indicates that additional binding to the backbone, over and above one equivalent binding per chelating branch, occurs in the derivative. Indeed, the difference between the theoretical maximum (100%) expected for binding to the iminodiacetate moieties, and the values determined for the derivatives is similar to the percent uptake of chitosan. This point was further examined by saturating both 151a and chitosan with copper(II) ions, and then dialyzing the result-

ing chelates against 0.1M iminodiacetic acid in distilled water for 48 hours. The copper content of chitosan decreased by only half to 1.15 g/mmol or 18.5%, while most of the copper (89%) was removed from 151a. Interestingly, the final % copper content of both 151a and chitosan after dialysis were very close. The major limitation in making comparisons of this sort arises from the fact that the physical form of the polymer can be very important. Table 16 shows two very different values for copper(II) uptake for chitosan flakes[92] and lyophilized chitosan.It must also be noted that in this study, the derivatives were dissolved prior to the addition of copper(II) acetate solution, while the lyophilized chitosan remained as a hydrated, particulate dispersion.

3.2.5 Viscometry

Since compound 151a formed viscous 1.0% (w/w) solutions, we felt it would be of interest to evaluate its rheological properties. We also felt viscometry could offer some insight into the interaction of the polymer with copper(II) ions. The steady shear viscometric determinations are shown in Fig. 49 for 1.0% (w/w) solutions of 151a in distilled water and in 1.0 mM aqueous copper(II) sulfate. Interestingly, the viscosity of the solution containing copper ions is less than that for the distilled water solution. This was contrary to the known behaviour of other metal chelating polysaccharides, such as sodium alginate which increases in viscosity or gels

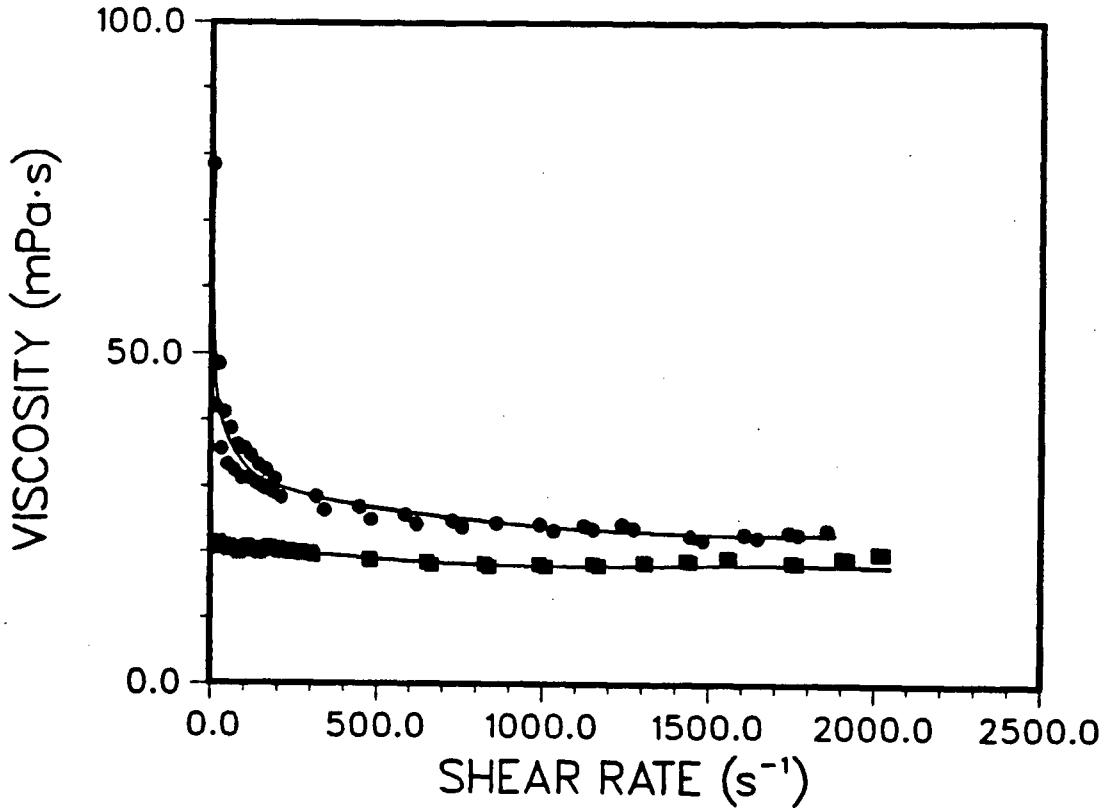


Figure 48. Rheograms on linear coordinates for derivative 151a as a 1.0% solution in water (●), and in 1.0 mM Cu(II)SO_4 (■) solution.

upon the addition of divalent metal ions such as calcium and copper[185-189]. This behaviour results from the crosslinking effect of the metal ions, something which is apparently not occurring in the samples described here. This leads us to the conclusion that each branch or residue is a self-contained chelate for one copper ion. Thus to satisfy the metal ion's ligand requirements, crosslinking is not necessary. The fact that the chelating results indicate >100%

binding supports the conclusion that each branch chelates one ion.

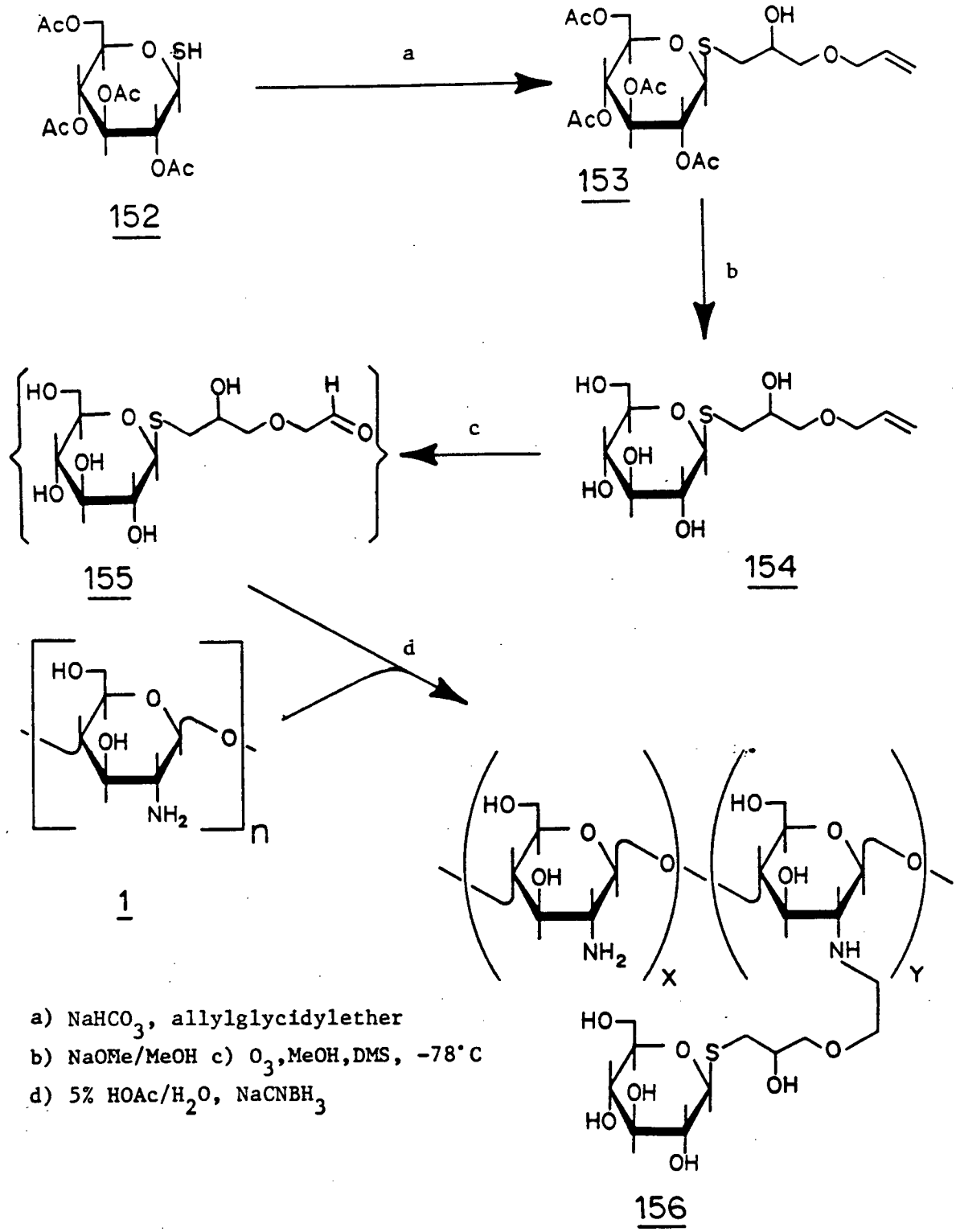
3.2.6 Conclusions

We are gratified by the range of properties exhibited by this chitosan derivative as they reflect the success of a rational approach to tailoring the properties of polysaccharides. Thus, in accord with previous experience, the presence of the high level of N-substitution by a hydrophilic side-chain resulted in a freely soluble derivative. Functional groups, in this case the chelating iminodiacetate, modifying the side-chain offer access to additional properties. This encourages further research into the rational tailoring of chitosan for structure/property studies.

3.3 AFFINITY CHROMATOGRAPHY DERIVATIVE

3.3.1 Synthesis and Characterization

The synthesis of the desired diastereomeric 1-thio-β-D-glucopyranoside 154, was accomplished from 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside (152) and 1-allyloxy-2,3-epoxypropane, as outlined in Scheme 22. Reductive ozonolysis of 154 provided the respective aldehyde 155, which was directly employed for coupling to chitosan. Concern over possible oxidation of the sulfur during ozonolysis prompted the use of a large excess of dimethyl sulfide during workup. The aldehyde 155 was then reductively aminated



a) $NaHCO_3$, allylglycidylether
b) NaOMe/MeOH c) O_3,MeOH,DMS, -78°C
d) 5% $HOAc/H_2O$, $NaCNBH_3$

Scheme 22

to chitosan according to standard methods, to afford the respective derivatives 156a-d, with characteristics listed in Table 16. The ^{13}C-spectrum of 156a showed the expected

Derivative	A/C	d.s. (±.05)	Crosslink (%)
156a	2.1	0.90	--
156b	2.1	0.90	5
156c	0.57	0.25	--
156d	0.57	0.25	15

Table 16. Characteristics of derivatives 156a-d.

resonances indicating attachment of the thio-β-D-glucopyranoside moiety.

As a prelude to testing of these materials as affinity supports it was necessary to determine whether the thioglycosidic linkage is indeed resistant to enzymic hydrolysis. The monomer 154 was utilised for these tests, thus avoiding problems associated with the handling of polymeric materials, yet still providing a stringent test of lability. An assay for glucose based upon the coupling of hexokinase and glucose-6-phosphate dehydrogenase was utilised to detect enzyme catalysed turnover, since glycosidase catalysed hydrolysis of thioglycosides produces glucose and the free aglycone thiol. Incubation of 154 (3.0 mM) with β-glucosidase (~5 units) in the presence of coupling enzymes and cofactors

overnight resulted in no significant increase in absorbance; thus no significant hydrolysis had occurred. The viability of the coupling enzymes at this stage was demonstrated by addition of a known amount of glucose and measurement of the expected response.

Binding of the thioglucoside 154 to the enzyme was then investigated kinetically using β-glucosidases from almond and from Alcaligenes faecalis. Inhibition of hydrolysis of p-nitrophenyl β-D-glucopyranoside by 154 was measured and K_i values of 35 mM and 1.5 mM determined for the β-glucosidases from almond and Alcaligenes faecalis respectively. The K_m values for p-nitrophenyl β-D-glucopyranoside for these two enzymes are 3 mM[263] and 0.08 mM[264] respectively. It therefore appears that both enzymes will bind the thioglucoside but since the β-glucosidase from Alcaligenes has the greater affinity it was used in assaying the affinity support for binding of enzyme.

A preliminary test of the ability of the derivatised polymer to bind β-glucosidase was performed using the cross-linked preparation, 156d. No specific attempts were made to optimise the flow properties of the polymer. Effluent from the column was monitored by assaying the enzyme activity released using p-nitrophenyl β-D-glucopyranoside, as shown in Figure 49. Clearly β-glucosidase is bound to the column initially and eluted at high salt concentrations, thus indicating the potential of this material as an affinity support.

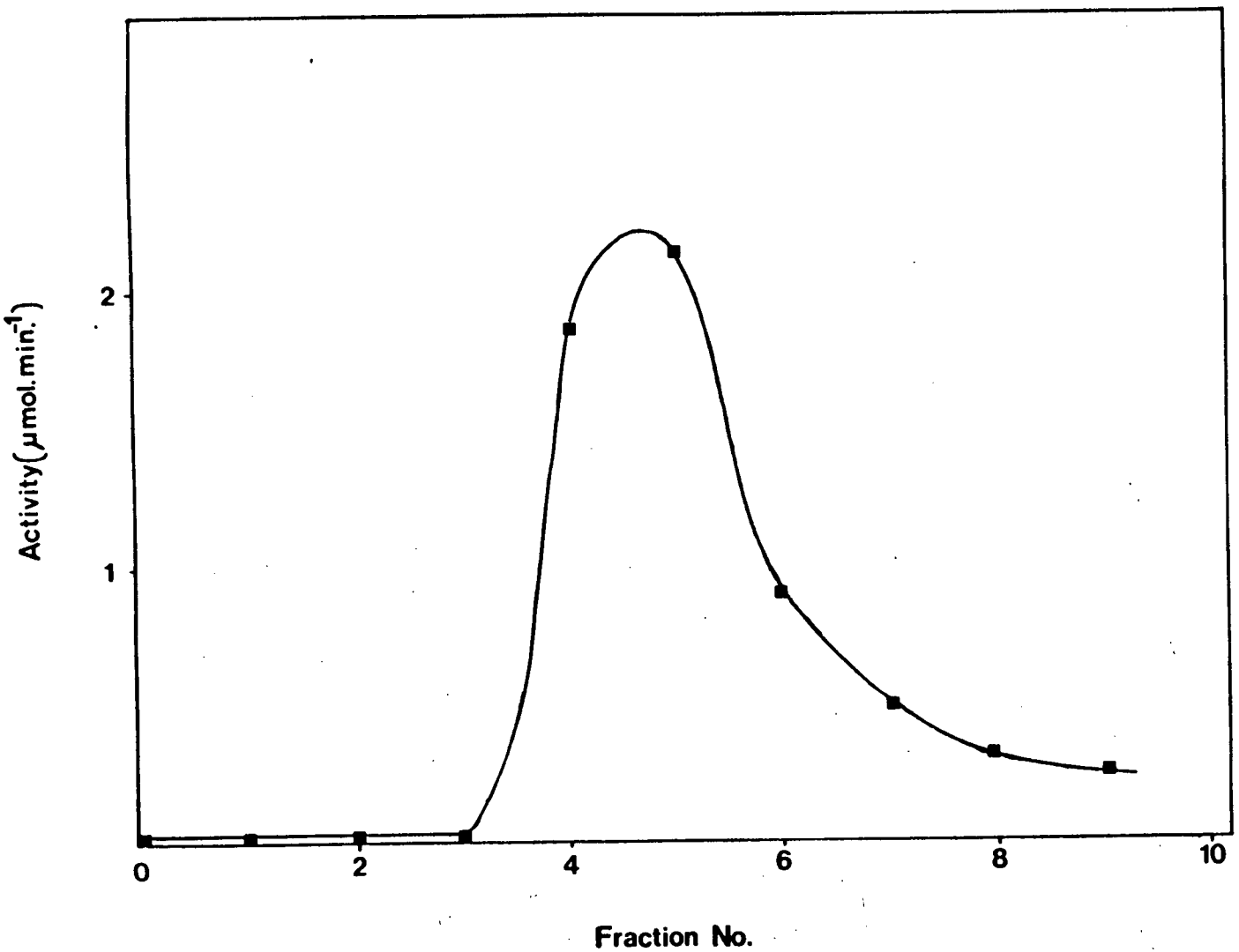


Figure 49. Profile of β-glucosidase activity in fractions eluted from a column of 156a.

CHAPTER 4

EXPERIMENTAL

4.1 GENERAL

4.1.1 Methods

All evaporations were performed under diminished pressure on a Buchi rotary evaporator. Melting points were determined using a Fisher-Johns melting point apparatus and are uncorrected. Infrared (ir) spectra were recorded on a Perkin-Elmer model 710B infrared spectrophotometer, and were calibrated using the 1601 cm^{-1} band of polystyrene film. Optical rotations were obtained using a Perkin-Elmer 141 polarimeter. Low resolution mass spectra (ms) were recorded on a Varian/MAT CH4B or Kratos/AEI MS 50 mass spectrometer. Analytical gas-liquid chromatography was performed on a Hewlett-Packard 5832A gas chromatograph with a 6 ft x 0.125 in stainless steel column packed with 5% OV-17 on 80-100 mesh Chromosorb W (HP). Carbon, hydrogen and nitrogen elemental microanalysis were performed by Mr. P. Borda, Microanalytical Laboratory, University of British Columbia. Copper microanalyses were done by Canadian Microanalytical Ltd., Vancouver, Canada. Analytical thin-layer chromatography (tlc) was done with 0.20 mm pre-coated aluminum back sheets of Silica Gel 60 F_{254} (E. Merck, Darmstadt, Germany). Solvent systems employed for tlc analyses were; A) ethyl acetate-hexane

(3:2), B) ethyl acetate- 2-propanol-water (9:4:2), C) chloroform-methanol (15:1), and D) chloroform-methanol (4:1). For detection of components, tlc sheets were sprayed with: a) 30% sulfuric acid in 95% ethanol followed by heating on a hot plate (for carbohydrates), b) 2.0% ammonium molybdate in 10% sulfuric acid-ethanol solvent, and subsequent heating on a hot plate, c) 2% ninhydrin in acetone and heating on a hot plate (for amino groups), and d) 1% neutral, aqueous potassium permanganate solution (for unsaturated moieties). Flash column chromatography was performed using 230-400 mesh silica gel (Kieselgel 60, E. Merck, Darmstadt, Germany) according to the procedure of Still *et al.*[266]

Workup and purification of reactions involving polysaccharides generally involved exhaustive dialysis (Spectrapor, membrane tubing, M.W. cutoff 6,000-8,000) against distilled water, followed by freeze-drying. Polysaccharide samples were dried for 48 hours at 70°C *in vacuo* (0.05 mm Hg) and stored in Schlenk tubes under nitrogen atmosphere prior to elemental microanalysis.

Ozonolyses were performed at -78°C, using a Welsbach Ozonator (90V, 2 psi input O_2 pressure) ozone source. The ozone was bubbled into the cooled solution *via* a sintered glass bubbling tube until the reaction mixture turned pale blue. The ozone source was turned off and the solution purged with O_2 (g) until colourless. Two equivalents of dimethyl sulfide were added to the reaction mixture, which was allowed to warm to room temperature with stirring for 2 hours.

4.1.2 Nuclear Magnetic Resonance Spectroscopy

^{1}H-nmr: Proton nmr spectra were typically measured at 270 MHz using a home-built unit comprised of an Oxford Instruments 63.4 KG superconducting solenoid, a Nicolet Model 1180 computer (32K) and a Bruker WP-60 console. Where indicated, 400 MHz spectra were recorded on a Bruker WH-400 spectrometer, and 300 MHz spectra on a Varian XL-300 spectrometer. Samples dissolved in deuterated chloroform were referenced relative to internal tetramethylsilane (TMS), those dissolved in deuterium oxide relative to internal sodium 3-trimethylsilylproprionate-2,2,3,3-d^4 (TSP).

^{13}C-nmr: Proton-decoupled carbon-13 nmr spectra were recorded at 100.6 MHz on a Bruker WH-400 spectrometer, or at 75.5 MHz with a Varian XL-300 spectrometer. Spectra were typically obtained at temperatures of 305-310 K, unless otherwise specified. Polysaccharide samples were prepared directly in the 10 mm nmr tube to avoid handling of the viscous or gelatinous materials. Concentrations were typically 5% (w/w), unless further dilution was necessary for dissolution, in which case the concentrations are specified.

4.1.3 Materials

Chemicals and reagents were purchased from suppliers as follows. 1-Allyloxy-2,3-epoxypropane, boron trifluoride-methanol (50%) complex, dimethylsulfide, Dowex 50x8, H^+ (100-200 mesh) ion-exchange resin, hydrogen bromide in acetic

acid (30% w/w), iminodiacetic acid, sodium cyanoborohydride, sodium hydride (60% dispersion in oil) and 10-undecenyl alcohol were obtained from Aldrich Chemical Co. D-Glucosamine hydrochloride was purchased from Sigma Chemical Co. Allyl alcohol and epichlorohydrin were supplied by MC/B Chemical Co. Mercuric cyanide was from ICN Pharmaceuticals Inc., D-lactose from Eastman Kodak Co., and D-glucouronolactone from Eastman Organic Chemicals Ltd. N-Acetyl-D-glucosamine and 1,2:3,4-di-O-isopropylidene-D-galactose were obtained from Koch-Light Laboratories, D-glucose from Fischer Scientific Co., and D-galactose from Merck and Co. BDH supplied the pyridine and Mallinckrodt, Inc. supplied the acetic anhydride. Purification and distillation of reagents were performed according to standard procedures.[267]

Chitosan (from crab shell, N-acetyl <5%) was purchased from Sigma Chemical Co. 2-Hydroxyethylcellulose (medium viscosity, 4500-6500 cPs, 2.0% solution) was obtained from Polysciences, Inc. Xanthan gum (Keltrol) and sodium alginate (Keltone) and locust bean gum were obtained from Kelco Co.

4.2 EXPERIMENTAL FOR CHAPTER 2

4.2.1 General Synthetic Procedures

Synthesis of Acetobromo Sugar Precursors (91-94)

The sugar was stirred in a 3:2 mixture of acetic anhydride/pyridine (10 mL/g sugar) at room temperature overnight

under a drying tube. The bulk of the solvent was then removed, ethanol was added and allowed to react for 15 min to remove residual acetic anhydride. The solvent was removed and the ethanol treatment was repeated. The resulting syrup was crystallized from ethanol to give a mixture of α- and β-peracetylated sugars in yields of 80-95%.

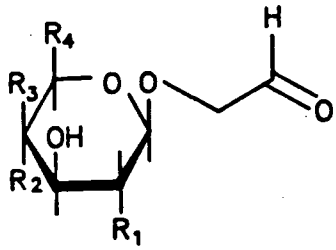
The sugar peracetates, either the crude syrup or the crystalline compound, of either anomer, were then treated with 30% (w/w) hydrogen bromide in acetic acid (@ 4 mL/g of peracetate) for 45 min at room temperature under a drying tube. The reaction mixture was poured into ice-water and extracted twice with equal volumes of chloroform. The chloroform layer was washed with saturated sodium bicarbonate to neutrality, then dried over magnesium sulfate, filtered, and the solvent removed. The resultant syrup was generally easily crystallized with either anhydrous ether or with anhydrous ether/petroleum ether mixtures. These compounds were stored with minimum decomposition for months in a desiccator over sodium hydroxide pellets. Typical yields were 85-90%.

Methyl (1,2,3,4-tetra-O-acetyl)-D-glucuronate, was prepared by a literature method from D-glucurofuranose-3,6-lactone,[272] giving mixtures of the α and β acetates (1:2) in 70-75% yields. Methyl (2,3,4-tri-O-acetyl-1-bromo)-α-D-glucuronate (94) was prepared from the peracetate mixture, using the standard hydrogen bromide-acetic acid (30%) treatment, in 61% yield.

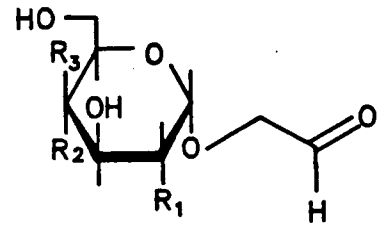
2-Acetamido-2-deoxy-3,4,6-tri-O-acetyl-α-D-glucopyranosyl

chloride (103) was prepared via a standard preparation in which 2-acetamido-2-deoxy-1,3,4,6-tetra-O-acetyl -α-D-glucopyranose was treated with hydrogen chloride saturated acetic anhydride.[268] Workup and storage of the product was similar to that of the acetobromosugars. Yield after crystallization was 79%, m.p. 126-127°C (lit. m.p. 126-127°C).

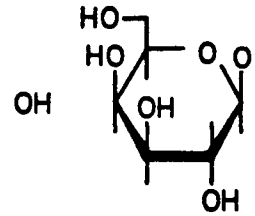
Synthesis of Formylmethyl Glycosides (112-119)



112-116

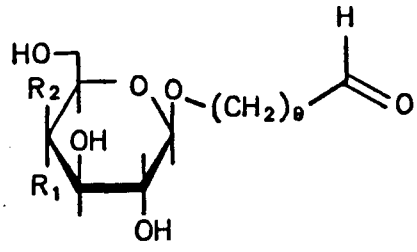


117-119

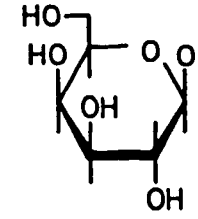
	R_1	R_2	R_3	R_4
112,117	OH	OH	H	CH_2OH
113,118	OH	H	OH	CH_2OH
114	OH	OH	H	CO_2H
116,119	NHAc	OH	H	CH_2OH
115			H	CH_2OH

A solution of the allyl D-glycoside in methanol (with the exception of allyl-β-D-lactose which required methanol-water, 2:1), (@ 5-10 mL/mmol) was cooled to -78°C and saturated with ozone. The pale blue solution was then purged with oxygen until colourless and treated with excess (2-4 equiv.) of dimethylsulfide. The stirred reaction mixture was warmed to ambient temperature over 2 h and concentrated. The syrupy residue was taken up in ethanol, precipitated with ether and decanted. This procedure was repeated, and the resultant gummy precipitate was dried <u>in vacuo</u> (0.05 mm Hg) to give a foamy solid which gave a streak by tlc analysis at R_f (solvent B) lower than starting material. Recovery of material was typically 85-95%, and the product was used directly in the subsequent reaction without further characterization.

Synthesis of 10'-Formylnonyl β-D-glycosides (<u>137</u>-<u>139</u>)



<u>137</u>-<u>139</u>

	R_1	R_2
<u>137</u>	OH	H
<u>138</u>	H	OH
<u>139</u>		H

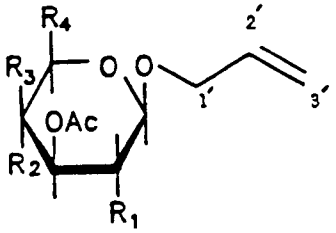
The starting 10'-undecenyl β-D-glycoside was dissolved in methanol (@ 5-10 mL/mmol), (methanol-chloroform, 4:1 for undecenyl β-D-lactose), cooled to -78°C and saturated with

ozone* (until the solution was pale blue). The stirred solution was then purged with oxygen until colourless, treated with excess dimethyl sulfide and warmed to room temperature over 2 h. The solvent was removed and the residue was repeatedly taken up in a minimum of ethanol and precipitated with ether. The gummy residue was dried in vacuo to give a waxy solid, usually in 85-95% yields. It was found that the aldehyde was best used immediately (within 24 h) because a methanol-insoluble residue formed when the aldehyde was left standing at room temperature. This residue dissolved slowly on addition of acetic or dilute hydrochloric acid.

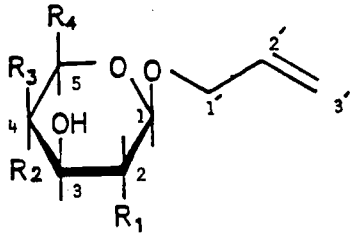
4.2.2 Synthesis of Allyl Glycosides

Allyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (95)

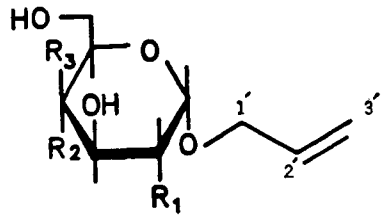
A mixture of 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (91, 18.0 g, 43.8 mmol), mercuric cyanide (12.8 g, 50.4 mmol) and Drierite (14.0 g) in allyl alcohol (90 mL), was stirred at ambient temperature for 24 h. Excess allyl alcohol was evaporated, the residue taken up in chloroform (250 mL) and filtered. The filtrate was washed with saturated brine solution (2x100 mL), dried over magnesium sulfate, filtered and concentrated. The residue was dissolved in methanol, filtered and allowed to crystallize to give 14.5 g (85%) of 95, as a white solid, having a single spot by tlc (solvent A), m.p. 87-88°C (lit. m.p. 86°, 88°C); ^{1}H-nmr (270 MHz): δ ($CDCl_3$), 5.84 (m, 1 H, H-2'), 5.29 (br d, 1 H, J 17.0



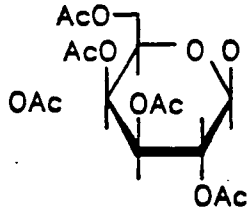
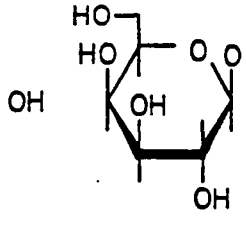
95-98,104



99-102,105



108,109,111

	R_1	R_2	R_3	R_4
95	OAc	OAc	H	CH_2OAc
96	OAc	H	OAc	CH_2OAc
97	OAc	OAc	H	CO_2CH_3
104	NHAc	OAc	H	CH_2OAc
98			H	CH_2OAc
99,108	OH	OH	H	CH_2OH
100,109	OH	H	OH	CH_2OH
101	OH	OH	H	CO_2H
105,111	NHAc	OH	H	CH_2OH
102			H	CH_2OH

Hz, H-3'a), 5.22 (br d, 1 H, J 12.0 Hz, H-3'b), 5.21 (t, 1 H, J 9.5, 9.5 Hz, H-4), 5.11 (t, 1 H, J 9.5, 9.5 Hz, H-3), 5.04 (dd, 1 H, J 9.5, 8.0 Hz, H-2), 4.48 (d, 1 H, J 8.0 Hz, H-1), 4.35 (dd, 1 H, J 14.0, 5.0 Hz, H-1'a), 4.28 (dd, 1 H, J 13.0, 2.0 Hz, H-6), 4.14 (dd, 1 H, J 13.0, 4.5 Hz, H-6a), 4.11 (dd, 1 H, J 14.0, 6.5 Hz, H-1'b), 3.71 (m, 1 H, H-5), 2.10, 2.06, 2.04, 2.02 (4s, 4x3 H, 4 OAc).

Allyl β-D-glucopyranoside (99)

Allyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (95, 12.0 g, 31.0 mmol) was dissolved in dry methanol (75 mL) and the resultant stirred solution was treated with 0.5N methanolic sodium methoxide (10 mL). The reaction was monitored by tlc (solvent B). When the reaction was complete, Dowex 50x8 (H^+, 100-200 mesh) and water (25 mL) were added to neutralize the reaction mixture, which was then filtered and concentrated. The residue was recrystallized from ethanol to give 5.25 g (77%) of 99, m.p. 99-100°C, $[\alpha]_D^{25}$ -39.0°(c 1.10, water) (lit. m.p. 100-101°C, $[\alpha]_D^{25}$ -40.0°in water); ^{1}H-nmr (270 MHz): δ(D_2O), 5.85 (m, 1 H, H-2'), 5.39 (d, 1 H, J 17.0 Hz, H-3'a), 5.30 (d, 1 H, J 10.0 Hz, H-3'a), 4.51 (d, 1 H, J 8.0 Hz, H-1), 4.39 (dd, 1 H, J 13.0, 5.0 Hz, H-1'a), 4.21 (dd, 1 H, J 13.0, 6.5 Hz, H-1'b), 3.93 (br d, 1 H, J 12.0, 2.0 Hz, H-6), 3.71 (dd, 1 H, J 12.0, 6.0 Hz, H-6a), 3.51 (t, 1 H, J 9.0, 9.0 Hz, H-3), 3.45 (dd, 1H, J 9.0, 6.0 Hz, H-5), 3.37 (t, 1 H, J 9.0, 9.0 Hz, H-4), 3.28 (t, 1 H, J 9.0, 8.0 Hz, H-2); ^{13}C-nmr (100.6 MHz): δ(D_2O), 131.8 (C-2'), 117.0 (C-3'), 99.6 (C-1), 74.21 (C-5), 74.17 (C-3), 71.5 (C-2),

68.0 (C-4 or 1'), 68.9 (C-1' or 4), 59.2 (C-6).

Allyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside (96)

2,3,4,6-Tetra-O-acetyl-α-D-galactopyranosyl bromide (92, 10.00 g, 24.3 mmol) was stirred in anhydrous allyl alcohol (50 mL) with mercuric cyanide (6.76 g, 26.6 mmol) and Drierite (7.0 g) under a drying tube, at ambient temperature for 24 h. Ether (50 mL) was added, the suspension was filtered and the solvent was removed. The residue was taken up in chloroform (100 mL), and the organic layer was washed with saturated brine (2x100 mL), dried over magnesium sulfate, filtered and concentrated. Flash column chromatography of the resultant residue gave 8.01 g (85%) of 96 as a syrup, which was homogeneous by tlc (solvent A); ^{1}H-nmr (270 MHz): $\delta(CDCl_3)$, 5.85 (m, 1 H, H-2'), 5.40 (d, 1 H, J 3.5 Hz, H-4), 5.34-5.16 (m, 3 H, H-2,3'a,3'b), 5.04 (dd, 1 H, J 10.0, 3.5 Hz, H-3), 4.53 (d, 1 H, J 8.0 Hz, H-1), 4.36 (dd, 1 H, J 13.0, 4.5 Hz, H-1'a), 4.23-4.05 (m, 3 H, H-1'b,6,6a), 3.91 (t, 1 H, J 7.0, 7.0 Hz, H-5), 2.16, 2.07, 2.00 (3s, 12 H, 4 OAc).

Allyl β-D-galactopyranoside (100)

To a stirred solution of allyl 2,3,4,6-tetra-O- acetyl-β-D-galactopyranoside (96, 7.90 g, 20.4 mmol) in anhydrous methanol (50 mL), was added a solution of 0.5N sodium methoxide/methanol (5 mL). When the reaction was complete (tlc), the mixture was neutralized with Dowex 50x8 (H^+, 100-200 mesh) ion-exchange resin, filtered and concentrated. The residue was crystallized from ethanol-ether to

give 4.30 g (96%) of 100 as a white solid, which was homogeneous by tlc (solvent A), m.p. 100-101°C, $[\alpha]_D^{25}$ -11.0° (c 1.02, water) (lit. m.p. 102-103°C, $[\alpha]_D^{25}$ -10.9° in water); ^{1}H-nmr (270 MHz): $\delta(D_2O)$, 5.97 (m, 1 H, H-2'), 5.39 (d, 1 H, J 16.0 Hz, H-3'a), 5.28 (dd, 1 H, J 10.0, H-3'b), 4.43 (d, 1 H, J 8.0 Hz, H-1), 4.40 (dd, 1 H, J 13.0, 6.0 Hz, H-1'a), 4.22 (dd, 1 H, J 13.0, 7.0 Hz, H-1'b), 3.92 (d, 1 H, J 3.0 Hz, H-4), 3.77 (dd, 1 H, J 11.0, 7.0 Hz, H-6), 3.75 (dd, 1 H, J 11.0, 4.0 Hz, H-6a), 3.67 (dd, 1 H, J 4.0, 7.0 Hz, H-5), 3.64 (dd, 1 H, J 10.0, 4.0 Hz, H-3), 3.53 (dd, 1 H, J 10.0, 8.0 Hz, H-2); ^{13}C-nmr (100.6 MHz): $\delta(D_2O)$, 131.9 (C-2'), 116.9 (C-3'), 100.2 (C-1), 73.4 (C-5), 71.2 (C-3), 69.1 (C-2), 68.9 (C-1'), 67.0 (C-4), 59.3 (C-6).

Methyl (allyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (97)

Methyl (2,3,4-tri-O-acetyl-α-D-glucopyranosyl bromide)uronate (93, 16.0 g, 40.0 mmol) was stirred under anhydrous conditions with mercuric cyanide (12.5 g, 49.0 mmol) and Drierite (13.0 g) in dry allyl alcohol (100 mL) for 19 h. The reaction mixture was filtered and concentrated, and the resultant syrupy residue was dissolved in chloroform (150 mL). The chloroform solution was washed with saturated brine (2x100 mL), dried over magnesium sulfate, filtered and concentrated. The solid residue was recrystallized from methanol to give crystalline 97 (13.8 g, 92%), m.p. 137-138°C, $[\alpha]_D^{25}$ -33.3° (c 1.14, water); ^{1}H-nmr (270 MHz): $\delta(CDCl_3)$, 5.74 (m, 1 H, H-2'), 5.32-5.16 (m, 4 H,

H-3,4,3'a,3'b), 5.04 (t, 1 H, J 9.0, 8.0 Hz, H-2), 4.60 (d, 1 H, J 8.0 Hz, H-1), 4.36 (dd, 1 H, J 13.5, 5.0 Hz, H-1'a), 4.09 (dd, 1 H, J 13.5, 6.5 Hz, H-1'b), 4.03 (d, 1 H, J 9.0 Hz, H-5), 3.76 (s, 3 H, CO_2CH_3), 2.06, 2.01 (2s, 9 H, 3 OAc); Anal. Calcd. for $C_{16}H_{22}O_{10}$: C, 51.34; H, 5.92. Found: C, 51.39; H, 5.90.

Allyl β-D-glucopyranuronic acid (101)

Methyl (allyl 2,3,4-tri-O-acetyl-β-D-glucopyranosid)uronate (97, 8.5 g, 25.5 mmol) was dissolved in dry methanol (100 mL) and treated with 0.5N sodium methoxide in methanol (5.0 mL). After one hour, all starting material was consumed, as shown by tlc (solvent B), and two new spots were evident (R_f 0.62, major and R_f 0.43, minor). After the addition of 2N sodium hydroxide (aq) solution (15 mL, 30 mmol) and the resultant reaction mixture was stirred for 1 h, a single spot (R_f 0.43) was observed by tlc. Dowex 50x8 (H^+, 100-200 mesh) ion-exchange resin was added to acidify the reaction mixture, which was then filtered, decolourized and concentrated. Attempts to crystallize the residue were unsuccessful, and after drying in vacuo, 5.2 g (87%) of a discoloured, foamy solid was obtained, ^{13}C-nmr (100.6 MHz): δ(D_2O), 170.4 (C-6), 131.7 (C-2'), 117.1 (C-3'), 99.7 (C-1), 73.7 (C-3), 72.8 (C-5), 71.1 (C-2), 69.6 (C-1' or C-4), 69.2 (C-4 or C-1').

Allyl 2',3',6'-tri-O-acetyl-4'-O-(2,3,4,6-tetra-O-acetyl β-D-galactopyranosyl)-β-D-glucopyranoside, (allyl 2,2',3,3'4,6,6'-hepta-O-acetyl-β-D-lactoside) (98)

2',3',6'-Tri-O-acetyl-4'-O-(2,3,4,6-tetra-O-acetyl-β-D-

galactopyranosyl)-α-D-glucopyranosiyl bromide (94, 1.60, 2.3 mmol) was stirred in the dark with mercuric cyanide (0.64 g, 2.5 mmol) and Drierite (1.0 g) in dry allyl alcohol (20 mL) for 24 h. Excess alcohol was removed and the residue taken up in dichloromethane chloride (50 mL), washed with saturated brine (2x25 mL), dried over magnesium sulfate, filtered and concentrated, to yield 1.50 g of a crude syrup. Tlc (solvent A) showed a major spot (R_f 0.70) and some minor lower R_f spots. Flash column chromatography gave 1.12 g (72%) of 98 as a foamy solid, which consisted of a single component by tlc. Large scale preparations with 10 g of "acetobromolactose" gave equally good or better yields of 98; ^{1}H-nmr (270 MHz): $\delta(CDCl_3)$, 5.94 (m, 1 H, H-2"), 5.35 (d, 1 H, J 3.0 Hz, H-4), 5.32-5.16 (m, 3 H, H-3',3"a,3"b), 5.12 (dd, 1 H, J 10.0, 8.0 Hz, H-2), 4.97 (dd, 1 H, J 10.0, 3.0 Hz, H-3), 4.94 (t, 1 H, J 9.0, 8.0 Hz, H-2'), 4.53 (d, 1 H, J 8.0 Hz, H-1 or 1'), 4.50 (d, 1 H, J 8.0 Hz, H-1' or 1), 4.47 (dd, 1 H, J 12.0, 2.0 Hz, H-6'), 4.31 (dd, 1 H, J 13.0, 5.0 Hz, H-1"a), 4.15-4.04 (m, 4 H, H-6,6a, 6'a, 1"b), 3.90 (t, 1 H, J 7.0, 7.0 Hz, H-5), 3.82 (t, 1 H, J 9.0, 9.0 Hz, H-4'), 3.61 (m, 1 H, H-5'), 2.16, 2.13, 2.07, 2.05, 1.96 (5s, 21 H, 7 OAc).

Allyl 4'-O-(β-D-galactopyranosyl)-β-D-glucopyranoside, (Allyl β-D-lactoside) (102)

Allyl 2,2',3,3',4,6,6'-hepta-O-acetyl-β-D-lactoside (98, 11.0 g, 16.0 mmol) was dissolved in anhydrous methanol (50 mL) and the resultant stirred solution was treated with 0.5N sodium methoxide/methanol solution (5.0 mL). When the reac-

tion was complete (tlc, solvent B), water (25 mL) and Dowex 50x8 (H^+, 100-200 mesh) were added until the solution was neutral. The suspension was filtered, the filtrate was concentrated and the residue was recyrstallized from ethanol-water to give 4.9 g (80%) of a white solid, which was homogeneous by tlc (solvent B); m.p. 169-170°C (lit. m.p. 170-171°C); ^{1}H-nmr (270 MHz): $\delta(D_2O)$, 5.94 (m, 1 H, H-2"), 5.33 (br d, 1 H, J 17.0 Hz, H-3'a), 5.24 (br d, 1 H, J 10.0 Hz, H-3'b), 4.49 (d 1 H, J 8.0 Hz, H-1 or 1'), 4.40 (d, 1 H, J 7.5 Hz, H-1' or 1), 4.35 (dd, 1 H, J 12.5, 5.5 Hz, H-1"a), 4.19 (dd, 1 H, J 12.5, 6.0 Hz, H-1"b), 3.98-3.48 (m, 11 H, H-2,2',3,3',4,4',5',6,6a,6',6'a), 3.30 (br t, 1 H, J 7.0, 6.0 Hz, H-5); ^{13}C-nmr (100.6 MHz): $\delta(D_2O)$, 131.8 (C-2"), 117.0 (C-3"), 101.2 (C-1), 99.4 (C-1'), 76.8 (C-4'), 73.6 (C-5), 73.0 (C-5' or 3'), 72.7 (C-3' or 5'), 71.1 (C-2' or 3), 70.9 (C-3 or 2'), 69.2 (C-2), 68.9 (C-1"), 66.8 (C-4), 59.5 (C-6'), 59.3 (C-6).

Allyl 2-acetamido-2-deoxy-3,4,6-tri-O-acetyl -β-D-glucopyranoside (104)

2-Acetamido-2-deoxy-3,4,6-tri-O-acetyl-α-D-glucopyranosyl chloride (103, 14.0 g, 38.3 mmol) was stirred with mercuric cyanide (10.8 g, 42.5 mmol) and Drierite (10 g) in dry allyl alcohol (75 mL) for 16 h under anhydrous conditions. The reaction mixture was diluted with chloroform (50 mL), filtered and concentrated to a thick syrup. The syrup was taken up in chloroform (150 mL), filtered and the organic phase was washed with saturated brine solution (2x100 mL), dried over

magnesium sulfate, filtered and concentrated. The residue was crystallized from ethyl acetate/petroleum ether to give 11.17 g (75%) of a white crystalline solid, which consisted of one component by tlc analysis (solvent A); m.p. 161-162°C (lit. m.p. 160°, 162-163°C); ^{1}H-nmr (270 MHz): $\delta(CDCl_3)$, 5.84 (m, 1 H, H-2'), 5.76 (d, 1 H, J 8.5 Hz, NHAc), 5.31 (t, 1 H, J 10.0, 10.0 Hz, H-4), 5.28 (br d, 1 H, J 18.0 Hz, H-3'a), 5.21 (br d, 1 H, J 11.0 Hz, H-3'b), 5.08 (t, 1 H, J 10.0, 10.0 Hz, H-3), 4.73 (d, 1 H, J 8.0 Hz, H-1), 4.35 (dd, 1 H, J 13.0, 5.0 Hz, H-1'a), 4.28 (dd, 1 H, J 13.0, 5.0 Hz, H-6), 4.15 (dd, 1 H, J 13.0, 2.0 Hz, H-6a), 4.10 (dd, 1 H, J 13.0, 6.0 Hz, H-1'b), 3.90 (dt, 1 H, J 10.0, 8.5, 8.0 Hz, H-2), 3.72 (m, 1 H, H-5), 2.10, 2.04, 2.03 (3s, 3 H each, 3 OAc), 1.95 (s, 3 H, NHAc).

Allyl 2-acetamido-2-deoxy-β-D-glucopyranoside (105)

De-O-acetylation of allyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside (104, 10.0 g, 25.8 mmol) was done in methanol with 0.5N methanolic sodium methoxide (5.0 mL). When the reaction was complete (tlc), the mixture was neutralized with Dowex 50x8 (H^+, 100-200 mesh) ion-exchange resin, filtered and concentrated. The residue was recrystallized from methanol to give 4.96 g (74%) of 105 as a solid, which consisted of one component by tlc (solvent B), m.p. 170-171°C, $[\alpha]_D^{25}$ -31.9° (c 0.58, water), (lit. m.p. 171-172°C, $[\alpha]_D^{25}$ -33.9° in water); ^{1}H-nmr (270 MHz): $\delta(D_2O)$, 5.90 (m, 1 H, H-2'), 5.31 (d, 1 H, J 17.0 Hz, H-3'a), 5.26 (d, 1 H, J 10.0 Hz, H-3'b), 4.56 (d, 1 H, J 8.0 Hz, H-1),

4.34 (dd, 1 H, J 13.0, 5.0 Hz, H-1'a), 4.16 (dd, 1 H, J 13.0, 6.0 Hz, H-1'b), 3.93 (d, 1 H, J 12.0 Hz, H-6), 3.74 (dd, 1 H, J 12.0, 5.0 Hz, H-6a), 3.72 (br t, 1 H, J 9.0, 8.0 Hz, H-2), 3.43-3.58 (m, 3 H, H-3,4,5); ^{13}C-nmr (100.6 MHz): δ(D_2O), 173.0 ($\underline{C}OCH_3$), 132.3 (C-2'), 116.5 (C-3'), 98.8 (C-1), 74.5 (C-5), 72.5 (C-3), 68.9 (C-1' or 4), 68.7 (C-4 or 1'), 59.5 (C-6), 54.2 (C-2), 20.8 ($CO\_C\underline{H}_3$).

Allyl α-D-glucopyranoside (108)

Anhydrous D-glucose (106, 20.0 g, 110 mmol) was stirred with Dowex 50x8 (H^+, 100-200 mesh) in dry allyl alcohol (200 mL) at reflux temperature for 90 min. The reaction mixture was filtered and concentrated to afford a syrupy residue. Crystallization of this material from absolute ethanol gave 4.1 g (17%) of 108. Decolourization and reprocessing of the mother liquor provided an additional 4.4 g (18%), giving an overall 8.5 g (35%) of a product, which exhibited one spot by tlc analysis (R_f 0.486, solvent B); m.p. 97-98°C, $[\alpha]_D^{25}$ +136.4° (c, 1.06, water), (lit. m.p. 95-97°C; $[\alpha]_D^{25}$ +133.8° in water); ^{1}H-nmr (270 MHz): δ(D_2O), 5.99 (m, 1 H, H-2'), 5.38 (br d, 1 H, J 17.0 Hz, H-3'a), 5.27 (br d, 1 H, J 10.0 Hz, H-3'b), 4.98 (d, 1 H, J 3.5 Hz, H-1), 4.24 (dd, 1 H, J 12.0, 5.0 Hz, H-1'a), 4.07 (dd, 1 H, J 12.0, 6.0 Hz, H-1'b), 3.89-3.66 (m, 4 H, H-3,5,6,6a), 3.57 (dd, 1 H, J 10.0, 3.5 Hz, H-2), 3.42 (t, 1 H, J 10.0, 10.0 Hz, H-4); ^{13}C-nmr (100.6 MHz): δ(D_2O), 132.1 (C-2'), 116.5 (C-3'), 95.7 (C-1), 71.5 (C-3), 70.2 (C-5), 69.6 (C-2), 68.0 (C-4), 66.8 (C-1'), 59.0 (C-6).

Allyl α-D-galactopyranoside (109)

D-Galactose (107, 20.0 g, 110 mmol) was suspended in dry allyl alcohol (200 mL) and stirred with Dowex 50x8 (H^+, 100-200 mesh, 12.0 g) at reflux temperature for 90 min. The reaction mixture was filtered and concentrated, and the resultant syrupy residue was crystallized from absolute ethanol to give 10.2 g (42%) of a white crystalline solid. Further recrystallization from absolute ethanol gave material homogeneous by tlc analysis (solvent B); m.p. 146-147°C; $[\alpha]_D^{25}$ +178.0° (c 0.99, water); (lit. m.p. 143-145°C, $[\alpha]_D^{25}$ +181.3°, in water); ^{1}H-nmr (270 MHz): $\delta(D_2O)$, 5.99 (m, 1 H, H-2'), 5.39 (d, 1 H, J 17.0 Hz, H-3'a), 5.28 (d, 1 H, J 11.0 Hz, H-3'b), 4.89 (d, 1 H, J 2.0 Hz, H-1), 4.25 (dd, 1 H, J 13.0, 5.0 Hz, H-1'a), 4.08 (dd, 1 H, J 13.0, 6.0, H-1'b), 3.98 (br s, 1 H, J 1.0 Hz, H-4), 3.96 (d, 1 H, J 8.0 Hz, H-5), 3.89-3.80 (m, 2 H, H-2,3), 3.77-3.63 (m, 2 H, H-6,6a); ^{13}C-nmr (100.6 MHz): $\delta(D_2O)$, 132.2 (C-2'), 116.5 (C-3'), 96.1 (C-1), 69.3 (C-5), 68.0 (C-3), 67.7 (C-4), 66.9 (C-2 or 1'), 66.7 (C-1' or 2), 59.6 (C-6).

Allyl 2-acetamido-2-deoxy-α-D-glucopyranoside (111)

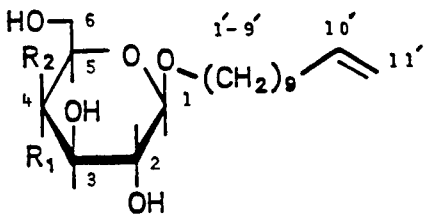
2-Acetamido-2-deoxy-glucose (110, 25 g, 113 mmol) was suspended in dry allyl alcohol (350 mL) and the resultant mixture was treated with boron trifluoride etherate complex (2.5 mL). The reaction mixture was refluxed for 2 h and allowed to stand at room temperature for 24 h. Removal of solvent left a crystalline residue which was recrystallized from ethanol/ether to give 13.9 g (47%) of a white crystal-

line solid, which consisted of a major spot (R_f 0.417, solvent B) and a minor spot (R_f 0.33) by tlc analysis. The lesser component was shown by ^{1}H-nmr to be the β-isomer (allyl 2-acetamido-2-deoxy-β-D-glucopyranoside), which was present in ~1:8 ratio with the α-isomer. Subsequent recrystallizations provided 111 as a single component by tlc analysis (R_f 0.417, solvent B); m.p. 172-173°C; $[\alpha]_D^{25}$ +147.6° (c 1.12, water); (lit. m.p. 172-174°C, $[\alpha]_D^{25}$ +148.8° in water); ^{1}H-nmr (270 MHz): δ(D_2O), 5.94 (m, 1 H, H-2'), 5.36 (d, 1 H, J 16.5 Hz, H-3'a), 5.27 (d, 1 H, J 11.0 Hz, H-3'b), 4.93 (d, 1 H, J 3.0 Hz, H-1), 4.23 (dd, 1 H, J 13.0, 5.5 Hz, H-1'a), 4.03 (dd, 1 H, J 13.0, 6.5 Hz, H-1'b), 3.95-3.63 (m, 5 H, H-2,3,5,6,6a), 3.49 (t, 1 H, H-4); ^{13}C-nmr (100.6 MHz) δ(D_2O), 172.7 ($\underline{C}OCH_3$), 132.1 (C-2'), 116.2 (C-3'), 94.5 (C-1), 70.3 (C-5), 69.4 (C-3), 68.5 (C-4), 66.8 (C-1'), 59.9 (C-6), 52.0 (C-2), 20.3 ($CO\underline{C}H_3$).

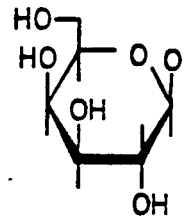
4.2.3 Synthesis of 10'-Undecenyl β-D-Glycosides

10'-Undecenyl β-D-glucopyranoside (134)

A suspension of 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (91, 20.0 g, 48.7 mmol), 10-undecenol (16.58 g, 97.3 mmol), mercuric cyanide (13.53 g, 53.6 mmol) and Drierite (14 g) were stirred in chloroform (125 mL) at reflux for 10 h. The reaction mixture was filtered, washed with saturated brine solution (2x75 mL), dried over magnesium sulfate, filtered and concentrated. The residue was taken up in



134-136

	R_1	R_2
134	OH	H
135	H	OH
136		H

anhydrous methanol and treated with 0.5N methanolic sodium methoxide (10.0 mL). When the reaction was complete (tlc, solvent D), Dowex 50x8 (H^+, 100-200 mesh) ion-exchange resin was added to neutralize the solution, which was then filtered. The filtrate was concentrated and the resultant residue was subjected to liquid chromatography (chloroform-methanol, 5:1) to afford 12.9 g (80%) of a waxy solid, 134, ^{1}H-nmr (270 MHz): $\delta(CD_3OD)$, 5.79 (m, 1 H, H-10'), 4.96 (br d, 1 H, J 17.0, 1.5 Hz, H-11'a), 4.90 (d, 1 H, J 10.0 Hz, H-11'b), 4.26 (d, 1 H, J 8.0 Hz, H-1), 3.86 (dd, 1 H, J 11.5, 2.4 Hz, H-6a), 3.69 (dd, 1 H, J 11.5, 5.2 Hz, H-6b), 3.55 (m, 2 H, H-1'a,b), 3.38 (t, 1 H, J 9.0, 8.5 Hz, H-3), 3.32 (t, 1 H, J 9.0, 8.5 Hz, H-4), 3.27 (m, 1 H, H-5), 3.19 (t, 1 H, J 8.5, 8.0 Hz, H-2), 2.04 (m, 2 H, H-9'a,b), 1.63 (m, 2 H, H-2'a,b), 1.43-1.29 (m, 12 H, alkyl protons); ^{13}C-nmr (100.6

MHz): $\delta(CD_3OD)$, 138.3 (C-10'), 112.8 (C-11'), 102.5 (C-1), 76.3 (C-5), 76.0 (C-3), 73.3 (C-2), 70.0 (C-4), 69.1 (C-1'), 61.1 (C-6), 32.9 (C-9'), 29.0-28.2 and 25.4 (C-2' to 8'); ms (fab), m/z: 333 $(M+H)^+$.

10'-Undecenyl β-D-galactopyranoside (135)

A mixture of 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide (92, 17.9 g, 43.6 mmol), 10-undecenal (14.8 g, 87.6 mmol), mercuric cyanide (12.1 g, 48.0 mmol) and Drierite (18 g) in chloroform (125 mL) was stirred at reflux for 9 h under anhydrous conditions. The reaction mixture was cooled, diluted with ether (60 mL), filtered and concentrated. The residue was dissolved in chloroform (200 mL), and the chloroform layer was washed with saturated brine (2x100 mL), dried over magnesium sulfate and filtered. After the solvent was removed, the residue was dissolved in anhydrous methanol (80 mL) and deacetylated with 0.5N sodium methoxide in methanol (10.0 mL). When the reaction was complete (tlc, solvent C), Dowex 50x8 (H^+, 100-200 mesh) was added and the mixture was filtered and concentrated. The crude product (16.0 g) was chromatographed (chloroform-methanol, 5:1) to give 12.2 (84%) of a waxy solid having a single spot by tlc analysis (solvent D), ^1H-nmr (270 MHz): $\delta(CD_3OD)$, 5.83 (m, 1 H, H-10'), 4.99 (br d, 1 H, J 17.0 Hz, H-11'a), 4.93 (br d, 1 H, J 17.0 Hz, H-11'b), 4.24 (d, 1 H, J 7.2 Hz, H-1), 3.90 (m, 3 H, H-1'a,1'b,5), 3.77 (d, 1 H, J 6.2 Hz, H-4), 3.63-3.45 (m, 4 H, H-6,6b,2,3), 2.04 (m, 2 H, H-9'a,b), 1.63 (m, 2 H, H-2'a,b), 1.43-1.30 (br s, 12 H, alkyl protons); ^{13}C-nmr

(100.6 MHz): δ(CD_3OD), 138.2 (C-10'), 112.8 (C-11'), 103.1 (C-1), 74.65 (C-5), 73.3 (C-3), 70.8 (C-2), 69.0 (C-4 or 1'), 68.5 (C-1' or 4), 60.7 (C-6), 32.9 (C-9'), 29.0-28.2 and 25.2 (C-2' to 8'); ms (fab), m/z: 333 $(M+H)^+$.

10"-Undecenyl 4'-O-(β-D-galactopyranosyl)-β-D-glucopyranoside, (10"-Undecenyl β-D-lactoside) (136)

"Acetobromolactose" (94, 25.0 g, 35.8 mmol), 10-undecenol (12.2 g, 72.0 mmol), mercuric cyanide (9.94 g, 39.4 mmol) and Drierite were stirred together in chloroform (100 mL) at reflux for 11 h. The reaction mixture was cooled and filtered, and the filtrate was washed with saturated brine solution (2x75 mL), dried over magnesium sulfate and concentrated. The residue was dissolved in anhydrous methanol and the resultant stirred solution was treated with 0.5N sodium methoxide in methanol (10.0 mL). When the reaction was complete (tlc, solvent D), the reaction was neutralized with Dowex 50x8 (H^+, 100-200 mesh) ion-exchange resin and filtered. The solvent was removed and the residue was precipitated from methanol to yield 14.9 g (84%) of 136 as a waxy solid, ^{1}H-nmr (270 MHz): δ(CD_3OD), 5.80 (m, 1 H, H-10"), 4.98 (br d, 1 H, J 17.0 Hz, H-11"a), 4.93 (d, 1 H, J 10.5 Hz, H-11"b), 4.42 (br d, 1 H, J 7.5 Hz, H-1'), 4.28 (d, 1 H, J 8.0 Hz, H-1), 3.94-3.83 (m, 3 H, H-6a,6b,4), 3.80 (br d, 1 H, J 11.5 Hz, H-6'a), 3.74 (dd, 1 H, J 11.5, 4.5 Hz, H-6'b), 3.70-3.49 (m, 5 H, H-2,3,3',4',5), 3.46 (m, 1 H, H-5'), 3.30 (t, 1 H, J 9.0, 8.0 Hz, H-2'), 2.04 (m, 2 H, CH_2-9"), 1.63 (m, 2 H, CH_2-2"), 1.42-1.29 (br s, 12 H, alkyl protons);

^{13}C-nmr (100.6 MHz): δ(CD_3OD), 138.3 (C-10"), 113.2 (C-11"), 103.0 (C-1), 102.2 (C-1'), 79.0 (C-4'), 75.1 (C-5 or 5' or 3'), 74.5 (C-5' and 3' or 5), 72.8 (C-2' or 3), 72.78 (C-3 or 2'), 70.8 (C-2), 69.7 (C-4), 68.4 (C-1"), 60.7 (C-6), 60.2 (C-6'), 32.9 (C-9'), 28.9-28.2 and 25.1 (C-2" to 8"); ms (fab), m/z: 495 $(M+H)^+$, 333.

4.2.3 Synthesis of Branched Chitosan Derivatives

General Procedure for the Preparation of N-[2'-O-(D-glycopyranosyl)ethyl]chitosans

Chitosan flakes were dissolved in 5.0% aqueous acetic acid (@ 10 mL/1.0 mmol) with stirring. A solution of the aldehyde (0.5-3.0 molar equivalents) in 5.0% aqueous acetic acid (10-15 mL) was added, followed by treatment with sodium cyanoborohydride (@ 4.0 molar equivalents) for 24 h. The reaction mixture was then dialyzed against distilled water (6x2 L) for 6 days, filtered through a medium pore glass frit filter and freeze dried. Yields varied between 55 and 95%.

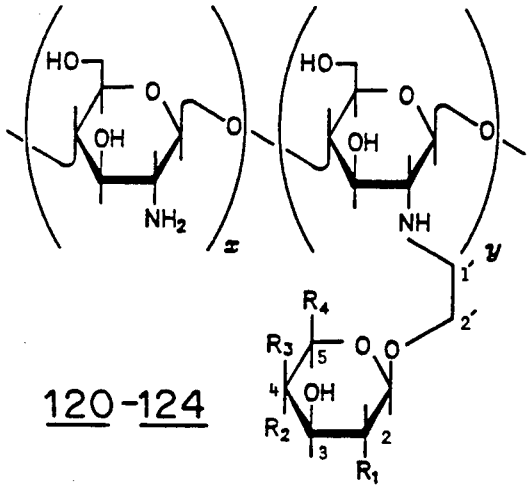
N-[2'-O-(β-D-glucopyranosyl)ethyl]chitosan (120)

a) Chitosan (0.50 g, 3.11 mmol) reacted with formylmethyl β-D-glucopyranoside (112, 2.10 g, 9.50 mmol) to give 1.08 g, (95%) of compound 120a.

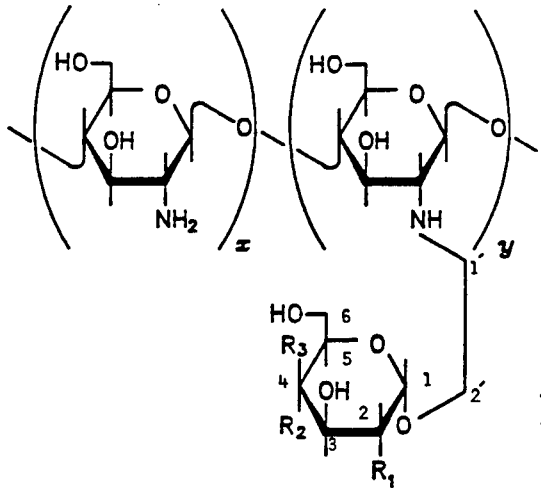
Anal. Calcd. for $[(C_{14}H_{25}NO_{10})_{1.00}]\cdot 0.51H_2O$: C, 44.66; H, 6.91; N, 3.72. Found: C, 44.67; H, 6.92; N, 3.62.

N-[2'-O-(β-D-galactopyranosyl)ethyl]chitosan (121)

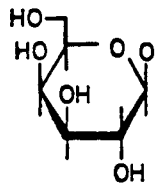
a) Chitosan (0.65 g, 4.04 mmol) was treated with the

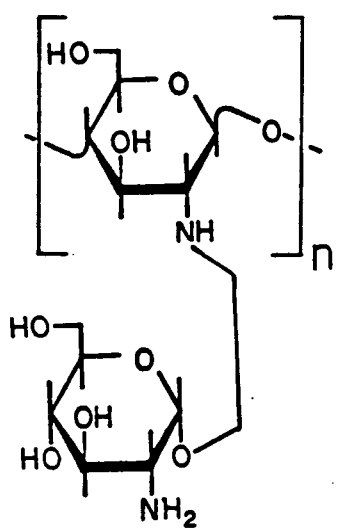


120-124

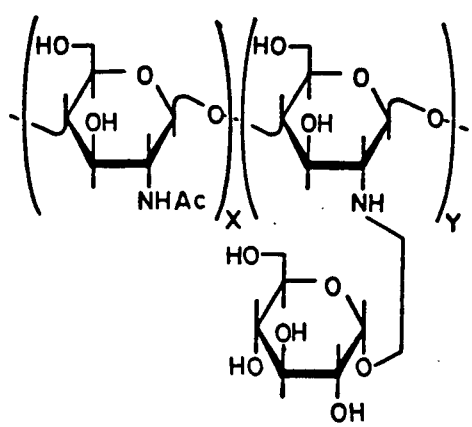


125-127

	R_1	R_2	R_3	R_4
120,125	OH	OH	H	CH_2OH
121,126	OH	H	OH	CH_2OH
122	OH	OH	H	CO_2H
124,127	NHAc	OH	H	CH_2OH
123	OH		H	CH_2OH



129



130

acetaldehydo-glycoside 113 (2.7 equiv) to give 0.86 g (58%) of derivative 121a.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.04}(C_{14}H_{25}NO_{10})_{0.96}] \cdot 2.3 H_2O$: C, 41.12; H, 7.25; N, 3.43. Found: C, 41.12: H,6.94: N, 3.51.

b) When chitosan (0.55 g, 3.42 mmol) was reacted with the aldehyde 113 (1.00 g, 4.50 mmol), 0.86 g (83%) of lyophilized product 121b was obtained.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.33}(C_{14}H_{25}NO_{10})_{0.67}] \cdot 0.50 H_2O$: C, 43.34; H, 7.12; N, 4.45. Found: C, 43.35; H, 7.11; N, 4.46.

c) Chitosan (0.65 g, 4.04 mmol) reacted with aldehydo sugar 113 (0.67 g, 3.00 mmol) to yield 0.82 g (81%) of compound 121c.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.62}(C_{14}H_{25}NO_{10})_{0.38}] \cdot 0.63 H_2O$: C, 43.31; H, 7.03; N, 5.59. Found: C, 43.31; H, 6.86; N, 5.59.

N-[2'-O-(β-D-glucuronopyranosyl)ethyl]chitosan (122)

a) Coupling of the aldehyde 114 (1.75 g, 7.5 mmol) to chitosan (0.40 g, 2.5 mmol) gave a precipitated product after 15 min. The solution was made basic (pH~8), dialyzed as usual and freeze-dried. The lyophilized product was dissolved in 0.5N sodium hydroxide (10 mL), precipitated with ethanol, filtered and washed with methanol to give, after drying, 0.67 g (67%) of 122a.

Anal. Calcd. for $[(C_{14}H_{22}NO_{11}Na)] \cdot 0.32H_2O$: C, 41.40; H, 5.40; N, 3.45. Found: C, 41.40; H, 5.40; N, 3.18.

b) Chitosan (0.40 g, 2.5 mmol), when treated with the aldehydo-sugar 114 (0.60 g, 2.6 mmol), produced a viscous solution after 24 h. The solution was dialyzed and freeze-dried to yield 0.67 g (81%) of derivative 122b.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.33}(C_{14}H_{23}NO_{11})_{0.67}]\cdot 1.13$ H_2O: C, 41.61; H, 6.47; N, 4.26. Found: C, 41.61; H, 6.43; N, 4.30.

N-{2"-O-[4'-O-(β-D-galactopyranosyl)-β-D-glucopyranosyl] ethyl}chitosan (123)

a) Chitosan (0.40 g, 2.5 mmol) was treated with acetaldehydo β-D-lactoside 115 (3.0 g, 7.8 mmol) to give 1.30 g (95%) of derivative 123a.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.11}(C_{20}H_{36}NO_{15})_{0.89}]\cdot 0.22H_2O$: C, 44.90; H, 6.83; N, 2.84. Found: C, 44.89; H, 6.84; N, 2.83.

b) When chitosan (0.65 g, 4.0 mmol) was treated with compound 115 (2.30 g, 6.00 mmol), 1.52 g (86%) of derivative 123b was obtained.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.24}(C_{20}H_{36}NO_{15})_{0.76}]>0.66$ H_2O: C, 44.12; H, 6.92; N, 3.09. Found: C, 44.12; H, 7.17; N, 3.09.

c) When chitosan (0.65 g, 4.04 mmol) was reacted with the aldehyde 115 (1.15 g, 3.00 mmol), 1.21 g (97%) of compound 123c was isolated.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.65}(C_{20}H_{36}NO_{15})_{0.35}]\cdot 0.74$ H_2O: C, 43.15; H, 7.00; N, 4.62. Found: C, 43.15; H, 7.19; N, 4.63.

d) Chitosan (0.80 g, 5.00 mmol) and 115 (1.00 g, 2.60 mmol) were reacted to give 1.36 g (93%) of derivative 123d. Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.67}(C_{20}H_{36}NO_{15})_{0.33}]\cdot 0.44H_2O$: C, 43.84; H, 6.92; N, 4.82. Found: C, 43.84; H, 7.27; N, 4.84.

e) Chitosan (0.60 g, 3.70 mmol) and 115 (0.50 g, 1.30 mmol) were reacted to give 0.80 g (87%) of 123e.

Anal. Calcd for $[(C_6H_{11}NO_4)_{0.76}(C_{20}H_{36}NO_{15})_{0.24}]\cdot 0.49H_2O$: C, 43.47; H, 6.97; N, 5.41. Found: C, 43.46; H, 7.29; N, 5.37.

N-[2'-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl) ethyl]chitosan (124)

a) Reaction of chitosan (0.30 g, 1.9 mmol) with the acetaldehydo-β-D-glycoside 116 (1.50 g, 5.6 mmol) gave 0.67 g (85%) of the derivative 124a.

Anal. Calcd. for $[(C_{16}H_{28}N_2O_{10})]\cdot 0.85H_2O$: C, 45.36; H, 7.02; N, 6.61. Found: C, 45.36; H, 7.33; N, 6.43.

N-(2'-O-(α-D-glucopyranosyl)ethyl]chitosan (125)

a) Chitosan (0.65 g, 4.04 mmol) when treated with acetaldehydo α-D-glucopyranoside 117 (2.65 g, 11.9 mmol) gave 1.42 g (96%) of derivative 125a.

Anal. Calcd. for $[(C_{14}H_{25}NO_{10})_{1.00}]\cdot 0.61H_2O$: C, 44.44; H, 6.94; N, 3.70. Found: C, 44.45; H, 6.85; N, 3.40.

b) Chitosan (0.60 g, 3.73 mmol) was coupled with the aldehyde 117 (1.24 g, 5.59 mmol) to give 0.66 g (61%) of 125b.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.41}(C_{14}H_{25}NO_{10})_{0.59}]\cdot 0.87 H_2O$: C, 43.14; H, 7.04; N, 4.69. Found: C, 43.14; H, 7.10; N,

4.69.

c) Chitosan (0.60 g, 3.73 mmol) was reacted with the aldehyde 117 (0.62 g, 2.80 mmol) to give 0.67 g (72%) of compound 125c.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.62}(C_{14}H_{25}NO_{10})_{0.38}]\cdot 0.62 H_2O$: C, 43.30; H, 7.00; N, 5.59. Found: C, 43.30; H, 7.01; N, 5.60.

d) Chitosan (0.80 g, 5.0 mmol) was treated with aldehyde 117 (1.11 g, 5.00 mmol) to yield 0.91 g (80%) of 125d.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.74}(C_{14}H_{25}NO_{10})_{0.26}]\cdot 0.66H_2O$: C, 42.82; H, 7.05; N, 6.18. Found: C, 42.82; H, 7.15; N, 6.17.

N-[2'-O-(α-D-galactopyranosyl)ethyl]chitosan (126)

a) Reaction of chitosan (0.45 g, 2.8 mmol) and the aldehyde (118, 1.90 g, 8.64 mmol) gave 0.60 g (57%) of lyophilized product.

Anal. Calcd. for $[(C_{14}H_{25}NO_{10})]\cdot 0.63H_2O$: C, 44.40; H, 6.94; N, 3.70. Found: C, 44.41; H, 7.01; N, 3.74.

b) Reaction of the aldehyde (118, 1.67 g, 7.52 mmol) and chitosan (0.60 g, 3.73 mmol) afforded 0.68 g (54%) of a white fluffy product 126b.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.14}(C_{14}H_{25}NO_{10})_{0.86}]\cdot 1.06H_2O$: C,43.27; H, 7.03; N, 3.92. Found: C, 43.27; H, 7.12; N, 3.91.

c) When chitosan (0.80 g, 5.0 mmol) was reacted with the aldehyde 118 (1.11 g, 5.00 mmol), 1.34 g (96%) of product 126c was obtained.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.52}(C_{14}H_{25}NO_{10})_{0.48}]\cdot 0.49$ H_2O: C, 43.95; H, 6.97; N, 5.21. Found: C, 43.95; H, 7.19; N, 5.20.

d) When chitosan (0.80 g, 5.0 mmol) was treated with the aldehyde 118 (0.83 g, 3.74 mmol), 1.10 g (95%) of product 126d was obtained.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.68}(C_{14}H_{25}NO_{10})_{0.32}]\cdot 0.41H_2O$: C, 43.85; H, 6.96; N, 5.98. Found: C, 43.85; H, 7.30; N, 5.98.

N-[2"-O-(2-acetamido-2-deoxy-α-D-glucopyranosyl) ethyl]chitosan (127)

a) Chitosan (0.60 g, 3.7 mmol) was treated with the aldehydo sugar 119 (3.05 g, 11.5 mmol) to give 1.35 g (92%) of lyophilized product 127a.

Anal. Calcd. for $[(C_{16}H_{28}N_2O_{10})\cdot 0.36H_2O]$: C, 46.32: H, 6.92; N, 6.76. Found: C, 46.32; H, 6.61; N, 6.78.

b) Reaction of chitosan (0.60 g, 3.7 mmol) with the acetaldehydo-glycoside 119 (3.00 g, 11.4 mmol) gave 1.25 g (85%) of product after freeze-drying.

Anal. Calcd. for $[(C_{16}H_{28}NO_{10})\cdot 1.53H_2O]$: C, 44.08; H, 7.12; N, 6.43. Found: C, 44.08; H, 7.05; N, 6.23.

c) Coupling of the aldehyde 119 (1.60 g, 6.0 mmol) to chitosan (0.65 g, 4.0 mmol) yielded 1.0 g (95%) of derivative 127c.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.63}(C_{16}H_{28}N_2O_{10})_{0.37}]$ $\cdot 0.52H_2O$: C, 44.47; H, 7.01; N, 7.33. Found: C, 44.47; H, 6.86; N, 7.31.

d) Treatment of chitosan (0.65 g, 4.0 mmol) with the aldehydo-glucoside 119 (0.79 g, 3.0 mmol) gave 0.80 g (93%) of compound 127d.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.83}(C_{16}H_{28}N_2O_{10})_{0.17}]\cdot 0.58$ H_2O: C, 43.30; H, 7.05; N, 7.68. Found: C, 43.30; H, 7.10; N, 7.71.

e) Chitosan (0.80 g, 5.0 mmol) was reacted with 119 (0.70 g, 2.5 mmol) to produce 1.03 g (95%) of product 127e.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.81}(C_{16}H_{28}N_2O_{10})_{0.19}]\cdot 0.46$ H_2O: C, 43.84; H, 7.00; N, 7.70. Found: C, 43.83; H, 7.18; N, 7.70.

Synthesis of N-[2'-O-(2-amino-2-deoxy-β-D-glucopyranosyl) ethyl]chitosan (129)

Derivative 127b (1.25 g, 3.10 mmol) was suspended in 40% aqueous sodium hydroxide (60 mL) and heated at 100 C under a nitrogen atmosphere for 6 h. The reaction mixture was then cooled, neutralized, dialysed and freeze dried to give 1.1 g (97%) of 129.

Anal. Calcd. for $[(C_{14}H_{26}N_2O_9)_{1.0}]\cdot 3.0H_2O$: C, 40.00; H, 7.63; N, 6.67. Found: C, 40.00; H, 7.97; N, 6.76.

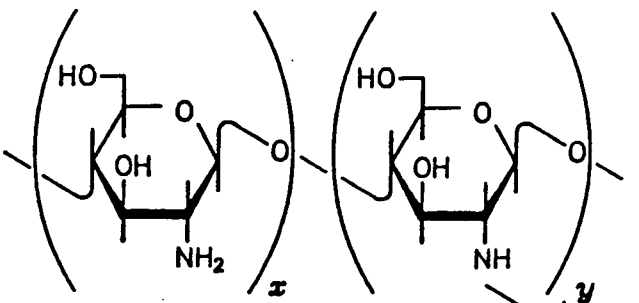
Synthesis of N-[2'-O-(α-D-glucopyranosyl)ethyl]chitin (130)

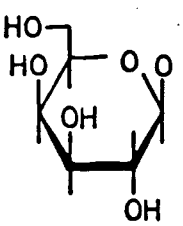
Derivative 125c (160 mg, 0.67 mmol) was dissolved in a water-methanol mixture (1:1, 13 mL) and stirred with acetic anhydride (70 mg, 65 mL, 0.67 mmol) for 24 h. Exhaustive dialysis against distilled water gave derivative 130 (95%).

Anal. Calcd. for $[(C_8H_{13}NO_5)_{0.62}(C_{14}H_{25}NO_{10})_{0.38}]\cdot 0.83$ H_2O: C,44.03; H, 6.86; N, 5.00. Found: C, 44.03; H, 6.84; N,

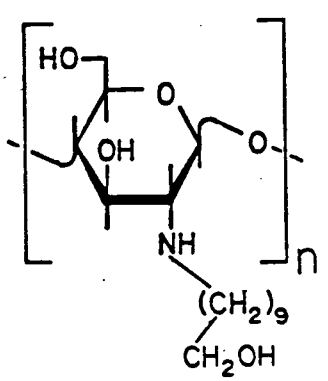
4.80.

General Procedure for the Preparation of N-[10'-O-(β-D-glycopyranosyl)decyl]chitosan Derivatives

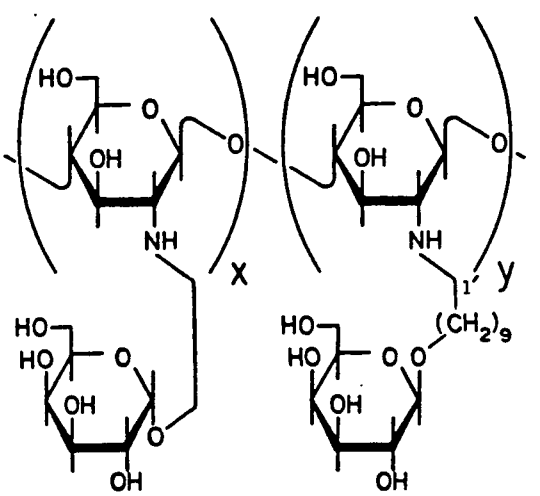


	R_1	R_2
140	OH	H
141	H	OH
142		H

140-142



143



144

A stirred solution of chitosan flakes in a mixture of 5% aqueous acetic acid-methanol (1:1, @ 15 mL/mmol) was treated with a solution of the 10-decanalyl β-D-glycoside (1.5-3.0 equiv.) in the reaction media (10-15 mL) and subsequently with sodium cyanoborohydride (@ 4.0 molar equiv). The resultant mixture was stirred for 24 h and then dialyzed for 3 days against methanol-water, 1:1 (3x1 L) and 3 days against distilled water (3x1 L). The solution was filtered through a medium pore sintered glass filter and freeze-dried. Yields were 50-90%.

N-[10'-O-(β-D-glucopyranosidyl)decyl]chitosan (140)

a) Chitosan (0.65 g, 4.0 mmol) was treated with the aldehyde 137 (2,8 g, 8.4 mmol) to give, after workup, 0.93 g (54%) of derivative 140a.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.19}(C_{22}H_{41}N)_{10})_{0.81}]\cdot 0.83\ H_2O$: C, 52.49; H, 8.53: N, 3.23. Found: C, 52.49; H, 8.20; N, 3.23.

b) When chitosan (0.45 g, 2.8 mmol) was reacted with the decanalyl β-D-glycoside 137 (2.85 g, 8.5 mmol), 1.64 g (91%) of product 140b was obtained.

Anal. Calcd. for $[(C_{22}H_{41}NO_{10})_{0.53}(C_{38}H_{71}NO_{16})_{0.47})]\cdot 1.0\ H_2O$: C, 54.80; H, 8.83; N, 2.17. Found: C, 54.80; H, 8.60; N, 2.17.

N-[10'-O-(β-D-galactopyranosyl)decyl]chitosan (141)

a) Treatment of chitosan (0.65 g, 4.0 mmol) with the glycoside 138 (4.0 g, 12.0 mmol) provided 1.43 g (58%) of 141a.

Anal. Calcd. for $[(C_{22}H_{41}NO_{10})_{0.63}(C_{38}H_{71}NO_{16})_{0.37}]_{0.82}$ H_2O: C, 54.79; H, 8.79; N, 2.29. Found: C, 54.79; H, 8.89; N, 2.29.

b) Chitosan (0.65 g, 4.0 mmol) was reacted with the aldehyde 138 (2.0 g, 6.0 mmol) to yield 0.89 g (90%) of derivative 141b.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.78}(C_{22}H_{41}NO)]\cdot 1.55H_2O$: C, 44.13; H, 8.00; N, 5.41. Found: C, 44.13; H, 7.83; N, 5.46.

N-[10"-O-(β-D-galactopyranosyl)-β-D-glucopyranosyl) decyl]chitosan or N-[10"-O-(β-D-lactosyl)decyl]chitosan (142)

a) Reaction of chitosan (0.65 g, 4.0 mmol) with the aldehyde 139 (5.8 g, 11.7 mmol) provided 1.95 g (67%) of product 142a.

Anal. Calcd. for $[(C_{28}H_{51}NO_{15})_{0.90}(C_{50}H_{91}NO_{26})_{0.1}]\cdot 2.04$ H_2O: C, 49.94; H, 8.14; N, 1.93. Found: C, 49.94; H, 8.11; N, 1.93.

b) Treatment of chitosan (0.65 g, 4.0 mmol) with the aldehyde 139 (3.10 g, 6.3 mmol) yielded 1.18 g (69%) of lyophilized 142b.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.50}(C_{28}H_{51}NO_{15})_{0.50}]\cdot 1.45$ H_2O: C, 47.77; H, 7.94; N, 3.28. Found: C, 47.77; H, 8.05; N, 3.26.

N-[10-hydroxydecyl]chitosan (143)

a) A solution of chitosan (0.65 g, 4.0 mmol) in 5% aqueous acetic acid-methanol-i-propanol (5:4:1, 60 mL) was treated with 10-hydroxydecanal (2.1 g, 12.0 mmol) and subsequently with sodium cyanoborohydride (1.0 g, 16.0 mmol)

for 24 h. A flocculent precipitate formed rapidly, which upon completion of reaction, was collected by filtration and washed with water to give 1.30 g (76%) of derivative 143a.

Anal. Calcd. for $[(C_{10}H_{31}NO_5)_{0.27}(C_{26}H_{51}NO_6)_{0.73}]\cdot 0.23 H_2O$: C, 64.26; H, 10.59; N, 3.22. Found: C, 64.26; H, 10.62; N, 3.22.

b) Treatment of chitosan (0.65 g, 4.0 mmol) as in part (a), with 10-hydroxydecanal (0.70 g, 4.0 mmol) and sodium cyanoborohydride (1.0 g, 16.0 mmol) for 24 h, provided a solution which after dialysis against methanol-water (2:1) for 2 days (2x1 L), methanol-water (1:1) for 2 days (2x1 L) and finally water for 2 days (2x2 L), gave 1.04 g (82%) of freeze-dried product 143b.

Anal. Calcd. for $[(C_{16}H_{31}NO_5)]\cdot 0.42H_2O$: C, 59.17; H, 9.81; N, 4.31. Found: C, 59.17; H, 9.65; N, 4.52.

Synthesis of mixed N-ethyl and N-decyl-D-glycopyranosyl chitosan derivative

Derivative 126d (150 mg, 0.68 mmol) in 5% aqueous acetic acid-methanol (1:1) was stirred with the aldehyde 138 (0.65 g, 1.95 mmol) and sodium cyanoborohydride (0.30 g, 4.80 mmol) for 24 h. The reaction mixture was transfered to a dialysis sack and dialysed against water-methanol (1:1) for 3 days (3x1 L), against distilled water for 3 days (3x1 L), and freeze dried to give 220 mg (63%) of 144.

Anal. Calcd. for $[(C_{14}H_{25}NO_{10})_{0.48}(C_{38}H_{71}NO_{16})_{0.52}]\cdot 1.28H_2O$: C, 51.79; H, 8.39; N, 2.28. Found: C, 51.78; H, 8.56; N, 2.29.

4.2.4 Viscometry

Polysaccharide solutions for viscometry were prepared by dissolving the sample in distilled water (5.0 mL) containing 50 ppm sodium benzoate as stabilizer. Typically 2.0% (w/w) solutions of all synthetic derivatives were prepared, except where otherwise indicated. Commercial polysaccharide derivatives were prepared in a similar manner, at concentrations of 1.0 and 2.0%. All samples were given at least 24 hours to disperse, with intermittent mixing on a Vortex-Genie^{TM} test tube mixer. Any samples which contained entrapped air bubbles were centrifuged on a bench-top serum centrifuge for 30 min.

The steady shear viscometric measurements were performed on a rotational viscometer (Visco-Elastic Analyzer, Sangamo Transducers, W. Sussex, England) with truncated cone and plate fixtures (d 50.0 mm, α 2.5°, gap 90 μm). A controlled temperature glycol bath under the plate provided temperature control. All measurements were recorded at 20.0° ($\pm$ 0.2°C). The gap setting was zeroed with no sample present by lowering the cone fixture until contact was just made with the plate. The cone fixture was then raised $\sim$ 2 mm to allow sample loading.

A sample solution (~1.5 mL) was loaded into a syringe with an 18 gauge needle or, for very viscous samples, a 7" Pasteur pipette. The sample was then discharged onto the center of the plate, taking care to avoid the formation of

air bubbles. The cone fixture was lowered onto the sample to the desired 90 μm gap setting. The sample was given five minutes to equlibrate to 20.0 (+ 0.5) C, as verified by measurement with a thermocouple. The sample was then stepped through a series of increasing torque settings, applied to the rotating cone fixture, typically from 0.1 to 60 G·cm. For each torque setting, the resultant angular velocity (radians·s^{-1}) was recorded on a strip chart, allowing the equilibrium value to be reached (usually within 30 s for the majority of samples). When either the maximum torque (60 G·cm) or maximum angular velocity (100 radians·s^{-1}) was approached, the torque was stepped through a similar decreasing series, and angular velocity was recorded. Duplicate measurements were performed on all samples. Three Newtonian standard oil samples were subjected to identical measurement, at all measured torque values, in order to calibrate the torque settings over the full range of observed viscosities.

Shear stress (σ) is related to torque (T) according to equation 30 :

$$\sigma = \frac{3T}{2\pi r^3} \qquad [30]$$

where T is torque in dynes·cm and r is radius; and shear rate ($\dot{\gamma}$) to angular velocity (ω) according to Eq. 31:

$$\gamma = \frac{\omega}{\tan \delta} \qquad [31]$$

Substitution of values from cone geometry can provide shear

stress and shear rate factors as seen in equations 32 and 33.

$$\sigma = 2996.7T \qquad (mPa) \qquad [32]$$

$$\dot{\gamma} = 22.92\omega \qquad (s^{-1}) \qquad [33]$$

Apparent viscosity will then be given by equation 34:

$$\eta = \frac{\sigma}{\dot{\gamma}} = 130.76\left(\frac{T}{\omega}\right) \qquad (mPa \cdot s) \qquad [34]$$

Corrected torque values (T_{corr}) were calculated from standard oil measurements by rearranging equation 34, to give:

$$T_{corr} = \frac{\eta_{oil}\omega}{130.76} \qquad [35]$$

where η_{oil} is the known standard oil viscosity, and ω was the measured angular velocity. Corrected torque values and determined angular velocities provided shear stress, shear rates and apparent viscosities from equations 32, 33 and 34. Logarithm of shear stress, shear rate and apparent viscosity were calculated. Linear regression of logarithm of shear stress against logarithm of shear rate, gave power-law parameters according to equation 36:

$$\log(\sigma) = \log(m) + n\log(\dot{\gamma}) \qquad [36]$$

Rheograms of steady shear viscometric data were plotted on both linear and logarithmic coordinates as apparent viscosity vs shear rate.

Rheometric evaluations reported in section 2.3.3 were done using a different rheometer, which was also a controlled

stress instrument, capable of elevated temperature measurments (Controlled Stress Rheometer, Carri-Med Ltd. , Dorking, Surrey, England). The data was obtained in the same way as described previously, giving torque (dyne·cm) and angular velocity (ω) values, and converted to shear stress and shear rate using Eq.s 30 and 31. The cone-plate dimensions were r 20.0 mm, and α 2.0°. Data treatment followed exactly the procedure already outlined.

4.2.5 Intrinsic Viscosity

Solutions of 0.075, 0.050, 0.025 and 0.010% (w/w) for intrinsic viscosity determinations were prepared by dilution of 0.10% w/w stock solutions (100 mg/100 mL). The solutions were loaded into the Canon-Fenske capillary viscometer, according to standard procedures,[219] and the viscometer was placed in a temperature-controlled jacket and allowed to equilibrate to 25 (± 0.5)°C for 10 min. Duplicate determinations of efflux time were recorded for each solution. Canon-Fenske #50 or #100 viscometers were used accordingly in order to keep efflux times in the optimal 200-800 s range. The viscometers were calibrated with water and standard oil (7.798 mPa·s at 25°C) to give the constant k according to equation 37;

$$\eta = k_1 t\rho \qquad [37]$$

where η is viscosity (mPa·s), t is efflux time and ρ is

density. From equation 37, viscosities for all sample solutions were determined and used to calculate relative viscosity (η_{rel}) according to Eq. 38;

$$\eta_{rel} = \frac{\eta}{\eta_s} \qquad [38]$$

where η_s is the solvent viscosity. Intrinsic viscosities [n] were obtained according to the Kraemer [Eq. 7] relationship:

$$\frac{Ln(\eta_{rel})}{c} = [\eta] + k'[\eta]^2 c \qquad [7]$$

where c is the concentration (g/100 mL) and k_1' is a constant. Linear regression of ln η_{rel}/c against concentration provided, intrinsic viscosities for the sample solutions.

4.3 EXPERIMENTAL FOR CHAPTER 3

4.3.1 General Procedures

pH Titration curves were determined on solutions of ~0.25 g of polysaccharide derivative in 0.1N HCl (aq) solution (25.0 mL) with a pH meter. Copper chelation samples were prepared by dissolving the derivative (~200 mg) in distilled water (15 mL) and treating with 2.0M copper(II) acetate (aq) solution (15 mL). After stirring for 24 h, the suspensions were dialyzed against 1) distilled water exhaustively (6x1 L)

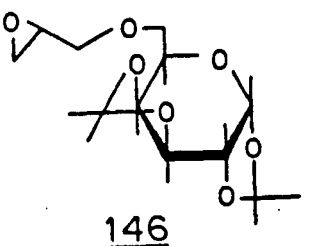
or 2) distilled water for 3 days (3x1 L), 0.1N iminodiacetic acid (250 mL) for 48 h and then exhaustively with distilled water (6x1 L). The samples were freeze-dried and then dried *in vacuo* (70°C, 0.05 mm Hg).

For viscometry, sample solutions of 1.0% (w/w) concentration were prepared in distilled water (containing 5 ppm sodium benzoate as stabilizer) or in 0.1mM copper(II) sulfate (aq) solution. Dissolution was aided by periodic mixing (Vortex-Genie) over a 24 h period. Entrapped air bubbles were removed by centrifugation on a bench-top centrifuge for 30 min. Viscometric measurements were performed with a rotational, controlled stress rheometer (Visco-Elastic Analyzer) as described in section 4.2.4.

4.3.2 Synthesis of Chelating Chitosan Derivatives

Dimethyl iminodiacetate (Iminodiacetic acid dimethylester), (147)

Iminodiacetic acid (5.15 g, 38.7 mmol) was stirred in anhydrous methanol (25 mL) to which was added 50% boron trifluoride-methanol complex (10 mL). The reaction mixture was refluxed for 9 h, cooled to room temperature, and poured into 150 mL of chloroform and 75 mL saturated sodium bicarbonate solution. Additional solid sodium bicarbonate was added until the aqueous layer was slightly basic (pH paper). The chloroform layer was separated, the aqueous layer was extracted twice more with chloroform (2x150 mL), and the



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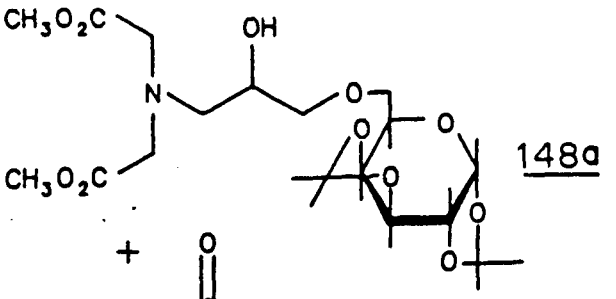

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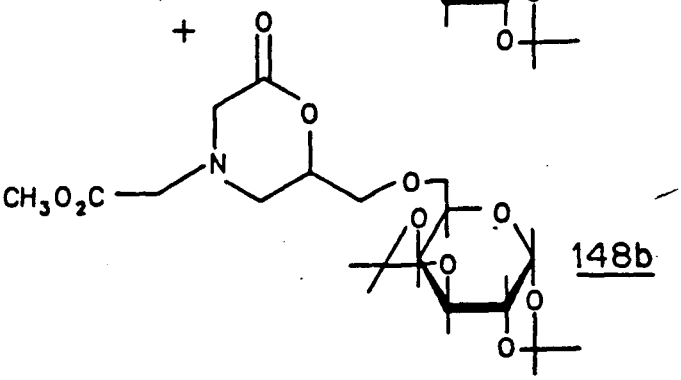
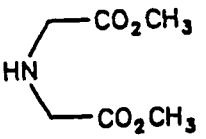


148b



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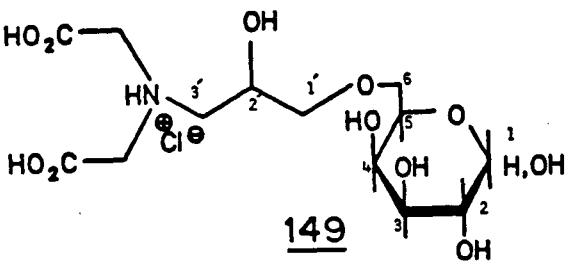

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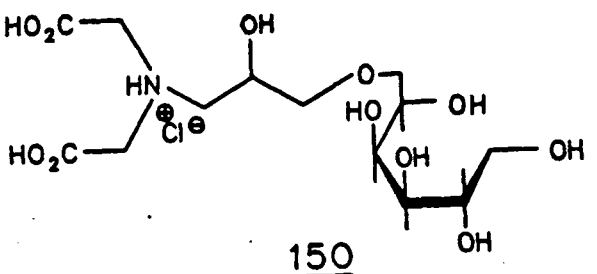

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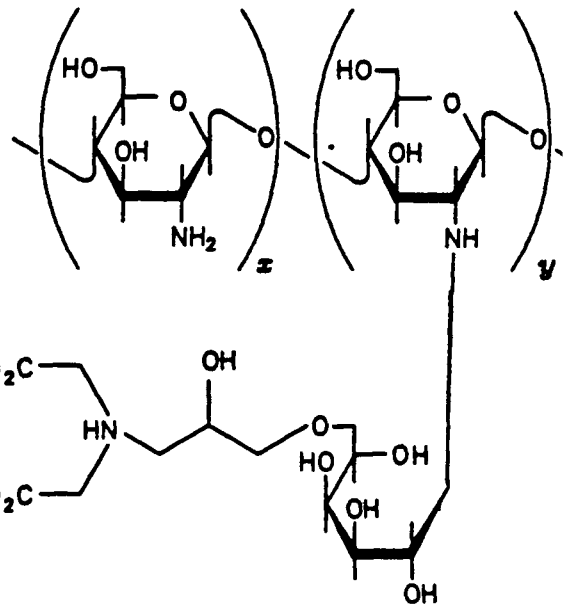


151

chloroform extracts were combined. The chloroform solution was then dried over magnesium sulfate and filtered. Concentration gave 5.96 g (96%) of a liquid, which corresponded to the diester 147. This material was one component by tlc analysis (R_f 0.27, solvent A); ir (film): ν_{max} 1750 cm^{-1}; ^1H-nmr (270 MHz): $\delta(CDCl_3)$, 2.07 (s, 1 H, NH), 3.49 (s, 4 H, $-CH_2-$), 3.74 (s, 6 H, CO_2CH_3); ms, m/z (rel. intensity): 161 (15), 129 (6), 103 (7), 102 (94), 74 (37).

6-O-(2,3-Epoxypropyl)-1,2:3,4-di-O-isopropylidene-α-D-galactopyranose (146)

1,2:3,4-Di-O-isopropylidene-α-D-galactopyranose, 145 (10.00 g, 38.4 mmol) and epichlorohydrin (3.55 g, 38.4 mmol) were dissolved in anhydrous tetrahydrofuran (THF, 100 mL). Sodium hydride (1.84 g, 60% oil dispersion, 46.1 mmol) was washed with three portions of hexane (10 mL) and added to the THF solution. The reaction mixture was refluxed for 9 h, and after dilution with methanol (2 mL), was cooled and poured into water (50 mL). The aqueous layer was extracted with three portions of ether (100 mL) and the combined ethereal extracts were washed twice with saturated brine solution (50 mL). The ether layer was dried with magnesium sulfate, filtered and concentrated to give 11.40 g of a crude syrup. Tlc analysis (solvent A) of this material showed a major component at R_f 0.55 and minor components at lower R_f values. Flash liquid chromatography (2:1 ether-petroleum ether) afforded 6.88 (57%) of a white crystalline solid

corresponding to diastereomeric 146; Further processing of mixed fractions gave additional product. Ir (KBr): ν_{max} 1450 (CH_3 deform), 1380 [$-C(CH_3)_2$ sym deform], 1250 cm^{-1} (C-O epoxide); ^{1}H-nmr (270 MHz): $\delta(CDCl_3)$, 1.34 (s, 6 H, CH_3), 1.45 (s, 3 H, CH_3), 1.54 (s, 3 H, CH_3), 2.63 (m, 1 H, J 5.5, 2.5 Hz, O-CH), 2.78 (t, 1 H, J 5.5, 5.5 Hz, O-CH), 3.17 (m, 1 H, -CH-C), 3.41, 3.49 (2dd, 1 H, J 1.0, 5.5 Hz, H-1'aR,S), 3.59-3.83 (m, 3 H, H-6a,6b, H-1bR,s), 3.98 (br t, 1 H, J 5.5, 5.5 Hz, H-5), 4.24 (ddd, 1 H, J 8.0, 2.0, 2.0 Hz, H-4), 4.30 (dd, 1 H, J 5.5, 3.0 Hz, H-2), 4.57 (dd, 1 H, J 8.0, 3.0 Hz, H-3), 5.52 (d, 1 H, J 5.5 Hz, H-1); ms, m/e (rel. intensity): 316 (0.1), 301 (66), 258 (3), 243 (3), 229 (9);

Anal. Calcd. for $C_{15}H_{24}O_7$: C, 56.95; H, 7.65. Found: C, 56.95; H, 7.78.

6-O-[2-Hydroxy-3-(methyliminodiacetate)propyl]-1,2:3,4-di-O-isopropylidene-α-D-galactopyranose (148a)and the morpholin-3-one 148b

6-O-(2,3-epoxypropyl)-1,2:3,4-di-O-isopropylidene-α-D galactopyranose (146, 2.00 g, 6.33 mmol) and dimethylimino-diaacetate (147, 1.00 g, 6.2 mmol) were dissolved in ethanol (50 mL) and refluxed for 8 h. The ethanol was removed and the resultant syrup dissolved in chloroform (50 mL). The chloroform layer was washed with two portions (50 mL) of saturated brine, dried over magnesium sulfate, filtered and concentrated to give 2.95 g of a syrup. Tlc analysis confirmed the absence of starting material and showed two major components

(R_f 0.41, 0.34, solvent A). Glc analysis of the product showed one major peak (>95% peak area). The ^{1}H-nmr spectrum of the syrup indicates a mixture of products 148a and 148b in a ratio of 2:1 respectively. After flash liquid chromatography purification, 2.45 g of a 1:4 mixture of 148a, 148b was obtained (86%). Subsequent short path distillation (200°C, 0.05 mm Hg) gave 1.85 g of pure 148b by ^{1}H-nmr spectroscopy. Glc analysis of this component gave one peak at the same retention time observed for the product mixture. 148a: ir (film): ν_{max} 1755 (C=O ester), 3500 cm^{-1} (OH); ^{1}H-nmr (270 MHz): $\delta(CDCl_3)$, 1.33 (s, 6 H, $2CH_3$), 1.45 (s, 3 H, CH_3), 1.54 (s, 3 H, CH_3), 2.64 (ddd, 1 H, J 12.5, 10.0, 2.0 Hz, -CH-N), 3.00 (dd, 1 H, J 12.5, 2.0 Hz, -CH-N), 3.39-3.79 (m, 8 H, H-6, H-6', $-CH_2O$, 2 $-CH_2CO_2Me$), 3.72 (s, 6 H, 2 CO_2CH_3), 3.83 (m, 1 H, CHOH), 3.97 (br s, 1 H, H-5), 4.25 (dd, 1 H, J 8.0, 1.0 Hz, H-4), 4.33 (dd, 1 H, J 5.0, 2.0 Hz, H-2), 4.61 (dd, 1 H, J 8.0, 2.0 Hz, H-3), 5.54 (d, 1 H, J 5.0 Hz, H-1). 148b: ir (film): ν_{max} 1755 cm^{-1} (C=O ester, morpholone); ^{1}H-nmr (270 MHz): $\delta(CDCl_3)$, 1.33 (s, 6 H, 2 CH_3), 1.44 (s, 3 H, CH_3), 1.54 (s, 3 H, CH_3), 2.86 (ddd, 1 H, J 12.5, 10.0, 2.0 Hz, CH-N), 3.08 (dd, 1 H, J 12.5, 2.0 Hz, -CH-N), 3.39 (s, 2 H, $-CH_2CO_2Me$), 3.40-3.79 (m, 6 H, H-6, H-6', $-CH_2CO_2Me$, $-CH_2O$), 3.73 (s, 3 H, CH_3), 3.97 (br t, 1 H, J 5.0, 1.0 Hz, H-5), 4.25 (d, 1 H, J 8.0, 1.0 Hz, H-4), 4.34 (dd, 1 H, J 5.0, 2.0 Hz, H-2), 4.63 (dd, 1 H, J 8.0, 2.0 Hz, H-3), 4.68 (m, 1 H, CHOR), 5.54 (d, 1 H, J 5.0 Hz, H-1). 148a, 148b mixture: ms, m/z (rel. intensity): 477 (5), 462 (10), 445 (2), 430 (6),

418 (30), 387 (12), 386 (14).

6-O-(2-Hydroxy-3-iminodiaceto-propyl)-D-galactose (149)

Compound 148 (2:1 mixture of 148a and 148b, 10.0 g, 21.4 mmol) was dissolved in 2N sodium hydroxide in 9:1 methanol-water (100 mL). The reaction mixture was refluxed for 4 h and cooled in an ice bath. 6N hydrochloric acid (60 mL) was added to give a 1N solution, which was then refluxed for 5 h. After cooling the solution was filtered and the filtrate was concentrated to a volume of 10 mL. Ethanol (20 mL) was added and the mixture was cooled in ice, filtered and concentrated. The residue was taken up in ethanol and precipitation was achieved by the addition of acetone. The gummy precipitate was taken up in ethanol and treated with decolourizing carbon, and the filtrate was treated with acetone to give a gummy precipitate. This material was a foamy hydroscopic solid (7.15 g, 86%) after drying in vacuo (25 C, 0.05 mm Hg) and consisted of a mixture of α and β 6-O-D-galactose isomers 149. ^{13}C-nmr (100.6 MHz, main isomer): $\delta(D_2O)$, 166.7 (COOH), 98 (C-1), 71.8, 71.2 (C-5,2), 69.3, 69.2 (CHOH), 67.9, 67.2 (C-3,4), 66.7 (C-6), 63.4 (CH_2O), 57.1, 57.2 ($-CH_2NH$), 54.3 ($NHCH_2CO_2H$).

6-O-[2-hydroxy-3-(iminodiacetic acid hydrochloride) propyl]-D-galactitol (150)

Compound 149 (0.90 g, 2.3 mmol) was dissolved in 95% ethanol (10 mL) to which was added an aqueous solution of saturated sodium bicarbonate (2 mL). To this mixture, sodium borohydride (0.50 g, 13.2 mmol) was added and allowed to stir

for 2 h at room temperature. The reaction was then acidified to pH 2 (pH paper) by dropwise addition of 6N hydrochloric acid. The solution was filtered and the solvent removed. The residue was taken up in methanol and concentrated thrice more to remove borate salts. The residue was then dissolved in ethanol-methanol (1:1), and the resultant solution was filtered and precipitated with acetone to give 0.75 g (83%) of a foamy hygroscopic solid. Attempts to crystallize the diastereomic mixture were unsuccessful. ^{13}C-nmr (100.6 MHz): $\delta(D_2O)$, 167.3 (COOH), 71.7, 71.3, 69.1 (C-5,4,3,2), 68.8, 68.6 (CHOH), 67.3 (C-6), 63.3, 63.0 (CH_2O-), 62.0 (C-1), 56.37, 56.07 (CH_2NH), 54.1, 53.9 ($NHCH_2CO_2H$).

Chelating Chitosan Derivative (151)

a) Chitosan (0.55 g, 3.4 mmol) was dissolved in 5% aqueous acetic acid (50 mL) and the galactose derivative 149 (2.0 g, 4.9 mmol) in 5% aqueous acetic acid (10 mL) was added to the resultant stirred solution. After 15 min, sodium cyanoborohydride (1.25 g, 20.0 mmol) in 5 mL of reaction solvent was added. After stirring for 24 h, the mixture was poured into a dialysis sac and dialyzed for 6 days (6x2 L) against distilled water and subsequently freeze-dried to give 1.62 g (84%) of a white fluffy solid; ^{13}C-nmr (100.6 MHz): $\delta(D_2O)$, 168.6 (COOH), 98.4 (C-1'), 75.9 (C-4'), 73.2 (C-5'), 71.2, 71.1 (C-5,2), 68.9 (=CHOH), 67.9, 67.4 (C-3,2), 66.7 (C-6), 63.2 (-CH_2O-), 58.7 (-CH_2NH), 56.3 ($NHCH_2CO_2H$), 54.7 (C-1).

Anal.Calcd for $(C_{19}H_{34}N_2O_{14})\cdot 1.56H_2O$: C,42.06; H, 6.85;

N, 5.16.Found: C, 42.06; H,7.10; N, 4.81.

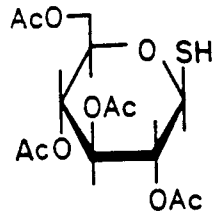
b) The procedure used for the preparation of 151b was as described for the preparation of 151a, with the exception that 1.00 g (2.45 mmol) of the galactose derivative 149 was used. The resultant yield was 1.16 g (63%) of a fluffy white solid.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.50}(C_{19}H_{34}N_2O_{14})_{0.50}]\cdot 1.1$ H_2O: C, 41.98; H, 6.91; N, 5.88. Found: C, 41.98; H, 7.17; N, 5.85.

4.3.3 Synthesis of Thio Glycoside Affinity Conjugate and Precursors

(3'-O-Allyl-2'-hydroxypropyl) 2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranoside (153)

2,3,4,6-Tetra-O-acetyl-1-thio-β-D-glucopyranose (152, 10.0 g, 27.5 mmol), 1-allyloxy-2,3-epoxypropane (3.14 g, 27.5 mmol) and sodium bicarbonate (2.31 g, 27.5 mmol) were stirred together in ethanol (60 mL) at reflux temperature for 4 h. The solution was cooled, poured into chloroform (200 mL), washed with saturated sodium bicarbonate aqueous solution (100 mL), saturated aqueous brine (2x100 mL), dried over magnesium sulfate, filtered and concentrated. Liquid chromatography afforded 7.56 g (58%) of diastereomeric 153, ^{1}H-nmr (270 MHz): δ($CDCl_3$), 5.87 (m, 1 H, H-2"), 5.32-4.96 (m, 5 H, H-2,3,4,3"a,3"b), 4.55 (d, 1 H, J 10.0 Hz, H-1), 4.30-4.05 (m, 2 H, H-6a,b), 4.00 (d, 2 H, J 5.0 Hz, H-3'),



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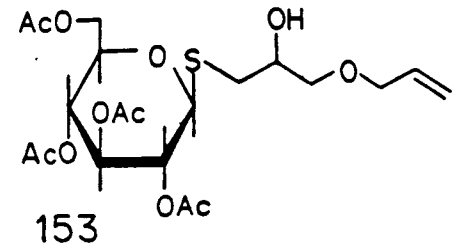

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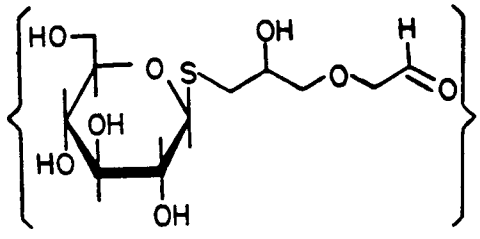

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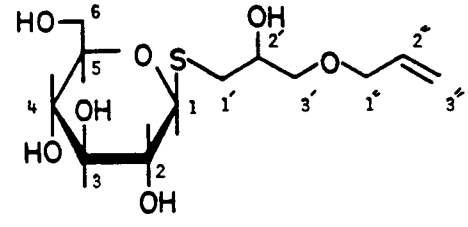

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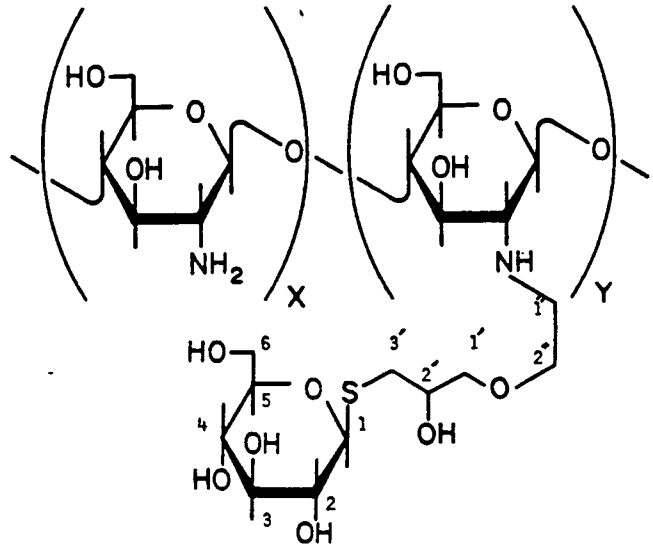

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3.93 (m, 1 H, H-2'), 3.73 (m, 1 H, H-5), 3.47 (m, 2 H, H-1'a,b), 3.15-2.58 (m, 2 H, H-1"a,b), 2.10, 2.06, 2.04, 2.00 (4s, 3 H each, 4 OAc); ms, m/z 478 (M^+), 418, 358.

(3'-O-Allyl-2'-hydroxypropyl) 1-thio-β-D-glucopyranoside (154)

A solution of compound 153 (7.3 g, 15.3 mmol) in anhydrous methanol (75 mL) was treated with 0.5N sodium methoxide in methanol (5 mL) until the reaction was complete (tlc, solvent B). The solution was neutralized with Dowex 50x8 (H^+-form, 100-200 mesh) ion-exchange resin, filtered, decolourized and concentrated. Attempts to crystallize the syrupy residue were unsuccessful. Drying *in vacuo* (0.05 mm Hg) gave 4.5 g (95%) of the diastereomeric mixture 154 as a foamy solid, ^{1}H-nmr (270 MHz): δ(D_2O), 5.89 (m, 1 H, H-2"), 5.28 (br d, 1 H, J 17.0 Hz, H-3"a), 5.20 (br d, 1 H, J 12.0 Hz, H-3"b), 4.49, 4.47 (2d, 1 H, J 7.0 Hz, H-1), 4.02 (d, 2 H, J 6.0 Hz, H-3'), 3.99 (m, 1 H, H-2'), 3.83 (dd, 1 H, J 12.0, 1.0 Hz, H-6a), 3.64 (dd, 1 H, J 12.0, 3.0 Hz, H-6b), 3.60-3.31 (m, 3 H, H-3,4,5), 3.26 (t, 1 H, J 9.0, 7.0 Hz, H-2), 2.92 (dd, 1 H, J 14.0, 5.0 Hz, H-1"a), 2.82 (m, 2 H, H-1'), 2.70 (dd, 1 H, J 14.0, 8.0 Hz, H-1"b).

Ozonolysis of 154

The 1-thio-β-D-glucopyranoside 154 (3.42 g, 11.0 mmol) was dissolved in methanol (50 mL), cooled to -78 C and saturated with ozone. Dimethylsulfide (3.43 g, 4.1 mL, 55.0 mmol) was added, and the reaction was allowed to warm to ambient temperature for 2 h with stirring. Excess solvent was removed

and the syrupy residue dissolved in ethanol and precipitated by the addition of ether. After precipitating twice and drying *in vacuo*, 3.30 g (96%) of crude 155 was obtained.

1-Thio-β-D-glucopyranoside Affinity Conjugate (156)

a) A solution of chitosan (0.85 g, 5.3 mmol) in 5% aqueous acetic acid (50 mL) was treated with a solution of the thio-glucosealdehyde 155 (3.4 g, 10.9 mmol) in the reaction solvent (10 mL) and sodium cyanoborohydride (1.25 g, 20.0 mmol) for 24 h. The solution was dialyzed for 6 days against distilled water (6x1 L), filtered and freeze-dried to give 2.25 g (95%) of the derivative 156a; ^{13}C-nmr (100.6 MHz): δ(D_2O), 99.5 (C-1*), 87.0 (C-1), 78.5 (C-5), 75.5 (C-3), 72.3,71.8 (C-2 and C-4), 67.6,67.5,66.5 (C-1',2', and 2"), 59.1 (C-6), 49.0 (C-3'), 45.5 (C-1").

Anal. Calcd for $[(C_6H_{11}NO_4)_{0.10}(C_{19}H_{31}NO_{11}S)_{0.90}]\cdot 1.9H_2O$: C,41.21; H, 7.13; N, 3.02; S, 6.22. Found: C, 41.21; H, 7.00; N, 3.46; S, 6.83.)

b) A stirred dispersion of derivative 156a (0.50 g, 1.3 mmol) in 2.0% aqueous acetic acid was treated with glutaraldehyde (25% aqueous solution, 0.10 mL, 0.26 mmol) and sodium cyanoborohydride (0.25 g, 4.0 mmol) for 24 h. The suspension was filtered and the precipitate was washed with water. Drying *in vacuo* (0.05 mm Hg) provided 0.40 g (69%) of 156b.

Anal. Calcd. for $[(C_{17}H_{31}NO_{11}S)_{0.90}(C_{8.5}H_{16}NO_4)_{0.10}]\cdot).65 H_2O$: C, 45.68; H, 6.67; N, 3.16; S, 6.51. Found: C, 45.68; H, 6.92; N, 3.05; S, 6.40.

* denotes carbons on the chitosan backbone.

c) A stirred solution of chitosan (0.70 g, 4.35 mmol) in 5% aqueous acetic acid (50 mL) was treated with the thio-glycoside 155 (0.8 g, 2.56 mmol) and sodium cyanoborohydride (1.0 g, 16.0 mmol) for 24 h. The solution was dialyzed for 6 days (6x1 L) against distilled water and freeze-dried to give 0.67 g (61%) of 156c.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.75}(C_{17}H_{31}NO_{11}S)_{0.25}]\cdot 0.88 H_2O$: C, 42.06; H, 7.11; N, 5.61; S, 3.20. Found: C, 42.06; H, 6.85; N, 5.80; S, 3.29.

d) A stirred solution of chitosan (0.70 g, 4.35 mmol) in 5% aqueous acetic acid (50 mL) was treated with the thio-sugar 155 (0.80 g, 2.56 mmol) and sodium cyanoborohydride (1.0 g, 16.0 mmol) for 24 h. 25% Aqueous glutaraldehyde (0.40 mL, 1.0 mmol) and sodium cyanoborohydride (0.25 g, 4.0 mmol) were then added and allowed to stir for 24 h, affording a stiff, clear foamy gel. After dialysis 0.70 g (64%) of 156d was obtained.

Anal. Calcd. for $[(C_6H_{11}NO_4)_{0.45}(C_{17}H_{31}NO_{11}S)_{0.25}(C_{8.5}H_{16}NO_4)_{0.3}]\cdot 0.43H_2O$: C, 45.13; H, 7.27; N, 5.54; S, 3.17. Found: C, 45.14; H, 7.22; N, 4.96; S, 3.04.

4.3.4 Enzyme Studies

All buffer chemicals, substrates, cofactors and coupling enzymes were obtained from Sigma Chemical Co. Assays of glucose liberation were performed essentially as described previously[265,269] and standardized against a glucose standard

solution. Quantities of coupling enzymes employed were sufficient that all glucose had been consumed within 5 minutes. Inhibition constants for the thioglucoside (154) with β-glucosidases from almond emulsin and A. faecalis were determined using a range of inhibitor concentrations (0.2-2.0 times K_i) at a fixed concentration (5 mM and 0.1 mM respectively) of p-nitrophenyl glucoside in 50 mM sodium phosphate buffer pH 6.8 at 25° C. Data were analyzed by means of a plot of $(v_{uninhibited})/(v_{inhibited})$ versus inhibitor concentration. The slope of such a plot equals $K_m/[K_i(S+K_m)]$ from which the K_i values can be determined.

The effectiveness of the affinity support was evaluated as follows. A small column (5x35 mm) was packed with the cross-linked polymer (156d), equilibrated with buffer (5 mM sodium phosphate, pH 6.8) and a small sample of A. faecalis β-glucosidase loaded on. After washing with the same buffer (5 mL), elution was effected using a buffer containing sodium chloride (0.5 M) and sodium phosphate (5 mM), pH 6.8.

β-Glucosidase activity eluted from the affinity column was estimated spectrophotometrically by measuring the rate of hydrolysis of p-nitrophenyl glucoside (0.25 mM) in 50 mM sodium phosphate buffer, pH 6.8 upon addition of a fixed aliquot (10 μL) of column effluent.

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APPENDIX A

Calculation of d.s. from C and N elemental microanalysis

The C/N ratio of the polymer product can be generally expressed as a linear combination, as shown in Eq. 39:

$$\frac{C}{N} = \frac{M_c(\sum x_i C_i)}{M_n(\sum x_i N_i)} \qquad [39]$$

where M_c and M_n are the atomic weights of carbon and nitrogen respectively, C_i and N_i are the number of carbon and nitrogen atoms in the ith residue, and x_i is the degree of substitution (or molar ratio) of the ith residue. When only two different monomer units are to be considered, Eq. 39 is expanded to give Eq. 40. In cases where both units contain only a single nitrogen atom, the relationship is simplified to give Eq. 41.

$$\frac{C}{N} = \frac{12(x_1C_1+(1-x_1)C_2)}{14(x_1N_1+(1-x_1)N_2)} \qquad [40]$$

$$\frac{C}{N} = \frac{12(x_1C_1+(1-x_1)C_2)}{14} \qquad [41]$$