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Isolation and structural characterization of an immunostimulating polysaccharide from fuzi, *Aconitum carmichaeli*

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Abstract—A water-soluble polysaccharide named as FPS-1 was isolated from fuzi, the root of *Aconitum carmichaeli* Debx. by hot-water extraction, anion-exchange and gel-permeation chromatography and tested for its pharmacological activities. Its structural characteristics were investigated by FTIR, HPLC, NMR spectroscopy, methylation analysis and GLC–MS. Based on the data obtained, FPS-1 was determined to be an α-(1→6)-D-glucan, with a weight-average molecular weight of about 14,000 Da. The glucan is highly branched with a single glucose at the C-3 position every four residues, on average, along the main chain. In immunopharmacological studies, FPS-1 showed potent stimulating effects on murine lymphocyte proliferation induced by concanavalin A or lipopolysaccharide both in vitro and in vivo as well as on splenocyte antibody production.

Keywords: Fuzi; *Aconitum carmichaeli*; Ranunculaceae; Structure; α-D-Glucan; Lymphocyte proliferation

1. Introduction

Fuzi (also known as aconite, monkshood or bushi), is the daughter root of *Aconitum carmichaeli* Debx. (Ranunculaceae) and is mainly produced in southern China (e.g., Sichuan Province). As an herbal drug, it has been reputed to be an important remedy in oriental medicine for thousands of years and appeared in the 1977 edition of the Chinese Pharmacopoeia and in the seventh revision of the Japanese Pharmacopoeia.

According to traditional Chinese medicine (TCM), the preparations of fuzi and various herbal formulas containing it, usually combined with other Chinese herbal medicines such as radix ginseng (a mixture of ginseng and fuzi), *Rhizoma zingiberis* and *Radix glycyrrhizae*,[1] have been frequently prescribed by practitioners of TCM to promote blood circulation, strengthen the immune system, for the treatment of heart failure congestion, neuralgia, rheumatism and gout, etc., as well as for invigoration and retarding aging.[2,3]

Herbal plants have traditionally been used as an important source of medicines. Up to now, the most well-investigated pharmaceutical ingredients in aconite are a group of Aconitum alkaloids.[4] Little attention had been paid to the carbohydrate compounds in this herb until, in 1986, Konno et al.[5] reported four hypoglycemic polysaccharides isolated from *Aconitum carmichaeli* roots from Japan. This revealed another sort of bioactive component, which probably should contribute to a considerable part of the medicinal uses of Aconitum species. However, the structures of the polysaccharides in aconite remain unclarified. In the present study, we successfully characterize the water-soluble α-D-glucan isolated from Chinese fuzi for the first time and report its immunostimulating activities.

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2. Results and discussion

2.1. Isolation and structural analysis of FPS-1

The yield of the water-soluble polysaccharide from fuzi (FPS-1) was 4.6% of the dry material. The crude polysaccharide was separated and sequentially purified through DEAE-cellulose and Sephadex G-75, each giving a single elution peak, as detected by the phenol–sulfuric acid assay. The total sugar content of the crude polysaccharide was determined to be 97.1% with a purity >99.8%.

FPS-1 appeared as a white powder, $[\alpha]_D^{20}$ +200.7 (*c* 0.5, H_2O). It had a negative response to the Lowry test and no absorption at 280 nm or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid. Elemental analysis: Found C, 41.21; H, 5.72; N, 0.00. The GPC profile (Fig. 1) showed a single and symmetrically sharp peak, indicating that FPS-1 was a homogeneous polysaccharide, with a weight-average molecular weight of ~14,000 Da.

The FTIR spectrum of FPS-1 showed a strong band between 950 and 1160 cm^{-1} attributed to the stretching vibrations of pyranose ring. A characteristic absorption at 857 cm^{-1} was also observed, indicating the α-configuration of the sugar units. There was no absorption at 890 $cm^{-1}$for the β-configuration.[6]

FPS-1 was composed of only glucose monomers, as detected by GLC of the alditol acetate derivatives of the components of the FPS-1 hydrolyzate. The optical rotation of the FPS-1 hydrolyzate obtained was shown to be $[\alpha]_D^{20}$ +52 (*c* 0.2, H_2O) [compare authentic D-glucose $[\alpha]_D^{20}$ +52.7 (*c* 0.2, H_2O)], indicating the D-configuration of the glucosyl residues.[7]

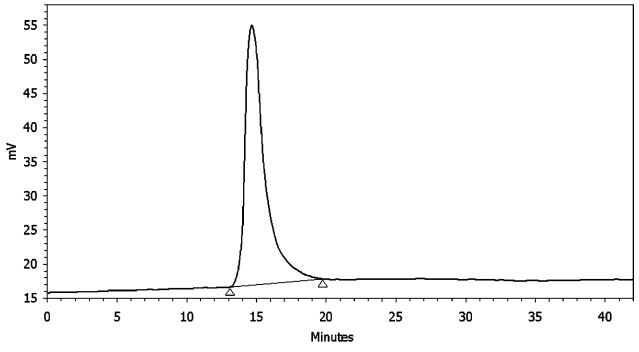


Figure 1. Profile of FPS-1 in HPGPC, with 0.1 mol/L NaCl at 0.6 mL/min.

Methylation analysis of FPS-1 by GLC–MS revealed three types of glucose derivatives in a relative molar ratio of 1.0:3.0:1.0 (Table 1). The identification of 2,4-di-*O*-methylglucitol acetate indicated that FPS-1 was a branched glucan.[8] The 2,3,4,6-tetra-*O*-methylglucitol acetate showed the presence of terminal non-reducing glucose, while 2,3,4-tri-*O*-methylglucitol was indicative of (1→6)-glucosyl residues which had the largest proportion of residues in the polysaccharide. Based on these results, an α-(1→6)-glucan with branches at C-3 position was indicated. However, methylation analysis was not sufficient for distinguishing the different surroundings of residues with the same linkage pattern. As there was no 2,4,6-tri-*O*-methylglucitol acetate detected (indicating the absence of (1→3)-glucosyl residues), it was postulated that the branching chains might be composed of a single terminal glucose or together with not more than three (1→6)-glucosyl residues.

NMR spectroscopy was used to complete the structural characterization of FPS-1. Chemical shifts of individual residue were assigned (Table 2) according to 1D (^1H, ^{13}C) and 2D ^1H–^1H COSY (not shown), HMQC (Fig. 2) and HMBC (Fig. 3) NMR data and references.[9–12] In the ^1H NMR spectrum, three signals appeared in the anomeric region at δ 5.22 (*d*, $^3J_{1,2}$ 3 Hz, residue **A**), 5.05 (*d*, $^3J_{1,2}$ 3 Hz, residue **B**) and 5.02 ppm (*d*, $^3J_{1,2}$ 3 Hz, residue **C**), and accordingly in the anomeric region of the ^{13}C NMR spectrum, three carbon resonances appeared at δ 101.4, 99.2 and 98.8 ppm. Both results confirmed the presence of three types of glucopyranose residues and their α-configurations, consistent with the GLC and FTIR data. In the ^{13}C NMR spectrum, the signal at δ 61.1 ppm was reasonably assigned to the unsubstituted C-6 of terminal residue **A**.[23] The C-3 signal of (1→3, 1→6)-linked residue **C** appeared at δ 81.4 ppm. The relatively downfield carbon chemical shifts at δ 67.8 and 67.5 ppm, caused by the α-glycosylation effect, were assigned to substituted C-6 of residue **B** and **C**. In the ^1H–^{13}C HMQC spectrum, the signals at δ 3.94/81.4 ppm, δ 3.82/61.1 and δ 3.69/61.1 ppm were unambiguously assigned to **C**3 and **A**6, respectively. These specific signals confirmed the presence of O-3 and O-6 substitutions, respectively, and

Table 1. GC–MS of alditol acetate derivatives from the methylated product of FPS-1

Methylated sugars (as alditol acetates)[a]	Type of linkage	Retention time[b]	Molar %	Mass fragments (*m*/*z*)
2,3,4,6-Me_4-Glc	Terminal Glc*p*	1.00	1.0	43,45,71,87,101,117,129,145,161,205
2,3,4-Me_3-Glc	1,6-Linked Glc*p*	2.46	3.0	43,87,99,101,117,129,161,189
2,4-Me_2-Glc	1,3,6-Linked Glc*p*	3.57	1.0	43,87,117,129,189

[a] 2,3,4,6-Me_4-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucose, etc.

[b] Relative retention times of the corresponding alditol acetate derivatives relative to 2,3,4,6-tetra-*O*-methyl-D-glucose on a column of DB-225 at 230 °C with He flow of 60 mL/min.

Table 2. ^{1}H and ^{13}C NMR chemical shifts of fuzi polysaccharide in D_2O and interresidue correlations from HMBC spectra

Residue	$\delta^{13}C/^{1}H$ (ppm)							Interresid. HMBC from H-1
	1	2	3	4	5	6 (6a)	6b	
A, α-D-Glc*p*-(1→	101.4	72.7	74.4	70.8	73.5	61.1		
	5.22	3.56	3.79	3.41	3.84	3.82	3.69	$H1_A/C3_C$
B, →6)-α-D-Glc*p*-(1→	98.8	72.6	74.4	69.7	72.2	67.8		
	5.05	3.63	3.77	3.49	3.94	4.02	3.75	$H1_B/C6_C$, $H1_B/C6_B$
→6)-α-D-Glc*p*-(1→								
3	99.2	71.7	81.4	70.5	72.8	67.5		
↑	5.02	3.68	3.94	3.56	3.92	3.91	3.78	$H1_C/C6_B$

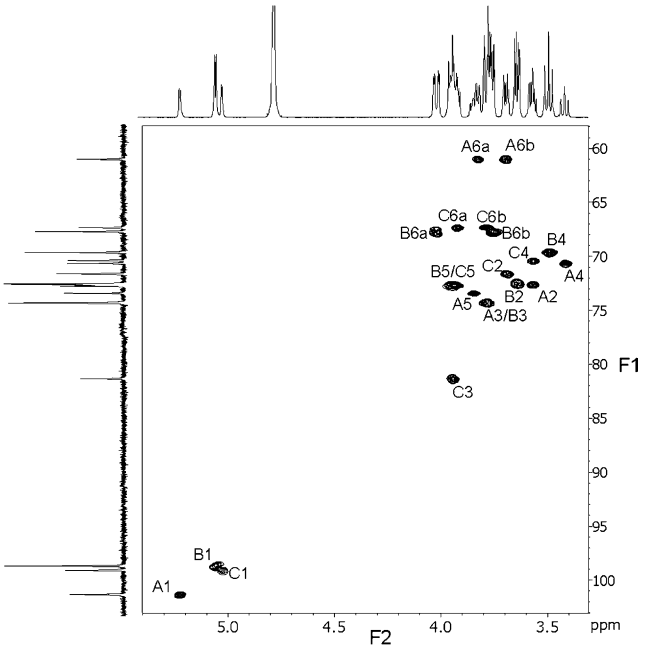


Figure 2. HMQC spectrum of FPS-1. A1 represents the cross-peak between H-1 and C-1 of residue **A**, etc.

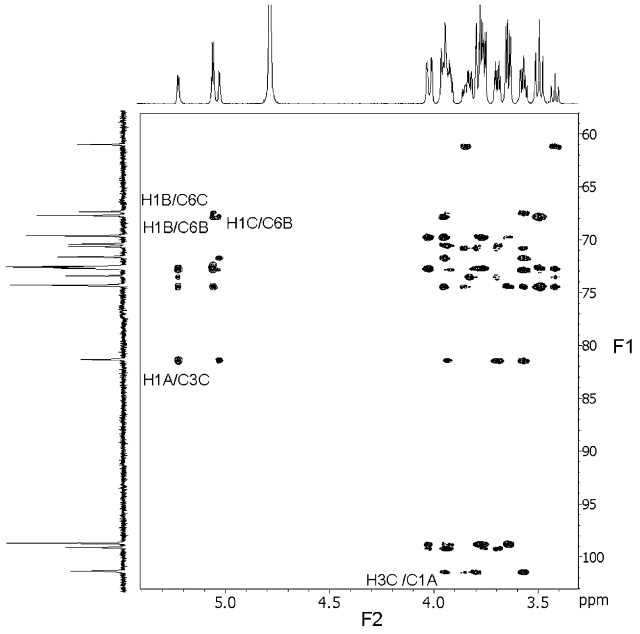
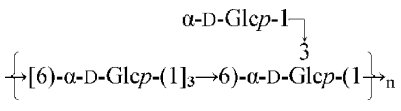


Figure 3. HMBC spectrum of FPS-1, interresidue connectivities identified. H1A/C3C represents the correlation between H-1 of residue **A** and C-3 of residue **C**, etc.

facilitated the sequential assignment of the other resonance signals. Then, the ^{1}H chemical shifts of each of the three residues were uniquely assigned from the ^{1}H–^{1}H COSY spectrum, and the HMQC spectrum allowed the corresponding assignments of the remaining ^{13}C chemical shifts of each residue. All residues were present as pyranoses as deduced from their C-4 carbon chemical shifts (Table 2, above 80 ppm for furanoses[13]), corroborated by the correlation signals from anomeric protons to intraresidue C-5 in the HMBC spectrum.

Correlations between the anomeric protons and the transglycosidic carbons could be observed in the HMBC spectrum (Fig. 3) that confirmed the monosaccharide sequence of FPS-1 and attachment sites. The interresidual $^{1}H/^{13}C$ cross-peaks $H1_A/C3_C$ (δ 5.22/81.4 ppm) and $H3_C/C1_A$ (δ 3.94/101.4 ppm) indicated the **A**-(1→3)-**C** sequence, which was the terminal residue linkage. The interresidual connectivity $H1_B/C6_B$ (δ 5.05/67.8 ppm) demonstrated the **B**-(1→6)-**B** sequence. Furthermore, the interresidual connectivities $H1_C/C6_B$ (δ 5.02/67.8 ppm) and $H1_B/C6_C$ (δ 5.05/67.5 ppm) were observed, thus establishing the presence of the **B**-(1→6)-**C**-(1→6)-**B** sequence, which formed the backbone chain of this polysaccharide. The NMR spectral analysis confirmed that, as supported by the conclusions drawn from methylation data, FPS-1 had a backbone chain of (1→6)-glucosyl residues, with one out of four residues being substituted at the C-3 position, on average, with one single glucose, since no evidence of NMR peaks attributable to the presence of any **A**-(1→6)-**B** sequence was detected. Taken together, the putative structure of FPS-1 was established as



2.2. Immunomodulating effects

Lymphocyte proliferation is a crucial event in the activation cascade of both cellular and humoral immune responses. The effects of FPS-1 on lymphocyte

proliferation induced by ConA or LPS were tested both in vitro and in vivo (Tables 3 and 4). Spleen lymphocyte proliferation induced by ConA in vitro may be used as a method to evaluate T lymphocyte activity, while that induced by LPS may be used to examine B lymphocyte activity.[14] FPS-1 produced statistically significant dose-dependent increases in ConA-induced splenocyte proliferation at 10 or 100 μg/mL in vitro, and in LPS-induced splenocyte proliferation at 25–100 mg/kg in vivo. These results suggested that FPS-1 could dose-dependently promote both murine T-and B-cell proliferation induced by specific mitogens.

In addition, as a model of primary T-cell-dependent splenocyte antibody production in response to antigenic stimulation, the antibody-producing capacity of splenocytes in response to sheep red blood cell (SRBC) was determined by quantitative hemolysis of SRBC.[14] The optical density values at 520 nm were significantly increased at the doses of FPS-1 from 25 to 100 mg/kg, indicating that FPS-1 could promote humoral immunity in vivo by stimulating antibody-secreting B-cells in the spleen. There was no observed cytotoxicity in cells as judged by cell viability within the experimental concentration range.

This is the first time such an immunostimulating polysaccharide has been isolated and purified from the Chinese herb, fuzi, and identified as a highly (1→3)-branched α-(1→6)-D-glucan. In the past decade, β-glucans consisting of (1→3)-glucopyranosyl units with (1→6)-linked side chains of varying distribution and length have been widely used as biological response modifiers (BRMs), which activate leukocytes, stimulating their phagocytotoxic and antimicrobial activities, and potentiate host responses against a variety of conditions, including tumor development and infections.[15–17] However, α-glucans with similar linkage patterns have also been found to posses potent immunological properties, such as those of a linear α-(1→3)-D-glucan,[18,19] a α-(1→6)-D-glucan from the roots of *Ipomoea batatas*,[20] and an α-D-glucan composed of a (1→4)-linked backbone and (1→6)-linked branches.[21] Recently, a novel (1→6)-branched (1→3)-glucan containing both α and fewer β linkages, isolated from *Pleurotus florida*, was reported.[22] This glucan was found to activate the phagocytic response of mice macrophages, although it is not of exactly the same structure as the glucan reported in this paper. Thus, FPS-1 may be explored as a potential natural immunomodulator, and further studies are needed to elucidate the structure–function relationships and mechanisms responsible for its immunological activities.

Table 3. Effects of FPS-1 from *Aconitum carmichaeli* on ConA- or LPS-induced proliferation activity of mouse splenocytes in vitro[a]

Preparation	Concentrations (μg/mL)	ConA (A_{570})	LPS (A_{570})
Control	—	0.33 ± 0.01	0.32 ± 0.01
FPS-1	1	0.35 ± 0.01	0.37 ± 0.04
	10	0.39 ± 0.02[b]	0.44 ± 0.01[c]
	100	0.41 ± 0.01[b]	0.47 ± 0.02[c]

[a] Results are represented as mean ± S.D. based on three independent experiments.
[b] $P < 0.05$, significantly different from the control.
[c] $P < 0.01$, significantly different from the control.

Table 4. Effects of FPS-1 from *Aconitum carmichaeli* on ConA- or LPS-induced spleen lymphocyte proliferation and antibody production in mice[a]

Preparation	Dose (mg/kg)	ConA (A_{570})	LPS (A_{570})	Antibody (A_{520})
Control	—	0.32 ± 0.01	0.33 ± 0.01	0.54 ± 0.09
FPS-1	25	0.33 ± 0.01	0.40 ± 0.02[b]	0.63 ± 0.01[c]
	50	0.41 ± 0.03[b]	0.43 ± 0.02[c]	0.66 ± 0.04[c]
	100	0.45 ± 0.01[c]	0.44 ± 0.01[d]	0.77 ± 0.06[d]

[a] Results are expressed as mean ± S.D. from 10 mice in each group.
[b] $P < 0.05$, significantly different from the control.
[c] $P < 0.01$, significantly different from the control.
[d] $P < 0.001$, significantly different from the control.

3. Experimental

3.1. Materials

Dried fuzi was purchased from Guangdong Traditional Chinese Medicinal Corp. and identified by Dr. Jinping Li, the Institute of Integrated Traditional Chinese and Western Medicine, Sun Yat-sen University. A voucher specimen (No.: 030427) was deposited in the Institute of Integrated Traditional Chinese and Western Medicine, Sun Yat-sen University. Sephadex G-75 was purchased from Pharmacia Biotech and DEAE-cellulose from Sigma Chemical Co. (St. Louis, MO, USA). Aqueous solutions were prepared with purified water from a Milli-Q system (Millipore, Bedford, MA, USA).

Trifluoroacetic acid (TFA) and Me_2SO were purchased from E. Merck. Concanavalin A (ConA) and lipopolysaccharide (LPS) were from Sigma, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Fluka. Medium RPMI-1640 was purchased from Gibco Laboratories. The RMPI-1640 medium, used for immunological tests, was supplemented with HEPES buffer 10 μmol/mL, penicillin 100 IU/mL, streptomycin 100 μg/mL, L-glutamine 2 μmol/mL, 2-mercaptoethanol 50 μmol/L and 10% newborn bovine serum, pH 7.2. All other reagents were of the highest available quality.

3.2. General methods

The specific rotation was determined at 20 °C with an automatic polarimeter (Model AA-10, Optical Activity Ltd, UK). UV–vis absorption spectra were recorded

with a UNICO UV-2000 spectrophotometer. The FTIR spectra (KBr pellets) were recorded on a Nicolet 550 FTIR spectrophotometer. Elemental analysis (C,H,N) was conducted on a Perkin–Elmer 2400 instrument. Total carbohydrate content was determined by the phenol–sulfuric acid method as D-glucose equivalents.[23] Protein was detected by the Lowry method.[24] Dialysis was carried out by using dialysis tubing (Spectra/Por MWCO: 3500).

3.3. Isolation and purification of FPS-1

The dried aconite root was crushed into fine particles (100 g) and defatted with 95% EtOH, then extracted with 1 L of double-distilled water for 2 h at 100 °C and filtered. The residue was further extracted with 750 mL of water for 1 h. The combined aqueous extracts were concentrated in a rotary evaporator under reduced pressure at 50 °C and filtered. Then the filtrate was mixed with 4 vol of 95% EtOH at 4 °C, followed by centrifugation at 5000 *g* for 20 min. The precipitate was dissolved in 300 mL of water and deproteinized three times with 60 mL of 5:1 $CHCl_3$–*n*-BuOH as described by Sevag.[25] The resulting aqueous fraction was extensively dialyzed against double-distilled water for 3 days and precipitated by addition of 3 vol of 95% EtOH. After centrifugation the precipitate was washed with anhydrous EtOH and then vacuum-dried at 40 °C to yield the crude polysaccharide (4.6 g). The crude polysaccharide (500 mg) was dissolved in distilled water, applied to a DEAE-cellulose column (2.0 × 40 cm) and eluted with water. Each fraction of 4 mL was collected at a flow rate of 40 mL/h and monitored by the phenol–sulfuric acid method at 490 nm.[23] The collected fractions were dialyzed and lyophilized. The product (50 mg) was further chromatographed on a Sephadex-G75 column (1.5 × 40 cm) with water and lyophilized to give 48 mg of white power, FPS-1, which was subjected to the subsequent analyses.

3.4. Gel-permeation chromatography (GPC) analysis

The homogeneity and molecular weight of FPS-1 was determined on a Waters HPLC system (UK6 injector and 510 HPLC pump, Waters, Milford, MA) equipped with a Waters Ultrahydrogel 250 column (7.8 × 300 mm) and a Waters 410 differential refractometer. A sample solution (20 μL of 0.1%) was injected in each run, with 0.1 mol/L NaCl as the mobile phase at 0.6 mL/min. The HPLC system was precalibrated with pullulan standards (Shodex Standard P-82, Waters).

3.5. Composition analysis

FPS-1 (5 mg) was dissolved in 6 mL of 2 mol/L TFA and hydrolyzed at 120 °C for 2 h, followed by evaporation and addition of MeOH to the residue. The hydrolyzate was reduced with $NaBH_4$ for 3 h at room temperature. The excess $NaBH_4$ was decomposed with HOAc and removed by repeated evaporation to dryness with the addition of 10% (v/v) HOAc in MeOH. Alditol acetates of the reduced sugars and authentic standards (D-glucose, D-mannose, D-galactose, D-fucose and D-arabinose with *myo*-inositol as the internal standard) were prepared with AC_2O at 100 °C for 1h and subjected to GLC analysis on an HP 6890 GC (Hewlett–Packard, Wilmington, USA) fitted with a capillary column DB-225 (30 m × 0.25 mm i.d., film thickness 0.25 μm) and a flame-ionization detector.[26] High-purity helium was used as the carrier gas at a flow rate of 1.2 mL/min. The oven temperature was kept for 3 min at 190 °C following injection of 1-μL samples in a split ratio of 1:10 and then raised at 4 °C min^{-1} to 230 °C. The injector and detector temperatures were 250 °C and 270 °C, respectively.

3.6. Methylation analysis

Methylation analysis was performed according to the procedures of Ciucanu and Kerek.[27] Briefly, the FPS-1 sample was dissolved in Me_2SO under nitrogen and permethylated by treatment with NaOH powder and iodomethane. Partially methylated alditol acetates were prepared from fully methylated samples by acid hydrolysis with 2 mol/L TFA at 120 °C for 1 h, reduction of the hydrolysates with $NaBH_4$, followed by acetylation with AC_2O. The alditol acetates were analyzed by GLC–MS on the HP 6890 GC equipped with an HP 5973 mass-selective detector. The GLC conditions were similar as described above, with interface and ion-source temperature both at 250 °C. EIMSs were recorded at 70 eV, with detector volts at 1.5 kV. The components were identified by a combination of the main fragments in the MS and relative retention times in the GC, and the molar ratios were estimated from the peak areas and response factors.[28,29] Data analyses were performed and controlled by the HP Chemstation software.

3.7. NMR spectroscopy

The samples were deuterium-exchanged several times by lyophilization from D_2O and then examined in 99.99% D_2O (4 mg/mL). Spectra were recorded at 300 K on a Bruker AMX-500 spectrometer operating at 500.13 MHz for ^1H and 125.75 MHz for ^{13}C. Chemical shifts are given in ppm, with acetone as an internal chemical shift reference (δ_H 2.225; δ_C 31.45). The 2D homonuclear ^1H–^1H COSY spectra were acquired using the double quantum filtered (DQF) method with a Bruker standard pulse sequence. The ^1H detected heteronuclear multiple quantum coherence (HMQC) spectra

were acquired with ^{13}C-decoupling by using the GARP (globally optimized alternating-phase rectangular pulses) sequence. The spectra were recorded with 256 experiments of 2048 data points and 64 scans per increment. The heteronuclear multiple-bond correlation (HMBC) experiment was performed with 256 increments of 2048 real points over a spectral width of 3000 Hz in the acquisition domain F2 and 26000 Hz in the time domain F1. A total of 192 scans were used per increment with a delay of 60 ms for the evolution of long-range couplings.

All spectra were processed with Bruker XWINNMR software. Squared sine-bell function was applied in both dimensions prior to Fourier transformation, and zero-filling was used in F1 to give a 512×2048 matrix.

3.8. Measurement of immunomodulating activity

3.8.1. Animals and preparation of mouse spleen cells. Female BALB/c mice, weighing 20 ± 2 g, were divided randomly into four groups: saline control group, 25, 50 and 100 mg/kg FPS-1 dose groups. The groups were immunized intraperitoneally (ip) with 0.2 mL/mouse of 5% SRBC suspension in 0.9% saline on day 0.[30] FPS-1 samples were prepared from 0.9% saline solution and injected ip into the mice of treatment groups from day 1 to day 6, respectively. The control mice were injected with an equal volume of sterile saline. The mice were sacrificed on day 7, and the spleens were removed, minced and passed through a sterilized ion mesh (200 mesh) to obtain single spleen cell suspensions. Erythrocytes in the cell mixture were destroyed by the rapid addition of H_2O. Then, the cells were washed twice with phosphate-buffered saline (PBS) and adjusted to a density of 5×10^6 cells/mL in the RPMI-1640 medium.

3.8.2. Immunomodulating effect on mouse splenocytes in vivo. An aliquot of 100 μL of splenocytes prepared from immunized mouse was seeded into each well of a 96-well plate in the presence of ConA (5.0 μg/mL) or LPS (10.0 μg/mL). After preincubation for 48 h at 37 °C in a humidified 5% CO_2 incubator, 10.0 μL of 0.4% MTT was added into each well.[31] The plate was incubated for another 4 h, and then the solution was dissolved in Me_2SO (100 μL/well). Absorbance at 570 nm was measured on an ELISA reader (Model EL310, Bio-TEK Instruments). Each experiment was performed in triplicate.

3.8.3. Immunomodulating effect on mouse splenocytes in vitro. An aliquot of 100 μL of normal mouse splenocytes, prepared following the procedure described above, was mixed with FPS-1 (1, 10, 100 μg/mL, final concentration) and incubated in the presence of ConA or LPS as described above. Absorbance at 570 nm was measured. The control experiments were performed without FPS-1. All experiments were performed three times independently.

3.8.4. Determination of the humoral immune response. The effect of FPS-1 on splenocyte antibody production in vivo was evaluated using the quantitative hemolysin spectrophotometry assay.[32,33] After the mice were sacrificed on day 7, a suspension of spleen cells (2×10^7 cells/mL) was prepared. SRBC was diluted with PBS (pH 7.2) to 1:20. Then 1.0 mL of splenocyte suspension was incubated with 1.0 mL of SRBC and 1.0 mL of 1:10 diluted guinea-pig serum at 37 °C for 1.5 h and centrifuged at 3000 *g* for 3 min. The extent of hemolysis of SRBC in the supernatant was determined at 520 nm.

3.8.5. Statistical analysis. The data were expressed as mean ± S.D. Significance of difference was evaluated with one-way ANOVA, followed by Dunnett's test to statistically identify differences between the control and treated groups.

Acknowledgements

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References

1. *The State Pharmacopoeia Commission of the People's Republic of China Pharmacopoeia of the People's Republic of China*; Chemical Industry Press: Beijng, 2000; Vol. 1, pp 149–150.
2. Qin, L.; Zhang, S. H.; Li, X. L. *Zhongguo Zhong Yao Za Zhi* **2002**, *27*, 541–544; MEDLINE, AN 2003253446.
3. Shan, B. E.; Zhang, J. Y.; Li, Q. X. *Zhongguo Zhong Xi Yi Jie He Za Zhi* **2001**, *21*, 768–772; MEDLINE, AN 2003065772.
4. Taki, M.; Niitu, K.; Omiya, Y.; Noguchi, M.; Fukuchi, M.; Aburada, M.; Okada, M. *Planta Med.* **2003**, *69*, 800–803.
5. Konno, C.; Murayama, M.; Sugiyama, K.; Arai, M.; Murakami, M.; Takahashi, M.; Hikino, H. *Planta Med.* **1985**, *51*, 160–161.
6. Bourne, E. J.; Stephens, R. *Annu. Rev. Biochem.* **1956**, *25*, 79–100.
7. Hara, C.; Kiho, T.; Ukai, S. *Carbohydr. Res.* **1983**, *117*, 201–213.
8. Sasaki, T. J. C.; Takasuka, N. *Carbohydr. Res.* **1976**, *47*, 99–104.
9. Olsthoorn, M. M.; Petersen, B. O.; Duus, J.; Haverkamp, J.; Thomas-Oates, J. E.; Bock, K.; Holst, O. *Eur. J. Biochem.* **2000**, *267*, 2014–2027.
10. Colson, P.; Jennings, H. J.; Smith, I. C. *J. Am. Chem. Soc.* **1974**, *96*, 8081–8087.
11. Agrawal, P. K. *Phytochemistry* **1992**, *31*, 3307–3330.
12. Bergstrom, N.; Jansson, P. E.; Kilian, M.; Skov Sorensen, U. B. *Eur. J. Biochem.* **2000**, *267*, 7147–7157.

13. Marino, C.; Varela, O.; de Lederkremer, R. M. *Carbohydr. Res.* **1989**, *190*, 65–76.
14. Han, S. B.; Kim, Y. H.; Lee, C. W.; Park, S. M.; Lee, H. Y.; Ahn, K. S.; Kim, I. H.; Kim, H. M. *Immunopharmacology* **1998**, *40*, 39–48.
15. Brown, G. D.; Gordon, S. *Immunity* **2003**, *19*, 311–315.
16. Pfeffer, P. E.; Osman, S. F.; Hotchkiss, A.; Bhagwat, A. A.; Keister, D. L.; Valentine, K. M. *Carbohydr. Res.* **1996**, *296*, 23–37.
17. Kulicke, W. M.; Lettau, A. I.; Thielking, H. *Carbohydr. Res.* **1997**, *297*, 135–143.
18. Kiho, T.; Yoshida, I.; Nagai, K.; Ukai, S.; Hara, C. *Carbohydr. Res.* **1989**, *189*, 273–279.
19. Bao, X.; Duan, J.; Fang, X.; Fang, J. *Carbohydr. Res.* **2001**, *336*, 127–140.
20. Zhao, G.; Kan, J.; Li, Z.; Chen, Z. *Int. Immunopharmacol.* **2005**, *5*, 1436–1445.
21. Raveendran Nair, P. K.; Rodriguez, S.; Ramachandran, R.; Alamo, A.; Melnick, S. J.; Escalon, E.; Garcia, J.; Wnuk, S. F.; Ramachandran, C. *Int. Immunopharmacol.* **2004**, *4*, 1645–1659.
22. Rout, D.; Mondal, S.; Chakraborty, I.; Pramanik, M.; Islam, S. S. *Carbohydr. Res.* **2005**, *340*, 2533–2539.
23. Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350–356.
24. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
25. Sevag, M. G.; Lackman, D. B.; Smolens, J. *J. Biol. Chem.* **1938**, *124*, 425–436.
26. Blakeney, A. B.; Harris, P. J.; Henry, R. J.; Stone, B. A. *Carbohydr. Res.* **1983**, *113*, 291–299.
27. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.
28. Tsumuraya, Y.; Misaki, A.; Takaya, S.; Torii, M. *Carbohydr. Res.* **1978**, *66*, 53–65.
29. Sweet, D. P.; Shapiro, R. H.; Albersheim, P. *Carbohydr. Res.* **1975**, *40*, 217–255.
30. Bao, X.; Liu, C.; Fang, J.; Li, X. *Carbohydr. Res.* **2001**, *332*, 67–74.
31. Wang, Y. P.; Li, X. Y.; Song, C. Q.; Hu, Z. B. *Acta Pharmacol. Sin.* **2002**, *23*, 263–266, CAPLUS, AN 2002:210385.
32. Heeg, K.; Reimann, J.; Kabelitz, D.; Hardt, C.; Wagner, H. *J. Immunol. Methods* **1985**, *77*, 237–246.
33. Kim, H. M.; Oh, G. T.; Hong, D. H.; Hyun, B. H.; Cha, Y. N.; Yoo, B. S.; Han, S. B. *Immunopharmacology* **1996**, *34*, 39–50.