University of Alberta

Design, Synthesis, and Screening of a Library of Peptidyl Bis-Boroxoles as Low Molecular Weight Receptors for Complex Oligosaccharides in Neutral Water: Identification of a Selective Receptor for the Tumour Marker TF-Antigen

by

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Abstract

The ability of boronic acids to bind reversibly to simple carbohydrates in neutral water can be exploited to address the problem of cell-surface recognition. Recently, it has been shown that benzoboroxole can complex hexopyranosides under physiological conditions. [1] These hemiboronate units were essential to the design of a small library of well-defined peptidyl-diboroxole receptors for complex oligosaccharides.

The library was synthesized using a combinatorial solid-phase approach with the Irori® technology, and it was screened in a biochemical assay for the selective recognition of the T-antigen disaccharide, a cancer-associated cell-surface marker. A few high-affinity receptors of low micromolar IC_{50} were identified, and their binding behavior in neutral water was characterized using competition experiments and a systematic evaluation of analogues. These results suggest that low molecular weight receptors for biologically relevant glycoconjugates could be made to rival the efficiency of Nature's carbohydrate-binding proteins.

References

[1] Dowlut, M.; Hall, D.G. *J. Am. Chem. Soc.* **2006**, *128*, 4226-4227.

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Table of Contents

List of Figures

List of Schemes

List of Tables

List of Abbreviations

Ac	Acetyl
Ala	Alanine
Alloc	Allyloxycarbonyl
Anal.	Elemental Analysis
Ar	Aryl group
9BBN	9-Borabicyclononane
Bn	Benzyl
BOC	*Tert*-butoxycarbonyl
br s	Broad singlet
BSA	Bovine serum albumin
Bu	*n*-Butyl
*t*Bu	*tert*-Butyl
^{o}C	Degree Celcius
Calcd	Calculated
cat	Catalytic amount
Cy	Cyclohexyl
dd	Doublet of doublets
ddd	Doublet of doublets of doublets
DIBAL-H	Diisobutylaluminum hydride
DCM	Dichloromethane

DMAP	Dimethylaminopyridine
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethylsulfoxide
dt	Doublet of triplets
EI	Electron Impact
equiv.	Equivalents
ESI	Electrospray Ionization
Et	Ethyl
HBTU	*O*-Benzotriazoyl-1-yl-*N*,*N*,*N'*,*N'*-tetramethylluronium
HOAt	1-Hydroxy-7-azabenzotriazole
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
ICD	Induced Circular Dichroism
IR	Infrared Spectroscopy
Hz	Hertz
J	Coupling constant
K_a	Binding association constant
K_d	Binding dissociation constant
m	Multiplet
Me	Methyl
mg	Milligrams
min	Minutes

mL	Milliliters
NMR	Nuclear magnetic resonance
Nu	Nucleophile
OAc	Acetoxy
OBn	Benzyloxy
OEt	Ethoxy
O*i*-Pr	*iso*-Propoxy
O*t*-Bu	*tert*-Butoxy
OMe	Methoxy
PBS	Phosphate buffered saline
Ph	Phenyl
PNA	Peanut agglutinin
PP	Polypropylene
Pr	*n*-Propyl
i-Pr	*iso*-Propyl
q	Quartet
qt	Quartet of triplets
quint	Quintet
R	Generic alkyl group
RT	Room temperature
t	Triplet
TBAF	Tetra *n*-butyl ammonium fluoride

TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin Layer Chromatography
TMB	Tetramethylbenzidine
TMEDA	*N*,*N*,*N*',*N*'-Tetramethylethylenediamine
UV	Ultraviolet

Chapter 1

Introduction

1.1 Carbohydrates

Carbohydrates are amongst the most abundant classes of organic molecules found in living organisms, and exhibit a wide structural diversity in nature. They are defined as polyhydroxyaldehydes (aldoses) or polyhydroxyketones (ketoses).

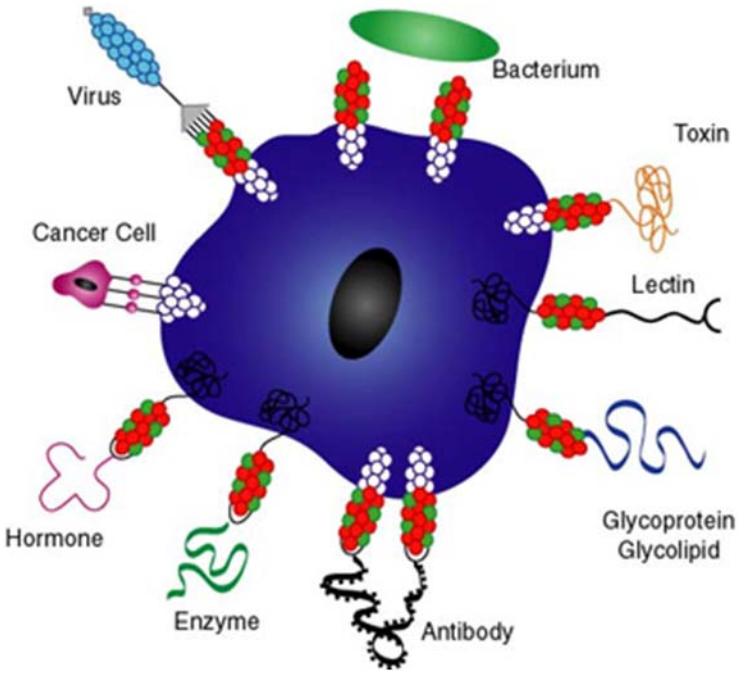


Figure 1.1: Cell-surface carbohydrates are involved in molecular recognition (Reprinted from reference 1).

In living cells, carbohydrates reside in complex structures such as glycolipids, glycoproteins and proteoglycans, etc. Interactions between carbohydrates and proteins play a key role in many biological functions. Figure 1.1 represents how cell-surface carbohydrates are involved in molecular recognition.[1] The recognition of carbohydrates as a medicinally important class of biomolecules has led to the investigation of therapeutic agents based on glycan structure.[2,3]

1.2 Lectins: carbohydrate binding proteins: peanut agglutinin

Lectins are carbohydrate-binding proteins that are highly specific for sugar moieties, and possess no catalytic activity.[4,5] Concanavalin A was the first lectin to be isolated,[6] sequenced and purified on a large scale and available on a commercial basis. Lectins mediate a range of biologically processes such as cell-cell interactions. They are usually oligomeric proteins composed of subunits and each unit has one carbohydrate-binding unit. Lectins are used in detecting and studying carbohydrates in solution and cell surfaces. Based on the specificity of monosaccharides towards lectins, they are categorized into five groups (mannose, galactose/N-acetylgalactosamine, N-acetylglucosamine, fucose and N-acetylneuraminic acids). In general, lectins bind reversibly to monosaccharides and oligosaccharides with normally modest binding constants in the range of 10^{-3} to 10^{-6} M. [7-9]

PNA (Peanut Agglutinin) is a 110,000 molecular weight lectin isolated from peanuts (*Arachis hypogea*),[10] and first characterized by Sharon and co-workers.[11] It is composed of four identical subunits of approximately 27,000 Da each,[12] and each subunit is a carbohydrate binding site containing one divalent cation of Ca^{+2} and Mg^{+2}.[12] PNA binds preferentially to β-galactopyranosyl-(1→3)-D-N-acetylgalactopyranosamine (**1**), also known as the Thompson-Friendenreich antigen, "TF-antigen" (Figure 1.2), with a binding constant of 1 x 10^{-7} M.[13-14]

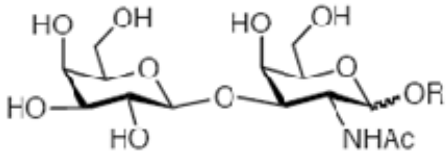


Figure 1.2: β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactopyranose, TF antigen disaccharide (**1**).

The TF-antigen is associated with the 90% of all types of human cancer cells, including many glycoconjugates such as M and N blood groups and many other soluble and membrane-associated glycoproteins and glycolipids.[15] As depicted in Figure 1.3, the galactoside ring in the TF antigen forms hydrogen bonds either directly or via water molecules (**w**) with the amino acids of the PNA lectin. On the other hand, the GalNAc ring is hydrogen bonded mainly to the extended site of the lectin.

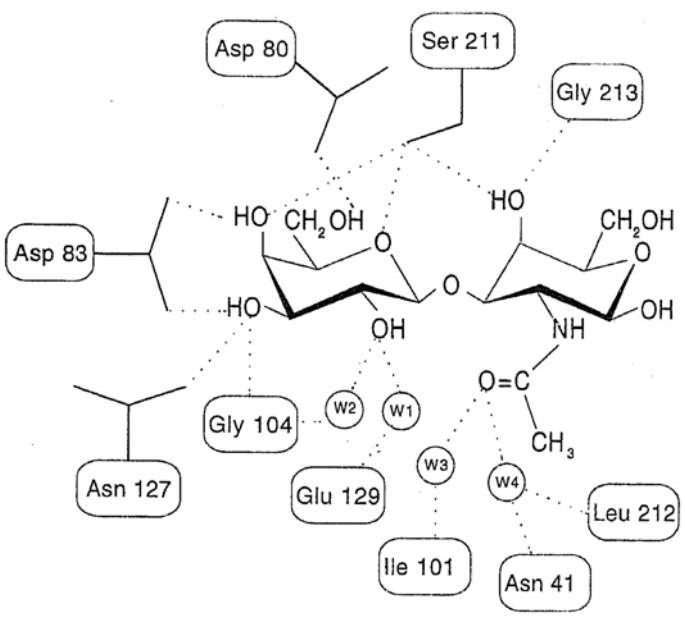


Figure 1.3: β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactopyranose in the combining site of PNA lectin

(Reproduced from reference 16).

1.3 Synthetic carbohydrate receptors

Cell surface carbohydrates are responsible for the majority of communication events between the cell and its surroundings. Consequently, synthetic receptors and sensors will aid in the understanding of the roles of carbohydrates in cells and allow for the elucidation of the mechanism of different biological processes that occur in the cell through recognition of specific carbohydrates. This will eventually open the door for the development of diagnostic probes for the detection of deadly diseases such as cancer, plague, etc.

In the area of medicinal and clinical chemistry, there is always a demand for small receptor molecules that bind with high affinity and selectivity with biomolecules. Carbohydrate receptors play an important role in addressing the fundamental problem of comprehending binding and selectivity. Tremendous amounts of work have been accomplished in targeting biomolecules such as polypeptides (proteins and amino acids), and nucleic acids (DNA/RNA). However, comparatively very little research has been done on the development of receptors for oligosaccharides. This can be rationalized through the lack of variety in functionalization in carbohydrates, as carbohydrates are mainly polyols.

It is very challenging to design small organic receptors that are able to bind simple, nonionic sugars via noncovalent interations.[17] Synthetic receptors can display different binding affinity depending on the choice of solvent. Use of protic solvents can modify or enhance intermolecular interactions like hydrogen bonding or charge-pairing.[18] It is also important to target sugars in aqueous medium for the long term plan of in vivo biological applications. Synthetic receptors, however, suffer from low solubility in aqueous solvents. However, Davis and co-workers recently showed that it is not impossible to bind carbohydrates in water.[19] They synthesized a cage like receptor that binds the disaccharide cellobiose in water with a K_d of 1.18 mM. Their receptor includes roof and floor motifs composed of aromatic hydrocarbons, which can promote hydrophobic attractions reinforced by CH–π interactions. These aromatic regions are composed of

polar groups that can undergo hydrogen bonds to the carbohydrate hydroxyl groups. The binding includes non-covalent interactions.

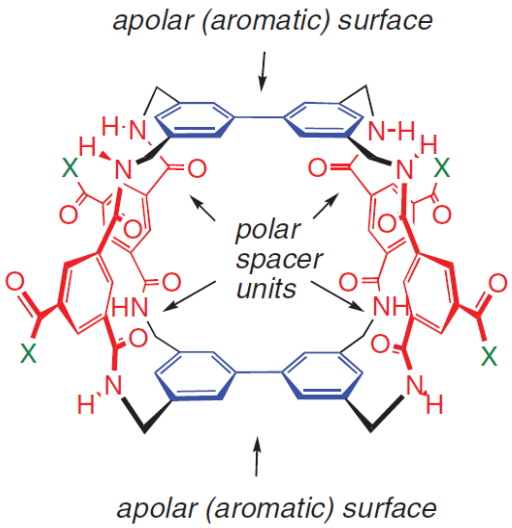


Figure 1.4: Synthetic lectin devised by Davis and co-workers.[19]

Miller and co-workers reported selective recognition of alkyl pyranosides in protic and aprotic solvents.[20] They synthesized and analyzed three novel tripodal receptors for monosaccharides using a *cis*-1,3,5- trisubstituted cyclohexane as a core structural element (Figure 1.5). According to their study, compounds **2** and **3** were the most potent receptors compared to **1** in targeting pyranosides. Importantly, compound **2** showed the highest affinity for noncovalent recognition of monosaccharides in a protic solvent (methanol).

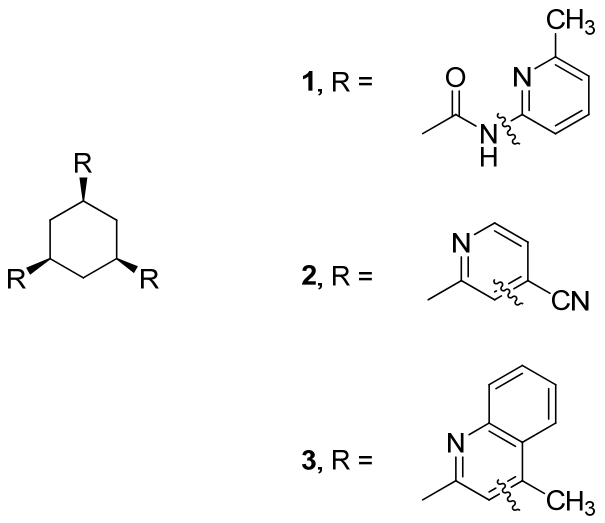
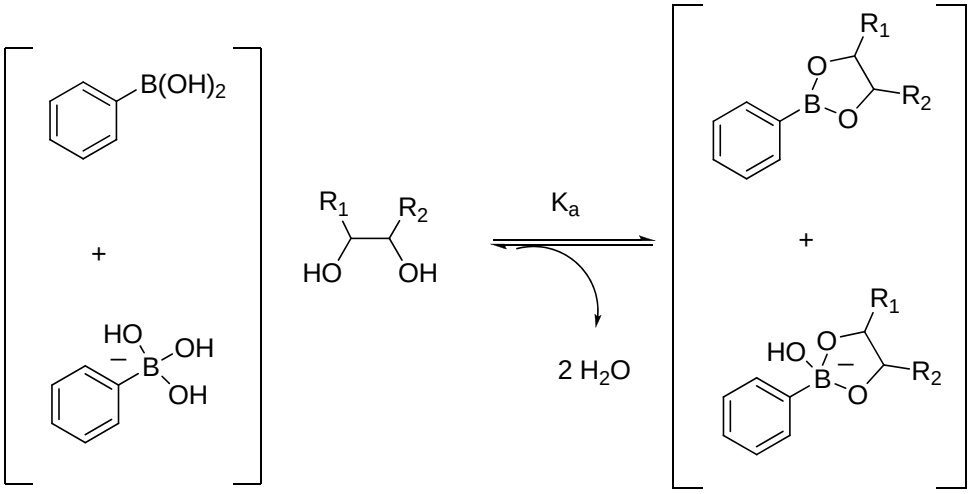


Figure 1.5: Structure of cyclohexane based receptors by Miller and co-workers.[20]

1.4 Boronic acid receptors and sensors for saccharides

Over the last few decades, boronic acids have become an extremely important class of compounds in chemistry. In synthetic medicinal chemistry, boronic acids have been used as important building blocks and intermediates in the Suzuki cross-coupling reaction,[21] Diels-Alder reactions,[22] and carboxylic acid activation.[23,24] Additionally, boronic acids have been employed in a variety of biological and medicinal applications including carbohydrate recognition,[25] enzyme inhibition,[26] and neutron capture therapy for cancer.[27] The increasing popularity of the boronic acids can be ascribed to their stability and ease of handling. Boronic acids can be regarded as "green" for their lack of toxicity and their ultimate degradation into the environmentally friendly boric acid. The recent FDA approval of Velcade® as an anticancer agent confirms the new status of boronic acids as an essential class of compounds in chemistry and medicine.[28]

In 1957, Kuivila and co-workers were first to describe the phenomenon of complex formation between an aromatic boronic acid and different diols.[29] Since this seminal paper, extensive work has been accomplished on complexation of carbohydrates.[30] Boronic acid receptors can also be used in industry, for analyzing fermentation processes. The main characteristic of boronic acids as receptors is that they bind reversibly in a covalent fashion with diol functionalities.



Scheme 1.1: Boronic acid - diol equilibrium.

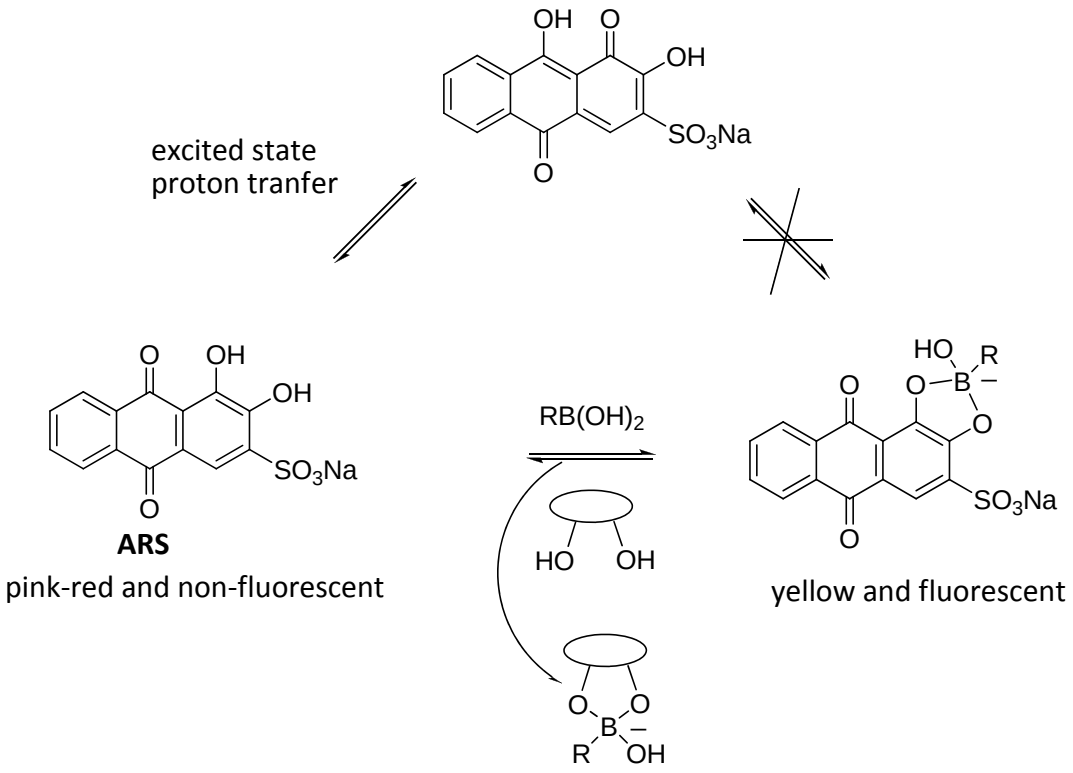
In 1959, Lorand and co-workers examined the binding affinity between various diols and phenylboronic acid in water.[31] They noticed that the lowering of pH in the solution correlates nicely with the decrease of the binding constant. However, the binding constants measured by this method were later showed to differ from the binding constants

obtained by spectroscopic methods. This inconsistency was explained to be a result of discrepancies arising from the proper definition of "binding constants".[32]

There are several methods for measuring binding constants between diols and boronic acids. Among all these, ^{11}B NMR[33-34] and fluorescence[32,35-36] methods are the most conventional. The ^{11}B NMR method is based on the fact that addition of a diol to a boronic acid solution results in a complex with a tetrahedral sp^3 boron center distinct from the trigonal form of free boronic acid. The ^{11}B NMR method tends to give binding constants similar to the pH depression method. However, the ^{11}B NMR method suffers from low sensitivity (a result of quadrupolar line broadening), difficulties with peak resolution, and the necessity of high concentrations of boronic acid.

Additionally, spectroscopic methods such as circular dichroism (CD)[37-38] ultra-violet absorption[39] and fluorescence are generally more sensitive than the NMR or pH depression methods. They operate by detecting the variations in the spectroscopic behavior of boronic acid upon binding with diols. Fluorescent methods cannot be applicable for those boronic acids that are not fluorescent. To overcome this drawback, Springsteen and Wang came up with a competitive dye displacement assay, which is more general.[32] This method involves the equilibrium shown in Scheme 1.2 where a boronic acid forms a yellow fluorescent complex with the pink red dye Alizarin Red S. This yellow colored fluorescent complex can revert back to the free dye upon the addition

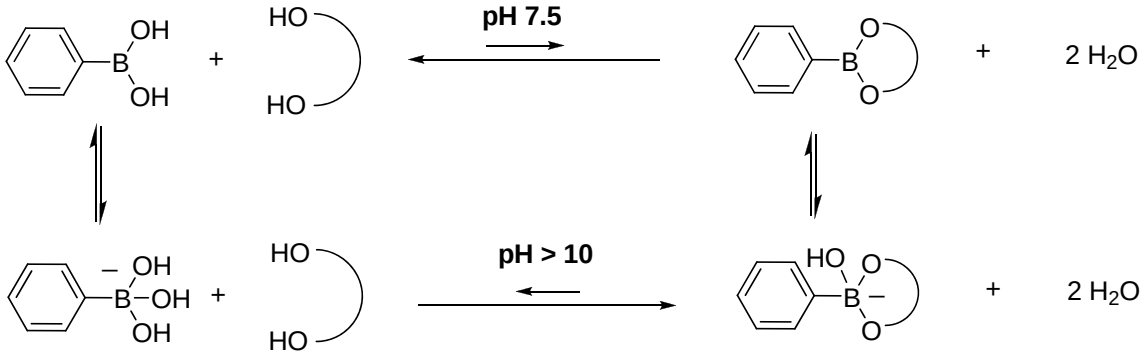
of a diol (or a carbohydrate). This whole procedure can be observed visually and can also be measured spectrophotochemically to deduce the binding constant between a given boronic acid and a carbohydrate.



Scheme 1.2: Representative equilibrium between Alizarin Red S, a boronic acid, and a diol.

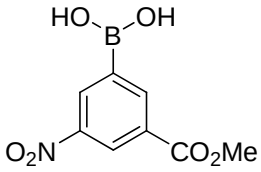
1.4.1 Effect of pH on boronic acid complexation of diols

Phenylboronic acid mainly exists as a free acid at neutral pH 7.4. In the complexation of a 1,2 diol, a high pH (>10) is generally required in order to favor the equilibrium (Scheme 1.3) toward the dialkoxyboronate anion.[30,31] This is due to the fact that at higher pH, the formation of sp^3 centers leads to the release of ring strain in the resulting 5-membered complex. Obviously, a neutral pH is required to recognize sugars in blood. It was acknowledged that reducing the pKa of the boronic acid can probably address this issue of unfavored equilibrium.



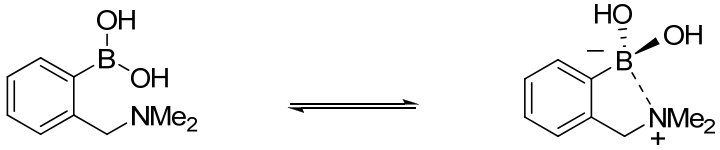
Scheme 1.3: Complexation between aryl boronic acids and diols in water**.**

Introducing electron-withdrawing groups can bring down the pKa of arylboronic acids by stabilizing the developing negative charge during the formation of a boronate ion. For example, when compared to phenylboronic acid (pK_a ~ 9), it can be noticed that pKa of the following boronic acid (Figure 1.6) is affected significantly with the electron-withdrawing substituent.



pK_a = 7.9

Figure 1.6: Arylboronic acid with low pK_a value**.**



Scheme 1.4: Putative B–N dative bond in Wulff-type boronic acid.

Wulff and co-workers suggested that the putative B–N dative bond formation depicted in Scheme 1.4 is responsible for the decrease of the apparent pKa of the boronic acid.[40] It was assumed that the Lewis acidity of the boron increases through the formation of the boronate ester, with consequential increase in B–N dative bond strength, therefore a shorter bond. On the contrary, Wang and co-workers have discovered that due to steric reasons, the dative B–N bond is longer in certain boronic esters compared to the corresponding free boronic acids.[41] Using ^{1}H NMR Anslyn and co-workers have reported that the extent of B–N coordination varies depending on (boronic acid) structure and the solvent.[42] In their study they reported that in the presence of methanol, a methoxy group coordinates to the boron atom while the nitrogen is protonated, resulting in the complex

depicted Figure 1.7. Coversely, they established that in aprotic solvents, when a solvolysis pathway is absent, B–N coordination is commonly observed.

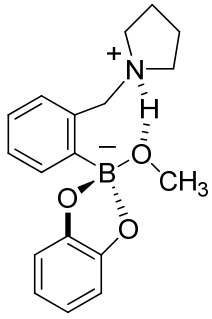


Figure 1-7: Representation of complex between catechol and Wulff-type aromatic boronic acid in methanol**.**

1.4.2 Structure of saccharides bound to boronic acids

It is well known that under aqueous conditions, monosaccharides maintain an equilibrium between their six membered pyranose and five membered furanose forms, and there have been arguments as to which of these two forms boronic acids bind predominantly.[43] In 1995, Eggert and Norrild[43] showed that glucose binds mostly in its weakly populated furanose form when it was mixed with *p*-tolylboronic acid. Later, they reported again that *p*-tolylboronic forms different complexes with D-fructose with an abundance of 2,3,6 tridentate complex A.[44]

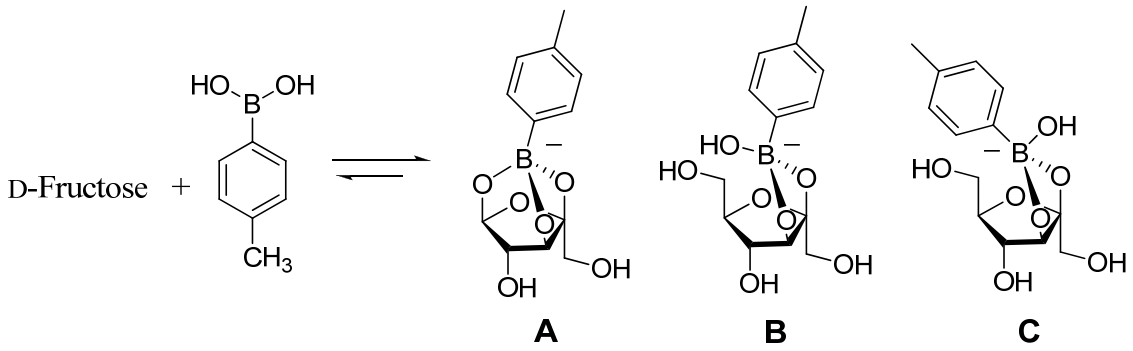


Figure 1.8: Complex formation between D-fructose and *p*-tolylboronic acid.[44]

Thus, it appears that boronic acids have an intrinsic preference to complex 5-membered carbohydrates. Even when designing libraries of diboronic acids with the combined effect of the "Wulff-type" arylboronic acids and electron-withdrawing cyano substituents into the arylboronic acid, no binding was observed with disaccharides that cannot isomerize to their five membered furanose rings (Figure 1.9).[45] In contrast, an increase in binding affinity was noticed with a disaccharide (lactulose) that can isomerise to a furanose form. In light of these results, it is clear that there is a need for a monoboronic acid unit that can form esters with adjacent diols of hexopyranosides.

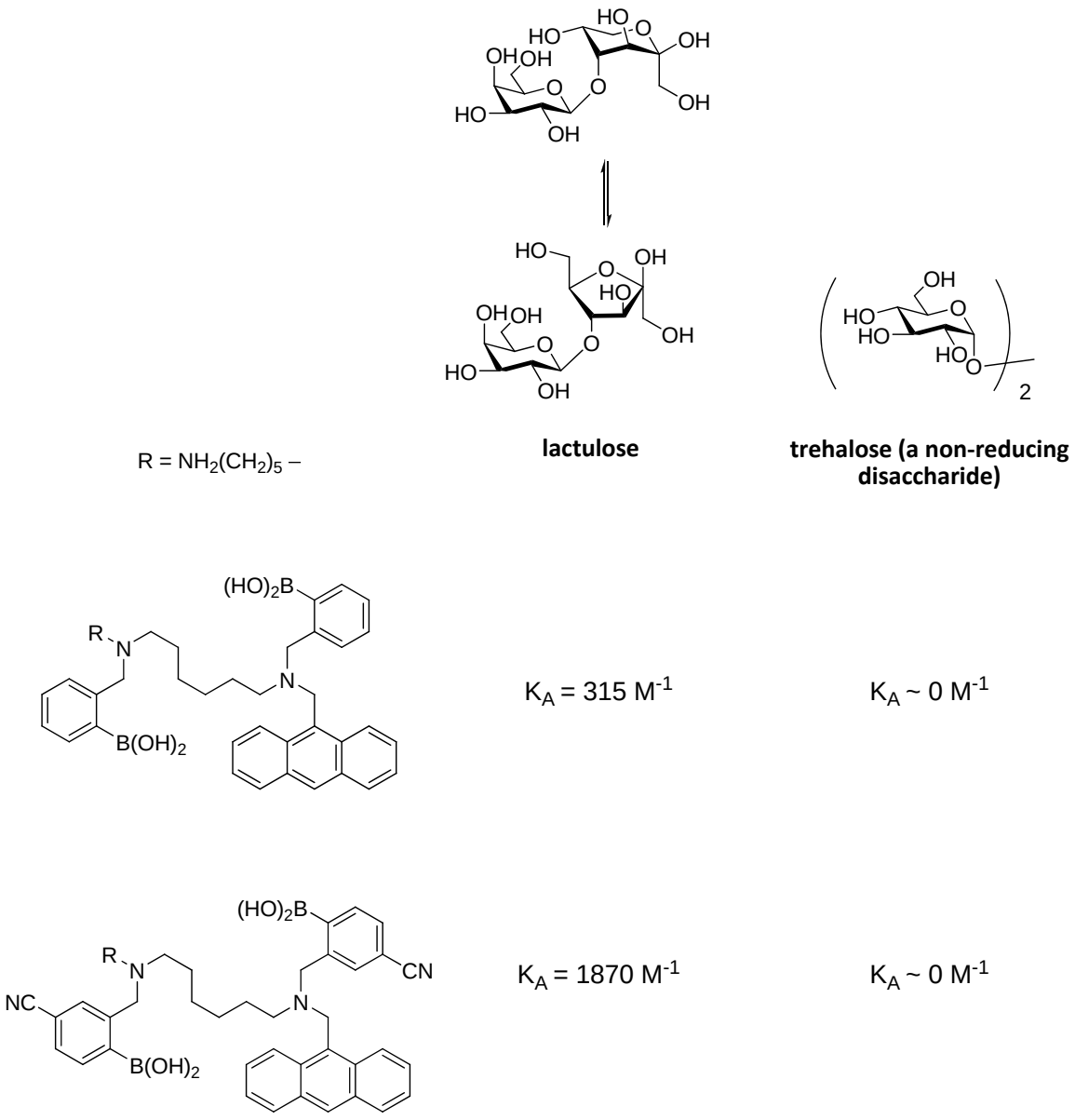


Figure 1.9: Binding constants of bis-boronic acid receptors with disaccharides.[45]

Figure 1.10 depicts examples of known receptors and sensors containing boronic acid units; however, the main problem with these receptors is that they are selective only towards reducing monosaccharides.

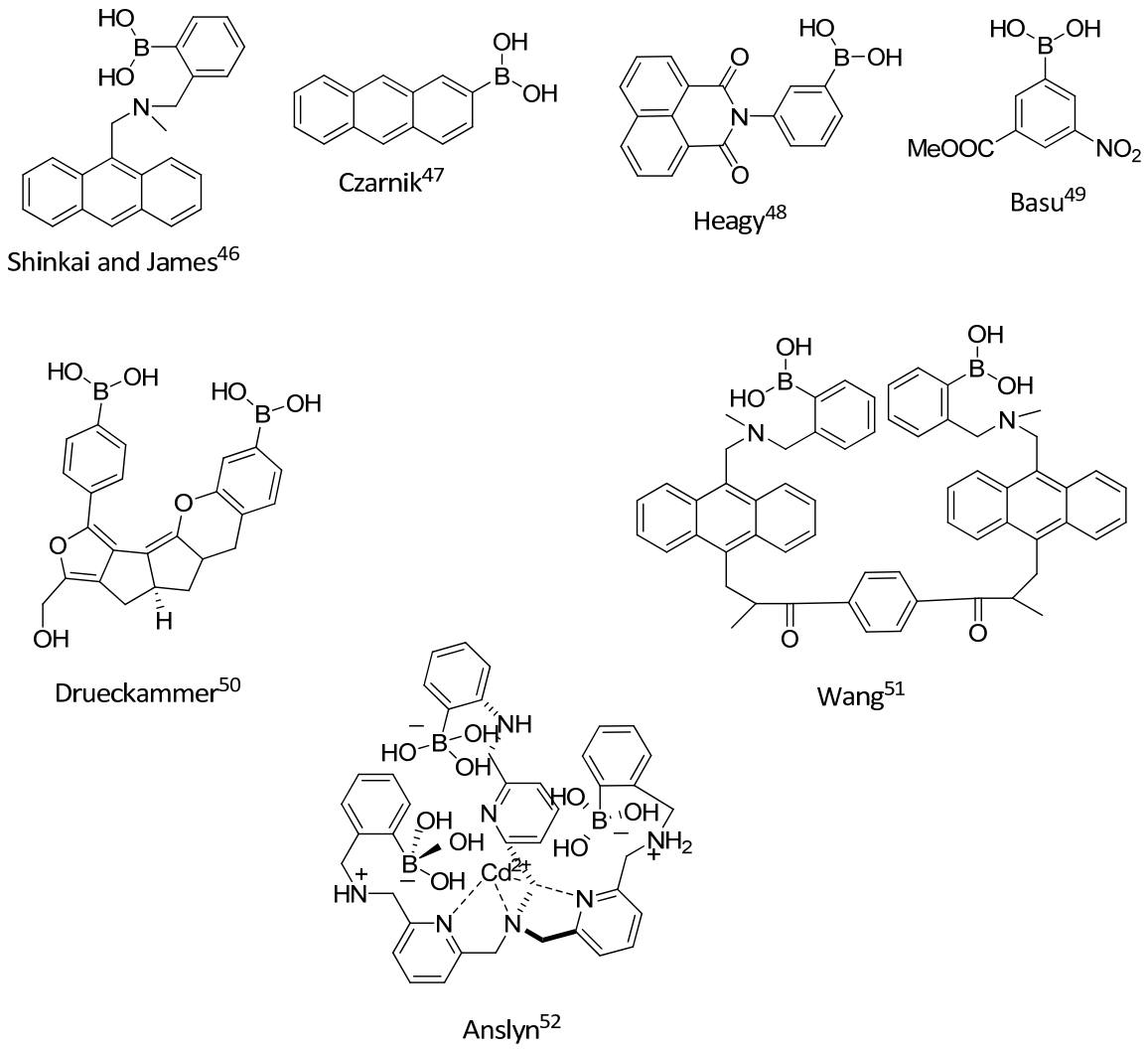


Figure 1.10: Examples of boronic acid sensors and receptors for monosaccharides.

The preference for furanose sugars is no different with oligomeric acid receptors.[45] Shinkai and co-workers[46] suggested that the bis-boronic acid anthracene sensor of Figure 1.8 binds to the pyranose form of glucose (B) in methanol. On the other hand, Norrild and co-workers[53] proved experimentally that this bis-boronic acid sensor binds with the furanose form of the monosaccharide in water.

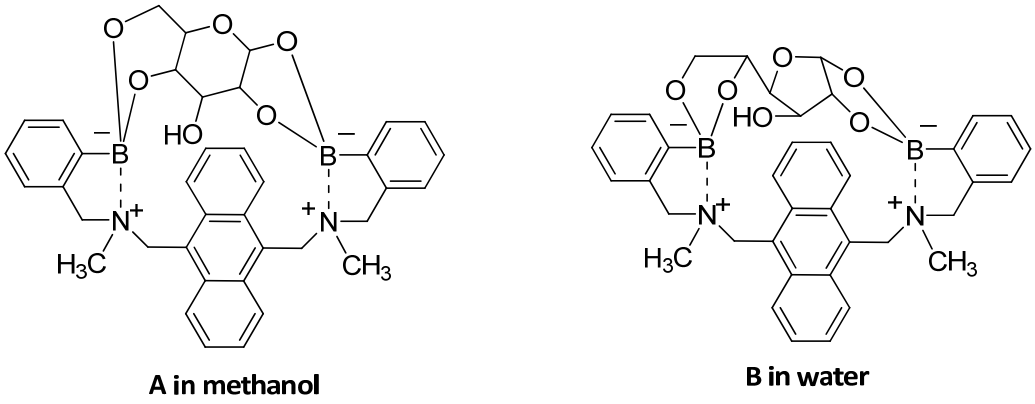


Figure 1.11: Complexation of glucose with a diboronic acid sensor in different conditions.

Exceptionally, Drueckhammer and co-workers[50] reported with a bis-boronic acid receptor based on computational design and the selectivity and the binding affinity were found to be higher for the pyranose form of glucose.

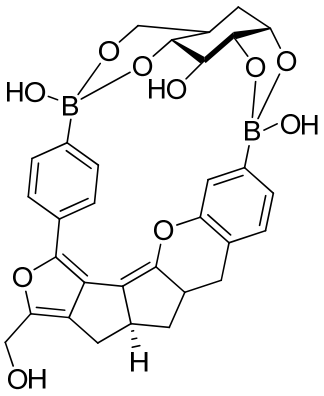
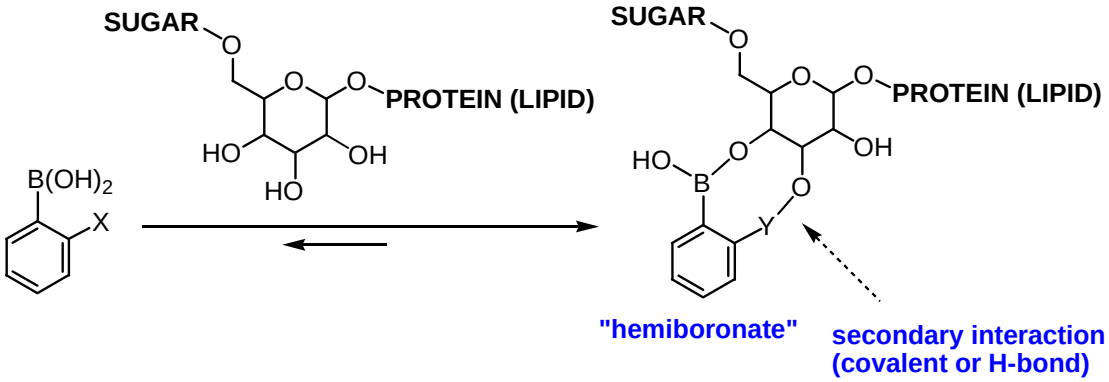


Figure 1.12: Receptor-complex devised by Drueckammer and co-workers.[50]

This example, however, was obtained when targeting a reducing monosaccharide. It is important to realize that biologically important glycoconjugates are hexopyranosides.

Thus, in view of examining biological applications, there is a need for a boronic acid unit capable of binding to hexapyranosides at pH 7.4. To address this problem and achieve recognition pyranosides of the type expressed on cell surfaces, our group envisioned that the neighboring group participation of an ortho group on the arylboronic acid could favor secondary interactions with the adjacent hydroxyl groups of hexapyranose.[54] The proposed concept is depicted below (Scheme 1.5). The indicated secondary interaction in the "hemiboronate" could be either a covalent bond or a hydrogen bond.



Scheme 1.5: Possible secondary interactions afforded by ortho-substituted arylboronic acids.

To verify the assumption, it was desirable to screen many ortho-substituted boronic acids using a quick and easy method. Accordingly, Wang's qualitative three-component colorimetric assay based on the competitive displacement of alizarin red S (ARS) was chosen to screen several ortho-substituted arylboronic acids. A series of over a dozen arylboronic acids (Figure 1.13) were tested at neutral pH in water. Benzoboroxole, i.e. *o*-hydroxymethylphenylboronic acid, was found to be the strongest binder to both D-

glucose and D-fructose.[54] This assay was again confirmed by measurements of binding constants. All the other boronic boronic acids, including the "Wulff-type" model, failed to show any binding in this assay, even with a large excess of glycoside. The benzoboroxole mainly stays in its cyclic dehydrated form.

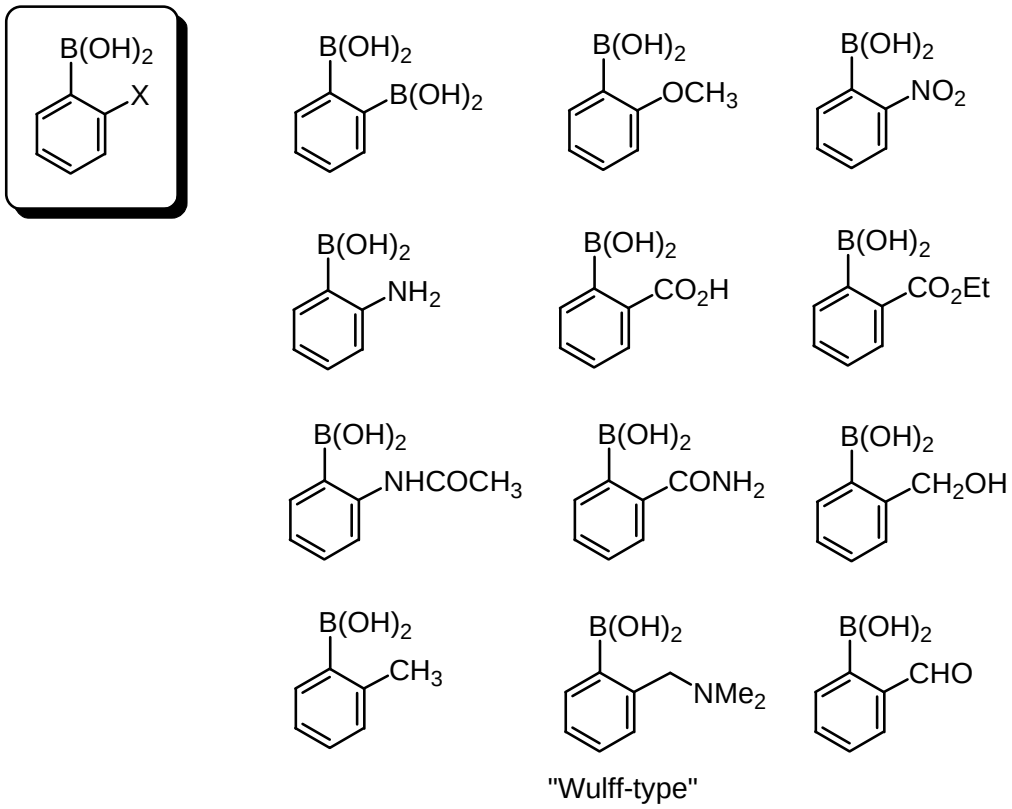


Figure 1.13**:** Ortho-substituted aryl boronic acids screened for binding hexopyranosides.

1.4.3 Benzoboroxole as an efficient glycopyranoside-binding agent

The binding selectivity of benzoboroxole was examined. It was not possible to measure binding constants by NMR for certain carbohydrates due to the lack of change in

chemical shifts upon binding. Therefore, the ARS-based quantitative UV method described by Wang and co-workers was employed.

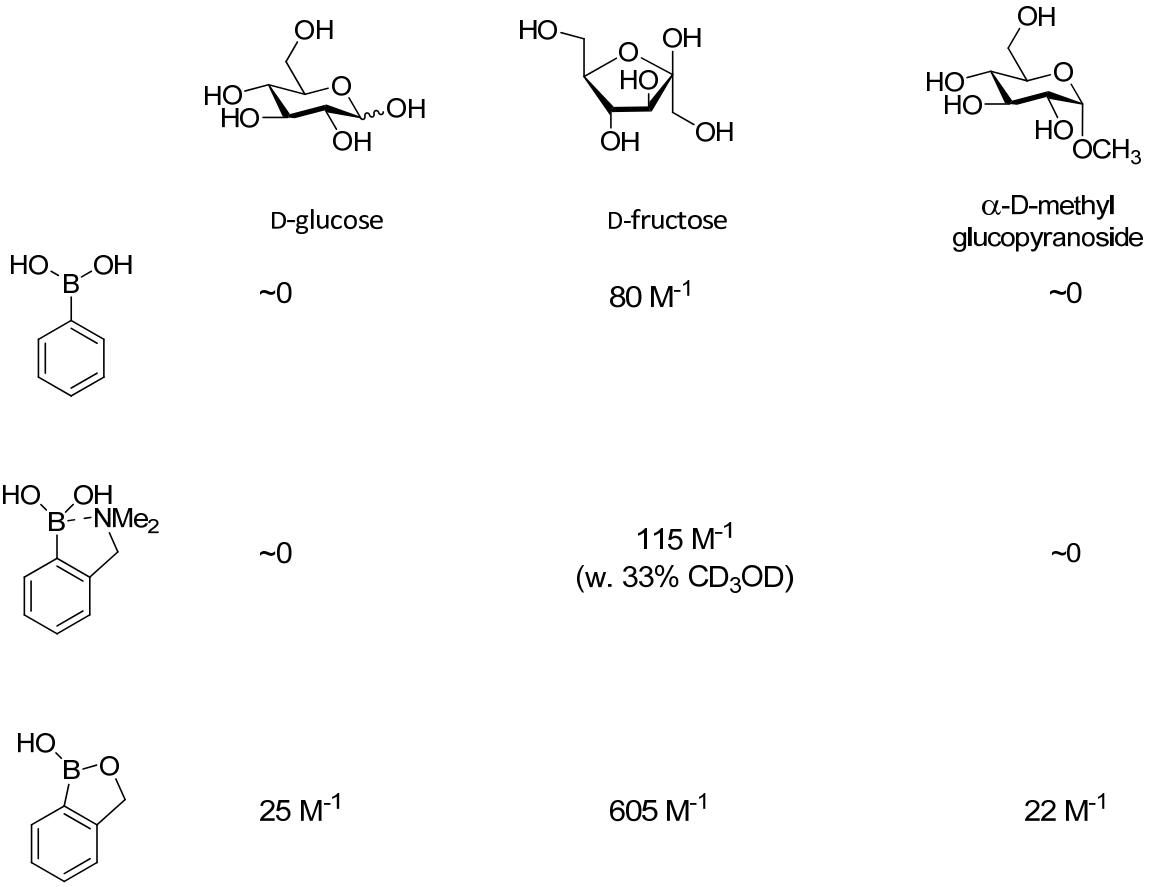


Figure 1.14: Comparison of binding constants between benzoboroxole and other arylboronic acids with D-glucose and D-fructose and methyl α-D-glucopyranoside.

These binding experiments confirmed that benzoboroxole is a superior complexing agent for reducing monosaccharides compared to phenylboronic acid and the Wulff-type *o*-dialkylaminomethylphenylboronic acids.[54,55] Additionally, benzoboroxoles are more soluble in water (no need for organic co-solvent) when compared to those boronic acids.

Most interestingly, benzoboroxole binds strongly to galactopyranoside compared to glucopyranoside. Tha data in Figure 1.15 proves that the binding constant value of benzoboroxole is more with α-D-galactopyranoside compared to α-D-glucopyranosides.[54]

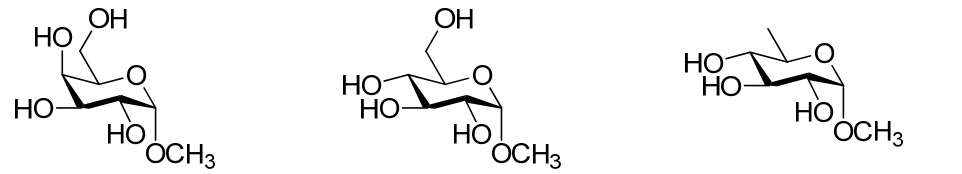


Figure 1.15: Comparison of binding constants of benzoboroxole with galactopyranoside and glucopyranosides.

Our group has also studied the effect of pH on the complexation equilibrium,[54] and the results indicate that fructose shows maximum binding affinity at pH 7.4 (rather than pH 7.0 or pH 7.8). It was proposed that the bimolecular benzoboroxole-diol complex exists in its stable ionized form **I** at near neutral pH, which is in contrast with most normal arylboronic acids that are ionized only at a higher pH (Figure 1.16). On the other hand, at a lower pH, the less stable neutral complex **II** may possibly compete and at a higher pH, the typical arylboronic acid complex **III** may dominate.

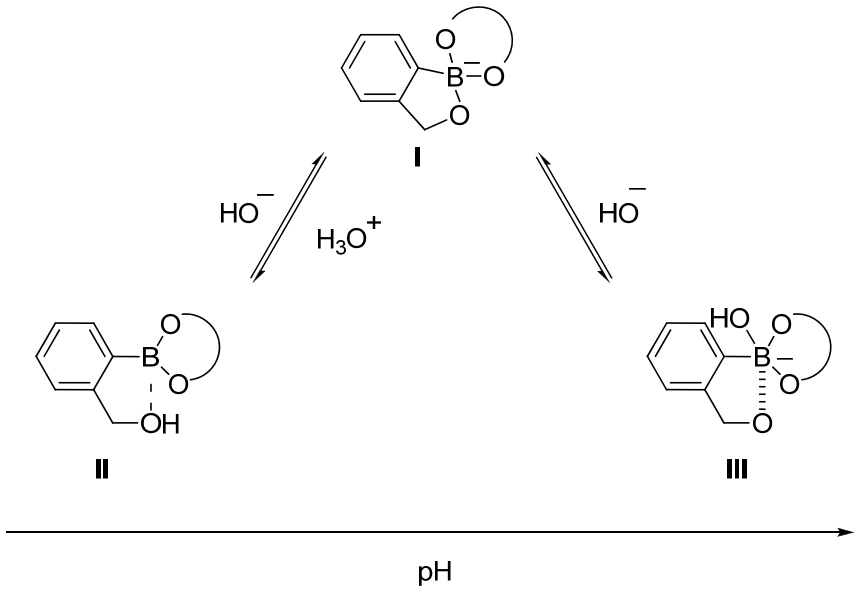


Figure 1.16: Effect of pH on the putative structure of benzoboroxole-diol complex.

It has been showed by Hall and co-workers that benzoboroxole binds preferentially to the pyranosides, 3,4-cis-diol (equatorial-axial) over the 4,6-diol as suggested by the stronger complexation with galactopyranosides over glucopyranosides (Figure 1.17).[55]

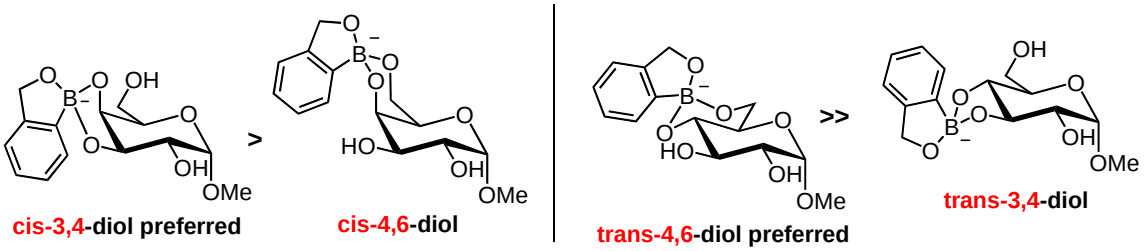


Figure 1.17: Favored diol binding modes between benzoboroxole and glycopyranosides.

Although the binding affinities observed between benzoboroxole and monopyranosides are still too weak for targeting complex oligosaccharides, it was hoped that exploring the multivalency effect with oligomeric benzoboroxole receptors would increase the binding affinities significantly. This concept is at the heart of the research project described in this thesis.

1.5 Thesis objectives

In the past decade, boronic acids have emerged as an important class of boron compounds that demonstrate excellent stability to air and aqueous media. The ability of boronic acids to interact reversibly with alcohols can also be exploited in chemical biology to address the problem of cell-surface recognition. Cells are covered with complex sugar molecules in the form of glycolipids and glycoproteins. The potential applications of small oligosaccharide-binding molecules are numerous, including therapeutic uses such as diagnosis and selective drug delivery, biological probes or analytical biosensors, and supports for affinity purification. Researchers have been successful in designing small molecules that bind selectively to proteins and enzymes, but efforts to target polysaccharides remain unrealized. Although antibodies can be generated for binding to cell surface oligosaccharides, they are not always as selective as desired. Our long term goal is to address this problem and come up with a general approach to the development of small synthetic molecules that can bind selectively to any desired carbohydrate.

Boronic acids such as phenylboronic acid have long been known to bind to simple sugars like glucose. However, none were ever shown to bind to the hexopyranosides of the type found on cell surfaces until our laboratory's recent breakthrough report of a "forgotten" class of hemi-boronic acids: benzoboroxoles.

Our objective was to develop a systemic and general approach with unambiguous and controlled recognition of diols on non-reducing hexopyranosides using structurally-defined receptors. Although benzoboroxole binds modestly to glycosides, receptors equipped with multiple units of benzoboroxole will be assembled in order to increase the binding affinity of these receptors.

As a model study, we first wanted to target β-Gal-(1→3)-α-GalNac disaccharide, known as the cancer-associated TF-antigen.[56] This disaccharide is associated with 90% of all types of human cancer cells, and, despite a number of limitations, antibodies against this antigen have been used as a diagnostic tool to detect cancers from human tissues. Since boroxole has a preference to complex galactose-like cis-3,4-diols, the TF antigen is an ideal model to test our approach.[57]

1.6 References

(1) http://www.scripps.edu/chem/wong/ (4/9/09)

(2) Koeller, K. M.; Wong, C. *Glycobiology* **2000**, *10*, 1157-1169.

(3) Chen, X.; Andreana, P. R.; Wang, P. G. *Curr. Opin. Chem. Biol.* **1999,** *3*, 650-658.

(4) Goldstein, I.J.; Hughes, R.C.; Monsigny, M.; Osawa, T.; Sharon, N. *Nature* **1980**, *285*, 66-66.

(5) Sharon, N; Lis, H. *Lectins*; Chapman and Hall: New York, **1989**.

(6) Sumner, J. B. *J. Biol. Chem.* **1919,** *137,* 137-142.

(7) Brewer, C. F.; Bhattacharya, L. *J. Biol. Chem.* **1986,** *261*, 7306-7310.

(8) Mandal, D. K.; Kishore, N.; Brewer, C. F. *Biochemistry* **1994,** *33,* 1149-1156.

(9) Dam, T.K.; Roy, R.; Das, S. K.; Oscarson, S.; Brewer, C. F. *J. Biol. Chem.* **2000,** *275,* 14223-14230.

(10) Bird, G. W. G. *Vox Sang.* **1964,** *9,* 748-749.

(11) Lotan, R.; Skutelsky, E.; Danon, D.; Sharon, N. *J. Biol. Chem.* **1975,** 250-251.

(12) Bannerjee, R.; Mande, S. C.; Ganesh, V.; Das, K.; Dhanraj, V.; Mahanta, S. K.; Suguna, K.; Surolia, A.; Vijayan, M. *Proc. Natl. Acad. Sci.* **1994,** *91,* 227-231.

(13) Neurohr, K. J.; Young, N. M.; Mantsch, H. H. *J. Biol. Chem.* **1980,** *255,* 9205-9209.

(14) Pereira, M. E. A.; Kabat, E. A.; Lotan, R.; Sharon, N. *Carbohydr. Res.* **1976,** *51,* 107-118.

(15) Ravn, P.; Stahn, R.; Danielczyk, A.; Faulstich, D.; Karsten, U.; Goletz, S. *Cancer Immunol. Immunother.* **2007**, *56*, 1345-1357.

(16) Ravishankar, R.; Ravindran, M.; Suguna, K.; Surolia, A.; Vijayan, M. *Curr. Sci.* **1997,** *72,* 855-861.

(17) Wiskur, S. L.; Lavigne, J. J.; Metzger, A.; Tobey, S. L.; Lynch, V.; Anslyn, E. V. *Chem. Eur. J.* **2004**, *10*, 3792–3804.

(18) Sebo, L.; Schweizer, B.; Diederich, F. *Helv. Chim. Acta* **2000,** *83,* 80-92.

(19) Ferrand, Y.; Crump, M. P.; Davis, A. P. *Science* **2007,** *318,* 619-622.

(20) Palde, P. B.; Gareiss, P. C.; Miller, B. L. *J. Am. Chem. Soc.* **2008**, *130*, 9566–9573.

(21) (a) Miyaura, N.; Suzuki, A.; *Chem. Rev.* **1995,** *95,* 2457-2483. (b) Suzuki, A. *J. Organomet. Chem.* **1999,** *576,* 147-168.

(22) Aggarwal, V. K.; Anderson, E.; Giles, R.; Zaparucha, A. *Tetrahedron: Asymmetry* **1995,** *6,* 1301-1306.

(23) Ishihara, K.; Ohara, S.; Yamamoto, H. *J. Org. Chem.* **1996,** *61,* 4196 – 4197.

(24) Al-Zoubi, R. M.; Marion, O.; Hall, D. G. *Angew. Chem. Int. Ed.* **2008,** *47,* 2876 – 2879.

(25) James, T. D.; Sandanayake, S.; Shinkai, S. *Angew. Chem. Int. Ed. Engl.* **1996,** *35,* 1910-1922.

(26) Yang, W. Q.; Kim, K. B.; crews, C. M. *Med. Res. Rev.* **2003,** *23,* 346-368.

(27) Soloway, A. H.; Tjarks, W.; Barnum, B.; Rong, F. G.; barth, R. F.; Codogni, I. M.; Wilson, J. G. *Chem. Rev.* **1998,** *98,* 1515-1562.

(28) *Boronic Acids—Preparation and Applications in Organic Synthesis and Medicine* (Ed.: D. G. Hall), Wiley-VCH, Weinheim, **2005.**

(29) Kuivila, H. G.; Keough, A. H.; Soboczenski, E. J. *J. Org. Chem.* **1954,** *8,* 780-783.

(30) Wang, W.; Gao, X.; Wang, B. *Curr. Org. Chem.* **2002,** *6,* 1285-1317.

(31) Lorand, J. P.; Edwards, J. O. *J. Org. Chem.* **1959,** *24,* 769-774.

(32) Springsteen, G.; Wang, B. *Tetrahedron Lett.* **2002,** *58,* 5291-5300.

(33) Vanduin, M.; Peters, J. A.; Vanbekkum, H. *Carbohydr. Res.* **1994,** *253,* 1-12.

(34) Vanduin, M.; Peters, J. A.; Vanbekkum, H. *Tetrahedron* **1985,** *41,* 3411-3421.

(35) Yan, J.; Fang, H. Wang, B. *Med. Res. Rev.* **2005,** *25,* 490-520.

(36) Sandanayake, K. R. A. S.; James, T. D.; Shinkai, S. *Chem. Commum.* **1997,** 71-72.

(37) Tacheuchi, M.; Taguchi, M.; Shinomori, H. Shinkai, S. *Tetrahedron* **2000,** *56,* 6193-6198.

(38) Yamamoto, H.; Tackeuchi, M.; Shinkai, S. *Tetrahedron* **2002,** *58,* 7251-7258.

(39) Ni, W.; Fang, H.; Springsteen, G.; Wang, B. *J. Org. Chem.* **2004,** *69,* 1999-2007.

(40) Wulff, G. *Pure Appl. Chem.* **1982,** *54,* 2093-2102.

(41) Frazen, S.; Ni, W.; Wang, B. *J. Phys. Chem.* **2003,** *107,* 12942-12948.

(42) Zhu, L.; Shabbir, S. H.; Gray, M.; Lynch, V. M.; Sorey, S.; Anslyn, E. V. *J. Am. Chem. Soc.* **2006,** *13,* 1019-1022.

(43) Norrid, J. C.; Eggert, H. *J. Am. Chem. Soc.* **1995,** *117,* 1479-1484.

(44) Norrid, J. C.; Eggert, H. *J. Chem. Soc. Perkin Trans. 2* **1996,** 2583-2588.

(45) Stones, D.; Manku, S.; Lu, X.; Hall, D. G. *Chem. Eur. J.* **2004,** *10,* 92-100.

(46) James, T. D.; Sandanayake, K. R. A. S.; Iguchi, R. Shinkai, S. *J. Am. Chem. Soc.* **1995,** *117,* 8982-8987.

(47) Yoon, J.; Czarnik, A. W.; *J. Am. Chem. Soc.* **1993,** *1,* 267-271.

(48) DiCesare, N.; Adhikari, D. P.; Heynekamp, J. J.; Heagy, M. D.; Lakowicz, J. R. *J. Fluoresc.* **2002,** *12,* 147-154.

(49) Mulla, H. R.; Agard, N. J.; Basu, A. *Bioorg. Med. Chem. Lett.* **2004,** *14,* 25-27.

(50) Yang, W.; He, H.; Drueckhammer, D. G. *Angew. Chem. Int. Ed.* **2001,** *40,* 1714-1718.

(51) Yang, W.; Gao, S.; Gao, X.; Karnati, V. V. R.; Ni, W.; Wang, B.; Hooks, W. B.; Carson, J.; Weston, B. *Bioorg. Med. Chem. Lett.* **2002,** *12,* 2175-2177.

(52) Zhang, T.; Anslyn, E. V. *Org. Lett.* **2008,** *8,* 1649-1652.

(53) Bielecki, M.; Eggert, H.; Norrild, J. C. *J. Chem. Soc. Perkin Trans. 2* **1999,** 449-455.

(54) M. Dowlut, D. G. Hall; *J. Am. Chem. Soc.* **2006**, *128*, 4226-4227.

(55) M. Bérubé, M. Dowlut, D. G. Hall *J. Org. Chem.* **2008,** *73,* 6471-6479.

(56) Yu, L.-G. *Glycoconj. J.* **2007**, *24*, 411-420.

(57) Moran, E. J.; Sarshar, S.; Cargill, J. F.; Shahbaz, M. M.; Mjalli, M. M.; Armstrong, R. W. *J. Am. Chem. Soc.* **1995**, *117*, 10787.

Chapter 2

Design and Synthesis of Peptidyl-bis(boroxole) Library

The synthesis of the library has been performed by my former labmate Dr. Marie Bérubé. It is being described in this chapter for the clarity and integrity of this Master's thesis. I have synthesized library compounds **1*{17,29}***, **1*{19,38}***, **1*{15,23}*** (library nomenclature is described in section 2.4) and two control compounds (**3** and **4**, Chapter 3) using the same procedure but in comparatively larger scale.

2.1 Combinatorial chemistry

Combinatorial chemistry gained prominence in the 1980's by facilitating the synthesis of large numbers of peptides over a short period of time. Combined with advanced high-thoughtput screening technologies, these libraries can be screened against a target of interest. Combinatorial chemistry has been paralleled with solid-phase organic synthesis to improve the synthesis of libraries.[1]

2.2 Solid-phase synthesis

Solid-phase synthesis is the most commonly utilized method for peptide synthesis. This technique is the preferred method due to the ease with which it allows one to derivatize the intermediates without resorting to tedious purification and isolation procedures. In solid-phase synthesis, the synthesis of desired compounds is performed with starting materials and intermediates immobilized onto an insoluble support. These supports are in the form of polymeric resin beads (between 10 μm and 500 μm in diameter) that are

comprised of polystyrene cross linked with 1 to 20% divinylbenzene, or polystyrene-polyethylene glycol co-polymers.[2, 3] During the reaction, solvent is added to resin beads to swell and expose their inner reactive sites. This swelling permits reagents to enter the polymeric matrix and react with the resin-bound substrate. After completion of the reaction, the resin is rinsed with solvent to wash off the excess reagents. A large excess of reagents can be used to drive the reaction to completion. Solid-phase synthesis suffers from the difficulties in monitoring the progress of the reaction and in characterizing each intermediate. At the end of the reaction, a small amount of the resin is cleaved to confirm the completion of the reaction and perform characterization. As a result, there is always some loss of resin when one wants to identify the intermediates.

Completion of a peptide coupling reaction can be monitored using the Kaiser test.[4] This test is a qualitative assay for the detection of free primary terminal amino groups in a solid phase peptide synthesis. The chemistry is based on the reaction of ninhydrin with primary amines, which gives a characteristic dark blue colour and the coupled amine yields a yellow color. The Kaiser test kit contains phenol, ~ 80% ethanol, KCN in H_2O/pyridine and ninhydrin, 6% in ethanol.

At each step, the resin should react in high yield to give the corresponding product. Otherwise, unreacted intermediates remain attached to the resin and can be responsible for impurities in the final product, which can be difficult to purify by flash chromatography.[5]

Solid-phase combinatorial synthesis can be divided into two categories: parallel synthesis and split-pool synthesis. Figure 2.1 depicts a general scheme of parallel synthesis, which

involves keeping each library member separate from others.[6] Therefore, every member can be identified using standard analytical methods at the end of the reactions. However, the synthesis scheme does not really reduce the total number of steps involved in the library purification.

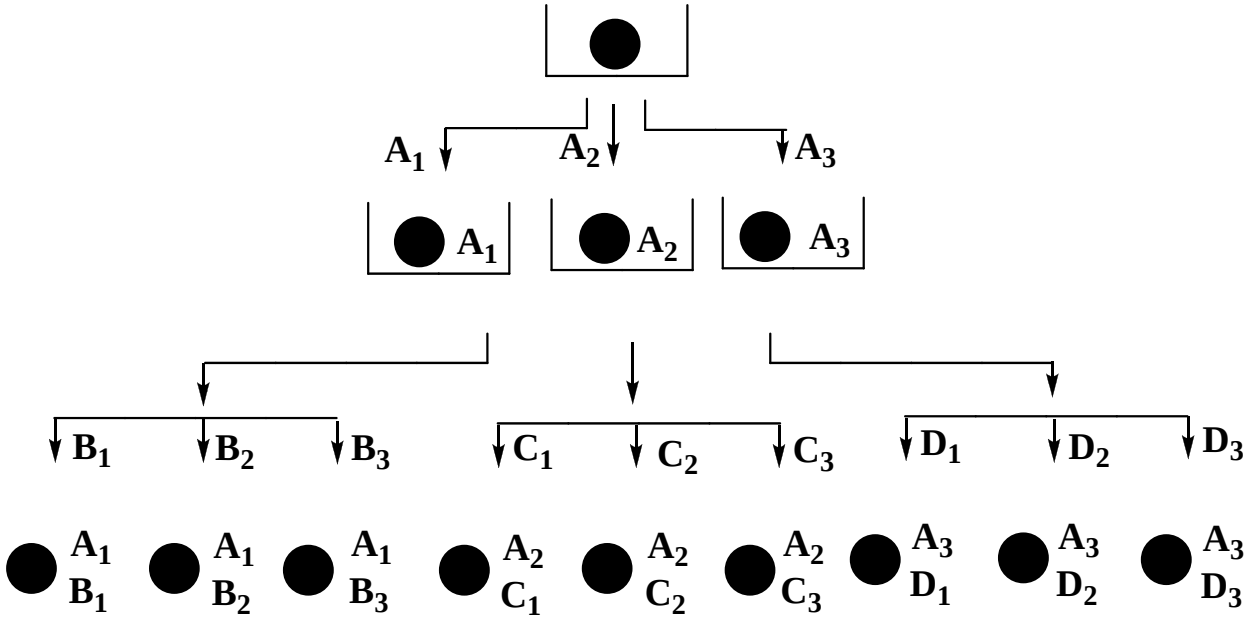


Figure 2.1: Representative diagram of parallel library synthesis.

Presently, the split-pool strategy is one of the best methods for the synthesis of large combinatorial libraries of compounds. This method allows one to carry out a combinatorial library synthesis in few reaction vessels. In the first step, a quantity of resin beads is split into multiple, equal sized portions, each of which is then coupled with different building blocks.[7] After the first reaction step, the resin-bound compounds from all the reactions are pooled together into one vessel where all the common steps such as

resin washing, and deprotection of the protecting groups are performed. This sequence is repeated until the entire compounds for the whole library have been assembled, and ideally each resin bead should contain only one distinct compound at the end of the synthesis.

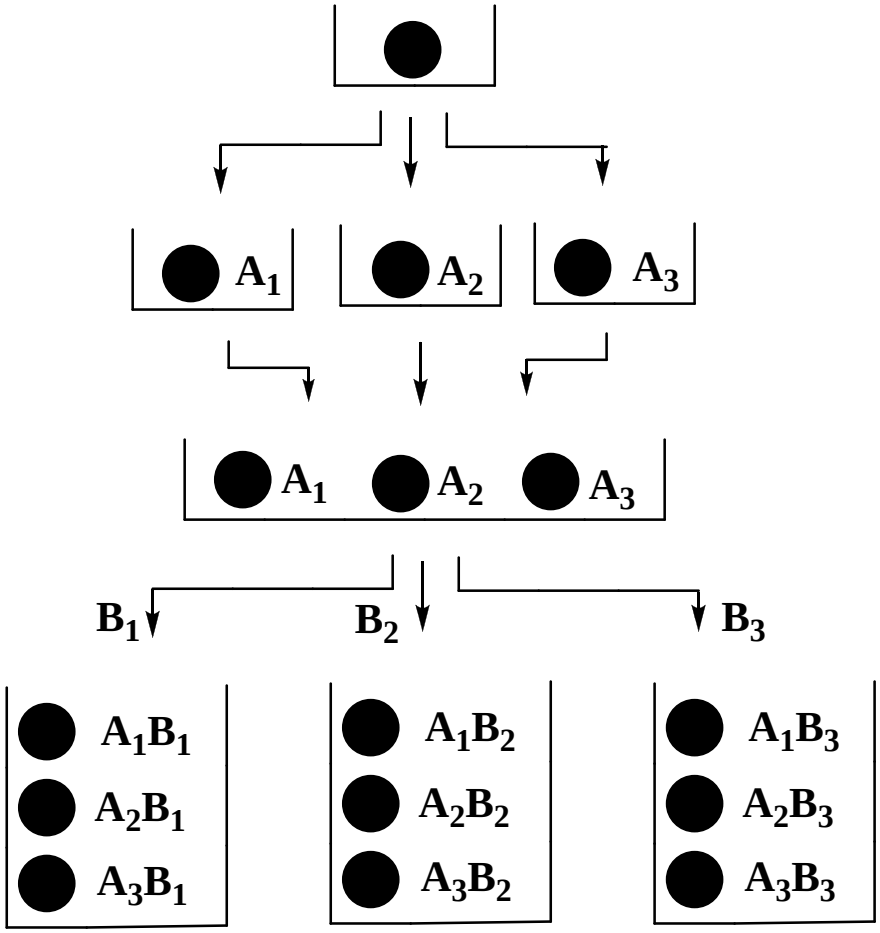


Figure 2.2: Split-pool synthesis scheme to prepare a combinatorial library.

2.3 Library generation using Irori® MiniKan technology

The Irori® MiniKan technology combines the advantages of parallel solid-phase synthesis and split-pool strategy while using traditional glassware for conducting reactions.[7]

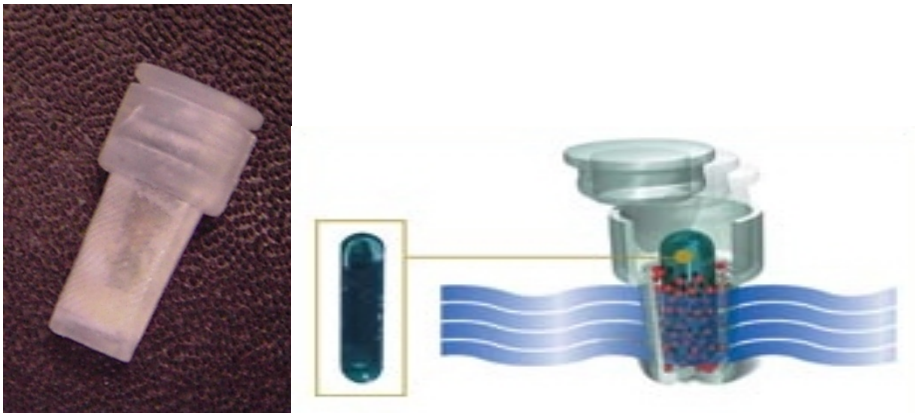


Figure 2.3: Irori® MiniKan technology.

Nicolaou and Xiao,[8] as well as Moran and co-workers[9] came up with a radiofrequency encoding system. Glass-encased semiconductor memory microchips are able to receive, store and emit radiofrequency signals. Taking advantage of these microchips, they designed polypropylene vessels that can encapsulate the resin (and the microchip) and undergo split-pool solid-phase synthesis (Figure 2.3). After distinguishing all the kans with different radio frequency, they were subjected to different reaction conditions in out the next step. MicroKan reactors can deliver the final compounds after cleavage of the resin, with the amount depending on the size of the Kan and the loading of the resin.

2.4 Design of the library

As mentioned in Chapter 1, Section 1.7, we wished to target one glycan of interest and identify a single small molecular weight receptor of defined composition. In this approach, benzoboroxoles were envisioned to be exploited as hexopyranoside-binding agents.[10] Our strategy couples boronate formation with other modes of molecular recognition inspired from the natural carbohydrate-binding proteins, lectins. In this model study, the library will be targeted against an important tumor-associated carbohydrate antigen, the Thomsen-Friedenreich (TF) disaccharide (β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactopyranose, Figure 2.4).[11] It is claimed that anti-TF antibodies show lower than desired affinity and selectivity, thus synthetic receptors are of great interest.[3]

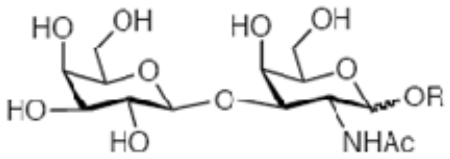


Figure 2.4: TF antigen disaccharide (β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactopyranose), R = lipid or protein.

The TF antigen possesses two 4,6-diol and one cis-3,4-diol units that bind preferentially with benzoboroxoles. Accordingly, we chose to include two boroxole units on the receptor structure.[12] Because it is difficult to predict the ground state conformation of oligosaccharides, a solution to address this issue is to rely on a combinatorial approach.

Subsequently, we planned to synthesize a library of bis(boroxoles) using a combinatorial solid-phase approach. In designing the library, we chose a peptide backbone due to the ease of synthesis. Additionally, peptides are known to possess of hydrogen bonding donor and acceptor capabilities that can contribute to the recognition of oligosaccharides. The central amino acid position, flanked by two diaminopropionic acid residues for the attachment of the benzoboroxole, was randomized with 20 natural and unnatural amino acids offering functional and geometrical diversity (Figure 2.6).[12]

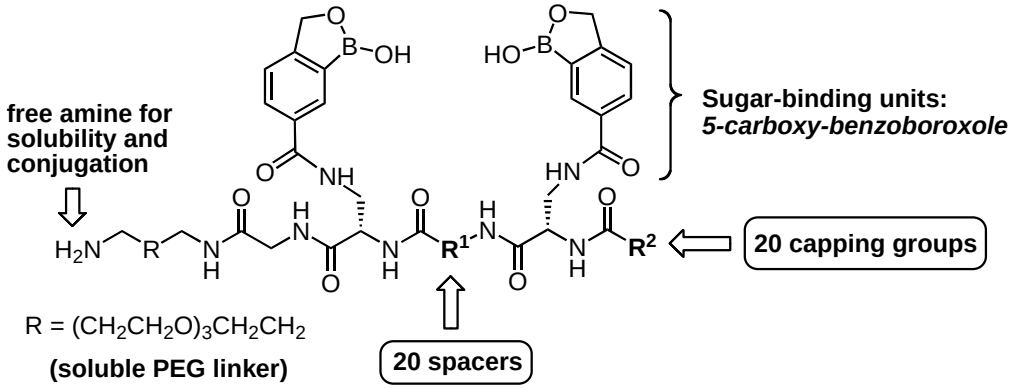


Figure 2.5: Design of peptidyl bis(boroxole) library **1.**[12]

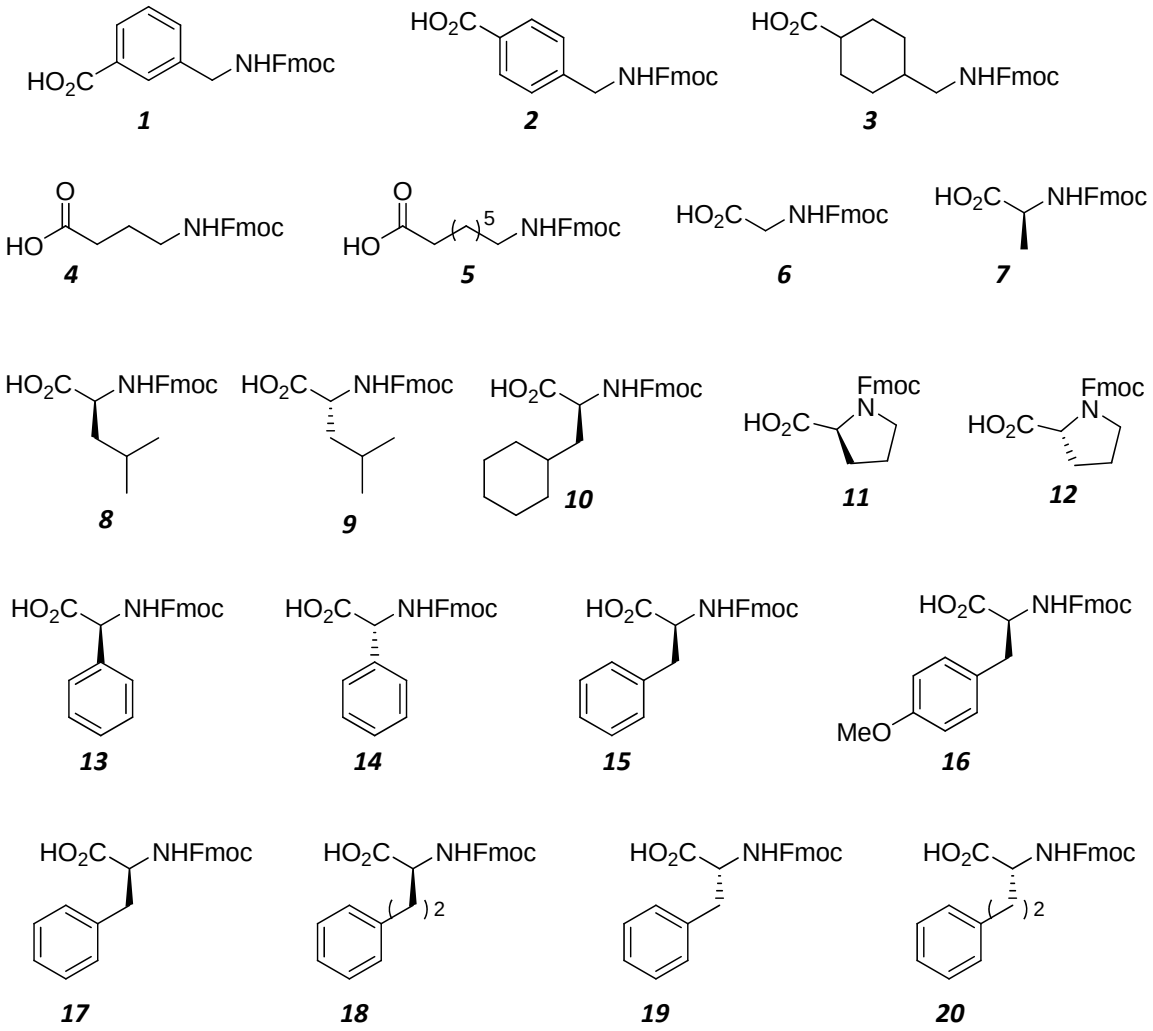


Figure 2.6: Amino acid building blocks ***1-20*** for the library of receptors.

The acyl capping group would consist in a selection of 20 carboxylic acids (Figure 2.8). Several library components for both the spacer and terminal positions include aromatic subunits because they are known in carbohydrate-binding proteins to promote

hydrophobic (or C-H---π) interactions with the non-polar face of the saccharides (Figure 2.7).[13]

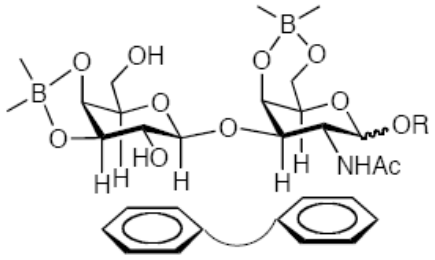


Figure 2.7: Possible interactions between library compound and TF antigen disaccharide: boronate formation, hydrophobic packing and C-H---π interactions.

The library would also include aliphatic spacers displaying range of geometries, and arene derivatives with different electronegative and electropositive groups. Additionally, the library includes epimeric spacers to examine the effect of spacer's stereochemistry. The PEG linker present in the library was included to increase the solubility in water and the terminal amine group was thought to allow for further conjugation after cleavage from the resin. The general name of a library compound is represented as **1{*spacer,capping group*}** where spacer and capping groups vary from ***1-20*** and ***21-40*** respectively (c.f. Figures 2.6 and 2.8).

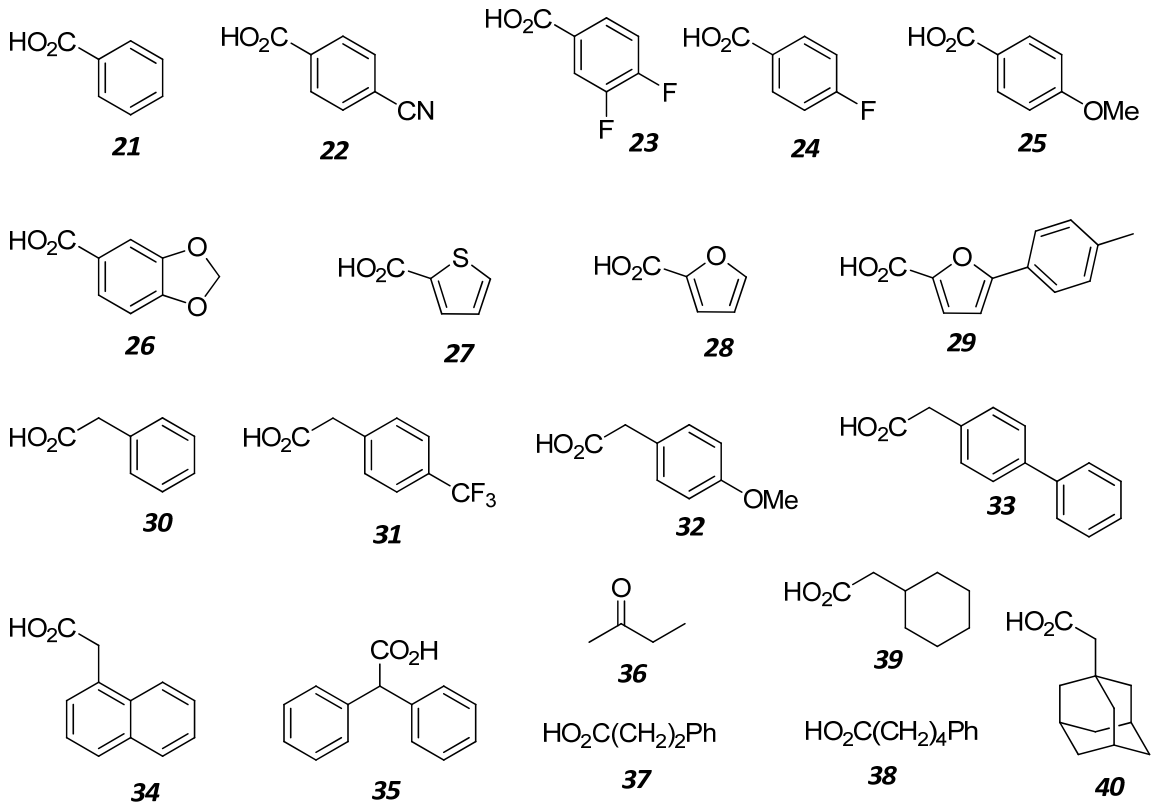
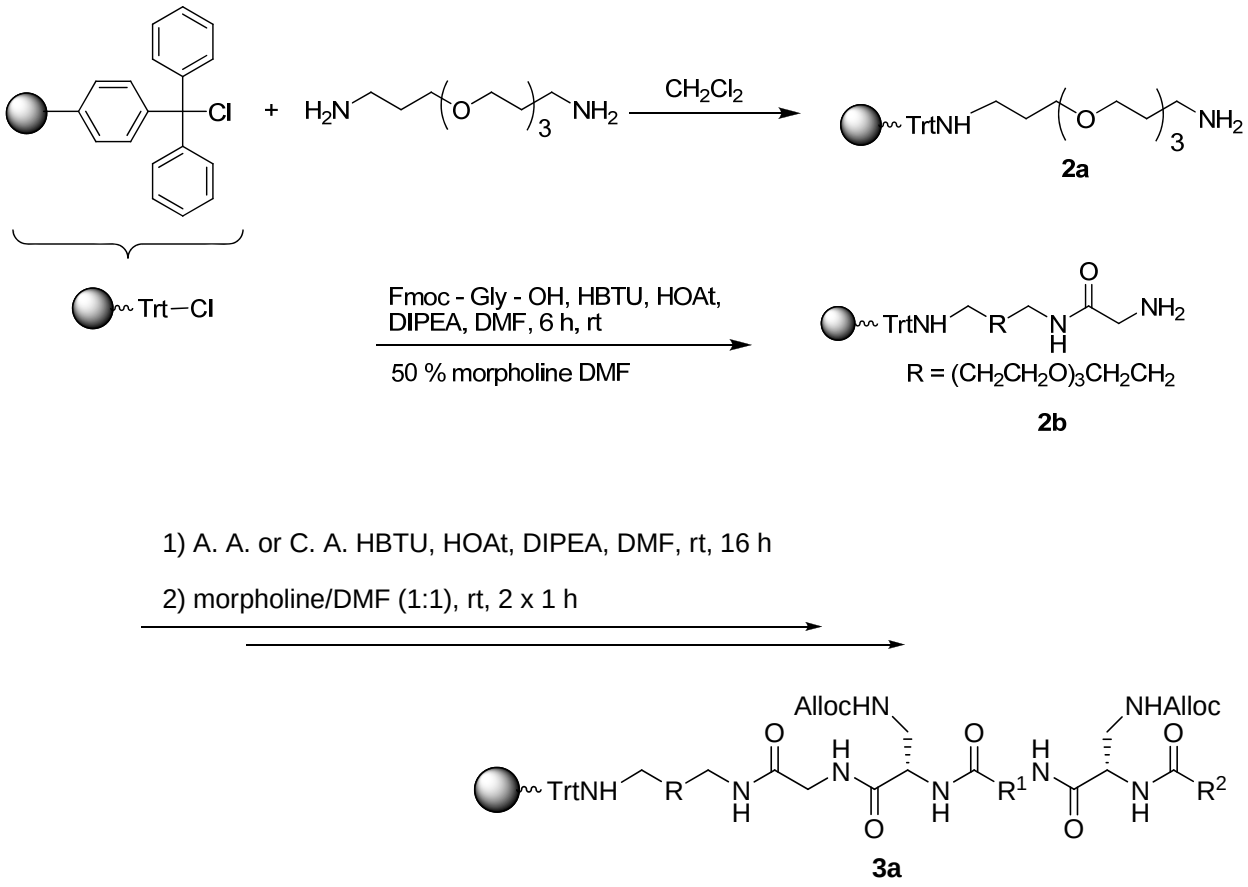


Figure 2.8: Carboxylic acid capping group for the library of receptors.

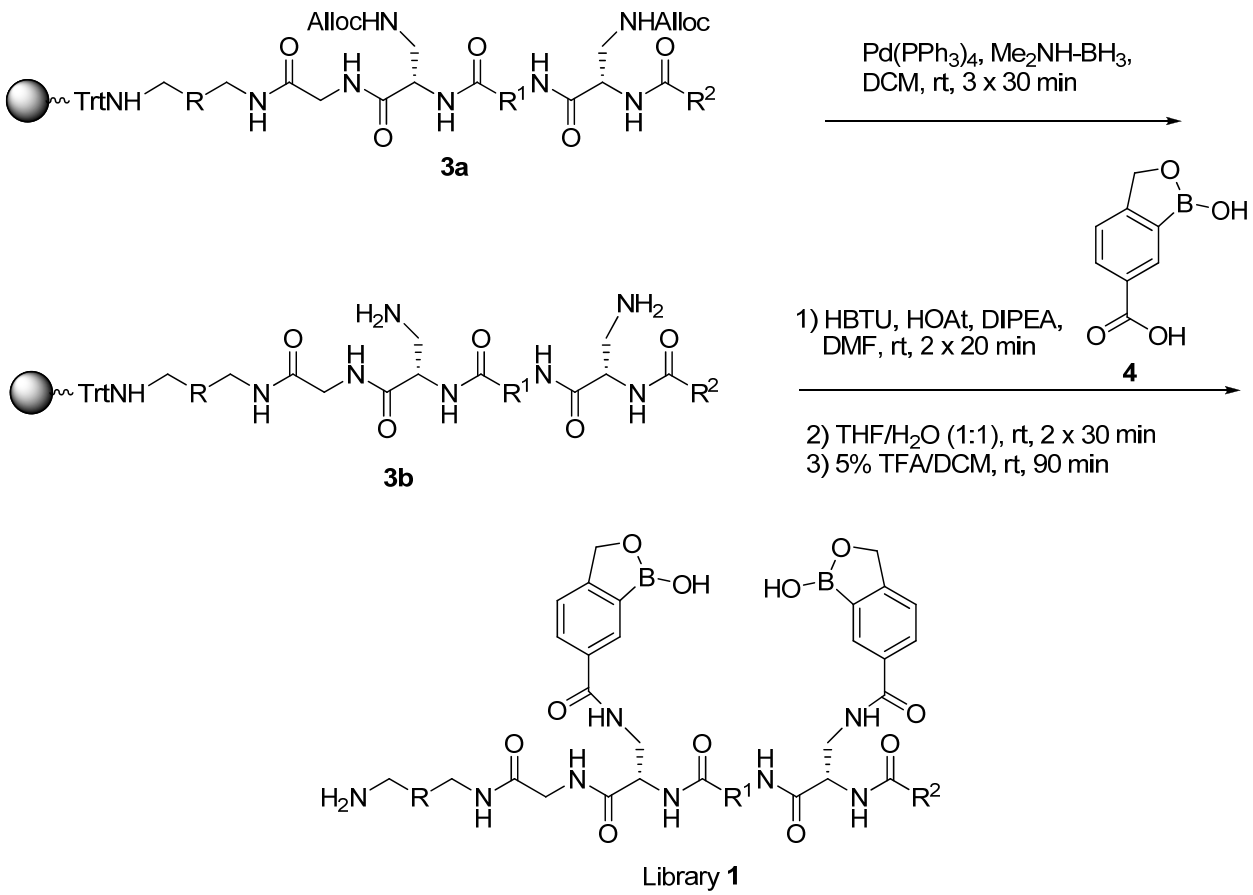
2.5 Synthesis of the library[12]

The library was assembled by solid-phase peptide synthesis starting from trityl resin including a short triethyleneglycol spacer followed by an anchoring primary amine **2a** (Scheme 2.1).



Scheme 2.1: Synthesis of peptide precursor to library **1.**

The free amine **2a** was first coupled with Fmoc-Gly-OH to obtain **2b,** which is the precursor for Fmoc-peptide-synthesis to achieve the required peptide skeleton. Then the free amine **2b** was coupled with Fmoc-Dap(Alloc)OH via an optimized coupling protocol using HBTU/HOAt as coupling reagents.[11] Thereafter, the Fmoc group was deprotected using morpholine/DMF in order to carry out further couplings with the amino acids depicted in Figure 2.6.



Scheme 2.2: Side chain deprotection, coupling and final cleavage of the resin.

This Fmoc peptide synthesis protocol was carried out to couple Dap(Alloc)OH and the different carboxylic acids of Figure 2.8 in order to obtain **3a** (Scheme 2.1). The Alloc group in compound **3a** was deprotected via a standard method using $Pd(PPh_3)_4$ and $Me_2NH\text{-}BH_3$. At last, the free amines in compound **3b** were coupled with 3-carboxy-2-hydroxymethylphenyl boronic acid (**4**) using a similar coupling protocol. The Kaiser test showed the presence of free amine even after the coupling reaction. So, double coupling was needed to couple both benzoboroxole units. Cleavage from the resin provided the crude library **1** (Scheme 2.2) in 30–40% overall yields from the trityl chloride resin. The

crude compounds were 70% to 90% pure according to HPLC analyses using detection at 254 nm. Subsequently, all 400 compounds were purified (by Eric Pelletier) using semi-preparative HPLC equipped with an electrospray detector.

2.6 Conclusion

This chapter focused on the design and preparation of new boroxole-containing peptide based receptors for the selective recognition of the TF antigen disaccharide under physiological conditions. In order to optimize appropriate spacers within a peptide skeleton, a library of 400 compounds containing two benzoboroxole units was synthesized and it was found that certain ones were able to bind selectively to the TF disaccharide. The chapter further describes a detailed description of the synthesis of the library using the Irori® MiniKan technology along with the purification of the whole library.

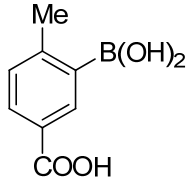
2.7 Experimental section[12]

2.7.1 General experimental conditions

Unless otherwise noted all reactions were performed under argon or N_2 atmosphere. Fmoc-protected amino acids were purchased from NovaBiochem (La Jolla, California), Advanced Chemtech (Louisville, Kentucky) or Iris Biotech [(Marktredwitz, Germany) Fmoc-Dap(Alloc)-OH]. Polystyrene trityl chloride resin (100–200 mesh) was purchased from Rapp Polymere (Tübingen, Germany). The loading specified by the supplier was used. DIPEA (Aldrich, purified by redistillation, 99.5% grade) was used. *N*-Bromosuccinimide was recrystallized from water and dried before use. THF (used for reaction and resin washing) was dried by distillation over sodium/benzophenone and used the same day. Toluene and methylene chloride were dried by distillation over CaH_2. Anhydrous DMF was obtained from Aldrich. Alizarin red S was purchased from Aldrich and used as received. Analytical thin layer chromatography (TLC) were conducted on plates covered with silica gel 60-F_{254}. Plates were visualized with cerium ammonium molybdate (CAM) or a potassium permanganate ($KMnO_4$) stain followed by heating. The library was prepared with IRORI MicroKan^{TM} Reactors (Discovery Partners, La Jolla, California) fitted with a radio frequency (RF) tag. The reactions were sorted with the IRORI AccuTag 100 sorting system. Compounds obtained from the library were purified with an Agilent 1100 MSD semi-preparative HPLC system. ^{1}H and ^{13}C NMR were recorded on Varian 300, 400 or 500 MHz spectrometers. Chemical shifts are reported in δ (ppm) units using ^{13}C and residual ^{1}H signals from the deuterated solvents.

A micromass TOF mass spectrometer provided the high resolution electrospray ionization mass spectra. Water used for the determination of K_a was distilled and further purified with a filtration system (Milli-Q). Quartz cuvettes were used for the quantitative ARS assay. All data were plotted on Microsoft Excel. Absorbance for ELISA tests were performed on a Molecular Devices Spectra Max 190 Microplate Reader. Peanut lectin from Arachis hypogaea (peroxidase labelled) was purchased from Sigma.

2.7.2 5-Carboxy-2-methylphenylboronic acid (5)



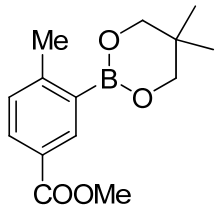
5

3-Bromo-4-methylbenzoic acid (5.00 g, 23.3 mmol) was dissolved in dry THF (75 mL) under N_2. The solution was cooled to –100 °C (ether/2-propanol (1:1) and liquid N_2) and *n*-BuLi (1.5 M in hexanes, 47.0 mL, 69.7 mmol) was added dropwise to the solution (60 minutes for addition). After completion of the addition, the solution was stirred at –100 °C for 1 h. After the elapsed time, $B(OMe)_3$ (13 mL, 116 mmol) was added slowly using syringe pump. The mixture was stirred 1 h at –100°C and then warmed up to room temperature overnight. The solvent was then evaporated under reduced pressure. The crude product was dissolved in ether and poured into HCl (1 M aqueous solution). The mixture was extracted with ether (3x), and the combined organic layers were washed with brine, dried over Na_2SO_4, filtered and concentrated to dryness. The crude product was

purified by flash chromatography (MeOH/ethyl ether 5:95) to give 5-carboxy-2-methylphenylboronic acid **5** (2.24 g, 54% yield) as a white solid, m.p. 191 °C. IR (microscope, cm^{-1}) 3293; ^{1}H NMR (300 MHz, acetone-d_6) δ 8.24 (d, J = 2.1 Hz, 1H), 7.84 (dd, J = 1.9, 8.0 Hz, 1H), 7.21 (dd, J = 0.6, 8.1 Hz, 1H), 2.51 (s, 3H); ^{13}C NMR (100 MHz, acetone-d_6) δ 168.1, 148.7, 135.9, 131.0, 130.3, 127.6, 22.7; ^{11}B NMR (128 MHz, acetone-d_6) δ 29.5; HRMS (ESI) Calcd. $C_8H_8BO_4$: 179.0510. Found: 179.0508.

Note: The carbon attached to boron does not show up in ^{13}C NMR.

2.7.3 2,2-Dimethyl-1,3-propanediol-(5-methoxycarbonyl-2-methylphenyl) boronate ester (6)

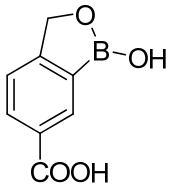


6

To a solution of boronate **5** (8.03 g, 44.6 mmol) in MeOH (250 mL) was added 12 N H_2SO_4 (5 mL). The mixture was stirred under reflux overnight. After cooling down to room temperature, the solvent was evaporated in vacuo. Water was added and the product was extracted with ethyl ether (3x), and the combined organic layers were washed with brine , dried over Na_2SO_4, filtered and evaporated to dryness to give methyl ester of compound **5** as a white solid (8.06 g) without further purification. 2,2-Dimethyl-1,3-propanediol (6.49 g, 62.3 mmol) was added to the methyl ester of compound **3** (8.06 g, 41.6 mmol) dissolved in dry toluene (200 mL). The reaction was heated to reflux in a Dean-Stark trap overnight. After cooling down, the solvent was removed in vacuo, and

the crude product was purified (EtOAc/hexanes 2:8) to afford compound **6** (10.4 g, 89% for two steps) as a white solid, m.p. 77-79 °C. IR (microscope, cm^{-1}) 3029, 2958, 2900, 1713, 1603,; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.40 (d, *J* = 1.8 Hz, 1H), 7.93 (dd, *J* = 2.3, 8.0 Hz, 1H), 7.21 (dd, *J* = 0.3, 8.1 Hz, 1H), 3.90 (s, 3H), 3.79 (s, 4H), 2.57 (s, 3H), 1.04 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.5, 149.7, 136.2, 131.1, 130.2, 126.7, 72.4, 51.8, 31.7, 22.6, 21.9; ^{11}B NMR (128 MHz, $CDCl_3$) δ 27.2; HRMS (ESI) Calcd. $C_{14}H_{20}BO_4$: 263.14492. Found: 263.14480.

2.7.4 3-Carboxy-2-hydroxymethylphenyl boronic acid (4)

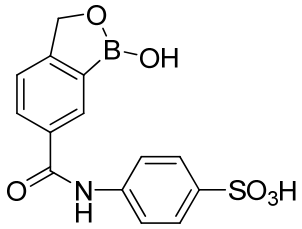


4

To a solution of compound **6** (2.53 g, 9.66 mmol) in benzene (50 mL) was added freshly recrystallized N-bromosuccinimide (1.80 g, 10.1 mmol) and AIBN (79.0 mg, 0.483 mmol). The mixture was stirred under reflux (oil bath at 100 °C). After 4 h, most of the solvent was evaporated in vacuo. The crude product was dissolved in ether and filtered. The filtrate was extracted with KOH (15% w/v in H_2O) (3x). The aqueous phase was stirred 1 h at room temperature. The solution was cooled at 0 °C and HCl (6N in H_2O) was added dropwise to a pH < 2. The white precipitate was collected by filtration through a medium fritted glass funnel, to afford 3-carboxy-2-hydroxymethylphenyl boronic acid (**4**) (1.17 g, 68% yield) as a white solid powder, m.p. 240-242°C. IR (microscope, cm^{-1}) 3195, 2960, 2650, 2474, 2228, 2067, 1681, 1619, 1456, 1407, 1265,

1185, 1115, 1003, 970, 846, 724; ^{1}H NMR (400 MHz, acetone-d_6) δ 8.39 (d, J = 0.8 Hz, 1H), 8.08 (dd, J = 1.6, 8.0 Hz, 1H), 7.50 (dd, J = 1.0, 8.2 Hz, 1H), 5.04 (s, 2H); ^{13}C NMR (126 MHz, acetone-d_6) δ 168.1, 159.9, 132.9, 132.7, 130.5, 122.3, 71.2; ^{11}B NMR (128 MHz, acetone-d_6) δ 32.1; HRMS (ESI) Calcd. $C_8H_6BO_4$: 177.03537. Found: 177.03540.

2.7.5 Synthesis of amide derivative of 4 (7)



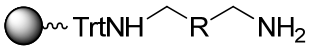
7

To a solution of 3-carboxy-2-hydroxymethylphenyl boronic acid (**4**) (200 mg, 1.12 mmol) in dry DMF (10 mL) under a N_2 atmosphere was added HBTU (852 mg, 2.25 mmol), HOAt (306 mg, 2.25 mmol) and DIPEA (782 µL, 4.49 mmol). The mixture was stirred for 5 min before the addition of sulfanilic acid (215 mg, 1.12 mmol). The reaction mixture was stirred at room temperature. After 3 days, the solvent was evaporated under reduced pressure (using a high vacuum rotavap). The crude mixture was partially dissolved in ether and extracted with KOH (15% wt/v) (3x). The aqueous phase was cooled down at 0 °C. Under stirring, HCl conc. was added dropwise until pH < 2. The precipitate was filtered on a medium fritted glass, washed with H_2O and dried under vacuum to give the title compound (336 mg, 90% yield) as a white solid. IR (microscope, cm^{-1}) 3462, 3284, 1662, 1596, 1502, 1538, 1405, 1176, 1130, 1038, 1010, 980, 833, 709; ^{1}H NMR (300 MHz, DMSO-d_6) δ 5.06 (s, 2H), 7.53-7.59 (m, 3H), 7.70-7.73 (m, 2H),

8.01-8.04 (dd, J = 1.8, 8.1 Hz, 1H), 8.30 (d, J = 1.2 Hz, 1H), 10.4 (s, 1H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 166.1, 157.4, 143.4, 139.4, 133.9, 130.3, 126.2, 121.7, 119.4; HRMS (ESI, *m/z*) calcd for $C_{14}H_{11}BNO_6S$ 332.03947, found 332.03977.

2.7.5 Synthesis of the library

2.7.5.1 4,7,10-Trioxy-1,13-tridecanediamine trityl polystyrene (8)

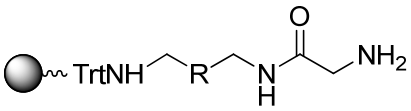


R = $(CH_2CH_2O)_3CH_2CH_2$

8

In a 500 mL round-bottomed flask, 4,7,10-trioxa-1,13-tridecanediamine (54 mL, 247 mmol) was charged into and diluted with dry DCM (300 mL) and stirred gently. To the solution was added chlorotrityl polystyrene resin (13.0 g, 0.950 mmol/g, 100–200 Mesh, 12.4 mmol) in portions (2–3 g per addition, 20-30 min between additions). After complete addition of the resin, the suspension was let to stir gently overnight. The resin was then transferred to a 100 mL PP vessel, washed with MeOH, DMF/Et_3N 4:1, MeOH and DCM (3x each), and dried overnight under high vacuum.

2.7.5.2 Synthesis of resin-bound Gly-NH_2 (2a)



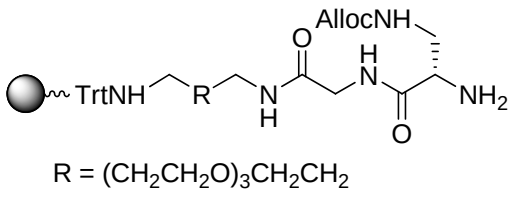
R = $(CH_2CH_2O)_3CH_2CH_2$

2a

Resin **8** (9.00 g, 7.34 mmol) and Fmoc-Gly-OH (4.80 g, 16.1 mmol) were charged into a 250 mL round-bottomed flask, and suspended in dry DMF (70 mL) and stirred gently

under N_2. This was followed by the addition of a solution of HBTU (6.07 g, 16.1 mmol) in dry DMF (10 mL) and a solution of HOAt (2.18 g, 16.1 mmol) in dry DMF (10 mL) and the resulting mixture was stirred for 5 min, after which DIPEA (5.60 mL, 32.2 mmol) was added. The mixture was stirred at room temperature for 6 h, and the resin was filtered, washed sequentially with DMF (3x), MeOH (3x) and DCM (3x) and dried under high vacuum. A negative Kaiser test was obtained. The Fmoc protecting group was removed by two treatments (20 min each) with a solution of morpholine in DMF (1:1) (2 x 110 mL solution). The mixture was filtered and washed sequentially with DMF (3x), MeOH (3x) and DCM (3x), and dried overnight under high vacuum.

2.7.5.3 Synthesis of resin-bound Gly-Dap(Alloc)-NH_2 (9) in IRORI MicroKan™ reactor

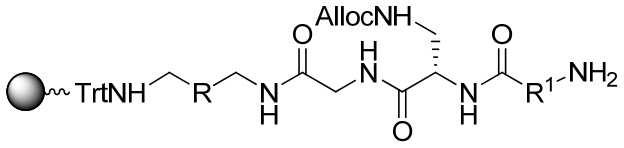


9

400 IRORI MicroKan™ reactors (referred to as Kans from here on) were fitted with a radio frequency (RF) tag followed by 25.0 mg of dry resin **2a** (0.0195 mmol of resin **2a**/Kan). The 400 Kans (7.80 mmol) were capped and put into a 1 L round-bottomed flask containing a magnetic bar. Fmoc-Dap(Alloc)-OH (9.59 g, 23.4 mmol) and dry DMF (300 mL) were added to the flask under N_2. A solution of HBTU (8.87 g, 23.4 mmol) in dry DMF (50 mL) and HOAt (3.18 g, 23.4 mmol) in dry DMF (50 mL) were added to the Kans. In order to remove any air bubbles from the Kans, the mixture was put

under high vacuum for 20 sec and then under N_2. This process was repeated until there were no air bubbles in the Kans (3 times). DIPEA (8.15 mL, 46.8 mmol) was added to the flask and the mixture was gently stirred (200 rpm) overnight at room temperature. The flask contents were filtered to separate the Kans from the reaction mixture. The Kans were placed back in the 1 L round-bottom flask and washed with DMF (3x), DCM (3x) and MeOH (3x) and twice with diethyl ether. The Kans were gently stirred 5 to 10 min prior to each washing. Finally, the Kans were dried under high vacuum. A negative Kaiser test was obtained on a few beads of resin picked randomly from two Kans. The Fmoc protecting group was removed by two treatments of 1 h each with 50% morpholine in DMF (2 x 400 mL solution). The reaction was filtered to separate the Kans from the reaction mixture, and the Kans were washed with DMF (3x), DCM (3x) and MeOH (3x) and twice with diethyl ether. Thus obtained Kans were gently stirred (5 to 10 min) during each washing. Finally, the Kans were dried under high vacuum. Using the software AccuTag Synthesis Manager, each Kan was assigned to a specific member of the library **1**{***1-20***, ***21-40***}.

2.7.5.4 Synthesis of resin-bound Gly-Dap(Alloc)-AA-NH_2 (10) in Kans



R = $(CH_2CH_2O)_3CH_2CH_2$

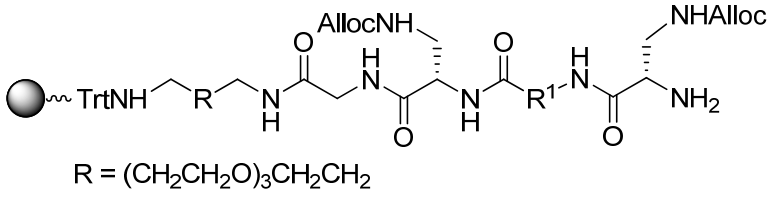
10

The Kans from above were separated into 20 different groups (***1-20***) by scanning each Kan with the IRORI AccuTag^{TM}-100 system. In a 50 mL round-bottomed flasks were

weighed the appropriate amino acid (1.56 mmol) [***1***: Fmoc-3-Amb-OH (583 mg); ***2***: Fmoc-4-Amb-OH (583 mg); ***3***: Fmoc-Amc-OH (592 mg); ***4***: Fmoc-4-Abu-OH (508 mg); ***5***: Fmoc-8-Aoc-OH (595 mg); ***6***: Fmoc-Gly-OH (464 mg); ***7***: Fmoc-Ala-OH (486 mg); ***8***: Fmoc-Leu-OH (551 mg); ***9***: Fmoc-D-Leu-OH (551 mg); ***10***: Fmoc-Cha-OH (614 mg); ***11***: Fmoc-Pro-OH (526 mg); ***12***: Fmoc-D-Pro-OH (526 mg); ***13***: Fmoc-Phg-OH (583 mg); ***14***: Fmoc-D-Phg-OH (583 mg); ***15***: Fmoc-Phe-OH (604 mg); ***16***: Fmoc-D-Phe-OH (604 mg); ***17***: Fmoc-Tyr(Me)-OH (651 mg); ***18***: Fmoc-Phe(4-F)-OH (632 mg); ***19***: Fmoc-Hfe-OH (626 mg); ***20***: Fmoc-D-Hfe-OH (626 mg)]. Under N_2 a magnetic bar and 20 Kans (0.39 mmol) were added in each appropriate flask followed by dry DMF (15 mL). Then, HBTU (592 mg, 1.56 mmol) and HOAt (212 mg, 1.56 mmol) dissolved both in dry DMF (5 mL each) were added to each flask. Air bubbles in the Kans were removed as described in section 1.3.3. DIPEA (543 uL, 3.12 mmol) was added in each flask. The mixtures were stirred gently (200 rpm) overnight at room temperature. The mixtures were filtered to separate the Kans from the reaction mixture. The Kans were pooled all together in a 1 L round-bottomed flask and washed sequentially with DMF (3x), DCM (3x) and MeOH (3x) and twice with diethyl ether. The Kans were gently stirred 5 to 10 min for each washing. Finally, the Kans were dried under high vacuum. A negative Kaiser test was obtained on few beads of resin picked randomly from one Kan of each group. The Fmoc protecting group was removed as described previously for the synthesis of resin-bound Gly-Dap(Alloc)-NH_2 (section 1.3.3) in IRORI MicroKan^{TM} reactor and the reaction took place in a 1 L flask with all the Kans in the same pot.

Note: Phenylglycine isomerized during the reactions on solid-phase. Both diastereoisomers were obtained (confirmed by HPLC) but these two diastereomers were not seperable. So, those receptors were tested as a mixture of diastereoisomers.

2.7.5.5 Synthesis of resin-bound Gly-Dap(Alloc)-AA-Dap(Alloc)-NH_2 (11) in Kans

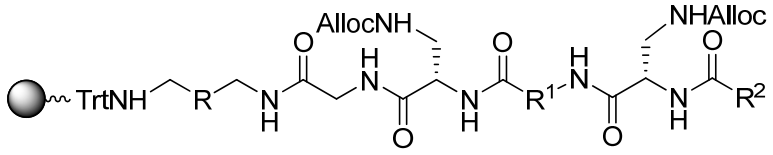


11

Dry DMF (300 mL) was added to a 1 L round-bottomed flask equipped with a magnetic bar, and the 400 Kans of **10** (7.80 mmol) and Fmoc-Dap(Alloc)-OH (12.8 g, 31.2 mmol). HBTU (11.8 g, 31.2 mmol) and HOAt (4.25 g, 31.2 mmol) dissolved in dry DMF (50 mL each) were added to the mixture. Air bubbles in the Kans were removed as described previously. DIPEA (11 mL, 62.4mmol) was added and the mixture was stirred gently (200 rpm) overnight at room temperature. The mixtures were filtered to separate the Kans from the reaction mixture. The Kans were put back in the 1 L round-bottomed flask and washed sequentially with DMF (3x), DCM (3x), MeOH (3x) and ethyl ether (2x). The Kans were gently stirred 5 to 10 min during each washing. Finally, the Kans were dried under high vacuum. A negative Kaiser test was obtained for a few beads of resin picked randomly from 5 Kans. The Fmoc protecting group was removed as described above for the synthesis of resin-bound Gly-Dap(Alloc)-NH_2 (section 1.3.3) in

IRORI MicroKan^{TM} reactor and the reaction took place in a 1 L flask with all the Kans in the same pot.

2.7.5.6 Synthesis of resin-bound Gly-Dap(Alloc)-AA-Dap(Alloc)-CA (2b) in Kans



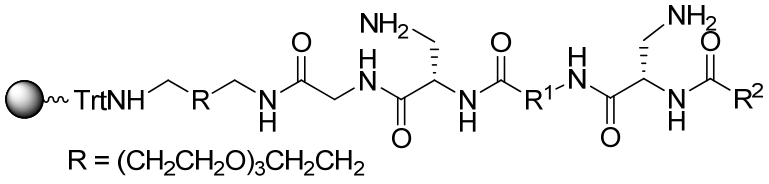
R = $(CH_2CH_2O)_3CH_2CH_2$

2b

The Kans of intermediates **11** were separated into 20 different groups (***21*** to ***40***) by scanning each Kan with the IRORI AccuTag^{TM}-100 system. In a 50 mL round-bottomed flask was weighed the appropriate carboxylic acid (1.56 mmol) [***21***: benzoic acid (191 mg); ***22***: 4-cyanobenzoic acid (230 mg); ***23***: 3,4-difluorobenzoic acid (247 mg); ***24***: 4-fluorobenzoic acid (219 mg); ***25***: 4-methoxybenzoic acid (237 mg); ***26***: piperonylic acid (259 mg); ***27***: thiophene-2-carboxylic acid (200 mg); ***28***: 2-furoic acid (175 mg); ***29***: 5-(4-methylphenyl)-2-furoic acid (316 mg); ***30***: phenylacetic acid (212 mg); ***31***: 4-(trifluoromethyl)phenylacetic acid (318 mg); ***32***: 4-methoxyphenylacetic acid (259 mg); ***33***: 4-biphenylacetic acid (331 mg); ***34***: 1-naphtaleneacetic acid (290 mg); ***35***: diphenylacetic acid (331 mg); ***36***: propionic acid (116 μL); ***37***: hydrocinnamic acid (234 mg); ***38***: 5-phenylvaleric acid (278 mg); ***39***: cyclohexylacetic acid (222 mg); ***40***: 1-adamantaneacetic acid (303 mg)]. A magnetic bar and the 20 Kans (0.390 mmol) were added in each appropriate flask followed by dry DMF (15 mL). HBTU (592 mg, 1.56 mmol) and HOAt (212 mg, 1.56 mmol) dissolved in dry DMF (5 mL each) were added to

each flask. Air bubbles in the Kans were removed as described in section 1.3.3. DIPEA (543 μL, 3.12 mmol) was added in each flask. The mixtures were stirred gently (200 rpm) overnight at room temperature. The mixtures were filtered to separate the Kans from the reaction mixture. The Kans were pooled together into a 1 L round-bottomed flask and washed sequentially with DMF (3x), DCM (3x), MeOH (3x) and diethyl ether (2x). The Kans were gently stirred 5 to 10 min during each washing. Finally, the Kans were dried under high vacuum. A negative Kaiser test was obtained on a few beads of resin picked randomly from one Kan of each group.

2.7.5.7 Synthesis of resin-bound Gly-Dap(NH_2)-AA-Dap(NH_2)-CA (12) in Kans

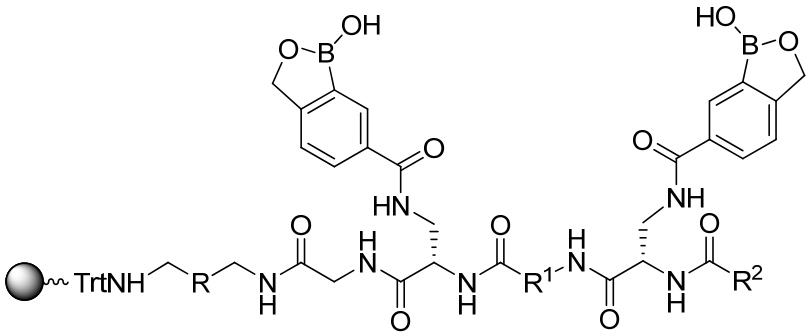


12

The 400 Kans of intermediate **2b** (7.80 mmol) were pooled together into a 1 L round-bottomed flask and underwent the final reaction steps in the same pot. To this flask equipped with a magnetic bar was added $Me_2NH{\cdot}BH_3$ (78.0 mmol, 4.60 g) and dry DCM (320 mL). A solution of $Pd(PPh_3)_4$ (1.17 mmol, 1.35 g) in dry DCM (80 mL) was added to the mixture. Air bubbles were removed by shaking vigorously the flask for 30 sec. The Kans were stirred gently for 30 min at room temperature. The mixture was filtered to separate the Kans from the reaction mixture. The Kans were washed 3 times with dry DCM and dried under high vacuum for 15 minutes before repeating the deprotection step twice. After the three-deprotection treatment, the Kans were washed with a 0.5%

solution of sodium diethyldithiocarbamate in DMF (5x), DCM (3x) and MeOH (3x) and twice with diethyl ether. The Kans were gently stirred 5 to 10 min for each washing. Finally, the Kans were dried under high vacuum overnight.

2.7.5.8 Synthesis of resin-bound Gly-Dap(3-carboxybenzoboroxole)-AA-Dap(3-carboxybenzoboroxole)-CA (13) in Kans

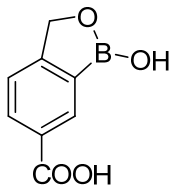


13

Dry DMF (300 mL) was added to a 1L round-bottomed flask equipped with a magnetic bar containing the 400 Kans of intermediate **12** (7.80 mmol) and 3-carboxy-2-hydroxymethylphenylboronic acid (**2**) (11.1 g, 62.4 mmol). HBTU (23.6 g, 62.4 mmol) in dry DMF (100 mL) and HOAt (8.50 g, 62.4 mmol) in dry DMF (50 mL) were added to the mixture. Air bubbles in the Kans were removed as described above. DIPEA (21.7 mL, 125 mmol) was added. The mixture was gently stirred (200 rpm) overnight at room temperature. The mixtures were filtered to separate the Kans from the reaction mixture. The Kans were put back in the 1 L round-bottomed flask and washed with DMF (3x), DCM (3x) and MeOH (3x) and twice with diethyl ether. Then the Kans were gently stirred 5 to 10 min for each washing. The Kans were dried under high vacuum. The

coupling of 3-carboxy-2-hydromethylphenylboronic acid (**2**) was repeated as described above, except that half the quantity of which was used. A negative Kaiser test was obtained on a few beads of resin picked randomly from nine Kans. Then the 400 Kans were treated three times with a H_2O/THF (50:50) solution for 30 min. The Kans were then washed with a H_2O/THF (50:50) solution (3x), THF (3x), DCM (1x), MeOH (1x) and diethyl ether (2x). The Kans were gently stirred 5 to 10 min during each washing. The Kans were dried under high vacuum overnight.

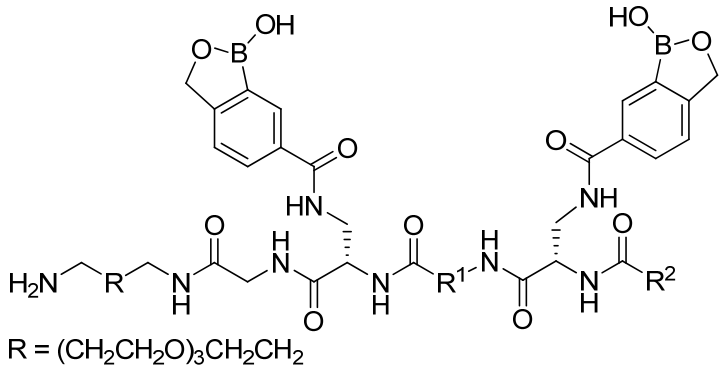
2.7.5.9 Recovery of unreacted 3-carboxy-2-hydroxymethylphenylboronic acid (4)



4

The organic solvent used for washing the Kans (containing the resin) was evaporated (a high vacuum rotavap was used to evaporate DMF). The crude mixture was partially dissolved in ether and extracted with aqueous KOH (15% wt/v) (3x). The aqueous phase was cooled to 0 °C. While stirring, conc. HCl was added dropwise to a pH < 2. The white precipitate was obtained by filtration through a medium fritted glass funnel, and washed with H_2O and dried under vacuum. Using this procedure, 82% of the unreacted boronic acid (**4**) was recovered.

2.7.5.10 Cleavage of resin-bound Gly-Dap(3-carboxybenzoboroxole)-AA-Dap(3-carboxybenzoboroxole)-CA (Library 1)



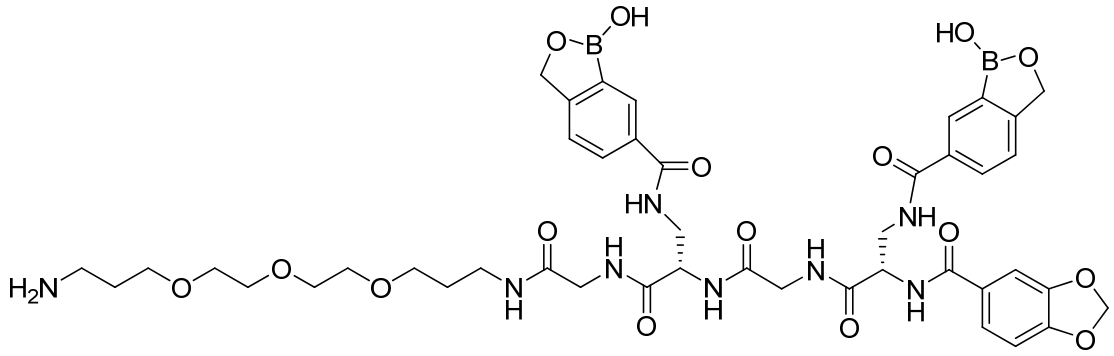
Library 1

The Kans were scanned with the IRORI AccuTag^{TM}-100 system and placed in the appropriate well of a microreactor. Five microreactor carriers containing 80 kans were used and the following procedure was repeated for each carrier. The rack was put in the AccuCleave-96 cleaving station and clipped to the vacuum chamber containing 8-mL vials. Into each well was added 1 mL of cleaving solution (5% TFA/DCM). The AccuCleave-96 cleaving station was covered and stacked on the IRORI vibratory shaker. After 90 min, vacuum was applied in the vacuum chamber for 2 minutes to empty the wells. The Kans were rinsed with MeOH (1 mL). After 10 min, vacuum was applied in the vacuum chamber for 2 minutes. The cleaving solutions were transferred in 3-mL vials and evaporated with an Atlas Evaporator Genevac. Each member of the library was purified by HPLC.

HPLC conditions: Semiprep: Zorbax R_x – C8, 9.4 X 250 mm, 40 ºC, 80% water + 0.05% formic acid and 20% acetonitrile, 1 mL/min., UV detection at 254 nm.

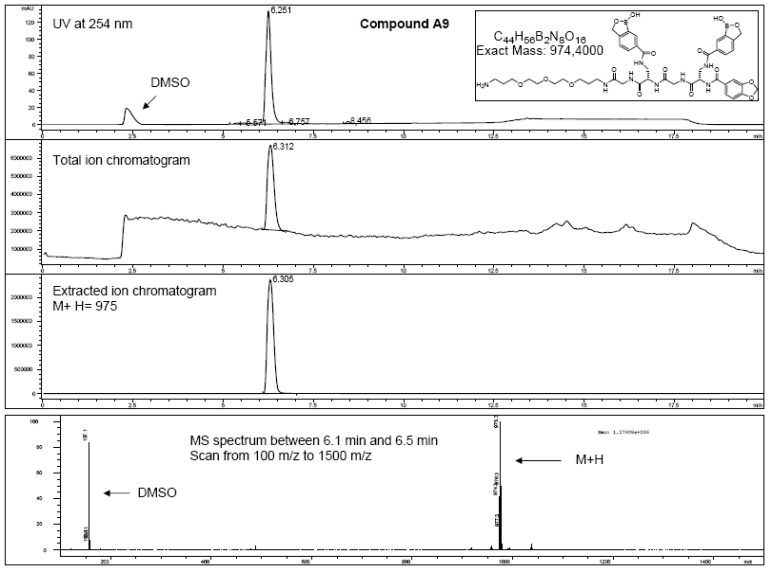
2.7.6 Characterization of a random sampling of the library

Library member 1{*6,26*}

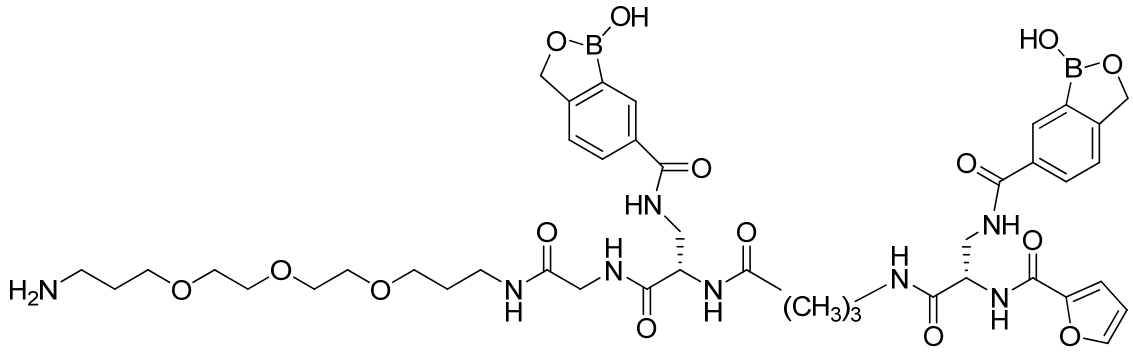


^{1}H NMR (400 MHz, MeOH-d_4) δ 7.96 (s, 1H), 7.94 (s, 1H), 7.77 (d, *J* = 9.6 Hz, 2H), 7.44 (dd, *J* = 1.6, 8.2 Hz, 1H), 7.31 (d, *J* = 6.8 Hz, 1H), 7.30 (d, *J* = 1.6 Hz, 1H), 7.26 (d, *J* = 7.6 Hz, 1H), 6.82 (d, *J* = 8.0 Hz, 1H), 6.00 (dd, *J* = 1.2, 2.0 Hz, 2H), 4.99 (s, 2H), 4.97 (s, 2H), 4.64 (t, *J* = 5.6 Hz, 1H), 4.52 (t, *J* = 5.6 Hz, 1H), 3.93 (d, *J* = 4.8 Hz, 2H), 3.91 (s, 2H), 3.86 (dd, *J* = 3.2, 6.0 Hz, 2H), 3.82 (s, 2H), 3.58-3.63 (m, 8H), 3.52-3.54 (m, 2H), 3.45 (t, *J* = 6.4 Hz, 2H), 3.25 (t, *J* = 6.4 Hz, 2H), 3.04 (t, *J* = 6.4 Hz, 2H), 1.87 (quint, *J* = 6.0 Hz, 2H), 1.72 (quint, *J* = 6.4 Hz, 2H); HRMS (ESI) Calcd. $C_{44}H_{57}B_2N_8O_{16}$: 975.40732. Found: 975.40744.

HPLC Chromatogram:



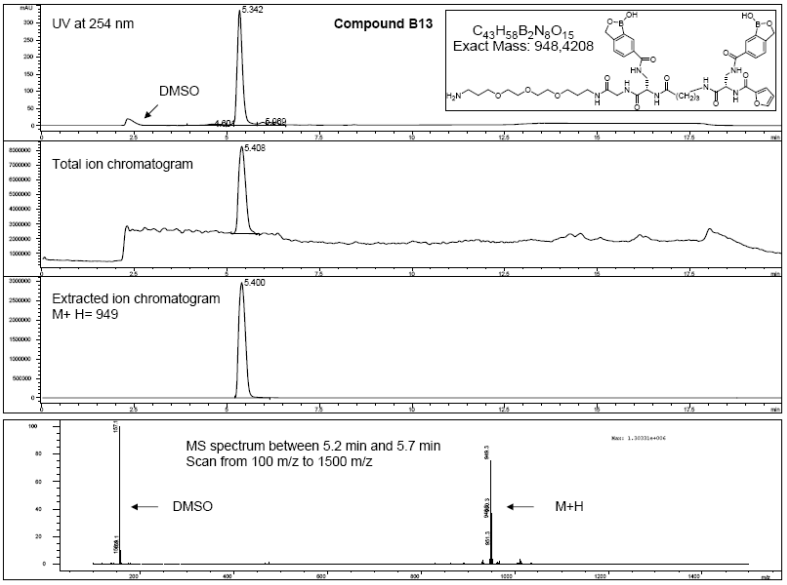
Library member 1{*4,28*}



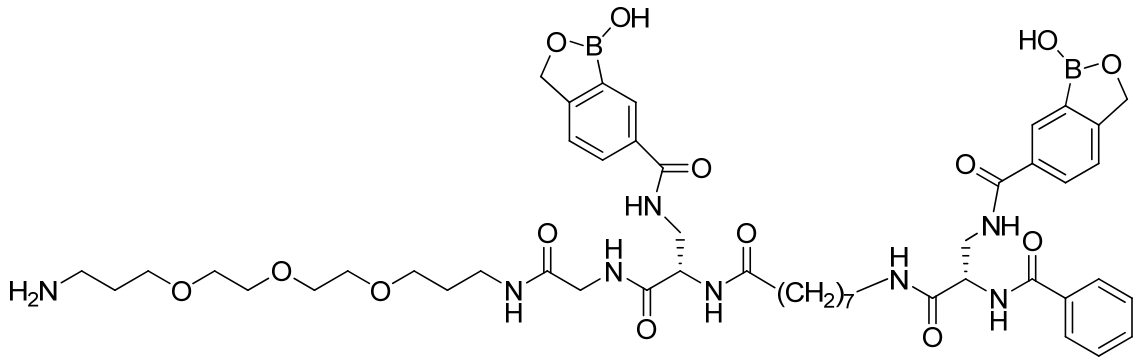
^{1}H NMR (400 MHz, MeOH-d_4) δ 8.04 (s, 1H), 7.97 (s, 1H), 7.84 (d, *J* = 8.0 Hz, 1H), 7.80 (d, *J* = 6.4 Hz, 1H), 7.67 (dd, *J* = 0.8, 1.6 Hz, 1H), 7.35 (brs, 1H), 7.33 (br s, 1H), 7.14 (dd, *J* = 0.8, 3.6 Hz, 1H), 6.57 (dd, J = 1.8, 3.6 Hz, 1H), 5.02 (s, 2H), 5.01 (s, 2H), 4.63 (dd, *J* = 5.4, 7.0 Hz, 1H), 4.46 (t, *J* = 6.4 Hz, 1H), 3.78-3.84 (m, 6H), 3.60-3.65 (m, 8H), 3.52-3.56 (m, 2H), 3.47 (t, *J* = 6.0 Hz, 4H), 3.27 (t, *J* = 7.2 Hz, 2H), 3.06 (t, *J* = 6.4 Hz, 2H), 2.30 (t, *J* = 6.8 Hz, 2H), 1.88 (quint, *J* = 6.0 Hz, 2H), 1.78-1.82 (m, 2H), 1.75

(quint, J = 6.8 Hz, 2H);HRMS (ESI) Calcd $C_{43}H_{59}B_2N_8O_{15}$: 949.42805. Found: 949.42767.

HPLC Chromatogram:



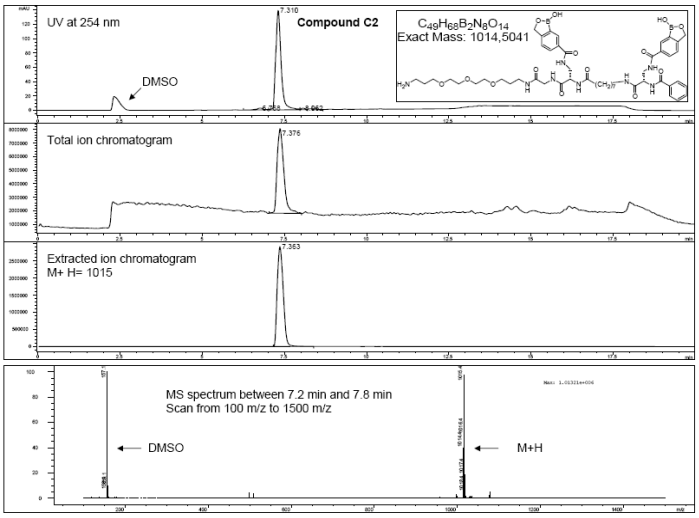
Library member 1{*5,21*}



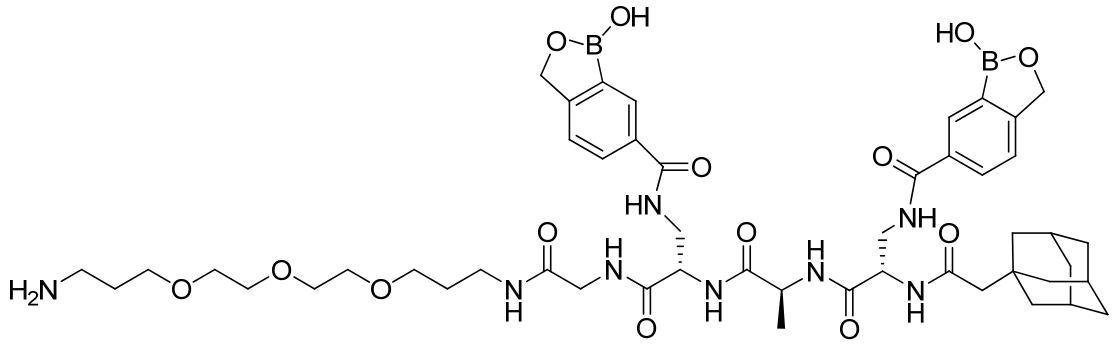
^{1}H NMR (400 MHz, MeOH-d_4) δ 7.98 (br s, 2H), 7.88 (dd, J = 1.2, 7.6 Hz, 2H), 7.79-7.82 (m, 2H), 7.53 (d, J = 7.2 Hz, 1H), 7.46 (t, J = 7.2 Hz, 2H), 7.30-7.34 (m, 2H), 5.00 (s, 4H), 4.74 (dd, J = 5.2, 6.8 Hz, 1H), 4.45 (t, J = 6.2 Hz, 1H), 3.75-3.84 (m, 6H), 3.60-3.64 (m, 8H), 3.54-3.56 (m, 2H), 3.47 (t, J = 6.0 Hz, 4H), 3.17 (t, J = 7.0 Hz, 2H), 3.04 (t,

J = 6.4 Hz, 2H), 2.23 (t, *J* = 7.6 Hz, 2H), 1.88 (quint, *J* = 6.0 Hz, 2H), 1.75 (quint, *J* = 6.4 Hz, 2H), 1.52-1.56 (m, 2H), 1.42-1.46 (m, 2H), 1.23 (br s, 6H); HRMS (ESI) Calcd $C_{49}H_{69}B_2N_8O_{14}$: 1015.51139. Found: 1015.51113.

HPLC Chromatogram:



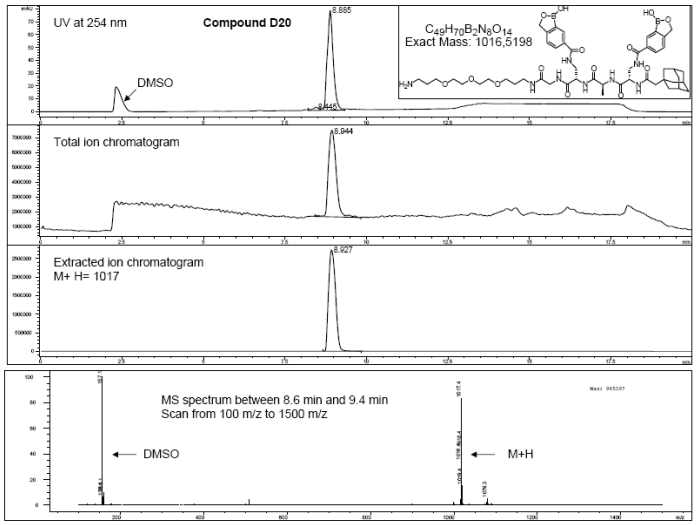
Library member 1{*7,40*}



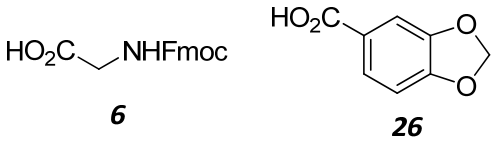
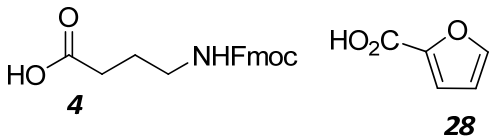
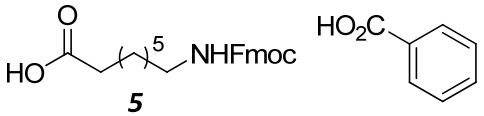
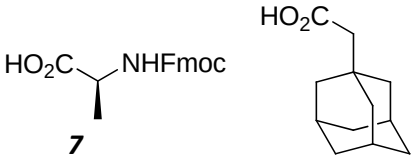
^{1}H NMR (400 MHz, MeOH-d_4) δ 8.00 (s, 1H), 7.95 (s, 1H), 7.81 (d, *J* = 7.2 Hz, 1H), 7.77 (d, *J* = 8.0 Hz, 1H), 7.29-7.31 (m, 2H), 4.99 (s, 2H), 4.97 (s, 2H), 4.53 (t, *J* = 6.0 Hz, 1H), 4.50 (dd, *J* = 4.4, 6.4 Hz, 1H), 4.29 (q, *J* = 7.2 Hz, 1H), 3.73-3.94 (m, 6H), 3.60-3.64 (m, 8H), 3.52-3.55 (m, 2H), 3.45 (t, *J* = 6.0 Hz, 2H), 3.25 (t, *J* = 7.0 Hz, 2H), 3.04 (t,

J = 6.4 Hz, 2H), 2.00 (s, 2H), 1.87 (t, *J* = 5.8 Hz, 2H), 1.80-1.84 (m, 3H), 1.73 (t, *J* = 6.8 Hz, 2H), 1.51-1.68 (m, 10H), 1.38 (d, *J* = 7.2 Hz, 3H); HRMS (ESI) Calcd. $C_{49}H_{71}B_2N_8O_{14}$: 1017.52704 Found: 1017.52631.

HPLC Chromatogram:



Purity and yield table for the compounds characterized above:

Entry	Compound Name	% of purity by UV after HPLC purification (254 nm)	Overall yield from trityl resin
1	**1 {*6,26*}**  ***6*** ***26***	94.7	33%
2	**1 {*4,28*}**  ***4*** ***28***	90.6	45%
3	**1 {*5,21*}**  ***5*** ***21***	95.2	40%
4	**1 {7,40}**  ***7*** ***40***	97.0	36%

2.8 References

1. Edwards, N. Y.; Sager, T. W.; McDevitt, J. T.; Anslyn, E. V. *J. Am.Chem. Soc.* **2007**, *129*, 13575-13583. (b) Manku, S.; Hall. D. G. *Aust. J.Chem.* **2007**, *60*, 824-828. (c) Duggan, P. J.; Offerman, D.A. *Tetrahedron* **2009**, *65*, 109-114.

2. Vanio, A. R.; Janda, K. D. *J. Comb. Chem.* **2000,** *2,* 579-596.

3. Yan, B. *Comb. Chem. High Throughput. Scr.* **1998,** *1,* 215-229.

4. Kaiser, E.; Colescot, R. L.; Bossinge, C. D.; Cook, P. I. *Anal. Biochem.* **1970,** *34,* 595-598.

5. Wentworth, P.; Janda, K. D. *Chem. Commun.* **1999,** 1917-1924.

6. Pirrung, M. C. *Chem. Rev.* **1997,** *97,* 473-488.

7. Plunkett, M. J.; Ellman, J. *J. Am. Chem. Soc.* **1995,** *117,* 3306-3307.

8. Moran, E. J.; Sarshar, S.; Cargill, J. F.; Shahbaz, M. M.; Loi, A.; Mjalli, A. M. M.; Armstrong, R. W. *J. Am. Chem. Soc.* **1995**, *117*, 10787-10788.

9. Nicolaou, K. C.; Xiao, X.-Y.; Parandoosh, Z.; Senyei, A.; Nova, M. P. *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 2289.

10. (a) Dowlut, M.; Hall, D. G. *J. Am. Chem. Soc.* **2006**, *128*, 4226-4227. (b) Bérubé, M.; Dowlut, M.; Hall, D. G. *J. Org. Chem.* **2008**, *63*, 6471-79.

11. Yu, L.-G. *Glycoconj. J.* **2007**, *24*, 411-420.

12. Pal, A.; Bérubé, M.; Hall, D. G. Submitted.

13. Laughrey, Z. R.; Kiehna, S. E.; Riemen, A. J.; Waters, M. L. *J. Am. Chem. Soc.* **2008**, *130*, 14625-14632.

Chapter 3

Screening of the Library of Peptidyl-bis(boroxoles)

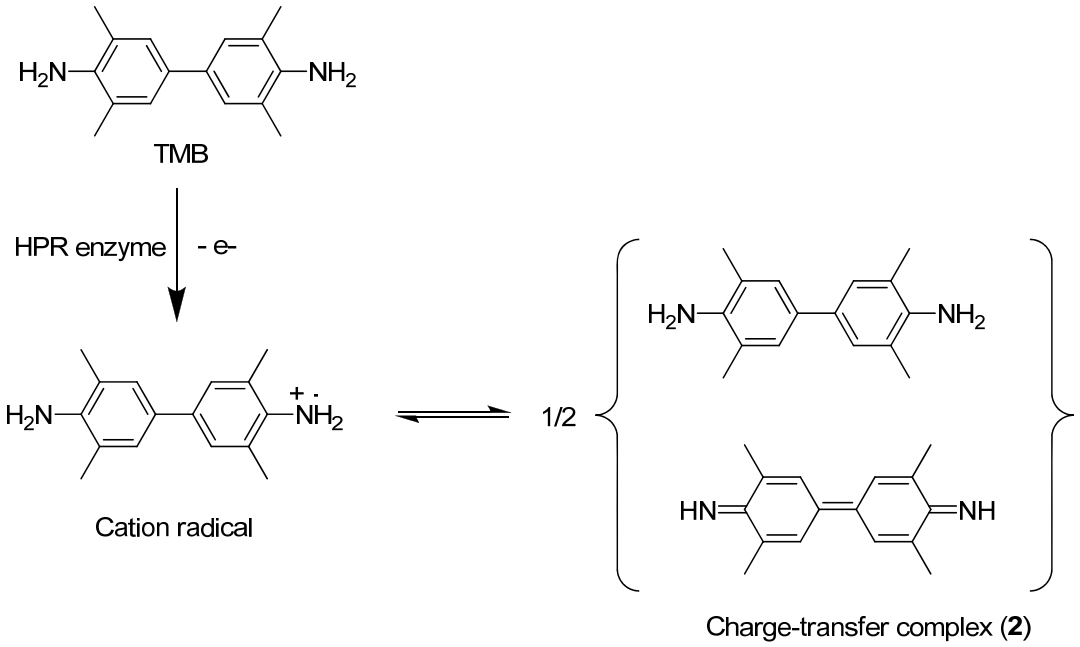
The synthesis of the library of 400 peptidyl bis-boroxole receptors was discussed in Chapter 2. Having this library of compounds in hand, we planned the screening for binding to the TF antigen disaccharide using a competitive ELISA.

3.1 ELISA (Enzyme-linked immunosorbent assay)

ELISA is a biochemical technique used primarily in immunology for detecting the presence of an antibody or an antigen in a sample.[1] This method has been used as a diagnostic tool in medicine and plant pathology, as well as a quality control check in various industries. Typically in an ELISA assay, an unknown amount of antigen sample is immobilized on a solid surface (a polystyrene microtiter plate). Then, the antibody which is covalently linked to a receptor enzyme is added to the plate. The plate is washed with buffer in between each step to remove all the nonspecifically bound protein or antibody. After the last step of the washing operations, the plate is treated with an enzyme substrate to obtain a signal that can indicate binding to the unknown antigen sample. The output signal could be UV-visible or fluorescence spectrophotometry, depending on chromogenic or fluorogenic substrates.

In our screening procedure we were interested in setting up a competition ELISA assay.[2]

Competition assays basically include two reactants that are trying to bind to a third reactant. In other words, the test scheme involves the reaction of two antibodies with an antigen attached to a solid support. Usually, the higher the original concentration of immobilized antigen, the weaker the eventual signals in a competition assay. The chromogenic substrate used in our assays is 3,5,3',5'-tetramethylbenzidine (TMB).



Scheme 3.1: Chemical structure of TMB and its oxidation according to Josephy *et al*.[3]

The oxidation of TMB by horseradish peroxidase (HPR)/H_2O_2 was reported by Josephy *et al*.[3] At acidic pH, the stable product of TMB oxidation, the TMB radical cation radical, is in equilibrium with the charge-transfer complex **2**, which is responsible for the blue

color in the plate. Thus, the higher the color, the higher the amount of antibody-antigen complexation.

3.2 Screening of the library

The library of 400 compounds was screened using the competition ELISA depicted in Figure 3.1. In this assay, first the 96-well plate was coated with β-D-Gal-1→3-D-GalNAc–$O(CH_2)_8CO$–BSA by incubating overnight. Then, PNA (peanut agglutinin) lectin and the library compound were premixed and incubated at a known concentration in the washed plate for two hours. A final 4% proportion of DMSO in phosphate buffer (pH = 7.2) was used to dissolve the library compounds because of their poor solubility in full aqueous buffer. The working concentration of the library compound was optimized to 400 μM after trying several concentrations. For example, the optical density was very low at lower concentration of the library compound, which made hit identification difficult. The working concentration of the PNA was also optimized to 1 nM in the same fashion. After washing of the plate with PBST {0.01 M phosphate buffer (pH = 7.2) containing 0.05% (v/v) Tween 20} three times, the optical density was measured at 450 nm upon treatment with the chromogenic substrate, i.e. 3,3',5,5' tetramethyl benzidine (TMB), followed by phosphoric acid.

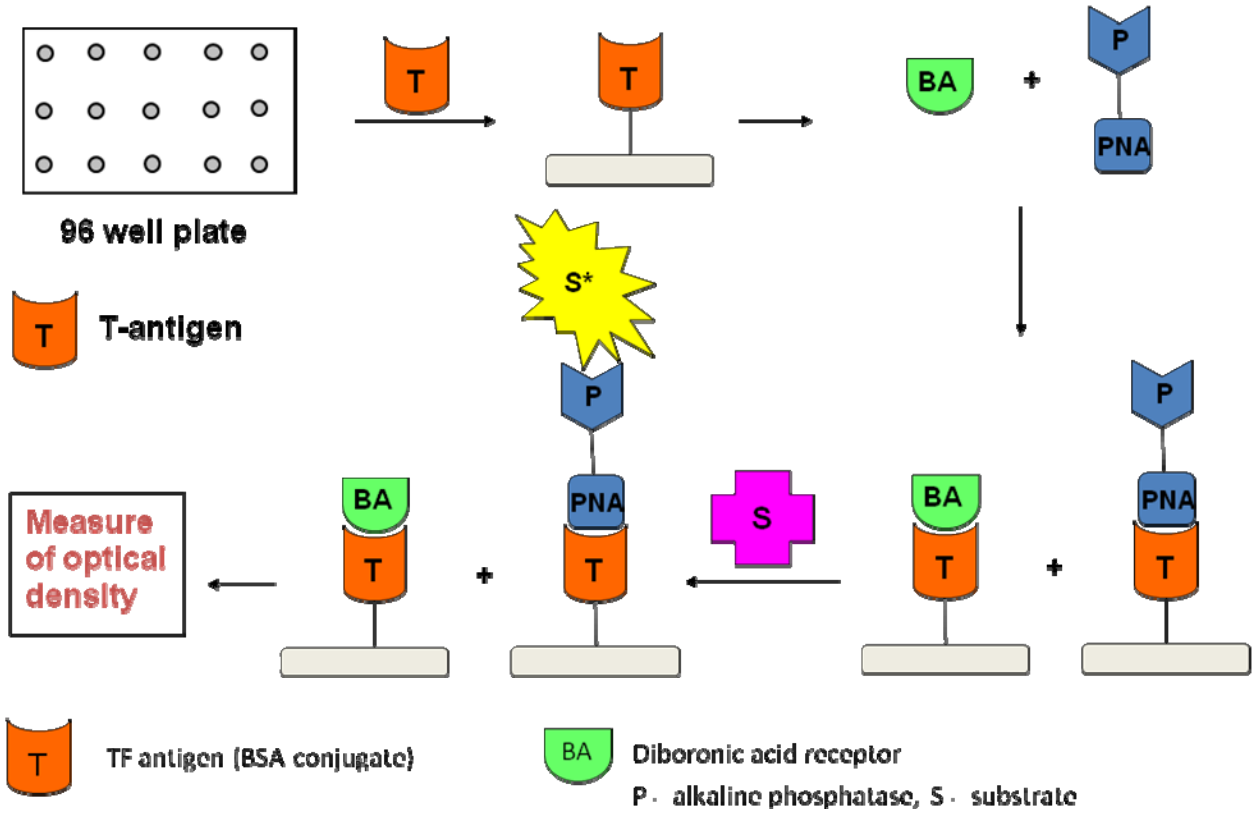


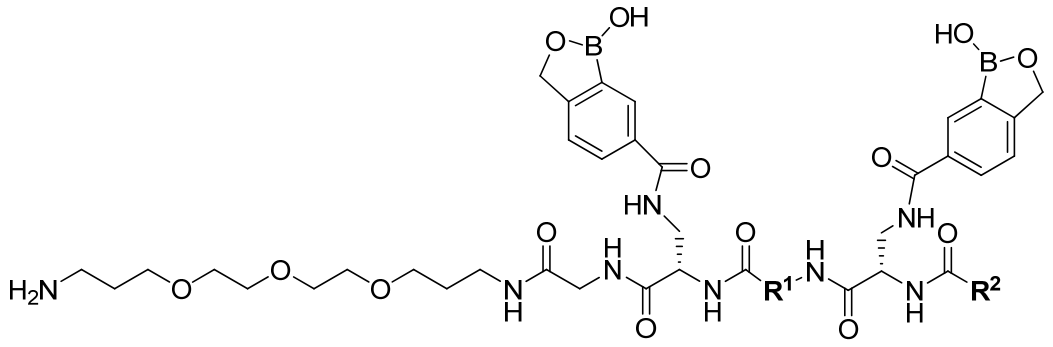
Figure 3.1: Pictorial description of the competition ELISA.

The complete set of data from the screening can be found in Section 3.7.2. Optical density was measured at 450 nm. Hits were identified by comparing the relative optical density to the control [well containing only PNA solution (i.e. no library compound)]. A compound was deemed a hit when the absorbance value was < 0.75, whereas, the control values are typically between 2 to 3. A total of 17 hits were identified and confirmed among the 400 compounds. The identified hits were:

1{8,35}, 1{15,23}, 1{15,29}, 1{18,29}, 1{17,21}, 1{17,37}, 1{17,33}, 1{17,29}, 1{17, 35}, 1{19,37}, 1{19,38}, 1{10,31}, 1{10,35}, 1{10,33}, 1{3,35}, 1{1,29}, 1{2,29}.

3.3 IC_{50} measurements

After comparing all the hits, IC_{50} measurements were obtained on the four most promising peptidyl bis(boroxole)s to confirm and quantify their potency (Table 3.1). A final proportion of 4% DMSO in phosphate buffer solution was used for dissolving the library compounds when measuring the IC_{50} values because of their poor solubility. The stock solution of the library compound (1 mM) was diluted serially to obtain several concentrations (0.316 mM, 0.10 mM, 0.031 mM, 0.01 mM, etc). It was difficult to prepare more concentrated (2 mM) stock solutions because of their poor solubilities. The serial dilution was triplicated in the same plate and averaged to get the IC_{50} values. The dose-response curve was drawn using the Origin software, which gives the IC_{50} value as an output.

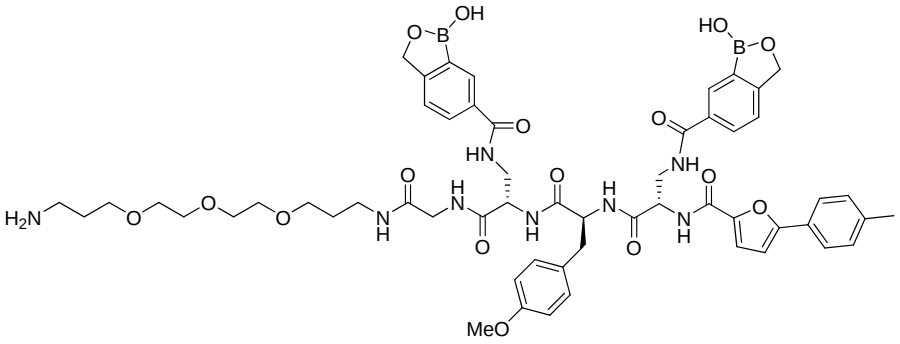


Library **1{*R*^{1},*R*^{2}}**

Compound number	**IC_{50} value**
1{***17***,***29***}	20 μM
1{*15,23*}	35 μM
1{*16,23*}	> 400 μM
1{*19,37*}	85 μM
1{*19,38*}	46 μM

Table 3.1: Most efficient receptors identified in the competition ELISA.

The most potent receptor, **1**{***17***,***29***}, showed an IC_{50} of 20 μM (Figure 3.2).



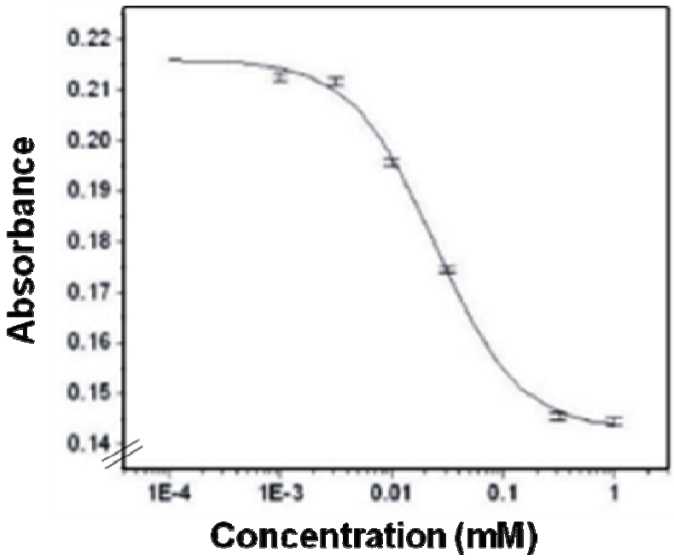


Figure 3.2: Structure and IC_{50} curve for the best receptor, **1{*17,29*}**.

The IC_{50} of **1{*16,23*}**, which is epimeric to **1{*15,23*}**, was also measured. The surprising disparity between receptors **1**{***15***,***23***} (IC_{50} = 35 μM) and **1**{***16***,***23***} (IC_{50} > 400 μM) differing only by the spacer's (R^1) stereochemistry, provided an early hint that selective and subtle molecular recognition is taking place with the TF antigen disaccharide.

3.4 Estimation of K_d of 1{*17,29*}·TF antigen by comparison with K_d of PNA·TF antigen

The K_d value between PNA and TF antigen is known to be 1.0 x 10^{-7} M.[4] The concentration of the PNA used in our experiment was 1 x 10^{-9} M. Since PNA has four potential binding sites for the TF antigen disaccharide, we can consider [PNA] to be ~ 4 x 10^{-9} M.

Hence,

$$\frac{K_{d\ Receptor}}{K_{d\ PNA}} \quad \alpha \quad \frac{[IC_{50}]}{[PNA]}$$

$$\frac{K_{d\ Receptor}}{1 \times 10^{-7}\ M} \quad \alpha \quad \frac{20 \times 10^{-6}\ M}{4 \times 10^{-9}\ M}$$

$K_{d\ Receptor}$ **~ 0.5 mM**

3.5 Methods for measuring binding constants

Conventional methods for measuring binding constants between boronic acids and polyols include ^{11}B NMR, ^{1}H NMR and pH depression experiments. Unfortunately, there was no distinct chemical shift change in NMR experiments for our system. On the other hand, spectroscopic methods, such as ICD (Induced Circular Dichroism), absorption, and fluorescence are generally more sensitive than NMR or pH determination

methods and have also been employed in carbohydrate recognition. In our case, ICD yielded a value of K_d comparable to the theoretically estimated binding constant.

In a typical ICD experiment, the concentration of the carbohydrate is varied, keeping a constant receptor concentration. The changes in ellipticity for the peptidyl bis(boroxole) receptor can be plotted against different concentration of the TF disaccharide at the optimal wavelength of 450 nm. Then the curve can be fitted according to a 1:1 fit using a non-linear least squares curve-fitting program to yield the association constant.

3.5.1 Methodology for measuring binding constant (K_d) using ICD[5]

The ICD experiment was done in methanol as the solvent because of the poor solubility of the best receptor when highly concentrated in the buffer (pH = 7.2). The following equation was used to estimate the association constant K_a

$$\Delta CD = CD_{max}[(K_a[s]_0 + K_a [R]_0 + 1) - \{(K_a [s]_0 + K_a [R]_0 + 1)^2 - 4K_a{}^2[R]_0[s]_0\}^{0.5}]/(2K_a) \quad \textbf{[1]}$$

Eq. **1** was derived from the following:

K_a = [complex]/[R][sugar]

$[R] = [R]_0 - [complex]$

$[sugar] = [s]_0 - [complex]$

$\Delta CD = CD_{obs} - CD_{receptor}$

$\Delta CD = CD_{max}[complex]$

In this equation, R represents the peptidyl bis(boroxole) receptor, S represents the sugar, CD_{max} is the maximum observed CD value for the saturated complex, CD_{obs} is the observed CD intensity, $CD_{receptor}$ is the CD intensity in the absence of sugar, and $[s]_0$ is the concentration of the added sugar.

The plotted curve obtained after the titration is represented by Figure 3.3. From this, curve-fitting using SigmaPlot software yielded a K_d of 0.90 mM. This value is in close agreement with the estimated value of Section 3.4.

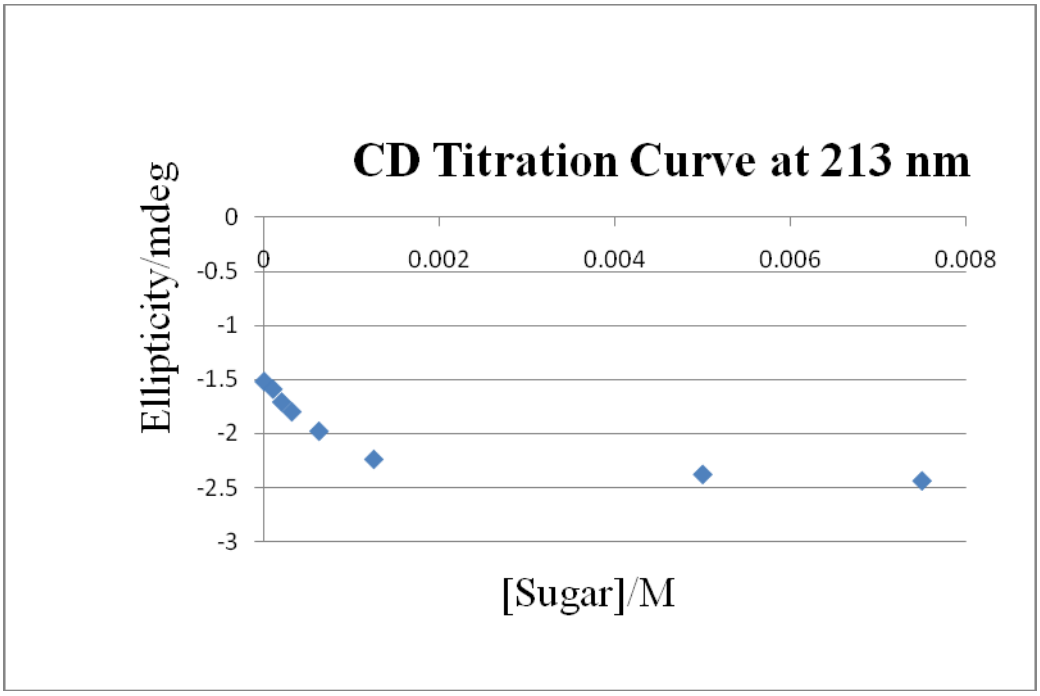


Figure 3.3: Titration curve for determination of K_d of **1{*17,29*}**.

3.6 Control experiments for binding selectivity

After measuring the binding constant between our best hit and the TF-antigen disaccharide, we were interested to know how important the boroxole unit is for the complexation. Therefore, control compounds **3** and **4** were synthesized in which the boroxole moiety of **1{*17,29*}** was replaced by a normal boronic acid moiety [bis(arylboronic acid)] and phenyl ring [bis(phenylamide)] respectively (Figure 3.4). These compounds were synthesized in the same manner as discussed in Section 2.9. A detailed description of the synthesis can be found in the following experimental section.

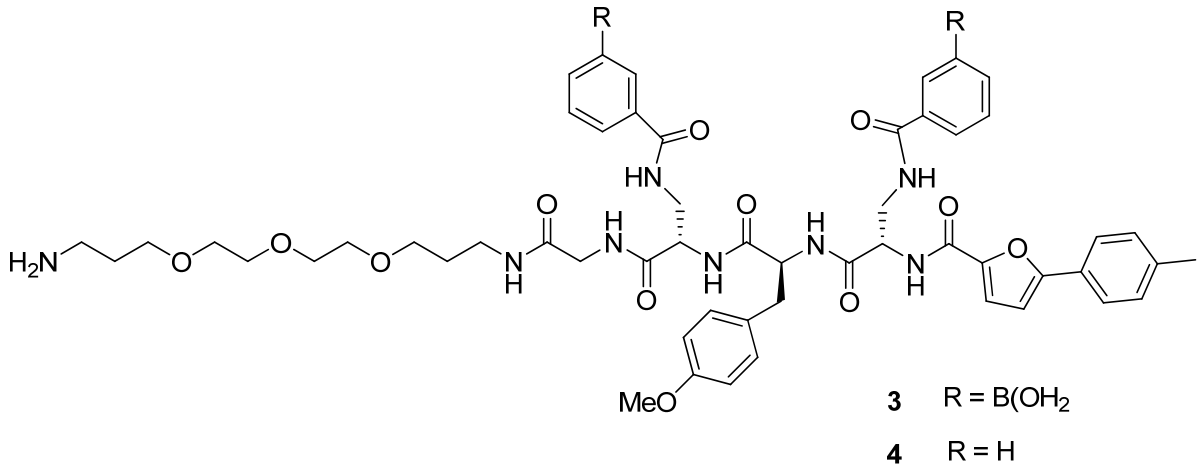
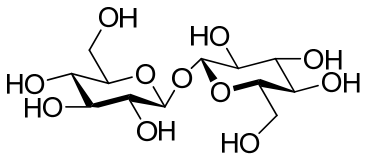


Figure 3.4: Control compounds **3** and **4** with modified Dpr side chains.

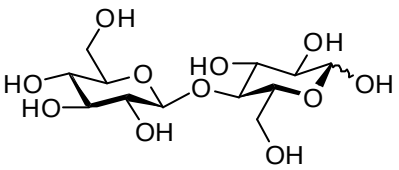
The IC_{50} values obtained for the above control compounds **3** and **4** were 43 μM and 90 μM respectively. It is obvious from these values that boroxoles are more favorable than normal boronic acids. With a less than 5-fold difference between **4** and **1**{***17***,***29***}, it

seems unlikely, however, that both boroxole units of **1**{***17***,***29***} are involved in strong covalent interactions with the two accessible diols of Gal-β-1→3-GalNAc. Other interactions from the peptide backbone such as H-bond donor/acceptor, or hydrophobic packing resulting from the aromatic R^1 and R^2 components, must be significant. At this stage we are not quite sure whether both of the benzoboroxoles are involved in the complex or not. Replacing each benzoboroxole selectivity by a phenyl group in the receptor could indicate whether one boroxole unit is sufficient for a moderate binding constant.

In the second part of our control experiments, the selectivity of receptor **1**{***17***,***29***} for the β-D-Gal-1→3-D-GalNAc disaccharide was assessed by monitoring the effect of adding various concentrations of other carbohydrates incubated under similar assay conditions in the presence of a fixed 20 μM concentration of **1**{***17***,***29***}, followed by washing and addition of the PNA lectin. As depicted in Figure 3.6, soluble β-D-Gal-1→3-D-GalNAc – $O(CH_2)_8CO_2Et$ interfered very strongly with the binding of **1**{***17***,***29***} to the β-D-Gal-1→3-D-GalNAc–$O(CH_2)_8CO$–BSA coated plates.

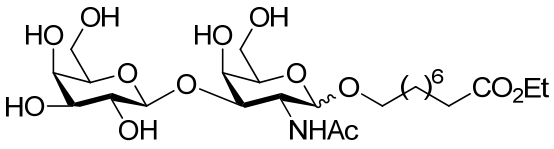


Trehalose

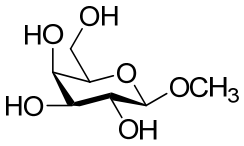


Cellobiose

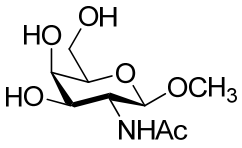
Figure 3.5: Unrelated oligosaccharides that do not show any competition.



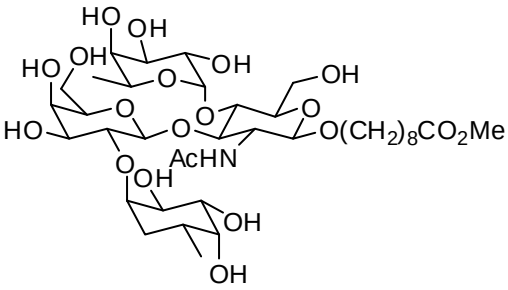
TF-Antigen glycoside



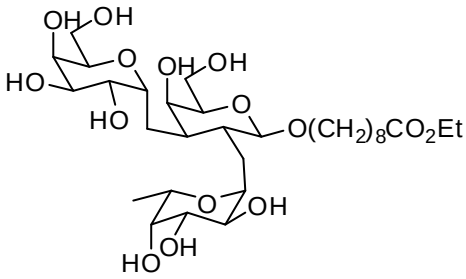
Methylgalactoside



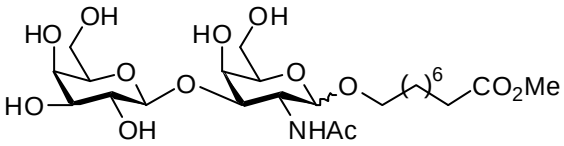
GalNAc methylglycoside



Lewis B tetrasaccharide



Blood group B trisaccharide



α-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactopyranose (TF antigen)

Figure 3.6: Oligosaccharides that show competition.

The individual Gal and GalNAc glycosides competed only to a small extent at high concentrations, while the unrelated oligosaccharides trehalose and cellobiose (Figure 3.5) had no effect on the binding of **1**{***17***,***29***} to the β-D-Gal-1→3-D-GalNAc–O$(CH_2)_8$CO–BSA coated plates. Lewis tetrasaccharide did not show much competition where as blood

group B trisaccharide showed quite similar competition like soluble soluble β-D-Gal-1→3-D-GalNAc–$O(CH_2)_8CO_2Me$ (Figure 3.7).

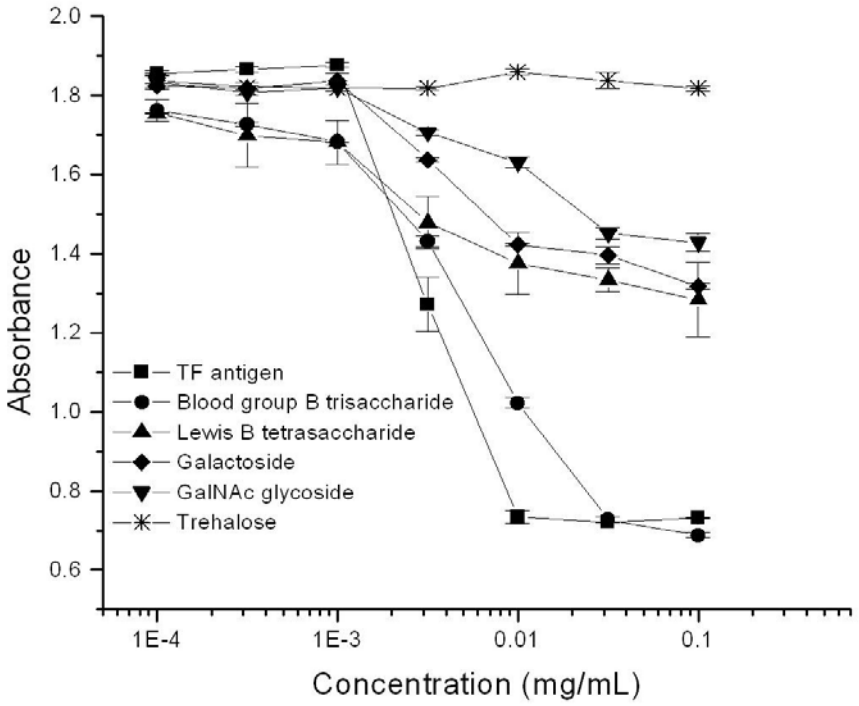


Figure 3.7: Control competition experiments to assess the selectivity of receptor **1**{***17***,***29***}. TF-antigen: β-D-Gal-1→3-D-GalNAc–$O(CH_2)_8CO_2Me$.

This results suggests that the best receptor **1**{***17***,***29***} is quite selective towards the TF antigen disaccharide compared to other disaccharides. It is also remarkable that the receptor does not bind appreciably to Gal and GalNAc glycosides.

3.7 Conclusion

In this chapter, a general screening protocol was described for identifying synthetic receptors of the TF antigen disaccharide. This competitive ELISA was carried out to screen the whole library of 400 peptidyl bis(boroxole) receptors. Among the preliminary seventeen screening hits, four showed relatively low IC_{50} values. The best receptor **1{*17,29*}** demonstrated an IC_{50} value of 20 μM. Its binding ability was tested by measuring a binding constant with a TF antigen derivative. Several sets of control experiments were carried out to confirm the binding affinity and selectivity of **1{*17,29*}**.

3.8 Experimental section

3.8.1 General experimental details

Unless otherwise noted all reactions were performed under argon or N_2 atmosphere. Fmoc-protected amino acids were purchased from NovaBiochem (La Jolla, California), Advanced Chemtech (Louisville, Kentucky) or Iris Biotech [(Marktredwitz, Germany) for Fmoc-Dap(Alloc)-OH]. Polystyrene trityl chloride resin (100-200 mesh) was purchased from Rapp Polymere (Tübingen, Germany). The loading specified by the supplier was used. DIPEA (Aldrich, purified by redistillation, 99.5% grade) was used. THF (used for reaction and resin washing) was dried by distillation over sodium/benzophenone and used the same day. Methylene chloride was dried by distillation over CaH_2. Anhydrous DMF was obtained from Aldrich. Compounds were purified with an Agilent 1100 MSD semi-preparative HPLC system. ^{1}H and ^{13}C NMR

were recorded on Varian 300, 400 or 500 MHz spectrometers. Chemical shifts are reported in δ (ppm) units using ^{13}C and residual ^{1}H signals from the deuterated solvents. A micromass TOF mass spectrometer provided the high resolution electrospray ionization mass spectra. Absorbance for ELISA tests were performed on a Molecular Devices Spectra Max 190 Microplate Reader. Peanut agglutinin lectin (PNA) from Arachis hypogaea (peroxidase labelled) was purchased from Sigma.

3.8.2 General procedure for screening of library using competition ELISA

Linbro microtiter plates were coated overnight with 100 μL of BSA (bovine serum albumin) conjugated with TF-Antigen [1 mg/mL stock solution in 0.01 M phosphate buffer (pH = 7.2)] at room temperature. The wells were then washed 3 times with PBST (0.01 M phosphate buffer (pH = 7.2) containing 0.05% (v/v) Tween 20). Library compound solution (final concentration = 400 μM solution in PBS and 4% DMSO) and PNA solution (final concentration 1.0 nM) were premixed and added to each well and incubated for 2 hours at room temperature. After excess compound and PNA were washed out with PBST (3x), each well was filled with 100 μL of TMB peroxidase substrate reagent (TMB = 3,3',5,5' tetramethyl benzidine). The reaction was stopped after 20 min by the addition of 100 μL/well of 1 M aqueous phosphoric acid solution. Optical density was measured at 450 nm. Hits were identified by comparing the relative

optical density to the control [well containing only PNA solution (i.e. no library compound)].

The charts below represent the obtained optical density after screening the library and all the hits were highlighted.

	21	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**control**
1	**1.789**	**1.821**	**1.87**	**2.05**	**2.11**	**2.01**	**1.99**	**2.11**	**0.51**	**2.23**	**1.889**
	31	***32***	***33***	***34***	***35***	***36***	***37***	***38***	***39***	***40***	
1	**2.0**	**1.95**	**1.89**	**2.05**	**1.91**	**1.93**	**1.93**	**2.17**	**1.97**	**2.10**	

	21	***22***	***23***	***24***	***25***	***26***	***27***	***28***	***29***	***30***	**control**
2	**1.415**	**1.17**	**2.21**	**2.61**	**2.91**	**1.71**	**1.95**	**1.45**	**0.72**	**2.95**	**3.014**
	31	***32***	***33***	***34***	***35***	***36***	***37***	***38***	***39***	***40***	
2	**2.35**	**2.91**	**3.01**	**3.14**	**3.15**	**1.65**	**2.38**	**3.21**	**2.98**	**3.05**	

	21	***22***	***23***	***24***	***25***	***26***	***27***	***28***	***29***	***30***	**control**
3	**1.68**	**2.35**	**2.95**	**3.22**	**3.42**	**3.01**	**1.37**	**2.95**	**2.75**	**1.81**	**3.12**
	31	***32***	***33***	***34***	***35***	***36***	***37***	***38***	***39***	***40***	
3	**1.64**	**1.91**	**3.14**	**3.21**	**0.73**	**3.01**	**1.95**	**3.42**	**1.92**	**3.43**	

	21	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	**control**
4	**2.42**	**2.51**	**2.50**	**2.49**	**2.76**	**2.54**	**2.58**	**2.72**	**2.61**	**2.13**	**2.603**
	31	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	
4	**2.51**	**2.51**	**2.22**	**2.49**	**2.59**	**2.52**	**2.58**	**1.39**	**2.64**	**2.88**	

	21	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	**control**
5	**1.91**	**2.14**	**1.95**	**1.98**	**2.55**	**2.25**	**2.26**	**1.45**	**1.61**	**0.81**	**2.26**
	31	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	
5	**1.71**	**.91**	**1.32**	**1.41**	**1.75**	**1.35**	**1.36**	**2.55**	**1.11**	**1.45**	

	21	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	**control**
6	**1.91**	**2.14**	**1.95**	**1.98**	**2.55**	**2.25**	**2.26**	**1.45**	**1.61**	**1.88**	**2.51**
	31	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	
6	**2.51**	**2.51**	**2.22**	**2.49**	**2.59**	**2.52**	**2.58**	**1.39**	**2.64**	**2.88**	

	21	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	**control**
7	**2.31**	**2.27**	**2.44**	**2.54**	**2.28**	**2.41**	**2.41**	**2.68**	**1.76**	**2.75**	**2.379**
	31	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	
7	**2.68**	**1.98**	**2.09**	**2.49**	**2.19**	**2.09**	**2.29**	**1.71**	**2.21**	**1.72**	

	21	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	**control**
8	**1.94**	**1.95**	**2.17**	**2.31**	**2.48**	**1.92**	**1.99**	**2.28**	**2.30**	**2.77**	**2.78**
	31	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	
8	**1.97**	**2.99**	**0.88**	**2.05**	**0.65**	**1.85**	**1.76**	**2.48**	**2.21**	**2.45**	

	21	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	**control**
9	**2.21**	**2.25**	**2.26**	**2.42**	**2.81**	**1.82**	**2.41**	**1.85**	**2.52**	**1.98**	**2.379**
	31	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	
9	**3.06**	**1.79**	**2.26**	**2.47**	**2.24**	**2.21**	**2.36**	**2.21**	**1.35**	**2.66**	

	21	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	**control**
10	**2.32**	**2.29**	**2.45**	**2.52**	**2.33**	**2.39**	**2.36**	**2.49**	**2.53**	**1.11**	**2.341**
	31	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	
10	**0.62**	**2.12**	**0.68**	**2.49**	**0.71**	**2.27**	**3.32**	**2.59**	**2.90**		

	21	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	**control**
11	**2.34**	**2.43**	**2.45**	**2.08**	**1.55**	**2.44**	**2.33**	**2.306**	**2.05**	**1.06**	**2.301**
	31	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	
11	**1.91**	**1.81**	**2.27**	**2.32**	**2.42**	**2.38**	**2.25**	**3.13**	**1.85**	**2.45**	

	21	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	**control**
12	**1.95**	**1.61**	**2.12**	**2.08**	**1.01**	**1.71**	**1.88**	**1.65**	**1.71**	**0.99**	**1.98**
	31	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	
13	**1.21**	**1.88**	**1.96**	**1.777**	**1.79**	**1.49**	**1.79**	**1.60**	**1.28**	**1.02**	

	21	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	**control**
14	**2.71**	**.91**	**1.94**	**1.83**	**.91**	**1.31**	**2.31**	**1.32**	**1.65**	**1.17**	**1.98**
	31	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	
14	**1.26**	**1.36**	**2.31**	**1.46**	**.91**	**1.16**	**1.75**	**1.62**	**1.05**	**1.11**	

	21	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	**control**
15	**1.61**	**1.97**	**0.69**	**1.65**	**1.19**	**1.19**	**1.03**	**0.78**	**0.62**	**1.72**	**1.99**
	31	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	
15	**0.997**	**.85**	**1.61**	**1.91**	**0.85**	**0.88**	**1.88**	**1.46**	**1.48**	**1.16**	

	21	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	**control**
16	**2.52**	**2.21**	**2.12**	**2.32**	**1.21**	**1.88**	**2.01**	**1.95**	**2.05**	**1.82**	**2.588**
	31	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	
16	**2.21**	**2.25**	**2.26**	**2.42**	**2.81**	**1.82**	**2.41**	**1.85**	**2.52**	**1.98**	**2.379**

	21	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	**control**
17	**0.44**	**2.31**	**2.48**	**1.062**	**1.71**	**2.51**	**2.41**	**2.39**	**0.38**	**2.45**	**2.23**
	31	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	
17	**2.19**	**2.81**	**0.51**	**1.71**	**0.72**	**1.02**	**0.71**	**2.81**	**2.62**	**2.42**	

	21	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	**control**
18	**2.18**	**2.23**	**2.31**	**2.11**	**.088**	**1.81**	**2.11**	**1.08**	**0.51**	**1.09**	**2.25**
	31	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	
18	**2.51**	**2.15**	**1.71**	**.833**	**1.42**	**.92**	**1.52**	**1.81**	**1.32**	**2.72**	

	21	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	**control**
19	**1.85**	**1.95**	**2.35**	**2.59**	**2.39**	**1.81**	**2.43**	**2.09**	**1.47**	**1.24**	**2.35**
	31	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	
19	**1.85**	**1.95**	**2.35**	**2.59**	**2.39**	**1.81**	**0.43**	**0.59**	**1.47**	**1.24**	

	21	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	**control**
20	**2.88**	**2.81**	**2.35**	**2.09**	**1.85**	**2.41**	**2.52**	**2.12**	**2.55**	**2.01**	**2.59**
	31	*32*	*33*	*34*	*35*	*36*	*37*	*38*	*39*	*40*	
20	**2.21**	**1.65**	**2.72**	**2.01**	**2.02**	**1.82**	**2.12**	**1.03**	**1.52**	**2.61**	

Compounds which were considered as hits:

A library member was considered a hit when the absorbance value was <0.75.

So from the above screening table we obtained 17 hits that were further investigated. The hits were:

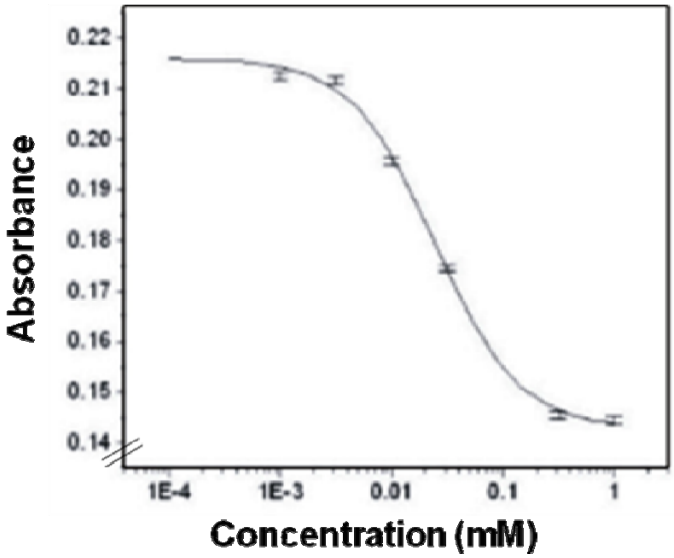
1*{8,35}*, 1*{15,23}*, 1*{15,29}*, 1*{18,29}*, 1*{17,21}*, 1*{17,37}*, 1*{17,33}*, 1*{17,29}*, 1*{17, 35}*, 1*{19,37}*, 1*{19,38}*, 1*{10,31}*, 1*{10,35}*, 1*{10,33}*, 1*{3,35}*, 1*{1,29}*, 1*{2,29}*.

3.8.3 Protocol for IC_{50} measurements using competition ELISA

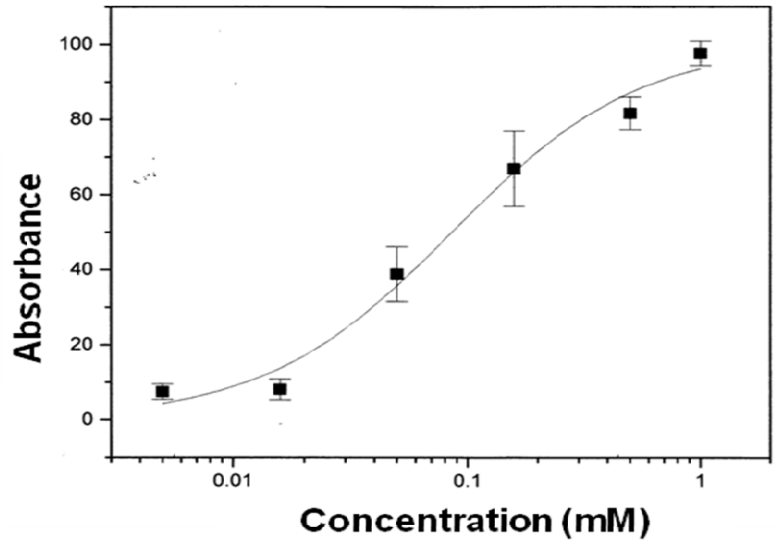
Linbro microtiter plates were coated overnight with 100 µL of BSA (bovine serum albumin) conjugated with TF antigen [1 mg/mL stock solution in 0.01 M phosphate buffer (pH = 7.2)] at room temperature. The wells were then washed 3 times with PBST (0.01 M phosphate buffer (pH = 7.2) containing 0.05% (v/v) Tween 20). PNA solution (final concentration 1.0 nM) and library compound solution in PBS with varying concentration from 1 M to 0.0001 M [prepared by twofold serial dilution (4% DMSO + PBS)] were premixed and added to each well and incubated for 2 h at room temperature. Library compounds were first dissolved in DMSO and then in buffer solution and they were sonicated afterwards at 35 °C. After excess compound and PNA were washed out with PBST, each well was filled with 100 µL of the TMB substrate (TMB = 3,3',5,5' tetramethyl benzidine). The reaction was stopped after 20 min by the addition of 100 µL/well of 1 M aqueous phosphoric acid solution. Optical density was measured at 450

nm. IC_{50}s were calculated (using Origin software) as the concentration required for 50% inhibition of the coating antigen.

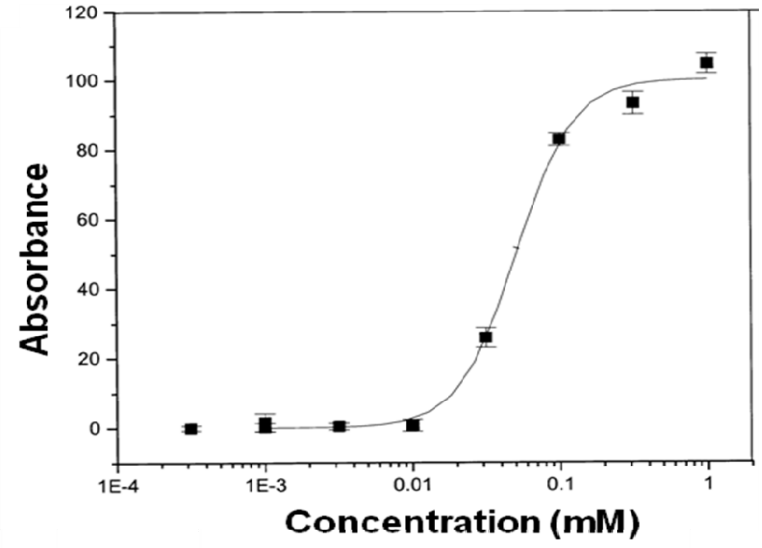
IC_{50} curve ***1{17,29}***: IC_{50} value = 20 μM



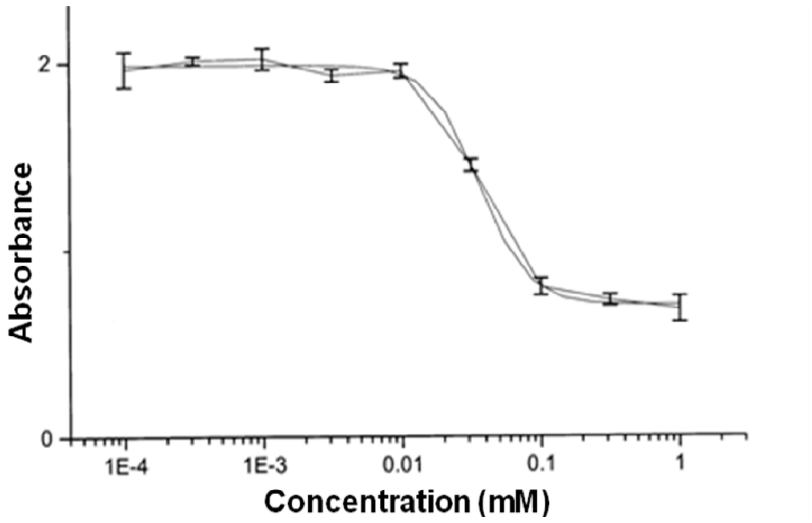
IC_{50} curve for **1*{19,37}***: IC_{50} value = 85 μM



IC_{50} curve for **1*{19,38}***: IC_{50} value = 54 μM

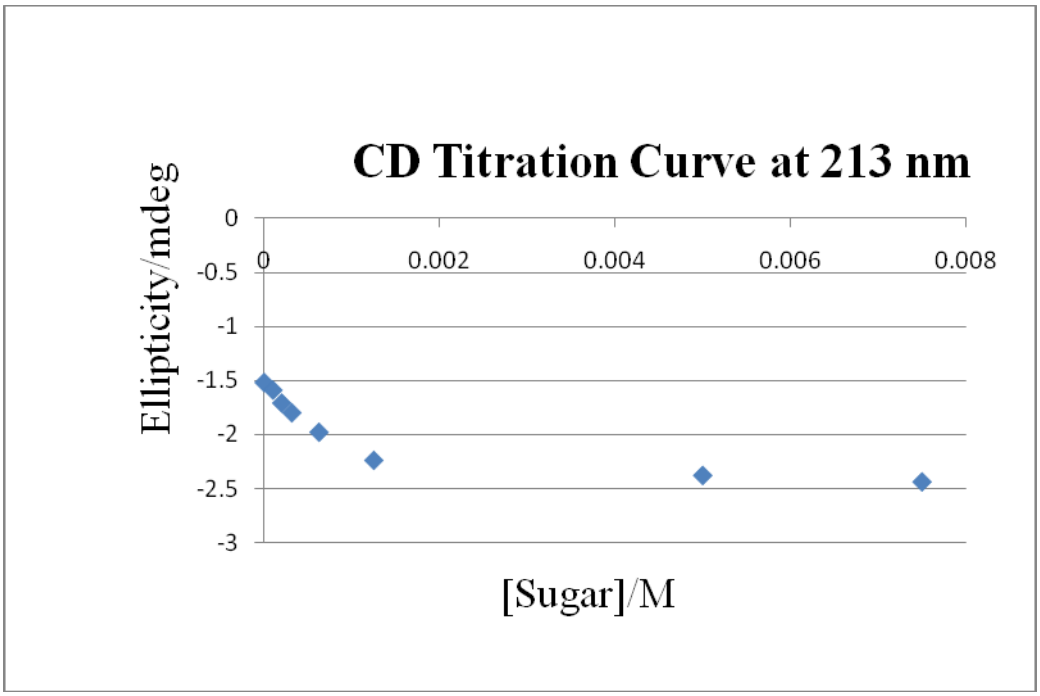


IC_{50} curve for ***1{15,23}***: IC_{50} value = 36 μM



3.8.4 Dissociation constant by induced circular dichroism titrations

Stock solutions of carbohydrates (10 mM) were made up in HPLC grade methanol and allowed to equilibrate overnight prior to use. Additions to receptor ***1{17,29}*** were performed using a procedure that kept [***1{17,29}***] = 0.2 mM in methanol and the total volume constant while raising [carbohydrate] from 0 mM to 7.50 mM in methanol (keeping the volume constant). After mixing by shaking, the CD spectrum was recorded on a Olis circular dichroism spectropolarimeter at 298 K. Changes in ellipticity were analysed and fitted using a non-linear least squares curve-fitting program present in software Sigma-plot. The program yields binding constants K_a as output. The resulting titration curve is shown below.



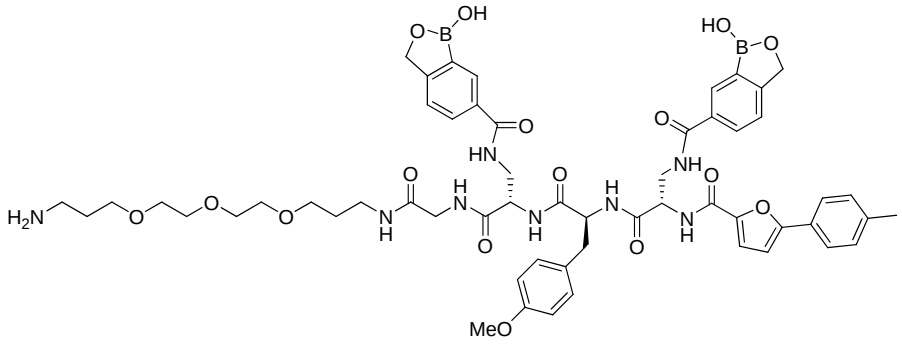
Data details:

sugar (M)	CD data (Ellipticity/mdeg)
0	-1.52
0.0001	-1.59
0.0002	-1.71
0.000315	-1.8
0.000625	-1.98
0.00125	-2.24
0.005	-2.38
0.0075	-2.44

$K_a = 1130\ M^{-1}$

i.e. $K_d = 0.90$ mM

3.8.5 Characterization of best receptor 1 *{17, 29}*

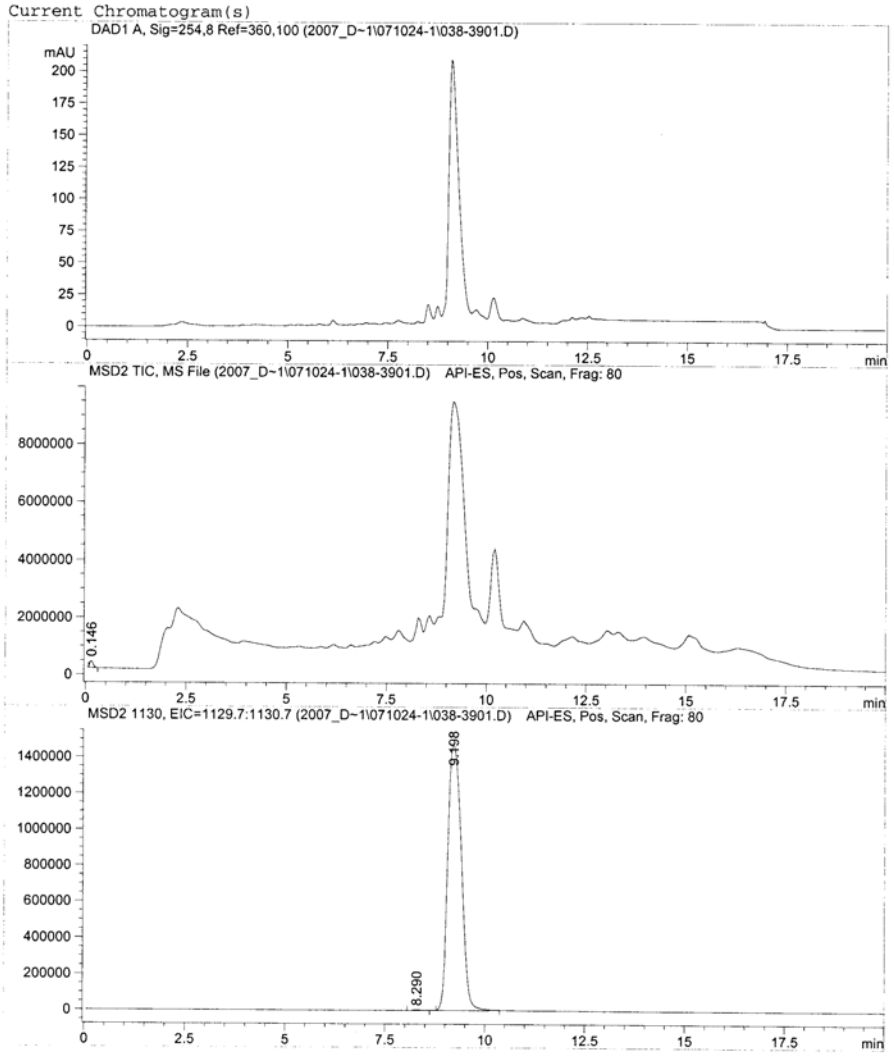


2.68 mg (19% overall yield), white solid; ^{1}H NMR (600 MHz; MeOH-d_4): δ 8.44 (s, 1H), 8.07 (s, 1H), 8.02 (s, 1H) 7.86 (ddd, *J* = 16.9, 8.0, 1.5, 2H), 7.79 (d, *J* = 8.2, 2H), 7.37 (d, *J* = 8.0, 1H), 7.28 (t, *J* = 7.8, 2H), 7.18 (d, *J* = 3.6, 1H), 7.07 (d, *J* = 8.6, 2H), 6.85 (d, *J* = 3.6, 1H), 6.65 (d, *J* = 8.7, 2H), 5.05 (s, 2H), 4.95 (s, 2H), 4.62 (dd, *J* = 6.4, 4.3, 1H), 4.56 (dd, *J* = 8.7, 5.6, 2H), 4.49 (dd, *J* = 6.8, 4.5, 1H), 3.89-3.85 (m, 2H), 3.82 (s, 2H), 3.80-3.76 (m, 2H), 3.61-3.56 (m, 6H), 3.52-3.51 (m, 2H), 3.45 (t, *J* = 6.1, 2H), 3.25 (t, *J* = 6.9, 2H), 3.10 (dd, *J* = 14.1, 5.6, 1H), 3.04 (t, *J* = 6.3, 1H), 2.93 (dd, *J* = 14.1, 8.7, 1H), 2.38 (s, 2H), 1.86 (dt, *J* = 11.5, 6.2, 2H), 1.72 (quint, *J* = 6.5 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.0, 169.9, 169.5, 168.4, 167.8, 166.9, 165.0, 157.9, 157.5, 156.6, 154.8, 145.9, 138.1, 132.6, 130.0, 129.7, 129.3, 126.4, 124.1, 120.9, 116.1, 113.2, 106.8, 69.7, 69.3, 67.8, 67.3, 54.5, 54.2, 53.1, 38.8, 36.7, 35.8, 29.0, 27.4, 20.7; HRMS (ESI) Calcd. $C_{51}H_{65}B_2N_8O_{15}$: 1130.49500. Found: 1130.49596.

HPLC conditions:

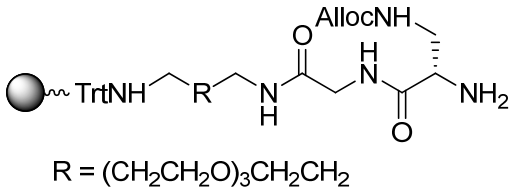
Zorbax SB – C8, 4.6 X 150 mm, 40 ºC, 80% water + 0.05% formic acid and 20% acetonitrile, 1 mL/min, UV detection at 254 nm.

HPLC (ESMS) chromatogram after purification



3.8.6 Synthesis of control compounds 3 and 4

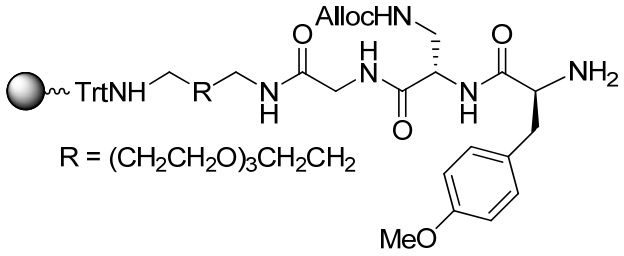
3.8.6.1 Synthesis of resin-bound Gly-Dap(Alloc)-NH_2 (5):



5

Resin-bound Gly-Dap(Alloc)-NH_2 (Chapter 2) (250 mg, 0.195 mmol) was weighed into a 10 mL PP vessel and suspended in dry DMF (0.5 mL). Fmoc-Dap(Alloc)-OH (240 mg, 0.585 mmol), HBTU (222 mg, 0.585 mmol) and HOAt (80 mg, 0.585 mmol) were weighed in a 3-mL vial, dissolved in dry DMF (2 mL) and added to the resin. After vortexing for 5 min, DIPEA (204 µL, 1.17 mmol) was added. The resin was vortexed for 7 h at room temperature. The resin was then filtered, washed sequentially with DMF (3x), DCM (3x), MeOH (3x) and diethyl ether (3x) and dried under high vacuum overnight. A negative Kaiser test revealed a complete coupling of the amino acid. The Fmoc protecting group was removed by two treatments with 50% morpholine in DMF (2.5 mL, 20 min). The resin was then filtered, washed sequentially with DMF (3x), DCM (3x), MeOH (3x) and diethyl ether (3x) and dried under high vacuum overnight.

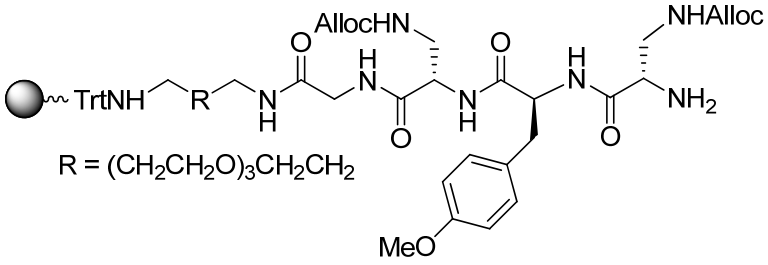
3.8.6.2 Synthesis of resin-bound Gly-Dap(Alloc)-Phe-NH_2 (6):



6

This coupling was carried out according to the procedure described for the synthesis of resin-bound Gly-Dap(Alloc)-NH_2 (**5**)using Fmoc-Tyr(Me)-OH (227 mg, 0.585 mmol) as amino acid.

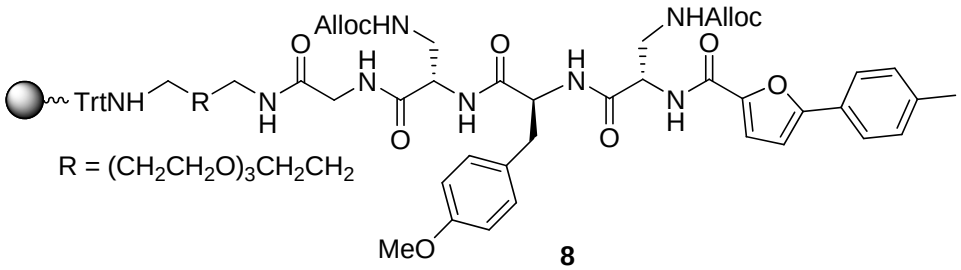
3.8.6.3 Synthesis of resin-bound Gly-Dap(Alloc)-Phe-Dap(Alloc)-NH_2 (7):



7

This coupling was carried out according to the procedure described for the synthesis of resin-bound Gly-Dap(Alloc)-NH_2 (**5**) except that the reaction was vortexed overnight.

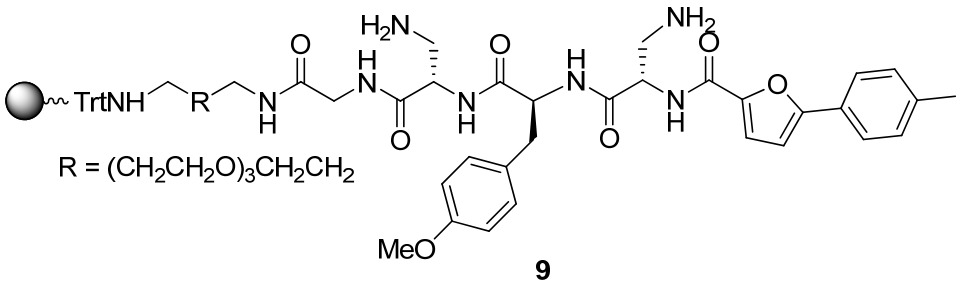
3.8.6.4 Synthesis of resin-bound Gly-Dap(Alloc)-Phe-Dap(Alloc)-5-(4-methylphenyl)-2-furanyl (8):



8

This coupling was carried out according to the procedure described for the synthesis of resin-bound Gly-Dap(Alloc)-NH_2 using 5-(4-methylphenyl)-2-furoic acid (44 µL, 0.585 mmol) as carboxylic acid.

3.8.6.5 Synthesis of resin-bound Gly-Dap(NH_2)-Phe-Dap(NH_2)-5-(4-methylphenyl)-2-furanyl (9):

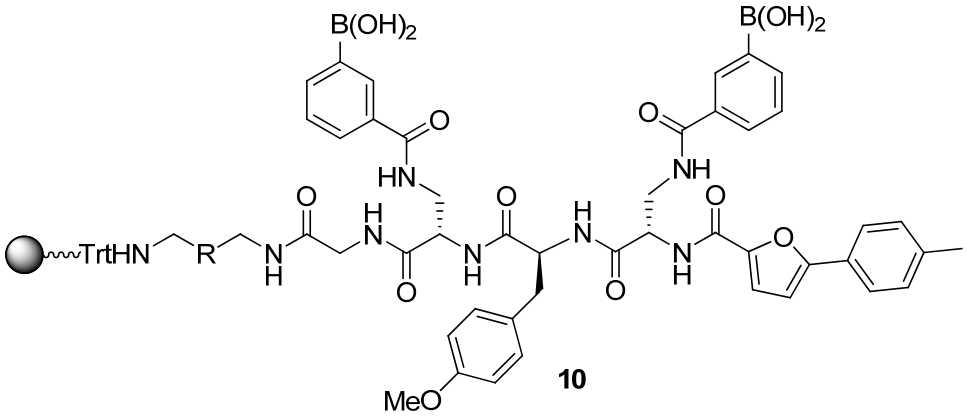


9

The protected resin **8** (0.195 mmol) and $Me_2NH{\cdot}BH_3$ (115 mg, 1.95 mmol) were weighed in a 10 mL PP flask equipped with a septum and suspended in dry DCM (2 mL) under N_2. A solution of $Pd(PPh_3)_4$ (0.029 mmol, 34 mg) in dry DCM (0.5 mL) was added to the reaction. The resin was vortexed at room temperature. After 30 min, the resin was filtered and washed with dry DCM (3x 5 mL). After three deprotection treatment, the resin was

filtered and washed with a 0.5% solution of sodium diethyldithiocarbamate in DMF (5x), DCM (3x), MeOH (3x) and diethyl ether (2x), and dried under high vacuum.

3.8.6.6 Synthesis of resin-bound Gly-Dap(NHBenzoyl)-Phe-Dap(NHBenzoyl)-5-(4-methylphenyl)-2-furanyl (10):

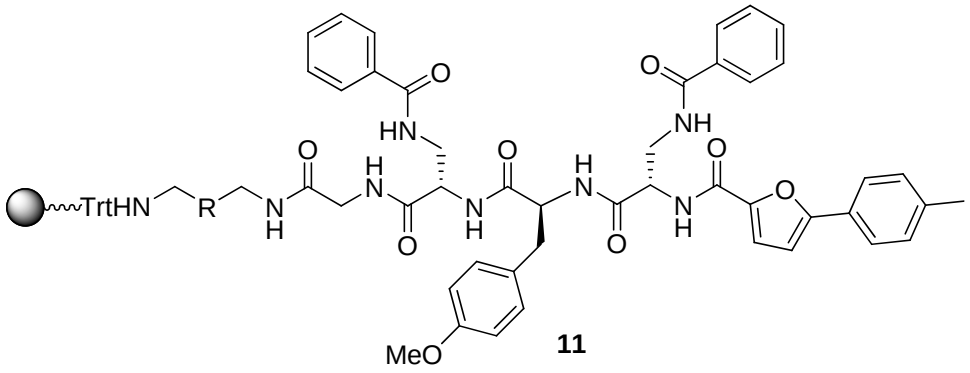


10

This synthesis was carried out according to the procedure described for the synthesis of resin-bound Gly-Dap(NHBenzoyl)-Phe-Dap(NHBenzoyl)-5-(4-methylphenyl)-2-furanyl (**10**) using 3-carboxyphenylboronic acid (39 mg, 0.234 mmol) as carboxylic acid. Then the resin was treated three times with a H_2O/THF (50:50) solution for 15 minutes. The resin was then washed with a H_2O/THF (50:50) solution (3x), THF (3x), DCM (2x), MeOH (2x) and diethyl ether (2x). The resin was finally dried under high vacuum overnight.

.

3.8.6.7 Synthesis of resin-bound Gly-Dap(NH-3-carboxyphenylboronic acid)-Phe-Dap(NH-3-carboxyphenylboronic acid)-5-(4-methylphenyl)-2-furanyl (11):



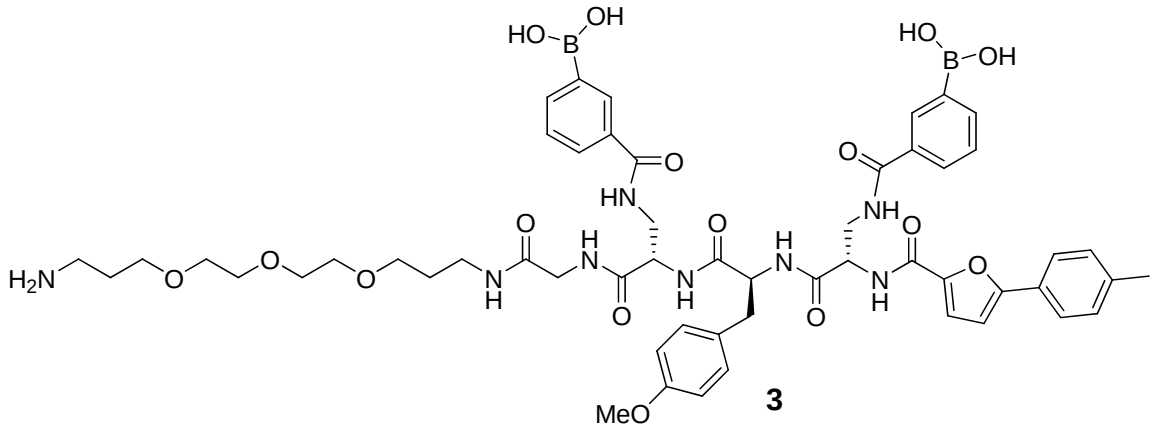
11

Resin **10** (50 mg, 0.039 mmol) was weighed into a 1 mL PP vessel and suspended in dry DMF (0.25 mL). Benzoic acid (29 mg, 0.234 mmol), HBTU (88 mg, 0.234 mmol) and HOAt (32 mg, 0.234 mmol) were weighed in a 3-mL vial, dissolved in dry DMF (0.25 mL) and added to the resin. After vortexing for 5 min, DIPEA (82 μL, 0.468 mmol) was added. The resin was vortexed for 24 h at room temperature. The resin was then filtered, washed sequentially with DMF (3x), DCM (3x), MeOH (3x) and diethyl ether (3x) and dried under high vacuum overnight. A negative Kaiser test revealed a complete coupling of the carboxylic acid

3.8.6.8 Cleavage of resin-bound to obtain Gly-Dap(NH-3-carboxyphenylboronic acid)-Phe-Dap(NH-3-carboxyphenylboronic acid)-5-(4-methylphenyl)-2-furanyl (3)

The cleavage of the resin **10** was carried out according to the procedure described for the cleavage of resin-bound Gly-Dap(NHBenzoyl)-Phe-Dap(NHBenzoyl)- 5-(4-methylphenyl)-2-furanyl (**10**).

Characterization of 3



3

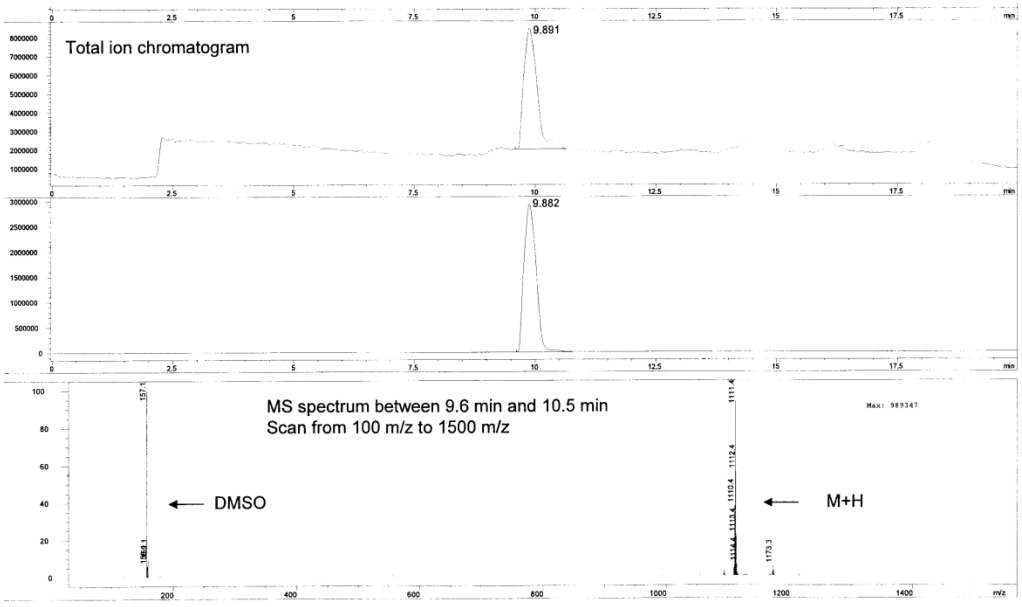
5.3 mg (29% overall yield), white solid; ^{1}H NMR (500 MHz, MeOH-d_4) δ 8.43 (s, 1H), 8.07 (s, 1H), 8.01 (1H), 7.85 (dd, *J* = 1.7, 7.8 Hz, 2H), 7.83-7.81 (m, 2H), 7.78-7.75 (m, 2H), 7.37 (d, *J* = 8.02 Hz, 1H), 7.36-7.27 (m, 3H), 7.17 (d, *J* = 4.0 Hz, 1H), 7.11-7.04 (m, 2H), 6.85-6.83 (m, 2H), 5.04 (1s, 2H), 4.95 (1s, 2H), 4.64-4.60 (m, 2H), 4.57-4.54 (m, 2H), 3.85-3.77 (m, 8H), 3.61-3.50 (m, 9H), 3.26-3.24 (m, 2H), 3.04-3.03 (m, 4H), 2.38

(s, 3H), 1.86 (quint, J = 6.1, 2H), 1.72 (quint, J = 6.3, 2H); HRMS (ESI, *m/z*) calcd. $C_{44}H_{63}B_2N_8O_{14}$: 1106.49444. Found: 1106.49393.

HPLC conditions:

Zorbax SB – C8, 4.6 X 150 mm, 40 ºC, 80% water + 0.05% formic acid and 20% acetonitrile, 1 mL/min, UV detection at 254 nm.

HPLC chromatogram after purification

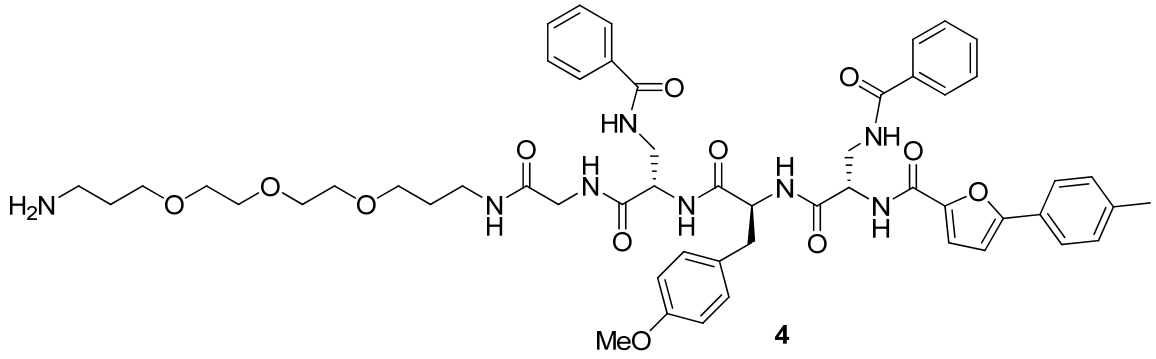


3.8.6.9 Cleavage of resin-bound to obtain Gly-Dap(NHBenzoyl)-Phe-Dap(NHBenzoyl)- 5-(4-methylphenyl)-2-furanyl (4)

The resin **11** (16 mg, 0.0195 mmol) was weighed in a 3-mL vial and suspended in a 5% TFA in DCM solution (1 mL). After vortexing for 45 min, the resin had turned a deep red color. The resin was filtered through a cotton plug in a pipette and washed with MeOH (3x). The combined filtrates were evaporated in a 3-mL vial with an Atlas Evaporator GeneVac. The crude product was purified by semipreparative HPLC.

HPLC conditions: Zorbax SB – C8, 4.6 X 150 mm, 40 ºC, 80% water + 0.05% formic acid and 20% acetonitrile, 1 mL/min, UV detection at 254 nm.

Characterization of 4:



4

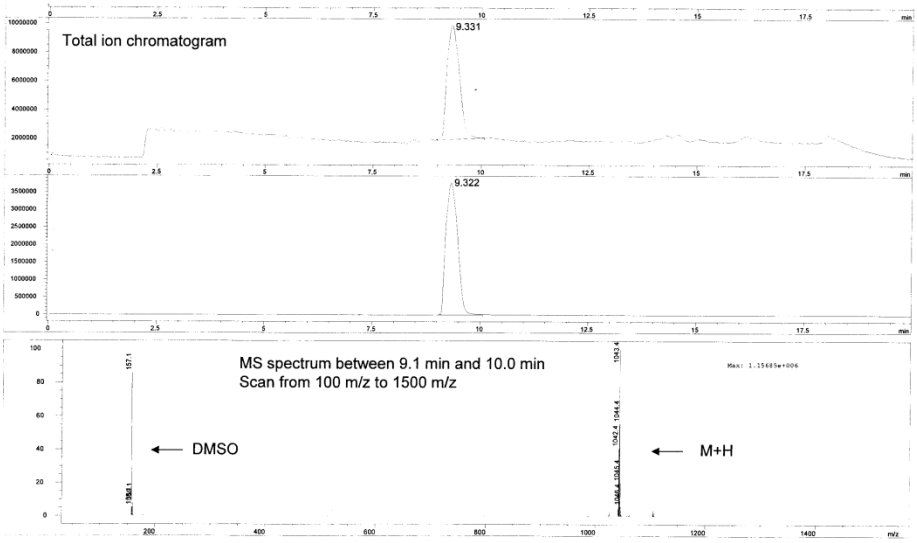
2.35 mg (14% yield), white solid; ^{1}H NMR (600 MHz, MeOH-d_4) δ 8.05 (s, 1H), 7.99 (1H), 7.85 (dd, *J* = 1.7, 7.9 Hz, 2H), 7.83 (dd, *J* = 1.6, 7.8 Hz, 2H) 7.78 (s, 1H), 7.78 (d, *J* = 8.2 Hz, 2H), 7.37 (d, *J* = 8.0 Hz, 1H), 7.36-7.28 (m, 3H), 7.17 (d, *J* = 3.6 Hz, 1H), 7.10-7.06 (m, 2H), 6.85 dd, *J* = 3.5 Hz, 1H), 6.65-6.64 (m, 2H), 5.04 (1s, 2H), 4.94 (1s, 2H),

4.62-4.60 (m, 1H), 4.57-4.54 (m, 2H), 4.49-4.47 (m, 1H), 3.85-3.78 (m, 7H), 3.61-3.56 (m, 8H), 3.45 (t, *J* = 5.8 Hz, 1H), 3.52-3.51 (m, 3H), 3.26-3.24 (dd, *J* = 5.7 Hz, 2H), 3.04 (t, *J* = 5.3 Hz, 2H), 3.03 (s, 1H), 2.88-2.90 (m, 1H), 2.38 (s, 3H), 1.86 (quint, *J* = 6.4 Hz, 2H), 1.71 quint, *J* = 6.3 Hz, 2H) ; HRMS (ESI, *m/z*) calcd. $C_{41}H_{61}N_8O_{10}$: 1018.48052. Found: 1018.48026.

HPLC conditions:

Zorbax SB – C8, 4.6 X 150 mm, 40 ºC, 80% water + 0.05% formic acid and 20% acetonitrile, 1 mL/min, UV detection at 254 nm.

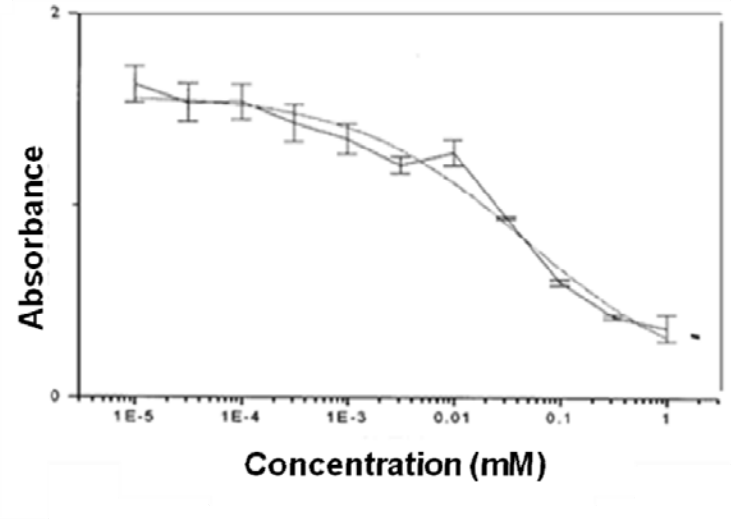
HPLC chromatogram after purification



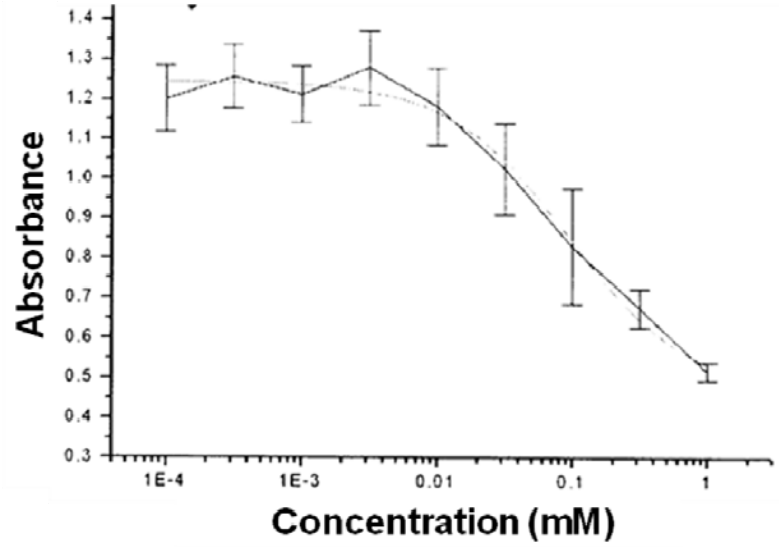
3.8.7 IC_{50} measurements for control compounds 3 and 4

The measurements were carried out as previously discussed in Section 1.8.

IC_{50} for **3**: 43 μM

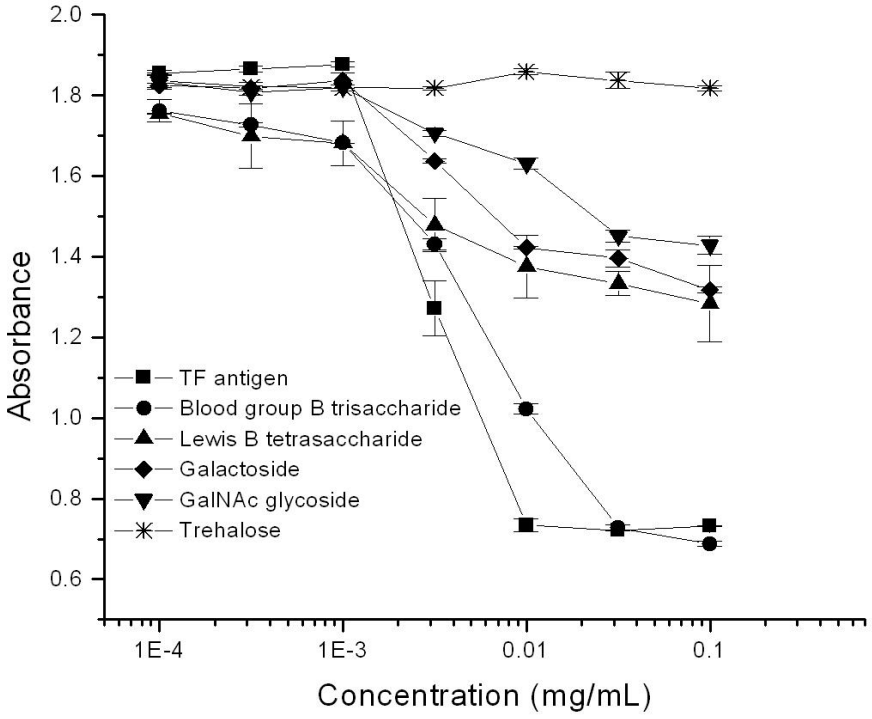


IC_{50} for **4**: 90 μM



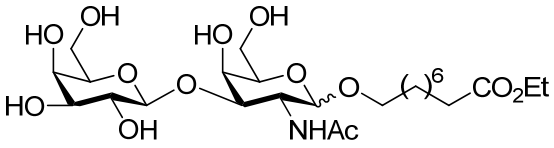
3.8.9 General procedure for control experiments using competition ELISA

Microtiter plates were coated overnight with 100 μL of BSA (bovine serum albumin) conjugated with TF-Antigen [1 mg/mL stock solution in 0.01 M phosphate buffer (pH = 7.2)] at room temperature. The wells were then washed 3 times with PBST {0.01 M phosphate buffer (pH = 7.3) containing 0.05% (v/v) Tween 20}. Library compound solution (final concentration = IC_{50} concentration of the hit = 20 μM) and control compound solution in PBS with varying concentration from 1 M to 0.0001 M [prepared by twofold serial dilution (4% DMSO / PBS)] were premixed and added to each well and incubated for 2 hour at room temperature. After excess compound and PNA were washed out with PBST, each well was filled with 100 μL of PNA solution (1 ng/mL) and incubated. After 1 hour, the plate was washed with PBST and each well was filled with TMB peroxidase substrate (TMB = 3,3',5,5' tetramethyl benzidine). The reaction was stopped after 20 min by the addition of 100 μL/well of 1 M aqueous phosphoric acid solution. Optical density was measured at 450 nm.

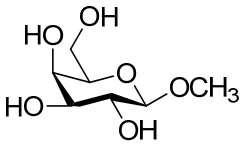


Structure of the control compounds

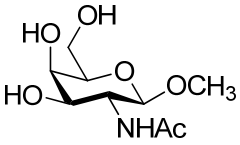
Related oligosaccharides:



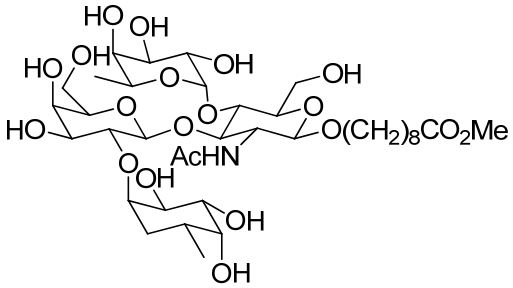
TF-Antigen glycoside



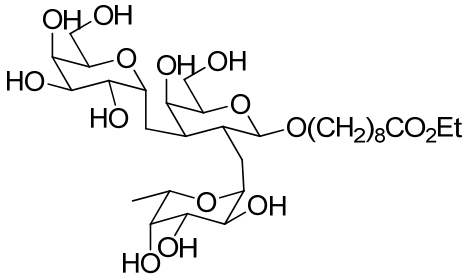
Methyl-D-galactoside



GalNAc methylglycoside

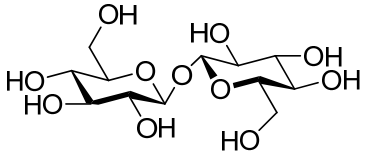


Lewis B tetrasaccharide

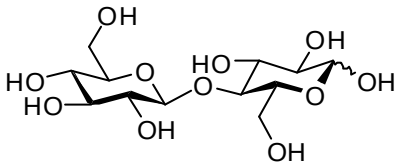


Blood group B trisaccharide

Unrelated oligosaccharides that do not show any competition:



Trehalose



Cellobiose

3.9 References

1. Crowther, J. *R. Methods in Molecular Biology,* **1995**.
2. Baek, M.; Roy, R. *Bioorg. Med. Chem.* **2002,** *10,* 11-17.
3. Josephy, P. D.; Eling, T.; Mason, R. P. *J. Biol. Chem.* **1982,** *257,* 3669-3675.
4. Neurohr, K.J.; Young, N.M.; Mantsch, H.H. *J. Biol. Chem.* **1980,** *255*, 9205-9209.
5. Tamaru, S.; Shinkai, S.; Khasanov, A. B.; Bell, T. B. *PNAS,* **2002,** *99,* 4972-4976.
6. Ferrand, Y.; Crump, M.; Davis, A. P. *Science* **2007,** *318,* 619-622.

Chapter 4

Thesis Summary and Future Direction

This thesis described a systematic design, synthesis, and screening of receptors for complex carbohydrates. It was segmented into parts; in Chapter 1, the glycoconjugate libraries are introduced and their utility was demonstrated as they have been gaining incredible popularity due to the importance of protein-carbohydrate interactions in nature. Amongst a limited number of classes of synthetic lectins are boronic acid based receptors and sensors, which are thoroughly discussed in the literature, as they engage in reversible interactions and can thus serve as protein (lectin) mimics. Furthermore, the importance and discovery of benzoboroxoles, a new class of hexopyranoside binding unit, was briefly discussed.

In Chapter 2, our research efforts focused on the design of new boroxole-containing peptide based receptors that strongly bind under physiological conditions to the TF antigen disaccharide, an important cancer marker. In order to choose appropriate spacers within a peptide skeleton, a library of 400 compounds containing benzoboroxole units was synthesized and it was found that certain molecules were able to bind specifically to the TF disaccharide. The chapter further outlined a detailed description of the synthesis of the library using the Irori® MiniKan technology. Using this technology, the library was prepared in roughly three weeks, demonstrating the efficiency and utility of this

combinatorial approach. Lastly, it was also important to note that the compounds in the library exhibited good to excellent purity prior to HPLC purification.

The bulk of the Chapter 3 dealt with the general screening protocol and identification of the best receptor. A competitive ELISA assay was carried out to screen the whole library. The first round of screens led to seventeen hits. Among the seventeen, four showed promising IC_{50} values. The best receptor demonstrated an IC_{50} value of 20 μM in the assay conditions. The binding ability of this receptor was tested by measuring a binding constant between the best receptor and the TF antigen derivative. The binding behavior in neutral water was again characterized using competition experiments and a systematic evaluation of analogues The approach was successful in the identification of a low molecular weight receptor effective in neutral water and selective for the TF disaccharide. Although the low binding affinity of this receptor is similar in efficiency to some lectins and the best synthetic receptors reported in the literature, it is nevertheless unsuitable toward many applications.

In the future, it would be very informative to investigate the binding mechanism of our synthetic lectins. In particular, we are interested to assess whether one or two benzoboroxole units are strongly involved in the binding, and to which hydroxyls of the TF antigen disaccharide. A change in chemical shift of the carbohydrate ^{13}C NMR spectra in the presence of excess receptor could reveal this information easily. In order to

investigate this hypothesis, synthesis of compound **1** and **2** could help address whether one boroxole unit is sufficient for a moderate binding constant

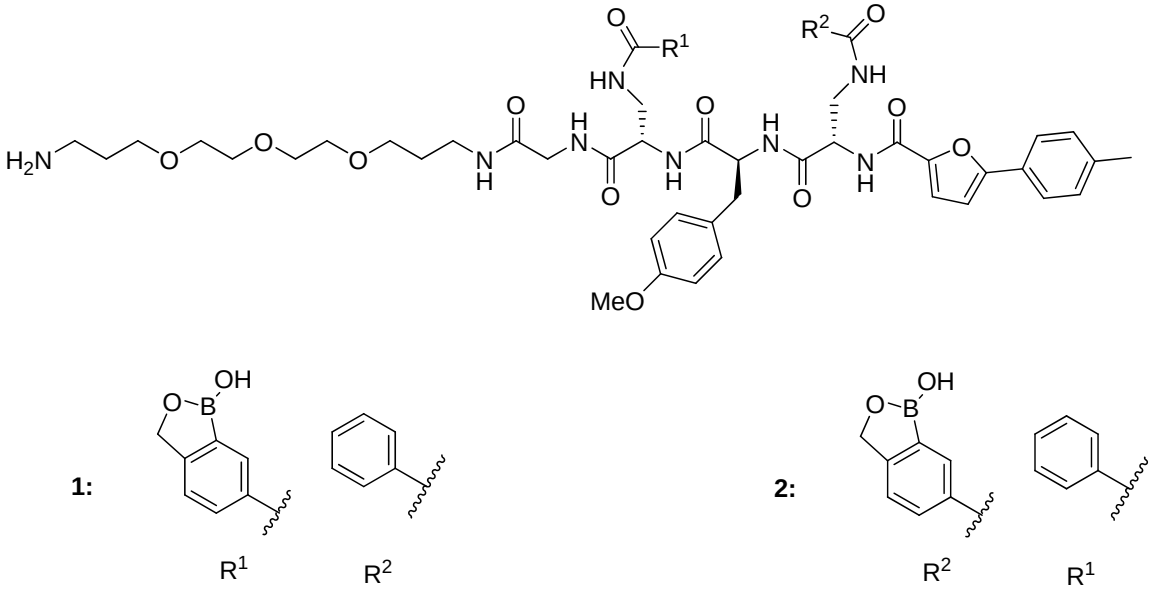


Figure 4.1: Proposal for modified structures of the best receptor.

To allow applications of our best receptors, we would also like to investigate the concept of multivalency, where the increased binding of oligomeric receptors could consequently result in an increase in the binding affinity (Figure 4.2). It would also be important to make microarrays with our receptor to screen several carbohydrates in a small time because the current assay is slow and cumbersome. Furthermore we could also assess their efficiency by conducting labeling studies of TF-specific tumour cell lines. In the long term, this project could be extended to applications in diagnostic studies and in the discovery of drug delivery agent.

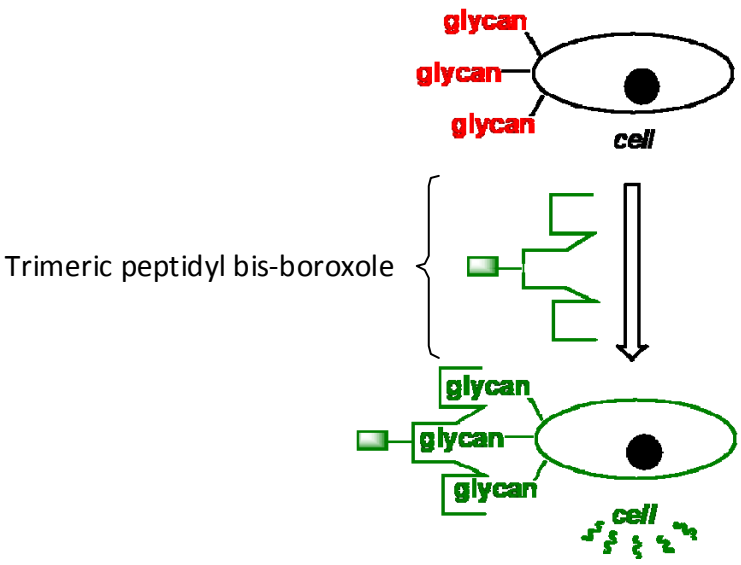
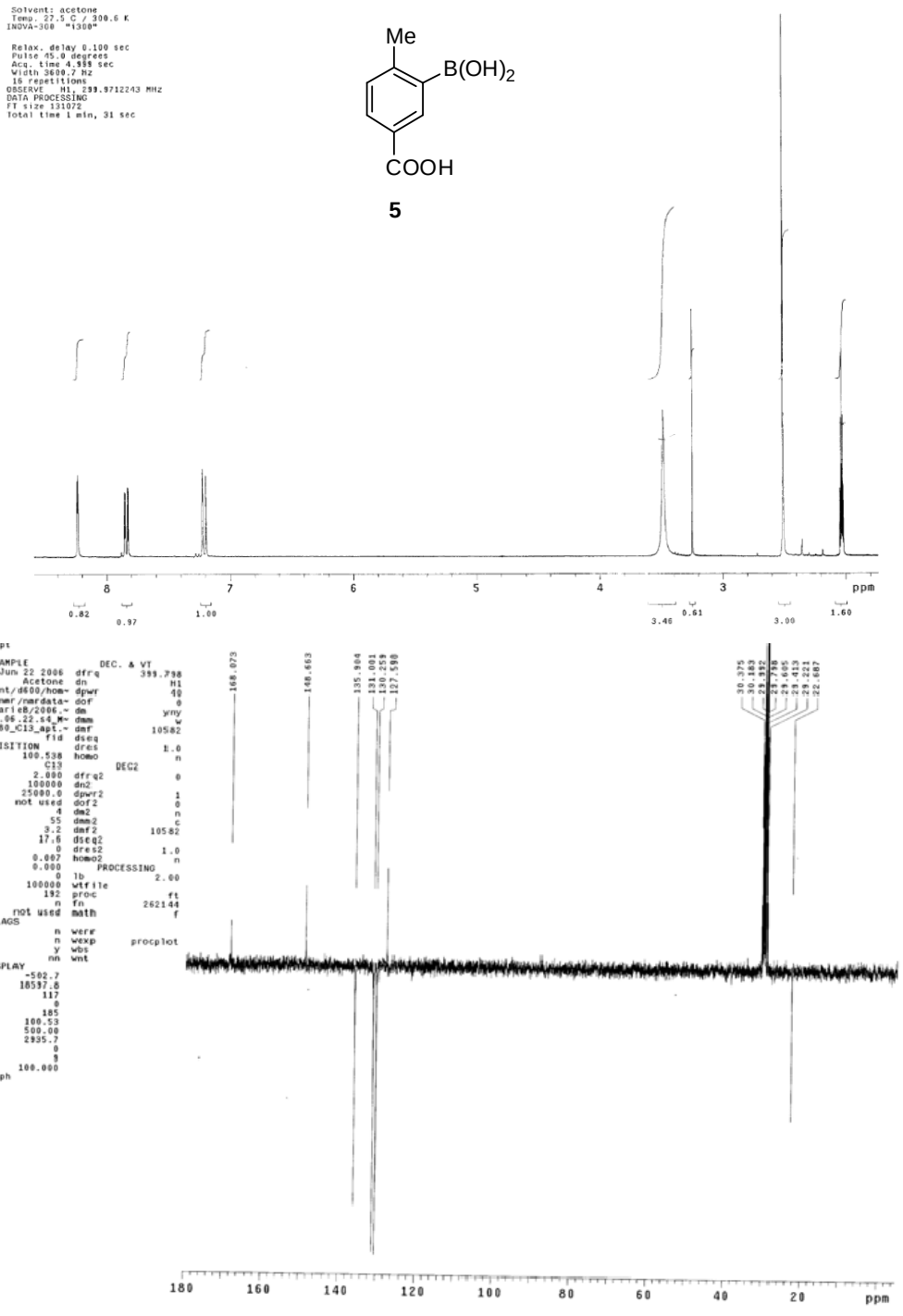
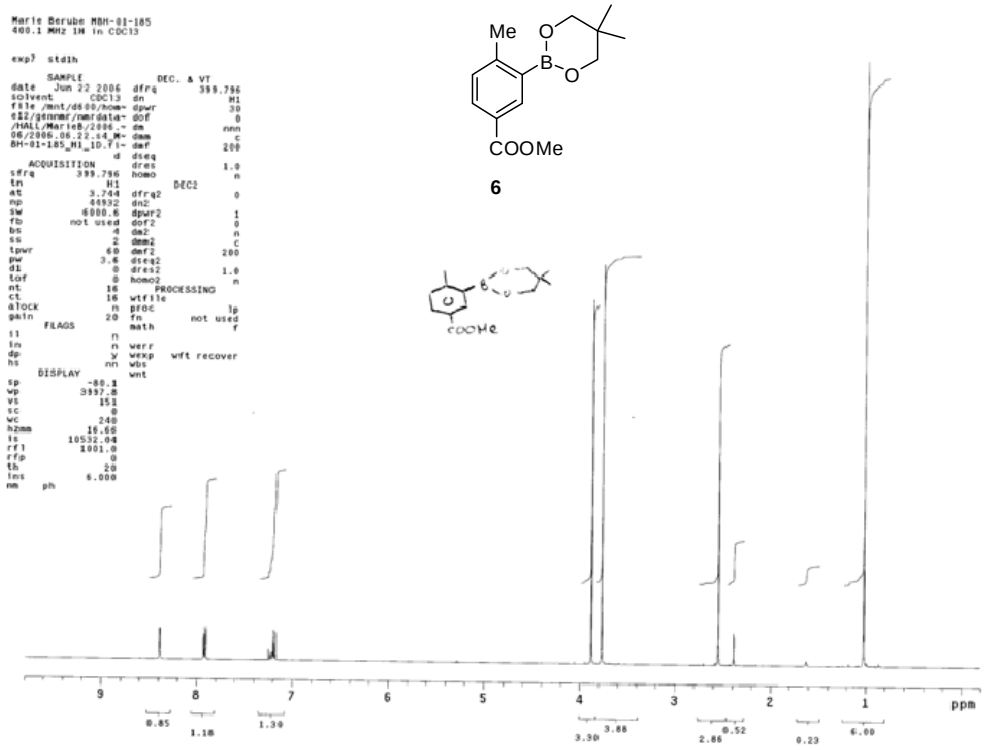


Figure 4.2: Multivalent receptor in cell marking.

Appendix

NMR spectra

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solvent CDCl3
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e12/gennmr/nmrdata~
/HALL/Marie8/2006.~
06/2006.06.22.s4_M~
BH-01-185_H1_1D.fi~
ACQUISITION
sfrq 399.796
tn H1
at 3.744
np 44932
sw 6000.6
fb not used
bs 4
ss 2
tpwr 60
pw 3.6
d1 0
tof 0
nt 16
ct 16
alock n
gain 20
FLAGS
DISPLAY
sp -80.1
wp 3997.8
vs 151
sc 0
wc 240
hzmm 16.66
ts 10532.04
rf1 1001.0
rfp 0
th 20
lns 6.000
nm ph
DEC. & VT
dfrq 399.796
dn H1
dpwr 30
dof 0
dm nnn
dmm c
dmf 200
dseq
dres 1.0
homo n
DEC2
dfrq2 0
dn2
dpwr2 1
dof2 0
dm2 n
dmm2 c
dmf2 200
dseq2
dres2 1.0
homo2 n
PROCESSING
wtfile
proc lp
fn not used
math f
werr
wexp wft recover
wbs
wnt
Me
O
B
O
COOMe
6
COOMe
9
8
7
6
5
4
3
2
1
ppm
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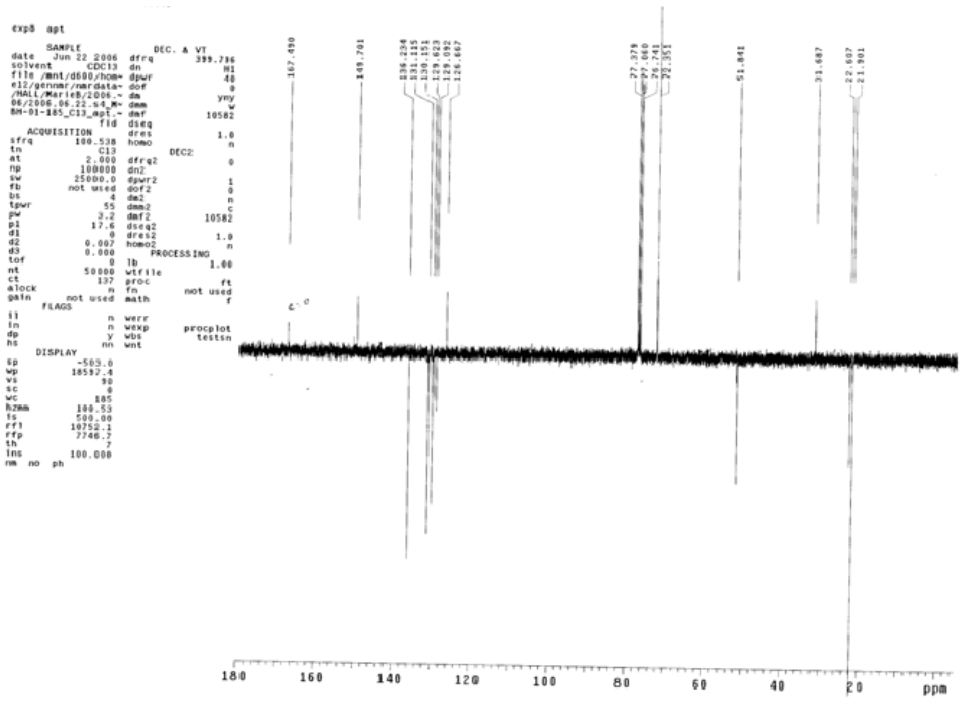
1.18
1.30
3.30
3.88
2.86
0.52
0.23
6.00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e12/gennmr/nmrdata~
/HALL/Marie8/2006.~
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BH-01-185_C13_apt.~
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sfrq 100.538
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at 2.000
np 100000
sw 25000.0
fb not used
bs 4
tpwr 55
pw 3.2
p1 17.6
d1 0
d2 0.007
d3 0.000
tof 0
nt 50000
ct 137
alock n
gain not used
FLAGS
DISPLAY
sp -565.6
wp 18592.4
vs 90
sc 0
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hzmm 188.53
ts 500.00
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rfp 7748.7
th 7
lns 100.000
nm no ph
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dfrq 399.796
dn H1
dpwr 48
dof 0
dm yny
dmm CWC
dmf 10582
dseq
dres 1.0
homo n
DEC2
dfrq2 0
dn2
dpwr2 1
dof2 0
dm2 n
dmm2 c
dmf2 10582
dseq2
dres2 1.0
homo2 n
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proc ft
fn not used
math f
werr
wexp procplot
wbs testsn
wnt
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149.701
136.234
131.115
130.451
129.203
129.092
126.667
77.378
77.060
76.741
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31.687
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21.901
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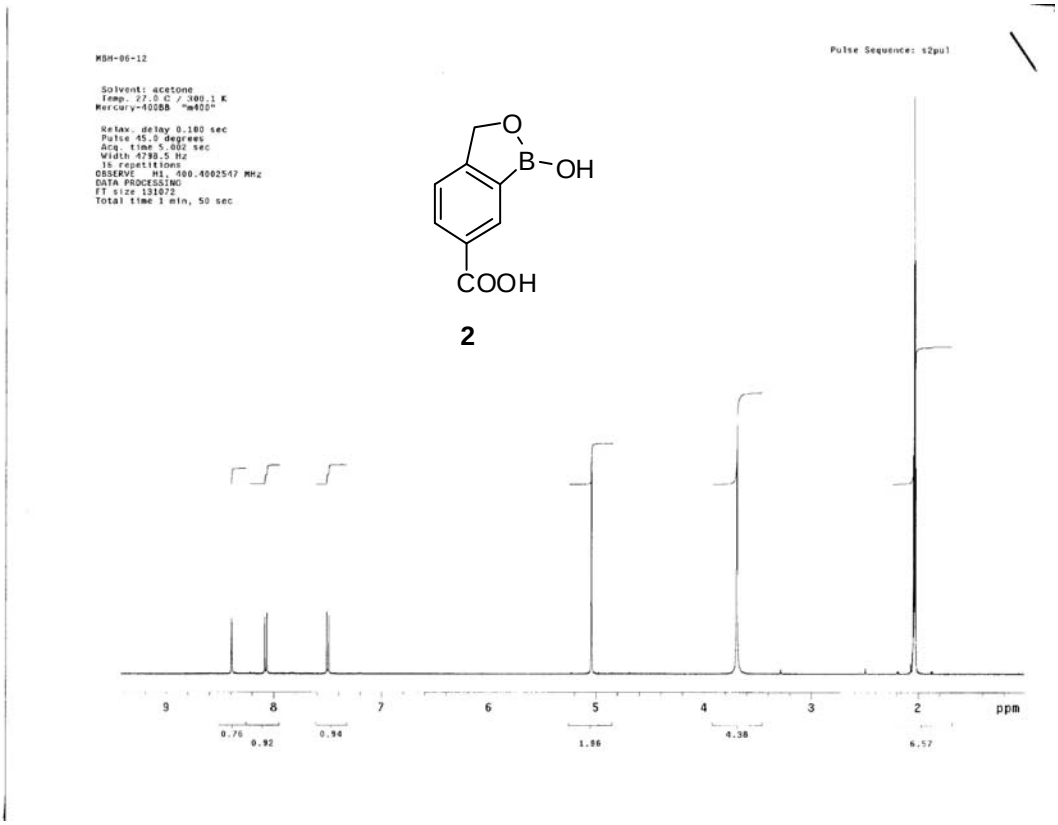
140
120
100
80
60
40
20
ppm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Pulse 45.0 degrees
Acq. time 5.002 sec
Width 4798.5 Hz
16 repetitions
OBSERVE H1, 400.4002547 MHz
DATA PROCESSING
FT size 131072
Total time 1 min, 50 sec
O
B
OH
COOH
2
9
8
7
6
5
4
3
2
ppm
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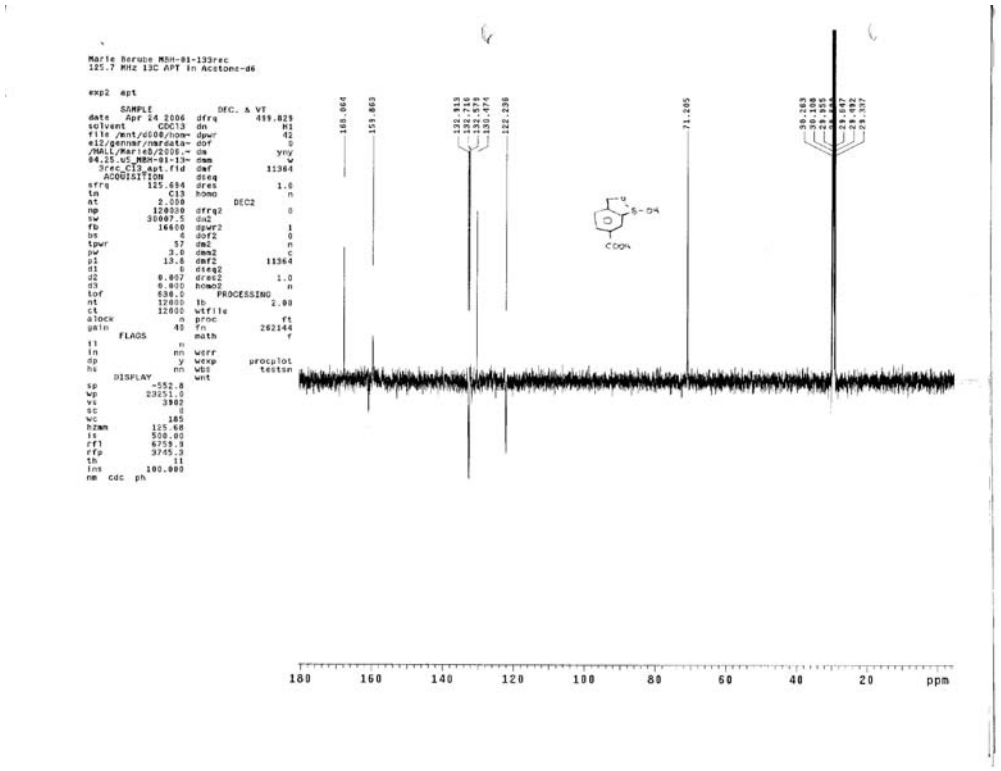
0.92
0.94
1.86
4.38
6.57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exp2 apt
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date Apr 24 2006
solvent CDCl3
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/MALL/Marie3/2006.
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bs 6
tpwr 57
pw 13.8
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d1 0.007
d2 0.000
d3 0.000
tof 630.0
nt 12000
ct 12000
alock n
gain 40
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wp 23251.0
vs 3982
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wc 185
hzmm 125.68
is 500.00
rf1 6759.9
rfp 3745.3
th 11
ins 100.000
nm cdc ph
DEC. & VT
dfrq 499.829
dn H1
dpwr 42
dof 0
dm yny
dmm w
dmf 11364
dseq
dres 1.0
homo n
DEC2
dfrq2 0
do2
dpwr2 1
dof2 0
dm2 n
dmm2 c
dmf2 11364
dseq2
dres2 1.0
homo2 n
PROCESSING
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wtfile
proc ft
fn 262144
math f
werr
wexp procplot
wbs testsn
wnt
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159.863
132.313
132.716
132.579
130.474
122.236
71.205
30.263
30.108
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29.647
29.492
29.337
COOH
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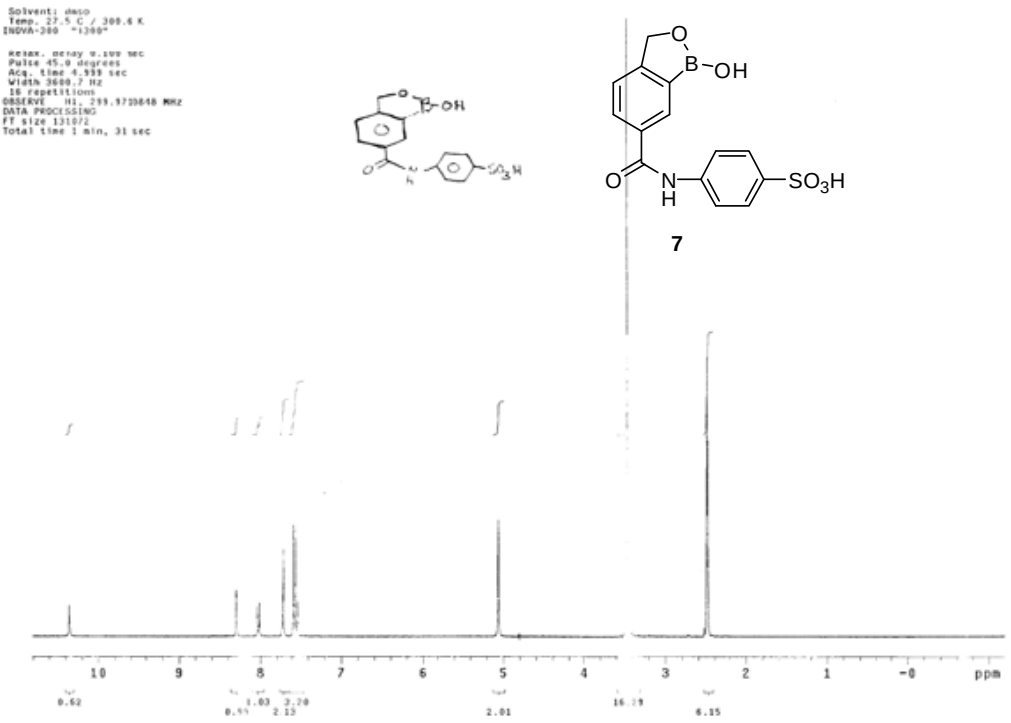
140
120
100
80
60
40
20
ppm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Pulse 45.0 degrees
Acq. time 4.999 sec
Width 3600.7 Hz
16 repetitions
OBSERVE H1, 299.9730648 MHz
DATA PROCESSING
FT size 131072
Total time 1 min, 31 sec
B-OH
O
N
H
SO3H
7
10
9
8
7
6
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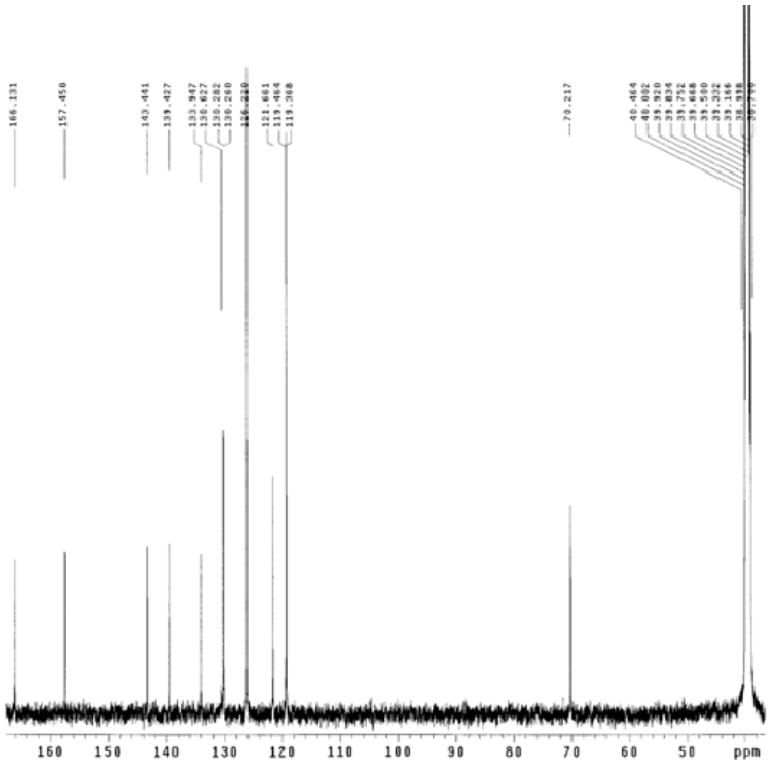
4
3
2
1
-0
ppm
0.62
1.03
3.20
2.01
16.19
6.15


166.131
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143.441
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133.947
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130
120
110
100
90
80
70
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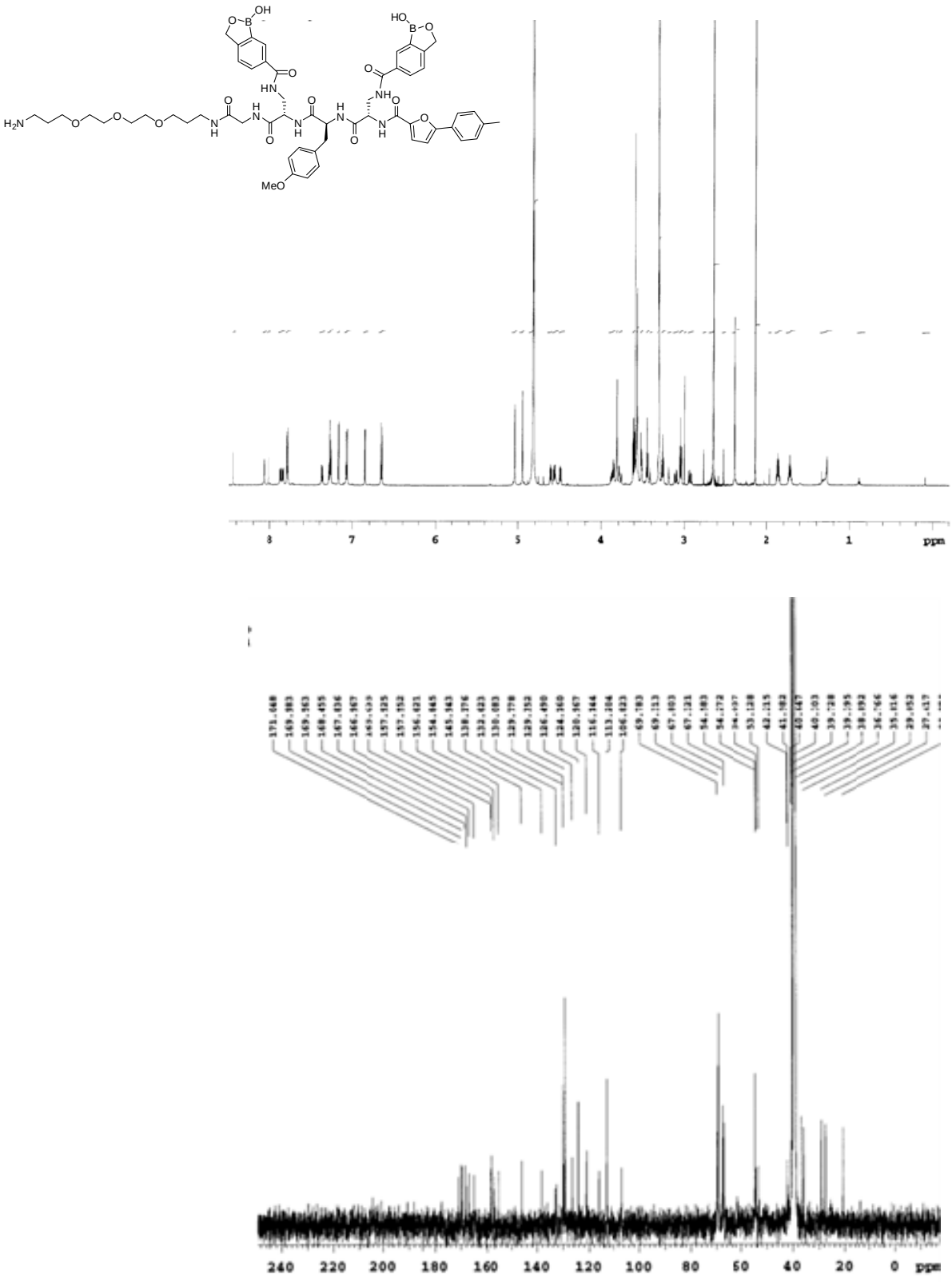
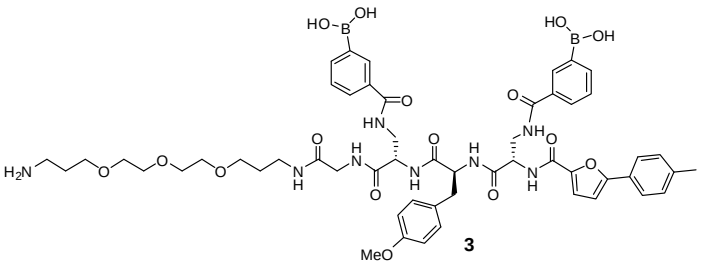
50
ppm





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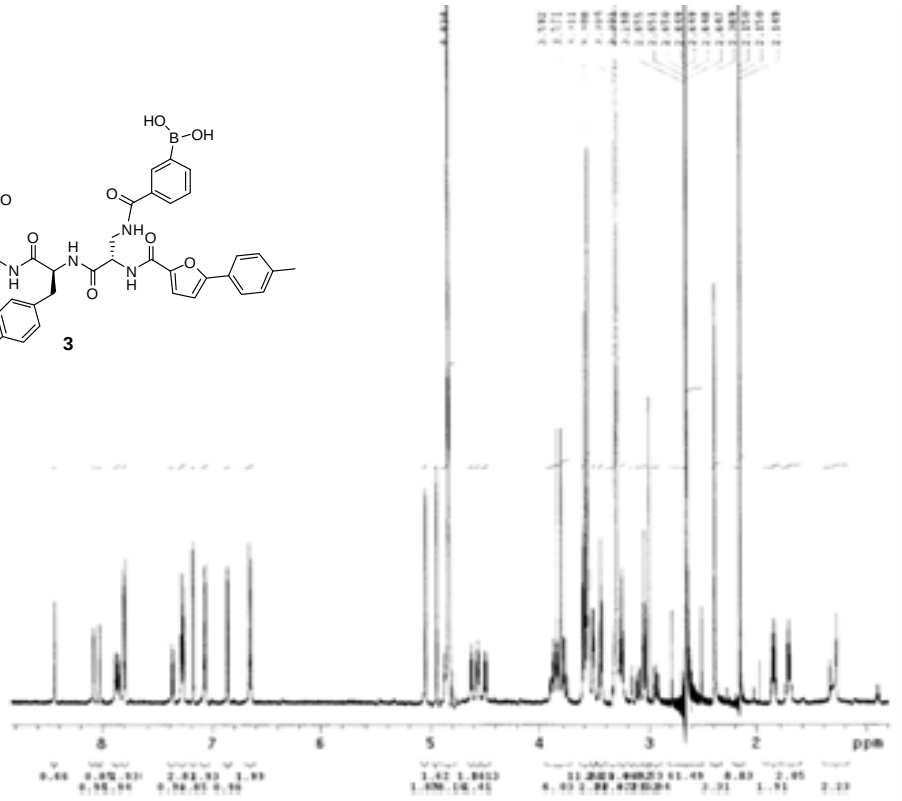

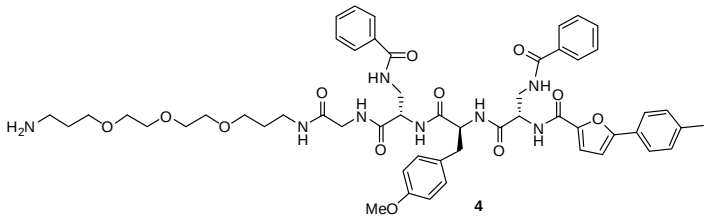

4

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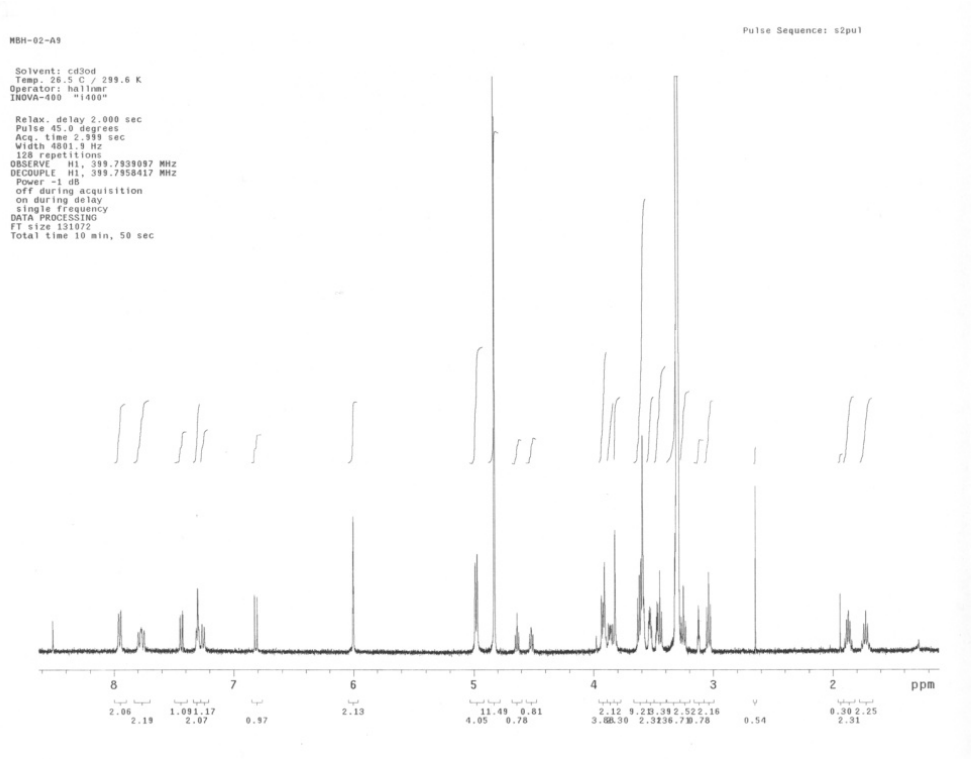

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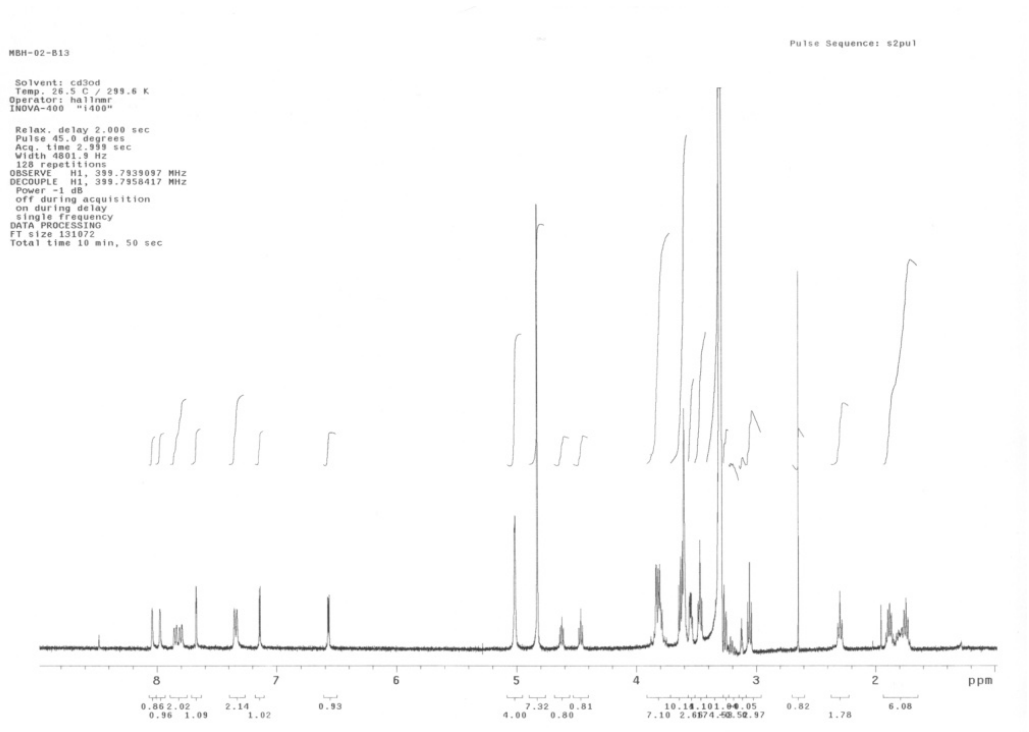

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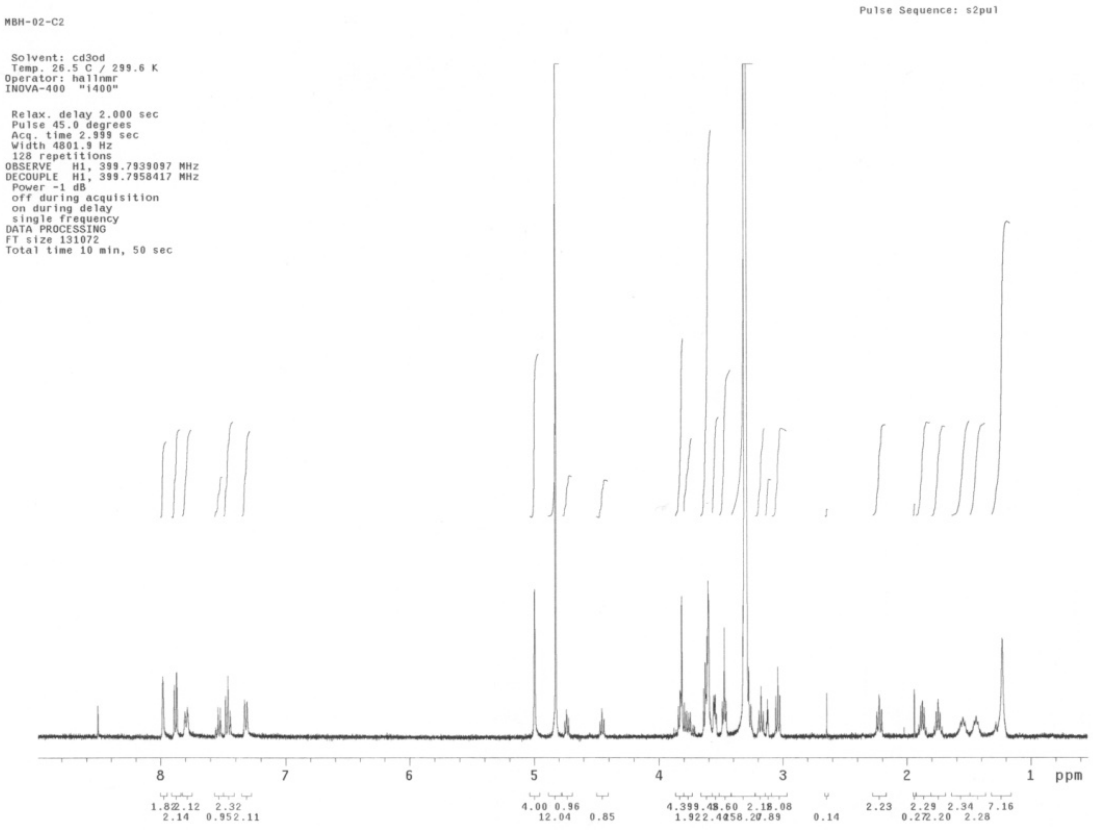

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