Carbohydrate Polymers 105 (2014) 152–160

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Structure of a homofructosan from *Saussurea costus* and anti-complementary activity of its sulfated derivatives

Hongwei Fan [a], Fei Liu [a], S.W. Annie Bligh [b,**], Songshan Shi [a], Shunchun Wang [a,*]

[a] The MOE Key Laboratory for Standardization of Chinese Medicines and the SATCM Key Laboratory for New Resources and Quality Evaluation of Chinese Medicines, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China
[b] Department of Complementary Medicine, Faculty of Science and Technology, University of Westminster, London W1W 6UW, UK

ARTICLE INFO

Article history:
Received 12 December 2013
Received in revised form 23 January 2014
Accepted 25 January 2014
Available online 2 February 2014

Keywords:
Polysaccharide
Saussurea costus
Homofructosan
Sulfated derivatives
Anti-complement

ABSTRACT

A homogeneous water-soluble polysaccharide APS-W1, (2 → 1)-β-D-fructofuranosan, with an average molecular weight of 3.9 kDa, was isolated and characterized from the roots of *Saussurea costus*. Five sulfated derivatives of APS-W1 with different degrees of sulfation were prepared and they showed strong inhibitory effect on the complement activation through the classical pathway (CP_{50}: 2.2–18.9 μg/mL; 8.3 μg/mL for heparin) and alternative pathway (AP_{50}: 11.4–115.8 μg/mL; 89.2 μg/mL for heparin). Mechanism studies by using complement-depleted sera indicated that sulfated derivatives with different positions of sulfation targeted to different complement proteins. Meanwhile the sulfated derivatives have limited anticoagulant effect based on re-calcification time and thrombin time. These results suggested that the sulfated derivatives prepared from APS-W1 could be promising potential complement inhibitors for the treatment of diseases caused by an over-activated complement system.

1. Introduction

The serum complement system, as a key system for immune surveillance and homeostasis, which is considered a chief component of innate immunity (Carroll, 2004; Ricklin, Hajishengallis, Yang, & Lambris, 2010), has distinct effect on altered host cells and foreign invaders. The system represents a complex pathway of more than 30 serum proteins and cell surface receptors that interact in a range of functions from direct cell lysis to the enhancement of B and T cell responses (Fearon & Carroll, 2000; Reid & Porter, 1981). It is activated by three different pathways: classical, lectin and alternative. However, any trigger that alters the delicate balance between complement activation and regulation can induce self-attack, and results in various diseases such as system lupus erythematosus (SLE), rheumatoid arthritis (RA), and acute respiratory distress syndrome (ARDS) (Kirschfink, 1997; Zhi, Zhang, Zhang, & Chen, 2008).

Heparin, a sulfated polysaccharide with O-sulfation, has been found to inhibit complement activation (Kazatchkine, Fearon, Metcalfe, Rosenberg, & Austen, 1981; Ormsby et al., 2006; Pangburn, Atkinson, & Meri, 1991). Structural and functional studies have shown that sulfated polysaccharides are potential candidates for screening as inhibitors of an over-activated complement system for therapeutic purpose. Examples of sulfated polysaccharides have good anti-complement activity including natural sulfated polysaccharides, such as fucans which were extracted from brown seaweed (Berteau & Mulloy, 2003; Blondin, Chaubet, Nardella, Sinquin, & Jozefonvicz, 1996; Blondin, Fischer, Boissonvidal, Kazatchkine, & Jozefonvicz, 1994), and synthetic sulfated polysaccharides (Wang H.J., Wang H.W., Shi, Duan, & Wang S.C., 2012). However, most of sulfated polysaccharides also have anticoagulant effect according to the relationship studies between sulfate groups and anticoagulant activity (Farias, Valente, Pereira, & Mourao, 2000; Shanmugam & Mody, 2000), which has limited their potential exploitation as anti-complement drugs. According to the study of Haroun-Bouhedja, Ellouali, Sinquin, and Boisson-Vidal (2000), Maillet, Petitou, Choay, and Kazatchkine (1988) and Agarwal and Danishefsky (1986), low molecular weight sulfated polysaccharides have less anticoagulant activity, and these polysaccharides may be more suited as drug candidates for inhibiting complement system.

Costus is the root of *Saussurea costus* (Falc.) Lipschitz, and it is a well known and an important medicinal plant. It is widely used in several indigenous systems of medicine for the treatment of various ailments. Several bioactive chemical constituents, such as cynaropicrin, costunolide, and dehydrocostus lactone, have been found in the *S. costus* (Pandey, Rastogi, & Rawat, 2007). Cynaropicrin has been identified as the principal inhibitor of TNF-alpha and

* Corresponding author. Tel.: +86 21 51322511; fax: +86 21 51322519.
** Corresponding author. Tel.: +44 2079115038; fax: +44 2079115038.
E-mail addresses: a.bligh@westminster.ac.uk (S.W.A. Bligh), shunchunwang@126.com (S. Wang).

0144-8617/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2014.01.084

a potential anticancer agent (Cho et al., 1998). Costunolide has been identified as potential anticancer and anti-HBV agents (Jeong et al., 2002). Dehydrocostus lactone has been reported to have the potential to be developed as anti-HBV drugs (Chen, Chou, Lee, Wang, & Yeh, 1995). Besides, there are also several other reports which indicate that it has antidiabetic, hypolipidemic, CNS depressant and antiparasitic activities.

However, up to now there are few reports on isolation and bioactivity of polysaccharide from costus root. In da Silva's study, three glucans with anti-inflammatory activity prepared from stems of *Costus spicatus* were reported (da Silva & Parente, 2003). Here we report the extraction and purification of a homogeneous polysaccharide APS-W1 from *S. costus*. APS-W1 was characterized by composition and methylation analysis, GC–MS, IR and NMR spectroscopy. As its molecular weight was relatively low, about 3.9 kDa, its sulfated derivatives may have complement inhibitory activity as well as limited anticoagulant activity. Therefore, five sulfated derivatives of APS-W1 were prepared under different conditions. The degree of substitution (DS) and the sulfate group position of the derivatives were elucidated, and the anti-complement activity through classical pathway and alternative pathway were also investigated.

2. Experimental

2.1. Materials

Costus was purchased from Bozhou city in Anhui province, China and was authenticated by Prof. Lihong Wu, Shanghai University of Traditional Chinese Medicine, PR China. Monosaccharides (D-Gal, D-Ara, L-Fuc, L-Rha, D-Man, D-Fruc, D-Xyl and D-Glc) and 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimidemetho-p-toluenesulfonate (CMC), trifluoroacetic acid (TFA) were purchased from Sigma (St Louis, MO). Pullulans of different molecular weights were purchased from the Shodex Co. (Tokyo, Japan). DEAE-cellulose and Sephacryl^{TM} S-100 HR (100 cm × 2.6 cm) were purchased from GE Healthcare Bio-Sciences (AB, Uppsala, Sweden). All other reagents were analytical grade. Singly-distilled water was used in chemical analysis and triply-distilled water was used in bioassay.

2.2. General methods

IR spectra were measured with a Perkin Elmer 2000 FTIR spectrometer as KBr pellets. HPGPC was performed with an Agilent 1100 instrument with the GPC software, and HPLC columns KS-804 and KS-802 (Shodex Co., Tokyo, Japan) connected in series were used. NMR spectra were recorded with a Bruker AM 600 spectrometer at room temperature. All chemical shifts were reported relative to acetone (^{1}H: 2.10 ppm, ^{13}C: 31.50 ppm). GC–MS was analyzed with a Thermo TRACE DSQ apparatus equipped with a TR-5ms column (length: 60 m × 0.25 mm, thickness of liquid phase: 0.25 μm).

2.3. Preparation of homogeneous polysaccharide

Dried costus roots (5 kg) were washed by distilled water and then were soaked with 95% ethanol for 6 days to remove small lipophilic components. After dried in air, the residue was extracted three times with 50 L distilled water for 3 h at 100 °C. The extracts were combined and concentrated to 10 L. After centrifugation, the supernatant was kept, 95% EtOH was added until the final EtOH concentration reached to 75%, to give a precipitate which was then lyophilized to give a crude polysaccharide APS (425.5 g, yield 8.51%).

The crude polysaccharide APS was applied to a DEAE-cellulose column (50 cm × 5 cm, Cl-form) and eluted with distilled water and different concentrations of NaCl solution. After concentration and lyophilization, the water fraction APS-W was obtained (43.2 g, yield 10.8%). APS-W was further purified on a column of Sephacryl^{TM} S-100 HR (100 cm × 2.6 cm) and eluted with 0.2 M NaCl solution to give the homogeneous polysaccharide APS-W1 (11.8 g, yield 58.8%).

2.4. Determination of homogeneity and molecular weight

Measurements were performed by HPGPC on two columns of KS-804 and KS-802 in series (Wang H.J. et al., 2012; Yu et al., 2012). The column was calibrated by standard pullulans with known molecular weight (P-5, P-10, P-20, P-50, P-100, P-200, P-400, P-800, Shodex Co., Tokyo, Japan). The column temperature was kept at 40.0 ± 0.1 °C. 0.2 M NaCl solution was used as eluant and the flow rate was kept at 0.8 mL/min. All samples were finally prepared as 2.0 mg/mL solutions, and 20 μL aliquot was injected for each run.

2.5. Monosaccharide composition analysis

APS-W1 was hydrolyzed with 2 M TFA at 120 °C for 2 h. After evaporation to completely remove TFA, the partial residue was dissolved in distilled water (0.1 mL) and analyzed by thin-layer chromatography (TLC), developed with 5:5:1:3 (v/v) EtOAc-pyridine-HOAc-water. The plate was visualized by spraying with O-phthalic acid reagent and heating at 100 °C for 5 min (Duan, Zheng, Dong, & Fang, 2004).

The remaining hydrolyzed residue was reduced with $NaBH_4$ at room temperature for 3 h. After neutralization with AcOH and evaporation to dryness, the residue was acetylated with Ac_2O for 1 h at 100 °C. The resulting alditol acetates were subjected to GC–MS analysis.

2.6. Methylation analysis

APS-W1 (5 mg) was methylated three times with methyl iodide and powdered sodium hydroxide in dimethyl sulfoxide as described by Needs (Needs & Selvendran, 1993). The sample APS-W1 was dried overnight in vacuo (P_2O_5) at room temperature, then dissolved in 4 Å molecular sieve-dried DMSO (1.5 mL), and methylated with the Need's method. The permethylated polysaccharide was hydrolyzed and converted into the partially acetylated, partially methylated alditol acetates and analyzed by GC–MS (Wang S.C. et al., 2008).

2.7. Preparation of sulfated polysaccharide

The sample (APS-W1) was suspended in dry formamide and kept at room temperature for 15 min followed by addition of chlorosulfonic acid (0.2–2 mL) and pyridine (1–2 mL). The mixture was maintained at 0 °C for 2–5 h (Table 1). After the reaction, the mixture was adjusted to pH 7–8 by 5 M NaOH solution, dialyzed first against saturated $NaHCO_3$ overnight, then against distilled water extensively. The retentates were lyophilized to give the sulfated derivatives (Table 1), which were named as APS-0.2, APS-0.5, APS-1a, APS-1b and APS-2 according to the volume of chlorosulfonic acid. The DS was measured by the $BaCl_2$ gelatin method (Chaidedgumjorn, Suzuki, et al., 2002).

2.8. NMR spectra

Native polysaccharide APS-W1 and sulfated polysaccharides (50 mg) were deuterium-exchanged and dissolved in 0.4 mL of D_2O (99.8%). The ^{1}H-, ^{13}C-, DEPT-^{13}C, HSQC, HSBC and H-H COSY NMR spectra were measured using a Bruker AM-600 spectrometer at ambient temperature. The chemical shifts of ^{13}C-NMR

Table 1
The degree of sulfation (DS), substitution position, CP_{50} and AP_{50} of the sulfated derivatives of APS-W1.

Sample name	Reaction conditions ($T = 0\,^{\circ}C$)			DS	Position of sulfation	CP_{50} (μg/mL)	Concentration (μg/mL)/percent (%)[a]	AP_{50} (μg/mL)
	$ClSO_3H$ (mL)	Pyridine (mL)	Time (h)					
APS-0.2	0.2	2	2	0.40	6	18.9 ± 0.7**	36.0/93.19 ± 0.70	115.8 ± 2.6**
APS-0.5	0.5	2	3	1.09	6	10.2 ± 0.9*	17.5/91.21 ± 0.90	74.3 ± 2.3**
APS-1a	1	2	3	1.57	4,6	6.9 ± 0.8**	11.0/93.27 ± 0.80	47.9 ± 1.7**
APS-1b	1	1	5	1.98	4,6	4.6 ± 0.4**	9.5/92.13 ± 0.60	23.2 ± 1.8**
APS-2	2	1	5	2.18	4,6	2.2 ± 0.3**	5.5/94.45 ± 0.70	11.4 ± 1.3**
Heparin	–			–	–	8.3 ± 0.5	–	89.2 ± 2.8

The native polysaccharide APS-W1 has no anti-complement activity through classical pathway or alternative pathway.
[a] The lowest concentrations that inhibit (percent inhibition > 90%) hemolytic activity and their percent inhibition.
** $p < 0.01$, significantly different from the heparin.
* $p < 0.05$, significantly different from the heparin.

are expressed in ppm using acetone as the internal standard at 31.50 ppm.

2.9. Anti-complementary activity through the classical pathway

According to the modified method described by Xu and Chen (2007), sensitized erythrocytes (EAs) were prepared by incubation of sheep erythrocytes (4.0×108 cells/mL) with equal volume of rabbit anti-sheep erythrocyte antibody in barbital buffered saline (BBS). Samples and heparin (positive control) were dissolved in BBS. Normal human serum (NHS) was used as the complement source. The diluted NHS was chosen to give sub-maximal lysis in the absence of complement inhibitors. Various dilutions of tested samples (100 μL) were pre-incubated with 100 μL NHS and 200 μL BBS at 37 °C for 10 min. Then 100 μL EAs were added and the mixture was incubated at 37 °C for 30 min. A range of different assay controls were incubated in the same conditions: (1) vehicle control, 100 μL EAs in 500 μL BBS; (2) 100% lysis, 100 μL EAs in 500 μL water; (3) sample control, 100 μL dilution of each sample in 500 μL BBS. The reaction mixture was centrifuged at 4 °C immediately. Optical density of the supernatant was measured at 405 nm using a UV-VIS spectrophotometer (Wellscan MK3, Lab systems Dragon Co., MA, USA). Corrected absorbance was obtained by subtracting sample control absorbance from corresponding samples and the percentage inhibition was calculated with the 100% lysis control as reference. Anti-complementary activity through the classical pathway was determined as a mean of triplicate tests per concentration and expressed as the 50% inhibitory concentration value (CP_{50} value) from complement-dependent hemolysis of the control.

2.10. Anti-compliment activity through the alternative pathway

According to the method of Xu and Chen (2007), each sample was dissolved in Mg-EGTA-GVB, and various dilutions of which were made. After pre-incubation of dilutions of each sample (150 μL) with 1:10 diluted NHS (150 μL) at 37 °C for 10 min, 200 μL rabbit erythrocytes (ERs 0.5%, V/V) were added. Following a second incubation step at 37 °C for 30 min, cell lysis was determined as described in the section anti-complementary activity through the classical pathway. Controls for vehicle, 100% lysis and sample were included. Anti-complement activity through the alternative pathway was determined as a mean of triplicate tests per concentration and expressed as the 50% inhibitory concentration value (AP_{50} value) from complement-dependent hemolysis of the control.

2.11. Preparation of complement-depleted serum with complement antibody

This experiment was conducted according to the modified method of Zhu et al. (2013). Various dilutions of each antiserum were incubated with the same volume of NHS (1:10) at 37 °C for 15 min. After centrifugation, supernatant (200 μL) was incubated with 200 μL BBS and 200 μL EAs at 37 °C for 30 min. Cell lysis was measured as described through the classical pathway. The antiserum dilution against the NHS hemolytic capacity was then determined. The optimal dilutions of complements (1:8 for C1q, 1:4 for C1r, 1:3 for C1s, 1:1 for C2, 1:32 for C3, 1:1 for C4, 1:6 for C5 and 1:32 for C9, respectively) were incubated with the same volume of NHS (1:10) at 37 °C for 15 min, followed by centrifugation and supernatants were stored as C-depleted sera in aliquots at −80 °C before use in hemolytic assays.

2.12. Interaction with individual complement components

The capacity of depleted sera to lyse EAs through the classical pathway was assessed in the presence or absence of sulfated derivatives of APS-W1 (APS-0.2, -0.5, -1a, -1b, -2) treated NHS, which were obtained by incubating optimally diluted the sulfated derivatives of APS-W1 (36.0 μg/mL for APS-0.2, 17.5 μg/mL for APS-0.5, 11.0 μg/mL for APS-1a, 9.5 μg/mL for APS-1b and 5.5 μg/mL for APS-2) with the equal volume of 1:10 diluted NHS at 37 °C for 10 min. The concentration of sulfated derivatives of APS-W1 which was just sufficient to cause complete loss of hemolytic activity of 1:10 diluted NHS was determined in the section of anti-complementary activity through the classical pathway. For the target complement group (the assay of the capacity of various depleted sera to restore the hemolytic capacity of sulfated derivatives of APS-W1 treated serum), 200 μL EAs and 200 μL individual depleted serum of C1q, C1r, C1s, C2, C3, C4, C5 or C9 were added to 200 μL sulfated derivatives of APS-W1 treated NHS and the mixture was incubated at 37 °C for 30 min. After centrifugation and measurement of the optical density of the supernatant, the percent hemolysis was calculated. For the assay of individual depleted serum group, C-depleted serums were directly incubated with EAs under the same conditions, and the hemolytic activities

Table 2
Linkage analysis by methylation and GC–MS of APS-W1 from costus root.

Methylation sugars	Deduced unit and substitution	Time (min)	Molar ratio
2,3,4,6-Tetra-Me-Glc/Man	Glc(1 → Or Man(1 →	26.29	5.35
3,4,6-Tri-Me-Man	→1)Man(2→	31.21	47.22
3,4,6-Tri-Me-Glc	→1)Glc(2→	31.43	47.43

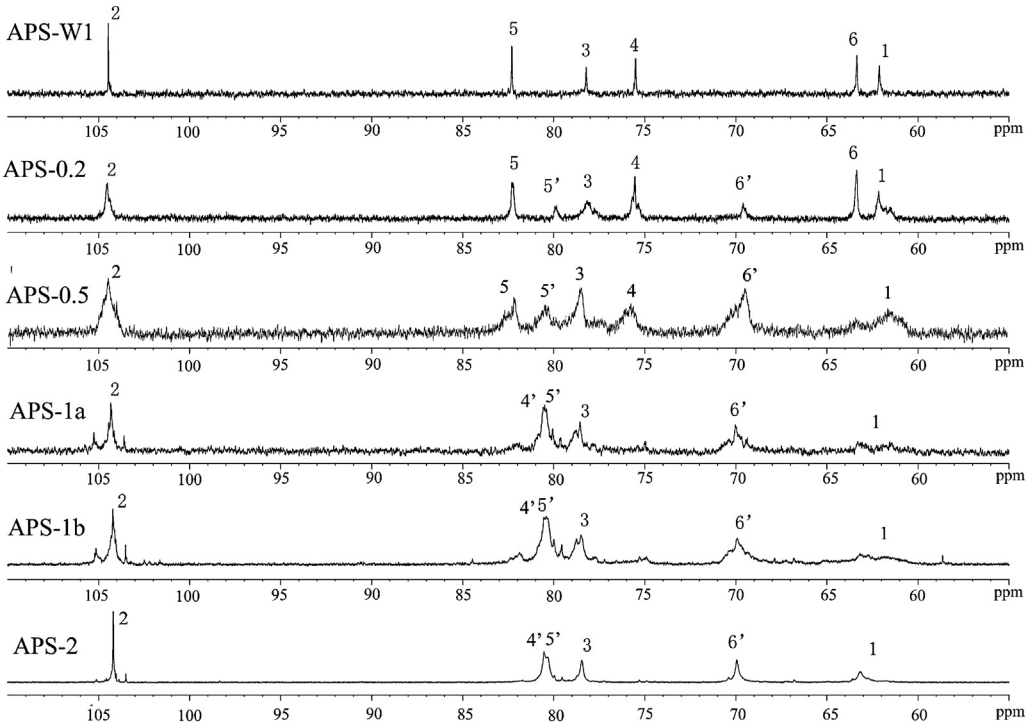

Fig. 1. ^{13}C NMR spectra of APS-W1 and its sulfated derivatives. APS-W1: the anomeric C-2 at 104.47 ppm indicating a β linkage. Signals at 82.31, 78.22, 75.51, 63.36 and 62.12 ppm corresponded to C-5, C-3, C-4, C-6 and C-1 of (1,2)-linked-Fruc, respectively. After sulfation of APS-W1, APS-0.2 and APS-0.5: C-6 (down-shifted 6.2 and 5.5 ppm, respectively) and C-5 (up-shifted 2.4 and 3 ppm, respectively) indicating substitution at C-6 of Fruc*f* residues; APS-1a, APS-1b and APS-2: C-4 and C-6 (typically down-shifted at about 5 and 6 ppm, respectively) and C-5 (all up-shifted 2.7 ppm) indicating substitution at C-4 and C-6 of Fruc*f* residues.

were calculated. The controls: (1) vehicle control, 200 μL EAs in 400 μL BBS; (2) 100% lysis, 200 μL EAs in 400 μL water; (3) complement control, 100 μL NHS (1:10) and 200 μL EAs in 300 μL BBS; (4) sample, 200 μL sulfated polysaccharide-treated NHS and 200 μL EAs in 200 μL BBS; and (5) sample control, 200 μL sample in 400 μL BBS were incubated in the same conditions.

2.13. Influence on re-calcification time (RT) and thrombin time (TT)

According to the method of Zhu et al. (2013), 150 μL of platelet poor plasma (PPP) (obtained from the heart of New Zealand Rabbits) was added into 15 μL of different concentrations of the derivatives (500, 50 and 5 μg/mL, diluted with BBS) and heparin (5.2 μg/mL, used as positive control), the mixture was incubated at 37 °C for 5 min, then 150 μL of 0.025 mol/L $CaCl_2$ solution was added. The time from addition of $CaCl_2$ to clot formation was recorded as plasma re-calcification clotting time, briefly called re-calcification time (RT). The determination of thrombin time (TT) was identical with RT except substitute $CaCl_2$ with thrombin.

3. Results and discussion

3.1. Isolation and purification of APS-W1

Crude polysaccharide APS (425.5 g, yield: 8.51%) was prepared from costus root by boiling water extraction and EtOH precipitation. APS was fractionated by anion-exchange chromatography and the water fraction APS-W (43.2 g, yield: 10.8%) was obtained. As the water fraction gave the highest yield, it was further purified by size-exclusion chromatography, and the final polysaccharide APS-W1 (11.8 g, yield: 58.8%) was obtained. APS-W1 was homogeneous, as it gives a single symmetrical peak in HPLC, with a molecular weight of 3.9 kDa as determined by HPGPC.

3.2. Monosaccharide composition analysis

According to TLC, APS-W1 was composed of fructose. The results of GC–MS gave two peaks (at 42.16 and 42.39 min) with a ratio of 1:1, which were identified as mannose and glucose, respectively, when compared with standard monosaccharides. The results of GC–MS agreed to that of the TLC. Reduction of fructose in APS-W1 gave mannose and glucose (Xu et al., 2005), hence APS-W1 was identified as a homofructosan.

3.3. Linkage analysis of APS-W1

APS-W1 was methylated three times to give a completely methylated polysaccharide. GC–MS analysis indicated that APS-W1 was linear (2→1)-linked mannan and linear (2→1)-linked glucan (Table 2), inferring that the native APS-W1 was linear (2→1)-linked homofructosan according to monosaccharide composition analysis.

3.4. NMR analysis of APS-W1

The signals in the ^{1}H NMR (not shown) and ^{13}C NMR (Fig. 1) spectra of APS-W1 were assigned based on literature values (Angyal, 2009; Cooper & Petrovsky, 2011; Heyer et al., 1998). The anomeric carbon signal at δ_C 104.47 indicates β-linkage (Barrow, Collins, Rogers, & Smith, 1984), and its disappearing in the DEPT-135 ^{13}C NMR indicates APS-W1 is a ketose. The identity of each resonance (methine or methylene) was determined by DEPT-135 and 2D NMR spectroscopy (Fig. 2). The signals at δ_H 4.15–4.19 were assigned to H-3 by HMBC, and then in HMQC the signal at δ_C 78.22 was assigned to C-3. The resonances at δ_C 62.12 and 63.36 were assigned to C-1 and C-6 respectively from DEPT-135 and HMQC spectra, and then C-4 (δ_C 75.51) was assigned from ^{1}H–^{1}H COSY and HMBC spectra (Fig. 2). The ^{13}C DEPT-135 NMR spectrum (not

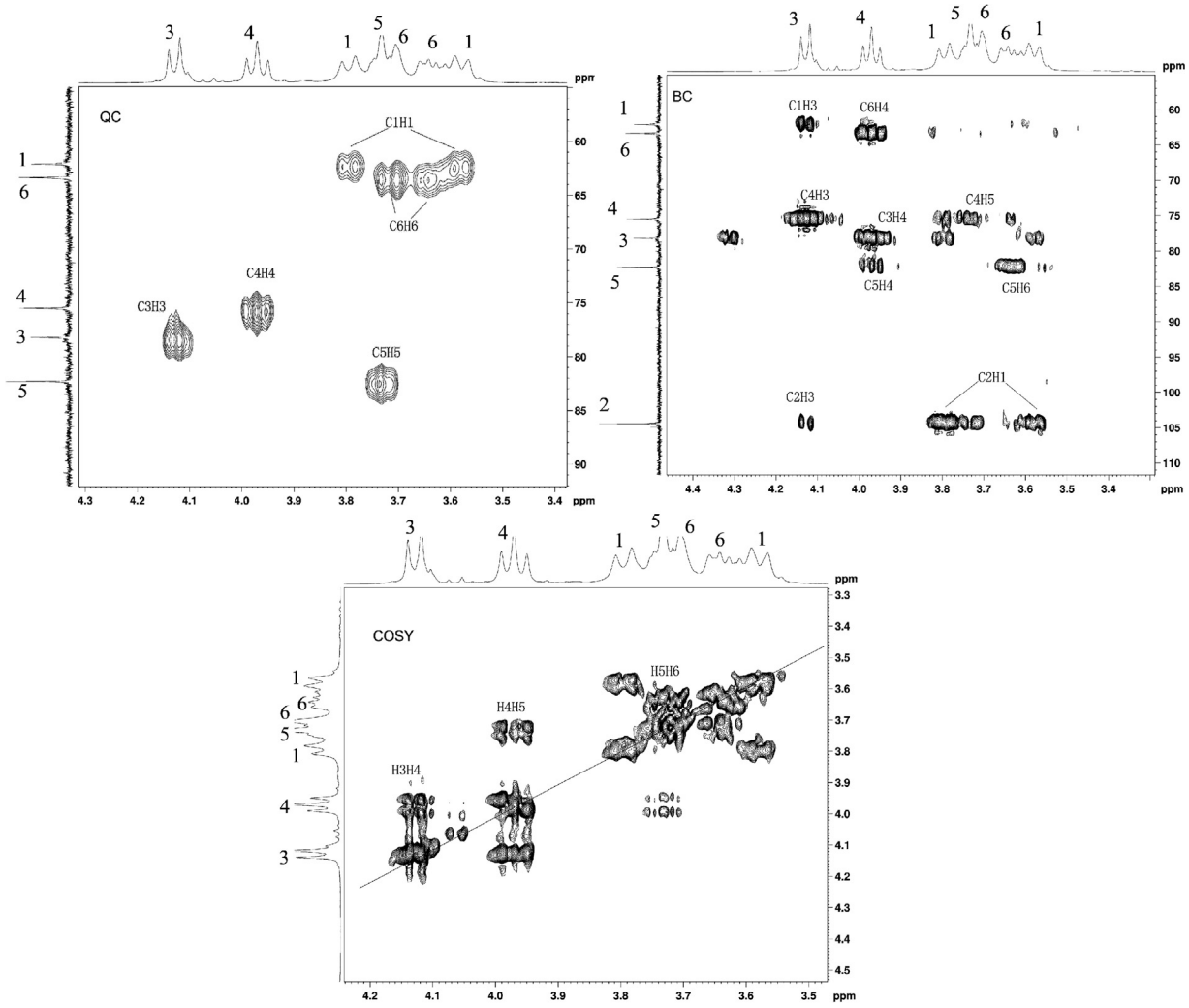

Fig. 2. 2D NMR spectra of APS-W1: $^{1}H/^{13}C$ HSQC, $^{1}H/^{13}C$ HSBC and $^{1}H/^{1}H$ COSY.

shown) gave the expected results in that the three upfield resonances were methylene while the remaining resonances were methine, which indicate that the fructose residues must be in the furanose form, rather than the pyranose form, a fact which is also supported by the chemical shift of the anomeric carbon (Barrow et al., 1984). Hence APS-W1 was characterized as β-D fructofuranosan.

3.5. Preparation and characterization of sulfated derivatives of APS-W1

The sulfated derivatives of APS-W1 were successfully prepared and was evidenced by their IR spectra (Fig. 3) showing an S=O vibration at approximately 1240 cm^{-1} (Yu et al., 2012). Interestingly, molecular weights of them were reduced to about one third of the native polysaccharide, which might be due to the partial acid hydrolysis, or to the high negative-charge density condition for sulfation (Komatsu, Takahata, Tanaka, Ishimitsu, & Okada, 1993). The degree of substitution (DS) was identified (Table 1), and they were in the range from 0.40 to 2.18, based on Chaidedgumjorn's method (Chaidedgumjorn, Toyoda, et al., 2002). The signals in ^{13}C NMR (Fig. 1) spectra of the sulfated derivatives were assigned from DEPT-135 ^{13}C NMR and 2D NMR (not shown). According to chemical shifts in ^{13}C-NMR spectra described by others (Vityazev et al., 2010; Wang H.J. et al., 2012), the substitution position of sulfate groups of the derivatives was assigned. The sulfate groups of APS-0.2 and APS-0.5 were substituted at C-6, and the others were substituted at C-4 and C-6.

3.6. Inhibition of the derivatives against the classical pathway of complement system

The effect of APS-W1 and the sulfated derivatives on human complement system through the classical pathway was examined in 1:10 diluted NHS, and heparin was used as a reference (Xu & Chen, 2007). The percentage of activation that 1:10 diluted NHS occurred in the classical pathway was 96.20 ± 7.62% in the complement control group. The native polysaccharide APS-W1 has no anti-complement activity through classical pathway. The anti-complementary activities of the sulfated derivatives and heparin on the classical pathway were shown in Fig. 4A. The concentrations that resulted in 50% inhibition and the concentrations that almost abolished all of the hemolytic activity (percent inhibition >90%) of NHS were shown at Table 1. The CP_{50} of the sulfated derivative APS-2 is 2.2 ± 0.4 μg/mL, showing it has the strongest anti-complement activity and it is better than the positive control heparin (CP_{50}: 8.3 ± 0.3 μg/mL). At a concentration of 5.5 μg/mL, APS-2 almost abolished all of the hemolytic activity of NHS (percent inhibition: 94.45 ± 0.70%). Thus apart from APS-0.2 and APS-0.5 the sulfated derivatives of APS-W1 exhibited strong inhibitory effects on the complement activation through the classic pathway.

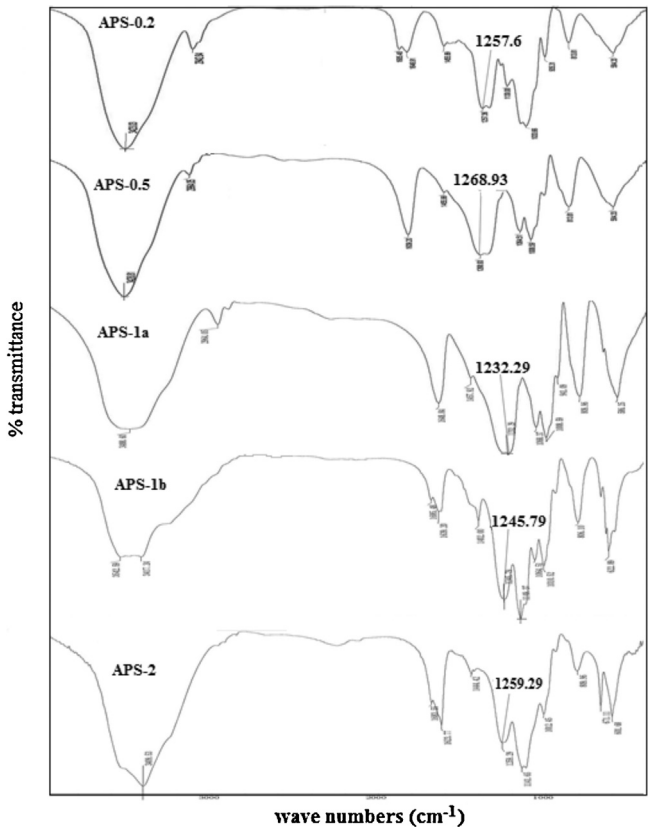

Fig. 3. IR spectra of the derivatives of APS-W1. S=O vibration bands at about 1240 cm^{-1} indicating that all of the derivatives were successfully sulfated.

3.7. Inhibition of the derivatives against the alternative pathway of complement system

The percentage of activation that 1:10 diluted NHS occurred in the alternative pathway was 94.35 ± 5.14% in the complement control group. The native polysaccharide APS-W1 has no anti-complement activity through alternative pathway. The anti-complementary activities of the sulfated derivatives and heparin on the alternative pathway are shown in Fig. 4B. They both blocked hemolysis of ERs in a dose-dependent manner. In the AP_{50} assay, the sulfated derivatives except for the APS-0.2 possess better 50% hemolysis inhibition of ERs than heparin (Table 1). They have strong inhibitory effects on the complement activation through the alternative pathway.

3.8. Identification of targets of derivatives in the complement activation cascade

Effects of the sulfated derivatives on individual complement components were investigated in the system with complement-depleted reagents and a limited amount of human serum. The capacities of various depleted sera to restore the hemolytic capacity of sulfated derivatives treated serum were examined. Under these conditions, the complement component under investigation is the limiting factor in the component-mediated hemolysis assay. Thus, the failure of restoring the hemolysis could be attributed to the interaction between the tested sample and corresponding complement component. As shown in Fig. 5, the percentage of the 1:10 NHS-induced hemolysis through the classical pathway was 96.36 ± 9.10% in the complement control group. The concentration of the derivatives that exhibited a strong inhibitory effect on this hemolysis (the hemolysis percentage <8.0%) were 18.9, 10.2, 6.9, 4.6 and 2.2 μg/mL for APS-0.2, APS-0.5, APS-1a, APS-1b and APS-2, respectively. For APS-0.2 and APS-0.5, the hemolysis percentages of the C-depleted sera that lysed EAs independently (including C1q, C1r, C1s, C2, C3, C5, C9) were no more than 10%, and the hemolysis percentages of C4 were 78.23 ± 5.50% and 86.34 ± 6.10%, respectively. Therefore, APS-0.2 and APS-0.5 inhibit complement activation by blocking C1q, C1r, C1s, C2, C3, C5 and C9 but not C4 in this assay. For APS-1a, APS-1b and APS-2, the hemolysis percentages of the C-depleted sera that lysed EAs independently (including C1q, C1r, C1s, C3, C5, C9) were no more than 10%, and the hemolysis percentages of C2 and C4 was 89.57 ± 3.40% and 75.21 ± 5.60% (APS-1a), 84.56 ± 5.30% and 92.73 ± 2.10% (APS-1b), 89.09 ± 2.90% and 88.23 ± 4.20% (APS-2), respectively. Hence, APS-1a, APS-1b and APS-2 inhibit complement activation by blocking C1q, C1r, C1s, C3, C5 and C9 but not C2 and C4 in this assay. According to these results, the sulfated group on the C-4 may play an important role on the target of C2.

In this work, the capacity of the sulfated derivatives to inhibit complement-mediated hemolysis of both classical and alternative pathways increased with increasing DS of the derivatives (Table 1). However, in previous study by Wang H. et al. (2013), DS have not significantly influenced the anti-complement activity. In Wang's study, the sulfated group of sulfated polysaccharides substituted at carbon-2 or carbon-3, selectively blocked C1q, C1r, C1s, C2, C5 and C9, and the monosaccharide composition was galacturonic acid. However in this study, the sulfate group of sulfated polysaccharides substituted at carbon-6 or/and carbon-4, selectively blocked C1q, C1r, C1s, C2, C3, C5, C9 or C1q, C1r, C1s, C3, C5, C9, and the monosaccharide composition was fructose, also the molecular weight of our sulfated polysaccharides were different from Wang's.

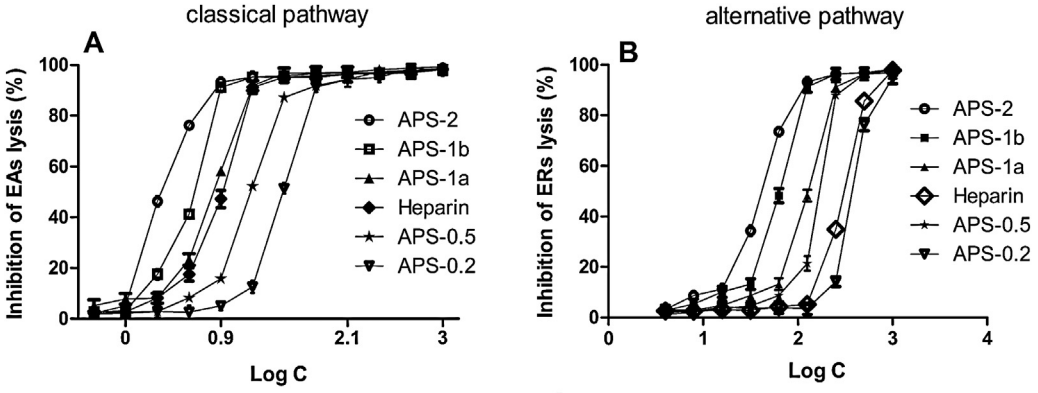

Fig. 4. Anti-complement activity of sulfated derivatives through (A) classical pathway and (B) alternative pathway. Inhibition of classical pathway-mediated hemolysis of EAs and alternative pathway-mediated hemolysis of ERs in 1:10 diluted NHS in the presence of increasing amounts of sulfated derivatives. Heparin was used as reference. Results are expressed as inhibition percentage, and concentration C is in μg/mL. Data are mean from 3 determinations ± S.E.M.

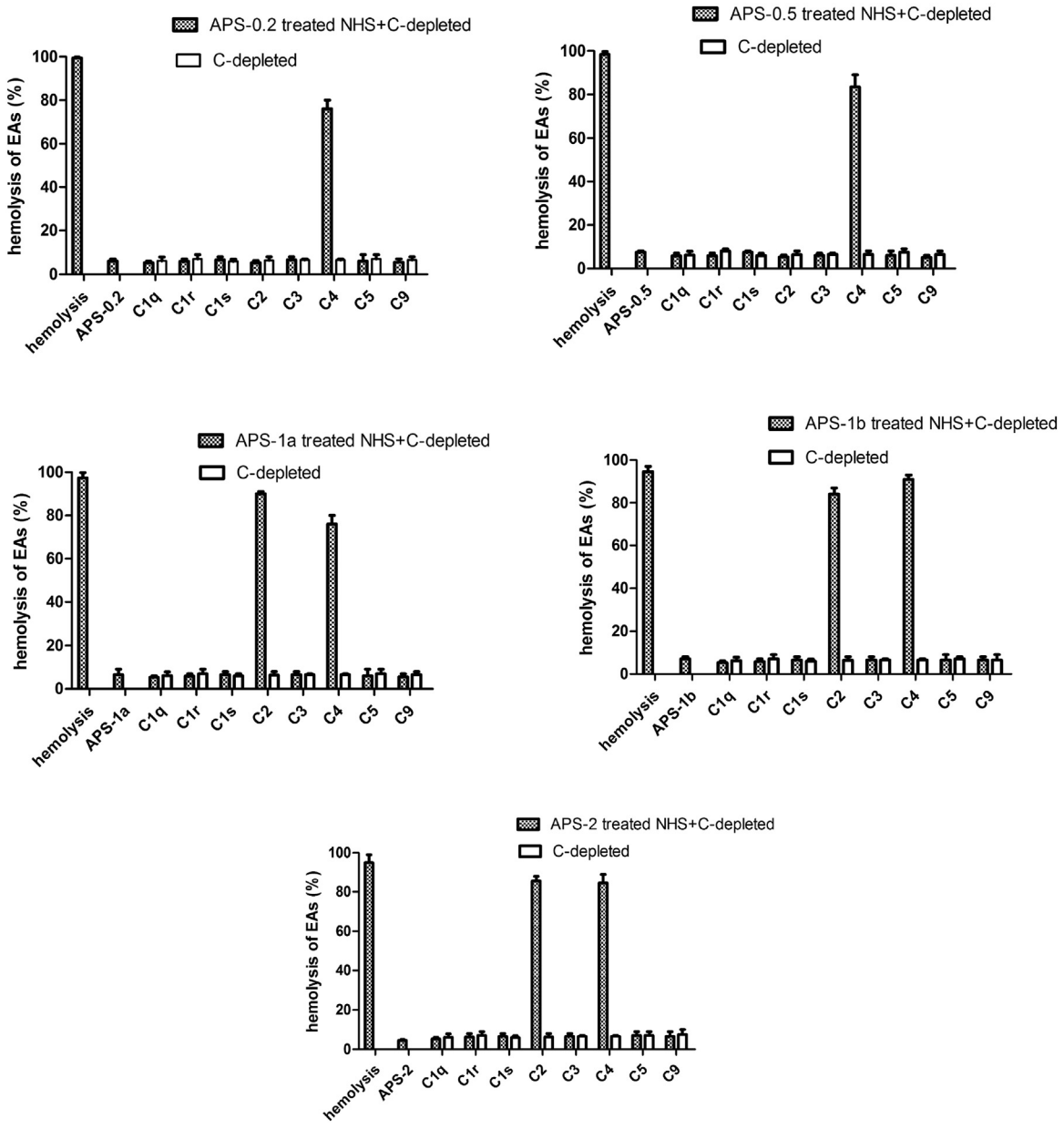

Fig. 5. Targets of the sulfated derivatives in the complement activation cascade. Sulfated derivatives-treated serum was mixed various depleted sera and the capacity of these depleted sera to restore classical pathway hemolytic capacity was estimated by adding sheep antibody-sensitized erythrocytes. Results are expressed as hemolytic percentage. Data are mean from 3 determinations ± S.E.M.

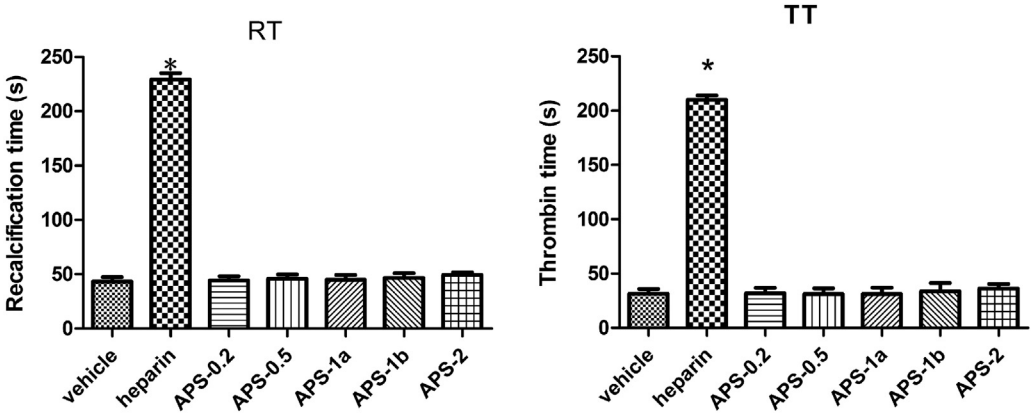

Fig. 6. Anti-coagulation effect of the sulfated derivatives. The anticoagulant activities of the derivatives were valued through re-calcification time (RT) and thrombin time (TT). BBS was used as blank control. Heparin (5.2 μg/mL) was used as positive control and the sulfated derivatives (500 μg/mL). Data are mean from 3 determinations ± S.E.M.

These differences indicated that the sugar composition, sulfated position and the molecular weight play an important role in the anti-complement activity of a sulfated polysaccharide.

3.9. Influence of the derivatives on coagulation system

As shown in Fig. 6, anti-coagulation activity of the derivatives was very limited versus heparin. For instance, at the concentration of 5.2 μg/mL, heparin markedly prolonged re-calcification time (RT: 235.5 ± 3.7 s) and thrombin time (TT: 210.1 ± 2.9 s) in contrast with vehicle control (RT: 42.0 ± 2.2 s, TT: 37.0 ± 1.8 s), whereas the sulfated derivatives had very little effect on both RT (APS-0.2: 41.8 ± 3.8 s, APS-0.5: 43.2 ± 2.9 s, APS-1a: 40.3 ± 3.5 s, APS-1b: 42.3 ± 2.1 s, APS-2: 44.1 ± 3.1 s) and TT (APS-0.2: 36.7 ± 2.7 s, APS-0.5: 35.2 ± 2.2 s, APS-1a: 35.8 ± 1.9 s, APS-1b: 38.1 ± 1.9 s, APS-2: 39.3 ± 1.8 s) compared to the vehicle control even at a higher concentration (500 μg/mL).

4. Conclusion

The present study has successfully extracted and characterized a novel homogeneous polysaccharide APS-W1, (2 → 1)-β-D-fructofuranosan, with an average molecular weight of 3.9 kDa from *S. costus*. Five sulfated derivatives with different degrees of sulfation (range from 0.40–2.18) of APS-W1 were prepared under different conditions. All sulfated derivatives demonstrated strong anti-complement activity through classical pathway and alternative pathway in a dose-dependent manner. In contrast, the native polysaccharide APS-W1 did not have such effects, which suggesting that sulfate groups on the derivatives should be essential for anti-complement activity. More interestingly, through re-calcification time (RT) and thrombin time (TT) assay, the sulfated derivatives have shown limited anticoagulant effects.

The average molecular weights of the highly sulfated derivatives were pretty low, which were only one third of the parent polymer (3.9 kDa). According to the study of Haroun-Bouhedja et al. (2000), Maillet et al. (1988) and Agarwal and Danishefsky (1986), low molecular weight sulfated polysaccharides have less anticoagulant activity, which was also confirmed in our study, meanwhile these highly sulfated derivatives have strong anti-complement activity. So low molecular weight sulfated polysaccharides will be potential complement inhibitor.

These sulfated polysaccharides from costus root can therefore be promising candidates of complement inhibitors in the treatment of diseases due to excessive activation of the complement system, such as system lupus erythematosus, rheumatoid arthritis, and acute respiratory distress syndrome.

Acknowledgments

This research was funded by the key New Drug Creation and Manufacturing Program in China (2012ZX09301001-003 to S.W.) and the National Natural Science Foundation of China (No. 81373951).

References

Agarwal, A., & Danishefsky, I. (1986). Requirement of free carboxyl groups for the anticoagulant activity of heparin. *Thrombosis Research*, *42*, 673–680.

Angyal, S. J. (2009). Carbohydrate research in the 20th century. *Australian Journal of Chemistry*, *62*, 501–502.

Barrow, K. D., Collins, J. G., Rogers, P. L., & Smith, G. M. (1984). The structure of a novel polysaccharide isolated from *Zymomonas mobilis* determined by nuclear magnetic resonance spectroscopy. *European Journal of Biochemistry*, *145*, 173–179.

Berteau, O., & Mulloy, B. (2003). Sulfated fucans, fresh perspectives: Structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. *Glycobiology*, *13*, 29R–40R.

Blondin, C., Chaubet, F., Nardella, A., Sinquin, C., & Jozefonvicz, J. (1996). Relationships between chemical characteristics and anticomplementary activity of fucans. *Biomaterials*, *17*, 597–603.

Blondin, C., Fischer, E., Boissonvidal, C., Kazatchkine, M. D., & Jozefonvicz, J. (1994). Inhibition of complement activation by natural sulfated polysaccharides (fucans) from brown seaweed. *Molecular Immunology*, *31*, 247–253.

Carroll, M. C. (2004). The complement system in regulation of adaptive immunity. *Nature Immunology*, *5*, 981–986.

Chaidedgumjorn, A., Suzuki, A., Toyoda, H., Toida, T., Imanari, T., & Linhardt, R. J. (2002). Conductivity detection for molecular mass estimation of per-O-sulfonated glycosaminoglycans separated by high-performance size-exclusion chromatography. *Journal of Chromatography A*, *959*, 95–102.

Chaidedgumjorn, A., Toyoda, H., Woo, E. R., Lee, K. B., Kim, Y. S., Toida, T., et al. (2002). Effect of (1 → 3)- and (1 → 4)-linkages of fully sulfated polysaccharides on their anticoagulant activity. *Carbohydrate Research*, *337*, 925–933.

Chen, H. C., Chou, C. K., Lee, S. D., Wang, J. C., & Yeh, S. F. (1995). Active compounds from *Saussurea lappa* Clarks that suppress hepatitis-b virus surface-antigen gene-expression in human hepatoma-cells. *Antiviral Research*, *27*, 99–109.

Cho, J. Y., Park, J. S., Yoo, E. S., Baik, K. U., Jung, J. H., Lee, J. S., et al. (1998). Inhibitory effect of sesquiterpene lactones from *Saussurea lappa* on tumor necrosis factor-alpha production in murine macrophage-like cells. *Planta Medica*, *64*, 594–597.

Cooper, P. D., & Petrovsky, N. (2011). Delta inulin: A novel, immunologically active, stable packing structure comprising beta-D-[2 → 1] poly(fructo-furanosyl) alpha-D-glucose polymers. *Glycobiology*, *21*, 595–606.

da Silva, B. P., & Parente, J. P. (2003). Bioactive polysaccharides from *Costus spicatus*. *Carbohydrate Polymers*, *51*, 239–242.

Duan, J. Y., Zheng, Y., Dong, Q., & Fang, J. N. (2004). Structural analysis of a pectic polysaccharide from the leaves of *Diospyros kaki*. *Phytochemistry*, *65*, 609–615.

Farias, W. R. L., Valente, A. P., Pereira, M. S., & Mourao, P. A. S. (2000). Structure and anticoagulant activity of sulfated galactans – Isolation of a unique sulfated galactan from the red algae *Botryocladia occidentalis* and comparison of its anticoagulant action with that of sulfated galactans from invertebrates. *Journal of Biological Chemistry*, *275*, 29299–29307.

Fearon, D. T., & Carroll, M. C. (2000). Regulation of B lymphocyte responses to foreign and self-antigens by the CD19/CD21 complex. *Annual Review of Immunology*, *18*, 393–422.

Haroun-Bouhedja, F., Ellouali, M., Sinquin, C., & Boisson-Vidal, C. (2000). Relationship between sulfate groups and biological activities of fucans. *Thrombosis Research*, *100*, 453–459.

Heyer, A. G., Schroeer, B., Radosta, S., Wolff, D., Czapla, S., & Springer, J. (1998). Structure of the enzymatically synthesized fructan inulin. *Carbohydrate Research*, *313*, 165–174.

Jeong, S. J., Itokawa, T., Shibuya, M., Kuwano, M., Ono, M., Higuchi, R., et al. (2002). Costunolide, a sesquiterpene lactone from *Saussurea lappa*, inhibits the VEGFR KDR/Flk-1 signaling pathway. *Cancer Letters*, *187*, 129–133.

Kazatchkine, M. D., Fearon, D. T., Metcalfe, D. D., Rosenberg, R. D., & Austen, K. F. (1981). Structural determinants of the capacity of heparin to inhibit the formation of the human amplification C3 convertase. *Journal of Clinical Investigation*, *67*, 223–228.

Kirschfink, M. (1997). Controlling the complement system in inflammation. *Immunopharmacology*, *38*, 51–62.

Komatsu, H., Takahata, T., Tanaka, M., Ishimitsu, S., & Okada, S. (1993). Determination of the molecular-weight distribution of low-molecular-weight heparins using high-performance gel-permeation chromatography. *Biological & Pharmaceutical Bulletin*, *16*, 1189–1193.

Maillet, F., Petitou, M., Choay, J., & Kazatchkine, M. D. (1988). Structure-function relationships in the inhibitory effect of heparin on complement activation: Independency of the anti-coagulant and anti-complementary sites on the heparin molecule. *Molecular Immunology*, *25*, 917–923.

Needs, P. W., & Selvendran, R. R. (1993). Avoiding oxidative-degradation during sodium-hydroxide methyl iodide-mediated carbohydrate methylation in dimethyl-sulfoxide. *Carbohydrate Research*, *245*, 1–10.

Ormsby, R. J., Jokiranta, T. S., Duthy, T. G., Griggs, K. M., Sadlon, T. A., Giannakis, E., et al. (2006). Localization of the third heparin-binding site in the human complement regulator factor H1. *Molecular Immunology*, *43*, 1624–1632.

Pandey, M. M., Rastogi, S., & Rawat, A. K. (2007). *Saussurea costus*: Botanical, chemical and pharmacological review of an ayurvedic medicinal plant. *Journal of Ethnopharmacology*, *110*, 379–390.

Pangburn, M. K., Atkinson, M. A., & Meri, S. (1991). Localization of the heparin-binding site on complement factor H. *Journal of Biological Chemistry*, *266*, 16847–16853.

Reid, K. B., & Porter, R. R. (1981). The proteolytic activation systems of complement. *Annual Review of Biochemistry*, *50*, 433–464.

Ricklin, D., Hajishengallis, G., Yang, K., & Lambris, J. D. (2010). Complement: A key system for immune surveillance and homeostasis. *Nature Immunology*, *11*, 785–797.

Shanmugam, M., & Mody, K. H. (2000). Heparinoid-active sulphated polysaccharides from marine algae as potential blood anticoagulant agents. *Current Science*, *79*, 1672–1683.

Vityazev, F. V., Golovchenko, V. V., Patova, O. A., Drozd, N. N., Makarov, V. A., Shashkov, A. S., et al. (2010). Synthesis of sulfated pectins and their anticoagulant activity. *Biochemistry (Moscow)*, *75*, 759–768.

Wang, H., Shi, S., Gu, X., Zhu, C., Wei, G., Bao, B., et al. (2013). Homogalacturonans from preinfused green tea: Structural characterization and anticomplementary

activity of their sulfated derivatives. *Journal of Agriculture and Food Chemistry*, *61*, 10971–10980.

Wang, H. J., Wang, H. W., Shi, S. S., Duan, J. Y., & Wang, S. C. (2012). Structural characterization of a homogalacturonan from *Capparis spinosa* L. fruits and anti-complement activity of its sulfated derivative. *Glycoconjugate Journal*, *29*, 379–387.

Wang, S. C., Bligh, S. W. A., Zhu, C. L., Shi, S. S., Wang, Z. T., Hu, Z. B., et al. (2008). Sulfated beta-glucan derived from oat bran with potent anti-HIV activity. *Journal of Agriculture and Food Chemistry*, *56*, 2624–2629.

Xu, H., & Chen, D. F. (2007). An anti-complementary polysaccharide from the roots of *Bupleurum smithii* and its mechanism of inhibition of the complement activation cascade. *Molecular Immunology*, *44*, 260.

Xu, D. S., Feng, Y., Lin, X., Deng, H. L., Fang, J. N., & Dong, Q. (2005). Isolation, purification and structural analysis of a polysaccharide MDG-1 from *Ophiopogon japonicus*. *Yao Xue Xue Bao*, *40*, 636–639.

Yu, Q. M., Yan, J., Wang, S. C., Ji, L. L., Ding, K., Vella, C., et al. (2012). Antiangiogenic effects of GFP08, an agaran-type polysaccharide isolated from *Grateloupia filicina*. *Glycobiology*, *22*, 1343–1352.

Zhi, H. W., Zhang, Y. Y., Zhang, J. W., & Chen, D. F. (2008). Isolation and characterization of an anti-complementary protein-bound polysaccharide from the stem barks of *Eucommia ulmoides*. *International Immunopharmacology*, *8*, 1222–1230.

Zhu, Z. Y., Liu, Y., Si, C. L., Yuan, J., Lv, Q., Li, Y. Y., et al. (2013). Sulfated modification of the polysaccharide from *Cordyceps_gunnii* mycelia and its biological activities. *Carbohydrate Polymers*, *92*, 872–876.