Carbohydrate Research 346 (2011) 1792–1800

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Preparation, characterization, and biological evaluation of $6^{I},6^{IV}$-di-*O*-[α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-cyclomaltoheptaose and 6-*O*-[α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-cyclomaltoheptaose

Takatoshi Nakagawa [a], Yuki Nishi [b], Akihiro Kondo [c], Yuko Shirai [b], Chie Honda [b], Michio Asahi [a], Toshiko Tanimoto [b,*]

[a] Department of Pharmacology, Faculty of Medicine, Osaka Medical College, 2-7 Daigaku-machi, Takatsuki 569-8686, Japan
[b] School of Pharmaceutical Sciences, Mukogawa Women's University, 11-68 Koshien Kyuban-Cho, Nishinomiya 663-8179, Japan
[c] Department of Molecular Diagnostics and Informatics, Osaka University Graduate School of Medicine, 1-7 Yamadaoka, Suita 565-0871, Japan

ARTICLE INFO

Article history:
Received 12 March 2011
Received in revised form 31 May 2011
Accepted 8 June 2011
Available online 15 June 2011

Keywords:
α-L-Fucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl-βCD
Nuclear magnetic resonance
Real-time cell sensing

ABSTRACT

$6^{I},6^{IV}$-Di-*O*-[α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-cyclomaltoheptaose (βCD) {$6^{I},6^{IV}$-di-*O*-[α-L-Fuc-(1→6)-β-D-GlcNAc]-βCD (**5**)} and 6-*O*-[α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-βCD {6-*O*-[α-L-Fuc-(1→6)-β-D-GlcNAc]-βCD (**6**)} were chemically synthesized using the corresponding authentic compounds, bis(2,3-di-*O*-acetyl)-pentakis(2,3,6-tri-*O*-acetyl)-βCD as the glycosyl acceptor and 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl-(1→6)-3,4-di-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-D-glucopyranosyl trichloroacetimidate as the fuco-glucosaminyl donor. NMR confirmed that α-L-Fuc-(1→6)-D-GlcNAc was bonded by β-linking to the βCD ring. To evaluate biological efficiency, the biological activities of the new branched βCDs were examined. The cell detachment activity of **5** was lower than that of **6** in real-time cell sensing (RT-CES) assay, indicating that **5** has lower toxicity. In SPR analysis, **5** had a higher special binding with AAL, a fucose-recognizing lectin. These results suggest that **5** could be an efficient drug carrier directed at cells expressing fucose-binding proteins.

1. Introduction

Cyclomaltooligosaccharides (cyclodextrins, CDs) can form inclusion complexes by taking various compounds into their interior cavities.[1] In addition to the natural, cyclomaltohexaose (αCD), cyclomaltoheptaose (βCD), and cyclomaltooctaose (γCD), variously modified CDs, that is, by alkylation and glycosylation, have been synthesized to modulate their physical, chemical, and biological characteristics. Among them, mono- or oligosaccharide-branched CDs have been documented for their potential as carriers of drug delivery systems.[2,3] In particular, positional isomers of di-branched glycosyl CDs, as with the glycoside cluster effect, are thought to possess molecular recognition characteristics arising from differences in their substitution positions.[4,5] We have chemically[6–10] and enzymatically[11–15] synthesized various homogeneous and heterogeneous branched CDs. The water solubilities of branched βCD derivatives linked with mono- or oligo-saccharides are approximately 50–100 times that of βCD.[16] Thus, branched βCD inclusion complexes can be used to increase the bioavailability of insoluble and unstable drugs.[2,3]

The α-L-fucopyranosyl residue has been observed in many glycolipids and glycoproteins.[17] We have recently reported the chemical synthesis of branched βCDs having either α-L- or β-L-fucopyranose (Fuc), which were recognized by the fucose-binding lectin, *Aleuria aurantia* lectin (AAL), as with fucosylated glycolipid and glycoproteins.[18,19] The binding affinity to AAL was $6^{I},6^{IV}$- > $6^{I},6^{III}$- > $6^{I},6^{II}$- di-*O*-(L-Fuc)-βCD, in both α- and β-configurations.[18,19] The hemolytic activities of these fucosylated βCD were lower than those of the native βCD.

An α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucopyranose [α-L-Fuc-(1→6)-D-GlcNAc] structure at the reducing end of N-glycan, called core-fucose, is involved in various biological processes.[20–22] Ablation of the core-fucose structure in mice leads to growth retardation and early death after birth.[23,24] Aberrant core-fucosylation has been documented in various tumors, that is, hepatocellular carcinoma and pancreatic carcinoma.[21–25] Though the core-fucose structure is enzymatically catalyzed by fucosyltransferase VIII in vivo, branched CDs having the α-L-Fuc-(1→6)-D-GlcNAc structure have not yet been synthesized chemically.

* Corresponding author. Tel.: +81 798 45 9950.
E-mail address: tanimoto@mukogawa-u.ac.jp (T. Tanimoto).

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doi:10.1016/j.carres.2011.06.013

Real-time cell sensing (RT-CES) is a noninvasive and sensitive technique for detecting cell adhesion, proliferation, and morphological changes. Higher cell adhesion is recorded as higher impedance, called the Cell Index. Generally, time courses of overall impedance during the biological processes are recorded for time intervals and are attributed to various cell behaviors, that is, growth, spreading, rounding, and detachment.

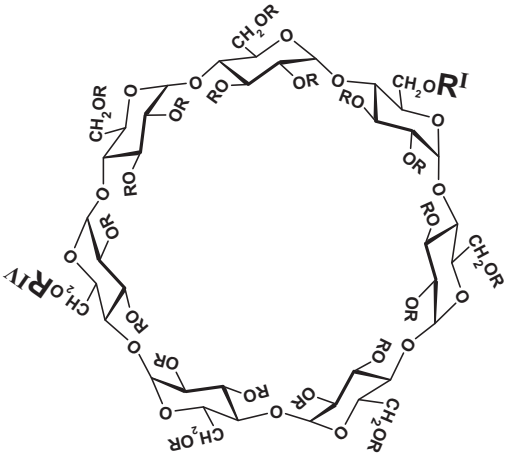
Here, we report the chemical synthesis of two new branched βCDs, $6^{I},6^{IV}$-di-*O*-[α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-βCD {$6^{I},6^{IV}$-di-*O*-[α-L-Fuc-(1→6)-β-D-GlcNAc]-βCD (**5**)} and 6-*O*-[α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-βCD {6-*O*-[α-L-Fuc-(1→6)-β-D-GlcNAc]-βCD (**6**) (Chart 1). RT-CES was used to measure their biological activities. In addition, surface plasmon resonance analysis was carried out to evaluate the binding abilities of **5** and **6** with AAL, a fucose-recognizing lectin.

We found higher specific binding to AAL and lower toxicity on comparing **5** with **6**, and discuss the biological efficiency of these new branched βCDs.

2. Results and discussion

2.1. Preparation of 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl-(1→6)-3,4-di-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-D-glucopyranosyl trichloroacetimidate (13)

For the synthesis of 6-*O*-(*tert*-butyldimethylsilyl)-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-α,β-D-glucopyranose (**8**), the free amine of D-glucosamine and the 6-*O*-hydroxyl group were protected with 2,2,2-trichloroethoxycarbonyl (Troc)[26,27] and *tert*-butyldimethylsilyl (*t*-$BuMe_2Si$) groups, respectively. Acetylation of **8** with Ac_2O–pyridine gave 6-*O*-(*tert*-butyldimethylsilyl)-1,3,4-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino) α-D-glucopyranose (**9α**) as crystals, then the crystalline 6-*O*-(*tert*-butyldimethylsilyl)-1,3,4-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranose (**9β**) was obtained from the filtrate at the **9α**:**9β** ratio of 7.7:1. O-Desilylation **9α** and **9β** with 50% acetic acid produced 1,3,4-tri-*O*-acetyl-6-*O*-(*tert*-butyldimethylsilyl)-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranose (**10α**) and 1,3,4-tri-*O*-acetyl-6-*O*-(*tert*-butyl-dimethylsilyl)-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranose (**10β**), respectively. Glycosylation of **10α** with 2,3,4-tri-*O*-benzyl-L-fucopyranosyl trichloroacetimidate[23] as the fucosyl donor in dichloromethane in the presence of trimethylsilyl trifluoromethanesulfonate (TMSOTf) as an acid catalyst gave disaccharide, 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl-(1→6)-1,3,4-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranose (**11α**) and 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl-(1→6)-1,3,4-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranose (**11β**) with **11α**:**11β** ratio of 2.2:1. Selective 1-O-deacetylation of **11α** with hydrazine acetate in *N*,*N*-dimethylformamide afforded 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl-(1→6)-3,4-di-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-D-glucopyranose (**12**). 2,3,4-Tri-*O*-benzyl-α-L-fucopyranosyl-(1→6)-3,4-di-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-D-glucopyranosyl trichloroacetimidate (**13**) was obtained by treatment of **12** with trichloroacetonitrile in the presence of 1,8-diazabicyclo[5,4,0] undec-7-ene (DBU) in dichloromethane (Scheme 1).



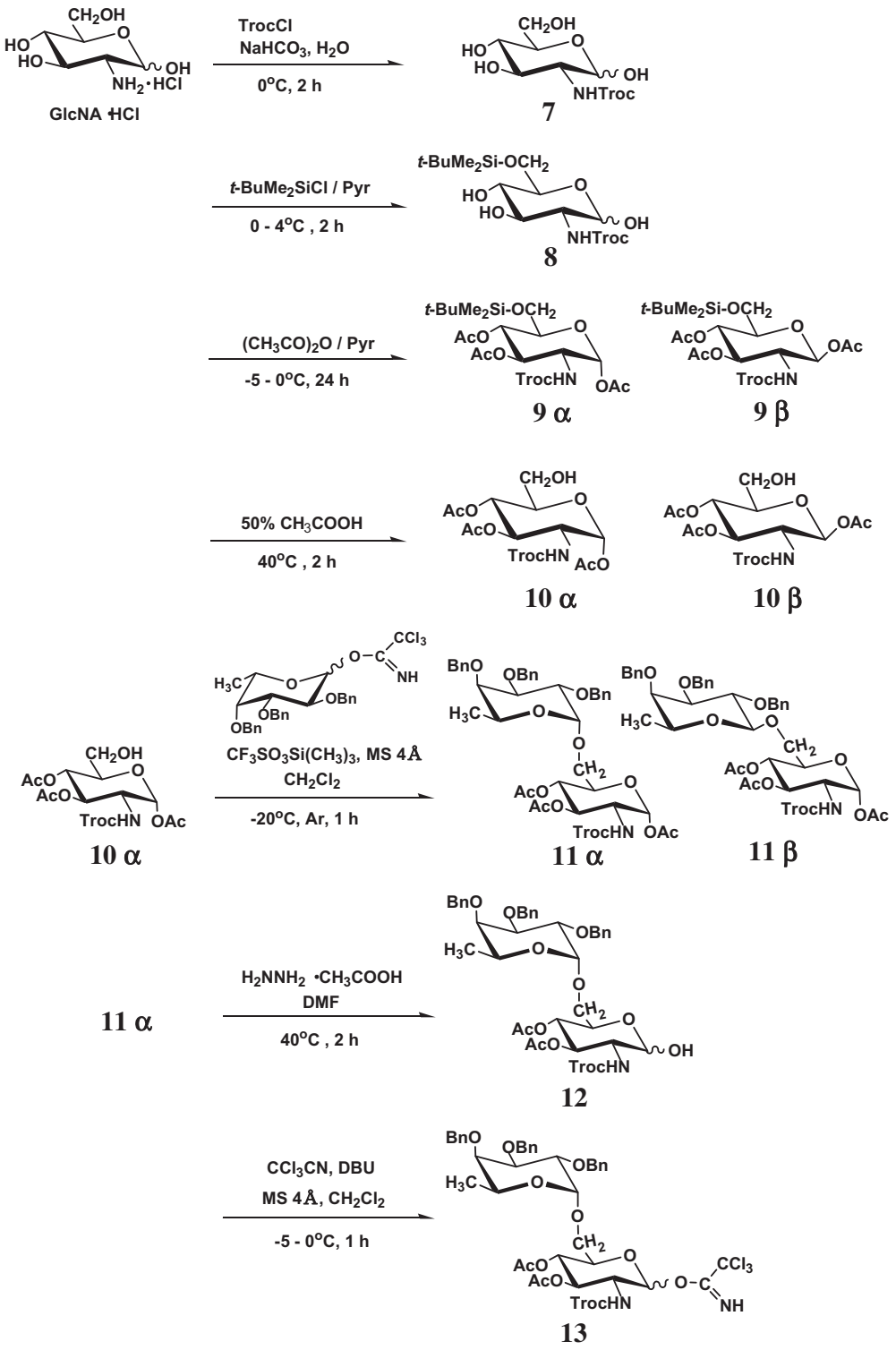
	R^{I}	R^{IV}	R
1	Si	Si	H
2	H	H	Ac
3	α-Fuc-GlcNAc	α-Fuc-GlcNAc	H
4	α-Fuc-GlcNAc	H	H
5	α-Fuc-β-GlcNAc	α-Fuc-β-GlcNAc	H
6	α-Fuc-β-GlcNAc	H	H

Si: *tert*-butyldimethylsilyl
α-Fuc-GlcNAc: α-L-Fuc-(1→6)-D-GlcNAc
α-Fuc-β-GlcNAc: α-L-Fuc-(1→6)-β-D-GlcNAc

Chart 1.

2.2. Preparation, separation and characterization of $6^{I},6^{IV}$-di-*O*-[α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-βCD (5) and 6-*O*-[α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-βCD (6)

To synthesize $6^{I},6^{IV}$-dibranched βCD, the glycosyl acceptor, bis(2,3-di-*O*-acetyl)-pentakis(2,3,6,tri-*O*-acetyl)-βCD (**2**), was obtained from $6^{I},6^{IV}$-di-*O*-(*t*-$BuMe_2Si$)-βCD peracetate (**1**), whose structure had been established in a previous study.[28,29] Glycosylation of **2** with **13** in dichloromethane in the presence of TMSOTf as an acid catalyst gave a dibranched βCD derivative having two disaccharides and a monobranched βCD derivative having one disaccharide. These compounds were fractionated by centrifugal chromatography. In the compounds of each fraction, the *N*-Troc group was replaced by the *N*-acetyl group with activated zinc powder in acetic acid/Ac_2O/tetrahydrofuran (1:1:1), and the product was hydrogenolyzed with 10% Pd–C in 10% formic acid–MeOH and O-deacetylated with methanolic sodium methoxide. $6^{I},6^{IV}$-Di-*O*-[α-L-fucopyranosyl-(1→6)-D-glucopyranosyl]-βCD {$6^{I},6^{IV}$-di-*O*-[α-L-Fuc-(1→6)-D-GlcNAc]-βCD} (**3**) and 6-*O*-[α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucopyranosyl]-βCD {6-*O*-[α-L-Fuc-(1→6)-D-GlcNAc]-βCD} (**4**) were purified by HPLC on a TSKgel Amide-80 column and Daisopak SP-ODS-BP columns (Scheme 2). High-resolution mass spectroscopic analysis of **3** (*m/z* 1833.6530 $[M+H]^+$) and **4** (*m/z* 1484.5151 $[M+H]^+$) indicated the molecular formulas of dibranched βCD and monobranched βCD, respectively. NMR analysis was performed using ^1H–^1H COSY, ^1H–^{13}C COSY, homo decoupling, and nuclear Overhauser effect (NOE) difference spectra, and all carbons in the spectrum of **4** were completely assigned (Fig. 1). The H-1 signal of GlcNAc was observed at δ 4.58, $J_{1,2}$ 8.20 Hz. Therefore, it was confirmed that α-L-Fuc-(1→6)-GlcNAc was bonded by β-linking to the CD ring. These results indicated that **3** and **4** were $6^{I},6^{IV}$-di-*O*-[α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-βCD (**5**) and 6-*O*-[α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-βCD (**6**), respectively. Figure 2 shows the elution profiles of compounds **5**, **6**, and βCD on a

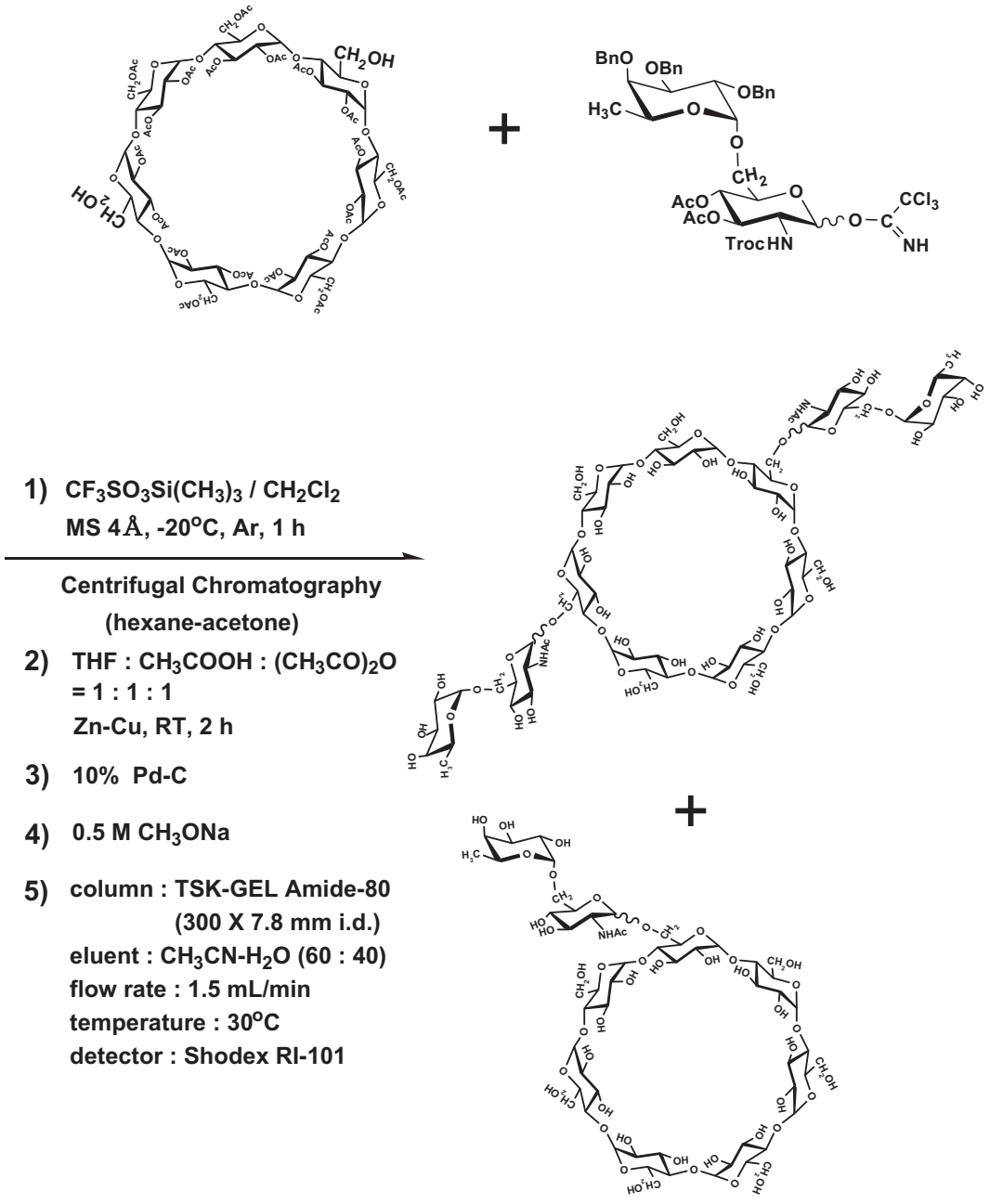


Scheme 1.

LiChroCART NH_2 column and on an ODS column XTerra RP_{18}. The difference in the separation mechanisms has been described in previous papers.[30–33]

2.3. Real-time cell sensing (RT-CES) analysis

The biological activities of $6^{I},6^{IV}$-di-*O*-[α-L-Fuc-(1→6)-D-GlcNAc]-βCD (**5**) and 6-*O*-[α-L-Fuc-(1→6)-D-GlcNAc]-βCD (**6**) were evaluated using RT-CES. Compared to hemolytic assay which is often utilized for biological cytotoxicity of CDs, RT-CES enables monitoring of the effects of CDs on cells in real time and evaluation of the short-time effects of CDs. RT-CES has been applied to the monitoring of cell adhesion, cell proliferation, and cell death.[34–37] HepG2 cells from a human hepatoma cell line were chosen as target cells. First, we monitored the biological activities of various methylated βCDs for 220 min in order to validate the usefulness of RT-CES (Fig. 3A). Changes of cell status, that is, adhesion, morphology, cell number, on the electrode were recorded as the 'Cell Index'.



Scheme 2.

For example, a decrease of the 'Cell Index' would indicate loss of cell adhesion, reduction of cell number, and cell shrinkage. As the most pronounced action of CDs on cells is cell-detaching activity partly via cholesterol depletion, we referred to the 'Cell Index' as 'Cell detachment activity' (Fig. 3). 2,6-Dimethyl-βCD (DMβCD) showed the highest cell detachment activity, with more than 50% of the cells becoming detached within 20 min at 5 mM (Fig. 3A). Methyl-βCD (MβCD) and 2,3,6-trimethyl-βCD (TMβCD) were similarly bio-active at 5 mM, but not as much (Fig. 3A); 1 mM of DMβCD was comparable to 5 mM of TMβCD. Collectively, the order of cell detachment activity was DMβCD ≫ MβCD > TMβCD > βCD, which was consistent with the hemolytic activity[38] as well as the water-solubility[39] of these βCDs. In addition, as early as 20 min after incubation with βCDs, the difference in cytotoxicity of these CDs became visible in RT-CES, suggesting that RT-CES can be an alternative assay for evaluating CD biological activity. Next, two compounds, **5** and **6**, were added to HepG2 cells at 5 mM (Fig. 3B) and at 10 mM (Fig. 3C), and the mixture was incubated for 180 min. DMβCD at 5 mM was used as a positive control. At 5 mM, the cell detachment activities of the two resulting products were almost equal, and were lower than that of βCD. In contrast, the cell detachment activity of **5** was slightly higher than that of **6** at 10 mM. Initially, we hypothesized that 6^{I},6^{IV}-linked βCD would exhibit higher cell detachment activity, because we thought that a higher branching number would facilitate the binding of this CD to the cell surface, which in turn would enhance CD lipid depleting activity. However, the data showed the opposite result. Collectively, RT-CES successfully evaluated the cell detachment activity of the two new branched-βCDs. The lower cell detachment activity of 6^{I},6^{IV}-di-branched βCD **5** compared to mono-branched βCD **6** suggests that **5** might be a safer drug carrier.

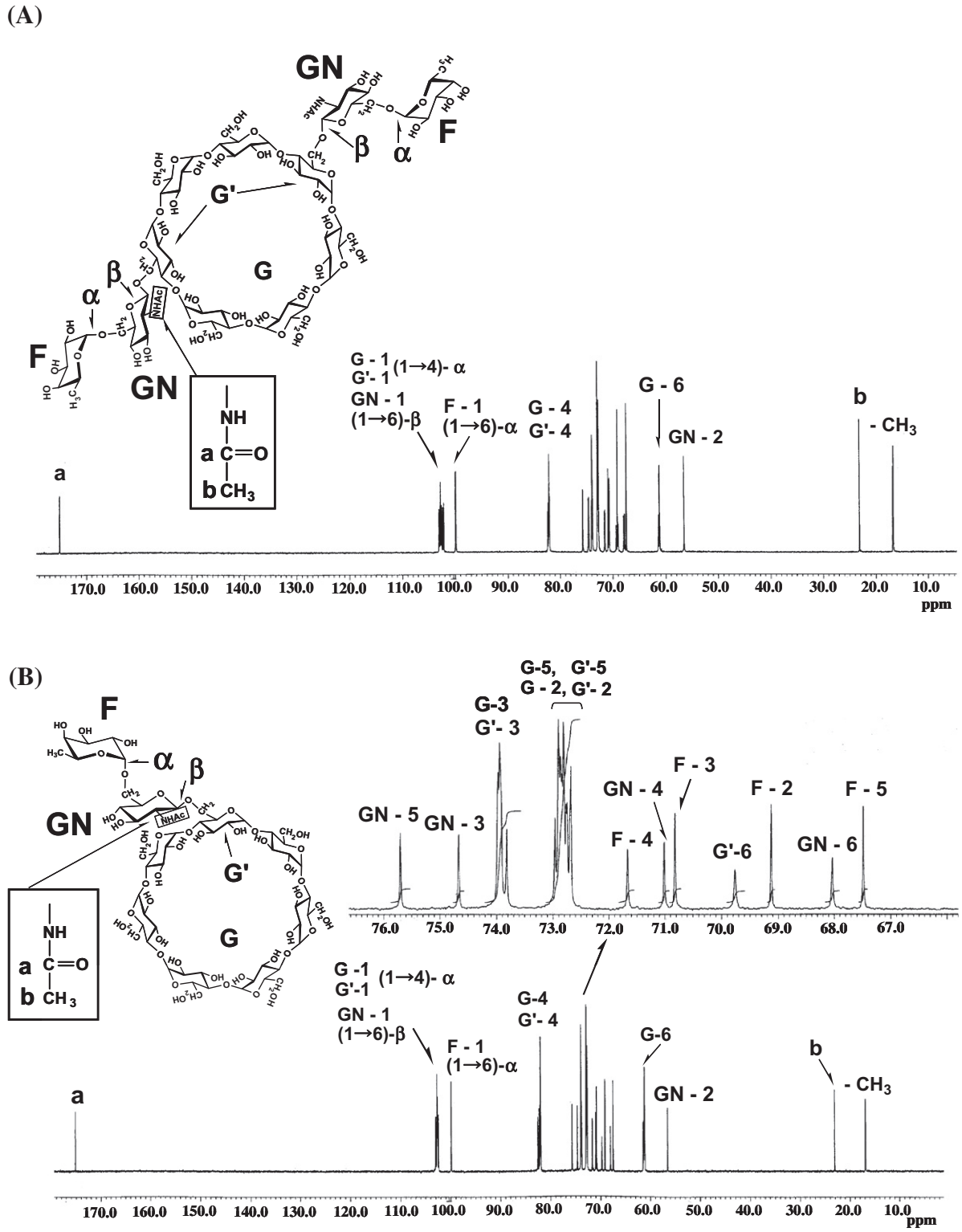


Figure 1. ^{13}C NMR spectra of (A) $6^{I},6^{IV}$-di-*O*-[α-L-Fuc-(1→6)-β-D-GlcNAc]-βCD (**5**) and (B) 6-*O*-[α-L-Fuc-(1→6)-β-D-GlcNAc]-βCD (**6**) measured in D_2O at 125 MHz.

2.4. Interactions of α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-βCD with AAL

AAL is a well-known fucose-specific lectin. We previously reported that β- and α-L-fucopyranosy-βCDs interact specifically with AAL lectin.[18,19] Because AAL has been reported to prefer the α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-*gluco*-pyranosyl structure, the so-called core-fucose, two α-1,6-linked fucosylated βCDs were evaluated for their affinity against AAL by the SPR method. The kinetic and equilibrium constants were listed in Table 1. As expected, the binding affinity of $6^{I},6^{IV}$-di-*O*-[α-L-Fuc-(1→6)-D-GlcNAc]-βCD (**5**) for AAL was much higher than that of 6-*O*-[α-L-Fuc-(1→6)-D-GlcNAc]-βCD (**6**) (*Ka* (M^{-1}) 21340 ± 0.19 vs 3570 ± 0.04).

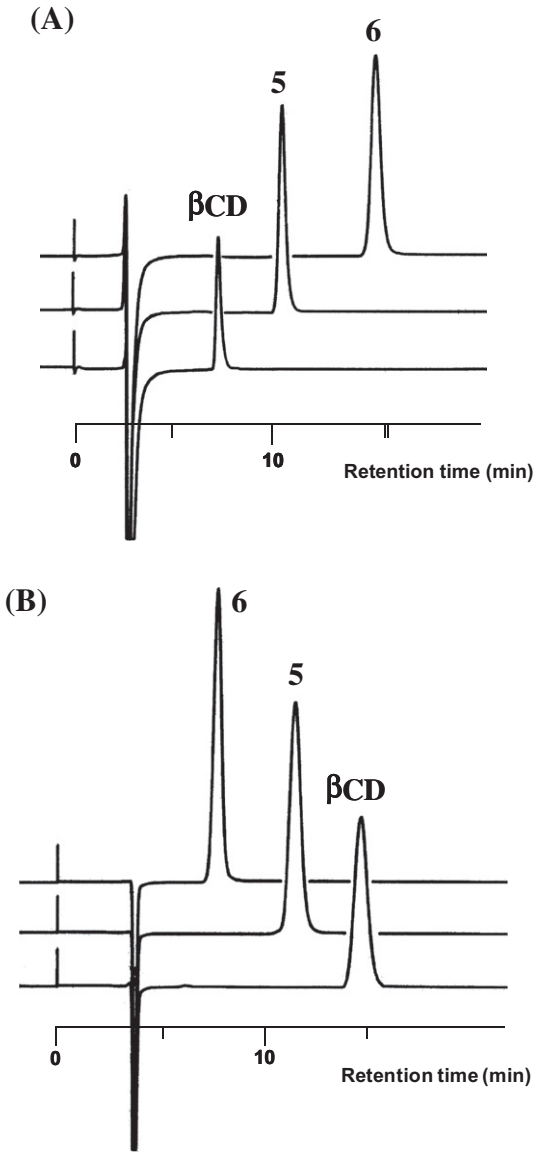


Figure 2. Elution profiles of $6^{I},6^{IV}$-di-*O*-[α-L-Fuc-(1→6)-β-D-GlcNAc]-βCD (**5**) and 6-*O*-[α-L-Fuc-(1→6)-β-D-GlcNAc]-βCD (**6**), and βCD. Chromatographic conditions: detector, Shodex RI-101; temperature, 30 °C; (A) column, LiChroCART NH_2 (250 × 4.6 mm i.d.); eluent, acetonitrile–water (62:38, v/v); flow rate, 1.0 mL/min, (B) column, XTerra RP_{18} (150 × 4.6 mm i.d.); eluent, MeOH–water (10:90, v/v); flow rate, 0.5 mL/min.

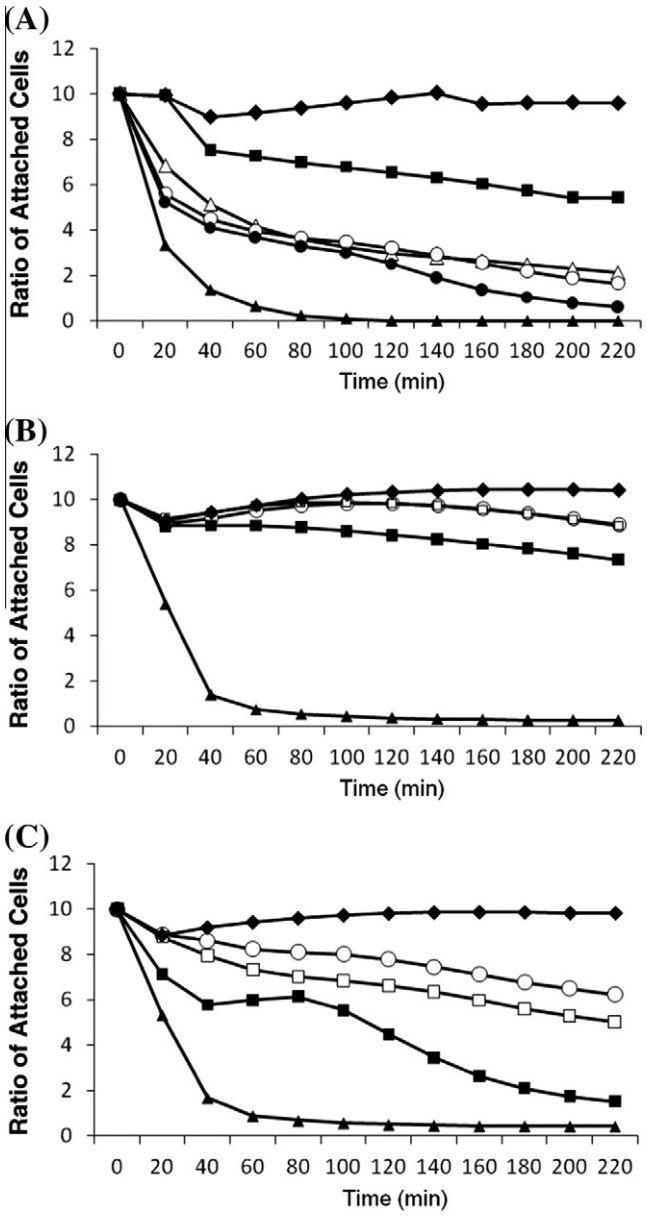


Figure 3. Real time cell sensing (RT-CES) assay using HepG2 cells. (A) RT-CES profiles of HepG2 cells in the presence of 1 and 5 mM βCD, and 5 mM of dimethyl-βCD (DMβCD), trimethyl-βCD (TMβCD), and methyl-βCD(MβCD). ■: 5 mM βCD, △: 1 mM DMβCD, ▲: 5 mM DMβCD, ○: 5 mM TMβCD, ●: 5 mM MβCD, ♦: blank. (B) RT-CES profiles of HepG2 cells in the presence of 5 mM of $6^{I},6^{IV}$-di-*O*-[α-L-Fuc-1→6)-β-D-GlcNAc]-βCD (**5**) and 6-*O*-[α-L-Fuc-(1→6)-β-D-GlcNAc]-βCD (**6**), βCD, and DMβCD. (C) RT-CES profiles of HepG2 cells in the presence of 10 mM of **5**, **6**, βCD, and 5 mM of DMβCD. RT-CES values are expressed as ratio of attached cells, which at time 0 are converted to 10 on a scale of 1–10. ○: 5 mM (B), 10 mM (C) **5**, □: 5 mM (B), 10 mM (C) **6**, ■: 5 mM (B), 10 mM (C) βCD, ▲: 5 mM DMβCD, ♦: blank.

We have reported the synthesis of $6^{I},6^{n}$-di-*O*-(α-L-Fuc)-βCD (n = II–IV), with comparison in terms of the fucose-binding position.[19] The results showed that α-L-Fuc-βCDs interacted with AAL with the binding affinities in the following order: $6^{I},6^{IV}$- > $6^{I},6^{III}$- > $6^{I},6^{II}$-di-*O*-α-L-Fuc-βCD. $6^{I},6^{IV}$-Di-branched βCD showed higher affinity than the other corresponding positional isomers. The binding affinity of **5** for AAL which was obtained this time, was strong compared to $6^{I},6^{IV}$-di-*O*-α-L-Fuc-βCD, *Ka* (M^{-1}), 21340 ± 0.19 (**5**) vs 18350 ± 0.10, respectively (Table 1). To circumvent the side effects of drugs, a cell-type-specific drug delivery system has been desired for a long time. Given that one such system relies on the application of a specific interaction between oligosaccharides and cell surface lectin,[40] CDs modified with specific structures of oligosaccharides would be good candidates as a drug carrier offering cell-type-specific drug delivery. Here, we have shown that **5** can bind to AAL, a fucose specific lectin, in a highly specific manner. This

Table 1
Kinetic and equilibrium constants of $6^{I},6^{IV}$-di-*O*-[α-L-Fuc-(1→6)-β-D-GlcNAc]-βCD and 6-*O*-[α-L-Fuc-(1→6)-β-D-GlcNAc]-βCD

Compound	Affinity constant *Ka* (M^{-1})
$6^{I},6^{IV}$-di-*O*-[α-L-Fuc-(1→6)-β-D-GlcNAc]-βCD	21340 ± 0.19
6-*O*-[α-L-Fuc-(1→6)-β-D-GlcNAc]-βCD	3570 ± 0.04
$6^{I},6^{IV}$-di-*O*-[α-L-Fuc]-βCD	18350 ± 0.10[a]

Data are expressed as mean ± SE.
[a] Our previous result.[24]

newly synthesized CD may serve as one of the specific drug-carriers to target cells and tissues harboring fucose-lectin-like molecules.

3. Experimental

3.1. General methods

HPLC was conducted with a PU-980 pump (Jasco) or a LC-10AD pump (Shimadzu), a Rheodyne 7125 injector, and a Shodex RI-71 or RI-101 refractive index monitor (Showa Denko), or a RI-2031 refractive index monitor (Jasco). HPLC analysis was conducted at constant temperature using a column oven CA-202 (Flom) or CTO-20AC (Shimadzu). The columns employed were YMC-Pack SH-343-7 ODS (250 × 20 mm i.d.), Daisopak SP-120-5-ODS-BP (250 × 10 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 Waters XTerra RP_{18} (150 × 4.6 mm i.d.). ^1H and ^{13}C NMR spectroscopic data were recorded for solutions in D_2O or $CDCl_3$, using a Jeol GSX-500 or Jeol JNM-ECP 500 spectrometer (^1H: 500 MHz, ^{13}C: 125 MHz). Chemical shifts are expressed in ppm downfield from the Me_4Si signal, with reference to external 1,4-dioxane (67.4 ppm). The conditions for ^1H–^1H COSY and ^1H–^{13}C COSY measurements were the same as those described in a previous paper.[41] HRFABMS was measured in positive-ion mode using a Jeol MS 700 mass spectrometer with xenon atoms, using glycerol as the matrix. MALDITOFMS was carried out on a Vision 2000 instrument (Thermo Bioanalysis), with 2,5-dihydroxybenzoic acid as the matrix. The instrument was operated in positive-ion reflection 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). Melting points were measured using a Yanagimoto micro melting-point apparatus and are uncorrected. A UVIDEC V-530 double beam spectrophotometer (Jasco) was used for determination of absorbances. Centrifugal chromatography was performed with a Harrison Centrifugal Thin-Layer Chromatotron 7924.

3.2. Real-time cell electronic sensing (RT-CES)

The cell detachment activity was measured with RT-CES. HepG2 cells were plated into wells of 16-well devices compatible with a W200 real-time cell electronic sensing (RT-CES) analyzer and 16× E plate station (Acea Biosciences, San Diego, CA). After 24 h, the medium was changed to serum-free DMEM, and HepG2 cells were cultured for another 24 h. Next, HepG2 cells were incubated with the indicated concentrations of CDs. The activity was monitored every 20 min for indicated durations via calculation of the 'Cell Index' (normalized impedance) for each well. Unless otherwise stated, experiments were replicated several times.

3.3. Surface plasmon resonance (SPR) analysis

3.3.1. Immobilization of AAL on an optical biosensor cuvette

AAL was immobilized as described previously.[18,19] Briefly, after equilibration and stabilization of the baseline with phosphate buffer saline (PBS, l0 mM KH_2PO_4/Na_2HPO_4, 0.15 M NaCl, pH 7.2), the optical biosensor cuvette coated with carboxylate was activated with a mixture of 400 mM l-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and 100 mM *N*-hydroxysuccinimide for 7 min. After washing with PBS, and 50 μL of AAL solution (1 mg/mL, in 5 mM maleate buffer, pH 6.0) was added. After 30 min, the remaining active sites were blocked with BSA solution (2 mg/mL in PBS). A baseline was established with PBS.

3.3.2. Binding of 6^{I},6^{IV}-di-*O*-[α-L-Fuc-(1→6)-D-GlcNAc]-βCD (5) and 6-*O*-[α-L-Fuc-(1→6)-D-GlcNAc]-βCD (6) to AAL

The strength of the interaction between (Fuc-GlcNAc)-βCDs and AAL was measured with an optical biosensor, IAsys (Fisons) at 25 °C, as described previously.[18,19] Binding of the ligand with the conjugated AAL was monitored in 50 μL of AAL solution. The association was terminated by aspiration of the AAL soln and replacement with PBS. The reaction was monitored for further several minutes.

3.4. Syntheses of substrates

3.4.1. 1,3,4-Tri-*O*-acetyl-6-*O*-(*tert*-butyldimethylsilyl)-2-deoxy-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranose (10α) and 1,3,4-tri-*O*-acetyl-6-*O*-(*tert*-butyldimethylsilyl)-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranose (10β)

A solution of D-glucosamine hydrochloride (30 g, 139 mmol) and $NaHCO_3$ (23.4 g, 279 mmol) in water (150 mL) was added trichloroethoxycarbonyl chloride (23 mL, 167 mmol), and the mixture was stirred 1 h at 0 °C. The reaction mixture was washed with cooled water and ethyl ether, and the solid residue was recrystallized from 80% CH_3OH to give 2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-α,β-D-glucopyranose (**7**) (32.1 g, 65%). Compound **7**: mp 191–192 °C, Anal. Calcd for $C_9H_{14}O_7NCl_3$: C, 30.49; H, 3.98, N, 3.95. Found: C, 30.19; H, 3.95, N, 3.87.

Compound **7** (8.3 g, 23.4 mmol) was stirred with *t*-$BuMe_2SiCl$ (4.25 g, 28.2 mmol) in dry pyridine (50 mL) for 1.5 h at 0–4 °C. The mixture was concentrated, the residue was extracted with EtOAc, and washed with aq NaCl and water. The organic layer was dried with $MgSO_4$ and concentrated to give 6-*O*-(*tert*-butyldimethylsilyl)-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-α,β-D-glucopyranose (**8**) (11.0 g, 100%).

Acetylation of compound **8** (11.0 g, 23.5 mmol) was carried out with a mixture of Ac_2O (20 mL) and dry pyridine (40 mL), which was stirred for 15 h at room temperature. The mixture was concentrated, the residue was extracted with $CHCl_3$, and the extract was washed with aq $NaHCO_3$ and water. The extract was dried ($CaCl_2$) and concentrated to give a colorless syrup (13.74 g, 96.7%). Crystallization from hexane gave 6-*O*-(*tert*-butyldimethylsilyl)-1,3,4-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloro-ethoxycarbonylamino)-α-D-glucopyranose (**9α**, 6.37 g, 45.6%) and 6-*O*-(*tert*-butyldimethylsilyl)-1,3,4-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranose (**9β**, 0.83 g, 5.9%). Compound **9α**: R_f 0.54 (8:1 benzene–acetone); mp 91–92 °C; $[\alpha]_D^{24}$ +82.0° (*c* 1.0, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ: 6.24 (1H, d, $J_{1,2}$ = 3.57 Hz, H-1), 5.27 (1H, t, H-3), 5.19 (1H, t, H-4), 5.13 (1H, d, –NH), 4.80 (1H, d, Troc-CH_2), 4.63 (1H, d, Troc-CH_2), 4.14 (1H, m, H-2), 3.84 (1H, m, H-5), 3.71 (1H, m, H-6), 3.67 (1H, m, H-6), 2.17 (3H, s, –$COCH_3$), 2.03 (6H, s, –$COCH_3$), 0.88 (9H, s, –$SiC(CH_3)_3$), 0.04, 0.03 (3H, s, –$Si(CH_3)_2$–); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 171.40, 168.98, 168.65 (–$OCOCH_3$), 154.09 (Troc-*C*O), 95.34 (Troc-*C*Cl_3), 90.58 (C-1), 74.71 (Troc-*C*H_2), 72.58 (C-5), 70.95 (C-3), 68.14 (C-4), 61.93 (C-6), 53.39 (C-2), 25.81 (3C, s, –$SiC(CH_3)_3$), 20.87, 20.68, 20.64 (–$COCH_3$), 18.28 (–$SiC(CH_3)_3$), −5.41, −5.43 (1C, s, –$Si(CH_3)_2$–). Anal. Calcd for $C_{21}H_{34}O_{10}NCl_3Si$: C, 42.39; H, 5.76; N, 2.35. Found: C, 42.32; H, 5.98; N, 2.60. Compound **9β**: R_f 0.61 (8:1 benzene–acetone); mp 141–142 °C; $[\alpha]_D^{24}$ +20.2° (*c* 1.0, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ: 5.71 (1H, d, $J_{1,2}$ = 8.70 Hz, H-1), 5.48 (1H, d, –NH), 5.26 (1H, t, H-3), 5.09 (1H, t, H-4), 4.73 (2H, s, Troc-CH_2), 3.94 (1H, dd, H-2), 3.75–3.72 (1H, m, H-6), 3.69–3.65 (1H, m, H-6), 3.68–3.65 (1H, m, H-5), 2.09 (3H, s, –$COCH_3$), 2.04 (6H, s, –$COCH_3$), 0.88 (9H, s, –$SiC(CH_3)_3$), 0.04, 0.02 (3H, s, –$Si(CH_3)_2$–); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 171.15, 169.33, 169.33 (–$OCOCH_3$), 154.29 (Troc-*C*O), 95.55 (Troc-*C*Cl_3), 92.44 (C-1), 75.43 (C-5), 74.53 (Troc-*C*H_2), 72.74 (C-3), 68.81 (C-4), 62.19 (C-6), 55.16 (C-2), 25.83 (3C, s, –$SiC(CH_3)_3$), 20.79, 20.70, 20.67 (–$COCH_3$),

18.34 (–SiC(CH$_3$)$_3$), −5.36, −5.41 (1C, s, –Si(CH$_3$)$_2$–). Anal. Calcd for $C_{21}H_{34}O_{10}NCl_3Si$: C, 42.39; H, 5.76; N, 2.35. Found: C, 42.32; H, 5.96; N, 2.64.

3.4.2. 1,3,4-Tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranose (10α)

Compound **9α** (1.1 g, 1.85 mmol) was stirred with 50% AcOH (40 mL) for 2 h at 40 °C. The mixture was diluted with $CHCl_3$, washed sequentially with ice-cold aq $NaHCO_3$ and water. The extract was dried with $CaCl_2$ and concentrated to give a colorless syrup (940 mg). Crystallization from hexane gave **10α** as colorless prisms (669 mg, 71.2%). Compound **10α**: mp 162–163 °C; $[\alpha]_D^{24}$ +90.9° (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.25 (1H, d, $J_{1,2}$ = 3.66 Hz, H-1), 5.32 (1H, t, H-3), 5.25 (1H, d, –NH), 5.16 (1H, t, H-4), 4.81 (1H, d, Troc-CH$_2$), 4.64 (1H, d, Troc-CH$_2$), 4.18 (1H, m, H-2), 3.83 (1H, m, H-5), 3.70 (1H, m, H-6), 3.59 (1H, m, H-6), 2.42 (1H, dd, –OH), 2.19, 2.07, 2.05 (–COCH$_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 171.29, 169.95, 168.77 (–OCOCH$_3$), 154.12 (Troc-*C*O), 95.30 (Troc-*C*Cl$_3$), 90.56 (C-1), 74.72 (Troc-*C*H$_2$), 72.12 (C-5), 70.32 (C-3), 68.07 (C-4), 60.92 (C-6), 53.36 (C-2), 20.89, 20.66, 20.60 (–COCH$_3$). Anal. Calcd for $C_{15}H_{20}O_{10}NCl_3$: C, 37.48; H, 4.19; N, 2.91. Found: C, 37.48; H, 4.15; N, 3.04.

3.4.3. 1,3,4-Tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranose (10β)

Compound **9β** (100 mg, 0.17 mmol) was desilylated as described for **9α**. The syrup (80 mg) was crystallized from hexane to give **10β** as colorless prisms (39 mg, 48.8%). Compound **10β**: mp 130–131 °C; $[\alpha]_D^{25}$ +17.5° (*c* 0.9, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 5.76 (1H, d, $J_{1,2}$ = 8.79 Hz, H-1), 5.51 (1H, d, –NH), 5.31 (1H, t, H-3), 5.08 (1H, t, H-4), 4.73 (2H, s, Troc-CH$_2$), 3.96 (1H, dd, H-2), 3.77 (1H, m, H-6), 3.69 (1H, m, H-5), 3.61 (1H, m, H-6), 2.37 (1H, dd, –OH), 2.11, 2.08, 2.05 (–COCH$_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 170.89, 170.24, 169.39 (–OCOCH$_3$), 154.29 (Troc-*C*O), 95.50 (Troc-*C*Cl$_3$), 92.44 (C-1), 75.06 (C-5), 74.56 (Troc-*C*H$_2$), 72.13 (C-3), 68.59 (C-4), 61.04 (C-6), 55.25 (C-2), 20.83, 20.65, 20.64 (CO*C*H$_3$). Anal. Calcd for $C_{15}H_{20}O_{10}NCl_3$: C, 37.48; H, 4.19; N, 2.91. Found: C, 37.45; H, 4.17; N, 3.01.

3.4.4. 2,3,4-Tri-*O*-benzyl-α-L-fucopyranosyl-(1→6)-1,3,4-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranose (11α)

A mixture of **10α** (2.52 g, 5.24 mmol) with 2,3,4-tri-*O*-benzyl-L-fucopyranosyl trichloroacetimidate (6.84 g, 15.75 mmol), along with dry 4 Å molecular sieves (type AW300, 5 g), in dry CH_2Cl_2 (20 mL) was stirred under argon at −20 °C, and TMSOTf (900 μL) was added. After stirring for 1 h, Et_3N (3 mL) was added to the mixture, which was diluted with $CHCl_3$, filtered through Celite, washed sequentially with 1 M H_2SO_4, aq $NaHCO_3$ and water, and then dried and concentrated. The residue was fractionated by centrifugal chromatography with 20:1 benzene-acetone to give fractions containing **11α** (2.43 g), and 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl-(1→6)-1,3,4-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranose (**11β**, 0.86 g). Compound **11α**: R_f 0.45 (8:1 benzene–acetone); $[\alpha]_D^{23}$ +23.6° (*c* 1.8, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.23 (1H, d, $J_{1,2}$ = 3.36 Hz, GN-1), 4.91 (1H, d, $J_{1,2}$ = 3.36 Hz, F-1); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 171.33, 169.14, 168.62 (–OCOCH$_3$), 154.04 (Troc-*C*O), 139.02, 138.86, 138.67 (Bn-1), 128.33–127.40 (15C, m, Bn-2-6), 98.00 (F-1), 95.33 (Troc-*C*Cl$_3$), 90.48 (GN-1), 79.05 (F-3), 77.77 (F-4), 76.38 (F-2), 74.84, 73.19, 72.75 (–*C*H$_2$–C$_6$H$_5$), 74.56 (Troc-*C*H$_2$), 71.47 (GN-5), 70.64 (GN-3), 68.50 (GN-4), 66.70 (F-5), 66.01 (GN-6), 53.24 (GN-2), 20.79, 20.65, 20.61 (–COCH$_3$), 16.65 (F-6). HR-FAB-MS *m/z*: 894.2071 (M−H)$^-$ (Calcd for $C_{42}H_{47}O_{14}NCl_3$: 894.2062). Compound **11β**: R_f 0.17 (8:1 benzene–acetone); $[\alpha]_D^{22}$ +63.4° (*c* 1.2, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ: 6.23 (1H, d, $J_{1,2}$ = 3.85 Hz, GN-1), 4.37 (1H, d, $J_{1,2}$ = 7.70 Hz, F-1); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 171.41, 168.94, 168.65 (–OCOCH$_3$), 153.87 (Troc-*C*O), 138.96, 138.68, 138.65 (Bn-1), 128.34–127.36 (15C, m, Bn-2-6), 103.90 (F-1), 95.38 (Troc-*C*Cl$_3$), 90.44 (GN-1), 82.52 (F-3), 79.17 (F-2), 76.56 (F-4), 74.96, 74.62, 74.62 (–*C*H$_2$–C$_6$H$_5$), 73.24 (Troc-*C*H$_2$), 71.42 (GN-5), 70.88 (GN-3), 70.44 (F-5), 68.27 (GN-4), 66.66 (GN-6), 53.32 (GN-2), 20.81, 20.67, 20.60 (–COCH$_3$), 16.78 (F-6). HR-FAB-MS *m/z*: 894.2063 (M−H)$^-$ (Calcd for $C_{42}H_{47}O_{14}NCl_3$: 894.2062).

3.4.5. 2,3,4-Tri-*O*-benzyl-α-L-fucopyranosyl-(1→6)-3,4-di-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-D-glucopyranosyl trichloroacetimidate (13)

Compound **11α** (2.26 g, 2.53 mmol) was stirred with hydrazine acetate (349 mg, 3.79 mmol) in dry DMF (50 mL) for 2 h at 40 °C. The mixture was then diluted with EtOAc and washed with aq NaCl and ice water, and organic layer was dried with $MgSO_4$ and concentrated to give 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl-(1→6)-3,4-di-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-D-glucopyranose (**12**) as a syrup (2.12 g, 98.8%). To a mixture of **12** (2.12 g, 2.48 mmol) with dry 4 Å molecular sieves (3 g) in dry CH_2Cl_2 (30 mL) was added 1.8-diazabicyclo[5.4.0]undec-7-ene (DBU) (75 μL, 0.50 mmol) and trichloroacetonitrile (2.5 mL, 24.9 mmol), and the mixture was stirred for 1 h at 0 °C. The reaction mixture was diluted with $CHCl_3$, washed with ice water, and then dried ($CaCl_2$) and concentrated. Centrifugal chromatography of the residue (4:1→3:1, hexane–acetone) gave **13** (1.79 g, 72.2%).

3.4.6. 6^{I},6^{IV}-Di-*O*-[α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-βCD (5) and 6-*O*-[α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-βCD (6)

Bis(2,3-di-*O*-acetyl)-pentakis(2,3,6-tri-*O*-acetyl)-βCD (**2**) (1.66 g, 0.86 mmol) with **13** (1.79 g, 1.79 mmol), along with dry 4 Å molecular sieves (type AW300, 2 g), in dry CH_2Cl_2 (30 mL) was stirred under argon at −20 °C, and TMSOTf (155 μL) was added. After stirring for 1 h at −20 °C, Et_3N (1 mL) was added to the mixture, which was diluted with $CHCl_3$, filtered through Celite, washed sequentially with 1 M H_2SO_4, aq $NaHCO_3$ and water, and then dried and concentrated. The residue was fractionated by centrifugal chromatography with 3:1→2:1→3:2→1:1 hexane–acetone to give three fractions [**I** (1.60 g), **II** (0.60 g), and **III** (94 mg)] containing 6^{I},6^{IV}-di-*O*-[2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-β-D-glucopyranosyl]-βCD and mono-glycosylated βCD derivative. Each fraction of **I** (1.60 g), **II** (0.60 g), or **III** (94 mg) was individually dissolved in AcOH (3–20 mL), Ac_2O (3–20 mL), and THF (3–20 mL), and then freshly activated zinc–copper couple (1.5–15 g) was added. After 2 h, each reaction mixture was filtered through Celite, neutralized with aq NaOH, concentrated lightly. Each of the residues was washed sequentially with aq $NaHCO_3$ and water, and then dried and concentrated to give **I**′ (1.47 g), **II**′ (0.54 g), and **III**′ (80 mg). Each solution of **I**′ (1.47 g), **II**′ (0.54 g), or **III**′ (80 mg) and 10% Pd–C (0.4–2.3 g) in MeOH containing 10% formic acid (15–50 mL) was individually stirred under argon for 2 h at room temperature, filtered through Celite, and concentrated, to give crude debenzylated products **I**″ (1.23 g), **II**″ (0.48 g), or **III**″ (70 mg), respectively. These crude products were treated with methanolic 0.5 M sodium methoxide (0.7–6.4 mL) for 1 h at room temperature, neutralized with Amberlite IR-120 (H^+) resin, filtered, and concentrated to give **I**‴ (0.68 g), **II**‴ (0.27 g), or **III**‴ (35 mg), respectively. The desired compounds 6^1,6^4-di-*O*-[α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucopyranosyl]-βCD (**3**), and 6-*O*-[α-L-fucopyranosyl-(1→6)-2-acetamido-2-deoxy-D-glucopyranosyl]-βCD (**4**) were isolated from **I**‴, **II**‴, or **III**‴, respectively, by HPLC on a column of TSK-gel Amide-80 (300 × 7.8 mm i.d.) with 60:40 acetonitrile–water, giving mixture of **3** (420 mg) and **4** (87 mg). The crude compounds of **3** and **4** were isolated by HPLC on a column of DAISOPAK SP-120-5-ODS-BP (250 × 10 mm i.d.)

with 14:86 MeOH–water, giving mixture of **5** (323 mg) and **6** (54 mg). Compound **5**: $[\alpha]_D^{25}$ +47.1° (*c* 1.0, H_2O); ^1H NMR (500 MHz, D_2O) δ: 4.91 (1H, d, $J_{1,2}$ = 3.80 Hz, F-1), 4.58 (1H, d, $J_{1,2}$ = 8.20 Hz, GN-1); ^{13}C NMR (125 MHz, D_2O) δ: 175.15, 175.09 (–NH–$COCH_3$), 103.03–102.14 (9C, m, GN-1, G1, G′-1), 99.91, 99.85 (F-1), 82.36–82.02 (7C, m, G-4, G′-4), 75.69, 75.66 (GN-5), 74.64, 74.55 (GN-3), 74.01–73.80 (7C, m, G-3, G-3), 72.94–72.68 (14C, m, G-2, G′-2, G-5, G′-5), 71.59, 71.49 (F-4), 70.91 (2C, s, GN-4), 70.75, 70.66 (F-3), 69.38 (G′-6), 69.12 (2C, s, F-2), 68.98 (G76), 67.97, 67.80 (GN-6), 67.47 (2C, s, F-5), 61.40–61.16 (6C, m, G-6), 56.57 (2C, s, GN-2), 23.17 (2C, s, –NH–$COCH_3$), 16.75, 16.66 (F-6). HR-FAB-MS *m/z*: 1833.6530 $(M+H)^+$ (Calcd for $C_{70}H_{116}O_{53}N_2$: 1833.6522). Compound **6**: $[\alpha]_D^{25}$ +91.5° (*c* 1.0, H_2O); ^1H NMR (500 MHz, D_2O) δ: 4.91 (1H, d, $J_{1,2}$ = 3.80 Hz, F-1), 4.58 (1H, d, $J_{1,2}$ = 8.20 Hz, GN-1); ^{13}C NMR (125 MHz, D_2O) δ: 175.16 (–NH–$COCH_3$), 102.98 (G′-1), 102.74–102.44 (6C, m, GN-1, G1), 99.87 (F-1), 82.52 (G′-4), 82.27–81.95 (6C, m, G-4), 75.70 (GN-5), 74.67 (GN-3), 73.94–73.82 (7C, m, G-3, G-3), 72.97–72.69 (14C, m, G-2, G′-2, G-5, G′-5), 71.66 (F-4), 71.00 (GN-4), 70.81 (F-3), 69.74 (G′-6), 69.13 (F-2), 68.02 (GN-6), 67.49 (F-5), 61.42–61.12 (6C, m, G-6), 56.53 (GN-2), 23.14 (–NH–$COCH_3$), 16.90 (F-6).

HR-FAB-MS *m/z*: 1484.5160 $(M+H)^+$ (Calcd for $C_{56}H_{93}O_{44}N$: 1484.5149).

Acknowledgments

We thank Wako Pure Chemical Industries, Ltd (Osaka, Japan) for technical support of the RT-CES (ACEA) study.

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