Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

69451 Weinheim, Germany

Carbohydrate Wheels: CB[6]-based Carbohydrate Clusters

Jeeyeon Kim, Youngjoo Ahn, Kyeng Min Park, Youngkook Kim,
Young Ho Ko, Dong Hyun Oh, Kimoon Kim*

National Creative Research Initiative Center for Smart Supramolecules, Department of Chemistry, and School of Environmental Science and Engineering
Pohang University of Science and Technology
San 31 Hyoja-dong, Pohang 790-784, Republic of Korea.
E-mail: kkim@postech.ac.kr

Materials and Methods.
All the reagents and solvents employed were commercially available and used as supplied without further purification. $(Allyloxy)_{12}CB[6]$,[1] 2,3,4,6-tetra-*O*-acetyl-β-D-1-thioglucoopyranose (**1**),[2] 2,3,4,6-tetra-*O*-acetyl-β-D-1-thiogalactopyranose (**2**),[2] 2,3,4,6-tetra-*O*-acetyl-α-D-1-thiomannopyranose (**3**),[2] and FITC-spermine (**10**)[1] were synthesized according to the literature. Concanavalin A (ConA) was purchased from Sigma and methyl-α-D-mannopyranose (Me-αMan) and methyl-β-D-galactopyranose were purchased from TCI (Japan). Photoreaction was performed in a quartz flask by irradiating UV light using a Rayonet photochemical reactor (Model: RMR-600) equipped with four 254 nm lamps and four 300 nm lamps. All the NMR data were recorded on a Bruker DRX500 spectrometer. UV-visible absorption measurement was performed on a Hewlett-Packard 8453 diode array spectrophotometer. Optical density for the turbidimetric assay was measured on a Victor plate reader from Wallac. Isothermal titration calorimetry (ITC) experiments were performed using a VP-ITC calorimeter from Microcal. Fluorescent and differential interference contrast (DIC) images were acquired by an Olympus Fluoview 1000 confocal laser scanning microscope under a 40× oil immersion objective. Human hepatocellular carcinoma cell (HepG2 cell) line was obtained from the Korean Cell Line Bank (KCLB).

Synthesis of CB[6]-based *O*-Acetylglucose Cluster (4).
To a solution of $(allyloxy)_{12}CB[6]$[1] (0.33 g, 0.20 mmol) in MeOH (60 mL) was added 2,3,4,6-tetra-*O*-acetyl-β-D-1-thioglucopyranose **1**[2] (3.5 g, 9.6 mmol). After degassing with N_2, the mixture was irradiated with UV light (254 nm and 300 nm) for 2 d. After the reaction, the solvent was removed under a reduced pressure and the remaining solid was washed with diethyl ether and dried in vacuo to give CB[6]-based *O*-acetylglucose cluster **4** (0.92 mg, 76%). The product was a mixture of partially substituted **4** with a different degree of substitution. The NMR and mass data indicates that 9 – 12 *O*-acetylglucose groups are attached to a CB[6] core with an average of ~11 *O*-acetylglucose groups per CB[6]. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.15–5.50 (br, 15H), 5.27 (m, 11H), 5.11 (m, 11H), 5.06 (m, 11H), 4.80–4.45(br, 14H), 4.28 (m, 14H), 4.17 (m, 14H), 3.83 (br, 20H), 3.70–3.30 (br, 18H), 3.10–2.45 (m, 22H), 2.45–1.80 (m, 154H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.96, 170.46, 169.88, 153.24, 96.82, 84.36, 76.33, 74.15, 80.13, 68.73, 62.45, 42.17, 29.81, 26.38, 21.25, 21.20, 21.00; MS

(MALDI-TOF): $[M + Na]^+$ ($M = C_{72}H_{84}N_{24}O_{24}(C_{14}H_{20}O_9S)_n$): *m/z* 6060.1 (n = 12), 5696.6 (n = 11); 5332.5 (n = 10), 4968.6 (n = 9).

Synthesis of CB[6]-based Glucose Cluster (5).
Methanolic NaOMe (25%) (400 μL) was added to a stirred solution of **4** (0.90 g, 0.15 mmol) in dry MeOH (50 mL), and the reaction mixture was allowed to stand at RT. A precipitate formed during this period of time. After 2 h, the solid was filtered, redissolved in water and neutralized with Amberlite IRC-50 (H^+ form) ion-exchange resin. After filtration, the filtrate was freeze-dried and the crude product was purified by reverse-phase HPLC (Vydac protein/peptide C18, 22 × 250 mm column, 9 mL/min, A: 0.1% trifluoroacetic acid (TFA)/water, B: 0.1% TFA/acetonitrile) to give CB[6]-based glucose cluster **5** (0.51 g, 85%). The isolated product was a mixture of partially substituted **5** with different degree of substitution. The MALDI-TOF mass spectrum of **5** revealed species with 9 – 12 glucose units attached to a CB[6] core. The N/S ratio in elemental analysis suggested that the average degree of substitution is 10.7, which was consistent with the ^{1}H-NMR integration. ^{1}H-NMR (500 MHz, D_2O): δ 5.64 (m, 15H), 4.57 (m, 11H), 4.40 (m, 12H), 3.90 (m, 11H), 3.89–3.60 (m, 35H), 3.60–3.40 (m, 33H), 3.35 (m, 11H), 2.94 (m, 22H), 2.12 (br, 22H); ^{13}C NMR (125 MHz, D_2O): δ 154.12, 96.51, 85.65, 80.38, 77.70, 72.80, 69.99, 64.05, 61.41, 41.68, 30.04, 26.67; MS (MALDI-TOF): $[M + Na]^+$ ($M = C_{72}H_{84}N_{24}O_{24}(C_6H_{12}O_5S)_n$): *m/z* 4044.1 (n = 12), 3848.0 (n = 11), 3652.0 (n = 10), 3456.0 (n = 9); Elemental analysis data were calculated based on the average degree of substitution (n = 10.7). Anal. Calcd. for **5** $[C_{72}H_{84}N_{24}O_{24}(C_6H_{12}O_5S)_{10.7}(H_2O)_{13}]$: C, 40.87; H, 6.00; N, 8.40; S, 8.57. Found: C, 40.60; H, 5.61; N, 8.06; S, 8.25.

Synthesis of CB[6]-based *O*-Acetylgalactose Cluster (6).
6 was synthesized in 77% yield using the same procedure for **4**. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.10–5.55 (br, 15H), 5.45 (br s, 11H), 5.17 (m, 11H), 5.11 (m, 11H), 4.90–4.45 (m, 23H), 4.45–3.95 (m, 33H), 3.80 (br, 24H), 2.77 (m, 22H), 2.17–1.65 (m, 154H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.47, 170.34, 170.10, 169.80, 154.18, 96.28, 83.99, 74.45, 71.80, 67.31, 61.12, 41.47, 29.53, 26.37, 21.08, 20.93, 20.76; MS (MALDI-TOF): $[M + Na]^+$ ($M = C_{72}H_{84}N_{24}O_{24}(C_{14}H_{20}O_9S)_n$): *m/z* 6060.7 (n = 12), 5696.8 (n = 11); 5332.2 (n = 10), 4968.4 (n = 9).

Synthesis of CB[6]-based Galactose Cluster (7).
7 was synthesized in 83% yield using the same procedure for **5**. ^{1}H-NMR (D_2O, 500 MHz): δ 5.64 (m, 15H), 4.51 (m, 11H), 4.40 (br, 12H), 4.01 (s, 11H), 3.95–3.65 (m, 57H), 3.60 (m, 11H), 2.96 (m, 22H), 2.14 (m, 22H); ^{13}C NMR (125 MHz, D_2O): δ 154.15, 96.50, 86.17, 79.34, 74.41, 70.11, 69.18, 64.13, 61.45, 41.71, 30.06, 26.70; MS (MALDI-TOF): $[M + Na]^+$ ($M = C_{72}H_{84}N_{24}O_{24}(C_6H_{12}O_5S)_n$): *m/z* 4044.1 (n = 12), 3848.1 (n = 11), 3652.0 (n = 10), 3455.9 (n = 9); Elemental analysis data were calculated based on the average degree of substitution (n = 11.4). Anal. Calcd. for **7** $[C_{72}H_{84}N_{24}O_{24}(C_6H_{12}O_5S)_{11.4}(H_2O)_{20}]$: C, 39.53; H, 6.16; N, 7.88; S, 8.57. Found: C, 39.23; H, 5.87; N, 8.01; S, 8.72.

Synthesis of CB[6]-based *O*-Acetylmannose Cluster (8).
8 was synthesized in 83% yield using the same procedure for **4**. ^{1}H NMR (500 MHz,

$CDCl_3$): δ 6.10–5.55 (br, 15H), 5.29 (m, 33H), 5.19 (m, 11H), 4.31 (m, 34H), 4.10 (m, 11H), 4.90–3.30 (br, 24H), 2.82 (m, 22H), 2.20–1.80 (m, 154H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 170.66, 169.91, 154.20, 96.31, 84.27, 71.14, 69.54, 66.26, 60.01, 62.49, 41.53, 29.56, 26.36, 21.04, 20.93, 20.76; MS (MALDI-TOF): $[M + Na]^+$ (M = $C_{72}H_{84}N_{24}O_{24}(C_{14}H_{20}O_9S)_n$): *m/z* 6060.5 (n = 12), 5696.6 (n = 11); 5332.5 (n = 10), 4968.3 (n = 9).

Synthesis of CB[6]-based Mannose Cluster (9).

9 was synthesized in 75% yield using the same procedure for **5**. ^{1}H-NMR (D_2O, 500 MHz): δ 5.59 (m, 15H), 5.28 (br s, 11H), 4.42 (m, 12H), 4.02 (m, 11H), 3.80 (m, 11H), 3.90–3.55 (m, 68H), 2.80 (m, 22H), 2.06 (m, 22H); ^{13}C NMR (125 MHz, D_2O): δ 153.94, 96.46, 85.12, 73.76, 72.13, 71.60, 67.36, 64.00, 61.22, 41.48, 29.55, 27.44; MS (MALDI-TOF): $[M + Na]^+$ (M = $C_{72}H_{84}N_{24}O_{24}(C_6H_{12}O_5S)_n$): *m/z* 4043.8 (n = 12), 3848.0 (n = 11), 3651.8 (n = 10), 3456.2 (n = 9); Elemental analysis data were calculated based on the average degree of substitution (n = 11.2). Anal. Calcd. for **9** $[C_{72}H_{84}N_{24}O_{24}(C_6H_{12}O_5S)_{11.2}(H_2O)_{19}]$: C, 39.72; H, 6.14; N, 7.99; S, 8.53. Found: C, 39.54; H, 5.83; N, 8.22; S, 8.82.

2D NMR Experiments.

COSY, ROESY and ^{1}H-^{13}C HSQC experiments were performed to assign the proton and carbon resonances of **5**, **7**, and **9** on a Bruker DRX500 NMR spectrometer operating at the proton Larmor frequency of 500.23 MHz at 298K.

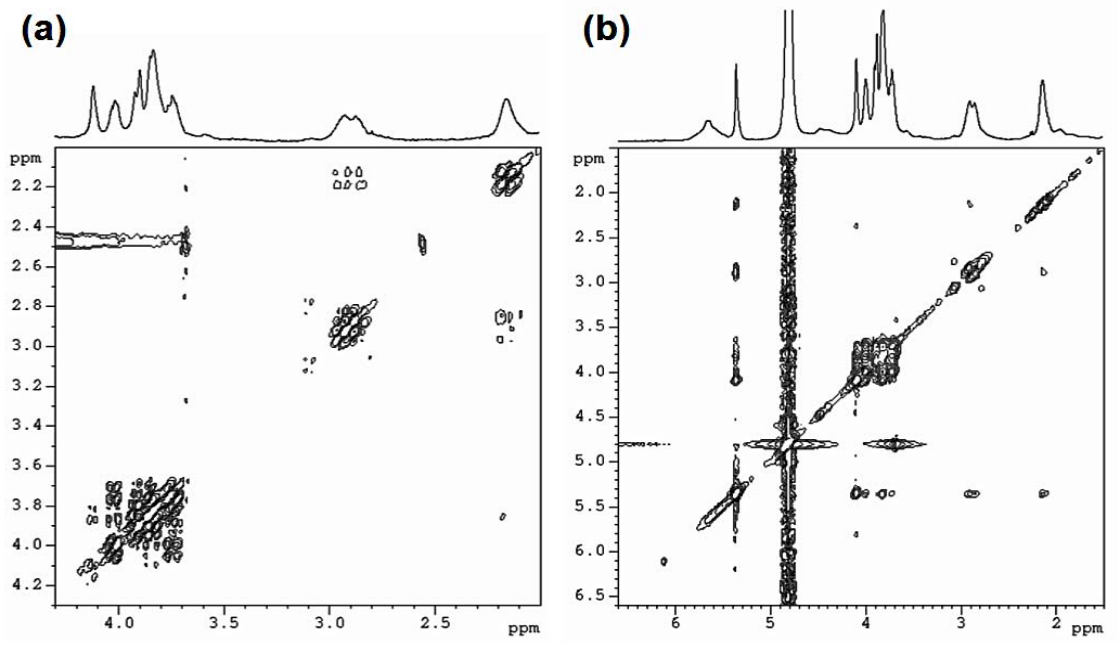

Figure S1. (a) COSY and (b) ROESY NMR spectra of **9**.

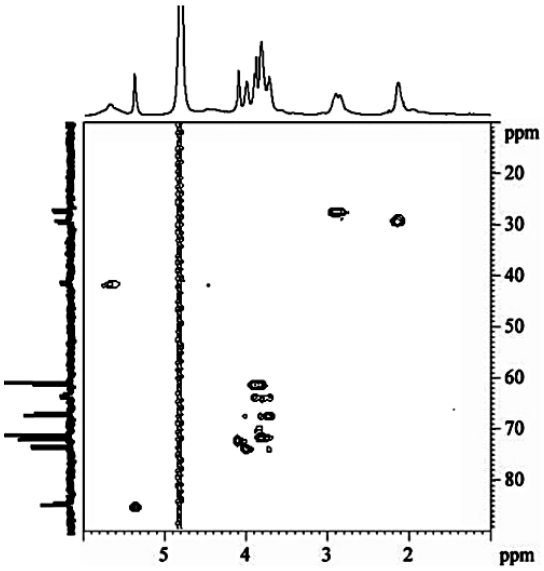

Figure S2. ^{1}H-^{13}C HSQC NMR spectrum of **9**.

Pulsed-field Gradient (PFG) NMR Experiments.

The diffusion coefficient measurements were carried out using a 5 mm Bruker QNP probe with an actively shielded z gradient coil. Diffusion coefficients were extracted from a series of ^{1}H NMR spectra measured by the bipolar pulse longitudinal encode-decode (BPPLED) pulse sequence as a function of gradient amplitude. In each experiment, the gradient duration time was 2.0 or 2.5 ms and the amplitudes of gradient pulses ranged from 1 to 40 G/cm. The diffusion time was 50 to 100 ms. Diffusion coefficients were calculated from the data obtained by 2D diffusion-ordered spectroscopy (DOSY). The diffusion coefficient of **5** was measured to be 1.55×10^{-10} m/s^2, from which the hydrodynamic diameter of **5** was estimated to be 2.6 nm with the Stokes-Einstein equation.

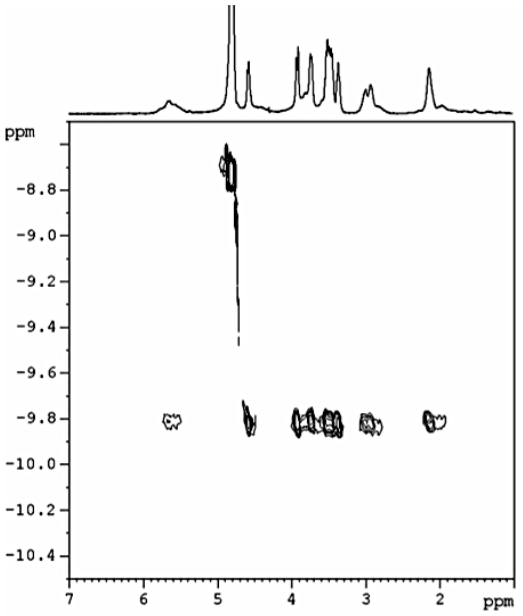

Figure S3. DOSY NMR spectrum of **5**.

Turbidimetric Assay.

Turbidimetric assay was performed on 96-well microtiter plates. Each assay was done in triplicate. For the selective binding test, stock solutions of **5**, **7**, or **9** (128 μM in HBS (0.01 M HEPES with 0.15 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$, pH 7.3)) were used. Each compound was added in serial two-fold dilution (50 μL/well) to 150 μL/well of ConA solution (2.7 mg/mL in HBS) on microtiter plates and incubated at RT for 10 min. The turbidity of the solutions was monitored by measuring the optical density (OD) at 405 nm (Figure S4).

To monitor the turbidity change over time, a solution of ConA (100 μL/well, 4.1 mg/mL in HBS) was mixed with solutions of **5**, **7**, or **9** (50 μL/well, 29 μM in HBS). The total volume of each well was brought to 200 μL by addition of 50 μL of HEPES buffer. The solutions were then incubated at RT and the turbidity was monitored by reading the OD at 405 nm for 3 h.

For the inhibition test, a solution of ConA (100 μL/well, 4.1 mg/mL in HBS) was mixed with a solution of **9** (50 μL/well, 29 μM in HBS) and incubated at RT for 3 h. To the mixed solution was added 0.32, 3.2, or 32 mM Me-αMan (50 μL/well), which corresponds to an 11-, 110-, or 1100-fold molar amount to the mannose cluster and an 1-, 10-, or 100-fold molar amount to the mannose on the CB[6] (CB[6] has an average of ~11 mannose groups). The change in turbidity of the solutions was monitored by reading the OD at 405 nm for 1 h.

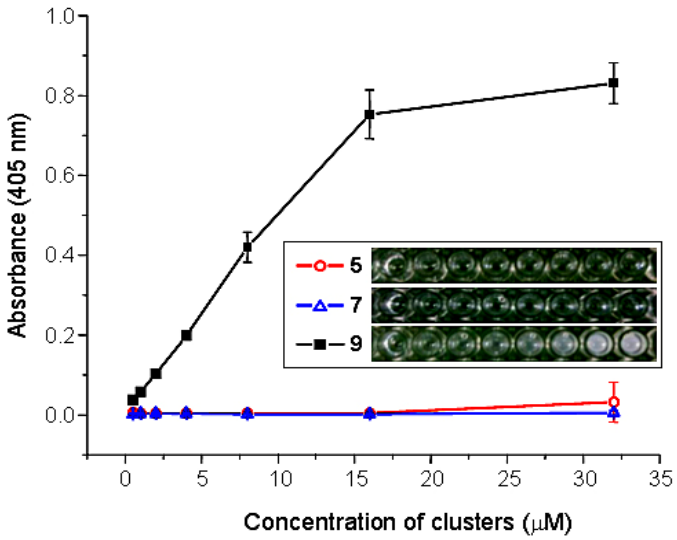

Figure S4. Turbidimetric assay of binding of **5**, **7**, and **9** to ConA.

Turbidimetric Job Plot.[3]

Stock solutions of ConA (100 μM as monomer) and **9** (100 μM) in buffer (0.1 M sodium acetate with 0.1 M NaCl, 5 mM each of $CaCl_2$ and $MnCl_2$, pH 5.2) were prepared. For a Job plot, the stock solution of ConA and **9** were mixed in twelve different ratios from 1:0 to 0:1 in a total volume of 200 μL. After 3 h, the turbidity was measured by reading OD at 405 nm. The resulting Job plot is shown in Figure S5. The maximum OD was observed at a ConA fraction of 0.75, indicating the binding stoichiometry of ConA to **9** is 3:1.

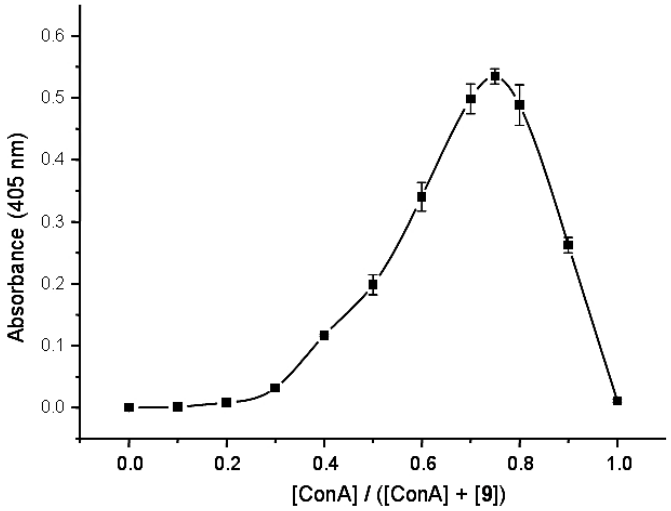

Figure S5. Job plot of ConA binding to **9**.

Isothermal Titration Calorimetry (ITC).[4]

ITC experiments were carried out on a VP-ITC calorimeter from Microcal following the well-established protocol in the literature.[4] A ConA solution (0.030 mM) in buffer (0.1 M sodium acetate with 0.1 M NaCl, 5 mM each of $CaCl_2$ and $MnCl_2$, pH 5.2) was placed in the reaction cell (volume = 1.398 mL). The concentration of ConA was determined spectrophotometrically at 280 nm using $A^{1\%,\ 1cm}$ = 12.4 at pH 5.2 and expressed in terms of monomer (M_r = 25,600). Sixteen injections of **9** solution (each injection, 17 μL, 0.10 mM) were added from a 250-μL microsyringe at an interval of 4 min into the ConA solution with stirring 459 rpm at 27 ^oC. For comparison, a similar experiment was performed with Me-αMan. Twenty injections of Me-αMan solution (each injection, 7 μL, 2.1 mM) were added into a ConA solution (0.125 mM). The data were fitted to a theoretical titration curve (Figure S6) using software supplied by Microcal, with ΔH (kcal mol^{-1}), K_a (M^{-1}) and n (number of binding sites per ConA monomer) as adjustable parameters. The thermodynamic parameters associated with the binding are given in Table S1.

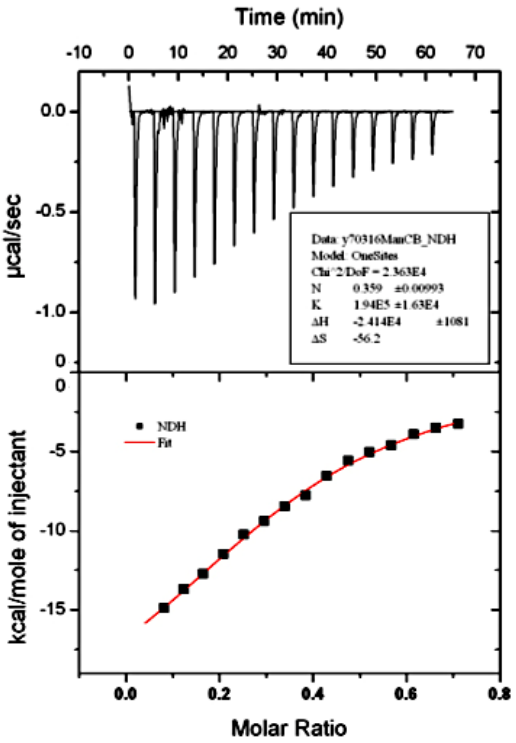

Figure S6. ITC profile of ConA (0.030 mM) with **9** (0.10 mM) at 27 °C.

Table S1. Thermodynamic binding parameters for ConA with Me-αMan and **9** at 27 °C.

	K_a	$-\Delta G$	$-\Delta H$	$-T\Delta S$	n
	M^{-1}		kcal/mol		No. of binding sites /ConA monomer
Me-αMan	$(7.6 \pm 0.8) \times 10^3$	5.3 ± 0.1	8.0 ± 0.7	2.7 ± 0.7	1.00 ± 0.06
9	$(1.9 \pm 0.2) \times 10^5$	7.2 ± 0.1	24 ± 1	17 ± 1	0.36 ± 0.01

Preparation of 10@5, 10@7 and 10@9.
To a solution of **5**, **7**, or **9** (4.1 mg) in water (1.0 mL), **10** (0.77 mg, 1.1 equiv.) was added, and the resulting solution was gently shaken for 2 h and dialyzed using Spectra/Por® cellulose membrane (MWCO: 1,000) for 1 d to remove unbound **10**. The formation of the complexes **10**@**5**, **10**@**7** and **10**@**9** was confirmed by ^{1}H NMR spectroscopy. The proton signals of the butyl unit (spermine) of **10** were shifted upfield from 3.1 and 1.7 ppm to 2.4 and 0.2 ppm, respectively, which indicated that the butyl unit of **10** was located inside the CB[6] cavity upon formation of the inclusion complexes.

Intracellular Translocation Experiments.
HepG2 cells were seeded on a poly-lysine coated cover glass in a 24-well plate at a density of 5×10^4 cells per well in DMEM medium containing 10% FBS and 1% penicillin/streptomycin (PS) and incubated in a humidified 5% CO_2 atmosphere at 37 °C for 24 h. The culture medium was replaced with 180 µL of DMEM medium including 20 µL (1.0×10^{-5} M) of **10**, **10**@**5**, **10**@**7**, **10**@**9**, or just 20 µL of distilled water. The

cells were incubated for 1 h at 37 °C. The incubated cells were washed three times with DMEM medium, fixed with 1% (w/v) *para*-formaldehyde solution and examined by a confocal laser scanning microscope using an excitation wavelength of 488 nm. To understand the mechanism of the intracellular translocation, the same experiments were performed at 4 ^{o}C, instead of 37 ^{o}C.

References

[1] S. Y. Jon, N. Selvapalam, D. H. Oh, J.-K. Kang, S.-Y. Kim, Y. J. Jeon, J. W. Lee, K. Kim, *J. Am. Chem. Soc.* **2003**, *125*, 10186.

[2] a) D. A. Fulton, J. F. Stoddart, *J. Org. Chem.* **2001**, *66*, 8309; b) P. L. Durette, T. Y. Shen, *Carbohydr. Res.* **1980**, *81*, 261.

[3] K. A. Connors, *Binding Constants*, John Wiley & Sons, New York, **1987**.

[4] a) T. K. Dam, R. Roy, S. K. Das, S. Oscarson, C. F. Brewer, *J. Biol. Chem.* **2000**, *275*, 14223; b) J. B. Corbell, J. J. Lundquist, E. J. Toone, *Tetrahedron Asy.* **2000**, *11*, 95.