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Preparation and Characterization of Branched β-Cyclodextrins Having Manno-Oligosaccharide Side Chains Derived from Yeast Mannan and Study of Their Functions

TOSHIKO TANIMOTO*, YUKO KISHIMOTO, AKIKO IKUTA, YUKI NISHI and KEISHIRO MIYAKE

School of Pharmaceutical Sciences, Mukogawa Women's University, 11-68 Koshien Kyuban-Cho, Nishinomiya 663-8179, Japan (E-mail: tanimoto@mwu.mukogawa-u.ac.jp)

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Abstract

Branched β-cyclodextrins (β-CDs) having manno-oligosaccharide side chains were investigated. Three kinds of monobranched β-CDs and five kinds of dibranched β-CDs were chemically synthesized using the trichloroacetimidate method. Their structures were analyzed by HPLC, MS, and NMR spectroscopies. The specific interaction between those compounds and mannose-binding lectins (Concanavalin A and *Pisum sativum* agglutinin) was investigated by inhibition tests of hemagglutinating activity and by using an optical biosensor of the IAsys apparatus with a resonant mirror detector. The results showed that all branched β-CDs interacted with lectins. The binding affinity was $6^1,6^4$-$(Man_3)_2$- ≫ $6^1,6^4$-$(Man_2)_2$- > $6^1,6^4$-$(Man_4)_2$-β-CD when the derivatives were compared on the basis of side chain length and $6^1,6^3$- ≫ $6^1,6^4$- > $6^1,6^2$-$(Man_2)_2$-β-CD when compared on the basis of side chain position.

Introduction

Branched cyclodextrins (CDs) having mannose (Man) at the non-reducing ends of their side chains are expected to be useful as possible drug carriers for targeted drug delivery. In particular, dibranched CDs have been considered to be promising in view of their clustering effect. This study deals with the preparation and characterization of branched β-CDs having manno-oligosaccharides derived from yeast mannan in side chains and the examination of their functions.

Three kinds of monobranched β-CDs and five kinds of dibranched β-CDs, namely mannobiosyl (Man_2)-, mannotriosyl (Man_3)-, and mannotetraosyl (Man_4)-β-CDs (**7**, **11**, and **13**) and $6^1,6^2$-, $6^1,6^3$-, and $6^1,6^4$-$(Man_2)_2$-, $6^1,6^4$-$(Man_3)_2$-, and $6^1,6^4$-$(Man_4)_2$-β-CDs (**8**, **9**, **10**, **12**, and **14**), were chemically synthesized by the trichloroacetimidate method [1] using $6^1,6^n$-di-*O*-(*tert*-butyldimethylsilyl (*t*-$BuMe_2Si$))-β-CDs (n = 2–4) (**1–3**) [2] as the key glycosyl intermediates (Table 1). Their structures were elucidated by HPLC, MS, and NMR spectroscopies.

The specific interaction between those compounds and mannose-binding lectins, Concanavalin A (ConA) and *Pisum sativum* agglutinin (PSA), was investigated with conventional and new analytical methods, i.e. by inhibition tests of hemagglutinating activity [3] and by using an optical biosensor of the IAsys apparatus with a resonant mirror detector.

Experimental

Materials

$6^1,6^n$-Di-*O*-(*tert*-butyldimethylsilyl (*t*-$BuMe_2Si$))-β-CDs (n = 2–4) (**1–3**) were prepared as described in our previous paper [2]. Mannan was extracted from the cell wall of bakers' yeast, *Saccharomyces cerevisiae*. ConA and PSA were purchased from Seikagaku Kogyo Co., Ltd.

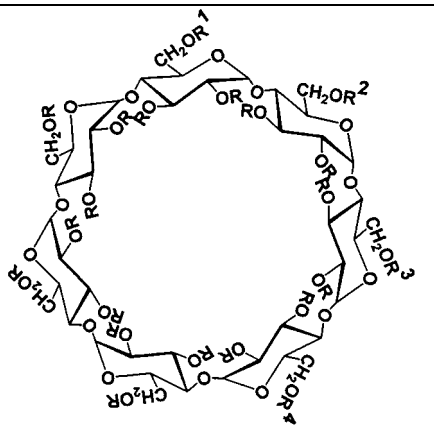
Analytical methods

TLC was performed on Silica Gel 60 plates (Merck). Centrifugal chromatography was performed with a Harrison Centrifugal Thin-Layer Chromatotron, model 7923. HPLC was conducted with a Jasco 980-PU pump and a Shodex RI-71 monitor. The columns used were Asahipak HIKARISIL C18-4D (150 × 4.6 mm i.d., Shodex), LiChroCART NH_2 (250 × 4 mm i.d., Kanto Chemical), Hypercarb (100 × 4.6 mm i.d. and 100 × 10 mm i.d., Thermo Hypersil), TSK-gel Amide-80 (300 × 7.8 mm i.d. and 300 × 21.5 mm i.d., Tosoh). TOF-MS was performed with a Vision 2000 (Thermo Bioanalysis) mass spectrometer. NMR spectra were recorded with a Jeol GSX-500 (125.65 MHz) spectrometer. Binding kinetics were determined using IAsys (Affinity Sensors).

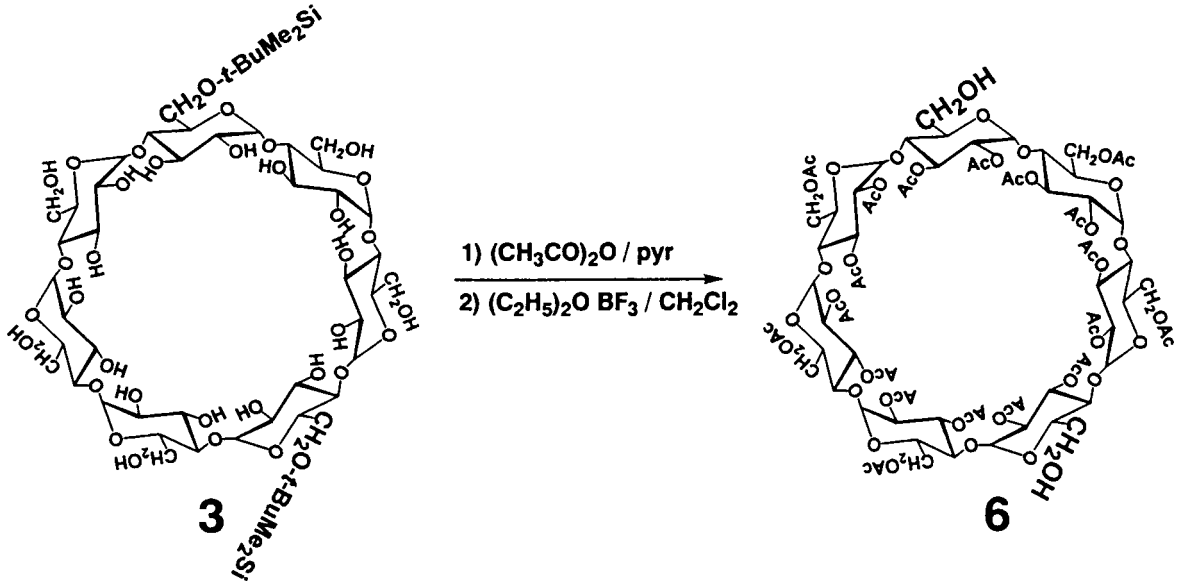
* Author for correspondence.

Table 1. Structures of compounds **1–14**

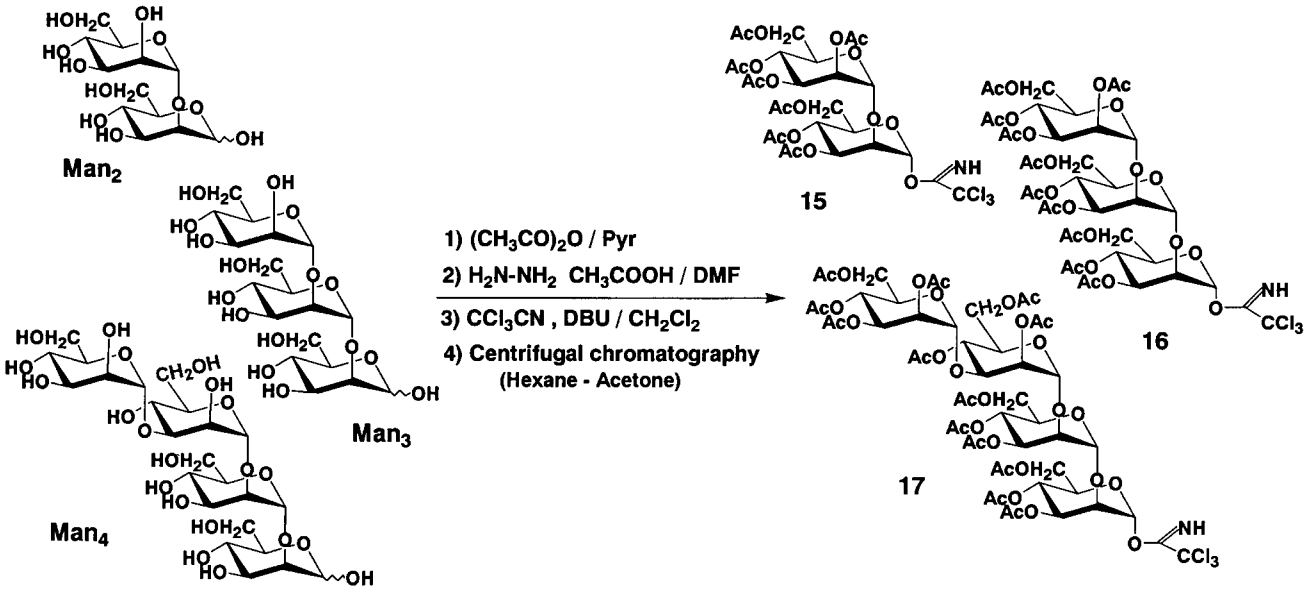
	R^1	R^2	R^3	R^4	R
1	X	X	H	H	H
2	X	H	X	H	H
3	X	H	H	X	H
4	H	H	Ac	Ac	Ac
5	H	Ac	H	Ac	Ac
6	H	Ac	Ac	H	Ac
7	Man_2	H	H	H	H
8	Man_2	Man_2	H	H	H
9	Man_2	H	Man_2	H	H
10	Man_2	H	H	Man_2	H
11	Man_3	H	H	H	H
12	Man_3	H	H	Man_3	H
13	Man_4	H	H	H	H
14	Man_4	H	H	Man_4	H



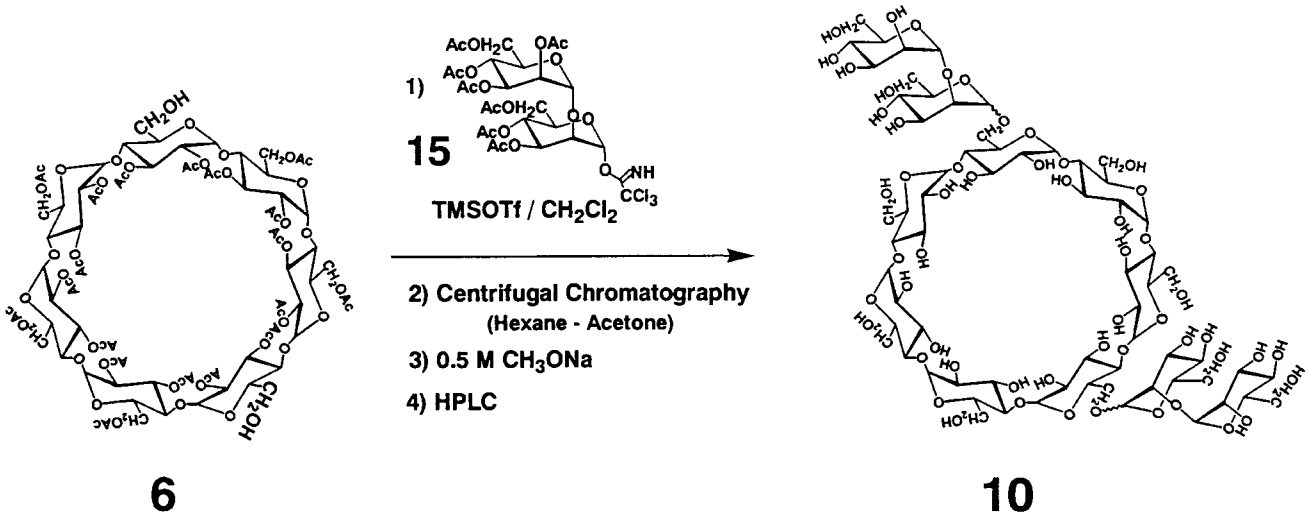
X = *t*-$BuMe_2Si$.



Scheme 1.



Scheme 2.



Scheme 3.

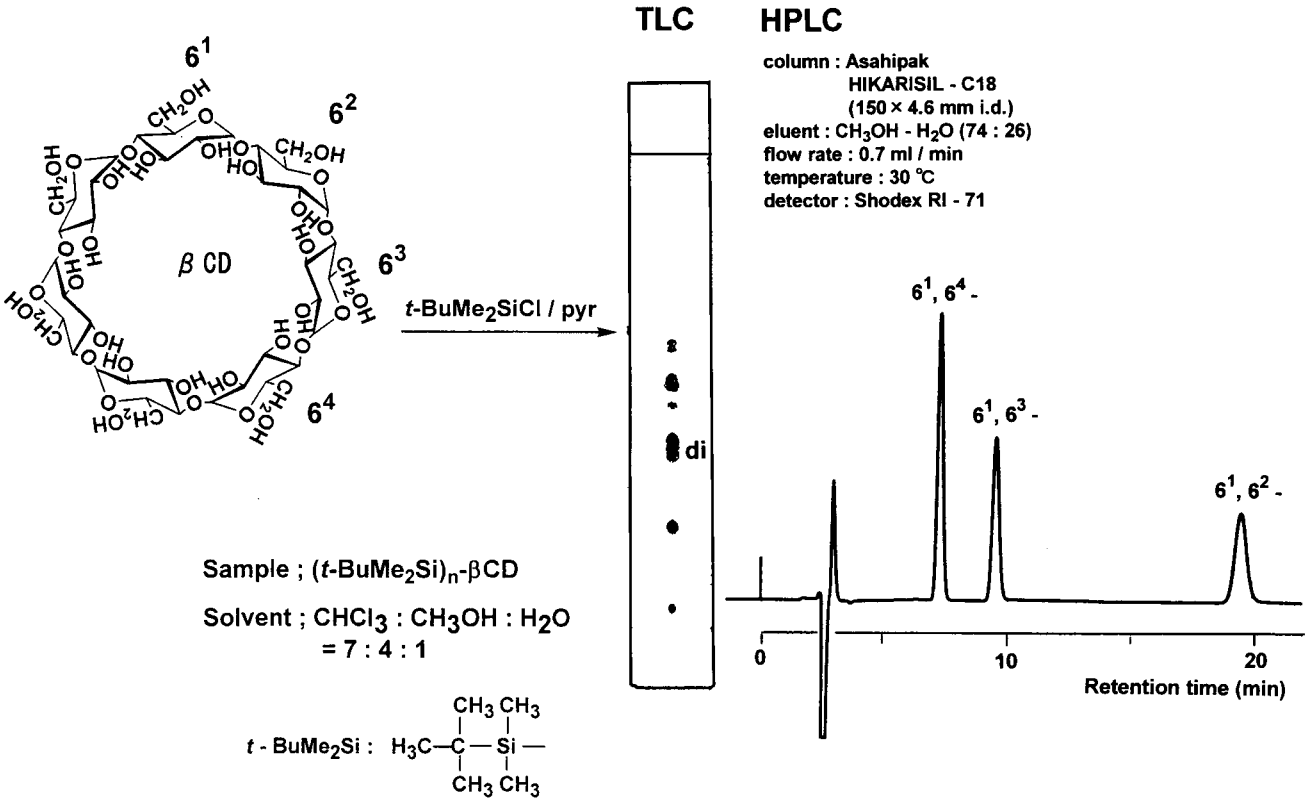


Figure 1. Preparation of 6^1,6^n-di-*O*-(*t*-BuMe_2Si)-β-CDs (n = 2–4) (**1–3**).

Preparation of β-CD glycosylation acceptors

Acetylation of **1**, **2**, or **3** with acetic anhydride-pyridine for 5 h at 100 °C, followed by *O*-desilylation with boron trifluoride etherate in dichloromethane afforded the glycosyl acceptor, bis(2,3-di-*O*-acetyl)heptaxis(2,3,6-tri-*O*-acetyl)-β-CDs (**4**, **5**, and **6**), after centrifugal chromatography (Scheme 1).

Preparation of mannosyl glycosylation donors

Mannan was obtained according to the method described by Peat *et al.* [4]. Mannan was acetolyzed in a mixture of acetic acid-acetic anhydride-sulfuric acid. The acetolysate obtained was deacetylated in the usual way. The main products, Man_2, Man_3s, and Man_4, were isolated and purified by HPLC on a TSK-gel Amide-80 column, and two Man_3s were isolated on a Hypercarb column. Their structures were analyzed by HPLC, MS, and NMR spectroscopies.

Acetylation of Man_2, Man_3 (Man_3-1), or Man_4 with acetic anhydride-pyridine, followed by 1-*O*-deacetylation with hydrazine acetate reacting with trichloroacetonitrile in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in dichloromethane, then afforded the glycosyl donors, **15**, **16**, and **17** after centrifugal chromatography (Scheme 2).

Glycosylation

Mannosylation of **4**, **5**, or **6** with **15** and **6** with **16** or **17** in dichloromethane in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) and molecular sieves for 1 h at –20 °C, followed by deacetylation, and isolation by HPLC on a TSK-gel Amide-80 column and an ODS column gave the desired main products **8**, **9**, **10**, **12**, and **14** and byproducts **7**, **11**, and **13** (Scheme 3). Their structures were analyzed by HPLC, MS, and NMR spectroscopies.

Affinity assay

ConA and PSA were covalently immobilized onto a carboxylate surface at a concentration of 0.5 mg/ml in 10 mM acetate buffer (pH 5.3) containing 1 mM $MnCl_2$ and 1 mM $CaCl_2$. All measurements were carried out in 10 mM phosphate buffered saline (PBS) (pH 6.3). Following analyte binding, the surface was regenerated with 20 mM HCl. The kinetics of the interaction between branched β-CDs and ConA or PSA were analyzed using the FASTfit software, and the association rate constant (Ka) and the dissociation rate constant (Kd) were calculated.

Hemagglutination assay

Human blood was collected in 3.8% sodium citrate. The erythrocytes were washed three times with PBS and then suspended at 3% concentration in PBS. A 10% suspension of erythrocytes in PBS (10 ml) was treated with pronase for 30 min at 45 °C, then washed three times with PBS and suspended at 3% concentration in PBS. Agglutination of 3% erythrocytes and inhibition of the agglutination by sugars were performed in microtiter U-plates. The titer was defined as the reciprocal of the end-point dilution exhibiting the hemagglutination. Inhibition was expressed as the minimum concentration of each sugar required for inhibition of hemagglutination of titer 4 of the lectin.

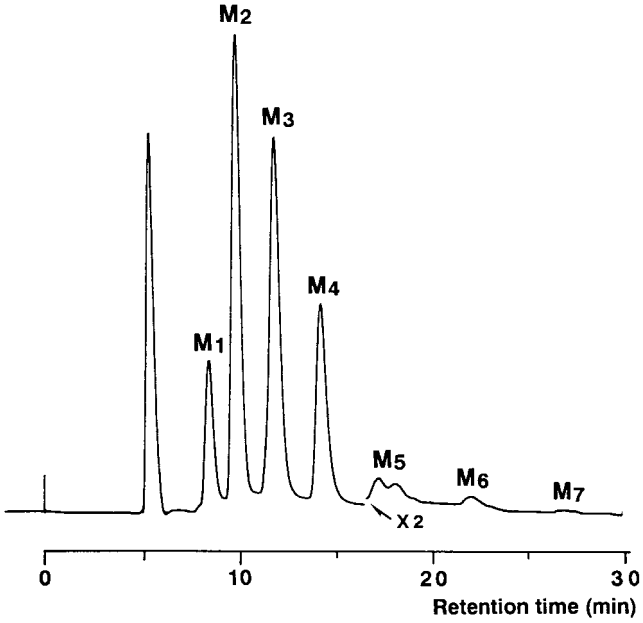


Figure 2. Elution profile of mannose oligosaccharides from *S. cerevisiae*. Chromatographic conditions: column, LiChroCART NH_2 (250 × 4.0 mm i.d.); eluent, 65 : 35 acetonitrile-water; flow rate, 0.5 ml/min; detector, Shodex RI-71.

Results and discussion

Preparation of β-CD glycosylation acceptors

Figure 1 shows the preparation of compounds **1–3** as the key glycosyl intermediates and chromatograms of TLC and HPLC. Synthesis of the glycosyl acceptor **6** from **3** as an example is shown in Scheme 1.

Preparation of mannosyl glycosylation donors

A portion of the acetolysate obtained was deacetylated in the usual way, and then HPLC analyses of manno-oligosaccharides were performed with an aminopropyl-silica column (LiChroCART NH_2) and a graphitized carbon column (GCC) (Hypercarb). The elution profiles are shown in Figures 2 and 3, respectively. Figure 2 shows the elution profile of the product of acetolysis; the ratio of Man : Man_2 : Man_3s : Man_4 : mannopentaose (Man_5) : mannohexaose (Man_6) was 14 : 44 : 44 : 38 : 2 : 1. In the analysis using this aminopropyl-silica column, the two Man_3s (Man_3-1 and Man_3-2) were not separated. Retention on an amino column is similar to that on an anion-exchange resin, and the retention rate increases with increasing molecular size. Figure 3 shows the elution profile of the same acetolysate on a GCC with 1.0% acetonitrile aqueous solution containing 1.0 mM sodium hydroxide. The ratio of Man : Man_2 : Man_3-1 : Man_3-2 : Man_4 : Man_5 : Man_6 was 36 : 92 : 79 : 21 : 83 : 2 : 1. Two Man_3s (Man_3-1 and Man_3-2) were clearly separated, and the ratio of Man_3-1 and Man_3-2 was 3.7 : 1.

Most of the acetolysate was fractionated by centrifugal chromatography with hexane-acetone, and then each fraction was deacetylated. The main products, Man_2, Man_3s , and Man_4, were isolated and purified by HPLC on a TSK-gel Amide-80 column (300 × 21.5 mm i.d.) with 60 : 40 acetonitrile-water, and two Man_3s were isolated on a Hypercarb column (100 × 10 mm i.d.) with 0.5 : 99.5 acetonitrile-water. The molecular weights of Man_2, Man_3s, and Man_4 were confirmed to be 365.4, 527.6, and 689.6, respectively, by TOF-MS. The NMR analysis was performed using ^{1}H-^{1}H COSY and ^{1}H-^{13}C COSY measurements, and all carbons in the spectra of Man_2, Man_3-1, Man_3-2, and Man_4 were completely assigned. The structures of Man_2, Man_3-1, Man_3-2, and Man_4 were identified as Man(1α)→2Man, Man (1α)→2Man (1α)→2Man, Man (1α)→3Man (1α)→2Man, and Man (1α)→3Man (1α)→2Man (1α)→2Man, respectively [5].

Acetylation of Man_2, Man_3 (Man_3-1), or Man_4, followed by 1-*O*-deacetylation, and reacting with trichloroacetonitrile in the presence of DBU in dichloromethane, then afforded the glycosyl donors, **15**, **16**, and **17** after centrifugal chromatography.

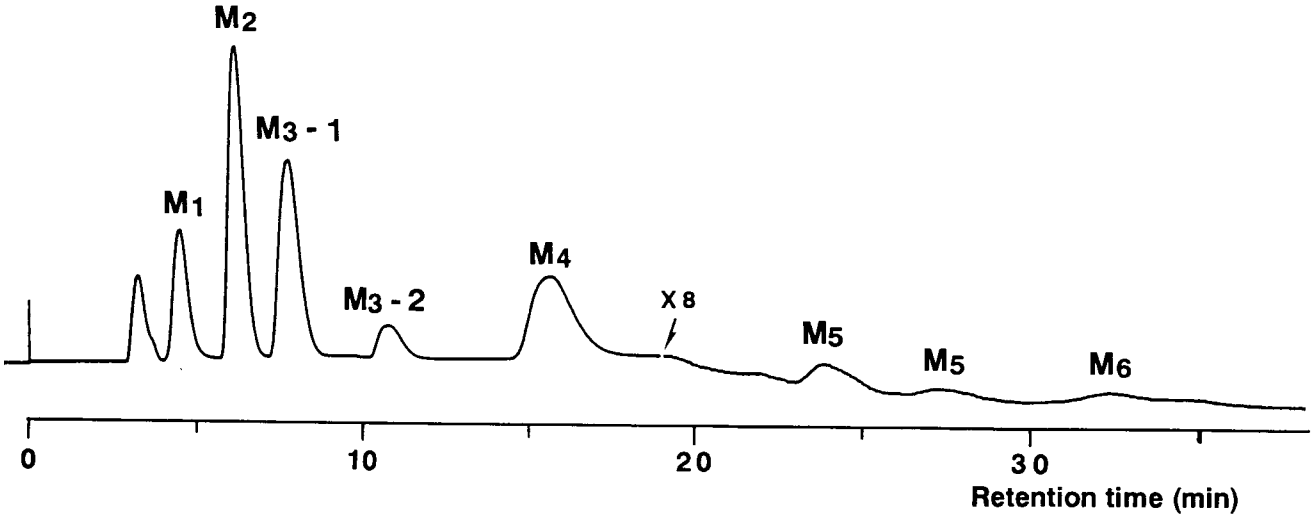


Figure 3. Elution profile of mannose oligosaccharides from *S. cerevisiae*. Chromatographic conditions: column, Hypercarb (100 × 4.6 mm i.d.); eluent, 1.0% acetonitrile aqueous solution containing 1.0 mM sodium hydroxide; flow rate, 0.5 ml/min; detector, Shodex RI-71; temperature, 20 °C.

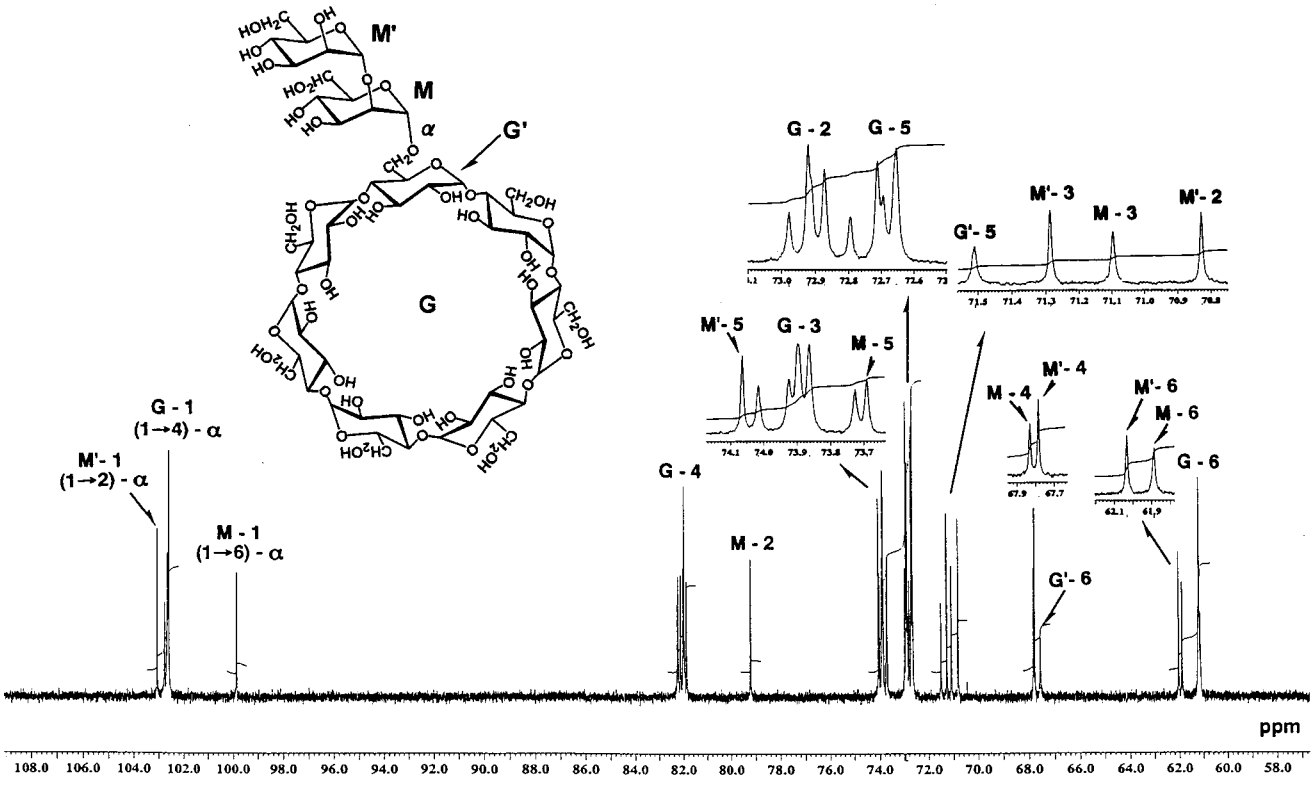


Figure 4. ^{13}C NMR spectrum of **7** measured in D_2O.

Glycosylation

Mannosylation of **4**, **5**, or **6** with **15** and **6** with **16** or **17** in dichloromethane in the presence of TMSOTf and molecular sieves for 1 h at −20 °C, followed by deacetylation, and isolation by HPLC on a TSK-gel Amide-80 column and an ODS column gave the desired main products **8**, **9**, **10**, **12**, and **14** and byproducts **7**, **11**, and **13**. The molecular weights of **7–14** were confirmed by TOF-MS. The structures of all those compounds were elucidated by NMR spectroscopy. The carbon signals for M-1 at 100 ppm in the spectra of **7–14** suggested only (1→6)-α-linkage. Thus all mannooligosaccharides became bound to the β-CD ring with an α-linkage. Figure 4 shows the ^{13}C NMR spectrum of **7** for which the ^{13}C resonances of all carbons in the spectrum were assigned using ^{1}H-^{1}H COSY and ^{1}H-^{13}C COSY methods.

Specific interaction between branched CDs and lectins

The specific interaction between the branched β-CD derivatives and mannose-binding lectins, ConA and PSA was investigated with conventional hemagglutination assay and new method based on optical resonance sensing with the IAsys apparatus.

Table 2. Association equilibrium constant (KA/M^{-1}) of surface plasmon resonance analysis and inhibition tests of hemagglutinating activity for various Man-βCDs with lectins (ConA and PSA)

	Surface plasmon resonance analysis		Inhibition tests of hemagglutinating activity	
	Binding constant (M^{-1})		Inhibitory concentration ($\times 10^{-4}$)	
	Lectin		Lectin	
Compounds	ConA	PSA	ConA	PSA
Man_2	N.D.	N.D.	160.0	120.0
Man_2-βCD	1,300	800	5.0	20.0
$6^1,6^2$-$(Man_2)_2$-βCD	2,700	N.D.	2.5	20.0
$6^1,6^3$-$(Man_2)_2$-βCD	11,000	700	1.3	10.0
$6^1,6^4$-$(Man_2)_2$-βCD	5,000	800	2.5	10.0
Man_3	2,500	700	0.6	20.0
Man_3-βCD	1,200	700	5.0	20.0
$6^1,6^4$-$(Man_3)_2$-βCD	18,000	16,000	1.3	5.0
Man_4	N.D.	N.D.	2.5	20.0
Man_4-βCD	3,200	4,000	1.3	5.0
$6^1,6^4$-$(Man_4)_2$-βCD	500	800	0.6	5.0

N.D. = Non Detect.

Affinity assay

The results showed that all branched β-CDs interacted with lectins (Table 2). The binding affinity was $6^1,6^4$-$(Man_3)_2$- $\gg$ $6^1,6^4$-$(Man_2)_2$- > $6^1,6^4$-$(Man_4)_2$-β-CD when the derivatives were compared on the basis of side chain length and $6^1,6^3$- $\gg$ $6^1,6^4$- > $6^1,6^2$-$(Man_2)_2$-β-CD when compared on the basis of side chain position.

Hemagglutination assay

Table 2 shows the results of the hemagglutination assay. All the branched β-CDs interact with both lectins. However the difference inhibitory concentration was not significant.

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