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6-Amino-6-deoxy-chitosan. Sequential chemical modifications at the C-6 positions of *N*-phthaloyl-chitosan and evaluation as a gene carrier

Taku Satoh,[a,e,*] Hiroshi Kano,[a] Mika Nakatani,[a] Nobuo Sakairi,[b] Seiji Shinkai[c,d] and Takeshi Nagasaki[a]

[a]*Department of Applied and Bioapplied Chemistry, Graduate School of Engineering, Osaka City University, Sumiyoshi, Osaka 558-8585, Japan*
[b]*Division of Bioscience, Graduate School of Environmental Earth Science, Hokkaido University, Kita-Ku, Sapporo 060-0810, Japan*
[c]*Department of Chemistry and Biochemistry, Graduate School of Engineering, Kyushu University, Nishi-ku, Fukuoka 819-0395, Japan*
[d]*Kyushu University Center for Future Chemistry, Nishi-ku, Fukuoka 819-0395, Japan*
[e]*SORST Program, Japan Science and Technology Corporation (JST), Japan*

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Abstract—The C-6 positions of chitosan were successively modified in a highly regioselective manner. The starting material, *N*-phthaloyl-chitosan, was successfully converted into the corresponding 6-deoxy-6-halo derivatives by reaction with *N*-halosuccinimides and triphenylphosphine in *N*-methyl-2-pyrrolidone. The resulting chloride and bromide derivatives were then substituted with azido groups by reaction with sodium azide at 120 and 80 °C, respectively. The azido groups were then reduced to amines via formation of the triphenylphosphinimine intermediate followed by hydrolysis using aqueous hydrazine, which also led to the removal of the *N*-phthaloyl groups at the C-2 positions. This sequence gave 6-amino-6-deoxy-chitosan, which, unlike chitosan, is soluble in water at neutral pH. The synthesized 6-amino-6-deoxy-chitosan derivative was evaluated as a gene carrier, and the transfection efficiency for COS-1 cells was shown to be superior to chitosan. In addition, the cytotoxicity was similar to chitosan.

Keywords: Chitin; Chitosan; 6-Amino-6-deoxy-chitosan; Drug delivery; Gene delivery; Polymeric gene carrier

1. Introduction

Chitosan is a structurally regular homopolymer composed of β-(1→4)-linked D-glucosamine (2-amino-2-deoxy-D-glucopyranose) residues. It is readily obtained from chitin, which is one of the most abundant natural polysaccharides. Chitosan and its derivatives have been used in medical,[1,2] food,[3] and chemical materials. Studies in the medical field have markedly expanded due to the suitable biological characteristics of chitosan, and a large number of medical applications have been reported such as wound healing,[4] use as hemostatic gels,[5] and drug, and gene delivery.[6–10] To modify the characteristics of chitosan, many chemical modifications have been reported. In particular, quarternized and aminated chitosan derivatives have been shown to have especially interesting potential as biomaterials[11–13] and functional materials.[14]

Chitosan is soluble in water containing organic acids, in which its amino groups are protonated. The insolubility of chitosan in organic solvents has restricted its modification; direct substitutions at the C-6 or C-3 positions have been either ineffective or nonselective. To perform selective modifications at these positions, stepwise approaches have been reported using some N-substituted derivatives including imido,[15] *N*-acyl,[16] and imino

* Corresponding author. Tel.: +81 6 6605 2696; fax: +81 6 6605 2785; e-mail: satoh@bioa.eng.osaka-cu.ac.jp

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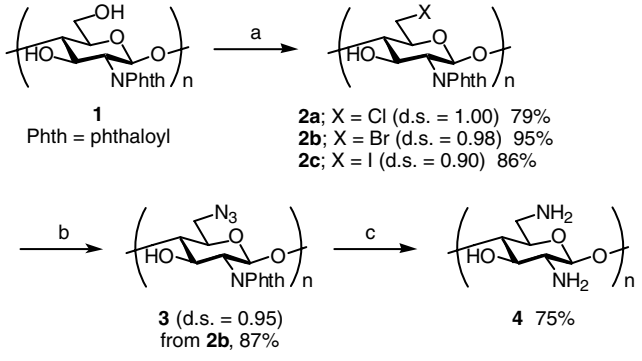
derivatives[17] as precursors. In particular, *N*-phthaloylchitosan (**1**) is regarded as one of the most useful precursors, due to its high solubility in polar aprotic solvents, which makes its modification straightforward. In addition, the free amino groups can be regenerated by dephthaloylation.[15] Interesting modifications, for example, 6-O-tritylation, 6-O-tosylation, and 6-O-trimethylsilylation, have been performed using **1**.[15,18] Furthermore, other derivatives have been prepared recently and have provided chitosan-based functional materials such as amphiphilic,[19] liquid crystalline,[20] site specific-sulfated,[21,22] and 6-O-glycosylated derivatives.[23]

Substitution of the hydroxyl groups of **1** by halogens was expected to provide more efficient precursors in terms of a reactivity and solubility in organic solvents. Deoxyhalogenated derivatives are versatile precursors in synthetic carbohydrate chemistry. Numerous investigations have been performed on the deoxyhalogenation of polysaccharides, for example, cellulose,[24–26] chitin,[27–29] and curdlan,[30] as well as mono- and oligosaccharides. Among the reported methods, reaction with *N*-halosuccinimides and triphenylphosphine (TPP) have shown a high site selectivity and a good reactivity.[25–28] We investigated the selective deoxyhalogenation of the C-6 positions of **1** using this method,[31] and the obtained halide derivatives were subsequently modified. As described above, chitosan and its derivatives have been already studied as a gene carrier. As a novel chitosan-based gene carrier, we prepared 6-amino-6-deoxy-chitosan (**4**) via azido and halo intermediates (Scheme 1).[32,33] This report describes the facile synthesis of **4** from **1** and its evaluation as a gene carrier.

2. Results and discussion

Deoxyhalogenation of **1** was carried out using the methods previously described for cellulose[25,26] and chitin.[27,28] Three *N*-halosuccinimides, *N*-chloro-, *N*-bromo-, and *N*-iodosuccinimide (NCS, NBS, and NIS, respectively), and TPP were used to introduce the halogens at the C-6 position of chitosan. A preliminary experiment was performed to examine the effect of the reaction temperature (40, 80, or 120 °C) and the amount of reagents (5 and 10 equiv of each reagent per sugar unit) on the degree of the substitution (d.s.) of the products. After acetylation of the unsubstituted hydroxyl groups in the products, the d.s. were roughly calculated by the peak area ratio between the aromatic and acetyl protons in the ^{1}H NMR spectra, although exact determinations are difficult due to the broad spectra. The introduction of halogen atoms proceeded in the order of chlorine > bromine > iodine, reflecting the nucleophilicities of halide ions in DMF[34] and DMSO.[35] Under the reaction conditions examined, the d.s. increased with an increase in the reaction temperature and the amounts of the reagents. Undesirable coloration and the lower product yields at 120 °C suggested the degradation of the polymer backbone under these harsh conditions. As a result of these studies we identified that carrying out the reaction at 80 °C and with 10 equiv of the halogenation reagent is optimal for achieving sufficient deoxyhalogenation of **1** (Scheme 1, path a). The exact d.s. of the products obtained under these conditions was determined by elemental analyses. The d.s. of chloro derivative **2a**, bromo derivative **2b**, and iodo derivative **2c** was 1.00, 0.98, and 0.90, respectively. The substitution by halogen atoms caused upfield shifts of the peaks attributed to C-6 carbons (C–Cl; 43.9, C–Br; 33.5, and C–I; 7.8 ppm) in the ^{13}C NMR spectra of **2a**–**c** (Fig. 1). These spectra demonstrate that the reaction proceeded site-selectively at the C-6 positions of **1**. The halogenated derivatives **2a**–**c** showed a good solubility in polar aprotic solvents such as *N*-methyl-2-pyrrolidone (NMP), DMSO, DMF, and pyridine.

It has been reported that the deoxyhalogenation of cellulose with NCS/TPP occurs to a small degree at



Scheme 1. Reagents and conditions: (a) *N*-halosuccinimide, triphenylphosphine, NMP, 80 °C, 2 h; (b) NaN_3, NMP, 80 °C, 4 h; (c) (i) triphenylphosphine, NMP, rt, 12 h; (ii) $NH_2NH_2{\cdot}H_2O$, NMP/water (1:1), 100 °C, 4 h.

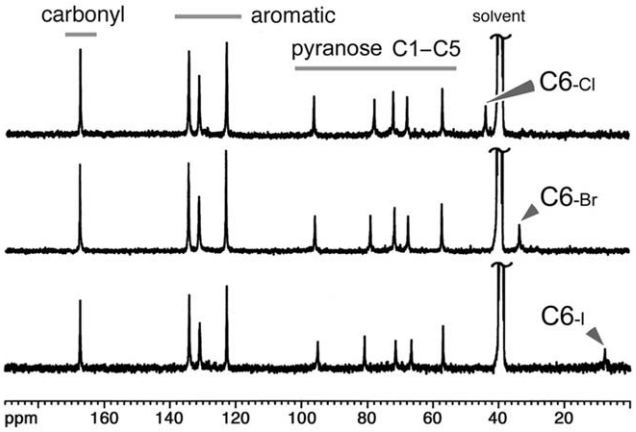


Figure 1. ^{13}C NMR (75.48 MHz) spectrum of **2a** (top), **2b** (middle), and **2c** (bottom) in DMSO-d_6 at 50 °C.

the C-3 positions in addition to the C-6 positions.[25] In contrast, similar reaction of chitin proceeded selectively at the C-6 positions.[22] This difference can be explained by consideration of the steric hindrance around the C-3 positions. Cellulose and chitin consist of the same β-(1→4)-D-glucan, however, the latter has acetamido groups at the C-2 positions. These large groups appear to inhibit reaction at C-3; for the same reason, the *N*-phthaloyl groups contribute to the C-6 selectivity of halogenation of **1**.

Azidation of the 6-bromodeoxy derivative **2b** with sodium azide in NMP readily proceeded at 80 °C (Scheme 1, path b). The IR spectra of the isolated products showed the characteristic azido group absorption band at 2100 cm^{-1}, and the absorption intensity became almost constant after 1 h (Fig. 2). The structure of the product obtained at the reaction time of 4 h was investigated by ^{13}C NMR spectroscopy. As shown in the spectrum (Fig. 3), the signal of the C-6 carbon of the product shifted downfield (50.1 ppm) compared to that of **2b** (33.5 ppm). Moreover, the small shifts of the other resonances suggested that the reaction proceeded selectively at the C-6 positions, as expected. These results, in addition to elemental analysis, supported the structure as 6-azido-6-deoxy-*N*-phthaloyl-chitosan (**3**). The exact d.s. of the azido groups calculated by elemental analysis was 0.95, with the bromide derivative corresponding to a d.s. of 0.03. When this reaction was applied to chloro derivative **2a** at 120 °C, a similar ^{13}C NMR spectrum to that of **3** was obtained; however the reaction with **2a** at 80 °C gave a product with a lower d.s. The efficient conversion of compound **2a**,**b** into **3** demonstrated the remarkable utility of these halide derivatives as reaction intermediates. A reaction temperature of 120 °C was required to complete azidation of **2a**, indicating the lower reactivity of this compound.

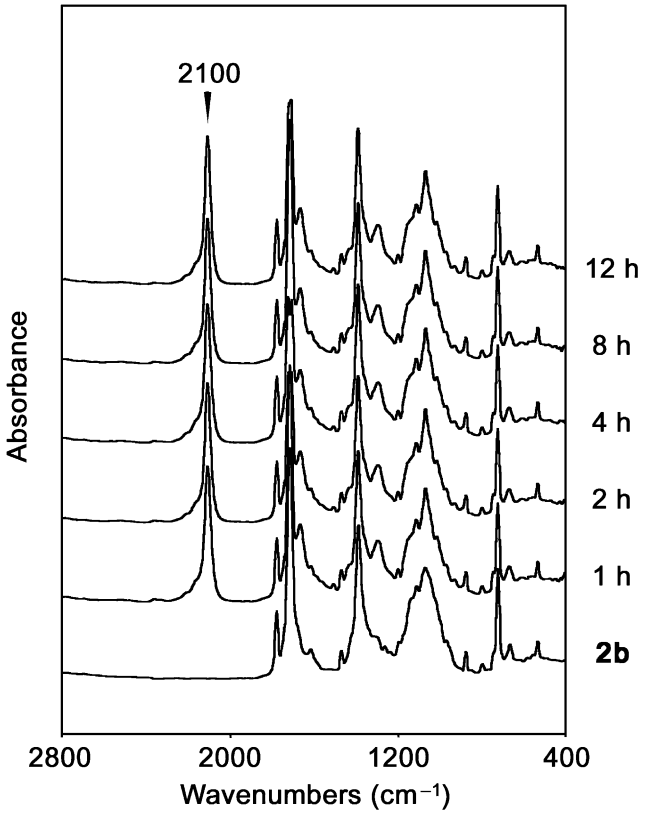


Figure 2. IR spectra of the products of azidation reaction of **2b** for 12, 8, 4, 2, and 1 h and original **2b** (top to bottom).

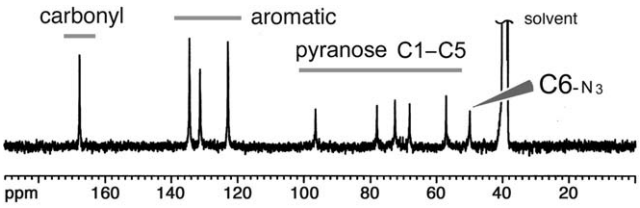


Figure 3. ^{13}C NMR (75.48 MHz) spectrum of **3** in DMSO-d_6 at 50 °C.

One of the useful routes to convert azido groups into amino groups involves treatment with TPP to form the corresponding phosphinimine intermediates and subsequent basic hydrolysis. However, the hydrolysis process can lead to the ring opening of the *N*-phthaloyl group in **3** as a side reaction and the resulting *N*-(2-carboxy)benzoyl groups are difficult to remove. The use of hydrazine monohydrate for the hydrolysis process is a convenient method for the preparation of 6-amino-6-deoxy-chitosan (**4**), because this reagent will promote both the hydrolysis of the phosphinimine intermediate and the *N*-phthaloyl group (Scheme 1, path c).

As a preliminary evaluation of this approach, treatment of **3** with hydrazine monohydrate was carried out to estimate the reaction time required for the complete dephthaloylation. In the absence of TPP, the reaction was performed in 4 M aqueous hydrazine monohydrate–NMP at 100 °C, and the reaction time was varied from 1 to 12 h. The IR spectra of the reaction mixture showed a decrease in the characteristic absorption bands of imide at 1780, 1710, 1390, and 720 cm^{-1} (Fig. 4) and an increase in the absorption band of the amine (3500–3300 cm^{-1}, data not shown). Small changes were observed in the range of 2000–400 cm^{-1} after 4 h and later. A small decrease in the azido group absorption band at 2100 cm^{-1} were shown at reaction times of 8 and 12 h, which was probably attributable to the reduction of the azido groups to amino groups due to the reducing ability of hydrazine monohydrate. The d.s. of *N*-phthaloyl groups as calculated from the ^{1}H NMR spectra suggested that the dephthaloylation was almost complete after 4 h (data not shown). These results agreed well with those from IR spectroscopy and thus the reaction was carried out for 4 h at 100 °C.

After these preliminary experiments, the conversion of **3** to **4** was examined. A solution of **3** in NMP was trea-

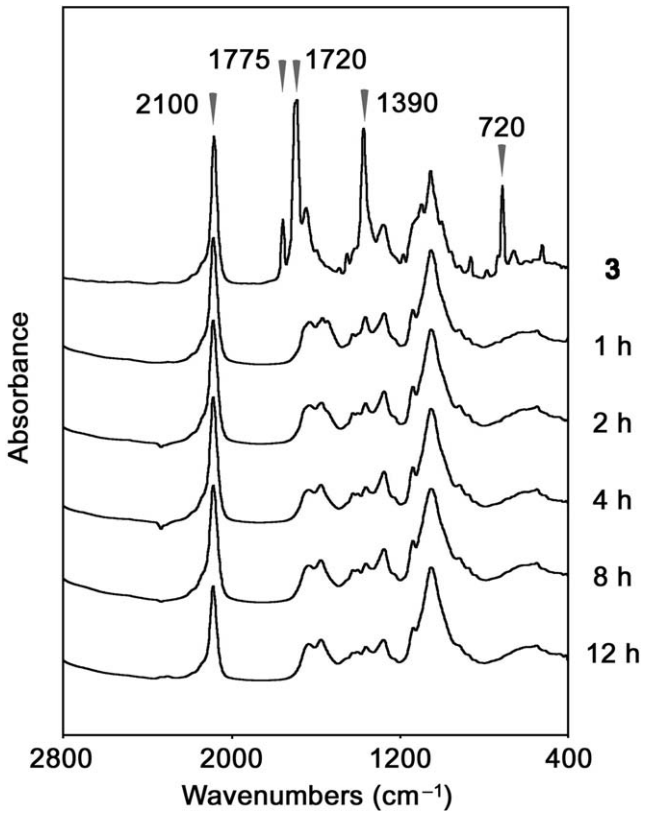


Figure 4. IR spectra of **3** and the products obtained by dephthaloylation with aqueous hydrazine for 1, 2, 4, 8, and 12 h (top to bottom).

ted with TPP to convert the azido groups into the corresponding triphenylphosphinimines. The resulting reaction mixture was successively treated with 4 M aqueous hydrazine monohydrate. The reaction mixture temporarily turned heterogeneous upon addition of the hydrazine monohydrate but returned to homogeneous after heating at 100 °C for 5 min.

The product was soluble in neutral water during the purification process; however, after lyophilization a small fraction was insoluble, even in acidic water. NMR spectra of the product were recorded in D_2O containing CD_3CO_2D after filtration of the insoluble material. The ^1H NMR spectrum suggested the presence of ~5% of the remaining phthaloyl derivative; however, the structure of the product was confirmed by ^{13}C NMR spectrum (Fig. 5). The IR spectrum of the product showed no characteristic absorption bands of the

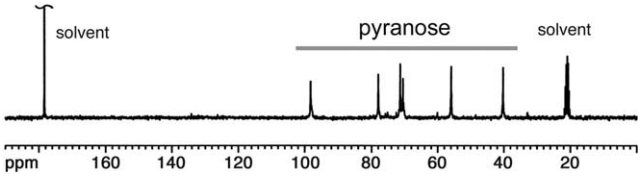


Figure 5. ^{13}C NMR (75.48 MHz) spectrum of **4** in D_2O containing CD_3CO_2D at 50 °C.

azido (2100 cm^{-1}) and imido groups (1775, 1720, 1390, and 720 cm^{-1}, data not shown). These results support the structure of the desired product as 6-amino-6-deoxy-chitosan (**4**). Although this compound with a 6-amino group with a d.s. of 0.7 was recently reported,[33] our synthetic route provides compound **4** with a well-defined structure.

Low cytotoxicity is one of the most important properties of biomaterials. The effects of the acetate salt of **4** on cell viability of COS-1 cells was evaluated by the WST assay and were compared with those of acetylated chitosan and polyethylenimine (PEI), which is a versatile polymer-based gene carrier.[36] As shown in Figure 6, cell viability decreased gradually with an increase in the concentration of the polyelectrolyte, 24 h after exposure. Upon exposure of the acetate salts of **4** and chitosan at a concentration of 150 μg/mL, 58% and 64% cell viability were observed, respectively. In contrast, 50% cell death was observed on exposure to 50 μg/mL of PEI. Serious cytotoxicity similar to PEI was not observed with the acetate salts of **4** or chitosan.

The complex formation of **4** with plasmid DNA (pGL3) was confirmed by an agarose gel retardation assay. Migration of pGL3 was not observed at an N/P of 2 and above (N/P is the ratio of the amino groups of **4** or chitosan, relative to the phosphate groups of pGL3), suggesting that pGL3 was completely complexed with **4** (Fig. 7a). A similar observation was shown for chitosan at an N/P of 2.5 and above (Fig. 7b). The small decrease in the N/P ratio required for sufficient complex formation was probably due to an increase in the amino groups on **4**.

The in vitro transfection efficiency of the **4**/pGL3 complexes was investigated using COS-1 cells as a model mammalian cell line and was compared with that of

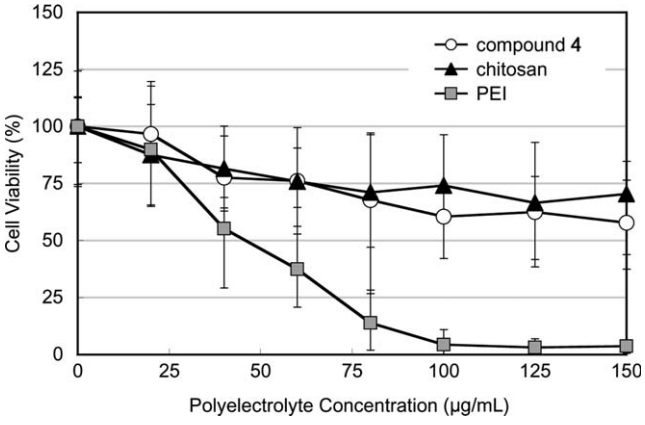


Figure 6. Evaluation of cytotoxicity of the acetate salt of **4** (open circles) and chitosan (closed triangles), and PEI (gray squares) on COS-1 cells by the WST assay. The cells were exposed to various concentrations of the cationic polyelectrolytes for 3 h and further incubated for 24 h. Cell Counting Kit-8 was added into the culture medium during the last 2 h.

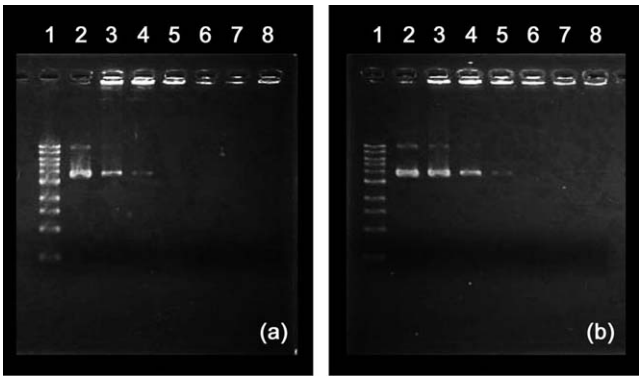


Figure 7. Agarose gel electrophoresis of the pGL3 solution mixed with **4** (a) and chitosan (b). Lane 1: 1 kb ladder. Lanes 2–8: The mixtures of polyelectrolyte and pGL3 in the N/P ratios 0, 1, 1.5, 2, 2.5, 3, and 5.

chitosan. The efficiency in the presence of 10% fetal bovine serum was evaluated by the luciferase assay. The expressed luciferase activity was represented as relative light units (RLUs) normalized against the total protein content. The complex formation and transfection process were achieved at pH 7.0. At this pH, chitosan demonstrates its activity as a gene carrier sufficiently, although the transfection efficiency of chitosan was significantly affected at medium pH and was almost suppressed under physiological conditions.[8] The maximum efficiency was found at an N/P of 2.5 in the **4**/pGL3 system, whereas that of the chitosan/pGL3 was observed at an N/P of 5 (Fig. 8). On comparison of efficiencies at each optimal N/P ratios, the luciferase activity in the cells transfected by **4** was 2.5 times higher than that with chitosan, although it was a quarter of that seen with the PEI complex (N/P = 10), which was used as a positive control (data not shown). These results indicate that **4** has superior properties to chitosan as a gene carrier.

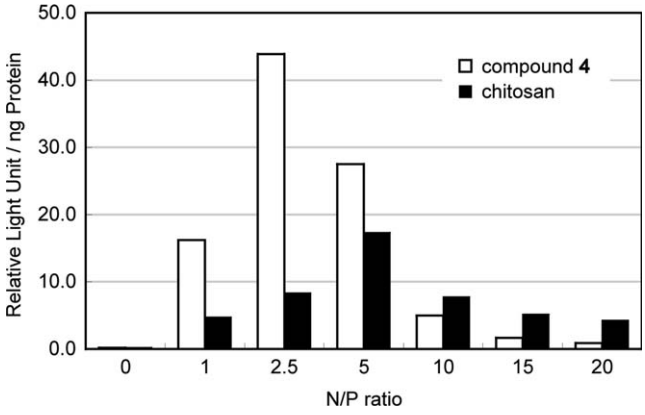


Figure 8. Relative transfection efficiency of **4** (open bars) and chitosan (closed bars) complexes with pGL3 for COS-1 cells. The complexes prepared at pH 7.0 were transfected in DMEM containing 10% fetal bovine serum at pH 7.0 for 3 h. Luciferase expression level was represented as a relative light unit (RLU) and was normalized against total protein content.

On the other hand, its transfection efficiency should be improved to make it practical.

In conclusion, deoxyhalogenation reactions of **1** with *N*-halosuccinimides and TPP in NMP progressed at the C-6 positions selectively and in good yield. The deoxychloro (**2a**) and deoxybromo (**2b**) derivatives were readily transformed to the corresponding azido derivative (**3**), with **2a** showing less reactivity than **2b**. The azido groups of **3** were reduced to amino groups by a reaction with TPP and successive hydrolysis. The use of hydrazine in the hydrolysis process simultaneously led to the regeneration of the amino groups at C-2 positions. The final product, compound **4**, has adequate solubility even at neutral and physiological conditions. The cytotoxicity of the acetate salt of **4** was similar to that of chitosan. Moreover, compound **4** has a more effective transfection efficiency than chitosan. Further modification of **4** is in progress to enhance its utility as a gene carrier.

3. Experimental

3.1. General methods

Completely deacetylated chitosan with a viscosity-average molecular weight of 110 kDa was prepared by N-deacetylation of chitosan from Flonac C (Kyowa Tecnos, Chiba, Japan). *N*-Phthahloyl-chitosan was prepared from fully deacetylated chitosan according to the literature.[37] Dulbecco's modified Eagle medium (DMEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Gibco BRL. Other reagents and solvents were purchased from Aldrich or Wako (Osaka, Japan) and were used as received except where specifically stated.

^{1}H and ^{13}C NMR spectra were recorded at 300.13 and 75.48 MHz, respectively, on a Bruker ASX-300 spectrometer (Bruker Japan, Ibaraki, Japan) at 50 °C using the solvent peaks in DMSO-d_6 (39.52 ppm), TMS in $CDCl_3$, or sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate (TSP) in D_2O as internal standards. IR spectra were recorded with a HORIBA FT-210 spectrometer (Horiba, Kyoto, Japan). Elemental analysis was performed at the Center for Instrumental Analysis, Hokkaido University and the Faculty of Science, Osaka City University.

The pGL3-control plasmid DNA (pGL3) was purchased from Promega (Madison, WI). Following amplification in *Escherichia coli*, pGL3 was purified using a QIAGEN Plasmid Mega Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions and stored in 7 mM Tris buffer containing 1 mM EDTA (TE buffer). The purity of pGL3 was confirmed by 1% agarose gel electrophoresis and the concentration was determined by UV absorption at 260 nm. African Green

Monkey Kidney fibroblast (COS-1) cells were obtained from the American Type Culture Collection (Rockville, MD). The COS-1 cells were cultured in DMEM containing 10% FBS, 100 units/mL penicillin, 100 mg/L streptomycin in an incubator supplied with 5% CO_2 at 37 °C.

3.2. Preparation of 6-deoxy-6-halo-*N*-phthaloyl-chitosans (2a–c)

To explore suitable reaction conditions, preliminary experiments were carried out as follows: To a mixture of **1** (10 mg/mL) and NMP was added the *N*-halosuccinimide (NCS, NBS, or NIS) and TPP (5 or 10 equiv per sugar unit) in an ice/water bath, and then the mixture stirred at a prescribed temperature (40, 80, or 120 °C) for 2 h under nitrogen. The reaction mixture was poured into EtOH, and the resulting precipitate was collected by centrifugation and filtration, before being washed with EtOH, acetone–water, and then acetone. After drying under a reduced pressure at 40 °C, the 6-deoxyhalo derivatives of **1** were obtained as light- or dark brown powders in 44–79 wt % yields. These deoxyhalo products were then treated with acetic anhydride in NMP/pyridine in the presence of trace amounts of 4-dimethylaminopyridine. After stirring at rt for over 8 h, the mixture was poured into Et_2O. The resulting precipitates were collected by filtration on Celite, and then washed with Et_2O and CH_3OH. The precipitates were dissolved in $CHCl_3$ and re-precipitated with EtOH, and then collected on Celite again. The products were re-dissolved in $CHCl_3$ after washing with CH_3OH, and the resulting solutions were concentrated and dried under reduced pressure. The O-acetylated derivatives of **2a**–**c** were obtained as light- or dark brown films (yields were not determined). The d.s. of the products were calculated by the peak area ratio between the aromatic and acetyl protons on ^{1}H NMR spectra measured in DMSO-d_6 or $CDCl_3$.

The typical procedure to prepare the 6-deoxy-6-halo-*N*-phthaloyl-chitosans was as follows: NCS and TPP (3.4 mmol each) were added to a mixture of **1** (100 mg, 0.34 mmol of sugar unit) and NMP (10 mL) in an ice/water bath, and then the mixture stirred at 80 °C for 2 h under nitrogen. The dark brown reaction mixture was poured into EtOH (~100 mL), and the resulting precipitate was collected by centrifugation and filtration, and then washed with EtOH, acetone–water, and then acetone. After drying under reduced pressure at 40 °C, the chlorodeoxy derivative **2a** was obtained as a light brown powder (84 mg). The bromodeoxy (**2b**) and iododeoxy derivatives (**2c**) were prepared by the same method.

3.2.1. 6-Chloro-6-deoxy-*N*-phthaloyl-chitosan (2a). D.s. 1.00; light brown powder; 79% yield; ^{13}C NMR (DMSO-d_6, 75.48 MHz): δ 43.9 (C-6), 57.2, 67.9, 72.1, 77.8, 96.3 (pyranose), 122.9, 131.1, 134.3 (aromatic), 167.6 (*C*=O). Anal. Calcd for $C_{14}H_{12}ClNO_5{\cdot}0.6H_2O$: C, 52.46; H, 4.15; N, 4.37; Cl, 11.06. Found: C, 52.10; H, 4.36; N, 4.61; Cl, 11.56.

3.2.2. 6-Bromo-6-deoxy-*N*-phthaloyl-chitosan (2b). D.s. 0.98; light brown powder.; 95% yield; ^{13}C NMR (DMSO-d_6, 75.48 MHz): δ 33.5 (C-6), 57.2, 67.5, 71.6, 79.0, 95.9 (pyranose), 123.0, 131.2, 134.3 (aromatic), 167.6 (*C*=O). Anal. Calcd for $(C_{14}H_{12}BrNO_5)_{0.98}(C_{14}H_{13}NO_6)_{0.02}{\cdot}0.7H_2O$: C, 46.01; H, 3.70; N, 3.83; Br, 21.42. Found: C, 45.80; H, 3.74; N, 4.22; Br, 21.34.

3.2.3. 6-Deoxy-6-iodo-*N*-phthaloyl-chitosan (2c). D.s. 0.90; light brown powder; 86% yield; ^{13}C NMR (DMSO-d_6, 75.48 MHz): δ 7.8 (C-6), 57.3, 66.7, 71.5, 80.9, and 95.2 (pyranose), 123.0, 131.2, 134.4 (aromatic), 167.5 (*C*=O). Anal. Calcd for $(C_{14}H_{12}INO_5)_{0.90}(C_{14}H_{13}NO_6)_{0.10}{\cdot}0.4H_2O$: C, 42.32; H, 3.27; N, 3.52; I, 28.74. Found: C, 42.56; H, 3.39; N, 3.64; I, 28.57.

3.3. Preparation of 6-azido-6-deoxy-*N*-phthaloyl-chitosan (3)

Sodium azide (926 mg, 14.2 mmol) was added to a solution of **2b** (d.s. of bromine 0.98, 500 mg, 1.42 mmol of sugar unit) in NMP (50 mL), and the mixture was stirred at 80 °C for 4 h under nitrogen. The mixture was filtered through cotton to remove the salts and the filtrate was poured into EtOH (500 mL). The resultant precipitate was collected by centrifugation (10^4 rpm, 7 min), and washed with EtOH–water, then acetone. After drying under a reduced pressure at 40 °C, 6-azido-6-deoxy-*N*-phthaloyl-chitosan (**3**) was obtained as a light brown powder (388 mg, 86% yield). 6-Azidation of **2a** (d.s. chloro 1.00) was carried out by the same method at 120 °C.

3.3.1. 6-Azido-6-deoxy-*N*-phthaloyl-chitosan (3). D.s. 0.95; IR (KBr): 2100 cm^{-1} (azido); ^{13}C NMR (DMSO-d_6, 75.48 MHz): δ 50.1, 57.2, 68.0, 72.5, 77.9, 96.5 (pyranose), 122.8, 131.1, 134.3 (aromatic), 167.5 (*C*=O). Anal. Calcd for $(C_{14}H_{12}N_4O_5)_{0.95}(C_{14}H_{12}BrNO_5)_{0.03}(C_{14}H_{13}NO_6)_{0.02}{\cdot}0.2H_2O$: 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.

3.4. Preparation of 6-amino-6-deoxychitosan (4)

As preliminary experiments, the cleavage of the *N*-phthaloyl groups was done as follows: A solution of **3** (d.s. azido 0.95, 100 mg, 0.32 mmol of sugar unit) in NMP (10 mL) was mixed with 4 M aqueous hydrazine monohydrate (10 mL), and the reaction mixture stirred at 100 °C under nitrogen. A 4 mL of aliquot was taken from the mixture at 1, 2, 4, 8, and 12 h. Each aliquot was poured into EtOH, and the resulting precipitate

was collected and washed with EtOH by centrifugation (3500 rpm, 5 min, five times). The precipitate was dispersed in a small amount of water and lyophilized. The products were obtained as ivory amorphous materials (2–12 mg yield for each aliquot).

A typical procedure to prepare 6-azido-6-deoxy-*N*-phthaloyl-chitosan was as follows: TPP (496 mg, 1.89 mmol) was added to a solution of **3** (200 mg, 0.63 mmol of sugar unit, d.s. azido 0.95) in NMP (20 mL), and the reaction solution was stirred at rt for 12 h under nitrogen. The reaction mixture was then treated with 4 M aqueous hydrazine monohydrate (20 mL) and stirred at 100 °C for 4 h. Following evaporation of the water, the suspended reaction mixture was poured into EtOH (180 mL). The resultant precipitate formed was collected by centrifugation (10^4 rpm, 7 min, three times) and washed with EtOH. The precipitate was dissolved in neutral water and purified by ultrafiltration using an ultrafilter with a cut-off of 10 kDa. The product was obtained by lyophilization as an ivory-colored amorphous material (76 mg, 75% yield). ^{13}C NMR (D_2O with CD_3CO_2, 75.48 MHz): δ 42.6, 58.3, 72.9, 73.7, 80.3, 100.6 (pyranose).

3.5. Molecular-weight fractionation of compound 4 and chitosan

Fractionation of **4** in 5% acetic acid was carried out by ultrafiltration using ultrafilters with a cut-off of 50, 30, and 10 kDa. The average molecular weight of the acetate salt of **4** was estimated to be 36 kDa by static light scattering using a Zetasizer Nano ZS (Malvern Instruments, Southborough, UK). Fully deacetylated chitosan acetate salt was prepared by dissolution of chitosan in 5% acetic acid and successive washing with ultra-pure water using ultrafiltration. Fractionation of the chitosan acetate salt was carried out in the same manner for the acetate of **4**.

3.6. Evaluation of cytotoxicity for COS-1 cells

The cytotoxicity of acetate salt of **4** and chitosan for COS-1 cells was evaluated using Cell Counting Kit-8® (Dojindo, Kumamoto, Japan) containing sodium 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (WST). The COS-1 cells were seeded at a density of 10^4 cells/well in 100 μL of the growth medium in 96-well microtitre plates. After a 20 h incubation, the medium was replaced with 50 μL/well of fresh medium containing the cationic polyelectrolytes. The cells were exposed to the polyelectrolytes at a range of concentrations upto 150 μg/mL for 3 h. After removal of the polyelectrolytes, the cells were further incubated in 100 μL/well of fresh medium for 22 h prior to the addition of Cell Counting Kit-8. The cells were treated with 10 μL/well of Cell Counting Kit-8 solution for 2 h in 110 μL/well of fresh medium. The absorbance of the medium was measured at 620 and 450 nm using a Multiskan Ascent BIF-S microplate reader (Thermo Labsystems, Helsinki, Finland). The percent of cell viability in the absence of a polyelectrolyte was normalized as 100%.

3.7. Agarose gel retardation assay

The acetate salt of **4** and chitosan dissolved in phosphate buffer were separately mixed with pGL3 in TE buffer. The mixing ratios are represented as the amino groups of **4** or chitosan relative to the phosphate groups of pGL3 (N/P ratio). The amount of pGL3 was 0.5 μg per sample solution (10 μL) and the N/P ratios of the mixtures were adjusted to 0, 1, 1.5, 2, 2.5, 3, and 5. The mixtures were left to stand for 20 min at rt. DNA retardation was confirmed by agarose gel electrophoresis (1% w/v agarose) in Tris–acetate–EDTA buffer run at 100 V. The 1 kb DNA ladder (New England Biolabs, Beverly, MA, USA) was used as a molecular weight standard. The retardation pattern was visualized by UV transillumination after ethidium bromide staining and photographed using an ATTO Printgraph (ATTO, Tokyo, Japan).

3.8. In vitro transfection for COS-1 cells

To prepare **4**/pGL3 complexes, a solution of the acetate salt of **4** in 10 mM HEPES buffer at pH 7.0 was mixed into the same volume and pH of pGL3 solution. The mixture was left to stand for 15 min at rt before use. Both the chitosan/pGL3 complexes were prepared in a similar manner. The N/P ratios of the complexes were adjusted for 1, 2.5, 5, 10, 15, and 20.

COS-1 cells were seeded at a density of 5×10^4 cells/well in 1 mL of the growth medium in 24-well plates and incubated for 24 h prior to transfection. At the time of transfection, the culture medium was replaced with 200 μL/well of the fresh medium. This medium had been 1.25-fold concentrated compared with a normal culture medium and pre-adjusted to pH 7.0 by addition of 1 M HCl. Either 50 μL of cationic polyelectrolyte/pGL3 complex or naked pGL3 solutions were added into each well. The amount of pGL3 was 1 μg/well. After 3 h of a contact time, the medium containing the polyplexes was removed, and 1 mL/well of fresh normal medium was supplied. The cells were further incubated for 48 h. The medium was removed prior to the measurement of luciferase activity and quantification of total protein. The luciferase activity in cells was measured by Steady-Glo® Luciferase Assay System (Promega) according to the manufacturer's instructions using a Fluoroskan Ascent FL microplate luminometer (Thermo Labsystems). Separately, the total protein content in cells was quantified by a Protein Quantification Kit (Dojindo, Japan)

according to the manufacturer's instructions using a Multiskan Ascent BIF-S microplate reader. The measurements of the luciferase activity and protein quantification were both carried out in triplicate.

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