STUDIES IN MOLECULAR STRUCTURE OF PLANT POLYSACCHARIDES

Exudates from Encephalartos species

A thesis submitted to the

UNIVERSITY OF CAPE TOWN

in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

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July 1990

SUMMARY

This project forms part of a general programme in which the molecular structure of plant polysaccharides, particularly those that occur as gummy exudates from trees, are studied. Variations in the molecular structure of the polysaccharides are linked with the taxonomy of the plant concerned; this link with taxonomy is of particular interest where the cycads are concerned as these plants originated during the Mesozoic era and can therefore be considered as "living fossils". The exudate gums from six different species of the genus Encephalartos were surveyed, and the detailed molecular structures of the gums from two of the species, viz. E. friderici-guilielmi and E. longifolius were examined. Of particular interest in the gums from this genus is the glucuronomannoglycan core structure. The determination of the core structure is made particularly difficult in this case by its being surrounded by a complex structure of sugars and uronic acid residues.

The polysaccharides, which exuded from the cones or from the crown of the tree after rain, were isolated and purified using standard techniques. Quantitative analysis of the sugar and uronic acid units, was performed on all of the species studied. Determination of the modes of linkage of the sugars in the two species mentioned, as well as in E.ghellinckii, was obtained by methylation analysis. This involved extensive use of g.l.c. and g.l.c.-m.s.. The gums from these three species were

compared further by performing base-catalysed β-elimination experiments on the methylated polysaccharides, from which it was deduced that →4)-GlcA is exterior to both mannose and galactose, linked to O-2 of mannose and O-6 of galactose, and interior to mannose and rhamnose. Galactose and mannose are in some cases further substituted at O-3 and at O-3 and O-4 respectively.

Detailed structural work on the two species was by various degradation methods, the products of degradation being isolated and analysed after each step. E.friderici-guilielmi gum was studied by partial acid hydrolysis, acetolysis and Smith degradation and E.longifolius by partial acid hydrolysis and a methylation study in which the higher molecular weight, partially methylated product obtained by fractionation of the partial hydrolysate of the methylated gum was isolated and characterised. During the course of the partial hydrolysis experiments, several oligosaccharides were isolated and characterised. Partial hydrolysis of low molecular weight degradation products, which were nearly galactose-free, gave oligomers of →4)-β-D-GlcpA-(1→2)-α-D-Man-(1-.

A general structure for the polysaccharide exudate from Encephalartos gums was proposed, having a glucuronomannoglycan core with 3-linked galactan chains onto O-3 of mannose, either directly or through →3)-Arap. O-4 of mannose is substituted by sugar residues that survive some acid hydrolysis and some of which survive Smith degradation, whereas the substituent on O-3

of glucuronic acid is an acid-labile sugar, probably Araf. In the peripheral regions, glucuronic acid is preponderant, onto O-6 of galactose and interior to rhamnose. The isolation of β-D-GlcpA-(1→6)-D-Gal and β-D-Galp-(1→3)-L-Ara as well as β-D-GlcpA-(1→6)-β-D-Galp-(1→3)-L-Ara from low molecular weight products after partial hydrolysis proved the existence of the linkages in these oligosaccharides in the gum.

Methyl ethers of galactose were isolated and their ^{1}H- and ^{13}C-n.m.r. assignments completed. This was done for a series of 2-O-methyl-galactopyranose methyl ethers. Changes in chemical shift on O-methylation were explained. Deviations from the normal O-methylation shift at C-1 and H-1 for the α-anomer and at C-4 and H-4 were explained in terms of a γ-gauche interaction between protons on 3-OCH_3 and C-4 and between 2-OCH_3 and C-1. These results were used as an aid in the characterisation of 3-O-methyl-L-rhamnose isolated from the gum.

Part of the work reported in this thesis has been published; viz.

3-O-Methyl-L-rhamnose as a constituent of plant polysaccharide gums - M.Kaplan, A. M. Stephen, and Daphne Vogt, S. A. Med. J., **40** (1966) 72.

The gum exudate of **Encephalartos longifolius** Lehm. (fem.). - A. M. Stephen and Daphne C. de Bruyn, Carbohydr. Res., 5 (1967) 256-265.

Exudates from Encephalartos cones as chemical taxonomic markers - Daphne C. Stephens and Alistair M. Stephen, S. Afr. J. Sci., **84** (1988) 263-266.

Exudates from Encephalartos cones - Daphne C. Stephens and Alistair M. Stephen, reviewed by Roy Osborne, Encephalartos, **No. 13** (1988) 22-24.

^{1}H-N.m.r. and ^{13}C-n.m.r. spectroscopy of methyl ethers of D-galactopyranose - Daphne C. Vogt, Alistair M. Stephen, and Graham E. Jackson, Carbohydr. Res., (1990), in press.

ACKNOWLEDGEMENTS

The author would like to thank the following:

Professor A. M. Stephen for guidance, support and continual encouragement.

Dr. S. C. Churms for many helpful discussions, encouragement and especially for imparting her expert knowledge on s.e.c. and advice on the preparation of this thesis.

The other members of the Carbohydrate group, in particular Dr. W. T. Mabusela for useful discussions and help and advice on g.l.c. and uronic acid determinations, Mr. P. F. K. Eagles for invaluable encouragement and help with practical problems, and Mr. E. Hanevil for general laboratory maintenance.

Professor G. E. Jackson for guidance and patient discussions in the n.m.r. work.

Professor K. R. Koch for help with n.m.r. operations.

Mr. Z. Brown and Mr. N. Hendricks for running n.m.r. experiments.

Mrs. J. van der Straaten (Mass Spectrometry Unit, Dept. of Chemistry, UCT) for performing g.l.c.-m.s. and m.s. experiments.

Dr. L. Parolis (School of Pharmaceutical Sciences, Rhodes University, Grahamstown) for coming to the rescue and providing expert g.l.c.-m.s. service when most needed.

Dr. A. Lawson (Clinical Research Centre, Mass Spectrometry Unit, Harrow, Middlesex, England) for carrying out f.a.b.-m.s. experiments and for helpful discussions.

Dr. E. H. Merrifield for gifts of β-galactosidase and advice on enzyme experiments.

Mr. J. Winter (National Botanic Gardens, Kirstenbosch) for help and advice on cycads and permission to collect gum.

Mr. Dirkie Petersen (National Botanic Gardens, Kirstenbosch) for help in collecting gum samples.

Dr. Roy Osborne (Dept. of Chemistry, University of Natal, Durban) for interest shown in the project and information on cycads.

Mrs. Denise Fourie (Botanic Research Institute, Pretoria) for samples of Encephalartos gums.

Cathie Lillie for drawing the figures and for much patience and understanding.

Malcolm Sole for help in processing the manuscript.

Andy Scholtz for much patience in the final layout and printing of the manuscript.

CSIR and the University Research Council for financial assistance and the University of Cape Town for facilities during the course of this work.

My daughters, Anthea and Sarah, for incredible support, encouragement and tolerance.

An amazing support group of friends without whom this work would never have been accomplished.

My brother, Dick, for encouragement and the provision of electronic equipment.

For Family and Friends

ABBREVIATIONS

~	approximately
a.a.	alditol acetate
Ara 	arabinose
c	centi-
ca.	approximately
d	day
D	deuterium
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DEAE	diethylaminoethyl
dimsyl	methylsulphinylmethanide
E.cycad.	Encephalartos cycadifolius
E.f-g.	Encephalartos friderici-guilielmi
E.ghel.	Encephalartos ghellinckii
E.long.	Encephalartos longifolius
eV	electron volt
Ery	erythritol
f	furanose
f.a.b.	fast atom bombardment
Fuc 	fucose
g	gram
Gal 	galactose
g.l.c.	gas liquid chromatography
Glc 	glucose
GlcA 	glucuronic acid
Gly	glycerol
h	hour

h.p.l.c.	high performance liquid chromatography
Hz	hertz
i.d.	internal diameter
i.r.	infrared
k	kilo-
LAD	lithium aluminium deuteride
l	litre
M	molar
m	metre
m	milli-
Man 	mannose
μ	micro-
min	minute
mol	mole
mol.wt.	molecular weight
m.p.	melting point
m.s.	mass spectrometry
$\overline{M}_W$	weight average molecular weight
n.d.	not determined
n.m.r.	nuclear magnetic resonance
p	pyranose
p.c.	planar chromatography
p.m.a.a.	partially methylated alditol acetate
p.p.m.	parts per million
Rha 	rhamnose
3Rha	3-O-methyl-L-rhamnose or 3-Me-Rha
$SD1_1$	first limit of the first Smith degradation
$SD1_L$	second limit of the first Smith degradation

s.e.c.		steric exclusion chromatography
T		terminal
TFA		trifluoroacetic acid
TMU		tetramethylurea
THF		tetrahydrofuran
tr		trace
v/v		volume per volume
vol		volume
w/w		weight per weight
Xyl	△	xylose

All other abbreviations are defined in the text.

NOTES TO READERS

1. Structure numbering is specific to the chapter in which the number appears.
2. The term "autohydrolysis" has been applied to partial hydrolysis of the gum at pH 2.
3. Generally, the condensed form of naming oligosaccharides has been used in the text, thus Galβ-3Ara is β-D-Galp--(1→3)-L-Ara or 3-O-β-D-galactopyranosyl-L-arabinose, etc.
4. Naming of methyl ethers: Me has usually been omitted, particularly in tables, thus 234 Rha is 2,3,4-tri-O--methyl-L-rhamnose or the appropriate derivative.

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1. INTRODUCTION

1.1 OCCURRENCE OF GLUCURONOMANNOGLYCAN CORE POLYSACCHARIDES

The occurrence of glucuronomannoglycan polymers in the higher plant kingdom, notably in plant gums of the type designated D, is now well documented[1]. Although conclusive evidence has not been obtained for the presence of such polymers having more than four consecutive units of the structure -4GlcAβ-2Manα-, it is generally believed that this core structure is likely to occur.

Other sugars found in association with -4GlcAβ-2Manα- have been predominantly galactose, arabinose and rhamnose. Although arabinose is often terminal as arabinofuranosyl residues, in some cases, notably gum ghatti (Anogeissus latifolia gum)[2] →3)-Arap has been proved to be the link sugar between the galactan side-chains and the glucuronomannoglycan core through O-3 of mannose.

The following constitutes a summary of some polysaccharide-containing plants and a seaweed, for which there is evidence of a glucuronomannoglycan core structure.

(a) Anogeissus latifolia (gum ghatti) and A.leiocarpus (leiocarpan) gum: Gums from these two species have similar basic structure[1,3] in which Man is substituted at positions 3 and 6 as shown in Figure 1.1.1.

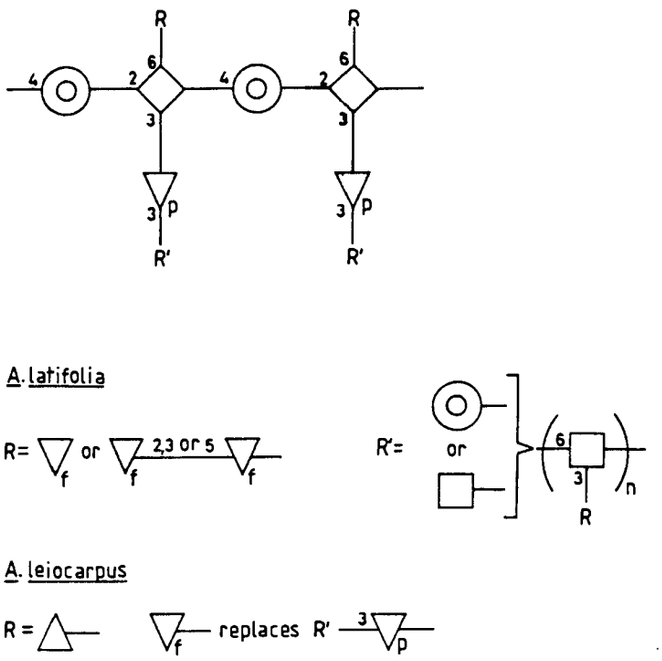


Figure 1.1.1 Structural features of gums from Anogeissus species.

In gum ghatti, the 6-linked Gal chain is terminated by GlcA or Gal. Furthermore, the 6-linked Gal is substituted at O-3 by Araf or short chains of Araf [2]. Evidence for substitution of Man at the 3-position by Arap was afforded by the isolation of the disaccharide 3-O-L-arabinopyranosyl-D-mannose after partial hydrolysis of the Smith degradation product[2]. Similarly, 3-O-β-D-galactopyranosyl-L-arabinose was isolated and characterised. Evidence for the glucuronomannoglycan core was obtained by acetolysis of the acetylated, reduced polysaccharide in gums from both species[3,4] . In leiocarpan, evidence for O-6 of Man as the site of attachment of the Xylp residue was obtained by base catalysed β-elimination studies of the methylated degraded gum, using the base, DBU

(1,5-diazabicyclo[5.4.0]undec-5-ene) as catalyst. 3,4-Di-O-methyl-6-O-(2,3,4-tri-O-methyl-β-D-xylopyranosyl)-D--mannitol was identified as a product by mass spectroscopy of its derivatives[5]. Further proof for the substitution pattern on A.leiocarpus gum was obtained by the decarboxylation--acetoxylation method[6].

(b) Gums from the Prunus species: There is the possibility of alternating residues of →4)-GlcA and →2)-Man in gums from cherry (P.cerasus)[7], apricot (P.armeniaca)[8] and blackthorn (P.spinosa)[9]. Again Man is substituted at O-3, largely with 6- and 3,6- linked Gal.

(c) Chorisia speciosa[10]: By a series of partial hydrolyses, base degradation, and periodate oxidation experiments, the structure of the gum from C.speciosa was postulated as comprising a glucuronomannoglycan core to which chains of 3-, 3,6- and 6-linked galactose residues are attached at O-3 of Man. Some galactan chains terminate in Rhaα-4GlcAβ-6Galβ-. Man is not further substituted.

(d) The carnivorous plants Drosera binata and D.capensis[11,12]: The polysaccharide mucins from the leaves of these two species were shown to have a glucuronomannoglycan structure with Galp present as non-reducing end-group linked to O-3 of the mannose residues. Araf and Xylp are linked to O-3 of the D-GlcA residues.

(e) Kiwi fruit (Actinidia deliciosa)[13,14,15]: The mucilage from the stem pith of kiwi fruit was shown, by partial acid hydrolysis of the methylated, carboxyl-reduced glucuronomannoglycan core to contain a -4GlcpAβ-2Manpα- backbone in which about 50% of the GlcA residues are substituted at O-3 with Araf and about 95% of the mannosyl residues are substituted at O-3 with oligosaccharides containing β-D-Gal, L-Ara, and α-L-Fuc. The neutral oligosaccharide substituents were shown to be 3-linked galactan chains with some Gal residues substituted at O-2 and/or O-6 with Ara or Fuc. A tentative structure for a portion of the kiwi fruit mucilage is shown in Figure 1.1.2. The characterisations were achieved by f.a.b.-m.s., e.i.-m.s., and g.l.c.-m.s. of the methylated alditol acetates that were isolated by reverse phase h.p.l.c..

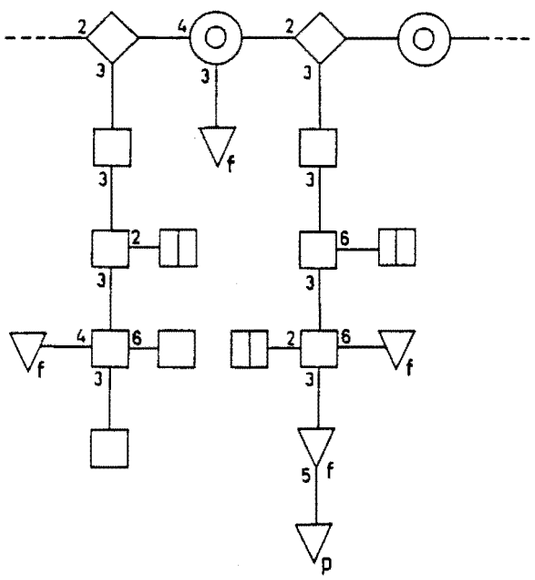


Figure 1.1.2 A tentative structure for the polysaccharide in kiwi fruit mucilage.

(f) Grevillea robusta[16], the silk oak: In the polysaccharide from this gum, the presence of -4GlcAβ-2Manα-4GlcAβ- was proven by DBU-catalysed β-elimination of a methylated Smith-degradation product. The identification of 3-O-acetyl-penta-O-methylmannitol by g.l.c.-m.s. after derivatisation confirmed the occurrence, immediately exterior to GlcA, of Man carrying a substituent on O-3. This substituent is largely 3-, 4-, or 6-linked Gal, which in turn is further substituted with the acid-labile sugars, Ara and Xyl.

(g) Hakea sericea and H.gibbosa[17] gums: The linear monomer, dimer and trimer of -4GlcAβ-2Manα- have been isolated after partial hydrolysis of the polysaccharide exudate from H.sericea. Smith-degradation studies on the polysaccharide have shown that Man is substituted at O-3 by galactan chains. There is also acid-labile Ara in the gum.

(h) Extracellular polysaccharides from suspension-cultured cells of tobacco (Nicotinia tabacum): The extracellular polysaccharides of suspension-cultured tobacco cells were shown to comprise a glucuronomannoglycan core[18] to which was attached L-arabinofuranosyl residues, α linked to O-3 of the GlcpA residues and β to O-3 of the Manp residues[19]. The detailed pattern of substitution by arabinose was determined by characterisation of a series of 4-O-α-D-mannopyranosyl-D-xylitol derivatives with and without L-arabinofuranosyl side-chain substituents, after decarboxylation-acetoxylation of

the saponified permethylated polysaccharide with lead tetraacetate[19].

(i) The brown seaweed, Lessonia nigrescens[20]: On partial hydrolysis the polysaccharide from L.nigrescens gave monosaccharides, neutral and acidic oligosaccharides, an insoluble residue and a soluble polymer. This soluble polymer which comprised 20% of the starting material and had a molecular weight of ca. 8000, had n.m.r. spectra indicative of a linear polymer of ca. 44 alternating units of GlcA and Man.

(j) Ornithogalum thyrsoides: The polysaccharide isolated from the mucilage of O.thyrsoides[21] appears to be a glucuronomannoglycan of exceptional homogeneity with Araf as single unit side chains attached to O-3 of GlcA. ^{13}C-n.m.r. of the partially hydrolysed polysaccharide from which Araf had been removed gave a spectrum comparable with that obtained for the polymer obtained from partial hydrolysis of Lessonia nigrescens. The monomer,dimer and trimer of -4GlcAβ-2Manα- were isolated and characterised, and there was also evidence of the tetramer and higher oligomers.

Polysaccharides from other sources, for example Brabium stellatifolium (van Riebeeck's hedge)[22,23], Virgilia oroboides[24], and Albyzzia zygia[25] gum have many structural features in common with the examples given above. The structure of the polysaccharide from V.oroboides gum has been

compared with that of gum ghatti, and that from A.zygia has, in addition to -4GlcAβ-2Manα-, prominent 3-linked galactan chains and the disaccharide Galβ-3Ara.

There is evidence from earlier work that the gum exudate from the cycad Encephalartos longifolius is a complex polysaccharide with a glucuronomannoglycan core. The Encephalartos gum exudates are particularly high in rhamnose and some, especially from E.longifolius, contain a high proportion of 3-O-methyl-L-rhamnose as well as galactose. Of particular interest in E.longifolius gum is the structural significance of the 3-O-methyl-L-rhamnose. From partial hydrolysis experiments, it appears that this sugar is close to the core as it remains with the higher molecular weight fraction and may be of use as a chemotaxonomic marker.

Welwitchia mirabilis, which like Encephalartos is a plant of ancient origin, but found in the Namib desert, exudes a gum from its leaves which appears to bear a close resemblance to that from Encephalartos species[26]. This is of some chemotaxonomic interest.

1.2 CYCADS

1.2.1 Introduction

Cycads[27], the most primitive living seed-bearing plants known, belong to the order Cycadales which flourished in the carboniferous period. Dyer, in "The Cycads of Southern Africa"[28] quoted Worsdell as saying in 1906 that "no Family of plants is exciting more interest at the present day than the Cycadaceae, and for the prime reason that they represent one of those forms of plant life which link the types of the far past with those of the present modern world".

The cycads have propagated themselves for over 50 million years, with little change in basic character. This does not imply that there has been no change in the species from one era to the next, but that new forms have evolved as and when environmental changes created sufficient stimulus. Apart from their long period of survival, the plants are slow-growing and have a long period of growth. The first cycad (a specimen of Encephalartos longifolius) collected in South Africa in 1772 is still flourishing in Kew Gardens and it is estimated that a cycad having a trunk 8m tall could be 500 years old.

The South African cycads are known to be toxic. However when E.longifolius was first discovered by Thunberg in 1772, he recorded that "It is out of the pith of this tree that the Hottentots contrive to prepare their bread". Young leaves of

all species are eaten by some animals, and the fruit is eaten by small animals, notably rodents, and some fruit-eating birds. However, the hardcoated kernel is discarded or regurgitated and is the only part of the plant known to be toxic.

1.2.2 Evolution of Cycads[29]

The first plants were, no doubt, the algae which grew abundantly in the oceans up to 3 X 10^{12} years ago. Their photosynthetic ability caused the oxygen and ozone levels to build up to the extent that it made possible the potential for life outside the marine environment. As a result of this the liverworts, mosses and primitive ferns evolved.

All of these required water for the reproductive process because of their motile sperm cells. During the evolutionary process some anachronisms have developed. The cycads have motile sperm cells but bear seeds like the most advanced flowering plants. Cycads have been likened to the reptiles of the plant world and have a mixture of some relatively advanced and some relatively primitive characteristics.
Cycads rose to abundance in the world's vegetation when the dinosaurs were at their peak.

The Mesozoic cycads were not the same as our modern cycads. There were two broad types, namely cycadaleans, the ancestors

of modern cycads, and cycadeoidaleans (also called Bennettitales) which later became extinct.

Geography of the world in the late Palaeozoic and Mesozoic era has influenced the distribution of the cycad species. The southern part of Africa, South America, India, Antarctica, and Australia were all joined to form the massive land mass of Gondwanaland. At the time when cycads were at their peak, cracks appeared in the land. The South Atlantic gradually widened, starting with the separation of the Falkland tip from the Agulhas escarpment late in the Mesozoic era. Only 65 million years ago India separated and moved up to collide with the Asian land-mass. Antarctica formed a bridge between South America and Australia until about 50 million years ago. 25 Million years ago Africa was already in its present position but the central plateau was 1800m lower than at present and the great escarpment had not yet formed. After major uplifts in the regions surrounding it, the central plateau became the arid Karoo basin.

During this time, the cycads were evolving and, although they presumably had one common ancestor, their evolution gave rise to the different genera in the following places:

in Africa: Encephalartos and Stangeria

in the lands bordered by the Indian Ocean: Cycas

in Australia: Bowenia, Macrozamia, and Lepidozamia

in the Americas: Ceratozamia, Chigua, Dioon, Microcycas, and Zamia.

There is only one species of Stangeria (FAMILY: STANGERIACAE) in South Africa but an estimated 52 different species[30] of Encephalartos (FAMILY: ZAMIACAE). Under the influence of local environmental conditions, various of the species have markedly similar characteristics.

Thus the cycads are not survivors as such from the Mesozoic era but are remarkable plants that have adapted and evolved through the millenia and are still capable of adapting and evolving to our changing environment. Their chemistry is therefore of interest not only because of their survival and evolution through the ages but also chemotaxonomically[31] as they are considered to occupy a key position in botanical hierarchy[32].

1.2.3 Gum Exudates from Cycads.

Although the n-alkane distribution patterns in leaf waxes in 42 taxa of Encephalartos have been reported[33], few studies of the gum exudates from cycads have been published. De Luca and coworkers[34] studied the monosaccharide composition of mucilages of Cycadales and found the proportions of sugars to be characteristic at generic level. The monosaccharide distribution of fourteen species of Encephalartos has been reported to be identical[35]. The monosaccharide composition of hydrolysates of the mucilage of individual specimens of E.longifolius was found to be independant of age, sex and

growth conditions[36]. However these results are not consistent with those obtained in the present study. Although the same monosaccharides are present in the different species, the proportions have been observed to vary from species to species; the general trend is the same throughout (see Chap. 4).

The presence of 3-O-methyl-L-rhamnose is a characteristic of the exudate gums from Encephalartos species, and this sugar has been isolated from a specimen of E.longifolius[37]. Although the total amount of rhamnose and 3-O-methyl-L-rhamnose appears to be consistent, a large difference in the proportions of these sugars in two samples of E.ghellinckii has been noted (see Chap. 4).

1.3 STRUCTURAL DETERMINATION OF GLUCURONOMANNOGLYCANS

The determination of the structure of gum exudate polysaccharides requires ultimately that monomer composition, sequence, position of linkage, and anomeric configuration be known. The standard methodology may be applied for the elucidation of polysaccharide structures[38], but those containing uronic acid residues, as do the gum exudate polysaccharides, pose a problem. Glycosiduronic acid linkages are well known to be resistant to acid-catalysed cleavage in hydrolysis and related reactions. It is therefore, not normally possible to isolate by hydrolytic procedures both acidic oligosaccharides and those bearing intact relatively

acid-labile substituents. For this reason, chemical reactions specific to the degradation of the glycosiduronic residue have been developed by Aspinall and coworkers[39,40]. The selective fragmentation of methylated glucuronomannoglycans has been discussed in recent reviews[41,42].

These reactions may take place more slowly in polysaccharide substrates than in related oligosaccharides which have been used as model compounds[8,39,43,44,45]. Completeness of reaction in structural modification is essential as it is not possible to separate modified from unmodified sugar residues in the same macromolecule. Where only single linkages in outer chains must be split to liberate the desired cleavage product, incomplete reaction will merely result in a low yield of that product. However, where it is necessary to split two or more linkages in inner chains before the cleavage product is removed, incomplete structural modification will result in drastically reduced yield and may even yield none of the required product. There is no guarantee that a specific degradation procedure will prove effective on a particular polysaccharide. The structural elucidation of polysaccharides that appear to be related can serve merely as a guide to possible techniques to be employed.

In the preliminary analyses of the cycad gum exudates under study, it was found that there was a high proportion of →4)-GlcA- and that much of the galactose was 3,6- linked. The procedures that would be likely to yield results are discussed here. In all cases some sort of controlled depolymerisation

has been employed to obtain intact segments of the polysaccharide.

Acid hydrolysis: As there is a preponderance of glucosiduronic acid residues in these gums, the technique of mild acid hydrolysis to cleave the glycosidic link should yield aldobiouronic acids and their oligomers. This technique was employed extensively in an attempt to obtain an intact section of polymeric -4GlcAβ-2Manα-.

Acetolysis: Acetolysis of the carboxyl-reduced polysaccharide serves as a complementary[3] procedure to acid-catalysed hydrolysis. Saponification of the acetolysed mixture releases oligosaccharides not normally obtained under conditions of acid hydrolysis, for example, a series of glucomannoglycans was obtained from leiocarpan[4].

Smith degradation[46,47]: Methylation analysis showed that the gum had a high proportion of sugar residues that contained 1,2-diols and would consequently be oxidised by periodate. The uptake of periodate is monitored[48,49] to ascertain when oxidation is complete and as a check on the expected uptake as calculated from the results of methylation analysis. Stoichiometrically, one mole is required per vicinal diol. The resulting polyal is then reduced to give the polyol, and this undergoes acetal cleavage under mild acid hydrolysis conditions at room temperature to yield low molecular weight polyols (mainly glycerol) and products of higher molecular weight

label it at C-1 of what is now the alditol. After mild hydrolysis to remove the residues of degraded acid, thereby exposing the site to which the acid had been linked, the degraded methylated gum is deuteriomethylated to label these sites and is then subjected to methylation analysis. In this way, a polysaccharide comprising alternate →4)-GlcA and →2)-Man residues would give only 1,2,5-tri-O-deuteriomethyl--3,4,6-tri-O-methyl mannitol, deuterium labelled at C-1 as shown in Figure 1.3.1.

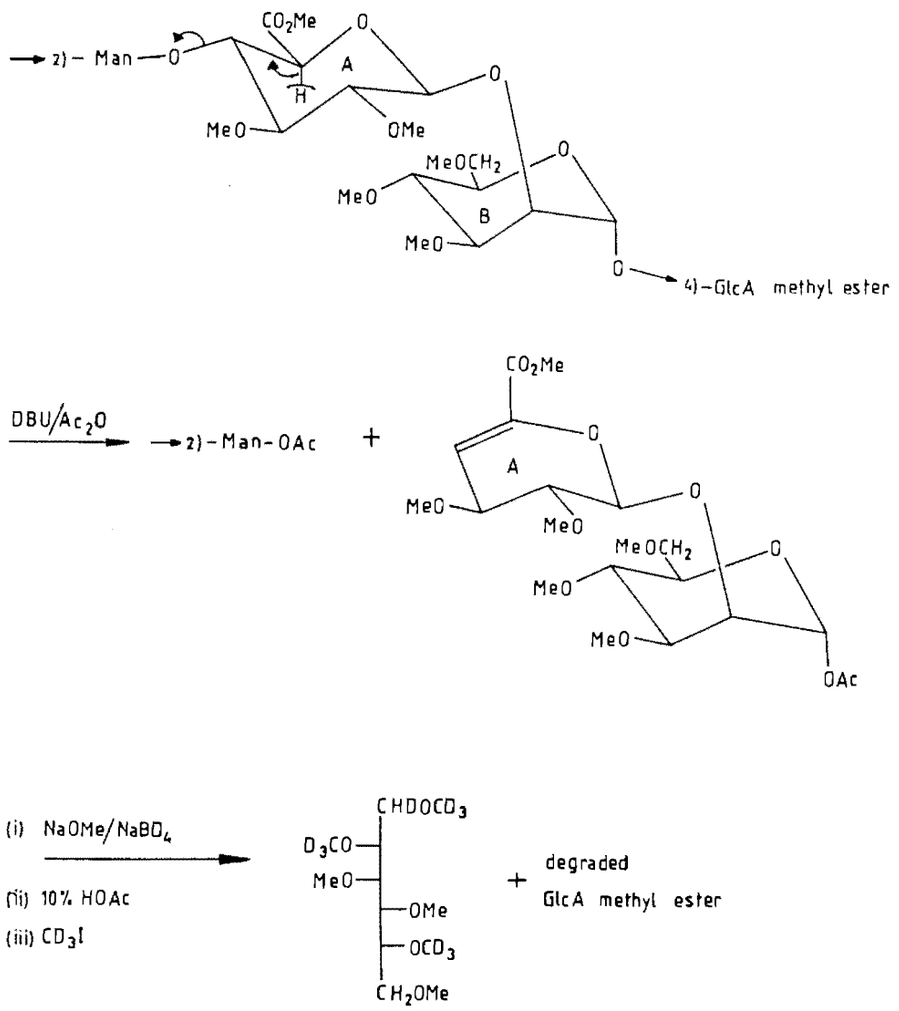


Figure 1.3.1 Base-catalysed β-elimination with concomitant protection of the newly released sugar, showing the mannitol derivative formed.

1.3.1 Characterisation of Products by N.m.r. Spectroscopy.

During the course of this work, it was necessary to characterise methyl ethers isolated on column fractionation and there was found to be a lack of n.m.r. data on methyl ethers of D-Gal. Chemical shifts of resonances of methoxyl protons had been published[51,52,53] but few corresponding results for ^{13}C. In an extensive review by Bradbury and Jenkins[54] there were data on skeletal ^{13}C signals for some compounds as well as a set of empirical rules relating substitution and changes in chemical shift. However, this was insufficient for complete characterisation of the methyl ethers isolated. Complete ^{1}H and ^{13}C assignments of a series of methyl ethers were made during the course of the present work, together with an analysis of the reasons for the changes in shift on substitution.

2. GENERAL EXPERIMENTAL CONDITIONS

2.1 PLANAR CHROMATOGRAPHY (p.c.)

The following solvent systems were used: (all v/v)

(i) For paper chromatography

a: butan-1-ol: acetic acid: water 2 : 1 : 1;

b: ethyl acetate: pyridine: water 8 : 2 : 1;

c: butan-1-ol: ethanol: water (upper phase) 4 : 1 : 5;

d: ethyl acetate: acetic acid: formic acid: water 18 : 3 : 1 : 4;

e: ethyl acetate: pyridine: water 10 : 4 : 3;

f: butan-2-one - water azeotrope;

g: benzene: ethanol: water (upper phase) 169 : 47 : 15;

(ii) For thin layer chromatography

h: chloroform: methanol: water 20 : 20 : 7;

i: chloroform: methanol 5 : 1.

Analytical paper chromatography was performed on sheets of Whatman No.1 paper by the descending method (two-dimensionally where required), and ascending thin-layer chromatography on Merck aluminium sheets coated with silica gel $60F_{254}$, thickness 0,2mm.

Preparative paper chromatography was performed on Whatman 3MM paper, pre-washed with deionised water then dried. The sample was dissolved in water and applied in a narrow band near the top of the sheet. Small aliquots were applied to narrow strips

of similar paper and eluted at the same time in the same tank; these were removed at intervals for spraying to determine the progress of the separation. After elution of the sheets and subsequent location of the components, suitable bands were cut from the sheets. Components were isolated by extraction with deionised water, filtration, concentration of the filtrate and freeze-drying.

Reagents for detecting components separated by p.c. were as follows:

(i) On paper

a: spraying with a solution of p-anisidinium hydrochloride in aqueous butan-1-ol, followed by heating at 110° for 5-10 minutes;

b: spraying with an aqueous solution of ammoniacal $AgNO_3$, followed by heating for 5 - 10 minutes at 110°.

(ii) On thin layer

c: spraying with p-anisaldehyde : sulphuric acid : ethanol (1:1:18, v/v) followed by heating at 110° for 5-10 minutes;

d: spraying with $\underline{\underline{M}}$ H_2SO_4 and heating at 110°.

2.2 GAS-LIQUID CHROMATOGRAPHY (g.l.c.)

2.2.1 Quantitative analysis

G.l.c. analysis of alditol acetates (a.a.'s), partially methylated alditol acetates (p.m.a.a.'s) or methyl glycosides[55] of partially methylated sugars was carried out using a Carlo Erba 4200 gas chromatograph (columns a and b) or a Carlo Erba GC 6000 VEGA SERIES 2 (column c) each coupled to a Spectra-Physics SP4290 integrator. Detection was by flame-ionisation.

The columns used were:

a: glass (2m x 3mm i.d.) column packed with 3% OV-225 on Chromosorb W-HP, 80-100 mesh;

b: glass (2m x 3mm i.d.) column packed with 3% ECNSS-M on Gas-Chrom Q, 100-120 mesh;

c: glass capillary OV-225 SCOT column (25m x 0,35mm i.d.).

The carrier gas was helium. Mixtures of a.a.'s and p.m.a.a.'s were analysed on columns a (isothermally at 175° for p.m.a.a.'s and 210° for a.a.'s) and c (programmed, from 100° at 4° per minute to 250°), and mixtures of methyl glycosides were analysed on column b (isothermally at 155°). Components in mixtures analysed were identified by comparison of relative retention times with those of standards run under identical conditions, by co-injection with standards in some cases, and by g.l.c.-m.s.. Quantitative analyses were achieved by using

the molar response factors of Sweet et al.[56] for mixtures of p.m.a.a.'s, and empirically determined molar response factors[3] for mixtures of a.a.'s.

2.2.2 Gas liquid chromatography - mass spectrometry[57] (g.l.c.-m.s.)

For g.l.c.-m.s., a Carlo-Erba 4200 gas chromatograph was coupled through a jet separator to a VG Micromass 16F mass spectrometer. Column c was used with temperature conditions identical to those used in quantitative analysis.

Some analyses were performed at the School of Pharmaceutical Sciences, Rhodes University, Grahamstown (by courtesy of Professor H. Parolis), on a Hewlett-Packard Model 5890A gas chromatograph coupled to a HO 5988A mass-spectrometer (quadrupole); the column used was a fused silica capillary column, 30m x 0,25mm, coated with OV-225, film thickness 0,25µm.

2.3 STERIC EXCLUSION CHROMATOGRAPHY (s.e.c.)

Average molecular weights ($\bar{M}_W$) were estimated by s.e.c.[58] on the following columns:

a: Sepharose 4B (60cm x 0,9cm), eluent M NaCl;

b: Bio-Gel P-10 (53cm x 1,5cm), eluent M NaCl.
Other columns used are described in the text.

The sample (1-2mg), dissolved in eluent ($1cm^3$), was applied to the column, and fractions ($1,5-2,5cm^3$) of the column effluent were collected. The emergence of carbohydrate in the fractions was monitored by the phenol-sulphuric acid method[59].

2.4 GENERAL CONDITIONS

Optical rotations were measured from aqueous solutions for underivatised samples, and from chloroform solutions for methylated products, on a Perkin-Elmer Model 141 polarimeter. X-Ray powder diffraction data, obtained by using a Philips model P.W. 1050 diffractometer, gave interplanar spacings (A) for Cu Kα radiation (peak intensities: vs, very strong; s, strong; m, moderate; w, weak; vw, very weak). Infra-red spectra of methylated derivatives in chloroform were recorded on Perkin-Elmer Model 237 or 983 spectrophotometers.

200MHz ^{1}H-n.m.r. and 50,3MHz ^{13}C-n.m.r. spectra were obtained on a Varian VXR-200 spectrometer. Deuterium exchange was effected by freeze-drying solutions in D_2O three times. Samples (11-32 mg) were examined at 25° (unless stated otherwise), as solutions in D_2O. ^{1}H and ^{13}C chemical shifts

were measured with reference to internal acetone, δH 2,21 and δC 31,0 downfield of tetramethylsilane.

Fast atom bombardment (f.a.b.)- mass spectrometry was carried out at the Clinical Research Centre, Mass Spectrometry Unit, Harrow, Middlesex, England (by courtesy of Dr. E. F. Hounsell). The samples were run on a VG Analytical ZAB2-E mass spectrometer operated at 8keV accelerating voltage and using a Cs ion gun (25keV, 0,5μA emission). The matrix was thioglycerol.

2.5 ORIGIN AND PURIFICATION OF SAMPLES

All samples, unless stated otherwise, were collected in Kirstenbosch Gardens after rain in the winter of 1987.

E.longifolius (female): Two samples were used. The first was collected in ca. 1964 and the second in 1987.

E.friderici-guilielmi (male): The first sample was collected near Cathcart in December, 1966 and the second in Kirstenbosch.

E.ghellinckii (sex unknown): The first sample was collected in Pretoria Botanical Gardens in 1966 and the second in Kirstenbosch.

Others were collected by the staff of Pretoria Botanical Gardens during 1987 (by courtesy of Mrs. D. Fourie).

Purification of old gum samples was effected by dissolving the hard gum nodules in water. This necessitated sonication in some cases. After filtration through muslin, the polysaccharide was precipitated by pouring the aqueous solution into ethanol (4 vols). After the precipitated polysaccharide had been washed with ethanol and dried, it was dissolved in water and the solution freeze-dried. Fresh gum samples were treated in much the same way, except that dissolution was easier and less sonication was necessary.

2.6 SUGAR ANALYSES

The proportions of neutral sugars were determined by g.l.c. as a.a.'s, prepared by the method of Albersheim et al.[60] or as aldononitrile acetates, prepared by the method of Morrison[61]. The proportions of neutral sugars constituting polymeric products were determined by hydrolysing the polysaccharide in 2M TFA at 100° for 8h or 18h, depending on whether the polysaccharide contained uronic acid (18h) or not (8h), prior to derivatisation. Smith-degradation products were hydrolysed in M TFA at 100° (14h).

Uronic acid was determined colorimetrically by the method of Blumenkrantz and Asboe-Hansen[62].

2.7 METHYLATIONS AND METHYLATION ANALYSES

Methylations of the purified polysaccharides of the native gums were carried out by the Haworth procedure[63] as little success was achieved by the Hakomori method.[86b] The polysaccharide was dissolved in the minimum of water to which was added a little $NaBH_4$ to protect the reducing end groups. Portions of 50% NaOH and dimethyl sulphate (DMS) were added dropwise over half-hour periods, with vigorous stirring, on the first day under N_2 at 0° and thereafter at room temperature, over a period of 8-10d. After dialysis, the non-dialysable product was freeze-dried.

After prior decationisation (Amberlite IR-120 (H^+)), methylation of the degradation products was performed under Ar, by the Hakomori method[64] as modified by Phillips and Fraser[65], using the base potassium methylsulphinylmethanide (potassium dimsyl) (2$\underline{\underline{M}}-3\underline{\underline{M}}$) to form the alkoxide, prior to treatment with excess MeI. The presence of excess base was tested for with Ph_3CH (which turns blood-red). After the addition of water, the Hakomori-methylated product was extracted into chloroform and the extract was evaporated to dryness, or, for products of higher molecular weight, the water-chloroform mixture was dialysed. This product was further methylated by the Purdie procedure[66] (refluxing in MeI in the presence of Ag_2O); this treatment was repeated until there was no deposit of yellow AgI on the black Ag_2O. Completeness of methylation in methylated gums was checked by

i.r. spectroscopy of the methylated product in chloroform, and by hydrolysis of a small sample followed by paper chromatography (solvent c). Purification was effected by passing the product through a column of Merckogel 2000 or, for products of higher molecular weight, Merckogel 20000. The eluent used was $CHCl_3$:EtOH (1:2), and the fractions were monitored using the anthrone reagent[67].

The methyl-esterified carboxylate groups were reduced using lithium aluminium deuteride (LAD) as reducing agent according to the method of Åman et al.[68]. The methylated polysaccharide and LAD were dissolved and suspended repectively, in dry tetrahydrofuran (THF). The LAD suspension was added to the polysaccharide solution under purified argon, then the reaction mixture was heated at 60° for 18h. Excess LAD was decomposed using moist EtOAc; aqueous tartaric acid was used to decompose any aluminium-polysaccharide complex formed and the reduced methylated product was extracted into $CHCl_3$.

Analyses of the methylated products were carried out after hydrolysis of the products. The hydrolysates were examined by p.c. (solvent c) using the partially methylated sugars from methylated Virgilia oroboides gum[24] as standards. The aldoses were derivatised to p.m.a.a.'s for g.l.c. analysis and g.l.c.-m.s.. Further analyses of the methylated products was carried out by g.l.c. of the glycosides produced by methanolysis of the methylated products.

2.8 BASE DEGRADATION EXPERIMENTS

Base-catalysed β-elimination degradations were performed using two methods.

2.8.1 Using potassium dimsyl[50] as catalyst:

A sample (5-10mg) of the polysaccharide containing methyl glucuronate was dissolved in DMSO and the solution was stirred with excess potassium dimsyl under argon for 18h. Thereafter the base-degraded product was cooled (ice/water) and methylated with excess MeI or CD_3I. Work up and analysis were as described for Hakomori methylations (2.7).

2.8.2 Using a non-nucleophilic base (Hünig's or DBU)[5,15] as catalyst:

The methylated uronic acid-containing polysaccharide (8-12mg) was dissolved in dry toluene ($1cm^3$). After the tube had been flooded with argon and cooled (ice/water), Ac_2O (0,7cm3) and the base, either DBU (1,8-diazabicyclo [5.4.0] undec-7-ene) or Hünig's base (N,N-diisopropylethylamine) ($1cm^3$), were added. Heat was evolved and the solution went green. The tube was sealed and the reaction allowed to proceed at 100° or 110° for times ranging from 24h to 48h. After the base had been removed by washing with 1M HCl, the toluene layer was monitored, by

hydrolysis and p.c., for completeness of degradation of uronic ester units . Thereafter the toluene extract was dried ($MgSO_4$), saponified (NaOMe) with simultaneous borodeuteride-reduction ($NaBD_4$), and hydrolysed with 10% HOAc to remove degraded acid residues preferentially. The product was then freeze-dried and deuteriomethylated, prior to complete hydrolysis and derivatisation to p.m.a.a.'s for methylation analysis.

2.9 SMITH DEGRADATION EXPERIMENTS

A freeze-dried sample of the substrate was dissolved in water and the solution was decationised with Amberlite IR-120 (H^+) resin, neutralised (pH 7) with $NaHCO_3$ and freeze-dried. Expected periodate consumption was calculated from the methylation analysis results and experiments were conducted with periodate in from 50% to 100% excess. The substrate was dissolved in 0,1 to 0,14 $\underline{\underline{M}}$ $NaIO_4$ and the solution was stored in the dark. A periodate solution, serving as a blank, was stored under identical conditions. Periodate consumption was monitored by the arsenite method[48,49,69] in both the blank and the experiment. Oxidation was allowed to proceed until the periodate consumption remained constant. Thereafter the reaction was terminated by the addition of either ethan-1,2-diol with subsequent dialysis against deionised

water, or $Ba(OAc)_2$ with subsequent centrifugation. The oxidised polysaccharide was reduced with $NaBH_4$, portions being added over 2 days until the total mass of $NaBH_4$ was equal to that of the substrate oxidised. The reaction mixture was allowed to stand for 6d. The reduced, oxidised polysaccharide was decationised in the cold (8°) with Amberlite IR-120 (H^+) and the solution was then freeze-dried. The freeze-dried product was dissolved in $\underline{\underline{M}}$ TFA and degradation allowed to proceed at room temperature. The course of the degradation was monitored by s.e.c. analysis[70] of aliquots removed at intervals, and in some cases, also by assay for glycolaldehyde[71] (see Chapter 5). The degradation was terminated by freeze-drying the solution. The Smith-degradation products were fractionated with either Me_2CO-MeOH (1:1) or propan-2-ol, into soluble (products of lower molecular weight) and insoluble (products of higher molecular weight) fractions. After evaporation of the solvent, the products were submitted to analysis.

3. MOLECULAR STRUCTURAL STUDIES ON E.longifolius PART I: EARLIER INVESTIGATIONS[37] INTO THE STRUCTURE OF THE GUM EXUDATE

3.1 DISCUSSION

The appearance of 3-O-methyl-L-rhamnose in the hydrolysate of E.longifolius gum exudate was of sufficient interest to warrant its isolation and identification. Its presence had been indicated in botanically related species[26] and it could therefore be important as a chemotaxonomic marker. The isolation of 3-O-methyl-L-rhamnose from E.long. exudate was achieved by partial acid hydrolysis and subsequent fractionation on a column of cellulose of the mixture of sugars released at an early stage and present in the hydrolysate.

In addition, inspection of the products of acid hydrolysis of the EtOH-precipitated polysaccharide from E.long. gum enabled some structural features of this highly complex material to be deduced[55]. Acid hydrolysis (0,25M H_2SO_4 for 11h, in a boiling water-bath) of the polysaccharide cleft the glycosidic bonds between neutral sugar residues but only a minimal proportion of those between uronic acid and sugar. In this way the following monosaccharides were released in the indicated approximate percentages (by weight) of the total carbohydrate in the polysaccharide: 3Rha (3), Rha (14), Fuc (0,2), Xyl (4), Ara (8), Man (0,5), and Gal (8). The Man residues released represent such a small proportion of the total (8) in the gum

that most, if not all, of the Man must be linked by glycosidic bonds from uronic acid residues which are still bound in polymeric material. This is not so for the Gal units, which constitute nearly one-quarter of the total Gal.

Most of the residual material (comprising 60% of the total carbohydrate in the gum) was found to consist of GlcAβ-6Gal, plus Man-containing acidic oligosaccharides of differing complexity. Further acid hydrolysis (0,25M H_2SO_4, 8h, boiling water-bath) released traces of Xyl (0,5), Ara (1), Man (2) and Gal (4), the latter two probably from the cleavage of biouronic acid residues. 4-MeGlcA (1) and GlcA (5) were also obtained free at this stage. The acidic oligosaccharides (comprising over 40% of total carbohydrate in the gum) that were found were as follows:

6-O-(4-O-methyl-β-D-glucopyranosyluronic acid)-D-galactose (I) (4), 2-O-(β-D-glucopyranosyluronic acid)-D-mannose (II) (8), and 6-O-(β-D-glucopyranosyluronic acid)-D-galactose (III) (23) together with fractions that appeared to contain in small quantities the dimer, trimer, and tetramer of II (8).

I had $[\alpha]_D$ -6° and was characterised by p.c., ionophoresis and hydrolysis. II ($[\alpha]_D$ -30°) was methylated to give the crystalline derivative which was (i) hydrolysed and (ii) methanolysed and submitted to g.l.c.. (Table 3.1.1). Methanolysis results showed that there was a one to one molar ratio of T-GlcA to →2)-Man in II. III had $[\alpha]_D$ -6° and was characterised in the same manner as II, although methanolysis

results gave a rather high ratio of 1:0,7 for T-GlcA to →6)-Gal.

Table 3.1.1 Methanolysis Results for Methylated II and III[a]

	t_R	II	III
2,3,4-GlcA	2,77;3,72	1	1
3,4,6-Man	3,71	1,02	
2,3,5-Gal	5,52	}	0,71
2,3,4-Gal	9,8	}	

a Molar ratios

Acid oligosaccharides containing GlcA and more than one Gal residue, together with material of higher molecular weight composed of GlcA and Man residues, constituted the rest of the hydrolytic products.

The characterisation of the polymeric series involving GlcA and Man was dependent on:
the linear relationship of log $[(1/R_{monomer}) -1]$ against suspected degree of polymerisation[72]; specific rotation and equivalent weight; and partial hydrolysis of each member of the series to give, predominantly, the biouronic acid.

Thus the acidic material from the polysaccharide exuded by E.long. was shown to consist predominantly of the aldobiouronic acids II and III. Preponderant sugars in the polysaccharide as a whole are Rha and Gal.

3.2 EXPERIMENTAL

The polysaccharide sample[26,37] had $[\alpha]_D$ -28°, g.(mol COOH)$^{-1}$ 600 and OMe 2%. After hydrolysis (0,25M H_2SO_4 for 11h at 95°) of the polysaccharide (8,4g), the neutralised ($BaCO_3$), syrupy product (7,0g) was transferred to a water-jacketed (30°) column (98 x 4,5cm) of powdered cellulose and eluted with water-saturated mixtures of light petroleum (b.p. 100-120°) and 1-butanol, wet 1-butanol, and finally, after elution of neutral sugars, with water. Fractions were collected, monitored by p.c. and combined as appropriate. All fractions were compared with known compounds by p.c. in solvents a, c, and d. Characteristics of fractions collected are displayed in Table 3.2.1.

Fraction 1 was identical by ionophoresis in 0,1M borate buffer with an authentic sample of 3Rha. Admixture with an authentic specimen did not depress the m.p.. The i.r. spectrum and X-ray powder diffractogram were identical with those of 3Rha. The d-spacings found were: 13,8w; 10,2m; 7,22s; 6,17s; 5,15s; 4,82m; 4,47s; 4,11m; 3,67m; 3,59m; 3,62w; 3,17s; 3,12m; 2,96w;

2,81w; 2,61w; 2,48w; 2,38w; 2,23m; 2,10w; 2,02vw; 1,97vw; 1,93vw; 1,88vw. De-O-methylation of the sugar with HBr gave Rha (p.c.), and reduction ($NaBH_4$) afforded 3-O-methyl-L-rhamnitol, which was identical (solvent g) with an authentic specimen and gave formaldehyde on periodate oxidation.

Table 3.2.1 Acid Hydrolysis (Stage One) of the Polysaccharide from E.longifolius

Fraction	Sugars	Yield(mg)	$[\alpha]_D$	M.p. (°C)
1	3Rha	142	+33°	112
2	Rha	666	+6,6°	92-92
3	Rha : Fuc (3:4)	17	-38°	-
4	Xyl	147	+19°	-[a]
5	Xyl : Ara (1:2)	141	+79°	-
6	Ara	242	+104,5→97°[b]	156-156,5[c]
7	Ara : Man (8:3)	112	+80°	-
8	Gal	367	+78,5°	-[d]
9	Acidic residues		3500	(as Ba salts)

a Di-O-benzylidene dimethyl acetal had m.p. and mixed m.p. 210-210,5°; b contained 10% (w/w) Xyl;c benzoylhydrazone had m.p. and mixed m.p. 198-200°; d 2-methyl-2-phenylhydrazone had m.p. and mixed m.p. 186-188,5°.

Fraction 9 contained acidic material moving more slowly than Gal on p.c., the major component travelling concurrently with II. As a considerable portion of the material had molecular

weight higher than that of the biouronic acid, the bulk (3,34g) of it was subjected to further hydrolysis, after trials, under similar conditions but for 8h.

After neutralisation and work-up, the acidic and neutral components were separated by successive treatments with Amberlite IR-120 (H^+) and Duolite A4 (OH^-) followed by elution from the latter, first with water and then with NaOH.

Neutral sugars (0,39g) were shown by p.c. and g.l.c. of a.a.'s to contain Gal (8); Man (4); Ara (2); Xyl (1) in the molar ratios indicated. The neutralised, acidic fraction (2,88g) was shown by p.c. to contain numerous components that were separated by transferring the bulk (2,24g) of this fraction to a cellulose column (56 x 3 cm) and eluting with 1-BuOH:HOAc:H_2O (3:1:1). As elution proceeded, content of 1-butanol in the solvent mixture was reduced to 2 parts and then to 1 part. Final washing of the column was with aqueous EtOH. The column performance is summarised in Table 3.2.2.

Fractions were routinely subjected to p.c. and ionophoresis and the later fractions were partially hydrolysed.

Table 3.2.2 Column Performance of E.longifolius. Stage Two: Acidic Components

Fraction	Yield(mg)[a]	$[\alpha]_D$	R_{gal}[b]	Identity[c]
1	31	+25°	1,2; 0,85	A + B
2	13	-	1,2; 0,85	A + B
3	100	+15°	0,85; 1,2	B + A
4	124	+3°	0,85; 0,6	B + C
5	145	-12°	0,85; 0,6; 0,5	B + C + D
6	118	-29°	0,6; 0,5	C + D
7	138	-29°	0,5	D
8	800	-6°	0,4	E
9	50	-15°	0,25	F + E (tr)
10	28	-3°	0,21	G + F (tr)
11	97	-22°	0,15	H + G (tr)
12	31	-4°	0,15	H + I
13	100	-23°	0,07	J
14	108	-14°	<0,05	K

a As Ba salts, except for fractions 1-3; b solvent a;
c A = 4-O-methyl-D-glucuronic acid; B = D-glucuronic acid;
C = 6-O-(4-O-methyl-β-D-glucopyranosyluronic acid)-D-galactose;
D = 2-O-(β-D-glucopyranosyluronic acid)-D-mannose;
E = 6-(β-D-glucopyranosyluronic acid)-D-galactose; F= dimer of acid D;
G = acid E linked to D-galactose; H = trimer of acid D; I = acid E linked to D-galactose; J = tetramer of acid D; K = mainly polymeric acid D.

4. SURVEY OF STRUCTURAL FEATURES OF GUM POLYSACCHARIDES FROM *Encephalartos* SPECIES

4.1 INTRODUCTION

Gum exudates available from various species of *Encephalartos* were surveyed for two reasons: firstly to ascertain whether there were any major chemical differences, particularly when the results were compared in the light of their proposed phylogenetic relationship among the species of origin[30], and secondly to determine which would be most suitable for use in further studies on the *Encephalartos* gums. In addition, the new samples were compared with the original ones. The sample of *E.ghellinckii* had been fractionated by Cetavlon precipitation.

4.2 DISCUSSION

The results obtained (Table 4.2.1) show that there is a general pattern in the distribution of monosaccharides in the gums from the genus. On comparing individual differences, it is apparent that only *E.latifrons* and *E.princeps* gums differ from the norm, the former having a higher proportion of total rhamnose and its 3-*O*-methyl ether and lower proportion of galactose and the latter having a lower proportion of the two rhamnoses and a higher proportion of galactose.

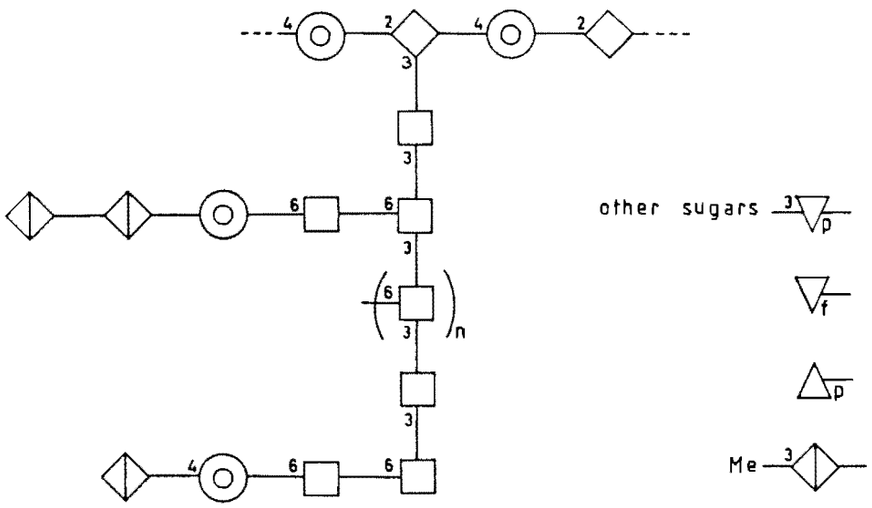


Figure 4.2.1 Proposed Encephalartos gum structure (major pieces only).

If the basic Encephalartos gum structure (Figure 4.2.1) comprises a glucuronomannoglycan core substituted with galactan chains which in turn are substituted with rhamnose chains linked onto glucuronic acid, then a high rhamnose content could mean that there are longer chains of rhamnose and a high galactose content could mean that the gum has longer galactan chains. It would be of benefit to scan samples of gums from as many species as possible to determine if there are similar differences in gums from any other species. (See Figure 4.2.2, showing the relationship between the six species studied here.)

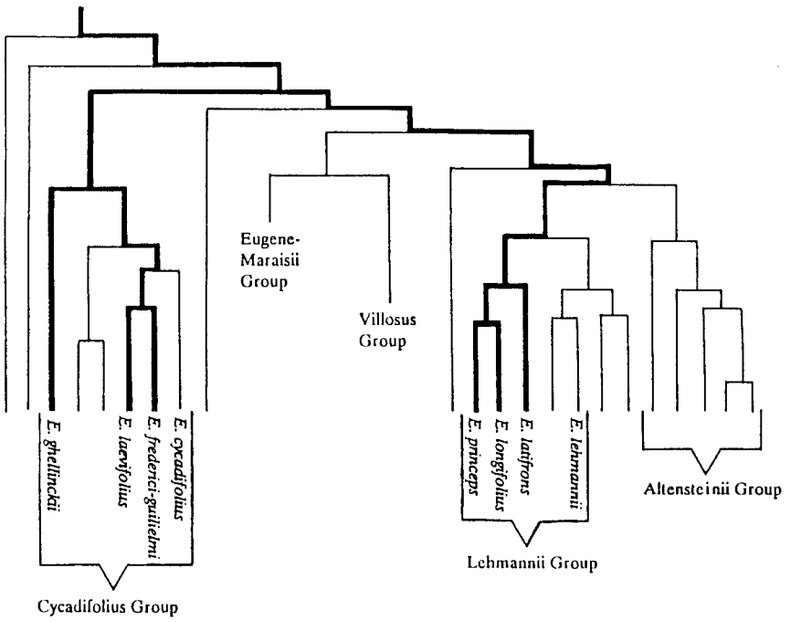


Figure 4.2.2 Showing the relationship of the six species studied here, contracted from a phenogram for the genus Encephalartos (by courtesy of Dr. R. Osborne).

Although the two samples of E.long. gum did not show any real difference in monosaccharide composition, the two samples of E.ghel. differed markedly in the proportions of 3Rha and Rha. However, the total amount of Rha and 3Rha together was constant. The two samples were collected from trees in different locations.

E.long. gum has a higher Man content than most of the others (cf. E.latifrons) and, as the earlier studies had indicated a probable glucuronomannoglycan structure, it was decided to undertake further work on this gum with the objective of approaching the polysaccharide core. Comparative studies would be carried out on E.friderici-guilielmi gum.

Although leiocarpan gum had been shown[73] to comprise two distinct fractions separable by Cetavlon precipitation, earlier work[74] on E.f-g. had given no evidence, using a similar method, of there being any different fractions in that gum. E.long. gum was chromatographed on DEAE-cellulose[74], >90% eluted with alkali and <8%, containing minimal acid, eluted earlier with phosphate buffer.

In this work, Cetavlon precipitation of E.ghel. and subsequent EtOH precipitation of the complex in NaCl solution caused 94% of the polysaccharide to be precipitated immediately (see 4.3.1). This precipitated gum was used for analysis. As it had been shown earlier that E.f-g. gum did not yield definable fractions on Cetavlon precipitation, the EtOH-precipitated gum was used for study. E.long. gum was used as described in Chapter 6.

Methylation analyses were performed on E.f-g., E.ghel. and E.long. gums (Table 4.2.2), the results of which indicated the probability of very similar monosaccharide composition. The main difference lay in the higher proportion of 3-linked Ara in E.long., which is not unreasonable considering the slightly higher proportion of Ara in that gum. The possibility of attachment of Arap to Man[75] is another important reason for further study of E.long. gum.

Table 4.2.1 (a) Monosaccharide Composition and Specific Rotation of Gums from Six Species of Encephalartos

	$[\alpha]_D$	Neutral sugars (mol%)							Uronic acid residues (mol%)
		3Rha	Rha	Fuc	Ara	Xyl	Man	Gal	
E.f-g.	-31°	<1	31	<1	7	2	4	32	24
E.long.[a]	-28°	2	16	<1	10	2	8	32	29
E.ghel.[b]	-30°	10	19	<1	6	1	3	35	26
E.latifrons	-17°	14	21	<1	10	<<1	6	20	29
E.laevifolius	gel	7	19	<1	7	<1	5	38	24
E.princeps	-21°	4	14	<1	4	<1	3	42	32

a original sample (1965);

b original sample (1966) CTAB precipitated.

In general, those sugars that are not in terminal positions in the gums, are mainly periodate invulnerable: only the 6-linked Gal and the 4-linked GlcA would be vulnerable to attack by periodate. The gums are therefore suitable for Smith degradation experiments (see Chapters 2 and 5) as Man, most Gal and in-chain Ara should survive. If these were linked together such that a 3-linked Gal chain was linked through Ara to Man as in leiocarpan[75] gum, a Smith-degradation product of high molecular weight, containing all these sugar residues would be obtained. (Figure 4.2.3)

Table 4.2.1(b) Neutral Sugar Composition of Gums from *E.longifolius* and *E.ghellinckii*: Comparison of different samples.

	Neutral sugars (mol% neutral sugars)						
	3Rha	Rha	Fuc	Ara	Xyl	Man	Gal
E.long.[a]	3	23	<1	14	3	11	45
E.long.[b]	3	25	<1	14	2	10	46
E.ghel.[c]	14	25	<1	8	2	4	47
E.ghel.[b]	1	38	<1	9	1	6	45

a original sample (1985); b fresh sample (1987); c original sample (1986) CTAB precipitated.

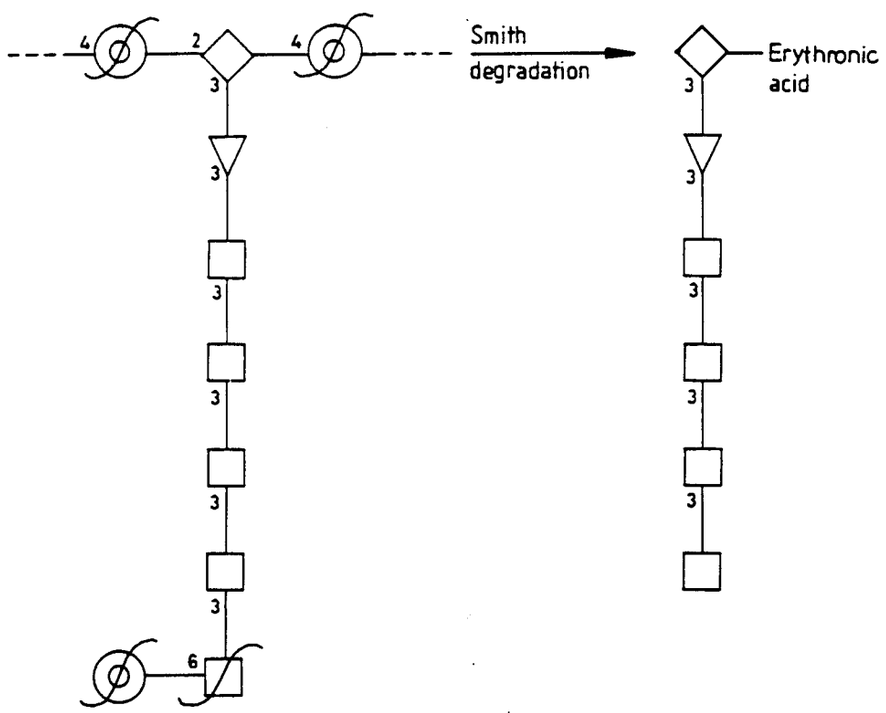


Figure 4.2.3 A proposed structure of *Encephalartos* gums showing →3)-Ara linking galactan chain to Man and Smith degradation thereof.

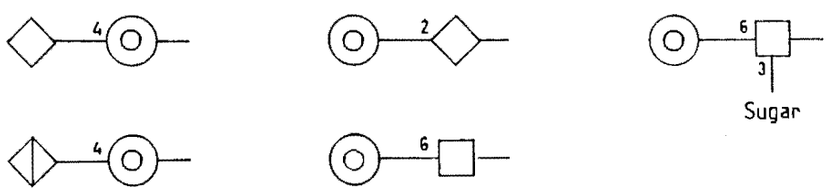
Table 4.2.2 Methylation Analysis Data for E.f-g., E.ghel., and E.long. Gums

	E.f-g.	E.ghel.	E.long.
T-GlcA	5	1	-
T-Rha + T-Ara_f[a]	20	24	16+4
T-Ara_p + T-Xyl_p	3	1	5
T-Gal	-	-	3
→4)-GlcA-	19	22	15[c]
→Rha-[b]	5	5	5
→3)-Ara-	3	2	7
→3)-Gal-	4	3	2
→6)-Gal-	8	6	9
→3,4)-GlcA-	5	3	7[c]
→3,6)-Gal-	22	23	19
→2,3)-Man-	<1	<1	<1
→3,4,6)-Gal-	2	4	2
→2,3,4)-Man-	3	2	8

a 2,3,4-Me_3Rha and 2,3,5-Me_3Ara did not always resolve;
b there was evidence from m.s. of all three di methyl ethers of Rha, although 2,3-Me_2Rha was predominant;
c from methanolysis/saponification results.

As a further check on their similarities, these three gums were subjected to base-catalysed β-elimination using potassium

dimsyl as catalyst (Table 4.2.3). These results showed that GlcA had been exterior to Gal, linked to the 6-position. Some, probably half of the 6-linked Gal was also 3-linked, i.e. it had a sugar residue glycosidically linked at O-3. In addition, the results showed that although minimal Man survived, it had GlcA exterior to it in the original polysaccharide. Because so little survived, it could be assumed that Man also had GlcA interior to it. Much of the Rha was destroyed and it was therefore assumed that Rha, too, was exterior to GlcA. This was borne out in all the dimsyl degradation experiments. Rha linked to the 4-position of GlcA is a common feature of plant gums[1]. Thus, from these results it is apparent that the following sub-structures exist in the Encephalartos gums:



As Man apparently has GlcA interior to it, it would be beneficial to attempt to perform base degradation experiments with concomitant protection of the newly released sugar exterior to the degraded GlcA residue[5]. It was decided to attempt this with low molecular weight substrates (see Chapters 5 and 6).

Table 4.2.3 Molar ratios of sugar residues before and after[a] dimsyl catalysed β-elimination on methylated gums from E.f-g., E.ghel. and E.long.

	ME.f-g.		ME.ghel.		ME.long.	
T-Ara$_f$	20	6	24	4	4	4
T-Rha		tr		3	16	9
→Rha-	5	-	5	1	5	4
T-Ara$_p$ + T-Xyl$_p$	3	<1	1	-	5	5
→3)-Ara-	3	1	2	2	7	3
T-Gal-[b]	-	7,5	<1	1	3	12
→6)-Gal-[b]	8	3,5	6	5	9	7
→3)-Gal-[b]	4	10	3	5	2	8
→3,6)-Gal-	22	9	23	20	19	6
→3,4,6)-Gal-	2	-	4	-	2	-
→2,3)-Man-	<1	-	<1	-	<1	-
→3)-Man-[c]	-	tr	-	-	-	-
→2,3,4)-Man-	3	-	2	-	8	-
→3,4)-Man[c]	-	tr	-	-	-	-

a Arrows indicate sugar residues before and after dimsyl degradation
b OCD_3 on C-6 after base degradation;
c OCD_3 on C-2

Partial hydrolysis experiments are necessary in attempts to degrade the polysaccharide to the point where only GlcA and Man residues remain in the polymeric material. As methylation analysis and base degradation studies had shown the gums to be remarkably similar, it was felt that it was necessary to compare at least two of the gums in greater detail, and therefore such experiments were performed on both "autohydrolysed" E.f-g. (E.f-g."A") and "autohydrolysed" E.long. (E.long."A").

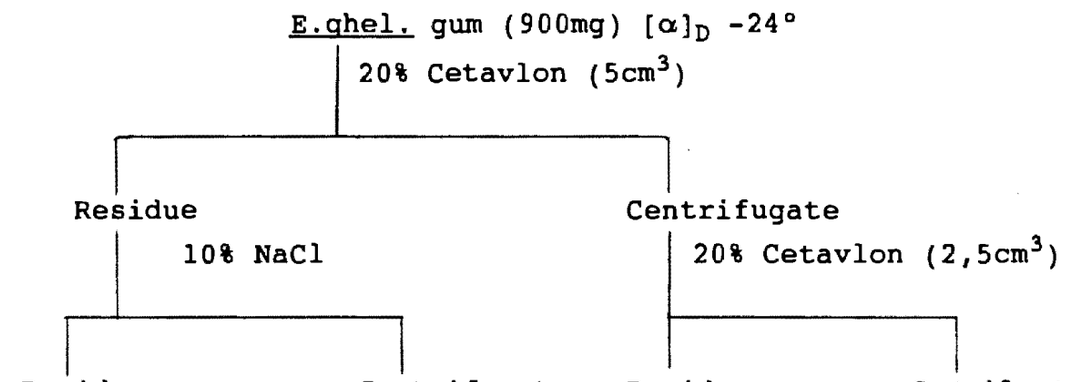
In a further attempt to isolate the core structure, methylated E.long. should be partially hydrolysed and the hydrolysate fractionated on a cellulose column, to investigate the high molecular weight acid fraction (see Chapter 6). Additional information gleaned from this study should be comparable with the methylation analysis results.

4.3 EXPERIMENTAL

Gum samples were prepared as described in Chapter 2. E.ghel. was precipitated with Cetavlon to determine if there were two distinct fractions, as with leiocarpan[73].

Impure E.ghel. exudate(900mg) had $[\alpha]_D$ -24° (brown murky solution). Cetavlon (1g as 20% solution) was added to precipitate the gum. After centrifugation, the

Cetavlon-polysaccharide precipitate was redissolved by addition of 10% NaCl. After brown debris had been removed by centrifugation, the polysaccharide was precipitated by pouring the solution into EtOH (4 volumes). The process was repeated on the centrifugate from the first precipitation, as shown in the scheme below.



	E.ghel.	E.ghel.I	E.ghel.II
Mass	560mg	27mg	8mg
% total CHO	94	4,5	1,5
$[\alpha]_D$	-30°	-24°	-19°
mol% Uacid	27		
Hydrolysis and p.c. (solvent a)			
GlcA	++	tr	faint tr
Gal	++++	+++	+++
Man	++	+	+
Ara	++	+	tr
Rha	+++	++	+
3Rha	++	+	tr

As such a low percentage of the total carbohydrate present was not precipitated by Cetavlon, it was felt that the whole EtOH-precipitated or EtOH-extracted gums would be suitable starting material for the experiments. A fresh clean sample of E.ghel. gum had 26 mol% uronic acid residues, in close agreement with the Cetavlon and EtOH-precipitated sample. In an attempt to determine the molecular weight of E.ghel. gum, a fresh clean sample was chromatographed on Sepharose 4B. It eluted at the void volume, showing the molecular weight to be $>2 \times 10^6$.

5. INVESTIGATION OF THE MOLECULAR STRUCTURE OF THE EXUDATE GUM FROM Encephalartos friderici-guilielmi

5.1 INTRODUCTION

E.friderici-guilielmi[76,77] is an attractive cycad growing in the Eastern Cape in the districts of Cathcart, Queenstown, and extending eastwards, to Kokstad. The stems of E.f-g. are up to 60 cm in diameter and up to 4 m tall; they may be branched from the base. Leaves are 1 to 1,5 m long including a petiole of 30 cm. A characteristic of E.f-g. is its densely woolly crown and, of the genus, E.f-g. bears the largest number of cones, males up to 12 and females up to 6.

This plant has been confused with E.cycadifolius in the past although the latter has almost no wool in the crown and is low-growing with a clustered stem. E.f-g., E.cycad. and E.ghellinckii are closely related but their areas of distribution do not overlap. (Figure 5.1.1)

5.2 ORIGIN, ISOLATION AND PREPARATION OF SAMPLES

The gum exudate from E.f-g. was collected in December 1966 near Cathcart as hard gum nodules. Further samples of the gum exudate were collected, after rain, during the winter of 1987 in Kirstenbosch Botanical Gardens.

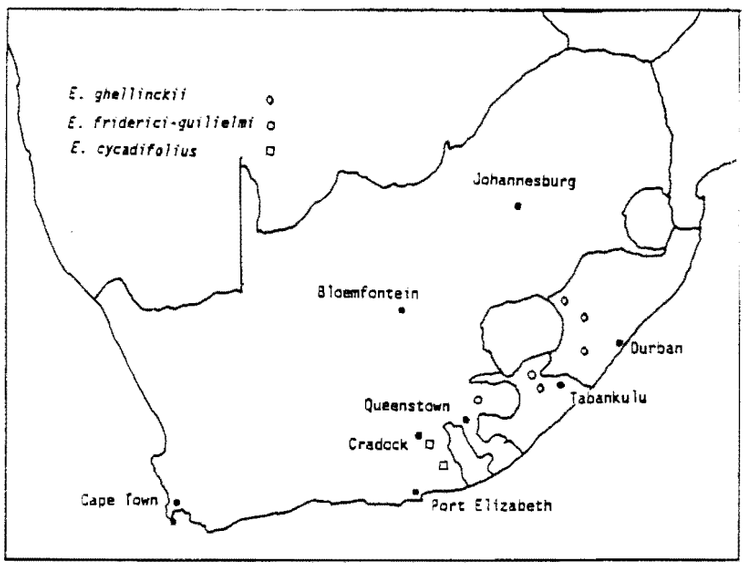


Figure 5.1.1 Map showing distribution of the three species, E.cycadifolius, E.friderici-guilielmi, and E.ghellinckii.

The gum was dissolved in water with the aid of sonication when necessary, freeze-dried and exhaustively extracted with ethanol. The ethanol-insoluble fraction E.f-g. was used in this study.

5.2.1 "Autohydrolysis"

In order to simplify studies on the core structure and, particularly, to facilitate Smith degradation studies, E.f-g. was hydrolysed at pH 2 ("autohydrolysis") to remove acid-labile Araf and Rhap residues. The hydrolysis was monitored by noting change in optical rotation of the solution. As the reaction

progressed, the rate of change of optical rotation decreased, as expected, but after 8h it increased again (Figure 5.2.1). The hydrolysis was therefore terminated after 10h. Some Gal and some GlcAβ-6Gal were observed in the ethanol-soluble extract of the resulting freeze-dried product.

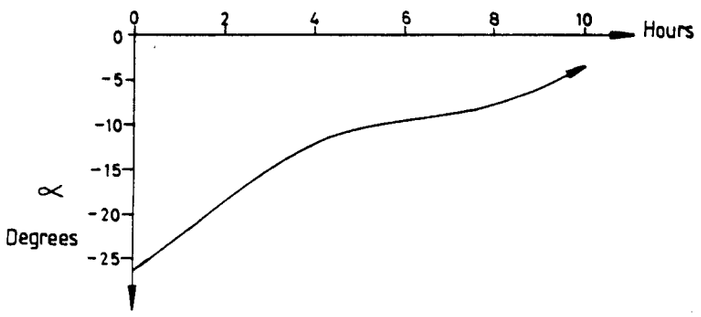


Figure 5.2.1 Graph of optical rotation vs. time for "autohydrolysis" of E.friderici-guilielmi gum.

This peculiar behaviour under "autohydrolysis" conditions could only be interpreted as being due to the presence of a fairly dense invulnerable section interior to the acid-labile (Araf and Rha) outer section, followed further towards the core by a fairly labile region (probably Arap, drawing analogy from the structure of Anogeissus leiocarpus gum[73]; see section 5.3) which is protected by the outer region of GlcAβ-6Gal. The sugar residues lost on "autohydrolysis" of E.f-g. are shown in Figure 5.2.2.

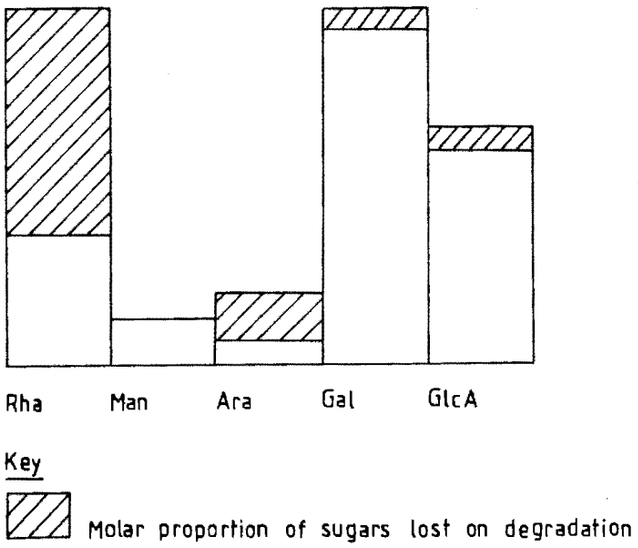


Figure 5.2.2 Composition of E.friderici-guilielmi gum showing loss of sugar residues on "autohydrolysis".

The ethanol-insoluble portion, E.f-g."A", was subjected to s.e.c. (Sepharose 4B) giving $\bar{M}_w$ ca. 60000 (Figure 5.2.3). This portion was studied further.

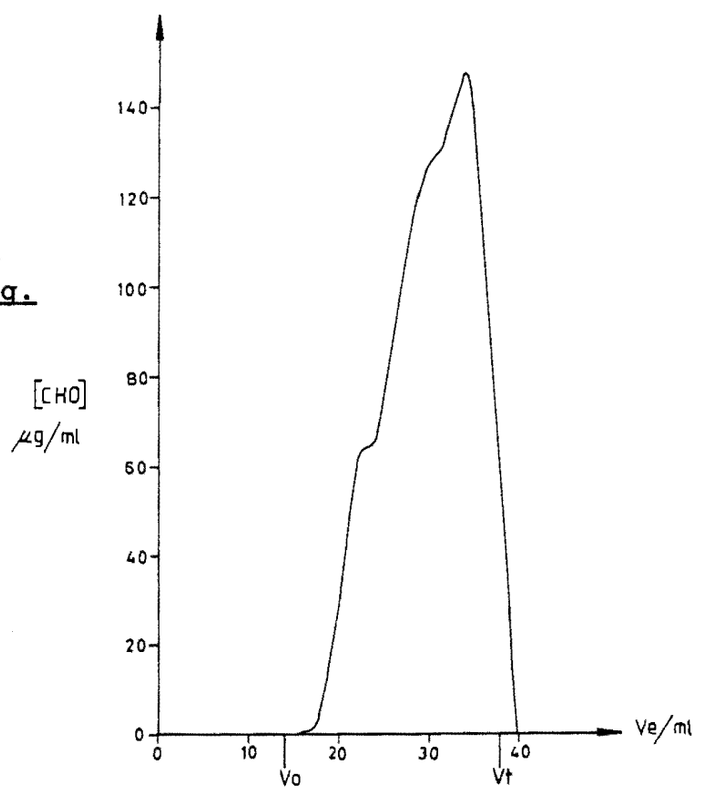


Figure 5.2.3 Molecular weight profile of E.f-g. "A" on Sepharose 4B.

5.2.2 Carboxyl reduction

It is known[2,78] that periodate-oxidised acids in a polysaccharide can pose problems during Smith degradation, owing to the formation of cyclic products that are resistant to acetal cleavage by hydrolysis. E.f-g. has a high content of uronic acid, mainly 4-linked (see Table 4.2.2). In Smith degradation studies, if a 4-linked D-GlcA residue is cleft by periodate, the resulting L-erythronic acid residue can lactonise with the newly formed glycolaldehyde residue, giving the 1,4-dioxane (δ-lactone) derivative shown (Figure 5.2.4).

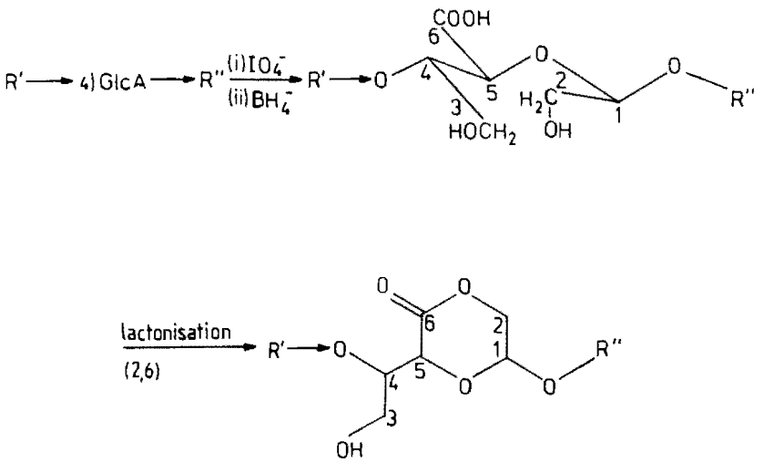


Figure 5.2.4 Lactonisation of the degraded GlcA residue.

This lactone is expected to be fairly stable under conditions used in Smith degradation (M TFA, room temperature, time, e.g. 5 days) and acetal cleavage to liberate glycolaldehyde does not readily take place. This lack of acetal cleavage was extreme in the Smith degradation studies on E.f-g., as

glycosidically-linked monosaccharides were released while glycolaldehyde was still bound. In order to overcome this, E.f-g. was carboxyl-reduced by the method of Taylor and Conrad[79,80]. After one such reduction, the proportion of uronic acid decreased to 8% (from 24%). On further reduction this fell to 2% but there was a marked decrease in the solubility of the polysaccharide. The carboxyl-reduced gum, based on a -4Glc-2Man- polymer, is presumably conformationally different to the substituted glucuronomannoglycan, the consequence of which is lowered solubility in water. The resulting carboxyl-reduced E.f-g., RE.f-g. was fractionated, giving water-soluble fraction RE.f-g.(S) (19%) and water-insoluble fraction RE.f-g.(IS) (81%). The sugar compositions of the two fractions (Table 5.2.1) were similar, although the water-insoluble fraction had a slightly higher Gal content. In view of the uncertainty of quantitative g.l.c. analysis, this difference was considered to be of little structural significance and both fractions were used, the water-soluble fraction in the Smith degradation experiment and methylation analysis, and the water-insoluble fraction in acetolysis.

Table 5.2.1 Characteristics of products from *E.f-g.*

	Gum	*E.f-g*	*E.f-g*"A"[a]	R*E.f-g*(S)[b]	R*E.f-g*(IS)[b]
$[\alpha]_D$	-28°	-32°	-15°	-8°	
M_W	n.d.	n.d.	60000	70000	n.d.
g/mol COOH	n.d.	736	580	n.d.	n.d.
mol% Uacid	n.d.	24%	29%	2%	2%
mol% neutral sugars					
Gal		33	44	32	40
Ara		7	4	9	7
Man		4	7	8	4
Rha		31	16	25	25
3Rha		<1	<1	-	-
Xyl		1	<1	-	-
Glc		-	-	25	24

a There was 72% recovery of "A" from *E.f-g.*; b there was 80% recovery of R*E.f-g.* from *E.f-g.*.

5.3 METHYLATION STUDIES

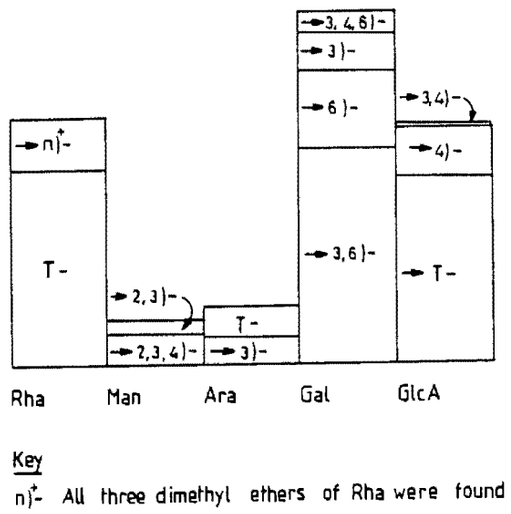
The sugar residue linkage composition of the *E.f-g.* polysaccharide, its autohydrolysis product and the carboxyl reduced product was investigated by methylation analysis.

Table 5.3.1 Methylation analysis results for E.f-g, E.f-g"A" and RE.f-g

	ME.f-g	RME.f-g	ME.f-g"A"	RME.f-g"A"[a]	MRE.f-g
$[\alpha]_D$	-38°	-46°	-41°	-20°	-38°
T-Rha	20	20	13	13	28
→Rha[b]	5	5	1,5	2	1
T-GlcA	-	5	-	16	4[c]
→4)-GlcA	-	19	-	12	20[c]
→3,4)-GlcA	-	n.d.[d]	-	1,5	1[c]
→2)-Man	-	-	1,5	1	1
→2,3)-Man	1	1	tr	tr	1
→2,3,4)-Man	3	3	6	6	6
→2,4)-Man	-	-	1	1	-
T-Gal	-	-	1,5	1,5	2
→3,6)-Gal	22	22	21	21	16
→3,4,6)-Gal	2	2	0,5	0,5	-
→3)-Gal	4	4	5	5	5
→6)-Gal	8	8	15	15	9
Arap	3	3	1,5	2	2
→3)-Ara	3	3	1,5	1,5	3

a There was a 72% yield of "A" from E.f-g.; b all three Me_2Rha ethers appeared to be present, not properly resolved, but predominantly 2,3-Me_2Rha; c Glc in RE.f-g.; d not detected.

From the methylation results for E.f-g. and E.f-g."A" (Table 5.3.1), it is apparent that the major acid labile peripheral sugar residue is Rha. Sugar linkages in E.f-g. and E.f-g."A" are better illustrated in the histograms (Figure 5.3.1(a) and (b)), which also show the loss of Ara, GlcA and Gal.



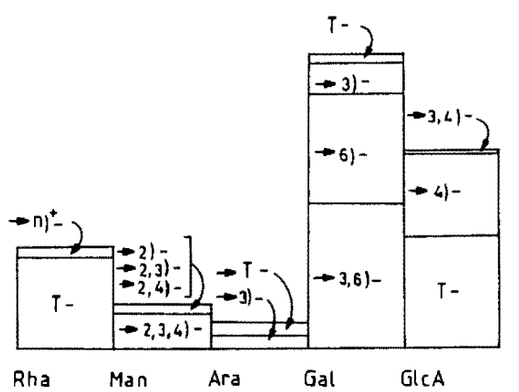


Figure 5.3.1 Histograms showing sugar linkages in (a) E.f-g., and (b) E.f-g."A".

GlcAβ-6Gal was identified in the ethanol-soluble fraction after "autohydrolysis" of E.f-g.. Both GlcA and Gal were cleft from the gum in similar molar ratios during hydrolysis at pH 2 (see Section 5.2.1).

That some degradation occurs during the carboxyl reduction of E.f-g. to RE.f-g. is indicated by the fact that, in RE.f-g. almost all Rha is terminal. In addition, if the 80% yield of the carboxyl reduced product is taken into account, it is concluded that approximately 9% GlcA and 10% Gal has been lost.

These methylation results show that the polysaccharide exuded from E.f-g. is suitable for

(i) base-catalysed β-elimination experiments on the methylated polysaccharide, as there is a high proportion of 4-linked GlcA in the gum;

(ii) Smith degradation studies of the polysaccharide, as there is a high proportion of 3-linked Gal. If the core structure is a polymer of alternating GlcA and Man, the Man would be largely periodate- invulnerable but the GlcA would be oxidized. Also, the only in-chain Ara is →3)-Arap; therefore, if, as in Anogeissus leiocarpus[75], there is Gal linked through →3)-Arap to the 3-position of Man, it would be possible to find a structural unit incorporating this (Figure 5.3.2).

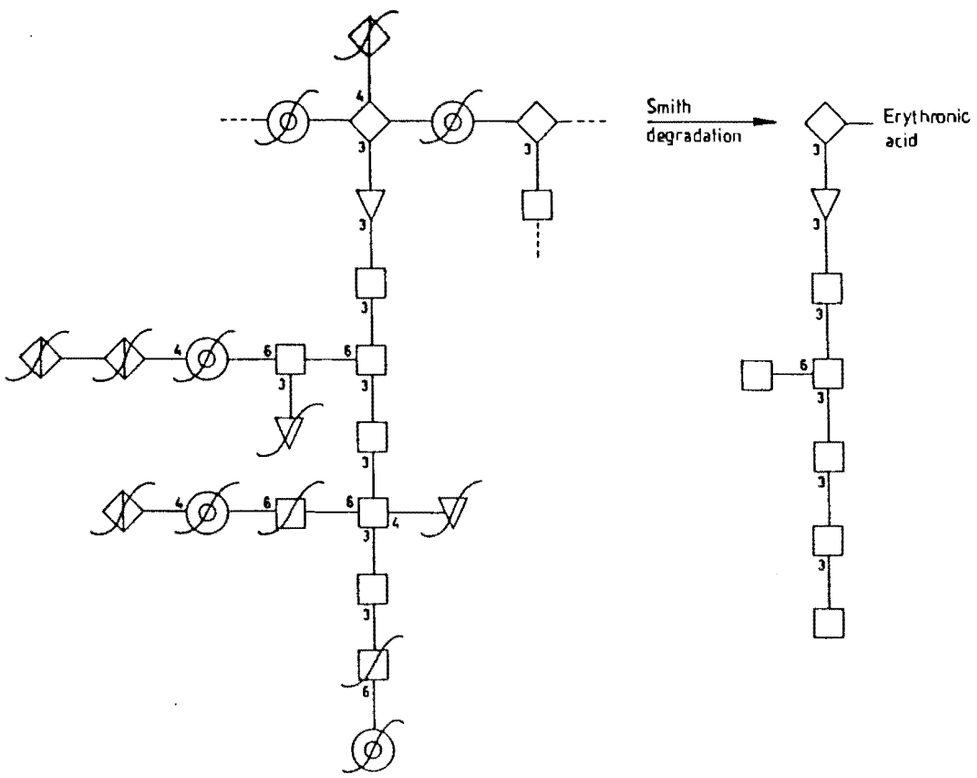


Figure 5.3.2 Showing the effect of Smith degradation of a proposed structure to yield the polymeric Gal-Ara-Man product.

However the reduced, oxidized GlcA residues would be expected to lactonise between C-6 and C-2, a difficulty overcome by repeating the experiment on the carboxyl-reduced polysaccharide.

(iii) As 1-6 linkages are most vulnerable under acetolysis conditions[81], acetolysis of the carboxyl-reduced gum could possibly give rise to oligosaccharides not obtained by partial acid hydrolysis.

5.4 BASE-CATALYSED β-ELIMINATION EXPERIMENTS

Base-catalysed β-elimination experiments using potassium dimsyl in DMSO[50] were performed on ME.f-g. and ME.f-g."A". It would be expected that sugars linked to the 4-position of GlcA and so exposed would be degraded under the strongly basic conditions of the experiment. For the sugar residue interior to the acid that had been degraded the position of linkage was labelled using CD_3I in the remethylation step. Thus in a structure such as

"-Sugar^1-4GlcA-Sugar^2-",

Sugar^1 would be degraded and Sugar^2 would be deuteriomethylated in the position at which the 4-linked uronic acid was attached. P.c. monitoring of the hydrolysate prior to derivatisation to p.m.a.a.'s showed a little undegraded acid for both substrates.

From the results in Table 5.4.1, it can be seen that 4-linked GlcA is linked to Man and Gal as shown below.

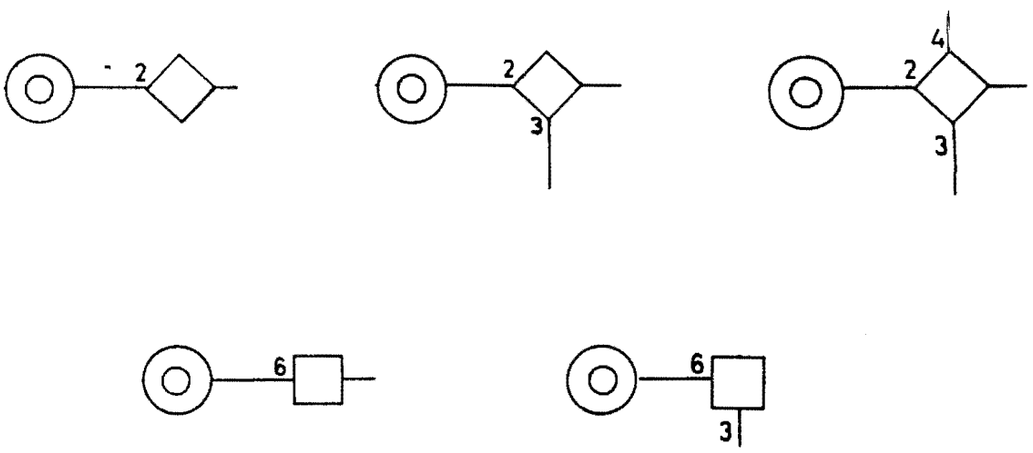
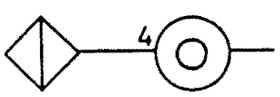


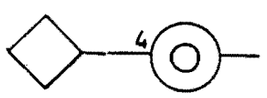
Table 5.4.1 Molar ratios of sugar residues before and after β-elimination of methylated E.friderici-guilielmi and E.f-g."A"

	ME.f-g.	BDME.f-g.	ME.f-g."A"	BDME.f-g."A"
Araf	20	6	13	tr
Rha		tr		1
→Rha	5	-	1,5	tr
→2)-Man	-	-	1	-
Man	-	tr[a]	-	tr[a]
→2,3)-Man	1	-	tr	-
→3)-Man	-	tr[a]	-	tr[a]
→2,3,4)-Man	3	-	6	-
→3,4)-Man	-	tr[a]	-	tr[a]
→2,4)-Man	-	-	1	-
Arap	3	<1	1,5	tr
→3)-Arap	3	1	1,5	1
Galp	-	7,5[b]	1,5	10[b]
→6)-Gal	8	3,5	15	2
→3)-Gal	4	10[b]	5	12[b]
→3,6)-Gal	22	9	21	6
→3,4)-Gal	-	tr[b]	-	-
→3,4,6)-Gal	2	-	0,5	-

a OCD_3 on C-2; b OCD_3 on C-6. Arrows in the table indicate sugar residues before and after degradation.

Also, from the decrease in Rha, both in-chain and terminal, it is apparent that Rha is exterior to acid. The only Man observed in the products of base degradation was in trace amounts and was deuteriomethylated at the 2 position, although most had been degraded. Thus Man residues are exterior to degraded acid as well as some, not necessarily the same Man, being interior to degraded acid. Rha and Man linkages to acid are shown below.





Gal survives base degradation conditions; it is thus not exterior to GlcA.

5.5 SMITH DEGRADATION STUDIES ON E.f-q.

Smith degradation experiments were conducted to find the size and structure of regions in which periodate-invulnerable sugar

residues remain linked together[82]. All of the E.f-g. samples showed a high proportion of periodate-invulnerable Gal; in addition Man is largely protected and the only substituted Ara is 3-linked. Thus in these experiments, Gal, Man and Ara residues should survive degradation.

5.5.1 Smith Degradation of E.f-g.

From the methylation analysis results, periodate uptake was calculated to be 5,9 mmol.g^{-1}. Monitoring by the arsenite method [48,49] showed that periodate uptake remained constant at 5,8 mmol.g^{-1} after 42h. Excess of periodate, and iodate formed, were precipitated by $Ba(OAc)_2$. After reduction ($NaBH_4$) of the solution, the product was Smith-degraded (M TFA, room temperature)[46,47]. Monitoring by s.e.c. (Sepharose 4B) showed the molecular weight of the degradation products to decrease initially from $\bar{M}_w > 2x10^6$ to 35000. After 4d, it decreased further to give a broad band of molecular weights having a major component at approximately 7000 and minor components of molecular weights 3000, 4000 and 15000 (s.e.c. on Bio-Gel P 10). After five days of degradation, half of the solution was freeze-dried to give the first limit product $SD1_1$E.f-g.. This was fractionated with MeOH/Me_2CO to give soluble(S) and insoluble(IS) fractions. The first limit can be explained by lactonisation between C-2 and C-6 of the degraded GlcA residue

to form the δ-lactone (see Figure 5.2.4). This lactone is resistant to acetal cleavage.

$SD1_1$E.f-g.(S) consisted of glycerol, glycolaldehyde and a component immobile in solvent c but having R_{gal} 1,1 in solvent a. This component was isolated by preparative p.c. but methylation analysis (Table 5.5.2) was not consistent with its being a single substance. However, its occurence does confirm that much highly substituted Man has small sustituents, either short chains of periodate-invulnerable sugar residues or single invulnerable sugars like 3-O-methyl rhamnose. 6-O-Methyl mannose is found on methylation analysis of this "spot"; therefore some Man in the gum is substituted on positions 2, 3, and 4 by periodate-invulnerable sugar residues, but the major proportion of Man has a degradable sugar residue on position 4. The observed 4-linked Gal could have come from previously 3,4,6-linked Gal.

Sugar analysis of the hydrolysate of $SD1_1$E.f-g.(S) showed the major, bound components to be Man and erythritol. This indicates that most Man has low molecular weight substituents (e.g. single or short chains of sugar residues) or periodate-vulnerable residues attached to it. As erythritol was shown to be formed by reduction of erythronolactone during the derivatisation to alditol acetates, it is likely that the erythritol present here originated from periodate-oxidised GlcA. The presence of Man and erythritol together in the low molecular weight fraction would therefore indicate that they

may be associated. If linked together, this would be evidence for Man-4GlcA.

P.c. indicated that most of the glycerol was free (Table 5.5.1). From the methylation results on ME.f-g. this would be from non-reducing Araf end-groups, and 6-linked Gal.

$SD1_1$E.f-g.(IS) showed no free glycolaldehyde on p.c., but glycolaldehyde assay was consistent with the presence of 1 mol of bound glycolaldehyde per 7 sugar residues. Uronic acid assay indicated only 1 uronic acid residue per 40 sugar residues, but equivalent weight was 1 COOH per 9 sugar residues. Thus, most of the acidic residues present must be erythronic acid, and there is approximately 1 glycolaldehyde per erythronic acid in this product. Sugar analysis of the hydrolysate showed the major sugar to be Gal (Table 5.5.1). Erythritol was shown to have been produced by the reduction of erythronic acid with borohydride during derivatization to alditol acetates. This was achieved by acetylating the mixture without prior reduction. The presence of tri-O-acetyl-glycerol and the absence of tetra-O-acetyl-erythritol showed that the latter was formed by reduction of the acid during reduction of the hydrolysate to alditols.

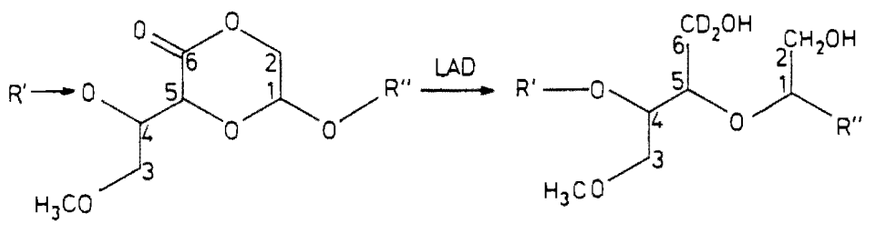
Table 5.5.1 Composition of $SD1_1$E.f-g. products (1st limit)

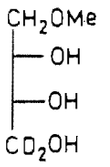
	$SD1_1$E.f-g.(IS)		$SD1_1$Ef-g.(S)
Yield	20%		
$\overline{M}_W$	7000(major), 10000,114000		
Glycolaldehyde	Bound 5% (w/w)		Free
Uronic acid	3% (w/w)		
g per mol COOH	1400		
mol % polyols in reduced hydrolysates			
Gly	0,6	tr[b]	48
Ery	4	0,5[b]	32
Ara	4	0,5[b]	1
Man	9	0,75[b]	14
Gal	79	7[b]	2
Rha	-	-	1
Glc[a]	3	0,25[b]	-

a Qrigin uncertain, possibly GlcA;
b residues per molecule.

From the methylation data (Table 5.5.2), it can be seen that the high molecular weight fraction $SD1_1$E.f-g(IS), which had coincidentally been fractionated during purification on Merckogel 2000 into Fr 1 (higher mol. wt.) and Fr 2 (lower mol. wt.), consists of a 3-linked galactan with one in five Gal

units being branched, and for each ten Gal units there is one 3-linked Ara. From p.c., Fr 2 appears to be low molecular weight 3-linked galactan. The presence of 1-O-methyl erythritol (2D on C-4) is significant in that it confirms the presence of the lactone, which would readily undergo reduction with LAD (Figure 5.5.1).



From D-GlcA,  is obtained

R' R'' are sugar residues

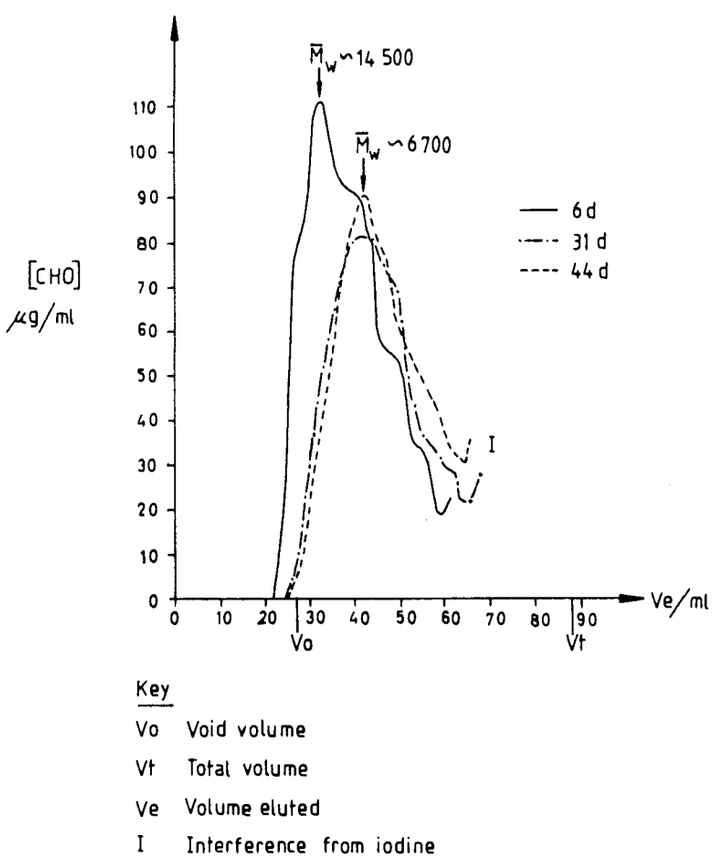
Figure 5.5.1 Shows the formation of 1-O-methyl erythritol from the methylated Smith-degradation product.

Table 5.5.2 Methylation analysis results of $SD1_1$E.f-q. products

	$SD1_1$E.f-q(IS)			$SD1_1$E.f-q(S)
	M Fr 1	RM Fr 1	M Fr 2	M 'spot'a ex pc
$[\alpha]_D$	-13°	-12°	+26°	-
T-Ara	-	-	-	4
→3)-Ara	15	8	-	5
T-Gal	7	11	+++++	20
→3)-Gal	53	52	++++	15
→4)-Gal	-	-	-	13
→6)-Gal	4	3	+	-
→3,6)-Gal	11	14	-	-
→3,4,6)-Gal	4	2	-	-
T-Man	-	-	-	21
→2,3)-Man	2	2	-	5
→2,3,4)-Man	-	-	-	4
T-Rha	-	-	-	15
→4)-Rha	2	2	-	-
T-GlcA	-	4	-	-
1-Me Ery	-	2[b]	-	tr
1,2-Me_2 Ery	-	-	-	tr

a The 'spot' referred to was that obtained from preparative p.c.;
b 2 D on C-4.

The remainder of the solution from which $SD1_1$E.f-g. had been removed was submitted to further, prolonged degradation, which was monitored as before by s.e.c.. Monitoring at 31d and 66d gave a major peak corresponding to $\bar{M}_w$ 6000 with minor peaks at 3500, 2500 and 2000 (Figure 5.5.2). At no time was there a clear indication of where to terminate the degradation.



Key

Vo Void volume

Vt Total volume

Ve Volume eluted

I Interference from iodine

Figure 5.5.2 S.e.c. monitoring of Smith degradation of E.f-g.: Molecular weight profiles of the solution after 6, 31, and 44 days, obtained on Bio-Gel P-10.

The course of degradation was also monitored by assay for free sugars and bound glycolaldehyde[71]. Even after 66d there were no free sugars. After 10 weeks, the solution containing the degrading product was heated to 45°. This caused cleavage of glycosidic linkages but glycolaldehyde was still bound (3%, i.e. 8 mol%) in the molecules. The degradation was terminated after 2 weeks at 45°.

Table 5.5.3 Composition of $SD1_L$E.f-g. products (2nd limit)a

	$SD1_L$E.f-g.(IS)	$SD1_L$E.f-g.(S)
Yield	11%	
Glycolaldehyde	Bound 3% (w/w)	Free
mol% polyols in reduced hydrolysates		
Gly	0,6	28[b]
Ery	5	23
Ara	2	3
Man	8	11
Gal	80	32
Rha	1	

a The molecular weights are not listed here as there was evidence (free Gal) of significant glycosidic cleavage;
b from p.c. before hydrolysis, this is free.

From Table 5.5.3 and the methylation analysis data (Table 5.5.4), the high molecular weight fraction is a 3-linked

galactan, one in seven Gal units being 3,6-linked. The ratio of Gal to Man approximated 10 to 1 in the high molecular weight fraction, and minimal Ara survives. The ratio of Man:Ara is approximately the same in both fractions.

Table 5.5.4 Methylation analysis results for $SD1_L$ E.f-g.(IS)

	$MSD1_L$ E.f-g.(IS)	$RMSD1_L$ E.f-g.(IS)
$[\alpha]_D$	-18°	
→3)-Ara	1	1
T-Gal	20	16
→3)-Gal	44	37
→6)-Gal	6	9
→3,6)-Gal	12	13
T-Man	2	2
→2)-Man	5	5
→2,3)-Man	2	4
1-Me Ery	tr	-
1,2-Me_2 Ery	tr	-

The size of the unattacked block could not be determined because of glycosidic cleavage. The molecular weight was determined as ca. 950. This would require ca. 17 mol% polyols in the higher molecular weight fraction whereas the proportion was found to be 5,6%. Also free Gal had been found in the

solution. Because of this excessive breakdown beyond acetal cleavage[78] in the Smith-degradation product, conclusions as to structure only, not size of any sub-unit, are given.

5.5.2 Smith Degradation of E.f-g"A"

The periodate consumption in this experiment was found to be a constant 9,6 mmol.g^{-1} after 2d. The oxidation was terminated by the addition of ethan-1,2-diol. After dialysis and reduction, the polyol was Smith-degraded with acid. Monitoring by s.e.c. on Bio-Gel P-10 indicated a molecular weight profile tending to $\bar{M}_w$ 2400 but with a wide range and minor peaks at 4000 and 5000 (Figure 5.5.3).

After 18d half of the solution was freeze-dried to yield the first limit product $SD1_1$E.f-g."A" which was fractionated with MeOH/Me_2CO to give solubles(S) and insolubles(IS). As before, degradation was allowed to proceed on the remainder, except that the entire degradation was at room temperature and the experiment was terminated after 12,5 weeks. Composition of SD products and methylation analysis data are summarised in Tables 5.5.5 and 5.5.6.

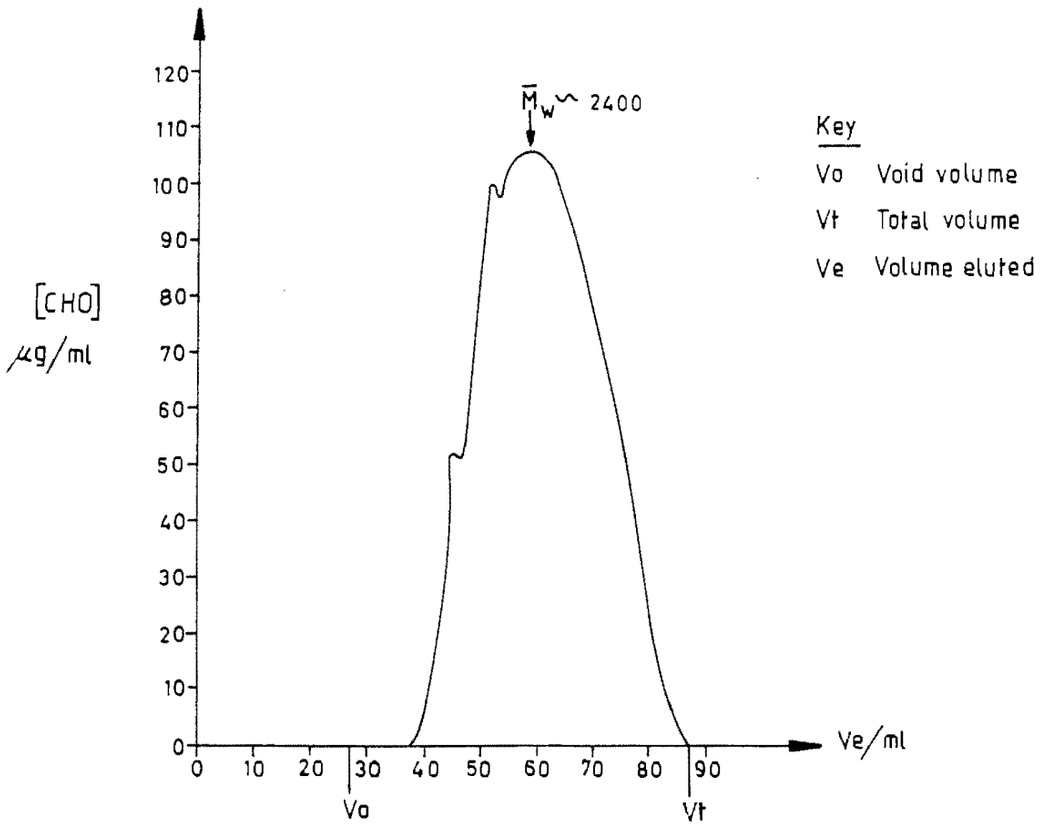


Figure 5.5.3 Smith degradation of E.f-g."A": Molecular weight profile of the solution after 14 days.

The molecular weight of the second limit product at 1250 gives a block size of seven sugar residues together with the necessary polyol. However, erythritol appears inexplicably high in $SD1_L$"A"(IS). On comparing the results for the first and second limits, the ratio of Gal to Man approximates to 6:1.

Table 5.5.5 Composition of SD products from E.f-g."A"

	1st Limit		2nd Limit		
	$SD1_1$"A"(S)	$SD1_1$"A"(IS)	$SD1_L$"A"(S)	$SD1_L$"A"(IS)	
Yield		39%		34%	
$\overline{M}_w$(major)		2400		1250	
Glycolald.	Free	Bound(12%)	Free	Bound	
Uronic acid		<1%			
g/mol COOH		2400			
mol% polyols in reduced hydrolysate					/mol
Glya	42	7	59	13	1[c]
Ery[b]	26	7	24	17	2[c]
Ara	3	5	1	5	0,5[c]
Man	15	8	9	7	0,7[c]
Gal	11	69	5	57	6[c]
Rha	4				

a From p.c. prior to hydrolysis, Gly was free;
b Ery was from erythronic acid;
c residues per molecule.

Table 5.5.6 Methylation analysis results of SD products from E.f-g"A"

	1st Limit $MSD1_1$ E.f-g."A"	2nd Limit $MSD1_L$ E.f-g."A"
$[\alpha]_D$	-19°	-7°
→3)-Ara	3	4
T-Gal	14	13
→3)-Gal	58	52
→6)-Gal	3	5
→3,6)-Gal	10	8
T-Man	1	4
→2,3)-Man	1	2

The results in Tables 5.5.5 and 5.5.6 are represented in the histograms (Figure 5.5.4). From this histogram and taking into account the molecular weight of the block, it is apparent that this periodate-invulnerable block comprises an average of six Gal residues, less than one Man and Ara. This agrees with a structure in which the galactan chain is linked onto Man in the glucuromannoglycan core, sometimes directly and sometimes through Ara.

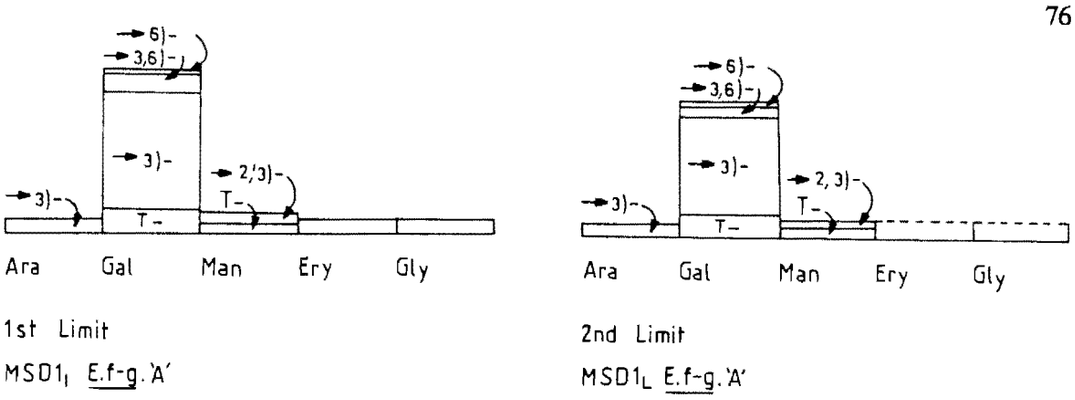


Figure 5.5.4 Histograms for the First and Second Limit Smith-degradation products of E.f-g."A".

5.5.3 Smith Degradation of RE.f-g.

Periodate uptake of 6,0 mmol.g^{-1} was in good agreement with that found for E.f-g.. As there was some degradation of E.f-g. during the carboxyl reduction (80% yield) to RE.f-g. more periodate-vulnerable sites were probably exposed. Excess periodate was destroyed with ethan-1,2-diol. After dialysis and reduction, solubility of the polyol decreased and the degradation was attempted with vigorous stirring. Monitoring (s.e.c. on Bio-Gel P-10) on days 3 and 6 showed no appreciable change in molecular weight distribution, viz. a broad molecular weight range with peaks at 5000, 2400, 1600 and 900. After freeze-drying, the product, SDRE.f-g., was fractionated by s.e.c. (Trisacryl GF05) to give three fractions. Hydrolysis and p.c. showed the presence of glycolaldehyde in the fraction

of lowest molecular weight but only faint traces in the other two fractions.

Table 5.5.7 Relative proportions of sugars and polyols in SDRE.f-g.

	Fr 1	Fr 2	Fr 3
Mol. wt. range	>3000	3000-800	<800
Polyols (100mmols)[a]			
Glyc	tr	3	36
Ery	2	5	58
Thr	-	-	2
Sugars (44mmols)[a]			
3Rha	1,5	0,5	0,5
Rha	1	0,5	1
Ara	2	1,5	1,5
Gal	17	3,5	1,5
Man	4,5	2,5	3,5
Glc	0,5	1	1

a Relative masses and hence molar ratios of polyols and sugars were based on the carbohydrate assay and total masses of the three fractions.

From Table 5.5.7, it can be seen that Gal is the predominant sugar in the high molecular weight fraction, Ara is equally distributed, Man:Ara is approximately 2:1 in all fractions and

remained elusive in all the earlier Smith degradation studies, is now apparent but distributed in all the fractions. These results support those obtained on E.f-g. and on E.f-g."A".

By considering the results of all three Smith degradation experiments, the following may be concluded:

(i) For E.f-g."A" the high molecular weight regions in which unattacked sugars remain linked together consist of a 3-linked galactan chain having average molecular weight of 1250. This molecular weight is consistent with a structure of 7 sugar residues and 1 polyol. Associated with this unattacked "block" are some Man and Ara (less than 10 and 5 mol% respectively) for both E.f-g. and E.f-g."A".

(ii) Although 3-O-methyl-L-rhamnose was found only in the Smith degradation products from RE.f-g., it had to survive the experiment and must therefore be considered. It was seen, together with Ara and Man, in all the fractions obtained on separation of the products from RE.f-g., with the three sugars in the same approximate proportions. This could imply that they are, in some way, associated with one another. However no such oligosaccharide was isolated, nor was an oligosaccharide comprising only Ara and Man found.

(iii) Although Man is generally highly substituted, the size of the substituent chains varies. This is deduced from the fact that Man appears in all fractions, soluble and insoluble, after Smith-degradation.

Thus the 3-linked galactan chain is associated with Man and Ara but these sugars may be cleft from it during degradation. This is borne out by the fact that the first-limit insoluble fraction ($SD1_1$E.f-g.) had a methylated sub-fraction that was pure galactan (from methylation analysis; Table 5.5.2). The analysis of the methylated "spot" obtained from the low molecular weight fraction of the first-limit product from E.f-g. (Table 5.5.2) also indicated that much of the highly substituted Man (observed as 6-O-methyl mannose on methylation analysis) is substituted by short periodate-invulnerable chains or single periodate-invulnerable sugar residues. Therefore it is essentially the size of the galactan chain that determines the size of the entire unattacked block. Furthermore this galactan chain is not likely to be attached to the 4-position of Man as there was no 6-O-methyl mannose found on methylation analysis of the higher molecular weight fractions. As it is known that Man is substituted on position 2 by GlcA, the galactan chain must be on position 3, either directly or indirectly, probably through →3)Arap. These conclusions are illustrated in Figure 5.5.5.

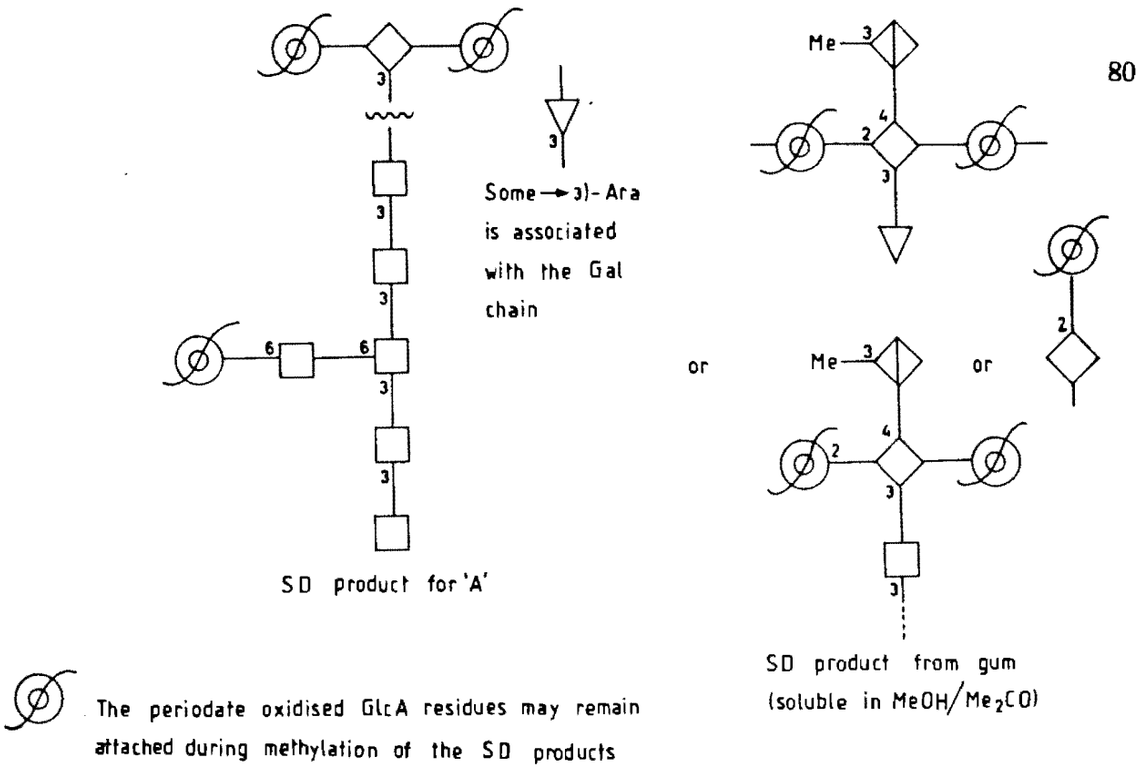


Figure 5.5.5 Illustrates the conclusions about the E.f-g. structure drawn from the Smith degradation experiments.

5.6 ACETOLYSIS OF RE.f-g.

Acetolysis was performed on the carboxyl-reduced gum using the method of Narui et al.[83]. Monosaccharides and oligosaccharides were separated on a short charcoal-Celite column to give three main fractions:

Fraction 1, monosaccharides, consisted of Gal(++++), Glc(+++), Rha(+++++), Ara(+), and trace amounts of Man.

Fraction 2, disaccharides (only 5% of that subjected to acetolysis); these were separated by preparative paper chromatography giving Galβ-3Gal and Galβ-3Ara, which were

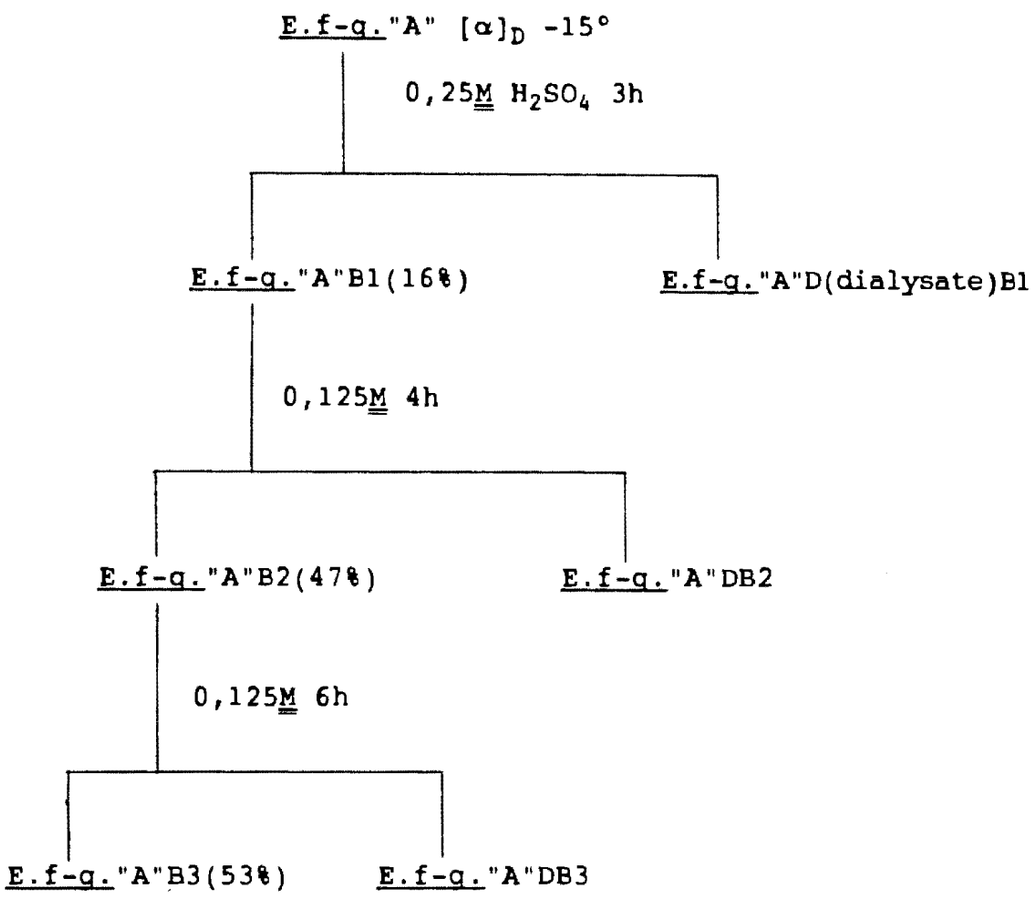
5.7 PARTIAL HYDROLYSIS OF E.f-g."A"

The purpose of the partial acid hydrolysis experiments was to isolate the proposed glucuronomannoglycan core. Additional information gleaned from any studies on the peripheral sections removed during the hydrolyses would aid in gross characterisation of the exudate, but because of the complexity of the gum, this was not the prime objective of the work.

E.f-g."A" was prepared as before (69% yield, $[\alpha]_D$ -15°) but without monitoring the hydrolysis. Characteristics of the product were in excellent agreement with those of the earlier preparation, except that both Gal and GlcA were 6 mol% higher. B1, B2, and B3 were prepared successively, starting with B1 from "A".

After trial experiments at each stage, the partial hydrolyses were achieved by hydrolysis at 100° in H_2SO_4 (B1 0,25M 3h; B2 0,125M 4h; B3 0,125M 6h) as illustrated below.

After neutralization of each of the hydrolysates, the supernatant was dialysed to give the non-dialysable fractions B1, B2 and B3, and the dialysates DB1, DB2 and DB3. The dialysates were monitored by p.c. for traces of Man in order to achieve a non-dialysable product from which as little Man as possible had been released.



The characteristics of the partial hydrolysis products are presented in Table 5.7.1. From these results, it is apparent that Man and GlcA are the major component monosaccharides resisting hydrolysis in the higher molecular weight fraction, which from its $\overline{M}_W$ as determined by s.e.c. on Bio-Gel P-10 (Figure 5.7.1) is approaching a 12-residue limit.

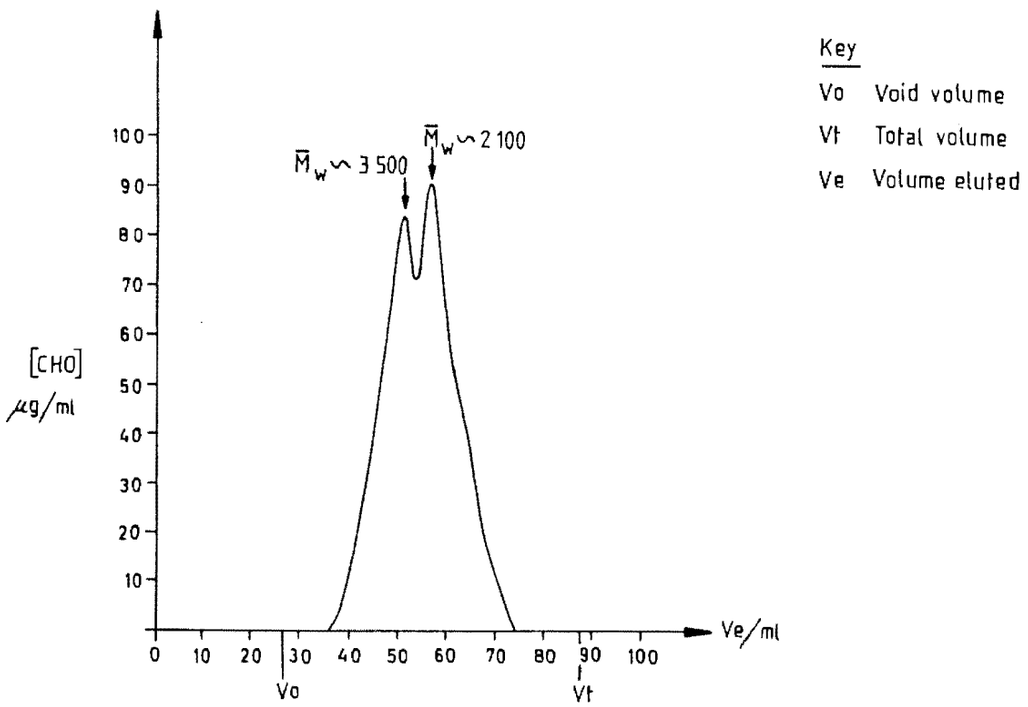


Figure 5.7.1 Molecular weight profile of E.f-g."A"B3 on Bio-Gel P-10.

The dialysate, DB1 was the only one to contain the disaccharide Galβ-3Ara; thereafter Galβ-3Gal was the only neutral disaccharide observed. This is in agreement with the hypothesis that there is a more acid-labile region of the polysaccharide interior to the preponderant GlcAβ-6Gal residues. However, although some Gal may be attached to the polysaccharide core ultimately through Ara, because of the fact that DB2 and DB3 showed no Galβ-3Ara, but still contained Gal, much of the Gal must be attached directly to the polysaccharide core.

Table 5.7.1 Characteristics of partial hydrolysis products of E.f-g."A"

	B1	B2	B3
$[\alpha]_D$	-	-	-9°
% yield	19	47	53
mol% Uacid	36	42	47
$\overline{M}_W$			2100,3500
mol% neutral sugars			
Gal	42	24	7
Man	22	31	45
Dialysate			
Neutral disaccharides			
Galβ-3Gal	+	+	+
Galβ-3Ara	+	-	-
Major acidic component			
GlcAβ-6Gal	+	+	+

Chemical shifts of resonances for anomeric protons in B3 (Table 5.7.2) are in agreement with those reported for the glucuronomannoglycan in the brown seaweed Lessonia nigrescens[20] and in Chorisia speciosa[10] (Table 5.7.2). The ratio of 5:1 for in-chain Man to reducing end Man supports a 12-residue glucuronomannoglycan molecule.

Table 5.7.2 N.m.r. results and assignments for E.f-g."A"B3

Chemical shift (δ ppm)	Assignment	Integration
5,40	-2 Man α-	5
5,27	-2 Man α-OH	1
4,86	-2 Man β-OH	small
4,50	GlcA-	]a
4,49	-4 GlcA β-	

a not distinguishable

Methylation data (Table 5.7.3) indicate that approximately 2 in 5 of the 2-linked Man residues are also 4-linked. Roughly half of the GlcA is terminal which is consistent with the presence of two mannose branch points. Sugar analysis indicated 7% Gal, in accordance with the occurrence of 1 Gal residue per 12-residue molecule. No attempt was made to label reducing terminal Man.

Table 5.7.3 Methylation Analysis Results[a] for E.f-q."A" B2 and B3

	MB2	RMB2	MB3	RMB3
$[\alpha]_D$	-23°		-18°	
→2)-Man	12	15	18	21
→2,4)-Man	16	13	20	17
T-GlcA	-	29	-	28
→4)-GlcA	-	24	-	29
→3)-Gal	3	3	-	-
→6)-Gal	7	8	2	2
→3,6)-Gal	9	7	1	1

a Mol%, allowing for uronic acid where necessary.

E.f-q."A"B3 was further hydrolysed with M TFA for 1,75h at 100° and chromatographed (p.c. in solvents a and d) against authentic specimens of GlcAβ-2Man, and the linear dimer, trimer, and tetramer of (-4GlcAβ-2Manα-). The hydrolysate had components with the mobility of all four oligomers of GlcAβ-2Man as well as that of GlcAβ-6Gal. In addition, there are three other components (Table 5.7.4).

Table 5.7.4 Results of Partial Hydrolysis of E.f-g."A"B3

R_{gal}	$R_{monomer}$	Amount	Identity
0,01	0,021	+	Tetramer[a]
0,05	0,080	+	Trimer
0,09	0,150	+	(ochre[b])
0,16	0,284	+	Dimer
0,22	0,376	tr	(yellow)
0,28	0,489	+	(ochre)
0,37	0,645	++	GlcAβ-6Gal
0,58	1,000	+++	Monomer
		++	Gal
		tr	Man

a Tetramer, Trimer, etc. refer to that of -4GlcAβ-2Man;
b Colours refer to the colours given by spray reagent a.

As expected, the graph of $\log[(1/R_{monomer}) - 1]$ vs n^{72}, the degree of polymerisation, gave a straight line for the oligomers of GlcAβ-2Man. The following structure can therefore be proposed for E.f-g"A"B3 (Figure 5.7.2).

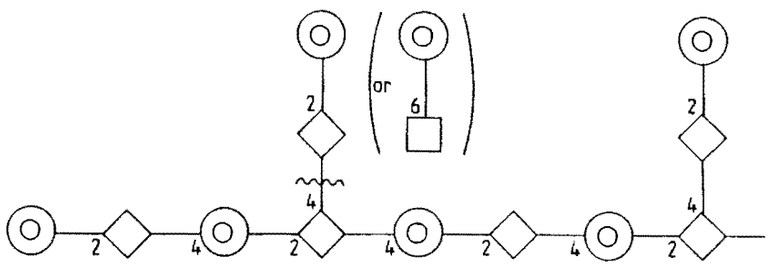


Figure 5.7.2 A proposed structure for E.f-g."A"B3.

5.7.1 Base-Catalysed β-Elimination Experiments on Partial Hydrolysis Products

Base-catalysed β-elimination experiments were conducted on B2 and B3 with potassium dimsyl and DBU as catalysts. The results with potassium dimsyl confirmed that GlcA is exterior to Gal and to Man. In the products, BDMB2 and BDMB3, after dimsyl-catalysed β-elimination, T-Man had CD_3 on C-2 and T-Gal and 3-linked Gal both had CD_3 on C-6. Thus GlcA is 6-linked to Gal and 2-linked to Man. Methylation analysis results of substrates, MB2 and MB3, and their degradation products, BDMB2 and BDMB3, are compared in Table 5.7.5.

From these results it is apparent that Man is degraded during the course of the experiment. In relation to Gal, the quantity of Man decreases, i.e. all Man that was 2-linked should appear as T-Man if there were no GlcA interior to it, but this is not the case. Furthermore no 2,3,6-tri-O-methyl mannose was observed; thus all →2,4)-Man had been degraded. There is no

evidence of Gal being exterior to GlcA as there is no apparent degradation of Gal.

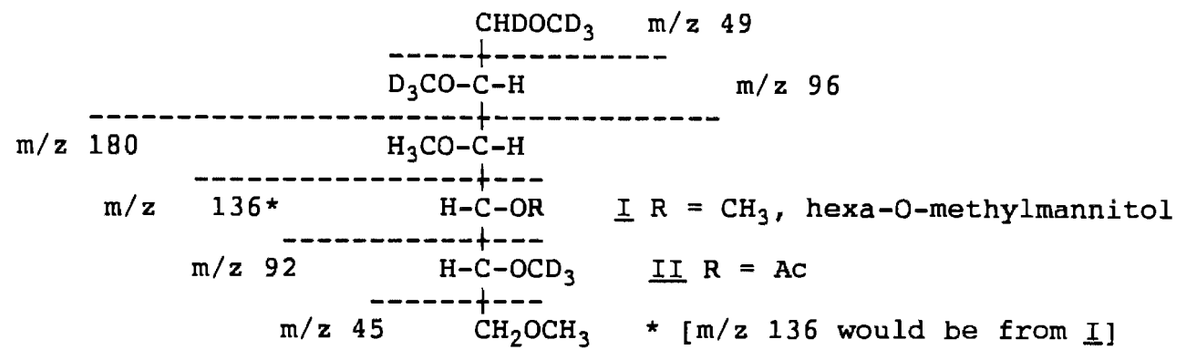
Table 5.7.5 Comparison of Methylation Analysis Data of Partial Hydrolysis Products and their Dimsyl Base Degradation Products[a]

	MB2	BDMB2	MB3	BDMB3
→2)-Man	12	-	18	-
T-Man	-	6	-	10
→2,4)-Man	16	-	20	-
→6)-Gal	7	2	2	<1
T-Gal	-	7	-	tr
→3,6)-Gal	9	1	1	tr
→3)-Gal	3	9	-	2

a Results are expressed as molar ratios keeping Gal total constant. Arrows indicate sugar residues before and after dimsyl degradation.

The fact that no →2,4)-Man appears to survive would indicate that this may be the predominant sugar residue in the proposed GlcA-Man core. For this reason, base-catalysed degradations were performed with bulky non-nucleophilic bases, DBU and Hünig's base, with concomitant protection by acetylation of the newly released sugar that had been exterior to the degraded acid[5,15]. This sugar will appear as the corresponding alditol after de-O-acetylation and reduction, the use of $NaBD_4$

introducing labelling at C-1. Thus if GlcA is linked to the 2-position of Man, under experimental conditions used (exposed hydroxyls are deuteriomethylated), the following p.m.a.a.'s would be expected, I from →2)-Man and II from →2,4)-Man.



The ions m/z 49 and 96 would be indicative of the presence of the mannitol. If there is a sugar exterior (at O-2) to the newly released Man or if for any experimental reason an acid residue remained on O-2, the Man would be acetylated on O-2 during alditol acetate formation and would appear as III from →2)-Man and as IV from →2,4)-Man.

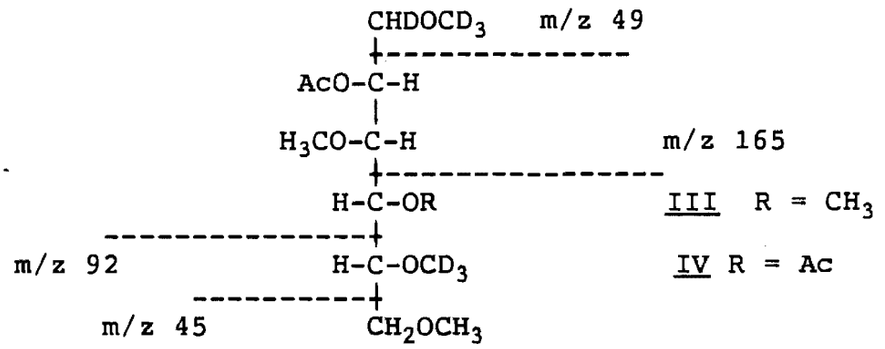


Table 5.7.6 Comparison of Methylation Analysis Data of Partial Hydrolysis Products and their DBU Base Degradation Products[a]

	MB2	DBUBDMB2	MB3	DBUBDMB3
→2)-Man	12	-	18	-
T-Man	-	4	-	3
→2,4)-Man	16	-	20	-
→6)-Gal	7	-	2	0,5
T-Gal	-	4	-	1
→3,6)-Gal	9	-	1	-
→3)-Gal	3	8	-	-
t_R 0,39[b]	-	8	-	15
T-Glc[c]	-	-	-	1
→4)-Glc	-	-	-	tr

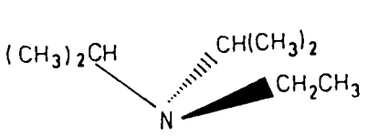
a Results are expressed as molar ratios;
b column c;
c CD_2OCD_3 at C-6.

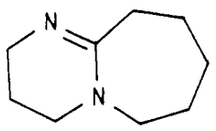
The results of the base-degradation experiments are shown in Table 5.7.6. The presence of T-Glc and a trace of →4)-Glc in the DBU product from MB3 confirmed the presence of undegraded acid. Any acid remaining after the base degradation would be reduced at C-6 by $NaBD_4$ in NaOMe/MeOH provided the acid remains as the ester. The early peak at t_R 0,39 had m/z 45(15%), 49(100%), 96(1%), 115(15%). These all agreed with expected

mass peaks for I as m/z 180 may lose CD_3OH and HCHO to form m/z 115. However it was not I as fully methylated mannitol eluted from column c at t_R 0,51.

There was no evidence for the presence of III or IV in the p.m.a.a.'s of either of DBUMB2 or DBUMB3. That some 2-linked Man survives as T-Man is indicative that there is a sugar residue (or undegraded acid) interior to some of the Man residues.

In these experiments two bases were used, namely Hünig's base (N,N-diisopropylethylamine) and DBU (1,8-diazabicyclo-[5.4.0]undec-7-ene).





Structural formulae for Hünig's Base and for DBU.

Several experiments were conducted on MB2 using both bases and monitoring the experiment by hydrolysis and p.c. after removal of the excess base. Hünig's base caused no appreciable degradation of the acid; and it was apparently too weak a base. It was hoped that the pyramidal shape of the molecule would enable it to act as a more efficient proton abstractor than DBU, but this was not the case. All degradations were

performed with DBU, this being the better proton abstractor for the substrate. Heating with base at 100° for 24h did not cause complete degradation and unless the degradation is complete the results cannot be conclusive. The reason for continuing with these experiments was in the hope of finding I or II as additional evidence for the GlcA-Man-GlcA sequence.

5.8 EXPERIMENTAL

5.8.1 "Autohydrolysis"

The purified gum (8,0g) was dissolved in 5m$\underline{\underline{M}}$ H_2SO_4 and the pH was adjusted to 2 by the addition of 0,5$\underline{\underline{M}}$ H_2SO_4. The course of the hydrolysis was monitored by measurement of the optical rotation of aliquots removed from the solution, and the reaction was terminated by neutralisation with $BaCO_3$. After centrifugation, the supernatant liquid was freeze-dried (7,77g). This freeze-dried product, dissolved in a minimum of water, was precipitated by pouring the aqueous solution into EtOH (4 vols.), and the precipitate was washed with EtOH; this yielded E.f-g."A" (5.71g) and EtOH-soluble fraction (1,62g).

5.8.2 Carboxyl Reduction[79,80]

Purified E.f-g. (790mg), $[\alpha]_D$ -32°, was dissolved in water (10cm^3) and the pH adjusted to 4,75 with 0,1M HCl. The solution was stirred continually while solid 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (1,6g = 8,3mmol) was added gradually during 1,5h, and pH was maintained at 4,75 (0,1M HCl). Thereafter 3M $NaBH_4$ (25cm^3) was added dropwise over a further 1,5h period while pH was maintained at 7,0 by dropwise addition of 4M HCl. The addition of octan-1-ol was necessary to control foaming. Excess borohydride was destroyed by making the solution slightly acid (pH 6,2) and the solution was dialysed against running tap water for 4d. The non-dialysable fraction was freeze-dried. A test for borate proved negative. The solid was dissolved in water giving a murky solution of low viscosity which was centrifuged. The centrifugate was retained and freeze-dried yielding a product (630mg) having a uronic acid content of 8%.

The major portion of the product (620mg), which was difficult to redissolve, was stirred overnight in water (50cm^3) with warming (35°). The reduction was repeated as before but using 1g (5,3mmol) of the carbodiimide. The non-dialysable material appeared to be largely in solution but after freeze-drying to yield the reduced product, RE.f-g., redissolution proved difficult and the material was fractionated by centrifugation into soluble [RE.f-g.(S), 113mg], $[\alpha]_D$ -8°, and insoluble

[RE.f-g(IS), 478mg] fractions. Hydrolysis (18h, 2M TFA, 100°) and p.c. showed no uronic acid but uronic acid assay indicated 2% for both fractions. Sugar ratios for the two fractions RE.f-g(S) and RE.f-g(IS) are given in Table 5.2.1.

5.8.3 Methylation Analyses

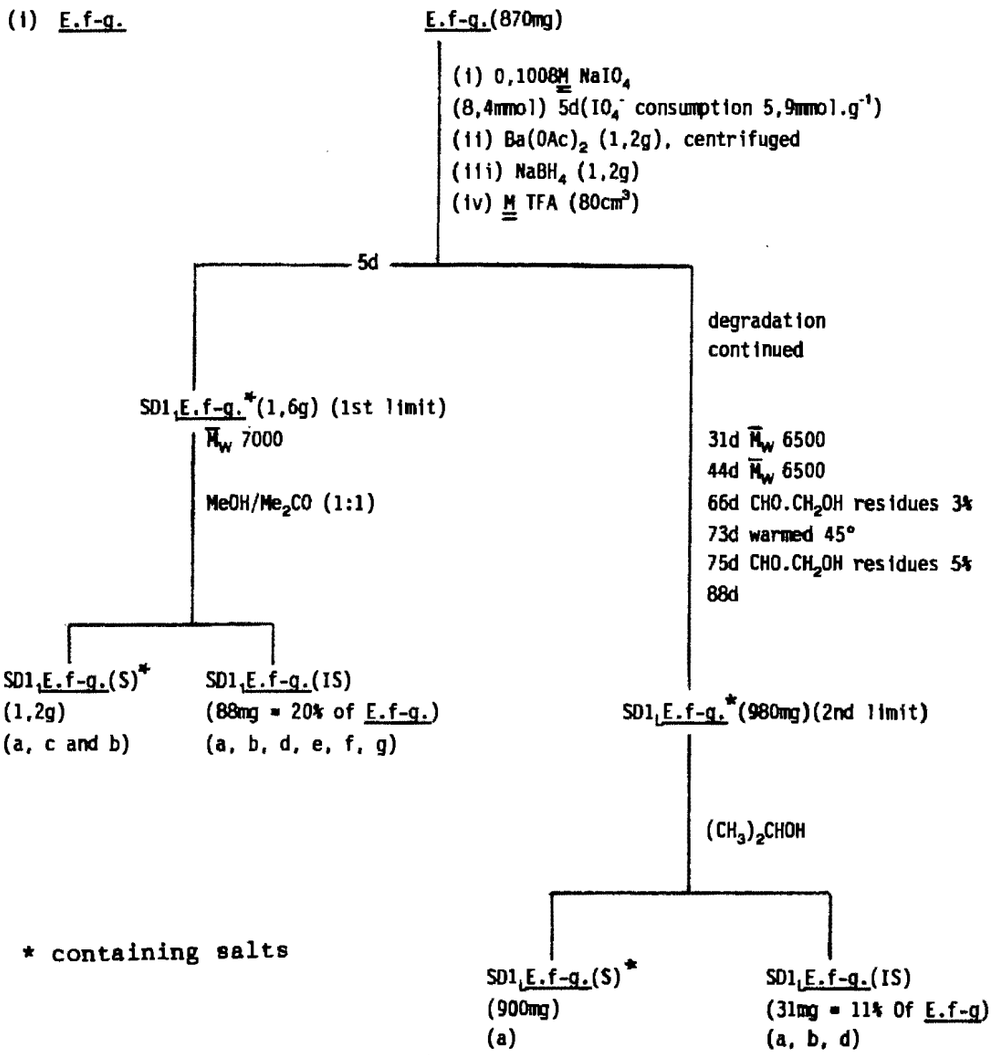
As described in Chapter 2, E.f-g. was methylated by the Haworth method, after protection of the reducing end groups by the reduction with $NaBH_4$. All degradation products were methylated by the Hakomori method as modified by Phillips and Fraser[65]. All methylated products were further methylated to completion by 3 to 4 Purdie methylations[66]. Mode of linkage of the uronic acid residues was determined by reducing the methyl carboxylate group to CD_2OH using LAD[68]. All methylated polysaccharides were hydrolysed and analysed by p.c.. The hydrolysates were derivatised to p.m.a.a.'s for analysis by g.c. and g.c.-m.s.. Molar sugar ratios were determined by correcting peak area response using the molar response factors of Sweet et al.[56].

5.8.4 Base Degradation Experiments

All base degradation experiments were performed as described in Chapter 2.

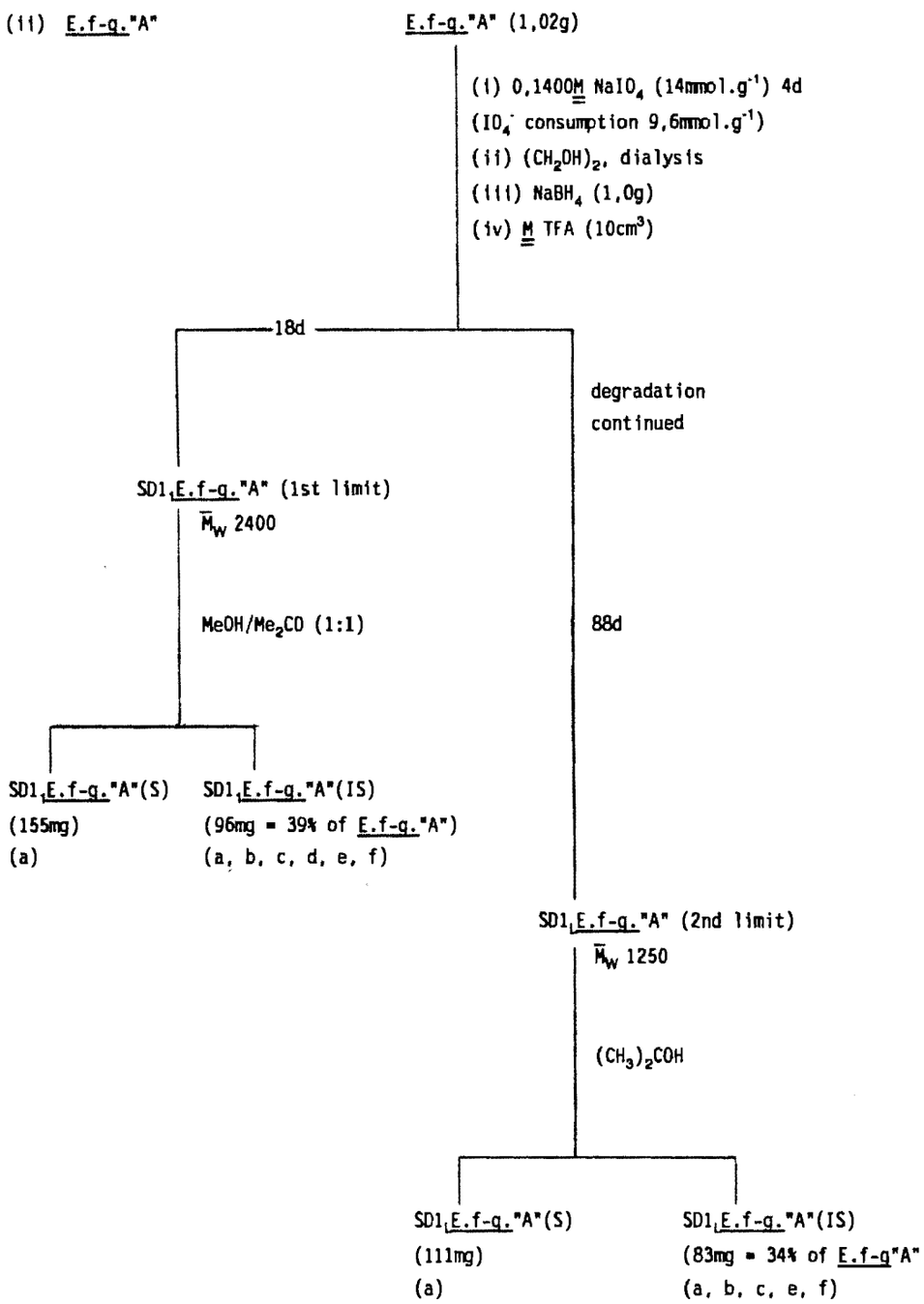
5.8.5 Smith Degradation Studies

Details that apply to a particular experiment are described, schematically where possible, in this section. General conditions are described in Chapter 2.



Analyses performed

(a) Hydrolysis, p.c., a.a.'s, acetates
(b) Methylation analysis
(c) Preparative p.c.
(d) Molecular weight determination
(e) Glycolaldehyde assay
(f) Equivalent weight (g/mol COOH)
(g) Uronic acid assay



Analyses performed

(a) Hydrolysis, p.c., a.a.'s, acetates
(b) Methylation analysis
(c) Molecular weight determination
(d) Glycolaldehyde assay
(e) Equivalent weight (g/molCOOH)
(f) Uronic acid assay

(iii) RE.f-g.

RE.f-g. (50mg)

(i) 0,1M $NaIO_4$ (5,0cm³ 0,5mmol) 5d
(IO_4^{-1} consumption 6,0mmol.g^{-1})
(ii) $(CH_2OH)_2$,dialysis
(iii) $NaBH_4$ (55mg)
(iv) M TFA (4,0cm³)

Monitoring by s.e.c. on Bio-Gel P-10
(1cm³ aliquots)
(i) 3d $\bar{M}_w$ 900, 1600, 2400, 5000, 10000
(ii) 6d $\bar{M}_w$ 900, 1600, 2400, 4800, 10000

7d 2cm³ was freeze-dried

SDRE.f-g. (12,5mg)

Trisacryl GF05
Eluent pyridinium acetate buffer pH 5,0
Fraction size 12,0cm³

	Fr 6-8	Fr 9-11	Fr 12-15
Mol. wt.	>3000	3000-800	<800
Mass	4,9mg	4,3mg	8,7mg
CHO (from PhOH-H_2SO_4 assay)			
	4,6mg	1,4mg	1,5mg
non-CHO	0,3mg	2,9mg	7,2mg

P.c. (solvent c, spray b) of the fractions prior to hydrolysis showed only fr 12-15 to contain substances having the mobility of Ery and Gly.

5.8.6 Acetolysis of RE.f-g

RE.f-g(IS) (192mg) was dissolved in 6cm^3 acetolysis mixture consisting of HOAc:Ac_2O:H_2SO_4:TMU (8:9,5:1,5:1,0(v/v))[83] and stirred in a stoppered flask for 15 minutes at 100°.The mixture was decomposed with ice water and the acetolysed mixture was extracted into $CHCl_3$. The extract was dissolved in dry MeOH (3cm^3) and de-O-acetylated with NaOMe (0,2M, 2mmol) for 45min at room temperature. MeOH was evaporated off, water was added and the mixture was decationised (Amberlite IR-120 (H^+)). The acetolysate was concentrated to a syrup (470mg). P.c. (solvent b) showed Gal(+++); Glc(+++); Man(tr); Ara(++); Rha(++); as well as R_{gal} 0,25(+); 0,43(+); 0,82-1,00(+++). The bulk (460mg) was applied to a short (2cm x 4cm) charcoal-Celite column. Monosaccharides were washed from the column with water and then 2,5% EtOH. 5% EtOH yielded disaccharides (9mg). The column was then washed with 10% EtOH to yield 2mg of a component having R_{gal} 0,1 (solvent b) and visualising as bright yellow fluorescence under UV (spray a). Hydrolysis and p.c. (solvents b and d) showed Man, and a streak from Glc to R_{gal} (solvent b) 0,65). The disaccharide fraction was separated by preparative paper chromatography, eluting with solvent c. Three fractions were isolated (Table 5.8.1).

Table 5.8.1 Acetolysis of RE.f-g.: Fractionation of the Disaccharides

	Fr A	Fr B	Fr C
Mass	3,5mg	4,0mg	1,3mg
R_{gal} (a)	0,45	0,53	0,67
R_{gal} (b)	0,20	0,27	0,42
R_{gal} (d)	0,29	0,33	0,50
Identification	--	Galβ-3Gal	Galβ-3Ara

Fraction A was divided into 2 portions.

(a) Half was hydrolysed and analysed by p.c. (solvent b) and by g.l.c. and g.l.c.-m.s. of the derived a.a.'s. Gal:Glc = 1,01:1,00

(b) The remainder was reduced ($NaBD_4$), hydrolysed, and analysed as before. P.c. showed Glc with only a faint trace of Gal remaining. G.l.c. of a.a.'s gave Gal:Glc = 1,06:1,00. G.l.c.-m.s. confirmed that Gal had D on C-1. (m/z 85,86; 115,116; 145,146; 157,158; 187,188; 217,218; 259,160; 289,290)

5.8.7 Partial Hydrolysis Experiments

In general, the substrate was dissolved in H_2SO_4 ($200cm^3$) at a suitable concentration and heated on a boiling water bath for

3h. The hydrolysate was neutralised with $BaCO_3$ and centrifuged. The centrifugate was dialysed (Spectrapor dialysis tubing, molecular weight cut-off 3500) against deionised water until the dialysate tested negative with Molisch reagent. The details are represented schematically in section 5.7. Masses used and reaped are listed below.

Used	Reaped Non-dialysable	Dialysate
E.f-g"A" (15g)	E.f-g"A"B1(2,4g)	E.f-g"A"DB1(13,0g)
E.f-g"A"B1(2g)	E.f-g"A"B2(830mg)	E.f-g"A"DB2(920mg)
E.f-g"A"B2(650mg)	E.f-g"A"B3(280mg)	E.f-g"A"DB3(250mg)

6. MOLECULAR STRUCTURAL STUDIES ON Encephalartos longifolius PART II: FURTHER INVESTIGATION INTO THE STRUCTURE OF THE GUM EXUDATE

6.1 INTRODUCTION

The elegant cycad, E.longifolius[84], grows in the Eastern Cape from Joubertina, through Humansdorp and Uitenhage to Somerset East. It has heavy stems up to 4m tall with diameter of 30 to 45 cm.. The dark glossy green leaves are 1 to 2 m long. Of the genus, this species has the largest cones, some weighing up to 30kg, although they only have 1 to 3. The gum exudes profusely, from the cones or from sites of injury on the plant, particularly after rain.

Preliminary work had shown that, of the species available for study, E.longifolius, because of its slightly higher Man content and with GlcAβ-2Man confirmed as a component, may yield the best gum on which to work towards the isolation of a glucuronomannoglycan. Methylation analysis and dimsyl-catalysed β-elimination experiments had shown that gums from the other two species available were likely to have structures very similar to that of E.longifolius gum (Chapter 4).

6.2 ORIGIN, ISOLATION, PURIFICATION AND ANALYSIS OF SAMPLES

New samples of gum exudate from E.longifolius were collected after rain during winter, 1987, as a clear gel. A portion of the new gum sample was purified by EtOH precipitation, and $[\alpha]_D$ and sugar ratios were found to agree with those of the earlier sample used[26,37]. As the prime objective in work on this gum was to reach a core structure, partial hydrolyses were performed with that as aim. Therefore the gum was hydrolysed at pH 2 ("autohydrolysis") and the EtOH-insoluble portion was studied further.

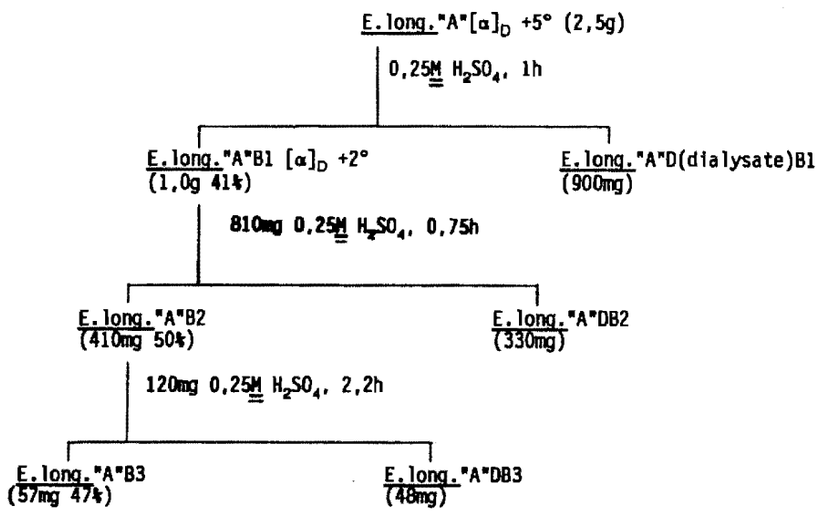
All methylation studies were performed on samples of E.longifolius gum that had been methylated earlier, using the Haworth[63] and Kuhn[85] methods. These were further methylated to completion by the Purdie[66] method. This was used for all methylation studies on E.long.. Comparative results for methylation analysis and dimsyl-catalysed β-elimination experiments on the three species compared, namely E.friderici-guilielmi, E.longifolius and E.ghellinckii, are tabulated and discussed in Chapter 4.

6.3 PARTIAL HYDROLYSIS STUDIES

The purpose of the partial acid hydrolysis studies was to remove successively the peripheral regions of the

polysaccharide. In addition the isolation of oligosaccharides would reveal more information on the gross structure of the gum. Trial experiments were performed, at all times, with the aim of achieving conditions for minimum loss of Man from the polymer. Enzymic hydrolysis[86a] to remove remaining Gal residues in E.long."A"B2 was considered. The only enzyme that could possibly achieve some degree of success was considered to be β-galactosidase, but incubation with this enzyme was unsuccessful. This corroborated the results of methylation analysis in demonstrating that Gal was not terminal. All hydrolyses performed were acid-catalysed.

The degradation of E.long"A", the "autohydrolysis" product, is shown schematically below.



Characteristics of the non-dialysable fractions, B1, B2, and B3, and of the dialysates, DB1, DB2, and DB3 are shown in Table 6.3.1. From these results, it can be seen that B3 consists of a polymer of GlcA and Man of approximately 14 sugar residues.

Gal, Ara, Xyl, and 3Rha had been progressively removed during the two hydrolysis steps. It is interesting to note that all Rha was removed during the first stage of partial hydrolysis of E.long."A" whereas 3Rha remained and was the second most abundant sugar in the dialysate DB3. Therefore, it must be associated with the core structure. Of the neutral disaccharides, Galβ-3Gal was liberated in increasing amounts, and Galβ-3Ara in decreasing amounts. The most abundant biouronic acid released was GlcAβ-6Gal. GlcAβ-6Galβ-3Ara was released in progressively lesser amounts.

Detailed work on the non-dialysable fractions is described in 6.3.1 and of the dialysates in 6.3.2.

Table 6.3.1.(a) Characteristics of Partial Hydrolysis Products of E.longifolius"A": The Non-dialysable Fractions

	B1	B2	B3
$[\alpha]_D$	+2°	-1°	-8°
%yield	41	50	47
mol% Uacid	42	44	47
mol% neutral sugars			
Gal	21	8	4
Man	31	44	49
Ara	2	1	-
Xyl	<1	tr	-
3Rha	4	2	-
Mol.wt.	-	2400;3800; 6600;10000	2500

Table 6.3.1(b) Characteristics of Partial Hydrolysis Products of *E.longifolius*"A": The Dialysates

Dialysate	DB1	DB2	DB3
Neutral disaccharides			
Galβ-3Gal	tr	tr	+
Galβ-3Ara	+	+	tr
Monosaccharides			
Gal	+++	++	++
Man	-	-	tr
Ara	+++++	++	tr
Xyl	++++	++	tr
Rha	++++++	-	-
3Rha	++	+	+
GlcA	-	-	tr
Acidic oligosaccharides[a]			
GlcAβ-6Gal	+++	+++	++
GlcAβ-6Galβ-3Ara	++	+	tr

a The dialysates all contained a series of acidic components similar to those in Fraction 9 of the stage 1 fractionation (Chapter 3)

6.3.1 The Non-dialysable Fractions, B1, B2, and B3.

6.3.1(i) Size and Sugar Linkage.

Molecular weight profiles for B2 and B3 were obtained on Bio-Gel P-10, (Fig 6.3.1) showing a tendency towards the 14-sugar unit. Integration from the ^{1}H n.m.r. spectrum of B3 would indicate a smaller average unit of approximately 10 sugars. Chemical shifts of resonances for anomeric protons in B3 are listed in Table 6.3.2, and agree with those obtained for E.f-g."A"B3, except that the ratio of end-group Man to in-chain Man is higher. There is a 1:1 ratio of total Man to total GlcA.

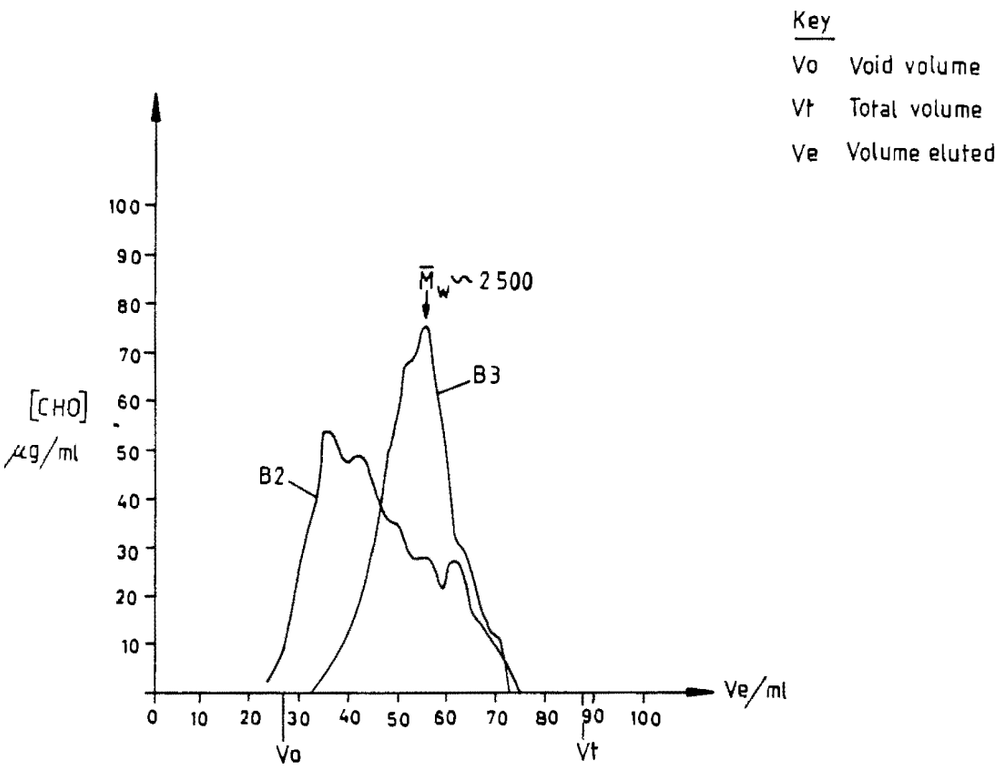


Figure 6.3.1 Partial hydrolysis of E.long."A": Molecular weight profiles of E.long."A"B2 and E.long."A"B3 on Bio-Gel P-10.

Table 6.3.2 N.m.r. results and assignments for E.longifolius"A"B3

Chemical shift (δ ppm)	Assignment	Integration
5,40	→2)-Man α-	4
5,27	→2)-Man α-OH	0,6
4,95	→2)-Man β-OH	0,4
4,51	GlcA β-	]
4,47	→4)-GlcA β-	] 5

The methylation analysis results for MB1, MB2, and MB3 are given in Table 6.3.3. These data show clearly that 2- and 2,4-linked Man and terminal and 4-linked GlcA comprise the polymeric acid-resistant core of the E.long. polysaccharide. These results would approximate to a possible 12-residue unit for B3 as shown in Figure 6.3.2.

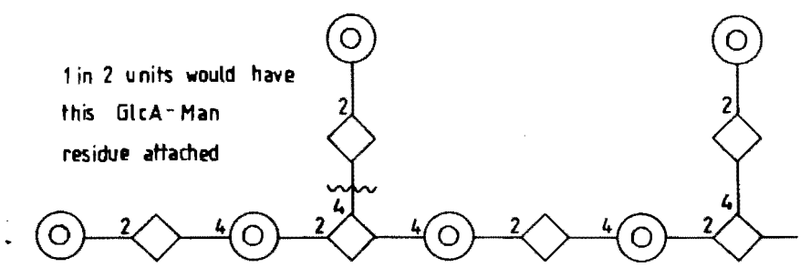


Figure 6.3.2 Possible 12-residue unit for E.long."A"B3.

Comparison of the results for MB3 (Table 6.3.3) with those obtained by methanolysis (Table 6.5.1) are in good agreement and on average agree with the proposed structure shown (Figure 6.3.2).

Table 6.3.3 Methylation Analysis Data for MB1, MB2, and MB3 of E.longifolius"A"

	MB1	MB2	MB3
T-Rha	4	2	-
T-Arap	2	1	-
T-Gal	2	<1	-
T-GlcA[a]	19	15	21
→3)-Ara	1	<1	-
→3)-Gal	3	<1	-
→6)-Gal	9	4	-
→4)-GlcA[a]	22	29	35
→2)-Man	10	19	30
→3,6)-Gal	5	4	-
→2,3)-Man	9	3	1
→2,4)-Man	8	13	12
→2,3,4)-Man	4	9	<1

a From LAD reduction of methylated substrate.

6.3.1(ii) Base catalysed β-elimination experiments.

B2 and B3 were subjected to base-catalysed β-elimination experiments to determine the sugars exterior to the 4-linked GlcA and, if possible, those sugars interior to that acid. The results of the dimsyl-catalysed[50] experiments (Table 6.3.4) show clearly that GlcA was exterior to Gal and to Man and linked to the 6 and 2 positions respectively, as those skeletal C atoms had been deuteriomethylated during the experiment. Some 6-linked Gal was substituted in the 3-position. On comparing the ratio of T-Man to T-Gal and →3)-Gal (4:4:4) formed after dimsyl degradation in BDMB2, with the ratio of →2)-Man to →6)-Gal and →3,6)-Gal (19:4:4) in the MB2 substrate, it can be seen clearly that, during the course of the experiment, the newly released Man had been degraded. Also there was no evidence for 2,3,6-tri-O-methyl mannose, that would have been formed from →2,4)-Man, giving further evidence that Man was exterior to GlcA in the substrates.

Table 6.3.4 Comparison of Methylation Analysis Data of E.long."A"B2 and B3 and their Dimsyl Degradation Products

	MB2	BDMB2	MB3	BDMB3
T-Rha	2	tr	-	-
T-Arap	1	tr	-	-
→3)-Ara	<1	tr	-	-
T-Gal[a]	<1	4	-	tr
→6)-Gal	4	tr	tr	-
→3)-Gal[a]	<1	4	-	tr
→3,6)-Gal	4	tr	tr	-
T-Man[b]	-	4	-	4
→2)-Man	19	-	30	-
→2,3)-Man	3	-	1	-
→2,4)-Man	13	-	12	-
→2,3,4)-Man	9	-	<1	-

a OCD_3 on C-6 after base degradation;
b OCD_3 on C-2. Arrows indicate sugar residues before and after dimsyl degradation.

The results of the dimsyl-catalysed degradation experiments on the partial hydrolysis products of E.long."A" were very similar to those on the E.f-g."A" products. Again, it was attempted to degrade the substrate using the bulky non-nucleophilic base, DBU, as for the E.f-g."A" hydrolysis products, with concomitant

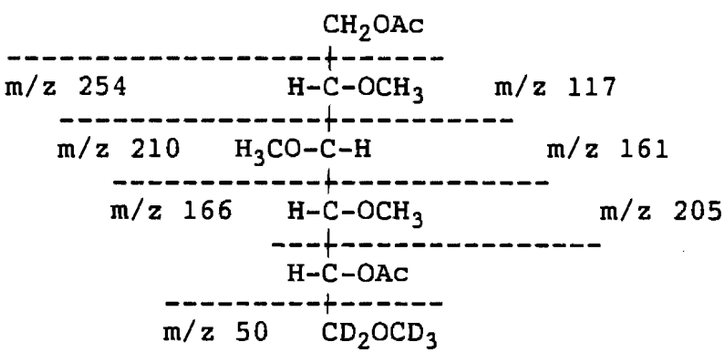
protection[5,15] of the newly released sugar that was exterior to the GlcA residue and should undergo degradation. The expected deuterium-labelled fully methylated and partially methylated alditol acetates are shown in Chapter 5 (Section 5.7.1). On monitoring the experiments after treatment with DBU for 48h at 110°, undegraded methylated acid was still visible on p.c. of the hydrolysate. However, one experiment on each of MB2 and MB3 was completed in the hope of obtaining the fully methylated mannitol, the 2-O-acetyl- or the 4-O-acetyl-penta-O-methyl mannitol. From the results (Table 6.3.5), it is apparent that MB2 yielded nothing on DBU degradation that had been borodeuteride reduced at C-1. DBUBDMB3 had a component eluting at t_R 0,38 (column c) as did the corresponding product from E.f-g."A". Characteristic ions present were m/z 49 and 115. M/z 49 corresponds to $CHDOCD_3$ at C-1, but this component was not hexa-O-methyl mannitol as the latter eluted from column c at t_R 0,51. However, the conditions of the saponification/ borodeuteride reduction step were such as to permit the reduction of the methyl carboxylate group on the non-degraded methyl glucuronate residues giving CD_2OCD_3 at C-6 on deuterio-methylation.

Table 6.3.5 Comparison of Methylaton Analysis Data of Partial Hydrolysis Products and their DBU-catalysed Degradation Products[a]

	MB2	DBUBDMB2	MB3	DBUBDMB3
T-Gal[b]	<1	5	-	tr
→6)-Gal	4	tr	tr	tr
→3)-Gal[b]	<1	4	tr	
→3,6)-Gal	4	-	tr	tr
T-Man[c]	-	20	-	5
→2)-Man	19	-	30	tr
→2,3)-Man	3	-	1	-
→4)-Man[c]	-	12	-	tr
→2,4)-Man	13	-	12	-
→2,3,4)-Man	9	-	<1	-
t_R 0,38[d]	-	-	-	1
T-Glc[e]	15	4	21	1
→4)-Glc[e]	29	-	35	1

a Results are expressed as molar ratios; only major components are included; in determining molar ratios, the likely survival of Gal is used as a basis for determining molar ratios of Man derivatives;
b OCD_3 on C-6 after degradation;
c OCD_3 on C-2;
d column c;
e CD_2OCD_3 at C-6; as GlcA in MB2 and MB3.

The presence of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl glucitol and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl glucitol was proof of incomplete degradation of GlcA in the substrates. These p.m.a.a.'s were identified because they had CD_2OCD_3 at C-6. The mass-spectral fragmentation of the tetramethyl glucitol is illustrated below:



Notable primary fragmentation ions were observed at m/z 50, 117, 161 and 166, and 205 and 210 with their secondary fragmentation ions at 145 and 150. Because not all the acid was degraded, no information additional to that gleaned from the dimsyl-catalysed experiments can be deduced from these experiments.

6.3.1(iii) Partial hydrolysis of E.long."A"B3.

Small scale partial hydrolysis was carried out on B3 to obtain, if possible, the oligomers of GlcAβ-2Man. Chromatographic standards were now available[21] for these compounds. Partially hydrolysed (1,5h M TFA, 100°) E.long."A"B3 had components with the mobility of the monomer and dimer of GlcAβ-2Man as well as traces of components having the mobility of the trimer and tetramer. This was confirmed in two solvent systems (solvents

a and d). In addition, the same components were found to conform to the plot of $\log[(1/R_{monomer})-1]$ *vs.* n^{72}, where monomer is defined as GlcAβ-2Man. Thus from chromatographic evidence, *E.long.*"A"B3 appears to contain at least the $(-4GlcA\beta-2Man\alpha-)_4$ structure.

6.3.1(iv) F.a.b.-m.s. of *E.long.*"A"B3.

Although B3 was expected, from its molecular weight profile, to have a range of molecular weights, its permethylated derivative, MB3, together with permethylated GlcAβ-2Man and the permethylated linear dimer of GlcAβ-2Man, was submitted to f.a.b.-m.s.. During the purification of MB3 on Merckogel 2000, MB3 was fractionated giving two fractions, Fraction 1 of higher molecular weight eluting first and Fraction 2 eluting second.

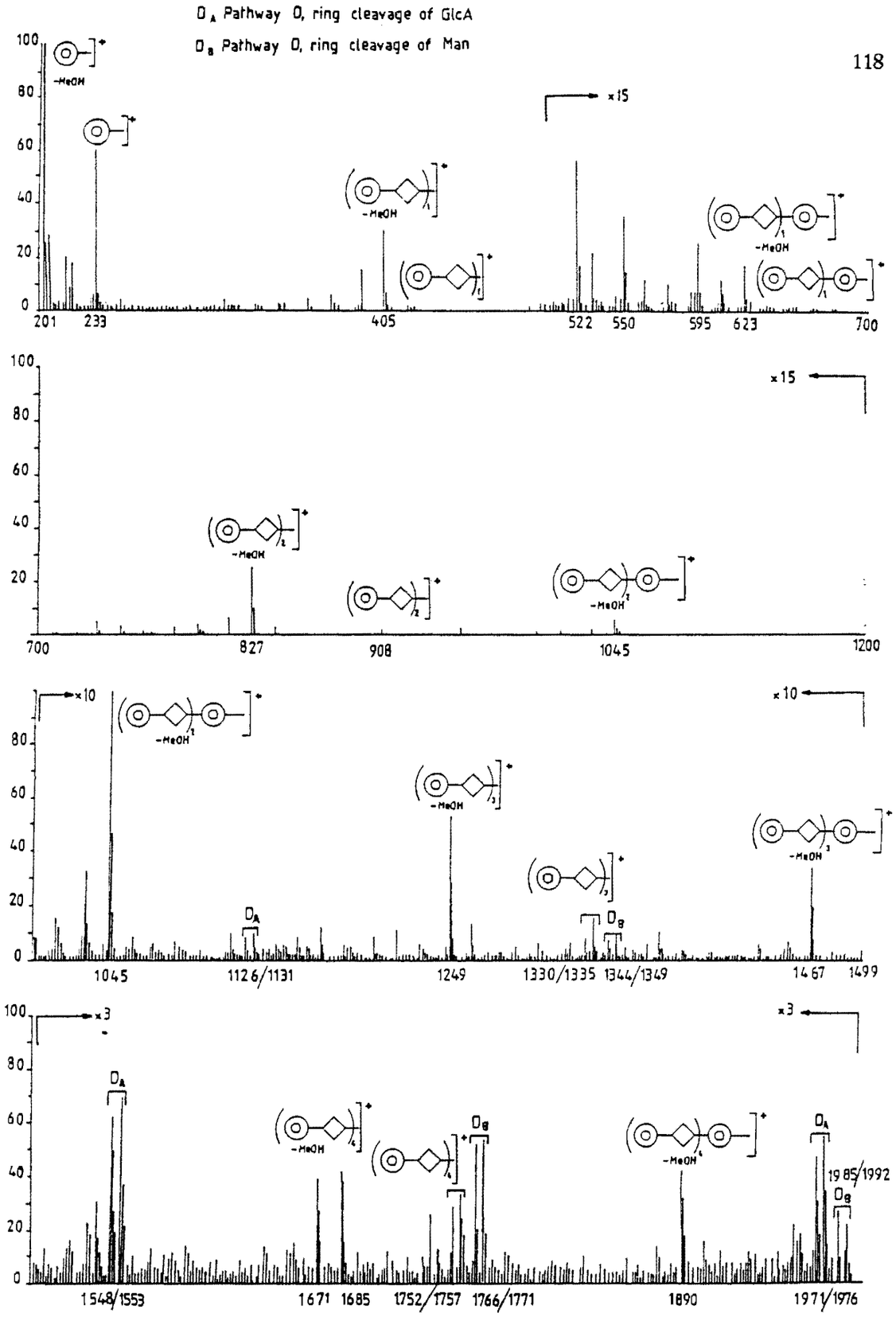


Figure 6.3.3 F.a.b.-m.s. spectrum of methylated E.long."A"B3 Fraction 1.

The f.a.b.-m.s. results (Tables 6.3.6 (a, b, and c) and Figure 6.3.3) show two main points:

(i) Cationised molecular ions (Table 6.3.6.(a))were observed corresponding to $GlcA_nMan_n$ for n=3 and 4 in Fraction 1 and for n=2, 3, and 4 in Fraction 2. This implies that MB3 is polymolecular, i.e. it consists of molecules having a range of molecular weights. This polymolecularity could have been due to degradation during Hakomori methylation of B3; but the two standards, GlcAβ-2Man and the linear dimer thereof, were both methylated under identical conditions and showed no evidence of degradation[17]. It is therefore probable that the range of molecular species observed was in the substrate, B3, although p.c. of B3 showed no evidence of low molecular weight oligomers of GlcAβ-2Man. These would be difficult to see because of the poor response of Man to spray reagent a exacerbated by decrease of the response decreasing with increasing degree of polymerisation.

(ii) Fragment ions for all fragmentations resulting from glycosidic cleavage with charge retention on the non-reducing end (pathway A^{87}) were observed (Table 6.3.6(b)). These fragment ions, usually with loss of MeOH, are consistent with alternating GlcA and Man residues, starting with GlcA at the non-reducing end.

Table 6.3.6(a) F.a.b.-m.s. results for MB3

(a) Cationised molecular ions

Permethylated	NH_4^+(m/z)	Na^+(m/z)
$GlcA_2Man_2$	908	913 (Fraction 2 only)
$GlcA_3Man_3$	1330	1335
$GlcA_4Man_4$	1752	1757

Table 6.3.6(b) F.a.b.-m.s. results for MB3

(b) Fragment ions, Pathway A cleavage

Permethylated	m/z	Fr 1	Fr 2	Permethylated	m/z	Fr 1	Fr 2
$GlcA^+$-MeOH	201	+	+	$GlcA^+$	233	+	+
$GlcAMan^+$-MeOH	405	+	+	$GlcAMan^+$	437	tr	+
$GlcA_2Man^+$-MeOH	623	+	+	$GlcA_2Man^+$	655	tr	+
$GlcA_2Man_2^+$-MeOH	827	+	+	$GlcA_2Man_2^+$	859	tr	+
$GlcA_3Man_2^+$-MeOH	1045	+	+	$GlcA_3Man_2^+$	1077	tr	+
$GlcA_3Man_3^+$-MeOH	1249	+	+	$GlcA_3Man_3^+$	1281	tr	+
$GlcA_4Man_3^+$-MeOH	1467	+	+	$GlcA_4Man_3^+$	1499	+	tr
$GlcA_4Man_4^+$-MeOH	1671	+	+	$GlcA_4Man_4^+$	1703	tr	tr
$GlcA_5Man_4^+$-MeOH	1890	+	+				

Other cationised species could be formed by a pathway D[87] type mechanism in which ring cleavage occurs with charge retention on the reducing end (Figure 6.3.4 and Table 6.3.6(c)).

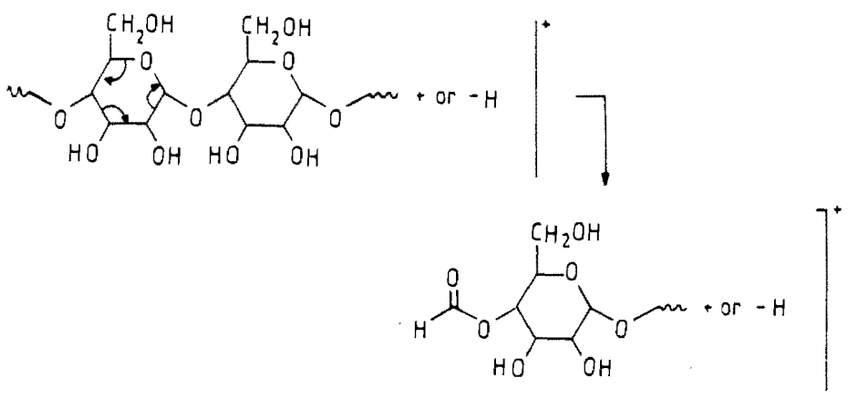


Figure 6.3.4 Pathway D mechanism[87]

Table 6.3.6(c) F.a.b.-m.s. results for MB3

(c) Cationised species, Pathway D cleavage

NH_4^+(m/z)	Na^+(m/z)	Fr 1	Fr 2	Assignment
1126	1131	+	++	A, n=2
1344	1349	+	++	B, n=3
1548	1553	++	++	A, n=3
1766	1771	++	+	B, n=4
1971	1976	++	+	A, n=4
1985	1992	+	+	B, n=5

A Ring cleavage of GlcA, possible species is Formate-Man-$(GlcA\text{-}Man)_n$-OMe
B Ring cleavage of Man, possible species is Formate-$(GlcA\text{-}Man)_n$-OMe

Thus the major cationised species and fragment ions can be explained in terms of the existence of a linear tetramer of GlcA-Man. However, the existence of prominent m/z 1685, equivalent to 1766-81 (MNH_4^+-NH_4^+-MeO-MeOH) in the spectrum of Fraction 1 is difficult to comprehend if m/z 1766 is assigned as suggested in Table 6.3.6(c), as it would mean ring cleavage within the polymer and glycosidic cleavage at the reducing end of the polymer leaving the charge on the non-reducing end with respect to the glycosidic cleavage. On the other hand, the prominent peaks at m/z 1045, 1467, and 1890, which could be the corresponding MNH_4^+-81, can be explained in terms of Pathway A glycosidic cleavage in a chain of alternating GlcA and Man residues.

In general, more prominent peaks having m/z <1500 can be explained in terms of Pathway A glycosidic cleavage. In addition m/z 1671 and corresponding cationised pair at 1752/1757 support the hypothesis that the core of E.long. does contain some linear tetramer of GlcAβ-2Man.

6.3.2 Examination of the dialysates DB1, DB2, and DB3.

In the first instance, dialysates were examined by p.c., comparing components with known substances and with Fraction 9 of the 1966 Stage 1 fractionation (Chapter 3). These results are represented in Table 6.3.7 and Figure 6.3.5 in which the 2-dimensional p.c. of DB1 is represented.

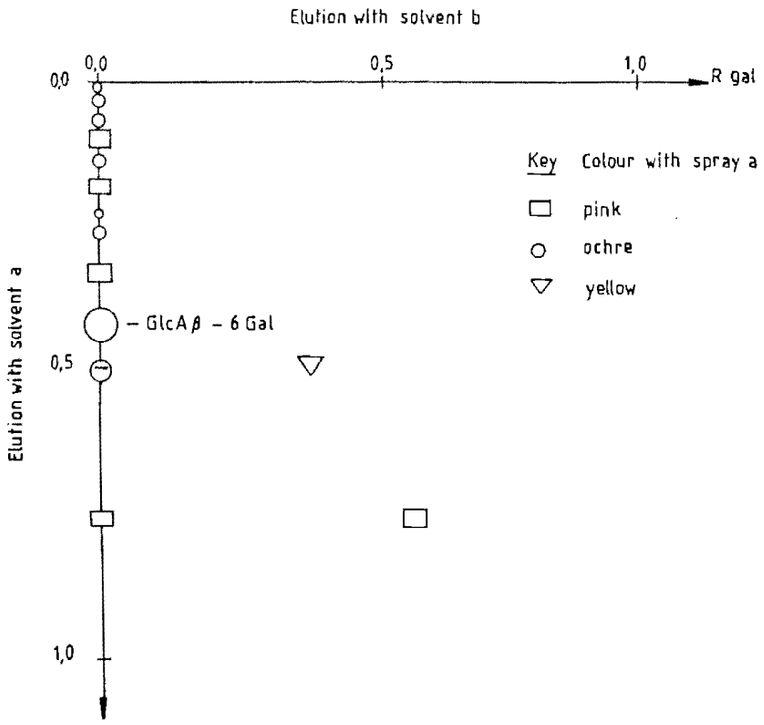


Figure 6.3.5 Two-dimensional p.c. of the dialysate DB1.

Table 6.3.7 Comparison of Oligosaccharides[a] in DB1, DB2, and DB3 with Fraction 9 (1965)

Oligosaccharides		DB1	DB2	DB3	Fr.9
R_{gal}[b]	Identification				
0,01y[c]		+	+		+
0,04y		+	+		+
0,07y		+	+	+	+
0,10p-o		+	+		
0,14y		+	+	+	+
0,19p-o		+	+		
0,23y		+	+	+	+
0,27y		+	+	+	+
0,33r-b	GlcAβ-6Galβ-3Ara	++	+	tr	+
0,44o	GlcAβ-6Gal	+++	+++	++	+++
0,58y	Galβ-3Gal/GlcAα-2Man	+	+	+	tr
0,77p-o	4GlcAβ-6Gal/Galβ-3Ara	+	+	+	+
R_{gal}[d]					
0,36y	Galβ-3Gal	+	+	+	tr
0,55r-b	Galβ-3Ara	+++	+	tr	tr

a Monosaccharides are compared in Table 6.3.1; b R_{gal} values in solvent a; c denotes colour with spray a, y = yellow fluorescence, p-o = pink-ochre, r-b = red-brown; d R_{gal} values in solvent b.

Two-dimensional p.c. of DB1, eluting first with the acidic solvent, a, and then with the basic solvent, b, shows that all but two of the oligosaccharide components are acidic. Those components appearing pink-ochre or red-brown with p-anisidinium chloride were of interest as they were likely to be Ara containing oligosaccharides. The two neutral components, Galβ-3Gal and Galβ-3Ara, were present in all three dialysates, but Galβ-3Ara was in decreasing amounts in each successive dialysate. This indicates that at least some Gal must be linked directly to the proposed glucuronomannoglycan core as Galβ-3Gal remains with the higher molecular weight component to a later stage of hydrolysis than the Galβ-3Ara does.

Fraction 9, the acid fraction from the Stage 1 hydrolysis of E.long. in 1965, was partially hydrolysed in 0,25M H_2SO_4 at 100° for 2h, 4h, and 8h, to compare the components released with the components in the dialysates. Monosaccharides released were the expected Gal(++), Ara (tr), and trace, but increasing, amounts of Man and GlcA. Of the neutral disaccharides, only Galβ-3Gal was noted, and only in the 2h hydrolysate. The oligosaccharides released are shown in Table 6.3.8.

Table 6.3.8 Partial Hydrolysis of Fraction 9: Oligosaccharides Released

R_{gal}[a]	Identification	2h	4h	8h
0,14y	Trimer[b]	+	tr	tr
0,23y	GlcAβ-6Gal-Gal[c]	tr	tr	tr
0,27y	Dimer[d]	+	+	tr
0,44o	GlcAβ-6Gal	+++	+++	+++
0,58y/o	Galβ-3Gal/GlcAα-2Man	tr(y)	+(y-o)	+(o)
0,77p-o	4GlcAβ-6Gal	+	tr	tr

a P.c. in solvent a, colours y = yellow, o = ochre, p = pink with spray a; b,d trimer and dimer of GlcAα-2Man; c identification of b and c is by comparison with standards and R_{gal} values quoted (Table 3.2.2) for the components identified in the Stage 2 hydrolysis.

Fraction 9 was the acid fraction from the hydrolysed gum and would therefore be expected to have GlcAβ-6Gal as a major component. However increased release of GlcAβ-2Man with increase in hydrolysis time is evident. It appears, as would be expected, that with longer hydrolysis times dimer and trimer produced are being hydrolysed to give monomeric GlcAβ-2Man .

DB1 dialysate, as Ba^{++} salts, was separated into EtOH-soluble (neutral) components and EtOH-insoluble fractions (Ba^{++} salts of the acidic components). The neutral components were

fractionated on a charcoal-Celite column, eluted with increasing concentrations of EtOH in water and the acidic components on cellulose, eluted with BuOH-HOAc-H_2O, decreasing the concentration of BuOH.

6.3.2 (i) The Neutral Components.

P.c. (solvent b) of the EtOH-soluble fraction confirmed the presence of two major oligosaccharides. Other major components were the monosaccharides, 3Rha, Rha, Xyl, Ara, and Gal. The properties of the fractions obtained are summarised (Table 6.3.9).

Table 6.3.9 Fractionation of Neutral Components of DB1 on Charcoal-Celite

Eluent	Fraction	Components	Mass	$[\alpha]_D$
H_2O	1-15	Gal, Ara, Xyl, Rha		
3% EtOH	16-20	3Rha	262mg	+34°
5% EtOH	24-26	Galβ-6Gal/Galβ-3Ara	17mg	
5% EtOH	27-29	Galβ-3Ara	33mg	+42°
5% EtOH	30-31	Galβ-3Ara/Galβ-3Gal	7mg	
5% EtOH	32-34	Galβ-3Gal	17mg	+30°

Detailed discussion is given for fractions 16-20 and 27-29; components in the remaining fractions were identified by p.c.

in three solvent systems (a, b, and c) against authentic specimens.

Fraction 16-20 crystallised spontaneously, m.p. 110°-114°, in agreement with the earlier value reported[37]. Chemical shifts of the ^{1}H and ^{13}C resonances are tabulated (Table 6.3.10). Complete assignment of the n.m.r. spectra was achieved as described in Chapter 7, except that the APT experiment would be of no value and was therefore not done. ^{1}H and ^{13}C chemical shifts were obtained for L-Rha as reference compound. Change in chemical shift ($\delta_{3Rha}-\delta_{Rha}$) of 9,1 p.p.m. ($\alpha$) and 9,4 p.p.m. ($\beta$) downfield on C-3 shows the presence of the methoxyl substituent on C-3. The chemical shift of the C-2 resonance is shifted 4,4 p.p.m. upfield for both α and β anomers. This is in agreement (see Chap. 7) with the γ-gauche interaction of the OCH_3 protons and the protons on C-2, thus showing the axial-equatorial relationship of the C-2 OH and C-3 OCH_3. There is a small (-1 p.p.m.) upfield change in the chemical shift for the resonance of C-4. In agreement with the change in chemical shift of C-2 and C-3 are the reciprocal changes in the chemical shifts of the H-2 (+0,25 p.p.m. downfield) and H-3 (-0,36 p.p.m. for α and -0,33 p.p.m. for β).

Table 6.3.10 N.m.r. Data for Fraction 16-20 (3Rha) and Rha

1H assignments (δ, p.p.m.) for 3Rha and Rha					Change in δ^a	
	α 3Rha	β 3Rha	α Rha	β Rha	α	β
H-1	5,13	4,83	5,09	4,84	0,04	-0,01
H-2	4,16	4,16	3,91	3,90	0,25	0,26
H-3	3,42	3,25	3,78	3,58	-0,36	-0,33
H-4	3,41-3,46	3,35	3,41	3,33	0	0,02
H-5	3,85	3,35	3,84	3,38	0,01	-0,03
H-6	1,25	1,27	1,25	1,27	0	0
OCH3	3,43	3,42				
$J_{1,2}$	1,99Hz	0,61Hz				
$J_{5,6}$	6,19Hz	5,11Hz				
13C assignments (δ, p.p.m.) for 3Rha and Rha					Change in δ_a	
	α 3Rha	β 3Rha	α Rha	β Rha	α	β
C-1	94,70	94,31	94,74	94,26	-0,04	0,05
C-2	67,15	67,67	71,56	72,08	-4,41	-4,41
C-3	80,16	82,62	70,70	73,50	9,46	9,12
C-4	71,82	71,49	72,94	72,57	-1,12	-1,08
C-5	69,02	72,75	69,04	72,79	-0,02	-0,04
C-6	17,56	17,53	17,53	17,48	0,03	0,05
OCH_3	56,83	56,74				

a Change in $\delta = \delta_{3Rha} - \delta_{Rha}$.

Table 6.3.11 ^{1}H and ^{13}C Data for Gal β-3Ara

	Gal moiety		Ara moiety		
^{13}C	Assign	^{1}H	^{13}C	Assign	^{1}H
104,95	1α	4,60[a]	97,24	1α	4,55[b]
104,85	1β		93,26	1β	5,24[c]
71,83	2	3,52-3,60 3,57[d]	71,74	2α	3,55-3,63
			68,24	2β	3,92-3,96
73,29	3	3,58-3,67	82,69	3α	3,76-3,80
			79,26	3β	3,98
69,32	4	3,85-3,94 3,91[d]	69,05	4α	4,15-4,20 4,17[d]
			69,21	4β	4,20-4,25 4,22[d]
75,77	5α	3,62-3,68	66,56	5α	3,82-3,86 3,84[d]
75,74	5β		62,74	5β	-[e]
61,68	6	3,70-3,73 3,72[d]			

a $J_{1,2}$=6,77Hz; b $J_{1,2}$=7,75Hz; c $J_{1,2}$=2,50Hz; d from J-resolved experiment; e there was no visible correlation in the HETCOR experiment.

Fraction 27-29 had $[\alpha]_D$ +42° and a 1:1 ratio of Gal to Ara on g.l.c. of a.a.'s. This component showed similar but not identical characteristics to Galα-3Ara on p.c. and therefore needed to be characterised. No Galβ-3Ara was available. Borohydride reduction of the disaccharide followed by hydrolysis showed only Gal on p.c.. Complete n.m.r. assignments for chemical shifts of 1H and ^{13}C resonances were made (Table 6.3.11) as described in Chapter 7. For additional elucidation

of the ^{1}H resonances, the J-resolved ^{1}H spectrum was used. Change in chemical shift of ^{13}C resonances was calculated using[54] Galβ-3Gal and Ara as reference compounds (Table 6.3.12).

C-1 and C-5 resonances of the Gal moiety both showed twinning[89], indicating the effect of equilibration of the α and β anomers of the Ara moiety. Ara C-3 resonances were deshielded by 9,2 (α) and 9,8 (β) p.p.m. respectively, while the C-2 resonances were shielded by 1,2-1,3 p.p.m. (Table 6.3.12). Deshielding at C-3 shows that there is a substituent on the 3-position of Ara and this is therefore the point of attachment of the Gal. Shielding at C-2 may be caused by hydrogen bonding between Ara 2-OH and Gal ring oxygen[88].

As shifts of both AraβOH α and β resonances were shown, Ara is at the reducing end. Also only Gal was visible on p.c. on reduction and hydrolysis of the disaccharide. Gal has the β anomeric configuration according to the shifts of the resonances of anomeric C and H.

Table 6.3.12 Change in δ of ^{13}C resonances of Galβ-3Ara

	Gal[a]	Ara α[b]	Ara β
C-1	-0,2	-0,8	-0,1
C-2	-0,2	-1,2	-1,3
C-3	-0,1	+9,2	+9,8
C-4	-0,1	-0,5	-0,3
C-5	-0,1	-0,6	-0,7
C-6	-0,1		

a Reference compound Galβ-3Gal; b reference compound Ara-OH.

On comparison of these results for the Ara moiety with those obtained for Galα-3Ara[89], the results for Galα-3Ara showed greater shielding (-2,0 p.p.m.) at C-2 of Ara and lesser deshielding (4,2 p.p.m. and 5,2 p.p.m. respectively) at C-3. Also, there was a ca. 4 p.p.m. shielding effect at C-4 which did not occur in Galβ-3Ara.

However, on comparing the chemical shifts of the ^{13}C resonances of C-2, C-3, and C-4 of the reducing sugar residue in Glcα-3Galβ-OH and Galβ-3Galβ-OH[54] with the results obtained for Galα-3Ara and Galβ-3Ara it is apparent that the differences can

be attributed to the glycosyl substituent being in the α or β configuration[90]. (See Table 6.3.13)

Table 6.3.13 Comparison of ^{13}C-n.m.r. results[a] for Galβ-3Ara with Literature Values[54,89]

	Galβ-3Galβ	Galβ-3Araα	Galα-3Araα	Glcα-3Galβ
C-1	97,0	97,24	97,19	97,7
C-2	71,8	71,74	70,94	71,5
C-3	83,3	82,69	77,71	78,8
C-4	69,4	69,05	65,44	66,3
C-5	75,6	66,56	66,62	76,1
C-6	61,8	-	-	62,2

a Results for skeletal carbon atoms of the reducing sugars are listed.

On comparing the results obtained for Galα-3Ara[89] and Galβ-3Ara, and considering a possible conformation of the latter, glycosylation at C-3 of Ara would appear to anchor the Ara moiety in the $^1C_4$ mode as for Galα-3Ara. In the conformation shown (Figure 6.3.6), there would be favourable intramolecular hydrogen bonding between the ring oxygen of Gal and the equatorial 2-OH of Ara. This is confirmed by the upfield change in chemical shift of the Ara moiety[88] Glycosylation of equatorial OH would cause an upfield change in chemical shift of the resonance of the neighbouring skeletal C atom if it carried equatorial H. This could be due to the

interaction of the protons on C-4 of Ara and C-1 of Gal if they were in γ-gauche conformation[91]. However, there is no change in chemical shift of the Ara C-4 resonance showing that there is no steric interaction of the Gal moiety with the atoms associated with that position. Thus the conformation of Galβ-3Ara shown (Figure 6.3.6) is in agreement with the results obtained. In Galα-3Ara, there is the possibility of hydrogen bonding of both the 4-OH and the 2-OH with the equatorial lobe of the ring oxygen of the Gal residue, whereas in Galβ-3Ara, the only possibility of hydrogen bonding is of the 2-OH with the axial lobe of the ring oxygen; this gives the chair/chair/chair conformation shown.

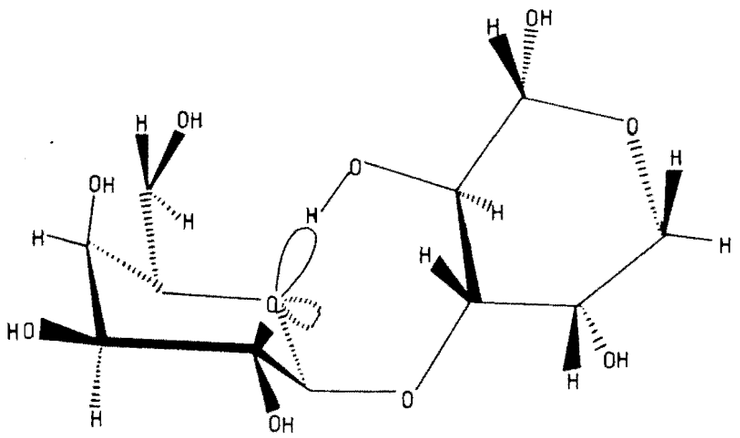


Fig. 6.3.6 Possible conformation of Galβ-3Ara.

6.3.2 (ii) The Acidic Components

The acidic components from the dialysate that were of interest were those appearing pink on spraying with p-anisidine

hydrochloride, as they would be expected to have Ara as a component. The major component conforming to this requirement was that having R_{gal} 0,33 in solvent a. Components moving more slowly than this were surveyed briefly by hydrolysis and p.c. to ascertain which sugars they contained. In considering the whole project, it was decided not to study all of the dialysate fractions in depth. The results are summarised (Table 6.3.14). Only Fraction 86-92 was characterised fully.

Table 6.3.14 Acidic Components[a] from Dialysate: Cellulose Column Fractions

Fraction	R_{gal}[b]	Identity, or Component Saccharides[c]
43-57	0,77	4MeGlcAβ-6Gal
57-73	0,58	GlcAβ-2Man
76-85	0,44	GlcAβ-6Gal
86-92	0,33	GlcAβ-6Galβ-3Ara
99-101	0,25	GlcAβ-6Gal(++),GlcA(+), Gal(+), Ara(+)
103-110	0,19	GlcAβ-6Gal(++), GlcA(+), Gal(++), Ara(+)
129-135	<0,1	GlcAβ-6Gal(++), GlcA(+), Gal(++), Ara(+)
151-158	<0,05	GlcAβ-6Gal(++), GlcA(tr), Gal(++), Ara(tr)
159-wash	0,00	GlcAβ-6Gal(+), GlcA(tr), Gal(++), Glc(+), Ara(tr), Xyl(+), GlcAα-2Man(tr), Man(tr)

a Only those fractions appearing to be homogeneous on p.c. were analysed;
b solvent a;
c saccharides identified on p.c. (solvents a anb b) after hydrolysis (M TFA, 8h, 100°).

The appearance of Glc in the hydrolysate of the final fraction was unexpected. It was the only occasion on which this monosaccharide appeared.

Fraction 86-92: Mild hydrolysis (2h 0,125$\underline{M}$ H_2SO_4, 100°) and p.c. (solvents a and b) showed unchanged 86-92(++++), GlcAβ-6Gal(+++), Galβ-3Ara(+), Gal(+), and Ara(++++). On further hydrolysis (2$\underline{M}$ TFA, 4h, 100°), some GlcAβ-6Gal remained and the only other components visible were GlcA, Gal and Ara. With reduction prior to hydrolysis under similar conditions, similar results were obtained but with a lesser response of Ara. Further reduction did not remove Ara completely. Analysis of the anomeric region of the ^1H n.m.r. spectrum for 86-92 (Table 6.3.15) indicated the presence of terminal β-linked GlcA, in-chain β-linked Gal, and Ara$\underline{p}$ at the reducing end. Fraction 86-92 was reduced ($NaBD_4$), methylated and hydrolysed. P.c. (solvent c) indicated T-GlcA, →6)-Gal, and traces of →3)-Ara$\underline{p}$ and a substance having mobility between that of 3,4,6-Me_3-Man and 2,3,4,6-Me_4-Gal.

A second portion of 86-92 was reduced ($NaBD_4$) and methylated, and the methyl carboxylate groups were reduced (LAD). Hydrolysis and p.c. showed the major components to be 2,3,4-Me_3Glc and 2,3,4,-Me_3Gal. The former would be from T-GlcA. There were the same two minor components as before.

Table 6.3.15 ^{1}H N.m.r. Spectrum of 86-92.

Resonances of Anomeric Protons.

86-92			Galβ-3Ara	
δ/p.p.m.	$J_{1,2}$/Hz	Assign	δ/p.p.m.	$J_{1,2}$/Hz
5,25	2,6	Araβ-OH	5,25	2,60
5,24			5,24	
4,62	7,6	Galβ-OR	4,62	6,80
4,58			4,58	
4,58	7,4	Araα-OH	4,57	7,60
4,54			4,54	
4,54[a]	7,6	GlcAβ-OR		
4,50				

a The values assigned to GlcAβ-OR agree with published data[10]

G.l.c. and g.l.c.-m.s. of the derived p.m.a.a.'s (Table 6.3.16) confirmed the presence of T-GlcA, →6)-Gal, some →3)-Ara and 3-O-acetyl-1,2,4,5-tetra-O-methylarabinitol deuterium labelled at C-1. From these results it is apparent that the ratio of Ara (total) to Gal to GlcA is 1:1:1. These results show that not all Ara was reduced to arabinitol during the borodeuteride reduction although this was not apparent on p.c. of the reduced trisaccharide. A possible explanation for this is the likely dimerisation of the disaccharide on standing[92]; 5-linked Ara which can exist only in the furanose form, dimerises readily and, from methylation analysis results, the →3)-Ara that was not reduced has been methylated in the furanose form. The proposed stucture for this tricaccharide is therefore

GlcAβ-6Galβ-3Ara.

Although not all the Ara had been reduced, the fact that the ratio of GlcA to Gal to Ara/arabinitol was 1:1:1 taken in conjunction with other chemical and spectroscopic evidence supports the proposed structure.

Table 6.3.16 Methylation Analysis Results for Fraction 86-92

Mol %	Identification	Deuterium Labelling
19	1,2,4,5-Me_4-Ara	1D on C-1
10	2,5-Me_2-Ara	
3	2,4-Me_2-Ara	
36	2,3,4-Me_3-Glc	2D on C-6
32	2,3,4-Me_3-Gal	

Thus the following oligosaccharides are confirmed components of the dialysate:

Galβ-3Gal Galβ-3Ara Galβ-6Gal

GlcAβ-6Gal Glcβ-6Galβ-3Ara

6.4 A METHYLATION STUDY ON METHYLATED E.longifolius

Methylated E.long was partially hydrolysed and then fractionated on cellulose. This large-scale separation of partially methylated sugars served two main purposes, firstly, the more important, to obtain a partially methylated polymeric fraction, and secondly as a source of partially methylated sugars. As a bonus this would serve as a rough check against the methylation analysis results obtained by g.l.c. and g.l.c.-m.s. of the p.m.a.a.'s derived from the hydrolysate of the methylated gum, although the conditions of hydrolysis in this experiment were necessarily milder than that for methylation analysis.

The column performance is summarised in the histogram (Figure 6.4.1) and details of identification of the sugars are described under experimental (6.5.4). A comparison of molar ratios of methylated sugars obtained from the column with methylation analysis results is summarised (Table 6.4.1).

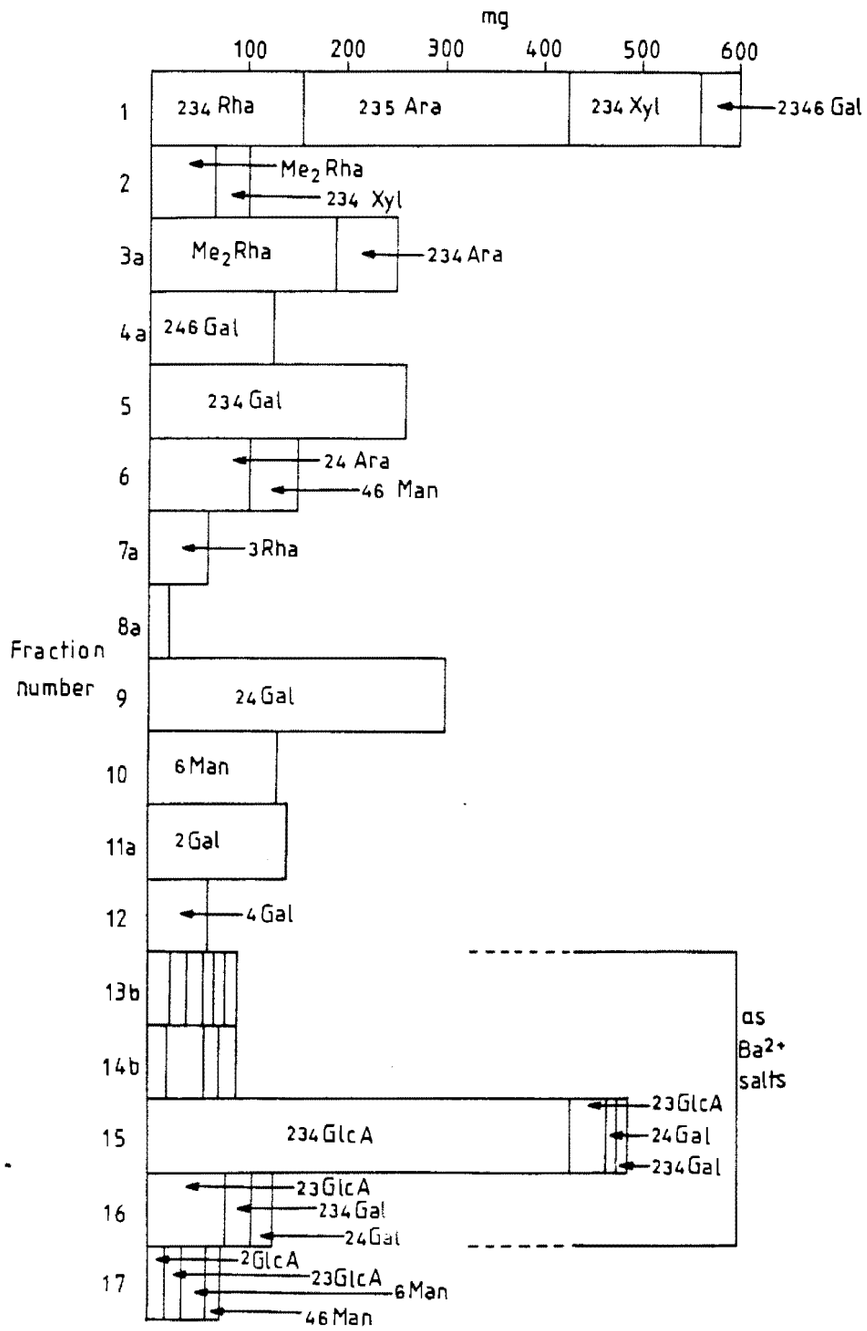


Figure 6.4.1 Fractionation of partially hydrolysed methylated E.longifolius: the histogram illustrating column performannce.

a,b See Tables 6.5.2(a) and (b) for detail of other components. Fractions 13 - 17 are polymeric. Hydrolysis products are indicated.

The later fractions contained T-GlcA and →4)-GlcA, much of which was oligomeric. It is thus not included in the comparison. Some Gal and Man methyl ethers were also in the later fractions; these additional quantities were estimated from the g.l.c. traces of the p.m.a.a.'s. However, the quantities of →2,3,4)-Man and →3,6)-Gal are still low. A possible reason for →2,3)-Man being higher in the column preparation than in analytical g.l.c. is that 4,6-Me_2Man and 2,3,4-Me_3Gal coelute on OV-225 and there is difficulty in their quantification.

Table 6.4.1 Comparison of Methylation Analysis Results for ME.long. and Quantities of Sugars obtained from the Column

Sugar	MA[a]	C	Sugar	MA	C
T-Rha	16	6	T-Araf	4	10
→Rha	5	8	T-Arap	5	3
T-Gal	3	1	T-Xyl_p		6
→3)-Gal	2	3	T-Fuc	-	1
→6)-Gal	9	9	→3)-Ara	7	5
→2,3)-Man	<1	5	→3,6)-Gal	19	13
→2,4)-Rha	tr	1	→3,4,6)-Gal	2	5
→2,3,4)-Man	8	4			

a MA = methylation analysis results, C = quantities from the cellulose column.

One of the purposes of obtaining the partially methylated sugars was to obtain and assign their n.m.r. spectra. N.m.r. assignments for fractions 4, 5, and 9 and discussion of the results are detailed in Chapter 7, and for fraction 10 the results are presented in Table 6.4.2.

Table 6.4.2 N.m.r. assignments for 6-O-methylmannose: Chemical Shifts of ^{1}H and ^{13}C Resonances

^{1}H δ/p.p.m.			^{13}C δ/p.p.m.		
α		β	α		β
5,14	H-1	4,86	94,68	C-1	94,35
3,9	H-2	3,9	71,30	C-2	71,85
3,8	H-3	3,63	70,82	C-3	73,66
3,65	H-4	3,53	67,66	C-4	67,36
3,9	H-5	3,46	71,46	C-5	75,27d
3,68-3,72	H-6	3,68-3,72	72,15	C-6	72,15
3,38	OCH_3	3,39	59,06	OCH_3	59,36
1,59Hz	$J_{1,2}$	1,09Hz			

On comparison of the ^{13}C chemical shifts with those for mannose[54], the changes in chemical shift for the C-6 resonance, which was identified by means of the APT experiment, are 9,55 p.p.m. each. This is in accordance with the empirical rules of

Bradbury and Jenkins[54], thus confirming the site of attachment of the methoxyl group.

6.4.1 The Partially Methylated Polymeric Fraction.

The polymeric fraction (17) was eluted from the column with BuOH-EtOH-H_2O (2:6:3,5) and hydrolysis yielded predominantly 6-Me-Man, 2-Me-GlcA and 2,3-Me_2GlcA, together with a lesser amount of 4,6-Me_2Man, 3,4,6-Me_3Man and 3,6-Me_2Man. The ratio of 6-Me to 4,6-Me_2Man was 7:1. Fraction 17 was characterised by reduction with $NaBD_4$ to label the reducing end, then deuteriomethylation to determine the linkages in the fraction as it came from the column (Fr 17 now named MR17). This should also indicate the site of attachment of the sugars released during partial hydrolysis of the methylated gum prior to fractionation. Thereafter, a portion of MR17 was reduced with LAD[68] to label the acidic components at C-6 (RMR17). Hydrolysis and p.c. of MR17 showed only 3,4,6-Me_3Man and acid, and after LAD reduction, RMR17 showed 2,3,4-Me_3Glc, 3,4,6-Me_3Man, 2,3-Me_2Glc and no acid.

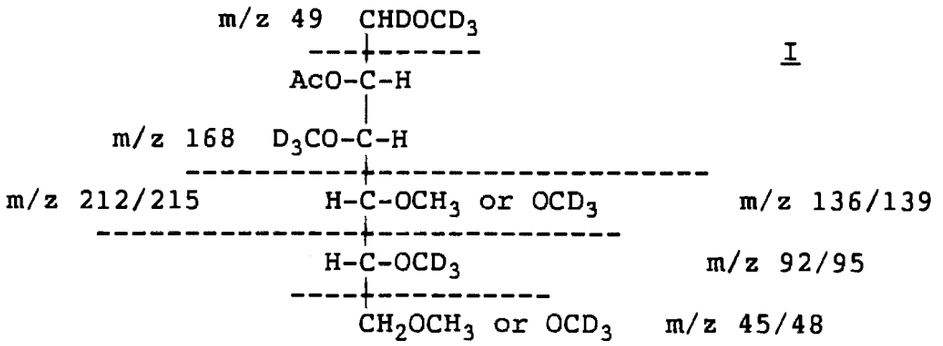
P.m.a.a.'s (Table 6.4.3) of both MR17 and RMR17 gave a ratio of 1:1,6 for reducing end Man to in-chain Man. The ratio of non-reducing end GlcA to in-chain GlcA was inexplicably high at 2:1. As well there was ±5 mol% 6-O-acetyl-1,2,3,4,5-penta--O-methyl glucose, deuterium - labelled at C-6.

Table 6.4.3 Methylation Analysis Results for MR17 and RMR17

		t_R[a]	MR17	RMR17
→2)-Mannitol	(I)	0,33	18	17
→2)-Man	(II)	1,51	29	27
Glucitol[b]	(III)	0,40	-	5
T-Glc[b]	(IV)	1,77	-	34
→4)-Glc[b]	(V)	3,10	-	17

a Retention relative to 2,3,4,6-Me_4-Gal on column a, isothermal at 175°;
b deuterium labelled at C-6, therefore all were from GlcA.

From these results MR17 is an oligomer with an average of 5 to 6 sugar residues. It comprises 2-linked Man and 4-linked GlcA with →2)-Man at the reducing end and GlcA as non-reducing end group. The glucitol obtained cannot be part of the oligomer as it is derived from T-GlcA. Analysis of the mass spectra of I to V indicated, by deuteriomethylation, the site of attachment of the sugars released during partial hydrolysis of the methylated gum, and by methylation, the site at which there had been no substituent sugar in the gum. This is illustrated by showing the probable fragmentations of I and its mass spectrum (Fig. 6.4.2).



I

Notable secondary fragmentations are the following:

m/z 136/139 - 32 (MeOH) = m/z 104/107

m/z 168 - 35 (CD_3OH) = m/z 133

m/z 212/215 - 60 (HOAc) = m/z 152/155

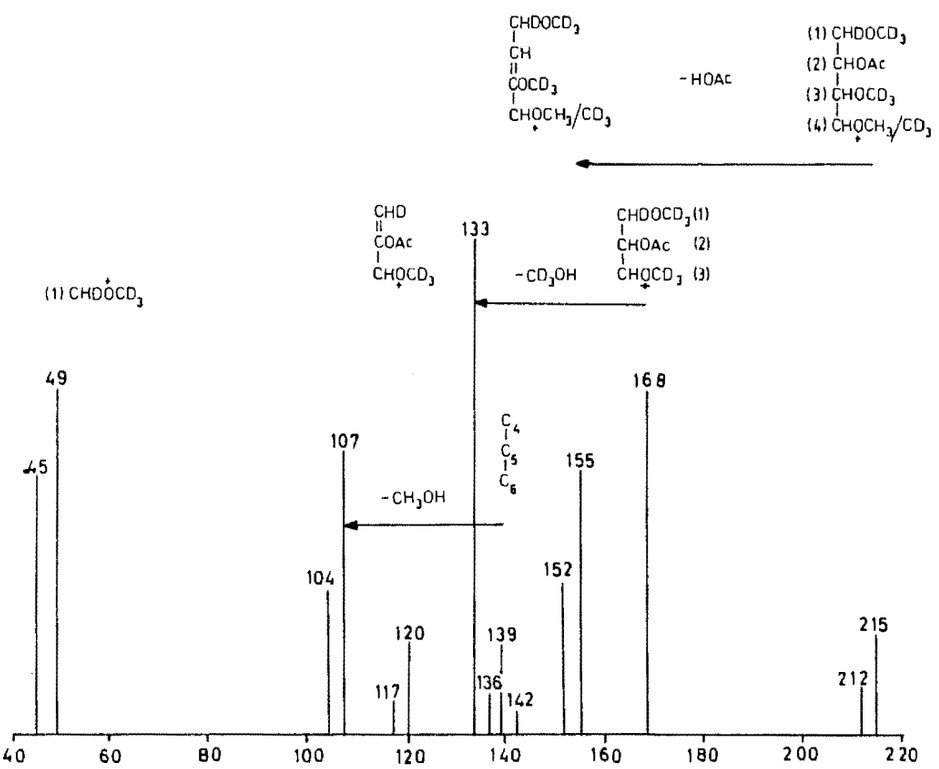


Figure 6.4.2 Mass spectrum ; 2-O-acetyl-penta-O-methyl mannitol.

By comparing the intensities of m/z 136 to 139 and 212 to 215 it was possible to estimate the ratio of OCH_3 to OCD_3 on C-4 and thus deduce the ratio of 4,6-Me_2Man to 6-MeMan originally in fraction 17. Similar analyses were carried out on the remaining four components. The results are summarised in Table 6.4.4.

Table 6.4.4 Methylation Analysis of RMR17:
Sites of Attachment and Ratios of CD_3 to CH_3

	CD_3 on	CH_3 on	Ratio CD_3 to CH_3	Deduction about source sugar
I[a]	1,3,4,5	4,6	C-4 2:1	6-Man[b] : 4,6-Man = 2:1
II	3,4	4,6	C-4 8:1	6-Man : 4,6-Man = 8:1
III	1,3,4,5	2		All from 2-GlcA
IV	3,4	2,3	C-3 2:1	2-GlcA : 2,3-GlcA = 2:1
V	3	2,3	C-3 1:2	2-GlcA : 2,3-GlcA = 1:2

a Numbers refer to components I to V in Table 6.4.3; b 6-O-methylmannose, etc.

As the 3-position is always deuteriomethylated in the p.m.a.a.'s of Man, it was concluded that the substituent sugars that had been on that position in the original gum were all released during partial hydrolysis of the methylated gum. Similarly, all GlcA had CH_3 on C-2 and was therefore never substituted in that position. Also all T-GlcA was

deuteriomethylated on C-4, showing that this had all been derived from 4-linked GlcA. This is summarised diagrammatically in Figure 6.4.3..

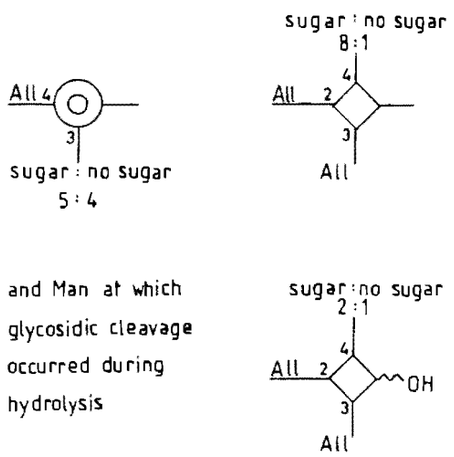


Figure 6.4.3 Diagrammatic summary of the sugar linkages in the original gum.

6.5 EXPERIMENTAL

6.5.1 Partial Hydrolysis Studies

To obtain E.long."A", E.long. (16g) was hydrolysed at pH 2 in 0,005$\underline{M}$ H_2SO_4 (500cm^3) on a boiling water bath for 13h. The solution was neutralised ($BaCO_3$) and centrifuged. The centrifugate was concentrated and poured into EtOH (4 vols.).

The precipitate was washed with EtOH, then with Me_2CO and dried in air (11,9g).

The partial hydrolyses to obtain B1, B2, and B3 were all carried out in 0,25$\underline{\underline{M}}$ H_2SO_4 on a boiling water bath for the times specified in 6.3. The hydrolysates were neutralised with $BaCO_3$ and centrifuged, and the centrifugates were dialysed (Spectrapor dialysis tubing, molecular weight cut-off 3500) against deionised water until the dialysate tested negative with Molisch's reagent. The dialysates were evaporated to dryness on a rotary evaporator and the non-dialysable fractions were freeze-dried. Molecular weight distributions were determined on Bio-Gel P-10. Specific rotations of the non-dialysable fractions were determined in H_2O. ^1H-N.m.r. spectra of B3 in D_2O were obtained at 25° and 80°. All p.c. was carried out in solvents a and d to identify the acidic components and in solvent b to identify neutral components, with authentic specimens as markers.

Methylations were carried out using the modified Hakomori method as described in Chapter 2, followed by three Purdie methylations. Typically 30mg of the non-dialysable fraction was decationised (Amberlite IR-120 (H^+)), freeze-dried, and then dried at 35° under vacuum. The dried polymer was dissolved in dry DMSO (1,5 cm^3) and K^+dimsyl (0,5cm^3 3$\underline{\underline{M}}$) was added. This was stirred for 10 minutes and tested for the presence of excess base with Ph_3CH. After cooling in ice/water, MeI (1,2

cm^3) was added and stirring was continued for 4h. After work up, the Hakomori-methylated product was dried under high vacuum to remove residual DMSO, and then subjected to three successive Purdie methylations. Thereafter the methylated product was purified by passage through Merckogel 2000, eluted with $CHCl_3$-EtOH (1:2). The methyl carboxylate groups were reduced with LAD as described in Chapter 2.

During purification on Merckogel 2000, B3 was separated into two fractions, fr.1 (24mg) eluting first and therefore having the higher molecular weight components, and fr.2 (11mg). These were analysed by methanolysis (2,4% methanolic HCl for 18h at 100° in a sealed tube) and g.l.c. as there was insufficient fr.2 for methylation analysis of the LAD-reduced product. Proportions of acidic components were determined by saponifying the methanolysate prior to reinjection. The saponification was carried out by adding 15µl 2$\underline{\underline{M}}$ NaOMe to 15µl methanolysate and allowing it to stand for 24h. This was neutralised to pH 8 with solid CO_2. Results are given in Table 6.5.1.

Portions of fractions 1 and 2 were submitted to f.a.b.-m.s..

Base catalysed β-eliminations using both K^+dimsyl and DBU were carried out as described in Chapter 2.

Table 6.5.1 Methanolysis Results of E.longifolius"A"B3

Sugar	t_R	Fr 1[a]	Fr 2[a]
T-GlcA	1,35; 1,81	2,1	1,1
→2)-Man	1,81	3,6	1,9
→4)-GlcA	5,57; 6,06; 7,68	2,9	1,0
→2,4)-Man	7,68	1,4	0,3
T-Man	0,77	-	0,3
T-Gal	1,00	-	tr
→6)-Gal	4,55; 4,85	-	0,3

a Molar ratio

6.5.2 The Dialysates

In order to isolate the neutral disaccharides and the trisaccharide having R_{gal} 0,33 in solvent a, the dialysate DB1 (6,43g) was extracted with EtOH to yield predominantly neutral components as EtOH-solubles (1,09g). P.c. (solvents a and b) showed two major disaccharides, one having mobility of

Galβ-3Gal and the other having mobility just slower than Galα-3Ara, as well as the monosaccharides listed in Table 6.3.1. The EtOH-insoluble fraction was dissolved in water and freeze-dried (4,35g). The acidic oligosaccharides as seen on p.c. (solvent a) are listed in Table 6.3.7.

Fractionation of EtOH-soluble (neutral) components: EtOH-soluble fraction (1,08g) was applied to a charcoal-Celite (1:1) column (17cm x 2cm) and eluted with water to remove monosaccharides, until Molisch's test proved negative, then with increasing concentration of EtOH, column progress being monitored with Molisch's reagent and individual Molisch-positive fractions by p.c. (solvent b). Disaccharides were eluted with 5% EtOH. Four fractions were obtained after the subfractions had been monitored by p.c. (solvent b) and those with similar components combined. Composite fractions were analysed by p.c..

Fraction 16-20, 3-O-methyl-L-rhamnose (262mg) was eluted with 3% EtOH, crystallised spontaneously, m.p. 110°-114°, and had $[\alpha]_D$ +34° (c = 3,72; H_2O). ^1H, ^{13}C, COSY and HETCOR n.m.r. experiments were done on a 0,25M solution in D_2O. Results are listed in Table 6.3.10.

Fraction 24-26 (17mg) comprised Galβ-6Gal (++) and Galβ-3Ara (+).

Fraction 27-29 (33mg) had $[\alpha]_D$ +42° (c = 1,5; H_2O). A portion was hydrolysed, showing Gal and Ara (solvents a and b) and g.l.c. of the derived a.a.'s gave molar ratio of Gal to Ara as 1,00:1,06. A further portion was reduced ($NaBH_4$) and

hydrolysed, showing only Gal on spraying with a. The substance was further characterised by running ^{1}H, ^{13}C, APT, COSY, and HETCOR n.m.r. experiments on a 0,17M solution in D_2O (Table 6.3.11). The J-resolved ^{1}H experiment provided additional information on the chemical shifts of the proton resonances.

Fraction 30-31 (7mg) comprised Galβ-3Ara (+) and Galβ-3Gal (++).

Fraction 32-34 (17mg) had $[\alpha]_D$ +30° (c = 1,0; H_2O) and was chromatographically identical with an authentic specimen of Galβ-3Gal (solvents a and b).

Fractionation of Acidic Components: The acidic components (3,8g) as Ba^{++} salts were transferred to a cellulose column (7 cm x 120 cm), eluted with BuOH-HOAc-H_2O (3:1:1), with gradual decrease in the proportion of BuOH in the eluent. The column performance was monitored by p.c. (solvent a) of concentrated aliquots from each fraction. Those having similar components were combined.(See Table 6.3.14) Fractions 43-57, 58-73, and 76-85 were identified by p.c. in solvents a and d against authentic specimens where possible (a specimen of 6-O-(4-O-methyl-β-D-glucopyranosyluronic acid)-D-galactose was not available but R_{gal} values agreed with those obtained earlier[37]). Those fractions eluting after 86-92 were hydrolysed (8h M TFA, 100°) and chromatographed (p.c. in solvents a and b).

Fraction 86-92 was hydrolysed as described in 6.3.2 (ii) with and without reduction ($NaBH_4$) and subjected to p.c. (solvents a and b). The success of the reduction was checked by p.c. using sprays a (negative) and b (positive). As p.c. after reduction and hydrolysis showed some Ara, the reduction was repeated but the hydrolysate still showed some Ara. Prior to methylation analysis, the remainder (12mg) of Fr.86-92 was reduced ($NaBH_4$, pH9, 72h, 25°), decationised (Amberlite IR 120 (H^+)) and freeze-dried. Thereafter R86-92 was methylated by the modified[65] Hakomori procedure with contact with base (0,3 cm^3 K^+dimsyl) for 20 mins. The methylated, reduced 86-92 (MR86-92) was subjected to Purdie methylation conditions for two 4d periods. The product was reduced[68] (LAD) to give RMR86-92, which was analysed by g.l.c. and g.l.c.-m.s. (Table 6.3.16).

6.5.3 The Methylation Study

Methylated E.long (ca 3,8g) that had had Haworth, Kuhn and Purdie methylations was subjected to three further Purdie[66] methylation treatments. A portion (21mg) was hydrolysed in HCOOH then 0,5M H_2SO_4 and analysed by p.c. (solvent c) for future reference. The bulk was then solubilised in hot HCOOH (±2h), water was added, and most acid removed in vacuo. The syrup was hydrolysed with 0,5M H_2SO_4, neutralised with $BaCO_3$,

separated on a cellulose column, at 30° using a gradient of petroleum ether (b.p. 120°) and butan-1-ol saturated with water; 7:3 to 1:3. Solvent was then half-saturated butan-1-ol, then EtOH-BuOH, and finally EtOH-H_2O. Fractions (2-hourly) were monitored by evaporation and p.c. (solvent c) and combined as appropriate. 17 such fractions were collected over a 23 day period. Identification of individual components is described briefly in Table 6.5.2. G.l.c. as methyl glycosides (column b) and as a.a.'s (column a) for quantifying, was used to ascertain proportions of the sugars present in each fraction. G.l.c.-m.s. (column c) was used as an aid to identifying the sugars. P.c. to identify sugars was performed in three solvent systems, c, f, and g. Only additional detail is given below. Identities and ratios of sugars in each fraction is best seen in the histogram (Figure 6.4.1)

Table 6.5.2 Fractionation of Partially Hydrolysed Methylated E.longifolius Gum: Identification of Cellulose Column Fractions.

(a) Neutral Fractions

Fraction	Mass(mg)	Identification	Methods Employed
1	600	234 Rha (8); 235 Ara (14); 234 Xyl (7); 2346 Gal (2).	a, b, c, d.
2	100	234 Xyl (1); Me_2Rha (2)*.	a, b, c.
3	250	234 Xyl (tr); 234 Ara (1); Me_2Rha** (3).	a, b, c.
4	125	246 Gal	a, b, c, f.
5	260	234 Gal	a, b, c, e, f.
6	150	24 Ara (3); 46 Man (1).	a, b, c, d, g.
7	60	3 Rha***	a, b, c, d, g.
8	20	****	a, c.
9	300	24 Gal	a, b, c, e, f.
10	130	6 Man	a, b, c, f.
11	140	2 Gal*****	a, b, c.
12	60	4 Gal	a, b, c.

Methods employed:
a p.c. (solvents c,f,g); b p.m.a.a.'s, g.l.c.-m.s.; c methyl glycosides, g.l.c.; d de-O-methylation[93], p.c. and a.a.'s of parent sugars; e crystallisation on seeding; f n.m.r.; g m.s. (probe); h hydrolysis; p.c. and derivatisation (a.a. and Me glycosides) and g.l.c.

* Traces 234 Fuc, 235 Ara, 346 Man, 34 Rha, 24 Rha.
** Mainly 23 Rha, traces 24 Rha,34 Rha, 346 Man,236 Gal, 23 Xyl.
*** Trace Me_2Man
**** Mixture of at least 4 components, including those in fraction 7, 23 GlcA, 23 Gal.
***** Traces 6 Man, Me GlcA.

Table 6.5.2(b) The Acidic Components

Fraction	Mass(mg as as Basalt)	Identification (methods a, b, c) of hydrolysates See Table 6.5.2 (a)
13	32	23 GlcA (++); 6 Man (++); 234 GlcA (+); 4 Gal (+); 234 Gal (+).
14	92	23 GlcA (++); 234 GlcA (++++); 46 Man (+); 234 Gal (+).
15	490	234 GlcA (++++++++); 23 GlcA (+); 234 Gal (tr); 24 Gal (tr).
16	128	23 GlcA (+++); 234 Gal (+); 24 Gal (+).
17	100	23 GlcA (+); 2 GlcA (+); 6 Man (+); 46 Man (+).

Fraction 17: (100mg as Ba salt) P.c., and g.l.c. of a.a.'s showed predominantly 6-Me Man with 4,6-Me_2Man, 3,6-Me Man and 3,4,6-Me_3Man (7:1:tr:tr). G.l.c. of methyl glycosides gave the ratio of 2,3,4-Me_3GlcA to 2,3-Me_2GlcA as 1:1,2. Fraction 17 was decationised (Amberlite IR-120(H^+) in MeOH-H_2O (1:1)) and freeze-dried (65mg). Decationised 17 was dissolved in a minimum of water, neutralised with $NaHCO_3$ and $NaBD_4$ was added to pH ca. 9. This was left overnight. After acidification with HOAc, borate was removed by distillation with MeOH. The residue was dissolved in water, decationised and freeze-dried (56mg). This

was insoluble in MeI, but soluble in DMSO, and was therfore methylated by the modified[65] Hakomori method. 27mg of decationised R17 was methylated as previously described with a contact time with base of 20 minutes and using CD_3I as methylating agent. The yield of MR17 after work-up was 28mg. 10mg of MR17 was LAD reduced to RMR17 in the normal way[68]. Both MR17 and RMR17 were analysed by hydrolysis (2M TFA, 8h, 100° and M TFA, 16h, 100°, respectively) and p.c., and g.l.c. and g.l.c.-m.s. as p.m.a.a.'s (Table 6.4.3). Mass spectra were used to determine the approximate ratios of sugar linkages in the original gum and in the gum after partial hydrolysis, prior to separation on the column. The following definitive ions were used.

For 3,4,6-Me_3Man: m/z 192 (22%) - 132 (100%) indicates CD_3 on C-3; 161 (3%) indicates CH_3 on C-4 and C-6; 164 (32%) indicates CD_3 on C-4 or C-6; 167 (4%) indicates CD_3 on C-4 and C-6; 45 (17%) indicates CH_3 on C-6; 48 (7%) indicates CD_3 on C-6.

For 2,3,4-Me_3Glc: m/z 194 (10%) - 134 (35%) indicates 2-D on C-6 and CD_3 on C-4; 161/164 (6%/14%) - 101/104 (24%/72%) indicates CH_3 to CD_3 on C-3 is 1:2,5; 117 (48%) indicates C-2 carries CH_3.

For 2,3-Me_2Glc: m/z 263/266 (6%/3%) indicates 2-D on C-6 and 1 in 2 CD_3 on C-3 [-60 (HOAc)] 203/206 (4%/2%) [-42 (CH_2CO)] 161/164 (6%/3%) [-60 (HOAc)] 101/104 (18%/10%). All the ratios

were consistent. 117 (69%) was prominent and consistent with CH_3 on C-2.

For 1,3,4,5,6-Me_5Man: m/z 215/212 (20%/9%) [-60 (HOAc)] 155/152 (53%/31%) indicates CD_3 on C-3 and CH_3 to CD_3 on C-4 is 1:2; 168 (69%) [-35 CD_3OH] 133(100%) indicates $CHDOCD_3$ at C-1 and CD_3 on C-3; 136/139/142 (8%/18%/5%) [-32 CH_3OH] 104/107/no110 (30%/57%) indicates CH_3/CD_3 on C-4 is 1:2 (There is CD_3 on C-5.); 49 (69%) indicates $CHDOCD_3$ at C-1; 45 (52%) indicates CH_3 on C-6; 48 (56%) is inconsistent with results indicating C-6 carries mostly CH_3.

For 1,2,3,4,5-Me_5Glc: m/z 122 (80%) 2-D on C-6 and CD_3 on C-5; 169 (20%) [-60 HOAc] 109 (65%) 2-D on C-6, CD_3 on C-4 and C-5; 187-32 = 155 (25%), 140 (14%), 93 (55%), 49 (35%) indicate $CHDOCD_3$ at C-1, CH_3 on C-2, CD_3 on C-3 and C-4.

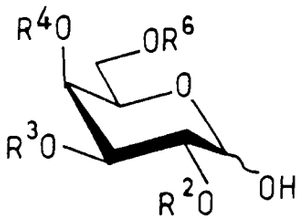
7. N.M.R. SPECTROSCOPY OF METHYL ETHERS OF D-GALACTOPYRANOSE

7.1 RESULTS AND DISCUSSION

Only incomplete n.m.r. data for methyl ethers of D-galactose are available in the literature. In earlier work, ^{1}H-n.m.r. spectroscopy of a range of methyl ethers of D-galactopyranose were studied with particular reference to the methoxyl protons[51-53]. The chemical shifts of the resonances of these protons were found to depend on the anomeric configuration and the presence and orientation of vicinal OH or OMe groups[94]. In addition, the chemical shifts of the MeO-2 and MeO-4 signals were found to be independent of their orientation, whether axial or equatorial[94].

Analysis of the ^{13}C-n.m.r. chemical shift data for mono-, di-, and oligo-saccharides allowed the postulation[54] of empirical rules that related changes in the chemical shifts of ring carbon atoms with substitution. Earlier it was proposed[95] that, for hexapyranoses, the presence of an OMe group causes an upfield shift of *ca.* 4,5 p.p.m. in the resonances of β-carbon atoms with axial OH groups. A similar effect was reported for inositols[96]. However, Usui *et al.*[88] found a 2,6 p.p.m. shift for the C-1α signal of 2-O-methyl-α-D-glucopyranose when compared with α-D-glucopyranose.

Since authentic specimens of a range of D-galactose methyl ethers, all having methoxyl on C-2, have become available, complete ^{1}H- and ^{13}C-n.m.r. spectral assignments have now been made for solutions in D_2O of α- and β-D-galactopyranose (1) and its 2- (2),2,3-di- (3), 2,4-di- (4), 2,3,4-tri- (5), 2,4,6-tri- (6), and 2,3,4,6-tetra-O-methyl (7) derivatives. Thus, the effect of methoxylation of an equatorial OH on the chemical shifts of the resonances of the neighbouring carbon carrying axial OH could be verified as the α-anomers have the required configuration between C-1 and C-2. Methoxylation should have a similar effect on the resonances of C-4, which also carry an axial OH.



1 R^2, R^3, R^4, R^6 = H
2 R^2 = Me, R^3, R^4, R^6 = H
3 R^2, R^3 = Me, R^4, R^6 = H
4 R^2, R^4 = Me, R^3, R^6 = H
5 R^2, R^3, R^4 = Me, R^6 = H
6 R^2, R^4, R^6 = Me, R^3 = H
7 R^2, R^3, R^4, R^6 = Me

The method for assigning chemical shifts for ^{1}H and ^{13}C resonances is illustrated using the spectra for compound 5. As shown in Figure 7.1.1, signals for anomeric protons in the ^{1}H-n.m.r. spectra were assigned readily and formed the basis

for assigning the H-2 signals *via* the COSY experiment. The correlation between H-1α and H-2α is shown in Figure 7.1.2. As illustrated in Figure 7.1.3, the APT experiment allowed assignment of the resonance of C-6, the methylene carbon. Literature values for the signals due to the ring carbon atoms and the empirical rules of Bradbury and Jenkins[54] were used as a guide in the assignment of the ^{13}C resonances. The ^{1}H assignments then followed *via* the HETCOR experiment. The ^{1}H chemical shifts of the OMe resonances determined by Rathbone *et al.*[51-53] were employed to assign those of the methoxyl carbons using the HETCOR experiments. Figure 7.1.4 shows the 2D HETCOR spectrum of compound 5, on which some correlations are indicated. Signal intensities were of little help, as the percentage at equilibrium of the less abundant α anomer of D-Gal is increased by O-methylation[97]. The ^{1}H and ^{13}C assignments are listed in Tables 7.1.1 to 7.1.4.

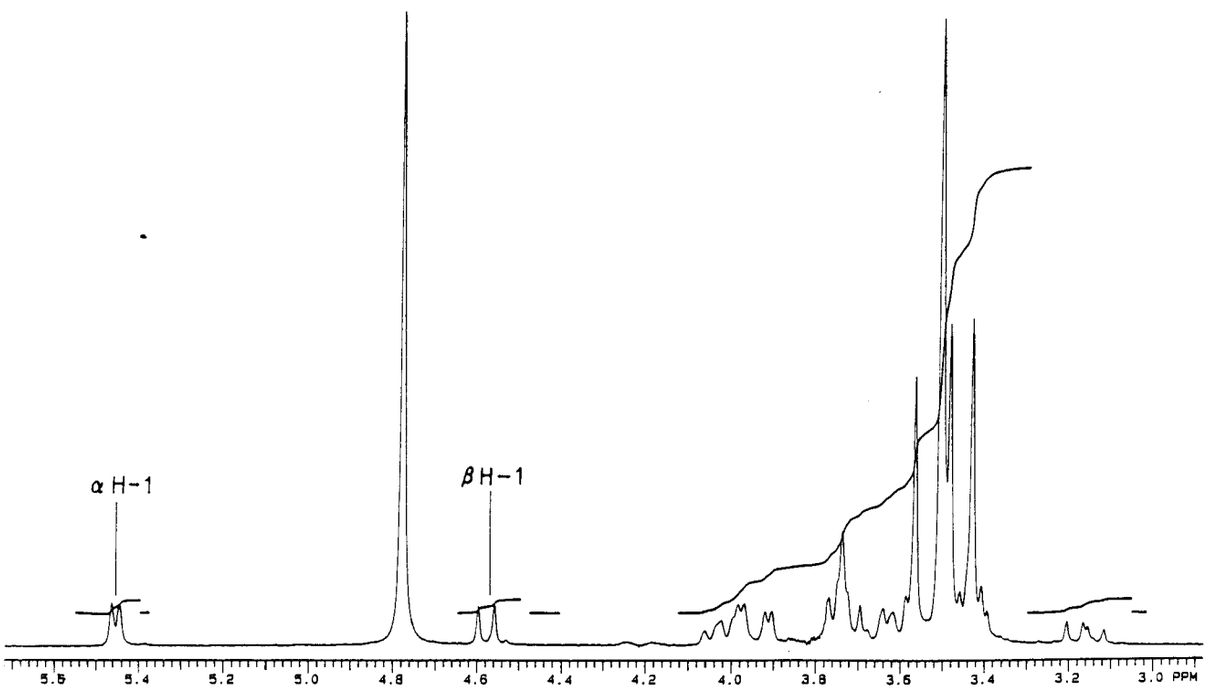


Figure 7.1.1 ^{1}H-n.m.r. spectrum for compound 5, showing assignment of the anomeric protons.

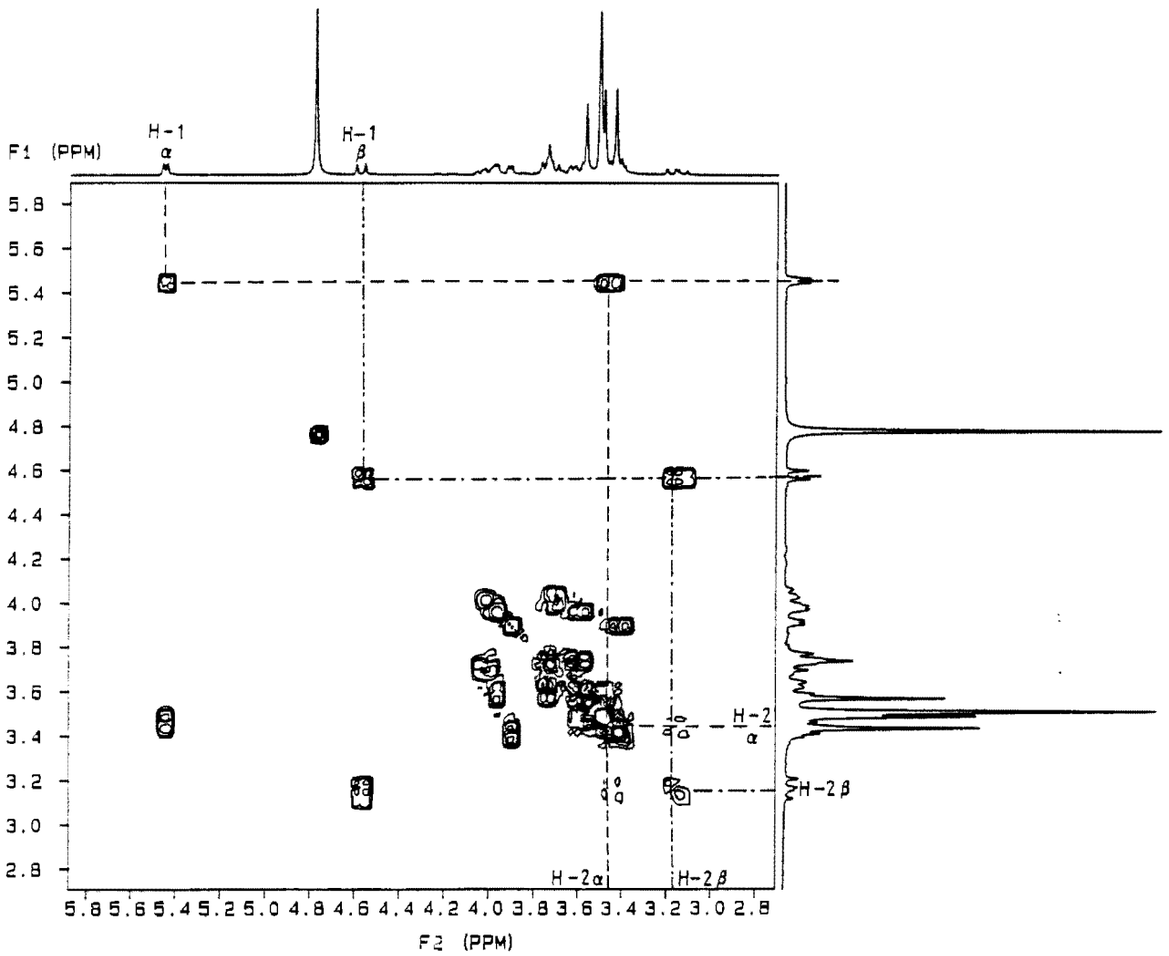


Figure 7.1.2 COSY Spectrum for Compound 5, Showing $H_{1,2}$ Correlations.

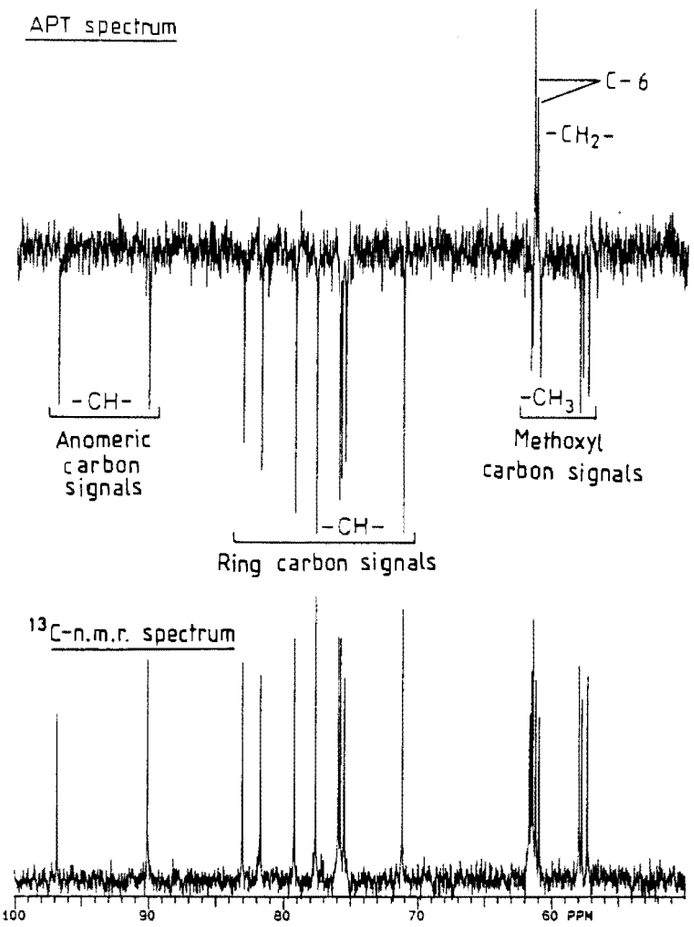


Figure 7.1.3 ^{13}C-n.m.r. and APT spectra for compound 5

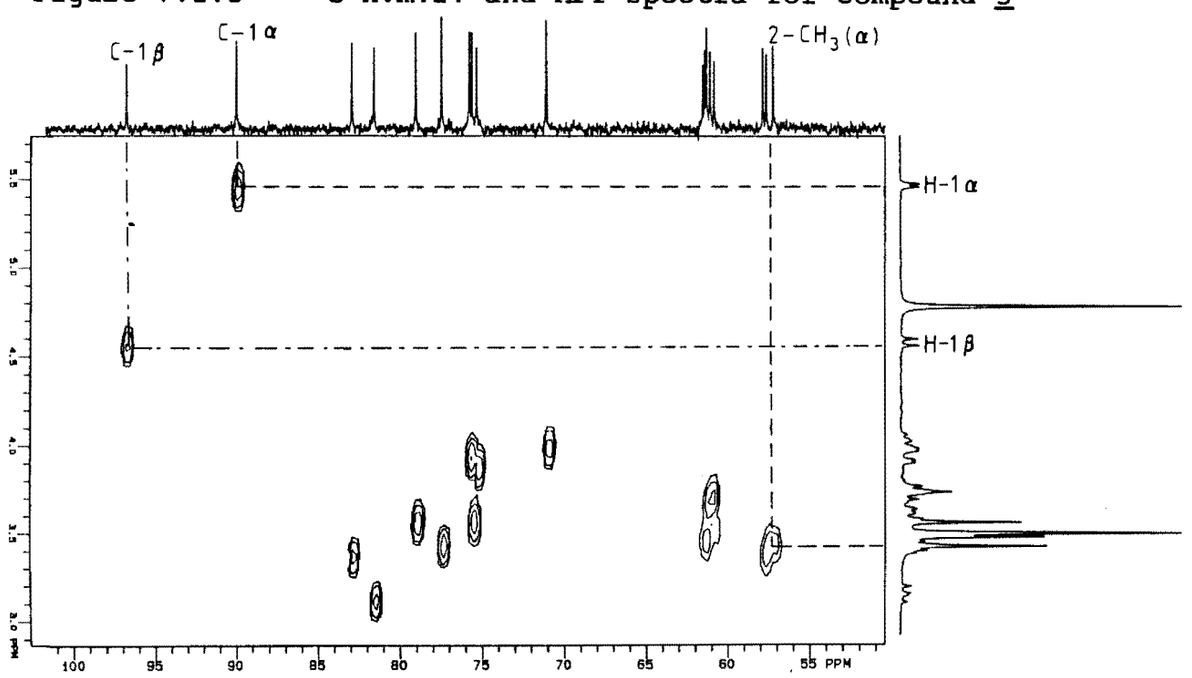


Figure 7.1.4 HETCOR spectrum for compound 5, some of the C,H correlations are shown

Table 7.1.1 ^{1}H assignments (δ, p.p.m.) for α-anomers of compounds 1 to 7

Compound	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*MeO-2*	*MeO-3*	*MeO-4*	*MeO-6*	$J_{1,2}$
1	5.24	3.78	3.81	3.95	4.06	3.72					3.26
2	5.48	3.51	3.91	3.96	4.05	3.72	3.45				3.84
3	5.48	3.52	3.53	4.25	4.03	3.72	3.43	3.40			2.41
4	5.37	3.34	3.83	3.59	3.96	3.65	3.38		3.44		3.30
5	5.45	3.45	3.58	3.96	4.01	3.73	3.43	3.48	3.50		3.65
6	5.43	3.42	3.92	3.65	4.19	3.64	3.45		3.50	3.38	3.66
7	5.43	3.45	3.58	3.92	4.15	3.62	3.43	3.48	3.49	3.39	3.59

Table 7.1.2 ^{1}H assignments (δ, p.p.m.) for β-anomers of compounds 1 to 7

Compound	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*MeO-2*	*MeO-3*	*MeO-4*	*MeO-6*	$J_{1,2}$
1	4.56	3.46	3.62	3.90	3.68	3.72					7.71
2	4.60	3.19	3.66	3.84	3.63	3.70	3.59				7.92
3	4.61	3.21	3.34	4.18	3.62	3.76	3.57	3.42			7.73
4	4.49	3.04	3.64	3.54	3.56	3.65	3.51		3.44		7.83
5	4.58	3.14	3.40	3.91	3.56	3.73	3.57	3.50	3.50		7.82
6	4.55	3.12	3.71	3.61	3.78	3.64	3.58		3.50	3.38	7.84
7	4.57	3.13	3.39	3.86	3.72	3.62	3.56	3.49	3.49	3.38	7.75

Table 7.1.3 ^{13}C assignments (δ, p.p.m.) for α-anomers of compounds 1 to 7

Compound	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*MeO-2*	*MeO-3*	*MeO-4*	*MeO-6*
1	93.03	69.10	69.92	70.06	71.22	61.93				
2	90.30	78.36	69.50	69.98	71.01	61.65[a]	58.15			
3	90.15	77.31	78.48	65.51	70.93	62.00	57.87	56.46		
4	90.23	78.64	69.62	80.43	71.15	61.32	58.24		62.01	
5	90.11	77.64	79.21	75.93	71.18	61.43	57.96	57.33	61.56	
6	90.33	78.61	69.55	80.76	69.18	71.99	58.31		62.07	59.01
7	90.15	77.56	79.13	76.26	69.12	72.03	57.98	57.41	61.58	59.01

[a] Or 61.90.

Table 7.1.4 ^{13}C assignments (δ, p.p.m.) for β-anomers of compounds 1 to 7

Compound	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*MeO-2*	*MeO-3*	*MeO-4*	*MeO-6*
1	97.20	72.62	73.55	69.50	75.89	61.73				
2	97.02	82.48	73.16	69.19	75.82	61.90[a]	61.00			
3	97.03	81.44	82.36	65.12	75.72	61.77	60.90	56.85		
4	96.88	82.70	73.54	79.86	75.82	61.09	61.05		62.09	
5	96.85	81.73	83.08	75.47	75.77	61.21	60.96	57.74	61.68	
6	96.89	82.66	73.48	80.19	73.88	71.76	61.13		62.15	59.15
7	96.82	81.63	83.00	75.78	73.71	71.80	60.95	57.81	61.69	59.15

[a] Or 61.65

In order to determine the effects of O-methylation on the chemical shifts of the skeletal carbon atoms and the ring hydrogens of D-Galp, change in chemical shift (relative to D-Galp) was calculated (Tables 7.1.5 to 7.1.8). These changes are shown graphically in Figures 7.1.5(a) and 7.1.5(b).

Table 7.1.5 Change in chemical shift for the α-anomer of compounds 2 to 7

	H-1	H-2	H-3	H-4	H-5	H-6
2	0,24	-0,27	0,10	0,01	-0,01	0,00
3	0,24	-0,26	-0,28	0,30	-0,03	0,00
4	0,13	-0,44	0,02	-0,36	-0,10	-0,07
5	0,21	-0,33	-0,23	0,01	-0,05	0,01
6	0,19	-0,36	0,11	-0,30	0,13	-0,08
7	0,19	-0,33	-0,23	-0,03	0,09	-0,10

Table 7.1.6 Change in chemical shift for the β-anomer of compounds 2 to 7

	H-1	H-2	H-3	H-4	H-5	H-6
2	0,04	-0,27	0,04	-0,06	-0,05	-0,02
3	0,05	-0,25	-0,28	0,28	-0,06	0,04
4	-0,07	-0,42	0,02	-0,36	-0,12	-0,07
5	0,02	-0,32	-0,22	0,01	-0,12	0,01
6	-0,01	-0,34	0,09	-0,29	0,10	-0,08
7	0,01	-0,33	-0,23	-0,04	0,04	-0,10

Table 7.1.7 Change in ^{13}C chemical shift for the α-anomers of compounds 2 to 7

	C-1	C-2	C-3	C-4	C-5	C-6
2	-2,73	9,26	-0,42	-0,08	-0,21	-0,28
3	-2,88	8,21	8,56	-4,55	-0,29	0,07
4	-2,80	9,54	-0,30	10,37	-0,07	-0,61
5	-2,92	8,54	9,29	5,87	-0,04	-0,50
6	-2,73	9,60	-0,37	10,70	-2,04	10,06
7	-2,88	8,46	9,21	6,20	-2,10	10,10

Table 7.1.7 Change in ^{13}C chemical shift for the β-anomers of compounds 2 to 7

	C-1	C-2	C-3	C-4	C-5	C-6
2	-0,18	9,86	-0,39	-0,31	-0,07	0,17
3	-0,17	8,82	8,81	-4,38	-0,17	0,04
4	-0,32	10,08	-0,01	10,36	-0,07	-0,64
5	-0,33	9,11	9,53	5,97	-0,12	-0,52
6	-0,31	10,04	0,07	10,69	-2,01	10,03
7	-0,39	9,01	9,45	6,28	-2,18	10,07

H-1α (but not for H-1β). The signal of H-6 is virtually unaffected by methylation of O-6, whereas the chemical shift of the H-5 resonance is unchanged, apart from minor variations when O-4 and O-6 are substituted.

These trends are mirrored in Figure 7.1.5(b) in which the change in chemical shift of the ^{13}C resonances are shown. O-Methylation results in an 8-10 p.p.m. downfield shift of the C-OMe resonance. For C-4 this downfield shift is opposed by a 4,5 p.p.m. upfield shift due to methylation of O-3 (cf. Voelter et al.[95]). O-Methylation at the 2-position results in an upfield shift of 2,7-2,9 p.p.m. of the C-1α signal but has no effect on that of C-1β. In contrast to the ^{1}H spectra, the ^{13}C spectra are sensitive to O-methylation at the 6-position, where a downfield shift of 10 p.p.m. is seen. There is minor shielding of C-5. The effects of O-methylation at C-2 upon the resonances of H-1 and C-1, and the effects of O-methylation at C-3 upon those of H-4 and C-4, are summarised in Figures 7.1.6 and 7.1.7 respectively. O-5 has an influence on the magnitude of these changes.

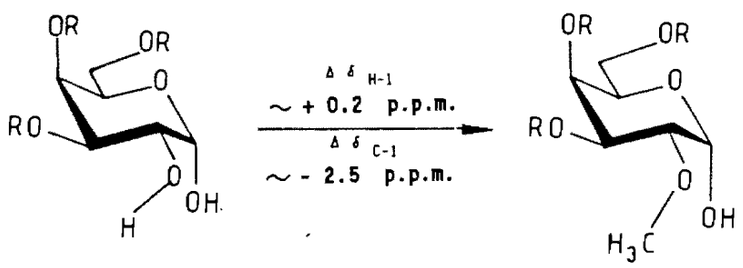


Figure 7.1.6 Effect of methylation of O-2 on chemical shifts of H-1 and C-1 of the α-anomer of D-Gal, R = H or Me; there are no changes for the β-anomer.

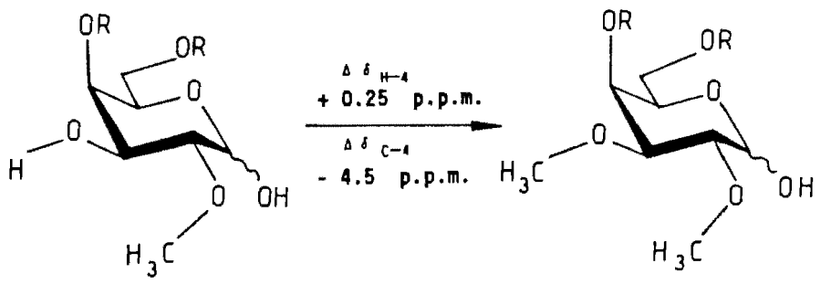


Figure 7.1.7 Effect of methylation of O-3 on the chemical shifts of the resonances of H-4 and C-4 of α- and β-D-Galp (R = H or Me).

The effects of O-methylation on the chemical shift of the α carbon resonance can be explained[98] by the changes in the paramagnetic and diamagnetic terms that give rise to the shielding tensor (σ). Cheney and Grant[99] showed that replacement of H by Me in saturated hydrocarbons leads to a contraction of the carbon 2p orbitals, which increases (0,7 p.p.m.) the diamagnetic (shielding) and decreases (10,6 p.p.m.) the paramagnetic (deshielding) terms of the chemical shift expression,

$$\sigma i = \sigma^{dia} + \sigma^{para} + \sigma^{N},$$

where σi is the magnetic shielding constant,

σ^{dia} is the diamagnetic term,

σ^{para} is the paramagnetic term, and

σ^{N} is the neighbour anisotropy term.

Similar results are seen for aliphatic alcohols, cf. $CH_3\underline{C}H_2OH$ (δ 57) with $CH_3\underline{C}H_2OCH_3$ (δ 67,9) and $\underline{C}H_3OH$ (δ 49) with $\underline{C}H_3OCH_3$ (δ 57,6). On the other hand, the upfield shift of 0,4 p.p.m. of the ^1H resonances upon O-methylation is due to the

neighbouring anisotropy effect[100,101], *cf.* $C\underline{H}_3OH$ (δ 3,4) and $C\underline{H}_3OCH_3$ (δ 3,2).

The protons in the γ-*gauche* (1,4) positions shield[102] the corresponding carbon atoms. With oxygen as part of the connecting sequence of atoms, the effect at C-4 of O-3 methylation is shown in Figure 7.1.7. Kotchetkov *et al.*[91] proposed that this effect stems from the spatial interaction of the protons attached to the carbon atoms that are in the 1,4-*gauche* conformation. According to Usui *et al.*[88] in the conformer shown (in Figure 7.1.8, looking along the O-2 - C-2 bond), hydrogen bonding between O-1 and O-2 is the cause of the enhanced shielding of C-1α on methylation of O-2. However, the rotamer displayed has the γ-*gauche* relationship , and this would lead to interaction between the 2-OMe protons and H-1. In compounds 3 hydrogen bonding between 4-OH and O-3 would lead to a satisfactory rotamer (Figure 7.1.9), but as O-4 methylation in compounds 5 and 7, in which there is no possibility for hydrogen bonding between O-3 and on OH on C-4, has no effect on the change in chemical shift for C-4 or for H-4 when O-3 is methylated, the explanation must be in the γ-*gauche* relationship.

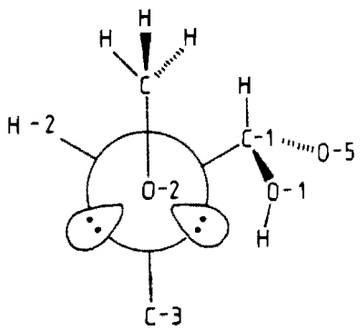


Figure 7.1.8 Newman projection along the O-2 - C-2 bond of the α-anomers of 2 to 7.

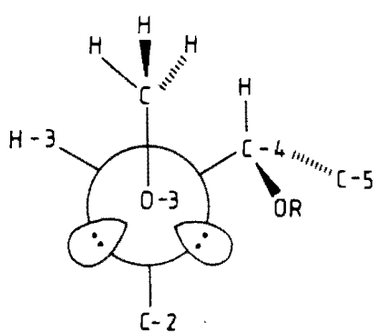


Figure 7.1.9 Newman projection along the O-3 - C-3 bond of 3 (R = H) or 5 and 7 (R = Me).

The signals for O<u>C</u>H$_3$ have been displayed (Figure 7.1.10) and fall into two distinct regions, namely upfield and downfield of 60 p.p.m.. The downfield signals reflect the lack of steric interactions with protons in the 1,4-<u>gauche</u> positions, whereas the signals that are upfield of 60 p.p.m. are due to those O<u>C</u>H$_3$ signals which could be affected by those interactions. The effect of methylation of O-3 on the resonance of C-4, was to

cause an upfield shift in the C-4 resonance. Thus the signals for MeO-3α and MeO-3β would be expected to be more shielded. This is seen clearly in Figure 7.1.10. Similarly the signal for MeO-2α would be expected to be upfield.

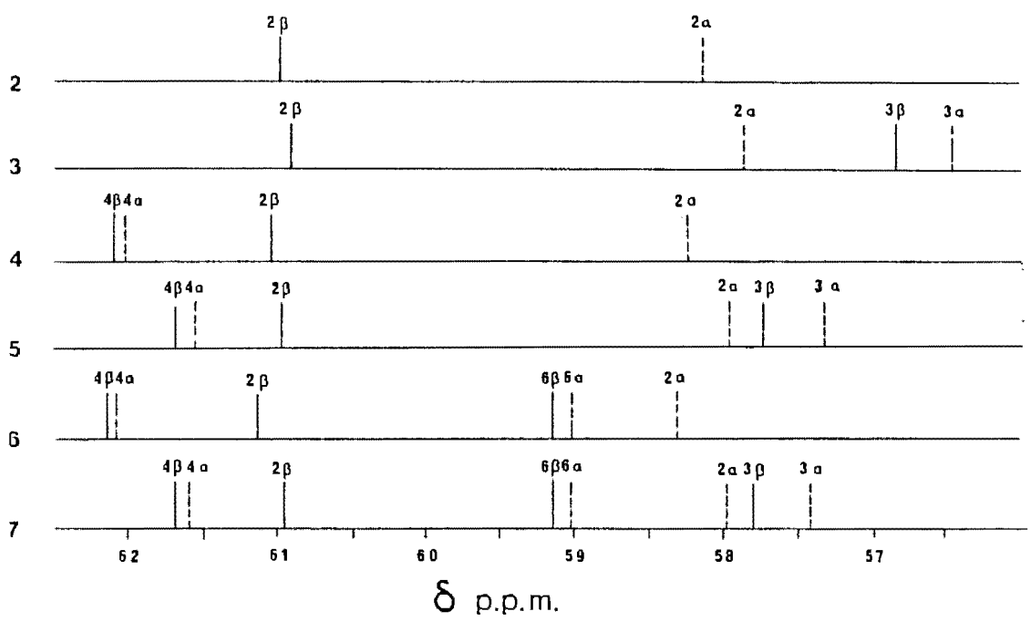


Figure 7.1.10 Chemical shifts of the methoxyl carbon atoms for 2 to 7

The crystal stuctures of α- and β-D-Gal[103] and 2,4-di-O-Me-D-Gal hydrate have been reported[104]. From the present study, it is apparent that 2,4-di-O-Me-D-Gal adopts different conformations in solution from that of the solid hydrate.

7.2 EXPERIMENTAL

Compounds 2 and 3 were isolated as previously described[24]; 4, 5, and 6 were collected as fractions 9, 5, and 4 from the cellulose column fractionation of the partial hydrolysate of methylated E. longifolius (Chapter 6, Section 6.4). To obtain compound 7 pure methyl-α-D-galactopyranoside was methylated (Hakomori, followed by two Purdie methylations), purified by passage through Merckogel 2000. This was shown to be pure by g.l.c. (column c), then the glycoside was hydrolysed (6h 2M TFA) and freeze-dried. Exchangeable protons were D-exchanged by freeze-drying samples, dissolved in D_2O, three times. Samples (11-32mg) were examined in D_2O solution (0,6ml) with Me_2CO as internal standard (^1H δ = 2,21 p.p.m., ^{13}C δ = 31,0 p.p.m.) at 25°.

8. CONCLUSION

8.1 INTRODUCTION

Compositions and structural features of exudate gums from Encephalartos species have been determined. Evidence of a glucuronomannoglycan core is apparent for the two species studied in detail, and there appears to be ca. 12%-17% branching of this core with the Man residue being the branch-point. No indication has been found for a linear chain of more than four -4Glcβ-2Manα- units linked together. Evidence for particular structural features as obtained from the various experiments is summarised below.

8.2 THE GENERAL SURVEY

The exudate gums all comprise the same range of monosaccharide components, the predominant sugars being 3-O-methyl-L-rhamnose, L-rhamnose, L-arabinose, D-mannose, D-galactose and D-glucuronic acid (including its 4-O-methyl ether), with lesser amounts of D-xylose and L-fucose. Ratios of the sugars vary from species to species, but the major constituents are always rhamnose, galactose and glucuronic acid, with arabinose and mannose occurring in consistently smaller amounts. Methylation analysis showed that galactose is predominantly 3,6-linked with some 3- and some 6- linked, arabinose is either →3)-Arap or

terminal (Araf and Arap), rhamnose is predominantly terminal but also exists in-chain, predominantly 4- linked. Mannose is always 2-linked but constitutes a branch point, mainly as →2,3,4)-Man, in the undegraded gum. Glucuronic acid is predominantly 4-linked, occurring as →3,4)-GlcA to a lesser extent.

8.3 PARTIAL HYDROLYSIS

In all cases, except when B3 products are hydrolysed, GlcAβ-6Gal is the major aldobiouronic acid produced on partial hydrolysis. During "autohydrolysis" of E.friderici-guilielmi gum, producing this aldobiouronic acid, most rhamnose and approximately half the arabinose are removed. From the rate of change of optical rotation with time as observed during the course of the hydrolysis, the polysaccharide appears to have a peripheral acid-labile region, followed by a zone of more stable glycosidic linkages, with another acid-labile region towards the interior of the polymer. This agrees with a structure comprising, from interior to exterior of the molecule, a glucuronomannoglycan core, chains of hexopyranose residues, aldobiouronic acid residues, and finally, acid-labile deoxy-hexoses and pentafuranose residues on the periphery.

During the course of the partial-hydrolysis experiments, Galβ-3Ara and GlcAβ-6Galβ-3Ara were isolated from the

dialysates and characterised, thus showing that the →3)-Ara is interior to Gal. In gum ghatti, 3-linked Ara was found[2] to be the link sugar between the galactan chains and the glucuronomannoglycan core. In the present work, it was shown to be interior to Gal, but at no stage could an oligosaccharide containing Man and Ara be isolated and characterised. Also, during the later stages of partial hydrolysis, Ara was removed before the last traces of Gal. From this it can be assumed that, although some galactan chains may be attached to the core through Ara, much galactan is attached directly to Man in the core. Unlike leiocarpan gum[6], in which Xyl is on O-6 of Man, there is no substituent in that position whereas O-4 of Man carries a substituent, as evidenced by the presence of 3,6-di-O-methyl mannose on methylation analysis of the partial hydrolysis products (see Tables 5.7.3 and 6.3.3) and the production of 6-O-methyl mannose in the gum (see Table 4.2.2). During the course of the hydrolyses on E.longifolius gum, 3-O-methyl-L-rhamnose survived with the higher molecular weight fraction (see Table 6.3.1); it is possible that this sugar could be on O-4 of Man. Alternatively, O-4 of Man could carry GlcAβ-2Man residues as proposed in the structure for B3 of both E.long."A" and E.f-q."A".

As well as Galβ-3Ara, Galβ-3Gal and Galβ-6Gal were isolated from the neutral fraction of the dialysate obtained on partial hydrolysis of E.long."A". The first two disaccharides (Galβ-3Ara and Galβ-3Gal) were also isolated after acetolysis of the carboxyl-reduced gum, which also yielded Glcβ-6Gal.

The "A"B3 products obtained from both E.friderici-guilielmi and E.longifolius gums yielded oligomers, up to the tetramer, of -4GlcAβ-2Manα- on further partial hydrolysis, and methylated "A"B3 of E.longifolius gave evidence of the existence of alternating GlcA and Man residues on f.a.b.-m.s..

8.4 SMITH DEGRADATION EXPERIMENTS

The purpose of these experiments was to isolate intact sections of the polymer containing periodate-invulnerable residues. From the preliminary work on the gum exudates, it was hoped to obtain a fragment containing a galactan chain linked through Ara to Man. However, the ratio of Ara to Man was always less than one and Ara could therefore not be the link from the galactan chain to Man in every case. From the methylation analysis results obtained for the "slow spot" isolated from a lower molecular weight Smith-degradation product (Table 5.5.2), it is apparent that when O-4 of Man is substituted, it is with a single periodate-invulnerable sugar unit e.g. 3-O-methyl--L-rhamnose, or a short chain of periodate-invulnerable residues, and that on the same Man the substituent on O-3 must also be a short chain of sugar residues or a single sugar residue. Terminal mannose and terminal rhamnose are all major products on methylation of the "slow spot". This is in agreement with the proposal that there could be 3-O-methyl--L-rhamnose or GlcAβ-2Man (see figures 5.5.5, 5.7.2, and 6.3.2)

on O-4 of Man in the core. In the methylation analysis results of the corresponding higher molecular weight Smith-degradation product, there was no terminal mannose, terminal rhamnose or →2,3,4)-Man, giving additional weight to the argument that Man (as GlcAβ-2Man-) and/or 3-O-methyl-L-rhamnose are the possible substituents on O-4 of Man in the core. In addition, when this occurs, there is likely to be a lower molecular weight periodate-invulnerable substituent or chain on O-3 of Man.

Terminal galactose is another major product of this methylation; this is easily accommodated by the possibility of there being short galactan chains interior to periodate-vulnerable GlcA (see figure 5.5.5).

8.5 BASE-CATALYSED β-ELIMINATION EXPERIMENTS

The base degradation experiments showed conclusively that GlcA is exterior to Man and Gal, linked to their 2- and 6- positions respectively. Both these sugars may be further substituted, at O-3 and O-4 in Man and at O-3 in Gal. From degradation of newly released sugars in the dimsyl-catalysed experiments, it is apparent that both Rha and Man are exterior to GlcA. Once the polymers had been degraded to such lower molecular weight products as "A"B3, it was hoped to degrade the polymer with a non-nucleophilic base, with concomitant protection of the newly released sugar to show conclusively whether Man was both

interior and exterior to GlcA or not. However no mannitol derivative was observed. It cannot be concluded from these experiments that Man is not exterior to GlcA, on two seperate grounds: firstly dimeric -4GlcAβ-2Manα- was isolated in earlier work and a range of oligomers having the mobility of standard oligomers of -4GlcAβ-2Manα- in two solvent systems was observed after partial hydrolysis of the B3 products, and, secondly, undegraded acid which would falsify the conclusion that neutral sugars occupied this position was observed in the dimsyl base degradation experiments and identified by g.l.c.-m.s..

8.6 A PROPOSED STRUCTURE FOR Encephalartos GUM EXUDATE

The structure below is a composite of the structural features of the Encephalartos gums studied, showing the methods employed to determine each feature.

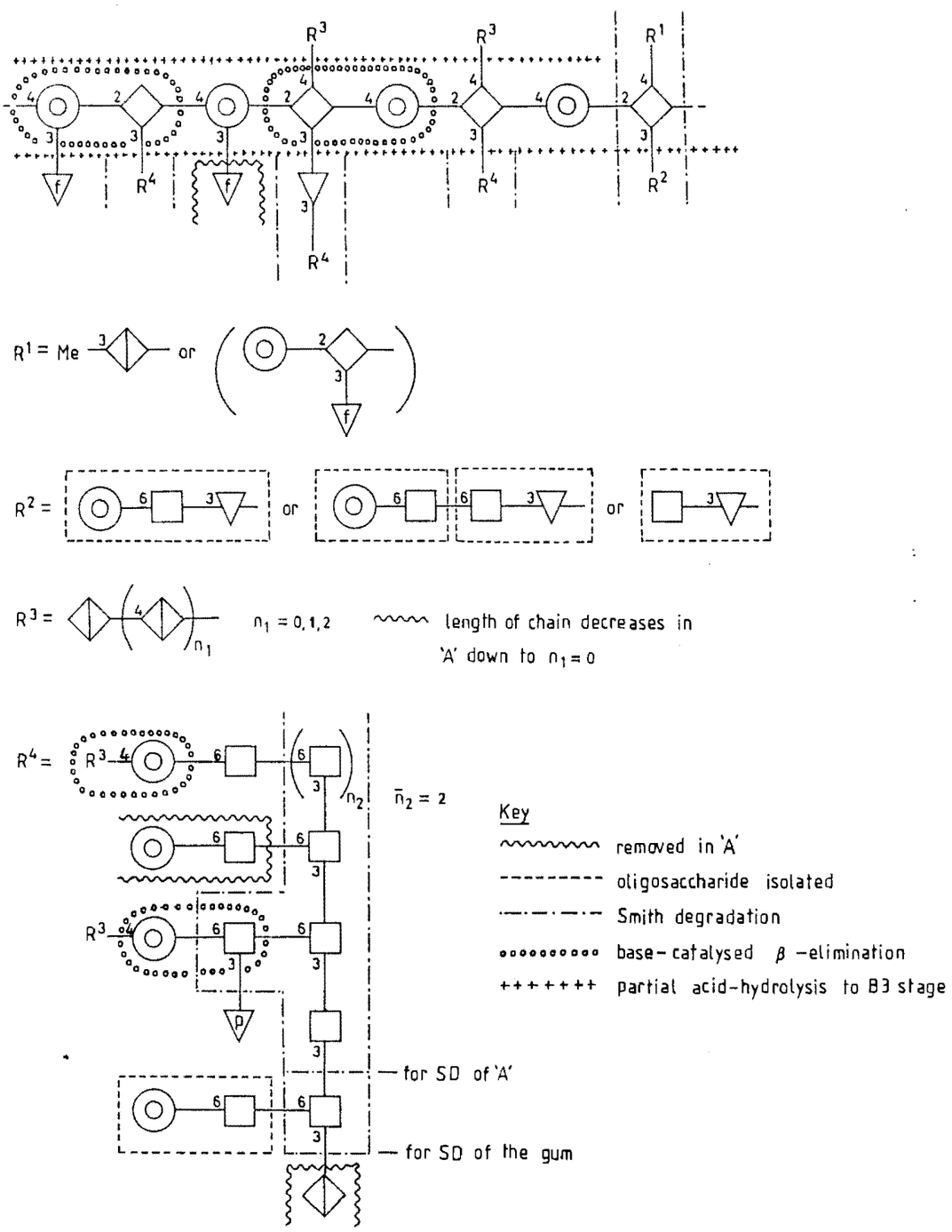


Figure 8.6.1 A proposed structure for <u>Encephalartos</u> gum exudate.

8.7 N.M.R. STUDIES

A range of methyl ethers of Gal all having a 2-O-methyl substituent has been studied and complete ^{1}H- and ^{13}C-n.m.r. spectral assignments have been made. In this range of methyl ethers, there are two situations in which an axial and equatorial OH are vicinal to each other, namely at C-3 and C-4, and at C-1 and C-2 for the α- anomer. This leads to a γ-gauche interaction between the protons of the methyl group and the proton attached to the ring carbon carrying the axial O, when the equatorial OH on C-3 and C-2 is methylated. This interaction causes an upfield shift of the ^{13}C resonance of the ring carbon carrying the axial O and a downfield shift of the ^{1}H resonance of the proton attached to it. This is in addition to the normal shifts observed on O-methylation.

Complete ^{1}H- and ^{13}C- assignments were made for 3-O-methyl--L-rhamnose, which was isolated from the dialysate during the partial acid hydrolysis of E.longifolius gum and also from the hydrolysis of methylated E.longifolius gum. This methyl ether has 2-OH axial and O-3, which is equatorial, is methylated. This leads to a γ-gauche interaction of the methoxyl protons and the proton on C-2. All observed chemical shifts were in agreement with this.

The disaccharide Galβ-3Ara was characterised fully by n.m.r. spectroscopy. The position of glycosylation was determined easily as C-3 of the Ara moiety experienced a ca. 10 p.p.m.

downfield shift. The fact that there was no upfield shift in the ^{13}C resonance of C-4, which carries axial OH, showed clearly that there was no interaction between the Gal moiety and the proton on C-4 of Ara. C-2 of Ara experienced an upfield shift which could be explained by H-bonding between HO on C-2 and the ring oxygen of the Gal moiety. This led to a proposed conformation for Galβ-3Ara and a comparison with the proposed structure for Galα-3Ara[89].

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