Preparation and Characterization of Novel Branched β-Cyclodextrins Having β-D-Galactose Residues on the Non-reducing Terminal of the Side Chains and Their Specific Interactions with Peanut (*Arachis hypogaea*) Agglutinin

Akiko IKUTA,[a] Naomi MIZUTA,[a] Sumio KITAHATA,[b] Takeomi MURATA,[c] Taichi USUI,[c] Kyoko KOIZUMI,[a,1)] and Toshiko TANIMOTO*,[a]

[a] *School of Pharmaceutical Sciences, Mukogawa Women's University; 11–68 Koshien Kyuban-cho, Nishinomiya 663–8179, Japan:* [b] *Department of Bioscience and Biotechnology, Shinshu University; 8304 Minamiminowa, Nagano 399–4598, Japan: and* [c] *Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University; Ohya 836, Shizuoka 422–8529, Japan.* Received August 28, 2003; accepted October 6, 2003

Six novel branched β-cyclodextrins (βCDs) having β-D-galactose residues on the non-reducing terminal of the sugar side chains, namely 6^1,6^4-di-*O*-(β-D-galactosyl)-βCD (10), 6-*O*-(β-D-galactosyl)-βCD (11), 6^1,6^4-di-*O*-(β-lactosyl)-βCD (14), 6-*O*-(β-lactosyl)-βCD (15), 6^1,6^4-di-*O*-(4′-*O*-β-D-galactosyl-β-lactosyl)-βCD (18), and 6-*O*-(4′-*O*-β-D-galactosyl-β-lactosyl)-βCD (19), were chemically synthesized using the trichloroacetimidate method. The reaction products were separated by HPLC on an amino column into dibranched and monobranched βCDs. Their structures were confirmed by mass spectrometry (MS) and two-dimensional (2D) NMR spectroscopic analysis. To study the length of the sugar side chains attached to the CD ring, which leads to differences in the functions of the branched CDs, interactions of these compounds with peanut (*Arachis hypogaea*) agglutinin (PNA) were investigated using an optical biosensor and an inhibition assay based on hemagglutination. The results showed that all branched βCDs interacted with PNA, and the binding affinity was 18>14>10 and 19>15>11 when the derivatives were compared on the basis of side chain length.

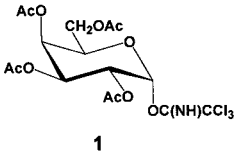
Key words branched β-cyclodextrin; β-D-galactosyl lactose; specific interaction; peanut (*Arachis hypogaea*) agglutinin; optical biosensor; inhibition assay based on hemagglutination

Cyclodextrins (CDs) are capable of forming inclusion complexes by taking various compounds into their interior cavities. It is well known that galactose plays important roles in recognition of receptor on the cell surface.[2,3)] Heterogeneous branched CDs having galactose residues on the non-reducing terminal of the sugar side chains are expected to be useful as drug carriers in targeted drug delivery systems. We hypothesized that the length of the sugar side chains attached to the CD ring leads to differences in the functions of the branched CDs. Therefore, we examined novel heterogeneous branched β-cyclodextrins (βCDs) having monosaccharides, disaccharides or trisaccharides for side chains. In particular, dibranched CDs have been considered to be promising in view of a cluster effect. In our previous work, we studied the chemical syntheses of glycosyl CDs, directly β-linked to the CD ring, which could not be synthesized by enzyme-catalyzed reactions. We prepared 6-*O*-(β-D-galactosyl)-γCD as a basic model compound using the trichloroacetimidate method.[4)] Here we describe the chemical syntheses of 6^1,6^4-di-*O*-(β-D-galactosyl)-βCD (**10**), 6^1,6^4-di-*O*-(β-lactosyl)-βCD (**14**), and 6^1,6^4-di-*O*-(4′-*O*-β-D-galactosyl-β-lactosyl)-βCD (**18**) using the trichloroacetimidate method and of monobranched βCDs obtained as their by-products *via* the same reactions, that is, 6-*O*-(β-D-galactosyl)-βCD (**11**), 6-*O*-(β-lactosyl)-βCD (**15**), and 6-*O*-(4′-*O*-β-D-galactosyl-β-lactosyl)-βCD (**19**).

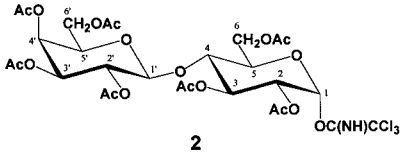
In order to evaluate the abilities of molecular recognition of these branched βCDs (**10**, **11**, **14**, **15**, **18**, **19**), their interaction with peanut (*Arachis hypogaea*) agglutinin (PNA), which is known as a terminal β-D-galactose-specific binding lectin,[5)] was investigated using an optical biosensor[6)] and inhibition assay based on hemagglutination.[7,8)]

Results and Discussion

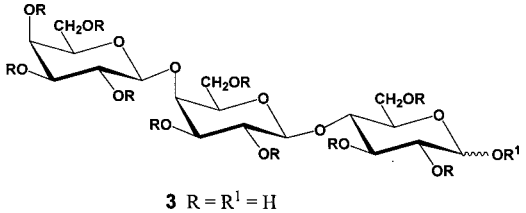
Preparation, Separation, and Characterization of 6^1,6^4-Di-*O*-(β-D-galactosyl)-βCD (10) and 6-*O*-(β-D-Galactosyl)-βCD (11) To synthesize 6^1,6^4-dibranched βCDs, the glycosyl acceptor, bis(2,3-di-*O*-acetyl)pentakis(2,3,6-tri-*O*-acetyl)-βCD (**6**),[9)] was obtained from 6^1,6^4-di-*O*-(*tert*-butyldimethylsilyl)-βCD (**7**),[10)] whose structure had been estab-



1



2



3 R = R^1 = H
4 R = Ac, R^1 = H
5 R = Ac, R^1 = C(NH)CCl$_3$

Chart 1. Structures of Compounds **1**—**5**

* To whom correspondence should be addressed. e-mail: tanimoto@mwu.mukogawa-u.ac.jp

	R	R^1	R^4
6	Ac	H	H
7	H	Si	Si
8	Ac	Gal(Ac)	Gal(Ac)
9	Ac	Gal(Ac)	H
10	H	β-Gal	β-Gal
11	H	β-Gal	H
12	Ac	Lac(Ac)	Lac(Ac)
13	Ac	Lac(Ac)	H
14	H	β-Lac	β-Lac
15	H	β-Lac	H
16	Ac	Gal-Lac(Ac)	Gal-Lac(Ac)
17	Ac	Gal-Lac(Ac)	H
18	H	β-Gal-Lac	β-Gal-Lac
19	H	β-Gal-Lac	H

Si: *tert*-butyldimethylsilyl
Gal(Ac): 2,3,4,6-tetra-*O*-acetyl-D-galactosyl
β-Gal: β-D-galactosyl
Lac(Ac): 2,3,6,2',3',4',6'-hepta-*O*-acetyl-lactosyl
β-Lac: β-lactosyl
Gal-Lac(Ac): 2,3,4,6-tetra-*O*-acetyl-β-D-galactosyl-(1→4')-2,3,6,2',3',6'-hexa-*O*-acetyl-lactosyl
β-Gal-Lac: 4'-*O*-β-D-galactosyl-β-lactosyl

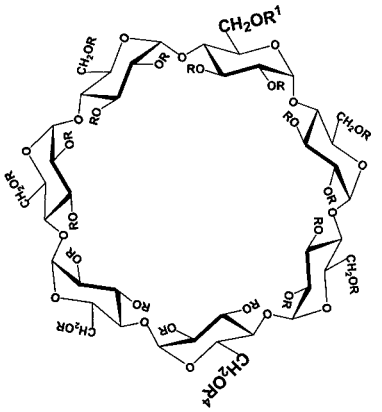


Chart 2. Structures of Compounds **6**—**19**

lished, in a previous study. 2,3,4,6-Tetra-*O*-acetyl-α-D-galactosyl trichloroacetimidate (**1**),[4,11—13] was obtained by treatment of 2,3,4,6-tetra-*O*-acetyl-D-galactose with trichloroacetonitrile in the presence of 1,8-diazabicyclo[5,4,0]-undec-7-ene (DBU)[14,15] in dichloromethane according to our reported method.[4] Galactosylation of **6** with **1** in dichloromethane in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) as an acid catalyst gave $6^1,6^4$-di-*O*-(2,3,4,6-tetra-*O*-acetyl-D-galactosyl)-βCD peracetate (**8**) and monogalactosylated βCD derivative (**9**). The fractions containing **8** and **9** were collected from the product by centrifugal chromatography, followed by deacetylation, and isolation by HPLC on a TSKgel Amide-80 column (300×7.8 mm i.d.) gave $6^1,6^4$-di-*O*-(β-D-galactosyl)-βCD (**10**) and 6-*O*-(β-D-galactosyl)-βCD (**11**).

High-resolution mass spectroscopic analysis of **10** (*m*/*z* 1459.4818; M^+) indicated a molecular formula of dibranched βCD. In the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrum of **11**, the $[M+Na]^+$ peak was clearly observed at *m*/*z* 1319.3. NMR analysis was performed using ^1H–^1H COSY and ^1H–^{13}C COSY measurements, and all carbons in the spectrum of **11** were completely assigned. It was confirmed that galactose was bonded directly by β-linking to the CD ring as observed when preparing 6-*O*-(β-D-galactosyl)-γCD.[4] In a similar manner, the structure of **10** was determined to be $6^1,6^4$-di-*O*-(β-D-galactosyl)-βCD.

Preparation, Separation, and Characterization of $6^1,6^4$-Di-*O*-(β-lactosyl)-βCD (14) and 6-*O*-(β-Lactosyl)-βCD (15) The reaction of **6** with 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-α-lactosyl trichloroacetimidate (**2**),[12,13] in dichloromethane in the presence of TMSOTf afforded $6^1,6^4$-di-*O*-(2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-lactosyl)-βCD peracetate (**12**) and monolactosylated compound (**13**). These compounds **12** and **13** were fractionated by centrifugal chromatography and then were deacetylated. $6^1,6^4$-Di-*O*-(β-lactosyl)-βCD (**14**) and 6-*O*-(β-lactosyl)-βCD (**15**) were purified by HPLC on a TSKgel Amide-80 column (300×7.8 mm i.d.). The structures of **14** and **15** were confirmed by MALDI-TOF-MS and NMR spectroscopic analysis.

Preparation, Separation, and Characterization of $6^1,6^4$-Di-*O*-(4′-*O*-β-D-galactosyl-β-lactosyl)-βCD (18) and 6-*O*-(4′-*O*-β-D-Galactosyl-β-lactosyl)-βCD (19) 4′-*O*-β-D-Galactosyl-lactose (**3**),[16] was synthesized from lactose by the galactosyl transfer action of *Bacillus circulans* β-D-galactosidase. The product was purified by an active carbon column and then by HPLC on a TSK gel Amide-80 column, and the trisaccharide was identified by TOF-MS. The structure was confirmed by NMR in which the D-galactose residue became bonded to lactose by the $\beta(1\rightarrow4)$-linkage (Fig. 1). All carbons in the NMR spectrum were completely assigned using ^1H–^1H COSY and ^1H–^{13}C COSY measurements. The assignments of the C-6 signals were detected by distortionless enhancement by the polarization transfer (DEPT)[17] method.

Acetylation of **3** with acetic anhydride-pyridine and then selective 1-*O*-deacetylation[14] of the fully acetylated trisaccharide with hydrazine acetate in dry *N*,*N*-dimethylformamide afforded 2,3,4,6-tetra-*O*-acetyl-β-D-galactosyl-(1→4′)-2,3,6,2′,3′,6′-hexa-*O*-acetyl-lactose (**4**). *O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactosyl)-(1→4′)-2,3,6,2′,3′,6′-hexa-*O*-acetyl-lactosyl trichloroacetimidate (**5**) was obtained by treatment of **4** with trichloroacetonitrile in the presence of DBU in dichloromethane.

Glycosylation of **6** with **5** in dichloromethane in the presence of TMSOTf gave dibranched βCD acetyl derivative having two trisaccharides (**16**) and a monobranched βCD acetyl derivative having one trisaccharide (**17**). As the isolation of **16** and **17** was difficult, eight fractions containing **16** and **17** were collected by centrifugal chromatography. Each fraction was *O*-deacetylated in the usual way and then $6^1,6^4$-di-*O*-(4′-*O*-β-D-galactosyl-lactosyl)-βCD [$6^1,6^4$-di-*O*-(Gal-Lac)-βCD] and 6-*O*-(4′-*O*-β-D-galactosyl-lactosyl)-βCD [6-

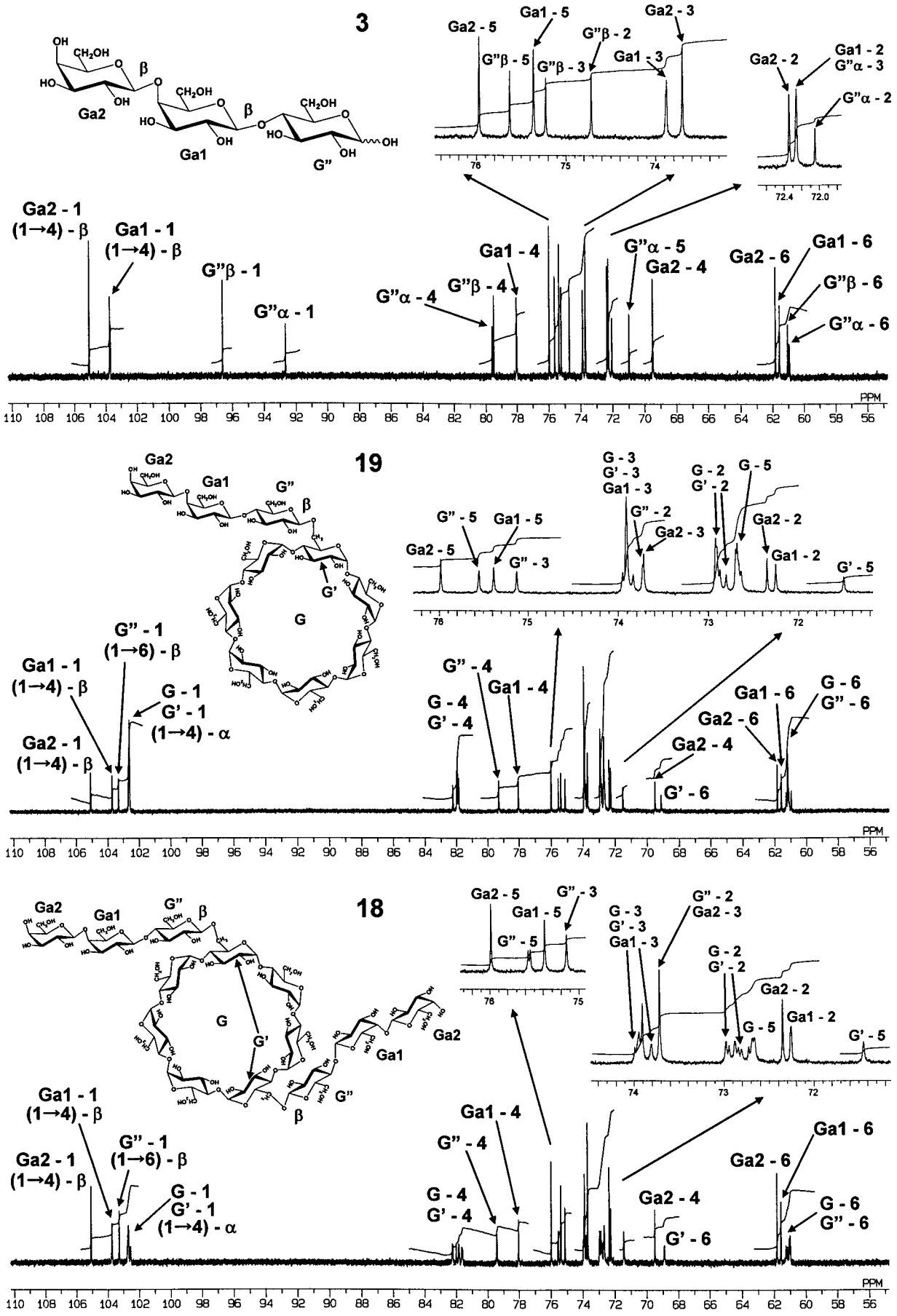


Fig. 1. ^{13}C-NMR Spectra of 4′-*O*-*β*-D-Galactosyl-lactose (**3**), 6^1,6^4-Di-*O*-(4′-*O*-*β*-D-galactosyl-*β*-lactosyl)-*β*CD (**18**), and 6-*O*-(4′-*O*-*β*-D-Galactosyl-*β*-lactosyl)-*β*CD (**19**) Measured in D_2O at 125.65 MHz

O-(Gal-Lac)-*β*CD] were isolated by HPLC on a TSKgel Amide-80 column (300×7.8 mm i.d.) with acetonitrile–water.

The branched trisaccharides of 6^1,6^4-di-*O*-(Gal-Lac)-*β*CD and 6-*O*-(Gal-Lac)-*β*CD were not determined for their bonding to *β*CD with either *α*(1→6)- or *β*(1→6)-linkage. The branched *β*CDs 6^1,6^4-di-*O*-(Gal-Lac)-*β*CD and 6-*O*-(Gal-Lac)-*β*CD were analyzed by TOF-MS and their molecular-ion peaks $[M+Na]^+$ were observed at *m*/*z* 2130.1 and 1644.2, respectively. Figure 1 shows the ^{13}C-NMR spectra of 6^1,6^4-di-*O*-(Gal-Lac)-*β*CD and 6-*O*-(Gal-Lac)-*β*CD. The ^{13}C signals of all carbons in the spectra of 6^1,6^4-di-*O*-(Gal-Lac)-*β*CD and 6-*O*-(Gal-Lac)-*β*CD were assigned using ^{1}H–^{1}H COSY and ^{1}H–^{13}C COSY methods and C-6 signals were confirmed by the DEPT method. In the spectrum of 6-*O*-(Gal-Lac)-*β*CD, the signal due to the *β*-glycosylated C-6 (G′-6, *δ* 69.1) shifted downward by 8 ppm, compared with C-6 signals (G-6, *δ* 61.0—61.3) of the six unsubstituted *β*CD ring glucoses. In addition, the C-1 signal (G″-1, *δ* 103.3) of glucose in the branched trisaccharide shifted downfield from the C-1 signals of the *β*CD ring (G-1, *δ* 102.6—102.7). In the case of glucose[18] or galactose[4,19] residue bonded to the CD ring with the *β*-(1→6)-linkage, the C-1 signal appeared downfield from C-1 signals of the CD ring, but also bonded to the CD ring with the *α*-(1→6)-linkage, the C-1 signal appearing further upfield than C-1 signals of the CD ring. Therefore 6^1,6^4-di-*O*-(Gal-Lac)-*β*CD was 6^1,6^4-di-*O*-(4′-*O*-*β*-D-galactosyl-*β*-lactosyl)-*β*CD (**18**), and similarly, 6-*O*-(Gal-Lac)-*β*CD was 6-*O*-(4′-*O*-*β*-D-galactosyl-*β*-lactosyl)-*β*CD (**19**).

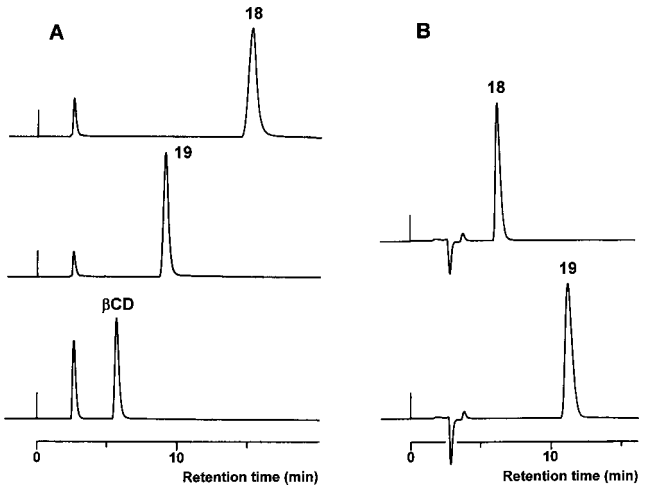


Fig. 2. Elution Profiles of 6^1,6^4-Di-*O*-(4′-*O*-*β*-D-galactosyl-*β*-lactosyl)-*β*CD (**18**) and 6-*O*-(4′-*O*-*β*-D-Galactosyl-*β*-lactosyl)-*β*CD (**19**)

Chromatographic conditions: (A) column, Hibar LiChroCART NH_2 (250×4.0 mm i.d.); eluent, 60 : 40 acetonitrile–water; flow rate, 1.0 ml/min; detector, Shodex RI-71; temperature, 30 °C. (B) Column, Hikarisil C18-4D (150×4.6 mm i.d.); eluent, 5 : 95 methanol–water; flow rate, 0.8 ml/min; detector, Shodex RI-71; temperature, 30 °C.

HPLC analyses of **18** and **19** were performed with an aminopropyl-silica column and an ODS column. Figure 2 shows the elution profiles of compounds **18** and **19** on an aminopropyl-silica column. The elution sequence with the NH_2-bonded silica and acetonitrile–water system follows the order of molecular size. The elution time gives qualitative information about the molecular size of sugar. Figure 2 also shows the elution profiles of **18** and **19** on an ODS column. In contrast, as C_{18} bonded silica is a reversed phase column, the separation mechanism is probably an example of hydrophobic chromatography, namely, increased retention with decreasing solubility in water. Thus, the retention times in Fig. 2 suggest that **18** would be more soluble in water than **19**.

Interactions of Branched *β*CDs with PNA In order to evaluate the molecular recognition abilities of branched *β*CDs (**10**, **11**, **14**, **15**, **18**, **19**), the interaction between those compounds and PNA which is known as terminal *β*-D-galactose-specific binding lectin was investigated using an optical biosensor and an inhibition assay based on hemagglutination.

Association constants of branched *β*CDs (**10**, **11**, **14**, **15**, **18**, **19**) or corresponding sugars with PNA were determined by the surface plasmon resonance method.[20] The lectin on the surface of biosensor cuvettes can be regarded as model of receptors displayed on the surface of cell membranes. We decided to immobilize PNA on the surface of cuvettes and inject the sugars over this surface. Kinetic and equilibrium constants of the interaction between PNA and the branched *β*CDs are listed in Table 1. The results showed that all branched *β*CDs interacted with PNA. Branched *β*CDs having longer side chains showed higher affinity, that is, the binding affinity was **18**>**14**>**10** and **19**>**15**>**11** when the derivatives were compared on the basis of side chain length. As anticipated, dibranched *β*CDs showed higher affinity than their corresponding monobranched *β*CDs.

Inhibition assay of hemagglutination was examined according to previous methods.[7,8] The effects of branched *β*CDs on hemagglutination caused by PNA are summarized

Table 1. Kinetic and Equilibrium Constants of Branched *β*CDs and Sugars against PNA and Inhibitory Effects on Hemagglutination of PNA by Branched *β*CDs and Sugars

Compound	Kinetic parameter			Inhibition assay
	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_A (M^{-1})	MIC[a] (mM)
6^1,6^4-(*β*-Gal)$_2$-*β*CD (**10**)	4.42×10^1	1.15×10^{-2}	3.86×10^3	4
(*β*-Gal)-*β*CD (**11**)	1.91×10^1	1.21×10^{-2}	1.58×10^3	16
D-Galactose	—	—	—[b]	64
6^1,6^4-(*β*-Lac)$_2$-*β*CD (**14**)	2.27×10^2	8.52×10^{-3}	2.67×10^4	4
(*β*-Lac)-*β*CD (**15**)	5.30×10^1	9.01×10^{-3}	5.89×10^3	8
Lactose	—	—	—[b]	32
6^1,6^4-(*β*-Gal-Lac)$_2$-*β*CD (**18**)	1.07×10^3	1.06×10^{-2}	1.01×10^5	2
(*β*-Gal-Lac)-*β*CD (**19**)	1.48×10^2	1.39×10^{-2}	1.07×10^4	8
3	—	—	—[b]	32

a) Minimum inhibitory concentration. *b*) Not detected ($K_A<10^3$ M^{-1}).

in Table 1 in comparison with those by the corresponding simple sugars. The minimum inhibitory concentration is expressed as the molar concentration of branched βCDs and sugars.

The results showed that all branched βCDs interacted with PNA. Though the minimum inhibitory concentration of branched βCDs were lower than that of the corresponding simple sugars used as a control, the difference in inhibitory concentration was not significant.

Experimental

General Methods HPLC was performed with a Jasco PU-980 pump, a Rheodyne 7125 injector, and a Shodex RI-71 refractive index detector. HPLC analyses at constant temperature were conducted with a column oven CA-202 (Flom). The columns employed were YMC-Pack SH-343-7 ODS (250×20 mm i.d.), TSKgel Amide-80 (300×7.8 mm i.d., Tosoh), Hibar LiChroCART NH_2 (250×4.0 mm i.d., Kanto Chemical) and Hikarisil C18-4D (150×4.6 mm i.d.). ^{1}H- and ^{13}C-NMR spectra were recorded on a Jeol GSX-500 or a Jeol JNM-ECP 500 spectrometer. Chemical shifts were expressed in ppm relative to 1,4-dioxane as the external standard. The other conditions for ^{1}H–^{1}H COSY and ^{1}H–^{13}C COSY measurements were the same as in the previous paper.[21] HR-FAB-MS was measured in the positive-ion mode using a Jeol MS 700 mass spectrometer with xenon atoms, and glycerol was used as the matrix. MALDI-TOF-MS was carried out on a Vision 2000 instrument (Thermo Bioanalysis). 2,5-Dihydroxybenzoic acid was used as the matrix. The instrument was operated in the positive ion reflectron mode with an accelerating potential of 7 kV. Optical rotations were determined with a Jasco P-1020 polarimeter at 25 °C. TLC was performed on Silica Gel 60 plates (E. Merck). Centrifugal chromatography was performed with a Harrison Centrifugal Thin-Layer Chromatotron 7924. The optical biosensor IAsys cuvette system[6] with IAsys software and IAsys cuvette coated with carboxylate were from Affinity Sensors.

Materials β-D-Galactosidase [EC 3.2.1.23] preparations from *Bacillus circulans* were obtained from Daiwa Kasei. Sialidase [EC 3.2.1.18] from *Arthrobacter ureafaciens* was purchased from Nacalai Tesque. PNA was purchased from Seikagaku Corp.

4′-*O*-β-D-Galactosyl-lactose (3) Lactose (10 g), 1.33 M, was dissolved in 100 μl of 1 M acetate buffer (pH 6.0), and incubated with *Bacillus circulans* β-D-galactosidase (1%, 2.2 ml) at 40 °C for 1 h. The reaction mixture was heated at 100 °C for 10 min to stop the enzyme action. Using an active carbon column (500×55 mm i.d.) with 7% aqueous ethanol solution, monosaccharides and lactose were removed from the reaction mixture, and then **3** was eluted from the column with 15% aqueous ethanol solution. Compound **3** was isolated by HPLC on a TSKgel Amide-80 column (300×7.8 mm i.d.) with 65 : 35 acetonitrile–water: $[\alpha]_D^{25}$ +35.8° (*c*=1.19, H_2O). MALDI-TOF-MS *m/z*: 527.5 $[M+Na]^+$ (Calcd for $C_{18}H_{32}O_{16}$: 504.4).

2,3,4,6-Tetra-*O*-acetyl-β-D-galactosyl-(1→4′)-2,3,6,2′,3′,6′-hexa-*O*-acetyl-lactose (4) To a solution of **3** (925 mg) in dry pyridine (30 ml) was added acetic anhydride (15 ml) and the mixture was stirred for 19 h at room temperature and then concentrated. The residue was extracted with chloroform, and the extract was successively washed with water, aqueous sodium hydrogen carbonate and water, dried and concentrated. A solution of residue in dry dimethylformamide (50 ml) was treated with hydrazine acetate (231 mg) and kept for 1.5 h at 50 °C, then the mixture was diluted with ethyl acetate and washed twice with satd. aqueous sodium chloride solution, then dried, and concentrated. Centrifugal chromatography (1 : 1 hexane–acetone) of the residue gave **4** (1267 mg, 75%): $[\alpha]_D^{26}$ +21.9° (*c*=2.00, $CHCl_3$). ^{1}H-NMR ($CDCl_3$): δ 2.18—1.85 (10s, 30H, $10CH_3$), 5.51—3.57 (m, 22H). ^{13}C-NMR ($CDCl_3$): δ 170.54, 170.48, 170.39, 170.32, 170.23, 170.17, 170.05, 169.80, 169.50, 169.00 (C=O), 101.29 (Ga2-1), 100.40 (Ga1-1), 95.44 (Gβ-1), 90.25 (Gα-1), 63.00, 61.38 (Ga1-6 and Ga2-6), 62.27 (G-6). δ 20.82—20.50 (10C, $10CH_3$). MALDI-TOF-MS *m/z*: 947.1 $[M+Na]^+$ (Calcd for $C_{38}H_{52}O_{26}$: 924.8).

***O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactosyl)-(1→4′)-2,3,6,2′,3′,6′-hexa-*O*-acetyl-lactosyl Trichloroacetimidate (5)** To a solution of **4** (1109 mg) in dry dichloromethane (10 ml), trichloroacetonitrile (1.4 ml) and DBU (35 μl) were added, and the mixture was stirred for 1 h at 0 °C. The reaction mixture was diluted with chloroform and washed with satd. aqueous sodium chloride solution, then dried, and concentrated. Centrifugal chromatography (3 : 2 hexane–acetone) of the residue gave **5** (1162 mg, 91%).

6^1,6^4-Di-*O*-(β-D-galactosyl)-βCD (10) and 6-*O*-(β-D-Galactosyl)-βCD (11) A mixture of **6** (856 mg), **1** (1259 mg), and dry powderd 4 Å molecular sieves (2.0 g) in dry dichloromethane (25 ml) was stirred under nitrogen at −20 °C. A solution of TMSOTf (80 μl) in dichloromethane (2 ml) was added. After stirring for 3 h at −20 °C, triethylamine (1 ml) was added to the mixture, which was diluted with chloroform, filtered through Celite, washed sequentially with 1 M sulfuric acid, aqueous sodium hydrogen carbonate and water, dried and concentrated. The residue was fractionated by centrifugal chromatography with 2 : 1→2 : 3 hexane–acetone, and then each fraction containing **8** and **9** was treated with methanolic 0.05 M sodium methoxide (3—10 ml) for 1 h at room temperature, neutralized with Amberlite IR-120B (H^+) resin, filtered, and concentrated. The fractions were mixtures of **10** and **11**. These compounds **10** (104 mg, 16% based on **6**) and **11** (143 mg, 25%) were isolated from the mixtures by HPLC on a TSKgel Amide-80 column (300×7.8 mm i.d.) with 60 : 40 acetonitrile–water: **10**, $[\alpha]_D^{26}$ +123.5° (*c*=1.18, H_2O). ^{13}C-NMR (H_2O): δ 104.6—104.5 (2C, Ga-1), 102.6 (7C, G-1 and G′-1), 82.4—81.9 (7C, G-4 and G′-4), 75.9 (2C, Ga-5), 73.9 (7C, G-3 and G′-3), 73.6 (2C, Ga-3), 72.9—72.8 (7C, G-2 and G′-2), 72.7 (5C, G-5), 71.7 (4C, G′-5 and Ga-2), 69.6 (4C, G′-6 and Ga-4), 61.8 (2C, Ga-6), 61.4—61.1 (5C, G-6). HR-FAB-MS *m/z*: 1459.4818 $[M+H]^+$ (Calcd for $C_{54}H_{91}O_{45}$: 1459.4832). MALDI-TOF-MS *m/z*: 1481.6 $[M+Na]^+$ (Calcd for $C_{54}H_{90}O_{45}$: 1458.5). **11**, $[\alpha]_D^{26}$ +122.5° (*c*=1.11, H_2O). ^{13}C-NMR (H_2O): δ 104.7 (Ga-1), 102.6 (7C, G-1 and G′-1), 82.4—81.9 (7C, G-4 and G′-4), 75.9 (Ga-5), 74.0—73.9 (7C, G-3 and G′-3), 73.7 (Ga-3), 72.9—72.8 (7C, G-2 and G′-2), 72.7 (6C, G-5), 71.8 (G′-5), 71.7 (Ga-2), 69.9 (G′-6), 69.6 (Ga-4), 61.8 (Ga-6), 61.4—61.1 (6C, G-6). MALDI-TOF-MS *m/z*: 1319.3 $[M+Na]^+$ (Calcd for $C_{48}H_{80}O_{40}$: 1297.1).

6^1,6^4-Di-*O*-(β-lactosyl)-βCD (14) and 6-*O*-(β-Lactosyl)-βCD (15) A mixture of **6** (844 mg), **2** (2210 mg), and dry powderd 4 Å molecular sieves (2.0 g) in dry dichloromethane (25 ml) was stirred under nitrogen at −20 °C. A solution of TMSOTf (78 μl) in dichloromethane (2 ml) was added. After stirring for 2.5 h at −20 °C, triethylamine (1 ml) was added to the mixture, which was diluted with chloroform, filtered through Celite, washed sequentially with 1 M sulfuric acid, aqueous sodium hydrogen carbonate and water, dried and concentrated. The residue was fractionated by centrifugal chromatography with 2 : 1→1 : 2 hexane–acetone, and then each fraction containing **12** and **13** was treated with methanolic 0.05 M sodium methoxide (3—10 ml) for 1 h at room temperature, neutralized with Amberlite IR-120B (H^+) resin, filtered, and concentrated. The fractions were mixtures of **14** and **15**. Therefore, compounds **14** (92 mg, 12% based on **6**) and **15** (101 mg, 16%) were isolated from the mixtures and purified by HPLC on a TSKgel Amide-80 column (300×7.8 mm i.d.) with 60 : 40 acetonitrile–water: **14**, $[\alpha]_D^{26}$ +100.6° (*c*=1.05, H_2O). ^{13}C-NMR (H_2O): δ 103.8 (2C, Ga-1), 103.3 (2C, G″-1), 102.7—102.5 (7C, G-1 and G′-1), 82.3—81.6 (7C, G-4 and G′-4), 79.4 (2C, G″-4), 76.2 (2C, Ga-5), 75.6 (2C, G″-5), 75.1 (2C, G″-3), 74.0 (5C, G-3), 73.8—73.7 (4C, G′-3 and G″-2), 73.5 (2C, G′-2), 73.0—72.6 (12C, G-2, Ga-3 and G-5), 71.8 (2C, Ga-2), 71.4 (2C, G′-5), 69.4 (2C, Ga-4), 68.9 (2C, G′-6), 61.8 (2C, Ga-6), 61.3—61.0 (7C, G-6 and G″-6). HR-FAB-MS *m/z*: 1783.5917 $[M+H]^+$ (Calcd for $C_{66}H_{111}O_{55}$: 1783.5889). MALDI-TOF-MS *m/z*: 1805.5 $[M+Na]^+$ (Calcd for $C_{66}H_{110}O_{55}$: 1782.6). **15**, $[\alpha]_D^{25}$ +123.2° (*c*=1.00, H_2O). ^{13}C-NMR (H_2O): δ 103.8 (Ga-1), 103.3 (G″-1), 102.7—102.6 (7C, G-1 and G′-1), 82.2—81.8 (7C, G-4 and G′-4), 79.3 (G″-4), 76.2 (Ga-5), 75.6 (G″-5), 75.1 (G″-3), 73.9 (6C, G-3) , 73.8 (G′-3), 73.7 (G″-2), 73.5 (G′-2), 72.9 (6C, G-2), 72.8 (Ga-3), 72.7—72.6 (6C, G-5), 71.8 (Ga-2), 71.5 (G′-5), 69.4 (Ga-4), 69.1 (G′-6), 61.8 (Ga-6), 61.3—61.1 (6C, G-6), 61.0 (G″-6). MALDI-TOF-MS *m/z*: 1481.6 $[M+Na]^+$ (Calcd for $C_{54}H_{90}O_{45}$: 1458.5).

6^1,6^4-Di-*O*-(4′-*O*-β-D-galactosyl-β-lactosyl)-βCD (18) and 6-*O*-(4′-*O*-β-D-Galactosyl-β-lactosyl)-βCD (19) A mixture of **6** (830 mg), **5** (1162 mg), and dry powderd 4 Å molecular sieves (2.0 g) in dry dichloromethane (30 ml) was stirred under nitrogen at −20 °C. A solution of TMSOTf (150 μl) in dichloromethane (2 ml) was added. After stirring for 1.5 h at −20 °C, triethylamine (1 ml) was added to the mixture, which was diluted with chloroform, filtered through Celite, washed sequentially with 1 M sulfuric acid, aqueous sodium hydrogen carbonate and water, dried and concentrated. The residue was fractionated by centrifugal chromatography with 1 : 1 hexane–acetone, and then each fraction containing **16** and **17** was treated with methanolic 0.05 M sodium methoxide (3—10 ml) for 1 h at room temperature, neutralized with Amberlite IR-120B (H^+) resin, filtered, and concentrated. The desired compounds **18** (81 mg, 9% based on **6**) and **19** (94 mg, 14%) were isolated and purified from each fraction by HPLC on a TSKgel Amide-80 column (300×7.8 mm i.d.) with 60 : 40—63 : 37 acetonitrile–water: **18**, $[\alpha]_D^{25}$ +85.2° (*c*=0.70, H_2O). HR-FAB-MS *m/z*: 2107.6946 $[M+H]^+$ (Calcd for $C_{78}H_{131}O_{65}$: 2107.6946). MALDI-TOF-MS *m/z*: 2130.1 $[M+Na]^+$ (Calcd for $C_{78}H_{130}O_{65}$: 2106.7). **19**, $[\alpha]_D^{25}$ +97.0° (*c*=1.03, H_2O). MALDI-TOF-MS *m/z*: 1644.2 $[M+Na]^+$ (Calcd for $C_{60}H_{100}O_{50}$: 1620.5).

Immobilization of PNA on Optical Biosensor Cuvettes Immobilization of PNA onto activated carboxylate surface was performed according to the manufacturer's specifications. Briefly, after equilibration and obtaining a stable baseline with phosphate buffer saline (PBS; 10 mM, pH 7.4), the optical biosensor cuvette coated with calboxylate was activated with a mixture of 400 mM *N*-ethyl-*N'*-(3-diethylaminopropyl)carbodiimide hydrochloride and 100 mM *N*-hydroxysuccinimide for 7 min. The activation solution was removed by washing with PBS, and 50 μl of PNA solution (1 mg/ml, in 10 mM acetate buffer, pH 5.0) was added for 30 min. The remaining active sites were blocked with BSA solution (2 mg/ml in PBS) for 10 min. A baseline was established with PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/CM, 10 mM, pH 7.2).

Interactions of Branched βCDs and Sugars with Immobilized PNA All binding experiments in the IAsys instrument were carried out using 10 mM PBS/CM at 25 °C. Binding of ligand onto the cuvette was monitored using 50 μl of sample solution. The association was ended by aspiration of the sample and replacing it with the same volume of PBS/CM. Monitoring was continued for a couple of minutes.

Inhibition Assay of Hemagglutination by Lectin The inhibitory activities of branched βCDs (**10**, **11**, **14**, **15**, **18**, **19**) and their corresponding sugars (D-galactose, lactose, and **3**) were examined using a 3% erythrocyte suspension treated with sialidase. The erythrocyte suspension (20 μl) was added to each well of the twofold dilution series of PNA (20 μl) in 96-well microtiter U-plates, and then incubated for 1 h, in order to determine the minimum concentration of PNA required for hemagglutination of erythrocytes. Its fourfold concentration solution was used for the hemagglutination inhibition assay. Twofold serial dilutions (10 μl) of branched βCDs and their corresponding sugars were prepared, PNA solutions (10 μl) were added, and the reaction mixtures were incubated for 1 h at room temperature. The erythrocyte suspension (20 μl) was added to each well and further incubated for 1 h at room temperature. Agglutination of erythrocytes was looked for and the minimum concentrations of the branched βCDs and sugars required to inhibit erythrocyte agglutination were identified.

Acknowledgments This work was partly supported by the Sasakawa Scientific Research Grant from The Japan Science Society. The authors thank Prof. M. Yamaki and her staff (Mukogawa Women's University) for measuring the NMR spectra.

References and Notes

1) Deceased August 16, 2000.
2) Morell A. G., Irvine R. A., Sternlieb I., Scheinberg I. H., Ashwell G., *J. Biol. Chem.*, **243**, 155—159 (1968).
3) Ashwell G., Morell A. G., *Adv. Enzymol.*, **41**, 99—128 (1974).
4) Ikuta A., Tanimoto T., Koizumi K., *J. Carbohydr. Chem.*, **22**, 297—308 (2003).
5) Goldstein I. J., Poretz R. D., "The Lectins: Properties, Functions, and Applications in Biology and Medicine," eds. by Liener I. E., Sharon N., Goldstein I. J., Academic Press, Orlando, 1986, pp. 33—247.
6) Cush R., Cronin J. M., Stewart W. J., Maule C. H., Molly J., Goddard N. J., *Biosensors & Bioelectronics*, **8**, 347—354 (1993).
7) Kawagishi H., Yamawaki M., Isobe S., Usui T., Kimura A., Chiba S., *J. Biol. Chem.*, **269**, 1375—1379 (1994).
8) Zeng X., Murata T., Kawagishi H., Usui T., Kobayashi K., *Biosci. Biotechnol. Biochem.*, **62**, 1171—1178 (1998).
9) Koizumi K., Okada Y., Nakanishi N., Tanimoto T., Takagi Y., Ishikawa M., Ishigami H., Hara K., Hashimoto H., *J. Carbohydr. Chem.*, **10**, 657—670 (1991).
10) Nakanishi N., Tsujikawa J., Hara N., Tanimoto T., Koizumi K., *Yakugaku Zasshi*, **110**, 477—483 (1990).
11) Schmidt R. R., Stumpp M., *Liebigs Ann. Chem.*, **1983**, 1249—1256 (1983).
12) Amvam-Zollo P. H., Sinay P., *Carbohydr. Res.*, **150**, 199—212 (1986).
13) Koeman F. A. W., Meissner J. W. G., van Ritter H. R. P., Kamerling J. P., Vliegenthart J. F. G., *J. Carbohydr. Chem.*, **13**, 1—25 (1994).
14) Sugimoto M., Numata M., Koike K., Nakahara Y., Ogawa T., *Carbohydr. Res.*, **156**, c1—c5 (1986).
15) Numata M., Sugimoto M., Koike K., Ogawa T., *Carbohydr. Res.*, **163**, 209—225 (1987).
16) Onishi N., Yamashiro A., Yokozeki K., *Appl. Environ. Microbiol.*, **61**, 4022—4025 (1995).
17) Doddrell D. M., Pegg D. T., *J. Am. Chem. Soc.*, **102**, 6388—6390 (1980).
18) Tanimoto T., Sakaki T., Koizumi K., *Carbohydr. Res.*, **267**, 27—37 (1995).
19) Ikuta A., Koizumi K., Tanimoto T., *J. Carbohydr. Chem.*, **19**, 13—23 (2000).
20) Fagerstam L. G., Frostell-Karlsson Å., Karlsson R., Persson B., Rönnberg I., *J. Chromatogr.*, **597**, 397—410 (1992).
21) Koizumi K., Tanimoto T., Okada Y., Takeyama S., Hamayasu K., Hashimoto H., Kitahata S., *Carbohydr. Res.*, **314**, 115—125 (1998).