Studies on Synthesis and Antitumor Activity of Phosphorylated *Achyranthes bidentata* Polysaccharide (P-AbPS)†

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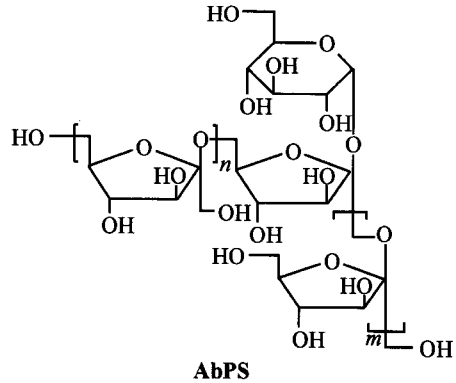
The synthesis of phosphorylated *Achyranthes bidentata* polysaccharide (P-AbPS) was reported based on different strategies. The P-AbPS with high degree of substitution (D.S. > 0.5) was obtained when phosphorus oxychloride ($POCl_3$) was used as a phosphorylating agent and trimethyl phosphate-pyridine or dimethyl formamide was used as solvent. The influences of different solvents and reaction conditions were discussed. The pharmacology assay shows that P-AbPS possesses antitumor activity against sarcoma 180 and Lewis lung cancer in mice.

Keywords phosphorylation, polysaccharide, *Achyranthes bidentata*, antitumor

Introduction

It is well known that the lipopolysaccharides are capable of revealing a variety of biological activities including tumor-growth inhibitory activity.[1] Phosphorylation of polysaccharides has attracted widespread attention because it was reported that they show antitumor activity and other important biological activities.[2,3] Due to the complex structure of polysaccharide, it is difficult to get phosphorylated polysaccharides with high degree of substitution (D.S.). The phosphorylation of polysaccharides was not fully studied. Up to now, there is no reported D.S. higher than 0.3.

Achyranthes bidentata is a famous traditional chinese herbal medicine which has functions of nourishing the liver and kidney, strengthening the tendons and bones, treating numbness of the waist and knee, *etc*.[4] A polysaccharide named **AbPS** was isolated from the root of *Achyranthes bidentata* B1 as described elsewhere.[5] The structure of **AbPS** can shown as follows:



AbPS

$m, n = 0-19,\ m+n = 2-19$

Fig. 1 Structure of **AbPS**.

Sulfated **AbPS** has been studied in detail, and showed high antivirus activity and low toxicity,[6] but no phosphorylated **AbPS** has been studied. In this paper, the phosphorylation of **AbPS** was reported, and the phosphorylation of polysacchride was studied to try to obtain products with high degree of substitution.

Experimental

Materials

AbPS was isolated from the root of *Achyranthes*

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bidentata B1 and purified on a Sephadex G-50 column according to the literature.[5] Sephadex G-50 and Sephadex A-50 were purchased from Ammershan Pharmacia Biotech.

Phosphorus oxychloride ($POCl_3$) and dimethyl formamide (DMF) were distilled before use. Pyridine and dimethyl sulfoxide (DMSO) were dried over CaH_2 and distilled at reduced pressure before use. Other solvents were of analytical grade.

Phosphorylation of **AbPS**

Phosphorus oxychloride (0.8 mL, 9.6 mmol) was added to anhydrous pyridine (10 mL) dropwise for 20 min with vigorous stirring and the reaction mixture was continued to stir for 10 min at 0—2 ℃ (ice-water bath). A pyridine solution (10 mL) containing **AbPS** (500 mg, 3.1 mmol) was then added. The mixture was stirred for 6 h at 0—2 ℃. The reaction mixture was poured into ice-water (20 mL) with stirring and adjusted to pH 7.0 with NaOH (2 mol/L). Then, acetone (200 mL) was added to the mixture which was kept at 4 ℃ overnight. The precipitate was collected and resolved in water. The solution was dialyzed against water for 36 h to remove inorganic salts and solvent. Then the lyophilized product named *Achyranthes bidentata* polysaccharide (**P-AbPS**) was obtained.

Alternatively, reaction time, temperature and solvent were changed. Molar ratio of $POCl_3$ to **AbPS** in the reaction mixture was also changed.

Purification of **P-AbPS**

P-AbPS was purified on a Sephadex A-50 column (2 cm × 50 cm) eluted with a gradient from 0.1 mol/L to 1 mol/L NaCl solution, monitored by phenol-sulphuric acid assay at 490 nm.[7] The eluate was further purified on a Sephadex G-50 column (1.5 cm × 150 cm).

The phosphorus content was determined by the reported method.[8]

Assay of antitumor activity

Assay of the antitumor activities of **P-AbPS** was done by the method of Yu.[9] C57BL/C mice were obtained from Shanghai Animal Center of Chinese Academy of Science, weighting about 20 g for the antitumor assay. Sarcoma 180 ascite cells (5×10^6 mL) were transplanted subcutaneously into the right groins of the mice. The test samples were dissolved in 0.9% NaCl solution and injected intraperitoneally daily for 8 d (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 weighted. The growth inhibitive ratio of tumor growth was calculated by the following equation:

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

Where A is the average tumor weight of the control group and B that of the treated group. Furthermore, the effect of **P-AbPS** on Lewis pulmonary carcinoma was investigated by the same method of Sarcoma 180.

Results and discussion

In our study, $POCl_3$ was used as a phosphorylating agent. A basic solvent was used to prevent **AbPS** from being degraded because **AbPS** is very sensitive to acid. The phosphorylation of **AbPS** was first examined in pyridine and the results are listed in Table 1. To obtain products with high degree of substitute and good yield, $POCl_3$ was added to pyridine first, then pyridine solution that dissolved most of **AbPS** was added. It was reported that the addition of an adequate amount of water can inhibit the formation of 2-(or 3-)-phosphate derivatives.[10,11] However we found that the selectivity of phosphorylation was very poor and the yields were low in this condition.

As shown in Table 1, when water was used in the reaction, the content of phosphorus in products was increased, while the yield was decreased (compare Entries 2 with 3, Entries 9 with 10). The reason is the appearance of the proton hydrate in this condition leads to the accelerating of reaction and the increased degrading of **AbPS**. The phosphorus content was increased gradually with reaction temperature.

From Table 1, when pyridine was used as solvent, the yields were low. The maximum phosphorylation can be acquired at 100 ℃ for 1 h (Entry 11).

Lewis[11] reported that the use of trialkylphosphates as solvent can enhance the rate of phosphorylation. But when trimethyl phosphate was used as solvent only, the yield was very poor. When trimethyl phosphate and pyridine was mixed as solvent, the good result was obtained. The results are listed in Table 2.

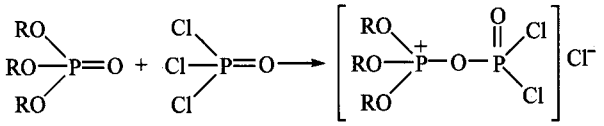
Table 1 Phosphorylation in pyridine

Entry	**AbPS**:$POCl_3$	T (℃)	t (h)	Yield (%)[b]	P (%)[c]	D.S.[d]
1	1:3	0—2	4	83.7	7.01	0.51
2[a]	1:3	0—2	6	46.2	8.53	0.67
3	1:3	0—2	6	75	7.25	0.53
4	1:5	0—2	6	92	7.70	0.58
5	1:5	45	2.5	55	7.75	0.59
6	1:3	45	4	43	8.51	0.67
7	1:3	75	1	66	10.12	0.88
8[a]	1:3	75	3	28	11.27	1.07
9[a]	1:3	100	1	46	8.12	0.63
10	1:3	100	1	88	11.19	1.06
11	1:5	100	1	51.2	12.11	1.23

[a] H_2O (0.5 equiv.) was added. [b] The yield of weight. [c] P: content of phosphorus. [d] D.S.: degree of substitution of phosphate, D.S. = $162 \times P/(3100(124 \times P)$.[7]

As shown in Table 2, when the mixture of trimethyl phosphate and pyridine was used as solvent, the D.S. and yields were high, especially, the yields increased greatly. Because the trimethyl phosphate may interact with the phosphorus oxychloride to form a complex[11] (Scheme 1) to enhance the activity of phosphorylating agent. This phosphorylating agent is milder as compared with $POCl_3$ and is able to reduce **AbPS** to be degraded in this condition.

Scheme 1



From Table 2, when pyridine was absent or the water was present, the yields were very low (compare Entries 1, 2 with 3, Entries 6 with 7), and most of **AbPS** was degraded. The yield was decreased gradually with the increase of reaction time.

When trimethyl phosphate and pyridine were used as solvent, the product with high D.S. and yield was acquired (50 ℃, 1 h) (Entry 5).

Kim[12] reported that the selective phosphorylating agent was obtained by mixing phosphoryl chloride, water, and pyridine in a molar ratio of 2:1:2 at a low temperature. The phosphorylation was carried out in acetonitrile. When this condition was used on phosphorylation of **AbPS**, D.S. is very low. Because few **AbPS** was dissolved in acetonitrile, the phosphorylation can not be carried out well. Attempt to study this agent in other solvents, no good results were obtained.

In addition, phosphorylation of **AbPS** in other solvents was studies. The results are listed in Table 3.

From Table 3, it was found that DMF is a good solvent when pyridine or triethylamine was used as base (Entries 8 and 9). When DMF was used as solvent and triethylamine as base, the yield was high. While pyridine was used as base, the degree of substitute was high. The product with the highest D.S. was prepared at 0—2 ℃ for 5 h in DMF (Entry 9).

Assignment of phosphorylated sites

The phosphorylated sites were determined by compared ^{13}C NMR spectra of **P-AbPS** (D.S. = 1) with those of **AbPS**, and the results are shown in Table 4.

As shown in Table 4, due to phosphorylation,[12] some C-1 and C-6 of fructose residues and C-6 of glucose residue were shifted downfield about δ 2. And these sites were linked with phosphate.

Antitumor activities of **P-AbPS**

P-AbPS was tested for antitumor activities against sarcoma 180 and Lewis lung cancer in mice. As shown in Tables 5 and 6, **P-AbPS** was found to be effective against mouse bearing sarcoma 180 and Lewis lung cancer. But there was no obvious difference between the phosphate with the diverse degree of substitution.

Table 2 Phosphorylation in trimethyl phosphate and pyridine

Entry	**AbPS**:$POCl_3$	T (℃)	t (h)	Yield (%)	P (%)	D.S.
1[a]	1:5	0—2	4	35.7	—	—
2[b]	1:5	0—2	3.5	42	10.8	0.99
3	1:5	0—2	4	118	10.5	0.95
4	1:5	50	0.5	144	9.6	0.81
5	1:5	50	1	210	10.6	0.96
6[a]	1:5	100	1	11.7	—	—
7	1:5	100	1	126.8	9.9	0.86
8	1:5	100	0.5	200	10.89	1.00
9	1:5	100	1.5	124	11.5	1.11

[a] Without pyridine. [b] H_2O (0.5 equiv.) was added.

Table 3 Phosphorylation in other solvents

Entry	Solvent	T (℃)	t (h)	Yield (%)	P (%)	D.S.
1	MeCN	0—2	4	80	2.3	0.13
2[a]	Propanediol	100	1	10	—	—
3	THF	0—2	11	12	—	—
4	DMSO	0—2	4	47	6.30	0.42
5	DMSO	100	1.5	23	7.25	0.53
6	DMSO	30	12	10	—	—
7[a]	DMSO	10	4	50	6.7	0.48
8[b]	DMF	0—2	5	145	11.9	1.18
9[a]	DMF	0—2	5	90	12.5	1.30
10	H_2O	45	3	92	5.3	0.35

[a] Pyridine as base. [b] Triethylamine as base.

Table 4 ^{13}C NMR data of **AbPS** and **P-AbPS**

	AbPS δ	**P-AbPS** δ
C-1[a]	63.60, 63.69, 63.95, **64.37**, **64.47**	63.30, 63.41, 63.61, **66.28**, **66.37**
C-2	107.20, 107.06, 106.91, 106.89, 106.62, 106.35, 106.24	107.02, 106.86, 106.69, 106.56, 106.38, 106.23, 106.10, 105.97
C-3	80.63, 80.48, 80.26, 80.13, 79.99, 79.94	80.52, 80.40, 80.18, 79.89, 79.76, 79.64
C-4	78.82, 78.74, 78.58, 78.46, 78.14, 78.10, 77.99, 77.93	78.41, 78.25, 78.14, 78.04, 78.01, 77.80, 77.56, 77.50
C-5	84.36, 84.32, 83.32	84.09, 83.08,
C-6	**65.47**, 65.71, 66.34, 66.43, 66.50	**67.02**, 65.34, 65.56, 66.25, 66.84
C-1[b]	95.63	95.47
C-2	74.32, 74.28	74.06
C-3	75.97, 75.93	75.47, 75.10
C-4	72.89, 72.76, 72.64, 72.30	72.06
C-5	75.60	75.00
C-6	**63.50**	**65.56**

[a] ^{13}C Chemical shifts of fructose residue. [b] ^{13}C Chemical shifts of glucose residue.

Table 5 Effect of **P-AbPS** on sarcoma 180

Sample	D.S.	Route	Dose (mg/kg × days)	Tumor weigh (X ± SD)	Inhibition ratio (%)
P-AbPS-1	1.30	i.p.	200 × 8	1.49 ± 0.14	36.32[a]
P-AbPS-1	1.30	i.p.	100 × 8	1.61 ± 0.17	31.20[a]
P-AbPS-2	0.86	i.p.	200 × 8	1.38 ± 0.15	41.03[a]
P-AbPS-2	0.86	i.p.	100 × 8	1.51 ± 0.15	35.47[a]
P-AbPS-3	0.35	i.p.	200 × 8	1.44 ± 0.15	38.46[a]
P-AbPS-3	0.35	i.p.	100 × 8	1.56 ± 0.18	33.33[a]
Control		i.p.		2.34 ± 0.29	

[a] $p < 0.01$

Table 6 Effect of **P-AbPS** on Lewis lung cancer

Sample	D.S.	Route	Dose (mg/kg × days)	Tumor weigh (X ± SD)	Inhibition ratio (%)
P-AbPS-1	1.30	i.p.	200 × 8	0.526 ± 0.06	47.40[a]
P-AbPS-1	1.30	i.p.	100 × 8	0.554 ± 0.06	44.60[a]
P-AbPS-2	0.86	i.p.	200 × 8	0.515 ± 0.07	48.50[a]
P-AbPS-2	0.86	i.p.	100 × 8	0.549 ± 0.05	45.10[a]
P-AbPS-3	0.35	i.p.	200 × 8	0.568 ± 0.04	43.20[a]
P-AbPS-3	0.35	i.p.	100 × 8	0.607 ± 0.03	39.30[a]
Control		i.p.		1.00 ± 0.14	

[a] $p < 0.01$

Conclusion

Under normal conditions, it is difficult to acquire the degree of substitution of the phosphorylated polysaccharide higher than 0.3. In our work, phosphorus oxychloride ($POCl_3$) was used as a phosphorylation agent and the reactions in different conditions were investigated. The high yield and degree of substitution of products were acquired when using trimethyl phosphate and pyridine or dimethyl formamide as solvent in the phosphorylation of **AbPS**. The higher degree of substitute can be obtained when DMF was used as solvent. The methods described here were simple and effective in phosphorylation of **AbPS**. It would be expected to use this reagent for the phosphorylation of other polysaccharides. The phospory-lated **AbPS** (**P-AbPS**) could inhibit growth of Sarcoma 180 and Lewis lung cancer implanted in mice.

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