Preparation of Novel (1→3)-β-D-Glucans Having Reducing Glucose Side Chains

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Novel (1→3)-β-D-glucans (GPBCD, GPECD, GP6CD, and GP3CD) having reducing glucose side chains were prepared from a linear (1→3)-β-D-glucan (curdlan: CD) with halogeno glucose isopropylidene derivatives in dimethyl sulfoxide containing dimsyl sodium, followed by treatment with 40% trifluoroacetic acid to remove protecting isopropylidene groups. The side chain glucose moiety was linked directly or through a spacer at various positions except for its anomeric carbon.

Key words branched (1→3)-β-D-glucan; curdlan; (3-*O*-glucopyranosyl)-1′-butylated glucan; 6-*O*-glucopyranosylated glucan

The effectiveness of polysaccharides as carriers in drug delivery systems for cancer chemotherapeutic agents has been proven.[1-3] We previously reported the preparation and antitumor activities of mitomycin C conjugated with carboxymethylated (1→3)-β-D-glucans.[4,5] Major problems in the use of glucans as carriers are the low reactivity of glucose residues with cancer chemotherapeutic agents and the decrease of the biological activities (antitumor and immunomodulating effects) that follows the conjugation process. For example, the highly ordered structure of schizophyllan is destroyed upon carboxymethylation, resulting in loss of its antitumor activity.[5] Therefore, we synthesized novel (1→3)-β-D-glucans substituted with reducing glucose moieties linked either through spacers or directly at position 3 or 6 with position 6 of the glucose units of a linear (1→3)-β-D-glucan, curdlan (CD).

The preparation of the (1→3)-β-D-glucan derivatives with reducing glucose side chains was performed in two steps, involving the modification of 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**1**) for introduction as side chains and the reaction of the modified glucofuranose with $NaBH_4$-reduced curdlan. For linking glucose residues to curdlan, butyl and ethoxyethyl as spacers were used. Compound **1** was modified with 1,4-dibromobutane and bis(2-chloroethyl)ether to yield 3-*O*-(1-bromobutyl)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**2**) and 3-*O*-(1-chloroethoxyethyl)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**3**), respectively. For direct linkage of glucose moieties to curdlan, compound **1** was brominated with *N*-bromosuccinimide (NBS) in *N*,*N*-dimethylformamide (DMF) or in chlorobenzene to yield 6-bromo-6-deoxy-1,2:3,5-di-*O*-isopropylidene-α-D-glucofuranose (**4**) and 3-bromo-3-deoxy-1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose (**5**), respectively. These glucose derivatives (1.2 g) were reacted with reduced curdlan (250 mg) in dimethylsulfoxide (DMSO) containing dimsyl sodium, and then the protecting isopropylidene group was removed to yield (3-*O*-glucopyranosyl)-1′-butylated curdlan (GPBCD, 243 mg), (3-*O*-glucopyranosyl)-1′-ethoxyethylated curdlan (GPECD, 236 mg), 6-*O*-glucopyranosylated curdlan (GP6CD, 210 mg), and 3-*O*-glucopyranosylated curdlan (GP3CD, 223 mg).

The ^{13}C-NMR spectra of the synthesized glucan derivatives in DMSO-d_6 showed two signals at 96.9 (C′-1, β) and 92.3 ppm (C′-1, α), in addition to the anomeric carbon signal at 103.1 ppm (C-1, β) due to curdlan, suggesting the presence of reducing glucose units. The degree of substitution (DS: the number of side chains per anhydroglucose) in the glucan derivatives was calculated from the value of reducing glucose measured by the Nelson–Somogyi method.[6] DS (0.12 to 0.24) of GPBCD and GPECD having a spacer was higher than that (0.03 to 0.10) of GP6CD and GP3CD. The results indicate that direct introduction was more difficult than introduction using a spacer. The molecular weights of GPBCD, GPECD, GP6CD, and GP3CD were estimated to be approximately 35000, 38000, 28000, and 25000, respectively, by gel filtration chromatography on Toyopearl HW-55F in 0.1 M NaOH. Since the molecular weight of reduced curdlan was about 72000 under the same conditions, the result suggests that these glucan derivatives

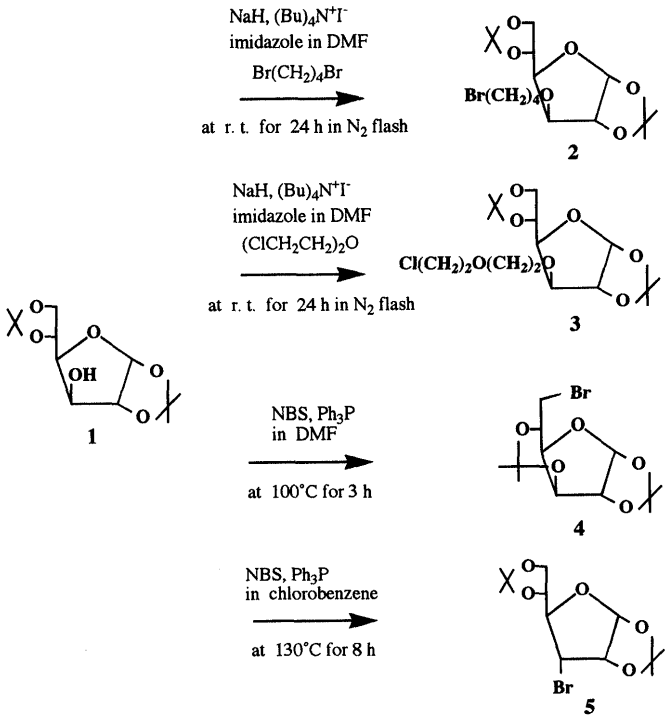

Chart 1

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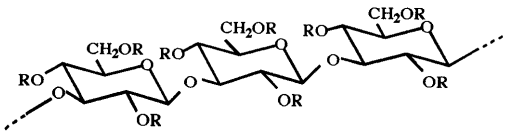
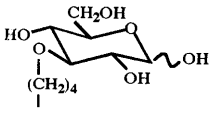
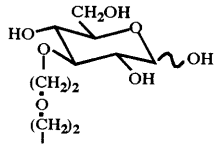
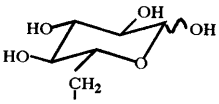


Curdlan derivative

GPBCD, R = H or 

GPECD, R = H or 

GP6CD, R = H or 

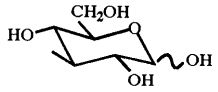
GP3CD, R = H or 

Chart 2

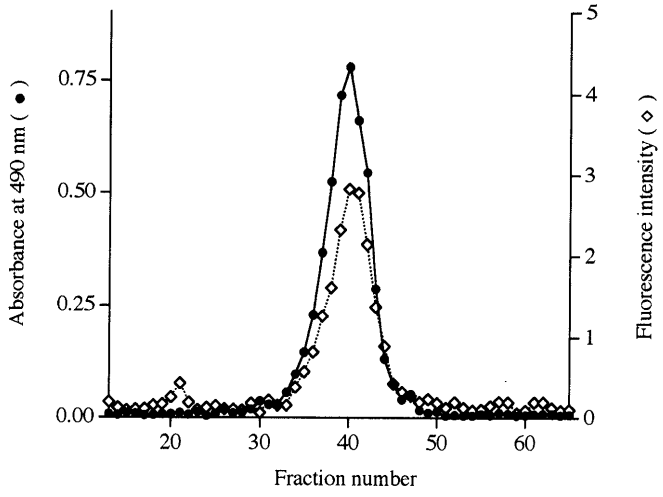


Fig. 1. Gel Filtration Pattern of Pyridylaminated GPECD in 0.1 M NaOH on Toyopearl HW-55F

are degraded during the process of preparation. However, high fluorescence intensities, over 80 for these derivatives compared to 100 for curdlan, were observed in the aniline blue test,[7] suggesting that the derivatives retain the high-ordered structure required for the antitumor activity of glucans.[8,9] To confirm the reactivity of the hemiacetal hydroxide groups in the glucose moieties introduced into curdlan, the derivatives were reacted with 2-aminopyridine. As glucose is reductively aminated with this fluorescence reagent,[10] the glucan derivatives were easily labeled. The labeled glucan derivative showed a homogeneous glucan peak in gel filtration chromatography on Toyopearl HW-55F (Fig. 1). Since the glucan derivatives show higher reactivity and solubility in water than natural (1→3)-β-D-glucans, the polymers seem to be available as useful pro-drug carriers.

Experimental

Materials Curdlan and 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**1**) were obtained from Wako Pure Chemical Industries, Ltd., Japan. Toyopearl HW-55F was from Tosoh Manufacturing Co., Ltd., Japan. Other chemicals were purchased commercially and were of the highest available grade.

Preparation of 3-*O*-(1-Bromobutyl)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (2) and 3-*O*-(1-Chloroethoxyethyl)-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (3) A DMF solution (5 ml) of **1** (1.5 g), n-$Bu_4N^+I^-$ (80 mg), and imidazole (200 mg) was dropped from a syringe into DMF solution (5 ml) containing sodium hydride (500 mg) and the mixture was allowed to stand for 30—60 min at room temperature. 1,4-Dibromobutane (2 ml) or bis(2-chloroethyl)ether (2 ml) was added and the reaction mixture was stirred overnight under ice cooling. The reaction mixture was dried and evaporated, and the residue was extracted with ethyl acetate. The product, **2** or **3**, was isolated by column chromatography on Wakogel C-300 in hexane–ethyl acetate (5:1) to yield 41 or 35%.

Preparation of 6-Bromo-6-deoxy-1,2:3,5-di-*O*-isopropylidene-α-D-glucofuranose (4) and 3-Bromo-3-deoxy-1,2:5,6-di-*O*-isopropylidene-α-D-allofuranose (5) NBS (1.8 g) and triphenylphosphine (Ph_3P) (2.5 g) were added to DMF (40 ml) under ice cooling and the mixture was stirred for 10 min. Compound **1** (1.5 g) was added, and the whole was stirred at room temperature for 3 h, then at 100 °C for 2 h. The reaction mixture was concentrated by evaporation and ethyl acetate (75 ml) was added to the residue. A 5% sodium bicarbonate solution was added, the mixture was vigorously shaken, and the organic layer was dried and evaporated. The residue was dissolved in ether (50 ml) and the solution was filtered, then the product was purified by column chromatography in hexane–ethyl acetate (3:1) to afford **4** in 75% yield.

Compound **5** was prepared according to the method reported by Hodosi *et al.*[11] Compound **1** (1.5 g), Ph_3P (2.7 g), and imidazole (0.7 g) were dissolved in chlorobenzene (75 ml) and the solution was refluxed at 130 °C. After 7 h, NBS (1.8 g) was added gradually and the mixture was refluxed for an additional 2 h. After the mixture had cooled, trichloromethane (75 ml) and 5% sodium bicarbonate were added and the organic layer was dried and evaporated. The residue was dissolved in ether (50 ml), the solution was filtered, and the product was purified by column chromatography in hexane–ethyl acetate (3:1) to afford **5** in 49% yield.

Preparation of Curdlan Derivatives Before curdlan was used in the linkage reaction with glucose derivatives, it was treated with sodium borohydride ($NaBH_4$).[12] Curdlan (1 g) was dissolved in 0.25 M NaOH (200 ml), $NaBH_4$ (500 mg) was added, and the mixture was stirred at room temperature for 5 h. It was neutralized with 1 M HCl, and dialyzed against water, then reduced curdlan was obtained by lyophilization.

Under a stream of nitrogen, reduced curdlan (250 mg) was dissolved in DMSO (25 ml) and 4 M dimsyl sodium (2 ml) was added slowly to the solution and thoroughly dissolved. Compound **2** or **3** (1.2 g) dissolved in DMSO (2 ml) was gradually added to the solution and the mixture was stirred at room temperature for 24 h. It was then diluted with water, dialyzed against water, and lyophilized. The lyophilysate was dissolved in 40% trifluoroacetic acid (TFA) to give a concentration of 5 mg/ml and the solution was stirred at room temperature for 5 h to remove

isopropylidene groups.[13] It was neutralized with 4 M NaOH and centrifuged, then the supernatant was dialyzed against water and lyophilized to obtain the product, GPBCD (243 mg) or GPECD (236 mg).

Reduced curdlan (250 mg) was dissolved 20 ml of DMF–DMSO (1 : 9) and the mixture was stirred at room temperature overnight under a stream of nitrogen. After 4 M dimsyl sodium (2 ml) had been added to the mixture and dissolved thoroughly, compound **4** or **5** (1.2 g) dissolved in DMSO (2 ml) was added and the reaction mixture was stirred at 15 °C for 48 h. It was diluted with water, dialyzed against water, and lyophilized. The lyophilysate was further reacted 3 times with compound **4** or **5** according to the procedure described above to increase the degree of substitution. The product, GP6CD (210 mg) or GP3CD (223 mg), was obtained after removing the protecting groups as described above.

Physicochemical Properties of Curdlan Derivatives The ^{13}C-NMR spectra were recorded on a JEOL-EX 400 FT NMR spectrometer using solutions in DMSO-d_6 (30 mg/0.5 ml) at room temperature.

For the determination of molecular weight, a sample (2 mg) was dissolved in 0.1 M NaOH (0.5 ml) and applied to a Toyopearl HW-55F column (1.5 × 90 cm) equilibrated with 0.5 M NaOH, then eluted with the same solution at a flow rate of 8 ml/h. Fractions (4 ml) were collected and the glucan was estimated by the phenol–sulfuric acid method.[14] The molecular weight standards used were Dextran T-70 (70000), Dextran T-40 (39500), and Dextran T-20 (22300) (Pharmacia Fine Chemicals).

The degree of helical conformation of curdlan derivatives was measured by the reported method using aniline blue.[7] The sample was dissolved at the concentration of 250 μg/ml in a solution consisting of 0.5 M NaCl, 0.1 M NaOH, and 10 μg/ml aniline blue. Fluorescence intensity was measured with excitation and emission at 395 and 495 nm, respectively.

Each curdlan derivative was reacted with 2-aminopyridine to confirm reducing glucose side chains in it. A curdlan derivative (5 mg) was dissolved in 0.1 M sodium phosphate buffer (pH 6.0) (1 ml), 2-aminopyridine (10 mg) was added and the mixture was stirred at room temperature overnight. It was neutralized with 1 M BaOH and filtered, then the filtrate was treated with $NaBH_4$ and dialyzed against water for 2 d. The dialysate was applied to a Toyopearl HW-55F column (1.5 × 90 cm) equilibrated with 0.1 M NaCl and subsequently eluted with the same solution. Fractions (2.5 mg) were collected and the glucan was determined as described above; in addition, the fluorescence intensity was measured with excitation and emission at 254 and 354 nm, respectively.

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