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Note

Molecular structure of fagopyritol A1 (*O*-α-D-galactopyranosyl-(1 → 3)-D-*chiro*-inositol) by NMR

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Abstract

The molecular structure of fagopyritol A1, a novel galactopyranosyl cyclitol from buckwheat seeds, was determined to be *O*-α-D-galactopyranosyl-(1 → 3)-D-*chiro*-inositol by ^{1}H and ^{13}C NMR. Fagopyritol A1 is a positional isomer of fagopyritol B1 (*O*-α-D-galactopyranosyl-(1 → 2)-D-*chiro*-inositol), representing a different series of fagopyritol oligomers. Trimethylsilyl derivatives of both compounds have similar mass spectra, but each may be identified by different abundance ratios of fragments with *m*/*z* 305/318 and 318/319.

Keywords: *Fagopyrum esculentum*; Buckwheat; Seed; Fagopyritol A1; Fagopyritol B1; D-*chiro*-Inositol; Galactosyl cyclitol

1. Introduction

Buckwheat (*Fagopyrum esculentum* Moench) seeds accumulate the soluble carbohydrates sucrose and fagopyritols in the embryo and aleurone tissues, instead of sucrose and the raffinose-series of oligosaccharides, as part of the normal seed maturation process. Fagopyritol A1 and fagopyritol B1 are the most prominent of the fagopyritols accumulated and are positively correlated to desiccation tolerance in buckwheat seeds [1]. Fagopyritols, D-galactopyranosyl derivatives of D-*chiro*-inositol, are structurally similar to a galactosamine derivative of D-*chiro*-inositol, a putative insulin mediator [2,3], and may be useful in the treatment of non-insulin dependent diabetes mellitus [1,4]. Fagopyritol B1 was recently confirmed to be *O*-α-D-galactopyranosyl-(1 → 2)-D-*chiro*-inositol using NMR [4]. Herein we report the molecular structure and absolute configuration of fagopyritol A1.

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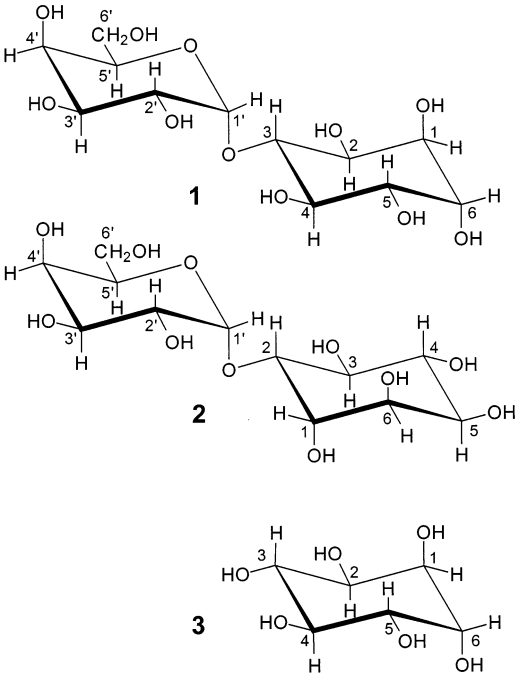
2. Results and discussion

Buckwheat bran was extracted with 50% ethanol, and the resulting extract chromatographed on 1:1 carbon–Celite (w/w), to purify fagopyritol A1 (**1**). Fagopyritol B1 (**2**) was also purified from the same sample for comparison. Per(pentafluoropropionates) of *chiro*-inositol from acid hydrolysis of **1** and **2** co-chromatographed on a chiral column with authentic D-*chiro*-inositol, but not with authentic L-*chiro*-inositol. Hydrolysis of **1** and **2** produced D-galactose and D-*chiro*-inositol in mole ratios of 1.01:1.00 for **1** and 1.03:1.00 for **2**. Free D-*chiro*-inositol (**3**) was obtained by acid hydrolysis of a mixture of **1** and **2** followed by purification on a carbon–Celite column. Compounds **1** and **2** were hydrolyzed by α-D-galactosidase, but not by β-D-galactosidase, demonstrating an α-D-galactopyranosyl linkage.

Table 1
^{1}H and ^{13}C NMR chemical shifts of fagopyritol A1 (**1**), fagopyritol B1 (**2**) and free D-*chiro*-inositol (**3**) in D_2O at 25 °C

Position	Chemical shifts (ppm)					
(no.)	**1**		**2**		**3**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
	Cyclitol ring					
1	3.85	72.56	4.04	68.46	3.85	72.43
2	3.67	69.51	3.64	76.61	3.58	71.22
3	3.54	81.77	3.53	72.04	3.41	73.52
4	3.61	73.61 [a]	3.44	73.48	3.41	73.52
5	3.61	71.06 [a]	3.60	71.19	3.58	71.22
6	3.83	72.08	3.87	71.85	3.85	72.43
	Galactopyranosyl ring					
1′	5.16	100.26	4.93	96.42		
2′	3.66	69.87	3.67	69.06		
3′	3.73	70.19	3.77	70.10		
4′	3.81	69.96	3.83	69.93		
5′	4.07	71.86	4.03	71.63		
6′a	3.55	61.80	3.55	61.69		
6′b	3.55		3.55			

[a] Assignment may be reversed.



The ^{13}C NMR spectra for **1** demonstrated 12 carbon resonances, and ^{1}H NMR spectra identified 13 ^{1}H resonances. The chemical shifts for the 12 carbons in **1** were each linked to those of the 13 hydrogens using GHMQC. The characteristically downfield anomeric carbon (δ 100.26) and anomeric ^{1}H (δ 5.16) of the D-galactopyranosyl residue served as the starting point for the analysis of the DQCOSY for connectivity within the spin systems. The two coincident H-6′ hydrogens on the galactosyl residue were also a useful marker, clearly identifiable by a doublet with double intensity at δ 3.55 in the ^{1}H spectrum, and attached to the most upfield carbon (δ 61.80) in the ^{13}C spectrum. The remaining resonance assignments for the galactosyl ring were straightforward. Once the ^{1}H signals for the galactose residue had been assigned (Table 1), the six remaining resonances could be assigned to the D-*chiro*-inositol ring. The hydrogen at 3.54 ppm was attached to the downfield carbon (δ 81.77) with a resonance shifted 8–10 ppm downfield in comparison to resonances for free D-*chiro*-inositol **3** (Table 1), thereby identifying the point of galactose substitution on the D-*chiro*-inositol ring. Additionally, GHMBC and NOESY experiments showed clear interactions between the hydrogen at δ 3.54 and the anomeric hydrogen (δ 5.16; H-1′) and anomeric carbon (δ 100.26; C-1′) of galactose, confirming the point of linkage.

Table 2
Estimated coupling constants derived from ^{1}H NMR spectral data of fagopyritol A1 (**1**), fagopyritol B1 (**2**) and free D-*chiro*-inositol (**3**)

Estimated proton–proton coupling constants (Hz)									
1				**2**				**3**	
Cyclitol		Galactose		Cyclitol		Galactose		Cyclitol	
$J_{1,2}$	3.2	$J_{1',2'}$	3.9	$J_{1,2}$	2.9	$J_{1',2'}$	3.9	$J_{1,2}$	2.6
$J_{2,3}$	9.7	$J_{2',3'}$	10.4	$J_{2,3}$	9.7	$J_{2',3'}$	10.4	$J_{2,3}$	9.8
$J_{3,4}$	9.7	$J_{3',4'}$	3.2	$J_{3,4}$	9.6	$J_{3',4'}$	3.4	$J_{3,4}$	9.8
$J_{4,5}$	9.7 [a]	$J_{4',5'}$	1.1	$J_{4,5}$	9.7	$J_{4',5'}$	1.1	$J_{4,5}$	9.8
$J_{5,6}$	3.2	$J_{5',6'}$	6.3	$J_{5,6}$	3.3	$J_{5',6'}$	6.3	$J_{5,6}$	2.6
$J_{6,1}$	4.0			$J_{6,1}$	3.9			$J_{6,1}$	3.7

[a] Expected value; confirmed by simulation of experimental data.

Coupling constants between adjacent hydrogens were calculated from the 1D ^{1}H signals. HOM2DJ aided in separating the multiplets in the region 3.5–3.9 ppm, which were formed from overlaid signals. The derived coupling constants (Table 2) allowed confirmation of the assignments made for the galactose ring of **1**. In particular, the small coupling constant $J_{1',2'}$ 3.9 Hz verified the α-anomeric linkage. The hydrogen at δ 3.54 on the linkage point of the D-*chiro*-inositol ring showed coupling constants of 9.7 Hz, indicating a *trans*-diaxial relationship with both adjacent hydrogens. Therefore, fagopyritol A1 was assigned the structure *O*-α-D-galactopyranosyl-(1→3)-D-*chiro*-inositol (**1**). Fagopyritol A1 is isosteric with *O*-(2-amino-2-deoxy)-α-D-galactopyranosyl-(1→3)-D-*chiro*-inositol [3] related to a putative insulin mediator [2,3].

Coupling constants between adjacent hydrogens on the D-*chiro*-inositol ring were important in numbering the ring. However, the coupling constants for H-4 and H-5 of **1** were not easily discernible. The intense interaction of these two strongly coupled hydrogens with coincident chemical shift (δ 3.61; H-4 and H-5), produced a complex multiplet with a maximum apparent splitting of 6.8 Hz. The three pairs of vicinal *trans*-diaxial hydrogens (H-2, H-3, H-4, H-5) in D-*chiro*-inositol should display coupling constants of 9–10 Hz, as observed for D-*chiro*-inositol in *O*-α-D-galactopyranosyl-(1→2)-D-*chiro*-inositol (**2**, Table 2) [4]. The contradiction was resolved by simulation of the ^{1}H spectrum for the C-3 substituted D-*chiro*-inositol, using chemical shifts (Table 1) and coupling constants (Table 2) calculated from experimental spectral line data, but with the expected value of 9.7 Hz for $J_{4,5}$. The simulated and experimental ^{1}H spectra were identical, confirming that the effect of the strong interaction between H-4 and H-5 resulted in a multiplet at 3.61 ppm with a maximum apparent splitting of 6.8 Hz. Thus, $J_{4,5}$ is given as 9.7 Hz (Table 2) based on simulation. Assignment of the D-*chiro*-inositol ring hydrogens was easier for **2** than for **1** because the galactosyl substitution at H-3 of **1** caused the adjacent H-4 of **1** (δ 3.61) to be shifted downfield from that in **2** (δ 3.44) and **3** (δ 3.41) such that it coincided with H-5 (δ 3.61). In **2**, H-4 (δ 3.44) was upfield of H-5 (δ 3.60) allowing the large coupling constant ($J_{4,5}$ 9.7 Hz) of a *trans*-diaxial interaction to be observed for **2** (Table 2). Simulation was also useful in the elucidation of coupling between hydrogens in free D-*chiro*-inositol (**3**). The ^{1}H NMR spectrum for **3** showed three multiplet patterns, each multiplet representing two identical hydrogens, two pairs of which had intrapair coupling. Simulation of calculated chemical shifts and coupling constants gave a spectrum consistent with the observed spectrum for **3** and confirmed the calculated coupling constants as shown in Table 2.

Structures **1** and **2** are positional isomers representing two different series of fagopyritol oligomers. The ^{13}C and/or ^{1}H resonances for **2**, *O*-α-D-galactopyranosyl-(1→2)-D-*chiro*-inositol, were in close agreement with those reported for this structure in buckwheat [4],

soybean [5] and jojoba bean [6]. Resonances of the galactopyranosyl ring were in close agreement also with those of galactosylononitol [7] and other disaccharides containing galactose α-substituted at the anomeric carbon [8].

Mass spectra for trimethylsilylated products of **1** and **2** were similar with ion fragment peaks at *m*/*z* 73, 103, 129, 147, 191, 204, 217, 305, 318, 319 and 361. Abundance ratios of fragments with *m*/*z* 305/318 were 0.9 for **1** and 2.6 for **2**, while abundance ratios of fragments with *m*/*z* 318/319 were 2.2 for **1** and 0.9 for **2**. These abundance ratios may distinguish trimethylsilyl derivatives of **1** and **2** by GC-MS.

3. Experimental

Extraction of fagopyritols from buckwheat.—The bran milling fraction of common buckwheat (*F. esculentum* Moench cv. Manor) seed was provided by Minn-Dak Growers, Ltd. (Grand Forks, ND). Buckwheat bran was extracted at pilot plant scale with 1:1 (v/v) EtOH–H_2O, 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 reduce non-carbohydrate components. The soluble carbohydrate extracts were finally filtered through a 10,000 MW cut-off hollow fiber filter cartridge 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) [9]. 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–H_2O and collected in 20-mL fractions. Samples of column eluate were taken periodically, phenyl α-D-glucoside was added as the internal standard, dried samples were derivatized with 1:1 (v/v) *N*-trimethylsilylimidazole–pyridine, and analyzed [10] 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 instrument was operated with a programmed initial temperature of 150 °C, adjusted to 200 °C at 3 °C/min, adjusted to 325 °C at 7 °C/min, 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**, buckwheat bran extract containing ~1.8 g of soluble carbohydrates (5% **1**) was chromatographed on a 25 × 900 mm bed of carbon–Celite. Following elution with 2.2 L of water to remove monosaccharides, and 2.5 L of 4% EtOH to remove galactinol and **2**, **1** was eluted with 5% EtOH, freeze-dried, and re-chromatographed (25 × 550 mm bed). Compound **1** (95% pure) was obtained by first removing **2** with 4% EtOH, and then eluting **1** with 5% EtOH. Fractions containing **1** were re-chromatographed a third time (10 × 370 mm bed; eluted with water and then 5% EtOH) to obtain pure **1** for NMR analysis.

For purification of **2**, buckwheat bran extract was incubated with baker's yeast to remove sucrose. A sample containing 3.15 g of soluble carbohydrates (25% **2**) was chromatographed on a 43 × 360 mm carbon–Celite bed. Following elution with 1.5 L water to remove monosaccharides, **2** and galactinol were eluted with 4% EtOH. Fractions containing **2** were freeze-dried and re-chromatographed on a 25 × 900 mm column. Galactinol co-eluted with the initial burst of **2**, allowing fractions from the tail of **2** elution to be combined to give pure **2** for NMR analysis.

Compound **3** was obtained by hydrolysis of partially purified **2** (106 mg of **2**;14 mg of **1**; 2.5 mg of galactinol) with 4 M HCl for 1 h at 95 °C, neutralized with NaOH, and the resulting mixture chromatographed on carbon–Celite (25 × 900 mm) and eluted with water.

Acid and enzymic hydrolysis.—Samples of **1** and **2** were hydrolyzed with 1 M CF_3CO_2H for 16 h at 80 °C and evaporated to dryness. Mole ratios of D-galactose and D-*chiro*-inositol were calculated after GC analysis of the trimethylsilylated products. Samples of **1** and

2 (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 by α- or β-D-galactosidase, respectively, confirming that both enzymes were active.

Separation of D-chiro-*inositol and* L-chiro-*inositol by chiral column GC.*—Authentic D-*chiro*-inositol and L-*chiro*-inositol standards (gift from A. Richter, University of Vienna) and the acid hydrolysis products (2 M CF_3CO_2H, 70 °C for 3 h) of pure **1** or pure **2** were derivatized with 1:1 pentafluoropropionic anhydride–MeCN (v/v) [11]. 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) [12] 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 (14.4 mg of **1**; 21 mg of **2**; 9 mg of **3**) were dissolved in 700 μL of 99.96% D_2O for analysis. A Varian VXRS-400 was used for 1D ^{13}C NMR at 100.5 MHz, 25 °C, with 1,4-dioxane (δ_C 67.4) as the external carbon standard. All other NMR experiments were performed at 499.9 MHz on a Varian Unity-500 instrument, standardized against the residual HOD (δ_H 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.

GC–MS analysis.—Trimethylsilyl-derivatized carbohydrates from buckwheat seeds containing **1** and **2** were chromatographed on a DB-5 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) in a Hewlett–Packard 5890 gas chromatograph coupled to a HP 5970 mass spectrometer. The temperature program was 140 °C for 4 min, increased at 3 °C min^{-1} to 270 °C, and held at 270 °C for 20 min. The carrier gas was helium at 1 mL/min. Mass spectra for GC peaks corresponding to derivatized **1** and **2** were compared with mass spectra for trimethylsilyl derivatives of authentic **3** and D-galactose.

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