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Full Length Research Paper

Isolation and structure determination of a new oligosaccharide from *Blume riparia*

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A water-soluble oligosaccharide (BROS) was obtained from *Blume riparia* DC. by hot water extraction, ethanol precipitation, and fractionated by Sephadex G-50 and Sephadex G-25 column filtration chromatography. BROS is a homogenous composition. Its molecular weight was 1.314×10^3 Da by MALDI-TOF-MS accurate determination. It was composed of 8 monosaccharides, namely Glc*p*, Fru*p* and Fru*f* in molar ratio of 1:1:6. The linkage type of all residues were determined by the means of methylation. A combination of ^{1}H NMR and ^{13}C NMR spectroscopy analysis, the BROS structure was confirmed finally as: *β*-*D*-Fru*p*-(2→1)-*β*-*D*-Fru*f*-1-(2→1)-*β*-*D*-Fru*f*-2-(2→1)-*β*-*D*-Fru*f*-3-(2→1)-*β*-*D* -Fru*f*-4-(2→1)-*β*-*D*-Fru*f*-5-(2→1)-*β*-*D*-Fru*f*-6-(2→1)-*α*-*D*-Glc*p*.

Key words: *Blumea riparia* (Blume) DC, oligosaccharide (BROS), purification, methylation, spectroscopy, structure.

INTRODUCTION

Blumea riparia (Blume) DC. (Asteraceae) is distributed mainly in Guangxi and Yunnan Provinces of China and is a well-known traditional Chinese herbal medicine (Huang, 1999; Huang et al., 2000; Li, 2006; Wiart, 2006). It has commonly been used as a folk herbal medicine to treat headache, alleviates colic, diuretic, hypertension, gynecological diseases including menorrhagia, puerperal metrorrhagia, peripheral edema, infertility and vulvar ulcer in China (Huang et al., 2000, 2010; Wiart, 2006).

During previous investigations many flavonoids, acetylenes, sesquiterpenes, phenolic acids, xanthenes, polysaccharide and proteins have been isolated from *B. riparia* (Li, 2006; Cao et al., 2007, 2008a, b; Zheng et al., 2007; Huang et al., 2010). The assay method developed for protocatechuic acid isolated from *B. riparia* was proposed to be useful in quality control testing of this herbal drug (Xie et al., 2000). Blumexanthene II of *B. riparia* was reported to be mildly cytotoxic while water soluble oligosaccharide exhibited procoagulant activity (Huang et al., 2009, 2010; Chen et al., 2010). Naturally occurring polysaccharide are important kind of carbohydrates. According to the structures and properties of constituent monosaccharide, polysaccharides show different chemical and biological activities (Jacob, 1985; Sun and Liu, 2008). Polysaccharides could be applied to health foods or medicines, which exhibit strengthening immunological ability, antioxidant, and antitumor effects (Peng et al., 2005; Sun and Liu, 2009; Chen et al., 2010). In addition, polysaccharides also engaged biochemical and nutritional researchers' attention. In continuation of our work on the screening for hemostatic constituents of *B. riparia*, we found a water-soluble oligosaccharide (BROS). The isolation of crude polysaccharides and structural determination of the newly isolated oligosaccharide BROS is presented in the current communication.

MATERIALS AND METHODS

Plant material

The whole herb of *B. riparia* was provided by Guixi Pharmacy Co. Ltd., Guangxi, China.

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Chemicals

Sephadex G-25 and Sephadex G-50 were purchased from Amersham (Sweden). T-series dextrans and standard sugars were purchased from Sigma Chemical Co.(USA). All the other chemicals used were of analytical grade.

General methods

Isolation and purification of the oligosaccharides

The dry branches of *B. riparia* (2 kg) were crushed to a particle size 0.5 to 1 mm in a mill and were extracted with 100% ethanol (3×5000 ml) at 70° for 3 h under atmospheric pressure. A reflux condenser was fixed to remove lipids. The residue left was then extracted with distilled water (10 L) at 90° for 3 times (2 h each time). After centrifugation (2000 g for 15 min, at 20°), the supernatant was concentrated to one-tenth of the volume, and precipitated with 4 volume of 95% ethanol at 4° for 24 h. The precipitate collected by centrifugation was deproteinated by proteninase digestion using Sevag method, followed by exhaustive dialysis with membrane WMCO: Nominal:1000 in flow water for 72 h. Then the concentrated dialyzate was precipitated with 4 volume of 95% EtOH at 4° for 24 h. The precipitate was the crude polysaccharide (40.4 g). The crude polysaccharide was dissolved in distilled water, centrifuged, and then the supernatant was purified by a Sephadex G-50 column (2.7×75 cm), equilibrated with ultrapure water. After loading with sample, the column was eluted with ultrapure water and then with stepwise gradient of NaCl aqueous solutions (0.1, 0.3 and 0.5 M) at a flow rate of 5 ml/min. Different fractions were collected using test tubes. Total carbohydrate content of each tube were measured at 480 to 490 nm by phenol-H_2SO_4 method (Dubois et al., 1956). The water-eluted solution was separated into one single fraction and then purified further on a Sephadex G-25 column (2.7×85 cm) by using ultrapure water (at a flow rate of 3 ml/min). After collecting the purified fraction, it was lyophilized. The yield thus obtained was 1.20 g.

UV-VIS and IR spectra analysis

UV-VIS absorption spectra were recorded with a Shimadzu MPS-2000 spectrophotometer between 190 and 290 nm. BROS (1 mg) was dissolved and diluted to 2 mg/ml. The diluted solutions were scanned from 190 to 290 nm with a MPS-2000 spectrophotometer (Sun et al., 2009). The IR spectrum of TFPSI was recorded with a Nicolet 5700 spectrometer with the range of 400 to 4000 cm^{-1}. The sample was analyzed as KBr pellets (Yang et al., 2008).

Homogeneity and molecular weight

High-performance gel permeation chromatography (HPGPC) was carried out with a Waters 515 instrument fitted with two columns in series (ultrahydrogel 250 and ultrahydrogel 2000 Waters). The eluent was distilled water and monitored with Waters 2410 refractive index detector. MALDI-TOF-MS had been shown to be an effective method for low molecular weight oligosaccharides determination (Bayerbach et al., 2006; Peterson et al., 2003). It was equipped with a SCOUT multiprobe inlet and nitrogen laser (337 nm, 3 ns pulse width with Pulse energy of 200 µJ). Acceleration voltage was 19 Kv and reflection voltage was 15.1 Kv with delayed extraction time of 200 ns.

Analysis of monosaccharide composition

Gas chromatography (GC) technique was used for identification and quantification of monosaccharides. BROS (4 mg) was hydrolyzed with 2 M trifluoroacetic acid TFA (4 ml) at 110° for 4h in a sealed glass tube (Erbing et al. 1995). The hydrolyzed product was converted into alditol acetates as described earlier (Jacob, 1985) and analyzed by GC. GC was performed on a Varian 3400 instrument (Hewlett-Packard Component, USA) equipped with DM-2330 capillary column (30 m × 0.32 mm × 0.2 µm) and flame-ionization detector (FID).

Methylation

The dry BROS (30 mg) was methylated 5 times using the modified Ciucanu method (Ciucanu and Kerek, 1984). The permethylated BROS was depolymerized with 90% formic acid (100°, 4 h), followed by hydrolysis with 2 M TFA (100°, 6 h). The hydrolysate was converted into methylated alditol acetate and analyzed by using GC-MS, Shimadzu QP Class-5000 instrument.

^1H NMR and ^{13}C NMR analysis

^1H and ^{13}C NMR spectra were measured using a Bruker AM-600 NMR instrument equipped with a dual probe in the FT model at 20°. BROS (30 mg) was dissolved in D_2O at a concentration of 30 mg/0.5 ml.

RESULTS AND DISCUSSION

Physicochemical properties and chemical compositions

BROS was obtained from the dry branches of *B. riparia*. The scan spectrum showed that there were no absorption at 260 and 280 nm in the UV spectra, indicating the absence of protein and nucleic acid in BROS. HPGPC showed only one symmetrical peak, indicating a homogenous fraction and the weight-average molecular mass was estimated to be 1.323×10^3 Da by reference to standard dextran. However, the molecular weight was 1.314×10^3 Da by MALDI-TOF-MS detection (Peterson et al., 2003). As was shown in Figure 1, the ion cleavage or breakage $[M-H]^-$ followed the $[M-H-(162)_n+Cl]^-$ cleavage rule. The M/S information of BROS is depicted in Table 1. As shown in Figure 1, the ion cleavage $[M-H]^-$ /z was of m/z 1.313×10^3 Da. It clearly indicated that the molecular weight of BROS was 1.314×10^3 Da (Bayerbach et al., 2006). The result showed the consistency with the result of detection molecular weight by HPGPC. Based on the molecular weight of BROS and following the ion cleavage rule, it was confirmed that BROS contained 8 hexose monosaccharides.

IR spectra of BROS showed that there was no absorption between 1680 and 1740 cm^{-1}, suggesting BROS to be contained no uronic acid component. Moreover, the characteristic absorption at 935.84 cm^{-1} indicated that furan-ring stretching type of vibration was present in BROS. GC analysis showed BROS was composed of three kinds of monosaccharides, namely Fru*p* (fructopyanose abbreviation Fru*p*), Fru*f* (fructofuranose

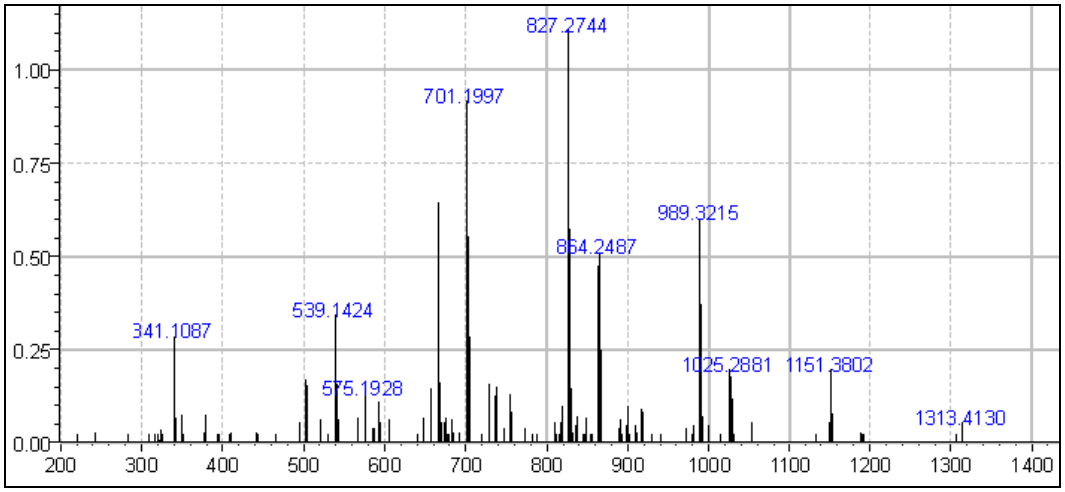

Figure 1. MALDI-TOF MS of BROS.

Table 1. MS information of BROS.

m/z	1313	1186/1188	1151
ion	$[M-H]^-$	$[HO[C_6H_{10}O_5]_7+Cl]^-$	$[HO[C_6H_{10}O_5]_7]^-$
group	M:$[HO[C_6H_{10}O_5]_8H)$	(M-$[C_6H_{10}O_5H]$+Cl)	(M-$[[C_6H_{10}O_5]_2H]$)
m/z	1024/1026	989	862/864
ion	$[HO[C_6H_{10}O_5]_6+Cl]^-$	$[HO[C_6H_{10}O_5]_6]^-$	$[HO[C_6H_{10}O_5]_5+Cl]^-$
group	(M-$[[C_6H_{10}O_5]_2H]$+Cl)	(M-$[[C_6H_{10}O_5]_2H]$)	(M-$[[C_6H_{10}O_5]_3H]$+Cl)
m/z	827	700/702	665
ion	$[HO[C_6H_{10}O_5]_5]^-$	$[HO[C_6H_{10}O_5]_4+Cl]^-$	$[HO[C_6H_{10}O_5]_4]^-$
group	(M-$[[C_6H_{10}O_5]_3H]$)	(M-$[[C_6H_{10}O_5]_4H]$+Cl)	(M-$[[C_6H_{10}O_5]_4H]$)
m/z	610	575	376
ion	$[HO[C_6H_{10}O_5]_3+Cl]^-$	$[HO[C_6H_{10}O_5]_3]^-$	$[HO[C_6H_{10}O_5]_2+Cl]^-$
group	(M-$[[C_6H_{10}O_5]_5H]$+Cl)	(M-$[[C_6H_{10}O_5]_5H]$)	(M-$[[C_6H_{10}O_5]_6H]$+Cl)
m/z	341		
ion	$[HO[C_6H_{10}O_5]_2]^-$		
group	(M-$[[C_6H_{10}O_5]_6H]$)		

abbreviation Fru*f*) and Glc (glucopyranose abbreviation Glc*p*) in molar ratios of 1:6:1.

Methylation product analysis

The dry BROS was methylated 5 times (Figure 2), according to the standard method as described above. The methylated products were extracted by $CHCl_3$, and showed no IR absorption peak between 3600-3300 cm^{-1} suggesting complete methylation. The data and results of methylation analysis are presented in Table 2; GC-MS results designated the backbone chains linkage of all residues (Bayerbach et al., 2006).

NMR analysis

The 1D ^1H spectra of BROS (Figure 3) showed that there was only an anomeric proton signal at δ_H 5.444 ($^2J_{H\text{-}1/H\text{-}2}$=3.6Hz) and no other anomeric proton from δ_H 4.3 to δ_H 5.500. It suggested that the proton signal was attributed to H-1 of α-Glc*p*. The ^{13}C NMR spectra (Figure 4) showed the presence of 8 anomeric carbons. In HMQC spectra of BROS (Figure 5), the proton signal had a cross with the anomeric carbon signal at δ_C 92.413. The δ_C 92.413 was assigned as C-1 of *α*-Glc*p*; while the rest 7 anomeric carbon signals at δ_C 97.728, δ_C 102.918, δ_C 103.021,δ_C 103.068,δ_C 103.110,δ_C 103.138 and δ_C 103.618,for there were no proton signal had a cross with them correlation.

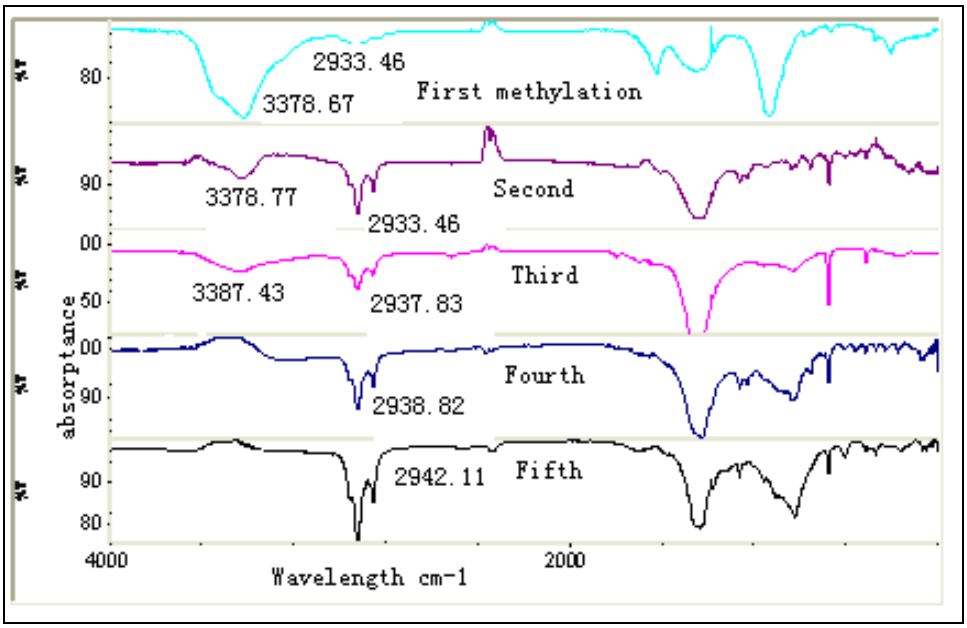

Figure 2. Five IR spectrum comparison of BROS methylation.

Table 2. Partially methylated alditol acetate derivatives from BROS.

Monosaccharide derivatives	Molar ratio	Linkage type
Methyl 1,3,4,5-tetra-O-methyl-*D*-fructopyranoside	1	Fru*p* (2→
Methyl 2,3,4,6-tetra-O-methyl-*D*-glucoside	1	→1)Glc*p*
Methyl 3,4,6-tri-O-methyl-*D*-fructofuranoside	6	→1) Fru*f* (2→

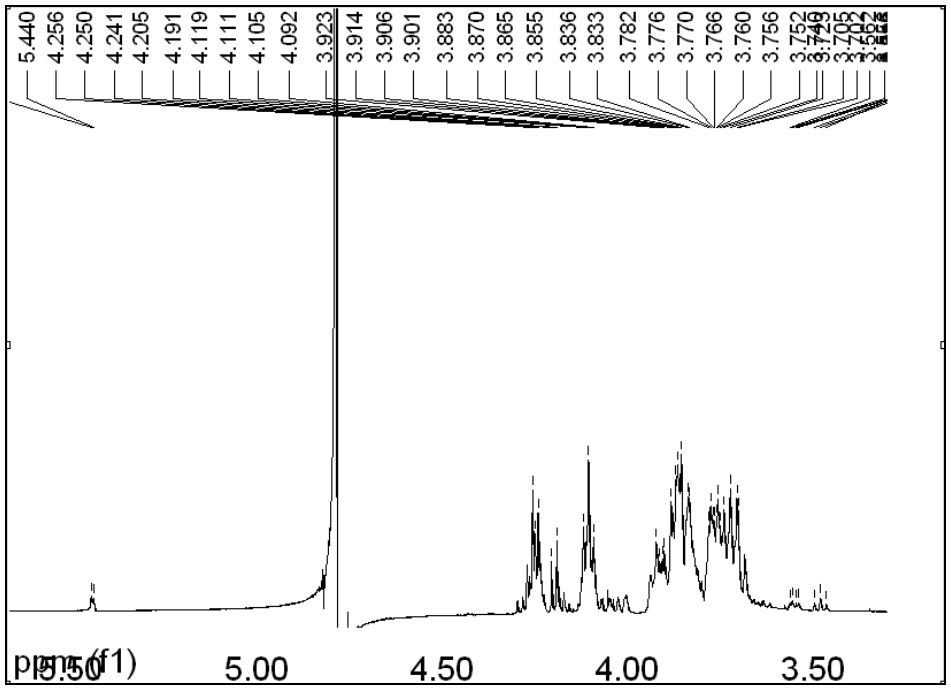

Figure 3. ^{1}H spectra of BROS.

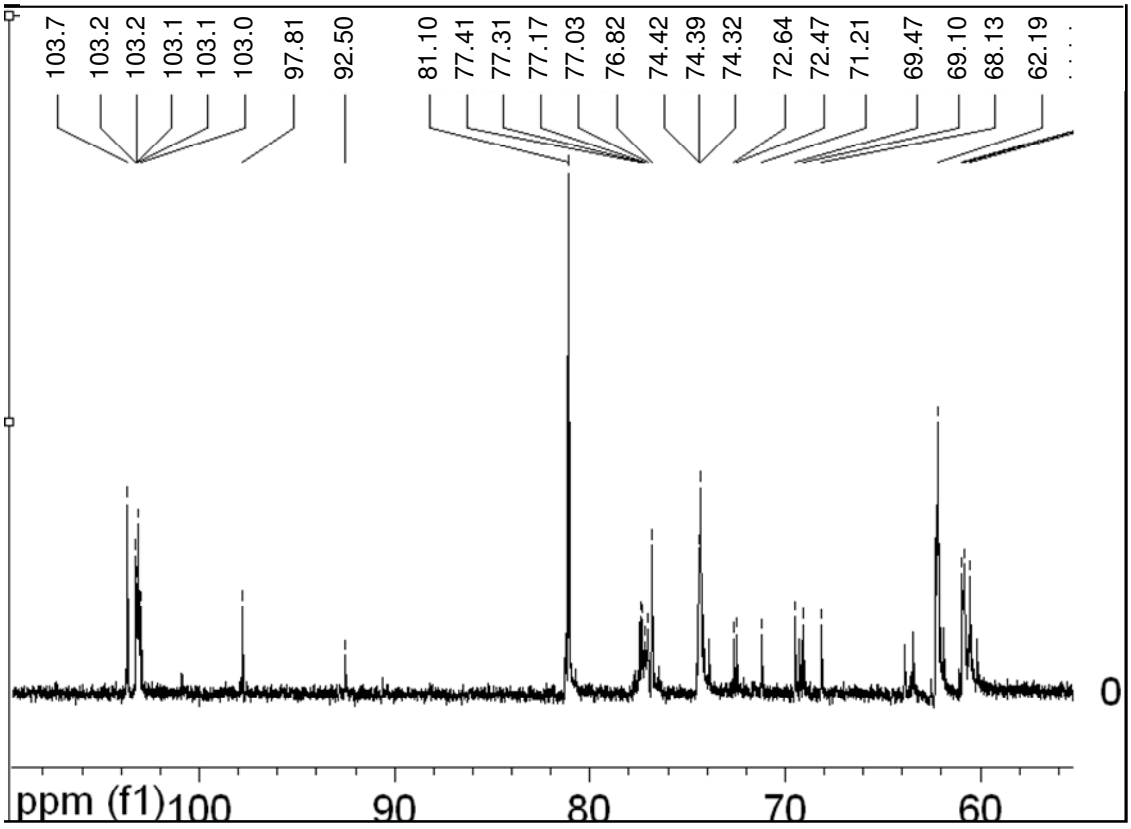

Figure 4. ^{13}C NMR spectra of BROS.

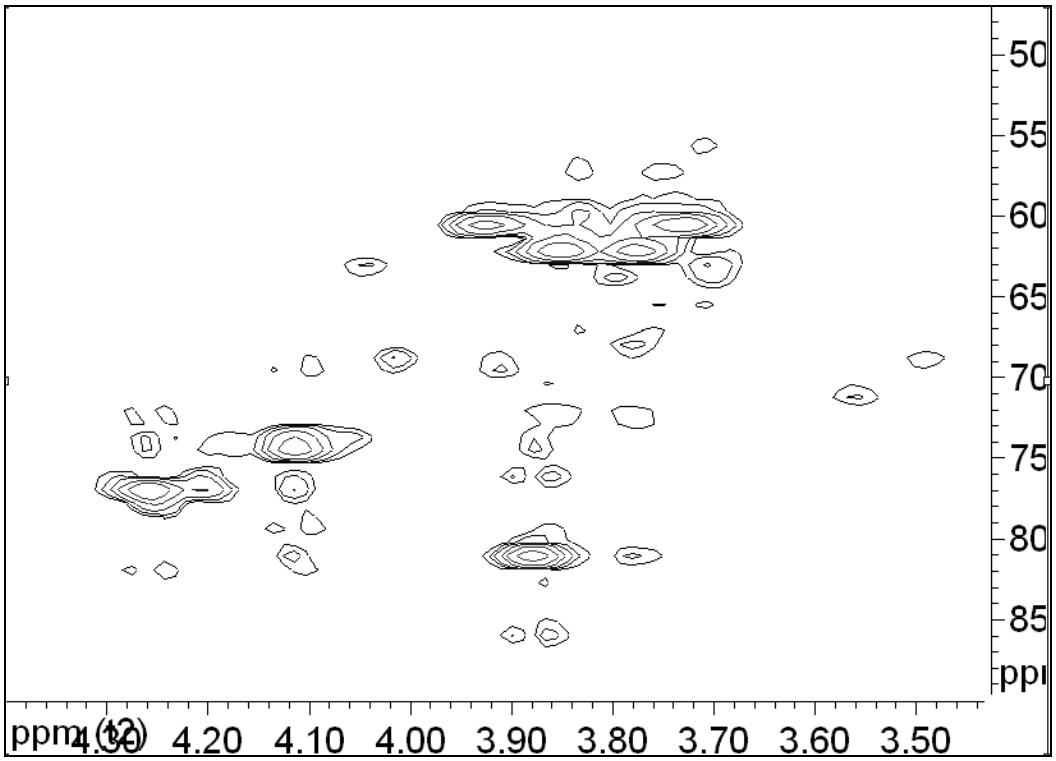

Figure 5. HMQC spectra of BROS.

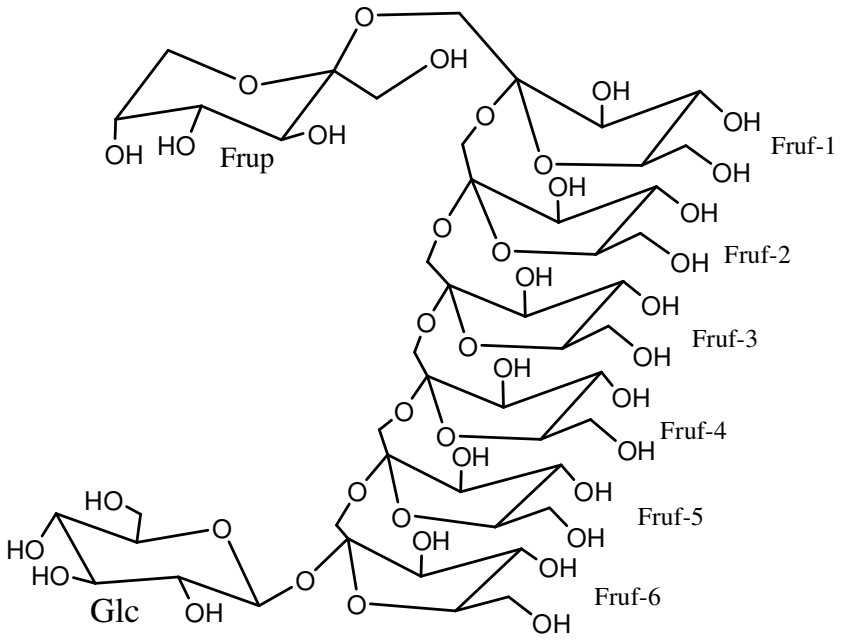

Figure 6. The chemical structure of BROS.

Fru*p* and the rest anomeric carbon signals from δ_C 102.918 to δ_C 103.618 were assigned as C-2 of 6 Fru*f*. The δ_C values of Fru indicated its *β* anomer form, by comparing those of *α* and *β* form of methy-*D*-fructopyranoside (Sinclair 1988; Hard et al., 1991).

Conclusion

According to the above comprehensive analysis, the structure of this oligosaccharide can be demonstrated and was determined to be as follows: *β*-*D*-Fru*p*-(2→1)-*β*-*D*-Fru*f*-1-(2→1)-*β*-*D*-Fru*f*-2-(2→1)-*β*-*D*-Fru*f*-3-(2→1)-*β*-*D* -Fru*f*-4-(2→1)-*β*-*D*-Fru*f*-5-(2→1)-*β*-*D*-Fru*f*-6-(2→1)-*α*-*D*-Glc*p*. The chemical structure of BROS is presented in Figure 6.

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