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Benzyl Benzoate Glycosides from *Oligoneuron rigidum*

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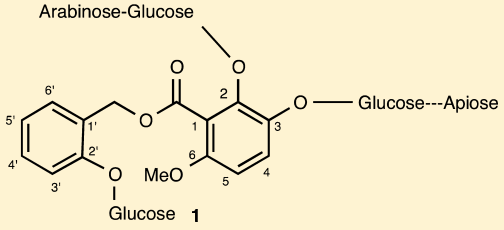
ABSTRACT: Two new benzyl benzoate glycosides were isolated from the leaves and stems of the native North American prairie plant *Oligoneuron rigidum* (stiff goldenrod). The glycosides were isolated as a mixture of benzoate and acetate esters, which were subjected to mild base hydrolysis to facilitate full structural characterization using LCMSMS and 1D and 2D NMR data.



The research interest of our laboratory is to examine North American prairie plants as a source of novel natural products. In addition to novel chemical structures, we are interested in identifying compounds that exhibit biological activity and may have commercial uses. We have previously identified compounds that show biological activity against HIV-1[1] and *Mycobacterium tuberculosis*.[2] In addition, we recently introduced a simple screen to identify prairie plants that may be sources of compounds useful as natural food preservatives.[3]

Herein we describe the isolation and structural elucidation of a novel benzyl benzoate glycoside from the North American prairie plant stiff goldenrod (*Oligoneuron rigidum*). Our interest in this plant was initiated by the crude extract's ability to inhibit the growth of microbes on the surface of cheese, which is the simple assay used by us for identifying natural food preservatives. The genus *Oligoneron* is a genus within the Asteracea family consisting of six species. *Oligoneuron rigidum* (stiff goldenrod) was originally classified in the *Solidago* genus (*S. rigida*).[4] The genus *Solidago* is an herbaceous, perennial species with a history of medicinal uses.[5]

RESULTS AND DISCUSSION

The methanol extract of *O. rigidum* was subjected to Sephadex LH-20 gel permeation chromatography followed by flash reversed-phase C_{18} chromatography. A ^{1}H NMR spectrum of the major fraction that was obtained from the C_{18} flash chromatography column (20% H_2O in MeOH) identified resonances associated with aromatic, anomeric, alcoholic, and acetate protons. This fraction was further purified using bonded-phase NH_2 HPLC to give two resolved peaks. The ^{1}H NMR spectra of these two peaks both contained signals observed for the initial fraction, but had subtle differences in the anomeric region of their spectra. Further, there were a series of sharp singlets between 1.96 and 2.05 ppm, which were attributed to acetate protons. Although these fractions appeared as single peaks in their chromatograms, the ^{1}H NMR spectra indicated that they were not pure compounds. The two fractions were subjected to an LC-MSTOF experiment to determine the molecular weight of the apparent mixture. Both fractions appeared as single peaks in the HPLC chromatogram and gave m/z values (negative ESI) of 1185.3568 (t_R = 4.40 min) and 1185.3470 (t_R = 4.70 min). These molecular weights indicate identical molecular formulas (M − 1) of $C_{52}H_{65}O_{33}$ (calculated 1185.3509). On the basis of the observed ^{1}H NMR spectra, mass spectrum, and HPLC chromatogram it was concluded that the two separable peaks represented regioisomeric compounds. Further, the presence of four peaks in the ^{1}H NMR spectra of the two compounds between 1.96 and 2.05 ppm suggested that the separable peaks were a set of inseparable regioisomers that differed in the position of the acetate groups. Further attempts to purify these peaks using other forms of bonded-phase HPLC were fruitless. Attempting to solve the structure of the two compounds as mixtures of acetates was not viable because the mixture gave a spectrum that could not be interpreted. To circumvent this problem, we subjected the compounds to mild base hydrolysis.

When the isomers associated with the peak at 4.40 min were treated with a methanolic solution of 0.5 M NaOH for 5 min followed by neutralization with HOAc and separated using HPLC (NH_2), a single peak (t_R = 9.1 min) was observed. An accurate mass (negative ESI) gave a molar mass of 1039.3229 corresponding to a molecular formula of $C_{43}H_{59}O_{29}$ (calculated 1039.3142). When the peak at 4.70 min was treated under the same conditions, a single peak (t_R = 8.00) was obtained. Accurate mass of that peak gave an m/z of 1039.3218, which corresponds to $C_{43}H_{59}O_{29}$. The loss of 146 amu from the native compounds upon hydrolysis was inconsistent with our initial hypothesis that these native compounds were mixtures of regioisomeric monoacetates (i.e., anticipating a loss of 42 amu). However, a decrease of 146 amu is consistent with the loss of an *O*-acetyl group with H-transfer (42 amu) and the loss of a benzoyl group with H-transfer (104 amu). The ^{1}H NMR spectrum of the hydrolysis product(s) now lacked the signals

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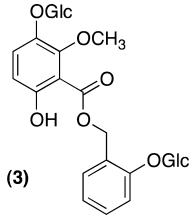
between 1.96 and 2.05 ppm (loss of the *O*-acetyl) and had three fewer aromatic signals (loss of a benzoyl group). Therefore, the native compounds are acetyl and benzoyl esters of **1** and **2**.

The ^{1}H NMR spectrum of the hydrolysis product from the peak at 4.40 min (**1**) showed six aromatic signals, which could be attributed to two aromatic rings. One aromatic ring had four adjacent protons (δ 7.51 dd, J = 7.6, 1.6 Hz; 7.08 dt, J = 7.6, 0.88 Hz; 7.30 dt, J = 7.6, 1.6 Hz; 7.23 dd, J = 7.6, 0.88 Hz), and the other had two adjacent protons [δ 7.27 d; 6.80 d, J = 9.23 Hz]. Two geminally coupled doublets (J = 12.8 Hz) at 5.2 and 5.68 ppm suggested that they were part of a rigid benzylic system. Additionally, there was a three-proton singlet at 3.78 ppm that could be attributed to an aromatic *O*-methyl group. These observations led to the conclusion that the aglycone portion of **1** was related to the previously isolated benzyl benzoate glycoside leiocarposide (**3**),[6] which had been previously isolated from *Solidago virgaurea*.



(3)

In addition to the six aromatic signals, the ^{1}H NMR spectrum of **1** indicated that there were five protons that could be attributed to carbohydrate anomeric protons (δ 5.31 d, J = 3.5 Hz; 5.02 d, J = 8.2 Hz; 4.94 d, J = 9.2 Hz; 4.91 d, J = 8.2 Hz; and 4.58 d, J = 6.5 Hz). On the basis of the molecular formula of $C_{43}H_{59}O_{29}$ obtained from the accurate mass ESI spectrum and the assumption that the aglycone portion of the molecule contained 15 carbons, the remaining 28 carbons would be required to be the carbohydrate of the molecule corresponding to three hexose and two pentose moieties. The positions of the five monosaccharide moieties on the benzyl benzoate aglycone were determined from a series of MS/MS experiments. Figure 2 shows the results of the ESI MS/MS spectrum of **1** in negative ion mode.

The LC/MS/MS indicated that the benzyl ring contained one hexose, while the benzoic acid portion contained two hexose and two pentose units. The loss of $C_{11}H_{18}O_9$ (giving m/z = 745.2193) indicated that there were two hexose−pentose disaccharide units on the benzoic acid ring.

The carbohydrate identity and glycosidic linkage positions were determined using the GC/MS reductive cleavage method originally developed by Gray.[7] The results of the GC/MS analysis identified C-1-linked glucose, arabinose, and apiose moieties. Additionally, there was a C-1, C-2-linked glucose and a C-1, C-3-linked glucose.

The final structure elucidation was accomplished through the use of HMBC, HSQC, 1D NOESY, COSY, and 1D TOCSY experiments (Tables 1 and 2). The position of glucose 3 on the benzyl ring was confirmed by the HMBC correlation of its anomeric proton (4.94 ppm) to C-2′ (156.85 ppm). The positions of the glucose units on the benzoic acid moiety were established by first assigning the aromatic carbons to their proper ^{13}C chemical shifts from the HSQC and HMBC correlations. Given in Table 1 are all the HMBC correlations for the resolved protons and carbons in compounds **1** and **2**. The HMBC correlation from the proton at 6.80 ppm to the

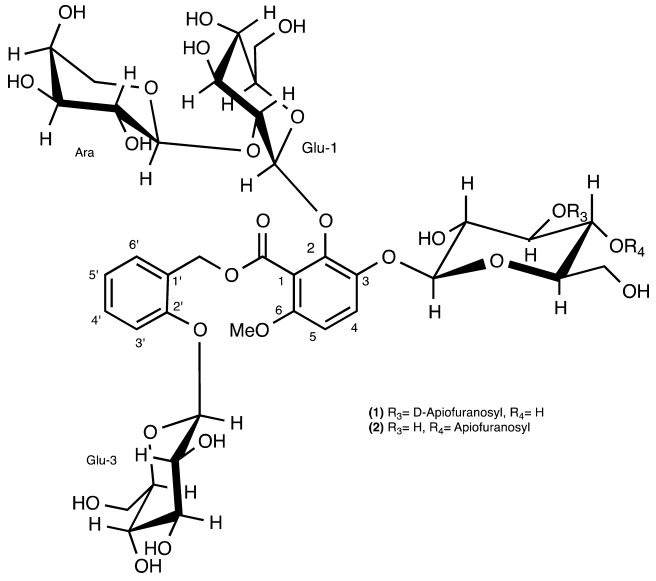


Figure 1. Base-hydrolyzed benzyl benzoate glycoside from *O. rigidum*.

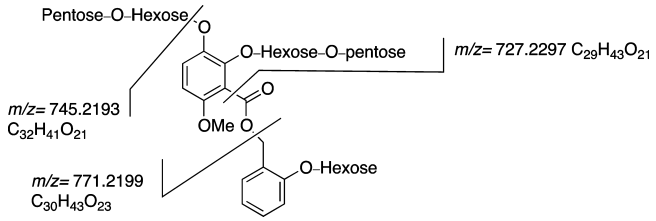


Figure 2. Results of LC/MS/MS (negative ESI) of m/z= 1039.3144. Fragmentation ions indicate that two hexoses and two pentoses are on the benzoate ring.

carbonyl at 168.03 (^4J correlation) initially suggested that the protons on the benzoic acid were *ortho* and *meta* to the carboxyl group, thus assigning C-6 to the proton at 6.78 ppm with a ^{13}C NMR chemical shift of 109.20 ppm. (This regiochemistry would have been consistent with the initially proposed structure for leiocarposide.[8]) Subsequent 1D and 2D NOESY experiments showed a strong enhancement from the proton at δ 6.80 to the OMe group at 3.77 ppm, thus indicating that the OMe group should be assigned to C-6 on the benzoic acid and the proton at 6.80 ppm assigned to C-5 (106.90 ppm). This regiochemistry is consistent with revised leiocarpiside structure.[9] The proton at 7.27 ppm showed an NOE enhancement to the anomeric proton at 4.91 ppm, thus establishing the position of one glucose at C-3 of the benzoic acid. An HMBC correlation from the same anomeric proton (4.91 ppm) to the aromatic carbon at 144.49 ppm established the chemical shift of C-3. A second glucose was established on C-2 of the benzoic acid by its anomeric proton (5.02 ppm) correlation to C-2 at 144.93 ppm.

The regiochemistry of the two pentose units was established from HMBC correlations of their anomeric protons to glucose carbons through their glycosidic linkages. The apiose anomeric proton (5.31 ppm) was identified via its correlation to the unique quaternary carbon (Api C-3) at 80.66 ppm. The apiose anomeric proton also showed a strong correlation to a carbon signal at 84.87 ppm, which was determined to be C-3 on glu-2. The anomeric proton of the arabinose moiety (4.57 ppm) was identified via correlation to its C-5 methylene carbon at 66.36 ppm. The regiochemistry of the arabinose was determined from

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Table 1. NMR Chemical Shift Assignments for 1 and 2 in Methanol-d_4 at 400 MHz (^{1}H) and 100 MHz (^{13}C)[a]

	1			2		
position	δ_C	δ_H (J in Hz)	HMBC	δ_C	δ_H (J in Hz)	HMBC
Api-1	111.35	5.31 d (3.6)	84.87 80.66 77.66 74.72	110.70	5.09 d (3.5)	74.67 80.26 79.35
2	77.66	4.05 d (3.6)	111.35 74.72	77.91	3.94 d (3.5)	74.67 64.67 110.70
3	80.60			80.26		
4	74.72	4.10 3.81 d (9.8)	111.35 80.60 77.66 64.95	74.67	4.15 3.82 d (9.8)	110.70 80.26 77.91 64.77
5	64.95	3.59 s	111.35 80.60 77.66 74.72	64.77	3.58 s	110.70 80.26 77.91
Ara-1	105.50	4.58 d (6.5)	83.23 74.17 72.84 66.36	105.20	4.57 d (6.2)	105.20 73.90
2	72.84	3.71 dd (6.5, 8.8)	105.50 74.17	72.84	3.72 dd (6.2, 8.8)	
3	74.17	3.57 dd (8.8 3.5)		73.90	3.63 dd (8.8, 3.5)	
4	69.40	3.74		68.99	3.79	
5	66.36	3.85 dd (12.7, 3.8)		66.23	3.85 dd, (12.7,3.8)	
		3.49 d (12.7)			3.49 d (12.7)	
Glu-1	102.20	5.02 d (8.4)	144.49 82.23 77.89	101.90	5.10 d (7.8)	143.82 82.51 77.55 74.60
2	83.23	3.30 dd (8.4, 8.8)	105.50 102.20 77.89	82.51	3.31 dd (7.8, 8.8)	105.20 101.90 77.55
3	77.89	3.54 t (8.8)		74.60	3.56 t, (8.8)	
4	71.43	3.25 t (8.8)	77.89 82.23 62.85	71.13	3.26 t (8.8)	77.55 62.97
5	77.91	3.13 ddd (8.8, 2.5,4.6)		77.55	3.15 ddd (8.8, 2.5, 4.6)	82.51 71.13
6	62.85	3.68 dd (2.5 12.2)		62.79	3.68 dd (2.5, 12.2)	
		3.54 dd (4.6, 12.2				
		3.54 dd (4.6, 12.2)			3.54 dd (4.6, 12.2)	
Glu-2	103.30	4.91 d (8.2)	144.93 78.05	102.50	4.93 d (8.4)	144.27 76.68
2	74.59	3.64 dd (7.4, 8.2)	103.30 84.87 78.05	76.68	3.58 dd (7.2, 8.4)	
3	84.87	3.58		75.58	3.64	
4	78.05	3.50		79.35	3.55	110.70
5	70.00	3.40		77.19	3.45	
6	62.40	3.87 d (12.3)		61.30	3.86 d (12.3)	
		3.75 dd (12.3, 2.5)			3.76 dd (12.3, 2.5)	
Glu-3	103.10	4.94 d (9.2)	156.85 78.31	102.60	4.97 d (8.4)	156.60 77.80
2	78.31	3.52 t (9.2)		77.83	3.57 t (8.4)	
3	78.08	3.44		77.80	3.48	
4	71.30	3.42		71.23	3.48	
5	74.72	3.52		74.42	3.52	
6	62.50	3.87 d (12.3)		62.20	3.89 d (12.3)	
		3.72 dd (12.3, 2.5)			3.75 dd (12.3, 2.5)	
1′	126.87			126.54		
2′	156.85			156.50		
3′	116.72	7.23 dd (7.6, 0.9)	156.85 126.87 123.65	116.50	7.22 dd (7.6, 0.9)	156.50 126.54 123.50 63.00
4′	130.80	7.30 dt (7.6, 1.6)	156.85 131.08	130.70	7.30 dt (7.6 1.6)	156.50 131.00
5′	126.65	7.08 dt (7.6, 0.9)	126.87 116.72	123.50	7.07 dt (7.6, 0.9)	126.50 116.50
6′	131.08	7.51 dd (7.6, 1.6)	156.85 130.80 63.27	131.00	7.50 dd (7.6, 1.6)	156.50 63.00 130.70
1	121.68			121.33		
2	144.93			144.27		
3	144.49			143.82		
4	120.74	7.27 d (9.2)	153.30 144.49	120.33	7.22 d (9.2)	153.45 143.83
5	109.20	6.80 d (9.2)	144.93 121.68 153.30 168.03	106.90	6.78 d (9.2)	167.70 144.27 153.45 121.30
6	153.50			153.45		
OMe	56.90	3.77	153.50	56.70	3.78	153.45
carbonyl	168.03			167.70		
benzylic	63.27	5.68 5.31 d (12.8)	168.03 156.85 131.08 126.87	63.00	5.69 5.29 d (12.8)	167.70 156.50 131.00 126.50

[a]Unresolved proton assignments were made based on 1D TOCSY correlations from anomeric protons to C-6 (or C-5) protons. Proton multiplicities and coupling constants were determined from 1D TOCSY experiments. Coupling constants not reported could not be resolved.

the HMBC correlation of its anomeric proton to a signal at 82.32 ppm, which was determined to be C-2 on glu-1.

At 400 MHz a number of ^{1}H NMR signals were not resolved. However, all five of the anomeric signals could be resolved, which allowed for the application of the 1D TOCSY experiment (along with the three-bond COSY data) to determine the chemical shifts of the five isolated spin systems associated with each monosaccharide with reasonable certainty. Given in Figure 3 is the result from the 1D TOCSY experiment with irradiation at 5.02 ppm for glu-1 with increasing mixing times (30, 60, 90, 120 ms).

The same experimental procedure as used for H-1 of glu-1 was applied to the other four anomeric signals. These relayed couplings along with COSY, HSQC, and HMBC data were used to make the ^{1}H and ^{13}C assignments given in Table 1.

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Table 2. HMBC Correlations To Anomeric and Glycosidic Carbons

carbon	H-correlation to carbon (**1**)	H-correlation to carbon (**2**)
Api C-1	4.05 3.81 3.59	4.15 3.94 3.82 3.56
Ara C-2	3.30 3.49 3.71 3.84	3.85 3.72 3.48 3.31
Glu-1 C-1	3.30	3.31 3.56
Glu-2 C-1	3.64	3.57
Glu-3 C-1	3.55	3.58
Glu-1 C-2	4.58 3.53 3.11	4.57 3.56
Glu-2 C-3	5.31 3.73 3.64	
Glu-2 C-4		5.09 3.58 3.76

Compound **2** had the same molecular formula as **1** and a similar ^{1}H NMR spectrum with two notable exceptions. The anomeric signal of the apiose moiety was substantially upfield, relative to **1** (5.31 ppm versus 5.09 ppm), and the signal for H-2 was further upfield as well (4.10 ppm versus 3.95 ppm). This suggested that **2** was a regioisomer of **1** that differed in the position of the apiosyl attachment to the glucosyl moiety. Following the same experimental procedures for the structural determination of **1**, it was determined that the apiose moiety was located at C-4 of glu-2. This was determined from the HMBC correlation of the anomeric proton of apiose (5.09 ppm) to the carbon at 79.35 ppm (C-4 glu-2). Similarly, the C-4 proton (3.55 ppm) on glu-2 showed a correlation to the anomeric carbon (110.70 ppm) on apiose.

The absolute configuration of glucose was assumed to be D based on previous glycosides isolated from other species of *Solidago*.[4] The absolute configuration of arabinose was determined by synthesizing the (*R*)-2-butanol permethyl glycoside of both D and L arabinose and comparing their GC chromatograms. When **1** or **2** was permethylated followed by strong acid exchange catalyzed butanolysis, the resultant chromatogram gave peaks consistent with the L-arabinose isomer. The absolute configuration of apiose was assigned to be D based on the isolation and characterization of an apioside previously isolated from *Solidago canadensis*[10] and the (*R*)-2-butanolysis results.

Structure Elucidation of the Native Compounds. The structural elucidation described above was for the base-hydrolyzed glycosides. Small quantities of **1** and **2** were found in the initial extract but in insufficient quantities to allow for a complete structural elucidation. The compounds (**1** and **2**) were initially isolated as a mixture of benzoate−acetate regiosiomers, which could not be separated into pure compounds. With the skeletal structure of the glycoside established, an attempt to interpret the NMR spectra of the native compounds was initiated. An HMBC experiment on the native compounds (data not shown) indicated that there was a single benzoate group at C-6 of one of the glucose units. The ^{1}H NMR spectra of the native compounds each had four resolvable singlets between 1.95 and 2.05 ppm, suggesting that each peak represented a mixture of (at least) four acetate regioisomers. The ^{13}C and ^{1}H NMR spectra of the mixtures had too many nonresolvable peaks to unequivocally assign the regiochemistry of the four acetates using NMR spectroscopy at 400 MHz.

Biological Activity. Plants of the genus *Solidago* have been shown to have a variety of biological activities.[5] The screen employed in this study was designed to determine the ability of the plant extract to inhibit the growth of microorganisms associated with the spoilage of fresh foods. In our assay we employed fresh cheese as a food source and monitored microorganism growth over the course of one week. Briefly, 10 μL of a DMSO solution of the test compound was rubbed on two pieces of similarly sized slices of mozzarella cheese, and the microbial growth monitored over a period of one week. The growth on the test compound was compared to the growth on cheese treated with only DMSO (negative control) and cheese treated with clortrimazole and cycloheximide (positive controls). Both pure **1** and **2** and the native benzoate−acetate isomeric mixtures were tested for their ability to inhibit growth. Visual inspection suggested that they inhibited growth relative to DMSO but not as effective as the two positive controls. We attempted to quantitate this activity using disk assays with pure cultures of *C. albicans* and *A.niger*. In these assays, compounds **1** and **2** and their benzoate−acetate precursors failed to show any growth inhibition.

EXPERIMENTAL SECTION

General Experimental Procedures. All solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). All reagents (standard monosaccharides and derivatizing agents) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Deuterated solvents were purchased from Cambridge Isotopes (Andover, MA, USA). Optical rotations were obtained using a Rudolph Instruments Digipole 781 polarimeter using the D-line from a sodium light source. Infrared spectra were obtained using a Thermo IR 200. All NMR experiments were performed on a Varian Inova 400 MHz NMR spectrometer using the standard Varian software and pulse programs without modification from VNMRJ 1.1D. All solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). All reagents (standard monosaccharides and derivatizing agents) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Deuterated solvents were purchased from Cambridge Isotopes (Andover, MA, USA). All NMR experiments were performed on a Varian Inova 400 MHz NMR spectrometer using the standard Varian software and pulse programs without modification from VNMRJ 1.1D. Accurate mass ESI-LC-MS were performed using an Agilent LC-MS-TOF equipped with a DAD UV−vis detector and an Agilent model 1100 LC system. LC-MS-MS experiments were performed as described below.

HPLC columns were either Zorbex (analytical and LC-MS) or Varian Dynamax (preparatory HPLC). Preparatory HPLC was performed using an ISCO model 2350 HPLC equipped with a UV detector. Peaks were monitored at 225 nm. TLC plates and flash chromatography sorbents were purchased from Sorbent Technologies (Atlanta, GA, USA).

Plant Material. Fresh plant parts of *O. rigidum* were collected in September 2009 at Clinton Lake State Park in Douglas, Kansas (latitude 38.9388, longitude 95.37725, elevation 303 m) by Hiliary Loring. It was identified by plant taxonomist Dr. Kelly Kindscher at the Kansas Biological Survey, University of Kansas. A voucher specimen (Hillary Loring 3630) was deposited in the R.L. McGregor Herbarium of the University of Kansas.

Extraction and Isolation. The air-dried leaves and stems of *O. rigidum* were ground in a Wiley mill. A sample (70 g) of the dried, ground plant was extracted with a 1:1 mixture of CH_2Cl_2/MeOH overnight. The extraction solvent was decanted from the plant

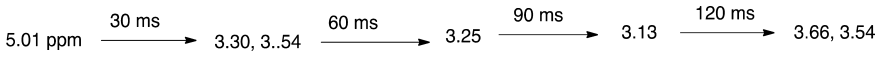

Figure 3. Results of 1D-TOCSY experiment for irradiation of the anomeric proton for glu-1.

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material, which was then extracted with MeOH for 12 h. The MeOH extract was combined with the CH_2Cl_2/MeOH, and the solvents were removed at reduced pressure (8 g). A sample (1.16 g) of the crude organic extract was dissolved in 1:1 CH_2Cl_2/MeOH and placed on an LH-20 column (50 cm × 2.5 cm). Samples (4 mL) were collected using a fraction collector (ISCO; Foxy 500, Lincoln, NB, USA). The fractions were analyzed using TLC (bonded phase amino, Sorbent Technologies, Atlanta, GA, USA). Similar fractions were combined and subjected to the inhibition assay and ^{1}H NMR spectroscopy. A fraction (eluting from 28 to 40 mL after the void volume; 187 mg) appeared to inhibit growth and showed signals that could be attributed to aromatic and carbohydrate compounds. This fraction was then adsorbed onto C_{18} bonded flash silica gel (Sigma-Aldrich, Milwaukee, WI, USA) and eluted using 50% aqueous MeOH, 20% aqueous MeOH, 100% MeOH (30 mL of C-18 in a 50 mL glass syringe employed as the column). The fraction that eluted with 20% aqueous MeOH was filtered, dissolved in a minimum amount of 80% CH_3CN/20% H_2O, and further separated with bonded phase amino HPLC (Varian Dynamax, Palo Alto, CA, USA; 25 cm × 1.0 cm; 70%CH_3CN/30%H_2O). Two symmetrical peaks (suggesting pure compounds) were observed in the chromatogram (t_R = 4.40 min; t_R = 4.70 min). The ^{1}H NMR spectra of these two fractions were similar and also appeared to be a mixture of compounds. Attempts at using other bonded phase HPLC columns (C_{18}; C_8; CN) still resulted in a single peak in the chromatogram but appeared as a mixture of compounds in the ^{1}H NMR spectrum. The individual peaks were subjected to LC-MS-ESI-TOF (Agilent Technologies; Santa Clara, CA, USA). Both compounds yielded [M − 1] ions in their negative ESIMS, which corresponded to a molecular formula of $C_{52}H_{65}O_{33}$ (calculated m/z 1185.3509; t_R = 4.40: m/z 1185.3568, t_R = 4.70: m/z 1185.3470). On the basis of the appearance of each ^{1}H NMR spectrum it appeared that both compounds were inseparable mixtures of acetates and benzoates. In an attempt to identify the glycosidic structure of the compounds, a mild base hydrolysis was performed to convert the esters to the free alcohols.

A sample of the early eluting peak (t_R = 4.40 min; 46.1 mg) was dissolved in 1 mL of MeOH and treated with 0.250 mL of 0.5 M NaOH. The sample turned a light yellow, and the reaction progress was monitored using TLC. When no starting material could be observed, the reaction was quenched with excess HOAc and passed through an AG 501-X8 (BIO-RAD, Hercules, CA, USA) mixed ion-exchange resin that had been washed with 5 volumes of MeOH. The reaction mixture was eluted with 5 volumes of MeOH (5 mL). The MeOH was removed at reduced pressure. The resultant material was dissolved in a minimum amount of MeOH and eluted on an amino bonded phase HPLC column (25 cm × 1.0 cm; 70%CH_3CN/30% H_2O). A large peak was collected after 8.0 min (26.3 mg) and subjected to ^{1}H NMR spectroscopy and LC-MS-TOF. The LC-MS-TOF (negative ESI) gave an [M − 1] molecular ion at m/z 1039.3229, corresponding to a molecular formula of $C_{43}H_{59}O_{29}$ (calculated m/z 1039.3142). The loss of 146 amu corresponds to the loss of one acetate (42) and one benzoate (104). The ^{1}H NMR spectrum appeared to be similar to that for the prehydrolyzed compound, but lacked any signals corresponding to acetate protons (three peaks between 1.95 and 2.05) and displayed three fewer signals corresponding to aromatic protons (**1**). The same procedure was performed on the later eluting compound (t_R = 4.70 min; 59.0 mg), which resulted in the isolation of **2** (22.7 mg; negative ESI m/z 1039.3218).

We note that under the reaction conditions employed for the hydrolysis, there was no evidence for the cleavage of the benzyl ester. We attribute this to the apparent rigid structure of the compound, rendering the ester carbonyl inaccessible to base hydrolysis at ambient temperatures.

2-[2-*β*-L-Arabinopyranosyloxy-*β*-D-glucopyranosyloxy]-3-[3-D-apiofuranosyloxy-*β*-D-glucopyranosyloxy]-6-methoxybenzoic acid 2′-*β*-D-glucopyranosyloxybenzyl ester (1): white powder; $[\alpha]_D$ −36.5 (*c* 0.6, MeOH); IR ν cm^{-1} 3290, 2887, 1713,1488, 1255, 1066; HRESIMS (−) m/z 1039.3229 (calcd for $C_{43}H_{59}O_{29}$ = 1039.3142); ^{1}H and ^{13}C NMR data, Table 1.

2-[2-*β*-L-Arabinopyranosyloxy-*β*-D-glucopyranosyloxy]-3-[4-D-apiofuranosyloxy-*β*-D-glucopyranosyloxy]-6-methoxybenzoic acid 2′-*β*-D-glucopyranosyloxybenzyl ester (2): white powder; $[\alpha]_D$ −28.5 (*c* 0.3, MeOH); IR ν cm^{-1} 3302, 2923, 1722, 1488, 1255, 1066; HRESIMS (−) m/z 1039.3218 (calcd for $C_{43}H_{59}O_{29}$ = 1039.3142); ^{1}H and ^{13}C NMR data, Table 1.

GC-MS Determination of Carbohydrate Composition. Following a procedure described by Gray,[11] the identity of the monosaccharides was determined by permethylating the intact glycoside followed by reductive cleavage to reduce the resultant monosaccharides to their permethyl-1,5-anhydro derivatives. Compound **1** (1 mg) was permethylated by dissolving the glycoside in a NaOH/DMSO dispersion (0.20 mL) followed by the addition of 0.10 mL of CH_3I (Sigma-Aldrich, Milwaukee, WI, USA) in a small vial.[12] The mixture was allowed to stir for 1 h and diluted with 1 mL of water and 2 mL of $CHCl_3$. The $CHCl_3$ was extracted (×3)with 1 mL of H_2O. The $CHCl_3$ was removed under a stream of nitrogen, and the residue dried under vacuum for 30 min.

The permethylated glycoside was dissolved in 0.250 mL of $CHCl_3$, followed by the addition of 8.5 μL of $(CH_3)_3SiOSO_2CH_3$, 8.8 μL of Et_3SiH, and 1.5 μL of $BF_3{\cdot}O(Et)_2$. After stirring the solution overnight at room temperature 1 mL of MeOH was slowly added to the solution and stirred for an additional 30 min. The sample was passed through an AG 501-X8 mixed ion-exchange resin. The MeOH and $CHCl_3$ were removed at reduced pressure. The sample was acetylated using 5 equivalents of Ac_2O and 1-methylimidazole in 0.20 mL of $CHCl_3$ for 0.5 h. The mixture was washed with H_2O and aqueous $NaHCO_3$. The $CHCl_3$ was removed under nitrogen and subsequently under vacuum. The above procedure was repeated with D-glucose, D-arabinose, L-arabinose, and D-apiose. The sample was analyzed using an Agilent GC-MSD (Agilent 6890 GC and 5973 MSD) equipped with a Restek RTx-5MS capillary GC column (0.25 mm i.d. × 30 m; initial temperature held at 60 °C for 2 min and then ramped to 240 °C at a rate of 7 °C/min; flow rate 24 mL/min, 10.25 psi). Retention times for standards: apiose (10.58 and 10.70 min); arabinose (10.28 and 10.92); glucose (13.20 and 13.80 min). The GC-MS retention times for **1** were 10.58 min (apiose), 10.92 min (arabinose), and 13.80 min (glucose). In addition, peaks at 16.95 and 17.48 min were obtained. The mass spectra of these peaks matched with the published EIMS for 1,5-anhydro-2-*O*-acetyl-3,4,6-tri-*O*-methylglucitol (16.95 min) and 1,5-anhydro-3-*O*-acetyl-3,4,6-tri-*O*-methylglucitol (17.48).[13]

LC-MS-MS Analysis. Reconstituted extracts were analyzed by LC/MS and MS/MSESI in negative ion polarity. Liquid chromatography was performed using an Infinity 1290 series (Agilent Technologies, Waldbronn, Germany) device with a Zorbax SB-Aq column (2.1 × 50 mm, 1.8 μm) at 50 °C. Mobil phases consisted of A (10 mM ammonium acetate) and B (MeOH) employed at a flow of 0.4 mL/min with the following gradient: 0 min 5% B, linear to 95% B in 4 min, 2 min hold and 2 min conditioning, overall time was 8 min. MS and MS/MS measurements were performed on a 6540 Q-TOF LC/MS (Agilent Technologies, Santa Clara, CA, USA). The QTOF-MS instrument was operated under the following conditions: electrospray negative ionization, the quadrupole was operated as an ion guide in MS mode and utilized for selection of precursor ions with an isolation width of m/z = 1.3 in MS/MS mode, CID of precursor ions in MS/MS mode were performed at 35 V energy, and the TOF-MS was maintained at a mass range of 25−1000 m/z. Acquisition rate was 5 Hz for MS and MS/MS experiments. Source parameters were as follows: gas temperature 320 °C, gas flow 9 L/min, nebulizer pressure 30 psi, VCap voltage 3250 V, and fragmentor voltages 120 and 300 V (induce in source fragmentation) were employed. The Agilent 6540 was operated with MassHunter Acquisition B.04.00, and data analyses of MS and MS/MS data were performed with Masshunter Qualitative Analysis B.05.00.

Determination of Carbohydrate Configuration. To determine the absolute configuration of arabinose, a modification of the procedure based on the formation of (*R*)-2-butylglycosides originally described by Vliegenthart was employed.[14] Samples (1 mg) of D- and L-arabinose (Sigma-Aldrich, Milwaukee, WI, USA) were permethylated as described above. The permethylated samples were placed in a small

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microwave reaction tube along with 5 mg of Dowex-50W-4X strong cation exchange resin (Baker) and 250 μL of *R*-(−)-2-butanol (Sigma-Aldrich, Milwaukee, WI, USA) and placed in a CEM (Matthews, NC, USA) Discover SP microwave reactor (125 °C, 15 min).[15] The sample was transferred to a vial and analyzed using GC-MS. The same procedure was performed on **1**, and the retention times of the arabinose standards were compared to those from **1**. D-Arabinose + (*R*)-(−)-2-butanol: t_R= 15.35, 15.85, 16.49, 16.60 min.; L-arabinose + (*R*)-(−)-2-butanol: 15.20, 15.93, 16.19, 16.29 min; **1** + (*R*)-(−)-2-butanol 15.20, 15.93, 16.19, 16.29 min. On the basis of the GC-MS retention times it was concluded that the absolute configuration of the arabinose was L. D-Glucose was reacted in the same manner as for the reaction between arabinose and (*R*)-(−)-2-butanol. Two peaks were observed when the (*R*)-2-butanol enantiomer was reacted with D-glucose (t_R = 17.88 and 18.40 min). The two peaks were observed when **1** was reacted with the (*R*)-(−)-2-butanol enantiomer. The same reactions with D-apiose and **1**: t_R = 15.18, 15.65, 15.70, 16.15 min for (*R*)-(−)-2 butanol + D-apiose.

Biological Activity. Compounds **1** and **2** and their benzoate−acetate precursor mixture were assayed for inhibition of microorganisms on fresh food and against *C. albicans* and *A. niger*.

Antimicroorganism Growth on Foods. Mozzarella cheese was purchased from a local grocery store and cut into small slices (2 cm × 2 cm × 0.3 cm). Crude fractions were dissolved in DMSO to give a final concentration of 200 μg/10 μL DMSO; pure compounds were tested at a concentration of 100 μg/10 μL of DMSO. The positive controls cycloheximide and clotrimazole were tested at 10 μg/10 μL and 6 μg/10 μL, respectively. Cycloheximide was obtained from Dr. James Jorgenson of the UNI biology department. The clotrimazole was obtained from a local pharmacy and prepared from the commercial 1% ointment. A 10 μL sample was placed on one slice of the cheese and rubbed with a second slice to give an even distribution of the sample on two slices. The two samples were placed side by side (sample side up) in a baking dish and covered with a piece of glass. The growth of microorganism was visibly apparent after 3 days. The amount of growth on the cheese was visually inspected every day and ended after 7 days. The positive controls would show inhibition of growth for 5 days, but by the seventh day growth would be apparent. The DMSO sample showed growth after 3 days. Visual inspection was used to determine if a sample was active. Active samples tended to have the centers devoid of growth with growth increasing from the outer fringe of the sample and moving toward the center over the course of the assay. The DMSO control tended to have growth throughout the entire sample. No attempts were made to quantitate the growth inhibition.

Antifungal Assay. A commercial sample of *C. albicans* (Microbiologics, St. Cloud, MN, USA; ATCC 90028) was grown on a solid malt agar sample. A colony was placed in 10 mL of fresh malt broth and placed on a shaker at room temperature overnight. The broth sample was poured onto a Petri dish containing malt agar. The 10 mL liquid was quickly poured off the Petri dish. This inoculated media was allowed to grow for 2 days at room temperature. This procedure produced a "lawn" of evenly distributed *C. albicans*. To test various samples, 5 μL of the test sample was placed on a small piece of filter paper (0.3 cm). The saturated samples of filter paper were distributed on the inoculated plate. In a typical assay, clotrimazole and cycloheximide showed zones of growth inhibition (0.5 and 0.9 cm, respectively). The nonhydrolyzed precursors, **1** and **2**, failed to show any growth inhibition.

An additional antifungal assay was performed using *Aspergillus niger* (a gift from Prof. Michael Walter, UNI biology department). The *A. niger* sample was grown as described above. However it did not readily produce an even "lawn" as observed for *C. albicans*. After a number of trials a lawn of growth was produced that covered the entire agar plate. If a plate containing a lawn of *A. niger* was rapidly placed on top of a fresh agar plate and gently tapped to release spores onto the new agar plate, a fresh lawn was consistently produced. An assay using *A. niger* test organism was performed in the same manner as for *C. albicans*. Both clotrimazole and cycloheximide showed zones of growth inhibition after 3 days. The nonhydrolyzed precursors, **1** and **2**, failed to show any growth inhibition.

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Notes

The authors declare no competing financial interest.

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