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SYNTHESIS OF SUGAR-BRANCHED CYCLODEXTRINS AND THEIR DUAL ASSOCIATION WITH PROTEINS AND DRUGS OBSERVED BY SPR ASSAY

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The efficient synthesis of the sugar-branched cyclodextrins was carried out. Evaluation of the dual association consisting of saccharide-recognition with concanavalin A and PNA of the lectin protein, and the inclusion interactions with immobilized cholic acid and doxorubicin as a model drug analyzed by surface plasmon resonance (SPR) assay. The high mannosyl type oligosaccharide-branched cyclodextrins showed a tight interaction both with concanavalin A and cholic acid, showing a K_a of 10^7 M^{-1} or more. It was observed that the number of sugar antennas and the length of the spacer arm are quite important for the dual association with proteins and drugs. We can foresee applications of the sugar-branched cyclodextrins to a targeting drug delivery system.

Осуществлен эффективный синтез сахар-разветвленных циклодекстринов. Методом поверхностного плазмон резонанса (ППР) произведена оценка двойственной ассоциации, содержащей сахарид-познающий участок с конканавалином А и с ПНК лектин - протеином, а также анализ реакции включения между иммобилизованной холиновой кислотой и доксорубицином, как модели лекарства. Олигосахарид-разветвленные циклодекстрины с высокой степенью типа манозил показали тесное взаимодействие как с конканавалином, так и с холиновой кислотой, представляя значения Ка 10^7 M^{-1} или более. Выявлено, что количество антен и длина пространственной ветви сахаров весьма важны для двойственной ассоциации с протеинами и лекарствами. Можно предположить, что сахар-разветвленные циклодекстрины могут применяться в качестве мишени для изыскания лекарств.

Իրականացվել է շաքար-ճյուղավորված ցիկլոդեքստրինների արդյունավետ սինթեզը: Սակերեսային պլազմոն ռեզոնանսի մեթոդով (ՍՊՄ) կատարվել է երկակի համակեցության գնահատումը, կազմված կոնկանավալին A-ի և լեկտին-պրոտեինի ՊՆԹ -ի հետ սախարիդ-ճանաչող հատվածից, ինչպես նաև իմոբիլիզացված խոլինաթթվի և դոկսոռուբիցինի որպես դեղերի մոդելի, ներառման միջռեակցիաների անալիզը: Հիմնականում մանոզիլ տիպի օլիգոսախարիդ-ճյուղավորված ցիկլոդեքստրինները ցուցաբերել են սերտ փոխազդեցություն ինչպես կոնկանավալինի, այնպես էլ խոլինաթթվի հետ, ցուցաբերելով Ka-ի 10^7 M^{-1} և ավելի արժեքներ: Բացահայտվել է, որ շաքարների անտենաների քանակը և տարածական արմունկի երկարությունը չափազանց կարևոր են պրոտեինների և դեղերի հետ երկակի համակեցության համար: Կարել է կանխատեսել, որ շաքար-ճյուղավորված ցիկլոդեքստրինները կարող են կիրառվել որպես թիրախ դեղերի որոնման համար:

Introduction

Among so many biological events, the interaction between a saccharide and protein has an important role in recognition phenomena [1-4]. The driving force for the association of saccharides with proteins is water solvation, van der Waals force and hydrogen bonding. A typical example of a structure scheme was known between the PNA lectin protein and a saccharide, galactose-β-1-3-*N*-acetylgalacosamine [5]. The saccharides bind with specific

lectin proteins through hydrogen bonding, sometimes using water. PNA lectin forms a tetramer of the subunit having a molecular weight of 26 kD [6]. The binding site with saccharide is at the four corners.

By the wide application of the saccharide-protein interaction, there are many examples of saccharide-conjugates in the field of polymers [7], oligomers [8], dendrimers [9-15], calixarenes [16-22], surfactants [23] and cyclodextrins (CDs) [24-39]. Table 1 shows the previous studies on the synthesis of sugar-branched CDs.

Table 1. Previous studies on the synthesis of sugar-branched cyclodextrins

Authors	Oligosaccharide-branched CD	References
1) Parrot-Lopez, Leray, Col" eman (1993)	*Mono*-Glc, Gal, Man, Fucose-CD	26
2) Hattori, Takahashi, Kosikawa (1994)	*Bi*-Gal-CD	24
3) Robertis, Lancelon-Pin, Driguez, Attioui, Bonaly, Marsura(1994)	*Hepta*-Gal-CD	36
4) Matsuda, Inazu, Haneda, Mizuno, Yamanoi, Hattori, Yamamoto, Kumagai (1997)	High mannose type oligosaccharide-CD Srato complex type oligosaccharide-CD Asialo complex type oligosaccharide-CD	31, 32, 33
5) Imata, Kubota, Hattori, Aoyagi, Jindoh (1997)	*Mono*-Glc-CD, *Mono*-Gal-CD	34, 35
6) Mellet, Fernabdez, Benito, Law, Chmurskie, Defaye (1998)	*Hepta*-Gal-CD	30
7) Ikuta, Tanimoto, Koizumi, Murata, Usui, Kitahata, Fujita, Hashimoto, Nakagawa, Shimoda, Kagatani, Maeda, Konnno, Hara, Fujito, Sonobe (1999)	*Bi*-Gal-Lac-CD *Bi*-Man_3-CD *Mono*-Gal-CD *Mono*-Glc-CD	28, 29, 59
8) García-Lopez, Hernández-Mateo, Isac-García, Kim, Roy, Santoyo-Gonzalez, Vargas-Berenguel (1999)	*Hepta*-Gal-CD *Hepta*-Glc-CD *Hepta*-GlcNAc-CD	25
9) Fruike, Aiba(1999):	*Hepta*-Gal-CD	38
10) Yasuda, Aoki, Abe, Hattori (2000)	*Hepta*-Gal-CD	37
11) Fulton, Stoddart (2000)	*Tetradeca*-Glc-CD *Hepta*-Glc-CD	39

Koizumi's group prepared glycosyl-branched CDs enzymatically and purified by HPLC. Many glycosylated CDs were obtained. These compounds are very interesting because they showed the binding to corresponding lectin to be 10^4-10^5 M^{-1} [28]. Galactose-branched CD was proved *in vivo* to be a good carrier to the liver for the practical drugs [29].

In recent years, *multi*antennary sugar-cluster type of cyclodextrins have been synthesized and evaluated. Hattori [37], Furuike [38] and Stoddart [39] prepared various *per*-glycosylated CDs having galactose at the terminal unit. Garcia-Lopez and the colleague did recent study about the synthesis of *per*-glycosylated CD and interaction with lectins. Glucose, galactose, N-acetylglucosamine, and mannose were attached on β-CD with spacer of sulfur atom, sufomethleneamide, and oxyphenylene-hexamethylenediamide-methylenesulfur. These spacer arms were consisted one, four and 18 atoms between sugar and C-6 of β-CD [25]. They showed the relative association with lectins by the inhibition assay.

Generally speaking, a galactosylated carrier will target the liver parenchymal cell carrying drug to cure an illness. Also, the mannosylated carrier will target the liver Kuffer cell, the macrophage and other cells [40-42].

We can suggest a schematic scheme of the targeting DDS by the application of the sugar-branched CD on a specific cell surface as shown in Figure 1. The lectin proteins or

receptor proteins is recognizing the saccharide, and is associating with a specific sugar-branched CD. CD cavity shows the inclusion of a drug as a guest compound [53-57].

Also, we can imagine another case where the lectin is connected between the sugar-branched CD and the saccharide on the cell surface as in the left side in the Figure.

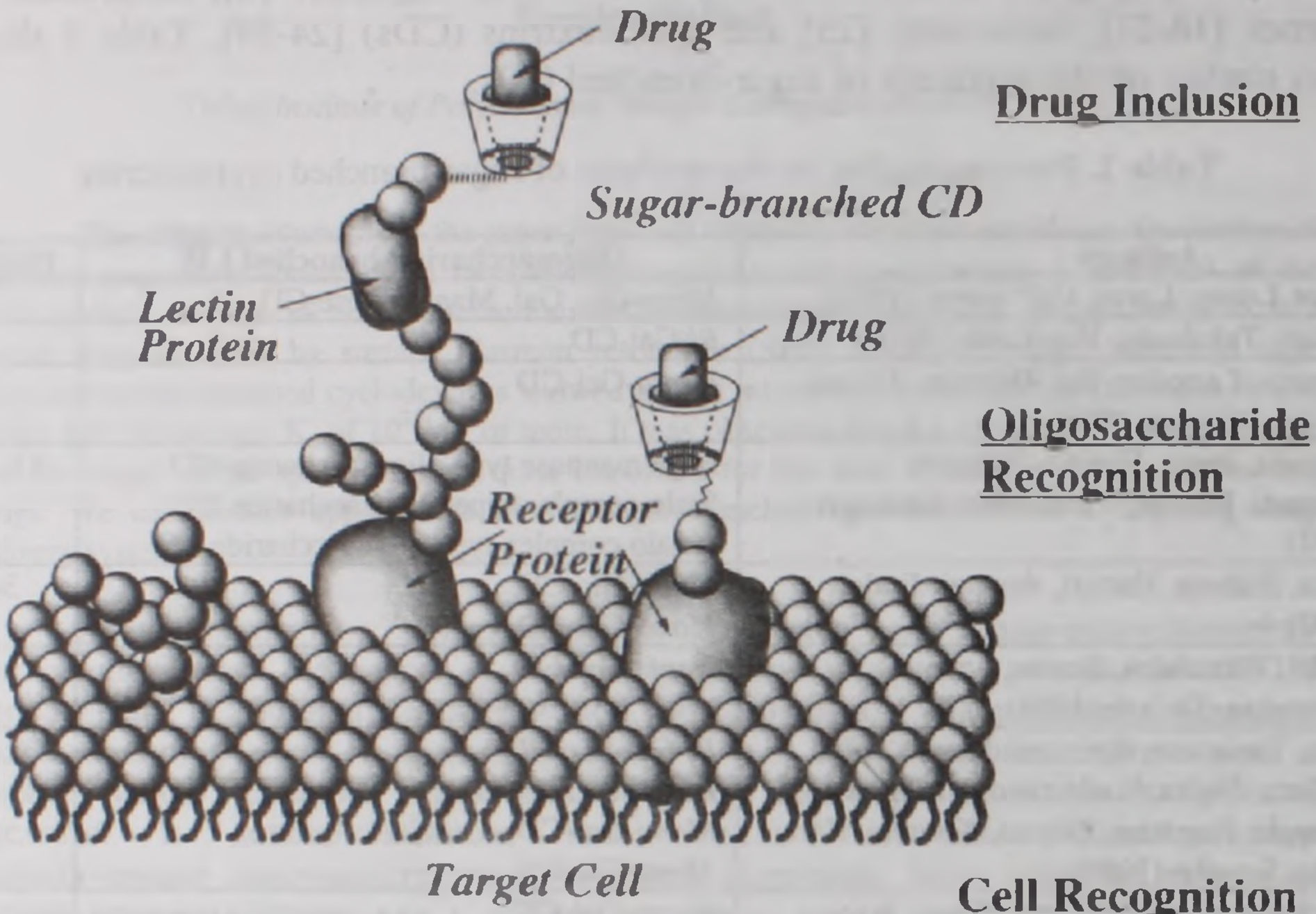

Figure 1. A concept of sugar-branched cyclodextrins for targeting drug delivery system.

The main purposes of the present review are focused on the following items.

1) The efficient synthesis of the sugar-branched CD.

2) Evaluation of the dual molecular recognition consisting of saccharide-recognition with lectin proteins, and the inclusion interactions with model drugs observed by SPR apparatus.

3) The observation results for the number of sugar antennas and the length of the spacer arm between sugar and CD in the structure of sugar-branched CDs shows quite important for the dual association with proteins and drugs.

4) The applications of the dual recognition of sugar-branched CD to a targeting drug delivery system is hopeful.

Synthesis of sugar-branched cyclodextrins

The *hexa*mannosyl asparagine (M6) was obtained according to the literature [43]. Attachment of fluorenyl-methylene-oxycarboxylate (Fmoc) group on asparagine of the oligosaccharides gave an 88% conversion for the high mannose type. The connection reaction of the Fmoc-mannosyl asparagine and *mono*amino-β-CD was tested in various condensing agents and solvents. The best reaction condition was to use PyBOP in *N*-methylpyrrolidinone solvent. We succeeded in the uptake of $Man_6GlcNAc_2$FmocAsn-CD (M6CD) and $Man_7GlcNAc_2$FmocAsn-CD (M7CD) in a similar manner. The HPLC and TOF-MS spectrum supported the structure. Three kinds of aminosugars (glucosamin,

galactosamin and mannosamin) were attached to glucuronylgluconyl-β-CD (GUG-CD). Other sugar-branched CDs (*bi*-antennary galactose-branched CD, *mono*-antennary galactose-branched CD, and *hpta*antennary galactose-branched CD) were prepared starting from the corresponding amino- or iodo-β-CDs.

Evaluation method for the association

SPR assay for the association kinetics.

The selection of the analytical method for the interaction analysis was considered. Various approaches are known for the interaction analysis. There are three broad categories. The indirect method such as competition inhibition and ELISA assay is used to define the relative binding activity. However, it does not provide direct information about the association constants. The kinetic method gives the association constant. Especially the SPR technique has the following advantages [4].

1) large range for analyte
2) association and dissociation rate measurement
3) small immobilized ligand sample
4) small analyte concentration
5) rapid measurement

SPR is a technique for the analysis of the association of free analytes with an immobilized ligand on a sensor metal that induces a change in the refractive index of the biosensor surface [44-46]. Changes in the refractive index (which is termed Response in arc sec unit) depend on the interacting mass with a ligand at the surface irrespective of the type of molecules. The information about the association and dissociation kinetics of the binding and the overall K_a is obtained.

We selected SPR and the immobilization of the protein and the drug on the biosensor cuvette were done.

Immobilization of ligands on the SPR optical biosensor [34,35]

The immobilization of concanavalin A (ConA) on the sensor cuvette was carried out by the reaction of a reactive linker molecule with the cuvette surface having an aminobutyl group. ConA in an acetate buffer of *p*H 5.3 was added to the cuvette. From the increase in response, 11 ng/mm^2 of ConA in dimeric subunit were immobilized. The immobilization of cholic acid on the cuvette surface was done with *N*-hydroxysuccinic amide, and a water-soluble carbodiimide in water. The amount of immobilized cholic acid and DXR was 1.23 and 0.67 ng/mm^2, respectively.

Interaction analysis by association kinetic assay [47,48]

The specific binding of a saccharide to a protein is governed by this equation. K_a is defined as the association constant in M^{-1} unit. K_a is equal to k_a/k_d, where k_a is the association rate constant and k_d is the dissociation rate constant. K_a is related to the standard free energy change in the binding.

In case to obtain the kinetic plots, several time-response curves are determined by changing the analyte concentration. The association kinetic constants were obtained from the linear plots between on-rate-constant, k_{on} and the concentration of the sugar-branched CD. The slope of the line gave k_a and they-intercept gave k_d. The k_{on}, was obtained from the slope of the plots between dR/dt *vs.* R from the saturation curves [54-55].

Natural oligosaccharide-branched cyclodextrins

Synthesis of *hexa*mannosyl and *hepta*mannosyl saccharide-branched CDs [31-33]

The synthesis of the natural oligosaccharide-branched CD were done using the procedure:

A) preparation of natural oligosaccharide sugar chain from chicken

B) protection of *N*-position of asparagine

C) connection of the oligosaccharide sugar with CD

From the egg white, ovalbumin was extracted by phenol and then centrifuged. To cut the peptide thoroughly, protease hydrolysis and then gel and ion-exchange chromatographies were repeated. From ovalbumin, *hexa*mannosyl asparagine was obtained according to the literature [43]. From egg yolk, the sialo-type oligosaccharide-asparagines, was obtained [60]. Enzymatic removal of the sialic acid group produced the asialo type oligosaccharide-asparagine.

Attachment of Fmoc group on asparagine of the oligosaccharides with Fmoc-OSu and hydrophobic adsorbent gave an 88 % in conversion for the high mannose type, 74 % for the sialo type and 88 % for the asialo type oligosaccharides.

We tried two route for the preparation of the high mannose oligosaccharide (termed FmocM6). The old route is straight separation in the first step. It gave only 8 % yield. However, the new route to attach Fmoc group on the *N*-position of the mixture was tried. It gave 30 % separation yield of Fmoc-M6 from the mixed Fmoc-oligosaccharides. Finally, 16.7 mg of the FmocM6 was able to prepare from one egg [32-33].

The connection reaction of the Fmoc-M6 and *mono-C6*-amino-β-CD was tested in various condensing reagents and solvents. The best reaction condition was to use PyBOP in *N*-methylpyrrolidinone solvent. This gave a quantitative yield of the M6CD ($Man_6GlcNAc_2$FmocAsn-β-CD). Other sialo-type and asialo-type oligosaccharides gave conversion yields of 30 % and 97 %, respectively. The HPLC chromatogram of M6CD showed it to be quite pure. The TOF-MS spectrum also supported the structure.

It was suggested that synthesizing the M7 oligosaccharide-CD in place of M6CD in hope of better association. M7CD ($Man_7GlcNAc_2$FmocAsn-β-CD) has an longer mannosyl oligosaccharide. Additional work on a *hepta*mannosyl derivative was carried out. The analytical HPLC chromatogram of mixed Fmoc-mannosylasparagines using an ODS column contains at least six fractions. We succeeded in the uptake of the *hepta*mannosyl type oligosaccharide, FmocM7 in 18 % yield from a peak after ODS column and amide column chromatographies. In a similar manner as the M6CD, also M7CD was synthesized and identified by TOF-MS.

Competitive inhibition assay.

The interaction of the synthesized sugar-branched CDs with the immobilized ConA was observed in the acetate buffer of *p*H 5.3 using the SPR apparatus (IAsys). The amount of association on the ligand was indicated when the response R increased with time and showed a saturation curve. In the presence of a mannose molecule as an epitope which blocks the binding site of ConA, the curve showed only the physiochemical adsorption at the short initial period and no saturation curve due to the competitive inhibition between the oligosaccharide and ConA. This result demonstrates that ConA recognized the mannosyl residues of the oligosaccharide-CD. Similarly, the response curve between the immobilized cholic acid and the oligosaccharide-CD was observed. In the presence of cyclohexanol, and

because of a competitive inhibitor only during the initial period, a physiochemical adsorption was observed, and not the saturation curve based on the inclusion association. This proved that the inclusion phenomena between the cyclodextrin cavity and the cholic acid.

Dual association of *hexa*mannosyl-branched cyclodextrin (M6CD)

M6CD shows the association constant K_1 on the order of 1.3×10^7 M^{-1} during the binding with ConA. This is reasonable in comparison with the reported value using the equilibrium dialysis method [49,50]. K_2 between the immobilized ConA in the presence of excess cholic acid is 4.7×10^6 M^{-1}. K_3 is the association constant of 1.3×10^7 M^{-1} between M6CD and the immobilized cholic acid. The unexpected large association constant of K_3 may be due to the induced fit effect by the branched group such as Fmoc and high mannosyl oligosaccharide. The association constant of K_4 between the complexed M6CD with ConA and free cholic acid was calculated to be 4.7×10^6 M^{-1} from the relation using rough assumptions. Also, the association constant with DXR showed a larger value of 4×10^7 M^{-1}. These values seem to be expected to provide sufficient adhesion to interact with a receptor on biomaterials and indicate the release of the drug on the receptor protein.

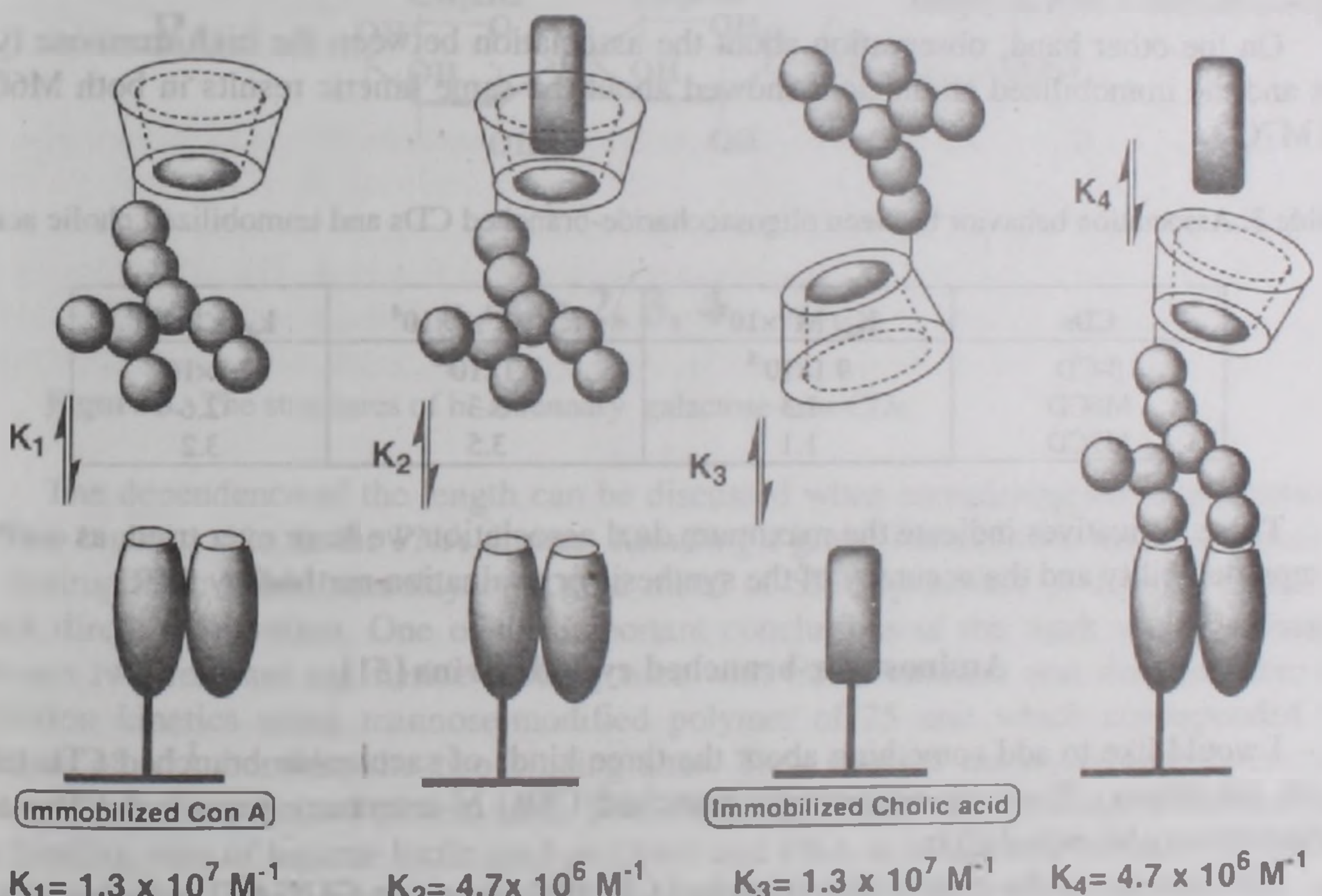

Figure 2. A schematic scheme of the association constants concerning dual association of hexamannosyl oligosaccharide-branched CD with Con A and cholic acid.

Comparison of heptamannosyl CD with hexamannosyl CD.

The results of the association constant, association rate constant, and the dissociation rate constant of two kinds of oligosaccharide-CDs having 6 and 7 mannose residues with ConA are summarized in the Table 2.

Table 2. Association behavior between oligosaccharide-branched CDs and immobilized ConA

CDs	K_a / $M^{-1}\times10^7$	k_a / $M^{-1}s^{-1}\times10^4$	k_d / $s^{-1}\times10^{-3}$
β-CD	0	0	0
FmocM6	1.2	5.8	5.0
M6CD	1.3	2.3	1.8
FmocM7	8.4	6.8	0.81
M7CD	8.1	5.4	0.67

Without a sugar branch, β-CD itself does not show any association with the ConA protein. The M6 type and M7 oligosaccharide itself without a CD cavity shows almost the same values of M6CD and M7CD, respectively. This means that the CD moiety is not involved with the interaction. The association constant of M7CD shows approximately a 7-fold increase due to the decrease in k_d. Naimith and his colleague[61] obtained an X-ray diffraction of the complex between ConA and trimannose. The recognition was induced by a number of hydrogen bonds between the amino acid residues of ConA and trimannose. These facts may demonstrate that the larger mannosyl oligosaccharide will resist removal from the oligosaccharide-ConA complex.

On the other hand, observation about the association between the high mannose type CDs and the immobilized cholic acid showed about the same kinetic results in both M6CD and M7CD.

Table 3. Association behavior between oligosaccharide-branched CDs and immobilized cholic acid

CDs	K_a / $M^{-1}\times10^7$	k_a / $M^{-1}s^{-1}\times10^4$	k_d / $s^{-1}\times10^{-3}$
β-CD	9.1×10^{-5}	7.1×10^{-6}	7.8×10^{-2}
M6CD	1.3	3.3	2.6
M7CD	1.1	3.5	3.2

These derivatives indicate the maximum dual association we have ever tried, as well as the reproducibility and the accuracy of the synthesis or evaluation method by SPR.

Aminosugar-branched cyclodextrins [51]

I would like to add something about the three kinds of saccharide-branched CDs tried in our laboratory. They are aminosugar-branched CDs, *bi*-antennary branched CDs, and *hepta*antennary branched CDs.

The first one is the aminosugar-branched CD made by using GUG-CD, which contains a carboxylic acid in the terminal glucose unit of *mono*-C6 maltosyl-β-CD. In quantitative yields, three kinds of aminosugars, that are mannosamine, glucosamine, and galactosamine, are attached to GUG-CD.

The evaluation of dual recognition with the corresponding lectin, cholic acid and anticancer DXR was done. Moderate K_a values of around 10^4 M^{-1} were obtained from the association with lectins. The association constants for the inclusion of cholic acid were also on the order of around 10^4 M^{-1}. Association with the anticancer drug, DXR, was also in the same order of 10^4 M^{-1}.

Bi-Antennary galactose-branched cyclodextrin [51]

Bi-antennary galactose-branched β-CD at *A, D* positions of the CD ring unit having various lengths of the spacer arm were prepared. The number of aminohexanoic acid units ranged from two to four as spacer arm between galactose and CD cavity. In the association constant with the PNA lectin, the K_a values increased depending on the arm length as 1.0, 2.6 and 4.6 ($\times 10^6$ M^{-1}) corresponding the arm length (n= 2, 3 and 4, respectively).

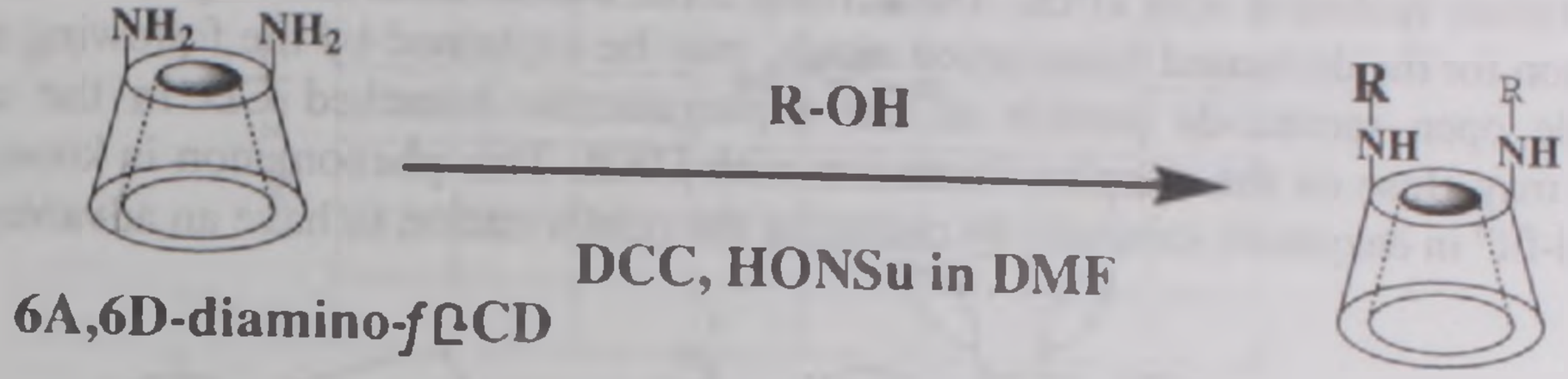

Figure 3. The structures of bi-antennary galactose-arm-CDs.

The dependence of the length can be discussed when considering the length between the two binding sites in the PNA dimers. Kiessling's group immobilized mannose-lipoid on the hydrophobic lipoid monolayer on gold metal of SPR tip surface as a probe ligand for ConA direct observation. One of the important conclusions of the work was the distance between two relevant saccharide binding sites with ConA tetramer was detected from the inhibition kinetics using mannose-modified polymer of 25 unit which corresponded the distance of 65Å between the two binding sites. They stressed the multivalent interaction between carbohydrate and protein [52]. The X-ray data suggested the distance between the two binding sites of legume lectin such as ConA and PNA to be about 65 Å [61]. Therefore, the *bi*-antennary galactose-branched CD derivative having a larger arm between the two galactoses may reach two binding sites on PNA, and the sugar-cluster effect [58] may appear in this case. Compounds with an arm shorter than 65 Å may bind at only one binding site. However, the depth of the binding site should need a minimum length to fit and form stable enough complexes. The association constant for the inclusion of DXR indicated that the cavity associated with DXR tighter than the *mono*antennary derivatives at the level of $3.4.10^5$ M^{-1}.

HEPTA-Antennary galactose-branched cyclodextrins [51]

The third experiment of the derivative is the comparison of the *hepta*antennary galactose-CD with the *mono*antennary galactose-branched CD. We prepared these structures starting *mono*- and *hepta*-C6-iodo-β-CDs using a thiol derivative of galactose unit. The K_a value of *hepta*galactose-branched CD with PNA lectin showed a 16-fold larger value than the *mono*antennary CD. In this case, the association constant of *hepta*galactose CD showed a 20-fold tighter inclusion with DXR. The driving force comes from the large decrease in k_d. The reason for the decreased dissociation rate k_a may be explained by the following scheme. The wide open saccharide portion of the *hepta*galactose-branched CD in the aqueous solution may close on the complex formation with DXR. This phenomenon is known as an "induced-fit" in enzymatic catalysis by changing the conformation to have an advantage.

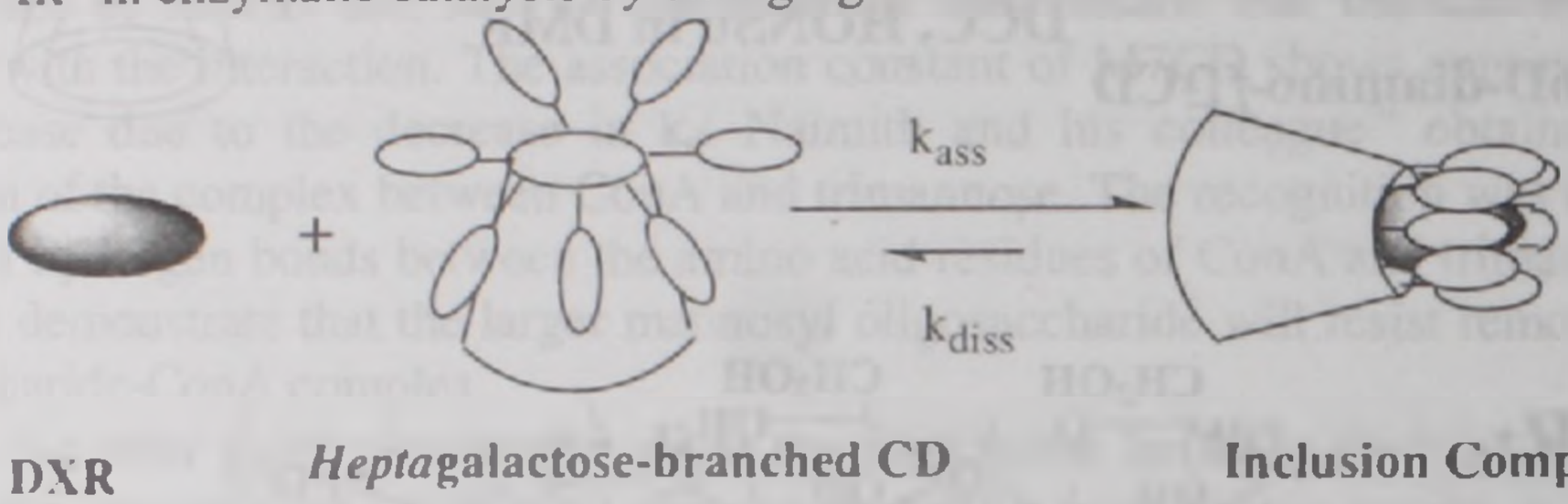

Figure 4. Induced-fit type conformational change at the association of heptagalactose-branched CD with DXR.

The computer-assisted molecular modeling supported this idea. The status before the complex formation may exist in a minimum potential energy. For the status of the complex formation with DXR, the conformation of the branch became closed with a decreased steric energy.

A two dimensional map for dual association of sugar-branched CD [51]

In this map Figure 5, the x-axis is log K_a of inclusion and, the y-axis is log K_a of oligosaccharide recognition. Almost all the aminosugar-branched CD exists in the area around 3-5 for the inclusion constant and around 3-5 for the saccharide recognition. The *mono*antennary galactose-branched CD goes up to the right and increase in this map when changed to the *hepta*antennary galactose-branched CD. The longer arm of *bi*antennary galactose-branched CD (n= 3) made the position upward (x= 4.6, y= 6.4). However, a natural high mannosyl CD existed in such an advantageous position (x= 7, y= 7-8) of a higher association constant with both the lectin and drug.

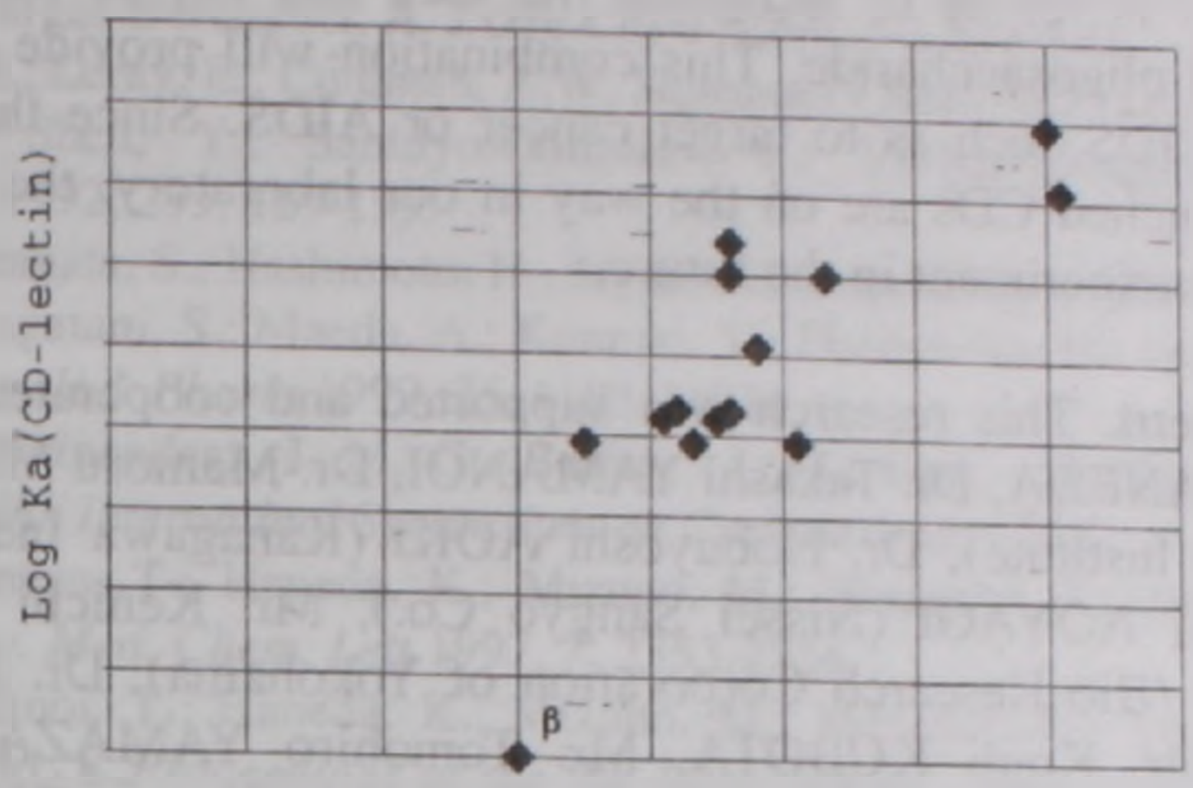

Figure 5. A two-dimensional map for dual association. K_a(CD-protein) means the association constant between oligosaccharide-branched CD and lectin protein. K_a(CD-drug) means the association constant between oligosaccharide-branched CD and cholic acid or DXR. *Octa*-Glc-calixarene means an *octa*glucose-branched calixarene [31].

In comparison with other carrier systems such as the calixarene-saccharide conjugates made by Aoyama's group, their compound is positioned around (x= 5.4, y= 6.0). They synthesized *octa*antennary glucose cluster also that has alkyl chain.[22] This associate with ConA at 10^6 M^{-1} and guest compound dye eosin around 1.8×10^5 M^{-1}.

All the other systems in the literature were not evaluated by the dual association constant, so they can not be exactly compared in this map.

Conclusion

In conclusion, we verified the following points.

A) The high mannosyl type oligosaccharide-branched CD (that is, M6CD and M7CD) showed a tight interaction both with lectin and a drug, showing a K_a of 10^7 M^{-1} or more by SPR assay.

B) The number of antennas attached on CD and the length of the spacer arm between two recognizing sites are quite important for the dual association with protein and drug.

C) In order to obtain an overview of the dual association, a two-dimensional map can be suggested.

Scope

I would like to add the scope of this review.

A) This will make the better universal carrier for various drugs ever studied. The sugar-branch itself may enforce the complex formation by a sugar-cluster effect and make a stable inclusion with drugs accompanying the induced-fit type conformational change.

B) The mannosyl group will associate with the receptors on the Kuffer cell in the liver, or on a macrophage. Also the galactosyl unit can target the liver parenchymal cell. As a step in the development of this research, we have to find the *in vitro* interaction with liver

cells, bacteria, and viruses. It will then, be necessary to proceed to the practical tests *in vivo*, followed by clinical tests.

C) Another key is for us to recognize the lock and key in the structural relation between a protein and oligosaccharide. This combination will provide us new strategy to design for effective TDDS such as to target cancer or AIDS. Since the study on various structures of sugar-branched CDs are on the way in our laboratory, the hopeful possibility should be proven in the experiment in the future.

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