6-Amino-6-deoxychitosan. Preparation and Application as Plasmid Vector in COS-1 Cells[1]

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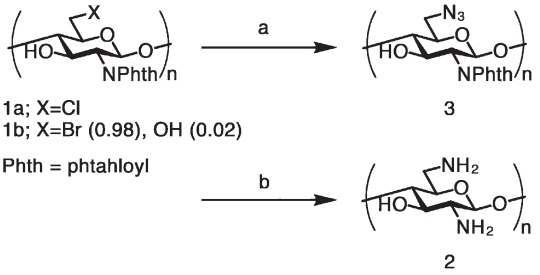
6-Amino-6-deoxychitosan was prepared from 6-deoxy-6-halo-*N*-phthaloylchitosan via 6-azidation. The product has high stereoregularity because of the effective and regioselective reactions. To evaluate 6-amino-6-deoxychitosan as a gene transfer agent, transfection efficiencies of its complexes with plasmid DNA in COS-1 cells were examined.

Aminopolysaccharides such as chitin and chitosan have attracted much attention because of their biological properties.[2,3] In order to add or enhance the properties, synthetic aminopolysaccharides have been prepared by modifying chitin, chitosan,[4] dextran,[5] and cellulose,[6] and by polymerizing aminosugars.[7] Stereochemical control during modification and synthesis of polysaccharides is generally difficult because these compounds possess numerous functional groups as reaction sites. The disordered structure of such derivatives results in analytical problems, and as a result, the actual structures of these products remain abstruse. Thus, precise control of the reaction site and degree of substitution are required in order to obtain derivatives having well-defined structures. In our previous report, we prepared 6-deoxy-6-halo-*N*-phthaloylchitosans (**1a–b**).[8] These chitosan derivatives possess structural regularity, high solubility in aprotic polar solvents, and high reactivity for nucleophilic substitutions, all of which are suitable properties for achieving the sophisticated chemical modifications described above. In this report, we describe the preparation of a novel non-natural aminopolysaccharide, 6-amino-6-deoxychitosan (**2**). In addition, **2** is evaluated as a non-viral plasmid vector.



Scheme 1. a) NaN_3, in NMP, at 120 or 80 °C. b) i; PPh_3, in NMP, at rt. ii; $NH_2NH_2{\cdot}H_2O$, in NMP–H_2O (1:1), at 100 °C.

Preparation of **2** was carried out by a) azidation of the C-6 positions of **1a–b** followed by b) reduction of the azido groups and deprotection of the C-2 amino groups as shown in Scheme 1. Sodium azide (926 mg, 14.2 mmol) was added to a solution of **1b** (degree of substitution (d.s.) of bromine 0.98, 500 mg, 1.42 mmol of sugar unit)[9] in *N*-methyl-2-pyrrolidone (NMP, 50 mL), and the mixture was stirred at 80 °C for 4 h under a nitrogen atmosphere. The reaction mixture was filtered through cotton wool to crudely remove the salts and the filtrate was poured into ethanol (500 mL). The resulting precipitate was collected by centrifugation (10^4 rpm, 7 min), and washed with ethanol–water, then acetone on a filter. After drying under reduced pressure at 40 °C, 6-azido-6-deoxy-*N*-phthaloylchitosan (**3**) was obtained as a light brown powder (388 mg, 86% yield). The IR spectrum of the product showed an absorption band characteristic of azido group at 2100 cm^{-1} (data not shown). Moreover, the structure of the product was investigated by ^{13}C NMR spectroscopy. The signal of the C-6 carbon shifted downfield (50.1 ppm) from that of **1b** (33.5 ppm) (Figure 1, top). In addition, slight shifts of other peaks suggested that the reaction selectively proceeded at the C-6 positions, as expected. These results supported the predicted structure **3**.[10] The exact d.s. as calculated from the results of elemental analysis was 0.95 for azido groups, with some remaining bromine (d.s. 0.03). Although the same reaction with **1a**[9] gave partially substituted product, an increase in reaction temperature to 120 °C gave a product having a similar ^{13}C NMR spectrum to that of **3**.

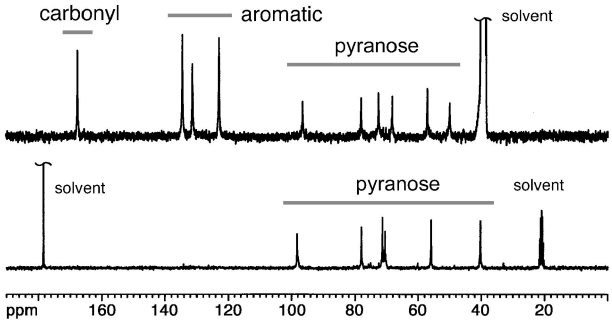


Figure 1. ^{13}C NMR spectra of compound **3** in DMSO-d_6 at 50 °C (top) and **2** in D_2O containing acetic acid-d_4 at 50 °C (bottom).

One of the more practical routes for converting azido groups into amino groups involves reacting the former with triphenylphosphine (TPP) to form the corresponding phosphine imido intermediate followed by basic hydrolysis. However, in this process, hydrolysis of **3** will lead to ring opening of the *N*-phthaloyl groups as a side reaction, and the resulting *N*-(2-carboxy)benzoyl groups are difficult to remove. Therefore, use of hydrazine monohydrate for hydrolysis was considered to be more suitable for the preparation of **2** because this reagent promotes hydrolysis of the phosphine imido and simultaneously removes the *N*-phthaloyl groups (Scheme 1b). After reaction of **3** (d.s. azido 0.95, 200 mg, 0.63 mmol of sugar unit) with TPP (496 mg,

1.89 mmol) in NMP (20 mL) for 12 h at room temperature under a nitrogen atmosphere, the reaction solution was treated with 4 mol/L aqueous hydrazine monohydrate for 4 h at 100 °C without a purification step. The reaction mixture became heterogeneous on addition of aqueous hydrazine monohydrate and then became homogeneous again within 5 min of heating at 100 °C. After the water was evaporated off, the suspended reaction mixture was poured into ethanol. The precipitate formed was collected by centrifugation (10^4 rpm, 7 min, three times) and washed with ethanol. The precipitate was dissolved in neutral water and purified by ultrafiltration using a filter with a cut-off of 10^4. After lyophilization, the product was obtained as an ivory amorphous material (76 mg, 75% yield). The product was soluble in neutral water during the purification process. However, after lyophilization, a small part was insoluble in even acidic water. The NMR spectra of the product were recorded in deuterium oxide containing acetic acid-d_4 after removal of the insoluble material by filtration. The well-defined structure of the product was confirmed from its ^{13}C NMR spectrum (Figure 1, bottom). In addition, the IR spectrum of the product showed the disappearance of the characteristic absorption bands for the azido (2100 cm^{-1}) and imido groups (1775, 1720, 1390, and 720 cm^{-1}) (data not shown). These results supported the predicted structure of the product as **2**.[11]

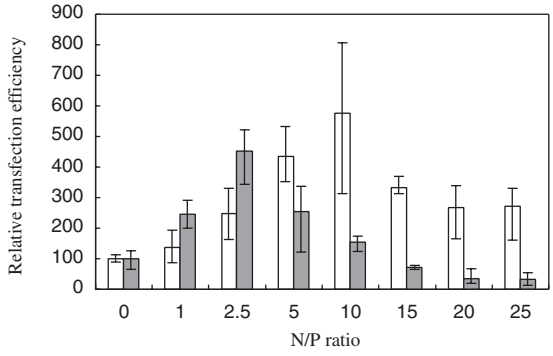


Figure 2. Transfection efficiencies of **2** and chitosan/pGL3 complex were evaluated by luciferase assay using COS-1 cells in DMEM containing 10% FBS. Unshaded bars, **2**; shaded bars, chitosan. Molecular weight of vectors ranged from 10 to 30 kDa.

Because cationic polyelectrolytes, including chitosan,[12] are used to transfer gene into tumor cells, we evaluated **2** as a non-viral gene vector. The gene transfer efficiency of **2** was examined by luciferase assay using COS-1 cells in DMEM containing 10% FBS, and the efficiency was compared with that of chitosan. The molecular weight of **2** and chitosan was estimated to be 36 kDa by static light scattering. The acetate and hydrochloride salts of **2** and chitosan were used, respectively. The **2**/pGL3 complexes were prepared by mixing of pGL3-control solution (17.5 μL, 0.616 mmol/L) with a given amount of solution of **2** (7.7 mmol/L). The complex solution contained 0.2% (v/v) of 10 mmol/L hydrochloric acid, because the transfection efficiency of chitosan at pH 6.9 is reportedly higher than that at pH 7.6.[12] The ratios of the amino groups in **2** to pGL3 phosphate (N/P ratio) were 1, 2.5, 5, 10, 15, 20, and 25. The chitosan/pGL3 complexes were prepared by the same methods. The measurement of transfection efficiency was performed in triplicate. The results of luciferase assay are shown in Figure 2. The preliminary results indicate that the transfection efficiency of **2** was significantly higher than that of chitosan. The highest activity for **2** was observed at an N/P ratio of 10. Agarose gel shift assay showed that **2** possessed a higher affinity than chitosan for pGL3 (data not shown). The increase in the density of amino groups along the polysaccharide chain is believed to have led to these results as reported with oligoamine-dextran conjugates.[13]

In conclusion, a synthetic β(1,4)-D-glucan composed of the D-2,6-diamino-2,6-dideoxyglucose **2** was prepared by stereoselective modification of the 6-halodeoxy chitosan derivatives **1a–b** via the 6-azidodeoxy derivative **3**. When compared to chitosan, this cationic polyelectrolyte exhibited higher transfection efficiency in COS-1 cells. We are now proceeding not only to examine the optimal conditions for transfection with **2** but also to modify **2** chemically to improve the transfection efficiency.

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References and Notes

1 This is the 28th paper in the series of "polysaccharide-polynucleotide complexes".
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9 **1a**–**b** were prepared from chitosan (Mv 1.1×10^5, 99% deacetylaiton) according to Ref. 8.
10 6-Azido-6-deoxy-*N*-phthaloylchitosan (**3**), d.s. 0.95. ^{13}C NMR (75.48 MHz): δ (DMSO-d_6) 50.1, 57.2, 68.0, 72.5, 77.9, and 96.5 (pyranose), 122.8, 131.1, and 134.3 (aromatic), and 167.5 ppm (C=O). ν_{max} (KBr) 2100 cm^{-1} (azido). Anal. Calcd. for $(C_{14}H_{12}N_4O_5)_{0.95}$ $(C_{14}H_{12}BrNO_5)_{0.03}$-$(C_{14}H_{13}NO_6)_{0.02}\cdot 0.2\ H_2O$: C, 52.46; H, 3.91; N, 16.83; Br, 0.75. Found: C, 52.93; H, 4.21; N, 16.20; Br, 0.48%.
11 6-Amino-6-deoxychitosan (**2**). ^{13}C NMR (75.48 MHz): δ (deuterium oxide containing acetic acid-d_4) 42.6, 58.3, 72.9, 73.7, 80.3, and 100.6 ppm. Anal. as acetate salt. Calcd. for $(C_6H_{12}N_2O_3)_{1.00}(C_2H_4O_2)_{1.00}\cdot 2.30\ H_2O$: C, 36.72; H, 7.94; N, 10.71. Found: C, 36.56; H, 7.69; N, 10.52%.
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