MONTANA STATE UNIVERSITY

Carbohydrate chemistry : synthetic and structural investigation of the phytotoxins found in Helminthosporium sacchari, and Rhynochosporium secalis
by Ross Carlton Beier

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Chemistry
Montana State University
© Copyright by Ross Carlton Beier (1980)

Abstract:
This thesis is comprised of four sections. In section 1 synthetic carbohydrate chemistry was extensively used during an attempt to verify a previously proposed structure for helminthosporoside, 11 Sec. 1 a host-specific toxin isolated from Helminthosporium sacchari. This previously proposed structure was determined not to be the actual structure of the toxin. Additional procedures were developed for the isolation of the toxin. PMR, CMR, and mass spectral analyses were obtained on the isolated toxin. The toxin appears to be a disaccharide with a large aliphatic aglycone.

During structural verification of a previously suggested structure for rhynchosporoside, 2 Sec. 2 as discussed in section 2, synthetic carbohydrate chemistry was extensively utilized. The previously proposed structure was determined not to be the actual structure of the toxin. Rhynchosporoside is a phytotoxin found in the fungal extracts of Rhynchosporium secalis. It is suggested that a number of phytotoxins may exist, i.e., a glucoide, cellobioside, and a cellotrioside, all having a glycosidic linkage to carbon-1 of 1,2-propanediol.

A 13C-NMR method was developed in section 3 for the identification of the carbohydrate residue in galacto- and glycopyranosides, and furanosides, with the capability of distinguishing the structural configuration of the carbohydrate residue. This method works for all reported CMR spectra of non-aromatic galactosides and glucosides, and for synthetic compounds reported in this paper.

The designs for a number of previously non-available laboratory apparatus are presented and discussed in section 4. A method for building and operating a 295.5 cm heated chromatography column is described. The separation of this instrument appears to be superior to comparable instruments previously described.

TRUTH:

Truths too fine spun are subtle fooleries.

H. G. Bohn

HONESTY:

To state the facts frankly is not to despair for the future nor indict the past.

John F. Kennedy
State of the Union Message, Jan. 29, 1961.

LEARNING:

Learning without thought is useless; thought without learning is dangerous.

Confucius

To JANET

JOSHUA

and SAMUEL

CARBOHYDRATE CHEMISTRY. SYNTHETIC AND STRUCTURAL INVESTIGATION OF THE PHYTOTOXINS FOUND IN *Helminthosporium sacchari*, and *Rhynchosporium secalis*.

by

Ross Carlton Beier

A thesis submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Chemistry

Approved:

Bradford P. Mundy
Chairman, Graduate Committee

Edwin H. Abbott
Head, Major Department

Michael P. Malone
Graduate Dean

MONTANA STATE UNIVERSITY

Bozeman, Montana

May, 1980

ACKNOWLEDGMENT

With humbleness, sincere gratitude is extended to all people who have touched my life. Special thanks are extended to those people of recent vintage who, in particular, have contributed in any way during this advanced study; specifically, to Dr. Arnold C. Craig for discussions concerning problems in stereochemistry and synthesis; to Dr. Patrik R. Callis for many helpful discussions and his fundamental understanding of molecular properties; to Dr. Gary A. Strobel for patience and assistance while working in his plant pathology laboratory; to Dr. Alois A. Bell and Dr. Robert D. Stipanovic for stimulating discussions; to the Herman Frasch Foundation for financial support; to my advisor, Dr. Bradford P. Mundy, for his continuous sincere help and support during my development as a researcher; to my parents, Carl and Jean, and Robert and Dorene Bauknecht, for their moral support and understanding; to the people of Christ the King for extending a helping hand and love during the time of need, to my wife, Janet, for her unceasing moral support and strength; to Joshua and Samuel for having to wait for a late Daddy.

TABLE OF CONTENTS

	PAGE
FRONTISPIECE	i
DEDICATION	ii
TITLE PAGE	iii
VITA	iv
ACKNOWLEDGEMENT	v
TABLE OF CONTENTS	vi
ABSTRACT	x
SECTION	
1 STRUCTURAL INVESTIGATIONS OF HELMINTHOSPOROSIDE (one of the phytotoxins found in *Helminthosporium sacchari*)	1
LIST OF TABLES FOR SECTION 1	2
LIST OF FIGURES FOR SECTION 1	4
INTRODUCTION	9
RESULTS AND DISCUSSION	20
SUMMARY	93
EXPERIMENTAL	95
APPENDIX A. Tabulated Results for Figure 26, Percent Decrease of 3-propeneoxyoxetane with time	142
APPENDIX B. GC-Minigrator Studies of the Hydrogenolysis of Benzylcyclohexylether in the Presence of 3-oxetanol	143

PAGE

REFERENCES AND NOTES. 147

SECTION

2 STRUCTURAL INVESTIGATIONS OF THE RHYNCHOSPOROSIDES (a series of phytotoxins found in *Rhynchosporium secalis*). . 155

LIST OF TABLES FOR SECTION 2 156

LIST OF FIGURES FOR SECTION 2 157

INTRODUCTION. 161

RESULTS AND DISCUSSION. 166

SUMMARY 220

EXPERIMENTAL. 223

APPENDIX A. R_F Values and Data Reduction for Some Oligomers From the Hydrolysate of Cellulose. . . . 257

APPENDIX B. Elution Volumes and Data Reduction for Some β(1→4) Oligoglucose Units Chromatographed on Bio-gel. 258

APPENDIX C. Data Reduction for A Bio-gel P-2 Column Separation of α(1→4) Oligoglucose Units. . . . 259

REFERENCES AND NOTES. 260

SECTION

3 THE USE OF ^{13}C-NMR FOR IDENTIFICATION AND ASSIGNMENT OF ANOMERIC CONFIGURATION FOR THE CARBOHYDRATE RESIDUE IN GALACTO- AND GLUCO- PYRANOSIDES AND FURANOSIDES 264

PAGE

LIST OF TABLES FOR SECTION 3 265

LIST OF FIGURES FOR SECTION 3 266

INTRODUCTION. 267

RESULTS AND DISCUSSION. 269

SUMMARY 286

EXPERIMENTAL. 287

APPENDIX A. CMR Data for α- and β-D-galactopyranosides . 288

APPENDIX B. CMR Data for α- and β-D-glucopyranosides . . 290

APPENDIX C. CMR Data for Synthesized α- and β-D-galacto-pyranosides. 295

APPENDIX D. CMR Data for Synthesized α- and β-D-gluco-pyranosides. 296

APPENDIX E. CMR Data for α- and β-D-galactofuranosides . 297

APPENDIX F. CMR Data for α- and β-D-glucofuranosides . . 299

REFERENCES AND NOTES. 300

SECTION

4 THE DESIGN OF PREVIOUSLY UNAVAILABLE LABORATORY APPARATUS 302

LIST OF TABLES FOR SECTION 4 303

LIST OF FIGURES FOR SECTION 4 304

INTRODUCTION. 306

RESULTS AND DISCUSSION. 307

SUMMARY 331

PAGE

EXPERIMENTAL 332

REFERENCES AND NOTES 335

ABSTRACT

This thesis is comprised of four sections. In section 1 synthetic carbohydrate chemistry was extensively used during an attempt to verify a previously proposed structure for helminthosporoside,[1] Sec. 1 a host-specific toxin isolated from *Helminthosporium sacchari*. This previously proposed structure was determined not to be the actual structure of the toxin. Additional procedures were developed for the isolation of the toxin. PMR, CMR, and mass spectral analyses were obtained on the isolated toxin. The toxin appears to be a disaccharide with a large aliphatic aglycone.

During structural verification of a previously suggested structure for rhynchosporoside,[2] Sec. 2 as discussed in section 2, synthetic carbohydrate chemistry was extensively utilized. The previously proposed structure was determined not to be the actual structure of the toxin. Rhynchosporoside is a phytotoxin found in the fungal extracts of *Rhynchosporium secalis*. It is suggested that a number of phytotoxins may exist, i.e., a glucoide, cellobioside, and a cellotrioside, all having a glycosidic linkage to carbon-1 of 1,2-propanediol.

A ^{13}C-NMR method was developed in section 3 for the identification of the carbohydrate residue in galacto- and glycopyranosides, and furanosides, with the capability of distinguishing the structural configuration of the carbohydrate residue. This method works for all reported CMR spectra of non-aromatic galactosides and glucosides, and for synthetic compounds reported in this paper.

The designs for a number of previously non-available laboratory apparatus are presented and discussed in section 4. A method for building and operating a 295.5 cm heated chromatography column is described. The separation of this instrument appears to be superior to comparable instruments previously described.

SECTION 1

STRUCTURAL INVESTIGATIONS OF HELMINTHOSPOROSIDE

(one of the phytotoxins found in *Helminthosporium sacchari*)

LIST OF TABLES FOR SECTION 1

TABLE		PAGE
1	R_F values of helminthosporoside	15
2	Assignments of signals, in the NMR spectrum, of helminthosporoside.	17
3	Cyclopropyl derivatives treated with antimony trichloride	23
4	^{13}C resonances of helminthosporoside.	31
5	Runner length for the amount of allyl α-D-galactopyranoside placed on sugarcane clone 51NG97	37
6	Galactosides produced by a mercury-ion assisted glycosidation, their R_F and R_{Gal} values obtained from thin-layer chromatography on silica-gel using the solvent system (ethylmethylketone-ethanol-water, 75:12:6).	52
7	Bioassays on 51NG97 with some α-D-galactosides having two and three carbon aglycones; runner length was determined 3 days after application.	53
8	Elution volume and estimated number of residues for the toxin and ally α-D-galactopyranoside.	74
9	R_F and R_{Gal} values of allyl α-D-galactopyranoside and helminthosporoside on Whatman #541, with the solvent system (1-propanol-ammonium hydroxide, 66:33)	74
10	Bioassays with punctures between the veins and in the veins, read at 42 hours	79

TABLE		PAGE
11	Signals observed in the proton NMR spectrum; the reference is DSS substituted.	86
12	CMR resonances of the helminthosporoside preparation; the reference is DSS substituted.	90

LIST OF FIGURES FOR SECTION 1

FIGURE		PAGE
1	Flow sheet summarizing the isolation procedure used by Steiner and Byther[5]	11
2	The first proposed structure for helminthosporoside	13
3	Hydrolysis mechanism producing acrolein	18
4	A double S_N2 reaction on perbenzylated galactosyl chloride	21
5	Acyloin condensation	22
6	Fischer method of producing methyl galactosides	24
7	Benzyl ether protection	25
8	Aglycone removal by acid hydrolysis	25
9	Graph of 1-R_F value vs. the number of benzyl groups on galactose	27
10	Vinylation of 2,3,4,6-tetra-O-benzyl-α-D-galactopyranose.	28
11	Formation of (2-O-t-butyratecyclopropyl) 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside by a carbene reaction.	29
12	Benzyl ether and ester cleavage by a dissolving metal reduction	30
13	Floridoside, (15), and isofloridoside, (16)	31
14	The second proposed structure for helminthosporoside.	32
15	S_N2 reaction by allyl alcohol	32
16	Allyl α-D-galactopyranoside by the Fischer method	33

FIGURE		PAGE
17	Acetylation of allyl α-D-galactopyranoside.	33
18	Epoxidation of allyl 2,3,4,6-tetra-O-acetyl-α-D-galacto-pyranoside.	34
19	Deacetylation by a catalytic amount of sodium methoxide .	35
20	Epoxide opening to form the diol.	36
21	The third proposed structure for helminthosporoside . . .	38
22	Reaction of thionyl chloride on 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside	38
23	2-allyloxy-3-chloropropanol from allyl alcohol.	39
24	Double bond rearrangement	39
25	Propene hydrolysis to form 3-oxetanol	40
26	Percent decrease of 3-propeneoxyoxetane with time	41
27	Formation of oxetanyl perbenzylated galactoside	42
28	Expected hydrogenolysis reaction.	42
29	Benzylation of cyclohexanol	43
30	Hydrogenolysis of methyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside	44
31	Percent hydrogenolysis vs. moles of 3-oxetanol.	45
32	Bromo-acetylation of D-galactose.	46
33	Koenigs-Knorr glycosidation	47
34	Deacetylation to produce (3-oxetanyl) β-D-galacto-pyranoside.	48

FIGURE		PAGE
35	Attempted anomerization with titanium tetrachloride . . .	48
36	Attempted anomerization with boron trifluoride-etherate .	49
37	Oxetane polymerization mechanism.	50
38	Mercury-ion assisted glycosidation.	50
39	Mercury-ion assisted glycosidation with 3-oxetanol. . . .	54
40	The fourth proposed structure for helminthosporoside. . .	54
41	Solvomercuration-demercuration with mercuric acetate. . .	55
42	Solvomercuration-demercuration with mercuric sulfate. . .	56
43	Block diagram method for comparison of CMR data	58
44	The fifth proposed structure for helminthosporoside . . .	59
45	Flow sheet summarizing the isolation procedure for obtaining the helminthosporoside preparation.	60
46	The carbohydrates used for comparison on HPLC	61
47	HPLC separation characteristics of some glycosides and helminthosporoside on a μ-bondapak carbohydrate column with solutions of acetonitrile-water.	63
48	HPLC separation of the helminthosporoside preparation . .	64
49	Ultraviolet absorption spectrum of the HPLC preparation .	65
50	Bio-gel P-2 column separation of the helminthosporoside preparation (after HPLC), and total carbon analysis of the fractions containing toxicity	67
51	Re-run of the toxic fractions over the Bio-gel P-2 column	68

FIGURE		PAGE
52	Ultraviolet spectrum of the Bio-gel P-2 purified toxin. .	69
53	Separation of the α-glucosides in corn syrup.	70
54	Plot of Ve/Vo vs. log(molecular weight) for the α-glucosides in corn syrup	71
55	Elution curve of allyl α-D-galactopyranoside on Bio-gel P-2	73
56	Cross section of a sugarcane leaf	75
57	The puncturing tool machined from a sewing needle	77
58	Preliminary bioassay to test the hypothesis of injecting the larger veins for better runners	78
59	A preliminary proposed structure for helminthosporoside .	79
60	Fragmentations from the peracetylated toxin	81
61	Major cleavages of the peracetylated toxin.	82
62	Fragmentations from the free toxin.	82
63	Major cleavages of the free toxin	83
64	Prehelminthosporol, (44a), and 9-hydroxyprehelminthosporol, (44b)	83
65	The proton NMR spectrum of the Bio-gel P-2 purified helminthosporoside preparation.	85
66	Proton NMR and assignments, of a derivative of prehelminthosporol, made by P. DeMayo et al.[27]	87
67	The ^{13}C-NMR spectrum of the Bio-gel P-2 purified helmin-	

FIGURE		PAGE
	thosporoside preparation.	89
68	A tracing of the thin-layer chromatogram showing reaction products of the transvinylation reaction.	105
69	A thin-layer chromatogram comparing α-methyl, and α-hydroxyl, to 2,3,4,6-tetra-O-benzyl-D-galactosyl chloride . .	107
70	A thin-layer chromatogram of allyl 2,3,4,6-tetra-O-benzyl -α-D-galactopyranoside.	108
71	Standard curve for the determination of glucose, by phenol-sulfuric acid.	139

INTRODUCTION

A disease of sugarcane (*Saccharum sp.*) caused by the fungus, *Helminthosporium sacchari* (Van Breda de Hann) Butler, is widespread.[1] From 1985-1934 it was known to exist in regions of Hawaii, India, Java, Puerto Rico, Cuba, Australia, Taiwan and Florida. In 1890 Kruger[2] named this disease 'eye spot' because the first symptoms are elongated leaf lesions showing red centers surrounded by a narrow margin of chlorotic tissue. After the lesions are formed, reddish-brown streaks (runners) initiating from the point of infection reach toward the tip of the leaf. It is possible to have runners attaining a length of 3 feet. Also, the fungus can be easily isolated from the lesions but not from the runner area.[1]

In 1929 Lee,[2] acting under the assumption that the runners on sugarcane leaves infected with *H. sacchari* were caused by a toxin produced by the fungus, proceeded to show that the culture filtrated from *H. sacchari* was toxic to excised leaves. The toxin was shown to be heat stable after three treatments in the autoclave (15 lbs. steam for 20 minutes). The toxin was thought to destroy the chlorophyll and reduce the iron in the leaves; as well as reduce nitrates to nitrites which are also toxic.

In 1968 Liu[4] demonstrated that *H. sacchari* is more pathogenic at 20°C than at 30°C, even though the growth of the fungus is more rapid at the higher temperature. Also, in a personal communication to Stein-

er and Byther,[5] C. A. Wismer in Hawaii noted that cool, wet climates contributed to greater incidences of eye spot disease. It has been suggested by Strobel[6] that Mg^{++}-K^{+} ATPase is required for the action of the toxin, and above 30°C Mg^{++}-K^{+} ATPase has a distinctive change in it's activity. The decrease in apparent pathogenicity at 30°C seems to be due not to the fungus, but to the sugarcane.

Steiner and Byther in 1971[5] initiated studies to determine the cause for toxicity of the culture filtrate, check for host specificity of the toxin, and determine the possibility of using the toxin in screening sugarcane clones for resistance to eye spot disease. During purification of the toxin, methods similar to those used by Pringle and Scheffer[7] were utilized. A flow sheet summarizing the steps used by Steiner and Byther is presented in Figure 1. It is important to note the results obtained through the use of Sephadex G-15. Two areas of toxin activity were observed; Peak 1, containing most toxic activity and being of larger molecular weight than the substance in peak 2, which contained less toxicity.

A dual bioassay was used by Steiner and Byther:

A. "In the first, seven inch excised leaves were used. A 1-µl drop of toxin solution was placed on a needle puncture spot near the base of the leaf. Injected leaves were incubated in a moist chamber at room temperature. The length of the resulting runner

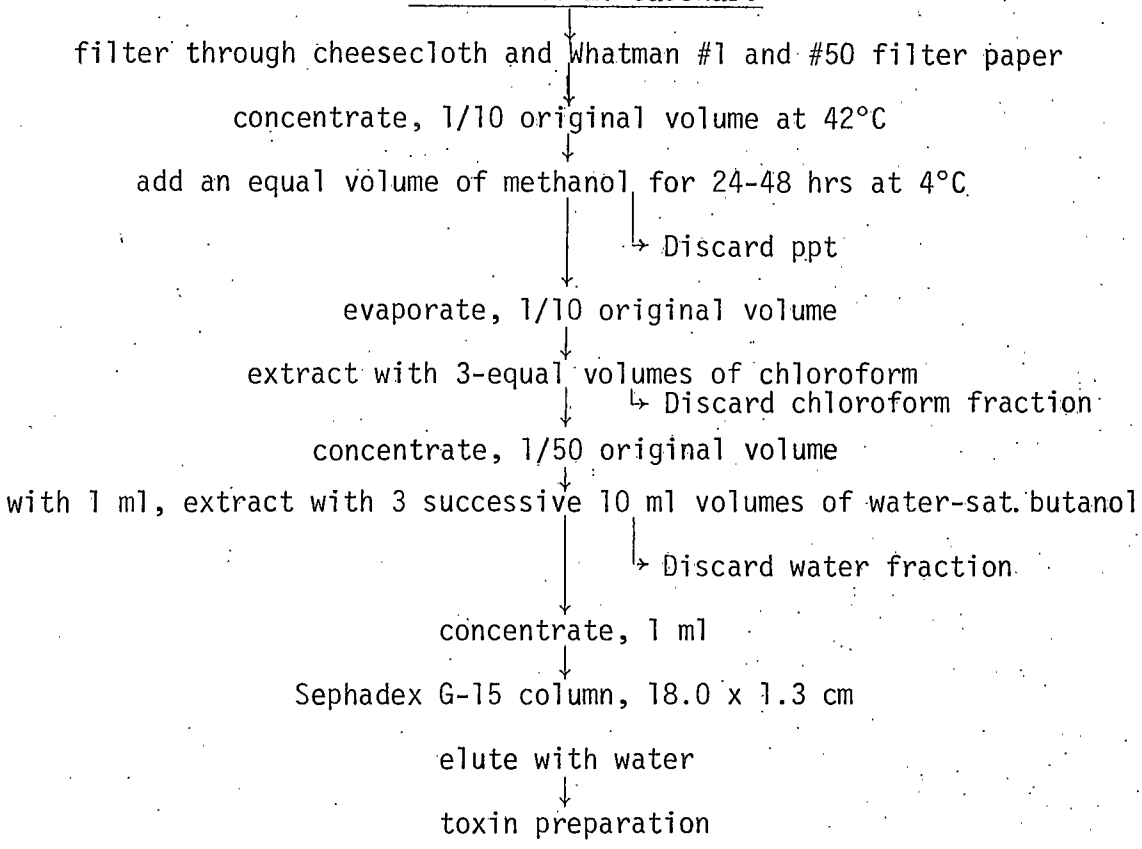


Figure 1. Flow sheet summarizing the isolation procedure used by Steiner and Byther.[5]

from each of several toxin dilutions was an indication of toxin activity. The measurements were made from 24-48 hours after inoculations."

B. "In the second, 0.2 ml of toxin solution was injected into the leaf spindle immediately above the apical

meristem of the intact plants. Degrees of clonal resistance could be determined by using several dilutions of toxin and rating the intensity of the symptoms from each dilution. Ratings were determined 24-30 hours after injection."

A total of 182 clones, 47 from Hawaii and 135 imported, were tested in the process of screening with the toxin for resistance to eye spot disease. A significant correlation, ($r=0.88$, $p<0.01\%$) between the reaction of the toxin and reaction of fungal inoculations was obtained. Because of the similarities with other host-specific toxins,[8,9] the toxin produced by *H. sacchari* was considered host-specific. The following characteristics of the toxins produced by *H. sacchari*, which are in common with other host-specific toxins include:[5]

A. The host range of the toxin is similar to the fungus.

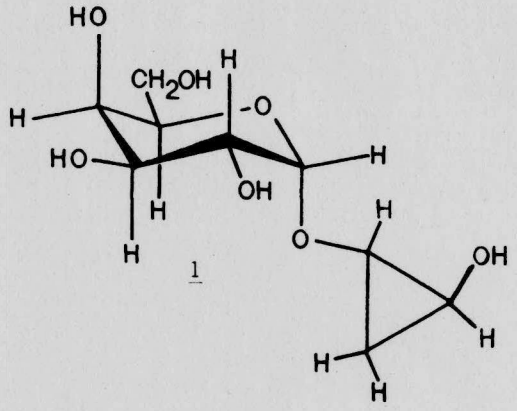
B. The symptoms produced by the toxin and fungus are similar.

C. The toxin has a low molecular wt., shown by its elution on a Sephadex G-15 column.

From these observations, Steiner and Byther were able to make a major contribution to the unfolding of the *Helminthosporium sacchari* story. They were able to show that not only did a toxic material exist, but at least two toxic components were present in culture filtrates and were stable to 144°C The toxins were actually used to advantage in screening clones for resistance to the eye spot disease. A subse-

quent paper by Byther and Steiner[10] in 1972 dealt with the problem of selecting sugarcane seedlings resistant to eye spot disease by utilization of partially purified culture filtrates.

Steiner and Strobel[11] subsequently extended the earlier isolation procedure by two steps. After the Sephadex G-15 column chromatography step, two paper chromatography steps were added, the first being paper chromatography with the solvent system (1-butanol-acetic acid-water, 4:1:5) and the second with the solvent system (1-propanol-ammonium hydroxide-water, 6:3:1). With the resulting purified preparation they proceeded to characterize the major component and suggested the structure, 2-hydroxycyclopropyl α-D-galactopyranoside, (1), shown in Figure 2.



1

Figure 2. The first proposed structure for helminthosporoside.

The following is a summary of the reported data which enabled the authors to characterize helminthosporoside:

A. Properties of helminthosporoside.

1. A relationship between runner length and toxin concentration was developed. For example, approximately 1.5 µg toxin correlated with an 8.1 cm runner in 22 hrs. The runner length was directly proportional to log(helminthosporoside). The method of application was the same as that used by Steiner and Byther.[4] A 1-µl drop of toxin solution was placed on a needle-puncture spot near the base of an excised leaf.
2. "At room temperature the toxin preparation was a yellow syrup. This syrup became crystalline after desication in a partial vacuum over phosphorus pentoxide."
3. Table 1 shows R_F values of helminthosporoside in several solvent systems. Since it moved well in polar solvents, it was suggested to be a polar compound.

B. Infrared spectrum.

1. "A strong absorption band at 3.0 µ indicating the presence of -OH groups on the molecule."
2. "The lack of a strong band around 6.0 µ assured the absence of a carbonyl group either as an acid or an aldehyde."
3. "A weak but detectable band in the region of 9.8 µ," supported the presence of a cyclopropyl ring.
4. "The remainder of the spectrum initially suggested that the toxin could be a glycoside."

Table 1. R_F values of helminthosporoside.
Solvents a through e were used on descending paper chromatography; f through k were used with thin-layer chromatography.

	Solvent	R_F value
a.	1-Butanol-acetic acid-H_2O, 4:1:5	0.30
b.	1-Propanol-NH_4OH-H_2O, 6:3:1	0.80
c.	1-Butyric acid-1-butanol-H_2O,2:2:1	0.14
d.	1-Butanol-ethanol-H_2O, 5:1:4	0.34
e.	Ethyl acetate-formic acid-acetic acid-H_2O 18:3:1:4	0.54
f.	Hexane-ethylacetate, 85:15	0.0
g.	Benzene-methanol, 95:5	0.0
h.	1-Butanol-acetic acid-H_2O, 3:1:1	0.70
i.	1-Butanol-acetone-H_2O, 4:5:1	0.54
j.	Methylethyl ketone-acetic acid-methanol 6:2:2	0.53
k.	Benzene-acetic acid-methanol, 2:2:6	0.56

C. Ultraviolet spectrum revealed no absorption bands above 210 mμ, eliminating the presence of aromatic and conjugated systems in the molecule."

D. Optical Rotation.

$[\alpha]_D^{20} = -49.0$

E. Mass spectroscopy-membrane osmometry.

1. "The toxin gave a discernible molecular ion peak of 236 m/e."

2. "That the peak of 236 represented the molecular ion was confirmed by a molecular weight determination by membrane osmometry near 250."

F. Elemental Analysis.

Calculated for $C_9H_{16}O_7$:	C, 45.8; H, 6.8; O, 47.4
Found:	C, 45.7; H, 7.3; O, 46±1

A trace of nitrogen was found in the samples.

G. NMR spectroscopy.

Table 2 summarizes peaks found in the NMR spectrum.

H. Carbohydrate moiety.

1. After acid hydrolysis with 0.8N H_2SO_4 at reflux for two hrs and then silylation treatment, the reaction mixture was subjected to gas chromatography, two peaks with the same retention time as α- and β-D-galactopyranoside were observed.

2. The identity of galactose was confirmed by paper chromatography in solvent a (see Table 1) and in ethyl acetate-pyridine-water, 8:2:1 v/v.

3. After refluxing the toxin in acid the neutralized solutions were not phytotoxic.

I. To support the cyclopropyl possibility the following was done:

Table 2. Assignments of signals in the NMR spectrum of helminthosporoside.

Peak	Shift, ppm	No. Protons	Assignments
A	5.32	1	Proton on the anomeric carbon.
B	4.19	4	1 proton from cyclopropyl, 3 protons from galactose.
C	4.10-3.75	3	Protons from C-5 and C-6 of galactose.
D	3.0-2.3	1	Protons on the aglyconic carbon in cyclopropyl.
E	1.8	1	Hydroxyl proton from the cyclopropyl moiety.
F	1.60-1.35	1	Cyclopropyl methylene.
G	1.35-1.2	1	
H	0.9	0	Impurity.

After acid hydrolysis a compound with the same retention time as acrolein was observed. Also, other major peaks were observed. The acrolein peak was estimated to be approximately 25% of the products. Mechanistically, this can arise from the

reaction delineated in Figure 3.

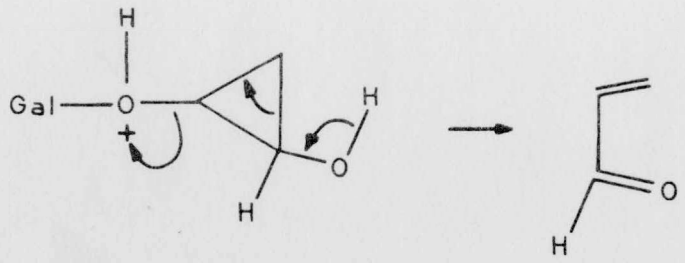


Figure 3. Hydrolysis mechanism producing acrolein.

J. Galactosidic linkage.

1. β-galactosidase from *E. coli*, when allowed to react with the toxin for 18 hrs yielded no free galactose.
2. α-galactosidase with the toxin for 18 hrs yielded less than 1% galactose.

"From these results it is concluded that the galactosidic linkage in the toxin is α."

It appeared from this work that the two additional chromatographic steps resulted in a pure sample of helminthosporoside.

There was a period of 5 years when no further work was attempted toward verification of the structure of helminthosporoside. However, in 1976 the work described here was initiated. The following questions were the intent of the study:

1. Can the proposed structure for helminthosporoside be verified by synthesis?

2. Can methods be devised to aid in the toxin purification procedure, if the toxin preparation is not pure?
3. Will other related molecules, obtained by synthesis, cause symptoms on sugarcane leaves similar to helminthosporoside?

RESULTS AND DISSCUSION

The primary focus of this work was to verify by synthesis, the proposed structure of helminthosporoside. Since the conclusions of the work presented in the introduction suggested an α-galactoside, the approach taken was to utilize existing methodologies for synthesis of α-D-galactosides.[12-17] Consideration was given to selection of methods for obtaining the carbohydrate and aglycone moieties, which might be applied to other related galactosides. With these goals, synthesis of 2-hydroxycyclopropyl α-D-galactopyranoside and related compounds was initiated.

The initial strategy for preparation of helminthosporoside was to utilize perbenzylated galactosyl chloride and a cyclopropanediol. Specifically, it was planned, as delineated in Figure 4, to react 2,3,4,6-tetra-O-benzyl-α-D-galactosyl chloride, (2), with 3,3-dimethyl-1,2-cyclopropanediol, (3), to produce the galactoside (4).[12]

This series of reactions was not designed to give helminthosporoside, but was rather to test out the methodology using compounds that were more readily available. The product, (4), would be an analogue of helminthosporoside. Analogues of helminthosporoside would be of interest, because phytotoxicity could be related to structure, thereby leading toward an understanding of what structural characteristics are essential for toxicity.

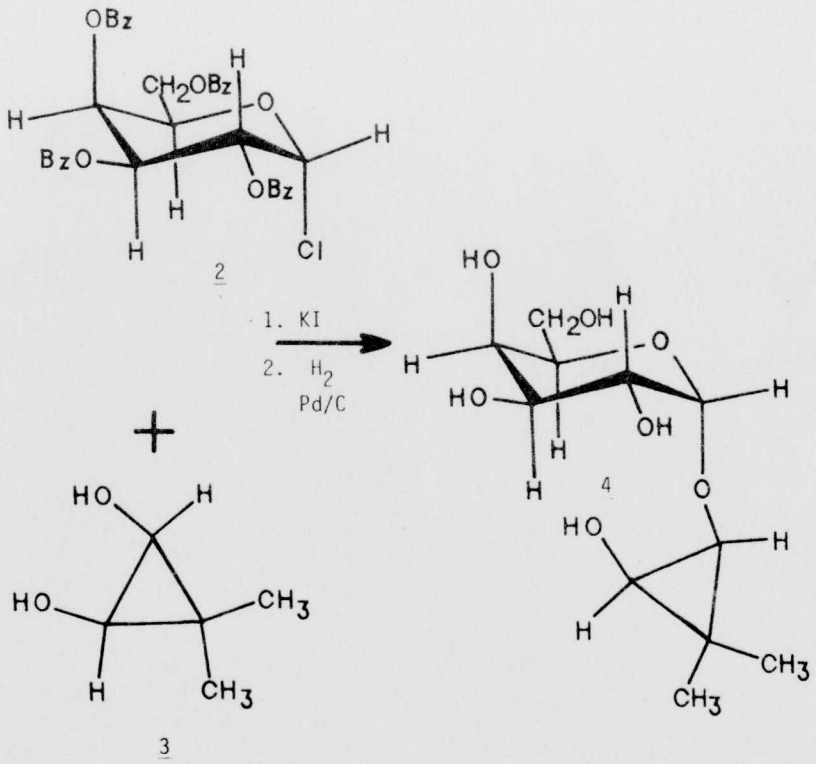
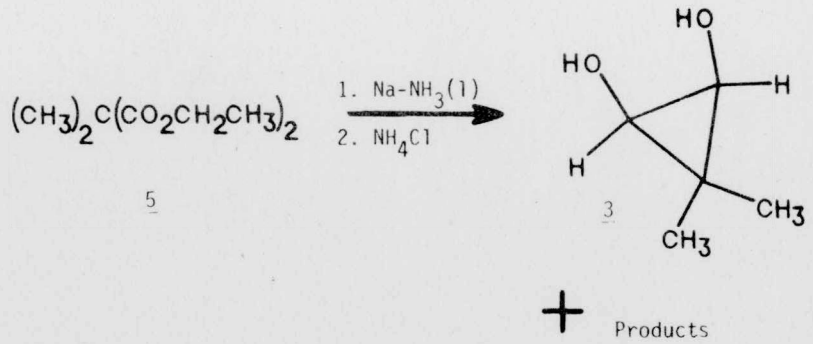


Figure 4. A double S_N2 reaction on perbenzylated galactosyl chloride.

Without literature precedent for 1,2-cyclopropanediol, the analogue, (3), was considered a good possibility to work out the described methodology. As shown in Figure 5, Chen and Ainsworth[18] made 3 from diethyl-dimethylmalonate, (5), by the acyloin condensation. The procedure for making diethyl-dimethylmalonate was the same as used by Adams and Kamm[19] for making ethyl n-butylmalonate.

The method for making diethyl-dimethylmalonate worked well with an overall yield of 66%; however, the reaction to make the cyclopropyl



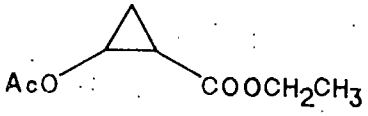
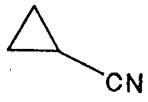
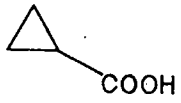
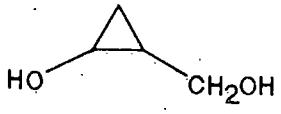
5 3

Figure 5. Acyloin condensation.

derivative did not work as expected. The reported method[18] utilized sodium in liquid ammonia, followed by removal of the ammonia and adding ammonium chloride. Upon inspection of the reaction mixture by glc the major portion of diethyl-dimethylmalonate had not reacted and there were at least nine products. Changes were made in the procedure in an attempt to improve the reaction. Lithium was used instead of sodium and the ammonium chloride was added before the ammonia was evaporated. Upon inspection of the reaction mixture by glc very little starting material remained, but there were at least twelve products. Seven of the most dominant products were collected by glc and tested with antimony trichloride, since it has been observed that a number of cyclopropyl compounds react with this reagent to give a pink flouresence under ultraviolet light. The results of testing these cyclopropyl compounds are represented in Table 3.

The material from only two peaks gave a positive test. The NMR

Table 3. Cyclopropyl derivatives treated with antimony trichloride.

Compound	Flouresence, Color
	Yes, pink
	None
	Yes, pink
	Yes, pink

spectrum was obtained from the most abundant of the two compounds; however, it was not consistent with the theoretical spectrum. There was not enough of the second compound to obtain an NMR spectrum. NMR spectra were taken of all the other products that existed in large enough quantity. But, these also proved not to correlate with the theoretical spectrum.

At this juncture correspondence with the authors of this work was made, lending no additional information or samples for comparison.

Therefore, our inability to repeat this work and the lack of samples for comparison prompted us to discontinue this approach.

As an alternate approach, a carbene reaction with a vinyl galactoside would result in a cyclopropyl galactoside. By using the appropriate carbene, 1 would be generated.

The following sequence of reactions demonstrates the synthetic methodology. The first products in this series of derivatives were methyl α-, (7), and β-D-galactopyranoside, (8). These result from an acid catalyzed attack of the methanolic oxygen on the anomeric carbon of D-galactose, (6),[20] as depicted in Figure 6.

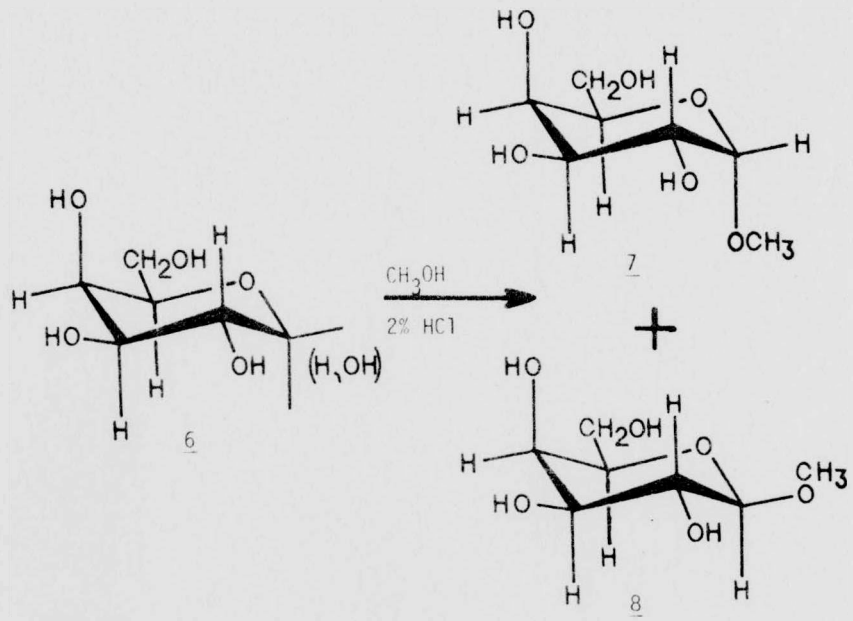


Figure 6. Fischer method of producing methyl galactosides.

Treatment of 7 with benzyl chloride and potassium hydroxide produces methyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside, (9), protecting all free hydroxyl groups.[21] This protection reaction is delineated in Figure 7.

The next step is the removal of the aglyconic methyl group by acid hydrolysis. The only method available for this reaction was that reported by Austin et al.;[22] however, it required a reflux for 10 days.

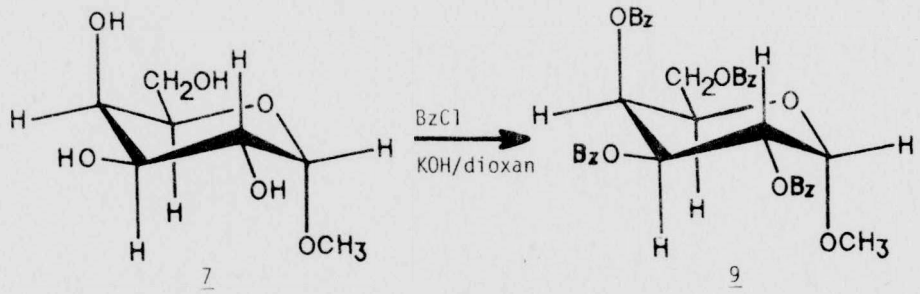


Figure 7. Benzyl ether protection.

Figure 8 describes this reaction resulting in 2,3,4,6-tetra-O-benzyl-α-D-galactose, (10).

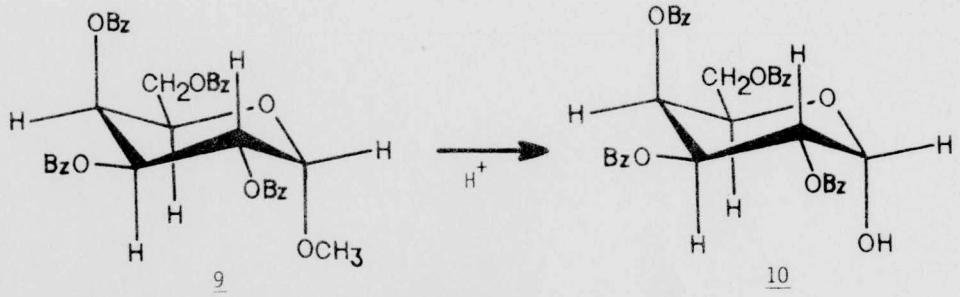


Figure 8. Aglycone removal by acid hydrolysis.

Purification by thin-layer chromatography (ethylacetate-methanol, 4:1) on silica-gel revealed a large amount of one product. By NMR, the ratio of aromatic to non-aromatic hydrogens was not consistent with the theoretical values. Therefore, a chromatographic system with better resolving power was found; namely (carbontetrachloride-methanol, 9:1) on silica-gel. With this new solvent system, six products could be resolved from the starting material. The required product, (10), was also the major product. By making a plot of $(1-R_F)$ vs. (number of benzyl groups on galactose), a straight line was obtained with a correlation coefficient of 0.9993, as represented in Figure 9. These extraneous products appear to result from degradation of 10 by successive removal of benzyl groups. Also, the column chromatography method suggested by Austin et al.[22] for purifying the products failed to work. Since this method is very laborious and does not work well, a different method was required.

A method by Glaudemans and Fletcher[21] was tried, even though it had been used only for hydrolysis of the glucose counter part. This method required using a mixture of acetic and sulfuric acid at 95°C for 24 hrs. Separation of the reaction mixture by thin-layer chromatography revealed at least five products, corresponding to those obtained by the first method. However, with this procedure the major products were due to the degradation of 10. A timed hydrolysis study was undertaken at three different temperatures, 75°C, 80°C and 85°C. The reac-

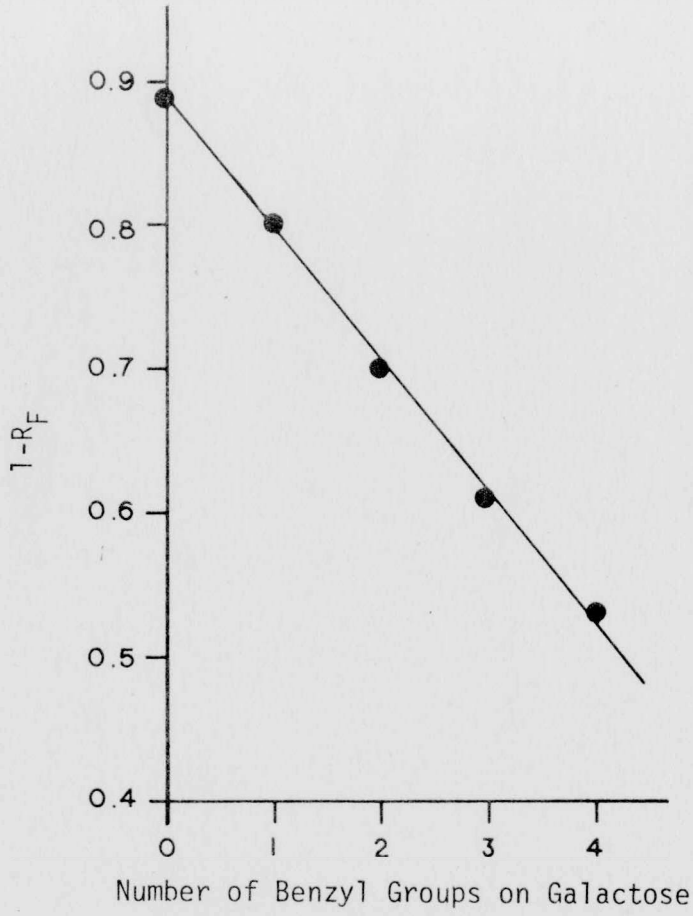


Figure 9. Graph of 1-R_F value vs. the number of benzyl groups on galactose.

tion was followed by thin-layer chromatography. At a temperature of 85°C, after a period of 5 hrs there was nearly a complete conversion of starting material to products. Also, essentially only 10 was obtained. However, when the temperature exceeded 90°C, degradation was initiated.

In a transvinylation reaction, 10 was converted to vinyl 2,3,4,6-

tetra-O-benzyl-α-D-galactopyranoside, (12), by reaction with ethyl vinyl ether, (11), and mercuric acetate (Figure 10).[23] However, after 55 hrs it was observed by thin-layer chromatography that most of the starting material remained. Therefore, 4Å molecular sieves were added to trap the released ethanol, forcing the reaction to completion.

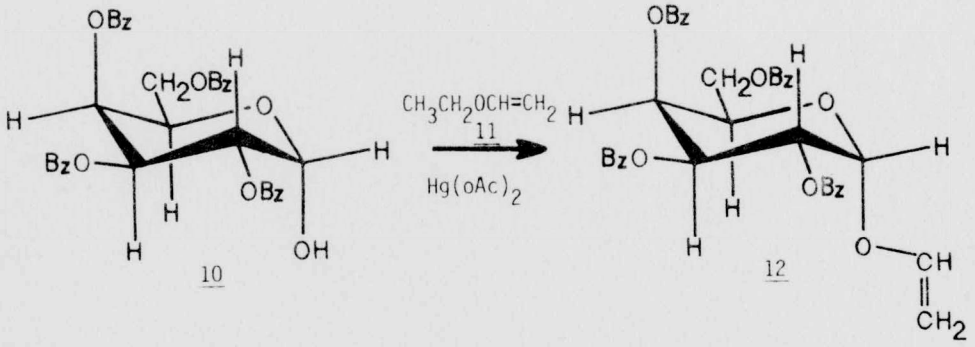


Figure 10. Vinylation of 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside.

Within 21.5 hrs five products were observed. By examining the NMR spectra of the products, it was apparent that one was the required derivative, (12); but, others lacked benzyl groups. The reaction was attempted again, adding the 4Å molecular sieves at the begining of the reaction. After 12 hrs the reaction was monitored by thin-layer chromatography, revealing the same products, with 12 as the major one. It appears that mercuric acetate was catalyzing removal of benzyl groups.

The next two steps in the methodology for obtaining 1 are shown in

Figure 11. The preparation of (2-O-t-butyrate cyclopropyl) 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside, (14), requires attack of carbene, (13), on 12.[24]

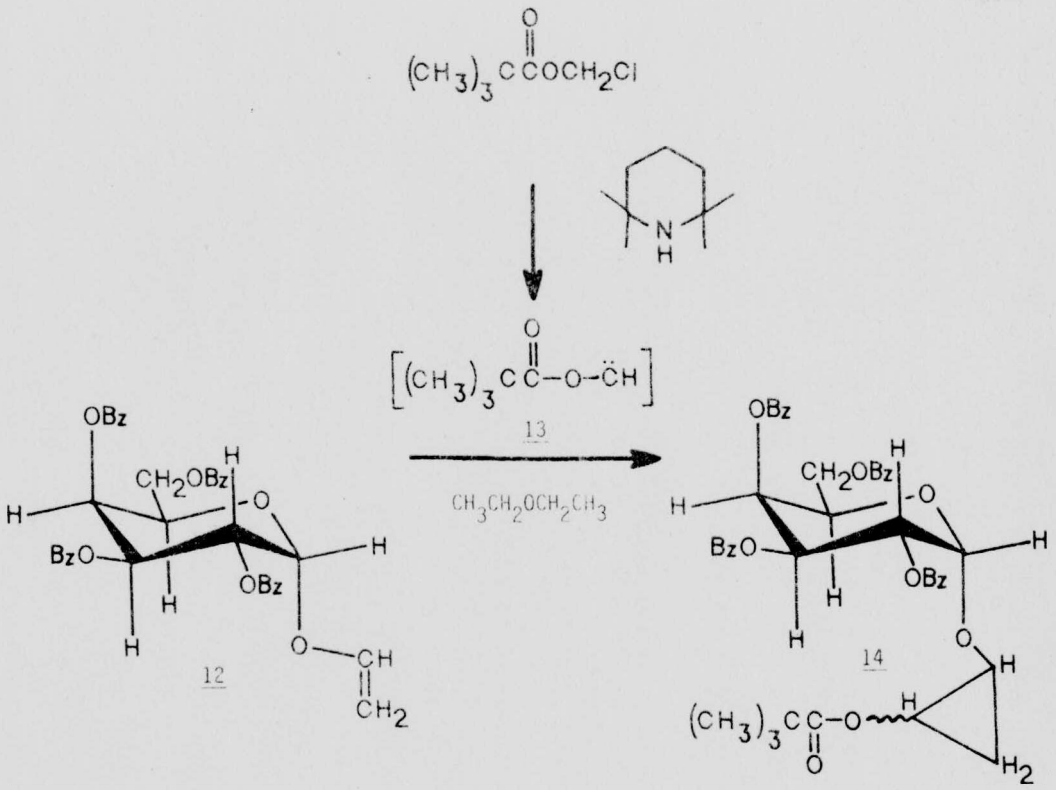


Figure 11. Formation of (2-O-t-butyrate cyclopropyl) 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside by a carbene reaction.

Benzyl ether and ester cleavage from 14 can be carried out by a sodium-liquid ammonia reduction, followed by addition of ammonium chloride, resulting in the derivative, (1), as shown in Figure 12.[23]

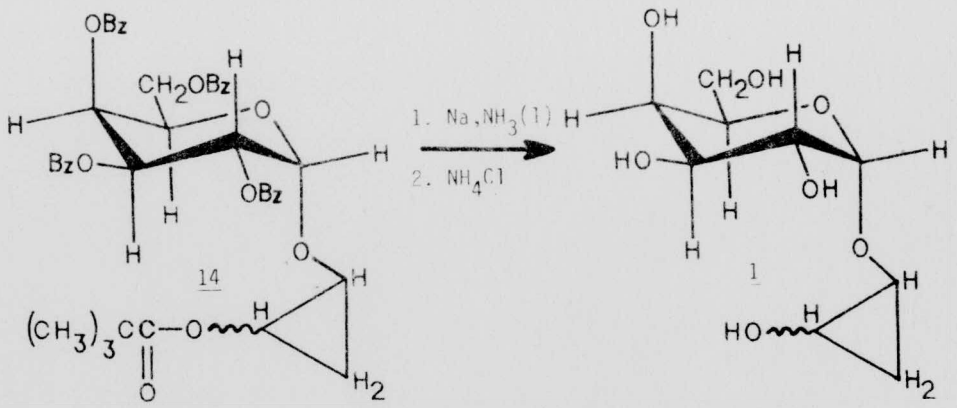


Figure 12. Benzyl ether and ester cleavage by a dissolving metal reduction.

Before initiation of the last two steps, a ^{13}C-NMR spectrum of helminthosporoside was obtained, and the values of the resonances are listed in Table 4. Since the ^{13}C-NMR spectrum ruled out the possibility of a cyclopropyl residue within the molecule, attempted syntheses of cyclopropyl derivatives were terminated. The ^{13}C-NMR spectrum of helminthosporoside had only nine major resonances, this number agreed with the original postulate that helminthosporoside had only nine carbons. Therefore, syntheses of nine carbon derivatives were still considered.

It was suggested that a glycerol moiety may be the aglycone of galactose. This would be consistent with the CMR resonances (63.14, 63.51, and 81.91 ppm), but would not be consistent with the chromatography data; where it is observed that the polarity of (1,3-propanediol-

Table 4. ^{13}C resonances of helminthosporoside.

PPM(TMS)	Intensity	Multiplicity	Carbon # Assignment
107.85	138	d	1
83.3	127	d	2
81.91	225	d	
77.17	162	d	5
76.79	98	d	3
71.14	152	d	4
63.51	133	t	
63.14	121	t	
61.89	99	t	6

2-0) α-D-galactopyranoside (isofloridoside), (16), would be greater than that of the toxin (Figure 13). These two compounds were aquired[25] and bioassayed, and exhibited no observable toxic activity on susceptable sugarcane leaves.

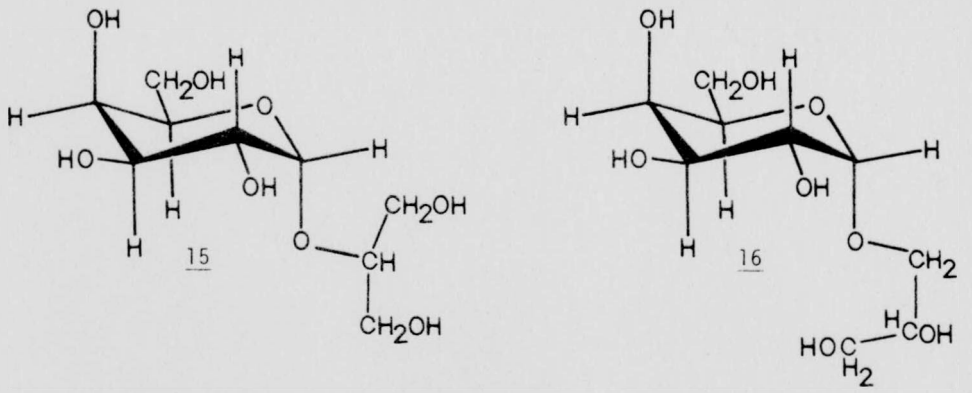


Figure 13. Floridoside, (15), and isofloridoside, (16).

Based upon the CMR data, a second structure, (2,3-epoxy propene-

1-O) α-D-galactopyranoside, (17), for helminthosporoside was proposed, and is shown in Figure 14.

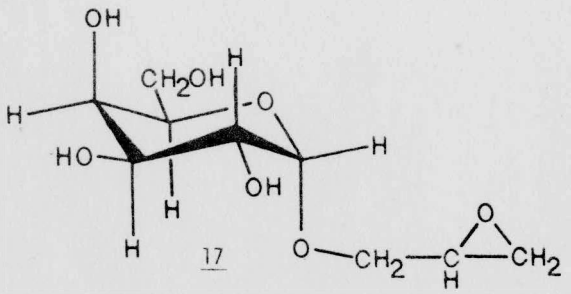


Figure 14. The second proposed structure for helminthosporoside.

A methodology for obtaining 17 requires the preparation of allyl α-D-galactopyranoside, (20), followed by its epoxidation. Two methods for synthesizing the allyl derivative were considered, and were simultaneously carried out. Figure 15 shows the first method utilizing a double S_N2 reaction on the chloride, (2), by potassium iodide and allyl alcohol, (18), to produce the allyl derivative, (19).[12] The reaction was observed, by thin-layer chromatography, to go to completion.

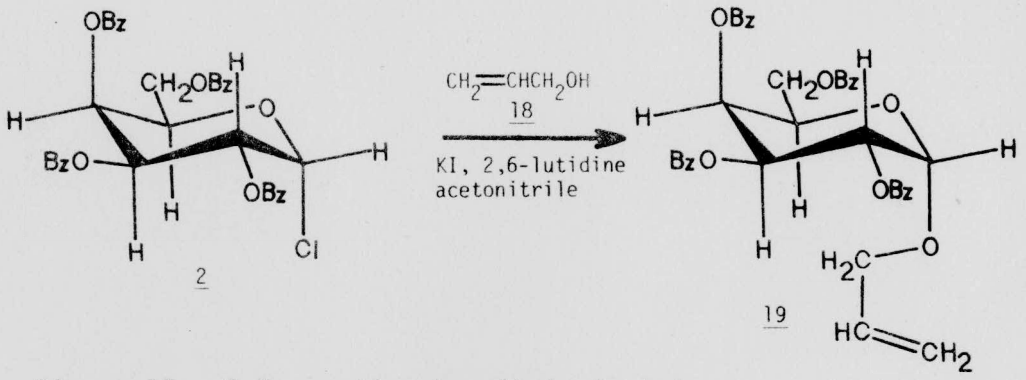


Figure 15. S_N2 reaction by allyl alcohol.

A dissolving metal reduction would remove the benzyl ethers. The second procedure, known as the Fischer method,[26] gave large amounts of crystalline 20 by a more direct route. This sequence, adopted from Talley et al. (Figure 16), consists of an acid catalyzed attack of 18 on 6 to form the alpha anomer, (20).[27]

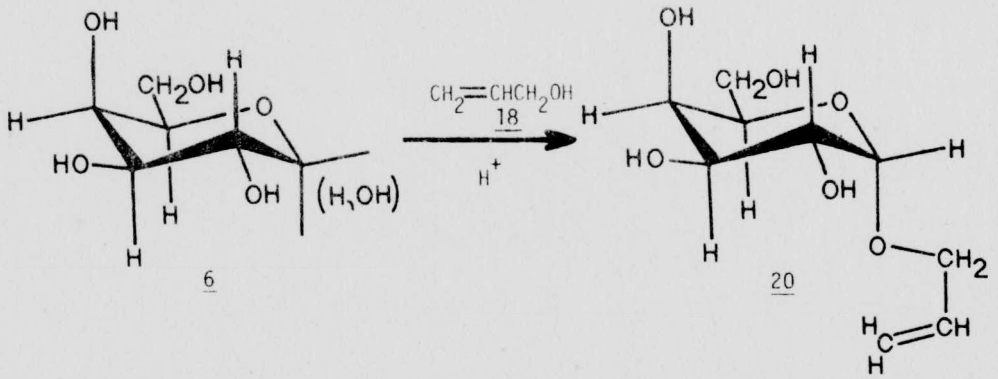


Figure 16. Allyl α-D-galactopyranoside by the Fischer method.

Derivative 20 was acetylated with acetic anhydride and pyridine at 0°C to give crystalline allyl 2,3,4,6-tetra-O-acetyl-α-D-galactopyranoside, (21),[28] as shown in Figure 17.

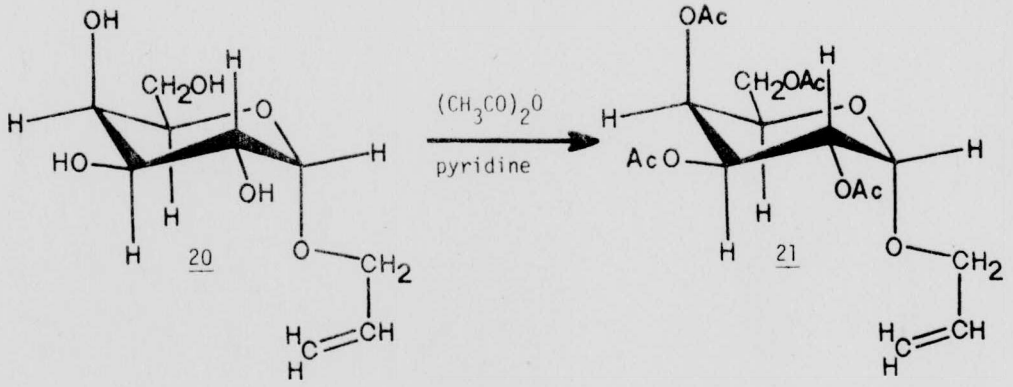


Figure 17. Acetylation of allyl α-D-galactopyranoside.

Methods of acetylation involving reflux had been tried; however, they produced colored products which would not readily crystallize.

An attempt to epoxidize 20 by perbenzoic acid was unsuccessful. However, epoxidation of 21 by m-chloroperoxybenzoic acid produced (2,3-epoxypropene-1-O) 2,3,4,6-tetra-O-acetyl-α-D-galactopyranoside, (22), as shown in Figure 18.[29]

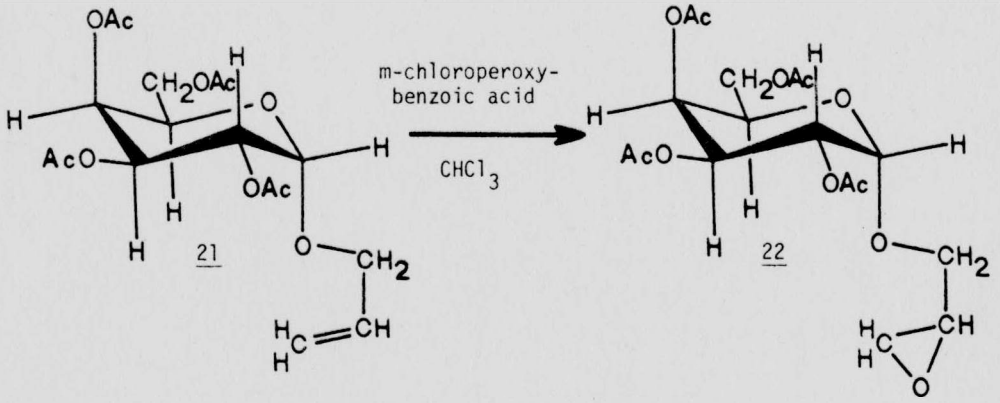


Figure 18. Epoxidation of allyl 2,3,4,6-tetra-O-acetyl-α-D-galactopyranoside.

The acetyl protection was removed from 22 with a catalytic amount of sodium methoxide to produce 17, as delineated in Figure 19.[29b]

It is of interest to note that isofloridoside, (16), can be synthesized from 17 with the addition of perchloric acid.[30] Isofloridoside is a naturally occurring glycoside isolated from red alga (*Porphyra umbilicalis*,[31] and this method (as shown in Figure 20) represents one

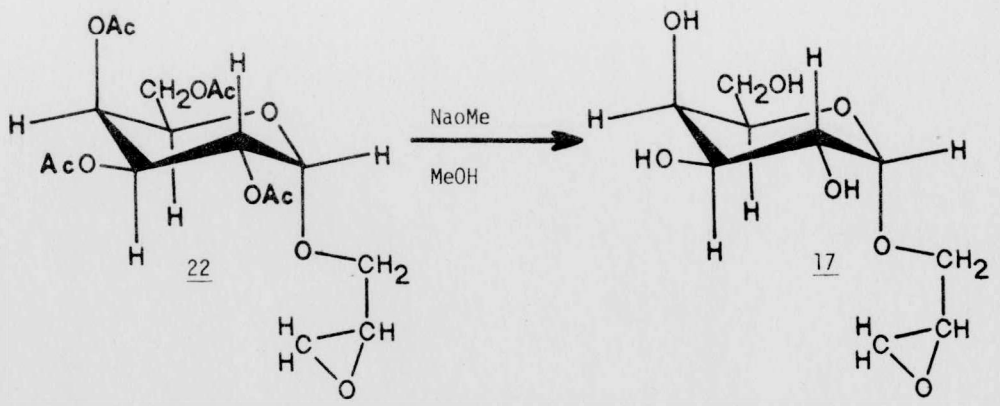


Figure 19. Deacetylation by a catalytic amount of sodium methoxide.

way of synthesizing isofloridoside.

When 17 was tested on sugarcane leaves (clone 51NG97) susceptible to helminthosporoside, no runners or lesions were formed. When the intermediate, 20 was placed on susceptible sugarcane leaves, lesions formed. Placing 20 on sugarcane leaves (clone 7209), resistant to helminthosporoside, resulted in no lesions being formed. Allyl α-D-galactopyranoside causes lesions and runners, but large quantities were required.

Bioassays with known amounts of allyl α-D-galactopyranoside were conducted on sugarcane leaves, both susceptible and resistant to helminthosporoside. The method for sample introduction was similar to that of Steiner and Byther,[5] that is a drop of toxin was administered to a needle puncture near the base of an excised leaf. There was no

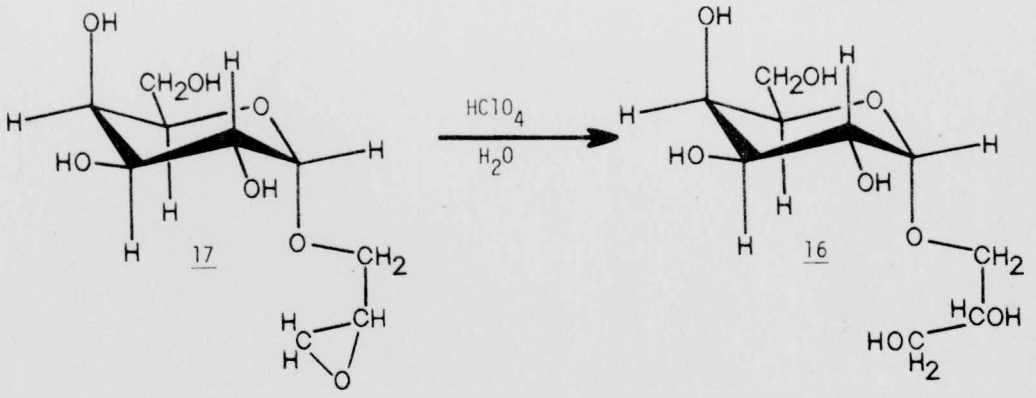


Figure 20. Epoxide opening to form the diol.

reaction on resistant sugarcane leaves to varying amounts of allyl α-D-galactopyranoside up through 530 μg per spot, at 115 hrs. By administering 150-600 μg of allyl α-D-galactopyranoside per spot, runners formed within 36 hrs on the susceptible clone, 51NG97, as shown in Table 5. It was noted that runner length varied considerably from leaf to leaf as well as within the same leaf. This same effect was also observed when helminthosporoside was placed on sugarcane leaves. Therefore, the large variation in response makes quantitation of amount vs. runner length unreliable.

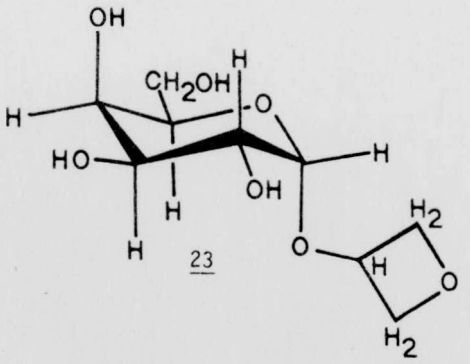
Since allyl α-D-galactopyranoside mimicked helminthosporoside in its reaction with sugarcane leaves, it was of interest to investigate if it biochemically bound to the same site as helminthosporoside. Halves of sugarcane leaves (clone 51NG97) were placed in a solution of allyl α-D-galactopyranoside (22 mg/ml) and the other leaf halves were

Table 5. Runner length for the amount of allyl α-D-galactopyranoside placed on sugarcane clone 51NG97.

Allyl α-D-galactoside, μg	Runner length at 36 hrs, cm
600	4.9
500	4.0
450	2.1
400	0.4
350	2.9
300	1.9
250	0.9
200	1.9
150	1.1
70	0.2
35	0.4
15	0.0

placed in water for five hours. Both halves of each leaf were then injected with a solution of helminthosporoside. Those halves soaking in water obtained runners, while the halves soaking in allyl α-D-galactopyranoside were unaffected. However, after two days the sections of leaves soaking in allyl α-D-galactopyranoside became a slight yellow-brown color.

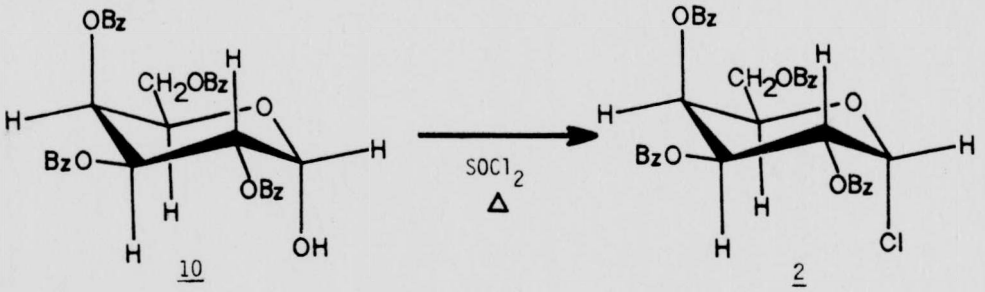
With the observation that the epoxide, (17), was not phytotoxic, a new working structure, (3-oxetanyl) α-D-galactopyranoside, (23), for helminthosporoside was proposed, as shown in Figure 21.



23

Figure 21. The third proposed structure for helminthosporoside.

The carbohydrate moiety, (2), required for a coupling reaction[12] was prepared from 10 and thionyl chloride,[22] as shown in Figure 22.



10 2

Figure 22. Reaction of thionyl chloride on 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside.

The aglycone moiety required for the attachment reaction was 3-oxetanol, (27). The method of obtaining 27 required a four step reaction sequence. Chlorine gas was introduced into allyl alcohol, (18), producing 2-allyloxy-3-chloropropanol, (24). After removal of excess

18, aqueous sodium hydroxide was added and when the solution was heated, 3-allyloxyoxetane, (25), was obtained,[32] as shown in Figure 23.

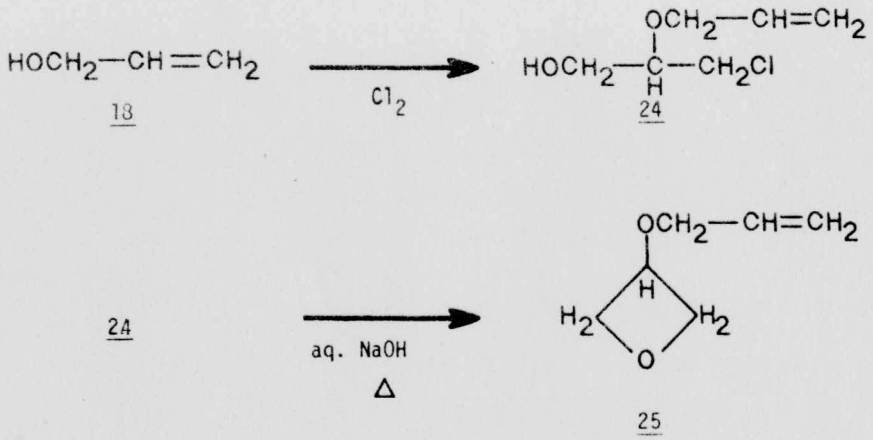


Figure 23. 2-allyloxy-3-chloropropanol from allyl alcohol.

Double bond rearrangement within 25 by potassium t-butoxide in dimethyl sulphoxide produced 3-propeneoxyoxetane, (26),[33] as delineated in Figure 24.

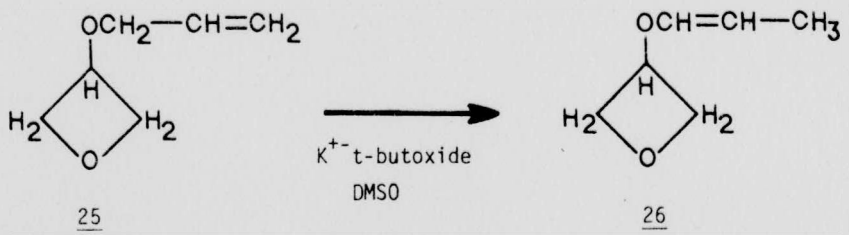
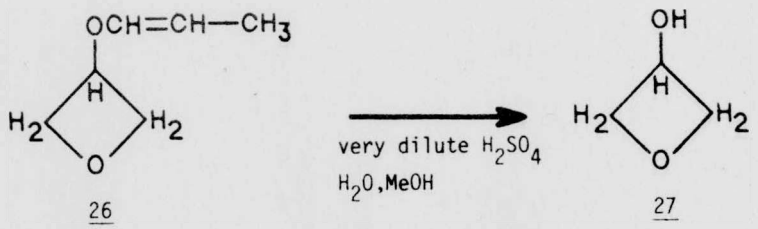


Figure 24. Double bond rearrangement.

The aglycone moiety, (27), was obtained from 26 by a five day, mild, acid hydrolysis (Figure 25).[33]



26 27

Figure 25. Propene hydrolysis to form 3-oxetanol.

The reaction described by Figure 25 was monitored by glc at timed intervals to evaluate the necessity for a five day time period. A plot of the data (listed in Appendix A) is depicted in Figure 26, showing that at five days, 90% of 26 was converted to 27. This result corresponded quite closely to a reported five day hydrolysis, with an 85% conversion.[33]

The reaction[12] to couple 27 with 2 (Figure 27) in an attempt to produce (3-oxetanyl) 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside, (28), actually produced the β-anomer, (29).

Hydrogenolysis of 29 would be expected to yield (3-oxetanyl) β-D-galactopyranoside, (30), the opposite anomer of the proposed structure, (23), as shown in Figure 28. However, the hydrogenolysis did not go to completion. Since amines can poison catalysts, 29 was prepared in a way to be certain that all 2,6-lutidine was completely removed, still

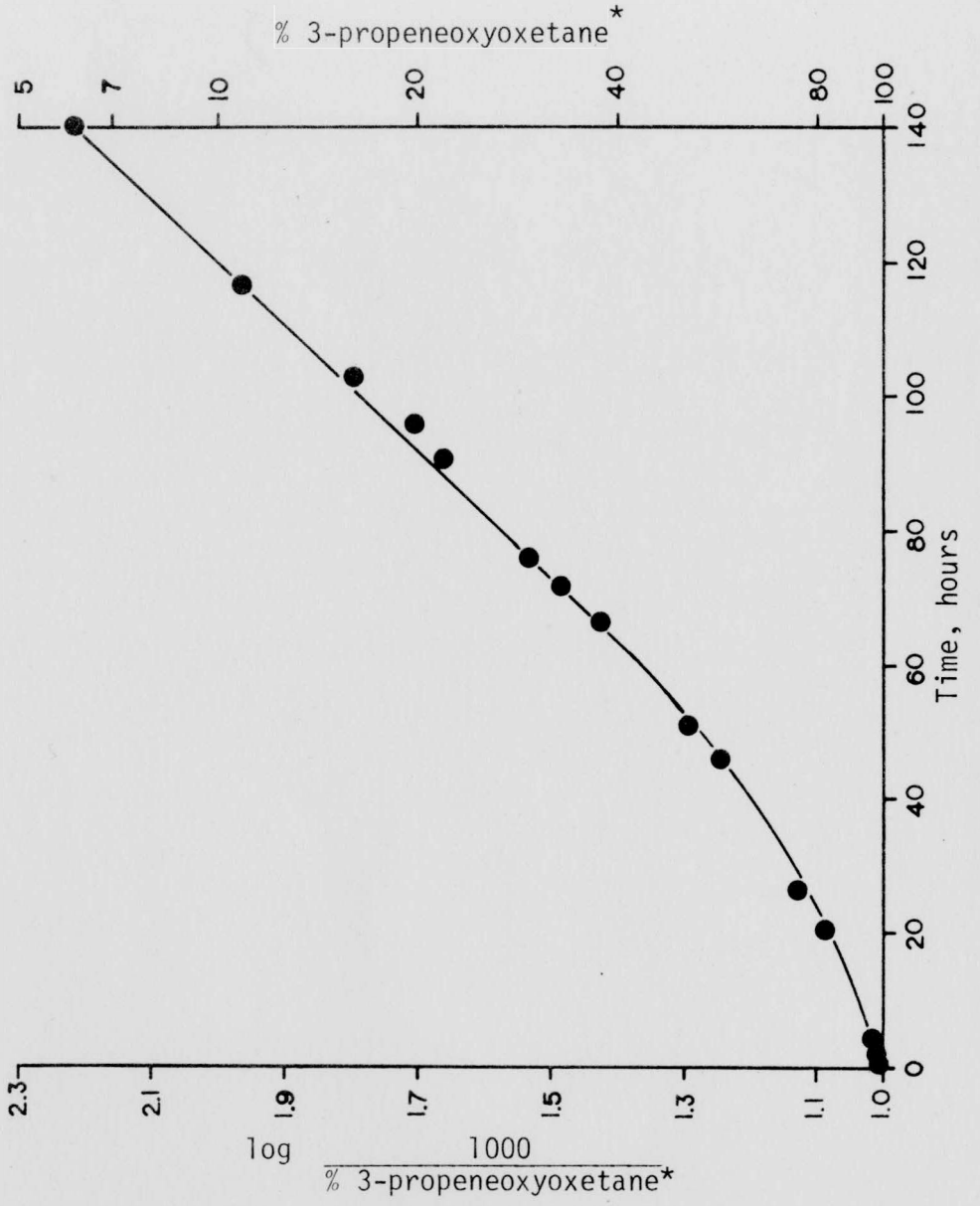


Figure 26. Percent decrease of 3-propeneoxyoxetane with time.

*Yields are normalized to 100%

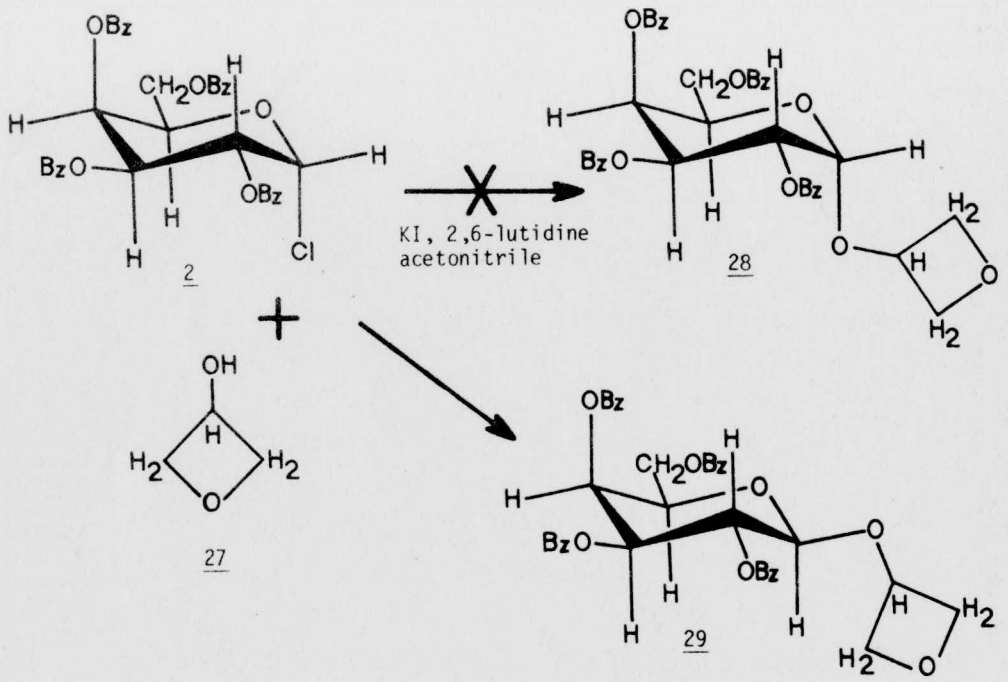


Figure 27. Formation of oxetanyl perbenzylated galactoside.

no complete hydrogenolysis was observed. Since acid can increase the rate of hydrogenolysis, acetic acid was added. Again, no further reaction was observed.

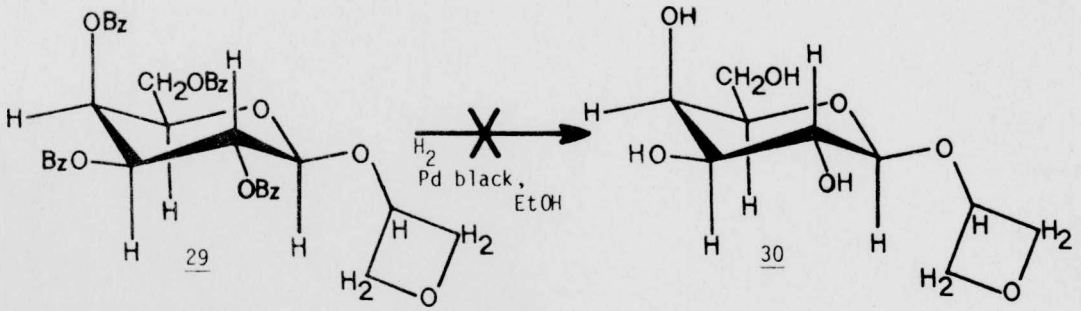


Figure 28. Expected hydrogenolysis reaction.

A model compound, benzyl cyclohexylether, (32), was synthesized from cyclohexanol, (31), with the addition of sodium hydroxide and benzyl chloride, as shown in Figure 29.[34]

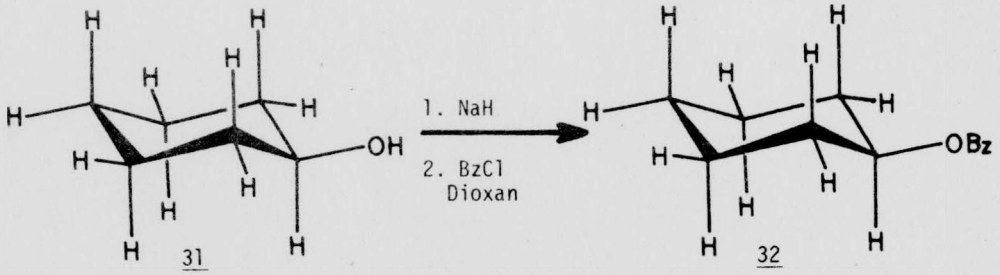


Figure 29. Benzylation of cyclohexanol.

Hydrogenolysis of 29 was attempted again without success, then the catalyst was filtered from the mixture, washed with methanol and placed in an ethanolic solution of 32. Hydrogenolysis of this mixture was attempted for two days, by glc analysis no reaction took place. Hydrogenolysis of 32 with fresh catalyst was complete within one hour. Therefore, it appeared that the catalyst was poisoned by 29. To confirm the carbohydrate moietys ability to be hydrogenolized, methyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside, (9), was completely hydrogenolized to methyl α-D-galactopyranoside, (7), in one hour, as shown in Figure 30.

It was then necessary to look at the effects of the oxetanyl ring on hydrogenolysis. Different amounts of 3-oxetanol, (27), were added to ethanolic solutions of 32 and hydrogenated for one hour. The results

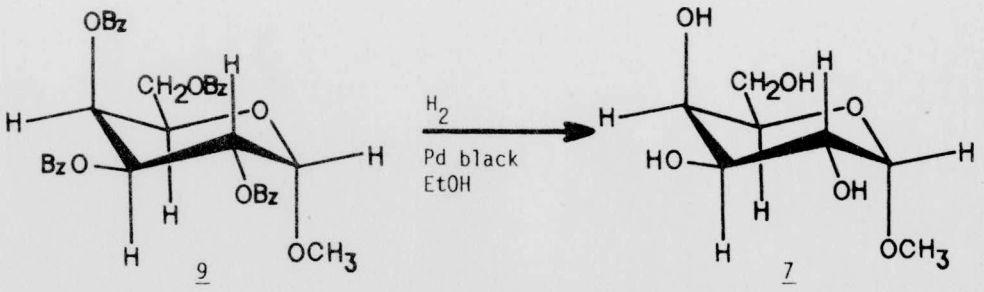


Figure 30. Hydrogenolysis of methyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside.

are tabulated in Appendix B. When the amount of 27 increased, the amount of hydrogenolysis decreased, as shown in Figure 31.

Since the pressure of hydrogen may have an effect on hydrogenolysis, 29 was hydrogenolized with 60 psi of hydrogen. There was no observable enhancement of cleavage. To increase the hydrogen pressure over 60 psi, a Parr bomb was used. With a sample of 29 in ethanol, 1000 psi of hydrogen was applied for 2 hrs. The result of this experiment, as seen by thin-layer chromatography, was the same as with lower hydrogen pressure. Since the benzyl protection could not be totally removed by hydrogenolysis, a dissolving-metal reduction was attempted. Sodium-ethanol reduction did not remove all of the benzyl groups. It was concluded that this molecule, (29), containing the oxetane ring was not suited for easy benzyl group removal. Therefore, another method was required for obtaining 23.

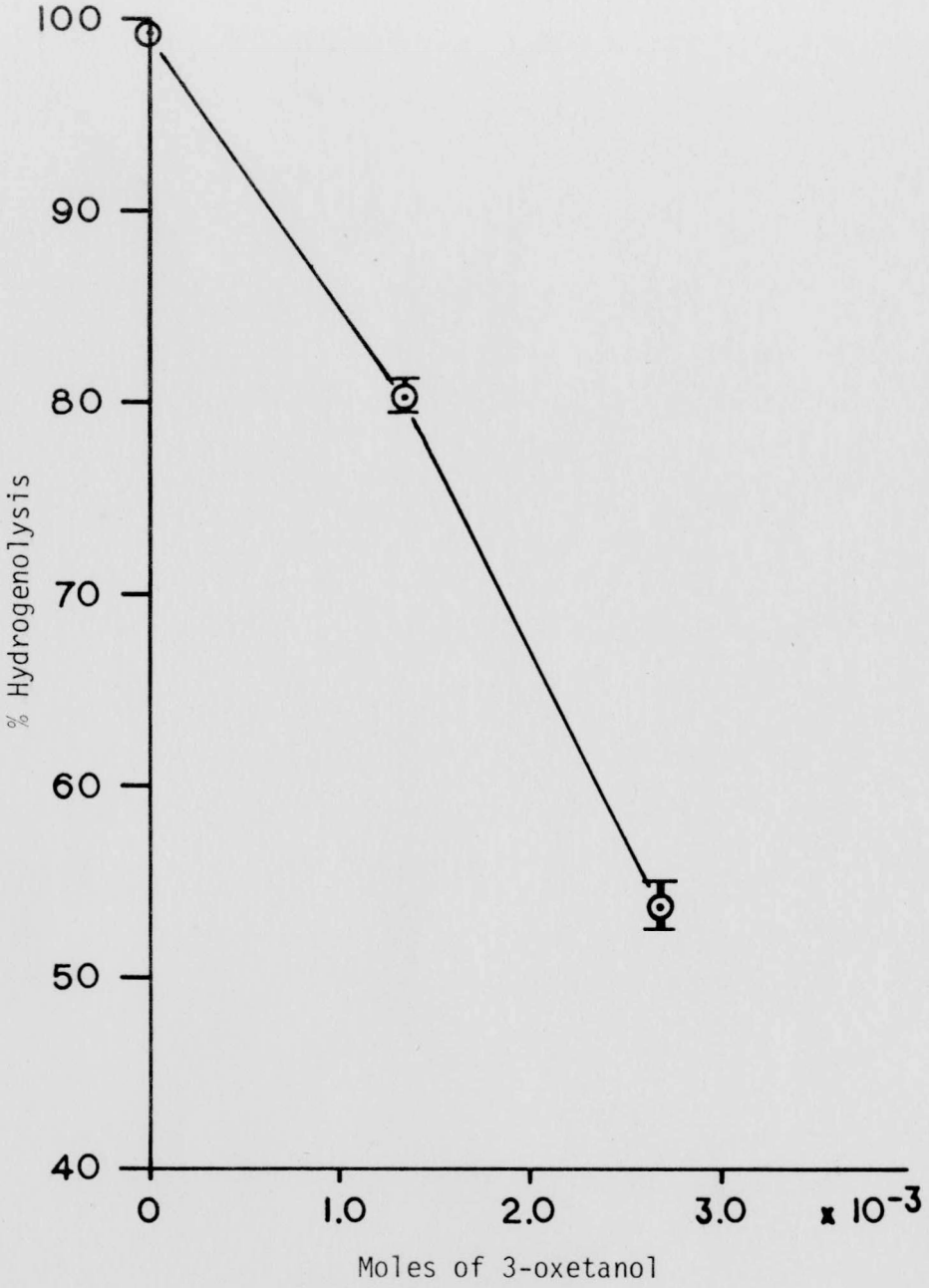


Figure 31. Percent hydrogenslysis vs. moles of 3-oxetanol.

*Yields are normalized to 100%.

A second methodology for making 23 utilized an anomer rearrangement (anomerization) of the β- to the α-anomer. A peracetylated-β-galactoside is commonly anomerized by either titanium tetrachloride or boron trifluoride-etherate.[16] To obtain starting material for the required Koenigs-Knorr glycosidation reaction, a multiple step, one-pot reaction was utilized, with the preparation of 2,3,4,6-tetra-O-acetyl-α-D-galactosyl bromide, (33), from D-galactose, (6),[35] as shown in Figure 32.

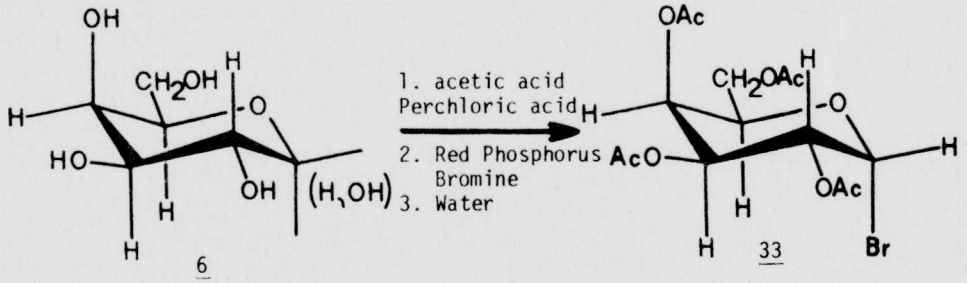


Figure 32. Bromo-acetylation of D-galactose.

The coupling of 27 to 33 (Figure 33) was accomplished with the aid of silver carbonate, Drierite and 3Å molecular sieves, giving (3-oxetanyl) 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside, (34). Proton NMR spectroscopy gave no indication for the alpha anomer, as evidenced by the lack of a doublet from 5.0-5.4 ppm. However, with this type of reaction (Figure 33), one generally anticipates obtaining 1-5% alpha anomer. In a concentration of 1-5%, the alpha anomer might not be observed by NMR spectroscopy. The reaction mixture was subjected to glc

and two components were collected, one being present in considerably larger quantity than the other. When these components were compared by thin-layer chromatography, their R_F values were identical and equal to that of the product, (34). Therefore, it is suggested that the reaction mixture contains both alpha and beta anomers.

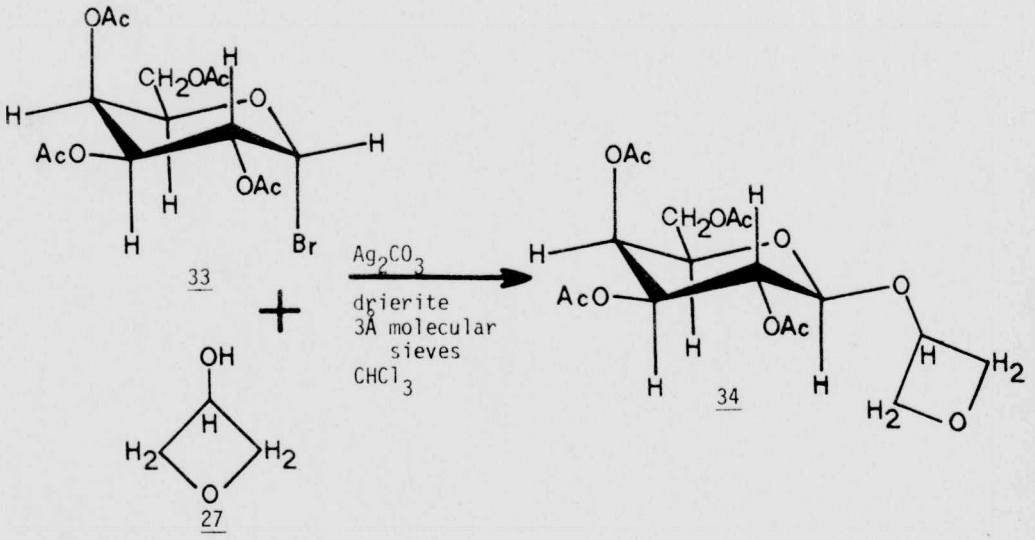


Figure 33. Koenigs-Knorr glycosidation.

With a catalytic amount of sodium methoxide, 34 was deacetylated— as depicted in Figure 34— to produce (3-oxetanyl) β-D-galactopyranoside, (30).[36] The product mixture was bioassayed on susceptible sugarcane leaves, but, no lesions formed. After deacetylation, paper chromatography was used to separate the reaction mixture into two products. Neither of these were active on susceptible sugarcane leaves.

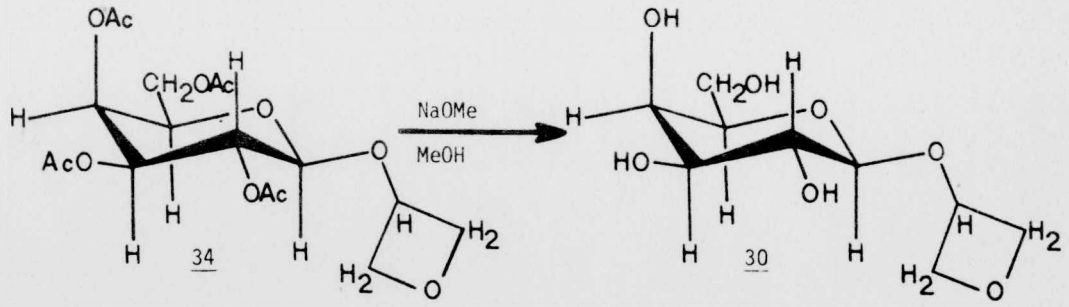


Figure 34. Deacetylation to produce (3-oxetanyl) β-D-galacto-pyranoside.

To obtain a larger proportion of the alpha anomer, titanium tetrachloride was used in an anomerization reaction,[16c] as shown in Figure 35. Rather than obtaining the expected alpha anomer, (35), the chloride, (36), was obtained. It is known that titanium tetrachloride can displace a glycosidic acetyl group, to produce an alpha chloride.[37]

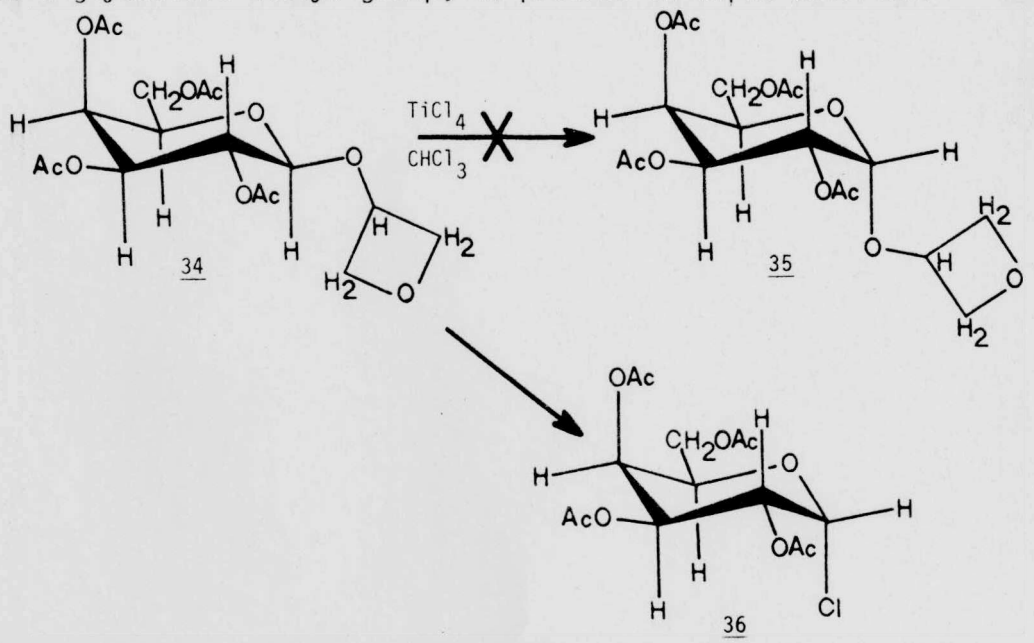


Figure 35. Attempted anomerization with titanium tetrachloride.

Since titanium tetrachloride did not facilitate the expected anomerization, boron trifluoride-etherate was used,[16ab] as described in Figure 36. With these reaction conditions, only polymeric materials were produced.

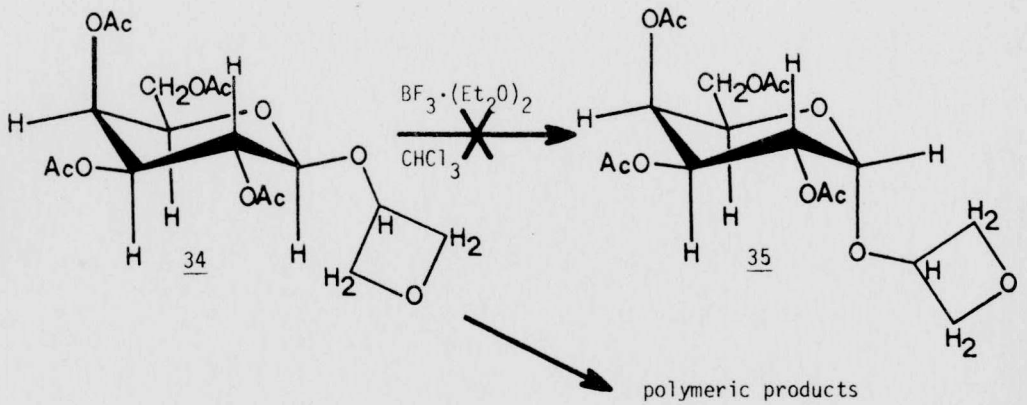


Figure 36. Attempted anomerization with boron trifluoride-etherate.

Polymerization is often observed from compounds containing the oxetane ring. It is especially favorable when using a nonpolar solvent and boron trifluoride-etherate, with no nucleophilic agent present.[38] A possible reaction mechanism is delineated in Figure 37.[39]

While the reaction in Figure 33 was being carried out to produce the intermediate, (34), it was of interest to attempt the synthesis of 23 by a reaction of phenyl 1-thio-β-D-galactopyranoside, (37), with the appropriate alcohol, in the presence of mercuric acetate (Figure 38).[15]

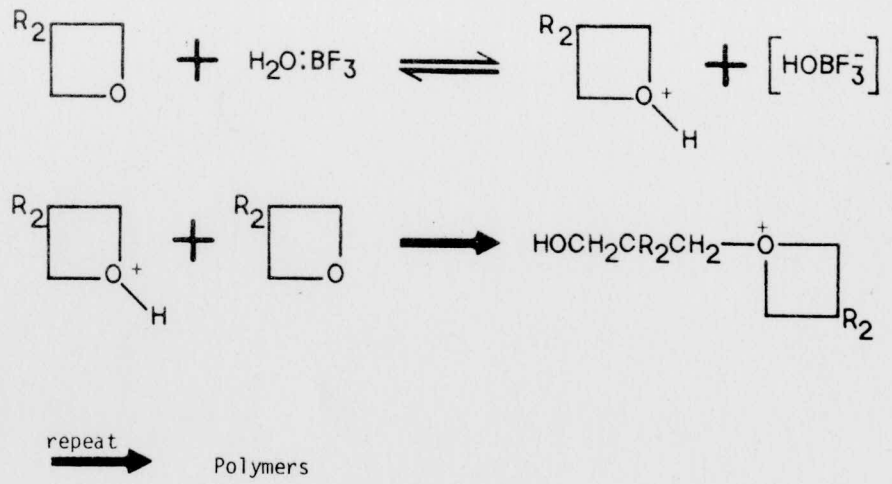


Figure 37. Oxetane polymerization mechanism.

The thio-derivative, (37), was a gererous gift from Dr. Richard Furneaux.[40] A number of simple derivatives were synthesized as model compounds in order to become familiar with the reaction. Table 6 gives these derivatives with respect to the aglycones, listing the R_F and R_{Gal} of each, using silica-gel with the solvent system, (ethyl methyl ketone-ethanol-water, 75:12:6).

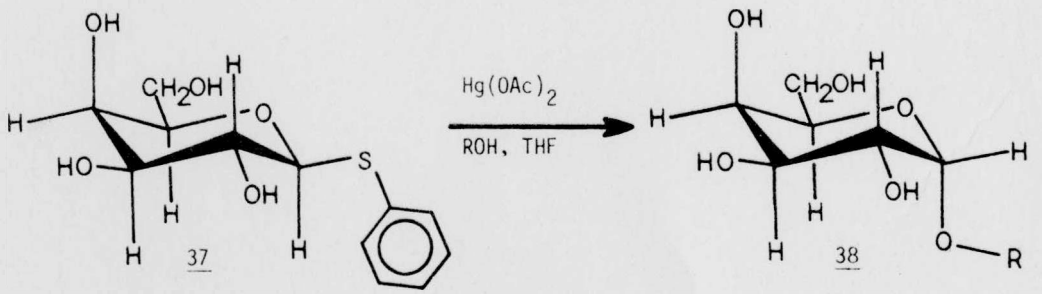


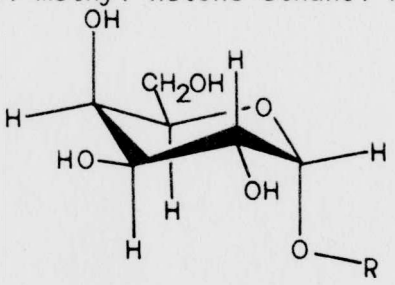
Figure 38. Mercury-ion assisted glycosidation.

The reaction mixture for each product (as shown in Table 6) was bioassayed on susceptible sugarcane leaves. Other than allyl α-D-galactopyranoside, the only derivatives exhibiting activity were the propyl's and ethyl α-D-galactopyranosides. Table 7 lists these compounds, including the amounts assayed and lesions formed. The only derivative exhibiting activity at the 350-700 μg level, was n-propyl α-D-galactopyranoside, which exhibits about one-tenth the activity of allyl α-D-galactopyranoside (Table 5).

Since all reactions for the simple galactosides were well-behaved, except the allyl alcohol experiment— where the double bond reacted with mercuric acetate, the reaction with 3-oxetanol was next attempted. When the mercury-ion assisted reaction of 27 and 37 was carried out (Figure 39), no product was observed. However, when two equivalents of mercuric acetate were used, four products were observed by thin-layer chromatography. The mixture was bioassayed, and no activity was observed. This mixture was not subjected to further separation procedures.

As a consequence of the observation that no oxetanyl derivatives were active, a new working structure was suggested, (39), for helminthosporoside, as depicted in Figure 40. The two isomers, (39a), and (39b), are the last possible combination of carbon, hydrogen, and oxygen atoms that would satisfy the previous physical and chemical data obtained for helminthosporoside.

Table 6. Galactosides produced by a mercury-ion assisted glycosidation, their R_F and R_{Gal} values obtained from thin-layer chromatography on silica-gel using the solvent system (ethyl methyl ketone-ethanol-water, 75:12:6).



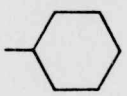
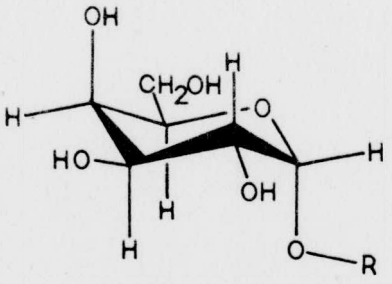
R	R_F	R_{Gal}
—H	0.074	1.0
$-CH_2CH_2CH_2OH$	0.23	3.1
$-CH_3$	0.32	4.3
$-CH_3$ (authentic)	0.29	3.9
$-CH_2CH_3$	0.48	6.49
$-\overset{CH_3}{\underset{H}{C}}-CH_3$	0.58	7.84
$-CH_2CH_2CH_3$	0.60	8.11
$-CH_2-\underset{H}{C}=CH_2$	0.59	7.97
$-CH_2-\underset{H}{C}=CH_2$ (authentic)	0.58	7.34
$-\overset{CH_3}{\underset{H}{C}}-CH_2CH_3$	0.64	8.65
$-CH_2CH_2CH_2CH_3$	0.65	8.78
	0.62	8.38

Table 7. Bioassays on 51NG97 with some α-D-galactosides having two and three carbon aglycones; runner length was determined 3 days after application.



R	length of lesion at 350μg per spot (mm)	length of lesion at 700μg per spot (mm)
$-CH_2CH_3$	1.5	1.5
$-CH_2CH_2CH_3$	3.7	7.0
$-\underset{H}{\overset{CH_3}{\overset{\vert}{C}}}-CH_3$	1.0	2.5
$-CH_2CH_2CH_2OH$	0.0	1.0

The synthetic strategy considered for obtaining the derivatives, (39a), and (39b), was very lengthy and required a large number of intermediates. The dioxan like structure of these compounds was potentially available by the reaction of allyl α-D-galactopyranoside,

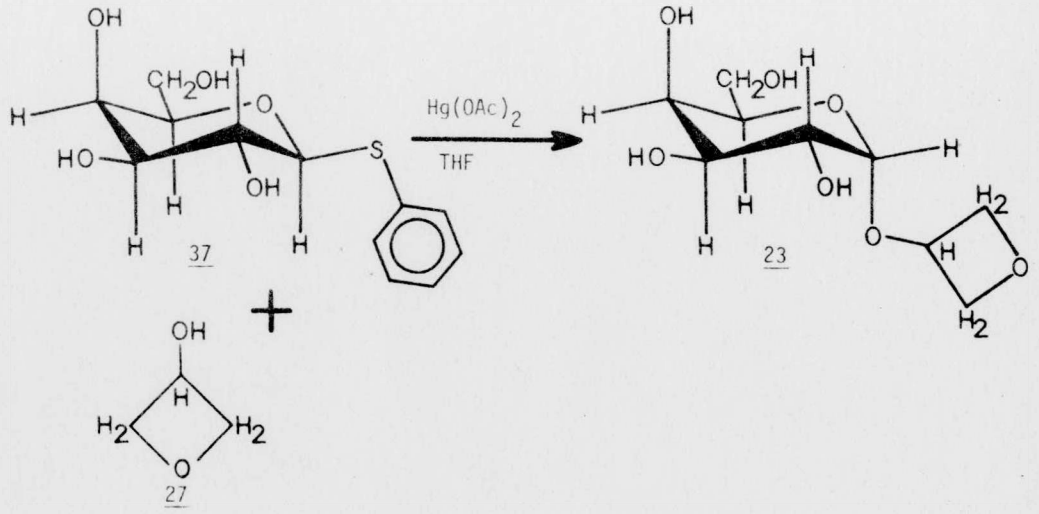


Figure 39. Mercury-ion assisted glycosidation with 3-oxetanol.

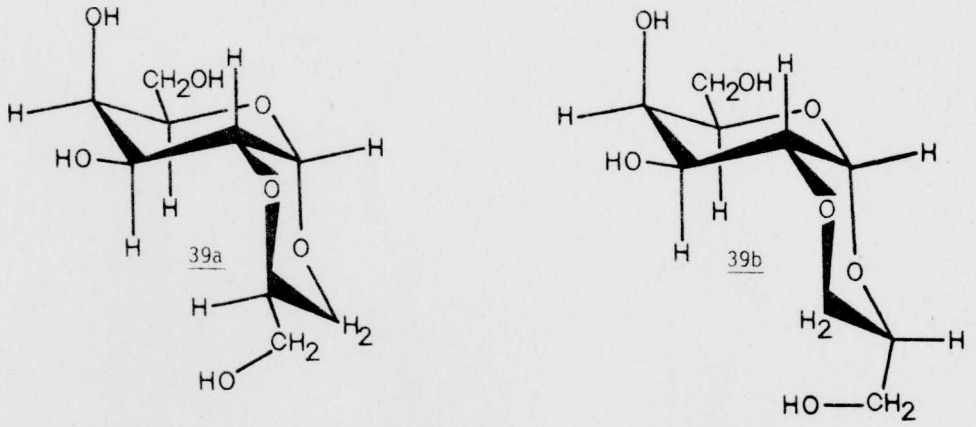


Figure 40. The fourth proposed structure for helminthosporoside.

(20), with a mercury (II) salt via a solvomercuration-demercuration, obtaining 40. Mercuric acetate is known to better facilitate formation of the tetrahydrofuran ring, than the tetrahydropyranyl ring adduct, through an internal solvomercuration-demercuration reaction. Mercuric sulfate, on the other hand, facilitates a larger percentage of the tetrahydropyranyl ring formation.[41] When the reaction was carried out with mercuric acetate, the only product observed was 41, as indicated by Figure 41.

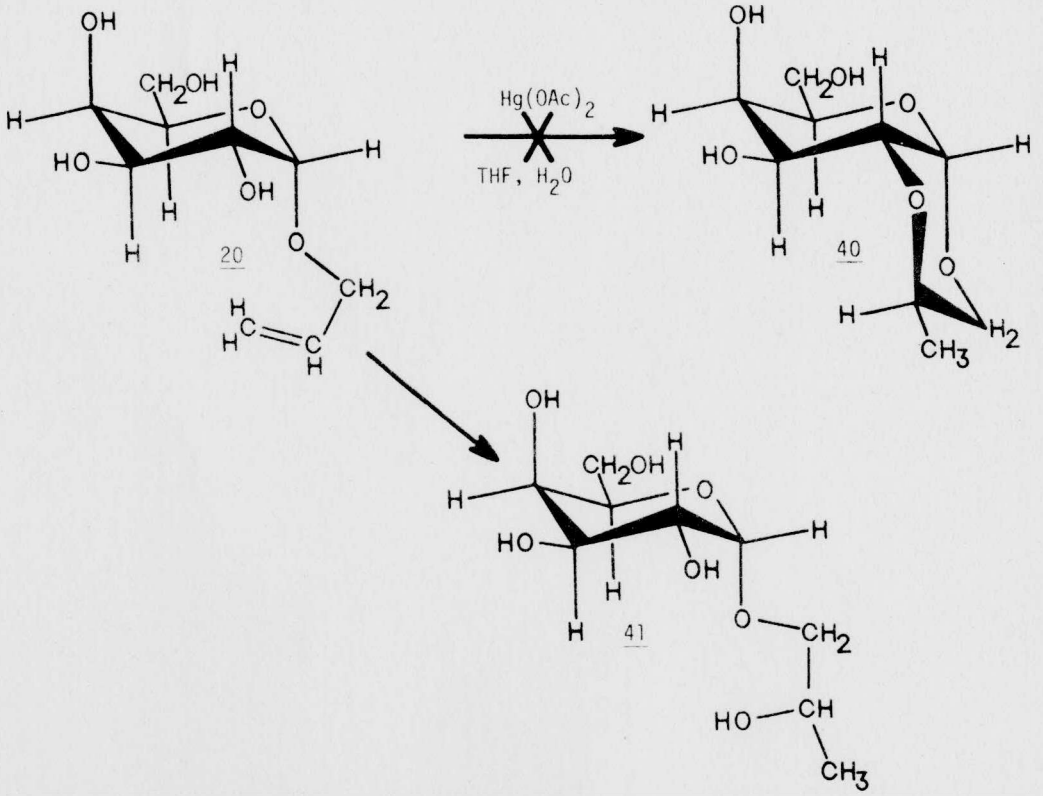


Figure 41. Solvomercuration-demercuration with mercuric acetate.

Upon reacting 20 with mercuric sulfate, two products were observed (Figure 42), the lesser mobile, by thin-layer chromatography, was identical to the product; (41), obtained with the mercuric acetate reaction. The other product had a chromatographic mobility consistent with that expected for 40. This mixture was observed not to be phytotoxic to susceptible sugarcane leaves. Other than the lack of phytotoxicity of these derivatives, an additional reason for discontinuing this line of investigation presented itself after re-examining the CMR spectrum of helminthosporoside.

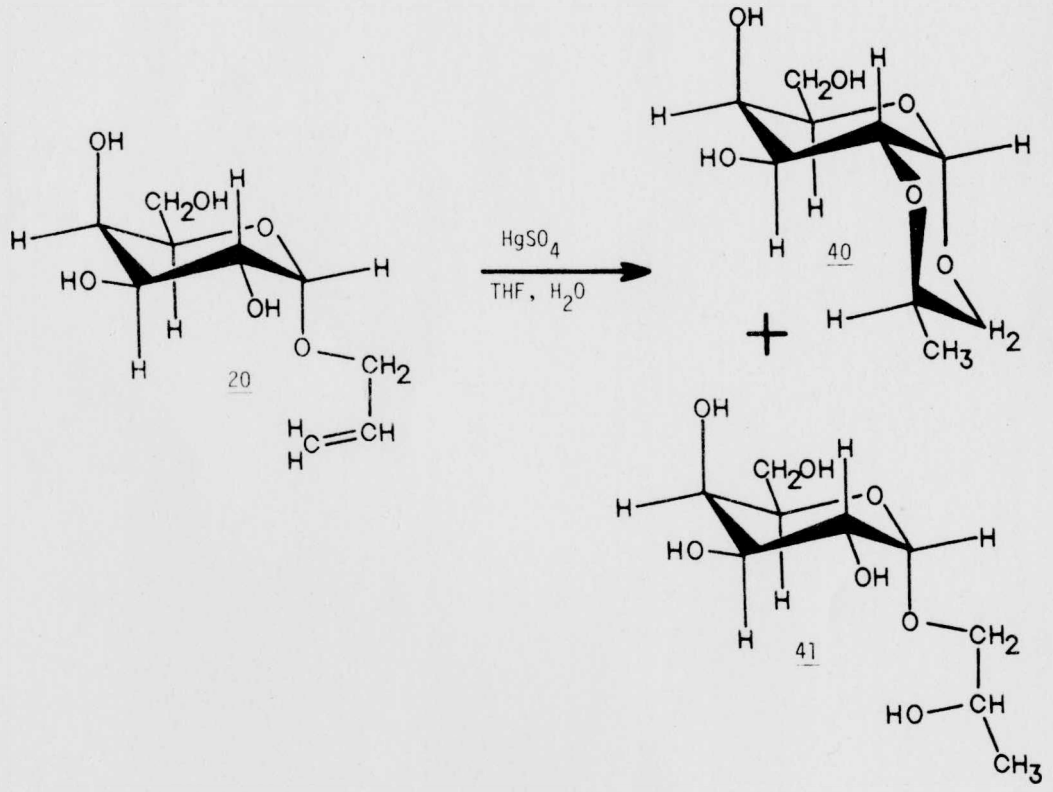


Figure 42. Solvomercuration-demercuration with mercuric sulfate.

In an attempt to verify the presence of a C-2 linkage in the toxin, a method utilizing reported CMR data was investigated. The method will be discussed in detail in Section 3. The CMR resonances from helminthosporoside are found in Table 4 (page 31). Figure 43 shows the method of representing CMR data for easy comparison of helminthosporoside to the two glucoside anomers. The only types of molecule that compared with the resonances of helminthosporoside were the β-D-glucopyranosides, since α- and β-D-galactopyranosides did not favorably compare to any of the nine major CMR resonances obtained for helminthosporoside. Also, from this type of comparison, the molecule does contain a C-2 linkage.

After the latest CMR data analysis, the glucosides, (42), were suggested as possible structures for helminthosporoside, as shown in Figure 44. This structure was contrary to the previous data, i.e., a galactose molecule containing an alpha linkage. In light of this anomaly, it was necessary to abandon all synthetic approaches and concentrate on re-isolation of helminthosporoside, checking for purity and possible ways of advancing the techniques for its isolation and purification.

A flow sheet summarizing the method utilized for isolation of helminthosporoside is given in Figure 45. This method has been used by Strobel's laboratory, and resulted in the isolation of a yellow-brown syrup, indicative of an impure carbohydrate mixture.

None of the purification steps had included activated charcoal,

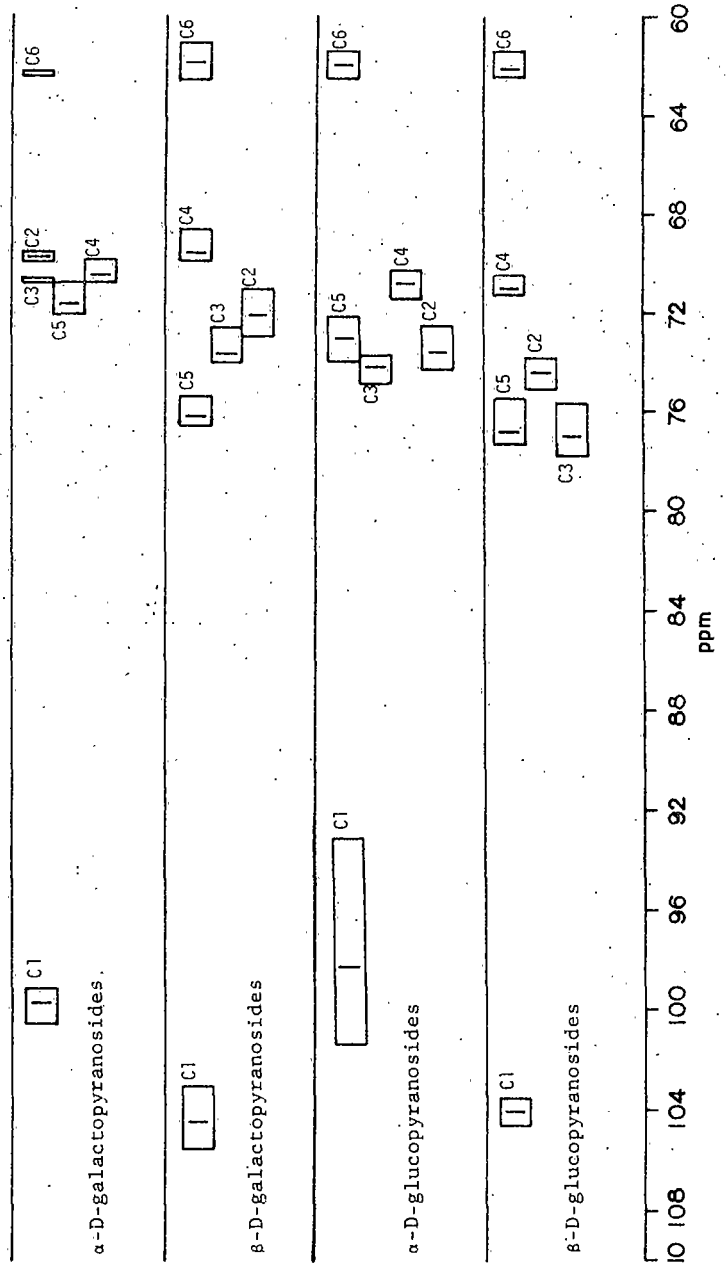


Figure 43. Block diagram method for comparison of CMR data.

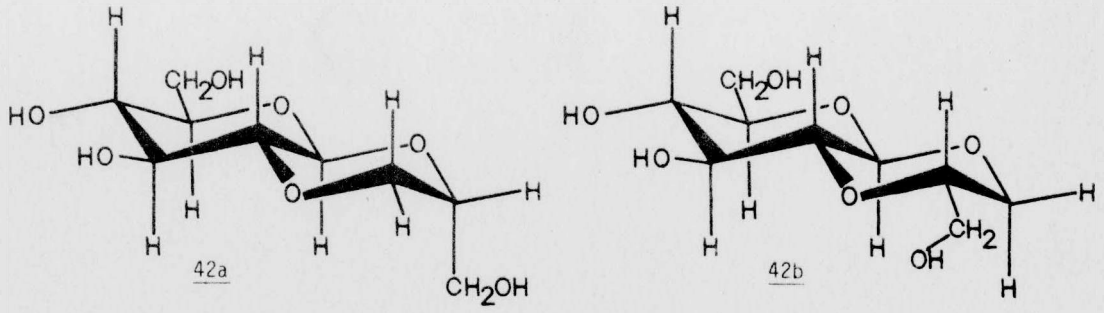


Figure 44. The fifth proposed structure for helminthosporoside.

and thus this seemed a reasonable procedure to include. An ethanolic solution of the toxin preparation was heated to boiling with activated charcoal. The mixture was then filtered, and the filtrate was taken to dryness at 40°C. When the resulting material was bioassayed, there appeared to be no loss of toxic activity. However, even though the physical appearance of the preparation improved, it still had a slight color. The next step pursued in further purification of the toxin preparation involved high presure liquid chromatography, using a μ-bondapak carbohydrate column. To select the proper solvent system, it was important to understand how the toxin behaved relative to other carbohydrates. A series of solvent systems was selected that varied in the percentage of acetonitrile to water. Various carbohydrates, methyl α-D-glucopyranoside, glucose, maltose and maltotriose (Figure 46) were compared to the toxin in these solvent systems.

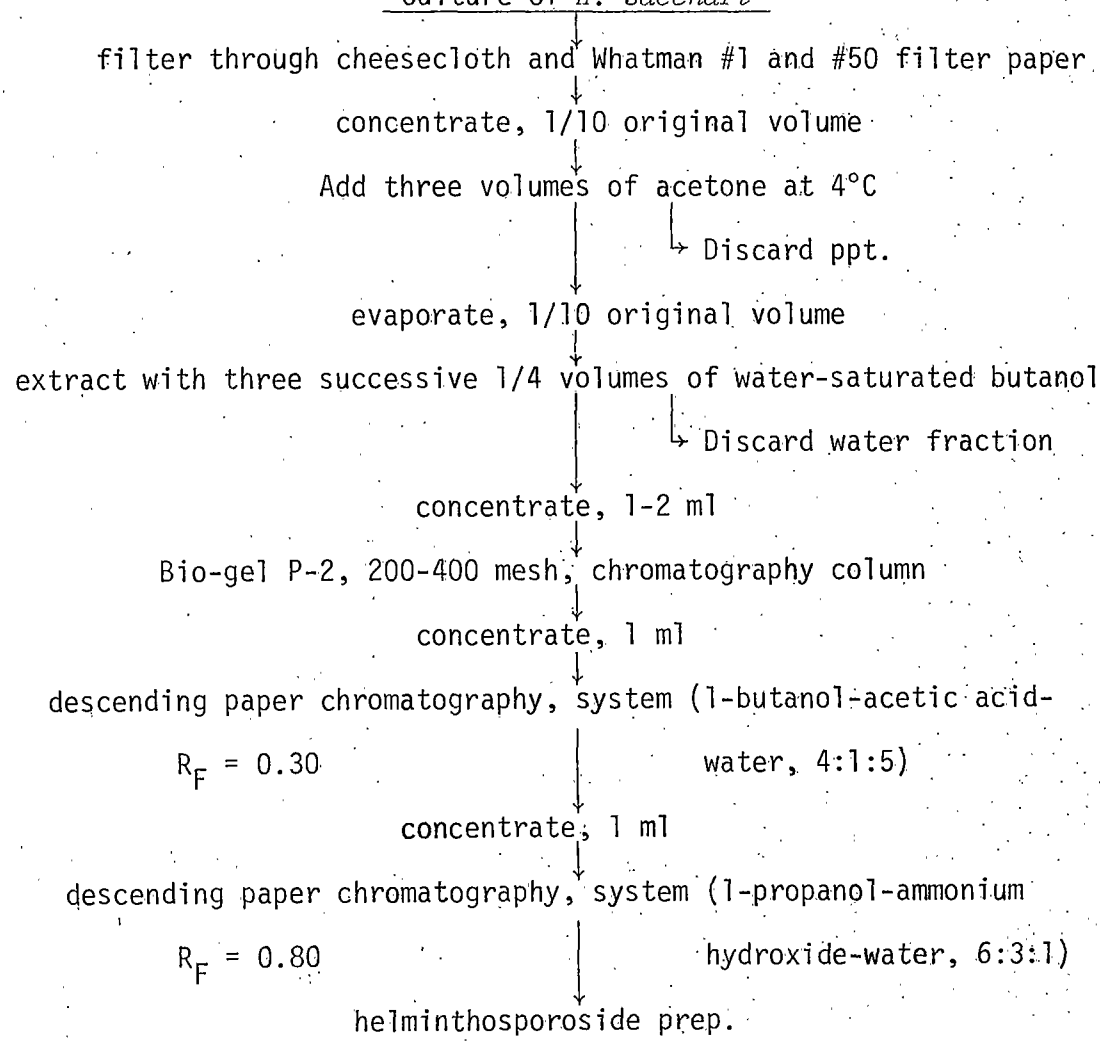
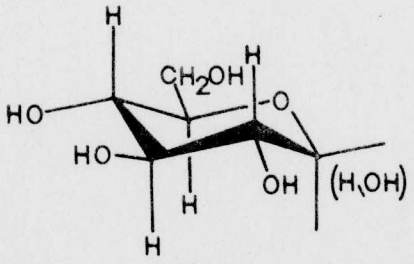
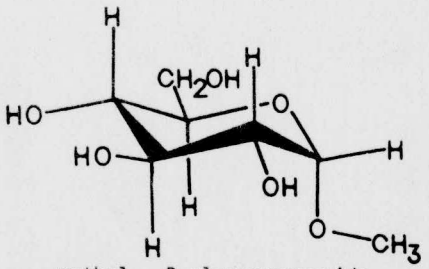


Figure 45. Flow sheet summarizing the isolation procedure for obtaining the helminthosporoside preparation.

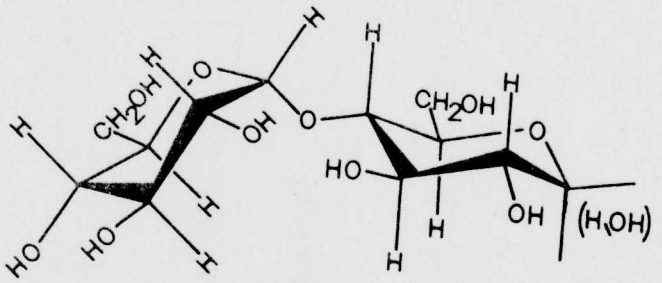
A plot of the data, as seen in Figure 47, shows the toxin midway between the monosaccharide, glucose, and the disaccharide, maltose. Interestingly enough, if the toxin had any one of the earlier proposed structures, it might be expected to have a retention time near that of



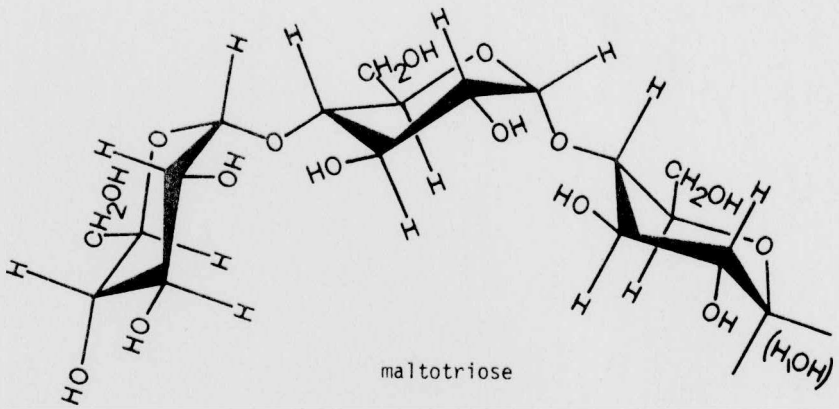
glucose



methyl α-D-glucopyranoside



maltose



maltotriose

Figure 46. The carbohydrates used for comparison on HPLC.

methyl α-D-glucoside, but not greater than glucose. It is important to note that all of the plotted lines, except for methyl α-D-glucopyranoside, intersect at a single point coinciding with the void volume. This occurs at 62.5% acetonitrile. With percentages of acetonitrile less than 62.5, no separation can occur.

The solvent system, (acetonitrile-water, 78:22), was used in the separation of components from the helminthosporoside preparation. A plot of the charcoal-treated helminthosporoside preparation after separation on the μ-bondapak column is shown in Figure 48. The curve represents the detector (refractive index) response. The bar graph represents a measure of a phenol-sulfuric acid analysis for each tube. This analysis was utilized for determining total carbohydrate.[41] Only one region of toxicity was found, and it was always present at about 14-15 minutes from injection, depending on the run. The toxicity is indicated on the bar graph by using solid bars to indicate the distribution of the activity. It was important to wait at least one and two-thirds hours between injections, because of the slow bleed-off of materials from the sample.

The ultraviolet spectrum of the HPLC toxin preparation was also obtained, and is shown in Figure 49. Since the curve is broad (extending from 190-250 nm), there probably is more than one ultraviolet active substance in the preparation.

After separation by HPLC, the active material was pooled and

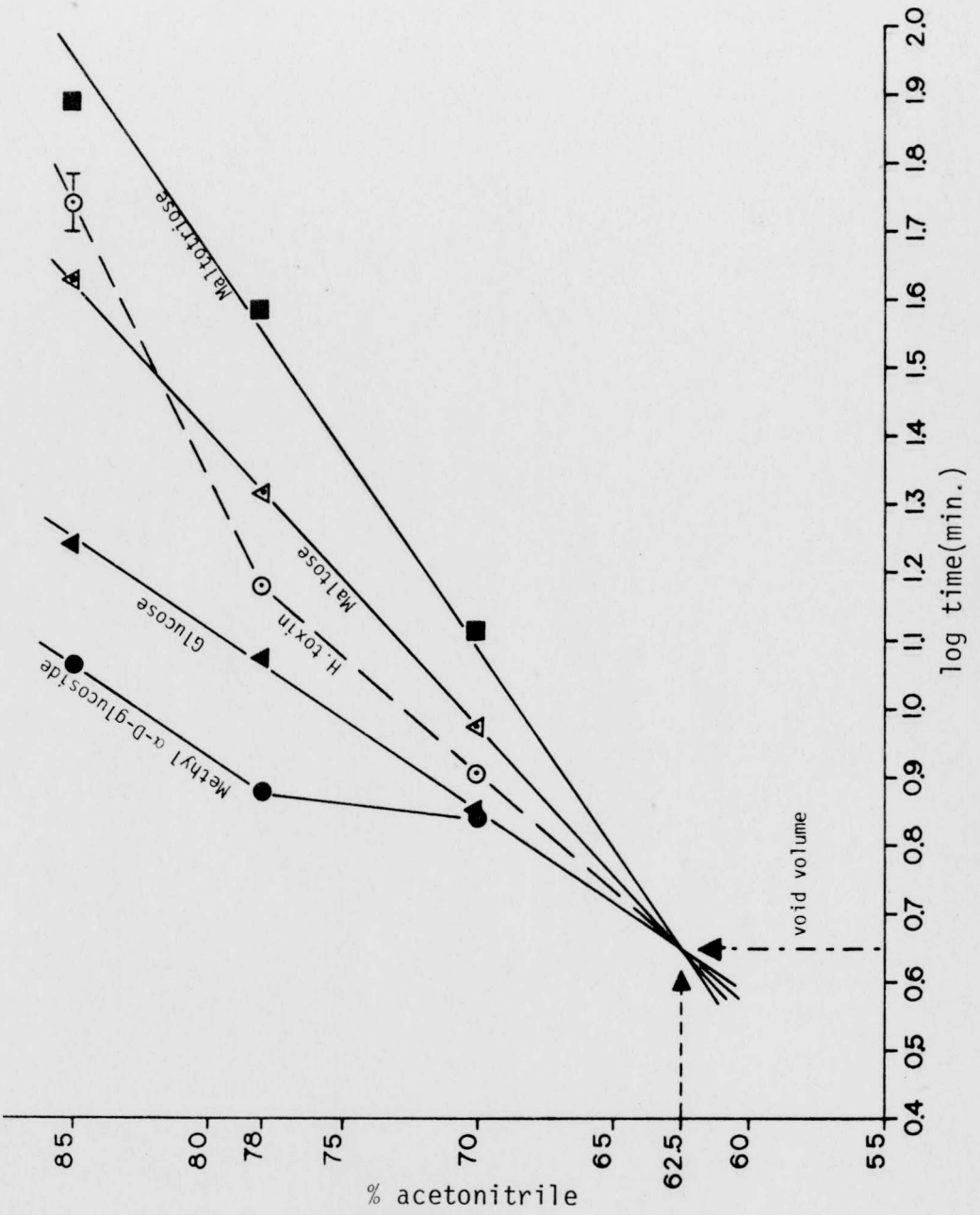


Figure 47. HPLC separation characteristics of some glycosides and helminthosporoside on a μ-bondapak carbohydrate column with solutions of acetonitrile-water.

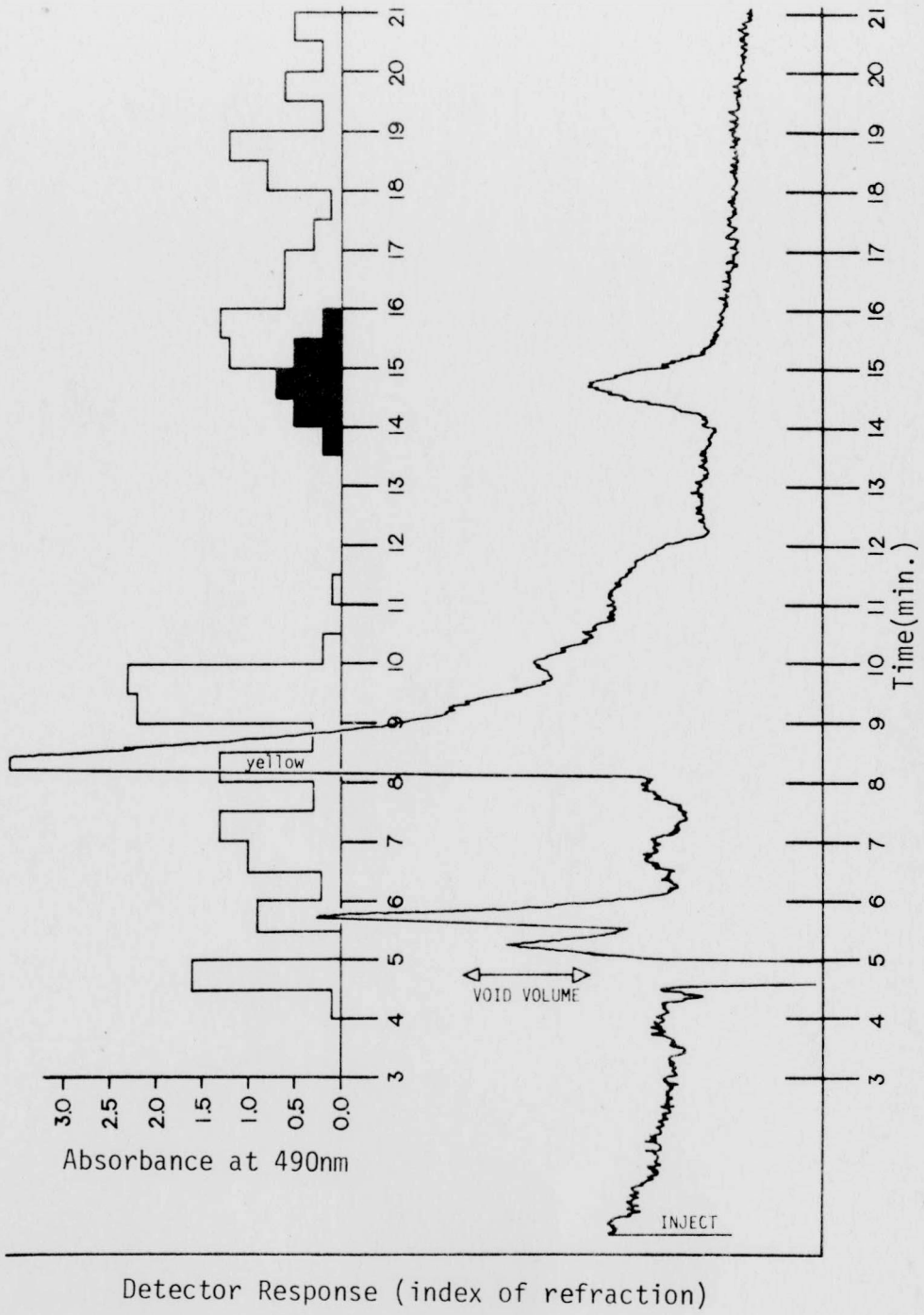


Figure 48. HPLC separation of the helminthosporoside preparation.

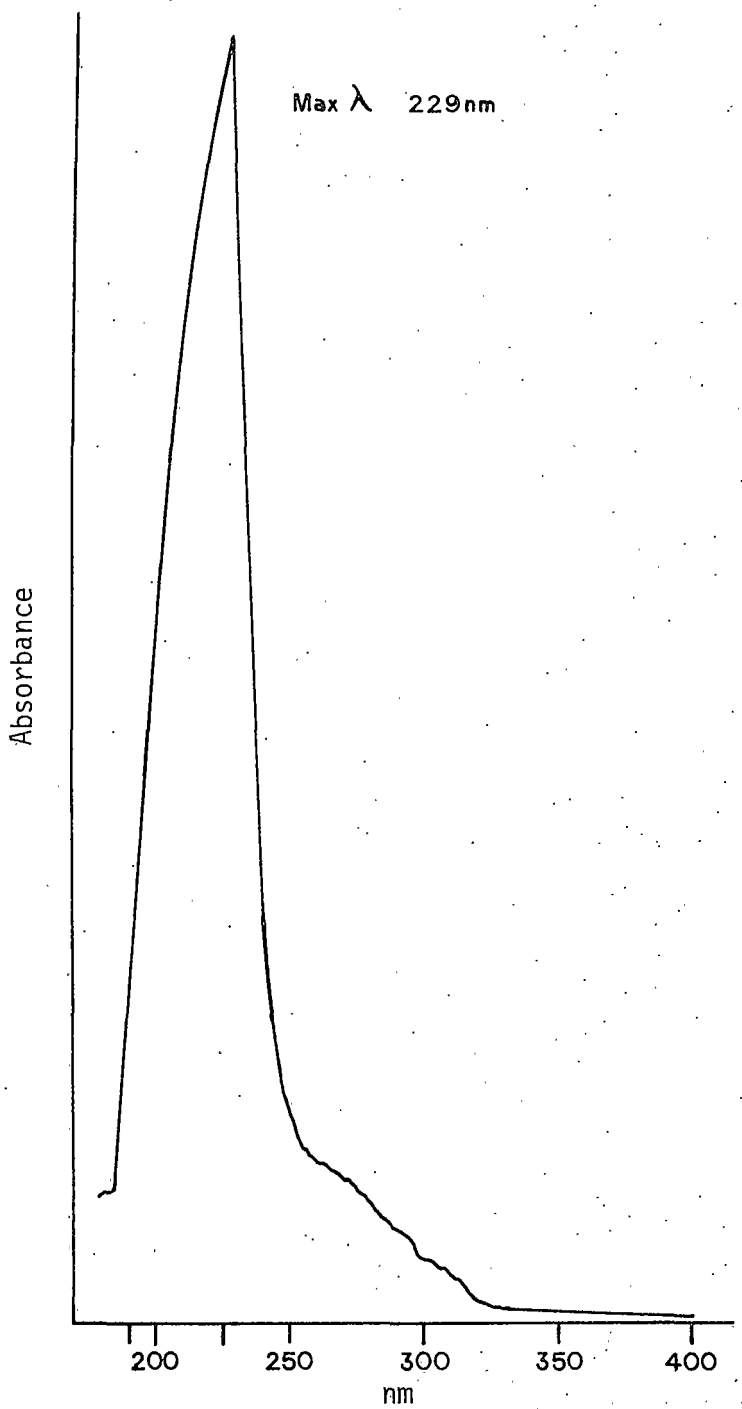


Figure 49. Ultraviolet absorption spectrum of the HPLC preparation

placed on a 295 x 0.8 cm, Bio-gel P-2 (-400 mesh) chromatography column, described in Section 4. The column effluent was monitored by a flow-thru cell at 196 nm, and all peaks were subsequently bioassayed for phytotoxicity. For a typical run, all peaks were checked for primary amine functionality by reacting the fractions with fluorescamine.[43] The material with toxic activity had no reation with fluorescamine, while 75 ng of glycine gave a positive reaction. With a typical run, all fractions around the peak containing toxic activity were analyzed for total carbon, as shown in Figure 50.

To better observe the symmetry of the toxin peak by the ultraviolet monitor and determine whether the toxin would deteriorate by degradation to other components, the fractions within the U.V. absorbing peak, containing toxic activity, were pooled and placed back over the same column (Figure 51). The toxin gave one major peak at 92.9 ml, and did not appear to have degraded during the various manipulations, as evidenced by the lack of other peaks in the elution profile, typical of smaller molecular weight compounds.

An ultraviolet spectrum was then obtained from the pooled toxic fractions encompassing four areas of the peak. A representative ultraviolet spectrum is found in Figure 52. It is interesting to note that simple carbohydrates like glucose, cellobiose, maltotriose and even dextran T-40, have ultraviolet spectra with absorption maxima at 192 nm. But, allyl α-D-galactopyranoside has an ultraviolet spectrum with the

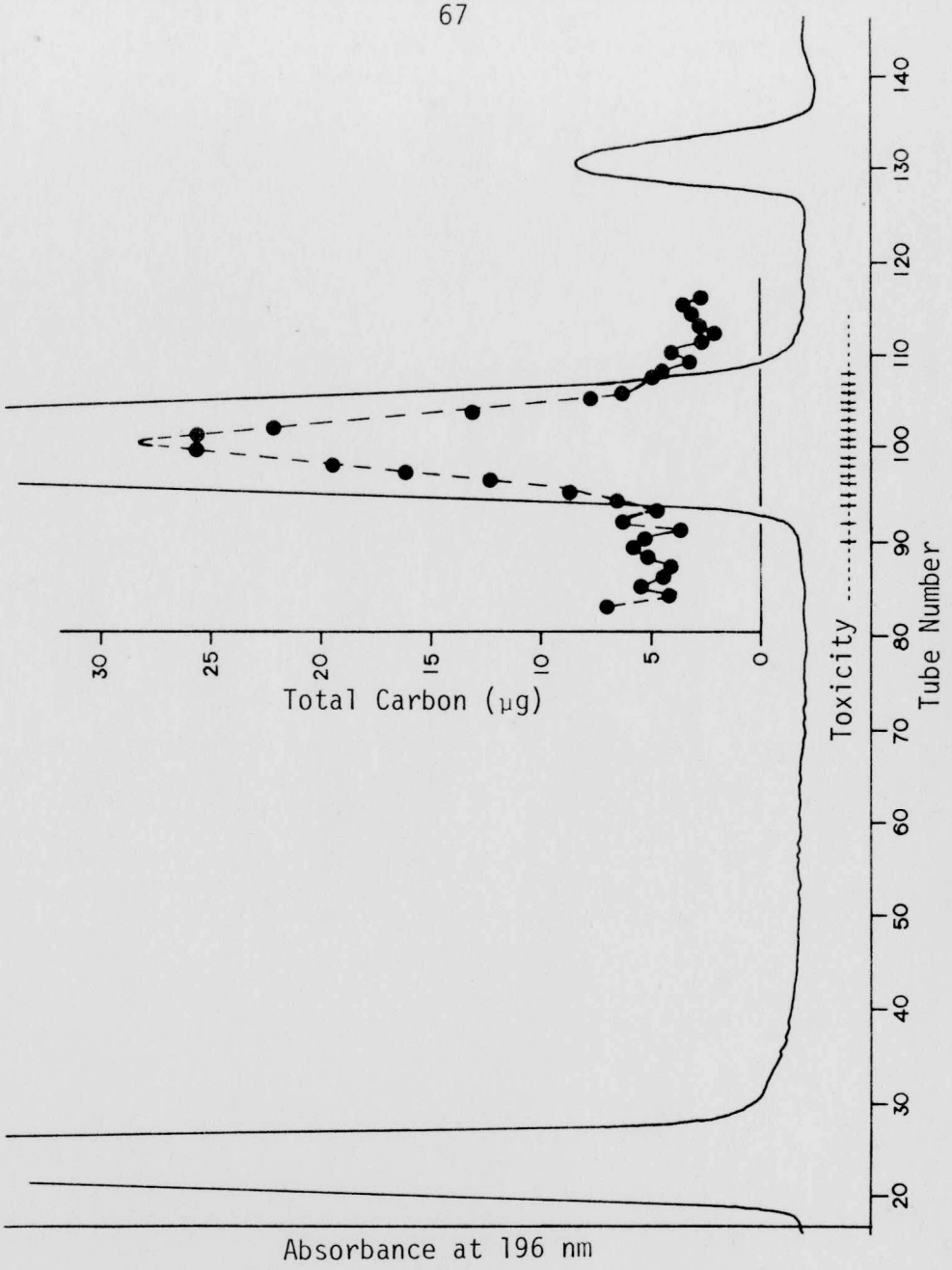


Figure 50. Bio-gel P-2 column separation of the helminthosporoside prep. (after HPLC), and total carbon analysis of the fractions containing toxicity.

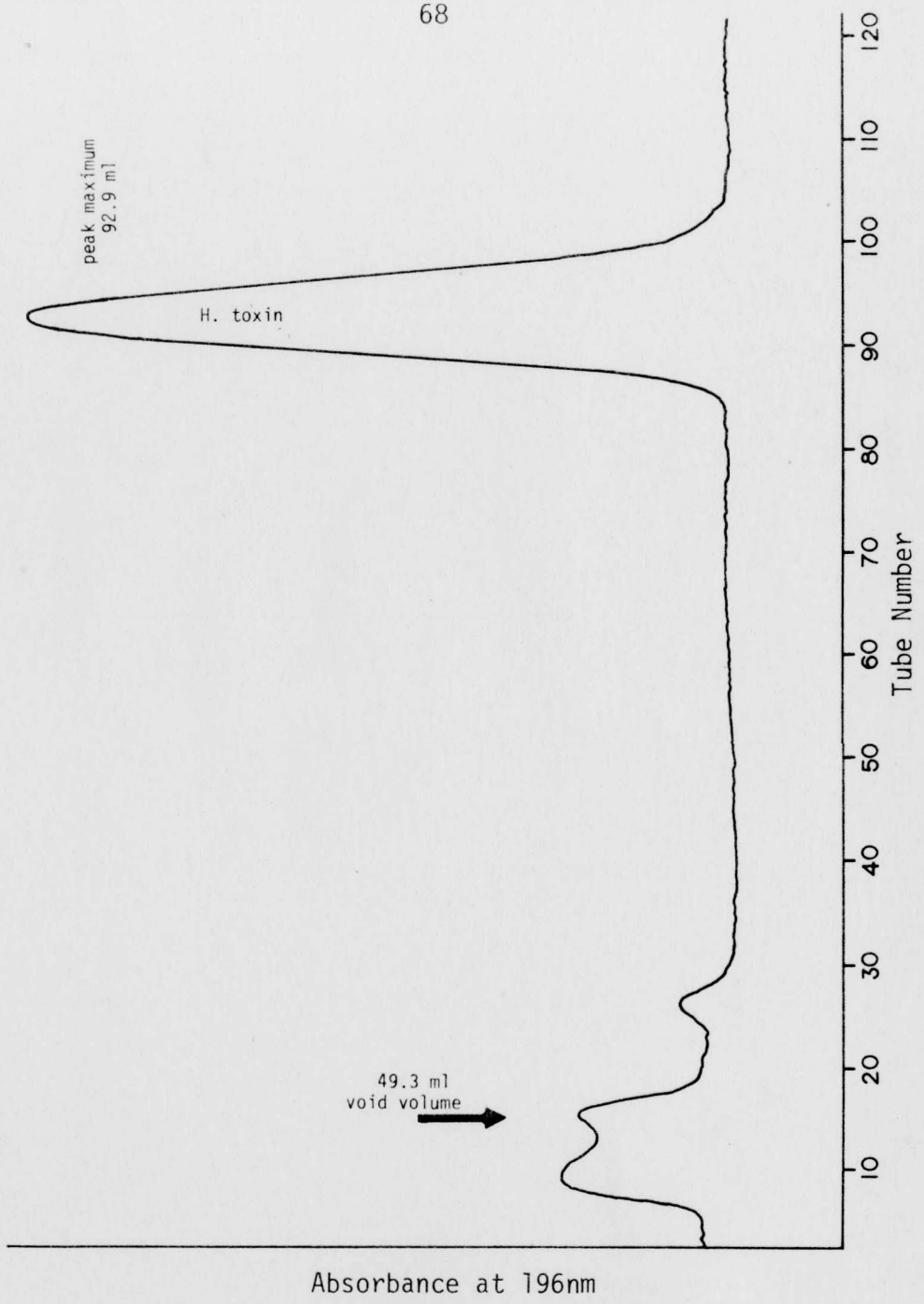


Figure 51. Re-run of the toxic fractions over the Bio-gel P-2 column.

absorption maximum at 195.5 nm. This result is comparable to the absorption maximum of the toxin.

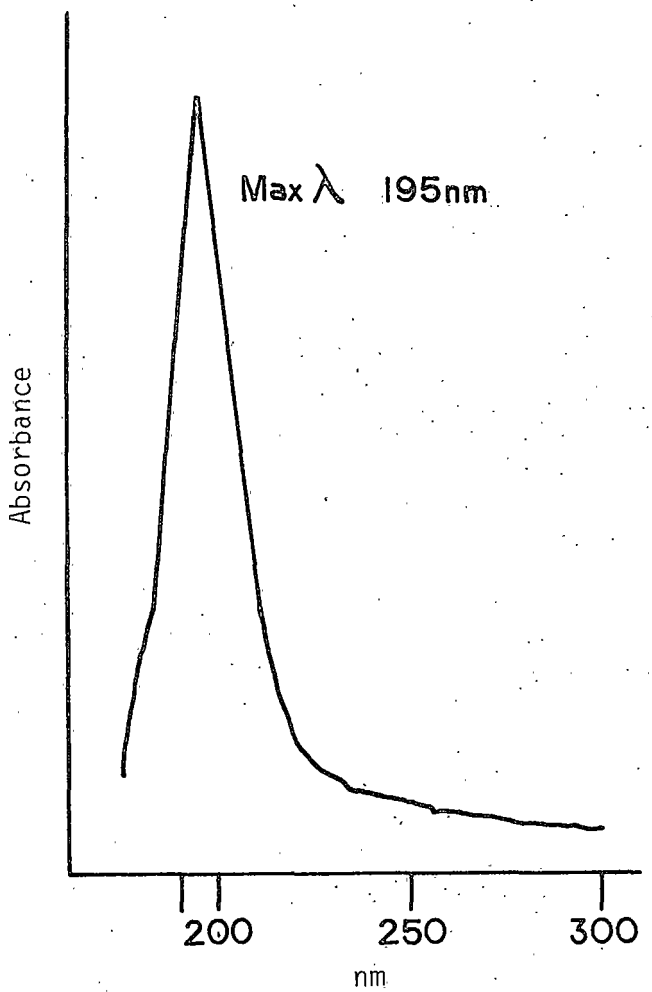


Figure 52. Ultaviolet spectrum of the Bio-gel P-2 purified toxin.

The elution volume obtained for the toxin was compared with values of other α-glycosides. Figure 53 shows the sizing distribution of the α-glucosides in corn syrup on the Bio-gel P-2 column. In the Figure,

the G's represent glucose and subscript represents the number of residues in the oligomer.

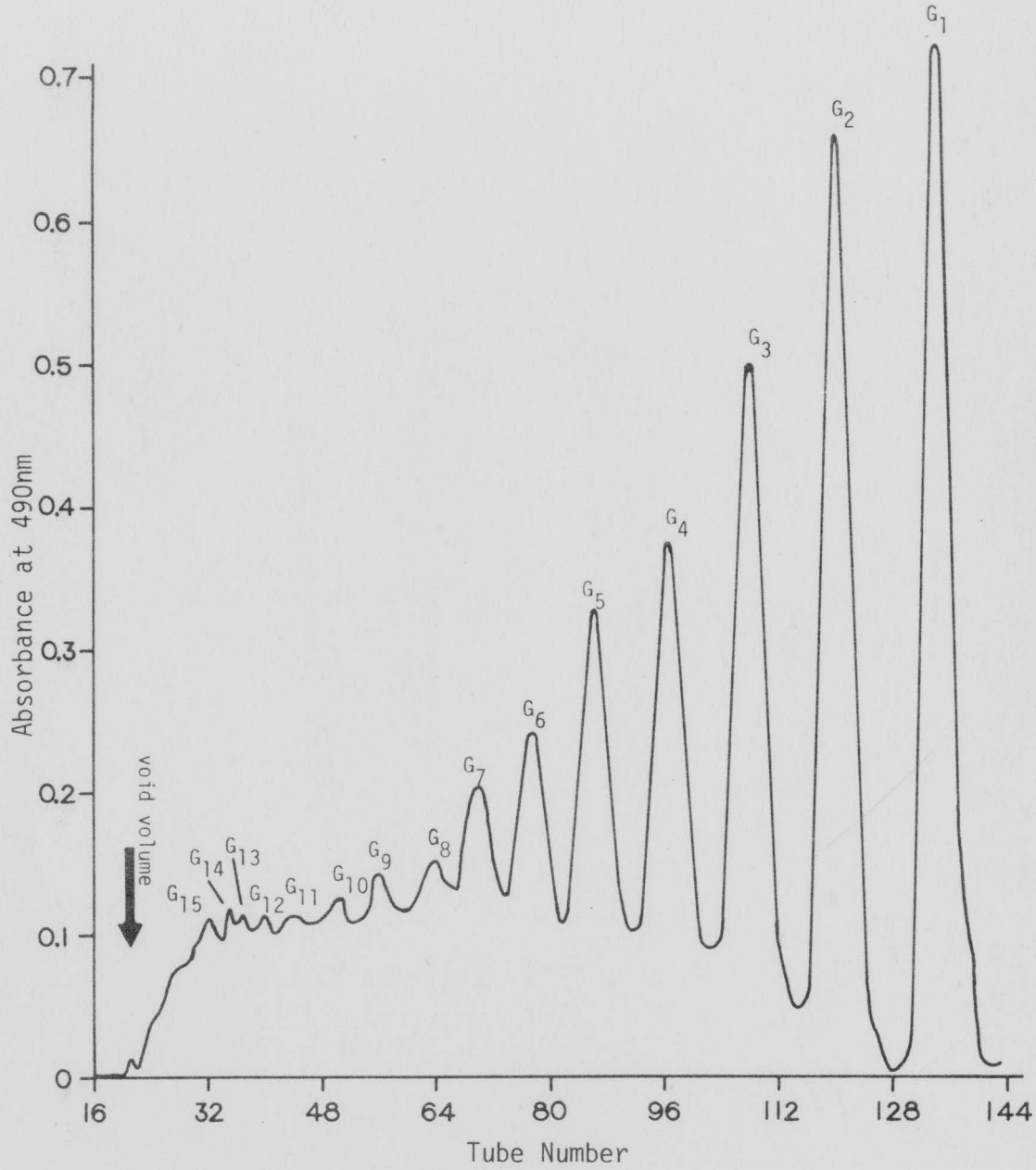


Figure 53. Separation of the α-glucosides in corn syrup.

The plot in Figure 54 is a straight line obtained from plotting (Ve/Vo) vs. log(molecular weight) of the α-glucosides from Figure 53.[44] By comparing the elution volume of the toxin to this plot, the number of residues may be estimated to be 3.65.

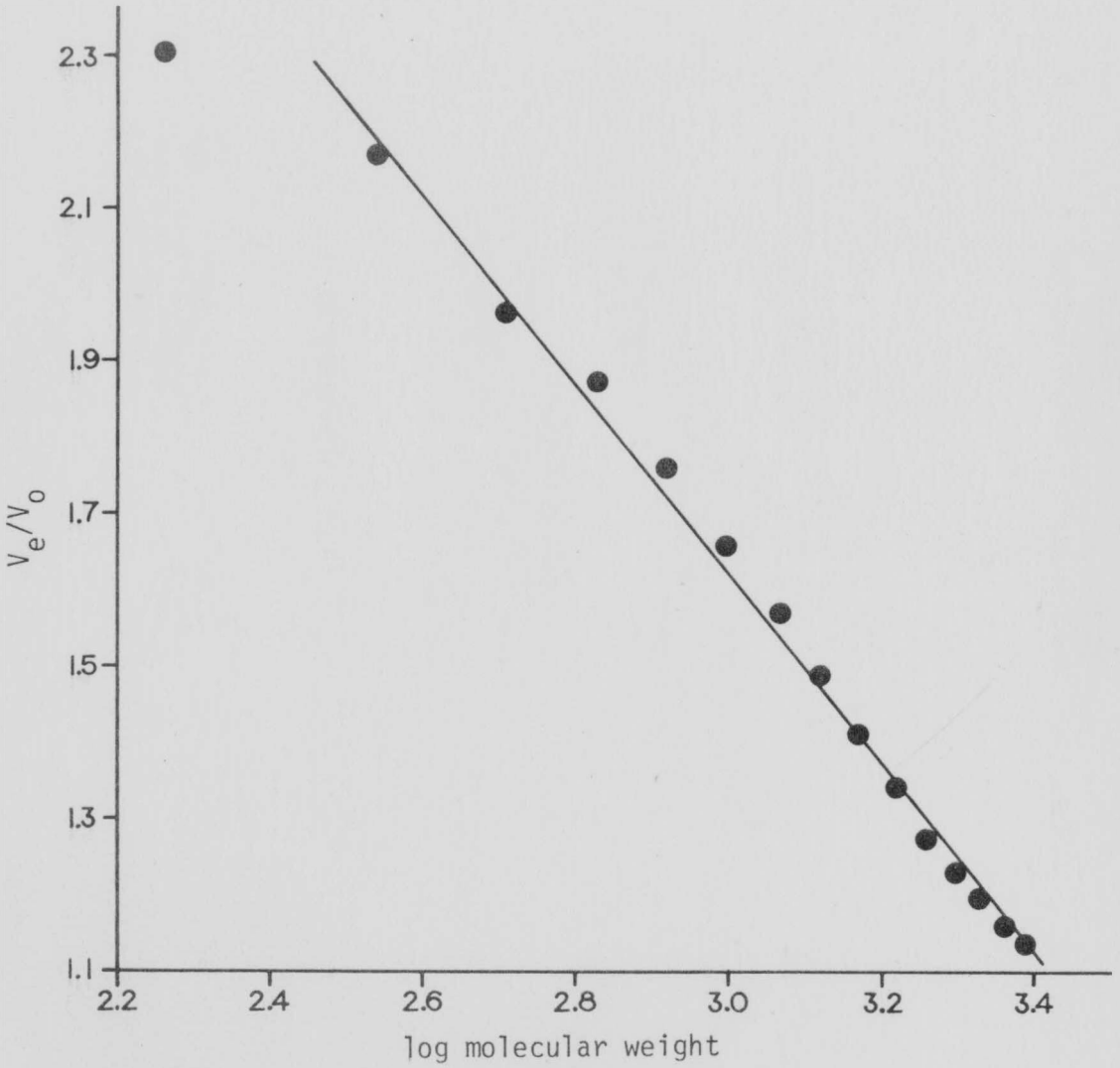


Figure 54. Plot of Ve/Vo vs. log(molecular weight) for the α-glucosides in corn syrup.

It was of interest to compare the elution volume of an α-galactoside, having a three carbon aglycone, to the data for the toxin. Allyl α-D-galactopyranoside was placed on the Bio-gel P-2 column; the elution profile obtained by monitoring at 196 nm is seen in Figure 55. The results obtained for allyl α-D-galactopyranoside are tabulated with the toxin data in Table 8. Thus, it appears that the toxin is approximately 1.25 residues larger than allyl α-D-galactopyranoside. This is interesting since they both have nearly the same R_F values on paper chromatography (Table 9). The difference in size can not be explained by merely adding more carbohydrate residues, since the polarity increase associated with more residues would severly slow the molecule down in paper chromatography systems.

At this juncture it seemed important to look into the development of a better bioassay system to quantitate helminthosporoside. The first consideration was a pollen bioassay; however, after some attempts it seemed an impossibility to obtain sugarcane pollen. The next possibility was to look more closely at the existing method for bioassaying the toxin, with the hope of finding a way to improve on the system. It seemed very important to consider the structure of the leaf while searching for a bioassay system. The sugarcane leaf has its structural base, a midrib, found at the center of the leaf and running from the stalk to the leaf tip. On either side of the midrib are large veins evenly spaced from the center to the edge of the leaf. Between these

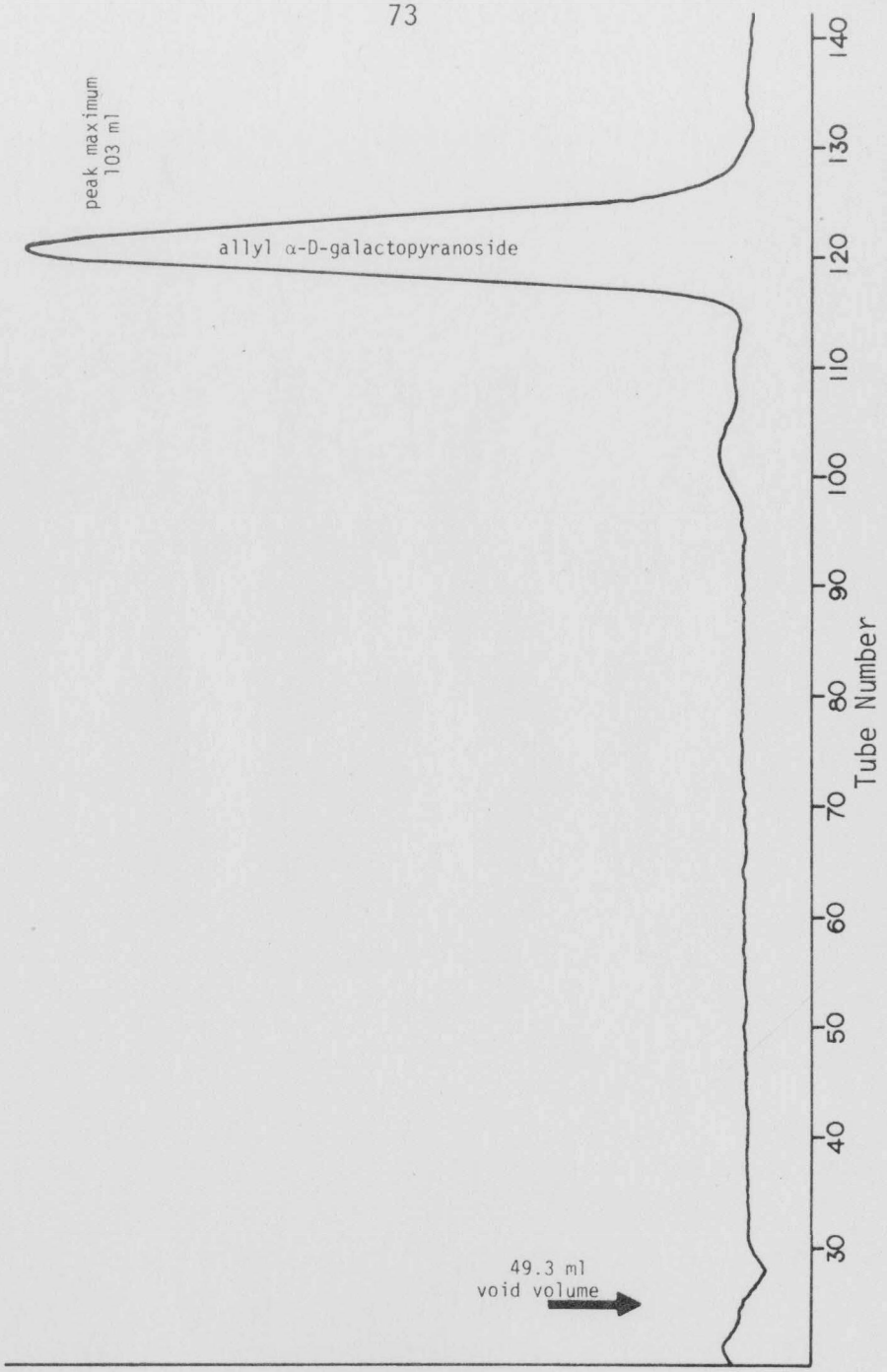


Figure 55. Elution curve of allyl α-D-galactopyranoside on Bio-gel P-2.

Table 8. Elution volume and estimated number of residues for the toxin and allyl α-D-galactopyranoside.

Compound	Elution Volume(ml)	Ve/Vo	No. of Residues
toxin	92.9	1.88	3.65
allyl α-D-galacto-pyranoside	103.0	2.09	2.4

Table 9. R_F and R_{Gal} values of allyl α-D-galactopyranoside and helminthosporoside on Whatman #541, with the solvent system (1-propanol-ammonium hydroxide, 66:33).

Compound	R_F	R_{Gal}
galactose	0.4	1.0
helminthosporoside	0.67	1.67
allyl α-D-galactopyranoside	0.77	1.92

large veins are 8-12 smaller veins. A cross-section of the leaf is found in Figure 56.

By observing toxic activity on sugarcane leaves, it became apparent that the large veins were generally the site for the long runners, i.e., when a puncture was made away from a large vein, crossing over would occur, enabling the toxin to eventually find its way to the large

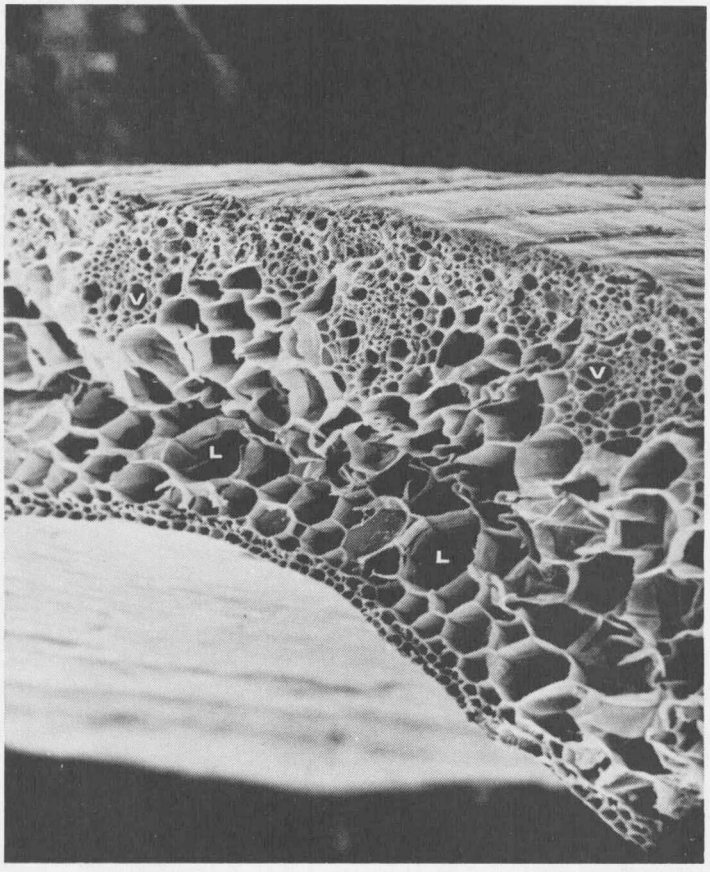


Figure 56. Cross section of a sugarcane leaf.
(This photo, taken of a scanning electron micrograph, was generously provided by G. A. Strobel. The letter V indicates the position of the veins.)

vein and subsequently traverse the leaf, making a long runner. It seemed reasonable that punctures close to and/or on the large veins may cause more reproducible results. By using a syringe, which is flexible, the rigidity of the veins precluded direct puncture. Therefore, a non-flexible tool was required for puncturing the veins. Puncturing tools were machined (in mechanical engineering) from sewing needles, size 1/10, to obtain a razor edge 350-500 microns in width, as shown in Figure 57.

The following experiment was performed to check the importance of puncture position. A sugarcane leaf was punctured between and on large veins (Figure 58), at positions where a microliter of toxin solution had been placed. Within 45 minutes and after 2.5 hrs data were obtained as described in Figure 58. The runners obtained from punctures on veins were much larger than those obtained from punctures between the veins. It also was seen, (puncture #3), that crossing over to a large vein improved the runner length. The crossing over phenomena is thus potentially the source of large variance in the bioassay data.

To verify the previous results, a series of 63 punctures were made in sixteen leaves. Most punctures resulted in runners extending the entire length of the excised leaves; therefore, runner length could not be used as the only criteria for analyzing the leaf's response to the different puncture positions. Runner intensity and runner length were observed, as tabulated in Table 10. There were three times as many

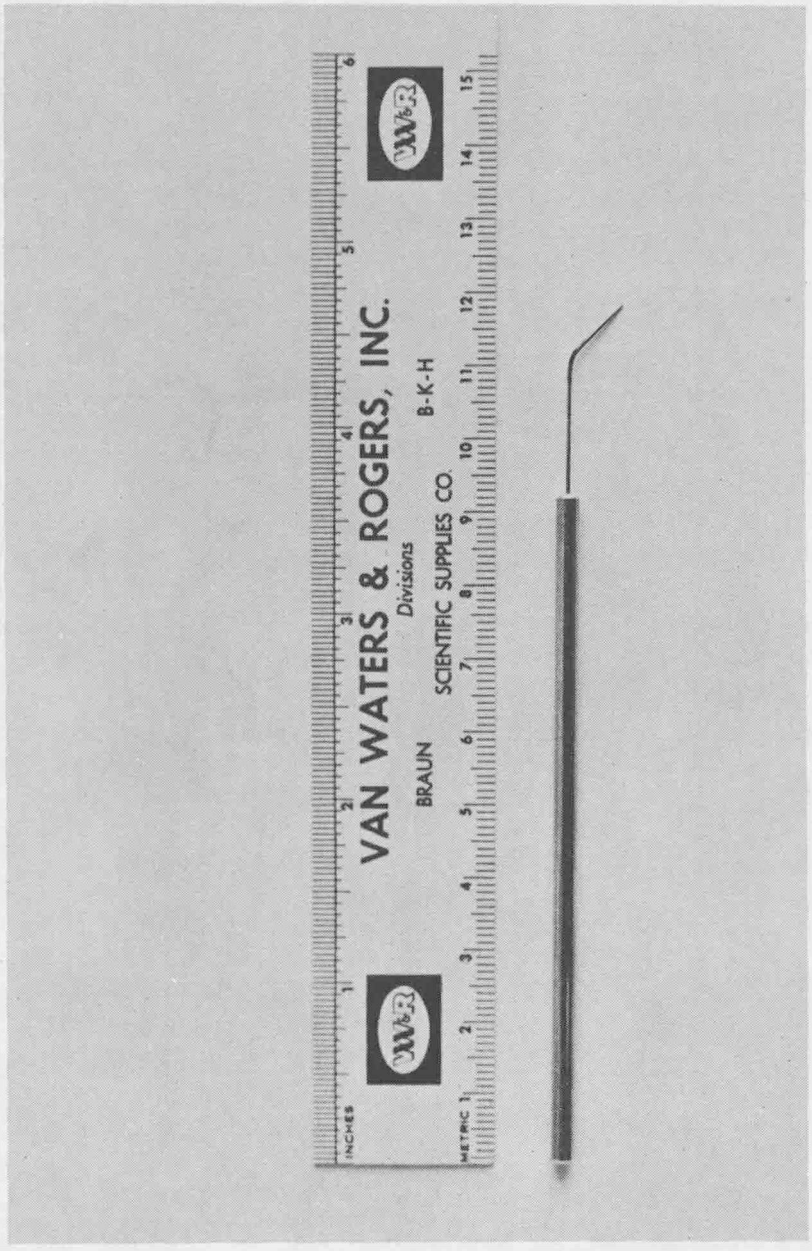
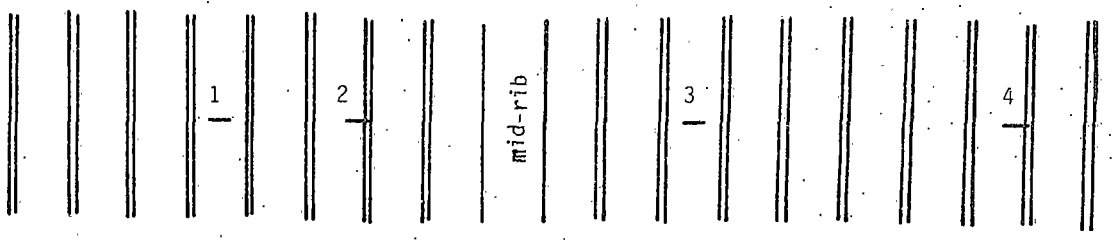


Figure 57. The puncturing tool machined from a sewing needle.

Position of Punctures on the Leaf



Puncture number	1	2	3	4
Runner Length at 45 min.	5cm	15.5cm	6cm	14cm
Runner Length at 2.5 hrs.	6.5cm	18cm	9.5cm (Crossing over)	16.5cm

Figure 58. Preliminary bioassay to test the hypothesis of injecting the larger veins for better runners.

runners with poor intensity from punctures between the veins as from punctures in the vein. The average length of the runners with poor intensity resulting from punctures between the veins was 26% less than those runners with poor intensity from punctures in the vein.

Even though this system looked very promising, it was not used for quantitation, because available time and other research commitments did not allow development of a quantitation scheme for this assay system. Therefore, chemical and instrumental analysis were performed on the toxin preparation obtained from the Bio-gel P-2 column, without quantitation of biological activity.

As a result of these analyses, a preliminary structure for helmin-

Table 10. Bioassays with punctures between the veins and in the veins, read at 42 hours.

Percentage Of Punctures With No Runners		Percentage Of Punctures With Runners Having Poor Intensity	
In Veins	Between Veins	In Veins	Between Veins
1.5	0.0	3.2	9.5

Percentage Of Total Possible Lesions, For Runners With Poor Intensity	
In Vein	Between Veins
100.0	73.9

thosporoside, (43), was formulated; this structure is presented in Figure 59. This structure will be used through out the remaining discussions of chemical and instrumental data.

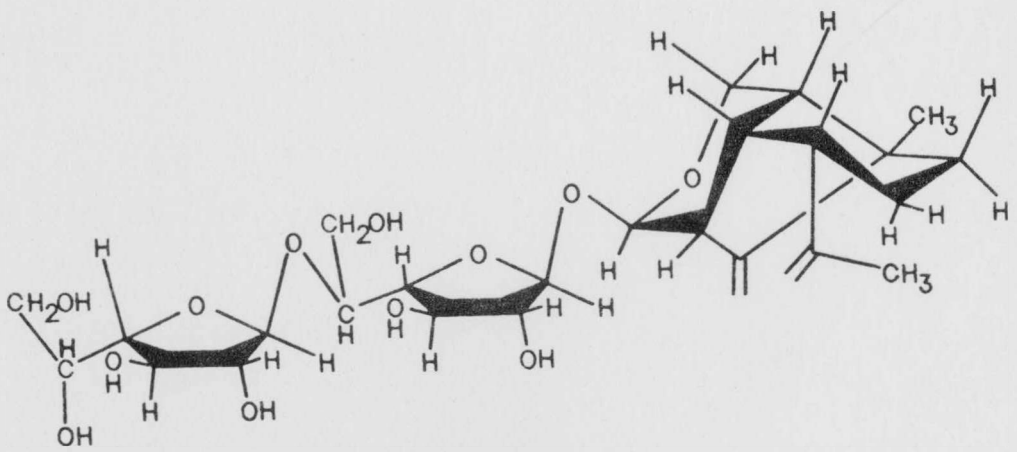


Figure 59. A preliminary proposed structure for helminthosporoside.

A sample of the toxin, obtained from the Bio-gel P-2 column, was sent to MIT for mass spectral analysis. High resolution field desorption spectra were obtained of the free and peracetylated toxin. A flow sheet showing the fragments of the peracetylated toxin is presented in Figure 60, and a drawing showing the major cleavages is presented in Figure 61. From these data, it is clear that at least two carbohydrate residues are present. Also, an aglycone is present having the formula, $C_{15}H_{22}O_2$. No molecular ion was observed in this spectrum, but the [M + H] ions were observed. It is quite common in field desorption mass spectroscopy to find the $[M]^+$ and/or $[M + H]^+$ ions formed.[45]

Interesting information concerning the carbohydrate linkages may be obtained from the mass spectrum. It was shown by lehmann et al.[46] that field desorption mass spectrometry can be used to differentiate α- and β-stereoisomeric glycosides. There was a difference in the signal intensities for the carbohydrate moiety due to a cleavage between C-1 of the sugar and the glycosidic oxygen. In one case, the α-anomer fragmentation was enhanced by a factor of 2 or 3 compared to the β-anomer. In another case, the α-anomer fragmentation was enhanced by a factor of 5 compared to the β-anomer. Since the intensities of the two fragments at 331 and 619 m/e are similar, it seems reasonable that both residues have the same configuration at C-1.

A field desorption mass spectrum was also obtained of the free toxin. The fragmentations are shown in Figure 62, and the major

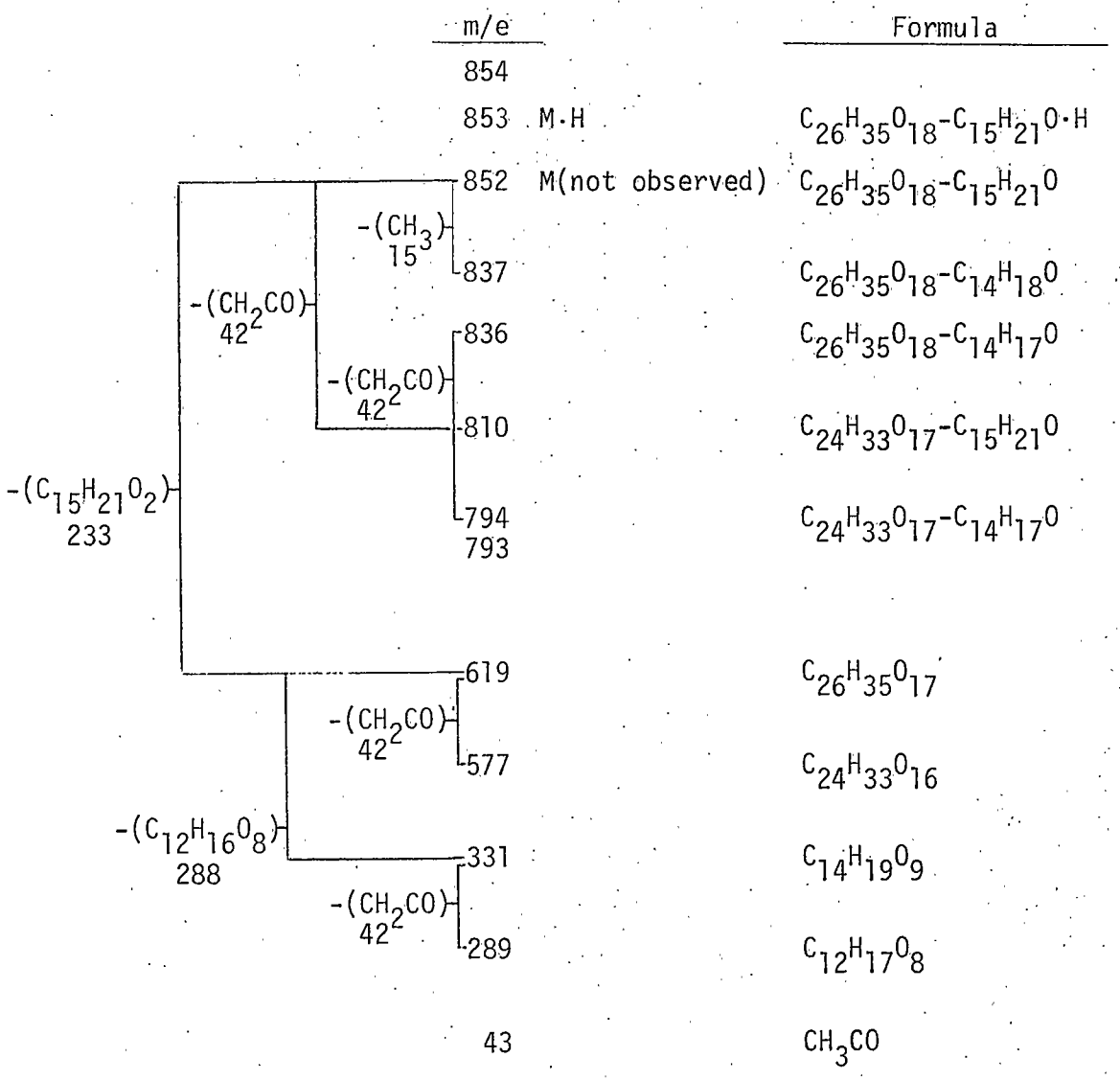


Figure 60. Fragmentations from the peracetylated toxin.

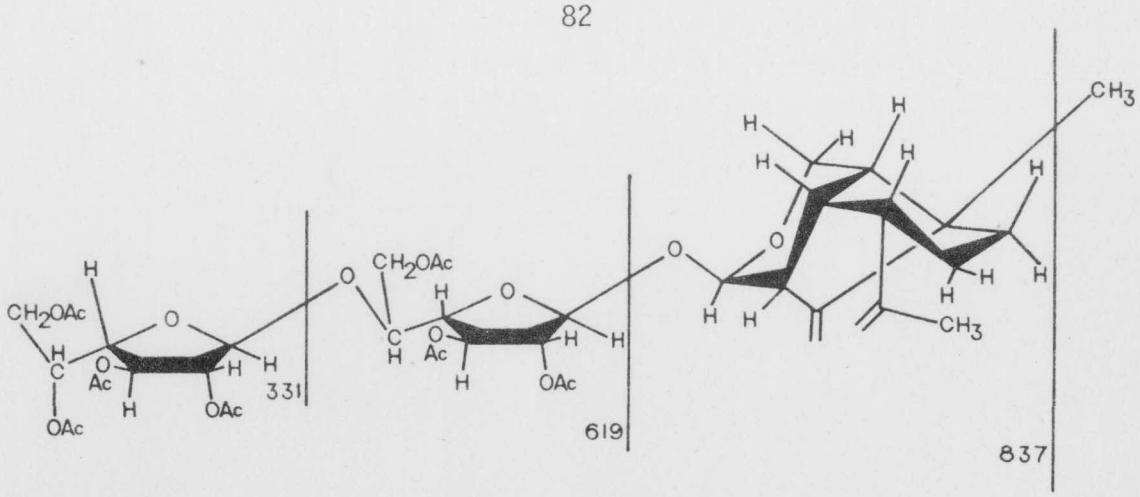


Figure 61. Major cleavages of the peracetylated toxin.

cleavages are represented in Figure 63. The molecular ion is not observed in this spectrum, but signals at 584 and 585 are observed; these may be due to impurities. Again the aglycone portion was observed, with a total molecular formula of $C_{15}H_{22}O_2$. Comparison of the signal intensities can not be used in this spectrum due to the cleavage position.

m/e		Formula
585	?	
584	?	
558	M(unobserved)	$C_{12}H_{21}O_{11}$-$C_{15}H_{21}O$
379		$C_6H_{10}O_5$-$C_{15}H_{21}O$
-($C_6H_{10}O_5$) 162		
218		$C_{15}H_{21}O{\cdot}H$
217		$C_{15}H_{21}O$

Figure 62. Fragmentations from the free toxin.

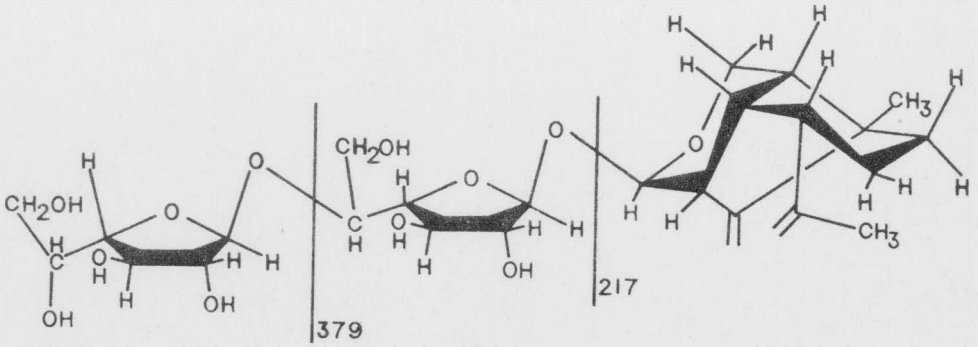
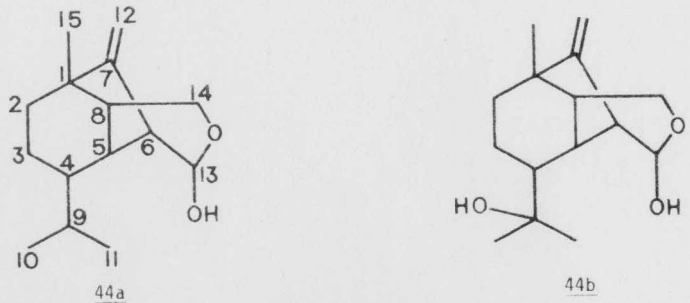


Figure 63. Major cleavages of the free toxin.

At this juncture, it is interesting to note that another fungus of the same genus, *Helminthosporium sativum*, was shown to contain two similar toxins.[47,48] Figure 64 shows these toxins, prehelminthosporol, (44a), $C_{15}H_{24}O_2$, and 9-hydroxyprehelminthosporol, (44b), $C_{15}H_{24}O_3$. The aglycone of helminthosporoside must have at least 5 degrees of unsaturation according to the molecular formula. If the toxin, (44a), had one more degree of unsaturation it would fit the molecular formula.



44a 44b

Figure 64. Prehelminthosporol, (44a), and 9-hydroxyprehelminthosporol, (44b).

A proton NMR spectrum of the Bio-gel P-2 purified toxin (Figure 65) was obtained and closely resembles the earlier obtained proton NMR spectrum. Table 11 lists all the resonances and their intensities. The resolution of this NMR spectrum appears to be better than the earlier NMR spectrum, allowing one to interpret the signals at 0.829 and 0.764 ppm to be most likely signals from the toxin and not due to an impurity, as earlier suggested.[11] The signal at 5.14 ppm was suggested earlier to be due to the anomeric proton; but upon inspection, there are actually six major signals in the region down-field from the DHO peak. The signals appear too complex to result from only an anomeric proton.

No signal in the NMR spectrum can be assigned to a cyclopropyl residue, which rules out the cyclopropyl group being part of the molecule. On the whole, the proton NMR spectrum is far too complex to be the result of a nine carbon glycoside. A Figure in reference 47 depicted the proton NMR spectrum of a derivative of 44a. This NMR spectrum with assignments,[47] is shown in Figure 66. By comparison of the NMR spectrum of helminthosporoside (Figure 65), nearly all the signals of the spectrum in Figure 66 can be accounted for in the spectrum of helminthosporoside. The doublet at 4.537 ppm has a coupling constant of 3 Hz and could be due to the proton H_e; the signals at 3.573 and 3.607 ppm could be a doublet due to the protons H_f; the broad signal at 2.548 could be due to the proton H_g; depending on the derivative, the

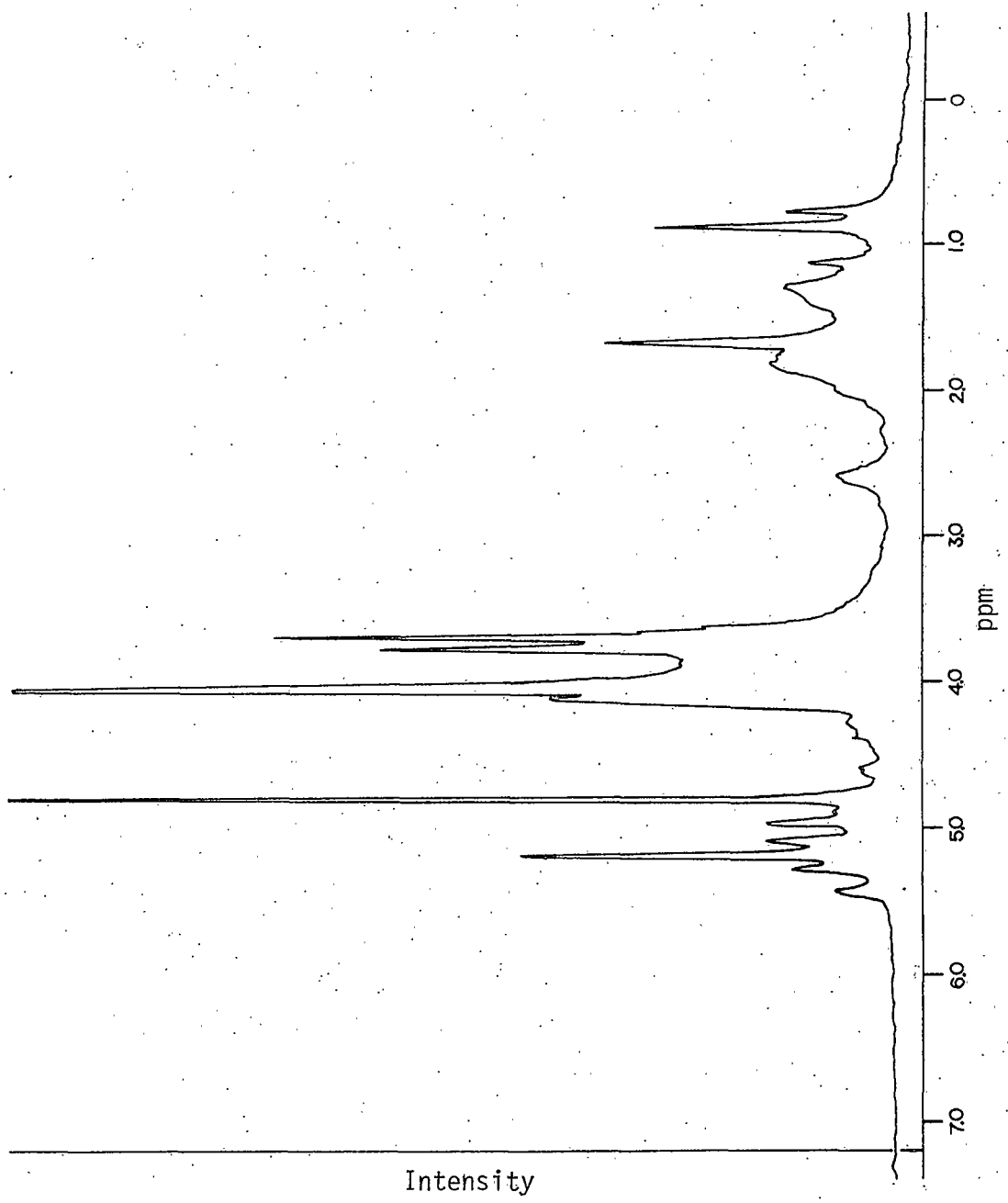


Figure 65. The proton NMR spectrum of the Bio-gel P-2 purified helminthosporoside preparation.

Table 11. Signals observed in the proton NMR spectrum; the reference is DSS substituted.

PPM	Intensity	PPM (Corrected to 4.758 ppm for DH)
5.453	183	5.405
5.291	304	5.243
5.188	1048	5.14
5.090	377	5.042
5.027	150	4.979
4.973	372	4.925
4.914	164	4.866
4.89	185	4.842
4.806	5994 (DHO)	4.758
4.6	103	4.552
4.57	91.5	4.522
4.39	142	4.342
4.287	147	4.239
4.12	967	4.072
4.057	2445	4.009
3.870	600	3.822
3.772	1433	3.724
3.694	1727	3.646
3.655	728	3.607
3.621	549	3.573
3.513	164	3.465
3.488	144	3.44
2.596	175	2.548
2.023	173	1.975
2.004	178	1.956
1.822	357	1.774
1.773	330	1.725
1.670	812	1.622
1.504	184	1.456
1.494	184	1.446
1.401	254	1.353
1.298	320	1.25
1.195	161	1.147
1.122	257	1.074
0.877	686	0.829
0.764	320	0.716

signal for proton H_h can drastically move in the proton spectrum; the large signal at 1.622 ppm may be due to the protons H_j; and the large signal at 0.829 ppm may be due to the protons H_i. Since there is only one major signal in the region for the H_i protons, it seems possible that the unsaturation may be between C-9 and C-10 or C-11.

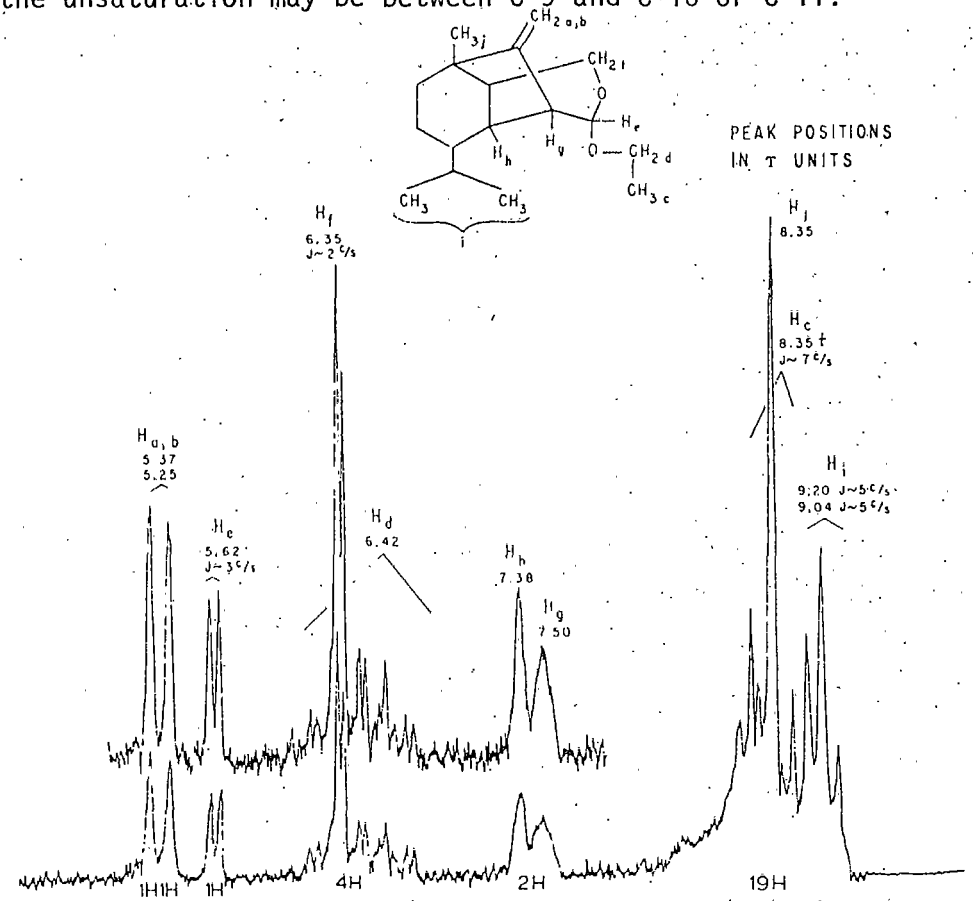


Figure 66. Proton NMR and assignments, of a derivative of prehelminthosporol, made by P. DeMayo et al.[27] Reproduced by permission of the National Research Council of Canada from the Canadian Journal of Chemistry, Volume 43, pp. 1357-1365, 1965.

Since mass spectroscopy showed the presence of two carbohydrate moieties, samples of the Bio-gel P-2 purified toxin were sent to two different groups for methylation analysis. The only carbohydrate found was galactose. Reihold[49] concluded, after methylation analysis, that the toxin contained a terminal galactofuranosyl residue and some 2,3,6- and 2,3-methylated galactose. He felt the 2,3-methylated galactose may arise from incomplete methylation. The ratio was approximately one terminal to one internal residue. The second group[50] concluded, after methylation analysis, that the compound contained a terminal galactofuranosyl residue and 2,3,6-methylated galactose, in a ratio of either 1:1 or 2:1. These two analyses agreed with the result of a terminal galactofuranosyl residue and a 2,3,6-methylated galactose. The internal galactose could either be a 4-linked galactopyranosyl or a 5-linked galactofuranosyl residue.

A CMR spectrum was obtained on the same sample, and is depicted in Figure 67. Table 12 lists all the resonances (twenty-one) and their intensities. This spectrum clearly shows thirteen more resonances than were distinguished in the earlier spectrum. One resonance (63.14 ppm) is either not present in this spectrum, or is too close to another resonance to be resolved. Other than the one missing resonance, the other eight are identically present, Including the ratio of their intensities. When the earlier spectrum was compared to this spectrum, the thirteen signals not previously recognized can be located; but the ratio

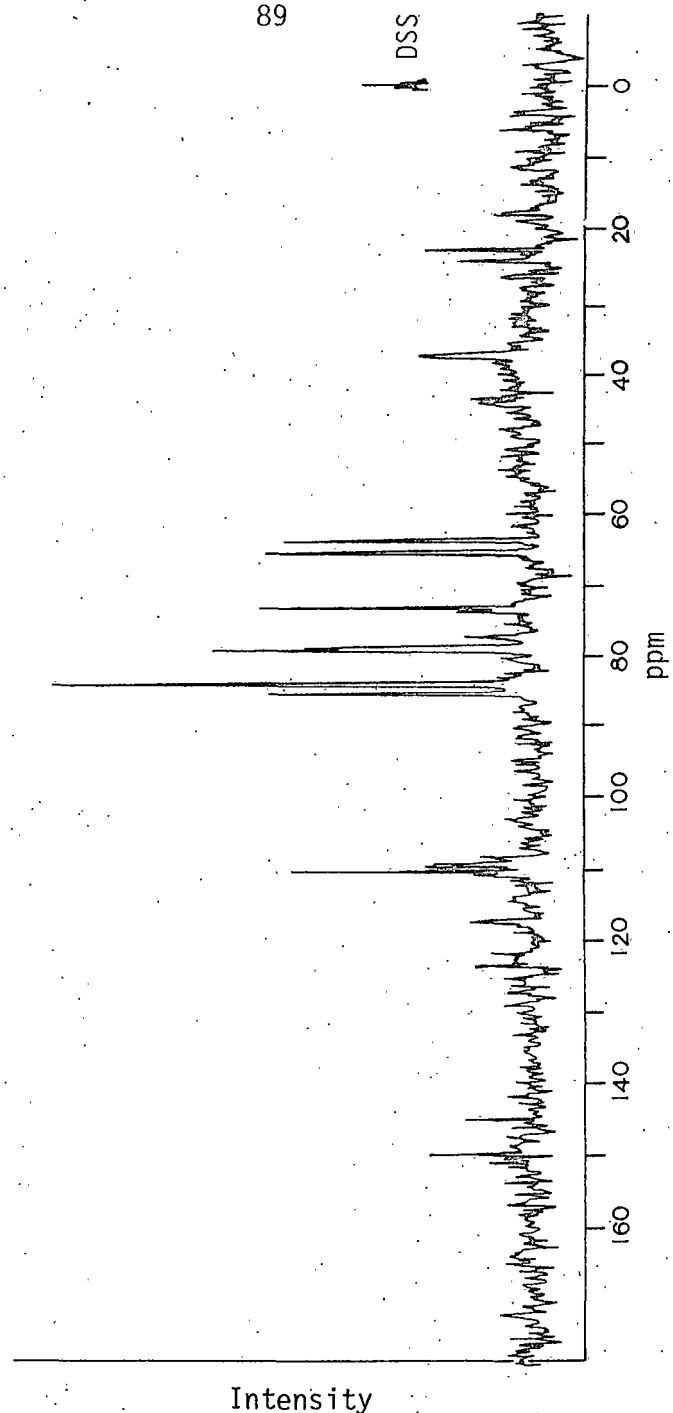


Figure 67. The ^{13}C-NMR spectrum of the Bio-gel P-2 purified helminthosporoside preparation.

Table 12. CMR resonances of the helminthosporoside preparation; the reference is DSS substituted.

ppm	Intensity	ppm corrected to external TMS
149.117	4042	147.185
144.212	2830	142.28
123.56	2420	121.628
121.78	1686	119.848
116.829	2526	114.897
109.706	8480	107.774
109.006	4135	107.074
107.838	2272	105.906
85.184	9143	83.252
83.842	16166	81.91
79.054	11022	77.122
78.703	7910	76.771
77.127	2718	75.127
73.507	3105	71.575
73.099	9520	71.167
65.450	9292	63.518
63.815	8108	61.883
44.022	2595	42.09
37.95	4124	36.018
24.638	2950	22.706
23.004	4160	21.072

of signal to noise is such that the signals might be considered noise. The intensities of these smaller signals are discussed in Section 3. Since the intensities are dependent on relaxation times, a direct comparison can not be made with the larger signals of the carbohydrate moieties.

The resonances at 119.8, 121.6, 142.3, and 147.2 ppm could be due to double bonded carbons. The intensity of these signals correlate with the other signals of the aglycone, giving more substance to the possibility that the aglycone has two double bonds.

From analyses of the ^{13}C-NMR spectrum of the toxin (Section 3), it was observed that galactose was present in two forms, a terminal β-D-galactofuranosyl residue, and internal 5-linked β-D-galactofuranosyl residue.

At this juncture a molecular formula may be written for the toxin, $C_{27}H_{42}O_{12}$, and the molecular weight would then be 558.62859. The following elemental relationship can be written:

C, 58.05; H, 7.58; O, 34.37.

A carbon-hydrogen analysis was obtained from Galbraith Laboratories on the toxin, and gave the following elemental relationship:

C, 42.88; H, 6.97; N, <0.5.

This result is indicative of a number of possibilities: The toxin may not be the structure derived in this paper, or there may be impurities in the toxin preparation, which would cause the values to be inconsis-

tant, or both of the above. A problem also appeared in the proton NMR. The integration of the NMR spectrum was not consistant with the proposed structure. There were too many protons in the carbohydrate region (3.5-4.2 ppm) to favorably compare to the number of protons in the aliphatic region. Also, the total carbon analysis of the Bio-gel P-2 column effluent appeared to reflect a constant bleed off of material. It is interesting to compare the molecular weight obtained on the Bio-gel P-2 column to the molecular weight of the postulated structure. The Bio-gel P-2 column gave an elution volume corresponding to 3.65 residues, which is equal to a molecular weight of 621. For this method, the value is as close as can be expected.

Therefore, the structure, (43), in Figure 59 is a preliminary postulated working structure for helminthosporoside. We submit, that this structure is only a working structure, and does not necessarily depict an exact structure of the toxin.

SUMMARY

All compounds that were made, with the aid of organic synthesis, having the originally postulated molecular formula of $C_9H_{16}O_7$, were not similar to helminthosporoside, with respect to chromatographic mobility, and toxicity. One compound, allyl α-D-galactopyranoside, was toxic (at high levels) to sugarcane leaves (clone 51NG97). Another compound, propyl α-D-galactopyranoside appeared to have some toxicity.

After using the three additional isolation steps, activated charcoal, HPLC, and a Bio-gel P-2 (-400 mesh) column, the toxin was obtained in a white powdery form. The elemental analysis of the toxin did not correlate with the mass spectral data; this may be due to possible impurities eluting from the Bio-gel column.

From high resolution mass spectral data, which correlated with PMR and CMR data, a molecular formula was suggested, $C_{27}H_{42}O_{12}$. A terminal β-D-galactofuranosyl residue, and an internal 5-linked β-D-galactofuranosyl residue were shown to be present. A strong correlation was made of the aglycone moiety to prehelminthosporol. A working structure for the toxin was proposed, as seen in Figure 59. It is felt that the native toxin may have a tertiary hydroxyl at C-9 of the aglycone. If the hydroxyl were eliminated during isolation, the resultant product would be identical to the proposed structure. At this juncture, more experimental work is required for the evaluation of the uncertain points

that remain in the working structure. There are at least two methods that could be used to verify the anomeric configuration of the carbohydrates. Chemical oxidation by CrO_3 will preferentially oxidize the α-galactofuranoside compared to the β-galactofuranoside.[51] An exo-β-galactofuranosidase has been isolated from *Penicillium charlesii*.[52] This enzyme will only hydrolyze a β-galactofuranoside. It will not hydrolyze an alpha derivative, and it will not hydrolyze an α- or β-galactopyranoside.

EXPERIMENTAL

Throughout this work, absorbances, for phenolsulfuric acid determinations, were obtained on a Bausch and Lomb spectronic 70, ultaviolet spectra were obtained on a Beckman model 25 spectrophotometer, and column effluents were monitored on a Gilson Hollochrome variable wavelenth monitor. High pressure liquid chromatography was accomplished on a Waters Associates liquid chromatograph using a differential refractometer, and equipped with a single minipump. Optical rotations were obtained on a Carl Ziess Circle Polarimeter 0.01°, using a sodium lamp. Infrared spectra were obtained on both the Beckman-IR5A infrared spectrophotometer and Beckman-IR20 infrared spectrophotometer. NMR were obtained on a Varian model T-60 NMR spectrometer, using carbon tetrachloride or deuterated chloroform for non-aqueous compounds, and deuterium oxide for aqueous compounds. An asterisk (*) appears after all CMR or NMR obtained by James S. Frye at the CSU Regional NMR Center, Department of Chemistry, Colorado State University, Fort Collins, CO 80523. All results obtained were with respect to DSS, substituted. Mass spectral analyses on samples of helminthosporoside, were obtained from C. E. Costello, Department of Chemistry, MIT, Cambridge, MA 92139. All thin layer plates were developed by spraying them with a solution of 1% potassium dichromate (w/v) in 50% sulfuric acid (v/v), and subsequently heating the plates at 100°C, for 5-10 minutes.[53] All melting points

and boiling points are uncorrected. All elemental analyses were obtained from Galbraith Laboratories Inc., Knoxville, TN 37921.

Throughout this work, the following solvent systems were used for chromatographic purposes: a. (carbon tetrachloride-methanol, 9:1); b. (carbon tetrachloride-ethyl acetate, 80:20); c. (carbon tetrachloride-ethyl acetate, 80:17); d. (carbon tetrachloride-ethyl acetate, 80:15); e. (2-butanone-ethanol-water, 75:8:3); f. (ethyl acetate-light petroleum ether 35-60°C, 3:2); g. (ethyl acetate-water-methanol, 84:18:28); h. (1-butanol-ethanol-water-ammonium hydroxide, 40:10:49:1); i. (carbon tetrachloride-ethyl acetate, 80:30), j. (1-propanol-ammonium hydroxide, 66:33); K. (carbon tetrachloride-ethyl acetate, 80:40); l. (diethyl ether-toluene, 2:1); m. (diethyl ether-petroleum ether 35-60°C, 22:3); n. (diethyl ether-petroleum ether 35-60°C, 22:6), o. (2-butanone-ethanol-water, 75:12:6).

Preparation of diethyl dimethyl malonate 5

diethyl methyl malonate:

In a 1 liter, 3-necked flask, equipped with a reflux condenser, addition funnel, and placed over a large dish (for ice water) on a magnetic stirrer, was slowly added diethyl malonate (73.3 g, 0.46 mol) to a cooled (20°C) solution of sodium ethoxide prepared from sodium (10 g, 0.44 mol) and dry ethanol (200 ml). To this reaction mixture methyl iodide (61.1 g, 0.43 mol) was gradually added. The resulting white so-

lution was cooled in an ice bath. After the reaction was under control, it was refluxed for 2 hrs. Ethanol was then distilled from the yellow solution. Water (160 ml) was added and the mixture was separated. The products were distilled to give a mixture of diethyl malonate and diethyl methyl malonate (63.7 g, 85% yield).

bp_{20} 98.5-102°C Literature[54] bp_{760} 201°C

diethyl dimethyl malonate:

The product obtained from the previous reaction was added to a cooled (20°C) solution of sodium (10.4 g, 0.45 mol) and dry ethanol (200 ml). Methyl iodide (63.8 g, 0.45 mol) was added to this solution. The apparatus and work up are the same as the preceding reaction. The separated material was distilled to give a clear liquid (54.6 g, 66% yield overall).

bp_{22} 96-9°C Literature[54] bp_{22} 97-8°C

NMR– 1.25 (triplet, 6H), 1.37 (singlet, 6H), 4.11 (quartet, 4H) ppm.

Sodium-ammonia reduction of diethyl dimethyl malonate

The reaction was conducted in a 500 ml, 3-necked flask equipped with an addition funnel, reflux condenser connected to a mercury trap, and a rotating sample tube (used for introduction of ammonium chloride).

Sodium (0.92 g, 0.04 mol) was added to liquid ammonia (75 ml) and ammonia vapors were removed with alternate repeated evacuations and nitrogen pressure. Over a period of 2 hrs, a solution of diethyl dimethyl malonate (1.9 g, 0.011 mol) in anhydrous ether (100 ml) was added.[18] At the disappearance of the blue color, the ammonia was allowed to evaporate and the reaction vessel was flushed with nitrogen. Ammonium chloride (1.07 g, 0.02 mol) was added with the aid of the rotating sample tube, the mixture was stirred for 0.5 hr and filtered. The solution was analyzed by glc using a 10', SE-30 column at 120°C, with a flow rate of 30 cc/min.

Lithium-ammonia reduction of diethyl dimethyl malonate

Lithium (1.388 g, 0.2 mol) was added to liquid ammonia (300 ml) as described previously. Over a period of 2 hrs, a solution of diethyl dimethyl malonate (9.4 g, 0.05 mol) in anhydrous ether (100 ml) was added. After 2 hrs ammonium chloride (5.35 g, 0.1 mol) was added to quench the reaction, and after stirring for 0.5 hr the ammonia was allowed to evaporate. The last traces of ammonia were removed by flushing with nitrogen, and the reaction mixture was filtered. The filtrate was analyzed on a 10', SE-30 column at 120°C, with a flow rate of 30 cc/min.

Visualization of the cyclopropane ring with antimony trichloride

The compound was first spotted on filter paper or silica-plates.

A solution of antimony trichloride in chloroform was then sprayed on the compound. To facilitate a reaction, the paper or plates were heated at 90°C for 3-4 min.[53] Both a particular color, and fluoresence in the U.V. may be distinguishing factors.

Preparation of methyl α- and β-D-galactopyranosides 7, 8[20]

Anhydrous D-galactose (250 g, 1.39 mol) was refluxed in a 3-necked, 3 liter flask, fitted with a reflux condenser and mechanical stirrer, containing a solution of hydrogen chloride gas (40 g) in dry methanol (2% w/v, 2000 ml) until it dissolved (20-30 min). This solution was refluxed for an additional 7 hrs and then cooled. After adding lead carbonate (200 g), the solution was stirred for 3 hrs, at which time it was neutral to moist litmus paper. The lead salts were removed with aid of celite and washed with methanol (200 ml). Upon evaporation of the methanol, a yellow syrup remained, which crystallized upon addition of water (80 ml). For recrystallization, the α-anomer was dissolved in boiling water (2 g/ml H_2O), and filtered. The solution was allowed to cool to 50°C and was seeded. The mixture was allowed to stand for 1 day at room temperature and 1 day at 5°C. The crystals were collected by suction filtration, rinsed with 85% ethanol, followed by absolute ethanol, to give the α-anomer, (7), (84.4 g, 31% yield).

mp 97.5°C Literature[20] mp 116-116.5°C**

NMR— 3.5 (singlet, 3H), 3.7-4.1 (envelope, 6H), 4.92 (doublet, 1H) ppm.

CMR*— 101.6964 (C-1), 73.0873 (C-5), 71.8612 (C-3), 71.5693 (C-4), 70.5-768 (C-2), 63.5705 (C-6), 57.4400 (methyl) ppm.

Elemental Analysis— for $C_7H_{14}O_6 \cdot H_2O$

Calculated:	C, 39.62; H, 7.6
Found:	C, 39.99; H, 7.72
Found:	C, 40.16, H, 7.68

**The article[20] reported 12 different melting points for this compound, ranging from 75°C-116.5°C. The final melting point, 116-116.5°C, was obtained after dehydration; however, after rehydration occurred, the melting point was still 116-116.5°C.

β-anomer:

From above, after addition of water, the mother liquor and washings were dried using the rotary evaporator with three additions of dry ethanol (75 ml each). Crystals formed during the final drying. For recrystallization, the crude β-anomer was dissolved in boiling absolute ethanol (30 ml/gm). The solution was decolorized with Darco G-60 and filtered. The filtrate remained at room temperature for 12 hrs, and 1 day at 5°C. The crystals were collected by suction filtration and rinsed with 95% ethanol (0°C), to give the β-anomer, (8), (36.5 g, 14% yield).

mp 176.5-7.5°C Literature[20] mp 177-80°C

NMR— 3.3-4.2 (envelope, 6H), 3.67 (singlet, 3H), 4.42 (doublet, 1H) ppm.

CMR*— 104.86 (C-1), 71.83 (C-2), 73.92 (C-3), 69.79 (C-4), 76.16 (C-5), 62.1 (C-6), 59.11 (methyl) ppm (in reference to external TMS).

Preparation of methyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside 9[21]

Dry methyl α-D-galactopyranoside (20 g, 0.1 mol) was suspended in dry dioxan (100 ml), in a 3-necked, 500 ml flask, equipped with a reflux condenser and mechanical stirrer. With continuous stirring, powdered potassium hydroxide (100 g) was added. While the mixture was refluxed, benzyl chloride (175 g, 1.38 mol) was added over a 20 min period. The reaction mixture was refluxed for an additional 0.5 hr, after which time the dioxan was removed by distillation. Water was added to dissolve the crystalline mass, and the solution was extracted with diethyl ether. The ether extract was washed with water and dried with anhydrous sodium sulfate. The solution was concentrated, by rotary evaporation, to a syrup. Impurities were distilled from the above syrup at 0.5 torr and a pot temperature not greater than 200°C, to give the product as a syrup.

Attempted preparation of 2,3,4,6-tetra-O-benzyl-α-D-galactose 10[21]

The syrup obtained from the above benzylation was dissolved in hot glacial acetic acid (1.25 liter). Boiling 2N sulfuric acid (270 ml)

was added while the solution was maintained at 95°C. After a two hr period, boiling 2N sulfuric acid (270 ml) was again added, and the solution was maintained at 95°C for another 24 hrs. The solution was then cooled and decanted into 10 liters of distilled water from which the product oiled out. The oil was extracted with dichloromethane, and thin-layer chromatography on silica-gel in solvent system (a) revealed extensive degradation.

Thin-layer chromatographic separation of the degradation products Figure 9

Thin-layer chromatography on silica-gel, in solvent system (a), was accomplished on the products of a hydrolysis reaction, as previously described. Their R_F values with respect to assigned number of benzyl groups are the following:

Number of Benzyl Groups	R_F	$1-R_F$
0	0.11	0.89
1	0.2	0.8
2	0.3	0.7
3	0.39	0.61
4	0.47	0.53

Least Squares: 0.9993 correlation coefficient.

Hydrolysis study of methyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside

In each of three reactions, methyl 2,3,4,6-tetra-O-benzyl-α-D-gal-

actopyranoside (0.772 g, 0.0014 mol) was placed in a 2-necked, 50 ml flask. Glacial acetic acid (13.5 ml) was added and heated to the desired temperature. Boiling 2N sulfuric acid (2.92 ml) was added, and after 2 hrs another aliquot of boiling 2N sulfuric acid (2.92 ml) was added. Reactions were carried out at temperatures of 75, 80, and 85°C. Aliquots were removed from the reaction vessel at 30 min intervals for the reactions at 75 and 80°C, and 15 min intervals for the reaction at 85°C. Thin-layer chromatography on silica-gel in solvent system (b), was used to evaluate the extent of reaction. Visually, the intensities of the product (R_F = 0.15) and reactant (R_F = 0.376) spots were compared. The reaction at 75°C did not go to completion. The reaction at 80°C appeared to go to completion after 11 hrs. The reaction at 85°C went to completion after 5 hrs. Only one product was observed in all three reactions. When the reaction mixture was heated above 90°C, degradation was observed.

Preparation of 2,3,4,6-tetra-O-benzyl-α-D-galactose 10

Methyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside (20 g, 0.036 mol) was placed in a 3-necked, 1 liter flask equipped with a reflux condenser and mechanical stirrer. Glacial acetic acid (350 ml) was added and the temperature was brought to 85°C. Boiling 2N sulfuric acid (75.5 ml) was added, and after 2 hrs another portion (75.5 ml) was added. The reaction was allowed to stir for 3 hrs at 85°C (Note: 5 hrs

should be allowed for more complete hydrolysis). Water (425 ml) was added and the solution was extracted three times with 150 ml portions of diethyl ether. The ether was removed by rotary evaporation (temperature not greater than 40°C). Two equal volumes of water were added and the mixture was neutralized with sodium bicarbonate, extracted three times with dichloromethane and the organic layer was dried over anhydrous sodium sulfate. The dichloromethane was removed, yielding a syrup. The syrup was placed on a low pressure liquid chromatography system using silica-gel, and solvent system (c). The chromatography column was 100 x 2.5 cm, with a void volume of 325 ml. Three runs were required to separate all the material. A 300 ml fraction was collected at the beginning of each run, then 19.8 ml fractions were collected. The product was observed in fractions 27-85 (6.7 g, 34% yield).

NMR— 3.33-5.03 (envelope, 15.6H), 5.13 (multiplet, 1H), 7.2 (multiplet, 20H) ppm.

Attempted preparation of vinyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside 12[23]

Mercuric acetate was recrystallized from 95% ethanol. Ethyl vinyl ether was redistilled (bp_{760} 36-37°C). To a 200 ml flask, 2,3,4,6-tetra-O-benzyl-α-D-galactose (1.7 g, 0.003 mol), ethyl vinyl ether (50 ml), mercuric acetate (1 g, 0.0031 mol), and 4Å molecular sieves (25 ml)

were added. The solution was stirred by a magnetic stirrer for 12 hrs at room temperature. Thin-layer chromatography on silica-gel in solvent system (b), was used to monitor the reaction. A tracing of the thin-layer plate is shown in Figure 68.

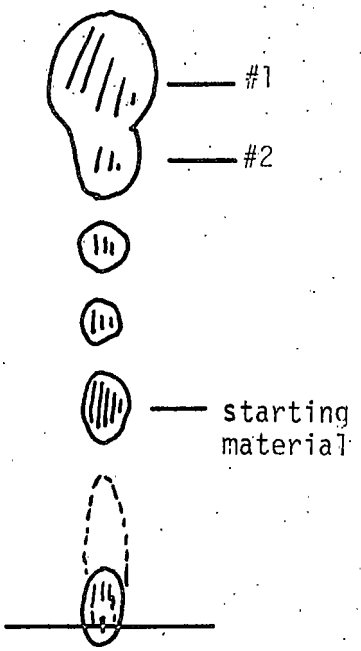


Figure 68. A tracing of the thin-layer chromatogram showing reaction products of the transvinylation reaction.

Column chromatography with a 40 x 1.2 cm column was successively eluted with (EtOAc-CCl_4, 1:20) 45 ml, (EtOAc-CCl_4, 1:12) 90 ml, and (EtOAc-CCl_4, 1:4) 90 ml, and 3 ml samples were collected. By NMR, sample #1 (as shown on the thin-layer plate) contained no benzyl groups. Sample #2, did not contain the vinyl group, but they appeared to contain

the ethyl groups, in place of the benzyl groups.

Preparation of 2,3,4,6-tetra-O-benzyl-D-galactosyl chloride 2

Thionyl chloride was distilled before use, using glass or teflon beads for boiling chips during reaction. The 2,3,4,6-tetra-O-benzyl-α-D-galactose (6.5 g, 0.012 mol) was dissolved in thionyl chloride (2.1 ml).[22] This solution was stirred for 2 hrs at room temperature and then at reflux for 30 min. Thionyl chloride was removed by rotary evaporation, with successive additions of toluene (3 times) to remove traces of thionyl chloride. The residual yellow syrup was used directly in the coupling reactions. Figure 69 depicting the thin-layer plate tracing shows a comparison of the reaction product to methyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside, and 2,3,4,6-tetra-O-benzyl-α-D-galactose, run on silica-gel in solvent system (b).

Preparation of allyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside 19

In a 25 ml flask, 2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl chloride (2.9 g, 5.23 mol), potassium iodide (3.47 g, 20.9 mmol, 4 eq.), 2,6-lutidine (0.146 ml), dry acetonitrile (2.9 ml), and allyl alcohol (0.61 g, 10.46 mmol, 2 eq.), were stirred at room temperature for 16 hrs.[12] The reaction mixture was poured into water, extracted with carbon tetrachloride and the extracts were dried over sodium sulfate. The mixture was passed through a 4" silica-gel column, and the eluate

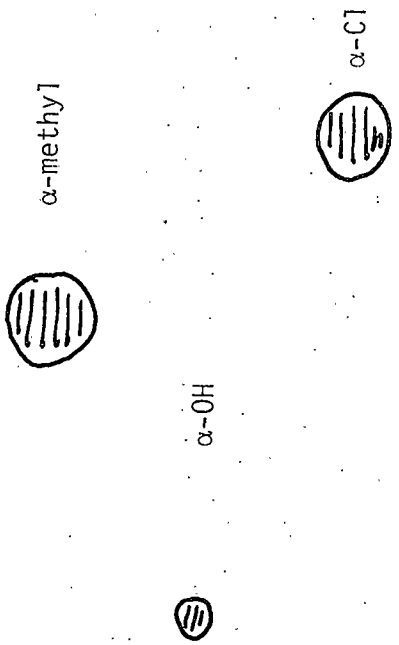


Figure 69. A thin-layer chromatogram comparing α-methyl, and α-hydroxyl, to 2,3,4,6-tetra-O-benzyl-D-galactosyl chloride.

was evaporated to a syrup. The thin-layer chromatogram tracing in Figure 70 shows the product in comparison with the methyl, chloro, and the free hydroxyl form of 2,3,4,6-tetra-O-benzyl-α-D-galactopyranose, in

solvent system (b).

The product was the only material reacting with dilute potassium permanganate.

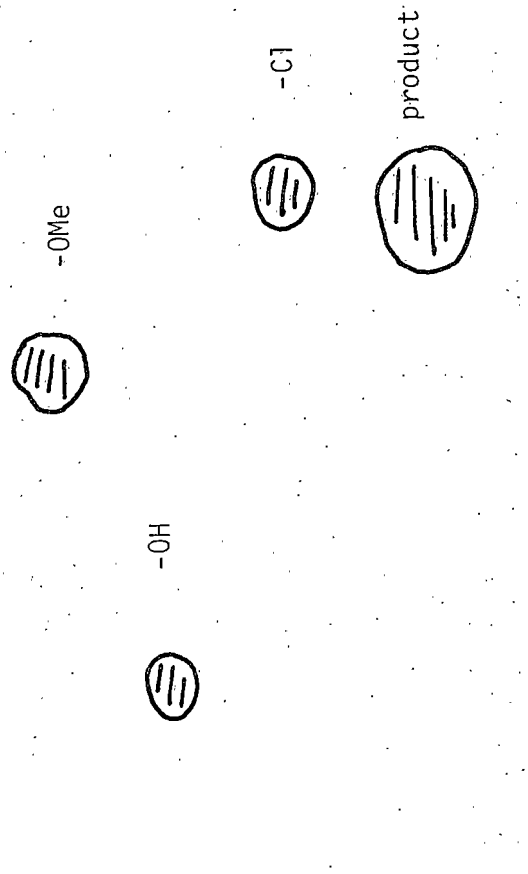


Figure 70. A thin layer chromatogram of allyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside.

Preparation of allyl α-D-galactopyranoside 20[27]

In a 500 ml, 2-necked flask, equipped with a mechanical stirrer,

hydrogen chloride (gas) (2 g, 0.055 mol) was bubbled into allyl alcohol (66.7 g, 1.15 mol). Dry D-galactose (33.3 g, 0.185 mol) was stirred into the solution which was then maintained at 70°C for 5.5 hrs. The temperature was then increased to 80°C with continued stirring for 4 additional hrs. After heating, concentrated ammonium hydroxide (7.67 ml) was added and the solution was decolorized with activated charcoal. Product 20 was observed by thin-layer chromatography on silica-gel in solvent system (e), R_F = 0.37. The solution was concentrated to a syrup by rotary evaporation, and extracted eight times with 200 ml portions of dry acetone. The concentrated acetone solution was cooled at 5°C, and within 1.5 days, crystals formed. After recrystallization from hot ethanol, crystalline 20 was obtained (12 g, 30% yield).

mp 145.5-6.5°C Literature[27] mp 145-6°C (cor)

$[\alpha]_D^{26}$ = +184.5° in water(c, 2.0) $[\alpha]_D^{25}$ = +185.0° in water(c, 5.8)

Infrared (KBr disc)— 3550, 3360, 2950, 1462, 1360, 1145, 1120, 1070, 1020, 920, 803 cm^{-1}.

NMR— 3.71-4.5 (multiplet, 8H), 5.1 (doublet, 1H), 5.2-5.68 (multiplet, 2H), 5.74-6.64 (multiplet, 1H) ppm.

CMR*— 134.802 (C-3, doublet), 119.432 (C-9, triplet), 98.761 (C-1, doublet), 72.252 (C-5, doublet), 70.793 (C-3, doublet), 70.55 (C-4, doublet), 69.82 (C-7, triplet), 69.529 (C-2, doublet), 62.525 (C-6, triplet), ppm.

Elemental Analysis– for $C_9H_{16}O_6$

Calculated: C, 49.09; H, 7.32

Found: C, 49.14; H, 7.47

Preparation of allyl 2,3,4,6-tetra-O-acetyl-α-D-galactopyranoside 21

Acetic anhydride was dried over 4Å molecular sieves. Pyridine was dried by distillation from barium oxide, after, refluxing for 4 hrs, then stored over Drierite.

A solution of acetic anhydride (1.4 ml) and pyridine (1.98 ml) was cooled to 0°C, and powdered dry allyl α-D-galactopyranoside (0.32 g, 1.45 mmol) was added to the stirred solution.[28] The suspension was stirred at 0°C until the galactoside dissolved (1.5 hr). The solution was allowed to stir at room temperature for 19 hrs and then poured into 6.5 ml of ice water. The reaction may be monitored by thin-layer chromatography on silica-gel, in solvent system (f), R_F = 0.5. The mixture was extracted with dichloromethane, and the extracts were washed successively with 1N hydrochloric acid, saturated sodium bicarbonate, water, and dried over anhydrous magnesium sulfate. After filtration, the solution was concentrated by rotary evaporation and crystallized at 5°C (0.55 g, 97%). Recrystallization can be effected from 95% ethanol (80% yield).

mp 82-3°C

$[\alpha]_D^{27.5}$ = +146.9° in $CHCl_3$(c, 4.7)

Infrared (KBr disc)— 2985, 2940, 1743, 1370, 1230, 1130, 1075, 1030, 975, 930, 905, 835 cm^{-1}.

NMR— 1.95-2.2 (multiple singlets, 12H), 3.8-4.4 (envelope, 5H), 4.75-5.5 (envelope, 6H), 5.6-6.23 (multiplet, 1H) ppm.

Elemental Analysis— for $C_{17}H_{24}O_{10}$

Calculated:	C, 52.57; H, 6.23
Found:	C, 52.35; H, 6.14

Preparation of (2,3-epoxypropene-1-O) 2,3,4,6-tetra-O-acetyl-α-D-galactopyranoside 22

At room temperature, 21 (0.5 g, 0.00129 mol) was added to a solution of m-chloroperoxybenzoic acid (0.783 g, 0.00454 mol) in chloroform (10 ml).[29] During 47 hrs, the reaction was intermittently monitored by thin-layer chromatography on silica-gel, in solvent system (f), R_F = 0.33. The reaction mixture was washed twice with 0.5M potassium bicarbonate and dried over anhydrous magnesium sulfate. After concentration, the syrup was placed on a 17 x 2.3 cm, silica-gel column, eluting with variations of solvent system (f), (1:4, 100 ml); (2:3, 50 ml); (3:2, 200 ml). During concentration of the fractions containing 22, the product crystallized (0.4 g, 77% yield). The product, (22), may be recrystallized from absolute ethanol.

mp 91.5-2.5°C

$[\alpha]_D^{27.1}$ = +126.5° in $CHCl_3$(c, 1.8)

Infrared (KBr disc)— 2980, 1760, 1450, 1380, 1240, 1145, 1065, 963, 913, 860 cm^{-1}.

NMR— 1.95-2.2 (multiple singlets, 12H), 2.5-4.5 (envelope, 8H), 5.0-5.6 (envelope, 4H) ppm.

Elemental Analysis— for $C_{17}H_{24}O_{11}$

Calculated:	C, 50.50; H, 5.98
Found:	C, 50.32; H, 5.77

Preparation of (2,3-epoxypropene-1-O) α-D-galactopyranoside 17

The tetra-acetate, (22), (127 mg, 0.314 mmol), was suspended in dry methanol (1.27 ml), to which 1.0M methanolic sodium methoxide (0.08 ml) was added. The suspension was shaken until the solid dissolved, and was left for another 15 min. Solid carbon dioxide was added, and when the solution reached room temperature, it was rotary evaporated to dryness.[29b] The reaction mixture was examined by thin-layer chromatography on silica-gel, in solvent system (g), R_F = 0.46 or in system (e), R_F = 0.14 and on descending paper chromatography with Whatman #1, in solvent system (h), R_{Gal} = 1.88. The product was separated on a 17 x 2.3 cm, silica-gel column, eluting with variations in solvent system (e), (75:6:1, 100 ml); (75:8:3, 100 ml); (75:12:6, 150 ml). After concentrating the fractions containing the product, a syrup was obtained (58 mg,

78% yield). Note: the NMR spectra obtained from this sample were the same as that for compound 16, epoxide opening occured in the NMR tube.

Preparation of (2,3-propanediol-1-0) α-D-galactopyranoside(isofloridoside) 16

(2,3-epoxypropene-1-0) α-D-galactopyranoside (2 mg, 0.0085 mmol) was dissolved in water (1 ml), to which perchloric acid (1 drop) was added.[30] The solution remained at room temperature overnight, and was then passed through a small silica-gel column. The product was identical to authentic isofloridoside when compared by thin-layer chromatography on silica-gel in solvent system (g), R_F = 0.25. The reaction produced only a single product, and went to completion.

NMR* 4.799, 4.78 (HDO), 4.337 (1735), 4.219 (9105), 3.361 (3579), 3.251 (4530), 3.153 (5083), 3.09 (3289) ppm.

CMR* 101.266 (3207), 73.493 (5823), 73.007 (3079), 71.791 (9657){2-3 carbons}, 70.964 (6342), 65.030 (3024), 63.766 (5668) ppm.

Bioassay of allyl α-D-galactopyranoside, (20), on sugarcane leaves

Standard solutions of allyl α-D-galactopyranoside were prepared by a serial dilution:

Dilution	µg/µl	µmol/µl
A	300.53	1.36
B	250.04	1.14
C	200.03	0.91
D	150.02	0.68
Two other standards were also prepared:		
E	35.26	0.16
F	7.05	0.03

Each of the following samples were administered to two excised leaves of sugarcane (clone 51NG97), by placing the sample on a needle puncture near the base of the leaf. The leaves were kept in a moist chamber, at room temperature. The lesions were measured after 36 hrs of incubation.

The following shows the amounts of each sample, that was administered to needle punctures:

Sample Number	Standard	µl	µg	µmol
1	A	2	601.0	2.73
2	B	2	500.1	2.27
3	B	1	450.1	2.04
	C	1		
4	C	2	400.1	1.82
5	C	1	350.1	1.59
	D	1		
6	A	1	300.5	1.36
7	B	1	250.0	1.14
8	C	1	200.0	0.91
9	D	1	150.0	0.68
10	E	2	70.5	0.32
11	E	1	35.3	0.16
12	F	1	7.1	0.03

The following is a list of the results obtained after 26 hours incubation:

Sample number	Average lesion length at 36 hrs (cm)	Lesion length after corrected for water controls (cm)
1	5.25	4.85
2	4.4	4.0
3	2.5	2.1
4	0.75	0.35
5	3.25	2.85
6	2.25	1.85
7	1.25	0.85
8	2.25	1.85
9	1.5	1.1
10	0.6	0.2
11	0.8	0.4
12	0.3	0.0

Four water controls were conducted in the same manner:

Sample number	Lesion length at 36 hrs (cm)	Average lesion length (cm)
1	0.3	0.4
2	0.4	
3	0.4	
4	0.5	

Sugarcane leaves (7209) resistant to helminthosporoside were administered various amounts of allyl α-D-galactopyranoside, 176.3 μg, 352.6 μg, and 528.9 μg. The leaves showed no runner formation up to 115 hrs, but had a slight yellowing (0.8 cm for 528.9 μg) around the injection sight.

Sugarcane leaf protection from the toxin by allyl α-D-galactopyranoside

Halves of two sugarcane leaves (clone 51NG97) were placed, for 5 hrs, in a solution of allyl α-D-galactopyranoside containing 21.97mg/ml. The other half of each leaf was placed in water. The leaves were not totally separated at the midrib. One micro-liter of a toxin solution was placed on a needle puncture near the middle of each leaf-half. Runners formed on the control leaves (those soaking in water), but no runners formed on the leaves soaking in ally α-D-galactopyranoside. The leaves soaking in the galactoside solution appeared yellow after two days.

Preparation of 3-allyloxyoxetane 25[32]

Three simultaneous reactions were carried out to prepare the intermediate. Batch 1: Chlorine gas (258.2 g, 3.64 mol) was introduced at 4 g/min into allyl alcohol (1.667 Kg, 24.5 mol). Batch 2: Chlorine gas (259.1 g, 3.65 mol) was introduced at 4 g/min into allyl alcohol (1.667 Kg, 24.5 mol). Batch 3: Chlorine gas (265.4 g, 3.74 mol) was

introduced at 4 g/min into allyl alcohol (1.667 Kg, 24.5 mol). These three batches were added together and distilled to remove unreacted allyl alcohol. The weight of the crude product was 1637 g. Aqueous sodium hydroxide (535 g in 957 ml water) was slowly added to the crude product, and the temperature was maintained below 30°C by an ice-water bath. The mixture was stirred with a mechanical stirrer and heated to 100°C for 10.5 hrs. The product, (25), was monitored on a 20' x 1/4", 30% SE-30 (on chromsorb G) column, at 85°C. Sufficient water was added to dissolve the salts, and the two layers were separated. The aqueous layer was extracted three times with carbon tetrachloride. These extracts were added to the original organic layer. This combined crude preparation was distilled, and then re-distilled with an 8" column packed with glass helicies at an oil bath temperature of 87-9°C, to obtain (101.1 g), 96% of the amount obtained by the reported method.[32]

bp_{25} 64-6°C	Literature[32] bp_{25} 64°C
Infrared— 1.633, 2.113, 6.07, 8.9, 10.2, 10.7 μ.	Infrared— 1.633, 2.113, 6.07, 8.9, 10.2, 10.7 μ.
NMR— 3.9 (multiplet, 2H), 4.6 (multiplet, 5H), 5.2 (multiplet, 2H), 5.9 (multiplet, 1H) ppm.	NMR— 3.9 (multiplet, 2H), 4.6 (multiplet, 5H), 5.2 (multiplet, 2H), 5.9 (multiplet, 1H) ppm.

Preparation of 3-propenoxyoxetane 26[33]

To distilled dimethylsulfoxide (61.3 g) in a 200 ml, 2-necked flask, was added 3-allyoxyoxetane (40 g, 0.35 mol). Potassium t-butoxide (4.47 g, 0.0398 mol) was added at an initial pot temperature of 18°C, and within thirty minutes, the temperature rose to 36°C. The product mixture was monitored by glc using a 10' x 1/4", 15% carbowax column, at 140°C. The mixture was filtered and distilled, having a conversion of 96.4% by glc (26.4 g, 64% yield).

bp_{50} 74-7°C	Literature[33] bp_{50} 73-7°C
Infrared— 3.41, 6.0, 7.26, 7.36, 7.98, 8.45, 8.8, 9.76, 10.27, 11.5, 13.8 μ.	Infrared— 3.27, 5.97, 7.25, 7.35, 8.86, 10.23, 13.7 μ.
NMR— 1.5-1.75 (multiplet, 3H), 4.25-4.9 (multiplet, 6H), 5.7-5.95 (multiplet, 1H) ppm.	NMR— 1.62 (multiplet, 3H), 4.5 (multiplet, 1H), 4.5-4.9 (multiplet, 5H), 5.83 (multiplet, 1H) ppm.

Preparation of 3-oxetanol 27[33]

Water (11.7 g), 3-propenoxyoxetane (12 g, 0.11 mol), methanol (8.57 g), and sulfuric acid (17.7 μl) were mixed in a 100 ml flask, at room temperature. The reaction was stirred for 5.5 days, and was then neutralized with 1N sodium hydroxide. The products were analyzed by glc on a 10' x 1/8", 15% carbowax (on chromsorb G) column, at a temper-

ature of 170°C and flow rate of 28 cc/min. The solution was rotary evaporated at a temperature less than 50°C, and distilled (5.35 g, 69% yield).

bp_{11} 84°C	Literature[33] bp_9 72-3°C
Infrared— 2.98, 3.41, 3.51, 7.0, 7.5, 8.85, 9.25, 10.4, 11.65 μ.	Infrared— 2.94, 10.36 μ.
NMR** 4.03 (doublet, 1H), 4.4-5.1 (multiplet, 5H) ppm.	NMR— 3.37 (doublet, 1H), 4.3-5.0 (multiplet, 5H) ppm.

**The position of the hydroxyl proton doublet is concentration dependent.

Timed hydrolysis of 3-propenoxyoxetane

Water (12.8 g), 3-propenoxyoxetane (13.13 g, 0.115 mol), methanol (9.39 g), and concentrated sulfuric acid (19.4 μl), were added, at room temperature, to a 100 ml flask. To observe the progress of the reaction, glc on a 10' x 1/8", 15% carbowax (on chromsorb G) chromatography column was used at a temperature of 170°C and a flow rate of 28 cc/min. The starting material, (26), had a retention time of 4.67 min, and the product, (27), had a retention time of 14.68 min. The proportions of 26 and 27, were normalized to 100%. A minigrator was used to evaluate retention times and peak areas, the settings used were the following:

PW = 12, SS = 18, BL = 5, TP = 20

The reaction was monitored for 140 hrs, and the results are listed in Appendix A. A graph depicting these results is shown in Figure 26. At five days, 90% of 26 was converted to 27.

Attempted preparation of (3-oxetanyl) 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside 28

The 2,3,4,6-tetra-O-benzyl-D-galactopyranosyl chloride (6.7 g, 0.012 mol), 3-oxetanol (1.91 g, 0.026 mol), sodium iodide (6.65 g, 0.0444 mol, 3.7 eq.), acetonitrile (67.2 ml), and 2,6-lutidine (1.29 g, 0.012 mol), were added, at room temperature, to a 200 ml flask.[12] The reaction was allowed to progress for 20 hrs while being monitored by thin-layer chromatography on silica-gel, in solvent system (b): 28 has an R_F = 0.24, while 2,3,4,6-tetra-O-benzyl-α-D-galactopyranose has an R_F = 0.16. The reaction mixture was poured into water (500 ml), and saturated sodium chloride (100 ml) was added. This mixture was extracted three times with carbon tetrachloride, and the organic layer was dried with anhydrous sodium sulfate. After filtration, carbon tetrachloride was removed by rotary evaporation. The resultant syrup was placed over a 4 x 2.3 cm silica-gel column, and eluted with solvent system (b) (crude product— 8.4 g). The crude product (1 g) was subjected to overnight hydrogenolysis, by hydrogen at a pressure of 30 psi, and 10% Pd/C (91 mg) in ethanol (30 ml). No products were observed by thin-layer chromatography.

Infrared– 3300, 2950, 2875, 1450, 1340, 1208, 1090, 1050, 1025, 988, 920, 760, 702 cm^{-1}.

NMR– 3.1 (2H), 3.3-5.0 (envelope, 20H), 7.23 (singlet, 20H) ppm. The alpha-anomer was not present, since there was an absence of a signal at 5.2 ppm.

Treating the preparation of 28 to remove 2,6-lutidine

The syrup obtained from the preparation of 28 was dissolved in carbon tetrachloride and twice washed with 5% hydrochloric acid, twice washed with sodium bicarbonate, and with water. The resultant organic layer was dried over anhydrous sodium sulfate. The volume was reduced to minimum and was placed over a 4 x 2.3 cm silica-gel column. The column was eluted with solvent system (i). After the solvent was removed, the syrup (1 g) was dissolved in ethanol (30 ml), and was subjected to 10% Pd/C (0.1 g) overnight at 45 psi of hydrogen. No hydrogenolysis occured, as seen by thin-layer chromatography.

Hydrogenolysis of 28 for an extended period, and with addition of acetic acid

To a sample of 28 (0.25 g) prepared as previously described, was added palladium black (0.1 g) in ethanol (30 ml). This mixture was hydrogenolyzed for three days at a hydrogen pressure of 60 psi. The hydrogenated sample was compared to the starting material, (28), by

thin-layer chromatography on silica-gel, in solvent system (i): starting material, R_F = 0.35; product, R_F = 0.195. In this solvent system, a completely debenzylated glycoside, i.e., allyl α-D-galactopyranoside, would not move away from the origin.

All of the previous hydrogenated sample from above was placed in methanol (100 ml), with addition of acetic acid (30 µl). This solution was hydrogenolyzed overnight with palladium black (0.1 g) at a hydrogen pressure of 60 psi. There was no change in the R_F of the sample when chromatographed in solvent system (i).

Preparation of benzylcyclohexylether 32[56]

Sodium hydride (12.0 g, 0.5 mol) was added to a solution of cyclohexanol (20 g, 0.2 mol) and dioxan (125 ml) in a 500 ml, 3-necked flask, equipped with a reflux condenser, dropping funnel, and mechanical stirrer. After the dropwise addition of benzylchloride (50.6 g, 0.4 mol), the mixture was refluxed for 4 hrs.[34] After cooling, the mixture was centrifuged at 13,000 x g, and the supernatant was subsequently filtered This solution was concentrated and distilled (26.6 g, 70% yield).

bp_{11} 141.5-6.5°C Literature[56] bp_{37} 165.5-6.5°C

Infrared— 3050, 2942, 2370, 1505, 1459, 1370, 1100, 1033, 955, 738, 698 cm^{-1}.

NMR— 1.05-2.1 (envelope, 10H), 3.24 (multiplet, 1H), 4.43 (singlet, 2H),

7.21 (singlet, 5H) ppm.

Hydrogenolysis of benzycyclohexylether

Benzylcyclohexylether (0.2 g) was dissolved in ethanol (10 ml), and 10% Pd/C (0.05 g) was added. After shaking for one hr at a hydrogen pressure of 20 psi, 99.3% of the sample was converted to toluene, by glc analysis on a 10' x 1/4", 15% SE-30 (on chromsorb B) column, with a temperature of 95°C until cyclohexanol appears, then program at 15°C/min to 200°C, at a flow rate of 50 cc/min.

Attempted hydrogenolysis of benzylcyclohexylether with the same catalyst that was used for [29]

The same hydrogenolysis mixture in which acetic acid was added, was filtered, rinsed with methanol, and the catalyst was taken up to 100 ml with ethanol. Benzylcyclohexylether was added to the solution, and it was shaken for two days at a hydrogen pressure of 60 psi. With the glc procedure previously described, no hydrogenolysis was observed to have taken place.

Hydrogenolysis of methyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside

Methyl 2,3,4,6-tetra-O-benzyl-α-D-galactopyranoside (0.5 g) was added to ethanol (30 ml) and palladium black (0.25 g). There was an original hydrogen pressure of 63.25 psi, but after 1 hr 6.75 psi was

used up, and the reaction components were observed by thin-layer chromatography on silica-gel, in solvent system (i). There was no benzyl derivative remaining in the reaction medium. On paper chromatography in solvent system (j), the product was identical to authentic methyl α-D-galactopyranoside.

GC-minigrator studies of the hydrogenolysis of benzylcyclohexylether in the presence of 3-oxetanol

All samples were injected into a 10' x 1/4", 15% SE-30 (on chromsorb B) column. At injection the column temperature was 95°C and was held at that temperature until cyclohexanol was detected, then the temperature was programmed at 15°C/min to 200°C. The flow rate was 50 cc/min. All reactions were conducted in the following manner:

Benzylcyclohexylether(0.2 g) was placed in ethanol (10 ml) and the quantity of 3-oxetanol was added. 10% Pd/C (0.05 g) was added and the mixture was shaken with an initial hydrogen pressure of 20 psi. After 1 hr, the hydrogen was removed, the mixture was filtered, and glc analysis commenced. The results are listed in Appendix B, and Figure 31 presents the graphical interpretation.

Parr bomb hydrogenolysis of 29

A Parr bomb was used to achieve a hydrogen pressure of 1000 psi. Since the Parr bomb is large and used for hydrogenating crude samples,

a sample vial and holder was needed. "The Parr bomb micro sample holder" was constructed as shown in Section 4. Into the sample vial was placed 29 (125.6 mg), ethanol (4 ml), and palladium black (125.4 mg). The sample was hydrogenolyzed for 2 hrs at a hydrogen pressure of 1000 psi. A comparison, by thin-layer chromatography (silica-gel in solvent system (k)), of the product having R_F = 0.26, and starting material with R_F = 0.52, demonstrated no experimental difference from the product obtained at a hydrogen pressure of 60 psi.

Attempted debenzylation with a sodium-ethanol reduction

To a 50 ml flask, ethanol (20 ml) and 29 (0.15 g) was added. Sodium was then dropped into the solution,[57] and because the solution became so syrupy, one drop of water was added. Thin-layer chromatography on silica-gel in solvent system (b), revealed a minute amount of material near the origin, the starting material, and a product migrating in an experimentally equivalent manner to the product obtained by hydrogenolysis.

Preparation of 2,3,4,6-tetra-O-acetyl-α-D-galactosyl bromide 33[35]

After cooling acetic anhydride (200 ml) in an ice-water bath, it was introduced into a 500 ml, 3-necked flask, equipped with a mechanical stirrer and a thermometer. Perchloric acid (1.2 ml) was added dropwise, and the solution was brought to room temperature. Anhydrous

D-galactose (50 g) was added to the solution over a 0.5 hr period (the reaction must be kept at a temperature between 30 and 40°C). After cooling the reaction mixture to 20°C, red phosphorus (15 g) was added. With the reaction temperature held below 20°C, bromine (90 g) was added. Water (18 ml) was then added dropwise to the continuously stirred and cooled (below 20°C) mixture over a 0.5 hr period. The mixture was then stirred for 2 hrs at room temperature. Chloroform (150 ml) was added and the mixture was filtered thru a bed of glass wool. The filtrate was poured into water (400 ml) near 0°C. The mixture was separated and the organic layer washed with water (150 ml). The original aqueous mixture was extracted with chloroform (50 ml), all chloroform extracts were combined, placed in a 1 liter beaker, and neutralized with sodium bicarbonate. The mixture was separated and the chloroform layer was dried over anyhdrous sodium sulfate. The chloroform solutions were filtered and concentrated to a syrup, which crystallized upon digestion with small amounts of (petroleum ether 35-60°C-ether, 1:1), to give a crude product (90.8 g, 80% yield), melting point 73-4°C. A lot of material was lost in attempts to obtain a clear crystalline product. The crude product was heated with activated charcoal in diisopropyl ether, filtered, and recrystallized from diisopropyl ether.

mp 78-80.5°C Literature[35] mp 80-2°C

Infrared— 3425, 2980, 1750, 1435, 1375, 1220, 1080, 952, 916, 605,

545 cm^{-1}.

NMR** 1.93-2.17 (multiple singlets, 12H), 3.75-5.5 (envelope, 6.17H), 6.67 (doublet, 0.83H) ppm.

**These values could be rounded to 6H and 1H, but the value at 6.67 ppm with J = 4Hz, might represent the percentage of alpha-anomer, 83%.

Note: This compound is very unstable and should be made immediately before use in further reactions.

Preparation of (3-oxetanyl) 2,3,4,6-tetra-O-acetyl-β-D-galactopyranoside 34

3-oxetanol (3.65 g, 0.0493 mol) was dried by stirring in dry chloroform (25 ml) with 3Å molecular sieves (2 g) for 1 hr. This solution was filtered directly into a 250 ml reaction flask containing dry chloroform (50 ml), Drierite (40 g), 3Å molecular sieves (4 g), and silver carbonate (12 g, 0.044 mol). After one hr of stirring, a solution of 33 (13.6 g, 0.033 mol) in dry chloroform (25 ml) containing Drierite (5 g) was added. The reaction was stirred with a mechanical stirrer and kept from exposure to light by covering with black paper and aluminum foil. The reaction continued for 21 hrs, at which time it was nearly complete, by thin-layer chromatography on silica-gel, in solvent system (1), R_F = 0.2. The mixture was filtered with celite filter aid and concentrated by rotary evaporation to obtain a crude product (14.5 g). Part of the syrup (1.32 g) was placed on a 17 x 2.3 cm silica-gel

column, and eluted with solvent system (m) in 20 ml fractions. Fractions 4-8 were pooled and concentrated to a syrup (0.852 g, 70% yield).

NMR– 1.93-2.23 (multiple singlets, 12H), 3.16 (multiplet, 1H), 3.46 (envelope, 1.7H), 3.8-4.8 (envelope, 7H), 4.9-5.5 (envelope, 3H).

Glc study of products from the preparation of 34

The product mixture obtained for 34, before column separation, was submitted to glc on a 10' x 1/4", 20% SE-30 (on anakrom) column. The column temperature was 220°C with a pressure of 30 psi of helium. Two peaks were collected, one with a retention time of 68.3 min (#1), and the other with a retention time of 86.7 min (#2). By observation, #2 contained much more material than did #1. Thin-layer chromatography on silica-gel in diethylether, revealed that both peak #1 and #2 had the same R_F (0.334) as did the main product of the reaction. This could be indicative of an α-, β-mixture, where β- would be #2, and α-, #1.

Deacetylation of 34 to produce (3-oxetanyl) β-D-galactopyranoside 30

Deacetylation was accomplished in a similar manner as used for 22 in preparation of 17.[29b,36] The reaction mixture was separated by paper chromatography on prewashed Whatman #541 in solvent system (j), R_{Gal} = 1.52.

NMR— 2.7-3.1 (multiplet, 1H), 3.2-4.2 (envelope, 10H), 4.43 (doublet, 1H).

Both the crude deacetylated mixture and the paper chromatography isolated material were bioassayed on sugarcane leaves (clone 51NG97); no activity was observed.

Attempted anomerization of 34 by titanium tetrachloride

Freshly distilled titanium tetrachloride (0.4 g, 0.0021 mol) in dry chloroform (20 ml), was added to a solution of (3-oxetanyl) 2,3,4,-6-tetra-O-acetyl-β-D-galactopyranoside (0.852 g, 0.0021 mol) in dry chloroform (40 ml). The solution appeared to be light creamy yellow. This mixture was refluxed for 75 min, and poured into a separatory funnel containing ice and water (250 ml).[16c] After extraction, the chloroform layer was washed with saturated sodium bicarbonate (100 ml), and three times with water (100 ml), dried over anhydrous calcium sulfate and sodium sulfate. The mixture was filtered and concentrated. An NMR spectrum of this material revealed a doublet at 6.37 ppm with J = 3-4Hz. This was consistent with an α-halogenated acetylated glycoside. By thin-layer chromatography on silica-gel in solvent system (n), R_F = 0.57, with tetra-O-acetyl-α-D-galactosyl bromide, R_F = 0.59, the product showed very similar chromatographic mobilities. In the NMR spectrum, the acetyl methyl protons have the same resonance distribution as they do in the bromo compound.

Attempted anomerization of 34 by boron trifluoride etherate

The galactoside (0.43 g, 0.0011 mol), (34), was dried by stirring overnight over 3Å molecular sieves in dry chloroform (2 ml). This mixture was filtered into a 500 ml, 2-necked flask, containing dry chloroform (4 ml). The flask was sealed with a serum cap and purged with nitrogen. Freshly distilled boron trifluoride etherate (0.156 g, 0.0011 mol) was injected and the solution was stirred for 24.5 hrs, at which time the solution was a clear yellow color.[16ab] The reaction mixture was washed twice with saturated sodium bicarbonate (solution turned clear), three times with water, dried over anhydrous sodium sulfate, and concentrated. By thin-layer chromatography on silica-gel in diethylether, there are 7-8 separable products, which span the entire thin-layer plate. The NMR spectrum of the syrup shows signals from 0.9-1.5 ppm.

Coupling reactions with phenyl 1-thio-β-D-galactopyranoside[15]

All reactions were carried out in small vials. A spatula tip-full of phenyl 1-thio-β-D-galactopyranoside was placed in the vial. An excess of mercuric acetate was added, and the alcohol to be coupled was used as solvent. Tetrahydrofuran was added in a volume equal to 1/3 of the reaction mixture, and the final mixture was left overnight at room temperature. The products were chromatographed by thin-layer on silica-gel, in solvent system (o). The following is a list of the alcohols

used: 1,3-propanediol, methanol, ethanol, 2-propanol, 1-propanol, allyl alcohol, 2-butanol, 1-butanol, and cyclohexanol. Their R_F and R_{Gal} values are presented in Table 6. By thin-layer chromatography, all reactions went to completion giving one product, except the allyl alcohol reaction, which gave two products, one having an R_F value equal to that of galactose, the other having an R_F value identical to authentic allyl α-D-galactopyranoside. The product of the methanol reaction was compared to authentic methyl α-D-galactopyranoside, the authentic methyl derivative was too concentrated to directly compare, but the fronts of the spots were identical. Some of the reactions were passed through small silica-gel columns and an NMR spectrum was obtained of the product. For ethyl α-D-galactopyranoside, a triplet was observed at 1.3 ppm. For (1-propanol-3-O) α-D-galactopyranoside, a multiplet was observed at 1.95 ppm, and a signal at 5.05 ppm for the anomeric proton. For (1-propyl) α-D-galactopyranoside, an envelope from 1.0-1.6 ppm and a signal for the anomeric proton at 5.08 ppm. For (2-propyl) α-D-galactopyranoside, an envelope was observed from 1.1-1.5 ppm.

Bioassay of the products from the reaction with phenyl 1-thio-β-D-galactopyranoside

Aliquots (1 and 2 μl) of each reaction mixture was placed on needle punctures near the base of excised sugarcane leaves (clone 51NG97). The leaves were kept in a moist chamber at room temperature, and the

symptoms were recorded after 3 days.

Compound	Lesion length after 3 days, (mm)
methyl α-D-galactopyranoside	None
ethyl " " "	5.0
2-propyl " " "	7.0
1-propyl " " "	2.5
allyl " " "	14.0
2-butyl " " "	3.5
1-butyl " " "	2.5
cyclohexyl " " "	None
phenyl 1-thio-β-D-galactopyranoside	None

To obtain an accurate value of lesion length with a known amount of product, ethyl, 1-propyl, 2-propyl, and (3-propanol-1-O) α-D-galactopyranoside were chromatographed on pre-washed Whatman #541 paper, in solvent system (j). Weights were determined on a Cahn Gram Electrobalance and solutions were made having concentrations of 350 µg/µl. One and two microliters of each solution were placed on 2 or 3 needle punctures near the base of excised sugarcane leaves (clone 51NG97). The leaves were kept in a moist chamber at room temperature for three days. The results are presented in Table 7.

Reaction of 3-oxetanyl with phenyl 1-thio-β-D-galactopyranoside

Phenyl 1-thio-β-D-galactopyranoside (0.004 g, 1.44×10^{-5} mol),

mercuric acetate (0.0165 g, 5.19 x 10^{-5} mol), and 3-oxetanol (0.00185 g, 2.5 x 10^{-5} mol) were placed in a small vial. Tetrahydrofuran (0.2 ml) was added and the reaction mixture was shaken overnight at room temperature.[15] Four products were obtained, by thin-layer chromatography on silica-gel, in solvent system (o). After 3 days most of the starting material had reacted. The reaction mixture was bioassayed before and after a silica-gel column, and no activity was observed. These products do not favorably compare, by thin-layer, with those obtained in the preparation of 30.

Solvomercuration-demercuration of allyl α-D-galactopyranoside with mercuric acetate 41

Allyl α-D-galactopyranoside (0.4 g, 0.002 mol) was dissolved in a solution of tetrahydrofuran (30 ml) and water (1 ml). To this solution was added mercuric acetate (0.6 g, 0.002 mol), and the solution turned yellow. After two hours, the solution had only a faint yellow color, and the reaction was stopped by addition of a potassium hydroxide solution (25 ml, 3M) followed by addition of a sodium borohydride solution (0.036 g $NaBH_4$ in 25 ml of 3M KOH).[41] The mercury was allowed to precipitate, and the solution was vacuum filtered through celite filter aid, and concentrated by rotary evaporation. The reaction mixture was observed by thin-layer chromatography on silica-gel, in solvent system (o), allyl α-D-galactopyranoside R_F = 0.57, product R_F = 0.25. A com-

peting reaction with the internal solvomercuration would be the Markovnikov hydroxylation of allyl α-D-galactopyranoside. That product would be expected to migrate with similar mobilities as the product obtained. Compound 40 would be expected to migrate as far, or farther, than allyl α-D-galactopyranoside.

Solvomercuration-demercuration of allyl α-D-galactopyranoside with mercuric sulfate 40, 41

Allyl α-D-galactopyranoside (0.1 g, 0.0005 mol) was added to a mixture of mercuric sulfate (0.14 g, 0.00047 mol) in aqueous tetrahydrofuran (80 ml; 70 ml THF, 10 ml H_2O) at room temperature. The solution was yellow, and was stirred for 0.5 hr (one should stir the solution for 1.5-2 hrs), at which time the color was faint yellow. The reaction was stopped by adding a potassium hydroxide solution (65 ml, 3M), followed by a sodium borohydride solution (0.01 g, $NaBH_4$, 0.003 mol; in 65 ml 3M KOH).[41] The mercury was allowed to precipitate and the mixture was vacuum filtered through celite filter aid, and concentrated by rotary evaporation. This material was passed through a Dowex 50W-X8 column (17 x 2.3 cm). The reaction components were separated on a 17 x 2.3 cm silica-gel column, by using variations in solvent system (e), (75:6:1, 100 ml); (75:8:3, 75 ml); (75:12:6, 225 ml). Three components were observed: component #1 migrated faster than allyl α-D-galactopyranoside, R_F = 0.47. Component #2 was identical to allyl α-D-galactopyran-

oside, R_F = 0.28. Component #3 was identical to the product obtained from the mercuric acetate reaction, (41), R_F = 0.11.

Component #1 40

The NMR of a mixture of component #1 and #2, revealed a doublet at 1.18 ppm, indicative of 40.

CMR* 96.5488 (C-1), 75.6826 (C-2), 74.2234 (C-5), 73.9315 (C-3), 71.7428 (C-4), 66.3925 (C-7), 65.9061 (C-8), 63.4741 (C-6), 17.5587 (C-9) ppm.

Component #3 41

$[\alpha]_D^{26.8}$ = +229.4 in water(c, 0.435)

NMR— 1.28 (doublet, 3H), 3.3-4.3 (envelope, 9H), 5.04 (doublet, 1H) ppm.

CMR* 101.12 (6813, C-1), 100.59 (9091, C-1), 75.39 (7677, C-7), 74.90 (7503, C-7), 73.35 (12097, C-5), 71.94 (17034, C-3), 71.74 (14359, C-4), 70.92 (12136, C-2), 69.02 (6823, C-8), 68.68 (9675, C-8), 63.67 (13021, C-6), 20.62 (14302, C-9) ppm.

Note: values for external TMS may be obtained by subtracting 1.37 ppm from each resonance.

Treatment of the toxin with activated charcoal

A toxin preparation (4-5 mg), as obtained with the method described by the flow sheet in Figure 45, was diluted with water (4 ml). Two microliters of this solution caused observable runner lesions on sugarcane leaves (clone 51NG97) within 25 min. An aliquot (0.2 ml) of this solution was diluted with 95% ethanol to 2 ml, a spatula tip-full of activated charcoal was added, and the solution was heated until bubbles formed. The mixture was filtered through Whatman #2 filter paper and taken to dryness at 40°C by rotary evaporation. The sample was taken up to 0.2 ml with water and bioassayed. Runners were observed within the same time, and had similar runner intensity. Colored material had been removed by this treatment.

High pressure liquid chromatography (HPLC) of standards in various proportions of acetonitrile-water Figure 47

The carbohydrates, depicted in Figure 46, glucose, methyl α-D-glucopyranoside, maltose, and maltotriose, were used as standards. Solutions of the standards (6-10 μg/μl) were injected in 20 μl aliquots on a μ-Bondapak carbohydrate column (P/N 84038), and was monitored by refractive index. The flow rate was 0.86 cc/min, and the void volume was at 4.5 min. Three solvent systems were utilized consisting of acetonitrile-water, (70:30), (78:22), and (85:15). Injections of 20 μl of the previous charcoal treated toxin were also made with each solvent system.

Fractions were collected at each half-minute, evaporated to dryness at 40°C, taken up with water (20 µl), and bioassayed on sugarcane leaves (clone 51NG97). The results are observed in Figure 47.

Preparative high pressure liquid chromatography of the toxin mixture Figure 48

A concentrated toxin mixture (after charcoal treatment) was injected on a µ-Bondapak carbohydrate column (P/N 84038) in 20 µl aliquots. The solvent system used was (acetonitrile-water, 78:22), at a flow rate of 0.87 cc/min, void volume was at 4.5 min. The entire column run was bioassayed (for at least 6 runs), and only one area of toxicity was found, this toxic area coincided with the peak (by refractive index) at 14-15 min. A typical curve for this separation is represented in Figure 48.

Carbohydrate determination by phenol-sulfuric acid[41]

The phenol-sulfuric acid analysis was conducted by placing an aliquot (140 µl), of a carbohydrate solution in the bottom of a 20 ml test tube. A 5% phenol-water solution (1 ml) was then added to the tube, with subsequent addition of concentrated sulfuric acid (5 ml).[41] After a ten minute cooling period, the tube was placed in a spectrophotometer to obtain its absorbance at 490 nm. A glucose standard with a concentration of 135.7 µg/ml was used. A standard curve was generated, accor-

ding to the following data:

µg glucose	Absorbance (corrected to a 140 µl addition)	Absorbance 18 µg glucose
9.499	0.159	0.302
9.499	0.156	0.297
18.998	0.321	0.304
28.497	0.463	0.293
37.996	0.629	0.299

mean = 0.299 A/18 µg Glc
s = 0.0043

The standard curve for this glucose determination is presented in Figure 71, and has a correlation coefficient of 0.9997.

U.V. spectrum of the HPLC toxin preparation Figure 49

The amount of toxin used in the determination was obtained from a 5-liter fungal preparation. This was treated with activated charcoal, and separated by HPLC. The resultant material was taken up in water (1 ml) and scanned against a water blank. Chart rate = 1 in/min, and span = 2 absorbance units. Wavelengths were scanned from 600 nm to 190 nm. The observed scan is presented in Figure 49.

Bio-gel P-2 (-400 mesh) column separation of the toxin preparation (after HPLC) Figure 50

Toxin preparations obtained from HPLC (not greater than 1 mg) were

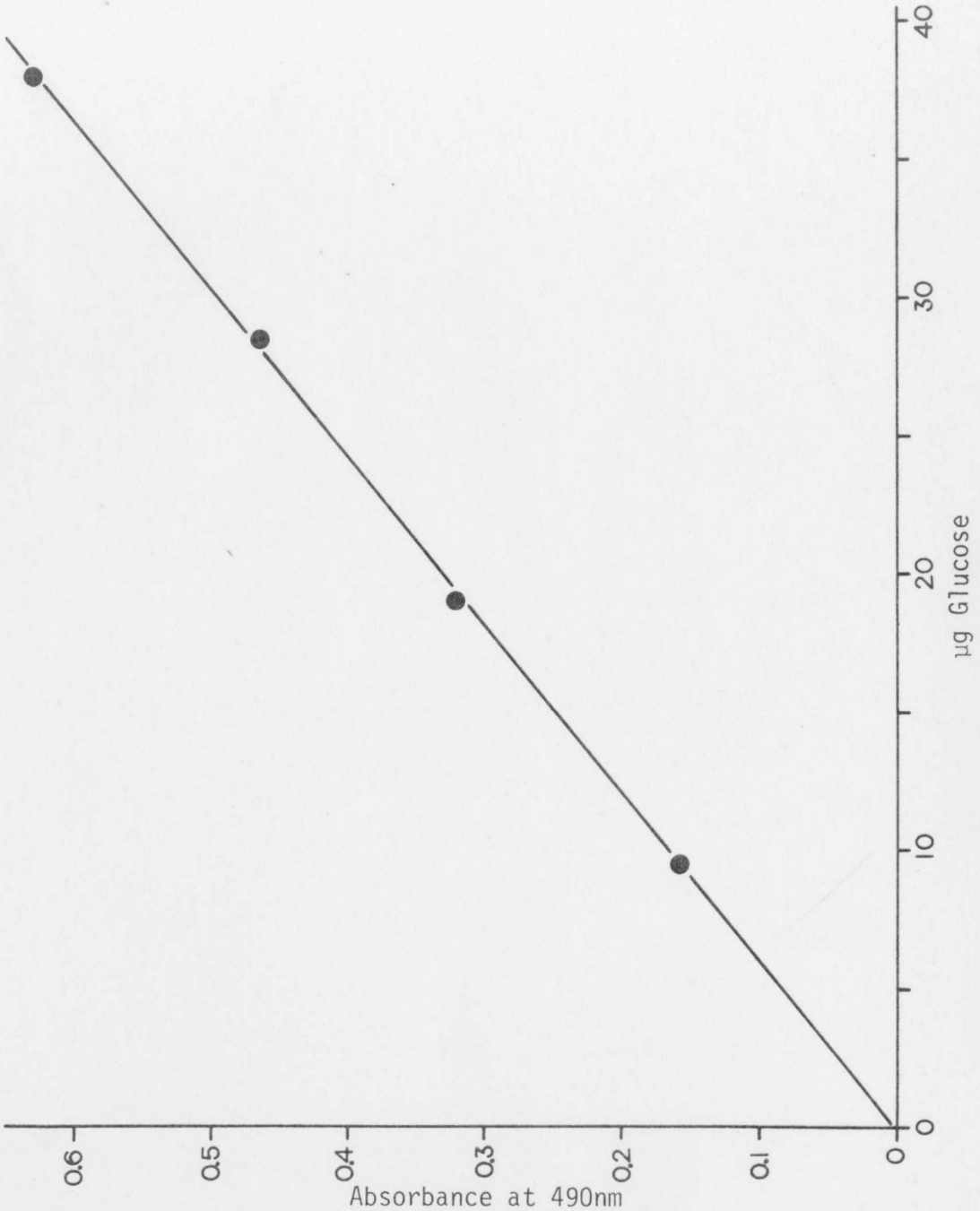


Figure 71. Standard curve for the determination of glucose, by phenol-sulfuric acid.

injected as a 0.25 ml sample onto a Bio-gel P-2 (-400 mesh) column. The flow rate was 1 drop/12 secs, collecting 13 drops or 0.56 ml per tube. The column was run at a temperature of 65°C (see Section 4), and the effluent was monitored at 192-196 nm in a 40 µl flow cell. A graduated cylinder was used to collect 36 ml prior to fraction collection. A carbon analysis was obtained (P. W. Jennings, Chemistry Department of MSU) on a portion (0.3 ml) from each tube, and normalized for total content. Each fraction was bioassayed on sugarcane leaves (clone 51NG-97), by placing equal quantities on a needle puncture near the base of excised leaves. The leaves were kept in a moist chamber at room temperature, and evaluated after three days.

Bio-gel P-2 (-400 mesh) column separation after the previous Bio-gel separation Figure 51

With conditions identical to the previous Bio-gel separation, 50µg of toxin preparation was re-loaded onto the column. A graduated cylinder was used to collect the first 41 ml of the run, then 0.56 ml fractions were collected. The resultant profile is seen in Figure 51.

Bio-gel P-2 (-400 mesh) column separation of allyl α-D-galactopyranoside Figure 55

Allyl α-D-galactopyranoside (50 µg) was injected (0.25 ml sample) onto a 295.5 x 0.8 cm, Bio-gel P-2 (-400 mesh) column. The flow rate

was 1 drop/12 secs, collecting 13 drops per tube or 0.56 ml/tube. The column was run at a temperature of 65°C and monitored at 196 nm in a 40 µl flow cell. A graduated cylinder was used to collect 35.4 ml prior to fraction collection. The peak as observed in Figure 55, reacted with potassium permanganate.

Bioassay to determine the importance of puncture position on sugarcane leaves Figure 58

Sugarcane leaves were excised and placed in water. Prior to puncturing, 1 µl of a toxin solution was placed on the reverse side of the leaf at the proposed puncturing site. The puncturing tool (shown in Figure 57) was used to make uniform punctures, and the leaves were placed in a moist chamber. The symptoms could be observed within 45 min, and runner length determined after 2.5 hrs. The results are presented in Figure 58.

Bioassay evaluating lesion length, and intensity of lesion, with respect to puncture postion

All punctures were treated as described previously. Sixty-three punctures were made in sixteen excised leaves. Since most runners extended the entire length of the leaves, the intensity of runners (visual comparison), and the length of runners having poor intensity, were evaluated. All symptoms were evaluated after 42 hrs of incubation in a moist chamber at room temperature. The results are tabulated in Table 10.

APPENDIX A

Tabulated Results for Figure 26, Percent Decrease of 3-propeneoxyoxetane with Time.

Time (hr)	Area of 3-propene-oxyoxetane	Area of 3-oxetanol	% 3-pro-peneoxy-oxetane	Log $\frac{1000}{\%\ 3\text{-propene oxyoxetane}}$
0.5	514697	2887	99.44	1.003
1.75	65980	690	98.97	1.004
4.58	82110	3140	96.32	1.017
20.5	24166	5409	81.71	1.086
26.78	23147	8103	74.07	1.130
46.08	13401	10244	56.68	1.246
51.05	15894	15478	50.66	1.294
66.52	11424	18959	37.6	1.425
71.63	9717	20031	32.66	1.486
76.38	8030	19377	29.3	1.533
90.83	5945	21219	21.89	1.66
95.88	5808	23872	19.57	1.708
103.0	3191	16795	15.97	1.797
116.5	2526	20848	10.8	1.967
140.08	737	11454	6.05	2.218

APPENDIX B

GC-Minigrator Studies of the Hydrogenolysis of Benzylcyclohexylether in the Presence of 3-oxetanol.

GC: The samples were injected onto a 10' x 1/4", 15% SE-30 (on chromsorb B) column. At injection the column temperature was 95°C and was held at that temperature until cyclohexanol was detected, then the temperature was programmed at 15°C/min to 200°C. The flow rate was 50 cc/min.

Minigrator: The following are the settings used during all determinations:

PW = 12	TP = 20
SS = 18	T1 = 130
BL = 5	T2 = 1130

The following symbols are used throughout this Appendix:

1 = toluene	2 = benzylcyclohexylether
m = mean	s = standard deviation

Determination of the detector response. Equimolar quantities of toluene and benzylcyclohexylether were used to make the standard solution.

Area			
1	2	(2/1) Ratio	Analysis
3691	10236	2.77	m = 2.71
3967	10764	2.71	s = 0.043
3650	9985	2.74	
3893	10470	2.69	
3783	10070	2.66	

Hydrogenolysis of Benzylcyclohexylether

Hydrogenation conditions

0.2 g of (2)
10 ml ethanol (absolute)
0.05 g 10% Pd/C
Time = 1 hr
Pressure = 20 psi H_2

Areas			% Production	
1	2	1 x R	of (1)	Analysis
3423	61	9276	99.3	m = 99.3
7785	123	21097	99.4	s = 0.05
7653	155	20740	99.3	
716	132	19404	99.3	
7513	142	20360	99.3	

Hydrogenolysis of Benzylcyclohexylether in the Presence of 3-oxetanol #1

Hydrogenation conditions

0.2 g of (2)
0.1 g of 3-oxetanol
10 ml ethanol (absolute)
0.05 g 10% Pd/C
Time = 1 hr
Pressure = 20 psi H_2

Areas			% Production	
1	2	1 x R	of (1)	Analysis
3717	2323	10073	81.3	m = 80.2
4209	2876	11406	79.9	s = 0.7
4061	2794	11005	79.8	
4109	2863	11135	79.5	
3994	2657	10824	80.3	

Hydrogenolysis of Benzylcyclohexylether in the Presence of 3-oxetanol #2

Hydrogenation conditions

0.2 g of (2)
0.2 g of 3-oxetanol
10 ml ethanol (absolute)
0.05 g 10% Pd/C
Time = 1 hr
Pressure = 20 psi H_2

Areas			% Production	
1	2	1 x R	of (1)	Analysis
3042	7098	8284	53.9	m = 53.6
2984	7076	8087	53.3	s = 0.846
3305	7728	8957	53.7	
3567	7925	9667	54.95	
3242	7795	8786	53.0	
3218	7905	8721	52.5	

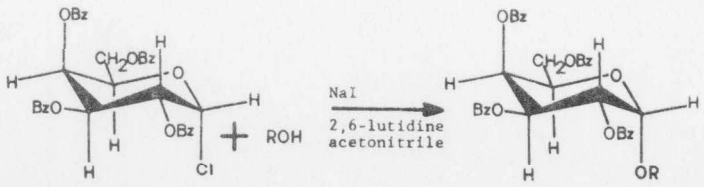
REFERENCES AND NOTES

1. J. P. Martin, E. V. Abbott and C. G. Hughes, *Sugarcane diseases of the World* 1, Elsevier, New York (1961) pp. 166-202.
2. a. This work quotes the original paper of Kruger[2b]; reference 1.

   b. W. Kruger, *Uber Krankheiten und Feinde des Zuckerrohres*, Berichte der versuchsstation für Zuckerrohr in West-Java, Kagok-Tegal (Java) Heft I. Dresden (Schönfeld), (1890) pp. 50-179.
3. A. Lee, *Plant Physiol.* 4, 193-212 (1929).
4. L. J. Liu, *Int. Soc. Sugarcane Technol. Proc.* 13, 1212-18 (1968).
5. G. W. Steiner and R. S. Byther, *Phytopath.* 61, 691-5 (1971).
6. G. A. Strobel, *Biochim. Biophys. Acta* 554, 460-8 (1979).
7. R. B. Pringle and R. P. Scheffer, *Phytopath.* 57, 1169-72 (1967).
8. R. B. Pringle and R. P. Scheffer, *Annu. Rev. Phytopath.* 2, 133-56 (1964).
9. a. R. P. Scheffer and K. R. Samaddar, *Recent Advan. Phytochem.* 3, 123 (1970).

   b. R. P. Scheffer and R. B. Pringle, *The Dynamic Role of Molecular Constituents in Plant-Parasite Interaction*, Edited by C. Mirocha and I. Uritorri, Bruce Publishing Company, St. Paul, Minn. (1965) p. 372.
10. R. S. Byther and G. W. Steiner, *Phytopath.* 62, 466-70 (1972).
11. G. W. Steiner and G. A. Strobel, *J. Bio. Chem.* 246, 4350-7 (1971).
12. F. J. Kronzer and C. Schuerch, *Carbohyd. Res.* 34, 71-8 (1974).

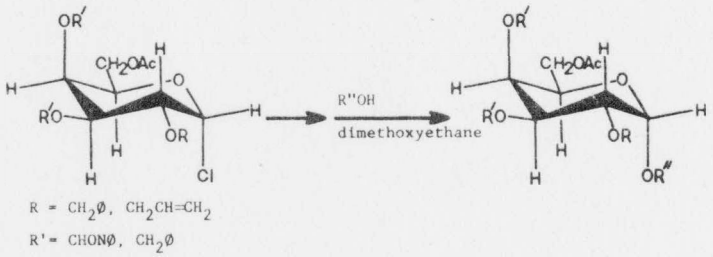
The following generalized reaction was utilized:



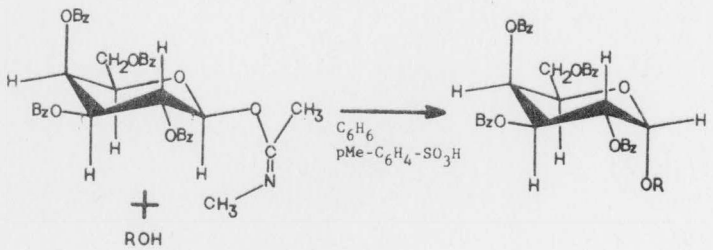
13. a. R. Eby and S. Schuerch, *Carbohyd. Res.* 34, 79-90 (1974).

b. T. J. Lucas and C. Schuerch, *Carbohyd. Res.* 39, 39-45 (1975).

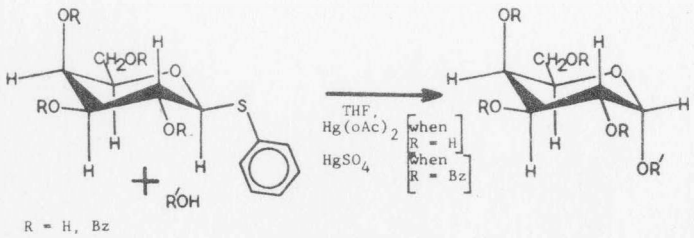
c. C. V. Maroušek, T. J. Lucas, P. E. Wheat and C. Schuerch, *Carbohyd. Res.* 60, 85-96 (1978). The following generalized reaction was utilized thru the tosylate or sulfonate:



14. J. R. Pougny, J. C. Jacquinet, M. Nasser, D. Duchet, M. L. Milat and P. Sinaÿ, *J. Amer. Chem. Soc.* 99, 6762-3 (1977). The following generalized reaction was utilized:

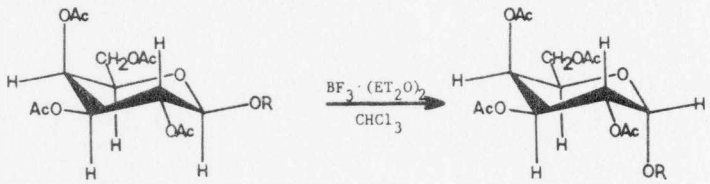


15. R. J. Ferrier, R. W. Hay and N. Vethaviyasar, *Carbohyd. Res.* 27, 55-61 (1973). The following generalized reaction was utilized:

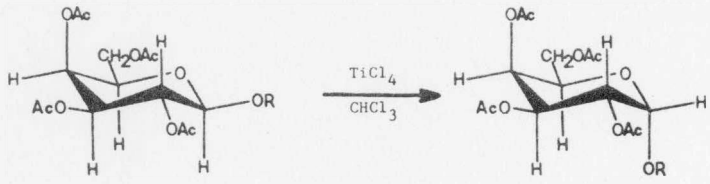


16. a. B. Lindberg, *Acta Chem. Scand.* 2, 429 (1948).

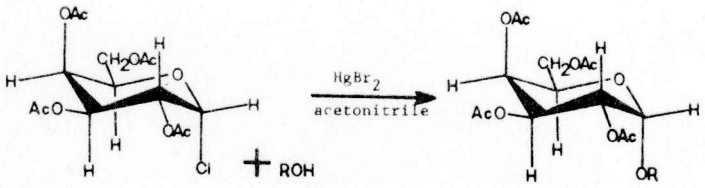
b. R. L. Whistler, K. A. Kimwell and D. F. Durso, *J. Amer. Chem. Soc.* 73, 3530 (1951). The following is a typical boron trifluoride-etherate anomerization:



c. E. Pacsu, J. Janson and B. Lindberg, *Methods in Carbohydrate Chemistry* 2, Academic Press, New York (1963) pp. 376-85. The following is a typical titanium tetrachloride anomerization:



17. G. Wueff and G. Rohle, *Angew. Chem. Int. Edn. Eng.* 13, 157 (1974). The following Koenigs-Knorr glycosidation is unpredictable and produces small amounts of the alpha anomer:



18. F. Chen and C. Ainsworth, *J. Amer. Chem. Soc.* 94, 4037-8 (1972).

19. R. Adams and R. M. Kamm, *Organic Synthesis* 1, John Wiley and Sons, New York (1967) pp. 250-1.

20. J. L. Frahn and J. A. Mills, *Aust. J. Chem.* 18, 1303-5 (1965).

21. C. P. J. Glaudemans and H. G. Fletcher, Jr., *Methods in Carbohydrate chemistry* VI, Academic Press, New York (1972) pp. 374-5.

22. P. W. Austin, F. E. Hardy, J. E. Buchanan and J. Baddiley, *J. Chem. Soc.*, 1419 (1965).

23. T. D. Perrine, C. P. J. Glaudemans, R. K. Ness, J. Kyle and G. Fletcher, Jr., *J. Org. Chem.* 32, 664-9 (1967).

24. R. A. OLafson, K. D. Lotts and G. N. Barber, *Tet. Lett.*, 3381-4 (1976).

25. Floridoside and isofloridoside, were a generous gift from J. S. Craigie and J. McLachlan, Atlantic Regional Laboratory, National Research Council of Canada, Halifax, Nova Scotia.

26. a. E. Fischer and L. Beensch, *Ber.* 27, 2478 (1894).

b. E. Fischer, *Ber.* 28, 1145 (1895).

27. E. A. Talley, M. D. Vale and E. Yanovsky, *J. Amer. Chem. Soc.* 67, 2037-9 (1945).

28. M. L. Wolfrom and A. Thompson, *Methods in Carbohydrate Chemistry* 2, Academic Press, New York (1963) p. 211.

29. a. G. Legler and E. Bause, *Carbohyd. Res.* 28, 45-52 (1973).

b. J. E. G. Barnett and A. Ralph, *Carbohyd. Res.* 17, 231-3 (1971).

30. M. Fieser and L. Fieser, *Reagents for Organic Synthesis* 1, John Wiley and Sons, New York (1967) p. 796.

31. B. Lindberg, *Acta Chem. Scand.* 9, 1097-99 (1955).

32. J. A. Wojtowicz, R. J. Polak and J. A. Zaslowsky, *J. Org. Chem.* 36, 2232-6 (1971).

33. J. A. Wojtowicz and R. J. Polak, *J. Org. Chem.* 38, 2061-6 (1973).

34. a. M. Fieser and L. Fieser, *Reagents for Organic Synthesis* 1,John Wiley and Sons, New York (1967) pp. 1079-80.

b. M. E. Tate and C. T. Bishop, *Can. J. Chem.* 41, 1801 (1963).

c. P. A. Gent, R. Gigg and R. Conant, *J. Chem. Soc. Perkin Trans.* 1, 1535-42 (1972).

d. R. Cornubert and H. LeBchan, *Bull. Soc. Chim.* 43, 74-8 (1928).

35. R. U. Lemieux, *Methods in Carbohydrate Chemistry* 2, Academic Press, New York (1963) p. 221.

36. A. Thompson and M. L. Wolfrom, *Methods in Carbohydrate Chemistry* 2, Academic Press, New York (1963) pp. 215-20.

37. F. J. Bates and Associates, *Polarimetry, Saccharimetry, and the Sugars*, Circular of the National Bureau of Standards C440 (1942) p. 498.

38. a. A. C. Farthing, *Brit. Pat.* 723,777 (Feb. 9, 1955).

b. A. C. Farthing, *J. Chem. Soc.*, 3648 (1955).

c. A. C. Farthing, *Chem. Ind. (London)*, 594 (1957).

d. R. Koehler and H. Pietsch, *Brit. Pat.* 797,276 (July 2, 1958).

39. A. Weissberger, Editor, *Heterocyclic Compounds with Three- and Four Membered Rings, Part Two*, John Wiley and Sons, New York (1964) pp. 1000-4.

40. Personal communication with R. H. Furneaux prompted interest in using phenyl 1-thio-β-D-galactopyranoside as a synthetic tool. He Kindly sent one gram of the compound for trial purposes. Reference 15 sites the method for synthesizing this galactoside.

41. F. G. Bordwell and M. L. Douglass, *J. Amer. Chem. Soc.* 88, 993 (1966). The following shows the oxymercuration reaction and some of the results:



42. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.* 28, 350 (1956).

43. S. Udenfriend, S. Stein, P. Böhlen, W. Dairman, W. Leimgruber and M. Weigele, *Science* 178, 871 (1972).

44. H. Determann and W. Michel, *J. Chromatog.* 25, 303 (1966).

45. B. J. Millard, *Quantitative Mass Spectroscopy*, Heyden and Son Ltd. (1978) p. 9.

46. W. D. Lehmann, H. -R. Schulten and H. D. Beckey, *Org. Mass Spectrom* 7,1103-8 (1973).

47. P. DeMayo, R. E. Williams and E. Y. Spencer, *Can. J. Chem.* 43, 1357-65 (1965).

48. D. C. Aldridge and W. B. Turner, *J. Chem. Soc. (C)*, 686-8 (1970).

49. Methylation analysis was completed by Vernon N. Reihold, Harvard Medical School, Boston, Mass.

50. Methylation analysis was completed by Peter Albersheim and Michael McNeil, Department of Chemistry, University of Colorado, Boulder,CO.

51. M. Oshima and T. Ariga, *Febs. Letters* 64, 440-2 (1976).

52. M. Rietschel-Berst, N. H. Jentoft, P. D. Rick, C. Fletcher, F.Fang and J. E. Gander, *J. Biol. Chem.* 252, 3219-26 (1977).

53. Egan Stahl, Editor, *Thin-Layer Chromatography*, Springer-Verlug, New York (1969).

54. These physical constants were obtained from the *Handbook of Chemistry and Physics* 47 ed., R. C. Weast editor, The Chemical Rubber Co. Cleveland, Ohio (1966-7).

55. B. Wickberg, *Acta Chem. Scand.* 12, 1187-1201 (1958).

56. R. Cornubert and H. LeBchan, *Bull. Soc. Chim.* 43, 74-8 (1928).

57. D. J. Bell and J. Lorber, *J. Chem. Soc.*, 453 (1940).

SECTION 2

STRUCTURAL INVESTIGATIONS OF THE RHYNCHOSPOROSIDES

(a series of phytotoxins found in *Rhynchosporium secalis*)

LIST OF TABLES FOR SECTION 2

TABLE		PAGE
1	Assignments of signals in the NMR spectrum of rhyncho-sporoside	165
2	Mass spectral data for the two isomers of benzyloxy-propanol.	177
3.	R_F values of both (2-propanol-1-O) α-D-galactopyranoside and α-cellobioside, and the native toxin in the solvent system, (ethylacetate-water-methanol, 84:18:35)	188
4	Methylation analysis of the toxin fraction.	192
5	Isolation steps including weights and amounts required for toxicity.	197
6	Symptoms observed after 3.5 days on barley cultivar var. Hannchen C. I. 531, from areas on paper chromatograms . .	198
7	Resonances from NMR spectra of all samples isolated from *R. secalis* extracts	214
8	Analysis by glc (on SE-30) for the isomers of benzyloxy propanol, in all samples that were determined, by NMR, to possibly contain 1,2-propanediol.	215
9	Samples from extracts of *R. secalis* determined to possess a glycosidic linkage to 1,2-propanediol	216
10	Methylation analysis of the sample, cell. #1.	218
11	Methylation analysis of the sample, cell. #2.	219

LIST OF FIGURES FOR SECTION 2

FIGURE		PAGE
1	Flow sheet summarizing the isolation procedure	163
2	Proposed structure for rhynchosporoside.	164
3	The 1-O-benzyl isomer of propanediol	167
4	Benzylation of allyl alcohol	167
5	Oxymercuration of allylbenzylether.	168
6	Benzylation of methyl α-D-glucopyranoside.	169
7	Hydrolysis of the anomeric linkage	169
8	Reaction of thionyl chloride on perbenzylated glucose. .	170
9	Coupling reaction forming the 2-O linkage.	170
10	Benzyl protection removed by hydrogenolysis.	171
11	Radio-active profile of a partially purified region containing carbohydrates, on paper chromatography, with solvent system (1-propanol-ammonium hydroxide, 66:33). .	173
12	Reaction scheme for obtaining the benzylated aglycone. .	174
13	2-benzyloxy-1-propanol	174
14	Hydroxyl protection of allyl alcohol by dihydropyran . .	175
15	Oxymercuration of 2-(allyloxy) tetrahydropyran	175
16	Benzylation, producing a double protected 1,2-propanediol	175
17	Removal of the tetrahydropyranyl moiety by acid hydrolysis.	176

FIGURE		PAGE
18	Acetylation of cellobiose.	178
19	Bromination of octa-acetyl cellobiose.	179
20	Bromide displacement by thiophenol	179
21	Deacetylation by ammonia in methanol	180
22	Benzylation of phenyl 1-thio-β-cellobioside.	180
23	Mercuric sulfate facilitated coupling of 2-benzyloxy-1-propanol to phenyl 2,3,6,2',3',4',6'-hepta-O-benzy-1-thio-β-cellobioside.	181
24	Hydrogenolysis of (2-benzyloxypropane-1-O) 2,3,6,2',3',4',6'-hepta-O-benzyl-cellobioside.	182
25	Mercuric acetate facilitated coupling of 1-benzyloxy-2-propanol to phenyl 2,3,6,2',3',4',6'-hepta-O-benzyl-1-thio-β-cellobioside.	183
26	Mercuric sulfate facilitated coupling of 1-benzyloxy-2-propanol to phenyl 2,3,6,2',3',4',6'-hepta-O-benzyl-1-thio-β-cellobioside.	184
27	Hydrogenolysis of (1-benzyloxypropane-2-O) 2,3,6,2',3',4',6'-hepta-O-benzyl-cellobioside.	185
28	Thin-layer chromatography of the (1-O) and (2-O) α-cellobiosides of 1,2-propanediol	186
29	Thin-layer chromatography of the (2-propanol-1-O) α-cellobioside hydrolysate and natural toxin on silica-gel in	

FIGURE		PAGE
	solvent system (ethyl acetate-water-methanol, 84:18:35).	187
30	Plot of subunits vs. $-\log(R_F/1-R_F)$ for (2-propanol-1-0) α-glucoside, α-cellobioside, and the natural toxin . . .	189
31	Plot of subunits vs. $-\log(R_F/1-R_F)$ for hydrolysis products of cellulose.	191
32	Comparitively chromatographed (1-propanol-2-0) α-D-glucopyranoside and the (2-propanol-1-0) α-cellobioside hydrolysate on silica-gel, in solvent system (ethyl acetate-water-methanol, 84:18:35)	193
33	Flow sheet summarizing the isolation procedures utilized for obtaining carbohydrates from *R. secalis* extracts . .	195
34	Areas bioassayed from paper chromatography, run in solvent system (1-butanol-pyridine-water, 9:5:4)	198
35	Cello-oligomers on a 95 x 1.5 cm Bio-gel P-2 (200-400 mesh) column	200
36	Plot of (Ve/Vo) vs. log(molecular weight) for cello-oligosaccharides	201
37	Malto-oligomers on a 95 x 1.5 cm Bio-gel P-2 (200-400 mesh) column	202
38	Plot of (Ve/Vo) vs. log(molecular weight) for malto-oligosaccharides	203

FIGURE		PAGE
39	Synthetic (2-propanol-1-O) α-cellobioside on a 95 x 1.5 cm Bio-gel P-2 (200-400 mesh) column	204
40	Observed toxicity for the Bio-gel P-2 (200-400 mesh) column separation on the toxin + origin section.	206
41	Elution profile of carbohydrates from the cellobioside section.	207
42	Elution profile of sample, Cell. #1.	208
43	Elution profile of sample, Cell. #2.	210
44	Elution profile of sample, Cell. #3.	211
45	Elution profile of the glucoside section	212
46	The proposed structures of the Rhynchosporosides	222

INTRODUCTION

Scald is a disease of barley and many other grasses. It is found in the large barley growing areas of the world. The incidence and economic importance have been increasing, particularly under conditions of continuous cropping in no-till agriculture.[1]

The affected areas of leaves initially appear grey, water soaked areas, 8-12 days after inoculation.[2] These affected areas may become dry but retain a grey-green color. Also, these areas or lesions may become straw-colored and surrounded by dark-brown margins. When these lesions become too numerous, photosynthesis is reduced, resulting in poor yields.

Ayesu-Offei and Clare[2] in 1971 were the first to apply sterile culture filtrates of *R. secalis* to cut stems of clipper barley seedlings and cause symptoms similar to those produced on infected leaves. The culture filtrates were prepared by straining the cultures through a double layer of muslin, centrifuging the filtrate at 110,000 x g for 1 hr at 15°C, and then sterilizing the supernatant by passing it through a 0.5 μm millipore filter. The following observations were made subsequent to treatment with the sterile culture filtrates. Within about 1 hr of treatment, large, dry, grey-green areas formed towards the leaf tips. These areas retained their color after the rest of the leaf had dried and turned a straw color. The effectiveness of sterile culture filtrates was not reduced by dialysis or autoclaving.

The affected parts of the leaves treated with culture filtrates

were examined microscopically. The mesophyll and anticlinal walls of the epidermis had collapsed so that the outer and inner periclinal walls were in contact. This damage was similar to that observed in infected leaves.[3]

Ayesu-Offei and Clare[2] concluded that toxic metabolites of *R. secalis* were reponsible for the disease symptoms in infected plants. They suggested that the *R. secalis* toxins may be glyco- or polypeptides, since the toxic effect was not removed by dialysis or autoclaving.

In 1978 Auriol et al. isolated a metabolite of *R. secalis* with phytotoxic activity. This metabolite caused symptoms in the barley cultivar var. Hannchen C. I. 531, similar to those observed upon infection with *R. secalis*. Contrary to the postulate of Ayesu-Offei and Clare,[2] that the toxins appeared to be glyco- or polypeptides; it was found that the isolated phytotoxic material was a simple carbohydrate. These authors named the phytotoxic material 'rhynchosporoside'.

A flow sheet summarizing the steps used in the isolation of rhynchosporoside is presented in Figure 1.

After acid hydrolysis, rhynchosporoside was determined to contain glucose by gas-liquid chromatography (glc), and thin-layer techniques. It was also shown to contain the elements of 1,2-propanediol by paper chromatography, thin-layer chromatography, glc and mass spectroscopy.

After treatment of rhynchosporoside by β-glucosidase, the primary products were glucose and a compound that had an R_F (in one solvent

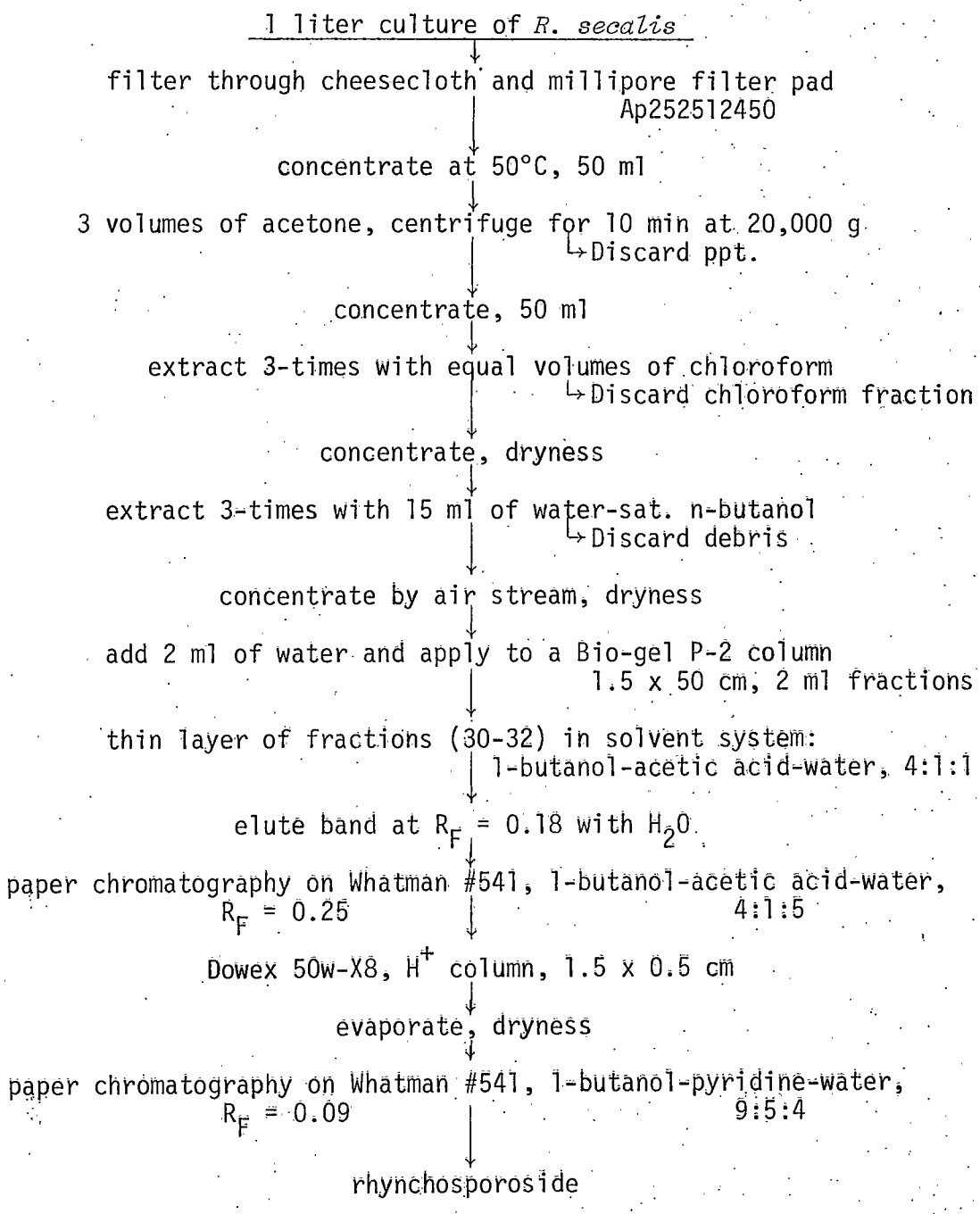
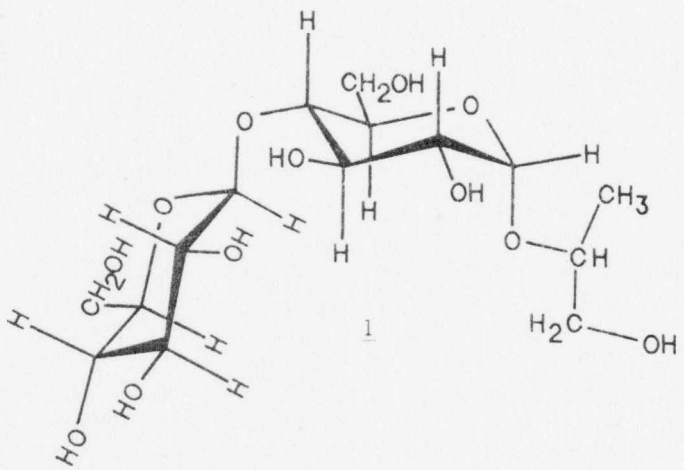


Figure 1. Flow sheet summarizing the isolation procedure.

system) identical to (1-propanol-2-O) α-D-glucopyranoside. After mild acid hydrolysis the reaction mixture contained cellobiose, glucose, 1,2-propanediol and a glucoside. However, upon treatment of the toxin with α-glucosidase, no new products were observed.

With the added information obtained from the NMR spectrum of rhynchosporoside (Table 1) a structure was proposed, (1-propanol-2-O) α-D-cellobioside, (1), as shown in Figure 2.



1

Figure 2. Proposed structure for rhynchosporoside.

The work to be described, concerning the synthesis of rhynchosporoside, and later the isolation of phytotoxic metabolites of *R. secalis*, was initiated. The following questions were the intent of this study:

1. Can (1-propanol-2-O) α-D-glucopyranoside be synthesized, and in a quantity suitable for use in subsequent enzymatic reactions?

Table 1. Assignments of signals in the NMR spectrum of rhynchosporoside.

Shift, ppm	J, Hz	Assignments
1.28	7.35	$-CH_3$ from Aglycone
3.4-4.08	-	sugar protons and protons on the aglycone carbons bearring oxygens
4.68	7.0	anomeric proton, β-glycosidic bond
5.37	2.5	anomeric proton, α-glycosidic bond

2. What is the nature of the α-linkage from cellobiose to 1,2-propanediol?
3. Can a compound having the proposed structure, (1-propanol-2-0) α-D-cellobioside, (1), be synthesized?
4. By looking at other phytotoxic metabolites of *R. secalis*, is it possible to understand any relationships between metabolites and their role in the disease?

RESULTS AND DISCUSSION

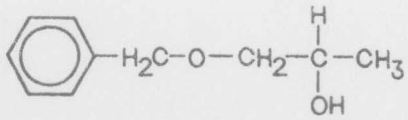
The origins of our involvement with the rhynchosporoside project can be traced to our interest in synthesizing the proposed cellobioside, (1). This is not a trivial problem since this cellobioside has an alpha linkage. The only reported method for synthesizing α-cellobiosides utilized titanium tetrachloride in an anomerization reaction on a per-acetylated-β-cellobioside.[4] However, the yields were very poor, owing to the susceptibility of both linkages to anomerization. Maximum yields could not be expected to be greater than 50%, and were usually not greater than 10-30%.

Since a primary reason for obtaining a synthetic product included the incorporation of ^{14}C radio-carbon label, it was considered advantageous to have a better method.

Pio Beltran, already working on the rhynchosporoside project, endeavored to synthesize radio-labeled toxin. His method utilized an enzyme, β-glucosidase, to facilitate a β-glucosy linkage with an appropriate α-glucopyranoside.[5] Our efforts then focused upon synthesizing the appropriate α-glucopyranoside.

The methods for producing alpha anomers were described in Section 1, reference 12-17. The method of choice was that of Kronzer and Schuerch.[6] The important point concerning this synthesis was to utilize an appropriately protected alcohol, which would guarantee only one aglyconic isomer. Figure 3 shows the alcohol of choice, 1-O-benzyl-2-propanol,

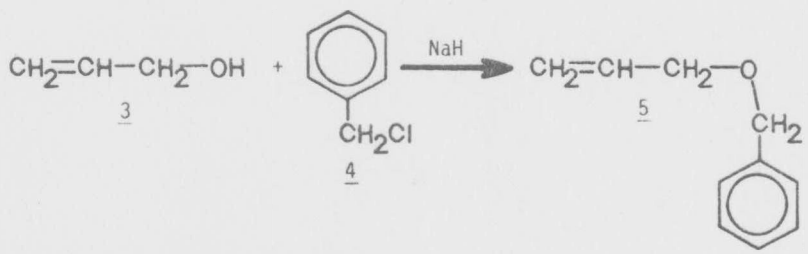
(2).



2

Figure 3. The 1-O-benzyl isomer of propanediol.

A two step synthesis was required to obtain 2. Allyl alcohol, (3), was reacted with benzyl choloride, (4), in the presence of sodium hydride, producing allyl benzyl ether, (5), as shown in Figure 4.[7]



3 4 5

Figure 4. Benzylation of allyl alcohol.

An exceptionally good method for obtaining the required functionality at C-2 and a proton at C-3 in one step, utilized an oxymercuration reaction, in which the mercuric-ion attacked, very specifically, the lesser substituted double bonded carbon. Figure 5 depicts the oxymercuration of 5 producing 1-benzyloxy-2-propanol, (2).[8]

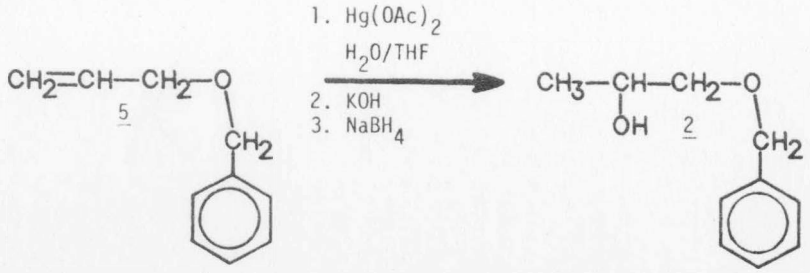


Figure 5. Oxymercuration of allyl benzyl ether.

A very interesting observation was made while doing oxymercuration reactions. By accident, dioxan was used instead of THF. A brilliant yellow color developed when mercuric acetate was added, unlike the light yellow color observed when THF-water was the solvent. It was found that upon acidification the yellow color disappeared, and reappeared upon making the solution basic. Also, if the solution was acidified before addition of mercuric acetate, the solution remained clear. The reaction mechanism requires that acetic acid be generated during the course of reaction, which may be responsible for the loss of the yellow color. Brown and Geoghegan[9] have used the disappearance of yellow color to signal the end of reaction, as well as a quantitative measure of reaction rate. However, they did not establish the cause of the color nor the reasons for its disappearance as the reaction progressed. At this juncture, it is suggested that further work be pursued to analyze these causes, and to examine solvent and pH effects on both reaction rates and product distribution.

The next three synthetic steps were used to prepare the carbohy-

drate derivative required for the coupling reaction. The hydroxyl groups on methyl α-D-glucopyranoside, (6), were totally protected by reaction with benzyl chloride, (4), producing methyl 2,3,4,6-tetra-O-benzyl-α-D-glucopyranoside, (7), as shown in Figure 6.[10] The benzyl derivative,

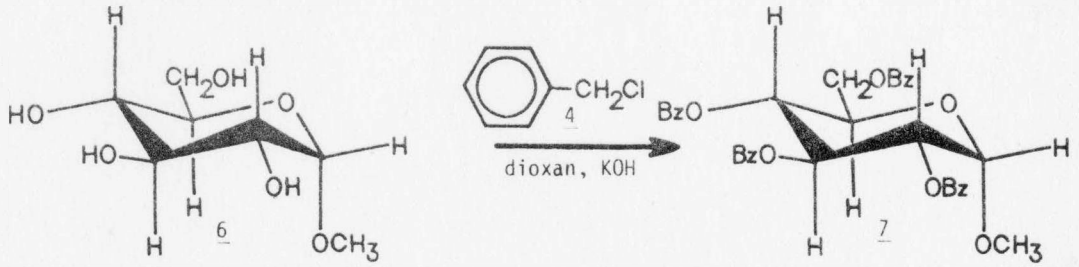


Figure 6. Benzylation of methyl α-D-glucopyranoside.

(7), was hydrolyzed with acetic and sulfuric acid (Figure 7), producing 2,3,4,6-tetra-O-benzyl-α-D-glucopyranoside, (8).[10]

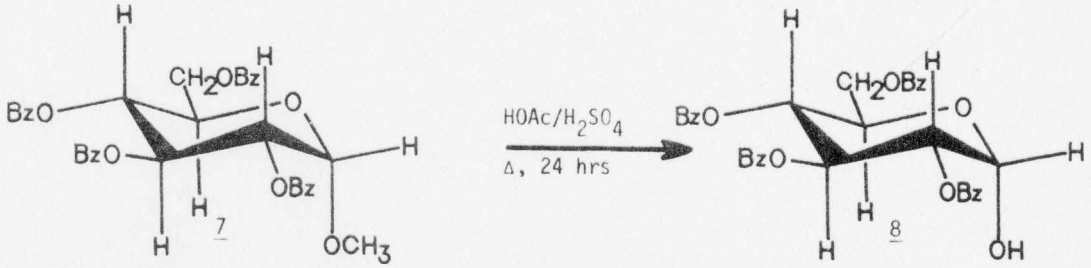


Figure 7. Hydrolysis of the anomeric linkage.

The derivative required for coupling was made by treating 8 with thionyl chloride, producing 2,3,4,6-tetra-O-benzyl-D-glucosyl chloride, (9), as

delineated in Figure 8.[11]

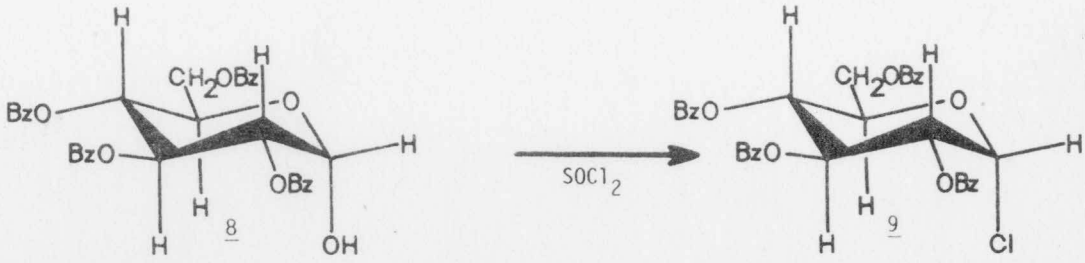


Figure 8. Reaction of thionyl chloride on perbenzylated glucose.

The iodide ion displaces the chloride of 9, which is itself subsequently displaced by the alcohol of 2, generating (1-benzyloxypropanol-2-O) 2,3,4,6-tetra-O-benzyl-α-D-glucopyranoside, (10), as shown in Figure 9.[6] The proportion of alpha-anomer in the sample purified by silica gel chromatography, was determined by proton NMR to be 75%.

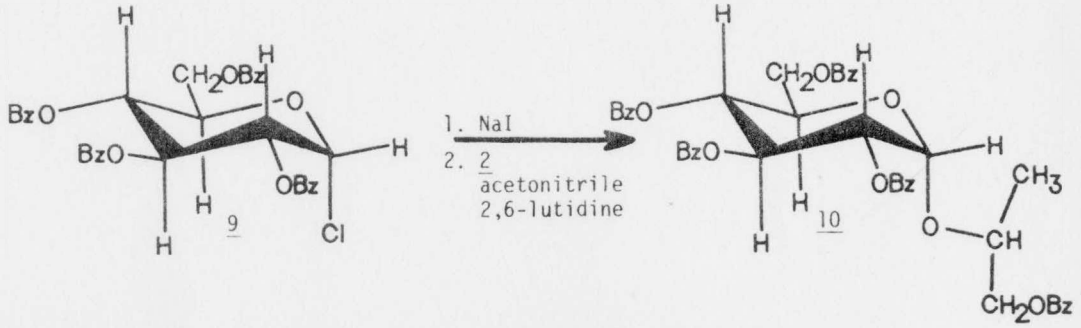


Figure 9. Coupling reaction forming the 2-O linkage.

Benzyl group protection was removed from 10 by a hydrogenolysis reaction using 10% Pd/C in ethanol, producing (1-propanol-2-O) α-D-glucopyranoside, (11), as depicted in Figure 10.[12] This derivative was

bioassayed on barley leaves (cultivar var. Hannchen C. I. 531) and activity was observed at approximately 400 μg/ml.

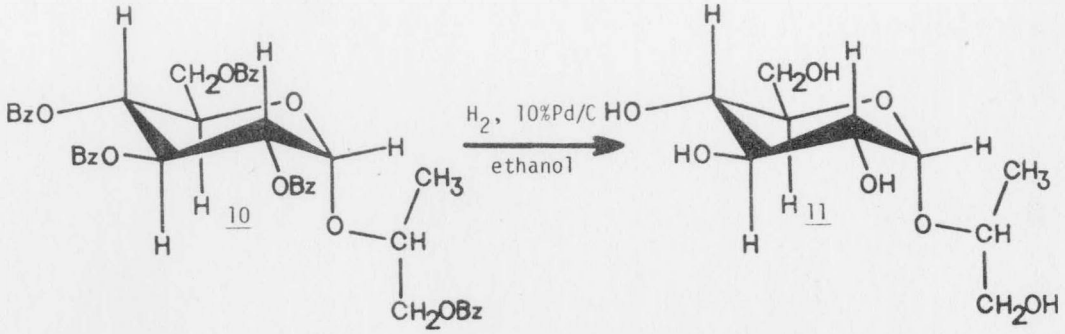


Figure 10. Benzyl protection removed by hydrogenolysis.

After using paper chromatography to separate the α- and β-anomers, the product was ready for the enzymatic reaction.[5]

Since we now had this glucoside, it was of interest to compare it to the carbohydrates isolated from *R. secalis*. Previously, Beltran had prepared ^{14}C-labeled carbohydrates from *R. secalis* by incubating the fungal mycelium with ^{14}C-glucose. With this radio-labeled mixture at hand, comparison of the synthetic glucoside would be straight-forward. To increase the proportion of radio-label in the compounds chromatographing in the region of the synthetic glucoside, the labeled carbohydrate mixture was submitted to preparative paper chromatography. Most of the glucose and slower moving compounds were removed, and the region of interest was concentrated. Figure 11 shows the radioactive profile of the concentrated mixture, with comparison to glucose and the synthetic (1-propanol-2-O) α-D-glucopyranoside, on paper chromatography in solvent

system (1-propanol-ammonium hydroxide, 66:33). It is obvious that a ^{14}C-containing molecule chromatographs very close to the synthetic glucoside, but is not identical. The radioactive component has a greater mobility in this solvent system than does the synthetic glucoside. In the solvent system (1-butanol-pyridine-water, 9:5:4), the separation is not as obvious, but the radioactive component does migrate a little faster than the synthetic glucoside.

Since radioactive material is found in the fungal extracts of *R. secalis* with chromatographic mobility nearly identical to the synthetic glucoside, and since ^{14}C-glucose was used as the labeled substrate, it seemed possible that the fungus produced a glucoside. However, with slightly different mobilities, it is necessary to conclude that the compounds are not identical. The isolated glucoside region possessed phytotoxic activity on barley leaves (personal communication with Pio Beltran).

The question that arises at this juncture is whether the linkage to 1,2-propanediol might actually be thru C-1 instead of C-2, as earlier postulated.[1] The only data, which could lead one to conclude the C-2 linkage, was comparison of the synthetic glucoside to the non radioactive carbohydrate mixture in solvent system (1-butanol-pyridine-water, 9:5:4). This procedure would certainly be misleading, since the solvent system does not separate the synthetic glucoside from the radioactive glucoside. Therefore, it was necessary to determine the exact linkage

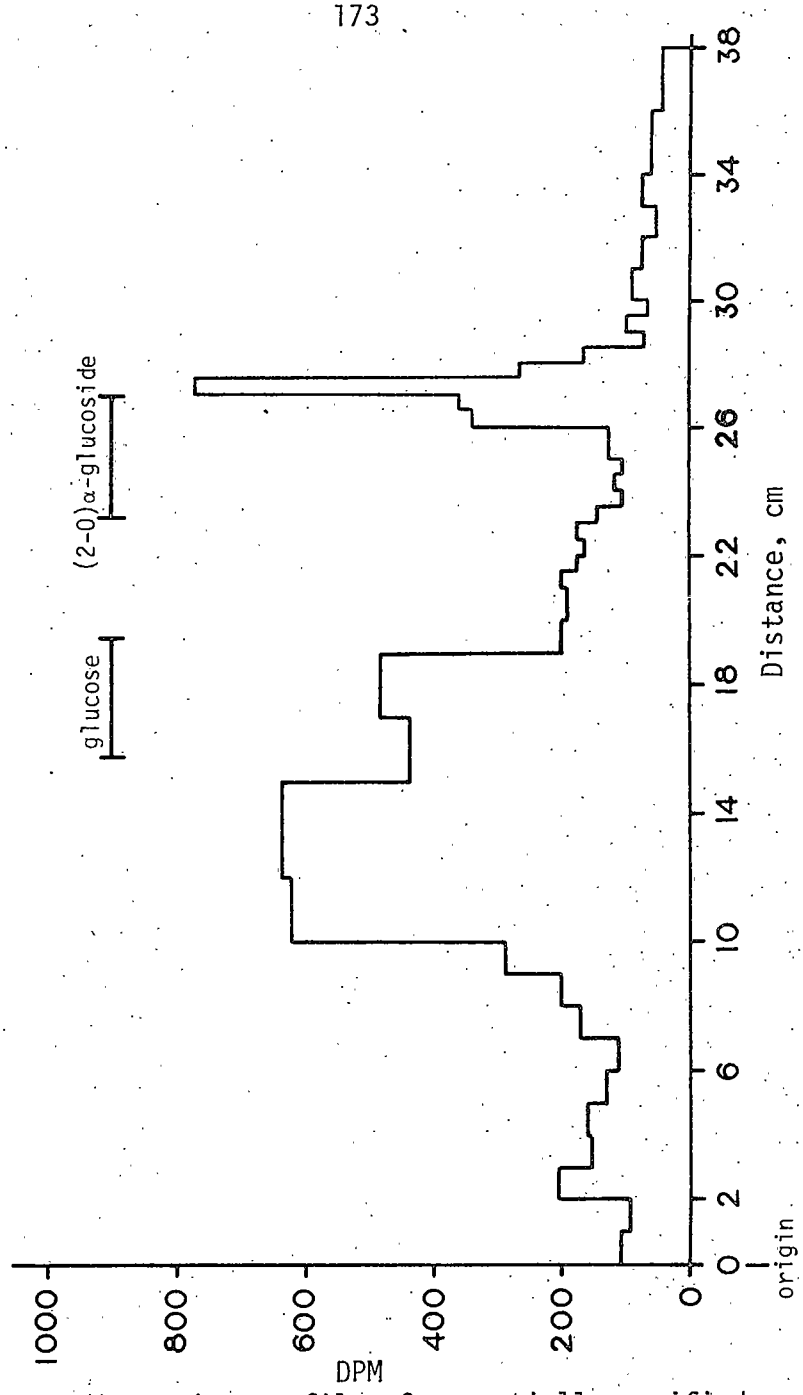
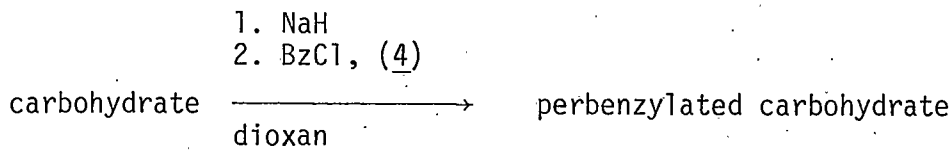


Figure 11. Radio-active profile of a partially purified region containing carbohydrates, on paper chromatography, with solvent system (1-propanol-ammonium hydroxide, 66:33).

to 1,2-propanediol. The proposed plan for determining the linkage involved preparing the benzyl derivative[7] of the toxin and then hydrolyzing the anomeric linkages (Figure 12),[10] producing benzyloxy propanol. The isomer obtained would be indicative of the original linkage. For this comparison, compound 2 and its isomer, 2-benzyloxy-1-propanol,(12), (Figure 13), would be required.



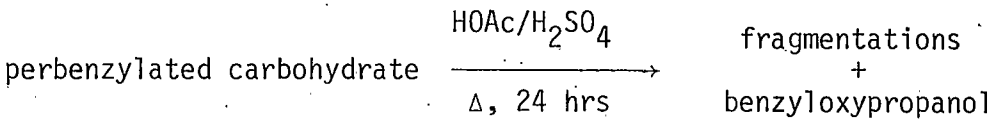
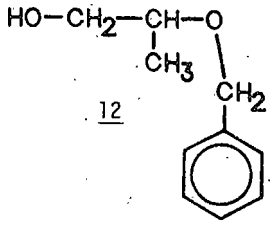


Figure 12. Reaction scheme for obtaining the benzylated aglycone.

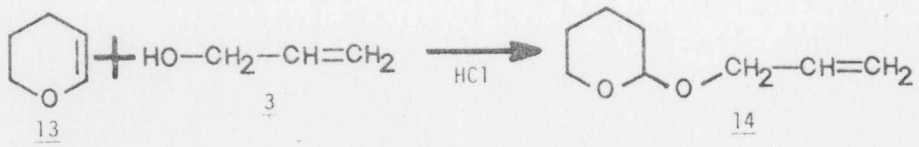


12

Figure 13. 2-benzyloxy-1-propanol.

A series of four reactions were required to prepare the other isomer, (12). Allyl alcohol, (3), was reacted with dihydropyran, (13), in the presence of a catalytic amount of HCl producing 2-(allyloxy) tetra-

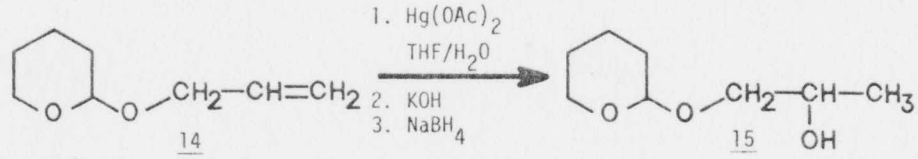
hydropyran, (14), as shown in Figure 14.[13]



13 3 14

Figure 14. Hydroxyl protection of allyl alcohol by dihydropyran.

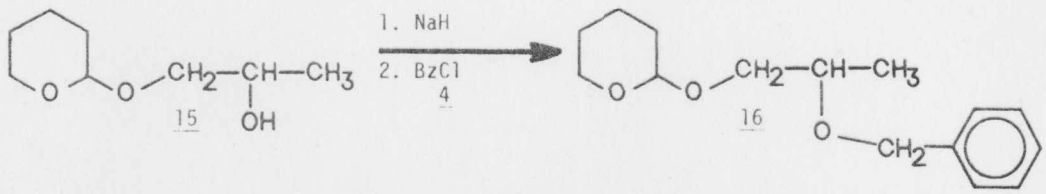
An oxymercuration reaction on 14,[8] produced 1-[(tetrahydropyran-2-yl) oxy]-2-propanol, (15), as shown in Figure 15.



14 15

Figure 15. Oxymercuration of 2-(allyloxy)tetrahydropyran.

With benzylation of 15,[7] the C-2 hydroxyl group was protected, resulting in the doubly-protected product, 1-[(tetrahydropyran-2-yl)oxy]-2-benzyloxypropane, (16), as delineated in Figure 16.



15 4 16

Figure 16. Benzylation, producing a double protected 1,2-propanediol.

The last step, was removal of the tetrahydropyranyl moiety from 16 by acid hydrolysis,[14] producing 2-benzyloxy-1-propanol, (12), the required isomer (Figure 17).

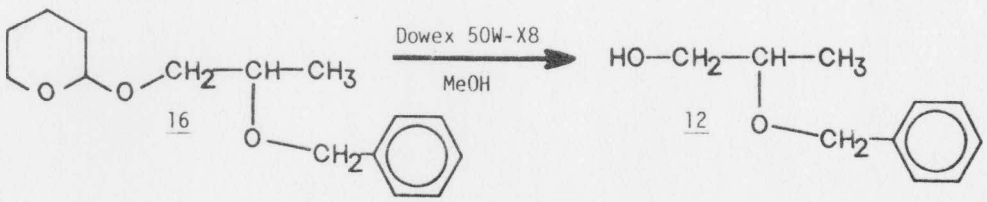
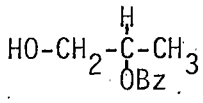


Figure 17. Removal of the tetrahydropyranyl moiety by acid hydrolysis.

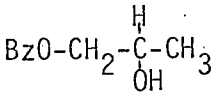
Mass spectra were obtained on the two isomers, (2) and (12), as listed in Table 2. This was done to confirm that 2 was a secondary alcohol, and that 12 was a primary alcohol.

By glc analysis, both isomers separated on a carbowax column as well as on a SE-30 column. The toxin, obtained from G. Strobel, after benzylation and hydrolysis, contained material that had a retention time on both the carbowax and SE-30 columns, equal to 2-benzyloxy-1-propanol. With this result, the linkage was determined to be thru C-1 of 1,2-propanediol. With the understanding about the nature of the carbohydrate attachment to 1,2-propanediol, it was now of interest to attempt synthesis of the α-cellobioside. Cellobiose was previously suggested as the carbohydrate moiety of the toxin.[1]

Table 2. Mass spectral data for the two isomers of benzyloxypropanol.



m/e		Intensity
166	Molecular ion	0.4
148		0.35
135		2.15
107		1.41
92		4.8
91	Base peak (tropylium ion)	28.8
65		2.6
39		1.2
31	Primary alcohol	1.4
28		1.8



m/e		Intensity
166	Molecular ion	0.4
107		0.7
92		2.1
91	Base peak (tropylium ion)	4.15
65		0.85
45	Secondary alcohol	2.55
28		1.95

The only known method for obtaining α-cellobiosides was by an anomerization reaction on the β-cellobioside by titanium tetrachloride,[4] as already mentioned. Since the yields can be very small and unpredictable, an alternate method was sought. The only reasonable approach that could be found required a reaction sequence that terminated in an attempted coupling of phenyl perbenzylated-1-thio-β-cellobioside with the alcohol, (12). This would be similar to the method reported by Ferrier et al.[15] for phenyl 2,3,4,6-tetra-O-benzyl-1-thio-β-D-glucopyranoside.

There were five intermediate steps to obtain the phenyl 1-thio-cellobioside required for coupling. Octa-acetyl-cellobiose, (18), was obtained by reaction of acetic anhydride and perchloric acid on cellobiose, (17), as shown in Figure 18.[16]

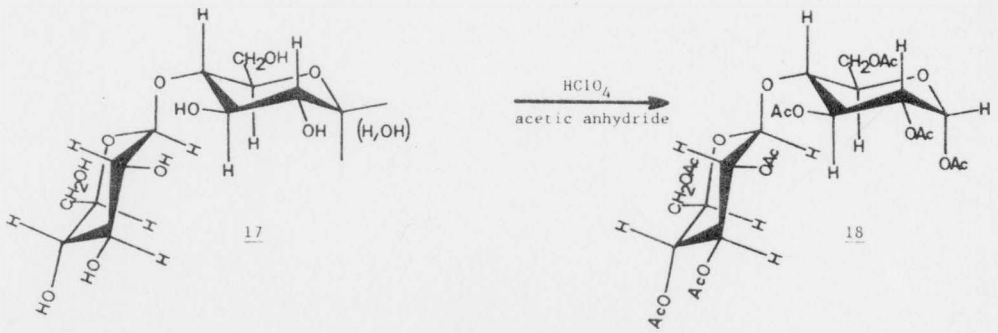


Figure 18. Acetylation of cellobiose.

The acetyl derivative, (18), was brominated by reaction with red-phosphorus, bromine, and water (Figure 19), producing 2,3,6,2',3',4',6'-hepta-

O-acetyl-cellobiosyl bromide, (19).[16] Phenyl 2,3,6,2',3',4',6'-hepta-O-acetyl-1-thio-β-cellobioside, (20), was obtained by reacting thiophenol with sodium hydride, and then displacing the bromide of 19, with the thiolate,[17] as seen in Figure 20. All acetyl protecting groups were removed from 20 by liquid ammonia in methanol,[17] producing phenyl 1-thio-β-cellobioside, (21), as depicted in Figure 21.

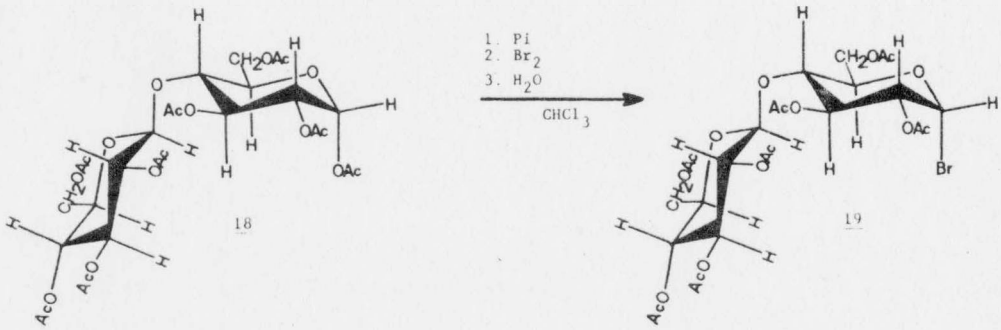


Figure 19. Bromination of octa-acetyl cellobiose.

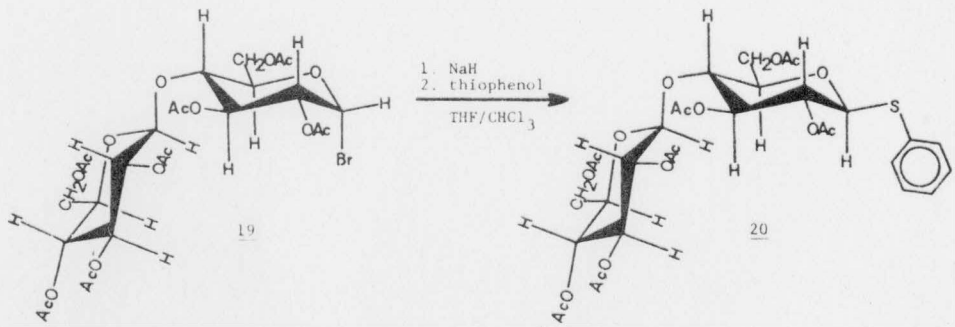


Figure 20. Bromide displacement by thiophenol.

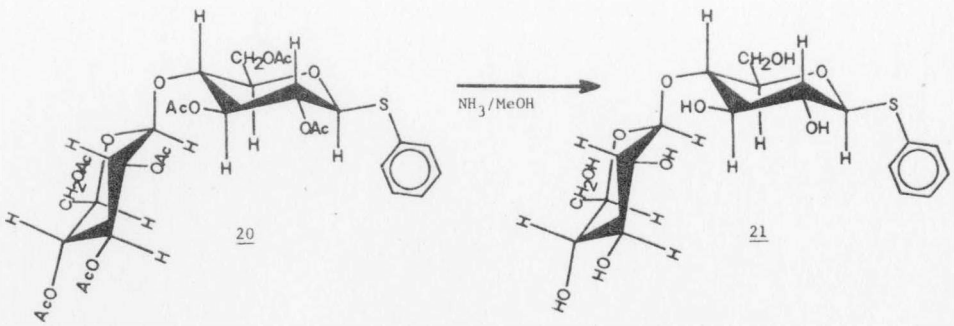


Figure 21. Deacetylation by ammonia in methanol.

Benzylation of 21 by addition of sodium hydride and benzyl chloride, (4), in dioxan, produced phenyl 2,3,6,2',3',4',6'-hepta-O-benzyl-1-thio-β-cellobioside, (22),[15] (Figure 22).

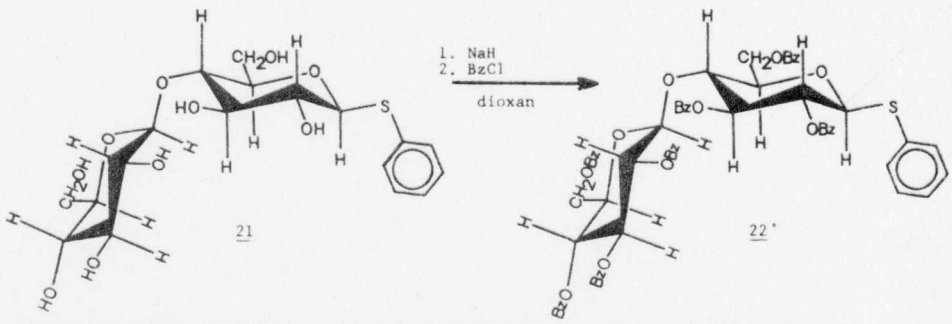


Figure 22. Benzylation of phenyl 1-thio-β-cellobioside.

Having the requisite derivative of cellobiose, the proposed structure of the toxin could be obtained by a two step reaction sequence.

The thio-acetal, (22), was converted to (2-benzyloxypropane-1-O) 2,3,6, 2',3',4',6'-hepta-O-benzyl-cellobioside, (23), by addition of 2-benzyl-oxy-1-propanol, (12), in the presence of mercuric sulfate (Figure 23).

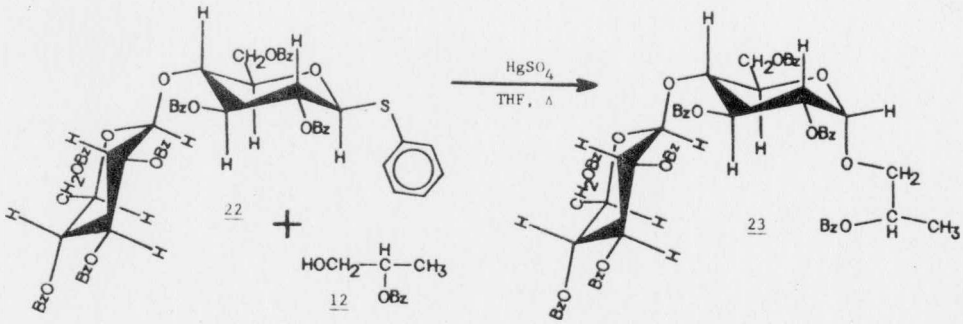


Figure 23. Mercuric sulfate facilitated coupling of 2-benzyloxy-1-propanol to phenyl 2,3,6,2',3',4',6'-hepta-O-benzyl-1-thio-β-cellobioside.

Mercuric sulfate has been shown to be the mercury (II) salt of choice for doing displacements on phenyl 2,3,4,6-tetra-O-benzyl-1-thio-β-D-glucopyranoside.[15] These workers did not use mercuric acetate; because, "the acetate appeared to give the glycosyl acetate."[15] Debenzylation of 23 was accomplished by a hydrogenolysis reaction using hydrogen and 10% Pd/C in ethanol,[12] producing (2-propanol-1-O) α-cellobio-

side, (24), as shown in Figure 24.

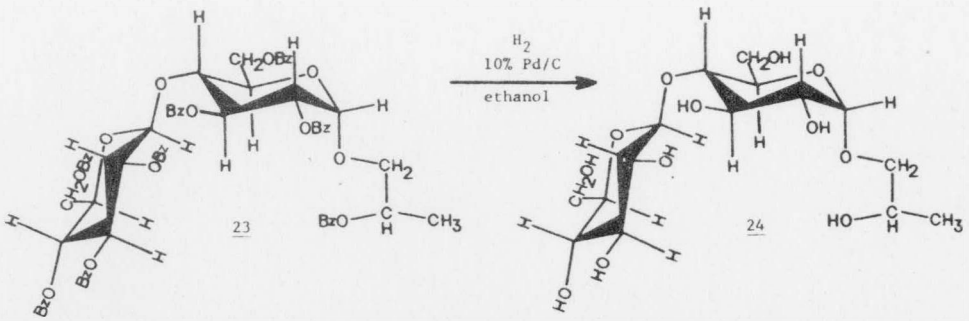


Figure 24. Hydrogenolysis of (2-benzyloxypropane-1-O) 2,3,6,2', 3',4',6'-hepta-O-benzyl-cellobioside.

It was of interest, for two reasons, to attempt coupling 1-benzyloxy-2-propanol, (2), with 22 using mercuric acetate. First of all, to obtain the other linkage with 1,2-propanediol, and secondly, to see if mercuric acetate would work with phenyl perbenzylated-1-thio-cellobioside. The reaction, as seen in Figure 25, did not couple the alcohol to give the desired product, (25), but did form the acetates, (26), in a quantitative yield. This corraborates for the cellobiose derivatives, the observation that was made with the glucose derivatives, that mercuric acetate appeared to give the glycosyl acetates.[15]

Reactions seem to proceed quite slowly with mercuric sulfate, possibly because it is not very soluble in the reaction media. It was thought that phenyl mercuric acetate might be a good reagent if it did

not cause formation of the acetate. This mercury compound should have good solubility in the solvents used during synthesis. A reaction was attempted utilizing 22 and 2 with phenyl mercuric acetate in THF. After refluxing the reaction mixture for two weeks, only a minute amount of product was observed.

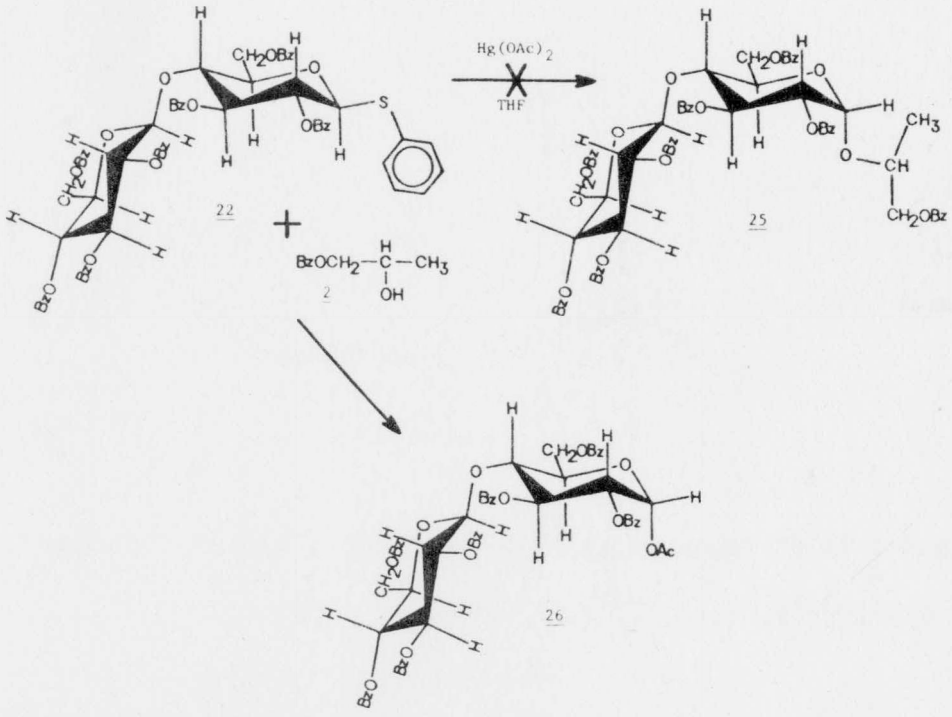


Figure 25. Mercuric acetate facilitated coupling of 1-benzyloxy-2-propanol to phenyl 2,3,6,2',3',4',6'-hepta-O-benzyl-1-thio-β-cellobioside. The acetate, (26), could be hydrogenated to give 1-O-acetyl cellobioside.

Since the reaction in Figure 25 did not produce the appropriate product, and phenyl mercuric acetate did not react, a third reaction was attempted using mercuric sulfate. A reaction of 2 with 22, facilitated by mercuric sulfate, produced (1-benzyloxypropane-2-O) 2,3,6,2',3',4',6'-hepta-O-benzyl-α-cellobioside, (25), as shown in Figure 26.

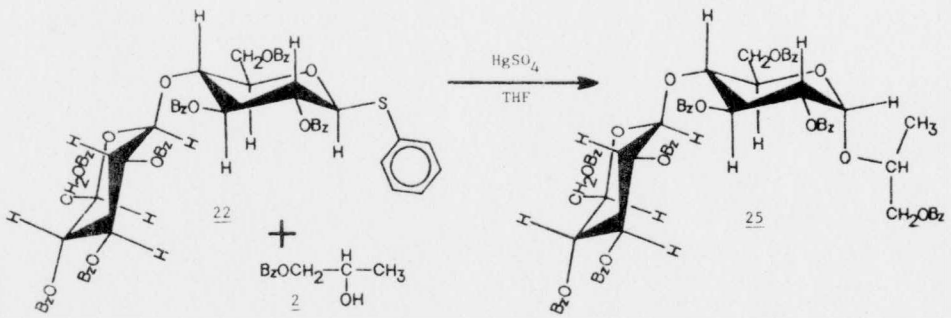


Figure 26. Mercuric sulfate facilitated coupling of 1-benzyloxy-2-propanol to phenyl 2,3,6,2',3',4',6'-hepta-O-benzyl-1-thio-β-cellobioside.

Debenzylation of 25 was accomplished by a hydrogenolysis reaction using hydrogen and 10% Pd/C in ethanol,[12] producing (1-propanol-2-O) α-cellobioside, (1), as shown in Figure 27.

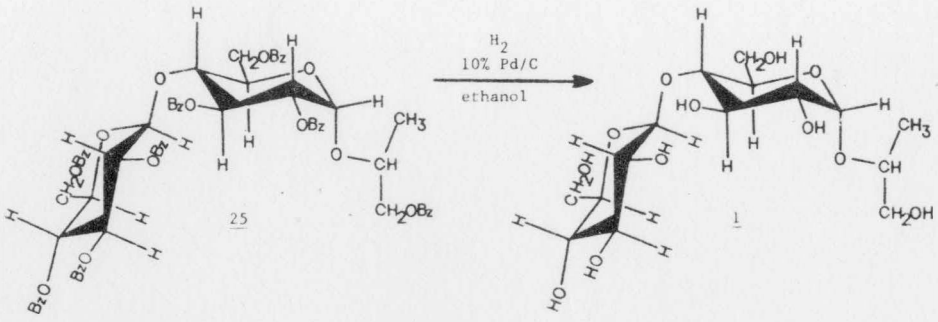


Figure 27. Hydrogenolysis of (1-benzyloxypropane-2-O) 2,3,6,2', 3',4',6'-hepta-O-benzyl-cellobioside.

Concentrated solutions of 1 and 24 were bioassayed on cuttings of barley (cultivar var. Hannchen C. I. 531) resulting in only slight phytotoxicity. This result was a striking setback. To be certain that the synthetic toxin migrated in chromatography systems similar to the native toxin, the silica-gel plate, represented in Figure 28, was run in solvent system, (ethyl acetate-water-methanol, 84:18:35). To our surprise, the R_F's of the (1-O), and (2-O) α-cellobiosides were experimentally equivalent to that of glucose (results were the same on paper chromatography). However, the toxin is known to migrate only a little faster than cellobiose, and not within the area of the glucose spot. With these observations one is naturally led to the conclusion that the toxin is not a cellobioside, as previously postulated.[2]

At this juncture it was of interest to see if the toxin correspon-

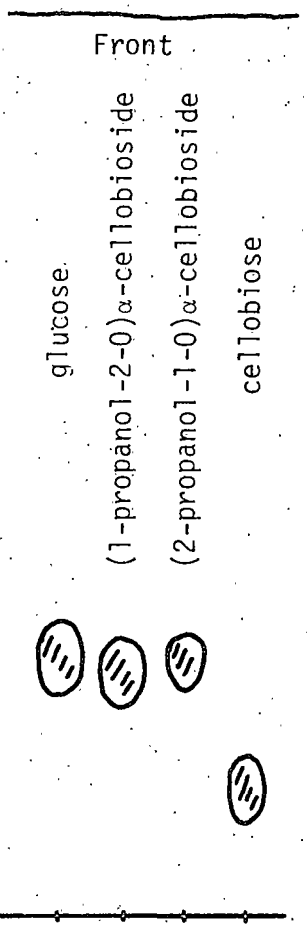


Figure 28. Thin-layer chromatography of the (1-0) and (2-0) α-cellobiosides of propanediol.

ded to a trisaccharide. The (2-propanol-1-0) α-cellobioside was submitted to mild acid hydrolysis, using 2.1N TFA at 80°C for 1.5 hrs. The resultant product mixture was comparatively chromatographed against an isolated toxin fraction (obtained from Strobel), as seen in Figure 29. The thin-layer plate shows that cellobiose was a probable major contam-

inate in the preparation, and only one other spot was observed. The other spot migrated a little further than cellobiose, but did not move into the glucose spot. This result confirmed the earlier idea that the cellobiosides can not be structurally identical to the toxin.

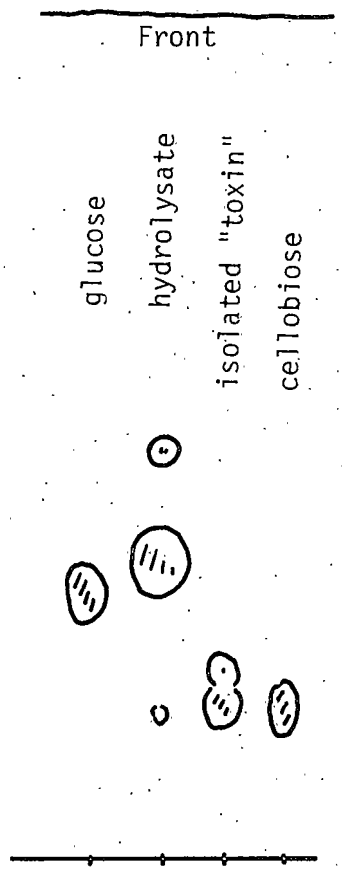


Figure 29. Thin-layer chromatography of the (2-propanol-1-0) α-cellobioside hydrolysate and natural toxin on silica-gel in solvent system (ethyl acetate-water-methanol, 84:18:35).

This chromatography was repeated three times and R_F values were obtained of the glucoside, cellobioside and toxin (Table 3). For graphical purposes, the number of residues assigned to each component were the following: glucoside = 1, cellobioside = 2, and toxin = 3 residues.

Table 3. R_F values of both (2-propanol-1-0) α-D-glucopyranoside and α-cellobioside, and the native toxin in the solvent system, (ethyl acetate-water-methanol, 84:18:35).

Number of Residues	R_F Values	$-\log(R_F/1-R_F)$
1	0.466	0.059
	0.466	0.059
	0.488	0.0209
2	0.328	0.312
	0.332	0.304
	0.332	0.304
3	0.209	0.578
	0.205	0.588
	0.202	0.597

Least squares treatment of these data gave a straight line (Figure 30), having a correlation coefficient of 0.998. This graphical treatment,[18] with its linear result is indicative of a series of oligosaccharides, α-glucoside, α-cellobioside and α-cellotrioside.

For a comparison analysis of the graphical method, with a different set of oligomers, Bio-gel P-2 isolated oligomers of a cellulose

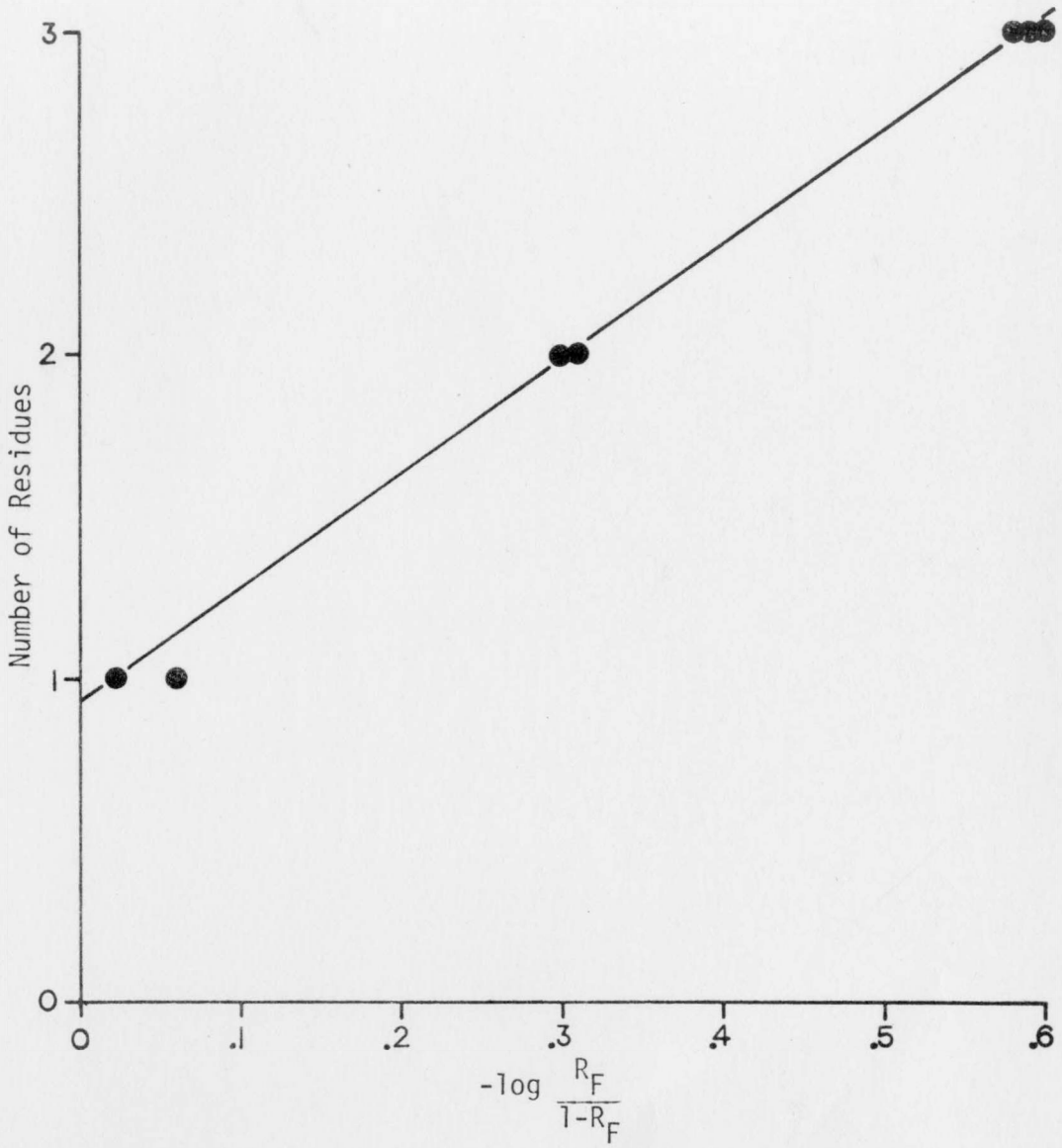


Figure 30. Plot of subunits vs. $-\log(R_F/1-R_F)$ for (2-propanol-1-O) α-glucoside, α-cellobioside, and the natural toxin.

hydrolysate were obtained. These oligomers were chromatographed on thin-layer plates in the solvent system, (ethyl acetate-water-methanol, 84:18:35), and their R_F's with data reduction are found in Appendix A. The least squares treatment of the data gave a straight line (Figure 31), with a correlation coefficient of 0.9988. Therefore, the interpretation of this type of plot is consistent for comparing the number of residues in oligomeric species.

With these empirical results, suggesting that the toxin may be a trisaccharide, other data were required. A highly purified (by paper chromatography-Strobel) toxin preparation was sent for methylation analysis,[19] and the results are presented in Table 4. Upon analysis of the data, cellotriose could very possibly be in the mixture, but would only be 15% of the total, with at least five other carbohydrates being present. For cellotriose to be present with these other carbohydrates, and at this R_F (on paper or thin-layer chromatography), it must have an aglycone. To be consistent with the residue data in Figure 30, and the glc data (showing the presence of (1-O) linked 1,2-propanediol), its possible that the trisaccharide might contain the (1-O) linked 1,2-propanediol.

The (2-propanol-1-O) α-cellobioside hydrolysate was also comparatively chromatographed on thin-layer with synthetic (1-propanol-2-O) α-D-glucopyranoside (Figure 32). The (1-O) α-D-glucopyranoside in this and other chromatograms travels a little faster than (2-O) α-D-glucopy-

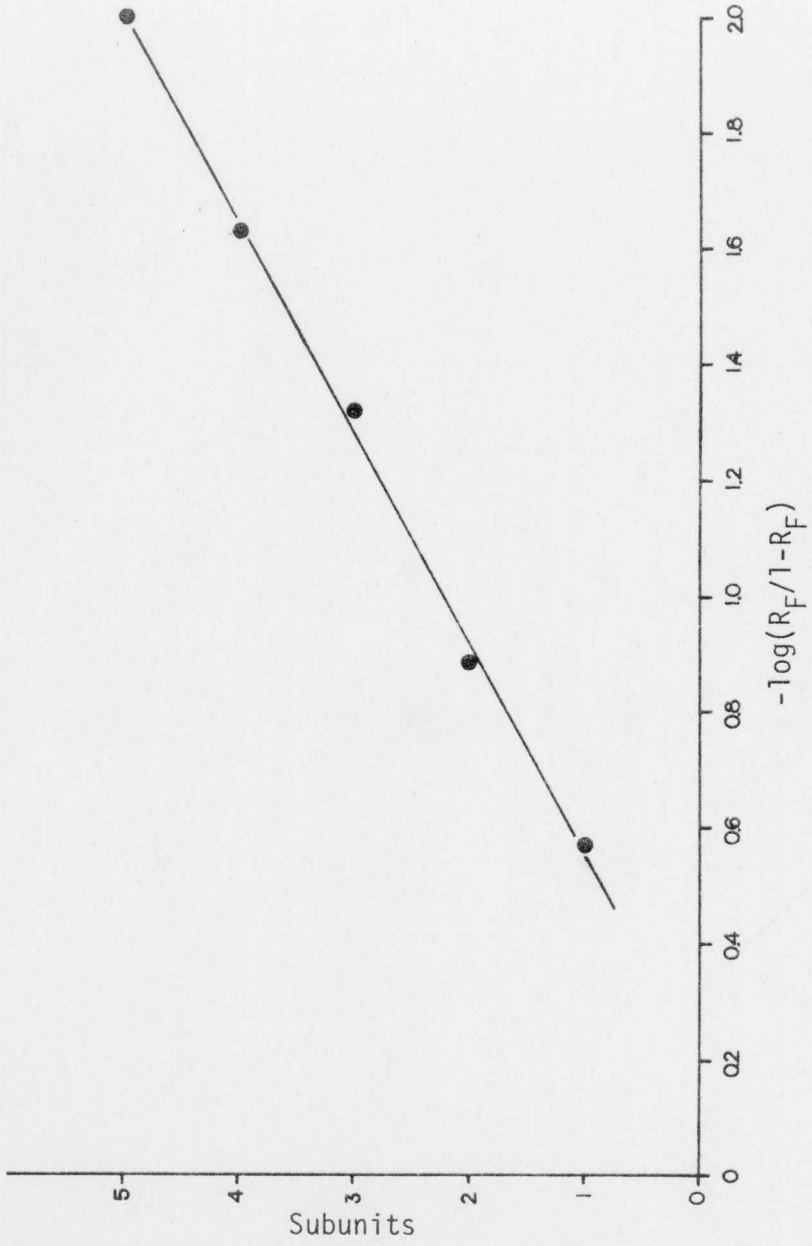


Figure 31. Plot of subunits vs. $-\log(R_F/1-R_F)$ for hydrolysis products of cellulose.

Table 4. Methylation analysis of the toxin fraction.

95% of total carbohydrate was glucose

5% was Xylose (4-linked)

41% of total glucose was terminal

42% { 10% 2 or 3 linked, mostly 3-linked
27% 4-linked
5% 6-linked

Analysis of the above data			Mole % of total
5%	5%		
gluc (1→4)	Xylose	5% terminal 5% Xylose	12.2
10%	10%		
gluc (1→2) (1→3)	gluc	10% terminal 10% (1→2), (1→3)	24.4
5%	5%		
gluc (1→6)	gluc	5% terminal 5% (1→6)	12.2
15%	15%		
gluc (1→4)	gluc	15% terminal 15% (1→4)	36.6
(known cellobiose contamination)			
6%	6%	6%	
gluc (1→4)	gluc (1→4)	gluc 6% terminal 12% (1→4)	14.6

ranoside. These chromatograms looked very similar to the ones obtained by comparison of the (2-0) α-D-glucopyranoside and the radio-labeled fungal carbohydrates (Figure 11).

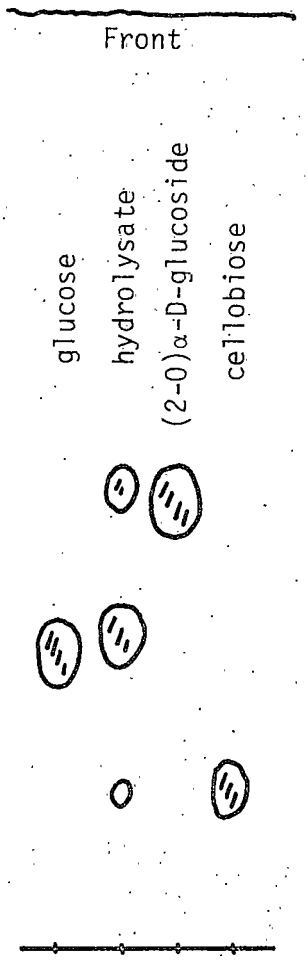
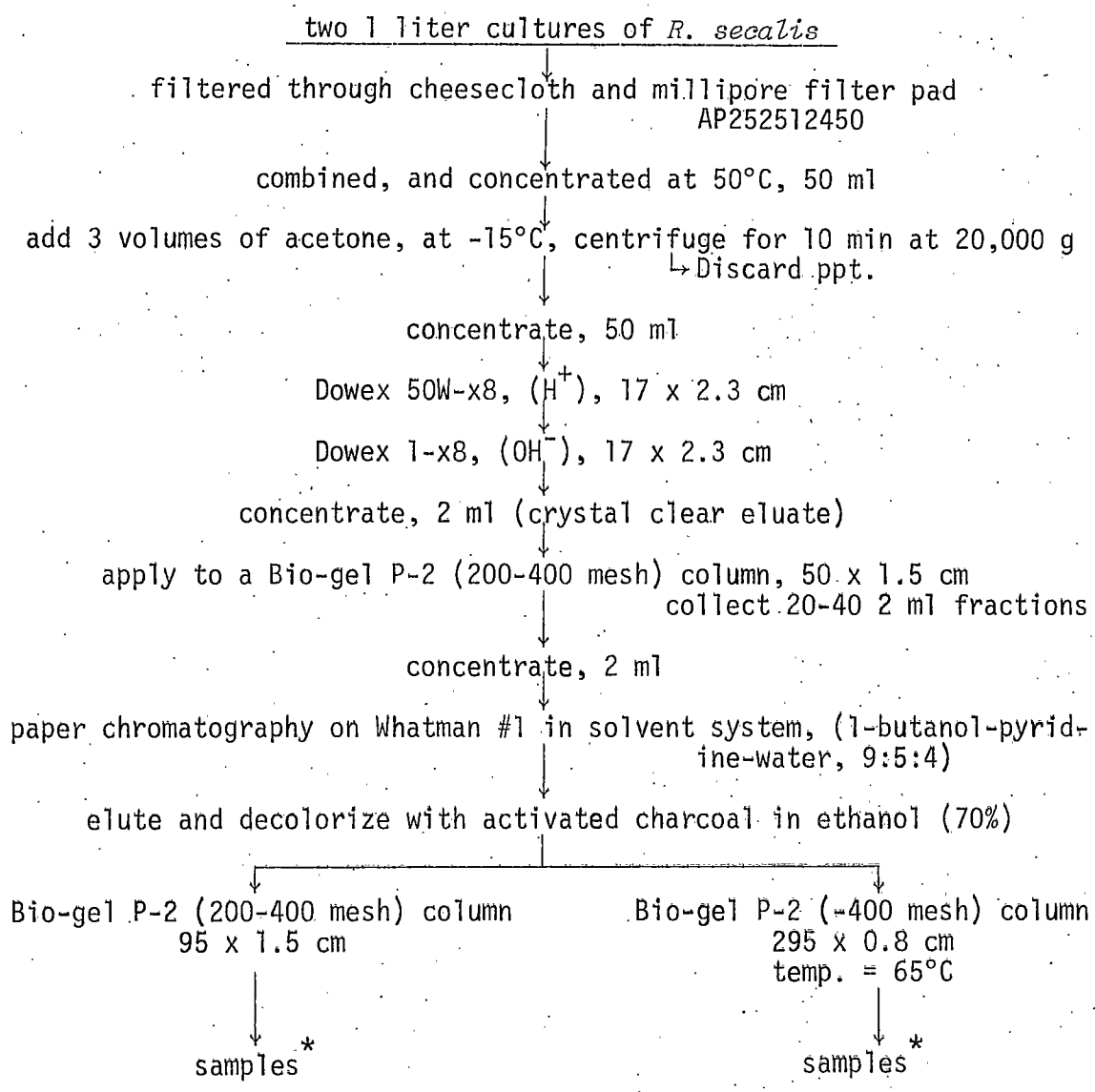


Figure 32. Comparitively chromatographed (1-propanol-2-0) α-D-glucopyranoside and the (2-propanol-1-0) α-cellobioside hydrolysate on silica-gel, in solvent system (ethyl acetate-water-methanol, 84:18:35).

It now looks very probable that a series of carbohydrates (toxins) are present instead of a single toxic component. The (2-propanol-1-0) α-D-glucopyranoside appears to be present, the isolated fraction containing it, is phytotoxic to barely leaves (var. Hannchen C. I. 531). The synthetic (1-propanol-2-0) α-D-glucopyranoside is also toxic. The synthetic α-cellobiosides are toxic; and the mixture which appears to contain the trisaccharide, is phytotoxic. In order to firm up the above data, the (1-0) linked aglycone should be present in all fractions corresponding to the compounds in question. Also, the presence of the cellobioside in *R. secalis* extracts must be substantiated. The question also arises, are there disaccharides having linkages other than (1→4) present in the extracts, that contain this aglycone? To obtain these kinds of data required us to test the hypothesis of a series of toxins, and to answer the question concerning the existance of other carbohydrates within the series, isolation procedures must be pursued.

Before pursuing the isolation of carbohydrates from *R. secalis* extracts, changes were made in the isolation procedure (presented in Figure 1). The procedure used is summarized in the flow sheet presented in Figure 33. The procedure used for the bioassay, was the same as that used by Auriol et al.[1] To facilitate bioassaying samples on a smaller scale, miniature conical tubes were used, enabling volumes as small as 0.2 ml to be assayed.

In an attempt to evaluate the possiblity of having a number of



*Note: The methods included here do not give complete separation of all carbohydrates.

Figure 33. Flow sheet summarizing the isolation procedures utilized for obtaining carbohydrates from *R. secalis* extract

toxins present in the extracts, isolation was pursued. Throughout the isolation, an account was made of the minimum amount of carbohydrate required to cause symptoms of barely scald (Table 5). For quantitation of carbohydrate material, the phenol-sulfuric acid analysis was used.[20] It is interesting that all steps thru the Bio-gel P-2 column do not separate the various carbohydrates earlier described as possible toxins. However, paper chromatography will separate carbohydrates. Figure 34 indicates the sections, on paper chromatography, that will be referred to in the following discussion. If the so called toxin, "rhynchosporoside," was the only toxin in the fungal extracts, after the paper chromatography step, it would be expected to have activity at a carbohydrate concentration of at least 6 mg/ml or less. Obviously this is not the case.

The other sections of the paper chromatograms were then checked for toxicity (Table 6). The "toxin" section had activity at a higher concentration, 40.5 mg/ml. Activity was also observed in the origin and cellobioside sections of the chromatograms. Previously, the glucoside section was observed to have toxicity.

It was next attempted to see whether some active components could be isolated by placing the toxin and origin sections over a Bio-gel P-2 column. A Bio-gel P-2 (200-400 mesh) 95 x 1.5 cm column (refered to as Bio-gel P-2 #1), was used, and a series of standards were placed on the column.

Table 5. Isolation steps including weights and amounts required for toxicity.

isolation step	total weight (mg)	carbohydrate (mg)	ratio: carbohydrate / total weight	minimum amount of carbohydrate needed for toxicity. (mg/ml)
Acetone precipitation	3800	1918.4	0.505	113.2
Dowex 1-x8	1370	1026.4	0.75	66.2
Dowex 50-x8	872	675.5	0.775	61.4
Bio-gel P-2	612.6	556.2	0.908	58
Paper chromatography				
1-butanol-pyridine-water,9:5:4 "toxin section"	——	(78.9)* 82.8	——	no activity at 20.2 activity at 40.5

* This smaller value was observed, due to a loss by cutting the paper and visualizing the bands of materials.

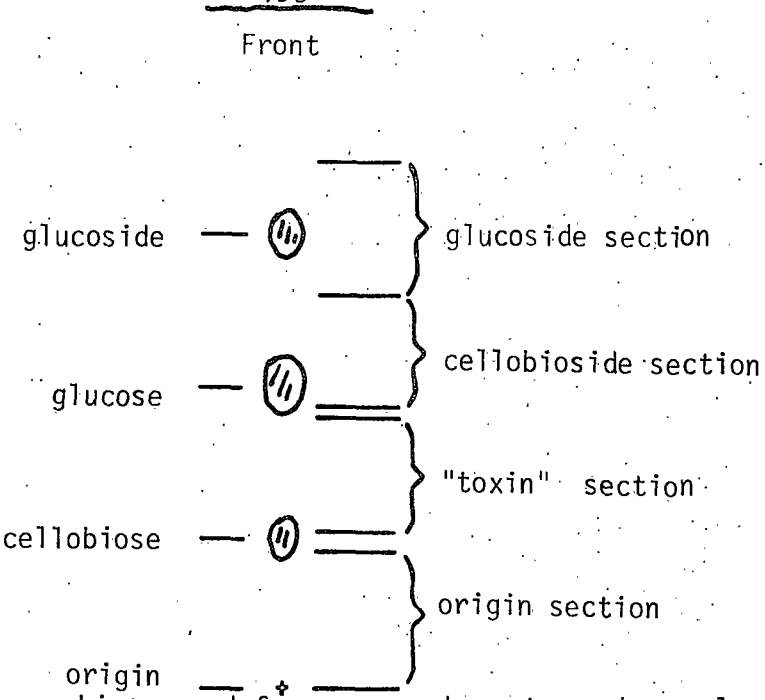


Figure 34. Areas bioassayed from paper chromatography, solvent system (1-butanol-pyridine-water, 9:5:4).

Table 6. Symptoms observed after 3.5 days on barley cultivar var. Hannchen C.I. 531, from areas on paper chromatography.

Fraction	Symptoms	Carbohydrate (mg/ml)
"Toxin" section	tip necrosis, yellowing of leaf margin	40.5
Origin section	slight tip necrosis, slight yellowing of leaf margin	-
Cellobioside section	yellowing of entire leaf	-
Water	none, after 6 days	-

Cello-oligosaccharides [β(1→4)] were prepared by hydrolyzing cellulose with 3N TFA, for 6 hrs at 100°C. The mixture, after paper chromatography, was placed on the Bio-gel P-2 #1 column, and the profile is seen in Figure 35. The data reduction from this elution curve is found in Appendix B.

Figure 36 shows the plot of elution volume/void volume (Ve/Vo) vs. log(molecular weight), to be a straight line having a correlation coefficient of 0.9994. Note: glucose was not included in the plot, since it is known to always fall off the line produced by the oligomers.

A corn syrup hydrolysate was obtained,[21] and its oligomers, [α(1→4)], were passed over the Bio-gel P-2 #1 column, and the profile is presented in Figure 37. The data reduction for this elution curve is found in Appendix C. Figure 38 shows the plot of (Ve/Vo) vs. log(molecular weight), to be a straight line having a correlation coefficient of 0.9893.

The synthetic (2-propanol-1-O) α-cellobioside, (24), was placed on the Bio-gel P-2 #1 column, and the resulting elution profile is presented in Figure 39. The β-anomer of 24 elutes at a slightly greater elution volume, 89 ml, while glucose elutes at the same volume as the previous run, 111.4 ml.

The "toxin" and origin sections from paper chromatography (Figure 34), were combined and placed over the Bio-gel P-2 #1 column. The collected fractions were pooled in groups of ten and bioassayed.

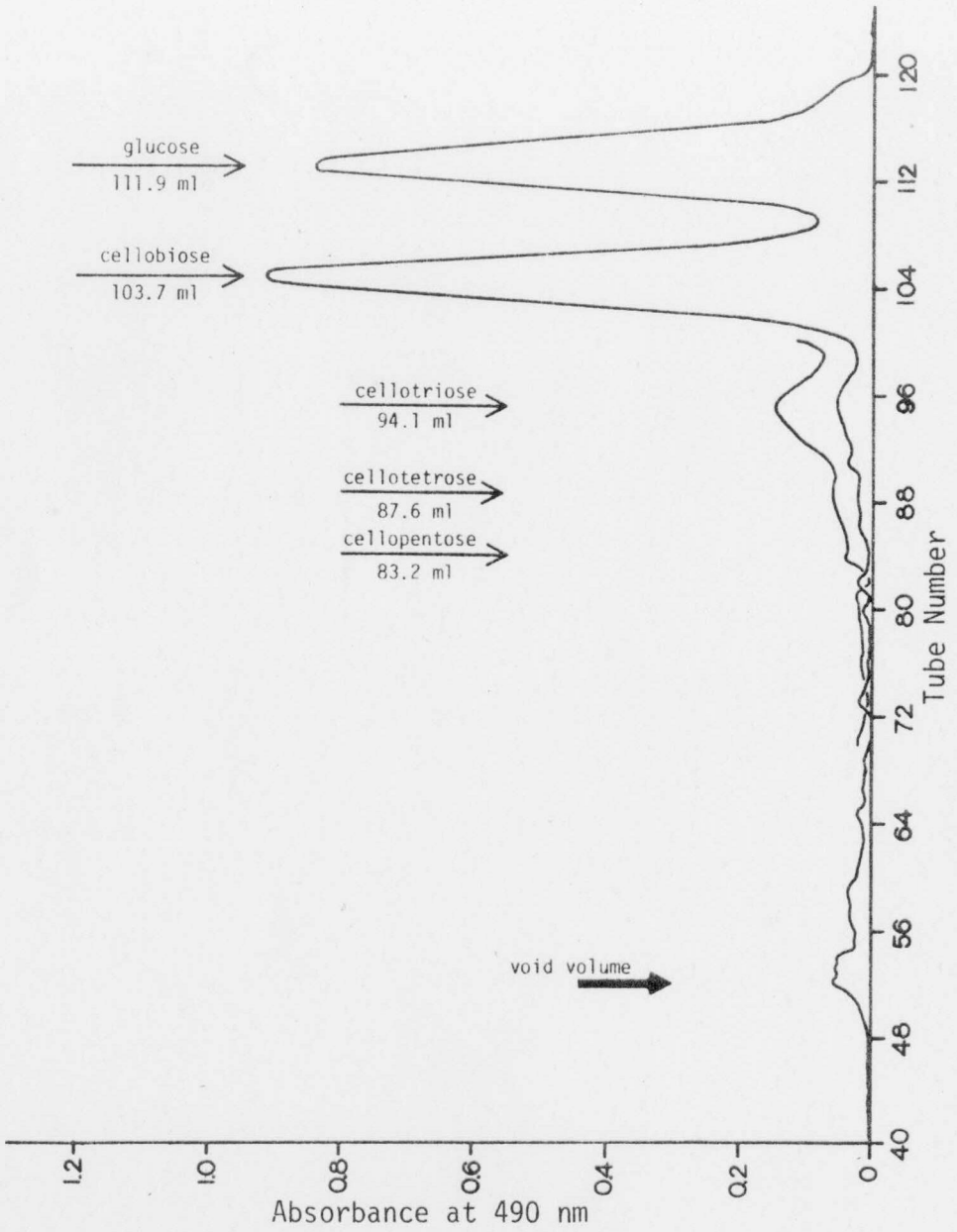


Figure 35. Cello-oligomers on a 95 x 1.5 cm Bio-gel P-2 (200-400 mesh) column.

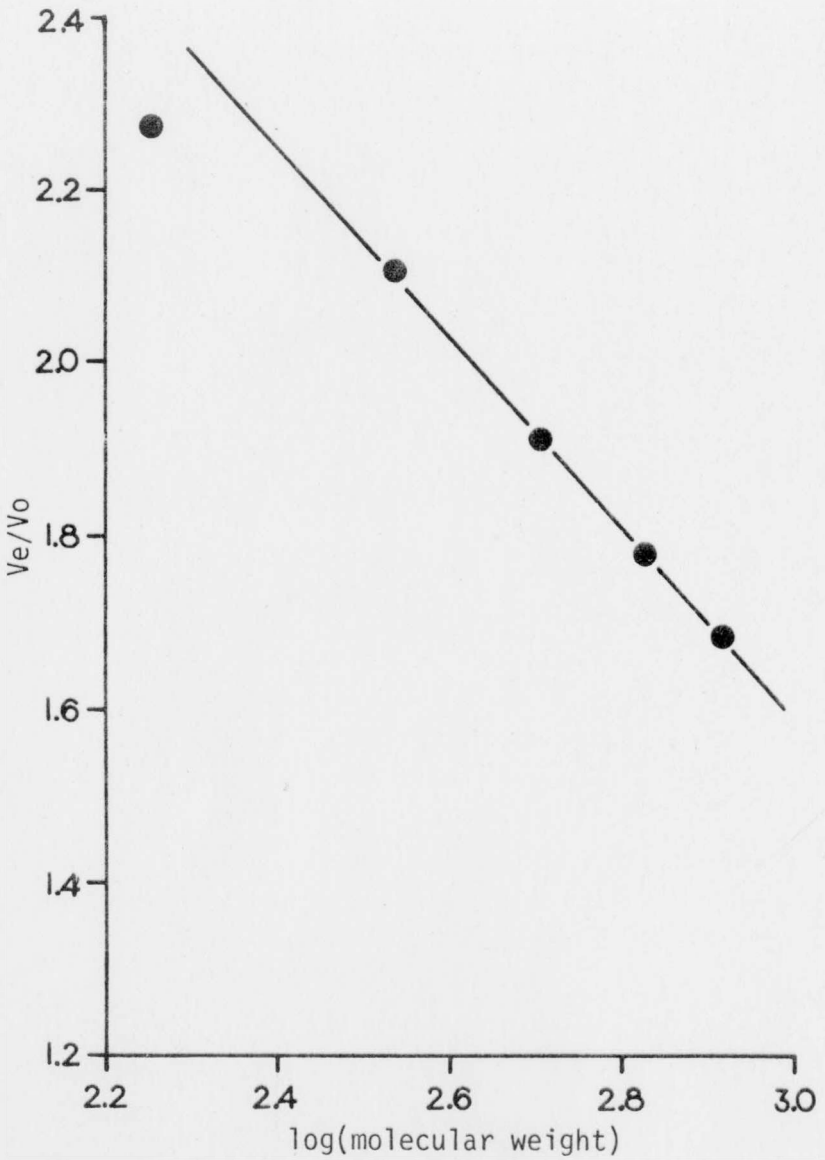


Figure 36. Plot of (Ve/Vo) vs. log(molecular weight) for cello-oligosaccharides.

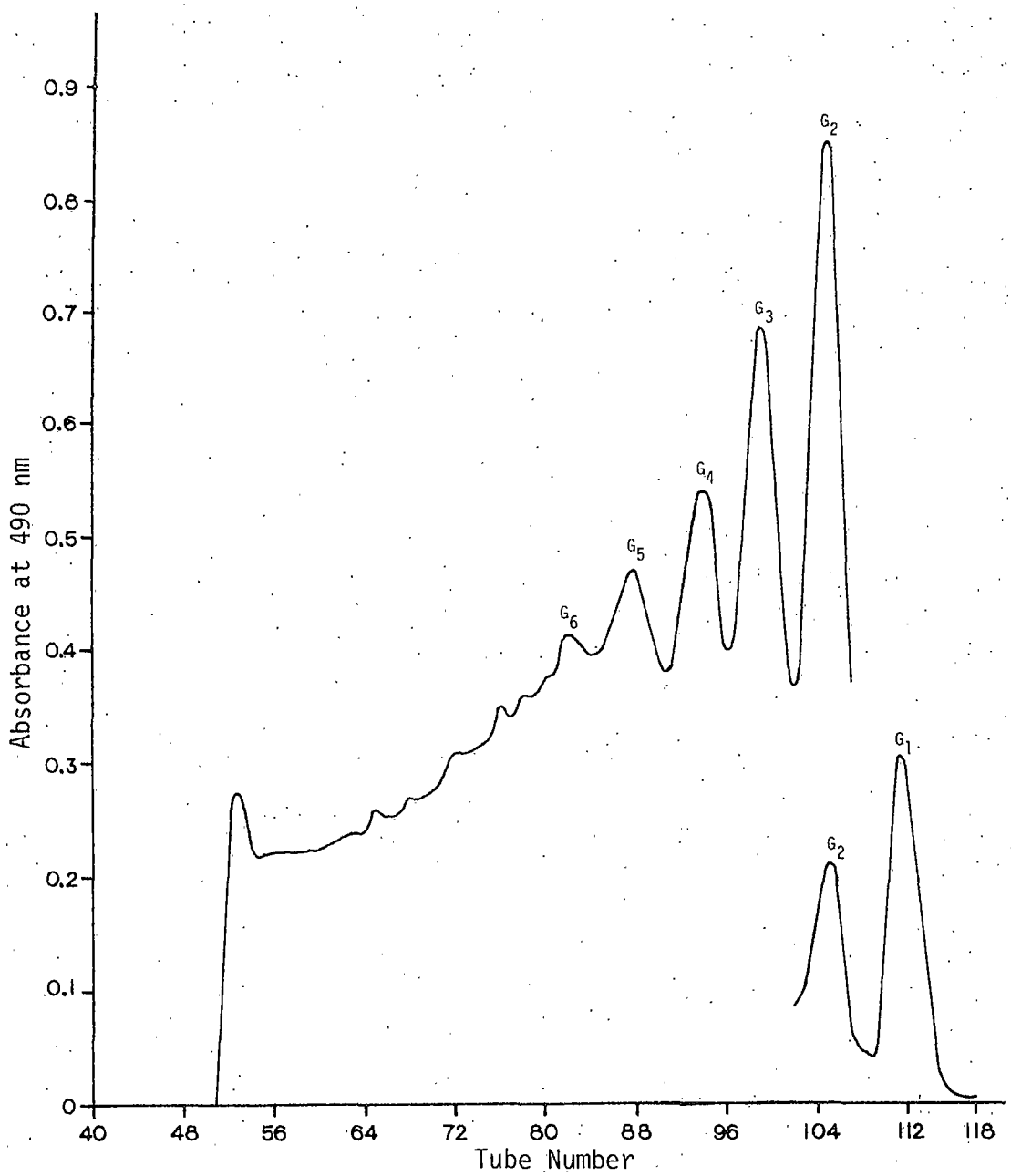


Figure 37. Malto-oligomers on a 95 x 1.5 cm Bio-gel P-2 (200-400 mesh) column.

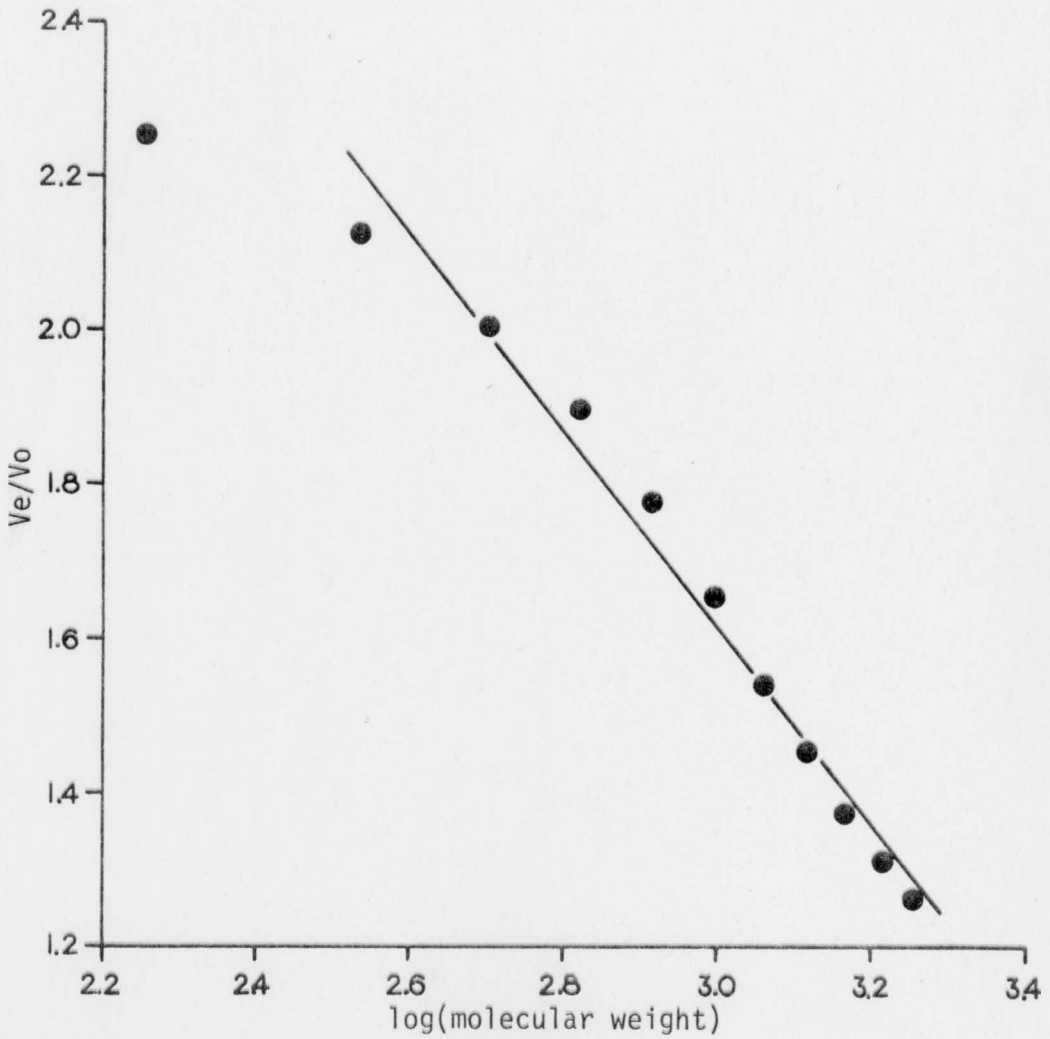


Figure 38. Plot of (Ve/Vo) vs. log(molecular weight) for malto-oligosaccharides.

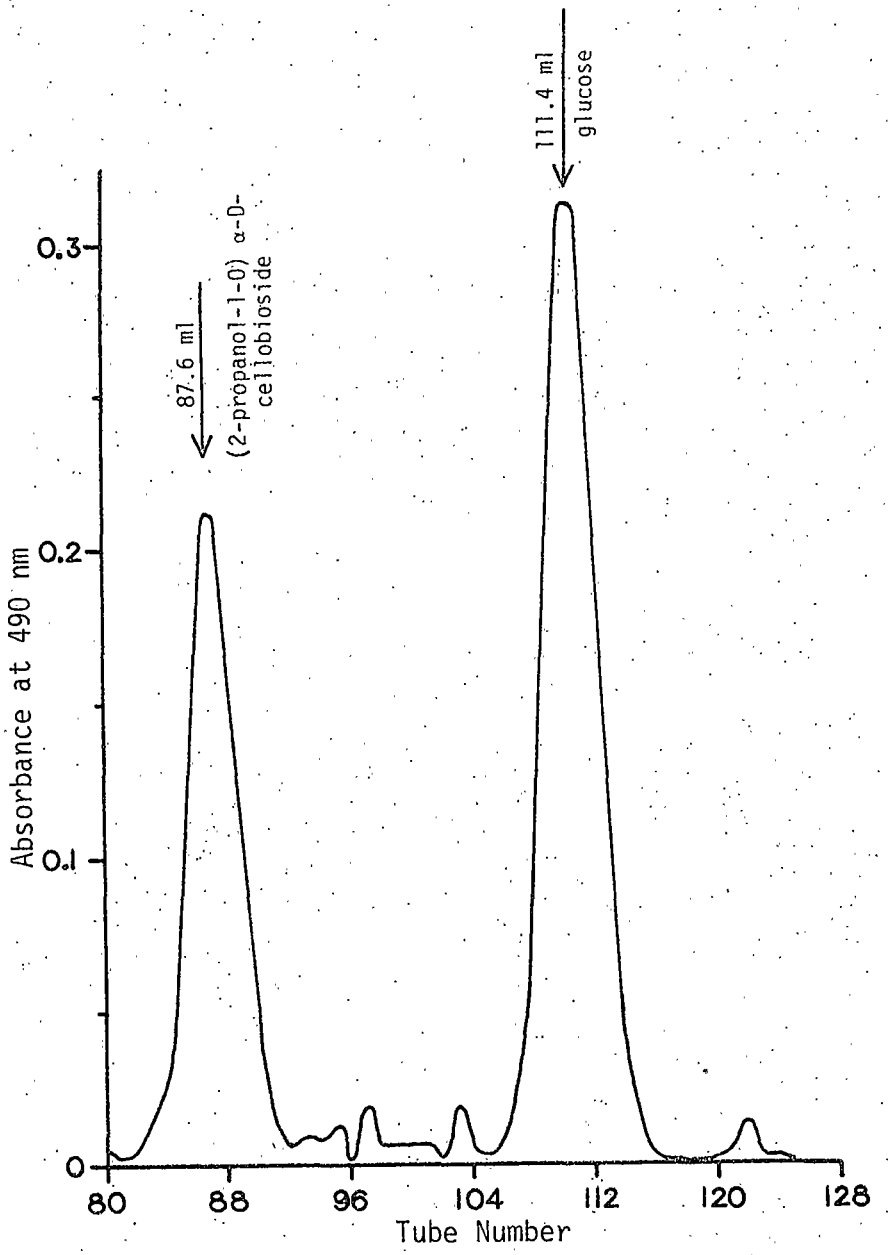


Figure 39. Synthetic (2-propanol-1-0) α-cellobioside on a 95 x 1.5 cm Bio-gel P-2 (200-400 mesh) column.

Results of the bioassay are presented (Figure 40) in a way that indicates which pooled fractions contained activity. There are a number of samples (pooled fractions) that contain activity, i.e., E-H. This distribution might be representative of a large number of different toxic compounds. Upon comparison of the elution diagram of the cello-oligosaccharides (Figure 35) to that of (2-propanol-1-O) α-cellobioside (Figure 39), the trisaccharide would be expected to elute in the region of fractions 82-88. This region does have toxic activity, correlating with earlier data that suggests a trisaccharide unit in the "toxin".

The cellobioside section (region around glucose on paper chromatography) was now investigated on the Bio-gel P-2 #1 column. Figure 41 shows the observed elution profile of the carbohydrates in the cellobioside section. The compound of interest was the cellobioside, (24), known to elute at 87.6 ml. There was no peak observed in Figure 41 where the cellobioside was expected to be found. However, if it was present in small quantities, it would be expected to elute with the material labeled, cell. #1.

The fractions within the peak area of cell. #1 were combined and replaced on the column, whose elution profile is shown in Figure 42. Interestingly, the material near 86 ml did not comprise a single symmetrical peak. A break-point was located at 87.8 ml, which is experimentally identical with the elution volume of the synthetic cellobioside. With this observation, one may suggest that the cellobioside

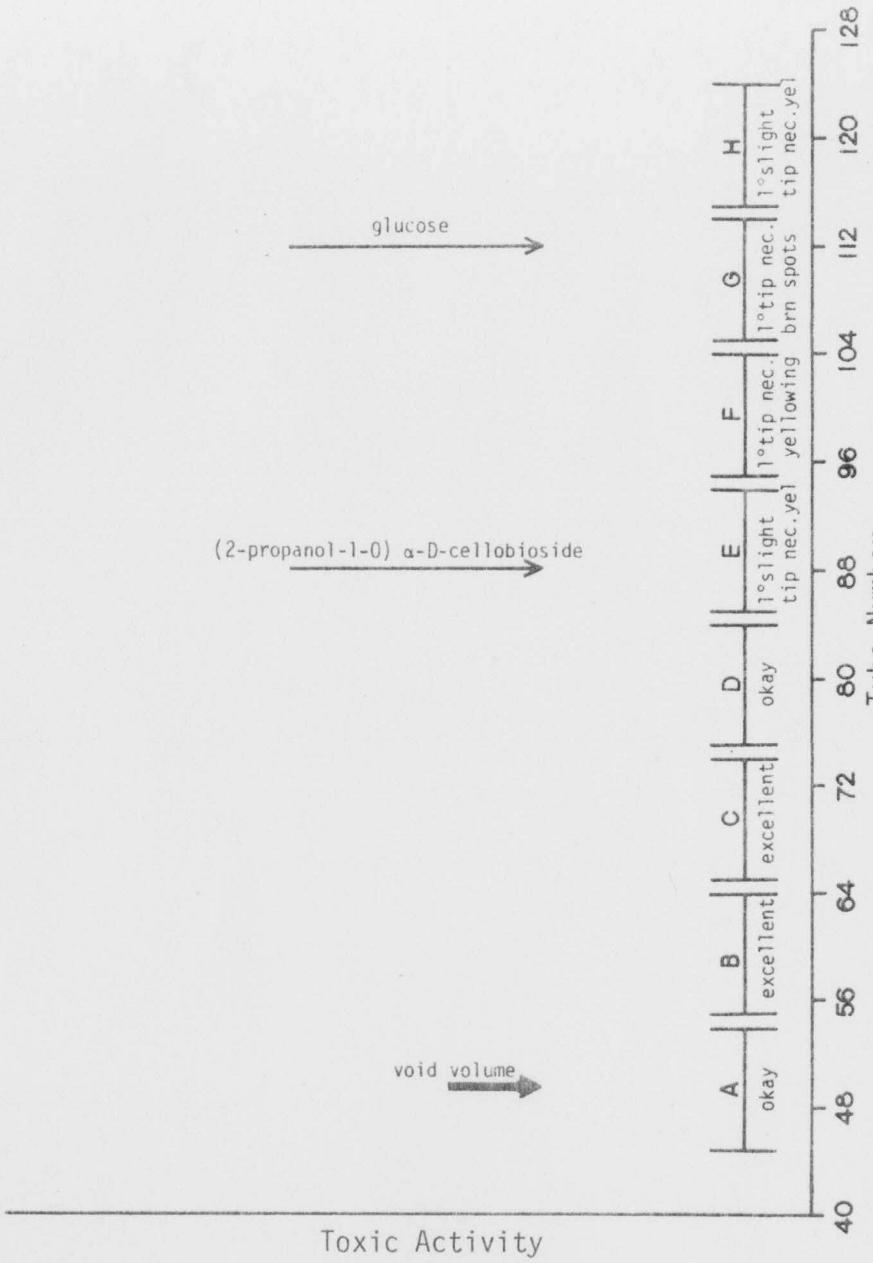


Figure 40. Observed toxicity for the Bio-gel P-2 (200-400 mesh) column separation of the toxin + origin sections.

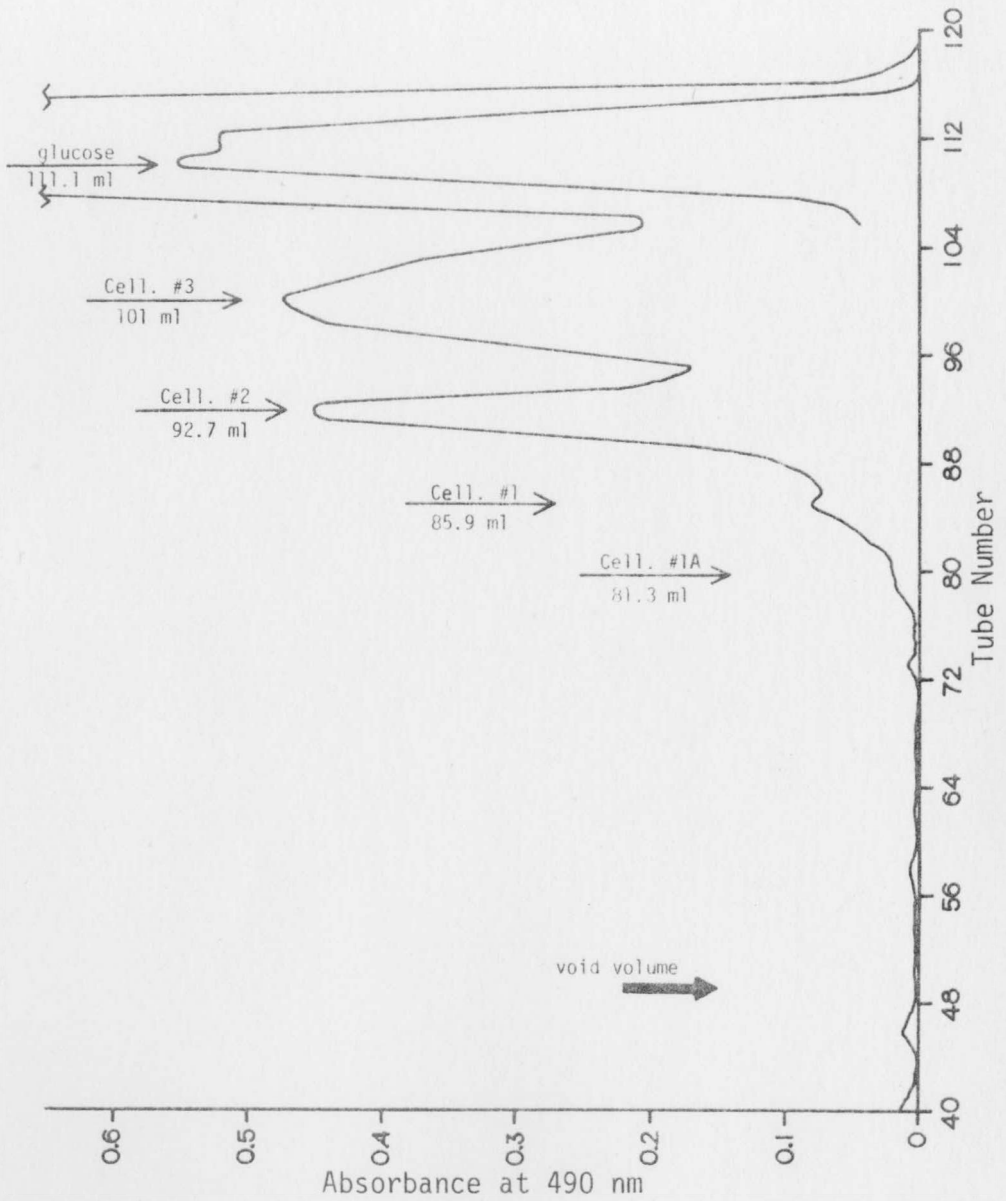


Figure 41. Elution profile of carbohydrates from the cellobioside section.

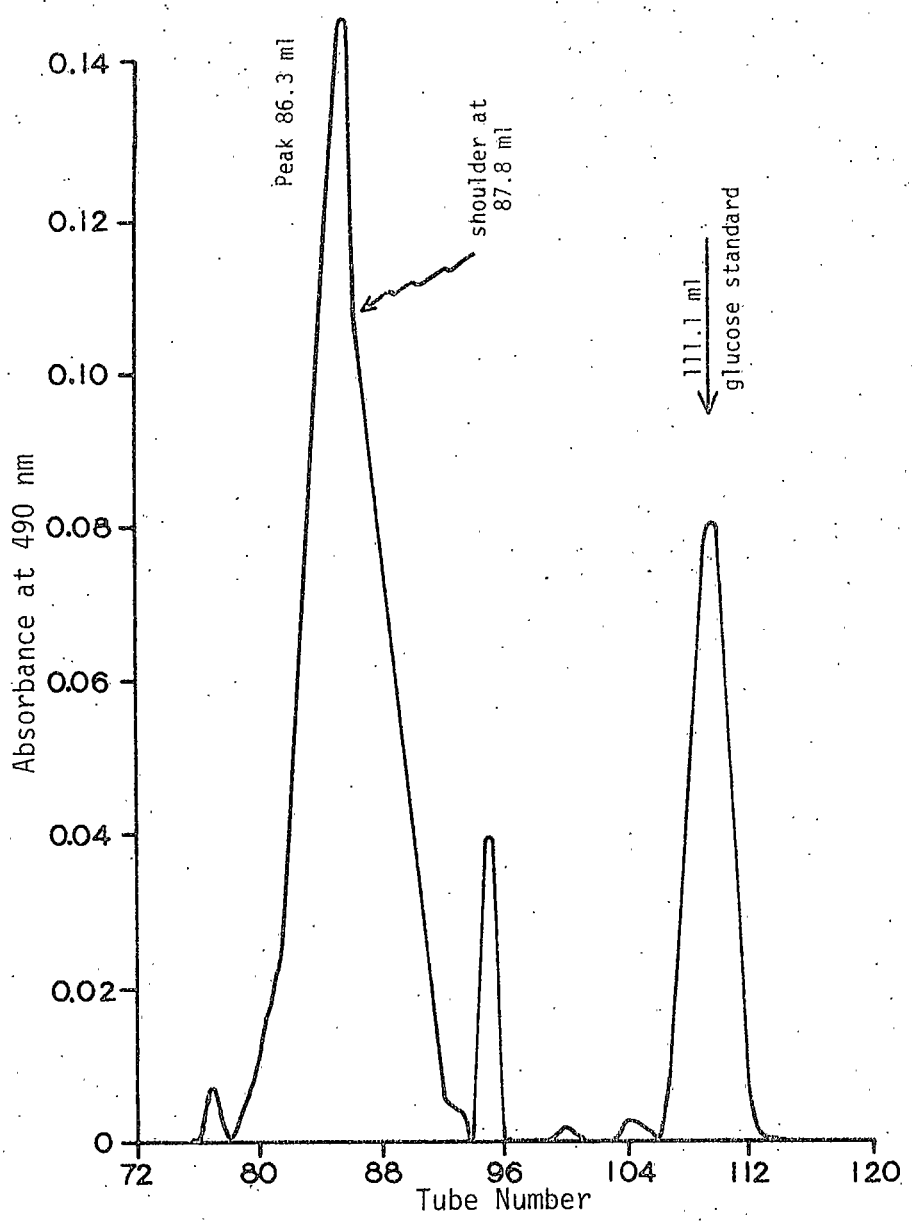


Figure 42. Elution profile of sample, cell. #1.

could be in this mixture.

The material within the peak area of cell. #2 was placed back over the column, and its elution profile is presented in Figure 43. The material in this peak eluting as one symmetrical peak, is indicative of a single component.

The material in sample, cell. #3, was seen to consist of at least three components, after separation on the Bio-gel P-2 (200-400 mesh) column. To obtain samples from each peak, cell. #3 was passed over a 295.5 x 0.8 cm, Bio-gel P-2 (-400 mesh) column {referred to as Bio-gel P-2 #2 column} (Figure 44). From a series of column runs, fractions were pooled producing three samples, cell. #3A, cell. #3B, and cell.#3C.

The glucoside section (from paper chromatography) gave the elution profile in Figure 45 after being subjected to the Bio-gel P-2 #2 column. There were four samples isolated from this column, Gluc. #2, #3, #4,and #5. Radioactive glucose was used as a marker during the column run. For comparison purposed, allyl α-D-galactopyranoside was also examined on the Bio-gel P-2 #2 column, and it eluted at 103 ml. Allyl α-D-galactopyranoside would be expected to elute in the same region as (2-propanol-1-0) α-D-glucopyranoside. There was a large peak at 104 ml, which contained at least two compounds. It is possible that one of these components may be the glucoside.

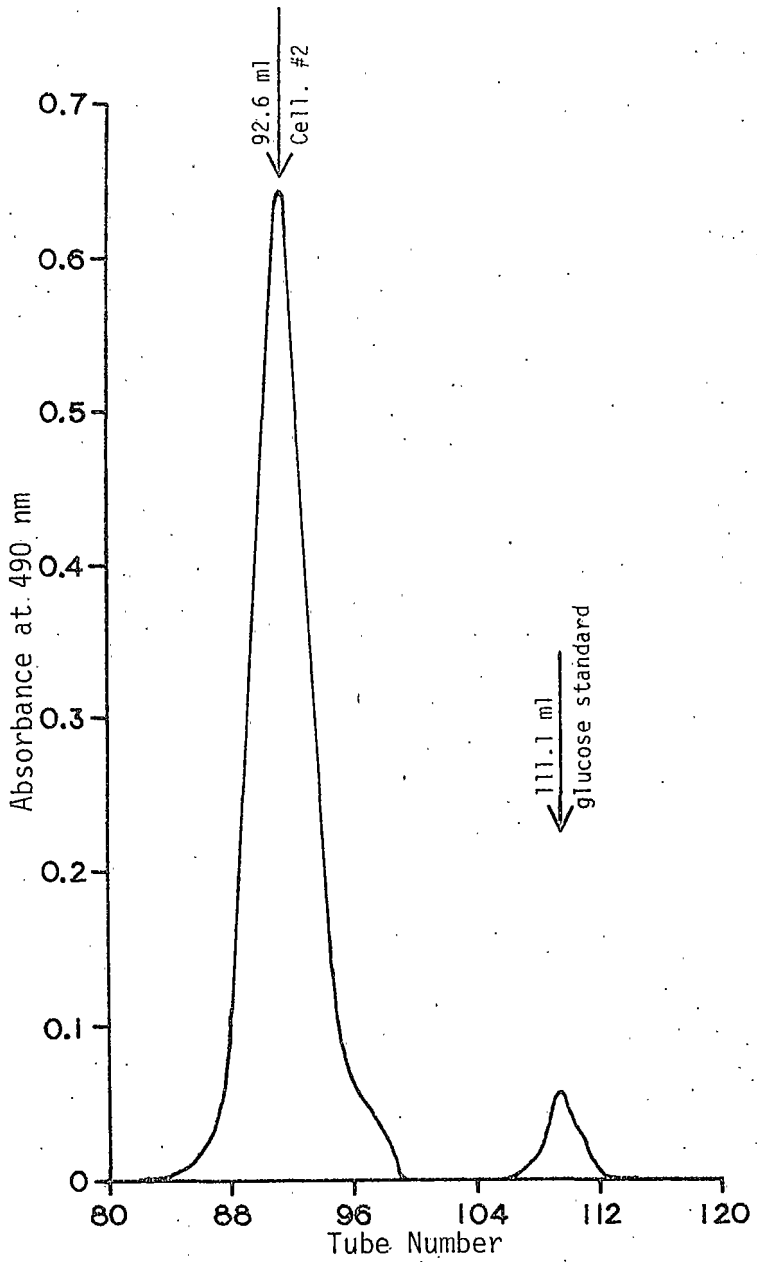


Figure 43. Elution profile of sample, cell. #2.

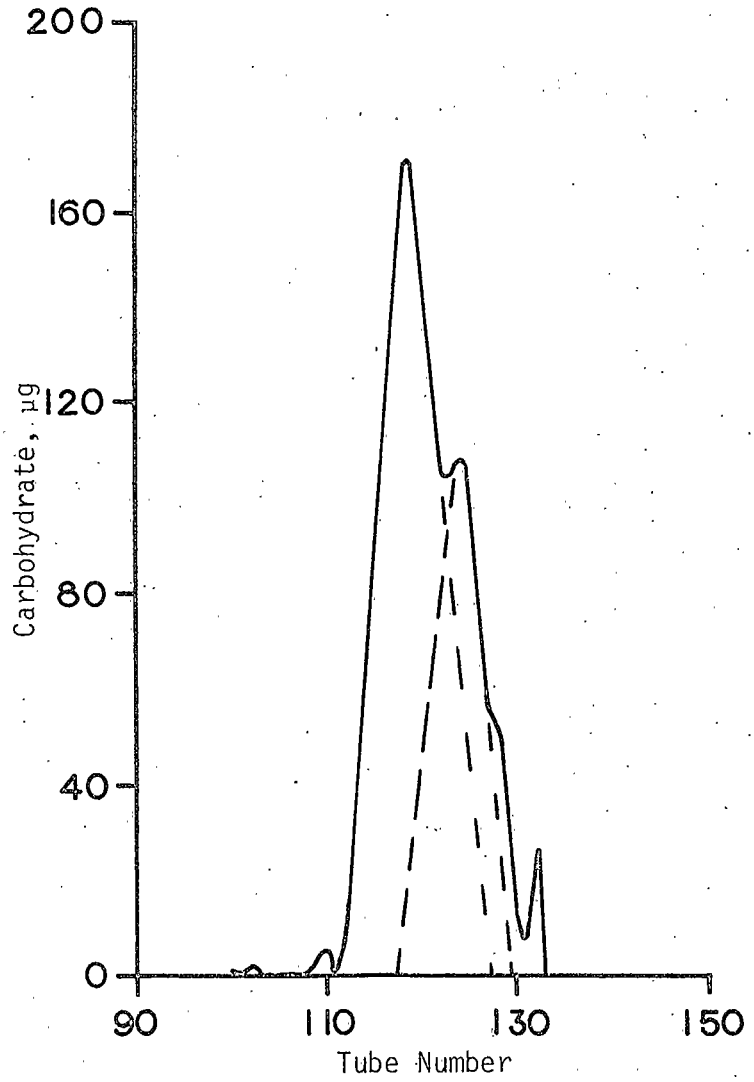


Figure 44. Elution profile of sample, cell. #3.

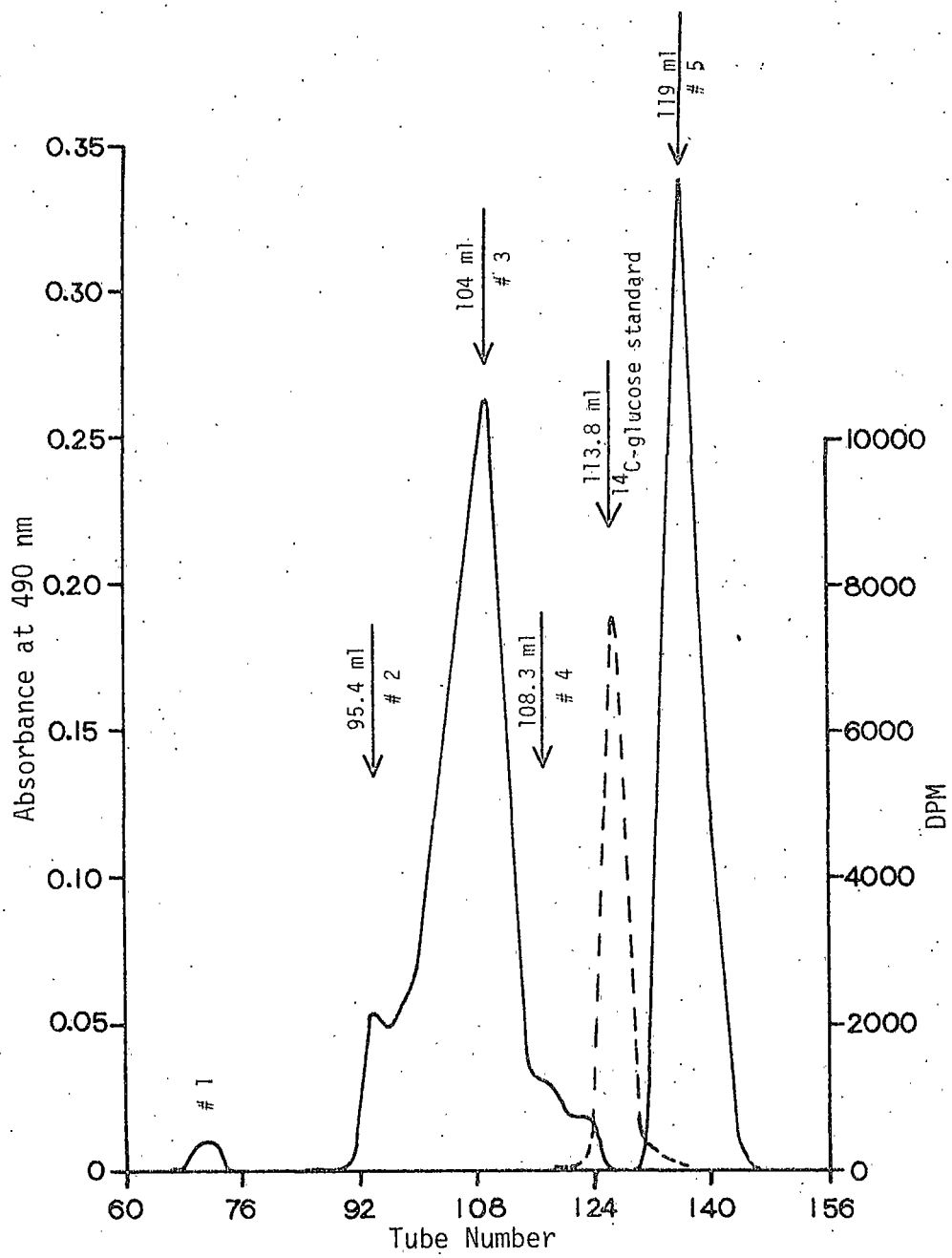


Figure 45. Elution profile of the Glucoside section.

The next step toward the verification of the possible existence of a series of 1,2-propanediol glycosides was an experiment utilizing NMR spectroscopy. The NMR experiment was used as a probe to look for resonances typical of the aglycone methyl doublet. The doublet, arising from the methyl protons on 1,2-propanediol, would be expected to be near 1.0-1.35 ppm. Coupling constants were also useful for comparison purposes; their values were expected to be between 6.2-7.4 Hz. Thirteen samples were submitted for proton NMR spectroscopy on an FX 100 FT NMR, and the results from these analyses are compiled in Table 7. By the criteria used, eight samples were determined to be candidates for possession of 1,2-propanediol. All samples that were determined to possibly contain 1,2-propanediol were considered for the next step; that is, the benzylation experiment. This experiment would be able to prove three points: (1) which samples contained the elements of 1,2-propanediol, (2) was 1,2-propanediol linked glycosidically, and (3) at which carbon of 1,2-propanediol the glycosidic linkage occurred. All samples were benzylated and hydrolyzed, as earlier described (Figure 12), and the results of the glc analysis are listed in Table 8. Glucose and (1-benzyloxy-2-0) 2,3,4,6-tetra-0-benzyl-α-D-glucopyranoside, (10), were used as controls. The glucoside was hydrolyzed under identical conditions, but was not placed through the benzylation reaction. All samples were analyzed using an SE-30 glc column. Contaminating material from the benzylation process was observed, on the carbowax column, at the same retention time

Table 7. Resonances (near 1.2 ppm) from NMR spectra of all samples isolated from *R. secalis* extracts.

Sample	Resonances*,(ppm)	All prospective coupling constants(J, Hz)	Possibility for aglycone moiety
Glucoside section	1.11,1.037,0.973	7.27,6.38	+
Gluc. #2	not a glycoside	-	-
Gluc. #3	1.259,1.188,1.115	7.097,7.297	+
Gluc. #4	none	-	-
Gluc. #5	none	-	-
Cellobioside section	1.336,1.267,1.154, 1.13,1.066	6.88,6.38,8.77	+
Cell. #1	1.252,1.183,1.115	6.86,6.78	+
Cell. #2	1.257,1.184,1.115	7.27,6.88	+
Cell. #3A	none	-	-
Cell. #3B	none	-	-
Cell. #3C	1.311,1.242	6.86	+
"Toxin"**	1.317,1.243	7.35	+
Origin Section	1.335,1.267,1.13, 1.071	6.78,5.88	+

* All resonances are relative to DHO at 4.78 ppm, and only resonances from 1.0-1.35 are reported.

** Spectrum obtained on a Bruker 270 MHz instrument.

Table 8. Analysis by glc (on SE-30) for the isomers of benzyloxypropanol, in all samples that were determined, by NMR, to possibly contain 1,2-propanediol.

Sample	HO—CH_2—C(H)(OBz)—CH_3	Bzo—CH_2—C(H)(OH)—CH_3
glucose control	-	-
(2-O) glucoside control	-	+
glucoside section	+	-
gluc. #3	+	+
cellobioside section	+	-
cell. #1	+	-
cell. #2	+	-
cell. #3	-	-
"Toxin"	+	-
origin section	-	-

as 2-benzyloxy-1-propanol. This was not the case on the SE-30 column. The only isomer present in all cases, except the sample labeled Gluc.#3, was 2-benzyloxy-1-propanol. Gluc. #3 appeared to contain both isomers. But, since the isomer, 1-benzyloxy-2-propanol, was not observed in the

overall glucoside section, its existence is questionable. With these results, the presence of glycosides linked (1-O) to 1,2-propanediol was confirmed, as listed in Table 9. There are at least four separate fractions having components that contain a glycosidic linkage to carbon-1 of 1,2-propanediol. This extra sample (determined to contain glycosidically linked 1,2-propanediol), Cell. #2, and even Cell. #1, which contains at least two compounds, may be other disaccharide derivatives of 1,2-propanediol. These other possible 1,2-propanediol linked disaccharides may be important for the understanding of the overall picture describing the interactions of *R. secalis* on barley plants.

Table 9. Samples from extracts of *R. secalis* determinded to possess a glycosidic linkage to 1,2-propanediol.

Sample	Carbohydrate $O\text{-}CH_2\text{-}\underset{\displaystyle OH}{\overset{\displaystyle H}{\underset{|}{\overset{|}{C}}}}\text{-}CH_3$	Carbohydrate $O\text{-}\underset{\displaystyle CH_2OH}{\overset{\displaystyle CH_3}{\underset{|}{\overset{|}{C}}}}\text{-}H$
Glucoside section	+	-
Gluc. #3	+	+
Cellobioside Section	+	-
Cell. #1	+	-
Cell. #2	+	-
Toxin	+	-

Therefore, another step toward the verification of the structure was attempted. The isolated samples, Cell. #1 and Cell. #2, were submitted for methylation analysis.[19] Neutral sugar analyses showed that Cell. #1 contained predominantly glucose with small amounts of other sugars, and Cell. #2 contained only glucose. The methylation analysis of Cell. #1 is presented in Table 10. This sample, which had a component eluting from a P-2 column at the same volume as (2-propanol-1-O) α-cellobioside, appears to contain two major disaccharide components. One component has a 4-linked glucose, and the other component has a 6-linked glucose. Small amounts of the 2- and 3-linked, and 4,6-linked residues were also noted. This result correlates with the possibility of having (2-propanol-1-O) α-cellobioside in the fungal extracts. It also presents the possibility of having all the possible linkage sites within the disaccharide moiety, i.e., gluc (1→2) gluc, gluc (1→3) gluc, gluc (1→4) gluc, and gluc (1→6) gluc.

The methylation analysis of Cell. #2 is presented in Table 11. This sample, which appeared to be a single component by column chromatography, has only one major disaccharide moiety. This disaccharide has a 4-linked glucose, and by mass spectroscopy, the major signals may be due to the fragmentation of an ethyl cellobioside. Therefore, Cell. #2 may not be important to the overall toxin picture.

Table 10. Methylation analysis of the sample, cell.#1.

Method: methylation, hydrolysis, reduction and acetylation.

Type of moiety found	Percent*
Terminal glucose	52
2- or 3-linked	4
4-linked	21
6-linked	16
4,6-linked	7

Analysis of the above data		Mole % of total
4% 4% glc(1→2)glc-aglycone or(1→3)	4% terminal 4% 1- or 2-linked	8.4
21% 21% glc(1→4)glc-aglycone	21% terminal 21% 4-linked	43.8
16% 16% glc(1→6)glc-aglycone	16% terminal 16% 6-linked	33.3
7% 7% glc(1→4,6)glc-aglycone	7% terminal 7% 4,6-linked	14.6

*The percentage is normalized to 100%.

Table 11. Methylation analysis of the sample, Cell. #2.

Method: methylation, hydrolysis, reduction and acetylation.

Type of moiety found	Percent*
Terminal glucose	52
2- or 3-linked	4
4-linked	40
6-linked	4

Analysis of the above data		Mole % of total
4% gluc (1→2) or (1→3) 4% gluc-aglycone	4% terminal 4% 2- or 3-linked	8.4
40% gluc (1→4) 40% gluc-aglycone	40% terminal 40% 4-linked	83.3
4% gluc (1→6) 4% gluc-aglycone	4% terminal 4% 6-linked	8.4

*The percentage is normalized to 100%.

SUMMARY

With the use of the protected alcohol, 1-benzyloxy-2-propanol,(2), the synthesis of (1-propanol-2-0) α-D-glucopyranoside, (11), for use in a subsequent enzymatic reaction was straight forward. This glucoside, although shown not to be important in the natural system, was of interest for a number of reasons. First, 11 was found to be toxic (at high levels) to barley leaves, showing symptoms similar to those of the natural toxin. Second, as a consequence of its structure, its NMR spectrum was of interest. The molecule was observed to contain two distinctly different carbon-1, β-protons. The aglycone moiety, in an alpha-linkage, contained two distinctly different methyl groups. This result could be due to the two possible isomers at C-2, of 1,2-propanediol, with either hydrogen bonding between the aglycone hydroxyl and the carbohydrate residue, or spatial crowding, stopping free rotation of carbon 2. It was shown by CMR (Section 3), that there was hydrogen bonding through the hydroxyl at C-2 of the glucose residue.

In accordance with the benzylation, hydrolysis, and glc study of the toxin and other isolated components of *R. secalis*, there was evidence for only 1-linked, 1,2-propenediol as an aglycone moiety. This rules out the earlier suggested 2-linked, 1,2-propanediol.[1]

As a result of the synthetic work, Rhynchosporoside was shown not

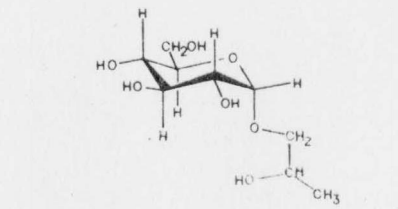
to be a cellobioside. However, by three independent methods including column chromatography, thin-layer chromatography, and methylation analysis, the "toxic" material may be a cellotrioside. The synthetic method used in the preparation of the cellobiosides has only previously been reported for glycoside syntheses.[15] This method is straight forward, and could possibly be applicable for cellotrioside syntheses.

After using syntheses, isolation techniques, NMR analyses, and benzylation-hydrolysis studies, it appears quite certain that the glucoside, cellobioside, and cellotrioside with an anomeric linkage to carbon-1 of 1,2-propanediol, does exist. Also, there is evidence that would enable one to suggest one or more potential disaccharides, other than the cellobioside, having an anomeric linkage to carbon-1 of 1,2-propanediol. Therefore, it seems clear that an entire family of toxins exist, at least the β(1→4) oligosaccharide family. Since the isolation and characterization was approached to ultimately aid in plant breeding and selection, it is advised that all selection experiments utilize not just one fragment of the *R. secalis* toxins, but in some manner take into account all possible toxins.

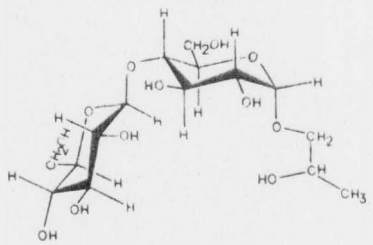
We submit that this work is in no way a complete picture of the phytotoxins produced by *R. secalis*; but rather, only a step toward opening the door. Further work is required to obtain the various toxic compounds in a pure state, and all samples should be checked individually, and in multiples, for toxicity. Also, a search for a possible

broader family of toxins (as was suggested) than in the cellulose family, should be pursued.

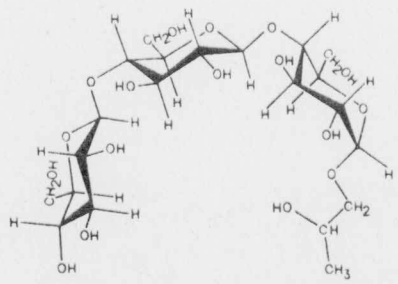
As a last note, we would like to recommend that the trivial name "Rhynchosporoside", given to one of the *R. secalis* toxins,[1] be changed to "Rhynchosporosides", meaning all of the toxins of *R. secalis*. And any particular toxin may be called "Rhyncosporoside$_b^{\{1\rightarrow a\}(b\text{-}1)}$", where the subscript, b, indicates the number of sugar residues, and superscript indicates the linkage assingments (a being the carbon of attachment). Figure 46 shows the proposed structures of the toxins, with their suggested trivial names.



Rhynchosporoside$_1$



Rhynchosporoside$_2^{\{1'\rightarrow 4\}}$



Rhynchosporoside$_3^{\{1''\rightarrow 4'\}\{1'\rightarrow 4\}}$

Figure 46. The proposed structures of the Rhynchosporosides.

EXPERIMENTAL

Throughout this work, absorbances, for phenol-sulfuric acid determinations, were obtained on a Bausch and Lomb Spectronic 70, and column effluents were monitored on a Gilson Hollowchrome variable wavelength monitor. Optical rotations were obtained on a Carl Zeiss Circle Polarimeter 0.01°, using a sodium lamp. Infrared spectra were obtained on both the Beckman-IR5A infrared spectrometer and Beckman-IR20 infrared spectrometer. NMR were obtained on a Varian model T-60 NMR spectrometer, using carbontetrachloride or deuterated chloroform for non-aqueous compounds, and deuterium oxide for aqueous compounds. An asterisk (*) appears after all CMR or NMR obtained by James S. Frye at the CSU Regional NMR Center, Department of Chemistry, Colorado State University, Fort Collins, CO 80523. All results obtained, were with respect to DSS substituted.

All melting points and boiling points are uncorrected. All elemental analyses were obtained from Galbraith Laboratories, Inc., Knoxville, TN 37921.

Throughout this work, the following solvent systems were used for chromatographic purposes: a. (carbon tetrachloride-ethyl acetate, 80:15); b. (carbon tetrachloride-ethyl acetate, 80-17); c. (carbon tetrachloride-chloroform, 80:20); d. (carbon tetrachloride-chloroform, 80:30); e. (carbon tetrachloride-chloroform, 80:40); f. (carbon tetrachloride-chloroform, 80:50); g. (carbon tetrachloride-ethyl acetate, 80:8);

h. (ethyl acetate-water-methanol, 84:18:35); i. (1-butanol-pyridine-water, 9:5:1); j. (chloroform-ethanol, 150:1); k. (2-butanone-ethanol-water, 75:12:6); l. (carbon tetrachloride-ethyl acetate, 80:20); m. (carbon tetrachloride-ethyl acetate, 80:10); n. (1-butanol-pyridine-water, 9:5:4); o. (1-propanol-ammonium hydroxide, 66:33).

Prepatation of allyl benzyl ether 5[22]

Sodium hydride (24.8 g, 1.03 mol) was added to a solution of allyl alcohol (30 g, 0.52 mol) and dioxan (50 ml) in a 500 ml, 2-necked flask, equipped with a mechanical stirrer and reflux condenser. Benzyl chloride (110 g, 0.87 mol) was added dropwise to the rigorously stirred mixture.[7] After the addition was completed, the mixture was refluxed for 4 hrs. The resultant mixture was centrifuged at 13,000 x g, and the supernatant was decanted. The pellet was washed with benzene, centrifuged, decanted and added to the previous supernatant. The combined supernatants were filtered, and the solvent was removed by rotary evaporation. The product can be observed by glc on a 10' x 1/4", 15% carbowax (on chromsorb G, base washed) column, at a temperature of 150°C, with a helium flow rate of 68 cc/min. The retention time was 26.7 min in this system. The resultant solution was distilled (40 g, 52% yield).

bp_{25} 105-7°C Literature[22] bp_{760} 204-5°C

Infrared– 3100, 3050, 2882, 1510, 1468, 1372, 1270, 1220, 1100, 1010,

749, 708 cm^{-1}.

NMR– 3.65-4.0 (multiplet, 2H), 4.38 (singlet, 2H), 4.9-5.43 (multiplet, 2H), 5.58-6.23 (multiplet, 1H), 7.23 (singlet, 5H) ppm.

Elemental Analysis– for $C_{10}H_{12}O$

Calculated:	C, 81.04; H, 8.16
Found:	C, 81.34; H, 7.96

Preparation of 1-benzyloxy-2-propanol 2

Allyl benzyl ether (10 g, 0.068 mol) was added to a stirred mixture of mercuric acetate (21.0 g, 0.068 mol) in aqueous tetrahydrofuran (80 ml, 50:50), at room temperature.[8] After the yellow color had disappeared, the reaction was stirred for 15 min, and then a potassium hydroxide solution (67.5 ml, 3M) was added. This was followed by a solution of sodium borohydride (1.28 g of $NaBH_4$, in 67.5 ml of 3M KOH). The mercury was allowed to precipitate and a solution of saturated sodium chloride (100 ml) was added. The solution was filtered through celite filter aid, extracted with methylene chloride, and dried over anhydrous magnesium sulfate. The product, (2), was monitored on a 10' x 1/4", 15% carbowax (on chromsorb G, base washed) column. With the oven temperature at 195°C, and a flow rate of 68 cc/min, the retention time was 36.7 min. The crude mixture was filtered, concentrated and run on a low pressure liquid chromatographic system utilizing a 100 x 2.5 cm column, packed with silica-gel (60-200 mesh) using solvent system (a), R_F = 0.21.

The void volume of the column was 325 ml, and after 300 ml of effluent passed, 19.6 ml fractions were collected. Tubes 23-68 contained the derivative (7.0 g, 62% yield).

** Literature[23] bp_{10} 123°C

Infrared– 3420, 2845, 1465, 1105, 745, 705 cm^{-1}.

NMR– 1.0-1.26 (doublet, 3H), 3.03-3.5 (multiplet, 2H), 3.63-4.2 (multiplet, 1H), 4.47 (singlet, 2H), 7.27 (singlet, 5H) ppm.

MS– molecular ion at 166 m/e.

Elemental Analysis– for $C_{10}H_{14}O_2$

Calculated: C, 72.26; H, 8.49

Found: C, 72.00; H, 8.45

**Distillation was not used during this procedure.

Preparation of 2,3,4,6-tetra-O-benzyl-α-D-glucopyranoside 8[10]

Methyl α-D-glucopyranoside (50 g, 0.26 mol), powdered potassium hydroxide (250 g), and dioxan (183 ml) were stirred and slowly warmed to the boiling point in a 1 liter, 3-necked flask, equipped with a reflux condenser, mechanical stirrer, and an addition funnel. Benzyl chloride (275.3 g, 2.18 mol) was added dropwise to the boiling and vigorously stirred mixture. After the addition, the mixture was refluxed for an additional 3 hrs. The resulting cooled mixture was steam-distilled, collecting 5 liters of distillate. The organic layer was separated and

washed twice with water, and sodium chloride was added to break the emulsion. The organic layer was mixed with glacial acetic acid (2.1 l) and 4N sulfuric acid (1.2 l) at 85°C in a 4 liter, 3-necked flask, equipped with a thermometer, reflux condenser, and mechanical stirrer. The solution was heated at 85-87°C for 24 hrs. Water (525 ml) was slowly added, and crystallization began. After all the water was added, the mixture was cooled in an ice bath. The product was removed by filtration, and washed successively with aqueous acetic acid (400 ml, 50% v/v), water, and aqueous methanol (400 ml, 75% v/v), and then the crystals were dried (102.3 g, 73% yield). The product was recrystallized twice from n-propanol (62.2 g, 45% yield). NOTE: The article[10] states that upon recrystallization there was little loss of material; however, we found a 22% loss for each recrystallization. The product can be observed by thin-layer chromatography on silica-gel, in solvent system (b), R_F = 0.15.

mp 151-1.8°C Literature[10] mp 151-2°C

$[\alpha]_{Hg}^{31.5}$ = +22.5° in $CHCl_3$ (c, 2.194); $[\alpha]_D^{20}$ = +21.7° in $CHCl_3$ (c, 2.19)

Infrared— 3400, 3040, 2910, 2865, 1458, 1360, 1145, 1085, 1045, 1030, 1005, 743, 693 cm^{-1}.

NMR** — 3.3-4.23 (envelope, 8H), 4.35-5.13 (envelope, 9H), 5.23 (multiplet, 1H), 7.3 (multiple singlets, 20H) ppm.

Elemental Analysis— for $C_{34}H_{36}O_6$

Calculated: C, 75.53; H, 6.71

Found: C, 75.48; H, 6.65

**Obtained from the mono-hydrated species.

Preparation of 2,3,4,6-tetra-O-benzyl-D-glucosyl chloride 9

Thionyl chloride must be distilled prior to use. The 2,3,4,6-tetra-O-benzyl-α-D-glucopyranose (4 g, 0.007 mol), and thionyl chloride (8.3 g, 0.07 mol) was added to a 50 ml flask.[11] After refluxing for 15 min (NOTE: It would be good not to reflux, but to heat to 40-50°C until the reaction was complete.), the solution was concentrated by rotary evaporation, and three consecutive aliquots of toluene were evaporated to remove traces of thionyl chloride. The product can be observed by thin-layer chromatography on silica-gel, in solvent system (b), R_F=048. A silica-gel column eluted with chloroform, R_F = 0.76, can be used to clean the product just prior to reaction.

Preparation of (1-benzyloxypropanol-2-O) 2,3,4,6-tetra-O-benzyl-α-D-glucopyranoside 10

Dry acetonitrile (122 ml), and sodium iodide (12.1 g, 0.08 mol), were added to 2,3,4,6-tetra-O-benzyl-D-glucosyl chloride (12.2 g, 0.0218 mol). After 10 min, 1-benzyloxy-2-propanol (4 g, 0.02 mol) and 2,6,-lutidine (2.58 g, 0.024 mol) {1.1 mol equivalent}, was added to the above solution. The reaction was continued for 18 hrs at room temperature.

The product can be monitored by thin-layer chromatography on silica-gel, in solvent system (c), R_F = 0.04; (d), R_F = 0.13; (e), R_F = 0.19; (f), R_F = 0.26; and in solvent system (g), R_F = 0.39, the product is beginning to separate into two spots (these may be α- and β-anomers). The reaction mixture was poured into water (1000 ml) and saturated sodium chloride was added. The mixture was extracted three times with carbon tetrachloride, washed twice with 5% hydrochloric acid, washed twice with saturated sodium bicarbonate, washed with water, and dried over anhydrous sodium sulfate. The solution was concentrated by rotary evaporation, and the by-product, 2,3,4,6-tetra-O-benzyl-D-glucopyranose, crystallized. After filtration, to remove sodium iodide and 2,6-lutidine the solution was passed over a 22 x 1.4 cm neutral aluminum oxide column with chloroform as eluent. The product was isolated by passing the resultant syrup over a silica-gel column using solvent system (e).

NMR— 1.07-1.3 (multiplet, 3H), 3.25-4.3 (envelope, 20H), 5.27 (doublet, 0.71),** 7.3 (multiple singlets, 25H) ppm.

Elemental Analysis— for $C_{44}H_{48}O_7$

Calculated:	C, 76.72; H, 7.02
Found:	C, 76.60, H, 7.22

**This result was evaluated to be 71% alpha anomer.

Preparation of (1-propanol-2-O) α-D-glucopyranoside 11

Hydrogenolysis of 10 was carried out overnight with an equal weight of 10% Pd/C at a hydrogen pressure of 30 psi, in 95% ethanol. Thin-layer chromatography on silica-gel, in solvent system (h), R_F = 0.476, indicated the hydrogenolysis was complete. The catalyst was removed by filtration, the solution was concentrated, and separated by descending paper chromatography on pre-washed Whatman #541, in solvent system (i). The separated material was decolorized with activated charcoal in 95% ethanol. From a fraction of the crude benzylated product (1.4 g, 0.002 mol), after hydrogenolysis and separation on paper chromatography, the β-anomer was obtained (24 mg, 5% yield), and the α-anomer was also obtained (198.9 mg, 42% yield). Therefore, by isolation, 89% of the products was the α-anomer.

NMR— 1.26 and 1.2 (doublets, 3H), 3.1-4.1 (envelope, 9H), 5.1 (multiplet, 1H) ppm.

NMR*— 1.125, 1.189, 1.255 (triplet), 3.638, 3.798 (envelopes), 5.064(multiplet) ppm.

CMR*— 100.633 (4556), 97.958 (5665), 78.21 (4772), 75.487 (14063), 74.368 (5932), 74.125 (8388), 73.736 (7561), 72.082 (10041), 67.753(5842), 67.121 (4438), 63.035 (9689), 19.504 (5043), 16.974 (6374) ppm.

From the NMR-CMR data, one can suggest that equal amounts of the two isomers at C-7 (carbon-2 of propanediol) exist.

Bioassay of (1-propanol-2-0) α-D-glucopyranoside on barley leaves

This experiment was done in conjuction with Pio Beltran. Excised barley leaves (cultivar var. Hannchen C. I. 531) were placed in water solutions of 11, having concentrations of 400 µg/ml and 250 µg/ml. After 3 days, the solution having a concentration of 250 µg/ml had very little or no effect on the barley leaves, but the solution having a concentration of 400 µg/ml caused symptoms typical of barley scald; wilting, marginal necrosis and yellowing.

Comparison of ^{14}C-glucose labeled fungal carbohydrates with (1-propanol-2-0) α-D-glucopyranoside Figure 11

A carbohydrate mixture, radio-labeled with ^{14}C-glucose, was obtained from Pio Beltran. This mixture was submitted to paper chromatography in solvent system (n), and a large section, including the area from the front to within the glucose region, was eluted with distilled water and concentrated. This syrup was then comparatively chromatographed on prewashed Whatman #541 paper, with (1-propanol-2-0) α-D-glucopyranoside, in solvent system (o). The glucoside was added in equal quantities to the radioactive mixture. Regions of the chromatogram were cut into 1 cm slices, except for the region around the glucoside, this region was cut into 0.5 cm slices, and each slice was counted in a scintillation counter. The glucoside, placed in equal quantities with the radioactive material, was exposed by the method of Trevelyan.[24] The results are pre-

sented in Figure 11.

Preparation of 2-(allyloxy)tetrahydropyran 14

Concentrated hydrochloric acid (2 drops) was added to a solution of distilled dihydropyran (30 g, 0.36 mol) and distilled allyl alcohol (20.7 g, 0.357 mol), in a 100 ml flask, equipped with a calcium chloride drying tube.[13a] Since the reaction is exothermic, it must be cooled with ice-water. The reaction was stirred with a magnetic stirrer for 3 1/3 hrs. The product was monitored by glc on a 10' x 1/4", 15% carbowax (on chromsorb G) column, at a temperature of 120°C, and a flow rate of 108 cc/min. The retention time was 12.5 min. The solution was neutralized with 10N sodium hydroxide, and dried with anhydrous potassium carbonate and magnesium sulfate in the presence of sodium bicarbonate. The mixture was filtered and distilled from sodium bicarbonate (35.9 g, 71% yield).

bp_{740} 156-9°C Literature[13b] bp_{760} 165-7°C

Infrared— 3095, 3025, 2950, 2885, 1653, 1460, 1448, 1388, 1350, 1328, 1268, 1205, 1188, 1138, 1122, 1082, 1060, 1030, 1000, 965, 921, 902, 870, 843, 815 cm^{-1}.

NMR— 0.9-2.2 (envelope, 6H), 3.2-4.39 (envelope, 4H), 4.59 (singlet, 1H), 4.9-5.4 (envelope, 2H), 5.56-6.26 (multiplet, 1H) ppm.

Preparation of 1-[(tetrahydropyran-2-yl)oxy]-2-propanol 15

2-(allyloxy)tetrahydropyran (26 g, 0.18 mol) was added to a stirred mixture of mercuric acetate (59.4 g, 0.183 mol) in aqueous tetrahydrofuran (220 ml, 50:50), in a 500 ml flask, at room temperature.[8] The yellow solution lost its color very quickly, and the reaction was stirred for another 1/2 hr. A potassium hydroxide solution (180 ml, 3M) was added, followed by a sodium borohydride solution (3.48 g of $NaBH_4$, in 180 ml of 3M KOH). The solution was left overnight while the mercury precipitated out. A saturated solution of sodium chloride (200 ml) was added, and the mixture was extracted 4 times with dichloromethane. The product was monitored by glc on a 9' x 1/4", SE-30 (on chromsorb-G) column, at a temperature of 125°C, and a flow rate of 50 cc/min. The product, (15), had a retention time of 19.6 min. The organic layer was dried over anhydrous sodium carbonate and sodium sulfate in the presence of sodium bicarbonate. The mixture was filtered, concentrated, and distilled (22.9 g, 78% yield).

bp_{24} 121.5-2.5°C Literature[25] $bp_{0.3-0.4}$ 60-4°C

Infrared– 3430, 2950, 2880, 1450, 1380, 1270, 1204, 1140, 1125, 1080, 1065, 1038, 978, 904, 870, 810 cm^{-1}.

NMR– 1.15 (doublet, J = 6.4 Hz, 3H), 1.3-2.3 (envelope, 6H), 3.0-4.2(envelope, 6H), 4.6 (singlet, 1H) ppm.

Preparation of 1-[(tetrahydropyran-2-yl)oxy]-2-benzyloxypropane 16

Sodium hydride (6.48 g, 0.27 mol) was added to a solution of 15 (21 g, 0.131 mol) and dioxan (40 ml), in a 250 ml, 3-necked flask equipped with a reflux condenser, mechanical stirrer, and an addition funnel. With vigorous stirring, benzyl chloride (24.9 g, 0.197 mol) was added dropwise. After the addition was completed, the mixture was refluxed for 4 hrs.[7] The product was observed by glc on a 10' x 1/4", 20% SE-30 (on anakrom) column, at a temperature of 210°C and flow rate of 108 cc/min. The product had a retention time of 22.9 min. The resulting mixture was centrifuged at 15,000 x g, decanted and the pellet was rinsed with dioxan. The combined supernatants were vacuum filtered, concentrated by rotary evaporation, and distilled from sodium bicarbonate (26 g, 79% yield).

bp_{13} 186-7°C

Infrared– 3510, 3062, 2970, 2900, 1513, 1470, 1390, 1365, 1276, 1218, 1200, 1138, 1080, 986, 915, 881, 828, 747, 708 cm^{-1}.

NMR– 1.16 (doublet, J = 6 Hz, 3H), 1.3-2.2 (envelope, 6H), 3.1-4.1 (envelope, 5H), 4.57 (singlet, 3H), 7.25 (singlet, 5H) ppm.

Elemental Analysis– for $C_{15}H_{22}O_3$

Calculated:	C, 71.97; H, 8.86
Found:	C, 71.83; H, 8.96

Preparation of 2-benzyloxy-1-propanol 12

1-[(tetrahydropyran-2-yl)oxy]-2-benzyloxypropane (1 g, 0.004 mol) was added to methanol (10 ml) containing Dowex 50W-X8 resin (1.5 g). The heterogeneous mixture was shaken at room temperature for about 1 hr.[14] The product was monitored on a 10' x 1/8", 15% SE-30 (on chromsorb G) column, at a temperature of 200°C, and a flow rate of 11 cc/min. The reaction mixture was filtered, concentrated by rotary evaporation and distilled. By glc, a 98.6% conversion was achieved.

bp_{11} 134-6°C Literature[26] $bp_{0.8}$ 88-9°C

Infrared– 3460, 3060, 3000, 2960, 2898, 1512, 1469, 1378, 1355, 1220, 1160, 1100, 1075, 1000, 920, 868, 805, 745, 705 cm^{-1}.

NMR– 1.09 (doublet, J = 6 Hz, 3H), 2.2 (multiplet, 1H), 3.42 (multiplet, 3H), 4.48 (singlet, 2H), 7.23 (singlet, 5H) ppm.

MS– molecular ion at 166 m/e.

Elemental Analysis– for $C_{10}H_{14}O_2$

Calculated:	C, 72.26; H, 8.49
Found:	C, 72.34; H, 8.43

Glc systems for analysis of 1-benzyloxy-2-propanol, (2), and 2-benzyloxy-1-propanol, (12)

For separation of 1-benzyloxy-2-propanol, and 2-benzyloxy-1-propanol, the following column and conditions were used: a 10' x 1/8", 15%

carbowax 20M (on acid washed chromsorb G, 60/80 mesh) column, at a temperature of 213°C, injector temperature of 273°C, detector temperature of 302°C, and a flow rate of 25 cc/min. The observed retention time for 2 was 1993 seconds, and the retention time for 12 was 2467 seconds. Minigrator settings were as follows:

PW = 50, SS = 100, Bl = 5, TP = 20, and Tl = 500.

A 4.5' x 1/8", 15% SE-30 (on acid washed chromsorb W, 80/100 mesh) column was used with the following conditions: injection was made at a column temperature of 85°C, after 200 seconds elapsed, the temperature was programmed at 1°C/min up to 140°C. The injector temperature was 273°C, the detector temperature was 302°C, with a flow rate of 12 cc/min. The observed retention time for 2 was 2893 seconds, and the retention time for 12 was 3023 seconds. Confirmation of the identity of peaks having retention times similar to those of 2 and 12, was obtained by co-injection of standards. Only peaks that exactly co-injected with standards were determined positive.

Preparation of octa-acetyl-cellobiose 18

Cellobiose (50 g, 0.15 mol) was added during the course of 0.5 hr to a solution of acetic anhydride (200 ml) and perchloric acid (1.2 ml). The temperature of the reaction mixture should not rise above 40°C or fall below 30°C.[16] After the temperature stabilized, chloroform (150 ml) was added and the mixture was poured into 400 ml of ice and water,

and the mixture was separated. The organic layer was washed twice with ice water, and the washings were extracted twice with chloroform. The combined organic layers were washed with a saturated sodium bicarbonate solution. The solution was dried over a mixture of calcium chloride, calcium carbonate, sodium bicarbonate, and activated charcoal. The mixture was filtered and taken up to 800 ml with chloroform. Diethyl ether (1000 ml) was added and the mixture was kept at 5°C overnight, and filtered. The crystals were taken up with chloroform (500 ml), and diethyl ether (300 ml) was added. The mixture was kept at 5°C overnight, and filtered (57 g, 58% yield).

mp 200-10°C Literature[27] α-mp 229.5
β-mp 202

NMR— 1.94-2.3 (multiplet, 24H), 3.5-4.76 (envelope, 8H), 4.76-5.8(envelope, 5H), 6.27 and 6.56 (doublets, 1H) ppm.

Preparation of 2,3,6,2',3',4',6'-hepta-O-acetyl-cellobiosyl bromide 19

Amorphus phosphorous (4.5 g) was added to a chloroform solution of 18 (30 g, 0.04 mol), and cooled in an ice bath. While the mixture was being vigorously stirred by a mechanical stirrer, bromine (29 g) was added, then water (5.5 g) was slowly added. The vessel was stoppered, and stirred at room temperature for 2 hrs. Chloroform (50 ml) was added and the mixture was poured into ice water (125 ml), and separated.[16]

The product was observed by thin-layer chromatography on silica-gel, in solvent system (j), R_F = 0.21. The chloroform layer was filtered, washed twice with water, and the water washings were also extracted twice with chloroform. The combined chloroform extracts were washed with a solution of saturated sodium bicarbonate, and dried over a mixture of anhydrous sodium sulfate and calcium carbonate. The mixture was filtered and concentrated to 75 ml. Diethyl ether (150 ml) was added, and the mixture was kept at 5°C overnight. The crystalline mass collected by filtration , crystallized from a solution of chloroform (75 ml) and diethyl ether (75 ml) (15.3 g, 50% yield).

mp 182-3°C Literature[28] mp 176°C[28b] mp 182°C[28d]

NMR- 1.9-2.26 (multiplet, 21H), 3.5-5.8 (envelope, 13H), 6.55 (doublet, J = 3.8 Hz, 1H)ppm.

Preparation of phenyl 2,3,6,2',3',4',6'-hepta-O-acetyl-1-thio-β-cellobioside 20[17]

The 2,3,6,2',3',4',6'-hepta-O-acetyl-cellobiosyl bromide was prepared from 18 (25 g, 0.036 mol) according to the previous method. After the chloroform solution was dried with a mixture of anhydrous sodium sulfate, and calcium carbonate, it was directly used in the following steps. Tetrahydrofuran (50ml), and a solution of sodium hydride (1.92 g,

0.04 mol), and 10% excess thiophenol (4.41 g, 0.04 mol) were added slowly to the chloroform solution. The mixture was warmed to 45°C for 30 min to provide nearly a complete condensation. The mixture was filtered and washed three times with an aqueous solution of saturated sodium bicarbonate. The chloroform layer was washed with water, and dried over anhydrous calcium chloride. The chloroform was removed by rotary evaporation to obtain a crude crystalline mass (33.5 g). This material could be purified by the method of Purves.[17] The product can be observed by thin-layer chromatography on silica-gel, in solvent system(j), R_F = 0.23. This product was used directly in the next step.

Literature[17] mp 295°d

$[\alpha]_D^{20}$ = -28.5° in chloroform

Preparation of phenyl 1-thio-β-cellobioside 21[17]

The product from the previous reaction was dissolved in 5 times its weight of ammonical methanol. The ammonia-methanol solution was obtained by saturating methyl alcohol with dry ammonia gas, at room temperature.[17] This solution was allowed to stir overnight at room temperature, the solvent was then removed by rotary evaporation, and the residue was extracted with warm dry ether (to remove the acetamide). The product was observed by thin-layer chromatography on silica-gel, in solvent system (k), R_F = 0.3 (13.9 g, 89% yield). This product was

used directly in the next reaction.

Literature[17] mp 230°C

$[\alpha]_D^{17}$ = -59.2° in water (c, 1.5)

Preparation of phenyl 2,3,6,2',3',4',6'-hepta-O-benzyl-1-thio-β-cellobioside 22[15]

Phenyl 1-thio-β-cellobioside (13.1 g, 0.03 mol) {from the previous preparation} was dissolved in dry dioxan (50 ml) in a 250 ml, 3-necked flask, equipped with a reflux condenser, mechanical stirrer, and a dropping funnel. Sodium hydride (7.3 g, 0.31 mol) was added to the solution, and the mixture was stirred for 15 min. Benzyl chloride (38.7 g, 0.306 mol) was added dropwise while the mixture was refluxing. Reflux was continued for 3 hrs, then the cooled mixture was poured onto ice (200 ml), and ethanol (130 ml) was added. Upon stirring, a solid separated, and water (100 ml) was added. The product was observed by thin-layer chromatography on silica-gel, in solvent system (1), R_F = 0.75. Recrystallization from ethanol gave the product (7.5 g, 23% yield). Overall yield from octa-O-acetyl-cellobiose was 20%.

mp 152°C

Infrared– 3440, 3050, 2880, 1463, 1362, 1220, 1072, 915, 740, 698 cm^{-1}.

NMR– 3.2-4.2 (envelope, 13H), 4.3-5.1 (envelope, 14.8H), 5.27 (0.33H),

7.3 (multiplet, 40H) ppm. {67% β-anomer}

NMR** 7.53 (375), 7.553 (410), 7.517 (502), 7.474 (532), 7.269 (8462), 7.24 (10158), 7.221 (5735), 7.207 (7423), 7.112 (690), 5.169 (305), 5.044 (480), 4.828 (957), 4.801 (865), 4.73 (1851), 4.654 (488), 4.548 (866), 4.504 (983), 4.474(1042), 4.365 (1720), 3.613 (831), 3.523 (856), 3.398 (629)ppm{64% β-anomer}.

Elemental Analysis— for $C_{67}H_{68}O_{10}S$

Calculated: C, 75.54; H, 6.43; S, 3.01

Found: C, 75.49; H, 6.57; S, 3.12

By NMR spectroscopy, the percentage of the β-anomer was 65.5%.

**This NMR spectrum was obtained on a 90 MHz Jeol instrument.

Preparation of the (2-benzyloxypropane-1-O) 2,3,6,2',3',4',6'-hepta-O-benzyl-cellobiosides 23

Prior to reaction, the alcohol was purified by chromatography on a 17 x 2.3 cm column, packed with silica-gel, and eluted with solvent system (a), R_F = 0.18.

Mercuric sulfate (0.6 g, 0.002 mol) was added to a solution of 2-benzyloxy-1-propanol (0.269 g, 0.00162 mol), and 22 (1 g, 0.0009 mol)in dry tetrahydrofuran (19 ml).[15] The mixture was refluxed overnight, and by thin-layer chromatography on silica-gel, in solvent system (a), there were three products: hepta-O-benzyl-cellobiose, R_F = 0.19; (2-benzyloxypropane-1-O) 2,3,6,2',3',4',6'-hepta-O-benzyl-α-cellobio-

side, R_F = 0.52; and the β-anomer, R_F = 0.62. The material with R_F = 0.19, was separated from the other two products by chromatography on a 17 x 2.3 cm column, packed with silica-gel, and eluted with solvent system (a). The material from the two more-mobile spots were collected, and formed a solid upon concentration (0.62 g, 59% yield).

NMR— 1.2 (doublet), 3.2-4.1 (envelope), 4.2-5.1 (envelope), 5.24 (resonance typical of an α-anomer), 7.26 (multiplet) ppm.

Elemental Analysis— for $C_{71}H_{76}O_{12}$

Calculated:	C, 76.05; H, 6.83
Found:	C, 76.37; H, 7.06

The mixture of material from above, was separated on a 17 x 2.3 cm, silica-gel column, with solvent system (m), to give the β-anomer (0.368 g, 35% yield).

NMR— 1.21 (multiplet, 3H), 2.9-5.1 (envelope, 33H), 7.26 (multiplet)ppm.

The α-anomer was also obtained (0.249 g, 24% yield).

NMR— 1.21 (doublet), 3.2-4.1 (envelope, 17H), 4.2-5.3 (envelope, 16H), 7.26 (multiplet) ppm.

NOTE: By NMR, the free cellobiose product appeared to be totally alpha. Since the yield of this by-product was approximately 41%, and the yield of the alpha anomer was 24%, the total yield of alpha products was 65%. This is interesting, since the percentage of the starting material,

phenyl 2,3,6,2',3',4',6'-hepta-O-benzyl-β-1-thio-cellobioside, by NMR, was 65.5%.

Preparation of the (2-propanol-1-O) cellobiosides 24

Debenzylation of both anomers was accomplished by dissolving the product in ethanol, in a micro-hydrogenation flask (Section 4), and adding an equal weight of 10% Pd/C. The mixture was hydrogenolyzed overnight with hydrogen, at 7 1/2 psi. After the hydrogenolysis was complete, with comparison by thin-layer chromatography on silica-gel, in solvent system (h), the α-anomer had an R_G value of 1.03, and the β-anomer had an R_G value of 1.11. However, the toxin has an R_G value of 0.72 in solvent system (h). NMR spectra were obtained confirming the products, but the sample size was small and resolution was not good enough for integration.

Attempted preparation of (1-benzyloxypropane-2-O) 2,3,6,2',3',4',6'-hepta-O-benzyl-cellobiosides with mercuric acetate 26

A chromatography column was used to purify the alcohol, similar to that used in the preparation of 23. Mercuric acetate (0.246 g, 0.000769 mol, 2 eq.) was added to a solution of 1-benzyloxy-2-propanol (0.141 g, 0.000846 mol, 1.1 eq.) and phenyl 2,3,6,2',3',4',6'-hepta-O-benzyl-1-thio-cellobioside (0.85 g, 0.00077 mol), in dry tetrahydrofuran (15 ml). The reaction went to completion when it was heated at 50°C for 1.5 days.

The reaction product was observed by thin-layer chromatography on silica-gel, in solvent system (a), and a single product was observed. The reaction mixture was filtered, concentrated by rotary evaporation, placed on a 17 x 2.3 cm, silica-gel column, and eluted with solvent system (a). Upon concentration, the product crystallized. Fractional crystallization was then carried out. The crystalline mass was dissolved in boiling ethanol, after the ethanol cooled some, 36% of the material oiled out (Fraction #1). The remaining solution was left at room temperature, and 23% of the material crystallized (Fraction #2). The mother liquor was kept at 5°C, and 5% crystallized (Fraction #3), (0.25 g, 64% yield overall). The following table lists these fractions with NMR data that estimates the percentage of the two observed acetyl proton signals (2.01 and 2.13 ppm).

	Percentage Peak Height		Anomeric Proton	
	2.01 PPM	2.13 ppm	6.33 ppm J = 3 Hz α-anomer	5.6 ppm J = 7 Hz β-anomer
original mixture	85	100	strong	strong
Fraction #1	71	100	strong	weak
Fraction #2	48	100	strong	-
Fraction #3**	100	93	+	+

**The spectrum for this sample had poor resolution.

Therefore, the alpha anomer could be, in principle, fractionally crystallized from the mixture of the two acetyls. The following data were obtained from Fraction #2:

mp 100-10°C

NMR— {for the α-anomer} 2.13 (singlet, 3H), 3.1-4.1 (envelope, 12H), 4.2-5.3 (envelope, 15H), 6.33 (doublet, J = 3 Hz, 1H), 7.27 (multiplet, 35H) ppm.

NMR— {for the β-anomer} 2.01 (singlet, 3H), 3.1-4.1 (envelope, 12H), 4.2-5.3 (envelope, 15H), 5.6 (doublet, 1H), 7.27 (multiplet, 35H) ppm.

Elemental Analysis— for $C_{63}H_{66}O_{12}$

Calculated:	C, 74.54; H, 6.55
Found:	C, 74.30; H, 6.72

Therefore, the only products obtained were the acetyl 2,3,6,2',3',4',6'-hepta-O-benzyl-cellobiosides, (26).

After overnight hydrogenolysis with 10% Pd/C in ethanol at a hydrogen pressure of 7.5 psi, the acetyl derivative was observed by thin-layer chromatography on silica-gel, in solvent system (h), R_F = 0.4, R_G = 1.29.

Attempted preparation of (1-benzyloxypropane-2-0) 2,3,6,2',3',4',6'-hepta-0-benzyl-cellobiosides with phenyl mercuric acetate

Phenyl mercuric acetate (0.312 g, 0.001 mol) was added to a solution of 1-benzyloxy-2-propanol (0.164 g, 0.000987 mol), and phenyl 2,3,6,2',3',4',6'-hepta-0-benzyl-1-thio-cellobioside (0.96 g, 0.0009 mol), in dry tetrahydrofuran (10 ml). The reaction mixture was refluxed, and it was periodically monitored by thin-layer chromatography on silica-gel, in solvent system (a). After a two-week period of reflux, without formation of a product, the refluxing was terminated. Mercuric acetate was added, and upon an overnight reflux, a single product was observed, which corresponded to the acetate, previously obtained.

Preparation of (1-benzyloxypropane-2-0) 2,3,6,2',3',4',6'-hepta-0-benzyl-cellobioside with mercuric sulfate 25

Mercuric sulfate (0.6 g, 0.001 mol) was added to a solution of 1-benzyloxy-2-propanol (0.54 g, 0.0032 mol), and phenyl 2,3,6,2',3',4',6'-hepta-0-benzyl-1-thio-cellobioside (1 g, 0.0009 mol), in dry tetrahydrofuran (20 ml). The mixture was stirred overnight at room temperature, and partially went to completion, by observation with thin-layer chromatography on silica-gel, in solvent system (a), the β-anomer has an R_F = 0.54, and the α-anomer has an R_F = 0.47. The reaction mixture was refluxed for seven days (this time was arbitrary), and the reaction was complete. The reaction mixture was filtered and concentrated by rotary

evaporation. All material was separated on a 17 x 2.3 cm silica-gel column, with variations of solvent system (a): (80:8, 200 ml); (80:10, 100 ml); and (80:15).

NMR— {of the β-anomer} 1.28 (doublet, 3H), 3.1-5.3 (envelope, 33H), 7.28 (multiplet, 40H) ppm.

NMR— {of the α-anomer} 1.07-1.33 (two doublets, 3H), 3.1-5.2 (envelope, 32H), 5.28 (doublet, J = 4 Hz, 1H), 7.28 (multiplet, 40H) ppm.

Both the α- and β-anomers were hydrogenolyzed overnight with equal weights of 10% Pd/C, in ethanol at a pressure of 7.5 psi. They were observed by thin-layer chromatography on silica-gel, in solvent system (h), the β-anomer had an R_F = 0.291, R_G = 0.96; the α-anomer had an R_F = 0.317, R_G = 1.05.

Mild acid hydrolysis of (2-propanol-1-0) α-cellobioside, with comparison thin-layer chromatography to the toxin Figure 29

(2-propanol-1-0) α-cellobioside was added to a round-bottom glass tube, and taken up to 0.1 ml with water. Trifluoroacetic acid (0.02ml) was added, the solution was cooled in an ice bath, and the open end of the tube was then sealed with a torch. The sealed tube was heated at 80°C for 1.5 hrs, cooled, opened, and rotary evaporated to remove the trifuoroacetic acid. The reaction mixture was comparatively chromatographed bythin-layer chromatography on silica-gel, in solvent system (h)

to give the following R_F and R_G values:

Compound	R_F	R_G
glucose	0.308	1.0
cellobiose	0.168	0.55
toxin	0.205	0.666
(2-propanol-1-0) α-cellobioside	0.332	1.08
(1-propanol-2-0) α-glucoside	0.476	1.55
(2-propanol-1-0) α-glucoside	0.488	1.58

Mild hydrolysis of starch to obtain β(1→4) gluco-oligomers, and their thin-layer chromatography Figure 35

Cellulose (3 spatula tips-full) was placed in a round bottom tube, and water (0.2 ml) was added. Trifluoroacetic acid (0.07 ml) was added, the solution was cooled in an ice bath, and the open end of the tube was sealed with a torch. The sealed tube was heated at 80°C for 3 hrs, cooled, opened, and rotary evaporated to remove the trifluoracetic acid. The reaction mixture was separated by paper chromatography on Whatman #1 in solvent system (n). All glucose was removed, and half of the cellobiose was removed, all other materials extending to the origin were combined and a small amount of glucose was added. The mixture was placed

on a Bio-gel P-2 chromatography column, as shown in Figure 35. Fractions that corresponded to the various oligomers were collected and pooled. These pooled fractions were then submitted to thin-layer chromatography on silica-gel, in solvent system (h), giving the R_F values listed in Appendix A.

Cultures of *R. secalis*

The *R. secalis* used throughout this study was isolated from naturally infected barley in Montana and was designated the CA 75 isolate. The isolate was grown and harvested according to the method of Auriol et al.[1]

Bioassay tests

Barley cultivar var. Hannchen C. I. 531, was used for all bioassay tests. When the seedlings were 8-9 days old (second leaf stage), the plants were cut with a sharp razor blade 1-2 cm above the ground, and each cutting was placed in a miniature conical tube, containing 0.2-0.6 ml of the solution to be tested. Water blanks were simultaneously run with all bioassay tests. Results of all tests were obtained after a 3 day incubation period, at room temperature. Bioassays were conducted in the plant growth chamber, being environmentally controlled at 15-24° diurnal cycle with 26,900 lx of combined incandescent and cool fluorescent light utilizing a 12 hr photoperiod.[1]

Phenol-sulfuric acid analysis of carbohydrates

The phenol-sulfuric acid analysis was conducted by placing an aliquot of a carbohydrate solution in the bottom of a 20 ml test tube. A 5% phenol-water solution (1 ml) was then added to the tube, with subsequent addition of concentrated sulfuric acid (5 ml).[41] After a ten min cooling period, the tube was placed in a spectrophotometer to obtain its absorbance at 490 nm. According to the results obtained from the standard curve (Section 1), 18 μg of glucose has an absorbance of 0.299.

Acetone precipitation

To a concentrated extract (50 ml), 3 volumes of acetone (150 ml) at -15°C were added. The precipitate was removed by centrifugation for 10 min at 20,000 x g. The acetone was removed from the supernatant by rotary evaporation.

Dowex 50W-X8, (H^+) column

A Dowex 50W-X8, (H^+), 17 x 2.3 cm column was prepared by first rinsing the resin with water and pouring off the fine material. The rinsed resin was poured into the column, and washed with 6N hydrochloric acid (300 ml). The acid was removed by washing the column with distilled water (600 ml). The material from the acetone precipitation step was applied to this column. The column was eluted with distilled water (150 ml).

Dowex 1-X8, (OH^-) column

A Dowex 1-X8, (OH^-), 17 x 2.3 cm column was prepared by first rinsing the resin with water and pouring off the fine material. The rinsed resin was poured into the column, and washed with 3N sodium hydroxide (300 ml). The base was removed by washing the column with distilled water (600 ml). The material from the Dowex 50W-X8, (H^+) column was applied directly to this column. The column was eluted with distilled water (150 ml).

Bio-gel P-2 (200-400 mesh) column, 50 x 1.5 cm

The solution from the preceeding step was reduced in volume by rotary evaporation to 2 ml. This solution was placed on a 50 x 1.5 cm, Bio-gel P-2 (200-400 mesh) column, and eluted with distilled water. Fractions containing 2 ml each were collected, and fraction numbers 20-40 were pooled. This solution was concentrated by rotary evaporation, with the aid of ethanol addition, obtaining a 2 ml solution.

Paper chromatography in solvent system (n)

The solution from the preceeding step was applied to 6-8 Whatman #1 papers, and run in descending fashion using solvent system (n). The areas on the paper chromatogram of interest are outlined in Figure 34. The glucoside, in this system, has an R_G value of 1.6.

Decolorization with activated charcoal

The areas of interest were eluted with distilled water, and the volume was reduced by rotary evaporation. The syrup was taken up with 70% ethanol, and activated charcoal was added. The mixture was heated until just boiling, and then filtered through Whatman #2 filter paper. The charcoal was rinsed with 95% ethanol. The ethanolic solution was concentrated to a syrup by rotary evaporation.

Bio-gel P-2 (200-400 mesh) column, 95 x 1.5 cm (Bio-gel P-2 #1)

Samples (0.7 ml) were placed on a 95 x 1.5 cm, Bio-gel P-2(200-400 mesh) column, and eluted with distilled water at room temperature. Each fraction was comprised of 24 drops or 0.99 ml, and were collected at a rate of 1 drop/13 seconds. The void volume was 49.25 ml.

Bio-gel P-2 #1 column separation of (2-propanol-1-O) α-cellobioside (synthetic) Figure 39

The run was carried out in identical fashion as that described for the operation of the Bio-gel P-2 #1 column. The fraction volume for this run was 1.0 ml/tube. About 0.3 mg of the cellobioside was placed on the column, and 0.37 mg of glucose was added for a marker. The fractions were monitored for carbohydrate by using phenol sulfuric acid analyses.[20] The β-anomer was run in identical manner as the above, having an elution volume of 89 ml.

Bio-gel P-2 #1 column separation of the combined "toxin" and origin sections Figure 40

The combined "toxin" (53 mg of carbohydrate) and origin sections (from paper chromatography), were placed on the Bio-gel P-2 #1 column, and eluted with water, at room temperature. The column flow rate was 1 drop/9 seconds, and 24 drops or 1.0 ml, were collected per tube. The fractions were combined, ten to a sample, and freeze-dried. Each sample was diluted to 0.6 ml, and 0.2 ml was placed in each of two tubes for bioassaying. Figure 40 shows the results of this experiment.

Bio-gel #1 column separation of the cellobioside section Figure 41

The cellobioside section (from paper chromatography), after combining the eluents of many papers, was placed on the Bio-gel P-2 #1 column, and eluted with distilled water, at room temperature. A flow rate of 1 drop/9 seconds was used, and 24 drops or 1.01 ml per tube were collected. Twenty micro-liters from each tube was analysed with phenol-sulfuric acid,[20] for total carbohydrate. The elution profile is presented in Figure 41.

Bio-gel P-2 #1 column separation of sample cell. #1 Figure 42

The fractions with-in the peak designated as cell. #1 (Figure 41), were pooled and placed on the Bio-gel P-2 #1 column. The column run was completed as previously described for the cellobioside section.

Bio-gel P-2 #1 column separation of sample cell. #2 Figure 43

The fractionswithin the peak designated as cell. #2 (Figure 41), were pooled and placed on the Bio-gel P-2 #1 column. The column run was completed as previously described for the cellobioside section.

Bio-gel P-2 (-400 mesh) column, 295.5 x 0.8 cm (Bio-gel P-2 #2 column) separation of the sample cell. #3 Figure 44

The fractions within the peak designated as cell. #3 (Figure 41), were pooled and placed on a 295.5 x 0.8 cm, Bio-gel P-2 (-400 mesh) column, and eluted with 0.22 micron filtered distilled water, at a temperature of 64°C. The flow rate was 1 drop/12seconds, and the fraction volume was 13 drops or 0.56 ml per tube. The column eluent was monitored at 192 nm, by passage through a 40 µl flow cell. A 30 µl sample, from each tube, was analyzed for total carbohydrate by phenol-sulfuric acid.[20]

Bio-gel P-2 #2 column separation of the glucoside section Figure 45

The glucoside section (combined from paper chromatography of at least 10 liters of initial fungal media) was placed on the Bio-gel P-2 #2 column, and eluted in like manner as described for sample cell #3. Labeled ^{14}C-glucose was added for a marker during the column run. On this same column, allyl α-D-galactoside was eluted at a volume of 103 ml (Section 1).

Benzylation of a typical carbohydrate sample isolated from *R. secalis* extracts

Sodium hydride (0.084 g, 0.0035 mol) was added to a solution of the carbohydrate sample (4.17 mg) and dioxan (10 ml). To this vigorously stirred solution, benzyl chloride (0.15 g, 0.0012 mol) was added.[7] The sample was refluxed overnight, and when cooled, the mixture was filtered by suction through Whatman #1 filter paper. The solution was then filtered through Whatman #2 filter paper, and rotary evaporated at 95°C.

Hydrolysis of the derivatized carbohydrates

The syrup from the previous reaction was transfered, with the aid of chloroform, to a 2-necked flask containing glacial acetic acid(56 ml) at a temperature of 90°C. Boiling 2N sulfuric acid (3 ml), and concentrated sulfuric acid (0.84 ml) was added, and solution was stirred for 24 hrs at 90±2°C.[10] After cooling, water (50 ml) was added, and the mixture was extracted 3 times with dichloromethane (30 ml). The dichloromethane was removed by rotary evaporation, and the residue was neutralized with a solution of saturated sodium bicarbonate. This mixture was extracted 3 times with dichloromethane (20 ml). The combined organic layers were dried over anhydrous sodium sulfate, filtered, and rotary evaporated at 95°C. This sample was now submitted for glc analysis.

Glc analysis of the derivatized *R.secalis* samples

Glc analyses of the derivatized samples (obtained from the previous reaction), were accomplished according to the methods previously described. The results obtained from the carbowax column were inconclusive, since impurities in the system had similar retention times as the two isomers. The results obtained from the SE-30 column are tabulated in Table 8 and 9. Only peaks that exactly co-injected with standards were determined to be positive.

APPENDIX A

R_F Values and Data Reduction for Some Oligomers From the Hydrolysate of Cellulose

The R_F values were determined on silica-gel by thin-layer chromatography in solvent system (ethyl acetate-water-methanol, 84:18:35).

Compound	Subunits	R_F	$R_F/1-R_F$	$-\log(R_F/1-R_F)$
glucose	1	0.212	0.269	0.57
cellobiose	2	0.116	0.131	0.883
cellotriose	3	0.045	0.0475	1.32
cellotetrose	4	0.023	0.0235	1.63
cellopentose	5	0.0098	0.0099	2.004

APPENDIX B

Elution Volumes and Data Reduction for Some β(1→4) Oligoglucose Units Chromatographed on Bio-gel

Figure 35 presents the elution profile.

Elution volume = Ve Void volume = Vo = 49.25 ml

Subunits	Ve (ml)
1	111.9
2	103.7
3	94.1
4	87.6
5	83.16

The data reduction for the plot in Figure 36 is listed below.

Subunits	Ve/Vo	log(molecular weight)
1	2.272	2.256
2	2.106	2.534
3	1.911	2.703
4	1.779	2.824
5	1.689	2.918

APPENDIX C

Data Reduction for A Bio-gel P-2 Column Separation of $\alpha(1\rightarrow4)$ Oligoglucose Units

Figure 37 shows the column separation, and the plot of the data is presented in Figure 38

Elution volume = Ve Void volume = Vo = 49.5 ml

Subunits	Ve (ml)	Ve/Vo	log(molecular weight)
1	111.5	2.253	2.256
2	105.25	2.126	2.534
3	99.25	2.005	2.703
4	94.0	1.899	2.824
5	88.0	1.778	2.918
6	82.0	1.657	2.996
7	76.25	1.54	3.062
8	72.0	1.455	3.119
9	68.0	1.374	3.169
10	65.0	1.313	3.215
11	62.5	1.263	3.256

REFERENCES AND NOTES

1. P. Auriol, G. Strobel, J. P. Beltran and G. Gray, *Proc. Natl. Acad. Sci.* 75, 4339-43 (1978).

2. E. N. Ayesu-Offei and B. G. Clare, *Aust. J. Biol. Sci.* 24, 169-74 (1971).

3. E. N. Ayesu-Offei and B. G. Clare, *Aust. J. Biol. Sci.* 23, 299 (1970).

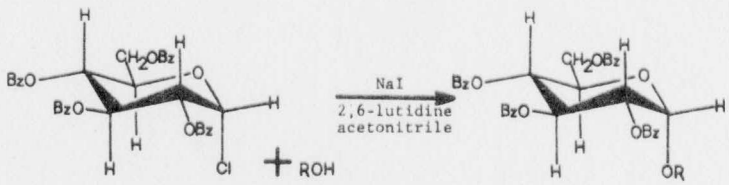
4. a. E. Pacsu, *J. Amer. Chem. Soc.* 52, 2571-5 (1930).

   b. E. Pacsu, J. Janson and B. Lindberg, *Methods in Carbohydrate Chemistry* 2, Academic Press, New York (1963) pp. 376-85.

5. J. Beltran, G. A. Strobel, R. Beier and B. P. Mundy, *Plant Physiol.* 65, 554-556 (1980).

6. F. J. Kronzer and C. Schuerch, *Carbohyd. Res.* 34, 71-8 (1974).

   The following generalized reaction was utilized:



7. a. M. Fieser and L. Fieser, *Reagents for Organic Synthesis* 1, John Wiley and Sons, New York (1967) pp. 1079-80.

   b. M. E. Tate and C. T. Bishop, *Can. J. Chem.* 41, 1801 (1963).

c. P. A. Gent, R. Gigg and R. Conant, *J. Chem. Soc., Perkin Trans. 1*, 1535-42 (1972).

8. R. D. Otzenberger, *Ph. D. Thesis*, Montana State University, Bozeman, MT 59717 (1971).

9. H. C. Brown and P. J. Geoghegan, Jr., *J. Org. Chem.* 35, 1844-50 (1970).

10. T. D. Perrine, C. P. J. Glaudemans, R. K. Ness, J. Kyle and H. G. Fletcher, Jr., *J. Org. Chem.* 32, 664-9 (1967).

11. E. L. Eliel, M. T. Fisk and T. Prosser, *Organic Synthesis*, collective volume 4, John Wiley and Sons, New York (1963) pp. 169-72.

12. P. W. Austin, G. E. Hardy, J. G. Buchanan and J. Baddiley, *J. Chem. Soc.*, 2128-37 (1964).

13. a. J. S. Cowie, P. D. Landor and S. R. Landor, *J. Chem. Soc., Perkin 1*, 720 (1973).

b. P. J. Barry and B. M. Craig, *Can. J. Chem.* 33, 716-21 (1955).

14. R. Beier and B. P. Mundy, *Synth. Comm.* 9, 271-3 (1979).

15. R. J. Ferrier, R. W. Hay and N. Vethaviyasar, *Carbohyd. Res.* 27, 55-61 (1973).

16. M. Bárczai-Martos and F. Körösky, *Nature* 165, 369 (1950).

17. C. B. Purves, *J. Amer. Chem. Soc.* 51, 3619-27 (1929).

18. a. E. C. Bate-Smith and R. G. Westall, *Biochem. et Biophys. Acta* 4, 427-40 (1950).

b. M. F. Levy, *Anal. Chem.* 26, 1849-50 (1954).

19. Methylation analyses were completed by Peter Albersheim and Michael McNeil, Department of Chemistry, University of Colorado, Boulder, CO.

20. M. Dubois, K. A. Gilles, J. K. Hamilton, D. A. Rebers and F. Smith, *Anal. Chem.* 28, 350 (1956).

21. The corn syrup hydrolysate was a generous gift from John (Jack) Nordin, Department of Biochemistry, University of Massachussets, Amherst, MA.

22. H. Baker, K. H. Cornell and M. J. Cron, *J. Amer. Chem. Soc.* 70, 1490-2 (1948).

23. a. This reference was obtained from *Chem. Abs.* 70, 106674h.[23b]

b. A. Kazvo, *Kitami Kogyo Tanki Dalgaku, Kenbyu, Hokoku* 2, 97-101 (1967).

24. W. E. Trevelyan, D. P. Procter and J. S. Harrison, *Nature* 166, 444 (1950).

25. J. B. Martin and E. S. Lutton, *J. Amer. Oil Chem. Soc.* 42, 529-33 (1965).

26. K. Mislow, R. E. O'Brien and H. Schaefer, *J. Amer. Chem. Soc.* 84, 1940-4 (1962).

27. These physical constants were obtained from the *Handbook of Chemistry and Physics 47ed,* R. C. Weast, editor, The Chemical Rubber Co., Cleveland Ohio (1966-7).

28. a. The reference for Danilov and Pastukhov,[28b] was obtained from *Chem. Abs.* 41, 2005i (1947).

b. S. N. Danilov and P. T. Pastukhov, *J. Gen. Chem. (USSR)* 16, 923-32 (1946).

c. The reference of Gakhokidze,[28d] was obtained from *Chem. Abs.* 41, 6209a (1947).

d. A. M. Gakhokidze, *J. Gen. Chem. (USSR)* 16, 1907-13 (1946).

SECTION 3

THE USE OF ^{13}C-NMR FOR IDENTIFICATION AND ASSIGNMENT OF ANOMERIC CONFIGURATION FOR THE CARBOHYDRATE RESIDUE IN GALACTO- AND GLUCO-PYRANOSIDES AND FURANOSIDES

LIST OF TABLES FOR SECTION 3

TABLE		PAGE
1	Resonance interval at each carbon atom for galacto- and glucopyranosides, in ppm with external TMS as reference	270
2	Resonance interval at each carbon atom for galacto- and glucofuranosides, in ppm with external TMS as reference	278

LIST OF FIGURES FOR SECTION 3

FIGURE		PAGE
1	A plot of the CMR resonance intervals for all carbon atoms in the galactopyranoside and glucopyranoside systems	271
2	Comparison of the CMR resonances for some synthetic galactopyranosides with the pyranose interval diagram	273
3	Comparison of the CMR resonances for some synthetic glucopyranosides with the pyranose interval diagram	274
4	Comparison of the ^{13}C-NMR spectra of methyl α-D-galactopyranoside and methyl α-D-glucopyranoside	275
5	Comparison of the CMR resonances (spectrum #1) for helminthosporoside to the pyranose interval diagram	277
6	A plot of the CMR resonances or resonance intervals for all carbon atoms in the D-galactofuranoside and D-glucofuranoside systems.	279
7	A plot of the CMR resonances or resonance intervals for all carbon atoms in the pyran and furan ring systems of the D-galactosides and D-glucosides	280
8	Comparison of the CMR resonances (spectrum #2) for helminthosporoside to the interval diagram for D-galactopyranosides and furanosides.	282
9	Tentative CMR assignments of the carbons in the two galactose residues in helminthosporoside.	284

INTRODUCTION

Carbon-13 NMR spectroscopy, with many recent advances, has become a useful tool for the carbohydrate chemist. There are three texts on ^{13}C-NMR (CMR),[1-3] that encompass carbohydrates. There are also some recent reviews,[4-6] which discuss the use of this technique as a tool for structural analysis. An article that lists nearly a complete compilation of CMR data for carbohydrates[7] is of tremendous help to the researcher.

The assignment of configuration to glycopyranosides has been an on-going problem in the field of carbohydrate chemistry. PMR has been used extensively for assignment of anomeric configuration (a review by Bundle and Lemieux[8] discusses PMR), but it can not be relied upon. It has also been suggested,[8] that like PMR, the assignment of anomeric configuration by CMR requires more input than just a single parameter, i.e., the resonance of the anomeric carbon.

Some[9,10] have investigated more rigorous methods for evaluating the anomeric configuration of glycopyranosides, by utilizing coupling constants. The two-bond ^{13}C-^{1}H coupling constant has an opposite sign for the alpha (+), than for the beta (-) anomer.[9] This sign difference seems to be a most definitive way of assigning configurations. However, there seem to be many problems associated with the technique, and improvements are needed to afford a general applicable method. Two and three bond ^{13}C-^{1}H coupling constants have now been obtained from natural

abundance ^{13}C NMR spectra.[10] The three-bond coupling constant is very difficult to obtain, only under the correct set of circumstances and with the proper derivatives, can this method be useful.

Therefore, since we were confronted with natural products that contained glucose and galactose residues, it was of interest to search for a simple method to determine anomeric configuration. But more than that, we were interested in determining which glycosides were present, those of glucose or galactose.

RESULTS AND DISCUSSION

The interest in assigning the carbon atoms in the pyranose ring to CMR resonances developed as the result of two happenings. First, the fourth proposed structure (Figure 40 in Section 1) for helminthosporoside had just been developed. Second, we obtained a CMR spectrum of helminthosporoside. Before one could look for an ether linkage in the carbohydrate moiety, as suggested by the fourth structure, a technique had to be employed for assigning the resonances to the appropriate carbons. A well-developed technique did not appear to exist. Therefore, we set out to examine the possibility of arriving at an acceptible technique for assigning resonances in the CMR spectra to carbons of the pyranose ring system.

To approach the problem, the literature was searched for all compounds containing galactopyranosides and glucopyranosides. All the CMR resonance values were compared for each carbon atom. A very striking relationship did exist; therefore, this approach was pursued.

Appendix A lists all values found for α- and β-galactopyranosides. Appendix B lists all values found for α- and β-glucopyranosides. For each compound, a value was tabulated for every carbon atom of the pyranose ring system (C-1 thru C-6). The mean values and standard deviations are also included in Appendicies A and B.

The interval (max. and min. values) for the resonance values at each carbon atom for α- and β-galactopyranosides and α- and β-glucopy-

ranosides are listed in Table 1.

A plot of these intervals is presented in Figure 1. It is clear, when observing Figure 1, that each glycopyranoside has a definite set of intervals, each set being different from the other. Therefore, by using these intervals, one could, in principle, not only predict the nature of the carbohydrate moiety, but determine whether it was the alpha or beta anomer.

Table 1. Resonance interval at each carbon atom for galacto- and glucopyranosides, in ppm with external TMS as reference.

	C1	C2	C3	C4	C5	C6
α-galactopyranosides	99.1-100.5	69.4-69.8	70.6-70.7	69.8-70.7	70.7-72.0	62.2-62.3
β-galactopyranosides	103.0-105.5	71.05-72.9	72.6-74.0	68.6-69.9	75.4-76.55	61.1-62.55
α-glucopyranosides	93.0-101.1	72.5-74.3	73.7-74.8	70.3-71.4	72.1-73.9	61.3-62.5
β-glucopyranosides	103.5-104.6	73.8-75.1	75.7-77.8	70.5-71.2	75.5-77.3	61.4-62.5

In order to explore the range of usefullness for this method, the CMR spectrum was obtained for a number of synthetic derivatives (which were discussed in Sections 1 and 2). Appendix C lists the CMR resonances for carbons of the pyranose system for synthetic α- and β-D-galactopyranosides. A plot of these resonance values is presented in Figure 2. It is very interesting that all resonances for both the alpha and beta compounds fall on the appropriate interval boxes. Note: the compounds,

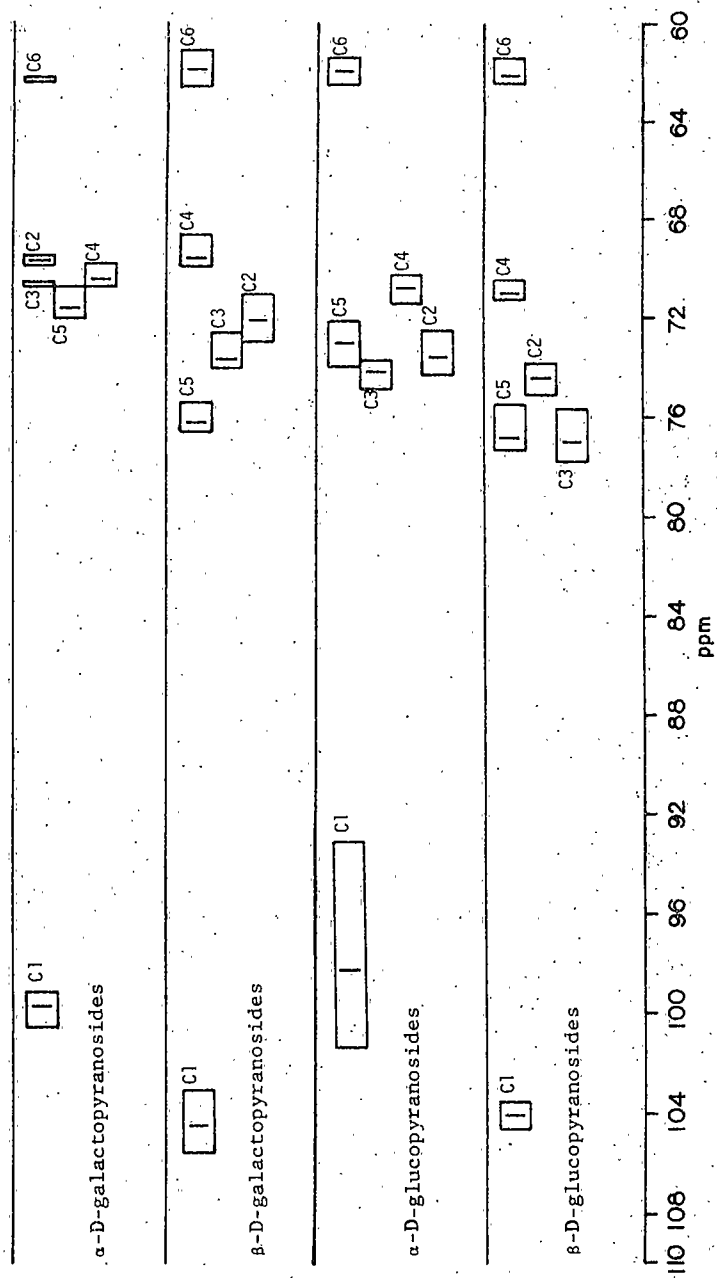


Figure 1. A plot of the CMR resonance intervals for all carbon atoms in the galactopyranoside and glucopyranoside systems.

allyl, (2,3-propanediol-1-O), and (2-propanol-1-O) were not included in the set of resonances used for obtaining the intervals, since the CMR spectra of these compounds are not in the literature.

Appendix D lists the CMR resonances for carbons of the pyranose system for synthetic α- and β-D-glucopyranosides. A plot of these resonance values is presented in Figure 3. Again, all resonances fall on the appropriate interval boxes, except for that of C-2 in (1-propanol-2-O) α-D-glucopyranoside. It seems reasonable that since two resonances are observed for the three carbons of the aglycone, C-1, and C-2 of the pyranose ring, that the hydroxyl group of the aglycone is probably hydrogen bonded to the C-2 hydroxyl of the pyranose ring system. Since there is an asymmetric center in the aglycone, two conformers would be expected to be observed.

To observe a comparison of the ^{13}C-NMR spectra for an α-D-galactopyranoside and an α-D-glucopyranoside, spectra of the methyl derivatives are shown in Figure 4. Even though the resonances of the two compounds are some what similar, by observation, the differences are quite clear. As shown earlier, by standard deviations (shown in Appendicies) and with the graphical treatments, these differences in resonance values are significantly different.

At this juncture, the resonances obtained from the first CMR spectrum of helminthosporoside were compared with the pyranose interval diagram (Figure 5). The only possible match, was that of a β-D-glucopyran-

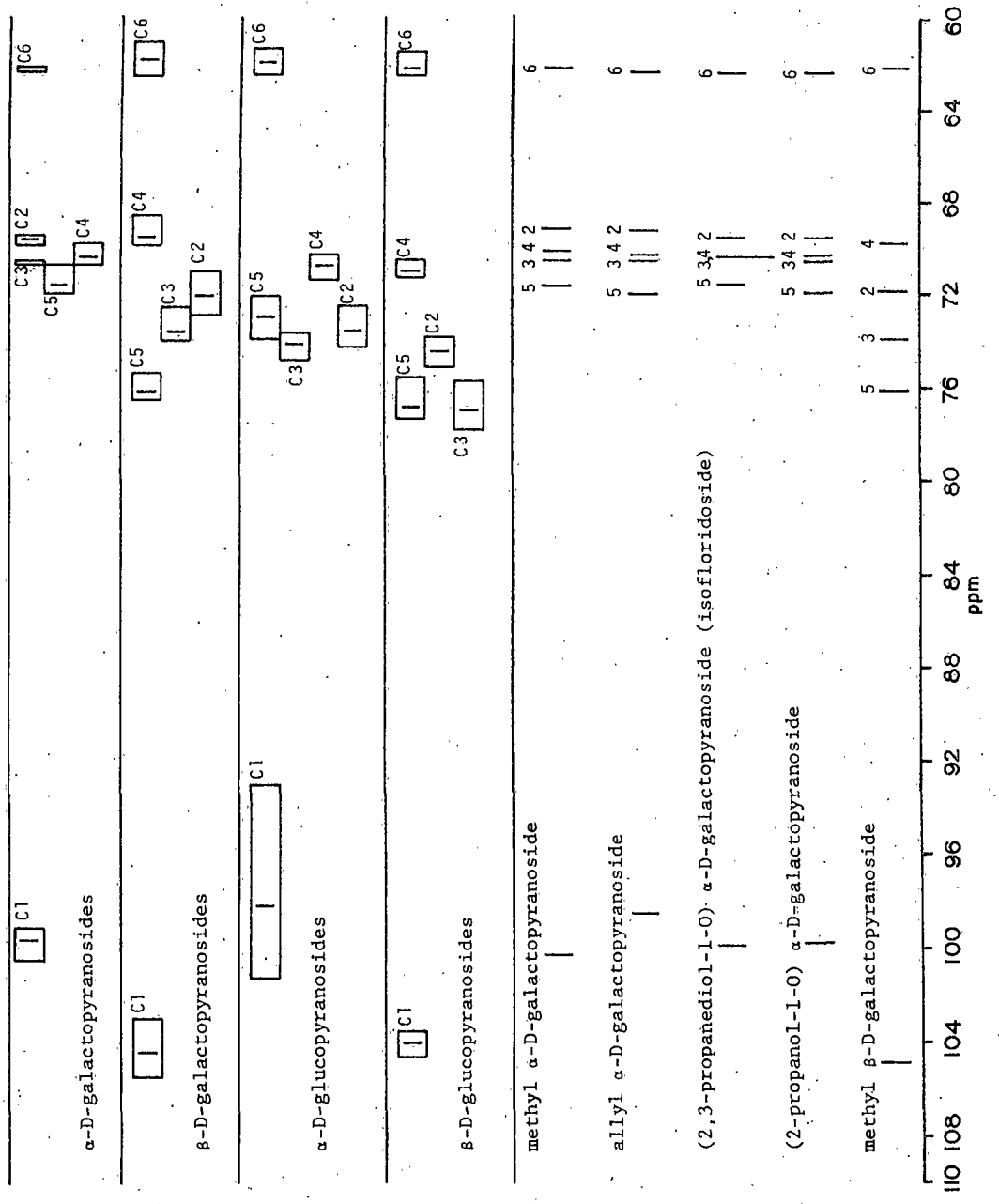


Figure 2. Comparison of the CMR resonances for some synthetic galactopyranosides to the pyranose interval diagram.

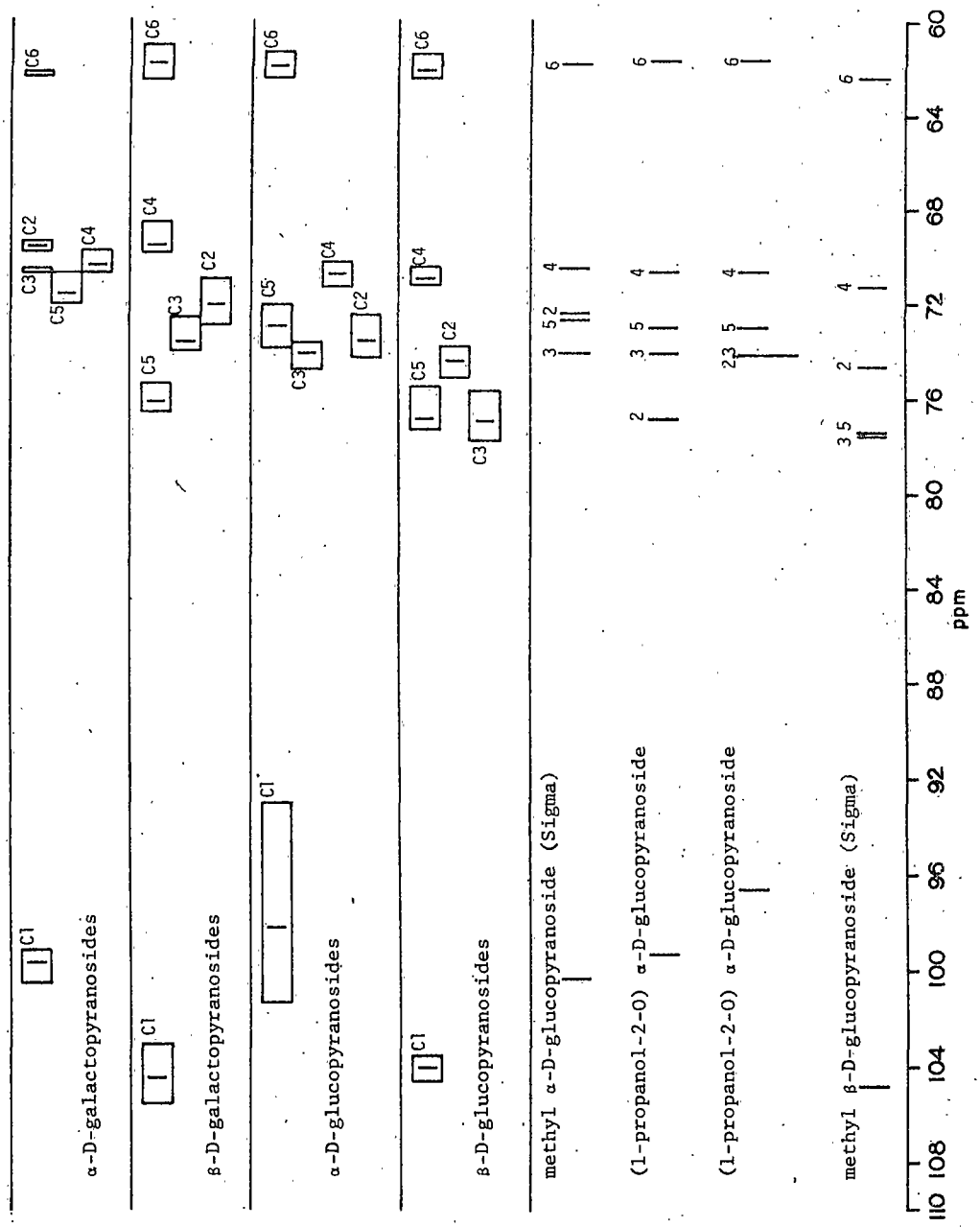


Figure 3. Comparison of the CMR resonances for some synthetic glucopyranosides to the pyranose interval diagram.

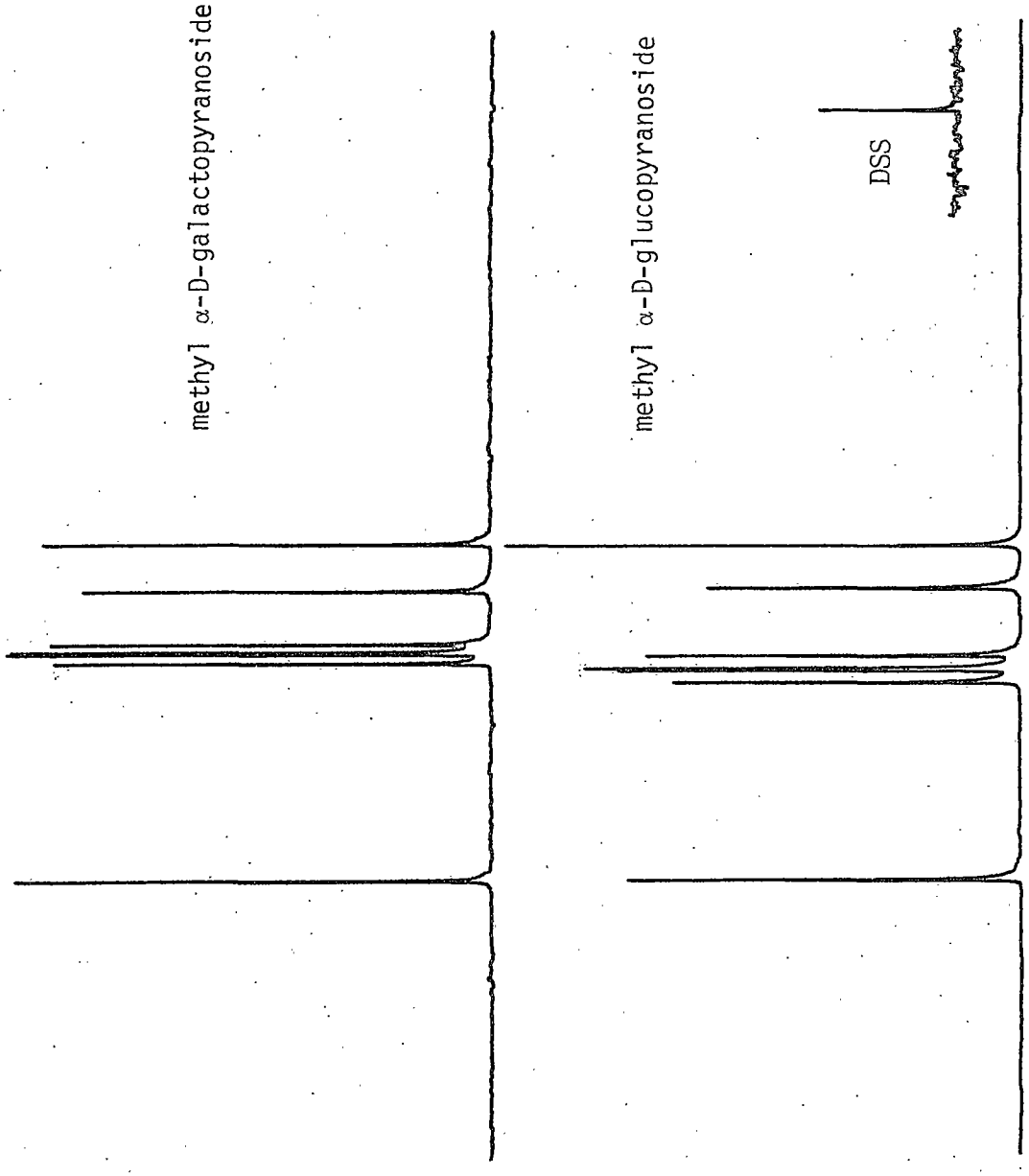


Figure 4. Comparison of the ^{13}C-NMR spectra of methyl α-D-galactopyranoside and methyl α-D-glucopyranoside.

oside, with a substitution at C-2. The effect observed (6-8 ppm shift down field) by substitution on the pyranose system, forming an ether has previously been noted.[6,11] However, this result seemed to be contrary to the accepted fact that galactose was present. This observation is what led us into a program for re-isolation of helminthosporoside.

After re-isolation, a CMR spectrum again was obtained for helminthosporoside. More resonances (with less intensity) were then observed, along with most of the resonances observed in spectrum #1 (Section 1). Since mass spectroscopy revealed two carbohydrate residues, linkage analysis was obtained (Section 1). This analysis, undertaken by two independent groups, showed the presence of a terminal galactofuranosyl residue, and either a 4-linked galactopyranosyl or a 5-linked galactofuranosyl residue.

The question at this juncture was, are the CMR resonances for the D-galactofuranosides enough different from those of the D-galactopyranosides, so that they may be used for structure verification and anomeric configuration assignment? After searching the literature, all compounds containing α- or β-D-galactofuranosides are listed in Appendix E, and all compounds containing α- or β-D-glucofuranosides are listed in Appendix F. For each compound, a value was tabulated for every carbon of the furan ring system (C-1 thru C-6). The mean values and standard deviations (where applicable) are also included in Appendicies E and F.

The interval (max. and min. values) for the resonance values at

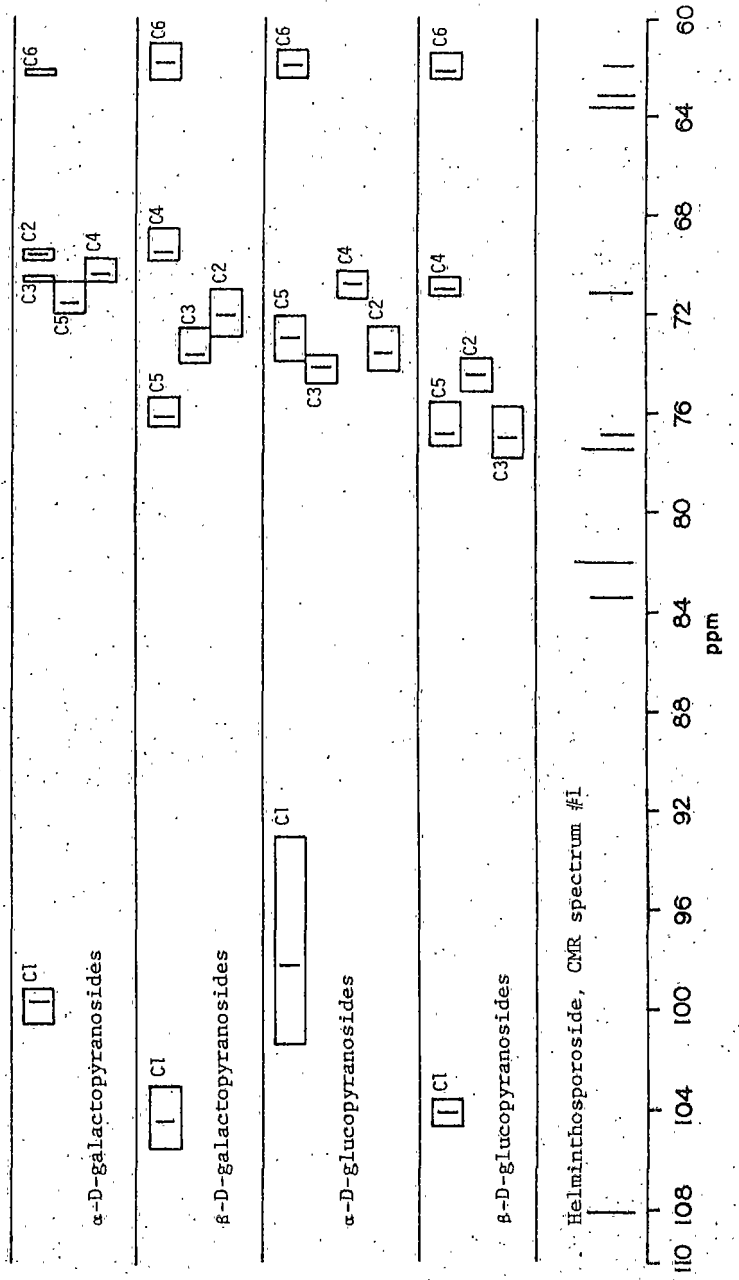


Figure 5. Comparison of the CMR resonances (spectrum #1) for helminthosporoside to the pyranose interval diagram.

each carbon atom for α- and β-D-galactofuranosides, and α- and β-D-glucofuranosides are listed in Table 2.

Table 2. Resonance interval at each carbon atom for galacto- and glucofuranosides, in ppm with external TMS as reference.

	C1	C2	C3	C4	C5	C6
α-D-galactofuranosides	103.1-103.8	77.4-78.2	75.5-76.2	82.3-83.1	73.7-74.5	63.4-64.1
β-D-galactofuranosides	108.0-109.9	81.3-82.4	77.5-78.4	82.4-84.7	71.6-72.0	60.9-63.9
α-D-glucofuranosides	104.1	77.7	76.6	78.8	70.7	64.2
β-D-glucofuranosides	110.0	80.6	75.8	82.3	70.7	64.7

A plot of these intervals is presented in Figure 6. It is clear, when observing Figure 6, that each glycofuranoside has a definite set of intervals or resonances, each set being different from the other. This is the same situation as observed for the glycopyranosides. Therefore, with the glycofuranosides as with the glycopyranosides, one could, in principle, by using these intervals not only predict the nature of the carbohydrate moiety, but determine whether it was the alpha or beta anomer. To show the correlation of CMR resonances for the pyran and furan ring system of the D-galactosides and D-glucosides, a diagram was constructed, as shown in Figure 7. When observing Figure 7, it is clear that each of the eight structural systems has a different set of signals. Therefore, in principle, this CMR method could verify the presence

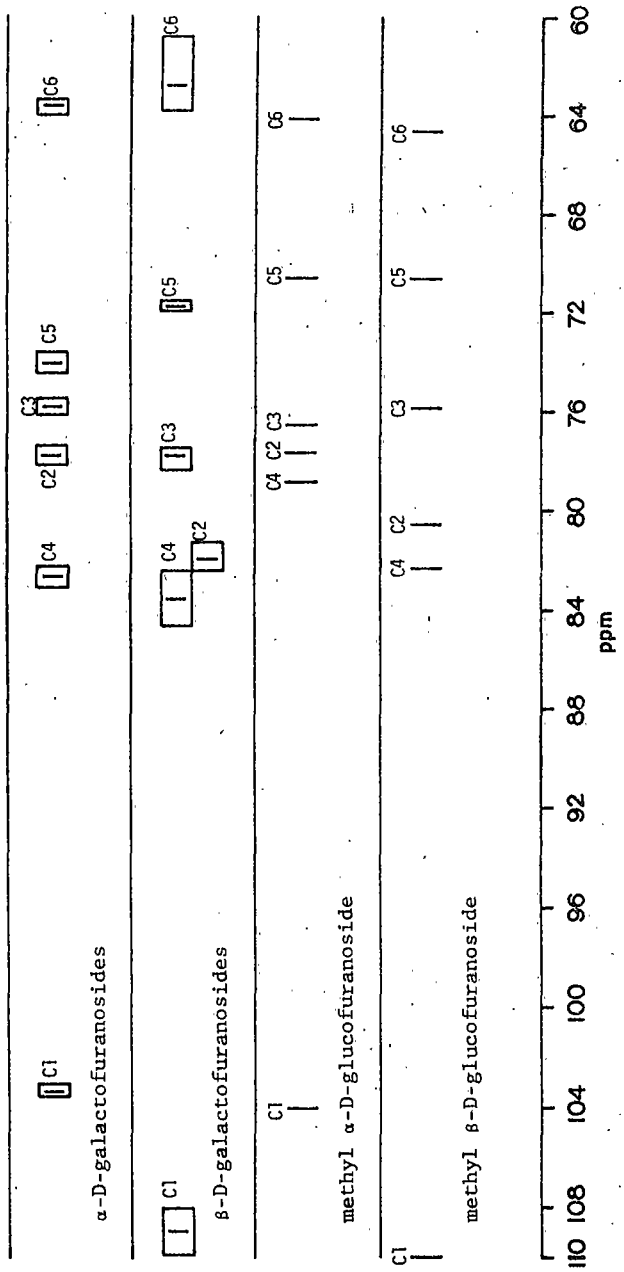


Figure 6. A plot of the CMR resonances or resonance intervals for all carbon atoms in the D-galactofuranoside and D-glucofuranoside systems.

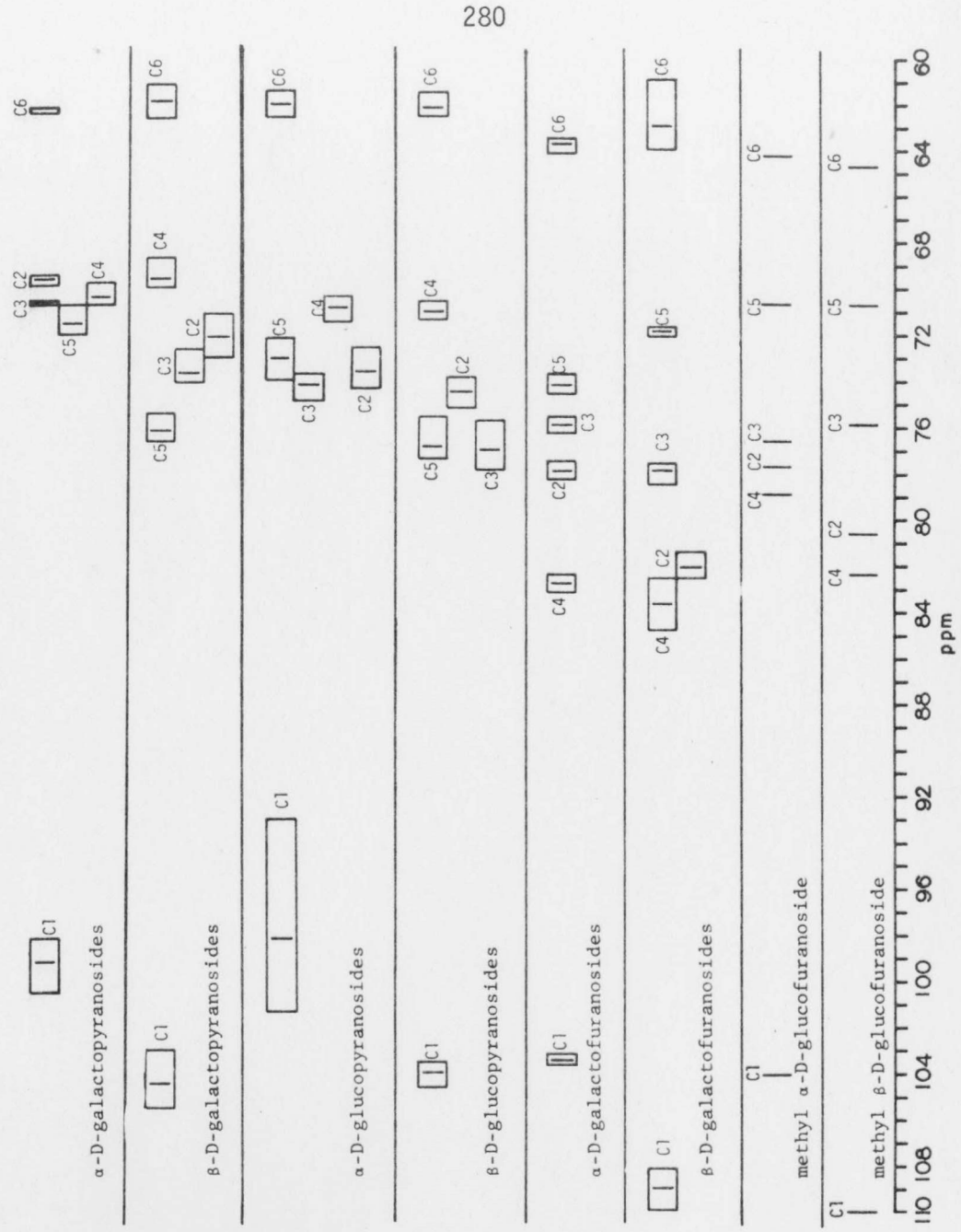


Figure 7. A plot of the CMR resonances or resonance intervals for all carbon atoms in the pyran and furan ring systems of the D-galactosides and D-glucosides.

of galactose or glucose, be able to determine its ring structure, and anomeric configuration.

Since the only carbohydrate found in helminthosporoside was galactose, and since no D-glucoside structure would fit the resonances, the resonances pertaining to carbohydrates in the CMR spectrum #2 were compared only to the D-galactoside structures (Figure 8). Spectrum #2 contains all the resonances observed in the carbohydrate region. The next line, spectrum #2 minus the resonances from the aglycone, contain all resonances from spectrum #2 minus the small resonances (which have intensities similar to the aglycone). By these comparisons, it seems reasonable that galactose exists in helminthosporoside in only the furan ring system. This in part, correlates with the methylation studies, that determined a terminal galactofuranosyl residue as one of the two residues. One can also conclude from this comparison that only the beta configuration is present.

To help make this more clear, since the combined resonances of a disaccharide can be complex, the tentative assignments of the carbons in the two galactose residues are presented in Figure 9. The assignments for the internal and terminal β-D-galactofuranosyl residues depend on the possible grouping of three resonances at two different possitions in the CMR spectrum. The resonance observed at 81.91 ppm, has the largest intensity of any resonance, and is broader than any single resonance. Therefore, one can easily suggest the possibility that this res-

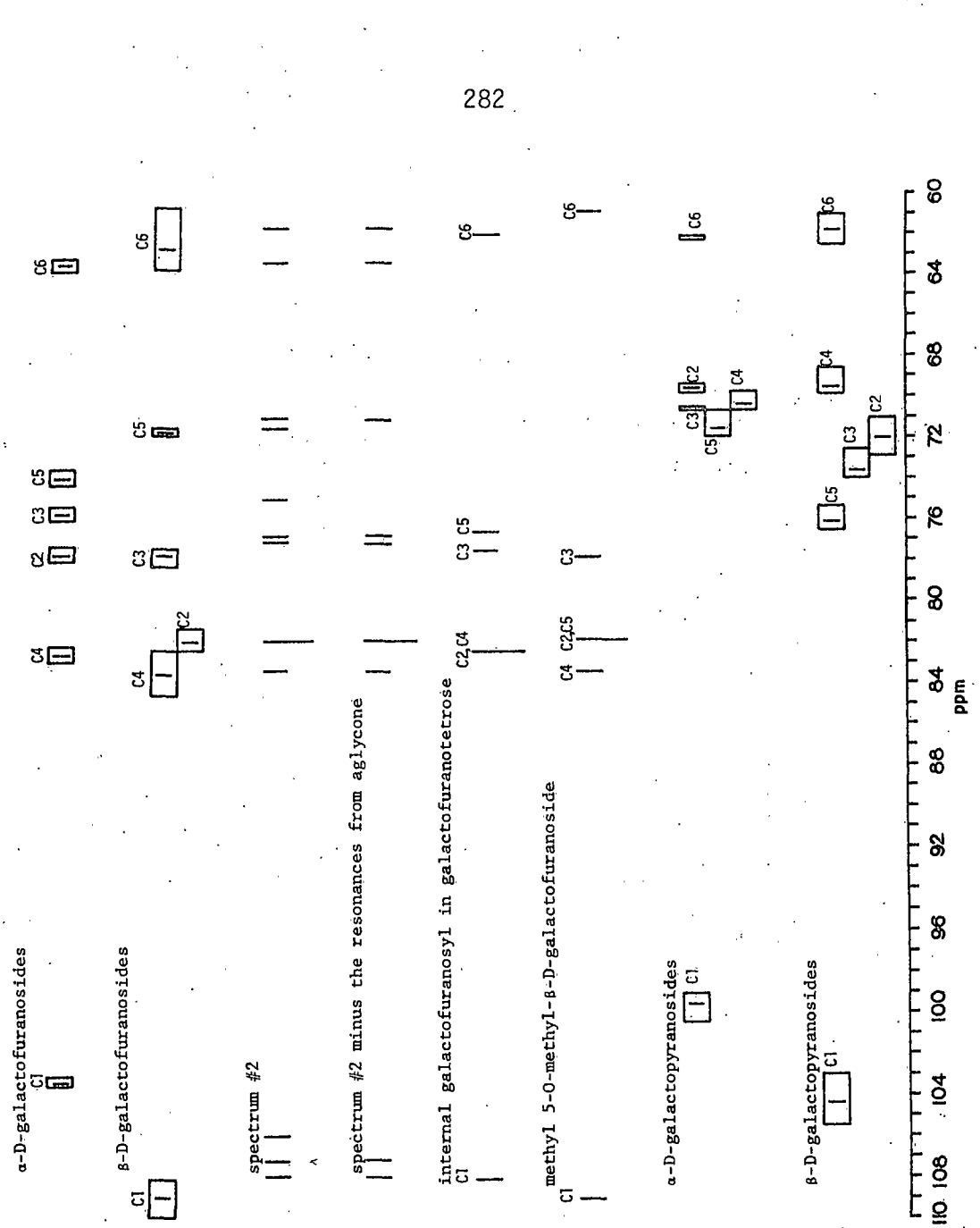


Figure 8. Comparison of the CMR resonances (spectrum #2) for helminthosporoside to the interval diagram for D-galactopyranosides and furanosides.

onance is a combination of C-2 and C-4 of the internal residue, and C-2 of the terminal residue. Also, three resonances must be assigned for the two observed resonances at 76.771 and 77.122 ppm. The intensity of the resonance at 77.122 is the second largest in the entire spectrum, and the combined peak at 76.771 and 77.122 ppm is very broad, more than would be expected for only two resonances. By comparison of observed resonances and by tentative assignment, the resonances for the two galactose residues fit only for that of β-D-galactofuranosyl residues, one being a terminal β-D-galactofuranosyl, and the other being a 5-linked β-D-galactofuranosyl residue.

However, there is a problem with the overall interpretation; that is, the signal intensities for the aglycone are much less than that for the carbohydrate signals. There is literature precedent for this phenomenon; for example, variations in segmental motion have been associated with differences in spin-lattice relaxation times (T_1) of the nuclei. In the "partially relaxed" FT spectrum of stachyose,[12a] the nuclei of the terminal galactose residue have longer T_1 values than do those of the internal galactose. By using the difference in T_1 values, an experiment was conducted with a τ value of 0.34 secs, with the result that one set of galactose resonances (terminal) was nulled. This was ascribed to the free rotation and greater mobility of the terminal galactose. In the CMR spectra of 1-octanol[12b] and 1-decanol[12c], there was a gradual increase in T_1 moving from the hydroxylated end to the CH_3 group end.

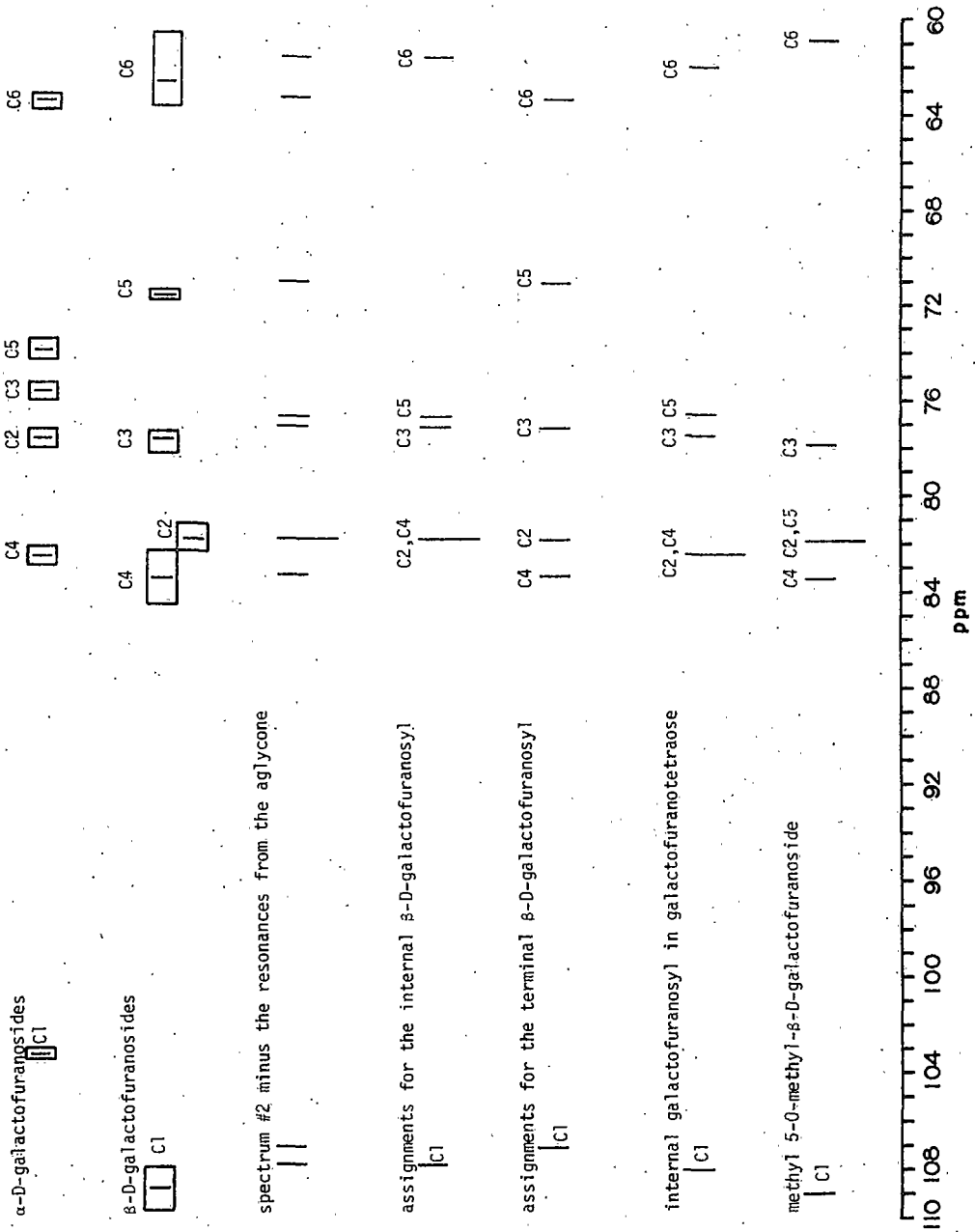


Figure 9. Tentative CMR assignments of the carbons in the two galactose residues in helminthosporoside.

This is indicative of slower movement of the hydroxylated end due to hydrogen bonding whereas the non-hydroxylated end would be free to move or "wiggle". This same effect would be expected in helminthosporoside, with deuterium oxide as solvent, hydrogen bonding to the hydroxyls of the carbohydrate moietys should cause these moietys to have restricted movement, leading to short (T_1)s. The very non-polar aglycone would be expected to have a lot of movement, leading to long (T_1)s.

SUMMARY

The ^{13}C-NMR method proposed here, for identification of gluco- and galactopyranosides and furanosides, and for assignment of anomeric configuration, appears to be a simple but useful method. The most practical area of use for this method would be that of natural products structure determination. This technique would also be useful for anomer verification in synthetic studies.

By comparing the CMR resonances of helminthosporoside to the interval diagrams, assignments can be made that allow one to suggest the presence of a terminal β-D-galactofuranosyl residue, and an internal 5-linked β-D-galactofuranosyl residue.

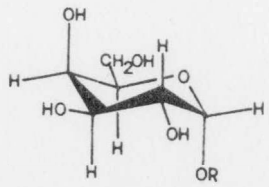
EXPERIMENTAL

All CMR spectra were obtained on an FX 100 FT NMR, by James S. Frye at the CSU Regional NMR Center, Department of Chemistry, Colorado State University, Fort Collins, CO 80523.

All data, were obtained with respect to DSS substituted. For comparison on the interval diagrams, all values were converted to external TMS.

APPENDIX A

CMR Data for α- and β-D-galactopyranosides

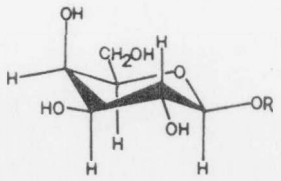


α-D-galactopyranosides

Compound	Ref.	C1	C2	C3	C4	C5	C6
methyl α-D-galactopy-ranoside	13	100.5	69.4	70.6	70.4	71.8	62.3
terminal galactose in raffinose	12	99.5	69.6[a]	70.6[b]	70.6	71.9	62.2
terminal galactose in stachyose	12	99.1	69.6[a]	70.7[b]	70.7	72.0	62.2
internal galactose in stachyose	12	99.4	69.8[a]	70.7[b]	69.8	70.7	67.5[c]
mean		99.6	69.6	70.6	70.4	71.6	62.2
s		0.6076	0.1633	0.0577	0.4031	0.6055	0.0577

[a]These numbers have been interchanged with those designated (b), since their reported assignments were similar to those of Perlin et al.[14]

[c]The downfield shift of this resonance is due to substitution.

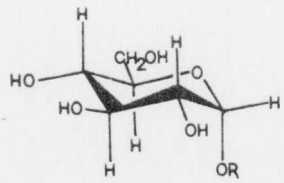


β-D-galactopyranosides

Compound	Ref.	C1	C2	C3	C4	C5	C6
methyl β-D-galactopyranoside	13	104.9	71.8	73.9	69.8	76.2	62.1
terminal galactose linked (1→4) to rhamnose	16	104.9	72.9	74.0	69.8	76.4	62.1
terminal galactose linked (1→3) to rhamnose	16	105.5	72.4	73.8	69.9	76.3	62.3
terminal galactose in lactose	17	104.0	72.2	73.9	69.8	76.3	62.1
terminal galactose in lactose	18	103.0	71.05	72.6	68.6	75.4	61.1
terminal galactose in methyl β-lactose	17	104.1	72.1	73.9	69.8	76.4	62.1
terminal galactose in methyl β-lactose	18	104.14	72.2	73.8	69.8	76.55	62.55
mean		104.4	72.1	73.7	69.6	76.2	62.1
s		0.8143	0.5689	0.4899	0.4614	0.3784	0.4518

APPENDIX B

CMR Data for α- and β-D-glucopyranosides

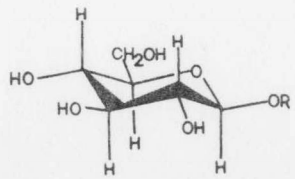


α-D-glucopyranosides

Compound	Ref.	C1	C2	C3	C4	C5	C6
methyl α-D-glucopyranoside	13	100.3	72.5	74.2	70.6	72.7	61.7
methyl α-D-glucopyranoside	2	100.1	72.5	74.2	70.7	72.3	61.7
methyl α-D-glucopyranoside	19	100.5	73.1	74.8	71.4	72.8	62.3
terminal glucose in α-maltose	17	100.9	73.8	73.8	70.7	73.0	61.9
terminal glucose in α-maltose	19	101.0	74.3	74.6	71.0	73.4	62.5
terminal glucose in β-maltose	19	101.0	74.3	74.6	71.0	73.4	62.5
terminal glucose in methyl β-maltoside	17	100.8	74.1	74.1	70.6	72.9	61.9
terminal glucose in methyl β-maltoside	19	101.1	74.3	74.6	70.9	73.4	62.3
glucose in sucrose	17	93.1	73.4	73.9	70.5	72.2	61.6
glucose in sucrose	12	93.0	73.3	73.7	70.3	72.1	61.3

Compound	Ref.	C1	C2	C3	C4	C5	C6
ring B glucose in stachyose	12	93.1	73.9	73.9	70.7	73.9	67.1[a]
ring B glucose in raffinose	12	93.0	73.9	73.9	70.6	73.9	67.2[a]
mean		98.2	73.6	74.2	70.8	73.0	61.97
s		3.7843	0.6562	0.3729	0.2876	0.6164	0.4111

[a]The downfield shift of this resonance is due to substitution.



β-D-glucopyranosides

Compound	Ref.	C1	C2	C3	C4	C5	C6
methyl β-D-glucopyranoside	13	104.3	74.2	76.9	70.8	76.9	61.9
methyl β-D-glucopyranoside	2	104.2	74.1	76.9	70.7	76.8	61.9
methyl β-D-glucopyranoside	19	104.5	74.6	77.3	71.2	77.3	62.4
terminal glucose in α-cellobiose	17	103.5	74.3	76.9	70.7	76.9	61.8
terminal glucose in α-cellobiose	19	103.9	74.7	77.2	71.1	77.2	62.4
terminal glucose in β-cellobiose	17	103.5	74.3	76.6	70.7	76.9	61.8
terminal glucose in β-cellobiose	19	103.9	74.7	77.2	71.1	77.2	62.4
terminal glucose in methyl β-cellobioside	17	103.7	74.3	77.0	70.7	76.9	61.9
terminal glucose in methyl β-cellobioside	19	103.9	74.6	77.5	71.2	77.2	62.4
internal glucose in methyl β-cellobioside	17	104.2	74.0	75.7	80.1[a]	75.5	61.4
internal glucose in methyl β-cellobioside	19	104.5	74.2	76.4	80.3[a]	75.9	61.8

Compound	Ref.	C1	C2	C3	C4	C5	C6
internal glucose in methyl β-maltoside	17	104.1	73.8	75.7	78.4[a]	77.3	61.7
internal glucose in methyl β-maltoside	19	104.4	74.6	77.8	78.7[a]	76.1	62.3
internal glucose in methyl β-lactose	17	104.1	73.9	75.8	79.8[a]	75.6	61.4
terminal glucose in α-laminaribiose	19	103.9	74.8	77.1	71.2	77.1	62.4
terminal glucose in β-laminaribiose	19	103.9	74.8	77.1	71.2	77.1	62.4
terminal glucose in α-gentiobiose	19	103.8	74.5	77.1	71.1	77.1	62.5
terminal glucose in β-gentiobiose	19	103.8	74.5	77.1	71.1	77.1	62.5
terminal glucose in methyl β-gentiobiose	19	104.0	74.0	77.2	71.0	77.2	62.5
internal glucose in α-gentiotriose	19	103.7	74.6	77.1	71.2	76.1	70.0[a]
terminal glucose in α-gentiotriose	19	103.7	74.6	77.1	71.0	77.1	62.4
internal glucose in β-gentiotriose	19	103.7	74.6	77.1	71.0	76.1	70.0[a]
terminal glucose in β-gentiotriose	19	103.7	74.6	77.1	71.0	77.1	62.4
terminal glucose (1→3) to rhamnose	16	105.0	74.7	76.9	70.8	76.9	61.9
terminal glucose (1→4) to rhamnose	16	104.4	75.1	77.2	70.8	77.0	61.9

Compound	Ref.	C1	C2	C3	C4	C5	C6
terminal glucose (1→2) to rhamnose	16	104.6	74.5	77.0	70.5	76.7	61.7
mean		104.0	74.4	76.9	71.0	76.8	62.1
s		0.3698	0.3140	0.5078	0.2158	0.5359	0.3615

[a]The downfield shift of the resonance is due to substitution.

APPENDIX C

CMR Data for Synthesized α- and β-D-galactopyranosides

Compound	Stand.	C1	C2	C3	C4	C5	C6
methyl α-D-galactopyranoside	A[a]	101.7	70.6	71.9	71.6	73.1	63.6
	B[b]	100.3	69.2	70.5	70.2	71.7	62.2
allyl α-D-galactopyranoside	A	99.9	70.6	71.9	71.7	73.4	63.7
	B	98.5	69.2	70.6	70.3	72.0	62.3
(2,3-propanediol-1-O) α-D-galactopyranoside	A	101.3	71.0	71.8[c]	71.8[c]	73.0	63.8
	B	99.9	69.6	70.4[c]	70.4[c]	71.6	62.4
(2-propanol-1-O) α-D-galactopyranoside	A	101.1[d] 100.6	70.9	71.9	71.7	73.3	63.4
	B	99.8[d] 99.2	69.6	70.6	70.4	72.0	62.3
methyl β-D-galactopyranoside	B	104.9	71.8	73.9	69.8	76.2	62.1

[a]Reference standard is DSS substituted.

[b]Reference standard is external TMS.

[c]These signals were not resolved.

[d]These values resulted from diasteriomers, at C-2 of the aglycone.

APPENDIX D

CMR Data for Synthesized α- and β-D-glucopyranosides

Compound	Stand.	C1	C2	C3	C4	C5	C6
methyl α-D-glucopyranoside (sigma)	A[a]	101.7	73.7	75.6	72.0	74.0	63.2
	B[b]	100.3	72.4	74.2	70.6	72.7	61.8
(1-propanol-2-0) α-D-glucopyranoside	A	100.6[c] 98.0	78.2[cd] 75.5	75.5	72.1	74.4	63.0
	B	99.3[c] 96.6	76.8[cd] 74.1	74.1	70.7	73.0	61.7
methyl β-D-glucopyranoside (sigma)	A	106.2	76.1	78.84	72.7	78.75	63.8
	B	104.8	74.7	77.5	71.3	77.4	62.4

[a]Reference standard is DSS substituted.

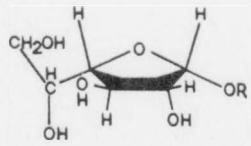
[b]Reference standard is external TMS.

[c]These values resulted from the diasteromers at C-2 of the aglycone.

[d]These values could not be unambiguously assigned by comparison of intensities, since one signal coincided with the resonance for C-3.

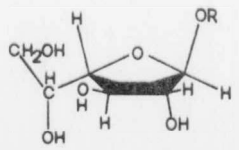
APPENDIX E

CMR Data for α- and β-D-galactofuranosides



α-D-galactofuranosides

Compound	Ref.	C1	C2	C3	C4	C5	C6
methyl α-D-galacto-furanoside	20	103.1	77.4	75.5	82.3	73.7	63.4
methyl α-D-galacto-furanoside	21	103.8	78.2	76.2	83.1	74.5	64.1
mean		103.4	77.8	75.8	82.7	74.1	63.7



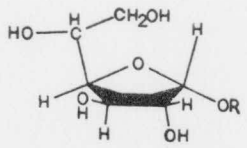
β-D-galactofuranosides

Compound	Ref.	C1	C2	C3	C4	C5	C6
methyl β-D-galacto-furanoside	20	109.2	81.9	77.8	84.0	72.0	63.9
methyl β-D-galacto-furanoside	21	109.9	81.3	78.4	84.7	71.7	63.6
terminal residue in galactofuranotetraose	22	108.2	82.4	77.5	83.7	71.6	63.8
internal residue in galactofuranotetraose	22	108.0	82.4	77.5	82.4	76.6[a]	62.1
methyl 5-methyl-β-D-galactofuranoside	22	109.0	81.8	77.8	83.4	81.8[a]	60.9
mean		108.9	82.0	77.8	83.6	71.8	62.9
s		0.7733	0.4615	0.3674	0.8444	0.2082	1.3160

[a]The downfield shift of the resonance is due to substitution.

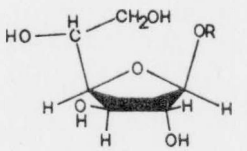
APPENDIX F

CMR Data for α- and β-D-glucofuranosides



α-D-glucofuranosides

Compound	Ref.	C1	C2	C3	C4	C5	C6
methyl α-D-gluco-furanoside	21	104.0	77.7	76.6	78.8	70.7	64.2



β-D-glucofuranosides

Compound	Ref.	C1	C2	C3	C4	C5	C6
methyl β-D-gluco-furanoside	21	110.0	80.6	75.8	82.3	70.7	64.7

REFERENCES AND NOTES

1. G. C. Levy and G. L. Nelson, *Carbon-13 Nuclear Magnetic Resonance for Organic Chemists*, Wiley-Interscience, New York (1972).
2. J. B. Stothers, *Carbon-13 NMR Spectroscopy*, Academic Press, New York (1972).
3. E. Wehrli and T. Wirthlin, *Interpretation of Carbon-13 NMR Spectra*, Heyden and Son, London (1976).
4. R. U. Lemieux and S. Koto, *Tetrahedron* 30, 1933-44 (1974).
5. H. A. Nunez, T. E. Walker, R. Fuentes, J. O'Connor, A. Serianni and R. Barker, *J. Supramolecular Structure* 6, 535-50 (1977).
6. A. S. Perlin, *Organic Chemistry, Series 2*, Butterworths, London (1975-76) pp. 1-34.
7. S. N. Rosenthal and J. H. Fendler, *Advan. Phys. Org. Chem.*, edited by V. Gold and D. Bethell, Academic Press, New York (1976) pp. 280-424.
8. D. R. Bundle and R. U. Lemieux, *Methods in Carbohydrate Chemistry VII*, Academic Press, New York (1976) pp. 79-86.
9. J. A. Schwarcz, N. Cyr and A. S. Perlin, *Can. J. Chem.* 53, 1872-5 (1975).
10. K. Bock and C. Pedersen, *Acta Chem. Scand.* B31, 354-8 (1977).
11. C. Laffite, A. M. N. PhuocDu, F. Winternitz, R. Wylde and F. Pratviel-Sosa, *Carbohyd. Res.* 67, 105-15 (1978).

12. a. A. Allerhand and D. Doddrell, *J. Amer. Chem. Soc.* 93, 2777 (1971).

b. D. M. Doddrell, *Pure and Appl. Chem.* 49, Pergamon Press, Great Britain (1977) pp. 1385-1401.

c. D. M. Doddrell and A. Allerhand, *J. Amer. Chem. Soc.* 93, 1558 (1976).

13. P. A. J. Gorin, *Can. J. Chem.* 52, 458 (1974).

14. A. S. Perlin, B. Casu and H. J. Koch, *Can. J. Chem.* 48, 2596 (1970).

15. E. Breitmaier, W. Voelter, G. Jung and C. Tanzer, *Chem. Ber.* 104, 1147 (1971).

16. P. Colson and R. R. King, *Carbohyd. Res.* 47, 1-13 (1976).

17. D. E. Dorman and J. D. Roberts, *J. Amer. Chem. Soc.* 93, 4463 (1971).

18. W. Voelter, U. Bilik and E. Breitmaier, *Coll. Czech. Chem. Comm.* 38, 2054 (1973).

19. T. Usui, N. Yamaoka, K. Matsuda, K. Tuzimura, H. Sugiyama and S. Seto, *J. Chem. Soc., Perkin I*, 2425 (1973).

20. P. A. J. Gorin and M. Mazurek, *Can. J. Chem.* 53, 1212-23 (1975).

21. R. G. S. Ritchie, N. Cyr, B. Korsch, H. J. Koch and A. S. Perlin, *Can. J. Chem.* 53, 1424-33 (1975).

22. P. A. J. Gorin and M. Mazurek, *Carbohyd. Res.* 48, 171-86 (1976).

SECTION 4

THE DESIGN OF PREVIOUSLY UNAVAILABLE LABORATORY APPARATUS

LIST OF TABLES FOR SECTION 4

TABLE		PAGE
1	Elution volumes and their data reduction from the column separation of a corn syrup hydrolysate	329

LIST OF FIGURES FOR SECTION 4

FIGURE		PAGE
1	A drawing of the exterior of the IR Pellet Transporter .	308
2	Top and bottom views for the Top Unit of the IR Pellet Transporter.	309
3	Side views for the Top Unit of the IR Pellet Transporter	310
4	Top and bottom views for the Bottom Unit of the IR Pellet transporter	311
5	Side views for the Bottom Unit of the IR Pellet Transporter	312
6	Legend and dimensions of the Disc-Gel Band Enhancement Apparatus.	314
7	A drawing of the exterior of the DG-BEA.	315
8	A drawing of the interior and back of the DG-BEA	316
9	A drawing of the Parr Bomb Micro Sample Holder	318
10	A drawing of the Micro-Hydrogenation Apparatus	319
11	A photo of the 295.5 x 0.8 cm, Bio-gel P-2 (-400 mesh) chromatography column.	321
12	A drawing of the column, depicting the distribution of the water jackets.	322
13	Column separation at a temperature of 44°C, and a flow rate of 1 drop/6 secs.	323

FIGURE		PAGE
14	Column separation at a temperature of 44°C, and a flow rate of 1 drop/13 secs.	325
15	Column separation at a temperature of 64°C, and a flow rate of 1 drop/13 secs.	326
16	Column separation of carbohydrates, monitored at 192 nm.	327
17	Separation of the glucosyl α(1→4) oligosaccharides in corn syrup	328
18	Plot of Ve/Vo vs. log(molecular weight) for the oligosaccharides in corn syrup.	330

INTRODUCTION

During the investigative process, described in Sections 1 and 2, and through efforts in other unrelated projects, various kinds of non-standard laboratory apparatus were required. This Section describes, in some detail, the different pieces of apparatus that had been constructed during the course of this study.

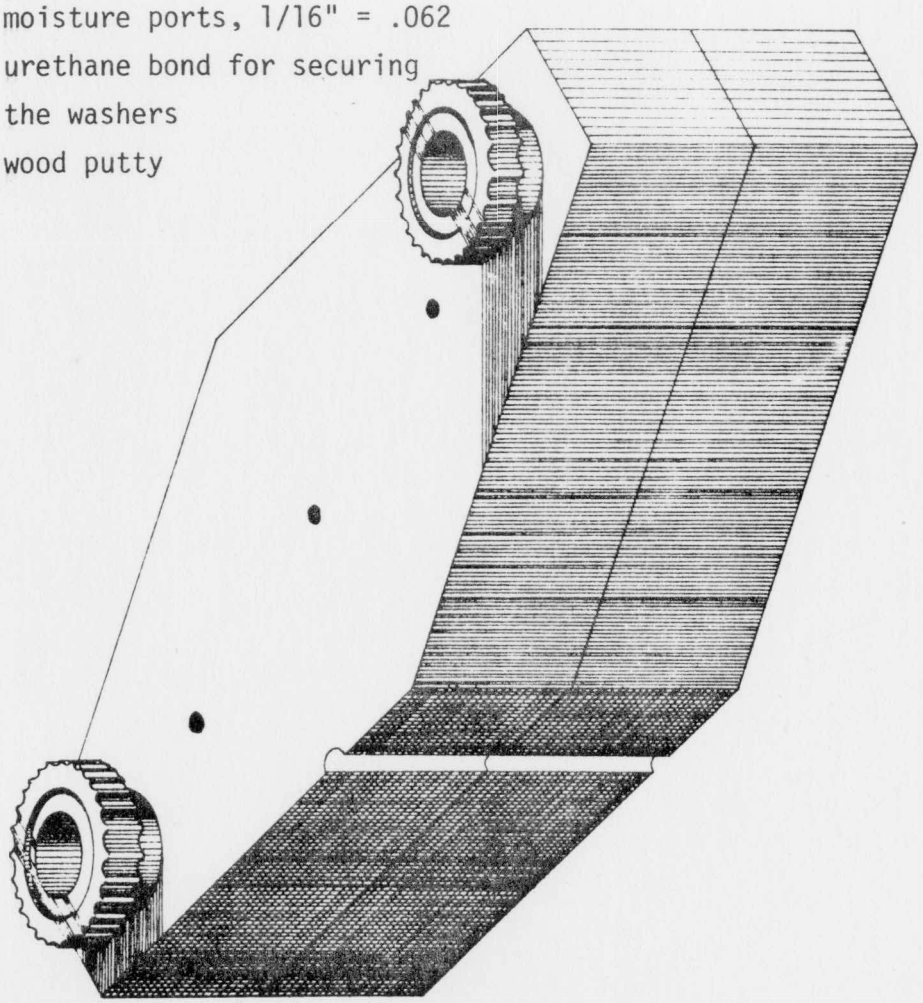
RESULTS AND DISCUSSION

Since we often dealt with small amounts of natural products, it became necessary to obtain IR spectra of KBr pellets containing not more than 100 μg of a particular compound. To obtain high resolution spectra, the pellets were shipped through the mail, and run on an FT IR spectrometer. To be certain that the pellet would arrive at its destination in one piece, the IR Pellet Transporter was built. A drawing of the exterior is presented in Figure 1. Figures 2 and 3 show the drawings of the top and bottom and side views for the Top Unit of the IR Pellet Transporter. Figures 4 and 5 show the drawings of the top and bottom, and side views for the Bottom Unit.

The pellets were pressed into small paper discs, (obtained from Varian), 1.3 cm in diameter, which have a rectangular hole in the center. After the pellet was mounted in the IR Pellet Transporter, the Transporter was placed in a box (containing a desiccant), and surrounded by foam. During the shipping process, no pellets were broken.

LEGEND: *Scale: 1.5" = 1"*

1. 5/16" x 1" brass machine screws
2. 3/8" brass washers
3. groove-optional
4. moisture ports, 1/16" = .062
5. urethane bond for securing the washers
6. wood putty



I.R. Pellet Transporter / designed by Ross Beier drawn by Mitchel Thompson

Figure 1. A drawing of the exterior of the IR Pellet Transporter.

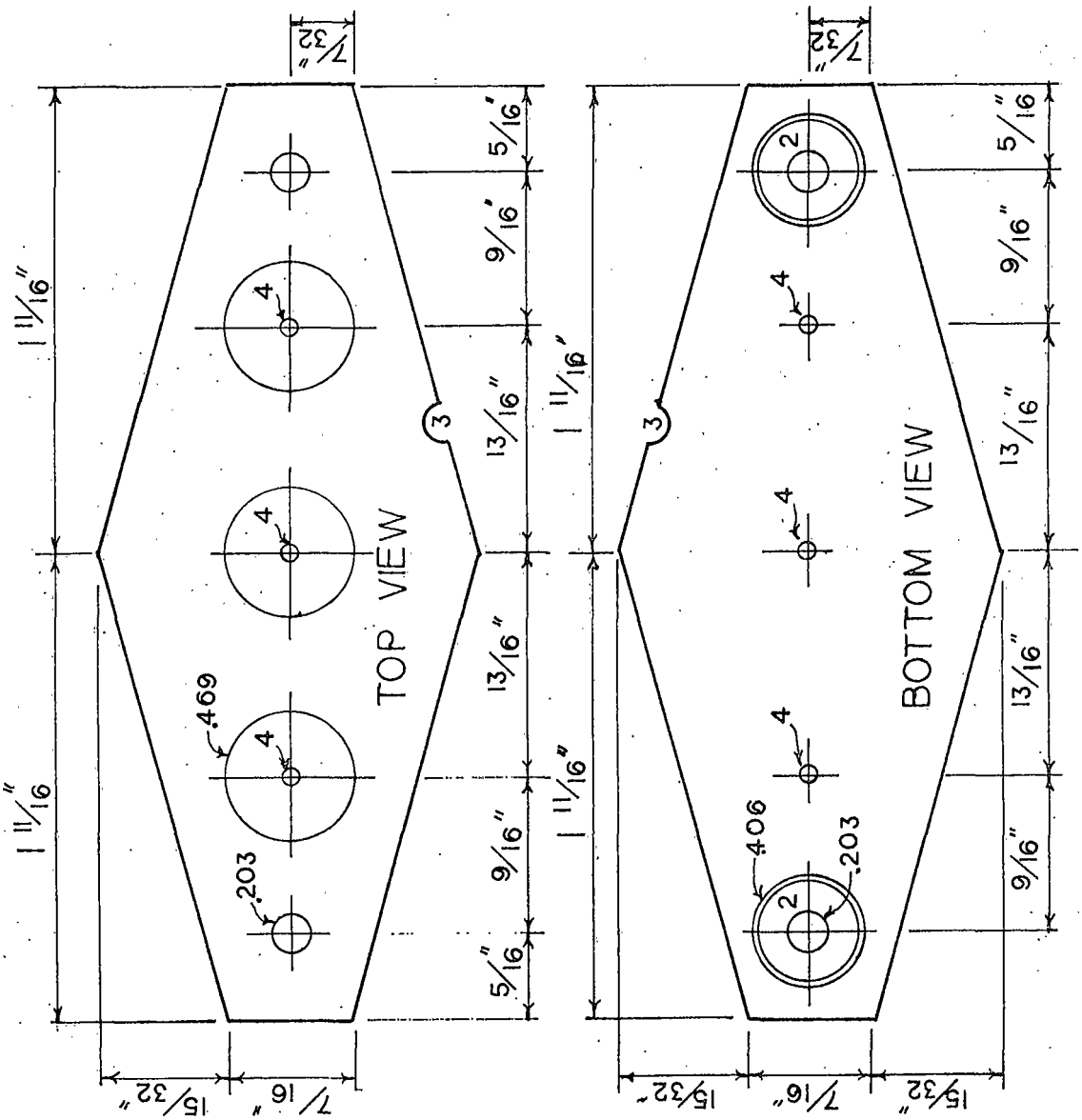


Figure 2. Top and bottom views for the Top Unit of the IR Pellet Transporter.

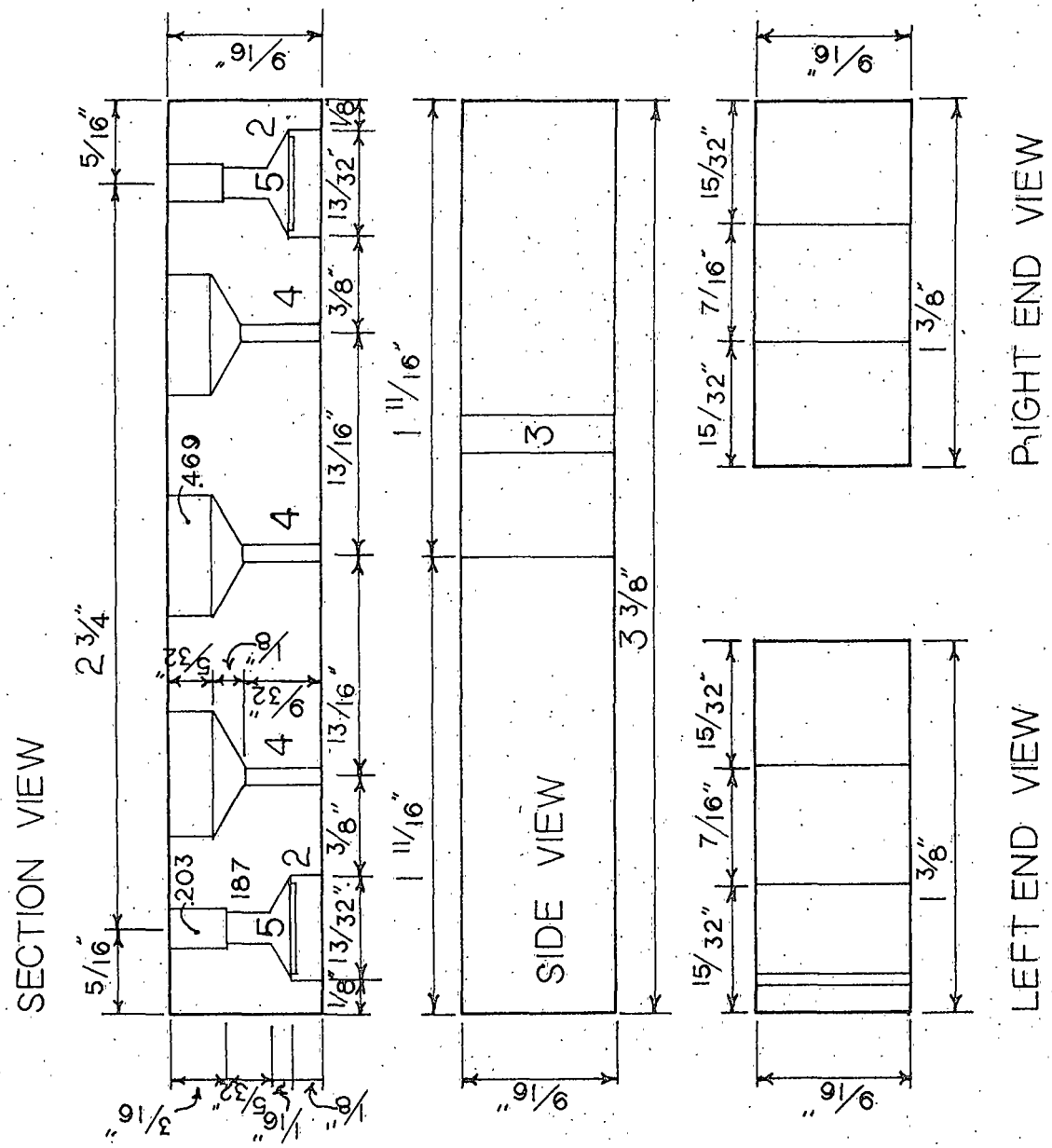


Figure 3. Side views for the Top Unit of the IR Pellet Transporter.

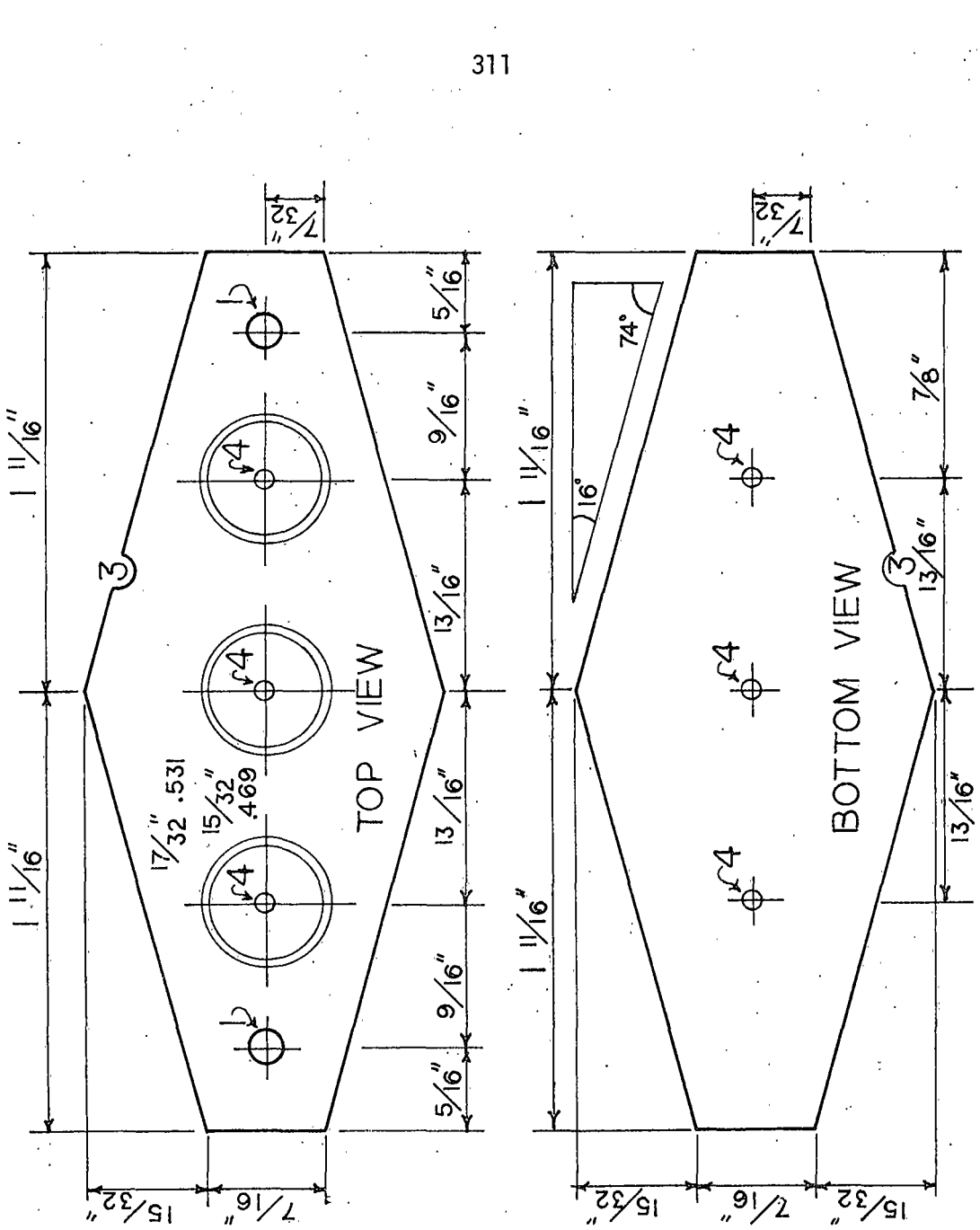


Figure 4. Top and bottom views for the Bottom Unit of the IR Pellet Transporter.

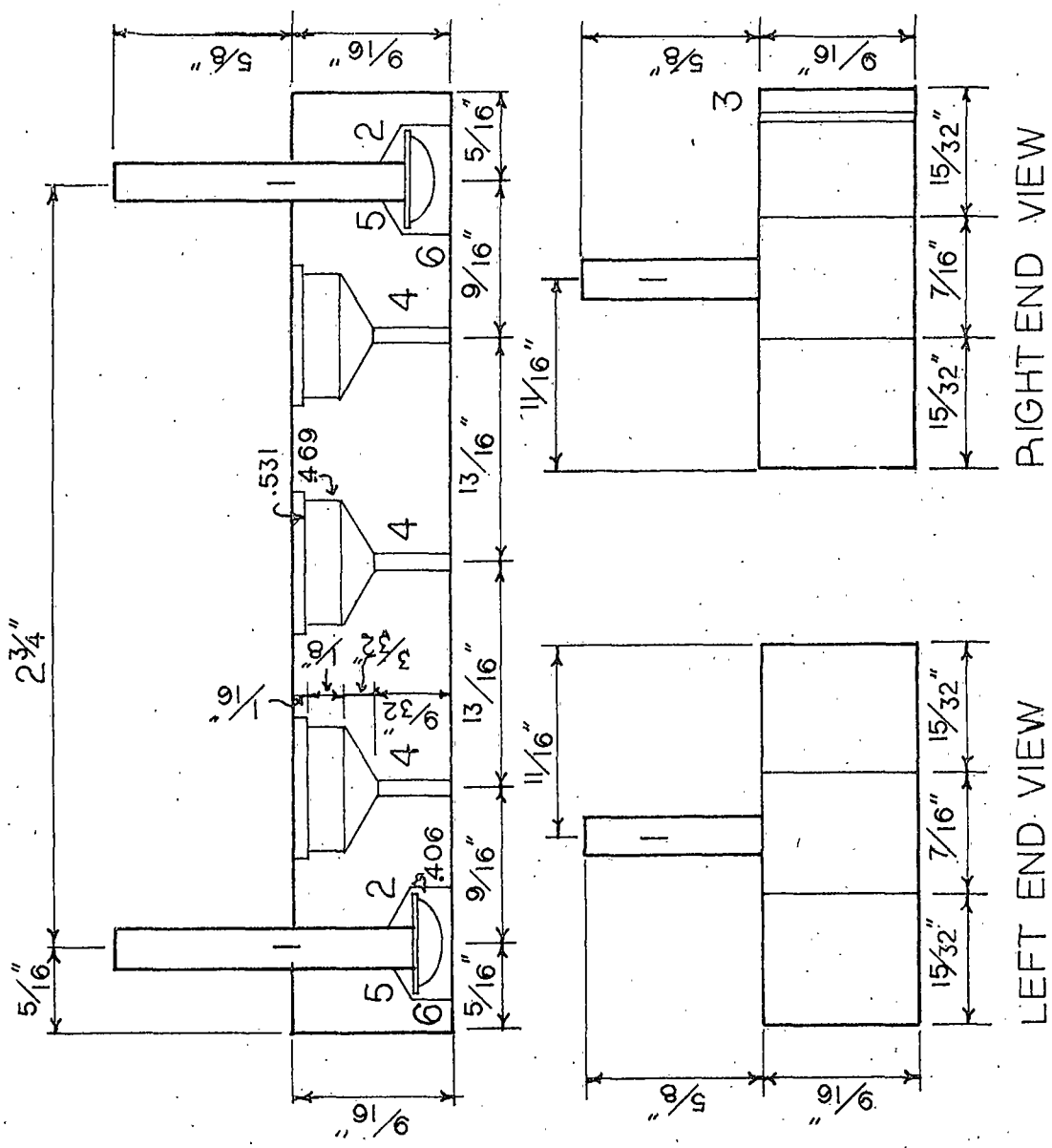


Figure 5. Side views for the Bottom Unit of the IR Pellet Transporter.

The Disc-Gel Band Enhancement Apparatus (DG-BEA), was built to aid in the visualization and photography of bands within disc-gels. The legend and dimensions of the apparatus are presented in Figure 6. Figure 7 shows a drawing of the exterior of the DG-BEA, and Figure 8 shows a drawing of the interior and back of the apparatus. One can observe or photograph 21 separate disc-gels at a time. The window of the box was made of transleucent plexiglass, with transparent rails (for holding tubes) above and below the window. A shutter assembly was included, that would slide in front of the window, through tracks provided in the rails. This shutter would stop the extraneous light between tubes from washing-out the small bands. Solid shutters made of colored plastics could be employed, to aid in photographing particular colored bands.

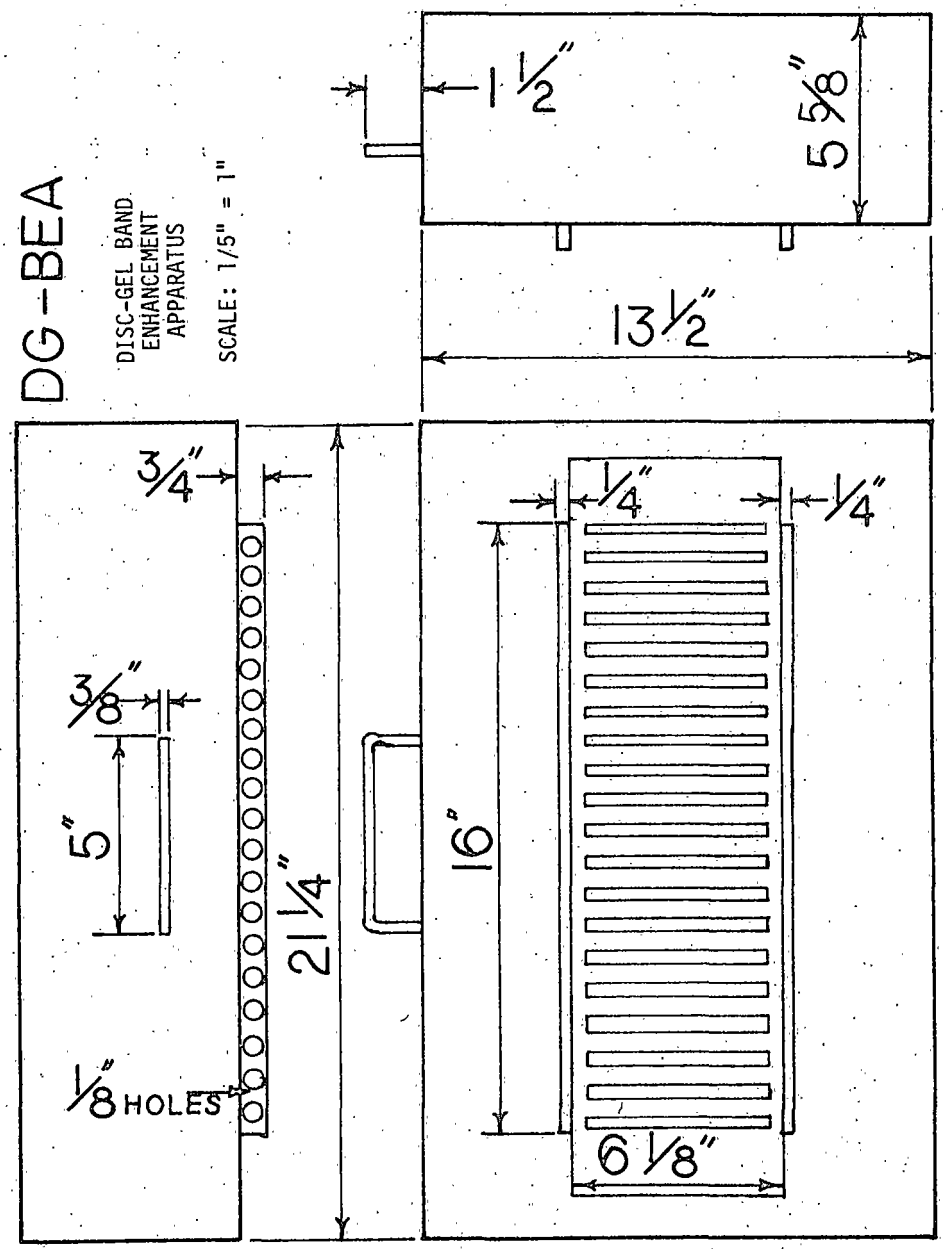


Figure 6. Legend and dimensions of the Disc-Gel Band Enhancement Apparatus.

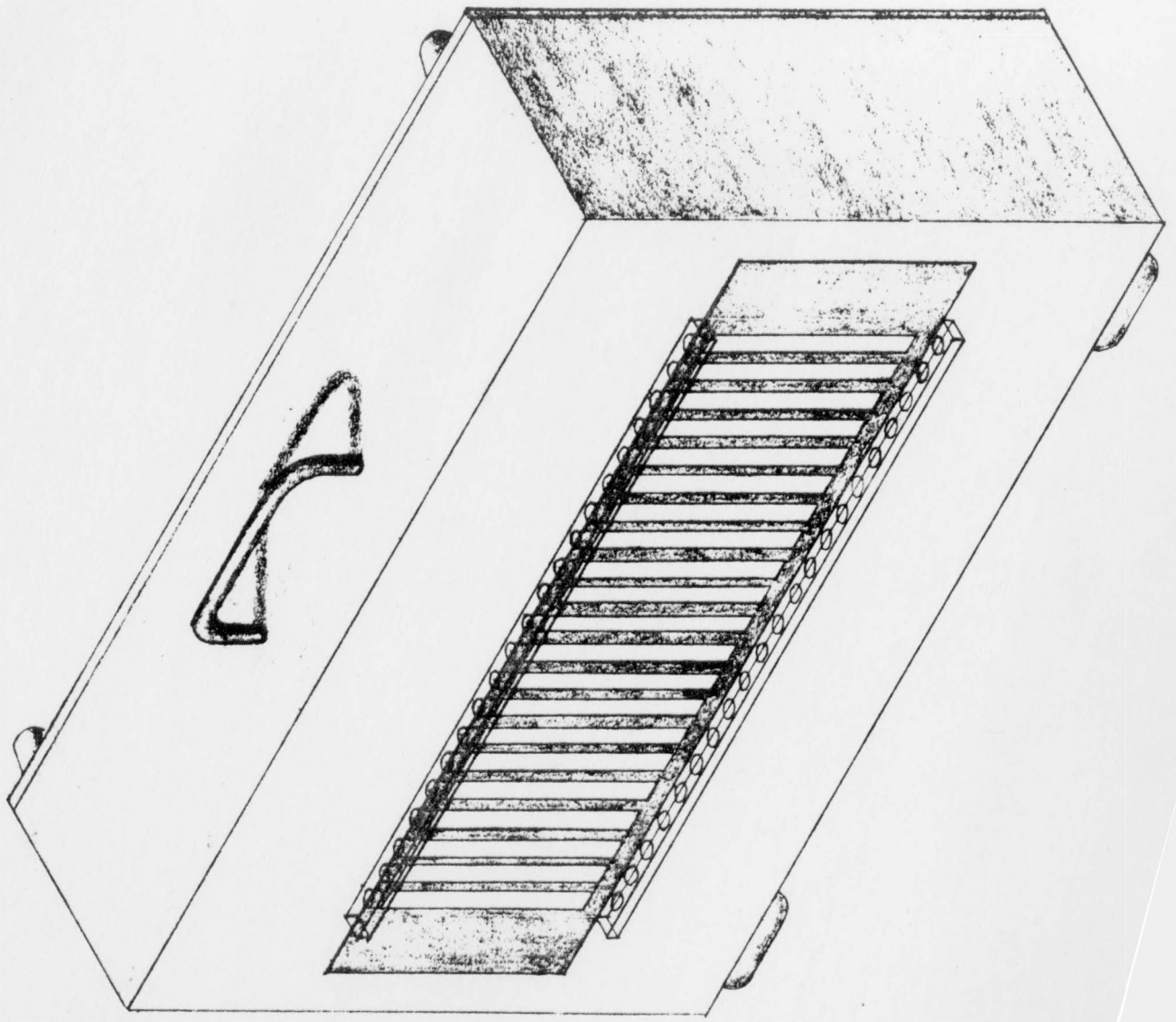


Figure 7. A drawing of the exterior of the DG-BEA.

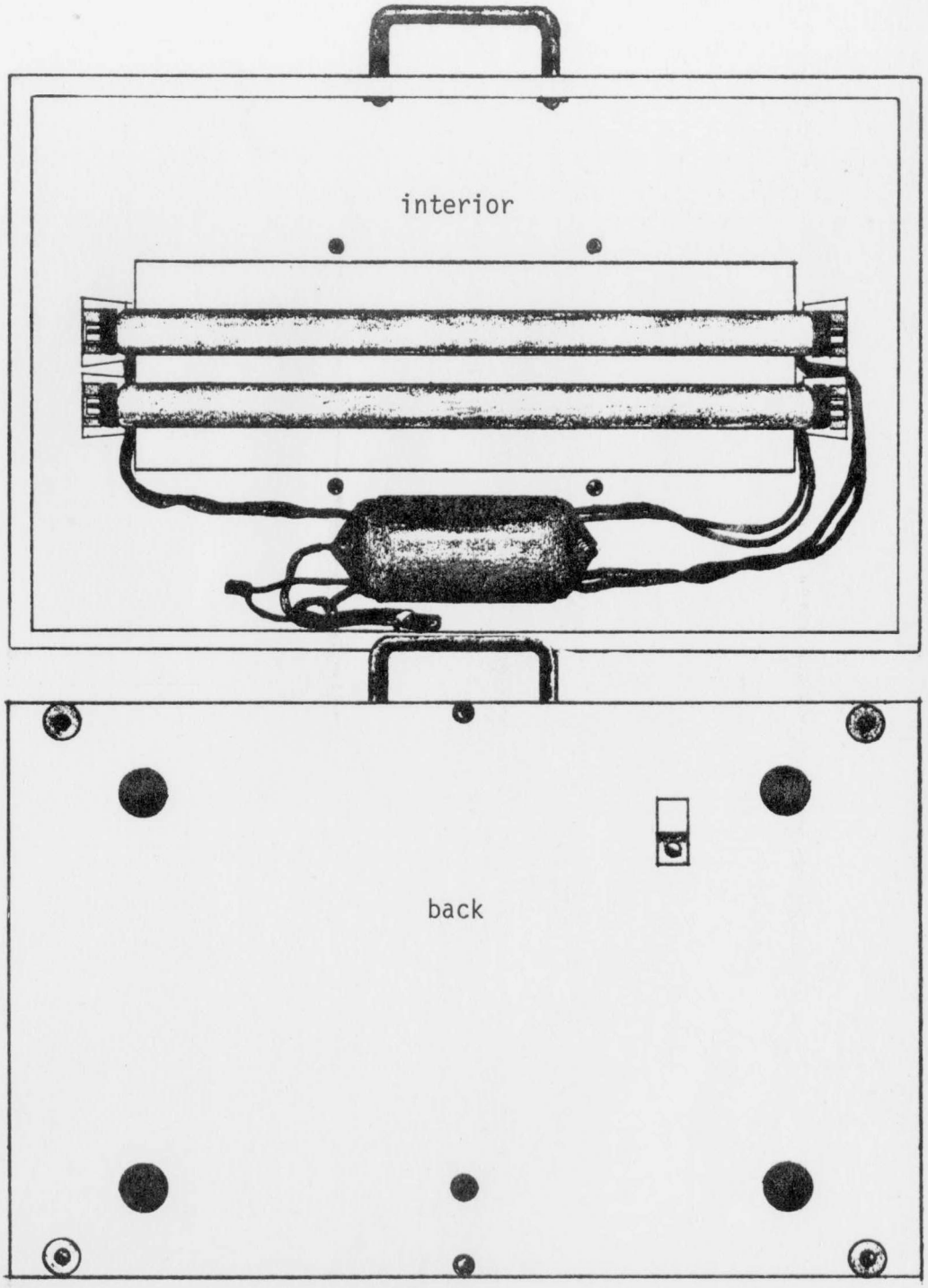


Figure 8. A drawing of the interior and back of the DG-BEA.

Because of problems we encountered during an attempted hydrogenolysis reaction of (3-oxetanyl) 2,3,4,6-tetra-O-benzyl-β-D-galactopyranoside, (29), (Section 1), it was decided to attempt a high pressure (1000 psi) hydrogenolysis. This reaction would have to be under taken in a Parr Bomb. Since our sample size was only about 5 ml total, and the interior of the Parr Bomb was grossly coated with tar and debris, a reaction vessel and holder was required. Figure 9 shows a drawing of the Parr Bomb Micro Sample Holder. The glass reaction vessel was blown from a glass tube (I. D. = 19 mm). The supporting holder was made from kiln dried wood. The cut-outs marked A, are drawings of the top and bottom surfaces of the base plate. The cut-outs marked B, are drawings of the top and bottom surfaces of the top plate. The two dowels, marked C and D, pass through both plates, holding the apparatus together. The dowel marked C, is stationary, and was used only as a support. But the dowel marked D, is free to rotate, and holds the sample vessel in position.

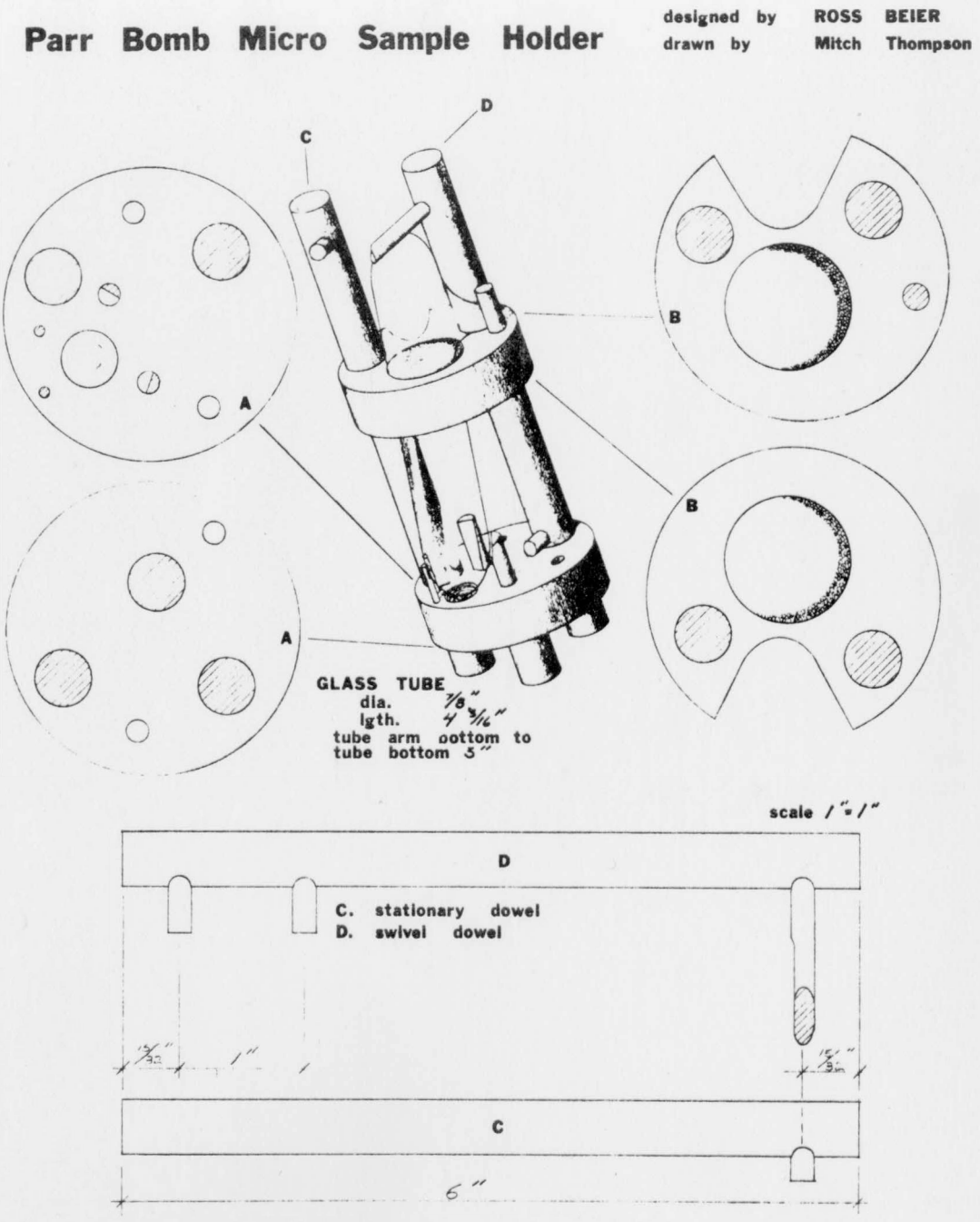


Figure 9. A drawing of the Parr Bomb Micro Sample Holder.

When dealing with small quantities of reaction products that required subsequent hydrogenolysis, to be able to observe a change in hydrogen pressure, a small hydrogenation apparatus was required. A drawing of the Micro Hydrogenation Apparatus is depicted in Figure 10. Letter A represents a thick-walled rubber tube, B is a tube of polycarbonate used as a shield, C is the reaction vessel, and D represents a magnetic stirrer. The reaction vessel was made from a thick-walled connecting tube. A flat bottom was blown, to facilitate the use of a 9 mm stir bar.

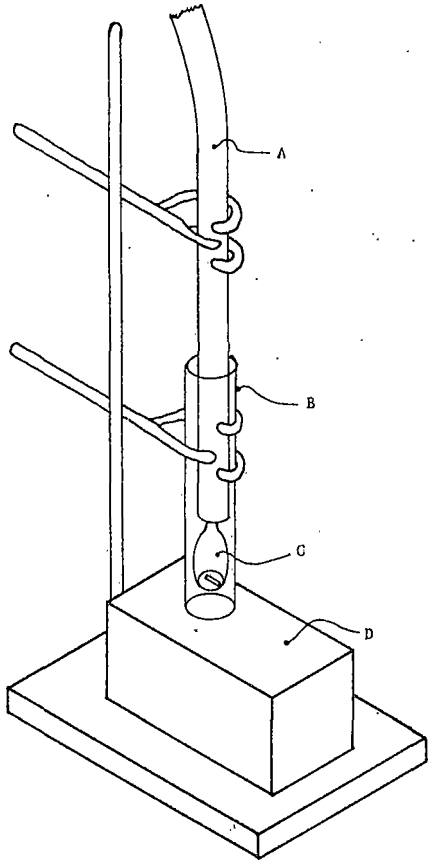


Figure 10. A drawing of the Micro Hydrogenation Apparatus.

It was considered that a good analytical Bio-gel column could be of use during the isolation of helminthosporoside and various rhynchosporosides. Therefore, a 295.5 x 0.8 cm, Bio-gel P-2 (-400 mesh) column was constructed. A photograph of this column, mounted in working condition, is shown in Figure 11. This column was mounted on a kiln dried wooden frame, so that it could be released from verticle and transported on the frame. Figure 12 is a drawing of the column, showing the distribution of the water jackets. With the water jackets placed in this manner, one can achieve a column run at one temperature, or with a temperature gradient. Each jacket had a separate line, which came from a 6-way stream splitter, and each line had a clamp to regulate the flow of water. The water was held at a particular temperature by using a circulating water bath.

The water used to elute the column, was kept in a 3-necked, 1-liter flask, and heated with a heating mantel to the temperature of the column. The water was pumped vertically through the column by a mini pump, and prior to entering the column, the water passed through a millipore 0.22 μ filter.

To check the separation capabilities of the column, a solution containing Dextran T40, maltotriose, cellobiose, and glucose was passed over the column (Figure 13). This separation occured at a temperature of 44°C, and a flow rate of 1 drop/6 secs. If the flow rate was decreased to 1 drop/13 secs, the separation improved, as shown in Figure 14.

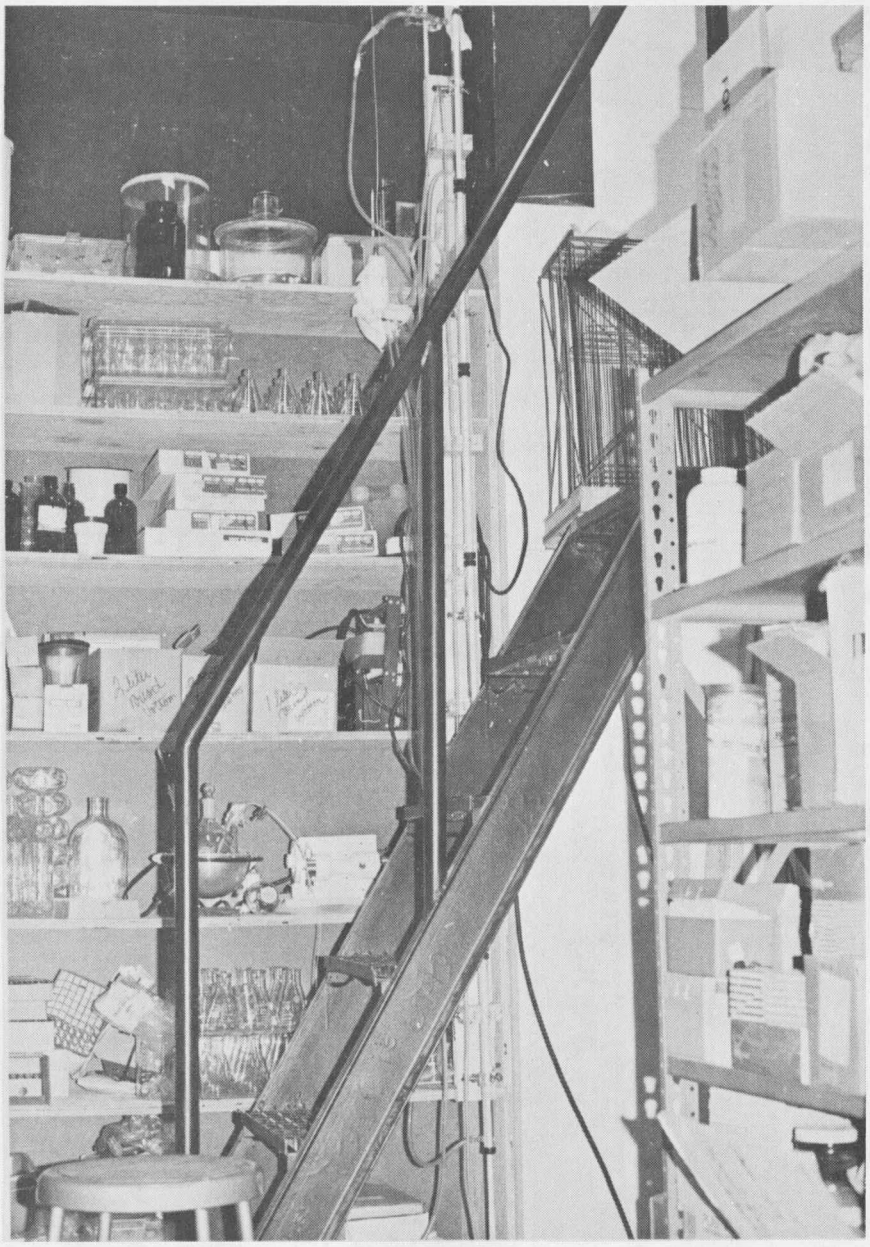


Figure 11. A photo of the 295.5 x 0.8 cm, Bio-gel P-2 (-400 mesh) chromatography column.

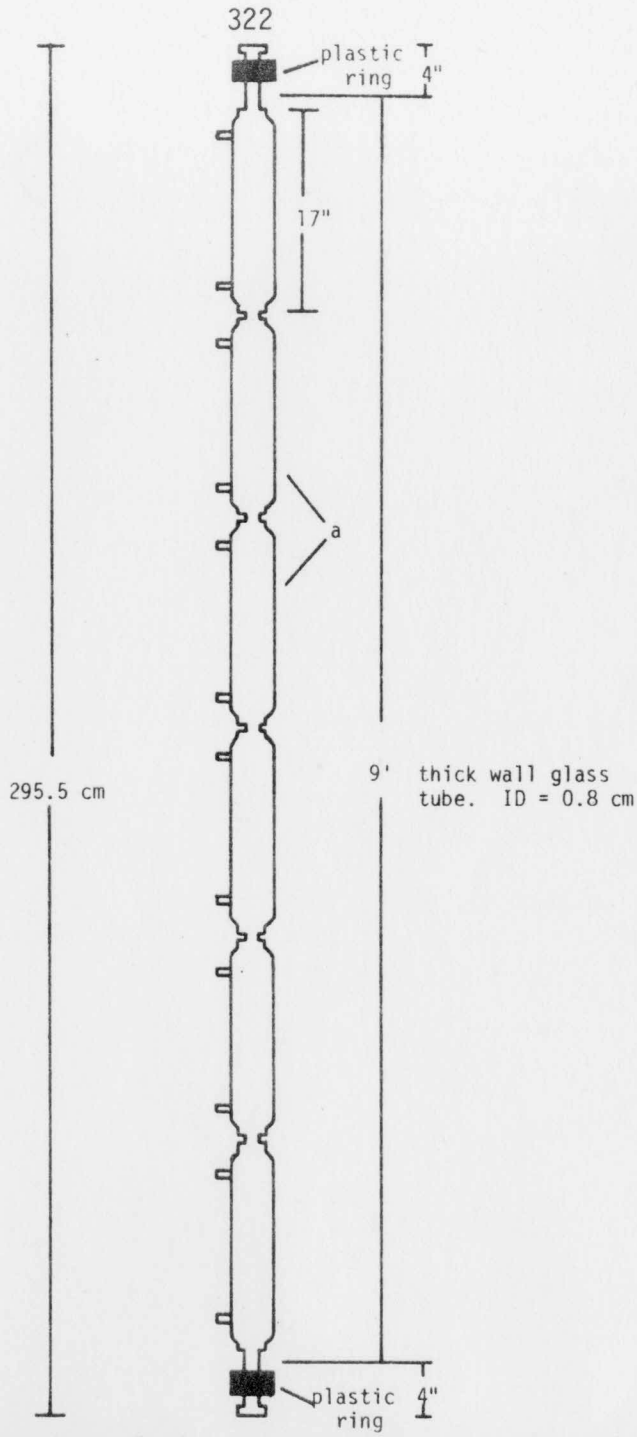


Figure 12. A drawing of the column, depicting the distribution of the water jackets.

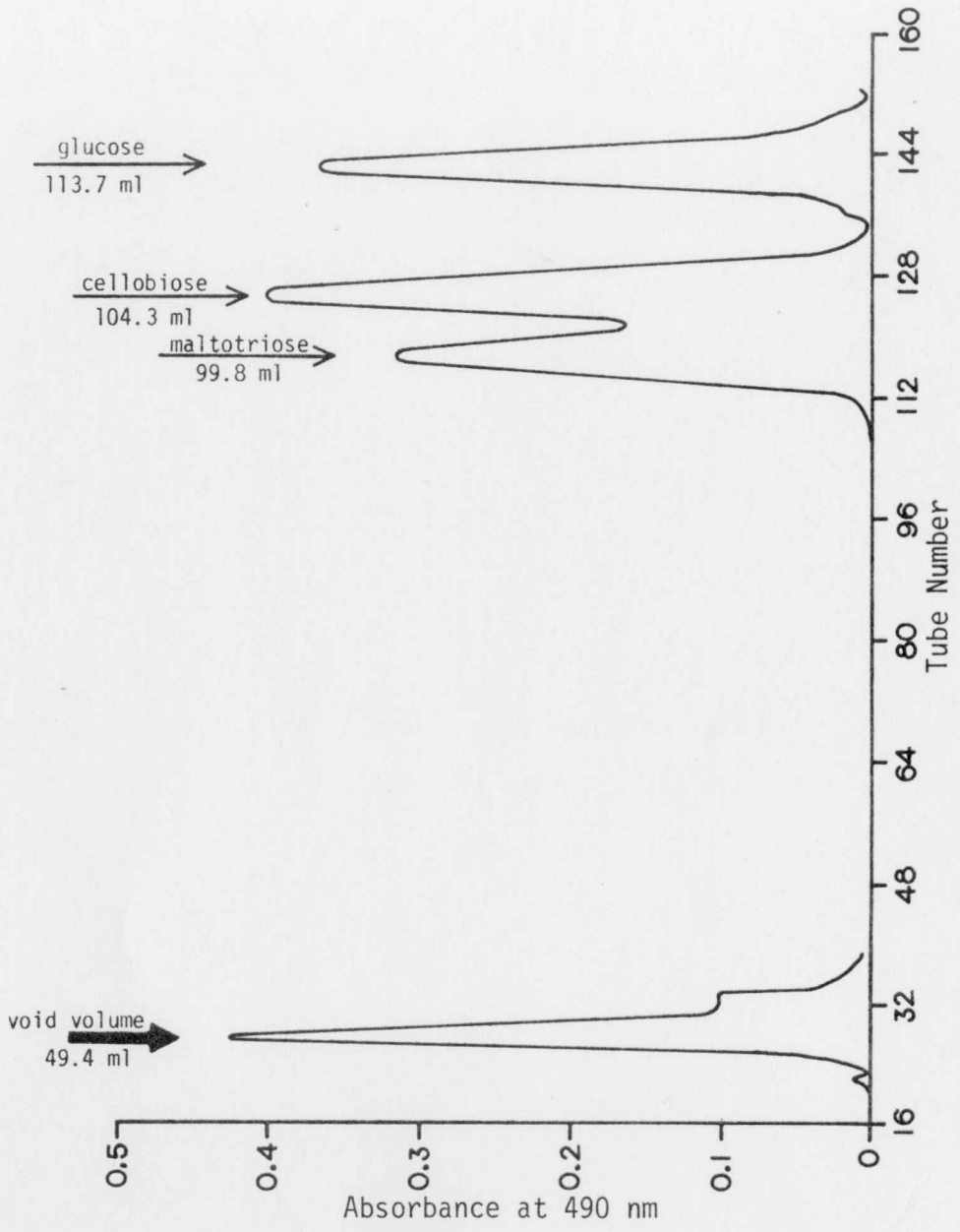


Figure 13. Column separation at a temperature of 44°C, and a flow rate of 1 drop/6 secs.

When the temperature was increased to 64°C, the separation continued to improve, as shown in Figure 15. For comparison, a column separation on a 1 meter, Bio-gel P-2 (200-400 mesh) column, gives a single broad peak for maltotriose and cellobiose.

Because we wanted to use UV detection during separation of the toxins, a UV detector having a 40 µl flow-cell was used. A number of runs were made using this dectector, allowing us to become familiar with the system. Figure 16 depicts a column run of the standard solution, monitored at 192 nm. There was approximately 1 mg of each compound in the standard solution.

The series of glucosyl $\alpha(1\rightarrow 4)$ oligosaccharides were of interest to separate on the column, for comparison with carbohydrates isolated from fungal cultures. A corn syrup hydrolysate was obtained,[1] and separated on the column (Figure 17). In the Figure, the G's represent glucose and the subscript represents the number of residues in the oligomer. The plot of Ve/Vo vs. log(molecular weight) for this column run is delineated in Figure 18. It is interesting that carbohydrates having up to 15 subunits are observed.

There have been two reports discussing separation of homologous glucose oligomers with polyacrylamide gels.[2,3] In both cases, the largest observed oligomer on a Bio-gel P-2 (-400 mesh) column was G_{11}. Only when a Bio-gel P-4 column was used, did G_{15} appear.[3] Separation of these oligomers, was also attempted by high-pressure liquid chromatography

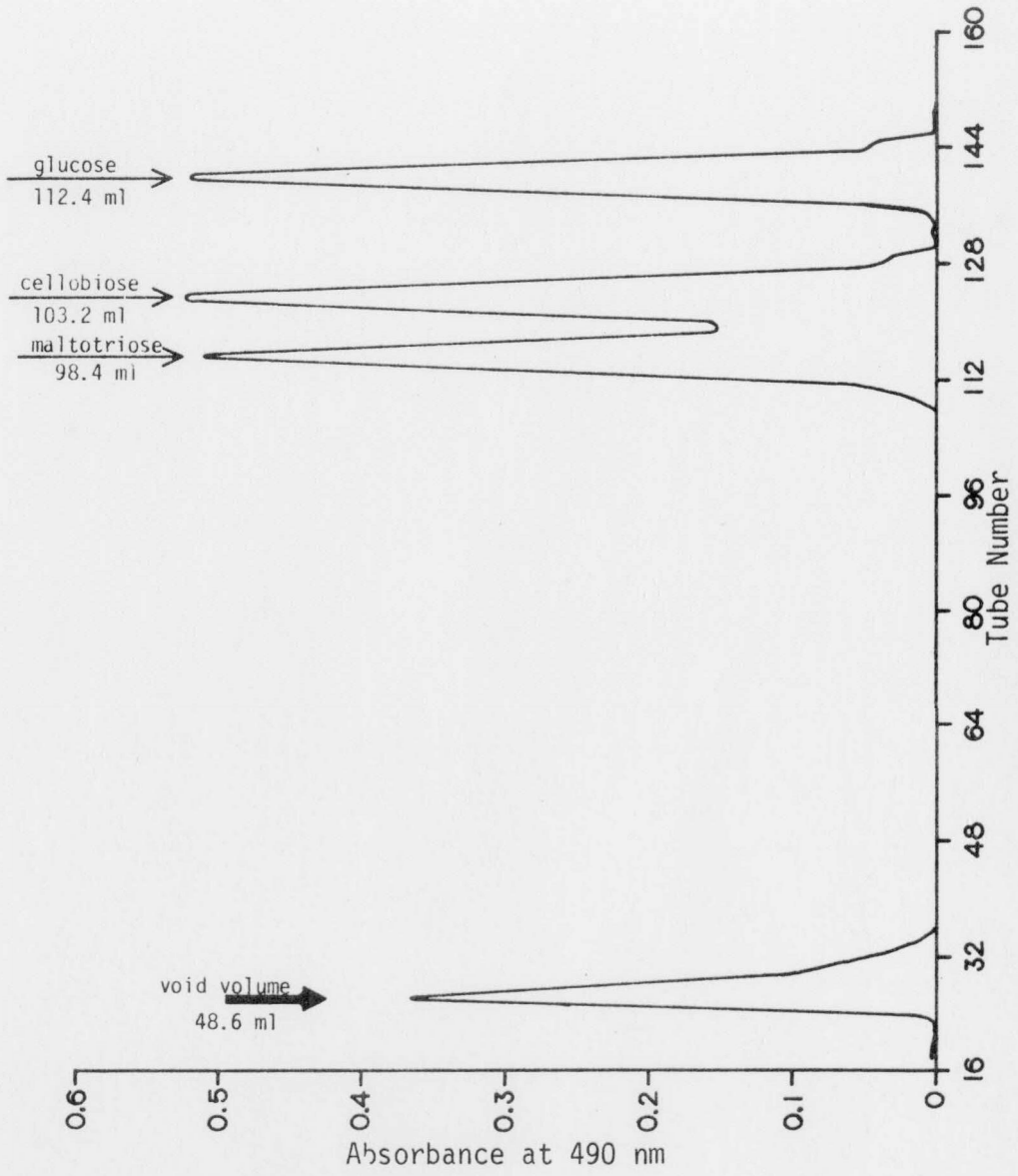


Figure 14. Column separation at a temperature of 44°C, and a flow rate of 1 drop/13 secs.

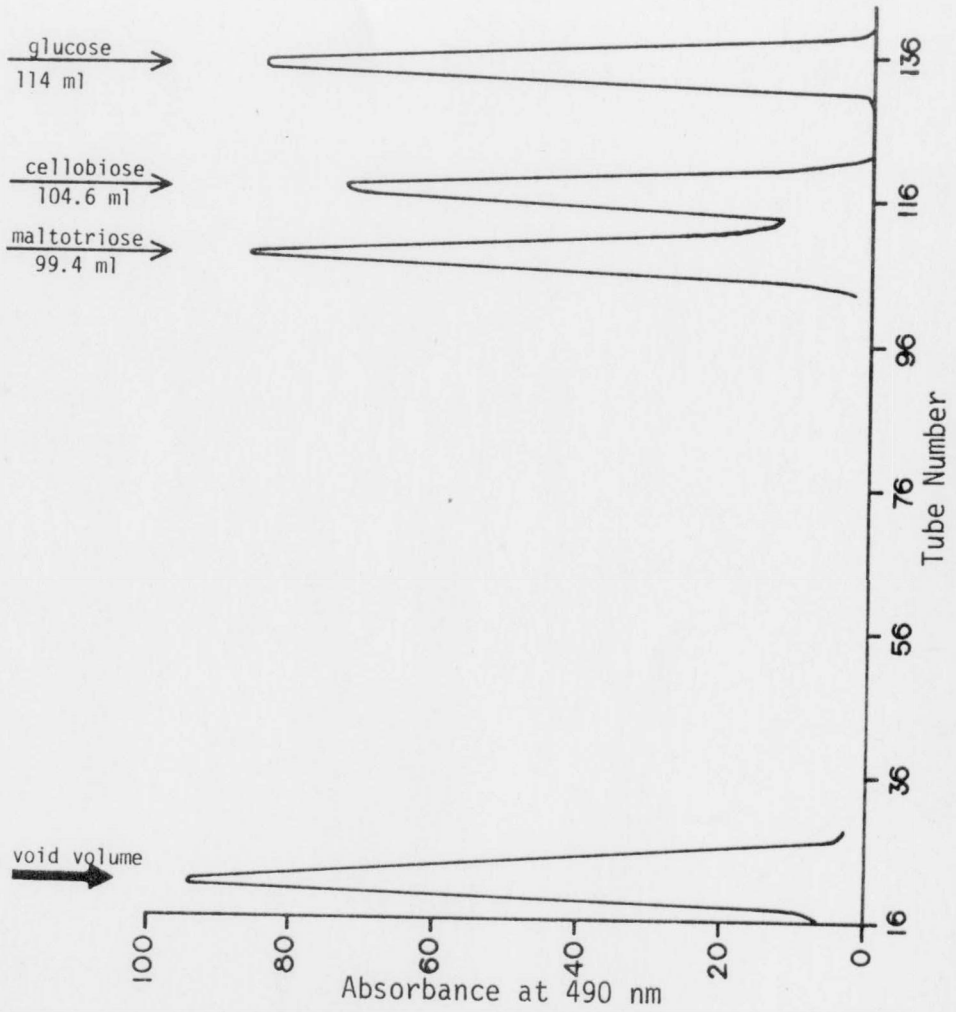


Figure 15. Column separation at a temperature of 64°C, and a flow rate of 1 drop/13 secs.

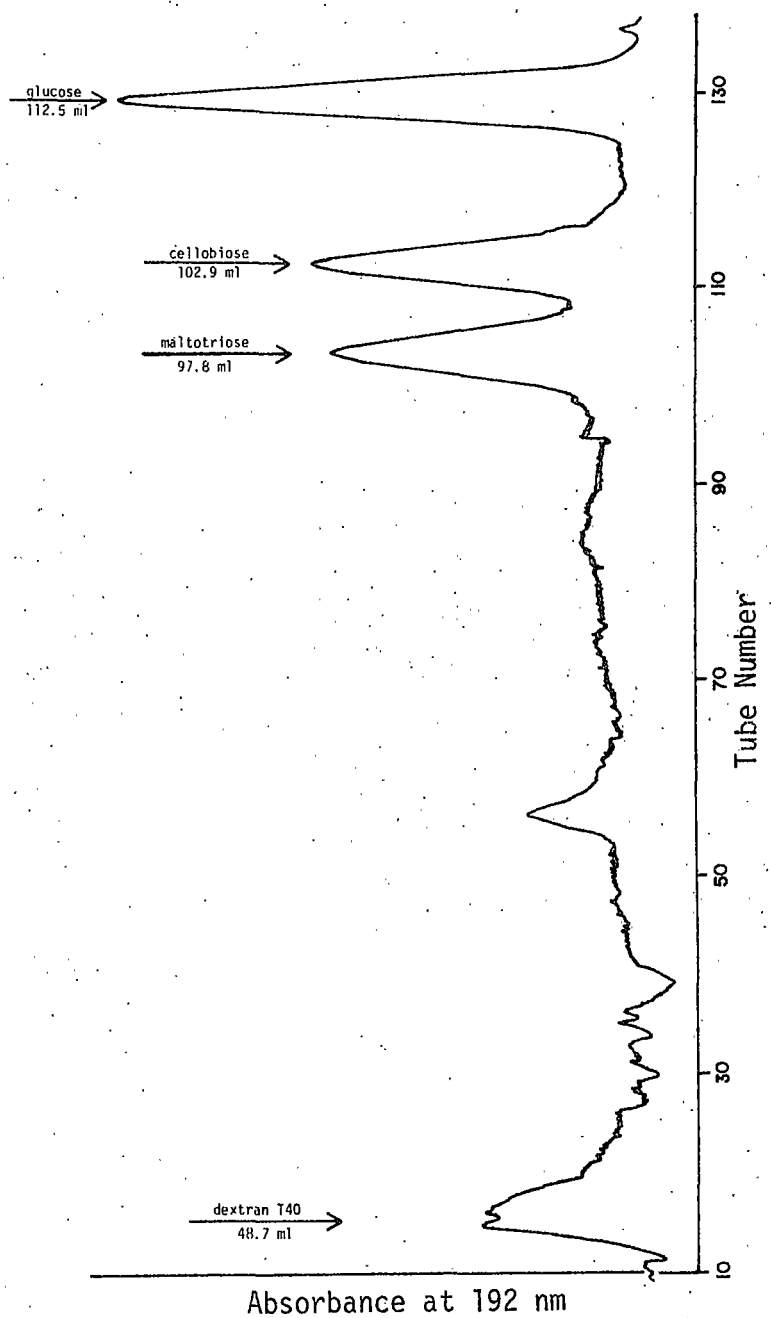


Figure 16. Column separation of carbohydrates, monitored at 192 nm

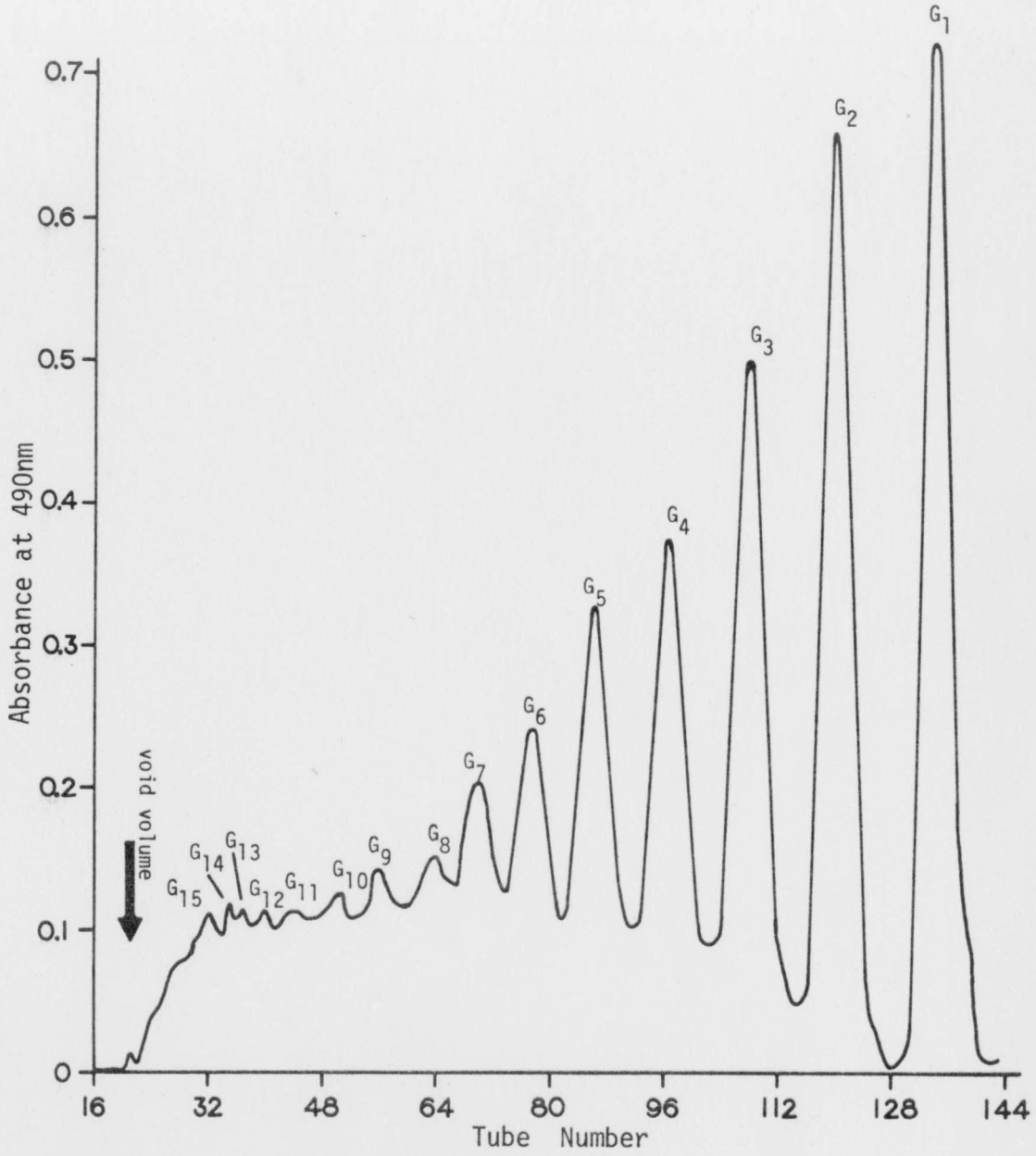


Figure 17. Separation of the glucosyl α(1→4) oligosaccharides in corn syrup.

Table 1. Elution volumes and their data reduction from the column separation of a corn syrup hydrolysate.

Oligomer	Ve(ml)	Ve/Vo	log(molecular weight)
G_1	113.4	2.3	2.256
G_2	105.2	2.13	2.534
G_3	98.4	1.96	2.703
G_4	92.0	1.87	2.824
G_5	86.3	1.75	2.918
G_6	81.4	1.65	2.996
G_7	77.2	1.57	3.062
G_8	73.3	1.49	3.119
G_9	69.4	1.41	3.169
G_{10}	66.0	1.34	3.215
G_{11}	62.6	1.27	3.256
G_{12}	60.4	1.22	3.293
G_{13}	58.7	1.19	3.328
G_{14}	57.0	1.16	3.359
G_{15}	55.9	1.13	3.389

(HPLC), using Partisil-10 PAC,[4] but the separation was not as good as with the polyacrylamide water-eluted columns.

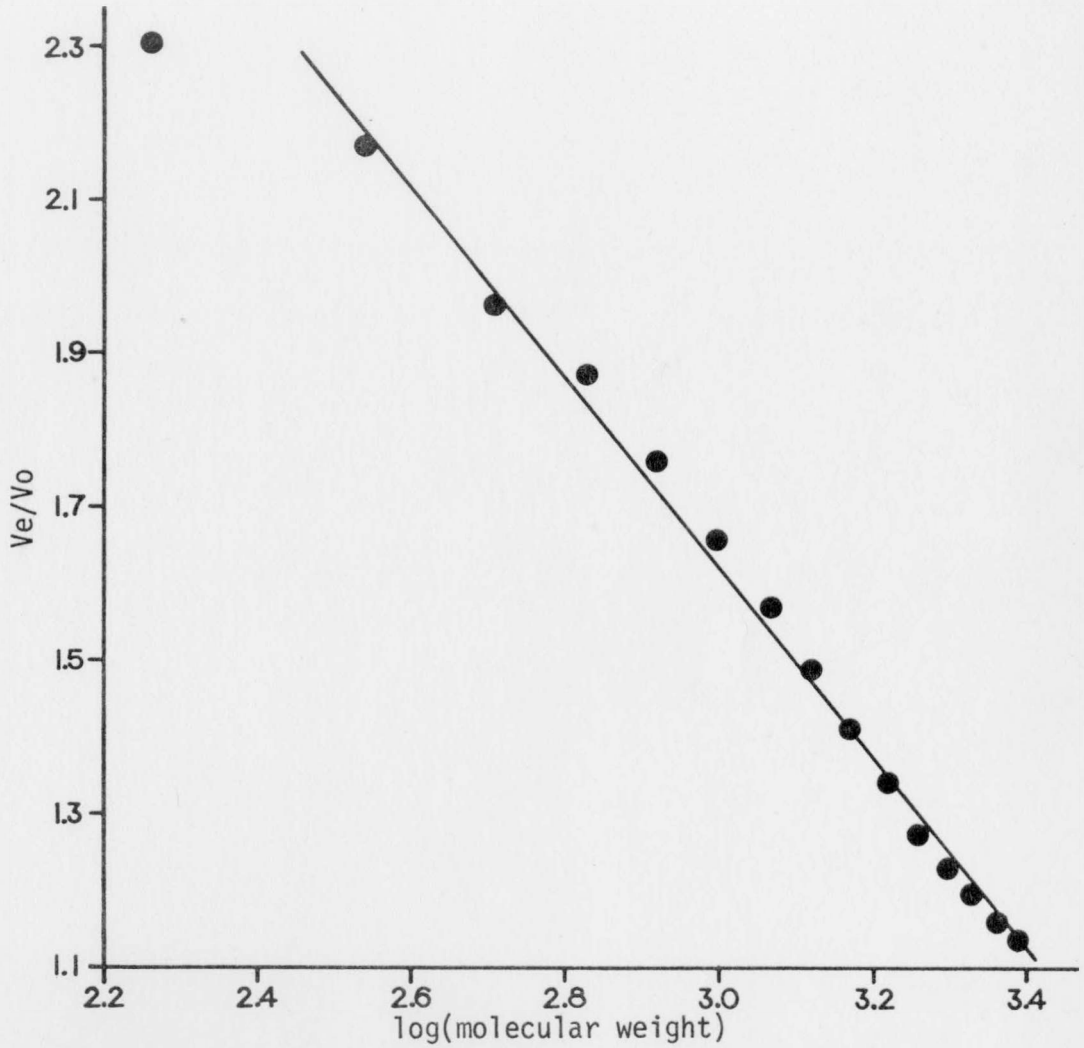


Figure 18. Plot of Ve/Vo vs. log(molecular weight) for the oligosaccharides in corn syrup.

SUMMARY

All apparatus discussed here, except for the Disc-Gel Band Enhancement Apparatus, adequately met the intended need. The DG-BEA functioned satisfactorily, and was indeed used, but three flourescent lamps behind the window, instead of only two, would be better.

The Bio-gel P-2 (-400 mesh) column was a good example of an inexpensive way to obtain an excellent separation instrument. Its separation potential appears to exceed that of any previously reported method.

EXPERIMENTAL

Throughout this work, absorbances, for phenol-sulfuric acid determinations, were obtained on a Bausch and Lomb Spectronic 70, and column effluents were monitored on a Gilson Hollochrome variable wavelength monitor.

Construction of the Bio-gel column

The column was made by joining thick-wall glass tubing (ID = 0.8 cm) together forming a nine foot column. Two, 4" pieces of glass tubing (ID = 0.9 cm) were joined at the ends of the nine foot tube, to allow the proper screw-on connections to be made. Prior to attaching the last 4" piece, six-17" water jackets with rubber seals (obtained from old condensers), were slipped on the column. The column was mounted on a bracket made of kiln dried wood, and this assembly was mounted vertically on a wall. The flow rate of the column was obtained with the help of a mini pump, which can be obtained from the Milton Roy Company, St. Petersburg, Florida (D-19-60029-001).

Carbohydrate determination by phenol-sulfuric acid

The phenol-sulfuric acid analysis was conducted by placing an aliquot of carbohydrate solution in the bottom of a 20 ml test tube. A 5% phenol-water solution (1 ml) was then added to the tube, with subsequent addition of concentrated sulfuric acid (5 ml).[41] After a ten min

cooling period, the tube was placed in a spectrophotometer to obtain its absorbance at 490 nm. According to the results obtained from the standard curve (Section 1), 18 μg of glucose has an absorbance of 0.299.

Determination of the included volume

A solution (0.25 ml) containing about 0.5 mg of glucose and tritiated water (a few microliters), was placed on the column. The flow rate was 1 drop/12 secs, collecting 13 drops or 0.56 ml/tube. The column was run at a temperature of 65°C. Glucose was detected by phenol-sulfuric acid determination, and tritium was counted using a scintillation counter. The elution volume of glucose was 113.2 ml, and that of the tritiated water was 131.7 ml.

Column separation at a temperature of 44°C, and a flow rate of 1 drop/6 secs Figure 13

A standard solution containing about 1 mg each of Dextran T40, maltotriose, cellobiose, and glucose in 0.25 ml water, was placed on the column. The flow rate was 1 drop/6 secs, collecting 13 drops or 0.56 ml per tube. The column was run at a temperature of 44°C. Each tube was monitored for total carbohydrate by the phenol-sulfuric acid method.

Column separation at a temperature of 44°C, and a flow rate of 1 drop/13 secs Figure 14

A standard solution containing about 1 mg each of Dextran T40, maltotriose, cellobiose, and glucose in 0.25 ml water, was placed on the column. The flow rate was 1 drop/13 secs, collecting 13 drops or 0.56 ml per tube. The column was run at a temperature of 44°C. Each tube was monitored for total carbohydrate by the phenol-sulfuric acid method.

The column run observed in Figure 15 was obtained in identical manner, except the temperature of the column was 64°C.

Column separation monitored at 192 nm Figure 16

A standard solution containing about 1 mg each of Dextran T40, maltotriose, cellobiose, and glucose in 0.25 ml water, was placed on the column. The flow rate was 1 drop/14 secs, collecting 13 drops or 0.56 ml/tube. The column was run at a temperature of 55°C. The column eluate was passed through a 40 µl flow-cell, being monitored at 192 nm.

Separation of the glucosyl α(1→4) oligosaccharides in corn syrup Figure 17

A solution of a corn syrup hydrolysate[1] in water (0.25 ml total) was placed on the column. The flow rate was 1 drop/14 secs, collecting 13 drops or 0.56 ml per tube. The column was run at a temperature of 55°C. Each tube was monitored for total carbohydrate by the phenol-sulfuric acid method.

REFERENCES AND NOTES

1. The corn syrup hydrolysate was a generous gift from John (Jack) Nordin, Department of Biochemistry, University of Massachusetts, Amherst, MA.
2. M. John, G. Trénel, and H. Dellweg, *J. Chromatog.* 42, 476-84 (1969).
3. N. K. Sabbagh and I. S. Fagerson, *J. Chromatog.* 86, 184-9 (1973).
4. F. M. Rabel, A. G. Caputo and E. T. Butts, *J. Chromatog.* 126, 731-40 (1976).

MONTANA STATE UNIVERSITY LIBRARIES
arch D378.B397 RL
Carbohydrate chemistry : synthetic and s
3 1762 00185961 8

D378
B397 Beier, Ross C
cop.2 Carbohydrate chemistry

DATE	ISSUED TO
2 WEEKS	USE INTERLIBRARY LOAN 7-1-85

D378
B397
cop. 2