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
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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

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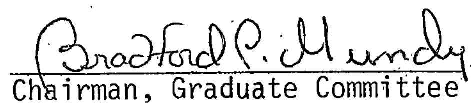
Approved:



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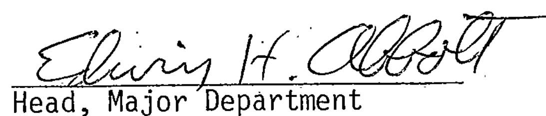

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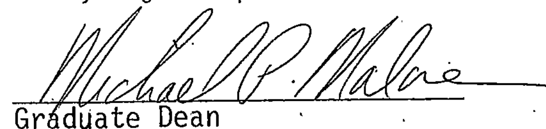

Graduate Dean

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.

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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 ^{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.

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SECTION 1

STRUCTURAL INVESTIGATIONS OF HELMINTHOSPOROSIDE

(one of the phytotoxins found in

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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

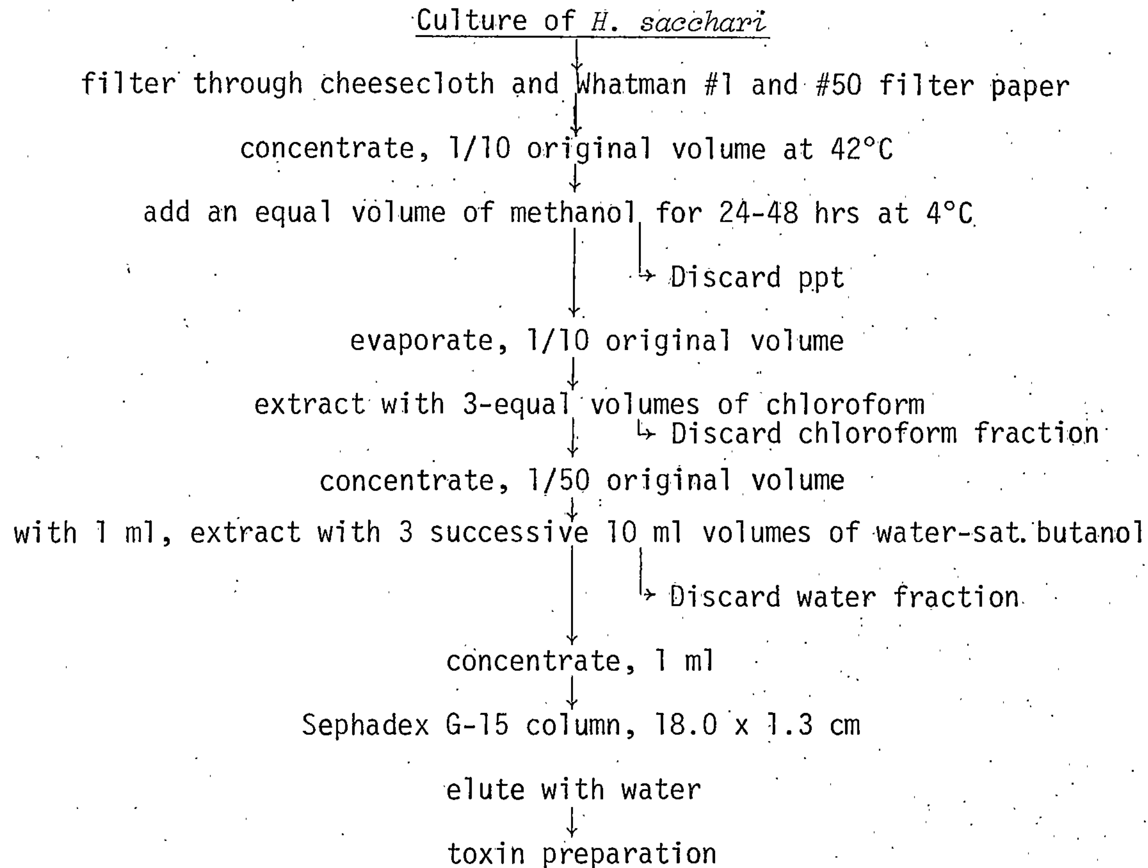

Figure 1. Flow sheet summarizing the isolation procedure used by Steiner and Byther.[5]

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-