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Arabinoxyloglucan Oligosaccharides May Contribute to the Antiadhesive Properties of Porcine Urine after Cranberry Consumption

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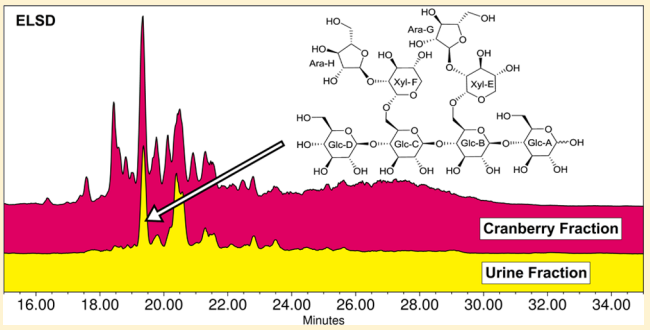
Supporting Information

ABSTRACT: Cranberry (*Vaccinium macrocarpon*) juice is traditionally used for the prevention of urinary tract infections. Human urine produced after cranberry juice consumption can prevent *Escherichia coli* adhesion, but the antiadhesive urinary metabolites responsible have not been conclusively identified. Adult female sows were therefore fed spray-dried cranberry powder (5 g/kg/day), and urine was collected via catheter. Urine fractions were tested for antiadhesion activity using a human red blood cell (A+) anti-hemagglutination assay with uropathogenic P-fimbriated *E. coli*. Components were isolated from fractions of interest using Sephadex LH-20 gel filtration chromatography followed by HPLC on normal and reversed-phase sorbents with evaporative light scattering detection. Active urine fractions were found to contain a complex series of oligosaccharides but not proanthocyanidins, and a single representative arabinoxyloglucan octasaccharide was isolated in sufficient quantity and purity for full structural characterization by chemical derivatization and NMR spectroscopic methods. Analogous cranberry material contained a similar complex series of arabinoxyloglucan oligosaccharides that exhibited antiadhesion properties in preliminary testing. These results indicate that oligosaccharides structurally related to those found in cranberry may contribute to the antiadhesion properties of urine after cranberry consumption.



Cranberry [*Vaccinium macrocarpon* Ait., (Ericaceae)] products are popular and widely available in U.S. food, juice, and dietary supplement markets.[1] Cranberry juice is commonly used as a folk remedy for the prevention of urinary tract infections (UTIs). P-Fimbriated *Escherichia coli* have been found to cause at least 80% of uncomplicated, community-acquired UTIs,[2,3] and recurrent UTIs are an increasingly recognized problem.[4] The overall high incidence of UTIs results in estimates of billions of dollars spent annually ($2.3 billion in 2010) for direct health care and other societal costs.[4,5] The prevention of UTIs is preferable to treatment with antimicrobial agents, as the use of preventive strategies would not provide a selective pressure for the development of antibacterial drug resistance.[6,7] Preventative therapies are also generally equally effective against both drug-susceptible and drug-resistant strains of bacteria.[6] As the initiation of a UTI requires the initial step of bacterial adhesion to uroepithelial membranes, compounds or products that interfere with bacterial adhesion are of great medical interest.[6] Anecdotal and clinical evidence supports the ability of cranberry products to help prevent UTIs by inhibiting bacterial adhesion in the urinary tract after consumption of cranberry.[8−11] Some clinical reports, however, are inconclusive or do not directly support the use of cranberry for preventing UTIs.[12,13] This controversy prompts questions regarding which types of UTIs can be prevented by cranberry and highlights the essential need for a better understanding of cranberry antiadhesive constituents.[14−17]

Cranberry juice and various extracts and other preparations of cranberry have shown the ability to prevent the adhesion of P-fimbriated *E. coli* to surfaces in several studies and in vitro biological assays.[18−22] Cranberry proanthocyanidins (PACs), specifically PAC dimers, trimers, and oligomers that contain at least one A-type linkage (Figure 1), have been isolated from cranberry and shown in vitro to prevent the adhesion of P-fimbriated *E. coli* to surfaces.[23−25] The predominant hypothesis

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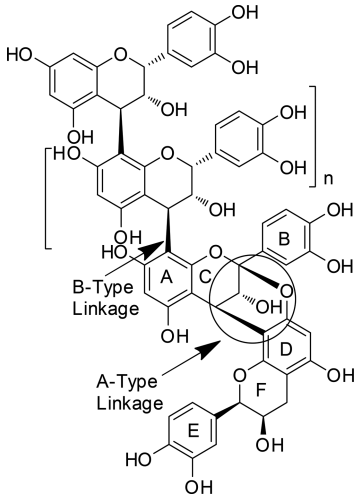

Figure 1. Structure for an example of an oligomeric PAC with indicated A- and B-type linkages.

regarding cranberry antiadhesion properties since 1998 has therefore been that PACs are the primary compounds responsible for the antiadhesion properties of cranberry and for the ability of cranberry materials to prevent UTIs.

In spite of the recognized antiadhesion properties of PACs, questions exist regarding the role of these compounds in the antiadhesion properties of urine after cranberry consumption. PACs are known to form nonspecific, covalent bonds with proteins[26] and recent studies have indicated that PACs have a low absorption and bioavailability, with limited transport across Caco-2 intestinal epithelial cell monolayers.[27] A PAC-type drug (crofelemer) has been approved by the U.S. FDA for the treatment of noninfectious diarrhea in HIV patients,[28] and it has been proposed that PACs could have protective effects for intestinal epithelial cells by preventing extraintestinal *E. coli* invasion.[29] Studies that have been specifically designed to detect the presence of low levels of PACs in human urine and blood plasma have failed to identify these compounds at biologically relevant concentrations, although some known PAC metabolites have been identified.[30−32] These data support the hypotheses that PACs are primarily eliminated by the fecal route in humans, that intact PACs are not present in urine at biologically relevant concentrations after cranberry consumption, and that PACs are therefore unlikely to be directly or solely responsible for the antiadhesion properties of urine produced after cranberry consumption.

Human volunteers who consume cranberry juice produce urine that has antiadhesive properties.[10,11,31−35] Antiadhesion activity appears shortly after consumption of the juice until about 8 h later, and maximum antiadhesion effects are observed within 4 to 6 h of ingestion.[31,34,35] This suggests rapid absorption and excretion of antiadhesive compounds into urine.[36] Atomic force microscopy (AFM) adhesion mechanism studies have shown that cranberry compounds are able to directly interfere with specific ligand−receptor binding interactions between bacteria and epithelial cells,[18] that compounds in addition to PACs are present in cranberry that can affect bacterial adhesion,[37,38] and that antiadhesive components reach the urine after cranberry consumption.[35] Studies on cranberry fractions containing nondialyzable material (NDM) also show antiadhesive activity for fractions that do not contain PACs.[39] Together, these data further support the hypothesis that additional, non-PAC compounds may be responsible for or may contribute to the antiadhesion properties of urine after the consumption of cranberry products.

Identification of the antiadhesive urinary compounds that are present as a result of cranberry consumption is essential for future studies regarding the bioactive constituents of cranberry products and the clinical efficacy of cranberry products for the prevention of UTIs. The initial objective of this study was therefore the isolation and identification of antiadhesive urinary compounds produced as a result of cranberry consumption. When the compounds isolated were found to be unrelated to PACs, we also pursued the secondary objective of determining if cranberry compounds could be a possible source of the urinary compounds identified.

The newest hypothesis regarding cranberry antiadhesion compounds is that complex carbohydrates, specifically arabinoxyloglucans related to those found in cranberry, may be at least partly responsible for the post-cranberry consumption antiadhesion properties of urine. This hypothesis was first presented publicly by us in July 2010 at the joint meeting of the American Society of Pharmacognosy and the Phytochemical Society of North America in St. Petersburg, Florida, USA, and is reported in the abstract for that meeting.[40] Since the date of this initial presentation, and later disclosures in 2011−2013[41] and in 2014,[42] additional researchers have pursued investigations of cranberry oligosaccharides and their biological properties.[43−46] Recent publications on this subject have provided additional support for some of our initial findings presented here, including the presence of arabinoxyloglucans in cranberry materials[43,44] and the possible antiadhesion properties of these compounds.[39,44,45] Additional work has focused on the complete structural elucidation of oligosaccharides from cranberry.[47,48] To date, no other studies have yet to connect cranberry oligosaccharides to post-cranberry-treatment urinary antiadhesion compounds. This report describes the isolation and full structural characterization of an arabinoxyloglucan oligosaccharide from a porcine urine fraction with antiadhesion properties and presents evidence that supports the hypothesis that cranberry oligosaccharides are the source of this compound.

Table 1. Details of the Swine Urine Collections Used as Source Material

sample code	urine collection dates	received by UM	animal number	urine volume[a] (L)	solute concentration[a] (g/L)
FC	2006/10/27 (control, 1 day)	2006/12	5081	2.0	18
F	2006/10/31−2006/11/02 (3 days treatment)	2006/12	5081	7.0	20
H	2008/06/24−2008/06/27 (4 days treatment)	2008/07	5023	3.5	17
I	2008/06/24−2008/06/27 (4 days treatment)	2008/07	8401	2.5	18

[a]Approximate values.

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Table 2. Anti-Hemagglutination Assay Results for Urine and Cranberry Samples

sample type					sample relative activity					
raw material	F	+	FC	− −	H	+/−	I	+/−	CJ	+ +
solids	FG	− −			HG	− −				
EtOAc	FB	−	FCE	+/−	HCE[a]	+	ICE[a]	+ +		
					HCM[b]	− −	ICM[b]	− −		
EtOAc/MeOH					HD	− −	ID	− −		
aqueous	FF	+	FC1	−	HF	+	IF	+/−	CJA	+ +
Sephadex LH-20 fractions	FA	+/−	FC1-1	− −	HF1-1	+ +	HI1[c]	+ +	CJA1-02	+ +
	FA1	+ +	FC1-2	− −	HF1-2	+/−	HI2[d]	+	CJA2-02[f]	+ +
	FA2	+	FC1-3	− −	HF1-3	−	HI3[e]	− −	CJA2-03[f]	+/−
	FA3	− −			HF1-4	−			CJA2-04[f]	+/−
	FA4	+/−			HF1-5	− −			CJA2-05[f]	−
	FA5	− −			HF1-6	−				
					HF1-7	− −				

[a]Water-soluble portion of EtOAc fraction. [b]Water-insoluble portion of EtOAc fraction soluble in MeOH or EtOAc. [c]HI1 = HF2-1 + IF1-1. [d]HI2 = HF1-1 + HF2-2 + IF1-2. [e]HI3 = HF1-2 + IF1-3 + HF2-3. [f]Fractions of the CJA2 Sephadex LH-20 column are described in the accompanying article.[47]

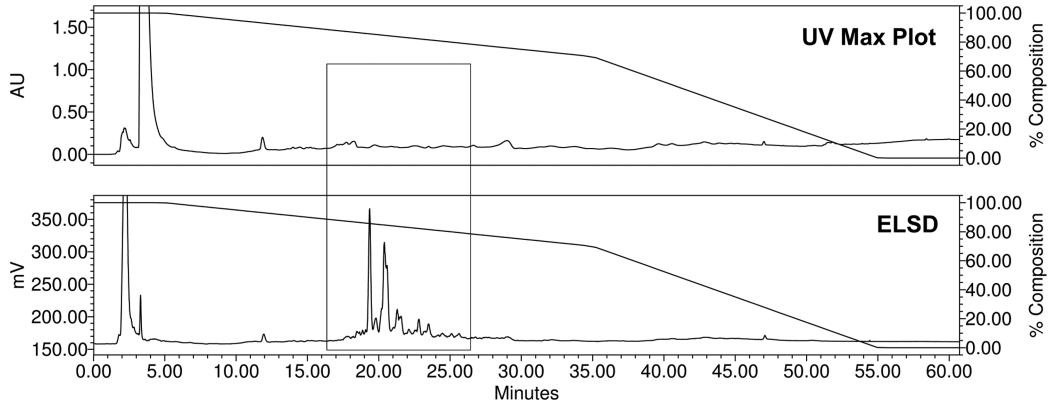

Figure 2. Analytical HPLC-ELSD and UV (PDA Max Plot) chromatograms for active fraction HF1-1. Comparison of the ELS and UV chromatograms for the active urine fraction HF1-1 (0.52 mg in 20 μL injection; 26 mg/mL) indicates that a set of major fraction components (box) is not visible by UV detection methods. Similar UV-transparent components were present in the active FA1 fraction. The semiquantitative nature of ELSD makes it possible to determine that the UV−visible component of HF1-1 is of relatively low concentration. Line overlay is % composition of water; counter solvent is MeOH.

RESULTS AND DISCUSSION

This study was initiated in 2004 and isolation efforts began in 2006. At the time, little was known regarding the bioavailability of PACs, and PACs were the primary recognized antiadhesive compounds associated with cranberry. Initial isolation efforts therefore focused on attempts to isolate PACs from treatment urine materials.[42] As work progressed, evidence accumulated that PACs were not the target compounds in the bioactive fractions, and isolation efforts continued with the target of an unknown, at that time, compound. Ultimately, complex carbohydrates, specifically oligosaccharides, were identified as the probable target compounds responsible for the anti-adhesion activity of urine fractions.

Composition of Active Urine Fractions. *Characterization.* The source material for this study was obtained over several years from multiple collections of swine urine after cranberry feeding (Table 1). Independent of the urine collection set, bioactive urinary components were consistently present in aqueous urine fractions after extraction with organic solvents, and urinary profiles of putative active constituents were consistent across samples. Antiadhesion activity (Table 2) was detected in some EtOAc fractions (HCE, ICE) but not others (FB, HCM, ICM, HD, ID), and insufficient amounts of these materials prevented further investigation. The control urine EtOAc fraction (FCE) also displayed possible bioactivity, indicating that constitutive urinary metabolites or dietary sources other than cranberry may be responsible for the activity observed in some EtOAc fractions. Efforts were therefore focused on the isolation of possible bioactive components from aqueous fractions.

Relative bioactivity results for selected samples are shown in Table 2 and Table S1 (Supporting Information). Assay results are qualitative (Section S1, Supporting Information), but provided sufficient information to guide isolation efforts. Through the submission of multiple samples and sample replicates, it was found that a minimum starting amount of 10 mg of material was required to avoid possible false negative results for fractions with unknown constituents.[42] At the time of this study, alternative antiadherence assays were unavailable.[21,22] Sample limitations therefore dictated that priority be given to the isolation and characterization of putative bioactive components in preference to comprehensive bioassay testing of urine fractions at each step of the isolation protocol.

The majority of the material from the F urine collection was consumed by method development and bioassay testing. The active FF material yielded active Sephadex LH-20 fractions

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that consistently eluted as the first 1−3 fractions (Tables S1 and S2, Supporting Information), permitting enrichment for the active 1−2% of total F urine materials into fraction FA (Section S2, Supporting Information). This material was further fractionated by Sephadex LH-20 (Table S3, Supporting Information) to give the active fractions FA1 (0.030 g) and FA2 (0.887 g).[42] Active components were found to be relatively stable under different storage and handling conditions for more than four years (data not shown).[42]

Analytical HPLC analyses of the enriched FF fractions FA1 and FA2 using multiple stationary phases and solvent systems eventually led to the observation of multiple UV-transparent components using evaporative light scattering detection (ELSD) (Section S3, Supporting Information). LC-micro-TOFMS analyses of fractions FA1 and FA2 were inconclusive (data not shown, Section 2b, Supporting Information), possibly due to a lack of method optimization, as the target compounds at the time of analysis were completely unknown. While ELSD is a destructive detection method and resulted in mass losses during split detection−collection preparative separations, it allowed for the detection of nonvolatile and semivolatile compounds regardless of the presence of specific structural features such as chromophores. ELSD was therefore the ideal detection method for this project, especially during the initial method development phase when insufficient information was available about the chemical nature of the target compounds to allow for the selection of a more customized or compound-specific approach.[42]

Further HPLC method optimization was pursued using comparisons of FA1, FA2, HF1-1, HF1-2, and other active samples. A complex profile of UV-transparent components (e.g., HF1-1; Figure 2) was consistently present in antiadhesive fractions of urine collected from three different swine after cranberry consumption, but not in control fractions (Sections S2 and S3, Supporting Information).

Comparisons between ^{1}H NMR spectra of active fractions FA1, FA2, HF1-1, HI1, and HI2 (Figure 3) indicated the presence of similar components. Comparisons of these spectra with those of synthesized trimeric and tetrameric PACs (Figure S1, Supporting Information), however, indicated that the ^{1}H NMR spectra of active urine fractions did not contain the characteristic overlapping aromatic resonances of the A- and B-rings of PAC constituent units. Although ^{1}H NMR spectra of crude active urine fractions did contain resonances in the aromatic region, these resonances were absent from enriched fractions with higher activity, were present in analogous inactive control fraction FC1-1, and were therefore likely due to simple aromatic acids commonly found in mammalian urine (Figures S2 and S3, Supporting Information).[49,50] The use of TLC spray reagents that produced color reactions characteristic of flavonoids (e.g., vanillin, *p*-anisaldehyde) further indicated that PACs and other flavonoids were not present in the active FA1 and FA2 fractions (data not shown; Section S2b, Supporting Information).

^{1}H NMR and TLC-based comparisons of active urine fractions FA1 and FA2 with various carbohydrate standards indicated that the components of these fractions were carbohydrates even though the possible presence of monosaccharides within these fractions had been eliminated through chromatography (Section S2c, Supporting Information). The dominant ^{1}H NMR resonances in D_2O were indicative of carbohydrates (Figure 3 and Figure S29, Supporting Information)[51] and reminiscent of those of apple pectin

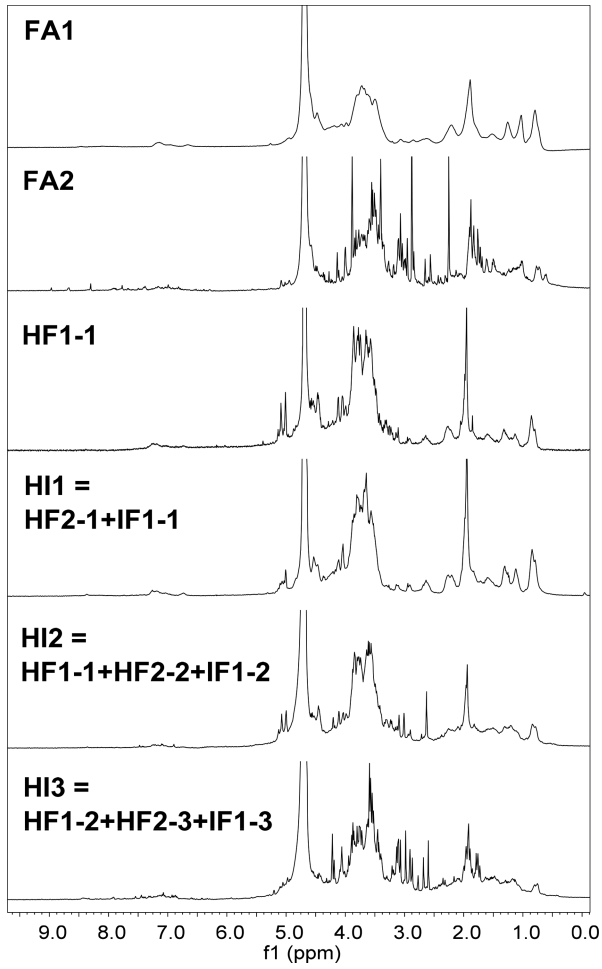

Figure 3. ^{1}H NMR spectra of Sephadex LH-20 urine fractions of interest, 400 MHz, D_2O. FA, HF, and IF materials are the aqueous fractions from three different source animals (see Tables 2 and 3 and Figures 4 and 6). Spectra were acquired on ∼10 mg of each material at similar concentrations. See Section S3 (Supporting Information) for additional details on the composition and bioassay testing of HI Atlantis dC_{18} subfractions. Fractions FA2 and HI3 had the lowest antiadhesion activity of the fractions shown in this figure.

(Figure S19, Supporting Information), but the active urine fractions had significantly different characteristics in aqueous solution than pectin. Comparisons with standard samples of D-glucose, sucrose, fructose, corn starch, and sodium citrate by TLC, using various carbohydrate-specific spray reagents (e.g., ninhydrin, *p*-anisidine, bromocresol green), further supported the absence of mono- or disaccharides (data not shown). It was therefore concluded that the components of interest in enriched urine samples were complex oligosaccharides.

Separations. The H and I urine collections provided sufficient material to permit further characterization and separation of the active fraction components. These materials were separately extracted and fractionated following the protocols developed with FF and earlier urine samples (Figure 4; Tables S2−S4, Supporting Information). As with the FF fractions, the active constituents remained in the aqueous layer after EtOAc extraction and eluted early from Sephadex LH-20 columns, with fractions HF1-1, HF2-1+IF1-1 (as HI1), and HF2-2+IF1-2 (as HI2) giving the best bioactivity results for the enriched samples tested (Table 2). All subsequent fractions were not tested in order to preserve material for isolation

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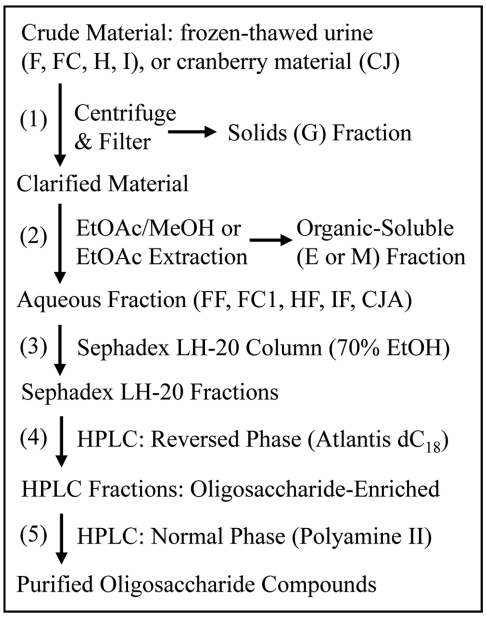

Figure 4. General purification protocol used for the isolation of urine and cranberry oligosaccharides.

efforts, and comparisons of the analytical HPLC-UV/ELSD profiles of active and inactive fractions were used to guide further method development and isolation (Figure 5; Figure S4 and Section S3, Supporting Information). The HPLC-ELSD comparative analyses indicated that a series of UV-transparent components eluting between 16 and 25 min (Figure 5) with an

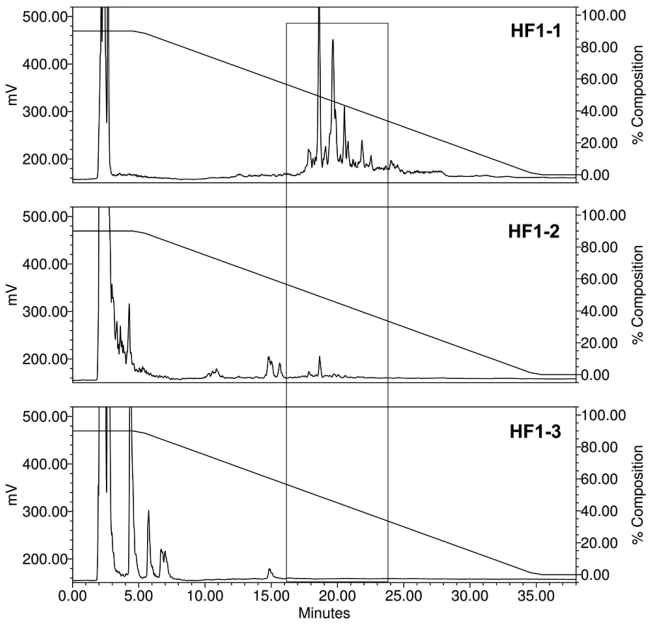

Figure 5. Analytical HPLC-ELSD chromatograms for Sephadex LH-20 fractions HF1-1, HF1-2, and HF1-3 (Atlantis dC_{18}). UV transparent components of interest (box), identified in the active fraction HF1-1 between 16 and 25 min, are detectable at low concentrations in the moderately active fraction HF1-2 and are absent from the inactive fraction HF1-3. All three samples were injected at the same concentration (50 μL injection, 10 mg/mL). The boxed components visible in the HF1-1 chromatogram are undetectable by UV (Figure 2; Figure S4, Supporting Information). Line overlay is % composition of water; counter solvent is MeOH.

optimized reversed-phase HPLC method were consistently present in active samples across multiple feedings and separations and absent from control samples.

Materials from the H and I treatment urines were handled separately through step 3 of the general isolation protocol (Figure 4). Sephadex LH-20 fractions from the two source materials were combined for preparative reversed-phase separations (step 4, Figure 4) and for subsequent semi-preparative normal-phase separations (step 5, Figure 4) based on similarities of their oligosaccharide elution profiles as observed by analytical HPLC-ELSD (Sections S3, Supporting Information). These combinations provided increased amounts of material for subsequent separations, but difficulties with resolution between the various individual oligosaccharides in the series made it difficult to meaningfully recombine the final fractions obtained (Table S12 and Section S3, Supporting Information). An overview of the fractions generated during the HPLC purification steps, showing their relationships to each other and to the various parent fraction materials, is presented in Figure 6.

Two major fractions of the UV-transparent series of compounds detectable in HF2-2 (Figure 7, Table S9, Supporting Information), HF2-2P1t20 (11.9 mg) and HF2-2P1t22 (17.1 mg), contained closely related complex oligosaccharides as determined by chromatography and NMR data analyses. Further separation of the HF2-2P1t20 fraction after combination with the closely related fraction IF1-3P1t20 yielded the primary component of this fraction as HF2-2P1t20A17 (8.8 mg) (Table S19, Figures S40 and S41, and Section S3, Supporting Information). A sufficient amount of this material was available to allow for the full structural characterization of compound **1** by MS and classical carbohydrate structural analyses (Section S4, Supporting Information) and to obtain supporting data for structural assignments via NMR spectroscopy (Figures 8–11; Figures S5–S13, Supporting Information). An insufficient amount was available, however, for reliable bioassay testing. The HF2-2P1t22 fraction yielded a series of at least six closely related oligosaccharides as visible by HPLC-ELSD (Figure 12; Figures S39 and S41, Supporting Information). Three of these compounds, HF2-2P1t22A17 (1.9 mg), HF2-2P1t22A18 (1.8 mg), and HF2-2P1t22A19 (1.9 mg), could be isolated in sufficient amounts for analysis by ^{1}H NMR spectroscopy in D_2O (Figure 13; Figure S42, Supporting Information), but insufficient amounts were available for further purification efforts or bioassay testing. Full structural assignments could not be made from the data obtained, but characteristic resonances could be detected at δ_H 4.4, 4.6, 5.0, 5.1, and 5.2 in the ^{1}H NMR spectra. These resonances were later determined to correspond to the anomeric protons for arabinoxyloglucan-type oligosaccharides similar to **1** and purified cranberry oligosaccharides.[47,48] Comparisons of 1D and 2D NMR data obtained (in DMSO-d_6) for the parent fractions HF2-2P1t20 and HF2-2P1t22 indicated that the mixed compounds present in fraction HF2-2P1t22 were all structurally related to the primary component of HF2-2P1t20, compound **1** (Figure 14; Figures S32 and S33, Supporting Information), and that this series of compounds likely contains structures with 7–9 monosaccharide units (DP 7–9) with variations in linkage positions and monomer composition.

The Oligosaccharide Series. The oligosaccharide series present in swine urine samples is highly complex, with as many as 50+ possible distinct compounds, and this series could not

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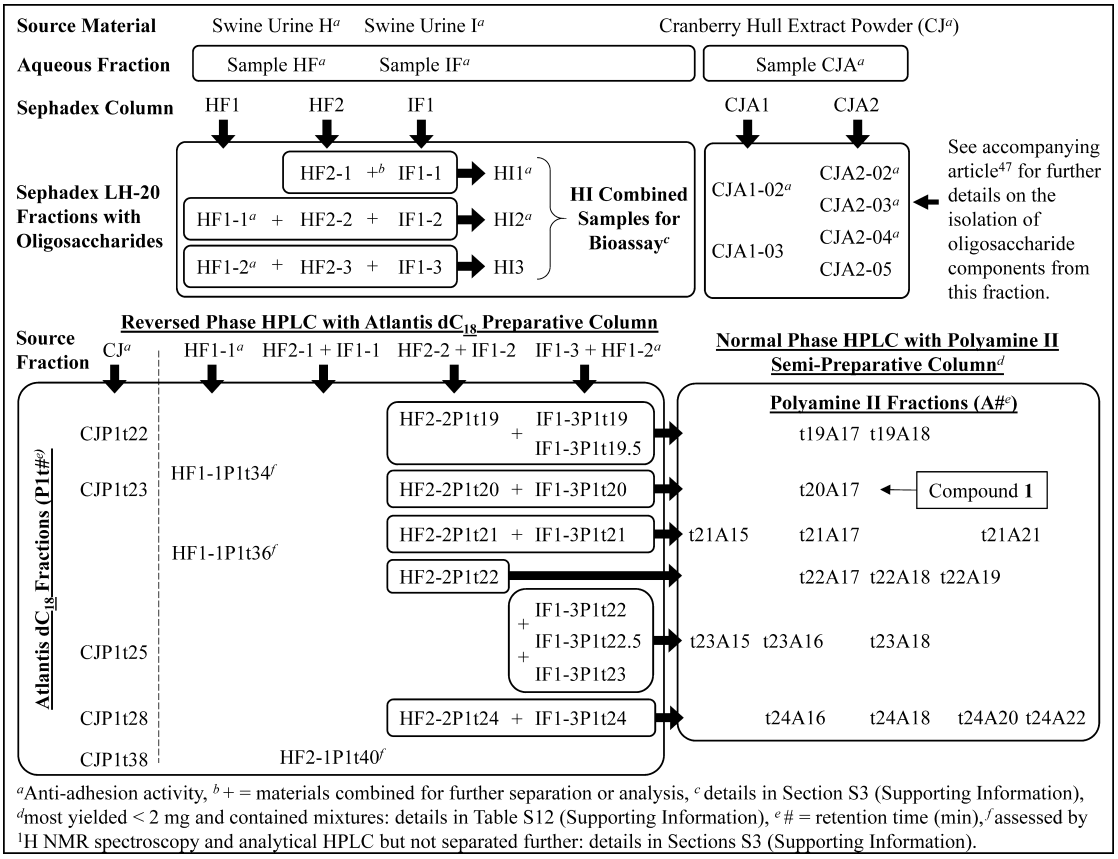


Figure 6. Overview of the various samples and fractions of interest showing their origin materials, relationships, and similarities of composition. All materials and fractions shown contain ELS-detectable oligosaccharides. Rounded boxes, rows, and columns indicate materials with similar methodological origins (e.g., sets of column fractions) or chemical characteristics as determined by HPLC-ELSD and ^{1}H NMR spectroscopy. For example, CJP1t23 is an Atlantis dC$_{18}$ column fraction derived from CJ material that is similar in composition to HF2-2P1t20 and IF1-3P1t20. The fraction HF2-2P1t20 was derived from the parent materials HF2-2 + IF1-2 when they were separated on the Atlantis dC$_{18}$ sorbent. HF2-2P1t20 was combined with IF1-3P1t20 and separated on the Polyamine II sorbent to yield HF2-2P1t20A17, a fraction that was determined to be pure enough to yield full structural elucidation of compound **1**.

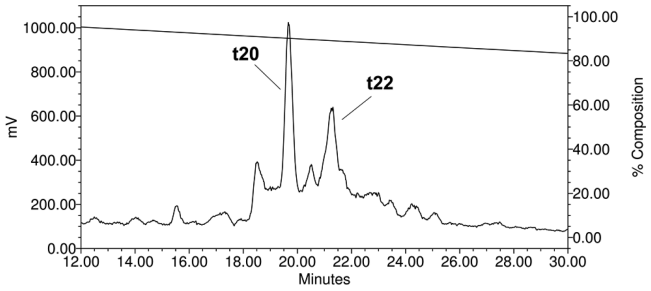
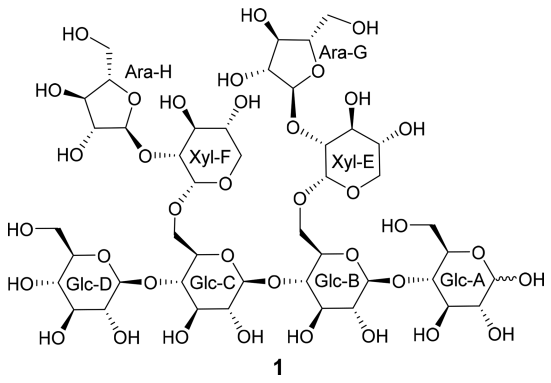


Figure 7. Portion of the preparative HPLC-ELSD chromatogram for HF2-2, R5 (Atlantis dC$_{18}$). Resolution of this separation is representative of the series of six preparative-scale separations for this sample. Fractions were combined across all six separations (runs 1−6) (Table S9 and Figure S30, Supporting Information). Line overlay is % composition of water; counter solvent is MeOH.



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be fully resolved or characterized with the resources available for this study. Multiple minor compounds were observed in HPLC-ELSD chromatograms, but collection attempts via preparative and semipreparative separations yield less than 2 mg each for most of these fractions (Table S12 and Section S3, Supporting Information). Subsequent injections on alternate stationary phases with different chromatographic properties (e.g., Atlantis dC$_{18}$ fraction injected onto a Polyamine column) often yielded multiple compounds from what had previously appeared to be a single eluting component. This chromatographic characteristic indicated the presence of compounds with closely related structures and properties, but confounded isolation efforts. Further discussion of this observation as applied to the isolation attempts concerning fraction IF1-3 and its subfractions is provided in the Supporting Information (Section S3c, Figures S43−S46). While the oligosaccharide nature of many of these fractions could be established by ^{1}H NMR spectroscopy in both DMSO-d_6 and D$_2$O, the data quality was insufficient to reliably distinguish or meaningfully compare any two subfractions with different retention times and different parent materials (data not shown), thereby

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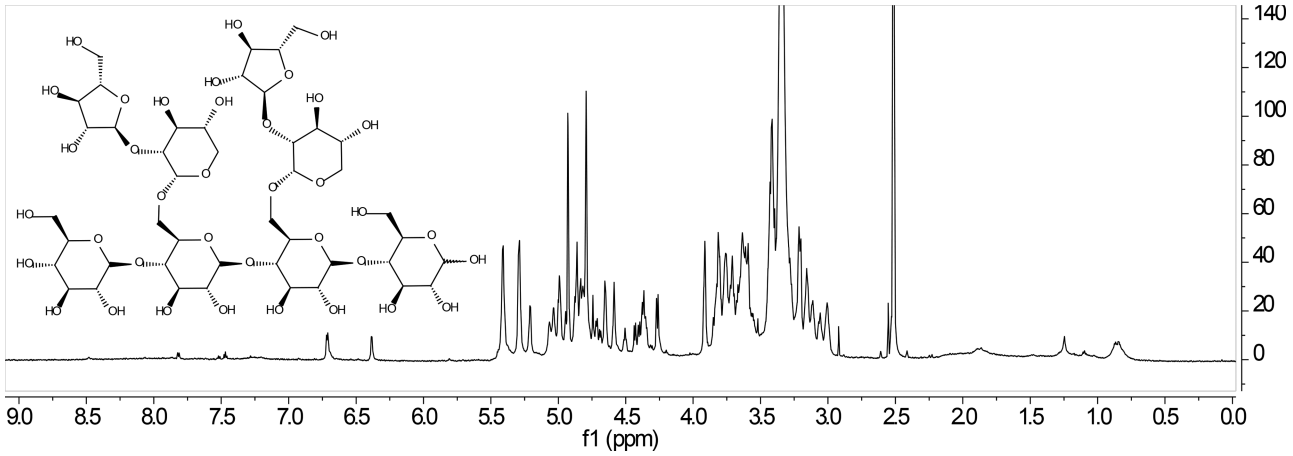

Figure 8. ^{1}H NMR spectrum of compound **1** in DMSO-d_6, 700 MHz. See Figure S5 (Supporting Information) for expansions.

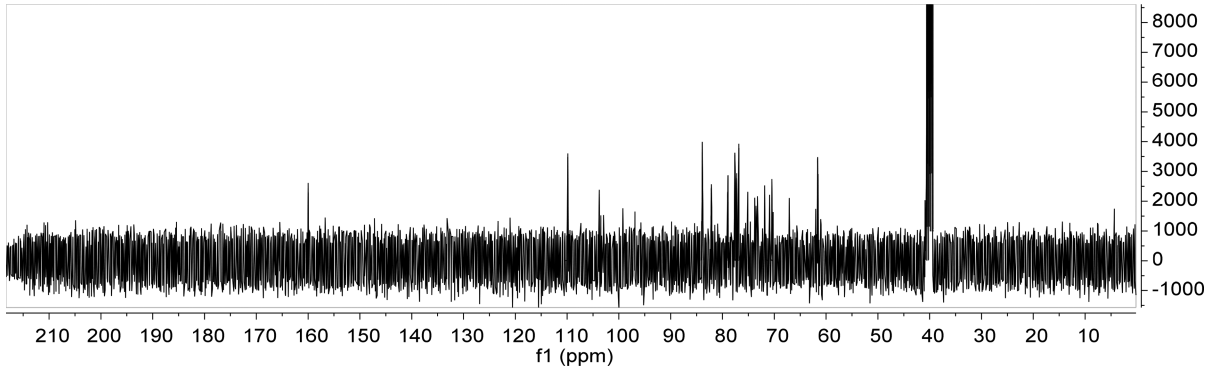

Figure 9. ^{13}C NMR spectrum of compound **1** in DMSO-d_6, 175 MHz. See Figure S6 (Supporting Information) for expansions.

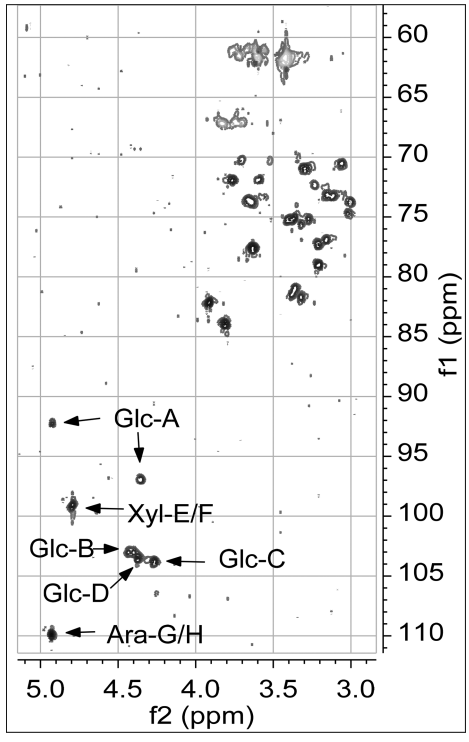

Figure 10. Multiplicity-edited HSQC spectrum of compound **1** with anomeric correlations indicated, DMSO-d_6, 700/175 MHz. See Figures S7 and S8 (Supporting Information) for expansions.

preventing rational fraction recombinations and further separation efforts. These observations and more recent MS-based studies on cranberry arabinoxyloglucans[43] support the hypothesis that the series of compounds detected in urine during this study is a complex series of oligosaccharides based on a core structural theme with similar monosaccharide composition and variations in connectivity and monomer unit ratios.

Complete structural elucidation of all oligosaccharides present in the urine was beyond the scope of this project due to the limited quantities of material isolated, the complexity of the probable compound structures, and a limited availability of general resources for continuing the investigation. Preliminary, approximate, mass recovery calculations indicated that oligosaccharide urinary metabolites are likely to be within a reasonable range for bioactivity,[49] with single components present at a minimum concentration of ~1−5 μg/mL urine.

Structure Elucidation. *Carbohydrate Structural Analyses.* The structure of **1** was established using standard carbohydrate derivatization and analysis procedures (Section S4, Supporting Information), MS^n fragmentation data (Section S5, Supporting Information), and 1D and 2D NMR experiments (Figures 8−11; Figures S5−S13, Supporting Information). Glycosyl composition analysis by GC-MS of the TMS methyl glycosides of **1** indicated it was composed of arabinose, xylose, and glucose with a molar ratio of 1:1:2 (Table S5, Supporting Information). The mass fragments of the TMS methyl glycosides at m/z 204 and 217 indicated that **1** contained neutral sugars. MALDITOFMS (Figure S14,

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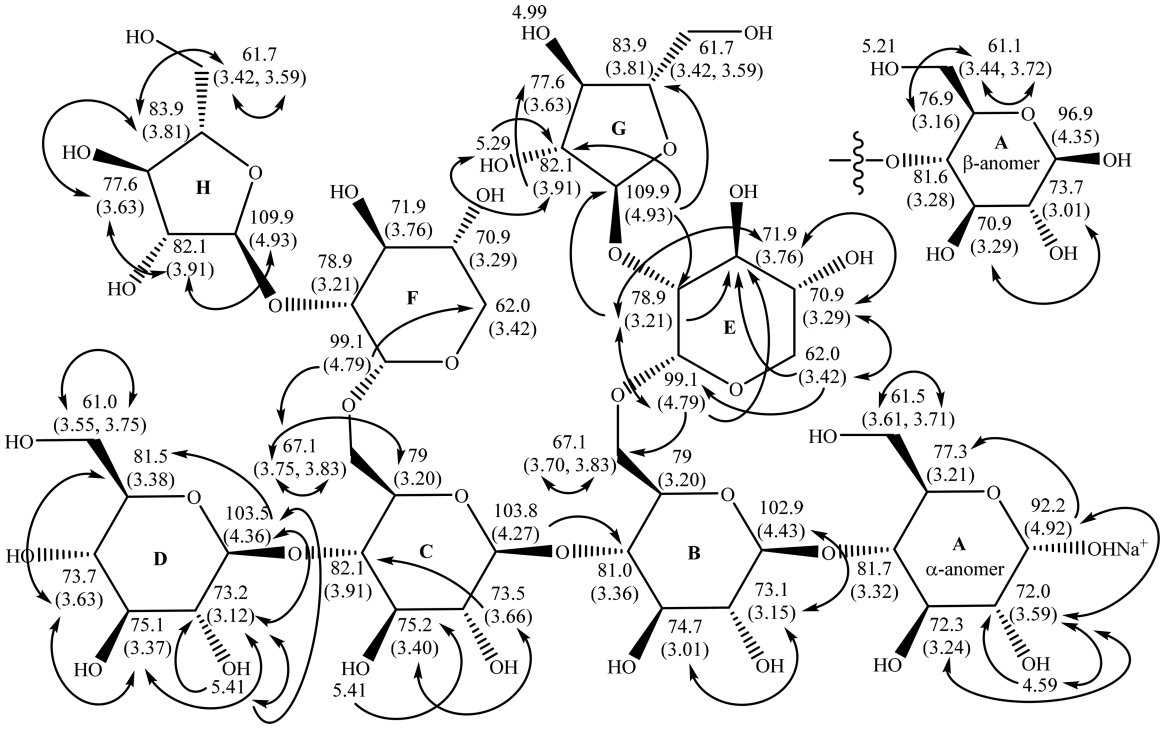

Figure 11. Structure of compound **1** showing indicative 2D COSY and HMBC correlations with ^{13}C and ^{1}H chemical shifts indicated at each position [δ_C (δ_H)]. Single-headed arrows indicate COSY correlations; double-headed arrows indicate HMBC correlations. For clarity, selected correlations are shown on individual Ara-G/Ara-H and Xyl-E/Xyl-F glycosyl units. These resonances are duplicated for both Ara-Xyl side chains.

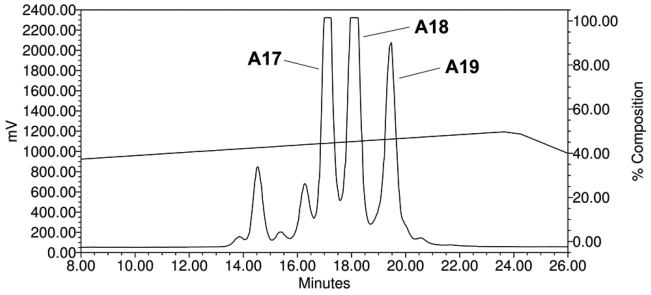

Figure 12. Portion of the semipreparative HPLC-ELSD chromatogram for HF2-2P1t22 (Polyamine), showing the resolution of isolated components. Intensity of the A17 peak in this separation is greater than that for other HF2-2P1t22 separations (Figure S39, Supporting Information) due to co-injection of a portion of HF2-2P1t20. Compounds eluting at A17, A18, and A19 were recovered in low quantities and analyzed by analytical HPLC-ELSD on Atlantis dC_{18} (Figure S41, Supporting Information) and by ^{1}H NMR spectroscopy (Figure 13 and Figure S42, Supporting Information). Line overlay is % composition of MeCN; counter solvent is water.

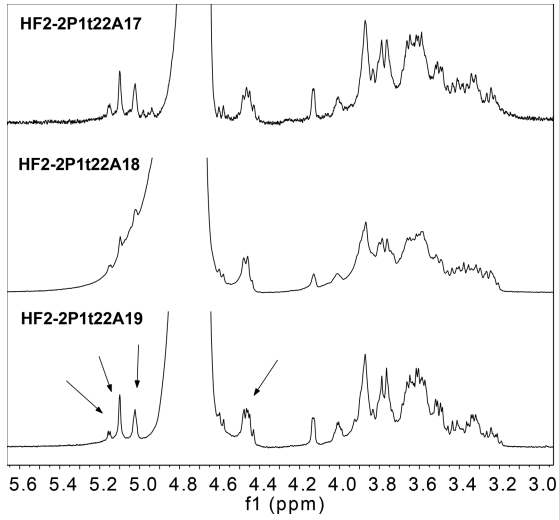

Figure 13. Comparison of the ^{1}H NMR spectra for HF2-2P1t22A fractions eluting at 17, 18, and 19 min, D_2O, 400 MHz. Chromatographic profile of these fractions is shown in Figure 12 and Figures S39 and S41 (Supporting Information). Arrows on the A19 spectrum indicate characteristic arabinoxyloglucan anomeric resonances. These spectra do not contain resonances in the regions that are not displayed. The full spectrum and expansions for the HF2-2P1t22A17 sample, of which **1** or a closely related compound may be a primary component, are included in Figure S42 (Supporting Information).

Supporting Information) analysis showed an $[M + Na]^+$ ion at m/z 1218.117, consistent with an octasaccharide composed of two arabinosyl, two xylosyl, and four glucosyl residues. GC-MS analysis of the (*S*)-(+)-butyl glycoside TMS derivatives of **1** as compared to standards (Figure S15, Supporting Information) indicated D-glucose, D-xylose, and L-arabinose absolute configurations, consistent with the natural abundances of these monosaccharides.

The interglycosidic linkage positions were determined using partially methylated alditol acetate derivatives of **1**. These data, evaluated in conjunction with the molar ratio and mass results, indicated that **1** contained two terminally linked arabinofuranosyl residues, two 2-linked xylopyranosyl residues, one terminally linked glucopyranosyl residue, one 4-linked glucopyranosyl residue, and two 4,6-linked glucopyranosyl residues.

Indicative partial fragmentation patterns from NSI-MS^n analyses of per-*O*-methylated derivatives of **1** were used to

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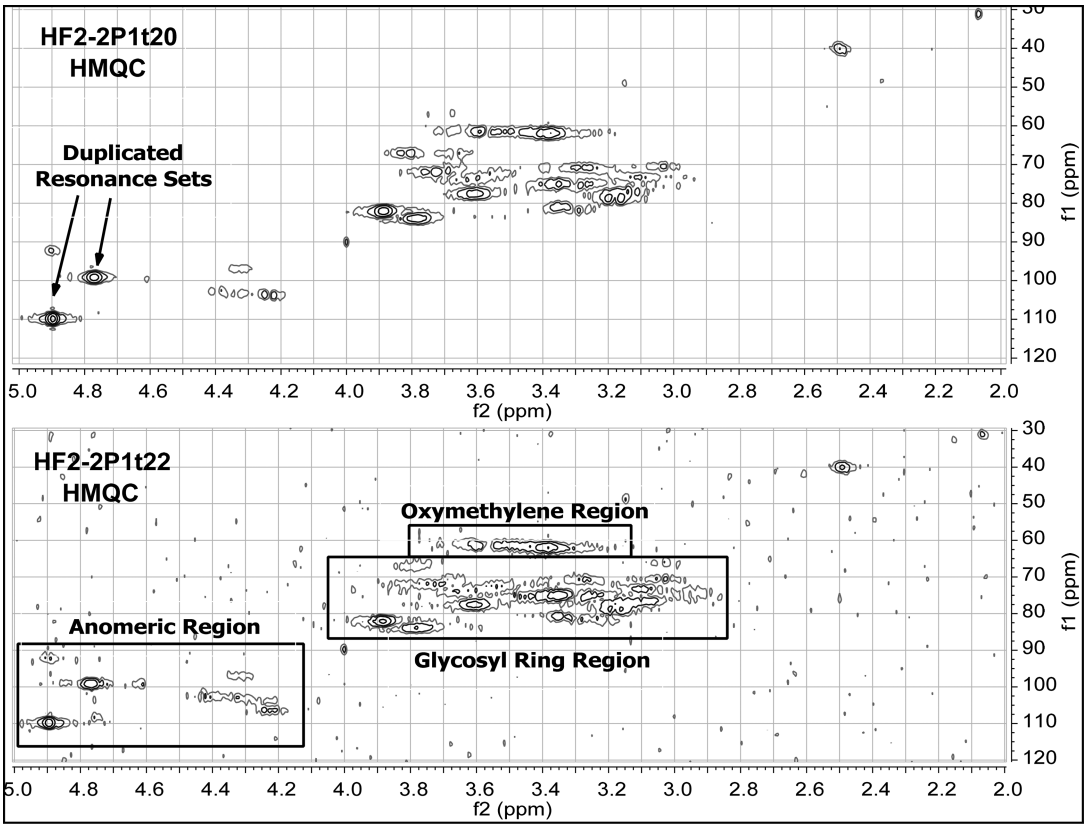

Figure 14. Comparison of the HMQC spectra for HF2-2P1t20 and HF2-2P1t22 showing the similarity of composition for these fractions, DMSO-d_6, 400 MHz. Duplicated resonance sets indicated are for Xyl ($\delta_{H/C}$ 4.79/99.1) and Ara ($\delta_{H/C}$ 4.93/109.9) glycosyl residues. Oxymethylene, glycosyl ring, and anomeric regions are indicated on the spectrum for HF2-2P1t22. ^{1}H and ^{13}C NMR spectra for these fractions are included in Figures S32 and S33 (Supporting Information).

confirm linkage positions and monomer connectivity. The observation of key fragments produced from the partial fragmentation of parent ions supported the presence of a reducing glucosyl moiety connected via a single glycosidic bond to the remainder of the molecule. It also permitted placement of the two Xyl-Ara units on the two internal glucosyl residues of the tetrameric glucosyl backbone. The assignment of the side chains on the two internal glucosyl residues is similar to that recently reported for the structure of a cranberry arabinoxyloglucan heptasaccharide.[44] Additional detailed discussion of the MS fragmentation patterns in support of structural assignments can be found in the Supporting Information (Section S5 with associated Figures S47−S61). All data obtained from the carbohydrate derivatization analyses were used to assign the structure of **1** as shown, with a D-glucopyranosyl-(1→4)-D-glucopyranosyl backbone and two L-arabinofuranosyl-(1→2)-D-xylopyranosyl side chains connected via D-xylopyranosyl-(1→6)-D-glucopyranosyl linkages.

NMR Spectroscopy. The use of NMR spectroscopy alone was initially attempted for full, de novo, structural elucidation of compound **1** employing spectra acquired at 400 MHz (data not shown). As compound **1** was an unknown when structural analysis began, DMSO-d_6 was selected as the solvent, as it provided better resolution across all proton resonances beyond the anomeric region, and anomeric resonances could still be identified using HSQC data (Figure 10; Figures S7 and S8, Supporting Information). In general, the preferred solvent for known or suspected carbohydrate compounds is D_2O, as it provides resolution of anomeric proton resonances without overlap from those of hydroxy protons and allows for comparisons to existing xyloglucan reference spectra.[51] The utility of D_2O as an NMR solvent and the appropriate application of associated reference spectra, however, depends upon the availability of additional knowledge regarding compound structure from non-NMR sources such as derivatization techniques and MS. These approaches were ultimately required for full structural elucidation of **1**, thereby establishing the xyloglucan nature of the compound and other structural features. Additional acquisitions of NMR spectra for **1** at 700 MHz were, however, also obtained in DMSO-d_6 to allow for direct comparisons with previously obtained and interpreted data for both compound **1** and related fractions (e.g., HF2-2P1t22 comparison, Figure 14). Once the xyloglucan oligosaccharide nature of **1** and the related series of compounds had been firmly established, future studies of cranberry oligosaccharides by our group were pursued using D_2O as the NMR solvent of choice.[47,48,52] The final structure established for **1** was compared to those of purified cranberry oligosaccharides,[47,48] and the observation of strikingly similar structures, in spite of the use of different structural elucidation approaches, supported our preliminary hypotheses regarding the probable presence of cranberry oligosaccharides in urinary materials. ^{1}H NMR spectra for the HF2-2P1t22A series (A17, A18, and A19) in D_2O (Figure 13; Figure S42, Supporting Information) further supported the conclusion that the oligosaccharide components present were xyloglucans and

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Table 3. ^{1}H and ^{13}C NMR Assignments for 1, DMSO-d_6, 700/175 MHz

monomer	position	δ_C	δ_H (mult;[a] J_{HH})	monomer	position	δ_C	δ_H (mult[a]; J_{HH})
Glc*p*-A	1	92.2	4.92 (3.9)	Glc*p*-D	1	103.5	4.36 (8.0)
α-anomer	2[b]	72.0	3.59		2[b]	73.2	3.12
	3[b]	72.3	3.24		3[b]	75.1	3.37
	4	81.7	3.32		4	73.7	3.63
	5	77.3	3.21		5	81.5	3.38
	6	61.5	3.61, 3.71		6	61.0	3.55, 3.75
Glc*p*-A	1	96.9	4.35 (6.8)	Xyl*p*-E	1	99.1	4.79 (3.3[c])
β-anomer	2[b]	73.7	3.01		2	78.9	3.21[b]
	3[b]	70.9	3.29		3	71.9	3.76
	4	81.6	3.28		4	70.9	3.29
	5	76.9	3.16		5	62.0	3.42
	6	61.1	3.44, 3.72	Xyl*p*-F	1	99.1	4.79 (3.3[c])
Glc*p*-B	1	102.9	4.43 (8.1)		2	78.9	3.21
	2[b]	73.1	3.15		3	71.9	3.76
	3[b]	74.7	3.01		4	70.9	3.29
	4	81.0	3.36		5	62.0	3.42
	5	79.0	3.20[b]	Ara*f*-G	1	109.9	4.93[d]
	6	67.1	3.70, 3.83		2	82.1	3.91
Glc*p*-C	1	103.8	4.27 (7.8)		3	77.6	3.63
	2[b]	73.5	3.66		4	83.9	3.81
	3[b]	75.2	3.40		5	61.7	3.42, 3.59
	4	82.1	3.91	Ara*f*-H	1	109.9	4.93[d]
	5	79.0	3.20[b]		2	82.1	3.91
	6	67.1	3.75, 3.83		3	77.6	3.63
					4	83.9	3.81
					5	61.7	3.42, 3.59

[a]All protons appeared as overlapping multiplets unless otherwise indicated. [b]Numerical assignments are tentative due to signal overlap. [c]Approximate J value. [d]A reliable numerical J value assignment could not be made due to signal overlap.

allowed for later comparisons to cranberry fraction spectra acquired in D_2O.[47,52]

NMR spectra (at 700 MHz in DMSO-d_6) indicated the presence of minor impurities, but, in conjunction with the HSQC spectrum, permitted assignment of ^{1}H and ^{13}C NMR data for compound **1** (Table 3, Figures S5−S8, Supporting Information), and further interpretations of 2D data (Figures S9−13, Supporting Information) provided partial structures that were consistent with the results from chemical carbohydrate analyses (Figure 11). NMR data were also used to determine anomeric configuration and to support the linkage positions and monomer connectivity indicated by the mass fragmentation pattern data.

The use of DMSO-d_6 as the NMR solvent reduced the exchange of hydroxy protons with deuterium, limited hydrogen bonding, and restricted the overall conformational flexibility of the molecule, thereby allowing for the detection of COSY and HMBC correlations between hydroxy protons and glycosyl ring protons and carbons, respectively. These correlations were used to support structural assignments and interglycosyl connectivity (Figure 11). In particular, the proton (δ_H 4.59) for the 2-hydroxy group of Glc-A allowed definition of the partial structure for this ring. The proton resonance at δ_H 5.41 was determined to be a duplicated signal and was assigned to the 3- and 2-hydroxy groups of Glc-C and Glc-D, respectively, and HMBC correlations to this resonance permitted assignment of the partial structures of both rings. The proton resonance at δ_H 5.29 was also a duplicated signal for the 2-hydroxy groups of both Ara-G and Ara-H units, and HMBC and COSY correlations to this resonance permitted confirmation of Ara-Xyl interconnectivity.

The anomeric carbons of Glc-B, Glc-C, and Glc-D were assigned β-configurations based on their shielded (δ_H < 4.70) chemical shifts and coupling constants of $J_{H1,H2}$ > 5 Hz, while Xyl-E and Xyl-F were assigned α-configurations due to their deshielded (δ_H > 4.70) chemical shifts and coupling constants of $J_{H1,H2}$ < 5 Hz.[51] The anomeric centers of the Ara-G and Ara-H residues were assigned α-configurations based on their chemical shifts and $J_{H1,H2}$ values of 1−2 Hz; however, signal overlap prevented accurate J-value assignments.[51,53] The reducing glucosyl residue was assigned to Glc-A, and its presence confirmed by duplicate resonance sets for the α- and β-anomeric protons at δ_H 4.92 and 4.35, respectively. COSY, TOCSY, and HMBC (Figure 11; Figures S9−S13, Supporting Information) correlations were used to confirm the identities and connectivity of the constituent monosaccharide units. HMBC correlations between H-1 of Ara*f*-G and -H (δ_H 4.93) and C-2 of Xyl*p*-E and -F (δ_C 78.9) and between H-2 of Xyl*p*-E and -F (δ_H 3.21) and C-1 of Ara*f*-G and -H (δ_C 109.9) directly supported the assignment of α-L-arabinofuranosyl-(1→2)-α-D-xylopyranosyl side chains. These side chains were linked to C-6 of the two internal glucosyl residues Glc*p*-B and -C through HMBC correlations between H-1 of Xyl*p*-E and -F (δ_H 4.79) and C-6 of the two glucosyl units at δ_C 67.1. The connectivity of the two internal glucosyl residues, Glc*p*-B and -C, was confirmed through HMBC correlations between H-1 of Glc*p*-C (δ_H 4.27) and C-4 of Glc*p*-B (δ_C 81.0).

Thus, the structure of **1** was assigned as β-D-glucopyranosyl-(1→4)-[α-L-arabinofuranosyl-(1→2)-α-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-(1→4)-[α-L-arabinofuranosyl-(1→2)-α-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-(1→4)-β-D-glucopyranose. The ^{1}H NMR data for other urinary oligosac-

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charides isolated in low quantities (Figures 13 and 14; Figure S42, Supporting Information) indicate that the composition of **1** is representative of the complex oligosaccharide pool. Spectra collected in D_2O at 400 MHz (Figure 13; Figure S42, Supporting Information) contain anomeric resonances with deshielded ($\delta_H > 5$) chemical shifts, as are characteristic for arabinoxyloglucans.[47,48]

Composition of Cranberry Materials. Cranberry samples CJ and CJA contained similar analytical-scale HPLC-UV/ELSD profiles in the region of interest as active urine fraction HF1-1 and analogous HF2-2, with the presence of a series of UV-transparent components eluting between 16 and 25 min with the optimized HPLC method (Figures 15 and 16; Figure S16, Supporting Information). The cranberry material was injected at 2−4 times higher concentrations than the urine fractions, but retention times and relative peak ratios remained similar and cranberry profiles could be directly overlaid with those of the urine fractions from 16 to 25 min (Figure 15) without postcollection processing of chromatographic data. As ELSD is a semiquantitative, mass-sensitive method, these observations indicate that a subset of similar components appear to be present in both urine and cranberry materials at similar concentration ratios. The overlay also shows that not all components present in cranberry materials were detected in the urine fractions of interest.

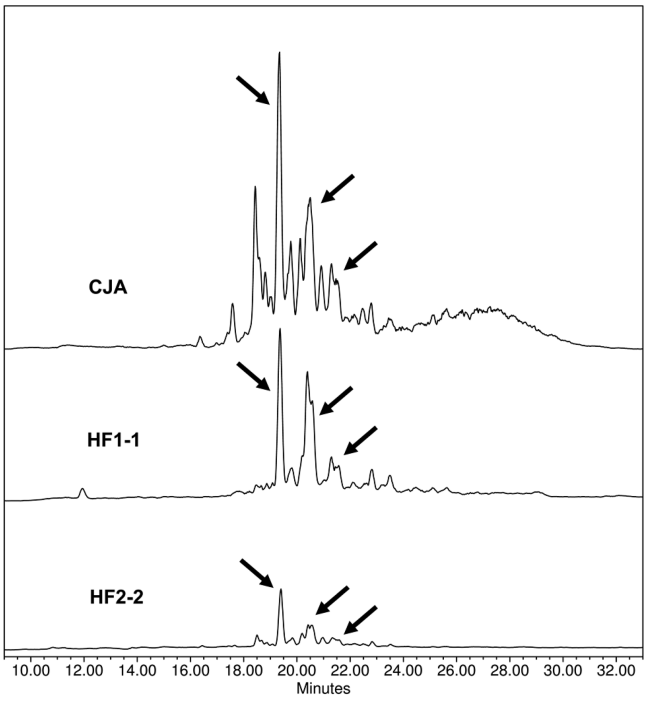

Figure 15. Analytical HPLC-ELSD plots of CJA compared to HF1-1 and HF2-2 (raw data overlay). This comparative profile indicates that urine and cranberry fractions have similar relative quantities of what appear to be similar components (arrows). Samples were injected at different concentrations as follows: CJA, 40 μL injection, 30 mg/mL; HF1-1, 20 μL injection, 26 mg/mL; HF2-2, 25 μL injection, 10 mg/mL. No retention time adjustments or other postacquisition processing methods were applied to this data.

Direct, preparative, reversed-phase HPLC separation of ~350 mg of CJ, using the methods applied to urine samples, led to the collection of 22.2 mg of total material from the region of interest (21.5−30 min, Figure 16). This material

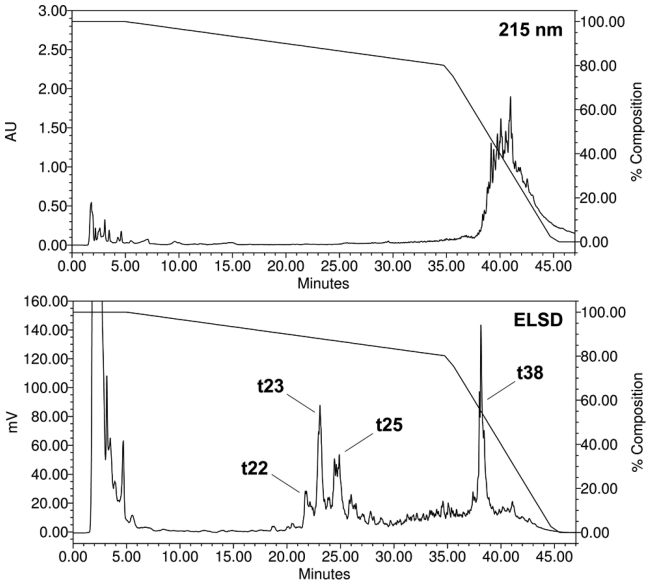

Figure 16. Preparative HPLC-UV/ELSD chromatograms for the CJ-P1 separation (Atlantis dC_{18}, R3). Comparison of these chromatograms indicates the presence of weak chromophores for a subset of the compounds eluting from 0 to 8 min and the absence of chromophores for the compounds eluting from 18 to 38 min. The fraction t28 is not shown on this figure, as it is a mixture of components eluting from 26 to 30 min. The absorbance profile from 38 to 46 min does not appear to correlate to the ELS profile of the fraction eluting at 38 to 39 min, suggesting that fraction CJ-P1t38 lacks a chromophore. The components eluting from 38 to 46 min showed a visible pink color in solution, suggesting the presence of known anthocyanins, but the low mV intensity of this region indicates relatively low concentrations of material. Line overlay is % composition of water; counter solvent is MeOH.

represented ~16% (w/w) of the total recovered CJ material (139.8 mg) as being composed of oligosaccharides. This value represents a minimum amount, as only 40% of the prepared material was recovered, probably due to mass losses from filtration and other handling steps, possible chromatographic retention, and destructive ELS detection. Most CJ-P1 fractions contained mixtures of compounds, but fraction CJ-P1t23 (4.0 mg) had similar HPLC-UV/ELSD characteristics to compound **1** isolated from urine samples. Comparison of this sample to that of the enriched fraction HF2-2P1t20, containing primarily **1**, via ^{1}H NMR spectroscopy in DMSO-d_6, confirmed the similar chemical composition of the two materials (Figure 17). Insufficient pure material of CJ-P1t23 was available for full structural characterization or bioassay testing, but ^{1}H NMR resonances in the anomeric region in DMSO-d_6 indicated the presence of an arabinoxyloglucan with eight monomeric units. Fractions CJ-P1t22 and CJ-P1t25 also contained resonances in the anomeric region indicative of arabinoxyloglucans (Figure S17, Supporting Information). In contrast, a corollary fraction to CJ-P1t38 was not detected in urine fractions, and this material had a distinct ^{1}H NMR profile from that of the urinary oligosaccharides, but it is also likely an oligosaccharide. These data indicate that the cranberry starting material contains a series of oligosaccharides with structural compositions similar to each other and to the isolated urinary oligosaccharides (Figure 6), but that additional oligosaccharides and related compounds may be present in cranberry materials that are not excreted into urine.

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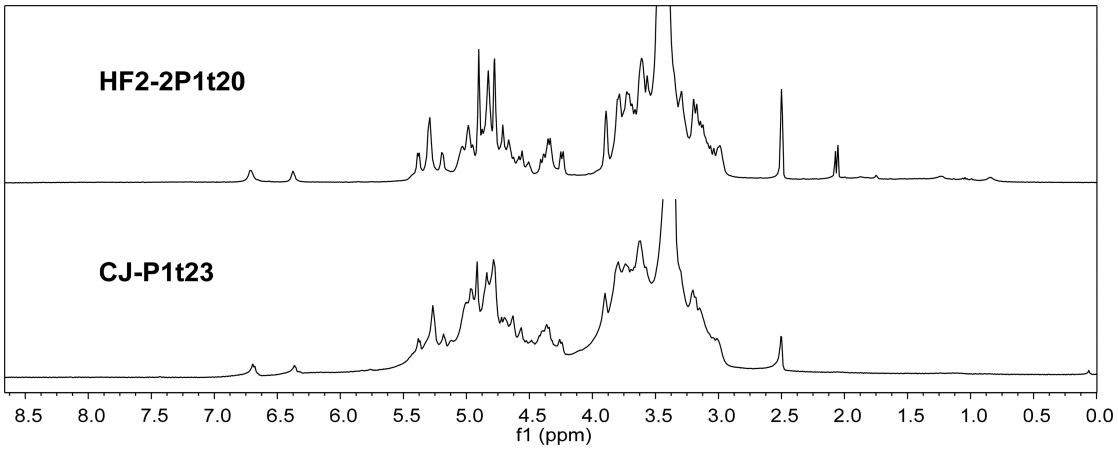

Figure 17. Comparison of the ^{1}H NMR spectra for the urinary fraction HF2-2P1t20, enriched for compound **1**, and an enriched cranberry oligosaccharide fraction CJ-P1t23 (4 mg), DMSO-d_6, 400 MHz. Spectra for both fractions were highly similar, indicating similar chemical composition. These data indicated that the cranberry material CJ-P1t23 was also an arabinoxyloglucan oligosaccharide.

Separation of the CJA material using Sephadex LH-20 via methods similar to those applied to urine samples yielded oligosaccharide-containing fractions (e.g., CJA1-02) that, combined, comprised ~25% (w/w) of the starting material and eluted at similar retention times as active urine fractions. ^{1}H NMR spectroscopy in D_2O indicated that these fractions were enriched for arabinoxyloglucans similar in composition to those found in active urine and in CJ-P1 fractions, as indicated by characteristic profiles of anomeric resonances (Figure 18).

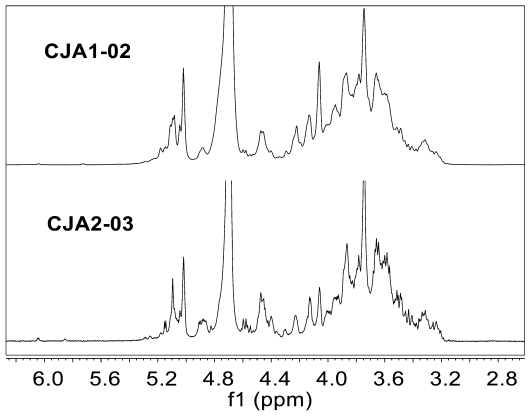

Figure 18. ^{1}H NMR spectra for active fractions CJA1-02 and CJA2-03, D_2O, 400 MHz. These spectra show the similarity of composition for oligosaccharide-enriched fractions from Sephadex LH-20 columns CJA1 and CJA2.[47] These spectra do not contain resonances in the regions that are not displayed.

A full description of the isolation and structure elucidation of two cranberry oligosaccharides from CJA Sephadex LH-20 column fractions and an additional source material is discussed in the accompanying article;[47] both compounds were found to have structures similar to that of **1**. Samples of CJ, CJA, and selected CJA1 and CJA2 Sephadex LH-20 column fractions were submitted to the bioassay used for testing urine fractions, and samples that contained oligosaccharides were found to have antiadhesion activity (Table 2).

Together, these data support the hypotheses that the UV-transparent oligosaccharides present in active urine fractions are directly derived from oligosaccharides present in the source cranberry material and that the oligosaccharides present in both urine and cranberry materials possess antiadhesion properties.

Oligosaccharide Biochemical Origins and Mammalian Digestion. The structure of **1** is closely related to those of two arabinoxyloglucans isolated from the same cranberry materials as used in this study (see accompanying article).[47] It is also similar to that of a cranberry arabinoxyloglucan heptasaccharide, isolated after pectinase treatment of cranberry as a part of commercial juice preparation processes.[43,44] The oligosaccharide series of interest has been detected in cranberry juice concentrate and in commercially obtained juice products, indicating that this group of compounds is present in products available to consumers.[52,54] Compound **1** and the reported cranberry arabinoxyloglucans are similar to fragments derived from polymeric xyloglucans identified in solanaceous plants.[55] Such compounds are hemicellulose polymers that serve as structural components of plant primary cell walls. These polymers are enzymatically cleaved and solubilized during the natural process of fruit ripening,[55−57] but may also be released from cell wall components into fruit products as a result of commercial fruit processing procedures.[58] Further research will be needed to investigate the biochemical origins and structural complexity of the cranberry oligosaccharide profile and the occurrence of these and similar compounds in various food products.

Direct absorption and rapid elimination of unmodified dietary oligosaccharides into mammalian urine would be consistent with the reported rapid appearance of antiadhesion activity in human urine after the consumption of cranberry juice.[33] Oligosaccharides with various structures have been previously isolated from normal human urine,[59] and reports have shown that mixtures of complex oligosaccharides ranging in size from trimers to heptamers and larger can be ingested and subsequently found in adult and infant human urine.[60,61] Mice gavaged with high doses of globotriose [Gal-(α1→4)-Gal-(β1→4)-Glc] were found to rapidly clear this trisaccharide from plasma ($t_{1/2}$ = 6 min) and excrete it primarily into urine without metabolic biotransformation at levels of >5 mg/mL for 4−12 h after administration, indicating that this compound is orally bioavailable and retained for later excretion over time.[62] Preliminary data from the present study indicate that a minimum of 0.13−0.25% of ingested cranberry oligosacchar-

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ides were excreted into urine, supporting the hypothesis that orally ingested cranberry oligosaccharides may also be excreted through feces or retained in body compartments and excreted into urine over time. As the swine used in this study were fed relatively high doses of cranberry material to ensure detection and isolation of possible antiadhesive components, further studies will be necessary to determine in vivo mammalian urinary excretion levels of oligosaccharides after the consumption of more typical quantities of cranberry materials.

Hemicellulose polymers and oligomers such as arabinoxyloglucans are generally considered to be a type of dietary fiber, as they are water-soluble, acid stable, and resistant to degradation during the early stages of mammalian digestion, as mammals lack β-glucosidase digestive enzymes.[63,64] While mammalian enzymes may not be able to metabolize the cellulosic β-(1→4)-linked glucose backbones of **1** and the cranberry oligosaccharides,[47,48] fecal microbes, such as specific *Bifidobacterium longum* subspecies, have been identified that are able to ferment cranberry xyloglucans.[65] It is therefore possible that oligosaccharides from plant sources may be excreted via both urinary and fecal routes, both with and without biotransformation. Furthermore, the fermentation of cranberry oligosaccharides by fecal microbes may have beneficial effects on gut composition and function such as those that have been observed for other dietary oligosaccharides.[66] Effects of cranberry oligosaccharides on the gut microbiome may therefore contribute to the reported systemic anti-inflammatory properties and other gut-associated health benefits attributed to cranberry products.[67] While this study did not directly investigate the fecal excretion of cranberry oligosaccharides, the observation that less than 1% of the ingested oligosaccharides were probably excreted via the urinary pathway suggests that the remaining material was eliminated by the fecal route, and further work will be needed to fully characterize the absorption, metabolism, and excretion of cranberry oligosaccharides in mammalian systems.

Oligosaccharide Antiadhesion Properties. Oligosaccharides in general are recognized to be natural interference ligands and are of significant medical interest for the prevention of infections.[58,68−72] Carbohydrates are well recognized as bioactive components that can prevent P-fimbriated *E. coli* adhesion, and globotriose [α-D-Gal(1→4)β-D-Gal(1→4)β-D-Gal] is able to inhibit adhesion both in vitro and in vivo by binding to P-fimbriae in place of cell surface receptors.[62] Previous studies have shown that mixtures of neutral oligosaccharides isolated from breast milk and the urine of breast-feeding babies are also able to interfere with *E. coli* adhesion.[60,61] Complex carbohydrates and oligosaccharide mixtures possess superior antiadhesion abilities as compared to mono- or disaccharides and may have increased efficacy at preventing adhesion due to their ability to mimic host cell carbohydrate receptors via simultaneous interactions with multiple bacterial adhesions.[70−72] Human milk oligosaccharides have been shown to have direct protective effects on human bladder epithelial cells in response to challenge by uropathogenic *E. coli*,[73] and such a mechanism may also contribute to the role cranberry compounds play in the prevention of UTIs. A cranberry oligosaccharide fraction has been shown to inhibit biofilm formation by *E. coli* in vitro,[45] and the bioassay results in this and our other studies[47,52] provide additional evidence that cranberry oligosaccharide mixtures can inhibit *E. coli* adhesion. Mixtures of cranberry oligosaccharides, rather than single compounds, may act as natural inhibitors of bacterial adhesion and may have additive or synergistic effects with other compounds from cranberry, but further investigations will be needed to elucidate the mechanisms involved.

Multiple compounds are likely to contribute to the overall antiadhesion properties of cranberry materials,[37−39,74] and additional studies will be required to fully elucidate the possible roles of oligosaccharides in the various biological properties attributed to cranberry. Further isolation and structural elucidation studies will be necessary to fully characterize the complex series of oligosaccharides present in various cranberry materials and in urine or feces after cranberry consumption. Future efforts will also need to focus on the development of analytical methods for the detection and quantification of this class of compounds in various cranberry products and in biofluids after the consumption of cranberry products. While other, possibly non-oligosaccharide, compounds were detected in some of the crude fractions with antiadhesion activity, attempts to isolate these compounds for further analysis were unsuccessful. The consistent presence of oligosaccharides in antiadhesive fractions combined with the results from three different bioassays that show antiadhesive properties for cranberry oligosaccharides mixtures[47,52] further supports the hypothesis that oligosaccharides are, in some way, involved in the antiadhesive properties of cranberry materials.

In conclusion, this study shows that a group of compounds found in antiadhesive swine urine fractions are oligosaccharides that are similar in structure to those found in analogous antiadhesive cranberry fractions. Cranberry-derived oligosaccharides are therefore potentially significant constituents that contribute to the antiadhesion effects of urine produced as a result of cranberry product consumption. This hypothesis provides a new guiding paradigm for future research regarding the antiadhesive constituents of cranberry.

EXPERIMENTAL SECTION

General Experimental Procedures. Regular solvents, HPLC solvents, and most reagents and general lab materials were obtained from Fisher Scientific, Inc. (Waltham, MA, USA), Sephadex LH-20 and additional materials (including apple pectin) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA), HPLC sample filters were obtained from Millipore, Inc. (Billerica, MA, USA), and NMR solvents were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA).

Source Materials. Spray-dried cranberry hull extract powder (*Vaccinium macrocarpon* Ait., Ericaceae) (a.k.a. cranberry powder, CJ) was supplied by Ocean Spray Cranberries, Inc. (Lakeville-Middleboro, MA, USA) and was used for direct separations and analytical studies and for the feeding of swine. This powdered material was created by spray-drying cranberry juice (cranberry hull extract) that had been produced via proprietary methods. The CJ powder was fully soluble in water and produced a dark burgundy solution that was similar in appearance (see accompanying article[47] for images) to commercially available cranberry juice products prepared from cranberry juice concentrate. Further studies by our research group have shown that the CJ material contains a similar oligosaccharide profile as that of commercially obtained cranberry fruit powder[47] and cranberry juice concentrate (provided to us by Ocean Spray Cranberries, Inc.).[52] Our studies have also found that commercially available cranberry juices contain detectable levels of a similar oligosaccharide series.[54]

Five separate feedings were performed in this study to produce sufficient amounts of cranberry-derived urine with antiadhesion activity;[42] materials from three of these feedings (designated F, H, and I; Table 1) are discussed in this publication and the Supporting Information. Animal protocols were approved by the University of Wisconsin Review Committee (approval number A3368-01, 04/25/

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2002). Adult female sows (ca. 160 kg each) were housed in gestation crates at the Livestock Laboratory on the University of Wisconsin−Madison campus and were offered a maintenance amount of a basal diet consisting of corn and soybean supplemented with tryptophan, methionine, lysine, and various vitamins and minerals as necessary to meet the National Research Council recommended requirements for maintenance.[75] Sows were chosen due to their similar metabolism to that of humans and because they can be fed a consistent diet supplemented with relatively large quantities of cranberry powder per day.[76] Sows also generate the volumes of urine (~1−2 L/d) necessary to provide sufficient material for bioassay-guided fractionation and metabolite purification.

Cranberry powder was mixed with basal diet components at an administration rate of 800 g powder/day/sow (~5 g/kg body weight) for 3−5 days prior to collection of treatment urine. Feeding was continued through the collection period of 3−4 days, and urine (2.0−6.5 L per sow) was collected via Foley catheters. Control urine (~2 L) was collected via catheter prior to initiating the feeding of cranberry powder. Urine was collected in containers placed over ice, containers were emptied at 4 h intervals, and urine was frozen at −20 °C immediately after collection. Animals were monitored twice daily and Foley catheters checked regularly by research staff during collection periods. Details of the urine collections that provided material used in this report are shown in Table 1. Aliquots (~5 mL each) of each urine collection were frozen and sent to Rutgers University for preliminary bioassay testing (results not shown), and remaining urine was shipped to the University of Mississippi as frozen liquid on dry ice.

Anti-Hemagglutination Assay. Anti-hemagglutination assays are commonly used to assess bacterial adherence to eukaryotic cells, as the agglutination of (A_1, Rh+) human red blood cells by P-fimbriated *E. coli* is due in part to the binding of the *E. coli* PapG adhesin to the α-Gal(1→4)β-Gal disaccharide binding epitope found on the surface of these cells.[77,78] The assay used for this study was performed as described previously with minor modifications.[24,25] This in vitro assay has been used to identify PACs as antiadhesive constituents of cranberry samples[24] and to detect antiadhesion properties of raw human urine samples.[33]

Bacterial Strains. Clinical strains of uropathogenic P-fimbriated *E. coli* (UPEC) were isolated and cultured as described previously,[24] with the modification that P-fimbriated bacteria were not washed to prevent fimbriae from breaking off, and were suspended directly in phosphate-buffered saline (PBS, pH 7.0) at a concentration of 5×10^8 bacteria/mL PBS for testing. Cultures were kept on agar slants at 4 °C for short-term use over several months, and strains were kept frozen at −70 °C in tryptose broth (30% glycerol) for long-term storage.

Hemagglutination Assessment and Relative Activity. Test samples were dissolved in different volumes of PBS based on the amount of sample provided (e.g., 150 μL for 10−30 mg, 200 μL for 40−70 mg, 400 μL for >80 mg) and adjusted to neutral, physiologic pH (between 5.5 and 7.0) with 1 N NaOH. Serial 2-fold dilutions of each test sample were prepared in PBS and were tested as described previously.[24,25] Hemagglutination was visually assessed using a scoring system to determine dilution end points (Section S1a, Supporting Information).

Dilution end point results were compared and interpreted using a qualitative scale (Section S1b, Supporting Information), modified from one used previously with this assay,[24] to determine the relative activity of a sample and whether it could possibly prevent UPEC−epithelial cell adhesion. Use of this scale allowed for comparisons across sample types and across submitted sets over time. This scale was based on four criteria: (1) the numerical dilution end point, (2) the number of 2-fold dilutions applied to the sample, (3) the activity of the sample relative to a parent fraction, and (4) a comparison of results from replicate samples, if available (Table S7, Supporting Information). Relative sample activity using this scale was assigned as follows: "+ +" = active; "+" = probably active; "+/−" = may or may not be active; "−" = probably not active; and "− −" = not active.

Bioassay Test Samples. Selected samples were coded, randomized, and submitted as dry powders (~10−400 mg per sample). Sample sets were shipped by overnight post on cold packs or dry ice. Duplicate or triplicate samples were submitted for parent materials and some fractions, but sample quantity limitations and the labor-intensive nature of the assay made it impractical to submit triplicate samples for every sample tested in every sample set. To account for assay variations over time, selected urine fractions were repeatedly tested with each set of samples submitted. These included control urine samples and fractions and the parent fractions for each separation step.

Isolation Methods for Urine Samples. Frozen liquid urine was thawed overnight at room temperature. A general purification protocol (Figure 4) was applied to the four batches of urine (FC, F, H, and I). Crude urine was centrifuged (5 min at 2500 rpm) or gravity filtered (Whatman #1) to remove casts and solids. A portion of each clarified urine batch was removed and lyophilized to serve as a pre-extraction control sample. Clarified urine aliquots (1 L each) were partitioned with EtOAc ((2−4) × 250 mL) or EtOAc/MeOH ((2−4) × 50:250 mL MeOH/EtOAc). EtOAc and aqueous urine fractions (FC1, FF, HF, IF) were evaporated under reduced pressure with a water bath temperature of ~40 °C. Dried EtOAc fractions were dissolved in water, aqueous samples were diluted as necessary, and all samples were frozen and lyophilized. Repeated dilution and lyophilization was necessary for highly hygroscopic samples and subsequent fractions. All samples were stored as dry powders or concentrated syrups (hygroscopic samples) in airtight containers at −20 °C. Control urine (FC) was partitioned using the procedures developed for the active urine samples, but was processed only to the stage of analytical-scale, reversed-phase HPLC (step 4, Figure 4; Section S2a, Supporting Information). Control urine fractions were submitted to the bioassay as negative controls.

Dried aqueous urine fractions (FF, HF, IF; 10−50 g per column) were chromatographed on Sephadex LH-20 (various column dimensions) using 50% or 70% EtOH for FF separations and 70% EtOH for HF and IF separations (isocratic) (Tables S2−S4, Supporting Information). Eluent was collected in tubes (5 to 25 mL each), and tubes were combined based on appearance and relative elution volume to give 5−8 total fractions per column. Fractions were dried as described above. Selected Sephadex LH-20 fractions were submitted for bioassay testing and further purified using HPLC-UV/ELSD. The optimum method involved preparative reversed-phase HPLC on Waters Atlantis dC_{18} sorbent followed by semipreparative, normal-phase HPLC on YMC Polyamine II sorbent (Section S3, Supporting Information).

Isolation Methods for Cranberry Samples. Cranberry powder (CJ) was directly separated by preparative HPLC-UV/ELSD (Atlantis dC_{18}) using a similar method to that applied to urinary fraction HF2-2 (Figure 16). Two batches of CJ (99.2 and 250 mg) were dissolved in water (0.80 and 1 mL, respectively), filtered (0.2 μm nylon filter), and separated (200 and 500 μL injections at 124 mg/mL and 1 mL injection at 250 mg/mL). Eluent was combined based on chromatographic profile and retention time to give fractions CJ-P1t22 (21.5−22.7 min, 3.3 mg), CJ-P1t23 (22.8−23.7 min, 4.0 mg), CJ-P1t25 (23.8−25.8 min, 7.3 mg), CJ-P1t28 (25.9−30.0 min, 7.6 mg), and CJ-P1t38 (6.5 mg), as well as others. These fractions were analyzed by ^{1}H NMR spectroscopy. Additional fractions collected did not yield enriched or purified compounds but accounted for 117.6 mg of additional material recovered from the two combined CJ-P1 separations.

Cranberry powder was also extracted and separated using the general protocol applied to urine samples (Figure 4). Aliquots of CJ (1.24 kg, in 80−120 g batches) were dissolved in water (450 mL/100 g) and extracted with EtOAc (10 × 1:3 v/v EtOAc/water). The EtOAc extracts (CJE) were combined (12.706 g; 1.02% w/w of CJ) and dried, and the residual amorphous burgundy solid was resuspended in a minimum amount of water and lyophilized. Aqueous material (CJA) was combined, evaporated under reduced pressure to remove residual organic solvent, diluted with water if necessary, frozen, and lyophilized in 1−2 L batches.

Fraction CJA was compared to urine fractions HF1-1 and HF2-2 by analytical HPLC-UV/ELSD (Figure 15) using the same method as

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applied to urine fractions (Atlantis dC_{18}, 4.6 × 150 mm, 5 μm). Samples of CJA (40 μL injection, 30.0 mg/mL), HF1-1 (25 μL injection, 10.0 mg/mL), and HF2-2 (20 μL injection, 26.0 mg/mL) were prepared in a single session and were sequentially separated using identical methods without flow interruptions between injections. The column was washed and re-equilibrated with a minimum of 10 column volumes of the starting solvent before each injection.

An aliquot of CJA (25.6 g) was fractionated on Sephadex LH-20 using protocols similar to those developed for urine samples. The CJA sample was dissolved in water, and EtOH (100%) was added gradually with intermittent sonication to give a final concentration of 70% EtOH (100 mL). The sample was briefly centrifuged to remove precipitate and was chromatographed on Sephadex LH-20 (7 × 37 cm) with 70% EtOH (isocratic) at 1.5−1.7 mL/min. Eluent was collected in 20−25 mL aliquots that were combined based on appearance and elution volume and dried as above to give 12 fractions including CJA1-02 (3.649 g), CJA1-03 (1.280 g), CJA1-04 (1.669 g), and others. Selected materials were submitted for bioassay testing and further characterized by ^{1}H NMR spectroscopy and HPLC-UV/ELSD. A second aliquot of CJA (CJA2) was later separated on Sephadex LH-20 and further purified by HPLC as discussed in the accompanying article.[47]

HPLC Separation Procedures. Reversed-phase samples were dissolved in a minimum amount of water, while normal-phase samples were dissolved in a minimum amount of either water or, at most, 60% MeOH. All samples were syringe-filtered with 0.2 μm filters (nylon or PTFE, 13 or 33 mm), and various amounts of material were injected per separation depending on the nature of the sample, the column, and the chromatographic conditions used (Section S3, Supporting Information). No postacquisition processing, retention time adjustments, or other modifications were made to the HPLC data shown in this report or associated materials.

Analytical-scale HPLC separations were performed on a Waters 2695 separations module equipped with a Waters 996 photodiode array (PDA) detector connected in series to a Polymer Laboratories PL-ELS2100 detector (ELSD). "Max plot" chromatograms (210−400 nm) were extracted from PDA data for detection of UV-active metabolites. Columns used included a Waters Atlantis dC_{18} (4.6 × 150 mm, 5 μm particle size) equipped with a guard column of the same sorbent (4.6 × 20 mm) and a YMC Polyamine II (Polyamine) (10 × 150 mm, 5 μm particle size, 12 nm pore size). The Atlantis dC_{18} column was used with a flow rate of 1 mL/min as follows: 5 min 100% water, 30 min gradient from 100% water to 30% MeOH, 20 min gradient from 30% to 100% MeOH, and 5 min 100% MeOH. The Polyamine column was used with a flow rate of 2.5 mL/min as follows: 5 min 100% MeCN, 20 min gradient from 100% to 60% MeCN, 10 min 60% MeCN, and 10 min gradient from 65% to 40% MeCN, with a counter solvent of water. Each column was allowed to equilibrate in the starting solvent conditions for a minimum of 10 column volumes prior to each sample injection.

Preparative-scale HPLC separations were performed using a Waters Delta Prep 4000 chromatographic system equipped with an LC controller and a 2487 dual-wavelength UV detector connected in series to a Polymer Laboratories PL-ELS1000 ELSD. Fractions were collected in parallel with ELS monitoring through the use of a flow splitter installed in the output line after the UV flow cell. A preparative Waters Atlantis dC_{18} column (19 × 250 mm, 10 μm particle size) was used with a flow rate of 25 mL/min as follows: 5 min 100% water, 30 min gradient from 100% water to 20% MeOH, 10 min gradient from 20% to 100% MeOH, and 3 min 100% MeOH. A semipreparative YMC Polyamine II column (10 × 150 mm, 5 μm particle size, 12 nm pore size) was used with a flow rate of 4 mL/min as follows: 5 min 65% MeCN and 25 min gradient from 65% to 45% MeCN, with a counter solvent of water. Guard columns were not used. Fractions were combined based on retention time and chromatographic profile and dried as above. Each column was allowed to equilibrate in the starting solvent conditions for a minimum of 10 column volumes prior to each sample injection.

ELSD settings varied based on the solvent systems used and the inlet solvent flow rate. Settings remained constant throughout each separation. The ELSD was allowed to equilibrate for at least 30 min at the specified settings prior to initial data acquisition for a given session and additionally for 10−20 min at the starting conditions for each separation. All eluent from analytical separations was directed through the ELSD. The HPLC instrument was configured for split detection−collection for semipreparative and preparative separations: for Polyamine separations, ∼0.5−1 mL/min of eluent was delivered to the ELSD with the remaining ∼2−2.5 mL/min delivered to a collection vessel, while for Atlantis dC_{18} separations, ∼1−3 mL/min of eluent was delivered to the ELSD with ∼22−24 mL/min delivered to a collection vessel. Typical ELSD settings for Atlantis dC_{18} separations were a gas flow rate of 0.8−1.2 SLM, an evaporator temperature of 100 °C, and a nebulizer temperature of 50 °C, while those for the Polyamine column were a gas flow rate of 1.0−1.2 SLM, an evaporator temperature of 90 °C, and a nebulizer temperature of 45 °C.

Structure Elucidation. *NMR Spectroscopy.* Bruker Avance III 400 MHz spectrometers equipped with either Ultrashield or Ultrashield Plus magnets and 3 mm probes were used to obtain ^{1}H and 2D NMR spectra for crude and enriched fractions. A Bruker Avance 700 MHz spectrometer with a 5 mm CPTCI cryoprobe was used to obtain the 1D and 2D NMR data used for assigning the structure of compound **1**. Samples for NMR analysis were prepared by dissolving 2−10 mg of material in a minimal amount of high-purity DMSO-d_6 (99.99%) or D_2O (99.99%).

Carbohydrate Structural Analyses. Compound **1** was found to be a complex carbohydrate and was submitted to the Complex Carbohydrate Research Center (CCRC) at the University of Georgia (Athens, GA, USA). Analyses necessary for full structural elucidation required derivatization of the sample and provided information on glycosyl composition, glycosyl linkage positions, high-resolution mass by MALDIMS, oligosaccharide sequence, and monomer configuration. Details of the standard methodologies employed are included in the Supporting Information (Section S4).

Compound 1. β-D-Glucopyranosyl-(1→4)-[α-L-arabinofuranosyl-(1→2)-α-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-(1→4)-[α-L-arabinofuranosyl-(1→2)-α-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-(1→4)-β-D-glucopyranose (**1**): white, amorphous powder; ^{1}H and ^{13}C NMR data, Figures 8 and 9, Figures S5 and S6 (Supporting Information), and Table 3; 2D NMR data, Figures 10 and 11 and Figures S7−S13 (Supporting Information); MALDIMS (positive ion mode) $[M + Na]^+$ at m/z 1218.117, to give a calculated molecular formula of $[C_{44}H_{74}O_{37}+Na]^+$ (Figure S14, Supporting Information).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b01043.

Additional tables and figures containing chromatographic, spectroscopic, and carbohydrate composition data; further description of the bioassay methods and scoring system; additional details and descriptions of isolation methods; supplementary results and discussion points; details of the methods used for carbohydrate structural analyses; detailed discussion of the partial fragmentation patterns from NSI-MS^n analysis used to confirm linkage positions and monomer connectivity (PDF)

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

Dedicated to Dr. Rachel Mata, of the National Autonomous University of Mexico, Mexico City, Mexico, and to Dr. Barbara N. Timmermann, of the University of Kansas, for their pioneering work on bioactive natural products.

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(41) Information regarding our findings pertaining to oligosaccharides was shared with employees of Ocean Spray Cranberries, Inc., through personal communications in 2011 and 2012 with the objective of obtaining funding for future research on the subject. Additional public disclosures of the cranberry oligosaccharide hypothesis were made at the Annual Meeting of the American Society of Pharmacognosy in St. Louis, MO, USA, on July 17, 2013.
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