Polymer Bulletin 39, 543–549 (1997)

Polymer Bulletin

Regioselective introduction of β-galactoside branches into chitosan and chitin

Keisuke Kurita*, Hirofumi Akao, Masayuki Kobayashi, Tomonori Mori, Yasuhiro Nishiyama

Department of Industrial Chemistry, Faculty of Engineering, Seikei University, Musashino-shi, Tokyo 180, Japan

Received: 31 July 1997/Revised version: 2 September 1997/Accepted: 19 September 1997

Summary

β-D-Galactoside branches have been introduced into chitosan and chitin regioselectively through a series of controlled modification reactions based on *N*-phthaloyl-chitosan. The glycosylation reaction between a chitosan derivative having a reactive group only at C-6 and an orthoester of D-galactose proceeded efficiently to give a protected product with a degree of substitution up to about 0.5. Deprotection gave a branched chitosan, and the subsequent N-acetylation afforded a branched chitin. Unlike chitosan and chitin, the resulting nonnatural branched polysaccharides were characterized by high affinity for solvents and readily soluble in neutral water. Furthermore, branched chitin was easily degraded by lysozyme.

Introduction

Chitin and the deacetylated form, chitosan, are structurally similar to cellulose, but because of amino polysaccharides, they are characterized by various interesting properties such as affinity for metal cations and biological functions including immunoadjuvant and wound-healing activities (1). However, both the basic and utilization studies on chitin have been delayed primarily due to the intractable nature, and chitin has remained an unutilized biomass resource.

Owing to the insolubility, the modification reactions are generally sluggish under heterogeneous conditions, and moreover, the products are structurally ambiguous in most cases. In order to explore the full potential of chitin, efficient chemical modifications based on organosoluble precursors are necessary leading to the preparation of derivatives with well-defined structures. *N*-Phthaloyl-chitosan is such a precursor and has proved to be suitable for conducting some modification reactions quantitatively in a controlled manner (2).

Regioselective modifications based on this key precursor would make possible sophisticated molecular design such as introduction of sugar branches into linear polysaccharides. Preparation of branched polysaccharides is quite interesting in view of the biological functions as exemplified by the significant immunoadjuvant activity of lentinan (3,4), and actually, the reaction of an *N*-phthaloyl-chitosan derivative with an orthoester of D-mannose gave rise to the introduction of α-mannoside branches into chitin at C-6 (5). This has prompted us to examine the possibility of introducing β-sugar branches into chitin. Glycosylation reaction between a galactose orthoester and a chitin derivative was thus examined to prepare branched chitosan and chitin having β-galactoside groups, and

* Corresponding author

the characteristic properties including solubility, hygroscopic nature, and biodegradability were elucidated.

Experimental

General

IR spectra were taken with a JASCO IR-700. ^{1}H NMR spectra were recorded on a JEOL JNM-GX270 at 270 MHz. Elemental analysis was performed with a Perkin Elmer 2400. Degree of deacetylation was determined by conductometric titration with a conductivity meter TOA CM-40S.

Chitin and chitosan

Chitin was isolated from shrimp shells and purified by treating with 1 mol/L aqueous sodium hydroxide at 100 °C for 8 h followed by washing with deionized water. The degree of deacetylation was 0.09. Chitin was then deacetylated with 40% sodium hydroxide at 120 °C for 5 h under nitrogen, filtered, and washed with water until neutral to give chitosan with a degree of deacetylation of 0.90. To attain complete deacetylation, chitosan was pulverized, and the deacetylation procedure was repeated three more times. The resulting chitosan had a degree of deacetylation of 1.00.

3-*O*-Acetyl-6-*O*-trimethylsilyl-2-*N*-phthaloyl-chitosan was prepared through five-step reactions starting from the fully deacetylated chitosan as reported in a previous paper (5).

Orthoester of D-galactose

2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl bromide, prepared from peracetylated β-D-galactopyranose, was treated with methanol in the presence of 2,6-lutidine in chloroform to give 3,4,6-tri-*O*-acetyl-1,2-*O*-methylorthoacetyl-α-D-galactopyranose according to the reported procedure (6,7). It was purified by column chromatography, and the overall yield was 20% from galactose.

Branching reaction

To a solution of 0.50 g (1.2 mmol pyranose units) of 3-*O*-acetyl-6-*O*-trimethylsilyl-2-*N*-phthaloyl-chitosan in 20 mL of dichloromethane were added 1.33 g (3.6 mmol) of the galactose orthoester and 0.02 mL (0.023 g, 0.1 mmol) of trimethylsilyl trifluoromethanesulfonate (TMSOTf). The solution was stirred in nitrogen at room temperature for 24 h and poured into methanol to precipitate the product. It was filtered and washed with methanol thoroughly to give 0.38 g of a pale brown powdery material. The degree of substitution was 0.46 as determined from the peak ratio of acetyl/phthaloyl groups in ^{1}H NMR and also from the C/N ratio of the elemental analysis. IR (KBr): ν 1778 (phthaloyl C=O), 1745 (ester C=O), 1721 (phthaloyl C=O), and 1150-1000 cm^{-1} (pyranose). ^{1}H NMR (DMSO-d_6): δ 1.5-2.1 (m, acetyl-H), 3.2-5.8 (m, pyranose-H), and 7.85 ppm (s, phthaloyl-H).

Chitosan having β-D-galactoside branches

To 50 mL of 1 mol/L sodium hydroxide was added 0.45 g of the above obtained branched product portionwise at 40 °C. The solid went into solution in 20 min. The resulting clear solution was cooled to room temperature and dialyzed against deionized water until the solution became neutral. The solution was concentrated under reduced pressure and freeze-dried to give 0.10 g of an off-white powdery product.

Hydrazine monohydrate, 30 mL, was added to the product, and the solution was heated at 100 °C for 24 h. The solution was dialyzed against deionized water until neutral, concentrated under reduced pressure, and freeze-dried to give 62 mg of chitosan having β-D-galactoside branches as a white powdery material. IR (KBr): ν 3400 (OH), 1638 (NH_2), and 1150-1000 cm^{-1} (pyranose).

Chitin having β-D-galactoside branches

The branched chitosan prepared above was suspended in 20 mL of methanol, and 0.06 mL (0.64 mmol) of acetic anhydride was added. The mixture was stirred at room temperature for 24 h and filtered with a sintered-glass filter. The product was washed thoroughly with methanol and filtered. It was air-dried and then dried in vacuo to give 60 mg of a white powdery material. IR (KBr): ν 3430 (OH), 1650 (amide I), 1559 (amide II), and 1150-1000 cm^{-1} (pyranose).

Hygroscopic nature

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

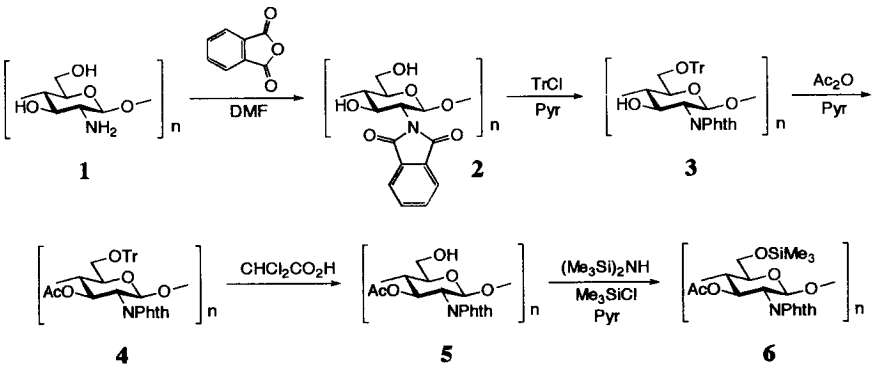
Biodegradation of branched chitin

Branched chitin was treated with lysozyme (from egg white) in 0.1 mol/L acetate buffer of pH 4.5 at 36 °C, and the amount of the resulting reducing ends was determined with ferricyanide by the method reported in a previous paper (8).

Results and discussion

Preparation of branched chitosan and chitin

Starting from *N*-phthaloyl-chitosan (**2**) as an organosoluble key intermediate, an acceptor (**5**) having free hydroxy groups only at C-6 was prepared according to the method reported in a previous paper (5) (Scheme 1). However, **5** was not soluble in low boiling solvents such as dichloromethane and dichloroethane suitable for glycosylation reactions and was therefore transformed into the corresponding trimethylsilyl derivative, 3-*O*-acetyl-6-*O*-trimethylsilyl-2-*N*-phthaloyl-chitosan (**6**) to further improve the solubility.

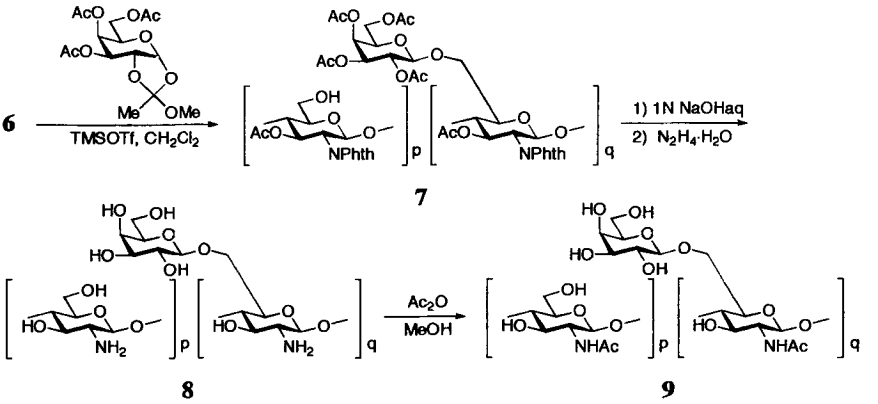


Scheme 1.

An orthoester, 3,4,6-tri-*O*-acetyl-1,2-*O*-methylorthoacetyl-α-D-galactopyranose, was derived from D-galactose, and the glycosylation reaction was carried out in dichloromethane at room temperature in the presence of TMSOTf as the catalyst to incorporate galactoside branches (Scheme 2). The reaction would result in the β-glycoside formation because of the use of the highly stereoselective catalyst (9) and as implied from the branching of other polysaccharides with an α-orthoester (10,11).

The reaction proceeded efficiently in homogeneous solution at room temperature to give fully protected β-galactoside-branched chitosan (**7**). The degree of substitution (ds)

could be determined by ^{1}H NMR spectroscopy from the acetyl/phthaloyl peak ratio and also by elemental analysis from the C/N ratio. As listed in Table 1, the ds was dependent on the amount of the orthoester and fairly reproducible. Furthermore, the ds values determined by the NMR method were confirmed to be almost the same as those by the elemental analysis method. The IR spectra showed a characteristic strong absorption band at 1745 cm^{-1} due to acetyl groups of the introduced peracetylated galactoside branches (Figure 1).



Scheme 2.

Table 1.
Glycosylation reaction between **6** and an orthoester of D-galactose

6 (g)	Galactose / Pyranose[a]	Yield (g)	ds NMR[b]	ds EA[c]	Elemental analysis (%) C	H	N
0.50	1	0.38	—	0.21	55.17	4.66	3.39
					(55.26	4.84	3.40)[d]
0.50	2	0.36	0.36	0.38	54.59	4.75	2.98
					(54.53	4.95	2.98)[e]
0.50	3	0.38	0.46	0.46	54.42	4.89	2.83
					(54.53	4.95	2.83)[f]
1.12	3	0.82	0.40	—	—	—	—
0.50	5	0.40	0.53	—	—	—	—
0.46	5	0.48	0.55	—	—	—	—

[a] Molar ratio.
[b] Determined from the peak ratio of Ac/Phth in ^{1}H NMR.
[c] Determined from the C/N ratio of elemental analysis.
[d] Calcd for $(C_{30}H_{33}NO_{16})_{0.21}(C_{16}H_{15}NO_7)_{0.79}\cdot 0.5H_2O$.
[e] Calcd for $(C_{30}H_{33}NO_{16})_{0.38}(C_{16}H_{15}NO_7)_{0.62}\cdot 0.6H_2O$.
[f] Calcd for $(C_{30}H_{33}NO_{16})_{0.46}(C_{16}H_{15}NO_7)_{0.54}\cdot 0.5H_2O$.

7 was then deacetylated with aqueous sodium hydroxide. Subsequent dephthaloylation could be effected with hydrazine hydrate to give chitosan having galactoside branches (**8**), but somewhat severe conditions were necessary compared to the dephthaloylation of a chitobiose derivative (12). The IR spectrum of **8** was quite similar to that of chitosan as shown in Figure 1. Selective N-acetylation of **8** was effected with acetic anhydride in methanol to give chitin having galactoside branches (**9**) (Scheme 2). The IR

spectrum of **9** was almost identical with that of the original chitin, but the bands due to pyranose rings became more evident as a result of the introduction of galactoside branches (Figure 1).

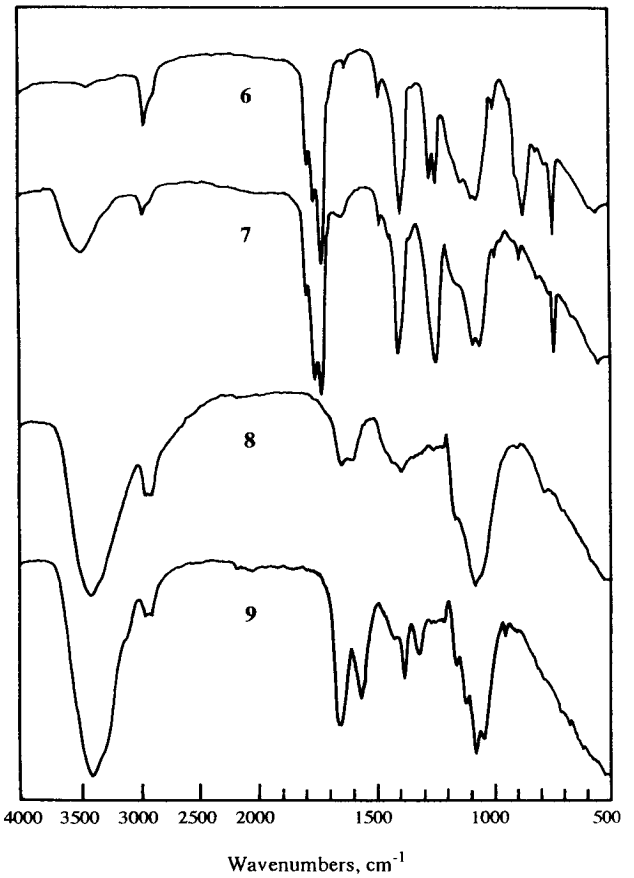


Figure 1. IR spectra of the chitin acceptor (**6**) and the branched derivatives (**7**, **8**, and **9**).

Characteristics of branched chitin and chitosan

Some characteristic properties were examined with **7** having ds 0.40 and the derived branched chitosan and chitin as compared with those of chitin. The branched products exhibited high affinity for solvents unlike insoluble chitin and chitosan. As summarized in Table 2, the protected product **7** was soluble in organic solvents. In sharp contrast, branched chitosan **8** and chitin **9** without protecting groups were readily soluble in neutral water and swelled highly even in common organic solvents, indicating that the introduction of sugar branches effectively increased the affinity for solvents.

The high affinity for water suggested that the branched chitin would be highly hygroscopic, and the sample was kept at 93% RH and then at 32% RH to see the moisture absorbency behavior. Figure 2 shows the weight change of the branched chitin **9** in comparison with that of the original linear chitin. The results indicated that **9** exhibited much higher absorption and retention of moisture than chitin. The much enhanced solubility and hygroscopicity are attributable to the disturbance of the tight arrangement of the macromolecular chains as a result of the introduction of hydrophilic bulky branches, as

suggested by the solubility of a partially deacetylated chitin (13) and tosyl- and iodo-chitins (14) in addition to various derivatives of *N*-phthaloyl-chitosan (2).

Table 2.
Solubility of chitin and the derived products[a]

	Solubility			
	H_2O	MeOH	$CHCl_3$	DMF
Chitin	–	–	–	–
Chitosan	–	–	–	–
7	–	–	+	+
8	+	±	±	±
9	+	±	±	±

[a] +, soluble; ±, partially soluble or swelled, –, insoluble.

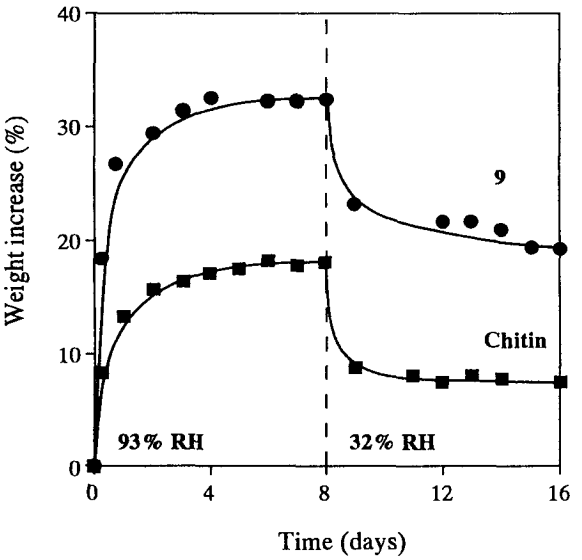


Figure 2. Hygroscopic nature of **9** and chitin.

The introduction of sugar branches into chitin would affect the biodegradability, and the susceptibility of **9** to lysozyme was examined. The formation of the reducing ends was followed by titration with ferricyanide, and the decrease in the absorbance of ferricyanide (ΔAbs) is illustrated in Figure 3. As evident in the figure, **9** turned out to be highly susceptible to lysozyme despite its nonnatural structure. Furthermore, the enzymatic degradation proceeded much more facilely than that of the original chitin. This is ascribable partly to the difference in the degradation conditions; **9** underwent degradation in solution whereas chitin was degraded under heterogeneous conditions.

Conclusion

A series of efficient and regioselective modification reactions based on *N*-phthaloyl-chitosan have made possible the preparation of β-galactoside-branched chitosan and chitin. The resulting nonnatural branched polysaccharides exhibited interesting characteristics significantly different from those of the linear chitosan and chitin, and the introduction of sugar branches into natural polysaccharides has proved to be a potentially useful tool for developing various new functions.

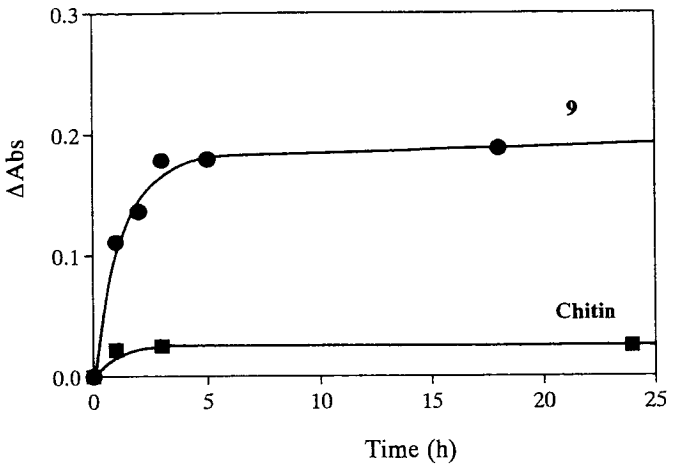


Figure 3. Susceptibility of **9** and chitin to lysozyme.

This work was supported in part by a Grant-in-Aid for Scientific Research (#08651061) from the Ministry of Education, Science, Sports, and Culture of Japan and by a grant from Towa Shokuhin Kenkyu Shinkoukai.

References

1. Muzzarelli RAA (1973) *Natural Chelating Polymers*, Pergamon, Oxford; (1977) *Chitin*, Pergamon, Oxford.
2. Nishimura S, Kohgo O, Kurita K, Kuzuhara H (1991) *Macromolecules* **24**: 4745.
3. Chihara G, Maeda Y, Hamuro J, Sasaki T, Fukuoka F (1969) *Nature* **222**: 687.
4. Chihara G, Maeda Y, Hamuro J (1982) *Int. J. Tissus. React.* **4**: 207.
5. Kurita K, Kobayashi M, Munakata T, Ishii S, Nishimura S (1994) *Chem. Lett.* 2063.
6. Kochetkov NK, Khorlin AJ, Bochkov AF (1967) *Tetrahedron* **23**: 693.
7. Garegg PJ, Lindberg B, Nilsson K, Swahn C-G (1973) *Acta Chem. Scand.* **27**: 1595.
8. Kurita K, Yoshino H, Nishimura S, Ishii S (1993) *Carbohydr. Polym.* **20**: 239.
9. Ogawa T, Beppu K, Nakabayashi S (1981) *Carbohydr. Res.* **93**: C6.
10. Matsuzaki K, Yamamoto I, Sato T, Oshima R (1985) *Makromol. Chem.* **186**: 449.
11. Hatanaka K, Hirobe T, Yoshida T, Yamanaka M, Uryu T (1990) *Polym. J.* **22**: 435.
12. Nishimura S, Kuzahara H (1990) *Carbohydr. Res.* **206**: 207.
13. Kurita K, Sannan T, Iwakura Y (1977) *Makromol. Chem.* **178**: 3197; Kurita K, Kamiya M, Nishimura S (1991) *Carbohydr. Polym.* **16**: 83.
14. Kurita K, Yoshino H, Yokota K, Ando M, Inoue S, Ishii S, Nishimura S (1992) *Macromolecules* **25**: 3786.