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Note

Molecular structure of lathyritol, a galactosylbornesitol from *Lathyrus odoratus* seeds, by NMR

Ralph L. Obendorf,[a,*] Christine E. McInnis,[a] Marcin Horbowicz,[b] Ivan Keresztes[c] and Lesław B. Lahuta[d]

[a]*Seed Biology, Department of Crop and Soil Sciences, Cornell University, Ithaca, NY 14853-1901, USA*
[b]*Research Institute of Vegetable Crops, Skierniewice, Poland*
[c]*NMR Facility, Department of Chemistry and Chemical Biology, Cornell University, Ithaca, NY 14853, USA*
[d]*Department of Plant Physiology and Biotechnology, University of Warmia and Mazury, Oczapowskiego 1A, 10-957 Olsztyn, Poland*

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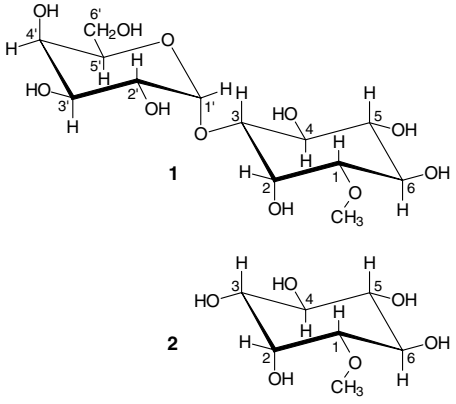
Abstract—The molecular structure of galactosyl-D-(−)-bornesitol, a novel compound isolated from sweet pea seeds, was determined to be α-D-galactopyranosyl-(1→3)-1-*O*-methyl-1D-*myo*-inositol by 1D and 2D NMR spectroscopy and is assigned the trivial name lathyritol.

Keywords: Sweet pea seed; Galactosyl-D-(−)-bornesitol; Galactosylcyclitol; NMR

Seeds of many species accumulate galactosylcyclitols as part of their normal seed maturation storage compounds.[1–4] Structural characterization is the first step to understand the metabolism and physiological role of galactosylcyclitols in seeds. Sweet pea (*Lathyrus odoratus* L.) seeds accumulate several soluble carbohydrates including the novel compound lathyritol (galactosyl-D-(−)-bornesitol; α-D-galactopyranosyl-(1→3)-1-*O*-methyl-1D-*myo*-inositol) (**1**) and free D-(−)-bornesitol (1D-1-*O*-methyl-*myo*-inositol) (**2**). The bornesitol moiety of galactosylbornesitol and free bornesitol from sweet pea seeds were confirmed to be the D configuration, as determined by their NMR spectra and optical rotation. The identification of free L-bornesitol in sweet pea flowers was probably incorrectly labeled.[5] Herein we report the molecular structure and absolute configuration of **1**, a novel compound.

Analysis by high-resolution gas chromatography of trimethylsilyl derivatives allowed the identification of chromatogram peaks corresponding to **1** and **2** in seed extracts, in selected fractions during purification, and in the analysis of hydrolysis products of **1**. Purified **1** was white powder and **2** was white crystalline solid. Acid hydrolysis of **1** produced D-galactose and **2** in a 1:1 mole ratio. Compound **1** was hydrolyzed by α-D-galactosidase, but not by β-D-galactosidase, demonstrating an α-anomeric linkage for the D-galactopyranosyl residue. Analysis of NMR spectra determined that the linkage



* Corresponding author. Tel.: +1 607 227 9313; fax: +1 607 255 2644; e-mail: rlo1@cornell.edu

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must be between carbon C-1′ on the galactopyranosyl ring and carbon C-3 on the bornesitol ring.

^{13}C NMR spectra identified seven carbon resonances and ^{1}H NMR spectra identified nine hydrogen resonances for **2** (Table 1). The ^{1}H NMR spectra of **2** showed a characteristic intense singlet at δ_H 3.28 corresponding to the *O*-methyl group. A highly deshielded triplet at δ_H 4.16 corresponded to the only equatorial hydrogen with the smallest coupling constants ($J_{1,2}$; $J_{2,3}$) characteristic of an H_{ax}–H_{eq}–H_{ax} system, and was assigned H-2 in accordance with published spectra for *O*-methyl ethers of *myo*-inositol.[6,7] The remaining hydrogens exhibited large coupling constants characteristic of H_{ax}–H_{ax}–H_{ax} transaxial systems on the cyclitol ring. The furthest downfield resonance in the ^{13}C NMR spectra at δ_C 80.80 (C-1) indicated the site of methylation.[1,6,7] By contrast, the methyl carbon was the furthest upfield, corresponding to δ_C 57.10. Assignments for the five remaining carbon (δ 68–75) and hydrogen resonances on the cyclitol ring were determined by 2D COSY and HSQC experiments (Table 1).

^{13}C NMR spectra identified 13 carbon resonances and ^{1}H NMR spectra identified 16 hydrogen resonances for **1** (Table 1). The ^{1}H NMR spectra for **1** showed the characteristic intense singlet at δ_H 3.29 and the furthest upfield carbon (δ_C 57.22) resonance in the ^{13}C NMR spectrum corresponding to the *O*-methyl group on the cyclitol ring. A highly deshielded triplet at δ_H 4.38 suggested that H-2 was equatorial. The small coupling constants corresponding to $J_{1,2}$ and $J_{2,3}$ confirmed that H-2 was indeed equatorial on **1** and **2**. The characteristically downfield anomeric carbon (δ_C 95.72) and anomeric hydrogen (δ_H 5.00) were useful for assigning resonances to the D-galactopyranosyl ring. The C-6′ on the galactopyranosyl ring was shielded the most and identified as the resonance at δ_C 61.45, the second furthest upfield. The two nearly coincident H-6′ hydrogens (δ_H 3.58 and 3.60) were characteristic of distal galactopyranosyl rings.[7–9] The remaining resonances could be assigned by coupling constants and 2D COSY and NOESY experiments. The hydrogen at 3.45 ppm was attached to the downfield carbon (δ_C 75.81) with the resonance shifted +4.3 ppm downfield in comparison to the corresponding resonance for free D-(−)-bornesitol **2** (Table 1), thereby identifying the point of galactose substitution on the bornesitol ring.[7–9] HMBC experiments showed clear interactions between the anomeric hydrogen (δ 5.00; H-1′) on the galactopyranosyl ring and the carbon at δ 75.81 (C-3) on the cyclitol ring, confirming the point of linkage. The small coupling constant $J_{1',2'}$ 3.9 verified the α-anomeric linkage.

2D NOSEY experiments showed interactions between the anomeric hydrogen (δ_H 5.00; H-1′) of the D-galactopyranosyl ring and hydrogens at δ_H 3.45 (H-3) and δ_H 4.38 (H-2) of the cyclitol ring, consistent with the assigned linkage. In addition, the observed correlation between H-5′ (δ_H 4.03) of the D-galactopyranosyl ring and H-3 (δ_H 3.45) of the cyclitol ring indicated close contact between those hydrogens. Molecular mechanics calculations predicted considerably shorter interatomic distance between H-5′ and H-3 for D-bornesitol (3.08 Å) than the L isomer (4.26 Å), which suggested that the cyclitol ring is in the D configuration.

Coupling constants between adjacent hydrogens were calculated from the 1D ^{1}H signals. The derived coupling

Table 1. ^{1}H and ^{13}C NMR chemical shifts and proton–proton coupling constants of lathyritol (**1**) and D-(−)-bornesitol (**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	3.05	80.70	3.06	80.80	$J_{1,2}$	2.7	2.8
2	4.38	63.98	4.16	68.00	$J_{2,3}$	2.7	2.9
3	3.45	75.81	3.35	71.50	$J_{3,4}$	10.0	10.0
4	3.60	71.30	3.47	72.70	$J_{4,5}$	9.4	9.4
5	3.18	74.68	3.13	74.80	$J_{5,6}$	9.4	9.4
6	3.54	71.98	3.49	72.00	$J_{6,1}$	10.0	10.0
OCH_3	3.29	57.22	3.28	57.10			
Galactopyranosyl ring							
1′	5.00	95.72			$J_{1',2'}$	3.9	
2′	3.70	68.74			$J_{2',3'}$	10.4	
3′	3.80	69.79			$J_{3',4'}$	3.3	
4′	3.86	69.63			$J_{4',5'}$	1.3	
5′	4.03	71.33			$J_{5',6'a}$	7.7[a]	
6′a	3.58	61.45			$J_{5',6'b}$	4.8[a]	
6′b	3.60				$J_{6'a,6'b}$	−11.6[a]	

[a] Determined by simulation.

constants (Table 1) allowed confirmation of the assignments made for cyclic bornesitols. The coupling constants for **1** indicated that the cyclitol ring is in the same configuration as **2**. The presence of small coupling constants ($J_{1,2}$ and $J_{2,3}$) for H-2 on **1** and **2** indicated the presence of the H_{ax}–H_{eq}–H_{ax} system, while the large coupling constants for the remaining hydrogens indicated they were all H_{ax}–H_{ax}–H_{ax} transaxial systems characteristic of the *myo*-inositol ring in bornesitol.

The optical rotation of **1** was $[\alpha]_D^{23}$ +96.5 (*c* 0.76, H_2O) and of **2** was $[\alpha]_D^{23}$ −28.4 (*c* 1.45, H_2O). Compound **2** was assigned the D configuration because of levorotation.[10] This configuration was also inferred from the NMR data for **1** (see above). The optical rotation value ($[\alpha]_D^{23}$ −28.4) for **2** is similar to published values ($[\alpha]_D^{30}$ −26.4; $[\alpha]_D^{18}$ −32.05; $[\alpha]_D^{20}$ −32.6),[4,7,8] and the value ($[\alpha]_D^{23}$ +96.5) for **1** is expected compared to those for galactinol (α-D-galactopyranosyl-(1→1)-1L-*myo*-inositol; $[\alpha]_D^{20}$ +135.6)[11] and galactosylononitol (α-D-galactopyranosyl-(1→3)-1D-4-*O*-methyl-*myo*-inositol; $[\alpha]_D^{20}$ +129.6).[7]

The GC mass spectrum of Me_3Si ethers of **2** (Fig. 1, top) was characteristic for bornesitol.[12] Mass spectra of Me_3Si ethers *O*-methyl inositols exhibit several characteristic ions[12] (present in Fig. 1) that are different than their corresponding inositols. The major fragment ions (*m*/*z* abundance relative to *m*/*z* 217) for **2** were *m*/*z* 89 (21), 159 (42), 217 (100), 247 (21), 260 (26), 374 (7.8), 375 (7.9), 432 (5.9), 433 (10), and 449 (4.4), and the abundance ratios, *m*/*z* to *m*/*z*, were 260/247 (1.24), 374/375 (0.99), and 432/433 (0.59). The GC–MS spectrum of Me_3Si ethers of **1** (Fig. 1, bottom) exhibited high abundance ions at *m*/*z* 191, 204, 217, and 361 from

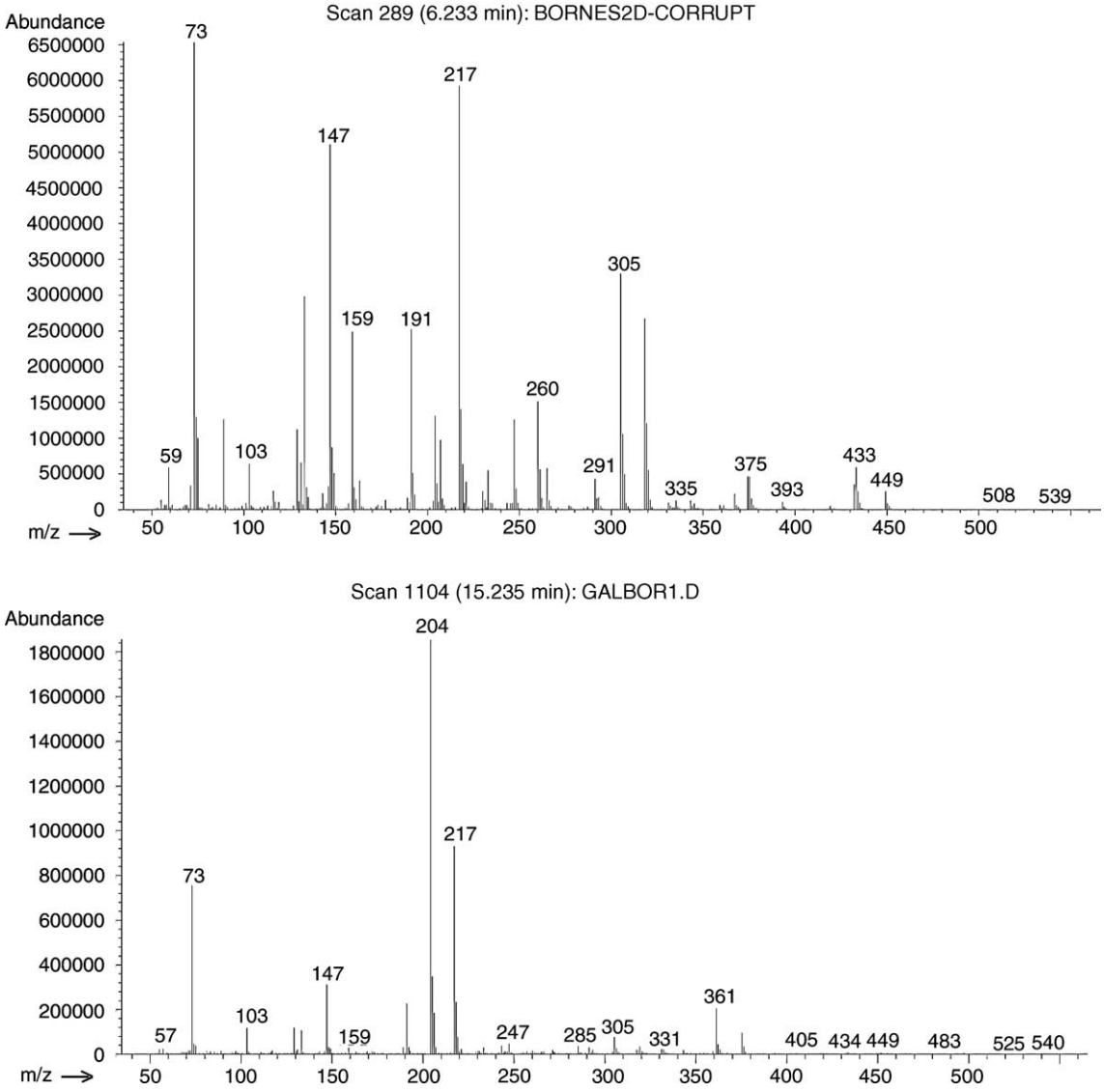

Figure 1. Mass spectrum of Me_3Si-D-(−)-bornesitol (**2**) (top) and of Me_3Si-α-D-galactopyranosyl-(1→3)-1D-1-*O*-methyl-*myo*-inositol (**1**) (bottom).

fragmentation of the Me_3Si ethers of the galactopyranosyl ring, while ions at *m/z* 159, 247, 375, and 449 resulted from fragmentation of the Me_3Si ethers of the *O*-methyl cyclitol ring.

Estimated molecular weights determined from negative-ion electrospray ionization mass spectra (ESI MS) were 356 for **1** and 194 for **2**.

Compound **1** is α-D-galactopyranosyl-(1→3)-1-*O*-methyl-1D-*myo*-inositol and assigned the trivial name of lathyritol, a novel galactosyl D-bornesitol that accumulates in *Lathyrus odoratus* L. seeds during late seed maturation and desiccation. Lathyritol accumulation began at 32 days after pollination (DAP; maximum seed fresh weight), increased to 1.5 mg g^{-1} dry weight at 34 DAP (maximum seed dry weight) and to 6.6 mg g^{-1} dry weight at 38 DAP when seeds had dried to 20% moisture. Lathyritol represents another methylated derivative of galactinol, the other compound being galactosyl ononitol.[7]

1. Experimental

1.1. Extraction and purification

Sweet pea (*Lathyrus odoratus* L.) seeds were purchased from Outsidepride.com, Inc. (Salem, OR, USA). Soluble carbohydrates in embryo tissues (cotyledons; axis) expressed as mg g^{-1} dry weight ± SE for three replicate samples were: D-(−)-bornesitol (**2**) (2.0 ± 0.2; 3.6 ± 1.1), lathyritol (**1**) (2.0 ± 0.2; 6.4 ± 1.6), *myo*-inositol (0.2 ± 0.0; 0.9 ± 0.2), galactinol (0.6 ± 0.0; 2.1 ± 0.4), sucrose (16.0 ± 0.2; 39.0 ± 6.5), raffinose (3.8 ± 0.2; 10.1 ± 1.7), stachyose (20.9 ± 1.0; 75.3 ± 14.0), and verbascose (1.4 ± 0.1; 6.1 ± 0.8). Approximately 300 g of embryos from de-coated seeds were pulverized to a fine powder in a coffee grinder, extracted with 2.5 L of EtOH–water (1:1, v/v) at 80 °C for 30 min, and cooled to 22 °C. Solids were removed by filtration through cheese cloth and centrifugation (25 samples). Clear supernatants were diluted 1:4 (v/v) with water, frozen at −80 °C, and freeze dried. Dry residues were dissolved in water, and **1** and **2** were purified 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] Freeze-dried samples were dissolved in minimal water for loading onto a water slurry packed column (100 mm × 200 mm), and soluble carbohydrates were eluted at 4 °C with stepwise increments of EtOH–water, v/v, (4 L of water; 4 L of 2:98; 4 L of 4:96; 2 L of 5:95; 2 L of 6:94) and collected in 500 mL fractions. Fractions were sampled (200 μL fraction + 25 μg of phenyl α-D-glucoside as internal standard), dried in a stream of nitrogen gas and stored overnight above P_2O_5, derivatized with 100 μL of *N*-trimethylsilylimidazole–pyridine (1:1, v/v) at 80 °C for 45 min, and analyzed[2,8,9] on a Hewlett–Packard 6890 GC (Agilent Technologies, Palo Alto, CA, USA) 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^{-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^{-1}. Typical retention times for trimethylsilyl derivatives were 23.8 min for **1** and 6.6 min for **2**.

Fractions 14–17 containing **2** (eluted with EtOH–water, 2:98, v/v) were freeze dried, pooled, loaded onto a carbon–Celite column (25 mm × 900 mm), and eluted with 2 L of water followed by 2 L of EtOH–water (2:98, v/v). Fractions containing **2** (90% pure) were pooled, freeze dried, and dissolved in hot (80 °C) MeOH. Crystals collected after evaporative cooling yielded 50 mg of **2** (99% pure). A second crystallization from MeOH did not increase purity.

Compound **1** eluted from the primary column with EtOH–water (4:96, 5:95, 6:94, v/v). Fractions 23–28 containing **1** were freeze dried, and dry residues were pooled (1785 mg). This pooled sample containing **1** was dissolved in water, loaded onto a Dowex 50Wx8 cation exchange column (25 mm × 600 mm), and eluted with water to remove contaminants. Fractions enriched in **1** were pooled and freeze dried. A sample containing **1** (266 mg, 85% pure) was loaded onto a carbon–Celite column (25 mm × 900 mm) and eluted stepwise with water (2 L) followed by EtOH–water (4 L of 3:97, 2 L of 3.5:96.5, 1 L of 4:96, v/v). Fractions 281–304 (eluted with EtOH–water, 3.5:96.5, v/v) yielded 95 mg of **1** (97% pure) that was taken for analysis by NMR, GC–MS, LC–MS, acid, and enzymatic hydrolysis, and optical rotation.

1.2. NMR analysis

Purified samples (60 mg of **1**, 100 mg of **2**) were dissolved in 1 or 2 mL of 99.96% D_2O (Cambridge Isotope Labs, Andover, MA) for NMR analysis. Hydrogen and 2D spectra were recorded on a Varian INOVA spectrometer operating at 499.92 and 125.72 MHz for hydrogen and carbon observation, respectively, using a Varian inverse triple resonance probe head equipped with a *z*-axis gradient coil. Hydrogen spectra were referenced relative to residual HOD at 4.63 ppm as an internal standard. Carbon spectra were recorded on a Varian INOVA spectrometer operating at 100.54 MHz for carbon observation using a Varian auto-switchable broadband probe and referenced relative to dioxane at 67.4 ppm as an external standard.

2D Spectra for **2** were collected with sweep widths of 1 kHz in the hydrogen and 5 kHz in the carbon dimen-

sion. For **1**, sweep widths of 1.2 and 6.3 kHz were used. Gradient-enhanced DQ-COSY spectra were acquired in phase-sensitive mode with 64 or 128 complex points in the indirectly detected dimension. One transient of 512 or 1k data points were collected for each increment. The data were zero filled to 2k complex data points and multiplied with 45° shifted squared sinusoidal window functions in both dimensions prior to Fourier transform. NOESY spectra were acquired in phase-sensitive mode with 200 complex points in the indirectly detected dimension and 500 ms mixing time. Sixteen transients of 512 or 1k data points were collected for each increment. The data were zero filled to 2k complex data points and multiplied with Gaussian window functions in both dimensions prior to Fourier transformation. Gradient enhanced HSQC spectra were acquired in phase sensitive mode with 128 complex points in the indirectly detected dimension and 3.57 ms evolution time. Two or four transients of 512 or 1k data points were collected for each increment. The data were zero filled to 2k complex data points and multiplied with Gaussian window functions in both dimensions prior to Fourier transformation. Gradient enhanced HMBC spectra were collected using a band selective constant time sequence[14] in absolute value mode with 400 points in the indirectly detected dimension and 62.5 ms evolution time for multiple bond couplings. Two or four transients of 512 or 1k data points were collected for each increment. Selective inversion of the carbon resonances was accomplished with Q3 Gaussian cascade pulses. The data were zero filled to 2k complex data points and multiplied with shifted sinusoidal window functions in both dimensions prior to Fourier transformation.

1.3. GC–MS and LC–MS analysis

GC–MS analyses of trimethylsilyl-derivatives of **1** and **2** (prepared as above) were with a 5971A Mass Selective Detector set at SCAN mode (ion source 70 eV) on a Hewlett–Packard 5890 GC. Separation was on an HP-1 capillary column (12 m × 0.2 mm i.d., 0.33 μm film thickness) using helium as carrier gas at 50 cm/s flow rate. Injection port temperature was 250 °C, split 1:10. Detector temperature was 280 °C. Column oven temperature was programmed at 150 °C for 2 min and then adjusted 8 °C/min to 300 °C.

Electrospray ionization mass spectra (ESI MS) in negative mode were performed on **1** and **2** using a Shimadzu HPLC-MS—QP 8000α system (Shimadzu, Kyoto, Japan). The curve desolvation line (CDL) temperature was 200 °C with −70 V CDL voltage, −5.0 kV probe voltage, −45 V defragmentation voltage, 2.8 mL min^{-1} nebulizer gas flow, 0.2 mL min^{-1} column flow rate, and a 5 μL injection loop. Samples were eluted with acetonitrile–formic acid–water, 40:0.25:59.75 (v/v/v).

1.4. Optical rotation analysis

Optical rotation values for **1** (90 mg) and **2** (45 mg) dissolved in water were assayed with a Autopol III polarimeter (Rudolph Research Analytical, Flanders, NJ, USA) at 589 μm and 23 °C using a 100 mm path length cell.

1.5. Acid and enzymic hydrolysis

Compound **1** (200 μg) was hydrolyzed with 1 M CF_3CO_2H for 16 h at 80 °C and evaporated to dryness. The mole ratio of D-galactose to **2** was calculated after GC analysis of the trimethylsilylated products. Compound **1** (200 μg) was incubated with 1.25 units of desalted green-coffee-bean α-D-galactosidase (EC 3.2.1.22) in 200 μL of water at 22 °C or with 0.5 units of bovine liver β-D-galactosidase (EC 3.2.1.23) in 200 μL of water at 37 °C for 24 h. Enzyme protein was removed by filtration (10,000 MW cut-off filter), the sample was 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.

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References

1. Schweizer, T. F.; Horman, I. *Carbohydr. Res.* **1981**, *95*, 61–71.
2. Horbowicz, M.; Obendorf, R. L. *Seed Sci. Res.* **1994**, *4*, 385–405.
3. Obendorf, R. L. *Seed Sci. Res.* **1997**, *7*, 63–74.
4. Szczecinski, P.; Gryff-Keller, A.; Horbowicz, M.; Lahuta, L. B. *J. Agric. Food Chem.* **2000**, *48*, 2717–2720.
5. Ichimura, K.; Kohata, K.; Mukasa, Y.; Yamaguchi, Y.; Goto, R.; Suto, K. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 189–191.

6. Angyal, S. J.; Odier, L. *Carbohydr. Res.* **1983**, *123*, 23–29.
7. Richter, A.; Peterbauer, T.; Brereton, I. *J. Nat. Prod.* **1997**, *60*, 749–751.
8. Obendorf, R. L.; Steadman, K. J.; Fuller, D. J.; Horbowicz, M.; Lewis, B. A. *Carbohydr. Res.* **2000**, *328*, 623–627.
9. Steadman, K. J.; Fuller, D. J.; Obendorf, R. L. *Carbohydr. Res.* **2001**, *331*, 19–25.
10. Bien, S.; Ginsburg, D. *J. Chem. Soc.* **1958** (Sep), 3189–3194.
11. Brown, R. J.; Serro, R. F. *J. Am. Chem. Soc.* **1953**, *75*, 1040–1042.
12. Binder, R. G.; Haddon, W. F. *Carbohydr. Res.* **1984**, *129*, 21–32.
13. Whistler, R. L.; Durso, D. F. *J. Am. Chem. Soc.* **1950**, *72*, 677–679.
14. Claridge, T. D. W.; Pérez-Victoria, I. *Org. Biomol. Chem.* **2003**, *1*, 3632–3634.