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Structural elucidation and antitumor activity of a fructan from *Cyathula officinalis* Kuan

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Abstract

A fructan named CoPS3 was isolated from *Cyathula officinalis* Kuan. The structure of CoPS3 was determined by methylation, by the reductive–cleavage method combined with GC–MS analysis, and both 1D and 2D ^{1}H and ^{13}C NMR spectroscopy. These results show that CoPS3 is a graminans-type fructan that is comprised of a β-D-fructofuranosyl backbone having residues linked (2→1)- and (2→6) with branches and an α-D-glucopyranose residue on the nonreducing end of the fructan chain. Each branch is terminated by a β-D-Fru*f* residue. Bioassay showed that it could inhibit growth of Lewis pulmonary carcinoma implanted in mice.

Keywords: Fructan; Reductive–cleavage; *Cyathula officinalis* Kuan; Antitumor

1. Introduction

Fructans exist as a wide range of oligo- and polysaccharides in many species of bacteria, fungi, and plants.[1] They are classified into different families on the basis of their glycosidic linkages, consisting of (2→1)-linked β-D-fructofuranosyl units such as inulin, or (2→6)-linked β-D-fructofuranosyl units such as levans, or highly branched structures comprised of both (2→1)- and (2→6)-linked β-D-fructofuranosyl units such as graminans.[2,3] However, there are few reports ever published on the bioactivity of fructans.

Cyathula officinalis Kuan is a well-known traditional Chinese herbal medicine that has been found to possess the abilities of 'promoting blood circulation to remove blood stasis, promoting diuresis and relieve stranguria etc'.[4] Chen and Liu[5] reported that a crude polysaccharide component isolated from *Cy. officinalis* Kuan has antitumor activity, but there were no reports about the structure of the polysaccharide. In this paper, the structure of the fructan (CoPS3) is reported, and we have used the reductive–cleavage method for analysis of the structure of the β-D-fructans because it enables a better discrimination of (2→1) and (2→6) linkages than traditional methods.

2. Results and discussion

CoPS3 was isolated from the root of *Cy. officinalis* Kuan and purified by CM-Sephadex C-50 and Sephadex G-50 column chromatography. The chemical homogeneity of CoPS3 was examined by High-performance liquid chromatography (HPLC) and Capillary electrophoresis (CE). The average molecular weight was 1400 Da, determined by electrospray-ionization mass spectrometry (ESIMS). Monosaccharide composition determined by HPLC gave fructose and glucose in a molar ratio of 21:1 (Fig. 1).

To determine the linkage of monosaccharides in the fructan, the methylated–acetylated derivatives of CoPS3 were analysed by GC–MS. The peak area ratio of methylated fragments was used as a molar ratio, and the integrated peak areas were corrected using the effective-carbon response method.[6] The gas chro-

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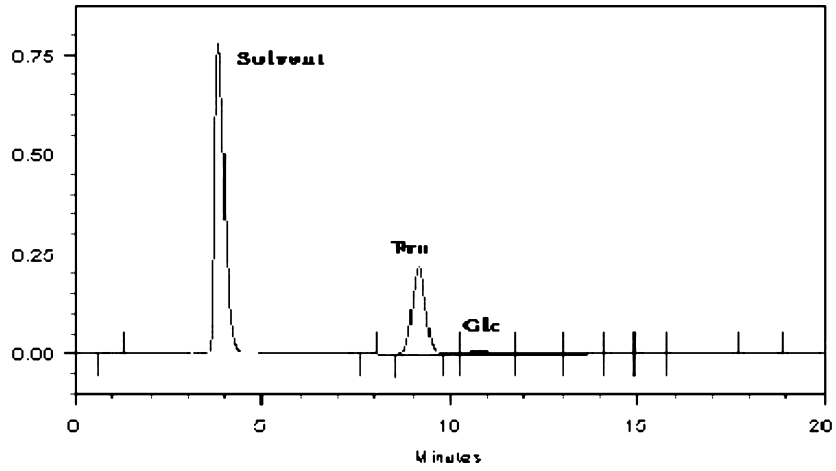

Fig. 1. The HPLC of the acid hydrolysates of CoPS3.

matogram of products is shown in Fig. 2. The fragmentation patterns (Table 1) of the methoxy derivatives were identical to those of the published data.[7–10]

The reductive–cleavage method can discriminate between (2→1)- and (2→6)-linked β-D-Fru*f* residues. The (2→1)- and (2→6)-linked β-D-Fru*f* residues give the same mannitol derivative **4**, but different glucitol derivatives **6** and **5**. The mannitol:glucitol ratio (4.1 ± 0.2) remains constant for inulin, where only (2→1)-linked β-D-Fru*f* residues were detected.[9] Thus, ($\mathbf{6} + 4.1 \times \mathbf{6}$) gives the proportion of (2→1)-linked residues and ($\mathbf{4} - 4.1 \times \mathbf{6}$) is added to **5** to give the proportion of (2→6)-linked residues. Therefore, the ratio of (2→1)- and (2→6)-linkages can be estimated. Table 2 shows the proportions of the several types of linked β-D-Fru*f* and α-D-Glc*p* residues.

According to Table 2, CoPS3 has a highly branched structure with a (2→1)- and (2→6)-linked backbone. 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* residue was in the nonreducing terminal positions, and was linked only at the 1-position.

^{13}C NMR spectroscopy was used to verify the linkages deduced by GC–MS. The chemical shifts of the carbons of CoPS3 were compared to those deduced for fructose, sucrose, bacterial and plant levans, and inulin.[11–14] The spectrum of CoPS3 (Fig. 3) contained six groups of intense signals due to the fructosyl carbon

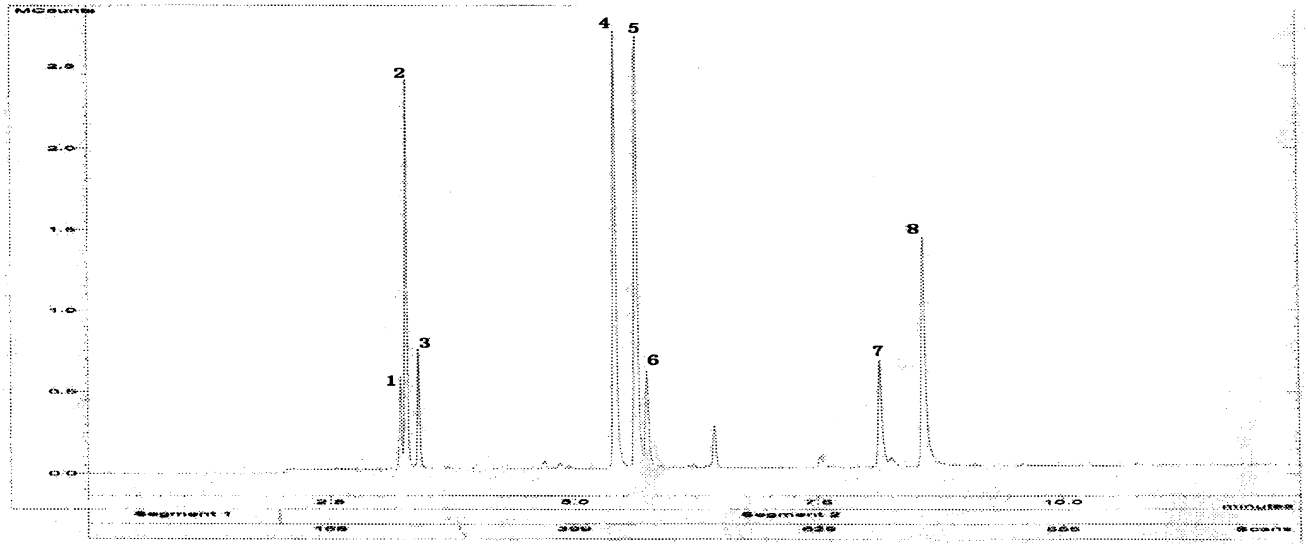

Fig. 2. The gas chromatogram of the products derived by the reductive–cleavage of the CoPS3: **1**, 1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol; **2**, 2,5-anhydro-1,3,4,6-tetra-*O*-methyl-D-mannitol; **3**, 2,5-anhydro-1,3,4,6-tetra-*O*-methyl-D-glucitol; **4**, 1-*O*-acetyl-2,5-anhydro-3,4,6-tri-*O*-methyl-D-mannitol; **5**, 6-*O*-acetyl-2,5-anhydro-1,3,4-tri-*O*-methyl-D-glucitol; **6**, 1-*O*-acetyl-2,5-anhydro-3,4,6-tri-*O*-methyl-D-glucitol; **7**, 1,6-di-*O*-acetyl-2,5-anhydro-3,4-di-*O*-methyl-D-mannitol; **8**, 1,6-di-*O*-acetyl-2,5-anhydro-3,4-di-*O*-methyl-D-glucitol.

Table 1
EIMS fragmentations of the derivatives produced by reductive–cleavage and acetylation [a]

1,5-anhydro-2,3,4,6-tetra-*O*-methyl-D-glucitol (**1**): *m*/*z* 41 (22%), 43 (23), 45 (53), 55 (8), 58 (10), 59 (13), 71 (70), 75 (50), 85 (15), 88 (33), 99 (22), 101 (100), 111 (17), 115 (15), 125 (18), 143 (37), 175 (8), 188 (12)

2,5-anhydro-1,3,4,6-tetra-*O*-methyl-D-mannitol (**2**): *m*/*z* 41 (35%), 43 (22), 45 (78), 55 (12), 59 (20), 71 (82), 75 (42), 83 (22), 85 (22), 89 (20), 99 (35), 101 (100), 111 (75), 115 (53), 125 (67), 126 (22), 143 (97), 155 (20), 156 (40), 157 (15), 175 (11), 187 (10), 221 (30)

2,5-anhydro-1,3,4,6-tetra-*O*-methyl-D-glucitol (**3**): *m*/*z* 41 (22%), 43 (16), 45 (63), 55 (10), 59 (17), 71 (32), 75 (18), 87 (22), 89 (19), 99 (23), 101 (100), 111 (38), 115 (22), 125 (7), 143 (50), 221 (6)

1-*O*-acetyl-2,5-anhydro-3,4,6-tri-*O*-methyl-D-mannitol (**4**): *m*/*z* 41 (28%), 43 (91), 45 (48), 53 (14), 55 (15), 59 (15), 69 (17), 71 (77), 75 (17), 83 (30), 85 (30), 87 (20), 99 (18), 101 (80), 111 (100), 114 (30), 115 (58), 117 (20), 125 (52), 126 (48), 143 (80), 156 (10), 157 (12), 171 (7), 188 (8), 203 (5), 216 (3), 249 (21)

6-*O*-acetyl-2,5-anhydro-1,3,4-tri-*O*-methyl-D-glucitol (**5**): *m*/*z* 41 (28%), 43 (82), 45 (47), 55 (11), 59 (13), 69 (20), 71 (72), 83 (18), 85 (20), 87 (23), 101 (100), 111 (92), 114 (45), 115 (38), 117 (72), 125 (17), 143 (43), 156 (15), 189 (7), 203 (5), 249 (30)

1-*O*-acetyl-2,5-anhydro-3,4,6-tri-*O*-methyl-D-glucitol (**6**): *m*/*z* 41 (37%), 43 (100), 45 (60), 55 (17), 59 (15), 69 (25), 71 (72), 75 (17), 83 (20), 85 (30), 87 (41), 99 (18), 101 (95), 111 (63), 114 (40), 115 (28), 117 (25), 125 (40), 126 (47), 143 (17), 157 (7), 188 (9), 249 (6)

1,6-di-*O*-acetyl-2,5-anhydro-3,4-di-*O*-methyl-D-mannitol (**7**): *m*/*z* 41 (20%), 43 (100), 45 (12), 71 (35), 83 (25), 85 (18), 87 (41), 101 (20), 111 (37), 115 (20), 117 (25), 124 (45), 143 (23), 156 (10), 216 (42)

1,6-di-*O*-acetyl-2,5-anhydro-3,4-di-*O*-methyl-D-glucitol (**8**): *m*/*z* 41 (22%), 43 (100), 45 (12), 71 (32), 83 (18), 85 (14), 87 (43), 101 (25), 111 (18), 115 (12), 117 (38), 124 (38), 125 (15), 143 (7), 156 (8), 216 (35)

[a] The mass number is given first, followed by the relative abundance in parentheses.

Table 2
Assignment and molar ratio of β-D-Fru*f* and α-D-Glc*p* residue per molecule of CoPS3

		GC (peak)	Molar ratio
β-D-Fru*f*	Terminal	2, 3	5.8
	1-linked	4, 6	7.6
	6-linked	4, 5	7.4
	1,6-linked	7, 8	6.2
α-D-Glc*p*	Terminal	1	1.0

atoms. There are at least eight different signals for C-2 of the β-D-Fru*f* residues. The signals at 105.8 and 105.9 ppm are typical for C-2 of (2→1)-linked residue, those at 106.1–106.6 ppm are assigned to terminal and branched residues. Those at 106.7 and 106.8 ppm to (2→6)-linked residues. The occurrence of (2→6)-linked residues is also supported by the signal at 82.9 ppm, which can be attributed to C-5 of such a unit or a branched residue. For residues not 6-substituted, the resonance for C-5 was at 83.7 ppm. Strong evidence for (2→6)-linked β-D-Fru*f* residues is the broad signal at 65.9 ppm, which is characteristic of 6-substitution. Table 3 shows the NMR data of CoPS3.

The structure of CoPS3 was further confirmed by its HMQC (Fig. 4) and HMBC spectra (Fig. 5). In the HMBC, 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 shown to be linked only at the 1-position.

In the anomeric region of the HMBC spectra (Fig. 5), 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. And 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, and 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.

There was reported that one glucose molecule can be present, but is not necessary in fructans.[15] On the basis of the results of monosaccharide composition (glucose is very small), GC–MS and ^{13}C NMR analysis suggest that the following structures of CoPS3 (G-Fn and Fn types) are reasonable. (See structure, next page.) Where *n* indicates the chain length of the sequences in the polymer.

Bioassay of CoPS3 showed that it inhibited the growth of Lewis pulmonary carcinoma implanted in mice at levels of 200 (44.17%) and 100 mg/kg (39.05%). The results, along with positive and neutral controls, are listed in Table 4.

3. Experimental

3.1. Material

Cy. officinalis Kuan was the product of Si Chuan Province, People's Republic of China. Sephadex G-50

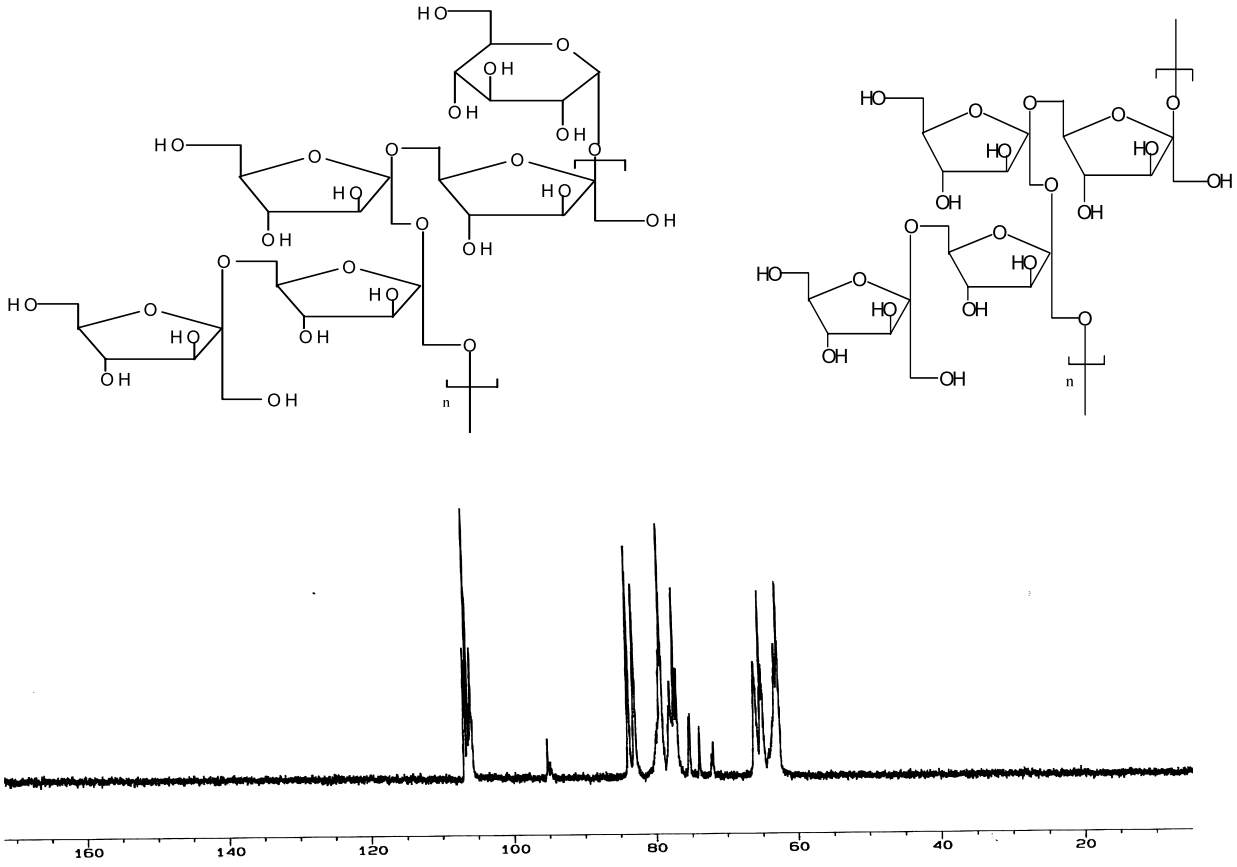

Fig. 3. ^{13}C NMR spectrum (100 MHz) of CoPS3 in D_2O.

Table 3
Chemical shift assignments (ppm) 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.

and CM-Sephadex C-50 were purchased from Ammersham Pharmacia Biotech.

3.2. General

HPLC was performed on a Shimadzu LC-10AD instrument equipped with a TSK-G2000SW exclusion column and water as eluant (1.0 mL/min); the eluate was monitored by an RI detector. CE was performed on a Waters Quanta 4000 E using 0.1 mol/L boric acid–KOH buffer (pH 10) as solvent, with detection at 254 nm. The infrared spectrum (IR) of the methylated polysaccharide was recorded on a Bio-Rad FTS 185 spectrometer. GC–MS was conducted with a Shimadzu QP 5000 instrument. The temperature program was 140–220 °C at 5 °C/min. For quantitative analysis, a HP HP-6890 and an OV-17 capillary column (0.2 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. ESIMS was

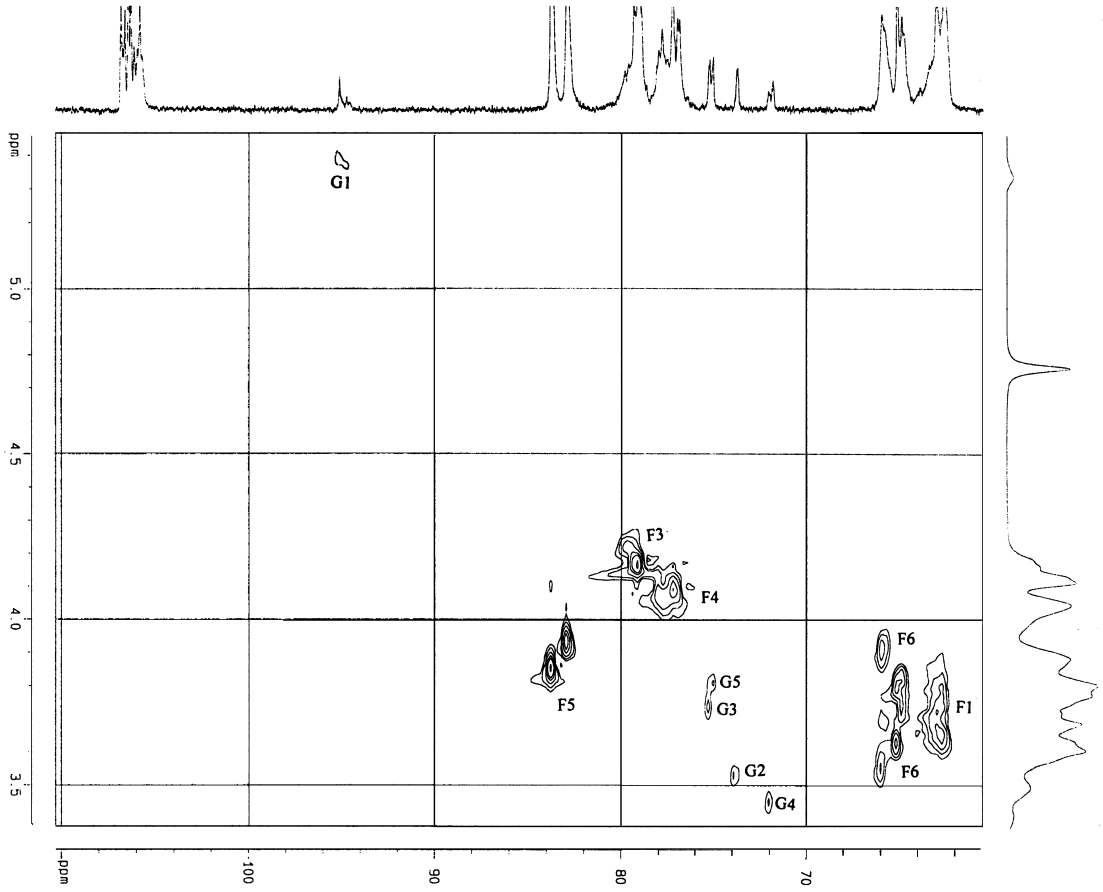

Fig. 4. HMQC spectrum of CoPS3. F: fructofuranose residues; G: glucopyanose residue; G1 connotes the cross-peak between C-1 and H-1 of glucopyanose; F5 connotes the cross-peak between C-5 and H-5 of fructofuranose.

conducted with a PE PerSeptive Mariner instrument, and the data were collected in the negative-ion mode. The NMR spectra were obtained on a Bruker-MX-400 spectrometer equipped with a dual probe, in the FT mode at 50 °C.

3.3. Isolation of the CoPS3

The stems of *Cy. officinalis* Kuan (100 g) were washed, then cut, 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 rpm. Acetone (40 mL) was again added to the solution, followed by centrifugation, 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.

3.4. Purification of CoPS3

CoPS3 was further purified on a CM-Sephadex C-50 column (2×60 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 it was again further purified using Sephadex G-50. The homogeneity of CoPS3 was determined by HPLC and CE.

3.5. Determination of monosaccharide composition

CoPS3 was hydrolyzed with 0.05 mol/L H_2SO_4 at 50 °C for 1 h, neutralized with $BaCO_3$, then centrifuged, filtered, and concentrated. HPLC was carried out on a Carbohydrate Analysis Column (Waters, 3.9 mm I.D. $\times$ 30 cm) with 82:18 MeCN–H_2O as eluent at a flow rate of 1.0 mL/min.

3.6. Methylation

CoPS3 was methylated using the Hakomori method.[16] Dimsyl carbanion was generated by adding hexane-ex-

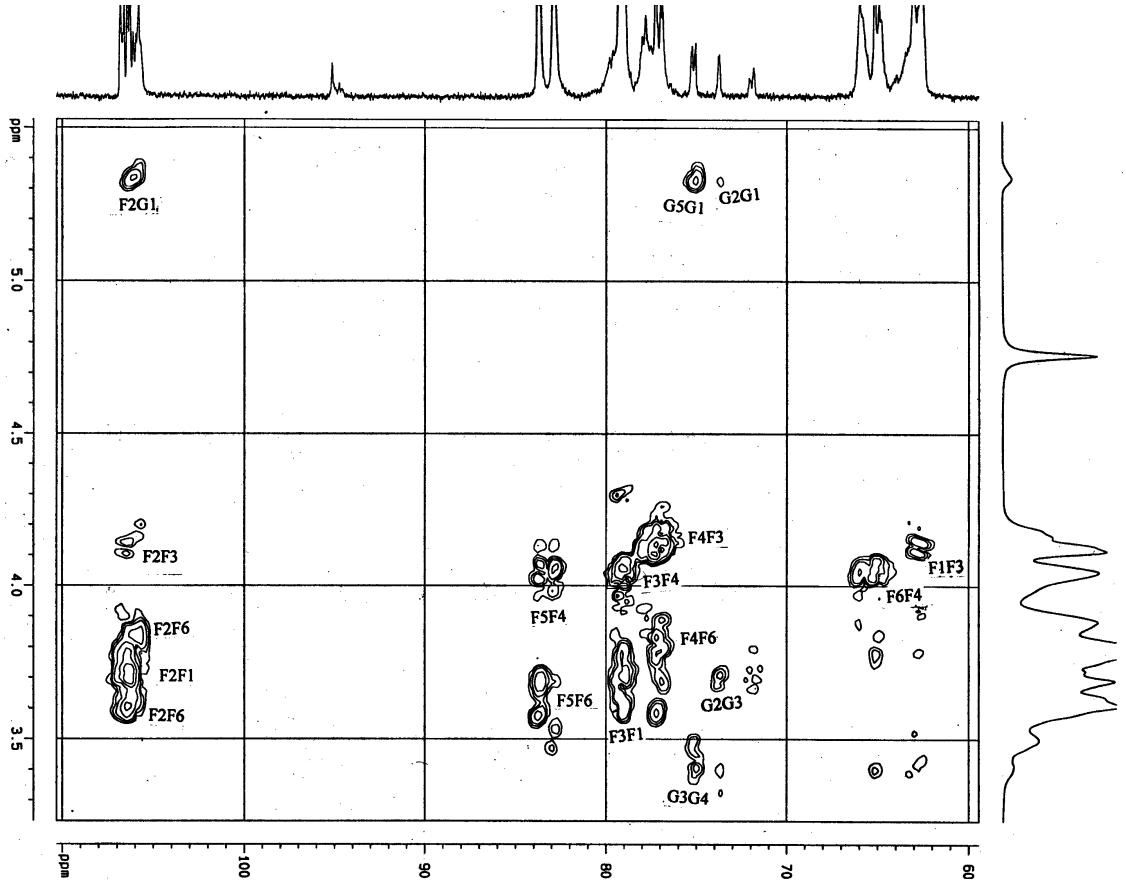

Fig. 5. HMBC spectrum of CoPS3. F: fructofuranose residues; G: glucopyanose residue; F2G1 connotes the cross-peak between C-2 of fructofuranose residues and H-1 of glucopyanose residue.

Table 4
Effect of CoPS3 on the inhibition of Lewis pulmonary carcinoma growth in mice

Sample	Administration method	No. of mice	Dose (mg/kg × days)	Tumor weight (X ± SD)	Inhibition ratio (%)
CoPS3	i.p.	10	200 × 10	0.536 ± 0.08	44.17 *
CoPS3	i.p.	10	100 × 10	0.640 ± 0.07	39.05 *
CTX	i.p.	10	30 × 10	0.124 ± 0.02	88.09 *
Control	i.p.	10		1.05 ± 0.16	

Control: 0.9% NaCl solution. CTX: cyclophosphamide.
* $P < 0.01$, significantly different from CoPS3 with contral.

tracted NaH (25 mg) to Me_2SO (0.5 mL, vacuum distilled at 70 °C and stored over CaH_2 under nitrogen). After warming the Me_2SO mixture for 30 min at 40 °C, the carbohydrate (10 mg) was added and allowed to react for 3 h at 25 °C. Iodomethane (0.8 mL) was added at 0 °C during 20 min and allowed to react overnight at room temperature. The reaction mixture was extracted three times with $CHCl_3$. The combined $CHCl_3$ layers were extracted with water. The dried (Na_2SO_4) $CHCl_3$ layer was evaporated. IR spectroscopy was used to determine the completeness of methylation.

3.7. Reductive–cleavage

The methylated polysaccharide was subjected to reductive–cleavage as described by Rolf and Gray.[17] The reducing agent was prepared from boron $BF_3{\cdot}OEt_2$ (310 μL), Et_3SiH (400 μL), CF_3COOH (64 μL) and CH_2Cl_2 (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 °C. Ac_2O (100 μL) was added, and the temperature was raised to 40 °C for 2 h.[7] The acetylated–methylated products were extracted

against water with CH_2Cl_2. The solution was extracted with water three times and dried down under nitrogen. CH_2Cl_2 was added, and the product was analyzed by GC–MS.

3.8. Assay of antitumor activity

Assay of the antitumor activities of CoPS3 was done by the method of Yu and Zhang.[18] C57BL/c mice were obtained from Shanghai Animal Center of the Chinese Academy of Science, weighting about 20 g for the antitumor assay. Lewis pulmonary carcinoma cells (5×10^6/mL) were transplanted into the toe of the mice. The test samples were dissolved in 0.9% NaCl soln and injected intra-peritoneally daily for 10 days (injection volume, 0.2 mL), starting 24 h after tumor implantation. All mice were kept under observation for 2 weeks and then killed for final evaluation of the effects of treatment on tumor growth. Tumors were excised and weighed. The growth inhibition ratio of tumor growth was calculated by the following equation:

$$\text{Inhibition ratio}\ (\%) = 100 \times [(A - B)/A]$$

where A is the average tumor weight of the control group and B that of the treated group. The results are shown in Table 4.

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