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SPECTROSCOPIC STUDY OF NEW SENSORS FOR ORGANOPHOSPHATES, METALS, AND CYANIDE BASED ON RHODAMINE AND CHOLIC ACID DERIVATIVES

by

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A Dissertation
Submitted to the
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requirements for the
Degree of Doctor of Philosophy
Department of Chemistry
Advisor: Ekkehard Sinn, Ph.D

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Despite the increasing interest in developing sensors for nerve gas agents, efficient detection remains challenging. Among the various sensors developed so far, fluorescence sensors play an important role due to their simplicity. We developed six new rhodamine-based compounds that can be used as fluorescent turn-on sensors. Compound 1 and 3 gave high fluorescent enhancement with diethyl chlorophosphate (DCP) compared to the other compounds. Very high selectivity and sensitivity were observed as these compounds did not show significant fluorescent enhancement with dimethyl methylphosphonate (DMMP), HCl and transition metal ions. The potential sensor can be used in solution as well as on a solid surface.

In addition, rhodamine-based compounds have been used as sensors for metal ions. We used the same strategy to develop turn-ON sensors for some environmentally and biologically important metal ions. Fluorescent sensors were developed for trivalent chromium which is considered a common environmental pollutant. The high affinity of Cr\textsuperscript{3+} towards CN\textsuperscript{-} was employed to develop a fluorescent turn-OFF sensor for toxic cyanide based on the metal displacement approach. Similarly, sensors were developed for Ni\textsuperscript{2+}, Hg\textsuperscript{2+}, Fe\textsuperscript{3+}, and Cu\textsuperscript{2+}. 
Siderophores are relatively small organic compounds with extremely high affinity for Fe$^{3+}$. We synthesized and characterized three new siderophore analogs bearing a cholic acid core (23, 27, and 30). Cholic acid is a biologically available compound with three hydroxyl groups that can be used to attach catechol arms. The salicylate mode of metal complexes (Fe$^{3+}$ and Al$^{3+}$) were synthesized successfully and their properties studied using UV-Vis, IR, and NMR spectroscopy.
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Arambe Gedara Aruna Weerasinghe
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BODIPY ........................................... 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene
CDCl₃ ............................................. Chloroform-d
CD₃CN ........................................... Acetonitrile-d₃
CH₂Cl₂ ........................................... Dichloromethane
COSY ............................................. Correlation Spectroscopy
d .................................................. Doublet
dd .................................................. Doublet of doublet
DCC ............................................... N,N’-Dicyclohexylcarbodiimide
DCl ............................................... Deuterium chloride
DCP ............................................... Diethyl chlorophosphate
DMAP ........................................... 4-(Dimethylamino)pyridine
DMMP ........................................... Dimethyl methylphosphonate
D₂O ............................................... Deuterium oxide
ESI-MS ....................................... Electro Spray Ionization - Mass Spectroscopy
EtOAc ......................................... Ethyl acetate
List of Abbreviations-Continued

Et$_3$N .......................................................... Triethylamine
FeCl$_3$ .......................................................... Iron(III) chloride
FRET .......................................................... Fluorescence Resonance energy Transfer
HETCOR ....................................................... Heteronuclear Correlation
HRESI .......................................................... High Resolution Electro Spray Ionization
ICP-AES ........................................................ Inductively Coupled Plasma Atomic Emission Spectroscopy
K$_2$CO$_3$ ......................................................... Potassium carbonate
KBr .............................................................. Potassium bromide
LMCT .......................................................... Ligand to Metal Charge Transfer
m ................................................................. Multiplet
mmol ............................................................. Millimole
MHz ............................................................. Megahertz
mM ............................................................... Millimolar
MeOH .......................................................... Methanol
NaBH$_4$ ........................................................ Sodium borohydride
NaOH .......................................................... Sodium hydroxide
List of Abbreviations-Continued

NMR ................................................................. Nuclear Magnetic Resonance

RT ................................................................. Room Temperature

s ................................................................. Singlet

SeO₂ ............................................................... Selenium dioxide

t ................................................................. Triplet

THF ................................................................. Tetrahydrofuran

Tris ................................................................. Tris(hydroxymethyl)aminomethane

UV ................................................................. Ultra Violet

q ................................................................. Quartet
CHAPTER 1

SYNTHESIS, CHARACTERIZATION, AND EVALUATION OF RHODAMINE BASED SENSORS FOR NERVE GAS MIMICS

1.1 Nerve Gas Agents

Nerve gas agents are highly toxic organophosphonates, which can inhibit acetylcholinesterase, a highly critical enzyme in nerve-muscle communication. Nerve gas agents irreversibly inhibit the serine esterase activity of acetylcholinesterase via the phosphorylation of the serine residue at the active site. This triggers rapid and fatal consequences such as paralysis of muscle and uncontrolled function at the organ level. The nerve agents of most concern include Sarin (GB), Tabun (GA), and Soman (GD) (Figure 1.1) which can be fatal in minutes when inhaled or absorbed through the skin. Different types of detection methods have been developed so far based on fluorescence, enzymes, interferometry, surface acoustic wave sensing and electrochemistry. However, most methods suffer certain drawbacks due to lack of selectivity, operational complexity or limited portability.
Figure 1.1: Nerve gas agents and mimics.

1.2 Fluorescence Spectroscopy

Fluorescence spectroscopy is basically concerned with electronic and vibrational energy states. As shown in figure 1.2, the initial process of fluorescence spectroscopy is the excitation of molecules by absorbing light at a particular wavelength. Once the molecule is excited, relaxation can take place via several processes. Fluorescence is one of such processes which involves the emission of light. Excited molecules in the highest vibrational level relax into the lowest vibrational level and then emit a photon to further relax into the ground state. Fluorescence has a short life time (~ $10^{-8}$ sec) compared to phosphorescence ($10^{-4}$ to 10 sec or more).^{21}
1.3 Fluorescent Sensors for Nerve Gas Agents

I would like to focus on certain fluorescent sensors developed to detect nerve gas agents as they have advantages over other types of sensors. Zhang et al.\(^7\) have reported sensors for nerve agents involving the formation of a phosphoester bond and later an intramolecular cyclized product which is fluorescent (Scheme 1.1). They found that the formation of phosphor ester is fast but the subsequent cyclization is very slow.\(^7\)
Scheme 1.1: Intra molecular cyclization which leads to the fluorescence change upon binding with DCP.$^7$

Scheme 1.2: Fluorescent sensor mechanism.$^8$
Later Dale et al.\textsuperscript{8} developed another fluorescent sensor based on a similar mechanism (Scheme 1.2). They also observed fluorescent enhancement upon the binding of nerve gas mimics.\textsuperscript{8} We decided to use rhodamine B based compounds to detect nerve agents as they are highly fluorescent and allow the naked eye detection because of clear color changes.

\textbf{1.4 Rhodamine Derivatives as Sensors}

Rhodamine-based compounds have properties, such as very high molar extinction coefficients and high fluorescent quantum yields, ideal for use as chemosensors.\textsuperscript{22} It is well known that the equilibrium between the nonfluorescent colorless ring-closed form and the highly fluorescent pink-colored ring-open form, provides a better model for the development of turn-on sensors. The equilibrium between the two forms is highly sensitive to the pH of the medium, the ring-open form being predominant in acidic conditions. Cations can trigger the change in structure between the spirocyclic and open-cycle form\textsuperscript{22} and therefore rhodamine-based compounds have been well established as sensors for metal ions such as Cu\textsuperscript{2+},\textsuperscript{24} Fe\textsuperscript{3+},\textsuperscript{25} Pb\textsuperscript{2+},\textsuperscript{28} Hg\textsuperscript{2+},\textsuperscript{30}\textsuperscript{36} and Cr\textsuperscript{3+}.\textsuperscript{37} Recently, Kang et al.\textsuperscript{38} have reported some rhodamine hydrazides as sensors for nerve agent mimics such as diethyl chlorophosphate (DCP) in the solid phase. They show that rhodamine hydrazides are much better sensors for DCP than rhodamine amides due to a proposed involvement of hydrazide nitrogen in binding with DCP.\textsuperscript{38} Here we report a series of rhodamine-based compounds as sensors for nerve gas agents in solution for the first time.
In order to avoid the interference from inorganic acids such as HCl or HBr, we decided to use a buffer system to study the binding of nerve gas mimics. Nerve gas agents such as Sarin, VX, Soman and Mustard Gas hydrolyze in aqueous systems with half-lives varying from minutes to days. For example, the half-life of Sarin is 2340 minutes at pH 7.5 while Soman has an even longer half-life.\textsuperscript{39, 40} As Sarin and Soman are the closest analogs to DCP, we can assume that DCP does not readily undergo hydrolysis in buffer systems. In addition, Sarin and Soman generate HF upon hydrolysis.\textsuperscript{41} Therefore all the compounds were tested with HCl to show that sensors should interact with the nerve gas and not the nerve gas degradation products. The compounds were also tested with DMMP to study the importance of the leaving group (Cl) of DCP in binding with sensors.
Experimental

Scheme 1.3: Synthesis of 1-6.
Scheme 1.4: Synthesis of 8.

Compound 1: A mixture of compound 7 (0.5 g, 1.09 mmol) and 2-furaldehyde (0.14 g, 1.45 mmol) in 10 ml of ethanol was refluxed for 12 hours. The precipitate formed during the reaction was filtered after cooling down to room temperature. The precipitate was washed with ethanol and dried to obtain 1 as a red solid (0.41 g, 70%). mp = 204 – 206 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 1.14 (12H, t, \(J = 6.9\) Hz), 3.31 (8H, q, \(J = 6.9\) Hz), 6.23 (2H, dd, \(J = 8.8\) Hz, 2.6 Hz), 6.33 (1H, dd, \(J = 3.3\), 1.4 Hz), 6.41 (2H, d, \(J = 2.6\) Hz), 6.53 (2H, d, \(J = 8.8\) Hz), 6.57 (1H, d, \(J = 3.4\) Hz), 7.05 (1H, d, \(J = 6.2\) Hz), 7.37 (1H, d, \(J = 1.1\) Hz), 7.43 (2H, m), 7.97 (1H, d, \(J = 8.1\) Hz), 8.19 (1H, s). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 12.7, 44.3, 65.6, 98.0, 105.5, 108.2, 111.6, 112.1, 123.5, 123.6, 127.9, 128.2, 133.5, 136.0, 143.9, 149.0, 150.7, 152.6, 152.8, 165.1. ESI MS: 535.42 [M+1]\(^+\). Anal. Calcd for C\(_{33}\)H\(_{34}\)N\(_4\)O\(_3\): C, 74.13; H, 6.41; N, 10.48. Found: C, 73.79; H, 6.80; N, 10.59.
Compound 2: A mixture of 7 (1.0 g, 2.2 mmol) and 3-furaldehyde (0.24 g, 2.5 mmol) in 20 ml of ethanol was refluxed overnight. The reaction mixture was allowed to cool down to room temperature and the precipitate was filtered. The precipitate was washed with ample amounts of ethanol and ether and dried to obtain 2 as a purple solid (0.2 g, 17%). mp = 182 – 184 °C; \(^1\)H NMR (400 MHz, Acetone-\(d_6\)): \(\delta\) 1.13 (12H, t, \(J = 6.9\) Hz), 3.37 (8H, q, \(J = 6.9\) Hz), 6.35 (2H, m), 6.47 (5H, m), 7.11 (1H, d, \(J = 7.6\) Hz), 7.47 (1H, s), 7.58 (2H, m), 7.80 (1H, s), 7.87 (1H, d, \(J = 7.3\) Hz), 9.23 (1H, s). \(^{13}\)C NMR (100 MHz, Acetone-\(d_6\)): \(\delta\) 12.0, 44.0, 66.1, 97.7, 106.8, 106.8, 107.9, 122.8, 124.0, 124.3, 127.8, 128.5, 130.4, 133.2, 142.0, 144.2, 144.8, 148.9, 151.2, 153.6, 163.9. ESI-MS: 535 [M+1]^+. Anal. Calcd for C\(_{33}\)H\(_{34}\)N\(_4\)O\(_3\): C, 74.13; H, 6.41; N, 10.48. Found: C, 73.74; H, 6.80; N, 10.50.

Compound 3: A mixture of 7 (1.0 g, 2.2 mmol) and 5-methyl-2-furaldehyde (0.29 g, 2.6 mmol) in 20 ml of ethanol was refluxed overnight. The reaction mixture was allowed to cool down to room temperature and the precipitate was filtered. The precipitate was washed with ample amounts of ethanol and dried to obtain 3 as a pink solid (0.85 g, 71%). mp = 193 – 194 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 1.14 (12H, t, \(J = 6.9\) Hz), 2.26 (3H, s), 3.31 (8H, q, \(J = 6.9\) Hz), 5.92 (1H, s), 6.23 (2H, d, \(J = 8.4\) Hz), 6.41 (3H, s), 6.55 (2H, d, \(J = 8.8\) Hz), 7.03 (1H, d, \(J = 6.9\) Hz), 7.41 (2H, m), 7.93 (1H, s), 7.97 (1H, d, \(J = 6.6\) Hz). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 12.7, 13.9, 44.3, 65.4, 98.0, 105.5, 108.0, 108.2, 114.3, 123.5, 127.9, 128.0, 128.1, 133.4, 135.7, 148.9, 149.0, 152.6, 152.8, 154.7, 165.1. ESI-MS: 549 [M+1]^+. Anal. Calcd for C\(_{34}\)H\(_{36}\)N\(_4\)O\(_3\): C, 74.43; H, 6.61; N, 10.21. Found: C, 74.06; H, 7.00; N, 10.43.
Compound 4: To a solution of 7 (1.0 g, 2.2 mmol) in 20 ml of ethanol was added 5-(2-nitrophenyl)-2-furaldehyde (0.57 g, 2.64 mmol). The resulting solution was refluxed overnight. The precipitate formed was filtered off after cooling down to room temperature. The precipitate was washed with ample amounts of ethanol and air dried to get 4 as a brown solid (0.94 g, 65%). mp = 107 – 110 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 1.14 (12H, t, \(J = 6.6\) Hz), 3.31 (8H, q, \(J = 6.6\) Hz), 6.25 (2H, d, \(J = 8.0\) Hz), 6.43 (2H, s), 6.53 (3H, m), 6.65 (1H, s), 7.09 (1H, d, \(J = 6.6\) Hz), 7.36 (1H, t, \(J = 7.5\) Hz), 7.46 (2H, t, \(J = 6.7\) Hz), 7.54 (1H, t, \(J = 7.5\) Hz), 7.60 (1H, d, \(J = 8.0\) Hz), 7.82 (1H, d, \(J = 7.3\) Hz), 7.98 (1H, d, \(J = 6.8\) Hz), 8.36 (1H, s). \(^13\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 12.7, 44.4, 66.0, 98.0, 105.5, 108.1, 112.1, 113.6, 123.4, 123.5, 123.7, 123.8, 127.9, 128.4, 128.7, 129.0, 131.8, 133.6, 135.8, 147.4, 148.5, 149.1, 151.7, 152.1, 153.0, 165.1. ESI-MS: 678 [M+Na]+.


Compound 5: To a solution of 7 (1.0 g, 2.2 mmol) in 20 ml of ethanol was added 5-nitro-2-furaldehyde (0.37 g, 2.64 mmol). The resulting solution was refluxed overnight. The precipitate formed was filtered off after cooling down to room temperature. The precipitate was washed with ample amounts of ethanol and air dried to get 5 as a brown solid (0.24 g, 19%). mp = 120 – 123 °C; \(^1\)H NMR (400 MHz, Acetone-d\(_6\)): \(\delta\) 1.14 (12H, t, \(J = 6.9\) Hz), 3.38 (8H, q, \(J = 6.9\) Hz), 6.38 (2H, d, \(J = 8.8\) Hz), 6.46 (2H, d, \(J = 1.8\) Hz), 6.50 (2H, d, \(J = 9.1\) Hz), 6.92 (1H, d, \(J = 3.6\) Hz), 7.11 (1H, d, \(J = 7.6\) Hz), 7.46 (1H, d, \(J = 4.0\) Hz), 7.58 (1H, t, \(J = 7.3\) Hz), 7.64 (1H, t, \(J = 7.3\) Hz), 7.93 (1H, d, \(J = 7.3\) Hz), 8.90 (1H, s). \(^13\)C NMR (100 MHz, Acetone-d\(_6\)): \(\delta\) 12.0, 44.0, 66.3, 97.8, 105.5, 108.2, 113.0, 113.6, 116.8, 123.2, 124.0, 127.8, 128.6, 128.8, 134.2, 134.6, 149.1, 152.0, 152.6, 153.2,

**Compound 6:** A mixture of 7 (1.0 g, 2.2 mmol) and 5-bromo-2-furaldehyde (0.45 g, 2.6 mmol) in 20 ml of ethanol was refluxed for 3 hours. The reaction mixture was allowed to cool down to room temperature and the precipitate was filtered off. The precipitate was washed with ample amount of ethanol and dried to obtain 6 as a pink solid (1.12 g, 83%).

Mp = 203 – 204 °C; ^1^H NMR (400 MHz, Acetone-d_6): δ 1.13 (12H, t, J = 6.9 Hz), 3.37 (8H, q, J = 6.9 Hz), 6.36 (2H, m), 6.43 (2H, s), 6.51 (3H, m), 6.70 (1H, d, J = 3.6 Hz), 7.06 (1H, d, J = 7.6 Hz), 7.56 (2H, m), 7.87 (1H, d, J = 6.9 Hz), 8.99 (1H, s). ^1^C NMR (100 MHz, Acetone-d_6): δ 12.0, 44.0, 66.0, 97.8, 106.1, 108.1, 114.1, 115.1, 116.8, 122.9, 123.8, 124.2, 127.8, 128.5, 129.1, 133.6, 137.2, 149.0, 152.1, 152.6, 153.2, 164.5. ESI-MS: 614[M+1]^+. Anal. Calcd for C_{33}H_{33}BrN_{4}O_{3}: C, 64.60; H, 5.42; N, 9.43. Found: C, 64.20; H, 5.62; N, 9.30.

**Preparation of 9:** A solution of rhodamine B (5.0 g, 10.4 mmol) in ethanol (100 mL) was treated with excess of ethylenediamine (17 mL, 0.25 mol) drop wise and triethylamine (4.3 mL, 30.9 mmol). The resulting solution was refluxed 5 hours until the solution became clear. The solution was evaporated to dryness and the crude product was dissolved in 1M HCl (200 mL). A solution of 10% NaOH was added until the pH reached 9-10 and then extracted with dichloromethane. The organic layer was separated, dried over MgSO_4 and evaporated to get 9 (3.8 g, 75%). ^1^H NMR (400 MHz, CDCl_3): δ 7.78 (1H, m), 7.43 (1H, m), 7.08 (1H, m), 6.42 (2H, d, J = 8.7 Hz), 6.35 (2H, d, J = 2.5 Hz), 6.25 (2H, dd, J = 8.8 Hz, 2.6 Hz), 3.32 (8H, q, J = 6.9 Hz), 3.19 (2H, t, J = 6.6 Hz), 2.44 (2H, t, J = 6.6 Hz)), 1.15 (12H, t, J = 6.9 Hz). ^1^C NMR (100 MHz, CDCl_3): δ 168.9,
Preparation of 8: A solution of furan-2-carboxylic acid (0.27 g, 2.5 mmol) in dichloromethane (20 ml) at 0 °C was treated with DCC (0.55 g, 2.7 mmol) and DMAP (0.30 g, 2.5 mmol). The resulting mixture was stirred for 15 minutes before the addition of compound 7 (1.0 g, 2.0 mmol). The mixture was stirred at room temperature for 12 hours and the precipitate was filtered off. The filtrate was evaporated and purified on a silica gel column using EtOAc as the mobile phase to obtain 8 as white solid (0.31 g, 27%).  

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 8.02 (1H, s), 7.93 (1H, dd, $J = $), 7.50 (1H, m), 7.44 (2H, dd, $J = $), 7.06 (1H, m), 7.03 (1H, d, $J = $ 3.2 Hz), 6.44 (3H, m), 6.36 (2H, s), 6.25 (2H, d, $J = $ 8.4 Hz), 3.38 (2H, m), 3.31 (8H, q, $J = $ 6.9 Hz), 3.17 (2H, m), 1.15 (12H, t, $J = $ 6.9 Hz).  

$^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 170.0, 158.5, 153.3, 149.0, 148.3, 144.1, 132.8, 130.5, 128.5, 128.2, 123.9, 123.0, 113.4, 111.6, 108.3, 104.7, 97.8, 65.7, 44.4, 40.6, 40.0, 12.6. Anal. Calcd for C$_{35}$H$_{38}$N$_4$O$_4$: C, 72.64; H, 6.62; N, 9.68. Found: C, 71.85; H, 7.12; N, 9.78.
Results and Discussion

1.5 Preparation of Solutions for UV Absorption and Emission Studies

The stock solutions of metal ions (0.034M) were prepared using nitrates \([\text{Cr(NO}_3\text{)}_3, \text{Zn(NO}_3\text{)}_2, \text{Ni(NO}_3\text{)}_2, \text{Pb(NO}_3\text{)}_2, \text{Cd(NO}_3\text{)}_2, \text{Hg(NO}_3\text{)}_2, \text{NaNO}_3, \text{KNO}_3]\) or chlorides \([\text{MnCl}_2, \text{CuCl}_2, \text{CoCl}_2, \text{FeCl}_2, \text{CaCl}_2]\). Stock solutions of DCP, DMMP and HCl (0.034 M) were prepared in acetonitrile. The stock solutions of compounds 1-7 (2 mM) were also prepared in 50% CH\(_3\)CN, 50% 0.01 M Tris HCl buffer (pH = 7.0). Fluorescence and UV absorption studies were performed using a 10 \(\mu\)M solution of the compounds and appropriate amounts of analytes. The solutions were shaken for 1 minute after each addition and allowed to stand 9 minutes before measuring the fluorescence. The fluorescence measurements were performed with 510 nm excitation. Both excitation and emission slit widths were 2 nm.

1.6 Association Constants

Association constants were calculated using the Benesi-Hildebrand equation.\(^{43}\)

\[
\frac{F_0}{F - F_0} = \frac{a}{b - a} \left(1 + \frac{[\text{substrate}]}{K}\right)
\]

where \(F_0\) is the fluorescence intensity of sensor at \(\lambda_{\text{max}}\) in the absence of analyte, \(F\) is the fluorescence intensity after addition of \(\text{Fe}^{3+}\) at \(\lambda_{\text{max}}\). \([\text{M}]\) is the concentration of the analyte.

\[
\frac{1}{A - A_0} = \frac{1}{K} \frac{[\text{M}^{\text{max}}]}{[\text{M}^{\text{max}}]} + \frac{A_{\text{max}} - A_0}{K} + A_{\text{max}} - A_0
\]

where \(A_0\) is the absorbance of the sensor without the analyte, \(A\) is the absorbance with the analyte and \(A_{\text{max}}\) is the absorbance with \([\text{M}^{\text{max}}]\).\(^{43}\)

The association constant \((K)\) can be obtained from the ratio of intercept/slope.
1.7 Synthesis of 1-8

I have synthesized six new rhodamine B derivatives with an electron rich furan moiety (Scheme 1.3). We used different substituent groups in the furan ring to study the effect of electronics and sterics on the sensitivity of the sensor towards DCP. We expected DCP to bind with sensors via the carbonyl O, and imine N similar to what Kang et al\textsuperscript{38} suggested. Scheme 1.4 shows the synthesis of compound 8 which has an additional two carbon units between two amide nitrogens. This design is used in order to better understand the binding mechanism between the sensors and nerve gas mimics. Chemosensors 1-6 were synthesized using Schiff-base condensation between the amine-containing compound 7\textsuperscript{24} and the corresponding aldehyde in ethanol. Compound 8 was synthesized in two steps that involve the formation of an amide bond between 9 and furan-2-carboxylic acid in the presence of DCC and DMAP. The yields were very high except for compound 2 and 5, which were obtained in only 17.0% and 19.0% yield respectively. Single crystals of 1, 2, 3, 4 and 6 were grown in acetonitrile at room temperature. The structures were well characterized using \textsuperscript{1}H NMR, \textsuperscript{13}C NMR, mass spectrometry and X-ray crystallography.

1.8 Spectroscopic Studies

All the spectroscopic studies were performed in 50% CH\textsubscript{3}CN, 50% 0.01 M Tris HCl buffer (pH = 7.0) medium and in acetonitrile in which compounds formed colorless solutions that were stable for over a week. Generally rhodamine-based compounds are protonated in acidic conditions and emit strong fluorescence. The colorless solutions were very weakly fluorescent and showed no absorption above 450 nm, properties that are characteristic of the ring-closed spirolactam. The predominance of the spirolactam
form was further confirmed by observation of the characteristic carbon resonance near 66 ppm for each of the compounds.

Compound 1 registered the highest emission intensity with DCP among all the compounds tested during the study (Figure 1.3). The fluorescence spectrum of 1 has a maximum at 583 nm after the addition of 34 equivalents of DCP, corresponding to delocalization in the xanthene moiety of rhodamine. There was a significant fluorescent intensity enhancement (>165 fold, Figure 1.4) as the solution turned pink, a color change clearly visible to the naked eye. Continuous addition of DCP resulted in increased fluorescence as shown in Figure 1.5. The linearity of the $F_0/(F-F_0)$ vs $1/[DCP]$ plot confirms the formation of a 1:1 complex between 1 and DCP (Figure 1.6). The binding constant was calculated using the Benesi-Hildebrand method and the values for all the compounds are summarized in Table 1.1.

Initially, compound 1 showed no absorption band above 400 nm, but a new absorption band around 550 nm with a shoulder at 520 nm appeared upon the addition of DCP (Figure 1.7). Interestingly, the addition of 34 equivalents of Cu$^{2+}$ also yielded a color change (Figure 1.8) but no fluorescence enhancement. Thus the compound senses copper, but copper is not an interferent for the detection of DCP, because of the different behavior of 1 with Cu$^{2+}$ and DCP.
Figure 1.3: Fluorescence spectra of compounds 1-7 (10 μM) with DCP (340 μM) in 50% CH$_3$CN, 50% 0.01 M Tris HCl buffer (pH = 7.0) ($\lambda_{ex} = 510$ nm).

Figure 1.4: Bar profiles of fluorescence changes of compounds 1-7 (10 μM) with DCP (340 μM) in 50% CH$_3$CN, 50% 0.01 M Tris HCl buffer (pH = 7.0) compared with fluorescence profiles of free compounds ($\lambda_{ex} = 510$ nm).
Figure 1.5: Compound 1 (10 μM) with DCP (0.255 mM - 0.85 mM) in 50% CH$_3$CN/50% 0.01 M Tris HCl buffer (pH = 7.0) ($\lambda_{ex} = 510$ nm).

Figure 1.6: $F_0/(F-F_0)$ vs $1/\text{[DCP]}$ for compound 1 ($K = 3.0 \times 10^3$ M$^{-1}$).
Figure 1.7: UV-Vis spectra of compounds 1-6 (10 μM) with DCP (340 μM) in 50% CH₃CN, 50% 0.01 M Tris HCl buffer (pH = 7.0).

Figure 1.8: UV-Vis spectra of compounds 1-6 (10 μM) with Cu²⁺ (340 μM) in 50% CH₃CN, 50% 0.01 M Tris HCl buffer (pH = 7.0).
Rhodamine derivatives are well known to give a color change in the presence of acids and metals.\textsuperscript{13-17} We tested our sensor systems with a range of potential interferents including HCl and metals such as Na\textsuperscript{+}, K\textsuperscript{+}, Ca\textsuperscript{2+}, Cr\textsuperscript{3+}, Fe\textsuperscript{2+}, Ni\textsuperscript{2+}, Cu\textsuperscript{2+}, Zn\textsuperscript{2+}, Cd\textsuperscript{2+}, Hg\textsuperscript{2+}, and Pb\textsuperscript{2+}. Furthermore we tested our compounds with dimethyl methylphosphanate (DMMP), another organophosphate interferent. Only Cr\textsuperscript{3+} showed a slight emission enhancement among the metals and other analytes used in the experiment (Figure 1.9). This indicates that compound 1 is highly selective towards DCP. As 1 did not give any response to DMMP, it is clear that the leaving group (chlorine) of DCP plays a key role in binding with sensor 1 to trigger the color change. In order to prove the importance of the furan moiety in sensing DCP, we tested compound 7, which lacks any furan fragments. It showed slight emission enhancement with DCP indicating that the furan moiety is important for DCP sensing.

The fluorescence studies of sensor 1 were also performed in acetonitrile and the results were completely different from those of the buffer system. There was a significant emission intensity enhancement (> 1400–fold) with 1.0 equivalent of Cr\textsuperscript{3+} (Figure 1.10). In addition, a significant fluorescence enhancement (ca 900–fold) was observed with Hg\textsuperscript{2+} for Sensor 1, while Pb\textsuperscript{2+}, Zn\textsuperscript{2+}, Cd\textsuperscript{2+}, and Fe\textsuperscript{2+} showed very slight interference at the same concentration. Compound 1 gave an instantaneous and far higher response with DCP in acetonitrile (Figure 1.11) but the sensitivity was low compared to the metal ions in acetonitrile. That motivated us to use an acetonitrile/buffer system which gave an excellent sensitivity towards DCP.
Figure 1.9: Fluorescence spectra of compound 1 (10 μM) with DCP, DMMP, HCl and metals (340 μM) in 50% CH<sub>3</sub>CN, 50% 0.01 M Tris HCl buffer (pH = 7.0) (λ<sub>ex</sub> = 510 nm).

Figure 1.10: Fluorescence changes of 1 (10 μM) with Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>, Cr<sup>3+</sup>, Mn<sup>2+</sup>, Fe<sup>3+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup>, Cu<sup>2+</sup>, Zn<sup>2+</sup>, Cd<sup>2+</sup>, Hg<sup>2+</sup>, Pb<sup>2+</sup> (10 μM) in CH<sub>3</sub>CN (λ<sub>ex</sub> = 510 nm).
Compound 2 was designed to understand the importance of the position of the furan oxygen in sensing DCP. Interestingly, the emission enhancement for 2 (13-fold, Figure 1.3) with DCP is significantly lower than that of compound 1. This example proves that 2–substitution of the furan produces a better DCP sensor than 3–substitution, possibly due to an electronic interaction during binding. However this was not directly confirmed. Compounds 4 - 6 bear electron-withdrawing groups which can lower the electron density of the furan ring. We selected a strong electron-withdrawing group (-NO₂) and a weak electron-withdrawing group (-Br) to compare their effectiveness. Compound 4 showed a fluorescence enhancement (> 23 fold) with DCP (34 equivalent), but the observed fluorescence intensity was much lower than that of compounds 1 and 3 with DCP. This behavior can be attributed to the effect of the electron withdrawing group at the 5\textsuperscript{th} position of the furan ring. In addition, the bulky 2-(2-nitrophenyl)furan moiety of compound 5 may sterically hinder the binding of DCP. Interestingly, compound 4 gave
a reasonable emission enhancement (10-fold) with Cu$^{2+}$ while none of the other compounds gave any significant fluorescence enhancement with Cu$^{2+}$ (Figure 1.12). Furthermore, the fluorescence enhancement observed with Cr$^{3+}$ was insignificant when compared to the 4-DCP and 4-Cu$^{2+}$ adducts. Compounds 5 and 6 produced extremely low emissive solutions compared to the other compounds, and as expected, both 5 and 6 yielded extremely low emission intensities with DCP due to the presence of the electron withdrawing group at the 5$^\text{th}$ position of the furan ring. In fact the binding constant for 5 with DCP was found to be very low (K = 13 M$^{-1}$). These observations indicate that a strong electron-withdrawing group (-NO$_2$) significantly lowers the sensitivity of the compound towards DCP. Bromine, a weak electron-withdrawing group, produces a similar yet smaller effect compared to the compound bearing the nitro group.

**Figure 1.12:** Compound 4 (10 μM) with DCP, DMMP, HCl and metals (0.340 M) in 50% CH$_3$CN/ 50% 0.01 M Tris-HCl buffer (pH = 7.0) ($\lambda_{\text{ex}}$ = 510 nm).
Conversely, compound 3 was designed to study the effect of an electron-donating group at the 5th position of the furan ring. There was a significant fluorescence intensity increase (> 150 fold) upon the addition of DCP. Furthermore, the calculated binding constant is $3.4 \times 10^3 \text{ M}^{-1}$ which is the highest of the compounds studied. This clearly indicates that an electron-donating group can improve the sensitivity of the sensor. In addition, 3 showed a slight emission enhancement with Cr$^{3+}$ which is however, insignificant compared with that for DCP.

**Table 1.1: Association constants of compounds 1-6 with DCP**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Association Constant, K / M$^{-1}$</th>
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<tbody>
<tr>
<td>1</td>
<td>$3.0 \times 10^3$</td>
</tr>
<tr>
<td>2</td>
<td>$1.69 \times 10^3$</td>
</tr>
<tr>
<td>3</td>
<td>$3.4 \times 10^3$</td>
</tr>
<tr>
<td>4</td>
<td>$2.1 \times 10^3$</td>
</tr>
<tr>
<td>5</td>
<td>17</td>
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<tr>
<td>6</td>
<td>$1.75 \times 10^3$</td>
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1.9 Kinetic Study

The fluorescence spectrum of 1 was recorded as a function of time, as the color development was not instantaneous in the buffer environment. As shown in Figure 1.13,
there is no fluorescence intensity enhancement during the first 4 minutes with 340 μM of DCP. The emission intensity was enhanced dramatically after 4 minutes and was static after 9 minutes. The color development accelerated with higher concentrations of DCP. The kinetics of the reaction were studied with respect to temperature and the concentration of DCP (Figure 1.14). As expected, reaction rates were increased with elevated temperature and concentration. The color development was observed in 40 seconds at 70 °C with 340 μM of DCP.

**Figure 1.13:** Change in fluorescence intensity of 1 (10 μM) in the presence of DCP (0.34 mM and 0.68 mM) with time in 50% CH₃CN, 50% 0.01 M Tris HCl buffer (pH = 7.0) (λₑₓ = 510 nm).
1.10 Sensing Mechanism

A $^1$H NMR titration was performed to shed light on the binding of DCP with compound 1 (Figure 1.15). Continuous addition of DCP resulted in a shortening and broadening of the imine hydrogen peak at $\delta 8.95$. Interestingly, the peaks corresponding to the furan hydrogens did not show any significant change with the addition of DCP, while the intensities of some of the xanthene ring hydrogens decreased and broadened. DCP is expected to trigger the formation of the highly fluorescent ring-open form involving the carbonyl oxygen and imine nitrogen as Kang et al.$^{38}$ postulated for the protonation of rhodamine hydrazides. As 1 did not interact with DMMP, it is clear that the chlorine leaving group of DCP plays a key role in binding with sensor 1 to trigger the color change (Figure 1.16). It is well known that ethylenediamine tetraacetic acid (EDTA) can remove metals from a rhodamine-metal complex thereby causing the

![Graph showing time required by 1 (10 μM) for the color change with DCP in 50% CH$_3$CN, 50% 0.01 M Tris HCl buffer (pH = 7.0).](image)
formation of the ring-closed non-fluorescent form. Based on an interaction between the phosphorous of DCP and the carbonyl of rhodamine, it is expected that a similar competitive interaction is occurring between the EDTA carbonyl groups and DCP causing a reduction in the fluorescence upon addition of EDTA (Figure 1.17).

**Figure 1.15:** $^1$H NMR (CD$_3$CN) spectra of 1 with DCP (0, 0.75, 1.5, 3, 4, 6 equiv. from bottom to top).
Figure 1.16: The possible DCP binding mechanism that triggers the formation of the colored ring-open form.

Figure 1.17: Compound 1 (10 μM) with DCP (680 μM) titrated against EDTA in 50% CH₃CN/ 50% 0.01 M Tris HCl buffer (pH = 7.0) (λₑₓ = 510 nm).

We used compound 8 to further strengthen the binding mechanism that contains two additional carbon units between two amide nitrogens. This structural change makes it difficult to adopt the previous binding conformation. As expected, compound 8 did not show any fluorescence enhancement upon the addition of 340 μM of DCP in acetonitrile (Figure 1.18). Based on these observations, it is crucial to position the carbonyl oxygen
and the nitrogen atom in close proximity to bind with DCP and form the highly fluorescent ring-open spirolactum.

**Figure 1.18:** Fluorescence spectra of compound 8 (10 μM) with DCP in acetonitrile.

### 1.11 Sensor Performance

Detection limits were determined for compound 1 with DCP in 50% CH$_3$CN, 50% 0.01 M Tris HCl buffer (pH = 7.0) as it registered the highest fluorescence enhancement with DCP. The detection limit was found to be 170 μM at room temperature and improved at 70 °C (147 μM). In addition, a low detection limit of 17 μM was found in CH$_3$CN. The visible color changes from colorless to pink also permit the identification of nerve gas agents with the naked eye. The new design clearly improves the detection of nerve gas agents, as the unmodified 7 showed a very weak signal compared to other compounds. Swager$^7$ and Rebek$^8$ have used an intramolecular cyclization mechanism which is irreversible to detect DCP so the sensor system can be used only once. In
contrast, our system is reversible and can be reused. In addition, our sensors are very selective as they do not give any fluorescence enhancement with DMMP, metal ions or in particular HCl, with which the reported sensors had problems. In terms of operational complexity and limited portability, our sensors are very easy to handle and easier to adapt for use in the field.

1.12 Gas Phase Sensing

A whatman filter paper was cut into 1 cm² pieces. Each piece was separately attached to a string. Filter paper pieces were immersed in a solution of compound 1 (10 mg) in CH₂Cl₂ (2 ml) for 10 minutes. Filter paper pieces were taken out and air dried for 30 minutes to remove dichloromethane.

DCP vapor was prepared by adding 1 drop of DCP into a 20 ml glass vial. Similarly, 2 drops of HCl were also added into a separate glass vial. Filter paper pieces loaded with 1 hung inside each vial as shown in Figure 1.19. Interestingly, filter paper inside the DCP vial turned red in a few seconds while the two in the other vials remained unchanged. This suggests that DCP produces enough vapor to react with 1 loaded into the filter paper (Figure 1.20). It seems HCl does not produce enough vapor under these circumstances. This experiment demonstrates the suitability of the sensor loaded into a solid material for use outside the lab for the detection of nerve gas agents.
Figure 1.19: Picture of 1 loaded into a filter paper in (a) air (b) DCP vapor (c) HCl vapor.

Figure 1.20: A filter paper loaded with 1 with (right) and without (left) exposing to DCP vapor for 5 minutes.
Conclusion

In conclusion, we have synthesized six new rhodamine-based compounds in high yields and determined the effect of the furan ring and its substitution upon DCP binding. In addition, compound 8 was synthesized to prove the imine nitrogen is important in binding mechanism. Introducing a furan ring increased the sensitivity towards DCP as compound 7 with no furan ring showed less fluorescence enhancement compared to all the other compounds. The study determined that compounds 1 and 3 are the best sensors of those studied. In addition, they show very high selectivity towards DCP over the common interferences used in the study. These results indicate that the presence of an electron-withdrawing group hampers the binding of DCP as it lowers the electron density of the furan ring and the imine nitrogen. The reaction rate increases dramatically with heating, so that 1 showed the color change with DCP in 40 seconds at 70°C. That none of the compounds showed any interaction with DMMP indicates the importance of the leaving group for the binding mechanism. Furthermore, the binding was found to be reversible, as the pink color disappears upon the addition of EDTA.
CHAPTER 2

RHODAMINE BASED TURN-ON SENSORS FOR Cr$^{3+}$ AND Ni$^{2+}$: DETECTING CN$^-$ VIA METAL DISPLACEMENT APPROACH

2.1 Importance of Detecting Ni$^{2+}$

The design and synthesis of sensors for metal ions is a rapidly developing part of many research areas including environmental, biological and waste management applications. Maximum sensitivity and selectivity is key. Selective detection of nickel is very important as it is a carcinogen and therefore considered a pollutant in the environment as well as a metal of biological interest. As Ni$^{2+}$ it causes diseases such as asthma and lung cancer. Nickel reaches the environment from its uses in ceramics, surgical and dental prostheses, Ni-Cd batteries, and rods for arc welding. Minute concentrations of Ni$^{2+}$ are now found in various food products including canned food, chocolates, raw meat, milk and milk-based products. Therefore it is important to detect low concentrations of Ni$^{2+}$ in biological samples. Several methods are available for the detection of nickel including flow injection spectrometry, flame and graphite furnace atomic absorption spectrometry, ICP-AES and flame photometry. The disadvantages of these techniques are the requirement of sample pretreatment and expert personnel to handle the instrument. We now present a “naked eye” sensor that is easy to operate even outside of the laboratory.
Several attempts have been made to develop turn-on sensors for Ni\(^{2+}\). Recently, Dodani et al have developed a fluorescent Ni\(^{2+}\) probe with a BODIPY dye with a N/O/S receptor to bind with the metal (Figure 2.1).\(^{49}\) They observed 25-fold fluorescence enhancement with 50 equivalents of Ni\(^{2+}\). The sensor is used to detect low Ni\(^{2+}\) levels in live cells. Live cells loaded with 1 mM NiCl\(_2\) in the growth medium were stained with the probe after 18 h and increased fluorescence was observed. Interestingly, this level of Ni\(^{2+}\) is not lethal to lung carcinoma cells but 2 – 10 mM Ni\(^{2+}\) does cause cell death.

![Figure 2.1: Structure of the fluorescent Ni\(^{2+}\) sensor.\(^{49}\)](image)

Recently, Kaur et al have developed anthracene-9,10-dione based sensors for Ni\(^{2+}\), Cu\(^{2+}\) and Co\(^{2+}\) (Figure 2.2). Sensor A produced two different bathochromic shifts

![Figure 2.2: Chemosensor A\(^{53}\) and B.\(^2\)](image)
upon binding with Cu$^{2+}$ and Ni$^{2+}$ allowing the selective identification of the two metal ions. In the continuation of their work on metal sensing, they have synthesized B which allows selective identification of Co$^{2+}$ and Cu$^{2+}$ or Co$^{2+}$ and Ni$^{2+}$. They observed color changes upon binding with metals and they were able to detect them in concentrations as low as 3 µM.$^{53}$

2.2 Detection of Cr$^{3+}$

Trivalent chromium (Cr$^{3+}$) is an important metal biologically and environmentally. It plays an important role in metabolism of carbohydrates, proteins, lipids and nucleic acids.$^{54}$ Chromium deficiency can increase the risk of diabetes and cardiovascular diseases.$^{55}$ Environmentally, it is a pollutant that accumulates due to industrial and agricultural activities.$^{56}$ Again we seek rapid and accurate detection. Extant Cr$^{3+}$ detection uses traditional methods such as electrochemical$^{57}$ and potentiometric.$^{58}$ In addition there are few reports on fluorometric detection$^{36, 59-63}$ of Cr$^{3+}$ which has a greater impact as it is simple and rapid. However, in most cases the selectivity for Cr$^{3+}$ is low.

Due to the paramagnetic nature of Cr$^{3+}$, the development of turn-on sensors still remains challenging. Scheme 2.1 shows the fluorescent turn-on sensor developed by Zhou et al.$^{60}$ The sensor was designed to observe the (Fluorescence Resonance Energy Transfer) FRET-based fluorescence enhancement upon the binding with Cr$^{3+}$ as shown in scheme 2.1. Initially, the sensor showed an absorption band at 380 nm and yellow fluorescence centered at 544 nm. When Cr$^{3+}$ was added, a new fluorescence band at 594 nm was observed upon excitation at 405 nm. Interestingly, the intensity of the
fluorescence band at 544 nm decreased due to the FRET behavior. A healthy association constant of $9.4 \times 10^3 \text{ M}^{-1}$ was found for Cr$^{3+}$.\textsuperscript{60}

**Scheme 2.1:** FRET-based Cr$^{3+}$ sensor.

Scheme 2.2 shows another selective and more sensitive Cr$^{3+}$ sensor developed by Mao et al.\textsuperscript{62} Comparing to the previous sensor, this one also has a rhodamine moiety and four coordinating sites that can bind with Cr$^{3+}$. Interestingly, they found a higher binding constant of $4.1 \times 10^4 \text{ M}^{-1}$. The binding mechanism remains the same as in the previous example and a visible color change was also observed.\textsuperscript{62}
2.3 Detection of CN⁻

Rhodamine-based Cr³⁺ or Ni²⁺ complexes can be used to detect CN⁻ as it can strongly bind with these metals. When the metal is removed by CN⁻, rhodamine goes back to its ring-closed spirolactum structure which is colorless. This drastic color change makes it a naked-eye sensor of CN⁻. Cyanide is an extremely toxic industrial waste. It is commonly used in electroplating, pharmaceutical production, the gas industry, and precious metal milling and refining. Cyanide binds to cytochrome a₃ and inhibits the electron transport chain, and therefore the production of ATP in all cells of an organism.⁶⁴ - ⁶⁶ Therefore the cyanide level in natural waters must be maintained below 0.1 ppm according to most regulatory agencies.⁶⁵ So far several detection methods have been reported for free cyanide, including electrometric,⁶⁷, ⁶⁸ chromatographic,⁶⁹ and fluorometric assays.⁷⁰, ⁷¹ The major drawback of most of these methods is the requirement of tedious sample preparation and expensive laboratory equipment.⁶⁴

Xu et al⁷² have reported a CN⁻ sensor based on the metal displacement approach of a 4,5-disubstituted-1,8-naphthalimide based Cu²⁺ complex. The free sensor showed a broad emission band at 534 nm which showed a decrease in intensity upon the addition of Cu²⁺. In addition a new emission band at 478 nm started to appear. The addition of CN⁻ resulted in the disappearance of the emission peak at 478 nm and the increase in the band at 534 nm due to the release of Cu²⁺ (Scheme 2.3).⁷²

2.4 Rhodamine Compounds as Chemosensors

Rhodamine-based compounds have been widely used as chemosensors due to their remarkable spectroscopic properties including high absorption coefficients, high
fluorescent quantum yields, and excitation and emission with the visible wavelength region.\textsuperscript{73} In addition, they can interconvert between a non-fluorescent ring-closed spirolactam form and a high fluorescent ring-open spirolactam form which makes them excellent candidates for chemosensors. We have recently developed a rhodamine-based single and multiphoton fluorescent sensor for Fe\textsuperscript{3+}\textsuperscript{26} Several other rhodamine-based sensors also have been reported for metal ions including Cu\textsuperscript{2+}\textsuperscript{24,25}, Fe\textsuperscript{3+}\textsuperscript{26-28}, Hg\textsuperscript{2+}\textsuperscript{30-35},\textsuperscript{74,75} and Pb\textsuperscript{2+}\textsuperscript{29} In all cases the mechanism involves the formation of ring-open spirolactum triggers by metal ions. Generally the ring-open form of rhodamine derivatives is pink in color with orange fluorescence even though green fluorescence is also reported.\textsuperscript{76}

Therefore, we decided to use rhodamine-based compounds to detect Cr\textsuperscript{3+}, Ni\textsuperscript{2+} and CN\textsuperscript{−}. We designed sensors 9, 10, and 11 for this purpose. We will discuss the synthesis, characterization and the optical properties of them as the discussion progresses.

\textbf{Scheme 2.3:} Detection of CN\textsuperscript{−} via the metal displacement approach.
Experimental

Scheme 2.4: Synthesis of 9 and 10.

Scheme 2.5: Synthesis of 11.
2.5 Synthesis

Compounds 9 and 10 were synthesized by reacting 7 with thiophene-2-carboxaldehyde and 3-formylchromone respectively in ethanol (Scheme 2.4). Both 9 and 10 obtained in very high yield, 81% and 88% respectively. Both formed precipitates upon cooling down to room temperature and were separated by filtration. Single crystals of 9 and 10 were grown in acetonitrile. Both have three coordination sites O/N/S and O/N/O respectively. Compound 7 was reacted with 2-methylquinoline-8-carboxylic acid in the presence of DCC and DMAP to produce compound 13 in a reasonably good yield (55%). Later the 2-methyl group on the quinoline ring was oxidized to aldehyde with SeO₂ and then the aldehyde was reduced back to the alcohol with NaBH₄ to obtain 11 with 60% yield (Scheme 2.5). Compound 11 has four coordination sites that can bind with metal atoms which is common for most reported Cr³⁺ sensors. Therefore, 11 is expected to be sensitive towards Cr³⁺ based on the knowledge from previous literature reports.

Compound 9: A solution of 7 (0.5 g, 1.1mmol) and thiophene-2-carboxaldehyde (0.16g, 1.4mmol) in 25 ml of ethanol was refluxed for 12 hours. The mixture was allowed to cool down to room temperature and the red crystals formed were separated by filtration, washed with ethanol and dried in air (0.49g), yield 81%. ¹H NMR (400MHz, CDCl₃): 1.14 (12H, t, J = 6.9 Hz), 3.31 (8H, q, J = 6.9 Hz), 6.23 (2H, dd, J = 8.8 Hz, 2.6 Hz), 6.43 (2H d, J = 2.2 Hz), 6.50 (2H, d, J = 8.8 Hz), 6.90 (1H, dd, J = 4.9 Hz, 3.7Hz), 7.06 (1H, d, J = 2.9 Hz), 7.12 (1H, dd, J = 6.2 Hz, 1.4 Hz), 7.22 (1H, d, J = 5.1 Hz), 7.47 (2H, m), 7.96 (1H, dd, J = 6.2 Hz, 1.4 Hz), 8.90 (1H, s). ¹³C NMR (100MHz, CDCl₃): ø 12.71, 44.41, 66.26, 97.98, 106.17, 108.00, 123.37, 123.98, 127.04, 127.88, 128.07, 128.39,
Compound 10: A solution of 7 (0.3 g, 0.65 mmol) and 3-formylchromone (0.114 g, 0.65 mmol) in ethanol (10 mL) was refluxed overnight. The mixture was allowed to cool down to room temperature and the precipitate was separated by filtration. The precipitate was washed with ethanol and air dried to obtain light brown powder (0.35 g, 88%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.14 (12H, t, $J = 6.5$ Hz), 3.30 (8H, q, $J = 6.9$ Hz), 6.23 (2H, d, $J = 8.4$ Hz), 6.46 (2H, s), 6.49 (2H, d, $J = 8.8$ Hz), 7.10 (1H, d, $J = 6.6$ Hz), 7.34 (1H, t, $J = 7.5$ Hz), 7.39 (1H, d, $J = 8.4$ Hz), 7.46 (2H, m), 7.59 (1H, t, $J = 7.5$ Hz), 7.98 (1H, d, $J = 6.9$ Hz), 8.14 (1H, d, $J = 8.0$ Hz), 8.43 (1H, s), 8.70 (1H, s). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 12.72, 44.39, 66.03, 98.14, 105.65, 108.02, 118.32, 119.95, 123.54, 123.95, 124.26, 125.49, 126.02, 127.96, 128.33, 128.86, 133.58, 133.69, 139.90, 149.03, 152.20, 153.26, 153.94, 156.18, 165.16, 175.63.

Compound 11: Compound 12 (50 mg, 0.078 mmol) was dissolved in 10 mL of ethanol and NaBH$_4$ (3 mg, 0.078 mmol) was added and the mixture stirred at room temperature for 5 hours. The solution was evaporated to dryness, the solid residue dissolved in ethyl acetate (25 ml) and extracted with water. The organic layer was separated, dried over MgSO$_4$ and evaporated to dryness to obtain 11 as pink solid (30 mg, 60%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 8.65 (1H, d, $J = 6.6$ Hz), 8.07 (1H, d, $J = 8.8$ Hz), 8.00 (1H, m), 7.78 (1H, d, $J = 8.4$ Hz), 7.47 (5H, m), 7.12 (1H, m), 6.90 (1H, d, $J = 8.8$ Hz), 6.38 (2H, dd, $J = 9.1$ Hz, 2.5 Hz), 6.29 (2H, d, $J = 2.5$ Hz), 4.27 (2H, s), 3.31 (8H, m), 1.13 (12H, t, $J = 6.9$ Hz). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 164.7, 164.0, 160.6, 153.6, 152.3, 149.0, 144.2, 138.4, 134.6, 132.9, 132.1, 129.4, 129.2, 128.2, 127.5, 127.4, 126.0, 123.9, 123.6, 118.8.
Compound 12: A solution of 12 (0.2 g, 0.32 mmol) in dioxane (20 mL) was heated to 60 °C and SeO₂ (70 mg, 0.64 mmol) was added. The mixture was heated at 80 °C for 4 hours. The reaction mixture was allowed to cool down to room temperature, filtered and evaporated to dryness. The crude product was purified on a silica gel column using EtOAc/Hexane (1:1) as the mobile phase to get 12 as a yellow solid (75 mg, 36%). 

H NMR (400 MHz, CDCl₃): 12.35 (1H, s), 9.03 (1H, s), 8.81 (1H, d, J = 7.3 Hz), 8.31 (1H, d, J = 8.4 Hz), 8.01 (1H, d, J = 5.8 Hz), 7.94 (1H, d, J = 8.0 Hz), 7.89 (1H, d, J = 8.8 Hz), 7.68 (1H, t, J = 8.0 Hz), 7.48 (2H, m), 7.14 (1H, d, J = 6.2 Hz), 6.90 (2H, d, J = 8.8 Hz), 6.39 (2H, d, J = 8.8 Hz), 6.26 (2H, d, J = 1.8 Hz), 3.31 (8H, m), 1.13 (12H, t, J = 6.9 Hz).

C NMR (100 MHz, CDCl₃): 192.4, 164.5, 163.1, 153.8, 152.1, 149.1, 144.5, 139.3, 135.9, 132.9, 132.2, 130.2, 129.4, 129.2, 129.0, 128.8, 128.2, 123.9, 123.6, 116.8, 107.9, 104.5, 97.4, 66.0, 44.3, 12.6. Analysis Calcd for C₃₉H₃₉N₅O₄.CHCl₃: C, 63.12; H, 5.30; N, 9.20. Found: C, 62.63; H, 5.49; N, 9.10. ESI-MS: 642.33 [M + H]⁺, 664.33 [M + Na]⁺, 680.25 [M + K]⁺.

Compound 13: A solution of 14 (0.49 g, 2.6 mmol) in dichloromethane (20 mL) at 0 °C was treated with DCC (0.82 g, 3.9 mmol) and DMAP (0.32 g, 2.6 mmol). The resulting mixture was stirred for 15 minutes before the addition of compound 7 (1.07 g, 2.3 mmol). The mixture was stirred at room temperature for 3 days and the precipitate was filtered off. The filtrate was evaporated and purified on a silica gel column using EtOAc/CHCl₃ (1:4) as the mobile phase to obtain 13 as white solid (0.78 g, 55%). 

Compound 14: To a refluxing solution of 2-aminobenzoic acid (2.25 g, 16.4 mmol) in 6 N HCl (34 mL), crotonaldehyde (1.6 mL, 19.4 mmol) was added drop wise over a period of 30 minutes. The resulting mixture was refluxed for another 2 h. After cooling down to room temperature, a solution of aqueous ammonia was added to raise the pH to 3 and the solution was extracted with CH$_2$Cl$_2$. The organic phase was evaporated to dryness and the crude sample was washed with ethanol to get 7 as a white solid (0.7 g, 23%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 8.75 (1H, dd, J = 7.3 Hz, 1.4 Hz), 8.27 (1H, d, J = 8.4 Hz), 8.03 (1H, dd, J = 8.0 Hz, 1.4 Hz), 7.68 (1H, t, J = 8.0 Hz), 7.44 (1H, d, J = 8.4 Hz), 2.83 (3H, s). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 167.6, 158.7, 145.0, 138.6, 135.2, 132.7, 126.5, 124.1, 122.8, 24.9.
Results and Discussion

2.6 Spectroscopic Studies

All the spectroscopic studies were performed in acetonitrile in which the sensors formed colorless solutions that were stable for more than one week. In addition, all the compounds were very weakly fluorescent at 580 nm (\(\lambda_{ex} = 510 \text{ nm}\)) in the absence of any analyte due to the predominant ring-closed spirolactone. Similarly, the absorption spectra showed no peak above 400 nm due to the predominance of ring-closed spirolactum.

The fluorescence spectrum of 9 showed a peak at 583 nm upon the addition of \(\text{Cr}^{3+}\) corresponding to the delocalization in the xanthene moiety of rhodamine. There was a significant emission intensity enhancement (> 1200 fold) with 1.0 equivalent of \(\text{Cr}^{3+}\) (Figure 2.6) which indicates that compound 9 is an excellent turn-on sensor for \(\text{Cr}^{3+}\). In addition, continuous addition of \(\text{Cr}^{3+}\) resulted in the enhancement of the emission band at 583 nm (Figure 2.3).

The absorption spectra also showed a new peak at 559 nm with a shoulder at 520 nm upon the addition of \(\text{Cr}^{3+}\) (Figure 2.4). Meanwhile the solution turned pink instantaneously as a result of the ring-open structure formation caused by \(\text{Cr}^{3+}\) binding. The Job’s plot (Figure 2.5) indicated a 1:1 binding stoichiometry between the sensor and \(\text{Cr}^{3+}\) with an association constant of 2.0 \(\times\) \(10^4\) M\(^{-1}\).
Figure 2.3: Fluorescence changes of 9 (10 μM) with Cr$^{3+}$ (0 – 13 μM) in CH$_3$CN.

Figure 2.4: UV absorption changes of 9 (10 μM) with Cr$^{3+}$ (0 – 12 μM) in CH$_3$CN.
It is very important for a good sensor system to have high selectivity. We tested our sensor for possible interferents including nitrate salts of Na$^+$, K$^+$, Ni$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$, Pb$^{2+}$ and chloride salts of Ca$^{2+}$, Mn$^{2+}$, Fe$^{3+}$, Co$^{2+}$, Cu$^{2+}$ (Figure 2.6). Sensor 9 showed a certain emission enhancement with Hg$^{2+}$ (< 600 fold, K = 1.0 x 10$^4$ M$^{-1}$) while Zn$^{2+}$ and Pb$^{2+}$ showed very weak responses (Figure 2.7). The fluorescent enhancement of sensor 9 with Cr$^{3+}$ was centered at 583 nm while with Zn$^{2+}$ has a maximum at 576 nm indicating a hipsochromic shift of 7 nm compared with Cr$^{3+}$. The hipsochromic shift w.r.t Cr$^{3+}$ is 10 nm for Pb$^{2+}$ where the fluorescent intensity centered at 573 nm. All these observations indicate that sensor 9 has high sensitivity and selectivity towards Cr$^{3+}$ over other metal ions tested.
Figure 2.6: Fluorescence changes of 9 (10 μM) with Na⁺, K⁺, Ca²⁺, Cr³⁺, Mn²⁺, Fe³⁺, Co²⁺, Ni²⁺, Cu²⁺, Cd²⁺, Hg²⁺, Pb²⁺ (10 μM) in CH₃CN (Excitation at 510 nm).

Figure 2.7: Fluorescence enhancement of 9 (10 μM) at 583 nm with metal ions (10 μM) in CH₃CN (Excitation at 510 nm).
Like most rhodamine-based spirolactam chemosensors, the binding of Cr\(^{3+}\) must be due to the ring opening mechanism. Chromium can chelate with carbonyl oxygen, imine nitrogen, and thiophene sulfur giving a pink color change (Figure 2.8). The other three coordination sites can be occupied by nitrate ligands. Furthermore, the sensing mechanism is found to be reversible as the pink color of the complex disappears with the addition of excess EDTA.

**Figure 2.8:** Photograph of compound 9 (10 \(\mu\)M) with different metal ions (6 \(\mu\)M), from left to right: 1 free, Na\(^+\), K\(^+\), Ca\(^{2+}\), Cr\(^{3+}\), Mn\(^{2+}\), Fe\(^{3+}\), Co\(^{2+}\), Ni\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\), Cd\(^{2+}\), Hg\(^{2+}\), Pb\(^{2+}\).

Introducing a formylchromone unit to compound 7 via an imine bond to produce 10 changed the sensing behavior. Figure 2.9 shows the absorption spectra of 10 in CH\(_3\)CN with 10 \(\mu\)M of various metal ions. The addition of 1 equivalent of Ni\(^{2+}\) showed a new absorbance peak at 550 nm with an immediate color change that can be visible to the naked eye. Interestingly, Ni\(^{2+}\) showed the highest absorbance enhancement (17 fold, Figure 2.10) while other metals showed no significant interference. This shows the excellent selectivity of 10 towards Ni\(^{2+}\).
**Figure 2.9:** UV-Vis spectra of compound 10 (10 μM) with metal ions (10 μM) in CH₃CN.

**Figure 2.10:** Absorbance enhancement of 10 (10 μM) at 550 nm with metal ions.
Figure 2.11: UV-Vis spectra of compound 10 (10 μM) with Ni$^{2+}$ (0 – 38 μM) in CH$_3$CN.

Figure 2.11 shows the absorbance titration of 10 with Ni$^{2+}$. Continuous addition of Ni$^{2+}$ resulted in increasing the absorbance peak at 550 nm. The sensor became saturated at 38 μM of Ni$^{2+}$ and showed no further absorbance enhancement. The binding constant was calculated based on the absorbance data using the Benesi-Hildebrand method by plotting $1/(A-A_0)$ against $1/[M^{2+}]$.\(^{43}\)

Interestingly, complex 10-Ni$^{2+}$ was not fluorescent at all due to the paramagnetic nature of Ni$^{2+}$. But, the absorption enhancement and the visible color change make 10 a selective sensor for Ni$^{2+}$. So finally we design a molecule, 11 that can detect both Cr$^{3+}$ and Ni$^{2+}$ simultaneously.

Fluorescence intensity changes of 11 (20 μM) upon the addition of metal ions (20 μM, 1 equiv.) in acetonitrile showed a remarkable sensitivity and selectivity towards Cr$^{3+}$ (Figure 2.12). The observed fluorescence enhancement at 580 nm ($\lambda_{ex} = 510$ nm) was
over 1100-fold, which is extremely high compared to other metals (Figure 2.13). Observed large fluorescence enhancement is attributed to the formation of ring-open spirolactum. In addition, the solution turned pink immediately, which is another advantage in that the sensor can be used as a naked eye sensor. Continuous addition of Cr$^{3+}$ resulted in increase in the fluorescent intensity until it became saturated with 36 μM of Cr$^{3+}$ (Figure 2.14).

High selectivity is always the key to a good sensor. In order to demonstrate the selectivity of this sensor system, we tested our sensors with other common metal ions including Na$^+$, K$^+$, Fe$^{2+}$, Fe$^{3+}$, Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, and Hg$^{2+}$. Interestingly, no significant fluorescence enhancement was observed with any of the metal ions tested during the experiment. Only Zn$^{2+}$ (54 fold) and Fe$^{3+}$ (40 fold) showed a slight fluorescence enhancement which is negligible compared to the huge enhancement observed for Cr$^{3+}$.

![Figure 2.12: Fluorescence spectra of 11 (20 μM) with metal ions (20 μM) in CH$_3$CN ($\lambda_e$ = 510 nm).]
Figure 2.13: Bar graph showing fluorescent enhancement of 11 (20 μM) at 580 nm (λ<sub>ex</sub> = 510 nm) with metals (20 μM).

Figure 2.14: Fluorescence spectra of 12 (20 μM) with Cr<sup>3+</sup> (0 - 36 μM) in CH<sub>3</sub>CN (λ<sub>ex</sub> = 510 nm).

Absorption data showed a completely different trend. The addition of 1 equivalent of Ni<sup>2+</sup> to a solution of 11 (20 μM) showed a new peak at 560 nm with a shoulder at 520 nm (Figure 2.17, 2.18). The absorption enhancement was dramatically high (183 fold)
with Ni$^{2+}$ compared to other metals (Figure 2.17). In addition, there is an immediate color change from colorless to pink (Figure 2.27). Absorption spectra of 11 were recorded with the continuous addition of Ni$^{2+}$ and showed a continuous increase in the absorption band at 560 nm (Figure 2.15). The linearity of the graph between $l/A-A_0$ vs $l/[Ni^{2+}]$ confirms the 1:1 binding between 11 and Ni$^{2+}$ and the association constant was found to be $4.25 \times 10^5$ M$^{-1}$ (Figure 2.16). Absorption data were used to calculate the binding constant for 11 with Cr$^{3+}$ (Figure 2.19) as it can be directly compared with the value for Ni$^{2+}$. The graph of $l/A-A_0$ vs $l/[Cr^{3+}]$ is a linear relationship confirming the 1:1 binding stoichiometry. The binding constant was found to be $3.47 \times 10^4$ M$^{-1}$. Apart from detecting Cr$^{3+}$ through the intense fluorescence change, 11 can be used as a very sensitive Ni$^{2+}$ sensor. To the best of our knowledge, this is the first rhodamine-based Ni$^{2+}$ sensor.

**Figure 2.15:** UV-Vis spectra of 11 (20 μM) with Ni$^{2+}$ (0 - 21 μM) in CH$_3$CN.
Figure 2.16: $1/A - A_0$ vs $1/[\text{Ni}^{2+}]$.

Figure 2.17: UV-Vis spectra of 11 (20 μM) with metal ions (20 μM) in CH$_3$CN.
**Figure 2.18:** Bar graph showing absorption enhancement of 11 (20 μM) at 560 nm with metals (20 μM).

**Figure 2.19:** Absorbance spectra of 11 (20 μM) with Cr³⁺ (0 - 36 μM) in CH₃CN.
Figure 2.20: Fluorescence spectra of 12 (20 μM) with metal ions (20 μM) in CH₃CN ($\lambda_{ex} = 510$ nm).

The fluorescence properties of 12 were also tested with metal ions in acetonitrile. It also formed a colorless solution in acetonitrile which is weakly fluorescent upon the excitation at 510 nm. The fluorescence profiles were very much similar to those for 11: once again Cr$^{3+}$ registered the highest fluorescence enhancement while other metals did not show any significant enhancement (Figure 2.20). Therefore both 11 and 12 are very selective and sensitive sensors for Cr$^{3+}$. It seems the hydroxyl functionality on the quinoline ring is not really important in Cr$^{3+}$ binding.

However, absorbance data showed an interesting trend. Compound 12 showed no absorbance band above 400 nm with any analyte in acetonitrile. The addition of 1 equivalent of Ni$^{2+}$ did not give any absorption enhancement, which is completely different than for 11. Oxidation of the methyl group on the quinoline ring to −CH$_2$OH dramatically changed the sensing behavior of the compound towards Ni$^{2+}$. Cu$^{2+}$ and Cr$^{3+}$ (20 μM) gave an absorption enhancement while other metals did not show any interaction.
(Figure 2.21). Both Cu$^{2+}$ and Cr$^{3+}$ showed an instantaneous color change with 12. Even though both Cr$^{3+}$ and Cu$^{2+}$ gave color change with 12, it is very easy to identify them separately due to the large fluorescence enhancement with Cr$^{3+}$.

![UV-Vis spectra of 12 (20 μM) with metal ions (20 μM) in CH$_3$CN.](image)

**Figure 2.21**: UV-Vis spectra of 12 (20 μM) with metal ions (20 μM) in CH$_3$CN.

As the 11-Cr$^{3+}$ complex is highly fluorescent, we choose it as a cyanide detector, via the metal displacement approach. In addition, cyanide complexes strongly with Cr$^{3+}$. Figure 2.22 shows the addition of 20 μM of CN$^-$ to a solution of 11-Cr$^{3+}$ (1:1) resulted in the complete quenching of the fluorescence at 580 nm due to the removal of Cr$^{3+}$ from 11. It seems the system is highly sensitive for CN$^-$ as the addition of 20 μM of CN$^-$ manages to chelate with all the Cr$^{3+}$ bound to 11 (Figure 2.23). Simultaneous absorbance quenching (Figure 2.24) is also observed with the addition of CN$^-$ which is compatible with the fluorescence quenching. The detection limit of 11-Cr$^{3+}$ for CN$^-$ calculated from fluorescence data was found to be 5.4 μM. Interestingly, the addition of 20 μM of CN$^-$ did not show any significant absorption change with non-fluorescent 11-Ni$^{2+}$ complex.
**Figure 2.22:** Fluorescence spectra of 11-Cr\(^{3+}\)  (1:1) with CN\(^-\), Cl\(^-\), Br\(^-\), I\(^-\), SO\(_4^{2-}\), Acetate (20 \(\mu\)M) (\(\lambda_{ex} = 510\) nm).

**Figure 2.23:** Fluorescence spectra of 11-Cr\(^{3+}\)  (1:1) with consecutive addition of CN\(^-\) (\(\lambda_{ex} = 510\) nm).
Figure 2.24: Absorbance spectra of 11-Cr$^{3+}$ (1:1) with consecutive addition of CN$^{-}$ (0 - 20 µM).

We have tested the 11-Cr$^{3+}$ for several other anions including Br$^{-}$, Cl$^{-}$, I$^{-}$, CH$_3$COO$^{-}$, and SO$_4^{2-}$, to determine the selectivity of the system towards CN$^{-}$ (Figure 2.22). None of these anions showed any significant fluorescence change, except acetate which showed slight quenching, indicating the sensor system is highly selective towards CN$^{-}$. In addition, the visible color change from pink to colorless allows the naked eye identification of CN$^{-}$ (Figure 2.28).

Both compound 11 and 12 showed approximately equal fluorescence enhancements with Cr$^{3+}$ indicating that the hydroxyl group on the quinoline ring is not very important in Cr$^{3+}$ binding. On the other hand, compound 11 showed a very high affinity towards Ni$^{2+}$ indicating the importance in Ni$^{2+}$ binding. Therefore, we studied the binding of Ni$^{2+}$ with compound 11 using $^1$H NMR. A mixture of CD$_3$CN and CDCl$_3$ was used due to the limited solubility of 11 in CD$_3$CN at higher concentrations. Initially a $^1$H-
$^1$H COSY experiment was performed to identify the relationship between peaks (Figure 2.25).

**Figure 2.25:** $^1$H-$^1$H-COSY spectrum of 11 and some important COSY relationships.
Addition of 1 equivalent of Ni$^{2+}$ resulted in the disappearance of the hydroxyl proton at δ 3.05 indicating the involvement of OH group in binding with Ni$^{2+}$. In addition, there was a broadening of peaks at δ 8.06, 7.76, and 7.43 corresponding to H$_b$, H$_c$, and H$_a$ respectively. After the addition of 4 equivalents of Ni$^{2+}$, the triplet at δ 7.35 corresponding to H$_d$ also broadened (Figure 2.26). This behavior is probably due to the binding of the ring nitrogen with Ni$^{2+}$. Subsequent addition of Ni$^{2+}$ also resulted in decreasing the intensities and broadening of the xanthene protons at δ 6.63 (H$_h$), 6.20 (H$_g$), and 6.07 (H$_f$) due to the formation of the highly fluorescent ring-open form (Scheme 2.6).
**Scheme 2.6:** Proposed binding mechanism of 11 with Ni$^{2+}$.

**Figure 2.27:** A photograph of 11 (20 μM) with 1 μM of metal ions (From left to right: 11 free, Ni$^{2+}$, Na$^+$, K$^+$, Cr$^{3+}$, Fe$^{2+}$, Fe$^{3+}$, Co$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Cd$^{2+}$, Hg$^{2+}$).

**Figure 2.28:** A photograph of 11 (20 μM), alone (left), with 20 μM of Cr$^{3+}$ (middle), with 20 μM of Cr$^{3+}$ and CN$^-$ (right).
Conclusion

In conclusion, we have synthesized three new fluorescent chemosensors 9, 10, and 11, which are very stable in CH$_3$CN for more than one week. Sensor 9 showed high sensitivity and selectivity towards Cr$^{3+}$ over other interference cations except Hg$^{2+}$, which shows a significant but smaller effect. We were able to use the ring opening mechanism of the new rhodamine B derivatives to develop a new sensor for Cr$^{3+}$. On the other hand, sensor 10 was very selective for Ni$^{2+}$ which forms a nonfluorescent 10-Ni$^{2+}$ complex. Even though it is nonfluorescent, it is highly colored and the high absorption enhancement enables the selective identification of Ni$^{2+}$ over other metal ions. Compound 11 is a selective sensor for both Cr$^{3+}$ and Ni$^{2+}$ as it forms a fluorescent complex with Cr$^{3+}$ while the 11-Ni$^{2+}$ complex is nonfluorescent. As both Ni$^{2+}$ complexes are nonfluorescent, we can expect they adopt tetrahedron or octahedron geometry. If the complexes are in the octahedron geometry, the rest of the coordination site has to be occupied by counter ions (Cl$^-$). The fluorescent 11-Cr$^{3+}$ complex can be used to detect CN$^-$ with the detection limit of 5.4 μM.
CHAPTER 3

RHODAMINE AND PYRENE BASED FRET-ON SENSOR FOR Hg$^{2+}$

3.1 Detection of Hg$^{2+}$

Mercury has long been known as a toxic metal for humans and the environment.\textsuperscript{78} Mercury is used in thermometers, barometers, even some electrical switches and as a preservative in vaccines and food. The toxicity of mercury led to reduced usage of mercury thermometers in clinical applications. Mercury contamination is widespread and results from natural sources like volcanic eruption as well as human activities.\textsuperscript{79} Elemental and ionic mercury released into the environment is frequently converted into the more toxic methylmercury by chemical and microbial activities. Methylmercury is extremely toxic and can be biomultiplied up the food chain until it bioaccumulates in the human body.\textsuperscript{80-82} Therefore, the detection of mercury is extremely important.

3.2 Fluorescence Resonance Energy Transfer (FRET) Based Sensors for Hg$^{2+}$

Recently, fluorescent chemosensors for the detection of mercury\textsuperscript{29 - 35, 83} have gained considerable interest due to their simplicity, sensitivity, and selectivity. In addition, they are easy to use and there is no need for trained personnel. In addition, several fluorescence resonance energy transfer (FRET)-based sensors for the detection of mercury have been developed in the recent past.\textsuperscript{74, 75, 84} FRET occurs when the excited donor fluorophore transfers energy to the acceptor fluorophore without photoemission.
There should be a certain degree of spectral overlap between the donor emission spectrum and acceptor absorption spectrum. In addition, the distance between the two chromophores is very critical and it has to be 10-100 Å.

Othman et al have developed a rhodamine and pyrene-based FRET sensor for the detection of Hg$^{2+}$ (Figure 3.1). Rhodamine and pyrene can form a FRET system due to their spectral overlap. The sensor is a complex molecule bearing two calix[4]arene molecules with one pyrene unit each condensed to two arms of the Tris(2-aminoethyl)amine. The third arm is occupied by a rhodamine moiety. Mercury-binding causes the FRET-ON due to the formation of a ring-open form of rhodamine. Calix[4]arene units work as the major frame work that holds the two fluorophores together. The sensor also can bind with Al$^{3+}$ but, it does not turn on the FRET process. That is mainly due to the binding mechanism which does not involve rhodamine.

Figure 3.1: Different complexation mechanism of the sensor with Hg$^{2+}$ and Al$^{3+}$. 
Similar to the previous example, Lee et al\textsuperscript{84} have developed another FRET-ON sensor for Hg\textsuperscript{2+} based on same building blocks (Figure 3.2). One striking difference is the use of one calix[4]arene unit which makes the system less complicated. Lee et al\textsuperscript{84} synthesized two different molecules with different orientations. One compound orients two pyrene units parallel to each other giving high excimer emission. The other compound orients two pyrene units far apart giving weak excimer emission. As shown in figure 3.2, the compound with high excimer emission binds with Hg\textsuperscript{2+} with 1:2 stoichiometry and gives a strong FRET signal. One Hg\textsuperscript{2+} atom binds in between two pyrene units bringing them closer while the other one binds with rhodamine. The other compound binds with Hg\textsuperscript{2+} with 1:1 stoichiometry, but gives a weak FRET signal.

![Figure 3.2: Complexation behavior of Hg\textsuperscript{2+}.](image)

Chen et al\textsuperscript{85} have developed a much simpler but effective sensor for Hg\textsuperscript{2+} as shown in figure 3.3. The synthesis is much less complicated compared to the previous two examples. The crystal structure of the sensor with Hg\textsuperscript{2+} proves the 2:1 binding mode.
between sensor and Hg$^{2+}$. The sensor is highly selective for Hg$^{2+}$ while the previous two had interference from Pb$^{2+}$.

Figure 3.3: Hg$^{2+}$ sensor by Chen et al.\textsuperscript{85}

After thorough investigation of the current Hg$^{2+}$ sensors, I decided to design a FRET-based sensor. As shown in Scheme 3.1, the sensor contains two pyrene units and a rhodamine moiety connected through a tren molecule. Again the synthesis is much less complicated compared to the first two FRET sensors. Compound 15 was obtained in two steps with a reasonable yield.
Scheme 3.1: Synthesis of 15.

3.3 Synthesis of 15

Compound 16: Compound 16 was synthesized based on a literature procedure. Rhodamine B (0.5 g, 1.04 mmol) and Tris(2-aminoethyl)amine (3.78 g, 25.9 mmol) were mixed in 10 ml of ethanol. The reaction mixture was refluxed for 5 hours. The solvent was evaporated under vacuo and the crude product was dissolved in CH$_2$Cl$_2$ (100 ml). The solution was extracted with 100 ml of water and the organic phase was separated, dried over MgSO$_4$, and evaporated to dryness to get 16 (0.41 g, 70%). $^1$H NMR (400
MHz, CDCl$_3$) $\delta$: 7.86 (1H, m), 7.46 (2H, m), 7.01 (1H, m), 6.37 (2H, d, $J = 2.2$ Hz), 6.36 (2H, d, $J = 4.0$ Hz), 6.25 (2H, m), 3.32 (8H, q, $J = 6.9$ Hz), 3.15 (2H, t, $J = 6.2$ Hz), 2.66 (4H, t, $J = 5.1$ Hz), 2.42 (4H, t, $J = 5.4$ Hz), 2.16 (2H, t, $J = 6.9$ Hz), 1.15 (12H, t, $J = 6.9$ Hz). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$: 168.2, 153.6, 153.0, 148.9, 132.6, 131.5, 128.9, 128.3, 123.9, 122.9, 108.2, 105.5, 97.7, 65.4, 54.3, 52.1, 44.4, 38.8, 37.9, 12.6.

Compound 15: A solution containing pyrene-1-carboxylic acid (1.3 g, 5.28 mmol) was treated with DCC (1.07 g, 5.3 mmol) and DMAP (0.63 g, 5.2 mmol). The resulting solution was stirred at 0 °C for 15 minutes. After 15 minutes, the solution was treated with compound 16 (1.19 g, 2.08 mmol) and the resulting mixture was stirred at room temperature for 12 hours. The solution was filtered and evaporated to dryness and the crude mixture was purified over a silica gel column using ethyl acetate as the mobile phase to get 15 as a white solid (0.56 g, 26%).$^1$H NMR (400 MHz, CDCl$_3$) $\delta$: 8.40 (2H, t, $J = 5.1$ Hz), 8.37 (2H, d, $J = 9.1$ Hz), 8.26 (2H, d, $J = 7.7$ Hz), 8.16 (2H, d, $J = 7.3$ Hz), 8.11 (2H, d, $J = 9.1$ Hz), 8.03 (2H, t, $J = 7.7$ Hz), 7.97 (2H, d, $J = 9.5$ Hz), 7.91 (2H, d, $J = 9.1$ Hz), 7.83 (4H, q, $J = 8.0$ Hz), 7.71 (1H, d, $J = 7.3$ Hz), 7.46 (2H, m), 7.03 (1H, d, $J = 6.9$ Hz), 6.33 (2H, d, $J = 2.2$ Hz), 3.36 (4H, m), 3.19 (10H, m), 2.67 (4H, t, $J = 5.3$ Hz), 2.29 (2H, m), 0.95 (12H, t, $J = 6.6$ Hz). $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$: 169.3, 167.0, 153.5, 153.4, 148.9, 133.1, 132.4, 131.7, 131.5, 131.1, 130.6, 128.9, 128.8, 128.5, 128.2, 128.1, 127.4, 126.8, 126.1, 125.9, 125.4, 125.2, 124.5, 124.2, 124.1, 124.0, 122.7, 108.6, 105.5, 97.9, 64.7, 53.5, 52.1, 44.1, 37.9, 12.8. Anal. Calcd for C$_6$H$_{62}$N$_6$O$_4$.H$_2$O. C: 78.14, H: 6.17, N: 8.04. Found. C: 78.38, H: 6.73, N: 8.35. ESI-MS: 1027.42 [M+H]$^+$
3.4 Synthesis and Characterization of 15

Compound 16 was synthesized according to a literature procedure. Compound 15 was obtained by reacting 16 with pyrene-1-carboxylic acid in the presence of DCC and DMAP. Purification of the crude mixture using column chromatography yielded 15 in 26% yield. The structure of 15 was confirmed by \(^1\)H NMR, \(^{13}\)C NMR, ESI-MS, and elemental analysis. All the spectroscopic studies were performed in acetonitrile in which 15 formed a colorless solution. Compound 15 showed two emission bands at 400 nm and 480 nm for pyrenyl monomer and excimer emission respectively. Initial fluorescence intensities of the two peaks were similar. That is due to the orientation of the two pyrenyl rings far apart from each other. Generally, this kind of system does not give strong FRET effect upon binding with analyte.

3.5 Spectroscopic Studies of 15

Fluorescence spectra of 15 were taken with 44 \(\mu\)M of metal ions, which upon the excitation at 340 nm showed an increase in intensity of both emission bands (Figure 3.4). The pyrenyl excimer emission was slightly higher in intensity compared to the pyrenyl monomer emission. In addition, there was a 20 nm red shift in the eximer band. There was a 4.6 fold emission enhancement at 400 nm while a higher enhancement (8.2 fold) was observed at 500 nm. In addition, \(\text{Cr}^{3+}\) also showed a slight emission enhancement at both 400 nm and 500 nm. These results suggest that \(\text{Al}^{3+}\) binds in between two pyrenyl units, making the system more rigid which results in the increase in pyrenyl monomer emission. It appears that the banding makes the two pyrenyl units come slightly closer as
the intensity of the excimer emission is higher than that of the monomer emission. No color change was observed, confirming that there is no interaction with the rhodamine moiety.

Figure 3.4: Fluorescence spectra of 15 (12.5 μM) with metal ions (44 μM) in acetonitrile ($\lambda_{ex} = 340$ nm).

Continuous addition of Al$^{3+}$ showed a continuous increase in intensity of both emission bands (Figure 3.5). Interestingly, at higher Al$^{3+}$ concentrations, both emission bands showed an almost similar intensity suggesting the effect of Al$^{3+}$ binding to bring the two pyrenyl units closer is minimal. The enhancement of both pyrenyl monomer and excimer emission is possible due to the chelation-enhanced fluorescence (CHEF). In the absence of Al$^{3+}$, the pyrenyl monomer and excimer emissions are quenched due to the PET process which involves the unshared electrons of tertiary nitrogen of tren.$^{14}$
The linearity of the graph between $F_0/F-F_0$ vs $1/[Al^{3+}]$ suggested the 1:1 binding stoichiometry between 1 and Al$^{3+}$. The association constant calculated using the Benesi-Hilderbrand$^{43}$ equation was found to be $1.07 \times 10^4$ M$^{-1}$.

![Fluorescence spectra of 15 (12.5 μM) with Al$^{3+}$ (0 – 67 μM) in acetonitrile ($\lambda_{ex} = 340$ nm).](image)

**Figure 3.5:** Fluorescence spectra of 15 (12.5 μM) with Al$^{3+}$ (0 – 67 μM) in acetonitrile ($\lambda_{ex} = 340$ nm).

The fluorescence spectra of 15 recorded with 2 mM of metal ions (Hg$^{2+}$, Pb$^{2+}$, Cr$^{3+}$, Zn$^{2+}$, Fe$^{3+}$, Co$^{2+}$, Ni$^{2+}$, Cu$^{2+}$) showed a new emission band at 579 nm for Hg$^{2+}$ (Figure 3.6). This is due to the formation of the ring-open spirolactum of the rhodamine moiety. The visual color change from colorless to pink also confirmed the formation of the highly fluorescent ring-open form of spirolactum. The addition of 2 mM of Pb$^{2+}$ also gave a slight fluorescence enhancement at 579 nm which, however, is very low compared to Hg$^{2+}$. The absorption spectrum also showed a new peak at 550 nm characteristic of the ring-open form of rhodamine on addition of Hg$^{2+}$. When 15 is excited at 343 nm, the pyrenyl units give rise to both monomer and excimer emission and the pyrenyl excimer
transfer to rhodamine to give a fluorescence band at 579 nm. That behavior is due to the FRET effect.

**Figure 3.6**: Fluorescence spectra of 15 (12.5 μM) with 2 mM of metal ions in acetonitrile (λ<sub>ex</sub> = 340 nm) after 10 minutes.

Continuous addition of Hg<sup>2+</sup> showed a continuous increase in intensity of the emission band at 579 nm (Figure 3.7). At the same time, the emission band at 500 nm showed a slight decrease in intensity due to the weak FRET effect. That observation is clear in Figure 3.10 which shows the emission change over time. The binding between 15 and Hg<sup>2+</sup> is not instantaneous, but happens over a period of time. This observation was monitored by measuring the fluorescence with time (Figure 3.10). Figure 3.11 shows the changing intensity of the two peaks (500 nm and 579 nm) with time while the Figure 3.12 shows the ratio of I<sub>579</sub>/I<sub>500</sub> changing with time. Both confirm the weak FRET interaction between two pyrenyl units and the rhodamine moiety. The enhancement of both pyrenyl monomer and excimer emission can be due to the CHEF effect upon binding with Hg<sup>2+</sup>. Therefore CHEF may be playing a bigger role than FRET in this sensor system.
The linearity of the graph between \( F_0/F - F_0 \) vs \( 1/[Hg^{2+}] \) confirmed the 1:1 binding stoichiometry between 15 and Hg\(^{2+}\) (Figure 3.9). The binding constant, calculated using the Benesi-Hilderbrand equation, was found to be 70 M\(^{-1}\).

The absorption spectra of 15 with Hg\(^{2+}\) showed the characteristic rhodamine absorption around 550 nm due to the ring-open spirolactum (Figure 3.8). The absorption intensity enhanced with the increasing concentration of Hg\(^{2+}\). This is due to the involvement of the carbonyl oxygen of the rhodamine with Hg\(^{2+}\) which leads to the formation of highly fluorescent ring-open form.

![Fluorescence spectra of 15 (12.5 \( \mu \)M) with Hg\(^{2+}\) (0 – 1.2 mM) in acetonitrile (\( \lambda_{ex} = 340 \) nm).](image)

**Figure 3.7:** Fluorescence spectra of 15 (12.5 \( \mu \)M) with Hg\(^{2+}\) (0 – 1.2 mM) in acetonitrile (\( \lambda_{ex} = 340 \) nm).
Figure 3.8: Absorption spectra of 15 (12.5 μM) with Hg$^{2+}$ (0 – 1.2 mM) in acetonitrile.

Figure 3.9: F$_0$/F-F$_0$ vs 1/[Hg$^{2+}$] for the binding of 15 with Hg$^{2+}$. 
Figure 3.10: Fluorescence spectra of 15 (12.5 μM) with Hg$^{2+}$ (1 mM) in acetonitrile ($\lambda_{ex} = 340$ nm) taken at 10 minute interval.

Figure 3.11: Change in fluorescence intensity of peaks at 500 nm and 579 nm over time.
Figure 3.12: $I_{579}/I_{500}$ over time.

The binding mechanism can be predicted based on literature reports\textsuperscript{94} and the fluorescence data. We can expect Hg$^{2+}$ to bind with the nitrogen atoms of tren and the carbonyl oxygen of the rhodamine unit. This type of binding can cause the formation of the ring-open form of the rhodamine and makes the two pyrene units be arranged slightly closer than before resulting in the weak FRET interaction. However, modifications are needed to get a strong FRET interaction upon binding with Hg$^{2+}$. Aluminum binding is completely different from Hg$^{2+}$ binding as it does not involve the rhodamine unit (Figure 3.13).
Figure 3.13: Sensing mechanism of 15.
Conclusion

A selective $\text{Hg}^{2+}$ sensor was synthesized successfully from a two step procedure with reasonable yield. The sensing mechanism involves FRET and CHEF processes. Clear visible color change from colorless to pink enables the use of 15 as a naked eye sensor. Aluminum binding between pyrenyl units results in the initiation of a CHEF process.
CHAPTER 4

SINGLE AND MULTIPHOTON TURN-ON FLUORESCENT SENSORS FOR \( \text{Fe}^{3+} \) AND \( \text{Cu}^{2+} \)

4.1 Detection of \( \text{Fe}^{3+} \) and \( \text{Cu}^{2+} \)

Detection of transition metals has become important due to their vital role in biological and environmental applications.\(^{28,45,86-88}\) Detection of trace amounts of \( \text{Fe}^{3+} \) is of great importance as it is an important transition metal for all organisms and disorders in its metabolism can cause anemia, liver and kidney damage, diabetes and heart failure.\(^{89,90}\) Very few sensors for \( \text{Fe}^{3+} \) have been reported despite its importance in many biochemical processes at the cellular level.\(^{27,91-95}\) \( \text{Fe}^{3+} \) is a well known fluorescence quencher due to its paramagnetic nature, which makes it difficult to develop a turn-on fluorescent sensor, especially a sensitive one. Several methods have been reported for detecting iron including atomic absorption spectroscopy,\(^{96}\) colorimetry,\(^{97}\) spectrophotometry,\(^{98-100}\) and voltammetry\(^{101}\) techniques, which generally require sophisticated equipment, tedious sample preparation procedures and trained operators.

Copper is also an important trace element in biological systems, involved in vital processes in many organisms.\(^{102-104}\) In fact, it is the third most abundant essential heavy [i.e not counting \( \text{Na}^+, \text{K}^+, \text{Ca}^{2+}, \text{Mg}^{2+} \) etc] metal (after \( \text{Fe}^{3+} \) and \( \text{Zn}^{2+} \)) present in the human body. However, the accumulation of large excesses of copper in various organs, including brain and liver, is highly toxic and is brought on by Wilson’s disease in humans.\(^{105}\)
Development of Fe$^{3+}$ sensors using rhodamine derivatives has increased in the recent past. Xiang et al$^{27}$ have developed a Fe$^{3+}$-selective sensor based on two rhodamine moieties linked together through a flexible linker. The sensor showed an excellent selectivity towards Fe$^{3+}$ (Scheme 4.1) while Cr$^{3+}$, Cu$^{2+}$, and Fe$^{2+}$ showed a slight interference.$^{27}$

\[ \text{X} + \text{Fe}^{3+} \rightarrow \text{X} + \text{Fe}^{3+} \]

\text{Scheme 4.1: Mechanism of Fe}^{3+} \text{ binding with X.}

"Naked eye" sensors for the detection of these metals have advantages over other methods in being easy to operate, portable and not requiring sophisticated instrumentation. In the recent past, several attempts have been made to develop chemosensors to detect paramagnetic species.$^{106} \cdot 112$ Among different sensors,
rhodamine-B derivatives and their ring-open reaction have received greater attention following the report of a rhodamine-B hydrazine sensor for Cu^{2+} by Czarnik et al\textsuperscript{25} (Scheme 4.2). The rate at which the hydrolysis takes place by Cu^{2+} is much greater than it is for other metal ions.\textsuperscript{25} This work triggered a whole new generation of metal ion sensors. Interestingly, compound Y has been used widely as the starting material for most of the metal ion sensors published after 1997.

![Scheme 4.2: Mechanism of Cu^{2+} binding with Y.](image)

4.2 Rhodamine Derivatives as Chemosensors

Rhodamine B derivatives have received a great deal of attention as chemosensors because of their useful properties such as high absorption coefficient, high fluorescent quantum yield for excitation and emission wavelength within the visible region. Moreover, rhodamine B derivatives can undergo equilibrium between their spirocyclic and ring-open forms, which have completely different fluorescent properties. This phenomenon gives them an excellent potential for the development of turn-on fluorescent sensors.\textsuperscript{22} In all these sensors, the mechanism involves the formation of a ring-opened form of the spirolactam upon cation binding, resulting in fluorescence enhancement (550-600 nm).

Later in 2006, Xiang et al\textsuperscript{24} developed a new Cu^{2+} sensor based on a rhodamine hydrazide which employed the same mechanism in Cu^{2+} binding. In this sensor design,
they linked a 2-hydroxybenzaldehyde group to compound Y through an imine linkage. The final sensor functions as a tridentate ligand which binds to Cu$^{2+}$ with OH, imine N, and carbonyl O (Scheme 4.3).$^{24}$

![Scheme 4.3: Mechanism of Cu$^{2+}$ binding with Z.](image)

We decided to use similar bis rhodamine sensors, but chose aromatic, relatively non flexible linkers in order to get higher fluorescence enhancements. Sensor 17 bears two rhodamine moieties linked together through a pyridine molecule at 2 and 6 positions while compound 18 and 19 used a benzene ring to attach two rhodamine groups at 1,3 and 1,2 positions respectively (Scheme 4.4).$^{26}$

Although fluorescence signals and their enhancement can be investigated with fluorescent molecular sensors, conventional fluorescence microscopy for imaging metal ions in biological systems with visible light is resolution-limited due to image blurring by out-of-focus light. This limitation can be rectified with two photon laser scanning microscopy, which needs good sensors to show enhanced two-photon excited fluorescence.$^{113,114}$ Further, multiphoton or two photon excitation-based sensing can be used to detect analytes in a stand-off excitation and detection approach which will be utilized to probe complex biological and environmental conditions. Multiphoton excitation uses near infrared light which has lower scattering and higher penetration depth, even in cloudy and biological environments.$^{115}$ Recently, multiphoton excitation
Two-photon turn-on sensors have also been designed for Zn\(^{2+}\) as well as for some biological molecules.\(^{117\text{--}119}\) In the present investigation, sensors are carefully engineered so that they are not only good sensors with one-photon excitation but also excellent two-photon turn-on sensors. Our sensors are based on derivatives of rhodamine, as these possess large two-photon cross-sections for commercially available dye molecules. We studied the single- and multi-photon properties of compound 17 and 18 and report very high sensitivity and selectivity towards Fe\(^{3+}\) over other metal ions.

**Experimental**

Scheme 4.4: Synthetic pathway of 17, 18, and 19.
4.3 Materials and Synthesis of 17, 18, and 19

Materials: Rhodamine B was purchased from Alfa Aesar. All the other chemicals were of reagent grade purchased from Sigma Aldrich and used without further purification. Nanopure water was used for making buffer solutions.

Synthesis of 17: A solution of 7 (0.74 g, 1.62 mmol) and pyridine-2,6-dicarboxaldehyde (0.1 g, 0.74 mmol) in 20 ml of ethanol was refluxed for 12 hours. The mixture was allowed to cool to room temperature. The solid formed was filtered off and air dried (0.64 g, 85.4%). Mp = 266-268 °C. 

\[ \text{\(^{1}H\ NMR\ (400 MHz, CDCl\textsubscript{3})\): } \delta 1.12 (24H, t, J = 6.9 Hz), \quad 3.29 (16H, q, J = 6.9 Hz), \quad 6.19 (4H, dd, J = 8.8 Hz, 2.6 Hz), \quad 6.43 (4H, d, J = 2.6 Hz), \quad 6.47 (4H, d, J = 8.8 Hz), \quad 7.11 (2H, d, J = 7.3 Hz), \quad 7.46 (5H, m), \quad 7.71 (2H, d, J = 7.7 Hz), \quad 7.96 (2H, d, J = 6.6 Hz), \quad 8.71 (2H, s). \]

\[ \text{\(^{13}C\ NMR\ (100 MHz, CDCl\textsubscript{3})\): } \delta 12.74, 44.39, 66.30, 98.20, 106.07, 107.94, 120.41, 123.56, 123.98, 127.80, 128.32, 129.16, 133.56, 135.94, 148.18, 148.97, 151.89, 153.29, 154.15, 165.09. \]

ESI-MS. 1034.67 [M+Na]. Analysis calcd: C, 74.75; H, 6.47; N, 12.45. Found: C, 74.83; H, 6.86; N, 12.65.

Synthesis of 18: A solution of 7 (1.0 g, 2.2 mmol) and isophthalaldehyde (0.13 g, 0.97 mmol) in 20 ml of ethanol was refluxed for 12 hours. The mixture was allowed to cool to room temperature. The solid was filtered off and air dried (0.78 g, 79.6%). Mp = 244-246 °C. 

\[ \text{\(^{1}H\ NMR\ (400 MHz, CDCl\textsubscript{3})\): } \delta 1.13 (24H, t, J = 6.9 Hz), \quad 3.29 (16H, q, J = 6.9 Hz), \quad 6.18 (4H, d, J = 8.8 Hz), \quad 6.42 (4H, s), \quad 6.49 (4H, d, J = 8.8 Hz), \quad 7.08 (2H, d, J = 7.3 Hz), \quad 7.17 (1H, t, J = 7.7 Hz), \quad 7.43 (5H, m), \quad 7.56 (2H, d, J = 7.7 Hz), \quad 7.97 (2H, d, J = 6.9 Hz), \quad 8.33 (2H, s). \]

\[ \text{\(^{13}C\ NMR\ (100 MHz, CDCl\textsubscript{3})\): } \delta 12.73, 44.39, 65.87, 98.19, 105.75, 108.03, 123.49, 123.74, 127.70, 127.90, 128.13, 128.23, 128.30, 128.59, 133.49, 135.45, 146.13, \]
Synthesis of 19: A solution of 7 (0.5 g, 1.1 mmol) and phthalaldehyde (0.058g, 0.44 mmol) in 20 ml of ethanol was refluxed for 5 hours. The mixture was allowed to cool to room temperature and filtered. The precipitate was washed with ethanol and dried in air (0.21 g, 47.2%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 1.11 (t, $J$ = 6.9 Hz, 24H), 3.28 (q, $J$ = 6.9 Hz, 16H), 6.21 (dd, $J$ = 8.8 Hz, 2.6 Hz, 4H), 6.43 (d, $J$ = 2.2 Hz, 4H), 6.49 (d, $J$ = 8.8 Hz, 4H), 7.11 (m, 2H), 7.14 (m, 2H), 7.47 (m, 4H), 7.59 (dd, $J$ = 5.8Hz, 3.3Hz, 2H), 7.99 (m, 2H), 9.84 (s, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 12.73, 44.39, 66.68, 98.08, 107.08, 107.85, 123.59, 124.12, 125.95, 128.18, 128.29, 129.18, 130.77, 133.03, 134.27, 146.83, 148.73, 153.66, 164.51. ESI-MS. 1012 [M+1$^+$]. Analysis calcd for C$_{64}$H$_{66}$N$_8$O$_4$H$_2$O: C, 74.68; H, 6.66; N, 10.89. Found: C, 74.66; H, 6.93; N, 11.07.

Results and Discussion

4.4 Preparation of Metal Ion Solutions for UV Absorption and Emission Studies

The stock solutions of metal ions (5x10$^{-3}$M) were prepared using nitrates [Cr(NO$_3$)$_3$, Zn(NO$_3$)$_2$, Ni(NO$_3$)$_2$, Pb(NO$_3$)$_2$, Cd(NO$_3$)$_2$, Hg(NO$_3$)$_2$, NaNO$_3$, KNO$_3$] or chlorides [MnCl$_2$, CuCl$_2$, FeCl$_3$, FeCl$_2$, CaCl$_2$]. All the solutions were prepared in water. The stock solutions of compounds 17, 18, and 19 (0.2mM) were also prepared in 75% CH$_3$CN, 25% 0.01 M Tris-HCl buffer. Fluorescence and UV absorption studies were performed using a 5 µM solution of 17, 18, and 19 in 75% CH$_3$CN, 25% 0.01 M Tris-HCl buffer with appropriate amounts of metal ions. The solutions were shaken for 1 minute and allowed to stand for another 2 minutes before measuring the absorption and fluorescence.
4.5 Steady State Optical Properties

As shown in Scheme 4.4, compounds 17, 18, and 19 were synthesized in high yield by refluxing \(7^{24}\) with pyridine-2,6-dicarboxaldehyde, isophthalaldehyde, and phthalaldehyde in ethanol respectively. Their structures were confirmed using \(^1\)H NMR, \(^{13}\)C NMR, ESI mass spectrometry and elemental analysis. All the spectroscopic studies were performed in 75% CH\(_3\)CN, 25% 0.01 M Tris-HCl buffer system (pH = 7.00). All three compounds were colorless and found to be very stable in the abovementioned solution system for more than one week. Furthermore, the stabilities of these compounds were tested over a wide range of pH and an increasing color change was observed with increasing acidity, indicating the formation of the ring-opened form (Figure 4.1). The absorption spectra of all compounds in buffer solutions did not show any peaks above 400 nm, indicating that the ring-closed spirolactone is predominant. In addition, a very weak fluorescence signal was observed at 580 nm upon excitation at 510 nm, confirming the presence of ring-closed spirolactone. The presence of the ring-closed form is further confirmed by the characteristic carbon resonances around δ 65 ppm. All the sensing and optical measurements were performed in buffer solution with a pH of 7 to keep the dye molecules in their ring-closed form.
**Figure 4.1:** Stability of compounds 17, 18, and 19 at different pH.

Figure 4.2 shows the absorption spectra of all three compounds (5 μM) in solution and in the presence of Cu$^{2+}$ and Fe$^{3+}$. There is no absorption observed above 500 nm in the absence of metal ions indicating that only the ring-closed form is present. Addition of Fe$^{3+}$ (200 μM) resulted in the appearance of the characteristic rhodamine B absorption at 560 nm with a shoulder at 520 nm due to the formation of the ring-opened spirolactam forms. Interestingly, all three compounds responded quite similarly with Fe$^{3+}$ (Figures 4.2, 4.3, 4.5, 4.7). On the other hand, Cu$^{2+}$ also yielded a significant absorption enhancement with 17 (Figures 4.2, 4.4) but a much lower enhancement with 18 (Figures 4.2, 4.6). However, the absorption increase at the same concentrations (200 μM) is lower than with Fe$^{3+}$. The results can be attributed to the differences in the association constants for 17 and 18 with Fe$^{3+}$ and Cu$^{2+}$. However, the behavior is very much different for 19 with Cu$^{2+}$ which showed the highest absorbance enhancement (180-fold) compared to
other two compounds. On the other hand, there is a clear blue shift in the absorbance from 563 nm to 551 nm.

**Figure 4.2:** UV-vis absorption spectra of 17, 18, and 19 (5 μM) with Fe$^{3+}$ and Cu$^{2+}$ (200 μM).

**Figure 4.3:** UV-Vis spectra of 17 with Fe$^{3+}$ (0 – 450 μM) in 75% CH$_3$CN, 25% 0.01 M Tris HCl buffer.
Figure 4.4: UV-Vis spectra of 17 with Cu$^{2+}$ (0 - 450 μM) in 75% CH$_3$CN, 25% 0.01 M Tris HCl buffer.

Figure 4.5: UV-Vis spectra of 18 with Fe$^{3+}$ (0 - 400 μM) in 75% CH$_3$CN, 25% 0.01 M Tris HCl buffer.
Figure 4.6: UV-Vis spectrum of 18 with Cu$^{2+}$ (0 – 350 μM) in 75% CH$_3$CN, 25% 0.01 M Tris HCl buffer.

Figure 4.7: UV absorbance changes of 19 (5 μM) in 75% CH$_3$CN 25% 0.01 M Tris-HCl buffer with Fe$^{3+}$. Inset: Absorbance change at 563 nm with the concentration of Fe$^{3+}$. 
The addition of 200 μM (40 equivalents) of Fe\(^{3+}\) immediately yielded a pink solution with a strong fluorescence signal at 580 nm (Excitation 510 nm) due to electron delocalization in the xanthene moiety of rhodamine. There was 33-fold fluorescent enhancement for sensor 17 with Fe\(^{3+}\) (Figure 4.8) while sensor 18 yielded a 48-fold enhancement (Figure 4.9). The emission enhancement for the compound 19 with Fe\(^{3+}\) (>13-fold) was large enough compared with that for the other metal ions tested in this experiment, but very low compared to 17, and 18 with Fe\(^{3+}\) (Figure 4.10). The fluorescence enhancement was much weaker than that of the absorbance. The plot of measured fluorescence \((F_0/F-F_0)\) against \(1/[Fe^{3+}]\) (Figure 4.12) showed a linear relationship confirming the formation of a 1:1 complex between 18 and Fe\(^{3+}\). Sensor 17 also showed similar behavior for concentrations of Fe\(^{3+}\) in the range 150 - 237.5 μM (Figure 4.11). Fluorescence data of 19 also showed a linear relationship \((F_0/F-F_0\) against \(1/[Fe^{5+}]\)) within the range of 0.3 mM to 0.5 mM, confirming 1:1 complex formation with Fe\(^{3+}\). The binding constants, calculated using the Benesi-Hilderbrand method for three compounds with Fe\(^{3+}\) were found to be 7.5 x 10\(^{3}\) M\(^{-1}\), 5.1 x 10\(^{3}\) M\(^{-1}\), and 9.75x 10\(^{2}\) M\(^{-1}\) for 17, 18, and 19 respectively.
Figure 4.8: Fluorescence spectra of 17 (5 μM) in 75% CH₃CN, 25% 0.01 M Tris HCl buffer with different metal ions (200 μM). Insert shows the photo of sensor 17 with different metal ions (200 μM).

Figure 4.9: Fluorescence spectra of 18 (5 μM) in 75% CH₃CN, 25% 0.01 M Tris HCl buffer with different metal ions (200 μM). Insert shows the photo of sensor 18 with different metal ions (200 μM).
Figure 4.10: Fluorescence changes of 19 (5 μM) in 75% CH₃CN 25% 0.01 M Tris-HCl buffer with metal ions (200 μM).

The association constant for 19 with Cu²⁺ was calculated using absorbance data as the complex itself was non-fluorescent. The Benesi-Hildebrand plot $I/(A-A_0)$ against $I/[M^{n+}]$ showed a linear relationship within the range of 0.225 mM to 0.525 mM, confirming the formation of a 1:1 complex between sensor 17 and Cu²⁺. The binding constant value was found to be $1.65 \times 10^3$ M⁻¹.

On the other hand, addition of Na⁺, K⁺, Ca²⁺, Mn²⁺, Fe²⁺, Ni²⁺, Zn²⁺, Cd²⁺, Hg²⁺ or Pb²⁺ did not yield any color change (insets of Figure 4.8 and Figure 4.9). Sensor 17 showed slight fluorescent enhancement (3 times) with Cr³⁺ while other metals used in the experiment showed none. Similarly, sensor 18 also showed a slight emission enhancement (>5 times) with Cr³⁺ while the other metals showed no interference at all. Compound 19 also showed a slight fluorescence enhancement with Cu²⁺ (2-fold) and Cr³⁺ (3-fold) while other metals showed no enhancement (Figure 4.10). The emission
enhancement of 19 with Cu$^{2+}$ is negligible compared to that of its absorption, as it gave the highest absorption enhancement of all the metals tested. These results indicate that all three compounds form fluorescent complexes with Fe$^{3+}$ and allow the selective identification of Fe$^{3+}$. On the other hand, all three compounds form non-fluorescent complexes with Cu$^{2+}$ with an immediately visible color change. However, compound 19 is very selective for Cu$^{2+}$, as it registered the highest absorbance enhancement over the other two compounds.

Figure 4.11: $I_0/I-I_0$ vs $1/[Fe^{3+}]$ for 17 ($K = 7.5 \times 10^3 M^{-1}$).
Fluorescence quantum yield measurements were carried out for the sensors 17 and 18 with Fe$^{3+}$ and Cu$^{2+}$ metal ions in order to understand the differences in fluorescence behavior observed, and the results show interesting trends. We assume a similar trend for compound 19 with Fe$^{3+}$ and Cu$^{2+}$. The fluorescence quantum yields for 17 with Fe$^{3+}$ and Cu$^{2+}$ are around 0.024 and 0.007 respectively, while the fluorescence quantum yields for 18 with Fe$^{3+}$ and Cu$^{2+}$ are around 0.048 and 0.018 respectively. It is observed that the fluorescence quantum yield is significantly lower for the sensors with both Fe$^{3+}$ and Cu$^{2+}$ when compared to free rhodamine B which has a quantum yield close to unity. This might be due to the inherent quenching of fluorescence with inter-rhodamine interactions. However, the fluorescence quantum yield is very low for the sensors 17 and 18 in the presence of Cu$^{2+}$ when compared to Fe$^{3+}$, and this is a possible reason for the lower enhancement of fluorescence for sensors with Cu$^{2+}$. Further time-
resolved fluorescence measurements with femtosecond fluorescence upconversion were carried out to resolve the conjecture.

4.6 Fluorescence Sensing: Single and Two Photon Excitation

As discussed above, the investigated sensors 17 and 18 show very good selectivity with turn-on fluorescence upon one-photon excitation. However, compound 19 showed very weak fluorescence enhancement with Fe$^{3+}$ compared to the other two compounds (Figures 4.10, 4.16). Two-photon fluorescence sensing measurements have been carried out for Fe$^{3+}$ and Cu$^{2+}$ with sensors 17 and 18 in order to reveal the differences between them. All the two-photon sensing measurements were carried out with excitation at 800 nm. Comparative enhancements observed for one-and two-photon excitations are shown in Figures 4.13 and 4.14, which indicate that the fluorescence enhancement has increased further with two-photon excitation than with one-photon excitation for both sensors in the presence of Fe$^{3+}$. However, the enhancement is minimal for Cu$^{2+}$ in both excitations. The larger enhancements observed with two-photon excitation can be attributed to two factors. Firstly, there is absorption interference from Fe$^{3+}$ in the visible region which influences the one-photon enhancement, while the two-photon enhancement is not influenced by such interference. Secondly, the presence of Fe$^{3+}$ increases the two-photon cross-section since the metal ions open up the ring and increase the inter-rhodamine interaction. This in turn enhances the two-photon cross-sections. Two-photon cross-section measurements have shown such inter-rhodamine interaction in the presence of metal ions and cross-section enhancement. Interestingly, the two-photon cross-section is larger for sensor 18 with Fe$^{3+}$ than that of sensor 17 because pyridine bridging negatively influences the conjugation between rhodamine. The present
results show that these new sensors can be efficiently utilized for multiphoton imaging of Fe\(^{3+}\) in biological systems and in complex environments because of their excellent two-photon excited fluorescence upon complexation. The histograms in Figure 4.13 and 4.14 clearly show that the sensors are highly selective for Fe\(^{3+}\) ion over all other metal ions. It is interesting to note that it is very easy to differentiate Fe\(^{2+}\) from Fe\(^{3+}\) with these sensors. The selectivity observed here is ascribed to the ability of Fe\(^{3+}\) to form a complex which can open the spirolactone ring. It is evident that other metal ions do not have the size advantage or the relative bonding strength to open up the spirolactone ring.

![Figure 4.13: Fluorescence histogram plot of 17 (5 μM) at 585 nm upon addition of different metal ions (200 μM) in 75% CH\(_3\)CN, 25% 0.01 M Tris HC1 buffer.](image)
**Figure 4.14:** Fluorescence intensity of 18 (5 μM) at 585 nm upon addition of metal ions (200 μM) in 75% CH$_3$CN, 25% 0.01 M Tris HCl buffer.

### 4.7 Sensitivity Measurements

Concentration dependent fluorescence measurements were carried out to monitor the sensitivity for Fe$^{3+}$ ion. Shown in Figures 4.15A and Figure 4.15B are the concentration dependent fluorescence measurements monitored after excitation at 510 nm. Association constants calculated from such measurements are higher for sensors 17 and 18 with Fe$^{3+}$ over other metal ions. It is observed that the sensitivities of 17 and 18 for Fe$^{3+}$ are around 50 μM and 70 μM respectively. Sensitivity is calculated by determining the concentration where the enhancement of fluorescence is three times that of the background. Similar sensitivity as that of one-photon excitation is observed for the two-photon excitation measurements. The present sensors are highly selective for Fe$^{3+}$, even differentiating it from Fe$^{2+}$ with sensitivity in the micromolar region.
Figure 4.15: (A) Fluorescence titration of 17 (5 μM) in 75% CH₃CN, 25% 0.01 M Tris HCl buffer with Fe³⁺ and (B) Fluorescent titration of 18 (5 μM) in 75% CH₃CN, 25% 0.01 M Tris HCl buffer with Fe³⁺ ions.
Figure 4.16: Fluorescence changes of 19 (5 μM) in 75% CH$_3$CN 25% 0.01 M Tris-HCl buffer with Fe$^{3+}$: Inset. Fluorescence change at 590 nm with the concentration of Fe$^{3+}$.

4.8 Fluorescence Upconversion Measurements

In an effort to understand the selectivity of the sensors 17 and 18 for Fe$^{3+}$ over the other metal ions especially that of Cu$^{2+}$, and the differences in fluorescence quantum yields, time-resolved fluorescence measurements were carried out. Time-resolved fluorescence measurements on the sensors 17 and 18 in presence of Cu$^{2+}$ and Fe$^{3+}$ were carried out with femtosecond fluorescence upconversion spectroscopy after excitation at 400 nm and monitoring the fluorescence at 580 nm (Figure 4.17). Fluorescence lifetime studies of sensor 17 with Cu$^{2+}$ (Figure 4.17A) demonstrated the decay, which was fitted to a multi-exponential function with lifetimes of 220 fs (65.5%), 1.2 ps (32.2%) and >20 ps (2.3%), where the average lifetime is around 540 fs, indicating a faster intersystem crossing rate. By comparison with Fe$^{3+}$, sensor 17 showed a double exponential decay of 290 fs (88.9%) and 350 ps (11.1%). The average lifetime of 17 with Cu$^{2+}$ is far shorter than that of 17 with Fe$^{3+}$, suggesting that the faster intersystem crossing caused by Cu$^{2+}$ is
the reason for observing little steady state fluorescence as well as lower fluorescence quantum yield. Interestingly, sensor 18 showed completely different behavior than did sensor 17 with both Cu$^{2+}$ and Fe$^{3+}$. Two major decay components (3.4 ps (80%) and >20 ps (20%)) were observed for 18 with Cu$^{2+}$, while with Fe$^{3+}$ it also yielded two lifetimes (3.7 ps (15.3%) and 330 ps (84.7%). Interestingly, the shorter lifetime component comprised 80% of the decay while the longer lifetime component comprised only 20%. Here again, differences in the average lifetimes for 18 with Fe$^{3+}$ and Cu$^{2+}$ can explain the shift to lower values of the fluorescence quantum yield. It has to be noted here that the free rhodamine does not give any shorter lifetime components in its decay. The observed shorter lifetime components for sensors 17 and 18 point to the presence of non-radiative decay pathways which can be ascribed to inter-rhodamine interactions, thus explaining the observed differences in fluorescence quantum yield of the sensors and rhodamine B.
Figure 4.17: Fluorescence decay traces for sensors 17 and 18 in presence of Fe$^{3+}$ and Cu$^{2+}$ respectively. The faster fluorescence decays with Cu$^{2+}$ indicate ultrafast deactivation pathways.
4.9 Mechanism of Fluorescent Sensing

We first did the $^1$H NMR titrations to understand the Cu$^{2+}$ binding with sensor 19. $^1$H NMR titration in CD$_3$CN is shown in Figure 4.18. Addition of one equivalent of Cu$^{2+}$ resulted in shifting downfield and broadening of the peak at $\delta$ 9.64, corresponding to the imine hydrogen. This indicates a decrease in electron density at imine nitrogen resulting from direct coordination with Cu$^{2+}$. When compared to the structural differences between 18 and 19, the only difference is the arrangement of two rhodamine moieties on the bridged benzene ring (1,2 positions (ortho) in 19 and 1,3 positions (meta) in 18). Therefore we can expect a similar binding mechanism for 18 with Cu$^{2+}$ or Fe$^{3+}$ involving imine N, and carbonyl O.

Figure 4.18: $^1$H NMR (400 MHz, CD$_3$CN) spectra of 19 with Cu$^{2+}$. 
It is reasonable to suggest that the Fe$^{3+}$ can bind with the amide oxygen which causes the ring opening (Figure 4.19). The pyridine nitrogen does not make much contribution for Fe$^{3+}$ binding, as indicated by the absence of a significant difference in emission enhancement for 17 and 18 with Fe$^{3+}$. Interestingly, there is remarkable difference in Cu$^{2+}$ binding with the sensors since 18 showed very small absorption enhancement compared to 17. This suggests that the pyridine nitrogen plays a key role in Cu$^{2+}$ binding. Furthermore, binding of Cu$^{2+}$ and Fe$^{3+}$ with 17 and 18 is reversible: the color disappears upon the addition of excess EDTA even in the presence of large amounts of Cu$^{2+}$; fluorescence quantum yields further distinguish Fe$^{3+}$ and eliminate false positives from Cu$^{2+}$.  

Figure 4.19: Possible mechanism of Cu$^{2+}$ and Fe$^{3+}$ binding with sensor 19, omitting coordination solvent.
Conclusion

Three new compounds bearing two rhodamine moieties have been synthesized. The selectivity of sensors for Fe$^{3+}$ and Cu$^{2+}$ varies with the substitution position of the bridging benzene or pyridine ring. Enhancements in both one- and two-photon excited fluorescence are observed for sensors 17 and 18 with Fe$^{3+}$, suggesting their application in biological imaging of Fe$^{3+}$-containing systems. Two photon fluorescence enhancement is greater than that of one photon excitation, which is ascribed to the greater two photon cross-sections upon complexation and lower interference from metal ion absorption. Bis(rhodamine) derivatives have greater association constants with Fe$^{3+}$ as they complex via the amide oxygen. This causes ring opening in the spirolactam, which gives rise to the luminescence. Since 17 and 18 behave similarly towards Fe$^{3+}$, it appears that the bridging pyridine is not of great importance in the complexation. On the other hand, Cu$^{2+}$ is able to open the ring, which is evident from optical absorption; the fluorescence enhancement is significantly smaller than that of Fe$^{3+}$. Lower fluorescence quantum yields and ultrafast fluorescence deactivation pathways with Cu$^{2+}$ are the reasons behind lower fluorescence enhancement when compared to Fe$^{3+}$. Compared to 17 and 18, sensor 19 showed higher absorption enhancement for Cu$^{2+}$. In addition, there was a greater blue shift in the absorption maxima for 19-Cu$^{2+}$ compared to the others. Furthermore, the sensing mechanism for all three sensors was found to be reversible, as the pink color of the sensor-metal complex disappears with the addition of excess EDTA.
CHAPTER 5

STRUCTURAL CHARACTERIZATION OF SALICYLATE MODE OF Fe$^{3+}$
BINDING IN NEW SIDEROPHORE ANALOGS

5.1 Importance of Fe$^{3+}$

Iron is required by all forms of life including microorganisms. Although iron is abundant for animals, its availability is limited to microorganisms that live in or on an animal host.\(^{120}\) Generally, microorganisms require 0.4 – 4 \(\mu\)M of iron to conduct their metabolic activities.\(^{121}\) But, the free iron available for them is as low as \(10^{-18}\) M due to the high binding constant of iron-binding proteins.\(^{122}\) The bioavailability of iron is likewise low in soil due to the low solubility of Fe (III).

The daily dietary supply of iron for humans is around 10 mg but, only 1-1.5 mg gets absorbed though the intestinal epithelium. Certain sugars, amino acids, and amines help the absorption process via the soluble complexes they form with iron. Also carbonates, oxalates, phosphates, and tannates form insoluble iron complexes which can reduce the iron absorption in the intestinal epithelium. About 95% of the iron in the plasma is bound by transferrin. When transferrin and other iron binding agents are saturated, the excess iron can bind with albumin and other serum proteins nonspecifically. This nonspecifically bound iron is readily available to microorganisms.\(^{123}\)
5.2 Transferrins

Transferrins are iron-binding glycoproteins that transport iron in the plasma. Transferrin binds with irons specifically with a very high binding constant (10^{24} \text{M}^{-1})^{124}$ but the affinity decreases with decreasing pH. Transferrin loaded with iron binds to the transferrin receptor on the cell surface and transports into the cell in a vesicle via receptor-mediated endocytosis. A Decrease in pH inside the vesicle causes the transferrin to release iron.$^{125, 126}$ The unloaded iron binds with ferritin inside the cell to be stored. Apotransferrin is transported back to the plasma, ready for another iron transport cycle.$^{127}$

In this environment, the concentration of free $\text{Fe}^{3+}$ is as low as $10^{-18}$ M due to its hydrolysis.$^{128}$ This solubility indicates the bio-unavailability of iron at physiological pH from the perspective of microorganisms. Microorganisms have developed an efficient iron transport mechanism which involves a class of highly specific iron chelating agents called siderophores.$^{129}$

5.3 Siderophores

Siderophores are relatively low molecular weight compounds with a very high binding affinity towards $\text{Fe}^{3+}$ used by both bacteria and fungi to uptake iron. There are a few cases of microbes, such as some lactic acid bacteria and fission fungi, which do not need iron and therefore do not produce siderophores.$^{130}$ Generally, siderophores have either catecholate or hydroxymate functional groups to chelate iron.$^{131, 132}$ However, siderophores with $\alpha$-hydroxy carboxylic acid$^{132}$ and $\beta$-hydroxyhistidine$^{133, 134}$ have also been reported. Siderophores form high-spin iron complexes with exceptional stability. Generally the association constant for a siderophore containing three bidentate ligands is
Enterobactin (Figure 5.1) is the strongest naturally occurring iron chelator with a stability constant of $10^{49}$. It has three catechol units that can encapsulate Fe$^{3+}$ in octahedral geometry.
5.4 Enterobactin

Enterobactin, the strongest known siderophore, can bind with Fe\(^{3+}\) with an exceptionally high affinity. It is primarily found in gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium*.\(^{139}\) It can steal iron from the environment where the soluble iron concentration is ultra-low. Pathogenic microbes can take iron from the host with the help of enterobactin. The enterobactin-Fe(III) complex can effectively transport into the cell through the receptor proteins on the outer membrane of the cell.\(^{140}\) The release mechanism of iron from the siderophore-iron complex is not yet known, but the release of iron from the enterobactin-Fe(III) complex involves the hydrolysis of the trilactone backbone by esterase.\(^{141}\) Raymond and coworkers have proposed a mechanism for iron release in catechol siderophore analogs based on the protonation of the 3-hydroxy oxygen, which shift the catecholate to salicylate mode of binding after the third protonation. Reduction of the salicylate mode of the complex with the help of a biological reductant could release the iron.\(^{142}\)

![Proposed mechanism for the release of Fe\(^{3+}\).](image)

**Figure 5.2:** Proposed mechanism for the release of Fe\(^{3+}\).

5.5 Siderophores as Drug Candidates

Siderophores have several potential therapeutic applications. For example, inhibition of the biosynthesis of siderophore can significantly lower the microbial growth.
Another application would be the use of them as a drug delivery agent.\textsuperscript{143} In addition, siderophores have been used as drugs to remove excess iron. Desferal is a siderophore from \textit{Streptomyces pilosus} used in iron-reduction therapy for thalassemia. The drug is available for intramuscular administration.\textsuperscript{144}

\begin{化学式}
\text{NH}_2(\text{CH}_2)_5\text{NC}(\text{CH}_2)_2\text{CNH}(\text{CH}_2)_5\text{NC}(\text{CH}_2)_2\text{CNH}(\text{CH}_2)_5\text{NCCH}_3.\text{CH}_3\text{SO}_3\text{H}
\end{化学式}

**Figure 5.3:** Structure of Desferal.

In the microbial transport of siderophores, the metal center is the key to microbial recognition. The receptors do not require the full siderophore structure for transportation.\textsuperscript{145 - 147} This behavior allows the modification of the siderophore structure to attach drug candidates as needed. The general siderophore-drug conjugate may consist of four parts: iron, ligand, linker and drug as shown in Figure 5.4.\textsuperscript{143}

\begin{化学式}
\text{Fe-Siderophore}
\end{化学式}

**Figure 5.4:** General structure of siderophore-drug conjugate.

We used biocompatible cholic acid as the core of the siderophore. Incorporation of three hydroxyl functionalities allows the introduction of three catechol arms. Initial esterification of the carboxylic acid group at the 24\textsuperscript{th} position improves the solubility and protects it from further reaction.
Experimental

Scheme 5.1: Synthesis of compound 20.
Scheme 5.2: Synthesis of compound 24.
Scheme 5.3: Synthesis of compound 28.
5.6 Synthesis and Characterization

Compound 21: A mixture of 2,3-dihydroxybenzoic acid (0.31 g, 2.01 mmol), benzyl bromide (2.07 g, 12.1 mmol), and K₂CO₃ (5.0 g, 36.2 mmol) was refluxed in acetone (40 mL) for 1 day. The reaction mixture was allowed to cool to room temperature, filtered and concentrated under vacuum. The residue was dissolved in methanol (120 mL) and a 5 M solution of NaOH (30 mL) was added before refluxing for 3 hours. The mixture was concentrated in vacuo to remove methanol and the residue was dissolved in water and acidified with 3 N HCl until pH reached 2. The precipitate was filtered and washed with plenty of water and dried to obtain 21 as a white solid (0.39 g, 58%). H NMR (400 MHz, CDCl₃): δ 11.33 (1H, s), 7.73 (1H, dd, J = 7.7 Hz, 1.8 Hz), 7.48 – 7.31 (9H, m), 7.25 (2H, m), 7.18 (1H, t, J = 8.0 Hz), 5.26 (2H, s), 5.18 (2H, s). C NMR (100 MHz, CDCl₃): δ 165.1, 151.3, 147.1, 135.9, 134.6, 129.4, 128.9, 128.9, 128.6, 127.8, 125.1, 124.5, 123.0, 119.0, 77.2, 71.6. ESI-MS. 357.17 [M + Na]+.

Compound 22: Compound 21 (6.74 g, 20 mmol) was dissolved in anhydrous THF (150 ml). Triethylamine (3.36 ml) was added, followed by 2,4,6-trichlorobenzoyl chloride (5.0 g, 20 mmol). The resulting mixture was stirred at room temperature for 1 h under nitrogen. The mixture was evaporated to dryness and dissolved in anhydrous diethyl ether. The precipitate was filtered off and the filtrate was dried under vacuum. The concentrated filtrate was dissolved in anhydrous THF (100 mL) and was added with methyl cholate (1.21 g, 2.86 mmol) and DMAP (1.6 g, 13.1 mmol). The reaction mixture was stirred at 50 °C for 3 days. The precipitate was filtered off and the filtrate was concentrated under vacuum. The product was isolated as a white solid (0.95 g, 24%) using a silica column chromatography (solvent gradient from 50% CHCl₃/Hexane to 10%
MeOH/CHCl₃). ¹H NMR (400 MHz, CDCl₃): δ 7.42-7.19 (32H, m), 7.05 (1H, dd, J = 8.0 Hz, 1.4 Hz), 7.00 (1H, dd, J = 7.7 Hz, J = 1.4 Hz), 6.95 (2H, d, J = 8.4 Hz), 6.86 (1H, t, J = 8.0 Hz), 6.80 (2H, m), 5.41 (1H, s), 5.24 (1H, s), 5.09 (2H, s), 5.08 (2H, s), 5.07 (2H, d, J = 2.2 Hz), 5.02 (2H, s), 5.00 (2H, s), 4.96 (2H, d, J = 5.1 Hz), 4.76 (1H, m), 3.59 (3H, s), 2.41-1.05 (24H, m), 1.00 (3H, s), 0.83 (3H, d, J = 6.6 Hz), 0.80 (3H, s). ¹³C NMR (100 MHz, CDCl₃): δ 174.6, 165.2, 164.9, 164.4, 153.0, 152.7, 148.8, 148.7, 148.4, 137.5, 137.3, 136.7, 136.6, 129.4, 128.9, 128.6, 128.5, 128.4, 128.2, 128.1, 128.0, 127.9, 127.7, 127.6, 127.5, 127.5, 124.5, 124.0, 123.9, 123.5, 122.7, 122.3, 122.0, 117.6, 77.7, 75.9, 75.7, 74.2, 71.6, 71.3, 71.3, 51.4, 47.6, 45.3, 43.4, 40.8, 38.4, 34.9, 34.8, 34.4, 31.5, 31.1, 30.7, 29.1, 26.6, 25.4, 23.1, 22.5, 17.5, 12.3. ESI-MS. 1393.42 [M + Na]⁺.

Compound 23: Compound 22 (0.1 g, 0.073mmol) was dissolved in 10 ml of methanol and 10% Pd/C (15 mg) was added. The mixture was stirred under hydrogen for 24 h, filtered and evaporated to dryness to obtain the product as a white solid (25 mg, 41%). ¹H NMR (400 MHz, CDCl₃): δ 11.07 (1H, s), 10.98 (1H, s), 10.81 (1H, s), 7.41 (1H, dd, J = 8.0 Hz, 1.4 Hz), 7.36 (1H, dd, J = 8.0 Hz, 1.4 Hz), 7.17 (1H, d, J = 8.1 Hz), 7.12 (1H, d, J = 8.1 Hz), 7.04 (1H, dd, J = 8.2 Hz, 1.3 Hz), 7.00 (1H, dd, J = 8.1 Hz, 1.5 Hz), 6.81 (1H, t, J = 8.0 Hz), 6.69 (2H, q, J = 7.7 Hz), 5.73 (2H, s), 5.60 (1H, s), 5.46 (1H, s), 5.32 (1H, s), 4.78 (1H, m), 3.58 (3H, s), 2.45 – 1.12 (24H, m), 1.03 (3H, s), 0.84 (3H, s), 0.82 (3H, d, J = 6.6 Hz). ¹³C NMR (100 MHz, CDCl₃): δ 174.5, 169.6, 169.6, 169.4, 149.5, 149.3, 148.9, 145.5, 145.4, 144.9, 120.5, 120.0, 120.0, 119.9, 119.8, 119.6, 119.5, 119.3, 118.9, 112.9, 112.7, 112.7, 74.7, 72.4, 51.5, 47.9, 45.5, 43.5, 40.4, 38.2, 34.8, 34.6, 34.5, 34.4, 31.4, 30.8, 30.6, 28.9, 27.2, 26.5, 25.6, 23.0, 22.4, 17.6, 12.3. Anal. Caled for C₄₆H₅₄O₁₄.H₂O: C, 65.08; H, 6.65. Found: C, 65.19; H, 6.55. ESI-MS. 829.33 [M - H]⁺.
Compound 20 (23-Fe\(^{3+}\)): Compound 23 (96 mg, 0.115 mmol) was dissolved in 7 ml of methanol and anhydrous FeCl\(_3\) (18 mg, 0.115 mmol) was added. Immediate precipitation was observed upon the addition of 0.3 mL of pyridine. The resulting dark purple solution was stirred an additional hour, filtered, washed with cold methanol and dried overnight to get a black solid (56 mg, 55%). Anal. Calcd for C\(_{46}\)H\(_{51}\)FeO\(_{14}\).H\(_2\)O.2HCl: C, 56.69; H, 5.69. Found: C, 56.50; H, 6.04. ESI-MS. 882.33 [M-H]\(^+\).

(23-Al\(^{3+}\)): Compound 23 (100 mg, 0.12 mmol) was dissolved in 7 ml of methanol and anhydrous AlCl\(_3\) (17 mg, 0.12 mmol) was added. Immediate precipitation was observed upon the addition of 0.3 mL of pyridine. The resulting white turbid solution was stirred an additional two hours, filtered, washed with cold methanol and dried overnight to light yellow solid (75 mg, 73%). Anal. Calcd for C\(_{46}\)H\(_{51}\)AlO\(_{14}\).CH\(_3\)OH.2H\(_2\)O.2HCl: C, 56.68; H, 6.17. Found: C, 56.81; H, 6.66.

Compound 25: A mixture of 2-hydroxy-3-methoxybenzoic acid (g, mmol), benzyl bromide (g, mmol), and K\(_2\)CO\(_3\) (g, mmol) was refluxed in acetone (mL) for 1 day. The reaction mixture was allowed to cool down to room temperature, filtered and concentrated under vacuum. The residue was dissolved in methanol (mL) and a 5M solution of NaOH (mL) was added before refluxing for 3 hours. The mixture was concentrated in vacuum to remove methanol. The residue was dissolved in water and acidified with 3N HCl until pH reached 2. The precipitate was filtered off and washed with plenty of water and dried to obtain 25 as a white solid. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 7.67 (1H, dd, \(J = 7.3\) Hz, 2.5 Hz), 7.43 (2H, m), 7.37 (3H, m), 7.16 (2H, m), 5.24 (2H, s), 3.95 (3H, s). \(^1\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 165.5, 152.2, 146.7, 135.0, 129.3, 129.2, 128.9, 125.0, 124.0, 123.3, 117.2, 77.0, 56.3. ESI-MS. 281.17 [M + Na]\(^+\).
Compound 26: Compound 25 (5.2 g, 20.0 mmol) was dissolved in anhydrous THF (150 mL). Triethyl amine (3.35 mL) was added, followed by 2,4,6-trichlorobenzoyl chloride (5.0 g, 20.0 mmol). The resulting mixture was stirred at room temperature for 1 h under nitrogen. The mixture was evaporated to dryness and dissolved in 100 mL of anhydrous diethyl ether. The precipitate was filtered and the filtrate was dried under vacuum. The concentrated filtrate was dissolved in anhydrous THF (100 mL) and was added methyl cholate (0.73 g, 1.72 mmol) and DMAP (0.84 g, 8.2 mmol). The reaction mixture was stirred at 50 °C for 3 days. The precipitate was filtered off and the filtrate was concentrated under vacuum. The product was isolated as a white solid (0.45 g, 23%) using a silica column chromatography using 30% EtOAc/Hexane as mobile phase. 

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.49 (2H, d, J = 7.4 Hz), 7.45 (2H, d, J = 7.2 Hz), 7.32-7.20 (11H, m), 7.06-6.96 (5H, m), 6.86 (1H, t, J = 7.6 Hz), 6.84 (1H, t, J = 7.6 Hz), 5.39 (1H, s), 5.22 (1H, s), 5.04 (4H, m), 4.95 (2H, d, J = 5.1 Hz), 4.95 (2H, d, J = 5.1Hz), 4.73 (1H, m), 3.86 (3H, s), 3.82 (3H, s), 3.81 (3H, s), 3.59 (3H, s), 2.39 – 1.05 (24H, m), 0.98 (3H, s), 0.82 (3H, d, J = 6.6 Hz), 0.78 (3H, s). 

$^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 174.6, 165.2, 164.9, 164.4, 153.9, 153.8, 153.7, 148.3, 148.2, 147.9, 137.7, 137.5, 128.4, 128.3, 128.2, 128.2, 127.8, 127.7, 127.4, 127.4, 124.0, 123.9, 123.5, 122.1, 121.7, 121.3, 115.7, 115.5, 77.7, 75.9, 75.6, 74.2, 71.3, 56.2, 56.1, 51.4, 47.6, 45.3, 43.5, 40.8, 38.3, 34.9, 34.8, 34.4, 31.5, 31.1, 30.7, 29.0, 27.1, 26.6, 25.4, 23.1, 22.5, 17.5, 12.3. ESI-MS. 1181.33 [M + K]$^+$.


Compound 27: Compound 26 (190 mg, 0.166 mmol) was dissolved in 10 ml of methanol and 10% Pd/C (30 mg) was added. The mixture was stirred under hydrogen for 24 h, filtered and evaporated to dryness to obtain the product as a white solid (130 mg, 89.8%).
\(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 11.19 (1H, s), 11.11 (1H, s), 10.99 (1H, s), 7.49 (1H, d, J = 8.0 Hz), 7.46 (1H, d, J = 8.0 Hz), 7.10 (1H, d, J = 6.9 Hz), 7.08 (1H, d, J = 7.3 Hz), 7.05 (1H, d, J = 8.0 Hz), 6.97 (1H, d, J = 7.7 Hz), 6.85 (1H, t, J = 8.0 Hz), 6.76 (1H, t, J = 8.0 Hz), 6.69 (1H, t, J = 8.0 Hz), 5.46 (1H, s), 5.33 (1H, s), 4.78 (1H, m), 3.90 (6H, s), 3.85 (3H, s), 3.85 (3H, s), 2.45-1.17 (24H, m), 1.03 (3H, s), 0.84 (3H, s), 0.81 (3H, d, J = 6.6 Hz).

\(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 174.4, 169.7, 169.7, 169.5, 152.8, 152.5, 152.2, 149.0, 148.9, 148.5, 121.1, 120.5, 120.4, 118.8, 118.6, 118.9, 118.5, 116.9, 116.5, 116.3, 113.1, 113.0, 74.8, 72.3, 56.3, 56.2, 51.5, 47.9, 45.5, 43.6, 40.5, 38.3, 34.8, 34.6, 34.5, 34.4, 31.4, 30.8, 30.6, 29.0, 27.2, 26.5, 25.6, 23.0, 22.4, 17.6, 12.3. ESI-MS. 895.25 [M + Na]\(^+\). Anal. Calcd for C\(_{49}\)H\(_{60}\)O\(_{14}\).H\(_2\)O: C, 66.05; H, 7.01. Found: C, 65.87; H, 7.03.

Compound 24 (27-Fe\(^{3+}\)): Compound 27 (50 mg, 0.057 mmol) was dissolved in 5 ml of methanol and anhydrous FeCl\(_3\) (10 mg, 0.06 mmol) was added. To the resulting solution was added 0.3 ml of pyridine and the mixture stirred for 3h. The white precipitate was filtered and dried in vacuo to get 24 as a black solid (25 mg, 47%). ESI-MS. 926.4 [M-H]\(^+\). HRESI m/z calcd for C\(_{49}\)H\(_{58}\)O\(_{14}\)Fe [M+H]\(^+\) 926.3176, found 926.3153.

Compound 29: Compound 21 (6.74 g, 20 mmol) was dissolved in anhydrous THF (150 ml). Triethylamine (3.36 ml) was added followed by 2,4,6-trichlorobenzoyl chloride (5.0 g, 20 mmol). The resulting mixture was stirred at room temperature for 1 h under nitrogen. The mixture was evaporated to dryness and dissolved in anhydrous diethyl ether. The precipitate was filtered off and the filtrate was dried under vacuum. The concentrated filtrate was dissolved in anhydrous THF (100 mL), followed by the addition of methyl cholate (1.21 g, 2.86 mmol) and DMAP (1.6 g, 13.1 mmol). The reaction mixture was stirred at 50 °C for 3 days. The precipitate was filtered off and the filtrate
was concentrated under vacuum. The product was isolated as a white solid (1.1 g, 36%) using a silica column chromatography (solvent gradient from 50% CHCl₃/Hexane to 10% MeOH/CHCl₃). ¹H NMR (400 MHz, CDCl₃): δ 7.43 – 7.20 (22H, m), 7.05 (1H, dd, J = 8.1 Hz, 1.5 Hz), 6.96 (3H, m), 5.39 (1H, s), 5.11 (2H, s), 5.08 (2H, s), 5.02 (4H, s), 4.74 (1H, m), 3.85 (1H, s), 3.62 (3H, s), 2.41 – 1.01 (24H, m), 0.92 (3H, s), 0.85 (3H, d, J = 6.6 Hz), 0.78 (3H, s). ¹³C NMR (100 MHz, CDCl₃): δ 174.8, 165.9, 165.5, 152.8, 152.7, 148.4, 148.1, 137.7, 137.5, 136.7, 136.6, 136.3, 132.5, 128.6, 128.6, 128.5, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 127.4, 124.1, 123.8, 122.8, 122.4, 117.7, 117.6, 76.3, 75.6, 75.0, 71.3, 71.2, 68.1, 51.5, 47.6, 45.3, 43.5, 41.2, 39.4, 35.3, 34.9, 34.7, 34.3, 31.0, 30.8, 27.9, 27.4, 26.7, 25.5, 23.0, 22.6, 17.5, 12.4. ESI-MS. 1077.33 [M + Na]⁺.

Compound 30: Compound 29 (0.8 g, 0.76 mmol) was dissolved in 10 ml of methanol and 10% Pd/C (150 mg) was added. The mixture was stirred under hydrogen for 24 h, filtered and evaporated to dryness and purified on a silica gel column using CH₂Cl₂ as the mobile phase to obtain 30 as a white solid (0.45 g, 85%). ¹H NMR (400 MHz, CDCl₃): δ 10.99 (1H, s), 10.97 (1H, s), 7.47 (1H, dd, J = 8.1 Hz, 1.4 Hz), 7.17 (1H, dd, J = 8.1 Hz, 1.4 Hz), 7.13 (1H, d, J = 6.9 Hz), 7.05 (1H, dd, J = 8.1 Hz, 1.4 Hz), 6.85 (1H, t, J = 8.1 Hz), 6.71 (1H, t, J = 8.1 Hz), 5.67 (1H, s), 5.62 (1H, s), 5.41 (1H, s), 4.76 (1H, m), 3.94 (1H, s), 3.61 (3H, s), 2.46 – 1.04 (..H, m), 0.95 (3H, s), 0.83 (3H, d, J = 5.5 Hz), 0.82 (3H, s). ¹³C NMR (100 MHz, CDCl₃): δ 174.5, 169.9, 169.9, 149.1, 148.9, 145.2, 144.9, 120.8, 120.5, 119.9, 119.6, 119.5, 118.9, 112.9, 77.3, 75.6, 68.0, 51.6, 47.9, 45.5, 43.7, 41.1, 39.3, 35.1, 34.8, 34.7, 34.6, 34.5, 30.9, 30.7, 27.8, 27.4, 26.5, 25.8, 23.1, 22.5, 17.6, 12.4. Anal. Calcd for C₃₉H₅₀O₁₁.CH₂Cl₂: C, 61.61; H, 6.72. Found: C, 61.58; H, 6.90. ESI-MS. 693.42 [M - H]⁺.
Compound 28 (30-Fe$^{3+}$): Compound 30 (50 mg, 0.07 mmol) was dissolved in 5 ml of methanol and anhydrous FeCl$_3$ (12 mg, 0.07 mmol) was added. The resulting solution was treated with 0.3 ml of pyridine and the mixture stirred for 3h. The white precipitate was filtered off and dried in vacuo to get 28 as a black solid (35 mg, 59%). Anal. Calcd for C$_{39}$H$_{48}$Cl$_2$FeO$_{11}$: C, 57.16; H, 5.90. Found: C, 57.42; H, 6.55. ESI-MS. 818.42 [M-H]$^+$.

(30-Al$^{3+}$): Compound 30 (42 mg, 0.06 mmol) was dissolved in 5 ml of methanol and anhydrous AlCl$_3$ (8.7 mg, 0.06 mmol) was added. To the resulting solution was added 0.3 ml of pyridine and the mixture stirred for 3h. The white precipitate was filtered and dried in vacuo to get the product as a white solid (30 mg, 63%). $^1$H NMR (400 MHz, Acetic acid-d$_4$): δ 7.58 (1H, d, $J$ = 8.0 Hz), 7.10 (2H, d, $J$ = 8.0 Hz), 7.02 (1H, d, $J$ = 8.0 Hz), 6.84 (1H, t, $J$ = 7.7 Hz), 6.70 (1H, t, $J$ = 8.0 Hz), 5.46 (1H, s), 4.76 (1H, m), 4.01 (1H, s), 3.59 (3H, s), 3.40 (1H, s), 2.49-1.04 (24H, m), 0.99 (3H, s), 0.86 (6H, s).$^{13}$C NMR (100 MHz, Acetic acid-d$_4$): δ 175.5, 169.9, 169.8, 149.4, 149.2, 145.4, 145.1, 120.4, 120.3, 120.1, 119.3, 118.8, 113.0, 112.9, 77.1, 75.5, 68.2, 51.2, 47.7, 45.3, 43.4, 41.0, 39.2, 34.7, 34.4, 34.3, 33.6, 30.5, 30.4, 27.7, 27.0, 26.1, 25.3, 22.6, 21.6, 16.9, 11.5. Anal. Calcd for C$_{39}$H$_{48}$Cl$_2$AlO$_{11.3}$H$_2$O: C, 55.45; H, 6.44. Found: C, 54.88; H, 7.31.
Figure 5.5: ESI Mass spectra of 20.

Figure 5.6: ESI Mass spectra of 24.
Results and Discussion

5.7 Synthesis

The synthesis of compound 20 is shown in Scheme 5.1. Two hydroxyl groups of 2,3-dihydroxybenzoic acid were protected by refluxing with benzyl bromide with excess K₂CO₃. Compound 21 was reacted with 2,4,6-trichlorobenzoyl chloride in the presence of triethyl amine to obtain 2,3-bis(benzyloxy)benzoic-2,4,6-trichlorobenzoic anhydride, which was then reacted with methyl cholate and the crude was purified on a silica column to get compound 22. The same reaction produced compound 29 with 36% yield (Scheme 5.3). The benzyl protecting groups of 22 and 29 were removed by hydrogenation in the
presence of Pd/C. The iron complexes of 23 and 30 were formed in methanol by reacting with anhydrous FeCl$_3$ in the presence of pyridine. Mass spectroscopic data (Figure 5.5 and 5.7) clearly showed the presence of salicylate mode iron complexes, 20 and 28. Aluminum complexes of 23 and 30 were also formed via similar methods by reacting with AlCl$_3$. Unfortunately, the aluminum complex of 23 was not soluble in any solvent, which restricted further studies.

Compound 24 which bears 3-methoxy functionality of each catechol arm was synthesised to improve the solubility of the metal complex. It can only display the salicylate mode of binding iron binding (Figure 5.6). As shown in Figure 5.2, a similar experimental procedure was used to get 24 starting from 2-hydroxy-3-methoxybenzoic acid.

5.8 UV-Vis Spectroscopic Study

![Graph](image.png)

**Figure 5.8:** UV-Vis absorbance of 20, 24, and 28 (12.5 µM) in water.
The spectroscopic properties of iron complexes were determined in a water/DMF mixture due to the limited solubility in water, but the amount of DMF is less than 5% (Figure 5.8). Introduction of a methoxy group dramatically improves the solubility of the metal complex. Compound 20 shows a strong absorbance band at 332 nm due to a $\pi - \pi^*$ transitions ($\varepsilon = 16800$) and a broad ligand to metal charge transfer band around 530 nm ($\varepsilon = 2400$). Similarly, 28 also showed a relatively weak absorption band at 343 nm due to a $\pi - \pi^*$ transitions ($\varepsilon = 5180$) and a LMCT band at 522 nm ($\varepsilon = 980$). Compound 24 which has methoxy groups on each catechol arm at 3rd position shows a $\pi - \pi^*$ band at 322 nm ($\varepsilon = 9600$) and a LMCT band at 517 ($\varepsilon = 1290$).

All three compounds adopt the salicylate mode of iron binding which involves the carbonyl and ortho hydroxyl group of each catechol arm. A titration was carried out to better understand the Fe$^{3+}$ binding mechanism. Initially compound 20 was titrated from pH 6.9 to pH 1.9 which displayed a decrease in intensity at the peak 332 nm (Figure 5.9). The intensity of absorption of LMCT band also decreased, which is similar to results reported by Abergel et al.\textsuperscript{148} Increasing the pH above 6.9 resulted in shifting the absorption band at 332 nm to 347 nm; this can be due to the base-catalyzed hydrolysis of the ester linkages (Figure 5.10). In addition the increase in intensity of LMCT band at the initial stage is due to the shifting of Fe$^{3+}$ from salicylate to catechol mode of binding.\textsuperscript{148} Similarly, 28 also displayed a similar behavior with the decreasing pH (Figure 5.11).
Figure 5.9: UV-Vis titration of Compound 20 from pH 6.9 to 1.9 in water.

Figure 5.10: UV-Vis titration of Compound 20 from pH 7.0 to 12.6 in water.
5.9 $^1$H NMR Study

$^1$H NMR studies of 30-Al$^{3+}$ were performed in acetic acid-d$_4$ due to the insolubility in any organic solvent. The structure was confirmed with $^1$H, $^{13}$C, $^1$H-$^1$H COSY (Figure 5.12, 5.13), and HETCOR (Figure 5.14) experiments. A $^1$H NMR titration was performed by adding DC1 solution (35%wt in D$_2$O) to lower the pH (Figure 5.15). Initially, the doublet corresponding to H$_c$ and H$_d$ appeared at 7.10 ppm. Lowering the pH with DC1 resulted in shifting the peak up field. At one stage, the peak resolved into two doublets, which finally merged into one doublet at 7.04 ppm. Other aromatic peaks did not show a considerable shift. Peaks at 5.46 (H$_h$) and 4.76 (H$_g$) also shifted up field ($\Delta \delta = 0.12$) and ($\Delta \delta = 0.13$) respectively. This observation could be due to the protonation of ortho hydroxyl groups and the metal center getting close to H$_g$ and H$_h$. Interestingly, the peak at 4.01 ppm, corresponding to H$_i$, did not show any movement. This observation
confirms that the hydroxyl group at the 7\textsuperscript{th} position is not involved in the complex formation with Al\textsuperscript{3+}. Finally, the complex started to precipitate below pH 2.0. Comparison of the proton NMR spectrum at pH 1.9 with the proton NMR of ligand confirms that the metal ion is still remaining attached to 30 (Figure 5.16).

![Diagram](image)

**Figure 5.12:** Some important $^1$H-$^1$H-COSY correlations of 30-Al\textsuperscript{3+}.

![Diagram](image)

**Figure 5.13:** $^1$H-$^1$H COSY spectrum of 30-Al\textsuperscript{3+} in acetic acid-d\textsubscript{4}. 

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Figure 5.14: HETCOR spectrum of $^{30}$-Al$^{3+}$ in acetic acid-d$_4$. 

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Figure 5.15: $^1$H NMR of 30-Al$^{3+}$ with decreasing pH in acetic acid-d$_4$ (from pH ~ 4.5 to 1.9).
**Figure 5.16:** Comparison of $^1$H NMR spectra of 30 and 30-Al$^{3+}$ in different conditions.

### 5.10 IR Spectroscopic Study

IR spectroscopy is a powerful tool that can be used to study the metal binding of siderophore analogs. Solid state spectra were taken in KBr of metal complexes and free ligands to study the changes upon binding with metal ions. Compound 23 showed a strong band at 1668 cm$^{-1}$ corresponding to three C=O on the catechol arms (Figure 5.17). Another band at 1734 cm$^{-1}$ was observed for the C=O on 24$^{th}$ position of cholic acid. Prominent change can be expected on carbonyl stretching upon binding with Fe$^{3+}$. The solid state IR spectrum of 20 showed a strong band at 1618 cm$^{-1}$ which could be due to the involvement of carbonyl group of catechol arms on iron binding. The shift of 50 cm$^{-1}$ to lower energy can be expected due to the salicylate mode of Fe$^{3+}$ binding.\textsuperscript{149}
The relatively weaker observed at 1668 cm\(^{-1}\) can be attributed to the asymmetric binding pattern of Fe\(^{3+}\) with three catechol arms. Asymmetric binding can occur as the environment of each catechol arm is unique.

Figure 5.17: FTIR spectra of 20 and 23.

Similarly, compound 30 also shows a strong IR band at 1666 cm\(^{-1}\) corresponding to two C=O groups on catechol arms (Figure 5.18). A 50 cm\(^{-1}\) of shift to the lower energy was observed upon binding with Fe\(^{3+}\).
Conclusion

Three siderophore analogs bearing a cholic acid core have been synthesized successfully. Iron and aluminum complexes of all three compounds were synthesized and found to be bound in the salicylate mode. Unfortunately, the insolubility of $\text{Al}^{3+}$ complexes in organic solvents hampered the opportunity of obtaining valuable information through NMR studies. The pH-dependant UV-Visible spectroscopic experiments showed that the compounds are stable in neutral to acidic conditions and the results are compatible with other reported data. The IR experiments further confirmed
that the Fe$^{3+}$ adopt the salicylate mode of binding as the carbonyl stretching shifts to lower energy. Introduction of the methoxy functionality at the 3$^{rd}$ position of the catechol arm clearly improved the solubility of the Fe$^{3+}$ complex.

**Future Studies**

The side chain of the cholic acid molecule allows further modification to use it as a siderophore-drug conjugate. A drug molecule can be easily attached to a side chain through an ester linkage or ether linkage, after the reduction of carboxylic acid to alcohol. In addition, better solubility of the iron complex allows further experimentation of the siderophore-drug conjugate (Figure 5.19).

![Figure 5.19: Possible siderophore-drug conjugate.](image)
REFERENCES


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Appendix A

$^1$H NMR, $^{13}$C NMR, ESI-MS, and X-ray crystallography data of rhodamine derivatives in chapter 1
$^1$H NMR of 1 in CDCl$_3$

$^{13}$C NMR of 1 in CDCl$_3$
$^1$H NMR of 2 in Acetone-$d_6$
$^1$H NMR of 3 in CDCl$_3$
$M^0 = 5000$

$\text{HNMR of 4 in CDCl}_3$

$\text{HNMR of 4 in CDCl}_3$
$^1$H NMR of 5 in Acetone-$d_6$
$^1$H NMR of 6 in CDCl$_3$
$^1$H NMR of 8 in CDCl$_3$
X-ray crystallography data

Sample and crystal data for compound 1. CCDC 730371

<table>
<thead>
<tr>
<th>Chemical formula</th>
<th>C_{35}H_{37}N_{5}O_{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formula weight</td>
<td>575.70</td>
</tr>
<tr>
<td>Temperature</td>
<td>296(2) K</td>
</tr>
<tr>
<td>Wavelength</td>
<td>0.71073 Å</td>
</tr>
<tr>
<td>Crystal size</td>
<td>0.25 x 0.35 x 0.40 mm</td>
</tr>
<tr>
<td>Crystal system</td>
<td>monoclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>P21/n</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
<td>a = 11.868(6) Å</td>
</tr>
<tr>
<td></td>
<td>b = 11.787(10) Å</td>
</tr>
<tr>
<td></td>
<td>c = 22.553(11) Å</td>
</tr>
<tr>
<td>Volume</td>
<td>3148.(3) Å³</td>
</tr>
</tbody>
</table>

X-ray crystal structure of 1
\[
\begin{align*}
Z & \quad 4 \\
\text{Density (calculated)} & \quad 1.215 \, \text{Mg/cm}^3 \\
\text{Absorption coefficient} & \quad 0.079 \, \text{mm}^{-1} \\
F(000) & \quad 1224 \\
\end{align*}
\]

Data collection and structure refinement for compound 1

\[
\begin{align*}
\text{Theta range for data collection} & \quad 1.81 \text{ to } 17.30^\circ \\
\text{Index ranges} & \quad -9 \leq h \leq 9, \, -9 \leq k \leq 9, \, -18 \leq l \leq 18 \\
\text{Reflections collected} & \quad 72199 \\
\text{Independent reflections} & \quad 1910 \, [R(\text{int}) = 0.0272] \\
\text{Coverage of independent reflections} & \quad 99.8\% \\
\text{Absorption correction} & \quad \text{multi-scan (SADABS)} \\
\text{Max. and min. transmission} & \quad 0.9805 \text{ and } 0.9691 \\
\text{Structure solution technique} & \quad \text{direct methods} \\
\text{Structure solution program} & \quad \text{SHELXS-97 (Sheldrick, 2008)} \\
\text{Refinement method} & \quad \text{Full-matrix least-squares on } F^2 \\
\text{Refinement program} & \quad \text{SHELXL-97 (Sheldrick, 2008)} \\
\text{Function minimized} & \quad \sum w(F_o^2 - F_c^2)^2 \\
\text{Data / restraints / parameters} & \quad 1910 / 0 / 521 \\
\text{Goodness-of-fit on } F^2 & \quad 1.026 \\
\Delta/\sigma_{\text{max}} & \quad 0.050 \\
\text{Final R indices} & \quad 1669 \quad \text{data R1} = 0.0403, \, \text{wR2} = 0.1089 \\
\end{align*}
\]
all data \( R_1 = 0.0458, \; wR_2 = 0.1150 \)

**Weighting scheme**
\[
w = \frac{1}{[\sigma^2(F_o^2) + (0.0655P)^2 + 2.3946P]}
\]
where \( P = (F_o^2 + 2F_c^2) / 3 \)

**Extinction coefficient**
\( 0.0018(5) \)

**Largest diff. peak and hole**
\( 0.189 \) and \( -0.139 \text{ eÅ}^{-3} \)

**R.M.S. deviation from mean**
\( 0.025 \text{ eÅ}^{-3} \)

---

Selected Bond lengths (Å), bond angles (°) and Torsion angles(°) for compound 1.

<table>
<thead>
<tr>
<th>Bond Lengths</th>
<th>Bond Angles</th>
<th>Torsion Angles</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1-C2</td>
<td>1.502(5)</td>
<td>N3-C1-C2-C3</td>
</tr>
<tr>
<td>C1-C13</td>
<td>1.511(5)</td>
<td>N3-C1-C13</td>
</tr>
<tr>
<td>N3-N4</td>
<td>1.402(5)</td>
<td>C9-C8-C13-C1</td>
</tr>
<tr>
<td>N3-C1</td>
<td>1.486(5)</td>
<td>C13-C1-C22-C27</td>
</tr>
<tr>
<td>N3-C28</td>
<td>1.369(6)</td>
<td>C22-C1-C13-C12</td>
</tr>
<tr>
<td>N4-N3-C28</td>
<td>130.0(4)</td>
<td>C2-C1-N3-C28</td>
</tr>
</tbody>
</table>
X-ray crystal structure of 2

Sample and crystal data for compound 2, CDCC 759587

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical formula</td>
<td>C$<em>{33}$H$</em>{34}$N$<em>{4}$O$</em>{3}$</td>
</tr>
<tr>
<td>Formula weight</td>
<td>534.64</td>
</tr>
<tr>
<td>Temperature</td>
<td>296(2) K</td>
</tr>
<tr>
<td>Wavelength</td>
<td>0.71073 Å</td>
</tr>
<tr>
<td>Crystal size</td>
<td>0.19 x 0.26 x 0.33 mm</td>
</tr>
<tr>
<td>Crystal habit</td>
<td>lustrous intense pink-red rectangular prism</td>
</tr>
<tr>
<td>Crystal system</td>
<td>monoclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>P 1 21/c 1</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
<td>$a = 17.4993$ Å, $\alpha = 90.000^\circ$</td>
</tr>
</tbody>
</table>
Sample and crystal data for compound 2

<table>
<thead>
<tr>
<th>Theta range for data collection</th>
<th>1.43 to 28.28°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Index ranges</td>
<td>-23&lt;=h&lt;=23, -15&lt;=k&lt;=15, -38&lt;=l&lt;=38</td>
</tr>
<tr>
<td>Reflections collected</td>
<td>221015</td>
</tr>
<tr>
<td>Independent reflections</td>
<td>14261 [R(int) = 0.0879]</td>
</tr>
<tr>
<td>Coverage of independent reflections</td>
<td>100.0%</td>
</tr>
<tr>
<td>Absorption correction</td>
<td>numerical</td>
</tr>
<tr>
<td>Max. and min. transmission</td>
<td>0.9853 and 0.9740</td>
</tr>
<tr>
<td>Structure solution technique</td>
<td>direct methods</td>
</tr>
<tr>
<td>Structure solution program</td>
<td>SHELXS-97 (Sheldrick, 2008)</td>
</tr>
<tr>
<td>Refinement method</td>
<td>Full-matrix least-squares on F^2</td>
</tr>
<tr>
<td>Refinement program</td>
<td>SHELXL-97 (Sheldrick, 2008)</td>
</tr>
</tbody>
</table>
Function minimized $\Sigma w(F_o^2 - F_c^2)^2$

Data / restraints / parameters $14261 / 0 / 730$

Goodness-of-fit on $F^2$ 1.014

$\Delta/\sigma_{\text{max}}$ 0.003

Final R indices

$I > 2\sigma(I)$

Data: $R_1 = 0.0712$, $wR_2 = 0.1906$

All data $R_1 = 0.1770$, $wR_2 = 0.2515$

Weighting scheme

$w = 1/[\sigma^2(F_o^2) + (0.1094P)^2 + 1.4870P]$ where $P = (F_o^2 + 2F_c^2)/3$

Extinction coefficient 0.0009(3)

Largest diff. peak and hole 0.429 and -0.327 eÅ$^3$

R.M.S. deviation from mean 0.047 eÅ$^3$

---

Selected Bond lengths (Å), bond angles (°) and Torsion angles(°) for compound 2

<table>
<thead>
<tr>
<th>Bond</th>
<th>Length (Å)</th>
<th>Bond Angle (°)</th>
<th>Torsion Angle(°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1-C2</td>
<td>1.507(4)</td>
<td>109.7(2)</td>
<td>-34.9(4)</td>
</tr>
<tr>
<td>C101-C102</td>
<td>1.509(4)</td>
<td>110.6(2)</td>
<td>-54.2(4)</td>
</tr>
<tr>
<td>C13-C113</td>
<td>1.509(4)</td>
<td>112.4(2)</td>
<td>98.8(2)</td>
</tr>
<tr>
<td>C101-C113</td>
<td>1.509(4)</td>
<td>111.5(2)</td>
<td>102.3(2)</td>
</tr>
<tr>
<td>N3-N4</td>
<td>1.368(3)</td>
<td>113.6(2)</td>
<td>125.0(3)</td>
</tr>
<tr>
<td>N103-N104</td>
<td>1.377(3)</td>
<td>112.3(2)</td>
<td>120.9(3)</td>
</tr>
<tr>
<td>C1-N3</td>
<td>1.489(3)</td>
<td></td>
<td>-76.2(4)</td>
</tr>
</tbody>
</table>

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X-ray crystal structure of 3

Sample and crystal data for compound 3. CCDC 759585

<table>
<thead>
<tr>
<th>Chemical formula</th>
<th>C_{34}H_{36}N_{4}O_{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formula weight</td>
<td>548.67</td>
</tr>
<tr>
<td>Temperature</td>
<td>296(2) K</td>
</tr>
<tr>
<td>Wavelength</td>
<td>0.71073 Å</td>
</tr>
</tbody>
</table>
Crystal size 0.15 x 0.23 x 0.54 mm

Crystal habit translucent light pink cut square needle

Crystal system orthorhombic

Space group P c a 21

Unit cell dimensions
\[ a = 20.7388(2) \text{ Å} \quad \alpha = 90^\circ \]
\[ b = 11.9816(2) \text{ Å} \quad \beta = 90^\circ \]
\[ c = 12.2015(2) \text{ Å} \quad \gamma = 90^\circ \]

Volume \[ 3031.88(8) \text{ Å}^3 \]

Z 4

Density (calculated) 1.202 Mg/cm\(^3\)

Absorption coefficient 0.078 mm\(^{-1}\)

F(000) 1168

Sample and crystal data for compound 3

Theta range for data collection 1.70 to 26.37\(^\circ\)

Index ranges \[-25 \leq h \leq 25, -14 \leq k \leq 14, -15 \leq l \leq 15\]

Reflections collected 82936

Independent reflections 6181 [R(int) = 0.0632]

Coverage of independent reflections 100.0%

Absorption correction numerical

Max. and min. transmission 0.9886 and 0.9590

Structure solution technique direct methods
Structure solution

program: SHELXS-97 (Sheldrick, 2008)

Refinement method

Full-matrix least-squares on F^2

Refinement program

SHELXL-97 (Sheldrick, 2008)

Function minimized

\[ \Sigma w(F_o^2 - F_c^2)^2 \]

Data / restraints / parameters

6181 / 1 / 376

Goodness-of-fit on F^2

1.159

\[ \Delta/\sigma_{\text{max}} \]

0.067

Final R indices

3670 data; R1 = 0.0593, wR2 = 0.1648

I>2\sigma(I)

R1 = 0.1049, wR2 = 0.1909

Weighting scheme

\[ w = 1/[(\sigma^2(F_o^2) + (0.0969P)^2 + 0.0000P)] \]

where P = (F_o^2 + 2F_c^2)/3

Extinction coefficient

0.0023(9)

Largest diff. peak and hole

0.425 and -0.162 eÅ^3

R.M.S. deviation from mean

0.035 eÅ^3

Selected Bond lengths (Å), bond angles (°) and Torsion angles(°) for compound 3

<table>
<thead>
<tr>
<th>Bond</th>
<th>Length (Å)</th>
<th>Bond</th>
<th>Angle (°)</th>
<th>Torsion Angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2-C1</td>
<td>1.499(5)</td>
<td>C2-C1-C13</td>
<td>110.9(3)</td>
<td>C3-C2-C1-N3</td>
</tr>
<tr>
<td>C1-C13</td>
<td>1.501(4)</td>
<td>C2-C1-N3</td>
<td>112.2(2)</td>
<td>C1-C13-C8-C9</td>
</tr>
<tr>
<td>N3-N4</td>
<td>1.373(4)</td>
<td>C13-C1-N3</td>
<td>112.0(2)</td>
<td>C13-C1-C22-C27</td>
</tr>
<tr>
<td>C1-N3</td>
<td>1.510(4)</td>
<td>N3-C1-C22</td>
<td>98.1(2)</td>
<td>C22-C1-C13-C12</td>
</tr>
</tbody>
</table>
X-ray crystal structure of 4

Sample and crystal data for compound 4. CCDC 759584

<table>
<thead>
<tr>
<th>Chemical formula</th>
<th>$\text{C}<em>{41}\text{H}</em>{40}\text{N}_6\text{O}_5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formula weight</td>
<td>696.79</td>
</tr>
<tr>
<td>Temperature</td>
<td>296(2) K</td>
</tr>
<tr>
<td>Wavelength</td>
<td>0.71073 Å</td>
</tr>
<tr>
<td>Crystal size</td>
<td>0.21 x 0.25 x 0.71 mm</td>
</tr>
<tr>
<td>Crystal habit</td>
<td>translucent intense orange-red rectangular prism</td>
</tr>
<tr>
<td>Crystal system</td>
<td>monoclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>P 1 21/n 1</td>
</tr>
</tbody>
</table>
**Unit cell dimensions**

\[ a = 11.8149(5) \text{ Å} \quad \alpha = 90^\circ \]
\[ b = 11.5722(5) \text{ Å} \quad \beta = 98.9570(10)^\circ \]
\[ c = 26.1223(11) \text{ Å} \quad \gamma = 90^\circ \]

**Volume**

\[ 3528.0(3) \text{ Å}^3 \]

**Z**

4

**Density (calculated)**

1.312 Mg/cm\(^3\)

**Absorption coefficient**

0.088 mm\(^{-1}\)

**F(000)**

1472

---

**Sample and crystal data for compound 4**

**Theta range for data collection**

1.58 to 27.10°

**Reflections collected**

7761

**Coverage of independent reflections**

99.8%

**Absorption correction**

numerical

**Max. and min. transmission**

0.9818 and 0.9405

**Structure solution technique**

direct methods

**Structure solution program**

SHELXS-97 (Sheldrick, 2008)

**Refinement method**

Full-matrix least-squares on \( F^2 \)

**Refinement program**

SHELXL-97 (Sheldrick, 2008)

**Function minimized**

\[ \Sigma w(F_o^2 - F_c^2)^2 \]
Data / restraints / parameters

Goodness-of-fit on $F^2$

Final R indices

Weighting scheme

Extinction coefficient

Largest diff. peak and hole

R.M.S. deviation from mean

Selected Bond lengths (Å), bond angles (°) and Torsion angles(°)

for compound 4

<table>
<thead>
<tr>
<th>Bond</th>
<th>Distance</th>
<th>Angle</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2-C1</td>
<td>1.515(2)</td>
<td>C13-C1-C2</td>
<td>110.12(14)</td>
</tr>
<tr>
<td>C13-C1</td>
<td>1.514(2)</td>
<td>N3-C1-C2</td>
<td>111.38(14)</td>
</tr>
<tr>
<td>N4-N3</td>
<td>1.390(2)</td>
<td>N3-C1-C13</td>
<td>110.59(14)</td>
</tr>
<tr>
<td>C1-N3</td>
<td>1.488(2)</td>
<td>N3-C1-C22</td>
<td>99.85(14)</td>
</tr>
<tr>
<td>C28-N3</td>
<td>1.382(2)</td>
<td>C28-N3-N4</td>
<td>126.43(15)</td>
</tr>
</tbody>
</table>
X-ray crystal structure of 6

Table 1.5.1 Sample and crystal data for compound 6. CCDC 759586

<table>
<thead>
<tr>
<th>Chemical formula</th>
<th>C\textsubscript{33}H\textsubscript{33}BrN\textsubscript{4}O\textsubscript{3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formula weight</td>
<td>613.54</td>
</tr>
<tr>
<td>Temperature</td>
<td>296(2) K</td>
</tr>
<tr>
<td>Wavelength</td>
<td>0.71073 Å</td>
</tr>
<tr>
<td>Crystal size</td>
<td>0.31 x 0.35 x 0.73 mm</td>
</tr>
<tr>
<td>Crystal system</td>
<td>orthorhombic</td>
</tr>
<tr>
<td>Space group</td>
<td>P c a 21</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
<td>\begin{align*} a &amp;= 20.5931(5) \text{ Å} \quad \alpha = 90^\circ \ b &amp;= 12.0025(2) \text{ Å} \quad \beta = 90^\circ \ c &amp;= 12.2360(4) \text{ Å} \quad \gamma = 90^\circ \end{align*}</td>
</tr>
<tr>
<td>Volume</td>
<td>3024.36(15) Å\textsuperscript{3}</td>
</tr>
<tr>
<td>Property</td>
<td>Value</td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>Density (calculated)</td>
<td>1.347 Mg/cm$^3$</td>
</tr>
<tr>
<td>Absorption coefficient</td>
<td>1.399 mm$^{-1}$</td>
</tr>
<tr>
<td>F(000)</td>
<td>1272</td>
</tr>
</tbody>
</table>

### Sample and crystal data for compound 6

- **Theta range for data collection**: 1.96 to 27.10°
- **Reflections collected**: 6674
- **Coverage of independent reflections**: 100.0%
- **Absorption correction**: numerical
- **Max. and min. transmission**: 0.6710 and 0.4279
- **Structure solution technique**: direct methods
- **Structure solution program**: SHELXS-97 (Sheldrick, 2008)
- **Refinement method**: Full-matrix least-squares on $F^2$
- **Refinement program**: SHELXL-97 (Sheldrick, 2008)
- **Function minimized**: $\sum w(F_o^2 - F_c^2)^2$
- **Data / restraints / parameters**: 6674 / 1 / 374
- **Goodness-of-fit on $F^2$**: 1.127
- **$\Delta/\sigma_{\text{max}}$**: 0.002
- **Final R indices**: 4417 data; $R1 = 0.0669$, $wR2 = 0.1644$
all data  

R1 = 0.0986, wR2 = 0.1756

**Weighting scheme**

\[
w = 1 / \left[ \sigma^2(F_o^2) + (0.0519P)^2 + 3.2678P \right]
\]

where \( P = (F_o^2 + 2F_c^2) / 3 \)

**Absolute structure parameter**

-0.0(0)

**Extinction coefficient**

0.0002(9)

**Largest diff. peak and hole**

0.478 and -0.712 eÅ⁻³

**R.M.S. deviation from mean**

0.055 eÅ⁻³

---

Selected Bond lengths (Å), bond angles (°) and Torsion angles (°) for compound 6

<table>
<thead>
<tr>
<th>Bond Lengths</th>
<th>Bond Angles</th>
<th>Torsion Angles</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1-C2</td>
<td>1.517(7)</td>
<td></td>
</tr>
<tr>
<td>C1-C13</td>
<td>1.513(6)</td>
<td></td>
</tr>
<tr>
<td>N3-N4</td>
<td>1.363(7)</td>
<td></td>
</tr>
<tr>
<td>C1-N3</td>
<td>1.491(6)</td>
<td></td>
</tr>
<tr>
<td>C28-N3</td>
<td>1.375(7)</td>
<td></td>
</tr>
<tr>
<td>C13-C1-C2</td>
<td>1.513(6)</td>
<td></td>
</tr>
<tr>
<td>N3-C1-C2</td>
<td>112.2(4)</td>
<td></td>
</tr>
<tr>
<td>N3-C1-C13</td>
<td>112.1(4)</td>
<td></td>
</tr>
<tr>
<td>N3-C1-C22</td>
<td>99.4(4)</td>
<td></td>
</tr>
<tr>
<td>N4-N3-C28</td>
<td>115.8(4)</td>
<td></td>
</tr>
<tr>
<td>N3-C1-C2</td>
<td>110.9(4)</td>
<td></td>
</tr>
<tr>
<td>C13-C1-C22</td>
<td>115.8(4)</td>
<td></td>
</tr>
<tr>
<td>C2-C1-N3-C28</td>
<td>117.8(4)</td>
<td></td>
</tr>
<tr>
<td>C9-C8-C13-C1</td>
<td>178.0(5)</td>
<td></td>
</tr>
<tr>
<td>C13-C1-C22-C27</td>
<td>-118.9(4)</td>
<td></td>
</tr>
<tr>
<td>C2-C1-N3-C28</td>
<td>117.8(4)</td>
<td></td>
</tr>
</tbody>
</table>

---

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Appendix B

$^1$H NMR, $^{13}$C NMR, ESI-MS, and X-ray crystallography data of rhodamine derivatives in chapter 2
$^1$H NMR of 9 in CDCl$_3$

$^{13}$C NMR of 9 in CDCl$_3$
$^{1}$H NMR of 10 in CDCl$_3$

$^{13}$C NMR of 10 in CDCl$_3$
$^{1}H$ NMR of 13 in CDCl$_3$

$^{13}C$ NMR of 13 in CDCl$_3$
$^1$H NMR of 12 in CDCl$_3$

$^{13}$C NMR of 12 in CDCl$_3$
$^1$H NMR of 11 in CDCl$_3$/CD$_3$CN

$^{13}$C NMR of 11 in CDCl$_3$
ESI mass spectra of 9
ESI mass spectra of 13

ESI mass spectra of 12
ESI mass spectra of 11
X-ray Crystal Structure of 9

Sample and crystal data for compound 9 CDCC 730370.

<table>
<thead>
<tr>
<th>Chemical formula</th>
<th>C&lt;sub&gt;33&lt;/sub&gt;H&lt;sub&gt;34&lt;/sub&gt;N&lt;sub&gt;4&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formula weight</td>
<td>550.70</td>
</tr>
<tr>
<td>Temperature</td>
<td>296(2) K</td>
</tr>
<tr>
<td>Wavelength</td>
<td>0.71073 Å</td>
</tr>
<tr>
<td>Crystal size</td>
<td>0.25 x 0.40 x 0.40 mm</td>
</tr>
<tr>
<td>Crystal habit</td>
<td>pink fragment</td>
</tr>
<tr>
<td>Crystal system</td>
<td>monoclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>P2&lt;sub&gt;1&lt;/sub&gt;/c</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
<td>a = 17.6430(10) Å, b = 11.5764(7) Å, c = 29.3069(18) Å</td>
</tr>
<tr>
<td>Volume</td>
<td>5837.0(6) Å&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Z</td>
<td>8</td>
</tr>
<tr>
<td>Density (calculated)</td>
<td>1.253 M g/cm&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Absorption coefficient</td>
<td>0.147 mm&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>F(000)</td>
<td>2336</td>
</tr>
</tbody>
</table>

Data collection and structure refinement for compound 9.

| Theta range for data collection | 1.64 to 22.90° |
| Index ranges                   | -17<=h<=18, -11<=k<=11, -30<=l<=30 |
Reflections collected 103849
Independent reflections 6261 \( [R(\text{int}) = 0.0485] \)
Coverage of independent reflections 78.0%
Absorption correction multi-scan (SADABS)
Max. and min. transmission 0.9641 and 0.9434
Structure solution technique direct methods
Structure solution program SHELXS-97 (Sheldrick, 2008)
Refinement method Full-matrix least-squares on \( F^2 \)
Refinement program SHELXL-97 (Sheldrick, 2008)
Function minimized \( \Sigma w(F_o^2 - F_e^2)^2 \)
Data / restraints / parameters 6261 / 0 / 730
Goodness-of-fit on \( F^2 \) 1.026
\( \Delta/\sigma_{\text{max}} \) 0.006
Final R indices
3997 data; I>2\( \sigma(I) \) \( R_1 = 0.0570, wR_2 = 0.1381 \)
all data \( R_1 = 0.1082, wR_2 = 0.1689 \)
Weighting scheme \( w = 1/[(\sigma^2(F_o^2)+(0.0707P)^2+7.8099P)] \)
where \( P = (F_o^2+2F_e^2)/3 \)
Extinction coefficient 0.0001(1)
Largest diff. peak and hole 0.432 and \(-0.497 \text{ eÅ}^{-3}\)
R.M.S. deviation from mean 0.045 \text{ eÅ}^{-3}

Selected Bond lengths (Å), bond angles (°) and Torsion angles(°)
for compound 9.

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>C1-C2</td>
<td>1.511(6)</td>
<td>C2-C1-C13</td>
<td>110.0(3)</td>
</tr>
<tr>
<td>C1-C13</td>
<td>1.511(6)</td>
<td>N3-C1-C2</td>
<td>113.4(3)</td>
</tr>
<tr>
<td>N3-N4</td>
<td>1.376(5)</td>
<td>N3-C1-C13</td>
<td>112.0(3)</td>
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<tr>
<td>N3-C1</td>
<td>1.491(5)</td>
<td>N3-C1-C22</td>
<td>98.8(3)</td>
</tr>
<tr>
<td>N3-C28</td>
<td>1.395(6)</td>
<td>N4-N3-C28</td>
<td>116.2(4)</td>
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<tr>
<td>C9-C8-C13-C1</td>
<td>-171.8(4)</td>
<td></td>
<td></td>
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<tr>
<td>C13-C1-C22-C27111.7(4)</td>
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<td></td>
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<tr>
<td>C22-C1-C13-C12-70.3(5)</td>
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<td></td>
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<tr>
<td>C28-N3-C1-C2</td>
<td>125.9(4)</td>
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<td></td>
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X-ray Crystal Structure of 10

Sample and crystal data for 10.

<table>
<thead>
<tr>
<th>Identification code</th>
<th>Aru25</th>
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<tbody>
<tr>
<td>Chemical formula</td>
<td>C_{38}H_{36}N_{4}O_{4}</td>
</tr>
<tr>
<td>Formula weight</td>
<td>612.71</td>
</tr>
<tr>
<td>Temperature</td>
<td>100(2) K</td>
</tr>
<tr>
<td>Wavelength</td>
<td>0.71073 Å</td>
</tr>
<tr>
<td>Crystal size</td>
<td>0.20 x 0.23 x 0.32 mm</td>
</tr>
<tr>
<td>Crystal habit</td>
<td>light orange rectangular prism</td>
</tr>
<tr>
<td>Crystal system</td>
<td>monoclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>P 1 2\overline{1}/n 1</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
<td>a = 15.464(4) Å ( \alpha = 90^\circ )</td>
</tr>
<tr>
<td></td>
<td>b = 12.117(4) Å ( \beta = 107.69(2)^\circ )</td>
</tr>
</tbody>
</table>
Volume: 3023.9(14) Å³

Z: 4

Density (calculated): 1.346 Mg/cm³

Absorption coefficient: 0.088 mm⁻¹

F(000): 1296

Data collection and structure refinement for 10.

Theta range for data collection: 1.56 to 30.03°

Index ranges: -21 ≤ h ≤ 21, -17 ≤ k ≤ 17, -23 ≤ l ≤ 23

Reflections collected: 122946

Independent reflections: 8851 [R(int) = 0.0729]

Coverage of independent reflections: 100.0%

Absorption correction: multi-scan

Max. and min. transmission: 0.9825 and 0.9723

Structure solution technique: direct methods

Structure solution: SHELXS-97 (Sheldrick, 2008)
program

Refinement method  Full-matrix least-squares on $F^2$

Refinement program  SHELXL-97 (Sheldrick, 2008)

Function minimized  $\Sigma w(F_o^2 - F_c^2)^2$

Data / restraints / parameters  8851 / 0 / 419

Goodness-of-fit on $F^2$  1.024

$\Delta/\sigma_{max}$  0.001

Final R indices

6099 data;  

R1 = 0.0466, wR2 =

I>2$\sigma$(I)  0.1320

R1 = 0.0803, wR2 =

all data  0.1566

Weighting scheme

$w = 1/[\sigma^2(F_o^2) + (0.1000P)^2 + 0.0000P]$

where $P = (F_o^2 + 2F_c^2)/3$

Largest diff. peak and hole

0.341 and -0.258 eÅ$^{-3}$

R.M.S. deviation from mean

0.054 eÅ$^{-3}$
Appendix C

$^1$H NMR, $^{13}$C NMR, $^1$H-$^1$H-COSY, and ESI-MS data of rhodamine derivatives in chapter 3
$^1$H NMR of 15 in DMSO-d$_6$

$^{13}$C NMR of 15 in DMSO-d$_6$
$^1$H-$^1$H COSY of 15 in DMSO-d$_6$

ESI mass spectrum of 15
Appendix D

$^1\text{H}$ NMR, $^{13}\text{C}$ NMR, and ESI-MS data of rhodamine derivatives in chapter 4
$^1$H NMR of 17 in CDCl$_3$

$^{13}$C NMR of 17 in CDCl$_3$
$^1$H NMR of 18 in CDCl$_3$

$^{13}$C NMR of 18 in CDCl$_3$
$^1$H NMR of 19 in CDCl$_3$

$^{13}$C NMR of 19 in CDCl$_3$
ESI mass spectrum of 17
ESI mass spectrum of 18

ESI mass spectrum of 19
Appendix E

$^1$H NMR and $^{13}$C NMR data of siderophore analogs in chapter 5
\(^1\)H NMR of 22 in CDCl\(_3\)

\(^{13}\)C NMR of 22 in CDCl\(_3\)
$^1$H NMR of 23 in CDCl$_3$
$^1$H NMR of 26 in CDCl$_3$

$^1$H NMR of 27 in CDCl$_3$
$^1$H NMR of 27 in CDCl$_3$

$^1$H NMR of 29 in CDCl$_3$

$^1$H NMR of 29 in CDCl$_3$
$^1$H NMR of 30 in CDCl$_3$