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Note

Complete NMR characterization of lychnose from *Stellaria media* (L.) Vill

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Abstract—Lychnose (α-D-Gal-(1→6)-α-D-Glc-(1→2)-β-D-Fru-(1→1)-α-D-Gal) was isolated from *Stellaria media*, a representative member of the Caryophyllaceae plant family. Weak acid hydrolysis, enzymatic hydrolysis and complete NMR characterization were performed to confirm the identity of the tetrasaccharide. All ^{1}H and ^{13}C resonances were unambiguously assigned and the conformation of the sugars was determined using one and two dimensional NMR techniques. Anomeric characterizations in lychnose were confirmed from HMBC and NOESY spectra.

Keywords: Lychnose; Caryophyllaceae; Hydrolysis; 2D NMR

Oligosaccharides from the raffinose family (RFOs) are the most widespread galactosyl-sucrose oligosaccharides in the plant kingdom. Raffinose, the smallest member of the family, is a trisaccharide formed by a galactosyl residue bound to sucrose at C-6 of the glucosyl moiety by an α-(1→6) linkage (Chart 1A). Further elongation is possible by additional transfer of galactosyl residues to the galactosyl moiety of raffinose by α-(1→6) linkages.[1] The tetrasaccharide stachyose (Chart 1B) and pentasaccharide verbascose are the next higher galactosyl-oligosaccharides in this series. Of the RFOs, raffinose and stachyose are nearly ubiquitous in higher plants.[2] Their biosynthesis is well established and are both galactinol (*O*-α-D-galactopyranosyl-(1→1)-L-*myo*-inositol)-dependent. Raffinose synthase (EC 2.4.1.82) catalyzes the reversible transfer of galactose units from galactinol to sucrose,[3] while stachyose synthase (EC 2.4.1.67) catalyzes the synthesis of stachyose from raffinose.[4] In contrast, synthesis of the higher DP RFOs 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.[5,6]

RFOs have multiple functions in plants. They play important roles in storage, translocation and utilization of carbon as well as protection against abiotic stress such as that caused by frost, drought and salt.[1,2]

In the Caryophyllaceae family however, two other types of galactosyl-oligosaccharides are found. The tetrasaccharides, lychnose (α-D-Gal-(1→6)-α-D-Glc-(1→2)-β-D-Fru-(1→1)α-D-Gal) and isolychnose (α-D-Gal-(1→6)-α-D-Glc-(1→2)-β-D-Fru-(1→3)α-D-Gal), which are not found in any other plant family, co-exist in all plant parts of the Caryophyllaceae other than seeds.[1] Sucrose, raffinose and lychnose are the most abundant oligosaccharides in Caryophyllaceae, while isolychnose is present in a proportion of only 5–10% as compared to lychnose.[7]

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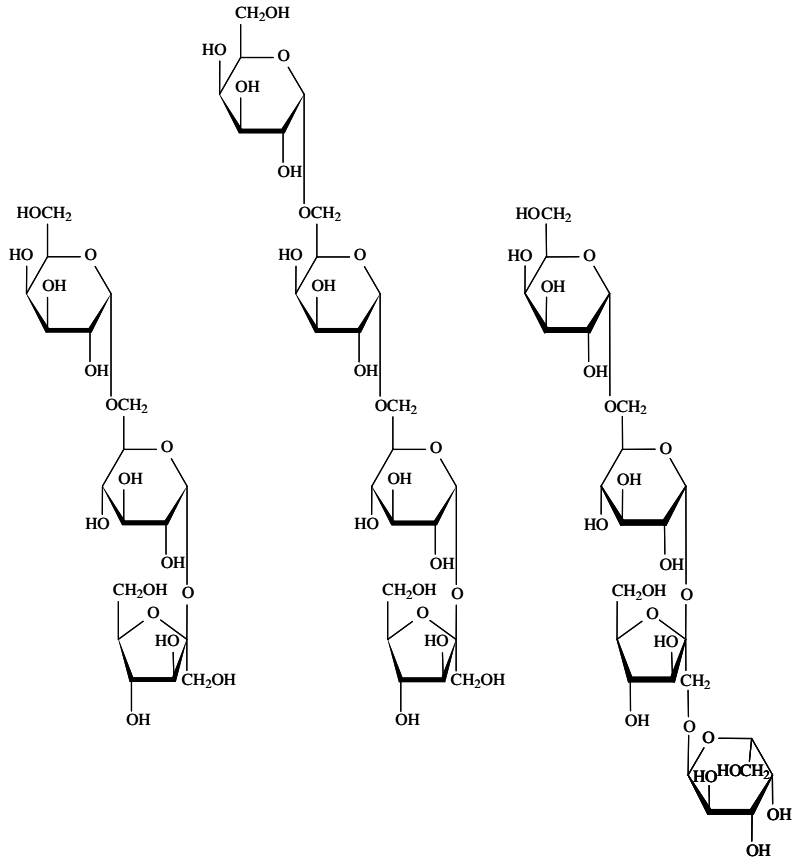

Chart 1. (A) Raffinose, the smallest member of the RFOs. (B) Stachyose, an α-(1,6) galactosyl elongation of raffinose, forming the next RFO. (C) Lychnose has a raffinose backbone, containing an α-(1→1) galactosyl residue linked to its fructosyl moiety.

Little is known about the biosynthetic pathways of both lychnose and isolychnose. It was suggested[7] that raffinose acts both as a donor and acceptor in a reaction catalyzed by the enzyme raffinose → raffinose α-galactosyltransferase (RRT; EC 2.4.1.166) via a galactinol-independent mechanism. Because of the importance of lychnose to the Caryophyllaceae family, further investigation is necessary to elucidate its metabolism. It is not clear whether lychnose should be considered as a reserve carbohydrate and its exact function has still to be elucidated, but there are strong indications that lychnose accumulation is correlated with frost-hardiness.[7]

Lychnose is a water-soluble, non-reducing tetrasaccharide first isolated from the roots of *Silene dioica* (L.) CLAIRV.[8] Its structure (Chart 1C) was elucidated by methods involving methylation and partial hydrolysis.[9] Since lychnose standards are not commercially available and the most recent research dates back to 1984, we decided to unambiguously determine its structure by two dimensional NMR spectroscopy. This is the first report of the complete assignment of the ^{1}H and ^{13}C NMR signals of lychnose.

Lychnose has only been detected in the vegetative parts of the Caryophyllaceae, whereas other plant families within the Caryophyllales only contain the classical RFOs. Therefore lychnose has been depicted as a chemotaxonomic marker of the Caryophyllaceae.[1] To confirm the presence of lychnose in the Caryophyllaceae, a screening of several species belonging to seven different genera was performed. Figure 1 shows that lychnose is present in all species investigated (see arrow), although it does not accumulate to the same extent. Interestingly, raffinose is ubiquitous in all species investigated, while stachyose was virtually absent. Thus, within the Caryophyllaceae, the tetrasaccharide stachyose seems to be replaced by the tetrasaccharide lychnose, a feature that might be used as a reliable chemotaxonomic marker for the family.

Because: (i) high concentrations of lychnose (20 mg g^{-1} FW in Fig. 1) can be found in *Stellaria media* (L) VILL; (ii) the plant can be easily found in the open fields the whole year round or can be easily cultivated within a few weeks and (iii) lychnose accumulates in the plant during cold periods, *S. media* was chosen as a model for our study on lychnose metabolism. By dissecting stems, leafs and flowers of *S. media*, it became clear that the highest lychnose concentration was found in the stems during the cold period (up to ca. 42 mg g^{-1}

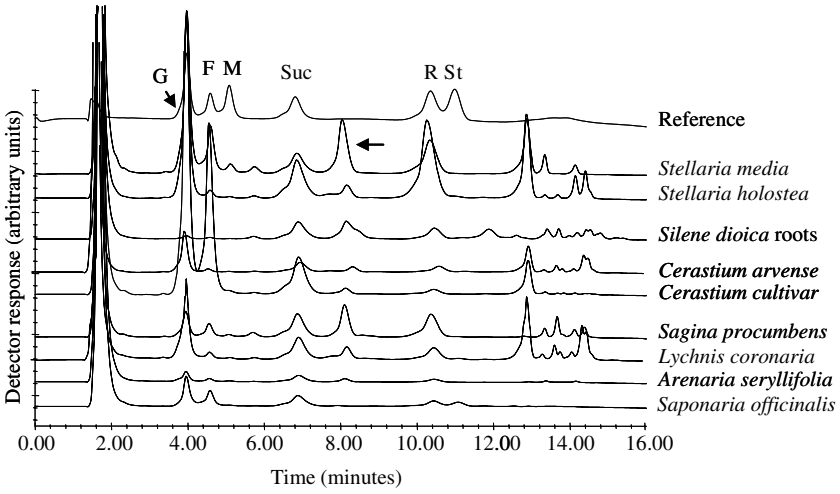

Figure 1. AEC-PAD chromatograms of the carbohydrate composition of different Caryophyllacean species belonging to different genera, as compared with a reference mixture (G = Gal + Glc, F = Fru, M = melibiose, Suc = sucrose, R = raffinose, St = stachyose). The horizontal arrow shows lychnose.

FW). Therefore, subsequent isolation and characterization of this galacto-oligosaccharide was initiated from 200 g of cold-induced stem material.

As a first step, we performed a weak acid hydrolysis (60 mM HCl) of sucrose, raffinose, stachyose and the purified galacto-oligosaccharide (presumably lychnose) from *S. media*. At different time intervals, the reaction mixture was neutralized by adding 10 mM Tris and the depletion of each compound was followed by HPAEC-PAD. All four carbohydrates were rapidly hydrolyzed, but those with a terminal fructosyl moiety (sucrose, raffinose and stachyose) were degraded at much higher rates (data not shown) than lychnose (Fig. 2), which contains an internal fructosyl moiety (Chart 1C).

Theoretically, lychnose degradation in weak acid should result in the formation of melibiose and the disaccharide α-D-galactopyranosyl-(1→1)-β-D-fructofuranoside. The latter disaccharide is not commercially available, so its retention time on HPAEC-PAD is unknown. The increase in melibiose after 30 min hydrolysis follows the increase in another disaccharide (see arrow in Fig. 2), presumably α-D-galactopyranosyl-(1→1)-β-D-fructofuranoside.

Additional enzymatic hydrolyses (with invertase, α-galactosidase and α-glucosidase) on all four carbohydrates were performed to further confirm the lychnose structure. Invertase showed a high affinity for sucrose, raffinose and stachyose, but lychnose degradation was minimal indicating that lychnose contains no terminal fructose (data not shown). From these data, it can be inferred that sensitivity to weak acid hydrolysis indicates the presence of a fructosyl moiety in the oligosaccharide,

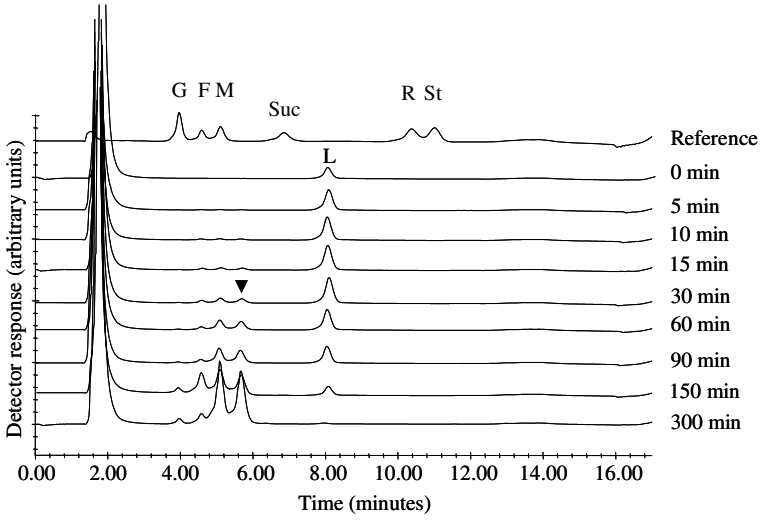

Figure 2. AEC-PAD chromatograms following weak acid hydrolysis of lychnose (L) at 60 °C for various lengths of time. The reference mixture contains Glc + Gal (G), Fru (F), melibiose (M), sucrose (Suc), raffinose (R) and stachyose (St).

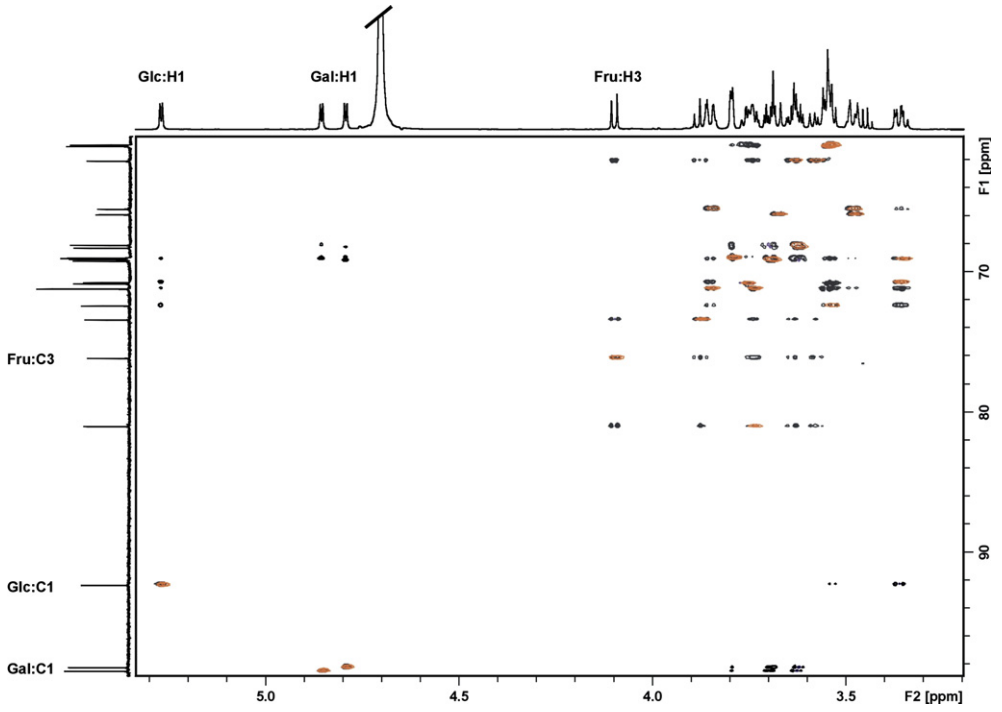

Figure 3. Overlay of the HSQC (red) and HSQC–TOCSY (black) spectra.

while degradation by invertase can be used as an indicator for its terminal position. As expected, α-glucosidase only degraded sucrose, since no terminal glucose residue is present in the RFOs. α-Galactosidase showed a high affinity for raffinose and lychnose but not for sucrose and stachyose.

NMR results: The structure of lychnose was unambiguously determined by NMR spectroscopy. Some of the characteristic signals could be readily assigned in the one-dimensional ^{13}C and ^{1}H spectra, but two-dimensional (2D) techniques were required to resolve signals in crowded regions.[10] The ^{13}C signals of the four anomeric carbon atoms within the polysaccharide can be easily recognized by their typical chemical shift. Starting from these, assignment in the six membered ring systems of galactose and glucose was possible using 2D HSQC–TOCSY and 2D HSQC spectra. While in a HSQC spectrum there are only crosspeaks between geminal carbon atoms and protons, the extra TOCSY transfer in HSQC–TOCSY allows to correlate anomeric atoms with their respective atoms in the sugar moiety. An overlay of both spectra is shown in Figure 3. The well defined signal for H-3 in fructose was used as a starting point to assign most of the signals in the HSQC–TOCSY spectra of the fructose oligosaccharides. The crosspeak of C-2 Fru to H-3 Fru and H-1a/b Fru in the HMBC spectrum allowed to unambiguously assign all fructose signals (Table 1).

The chair conformation of the six-membered ring residues was determined by the measured coupling constants listed in Table 2 and are in agreement with the observed contacts in the NOESY spectrum. The β-anomeric configuration of fructose was derived from the NOE-effect between H-3 Fru and H-1a/b Fru (Fig. 4).

For sequential assignment, crosspeaks in the HMBC spectrum that appear due to long-range coupling of ^{1}H and ^{13}C over the glycosidic bonds were used. Correlations between anomeric protons and trans-glycosidic carbon atoms were observed (Fig. 4). They confirm the monosaccharide sequence and are key parameters to differentiate between the two galactose residues in lychnose (Table 3).

Table 1. Proton chemical shifts for lychnose at 22 °C in D_2O

Residue	^{1}H Chemical shifts (ppm)							
	H-1(a)	H-1b	H-2	H-3	H-4	H-5	H-6a	H-6b
Gal	4.79		3.64	3.69	3.80	3.76	3.54	
Glc	5.27		3.36	3.54	3.35	3.85	3.48	3.84
Fru	3.68	3.49		4.10	3.88	3.74	3.64	3.57
Gal	4.86		3.63	3.70	3.80	3.74	3.55	

Table 2. H/H Coupling constants in lychnose

Residue	H/H Couplings (Hz)				
	$J_{1a,1b}$	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$
Gal		3.80	10.25	3.15	3.40
Glc		3.91	10.92	9.80	10.53
Fru	11.75			8.81	8.81
Gal		3.72	10.30	3.32	3.40

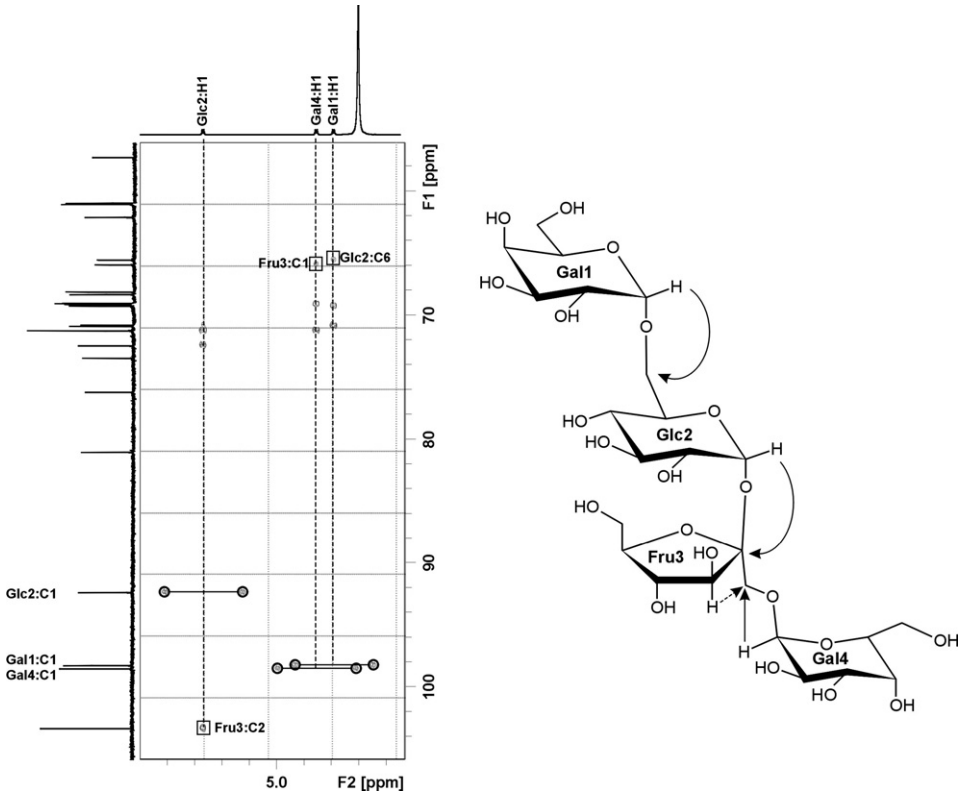

Figure 4. Part of the HMBC spectrum that contains the crosspeaks between anomeric protons and glycosidic carbon atoms in trans-disposition. Solid arrows in the formula drawing correspond to (□) signals in the spectrum. The dashed arrow indicates the observed NOE effect that is characteristic for β-fructose (data not shown).

Table 3. ^{13}C Chemical shifts for lychnose at 22 °C in D_2O

Residue	^{13}C Chemical shifts (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
Gal	98.25	68.31	69.24	69.01	70.86	60.95
Glc	92.37	70.77	72.44	69.14	71.23	65.53
Fru	65.92	103.33	76.19	73.44	81.02	62.1
Gal	98.51	68.1	69.11	69.01	71.23	61.04

1. Experimental

1.1. Plant materials

Plants belonging to seven different genera of the Caryophyllaceae were collected in the Leuven area in March, April and May, 2006. They included *S. media* (L.) VILL; *Stellaria holostea* L.; *Silene dioica* (L.) CLAIRV.; *Cerastium arvense* L.; a *Cerastium cultivar*; *Lychnis coronaria* (L.) DESR.; *Sagina procumbens* L.; *Arenaria seryllifolia* L. and *Saponaria officinalis* L.

1.2. Carbohydrate analysis

After washing, the fresh plant material (4 g) was ground in liquid N_2 and 1 g of the fine powder was further homogenized in a mortar with 2 mL NaN_3 (0.02% w/v). The homogenate was immediately boiled in a water bath for 10 min. After cooling, centrifugation (5 min at 16,100*g*) and mixed-bed ion exchange chromatography,[11] the neutral fraction was analyzed by HPAEC-PAD. For anion exchange chromatography, 25 μL of the carbohydrate solution was injected on a Carbo Pac™ PA-100 column (5 × 250 mm, Dionex, Sunnyvale, USA) previously equilibrated with 90 mM NaOH. Detection was achieved using pulsed amperometric detection (PAD). The flow rate was 1 mL min^{-1} and the temperature was 32 °C. A gradient of 24 min was used for elution. In the first 6 min, the gradient involved 10 mM NaOAc, after which a second gradient of 81 mM NaOAc followed during 8 min, while the NaOH concentration remained constant at 90 mM. After this period, the NaOAc concentration was kept at 500 mM for 5 min.

1.3. Mild acid hydrolysis

Various substrates (sucrose, raffinose, stachyose and lychnose) were subjected to acid hydrolysis. A 200-μL reaction mixture, containing 60 mM HCl and 1 mM of each substrate was heated at 60 °C. The hydrolysis was quenched by adding the hydrolysate (10 μL) to 10 mM Tris (pH 8.5, 90 μL) at different time points. The samples were analyzed by HPAEC-PAD.

1.4. Enzyme incubations

Sucrose, raffinose, stachyose and lychnose were incubated with several enzymes including 100 U cell-wall invertase (from *Arabidopsis thaliana* heterologously expressed in *Pichia pastoris*—in 300 μL 50 mM NaOAc buffer, pH 5); 60 U α-galactosidase (from green coffee beans—in 300 μL 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.5, Sigma) and 58 U α-glucosidase (bakers yeast type I—in 300 μL 25 mM MES buffer, pH 6.3, Sigma). The reaction mixture contained 10 μL enzyme and 3 μL 100 mM substrate in a total volume of 300 μL buffer and was kept at 30 °C. After several periods of time, the reaction was stopped by boiling for 5 min.

1.5. Isolation of lychnose

For lychnose isolation, the fresh cold-induced stem material from *S. media* (L.) VILL (200 g) was homogenized for 1 min in a blender with 200 mL of ice-cold NaN_3 (0.02% w/v). A neutral fraction was obtained by mixed-bed ion chromatography as described above. In contrast with sucrose and raffinose, lychnose is not degraded by invertase. Therefore, 500 μL of 1 μM heterologous cell-wall invertase (AtCWINV 1[12]) was added to the carbohydrate syrup. After 24 h at 37 °C, the enzyme incubation was stopped by boiling for 5 min.

After concentration (Speedvac), the mixture was further fractionated on a strongly acidic cation exchange column as described.[13] The fractions containing lychnose were collected between 120 and 140 min and were evaporated (Speedvac) to a 5-mL vol.

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, USA) 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 30 and 35 min, lychnose eluted and fractions were manually collected. Because of the high alkaline conditions of the solution, sufficient 1 M HCl was added to neutralize these fractions. To remove NaOH and NaOAc, the neutralized fractions were purified on an active charcoal column. The column was washed with water and finally lychnose was eluted with 40 mL of 25% (v/v) EtOH. The fractions with the highest concentration in lychnose were lyophilized prior to NMR analysis. Lychnose (18 mg) was dissolved in 0.750 mL D_2O to obtain the NMR sample.

1.6. NMR analysis

The pulse sequence for the 2D gradient enhanced ^{1}H-^{13}C HSQC was as described by Sattler et al.[14] The delays $\Delta 1$ and $\Delta 2$ in the inept transfer were set to 1.67 ms ($1/4J_{CH}$). The 2D HSQC–TOCSY consisted of an HSQC building block[15] ($\Delta 1 = \Delta 2 = 1.67$ ms) followed by a clean MLEV17 TOCSY transfer step[16] during a mixing time of 60 ms prior to detection. Decoupling during the acquisition of the HSQC and HSQC–TOCSY spectra was achieved by using the garp sequence.[17] Both spectra were recorded using two scans and 512/1024 complex data points and 9055/1836 Hz spectral widths in t_1 and t_2, respectively. The HMBC[18] spectrum was recorded with exactly the same spectral widths but using eight scans and 512/2048 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. 2D DQF-COSY[19] and NOESY[20] spectra were measured with a spectral width of 1836 Hz in both dimensions using 2 scans and 256/2048 complex data points in t_1 and t_2, respectively. A mixing time of 300 ms was used in the NOESY spectrum.

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 non-overlapping peaks were determined in a 1D proton spectrum, others were extracted from the DQFCOSY experiment. All 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 our spectra.

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