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Sweet Genies Hard to Catch
~ Molecular Recognition of Carbohydrates in Aqueous Media ~

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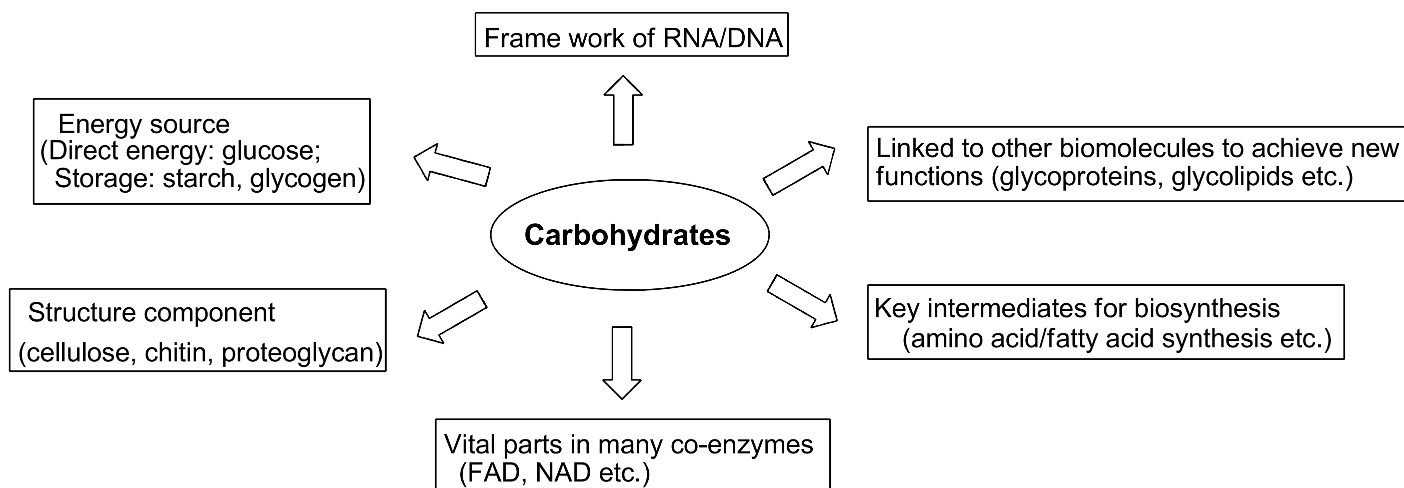
1. Background

1-1. Carbohydrates ABC

Defination:

Carbohydrates are compounds defined as **aldehydes and ketones with multiple -OH groups.** They are one of the four majar biomolecules along with proteins, nucleic acids, and lipids. They also share a synonym called "saccarides", or a more common name "sugar".

Biological and physiologycal importance:



Classification: (2)(3) are for monosaccharides

(1) Upon the number of component units:
Monosaccharides (U1), Disaccharides (U2), Oligosaccharides (U3~10), Polysaccharides (U>10)

(2) Upon functional groups: Aldoses (aldehyde group), Ketose (keto group)

(3) Upon the number of C-atom: Trioses, Tetroses, Pentoses, Hexoses etc. (Max C=9 in Nature)

Various forms of isomerism in monosaccharides:

(1) D/L isomers:

D/L are prefixes to distinguish the two mirror-images of a particular sacchrides. They designate the absolute configuraiton of **the asymmetric carbon atom farestest from the aldehyde of keto group**. When writhen by Fischer projections,**if the OH group is on the right it is designate as "D-", if on the left as "L".**

CHO
HO — H
H — OH
HO — H
HO — H
CH_2OH

L- glucose

enantiomer

CHO
H — OH
HO — H
H — OH
H — OH
CH_2OH

D-glucose

epimer

CHO
H — OH
HO — H
HO — H
H — OH
CH_2OH

D-galactose

(2) Epimers: Monosaccharides **differing in configuration at a single asymmetric centre.**

(3) Cyclic isomers:

In cases of sugars which possess more than 5 carbon atoms, they take the 5-membered of 6-membered ring forms rather than open-chain forms because of energetic reason. The 5-membered ring form is called furanose and 6-membered one called pyranose.

e.g D-glucose in water

Furanose form — Acyclic form — Pyranose form

<1%   <1%   99%

In case of glucose in solution, more than 99% is in pyranose form and both open-chain and furanose forms are less than 1%.

(4) α/β anomers:

The ring form of sugar creates a new stereocentre at the carbonyl carbon(or **anomeric carbon**). the newly generated OH group could take two position relative to the ring's midplane. When written by Haworth projection, if **the OH group comes on the opposite side of the ring from terminal CH_2OH it is "α", the same side is "β".**

e.g D-glucose in water

α   33%

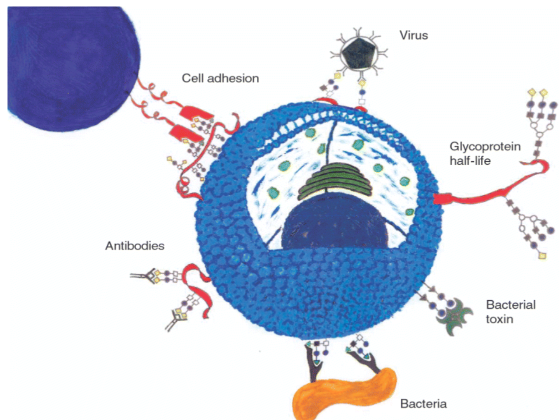
CHO
H — OH
HO — H
H — OH
H — OH
CH_2OH

<1%

β   66%

1-2. Studies on molecular recognition of carbohydrates in nature

The recognition of carbohydrates in nature is mainly represented in forms of protein-carbohydrate interactions, carbohydrate-carbohyadrate interactions and very rarely DNA-carbohydrate interactions. Amongst, protein-carbohydrates interactions play the most crucial role and most well studied.



Biological processes mediated by carbohydrates recognition:

Neuronal development	Pathogen infection
Hormonal activities	Intracellular transportaton
Fertilization	Degradation of proteins
Immune surveillance	Inflammatory responses
Tumor metastasis	

M.E. Breimer *et al. Immunology and Cell Biology*, **2005**, *83*, 694-708

Generally, there are three types of carbohydrate-binding proteins which are important in biological processes ----Lectin, antibody, and carbohydrate-specific enzymes.

Lectin is most major group and can be classified into 3 types:

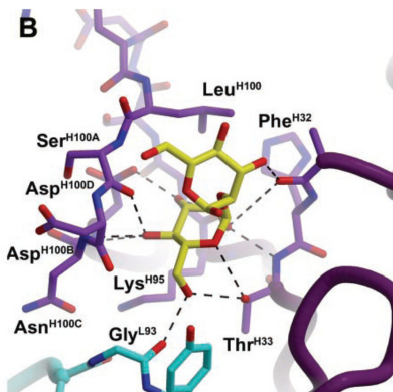
C-type: Ca^{2+} takes part in protein-carbohydrate binding by non-covalent bond

P-type: Special recognition towards mannose-6-phosphate

I-type: Possessing immunogloulin-like domain

<u>Binding patterns of protein-carbohydrate complexes:</u>

(1) Direct binding between protein and carbohydrates



Structure of binding domain between Fab 2G12 to disaccharide(Manα1-2Man)

12 hydrogen bonds devoted by a series of polor functional groups and van der Waals interactions devoted by Leu,Lys(hydrophobic),Phe(CH-π) constituent the stability of the complex.

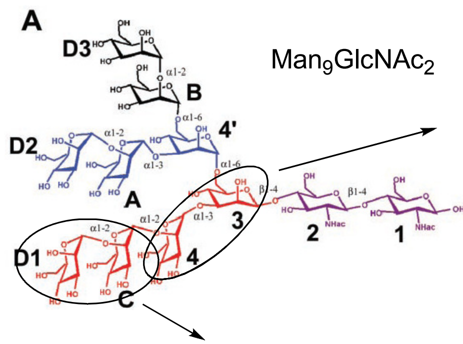
In this case, direct interaction between saccharide and protein is achieved.

Competition studies indicates:

(1) Additional interactions give grave influence upon the affinity

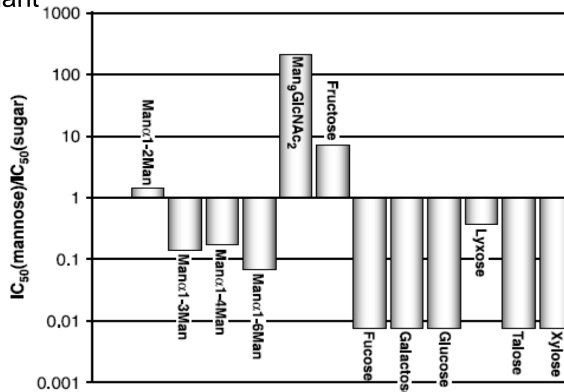
(2) Not all parts are involved in recognition

(3) Conformation is determinant



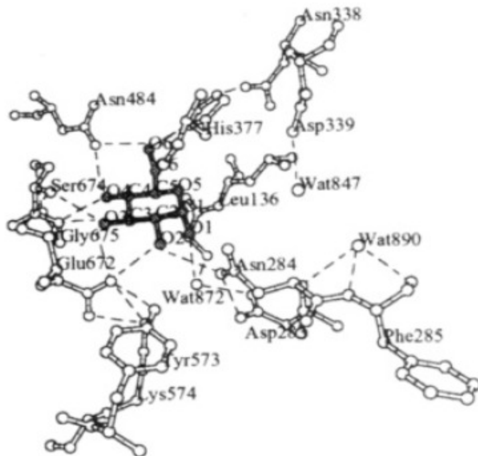
Additional interactions contributed by 3,4 mannose unit

Direct contacts with Fab 2G12 primary binding site



D.R. Burton *et al. Science*, **2003**, *300*, 2065-71

(2) Binding with assistance of small molecule (in most cases H_2O)



Binding of α-D-glucose to the catalytic site of glucose-phosphorylase *b* (GP*b*)

The polar contacts between glucose(analogue) ring OHs and the catalytic site residues of GP*b*

glucose atom	protein atom	compound and distance (Å)				
		α-D-glucose	1	2	11	12
O2	OH8 Wat872	–	–	2.9	–	–
	ND2 Asn284	3.2	3.3	–	3.1	3.1
	OE1 Glu672	2.9	3.2	–	2.9	3.0
	OH7 Wat890	3.0	3.2	–	3.1	3.1
	OH Tyr573	–	–	–	3.2	3.1
O3	OH7 Wat890	–	–	3.2	–	–
	OE1 Glu672	2.9	2.9	2.9	2.8	2.9
	N Ala673	–	–	2.8	–	–
	N Ser674	3.0	3.1	3.2	3.1	3.2
	N Gly675	3.0	3.2	–	3.0	3.0
O4	OD1 Asn484	3.1	–	–	–	3.3
	N Gly675	2.8	3.0	2.6	2.8	2.9
	OH1 Wat897	2.6	2.5	2.6	2.6	2.6
O6	ND1 His377	2.7	2.9	2.7	2.7	2.7
	OD1 Asn484	3.0	2.9	3.2	2.8	3.0

[a] Polar contacts between α-D-glucose and GP*b* are given for comparison.
–: indicates contact is greater than 3.3 Å.

Table 1: Glucose Analogue Inhibitors and Their Kinetic Constants for Glycogen Phosphorylase *b*[a]

compound	substituents at C1-position		concentrations used (mM)	K_i (mM)	*n*
	α	β			
1	C(=O)NH_2	H	1, 2, 3	0.37 ± 0.03	1.5
2	C(=O)NHCH	H	40	36.7 ± 5.6	1.4
3	C(=O)$NHCH_2CH_2OH$	H	20, 40	16.9 ± 4.4	1.4
4	C(=O)NHC_6H_5-4-OH	H	20	12.6 ± 2.0	1.3
5	C(=O)NH-4-OHC_6H_4	H	20, 30	5.6 ± 0.5	1.1
6	C(=O)$NHCH_2$-2,4-$F_2C_6H_3$	H	10	27.2 ± 5.2	1.0
7	C(=O)$NHNH_2$	H	5	3.0 ± 0.7	1.3
8	C(=O)NHNH-2,4-$(NO_2)_2C_6H_3$	H	1, 1.5, 2	0.7 ± 0.1	1.3
9	COOH	H	5, 10	1.62 ± 0.02	1.7
10	$COOCH_3$	H	40	24.2 ± 6.4	1.5
11	H	C(=O)NH_2	0.5, 1, 1.5, 2	0.44 ± 0.07	1.3
12	H	C(=O)$NHCH_3$	0.3, 0.5, 1	0.16 ± 0.03	1.2
13	H	C(=O)$NHCH_2CH_2OH$	5, 10	2.6 ± 0.2	1.1
14	H	C(=O)NHC_6H_5	15	5.4 ± 0.4	1.4
15	H	C(=O)NH-4-OHC_6H_4	10	4.4 ± 0.7	1.6
16	H	C(=O)$NHCH_2$-2,4-$F_2C_6H_3$	10	8.6 ± 1.2	1.0
17	H	C(=O)$NHNH_2$	2, 3	0.4 ± 0.1	1.1
18	H	C(=O)$NHNHCH_3$	5, 10	1.8 ± 0.3	1.7
19	H	C(=O)$NHCH_2CF_3$	10	8.1 ± 1.8	1.0
20	H	C(=O)$NHCHCH_2CH_2$	10	1.3 ± 0.3	1.5

K.A. Watson *et al. Biochem.*, **1994**, *33*, 5745-58

Conclusion:

1. Water molecules are involved in the hydrogen bondings formed between carbohydrates and protein.
2. Hydrogen bonds intermediated by water molecules are as strong as those without intervening water bridges.
   ⟹ Earned hint: Structural water could play as an extension of protein surface.
3. Subtle change on substitution or conformation could lead to drastic variation on binding affinity.

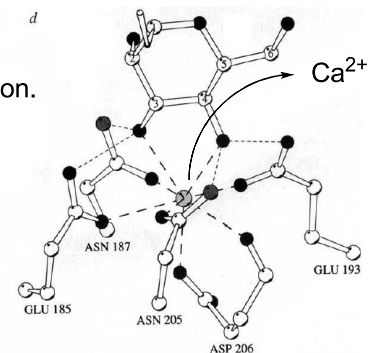
(3) Binding with assistance of metal ions:

In many cases, protein-carbohydrate recognition requires the assistance of metal ions, most commonly Ca^{2+}(such as C-lectin),sometimes Mg^{2+},Mn^{2+} or other divalent cation.

e.g. 1 mannose-binding site of MBP-A (Ca^{2+} assistance)

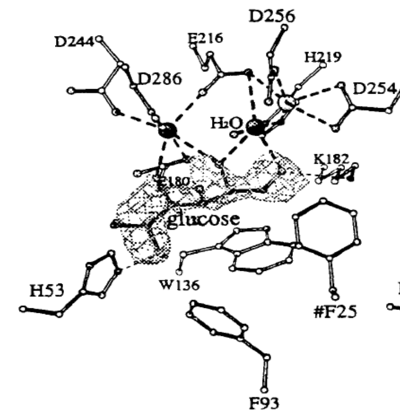
Bipyramid coordination of Ca^{2+} is formed, involving direct Ca-carbohydrate interaction.

Amino acid residues both accept hydrogen bonds from the carbohydrate ligand and act as coordination sites for Ca^{2+}, providing an interlocked lectin-Ca-carbohydrate complex.



W.I. Weis *et al. Nature,* **1992**, *360*, 127-34

e.g 2 glucose bound to active site of xylose isomerase (Mg^{2+} assistance)



Bi-nuclear type complex of protein-Mg-carbohydrate is formed.

The glucose substrate provides two ligands to each of two Mg^{2+}, and hydrogen-bonded only to His53 and Lys182.

D. Ringe *et al. Biochem.*, **1994**, *33*, 5469-80

B. Characteristics of carbohydrate recognition in nature

(1) The interactions in carbohydrate recognition are far too weaker than other biomolecular associations

Comparison between K_d of protein-protein interaction and protein carbohydrate interaction

Complex[a]	K_d (M)
PDEαβ:PDEγ	1.3×10^{-10}
Citrate synthase: malate dehydrogenase	1×10^{-6}
EGF:EGF receptor	4.1×10^{-7}
ras:raf	5×10^{-8}
NusA: core RNA polymerase	1×10^{-7}
Trypsin:pancreatic trypsin inhibitor	6×10^{-14}
PKA-C:PKA-R	2.3×10^{-10}
PRI:angiogenin	7×10^{-16}
TαGDP:PDEγ	3×10^{-9}
CAP cAMP:RNA polh	3×10^{-5}

Lectin	Carbohydrate	K_d (M)
Concanavalin A	αManOMe	$1.2*10^{-4}$
	β-GlcNAc(1→2)α-Man(1→6)\ ManOH / β-GlcNAc(1→2)α-Man(1→3)/	$6.5*10^{-7}$
	α-Glc(1→4)GlcOH	$9.6*10^{-4}$
	α-Glc(1→4)-α-Glc(1→4)GlcOH	$6.8*10^{-4}$
Se 155-4	α-Gal(1→2)[α-Abe(1→3)]α-4-deoxyManOMe	$1.9*10^{-5}$
	α-Gal(1→2)[α-Abe(1→3)]α-6-α-Abe(1→3)α-ManOMe	$3.8*10^{-5}$
Cholera toxin	G_{M1} pentasaccharide	$5.5*10^{-7}$

(2) The driving force for binding is unclear and unpredictable in aqueous media.

Assuming proteins are pre-binded with H_2O, the binding of carbohydrates therefore involves the replacement of H_2O to ROH. Thus binding should be entropy driven.

Value of free energy, enthalpy and entropy change in protein-carbohydrate bingding process

Lectin	Carbohydrate	ΔG	ΔH	TΔS (kcal)
Concanavalin A	αManOMe	−5.3	−6.6	−1.3
	α-GlcNAcOMe	−4.1	−6.2	−2.1
	α-Glc(1→4)GlcOH	−4.1	−6.2	−1.9
	α-Glc(1→4)-α-Glc(1→4)GlcOH	−4.3	−6.4	−2.1
	α-Man(1→2)-α-ManOH	−6.3	−9.9	−3.6
	α-Man(1→2)-α-ManOMe	−7.0	−10.5	−3.5
	α-Man(1→2)-α-Man(1→2)-ManOH	−7.6	−10.7	−3.1
Se 155-4	α-Gal(1→2)[α-Abe(1→3)]α-ManOMe	−7.3	−5.8	+1.5
	[(→3)α-Gal(1→2)[α-Abe(1→3)] -α-Man(1→4)α-Rha(1→)]$_2$	−7.8	−8.4	−0.6
	[(→3)α-Gal(1→2)[α-Abe(1→3)] -α-Man(1→4)α-Rha(1→)]$_3$	−7.8	−10.7	−2.9
	[(→3)α-Gal(1→2)[α-Abe(1→3)] -α-Man(1→4)α-Rha(1→)]$_4$	−8.2	−10.8	−1.6
	[(→3)α-Gal(1→2)[α-Abe(1→3)] -α-Man(1→4)α-Rha(1→)]$_5$	−7.8	−17.0	−9.2
Cholera toxin	G_{M1} pentasaccharide	−8.5	−22	−13.5

* In most cases, the enthalpy of binding is negative or equal to the free energy of binding.

⟹ The recognition(binding) process is driven by enthalpy!!

Possible reason:

1. The carbohydrate-binding process energetically surpass protein's desolvation from H_2O.
2. Some enthalpically driven "non-classical" hydrophobic effect may play a role in binding process. (e.g solvent reorganization, carbohydrate-carbohydrate interaction etc.)

C. The role of water in recognition

(1) Mediates hydrogen-bonding interactions between proteins and carbohydrates.

(2) May provide a favorable contribution to ΔH and ΔG through the release from solute surfaces.

R.U. Lemieux *et al. Acc. Chem. Res*. **1996**, *29*, 373

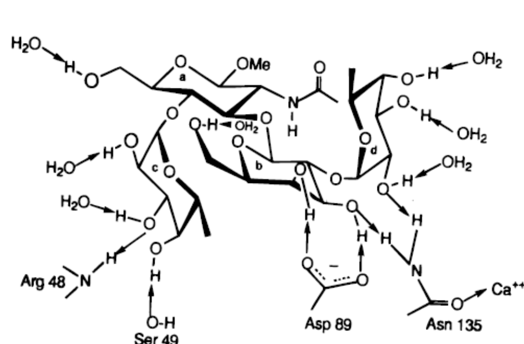


Figure 1. Hydrogen bonds formed between the epitope of Le[b]-OMe and the receptor site of GS-IV to illustrate[27-29] (1) the three key interactions (OH-4c to Ser 49, OH-3b to both Asp 89 and Asn 135, and OH-4b to Asp 89), (2) the bonding at the periphery of OH-3c to Arg 48 and water and OH-2d to Asn 135 and water, and (3) that the other five hydroxyl groups remain entirely bonded to water.

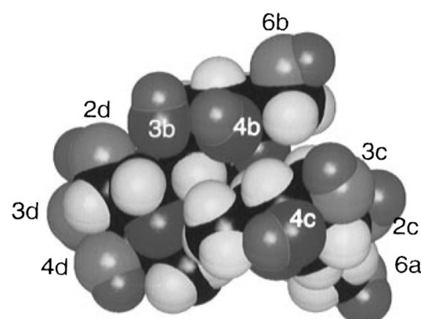


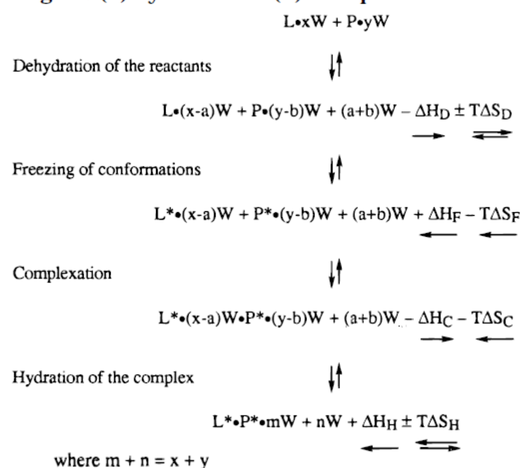
Figure 2. Polyamphiphilic topography of the epitope of Le[b]-OMe that is recognized by the lectin GS-IV.[2,28] Note that hydration of this surface must include the six hydroxyl groups (in orange) that, in the complex, are at or very near the periphery of the combining site (see Table 1) in a network of hydrogen-bonded water molecules that also includes hydrogen bonding to the three key hydroxyl groups (in red) at positions 3b, 4b, and 4c. Hydroxyl group hydrogens are green, and those attached to carbon are white.

Table 1. Thermodynamic Parameters[a] for the Binding of Le[b]-OMe Tetrasaccharide and Monodeoxy Congeners by the Lectin GS-IV at 298 °C

position deoxygenated[b]	$\Delta G°$ (kcal/mol)	$\Delta\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$\Delta\Delta H°$ (kcal/mol)	$T\Delta S°$ (kcal/mol)	internuclear distance[c] (Å)
none	−6.3 (−6.5)	0	−13.3 (−11.9)	0	−7.0	
6a	−6.3 (−7.0)	0	−13.1 (−10.7)	0.2	−6.8	6.44 (O-Tyr 105)
6b	−6.2 (−6.4)	0.1	−8.7 (−7.4)	4.6	−2.5	3.62 (C-Tyr 223)
2c	−5.7	0.6	−10.0	3.3	−4.3	3.32 (N-Arg 48)
3c	−5.5 (*d*)	0.8	−6.6 (*d*)	6.7	−1.1	2.97 (N-Arg 48)
2d	−5.7 (*d*)	0.5	−12.1 (*d*)	1.2	−6.4	2.96 (N-Asn 135)
3d	−5.6 (−6.5)	0.7	−8.6 (−12.4)	4.7	−3.1	3.01 (N-Trp 138)
4d	−6.4	−0.1	−7.4	5.9	−1.0	2.88 (N-His 114)

[a] The values in parentheses were obtained by microcalorimetry and provided by Dr. Eric Toone, Duke University. [b] The 3b-, 4b-, and 4c-monodeoxy congeners were too inactive for significant measurements. [c] The distance between the oxygen of the hydroxyl group that was replaced by hydrogen and the nearest non-hydrogen atom in GS-IV (identified in parentheses). [d] Because of the weak binding and paucity of materials, reproduced results were not obtained. Definitely, however, these reactions were also exothermic ($\Delta H \simeq -9$ kcal/mol) with a compensating decrease in entropy ($\Delta G \simeq -7$ kcal/mol).

Scheme 1. Artificial Expressions for the Binding of a Ligand (L) by a Protein (P) in Aqueous Solution[a]



[a] The hydration of the reactants and the product is expected to involve *x*, *y*, and *m* water (W) molecules that have thermodynamic parameters significantly different from those in bulk. The asterisks are to represent L and P in specific conformations. The arrows under the thermodynamic parameters are to indicate the direction that their change is expected to have on the various hypothetical equilibria. The $\pm T\Delta S_D$ and $\pm T\Delta S_H$ are to indicate both increases and decreases may contribute to the net $T\Delta S$.

Conclusion:

(1) The driving force of carbohydrate recognition is complicated. No specific factor always plays a dominant role.

(2) Binding of carbohydrates is usually weak, also an aggregate result.

Challenges in carbohydrate receptor design in aqueous media

A. The amount of targets is tremendous and subtle changes could have great influence in binding process.
   e.g 6 carbohydrate monomers can yield >10^{12} oligomeric structure (compared to 4096 for nucleotides and $6*10^7$ for peptides)

B. As no specific interaction accounts for the binding, both polar and apolar interactions are required in receptor design.

C. For the polyol moiety that carbohydrates hold, the differentiation between H_2O and carbohydrate molecule is difficult.

Put these challenges on mind, let's review how the scientists progressed in carbohydrate receptor design.

2. Molecular carbohydrate recognition via covalent bonds

This stratey is mainly relies on the reversible formation of covalent bonds from diol units and boronic acids, which is an interaction not employed in nature.

(1) Basis of boronic acid- carbohydrate interaction

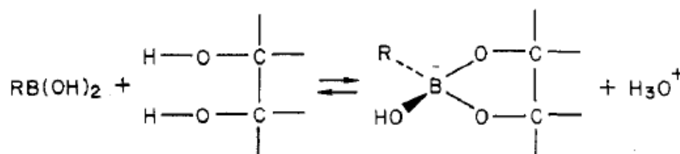


Table I. Stability Constants of Boron Acid Complexes

ligand	pK_a(ligand)	m-$NO_2PhB(OH)_2$, $pK_a = 6.96$	$PhB(OH)_2$, $pK_a = 8.72$	$B(OH)_3$, $pK_a = 8.98$	$CH_3B(OH)_2$, $pK_a = 10.40$
oxalic acid[9]	1.04		3.2		
malonic acid[10]	2.59		2.6×10^{-2}		
salicylic acid	2.83	1.1	6.8×10^{-2}	1.1×10^{-2} [a]	4.5×10^{-3}
tartaric acid[6]	2.89[b]			1.8×10^{-2} [c]	
mandelic acid	3.22	1.9×10^{-1}	1.5×10^{-2}		2.1×10^{-3}
lactic acid[7]	3.70		3.7×10^{-3}		
4-nitrocatechol	6.69	1.6×10^{-2}	9.5×10^{-4} [d]	1.5×10^{-4} [d]	4.3×10^{-5}
catechol	9.27		4.7×10^{-5} [d]	1.1×10^{-5} [d]	1.6×10^{-6}
4-methylcatechol	9.39	5.5×10^{-4}	3.0×10^{-5} [d]	6.3×10^{-6} [d]	
mannitol	13.5[e]		5.3×10^{-6} [d]		

[a] Reference 20. [b] V. Frei, *Collect. Czech. Chem. Commun.*, **30**, 1402 (1965). [c] L. I. Katzin and E. Gulyas, *J. Am. Chem. Soc.*, **88**, 5209 (1966); see also ref 6. [d] Reference 3. [e] J. Thamsen, *Acta Chem. Scand.*, **6**, 270 (1952).

Two trends can be observed:

a. For a given boronic acid, as the pKa of the diol decreases, the stability constant increases
b. For a given diol, as the pKa of the boronic acid decreases, the stability constant increases

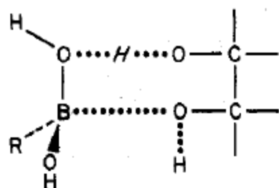
As in cases of carbohydrates, the pKa of the ligand are always quite high, which means the reaction is proceeded without ligand deprotonation

Table II. Rate Constants for the Reactions of $RB(OH)_2$ with Fully Protonated Ligands

	m-$NO_2PhB(OH)_2$		$PhB(OH)_2$		$B(OH)_3$		$CH_3B(OH)_2$	
ligand	k_f, $M^{-1}\ s^{-1}$	k_r, $M^{-1}\ s^{-1}$	k_f, $M^{-1}\ s^{-1}$	k_r, $M^{-1}\ s^{-1}$	k_f, $M^{-1}\ s^{-1}$	k_r, $M^{-1}\ s^{-1}$	k_f, $M^{-1}\ s^{-1}$	k_r, $M^{-1}\ s^{-1}$
oxalic acid[9]			2.0×10^3	6.2×10^2				
malonic acid[10]			3.5×10^2	1.3×10^4				
salicylic acid	6.5×10^2	5.9×10^2	2.3×10^2	3.3×10^3	1.4×10^2 [a]	4.5×10^3 [a]	5.5×10	1.2×10^4
tartaric acid[6]					4.8×10^2	2.6×10^4		
mandelic acid	2.5×10^3	1.3×10^4	1.8×10^2	4.7×10^4				
lactic acid			1.4×10^2	3.8×10^4				
4-nitrocatechol	2.0×10^3	1.3×10^5	6.5×10^2 [b]	6.8×10^5 [b]	2.5×10^2 [b]	1.7×10^6 [b]	4.5×10	1.0×10^6
catechol			1.1×10^2 [b]	2.3×10^6 [b]	6.0×10[b]	5.4×10^6 [b]	7.6	4.8×10^6
4-methylcatechol	1.5×10^3	2.7×10^6	1.2×10^2 [b]	4.0×10^6 [b]	5.4×10[b]	8.6×10^6 [b]		
mannitol			~50[b]	~10^7[b]				

A clear conclusion could be drawn that the complexation process under this condition is dominantly depended on ligands.

The plausible transition state for this process is supposed as below:



One proton(in italic) is transferred from fully protonated ligand to the leaving hydroxide on boron

The other one is displaced directly by boron.

The rate-limiting step is supposed to be ring closure process.

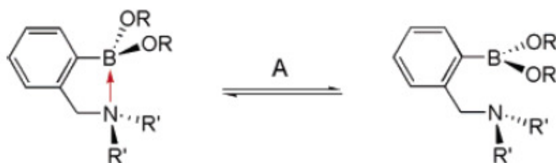
Because the simple boronic acids always have pKa value in a range from 8 to 10, considering the future application would mostly under neutral pH condition, the lowering of this value is highly demanded.

(2) Carbohydrate receptors developed under this concept

a.By means of employing B-N interaction

As introducing strong electro-withdrawing groups to aromatic ring of the boronic acid moiety requires high synthetic efforts. Here another practical strategy was employed.

The exchanges rate between free diols and diol esters of boronic acids can be greatly enhanced by neighbouring amino functionalities in boronic acids.



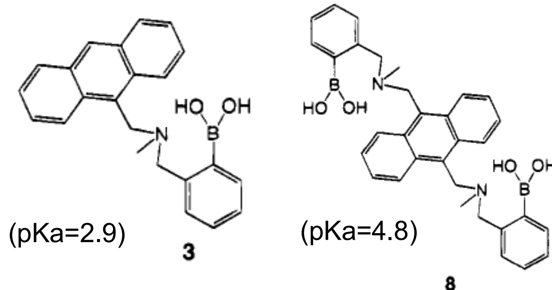
A: ligand replacement

S. Shinkai *et al. JACS,* **1995**, *117*, 8982-87

Table 1. Stability Constant (log K_a) for the Monosaccharide Complex with Boronic Acid **3** or **8**

saccharide or diol	boronic acid **3**: log *K* (r^2; data points)	boronic acid **8**: log *K* (r^2; data points)
D-glucose	1.8 (0.998; 9)	3.6 (0.998; 7)
D-fructose	3.0 (0.998; 9)	2.5 (0.999; 6)
D-allose	2.5 (0.995; 6)	2.8 (0.997; 9)
D-galactose	2.2 (0.998; 7)	2.2 (0.998; 11)
ethylene glycol	<0.4[a] (0.995; 4)	<0.2[a] (0.998;7)

[a] Upper limit calculated assuming that the observed $(I/I_0)_{max}$ is the saturation value.



The order of selectivity for monoboronic acid **3** is:

D-fructose> D-allose≈ D-galactose> D-glucose> ethyleneglycol

In comparison, the order is switched in diboronic acid **8** (log K_2-log K_1):

D-glucose(+1.8)> D-allose(+0.3)> D-galactose(0)> ethylene glycol(-0.2)> D-fructose(-0.5)

Reason for the reversion of selectivity probably due to the relative stability of **8b**

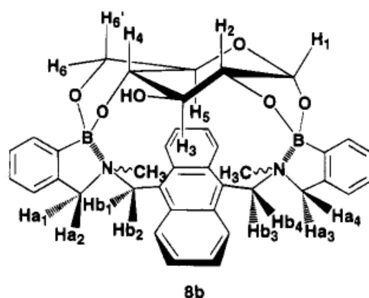


Table 2. ^{1}H NMR Assignment of D-Glucose Complex **8b**

assignment	chemical shift (ppm)	coupling constant (Hz)
	Complex	
H1	5.18	$J_{1,2} = 5.7$
H2	3.01	$J_{1,2} = 5.7$, $J_{2,3} = 7.5$
H3	−0.30	$J_{2,3} = 7.5$, $J_{3,4} = 7.5$
H4	2.68 (masked)	
H5	3.43	$J_{4,5} = 10.5$, $J_{5,6} = 9.3$ or 0, $J_{5,6'} = 9.3$ or 0
H6 and H6′	3.73	
Ha1 and Ha2 or Ha3 and Ha4	3.93 and 4.85 (masked)	$J_{1a,2a}$ or $J_{3a,4a} = 11.7$
Ha1 and Ha2 or Ha3 and Ha4	4.10 and 4.60	$J_{1a,2a}$ or $J_{3a,4a} = 11.7$
Hb1 and Hb2 or Hb3 and Hb4	5.66 and 6.80	$J_{1b,2b}$ or $J_{3b,4b} = 8.7$
Hb1 and Hb2 or Hb3 and Hb4	6.13 and 6.78	$J_{1b,2b}$ or $J_{3b,4b} = 8.7$
CH_3 or H_3C	2.42	
CH_3 or H_3C	2.68	
	Solvent	
$\mathit{CH_3}OH$	3.30	
$CH_3\mathit{OH}$	4.89	

1.Glucose was the best fits with the saccharide cleft and fructose was worst.

2. Molecular complementarity is important, and selectivity could be tuned by molecular design.

Chiral recognition has also been achieved by similar compound.

S. Shinkai *et al. Nature*, **1995**, *374*, 345-47

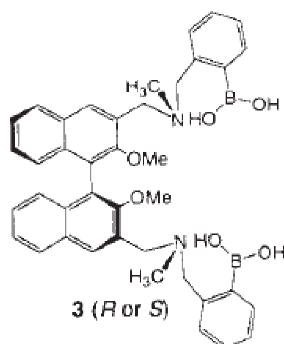
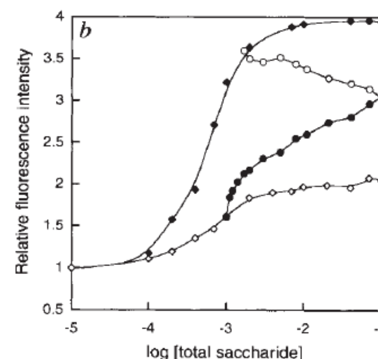


TABLE 1 Stability constants and fluorescence enhancements for saccharides with **3***R* (or *S*)

Saccharide	D log *K* (±0.05)	L log *K* (±0.05)	D/L fluorescence intensity ratio
Fructose	4.0 (3.7)	3.5 (4.0)	1.47 (0.69)
Glucose	3.3 (3.4)	3.1 (3.5)	1.93 (0.53)
Galactose	3.1	3.3	0.82
Mannose	<2.4	—	—

b, Chiral recognition for mixtures of enantiomers. The figure shows the fluorescence intensity log [total saccharide] profile of **3***R* at 25 °C; 1.0×10^{-5} M of **3***R* in 33.3% MeOH/H_2O buffer at pH 7.77, λ_{ex} 289 nm, λ_{em} 358 nm. D-Glucose (◆); L-glucose (◇); added D-glucose from initial condition of 0.001 M L-glucose (●); added L-glucose from initial condition of 0.001 M D-glucose (○).



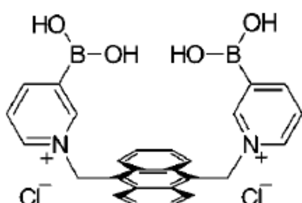
3R shows great stability of complexation with D-fructose, D-glucose, D-mannose and L-galactose

3S shows greater stability of complexation with L-fructose, L-glucose

This is one of few examples that could achieve chiarl carbohydrate recognition up to now.

b. Application of cationic moiety

The goal of lowering down boronic acid's pKa was also achieved by employing cationic moiety below.



*The cationic pyridinium not only lowered the pKa of boronic acid (down to 3.8), but also increased the solubility in water.

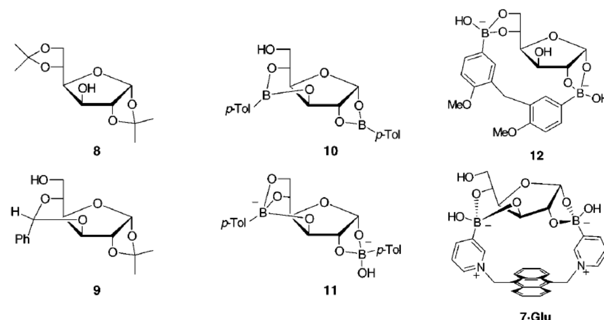
* Another intersting point observed in this compound is that binds to glucose in furanose form rather than pyranose form.

⇨ The recognition pattern would shift by subtle cleft change

Table 3. J_{H-H} Coupling Constants (Hz) for the Glucose Part of Boronic Acid Complexes and Model Compounds

compound	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6a}$	$J_{5,6b}$	$J_{6a,6b}$
7·Glu[a]	3.7	~0	4.4	7.3	7.5	3.7	11.3
7·Glu$_{6d}$[a]	3.5	~0	4.8	7.8	6.0	–	–
8[b]	3.6	~0	2.8	6.8	6.4	5.5	8.8
9[b]	3.8	~0	2.0	~0	6	6	11.5
10[b]	4.1	~0	2.4	~0	2.4	2.4	m.
11[c]	3.6	~0	2.8	2.6	~0	5.1	8.8
12[c]	4.0	~0	2.4	9.5	6.0	3.5	9.0
α-D-Glucopyranose[d]	3.8	9.9	9.6	9.6	2.2	5.5	12.3

[a] In D_2O at pD = 7.4. [b] In DMSO-d_6. [c] In D_2O at pD = 11–12. [d] In D_2O.[52]



J.C. Norrid et al. JOC, 1999, 64, 3846-52

Summary:

The method employing boronic acid-diol covalent interacion has successfully achieved carbohydrate recognition and is probably the most mature methodology in this field.

The reversible binding of boron to diol moieties in carbohydrate smartly avoid the competition of H_2O molecules because of stable ring formation.

However, there still remains several drawbacks in this strategy:

1. The pKa of boronic acid is a limitation.
2. The solubility of this group compound is mostly not so satisfying in neat water system.
3. Differentiation between carbohydrates is difficult as the recognizing part is not specific enough.
4. The boronic acid part is sensitive to other functional groups(e.g $-NH_2$), which limits its application scope.

3. Molecular recognition of carbohydrates via non-covalent interaction

Without the assistance of strong interactions like covalent bond, the recognizor design become much more chanllenging,because at this situation, the receptor must "fight" directly with H_2O molecules to catch carbohydrates.

Therefore, the real rational design of such receptors is highly demanded.

Criteria supposed to be met in recepor design:

a. Delicate array both polar and apolar functional groups to match the potential of a carbohydrate
b. Have rigidity to prevent intramolecular recognition or self-association
c. Show good solubility in water

Retrospecting the previous works, such receptors could be catagorized into two groups according to the molecule size---- Small molecule receptors and macromolecule templates.

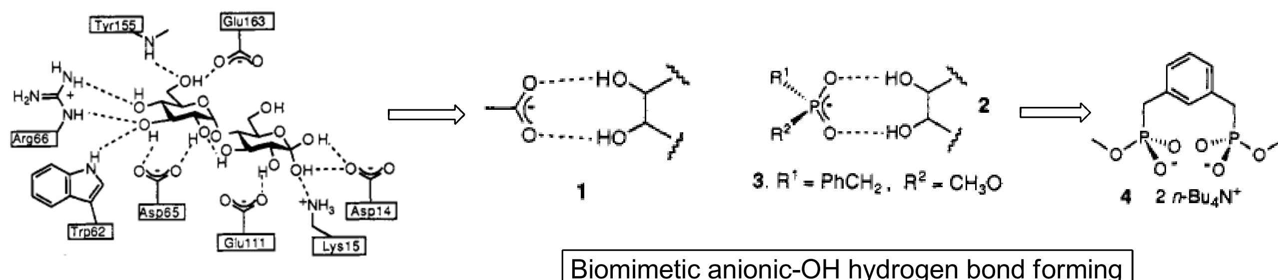
3-1. Designed small molecules as carbohydrate recognizor

Small molecule design is relatively easy to achieve because the interaction spots are limited and troubles like intramolecular recognition can be avoided.

A. Aromatic-centred compound

A.D. Hamillton *et al. JACS*, **1994**, *116*, 11139-40

Strategy:



Substrate recognition pocket of maltose binding protein

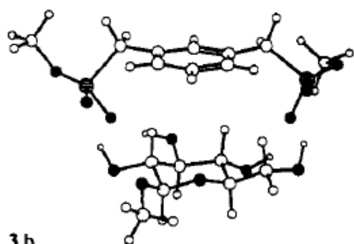
Design point:

1. Introduction of anionic function group ⟶ Selective recognition to diol moiety, increase solubility
2. Employing aromatic ring as backbone. ⟶ Introduce additional CH-π interaction
3. Bidentate form ⟶ Increase stablility of binding complex

Table 1. Association Constants ($K_{1:1}$)[a,b] (M^{-1}) of Tetrabutylammonium salts of Methyl Benzylphosphonate (**3**) and *m*-Xylene Bis(methyl phosphonate) (**4**) with Representative Substrates in CD_3CN at 20 °C

substrate	3	substrate	3	4
n-octanol	18	*cis*-cyclohexane-1,2-diol	2.1×10^2	
cyclohexanol	11	*trans*-cyclohexane-1,2-diol	3.3×10^2	
cyclopentanol	15			
3-hydroxytetrahydrofuran	42	1-*O*-octyl β-D-glucopyranoside	4.4×10^3	2.6×10^4
trans-2-methoxycyclopentanol	25	1-*O*-octyl α-D-glucopyranoside	4.2×10^3	1.8×10^4
cis-cyclopentane-1,2-diol	2.0×10^2	1-*O*-octyl β-D-galactopyranoside	3.9×10^3	2.5×10^4
trans-cyclopentane-1,2-diol	3.9×10^2	1-*O*-octyl α-D-mannopyranoside	4.0×10^3	3.6×10^4

[a] Results of ^{1}H NMR titrations performed by keeping the substrate concentration constant and varying the receptor concentration. All K_a's are the mean of at least two determinations. [b] Titration data analyzed using versions of the Hostest program. Errors for K_a's less than 10^4 were estimated at ±10%; for K_a's above 10^4, errors were estimated at ±20%.



structure for the complex between 4 and 1-O-methyl β-D-glucopyranoside

Achievement:

High affinity towards monosacchride--goals of design are almost achieved.

Drawbacks:

Only works in aprotic polar solvent.

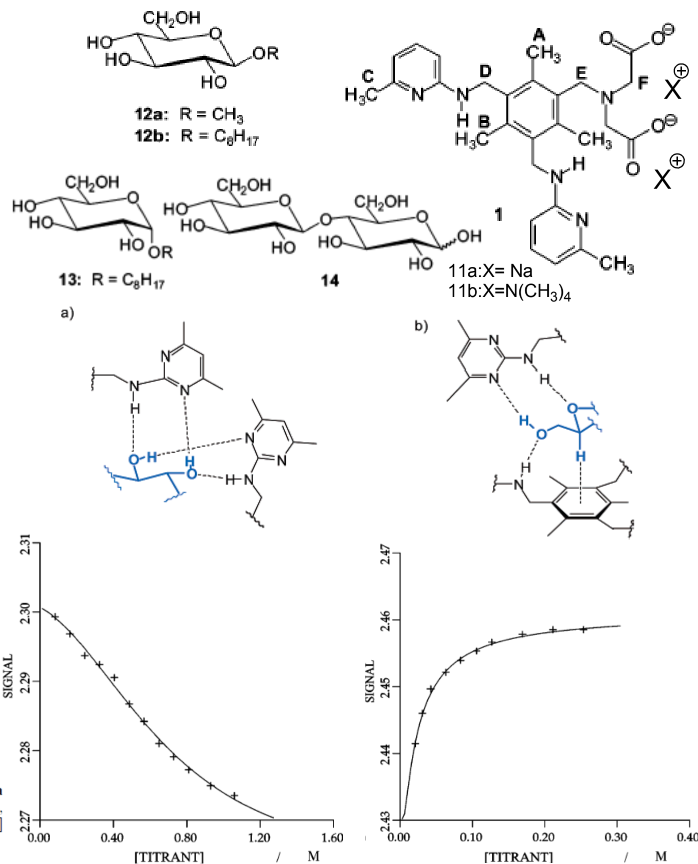
Differentiation between monosaccharides is impossible.

Does not show affinity towards unprotected monosaccharide

M. Mazik *et al. JOC*, **2006**, *71*, 2957-63

host−guest complex	solvent	K_{a1}	K_{a2}[c]	$\Delta\delta_{max}$[d] ($\Delta\delta_{obs}$)[e] [ppm]
11b·12b	$CDCl_3$[a]	119 420	4730	NH: 1.60 (1.60)[f]
11b·13	$CDCl_3$[a]	21 500	3900	NH: 1.62 (1.59)[f]
11a·12a	H_2O/D_2O[b]	2	72	CH: −0.04(−0.03)[g]
11a·14	H_2O/D_2O[b]	305	66	CH: 0.03 (0.03)[h]

[a] $CDCl_3$ was stored over activated molecular sieves and deacidified with Al_2O_3. For each system, at least three titrations were carried out. The error in a single K_a estimation was <10%. [b] H_2O/D_2O, 93:7, v/v. [c] Receptor/sugar complex, 1:2. [d] Change in chemical shift at saturation binding, values provided by HOSTEST.[13] [e] Largest change in chemical shift observed during the titration. [f] Complexation-induced shifts observed for the amine-NH of the receptor (the concentration of the receptor was kept constant and that of the sugar was varied). [g] Upfield complexation-induced shifts observed for the protons A of the receptor. [h] Downfield complexation-induced shifts observed for the protons C of the receptor. [i] Results from ref 10c.



1. Both hydrogen bonds and apolar interaction are successfully formed.((a)&(b))
2. The affinity dropped significantly in water system
3. The selectivity shifted from monosaccharide in organic solvent to dissacharide in water system.

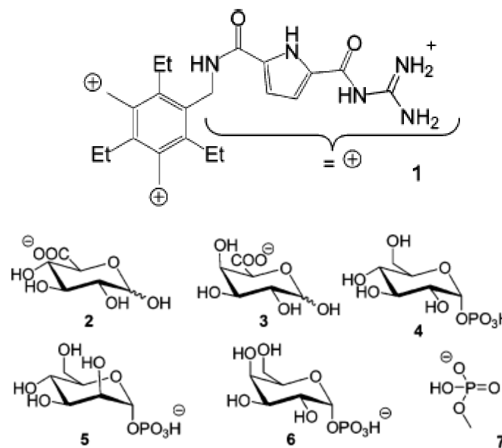
FIGURE 7. ^1H NMR titration of receptor **11a** with sugars **12a** and **14** in H_2O/D_2O (93:7, v/v). (a) Upfield chemical shifts of the protons A of **11a** are plotted against increasing *β*-D-glucopyranoside (**12a**) concentration; [**11a**] = 0.81 mM; equiv of **12a** = 70, 140, 215, 287, 358, 430, 502, 574, 645, 717, 820, and 900. (b) Downfield chemical shifts of the protons C of **11a** are plotted against increasing D-cellobiose (**14**) concentration; [**11a**] = 0.72 mM; equiv of **14** = 29, 44, 58, 87, 116, 145, 174, 233, 291, and 349.

C. Schmuck *et al. O.L.*, **2005**, *7,* 3517-20

Table 1. Association Constants K_{ass} (M^{-1}) Determined for the Binding of Anionic Substrates by Host **1** in Aqueous Solvents

substrate	K_{ass} (M^{-1})	pH	method[a]
glucuronic acid (**2**)	480		NMR
	3240	6.0	UV
galacturonic acid (**3**)	1550		NMR
	6160	6.0	UV
glucose-1-phosphate (**4**)	25 610	4.0	UV
	12 940	7.4	UV
galactose-1-phosphate (**5**)	21 150	4.0	UV
	12 160	7.4	UV
mannose-1-phosphate (**6**)	25 980	4.0	UV
	14 020	7.4	UV
methyl phosphate (**7**)	12 460	4.0	UV
	4850	7.4	UV

[a] NMR titration: 30% water in DMSO, error estimated to be ±10%; UV titration at pH = 4 (4 mM acetate buffer in 10% DMSO in water, $[host]_0$ = 20 μM) or at pH = 7.4 (10 mM bis-tris buffer in 70% DMSO in water, $[host]_0$ = 25 μM), error estimated to be ± 20%.



1. High affinity towards monosaccharide phosphate (Ka> 10^4 M^{-1}) was achieved.
   → Ion pairs formation levels up the affinity
2. Preference for saccharides to simple anions
   → Besides ion pair formation, H-bonds to the sugar also formed.
3. Preference for phosphate to carboxylate
   → Strongness of ion pair in this case is crucial
4. The affinity is pH depended
   → The protonation state of the receptor also plays key role in recognition

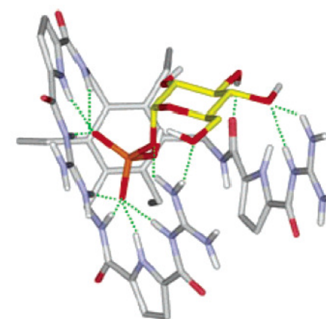


Figure 4. Calculated complex structure between **1** (gray) and **4** (yellow) showing the ion pair formation with the phosphate (left) and the additional H-bonds to the sugar OHs at C3, C4, and C6 (right). Nonpolar hydrogens have been omitted for clarity.

B. Metal complex

S. Striegler *et al.* *JACS*. **2003**, *125*, 11518-24

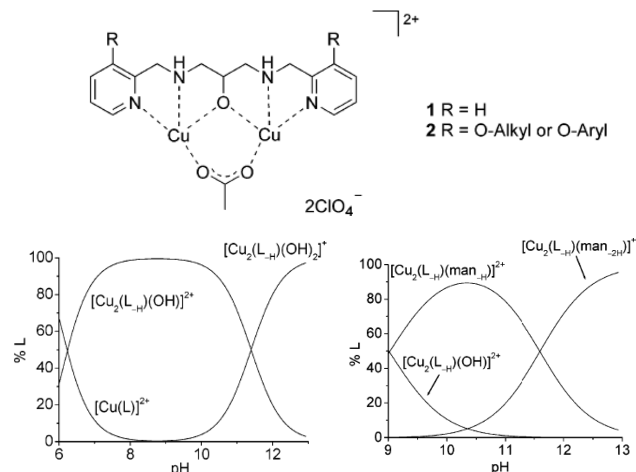


Table 1. Stability Constants for Monosaccharide−**1** Complexes, Determined in Aqueous Solution at pH 12.40 ± 0.01 and 25 °C[11]

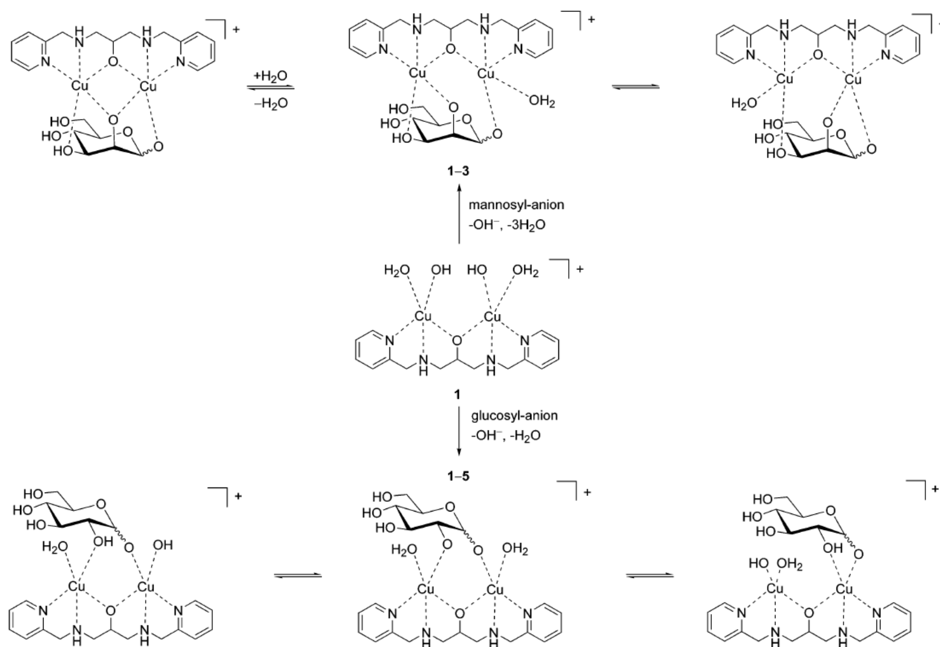
hexose	$pK_{app} \pm \Delta pK_{app}$	pentose	$pK_{app} \pm \Delta pK_{app}$
D-mannose (**3**)	4.06 ± 0.03	L-ribose (**9**)	4.11 ± 0.03
L-mannose (**21**)	3.98 ± 0.03	D-ribose (**6**)	4.07 ± 0.02
L-rhamnose (**22**)	3.75 ± 0.03	D-lyxose (**10**)	3.75 ± 0.04
D-fructose (**11**)	3.33 ± 0.04	L-lyxose (**12**)	3.75 ± 0.05
D-galactose (**4**)	3.02 ± 0.05	L-xylose (**13**)	3.58 ± 0.04
D-glucose (**5**)	2.56 ± 0.03	D-xylose (**7**)	3.55 ± 0.03
3-*O*-methyl-glucose (**19**)	2.52 ± 0.02	D-arabinose (**8**)	2.64 ± 0.02
		L-arabinose (**14**)	2.64 ± 0.03

Other experimental facts:

1. CD spectroscopy differs substantially between mannose and glucose.
2. Complex between mannose-**1** and glucose **1** showed differant absorption shift pattern in UV/vis.
3. Neither α-methyl mannose nor α-methyl glucose forms complex with **1**.
4. Removing C2-OH of either mannose or glucose fails the complex formation.
5. Methylation of C3-OH decrease the affinity to mannose significantly but slightly to glucose.

→ Differant binding pattern of **1** to mannose and glucose

Complex formation supposed by authors:



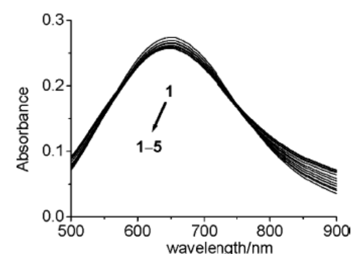


Figure 4. Selected UV/vis spectra observed during titration of binuclear copper(II) complex **1** (2 mM) with D-glucose (**5**) (0−12 mM).[12,13]

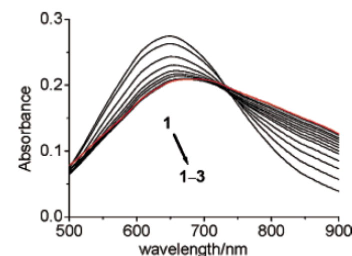


Figure 5. Selected UV/vis spectra obtained during titration of binuclear copper(II) complex **1** (2 mM) with D-mannose (**3**) (0−8 mM).[12,13]

This kind of recognizor is not "non-covalent" complex in the strict sense of the word, as the sugar OHs have got deprotonated and make ionic interaction to metal ions. However, this kind of recognizer has successfully employed the strategy of C-lectin. But at present this type recognizor is hardly reported.

3-2. Supramolecule as carbohydrate recognizor

A. Oligo-aromatic hosts

Strategy: Positive application of CH-π interaction as such hydrophobic forces are especially enhanced.

Y. Aoyama *et al*. *JACS*, **1992**, *114*, 10307-13

Table II. Binding Constants $(K)^a$ for the Complexation of Hosts **1b** and **1c**[b] with Various Guests and Saturation Shifts ($\Delta\delta_{sat}$) of the Aromatic 5-H of the Host[c]

	host			
	1b		**1c**	
guest	K (M^{-1})	$\Delta\delta_{sat}$ (ppm)	K (M^{-1})	$\Delta\delta_{sat}$ (ppm)
4t	19	0.42	24	0.39
6	125	0.41	64	0.42
7c	80	0.45	80	0.42
10	≤2.1	0.39	≤2.5	0.35
16	≤4.9	0.47	≤3.9	0.42
17	≤6.0	0.38	≤8.4	0.38
19β	<1[d]	0.38	≤2.4	0.26
23	68	0.30	47	0.28
24	≤4.6	0.30		
27	110	0.30		

[a] See ref 26 for the treatment of small binding constants. [b] [**1a**] = 2 mM and [**1b**], [**1c**] = 2, 1, or 0.5 mM in D_2O at 25 °C. [c] Positive value indicates a downfield shift. [d] The actual value obtained by the Benesi–Hildebrand analysis is 0.60.

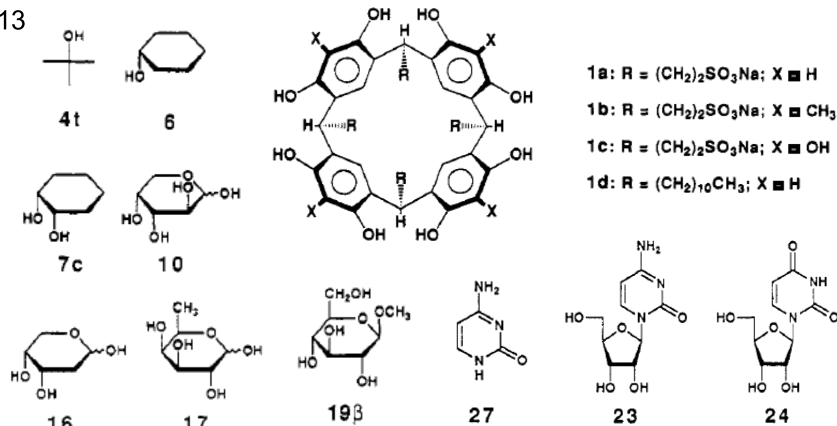


Table I. Binding Constants $(K)^a$ for the Complexation of Host **1a**[b] with Various Guests and Saturation Shifts ($\Delta\delta_{sat}$) for the Aromatic 5-H of the Host[c]

guest	K (M^{-1})	$\Delta\delta_{sat}$ (ppm)	guest	K (M^{-1})	$\Delta\delta_{sat}$ (ppm)
2	<1[d]	0.46	**14**	~0	
3	≤1.7	0.36	**15**	~0	
4n	≤2.7	0.41	**16**	≤1.2	0.38
4i	≤3.1	0.42	**17**	≤1.8	0.40
4s	≤3.5	0.37	**18β**	<1[d]	0.53
4t	≤4.2	0.42	**19α**	<1[d]	0.42
5	≤5.7	0.39	**19β**	<1[d]	0.47
6	16	0.43	**20α**	≤1.8	0.46
7c	14	0.46	**21**	≤2.3	0.41

guest	K (M^{-1})	$\Delta\delta_{sat}$ (ppm)	guest	K (M^{-1})	$\Delta\delta_{sat}$ (ppm)
7t	14	0.42	**22**	~0	
8	~0		**23**	26	0.30
9	<1[d]	0.26	**24**	≤1.2	0.30
10	<1[d]	0.27	**25**	≤3.6	0.32
10L	<1[d]	0.27	**26**	20	0.33
11	~0		**27**	29	0.38
12	~0		**28**	~0	
13	~0				

[a] See ref 26 for the treatment of small binding constants. [b] [**1a**] = 2 mM in D_2O at 25 °C. [c] Positive value indicates a downfield shift. [d] The actual values obtained by the Benesi–Hildebrand analyses are 0.27 (**2**), 0.44 (**9**), 0.85 (**10**), 0.85 (**10L**), 0.39 (**18β**), 0.41 (**19α**), and 0.40 (**19β**).

From the result several points could be concluded:

1. Apolar CH moieties provide the primary binding sites.
2. Ch-π interactions lay great influence upon binding affinity
3. The binding affinity are almost insensitive to the hydrophility of guest compounds
4. The affinity to all carbohydrates' cases is very low (Ka<10).

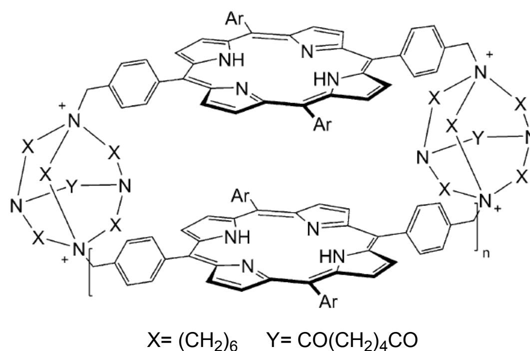
B. porphyline

F.P. Schmidtchen *et al*. *OL*, **2001**, *3*, 873-76

Table 1. Association Constants for Binding of Saccharides to Receptors **4–6** in Water monitored by UV–Vis[a]

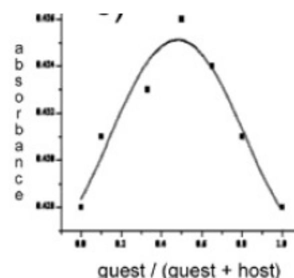
	association constant log K_a ± exp error range		
saccharide	**4**	**5**	**6**
D-galactose	3.10 ± 0.14	3.52 ± 0.07	3.32 ± 0.06
D-glucose	3.14 ± 0.13	3.63 ± 0.06	3.08 ± 0.17
methyl-α-D-glucoside	3.75 ± 0.06	3.89 ± 0.06	3.77 ± 0.08
methyl-β-D-glucoside	3.14 ± 0.15	3.36 ± 0.08	3.04 ± 0.11
octyl-α-D-glucoside	3.20 ± 0.11	3.86 ± 0.03	3.74 ± 0.11
D-trehalose	3.96 ± 0.05	3.98 ± 0.07	3.62 ± 0.06
D-lactose	4.45 ± 0.16	3.81 ± 0.07	3.74 ± 0.04
maltotriose	4.72 ± 0.06	4.24 ± 0.10	3.78 ± 0.12

[a] The formation constants (UV–vis determination) of sugar–receptor complexes. In a 1 cm square quartz cuvette was placed a 2.4×10^{-6} M solution of macrocycle **4**, **5**, or **6** in H_2O containing 5% of MeOH (v/v). Saccharide was added in aliquots of a stock solution (0–100 equiv; the solution contained the same concentration of receptor as in the cuvette). The absorbance changes at the position of the Soret band were measured (room temperature), and the data were evaluated with the aid of least squares curve fitting. The K_a was calculated for 1:1 complexes and averaged over four independent determinations.



X= $(CH_2)_6$ Y= $CO(CH_2)_4CO$

4: n=1 Ar= H 5: n=1 Ar= *p*-tolyl 6: n=0 Ar= *p*-tolyl



Job plot indicating a maximum at a mol fraction of 0.5 (i.e. 1:1 stoichiometry)

1. Ka shows in order of mono-< di-< tri-, but diminishes again with higher oligomers of glucose
2. Able to differentiate α- and β- anomers.
3. **4/5** is much affable to trisaccharide moiety.

C. Cyclodetrins

Cyclodextrins are also exploited as carbohydrate recognizor by taking advance of its fine solubility in water and probable carbohydrate-carbohydrate interactions.

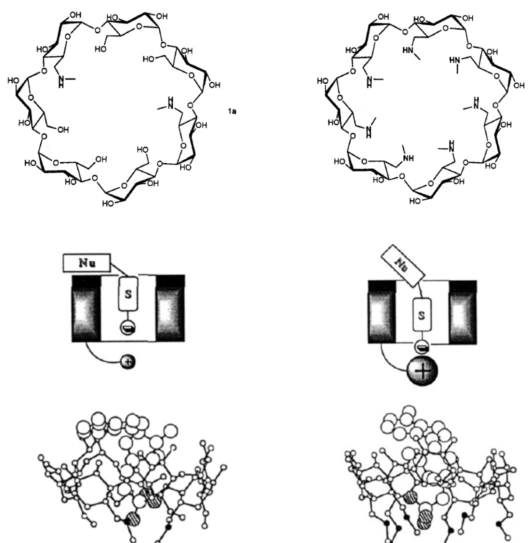
H. Schneider *et al. JACS*, **1994**, *116*, 6081-88

Table 1. Binding Constants K (M^{-1}) and ΔG(Kj/mol) in complexation

$$RPO_3^{2-} + CyD^{n+} = (RPO_3 \cdot CyD)^{(n-2)+}$$

Nr	guest	1a		1b		β-CyD
		10^{-3} K	-ΔG	10^{-5} K	-ΔG	K
1	5′-AMP	14.1	23.7	1.26	29.1	90[a]
2	3′-AMP	1.51	18.1	0.92	28.2	250[b]
3	d-5′-AMP	0.48	15.3	1.17	28.9	
4	5′-GMP	6.16	21.6	0.40	26.2	
5	d-5′-GMP		<15	5.89	32.9	
6	5′-CMP	0.83	16.6	0.20	24.5	
7	d-5′-CMP		<15	0.44	26.5	
8	5′-UMP	0.83	16.6	0.87	28.1	
9	d-5′-UMP		<15	0.38	26.1	
10	5′-ATP[c]	97.7	28.4	32.4	37.1	
11	RP	11.2	23.1	8.51	33.8	
12	d-RP	2.4	19.3	8.13	33.7	
13	PO_4^{3-}	0.20	13.1	0.037	20.3	
14	ribose			0.00026	8.1	1.0

[a] The value from ref 7a. The same constant as well as related ones measured elsewhere[34] by chromatography was found to be much higher, perhaps due to additional interactions with a stationary phase. [b] The value from ref 6b. [c] Tetraanion.



1. Nucleotides recognition is achieved at hight affinity (Ka>10^3 in **1a**, >10^5 in **1b**)
2. The electrostactic interactions play key role in complexation rather than the interaction to neutral carbohydrate moiety
3. High nucleabase selectivity is achieved in **1a**. Purine-based nucleatides (AMP,GMP) shows specially high affinity.

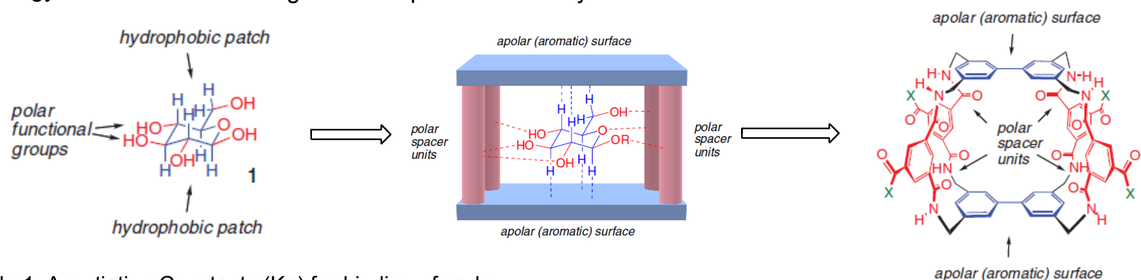
The effect of direct recognition to carbohydrates was also checked by Aoyama group at earlier time (*ACIE*, **1992**, *31*, 745-47)Using CyD template as recognizor is not so efficient as expectation.

D. Ab initio desigbed Cyclic cages

Original design of a cyclic molecule was synthesized by A.P. Davis' group , which provide an example for rational design of carbohydrate receptor.

A.P. Davis *et al*. *ACIE*, **2005**, *44*, 298-302

Strategy: Molecular design for all-equatorial carbohydrates

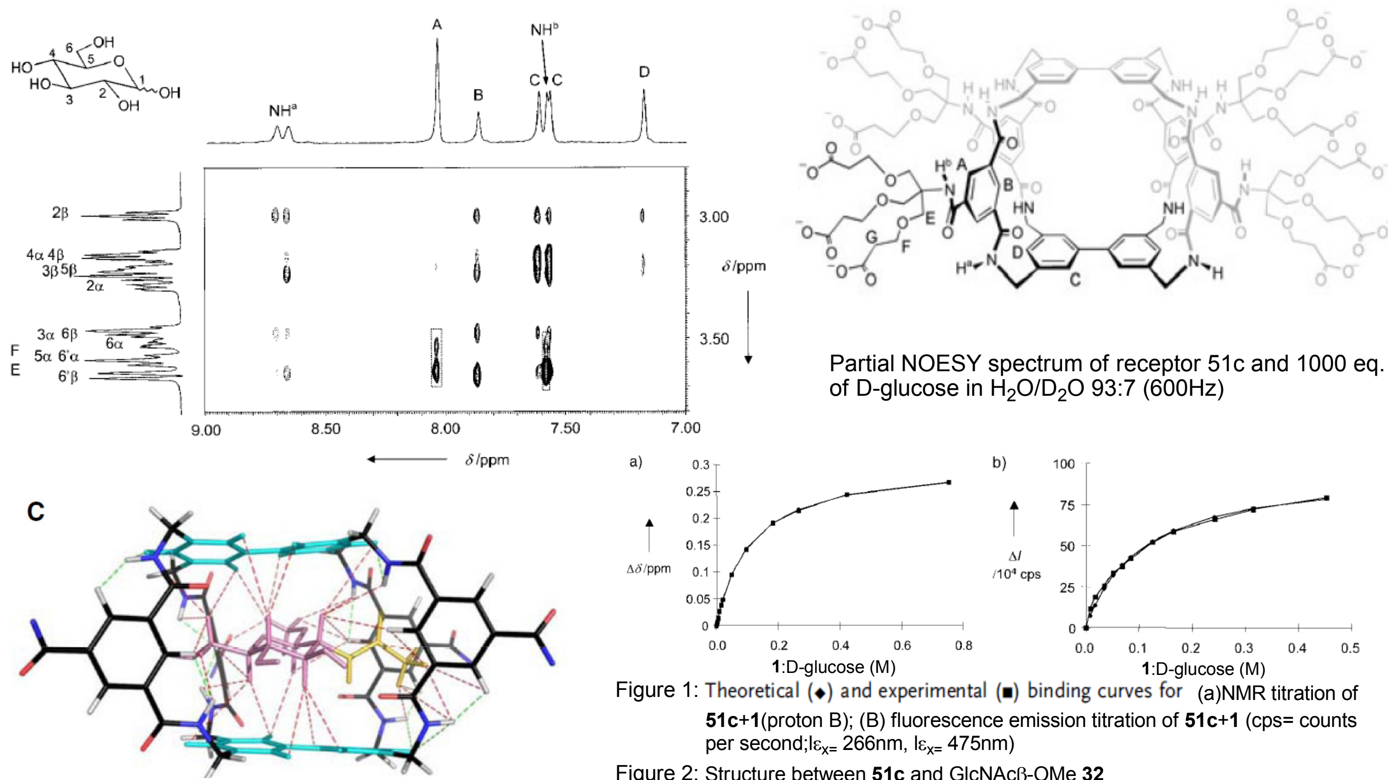


51c: X= $NHC(CH_2OCH_2CH_2COO^-)_3$

Table 1: Assotiation Constants (Ka) for binding of carbohyadrates in water to 51c

Substrate[a]	K_a (M^{-1}) for binding to 51c[b]	K_a (M^{-1}) for binding to whea germ agglutinin
GlcNAcβ-OMe **32**	630[d]	730
GlcNAc **15** (α:β = 64:36)	56	410
Methyl β-D-glucoside **27**	28	
GlcNAcα-OMe **33**	24[e]	480
D-Cellobiose **29**	17	
D-Glucose **1**	9	
2-Deoxy-D-glucose **14**	7	
Methyl α-D-glucoside **28**	7	
D-Xylose **6**	5	
D-Ribose **4**	3	
D-Galactose **9**	2	

Substrate[a]	K_a (M^{-1}) for binding to 51c[b]	K_a (M^{-1}) for binding to wheat germ agglutinin[c]
L-Fucose **11**	2	
N-acetyl-D-galactosamine **16**	2	60
N-acetyl-D-mannosamine **17**	2	60
D-Arabinose **5**	2	
D-Lyxose **7**	≤2	
D-Mannose **10**	≤2	
L-Rhamnose **12**	≤2	
D-Maltose **30**	≤2	
D-Lactose **34**	≤2	
N-acetyl-D-muramic acid **35**	0[f]	
N-acetyl-D-neuraminic acid **36**	0[f]	560
N,*N*′-diacetylchitobiose **37**	0[f]	5,300



Partial NOESY spectrum of receptor 51c and 1000 eq. of D-glucose in H_2O/D_2O 93:7 (600Hz)

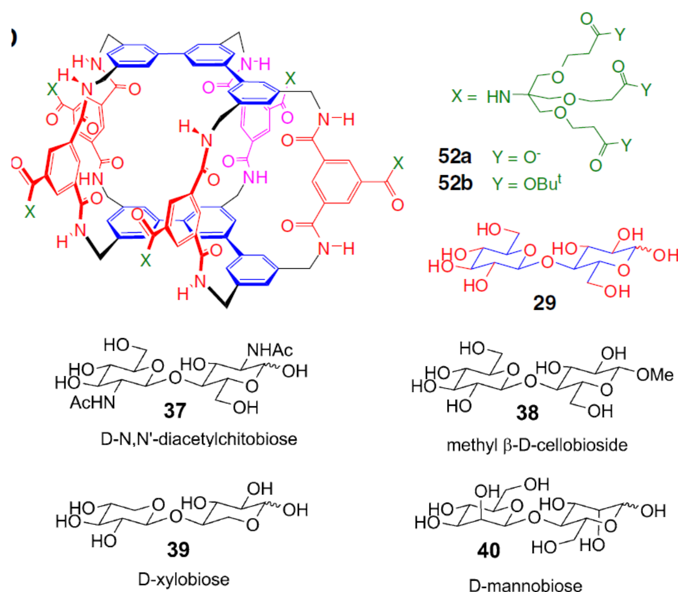
Figure 1: Theoretical (◆) and experimental (■) binding curves for (a)NMR titration of **51c**+**1**(proton B); (B) fluorescence emission titration of **51c**+**1** (cps= counts per second;$I\varepsilon_{x=}$ 266nm, $I\varepsilon_{x=}$ 475nm)

Figure 2: Structure between **51c** and GlcNAcβ-OMe **32**

A.P. Davis et al. Science, 2007, 318, 619-22

Based on the same idea, similar receptor was also developed to recognize oligosaccharides by extending the receptor sphere.

Substrate[a]	^{1}H NMR	ICD	Fluorescence
D-Cellobiose **29**	600	580	560
Methyl *β*-D-cellobioside **38**		910	850
D-Xylobiose **39**		250	270
D-*N,N'*-diacetylchitobiose **37**	120		120
D-Lactose **34**		11	14
D-Mannobiose **40**		13	9
D-Maltose **30**		15	11
D-Gentiobiose **41**		12	5
D-Trehalose **42**	0[b]	0[b]	
D-Sucrose **43**		0[b]	0[b]
D-Glucose **1**	11	12	0[b]
D-Ribose **4**		0[b]	0[b]
D-*N*-acetylglucosamine **15**	24		19



1. Good affinity to all-equatorial disaccharides especially **29**
2. This complexation process is enthalpy driven (ΔH= -3.22 kcal/mol) and a minor contribution from entropy (TΔS= 0.62 kcal/mol), which lies well within the range observed for lectins.

This kind of carbohydrate receptors achieved a high resembleness to natural lectins yet do not suffering denaturation problem. Also by tuning external functional groups, they can confer good solubility in almost any medium, which provides an ideal way to clarify the role of solvent in natural carbohydrate recognition.

Summary:

1. Carbohydrate recognition in aqueous media has been approached either from covalent interaction or non-covalent interaction strategy, and a number of such sugar-receptors have been developed.
2. Most of so far developed receptors still suffer a low affinity (Ka>100 is rare in rare)
3. Still no efficient methodology and clear conception in receptor design.
4. Far from realizing the recognition of much more complicated oligo-saccharide chain, which is most common form in various biological process contributed by carbohydrates.

As synthetic carbohydrate receptors could be used as drugs (eg. anti-infective agents) or drug tranportors to target at specific cell types, or just as sugar-chain sensor in diagnosis or medical treatment, the deveplopment in this field is highly demanded. However, how to conquer the limits listed above remains of much difficulty. Really looking forward to a breakthrough that scientists can catch this "sweet genie" out of "troubled water".