Structural Characterization And Bioactivity Of Cranberry Oligosaccharides

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STRUCTURAL CHARACTERIZATION AND BIOACTIVITY OF CRANBERRY OLIGOSACCHARIDES

A Thesis
presented in partial fulfillment of requirements
for the degree of Master of Science
in the Department of Pharmacognosy, School of Pharmacy
The University of Mississippi

by
Kimberly M. Auker

May 2013
ABSTRACT

Complex carbohydrates from plant sources, such as the fruits of cranberry (*Vaccinium macrocarpon* Aiton), promote human health in a variety of ways (1-4). However, complete studies of carbohydrate function often hinge on a detailed understanding and thorough elucidation of the chemical structure. Several methods have been used in the structure elucidation of cranberry oligosaccharides, such as derivatization followed by analysis using gas chromatography-mass spectrometry (GC-MS). In this manner, the identity and quantity of glycosyl units within two cranberry oligosaccharides were investigated through hydrolysis of the oligomers and subsequent synthesis of trimethylsilyl and alditol acetate derivatives. Further analysis using partially methylated alditol acetate derivatives revealed the linkage position on each glycosyl unit. A complete structure was assembled through the compilation of data from the analysis of 1D and 2D NMR experiments yielding two novel oligosaccharides, the heptasaccharide $\beta$-D-glucopyranosyl-(1→4)-[\(\alpha\)-D-xylopyranosyl-(1→6)]-$\beta$-D-glucopyranosyl-(1→4)-$\beta$-D-glucopyranosyl-(1→4)-[\(\alpha\)-L-arabinofuranosyl-(1→2)-\(\alpha\)-D-xylopyranosyl-(1→6)]-$\beta$-D-glucopyranose (1) and the octasaccharide $\beta$-D-glucopyranosyl-(1→4)-[\(\alpha\)-L-arabinofuranosyl-(1→2)-\(\alpha\)-D-xylopyranosyl-(1→6)]-$\beta$-D-glucopyranosyl-(1→4)-$\beta$-D-glucopyranosyl-(1→4)-[\(\alpha\)-L-arabinofuranosyl-(1→2)-\(\alpha\)-D-xylopyranosyl-(1→6)]-$\beta$-D-glucopyranose (2).

Cranberry products are known to contain several biological activities, such as antioxidant properties (5), antiproliferative properties against several cancer cell lines (6-8), and antimicrobial and antiviral properties (9, 10). In addition, several clinical trials and in vitro analyses have assessed the ability of cranberry products and extracts to aid in the treatment of
urinary tract infections via prevention of adhesion of bacteria to uroepithelial cells (11-14).

Cranberry extracts enriched with oligosaccharides, including compounds 1 and 2, were assessed for biological activity. Bioassays included an antimicrobial screen against 10 different pathogenic organisms, a breast cancer cell viability assay, and an anti-adhesion assay involving *E. coli* and human uroepithelial cells. While antimicrobial, anticancer, and anti-adhesion activity was not confirmed for purified compounds 1 and 2, the parent fraction exhibits activity which prevents the adhesion of P-fimbriated *E. coli* to uroepithelial cells. This activity suggests the importance of oligosaccharide-enriched fractions in the anti-adhesion activity of cranberry products.
DEDICATION

To my family, who provides continual support and encouragement while I pursue my passions.
ACKNOWLEDGEMENTS

This work would not have been possible without the assistance and support of my advisor, Dr. Daneel Ferreira, and fellow graduate student, Ms. Christina M. Coleman. Throughout my time here, Dr. Ferreira has provided a strong foundation in chemical expertise while also fostering a spirit of creativity, honesty, and pride in research. Likewise, Ms. Coleman has played a supportive role both in and out of the laboratory. I also present my appreciation to the members of my advisory committee, Dr. Jordan Zjawiony and Dr. Marc Slattery, for their evaluation and helpful critique of the research presented in this thesis.

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# TABLE OF CONTENTS

ABSTRACT........................................................................................................................................... ii  
DEDICATION.......................................................................................................................................... iv  
ACKNOWLEDGEMENTS......................................................................................................................... v  
LIST OF TABLES................................................................................................................................... viii  
LIST OF FIGURES.................................................................................................................................. ix  
CHAPTER 1. STRUCTURE ELUCIDATION OF CRANBERRY OLIGOSACCHARIDES .... 1  
  1.1 Introduction................................................................................................................................... 2  
  1.2 Experimental Methods .................................................................................................................. 8  
  1.3 Results and Discussion .................................................................................................................. 21  
CHAPTER 2. BIOACTIVITY OF CRANBERRY OLIGOSACCHARIDES............................ 53  
  2.1 Introduction................................................................................................................................... 54  
  2.2 Experimental Methods .................................................................................................................. 58  
  2.3 Results and Discussion .................................................................................................................. 60  
LIST OF REFERENCES.......................................................................................................................... 70  
LIST OF APPENDICES......................................................................................................................... 76  
  APPENDIX A: 2D NMR SPECTRA FOR COMPOUND 1................................................................. 77  
  APPENDIX B: 2D NMR SPECTRA OF COMPOUND 2................................................................. 86  
VITA......................................................................................................................................................... 94
LIST OF TABLES

1.1 Mobile phase method fractionation of SN-03 and CJ-03 .......................... 11
1.2 Exact mass and molecular formula of compounds 1 and 2 .......................... 23
1.3 Alditol acetate analysis of compounds 1 and 2 ........................................ 25
1.4 PMAA derivative analysis of compounds 1 and 2 ...................................... 28
1.5 $^1$H and $^{13}$C NMR spectroscopic data for compound 1 .......................... 36
1.6 $^1$H and $^{13}$C NMR spectroscopic data for compound 2 .......................... 46
2.1 Antimicrobial assay of compound 2 and SN-03 ....................................... 61
2.2 Breast cancer cell viability assay of compounds 1 and 2 ............................ 62
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Structure elucidation scheme</td>
<td>5</td>
</tr>
<tr>
<td>1.2</td>
<td>Chemical derivatizations of compounds 1 and 2</td>
<td>6</td>
</tr>
<tr>
<td>1.3</td>
<td>Chemical structure of compounds 1 and 2</td>
<td>7</td>
</tr>
<tr>
<td>1.4</td>
<td>ELSD chromatogram of fraction SN-03</td>
<td>10</td>
</tr>
<tr>
<td>1.5</td>
<td>ESI-QTOF-MS spectra of permethylated compounds 1 and 2</td>
<td>23</td>
</tr>
<tr>
<td>1.6</td>
<td>TIC chromatogram of trimethylsilyl derivatives of compound 2</td>
<td>24</td>
</tr>
<tr>
<td>1.7</td>
<td>TIC chromatograms of AA-derivatives of compounds 1 and 2</td>
<td>26</td>
</tr>
<tr>
<td>1.8</td>
<td>PMAA derivatives of compounds 1 and 2</td>
<td>28</td>
</tr>
<tr>
<td>1.9</td>
<td>TIC chromatogram of PMAA derivatives of compound 1</td>
<td>29</td>
</tr>
<tr>
<td>1.10</td>
<td>TIC chromatogram of PMAA derivatives of compound 2</td>
<td>29</td>
</tr>
<tr>
<td>1.11</td>
<td>HPLC-UV chromatogram of phenyl thiocarbamate standards</td>
<td>31</td>
</tr>
<tr>
<td>1.12</td>
<td>Phenyl thiocarbamate derivatives of SN-03-Fr. 6 spiked with L-xylose</td>
<td>31</td>
</tr>
<tr>
<td>1.13</td>
<td>Phenyl thiocarbamate derivatives of SN-03 spiked with L-xylose</td>
<td>32</td>
</tr>
<tr>
<td>1.14</td>
<td>Phenyl thiocarbamate of SN-03-Fr. 6</td>
<td>32</td>
</tr>
<tr>
<td>1.15</td>
<td>Phenyl thiocarbamate derivatives of SN-03</td>
<td>32</td>
</tr>
<tr>
<td>1.16</td>
<td>$^1$H and $^{13}$C NMR spectra of compound 1</td>
<td>38</td>
</tr>
<tr>
<td>1.17</td>
<td>HSQC spectrum of compound 1 indicating anomeric correlations</td>
<td>39</td>
</tr>
<tr>
<td>1.18</td>
<td>COSY and key H2BC correlations of compound 1</td>
<td>40</td>
</tr>
<tr>
<td>1.19</td>
<td>Key HMBC correlations of compound 1</td>
<td>41</td>
</tr>
</tbody>
</table>
1.20 Key TOCSY and HSQC-TOCSY correlations of compound 1................................. 41
1.21 Phase sensitive COSY spectrum of compound 1.................................................. 42
1.22 $^1$H and $^{13}$C NMR spectra of cellotetraose.......................................................... 42
1.23 NOESY correlations of compound 1........................................................................ 43
1.24 $^1$H and $^{13}$C-DEPT spectra of compound 2............................................................ 48
1.25 HSQC spectrum of compound 2 indicating anomeric correlations.......................... 49
1.26 COSY and key H2BC correlations of compound 2.................................................... 50
1.27 Key HMBC correlations of compound 2................................................................. 51
1.28 HSQC of compound 2 focusing on the oxymethylene C and H signals................... 51
2.1 Chemical structure of quercitin, myricetin, and an A-type proanthocyanidin............. 55
2.2 HPLC-ESLD chromatograms of SN-03 and a pig urine fraction............................... 57
2.3 Anti-adhesion activity of aq. SN, SN-03, SN-04, and myricetin............................... 64
2.4 Anti-adhesion activity of SN-03-Fr. 4, Fr. 5, and Fr. 6.............................................. 65
2.5 Anti-adhesion activity of compounds 1 and 2 ....................................................... 66
2.6 Anti-adhesion activity of SN-03-Fr. 1, Fr. 3, Fr. 7, Fr. 8, and Fr. 9......................... 67
CHAPTER 1

STRUCTURE ELUCIDATION OF CRANBERRY OLIGOSACCHARIDES
1.1 Introduction

Complex carbohydrates, such as protein-bound and free oligosaccharides, from natural sources are involved in human health in a variety of ways (1, 15). For instance, oligosaccharides are being investigated as anti-adhesion agents for use in the treatment or prevention of infectious disease (2, 16). Glycol portions of a mucosal cell glycoconjugate are often involved in bacterial adhesion to such cells. The mechanism to prevent adhesion of bacteria is through competitive attachment of free oligosaccharides that mimic the structure of the glycol portion of the glycoconjugate to which bacterial adhesins bind (16). Health benefits of oligosaccharides include immune effects such as the immunostimulatory properties of mushroom β-glucans (17) and inhibition of the human complement pathway by brown algal fucoidan oligosaccharides (3). Recently, much attention has been given to the stimulation of a beneficial gastrointestinal (GI) microbiota by oligosaccharides found in an assortment of natural sources such as grain products, Norway spruce (Picea abies), and wine (4, 18-20). Oligosaccharides selectively fermented by GI flora, resulting in either a compositional change in the microbiota or changes in their metabolic activity and generating a beneficial effect on health, are known as prebiotics (4).

Understanding the structure of an oligosaccharide is a key element to understanding its bioactivity. In the case of bacterial adhesins, specific bacterial species, even strains of the same species, bind to specific carbohydrate moieties on glycoconjugates present on a particular cell type. For example, the urinary pathogen *Escherichia coli* Type 1 has adhesins that bind to Manα3Manα6Man present on epithelial cells in the lower urinary tract, rendering it susceptible to the anti-adhesive properties of D-mannose. Conversely, P fimbriated *E. coli* adhesins bind to Galα4Gal present on the surface of upper urinary tract epithelial cells and are inherently resistant to D-mannose anti-adhesive effects. NMR and molecular modeling based structure-activity
studies of the fucoidan oligosaccharides have highlighted that the decrease in flexibility of the oligosaccharide backbone promotes binding to the C4 receptor protein in the classical complement pathway (3). It is the presence of side chains in some fucoidan oligomers that results in the decreased backbone flexibility. Further, the structural characteristics of prebiotic oligosaccharides can stimulate growth of specific types of GI flora, with bifidobacteria and lactobacilli most often effected (4). In vitro examinations indicate that glycosyl composition, for example the ratio of xylose to arabinose residues in xylo-oligosaccharides (18), degree of polymerization, and degree of branching effect the fermentation of oligosaccharides (4, 18, 19, 21). Overall, it is clear that a complete understanding of a compound’s bioactivity hinges on a thorough examination of the molecular structure.

Many techniques have been reported regarding structure elucidation of oligosaccharides. The complexity of compounds addressed range from di- and trisaccharides to molecules with over 50 degrees of polymerization (4, 21, 22). Analytical techniques include hydrolysis and analysis of glycosyl identity and quantity (22), permethylation (23, 24) and methanolysis combined with gas-liquid chromatography (GLC) (22, 25), and trimethylsilyl derivatization combined with gas chromatography-mass spectrometry (GC-MS) (26). Advanced NMR techniques aid in the full structural assignment of carbohydrates and are also utilized in structure-activity studies (3, 22, 27). Owing to the enormous variety of structures that are possible in complex carbohydrates, a combination of methods is necessary to assess the complete structure.

In the present work, two novel oligosaccharides isolated from cranberry fruit (Vaccinium macrocarpon Aiton, Ericaceae) products have been investigated. A variety of techniques employed in the structure elucidation (Figure 1.1) are utilized to provide absolute confidence in the structural assignment of these complex molecules. The process begins with the
determination of molecular weight, achieved via permethylation of the compounds and analysis with high resolution mass spectrometry (HRMS); this information subsequently provides the degree of polymerization (DP). The DP is applied to the results of the quantification and identification of glycosyl units assessed with alditol acetate derivatization (Figure 1.2 A) followed by GC-MS analysis. The alditol acetate derivatization involves the reduction of hydrolyzed glycosyl residues from the aldehyde to the alditol form and subsequent $O$-acetylation of the hydroxy groups. In this analysis, a molar ratio between the participating glycosyl units is produced and, in combination with the DP, the actual number of glycosyl types is determined. For example, if the ratio of monosaccharides is 2 Glc:1 Xyl then with a DP of 3, it is established that two Glc units and one Xyl unit are present, rather than four Glc and two Xyl units. At this point, several questions still remain about the structure of these complex carbohydrates, such as what is the ring size and stereochemistry of each monomer and how are they linked together? The partially methylated alditol acetate (PMAA, Figure 1.2 B) derivative analysis affords the details of ring size and linkage position for each individual monomer and the phenyl thiocarbamate derivative analysis (Figure 1.2 C) allows the assignment of D or L configuration based on the absolute configuration of C-4 and C-5 for pentose and hexose saccharides, respectively. These methods are further explained in the Results and Discussion section. Finally, 1D and 2D NMR experiments are employed to establish the configuration at the anomeric carbon as $\alpha$ or $\beta$ as well as establish the connectivity of the monomers (HMBC double-headed arrow in Figure 1.1). With the pieces of information from each experiment pulled together, each complexity of the oligosaccharide structure is addressed yielding a thorough elucidation of the two novel oligosaccharides presented herein (Figure 1.3).
**Figure 1.1.** Structure elucidation of a hypothetical trisaccharide employed for compounds 1 and 2.
Figure 1.2. Chemical derivatizations of compounds 1 and 2. Alditol acetate (A), partially methylated alditol acetate (B), and phenyl thiocarbamate (C) derivatizations.
Figure 1.3. Chemical structure of compounds 1 and 2.
1.2 Experimental Methods

Solvents and chemicals

Derivatization: Solvents were purchased from Fisher Chemicals and are ACS certified unless otherwise stated. DMSO and MeOH were both dried with a 3 Å molecular sieve. Chemical reagents include Tri-Sil reagent (Thermo Scientific), L-cysteine methyl ester (Aldrich), and phenyl isothiocyanate (Aldrich).

Standards: All carbohydrate standards were purchased from Sigma unless otherwise stated: cellotetraose (Dextra, Reading, UK), L-(+)-arabinose, D-(-)-arabinose, D-(+)-glucose, L-(-)-xylose, D-(+)-xylose, D-(+)-fucose, L-(+)-rhamnose monohydrate, myo-inositol (Sigma).

Isolation

Two starting materials were used: Sundown Naturals® brand cranberry fruit powder in 475 mg capsules (SN) and Ocean Spray® spray-dried cranberry juice powder (CJ) provided by Ocean Spray Cranberries, Inc. The SN (after removal from capsules) and CJ powders (40.0 g and 1 kg, respectively) were dissolved separately in 150 mL of H₂O using 30 min of sonication. The dissolved samples were centrifuged for 5 min at 1800 rpm and the supernatant of each was decanted into separate 1 L separatory funnels. The precipitate isolated upon centrifugation was washed twice with 100 mL and then 50 mL of H₂O and the wash was added to the supernatant of each respective sample, for a total of 300 mL of aq. solution for each sample. The samples were extracted with 150 mL of EtOAc four times and the resulting aqueous and EtOAc fractions were dried by rotary evaporation. A secondary extraction was carried out on the aqueous extract of SN using EtOH in order to precipitate proteins and pectins. EtOH (500 mL) was added to 250 mL of the aq. SN extract followed by sonication for 20 min. The resultant aq. extracts were
centrifuged for 5 min at 3000 rpm to remove precipitate. The supernatants were dried using a combination of rotary evaporation and lyophilization.

The aqueous SN and CJ extracts were fractionated using Sephadex LH-20 (320 g dry resin, Amberlite). The resin was equilibrated with 70% aq. EtOH resulting in a column with dimensions of 70 x 36 x 7 cm. The SN and CJ aq. extracts were dissolved in 70% aq. EtOH (13.7 g and 25.3 g, respectively) and centrifuged to remove any precipitate, resulting in 60 mL of the SN sample and 100 mL of the CJ sample which were then separately loaded onto the Sephadex LH-20 columns. Elution proceeded with 70% aq. EtOH at ~1.7 mL/min for the SN sample. Two fractions (SN-03 and SN-04) containing the oligosaccharides eluted between 320 and 620 minutes with a larger quantity of oligosaccharides eluting in fraction SN-03 (320-450 min). The SN-03 and SN-04 fractions were dried to yield a lavender-maroon powder (3.62 g and 2.44 g, respectively). Elution of the CJ extract proceeded with the same solvent and flow rate affording two oligosaccharide-containing fractions (CJ-03 and CJ-04) eluting between 350 and 600 minutes. The fractions were dried, yielding 3.98 g of CJ-03 and 1.74 g of CJ-04, a pink-lavender powder. The combined yields of the oligosaccharide fractions comprise 44.3 and 22.6% of the aq. SN and CJ extracts, respectively. When assessed by analytical scale HPLC-UV-ELSD, the oligosaccharide-containing fractions afforded a unique chromatographic profile as shown in Figure 1.4. The conditions employed for this analysis include: Waters 2695 Separations Module; Waters 996 Photodiode Array Detector; Polymer Laboratories PL-2100 ELS detector (ELSD); stationary phase, Atlantis dC18 column (4.6 x 150 mm, 5 µm); UV Maxplot 210-400 nm; ELSD gas flow 1.0 SLM, nebulizer temp. 45 °C, evaporator temp. 90 °C; mobile phase gradient 0-5 min H2O, 5-35 min H2O to 30% aq. MeOH, 35-45 min 30 to 100% MeOH.
Further fractionation occurred using a Waters Delta Prep 4000 HPLC equipped with a Waters 2487 Dual Wavelength Absorbance UV and Polymer Laboratories PL-1000 ELS detectors in series. An Atlantis dC$_{18}$ (19 x 250 mm, 10 µm) column was used as the stationary phase and the mobile phase was a gradient from H$_2$O to 40% aq. MeOH at 25 mL/min (Table 1.1). UV spectra were recorded at 210 and 254 nm and the ELSD conditions were as follows: carrier gas, N$_2$, gas flow 0.8 SLM, nebulizer temp., 50 °C, and evaporator temp. 100 °C. SN-03 (3.17 g) and CJ-03 (1.00 g) were dissolved in 9.2 and 4 mL of H$_2$O, respectively, and filtered through a 0.2 µm nylon membrane. Repeated injections were applied ranging from 400 to 600 µL each. Nine fractions were collected: fraction 4, collected from 25.5-26.8 min, contained compound 1 as its major component and fraction 5, collected from 26.8-28.2 min, contained compound 2 as its major component. SN-03 afforded 82.3 mg and 211.9 mg and CJ-03 afforded 55.7 mg and 96.6 mg of fractions 4 and 5, respectively. The quantity of fractions 4 and 5 constitutes 2.6 and 6.6% of SN-03, while fractions 4 and 5 constitute only 5 and 9% of CJ-03, respectively. Fraction SN-03 constitutes a much larger portion of the aq. SN extract (44.3%) as compared to CJ-03 which makes up 22.6% of the aq. CJ extract. This suggests that the yield of fractions 4 and 5 may be higher in the SN fruit powder product as compared to the CJ juice.
powder product. However, almost 50% of the dry weight of the original SN product was
removed through the EtOH precipitation process. The final yield of compounds 1 and 2 are
~0.13 and 0.29% of SN and ~0.33 and 0.52% of CJ, respectively.

**Table 1.1.** General mobile phase method used for preparative scale fractionation of SN-03 and
CJ-03 (Atlantis dC$_{18}$, 19 x 250 mm, 10 µm).

<table>
<thead>
<tr>
<th>Time</th>
<th>% Solvent: H$_2$O</th>
<th>% Solvent: MeOH</th>
<th>Curve*</th>
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<tr>
<td>0</td>
<td>100</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>0</td>
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<tr>
<td>65</td>
<td>100</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

*The curve profile of the gradient method indicates the change in solvent composition over the
time allotted. A curve profile of 6 is a gradual, linear change in the solvent composition from the
initial to the final solvent composition over the time segment of the gradient schedule. For
example, a linear change in solvent composition occurs from 10 min and 100% H$_2$O to 50 min
and 30% aq. MeOH.

The final purification was completed using the same HPLC and detector conditions with
a YMC Polyamine II (20 x 250 mm, 5 µm) stationary phase and an isocratic mobile phase of
63% aq. MeCN. SN-03 fraction 4 (80.1 mg) was injected over several runs (200 µL injections)
yielding 31.0 mg of compound 1 which eluted at 25 min. SN-03 fraction 5 (169.4 mg) was
injected over several runs (~200 µL injections) eluting compound 2 at 28 min with a yield of
65.0 mg. CJ-03 fraction 5 (~60 mg) was injected over two runs (200 µL injections), eluting
compound 2 at 28 min with a yield of ~20 mg.

Intensive structural studies were carried out using oligosaccharides isolated from the SN
product. Accidental sample loss of CJ oligosaccharide samples prevented thorough study;
however, the presence of compound 2 could be confirmed through chromatographic and NMR
spectroscopic similarities and the presence of compound 1 is suspected.
**Permethylation**

Compounds 1 and 2 (200-500 µg) were dissolved in 200 µL of dry DMSO in a screw-cap tube and stirred for 1 hr after purging the headspace with N₂. The base was freshly prepared using 4 mL of dry DMSO added to 100 µL of 50% sodium hydroxide (w/w). The solution was vortexted, centrifuged, and washed with dry DMSO repeatedly until salts and H₂O were removed. Dry DMSO (2 mL) was added to the washed hydroxide pellet. The resulting base (200 µL) was added to each carbohydrate sample and stirred briefly, after which iodomethane (100 µL) was added. The headspace of the screw-cap tube was purged with N₂ and the solution was stirred at room temperature for 40 min. This process was repeated followed by addition of H₂O (2 mL) to quench the reaction, yielding a cloudy solution. The remaining iodomethane was removed under a low flow of N₂. The permethylated carbohydrates were extracted into DCM (2 mL), washed with H₂O (2 x 2 mL), dried under N₂, and further purified by solid phase extraction (SPE; C₁₈ E Strata, Phenomenex). The SPE cartridge was conditioned with MeOH, then MeCN and finally H₂O and the dried carbohydrate extracts were loaded onto the cartridge with H₂O and eluted with MeCN. The samples were dried prior to ESI-QTOF-MS analysis.

**Trimethylsilyl Derivatization**

Compound 2 and two sets of standards were derivatized. Standard set 1 consisted of 50 µg each of arabinose, fucose, galacturonic acid, glucose, N-acetylglucosamine, and N-acetylmannosamine. Standard set 2 consisted of 50 µg each of rhamnose, xylose, glucuronic acid, mannose, galactose, and N-acetylglactosamine. *myo*-Inositol (20 µg) was added to 100 µg of sample 2 and to each standard set in individual screw-cap tubes. The derivatization began with methanolation by heating the samples to 85 °C overnight after the addition of 500 µL of 1 M
methanolic HCl (28). The samples were cooled and dried under N₂ in a H₂O bath at 40 °C during which an additional 200 µL of dry MeOH was added. MeOH (200 µL), pyridine (100 µL), and Ac₂O (100 µL) were added to the dried samples and left for 30 min at room temperature, after which the samples were dried as described above. Tri-Sil reagent (200 µL) was added and the samples were heated at 80 °C and stirred for 20 min. The samples were concentrated to a minute volume (~50 µL) and then 2 mL of hexane was added. The samples were centrifuged for 30 sec and the supernatant was removed and filtered through a glass wool-packed pipette and concentrated to a volume of ca. 100 µL. The remaining sample was transferred to a 1.5 mL vial with a septum-cap for GC-MS analysis.

Alditol Acetate Derivatization

The following samples were used to create alditol acetate derivatives: L-(+)-arabinose, D-(+)-glucose, L-(−)-xylose, D-(+)-fucose, L-(+)-rhamnose, D-(+)-galactose, myo-inositol, 1 and 2. The standards were prepared individually using the reported procedure (Whiton et al. 1985) modified as described below, to assess retention time and fragmentation by GC-MS analysis. A standard set of multiple monosaccharides (AA_{std}) was used to create a GC-MS calibration curve for quantification purposes. Individual monosaccharide samples (100 µg each) were combined with 20 µg of the internal standard, myo-inositol, in individual screw-cap tubes. The standard of combined monosaccharides consisted of 50 µg each of L-(+)-arabinose, D-(+)-glucose, and L-(−)-xylose, and 20 µg of myo-inositol. 1 and 2 (~100 µg each) were combined with 20 µg of myo-inositol in a screw-cap tube and were hydrolyzed through incubation in 2 M TFA at 121 °C for two hr, after which the samples were removed from heat and allowed to cool to room temperature. Isopropanol (200 µL) was added and the samples were dried under a stream of N₂
while sitting in a H₂O bath at 40 °C. The addition and subsequent drying of isopropanol was repeated twice. The hydrolysis and drying were followed by reduction of the resultant monomeric moieties from the oligosaccharides by incubating the samples in 200 µL of 10 mg/mL NaBD₄ in 1 M NH₄OH at room temperature overnight. The reduction reaction was neutralized with five drops of glacial HOAc, followed by the addition of 5 drops of MeOH and drying of the samples. Any remaining NaBD₄ was removed by repeated addition and drying of 9:1 MeOH:glacial HOAc. The final step of O-acetylation of the reduced monomeric moieties was carried out by the addition and brief mixing with 250 µL of Ac₂O followed by addition and incubation with 230 µL of TFA (99.9%) at 50 °C for 20 min. After the sample cooled, the reaction concluded with the addition of isopropanol, which was subsequently dried and followed by the addition of 0.2 M Na₂CO₃. The resulting alditol acetate derivatives were extracted from the final solution using DCM (2 mL), washed with H₂O (2 x 1 mL), and concentrated to ~100 µL in preparation for GC-MS analysis.

**Partially Methylated Alditol Acetate (PMAA) Derivatization**

*Preparation of standards:* The PMAA standards were created from the following monosaccharides: L-(+)-arabinose, D-(+)-glucose, and L-(−)-xylose, with the addition of myo-inositol as an internal standard. Partial methylation of each monosaccharide was carried out based on the methods described in Doares et al. (29). Briefly, methanolysis of each monosaccharide was performed with the addition of 2 mL of 1 M methanolic HCl in a screw-cap tube. Following incubation at 80 °C for two hours with stirring, 200 µL of t-butanol was added and the samples were dried under flowing N₂ while sitting in a H₂O bath at 40 °C. Subsequently, 1 mL of MeOH was added and the samples were re-dried under N₂ and finally in a vacuum oven.
The resulting methyl glycosides were treated with freshly prepared 1M dimsyl anion at a volume that provides the molar equivalence of the anion to 70% of the remaining free hydroxy groups in each of the methyl glycoside standards. After 10 min of stirring, iodomethane was added (130 µL) and the mixture was stirred for an additional 10 min. The remaining iodomethane was removed by placing the sample under a flow of nitrogen and eventually drying the samples. The resulting partially methylated monosaccharide standards each underwent O-acetylation using 200 µL of 4-(dimethylamino)pyridine (120 mg/mL pyridine) and subsequent addition of 2 mL of Ac₂O with stirring for 10 min. O-acetylation of the samples allows for more efficient extraction using DCM. The following treatment of the extracted and dried partially methylated standards mimicked the method described for alditol acetate derivatization. This method employed hydrolysis with 2 M TFA, reduction with NaBD₄ in 2 N NH₄OH, and O-acetylation with TFA 99.9% and Ac₂O.

To create the PMAA derivatives of samples 1 and 2 (400 µg each), the oligosaccharides were subjected to the permethylation procedure described above, which was immediately followed by the alditol acetate procedure. Derivatives were reduced to a volume of ~100 µL in DCM and injected into GC-MS.

Phenyl Thiocarbamate Derivatization

The determination of D or L configuration of the monomeric moieties was completed through the creation of their aryl thiocarbamate derivatives followed by comparison of retention times to corresponding standards using HPLC-UV analysis (30). In order to conserve the purified oligosaccharides, this analysis was carried out using the parent fraction (SN-03) and an enriched fraction known to contain both compounds (SN-03-Fr. 6). One mg and 23.7 mg of SN-03 Fr. 6
and SN-03, respectively, were hydrolyzed in 2 M HCl for 60 min, with stirring, in an oil bath at 100 °C. The acid was removed from the samples either by repeated addition of H2O and evaporation (SN-03-Fr. 6) or by the addition of Ag₂CO₃ (SN-03) and subsequent filtration to remove AgCl. Before proceeding with derivatization, the hydrolyzed SN-03 sample was extracted with EtOAc in an attempt to remove non-carbohydrate, colored compounds; however, these remained in aq. solution but did not interfere with further reactions. L-cysteine methyl ester (1-2 mg) and 100-200 μL of anhydrous pyridine (99%) was added to dried SN-03-Fr. 6 and SN-03 as well as to the following standards individually (except SN-03, to which 5 mg and 500 μL was added, respectively): D- (+)-glucose, L- (+)-arabinose, D- (-)-arabinose, L- (-)-xylose, D- (+)-xylose, L- (+)-rhamnose, D- (+)-galactose, and D- (+)-mannose. Samples were incubated for 60 min at 60 °C in an oil bath with a reflux condenser and stirring, after which 100-200 μL of phenyl isothiocyanate (10 mg/mL) in anhydrous pyridine was added and the samples incubated for 60 min at 100 °C in an oil bath with stirring and reflux condensation. The cooled samples were then analyzed by HPLC-UV.

**HPLC-UV for D/L Determination**

The liquid chromatographic system was a Waters 2695 Separations Module equipped with a Waters 996 Photodiode Array detector (Waters Corporation, Milford, MA, USA). The chromatographic conditions were as follows: stationary phase, Gemini C₁₈ (4.6 x 250 mm, 110 Å, 5 μm); mobile phase, flow rate 1 mL/min; mobile phase gradient, H₂O (0.1% formic acid): MeCN (90:10) to MeCN over 45 min with a gradient curve of 6. A five min wash period with MeCN and 15 min equilibration period was applied between each injection. The MaxPlot UV absorbance and 210 nm absorbance was monitored. Each sample was injected twice to provide
an average retention time for each peak. The injection volume of individual samples and standards was 2 µL, while the injection volume of samples spiked with the standard was 3 µL. An isocratic method with a mobile phase of 27% aq. MeCN was employed for samples spiked with L-(-)-xylose.

**MSD-TOF**

Samples were analyzed using an Agilent HPLC 1100 series equipped with a diode array detector and a time of flight mass detector (Model G1969A) in series (Agilent Technologies, Palo Alto, CA, USA). The mass detector contains an electrospray ionization interface and was controlled by Agilent software (Agilent MassHunter Work Station, A.02.01) with subsequent data acquisition and processing by the Analyst™ QS software (Analyst, QS 1.1). Mass spectra were acquired in both positive and negative ionization modes for 1, 2, and their permethylated derivatives using a capillary voltage of 3000 V. The nebulizer carrier gas was N₂ (30 psig) in combination with a drying gas at a flow of 10 mL/min and temperature of 325 °C. Full scan mass spectra were acquired from m/z 100-1500. All samples analyzed were dissolved in 1 mM NaOH in 50% aq. MeOH.

**UHPLC/QTOF-MS**

UHPLC-MS/MS analysis was carried out using an Agilent LC-1200 Infinity Series (1290) UHPLC system coupled to a QTOF-MS (Model #G6530A, Agilent Technologies, Palo Alto, CA, USA). The solid phase was an Agilent Bonus RP18 (2.1 x 100 mm, 1.8 µm) and a mobile phase gradient of 3% aq. MeOH to 100% MeOH was engaged over 5 min at 0.25 mL/min, after which MeOH was held for 3 min at the same flow rate. A five minute wash with MeOH followed by an equilibration period of 3 min was completed between each run. The
column temperature was set at 35 °C and the injection volumes ranged from 3-5 µL. TIC chromatogram peaks were assigned according to the mass of the compounds. The conditions of the QTOF-MS were as follows: ESI source with Jet Stream technology using a drying gas (N₂) at 11.0 L/min and 250 °C; nebulizer, 35 psig, sheath gas at 10 mL/min and 325 °C; capillary, 3500 V; skimmer 65 V; Oct RF V, 750 V; fragmentor voltage, 100 V. The sample collision energy was set to 65 V. The data acquisition and analysis were controlled by Agilent MassHunter Acquisition Software Ver. A.02.00 and processed by MassHunter Qualitative Analysis software Ver. B.02.00. The samples were analyzed in positive mode and spectra were acquired across the range m/z 100-1700 with accurate mass measurement. The accurate mass measurements were obtained through use of a reference ion correction using m/z 121.0509 (protonated purine) and 922.0098 [protonated hexakis(1H, 1H, 3H-tetrafluoropropoxy) phosphazine or HP-921]. The reference solution was introduced into the ESI source using a T-junction with an Agilent Series 1200 isocratic pump (Agilent Technologies, Santa Clara, CA, USA) containing a 100:1 splitter with a flow rate of 20 µL/min. All samples analyzed were dissolved in 1 mM NaOH in 50% aq. MeOH.

**GC-MS Analysis**

Gas chromatography-mass spectrometry was completed using an Agilent Packard 6890 Series GC system coupled with a Hewlett Packard 5973 Mass Selective detector. The conditions of operation for analysis of the AA and PMAA derivatives were as follows: Restek Rtx®-2330 30 m, 0.25 mm id, 0.2 µm film thickness (d_f) column; injector temperature of 250 °C, split ratio of 20:1, helium as the carrier gas, and a mass scan mode from m/z 50-500. The temperature program was set with an initial temperature at 80 °C for two min, a first ramp at 30 °C/min to a
final temperature of 170 °C and a second ramp at 4 °C/min to a final temperature of 240 °C, after which this temperature was held for 20 min. The injection volume was 5 µL and each injection was repeated three times. The conditions of operation for analysis of the trimethylsilyl derivatives were the same as above except for the following: Grace EC-1 30 m, 0.25 mm id, 0.2 µm df column and an injection volume of 1 µL. Data acquisition and analysis were completed with Agilent ChemStation and MestreNova software (Mestrelab Research, Escondido, CA, USA).

*Quantification of Alditol Acetate Derivatives*

In order to determine a relative molar quantity of each monomer in the oligosaccharides, a six-point calibration curve was created using the averaged peak areas (PA) from three injections of the standard (AA<sub>std</sub>). The detector Response Factor (RF; slope of the linear calibration curve) was used to calculate a Relative Response Factor (RRF) of each monosaccharide standard (S) relative to the internal standard (IS), as shown below:

\[
RRF = \frac{RF_S}{RF_{IS}}
\]

The concentration (C) of each monosaccharide moiety (S) in the oligosaccharide samples was calculated using the corresponding standard monosaccharide RRF (e.g. the glucose RRF was used to calculate the concentration of glucose in the oligosaccharide sample) with the following formula:

\[
C_S = \frac{C_{IS} \times PA_S}{RRF_S \times PA_{IS}}
\]
The concentration of each monomeric moiety in the oligosaccharide sample was used to calculate a relative molar ratio of the monomers in the sample. The concentration of the hexose moiety (glucose) was divided by its approximate molar mass (180) and, likewise, the pentose moieties (arabinose and xylose) were divided by 150. The resulting values were compared to determine the relative molar ratio of each monomeric moiety.

**NMR Analysis**

1D and 2D NMR experiments were performed on a Bruker Avance 400 MHz NMR equipped with a 3 mm z-gradient $^1$H/$^{13}$C dual-nucleus probe for sample 2 and on a Bruker 600 MHz NMR for sample 1. Purified samples were dissolved in deuterium oxide (99.99%) purchased from Cambridge Isotope Laboratories (Andover, MA, USA). The following spectra were collected for each sample $^1$H, $^{13}$C, COSY, HSQC, HMBC, H2BC, NOESY, TOCSY, and HSQC-TOCSY. A phase-sensitive COSY experiment was carried out for compound 1 using a pulse program with the optimized 90 degree pulse of 11µs and a relaxation time (d1) of 1 s.

$$\beta-D\text{-glucopyranosyl-(1→4)}[\alpha-D\text{-xylopyranosyl-(1→6)}]\beta-D\text{-glucopyranosyl-(1→4)}[\alpha-L\text{-arabinofuranosyl-(1→2)}\alpha-D\text{-xylopyranosyl-(1→6)}]-\beta-D\text{-glucopyranose (1)}$$

White amorphous powder; moderately hygroscopic; $^1$H NMR and $^{13}$C NMR data: see Table 1.5 and Figure 1.16; MSD-TOF (negative ion mode) [M-H] at m/z 1061.3121; ESI-QTOF-MS of permethylated compound (positive ion mode) [M+Na]$^+$ at m/z 1365.6545, see Table 1.2.
White amorphous powder; moderately hygroscopic; \(^1\)H NMR and \(^{13}\)C NMR data: see Table 1.6 and Figure 1.24; MSD-TOF (negative ion mode) [M-H] at \(m/z\) 1193.2786; ESI-QTOF-MS of permethylated compound (positive ion mode) [M+Na]+ at \(m/z\) 1525.7012, see Table 1.2.

1.3 Results and Discussion

The fractionation and isolation of cranberry products afforded two novel compounds (1 and 2, Figure 1.3) from the aqueous extract. Sequential fractionation and purification steps included Sephadex LH-20 size exclusion chromatography and HPLC with a C\(_{18}\) stationary phase, followed by a polyamine stationary phase for the final purification. The purity and carbohydrate nature of the isolated compounds was confirmed in the 1D NMR spectra and derivatization procedures ensued.

**MS Analysis**

Initial MSD-TOF spectra of the non-derivatized, purified oligosaccharides indicate the molecular ion; however, it is not present as a major ion peak. In all cases, the molecular ion is less than 50% of the relative intensity (data not shown). Further, compound 1 yields no molecular ion in the positive ion mode. Permethylation of the oligosaccharides provides confirmation of suspected molecular ion mass. Permethylated carbohydrates more readily form metal adducts as shown for tetramethylglucose (31), in addition, sodium has one of the highest affinities for oligosaccharides and sodium adducts produce increased fragmentation in tandem.
MS-MS analysis (31, 32). Samples were injected in a 1 mM NaOH solution to enhance metal-adduct formation and hence the ability to detect molecular ions. While sodiated ions are present, in this case, [M+NH₄]⁺ ions are present at the highest intensity which has been observed for olive pulp and other oligosaccharides (33, 34). Permethylated 1 elutes at 4.9 and 5.1 min and affords strong [M+NH₄]⁺ and [M+Na]⁺ molecular ions (m/z 1360.6652 and 1365.6263, respectively), with [M+NH₄]⁺ at 100% relative intensity in both eluted peaks (Figure 1.5). Permethylated 2 elutes at 5.2 and 5.4 min and affords the same type of molecular ions as permethylated 1 at m/z 1520.7398 and 1525.6988, respectively (Figure 1.5). It is likely that the appearance of two elution peaks is a result of the presence of both α- and β-anomers of the permethylated oligosaccharides. Additional ions at m/z 689.3450 and 769.3771 are apparent in the permethylated 1 and 2 spectra and represent the doubly charged molecular ions; this indicates the ability of the oligosaccharides to associate with two ammonium ions. This phenomenon is reported to occur with other xylan oligosaccharides (33). A secondary goal of permethylation and analysis by QTOF-MS is the investigation of molecular fragments to aid in identification of the connectivity of the monomers. However, in this analysis, minimal fragmentation is observed. Cancilla et al. (32) has shown an inverse correlation of degree of branching of oligosaccharides to fragmentation and the high level of branching in these oligosaccharides contributes to the lack of fragmentation observed. The exact masses of the oligosaccharides (Table 1.2) allow the calculation of the molecular formula and the assessment of the quantity of pentose and hexose sugar residues establishing the DP. Compound 1 contains four hexose and three pentose moieties and compound 2 contains four hexose and four pentose moieties.
Figure 1.5. ESI-QTOF-MS spectra of permethylated compounds 1 and 2.

Table 1.2. Exact mass, molecular formula, and DP of two oligosaccharides from cranberry.

<table>
<thead>
<tr>
<th></th>
<th>Compound 1</th>
<th>Compound 1-Permethylated</th>
<th>Compound 2</th>
<th>Compound 2-Permethylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exact Mass</td>
<td>1062.34</td>
<td>1342.6616</td>
<td>1194.39</td>
<td>1502.73</td>
</tr>
<tr>
<td>Molecular Formula</td>
<td>C_{39}H_{66}O_{33}</td>
<td>C_{59}H_{106}O_{33}</td>
<td>C_{44}H_{74}O_{37}</td>
<td>C_{66}H_{116}O_{37}</td>
</tr>
<tr>
<td>Degree of Polymerization</td>
<td>7</td>
<td>7</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Trimethylsilyl Derivatization

After obtaining the molecular formulae and DP, the identification of the sugar residues is pursued. Trimethylsilyl (TMS) derivatives are analyzed by GC-MS and the resulting retention times and fragmentation patterns in the MS spectra are compared to standard monosaccharide
derivatives. This particular derivatization is beneficial in that any acidic or amino-sugar residues are preserved. However, due to mutarotation during methanolysis of the oligosaccharide, each glycosyl residue yields four configurational isomers, including both α- and β-anomers of the furan and pyran rings. The TMS derivatives of 2 match the retention times of the major configurational isomers of arabinose, xylose, and glucose standards, indicating that only neutral glycosyl units are present (Figure 1.6). Owing to the similarity of NMR spectra, it is apparent that neutral sugar residues are also present in 1 and that the alditol acetate (AA) derivatization method, which is specific to neutral sugars, will better suit further analysis.

Figure 1.6. TIC chromatogram of trimethylsilyl derivatives of compound 2 from GC-MS analysis indicating the retention time (RT) and glycosyl identity.

AA

The AA derivatives produce a single product for each type of monomeric moiety present in a carbohydrate, therefore, this method of derivatization is better suited for quantification of the monomers (Figure 1.2 A). AAs of 1 and 2 are created through hydrolysis of the monomers, reduction to the alditol form, and O-acetylation of the hydroxy groups, which improves
volatilization in GC-MS analysis. The reduction using NaBD₄ provides an additional informative fragment in the MS spectra to allow confirmation of pentose or hexose residues.

Indicative fragment pairs of pentose and hexose residues appear at \( m/z \) 290/291 and \( m/z \) 361/362, respectively. The identities of the residues are confirmed by comparison of the retention times and MS spectra to the corresponding standards (Table 1.3). Through use of an internal standard, the molar ratio of the monomers in the compounds is established. By combining the information gained through MS analysis and the ratio of monomers present in the compounds, the exact number of each residue is identified as one arabinose, two xylose, and four glucose moieties in compound 1 and two each of arabinose and xylose, and four glucose moieties in compound 2 (Figure 1.7).

**Table 1.3** Alditol acetate analysis of compounds 1 and 2.

<table>
<thead>
<tr>
<th>Monomeric Moiety</th>
<th>RT of Standard (min)</th>
<th>RT of Sample (min)</th>
<th>Molar Ratio</th>
<th>RT of Sample (min)</th>
<th>Molar Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>19.45</td>
<td>19.82</td>
<td>1</td>
<td>19.90</td>
<td>1</td>
</tr>
<tr>
<td>Xylose</td>
<td>21.35</td>
<td>21.73</td>
<td>2</td>
<td>21.80</td>
<td>1</td>
</tr>
<tr>
<td>Glucose</td>
<td>26.19</td>
<td>26.88</td>
<td>4</td>
<td>27.06</td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 1.7. TIC chromatograms of alditol acetate derivatives of compounds 1 and 2 indicating the RT and glycosyl identity.

PMAA

The purpose of developing PMAA derivatives is to gain information about the ring size and location of the linkage(s) within a single glycosyl residue. This analysis answers two questions: 1) Is a particular monomeric moiety in the furanose or pyranose form? 2) How many residues is that monomer connected to and through which carbon is(are) the glycosidic bond(s) formed? The derivatization technique is similar to that of the AA preparation except that the samples are first methylated. This results in alditols that retain methoxy groups in the positions where free hydroxy groups exist in the original structure as shown in Figure 1.2 B with the exception of the reducing anomeric carbon. Acetoxy groups are present in the positions that are involved in either the linkage to an adjacent residue or in ring formation as highlighted by
corresponding colored boxes in Figure 1.2 B. The positions of the methoxy and acetoxy groups are determined by comparison of the relative retention times (RRT; relative to the internal standard) to that of PMAA derivatives of monosaccharide standards in GC-MS analysis. This comparison must be combined with the analysis of the fragmentation observed in the MS spectra and identification of distinguishing mass fragments (35) due to the potential overlap of RRTs.

The monomeric moieties are described in more detail with the information gained from this analysis (Table 1.4 and Figure 1.8). Each residue can be viewed as a puzzle piece containing a different shape and connectivity. Compound 1 contains one terminal arabinofuranoside, one terminal xylopyranoside, one 2-linked xylopyranoside, two 4- and 6-linked glucopyranosides, one terminal glucopyranoside, and one 4-linked glucopyranoside (Figures 1.8 and 1.9). Compound 2 contains two terminal arabinofuranosides, two 2-linked xylopyranosides, one terminal glucopyranoside, two 4- and 6-linked glucopyranosides, and one 4-linked glucopyranoside (Figures 1.8 and 1.10). The only residues that can definitively be placed in a terminal, non-reducing position are those that only contain an acetoxy group in the anomeric position. The residues listed above without the “terminal” denotation may or may not be linked through the anomeric position, even though the derivatives of all of these residues yield an acetoxy group in the anomeric position. This is a result of the replacement of a methoxy group in the anomeric position with an acetoxy group during derivatization. Accordingly, the 2-linked xylopyranoside may represent a xylopyranoside with two glycosidic linkages through C-1 and C-2 or it may represent a xylopyranose with a single substitution at C-2. It is apparent that, while a wealth of information is gained from this method, caution is necessary in the interpretation.

With the compilation of the data provided through derivatization and NMR analysis, steps can be taken towards arranging the monomeric moieties in their appropriate order.
Table 1.4. PMAA derivative analysis of compounds 1 and 2.

<table>
<thead>
<tr>
<th>TIC RT</th>
<th>Distinguishing Mass Fragments</th>
<th>Monomeric Moiety Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.05</td>
<td>118, 161, 162</td>
<td>terminal arabinofuranoside</td>
</tr>
<tr>
<td>12.28a</td>
<td>117, 118, 161, 162</td>
<td>terminal xylopyranoside</td>
</tr>
<tr>
<td>13.95</td>
<td>118, 161, 162, 205</td>
<td>terminal glucopyranoside</td>
</tr>
<tr>
<td>15.98</td>
<td>117, 190</td>
<td>2-linked xylopyranoside</td>
</tr>
<tr>
<td>18.38</td>
<td>118, 233</td>
<td>4-linked glucopyranoside</td>
</tr>
<tr>
<td>21.78</td>
<td>118, 261</td>
<td>4, 6-linked glucopyranoside</td>
</tr>
</tbody>
</table>

*aOnly present in compound 1

Figure 1.8. PMAA derivatives from compounds 1 and 2.

*Only present in compound 1.
Figure 1.9. TIC chromatogram of PMAA derivatives of compound 1 by GC-MS analysis indicating RT and glycosyl ring size and linkage. Minor peaks are contaminants and most are non-carbohydrate in nature based on analysis of MS fragmentation.

Figure 1.10. TIC chromatogram of PMAA derivatives of compound 2 by GC-MS analysis indicating RT and glycosyl ring size and linkage. Minor peaks are contaminants and most are non-carbohydrate in nature based on analysis of MS fragmentation.
**D/L Configuration**

Determination of the absolute configuration of the monomeric moieties in a carbohydrate is an essential aspect of understanding biological activity, as the presence of the enantiomer of a single residue may have a significant effect on the activity of a glycan-containing molecule. Through the reaction of L-cysteine methyl ester and phenyl isothiocyanate with hydrolyzed carbohydrate residues, phenyl thiocarbamates are created and provide optically active diastereomers of the enantiomeric carbohydrate reactants (Figure 1.2 C). These diastereomers are then readily analyzed using commonly available HPLC-UV systems (30). In order to conserve the purified oligosaccharide samples, two parent fractions containing both compounds 1 and 2 are included in the analysis, SN-03-Fr. 6 and SN-03. SN-03-Fr. 6 is a fraction enriched for the two compounds, while SN-03 contains several other compounds. Through comparison of retention times to standards (Figure 1.11) and spiking of the derivatized samples, the presence of D-(-)-arabinose is ruled out and the presence of D-(+)-glucose and D-(+)-xylose is confirmed. The remaining peak eluting at 18.65 min in SN-03-Fr. 6 closely overlaps with both L-(+)-arabinose and L-(−)-xylose standard RT (Figures 1.14 and 1.11). To provide definitive identification, an altered isocratic elution method is employed in addition to spiking both samples with L-(+)-arabinose and L-(+)-xylose on separate occasions (Figures 1.12 and 1.13). With this approach, it is clear that this peak is L-(+)-arabinose; thereby confirming the presence of a single enantiomer of each different sugar residue present in the oligosaccharides. Compounds 1 and 2 are both composed of D-(+)-glucose, L-(+)-arabinose and D-(+)-xylose (Figures 1.14 and 1.15).
**Figure 1.11.** HPLC-UV chromatogram of phenyl thiocarbamate derivatives of monosaccharide standards indicating RT.

![HPLC-UV chromatogram of phenyl thiocarbamate derivatives of monosaccharide standards indicating RT.](image)

**Figure 1.12.** HPLC-UV chromatogram of phenyl thiocarbamate derivatives of SN-03-Fr. 6 spiked with L-xylose indicating peak identity.

![HPLC-UV chromatogram of phenyl thiocarbamate derivatives of SN-03-Fr. 6 spiked with L-xylose indicating peak identity.](image)
Figure 1.13. HPLC-UV chromatogram of phenyl thiocarbamate derivatives of fraction SN-03 spiked with L-xylose indicating peak identity.

Figure 1.14. HPLC-UV chromatogram of the phenyl thiocarbamate of SN-03-Fr. 6 indicating RT and peak identity.

Figure 1.15. HPLC-UV chromatogram of phenyl thiocarbamate derivatives of fraction SN-03 indicating RT and peak identity.
The structural information gained from NMR experiments includes identification of the configuration at the anomeric carbon and of the linkage between glycosyl residues. The ability to confidently assign linkage of individual monomers relies upon the sound assignment of the key carbons involved in the glycosidic linkage. This section describes the identification of anomeric configuration of each monomer followed by the steps taken towards a full structural assignment of these oligosaccharides, including the glycosidic linkages.

The $^1$H NMR spectrum of compound 1 contains eight proton signals in the anomeric region at $\delta$ 4.38 (Glc-C), 4.42 (Glc-D), 4.43 (Glc-B), 4.53 ($\beta$-Glc-A), 4.83 (Xyl-F), 4.97 (Xyl-E), 5.04 (Ara-G), and 5.09 ($\alpha$-Glc-A) (Figure 1.16, Table 1.5). The corresponding $^{13}$C NMR chemical shifts to the anomeric protons presented in the HSQC spectrum (Figure 1.17) are $\delta$ 102.6, 102.51, 102.46, 95.68, 98.77, 98.56, 109.2, and 91.78, respectively. The reason for the appearance of eight anomeric signals, despite the presence of seven glycosyl residues, is attributable to the presence of one glycosyl residue (Glc-A) as both $\alpha$- and $\beta$-anomers. In general, anomeric signals with a relatively upfield chemical shift (e.g. > 4.70 ppm) are $\alpha$-anomers, while those with a relatively downfield chemical shift (e.g. < 4.70 ppm) are $\beta$-anomers (36) and can therefore be assigned as such. In addition, the $^3J_{H1,H2}$ values are used for further support. The $^3J_{H1,H2}$ coupling constant of glucose in a $^4C_1$ conformation has a value in the range of ca. 4-5 Hz for the $\alpha$-anomers due to a relatively small dihedral angle between H-1 and H-2. Likewise, for the $\beta$-anomers the value ranges from ca. 7-8 Hz due to a relatively larger dihedral angle. The $^3J_{H1,H2}$ of the glucose moieties of compound 1 are 7.13 Hz (Glc-C), 7.13 (Glc-D), 7.62 (Glc-B), 7.62 ($\beta$-Glc-A), and 3.63 ($\alpha$-Glc-A). This indicates $\beta$-anomeric glucose moieties, with the exception of Glc-A which, due to its reducing nature, undergoes mutarotation and hence is
present as both α- and β-anomers in solution. The \(^4\)C\(_1\) conformation of xylopyranose is similar to that of glucose and hence the same rules can be applied. The \(^3\)J\(_{\text{H1,H2}}\) values for the xylose residues are 2.74 and 3.32 Hz for Xyl-E and Xyl-F, respectively, which establishes the α-anomeric form for both. The \(^3\)J\(_{\text{H1,H2}}\) value of Ara-G is 1.92 Hz indicating its α-anomeric nature in agreement with published \(^3\)J\(_{\text{H1,H2}}\) values for α-L-arabinofuranosides (37, 38).

The remaining proton and carbon assignments are generated from the HSQC spectrum and further clarified with additional 2D NMR experiments. Many of the methine carbons are assigned using data provided by COSY and H2BC experiments (Figure 1.18). C-1 through C-3 and in some cases C-4 assignment is possible in this way for each glycosyl residue. The crowding of correlations in the δ 3.10-3.85 region masks many correlations which one would expect to observe. Although, Ara-G is fully elucidated with just these three 2D experiments, in combination with \(^1\)H and \(^13\)C NMR spectra, because most of the chemical shifts of this monosaccharide lie in less crowded regions. The HMBC spectrum provides further information to allow assignment of remaining carbons with the exception of C-5 and C-6 of Glc-A and C-6 of Glc-C (Figure 1.19). The HSQC-TOCSY experiment reveals the involvement of a carbon in the δ 79 region in the Glc-A ring system (Figures 1.20 and 1.16) and therefore allows the assignment of the δ 79 resonance to C-4; however a corresponding proton signal cannot be confirmed. The remaining C-6 resonances of Glc-A and Glc-C are known to have a similar chemical shift, δ 66.28 and 66.08, based on the fact that these two methylene carbons are the only two yet to be assigned. The position of both can be confirmed if a correlation is identified with just one of these two proton-carbon pairs. The proton signal at δ 3.88 (dd) corresponding to the carbon at δ 66.08 (Figure 1.16) is the optimal signal to pursue with a phase sensitive COSY experiment. With this experiment, a correlation between C-5 (δ 3.62) and C-6 (δ 3.88) of Glc-C
is identified (Figure 1.21), confirming the assignment of C-6 of Glc-C at δ 66.08 and C-6 of Glc-A at δ 66.28. Full 2D NMR spectra for compound 1 are reported in Appendix A.

With the structural assignment of compound 1 confirmed, the linkage of the monomeric moieties can be accurately assessed. The nature of the β-(1→4)-glucose backbone is determined through comparison of NMR spectra with that of cellotetraose (Figure 1.22) as well as through key NOESY correlations between H-3 (δ 3.52) of Glc-A and H-1 (δ 4.43) of Glc-B and between H-3 (δ 3.54) of Glc-B and H-1 (δ 4.38) of Glc-C (Figure 1.23). As for the side chains, the HMBC data (Figure 1.19) indicates a correlation between H-1 (δ 5.04) of Ara-G and C-2 (δ 78.78) of Xyl-E establishing a 1 → 2 linkage. The correlation between H-1 (δ 4.97) of Xyl-E and C-6 (δ 66.28) of Glc-A and between H-1 (δ 4.83) of Xyl-F and C-6 (δ 66.08) of Glc-C establishes a 1 → 6 linkage between the respective xylose and glucose residues. The assignment of C-6 of Glc-A and Glc-C is integral in determining the location the Xyl-E-Ara-G and Xyl-F side chains due to the attachment of each Xyl residues to one of the two methylene carbons at δ 66. Consequently, compound 1 is identified as the novel β-D-glucopyranosyl-(1→4)-[α-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→4)-[α-L-arabinofuranosyl-(1→2)-α-D-xylopyranosyl-(1→6)]-β-D-glucopyranose.
Table 1.5. \(^1\)H and \(^{13}\)C NMR data for compound 1.

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Figure 1.16. $^1$H and $^{13}$C NMR spectra of compound 1. $^1$H NMR B) 600 MHz spectrum; A) expanded anomeric region at 400 MHz; C) $^{13}$C NMR spectrum.
Figure 1.17. HSQC spectrum of compound 1 highlighting the anomeric region correlations.
Figure 1.18. COSY and key H2BC correlations of compound 1.
Figure 1.19. Key HMBC correlations of compound 1.

Figure 1.20. Key TOCSY and HSQC-TOCSY correlations of compound 1.
Figure 1.21. Phase sensitive COSY spectrum of compound 1 indicating a key correlation.

Figure 1.22. $^1$H and $^{13}$C NMR spectra of cellotetraose.
Figure 1.23. NOESY correlations of compound 1.

The $^1$H NMR spectrum for compound 2 contains eight signals in the anomeric region, similar to that of compound 1, with the major difference being the partial overlap of chemical shifts of the two xylose residues. This partial overlap is attributable to the increased similarity of the chemical environment of the residues, as compared to those in compound 1. The two doublets merge to form a signal resembling a triplet; a single chemical shift is used to indicate both xylose anomeric carbons because it is both unnecessary and unfeasible to distinguish between the two in further structural assignment. The anomeric protons resonate at δ 4.43 (Glc-C), 4.45 (Glc-D), 4.47 (Glc-B), 4.58 (β-Glc-A), 5.01 (Xyl-E and F), 5.09 (Ara-G and H), and 5.14 (α-Glc-A) (Table 1.6 and Figure 1.24). The corresponding $^{13}$C NMR signals are presented in the HSQC spectrum (Figure 1.25) and resonate at: δ 102.5, 102.5, 102.5, 95.6, 98.5, 109.2, and 91.7, respectively. As in compound 1, the reducing glucose (A) is present in both α- and β-
anomers with the α-anomer carbon and proton resonating at δ 91.7 and δ 5.14 ($^3J_{\text{H1,H2}}$ 3.90 Hz), respectively, and the β-anomer carbon and proton resonating at δ 95.6 and δ 4.58 ($^3J_{\text{H1,H2}}$ 7.99 Hz), respectively. The remaining glucose moieties are present as β-anomers as determined by the $^3J_{\text{H1,H2}}$ values of 8.21, 7.76, and 7.99 Hz for Glc-B, Glc-C, and Glc-D, respectively. The $^3J_{\text{H1,H2}}$ of 3.76 Hz for the two xylose moieties establishes these as α-anomers. The two Ara moieties are also defined as α-anomers, as in compound 1, by a $^3J_{\text{H1,H2}}$ value of 1.95 Hz.

The remaining protons and their respective carbons are identified using HSQC and the structural assignment of most signals is completed with COSY, H2BC, and HMBC data (Figures 1.26 and 1.27). HMBC resonances are identified between monosaccharide residues establishing the nature of the glycosidic linkages. The correlations between H-1 of Ara moieties (δ 5.09) and C-2 of Xyl moieties (δ 78.7) confirm the arabinoside-(1→2)-xyloside linkage which is identical to that observed in compound 1. The correlations between H-1 of Xyl moieties (δ 5.01) and C-6 of Glc-A and C (δ 66.3) also closely resemble the branching order in compound 1. Further 2D NMR experiments are presented in Appendix B (TOCSY, HSQC-TOCSY, and NOESY), however, no additional information is gained beyond that provided by the COSY, H2BC, and HMBC experiments. While NOESY or HMBC did not provide correlations indicating glycosidic linkage between glucose moieties, the β-(1→4)-glucose backbone was assigned as a result of the similarity of spectra of compound 1, compound 2, and cellotetraose.

It should be noted that proton and carbon signals with little or no apparent correlations were present in the data which suggest conformational flexibility of the oligosaccharide. The methylene carbons resonating at δ 60.0 and 60.5 correlated with a larger than expected number of protons in the HSQC spectrum (Table 1.6 and Figure 1.28) and all proton signals were considered in assignment. Two carbon signals were present at δ 59.85 and 60.0 which were
lumped together as the δ 60.0 signal. The presence of the two small carbon signals as well as the larger than expected number of proton correlations reveals variation in chemical shift values which may be a result of conformational changes. This change can be expected in such a large molecule containing sp³ hybridized carbons. Further, the location of the C-6 resonances at δ 60.0 and 60.5 as oxymethylene tails surrounded by neighboring disaccharide side chains may result in intramolecular interactions that influence electron shielding or deshielding of the methylene groups. Another assignment in which shifting, likely as a result of conformational change, is apparent is in C-3 of Glc-C. In this case, the vicinal C and H signals presented dual correlations with the carbons at both δ 75.4 (H, δ 3.42) and 75.9 (H, 3.40) in COSY, H2BC, and HMBC experiments (Figure 1.26). As a result, resonances at δ 75.4 and 75.9 are assigned to C-3 of Glc-C.

The NMR data for compound 2 contains a number of signals and correlations that are determined to be insignificant for the structural assignment. For example, a large singlet at δ 3.27 correlating with a carbon signal at δ 48.8 in the HSQC spectrum is an impurity that appeared subsequent to initial NMR examination. In addition, a doublet of doublets at δ 3.39 could not be assigned in any 2D experiments except for a single correlation in the COSY data, and a small group of correlations in the regions δC 78-79 and δH 3.49-3.59 are left inexplicable. Owing to the knowledge of sample contamination and the potential for conformational shifting, the structural assignment of compound 2 integrates reliable signals gained from the NMR data and chemical shift information obtained for compound 1, resulting in the fully elucidated structure. This establishes the structure of the novel octasaccharide 2 as β-D-glucopyranosyl-(1→4)-[α-L-arabinofuranosyl-(1→2)-α-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-(1→4)-β-D-
glucopyranosyl-(1→4)-[α-L-arabinofuranosyl-(1→2)-α-D-xylopyranosyl-(1→6)]-β-D-glucopyranose.

Table 1.6. $^1$H and $^{13}$C NMR data for compound 2.

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Figure 1.24. $^1$H and $^{13}$C NMR DEPT spectra of compound 2.
Figure 1.25. HSQC spectrum of compound 2 indicating anomeric correlations.
Figure 1.26. COSY and key H2BC correlations of compound 2.
Figure 1.27. Key HMBC correlations of compound 2.

Figure 1.28. HSQC of compound 2 focusing on the oxymethylene C and H signals.
The structure elucidation presented herein provides detailed structural characteristics of two novel oligosaccharides from cranberry. This is the first presentation of a thorough elucidation of oligosaccharides of this size and nature from plant sources. Oligosaccharides isolated from wine (Grignolino and Chardonnay) of similar structure have been suggested, however the extent of analysis did not provide confirmed structures (20). The structural similarity lies in the substituted tetraglycosyl backbone, however, the side chain locations and glycosyl composition are inconsistent with that presented here. Not one single method for structure elucidation of carbohydrates, for example, ESI-QTOF-MS or NMR alone, can fully describe each complexity of a compound class blessed with enormous potential for variety. The dynamic structure elucidation scheme presented herein provides a model to guide further investigation into complex oligosaccharide structures. In addition, the presentation of these novel compounds invites future investigation into biological activity. The relatively small degree of polymerization and molecular stability make these compounds potential candidates for prebiotic and anti-adhesion activities.
CHAPTER 2

BIOACTIVITY OF CRANBERRY OLIGOSACCHARIDES
2.1 Introduction

As seen in the first chapter, oligosaccharides have the potential to offer many benefits to human health including benefits to the immune system \(3, 17\), the gastrointestinal tract by providing a prebiotic effect \(4\), and the potential for use in the treatment and prevention of microbial infections \(2\). In addition, cranberry products have shown antimicrobial activity and antiviral activity. An extensive study against various foodborne bacterial pathogens indicates the importance of a water soluble phenolic extract in the inhibition of growth of several pathogens including two strains of \textit{Escherichia coli}, \textit{Staphylococcus aureus}, \textit{Listeria monocytogenes}, and \textit{Pseudomonas aeruginosa} \(9\). Antiviral activity includes inhibition of the influenza virus by a high molecular weight fraction of cranberry non-dialyzable material \(10\) and inhibition of norovirus by cranberry proanthocyanidins \(39\). Further, cranberry extracts are being assessed for anticancer effects and have been shown to be effective against several cancer cell types, including ovarian, prostate, colon, and neuroblastoma cancers \(6-8, 40\). The most studied fractions of cranberry assessed for anticancer activity are phenolic fractions \(6, 8\) and, more specifically, proanthocyanidin extracts \(7, 40\). Likewise, both antimicrobial and anticancer studies have focused on phenolic extracts from cranberry products leaving a void of information regarding cranberry carbohydrate biological activity.

In addition to recent evidence of antimicrobial and anticancer properties, cranberries are widely known for their traditional use in the treatment and prevention of urinary tract infections (UTIs) \(41, 42\). Although the true efficacy of the consumption of cranberry products to aid in the prevention of UTIs has been in question due to heterogeneity in clinical trials and patient populations \(43\), recent reviews of clinical trials support the efficacy in young and middle-aged women \(11, 42\). The pathogenesis of urinary tract bacterial infection is well understood,
especially for infections caused by *E. coli*, which is the causative pathogen of more than 80% of UTIs in young and adult women (44). Infection begins with the adhesion of bacteria to the epithelial cells lining the urinary tract. Adhesion is followed by biofilm formation and invasion into the uroepithelial cells (44). The site of attachment to uroepithelial cells has been identified for several strains of *E. coli*, including Type 1 which binds to the Manα3Manα6Man moiety of glycoproteins on the cell surface and P-fimbriated *E. coli* which binds to the Galα4Gal moiety of glycolipids on cell surfaces (2). While Type 1 fimbriae can be inhibited by a number of natural products or synthesized compounds containing D-mannose (2), cranberry products exhibit a unique effectiveness against the adhesion of P-fimbriated *E. coli* (12, 45). The effectiveness of cranberry products in the treatment of P-fimbriated *E. coli* UTIs has been assessed extensively with in vitro assays using cranberry juice cocktail (45), cranberry extracts (14, 46, 47), and urine collected from individuals after consumption of cranberry juice (13, 48, 49).

**Figure 2.1.** Chemical structure of quercitin, myricetin, and an A-type proanthocyanidin.
Chemical investigation of cranberry products has afforded bioactive compounds that exhibit an anti-adhesive effect against P-fimbriated *E. coli*. These compounds include A-type proanthocyanidins (PACs, Figure 2.1), which have been hailed as a unique and highly active group of polymers (14, 50), and other putatively active flavonoids (51). While it is reported that PACs are the most important compounds in cranberry that possess this bioactivity against bacterial UTIs, an even more important question is whether these compounds reach the urinary tract in their native form. As of yet, it has been difficult to identify non-metabolized phenolic cranberry compounds, such as myricetin and its isomer quercetin (Figure 2.1), in human urine. However, metabolites of phenolic compounds are readily identifiable (52). Recent work has established the tentative presence of myricetin and quercetin analogues in urine; albeit at low concentrations that may not be effective in the prevention or treatment of UTIs (53). This further implies the rarity of high molecular weight phenolic compounds, such as PACs, reaching the bladder in their native form. Previous work by Christina M. Coleman in this laboratory has revealed the presence of a putative bioactive oligosaccharide-containing fraction from pig urine, collected after the consumption of spray-dried cranberry juice powder (unpublished data). The chromatographic profile of this fraction closely resembles the chromatographic profile of the parent fraction (SN-03) to the oligosaccharides presented in this work (Figure 2.2). While this fraction could not be purified to its major constituents, NMR analysis suggests the presence of compound 2 as well as other oligosaccharides and phenolic compounds (unpublished data). Synthesized oligosaccharides, such as galabiose and derivatives of galabiose have been shown to possess anti-adhesive activity (54) which calls into question the potential bioactivity of compounds 1 and 2 as well as oligosaccharide fractions from cranberry in the prevention of adhesion of *E. coli* to uroepithelial cells.
Figure 2.2. HPLC-ESLD chromatograms of SN-03 (A) and an oligosaccharide-enriched fraction (B) from pig urine collected after spray-dried cranberry juice consumption. Chromatographic conditions: Waters Delta Prep 4000 and 2487 Dual Wavelength UV and PL-1000 ELS detectors in series. Stationary phase: Atlantis dC$_{18}$ (19 x 250 mm, 10 µm); mobile phase: (A) 0-10 min H$_2$O, 10-50 min H$_2$O to 70% aq. MeOH; (B) 0-5 min H$_2$O, 5-35 min H$_2$O to 70% aq. MeOH. The different gradient times result in different retention times of the peaks.

The research herein presents an analysis of the bioactivity of cranberry oligosaccharide fractions and compounds 1 and 2 (Figure 1.3). The contribution of cranberry oligosaccharides to antimicrobial activity is assessed as well as anticancer activity. Finally, an in vitro assay utilizing radio-labeled P-fimbriated E. coli and uroepithelial cells is used to assess anti-adhesion activity.
2.2 Experimental Methods

Antimicrobial Assay

This assay was completed in the antimicrobial screening program available at the National Center for Natural Products Research (NCNPR) according to previously reported methods (55, 56) which have been modified from the Clinical and Laboratory Standards Institute (CLSI) methods, formerly the National Committee for Clinical and Laboratory Standards (NCCLS) (57-60). Cranberry samples were tested against a panel of 10 pathogenic organisms; five of which were fungi (*Candida albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigates* ATCC 204305) and five of which were bacteria (*Staphylococcus aureus* ATCC 29213, *methicillin-resistant Staph aureus* ATCC 33591, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068). The inhibition of growth of each organism by compound 2 was assessed at 20, 4, and 0.8 µg/mL and the IC$_{50}$ was calculated using XLFit curve fitting software. The IC$_{50}$ of SN-03 was calculated in the same manner using test concentrations of 200, 40, and 8 µg/mL. The IC$_{50}$ represents the concentration (µg/mL) at which 50% inhibition of the organism is exhibited relative to positive and negative controls.

Breast Cancer Cell Viability Assay

Breast cancer cells T47D and MDA-MB-231 were seeded into a 96 well culture plate at 30,000 cells per well and incubated at 37 °C for 24 hrs in 200 µL of 10% FBS DMEM/F12 medium supplemented with 50 µg/mL penicillin/streptomycin. Compounds 1 and 2 (100 µL) were added into separate wells at 10, 30, and 100 µM concentrations and fraction SN-03 was added at 30 and 100 ppm and samples were tested in triplicate. Chlorhexamide (CHX; 100 µL,
10 µM) was used as a positive control in addition to a media control. The cultures were incubated at 37 °C for 48 hr under normoxic conditions. The cells were fixed with 10% trichloroacetic acid (TCA) for 1 hr at 4 °C and washed four times with H₂O then allowed to air dry. Sulforhodamine B (0.4%) in 1% HOAc was added and allowed to sit for 15 min at room temperature. The culture wells were washed four times with 1% HOAc and allowed to air dry. Tris (200 µL, 10 mM) was added and the plates were placed on a rotary table for 5 min and vortexed. Plates were read at 490/690 nm on a microplate reader. Percent inhibition was calculated as:

\[
\text{% Inhibition} = \frac{1 - \overline{X}_s}{\overline{X}_{ctrl}}
\]

Where \( \overline{X}_s \) is the average absorbance of the triplicate wells incubated with a sample and \( \overline{X}_{ctrl} \) is the average absorbance of the triplicate media control wells.

**Escherichia coli-Uroepithelial (EC-UEC) Cell Anti-adhesion Assay**

The EC-UEC anti-adhesion assay was recently developed in the laboratory of Dr. Boon Chew at Washington State University and a publication detailing the method is not available. Briefly, the procedure is as follows: human uroepithelial cells (UEC; HTB-4) were seeded at 1x10⁵ cells per well in a 96 well culture plate and rinsed with PBS. P-fimbriated *E. coli* (ATCC 700928) were subcultured in colony forming antigen (CFA) agar to promote expression of P-fimbriae (61) and incubated for 4 hr at 35 °C with \(^3\text{H}\)-uridine. The cells were rinsed with phosphate buffered saline (PBS) and incubated with cranberry samples for 30 min at 35 °C. \(^3\text{H}\)-*E. coli* and UECs were incubated together in a ratio of 400:1, respectively, for 1 hr at 37 °C and washed with PBS. Radioactivity of the wells was measured by liquid scintillation. Each
sample was tested in duplicate in addition to a media control, solvent control, and two positive controls (myricetin and A-type proanthocyanidin enriched cranberry extract). Inhibition curves were collected with four concentration points that resulted in serial dilutions of the highest concentration attainable, which varied depending on sample availability. IC$_{50}$ was calculated for positive controls but was unattainable for cranberry samples.

2.3 Results and Discussion

*Antimicrobial Assay*

Compound 2 and fraction SN-03 are both ineffective at inhibiting growth of 10 pathogenic micro-organisms, five fungi and five bacteria, as shown in the NCNPR antimicrobial screen (Table 2.1). The lack of antimicrobial activity supports a mechanism of microbial disease prevention or treatment that involves non-cytotoxic effects, such as anti-adhesion. It is important to assess several mechanisms of microbial inhibition when determining the mechanism with which a compound is involved. Other compounds present in cranberry, such as phenolic compounds and proanthocyanidins possess antimicrobial activity, irrespective of anti-adhesion activity, at µg concentrations (9) and this effect may confound results in an anti-adhesion assay.
Table 2.1. IC$_{50}$ of compound 2, fraction SN-03, and control against five fungal and five bacterial pathogenic species.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Compound 2</th>
<th>SN-03</th>
<th>Amp B$^a$</th>
<th>Cipro$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida albicans</em></td>
<td>NA$^c$</td>
<td>NA</td>
<td>1.20</td>
<td>ND$^d$</td>
</tr>
<tr>
<td><em>C. glabrata</em></td>
<td>NA</td>
<td>NA</td>
<td>0.71</td>
<td>ND</td>
</tr>
<tr>
<td><em>C. kreas</em></td>
<td>NA</td>
<td>NA</td>
<td>1.18</td>
<td>ND</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em></td>
<td>NA</td>
<td>NA</td>
<td>1.17</td>
<td>ND</td>
</tr>
<tr>
<td><em>Cryptococcus neoformans</em></td>
<td>NA</td>
<td>NA</td>
<td>0.31</td>
<td>ND</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>NA</td>
<td>NA</td>
<td>ND</td>
<td>0.09</td>
</tr>
<tr>
<td><em>MRSA</em></td>
<td>NA</td>
<td>NA</td>
<td>ND</td>
<td>0.07</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>NA</td>
<td>NA</td>
<td>ND</td>
<td>0.00</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginans</em></td>
<td>NA</td>
<td>NA</td>
<td>ND</td>
<td>0.06</td>
</tr>
<tr>
<td><em>Mycobacterium intracellulare</em></td>
<td>NA</td>
<td>NA</td>
<td>ND</td>
<td>0.33</td>
</tr>
</tbody>
</table>

$^a$ Amphotericin B, positive control for fungal pathogens; $^b$ Ciprofloxacin, positive control for bacterial pathogens; $^c$ Not active; $^d$ Not determined; $^e$ Methicillin-resistant *Staph aureus*

*Breast Cancer Cell Viability Assay*

A cell viability assay using two breast cancer cell types (T47D and MDA-MB-231) facilitates assessment of the anticancer activities of compounds 1 and 2 and fraction SN-03. Anti-proliferative effects against two colon cancer cell lines are observed with treatment of cranberry phenolic extracts (8). PACs also seem to be important in anti-proliferative activity against different cancer cell lines as well as human umbilical vein endothelial cells (HUVECs) (7, 40). However, the pure oligosaccharides and enriched fractions do not possess cytotoxic effects against the two breast cancer cell lines (Table 2.2). The majority of biological assays of cranberry are focused on phenolic extracts, however, assessing cranberry extracts of a different chemical class, such as carbohydrates, provides a more thorough understanding of potential bioactivity. This preliminary data suggests that the anti-proliferative effects of cranberry extracts against both a variety of cancer cell types and HUVECs are a result of phenolic compounds rather than carbohydrates.
Table 2.2. Percent inhibition of breast cancer cell viability (T47D and MDA-MB-231) in normoxic conditions by compounds 1 and 2, and fraction SN-03.

<table>
<thead>
<tr>
<th></th>
<th>T-47D % Inhibition (SE)</th>
<th>MDA-MB-231 % Inhibition (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 µm</td>
<td>30 µm</td>
</tr>
<tr>
<td>Compound 1</td>
<td>-3 (± 4)</td>
<td>-6 (± 4)</td>
</tr>
<tr>
<td>Compound 2</td>
<td>-3 (± 3)</td>
<td>-6 (± 11)</td>
</tr>
<tr>
<td>SN-03</td>
<td>5a (± 3)</td>
<td>2b (± 3)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>T-47D % Inhibition (SE)</th>
<th>MDA-MB-231 % Inhibition (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 µm</td>
<td>30 µm</td>
</tr>
<tr>
<td>Compound 1</td>
<td>0 (± 1)</td>
<td>-1 (± 2)</td>
</tr>
<tr>
<td>Compound 2</td>
<td>-2 (± 2)</td>
<td>-3 (± 1)</td>
</tr>
<tr>
<td>SN-03</td>
<td>0 (± 2)</td>
<td>1 (± 2)</td>
</tr>
</tbody>
</table>

a Tested at 30 ppm; b Tested at 100 ppm

EC-UEC Anti-adhesion Assay

This assay is under development and is performed at Washington State University by Dr. Boon Chew. The method involves radio-labeling P-fimbriated E. coli using [3H]-uridine, which is incorporated into the RNA of the bacterial cells. A similar method of labeling DNA with [3H]-thymidine shows the effectiveness of radio-labeling as a method to quantify bacteria and bacterial growth (62). The radio-labeled, washed E. coli cells are first incubated with the sample and the mixture is transferred to the UEC wells and incubated a second time to allow adhesion of the E. coli to the UECs, similar to a previously described method using unlabeled E. coli (51). Any bacterial cells that do not adhere to the UECs are washed away, leaving behind adhered, radio-labeled E. coli cells which are then quantified by liquid scintillation. Considering that this assay is still under development, it would be helpful to first address the issues that arose in the testing of this cranberry sample set. First, the intention of the assay was to develop a six point IC₅₀ curve in which a single IC₅₀ would be calculated for each sample to allow comparison between samples. A complete inhibition curve is attained for myricetin, the positive control, however, cranberry samples did not reach maximum inhibition due to a limitation of sample
quantity and hence a less than optimal maximum concentration. This limitation prevents the
calculation of the IC\textsubscript{50} and consequently limits a quantitative comparison between samples.
Second, due to the variation in sample matrix, the range of measured radioactivity (counts per
minute, cpm) varies from sample to sample; for example, myricetin ranges from \sim 6200 to 4300
cpm while aq. SN ranges from \sim 12000 to 2000 cpm. This variation also limits the ability to
compare activity between two samples and the ability to compare samples to the solvent control
(4383 cpm). Despite the problems presented, a classification of active or not active can be
assigned to each sample based on the shape of the dose response curves. Samples with an
overall negative slope (e.g. Figures 2.3 C, D) are considered active and samples with an overall
positive slope (e.g. Figure 2.4 A) are considered inactive. Samples in which the overall slope is
questionable (e.g. Figures 2.4 B, C) are considered potentially active in the sense that a negative
slope appears at higher concentrations and if samples could be tested at higher concentrations
than were reached in the data presented then increased anti-adhesion is possible.

The aq. SN extract, and fractions SN-03 and SN-04 that result from it, exhibit a dose
dependent anti-adhesive effect as seen by the decrease in radioactivity which parallels a
decreased number of \textit{E. coli} adhered to UECs (Figures 2.3 A, B, C). The slope of the dose
response curves of SN-03 and SN-04 resemble that of the positive control, myricetin (Figure 2.3
D) and are thus considered active in their ability to prevent adhesion of \textit{E. coli} to UECs. A
qualitative assessment of the curves suggests an enhanced anti-adhesive effect of aq. SN due to
the increased slope of the curve and lower final level of radioactivity. This result may be a
combination of anti-adhesion and antimicrobial activity of the sample, as cranberry juice
fractions have been shown to possess antimicrobial activity, specifically, against \textit{E. coli}.  

63
Figure 2.3. Dose response curves of aq. SN (A), SN-03 (B), SN-04 (C), and myricetin (D) in the EC-U2C anti-adhesion assay.

The fractionation of SN-03 yields three fractions containing compounds 1 and 2. SN-03-Fr. 4 contains compound 1 as its major constituent and SN-03-Fr. 5 contains compound 2 as its major constituent. SN-03-Fr. 6 contains both compounds 1 and 2 in addition to other minor constituents. Anti-adhesive activity appears to be lost in these fractions (Figure 2.4 A, B, C) as compared to the parent fraction. This lack of activity is also exhibited in purified 1 and 2 from SN-03-Fr. 4 and 5, respectively (Figure 2.5 A, B).
**Figure 2.4.** Dose response curves of SN-03-Fr. 4 (A), Fr. 5 (B), and Fr. 6 (C) in the EC-UEC anti-adhesion assay.
The progressive lack of anti-adhesive activity in the purification of compounds 1 and 2 from the parent fractions indicates that the oligosaccharides alone may not be the source of the bioactivity. Being that compounds 1 and 2 are present in the highest quantity in the active SN-03 fraction, one would assume their involvement in the bioactivity. It is possible that another compound is present in the SN-03 fraction that is solely responsible for the bioactivity, however, upon investigation of the bioactivity of the remaining SN-03 fractions (Figures 2.6 A, B, C, D, E) this is unlikely. SN-03-Fr.2 was not assayed due to sample limitation; this fraction was collected over a time period in which no chromatographic peaks eluted. All of the bioassayed fractions collected from SN-03 have similar dose response curves suggesting that a single compound does not stand out above the others in terms of bioactivity. Fractions 5, 6 (Figures 2.4 B, C) and 9 (Figure 2.6 E) exhibit curves with a negative slope above a test concentration of 7 mg/mL. It is possible that the concentrations that were examined are not high enough to exhibit activity and if these fractions were tested at higher concentrations, bioactivity may be seen.
Figure 2.6. Dose response curves of SN-03-Fractions 1 (A), 3 (B), 7 (C), 8 (D), and 9 (E) in the EC-UEC anti-adhesion assay.

A synergistic interaction between the compounds present in SN-03 is also important to consider. The low resolution of the major chromatographic peaks in SN-03 (Figure 1.4) suggest several compounds of similar composition; that is, several oligosaccharides constitute this fraction. The phenyl thiocarbamate analysis described in Chapter 1 reveals that four monosaccharides (Ara, Glc, Xyl, and Gal; Figure 1.15) are present in SN-03 after hydrolysis, of
which only three were identified in the purified compounds. In addition, a simple anthocyanidin test on SN-03 indicates the presence of a small amount of proanthocyanidin-type compound(s) (data not shown). This is also apparent in the lavender-maroon color of the dried SN-03 fraction, the color which eluted in SN-03-Fr. 9. Interactions have been shown to occur between proanthocyanidins, or other polyphenolic compounds, and carbohydrates (63, 64). The potential interaction between oligosaccharides present in this fraction with proanthocyanidin-type compounds may have a synergistic effect on the anti-adhesive activity of the fraction. NMR studies of purified oligosaccharides and purified compounds from fraction 9 would be helpful in elucidating potential interactions.

In order to fully understand the anti-adhesive bioactivity of the oligosaccharides in cranberry, continued isolation and analysis are necessary. Bioassay of higher concentrations of the purified compounds and fractions would confirm the presence of bioactivity, especially in SN-03-Fr. 5, 6, and 9. In addition, testing the fractions of SN-03 in different combinations may reveal a synergy or combined anti-adhesive effect that matches the activity of the parent fraction. For example, the combination and subsequent bioassay of SN-03-Fr. 5 and 9 may exhibit bioactivity.

In conclusion, the oligosaccharide-enriched fraction, SN-03, and the aq. SN extract are confirmed to possess anti-adhesive activity, inhibiting the adhesion of P-fimbriated E. coli to uroepithelial cells. Continued bioassay of SN-03 fractions will confirm the nature of anti-adhesion activity of the parent fraction which may be a result of synergistic interaction of the constituents. A lack of antimicrobial activity of SN-03 further supports the anti-adhesive activity by ruling out a cytotoxic effect on the bacterial cells. In addition, the absence of both
antimicrobial and anticancer activity of these oligosaccharide rich fractions and pure compounds indicate that free carbohydrates are not likely involved in this type of bioactivity.
LIST OF REFERENCES


60. NCCLS Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard, M38-A; National Committee on Clinical Laboratory Standards: 2002.


LIST OF APPENDICES
APPENDIX A: 2D NMR SPECTRA OF COMPOUND 1
APPENDIX B: 2D NMR SPECTRA OF COMPOUND 2
VITA

EDUCATION

Graduated Summa cum Laude
Humboldt State University, Arcata, CA, 95521

Central Oregon Community College, Bend, OR, 97701

RESEARCH POSITIONS

Graduate Research Assistant  Jan. 2011 – present
The University of Mississippi, Department of Pharmacognosy, Oxford, MS
- Extraction and isolation using various chromatographic techniques
- Structure elucidation of carbohydrates from Vaccinium macrocarpon Aiton using 1D and 2D NMR, GC-MS, and MS-MS
- Derivatization of complex carbohydrates
- Human urinary metabolite analysis
- Advisor: Dr. Daneel Ferreira

Visiting Scholar  June 2010 - Aug. 2010
National Center for Natural Products Research, The University of Mississippi, University, MS
- Composition analysis of the lichen Lobaria oregana
- Extraction and isolation using various chromatographic techniques
- Structure elucidation using 1D and 2D NMR and MS
- Supervisor: Ms. Julie Mikell

Field Botanist  June 2009- Aug. 2009
University of Washington, School of Forest Resources, Seattle, WA
- Ecological data collection along permanent transects in the Cascade Mountains of Oregon
- Plant identification and vegetation monitoring
- Supervisors: Dr. Charles Halpern and Dr. Ryan Haugo
TEACHING POSITIONS

**Graduate Teaching Assistant**  
Sept. 2011 – May 2013

The University of Mississippi, School of Pharmacy,  
Department of Pharmacognosy, Oxford, MS

**Instructor**  
March 2009 - Dec. 2010

COCC, Dept. of Natural and Industrial Resources, Bend, OR  
Plant Identification and Forest Ecology  
Department Chair and Supervisor: Dr. Michael Fisher

**COCC Tutor**  
Sept. 2008 - Dec. 2010

COCC, Department of Testing and Tutoring, Bend, OR

OTHER POSITIONS

**COCC Arboretum Project**  

COCC, Special Grant, Bend, OR  
- Inventory, identification, and mapping of all planted trees on the college campus  
- Supervisors: Jim Jones (VP & Chief Financial Officer), Dr. Christine Ott-Hopkins

AWARDS AND HONORS

**AWARDS**

- *Teaching Assistant of the Year for the PY2 class 2013*  
The University of Mississippi, School of Pharmacy

- *GSC Research Day Poster Award, 2nd Prize*  
The University of Mississippi, Graduate Student Concil

- *Best Student Poster Presentation*  
International Congress on Natural Products Research, New York City, August 2012

- *Best Podium Presentation*  
The University of Mississippi, Dept. of Pharmacognosy Annual Retreat, University, MS, August 2011
- **Dennis K. Walker Botany Award**  
  Humboldt State University, Dept. of Biological Sciences, Arcata, CA 95521, May 2008

- **Governor’s Scholars Award**  
  State of California, 2001

**HONORS**

- **Summa cum Laude graduate**  
  Humboldt State University, Dept. of Biological Sciences, Arcata, CA 95521, May 2008

- **Presidential Scholar**  
  Humboldt State University, Dept. of Biological Sciences, Arcata, CA 95521, Aug. 2004-May 2008

- **Dean’s List**  
  Central Oregon Community College, Bend, OR, 97701, Sept. 2002 – June 2004

**ORAL PRESENTATIONS**

“**Breaking it down: Approaches to complex carbohydrate structural analysis**”  
May 2012
Auker, K. and D. Ferreira. 39th Annual MALTO – Medicinal Chemistry-Pharmacognosy Meeting  
University of Louisiana at Monroe, LA

“**From HIV to cancer: The CXCR4 G-protein coupled receptor as a therapeutic drug target**”  
March 2012
Auker, K. Spring 2012 Pharmacognosy Seminar Series  
The University of Mississippi, University, MS

“**Breaking it down: Approaches to complex carbohydrate structural analysis**”  
August 2011
Auker, K. and D. Ferreira. Pharmacognosy Departmental Retreat 2011  
The University of Mississippi, University, MS