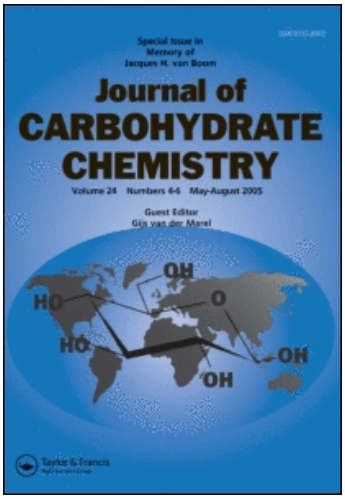

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Hydroxyethylation of *Achryranthes bidentata* Polysaccharide and Investigation of Their Antitumor Activity

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The hydroxyethylated *Achyranthes bidentata* polysaccharides (Abps) were synthesized. The distribution of substituents in these derivatives was studied using HPLC and NMR analysis. The results show that the substituents are not homogeneously distributed along the Abps chain and the reactivity of the hydroxyl groups in the sugar units is OH-4 $\gg$ OH-3 $>$ OH-6 $\approx$ OH-1. Bioassay showed that the derivative with a low degree of substitution (DS) was the best choice to inhibit the growth of Lewis pulmonary carcinoma.

Keywords *Achyranthes bidentata* polysaccharide, Hydroxyethylation, Structural analysis, Reactivity of the hydroxyl groups, Antitumor

INTRODUCTION

Achryranthes bidentata is a well-known traditional Chinese herb medicine, which has the ability of "strengthening the tendons and the bones, nourishing the liver and the kidney, and treating numbness of the waist and knee".[1] It also can promote blood circulation to remove blood stasis. Tian reported that a polysaccharide component, *Achryranthes bidentata* polysacchride (Abps), was isolated from this herb.[2,3] Abps possesses effects of immunomodulation, protection of liver, and inhibition of tumor.[4–6] The structure of Abps has been proved to be (2 $\rightarrow$ 1) and (2 $\rightarrow$ 6)-β-D-fructan with a D-glucose unit at the reducing end,[7]

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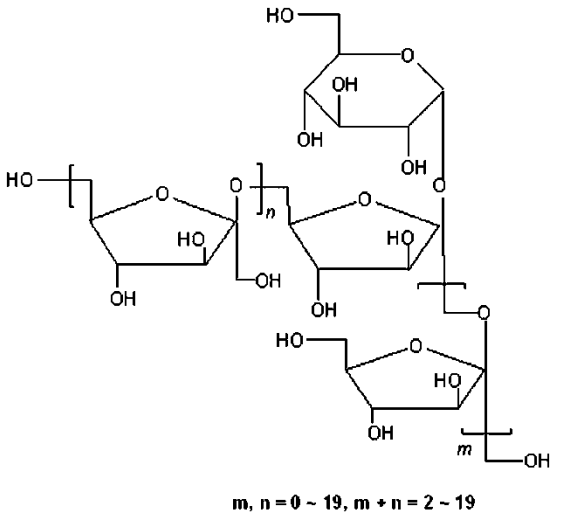
consisting of (2 → 1)-linked β-D fructofuranosyl units such as inulin and (2 → 6)-linked β-D fructofuranose units such as phlein (Sch. 1).

Besides the obvious use of polysaccharide, the new derivative showed interesting properties and biology activities,[8,9] and it might be useful to understand the structure–function relationship of polysaccharides. In this paper, we report the synthesis of hydroxyethylated Abps (HE-Abps). The distribution of substituents was studied using NMR, HPLC, and methylation analysis. The antitumor activity of these modified derivatives is discussed.

RESULTS AND DISCUSSION

Hydroxyethylation of Abps

Abps was hydroxyethylated using two etherification reagents (ERs), 2-chloroethanol and ethylene oxide. The molar ratio of ER to Abps was varied according to Table 1. The degree of substitution (DS) of the isolated reaction products was determined by monosaccharide composition analysis[10] (Table 1). From Table 1, it can be seen that with the increased temperature (entry 1 and entry 2) and NaOH concentration (entry 5 and entry 6), the DS will decrease from 0.39 to 0.30 and from 0.77 to 0.56, respectively. However, the time delay (entry 3 and entry 4) will favor DS little. It was suggested that during hydroxyethylation reactions of polysaccharide in an alkaline medium, there is a competition between the hydroxyethylation and the hydrolysis of 2-chloroethanol to glycol.[10] So the increase of temperature and NaOH concentration will favor the hydrolysis of 2-chloroethanol, thus decreasing ER efficiency and DS.



Scheme 1: Structure of Abps.

Table 1: Influence of reaction conditions on the hydroxyethylation of Abps.

Entry[a]	ER	ER/ Abps[b]	NaOH/ Abps[c]	Temp. (°C)	Time (hr)	Yield (%)	DS
1	$ClCH_2CH_2OH$	2.0	5.0	50	6	67.6	0.39
2	$ClCH_2CH_2OH$	2.0	5.0	80	6	59.2	0.30
3	$ClCH_2CH_2OH$	3.5	8.0	50	6	63.6	0.57
4	$ClCH_2CH_2OH$	3.5	8.0	50	12	56.2	0.62
5	$ClCH_2CH_2OH$	5.0	11.0	50	6	61.0	0.77
6	$ClCH_2CH_2OH$	5.0	15.0	50	6	69.6	0.56
7		6.6	1.6	0	24	88.0	0.34
8		20.0	5.0	0	24	96.0	1.46
9		60.0	15.0	0	24	119.4	1.71

[a]From Abps (0.5 g) in water (20 mL).
[b]Initial ratios of ER or NaOH to Abps monomer units.
[c]The mass of the product compared with the starting material.

The distribution of the substituent was also determined by HPLC analysis. An example of HPLC of the hydrolysates is shown in Figure 1. The hydrolysates were isolated by silica gel chromatography. The monosubstituted monosaccharide (fraction 2), disubstituted monosaccharide (fraction 1), and unsubstituted monosaccharide (fractions 3 and 4) were identified after electrospray-ionization mass spectrometry (ESIMS) analysis. Table 2 shows the distribution of the substituents for HE-Abps with various DS. The product

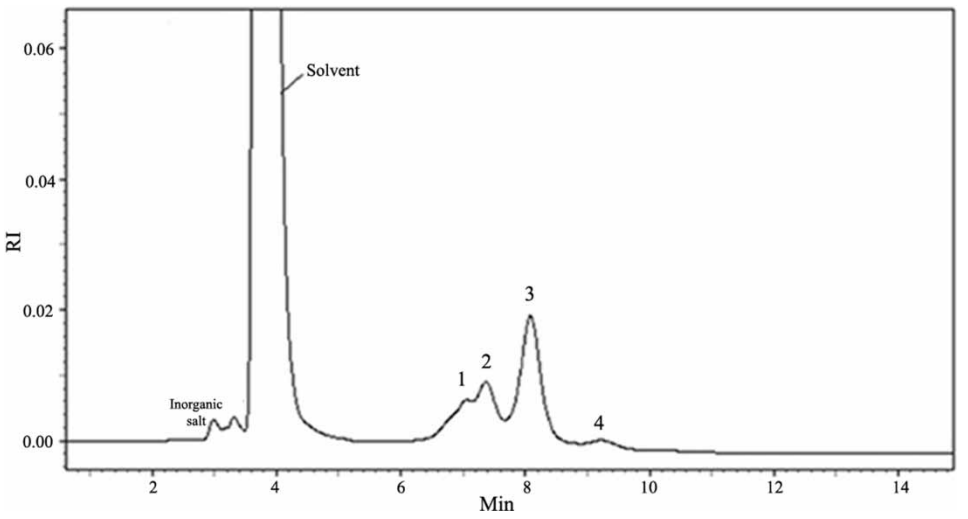


Figure 1: The HPLC of the hydrolysates of HE-Abps (DS 0.34).

Table 2: Distribution of the substituents in mono-, di- and unsubstituted monosaccharide units (mol %) as determined by HPLC analysis.

	Monosaccharide units (mol %)		
DS of HE-Abps	**Unsubstituted**	**Monosubstituted**	**Disubstituted**
0.30	75.8	14.1	8.1
0.39	71.9	17.4	10.8
0.56	60.5	22.9	16.6
0.62	57.1	24.2	18.8
0.77	47.9	27.2	25.0
1.46	17.4	18.8	63.8
1.71	14.6	0	85.4

with the lowest DS (DS 0.3) still contains disubstituted monosaccharide (8.1%), while the product with the highest DS (DS 1.71) still contains unsubstituted monosaccharide (14.6%). When the DS is <0.8, with the increase of DS, mono-, disubstituted units both increase, and the increase in proportion of disubstituted units is larger than that of monosubstituted units. When the DS is >0.8, the monosubstituted unit will decrease (Fig. 2). So, we can conclude that hydroxyethyl groups are not distributed homogeneously, which is due to the difference of steric hindrance of monosaccharide units.

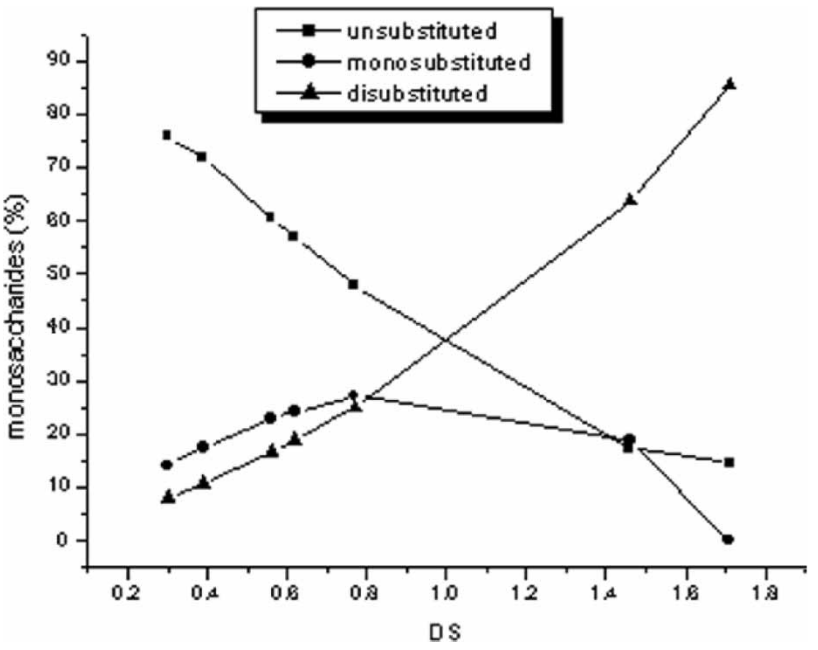


Figure 2: Monosaccharide composition of HE-Abps with Varied DS.

Reactivity of the Hydroxyl Groups of the D-Fructose Units in Abps During Hydroxyethylation

For the study of the reactivity of the hydroxyl groups, HE-Abps with a low degree of substitution (DS 0.34) was hydrolyzed in an acid medium. The structure of hydrolysates (fractions 1 and 2 in Fig. 1) was elucidated by NMR study. ^{13}C NMR spectrum revealed one predominant monosubstituted component, which showed that one of the hydroxyl groups of the D-fructose unit is much more reactive than the others during hydroxyethylation.

In ^{13}C NMR spectroscopy of the monosubstituted monosaccharide (fraction 2 in Fig. 1), the chemical shift of the anomeric carbon C-2 (99.1 ppm, 102.7 ppm) indicated major β-pyranose structure and minor β-furanose structure.[11] The ^{13}C chemical shifts of C-1 and C-6 were identified by comparison with unsubstituted β-D-fructopyranose (Table 3, Fig. 3). The chemical shifts of H-1, H-1' and H-6, H-6′ atoms were assigned by HMQC spectra (Fig. 4). The H-6, H-6′ atoms were used as a starting point in identification of C-5 on the pyranose ring by HMBC spectra. The cross-peaks of C-2 and H-3, C-4 and H-3 were also shown in HMBC spectra (Fig. 5). All data of the ^{13}C and ^{1}H shift are shown in Table 3 and Figure 3. The large upfield shift of H-4 (from 3.86 to 3.68) and downfield shift of C-4 (from 71.2 to 79.3), and the small opposite shifts of the neighbouring atom (H-3, H-5, C-3, C-5) demonstrate that the

Table 3: ^{1}H and ^{13}C chemical shifts of β-D-fructopyranose (A) and 4-*O*-hydroxyethyl-β-D-fructopyranose (B).

	Compound		
δ (ppm)	**A**	**B**	**$\Delta\delta$**
H-1	3.68[a]	3.70	+0.02
H-1′	3.53[a]	3.54	+0.01
H-3	3.76[a]	3.86	+0.10
H-4	3.86[a]	3.68	−0.18
H-5	3.96[a]	4.18	+0.22
H-6	4.00[a]	4.00	0
H-6′	3.68[a]	3.74	+0.06
CH_2		3.86,3.66	
CH'_2		3.76,3.73	
C-1	64.7[b]	64.9	+0.2
C-2	99.1[b]	99.1	0
C-3	68.4[b]	66.9	−1.5
C-4	70.5[b]	79.3	+8.8
C-5	70.0[b]	67.9	−2.1
C-6	64.1[b]	64.2	+0.1
CH_2		71.3	
CH'_2		61.9	

[a]Taken from ref. (12).
[b]Taken from ref. (11).

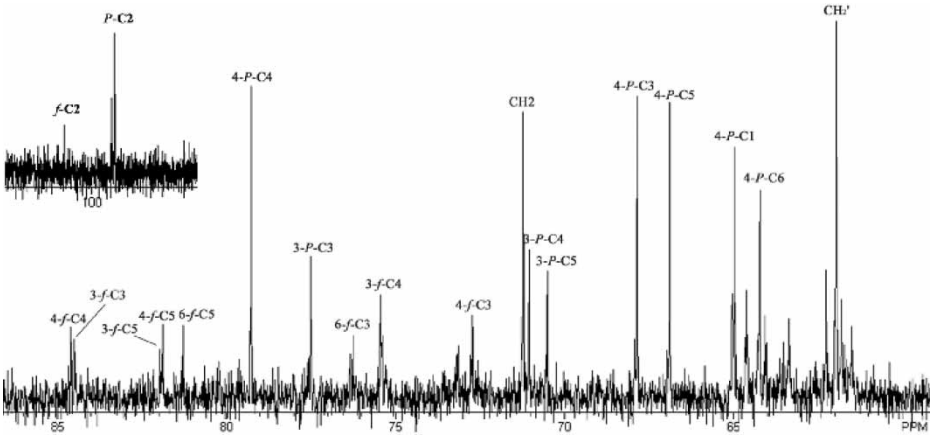


Figure 3: ^{13}C NMR spectrum (100 MHz) of hydrolysates of HE-Abps (fraction 2) in D_2O. The legend in the figure such as '*4-P*-C4' indicates C-4 of 4-*O*-hydroxyethyl-β-D-fructofuranose.

substituent is mainly located at the 4-position. From the similar NMR analysis, the chemical shifts of minor 4-*O*-hydroxyethyl-β-D-fructofuranose, 3-*O*-hydroxyethyl-β-D-fructopyranose, 3-*O*-hydroxyethyl-β-D-fructofuranose, and 6-*O*-hydroxyethyl-β-D-fructofuranose were identified (Fig. 3, Table 4).

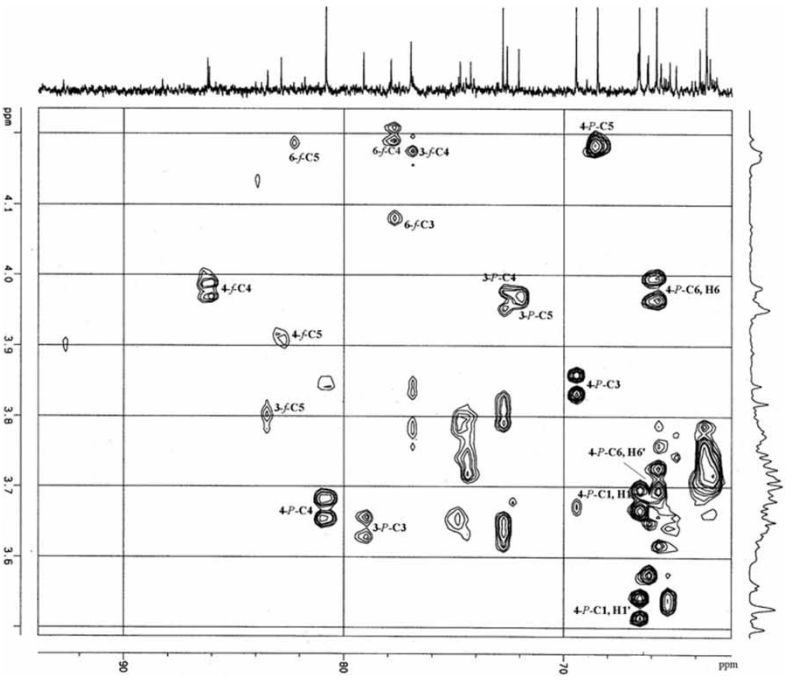


Figure 4: HMQC spectrum of hydrolysates of HE-Abps (fraction 2).

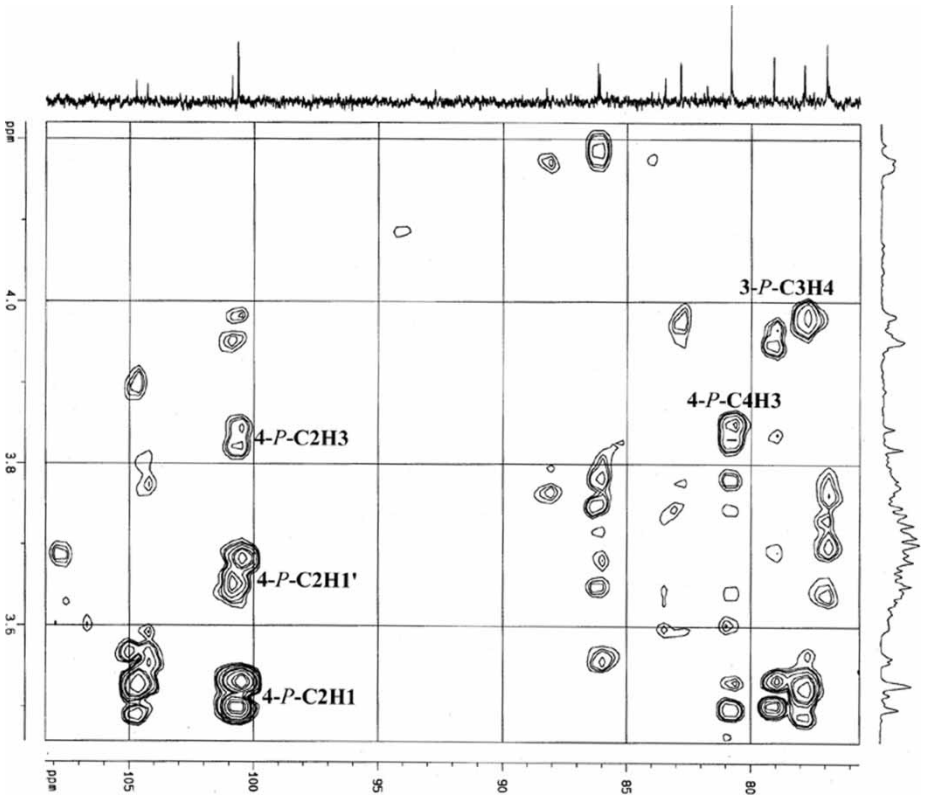


Figure 5: HMBC spectrum of hydrolysates of HE-Abps (fraction 2).

In conclusion, the main product formed by hydrolysis of HE-Abps with low DS (DS 0.34) is 4-*O*-hydroxyethyl-β-D-fructopyranose. All the data show that the hydroxyl group at the 4-position is the most reactive during hydroxyethylation.

The high preference for the 4-position might be because of two reasons: higher acidity and lower steric hindrance. The higher acidity was found for D-glucose units in starch and cellulose, and was confirmed to be responsible for the higher reactivity of the 2-position during etherification.[13,14] However, when the concentration of NaOH increases (Table 1, entries 5 and 6), the DS of HE-Abps will reduce from 0.77 to 0.56. It may be assumed that under these hydroxyethylation conditions (concentration of NaOH $\approx$1), all the hydroxyl groups have been dissociated. It was also reported that when methyl-α-D-fructofuranoside was etherified, there was no main product formed, and four regioisomers (1-, 3-, 4-, and 6- substituted monosaccharide) were formed in about equal amounts.[15] So the high preference for the 4-position in Abps during hydroxyethylation must be attributed to structural features of Abps in solution, rather than to differences in acidity of the

Table 4: ^{13}C NMR chemical shifts of *O*-hydroxyethyl-D-fructose.

	β-D-Fructopyranose						β-D-Fructofuranose[b]				α-D-Fructofuranose[b]			
Compound	**C-1**	**C-2**	**C-3**	**C-4**	**C-5**	**C-6**	**C-2**	**C-3**	**C-4**	**C-5**	**C-2**	**C-3**	**C-4**	**C-5**
Unsubstituted[a]	64.7	99.1	68.4	70.5	70.0	64.1	102.6	76.4	75.4	81.6	105.5	82.9	77.0	82.2
3-substituted	64.6	99.3	77.6	71.7	71.0	64.1	102.4	84.5	75.4	82.0	n.d.[c]			
4-substituted	64.9	99.1	66.9	79.3	67.9	64.2	102.7	72.7	84.6	81.9	n.d.[c]			
1-or 6-substituted							n.d.[c]	76.4	76.3	81.3	n.d.[c]			
3,4-substituted	64.8	99.1	76.1	79.4	67.2	64.0	103.1	84.1	83.9	81.3	105.9	88.6	82.4	79.5
4,6-substituted							103.2	75.9	84.7	78.8				

[a]Taken from ref. (11).
[b]The differences in chemical shift of C-1, C-6, and CH_2' are too small to make assignment.
[c]n.d.: not determined.

hydroxyl groups. The 3-position and 1-position are located close to the Abps backbone, so the ER will be difficult to approach the position. Furthermore, the hydroxyl groups at the 6-position is oriented *cis* with the hydroxyl group at the 3-position, so the steric effect will favor the 4-position, where the hydroxyl groups are particularly exposed.

^{13}C NMR, HMQC, and HMBC spectra of disubstituted monosaccharide (fraction 1 in Fig. 1) revealed that 3,4-di-*O*-hydroxyethyl-β-D-fructopyranose was the predominant product. The other minor products, such as 4,6-di-*O*-hydroxyethyl-β-D-fructofuranose, 3,4-di-*O*-hydroxyethyl-β-D-fructofuranose, and 3,4-di-*O*-hydroxyethyl-α-D-fructofuranose were also found. All the [13]C chemical shifts of disubstituted monosaccharides are shown in Table 4. Therefore, it can be concluded that the reactivity of the hydroxyl groups of sugar units in Abps is OH-4 $\gg$ OH-3 $>$ OH-6 $\approx$ OH-1.

Methylation Analysis

To determine the location of the HE groups in the glycan, HE-Abps (DS 0.34) was methylated and subjected to GC-MS linkage analysis (Table 5). From Table 5, some important structural conclusion can be drawn: (1) The HE groups were mainly located on terminal fructose (Fru2 $\rightarrow$) and (2) the substitution groups were mainly introduced to the 4-position of fructose, which was in accordance with the NMR study.

Antitumor Activity

The effects of HE-Abps with three DS on Lewis pulmonary carcinoma were tested in vivo (Table 6). HE-Abps (DS 0.34) with a low DS showed an excellent activity to inhibit the growth of the carcinoma. This effect decreased with the increased DS at the experimental concentration of 200 and 50 mg/kg. Only HE-Abps with DS 0.34 showed a higher inhibition ratio than the unmodified polysaccharide Abps.

Table 5: GC-MS data for HE-Abps (DS 0.34).

Peak	Fragment	Molar ratio	Linkages
1	1, 3, 4, 6–Me4–Fru	1.25	Fru*f*–(2 $\rightarrow$
2	2, 3, 4, 6–Me4–Glc	Trace	Glc*p*–(1 $\rightarrow$
3	1, 3, 4–Me3–Fru	15.50	$\rightarrow$ 6)–Fru*f*–(2 $\rightarrow$
	3, 4, 6–Me3–Fru		$\rightarrow$ 1)–Fru*f*–(2 $\rightarrow$
4	3, 4–Me2–Fru	1.00	1, 6)–Fru*f*–(2 $\rightarrow$
5	4–OMe–HE–1, 3, 6–Me3–Fru	4.75	4–O–HE–Fru*f*–(2 $\rightarrow$
6	*di*–OMe–HE–Me2–Fru	1.75	di–O–HE–Fru*f*–(2 $\rightarrow$

Table 6: Effect of HE-Abps on the inhibition of Lewis pulmonary carcinoma growth in mice.

Sample	Degree of substitution (DS)	No. of mice	Dose (mg/kg × days)	Tumor weigh (X ± SD)	Inhibition ratio (%)
HE-Abps-1	0.34	10	200 × 10	0.515 ± 0.06	46.91**
HE-Abps-1	0.34	10	50 × 10	0.690 ± 0.05	35.05**
HE-Abps-2	0.56	10	200 × 10	0.543 ± 0.07	44.02**
HE-Abps-2	0.56	10	50 × 10	0.648 ± 0.12	33.20**
HE-Abps-3	1.46	10	200 × 10	0.601 ± 0.14	38.04**
HE-Abps-3	1.46	10	50 × 10	0.708 ± 0.09	27.01**
Abps		10	200 × 10	0.540 ± 0.11	44.33**
Abps		10	50 × 10	0.649 ± 0.03	33.71**
CTX		10	100 × 2 (1, 3)	0.025 ± 0.03	97.42**
Control		10		0.970 ± 0.13	

Control: 0.9% NaCl solution.
CTX: cyclophosphamide.
**$P < 0.01$, significantly different from HE-Abps and Abps with control.

EXPERIMENTAL

Material. Abps was obtained from the traditional Chinese herb *Achryranthes bidentata* according to Yu's method.[7] Sephadex G-25 was purchased from Ammersham Pharmacia Biotech.

General. HPLC was performed on a Shimadzu LC-10AD instrument using an RI detector. Silica gel chromatography was performed on silica gel (10–40 μm). ESIMS was conducted with a PE PerSeptive Mariner instrument. The NMR spectra were obtained on a Bruker-MX-400 spectrometer equipped with a dual probe in the FT mode at 50°C.

Hydroxyethylation. Method (a): Abps (0.5 g, 3.1 mmol monomeric units) was dissolved in a solution of NaOH in water (20 mL). The amount of NaOH, 2-chloroethanol, and the reaction temperature and time were varied (Table 1). After the reaction was finished, the reaction mixture was neutralized with 1 mol/L HCl. Acetone (200 mL) was added, and the solution was centrifuged at 4500 rpm. The precipitate was dissolved in water, dialyzed (1000 MW cutoff) against water, and freeze-dried. The crude HE-Abps was further purified using Sephadex G-25 and eluted with distilled water, and the sugar fraction was monitored by phenol-H_2SO_4 method. The homogeneity of HE-Abps was determined by HPLC on a TSK-G2000SW exclusion column, using the following condition: eluent: distilled water; flow rate: 1 mL/min; detection: RI.

Method (b): Abps (0.5 g) was dissolved in water (20 mL) at 0°C. NaOH and ethylene oxide (Table 1) were added respectively. The stirring was continued for 24 hr at 0°C. The subsequent procedure was performed according to method (a).

HPLC analysis. HE-Abps was hydrolyzed with 0.1 mol/L H_2SO_4 at 50°C for 1 hr, neutralized with $BaCO_3$, then centrifuged. HPLC of the hydrolysate was performed on a Carbohydrate Analysis Column (Waters, 3.9 mm I.D. × 30 cm) with 82:18 CH_3CN-H_2O as eluent at a flow rate of 1 mL/min, and RI detected. The HPLC analysis yielded a good separation of di- and mono-substituted monosaccharide and unsubstituted fructose and glucose (retention time 7.1 min, 7.4 min, 8.1 min, 9.3 min, respectively).

NMR spectroscopy. The hydrolysate of HE-Abps (100 mg) was isolated by silica gel chromatography (1.2 cm × 20 cm) with 8.5 : 5.0 : 1.0 $CHCl_3$-MeOH-H_2O as eluent. The NMR spectra of pure monosubstituted monosaccharide (fraction 2 in Fig. 1) were recorded on a Bruker-MX-400 spectrometer. D_2O was used as solvent and ter-butanol as internal standard (δ_H 1.2 ppm, δ_C 31.2 ppm).

Methylation analysis. The sample was methylated with DMSO-NaOH-CH_3I method,[16] hydrolyzed in 2M CF_3COOH at 100°C for 1 hr, and then transferred into alditol acetate by Ac_2O/Py. GC-MS measurement of the partial methylated aiditol acetates was carried out on Shimadzu QP 5000.

Assay of antitumor activities. Assay of the antitumor activities of HE-Abps was done by the method of Yu and Zhang.[17] C57BL/C mice were obtained from Shanghai Animal Center of the Chinese Academy of Science, and weighed about 20 g for the antitumor assay. Lewis pulmonary carcinoma cells (5 × 106/mL) were transplanted into the toe of the mice. The test samples were dissolved in 0.9% NaCl solution and injected intraperitoneally daily for 10 days (injection volume, 0.2 mL), starting 24 hr 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 6.

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