Carbohydrate Research, 53 (1977) 223–229

PREPARATION AND ANTITUMOR ACTIVITY OF *O*-PALMITOYLDEXTRAN PHOSPHATES, *O*-PALMITOYLDEXTRANS, AND DEXTRAN PHOSPHATE*

MASUKO SUZUKI, TAKESHI MIKAMI, TATSUJI MATSUMOTO, AND SHIGEO SUZUKI
Department of Microbiology and Second Department of Hygienic Chemistry, Tohoku College of Pharmacy, Komatsushima 4-4-1, Sendai 983 (Japan)
(Received December 1st, 1975, accepted in revised form, September 1st, 1976)

ABSTRACT

Dextran was modified by introduction of palmitoyl and phosphate groups. The derivatives were analyzed by sequential periodate oxidation–sodium borohydride reduction. Only one of these derivatives showed a significant growth-inhibitory effect when administered alone, while a second derivative showed, in combination therapy with an ineffective dose of Mitomycin C, a marked synergistic effect.

INTRODUCTION

Polysaccharides have been esterified with high-molecular-weight fatty acid chlorides[1,2]. In the present study, a commercial dextran preparation (mol. wt. ~38,000) consisting of 93.7% of α-(1→6)-linked D-glucose units was esterified in formamide solution with palmitoyl chloride or polyphosphoric acid, or with both, to give *O*-palmitoyldextrans, dextran phosphate, or *O*-palmitoyldextran phosphates, respectively. The structures of the compounds that were water-soluble were determined by the Smith degradation procedure, and their antitumor activity was assayed with Sarcoma-180, ascites tumor-cells in mice.

RESULTS AND DISCUSSION

The commercial dextran preparation was esterified with an increasing proportion of palmitoyl chloride and a fixed proportion of polyphosphoric acid to give *O*-palmitoyldextran phosphates. For the synthesis of these derivatives, formamide was preferred as the solvent, although *N,N*-dimethylformamide, in which this polysaccharide dextran is practically insoluble, has been used[3,4]. The modified products were isolated from each reaction mixture by the addition of methanol, and fractionated by dialysis against water, followed by precipitation with methanol.

*This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan.

Three palmitoyldextran phosphates were obtained, designated as $PalDP_A$, $PalDP_B$, and $PalDP_C$. $PalDP_A$ is soluble in water and was purified by column chromatography on Sephadex G-100 and on DEAE-cellulose (AcO^-) to give the corresponding purified fractions, $PalDP_A$-I and $PalDP_A$-II (Fig. 1). Homogeneity of $PalDP_A$-II was shown by its ultracentrifugal pattern, which gave S_{20} 4.0. $PalDP_B$ and $PalDP_C$ were precipitated from a solution in hot formamide with methanol to give the corresponding purified fractions, $PalDP_B$-I and $PalDP_C$-I, insoluble in water and in most organic solvents (see Table I). All *O*-palmitoyldextran phosphates described

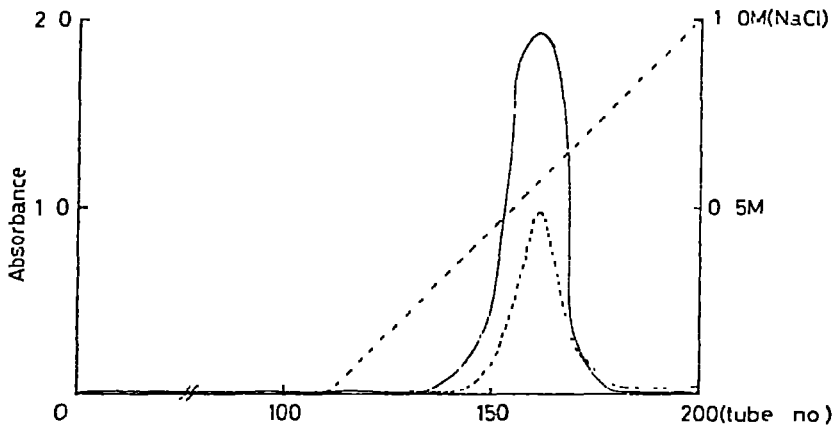

Fig. 1. Elution profile of $PalDP_A$-I from a column (2.64 × 57 cm) of DEAE-cellulose (AcO^-). Elution was performed at first with water (660 ml) and then with a gradient system varying from water to M NaCl (each 550 ml). Each fraction was 6 ml/tube. Carbohydrate content determined by the modified Molisch method at 550 nm (———); phosphorus content by the Allen–Nakamura method at 720 nm (— — —).

TABLE I

CHEMICAL COMPOSITION AND PROPERTIES OF DEXTRAN DERIVATIVES

Materials	*Carbohydrate*[a] (%)	*Phosphate*[b] (%)	*Palmitoyl groups*[c] (%)	$[\alpha]_D^{20}$ (°)[d]
Dextran	100.0			+198
$PalDP_A$-II	76.8	19.0	0.6	+129
$PalD_A$-I	85.6		0.6	+169
DP-II	81.0	18.3		+127
$PalDP_B$-I	71.3	22.1	3.0	
$PalDP_C$-I	41.7	7.3	47.8	
$PalD_B$-I	87.3		2.3	
$PalD_C$-I	62.8		35.9	

[a]Determined by the Molisch procedure as modified by Devor[6]. [b]Determined as phosphorus by the method of Allen as modified by Nakamura[7]. [c]Determined by g.l.c. with oleic acid as internal standard. [d]In water, *c* 1.0.

here were found to consist of three components only, namely, carbohydrate, phosphate, and fatty acid.

Three *O*-palmitoyldextrans ($PalD_A$, $PalD_B$, and $PalD_C$) having various fatty acid contents and corresponding to the *O*-palmitoyldextran phosphate preparations, were prepared by treatment of dextran with the same concentration of palmitoyl chloride as used in the preparation of *O*-palmitoyldextran phosphates. Only $PalD_A$ was soluble in cold water, but the other compounds were insoluble in most solvents, including water, and were purified by the same method as that used for the water-insoluble PalDP preparations.

The parent dextran was treated with polyphosphoric acid under the same conditions as for the synthesis of *O*-palmitoyldextran phosphates in order to obtain dextran derivatives modified only by the introduction of phosphate groups. A dextran phosphate preparation containing 81% of carbohydrate and 18.3% of phosphate groups was obtained readily. The properties of *O*-palmitoyldextrans and of a dextran phosphate are reported in Table I.

The results of periodate oxidation and of the Smith degradation of $PalDP_A$-II, DP-II, $PalD_A$-I, and the native dextran are reported in Table II. The last-named compound showed for the periodate consumption and the production of formic acid,

TABLE II

MAXIMUM CONSUMPTION OF PERIODATE, PRODUCTION OF FORMIC ACID, AND MOLAR PROPORTION OF COMPOUNDS FORMED IN THE SMITH DEGRADATION OF DEXTRAN DERIVATIVES

	Periodate oxidation		*Ratio of glycerol: erythritol: glucitol produced in the Smith degradation*
	IO_4^- *consumed*[a]	HCO_2H *production*[a]	
$PalDP_A$-II	1.71	0.71	89.7:1.7:8.5
DP-II	1.70	0.69	90.2:1.9:7.9
$PalD_A$-I	1.85	0.90	92.4:1.7:5.9
Dextran	1.89	0.91	92.6:1.6:5.8

[a]Mole per mole of anhydro-D-glucose unit

values of 1.89 and 0.91 mole/anhydro-D-glucose unit, respectively, relatively higher than the values observed for the other derivatives. Thus, it is evident that more than 90% of the D-glucose units of this dextran consist of (1→6)-linked residues. On the other hand, $PalDP_A$-II and DP-II gave relatively lower values of periodate uptake and release of formic acid than did the dextran preparation, indicating that the substitution with phosphate groups prevented an extensive oxidation of these modified polysaccharides. The results of the Smith degradation of the native and modified dextrans were consistent with the results of the periodate oxidation; the amounts of glycerol detected in the degradation products of both $PalDP_A$-II and DP-II were nearly equal to that derived from the native dextran, thus suggesting that the substitution by phosphate groups occurred for the largest part at the C-2 position of (1→6)-linked D-glucopyranose residues. The release of nearly equal proportions of erythritol by

all degradation products indicates that the substitution at C-4 of D-glucopyranose residues by phosphate groups may be excluded. The proportions of periodate-resistant sugar residues of two dextran derivatives were slightly higher than that of the native dextran. Thus, it may be assumed that a small proportion of phosphate groups are also linked at C-3. The location of *O*-palmitoyl groups in $PalDP_A$-II and $PalD_A$-I was found to be at C-6 of the nonreducing, terminal D-glucopyranose residues, as 1-*O*-palmitoylglycerol was detected in the product of the Smith degradation as the sole palmitic acid derivative.

One of the water-insoluble *O*-palmitoyldextran phosphates, $PalDP_C$, showed a significant growth-inhibition against sarcoma 180 ascites-tumor in mice, whereas the purified, water-soluble $PalDP_A$-II, exhibited only a borderline inhibition when administered alone (see Table III). The latter compound, however, revealed a marked synergistic effect against the same tumor in combination therapy with Mitomycin C,

TABLE III

SINGLE AND COMBINATION CHEMOTHERAPY OF DEXTRAN DERIVATIVES AGAINST SARCOMA-180, ASCITES TUMOR-CELLS BY TOTAL PACKED-CELL VOLUME METHOD

Materials	*Dose*[a]	*Inhibition ratio* (%)	*Number of complete regressions/ total number of mice*
$PalDP_A$-II	1	17	0/18
	20	21	0/18
$PalDP_C$-I	1	82	4/12
	20	92	6/12
$PalDP_A$-II + Mitomycin C	1		
	0.01	91	10/12
$PalDP_C$-I + Mitomycin C	1		
	0.01	83	9/12
Mitomycin C	0.01	9	0/36

[a] Mg/kg/day × 5.

showing an 83% regression of tumor by the total packed-cell volume method[5]. The observation that all *O*-palmitoyldextrans and dextran phosphates are practically inactive in the inhibition of this ascites tumor, even in combination therapy, indicates the necessity for the existence of both fatty acid and phosphate groups to manifest antitumor activity. Thus, simple polysaccharides without apparent biological activity, such as dextran, can be converted into derivatives displaying some antitumor activity.

EXPERIMENTAL

Materials. — The dextran (mol. wt. 38,000) was a commercial product "dextran 40" of Meito Sangyo Co., Nagoya, Japan, and palmitoyl chloride was purchased from Tokyo Kasei Co., Tokyo, Japan and was 98% pure, as examined by g.l.c. after its conversion to the corresponding methyl ester.

General methods. — Total carbohydrate and phosphorus contents were determined by the modified Molisch[6] and Allen–Nakamura methods[7], respectively. The palmitoyl group content was estimated by g.l.c., after methanolysis of the sample with 5% methanolic HCl, with oleic acid as the internal standard. Optical rotations were measured with an Applied Electric Automatic Polarimeter. Ultracentrifugal analysis was performed with a Hitachi UCA-6A ultracentrifuge at 60,000 r.p.m. G.l.c. was performed with a Shimadzu GC-5AM chromatograph, equipped with a flame-ionization detector. The products from the Smith degradation were analyzed as the *O*-acetyl or *O*-(trimethylsilyl) derivatives on a column (4 × 2,000 mm) of 3% ECNSS-M on Gas Chrom Q (80–100 mesh), at 100–210° (programmed at a rate of 4°/min) or on a column (4 × 1,500 mm) of 10% Silicone SE-30 on Gas Chrom Q (80–100 mesh), 100–300° (programmed at a rate of 4°/min); N_2 was the carrier gas at a flow rate[8–11] of 40–60 ml/min.

O-*Palmitoyldextran phosphate.* — Dextran (1 g, dried *in vacuo* for 24 h at 110° in the presence of P_2O_5) was suspended in anhydrous formamide (100 ml), and the mixture was stirred in a water-bath at 70° until the polysaccharide dissolved completely (1 h). Anhydrous triethylamine (20 ml) and crystalline palmitoyl chloride (0.2 g) were added, and warming and stirring were continued for a further 2 h. In other experiments, a larger amount of the acid chloride (0.5 and 5.0 g) was added to give the corresponding products having a higher fatty acid content. After the cloudy reaction mixture had been cooled to 20°, polyphosphoric acid (5 g) was added in one portion, and the mixture was stirred for 24 h. The resultant, clear solution was diluted with methanol (400 ml) and, after 30 min, the precipitate formed was collected by centrifugation. After being washed twice with methanol,the precipitate was dissolved (or suspended) in water (200 ml), the pH adjusted to 9.0 with aqueous 10% NaOH solution, and the solution (or suspension) dialyzed against running tap-water for 48 h. The nondialyzable fraction was concentrated to 50 ml *in vacuo* at a temperature below 40°, and poured into methanol (200 ml). The precipitate was collected by centrifugation and washed successively with methanol, benzene, and hexane (100 ml each), and dried over P_2O_5.

Purification of O-*palmitoyldextran phosphates.* — A solution of $PalDP_A$ (100 mg) in water (5 ml) was applied to a column (2.6 × 89 cm) of Sephadex G-100 (medium). The column was eluted with water, and each eluate (9 ml/tube) was assayed for the contents of carbohydrate and phosphorus. Eluates containing carbohydrate were combined, concentrated to a small volume *in vacuo*, and the polysaccharide derivative was precipitated by the addition of a large volume of methanol. The precipitate was collected, washed with methanol by centrifugation, and dried *in vacuo* over P_2O_5 (yield 98 mg). This fraction was designated as $PalDP_A$-I. The clear solution of $PalDP_A$-I (50 mg) in water (5 ml) was applied to a column (2.6 × 57 cm) of DEAE-cellulose (AcO^-). The column was first washed with water (670 ml) and eluted with a gradient from water to M NaCl solution (1100 ml). Only one carbohydrate-containing component was eluted from the column at an NaCl concentration of 0.4–0.6M. The eluate was concentrated and dialyzed, and the polysaccharide derivative was recovered

by methanol precipitation, as described for $PalDP_A$-I (yield 40 mg). This fraction was designated as $PalDP_A$-II.

Purification of $PalDP_B$ and $PalDP_C$ by precipitation. — Crude $PalDP_B$ (or $PalDP_C$) (100 mg) was dissolved in formamide (10 ml) by heating at 110°. The solution was filtered and poured into methanol (10 vol.). The precipitate formed was collected by centrifugation, washed thoroughly with methanol, and dried over P_2O_5 *in vacuo*. This fraction was designated as $PalDP_B$-I (or $PalDP_C$-I), and the yield was 96 mg (or 83 mg).

O-*Palmitoyldextran.* — This derivative was prepared by essentially the same method as that described for the preparation of PalDP, but without subsequent addition of polyphosphoric acid and keeping for 24 h at room temperature. The proportions of palmitoyl chloride added were the same as those for the preparation of PalDP. Three *O*-palmitoyldextrans ($PalD_A$, $PalD_B$, and $PalD_C$) were obtained, and $PalD_A$ was shown to be readily water-soluble, whereas the other two were sparingly soluble in water. A solution of $PalD_A$ was passed through a column of Sephadex G-100 under the same conditions as described for $PalDP_A$; only a single peak (designated $PalD_A$-I) containing a carbohydrate component was eluted. The other *O*-palmitoyldextrans ($PalD_B$ and $PalD_C$) were purified by precipitation with formamide and methanol as described for $PalDP_B$ and $PalDP_C$, and designated $PalD_B$-I and $PalD_C$-I. The chemical composition of the purified PalDs is given in Table I.

Dextran phosphate. — To a solution of dextran (1 g) in 1:5 (v/v) triethylamine–formamide, polyphosphoric acid (5 g) was added in one portion. The mixture was stirred until the polyphosphoric acid had dissolved, and the clear solution was kept for 24 h at room temperature. The solution was diluted with methanol (5 vol.), and the precipitate was collected by centrifugation. After being washed throughly with methanol, it was dissolved in water (100 ml). The pH of the solution was adjusted to 9.0 with 10% NaOH, and the solution was dialyzed against running tap-water overnight. After removal of a small amount of insoluble material by centrifugation, the dextran phosphate was recovered as a white precipitate by the addition of methanol (5 vol.). The precipitate was collected by centrifugation, washed with methanol, and dried over P_2O_5 *in vacuo* (yield 1.25 g).

Periodate oxidation and Smith degradation. — To a solution of dextran derivative (200 mg) in 3% NaCl (8 ml) was added 0.25M sodium periodate (15 ml), and the total volume was completed to 25 ml with[12] 3% NaCl. The oxidation mixture was kept in the dark at 15° and, at suitable time intervals for 24 h, the consumption of periodate and the production of formic acid were measured by the Fleury–Lange method[13] and by titration with 0.01M NaOH, respectively. An excess amount of 1,2-ethanediol was added to decompose the excess of periodate when the oxidation was complete, and the reaction mixture was dialyzed. The nondialyzable fraction was treated with $NaBH_4$ in the usual way. After the excess of $NaBH_4$ had been destroyed by adjusting the pH to 5.5 with acetic acid, the solution was heated with 0.5M H_2SO_4 for 6 h at 100°. The hydrolyzate was neutralized with $BaCO_3$, filtered, and the filtrate evaporated to dryness. No phosphorus could be detected in this residue, indicating that all phosphate

groups had been hydrolyzed and removed as insoluble Ba salt. H_3BO_3 was removed by treatment with a cation-exchange resin (Amberlite IR-120, H^+) followed by repeated additions and evaporations of methanol at room temperature.

Assay of antitumor activity. — Male ddY mice, five weeks old, were obtained from Shizuoka Animal Farm, Shizuoka (Japan). The antitumor activity against sarcoma-180, ascites tumor-cells was measured by the total packed-cell volume method[5], 7 days after sarcoma-180 tumor cells (1×10^7) had been inoculated intraperitoneally. A combination therapy against sarcoma-180 cells in the ascites form was performed with the dextran derivatives and Mitomycin C (Kyowa Hakko Kogyo Co., Tokyo, Japan). Mitomycin C (0.01 mg/kg/day) was injected intraperitoneally once daily for 5 days, 1 h after injection of the dextran derivative (1 mg/kg/day).

REFERENCES

1 U. Hammerling and O. Westphal, *Eur. J. Biochem.*, 1 (1967) 46–50
2 S. Hirano and Y. Ohe, *Carbohydr. Res.*, 41 (1975) C1–C2.
3 R. L. Whistler and G. A. Towle, *Arch. Biochem. Biophys.*, 135 (1969) 396–401.
4 F. Suzuki, N. Ishida, M. Suzuki, T. Sato, and S. Suzuki, *Proc. Soc. Exp. Biol. Med.*, 149 (1975) 1069–1075.
5 E. N. Sassenerath, *Ann. N.Y. Acad. Sci.*, 76 (1958) 601–609.
6 A. W. Devor, *Anal. Chem.*, 24 (1948) 1626.
7 M. Nakamura, *Nippon. Nogei Kagaku Zasshi*, 24 (1950) 1–5.
8 A. Misaki, T. Ito, and T. Harada, *Agric. Biol. Chem.*, 36 (1972) 761–771.
9 H. Miyaji and A. Misaki, *J. Biochem. (Tokyo)*, 74 (1973) 1131–1139.
10 R. Watts and R. Dils, *J. Lipid Res.*, 9 (1968) 40–51.
11 R. Watts and R. Dils, *J. Lipid Res.*, 10 (1969) 33–40.
12 S. Akiya and S. Okui, *Yakugaku Zasshi* 71 (1951) 865–869.
13 P. Fleury and J. Lange, *J. Pharm. Chim.*, 17 (1933) 107–113, 196–208.