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Isolation and Characterization of a Pentasaccharide from *Stellaria media*

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While classic raffinose family oligosaccharides (RFOs) such as raffinose and stachyose are common in plants, stachyose is absent in the Caryophyllaceae. Instead the tetrasaccharide lychnose α-D-Gal-(1→6)α-D-Glc-(1→2)β-D-Fru-(1→1)α-D-Gal can accumulate. *Stellaria media*, a representative member of this family, was used to isolate α-D-Gal-(1→6)-[α-D-Gal-(1→4)]α-D-Glc-(1→2)β-D-Fru-(1→1)α-D-Gal, a novel pentasaccharide with a lychnose backbone. Complete NMR characterization using COSY, HSQC, HSQC-TOCSY, HMBC, and NOESY experiments was performed to unequivocally resolve its structure. This is the first report of a natural compound containing a Gal α(1→4)Glc linkage. The trivial name stellariose is proposed for this new pentasaccharide.

Sucrose is the main product of photosynthesis and is therefore ubiquitous in higher plants.[1] Besides sucrose, most plants accumulate other water-soluble carbohydrates (WSC), highly diverse structurally and functionally. One important group are the sucrosyl-oligosaccharides (SOS), which include the fructans and raffinose family oligosaccharides (RFOs).[2]

Of eight possible α-galactosyl linkages to the sucrose backbone (at C-2, C-3, C-4, and C-6 of the glucose moiety and at C-1, C-3, C-4, and C-6 of the fructose moiety), the α(1→6) galactosyl extensions at the C-6 of glucose are the most widespread in the plant kingdom. They are typically called the RFOs. Among these RFOs, the trisaccharide raffinose and tetrasaccharide stachyose are common in higher plants.[3] Several plants use this type of carbohydrates as substitutes for starch and sucrose, as they fulfill similar physiological functions, such as storage and transport. They may also act as protecting agents during cold, drought, or salt stress and are believed to be involved in desiccation tolerance in seeds.[2]

Similar to fructans, α-galactosyl oligosaccharides are known as useful prebiotic oligosaccharides. Raffinose and stachyose are readily degraded by α-galactosidases from Bifidobacteria.[4,5] The *B. adolescentis* α-galactosidase degrades α(1→3) and α(1→4) linkages from galactobiose even at a faster rate than the α(1→6) bond in raffinose.[6]

The biosyntheses of raffinose and stachyose are galactinol [α-D-Gal-(1→1)-L-*myo*-inositol]-dependent. Raffinose synthase (EC 2.4.1.82) catalyzes the reversible transfer of a galactosyl unit from galactinol (as donor substrate) to sucrose (as acceptor substrate).[7] Subsequently, raffinose acts as an acceptor in the stachyose biosynthesis reaction catalyzed by stachyose synthase (EC 2.4.1.67).[8] In contrast, synthesis of the higher DP RFOs (>DP 4) is galactinol-independent. The enzyme galactan:galactan galactosyltransferase (GGT) catalyzes the direct transfer of a terminal galactosyl residue from one RFO molecule to another, resulting in the next higher and lower RFO oligomers, respectively.[9,10]

Besides the α(1→6) linkage, five other types of α-galactosyl SOS have been isolated. Contrary to RFOs, the other five isomers are restricted to one or a few plant families.[2] A first isomer is loliose [α-D-Gal-(1→3)α-D-Glc-(1→2)β-D-Fru]. This trisaccharide is typically accumulated in seeds of Festuca and Lolium, from which it was isolated and its chemical structure was elucidated.[11,12] A second type is umbelliferose [α-D-Gal-(1→2)α-D-Glc-(1→2)β-D-Fru], where a galactosyl residue is bound to sucrose at the glucosyl moiety by an α(1→2) linkage. Umbelliferose is restricted to the Apiaceae and the Araliaceae families, where it is found in both vegetative and reproductive plant parts.[13,14] The third isomer planteose [α-D-Glc-(1→2)β-D-Fru-(6→1)α-D-Gal] is formed by the transfer of a galactose to the fructosyl moiety of sucrose, forming an α(1→6) linkage.[15] Planteose was first believed to be restricted to seeds, but recently it was reported to act as a short-term storage carbohydrate in *Actinidia* leaves.[16] The last two types are the tetrasaccharides lychnose and isolychnose. They consist of a raffinose backbone with a galactosyl residue bound to the fructosyl moiety by an α(1→1) linkage (lychnose) or an α(1→3) linkage (isolychnose). Both tetrasaccharides have been detected only in the vegetative plant parts of Caryophyllaceae, for which they are considered to be a chemotaxonomic marker.[3] The biosynthesis of the two oligosaccharides is not yet entirely elucidated, but it was reported that it should be raffinose-dependent.[17] The structure of lychnose [α-D-Gal-(1→6)α-D-Glc-(1→2)β-D-Fru-(1→1)α-D-Gal] has recently been unambiguously determined by 2D NMR spectroscopy.[18]

Here we report the purification of a new pentasaccharide from *Stellaria media*. The structure of this water-soluble, nonreducing oligosaccharide was determined by NMR spectroscopy. This is the first report of a natural carbohydrate containing a Gal α(1→4)Glc linkage.

Results and Discussion

S. media L. VILL (common chickweed), a winter annual spread all over the world, is a representative species of the Caryophyllaceae.[19] This plant can be used as a model plant to study lychnose metabolism, as *S. media* accumulates lychnose in as high or even higher concentrations as raffinose.[18] Therefore, lychnose seems to be an important soluble carbohydrate in Caryophyllacean plants probably fulfilling functions similar to classic RFOs. Besides lychnose, a lychnose-derived series has been found in *Silene dioica* roots. The pentasaccharide of this series is believed to be formed by the transfer of an extra galactose to the C-6 of the galactosyl moiety bound to fructose.[20] High-resolution mass spectrometry (HRMS) showed that the mass $[M + Na]^+$ of the purified compound was 851.2612 (Figure S1), confirming its pentasaccharide nature. The calculated mass $[M + Na]^+$ was 851.2645.

First we believed that the new isolated pentasaccharide belonged to the lychnose series as described in *S. dioica*. Surprisingly, NMR analyses on the purified compound revealed that the galactosyl residue was not attached to the galactosyl end but at the C-4 of the glucosyl moiety of lychnose. This is the first report of an α(1→4)

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Table 1. ^{1}H NMR Shifts (ppm) for Stellariose at 22 °C in D_2O

residue	H-1a	H-1b	H-2	H-3	H-4	H-5	H-6a	H-6b
Gal1	4.87		3.66	3.73	3.81	3.80	3.57	3.57
Glc2	5.28		3.44	3.86	3.55	4.00	3.87	3.65
Fru3	3.50	3.70		4.12	3.92	3.77	3.65	3.62
Gal4	4.88		3.63	3.72	3.82	3.77	3.57	3.57
Gal5	5.27		3.68	3.68	3.80	3.88	3.57	3.57

Table 2. ^{13}C NMR Shifts (ppm) for Stellariose at 22 °C in D_2O

residue	C-1	C-2	C-3	C-4	C-5	C-6
Gal1	99.16	68.34	69.04−69.24	69.04−69.24	70.84	60.95
Glc2	92.09	70.50	72.26	77.93	70.26	66.93
Fru3	66.02	103.32	76.32	73.50	81.04	62.11
Gal4	98.54	68.16	69.04−69.24	69.04−69.24	71.26	61.07
Gal5	99.71	68.69	69.04−69.24	69.04−69.24	71.71	61.11

linked galactosyl residue bound to glucose in nature. In contrast, Gal β(1→4)Glc linkages are very common.[21]

The systematic name of this pentasaccharide is α-D-Gal-(1→6)-[α-D-Gal-(1→4)]α-D-Glc-(1→2)β-D-Fru-(1→1)α-D-Gal, or abbreviated Gal(α1,6)[Gal(α1,4)]Glc(α1,2β)Fru(α1,1)Gal. Rather than using this complicated terminology, we propose the trivial name "stellariose".

Some of the characteristic signals can be assigned from 1D ^{13}C and ^{1}H NMR and selective TOCSY spectra,[22−24] but the majority of signals required 2D homonuclear and heteronuclear NMR techniques, which included COSY,[25] HSQC,[26−28] HSQC-TOCSY,[29] HMBC,[30] and NOESY[31,32] experiments (Tables 1, 2). First, assignment and quantification of through-bond homonuclear couplings $J_{H,H}$ via 2D experiments such as COSY and TOCSY yielded the information required to assign each monosaccharide spin system to a characteristic configuration, including anomeric configuration and ring form. Second, observation of true through-bond correlations of nuclei between monosaccharide residues provided the information needed to establish the sites of linkages.

Fructose H-3 and C-2 have characteristic chemical shifts and are well-defined signals in corresponding 1D spectra, while H-4,5,6a,6b could be traced from H-3 in the COSY spectrum. Both H-1a and H-1b have strong cross-peaks with C-2 in HMBC. The ^{13}C NMR resonances of C-1,3,4,5,6 were then assigned from the HSQC experiment and further confirmed with HSQC-TOCSY (Figure S2). The NOE effect between Fru-H3 and Fru-H1a/H1b confirmed the β-anomeric configuration of fructose (Figures 1, S3). Starting from a well-defined anomeric proton at 5.28 ppm, H-2,3,4,5,6 can be assigned from the COSY experiment. On the basis of the HSQC spectrum, ^{13}C resonances of residues have been determined. According to high values of three-bond scalar coupling constants, there are three *axial-axial* systems, H-2/H-3 (10.14 Hz), H-3/H-4 (9.54 Hz), and H-4/H-5 (9.87 Hz), and one *equatorial-axial* system, H-1/H-2 (3.78 Hz), that allowed the assignment of the residue as a glucose with an α-anomeric configuration.[33]

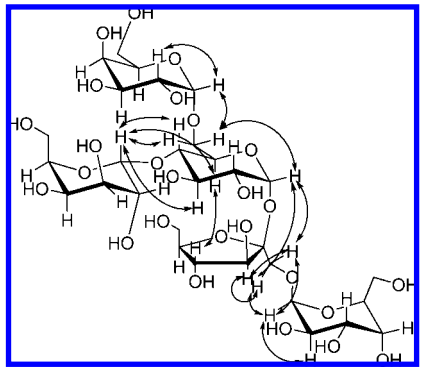

Figure 1. NOE connectivities. Trivial NOEs between vicinal 1-H pairs are not included.

Table 3. $^3J_{H,H}$ Coupling Constants in Stellariose

residue	1a, 1b	1, 2	2, 3	3, 4	4, 5
Gal1		3.84	10.64	3.54	3.52
Glc2		3.78	10.14	9.54	9.87
Fru3	11.52			8.70	8.70
Gal4		3.83	10.45	3.60	3.00
Gal5		3.44	nd	3.39	3.55

NMR signals of the galactose anomeric protons and carbons can be readily recognized on the basis of their typical chemical shifts. One of these anomeric protons is downfield shifted (5.27 ppm), which is indicative for a glycosidic linkage directly on a sugar ring system, while two other anomeric protons have a shift typical for a 1→CH_2 linkage.[34] Starting from H-1, the remaining protons H-2,3,4,5,6 for the galactose residues have been assigned from the COSY spectrum followed by C-2,3,4,5,6 found in the HSQC spectra. The chemical shifts of resonances of those galactose residues are very close, while H-5,6 and C-4,6 are overlapping. Therefore, HSQC-TOCSY and HMBC experiments were necessary to distinguish different residues. On the basis of coupling constants between vicinal protons extracted from the 2D COSY spectrum, the conformation of the two 1→CH_2 linked galactoses has been determined (Table 3). High values of $^3J_{H2,H3}$ (10.64 and 10.45 Hz)[33] suggest the presence of an *axial-axial* system for H-2,3 of both residues. Coupling constants between H-1/H-2, H-3/H-4, and H-4/H-5 are around 3−4 Hz, indicating the presence of *equatorial-axial* systems. This is only possible when galactose residues are in the synclinal orientation to the corresponding protons and there is an α configuration of the glycoside linkage. The conclusions on the conformation of the last galactose residue were derived from the shape of cross-peaks resulting from H-1 and H-2 protons (Figures 2, S4). The Glc-H-1/H-2 cross-peak pattern at 5.28/3.44 ppm displays splittings arising from three-bond scalar coupling between H-1 and H-2 (active coupling) and between H-2 and H-3 (passive coupling). The patterns of two 1→CH_2 linked galactoses H-1/H-2 cross-peaks are similar, but are less resolved because of overlapping. They possess a high passive coupling constant (10.64 and 10.45 Hz) that belongs to the H-2−H-3 scalar coupling and low active one (3.84 and 3.83 Hz, respectively) arising from the H-1−H-2 coupling. The last H-1/H-2 cross-peak pattern at 5.27/3.68 ppm is clearly different. The active coupling (3.44 Hz) can be measured, but the passive coupling constant is too small to be detected, typical for both H-2 and H-3 in an equatorial orientation. These results indicate a flipped chair conformation of the third galactose residue (Figure 2).

The linkages between the five residues were proven by long-range connectivity between anomeric protons and carbons through glycosidic bonds, allowing a sequential assignment of the individual sugars. The three-bond ^{13}C−^{1}H correlations between Gal1−H-1 and Glc2−C-6, Gal5−H-1 and Glc2−C-4, Gcl2−H-1 and Fru3−C-2, and Gal4−H-1 and Fru3−C-1 were observed in the 2D HMBC spectrum (Figures 3, S5) and are in agreement with the observed inter-residue NOEs (Figures 1, S3).

Through analysis of the resulting structure, we have found that the new branched pentasaccharide consists of lychnose with an additional galactose moiety attached to the glucose residue. A comparison of 1D ^{1}H and ^{13}C NMR spectra of these oligosaccharides is shown in Figure S6. From the 1D ^{13}C NMR spectra, it is evident that the downfield shifts of glucose C-4 arise from

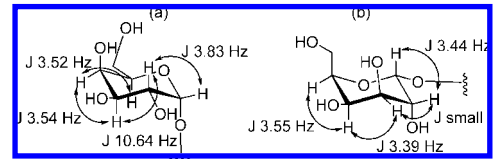

Figure 2. Residues Gal1 (a) and Gal5 (b) with ^3J constants.

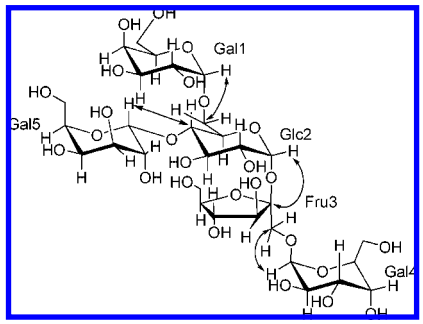

Figure 3. HMBC connectivities. Only inter-residue long-range couplings are shown.

galactosylation of 4-OH, as compared to that of glucose in lychnose. This provides additional evidence in favor of the linkage to C-4 of the glucose. The same effect has been observed for protons of the glucose residue (Figure S6).

Earlier, only six out of eight possible α-galactosyl SOS were described. The structural characterization of stellariose creates the starting point for further unravelling the metabolism of lychnose derivatives in the model plant *S. media*. Further objectives include the purification and characterization of the enzyme(s) involved in the synthesis of the Gal α(1→4)Glc linkage and the isolation and cloning of the cDNA(s). Moreover, the discovery of stellariose will stimulate further research focusing on detecting new SOS and other oligosaccharides in nature.

Besides their importance in plants, both raffinose and stachyose have been reported to possess prebiotic effects.[4] In *Bifidobacterium adolescentis*, higher specific growth rates and cell yields were attained on raffinose, lactose, and FOS (fructosyloligosaccharides) as compared to glucose and fructose.[5] Next to their affinity for α(1→6) linkages, the α-galactosidase of *B. adolescentis* DSM 20083 also hydrolyses α(1→3) and α(1→4) linkages.[6]

Owing to the high number of galactosyl residues (3 out of 5) and the presence of an additional α(1→4) linkage, stellariose might be a promising prebiotic compound as well. Comparable to raffinose, stellariose will probably also resist degradation by gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal absorption. To fulfill all three criteria of a prebiotic, stellariose should also be fermented by the intestinal microflora and selectively stimulate the growth and/or activity of intestinal bacteria associated with health and well-being.[35]

Experimental Section

Mass Analysis. Exact mass measurements were performed on a quadrupole/orthogonal-acceleration time-of-flight (Q/oaTOF) tandem mass spectrometer (qTof 2, Micromass, Manchester, UK) equipped with a standard electrospray ionization (ESI) interface. Samples were infused in an acetonitrile/water (1:1) mixture at 3 μL/min. Leucine enkephalin was mixed in the sample at a concentration of 5 μM and used as internal standard. The calculated mass $[M + Na]^+$ was 851.2645; the found mass was 851.2612 (Figure S1).

NMR Analysis. The pentasaccharide sample was deuterium exchanged by lyophilising three times from 99.6% D_2O and was then dissolved in 0.6 mL of 99.6% D_2O. Spectra were recorded at 22 °C on a Bruker Avance II 600 equipped with a 5 mm TCI HCN Z gradient cryoprobe.

The Bruker Topspin 1.3 software was used to process all spectra. The 2D pulse programs were as follows: (a) phase-sensitive 2D DQF-COSY with gradient pulses for selection was recorded using 16 scans and 256/4096 complex data points in t_1 and t_2, respectively and 1798 Hz spectral widths in both dimension; (b) phase-sensitive NOESY using a mixing time of 500 ms with exactly the same spectral widths and number of scans as for COSY; (c) 2D gradient-enhanced HSQC[27] using trim pulses and the delay τ_1 1.72 ($1/4J_{CH}$) ms, τ_2 3.34 ms ($1/2J_{CH}$) in the inept transfer was recorded using 16 scans and 512/1024 complex data points and 9057/1836 Hz spectral widths in t_1 and t_2, respectively; decoupling during the acquisition was achieved by using the garp sequence;[36] (d) HMBC without decoupling during acquisition using gradient pulses for selection with exactly the same spectral widths as HSQC but using 32 scans and 512/4096 complex data points in t_1 and t_2, respectively; a delay of 62.5 ms was used to allow long-range couplings to evolve; (e) HSQC-TOCSY consisted of an HSQC building block followed by a clean MLEV-17 TOCSY transfer step with 60 ms TOCSY mixing time just prior to the refocusing gradient with exactly the same spectral widths and number of points as HSQC but using 32 scans. Sine-bell-shaped gradients were applied along the *z*-axis during the sequences to obtain coherence selection and sensitivity enhancement. Prior to Fourier transformation, a squared sine-bell function was applied in both dimensions of 2D spectra. Coupling constants of nonoverlapping peaks were determined in a 1D proton spectrum; others were extracted from the phase-sensitive 2D DQF-COSY experiment. NMR results are given in Tables 1−3.

Isolation of Stellariose. For pentasaccharide isolation, fresh cold-induced stem material from *S. media* (200 g) was homogenized for 1 min in a blender with 200 mL of ice-cold NaN_3 (0.02% w/v). The homogenate was immediately boiled in a water bath for 30 min. After cooling, centrifugation (5 min at 40695*g*), and mixed-bed ion exchange chromatography, the neutral fraction was analyzed by HPAEC-PAD.[37] In contrast to sucrose and raffinose, the pentasaccharide is not degraded by invertase. Therefore, 500 μL of 1 μM heterologous cell wall invertase AtcwINV1[38] was added to the carbohydrate syrup. After 24 h at 37 °C, the enzyme incubation was stopped by boiling the sample for 5 min.

The concentrated mixture (Speedvac) was further fractionated on a strongly acidic cation exchange column as described.[39] The fractions containing lychnose were collected between 110 and 130 min and were evaporated (Speedvac) to a 5 mL volume.

Subsequently, preparative HPAEC-PAD was performed on these fractions. Therefore, 200 μL was repeatedly injected on a preparative Carbo Pac PA-100 column (22 × 250 mm, Dionex, Sunnyvale, CA) pre-equilibrated with 90 mM NaOH. After injection, the column was isocratically eluted with 15 mM NaOAc in 90 mM NaOH during 45 min. The flow was 5 mL min^{-1}. Between 42 and 48 min, the pentasaccharide eluted and fractions were manually collected. To remove NaOH and NaOAc, the neutralized fractions were purified on an active charcoal column. The column was washed with H_2O, and finally the pentasaccharide was eluted with 40 mL of 25% (v/v) EtOH. The fractions with the highest concentration of stellariose were lyophilised prior to NMR analysis.

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Supporting Information Available: Figure S1: HRMS data of stellariose. Figure S2: 2D HSQC-TOCSY spectrum of stellariose. Figure S3. Parts of NOESY spectrum of stellariose. Figure S4. Part of 2D DQF-COSY spectrum. Figure S5: Part of HMBC spectrum of stellariose. Inter-residue long-range couplings are shown. Figure S6.1: Comparison of ^{13}C NMR spectra of lychnose (a) and stellariose (b). Figure S6.2: Comparison of ^{1}H NMR spectra of lychnose (a) and stellariose (b). This material is available free of charge via the Internet at http://pubs.acs.org.

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