Synthesis and Some Properties of Nonnatural Amino Polysaccharides: Branched Chitin and Chitosan

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ABSTRACT: Regioselective introduction of *N*-acetyl-D-glucosamine and D-glucosamine branches at C-6 of chitin and chitosan has been accomplished. *N*-Phthaloylchitosan was used as an organosoluble key intermediate for a series of controlled modification reactions, and the derived 3-*O*-acetyl-2-*N*-phthaloylchitosan and 3-*O*-acetyl-2-*N*-phthaloyl-6-*O*-trimethylsilylchitosan proved to be suitable acceptors for glycosylation with an oxazoline from glucosamine. The resulting branched product had a substitution degree up to 0.63. It was deprotected to give branched chitosan, which was subsequently N-acetylated to afford branched chitin. The introduction of such branches markedly improved the affinity for solvents, and the product with protecting groups was soluble in common organic solvents in sharp contrast to the insoluble chitin and chitosan. Furthermore, both the branched chitin and chitosan were readily soluble in neutral water and showed high moisture absorption and retention. The branched chitin was degraded with lysozyme, and the susceptibility decreased with increasing extent of branching. The branched chitosan was characterized by marked antimicrobial activity and aggregation ability for bovine serum albumin.

Introduction

Some branched polysaccharides exhibit unique biological activities. Lentinan,[1] schizophyllan,[2] and pestalotan,[3] for example, have β-(1→3)-D-glucan backbone and β-(1→6)-glucoside branches and are attracting increasingly much attention owing to their substantial immunostimulating activity. These polysaccharides are, however, available only scarcely in nature. Introduction of sugar branches into linear polysaccharides may be a practical way to supply branched polysaccharides in quantity. Glycosylation of amylose, cellulose, and curdlan actually gave branched products,[4,5] but it is generally difficult to introduce sugar branches at a specific position because of the multifunctionality, limited solubility, and poor reactivity of the polysaccharides. Synthetic polysaccharides were also used for branching.[6−8]

Chitin and the deacetylated form, chitosan, are particularly attractive linear amino polysaccharides not only as abundant biomass resources but also as specialty biopolymers for preparing advanced functional materials.[9,10] It is, however, not soluble in common solvents, and this has caused serious difficulties in modification reactions to prepare well-defined derivatives. Therefore, it is generally hard to discuss the structure−property relationships. To fully develop the high potentials of chitin, it is necessary to introduce substituents at a specific position in a controlled manner. Under these circumstances, we have been interested in the regioselective and quantitative modification reactions to enable sophisticated molecular design and found some precursors suitable for modifications.[11−14] *N*-Phthaloylchitosan is a particularly convenient organosoluble precursor for efficient modifications.[12]

One of our targets is the regioselective introduction of sugar branches into linear polysaccharides to synthesize tailored branched polysaccharides, and some sugar branches such as mannose and galactose were introduced into chitin through multistep reactions.[15,16] In view of the distinctive biological and physicochemical properties of chitin and chitosan, it is important to establish a synthetic procedure for chitin and chitosan derivatives having *N*-acetyl-D-glucosamine and D-glucosamine branches, respectively, and we reported the possibility of branching in a preliminary communication.[17] Since the resulting branched chitin and chitosan have additional amino sugars in branches, they are expected to show much improved properties compared to those of the linear counterparts. Here we report the results of detailed studies on the regioselective introduction of the amino sugar branches into chitin and chitosan, and some properties of the resulting nonnatural branched amino polysaccharides are discussed.

Experimental Section

General. IR and UV−vis spectra were recorded on JASCO IRA-700 and JASCO Ubest-30 instruments, respectively. ^{1}H and ^{13}C NMR spectra were taken with a JEOL JNM-LA400D. Elemental analysis was performed with a Perkin-Elmer 2400. Gel permeation chromatography (GPC) was carried out with a JASCO 880-PU connected to a Shodex RI detector SE-61 (columns, TSK guard column + TSKgel Type GMPWXL no. PWMXE 0009 + TSKgel Type GMPWXL no. 0053; solvent, 0.1% aqueous lactic acid; flow rate 1.0 mL/min; standards, pullulan). The solution viscosity was measured with an Ubbelohde viscometer, and the molecular weight (M_v) was estimated with Mark−Houwink's relation for chitosan.[18] Solvents were purified in usual manners and stored over molecular sieves.

Chitin and Chitosan. Chitin was isolated from shrimp shells; the degree of deacetylation was 0.10 as determined by conductometric titration with a TOA conductivity meter CM-40S. It was pulverized and treated with 40% aqueous sodium hydroxide at 110 °C for 4 h three times to give chitosan with a degree of deacetylation of 1.0 as an almost colorless powdery material.[19]

Chitosan Acceptors for Glycosylation. 3-*O*-Acetyl-2-*N*-phthaloylchitosan and 3-*O*-acetyl-2-*N*-phthaloyl-6-*O*-trimeth-

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ylsilylchitosan were prepared starting from 2-*N*-phthaloylchitosan as reported.[16] Hydroxy groups at C-6 were often acylated to some extent in the phthaloylation,[16,20] but the O-phthaloyl groups could be substituted completely in the subsequent step as confirmed by ^{1}H NMR, IR, and elemental analysis.[21]

Oxazoline from Glucosamine. Chitin was hydrolyzed with concentrated hydrochloric acid to give D-glucosamine hydrochloride,[22] which was transformed into the corresponding oxazoline derivative according to a reported method.[23] It was purified by column chromatography with silica gel to give a light tan oil.

Glycosylation Reaction. To a solution of 9.87 g (30.0 mmol) of the oxazoline prepared above in 30 mL of 1,2-dichloroethane were added 1.00 g (3.00 mmol pyranose units) of 3-*O*-acetyl-2-*N*-phthaloylchitosan and 0.10 g of (+)-10-camphorsulfonic acid. The heterogeneous reaction mixture was stirred at 80 °C in nitrogen. After 60 h, the resulting clear solution was poured into methanol to precipitate the product, which was filtered, washed thoroughly with methanol, and dried to give 1.20 g of the product as a light tan powdery material. The degree of substitution (ds) was 0.63 as determined from the peak area ratio of acetyl/phthaloyl in the ^{1}H NMR spectrum in DMSO-d_6. The yield was calculated to be 74% on the basis of the ds value. IR (KBr): ν 3400 (OH and NH), 1775 (imide C═O), 1745 (ester C═O), 1721 (imide C═O), 1150−1000 (pyranose), and 725 cm^{-1} (arom). ^{1}H NMR (DMSO-d_6): δ 1.63, 1.88, and 1.97 (m, CO−CH_3), 3.2−5.5 (m, pyranose H), and 7.83 ppm (broad s, phth H). ^{13}C NMR (CDCl_3): δ 20.51 (OCO*C*H$_3$), 22.91 (NHCO*C*H$_3$), 55.30 (C-2,2′), 60.50 and 62.22 (C-6,6′), 68.69, 71.45, 72.84, and 74.89 (C-3,3′, C-5,5′, and C-4,4′), 96.12 (C-1,1′), 123.48, 131.30, and 134.21 (phth arom C), 167.58 (O*C*OCH$_3$), 169.24 (NH*C*OCH$_3$), and 170.61 ppm (phth C═O).

Anal. Calcd for $(C_{30}H_{34}N_2O_{15})_{0.63}(C_{16}H_{15}NO_7)_{0.37}\cdot 0.7H_2O$: C, 53.87; H, 5.17; N, 4.13. Found: C, 53.78; H, 5.03; N, 4.06.

Deprotection of the Branched Product. The branched product obtained above (0.50 g) was added to 30 mL of hydrazine hydrate, and the solution was stirred at 80 °C for 16 h in nitrogen. The solution was dialyzed in deionized water until neutral (3 days) and concentrated under reduced pressure. The resulting viscous solution was freeze-dried to give 0.23 g (96%) of the fully deprotected product, branched chitosan, as an almost colorless fluffy material. IR (KBr): ν 3400 (OH and NH$_2$), 1644 (NH$_2$), and 1150−1000 cm^{-1} (pyranose). ^{1}H NMR (D$_2$O): δ 3.5−4.2 ppm (m, pyranose).

Anal. Calcd for $(C_{12}H_{22}N_2O_8)_{0.63}(C_6H_{11}NO_4)_{0.37}\cdot 0.9H_2O$: C, 42.12; H, 7.13; N, 8.19. Found: C, 42.32; H, 6.84; N, 7.68.

N-Acetylation of Branched Chitosan. The branched chitosan obtained above (0.21 g) was dispersed in 15 mL of methanol, and 0.23 mL of acetic anhydride was added. The mixture was stirred at room temperature for 24 h and filtered. The solid was washed in methanol overnight, filtered, and dried to give 0.22 g (84%) of the N-acetylated product as a pale tan powdery material. IR (KBr): ν 3420 (OH and NH), 1652 (amide I), 1554 (amide II), and 1150−1000 cm^{-1} (pyranose). ^{1}H NMR (D$_2$O): δ 2.17 ppm (s, NHCOCH_3) and 3.5−4.3 ppm (m, pyranose).

Anal. Calcd for $(C_{16}H_{26}N_2O_{10})_{0.63}(C_8H_{13}NO_5)_{0.37}\cdot 1.6H_2O$: C, 43.50; H, 6.83; N, 6.34. Found: C, 43.50; H, 6.81; N, 6.28.

Moisture Absorption and Desorption. Pulverized samples were dried with phosphorus pentoxide and placed in a desiccator containing saturated ammonium dihydrogen phosphate solution at 25 °C (93% relative humidity (RH)). The weight increases were followed for 8 days. The samples were then placed in another desiccator containing saturated calcium chloride solution at 25 °C (32% RH), and the weight decreases were measured.

Enzymatic Degradation. Branched chitins with various ds values and the original chitin (degree of deacetylation, 0.10) were treated with lysozyme from egg white in pH 4.50 acetate buffer at 37 °C. The amount of the resulting reducing ends was determined with ferricyanide as reported.[24]

Antimicrobial Activity. The activity of a branched chitosan was measured by virtually the same method described in previous papers.[17,25] Precultures (50 μL) of microbes were mixed with 10 mL of a 5 ppm solution of the branched chitosan dissolved in 0.45% L-lactic acid buffer solution (pH 5.8). The mixtures were allowed to stand at room temperature for 1 min with occasional stirring. After appropriate dilution, they were cultured aerobically at 25 °C for 48 h, except *S. mutans* which was cultured anaerobically at 37 °C for 24 h. The colony forming units were counted, and the suppression percentage was determined on the basis of the control.

Aggregation of Bovine Serum Albumin. Formation of aggregates between branched chitosans and bovine serum albumin (BSA) was determined in a manner similar to that reported.[18] A solution containing 0.1% of a branched chitosan in 0.5% aqueous lactic acid buffer solution (pH 5.90) was prepared and diluted with the lactic acid buffer solution to give solutions of various concentrations. BSA was also dissolved in the buffer solution to prepare 0.2% solution. To 1 mL of a branched chitosan solution was added 1 mL of the BSA solution, and the mixture was left standing at room temperature for 10 min. The absorbance of the mixture was measured at 660 nm, and the control value was subtracted to give a net absorbance change due to aggregation.

Results and Discussion

Synthesis of Chitosan Acceptors for Glycosylation. *N*-Acetyl groups of chitin (**1**) were first thoroughly removed to prepare structurally uniform chitosan (**2**) for minimizing structural ambiguity of the derived products. Chitosan **2** was transformed into *N*-phthaloylchitosan (**3**), which was converted into 3-*O*-acetyl-2-*N*-phthaloylchitosan (**6**). To further improve the solubility, the C-6 hydroxy groups were trimethylsilylated to give the fully substituted derivative (**7**) as reported (Scheme 1).[16] All the reactions proceeded smoothly in solution and were quantitative in terms of ds.

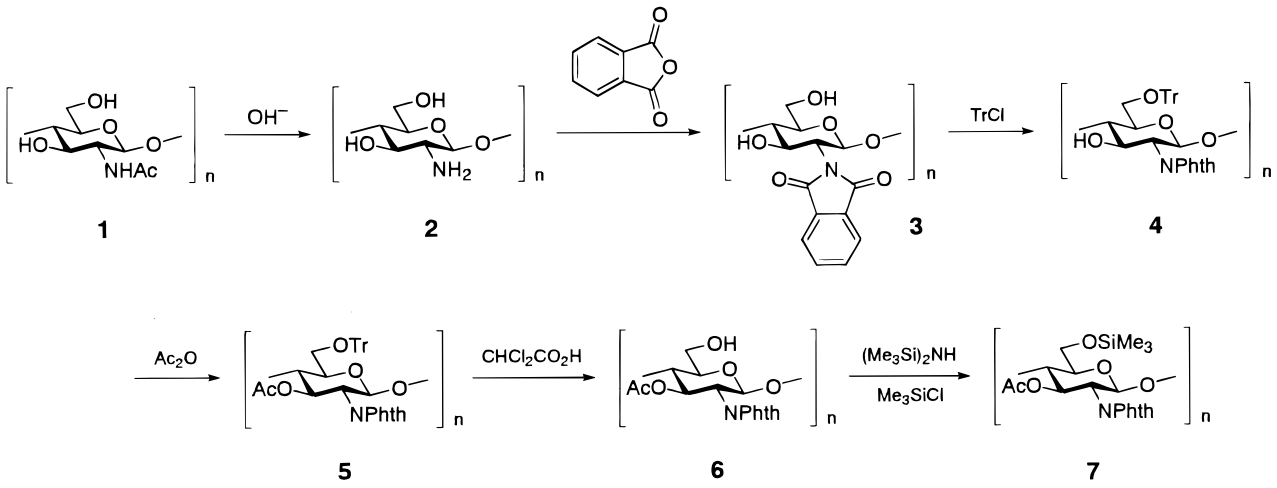
Glycosylation Reaction. Silylated derivative **7** would allow regioselective substitution at C-6 in solution and was thus subjected to the glycosylation with an oxazoline (**8**) derived from glucosamine. The reaction was carried out in 1,2-dichloroethane in the presence of 10-camphorsulfonic acid, which is a practical promoter to introduce *N*-acetyl-β-D-glucosaminide branches.[26]

The glycosylation reaction appeared very sluggish at 60 °C despite the homogeneous solution reaction, but it proceeded facilely at 80 °C to give the branched product (**9**) (Scheme 2). The ds, determined from the peak area ratio of acetyl/phthaloyl in ^{1}H NMR, increased with an increase in the amount of oxazoline and reached 0.6 with 5 equiv of oxazoline. The results are listed in Table 1.

The glycosylation was also examined with the C-6 hydroxy derivative **6**. The reaction was first attempted in *N*,*N*-dimethylformamide, since **6** was soluble in the solvent, but no substitution took place.[17] The solvent was then replaced with 1,2-dichloroethane. Though in this case the reaction mixture was heterogeneous in the initial stage since **6** was not soluble, it became a homogeneous solution as the glycosylation reaction proceeded (Scheme 2). As a consequence, similar results could be obtained from either of these precursors **6** and **7**, indicating that it is not necessary to trimethylsilylate **6** to use as an acceptor for this glycosylation.

As summarized in Table 1, the ds value of the resulting **9** was up to 0.63 under appropriate conditions. It increased with an increase in the amount of oxazoline. The effect of reaction time was also examined, and judging from the data obtained after 24, 48, and 72 h, 48 h appeared to be sufficient. The yields were in the range of 75−90%. The IR spectrum of **9** ensured the presence of peracetylated glucosamine branches as

Scheme 1



Scheme 2

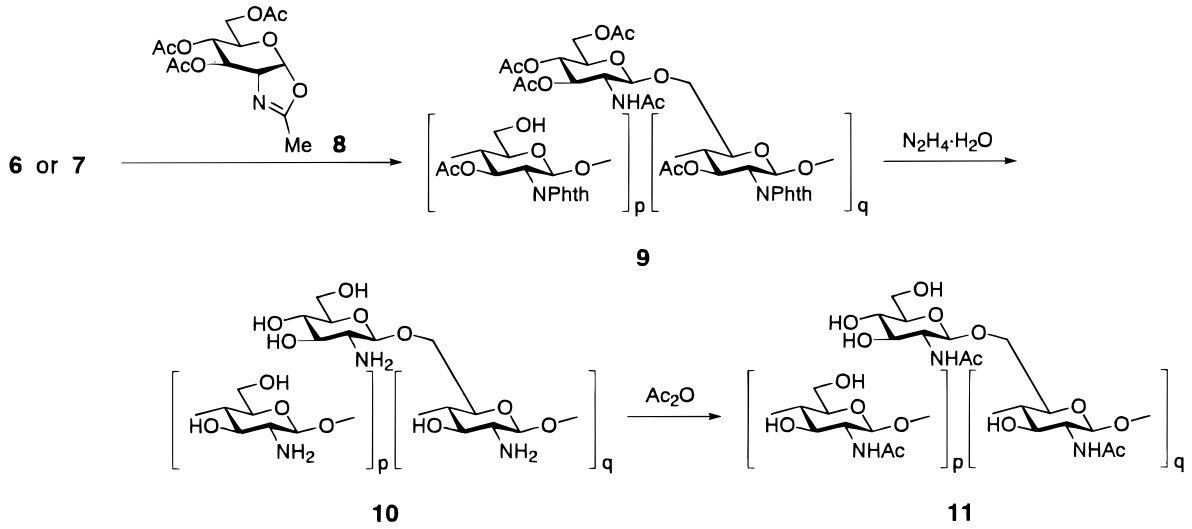


Table 1. Glycosylation of 6 and 7 with the Oxazoline to Form Branched Product 9

oxazoline/ pyranose[a]	temp (°C)	time (h)	ds of **9**[b] from **7**	ds of **9**[b] from **6**
3	60	24	0.06	0.03
1	80	24	0.21	0.13 (**9a**)[c]
2	80	24		0.33 (**9b**)[c]
3	80	24	0.31	0.37
3	80	48	0.40	0.45 (**9c**)[c]
3	80	72		0.42
5	80	24	0.61	0.56
5	80	48		0.60
10	80	24		0.60
10	80	60		0.63 (**9d**)[c]

[a] Mole ratio. [b] Determined from the peak ratio of Ac/Phth in ^{1}H NMR in DMSO-d_6. [c] Products used in the subsequent transformations.

evident from the strong absorption band at 1745 cm^{-1} due to the *O*-acetyl groups. The ^{1}H and ^{13}C NMR spectra in Figure 1 and elemental analyses[27] also support the structure of **9**.

Synthesis of Branched Chitosans and Chitins. The protecting acetyl and phthaloyl groups of **9** were then removed with aqueous sodium hydroxide and hydrazine hydrate, respectively, to give chitosan having glucosamine branches (**10**). The process, however, often caused partial degradation of the main chain as implied by low yield,[17] and alternative one-step deprotection with hydrazine hydrate at 80 °C was found suitable to give the corresponding **10** in high yield (80−95%). The regenerated free amino groups of **10** were acetylated with acetic anhydride in methanol to give branched chitin (**11**) in 85−90% yield (Scheme 2).

In the IR spectrum of **10**, the bands due to acetyl and phthaloyl groups of **9** disappeared completely, and the spectrum was quite similar to that of chitosan. On N-acetylation, amide I and II bands appeared, and the spectrum of **11** was almost identical with that of the original chitin.

The molecular weights of **10** and **11** were estimated by GPC calibrated with pullulan standards. As summarized in Table 2, the weight-average molecular weights (M_w), number-average molecular weights (M_n), and polydispersity values (M_w/M_n) were around 100 × 10^3, 4 × 10^3, and 3. Compared to the M_w and M_n of the original chitosan, these values imply the possibility of reduction in the molecular weights during the synthetic procedures, but the extent of degradation appeared not significant. The molecular weight (M_v) of branched chitosan **10c** determined by viscometry with a Mark−Houwink relation for chitosan was 100 000 and between M_w and M_n as observed for linear chitosans of various molecular weights.[18]

Solubility. The results of solubility test for **1**, **2**, **9** **10**, and **11** are summarized in Table 3. The branched

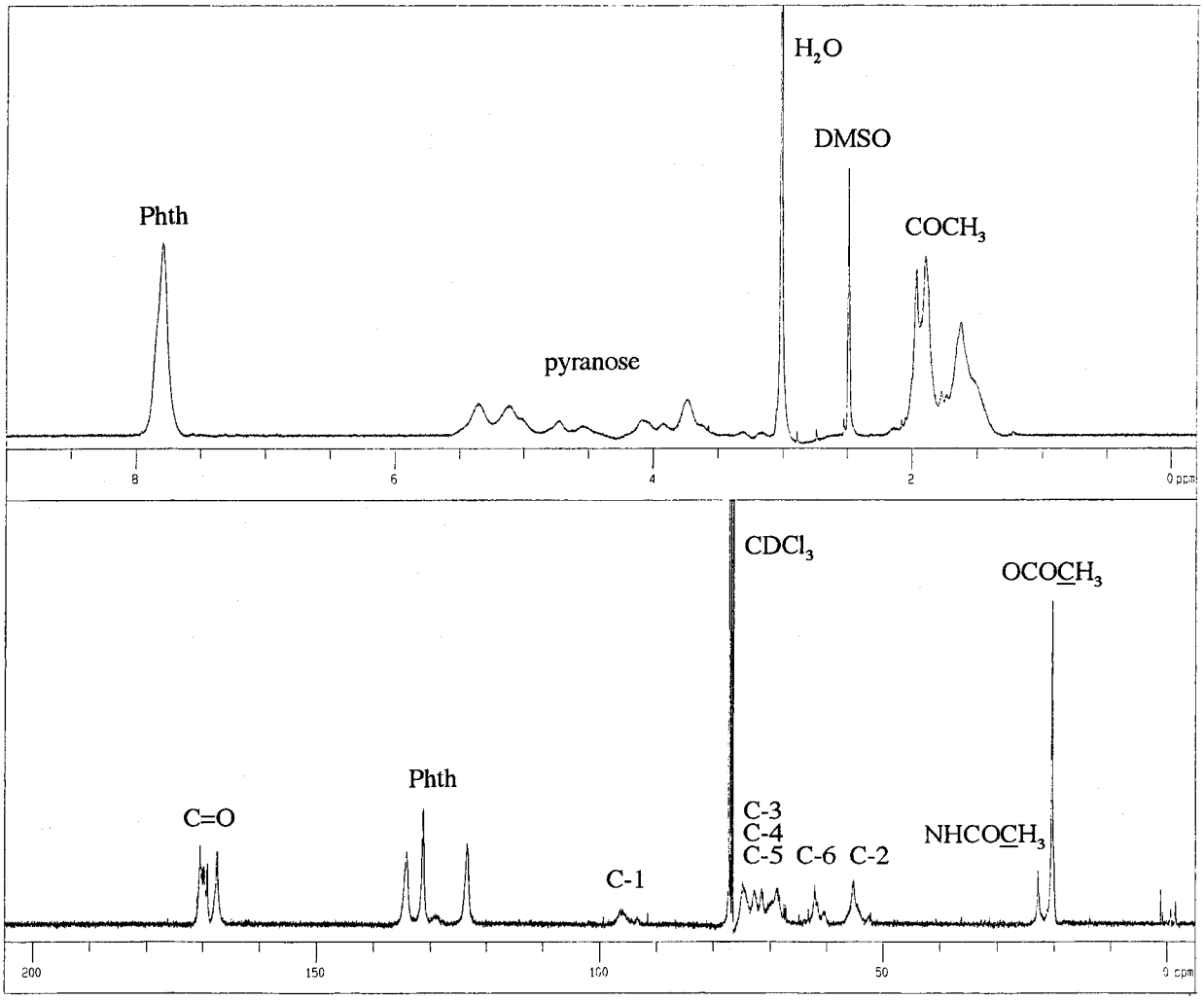


Figure 1. ^{1}H NMR (DMSO-d_6) and ^{13}C NMR ($CDCl_3$) spectra of **9c**.

Table 2. Molecular Weights of Chitosan 2, Branched Chitosans 10, and Branched Chitins 11[a]

sample[b]	$M_w \times 10^{-3}$	$M_n \times 10^{-3}$	M_w/M_n
2	411	93.3	4.41
10a	96	31.0	3.12
10b	114	38.9	2.94
10c[c]	128	46.0	2.77
10d	114	40.0	2.88
11a	121	30.1	4.02
11b	172	46.7	3.68
11c	115	33.3	3.44
11d	124	37.0	3.36

[a] Determined by GPC with pullulan standards. [b] Letters **a**, **b**, **c**, and **d** refer to the products derived from **9a**, **9b**, **9c**, and **9d**, respectively. [c] $M_v = 100\,000$.

derivatives prepared here were characterized by remarkable affinity for solvents. In sharp contrast to the linear chitin and chitosan, **9** was soluble even in low boiling organic solvents. Both **10** and **11** were readily soluble in neutral water when the ds was above 0.3 and swelled highly in common organic solvents. These data indicate that the introduction of sugar branches improves the solubility quite effectively.

Hygroscopicity. The high affinity for solvents suggested that **10** and **11** would be highly hygroscopic, and they were evaluated in terms of moisture absorption and retention. The dried powder samples were placed in an environment of 93% RH at 25 °C for 8 days and then placed under relatively dry conditions.

Table 3. Solubility of Chitin 1, Chitosan 2, Glycosylated Product 9, Branched Chitosans 10, and Branched Chitins 11[a]

	1	**2**	**9**	**10a**	**10b**	**10d**	**11a**	**11b**	**11d**
DMF[b]	−	−	++	±	±	±	±	±	±
CH_2Cl_2	−	−	++	±	±	±	±	±	±
MeOH	−	−	−	±	±	±	±	±	±
H_2O	−	−	−	+	++	++	+	++	++
5% AcOH	−	++	−	++	++	++	++	++	++

[a] ++, soluble; +, partially soluble; ±, swelled; −, insoluble. [b] *N*,*N*-Dimethylformamide.

Weight increases of the original chitin and chitosan were up to 17 and 23% at 93% RH as shown in Figure 2. Branched products **10** and **11** were confirmed to be much more hygroscopic, and the values were about 40%. Moreover, they exhibited high retention of moisture even in a dry environment at 32% RH.

Enzymatic Degradation. The introduction of sugar branches would affect the biodegradation of the original linear chitin, and the influence of the branching was elucidated. Branched chitins **11** as well as the original chitin were thus subjected to enzymatic degradation with lysozyme. The degradation was followed by the amount of the resulting reducing ends determined by titration with ferricyanide,[24] and the amount of consumed ferricyanide (Δabsorbance) was plotted against time. As shown in Figure 3, chitin was degraded slowly, and this may be ascribable partly to the heterogeneous

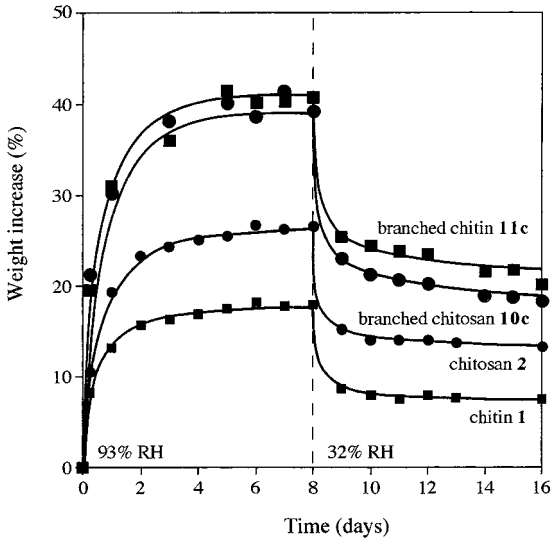


Figure 2. Moisture absorption and retention behavior of chitin **1**, chitosan **2**, branched chitosan **10c**, and branched chitin **11c**.

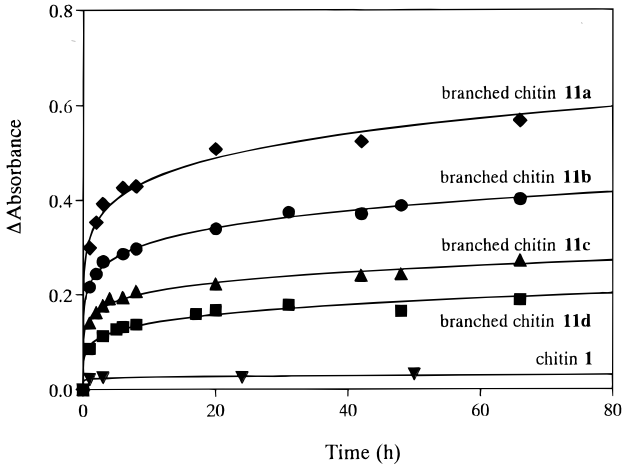


Figure 3. Lysozyme susceptibility of chitin **1** and branched chitins **11**.

reaction system in suspension. The decrease in absorbance of 0.03 after 50 h corresponds to the formation of 1.3 μmol of reducing end, as calibrated with *N*-acetylglucosamine, indicating about 1% of the glycosidic linkages were cleaved. In contrast, branched chitins **11** were degraded efficiently in aqueous solution. The susceptibility to lysozyme was dependent on the degree of branching, and the one with a low branching (**11a**) was degraded rapidly; the extent of hydrolysis of the glycosidic linkages in 66 h is as high as about 20%. The degradation rate decreased with an increase in the ds, and the decrease is probably due to the bulky nature of the branches. These results suggest that the β-(1→6) linkage in the side chain is resistive against the enzymatic hydrolysis compared to the β-(1→4) linkage of the main chain. It is noteworthy that all the branched chitins were degraded smoothly despite their nonnatural structures, and the biodegradability can be regulated finely by the extent of branching. This property is favorable for drug delivery systems and biomedical use.

Antimicrobial Activity. One of the most significant biological functions of chitosan is antimicrobial activity. Practical applications based on this activity are expected to be quite promising in various fields because of the considerable activity along with low toxicity[28] of this

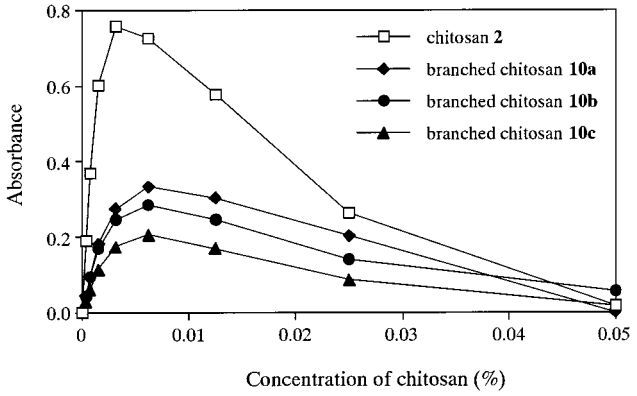


Figure 4. Aggregation of BSA with chitosan **2** or branched chitosans **10**.

Table 4. Antimicrobial Activity of Chitosan and Branched Chitosans[a]

	suppression of the growth (%)[b]		
	2	**10c**	man-chitosan[c]
Bacillus subtilis IAM1069	62 ± 5.0	96 ± 1.0	26 ± 5.4
Staphylococcus aureus IAM1011	81 ± 4.7	93 ± 1.5	17 ± 1.7
Escherichia coli IFO14249	21 ± 1.4	24 ± 3.9	26 ± 3.1[d]
Pseudomonas aeruginosa IAM1095	83 ± 5.5	84 ± 6.8	93 ± 7.9
Streptococcus mutans GS5	51 ± 5.1	56 ± 3.8	36 ± 7.9[d]
Candida albicans TIMM0613	72 ± 2.7	96 ± 1.5	0[d]

[a] **2**: M_v = 110 000. **10c**: M_v = 100 000. Concentration, 5 ppm. [b] Percentage of the colony forming units decreased by the treatment with chitosans. [c] Chitosan having mannose branches (data from ref 17). [d] Concentration of man-chitosan, 50 ppm.

amino polysaccharide. However, it is not soluble in neutral water, which may possibly limit the utilizations. In contrast, **10** is readily soluble in water, and the antimicrobial activity was measured to see the influence of branching.

Table 4 summarizes the results of antimicrobial activity with some typical microbes. The activities were evaluated in terms of the suppression percentage of the growth as a result of the treatment with a 5 ppm solution of **10**. As evident here **10** proved effective, and the activity was found higher against *B. subtilis*, *S. aureus*, and *C. albicans* than that of the original linear chitosan. Regarding *E. coli*, *P. aeruginosa*, and *S. mutans*, a similar level of activity was observed. Furthermore, **10** is much more potent than a chitosan having mannose branches,[16] indicating the importance of the free amino groups. Branched chitosan **10** therefore has a high possibility as a new type of water-soluble polymeric antimicrobial agent.

Aggregation of BSA. Chitosan is an effective coagulating agent for proteins, and some factors influencing the aggregation of BSA as a typical protein were discussed previously.[18] The effects of the presence of glucosamine branches and the extent of branching on the aggregation were examined by mixing BSA with **10**. As shown in Figure 4, **10** also aggregated BSA, but the efficiency was less than that of chitosan. Moreover, the efficiency decreased with increasing degree of branching, and this may be attributable again to the bulky nature of the branches.

Conclusions

N-Phthaloylchitosan is a useful key intermediate for controlled modifications of chitin, since it is soluble in common organic solvents and, moreover, easily deprotected to regenerate the free amino groups. Both the derived 3-*O*-acetyl-2-*N*-phthaloylchitosan and 3-*O*-acetyl-2-*N*-phthaloyl-6-*O*-trimethylsilylchitosan are convenient organosoluble precursors for site specific substitution at C-6, and a series of modifications have proved to proceed efficiently under mild conditions, giving rise to the synthesis of nonnatural branched amino polysaccharides. The important point is that the three kinds of functional groups of chitin and chitosan can be effectively discriminated by these procedures, and all the reactions proceed in well-controlled manners. The resulting branched chitins and chitosans were confirmed to exhibit various characteristics quite different from those of the original linear chitin and chitosan, including the affinity for solvents and hygroscopicity. The relationships between the molecular structures and some properties such as biodegradability, antimicrobial activity, and aggregation of BSA are also noteworthy. These characteristics of branched chitins and chitosans would be useful in various fields, for instance, as moisturizers for cosmetics, carriers for drug delivery systems, degradable materials for biomedical use, thickeners and preservatives for food processing, and antimicrobial agents for fiber and textile treatment.

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(27) **9a**, Calcd for $(C_{30}H_{34}N_2O_{15})_{0.13}(C_{16}H_{15}NO_7)_{0.87}\cdot 0.5H_2O$: C, 55.58; H, 4.83; N, 4.11. Found: C, 55.69; H, 4.81; N, 3.94. **9b**, Calcd for $(C_{30}H_{34}N_2O_{15})_{0.33}(C_{16}H_{15}NO_7)_{0.67}\cdot 0.6H_2O$: C, 54.70; H, 5.00; N, 4.11. Found: C, 54.69; H, 4.97; N, 4.01. **9c**, Calcd for $(C_{30}H_{34}N_2O_{15})_{0.45}(C_{16}H_{15}NO_7)_{0.55}\cdot 0.4H_2O$: C, 54.81; H, 5.02; N, 4.16. Found: C, 54.73; H, 5.04; N, 4.18. **9d**, in Experimental Section.
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