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Purification and molecular structure of two digalactosyl D-*chiro*-inositols and two trigalactosyl D-*chiro*-inositols from buckwheat seeds

Kathryn J. Steadman, [a] David J. Fuller, [b] Ralph L. Obendorf [a,*]

[a] *Department of Crop and Soil Sciences, Seed Biology, Cornell University Agricultural Experiment Station, Cornell University, 617 Bradfield Hall, Ithaca, NY 14853-1901, USA*

[b] *Department of Chemistry and Chemical Biology, NMR Facility, Cornell University, Ithaca, NY 14853-1301, USA*

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Abstract

Two digalactosyl D-*chiro*-inositols and two trigalactosyl D-*chiro*-inositols, members of the fagopyritol A series and fagopyritol B series, were isolated from buckwheat (*Fagopyrum esculentum* Moench) seeds. Structures of the first three were determined by ^{1}H and ^{13}C NMR. Fagopyritol B2 is α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→2)-1D-*chiro*-inositol, and fagopyritol A2 is α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→3)-1D-*chiro*-inositol. Fagopyritol A3, a trigalactosyl D-*chiro*-inositol, is α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→3)-1D-*chiro*-inositol. From analysis of hydrolysis products, the second trigalactosyl D-*chiro*-inositol, fagopyritol B3, is α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→2)-1D-*chiro*-inositol.

Keywords: *Fagopyrum esculentum*; Buckwheat; Seed; Fagopyritol A2; Fagopyritol A3; Fagopyritol B2; Fagopyritol B3; D-*chiro*-Inositol; Galactosyl cyclitol; NMR spectroscopy

1. Introduction

Buckwheat (*Fagopyrum esculentum* Moench) seeds accumulate galactosyl D-*chiro*-inositols during seed maturation.[1] Structures of two monogalactosyl D-*chiro*-inositols have been previously verified: fagopyritol B1 (α-D-galactopyranosyl-(1→2)-1D-*chiro*-inositol)[2,3] and its positional isomer fagopyritol A1 (α-D-galactopyranosyl-(1→3)-1D-*chiro*-inositol).[3] Fagopyritol A1 is isosteric with 2-amino-2-deoxy-α-D-galactopyranosyl-(1→3)-1D-*chiro*-inositol[4] related to a putative insulin mediator.[4,5] In buckwheat seeds fagopyritol accumulation has been positively correlated with tolerance to desiccation.[1]

Purification, molecular structure and absolute configuration by NMR of fagopyritol A2, fagopyritol A3, and fagopyritol B2, higher oligomers in the fagopyritol A series and fagopyritol B series present in buckwheat seeds, are described herein. The structure of fagopyritol B3 is based on analysis of hydrolysis products.

* Corresponding author. Tel.: +1-607-2551709; fax: +1-607-2552644..

E-mail address: rlo1@cornell.edu (R.L. Obendorf).

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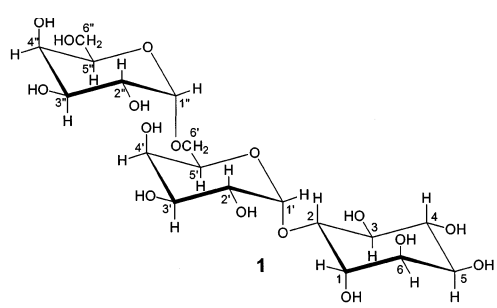
2. Results and discussion

Buckwheat bran was extracted with 50% EtOH and fagopyritols were purified using column chromatography with 1:1 (w/w) carbon–Celite. Fagopyritol B2 (**1**) was purified from fractions eluted with 6% EtOH, fagopyritol A2 (**2**) was eluted with 9% EtOH, and fagopyritol A3 (**3**) was eluted with 14% EtOH. Per(pentafluoropropanoates) of *chiro*-inositol from acid hydrolysis of **1**, **2** and **3** co-chromatographed on a chiral column with D-*chiro*-inositol but not with L-*chiro*-inositol. Acid hydrolysis of **1**, **2**, and **3** produced D-galactose and D-*chiro*-inositol in mole ratios of 2.02:1.00 for **1**, 2.03:1.00 for **2**, and 2.98:1.00 for **3**. GLC analysis of partial hydrolysis products of **1**, **2** and **3** identified fagopyritol B1 from **1**, fagopyritol A1 from **2**, **2** and fagopyritol A1 from **3**, and D-*chiro*-inositol and galactose from **1**, **2**, and **3**. Compounds **1**, **2** and **3** were hydrolyzed by α-D-galactosidase, but not by β-D-galactosidase, demonstrating α anomeric linkages for all D-galactopyranosyl residues.

^{13}C NMR spectra for **1** identified 18 carbon resonances, and ^{1}H NMR spectra identified 20 hydrogen resonances. Chemical shifts for the 18 carbons were each linked to those of the 20 hydrogens using GHMQC. All but four of the carbon chemical shifts were between 69 and 76 ppm. Two carbon resonances had chemical shifts downfield at 99.02 and 95.74 ppm, characteristic of substituted anomeric carbons. Two carbons were upfield at 62.03 and 67.72 ppm, and these were identified as CH_2 carbons by DEPT. All but three of the hydrogens had chemical shifts between 3.5 and 3.9 ppm. The two most downfield hydrogens (δ 4.81 and 4.99) had chemical shifts characteristic of anomeric hydrogens, and GHMQC showed attachment to anomeric carbons (δ 99.02 and 95.74, respectively). These served as the starting points for tracing connectivity within the galactose spin-systems by DQCOSY. Assignment of hydrogen resonances to the upfield CH_2 carbons allowed distinction between the two galactose residues; one of the CH_2 carbons (δ 62.03) had two hydrogens with accidentally equivalent resonances (δ 3.59), while hydrogens on the other CH_2 carbon (δ 67.72) had non-equivalent resonances (δ 3.72 and 3.54). The presence of non-equivalent resonances between CH_2 hydrogens is suggestive of further substitution at this carbon; thus, the spin system involving the non-equivalent hydrogens (δ 3.54, H-6′a; δ 3.72, H-6′b) was assigned to the galactose proximal (closest) to *chiro*-inositol, and the spin system involving the equivalent protons (δ 3.59, H-6″a, H-6″b) was assigned to the distal galactose residue (Table 1). Additional evidence for this assignment came from C-6′ (δ 67.72) which is deshielded by 5.5 ppm in comparison to C-6″ (δ 62.03), and the adjacent C-5′ (δ 69.98) is shifted upfield by a small amount in comparison to C-5″ (δ 71.71), indicative of substitution at C-6′. The small coupling constants ($J_{1',2'}$ 3.9; $J_{1'',2''}$ 3.9 Hz) calculated from the 1D ^{1}H spectra (Table 1) verified the α anomeric linkage for both galactosyl rings.

For **1**, the six remaining ^{1}H resonances, all between 3.47 and 4.08 ppm, were assigned to D-*chiro*-inositol, and connectivity delineated from the DQCOSY experiment. The carbon to which each hydrogen was attached was estimated from GHMQC. The most downfield of these carbons (δ 75.58) in the cyclitol ring was the point of linkage to galactose. Coupling constants ($J_{1,2}$ 3.0; $J_{2,3}$10.0 Hz) observed for the hydrogen (δ 3.73, H-2) attached to the substituted carbon (δ 75.58) and those observed for the hydrogen assigned H-1 ($J_{1,2}$ 3.0; $J_{1,6}$ 4.1 Hz) and H-3 ($J_{2,3}$ 10.0; $J_{3,4}$ 9.6 Hz), confirmed the assignment of 3.73 ppm to be an axial hydrogen with an axial and equatorial hydrogen adjacent (i.e., H-2 or H-5). Due to symmetry of the *chiro*-inositol ring, substitution at C-2 or C-5 results in equivalent structures, and the smaller number is assigned. Therefore, fagopyritol B2 is α-D-galactopyranosyl-(1 → 6)-α-D-galactopyranosyl-(1 → 2)-1D-*chiro*-inositol (**1**).



1

^{13}C NMR spectra for **2** identified 18 carbon resonances, and ^{1}H NMR spectra identified 20 hydrogen resonances. The two galactosyl residues of **2** were elucidated in the same way as described for **1** (above), and the chemical shifts and coupling constants are comparable to those of **1** (Table 1). For **2** (fagopyritol A2), the hydrogen (δ 3.54) on the substituted carbon (δ 81.78) in the D-*chiro*-inositol ring was observed to have only large coupling constants ($J_{2,3}$ 9.8; $J_{3,4}$ 9.8 Hz) from both ^{1}H NMR and HOM2DJ experiments, indicating trans-axial vicinal hydrogens (namely H-3 or H-4). Due to symmetry of the *chiro*-inositol ring, substitution at C-3 or C-4 results in equivalent structures, and the smaller number is assigned. The point of linkage in the D-*chiro*-inositol residue was C-3 in **2**, as in fagopyritol A1.[3] The only apparent GHMBC interaction for C-1′ (δ 100.33) was with H-3 (δ 3.54), confirming the presence of axial hydrogens at H-2 and H-4 of the D-*chiro*-inositol ring. Similarly, the interaction between C-3 and H-1′ but not H-2′ confirmed the (1′→3) connectivity, with H-2′ being axial to the ring. Fagopyritol A2 is α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→3)-1D-*chiro*-inositol (**2**).

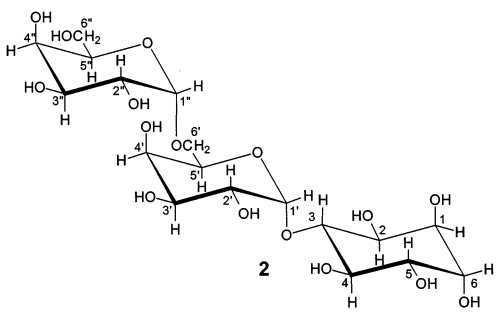


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Table 1
^{1}H and ^{13}C NMR chemical shifts and proton–proton coupling constants of fagopyritol B2 (**1**) and fagopyritol A2 (**2**) in D_2O at 25 °C

Position (no.)	Chemical shifts (ppm)				Coupling constants (J, Hz)		
	1		**2**		No.	**1**	**2**
	δ_H	δ_C	δ_H	δ_C			
Cyclitol ring							
1	4.08	68.14	3.86	72.57	$J_{1,2}$	3.0	3.2
2	3.73	75.58	3.67	70.14 [a]	$J_{2,3}$	10.0	9.8
3	3.59	71.97	3.54	81.78	$J_{3,4}$	9.6	9.8
4	3.48	73.59	3.61	73.65 [b]	$J_{4,5}$	9.8	9.8 [c]
5	3.64	71.25	3.61	71.05 [b]	$J_{5,6}$	3.2	3.2
6	3.90	71.76	3.83	72.12	$J_{6,1}$	4.1	4.0
Galactopyranosyl ring (proximal)							
1′	4.99	95.74	5.14	100.33	$J_{1',2'}$	3.9	3.9
2′	3.72	69.10 [d]	3.67	69.45	$J_{2',3'}$	10.4	10.4
3′	3.82	70.21 [e]	3.75	70.04	$J_{3',4'}$	3.4	3.2
4′	3.90	70.13	3.86	69.93	$J_{4',5'}$	1.2	1.3
5′	4.25	69.68	4.30	70.06 [a]	$J_{5',6'a}$	4.4	4.9
6′a	3.54	67.72	3.51	67.53	$J_{5',6'b}$	8.1	7.8
6′b	3.72		3.70		$J_{6'a,6'b}$	10.7	10.5
Galactopyranosyl ring (distal)							
1″	4.81	99.02	4.81	99.30	$J_{1'',2''}$	3.9	3.9
2″	3.65	69.08 [d]	3.63	69.12	$J_{2'',3''}$	10.4	10.3
3″	3.73	70.24 [e]	3.73	70.04	$J_{3'',4''}$	3.2	2.9
4″	3.79	70.13	3.79	69.93	$J_{4'',5''}$	1.2	1.2
5″	3.86	71.71	3.78	71.84	$J_{5'',6''a}$	6.2	6.1
6″a	3.59	62.03	3.56	62.02	$J_{5'',6''b}$	6.2	6.1
6″b	3.59		3.56				

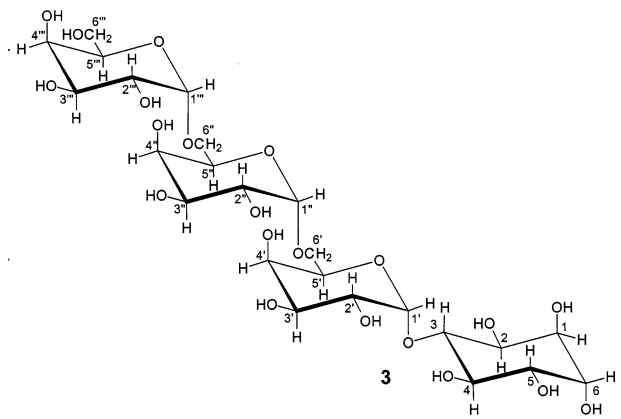
[a,b,d,e] Assignment may be reversed for values with the same letter.
[c] Expected value; confirmed by simulation of experimental data.

Table 2
^{1}H and ^{13}C NMR chemical shifts and ^{1}H coupling constants of fagopyritol A3 (**3**) in D_2O at 25 °C

Position (no.)	Chemical shifts (ppm)		Coupling constants (J, Hz)	
	δ_H	δ_C		
Cyclitol ring				
1	3.91	72.56	$J_{1,2}$	2.9
2	3.73	70.11 [a]	$J_{2,3}$	10.1
3	3.59	82.04	$J_{3,4}$	10.1
4	3.65	73.58 [b]	$J_{4,5}$	10.1 [c]
5	3.65	71.06 [b]	$J_{5,6}$	3.1
6	3.87	72.11	$J_{6,1}$	4.1
Galactopyranosyl ring (proximal)				
1′	5.20	100.36	$J_{1',2'}$	4.0
2′	3.72	69.45 [d]	$J_{2',3'}$	10.4
3′	3.84	70.11 [a]	$J_{3',4'}$	3.3
4′	3.88	70.03 [a]	$J_{4',5'}$	1.0
5′	4.34	69.95	$J_{5',6'a}$	4.1
6′a	3.50	67.61	$J_{5',6'b}$	8.1
6′b	3.78		$J_{6'a,6b}$	10.6
Galactopyranosyl ring (middle)				
1″	4.84 [e]	99.05	$J_{1'',2''}$	3.8
2″	3.68	69.10	$J_{2'',3''}$	10.4
3″	3.79	70.18 [a]	$J_{3'',4''}$	3.4
4″	3.88	70.03 [a]	$J_{4'',5''}$	1.0
5″	4.02	69.53 [d]	$J_{5'',6''a}$	4.8
6″a	3.57	67.26	$J_{5'',6''b}$	7.0
6″b	3.72		$J_{6''a,6''b}$	11.0
Galactopyranosyl ring (distal)				
1‴	4.85 [e]	98.85	$J_{1''',2'''}$	3.8
2‴	3.68	69.10	$J_{2''',3'''}$	10.4
3‴	3.79	70.23 [a]	$J_{3''',4'''}$	3.4
4‴	3.72	70.11 [a]	$J_{4''',5'''}$	1.2
5‴	3.87	71.74	$J_{5''',6'''a}$	6.4
6‴a	3.60	61.95	$J_{5''',6'''b}$	6.4
6‴b	3.60			

[a,b,d,e] Assignment may be reversed for values with the same letter.
[c] Expected value; confirmed by simulation of experimental data.

A clear differential can be seen between the fagopyritol A series and the fagopyritol B series in the chemical shift of the substituted carbon, which is the most downfield of the non-anomeric carbons. In the B series, this is at 76 ppm, whereas for the A series the substituted carbon is 6 ppm further downfield at 82 ppm. This pattern defined **3**, a trigalactosyl D-*chiro*-inositol, as a member of the fagopyritol A series, since it contains a carbon resonance at 82.04 ppm (C-3) (Table 2). Further, the hydrogen attached to the substituted carbon (δ 82.04) is observed to have a chemical shift of 3.59 ppm (H-3) with large coupling constants ($J_{2,3}$ 10.1, $J_{3,4}$ 10.1 Hz) calculated from ^{1}H NMR and HOM2DJ. This compound was previously predicted to be a trigalactosyl D-*chiro*-inositol of the fagopyritol B series;[1,6] since buckwheat seeds have more fagopyritol B1 than fagopyritol A1 and more fagopyritol B2 than fagopyritol A2, the same pattern was expected for trigalactosyl D-*chiro*-inositols. Herein we have determined this trigalactosyl D-*chiro*-inositol to be α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→3)-1D-*chiro*-inositol (**3**) and assigned the trivial name fagopyritol A3.

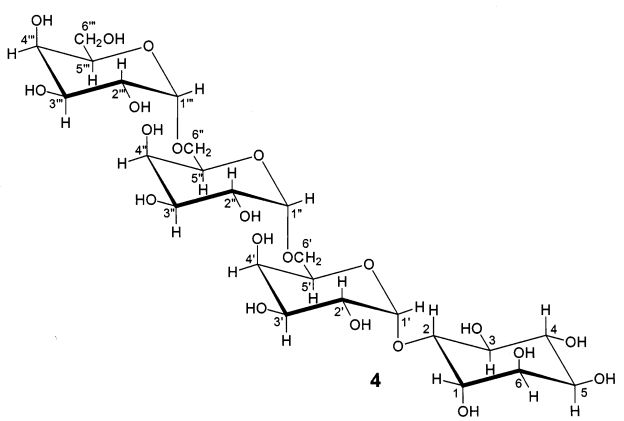


With four ring systems superimposed, spectra for **3** were complex. Once most of the 27 hydrogens were identified from the 1D ^{1}H and HOM2DJ spectra, DQCOSY was used to assign hydrogens as belonging to galactose or D-*chiro*-inositol. The hydrogens at the 2, 3, and 4 positions of the three galactose rings had very similar or identical resonances between rings, making assignment of hydrogens into each individual ring not immediately apparent. Three pairs of hydrogens (δ 3.50 and 3.78; δ 3.57 and 3.72; δ 3.60 and 3.60) were identified as 6 position in the galactose residue by their interaction in the GHMQC experiment with the carbons (δ 67.61; 67.26; 61.95) identified as CH_2 by DEPT. One pair of hydrogens was accidentally equivalent (δ 3.60), linked to the most upfield carbon (δ 61.95), and assigned as H-6‴a and H-6‴b. These hydrogens (δ 3.60) interacted with the hydrogen at 3.87 ppm (H-5‴) by DQCOSY.

Through its GHMBC interaction with H-5‴, the anomeric carbon at 98.85 ppm was assigned as C-1‴. This carbon (δ 98.85) also interacted with the CH_2 hydrogens with chemical shifts of 3.72 and 3.57 ppm, assigned H-6″a and H-6″b. Similarly, the carbon at 99.05 ppm (C-1″) interacted with the hydrogen at 4.02 ppm (H-5″), and with hydrogens at 3.78 and 3.50 ppm (H-6′a and H-6′b). GHMBC interaction of C-1′ (δ 100.36) with only H-3 (δ 3.59) of D-*chiro*-inositol confirmed H-3 as attached to the carbon at the point of substitution (C-3), with axial H-2 and H-4. With these assignments made, the remaining assignments could be made for each of the four spin-systems. Finally, GHMQC was used to assign carbons. With eight of the 24 carbon resonances occurring between 69.95 and 70.23 ppm, absolute assignment was not possible, leading to a number of assignments being potentially reversible (Table 2).

It was possible to calculate the coupling constants between hydrogens in the D-*chiro*-inositol ring for the fagopyritol B series from their 1D ^{1}H NMR spectra. However, in the fagopyritol A series chemical shifts of H-4 and H-5 coincide, and the strong coupling between H-4 and H-5 results in a complex multiplet in which it is not possible to find a coupling constant larger than 6.5 Hz. Simulation of the observed ^{1}H chemical shifts for D-*chiro*-inositol in **2** or **3** with coupling constants that would be expected for D-*chiro*-inositol based on those measured for the fagopyritol B series, results in a multiplet for H-4 and H-5 consistent with that observed in the fagopyritol A series. This confirms the coupling constants ($J_{4,5}$ 9.8 Hz) simulated for **2** (Table 1) and ($J_{4,5}$ 10.1 Hz) simulated for **3** (Table 2).

Typical retention times (min) on gas chromatograms for the trimethylsilyl derivatives of six fagopyritols were: fagopyritol A1 (24.19), fagopyritol B1 (24.75), fagopyritol A2 (**2**) (31.79), fagopyritol B2 (**1**) (32.23), fagopyritol A3 (**3**) (38.27), and fagopyritol B3 (**4**) (39.01). The abundance ratio of **3**:**4** was 4:1 in extracts from buckwheat bran. Compound **4** was hydrolyzed by α-D-galactosidase. Acid hydrolysis of **4** produced D-galactose and D-*chiro*-inositol in a mole ratio of 3:1. GLC analysis of products after partial hydrolysis of **4** identified fagopyritol B1 and fagopyritol B2 (**1**). Pure **4** was not in sufficient quantity for structure analysis by NMR. From analysis of hydrolysis products, **4** is α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→2)-1D-*chiro*-inositol and is assigned the trivial name fagopyritol B3 as the third member of the fagopyritol B series. In a previous report,[1] a peak incorrectly predicted to be fagopyritol B3 was confirmed herein to be fagopyritol A3 (**3**). Herein we report the first confirmation of the presence and putative structure of fagopyritol B3 (**4**).



Two members of the fagopyritol B series have been previously identified. Fagopyritol B1 is in seeds of buckwheat,[1–3] soybean,[7] lupin, lentil, and chickpea,[8] and jojoba bean.[9] Chemical synthesis of fagopyritol B1 has been reported.[10] Fagopyritol B2 (**1**) was identified by chemical methods in seed balls of sugar beet,[11] but its absolute configuration is first presented herein. The third member of this series, fagopyritol B3 (**4**), is confirmed herein. Fagopyritol A2 (**2**) and fagopyritol A3 (**3**), along with fagopyritol A1,[3] are members of a novel series of galactosyl cyclitols in buckwheat seeds.

3. Experimental

Extraction of fagopyritols from buckwheat.—The bran milling fraction[12] of common buckwheat (*Fagopyrum esculentum* Moench cv. Manor) seed was provided by Minn-Dak Growers, Ltd. (Grand Forks, ND). Typical concentrations (g/kg) of six fagopyritols in buckwheat bran were: fago-

pyritol A1 (3.08), fagopyritol A2 (**2**) (1.38), fagopyritol A3 (**3**) (0.77), fagopyritol B1 (18.85), fagopyritol B2 (**1**) (1.77), and fagopyritol B3 (**4**) (0.19).[12] Buckwheat bran was extracted at pilot plant scale with 1:1 (v/v) EtOH–water, the extract was filtered through a diatomaceous earth plate filter, evaporated to reduce the volume and remove EtOH, and treated with polyvinylpolypyrrolidone (PVPP), bentonite, and ion-exchange resin (mixed strong anionic and cationic) to remove non-carbohydrate components. The soluble carbohydrate extracts were finally filtered through 10,000 MW cut-off hollow fiber filter cartridges and evaporated to reduce the volume.

Purification of fagopyritols.—Purification was performed by preparative chromatography on a stationary phase of carbon (Darco G60; J.T. Baker, Phillipsburg, NJ) and Celite 545-AW (Supelco, Bellefonte, PA), 1:1 (w/w).[13] Columns were freshly slurry packed in water, freeze-dried samples were dissolved in minimal water for loading, and soluble carbohydrates were eluted at 4 °C with stepwise increments of EtOH–water and collected in 20-mL fractions. Samples of column eluate were taken periodically, phenyl α-D-glucoside was added as internal standard, dried samples were derivatized with 1:1 (v/v) *N*-trimethylsilylimidazole–pyridine, and analyzed[6] on a Hewlett–Packard 6890 GC equipped with a flame-ionization detector, split-mode injector (1:50), and a HP-1MS capillary column (15 m × 0.25 mm i.d., 0.25 μm film thickness). The GC was operated with a programmed initial temperature of 150 °C, adjusted to 200 °C at 3 °C min^{-1}, adjusted to 325 °C at 7 °C min^{-1}, and held at 325 °C for 20 min. The injector port was operated at 335 °C and the detector at 350 °C. The carrier gas was nitrogen at 2.5 mL/min.

For purification of **1**, **2**, and **3**, buckwheat bran extract was chromatographed on a 25 × 900 mm bed. Following successive elution with 2.2 L of water, 2.5 L of 4% EtOH, 3 L of 5% EtOH, **1** was eluted with 2 L of 7% EtOH, **2** with 1 L of 12% EtOH, and **3** with 1 L of 20% EtOH. Fractions containing **1** or **2** or **3** were freeze-dried and re-chromatographed.

Fractions of **1** were treated with invertase (EC 3.2.1.26) from *Candida utilis* for 1 h at 22 °C to hydrolyze sucrose, and then chromatographed on a 15 × 450 mm bed. Following elution of contaminants with 1 L of 5% EtOH, **1** was eluted with 6% EtOH.

Compound **2** was chromatographed on a 15 × 500 mm bed. Low levels of contaminants were eluted sequentially with 0.5 L of water, 1 L of 6% EtOH, and 0.5 L of 8% EtOH, and **2** was eluted with 9% EtOH.

Fractions containing **3** from four separate columns of buckwheat bran extract were combined and re-chromatographed (19 × 350 mm). Compound **3** was eluted with 14% EtOH following removal of contaminants by elution with 12% EtOH.

For purification of **4**, buckwheat bran extract was chromatographed on a 100 × 200 mm bed of carbon–Celite. Following successive elution with 4 L each of water, 2, 4, 5, 8, 10, and 14% EtOH, **4** was eluted with 2 L of 20% EtOH. Compound **4** was eluted between **2** and **3**. Fractions (500 mL) containing **4** from eight separate columns were freeze-dried and re-chromatographed on a 100 × 200 mm bed and then on a 25 × 750 mm bed.

Acid and enzymic hydrolysis.—Samples of **1**, **2**, **3** and **4** were hydrolyzed with 1 M CF_3CO_2H at 80 °C for 16 h and evaporated to dryness. Mole ratios of D-galactose and D-*chiro*-inositol were calculated after GLC analysis of the trimethylsilyl-products. Samples of **1**, **2**, **3** and **4** (200 μg) were incubated with 1.25 units of desalted green coffee bean α-D-galactosidase (EC 3.2.1.22) in 200 μL water at 22 °C or with 0.5 units of bovine liver β-D-galactosidase (EC 3.2.1.23) in 200 μL water at 37 °C for 24 h. Enzyme protein was removed by filtration (10,000 MW cut-off filter), samples were dried, and products assayed by GC analysis of trimethylsilyl derivatives. Raffinose and lactose (100 μg) were hydrolyzed only by α- or β-D-galactosidase, respectively, confirming that both enzymes were active.

Analysis of chirality of chiro-*inositol.*—Authentic D-*chiro*-inositol and L-*chiro*-inositol standards (Aldrich, Milwaukee, WI) and the acid hydrolysis products (2 M CF_3CO_2H, 70 °C for 3 h) of **1**, **2** and **3** were derivatized

with 1:1 (v/v) pentafluoropropanoic anhydride–MeCN.[14] Derivatized products were co-chromatographed with the derivatized standards by GC using a Chirasil–Val chiral capillary column (25 m × 0.25 mm i.d., 0.16 μm film thickness)[15] run at 100 °C isothermally on a Hewlett–Packard 5890 Series II GC. Injection port temperature was 130 °C and FID temperature was 130 °C. Helium was the carrier gas at 3 mL/min, split 1:50.

NMR analysis.—Purified samples (41 mg of **1**, 13.5 mg of **2**, 34.5 mg of **3**) were dissolved in 700 μL of 99.96% D_2O (Cambridge Isotope Labs, Andover, MA) for analysis. A Varian VXRS-400 was used for 1D ^{13}C NMR and DEPT at 100.5 MHz, 25 °C, with 1,4-dioxane (δ_C 67.4) as external carbon standard. All other NMR experiments were performed at 499.9 MHz on a Varian Unity-500, standardized against the residual HOD (δH 4.63) at 25 °C. These were 1D ^1H NMR, double-quantum filtered COSY, gradient-enhanced HMQC, homonuclear 2D J-spectroscopy (HOM2DJ), NOESY, and gradient-enhanced HMBC. Data were analyzed and simulations performed using Varian VNMR software, version 6.1A.

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