Structural Features of Fructans from the Root of *Cyathula officinalis* Kuan†

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Three fructans (CoPS1, CoPS2 and CoPS3) were isolated from the root of *Cyathula officinalis* Kuan, a traditional Chinese medicine. The structures of the fructans were determined by methylation, reductive-cleavage method combined with GC-MS analysis, and ^{13}C NMR spectroscopy. These results show that the fructans (CoPS1, CoPS2 and CoPS3) are graminan type fructans, and comprised of (2→1)- and (2→6)-linked β-*D*-fructofuranosyl backbone residues containing high branches.

Keywords fructans, reductive-cleavage, *Cyathula officinalis* Kuan, graminan

Introduction

Fructans exist as a wide range of oligo- and poly-saccharides in many species of bacteria, fungi, and plants.[1] They are classified into different families on the basis of their glycosides. Fructans with predominately (2→1)-linked β-*D*-fructofuranosyl residues are named inulin, with predominately (2→6)-linked β-*D*-fructofuranosyl residues are named levan, and containing both (2→1)-and (2→6)-linked β-*D*-fructofuranosyl residues are named graminan.[2,3]

Cyathula officinalis Kuan is a famous traditional Chinese herbal medicine, which has functions of "promoting blood circulation to remove blood stasis and dredging meridian, *etc*".[4] Chen and Liu[5] reported that a crude polysaccharide component isolated from *Cyathula officinalis* Kuan has antitumor activity, but there were no reports about the structure of the polysaccharide. In this paper, we reported the structures of the fructans isolated from the root of *Cyathula officinalis* Kuan for the first time.

Experimental

Materials

Cyathula officinalis Kuan was the product of Sichuan Province, China. Sephadex G-50 and CM-Sephadex C-50 were purchased from Ammersham Pharmacia Biotech. T-dextran series of different standard molecular weights were purchased from Fluka.

General experimental procedures

High performance liquid-chromatography (HPLC) was performed on Shimadzu LC-10AD equipped with TSK-G2000SW exclusion column and water as eluant (1.0 mL/min), the eluate was monitored by RI detector. Capillary electrophoresis (CE) was performed on Water Quanta 4000 E using 0.1 mol/L boric acid-KOH buffer (pH = 10) as solvent, detected at 254 nm. Optical rotations were determined on a Perklin-Elmer 241Mc digital polarization. The infrared spectrum (IR) was recorded on a Bio-Rad FTS 185 spectrometer. GC-MS was conducted with a Shimadzu QP 5000. The temperature program was 140→220 ℃ at the rate of 5 ℃/min. For quantitative analysis, a HP 6890 and an OV-17 capillary column (0.25 mm × 30 m) were used with the above temperature program and N_2 as the carrier gas. Mass spectra were acquired by scanning from m/z 40 to 400. NMR spectrum was obtained on a Bruker-DPX-400 spectrometer equipped with a dual probe, in the FT mode at 50 ℃.

Isolation of the fructans

The root of *Cyathula officinalis* Kuan (100 g) was sliced into sheets, and the carbohydrates were extracted with water. The extract was filtered and concentrated to 100 mL. Acetone (80 mL) was added, and the solution was centrifuged at 4500 r/min. The precipitate was dissolved in water, centrifuged, dialyzed (1000 MW cutoff) against water, and freeze-dried. The crude polysaccharide (1.3 g) was named CoPS1. Acetone (40 mL) was again added to the solution, followed by centrifugation, the precipitate was dissolved in water, centrifuged, dialyzed (1000 MW cutoff) against water, and freeze-dried. The

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crude polysaccharide (10 g) was named CoPS2. Then acetone (150 mL) was again added to the solution, and it was centrifuged. The precipitate was dissolved in water, centrifuged, dialyzed (500 MW cutoff) against water, and freeze-dried. The crude polysaccharide (4 g) was named CoPS3.

Purification of fructans

Fructans (CoPS1, CoPS2 and CoPS3) were purified on CM-Sephadex C-50 column (2 cm × 60 cm) eluted with 0.1 N NaCl at a flow rate of 0.5 mL/min, monitored by phenol-H_2SO_4 assay at 490 nm. The eluate was dialyzed and freeze-dried, then was further purified using Sephadex G-50. The homogeneity of fructans (CoPS1, CoPS2 and CoPS3) were detected by HPLC and CE.

Determination of monosaccharide composition

Fructans (CoPS1, CoPS2 and CoPS3) were hydrolyzed with 0.05 mol/L H_2SO_4 at 50 ℃ for 1 h, neutralized with $BaCO_3$, then centrifuged, filtrated, and concentrated. HPLC was carried out on a Carbohydrate Analysis Column (Waters, 3.9 mm I. D. × 30 cm) with $V(MeCN)/V(H_2O)$ (82∶18) as eluent at a flow rate of 1.0 mL/min.

Methylation

Fructans (CoPS1, CoPS2 and CoPS3) were methylated using the Hakomori method.[6] Dimsyl carbanion was generated by adding hexane-extracted NaH (25 mg) to Me_2SO (0.5 mL, vacuum distilled at 70 ℃ and stored over CaH_2 under nitrogen). After warming the Me_2SO mixture for 30 min at 40 ℃, the carbohydrate (10 mg) was added and allowed to react for 3 h at 25 ℃. Methyl iodide (0.8 mL) was added at 0 ℃ during 20 min and allowed to react overnight at room temperature. The reaction mixture was extracted three times with chloroform. The combined chloroform layers were washed by water. The chloroform layer was evaporated. IR spectroscopy was used to test for completeness of methylation.

Reductive cleavage

The methylated polysaccharide was subjected to reductive cleavage as described by Rolf and Gray.[7] The reducing agent was prepared using the following recipe: boron trifluoride etherate (310 μL), triethylsilane (400 μL), trifluoroacetic acid (64 μL) and dichloromethane (260 μL). The reducing agent (500 μL) mixture was added to the methylated product (1 mg) and was allowed to react for 24 h at 0 ℃. Acetic anhydride (100 μL) was added and the temperature was raised to 40 ℃ for 2 h.[8] The acetylated-methylated products were extracted against water with CH_2Cl_2. The organic layer was washed by water three times, and dried down under nitrogen. Dichloromethane was added and the product was analyzed by GC-MS.

Results and discussion

The fructans (CoPS1, CoPS2 and CoPS3) were isolated from the root of *Cyathula officinalis* Kuan, purified on a CM-Sephadex C-50 and Sephadex G-50 column. HPLC and CE were used to examine the chemical homogeneity of CoPS1, CoPS2 and CoPS3. Elemental analysis showed no nitrogen, phosphorus and sulfur. The average molecular weight was calculated to be 5200 (CoPS1), 3000 (CoPS2), and 1400 (CoPS3), determined by Sephadex G-50 column, using T-dextran as standard. And the molecular weight of CoPS3 was also determined by ESI-MS (Fig. 1). The optical rotation was $[\alpha]_D^{25} - 43.20$

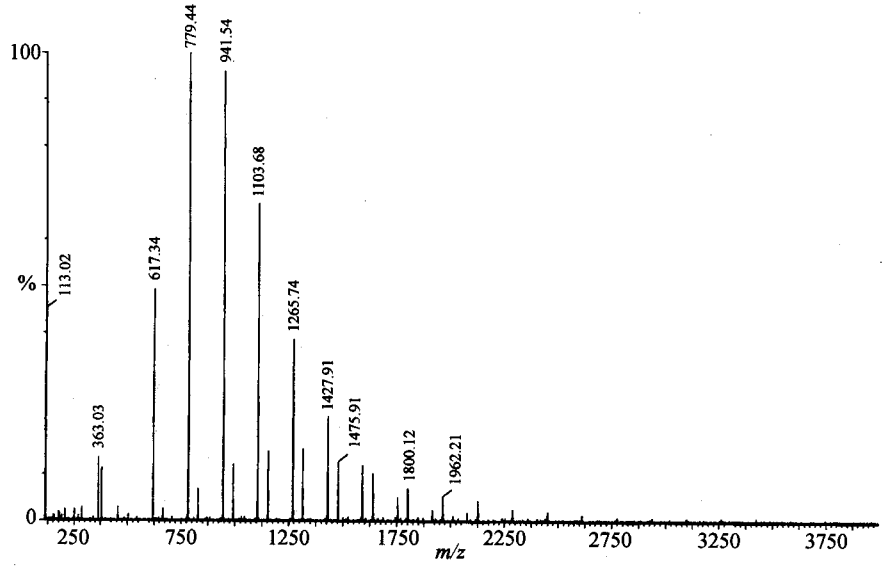

Fig. 1 ESI-MS of CoPS3.

(*c* 0.5, H_2O, for CoPS1), $[\alpha]_D^{25}$ -35.43 (*c* 0.5, H_2O, for CoPS2) and $[\alpha]_D^{25}$ -28.57 (*c* 0.5, H_2O, for CoPS3). The HPLC of the acid hydrolysates of the fructans reveal that the components are fructose and glucose (Fig. 2, Fig. 3 and Fig. 4), and their properties are shown in Table 1.

Table 1 Some properties of CoPS1, CoPS2 and CoPS3

	CoPS1	CoPS2	CoPS3
$[\alpha]_D^{25}$ (*c* 0.5, H_2O)	-43.20	-35.43	-28.57
M_w	5200	3000	1400
Fructose[a]	39	36	21
Glucose[a]	1	1	1

[a] mol%.

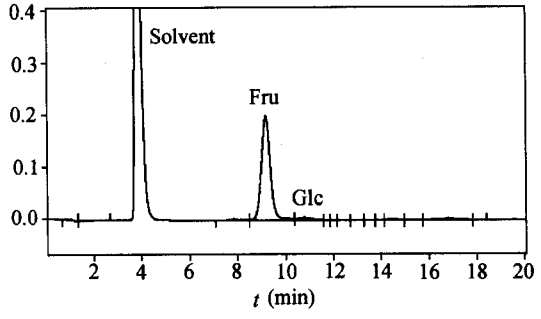

Fig. 2 HPLC of the acid hydrolysates of CoPS1. Column: carbohydrate analisis column (Waters, 3.9 mm I.D. × 30 cm). Eluent: $V(CH_3CN)/V(H_2O)$ (82:18). Flow rate: 1 mL/min.

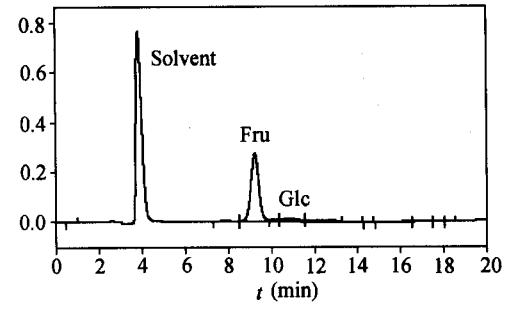

Fig. 3 HPLC of the acid hydrolysates of CoPS2. Column: carbohydrate analisis column (Waters, 3.9 mm I.D. × 30 cm). Eluent: $V(CH_3CN)/V(H_2O)$ (82:18). Flow rate: 1 mL/min.

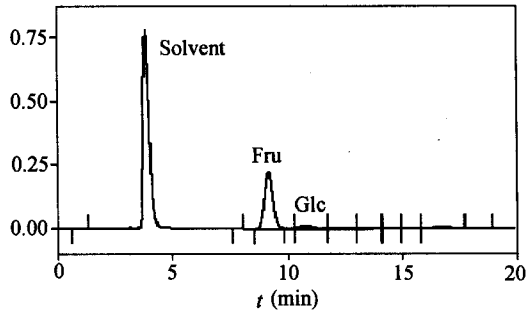

Fig. 4 HPLC of the acid hydrolysates of CoPS3. Column: carbohydrate analisis column (Waters, 3.9 mm I.D. × 30 cm). Eluent: $V(CH_3CN)/V(H_2O)$ (82:18). Flow rate: 1 mL/min.

^{13}C NMR spectra of various fructans are highly characteristic.[9-12] All resonances observed could be assigned to the carbons of the fructans. The structural features (inulin, levan and graminan) can be classified from the chemical shifts and the relative intensity of the ^{13}C NMR signals.

The resonances observed in the proton-decoupled ^{13}C NMR spectra of CoPS1 are assigned in Table 2, CoPS2 in Table 3 and CoPS3 in Table 4. The overlapping pattern of the ^{13}C NMR spectra of CoPS1, CoPS2 and CoPS3 reveal that the fructans are branched, containing both 2→1 and 2→6 linkages, since linear inulins or levans show much simpler ^{13}C NMR spectra.[13-17] The multiplet signals at δ 105—106 are assigned to C-2 of *β-D*-fructofuranosyl residues with different substitution.[12] The signals about δ 82 and 83 are typical of C-5 of the *β-D*-fructofuranosyl residues, and the former is from the *β-D*-fructofuranosyl residues carrying substituents at O-6, the latter is from others.[12] The ^{13}C NMR specta of CoPS1, CoPS2 and CoPS3 (Fig. 5, Fig. 6 and Fig. 7) are very similar to that of the fructan from *Achyranthes bidentata Blume*.[18] It is very infrequent that the structures of the fructans isolated from *Cyathula officinalis* Kuan and *Achyranthes bidentata Blume* (they are in the different genus) are so similar.

In the HMBC of CoPS1, The C-2 signal of (2→1)-linked *β-D*-Fru*f* residues showed cross peaks with the H-6 signal of (2→6)-linked β-D-Fruf residues. Therefore, it can be concluded that the C-2 atom of the (2→1)-linked *β-D*-Fru*f* residues are 2→6 linked to the (2→6)-linked *β-D*-Fru*f* residues.

Table 2 Chemical shift assignments (δ) for the ^{13}C NMR spectrum of CoPS1

	→6)-Fru*f*-(2→	→1)-Fru*f*-(2→	Fru*f*-(2→	→1,6)-Fru*f*-(2→	*α-D*-Glc*p*
C-1	62.35[a]	62.70[a]	62.35[a]	62.35[a]	94.83
C-2	106.23, 106.05	105.30, 105.18	105.58, 105.40	105.87, 105.76	73.28
C-3	78.68[a]	79.40, 79.23	78.99[a]	78.81[a]	74.85
C-4	77.58, 77.40	76.62, 76.53	76.90[a]	77.14[a]	71.41
C-5	82.32[a]	83.25[a]	83.25[a]	82.32[a]	74.57
C-6	65.41, 65.29[a]	64.63, 64.52	64.41, 64.30	65.41, 65.29[a]	61.92

[a] Unresolved from other signals.

Table 3 Chemical shift assignments (δ) for the ^{13}C NMR spectrum of CoPS2

	→6)-Fru*f*-(2→	→1)-Fru*f*-(2→	Fru*f*-(2→	→1,6)-Fru*f*-(2→	*α-D*-Glc*p*
C-1	62.70[a]	63.46, 63.40	63.12, 63.06[a]	63.12, 63.06[a]	95.15
C-2	106.83, 106.74	105.98, 105.85	106.32, 106.15	106.63, 106.46, 106.44	74.04
C-3	79.00[a]	79.89, 79.68	79.39[a]	79.18, 79.08	75.28
C-4	78.13, 78.05	77.34, 77.28	77.56, 77.59	77.74, 77.65	72.14
C-5	82.93[a]	83.79[a]	83.79[a]	82.93[a]	75.15
C-6	65.99, 65.87[a]	65.15, 65.05[a]	64.95, 64.84	65.99, 65.87[a]	62.70[a]

[a] Unresolved from other signals.

Table 4 Chemical shift assignments (δ) for the ^{13}C NMR spectrum of CoPS3

	→6)-Fru*f*-(2→	→1)-Fru*f*-(2→	Fru*f*-(2→	→1,6)-Fru*f*-(2→	*α-D*-Glc*p*
C-1	62.62, 62.98	63.90, 63.44	63.05[a]	63.05[a]	95.13
C-2	106.82, 106.73	105.96, 105.82	106.31, 106.13	106.62, 106.42	73.74
C-3	78.91, 78.63	79.80, 79.61	79.32[a]	79.10, 79.00	75.24
C-4	77.99, 77.81	76.99, 76.88	77.28[a]	77.22, 77.52	71.84
C-5	82.92[a]	83.77[a]	83.77[a]	82.92[a]	75.05
C-6	65.96[a]	65.12[a]	65.02, 64.90	65.96[a]	62.20

[a] Unresolved from other signals.

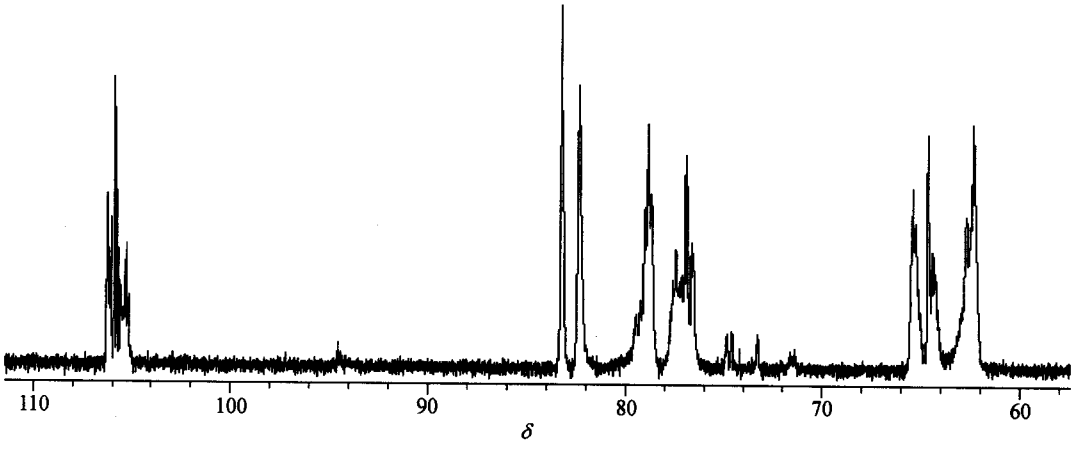

Fig. 5 ^{13}C NMR spectra of CoPS1.

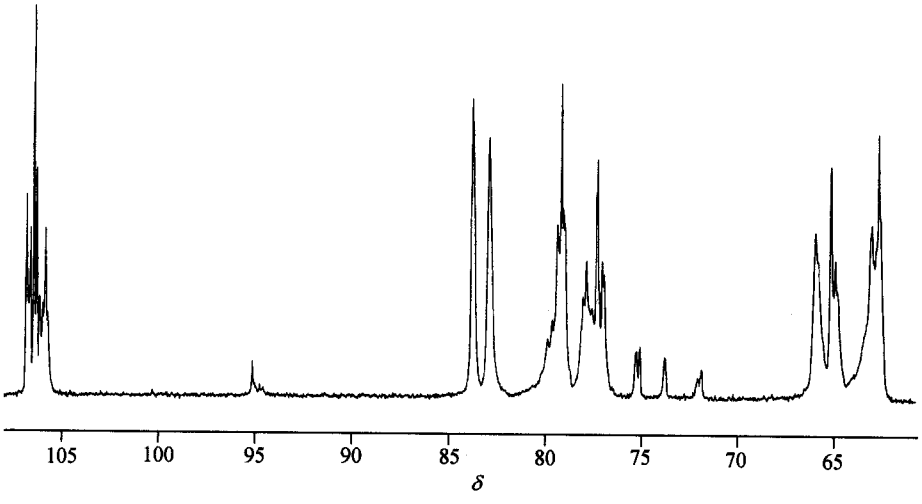

Fig. 6 ^{13}C NMR spectra of CoPS2.

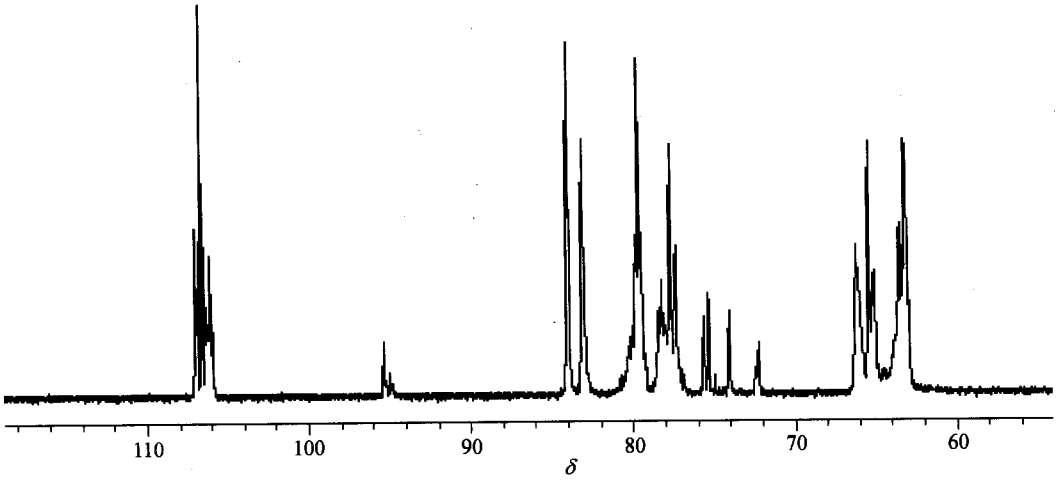

Fig. 7 ^{13}C NMR spectra of CoPS3.

In the HMBC of CoPS2, the cross peak was found showing a correlation between the C-2 signal of the β-*D*-Fru*f* residue with the H-1 signal of the α-*D*-Glc*p* residue, and there were no other cross peaks between the signal of the β-*D*-Fru*f* residue with the signal of the α-*D*-Glc*p* residue. The C-2 signal of nonreducing terminal β-*D*-Fru*f* residues showed cross peaks with the H-6 signal of (1,2→6)-linked β-*D*-Fru*f* residues. Therefore, it can be concluded that the C-2 atom of the nonreducing terminal β-*D*-Fru*f* residues are 2→6 linked to the (1,2→6)-linked β-*D*-Fru*f* residues.

In the HMBC of CoPS3, the cross peak was found showing a correlation between the C-2 signal of the β-*D*-Fru*f* residue with the H-1 signal of the α-*D*-Glc*p* residue, and there were no other cross peaks between the signal of the β-*D*-Fru*f* residue with the signal of the α-*D*-Glc*p* residue. Therefore, the α-*D*-Glc*p* residue was linked only at the 1 position. The C-2 signal of (2→1)-linked β-*D*-Fru*f* residues showed cross peaks with the H-6 signal of (2→6)-linked β-*D*-Fru*f* residues. Therefore, it can be concluded that the C-2 atom of the (2→1)-linked β-*D*-Fru*f* residues are 2→6 linked to the (2→6)-linked β-*D*-Fru*f* residues.

The results of the methylation analysis, which were performed by using the reductive-cleavage method,[7] confirm the linkages deduced by ^{13}C NMR spectra. GC-MS was used to identify the methylated, reductive-cleavage and acetylated derivatives of CoPS1, CoPS2 and CoPS3. And the integrated peak areas of GC were corrected using the effective-carbon response method.[19] The e.i. fragmentation patterns of the methoxy derivatives were identical to the published data.[8,20-22] The peaks in the chromatogram corresponded to the various methoxy derivatives of non-reducing ends, β-(2→1)-linked residues, β-(2→6)-linked residues, and the branch points. The reductive cleavage method can discriminate between (2→1)- and (2→6)-linked β-*D*-Fru*f* residues. The proportions of the several types of linked β-*D*-Fru*f* and α-*D*-Glc*p* residues are shown in Table 5.

According to Table 5, the fructans (CoPS1, CoPS2 and CoPS3) have a highly branched structure with a (2→1)- and (2→6)-linked backbone. The reductive cleavage gave 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-*D*-glucitol as the only glucose-derived unit; thus, the α-*D*-Glc*p* residues were in the non-reducing terminal positions.

By current definition, fructans are all oligo- or polysaccharides that contain two adjacent fructose units, and one glucose molecule can be present but is not necessary.[2,23,24] F_n means that the terminal glucose molecule is

Table 5 Analysis of the partially *O*-methylated acetates formed from the fructans

	Component separated	CoPS1 (mol)	CoPS2 (mol)	CoPS3 (mol)
Fru*f*-(2→	2,5-anhydro-1,3,4,6-tetra-*O*-methyl-*D*-mannitol	5.0	5.9	5.3
	2,5-anhydro-1,3,4,6-tetra-*O*-methyl-*D*-glucitol			
→1)-Fru*f*-(2→	1-*O*-acetyl-2,5-anhydro-3,4,6-tri-*O*-methyl-*D*-mannitol	11.0	10.6	6.1
	1-*O*-acetyl-2,5-anhydro-3,4,6-tri-*O*-methyl-*D*-glucitol			
→6)-Fru*f*-(2→	6-*O*-acetyl-2,5-anhydro-1,3,4-tri-*O*-methyl-*D*-mannitol	16.5	11.7	6.1
	6-*O*-acetyl-2,5-anhydro-1,3,4-tri-*O*-methyl-*D*-glucitol			
→1,6)-Fru*f*-(2→	1,6-di-*O*-acetyl-2,5-anhydro-3,4-di-*O*-methyl-*D*-mannitol	9.5	6.5	5.6
	1,6-di-*O*-acetyl-2,5-anhydro-3,4-di-*O*-methyl-*D*-glucitol			
α-*D*-Glc*p*	1,5-anhydro-2,3,4,6-tetra-*O*-methyl-*D*-glucitol	1.0	1.0	1.0

absent in the fructans, and G-F_n is the regular type fructans that contains glucose molecule. The results of molecular weight analysis, monosaccharide composition (glucose is very small), GC-MS and ^{13}C NMR analysis suggest that the fructans (CoPS1, CoPS2 and CoPS3) include the F_n type and G-F_n type fructan chains.

In conclusion, these fructans (CoPS1, CoPS2 and CoPS3) have a highly branched, short-chain bearing both (2→1)-linked *β-D*-fructofuranosyl residues and (2→6)-linked *β-D*-fructofuranosyl residues. And the CoPS1 bears more (2→6)-linked *β-D*-fructofuranosyl residues than (2→1)-linked *β-D*-fructofuranosyl residues.

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