Synthesis and study of new materials for the visual detection of biomolecules

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SYNTHESIS AND STUDY OF NEW MATERIALS FOR THE VISUAL DETECTION OF BIOMOLECULES

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DEDICATION

I dedicate this dissertation to my family. First of all, to my father He Yaokun and mother Ge Chunhua. Thank you for all your love, support and guidance that you have afforded ever since I was born. I hope that I have made you proud. Next, to my loving wife Lu Wenqian and daughter Michelle He. Xiao Wen, thank you for your love and support. I am lucky to have you as my life partner. Michelle, daddy loves you very much. Thank you for all the joy, happiness and inspiration that you have brought to daddy. Third, to my brother He Xin. Thank you for all your support and help in my graduate study in the United States. Finally, to the rest of my family, thank you all for your love and support. I love you all.
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<th>Abbreviation</th>
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<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene, 2,6-Di-<em>tert</em>-butyl-4-methylphenol</td>
</tr>
<tr>
<td>DCC</td>
<td>1,3-Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMAP</td>
<td>4-Dimethylaminopyridine</td>
</tr>
<tr>
<td>DMF</td>
<td><em>N,N</em>-Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>HOBT</td>
<td>1<em>H</em>-Benzotriazole</td>
</tr>
<tr>
<td>Ms</td>
<td>Mesylate, Methanesulfonate</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>THP</td>
<td>3,4-Dihydro-2<em>H</em>-pyran</td>
</tr>
<tr>
<td>Ts</td>
<td>Tosylate, <em>p</em>-Toluenesulfonate</td>
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ABSTRACT

The visual determination of saccharides has been of interest for well over a century. Sugars are a relatively challenging class of compounds to analyze. They exhibit great structural similarity as well as transparency in the visible region. The sensing of specific saccharides could aid the monitoring of disease or industrial fermentation products. Herein I present new methods allowing for simple and rapid visual detection of sialic acid, fructose and a homologous series of prototypical neutral oligosaccharides, the linear maltodextrins, containing up to seven glucose residues. The new methods allow for selective color detection of fructose in a large excess of glucose and simultaneously determination of glucose. The fundamental problem of diminished optical detection of larger oligosaccharides is overcome using simple colorimetric agents. In addition, the first evidence that xanthenes form and serve as the active chromophores in resorcinarene solutions is presented. Meanwhile, the saccharide-induced optical signal transduction mechanism is investigated. A synthesis of resorcinarene derivatives, which have potential application in liposome stabilization, is also described.
CHAPTER 1

INTRODUCTION

1.1 History of Resorcinarenes

Resorcinarenes were first synthesized by Adolf von Baeyer in 1872. He reported that upon addition of concentrated sulfuric acid, a mixture of benzaldehyde and resorcinol gave a red-colored product which changed to violet in alkaline solutions. When the mixture was heated, Baeyer obtained a crystalline compound in addition to the reddish resin. The crystalline compound was later determined to be an isomer of the red resin. Several years later, the correct elemental composition of this slightly soluble, high melting crystalline compound \((\text{C}_{13}\text{H}_{10}\text{O}_{2})_n\) as well as its acetyl derivative \((\text{C}_{13}\text{H}_8(\text{OCOCH}_3)_2)_n\), were determined by Michael. He also reported that the product was formed by combination of an equal number of benzaldehyde and resorcinol molecules and loss of an equal number of water molecules. However, it took several decades to determine the structure of the product. Michael initially suggested structure 1.1 for the phenolic compound. In 1940, Niederl and Vogel showed that the ratio between aldehyde and resorcinol in the product should be 4:4 according to molecular weight determinations. They proposed structure 1.2, a cyclic tetramer, which is a structural analogue to porphyrins. In 1968, with the aid of single crystal X-ray analysis, Erdtman and coworkers finally proved its structure. 1.4

The official IUPAC-name for resorcinarene is 2,8,14,20-tetraalkylpentacyclo-[19.3.1.1^{5,6,7,18}.1^{9,10}]octacosa-1-(25),3,5,7(28),9,11,13(27),15,17,19(26),21,23-dodecaene-4,6,10,12,16,18,22,24-octol. The name "resorcinarene" was recently suggested by Schneider. Gutsche and Böhmer referred to them as
calix[4]resorcinarenes or resorcinol-derived calix[4]arenes.\textsuperscript{1,6,1,12} Names like 'Högberg compounds'\textsuperscript{1,7} or "octols"\textsuperscript{1,13,1,14} can also be found in literature.

Figure 1.1. Structures of the crystalline compound obtained from Baeyer’s synthesis proposed by Michael (1.1), Nierdel and Vogel (1.2), R = aliphatic.

1.2 Synthesis of Resorcinarenes

Most resorcinarene syntheses involve a condensation reaction between resorcinol and an aliphatic or aromatic aldehyde. The reaction is catalyzed by acids. Lewis acids can be used in this reaction.\textsuperscript{1,9,1,15,1,16} Generally, a cyclotetramer crystallizes from the reaction mixture, but in some cases the isolation of the condensation product requires the addition of water.\textsuperscript{1,13,1,17} An unsubstituted resorcinol (1,3 dihydroxybenzene) is usually
used in the synthesis of resorcinarenes. The use of 2-methylresorcinol or pyrogallol (1,2,3-trihydroxybenzene) is reported to also yield isolable amounts of tetrameric products. The products won’t be formed if the resorcinol derivatives bear electron-withdrawing substituents such as NO$_2$ or Br at the 2-position or when the hydroxyl groups are partially alkylated. There is little limitation to the structure of both the aliphatic and aromatic aldehyde. The only exception to this rule are the use of very sterically crowded aldehydes, like 2,4,6-trimethylbezaldehyde or aliphatic aldehydes with functionalities too close to the reaction center, such as ClCH$_2$CHO or glucose.

The mechanism of the acid-catalyzed condensation reaction for the formation of resorcinarenes has been thoroughly studied (Figure 1.2). Under acidic conditions, the aldehyde is first protonated to serve as the initial electrophile which adds to resorcinol. The alcoholic hydroxyl of the subsequent adduct is protonated again generating a mole of water. Removal of water affords a carbocation intermediate, which undergoes second electrophilic addition to another resorcinol to form a dimer. Sequential coupling of the dimer with resorcinol units results in trimer, tetramer or higher oligomers containing more than four monomers. Since the condensation reaction is reversible under acidic conditions, most of the higher oligomers disappear towards the end of the reaction although they are present during the intermediate reaction time. The linear tetramers cyclize rapidly to form resorcinarenes once they are formed in the reaction mixture. The cyclization is too fast for tetramers to be isolated. The cyclization is favored, due to their lack of conformational strain, by the formation of hydrogen bonds between proximal phenolic hydroxyl groups of adjacent resorcinol units in the folded structures.
Figure 1.2. Mechanism of the acid-catalyzed synthesis of resorcinarenes.

1.3 The Stereochemistry of Resorcinarenes

The stereochemistry of resorcinarenes results from their non-planarity and, generally, it is defined based on three criteria.
The first criterion is the conformation of macrocyclic ring. As the macrocycle ring has five highly symmetrical conformations: crown ($C_{4v}$), boat ($C_{2v}$), chair ($C_{2h}$), diamond ($C_s$), and saddle ($D_{2d}$), resorcinarenes can have five corresponding stereoisomers (Figure 1.3).

Figure 1.3. Five possible stereoisomers of resorcinarene.

The second criterion is the relative configuration of the substitutes at the methylene bridges, giving the all-cis (ccc), cis-cis-trans (cct), cis-trans-trans (ctt) and trans-cis-trans (tct) resorcinarene stereoisomers (Figure 1.4).
Figure 1.4. Relative configuration of the substituents at methylene bridges.

The last criterion is the individual configuration of methylene bridge substituents, which may be either axial or equatorial in conformations of the macrocycle with C symmetry. Combination of these three criteria results in a great number of possible stereoisomers. However, only four have been reported experimentally so far. The boat conformation is usually reported as a crown conformation due partially to the presence of two boat isomers, which interconvert rapidly to give a time-averaged crown conformation. The interconversion between boat, chair and diamond isomers, however, does not occur since it requires the breaking of at least two covalent bonds. They are diastereomeric isomers. All of them can be produced in a reaction. Under homogeneous acidic conditions, the product ratio is mainly determined by the thermodynamic stability of the different isomer because the condensation reaction is reversible under such conditions.\(^{1,19}\) The relative solubility of the different isomers in the reaction solvent play a key role in determining the product ratio if the reaction is performed under heterogeneous conditions.\(^{1,20}\) The ratio of different diastereoisomers depends greatly on the reaction conditions used although there are many factors that may affect the presence or absence of a specific isomer.
1.4 Complexation of Polar Organic Molecules by Resorcinarenes

There are eight hydroxyl groups at the upper rim of the resorcinarenes, which can complex organic molecules containing polar substituents. This feature was first recognized by Aoyama et al. a decade ago. He has studied this phenomenon extensively. The complexation behavior of resorcinarenes for a variety of guest molecules such as sugars and steroids, amino acids, triethylamine and [2,2,2] cryptand has been studied.

After he studied the complexation of resorcinarenes with several cyclohexanediols, Aoyama found that, among all possible isomers, cis-1,4-cyclohexanediol was bound the most tightly and the binding is eight times stronger than that of the corresponding trans isomers. This cis/trans selectivity results from the preferred geometry of the cis-isomer where one of two related hydroxyl is equatorial and the other is axial. This 1,4-cis selectivity is also very important in the case of carbohydrate complexation. D-ribose was found to be readily extracted from a concentrated resorcinarenes solution in CCl₄ although it is almost insoluble in pure CCl₄. NMR investigations clearly showed that D-ribose is bound to resorcinarenes only in the α-pyranose form which has a cis orientation of the hydroxyl groups at C-1 and C-4 (Figure 1.5). Fucose and 2-deoxyribose were found more readily extracted than ribose, while xylose could not be extracted at all although only its configuration at C-3 is different. This suggests that a cis relationship between the C-3 and C-4 OH’s is also very important for complexation. A trans 3-OH simply gave unfavorable interactions with the aryl ring between the two binding sites. The OH at C-2 should also be cis to the C-3 and C-4 OHs or absent to avoid unfavorable exposure to the apolar solvent. The substituents
at C-5 should be as hydrophobic as possible because they interact only with the apolar solvent.

![Structure of the complex between Resorcinarene and D-ribose.](image)

**Figure 1.5.** Structure of the complex between Resorcinarene and D-ribose.

Aoyama and co-workers discovered that the major binding force for complexation in apolar, organic solvent is hydrogen bonding to four pair of hydroxyl groups of the resorcinarenes. In addition, the interaction between an aliphatic moiety at the guest and the electron-rich aromatic rings in the host (CH-π interactions) also contributes to the binding.\(^1\)\(^{24}\), \(^1\)\(^{29}\) In aqueous systems,\(^1\)\(^{26}\), \(^1\)\(^{30}\) the absence of hydrogen bonding as a driving force for complexation, the affinity of resorcinarenes for polar guests is mainly determined by hydrophobic interactions. Particularly, CH-π interactions play a very important role in the binding if the guest molecules are hydrophobic.\(^1\)\(^{29}\)

1.5 Complexation of Boronic Acid to Saccharides and Its Significance

Boronic acids have been known for over one century since Michaelis and Becker first synthesized phenylboronic acid.\(^1\)\(^{31}\) Although borates was already known to be able to bind polyhydroxyl compounds at the beginning of last century,\(^1\)\(^{32}\) it wasn’t until 1954 that Kuivila et al. first published the binding studies of diols with boronic acids.\(^1\)\(^{33}\) They
postulated the formation of a cyclic boronic ester after they observed that boronic acids could solubilize saccharides and polyols. It is well known now that boronic acids form covalent bonds with 1,2- or 1,3-diols to give five- or six-membered cyclic esters in both neutral nonaqueous and alkaline aqueous solutions (Figure 1.6). Norrid and Eggert reported the structures of the complexes of boronic acid with fructose and glucose under these conditions by $^1$H and $^{13}$C NMR spectroscopy.$^{1,34}$

![Figure 1.6. Boronate ester formation with phenylboronic acid in alkaline aqueous solution (top) and aprotic media (bottom).](image)

The function of many synthetic molecular receptors in the recognition of biologically important molecular species, including carbohydrates, is based on hydrogen-bonding interactions. In aqueous media, serious drawback arises from the competitive hydrogen bonding by the solvent. The ability of boronic acid to readily form cyclic ester with saccharides in aqueous media provides an important binding force in the recognition of saccharides and related molecular species. As saccharides play an important role in
the metabolic pathway of living organism, determination of the presence and concentration of sugars of biological importance (e.g., glucose, fructose) becomes necessary in the fields of medicine and industry.

Lorand and Edwards published the first quantitative evaluation of the interaction between boronic acid and saccharides in 1959.\textsuperscript{1,35} They also discovered the selectivity order of phenylboronic acid towards several monosaccharides: D-fructose \textgreater{} D-galactose \textgreater{} D-mannose \textgreater{} D-glucose. Another discovery they made is that the rigid, vicinal \textit{cis} diols of saccharides form more stable cyclic esters than simple acyclic diols such as ethylene glycol. Recently, a variety of receptors containing a boronic acid moiety have been synthesized in different laboratories and employed for the recognition of mono- and oligosaccharides.\textsuperscript{1,36} The industrial applications of these kind of studies range from the monitoring of fermenting processes to the establishment of the enantiomeric purity of synthetic drugs. The current enzymatic methods for sugar detection are limited in specificity. In addition, harsh conditions are used in some enzymatic methods which make enzyme-based sensors unstable. Therefore, the design and synthesis of a stable receptors based on boronic acid can potentially lead to useful detection of saccharides.

1.6 References


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1.21 As a result of this extensive study, resorcinarenes were awarded “reagent of the year in 1993” by Fluka: *J. Org. Chem.* **1993**, *58*, 2A.


CHAPTER 2

THE FORMATION AND STRUCTURE OF THE CHROMOPHORE IN RESORCINARENE SOLUTIONS*

2.1 Introduction

The colorimetric properties of resorcinarene solutions have not been investigated since Baeyer’s initial synthesis. The tetraarylboronic acid resorcinarene stereoisomers 2.1 and 2.2a can be obtained in one step in a combined 90% yield via the HCl-promoted condensation of commercially available 4-formylphenyl boronic acid and resorcinol.\textsuperscript{2,1} Separation of the two stereoisomers by fractional crystallization afforded white crystalline solids. X-ray quality crystals of the half-methyl tetraboronate ester of 2.1 was obtained upon slow crystallization from a 9:1 MeOH:EtOH solution. The solid state architecture has an infinite, antiparallel two-dimensional network of macrocycles, each of which exhibited twelve intermolecular hydrogen bonds.\textsuperscript{2,2}

\begin{align*}
2.1 & \\
2.2a & \text{R=CH}_3 \\
2.2b & \text{R=H}
\end{align*}

**Figure 2.1.** Chair (2.1) and crown (2.2a) stereoisomers of tetraarylboronic acid resorcinarenes.

It was recently reported that solutions containing resorcinarene macrocycles develop color upon heating or standing. The colorless DMSO solutions of freshly crystallized 2.1 or 2.2a, (5.2 mM), upon standing in solution for several hours or upon heating at 90 °C for 1 min, developed a pinkish-purple color. The color formation was monitored via UV-vis spectroscopy. The appearance of a new $\lambda_{\text{max}}$ at 535 nm was accompanied by a less intense absorbance at 500 nm.\textsuperscript{2,3} Strong evidence has been amassed to prove that the solution color is due to xanthene formation from macrocycle ring opening and oxidation.

2.2 Results and Discussion

Initial attempts at understanding the origin of the solution color involved heating solutions of 2.1 in the dark or in O\textsubscript{2} degassed conditions. In both cases, the color intensities diminished, as evidence by both visual inspection and UV-vis spectroscopy.\textsuperscript{2,3} For instance, heating a solution of 2.1 5.2 mM in DMSO) under O\textsubscript{2} degassed conditions led to a 61% decrease in absorbance at 536 nm. Light and O\textsubscript{2} apparently promote color formation. In addition, when the phenolic hydroxyls of 2.1 was acylated and a DMSO solution of the resultant octaacetate was heated to reflux, the solution remained colorless.\textsuperscript{2,4} The phenolic hydroxyls thus also play a key role in chromophore formation. It was reasoned that the chromophore arises via oxidation of a resorcinol moiety to a quinone\textsuperscript{2,3,2,4} (Figure 2.2)

![Figure 2.2. Dehydration and oxidation of methine-bridged resorcinol oligomers leading to xanthenes.](image-url)
If the solutions of resorcinol or phenylboronic acid were heated separately or as an equimolar mixture using the aforementioned conditions and concentrations, with and without added monosaccharides. Only very faint solution colors were observed by visual inspection. This result showed that a methine-bridged resorcinol/aldehyde condensation framework was needed for effective chromophore formation and optical sugar detection. Methine-bridged condensation product resorcinarene substructures, of which 2.5a-c (Figure 2.3) are examples, were noted as reaction intermediates in standard xanthene dye syntheses (e.g., the transformation of 2.3 to 2.4, n=m=0, Figure 2.2). 2.5

![Figure 2.3. Resorcinarene substructures of methine-bridged condensation products (2.5) and some xanthene dyes (2.6).]

Xanthenes are some of the oldest known synthetic dyes, such as fluorescein, rhodamine, 2.6a and 2.6b and ethyl eosin (2.6c) (figure 2.3) as well as many others. The colorimetric properties of xanthenes are a function of the ionization state of the C-6 moiety. 2.6 They typically exhibit two absorbance maxima in the visible region. 2.6 The absorption spectrum of 2.6b (5.0 x 10⁻⁶ M) in 9:1 DMSO:H₂O is shown in Figure 2.4. It exhibits a λ_max at 530 nm and a less intense λ_max at 500 nm. The λ_max absorbance values
and spectral features are strikingly similar to those observed for colored DMSO solutions of 2.1 as well as 2.2a, 2.2b and 2.5a which we previously reported.\textsuperscript{2,4}

![Absorbance vs Wavelength](image)

**Figure 2.4.** 2.2a (1.0 mg) and 2.5a (1.0 mg) each in 0.9 mL DMSO were heated to gentle reflux over two minutes and cooled to room temperature before 0.1 mL H\textsubscript{2}O was added to each solution. The final concentrations of 2.2a and 2.5a in 9:1 DMSO:H\textsubscript{2}O are 1.03 × 10\textsuperscript{-3} M and 1.96 × 10\textsuperscript{-3} M respectively. A solution of 2.6b (5.0 × 10\textsuperscript{-6} M) was prepared at rt in 9:1 DMSO:H\textsubscript{2}O.

Incorporation of a planar xanthene within a resorcinarene macrocycle framework via the transformation shown in Figure 2.2 would lead to a considerable increase in strain energy. Simulations (Sybil 6.6) show that an increase in strain energy of 34.2 kcal/mol would occur upon formation of a xanthene substructure within 2.2b. Prior studies of the related calixarenes (macrocycles formally derived from phenol/formaldehyde condensations) showed that xanthenes did not form in cyclic tetrameric structures.\textsuperscript{2,7}

Ring opening to acyclic oligomers could thus be a prerequisite for xanthene formation from resorcinarenes. It is known that the condensation reactions producing resorcinarenes are reversible under acidic conditions.\textsuperscript{2,8} The detailed mechanism of resorcinarene macrocycle genesis has been studied thoroughly by Weinelt and
They found that 2.2b and its macrocyclic stereoisomers interconverted via the intermediacy of acyclic oligomers. Their studies included the rapid quenching of condensation reactions between resorcinol and either acetaldehyde or paraldehyde in MeOH in the presence of anhydrous HCl. Since the opening of a resorcinarene ring has only been previously shown to occur upon the addition of strong acid, our hypothesis of acyclic oligomer formation in aqueous or neat DMSO solutions without added acid warrants further analysis.

It was observed that $^1$H and $^{13}$C NMR spectra of DMSO$_{d6}$ solutions of 2.1 (5.2 mM), heated at 90 °C for 3 min (initial sugar colorimetric detection conditions) exhibited no readily observable change in chemical shifts or peak area integrals compared to fresh, colorless samples. Xanthenes are strongly absorbing materials which need be only produced in trace amounts to afford solution colors under our conditions (ca. 0.5% conversion; see, for example, the concentrations and absorbances shown in Figure 2.4).

It was found that more vigorous thermolytic conditions are necessary to afford conversion to significant amounts of products. R. Johnson observed that heating a DMSO (10 mL) solution of freshly recrystallized 2.2b (18.4 mM) for 8 h at 120 °C followed by analysis via reversed phase HPLC revealed the formation of numerous new products representing a 74% conversion of 2.2b to products based on relative peak areas.

Strong literature precedent allows us to propose that acyclic oligomers arise from 2.2b via the in situ formation of strong acids. It is known that acid production from DMSO is promoted by the presence of O$_2$ and peroxides. In addition, in situ acid formation has been attributed as the cause of certain oxidations in DMSO. We have noted the effect of O$_2$ on resorcinarene solution color intensity (vide supra).
Acid formation observed during DMSO decomposition has been inhibited by free radical scavengers.\textsuperscript{2.11c} Under the same thermolysis conditions as noted above, but in the presence of free radical scavengers (either BHT or phenothiazine, 10 mol%), R. Johnson\textsuperscript{2.10} observed less than 28% conversion to products by HPLC analysis.

Important further evidence that strong acids form under our conditions was presented in our group’s recent report describing the first X-ray crystal structure of (CH\textsubscript{3})\textsubscript{3}S\textsuperscript{+}CH\textsubscript{3}SO\textsubscript{3}\textsuperscript{-}.\textsuperscript{2.12} The latter compound was obtained from a thermolysis reaction of 2.2b in DMSO. It is known that (CH\textsubscript{3})\textsubscript{3}S\textsuperscript{+}CH\textsubscript{3}SO\textsubscript{3}\textsuperscript{-} forms, along with CH\textsubscript{3}SO\textsubscript{3}H, CH\textsubscript{3}SO\textsubscript{2}H and CH\textsubscript{3}SOH (and several other products) via the radical and acid promoted decomposition of DMSO.\textsuperscript{2.13}

Attention was next turned to identifying the products of macrocycle ring opening. R. Johnson\textsuperscript{2.10} isolated 2.7 (Figure 2.5), a rarely observed "diamond" resorcinarene stereoisomer,\textsuperscript{2.8(d)} in 2.3% yield from the thermolysis of 2.2b in DMSO, via flash column chromatography. The structure of 2.7 was previously assigned (as the octabutyrate derivative) via NMR evidence during the acid-catalyzed condensation/isomerization studies of Schneider.\textsuperscript{2.9}

Single crystals suitable for X-ray analysis, grown via slow evaporation of a 9:1 CH\textsubscript{2}Cl\textsubscript{2}:MeOH solution of 2.7, allowed the first known crystal structure of a resorcinarene diamond isomer to be reported. The macrocyclic molecule lies on a mirror plane in the crystal, with three methyl groups (C10, C8, and its mirror-related equivalent) relatively \textit{syn}. The fourth methyl group, C18, lies on the mirror, and is anti to the other three. The resulting conformation is such that OH groups O1 and O4 both form intramolecular hydrogen-bonding contacts with their mirror-related equivalents on
adjacent aromatic rings. The H atoms in these contacts are disordered, with the two O atoms alternately donor and acceptor. The O...O distances are 2.712(2) Å for O1 and 2.671(2) Å for O4. There are two independent methanol molecules, both lying on mirrors and forming hydrogen bonds with external macrocyclic OH groups O2 and O3. Importantly, stereoisomer 2.7 can only form from 2.2b via bond rupture and reformation.\(^2\)\(^9\) If 2.7 were a conformer of 2.2b, the methyl group (C18), would reside outside, rather than above the plane of the macrocycle cavity.

![Figure 2.5. Compound 2.7 and ORTEP.](image)

It was also observed acyclic products during the thermolysis of 2.2b. A key product, 2.5b was found by R. Johnson and P. Beck in a broad HPLC fraction eluting from 18-20 min. The \(^1\)H NMR spectrum of the isolate exhibits several peaks including each of the resonances associated with 2.5b\(^2\)\(^9\) (CH\(_3\)OD δ 1.46, d, \(^3\)J = 7.3 Hz, 4.53, q, \(^3\)J = 7.3 Hz, 6.18-6.22, m, 6.89, d, \(^3\)J = 8.0 Hz). Overlay of the \(^1\)H NMR spectra of the HPLC isolate with a sample of independently synthesized and isolated 2.5b confirms the
assignment (Figure 2.6). In addition, the MALDI MS (anthracene matrix) of the HPLC fraction contains a peak at 245.59 amu (246.26 amu calcd). The production of compounds 2.5b and 2.7 under our conditions constitutes an important initial link between our investigations and the prior acid-catalyzed macrocycle genesis mechanism studies.  

![Figure 2.6](image)

**Figure 2.6.** Top: expansion of a $^1$H NMR spectrum of semipurified thermolysis reaction products of 2.2b showing the formation of 2.5b. Bottom: expansion of a $^1$H NMR spectrum of pure 2.5b.

R. Johnson also found evidence for higher order oligomer production under our conditions (*vide supra*) involving thermolysis of 2.2b in DMSO. At least five sets of doublets appear between 0.72 and 1.53 ppm in the $^1$H NMR of each of two flash column fractions (TLC $R_f = 0.54$ and 0.63, 9:1 CH$_2$Cl$_2$:CH$_3$OH, $\delta$ 1.53, 1.08, 1.01, 0.97, 0.83, 0.72 ppm, and $\delta$ 1.29, 1.15, 1.00, 0.89, 0.84 ppm, CH$_3$OD, respectively). In addition, the MALDI mass spectra (anthracene matrix) of other fractions ($R_f = 0.29$ and 0.44) exhibit peaks for higher homologues of 2.5b (entries 1 and 2, Table 2.1). MALDI MS evidence also suggests the formation of xanthene materials not previously reported in previous fragmentation and equilibration studies of 2.5b (entries 3-6, Table 1).  

2.14, 2.15
Table 2.1. MALDI MS evidence for the formation of acyclic oxidized and unoxidized products from the thermolysis of 2.2b.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Structure</th>
<th>TLC R_F</th>
<th>(m/z) calcd</th>
<th>(m/z) obsd</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.4, R=Me, m=1, n=0</td>
<td>0.29</td>
<td>382.41</td>
<td>381.89</td>
</tr>
<tr>
<td>2</td>
<td>2.4, R=Me, m=3, n=2</td>
<td>0.44</td>
<td>926.36</td>
<td>926.28</td>
</tr>
<tr>
<td>3</td>
<td>2.6a</td>
<td>0.44</td>
<td>226.23</td>
<td>225.61</td>
</tr>
<tr>
<td>4</td>
<td>2.5, R=Me, m+n=4</td>
<td>0.26</td>
<td>906.01</td>
<td>906.33</td>
</tr>
<tr>
<td>5</td>
<td>2.5, R=Me, m+n=3</td>
<td>0.84</td>
<td>770.79</td>
<td>770.82</td>
</tr>
<tr>
<td>6</td>
<td>2.5, R=Me, n=1, m=0</td>
<td>0.79</td>
<td>362.51</td>
<td>361.38</td>
</tr>
</tbody>
</table>

In order to study the formation of the oxidation products, compound 2.5b was heated, the parent acyclic unoxidized homolog attained via ring-opening of 2.2b. Heating an air saturated solution of 2.5b (0.880 g, 3.576 mmol) dissolved in DMSO (78 mL) at 100 °C for 28 h leads to the formation of several products. The complex ¹H NMR of the crude mixture reveals the presence of resorcinol as the predominant (90%) product as well as minor conversion to 2,4-dihydroxyacetophenone 2.8 (Figure 2.7, ratio of integrals of resorcinol triplet 6.94 ppm to 2.8 doublet at 7.76 ppm is 153:1, CH₃OD) and very small traces of xanthene 2.6a (d, 7.65 ppm).

![Figure 2.7](image)

Figure 2.7. Minor product of thermolysis of 5b in DMSO.
The production of resorcinol and 2.8 is consistent with the reversible opening and fragmentation of the resorcinarenes in acidic media.\textsuperscript{2.9} This result also complements our recent report describing the production of 4-formylphenylboronic acid from 2.5c.\textsuperscript{2.16} Furthermore, in acidic media, the addition of water at the methine carbon of 2.3 (R = Ar, n=0, m=0) followed by elimination has been described as an intermediate step in the synthesis of xanthenes.\textsuperscript{2.17}

K. K. Kim\textsuperscript{2.10} attained better conversion to xanthene 2.6a from 2.5b by limiting thermolysis time to 2 h. The \textsuperscript{1}H NMR spectrum (DMSO\textsubscript{d6}) of the crude reaction mixture clearly shows a doublet at 7.65 ppm characteristic of 2.6a with improved S/N compared to the 28 h experiment (\textit{vide supra}). Resonances centered at 5.26, 6.49 and 6.60 ppm are also discernable, overlaying with the \textsuperscript{1}H NMR of an analytical sample\textsuperscript{2.17} of 2.6a. Since oxidation to xanthenes can be promoted by peroxides and acid,\textsuperscript{2.17, 2.18} we should attain better conversion to xanthenes upon addition of these latter reagents. Indeed, K. K. Kim\textsuperscript{2.10} found that heating a solution of 2.5b (50 mg, 0.203 mmol), H\textsubscript{2}SO\textsubscript{4} (0.15 mL) and K\textsubscript{2}S\textsubscript{2}O\textsubscript{8} (1.0 mg) in 1.5 mL MeOH at reflux for 2h produces the most significant conversion (4\% yield) of 2.5b to 2.6a we have observed to date.

\textbf{2.3 Conclusions}

Xanthenes form in solutions containing resorcinarene macrocycles (Figure 2.2). The O\textsubscript{2}-induced radical decomposition of DMSO leads to \textit{in situ} strong acid formation. The acid catalyzes a reverse condensation reaction to afford acyclic oligomers. The acyclic oligomers undergo oxidation also via the action of acid and peroxide.
2.4 References


2.14 In the previous work (reference 2.9), acyclic oligomeric products (2.3b and two stereoisomeric trimeric compounds, three resorcinol rings, 2.4, R=Me, m=1, n=0, Scheme 1) were isolated and characterized. Higher order acyclic oligomers (e.g.,
pentamers and hexamers) were also observed as major reaction products. Methyl $^1$H NMR resonances, appearing as several doublets between 0.7 and 2.0 ppm (CH$_3$OD) that corresponded to neither 2.3b, 2.4 (R=Me, m=1, n=0), or resorcinarene macrocycles, thus were assigned to acyclics with five or more resorcinol moieties.

2.15 Flash column chromatography and TLC analysis of the thermolysis products of 2.2b were complicated by the multiple product formation and fraction streaking.


CHAPTER 3

SOLUTION OF COLOR CHANGES PROMOTED BY RECEPTORS POSSESsing BORON IN THE PRESENCE OF SUGARS*

3.1 Introduction

It is known that boronic acid-appended dyes can produce color changes in the presence of saccharides.\(^3.1\) Importantly, when saccharides form cyclic boronates the Lewis acidity of boron is enhanced.\(^3.2\) Thus, upon saccharide binding, sp\(^2\) hybridized neutral boron is more readily converted to an sp\(^3\)-hybridized anion via the addition of H\(_2\)O or HO\(^-\) as the fourth ligand. It was shown by several researchers that the spectral changes of boronic acid-appended chromophores and fluorophores induced by saccharide binding arise due to the change from a neutral, sp\(^2\) boronic acid to an sp\(^3\)-hybridized anionic boronate-saccharide complex.\(^3.1,\ 3.2b\) We thus set out to determine whether the formation of sp\(^3\)-hybridized sugar boronates occurs under our experimental conditions.

3.2 Evidence from \(^{13}\)C NMR

The complexation formation between \(3.1\) (Figure 3.1) and D-fructose was investigated using \(^{13}\)C NMR spectroscopy in a 9:1 DMSO\(_{d6}\):D\(_2\)O solvent system to see if the boronate-saccharide complex is formed and what kind of complexes are formed under our conditions. It is known that fructose exists in solution in a complex mutarotational equilibrium among five isomeric forms (Figure 3.2).\(^3.3\) Except for D-fructoketose, all other forms of D-fructose can form boronate-saccharide complexes with boronic acid.

Isotopically labeled D-fructose-2-\(^{13}\)C was employed to study complexation with 3.1. In the presence of 3.1 (40 mM), D-fructose-2-\(^{13}\)C (1 equiv) in 9:1 DMSO\(_{d6}\):D\(_2\)O exhibits several new \(^{13}\)C-2 resonances which correspond to cyclic sugar boronic esters (Figure 3.3).

**Figure 3.1.** Structures of boron-containing receptors 3.1 and 3.2.

**Figure 3.2.** Five isomeric forms of fructose.
The $^{13}$C chemical shifts (Table 3.1) are in agreement with the values obtained by Norrild for the analogous $p$-tolylboronic acid sugar complexes (see APPENDIX B for spectra). In addition, the resonances for three anionic complexes, which correspond to known $\beta$-D-fructofuranose esters (Figure 3.3), are also observed.

![Structures of the four $\beta$-D-fructofuranose complexes (3.3-3.6)](image)

**Figure 3.3.** Structures of the four $\beta$-D-fructofuranose complexes (3.3-3.6) observed in 9:1 DMSO$_{d6}$:D$_2$O. Boronates 3.5 and 3.6 are "ex" and "endo" isomers exhibiting $^{13}$C-2 chemical shifts of equal intensity at 114.4 and 114.3 ppm, respectively.
Table 3.1. $^{13}$C NMR evidence of boronate ester formation.

<table>
<thead>
<tr>
<th>Fructose mutarotational isomer bound to boron</th>
<th>$^{13}$C-2 chemical shift (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Literature value (p-tolylboronic ester)</td>
<td>Experimental value (boronic ester)</td>
</tr>
<tr>
<td>Fructose mutarotational isomer bound to boron</td>
<td></td>
</tr>
<tr>
<td>β-D-fructofuranose</td>
<td>$^{13}$C-2 chemical shift (ppm)</td>
</tr>
<tr>
<td>Literature value (p-tolylboronic ester)</td>
<td>Experimental value (boronic ester)</td>
</tr>
<tr>
<td>β-D-fructofuranose</td>
<td>113.4&lt;sup&gt;a&lt;/sup&gt; 113.8 (3.4)</td>
</tr>
<tr>
<td>β-D-fructofuranose</td>
<td>114.7&lt;sup&gt;a&lt;/sup&gt; 114.4 (3.5)</td>
</tr>
<tr>
<td>β-D-fructofuranose</td>
<td>114.6&lt;sup&gt;a&lt;/sup&gt; 114.3 (3.6)</td>
</tr>
<tr>
<td>α-D-fructofuranose</td>
<td>115.2&lt;sup&gt;a&lt;/sup&gt; 115.5 (3.3)</td>
</tr>
<tr>
<td>β-D-fructopyranose</td>
<td>105.0&lt;sup&gt;b&lt;/sup&gt; 105.0</td>
</tr>
<tr>
<td>β-D-fructopyranose</td>
<td>107.6&lt;sup&gt;b&lt;/sup&gt; 107.7</td>
</tr>
<tr>
<td>α-D-fructofuranose</td>
<td>99.8&lt;sup&gt;b&lt;/sup&gt; 99.9</td>
</tr>
<tr>
<td>α-D-fructopyranose</td>
<td>92.6&lt;sup&gt;b&lt;/sup&gt; 93.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>In D<sub>2</sub>O at pH = 11-12. <sup>b</sup>In DMSO<sub>d6</sub>. <sup>c</sup>Reference 3.3. <sup>d</sup>Experimental values are obtained in 9:1 DMSO<sub>d6</sub>:D<sub>2</sub>O.

3.3 $^{11}$B NMR study

Further proof that anionic sugar boronates are forming derives from $^{11}$B NMR spectroscopy. The $^{11}$B NMR chemical shifts of boronates change as a result of complexation with sugars due to differential electronic shielding of the $^{11}$B atom. An upfield shift of the $^{11}$B NMR signal accompanies the conversion of sp<sup>2</sup>-hybridized neutral
species to sp\textsuperscript{3}-hybridized boronate anions.\textsuperscript{3,4} At pH = 6.5, compound \textbf{3.1} (10 mM) 1:1 DMSO: \textsubscript{2}O (pH value refers to the buffered aqueous portion before mixing) exhibited a single broad resonance at -19.1 ppm which was assigned to the neutral sp\textsuperscript{2} hybridized boronic acid (3.7, Figure 3.4). At pH = 11.0, but in the presence of 0.5 equiv D-fructose, a new resonance appeared at -32.9 ppm which intensifies when the amount of D-fructose is increased to 5 equiv. The resonance at -32.9 ppm was thus assigned to D-fructose cyclic boronate anion \textbf{3.10}. A solution of \textbf{3.1} (20 mM) in DMSO also exhibited a resonance at -32.9 ppm upon D-fructose (3 equiv) addition. The observation of the resonance at -32.9 ppm corresponding to boronate \textbf{3.10} in DMSO is consistent with \textsuperscript{13}C NMR results.

\begin{align*}
\text{HO}_2B\text{OH} & \leftrightarrow \text{HO}_2B\Theta\text{OH} \\
\text{HO}_2B\Theta\text{OH} & \leftrightarrow \text{HO}_2B\Theta\Theta\text{OH} \\
\text{HO}_2B\Theta\Theta\text{OH} & \leftrightarrow \text{HO}_2B\Theta\Theta\text{OH}
\end{align*}

\textbf{3.7} \quad \textbf{3.8} \quad \textbf{3.9} \quad \textbf{3.10}

\textbf{Figure 3.4.} Equilibria for solutions of \textbf{3.1} and D-fructose.

The formation of the sugar boronate anion allows us to establish a mechanism for the sugar-induced color changes with our receptors. Anionic boronate formation, favored
in the presence of sugars, leads to the diminished acidity of the C-6 hydroxyl. One way to envision this is via examination of xanthene resonance forms 3.11 and 3.12. Structure 3.12 possesses a more stable cation than 3.11, rendering the C-6 hydroxyl of 3.12 relatively less ionizable.

![Figure 3.5. Xanthene resonance forms before and after the formation of boronate ester.](image)

### 3.4 Result from pH Titration Experiments: Color Changes in Buffered Media at Room Temperature

It has been reported that colored aqueous DMSO solutions of resorcinol condensation products exhibited enhanced absorbances at 532 nm upon raising solution pH.\(^3\) If sugar complexation results in a raising of receptor pKa, we should observe relatively diminished receptor absorbance in pH controlled media in the presence of sugars. Figure 3.6 shows the results of a pH vs. absorbance titration monitored at 534 nm. As expected, the absorbance of colored 9:1 DMSO:H\(_2\)O solutions containing 3.1 exhibited a sharp intensity onset beginning at ca. pH = 9. When the same pH titration experiment was run in the presence of D-fructose, the absorbance increase at and beyond pH = 9 is significantly lessened. This is evidence of the diminished ionizability of the C-6 hydroxyl induced by sugar complexation.
A similar pH titration profile was observed for colored solutions of 3.2 alone and with added D-fructose (Figure 3.6), demonstrating that this finding appears general to these materials. Solutions containing 3.1 or 3.2 each exhibited color changes from dark brown to pale orange when D-fructose is added beyond pH = 9 (of the aqueous portion of the solution mixture). We are thus able to observe monosaccharide-induced solution color changes at room temperature in buffered media using these methods.\textsuperscript{3,6}

\textbf{Figure 3.6.} Spectrophotometric study (λ=534 nm) of colored solutions containing 3.1 (top, 3.5 × 10^{-3} M) and 3.2 (bottom, 3.5 × 10^{-4} M) alone and with D-fructose (3.5 × 10^{-2} M) in buffered 9:1 DMSO:H_2O (pH values refer to the H_2O portion before mixing with DMSO).
3.5 Conclusion

In conclusion, solutions of resorcinarenes develop color upon heating due to the formation of new xanthene-like chromophores (CHAPTER 2). Saccharides promote solution color changes via boronate ester formation with chromophore-appended boronic acids. The boronate ester formation can be monitored by $^{11}$B NMR and the discrimination of different complexes between saccharides and the chromophore can be achieved on the basis of the chemical shift values of $^{13}$C NMR. UV-vis spectroscopic titration of the chromophore solution in the absence and presence of saccharides is also a direct and easy method to detect the formation of boronate esters. The understanding of the mechanism of the color changes in the solutions of resorcinarene possessing boronic acids provides us an excellent basis to develop methods to sense different saccharides with our new compounds.

3.6 References


3.6 Derivative analysis revealed slope maxima for the titration curves of colored solutions of 3.1 and 3.2 occur at pH values corresponding to 9.45 and 9.80, respectively. However, these values have no physical significance towards assigning a respective pKa to the materials since the pH values on the x-axis of Figure 3.6 refer only to the aqueous sugar-containing solutions prior to mixing with solutions derived from heated 3.1 and 3.2 in DMSO. A known method for the determination of resorcinarene pKa values in a mixed aqueous solvent system (Schneider, H.-J.; Güttes, D.; Schneider, U. J. Am. Chem. Soc. 1988, 110, 6449.) more appropriate for potentiometric titrations was applied to colored solutions of 3.1 and 3.2 with and without added fructose. Unfortunately, solubility problems precluded us from obtaining pKa data via this method. Although to date we have not been able to determine pKa values, the strikingly similar effect of fructose on the UV-vis spectra of distinct but related materials (colored solutions of 3.1 and 3.2) showed that the sugar-induced perturbation of the chromophore ionization state is apparently significant and reproducible within this class of materials.
CHAPTER 4

SOLUTION OF COLOR CHANGES PROMOTED BY RECEPTORS NOT POSsessING BORON IN THE PREsENCE OF POLAR ANALYTES*

4.1 Introduction

The mechanism accounting for the color changes in the solution of chromophore-appended boronic acids was explained in the previous chapter. Attention was next turned to the optical response to the colored solution of resorcinarenes not possessing boronic acids. In our study, it was noticed that compound 4.2b, which was synthesized from resorcinol and acetaldehyde, analogous to 4.1 and 4.2a, also exhibited color changes upon heating with specific sugars. Since the boronic acid moiety is no longer present in compound 4.2b, there is an alternative explanation for the color change promoted by the saccharides in this case.

![Figure 4.1](image_url)

Figure 4.1. Chair (4.1) and crown (4.2a) stereoisomers of tetraarylboronic acid resorcinarenes.

4.2 Results and Discussion

In order to understand the different optical responses to sugars observed with boronic acid and non-boronic acid containing compounds, the solutions containing 4.2b and reducing and non-reducing sugars were first examined. We found that non-reducing sugars did not promote significant optical changes when heated in the presence of 4.2b. For example, when a solution of 4.2b (5.2 mM) in 9:1 DMSO/H$_2$O (1 mL) in the presence of non-reducing sugars, either methyl-\(\alpha\)-glucopyranoside (4.3, 15.6 mM) or \(\alpha\)-methyl-mannopyranoside (4.4, 15.6 mM), was heated for 2.5 minutes to gentle reflux, each solution color and absorption spectrum was unchanged from the heated solution of 4.2b without sugar (Figure 4.3).

![Structures of sugars](image)

**Figure 4.2.** Structures of two reducing (4.3, 4.4) and three non-reducing sugars.

Reducing sugars underwent fragmentation to chain-shortened carbohydrates including aldonic acids, in the presence of peroxide.$^{4,3}$ The $^1$H NMR spectra of respective DMSO$_{d6}$ solutions containing three reducing sugars (28 mM), maltose, D-\((-\text{-fructose})\) and D-\((+\text{-glucose})\), heated until gentle reflux (2.5 min), exhibited at least five new aldehyde resonances between 9 and 10 ppm as well as a broad proton resonance in the carboxylic
acid region at 11.7 ppm. None of these observed resonances are present in the NMR spectrum of heated neat DMSO$_{d6}$. The region between 9 and 15 ppm in the spectra of the two non-reducing sugars 4.3 and 4.4 exhibited only very weak resonances which were also observed in the spectrum of heated neat DMSO$_{d6}$. We thus hypothesized that the color changes observed in 4.2b solutions are due to the new products formed upon reducing sugar thermolysis. When unheated solutions of either maltose, D-fructose or D-glucose were added to a colored solution of 4.2b at room temperature, no color change was observed.

![Absorbance heated solution of 4.2b (5.2 mM) in 9:1 DMSO/H$_2$O (1 mL) in the absence and presence of non-reducing sugars 4.3 (top) and 4.4 (bottom).](image)

**Figure 4.3.** Absorbance heated solution of 4.2b (5.2 mM) in 9:1 DMSO/H$_2$O (1 mL) in the absence and presence of non-reducing sugars 4.3 (top) and 4.4 (bottom).
If reducing sugar reaction products are a cause of the solution color changes in the presence of the resorcinol condensation products, then the addition of preheated reducing sugars to preheated colored solutions of 4.2b should produce a color change. We heated DMSO (0.9 mL) solutions of maltose, D-fructose and D-glucose each to a gentle reflux, cooled to room temperature and add 0.1 mL H₂O. We added each of the solutions to three preheated 9:1 DMSO:H₂O solutions of 4.2b. The final concentration was 5.2 × 10⁻³ M for 4.2b and 1.6 × 10⁻² M for all sugars. Each solution was stirred for 6 min prior to UV-vis analysis. The three sugar solutions exhibited color changes observable by visual inspection and exhibited UV-vis spectra that were different from that of a heated solution containing only 4.2b.⁴,⁵ The absorbance decreased (compared to the solution of 4.2b without sugar) at 530 nm for the solution containing D-(+)-glucose is (17.9±1.0)%, (23.7±1.3)% for D-(-)-fructose and (9.1±2.0)% for maltose (Figure 4.4).

![Figure 4.4. Absorbance of heated 9:1 DMSO:H₂O solutions of 4.2b (5.2 × 10⁻³ M) in the absence and presence three preheated reducing sugars (1.6 × 10⁻² M).](image-url)
We proposed therefore that acyclic, xanthene-containing oligomers, which we have demonstrated to be the active chromophores in resorcinarene solutions, function as colorimetric receptors for sugar-derived acids. In order to prove this hypothesis, we chose to study the interaction of 4.6 (Figure 4.5) with N-acetylneuraminic acid (the most naturally abundant sialic acid, 4.5), an important cell surface residue, in buffered media. Compound 4.6 is a xanthene dye with hydroxyls adjacent to the C-6-OH and carbonyl moieties. The structure of 4.6 is analogous to oxidized acyclic oligomers derived from resorcinarenes, in that it contains proximal potential hydrogen bonding sites for charged carboxylates (Figure 4.5).

![Structures of compounds 4.5, 4.6, and 4.7](image)

**Figure 4.5.** Top: Structures of compound 4.5 and 4.6. Bottom: Charged hydrogen bonding between carbohydrates and proximal hydroxyls of 4.6 and 4.7.
Addition of varying amounts of sialic acid 4.5 (1.9 \times 10^{-4} \text{ M} - 1.5 \times 10^{-3} \text{ M}) to a peach-colored solution of compound 4.6 (1.0 \times 10^{-5} \text{ M}) in a 9:1 DMSO:H_{2}O solution (HEPES buffer, 5.0 \times 10^{-3} \text{ M} \text{ final concentration, adjusted to pH = 7.5 in H}_{2}O \text{ before mixing with DMSO}) at room temperature resulted in a solution color change to yellow. The color changes monitored by UV-vis spectroscopy were concentration dependent. We also observed a color change to pink under the same conditions when NaOAc (2.2 \times 10^{-4} \text{ M}) instead of 4.5 was added to a solution of 4.6. This latter result suggests that the carboxylate moiety of the sugar is responsible for the change in solution color.\textsuperscript{4.6}

FT-IR absorption shifts served as evidence supporting a hydrogen bonding interaction between the carboxylate moiety of 4.5 and the hydroxyls of 4.6. The FT-IR spectrum of a 1.0 \times 10^{-2} \text{ M} \text{ solution of 4.5 in DMSO} \text{ exhibited a carbonyl absorption at 1726 cm}^{-1}. \text{ Upon addition of an equimolar amount of 4.6, a new absorption appeared at 1661 cm}^{-1} \text{ accompanied by significant weakening of the absorption at 1726 cm}^{-1}. \text{ This was in keeping with hydrogen bonding of 4.6 to the sialic acid carbonyl oxygen.\textsuperscript{4.7}} Further support for hydrogen bonding was the fact that solution color changes upon sialic acid addition to 4.6 were no longer observed when the proportion of H_{2}O to DMSO is \geq 50 \%.\textsuperscript{4.8}

4.3 Conclusions

We conclude that aldonic acids, formed from reducing sugars \textit{in situ} upon heating in DMSO, promoted solution color changes via a charged hydrogen bonding interaction with the xanthene chromophore. This latter interaction perturbed the ionization state of the C-6 hydroxyl of the dye. In contrast, heating reducing sugars in the presence of
boronic acids should not favor aldonic acid formation since anomeric hydroxyls are typically those involved in boronate ester formation.

4.4 References


4.4 Analysis of NMR integral ratios revealed that 59% of starting D-fructose was converted to new products under these conditions. The new product mixture contained 27% aldehydes and 0.9% carboxylic acids.

4.5 We heated solutions of D-(-)-fructose, maltose, or D-(+)-glucose in DMSO to a gentle reflux for 2 min and cooled to rt. A solution of 4.2b in DMSO was also heated to gentle reflux and cooled to room temperature. The three sugar solutions were added to the solution of 4.2b respectively followed by the addition of water to afford mixtures in 9:1 DMSO:H₂O. The final concentration was 5.2 × 10⁻³ M for 4.2b and 1.6 × 10⁻² M for all sugars. Each solution was stirred for 6 min prior to UV-vis analysis. The absorbance decreased (compared to the solution of 4.2b without sugar) at 530 nm for the solution containing D-(+)-glucose was (17.9±1.0)% , (23.7±1.3)% for D-(-)-fructose and (9.1±2.0)% for maltose (three trials).

4.6 Having shown that acyclic aldonic acids, rather than uncharged sugars, produced a solution color change at room temperature in the presence of our active, non-boronic acid-containing chromophores, we next chose to generalize this finding to include sialic acid 4.5 because it is a prototypical, readily available and bioactive cyclic sugar molecule possessing a carboxylate moiety. In addition, in our first communication in this study, we used boron-containing 4.1 to produce significant solution color change with 4.5 as well as other carboxylate-, phosphate- and amine-functionalized saccharides. The results reported herein thus show that the boronic acid moieties may not be required (see also reference 4.1) in order to produce solution color changes in the presence of charged sugars. The fact that acetate also induced a color change at room temperature in buffered media demonstrates that simpler carboxylic acids could also be determined under these conditions. Research is in progress in our laboratory to develop selective sensing conditions for bioactive charged phosphates and carboxylates, including sialic acids, based on these findings.
4.7 The IR absorption changes we observed were in keeping with those reported by Zhang and co-workers who have recently studied the formation of hydrogen bonds between anthracene carboxylic acid and xanthene dyes using several techniques including IR and $^1$H NMR: Zhang, H.; Zhang M.; Shen, T. *Dyes and Pigments*, 1999, 43, 15.

4.8 In addition, 4.6 (10 mM) in DMSO$_{d6}$ was titrated with 0.13 to 2.6 equiv 4.5. The $^1$H NMR spectra showed that a 4.6 hydroxyl resonance exhibited a successive downfield chemical shift from 10.53 ppm to 10.70 ppm upon increasing the concentration of 4.5.
CHAPTER 5

SIMPLE AND RAPID VISUAL SENSING OF OLIGOSACCHARIDES*

5.1 Introduction

The field of glycobiology has recently undergone great resurgence as a result of the exciting therapeutic potential of oligosaccharides.\(^1\) A vast number of oligosaccharides are found in glycoproteins and on cell surfaces. Most of the recent progress towards the color detection of sugars has, however, involved monosaccharide analysis.\(^2\,\,5.3\) The great variety of linear and branched oligosaccharides magnifies the problems associated with monosaccharide analysis.

New methods allowing for simple and rapid oligosaccharide detection are in demand. The colorimetric detection of oligosaccharides eluting from chromatographic columns is one of the biggest challenges in sugar analysis.\(^4\) There is an unfilled need for a simple method for the visible detection of oligosaccharides. There are currently no useful direct color tests for higher molecular weight oligosaccharides. Refractive index detectors are typically used to detect oligosaccharides in HPLC analyses, but refractive index detection is highly sensitive to the temperature used and the nature of the mobile phase employed, and refractive index detection is also susceptible to non-specific cross-reactivity. UV detection at wavelengths below 210 nm is another option, but UV detection limits solvent choice and requires expensive ultrapure solvents to reduce interferences. Electrochemical detection by pulsed amperometric detection (PAD)

requires high pH conditions. Mass spectrometry, coupled with chromatographic separations, requires specialized and expensive equipment. Aromatic or heterocyclic substituents have been used as chromogenic labels to facilitate UV detection of oligosaccharides. Radioactive labeling has also been used, but has the obvious disadvantage that it requires the handling of radioactive substances. The older color tests used for automated HPLC post-column detection of monosaccharides fail to effectively directly detect oligosaccharides containing more than three residues. For example, in a representative assay, maltohexaose's response is only 18% of that observed for the same weight of glucose.\textsuperscript{5,4} Colorimetric methods thus typically require prior complete hydrolysis to monosaccharides or covalent attachment of the oligosaccharides to a chromophore, which can lead to diminished separation.\textsuperscript{5,4,5.5}

We thus extend the scope and utility of our sensing methodology to include the color detection of a homologous series of prototypical neutral oligosaccharides, the linear maltodextrins, containing up to seven glucose residues. We address the fundamental problem of diminished optical detection of larger oligosaccharides using simple colorimetric agents.

**5.2 Visual Detection of Oligosaccharides by Different Chromophores**

The linear maltodextrins \textbf{5.3a-f} (Figure 5.2) are a prototypical class of readily available neutral oligosaccharides composed of glucose residues with α-1,4-glycoside linkages. We heated solutions (9:1 DMSO:H\textsubscript{2}O, 1 mL) containing specific maltodextrins \textbf{5.3a-f} (2 mg), in the presence of \textbf{5.2a} (Figure 5.1, 5 mg, 5.2 mM) until a temperature of 120 °C was reached in 3.0 min. In these studies the resorcinarenes were initially preheated in order to maximize chromophore formation.
Upon cooling the respective solutions containing mixtures of 5.2a and each of 5.3a-f we observed solution colors of deep orange yellow, yellow-gold, pale orange, pale orange, pink and peach for maltose 5.3a through maltoheptaose 5.3f, respectively (Figure 5.2). The UV-vis spectra of each of the sugar-containing solutions exhibited increased absorbances at 460 nm (and decreased absorbances at 530 nm) compared to a solution of 5.2a heated without sugar (Figure 5.3). Heated solutions containing 5.1 and 5.3a-5.3f also exhibited absorbance maxima at 460 nm that are greater than 5.1 heated alone. The boronic acid-containing resorcinol condensation products thus allowed for potentially useful colorimetric detection of larger oligosaccharides at 460 nm.
In analogous experiments to those above, but employing 5.2b instead of 5.1 or 5.2a, we found the absorbance changes in the oligosaccharide-containing solutions were greatly diminished compared to the solution containing 5.2b alone. The presence of boronic acid moieties is apparently a key to attaining enhanced absorbance values with larger oligosaccharides.

Figure 5.2. Colored solutions of 5.2a after heating alone or with maltodextrins 5.3a-f.
Figure 5.3. UV-vis spectra of solutions of 5.2a, 5.1 and 5.2b heated alone or in the presence of 5.3a-f. The boronic acid-containing receptors 5.1 and 5.2a exhibited larger absorbance responses to oligosaccharides.
5.3 Binding Constant Trends

It has been showed that oligosaccharides promote solution color changes via boronate ester formation with chromophore-appended boronic acids (CHAPTER 3). The pH-dependent sugar-complexation equilibrium is shown in Figure 5.4. Apparent equilibrium constant ($K_{\text{exp(app)}}$)\textsuperscript{5,6} data for the interaction of sugars and receptors derives from UV-vis spectroscopy. The equilibrium constants were evaluated via Benesi-Hildebrand treatment\textsuperscript{5,7} (equations 1 and 2) using UV-vis absorbance changes observed at 460 nm for the linear maltodextrin series in colored solutions of 5.2a (complexation coverage = 50-60%). In each case, the double reciprocal plots according to equation 1 afforded very good linearity with a correlation coefficient $r \geq 0.99$ (APPENDIX A).

\begin{align*}
\frac{1}{[A_0] - [A]} &= \frac{1}{[A_0] - [A_\infty]} + \frac{1}{[A_0] - [A_\infty]} \cdot \frac{1}{K} \cdot \frac{1}{[\text{sugar}]} \quad (1) \\
K &= \frac{y\text{-intercept}}{\text{slope}} \quad (2)
\end{align*}

$A_0$ and $A$ are UV-vis absorbance at 460 nm of chemosensor in the absence and presence of a sugar, respectively, while $A_\infty$ is that of the complex of sugar and chemosensor.

When we carried out a UV-vis spectroscopic titration to determine the binding constants of oligosaccharides, the linear maltodextrins 5.3a-f (0-7.5 $\times$ $10^{-2}$ M) were dissolved in 0.5 mL carbonate buffer (2.5 $\times$ $10^{-2}$ M, pH = 11) and mixed with 0.5 mL DMSO colored preheated solutions of 5.2a (5.0 $\times$ $10^{-4}$ M) respectively to furnish a 1:1 DMSO:H$_2$O solvent system. The solution colors changed from orange to yellow upon sugar addition in every case and the absorbance change was monitored by UV-vis
spectroscopy. We thus provided a simple and rapid method for the detection of neutral oligosaccharides in buffered media at room temperature by visual inspection.

\[ K_{\text{exp}} = \frac{[\text{Anionic Sugar-Chromophore complex}][H^\oplus]}{[\text{Chromophore}][\text{Sugar}]} = K_{\text{exp(app)}}[H^\oplus] \]

**Figure 5.4.** pH-dependent sugar-complexation equilibrium.

Prior studies (using fluorescence detection) showed that binding constants observed for the interaction of oligosaccharides and fluorophore-appended arylboronic acids increased with larger oligosaccharide size. This has been attributed to sugar binding via boronate formation involving the hydroxyls of the oligosaccharide reducing termini. The binding is enhanced by secondary sugar-dye contacts including hydrophobic and CH-π interactions. Larger oligosaccharides have greater flexibility and can thus more readily fold back and interact with the dye molecule when a boronate ester is formed. The expected trend of enhanced binding affinity correlating with longer oligosaccharide length was observed in our study (Figure 5.5).

### 5.4 Conclusions

The important problem of diminished optical response correlating with increasing oligosaccharide chain length may be overcome via the use of boronic acid-containing chromophores. This is apparently due to the enhanced binding affinities of
Figure 5.5. Plot of binding constant (three trials, monitored at 460 nm) vs molecular weight. Increasing molecular weight beginning with α-D-glucose through the series of linear maltodextrins 5.3a-f results in an increase of binding constants (65.0, 108.3, 145.0, 189.7, 246.0 and 275.0 M⁻¹) in colored solutions containing 5.2a. The binding constants are reproducible within ± 10%.

larger oligosaccharides for boronic acids.⁵,⁶ We also provided a simple and rapid method for the visual detection of neutral oligosaccharides in buffered media at room temperature. We conclude that the significant solution color changes observed for the oligosaccharides (5.3a-f) are due, in large part, to the formation of cyclic sugar boronate esters.

5.5 References


6.1 Introduction

Fructose is a major component of sweet foods and beverages. The worldwide production of high fructose syrup (HFS) is currently about $8 \times 10^9$ kg/year.\(^6\)\(^1\) The quality control of fructose levels in food and beverages is an ongoing concern. Fructose is also an important, common energy source and sweetener metabolized at a high rate in animals and humans. High D-fructose intake is implicated in the pathogenesis of hypertriglyceridaemia, atherosclerosis\(^6\)\(^2\) and insulin resistance.\(^6\)\(^3\) Non-enzymatic glycosidation products form more rapidly from fructose than from glucose.\(^6\)\(^4\) Further elucidation of the biochemical role of D-fructose requires better methods of analysis. For instance, the determination of the relatively low fructose levels in human plasma cannot be performed in a reliable manner due, in large part, to excess of glucose.\(^6\)\(^5\) Levels of fructose reported vary greatly among laboratories and on the technique employed, including enzymatic detection.\(^6\)\(^6\) Many methods are based on GC or HPLC determinations but often involve tedious sample preparation and derivation.\(^6\)\(^6\)

The AOAC (formerly the Association of Official Analytical Chemists) official methods for the analysis of fructose rely on gas (GC) and liquid chromatography (HPLC) with refractive index detection.\(^6\)\(^7\) Gas chromatography typically relies on prior derivatization (methylation, trimethylsilation) of the sugars.\(^6\)\(^7\) Refractive index detection exhibits significant cross-sensitivity to other materials.\(^6\)\(^8\) More recently, pulsed amperometric detection (PAD), an electrochemical method, has gained widespread use
for monosaccharide detection in conjunction with HPLC; however, it can be limited by the necessity to operate at high pH which, in turn, limits the choice of solvents and conditions.\textsuperscript{6.8} Mass detectors are very useful when coupled to HPLC or GC systems; however, this adds a further great degree of sophistication and specialization to the analysis.\textsuperscript{6.9} The simple reducing sugar assays are also used in automated postcolumn detection systems for monosaccharides including fructose;\textsuperscript{6.10} their apparent limitations are summarized in Table 6.1. Enzyme-based assays are also used for specific fructose determination;\textsuperscript{6.11} however, enzymes are expensive and degradable. The great number of patented methods for fructose determination highlight the great technological interests in fructose sensing on the international scene and are largely based on enzymatic, pH or chromatographic assays.\textsuperscript{6.12}

It is well-known that saccharides can be quantified by color assays.\textsuperscript{6.13} Colorimetric methods are of great importance because saccharides contain no inherent chromophore. The color assays based on synthetic molecules are typically less expensive than enzymatic methods and do not use readily degradable materials. Enzymatic assays offer much greater specificity compared to the simpler color tests; however, the non-enzymatic color tests are still often used (see Table 6.1) because of cost and stability. The standard color assays summarized in Table 6.1 are based upon the reaction of the carbonyl function of the sugar with a certain reagent to form a colored product. As can be seen in Table 6.1, most of the classical color tests employ relatively harsh conditions or toxic reagents.

More recent studies describe the visual color sensing of monosaccharides including fructose by boronic acid-appended dyes. These studies, pioneered mainly by
Table 6.1. Standard color assays for various analytes including sugars.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>general phenol-sulfuric acid assay for reducing sugar $^{6,14}$</td>
<td>Uses the toxic reagent phenol and corrosive $\text{H}_2\text{SO}_4$ and affords mixed responses for different classes of sugars. Interference from metals, proteins, cysteine and azide. Reproducibility is dependent upon the manner of $\text{H}_2\text{SO}_4$ addition.</td>
</tr>
<tr>
<td>dinitrosalicylic acid assay for reducing sugar $^{6,15}$</td>
<td>Interference by $\text{CO}_2$ and dissolved $\text{O}_2$ as well as certain metal ions and requires heating at 100 °C.</td>
</tr>
<tr>
<td>Nelson-Somogyi method for reducing sugar $^{6,16}$</td>
<td>Requires toxic $\text{Na}_2\text{HAsO}_4$ as well as $\text{H}_2\text{SO}_4$.</td>
</tr>
<tr>
<td>ferric-orcinol assay for pentose $^{6,17}$</td>
<td>Includes the use of corrosive $\text{HCl}$ and requires heating to 100 °C. Interference from hexoses can be significant and standards containing hexoses and pentoses in the expected amounts are needed.</td>
</tr>
<tr>
<td>phenol-boric acid-sulfuric acid assay for ketose $^{6,18}$</td>
<td>Uses the toxic reagent phenol and corrosive $\text{H}_2\text{SO}_4$. Different ketoses give different responses. Reproducibility is dependent upon the manner of $\text{H}_2\text{SO}_4$ addition.</td>
</tr>
<tr>
<td>Morgan-Elson assay for hexosamine $^{6,19}$</td>
<td>Uses corrosive acids. Prior acetylation of free hexosamines is often required. Heating at 100 °C required.</td>
</tr>
<tr>
<td>Carbazole assay for uronic acids $^{6,20}$</td>
<td>Interference from neutral carbohydrates, cysteine, other thiols and proteins. Different uronic acids afford different responses. Heating at 100 °C required.</td>
</tr>
<tr>
<td>Warren assay for sialic acid $^{6,21}$</td>
<td>Requires prior periodate oxidation and toxic sodium arsenate, redistilled cyclohexanone and heating at 100 °C. Interference from fucose (reduced absorbance) and fructose.</td>
</tr>
</tbody>
</table>

the Shinkai group, rely on color sensing promoted by either perturbation of an aggregation-deaggregation equilibrium (addition of saccharides caused deaggregation of
the boronic acid functionalized dye\textsuperscript{6,22} or the interaction of the boronic acid with a neighboring amine (attached to an azo dye)\textsuperscript{6,23,6,24} resulting in charge transfer effects.

Boronic acids react with the hydroxyl groups of saccharides to form five- or six-membered ring cyclic sugar boronate esters. Fructose forms the most stable of all complexes with boronic acids.\textsuperscript{6,25} Russell patented the reaction of diazotized \textit{m}-phenylboronic acid with glucose, although he reported that other common polyhydroxy compounds\textsuperscript{6,26} exhibited responses to his dye as well. The material was thus non-selective. Boronic acids are the basis of carbohydrate affinity chromatography and the resorcinarenes promoted the first known binding of sugars in apolar solvents.\textsuperscript{6,27d} Therefore, combining boronic acids and resorcinarenes into single molecular frameworks may afford unique and powerful new sugar receptors. We synthesized tetraarylboronic acid resorcinarene compound 6.1 and structure-related compound 6.2 (Figure 6.1) to demonstrate their utility as sugar visualization agents.

![Figure 6.1. Structures of boronic acid-containing sugar sensors.](image)

**6.2 Selective Fructose Color Sensing in neutral aqueous DMSO solution.**

Colorless chemosensor 6.1 (5.2 mM, 0.9 mL) in DMSO is heated to a gentle reflux during 3 minutes in air to afford a pink solution. After cooling to room
temperature, fructose (1 equiv) in 0.1 mL H₂O is then added. A color change from pink to orange-yellow is observed. The color change is monitored by observing the absorbance intensity decrease at 535 nm and increase at 464 nm. In addition, glucose, sucrose, maltose, lactose, xylose, and glucose, 3 equiv each, exhibit no significant color change at room temperature under the same conditions with 6.1 (Figure 6.3). This finding offers promise for the selective detection of the sweetener fructose in the presence of mixtures of several carbohydrates.

In addition, we find that 100 equiv of glucose in an equimolar solution of fructose and 6.1 (1 equiv each fructose and 6.1, 5.2 mM, 100 equiv glucose in 10% aqueous DMSO) affords virtually no change in the absorbance at 464 nm of fructose/I alone (Figure 6.2).

**Figure 6.2.** UV-vis spectra of a solution containing pre-heated (1.5 min at reflux) 1 (5.2 × 10⁻³ M) in 9:1 DMSO : H₂O (1 mL) (i) at room temperature alone, with (ii) 1 equiv D-(-)-fructose, (iii) 1 equiv fructose and 100 equiv glucose and (iv) 2 equiv fructose and 100 equiv glucose. Inset shows the equivalence of fructose and glucose to 6.4.
Figure 6.3. Color of preheated DMSO solution of 6.1 (5.2 × 10⁻³ M, unlabeled) in the absence and presence of 3 equivalence carbohydrates.

Addition of 1 equiv fructose to 6.1 9:1 DMSO : H₂O (1 mL) alone in solution at room temperature results in a 29% increase in the absorbance intensity at 464 nm (Table
6.1). Addition of 100 equiv glucose results in only a 0.28% decrease in the absorbance of the fructose/6.1 absorbance intensity at 464 nm (from $A = 0.4295$ to $A = 0.4283$). Addition of a second equiv of fructose to this latter solution results in an absorbance increase of 23% (Table 6.1). At 536 nm, the absorbance intensity for 6.1 is lowered by 25% upon addition of 1 equiv fructose. Addition of 100 equiv glucose lowers the intensity further by 8.3% (from $A = 0.7457$ to $A = 0.6835$). Addition of another equiv of fructose again lowers the absorbance by 19% (Table 6.2). This result indicates that it may be possible to determine fructose in the presence of 100 equiv glucose by monitoring at 464 nm and comparing to a calibration plot. In addition, one might be able to concurrently determine the glucose present in a sample via examining the ratio of the intensities at 536:464 nm, with a calibration plot after the fructose concentration is determined at 464 nm.

**Table 6.2.** Absorbance of pre-heated 6.1 (5.2 mM) in 9:1 DMSO:H$_2$O (i) at room temperature alone, with (ii) 1 equiv D-(-)-fructose, (iii) 1 equiv fructose and 100 equiv glucose and (iv) 2 equiv fructose and 100 equiv glucose.

<table>
<thead>
<tr>
<th></th>
<th>$A_{464 \text{ nm}}$</th>
<th>$A_{536 \text{ nm}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>i) 6.1</td>
<td>0.3337</td>
<td>0.9998</td>
</tr>
<tr>
<td>ii) 6.1 + 1 eq fructose</td>
<td>0.4295</td>
<td>0.7457</td>
</tr>
<tr>
<td>iii) 6.1 + 1 eq fructose + 100 eq glucose</td>
<td>0.4283</td>
<td>0.6835</td>
</tr>
<tr>
<td>iv) 6.1 + 1 eq fructose + 100 eq glucose + 1 eq fructose</td>
<td>0.5282</td>
<td>0.5526</td>
</tr>
</tbody>
</table>

**6.3 Selective Fructose Color Sensing in Alkaline Media**

Most of the color assays are based on the direct interaction of the chromophore and the analyte. It can often be difficult to perturb the microenvironment of the
chromophores sufficiently to cause a spectra change if absorbance spectroscopy is used. An alternative for changing the microenvironment of the chromophore significantly is its release from a complex.

It is well known that boronic acids react with the 1,2- or 1,3 cis diols to form five- or six-membered ring cyclic boronate esters. Among all sugars, fructose forms the most stable of all complexes with boronic acids.\textsuperscript{6,25} Therefore, fructose can displace the boronic acids from a weaker boronate ester to form a new stronger ester because cyclic boronate ester formation is reversible. If one of the components in a weaker ester is UV-active, the release of the chromophore can cause a color or absorbance change, which can be used for the visual sensing of fructose. An ideal chromophore should have (i) proximal cis diol and (ii) a large color or absorbance change upon the formation of boronic ester. Many commercial dyes with adjacent hydroxyl groups (catechols) have been studied. Boric acid\textsuperscript{6,28}, instead of boronic acid, can be used to bind these chromophores. We find that pyrocatechol violet 6.3 (Figure 6.4) is an excellent chromophore for this purpose. Compound 6.3 has previously been used to determine the presence of many metals such as bismuth and tin.\textsuperscript{6,29}

![Figure 6.4. Structures of dye pyrocatechol violet (6.3) and 4-(methylthio)phenylboronic acid (6.4).](image-url)
Figure 6.4 shows the results of a pH vs. absorbance titration monitored at 590 nm. Dye 6.3 (3.6 \times 10^{-5} \text{ M}, 1 \text{ mL}) in 2:8 MeOH:H\textsubscript{2}O (buffered with 5.0 \times 10^{-2} \text{ M phosphates}) exhibits biggest absorbance at ca. pH = 4.1 and second biggest absorbance at pH = 8.3. However, the biggest absorbance decrease (32.5\%, from 0.7392 to 0.4990) is observed at pH = 8.3 upon the addition of boric acid (1.8 \times 10^{-3} \text{ M}). The color changes from violet (\(\varepsilon_{\text{max}} = 590 \text{ nm}\)) to purple (\(\varepsilon_{\text{max}} = 500 \text{ nm}\)). Few dyes that we have studied exhibit this dramatic color or absorbance change at any pH value under same conditions.

Figure 6.5. Spectrophotometric study (\(\lambda = 590 \text{ nm}\)) dye 6.3 (3.6 \times 10^{-5} \text{ M}) alone and with boric acid (1.8 \times 10^{-3} \text{ M}) in buffered 2:8 MeOH:H\textsubscript{2}O (pH values refer to the H\textsubscript{2}O portion before mixing with MeOH).

A suitable boronic acid, which could cause a significant color or absorbance change upon complexation with dye 6.3, is searched for. It is found that, among more than twenty boronic acids that are tested, 4-(methylthio)phenylboronic acid 6.4 (Figure
6.6), together with several other aryl boronic acids, meets the requirement and 6.4 is arbitrarily selected for our next study.

The absorbance response of dye 6.3 upon complexation with 6.4 in aqueous alkaline media is shown in Figure 6.6. Addition of varying amounts of boronic acid 6.4 (3.6 × 10⁻⁵ M - 1.8 × 10⁻³ M) to a violet-colored solution of compound 3 (3.6 × 10⁻⁵ M) in a 2:8 MeOH:H₂O solution (phosphates buffer, 5.0 × 10⁻² M final concentration, adjusted to pH = 8.3 in H₂O before mixing with MeOH) at room temperature results in a solution color change to dark pink. An isosbestic point at 545 nm suggests a 1 : 1 binding between dye 6.3 and boronic acid 6.4.

![Figure 6.6. UV-vis spectra of 6.3 (3.6 × 10⁻⁵ M) in the absence and presence of 6.4 (3.6 × 10⁻⁵ M - 1.8 × 10⁻³ M) in 2:8 MeOH:H₂O (pH 8.3 phosphates buffer). Inset shows the concentration of 6.4.](image)

Varying amount of D-(-)-fructose is next added to this 6.3:6.4 complex and observe a color change from purple to violet (Figure 6.7). Five equiv 6.4, instead of 50
equiv, is used to form the 6.3:6.4 complex so that less D-(−)-fructose can be detected. The 6.3:6.4 complex is obtained by mixing a solution of 6.3 (3.6 × 10⁻⁵ M) in 0.8 mL H₂O (pH 8.3, phosphates buffer) with a solution of 6.4 (1.8 × 10⁻⁴ M) in 0.2 mL MeOH. D-(−)-fructose is added as a solution in 0.2 mL 2:8 MeOH:H₂O (pH 8.3 phosphates buffer) to achieve required concentration. The final concentration of 6.3, 6.4 and buffer are 3.0 × 10⁻⁵ M, 1.5 × 10⁻⁴ M and 5.0 × 10⁻² M respectively. The color change from purple back to violet indicates the displacement of dye 6.3 by D-(−)-fructose.

![Figure 6.7](image)

**Figure 6.7.** UV-vis spectra of 6.3:6.4 complex in the absence and presence of D-(−)-fructose (3.0 × 10⁻⁴ M - 1.5 × 10⁻² M) in 2:8 MeOH:H₂O (pH 8.3 phosphates buffer). Inset shows the concentration of D-(−)-fructose.

We next turn our focus on detecting D-(−)-fructose in the presence of large excess of D-(+)-glucose. The 6.3:6.4 complex is formed as above. Addition of 3.3 equiv fructose to a solution of complex 6.3:6.4 in 2:8 MeOH:H₂O (pH 8.3 phosphate buffer) alone results in a 6.2% decrease in the absorbance at 500 nm (Figure 6.8, Table 6.2).
Addition of 100 equiv glucose does not give a significant color or absorbance change (Figure 6.8). Addition of another 3.3 equiv fructose to this latter solution causes an absorbance decrease of 6.1% (Table 6.2). At 590 nm, the absorbance intensity for complex 6.3:6.4 is increased by 8.8% upon addition of 3.3 equiv fructose. Addition of 100 equiv glucose increases the intensity further by 4.2% (Table 6.2). Addition of another 3.3 equiv fructose again lowers the absorbance intensity by 4.9% (Table 6.2). This result is similar to that observed in the neutral aqueous DMSO solution system. Therefore, we have made it possible to detect D-(-)-fructose and simultaneous determination of D-(+)-glucose at buffered alkaline media. With this method, the detection limit \(6.30 \times 10^{-4}\) M for D-(-)-fructose is also obtained.

**Figure 6.8.** UV-vis spectra of complex 6.3:6.4 in 2:8 MeOH:H\(_2\)O (pH 8.3 phosphate buffer) (i) alone, with (ii) 3.3 equiv D-(-)-fructose, (iii) 3.3 equiv fructose and 100 equiv glucose and (iv) 6.6 equiv fructose and 100 equiv glucose. Inset shows the equiv of fructose and glucose to complex 6.3:6.4.
Table 6.3. Absorbance of complex 6.3:6.4 in 2:8 MeOH:H₂O (pH 8.3 phosphate buffer) (i) alone, with (ii) 3.3 equiv D-(−)-fructose, (iii) 3.3 equiv fructose and 100 equiv glucose and (iv) 6.6 equiv fructose and 100 equiv glucose.

<table>
<thead>
<tr>
<th></th>
<th>A₅₃₀ nm</th>
<th>A₅₉₀ nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>i) 6.3:6.4</td>
<td>0.4044</td>
<td>0.4331</td>
</tr>
<tr>
<td>ii) 6.3:6.4 + 3.3 eq fructose</td>
<td>0.3794</td>
<td>0.4712</td>
</tr>
<tr>
<td>iii) 6.3:6.4 + 3.3 eq fructose + 100 eq glucose</td>
<td>0.3745</td>
<td>0.491</td>
</tr>
<tr>
<td>iv) 6.3:6.4 + 3.3 eq fructose + 100 eq glucose + 3.3 eq fructose</td>
<td>0.3557</td>
<td>0.5148</td>
</tr>
</tbody>
</table>

6.4 Conclusion

We have overcome the problem of determining D-(−)-fructose in the presence of a large excess of D-(+)-glucose in either neutral aqueous DMSO solution or buffered alkaline media. The former is based on the direct attachment of sugars to the chromophore, which results in the formation of a cyclic boronate ester. The later is due to the release of chromophore from a weaker boronate, which changes the microenviroment of the chromophore and therefore causes a significant color change. The determination of D-(−)-fructose and D-(+)-glucose in plasma is ongoing in our laboratory.

6.5 References


6.9 Reference 6.8, p. 34.


6.17 Reference 6.8, p. 4.

6.18 Reference 6.8, p. 5.


CHAPTER 7

THE SYNTHESIS OF A RESORCINARENE DERIVATIVE FOR THE STABILIZATION OF LIPOSOMES

7.1 Introduction

The liposome drug-carrier concept was proposed and tested a quarter of a century ago. Liposomes have been used as a drug delivery system for the last twenty years. Liposomes are believed to have appreciable potential as drug carriers, especially in cancer therapy infection treatment. Some uses of liposomes as carrier of enzymes and DNA have also been described. Liposomes can incorporate into any type of drug, either in the polar vesicle interior or in the hydrophobic regions of the bilayers of the lipids. The targeting of liposomes towards specific tissues is severely limited by the rapid sequestration of liposomes by the reticuloendothelial system (RES). Therefore, many of the applications of liposome drug-delivery systems have been limited by their short circulation half-lives ($T_{1/2}$) as a result of rapid uptake into the RES. In an effort to optimize their therapeutic utility, it is necessary to determine how to keep them out of the RES. Scientists have been working to make various types of liposomes with a reduced affinity to cells in the RES and hence a prolonged circulation time. In those studies, ganglioside GM$_1$ was used as a molecule which endows the liposomes with a lower level of RES uptake and hence an increased $T_{1/2}$. Allen et al were the first to show this unusual activity of GM$_1$ and the name ‘stealth liposome’ (‘stealth liposome’ is a registered trademark of Liposome Technology, Inc.) has been given to this class of liposomes. A number of other natural and synthetic lipids have been examined for the stealth activity. For example, lipophilic polyethylene glycol (PEG) derivatives have been
extensively studied because it has been shown that the conjugation of PEG to proteins significantly prolongs the circulation $T_{1/2}$ of the protein.\textsuperscript{7,10} Drugs and conventional liposomes are rapidly cleared from the bloodstream by the RES and other nonspecific mechanisms.\textsuperscript{7,11} This clearance of liposomes can be significantly decreased if polyethylene glycol-linked lipids (molecular weight 2000 and 5000 Da) are incorporated. A picture of polymer grafted bilayer is shown in Figure 7.1. The mechanism of the stabilization is believed to be a physical one.\textsuperscript{7,12-7,15} Water soluble polymer creates a steric barrier to enhance the repulsive properties of the liposomal surface, stabilizing the lipid bilayer against close approach of other macromolecules and cells.

**Figure 7.1.** Schematic diagram showing a polymer-grafted bilayer at low (left) and high (right) grafting concentration.

The purpose of our study is to synthesize a novel reagent which can significantly increase the stabilization of liposome systems upon incorporation into the bilayers of specific liposomes. In the aforementioned example, the prolonged circulation time was achieved by casting phosphatidyl ethanolamine with a covalently attached polyethylene glycol into phosphatidylcholine (PC) liposomes. Resorcinarene is employed as an amphiphile (Figure 7.2). A polyethylene glycol (PEG) unit of a fixed length will be
covalently attached to the upper rim of the resorcinarene at one end of the PEG chain. At the other end of the chain, a carbon-carbon double bond is introduced.

![Resorcinarene Structure](image)

**Figure 7.2.** Structure of resorcinarene.

Subsequently, the functionalized resorcinarene will be incorporated into the bilayer of the liposome. Upon cross-linking a ‘screen’ will be formed outside of the liposome. We believe this type of liposome systems will significantly increase the circulation time because of the surface barrier present in the upper rim of the functionalized resorcinarene. In addition, the cross-linked ‘screen’ anchors itself at the hydrophobic area of the bilayer via the lower rim of the functionalized resorcinarene, which further stabilizes the liposome.

### 7.2 Synthesis of the Resorcinarene Derivatives

The resorcinarenes are unique three-dimensional cyclic aromatic tetramers. Their impact in the disciplines of molecular recognition, supramolecular chemistry and materials science has been extensively studied. The resorcinarene (7.1) has an eleven carbon aliphatic chain at the lower rim and eight hydroxyl groups at the upper rim. The length of the alkyl chain is similar to that of the lipids, which will be used to prepare
liposome. The polyethyleneglycol chain will be covalently attached to the resorcinarene via the polar hydroxyl groups of the resorcinarol units. The length of PEG chain can be varied for best liposome-stabilizing effect. The PEG that we have chosen consists of six repeating units. The PEG groups will increase the solubility of the macrocycle once attached to the parent macrocycle. Two hydroxyl groups at both ends of the PEG chain will enable us to conduct our synthetic pathway. It is an arduous task to put PEG groups on eight hydroxyl groups of the parent macrocycle. Thus one of the PEG hydroxyl groups is selectively protected, and a good leaving group is put at the other end via ether or ester linkage. Some of the leaving groups that are tried include Ms, Ts, Cl (THP-O-PEG-O-C(O)CH₂Cl). The bases in our system are NaH or DBU. However, none of our attempts are successful. This may be the result of the steric hindrance among eight hydroxy groups at the upper rim. Therefore, a spacer or linker was put between the macrocycle and PEG chains to reduce the steric hindrance at the upper rim so that there will be more space for the incoming PEG molecules to reach the reaction sites at the upper rim. Chloroacetyl chloride is tried first. However, the reaction of chloroacetyl chloride with macrocycle and sodium hydride in DMF turns out to be a failure. It is very difficult to separate the reaction mixture, and we did not find evidence of the desired product. Next attention is turned to implementing ethyl bromoacetate as a linker. The reaction of parent macrocycle 7.1 with ethyl bromoacetate in the presence of potassium carbonate and 18-crown-6 in dry acetone gives the product 7.2 in 90% yield. Then the ester functionality in 7.2 is successfully transformed to carboxylic acid via saponification using potassium hydroxide followed by the acidification with dilute hydrochloric acid to afford compound 7.3. This synthesis is shown in Scheme 7.1.
Scheme 7.1. Introduction of carboxylic acid functionality to the upper rim of resorcinarene

In the next step, the PEG is to be linked to macrocycle via the spacer. First, one of the hydroxyl groups of PEG, which is kept available for the introduction of polymerizable monomer later, is protected. Dihydropyran and trityl chloride are employed to introduce THP and trityl protective groups. Both methods are very successful for the purpose of protection, as shown in Scheme 7.2. The protected PEGs 7.4a and 7.4b undergo coupling reactions with 7.3 in the presence of DMAP, HOBT and DCC in dry DMF to give 7.5a and 7.5b respectively. $^1$H NMR and MALDI-MS data
confirmed the formation of 7.5a and 7.5b. However, it is difficult to perform the deprotection of THP and trityl groups in compound 7.5a and 7.5b without disturbing the ester linkage. Finally, the synthetic pathway is change by introducing the carbon-carbon double bond to the PEG groups first and then attaching the functionalized PEG chains to the macrocycle. Methacryloyl chloride is used to react with hexa(ethylene glycol) in the presence of DMAP to yield compound 7.4c, which is subjected to the coupling reaction under the same condition to afford 7.5c (Figure 7.3). The $^1$H NMR data matches the structure of compound 7.5c. MALDI-MS data further confirmed the formation of 7.5c. This series of reactions can be found in Scheme 7.2.

![Figure 7.3. Structure of target compound 7.5c.](image)
Scheme 7.2. Synthesis of resorcinarene derivatives.

In the future studies, compound 5c will be incorporated into the bilayer of the liposomes (Figure 7.4). Utilizing liposome as a template, the carbon-carbon double bonds, which are at the end of the PEG chains, will be cross-linked to form a two
dimensional polymer network above the surface of the liposome (Figure 7.5). The polymer network may also form in the interior of the liposome if the polymerizing condition is met there. It is projected that the polymer network will stabilize the liposome and hence a prolonged $T_{1/2}$. In addition, the polymer network will retain its structure to give polymer hollow sphere after the extracting the surfactant matrix. The polymer hollow sphere will lead to interesting future studies.

Figure 7.4. Proposed incorporation of compound 7.5c into the bilayer of the liposome.
7.3 Experimental Procedures

**Materials and Methods.** All chemicals were purchased from the Aldrich Chemical Company and used without further purification. Unless otherwise noted, all non aqueous reactions were carried out under dry nitrogen atmosphere in flame dried glassware. DCM, DMF and THF were distilled over CaH$_2$ prior to use. Analytical thin-layer chromatography (TLC) was performed using general purpose 60-Å g silica gel on glass (Aldrich). TLC plates were visualized with the powder mixture of iodide and silica gel. Flash chromatography columns were prepared with Kieselgel 60-Å silica gel 230-400 mesh (Merck). Proton ($^1$H) NMR spectra were acquired with Bruker AC-200, Bruker AC-250 spectrometers.

**Octa(ethyl acetate ) resorcinarene 7.2**  C-undecylcalix[4]resorcinarene monohydrate 7.1 (1.22 g, 1.1 mmol), anhydrous K$_2$CO$_3$ (3.60 g, 26.1mmol) and 18-crown-6 (0.69 g, 2.6 mmol) were added to dry acetone (80 ml). The mixture was refluxed for half an hour.

**Figure 7.5.** Sketch of two dimensional polymer network above the surface of the liposome
before dry ethyl bromoacetate (3 ml, 26.1 mmol) was added dropwise to the above mixture. The resulting mixture was refluxed for 18 h. The reaction mixture was filtered and the filtrate concentrated in vacuo to thick oil which was dissolved in 10% MeOH 90% DCM and purified by chromatography using a gradient of 25% EtOAc 75% Hexane to 45% EtOAc 55% Hexane to afford light yellow oil 7.2 (1.91g, 97%). \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 6.62 (s, 4H, ArH meta to OH), 6.25 (s, 4H, ArH ortho to OH), 4.61 (t, 4H), 4.29 (s, 16H), 4.19-4.28 (q, \(J = 7.5\) Hz, 16H), 1.86 (s, 24H), 1.25-1.30 (m, 104H), 0.89 (t, \(J = 7.5\) Hz, 12H).

**Octa(acetic acid) resocinarene 3**  To a solution of 2 (1.91 g, 1.1 mmol) in EtOH (40 ml) was added a solution of 1 N NaOH (150 ml). The mixture was set to reflux for 6h and concentrated in vacuo after it is cooled to room temperature. The resultant solid was dissolved in water and acidified with 3 M HCl. The precipitate was extracted three times with Et\(_2\)O and organic layer was dried with MgSO\(_4\) to give white solid 7.3. \(^1\)H NMR (DMSO-d\(_6\)): \(\delta\) 6.51 (s, 4H, ArH meta to OH), 5.77 (s, 4H, ArH ortho to OH), 4.53 (t, 4H), 4.20-4.46 (dd, 16H), 1.21 (m, 80H), 0.81-0.86 (t, \(J = 14\) Hz, 12 H).

**Hexa(ethylene glycol) monotetrahydropyran ether 7.4a**  Dihydropyran (1.51 g, 18 mmol) in dry DCM (80 ml) was added dropwise to a solution of Hexa(ethylene glycol) (8.5 g, 30 mmol) and PPTS (0.53 g, 2.1 mmol) in dry DCM (320 ml). The mixture was stirred at room temperature for 8 h and then washed with half saturated brine. The organic layer was concentrated in vacuo and the resultant was purified by chromatography using 11% MeOH 89% DCM to give compound 7.4a (3.5g, 53%) as a yellow viscous oil. \(^1\)H NMR (CDCl\(_3\)): \(\delta\) 4.63 (t, 1H), 3.8-3.6 (m, 26H), 3.1 (s, 1H), 1.7-1.5 (m, 6H).
Octa(hexaethylene glycol monotetrahydropyran ether) resorcinarene 7.5a

Compound 7.3 (0.55 g, 0.35 mmol), DMAP (0.37 g, 3.0 mmol) and HOBT (0.41 g, 3.0 mmol) were dissolved in dry THF (18 ml) and dry DMF (15 ml) and a solution of 7.4a (2.30 g, 6.3 mmol) in dry THF (12 ml) was then added to the previous mixture. DCC (0.86 g, 4.2 mmol) was finally added to the reaction mixture and the whole mixture was stirred at room temperature for 18 hours. The reaction mixture was then filtered and the solvent was removed in vacuo. The resultant was treated with water and extracted twice with chloroform. The solvent of combined organic layer was removed and the resultant was further purified by flash chromatography using 14% MeOH 86% DCM to give 7.5a (1.1g, 73%). 1H NMR (CDCl₃): δ 6.65 (s, 4H), 6.23 (s, 4H), 4.65 (t, 16H), 4.58 (t, 4H), 4.32 (broad, 16H), 3.90-3.62 (m, 160H), 1.9-1.5 (m, 48H), 1.30-1.24 (m, 80H), 0.88 (t, 12H). MALDI-MS: found 4378.48 (C₂₂₄H₃₈₄O₈₀ = 4357.47).

Hexa(ethylene glycol) monotriphenylmethyl ether 7.4b

To a solution of hexaethylene glycol (1.0 g, 3.5 mmol) and triethylamine (0.36 g, 3.6 mmol) in dry DCM (8 ml) was added triphenylmethyl chloride (0.92 g, 3.3 mmol) and the reaction mixture was stirred at room temperature for 5.5 h. The reaction mixture was washed with ice-cold water once and organic layer dried over MgSO₄. The resultant was concentrated in vacuo and further purified by flash chromatography using 5% MeOH 95% DCM to give product 7.4b (0.4g, 22%) as a viscous oil. 1H NMR (CDCl₃): δ 7.62-7.18 (m, 15H), 3.8-3.6 (m, 22H), 3.25 (t, 2H), 2.7 (broad, 1H).

Octa(hexaethylene glycol monotriphenylmethyl ether) resorcinarene 7.5b

To a solution of 7.3 (0.0565 g, 0.036 mmol), DMAP (0.0044 g, 0.036 mmol) and HOBT (0.0049 g, 0.036 mmol) in DMF (1.0 ml) was added 7.4b (0.37 g, 0.57 mmol) in dry DMF
(4ml) and the reaction mixture was heated gently to make a clear solution. DCC (0.0743g, 0.36 mmol) was then added to the above solution and the whole reaction mixture was stirred at room temperature for 2 days. The reaction mixture was filtered and solvent removed in vacuo. The residue was treated with chloroform and washed with water twice. The organic layer was dried over MgSO$_4$ and the resultant was further purified by flash chromatography using 10% MeOH 90% DCM to give the product 7.5b. No NMR data available. MALDI-MS found: 5734.6 (C$_{336}$H$_{432}$O$_{72}$ = 5623.00).

**Hexa(ethylene glycol) monomethacrylate 7.4c** To a solution of DMAP (0.41 g, 3.3 mmol) in dry DCM (150 ml) was added dropwise a solution of methacryloyl chloride (0.32 ml, 3.3 mmol) in dry DCM (60 ml) at 0°C. The solution was allowed to warm up to room temperature, diluted with ether (300 ml), and then washed with brine (300 ml). The organic phase was dried over MgSO$_4$ and then concentrated under reduced pressure. Chromatography of the residue with 20% MeOH 80% DCM afford 7.4c (0.48 g, 36%) as yellow oil. $^1$H NMR (CDCl$_3$): δ 6.15 (s, 1H), 6.56 (s, 1H), 4.33 (t, $J$ = 10 Hz, 2H), 3.61-3.77 (m, 22H), 2.48 (s, 1H) 1.97 (s, 3H).

**Octa(hexaethylene glycol methacrylate) resorcinarene 7.5c** Compound 7.3 (0.1g, 0.061mmol), DMAP (0.066 g, 0.54 mmol) and HOBT (0.072 g, 0.54mmol) were dissolved in 15 ml 1:2 dry THF/dry DMF (gentle heating was required). DCC (0.151g, 0.73mmol) was then added to the above mixture. Finally, 7.4c (0.48 g, 1.1 mmol) in dry THF (5 ml) was added to the above solution. The reaction mixture was stirred at room temperature for 90 h and filtered. The filtrate was concentrated in vacuo and the residue was treated with water, followed by the extraction with chloroform twice. The combined organic layer was dried over MgSO$_4$ and purified by flash chromatography using a
gradient of 20% MeOH 80% DCM to 30% MeOH 70% DCM to give compound 5c as an oil. \(^1\)H NMR (DMSO-\(d_6\)): \(\delta\) 6.59 (s, 4H), 6.30 (s, 4H), 6.14 (s, 8H), 5.58 (s, 8H), 4.58 (t, 4H), 4.39-4.24 (m, 48H), 3.77-3.64 (m, 160H), 1.96 (s, 24H), 1.30-1.24 (m, 80H), 0.88 (t, 12H).

7.4 References

7.2 Gregoriadis, G.; Ryman B. E. Eur. J. Biochem. 1972, 24, 485
7.4 Ostro, M. J. Liposomes from Biophysics to Therapeutics 1987, 277.
CHAPTER 8

FUTURE WORK

8.1 Introduction

The synthesis of several powerful resorcinarene receptors (Figure 8.1) for the visual sensing of mono- and oligosaccharides was reported recently. Sugars are very difficult to analyze because they are similar in structure and transparent in visible region. The development of more powerful color sensing reagents and methods will provide improved monitoring of disease status and the products of fermentation processes. The fundamental study of color sensing for linear malto-oligosaccharides with our receptors 8.1 and 8.2 was reported recently. One of the next aims is to detect oligosaccharides in post-HPLC column effluents. Great progress and exciting results on the fundamental study of visual sensing for fructose and sialic acid have been obtained. Both receptor structure and experiment conditions for the applied studies of those analytes, which are of great biological importance, are now being optimizing. Methods on the selective sensing of fructose in plasma and sweet foods and beverages are being tested. Research on the visual sensing of sialic acid in gangliosides is ongoing.

Figure 8.1. Structures of resorcinarene colorimetric sensor
8.2 Applied Study of Highly Selective Color Sensing Methods for Fructose Determination

Fructose is also an important, common energy source and sweetener metabolized at a high rate in animals and humans. High D-fructose intake is implicated in the pathogenesis of hypertriglyceridaemia, atherosclerosis\(^8.4\) and insulin resistance\(^8.5\). Non-enzymatic glycosidation products form more rapidly from fructose than from glucose\(^8.6\). Further elucidation of the biochemical role of D-fructose requires better methods of analysis. For instance, the determination of the relatively low fructose levels in human plasma cannot be performed in a reliable manner due, in large part, to excess of glucose\(^8.7\). Recently, Pitkänen reported a relatively laborious method whereby mannose and fructose concentrations were determined in human plasma via mass spectrometry\(^8.8\). The fructose concentration was determined at 23.30 µM (and 18.3 µM in fasting samples). Their values were lower than those obtained by enzymatic (86 µM) and other chromatographic methods. We are now working on the determination of fructose in human plasma in commercial plasma samples from Sigma. Both methods, which are developed for the determination of fructose in neutral or alkaline media (CHAPTER 6), will be used and result will be compared to each other and to that in the literature. The clean-up of the plasma is easy and simple. Ultrafiltration via centrifugation will be used to remove high molecular weight proteins in the plasma and by filtration through ion exchangers to remove potentially interfering charged species. Meanwhile, we are trying to optimize the detection limit of both methods in order to decrease the sample amount drawn from the plasma and/or increase the amount of solvent for dissolving the dried material. Fluorescence determination will also be studied.
Carbohydrates account for more than 98% of the total soluble solids in orange and apple juices.\textsuperscript{8,9} Fructose, glucose and sucrose are the major carbohydrate constituents of orange juice present in a ratio of 1:1:3. Fructose is present in 0.1 M concentrations.\textsuperscript{8,9} We will work with a commercial orange juice sample. The sample clean-up and methods employed are almost same to those in the determination of fructose in plasma. In a similar fashion we plan to analyze for fructose in other food and drink samples such as honey and wine after pre-sample treatment to remove charged substances. Competitive binding vs. glucose and sucrose (sucrose affords minute absorbance changes with our receptors) as well as checking our analysis with enzymatic kits and chromatographic techniques will be done as described above for the plasma studies.

\textbf{8.3 Applied Study of a Simple and Rapid Detection Methods for Sialic Acid Determination}

Sialic acids (Figure 8.2) are components of glycoproteins, glycopeptides and glycolipids. They play roles in cell to cell communication in the human body and increased virulence in some bacteria. Imbalances in sialic acid levels can have clinical manifestations such as alterations in cell adhesion, a condition implicated in some cancers and graft rejection. An increase in the levels of both soluble and cellular sialic acid can be a marker for cancer diagnosis.\textsuperscript{8,10} The most commonly occurring and important sialic acid is $N$-acetylneuraminic acid (NeuAc), followed by $N$-glycoloylneuraminic acid (NeuGc).\textsuperscript{8,10}

A simple and rapid method for the determination of sialic acid using commercial xanthene dye has already been discovered.\textsuperscript{8,3} It is also found that compound \textbf{8.3} (Figure 8.2) could potentially serve as a selective color sensing agent for sialic acid (Figure 8.3). It is planed to develop a method for sialic acid analysis on commercially available
samples of gangliosides. Mono-through tetra sialylated gangliosides are commercially available. The gangliosides embody relatively simpler structures compared to other sialic acid-containing biomolecules. Gangliosides, therefore, are an excellent place to start in developing an assay for naturally-occurring sialic acid.

\[ \text{Structure of receptor 8.3} \]

**Figure 8.2.** Structures of receptor 8.3 and sialic acid (8.4).

\[ \text{Structure of receptor 8.3} \]

**Figure 8.3.** Preheated $5.2 \times 10^{-3}$ M aqueous DMSO solutions of 8.3 (unlabeled) were cooled to room temperature and stirred in the presence of 3 equiv of carbohydrates for five minutes.
8.4 Applied Study of the Method for the Colorimetric Determination of Oligosaccharides

The automation of the classical saccharide color tests is of great current interest.\textsuperscript{8.11} Unfortunately, there are currently no useful direct color tests for higher molecular weight oligosaccharides.\textsuperscript{8.12} The current detection methods require prior complete hydrolysis to monosaccharides or covalent attachment to a chromophore.\textsuperscript{8.12}

The classical color tests for monosaccharides simply fail to directly detect oligosaccharides containing more than three residues.\textsuperscript{8.11} The color response is only related to the molar concentration of oligosaccharide, not the concentration by weight. For example, maltohexaose's response is only 18\% of the same weight of glucose.\textsuperscript{8.11}

"Detection of oligosaccharides eluting from HPLC columns is the biggest challenge and weakest link in the analysis of oligosaccharides."\textsuperscript{8.13} What is needed is a simple method for increasing the visible detection response to an oligosaccharide. We have reported a breakthrough simple color test for detecting oligosaccharides, as exemplified by our studies of the prototypical neutral maltose oligomers composed of glucose monomers.\textsuperscript{8.3} Oligosaccharide solutions (maltotriose through maltohexaose, each at the same weight, in the presence of our chemosensor, exhibit increased intensity absorbance responses in the visible region at three different wavelengths. No prior hydrolysis or chemical modification of the sugars with a chromogenic reagent is needed. Therefore, the problem of decreased absorbance with increased molecular weight is overcome.

Next focus will be turned on the implementation of an automated assay system for oligosaccharides after methods are optimized by manual assay techniques. The
automated system, in tandem with the appropriate HPLC columns and conditions based on well-known separation procedures, should be useful for detecting a wide variety of monosaccharides and oligosaccharides. It is planned to apply the method to the determination of malto- and isomalto-oligosaccharides. These efforts should lead to a general method for post-column oligosaccharide detection, as the maltose oligomers embody "prototypical" neutral glucose oligomers.

8.5 Conclusions

The visual determination of saccharides has been of great interest for more than a century. In 1887 Seliwanoff reported a resorcinol color test that was specific for ketoses.8,14 Other resorcinol-based color tests for sugars were later developed.8,15, 8,16 The versatility of our chemosensors was exemplified in our initial paper in this field which showed that our easily-prepared, one-step condensation products promoted rapid and dramatic, highly characteristic coloration of solutions of a variety of specific saccharides.8,1-8,3 We will continue to focus on the development of more powerful and sensitive receptors as well as more efficient method for the visual sensing of mono- and oligosaccharides and other analytes of great biological importance.

8.6 References


8.13 Reference 8.9, p. 62.


8.16 Kulka, R. G Biochem J. 1956, 63, 542.
APPENDIX A: BINDING CONSTANTS OF MALTODEXTRINS

Figure A.1: Binding constant determination of glucose according to Benesi-Hildebrand equation.

Figure A.2: Binding constant determination of maltose according to Benesi-Hildebrand equation.
Figure A.3: Binding constant determination of maltotriose according to Benesi-Hildebrand equation.

Figure A.4: Binding constant determination of maltotetraose according to Benesi-Hildebrand equation.
Figure A.5: Binding constant determination of maltopentaose according to Benesi-Hildebrand equation.

Figure A.6: Binding constant determination of maltohexaose according to Benesi-Hildebrand equation.
Figure A.7: Binding constant determination of maltoheptaose according to Benesi-Hildebrand equation.
APPENDIX B: $^{13}$C NMR EVIDENCE OF BORNATE ESTER FORMATION

Figure B.1: $^{13}$C NMR spectrum of Isotopically labeled D-fructose-2-$^{13}$C in DMSO$_{d6}$.
**Figure B.2:** $^{13}$C NMR spectrum of the mixture of compound 3.1 and Isotopically labeled D-fructose-2-$^{13}$C in DMSO$_{d6}$. Peaks at 93.2, 99.9, 105.0, 107.7, 113.8, 114.3, 114.4, 115.5 ppm are from the complexes between D-fructose-2-$^{13}$C and compound 3.1 (see text).
Figure B.3: Expanded $^{13}$C NMR spectrum of the mixture of compound 3.1 and Isotopically labeled D-fructose-2-$^{13}$C in DMSO$_{d6}$. Peaks at 93.2, 99.9, 105.0, 107.7, 113.8, 114.3, 114.4, 115.5 ppm are from the complexes between D-fructose-2-$^{13}$C and compound 3.1 (see text).
APPENDIX C: LETTERS OF PERMISSION

May 30, 2002

American Chemical Society
1155 16th St., N.W.
Washington, DC 20036

To Whom It May Concern:

I am writing to obtain permission for the use of my contributions to an article published in the Journal of the American Chemical Society. I am a graduate student in the Department of Louisiana State University. I am the first author on the article and would like to include my contributions to the article in my doctoral dissertation. The article is "Chromophore Formation in Resorcinarene Solutions and the Visual Detection of Mono- and Oligosaccharides". 2002, Vol. 124, pp. 5000-5009.

Thank you for your consideration of this request.

Sincerely,

Ming He
Phone: (225)-578-2706
Fax: (225)-578-3458
E-mail: minghe@rocketmail.com
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4/10/02
**VITA**

Ming He was born in Changzhou, Jiangsu Province of the People’s Republic of China, on October 10, 1970. He graduated in 1988 at Beijiao Middde School in Changzhou, Jiangsu Province. He was the recipient of many honors including Outstanding Student in the City of Changzhou and Excellent Student leader in the City of Changzhou. In the fall of 1988, he enrolled at Jiangsu Institute of Petrochemical Technology (JIPT) in Changzhou, Jiangsu Province majoring in Polymer Materials and Engineering. He received JIPT Top Scholarship four years in a row from 1988 to 1992. In 1992, he graduated and received a Bachelor of Science degree with the honor of JIPT Outstanding Graduate.

In 1996, after he worked in polymer industry in P. R. China for four years, Ming entered the graduate program in the Department of Chemistry at East Tennessee State University in Johnson City, Tennessee. A year later, he transferred to Louisiana State University (LSU) in Baton Rouge, Louisiana. While at LSU, he synthesized and studied new materials for the visual detection of biomolecules under the supervision of Dr. Robert M. Strongin. He received LSU Research Award in Chemistry and LSU Teaching Award in Chemistry and he was the only one to win both awards. He is a member of the American Chemical Society. Ming is currently a candidate for the degree of Doctor of Philosophy in organic chemistry, which will be awarded on the December 2002 Commencement.