Detection of Biologically Important Molecules

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DETECTION OF BIOLOGICALLY IMPORTANT MOLECULES

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

In

The Department of Chemistry

By
Kyukwang Kim
B.S., Hallym University, South Korea
December 2005
DEDICATION

I’d like to dedicate my doctoral dissertation to my family

Un-Sup Kim (김운섭), Young-Ja Lee (이영자), Kyu-Na Kim (김규나)

Sunjoo Lim (임선주) and Andrew Myungsuk Kim (김명석)

I love you all so much
ACKNOWLEDGEMENTS

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<th>Definition</th>
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<tbody>
<tr>
<td>A</td>
<td>Absorbance</td>
</tr>
<tr>
<td>pKₐ</td>
<td>Acid dissociation constant</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary Unit</td>
</tr>
<tr>
<td>amu</td>
<td>Atomic mass unit</td>
</tr>
<tr>
<td>TBSCI</td>
<td>tert-Butyl dimethyl silyl chloride</td>
</tr>
<tr>
<td>BHT</td>
<td>2,6-Di-tert-butyl-4-hydroxytoluene</td>
</tr>
<tr>
<td>calcd</td>
<td>Calculated</td>
</tr>
<tr>
<td>¹³C-NMR</td>
<td>Carbon-13 Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-Dimethyl formamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>ESI</td>
<td>ElectroSpray Ionization</td>
</tr>
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<td>Ethanol</td>
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<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
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<tr>
<td>ELSD</td>
<td>Evaporative Light Scattering Detection</td>
</tr>
<tr>
<td>FAB</td>
<td>Fast Atom Bombardment</td>
</tr>
<tr>
<td>GM</td>
<td>Monosialoganglioside</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-Performance Liquid Chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Hcy</td>
<td>Homocysteine</td>
</tr>
<tr>
<td>ID</td>
<td>Internal Diameter</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit Of Detection</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-Assisted Laser Desorption Ionization</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MV$^{2+}$</td>
<td>Methyl viologen dication</td>
</tr>
<tr>
<td>MV$^{+}$</td>
<td>Methyl viologen radical cation</td>
</tr>
<tr>
<td>M</td>
<td>Molar concentration</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts Per Million</td>
</tr>
<tr>
<td>$^{31}$P-NMR</td>
<td>Phosphorus-31 Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>pH</td>
<td>Potential of hydrogen</td>
</tr>
<tr>
<td>$^1$H-NMR</td>
<td>Proton Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>PAD</td>
<td>Pulsed Amperometric Detection</td>
</tr>
<tr>
<td>rt</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TPPO</td>
<td>Triphenyl phosphine oxide</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxylmethyl)aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet-Visible</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>$\lambda$</td>
<td>Wavelength</td>
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ABSTRACT

Anions play important roles in biological systems. The design of anion sensors has been of great interest. The selective detection of a specific anion is particularly challenging. In the first chapter, I describe the synthesis of a TBS-protected fluorescein dye (1.1) and its use for the selective detection of fluoride. Solutions of 1.1 either in DCM or in aqueous media at physiological pH show the excellent selectivity towards fluoride to afford color formation while solutions of other interfering anions remain colorless.

The monitoring of homocysteine (Hcy) and cysteine (Cys) levels is important since they are biomarkers associated with Alzheimer’s and cardiovascular disease, among many others. In the second chapter, the selective detection of Hcy and Cys over other amino acids using a library of chromogenic reagents and their application in an HPLC post-column system are described.

Saccharides are widely used in industrial processes and biological systems. The determination of a specific saccharide is difficult since sugars are colorless and structurally similar to each other. Furthermore, the colorimetric detection of oligosaccharides via conventional methods is difficult. In the third chapter, I have reported the synthesis of a boronic acid-appended rhodamine dye (3.1) and its application towards the detection of mono- and oligosaccharides via an HPLC post-column system. The selectivity of 3.1 towards fructose allows one to detect fructose in the presence of a 100-fold excess of glucose. The detection of sialic acid is also described.

In the fourth chapter, the elucidation of the mechanism of the origin of color formation in resorcinarene macrocyclic compounds is explained in detail. The in situ acid formation from DMSO decomposition can cause the ring-opening of resorcinarenes followed by the
condensation of resultant products to afford xanthene dyes. This discovery has influenced the development of other indicator molecules discussed in the previous chapters.

In the last chapter, I reported the synthesis of a cysteine-conjugated rhodamine dye and its application in the selective detection of α-dicarbonyl compounds and a DNA lesion marker. These ongoing research projects will continue. New studies including optimizing sensing conditions and the elucidation of selective binding mechanism include future work.
CHAPTER 1
SELECTIVE RECOGNITION OF FLUORIDE WITH A FUNCTIONALIZED
FLUORESCEIN VIA CHROMOPHORE FORMATION IN AQUEOUS MEDIA

1.1. Anion Recognition

The search for artificial anion receptors has been of great recent interest. Anions play important roles in biological systems and industrial processes.\(^1\) Fluoride detection has gained attention due to its importance in human health.

For the past few years, receptors based on hydrogen bonding interactions with anions have been intensively evaluated. Receptors are generally designed in combination with a responsive functional group and, for sensing, a signaling unit. Urea, thiourea or pyrrole groups have been shown to interact with anions to produce an electrochemical\(^1,2\) or an optical (UV-Vis/fluorescence) response.\(^3\) However, the nature of such receptors limits the ability for selective recognition towards fluoride in the presence of other anions since hydrogen bonding interactions could also occur with other strong electronegative ions. Typical interferents include halides and oxygen-containing species such as \(\text{CH}_3\text{CO}_2^-\), \(\text{H}_2\text{PO}_4^-\) and \(\text{H}_2\text{SO}_4^-\).

Efforts have been made to improve fluoride selectivity.\(^1,4,5\) However, these latter approaches restrict the choice of solvent, since aqueous or hydrogen bonding media compete with fluoride in binding the receptor.\(^1,3a,1,5\) Electrochemical signaling can be obtained in aqueous media upon the binding of boronic acids to fluoride. The ensuing negative charge on boron generates an electrochemical signal via a ferrocenyl subunit.\(^6\) Visible detection was also achieved indirectly in the same system using redox interactions between ferrocenylboronic acid and organic dyes.\(^7\) In addition, boronic acids conjugated to fluorogenic substrates such as benzene or naphthalene were introduced to produce optical signals at emission wavelengths below 350 nm over a moderate range of fluoride.
concentrations (50-70 mM). To the best of our knowledge, there has been no report of a simple indicator molecule that is selective towards fluoride in the presence of other anionic interferents in aqueous solution at physiological pH. Indicator molecules that interact with specific anion target molecules can afford significant and characteristic color and/or fluorescence signaling.

Fluorescein is a well-known chromo/fluorogenic compound which shows high extinction coefficient and fluorescence quantum yield. Fluorescein derivatives have been intensively utilized in numerous biological applications. Fluorescein derivatives can exist in two tautomeric forms; a colorless/non-fluorescent lactone or highly colored/fluorescent quinine (Scheme 1.1). Hence, the controlled manipulation of the tautomeric transition could afford a chromogenic and fluorogenic indicator. For example, some functionalized fluorescein dyes have been used as photoactivatable fluorophores and as peroxide probes.

1.2. Selective Detection of Fluoride

In recent work, I reported the synthesis of a boronic acid functionalized rhodamine dye and its application towards the detection of mono- and oligosaccharides. It is known that silicon has a strong affinity towards fluoride. Silyl-protected phenolic hydroxyl groups could be effectively cleaved by a reagent containing fluoride. Thus, conventional fluoride-induced deprotection would allow us to attain a very simple chromogenic and fluorogenic sensor. Silyl-protected fluorescein derivatives are readily synthesized in one step from commercially available fluorescein using a standard phenol protection protocol (Scheme 1.2) and evaluated as fluoride indicators in aqueous solutions. It has been reported that refluxing aryl trimethylsilyl ethers in aqueous MeOH could cause cleavage of the Si-O bond.
Figure 1.1. Selective detection of fluoride using a colorimetric receptor 1.1 (2.8 × 10^{-4} M) in aqueous media (75 mM triethanolamine buffer at pH 7.5 in 7:3 acetone:water). Each sample contains the same concentration of different anionic species as their corresponding tetrabutylammonium salt forms (1.5 × 10^{-3} M each). (a) no analyte, (b) fluoride, (c) chloride, (d) bromide, (e) iodide, (f) nitrate, (g) acetate, (h) cyanide, (i) hydrogensulfate.

Scheme 1.1. Tautomeric transition of fluorescein derivatives.

Greater steric hindrance of the silyl protecting group should impart enhanced resistance to hydrolysis. Indeed, compound 1.3 exhibits better stability in aqueous solutions. I observed that the solutions of 1.3 do not promote any significant change in aqueous solutions for overnight. However, we evaluate compound 1.1 as a selective fluoride indicator since Compound 1.1 shows balanced sensitivity towards fluoride and water resistance. We observe that compound 1.1 promotes the formation of a yellow color from a colorless solution with a $\lambda_{\text{max}}$ of 480 nm upon the addition of fluoride. Other anions including chloride, bromide, iodide, acetate, cyanide, hydrogensulfate and nitrate (all as tetrabutylammonium salts) do not promote any color formation (Figure 1.1). Fluoride has been used for the prevention of tooth caries$^{1,16}$ and in the treatment of osteoporosis.$^{1,17}$ However, elevated levels of fluoride exposure could lead to adverse consequences including dental fluorosis, skeletal fluorosis and
even acute fluoride poisoning. In addition, the threat of bio-terrorism utilizing chemical warfare reagents has gained enormous attention. For example, organophosphofluoridate derivatives including soman and sarin, which are extremely lethal nerve gas agents (Figure 1.2) and produce fluoride upon reaction with moisture, are well-known.

![Scheme 1.2. Synthesis of silyl-protected fluorescein derivatives.](image)

Figure 1.2. G-type chemical warfare agents.

It would be very convenient to develop a methodology to detect those chemical warfare reagents with an inexpensive and simple detection method. We are currently exploring this with a company that fabricates receptors for sarin, soman and VX (proprietary information).

The concept of using silyl deprotection of a phenolic moiety in fluorogenic fluoride sensors has been reported very recently. In the first step, silyl cleavage promoted by
fluoride occurs to free a phenolic hydroxyl which undergoes a cyclization to afford a coumarin derivative in the second step.

![Absorbance vs. concentration plot of 1.1 (2.5 × 10^{-4} M) upon the addition of various anions (0.15 M triethanolamine buffer at pH 7.5 in 7:3 acetone/water) at 490 nm. 1.0 μmol of each anion was added into the stock solution (7 mL) containing compound 1.1 and then stirred for 1 min prior to the UV-Vis spectrum observed.](image)

Figure 1.3. Absorbance vs. concentration plot of 1.1 (2.5 × 10^{-4} M) upon the addition of various anions (0.15 M triethanolamine buffer at pH 7.5 in 7:3 acetone/water) at 490 nm. 1.0 μmol of each anion was added into the stock solution (7 mL) containing compound 1.1 and then stirred for 1 min prior to the UV-Vis spectrum observed.

However, saturation of the fluorescence intensity requires a prolonged period of approximately 2 h, even with excess amount of fluoride since the cyclization reaction in the fluorescence-producing second step takes place relatively slowly. Figure 1.4 clearly shows that the absorbance signal of 1.1 can saturate upon the addition of fluoride in the non-aqueous media in which the previously reported fluoride receptor (*vide supra*) was studied. To enhance the interaction rate between 1.1 and fluoride, an 8-fold excess of fluoride to each silyl group is added. The same experiment is performed in aqueous media (Figure 1.4) at physiological pH. We observe similar yellowish solution forming shortly after fluoride is added. Saturation is reached more slowly than in organic media (approximately 18 min), presumably due to the stronger hydration of fluoride ion.
This is the first example of the application of this technique used directly in buffered media. We find that \chem{1.1} possesses high affinity towards fluoride over other anionic interferents present even in an excess amount either in DCM or in aqueous media.

**Figure 1.4.** (top) \chem{1.1} (1.0 \times 10^{-5} \text{ M}) exhibits instant saturation of the absorbance intensity upon fluoride addition (8-fold excess to each silyl unit of \chem{1.1}) in DCM. (bottom) \chem{1.1} (3.5 \times 10^{-4} \text{ M}) with 20 equivalents of fluoride (6.9 \times 10^{-3} \text{ M}) in 0.24 M triethanolamine buffer at pH 7.5 in a mixture of 7:3 acetone/water.

Figure 1.5 shows that \chem{1.1} (1.0 \times 10^{-5} \text{ M}) interacts selectively with fluoride (1.6 \times 10^{-4} \text{ M}) while a 56-fold excess of other anions affords no significant increase in absorbance signals in
DCM solution. Not surprisingly, specific recognition of fluoride with a solution containing 1.1 is also achieved in aqueous media (0.24 M triethanolamine buffer at pH 7.5 7:3 acetone:water).

![Graph showing absorbance intensity changes of solutions containing 1.1 with various anions in DCM (left) and in a mixture of acetone and water (right).]

**Figure 1.5.** Absorbance intensity changes of solutions containing 1.1 with various anions in DCM (left) and in a mixture of acetone and water (right).

![Graph showing log fluorescence emission intensity vs. concentration plot.]

**Figure 1.6.** log fluorescence emission intensity vs. concentration plot shows that the solution of 1.1 (3.4 × 10⁻⁴ M) in DCM can monitor fluoride concentration (in tetrabutylammonium form) as low as 1.66 × 10⁻⁶ M.

Compound 1.1 (1.75 × 10⁻⁴ M) combined with fluoride (2.1 × 10⁻³ M) develops an intense yellowish color from a colorless solution. Other anionic interferent-containing
solutions (10-fold excess to fluoride) remain colorless under the same conditions. We also find that solutions of compound 1.1 (3.4 × 10⁻³ M) in DCM can detect fluoride concentration levels as low as 1.6 × 10⁻⁶ M by fluorescence emission spectroscopy (Figure 1.6).

![Scheme 1.3. Synthesis of polymerizable TBS-protected fluorescein dye (1.5).](image)

We have also synthesized a polymerizable TBS-protected fluorescein derivative (1.5). 4-Acrylamidofluorescein (1.4) is obtained by the reaction of commercially available 4-aminofluorescein with acryloyl chloride according to the previously reported procedure. Upon protection of hydroxyls with t-butyldimethylsilyl chloride, compound 1.4 furnishes a polymerizable fluoride indicator (1.5). We are continuing to study applications of this polymerizable compound.

1.3. Conclusion

I have synthesized silyl-protected fluorescein derivatives and evaluated their application as a selective receptor for fluoride in mixed aqueous neutral buffer solution. Compound 1.1 can be easily made via a simple one step reaction in a high yield. Compound 1.1 shows
excellent selectivity towards fluoride over other interfering anionic compounds in aqueous as well as organic media by spectroscopic detection.

1.4. Experimental Section

Synthesis of compound 1.1; fluorescein (1.5g, 4.5 mmol), imidazole (1.23g, 18 mmol), and t-butyldimethylsilyl chloride (2.72g, 18 mmol) are combined in 5 mL of dry DMF. The solution is stirred at 65 °C for 24 h under N₂. The viscous liquid is dissolved in 100 mL EtOAc and the organic layer washed with H₂O (50 mL × 6), dried with MgSO₄, and filtered. The solvent is evaporated in vacuo to afford a crude product. For further purification, column chromatography is performed with EtOAc and hexanes (2:8 to 3:7). The fraction isolated is dried in vacuo to afford 2.18g of 1.1 (86 %). Analytical data for 1.1: ¹H NMR (300 MHz, CDCl₃) δ (ppm): 0.23 (s, 12H), 0.99 (s, 18H), 6.52 (dd, J = 8.6 and 2.3 Hz, 2H), 6.64 (d, J = 8.6 Hz, 2H), 6.73 (d, J = 2.3 Hz, 2H), 7.19 (d, J = 7.3 Hz, 1H), 7.59-7.70 (m, 2H), 8.02 (d, J = 6.87 Hz, 1H); ¹³C NMR (300 MHz, CDCl₃) δ (ppm): -4.21, 18.41, 25.81, 83.49, 107.83, 112.26, 116.80, 124.21, 125.15, 127.06, 129.19, 129.81, 135.13, 152.50, 153.38, 157.78, 169.67; MALDI-MS m/z (dithranol matrix) calculated for C₃₂H₄₀O₅Si₂ 560.2 M⁺, found 561.5 [M+H]⁺.

Synthesis of compound 1.2; To the solution of fluorescein (2.1 g, 6.3 mmol) and imidazole (1.72 g, 25.3 mmol) in 150 mL of dry DMF is added triisopropyl silyl chloride (4.0 mL, 18.7 mmol). After the mixture is stirred for 16 h at room temperature, the solvent is evaporated in vacuo. The resultant mixture is combined with 200 mL of distilled water, extracted with EtOAc (200 mL + 150 mL × 3). The organic layers are combined, washed with saturated NaCl solution (200 mL) and dried with MgSO₄ to afford crude 1.2. For further purification, column chromatography is performed with a mixture of hexanes, toluene and
EtOAc (8:1:1) to afford 562.5 mg of 1.2 (13.8 %). Analytical data for 1.2: $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ (ppm): 1.10 (d, $J = 6.68$ Hz, 36H), 1.26 (m, 6H), 6.55 (dd, $J = 8.6$ and 2.3 Hz, 2H), 6.63 (d, $J = 8.6$ Hz, 2H), 6.76 (d, $J = 2.3$ Hz, 2H), 7.17 (d, $J = 6.7$ Hz, 1H), 7.63 (m, 2H), 8.01 (d, $J = 6.48$ Hz, 1H).

Synthesis of compound 1.3; To the solution of fluorescein (3.0 g, 9.0 mmol) in 10 mL of dry DMF are added imidazole (2.464 g, 36.1 mmol) and $t$-butyldiphenylsilyl chloride (7.0 mL, 26.9 mmol). The mixture is stirred for 16 h at 65 °C under N$_2$ and poured in 100 mL of H$_2$O. The yellowish suspension in the aqueous solution is recovered by filtration and washed with H$_2$O again. The solid materials are dissolved in diethyl ether, dried with Na$_2$SO$_4$ and filtered. The solvent is removed by evaporation in vacuo to afford crude 1.3. For further purification, column chromatography is performed with EtOAc and hexanes (2:8) to attain 6.936 g of 1.3 (95 %). Analytical data for 1.3: $^1$H NMR (250 MHz, CDCl$_3$) $\delta$ (ppm): 1.08 (s, 18H), 6.37 (dd, $J = 8.7$ and 2.3 Hz, 2H), 6.43 (d, $J = 8.7$ Hz, 2H), 6.62 (d, $J = 2.3$ Hz, 2H), 7.06 (d, $J = 6.93$ Hz, 1H), 7.38 (m, 12H), 7.54 (m, 2H), 7.69 (d, $J = 6.9$ Hz, 8H), 7.91 (d, $J = 7.4$ Hz, 1H).

Synthesis of compound 1.5; 4-Acrylamidofluorescein (1.4) is synthesized according to the previously reported procedure.$^{1,19}$ 4-acrylamidofluorescein (800 mg, 2.0 mmol), imidazole (1.2 g, 8.0 mmol) and $t$-butyldimethylsilyl chloride (543 mg, 3.6 mmol) are dissolved in 5 mL of dry DMF. The mixture is stirred for 17 h under N$_2$. To the solution is added 100 mL of EtOAc. The organic layer is washed with H$_2$O (75 mL × 5), dried with MgSO$_4$, filtered and evaporated in vacuo. The resultant mixture is purified by column chromatography (EtOAc:Hexanes 6:4) to attain 392 mg of 1.5 (31.2 %). Analytical data for 1.5: $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ (ppm): 0.21 (s, 12H), 0.93 (s, 18H), 5.84 (d, $J = 9.3$
Hz, 1H), 6.33 (d, J = 17.1 Hz, 1H), 6.47 (dd, J = 17.1 and 9.3 Hz, 1H), 6.72 (m, 6H), 7.27 (d, J = 7.8 Hz, 1H), 7.89 (d, J = 7.8 Hz, 1H), 8.44 (s, 1H), 10.63 (s, 1H). MALDI-MS with dithranol m/z calculated for C_{35}H_{43}NO_{6}Si_{2} 629.26 for M+, found 630.84 [M+H]^+.

All UV-Visible spectra are recorded on a Spectramax Plus 384 spectrophotometer (Molecular Devices).

1.5. References


1.5 Black, C. B.; Andrioletti, B.; Try, A. C.; Ruiperez, C.; Sessler, J. L. J. Am. Chem. Soc. 1999, 121, 10438


1.15 See “1.4 Experimental Session”


2.1. Aminothiols in Human Health

Elevated levels of homocysteine (Hcy) in human plasma are associated with severe diseases. For instance, hyperhomocysteinemia is involved in cardiovascular disease, Alzheimer’s disease, complications during pregnancy and osteoporosis. Homocystinuria has also been linked to mental retardation and neurodegeneration. Cysteine deficiency is associated with numerous disorders including liver damage, slowed growth, hair depigmentation, skin lesions and fat and muscle loss. In addition, elevated levels of both Cys and Hcy are related to neurotoxicity. Glutathione (GSH) which is a tripeptide amino acid covalently linking cysteine, glycine and glutamic acid residues is known as a beneficial antioxidant.

2.2. The Detection of Hcy and Cys by Thiazolidine and Thiazinane Formation

It has been long-known that Cys can react with carbonyls. Aldehydes readily react with \(N\)-terminal Cys to form thiazolidine products. Thiazolidine formation has been employed to selectively label proteins with \(N\)-terminal Cys residues and in stereospecific ligations of peptides. Oxazolidine formation, in contrast, from the reaction of an amino alcohol and an aldehyde, is not highly favored in aqueous media. It has been found that thiazolidines are \(10^4\) times more stable than oxazolidines.

Lippard et al. initially obtained compound 2.6 via a multi-step synthesis. Recently, a co-worker in the Strongin group synthesized 2.6 in one step via a Reimer-Tieman reaction using commercially available fluorescein.

* Reproduced in part with permission from *Journal of the American Chemical Society*, 2005, volume 127, pages 15949-15958; Copyright 2005 American Chemical Society.
Figure 2.1. Some bioactive aminothiols.

Scheme 2.1 The reaction of 2.6 with Cys and Hcy to afford bis-thiazolidine (n=1) and bis-thiazinane (n=2).

Our group observed that a solution containing 2.6 (1.0 × 10⁻³ M) changes color from bright yellow to brownish-orange upon the addition of Cys or Hcy (1.0 × 10⁻⁶ M) in 0.01 M pH 9.5 carbonate buffer. In addition, similar observations were also attained when the solution of 2.6 (1.0 × 10⁻³ M) was co-spotted with or without various analytes (1.0 × 10⁻³ M) on C₁₈-bonded silica plate.

Hcy possesses an oxidizing thiol moiety which easily forms a disulfide bond in the presence of O₂. Only less than 1% of Hcy exists in a reduced form (free Hcy) in a biological system while the rest of Hcy is covalently bound to other sulfhydryl groups (e.g. Hcy, Cys and GSH) as disulfide forms. Therefore, the concentration of total Hcy (tHcy) has often been
used to indicate the concentration of Hcy in biological samples. The level of tHcy is found to be less than $1.2-1.5 \times 10^{-5}$ M in healthy human plasma, which is typically 20-30 times less than that of Cys. Absorbance changes in solutions of 2.6 were clearly observed in the range of $10^{-5}$-$10^{-6}$ M of Cys with a red-shift in wavelength of 25 nm (Figure 2.3).

**Figure 2.2.** (Left) solutions containing 2.6 with and without various analytes (a) control, (b) Cys, (c) Hcy, (d) bovine serum albumin, (e) glycine and (f) $n$-propylamine (Right) observations on C18-bonded silica plates exposed to visible and UV light.

**Figure 2.3.** Absorbance vs concentration plot for solutions containing 2.6 ($2.5 \times 10^{-6}$ M, pH 9.5 carbonate buffer) with added Hcy (○) or Cys (▲). The addition of Hcy and Cys ($5.0 \times 10^{-6}$ M) to 2.6 showed an absorbance decrease at 480 nm. An increasing absorbance was monitored at 505 nm with a Hcy or Cys concentration range from $1.0 \times 10^{-5}$ to $4.0 \times 10^{-5}$ M under the same conditions.

$^1$H NMR and mass spectroscopic studies confirmed the formation of thiazolidines and thiazinanes. Solutions of 2.6 in the presence of an equimolar amount of Hcy or Cys
showed similar absorbance changes while only a minimal change was monitored in the solutions of 2.6 with an excess of other analytes (8.0 \times 10^{-4} \text{ M}). Less than 15\% of an absorbance change was observed at 480 nm without any wavelength shift.

\[
\text{RS}' + \text{RS}^- \rightleftharpoons \text{RSSR}^- \quad (1)
\]

\[
\text{RS}' \rightleftharpoons \cdot \text{CR} \quad (2)
\]

**Figure 2.4.** Equilibrium reactions to form reducing radicals.

**Scheme 2.2.** Carbon-centered radical formation of aminothiols by intramolecular hydrogen abstraction.

Amines (glucosamine HCl and \textit{n}-propylamine), thiols (methionine, mercaptoethanol and GSH) and amino acids (glutamine, serine, glycine and glutamic acid) were included in the screening. This corroborates the high selectivity of 2.6 towards Hcy and Cys over other possible interferents.

In addition, a spectral change in deproteinized human plasma solutions containing 2.6 upon the addition of healthy-to-abnormal level of Cys or Hcy was also monitored via UV-Vis absorbance or fluorescence quenching.\textsuperscript{2,11}

**2.3. The Selective Detection of Hcy by MV\textsuperscript{2+} and Fluorone Black**

Thiyl radicals can be attained via the oxidation process of thiols in nature.\textsuperscript{2,12} The oxidizing thiyl radicals can either form the reducing \textit{\alpha}-amino carbon-centered radical via an
intramolecular hydrogen abstraction or react with a thiolate anion to afford a reducing disulfide radical anion (Figure 2.4). It has been reported that the $\alpha$-amino carbon-centered radical of Cys and GSH could be formed by the intramolecular hydrogen abstraction of a thyl radical.

**Figure 2.5.** The solution of 2.7 shows the selective response to Hcy; (a) control, (b) Cys, (c) Hcy and (d) GSH.

**Figure 2.6.** The electron transfer of the reducing $\alpha$-amino carbon-centered radical of Hcy to 2.7 and 2.8.

Recently, Zhao et al. reported that the formation of the $\alpha$-amino carbon-centered radical of Hcy should be more favorable due to a favored five-membered ring transition state during the intramolecular hydrogen abstraction process (Scheme 2.2).$^{2,13a}$

Methyl viologen dication (1,1'-Dimethyl-4,4'-bipyridinium dication; MV$^{2+}$ 2.7) is a moderate electron acceptor. When colorless 2.7 captures an electron, MV$^{3+}$ exhibits characteristic blue color. 2.7 was used in the study of the equilibrium kinetics of the reducing
α-amino carbon-centered radicals and the reducing disulfide radical anions derived from the thyl radicals of GSH, Cys and Hcy at pH 10.5.2.13

The concentration of thiolate anion is only minimal to that of sulfhydryl group (pKₐ ≈ 9.5) at physiological conditions. The predominant hydrogen abstraction process should warrant the selectivity for Hcy over other aminothiols. In addition, the reducing α-amino carbon-centered radicals should be readily formed under basic conditions since they can be captodatively stabilized by the rearrangement of the deprotonated amine moiety. Thus, no selectivity among GSH, Cys and Hcy would be observed.

![Figure 2.7](image)

**Figure 2.7.** UV-Vis spectra of the solutions of 2.8 with or without aminothiols.

We therefore envisioned that the selective detection of Hcy would be possible via the redox reaction of 2.7 at physiological conditions. We observed that heating the colorless solution containing 2.7 (4.0 mM in 0.1 M Tris buffer at pH 7.5) to reflux (5 min) turns to a bluish color upon addition of Hcy (17.0 mM). In contrast, no color formation with either GSH or Cys was observed at the same conditions. The new appearance of absorbances at 398 nm and 605 nm was monitored by UV-Vis spectrophotometer.2.14 We have also investigated
the commercially available fluorone black as an electron acceptor. The solution of 2.8 ($1.0 \times 10^{-5}$ M) in a mixture of methanol and water (7:3) at pH 7.3 exhibit an absorbance increase at 510 nm upon the addition of 10-fold excess Hcy ($1.0 \times 10^{-4}$ M) at room temperature. However, equimolar amount of other aminothiols including GSH, Cys and penicillamine (Figure 2.1) promote relatively smaller increase of an absorbance (Figure 2.7).

![UV-Vis spectra](image)

**Figure 2.8.** UV-Vis spectra of solutions containing 2.8 ($1.0 \times 10^{-5}$ M, pH 7.3 15 mM phosphate buffer, 3:7 H$_2$O:MeOH) with added PPh$_3$ ($5.0 \times 10^{-4}$ M) and aminothiols ($1.0 \times 10^{-5}$ M).

No significant change is observed in the presence of other amino acids (glycine, serine, methionine, alanine, glutamine, lysine, threonine and arginine) and homocystine (a disulfide form of Hcy).$^{2,14}$ We found that the added PPh$_3$ solutions containing 2.8 show the similar pattern of an absorbance increase at 510 nm as they do with the addition of Hcy. Interestingly, a phosphine is often used as a reductant to reduce biological disulfide bonds. $^{31}$P NMR study showed that the formation of triphenylphosphine oxide (TPPO) is promoted when the solution of 2.8 is combined with PPh$_3$. The formation of TPPO was not detected in the PPh$_3$
solution without added 2.8 for 19 h. In fact, it is well-known that TPPO can be formed via the radical cation of PPh₃. No spectral change is observed in the solutions containing fluorescein with added Hcy or Cys.

These observations suggest that the spectral changes of 2.8 upon the addition of aminothiols are caused by the formation of a semiquinone radical in 2.8.

The electron transfer of the reducing α-amino carbon-centered radicals to 2.8 raise the pKₐ of 2.8 to afford an absorbance increase at 510 nm. Identical spectral changes can be observed in the solutions of 2.8 under basic conditions (Figure 2.6). PPh₃ could compete with thiols reducing 2.8. Since Hcy can act as the best reducing agent among other aminothiols listed above (Figure 2.1), the controlled PPh₃ addition would suppress the interactions of 2.8 with other aminothiols except for Hcy. Indeed, we found that at least 45-fold excess PPh₃ to the solution of 2.8 is required to inhibit any absorbance changes from aminothiols except for Hcy which exceptionally affords a significant absorbance increase (Figure 2.8). In addition, we found that solutions of 2.8 (1.0 × 10⁻⁵ M) with added PPh₃ (1.5 × 10⁻³ M) can allow us to selectively detect a physiological level of Hcy (1.0 × 10⁻⁵ M) in the presence of 30-fold excess of Cys as compared to the spectrum of Hcy alone under identical conditions.²¹⁴ We successfully quantified Hcy in deproteinized human plasma samples via the standard addition method. The linear working range for Hcy was from 0 to 15 µM.²¹⁴

2.4. Introduction of HPLC Post-Column Detection of Aminothiols

The determination of biological thiols has been performed by high-performance liquid chromatography (HPLC) in conjunction with analyte derivatization. Most of the derivatization methods proceed by formation of disulfide bonds or electrophilic alkylations of activated halides with thiol-containing compounds. Most derivatization reagents are not
selective towards a specific thiol and have several limitations and drawbacks. For example, \(N\)-substituted maleimide (2.10) requires the complete conversion to hydrolysis products. Otherwise the peak from the reaction intermediates could be monitored. The self-hydrolysis of 2.10 could significantly compete with the thiol modification above pH 8.

![Chemical structures](image)

**Figure 2.9.** Common thiol-derivatization reagents.

![Chromatograms](image)

**Figure 2.10.** Chromatograms of mixtures of Cys and Hcy. Inset; (a) histidine, methionine and glutamine and (b) lysine, glycine and serine. Conditions: Mobile phase: 100 % of HPLC grade water; mobile phase flow rate: 1.5 ml/min; reagent: 2.8 \((1.25 \times 10^{-5} \text{ M})\) in 50/50 \((\text{v/v})\) mixture of MeOH and aqueous carbonate buffer \((0.25 \text{ M, pH 9.5})\); reagent delivery pressure: 40 psi; reactor temperature: 80 °C.; detector wavelength: 505 nm.
Monobromobimane (2.11) is unstable at room temperature. In addition, the choice of buffer components is limited due to their possible nucleophilic reactions with 2.11. The sample preparation with 7-fluoro-2,1,3-benzoxadiazole (2.12) or dansylaziridine (2.13) requires a prolonged reaction time (1 h at 60 °C). o-Phthalaldehyde (2.14) produces photo-unstable thiol-adducts and undergoes cross-reactivity with primary amines. Recently, I reported that a boronic acid functionalized rhodamine dye can serve in the detection of mono- and oligosaccharides via HPLC post-column system. I herein describe the application of our library of aminothiol selective reagents in an HPLC post-column detection system. In addition, our derivatization reagents absorb at visible wavelengths (> 500 nm) which are preferred because many biological interferents absorb below 400 nm.

![Figure 2.11](image)

**Figure 2.11.** Chromatograms of mixtures of Cys and Hcy using 2.6 as post-column reagent. (a) separation using HPLC grade water as mobile phase. (b) using TFA 6.5 mM to improve separation.

### 2.5. Experimental Results of HPLC Post-Column Detection of Aminothiols

In Figure 2.10 two sharp peaks are observed upon the injection of Cys and Hcy (28.8 nmol each) using the HPLC post-column system with 2.8 as a chromogenic reagent. Interestingly, equimolar amounts of six different amino acids including histidine, methionine,
glutamine, lysine, glycine, and serine shown on Figure 2.10 (inset) do not produce any absorbance change at the same wavelength. As shown in these chromatograms, Cys and Hcy can be selectively detected without any significant interference from other amino acids.

Compound 2.6 is also used as reagent for HPLC post-column detection. In Figure 2.11a, two sharp peaks are observed when 43 nmol each of Cys and Hcy are injected.

![Chromatogram of human plasma using 2.6 as post-column reagent](image)

**Figure 2.12.** (a) Chromatogram of a sample of human plasma using 2.6 as post-column reagent. Lyophilized plasma was reconstituted with water (1 mL) and mixed with 100 µL of TFA and then diluted five times with water and filtered before injection. The mobile phase flow rate was 1.0 mL/min. (b) chromatogram of a sample of human plasma spiked with Hcy using 2.6 as post-column reagent. The reconstituted plasma (1 mL) was mixed with 100 µL of TFA was diluted ten times with water, spiked with 9.5 nmol of Hcy and filtered before injection.

To obtain better separation, we switch the mobile phase from 100 % water to aqueous TFA (6.5 mM) (Figure 2.11b). Other conditions for both systems are as follows: mobile phase flow rate: 1.5 mL/min; reagent: 2.6 (6.4 × 10⁻⁶ M in carbonate buffer 0.125 M, pH 9.5); reagent delivery pressure: 40 psi (a: 100% HPLC water), 60 psi (b: aqueous TFA solution); reactor temperature: 80 °C; detector wavelength: 510 nm.

I also studied the same post-column reagent for the detection of Hcy in human plasma. 2.6 (3.2 × 10⁻⁶ M) is prepared in 0.125 M of pH 9.5 aqueous carbonate buffer and delivered
by 40 psi of pressure. The mobile phase is 6.5 mM aqueous TFA with a flow rate of 1.0 mL/min. The reactor temperature is set to 80 °C. Absorbance is monitored at 510 nm. 1 mL of human plasma (reconstituted) is mixed with 100 μL of TFA and passed through an Alltech Polypure ll 0.2 μm filter without any further purification. The plasma sample is freshly prepared before the HPLC injection.

**Figure 2.13.** Chromatogram of a mixture of Cys and Hcy (85 nmol each) using 2.7 as post-column reagent.

We observe that the peak corresponding to spiked free Hcy in plasma clearly appears at 5.3 min. The injection of twice the amount of plasma sample alone does not afford any significant peaks at 5.3 min. (Figure 2.12)

In addition, the peak for Hcy is well resolved from that of Cys (3.6 min) under the same conditions.219

Using 2.7 as a post-column detection reagent results in selective detection of both Cys and Hcy but is qualitatively less sensitive than the other reagents tested. Conditions: mobile phase: TFA (0.01 M); mobile phase flow rate: 1.5 ml/min; reagent: 2.7 (0.01 M) in 0.25 M carbonate buffer, pH 9.5; reactor temperature: 80°C; detector wavelength: 610 nm.
2.6. Conclusion

I have demonstrated the selectivity of compounds 2.6, 2.7, and 2.8 towards aminothiols and their potential utility for the selective detection of aminothiols in an HPLC post-column system.

2.7. Experimental Section

The general reaction conditions and equipment used in our post-column detection methods are as follows: An RDR-1 reagent delivery/reaction module (Timberline) equipped with a 110B solvent delivery module (Beckman) and a SpectroMonitor 3200 UV-Vis detector (LDC/Milton Roy), using a LiChrospher 100 RP-18 end-capped column (4.6 mm × 250 mm, Alltech Associates Inc.), particle diameter of 5 μm. The RDR-1 unit contains a reagent reservoir, a mixing tee, and a teflon reaction coil (0.02" ID × 3 m, with a nominal volume of 0.6 mL) cast in a tin alloy heating block connected to a temperature controller. Other conditions specific to the different experiments are described above.

All UV-Visible spectra are recorded on a Spectramax Plus 384 spectrophotometer (Molecular Devices).

2.8. References


2.10 Synthesized by Xiangyang Xu.


2.19 See “Appendix H”
CHAPTER 3
SELECTIVE SUGAR DETECTION USING A HPLC POST-COLUMN SYSTEM WITH A RHODAMINE-BASED BORONIC ACID*3.1

3.1. Introduction

Novel boronic acid-appended rhodamine dye (3.1) promotes the detection of mono- and oligosaccharides via an HPLC post-column system with a standard UV-Vis detector. These saccharides include fructose, glucose, maltotriose, maltohexaose and sialic acid. The use of selective, reversible binding agents in automated HPLC assays should allow for improved monitoring of specific analytes as well as material recovery.

![Figure 3.1](image)

**Figure 3.1.** Rhodamine-based boronic acid (3.1)

3.2. The Drawbacks of Common Saccharide Detection Methods

The monitoring of specific saccharides has been considered a great challenge in a molecular recognition due to their conformational diversity and similar functionality. The direct measurement of saccharides with UV-Vis detectors below 210 nm limits solvent choice and requires ultra-pure solvents. Measuring refractivity is very common for the detection of carbohydrates. However, it is not highly sensitive for the low concentration of samples (≤10 nmol).

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In addition, it is much liable to the change of solvent composition and column temperature. Electrochemical detection by pulsed amperometric detection (PAD) often requires elevated pH conditions (>pH 13). Mass spectrometry detection, coupled with chromatographic separations, requires very expensive and customized equipments. Radioactive labeling has also been used for radioactivity monitoring. It requires the handling of dangerous radioactive substances. Most recently, Evaporative light scattering detection (ELSD) has gained great attention for chromatographic sugar detection. However, low molecular weight compounds could have a drawback of the potential evaporation along with the mobile phase.

![Figure 3.2. Flow diagram of HPLC post-column system.](image)

### 3.3. The Detection of Saccharides via HPLC Pre/Post-column System

The direct measurement of carbohydrates with common optical instruments (UV-Vis or fluorescence spectrophotometer) is difficult since carbohydrates do not contain fluorogenic and/or chromogenic functionalities. Recently, chemical derivatization, also called reaction detection (transforming analytes to spectrophotometrically detectable forms), has been of
Carbohydrates may be derivatized with chromopohores or fluorophores prior to separation; however, this procedure can hamper separation via the appendage of moieties with intrinsically similar properties. Post-column derivatization techniques have thus attracted great attention in carbohydrate analysis.\textsuperscript{3.2,3.3,3.4}

The most important advantages of a HPLC post-column detection system are that the analytes, for instance, monosaccharides are separated through the appropriate column without destroying their original forms. In addition, the previously published separation procedures for analytes could be adopted.

Classical methods for carbohydrate derivatization include the conversion of carbohydrates to furfural derivatives. Upon treatment of carbohydrates with strong mineral acids, carbohydrates are converted into furfural which reacts with a proper chromogenic reagent (for instance, resorcinol,\textsuperscript{3.5} orcinol,\textsuperscript{3.6} phenol,\textsuperscript{3.7} etc.). In addition, periodate oxidation of carbohydrates produces aldehydes which are combined with other reagents such as amines and $\beta$-diketones or $\beta$–oxo esters compounds to attain pyridine derivatives by the Hantzsch pyridine synthesis.\textsuperscript{3.8} The resultant pyridine derivatives can be monitored in the UV-Vis region. However, the formation of pyridine derivatives might be hindered by the excess periodates which has to be reduced by the addition of sodium arsenite.\textsuperscript{3.9} Fe(III) or Cu(II) can be reduced by reducing carbohydrates to form Fe(II) or Cu(I) respectively. The use of the reagents which only chelates with the lower oxidation state of metal can be applied for the reducing carbohydrate derivatizations. For instance, the mixture of Cu$^{2+}$ and 2,2’-bicinchoninate has been used for this purpose.\textsuperscript{3.10} The direct reaction of derivatization reagents including alkylamines,\textsuperscript{3.11} 2-cyanoacetamide\textsuperscript{3.12} and benzamidines\textsuperscript{2.13} with reducing carbohydrates has also been studied.

However, many older automated detection systems based on a specific, post-column
reaction of sugars require specially designed acid-resistant reagent delivery and detection systems, cause excessive peak broadening and are incompatible with certain solvents used for separations.\textsuperscript{3.3,3.4}

![Scheme 3.1. Synthesis of 3.1](image)

More recently, milder reactions with fluorogens have been reported.\textsuperscript{3.14} The reagents currently used in post-column derivatization are typically selective for a family of compounds (for instance, aldoses, ketoses, uronic acids, aminosugars, etc.). The reactions are irreversible. The use of more selective synthetic chromogenic and/or fluorogenic receptors as post-column detection agents could significantly improve the analysis of a component of interest.

Moreover, if binding is via non- or reversible covalent interactions, recovery of expensive or scarce biomolecules should be possible. Herein we report the use of new rhodamine-derived boronic acid 3.1 as a post-column derivatization agent in an automated HPLC method towards the detection of a variety of sugars and related biomolecules.

### 3.4. Results

Recently, we reported that xanthene dye-functionalized boronic acids can be formed \textit{in situ} from resorcinarenes.\textsuperscript{3.15} These compounds were used towards the detection of several mono- and oligosaccharides.\textsuperscript{3.15,3.16} It is well-known that boronic acids can react with the diol moieties of sugars to form cyclic esters using strong covalent bonds.
The formation of cyclic ester is pH-dependant in aqueous media. Since phenylboronic acid has a pKₐ of ≈8.8, the significant formation of cyclic esters requires alkaline conditions to attain tetrahedral boronate esters. To monitor sugars at physiological pH, a lower pKₐ value of boronic acid is required.

Lowering the pKₐ of boronic acids can be attained in the modification of boronic acids involving electron-withdrawing effects. For examples, 4-carboxy-3-nitrophenylboronic acid and N-methylpyridiniumboronic acid exhibit pKₐs of 7.0 and 4.4 respectively. Wulff has introduced the concept of an intermolecular B-N interaction which substantially lowers the pseudo pKₐ of boronic acids. This concept has recently been adopted by S. Shinkai and co-workers in the development of saccharide sensor, 9,10-Bis[N-methyl-N-(o-boronobenzyl)amino]methyl] anthracene, which shows the pKₐ of ≈5.0.

In the current work, we functionalize a related rhodamine scaffold due to its high molar absorptivity in the visible region as well as the potential for favorable boron-nitrogen
interactions to promote sugar-boronate formation.\textsuperscript{3,25} Compound \textbf{3.1} is readily synthesized via a reductive amination reaction between commercially available rhodamine 110, 2-formylphenylboronic acid and NaBH\textsubscript{4} (Scheme 3.1).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3_3.png}
\caption{UV-Vis spectra of solutions of \textbf{3.1} (1.64 \times 10^{-5} M, 0.16 M pH 9.5 carbonate buffer in 1:2 MeOH:H\textsubscript{2}O) upon titration with D-fructose. The final concentration of fructose was 8.33 \times 10^{-4} M.}
\end{figure}

It exhibits selectivity for fructose over glucose in solution, in keeping with the behavior of most boronic acid derivatives.\textsuperscript{3,25} We observe a blue shift of the $\lambda_{\text{max}}$ from 550 nm to 530 nm upon addition of D-fructose or D-glucose (Figure 3.3). This observation of a blue shift in UV-Vis spectrum could be explained by the formation of partial positive charge on the nitrogen moiety of compound \textbf{3.1} through the B-N interaction. The apparent equilibrium constant for the interaction of \textbf{3.1} and D-fructose is 3,806 $M^{-1}$; for D-glucose the value is 375 $M^{-1}$.

Fructose is a 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\textsuperscript{3,26} and insulin resistance.\textsuperscript{3,27} Non-enzymatic glycosidation products form more
rapidly from fructose than from glucose.\textsuperscript{3.28} 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 is hampered, due, in large part, to excess (ca. 100-fold) glucose.\textsuperscript{3.29}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fructose_glucose_chromatograms.png}
\caption{(Top) chromatogram of a 1:1 mixture of D-fructose and D-glucose (20.0 μg, λ=560 nm). (Bottom) chromatogram of a mixture of D-fructose (4.5 μg) in the presence of a 100-fold excess of D-glucose.}
\end{figure}

Levels of fructose reported vary among laboratories and on the technique employed, including enzymatic detection.\textsuperscript{3.30} We can monitor mixtures of D-glucose and D-fructose via automated post-column HPLC detection with \textsuperscript{3.1}. We clearly observe a peak for D-fructose even in the presence of a 100-fold excess of D-glucose (Figure 3.4).
Figure 3.5. Continuous variation plot (Job’s plot) indicates 1:1 stoichiometry in the interaction of 3.1 with fructose.

Figure 3.6. UV-Vis spectra of solutions of 3.1 (4.2 × 10⁻⁵ M, 0.25 M pH 10.4 carbonate buffer in 1:1 MeOH:H₂O) with oligosaccharides. The final concentration of oligosaccharides was 8.0 × 10⁻³ M.

The stoichiometry of the interaction of compound 3.1 with fructose is measured by the continuous variation method (Job’s plot). The result clearly shows a 1:1 complex of compound 3.1 and fructose in 0.125 M pH 9.5 carbonate buffer in 1:1 MeOH:H₂O solution (Figure 3.5).
NMR studies of $^{13}$C-labelled D-fructose could be used to identify what isomers are involved in the complex formation with 3.1.$^{3,31}$

![Structure of maltotriose (n=1) and maltohexaose (n=4).](image)

**Figure 3.7.** Structure of maltotriose (n=1) and maltohexaose (n=4).

![Chromatogram of a 1:1 mixture of (A) maltohexaose and (B) maltotriose (80 μg, $\lambda=560$ nm).](image)

**Figure 3.8.** Chromatogram of a 1:1 mixture of (A) maltohexaose and (B) maltotriose (80 μg, $\lambda=560$ nm).

The new post-column detection method is also applicable to the monitoring of oligosaccharides. Current oligosaccharide colorimetric HPLC detection methods typically require prior complete hydrolysis to monosaccharides or pre-column covalent attachment to a chromophore.$^{3,32,3,33}$

The classical color tests used for HPLC post-column detection of monosaccharides cannot be used to directly detect oligosaccharides containing more than three residues.$^{3,32}$ The colorimetric response is only related to the molar concentration of oligosaccharide, not the
concentration by weight. Recently, we can apply these findings to the colorimetric HPLC
detection of oligosaccharides. Under similar conditions (reactor temperature set to 94 °C) to
those used for the monosaccharides above, mixtures of maltotriose and maltohexaose (80 g) can
be monitored (Figure 3.8).

![Figure 3.9. Structures of N-acetylneuraminic acid and GM1 ganglioside.](image)

This observation of higher oligomer detection can be confirmed by previous studies. The interaction of oligosaccharides with arylboronic acids shows an increase of binding
constants with higher oligomers. The formation of boronate esters occurs with the diol of the
reducing sugar termini. The binding of oligosaccharides is enhanced by the secondary CH-π
interactions with arylboronic acids. The higher oligomers possess greater flexibility in their
structure and can easily achieve the proper conformation to interact with the receptor molecule.
In addition, we reported that boronic acid-functionalized xanthenes allowed us to generate strong colorimetric responses for larger oligosaccharides.\textsuperscript{3,15}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{gm1_chromatogram}
\caption{Chromatogram of GM1 (220 μg, λ=520 nm).}
\end{figure}

Sialic acids are found in glycoproteins, glycopeptides and glycolipids. They play important roles in cellular communication in human body. Unbalanced sialic acid levels can lead to alterations in cell adhesion, which is implicated in certain cancers and graft rejection. An increase in the levels of both soluble and cellular sialic acid can be a marker for cancer diagnosis.\textsuperscript{3,36} Gangliosides are a natural source of sialic acids. The function of sialic acids in gangliosides is presently not completely understood.\textsuperscript{3,37} Improved methods of analysis would aid in the elucidation of their biochemistry.

The analysis of the sialic acid content of gangliosides is challenging. The common Warren\textsuperscript{3,38} and Svennerholm\textsuperscript{3,39} color tests (the most commonly used assays for sialic acid\textsuperscript{3,40}) employ high temperatures, harsh reagents, and suffer from interference with other carbohydrates.

In addition, many assays require prior liberation of the sialic acids which often results in decomposition.\textsuperscript{3,41} In the case of enzymatic hydrolysis, incomplete sialic acid liberation can be a
problem. Compound 3.1 can be used for the post-column HPLC detection of N-acetylneuraminic acid, the most commonly occurring sialic acid and GM1 (Figure 2.10), a commercially available ganglioside. The retention times and detection limits for N-acetylneuraminic acid, GM1, and the other analytes described above are summarized in Table 3.1.

<table>
<thead>
<tr>
<th>carbohydrate</th>
<th>retention time (min)</th>
<th>LOD (μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-fructose</td>
<td>10.1</td>
<td>2.3</td>
</tr>
<tr>
<td>D-glucose</td>
<td>12.0</td>
<td>7.1</td>
</tr>
<tr>
<td>maltotetraose</td>
<td>6.0</td>
<td>30.1</td>
</tr>
<tr>
<td>maltotriose</td>
<td>7.3</td>
<td>35.8</td>
</tr>
<tr>
<td>N-acetylneuraminic acid</td>
<td>7.2</td>
<td>45.4</td>
</tr>
<tr>
<td>GM1</td>
<td>5.5</td>
<td>26.8</td>
</tr>
</tbody>
</table>

3.5. Conclusion

We have synthesized new boronic acid probe 3.1 and demonstrated its use as a detection agent in an automated post-column HPLC system. The selectivity of 3.1 for fructose promotes fructose monitoring in the presence of a large excess of glucose. The affinity of 3.1 for oligosaccharides and sugar-containing biomolecules such as GM1 allows for colorimetric monitoring upon chromatographic elution. We are continuing to study the synthesis and properties of chromophoric and fluorophoric chemosensors and supramolecular materials as HPLC post-column reagents.

3.6. Experimental Section

3.6.1. Synthesis of Compound 3.1

Rhodamine 110 (0.1 g, 0.27 mmol) and 2-formylphenylboronic acid (0.082 g, 0.54 mmol) are mixed in absolute EtOH (20 mL) and PhCH₃ (3.1 mL). A Dean-Stark trap is fitted and the
reaction mixture is heated at reflux overnight (18-24 hrs). After cooling, the solvent is removed in vacuo to afford a yellow oil. Anhydrous MeOH (25 mL) is added and NaBH₄ (0.041 g, 1.08 mmol, 4 equiv) is added over 5 min. The reaction is stirred at rt. for 2 hr, and poured into ice-water (10 mL). A small amount of saturated NaHCO₃ (5 mg) is added. The aqueous solution is extracted with DCM (3 × 50 mL). The aqueous layer is collected, dried, and filtered. The solvent is removed in vacuo to afford (0.13 g, 81%) of product. To obtain an analytical standard for sensing work, further purification is performed by reversed-phase HPLC using a Dynamax 60Å C18 (21.4 mm ID × 25 cm L) column with a flow rate of 5 mL/min. and a gradient of 70/30 MeOH/H₂O to 100% MeOH in 30 min. Data for 3.1: ¹H NMR (300 MHz, DMSO-d₆): 4.53 (s, 4H), 6.74-6.86 (m, 4H), 7.11-7.23 (m, 8H), 7.58-7.63 (m, 2H), 8.05 (d, J = 7.2 Hz, 2H), 8.54 (s, 2H). ¹³C NMR (500 MHz, DMSO-d₆): 22.07, 28.66, 28.95, 46.45, 97.17, 105.85, 114.91, 118.83, 122.48, 124.05, 124.33, 125.24, 126.38, 128.13, 128.83, 129.73, 150.87, 152.33, 154.98, 168.87. FAB-MS m/z (glycerol matrix) calcd for C₃₄H₂₈B₂N₂O₇ 598.22 M⁺, found 710.1[M + 2 C₂H₆O₂ – 4 H₂O]⁺.

3.6.2. Experimental Conditions
HPLC experiments are performed on a CM4000 multiple solvent delivery system (LDC/Milton Roy) and a SpectroMonitor 3100 UV-Vis detector (LDC/Milton Roy) using a 700CH carbohydrate column (6.5 × 300 mm, Alltech Associates Inc.). The column is maintained at constant temperature using a CH-30 column heater (Eppendorf). The post-column detection system consists of a He cylinder connected to a RDR-1 Reagent Delivery/Reaction Module (Timberline). The RDR-1 unit contains a pressurized reagent reservoir, a mixing tee, and a thermostated reaction block with a Teflon reaction coil (0.02 in ID × 1 mL) with a nominal volume of 0.2 mL. The HPLC column is connected to the RDR-1. The RDR-1 is attached to a
SpectroMonitor 3100 UV-Vis detector (LDC/Milton Roy). The column temperature is set to 85 °C and the temperature of the reaction block to 50 °C. Absorbance is monitored at 560 nm except for sialic acid and GM1 which are monitored at 520 nm. The reagent consists of a solution of 3.1 in 0.05 M buffer (pH=10.5, carbonates). The final concentration of 3.1 is 4.0 x 10⁻⁶ M. The reagent is introduced at a flow rate of 0.5 mL/min and the reactor temperature kept at 50 °C. The mobile phase is 100 % deionized H₂O. All UV-Visible spectra were recorded on a Spectramax Plus 384 spectrophotometer (Molecular Devices).

3.7. References


3.5 Roe, V. H. J. Biol. Chem. 1934, 107, 15.


4.1. Introduction

Resorcinarenes have been employed in a wide range of applications including molecular recognition, materials science, and supramolecular chemistry. Recently, we reported that strong color formation can occur upon heating of solutions containing resorcinarene macrocycles and its use for the sensing of saccharides. These colorimetric properties were initially observed by von Baeyer in 1872 when he synthesized resorcinarene macrocycles during his studies on the condensation reaction of resorcinol and benzaldehyde in the presence of acid. He observed that the resultant solution underwent a color change from red to violet upon the addition of base. However, further investigation of this observation has not been pursued. In this section, the origin of color formation in resorcinarene macrocycles is discussed in detail.

4.2. Results

Davis and Lewis of the Strongin group reported that the acid promoted condensation reaction of commercially available 4-formylphenyl boronic acid and resorcinol produced compounds and in one step in a combined yield of 91%. White crystalline solids were obtained via the separation of the two stereoisomers by fractional crystallization.

Colorless DMSO solutions (5.2 mM) containing crystalline compounds and upon standing for several hours at room temperature or for 1 min at 90 °C developed a color change from colorless to pinkish-purple.

Davis and Lewis found that the presence of oxygen or light was directly related to the color promotion of DMSO solutions of resorcinarenes.

* Reproduced in part with permission from Journal of the American Chemical Society, 2002, Volume 124, pages 5000-5009; Copyright 2002 American Chemical Society.
Initial studies showed that heated solution containing 4.1 in DMSO under oxygen-degassed condition or in the dark produced relatively decreased intensity in color.\textsuperscript{4.2} For example, heating a solution containing 4.1 (5.2 mM) in oxygen-degassed DMSO, produced decreased absorbance at 535 nm up to 61%. Additionally, heating an acylated derivative of 4.1 on phenolic hydroxyls to reflux did not exhibit any color formation.\textsuperscript{4.5}
Figure 4.3. Overlaid UV-Vis spectra of the solutions of 4.2a and 4.3a each in DMSO (0.9 mL) were gently refluxed over 2 min and cooled to room temperature followed by the addition of H₂O (0.1 mL) to each solution. The final concentrations of 4.2a and 4.3a are 1.03 × 10⁻³ M and 1.96 × 10⁻³ M respectively. A solution of 4.6b (5.0 × 10⁻⁶ M) was prepared in 9:1 DMSO:H₂O at room temperature.

Thus, the phenolic hydroxyls also played an important role in the formation of chromophores. Heating solutions of resorcinol or phenylboronic acid individually or an equimolar mixture of those with or without the added sugars under the same reaction conditions produced only pale color changes by visible detection.⁴² Thus, they reasoned that effective chromophore formation also required the methine-bridge of resorcinol and aldehyde condensation.

Interestingly, methine-bridged condensation products of resorcinol/aldehyde were known as reaction intermediates in the syntheses of well-known xanthene dyes which include fluorescein, rhodamine, 4.6a, 4.6b, and ethyl eosin (4.6c).

Indeed, the UV-Vis spectrum of 4.6b exhibits an absorbance maximum at 530 nm and, less intensely, at 500 nm. Those xanthene spectral patterns are similar to those seen in DMSO solutions containing 4.1, 4.2a, 4.2b, and 4.3a.⁴⁵
A computer-simulated structure study of 4.1 by Sybyl® ver. 6.6 by Jorge Escobedo showed that the formation of planar xanthene substructure within resorcinarene macrocycles increases in strain energy by 34.2 kcal/mol. Thus, the ring opening of resorcinarene macrocycles to acyclic oligomers could be a prerequisite to promote xanthene dyes in solution. It was also well-documented that 4.2b and its macrocyclic stereoisomers could interconvert through their acyclic oligomer intermediates in acidic media. Since the ring opening of resorcinarene macrocycles had only been reported to occur in the presence of strong acid, the formation of acyclic oligomers in a mixture of DMSO and H₂O (9:1) without the addition of any acid required the further investigation.

Solutions containing 4.1 (5.2 mM) in DMSO-\(d_6\) were heated for 3 min at 90 °C (the same procedure as in the initial study of colorimetric sugar detection) and monitored by \(^1\)H and \(^{13}\)C NMR spectrophotometer. They did not show any noticeable change in peak integral and chemical shifts compared to those of pure and colorless samples. In fact, only a trace amount of xanthene dye needs to be produced since xanthene dyes are highly absorbing materials. Figure
4.3 shows that approximately 0.5% conversion of 4.1 affords detectable colorimetric properties in solution.

We observed that more vigorous conditions were required to afford significant amounts of conversion products. Fresh 4.2b (100 mg, 18.4 mM) in DMSO was heated at 120 °C for 8h and monitored by reversed phase high performance liquid chromatography (HPLC) which revealed a 74% conversion of 4.2b to numerous products in a basis of relative peak integrals.

![Figure 4.5. X-ray crystal structure of (CH₃)₃S⁺CH₃SO₃⁻](image)

D. Swern and co-workers found that DMSO by the presence of peroxides and oxygen could promote acidic products which lead to the certain oxidations.⁴.⁷ Free radical scavengers have been used to inhibit acid formation from DMSO decomposition.⁴.⁷c We observed that the same thermolytic condition as described above, but with added free radical scavengers (either phenothiazene or BHT, 10 mol %) promoted less than 28% conversion to products monitored by HPLC.

Our recent publication described the strong evidence of the acid formation from DMSO decomposition by determining the first X-ray crystal structure of (CH₃)₃S⁺CH₃SO₃⁻ from a thermolysis reaction of 4.2b in DMSO.⁴.⁸ The precedent literatures reported that the radical- and
acid-promoted decomposition of DMSO could afford (CH₃)₃S⁺CH₃SO₃⁻ along with other decomposition products including CH₃SOH, CH₃SO₂H, CH₃SO₃H.⁴.⁹

![Chemical structure](image)

**Figure 4.6.** X-ray crystal structure of a “diamond” stereoisomer (4.7).

We also found strong evidence to prove the ring opening of resorcinarene macrocycles. R. Johnson isolated a rarely found “diamond” stereoisomer⁴.¹⁰ from the thermolysis reaction of 4.2b in DMSO followed by column chromatography (2.3% yield). Slow evaporation of 4.7 in a mixture of CH₂Cl₂ and MeOH (9:1) afforded X-ray quality crystals. Transformation of 4.2b to a “diamond” structure of resorcinarene is only possible to form via the ring-opening/re-condensation of 4.2b. Weinelt and Schneider previously assigned the structure of 4.7 as in the octabutyrate form via NMR study during their studies of macrocyclic stereoisomer interconversions in acidic media.⁴.⁶

The thermolysis of 4.2b followed by preparative HPLC separation allows us to observe a key acyclic product, 4.3b. The comparison of the ^1H NMR spectrum (Figure 4.7) of the isolated compound with a sample which we independently synthesized shows each of the corresponding
resonances of 4.3b (CH3OD δ 1.46, d, $J = 7.3$ Hz, 4.53, q, $J = 7.3$ Hz, 6.18-6.22, m, 6.89, d, $J = 8.0$ Hz). In addition, the MALDI mass spectrometer data of the HPLC isolate shows a peak at 245.59 amu (246.26 amu calcd).

![Figure 4.7](image)

**Figure 4.7.** (Top) An expanded $^1$H NMR spectrum of semipurified thermolysis products with the resonances of 4.3b. (Bottom) An expanded $^1$H NMR spectrum of pure 4.3b.

![Figure 4.8](image)

**Figure 4.8.** 2,4-dihydroxyacetophenone

The formation of 4.3b and 4.7 under our thermolysis conditions supports our hypothesis of acid-promoted ring opening and acyclic interconversions of resorcinarenes.4.6 In a DMSO solution containing 4.3b (0.880g, 3.576 mmol) was saturated with air and heated at 100 °C for 28h. The thermolytic products of 4.3b were determined by relative peak integrals of $^1$H NMR spectrum. The major product was resorcinol (90%) along with several minor products including 2,4-dihydroxyacetophenone (4.8) and xanthene dye (4.6a). The ratio of peak integrals of resorcinol (triplet, 6.94 ppm in CH$_3$OH-$d_4$) to 2,4-dihydroxyacetophenone (doublet, 7.76 ppm) is
153:1. Just small traces of 4.6a (doublet, 7.65 ppm) were observed. The production of 4.8 provides a link with prior work by Weinelt and Schneider.4.6

My discovery of 4.6a afforded the first experimental proof that Baeyer’s reaction in 1872 afforded colored xanthene dyes and that this was the material responsible for the color in our resorcinarene solutions which allowed us to develop sugar sensors. Because acid and peroxides can promote oxidation of xanthenes,4.11 we should obtain a better conversion of xanthene dye (4.6a) from 4.3a by the presence of these reagents.

\[
\begin{align*}
\text{HO} & \quad \text{OH} & \quad \text{OH} & \quad \text{OH} \\
4.3b & \quad \text{H}_2\text{SO}_4, \text{K}_2\text{S}_2\text{O}_8 & \quad 2 \text{ h} & \quad \text{HO} & \quad \text{OH} & \quad \text{O} & \quad \text{HO} \\
& & & \text{4.6a}
\end{align*}
\]

**Figure 4.9.** An expanded $^1$H NMR spectra of the oxidation product of 4.3b showing the formation of 4.6a.

Indeed, I found that the most significant conversion (4% yield) to 4.6a was afforded by heating a solution of 4.3b (50 mg, 0.203 mmol), H$_2$SO$_4$ (0.15 mL), and K$_2$S$_2$O$_8$ (1.0 mg) in MeOH (1.5 mL) to reflux for 2 h.

### 4.3. Conclusion

We have elucidated the mechanisms of the origin of color formation from resorcinarene macrocycles originally observed in von Baeyer’s condensation reaction. Our mechanistic studies
suggest that the color formation of resorcinarenes occurs via acid-promoted ring opening/condensation followed by oxidation to xanthenes. The \textit{in situ} acid formation is attributed to the radical decomposition of DMSO.

4.4. Experimental Section

Matrix Assisted Laser Desorption Ionization mass spectra were recorded on a Bruker Proflex III MALDI mass spectrometer using either anthracene or dithranol matrices. UV-Visible spectra were acquired on a Spectramax Plus 384 spectrophotometer (Molecular Devices). Analytic and preparative HPLC separations were recorded on a Milton Roy CM4000 multiple solvent delivery system equipped with a LDC Analytical Spectromonitor 5000 photodiode array detector. A Dynamax 60Å C18 (21.4 mm ID \times 25 cm L) column was used. Experimental conditions were followed; a gradient elution from 50% H2O and MeOH to 100% MeOH in 20 min and a flow rate of 5 mL/min otherwise stated above. Compound 4.1, 4.2a, 4.2b, 4.3a, 4.3b, 4.6a and 4.6a were as prepared according to the previous literature. All other compounds were purchased from Sigma-Aldrich and used without further purification. 1H NMR spectra were recorded on a Bruker DPX-250, DPX-400, or AMX-500 spectrometer with a reference either by DMSO (2.45 ppm) or (CH3)4Si (0.00 ppm).

4.5. References


4.10 To see the possible structures of other resorcinarene stereoisomers, check, Timmerman, P.; Verboom, W.; Reinhoudt, D. N. Tetrahedron 1996, 52, 2663.


CHAPTER 5
ONGOING RESEARCH IN THE DETECTION OF BIOLOGICALLY IMPORTANT MOLECULES

5.1. Detection of $\alpha$-Dicarbonyl Compounds

The selective detection of $\alpha$-dicarbonyl compounds is of great importance. Advanced glycation end products (AGEs) have been linked to numerous diseases including Alzheimer’s disease, diabetes, rheumatoid arthritis and aging process.\textsuperscript{5.1,5.2} It is known that $\alpha$-dicarbonyl compounds are directly involved in AGEs. Glyoxal and methylglyoxal, $\alpha$-dicarbonyl compounds, are major contributors in the formation of AGEs.\textsuperscript{5.2} In addition, methylglyoxal is, in particular, the most reactive precursor for AGE formations.\textsuperscript{5.2} The elevated levels of reactive $\alpha$-dicarbonyl compounds including methylglyoxal and glyoxal have been found in diabetic patients.\textsuperscript{5.3} It is also known that the rate of AGE formations is increased by hyperglycemia in diabetes.\textsuperscript{5.4} Glyoxal along with arabinose has also been recognized as intermediates in glucose-induced protein crosslinking and autoxidative glycosylation.\textsuperscript{5.5} In addition, methyl glyoxal has been reported as a mutagenic compound towards \textit{Salmonella typhimurium TA 100} and \textit{Escherichia coli}.\textsuperscript{5.6}

5.2. Selective Detection of the DNA Lesion Compound

Recently the C1’-oxidized abasic DNA lesion (2-deoxyribonolactone) has attracted great attention owing to its mutagenicity.\textsuperscript{5.7}

\begin{center}
\includegraphics[width=\textwidth]{scheme51.png}
\end{center}

\textbf{Scheme 5.1.} Formation of butenolide.
The generation of 2-deoxyribonolactone in DNA has only been studied in model system by DNA-damaging agents including γ-radiolysis.\textsuperscript{5,7}

However, in vivo formation of 2-deoxyribonolactone and its biological function have been rarely studied.\textsuperscript{5,8} Only recently, a selective detection method towards 2-deoxyribonolactone has been introduced according to using a biotin-avidin strategy. The biotinylated cysteine reacts with the butenolide formed upon treatment of 2-deoxyribonolactone with \textit{N,N’}-dimethylethylenediamine (DMEDA). The resultant biotin adducts (Figure 5.1) binds to an avidin-horseradish peroxidase conjugate which can oxidize 1,2-diaminobenzene in the solution to afford optical signal at 490 nm.

\textbf{Figure 5.1.} Biotin-butenolide adduct.

\textbf{5.3. Results}

Therefore, we reasoned that cysteine-conjugated rhodamine dye (5.2) would warrant a simple and selective detection method towards 2-deoxyribonolactone. Compound 5.2 is synthesized by a simple carbodiimide method. The activated N-Boc-S-tritylcysteine is acylated with commercially available rhodamine 110. Both N-Boc and S-trityl protecting groups are then removed by a mixture of trifluoroacetic acid and triethylsilane to attain compound 5.2.

Initial studies show that solutions of 5.2 promote a significant increase in fluorescence emission upon addition of glyoxal (α-dicarbonyl compound) while malondialdehyde (β-
dicarbonyl compound) or other structurally similar compounds acrolein and crotonaldehyde show only minimal changes in fluorescence intensity. (Figure 5.2)

\[
\text{i: } N\text{-Boc-S-trityl-Cys-OH, } N\text{-ethyl morpholine,} \\
\text{N(3-Dimethylaminopropyl)-N'-ethyl-carbodiimide.HCl} \\
\text{ii: TFA, Et}_3\text{SiH}
\]

**Scheme 5.2.** Synthesis of compound 5.2.

**Figure 5.2.** Fluorescence emission spectrum of 5.2 (2.3 x 10^{-5} M) with various analytes (5 x 10^{-5} M) in 0.1 M pH 9.5 carbonate buffer.
We have also evaluated compound 5.2 as a potential indicator for the selective detection of 2-deoxyribonolactone. Upon addition of 5(2H)-furanone, a compound structurally similar to butenolide, the solution of 5.2 exhibits an exceptional selectivity over other analytes including DNA and RNA.

We continue to study with compound 5.2 for the optimization of sensing conditions as well as for elucidation of the mechanism for the selective reaction of compound 5.2 on α-dicarbonyl compounds.

![Graph](image)

**Figure 5.3.** Relative fluorescence emission intensity of 5.2 (1.5 x 10⁻⁸ M) with various analytes (2 x 10⁻³ M) in 0.1 M pH 9.5 carbonate buffer.

**5.4. Experimental Section**

Synthesis of compound 5.2; N-t-Boc-S-trityl-cysteine (695.4 mg, 1.5 mmol) and N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimide·HCl (287.55 mg, 1.5 mmol) are combined in 1 mL of dry DMF and stirred for 30 min at 0 °C under N₂. To the solution, are added rhodamine 110 (550.2 mg, 1.5 mmol) and N-ethylmorpholine (190.5 μL, 1.5 mmol). After stirring this solution for 30 min at 0 °C, the solution is stirred at room temperature for
overnight. The resultant mixture is poured into 90 mL of H₂O and extracted with EtOAc (60 mL × 3). The organic layer combined is filtered, dried with Na₂SO₄ and filtered again. The solvent is evaporated in vacuo to afford a crude product. For further purification, column chromatography is performed with EtOAc and hexanes (1:1) to attain 277.1 mg of 5.1 (23.8 %). To the solution of 5.1 (61 mg, 78.7 μmol) in 0.8 mL of DCM, are added triethylsilane (39 μL, 244 μmol) and trifluoroacetic acid (0.8 mL, 10.4 mmol). The mixture is stirred for 1 hr under N₂ at room temperature. After evaporating the solvent in vacuo, the resultant mixture is washed with hexanes (5 mL × 2) and the solvent is evaporated in vacuo to afford a crude product of 5.2. For further purification, C18 reverse phase HPLC is performed (100 % of H₂O to 100 % of acetonitrile for 30 min, 1 % TFA) to attain the compound 5.2.

Analytical data for 5.2: ¹H NMR (250 MHz, CD₃OD) δ (ppm): 3.08 (dd, J = 14.8 and 6.8 Hz, 1H), 3.20 (dd, J = 14.8 and 5.0 Hz, 1H), 4.25 (dd, J = 6.8 and 5.0 Hz, 1H), 6.93 (s, 1H), 6.95 (d, J = 9.8 Hz, 1H), 7.19 (d, J = 9.8 Hz, 1H), 7.23 (d, J = 8.8 Hz, 1H), 7.41 (d, J = 6.9 Hz, 1H), 7.48 (d, J = 8.8 Hz, 1H), 7.83 (m, 2H), 8.33 (d, J = 8.7 Hz, 1H), 8.37 (s, 1H). MALDI-MS m/z calculated for C₂₃H₁₉N₃O₄S 433.11 for M⁺, found 434.13 [M+H]⁺.

5.5. References


APPENDIX A: CHARACTERIZATION OF COMPOUND 1.1

Figure A.1. $^1$H NMR spectra of 1.1

Figure A.2. $^{13}$C NMR spectra of 1.1
Figure A.3. MALDI TOF MS spectra of 1.1
APPENDIX B: $^1$H NMR SPECTRA OF 1.2
APPENDIX C: $^1$H NMR SPECTRA OF 1.3
APPENDIX D: CHARACTERIZATION OF COMPOUND 1.5

Figure D.1. $^1$H NMR spectra of 1.5

Figure D.2. MALDI TOF MS spectra of 1.5
APPENDIX E: UV-VIS SPECTRUM OF COMPOUND 2.8 WITH VARIOUS AMINO ACIDS

Figure E.1. UV-Vis spectrum of the solution containing 2.8 (1.0 $\times$ 10$^{-5}$ M, pH 7.3 15 mM phosphate buffer, 3:7 H$_2$O:MeOH) was monitored with added alanine (1.0 $\times$ 10$^{-4}$ M). The same condition was applied for all other experiments in appendix B.

Figure E.2. UV-Vis spectra of 2.8 with glutamine
Figure E.3. UV-Vis spectra of 2.8 with lysine

Figure E.4. UV-Vis spectra of 2.8 with glycine
Figure E.6. UV-Vis spectra of 2.8 with methionine

Figure E.7. UV-Vis spectra of 2.8 with arginine
Figure E.7. UV-Vis spectra of 2.8 with threonine

Figure E.8. UV-Vis spectra of 2.8 with serine
APPENDIX F: UV-VIS SPECTRA OF FLUORESCEIN WITH ADDED Hcy

The added Hcy \((1.0 \times 10^{-4} \text{ M})\) to the solution of fluorescein \((1.0 \times 10^{-5} \text{ M}, 50 \text{ mM phosphate at pH 7.3})\) do not afford any significant increase in UV-Vis spectra.
APPENDIX G: UV-VIS SPECTRA OF COMPOUND 2.8 WITH ADDED PPh₃ AND Hcy

UV-Vis spectrum of the solution containing 2.8 (1.0 \times 10^{-5} \text{ M}, \text{pH} 7.3 15 \text{ mM phosphate buffer, 3:7 H}_2\text{O:MeOH}) with added PPh₃ (5.0 \times 10^{-4} \text{ M}) and Hcy (1.0 \times 10^{-5} \text{ M}) showed no absorbance increase upon the addition of 30 equivalent of Cys to Hcy.
APPENDIX H: CHROMATOGRAM OF Cys USING 2.6 AS A DERIVATIZATION REAGENT
APPENDIX I: CHARACTERIZATION OF COMPOUND 3.1

Figure I.1. $^1$H NMR spectra of 3.1

Figure I.2. FAB MS spectra of 3.1
Interconversions of macrocyclic stereoisomers. (D: pentamers, five resorcinol units)
APPENDIX K: THE IN SITU ACID FORMATIONS VIA THE DECOMPOSITION OF DMSO IN RADICAL AND ACID PATHWAYS

(a) \[ \text{CH}_3\text{SCH}_3 + \text{O}_2 \rightarrow \text{CH}_3\text{SCH}_3 + \text{O}_2^- \]  
DMSO

\[ \text{OH} + \text{CH}_3\text{SCH}_3 \rightarrow \text{OCH}_3 + \text{CH}_3\text{SOH} \]  
(Methanesulfenic Acid)

\[ \text{CH}_3\text{SOH} \rightarrow \text{CH}_3\text{SOH} + \text{CH}_3\text{SH} \]  
(Methanesulfonic Acid)

(b) \[ \text{CH}_3\text{SCH}_3 + \text{H}^+ \rightarrow \text{CH}_3\text{SOH} \]

\[ \text{OCH}_3 + \text{CH}_3\text{SOH} \]  
(Methanesulfenic Acid)

\[ \text{CH}_3\text{SOH} \rightarrow \text{CH}_3\text{SOH} \]  
(Methanesulfonic Acid)

The in situ acid formations via the decomposition of DMSO in (a) radical and (b) acid pathways.\textsuperscript{4,9}
APPENDIX L: CHARACTERIZATION OF COMPOUND 5.1

Figure L.1. $^1$H NMR spectra of 5.1

Figure L.2. ESI MS spectra of 5.1
APPENDIX M: CHARACTERIZATION OF COMPOUND 5.2

Figure M.1. $^1$H NMR spectra of 5.2

Figure M.2. MALDI TOF MS spectra of 5.2
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Kyu Kwang Kim was born in Seoul, South Korea, on October 26, 1973. He graduated in 2000 from Hallym University with the Bachelor of Science degree in chemistry. At the same year, he started a doctoral program in Louisiana State University. Under the supervision of Dr. Robert M. Strongin, he has been involved in many collaborative projects mainly focusing on the syntheses of indicator reagents for biomolecules and their application onto analytical instruments. Kyu Kwang Kim is currently a candidate for the degree of the Doctor of Philosophy in organic chemistry, which will be awarded in December, 2005.