Galactosylpinitols Isolated from Vetch (*Vicia villosa* Roth.) Seeds

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Three α-galactosides of D-pinitol: 1D-*O*-(α-D-galactopyranosyl)-(1→2)-4-*O*-methyl-*chiro*-inositol, **1**, 1D-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→2)-4-*O*-methyl-*chiro*-inositol, **2**, and 1D-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→2)-4-*O*-methyl-*chiro*-inositol, **3**, present in vetch seeds, were isolated, purified, and quantitatively determined using column and high-resolution gas chromatography. Their structures were established by ^{1}H and ^{13}C NMR 1D and 2D techniques.

Keywords: *NMR; α-galactosides; pinitol*

INTRODUCTION

Galactosides of sucrose are well-known and widely distributed in seeds of many plant species and are localized mainly in the embryos of cereals and axis tissue in legumes (Horbowicz and Obendorf, 1994). Legume seeds also contain, in relatively high concentrations, galactosides of cyclitols: D-pinitol, *chiro*-inositol, and *myo*-inositol (Obendorf, 1997). In some seeds, the level of α-D-galactopyranosylcyclitols reaches over 30% of the total amount of soluble carbohydrates (Horbowicz and Obendorf, 1994). Both types of galactosides are considered to be protective compounds for the stability and the viability of the organelles, membranes, enzymes, and proteins during development, desiccation, and storage of seeds (Chen and Burris, 1990; Blackman et al., 1992; Brenac et al., 1997). Several α-D-galactopyranosylcyclitols were identified in legume seeds (Schweizer and Horman, 1981; Quemener and Brillouet, 1983; Nicolas et al., 1984; Ganter et al., 1991; Bernabé et al., 1993; Chien et al., 1996). The main sources of cyclitol galactosides are seeds of lupin, lentil, chick pea, and soybean. Also, in buckwheat (*Fagopyrum esculentum*) seeds, fagopyritol B1, (1D-α-D-galactopyranosyl-(1→2)-*chiro*-inositol), has recently been found in high amounts (Horbowicz and Obendorf, 1998; Szczeciński et al., 1998). Vetch is an important fodder crop in Poland. It is sown together with the popular grains such as rye and wheat often also as a forecrop or an aftercrop. There is little information concerning the content of cyclitol derivatives in seeds of vetch. Preliminary studies indicated that they contain the unique set of cyclitol galactosides. We decided to check in detail what kind and what amounts of these compounds are present in the seed axis and cotyledons of *Vicia villosa* Roth. Such information could be useful for the determination of the physiological role of the galactosides present in plant seeds.

EXPERIMENTAL PROCEDURES

Extraction of D-Pinitol Galactosides from Vetch Seeds. Decoated, powdered seeds (150 g) were twice defatted with benzene−ethanol (300 mL; 2:1, v/v) by boiling the suspension under reflux for 1 h. After filtration was performed, the residue was twice extracted with ethanol−water (1000 and 500 mL; 6:4, v/v). The combined extracts were concentrated under vacuum to ca. 50 mL, and then 30 mg of invertase (Sigma) in 5 mL of distilled water was added. The invertase-catalyzed hydrolysis was allowed to proceed at 30 °C for 6 h. Then 3 vol of ethanol was added, and the mixture was centrifuged (6000 rpm, 2000*g*). The clear supernatant was evaporated under vacuum to a small volume (ca. 30 mL). The excess of monosaccharides and other carbohydrates was removed using charcoal-Celite 545 (1:1) column chromatography (22 mm i.d. × 500 mm). The monosaccharides were washed out from the column with water (3 L), and then the galactosides of cyclitols were eluted with ethanol−water (1.5 L; 5:95, v/v). After the solvents were evaporated, the crude, galactosides-containing syrup was obtained (5.7 g).

Chromatographic Separation of Galactosylcyclitols. Part of the syrup (2.8 g) was dissolved in a small volume of acetonitrile−water solution (6:4) and subjected to flush chromatography using a column (22 mm i.d. × 300 mm) filled with the 3-aminopropyl-functionalized silica gel (Aldrich, catalog no. 36,425-8). The column was first eluted with acetonitrile−water (500 mL; 80:20, v/v; solution A), then with acetonitrile−water (500 mL; 75:25, v/v; solution B), and finally with acetonitrile−water (500 mL; 70:30, v/v; solution C). Fractions of 20 mL volume were collected. The fractions obtained through the elution with solution A contained the underivatized cyclitols and the rest of monosaccharides. The elution with solution B gave compound **1** (150 mg; fractions 5−7), and the elution with soluton C gave compound **2** (400 mg; fractions 1−8) and compound **3** (70 mg; fractions 18−25) (Figure 1). The presence and the composition of carbohydrates in the obtained fractions were monitored by the gas chromatographic method (Górecki et al., 1997).

Determination of Carbohydrates in Vetch Seeds. Carbohydrates in vetch seeds were determined in cotyledons and axis separately using the previously described gas chromatographic method (Horbowicz and Obendorf, 1994; Gorecki et al., 1997). The standards of D-pinitol, *myo*-inositol, sucrose, raffinose, and stachyose were obtained from Sigma, and verbascose was obtained from Megazyme (Australia). The standard of ononitol was obtained from A. Richter (University of Vienna, Austria). The standards of galactosylo-pinitols A (**1**) and B were obtained from T. Schweizer (Nestle, Switzer-

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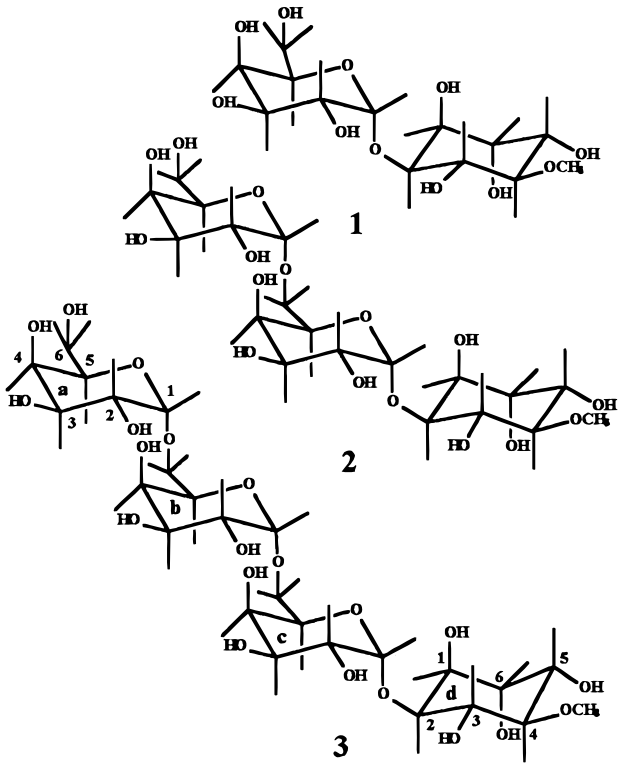

Figure 1. Structure of compounds **1**, **2**, and **3**. Sticks without atom symbols denote C−H bonds.

Table 1. Proton Chemical Shifts, $J_{H,H}$ Coupling Constants (DQF COSY), and Carbon Chemical Shifts (1D) for Compound 3 in D_2O at 30 °C

	chemical shifts [ppm]							
	ring a		ring b		ring c		ring d	
position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	5.019	98.33	4.959	98.65	5.142	95.38	4.212	69.08
2	3.830	69.00[a]	3.813	69.02	3.876	70.19	3.909	75.33
3	3.869	68.97[a]	3.910	70.27	3.978	70.08	3.828	71.37
4	3.989	69.94[a]	3.982	70.12[a]	4.037	70.31	3.381	83.57
5	3.994	71.63	4.215	67.75	4.376	70.12	3.851	70.49
6	3.751	61.87	3.862	66.89	3.929	67.91	4.041	71.66
6′	3.751		3.713		3.667			
	proton−proton coupling constants [Hz]							
1,2	3.6		3.9		3.9		2.8	
2,3	10.3		10.3		10.3		10.2	
3,4	2.9		3.3		3.3		9.1	
4,5	nd[b]		1.5		1.5		9.9	
5,6	6.4		4.7		3.3		3.1	
5,6′	6.4		7.7		8.4			
6,6′		−11.1		−10.5				
6,1				4.4				

[a] Tentative assignments. [b] nd, not determined.

land) and J. Streeter (USA). Galactinol was a gift from T. M. Kuo (Peoria, IL). The presence of di-galactosylo-pinitol B was tentatively assumed, and its content was calculated on the basis of ciceritol (**2**) standard, obtained in this work.

NMR Spectroscopy. A sample of compound **3** (70 mg) was dissolved in 5 mL of D_2O, and the solvent was evaporated under reduced pressure. The procedure was repeated twice. The residue was dissolved in 0.8 mL of D_2O and filtered through a Celite pad directly into a 5-mm NMR tube. The tube was sealed under vacuum. Proton chemical shifts were referenced to residual HOD at 4.71 ppm. The proton chemical shift values (Table 1) were given to the third decimal place to stress the accuracy of the measurements performed. It is, however, obvious that the reproducibility of the measurements of this kind depends strongly on the concentration and temperature and may not exceed 0.01 ppm. Carbon chemical shifts were referenced to external dioxane in D_2O at 67.19 ppm (Gottlieb et al., 1997).

All the NMR techniques applied in this work are well documented in the literature (Braun et al., 1998), and their standard implementations are included into the modern spectrometer software. The 1D ^{13}C NMR spectrum, carbon-detected $^{13}C-^{1}H$ correlation spectrum (HETCOR), the proton-detected $^{1}H-^{13}C$ correlation spectra, HSQC and HMBC, and the 2D spectra correlated by the nuclear Overhauser effect in the rotating frame, ROESY, were recorded using a Varian UNITY + spectrometer working at 500-MHz proton frequency. The 1D ^{1}H NMR spectrum, the 2D phase-sensitive double quantum filtered $^{1}H-^{1}H$ correlation spectrum, DQF COSY, and the 1D TOCSY spectra were recorded using a Brucker DRX 500 advanced spectrometer. The z-gradient assisted versions of both the COSY and 1D TOCSY techniques were used. In the latter type of spectra, one of the anomeric proton signals or the inositol signal lying at 3.381 ppm was selectively excited. The line widths and digital resolutions allowed the distinguishing of spectral lines separated by 1 and 3 Hz in the 1D ^{1}H and ^{13}C NMR spectra, respectively. The spectral windows in the 2D spectra were adjusted in both dimensions to cover the total range of proton (2 ppm) and/or carbon (40 ppm) signals. After the appropriate zero-filling, the data matrixes were of 2 × 2K dimension. Owing to the relatively high solute concentration and the high degree of deuteriation, the suppression of the residual HOD signal prior to the proton excitation was not necessary.

RESULTS AND DISCUSSION

The chromatographic properties of compounds **1**−**3** suggest that they belong to the galactosylcyclitol series. Indeed, compounds **1** and **2** were unambiguously identified by comparison of their ^{13}C NMR spectra with the literature data reported for compounds separated from other leguminous plants. After due referencing, the spectrum of compound **1** was identical within 0.05 ppm to the spectrum of 1D-*O*-α-D-galactopyranosyl-(1→2)-4-*O*-methyl-*chiro*-inositol, which was separated previously from soya bean (Schweizer and Horman, 1981). Similarly, a perfect agreement was observed for the ^{13}C NMR spectra of compound **2** and ciceritol, i.e., 1D-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→2)-4-*O*-methyl-*chiro*-inositol separated from lentils (Bernabe et al., 1993). Ciceritol was previously also found in lentil, white lupin, bean, soyabean, and chick pea (Quemener and Brillouet, 1983). The reported assignments of signals in the proton as well as the carbon NMR spectra of both the compounds are complete and unequivocal.

The situation was more complicated in the case of compound **3**. Inspection of its ^{1}H NMR (500 MHz, D_2O) spectrum allowed for the straightforward identification of the singlet of a methoxy group at 3.61 ppm and three doublets for anomeric protons at 5.14, 5.09, and 4.96 ppm. In the ^{13}C NMR spectrum of compound **3**, three anomeric carbon signals were distinguishable owing to their high chemical shifts caused by the neighborhood of two oxygen atoms. The overall spectrum contained 24 signals, 23 of them being of similar intensity and one approximately twice as strong, apparently due to the accidental overlap of two resonances. No signals attributable to quaternary carbon atoms were observed. The APT spectrum showed that three signals originated from CH_2 groups. A signal lying at the highest field was similarly assigned to a methyl group. All the above findings lead to the conclusion that compound **3** is a pseudotetrasaccharide composed of three hexopyranose and one cyclitol entities, one of them bearing a *O*-methyl group.

Table 2. Content of Carbohydrates in Axis and Cotyledons of Vetch Seeds (*Vicia villosa* Roth.)[a]

analyzed carbohydrate		axis	SD		cotyledons	SD
D-pinitol		7.67	1.29		7.03	0.40
ononitol		1.14	0.03		0.35	0.02
myo-inositol		3.81	0.07		1.32	0.05
sucrose		25.57	3.93		25.49	1.11
galactopyranosyl-pinitol A (**1**)		9.56	0.65		5.76	0.28
galactopyranosyl-pinitol B		5.23	0.06		2.66	0.11
galactinol (galactopyranosyl-*myo*-inositol)		5.46	0.17		2.95	0.15
raffinose		10.40	0.65		2.51	0.08
ciceritol (**2**) (di-galactopyranosyl-pinitol A)		63.49	2.67		51.77	1.42
di-galactopyranosyl-pinitol B[b]		4.98	0.53		3.99	0.24
stachyose		15.10	0.29		6.24	0.28
tri-galactopyranosyl-pinitol (**3**)		26.38	2.87		18.20	1.19
verbascose		51.80	1.69		38.26	1.90
total galactosides	(100.0%)	192.40		(100.0%)	132.34	
total sucrose galactosides	(40.17%)	77.30		(35.52%)	47.01	
total cyclitol galactosides	(59.82%)	115.10		(64.48%)	85.33	
total pinitol galactosides	(56.98%)	109.64		(62.25%)	82.38	

[a] mg/g of dry weight. [b] Di-galactopyranosyl-pinitol B content was calculated on the basis of ciceritol (**2**) standard.

Taking into account the structures of compounds **1** and **2**, one may expect by analogy that compound **3** is a trigalactosyl-D-pinitol. Until now, two isomeric compounds of that kind have been isolated and characterized. Ganter et al. (1991) isolated 1D-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→2)-3-*O*-methyl-*chiro*-inositol from the *Mimosa scabrella* seeds, but its ^{13}C NMR spectrum is different from that of compound **3**. The other isomer was isolated from chick pea by Nicolas et al. (1984), and its structure was determined to be 1D-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→2)-4-*O*-methyl-*chiro*-inositol using biochemical methods. Unfortunately, the reported spectral data were insufficient for proving whether this was, or was not, our compound **3**. However, as it will be shown below, a detailed interpretation of the ^{1}H and ^{13}C NMR spectra, with the aid of appropriate 2D techniques, enabled us to show them to be the same.

Taking the proton CH_3 signal as a starting point, one can identify the proton and carbon signals of the CH_3-OCH fragment using ROESY, HETCOR, and/or HMBC 2D spectra. The signal of the methine proton identified in this way was well separated from other signals in the 1D proton spectrum and had the form of triplet (degenerate dd) due to two large (ca. 10 Hz) vicinal spin−spin couplings. Such a spectral pattern denotes that this proton occupies an axial position, as do its two neighbors. Furthermore, TOCSY and phase-sensitive DQF COSY spectra showed that this proton belonged to a spin system of six vicinally coupled protons, none of them being of anomeric or methylene type. The splitting patterns of particular signals, which were visible in 2D ^{1}H−^{1}H and ^{13}C−^{1}H correlation spectra, showed that four consecutive protons of this system occupy axial positions, and the remaining two are equatorial. Such a spatial proton arrangement is characteristic for 4-*O*-methyl-*chiro*-inositol.

It was mentioned above that compounds **1**, **2**, and **3** exhibit very similar chromatographic properties. This similarity as well as general knowledge about oligosaccharides occurring in legumes strongly suggest that hexopyranoses comprising the building blocks of compound **3** are D-galactoses. Because of extensive overlap of proton signals in the 1D spectrum, a complete direct analysis of the spectral patterns of protons H-2−H-6 was not feasible. However, it was done using 2D spectra. The anomeric proton signals are well separated so that the identification of the corresponding H-2 and H-3 signals was possible in COSY spectra. Also, signals of the H-6 and H-6′ protons, which are partially visible in the 1D spectrum, allowed for the identification of the H-5 signals, using complementary information from HSQC and HMBC spectra. Using a similar method, tentative assignments for the H-4 resonances were proposed. It is to be stressed that the signal patterns can be seen, and the vicinal coupling constant values as well as chemical shift values can be estimated from phase-sensitive DQF COSY and/or some of ^{13}C−^{1}H correlation spectra for all protons in all three hexoses (Table 1). Thus, $J_{2,3}$ in all three cases is of the axial−axial type, whereas $J_{1,2}$ is of equatorial−axial type. Not all the relevant vicinal spin−spin coupling constants could be measured with accuracy, but the information is sufficient to prove the spatial arrangement of the H-1−H-5 pyranose protons to be of e−a−a−e−a type. Such a stereochemistry is characteristic for the α-galactoside moiety.

As mentioned above, the proton signals were strongly overlapped in the 1D NMR spectrum. It was, however, possible to identify all the individual lines originated from H-6, H-6′, and H-5 of the c-ring. The numerical analysis of the four spin system, using the iterative procedure based on a Laocoon-like program, gave following results: $\delta_5 = 4.38$, $\delta_6 = 3.93$, and $\delta_{6'} = 3.67$ ppm and $J_{4,5} = 1.1$, $J_{5,6} = 3.4$, $J_{5,6'} = 8.2$, and $J_{6,6'} = -10.4$ Hz ($\delta_4 = 4.037$ ppm was not fitted). A comparison of the above values with those shown in Table 1 illustrates the accuracy of the spectral parameters determined from DQF COSY spectrum.

The evidence for the position of the glycoside linkages between pinitol and three galactose residues originate from ROESY and HMBC spectra. These yielded information that were consistent and complementary. In the ROESY spectra, apparent correlations are observed in two cases between anomeric proton signals and signals of two CH_2 protons. These CH_2 protons are correlated with the appropriate C-1 carbons in the HMBC spectrum. Thus, the linking between galactoses is of the 1→6 type. The substitution position on the pinitol was not so evident from ROESY spectrum, since two weak cross-peaks of comparable intensity between the anomeric proton H-1c signal and H-1d and H-2d were observed. This ambiguity is, however, removed by the HMBC spectrum, in which C-2d rather than C-1d was correlated with the anomeric proton.

The only structural question that remained concerned the absolute configurations of the residues in the trigalactopinitol. It may be reasonably assumed that the galactose residues belong to the D series, as galactose of the opposite configuration have never been isolated from natural sources. If so, a reasoning identical to that performed in the case of ciceritol (based on the observed chemical shifts, and the ROESY spectrum) leads to the conclusion that the *chiro*-inositol moiety possesses the D configuration. An additional comment is needed to explain how the assignments of signals to particular galactose residues has been made. This differentiation was feasible because only one of the CH_2 groups gave no ROESY correlations to anomeric protons in another residues. This was obviously the CH_2 group of the galactose a. The signal of these protons appeared as a doublet. Obendorf et al. (1999) noted that this is characteristic for terminal α-galactosyl residues. This identification enabled unequivocal assignment of H-5 and H-1 of residue-a on the basis of HMBC correlations. Further assignments became then straightforward.

Thus three galactopinitols 1D-2-*O*-(α-D-galactopyranosyl)-4-*O*-methyl-*chiro*-inositol, compound **1**, 1D-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→2)-4-*O*-methyl-*chiro*-inositol, compound **2**, and 1D-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→2)-4-*O*-methyl-*chiro*-inositol, compound **3**, were isolated from vetch seeds (*Vicia villosa* Roth.). All of these compounds had been isolated earlier from other legumes. The amounts of these galactosides in the cotyledons and axis of vetch seeds are very high (Table 2). In fact, to the authors' knowledge, vetch seeds contain the highest concentration of galactosyl derivatives of D-pinitol thus far reported among plants.

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