Investigation of Zinc Metalloneurochemistry with Fluorescent Sensors Based on Fluorescein Platforms

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SUBMITTED TO THE DEPARTMENT OF CHEMISTRY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN INORGANIC CHEMISTRY AT THE MASSACHUSETTS INSTITUTE OF TECHNOLOGY

February 2003
September 2002

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Submitted to the Department of Chemistry on September 24, 2002, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Abstract

Chapter 1. Coordination Chemistry for the Neurosciences

Metal ions are integral components of numerous enzymes and proteins. Although the field of bioinorganic chemistry has not focused on the brain and central nervous system, metal ions are vital to many neurological functions and are implicated in several neurological disorders. In this chapter, I present a brief overview of the functions of metal ions in neurobiology and highlight recent advances in the use of fluorescent sensors to study the neurotransmitters zinc and nitric oxide.

Chapter 2. Fluorescent Sensors for Zn$^{2+}$ Based on a Fluorescein Platform: Synthesis, Properties and Intracellular Distribution

Two new fluorescent sensors for Zn$^{2+}$ that utilize fluorescein as a reporting group, Zinpyr-1 (ZP1) and Zinpyr-2 (ZP2), have been synthesized and characterized. ZP1 is prepared in one step via a Mannich reaction, and ZP2 is obtained in a multi-
step synthesis that utilizes 4',5'-fluorescein dicarboxaldehyde as a key intermediate. Both ZP sensors have excitation and emission wavelengths in the visible range (~500 nm), dissociation constants \( (K_{d1}) \) for \( \text{Zn}^{2+} \) of less than 1 nM, quantum yields approaching unity (\( \Phi = 0.9 \)), and cell permeability, making them well suited for intracellular applications. A 3- to 5-fold fluorescent enhancement occurs under simulated physiological conditions corresponding to the binding of the \( \text{Zn}^{2+} \) cation to the sensor, which inhibits a photo-induced electron transfer (PET) quenching pathway. The X-ray crystal structure of a 2:1 \( \text{Zn}^{2+}/\text{ZP1} \) complex has been solved. It is the first structurally characterized example of a complex of fluorescein substituted with metal binding ligands.

Chapter 3  Improved Synthetic Methods for Preparing Fluorescein-Based Sensors and Application to the Preparation of ZP3

The synthetic precursor to ZP2 is a fluorescein dialdehyde prepared by a low yielding oxidation reaction. Several pathways for accessing versatile fluorescein scaffolds for \( \text{Zn}^{2+} \) sensors have been explored. Although attempts to convert 4',5'-bis(bromomethyl)fluorescein dibenzoate to a diol were unsuccessful, substitution of the benzoate ester protecting groups of silyl ethers permitted the bromo groups to be activated toward nucleophilic substitution upon treatment with \text{AgNO}_3. This method has been applied to the synthesis of ZP3 (Zinpyr-3, 9-(o-carboxyphenyl)-2-chloro-5-[2-[bis(2-pyridylmethyl)aminomethyl]-N-(p-anisidine)]-6-hydroxy-3-xanthanone). ZP3 binds \( \text{Zn}^{2+} \), but exhibits only a modest enhancement of the quantum yield from 0.04 to 0.05.
Chapter 4. ZP4, an Improved Neuronal Zn$^{2+}$ Sensor of the Zinpyr Family

A second-generation fluorescent sensor for Zn$^{2+}$ from the Zinpyr family, ZP4, has been synthesized and characterized. ZP4 (Zinpyr-4, 9-(o-carboxyphenyl)-2-chloro-5-[2-[bis(2-pyridylmethyl)aminomethyl]-N-methylaniline]-6-hydroxy-3-xanthanone) is prepared by a convergent synthetic strategy developed from previous studies with these compounds. ZP4, like its predecessors, has excitation and emission wavelengths in the visible range (~500 nm), a dissociation constant ($K_d$) for Zn$^{2+}$ of less than 1 nM and a high quantum yields ($\Phi = 0.4$), making it well suited for intracellular applications. Zn$^{2+}$-binding to ZP4 inhibits a photo-induced electron transfer (PET) quenching pathway resulting in a 5-fold fluorescent enhancement under simulated physiological conditions. The metal-binding stereochemistry of ZP4 was evaluated through the synthesis and X-ray structural characterization of [M(BPAMP)(H$_2$O)$_n$]$^+$ complexes, where BPAMP is [2-[bis(2-pyridylmethyl)aminomethyl]-N-methylaniline]-phenol and M = Mn$^{2+}$, Zn$^{2+}$ (n = 1) or Cu$^{2+}$ (n = 0).

Chapter 5. Identification of Neuronal Cells Suffering Zinc Toxicity by Use of a Novel Fluorescent Sensor

During excitotoxic brain damage, injured neurons accumulate an anomalous, pathological burden of weakly bound, rapidly exchangeable Zn$^{2+}$ that diffusely fills the soma, nucleus and proximal dendrites. Mounting evidence indicates that this Zn$^{2+}$ is a major contributing factor in the subsequent demise of the damaged neurons. Thus, identifying, imaging, and characterizing zinc-filled cells has become an essential step in understanding excitotoxicity. ZP4 quite vividly labels zinc-filled neurons in frozen histologic sections. The method is more sensitive and selective that the existing TSQ, and simpler that the Timm-Danscher silver, staining techniques.
Chapter 6. Further Applications of ZP Sensors: Two-Photon Microscopy and Surface Attachment Strategy

Two-photon microscopy (TPM) is a powerful imaging technique that utilizes non-linear excitation of fluorophores. Initial measurements indicate that ZP1, ZP2 and ZP4 undergo two-photon excitation at wavelengths between 740 and 920 nm and the emission intensity of each is enhanced upon the addition of Zn$^{2+}$. In addition TPM can be used to monitor exogenously applied Zn$^{2+}$ in MCF-7 cells with ZP1. Efforts to develop methodology for attaching ZP derivatives to surfaces have been initiated. Toward this goal, the ZP derivative 9-(3,4,5,6-tetrafluoro-2-carboxyphenyl)-2,7-dichloro-4,5-bis[bis(2-pyridyl-methyl)-aminomethyl]-6-hydroxy-3-xanthanone (ZinpyrF-1, ZPF1) has been prepared and characterized. The fluorescence properties of ZPF1 are similar to those of previous ZP sensors. Nucleophilic aromatic substitution of a fluorine atom on the phthalate ring by thiol derivatives provides a potential strategy for affixing ZPF1 to a solid support.

Chapter 7. The Rhodafluor Family, an Initial Study of Potential Ratiometric Fluorescent Sensors for Zn$^{2+}$

A new class of ratiometric Zn$^{2+}$ sensors that employ a hybrid fluorescein and rhodamine fluorophore has been designed, and two members of the rhodafluor family of sensors, RF1 and RF2, have been synthesized. The preparation of RF1 (9-(o-carboxyphenyl)-2-chloro-6-[bis(2-pyridylmethyl)amino]-3-xanthanone, Rhodafluor-1), uses conventional synthetic methods. Elaboration of the RF1 synthesis in an effort to enhance the Zn$^{2+}$ affinity was unsuccessful, so palladium catalyzed aryl amination was applied to prepared RF2 (1-[9'-(o-car-boxyphenyl)-6'-amino-2'-chloro-3'-xanthanone]-4,10-(diethyl)-7-(2-pyridylmeth-yl)-1,4,7,10-tetraazacyclo-dodecane,
Rhodafluor-2). The key step in the synthesis of RF2 is coupling of a tri-protected tetraazamacrocycle (cyclen) to 3-bromoanisidine. RF2 binds Zn$^{2+}$ with a dissociation constant of 13.5 μM accompanied by a ~50% increase in quantum yield. Although only small shifts in absorption wavelength were observed, because protonation of the amino nitrogen atoms of the macrocycle prevents the uncomplexed sensor from adopting the desired mesomer, the intensity doubling makes the probe of value for immediate application in situations where our previous tight binding (< 1 nM) sensors are inadequate.
This thesis is dedicated to the memory of my father
Acknowledgements

A Ph.D. thesis is not so much a stack of papers and a collection of new compounds, but rather the culmination of a long learning process influenced by numerous people who do not always receive enough recognition for their contributions. As I put the finishing touches on this document, it is necessary to thank all those people who have helped me achieve all that follows.

First, I would like to thank my advisor Steve Lippard for his guidance over the past five years, and the opportunity to undertake an exciting new project in his lab. Since I had no prior experience in bioinorganic or biochemistry prior to coming to MIT, Steve had faith that somehow we could succeed in undertaking research in a challenging new field. I thank him for providing the resources necessary to do good science, staffing the lab with talented and helpful colleagues and guiding the project in many interesting directions. Steve is a great mentor with a genuine appreciation for the learning process, and great enthusiasm for chemistry and teaching people how to do science.

The Lippard Lab has always been a stimulating and enjoyable place to work. Since joining the group, I had the opportunity to interact with a number of talented postdocs including Shannon Stahl, Jack Mizoguchi, Justin DuBois, Seth Cohen, Chris Ziegler, George Gassner, Natalia Kaminska, Yuji Mikata, Heidi Börtzel, Josh Farrell, Ariel Haskel and many others both inside and outside of the lab. In particular I thank Justin for his expert advice in organic synthesis, and Jack, Seth and Chris for their discussions about photochemistry. My career has also overlapped with a trio of X-ray postdocs, and I thank Peter Fuhrmann, Bernhard Spingler and Weiming Bu for their assistance in solving structures. I also thank our technicians Alissa Dangle and Olga Burekova for their work with cells. Not to be overlooked are the numerous graduate students who have helped me out and have made my career in the Lippard lab more enjoyable. I thank Ann Valentine, Chuan He, Amy Barrios, Dongwhan Lee, Betsy Jamieson, Doug Whittington, Qing He and Dietrich Steinhuebel for stimulating science conversations, assistance in the lab and for their friendship. Also thanks to my lone Lippard group classmate Dan Kopp who has shared in the trials and tribulations over the past five years. Special recognition to the Neurochemistry subgroup of Carolyn Woodroffe, Scott Hildebrand, Matt Clark and Elizabeth Nolan who have had to endure my subgroup reports for varying numbers of years, and their contributions to the advancement of synthetic fluorescein chemistry. In addition I thank my UROP Jessica Harvey for her efforts toward making ZP5, and I wish Liz the best of luck as she takes over the project (if she does all the work I’ve suggested that should just about take care of her thesis!). Also best of luck to Dr. Chris Chang as he takes over the other vacated facet of the zinc-sensing project.

Much of the work describe here was done in collaboration with other groups at MIT and elsewhere. I thank Roger Tsien and Grant Walkup for their contributions to the early ZP work, and in particular Grant, who I’ve never met, but was instrumental in teaching me the techniques necessary to characterize our sensors. Also thanks to Chris Frederickson and his colleagues at NeuroBioTex for their efforts in applying our sensors to imaging. I thank Professor Peter So and Dr. Michael Previte for their efforts in performing two-photon microscopy.

I would like to thank my other colleagues at MIT. To my classmates Alan Heyduk, Christina Rudzinski and other members of the Nocera group for many helpful discussions about sensing as we initiated this project. I thank Prof. Dan
Nocera as well for his insightful questions and helpful suggestions. All of the inorganic chemistry faculty have been very helpful and friendly over my tenure at MIT. I thank Alan Davison who has served as my thesis committee chair for many entertaining conversations as well as his wisdom. I also thank Barbara Imperiali for being on my committee, following my career closely and for her suggestions concerning my work.

I'd like to recognize those people who contributed to my development as a scientist before I arrived at MIT. I thank John Argyropoulos, Steve McDonald, Arthur Doumaux and Tim Herzog for their guidance while I was an intern at Union Carbide. Also thanks to John Protasiewicz who gave me my start in academic research at Case Western Reserve University, and helped me through the process of applying to graduate school. Thanks as well to Connie Strickland and Mary Kay White for encouraging me to set my goals high, and Mary McKown for fostering my love of science early in life.

Finally I thank the members of my family for their support. I thank my Mom, Chad and Seth for always being there. Also thanks to Ellen and Mike for their support during my college years and afterwards. Thanks also to Emma for her love and support (and occasional proofreading). While I've chosen to dedicate my thesis to the memory of my Dad, I'd like to make a special dedication to my grandparents who are an inspiration to me for what they have accomplished and done in their lives. Granddad, who built a family business and kept in running for over 50 years, and whose work ethic even at 90 years old is an inspiration. Also to Mawmaw and Pawpaw, who is the original family chemist, for their love and support. My grandparents are members of the “Greatest Generation,” and their sacrifices and hard work have made it possible for me to achieve my goals.
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Chapter 1

Coordination Chemistry for the Neurosciences*
Bioinorganic Chemistry at the Beginning of the 21st Century

At the close of the 20th century, the fundamental importance of metal ions in biology was clearly established after more than fifty years of extensive research.1-5 Important developments in enzymology allowed metalloproteins to be expressed and studied by site directed mutagenesis. Technological advances in magnetic and electronic spectroscopy facilitated exploration of the properties of metal active sites. Innovative techniques macromolecular crystallography afforded atomic resolution structures of numerous metalloenzymes.6 In addition to their functions in proteins and enzymes, metals have emerged as important therapeutic and diagnostic agents in medicinal chemistry.7,8 New metalloenzymes continue to be discovered with novel functions and properties, and there is an increase in the number of metal ions being evaluated as remedies for human ailments.9-11

The last fifty years also has witnessed the use of small molecule model compounds as important tools for exploring the properties of systems that are difficult to study in their natural state.12 Reactive enzyme intermediates, as well as systems where detailed structural information is not available, can often be characterized only with the aid chemical models. Model studies can provide information to help direct work on the natural enzymes.

Of the areas at the interface between inorganic chemistry and biology that remain to be explored, the role of metal ions in neuroscience is perhaps the most prominent. Bioinorganic chemists have been attracted historically to systems involving transition metal ions because of their valuable magnetic and
spectroscopic properties. Despite the preponderance of Na⁺, K⁺, Mg²⁺ and Ca²⁺ in biological processes, the inorganic chemistry community has often ignored these metal ions because they lack the electronic and magnetic properties of their d-block counterparts. In a similar manner, neuroscientists have focused on group 1 and 2 metal ions and, until recently, have dismissed transition metals such as Mn, Fe, Cu, and Zn as inconsequential trace elements in the central nervous system. By combining the ability of bioinorganic chemists to evaluate the properties of metal ions with that of neuroscientists to explore the physiology of the nervous system, a powerful new alliance could emerge for understanding such complex processes as neurotransmission and synaptic plasticity. An important consequence would be to uncover the causes of, and develop treatments for, neurodegenerative disease.

**Inorganic Chemistry and Neurosciences**

The nervous system is a complex electrochemical computer linked to mechanical components. The extracellular medium that envelops nerve cells and the cytoplasm of these cells are replete with inorganic ions that help generate electrical currents required for movement, sensation, reflexes, learning, and memory. The unequal distribution of ions across cell membranes, and the differential permeability of these ions produced by pumps and channels, generates membrane potentials that provide the driving force for neurological events. The primary ions involved in the generation and regulation of the electric currents are the s-block alkali and alkaline earth metals Na⁺, K⁺, Mg²⁺, and Ca²⁺. The primary intracellular ion is K⁺, and Na⁺ is the major cation outside
the cell. The approximate concentrations of these ions are summarized in Table 1.1.

Recently our laboratory has initiated three projects in the neurosciences. One is to prepare structural and functional models for the K⁺ channel and the other two are to explore the neurological functions of ionic zinc (Zn²⁺) and nitric oxide (NO) by preparing new fluorescent sensors and applying them to biological systems. This chapter highlights recent advances in these areas with a focus on our progress in the synthesis and application of NO fluorescent sensors over the past three years. We also provide a brief overview of inorganic chemistry relevant to the brain and nervous system, as well as a perspective on our future goals including work on ion channel modeling.

*The Chemistry of s-Block Metal Ions and the K⁺ channel*

Cursory examination of the physical and chemical properties of Na⁺ and K⁺ reveals that the two ions are quite similar. Both are closed shell, hard ions that prefer oxygen donors; they have identical coordination geometries and display similar exchange kinetics for bound water molecules.¹ Despite these similarities, ion channels, a class of transmembrane proteins responsible for a host of biological events, can discriminate between Na⁺ and K⁺ ions with unprecedented selectivity. In addition to this selective binding property, channels facilitate the flow of ions at a rate near the diffusion limit. Perhaps the most significant recent breakthrough in understanding metal ion discrimination and ion channel function was the determination in 1998 of the X-ray crystal structure at 3.2 Å resolution of the KcsA K⁺ channel.¹⁵
The structural information provided by the crystal structure affords considerable insight into the mechanism by which the potassium channel discriminates for K⁺ over Na⁺ by a factor of $10^3$-$10^4$ and passes current at a rate of $10^8$ ions/s. The channel is a C₄-symmetric tetramer having a pore created at the interface of the four subunits. The key event in ion discrimination takes place at the extracellular end of the pore in a region termed the selectivity filter, a narrow passageway formed by the confluence of 12-16 oxygen atoms, contributed primarily from protein backbone carbonyl groups. The filter is just large enough to accommodate a partially dehydrated K⁺ ion (ionic radius 1.33 Å), but too large to allow passage of smaller Na⁺ ions (ionic radius 0.95 Å). An equatorial belt of four water molecules around an octahedral K⁺ ion is stripped away and perfectly compensated by four peptide backbone carbonyl oxygen atoms as potassium passes through the filter. For the smaller Na⁺ ion, only three such water molecules can be so compensated. The fourth water thus provides a steric block to passage of the partially hydrated sodium ion through the filter. Two octahedrally coordinated ions were located within the selectivity filter in the crystal structure analysis. Each was assumed to be equatorially coordinated by 4 oxygen atoms from the protein and 2 axial water molecules. A third hydrated ion appears at the intracellular side of the channel, presumably awaiting entry into the filter region. In the absence of excess K⁺ ions, the filter has an affinity for K⁺ of ~3 µM, but this value is lowered to ~0.1 M in the presence of other K⁺ ions. This dramatic difference illustrates how the protein balances the needs for selectivity and speed. At physiological concentrations, the channel contains several ions that diminish the inherent affinity for K⁺. In addition to the two ions that held in
the pore by chemical bonds, the third uncoordinated $K^+$ in the selectivity filter is presumably stabilized by protein electrostatics. The alignment of four helix dipoles acts as a stabilizing force for the third ion in the lipophilic membrane. The perfect positioning of these three $K^+$ ions allows coordination chemistry and electrostatic repulsion to work in concert permitting selectivity at high flow rates.

The $K^+$ channel structure is a conspicuous example of the importance of metal ion coordination chemistry in controlling the selection of metal ions and determining their role in molecular neurobiology. Other work includes studies of the Na$^+$-K$^+$ pump, of Ca$^{2+}$ pumps, and of the relationships between ion channels and neurological disease. The potential for growth of this field is enormous. Despite the wealth of opportunities, bioinorganic chemists have yet to make a significant contribution.

*Transition Metals including Zinc in the Brain*

The quantities of Fe, Zn, and Cu present in the brain are significantly lower than that of $K^+$ and Na$^+$, but similar to the concentration of Mg$^{2+}$, which is present at 0.1-0.5 mM. Recent work suggests that redox active metals including Mn generate and/or protect cells from dioxygen and protein radicals that are implicated in neurological disease. The dual capacity of transition metals both to regulate and to generate detrimental radicals requires that their homeostasis be tightly controlled.

Iron is involved in the synthesis of neurotransmitters, required as a cofactor in brain energy metabolism and suspected to play a role in neural communication. Copper enzymes also participate in several important
neurochemical processes. Included are peptidylglycine α-amidating monoxygenase that modifies neuropeptides, lysyl oxidase that cross-links elastin and collagen, and Cu,Zn-superoxide dismutase (SOD) that disproportionates harmful superoxide radicals.\textsuperscript{27} Although studies of the chaperone protein (CCS) for providing copper to superoxide dismutase suggest that there is essentially no free Cu\textsuperscript{2+} in the cytoplasm,\textsuperscript{28} there is a significant possibility that bound Cu\textsuperscript{2+} in neurons and glia, the two major cell types in brain, can be labilized and become cytotoxic. There may be a function for released Cu\textsuperscript{2+} during neurotransmission, but so far it has been difficult to establish such a role.\textsuperscript{13} The current interest in learning how metal ions are transported to their target proteins,\textsuperscript{25,29} and the increase in research implicating metals in Alzheimer’s disease (AD), Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS), and aging, indicate the need for bioinorganic chemists to help unravel the roles metal ions in neuroscience.\textsuperscript{13,30,31}

Although not redox-active like Fe, Cu, and other first row transition metals required for neurobiology, Zn\textsuperscript{2+} also plays an important role in the central nervous system (CNS).\textsuperscript{32-34} Whereas most Zn\textsuperscript{2+} in the CNS is tightly bound to proteins and enzymes, pools of free Zn\textsuperscript{2+} occur at high levels in the mossy fiber terminals of the hippocampus.\textsuperscript{35} Zinc ion has the ability to modulate a variety of ion channels,\textsuperscript{36} may play a role in neuronal death during seizures,\textsuperscript{37} is involved in neurodegenerative disorders,\textsuperscript{38} and may be vital to neurotransmission.\textsuperscript{39} Since many of the effects of Zn\textsuperscript{2+} have been demonstrated \textit{in vitro} by the addition of the exogenous metal ion, it is important to devise methods for interrogating how it functions under physiological conditions.\textsuperscript{32,35}
Concentrations of ionic Zn$^{2+}$ are estimated to vary widely throughout the CNS. The levels of Zn$^{2+}$ in the brain and elsewhere are regulated by three homologous Zn$^{2+}$ transport proteins (ZnT-1, ZnT-2, and ZnT-3)\textsuperscript{40-42} and by metallothioneins (MTs)\textsuperscript{43-45} including MT-III and MT-IV, which are expressed mainly in the brain.\textsuperscript{46-49} In addition, Zn$^{2+}$ can be released from synaptic vesicles\textsuperscript{35,50} and can enter postsynaptic cells through voltage-dependent Ca$^{2+}$ channels.\textsuperscript{32,51} Because of its diverse functions, Zn$^{2+}$ continues to be an interesting subject of research in neurobiology.

\textit{Neurochemistry of NO and other RNOS}

Nitric oxide (NO) became the focus of many biological studies following the discovery of its role as a signaling agent in the cardiovascular system,\textsuperscript{52} nervous system,\textsuperscript{53} and brain.\textsuperscript{54} The three isozymes of nitric oxide synthase (NOS) catalyze the biosynthesis of NO and L-citrulline from dioxygen and L-arginine. Neuronal NOS (nNOS), which is expressed in postsynaptic terminals of neurons in the brain, and endothelial NOS (eNOS), which is found in endothelial cells lining blood vessels, are activated by the binding of calmodulin at elevated levels of intracellular Ca$^{2+}$.\textsuperscript{55} Agonists like acetylcholine and glutamate generate increased levels of Ca$^{2+}$ that induce these two NOS enzymes to release small amounts of NO.\textsuperscript{56} Inducible NOS, which is regulated at the level of transcription,\textsuperscript{57} is expressed by macrophages and produces higher levels of NO over longer periods of time. Once formed under physiological conditions, NO can have a lifetime up to 10 minutes\textsuperscript{58} and can diffuse over a range of 100-200 µm.\textsuperscript{59} The relatively long lifetime and dispersion range permit NO to react with a
variety of targets. Reaction with O$_2$ forms reactive nitric oxide species (RNOS) such as NO$_2$ and NO$^+$, and with superoxide (O$_2^{-}$), peroxynitrite (ONOO$^-$), a potent oxidant, arises. In addition to reactions with oxygen species, NO can react with thiols, amines, and transition metal centers such as iron in oxyhemoglobin. Although certain of these reactions may be fairly innocuous, peroxynitrite appears to be the main culprit involved in nitration of tyrosine residues found in disease states, and can cause DNA strand breaks.

Over the past decade several possible roles for NO and RNOS in biological systems have been explored. NO activates soluble guanylyl cyclase, which catalyzes the formation of the intracellular messenger guanosine 3',5'-monophosphate, and NO is proposed to act as a retrograde neurotransmitter in the hippocampus during memory formation. In addition, NO damages DNA and inhibits its repair. It is also implicated in vasodilation, neurotoxicity, and neurotransmission. Although NO is necessary for many neurological signaling functions, its overproduction appears to be responsible for oxidative damage in the CNS. NO and RNOS species are suspected to play major roles in the pathogenesis of several neurological disorders, such as AD, ALS, PD, multiple sclerosis (MS), Huntington’s disease (HD), and stroke.

The study of the various functions of NO in biological systems is often hampered by the lack of a method to detect it directly and to provide temporal and spatial information about its distribution. Fluorescent sensors are ideally suited tools for performing these types of measurements. The development of an array of fluorescent sensors for Ca$^{2+}$ has facilitated the study and
understanding of its functions in the cell; however, few sensors exist for directly imaging NO.

**Fluorescent Sensors for NO and RNOS**

The stringent criteria required for an effective intracellular fluorescent sensor for detecting analytes, particularly an inorganic radical like NO, create design challenges. Sensors must be able to bind selectively and reversibly the species of interest in the presence of competitors that could produce a false signal, and should have an affinity (K_d) close to the median concentration of the analyte. The sensor should produce a positive fluorescence response or a significant change in the excitation or emission maximum upon binding of the analyte. The reporting group of the sensor should have an intense fluorescence with an excitation maximum above 340 nm to facilitate use with glass microscope lenses and to avoid UV-induced cell damage. An emission wavelength exceeding 500 nm helps to prevent interference by autofluorescence from native species in the cell. In addition to its spectral properties, the sensor must be water-soluble and have the ability to be passively and irreversibly loaded into cells. Selective binding of NO in the presence of O_2 presents a challenge, and NO is known to quench the fluorescence of fluorophores.76,77

**Griess Assay, Microsensors and Fiber Optic Sensors**

Several approaches to detecting biological NO are available that do not involve a fluorescent signal, including the Griess assay that measures nitrite, and electrochemical microsensors. These methods and others are invaluable
in studying NO biochemistry, but are often limited in sensitivity and ability to provide spatial information.\textsuperscript{82,83} Fiber optic fluorescent sensors based on soluble guanylate cyclase (sGC),\textsuperscript{84} cytochrome c\textsuperscript{85} vitamin B12,\textsuperscript{86} and 4-carboxy-2'-7'-difluorofluorescein succinimidyl ester adsorbed on a gold surface\textsuperscript{87} are capable of directly detecting NO, but can only measure NO localized near the tip of the fiber optic. Small molecule fluorescent sensors seem to provide the most powerful approach for detecting intracellular NO.

\textbf{FNOCTs}

Fluorescent sensors for directly detecting NO evolved from a class of \(\alpha\)-quinodimethanes developed for monitoring NO by EPR spectroscopy.\textsuperscript{88-90} The sensors called fluorescent nitric oxide cheletropic traps (FNOCTs, Scheme 1.1) incorporate a phenanthrene fluorophore into a \(\alpha\)-quinodimethane scaffold. Reaction of 1 with nitric oxide generates the nitroxide radical 2 which can be reduced to the corresponding hydroxylamine, 3. The dimethylamino group was incorporated into 1\textsubscript{b} to induce a red shift in the excitation and emission wavelengths compared to 1\textsubscript{a}. Reaction of NO with FNOC\textsubscript{b} \(1\textsubscript{b}\) produces an approximately 9-fold fluorescence increase (\(\Phi_{1\text{b}} = 0.03, \Phi_{3\text{b}} = 0.27\)) in the presence of ascorbate as a reductant. Compound 1\textsubscript{b} has optical properties amenable for intracellular applications (\(\lambda_{ex} = 460\) nm, \(\lambda_{em} = 600\) nm), and can detect NO at nM concentrations. The ability of FNOCT to detect biological NO was demonstrated in alveolar macrophages after functionallization of the carboxylic acids with acetoxymethylesters to permit cell loading. Although FNOCTs are capable of detecting intracellular NO, they also react with peroxynitrite. In addition, the
second-order rate constant (100 m$^{-1}$s$^{-1}$) of FNOCTs reaction with NO is slower than the reaction of NO with superoxide and iron complexes. FNOCTs also require a reductant to achieve maximum quantum yields, which may limit their use for certain applications. FNOCTs will soon become available to the biological community at large.

DAFs

The most widely used fluorescent probes for indirectly detecting intracellular NO are diaminofluoresceins (DAFs). DAFs were developed as NO sensors to overcome problems experienced using diaminonapthalenes at physiological conditions. Under aerobic conditions, DAFs react with NO at concentrations as low as 3 nM to form triazolofluoresceins (DAF-Ts, Scheme 1.2). Eleven variations of DAFs have been examined including the more stable N-methylated derivatives and a fluorinated derivative that has superior pH properties. The diarnino compounds have low quantum yields ($\Phi_{\text{DAF}} = 0.002$-$0.007$), presumably resulting from intramolecular PET (photoinduced electron transfer) quenching of the excited state by the free amines, which increase dramatically upon the formation of the triazole ring ($\Phi_{\text{DAF-T}} = 0.53$-$0.92$). DAFs can be loaded into cells by esterification of the phenols for detection of intracellular NO. Recently similar fluorescent probes based on rhodamine chromophores have been prepared. Despite their excellent optical properties owing to the use of fluorescein and rhodamine fluorophores, DAFs react with NO$^+$ equivalents such as nitric anhydride ($N_2O_5$) instead of directly with NO, so conclusions about NO based experimental results can be somewhat ambiguous.
Other NO Sensors

Several other approaches to detecting NO, such as the use of small molecule sensors based on the reaction of rhodamine B hydrazine (RBH) with nitrite\textsuperscript{97} or of dichlorodihydrofluorescein and dihydrorhodamine with a number of oxidants,\textsuperscript{98-101} have problems similar to those encountered in the application of DAFs. One possible solution to developing a sensor with the ability to bind NO selectively and reversibly is to use the formation of a transition metal nitrosyl as the signaling methodology. Such an approach would provide accurate concentration estimates by effectively competing with superoxide and metalloenzymes for NO, and eliminating misleading signals from RNOS species. Recently a quinoline pendant cyclam sensor that mimics the structure and activity of sGC has been used as a fluorescent NO sensor.\textsuperscript{102} In the absence of NO, the quinoline fluorophore is coordinated to the Fe\textsuperscript{2+} center similar to the distal histidine of sGC. In the proposed mechanism, an Fe-nitrosyl is formed upon exposure to NO under anerobic conditions, and the quinoline fluorophore is released (Scheme 1.3). The release of the quinoline results in a fluorescence decrease. Although the quinoline pendent cyclam sensor monitors NO by fluorescence quenching, a pH sensor that utilizes a similar strategy with a Ni\textsuperscript{2+} cyclam produces a positive response.\textsuperscript{103}

Co Tropocoronand Complexes

During the course of research in our laboratory on the reactivity of Fe,\textsuperscript{104} Mn\textsuperscript{105} and Co\textsuperscript{106-108} tropocoronand complexes, we reasoned that the related
aminotroponiminate ligands might be amenable to the development of Co(II)-based fluorescent NO sensors. A procedure for synthesizing \(N,N'\)-disubstituted aminotroponimine (HR\(_2\)ATI) ligands was modified to produce a series of differentially substituted derivatives incorporating a dansyl (5-dimethylaminonaphthalenesulfonamide) fluorophore with either an isopropyl, tert-butyl or benzyl group (Scheme 1.4).\(^{109}\) The 4-step synthetic route provided multi-gram quantities of the desired H\(^8\)DATI ligands in 20-50\% yields. The Co(II) complexes formed by reacting 2 equiv of the ligand with potassium or sodium hydride and CoCl\(_2\) are air stable crystalline solids that are sensitive to moisture only in solution.

X-ray structural studies of [Co\(^{tp}\)DATI\(_2\)] (14), [Co\(^{tr}\)DATI\(_2\)] (15), and [Co\(^{tb}\)DATI\(_2\)] (16) revealed that the steric bulk of the ligand favors bis(chelate) four-coordinate Co\(^{2+}\) rather than tris(chelate) octahedral Co\(^{3+}\) complexes (Fig. 1.1).\(^{110}\) The Co center in 14 is pseudo-tetrahedral with a dihedral angle, \(\Theta\), of 76.1\(^\circ\) between the two five-membered chelate rings. The differences in \(\Theta\) for the \(t\)-butyl (81.4\(^\circ\)) and benzyl (73.8\(^\circ\)) derivatives reflect the different steric requirements of the ligands.\(^{109}\) In addition, the dansyl groups of 16 are positioned with an appropriate orientation and the correct distance, 3.5(1) \(\AA\), for \(\pi-\pi\) stacking.\(^{111}\) Although the coordination chemistry of Co sulfonamides is limited to a few examples of octahedral\(^{112-114}\) and tetrahedral\(^{115,116}\) complexes, the bond distances and angles are consistent with expected values.

The HR\(_2\)ATI ligands 11-13, exhibit a broad fluorescence emission with a maximum around 500 nm when excited at 350 nm. The Co complexes 14, 15, and 16 display dramatically diminished fluorescence emission compared to the
corresponding free ligands. Figure 1.2 shows the relative fluorescence intensities of 11 and 14, measured at 40 μM in CH₂Cl₂. Transition metal ions, particularly those of the first row with unoccupied of partially filled d shells, can provide non-radiative relaxation pathways for the excited states of fluorophores by an energy- or electron-transfer mechanism.¹¹⁷,¹¹⁸

After several hours of exposure to NO, two new bands develop in the IR spectra of 14, 15, and 16 at 1838, 1760 cm⁻¹, 1833, 1760 cm⁻¹, and 1833, 1755 cm⁻¹ respectively. These absorption bands correspond to the symmetric and asymmetric stretching modes of metal dinitrosyl complexes found between 1750-1798 and 1820-1876 cm⁻¹.¹¹⁹-¹²⁶ A ¹H NMR experiment with 14 revealed that, upon exposure to NO, the paramagnetic resonances of 14 slowly disappear over a 4 day period, to be replaced by two sets of peaks corresponding to two separate diamagnetic compounds. Further studies demonstrated that one set of peaks arises from the formation of a Co-dinitrosyl species with the other set coming from H⁺DATI liberated from the Co center (eq 1).

\[
[\text{Co}^{(P)\text{DATI}}_2] + \text{NO}^+ \xrightarrow{e^-, H^+} [\text{Co(NO)}_2^{(P)\text{DATI}}_2] + \text{H}⁺\text{DATI} \quad (1)
\]

The formation of the dinitrosyl complexes of 14, 15, and 16 is accompanied by an intensity increase in fluorescence at 505 nm attributed to fluorescence from the liberated ligand fragment.¹⁰⁹ No fluorescence response occurs upon similar exposure of the complexes to O₂. The release of a ligand fragment as a signaling methodology led to the design DATI, an aminotroponimine that would remain coordinated after liberation of one of its dansyl components upon NO binding. The synthesis of H₂DATI-4 (18, Fig. 1.3) is similar to that of the unlinked ligands. The desired complex [Co(DATI-4)] (19) was readily obtained upon reaction of
H₂DATI-4 with potassium hydride and [Co(CH₃CN)₄](PF₆)₂. The crystal structure of 19 revealed the Co²⁺ center to have a distorted tetrahedral geometry with Θ = 62.2° and the dansyl groups 3.63(9) Å apart, arranged in a π-stacking type of interaction (Fig. 1.4). The tetramethylene linker chain restricts the N2-Co1-N3 angle to be 103.94(10)°, considerably smaller than the unrestricted N1-Co-N4 angle of 123.78(10)° on the opposite side of the complex.

One consequence of this geometry is that the reaction rate of 19 with NO is 50 times faster than [Co(⁴²⁴⁴DATI)₂] and other analogs in which the dansylated aminotroponimide moieties were not linked to one another. The distorted tetrahedral environment of 19 may permit better access of NO to the Co center, and the formation of the psuedotetrahedral dinitrosyl adduct may relieve geometric constraints in the starting complex, helping to drive the reaction. The formation of the desired dinitrosyl product was confirmed by the appearance of bands at 1835 and 1760 cm⁻¹, monitored by in situ IR spectroscopy. These peaks are analogous to those present in the unlinked compounds. NO stretching bands at 1827 and 1751 cm⁻¹ in the solid state spectrum shift to 1793 and 1719 cm⁻¹ following isotopic labeling experiments with ¹⁵NO, in agreement with calculated values.¹⁰⁹ ¹H NMR experiments reveal the complex to be diamagnetic with proton resonances indicative of two types of ligand arm environments.

The fluorescence emission of the Co complex 19 (Φ = 9 × 10⁻⁴) is dramatically quenched compared to that of the free ligand, 18 (Φ = 0.01). Purging of the headspace over a 40 µM solution of 19 in CH₂Cl₂ with NO results in a doubling of the emission intensity at 505 nm (λₑₓ = 350 nm) after 3 minutes; the intensity reaches a maximum 4-fold increase after 6 hours (Fig. 1.5a). Similar
exposure to air affords no such increase in fluorescence (Fig. 1.5b). Incremental addition of NO suggests the NO detection limit of 19 to be on the order of 50-100 μM.

The dissociation of a ligand arm from 19 to form the dinitrosyl cobalt complex removes one of the dansyl fluorophores from the quenching influence of the Co center and its ligands, which could account for the observed fluorescence increase (Scheme 1.5). In the Enemark-Feltham notation for nitrosyl compounds, the pseudo-tetrahedral cobalt dinitrosyl 20 is designated \([\text{Co(NO)}_2]^{10}\). The number of electrons, \(n = 10\), in an \([\text{MNO}]^{n}\) complex corresponds to the sum of d-electrons of the metal and electrons in the \(\pi^*\) orbitals of the NO ligands. The formation of such a diamagnetic cobalt dinitrosyl with a filled \(d^{10}\) shell may also remove a quenching mechanism and account for some of the fluorescence increase.\(^{127}\) The \(\pi\)-stacking of the dansyl rings in the structure of 19 does not appear to be sufficient to induce eximer formation;\(^{128}\) however, the radiative decay of the dansyl group involves a twisting of the dimethylamino group,\(^{129,130}\) and the proximity of two fluorophore rings in 19 may create a steric interaction that inhibits TICT (twisted internal charge transfer). Relaxation pathways involving unoccupied Co d-orbitals probably dominate the quenching mechanism.

The formation of a Co-dinitrosyl complex with fluorescent DATI ligands overcomes two important challenges for the detection of NO by small molecule sensors. It selectively reports NO over \(O_2\) and generates a positive fluorescence response. Although the chemistry provides an important proof of principle, the current systems are unsuitable for intracellular applications because of their low
sensitivity, water incompatibility, and irreversibility. Our current goals involve utilization of this new strategy for NO sensing to evolve probes for intracellular applications, specifically in neurobiology. Addressing the issues of reversible NO binding, water solubility, and better optical properties are the primary challenges.

Fluorescent Sensors for Zn$^{2+}$

Zinquin and Quinoline-based Probes

Several strategies have been devised to detect Zn$^{2+}$ by fluorescence including the use of peptides,$^{131-134}$ proteins,$^{135-137}$ and fluorophore-appended amines$^{138,139}$ and azamacrocycles,$^{140-142}$ however, none of these approaches are amenable to intracellular work.$^{143}$ The most widely used probes for detecting intracellular Zn$^{2+}$ are the aryl sulfonamide derivatives of 8-aminoquinoline (Fig. 1.6), TSQ (20, 6-methoxy-(8-p-toluenesulfonamido)quino-line),$^{144}$ Zinquin (21),$^{145,146}$ and TFLZn (22).$^{147}$ Recent investigations of the aqueous binding properties of these quinoline probes have clarified some discrepancies in the literature on these compounds.$^{148}$ Thermodynamic studies reveal that Zinquin analogues exhibit a high degree of cooperative binding to afford only the ZnL$_2$ complex under physiological conditions (Fig. 1.7). During Zn$^{2+}$ binding the sulfonamide nitrogen becomes deprotonated, yielding a neutral complex that has been characterized by X-ray crystallography.$^{149}$ The fluorescence of these probes is independent of pH, and the intensity increases 100-fold by addition of excess Zn$^{2+}$, owing to metal coordination to the quinoline nitrogen atom, which inhibits
a quenching pathway.\textsuperscript{149} Zinquin can detect Zn\textsuperscript{2+} at 100 pM to 10 nM concentrations and has been extensively used to study intracellular Zn\textsuperscript{2+}. Vesicle-like punctate staining patterns are observed widely in a variety of eukaryotic cells, suggesting that the concentration of free Zn\textsuperscript{2+} exceeds 100 pM in such compartments.\textsuperscript{149} Despite their ability to image intracellular Zn\textsuperscript{2+}, quinoline probes can form mixed complexes with partially coordinated Zn\textsuperscript{2+} in cells, generating uncertainty in measurements of free Zn\textsuperscript{2+}. In addition, quinoline probes have relatively dim fluorescence with quantum yields \( \approx 0.1 \) and extinction coefficients \( \approx 10 \times 10^{-3} \).\textsuperscript{150} Moreover, their excitation wavelengths are somewhat shorter than desired, so there is considerable room for improvement in sensors to facilitate the study of intracellular Zn\textsuperscript{2+}.\textsuperscript{151}

\textit{Cyclen Macrocycles}

In one approach, macrocyclic amines were applied to prepare dansylamidoethylcyclen (23), a sensor capable of detecting Zn\textsuperscript{2+} at sub-nanomolar concentrations (\( K_d = 5.5 \times 10^{-13} \)) and physiological pH (Scheme 1.6).\textsuperscript{152} Like the quinoline sulfonamides, compound 23 has an excellent selectivity for Zn\textsuperscript{2+} over Mg\textsuperscript{2+} and Ca\textsuperscript{2+}, two major competing divalent cations present in cells, owing to the use of a sulfonamide ligand. Compound 23 has a low quantum yield in aqueous solution (\( \Phi = 0.11 \)), however, exhibits only a 5-fold fluorescence intensity enhancement, and has an excitation wavelength somewhat lower than optimal for intracellular work. The application of cyclen as a receptor for Zn\textsuperscript{2+} in a sensor was extended to a pair of probes utilizing xanthene chromophores as the reporting groups (Fig. 1.8).\textsuperscript{153} The two sensors ACF-1 (24) and ACF-2 (25) have
excitation and emission wavelengths, \( \lambda_{ex}/\lambda_{em} \) 495/515 and 505/525 respectively, a range ideal for intracellular studies. At pH 7.5, the fluorescence intensity of 24 increases 14-fold upon saturation with Zn\(^{2+}\), and that of 25 increases 26-fold. Although these sensors have exceptional optical properties, they have a fairly low Zn\(^{2+}\) affinity (\( K_d = 5.7 \mu M \)),\(^{154}\) and require an intricate multi-step synthesis with low overall yields, 1.9% for 24 and 0.11% for 25. Sensors 23 - 25 are presented as fluorescent probes for monitoring intracellular Zn\(^{2+}\), but none has yet been successfully employed in biological studies.

Other Zn\(^{2+}\) Sensors

Several other sensors have been used with varying degrees of success to study Zn\(^{2+}\) in biological systems. Newport Green (26), a 2',7'-dichlorofluorescein-based sensor (Fig. 1.9), exhibits a 3.3-fold enhancement under physiological conditions, but has a relatively high dissociation constant (\( K_d = 1 \mu M \)).\(^{150}\) Traditional probes for Ca\(^{2+}\) and Mg\(^{2+}\) include fura-2 (27), mag-fura-2 (28), and mag-fura-5 (29, Fig. 1.10). The molecules intracellular Zn\(^{2+}\) sensors, but the Zn\(^{2+}\)-induced signals can be difficult to delineate from those arising from the alkali earth metal ions.\(^{51,155}\) With such probes the concentrations of chelatable Zn\(^{2+}\) have been estimated to range from sub-nM in undifferentiated mammalian cells\(^{149}\) to ~0.3 mM in hippocampal nerve synaptic vesicles.\(^{147}\) In addition to its roles in the CNS and involvement in neurodegenerative disorders,\(^{37,38}\) zinc is a critical cofactor for numerous cellular functions,\(^{39}\) and is necessary for gene expression\(^{156}\) and apoptosis.\(^{145}\) These many functions of Zn\(^{2+}\) are consistent with its postulated regulatory role in cellular metabolism.\(^{4,157}\) With such a wide range
of concentrations and functions, there is a great need for improved probes to investigate Zn\(^{2+}\) in vivo.

**Fluorescein-Based Sensors**

This thesis explores fluorescein-based sensors for zinc. Fluorescein, a xanthene-based fluorophore, is well suited for sensor applications because of its high extinction coefficient, a quantum yield approaching unity, membrane permeability, and the ready availability of optical filter sets for fluorescence microscopy. In addition to Newport Green and the ACF sensors, several other Zn\(^{2+}\) sensors that utilize xanthanone-based fluorophores have been reported. Included are ZnAF-1 (30), ZnAF-2 (31), ZnAF-1F (32) and ZnAF-2F (33), which utilize a di-2-picolylamine (DPA) group as the Zn\(^{2+}\)-binding ligand (Fig. 1.11).\(^{158,159}\) These sensors and their Zn\(^{2+}\) complexes have excitation and emission maxima near 490 nm and extinction coefficients of 70 \(\times\) 10\(^3\) M\(^{-1}\) cm\(^{-1}\). The emission intensity of 30, 31, 32 and 33 increases 17-, 51-, 69- and 60-fold respectively upon Zn\(^{2+}\)-binding. The binding affinity of these four probes range from 0.78 to 5.5 nM at pH 7.4. Although these probes have been applied successfully in biological imaging,\(^{159,160}\) their synthesis is laborious and therefore not applicable to preparing new sensors.

FluoZin-1 (34), FluorZin-2 (35), FluoZin-3 (36), RhodaZin-1 (37), as well as FuraZin-1 (38) and IndoZin-1 (39) that are based on non-xanthanone fluorophores, comprise a family of zinc sensors containing carboxylate donor groups (Fig. 1.12).\(^{150}\) With the exception of 36, which has a \(K_d\) for Zn\(^{2+}\) of 15 nM, these probes are sensitive to \(\mu\)M concentrations of Zn\(^{2+}\). The fluorescence of all
six increases >100-fold upon analyte binding. Although preliminary studies using FluoZin-3 to monitor secretions from pancreatic β-cells show promise,\textsuperscript{161} there are little available data by which to evaluate these probes as biological sensors.

**Conclusions and Future Prospects**

Interest in the sub-field of metalloneuroscience continues to grow in the bioinorganic chemistry community.\textsuperscript{11} The study of ion channels and pumps,\textsuperscript{17,19} transition metals in normal and disease states,\textsuperscript{13,31} brain enzymes like nerve growth factor\textsuperscript{162} and synaptotagmin,\textsuperscript{163} and the continuing need for new and better sensors\textsuperscript{67,68,143} highlight only some of the potential areas for future research. Understanding the inorganic chemistry of the brain is required for a full appreciation of memory formation, synaptic transmission, and neurodegenerative disorders.

**Acknowledgments.** Work in our laboratory reviewed in this chapter was supported by seed money from MIT as well as a grant from the McKnight Foundation for the Neurosciences.
References

(*) This work has appeared previously in slightly altered form in reference (†).


(64) Laval, F.; Wink, D. A. Carcinogenesis 1994, 15, 443-447.


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39, 1052-1054

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### Table 1.1. Concentrations (mM) of important metal ions in cells

<table>
<thead>
<tr>
<th>Cation</th>
<th>K⁺</th>
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<td>145</td>
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</tbody>
</table>
Scheme 1.1

1a, 2a, 3a \( R = H \)
1b, 2b, 3b \( R = N(CH_3)_2 \)
Scheme 1.2
Scheme 1.3
Scheme 1.4
Scheme 1.5
Scheme 1.6
Figure 1.1. ORTEP diagrams showing selected atom labels and 50% probability ellipsoids for all non-hydrogen atoms of [Co(iPrDATI)$_2$] (14·CH$_2$Cl$_2$), [Co(nBuDATI)$_2$] (15·CH$_2$Cl$_2$·THF), and [Co($
u$DATTI)$_2$] (16·0.5 CH$_2$Cl$_2$·0.5 THF). Solvent molecules are omitted.
Figure 1.2. Comparison of the fluorescence emission spectra of 46 μM CH₂Cl₂ solutions of H(PrDATI) and [Co(PrDATI)₂] (14). Excitation = 350 nm.
Figure 1.3. The linked tropocoronand ligand, H₂DATI-4 (18), prepared from a modified synthesis based on the unlinked compound preparation.
Figure 1.4. ORTEP diagram of [Co(DATI-4)] (19) showing 50% thermal ellipsoids and selected atom labels.
Figure 1.5. (a) Fluorescence emission spectra showing the increase in intensity at 505 nm (excitation = 350 nm) when a 40 μM CH₂Cl₂ solution of [Co(DATI-4)] (19) was exposed to 1 atm NO gas. The lowest intensity spectrum is that of the starting material, the next spectrum was recorded 3 minutes after addition of NO into the headspace. After 6 h (top spectrum), a greater than 4-fold increase in intensity over that of the starting material was observed. (b) The fluorescence emission spectra of 40 μM CH₂Cl₂ solution of 19 is unaffected by exposure to air over 6 hours.
Figure 1.6. The commonly employed quinoline-based fluorescent sensors for Zn$^{2+}$: TSQ (20), Zinquin (21), and TFLZn (22).
Figure 1.7. Graphical representation of the X-ray crystal structure of the Zn$^{2+}$ complex of a Zinquin derivative.
Figure 1.8. Xanthene-based fluorescent sensor for Zn$^{2+}$ that employs a cyclen macrocycle as the metal ion binding moiety.
Figure 1.9. The fluorescein-based fluorescent sensor for Zn$^{2+}$ Newport Green (26).
Figure 1.10. Traditional Ca$^{2+}$ sensor fura-2 (27), and Mg$^{2+}$ sensors mag-fura-2 (28) and mag-fura-5 (29) that have been used as Zn$^{2+}$ sensors.
Figure 1.11. Fluorescein-based sensors ZnAF-1 (30), ZnAF-2 (31), ZnAF-1F (32), ZnAF-2F (33).
Figure 1.12. Fluorescent probes for Zn$^{2+}$ available from Molecular Probes: FluoZin-1 (34), FluorZin-2 (35), FluoZin-3 (36), RhodaZin-1 (37), FuraZin-1 (38) and IndoZin-1 (39).
Chapter 2

Fluorescent Sensors for Zn²⁺ Based on a Fluorescein Platform: Synthesis, Properties and Intracellular Distribution*
Introduction

Zinc is a vital component in many cellular processes. Although traditionally the study of Zn\(^{2+}\) bioinorganic chemistry has focused on its structural role and catalytic functions in proteins, the neurobiology of Zn\(^{2+}\) has been a subject of increasing attention. Whereas most Zn\(^{2+}\) in biological systems is tightly bound in proteins, a pool of free Zn\(^{2+}\) has been imaged in cells. Included are sub-nanomolar concentrations in undifferentiated mammalian cells and higher concentrations, approaching 300 \(\mu\text{M}\), in the mossy fiber terminals of the hippocampus. The Zn\(^{2+}\) ion has the ability to modulate a variety of ion channels, may play a role in neuronal death during seizures, is pertinent to neurodegenerative disorders, and may be vital to neurotransmission. Because of these diverse functions, Zn\(^{2+}\) continues to be an interesting subject of research in neurobiology.

The levels of Zn\(^{2+}\) in the brain and other parts of the body are regulated by at least three homologous Zn\(^{2+}\) transport proteins (ZnT-1, ZnT-2, and ZnT-3) and by metallothioneins (MTs), including MT-III and MT-IV which are expressed mainly in the brain. ZnTs and MTs are probably responsible for distributing the required amounts of Zn\(^{2+}\) to proteins and enzymes, minimizing the amounts of free and potentially toxic levels of Zn\(^{2+}\) present in cells. In addition to protein regulators, Zn\(^{2+}\) can be released from synaptic vesicles and can enter cells through voltage-dependent Ca\(^{2+}\) channels, indicating that free Zn\(^{2+}\) is still available for neurological functions. Despite much research, many aspects of ionic Zn\(^{2+}\) in neurobiology remain unclear due to the limited detection methods currently available to the neuroscience community.
Fluorescent sensors are indispensable tools for visualizing ions at the molecular level without the need for special instrumentation. Practical fluorescent sensors must produce a perceptible luminescent signal upon the selective binding of the desired analyte. Careful engineering of the molecular properties of these probes can provide an effective methodology for studying relevant intracellular metal ions like Ca$^{2+}$ in biological systems. In addition to selectivity for the analyte, biosensors should have a $K_d$ value near the median concentration of the species under investigation to allow concentration changes to be monitored. Desirable intracellular fluorescent sensors should produce a signal with a high quantum yield ($\Phi$) and have excitation wavelengths exceeding 340 nm. The latter property permits use with glass microscope objectives and prevents UV-induced cell damage. A good sensor will also have emission wavelengths approaching 500 nm to avoid autofluorescence from species native to the cell and to facilitate use with typical fluorescence microscopy optical filter sets. Finally, sensors must have the ability to be passively and irreversibly loaded into cells.

The majority of studies of intracellular Zn$^{2+}$ with fluorescent sensors have been performed with TSQ, Zinquin, and TFLZn, all of which are aryl sulfonamide derivatives of 8-aminoquinoline. Recent investigations into the aqueous binding properties of these compounds have clarified many discrepancies found in the literature on these probes, promoting the accuracy of results for future application of these sensors. Although quinoline-based probes are useful, these sensors are not ideal because they require near ultraviolet excitation, and can form mixed complexes sensing partially coordinated Zn$^{2+}$. Several approaches to fluorescent Zn$^{2+}$ detection based on polypeptide, protein, and macrocyclic sensors have been
reported, but none has been applied successfully to sensing zinc in living cells. These alternative sensors are not suitable for intracellular work either because of unsatisfactory binding affinity, optical properties, cell toxicity, or the need to perform microinjections into cells. Several traditional Ca\textsuperscript{2+} and Mg\textsuperscript{2+} probes have been utilized to study Zn\textsuperscript{2+},\textsuperscript{22,37} but results from these applications can be ambiguous because Zn\textsuperscript{2+}-induced signals are difficult to distinguish from those involving alkaline earth metal ions. Recently the synthesis and characterization of ZnAF-1 and ZnAF-2, two amino-fluorescein-based Zn\textsuperscript{2+} sensors, were reported.\textsuperscript{38} The esterified forms of these new sensors are cell permeable, but details about their intracellular behavior were not described.

In an effort to facilitate the understanding of Zn\textsuperscript{2+} in cell biology and neurology, we recently described the preparation and preliminary characterization of ZP1,\textsuperscript{39} a new cell-permeable fluorescent sensor for Zn\textsuperscript{2+}.\textsuperscript{40} Our initial investigations indicated that ZP1 exhibits a 3.1-fold increase in integrated fluorescence intensity upon the formation of a 1:1 ligand:Zn\textsuperscript{2+} complex. ZP1 can be passively loaded into cells and used to detect Zn\textsuperscript{2+} introduced exogenously. ZP1 is particularly amenable to intracellular work because fluorescein is the reporting group. This xanthenone-based chromophore has a quantum yield approaching unity (\(\Phi = 0.95\)), and excitation and emission wavelengths exceeding 490 nm.\textsuperscript{41}

Previous approaches to preparing fluorescent sensors have often suffered from a lack of generality. We have therefore devised a general methodology for preparing fluorescent sensors based on the fluorescein framework and demonstrate here its utility through the synthesis of ZP2, a first-generation fluorescent probe
structurally similar to ZP1. In addition we describe a thorough investigation of the physical and structural properties of these probes related to their use as Zn$^{2+}$ sensors.

Experimental

Materials and Methods. Chlorobenzene and 1,2-dichloroethane (DCE) were distilled from calcium hydride under nitrogen. Dimethyl sulfoxide (DMSO) was vacuum distilled from CaH$_2$ and subsequently dried over 3 Å molecular sieves. Deuterated chloroform was dried over 3 Å molecular sieves. Zinc chloride was fused prior to each use. Di(2-picolyl)amine (DPA) was prepared as previously described.$^{42}$ All other reagents were purchased and used as received. Flash column chromatography was performed with silica gel-60 (230-400 mesh) or Brockman I activated neutral aluminum oxide (150 mesh). Thin layer chromatography (TLC) analysis was performed with Merck F254 silica gel-60 plates or Merck F254 aluminum oxide-60 and viewed by UV light, or developed with ceric ammonium molybdate, 2,4-dinitrophenyl hydrazine or iodine stain. Infrared spectra were recorded on a BTS 135 FTIR instrument as KBr pellets. NMR spectra were recorded on a Varian 500 MHz spectrometer at ambient probe temperature, 283 K, and referenced to the internal $^1$H and $^{13}$C solvent peaks. Fast atom bombardment (FAB) mass spectrometry was performed in the MIT Department of Chemistry Instrumentation Facility (DCIF) with the use of $m$-nitrobenzyl alcohol as the matrix. Melting points were recorded on a Thomas Hoover capillary melting point apparatus. CAUTION: One of the isolated compounds below contains perchlorate ion, which can detonate explosively and without warning. Although we have
encountered no incidents with the reported compound, all due precautions should be taken.

4',5'-Dimethylfluorescein Dibenzoate (1). Phthalic anhydride (16.7 g, 113 mmol) and 2-methylresorcinol (24.9 g, 201 mmol) were crushed and melted into a brown liquid at 150 °C. Fused ZnCl₂ (15 g, 110 mmol) was added slowly over 35 min, and the temperature was slowly increased to 230 °C over 30 min until the material solidified. The brick red solid was pulverized and boiled in 250 mL of 6 M HCl for 30 min. The red solid was collected on a frit, washed thoroughly with distilled water, and dried in vacuo at 50 °C for 2 h. The crude product was combined with benzoic anhydride (115 g, 509 mmol) in 400 mL of pyridine and refluxed at 140 °C for 2.5 h. The reaction mixture was diluted with 700 mL of distilled water, and a dark brown solid formed upon cooling. The solids were collected, washed thoroughly with water, and dried. The dark brown solids were dissolved in 550 mL of boiling toluene and decolorizing carbon was added. The hot mixture was filtered through Celite, and the Celite/carbon mixture was washed with 250 mL of boiling toluene. The product was crystallized from toluene and recrystallized (4:1 toluene/EtOH) to yield a white crystalline solid (32.3 g, 56.7%). TLC Rₖ = 0.41 (3:1 hexanes/EtOAc). Mp = 240-42 °C. ¹H NMR (CDCl₃, 500 MHz) δ 2.42 (6 H, s), 6.76 (2 H, d, J = 9.0 Hz), 6.93 (2 H, d, J = 8.5 Hz), 7.28 (1 H, d, J = 7.5 Hz), 7.55 (4 H, t, J = 7.5 Hz), 7.65-7.74 (4 H, m), 8.06 (1 H, d, J = 8.0 Hz), 8.25 (4 H, d, J = 7.5 Hz). ¹³C NMR (CDCl₃, 125 MHz) δ 9.85, 82.97, 116.60, 118.09, 119.63, 124.47, 125.39, 125.79, 126.60, 128.91, 129.14, 130.16, 130.48, 134.11, 135.35, 150.44, 150.80, 153.03, 164.74, 169.42. FTIR (KBr, cm⁻¹) 1771, 1763, 1738, 1599, 1592, 1452, 1421, 1267, 1222, 1099. HRMS (+FAB): Calcd for MH⁺, 569.1600; Found 569.1588.
4',5'-Bis(bromomethyl)fluorescein Dibenzoate (2). 4',5'-Dimethyl-fluorescein dibenzoate (5.00 g, 8.79 mmol) and 1,3-dibromo-5,5-dimethylhydantoin (3.87 g, 13.6 mmol) were combined in 550 mL of C₆H₅Cl, and acetic acid (133 μL, 2.32 μmol) and 1,1'-azobis(cyclohexanecarbonitrile (0.181 g, 0.740 mmol) were added to the solution with stirring. The solution was stirred at 40 °C for 72 h, and then washed four times with hot water (100 mL, 80 °C). Recrystallization (9:1 toluene/EtOH) and washing with n-pentane yielded the product as a white crystalline solid (6.27 g, 98.2%). TLC R₅ = 0.34 (7:3 hexanes/EtOAc). Mp = 300 °C dec. ¹H NMR (CDCl₃, 500 MHz) δ 4.87 (2 H, d, J = 10.0 Hz), 4.88 (2 H, d, J = 10.5 Hz), 6.92 (2 H, d, J = 8.5 Hz), 7.08 (2 H, d, J = 9.0 Hz), 7.32 (1 H, d, J = 8.0 Hz), 7.58 (4 H, t, J = 7.5 Hz), 7.68-7.78 (4 H, m), 8.08 (1 H, d, J = 8.5 Hz), 8.28 (4 H, d, J = 7.0 Hz). ¹³C NMR (CDCl₃, 125 MHz) δ 20.69, 81.60, 117.09, 119.21, 119.38, 124.47, 125.63, 126.36, 128.68, 128.99, 129.10, 130.58, 130.65, 134.50, 135.71, 149.63, 150.79, 152.48, 164.39, 169.04. FTIR (KBr, cm⁻¹) 1774, 1743, 1601, 1589, 1427, 1234, 1081, 1066. HRMS (+FAB): Calcd for MH⁺, 724.9811; Found 724.9824.

4',5'-Fluoresceindicarboxaldehyde (3). 4',5'-Bis(bromomethyl)fluorescein dibenzoate (2.00 g, 2.75 mmol) and NaHCO₃ (2.00 g, 23.8 mmol) were combined in 200 mL of DMSO and heated to 150 °C for 4 h. The dark red solution was cooled and then diluted into 700 mL of 2 M HCl and stirred for 2 h. The aqueous material was extracted thoroughly with CH₂Cl₂ (8 × 100 mL) and the solvents were removed to isolate an orange liquid. The orange solid that precipitated with the addition of 300 mL of water was collected on a frit and washed thoroughly with water. The orange solid was redissolved in CH₂Cl₂ and dried over Na₂SO₄. An orange solid was isolated after filtration and solvent removal. Flash chromatography on silica gel (33:1
CHCl₃/MeOH) yielded the product as a yellow powder (391 mg, 36.7%). TLC Rₜ = 0.46 (19:1 CHCl₃/CH₃OH). Mp = 301-303 °C dec. ¹H NMR (CDCl₃, 500 MHz) δ 6.74 (2 H, d, J = 8.5 Hz), 6.94 (2 H, d, J = 9.5 Hz), 7.18 (1 H, d, J = 7.5 Hz), 7.69 (1 H, td, J = 1.0, 7.5 Hz), 7.74 (1 H, td, J = 1.0, 7.5 Hz), 8.07 (1 H, d, J = 7.5 Hz), 10.67 (2 H, s), 12.13 (2 H, s). ¹³C NMR (CDCl₃, 125 MHz) δ 80.80, 109.23, 109.71 115.42, 123.90, 125.76, 126.73, 130.72, 135.86, 137.16, 151.87, 152.11, 164.87, 168.87, 192.00. FTIR (KBr, cm⁻¹) 1769, 1656, 1600, 1228, 1172, 1092. HRMS (+FAB): Calcd for MH⁺, 389.0661; Found 389.0674.

9-(α-Carboxyphenyl)-4,5-bis[bis(2-pyridylmethyl)aminomethyl]-6-hydroxy-3-xanthanone (Zinpyr-2, ZP2, 4). 4',5'-Fluorescein-dicarboxaldehyde (200 mg, 0.515 mmol) and acetic acid (120 µL, 2.1 mmol) were combined in 1,2-dichloroethane (DCE, 30 mL) and stirred. To the resulting solution DPA (215 mg, 1.08 mmol) in DCE (20 mL) was added dropwise and stirred for 30 min. Sodium triacetoxyborohydride (230 mg, 1.08 mmol) was added and the reaction mixture was stirred 12 h at room temperature. The reaction was chilled to 0 °C, and water was added to the solution with stirring. The aqueous layer was extracted with CH₂Cl₂ and the combined organic layers were washed twice with saturated NaCl to give an orange solid after solvent removal. The compound was dried by azeotroping with benzene. Flash chromatography on activated neutral alumina (24:1 CHCl₃/MeOH) yielded the product as an orange solid (108 mg, 27.8%). TLC Rₜ = 0.10 (alumina, 19:1 CHCl₃/CH₃OH). Mp = 195-197 °C dec. ¹H NMR (CDCl₃, 500 MHz) δ 3.98 (4 H, d, J = 15.0 Hz), 4.02 (4 H, d, J = 15.0 Hz), 4.18 (4H, s), 6.57 (2 H, d, J = 8.5 Hz), 6.64 (2 H, d, J = 8.5 Hz), 7.17-7.21 (5 H, m), 7.37 (4 H, d, J = 7.5 Hz), 7.58-7.67 (6 H, m), 8.00 (1 H, d, J = 7.5 Hz), 8.59 (4 H, d, J = 6.5 Hz). ¹³C NMR (CDCl₃, 125 MHz) δ 49.24, 59.45, 109.84,
110.24, 113.38, 122.57, 123.52, 124.38, 125.10, 127.69, 128.30, 129.70, 134.92, 137.16, 149.02, 150.59, 152.86, 158.19, 160.21, 169.64. FTIR (KBr, cm\(^{-1}\)) 3448, 1755, 1591, 1435, 760. HRMS (+FAB): Calcd for MH\(^+\), 755.2982; Found 755.2959.

9-(o-Carboxyphenyl)-2,7-dichloro-4,5-bis[bis(2-pyridylmethyl)aminomethyl]-6-hydroxy-3-xanthanone (Zinpyr-1, ZP1, 5). DPA (1.59 g, 7.99 mmol) and paraformaldehyde (0.224 g, 7.47 mmol) were combined in 20 mL of CH\(_3\)CN and refluxed for 30 min. 2',7'-Dichlorofluorescein (1.00 g, 2.49 mmol) in 30 mL of CH\(_3\)CN/H\(_2\)O (1:1) was added to the solution and the reaction mixture was refluxed for 24 h. The CH\(_3\)CN was removed and the product and residual water were triturated with 30 mL of boiling ethanol. The product was precipitated at \(-25^\circ\)C, filtered on a frit, washed thoroughly with ice cold water and dried. Flash chromatography on activated neutral alumina (45:1 CHCl\(_3\)/MeOH) yielded the product as a salmon pink solid (960 mg, 46.8\%). TLC R\(_f\) = 0.10 (alumina, 19:1 CHCl\(_3\)/CH\(_3\)OH). Mp = 185-187 \degree\)C dec. \(^1\)H NMR (CDCl\(_3\), 500 MHz) \(\delta\) 3.98 (4 H, d, \(J = 15.0\) Hz), 4.01 (4 H, d, \(J = 15.0\) Hz), 4.20 (4H, s), 6.64 (2 H, s), 7.19 (5 H, t, \(J = 7.5\) Hz), 7.36 (4 H, d, \(J = 8.0\) Hz), 7.64-7.73 (6 H, m), 8.04 (1 H, d, \(J = 7.0\) Hz), 8.60 (4 H, dq, \(J = 1.0, 5.0\) Hz). \(^{13}\)C NMR (CDCl\(_3\), 125 MHz) \(\delta\) 49.38, 59.31, 89.44, 110.26, 111.93, 117.72, 122.67, 123.43, 124.27, 125.54, 127.28, 127.86, 130.31, 135.41, 137.32, 148.99, 151.83, 156.09, 157.81, 169.13. FTIR (KBr, cm\(^{-1}\)) 3447, 1761, 1750, 1592, 1475, 1435, 761. HRMS (+FAB): Calcd for MH\(^+\), 823.2202; Found 823.2229.

\([\text{Zn}_2(\text{ZP1})(\text{H}_2\text{O})_2]_2(\text{ClO}_4)_2\) (6). ZP1 (50.6 mg, 61.5 \(\mu\)mol) was combined with zinc triflate (2.00 ml, 0.121 M, 243 \(\mu\)mol) and sodium hydroxide (2.00 ml, 0.121 M, 243 \(\mu\)mol) in CH\(_3\)CN (5 mL) and sonicated for 10 min to dissolve all the solids. Sodium perchlorate (0.500 g, 4.70 mmol) was dissolved in water (5 mL) and added to the
CH$_3$CN solution, then the combined solutions were filtered through Celite. The product partially crystallized at room temperature after 12 h. The remainder of the product was crystallized at -25$^\circ$C over 12 h. The orange crystalline material was filtered, powdered and dried under vacuum at 50$^\circ$C to yield 37.0 mg of dried product in 48.4% yield. Analogous small-scale reactions at 25$^\circ$C yielded orange square prisms suitable for X-ray crystallography. $^1$H NMR (DMSO-d$_6$, 500 MHz) $\delta$ 4.13-4.41 (12 H, m), 6.14 (2 H, s), 7.14 (1 H, d, $J = 8.0$ Hz), 7.54 (4 H, q, $J = 8.0$ Hz), 7.604 (4 H, t, $J = 8.0$ Hz), 7.70 (1 H, t, $J = 7.0$ Hz), 7.84 (1 H, t, $J = 7.5$ Hz), 7.92 (1 H, t, $J = 7.5$ Hz), 8.07 (4 H, quin.d, $J = 1.5$, 7.5 Hz), 8.68 (2 H, d, $J = 5.0$ Hz), 8.74 (2 H, d, $J = 5.0$ Hz). FTIR (KBr, cm$^{-1}$) 3441, 1748, 1610, 1571, 1457, 1363, 1121. Anal. Calcd for Zn$_2$C$_46$H$_{44}$N$_6$O$_{16}$Cl$_4$ (6-H$_2$O): C, 45.84; H, 3.34; N, 6.97. Found: C, 45.89; H, 3.13; N, 6.71.

Collection and Reduction of X-ray Data. Crystals were covered with Paratone-N oil and suitable specimens were mounted on the tips of glass fibers at room temperature and transferred to a Bruker (formerly Siemens) CCD X-ray diffraction system with a graphite-monochromatized MoK$_\alpha$ radiation ($\lambda = 0.71073$ Å) controlled by a Pentium-based PC running the SMART software package.$^{43}$ Data were collected at 188 K in a stream of cold N$_2$ maintained with a Siemens LT-2A nitrogen cryostat. Procedures for data collection and reduction have been reported previously.$^{44}$ The structure was solved by direct methods and refined by full matrix least-squares and difference Fourier techniques with SHELXTL.$^{45}$ Empirical absorption corrections were applied with the SADABS program,$^{46}$ and the structure was checked for higher symmetry by PLATON.$^{47}$ The space group was determined by examining systematic absences and confirmed by the successful solution and refinement of the structure. All non-hydrogen atoms were refined anisotropically.
Hydrogen atoms were assigned idealized locations and given isotropic thermal parameters 1.2 times the thermal parameter of the carbon atoms to which they were attached. In the structure of 6·6H₂O, one perchlorate anion is disordered and several water molecules are partially occupied; all were modeled and refined accordingly. Relevant crystallographic information is summarized in Table 2.2, and the 50% thermal ellipsoid plot is shown in Fig. 2.1. Table 2.3 contains selected bond distances and angles, and the atomic coordinates and equivalent isotropic displacement parameters are provided in Tables 2.4.

**General Spectroscopic Methods.** Ultrol grade HEPES (2[4-(2-hydroxyethyl)-1-piperazinyl]ethane-sulfonic acid) and PIPES (piperazine-\(N,N'\)-bis(2-ethanesulfonic acid)) from Calbiochem and KCl (99.997%) were purchased and used as received. All solutions were filtered through 0.2-μM cellulose filters before measurements. Except for the fluorescence titration experiment, Zn solutions were prepared by the addition of appropriate amounts of 1.0 M, 100 mM, 10 mM or 1 mM Zn\(^{2+}\) stocks that were checked by atomic absorption spectroscopy for concentration accuracy, or by titration with terpyridine and measurement of the absorption spectra. The titration was performed by treating a 70 μM solution of 2,2'·6'·2"-terpyridine in 10 mM PIPES, pH 7.0, with aliquots of 10 mM (nominal) ZnCl₂, and determining the equivalence point by monitoring the absorbance of the resulting complex at 335 nm (\(ε = 28.5 \times 10^3\) M\(^{-1}\) cm\(^{-1}\)). The Zn\(^{2+}\) stocks were prepared from 99.999% pure ZnCl₂. The purity of the ZP probes was verified to be greater than 99% by HPLC. ZP was introduced to aqueous solutions by addition of a stock solution in DMSO (0.67 mM). Graphs were manipulated and equations calculated by using Kaleidagraph 3.0. The pH values of
solutions were recorded with an Orion glass electrode that was calibrated prior to each use.

**UV-Visible Spectroscopy.** Absorption spectra were recorded on a Hewlett Packard 8453A diode array spectrophotometer under the control of a Pentium II-based PC running the Windows NT ChemStation software package, or a Cary 1E scanning spectrophotometer under the control of a Pentium PC running the manufacturer supplied software package. Spectra were routinely acquired at 25 °C, maintained by a circulating water bath in 1-cm path length quartz cuvettes with a volume of 1.0 or 3.5 mL.

**Fluorescence Spectroscopy.** Fluorescence spectra were recorded on a Hitachi F-3010 spectrofluorimeter under the control of a Pentium-based PC running the SpectraCalc software package. Excitation was provided by a 150 W Xe lamp (Ushio Inc.) operating at a current of 5 A. All spectra were normalized for excitation intensity via a rhodamine quantum counter, and emission spectra were normalized by the manufacturer-supplied correction curves. Spectra were routinely acquired at 25 °C, maintained by a circulating water bath in 1 cm × 1 cm quartz cuvettes using 3 nM slit widths and a 240 nm/min scan speed. Fluorescence emission measurements were also acquired in a 1 cm × 1 cm quartz cell using a Spex Fluorolog-2 instrument with 1-nm bandwidth slits. All spectra were corrected for emission intensity by using the manufacturer-supplied photomultiplier curves.

**(a) pH-Dependent Fluorescence Studies.** The apparent $pK_a$ was measured by plotting the integrated area of the fluorescence emission spectrum against pH recorded in the range from pH 12.5 to 2.5. A 1 µM solution of the ZP dye (10 mL) containing ~1 mM KOH and 100 mM KCl was adjusted to pH 12.5 and the UV-vis
and fluorescence spectra were recorded. The pH was lowered in steps of $\Delta \text{pH} = 0.5$ with the addition of appropriate amounts of $6 \text{ N}$, $2 \text{ N}$, $0.5 \text{ N}$, $1 \text{ N}$, $0.1 \text{ N}$ and $0.01 \text{ N}$ HCl, recording the absorption and emission spectra at each pH interval. The volume of the solution was controlled such that the overall change in volume was $<2\%$. Emission for ZP1 was integrated from 450 to 700 nm. Emission for ZP2 was integrated from 500 to 700 nm. The resulting integrated emission spectral areas were normalized, plotted against pH, and fit to the nonlinear expression in eq 1 to calculate the $pK_a$ value. In the equation, $\Delta F_{1\text{max}}$ and $\Delta F_{2\text{max}}$ are the maximum fluorescence changes associated with the corresponding $pK_a$ values.

$$\Delta F = \frac{\Delta F_{1\text{max}}}{(1+10^{(\text{pH} - pK_a)})} + \frac{\Delta F_{2\text{max}}}{(1+10^{(\text{pH} - pK_a)})}$$ (1)

(b) Quantum Yield. The quantum yields for fluorescence were obtained by comparison of the integrated area of the corrected emission spectrum of the samples with that of a solution of fluorescein in $0.1 \text{ N NaOH}$, which has a quantum efficiency of 0.95. The concentration of the reference was adjusted to match the absorbance of the test sample. The quantum efficiency of the metal-free ligand was measured by using a dilute sample of ZP dye ($\sim 1\times10^{-6} \text{ M}$, Abs $\leq 0.1$) in $10 \text{ mM PIPES}$, pH = 7.0, 100 mM KCl and 50 $\mu$M EDTA. The quantum efficiency of metal-bound ligand was measured by using dilute sample of ZP ($\sim 8\times10^{-7} \text{ M}$, Abs $\leq 0.1$) in $10 \text{ mM PIPES}$, pH = 7.0, 100 mM KCl and 100 $\mu$M ZnCl$_2$. The concentration of the reference was adjusted to match the absorbance of the test sample at the wavelength of excitation. Emission for ZP1 was integrated from 480 to 650 nm with excitation at 475 nm, and emission for ZP2 was integrated from 496 to 600 nm with excitation at 492 nm. The quantum
yields were calculated with the expression in eq 2.

\[
\Phi_{\text{sample}} = \Phi_{\text{standard}} \times \frac{I_{\text{emission sample}}}{I_{\text{emission standard}}} \tag{2}
\]

(c) Titration of Zn\(^{2+}\) Binding by Fluorescence Spectroscopy (\(K_{d}\)).

Calculation of free Zn\(^{2+}\) concentrations in buffers. Free Zn\(^{2+}\) concentrations were calculated by a published method\(^{48}\) with some minor modifications. In these calculations, the quantity \(P\) is identical to the more common \(\alpha\) value,\(^{49}\) but the equilibria are described as dissociation rather than association constants. Also, the method uses "concentration constants" and thus assumes that pH = -\(\log[H_3O^+]\), which does not take into the account the activity of H\(^+\) at 0.1 M ionic strength (0.78), as is used operationally in the biologists' definition of pH. This difference requires a 0.11 log unit adjustment in the stability constant calculation, which was made. Stability constants for the Ca\(^{2+}\) and Zn\(^{2+}\) complexes of EDTA were taken from the recent literature.\(^{50}\) Thus for EDTA (25 °C, 0.1 M KCl) \(pK_1 = 10.19\), \(pK_2 = 6.13\), \(\log K\) (CaL) = 10.65, \(\log K\) (ZnL) = 16.5. The zinc concentration is computed from eq. 3,

\[
[Zn] = \frac{\Delta - K_{D_{Zn,EDTA}}^{app}}{2(1+R)} + \sqrt{\left(\frac{\Delta - K_{D_{Zn,EDTA}}^{app}}{2(1+R)}\right)^2 + \left[\frac{[Zn] \times K_{D_{Zn,EDTA}}^{app}}{1+R}\right]} \tag{3}
\]

where \(K_{D_{Zn,EDTA}}^{app}\) is the pH-adjusted apparent \(K_d\) of the Zn\(^{2+}\)-EDTA complex, determined from the relationship in eq. 4,

\[
K_{D_{Zn,EDTA}}^{app} = \log 10^{10.19+0.11\cdot pH}\left[1+10^{(10.19+0.11\cdot pH)+10^{(10.19+6.13+0.22\cdot 2pH)}}\times 10^{-16.5}\right] \tag{4}
\]
and $K_{D_{\text{Zn}^{2+} \text{EDTA}}}$ is calculated similarly. The substitution of eq. 5 was made into eq. 3,

$$\Delta = [\text{Zn}]_t + [\text{Ca}]_t - [\text{EDTA}]_t$$  \hspace{1cm} (5)

The first approximation of free Zn$^{2+}$ was made by calculating $R$ using eq. 6,

$$R = \frac{K_{D_{\text{Zn}^{2+} \text{EDTA}}}}{K_{D_{\text{Ca}^{2+} \text{EDTA}}}} \times \frac{[\text{Ca} \cdot \text{EDTA}]}{[\text{Zn} \cdot \text{EDTA}]}$$  \hspace{1cm} (6)

This approximation was initialized with the value $[\text{Zn}] = 1$ nM, with $[\text{Ca}]_t$ set at 2 mM, $[\text{EDTA}]_t$ set at 1 mM, and $[\text{Zn}]_t$ was varied from 0.1 – 0.9 mM in 0.1 mM increments. The value of $[\text{Zn}]$ obtained from eq. 3 was then re-applied to eq. 7

$$\frac{[\text{Ca} \cdot \text{EDTA}]}{[\text{Zn} \cdot \text{EDTA}]} = \frac{[\text{EDTA}]_t}{[\text{Zn}]_t} - \frac{K_{D_{\text{Zn}^{2+} \text{EDTA}}}}{[\text{Zn}]} - 1$$  \hspace{1cm} (7)

until convergence was obtained, usually within three iterations. Calculations were performed using the software package Mathcad Plus 6.0 (Mathsoft). For 2 mM $[\text{Ca}]_t$, 1 mM $[\text{EDTA}]_t$, the free Zn$^{2+}$ values in Table 2.1 were obtained.

**Preparation of Ca$^{2+}$/Zn$^{2+}$/EDTA stock solutions.** Solution 1 (0.1 M EDTA, 0.2 M CaCl$_2$, 0.1 M ZnCl$_2$, pH 5) and Solution 2 (0.1 M EDTA, 0.2 M CaCl$_2$, pH 7) were prepared using similar procedures to those described for making Ca$^{2+}$-EGTA stock solutions. The only difference in these procedures is that the pH-metric titration used to prepare Solution 1 is carried out at pH ~5 to prevent precipitation of the Zn$^{2+}$-EDTA complex.

**Fluorescence Zn$^{2+}$-binding titrations.** For a typical titration, 11.0 µL of a 0.67 mM solution of ZP in DMSO was added to 15 mL of PIPES buffer (100 mM KCl, 50 mM PIPES, pH 7.00) and the solution was vortexed to assure complete mixing. From this solution, one aliquot of 5.00 mL was made, and 50 µL of Solution 2 (described above) was added. To a second 7.00 mL aliquot, 70 µL of Solution 1 was added. This
procedure prepares a matched pair of ZP solutions (0.5 μM), which also contain 2 mM total Ca\(^{2+}\), 1 mM total EDTA, and either 0 or 1 mM total Zn\(^{2+}\).

To acquire fluorescence spectra for solutions containing between 0 and 0.9 mM total Zn\(^{2+}\), the method of reciprocal dilutions was used. A 3.0 mL of the ZP solution containing 0.0 mM total Zn\(^{2+}\) was placed in a cuvette and its emission spectrum recorded. For the first iteration (n = 1), in order to prepare a solution containing 0.1 mM Zn\(^{2+}\) total, 300 μL of the solution was removed from the cuvette and replaced with 300 μL of the ZP stock containing 1 mM total Zn\(^{2+}\). The contents of the cuvette were mixed with a polypropylene transfer pipette, and the fluorescence spectrum recorded. Additional iterations (n = 2 to 9) were performed by removing and replacing [1/(11-n) × 3000] μL (8) of the solution in the cuvette with the 1 mM total Zn\(^{2+}\) ZP stock. Finally, to achieve saturation of the ZP Zn\(^{2+}\) binding, the cuvette was emptied, dried, and 3.00 mL of the 1 mM total Zn\(^{2+}\) ZP solution was added, followed by 7.5 μL of a 10 mM ZnCl\(_2\) stock solution. The fluorescence emission spectrum was then recorded. This last step (n=10, the eleventh spectrum) is required to overwhelm the Zn\(^{2+}\) buffering capacity of the Ca\(^{2+}/\)EDTA/Zn\(^{2+}\) system, resulting in a solution with ~25 μM free Zn\(^{2+}\).

To calculate the \(K_d\) value, the fluorescence response was obtained by integrating the emission spectra, subtracting the baseline (0 Zn\(^{2+}\)) spectrum, and normalizing to the full scale response obtained at 25 μM free Zn\(^{2+}\). The fluorescence response \(R\) was fit to eq. 9,

\[
R = \frac{B \cdot Zn}{K_d + Zn}
\]  

(9)
where B is the normalization parameter, numerically equal to 1 for pre-normalized data, Zn is the free Zn$^{2+}$ (known), and $K_d$ the calculated dissociation constant.

ZP1 spectra were acquired by exciting at 507 nm and integrating the emission from 475-650 nm. ZP2 spectra were acquired by exciting at 490 nm and collecting and integrating data from 500-575 nm. The measurements were performed in triplicate.

(d) Titration of Zn$^{2+}$ Binding by Absorption Spectroscopy ($K_{d}$). A 3.0 mL solution containing 10 μM ZP1 in PIPES buffer (100 mM KCl, 50 mM PIPES, pH 7) was prepared, and an initial absorption measurement was made. A portion of Zn$^{2+}$ was added from standardized stock solutions to give an equimolar solution of ligand and metal ion, and the absorbance was measured. Additional Zn$^{2+}$ aliquots were added to give final metal ion concentrations of 250 μM, 500 μM, 1 mM, 5 mM, 10 mM, and 15 mM. Calculation of the ΔA by subtracting the 10 μM Zn$^{2+}$ spectrum from the higher concentration spectra followed by plotting against wavelength revealed maximum changes at 497 (increase) and 520 (decrease) nm.

For ZP2, a 3.0 mL solution containing 19 μM ZP2 in PIPES buffer, pH 7 (100 mM KCl, 50 mM PIPES) was prepared, and an initial absorbance measurement was made. Zn$^{2+}$ aliquots were titrated into the solutions to give final concentrations of 1-20 μM (1 μM increments), 30, 40, 50, 150, 250, 500 and 750 μM, and the absorption spectra were recorded. The ΔA values were calculated by subtracting the 0 μM spectrum from the subsequent spectra and plotted against the wavelength. The maximum absorption changes occurred at 486 nm (increases) and 508 nm (decreases). The change at 508 nm corresponds to the first binding event, but the increase at 486 nm is not indicative of a single process.
The measurements were repeated at pH 7.5 in HEPES buffer (100 mM KCl, 50 mM HEPES) for both ZP1 and ZP2 using the methods described above. The absorbance change for ZP1 was fit to a binding curve, and the spectral response of ZP2 was corrected for dilution, and analyzed using SPECFIT, a non-linear least-squares fitting program.\textsuperscript{52}

(e) Metal Ion Selectivity. The fluorescence response of the ZP dyes was investigated with a variety of metal ions including Mn\textsuperscript{2+}, Fe\textsuperscript{3+}, Co\textsuperscript{2+}, Ni\textsuperscript{2+}, Cu\textsuperscript{+}, Cu\textsuperscript{2+}, and Cd\textsuperscript{2+}. For each tested ion, a 1 \(\mu\text{M}\) solution of ZP dye was prepared in a buffer containing 100 mM KCl, 50 mM PIPES and 10 \(\mu\text{M}\) EDTA. A 3.0 mL aliquot of the solution was removed and the emission spectrum was recorded exciting at 505 nm for ZP1 and 490 nm for ZP2. Subsequently, 6 \(\mu\text{L}\) of a 10 \(\mu\text{M}\) stock solution of the indicated metal ion was added to the sample (20 \(\mu\text{M}\) total, \(\sim 10 \mu\text{M}\) free metal), and the emission intensity was again recorded. Stocks of metal solutions were prepared by using the chloride salt, except for Cu\textsuperscript{2+}, which was prepared from the sulfate salt and Fe\textsuperscript{2+}, which was prepared from ferrous ammonium sulfate. For the redox labile species Fe\textsuperscript{2+} and Cu\textsuperscript{+}, stock metal solutions were prepared in degassed water immediately prior to use and the ZP pH buffered solution was sparged with Ar or N\textsubscript{2} for 0.5 h and kept under a nitrogen or argon blanket during the measurement. The ration of the fluorescence intensity after the metal addition with the initial measurement was then calculated.

Cell Preparation. COS-7 cells were plated onto ethanol-washed, UV-sterilized glass coverslips and grown to 60-80% confluence at 37 °C in DMEM (Gibco catalog # 31600-075) supplemented with 10% v/v fetal bovine serum. For labeling, the cells were washed once with HBSS (Gibco catalog # 11201-092) supplemented with 2 g/L
D-glucose and 20 mM HEPES pH 7.4, then incubated with 5 μM fluorophore in HBSS at 37 C for 0.5-1h. Prior to imaging, the labeling solution was aspirated and the cells were washed 3 times with HBSS.

**Cell Imaging.** Cells were imaged on a Zeiss Axiovert microscope equipped with a cooled CCD camera (Princeton Instruments, Trenton, NJ). Excitation light was provided by a 150 W Xe arc lamp transmitted through a 465-495 nm excitation filter, a 505 nm long-pass dichroic mirror, and fluorescence was measured after passage through a 513-558 nm emission filter. Filters were obtained from Omega Optical and Chroma Technologies (Brattleboro, VT).

**Results and Discussion**

**Synthesis.** The synthesis of ZP1 was accomplished by a Mannich reaction between bis(2-pyridylmethyl)amine (dipicolylamine or DPA), prepared from 2-pyridinecarboxaldehyde and 2-aminomethylpyridine according to published procedures, and 2',7'-dichlorofluorescein (DCF, Scheme 2.1). Formation of the imminum cation by condensation of paraformaldehyde and DPA and subsequent reaction with DCF afforded the desired compound in ~60% yield after trituration with boiling ethanol and washing with cold water. The crude material, which is >90% pure after trituration (1H NMR), can be further purified by chromatography on neutral alumina or reverse phase silica to give analytically pure material (HPLC). This synthesis provides easy access to gram quantities of the desired Zn²⁺ probe.

Although fluorescein undergoes electrophilic substitution preferentially at the 4' and 5' positions, a mixture of structural isomers with substitution at the 4', 5', 2' and 7' positions is obtained when the compound is subjected to the Mannich
conditions used to prepare ZP1. These isomers are inseparable by conventional flash chromatography. We therefore envisioned that a fluorescein compound functionalized at the 4' and 5' positions with a synthetically useful group such as an aldehyde could be employed as a scaffold for preparing both the ZP1 analog as well as a variety of related sensors. Scheme 2.2 outlines the 4-step synthetic route devised to prepare ZP2, where the dialdehyde 3 is a key intermediate.

The synthesis of 1 was achieved by modification of the published procedure that involves installation of benzoate protecting groups on the phenolic oxygen of 4',5'-dimethylfluorescein.\textsuperscript{55} In the initial report, 1 was prepared using benzoyl chloride and characterized only by its melting point. Unprotected fluoresceins are highly polar compounds that are only slightly soluble in most common organic solvents. The benzoate protecting groups provide a convenient method for purifying fluorescein compounds by chromatography or crystallization and facilitating subsequent chemical manipulation by enhancing their solubility in organic media. In addition, protecting the phenolic oxygen atoms forces the fluorescein to adopt the lactoid isomer, preventing isomerization between the quinoid and lactoid forms.

Compound 2 can be formed in multigram quantities by bromination of 1 under free radical conditions. The reaction proceeded under facile conditions to give 2 in \textasciitilde 95\% purity before recrystallization. No dibromination was observed. Compound 2 can be carried on without purification to the next step, or recrystallized to give highly pure material (\textasciitilde 99\%, TLC/\textsuperscript{1}H NMR). Attempts to prepare sensors directly from 2 by direct displacement of the bromides were unsuccessful. Side reactions involving the benzoate protecting groups and lactone ring are suspected to be the problems.
Oxidation of 2 with DMSO in the presence of NaHCO₃ yielded the dialdehyde 3. Rigorous drying of the DMSO by distillation from CaH₂ followed by storage over molecular sieves is required to obtain the product in ~40% yield. Reactions using undistilled DMSO reduced the isolated yields by >50%. The synthesis of 3 resulted in cleavage of the benzoate protecting groups, but H-bonding between the phenolic hydrogens and the aldehyde carbonyl oxygens enforces the lactoid isomer, as shown by the NMR chemical shift of the phenolic hydrogen atoms ($\delta = 12.2$), compensating for the loss of the protecting groups. Compound 3 can be condensed with primary amines to give imines, or aminated under reducing conditions to give amines. The synthetic chemistry of 3 and the use of the compound as a scaffold for sensors as well as for other purposes will be the subjects of future reports. ZP2 was prepared by reaction of 3 with DPA using NaBH(OAc)₃ as the reducing agent in fairly good yield. The zinc complex of ZP1 crystallizes within 2 hours from an aqueous solution of CH₂CN in the presence of excess NaClO₄. The orange crystalline compound is non-fluorescent under UV-light.

**Fluorescence Properties of ZP Sensors.** Under simulated physiological conditions (50 mM PIPES, 100 mM KCl) at pH 7 in the presence of EDTA to scavenge adventitious metal ions, ZP1 and ZP2 have quantum yields of 0.38 and 0.25 respectively. The quantum yields increase to 0.87 for ZP1 and 0.92 for ZP2 in the presence of 25 µM Zn²⁺. The excitation maximum of ZP1 shifts from 513 nm ($\varepsilon = 67.1 \times 10^3$ M⁻¹ cm⁻¹) to 507 nm ($\varepsilon = 77.8 \times 10^3$ M⁻¹ cm⁻¹) upon Zn²⁺ complexation. ZP2 exhibits a similar shift from 498 nm ($\varepsilon = 40.5 \times 10^3$ M⁻¹ cm⁻¹) to 490 nm ($\varepsilon = 52.6 \times 10^3$ M⁻¹ cm⁻¹) after the addition of Zn²⁺. The slight hypsochromic shift in the absorption wavelength is indicative of coordination of the donor group (phenol) to Zn²⁺.56 Since
the phenol is incorporated into the π-system of the fluorophore, coordination to a metal is expected to perturb the electronic structure of the system and produce a shift in the excitation wavelength. The fluorescence response is Zn$^{2+}$ selective. Ca$^{2+}$ and Mg$^{2+}$ concentrations as high as 5mM produce no change, and other first row transition metal ions including Cu$^+$, Cu$^{2+}$, Ni$^{2+}$, Co$^{2+}$, Fe$^{2+}$ and Mn$^{2+}$ quench the fluorescence (Fig. 2.2).

The fluorescent increase and binding affinity for Zn$^{2+}$ were characterized by using a dual-metal single-ligand buffer system$^{48}$ comprising 1 mM EDTA, 2 mM Ca$^{2+}$ and 0-1 mM total Zn$^{2+}$. The formation of the [Zn(EDTA)]$^{2-}$ complex, which has an apparent $K_d$ of 2.11 nM under these conditions, allowed the controlled variation of [Zn$^{2+}$]. Fig. 2.3a and 2.3b show the results of representative titrations for ZP1 and ZP2, respectively. Whereas the quantum yield of ZP1 increased by 2.25-fold, changes in the absorption properties of the bound and unbound probe afforded a 3.1 fold change in integrated emission. Similarly, the quantum yield of ZP2 increased by $\sim$3.7-fold, and the integrated emission increases 6.0-fold.

The Zn$^{2+}$ affinity of the ZP sensors was determined by performing these measurements in triplicate using different Ca$^{2+}$/EDTA/Zn$^{2+}$ buffers. The measurements indicated that the [(Zn)ZP2] complex has an apparent $K_d$ at pH 7.0 of 0.5 ± 0.1 nM (mean ± esd) that is slightly lower than that of ZP1, for which $K_d$ is 0.7 ± 0.1 nM. The [Zn(DPA)]$^{2+}$ has an apparent $K_d$ of 70 nM at pH 7, approximately 2 orders of magnitude weaker than the observed $K_d$ values for the ZP compounds.$^{57}$ The tighter binding of the latter suggests that there are additional groups involved in Zn$^{2+}$ chelation. The fluorescence response fit to a Hill coefficient of 1, consistent with the formation of a 1:1 ZP:Zn$^{2+}$ complex responsible for the enhancement. The initial
speculation that ZP1 might bind Zn\(^{2+}\) in a "TPEN-like" manner, where TPEN is the intracellular heavy metal ion chelator \(N,N,N',N'\text{-tetra(2-picolyl)ethylenediamine}\), was not consistent with the shifting of the excitation wavelengths or the binding affinity of TPEN (~1 fM at pH 7).

The fluorescence of ZP was also enhanced by protonation of the tertiary amines. ZP fluorescence is almost completely quenched at pH > 12, and reaches a maximum for both sensors near pH 5.5. The pH change fits to an apparent pK_a of 8.3 for ZP1 (Fig. 2.4a) and 9.4 for ZP2 (Fig. 2.4b). The plateau of the fluorescence corresponds to a state of the molecule where both tertiary amines are protonated. The difference in the pK_a values between the two sensors can be attributed to the chlorine atoms on the xanthenone ring, which decrease the pK_a of the phenols. The increased acidity of the phenol groups should have an effect on the fluorescence pK_a by modifying the local pH in the vicinity of the amines. In addition to the observed difference in pK_a values for the two sensors, each of the tertiary amines of the individual molecules is predicted to have a different pK_a value, because of the difference in environment between the amines on the phenolic and keto oxygen halves of the molecule. This difference is not manifest in a stepwise change in the fluorescence intensity; however, the amine adjacent to the keto oxygen should become protonated before the more acidic site adjacent to the deprotonated phenol. The fluorescence quenching observed for both molecules below pH 5.5, with a pK_a of 2.7 for ZP1 and 3.9 for ZP2, is attributed to the formation of the non-fluorescent diphenolic zwitterion isomer.\(^{58}\)

At high pH, because the unbound sensor is more efficiently quenched, the magnitude of Zn\(^{2+}\)-induced fluorescence change is more dramatic, increasing over
200-fold at pH 12. The quenching of the fluorophore at high pH and enhancement by H⁺ and Zn²⁺ are consistent with a photoinduced electron transfer (PET) mechanism from the benzylic amines. Inhibition of PET by coordination of d¹⁰ transition metals or protons to amines is commonly observed mechanism for fluorescent enhancement.²³,²⁴ The observed fluorescence increase with Cd²⁺ also supports the validity of this suggested mechanism. Although the fluorescence of the ZP sensors is enhanced by protons as well as Zn²⁺, the quantum yield experiment demonstrates that the emission intensity of the protonated form is significantly lower than that of the Zn complex. More importantly, the emission intensity over the physiologically relevant pH range (pH 5.5 to pH 8) does not vary significantly (Fig. 2.4), so Zn²⁺-induced changes can be readily measured.

**Stoichiometry, Kₐ₂ and Solution Behavior.** Small increases in the absorption spectrum of ZP1 can be observed by titrating more than 1 equivalent of Zn²⁺ per ligand into buffered solutions of ZP1 at pH 7. At Zn²⁺ concentrations in the µM–mM range spectral changes occur with maxima at 497 nm (increases) and 520 (decreases) (Fig. 2.5). Examination of the absorption changes suggested the occurrence of a second binding event with a weak Kₐ₂ in the vicinity of ~85 ± 10 µM. The affinity of this weaker binding event Kₐ₂ increased to ~35 µM at pH 7.5, consistent with the second Zn²⁺ binding associated with displacement of a single proton.

The binding of Zn²⁺ by ZP2 was accompanied by slightly different changes in the absorption spectra (Fig. 2.6). Titration through the first binding event showed that the absorption changes around 485 indicate the occurrence of more than one process. Addition of greater than one equivalent of Zn²⁺ produced small changes in the absorption spectra of ZP2, similar to the behavior of ZP1. Analysis of the spectral
data with SPECFIT suggest a $K_{d2}$ in the range of $9.0 \pm 1.0 \mu M$, which decreases to $\sim 2 \mu M$ at pH 7.5. Figure 2.7 shows the percentage of each species detected during the titration of ZP2 (19 \mu M) with increasing concentrations of total Zn$^{2+}$. The second binding event for both ligands was not associated with a measurable change in fluorescence at physiologically relevant pH. Formation of insoluble zinc(II) hydroxide species at high [Zn$^{2+}$] and pH 7 makes it difficult to estimate free metal ion concentrations, so the values reported apply only to the stated conditions. These experiments suggest, however, that the affinity of the second binding site for Zn$^{2+}$ is several orders of magnitude weaker than the first and is not accompanied by a fluorescence change at pH 7.

Scheme 2.3 shows our interpretation of the behavior of the ZP ligands in solution, which is consistent with all of the spectral measurements. At pH 7 both benzylic amines are expected to be largely protonated, inhibiting PET quenching of the unmetallated fluorophore. Binding of the first Zn$^{2+}$ more efficiently interrupts the PET process and increases the HOMO-LUMO gap between the $S_0$ and $S_1$ energy levels. The $K_{d1}$ value is lower than that of DPA because of additional coordination by the phenolic oxygen. It is difficult to measure accurately the affinity of the second binding event, because Zn$^{2+}$ is added from unbuffered solutions, and the Zn$^{2+}$ ion has limited solubility at pH 7-7.5; however, a second binding event is consistent with all our observations including the X-ray crystal structure determination. The measured $K_{d2}$ of both ligands is significantly weaker than $K_{d1}$, presumably because of loss of coordination by the phenol and electrostatic repulsion between the more positively charged metallated ligand, and the incoming Zn$^{2+}$ cation. The lack of a significant shift in the absorbance, and the persistence of a fluorescent signal at high
concentrations of Zn$^{2+}$, suggest that the lactone ring remains open in aqueous solution.

**Structural Studies.** The nature of the Zn$^{2+}$ complex of ZP1 in the solid state was investigated by X-ray crystallography. Attempts to crystallize 6 with Cl$^{-}$, I$^{-}$, NO$_3^{-}$, PF$_6^{-}$, BF$_4^{-}$, BPh$_4^{-}$, CF$_3$SO$_3^{-}$ and SO$_4^{2-}$ counterions in a variety of solvents failed to yield X-ray quality crystals. Only the ClO$_4^{-}$ complex yielded single crystals of suitable quality for structural analysis. The structure of 6 is displayed in Fig. 2.1 as an ORTEP diagram. Single crystal X-ray diffraction results are shown in Table 2.1, and selected bond distances and angles are furnished in Table 2.2. Two ClO$_4^{-}$ and 6 disordered H$_2$O molecules are also present in the asymmetric unit. The structure of 6 is to our knowledge the first X-ray structure of a metal bound to fluorescein molecule having appended donor moieties. A survey of the CSD (Cambridge Structural Database)$^{59}$ revealed only nine examples of crystal structures of any fluorescein-based molecules, and only one metal complex, that of Pb$^{2+}$. The relatively few examples of fluorescein crystal structures has been attributed to properties of the molecule that disfavor crystallization.$^{60}$

The Zn$^{2+}$ centers in [Zn$_2$(ZP1)(H$_2$O)$_2$](ClO$_4$)$_2$·6H$_2$O are trigonal bipyramidal, a relatively common coordination geometry for this metal ion. Each Zn$^{2+}$ ion is coordinated by the three nitrogen atoms of one DPA arm, a phenolic oxygen atom, and a water molecule. The tertiary amine atom and water molecule occupy the axial positions, with the pyridine and phenol oxygen atoms in the equatorial sites. The bond lengths and angles are similar to those in X-ray structures of zinc complexes of similar tripodal N$_3$O ligands.$^{61}$ The fluorescein fragment adopts the lactoid form, consistent with the lack of fluorescence of the orange crystals under UV light. Since
the complex fluoresces in solution, we conclude that crystallization induces formation of the lactone.

Attempts to crystallize a 1:1 ligand:metal complex were unsuccessful. Reactions of ZP1 in the presence of less than one equivalent of Zn$^{2+}$ afforded only the 2:1 complex. Complexation of Zn$^{2+}$ in the first binding site leaves a DPA arm uncoordinated, and the resultant multiple degrees of freedom might disfavor crystallization. The 2:1 complex is significantly more symmetric and rigid, leading to more favorable crystal packing opportunities.

Formation of mixed complexes with partially coordinated Zn$^{2+}$ is one of the perceived shortcomings of the quinoline-based sensors. Coordination of Zn$^{2+}$ by the ligand arm of the ZP sensor leaves one open coordination site that is occupied by a water molecule in the crystal structure. Although this open coordination site offers the possibility to form mixed complexes, binding at this open site should be relatively weak since the coordination sphere of the Zn$^{2+}$ metal ion is nearly saturated. The open site is also fairly crowded, which should hinder coordination of ligands significantly larger than water.

**Intracellular Staining with ZP1 and ZP2.** Incubation of Cos-7 cells with 5 μM ZP reagent produced bright punctate staining patterns similar to observations made with quinoline-based sensors.$^{5,12}$ These observations were made with both ZP1 and ZP2. The bright fluorescence co-localized with the acidic compartment probe LysoTracker (Molecular Probes, Eugene OR). The initial ZP fluorescence was not diminished by addition of TPEN, which suggests that the concentration of free Zn$^{2+}$ in these cells is lower than the detection limit of the sensor (~0.1 nM). The low free Zn$^{2+}$ may reflect buffering and chelation by exogenous ZP. In order to draw
conclusions about endogenous Zn^{2+}, further experiments will be necessary in which
the ZP concentration is varied and the observed free [Zn^{2+}] extrapolated to zero dye.
The fluorescence initially observed is most likely background induced by
protonation within vesicular membranes (Fig. 2.8a). The apparent propensity of
these molecules to become trapped in acidic compartments represents the principal
challenge in the measurement of Zn^{2+} within cells using ZP sensors. The nature of
the fluorescence staining suggested that the sensor localized in a specific cell
organelle. Additional double-labeling experiments with a galactosyl transferase-
enhanced cyan fluorescent protein fusion (GT-ECFP) that co-localizes in the
medial/trans-Golgi revealed that ZP stains the Golgi or a Golgi-associated vesicle.\textsuperscript{40}

A significant enhancement of the fluorescence in the puncta could be observed
when the cells were treated with exogenous Zn^{2+} (50 μM) using the zinc ionophore
pyrithione (2-mercapto-pyridine N-oxide, 20 μM, Fig. 2.8b). Addition of TPEN (100
μM) reversed the fluorescence in the organelles to levels observed before the
treatment with Zn^{2+} (Fig. 2.9). Although the background fluorescence from the
unmetallated sensor presents a significant difficulty in measuring the free [Zn^{2+}],
monitoring [Zn^{2+}] change is straightforward, and represents a significant application
for these sensors. The ability of these probes to image exogenously applied Zn^{2+}
demonstrates that ZP sensors can produce a discernible change in the fluorescence
signal even though the unmetallated/protonated sensor has a relatively bright
fluorescence. Examination of cell types that undergo fluxuations in endogenous
[Zn^{2+}] as a result of biochemical events would be of interest.
Conclusions

We have developed two new fluorescent Zn$^{2+}$ sensors, ZP1 (5) and ZP2 (4), utilizing fluorescein as the reporting group. These new sensors are amenable to intracellular studies owing to their favorable optical properties, binding affinity, water solubility, and their ability to be loaded passively into cells. The primary shortcoming of the ZP sensors is their sensitivity to protons and the relatively modest fluorescence enhancement upon binding of the analyte. Access to the fluorescein dialdehyde (3), the key intermediate in the synthesis of ZP2, is significant because it can be utilized as a starting point for the design of future sensors. In addition to sensor design, the aldehyde functionality of 3 provides a convenient synthetic means for introducing a fluorescein molecule passively into a variety of systems. The ability to modify synthetically commercially available substituted fluorescein compounds like fluorescein amine is often limited because the compounds are relatively unreactive, and require harsh conditions for introduction into the desired system.

The ZP dyes are the first generation of molecules in an ongoing effort to develop Zn$^{2+}$ sensors for the neurosciences. Our current efforts are focused on preparation of sensors that are completely quenched in the absence of Zn$^{2+}$, and developing a series of probes with a range of binding affinities to study free Zn$^{2+}$ at the different concentrations proposed to be present in living systems. The use and elaboration of the synthetic methodologies developed here to prepare the dialdehyde 3 are key steps in accomplishing these goals. In addition to new sensors, we are also continuing to apply the ZP sensors to investigating the roles of free Zn$^{2+}$ in neurophysiology.
Acknowledgements. This work was supported at MIT by seed funds to launch new projects in the neurosciences, and a grant from the McKnight Foundation for the Neurosciences. The NMR spectrometer at the MIT DCIF was purchased with support from the National Science Foundation under grant number CHE9808061. SCB and SJL thank J. Du Bois, K. J. Franz, D. A. Kopp, G. T. Gassner, and W. M. Davis for helpful suggestions. SCB thanks R. A. Binstead of Spectrum Software Associates for assistance with SPECFIT, and B. Spingler for his assistance with X-ray crystallography.
References

(*) This work has appeared previously in slightly altered form in reference (†). Some of the work on ZPI, and the confocal microscopy were carried out by Grant K. Walkup and Roger Y. Tsien.


(39) ZP in an abbreviation for Zinpyr, a name that indicates the structural composition of the ligand (four pyridyl groups) as well as its ability to "peer" into the Zn$^{2+}$ concentration of samples.


(43) SMART; 5.05 ed.; Bruker AXS, Inc.: Madison, WI, 1998.


(46) Sheldrick, G. M. *SADABS: Area-Detector Absorption Correction*; University of Göttingen: Germany, 1996.


Table 2.1. Calculated free Zn$^{2+}$ values for solutions containing 2 mM [Ca], 1 mM [EDTA], at different [Zn],

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Table 2.1. Crystallographic Parameters for [Zn₂(ZP1)(H₂O)₂](ClO₄)₂·6H₂O (6·6H₂O)

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⁴ Observation criterion: I>σ2(I). ³R = \( \frac{\sum | |F_o| - |F_c| |}{\sum |F_o|} \). ⁴c wR² = \( \frac{\sum [w(F_o^2 - F_c^2)^2]}{\sum [w(F_o^2)^2]} \)^{1/2}
Table 2.2. Selected Interatomic Distances (Å) and Angles (deg) for $[\text{Zn}_2(\text{ZPI})(\text{H}_2\text{O})_2](\text{ClO}_4)_2\cdot6\text{H}_2\text{O} (6\cdot6\text{H}_2\text{O})^a$

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$^a$Numbers in parentheses are estimated standard deviations in the last digit(s). Atom labels are provided in Fig. 2.1.
Table 2.3. Atomic coordinates \( (\times 10^4) \) and equivalent isotropic displacement parameters \( (\text{Å}^2 \times 10^3) \) for \([\text{Zn}_2(\text{ZP1})(\text{H}_2\text{O})_2(\text{ClO}_4)_2] \cdot 6\text{H}_2\text{O})\).

\(U(\text{eq})\) is defined as one third of the trace of the orthogonalized \(U_{ij}\) tensor.

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Scheme 2.1
Scheme 2.2
Scheme 2.3
Figure 2.1. ORTEP diagram of \([\text{Zn}_2(\text{ZP1})(\text{H}_2\text{O})_2(\text{ClO}_4)_2] \cdot 6(\text{H}_2\text{O}) \cdot 6\cdot 6\text{H}_2\text{O}\) showing 50% thermal ellipsoids and selected atom labels. Water molecules, hydrogen atoms and disordered perchlorates are omitted for clarity.
Figure 2.2. Fluorescence response of ZP1 (a) and ZP2 (b) to various metal ions. Bars represent the final fluorescence ($F_f$) over the initial fluorescence ($F_o$).
Figure 2.3. Fluorescence emission response of ZP1 (a) and ZP2 (b) to buffered Zn$^{2+}$ solutions. Spectra were acquired in 100 mM KCl, 50 mM PIPES, pH 7.00 at 25 °C. Excitation was provided at 490 nm with 0.25 mm slit widths. Emission data were corrected for the response of the detector, using the manufacturer supplied curve, and the emission data points at 490 nm, which were perturbed by scatter, has been removed for clarity. The spectra shown are for free zinc buffered at 0, 0.172, 0.424, 0.787, 1.32, 2.11, 3.34, 5.60, 10.2, and 24.1, nM respectively. For the final spectrum (containing 1 mM EDTA and 1 mM Zn$^{2+}$) additional ZnCl$_2$ was added to provide ~25 μM free Zn$^{2+}$. Inset: fluorescence response obtained by integrating the emission spectra between 500 and 575 nm, subtracting the baseline (0 Zn$^{2+}$) spectrum and normalizing to the full scale response (25 μM free Zn$^{2+}$).
Figure 2.4. Plot of the normalized integrated emission intensity versus pH for ZP1 (a) and ZP2 (b). The increase in fluorescence corresponds to protonation of both tertiary amines, and the decrease corresponds to the isomerization of the fluorescein to its non-fluorescent zwitterion isomer. The emission intensity only varies minimally of the physiological range which is an important property for measuring intracellular [Zn\(^{2+}\)] changes.
Figure 2.5. Plot of the difference in absorption between the 1:1 ZP1/Zn$^{2+}$ spectrum, and the absorption spectra after the addition of Zn$^{2+}$ to give concentrations of 250 μM, 500 μM, 1 mM, 5 mM, 10 mM and 15 mM. Inset: plot of the change in absorbance and the concentration of [Zn$^{2+}$].
Figure 2.6. Absorption spectra of ZP2 [19 µM] after the addition of Zn$^{2+}$ to give final concentrations of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, 40, 50, 150, 250, 500 and 750 µM. Addition of Zn$^{2+}$ induces a decrease and absorption at 508 nm and an increase at 486 nm.
Figure 2.7. Plot of the percentage of each species present in the titration of ZP2 with increasing concentrations of Zn$^{2+}$. The titration was performed with 19 µM ZP2 in PIPES buffer (pH 7, 100 mM KCl). The total [Zn$^{2+}$] represents the total concentration of Zn$^{2+}$ titrated into the solution. ZP2 (○) decreases upon the formation of the [(Zn)ZP2] complex (●). At higher concentrations of Zn$^{2+}$, [(Zn)$_2$ZP2]$^{2+}$ (□) forms. The formation of [(Zn)$_2$ZP2]$^{2+}$ is not detected until all the higher affinity 1/1 sites are occupied.
Figure 2A. a) Holograph of the test object. b) Holograph of the object illuminated by a metal vapour lamp and imaged through a pinhole. The bright spherical pattern reveals a metal droplet. The bright pattern on the left side is due to the bright permittivity contrast of the metal vapour.
**Figure 2.9.** Fluorescence intensity analysis of COS-7 cells shown in Fig. 2.8. The fluorescence intensity observed for two of the bright perinuclear punctate regions (Golgi), and one nucleus are shown. Exogenous Zn$^{2+}$ was added using the zinc ionophore pyrithione 5 min after the initial treatment with ZP1 causing a dramatic increase in fluorescence in the puncta. The enhancement was reversed by treatment with TPEN which was added at the 30 min mark.
Chapter 3

Improved Synthetic Methods for Preparing Fluorescein-Based Sensors, and Application to the Synthesis of ZP3
Introduction

The concentration of free zinc varies widely throughout the body.\textsuperscript{1} Estimates range from sub-pM concentrations that are transiently uncomplexed by proteins to 10-30 $\mu$M "puffs" that are released from synaptic vesicles containing 100-300 $\mu$M Zn$^{2+}$.\textsuperscript{2} In the absence of Zn$^{2+}$ homeostasis, an indeterminate amount of liberated metal ion can rapidly damage cells or initiate events leading to the onset of disease states.\textsuperscript{3,4} Since these concentrations differ significantly, a single fluorescent sensor is unlikely to have universal utility for studying the functions and consequences of free Zn$^{2+}$. Therefore, access to a diverse selection of sensors with wide range of binding affinities is required to undertake such investigations.

The preparation of a sensor with the desired properties requires screening numerous metal-binding units having different functionalities. A central synthetic scaffold for constructing the sensors as well as a synthetic route that affords multi-gram quantities of that synthon facilitates examining such a variety of ligands. The precursor to ZP2, 4',5'-fluoresceindicarboxaldehyde, is one such convenient and versatile starting material for preparing sensor candidates, but the synthesis of this dialdehyde requires a low-yielding oxidation step using DMSO.\textsuperscript{5} Maximizing the yield of this oxidation reaction also requires rigorous drying of DMSO by vacuum distillation followed by placement over 3 Å molecular sieves. Since the dialdehyde is a valuable synthetic precursor, an improved method for obtaining it in higher yield is desirable. Therefore, we have examined several synthetic routes to find an efficient synthesis of a useful synthetic intermediate.
Experimental

Materials and Methods. Chlorobenzene was distilled from CaH₂ under nitrogen. Acetonitrile was distilled from CaH₂ under nitrogen and dried over 3 Å molecular sieves. MeOH was distilled from Mg/I₂. DMF was dried over 3 Å molecular sieves. THF was dried on an aluminum oxide column followed by a column of molecular sieves. CDCl₃ was dried over 3 Å molecular sieves. DPA,⁶ 4',5'-bis(bromomethyl)fluorescein dibenzoate⁵ and 2'-chloro-5'-methylfluorescein di-t-butyl(dimethyl)silyl ether⁷ were prepared as previously described. All other reagents were purchased and used as received. Flash column chromatography was performed with silica gel-60 (230-400 mesh), octadecyl-functionalized silica gel (RP18), or Brockman I activated basic aluminum oxide (150 mesh). Thin layer chromatographic (TLC) analysis was performed with Merck F254 silica gel-60, Merck RP-18 F254S, or Merck F254 aluminum oxide-60 plates and viewed by UV light, or developed with ceric ammonium molybdate, ninhydrin or iodine stain. NMR spectra were recorded on a Varian 500 MHz or a Mercury 300 MHz spectrometer at ambient probe temperature, 283 K, and referenced to the internal ¹H and ¹³C solvent peaks. Infrared spectra were recorded on a BTS 135 or an Avatar 360 FTIR instrument as KBr pellets or thin films on NaCl plates. Electrospray ionization (ESI) mass spectrometry was performed in the MIT Department of Chemistry Instrumentation Facility (DCIF) with the use of m-nitrobenzyl alcohol as the matrix.

4',5'-Dimethoxymethylfluorescein (2). Fluorescein (1, 1.68 g, 5.02 mmol) and p-formaldehyde (0.982 g, 32.7 mmol) were added to 10 mL of concentrated HCl in a thick-walled glass tube. The tube was sealed and the orange slurry was
heated to 120 °C for 14 h with vigorous stirring. The reaction was cooled and diluted with distilled water. The resulting orange solids were collected by filtration, washed thoroughly with distilled water, and dried. The crude product was filtered through silica gel (9:1 CHCl₃/MeOH), and the solvents were removed. Flash chromatography on silica gel (9:9:2 CHCl₃/C₆H₅CH₂/MeOH) yielded the product as a yellow crystalline solid (109 mg, 5.16%). TLC Rᵢ = 0.25 (9:1 CHCl₃/MeOH). ¹H NMR (CD₂Cl₂) δ 3.59 (6 H, s), 5.05 (4 H, s), 6.573 (4 H, m), 7.16 (1 H, d, J = 7.2 Hz), 7.63-7.72 (2 H, m), 7.98 (1 H, d, J = 6.6 Hz). FTIR (KBr, cm⁻¹) 3278, 2937, 2831, 1757, 1637, 1600, 1498, 1467, 1433, 1378, 1288, 1254, 1221, 1165, 1104, 1015, 945, 873, 824, 799, 762, 696, 655. HRMS (ESI): Calcd for MH⁺, 421.1282; Found 421.1271.

4',5'-Diacetoxyethylfluorescein Dibenzoate (4). Potassium acetate (0.676 g, 6.90 mmol) and 4,5-dibromomethylfluorescein dibenzoate (3, 1.00 g, 1.37 mmol) were combined in DMF (50 mL) and stirred for 5 h. The reaction mixture was diluted with water (250 mL) to precipitate a pinkish white solid. The precipitate was collected on a frit, washed with water and dried. Recrystallization (9:1 C₆H₅CH₂/EtOAc) and washing with n-pentane yielded the product as a white crystalline solid (0.494 g, 52.4%). TLC Rᵢ = 0.39 (2:1:1 hexanes/C₆H₅CH₂/EtOAc). ¹H NMR (CDCl₃) δ 1.99 (6H, s), 5.48 (4 H, s), 6.93 (2 H, d, J = 8.7 Hz), 7.04 (2 H, d, J = 8.7 Hz), 7.34 (1 H, d, J = 7.5 Hz), 7.55 (4 H, t, J = 6.3 Hz), 7.65-7.80 (4 H, m), 8.08 (1 H, d, J = 6.0 Hz), 8.23 (4 H, d, J = 8.4 Hz). FTIR (KBr, cm⁻¹) 1744, 1600, 1426, 1223, 1092, 1051, 1023, 873, 764, 709. HRMS (ESI): Calcd for MH⁺, 685.1704; Found 685.1719.
4',5'-Dilevulinoyloximethylfluorescein Dibenzoate (5). The potassium salt of levulinic acid was prepared with potassium hydroxide in EtOH. Potassium levulinate (2.54 g, 16.5 mmol) and 4,5-dibromomethylfluorescein dibenzoate (3, 2.00 g, 2.74 mmol) were combined in DMF (250 mL) and stirred for 8 h. The reaction mixture was concentrated to 50 mL, and diluted with water (300 mL) to precipitate a brownish white solid. The solids were collected on a frit and dried. Flash chromatography (2:1 C₆H₅CH₃/EtOAc) yielded the product as a white crystalline solid (1.17 g, 53.4%). TLC Rᵣ = 0.27 (2:1 C₆H₅CH₃/EtOAc). ¹H NMR (CDCl₃) δ 2.11 (6 H, s), 2.53 (4 H, t, J = 6.6 Hz), 2.67 (4 H, t, J = 6.3 Hz), 5.50 (4H, s), 6.94 (2 H, d, J = 8.7 Hz), 7.03 (2 H, d, J = 8.7 Hz), 7.35 (1 H, d, J = 7.5 Hz), 7.55 (4 H, t, J = 7.2 Hz), 7.66-7.95 (4 H, m), 8.08 (1 H, d, J = 6.9 Hz), 8.23 (4 H, d, J = 7.2 Hz). ¹³C NMR (CDCl₃) δ 19.66, 28.02, 29.98, 38.07, 55.80, 117.09, 117.34, 119.18, 124.53, 125.57, 126.47, 128.83, 128.99, 129.28, 130.56, 134.29, 135.62, 150.54, 151.86, 152.51, 164.88, 169.11, 172.39, 206.59, 233.19. FTIR (KBr, cm⁻¹) 1741, 1718, 1426, 1224, 1154, 1093, 1022, 710. HRMS (ESI): Calcd for MH⁺, 797.2229; Found 797.2127.

4',5'-Dibenzoyloxycarbonylaminobutyroyloximethylfluorescein Dibenzoate (6). Benzyloxycarbonylaminobutyric acid was prepared as described, and the potassium salt was prepared with potassium hydroxide in EtOH. Potassium benzyloxycarbonylaminobutyrate (1.00 g, 4.24 mmol) and 4,5-dibromomethylfluorescein dibenzoate (3, 0.612 g, 0.843 mmol) were combined in DMF (50 mL) and stirred for 3 h. The reaction mixture was diluted with saturated NaCl solution (300 mL), and the aqueous layer was extracted with EtOAc. Removal of solvent followed by flash chromatography (3:1
C₆H₅CH₃/EtOAc) yielded a white crystalline solid (394 mg, 45.0%). TLC Rᵣ = 0.40 (3:1 C₆H₅CH₃/EtOAc). ¹H NMR (CDCl₃) δ 1.70 (4 H, quin., J = 7.2 Hz), 2.27 (4 H, t, J = 7.2 Hz), 3.12 (4 H, q., J = 6.6 Hz), 5.05 (4 H, s), 5.12 (2 H, b), 5.49 (4 H, d, J = 4.8 Hz), 6.94 (2 H, d, J = 8.7 Hz), 7.02 (2 H, d, J = 8.4 Hz), 7.30 (11 H, s), 7.52 (4 H, t, J = 7.8 Hz), 7.64-7.83 (4 H, m), 8.08 (1 H, d, J = 6.9 Hz), 8.20 (4 H, d, J = 7.2 Hz). ¹³C NMR (CDCl₃) δ -20.42, -19.53, 25.05, 31.19, 40.31, 55.72, 66.74, 81.92, 117.23, 117.32, 119.27, 124.49, 125.61, 126.50, 128.22, 128.66, 128.74, 129.03, 129.35, 130.49, 134.37, 135.65, 136.80, 150.47, 151.91, 152.37, 156.61, 164.99, 169.05, 172.86, 226.47, 233.17. FTIR (KBr, cm⁻¹) 3385, 2941, 1748, 1629, 1600, 1527, 1427, 1224, 1176, 1092, 752, 709. HRMS (ESI): Calcd for MH⁺, 1039.3284; Found 1039.3244.

4',5'-Dibenzoxyethylfluorescein (7). Hydrazine monohydrate (0.36 mL, 7.4 mmol) and acetic acid (3.0 mL, 52 mmol) were dissolved in 12 mL of pyridine to give a 0.50 M solution of hydrazine acetate. A 250 µL aliquot (0.125 mmol) of the hydrazine solution was added to a solution of 4',5'-dilevulinoyloximethylfluorescein dibenzoate (5, 50 mg, 0.063 mmol) in 5 mL of THF and MeOH (1/1 solution). The reaction mixture was stirred overnight at room temperature and diluted with water (25 mL). The aqueous layer was extracted with C₆H₅CH₃/CH₂Cl₂ (1/1) and washed with water. Removal of solvents followed by flash chromatography (9:1 CHCl₃/MeOH) yielded the product as an orange solid. TLC Rᵣ = 0.40 (2:1 C₆H₅CH₃/EtOAc). ¹H NMR (CDCl₃) δ 5.84 (4H, s), 6.71 (2 H, d, J = 9.0 Hz), 6.74 (2 H, d, J = 9.0 Hz), 7.18 (1 H, d, J = 6.0 Hz), 7.44 (4 H, t, J = 7.8 Hz), 7.59-7.70 (5 H, m), 8.02 (1 H, d, J = 6.0 Hz), 8.13 (4H, d, J = 8.1 Hz). FTIR (KBr, cm⁻¹) 1719, 1655, 1438, 1382, 1316, 1274, 1226, 1114, 1091, 712.
4',5'-Dimethylfluorescein Di-\(t\)-butyldiphenylsilyl Ether (9). Imidazole (2.83 g, 41.6 mmol) and 4',5'-dimethylfluorescein (8, 5.00 g, 0.138 mmol) were combined in DMF (200 mL) and stirred. To the resulting red slurry was added \(t\)-butyldiphenylsilyl chloride (7.60 mL, 29.2 mmol). The solution was stirred for 12 h at room temperature. The reaction mixture was concentrated to 50 mL and diluted with water (200 mL), after which the aqueous layer was extracted with EtOAc. The combined organic extracts were washed with saturated NaCl and dried over MgSO\(_4\) to give a brown solid after filtration and solvent removal. Flash chromatography (4:1:1 C\(_6\)H\(_5\)CH\(_3\)/hexanes/EtOAc) yielded a brown sticky solid (7.70 g, 66.0%). TLC \(R_f\) = 0.30 (4:1 hexanes/EtOAc). \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 1.15 (18 H, s), 2.63 (6 H, S), 6.14 (2 H, d, \(J = 8.7\) Hz) 6.16 (2 H, d, \(J = 8.7\) Hz), 7.11 (1 H, d, \(J = 7.5\) Hz), 7.33-7.57 (14 H, m), 7.68-7.76 (8 H, m), 7.85 (1 H, d, \(J = 7.2\) Hz). \(^{13}\)C NMR (CDCl\(_3\)) \(\delta\) -22.09, 9.69, 14.35, 22.87, 26.76, 31.80, 84.78, 111.98, 114.67, 116.14, 124.33, 124.95, 125.02, 127.44, 127.91, 128.02, 128.14, 129.50, 129.84, 130.15, 130.31, 132.41, 132.84, 134.69, 135.00, 135.52, 135.51, 151.12, 152.59, 155.24, 169.48, 233.17. FTIR (thin film, cm\(^{-1}\)) 2959, 2931, 2858, 1767, 1602, 1491, 1428, 1419, 1282, 1221, 1182, 1114, 873, 825, 779, 756, 741, 701, 613, 550, 503. HRMS (ESI): Calcd for MH\(^+\), 837.3426; Found 837.3428.

4',5'-Bis(bromomethyl)fluorescein Di-\(t\)-butyldiphenylsilyl Ether (10). 4',5'-Dimethylfluorescein di-\(t\)-butyldiphenylsilyl ether (9, 0.500 g, 0.597 mmol), 1,3-dibromo-5,5-dimethylhydantoin (0.256 g, 0.895 mmol), and 1,1'-azobis(cyclohexanecarbonitrile) (12 mg, 49 \(\mu\)mol) were combined in C\(_6\)H\(_5\)Cl (100 mL). Acetic acid (10 \(\mu\)L, 0.17 \(\mu\)mol) was added to the stirred solution, and the reaction mixture was stirred at 40\(^\circ\) C for 72 h. The solution was washed with
warm water, and the solvent was removed. Flash chromatography on (3:1 hexanes/EtOAc) yielded the product as a brown sticky solid (0.594 g, 87.7%). TLC R$_f$ = 0.50 (7:3 hexanes/EtOAc). $^1$H NMR (CDCl$_3$) δ 1.16 (18 H, s), 5.19 (4 H, S), 6.10 (2 H, d, J = 8.7 Hz), 6.24 (2 H, d, J = 9.0 Hz), 7.15 (1 H, d, J = 7.5 Hz), 7.33-7.59 (14 H, m), 7.69-7.74 (8 H, m), 7.84 (1 H, d, J = 7.5 Hz). FTIR (thin film, cm$^{-1}$) 3071, 2958, 2930m 2857, 1773, 1627, 1605, 1572, 1489, 1466, 1428, 1285, 1234, 1211, 1189, 1151, 1114, 1075, 1013, 879, 852, 832, 823, 788, 773, 752, 701, 670, 615, 547, 502. HRMS (ESI): Calcd for MH$^+$, 993.1636; Found 993.1630.

4',5'-Bis(nitrooxymethyl)fluorescein Di-t-butyldiphenylsilyl Ether (11). 4',5'-Dibromomethylfluorescein di-t-butyldiphenylsilyl ether (10, 500 mg, 0.503 mmol), 2,6-di-t-butyl-4-methylpyridine (120 mg, 0.584 mmol), and silver nitrate (200 mg, 1.18 mmol) were combined in 200 mL CH$_3$CN to give a yellow slurry. The reaction mixture was stirred for 12 h under Ar. The gray silver precipitate was removed by filtration through Celite and the solvents were evaporated. Flash chromatography on silica with a solvent gradient (5:1 hexanes/EtOAc→4:1 hexanes/EtOAc→3:1 hexanes/EtOAc) yielded the product as a yellow solid (38.5 mg, 7.99%). TLC R$_f$ = 0.24 (4:1 hexanes/EtOAc). $^1$H NMR (CDCl$_3$, 500 MHz) δ 1.11 (18 H, s), 6.06 (2 H, d, J = 11.0), 6.08 (2 H, d, J = 11.0), 6.17 (2 H, d, J = 9.0 Hz), 6.35 (2 H, dd, J = 1.0 8.5 Hz), 7.13 (1 H, dd, J = 0.5, 7.5 Hz), 7.35-7.49 (13 H, m), 7.51 (1 H, t, J = 7.5 Hz) 7.61 (1 H, t, J = 7.5 Hz), 7.67-7.69 (8 H, m), 7.85 (1 H, dd, J = 1.0, 8.0 Hz). $^{13}$C NMR (CDCl$_3$, 125 MHz) δ 19.65, 25.93, 26.45, 30.36, 65.21, 82.72, 109.90, 112.23, 115.69, 124.27, 125.21, 127.04, 127.95, 128.37, 130.27, 130.49, 130.68, 131.24, 131.53, 134.83, 135.40, 151.28, 151.79, 157.10, 169.03. FTIR (KBr,
$N,N'$-Diphenyl-4',5'-diaminomethylfluorescein Di-$t$-butyldiphenylsilyl Ether (12). $4',5'$-Dibromomethylfluorescein di-$t$-butyldiphenylsilyl ether (10, 500 mg, 0.503 mmol), 2,6-di-$t$-butyl-4-methylpyridine (225 mg, 1.10 mmol), silver nitrate (175 mg, 1.03 mmol), and aniline (100 µL, 1.10 mmol) were combined in 200 mL CH$_3$CN to give a yellow slurry. The reaction mixture was stirred for 12 h under Ar. An additional aliquot of aniline (200 µL, 2.20 mmol) was added and the reaction mixture was stirred for 5 h. The gray silver precipitate was removed by filtration through Celite and the solvents were evaporated. Flash chromatography on silica (7:3 hexanes/EtOAc) afforded the product as a yellow solid (142 mg, 27.7%). TLC $R_f = 0.24$ (4:1 hexanes/EtOAc). $^1$H NMR (CDCl$_3$, 500 MHz) $\delta$ 1.14 (18 H, s), 4.88 (2 H, d, $J = 11.5$ Hz), 4.90 (2 H, d, $J = 11.5$ Hz), 6.29 (4 H, dd, $J = 7.5$, 16.5 Hz), 7.11 (3 H, dquin, $J = 1.0$, 5.0 Hz), 6.81 (3 H, td, $J = 1.0$, 7.5 Hz), 6.88 (4 H, d, $J = 7.5$ Hz), 7.37-7.50 (13 H, m), 7.58 (1 H, td, $J = 1.0$, 7.0 Hz), 7.71-7.78 (8 H, m), 7.88 (1 H, d, $J = 6.5$ Hz). $^{13}$C NMR (CDCl$_3$, 125 MHz) $\delta$ 19.68, 26.65, 38.18, 83.74, 112.30, 113.58, 115.24, 115.48, 116.81, 117.81, 118.65, 124.34, 124.97, 127.22, 127.53, 127.83, 128.14, 128.23, 129.41, 129.73, 130.32, 130.48, 131.81, 132.22, 134.93, 135.42, 146.49, 148.50, 151.20, 152.06, 155.57, 169.22. FTIR (thin film, cm$^{-1}$) 3375, 3050, 2956, 2931, 2858, 1756, 1603, 1500, 1488, 1427, 1294, 1261, 1220, 1106, 872, 823, 747, 701. HRMS (ESI): Calcd for MH$^+$, 1019.4276; Found 1019.4270.

4-Nitro-3-bromomethylanisole (14). 3-Methyl-4-nitroanisole (13, 2.50 g, 15.0 mmol) and 1,3-dibromo-5,5-dimethylhydantoin (5.00 g, 17.5 mmol) were
combined in 250 mL of C₆H₅Cl, and acetic acid (75 µL, 1.31 mmol) and 1,1'-azobis(cyclohexanecarbonitrile) (VAZO 88, 150 mg, mmol) were added to the stirring solution. The solution was stirred at 40 °C for 72 h. The crude reaction mixture was washed three times with hot NaHCO₃ solution (75 mL, 80 °C), and once with water (75 mL). The organic portion was dried over MgSO₄ to give an orange solid after filtration and solvent removal. The solid was filtered through a plug of silica gel to yield a mixture of the brominated product and unbrominated starting material that was carried on to the next step without further purification. Pure material could be obtained from flash chromatography on silica (15:3:2 hexanes/EtOAc/C₆H₅CH₃) to give a brown oil (117 mg, 3.19%). TLC Rₗ = 0.34 (4:1 hexanes/EtOAc). ¹H NMR (CDCl₃, 500 MHz) δ 3.91 (3 H, s), 4.85 (2 H, s), 6.92 (1 H, dd, J = 4.5, 15.0 Hz), 7.02 (1 H, d, J = 5.0 Hz), 8.13 (1H, d, J = 15.5 Hz). ¹³C NMR (CDCl₃, 125 MHz) δ 30.19, 56.25, 114.11, 117.69, 127.61, 128.48, 135.72, 163.45. FTIR (thin film, cm⁻¹) 2942, 2845, 1726, 1606, 1582, 1515, 1463, 1438, 1420, 1339, 1295, 1263, 1085, 1030, 836, 758. HRMS (ESI): Calcd for MH⁺, 245.9766; Found 245.9760.

4-Nitro-3-[bis(2-pyridylmethyl)aminomethyl]anisole (15). DPA (950 mg, 4.77 mmol), K₂CO₃ (6.50 g, 47.0 mmol), 4-nitro-3-bromomethyl anisole (14, 1.12 g, 4.55 mmol; from a 3.56 g of a mixture containing ~68% 3-methyl-4-nitro anisole), and powdered 3 Å molecular sieves (750 mg) were combined in 20 mL of CH₃CN and stirred for 12 h under Ar. The crude reaction mixture was filtered through Celite to give a brown oil after solvent removal. Flash chromatography on basic alumina with a solvent gradient (9:1 CH₂Cl₂/EtOAc→4:1 CH₂Cl₂/EtOAc→7:3 CH₂Cl₂/EtOAc) yielded the product as an orange oil (954
mg, 57.4%). TLC Rf = 0.32 (3:1 CH2Cl2/EtOAc). 1H NMR (CDCl3, 500 MHz) δ 3.84 (4 H, s), 3.88 (3 H, s), 4.14 (2 H, s), 6.77 (1 H, dd, J = 2.5, 8.5 Hz), 7.13 (2 H, td, J = 1.5, 5.0 Hz), 7.43 (2 H, d, J = 7.5 Hz), 7.50 (1H, d, J = 3.0 Hz), 7.62 (2 H, td, J = 2.0, 7.5 Hz), 7.92 (1 H, d, J = 8.5 Hz), 8.51 (2 H, dq, J = 1.0, 5.0 Hz). 13C NMR (CDCl3, 125 MHz) δ 56.06, 56.40, 112.84, 115.65, 122.32, 123.18, 127.49, 136.68, 138.46, 142.50, 149.20, 158.90, 163.27. FTIR (thin film, cm⁻¹) 3066, 3010, 2926, 2841, 1589, 1513, 1433, 1340, 1284, 1236, 1080, 842, 763. HRMS (ESI): Calcd for MH⁺, 365.1614; Found 365.1608.

3-[Bis(2-pyridylmethyl)aminomethyl]-p-anisidine (16). Pd/C (1.0 g, 10% activated) and 4-nitro-3-[bis(2-pyridylmethyl)aminomethyl]anisole (15, 914 mg, 2.51 mmol) were combined in 200 mL of MeOH and stirred under a hydrogen atmosphere (1 atm) for 12 h. The reaction mixture was filtered through Celite to give a dark yellow oil after solvent removal. Flash chromatography on basic alumina with a solvent gradient (9:1 CH2Cl2/EtOAc→4:1 CH2Cl2/EtOAc→7:2:1 CH2Cl2/EtOAc/MeOH) yielded an impure orange oil. Additional flash chromatography on basic alumina (99:1 CHCl3/MeOH) yielded the product as an orange oil (466 mg, 55.5%). TLC Rf = 0.82 (4:1 CH2Cl2/EtOAc). 1H NMR (CDCl3, 500 MHz) δ 3.63 (2 H, s), 3.73 (3 H, s), 3.80 (4 H, s), 4.56 (2 H, bs), 6.57 (1 H, d, J = 8.5 Hz), 6.64-6.69 (2 H, m), 7.15 (2 H, t, J = 5.0 Hz), 7.39 (2 H, d, J = 8.0 Hz), 7.62 (2 H, td, J = 2.0, 7.5 Hz), 8.55 (2H, dt, J = 1.0, 5.0 Hz). 13C NMR (CDCl3, 125 MHz) δ 55.96, 58.02, 60.38, 113.94, 116.65, 117.26, 122.23, 123.61, 123.99, 136.54, 140.76, 149.32, 151.97, 159.34. FTIR (thin film, cm⁻¹) 3412, 3319, 3217, 2933, 2829, 1590, 1569, 1503, 1433, 1253, 1150, 1046, 764. HRMS (ESI): Calcd for MH⁺, 335.1872; Found 335.1794.
9-(o-Carboxyphenyl)-2-chloro-5-{2-[bis(2-pyridylmethyl)aminomethyl]-N-(p-anisidine)}-6-hydroxy-3-xanthanone (Zinpyr-3, ZP3, 20). 2’-Chloro-5’-bromomethylfluorescein di-t-butyldimethylsilyl ether (17, 340 mg, 494 μmol) and pyridine (304 mg, 1.48 mmol) were combined in 20 mL of CH$_3$CN. AgNO$_3$ (92 mg, 541 μmol) was added to the stirred solution causing the reaction to change from a yellow to a clear solution with the formation of a white precipitate. After 10 min, 3-[bis(2-pyridylmethyl)aminomethyl]-p-anisidine (16) in 10 mL of CH$_3$CN was added to the solution, and the reaction mixture was stirred for 12 h at room temperature. The crude reaction mixture was filtered through Celite and the solvents were removed to give TBS-protected ZP3 (19). The crude product was combined in 15 mL of THF with AcOH (30 μL, 5048 μmol) and 1.0 M tetra butylammonium fluoride (TBAF in THF, 350 μL, 350 μmol) and stirred at room temperature. Upon addition of the TBAF, the solution immediately changed from orange to deep red. After 24 h, the reaction was diluted with 75 mL of H$_2$O, and the aqueous solution was washed with hexanes (2×100 mL) then saturated with NaCl. The product was extracted into EtOAc, washed with H$_2$O (100 mL) and brine (3×100 mL) and dried over Na$_2$SO$_4$ to give a red solid after filtration and solvent removal. Medium pressure flash chromatography on RP18 silica (3:1 0.1 N HCl:CH$_3$CN) followed by removal of the CH$_3$CN afforded an acidic solution of ZP3. The solution was loaded onto a second RP18 silica column packed with millipure H$_2$O, and the column was washed with millipure water until the elutant from the column reached neutral pH (6-7, pH paper). The product was washed off the column (4:1 CH$_3$CN/H$_2$O) and the solvents were removed to give an orange powder (69 mg, 19.6%). $^1$H NMR (CD$_3$CN, 500 MHz)
δ 3.63 (2 H, d, J = 1.5 Hz), 3.66 (3 H, s) 3.69 (4 H, s), 4.64 (1 H, d, J = 14.5 Hz), 4.68 (1 H, d, J = 14.5 Hz), 6.52 (1 H, d, J = 8.5 Hz), 6.57 (1 H, d, J = 9.0 Hz), 6.67 (1 H, dd, J = 3.0, 9.0 Hz), 6.75-6.77 (2 H, m), 6.80 (1 H, s), 6.97 (1 H, s), 7.13 (2 H, td, J = 1.0, 4.0 Hz), 7.23 (1 H, d, J = 7.5 Hz), 7.27 (2 H, d, J = 8.0 Hz), 7.56 (2 H, td, J = 2.0, 7.5 Hz), 7.70 (1 H, td, J = 1.0, 7.5 Hz), 7.76 (1 H, td, J = 1.0, 7.5 Hz), 7.99 (1 H, d, J = 8.0 Hz), 8.42 (2 H, d, J = 4.0 Hz). ¹³C NMR (DMF-d₇, 125 MHz) δ 37.54, 55.43, 57.80, 60.35, 104.57, 110.56, 111.09, 111.76, 113.45, 113.47, 118.11, 122.37, 123.44, 124.42, 124.95, 125.47, 127.93, 128.49, 130.55, 135.61, 136.73, 136.82, 142.87, 149.16, 151.23, 151.49, 151.88, 159.16, 162.14, 162.37, 160.61, 169.05. FTIR (KBr, cm⁻¹) 3333, 3061, 2830, 1760, 1636, 1598, 1511, 1451, 1371, 1283, 1252, 1218,1152, 869, 763. HRMS (ESI): Calcd for MH⁺, 713.2167; Found 713.2188.

**General Spectroscopic Methods.** Ultral grade PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid)) from Calbiochem and KCl (99.997%) were purchased and used as received. All solutions were filtered through 0.2-μM cellulose filters before fluorescence measurements. Zn solutions were prepared by the addition of appropriate amounts of 1.0 M, 100 mM, 10 mM or 1 mM Zn²⁺ stocks that were checked by atomic absorption spectroscopy for concentration accuracy, or by titration with terpyridine and measurement of the absorption spectra. The titration was performed by treating a 100 mM solution of 2,2':6',2''-terpyridine in buffered solution (50 mM PIPES, 100 mM KCl, pH 7) with aliquots of 10 mM (nominal) ZnCl₂ and determining the equivalence point by monitoring the absorbance of the resulting complex at 321 nm (ε = 35.9 10³ M⁻¹ cm⁻¹). The Zn²⁺ stocks were prepared from 99.999% pure ZnCl₂. The purity of the ZP probes was verified by HPLC. ZP was introduced to aqueous solutions by addition of a
stock solution in DMSO (0.67 mM). Graphs were manipulated and equations calculated by using Kaleidograph 3.0. The pH values of solutions were recorded with an Orion glass electrode that was calibrated prior to each use. The experiments for measuring the quantum yield are described elsewhere.\textsuperscript{5}

**UV-Visible Spectroscopy.** Absorption spectra were recorded on a Cary 1E scanning spectrophotometer under the control of a Pentium PC running the manufacturer supplied software package. Spectra were routinely acquired at 25 °C, maintained by a circulating water bath in 1-cm path length quartz cuvettes with a volume of 3.5 mL.

**Fluorescence Spectroscopy.** Fluorescence spectra were recorded on a Hitachi F-3010 spectrofluorimeter under the control of a Pentium-based PC running the SpectraCalc software package. Excitation was provided by a 150 W Xe lamp (Ushio Inc.) operating at a current of 5 A. All spectra were normalized for excitation intensity via a rhodamine quantum counter, and emission spectra were normalized by the manufacturer-supplied correction curves. Spectra were routinely acquired at 25 °C, maintained by a circulating water bath in 1 cm × 1 cm quartz cuvettes using 3 nM slit widths and a 240 nm/min scan speed.

**Titration of ZP3 with Zn\textsuperscript{2+}.** A solution (50 mM PIPES, 100 mM KCl, pH 7) containing 13 μM ZP3 was titrated with 10 mM stock solutions of ZnCl\textsubscript{2}. The absorption spectrum for each trial was measured initially and following the addition of each aliquot of stock solution. Subtraction of the initial spectrum from subsequent ones generated absorption difference plots. The absorption changes were plotted as a function of [Zn\textsuperscript{2+}].
Results and Discussion

Synthetic Methods. The oxidation of 4',5'-bis(bromomethyl)fluorescein dibenzoate (3) with DMSO in the presence of sodium bicarbonate affords 4',5'-fluoresceindicarboxaldehyde in 37% yield. Numerous methods for converting benzyl halides to aldehydes are known. Attempts to oxidize 3 with hexamethylenetetraamine (Sommelet oxidation) and trimethylamine N-oxide, as well as chromium and selenium reagents, failed to convert 3 to a dialdehyde. In addition to oxidation of 3, several electrophilic aromatic substitution reactions, such as the Vilsmeier-Haak formylation, were examined as methods to convert fluorescein (1) directly to a dialdehyde. With the exception of the Mannich reaction used to synthesize ZP1, these reactions were unsuccessful in making useful fluorescein derivatives.

The oxidation of alcohols to aldehydes offers an alternative route to preparing the desired fluorescein compounds. The conversion of 3 to the corresponding benzylc diol requires a facile synthesis that proceeds in the presence of the benzoate protecting groups. Introduction of ester protecting groups orthogonal to the benzoate esters onto the 4' and 5' positions by the reacting 3 with carboxylate salts provides the means to introduce "masked" alcohols. Subsequent removal of the ester using conventional deprotection chemistry could provide the desired diol (Fig. 3.1). Reaction of 3 with the appropriate carboxylate salt affords the three ester derivatives 4',5'-diacetoxymethylfluorescein dibenzoate (4), 4',5'-dilevulinoyloxymethylfluorescein dibenzoate (5) and 4',5'-dibenzoxycarbonylaminobutyroyloximethylfluorescein dibenzoate (6) in high yield (Scheme 3.1). The deprotection
of both 5 and 6 occurs by intramolecular cleavage. The levulinate group of 5 condenses with NH₂NH₂ with the ketone group to generate a primary nitrogen atom. This nucleophile attacks the ester carbon to unmask the alcohol with concomitant release of a cyclic side product. Hydrogenation of 6 cleaves the benzyl group and liberates an equivalent of CO₂ to produce a primary nitrogen atom that performs an ester cleavage reaction similar to the levulinate removal. The product of these deprotection reactions was not the desired benzylic diol, however. Migration of the benzoate protection groups from the phenols to the benzylic positions yields 4',5'-dibenzoxymethylfluorescein (7, Scheme 3.2).

Although removal of both the benzoate groups and the 4',5'-esters would afford a highly polar product with multiple hydroxyl groups, transesterification should yield the completely deprotected diol. Both 4 and 5 react with sodium methoxide to give 4',5'-dimethoxyfluorescein (2) instead of the diol (Scheme 3.3). The formation of 2 occurs through a Michael reaction (Fig. 3.2). One equivalent of methoxide cleaves the phenolic benzoate. The loss of the carboxylate gives a quinone methide intermediate that reacts with a second equivalent of methoxide.¹⁰ The product of the transesterification reaction was prepared independently in low yield by reacting fluorescein with p-formaldehyde in HCl (Scheme 3.3).

The results of the studies with 4, 5 and 6 suggest that the lability of the phenolic benzoate groups interferes with the desired chemistry, so similar fluorescein compounds with more stable protecting groups are possible precursors to synthetically useful derivatives. Silyl protecting groups like the TBS (t-butyldimethylsilyl) and TBDPS (t-butyldiphenylsilyl) are more stable
toward nucleophiles than ester protecting groups. The reaction of 4',5'-dimethylfluorescein (8) with t-butyldiphenylsilyl chloride yields 4',5'-dimethylfluorescein di-t-butyldiphenylsilyl ether (9, Scheme 3.4). Free radical bromination of 9 affords 4',5'-(bis)bromomethylfluorescein di-t-butyldiphenylsilyl ether (10) in high yield. Unfortunately, the reaction of 10 with potassium levulinate and sodium trichloroacetate results in the cleavage of the silyl protecting groups. In addition to the lability of the benzoate protecting groups, the unexpected loss of the silyl protecting groups suggests that the lactone ring or another property of the fluorescein molecule destabilizes protecting groups on the phenolic oxygens.

Since the silyl protecting groups are unstable to the desired nucleophiles, silver reagents were examined as another possible method to prepare fluorescein diols by hydrolysis. Silver nitrate (AgNO₃) reacts with 10 in the presence of 2,6-di-t-butyl-4-methylpyridine, a proton scavenger, to yield 4',5'-bis(nitrooxy-methyl)fluorescein di-t-butyldiphenylsilyl ether (11, Scheme 3.4). Although nitrooxyalkyl compounds frequently react with water under facile conditions to give the corresponding alcohol, migration of the silyl groups prevents access to the diol. Although it decomposes in the presence of water, 11 appears to be stable under anhydrous conditions. The product decomposes on silica gel so isolated yields are low, but when 11 is generated in situ the formation of the nitrooxy group activates the 4' and 5' positions toward nucleophilic attack, even to relatively weak donors like aniline. The reaction of aniline with 10 in the presence of AgNO₃ provides N,N'-diphenyl-4',5'-diaminomethylfluorescein di-t-
butyldiphenylsilyl ether (Scheme 3.4, 12) in reasonable yield. The activation of these positions eliminates the need to prepare a diol or a dialdehyde derivative.

**Synthesis of ZP3.** In an effort to apply this new method to the synthesis of Zn$^{2+}$ sensors, 2′-chloro-5′-methylfluorescein di-t-butyldimethylsilyl ether (17) was prepared as a scaffold for a probe with a single metal-binding site. Scheme 3.5 outlines the synthesis of the Zn$^{2+}$-binding portion of the fluorophore. 3-Methyl-4-nitroanisole (13) was selected as the building block for the Zn$^{2+}$ ligand to install a p-methoxy group on the aniline ring. Aniline nitrogen atoms are typically poor metal-binding ligands; however, an electron-donating group on the para position of the aromatic ring should enhance binding affinity by increasing the electron density on the aniline nitrogen atom. Decomposition on silica and difficulty separating the product from the unbrominated starting material limited the amount of 4-nitro-3-bromomethylanisole (14) isolated. Nevertheless, the crude reaction mixture of 13 and 14 could be carried on without purification to provide adequate quantities of 4-nitro-3-[bis(2-pyridylmethyl)aminomethyl]-anisole (15). Hydrogenation of 15 gives the desired 3-[bis(2-pyridylmethyl)aminomethyl]-p-anisidine (16) metal-binding fragment of ZP3.

Generation of a nitroxyl compound in situ by adding AgNO$_3$ to 17 followed by addition of 16 provides the silyl protected precursor of ZP3 (Scheme 3.6). Subsequent removal of the TBS ethers with fluoride ion yields ZP3 after purification on C18 silica gel. This synthetic method is a significant improvement over that employed in the synthesis of ZP2 because it avoids the
low yielding oxidation step and provides access to sensors with a single binding site.

**Fluorescence and Zn²⁺-binding properties of ZP3.** In buffered solution (50 mM PIPES, 100 mM KCl, pH 7) using EDTA to scavenge adventitious metal ions, ZP3 has a quantum yield of 0.04 and an emission maximum at 521 nm. Upon the addition of 25 μM Zn²⁺ the quantum yield increases to 0.05 and the emission maximum shifts to 517 nm. The addition of Zn²⁺ is also accompanied by a shift in the excitation maximum from 505 nm (ε = 68.8 × 10³) to 495 nm (ε = 77.4 × 10³). Since the enhancement of fluorescence is negligible, Zn²⁺ binding by ZP3 was monitored by absorption spectroscopy. Addition of Zn²⁺ to a buffered solution of ZP3 demonstrates that the probe binds the metal ion with 1:1 stoichiometry (Fig. 3.3). The changes in absorption are similar to those observed for the structurally related sensor ZP47 (Fig. 3.4), indicating that ZP3 binds Zn²⁺ in an identical manner with similar affinity.

Although the chemical structure of ZP4 is nearly identical to that of ZP3, Zn²⁺ does not enhance the fluorescence of the latter. Zn²⁺-binding to ZP4 interrupts a photoinduced electron transfer (PET) quenching mechanism involving the lone pair of electrons on the aniline nitrogen atom and the excited state of the fluorophore. This difference suggests that a second quenching mechanism exists for ZP3. One possibility is PET from the ether oxygen to the fluorophore (Fig. 3.4). Several fluorescent sensors for alkali metal ions utilize PET from ether oxygen atoms as the quenching mechanism.¹¹ Alternatively, the entire aniline-based ligand may participate in PET (Fig. 3.4).¹² In order to understand this fluorescence behavior, additional structurally related ZP sensors
are being prepared with different functional groups in the \( p \)-position on the aniline ring.\(^\text{13} \) By varying the nature of this group, the fluorescence properties can be correlated with structural features. When a sufficient number of probes have been characterized, density functional theory (DFT) will be utilized to examine the electronic structure of these sensors. The combination of new probes and DFT studies will not only help interpret the fluorescence behavior of ZP3 but also assist in the rational design of sensors with superior physical properties.

**Conclusions**

In an effort to access increased quantities of precursors to fluorescein-based Zn\(^{2+} \) sensors, several synthetic approaches were examined to convert 4',5'-bis(bromomethyl)fluorescein dibenzoate (3) efficiently to a dialdehyde product. Most of these endeavors focused on the preparation of a diol that could be oxidized to a dialdehyde, but none was successful. Substituting the ester protecting groups on the phenolic oxygen atoms with silyl ethers, however, permits the 4' and 5' positions of the brominated derivative to be activated toward nucleophilic substitution by generating a nitrooxy compound by treatment with AgNO\(_3 \). This new methodology was applied to the synthesis of ZP3, a second-generation fluorescent sensor for Zn\(^{2+} \). Unexpectedly, ZP3 shows only a negligible enhancement of the emission intensity upon Zn\(^{2+} \)-binding. Although the fluorescent behavior of ZP3 is not fully understood, the mostly likely explanation is the presence of a second quenching mechanism. Synthesis
of structurally related sensors and DFT calculations will be employed in future investigations to resolve the nature of the fluorescence quenching.
References

(13) ZP5 has been synthesized by Jessica Harvey and Elizabeth Nolan, and Nolan is currently preparing additional derivatives.
Scheme 3.1

4: R = CH₃(Ad)
5: R = CH₃CH₂COCH₃ (Lev)
6: R = CH₃CH₂CH₂NHCCOCH₂C₆H₄(GABA)
Scheme 3.2
Scheme 3.3
Scheme 3.4
Scheme 3.5
Scheme 3.6
Figure 3.1. Reaction of carboxylate salts with 3 introduces a "masked" alcohol. Removal of the ester provides a diol.
Figure 3.2. Mechanism of the formation of the benzylic ether. The cleavage of the phenolic ester by NaOMe eliminates the benzylic ester to generate a quinone methide. A Michael reaction with a second equivalent of NaOMe yields the ether.
Figure 3.3. Plot of the difference in absorption between the ZF3 (13 μM) and the absorption spectra after the addition of Zn2+ to give concentrations of 1.9, 3.7, 5.6, 7.4, 9.3, 11.1, 13.0 and 14.8 μM. The addition of Zn2+ is accompanied by a decrease in absorption at 512 nm and increases at 490 and 462 nm. Inset: plot of the change in absorption at 512 and 490 nm, and the concentration of Zn2+. Note the complete formation of the 1:1 complex at 13 μM Zn2+. 
Figure 3.4. Structure of ZP4, a compound related to ZP3 that shows an enhancement of fluorescence upon the addition of Zn$^{2+}$. The quantum yield increases from 0.06 to 0.34. Further details are provided in Chapter 4.
Figure 3.5. PET quenching pathways in ZP3. Both the aniline and aliphatic nitrogen atoms can quench the fluorophore. In addition, PET from the methoxyether oxygen atom and/or the entire ligand ligand fragment may be responsible for quenching the Zn$^{2+}$-bound sensor.
Chapter 4

ZP4, an Improved Neuronal $\text{Zn}^{2+}$ Sensor of the Zinpyr Family
Introduction

Zinc is one of many transition metal elements that sustain life. Once postulated to be readily available from a cytosolic pool of free metal ion, mounting evidence suggests that Zn$^{2+}$ uptake and distribution are regulated by a complex intracellular mechanism. Disruption of Zn$^{2+}$ homeostasis after ischemia, seizures, and following head injury induces neuronal death. Although methods for detecting zinc show no evidence of perikaryal Zn$^{2+}$ in healthy cells, following these various traumas, damaged neuronal cells are stained intensely for this metal ion.

In addition to acute toxicity, disruption of Zn$^{2+}$ homeostasis may play a role in the pathology of several neurodegenerative disorders like the formation of amyloid plaques in Alzheimer’s disease. The highly regulated delivery of zinc to intracellular targets and the deleterious effects observed during overexposure to the free metal ion suggest that healthy cells contain no unbound Zn$^{2+}$; however, free Zn$^{2+}$ may play a vital role in communication between cells. Zinc released from glutamatergic nerve terminals plays a role in neurotransmission, but the precise function of this synaptic Zn$^{2+}$ remains unclear. Extracellular Zn$^{2+}$ has been implicated in the modulation of cellular processes through a zinc-sensing receptor.

Fluorescent sensors facilitate study of spectroscopically silent metal ions like Zn$^{2+}$ in biological systems because specific properties can be engineered into probes through chemical synthesis. Moreover, fluorescence microscopy is
both a sensitive and an innocuous investigative technique. In order to advance our studies of Zn\textsuperscript{2+} in neurobiology, we have designed and reported on a new family of Zn\textsuperscript{2+}-sensitive fluorescent probes (Fig. 4.1).\textsuperscript{38,39} ZP1 and ZP2 were the first in a series of fluorescein-based sensors designed to induce a positive fluorescence response upon complexation of Zn\textsuperscript{2+}. Although these two dyes offer several advantages over traditional Zn\textsuperscript{2+} sensors, improvements in the both the synthetic methodology and the physical properties of the sensor are desirable for an even more efficient investigation of Zn\textsuperscript{2+} ion in neurochemistry.

ZP1 can be prepared by a Mannich reaction in one step,\textsuperscript{40} but the conditions limit the variety of metal-binding ligands that can be incorporated into the sensor. The preparation of ZP2 is more versatile,\textsuperscript{38} but a low-yielding synthesis of the key intermediate reduces the rate at which compounds can be produced. In addition to these synthetic restrictions, both compounds have two distinct Zn\textsuperscript{2+} coordination sites. Although the binding affinities of the two sites for Zn\textsuperscript{2+} differ significantly, and only the first binding event is accompanied by a fluorescence change,\textsuperscript{38} these properties may not apply to future sensors. Multiple metal ion binding events under physiological conditions complicate the analysis and can lead to ambiguous conclusions. One of the advantages of ZP sensors is the brightness of the Zn\textsuperscript{2+}-bound complex, yet the tertiary amines responsible for the fluorescence increase are susceptible to protonation at physiological pH. The inhibition of a photoinduced electron transfer (PET) pathway either by coordination to a closed-shell metal ion or protonation of an amine is a common strategy utilized in the design of intensity-based sensors.\textsuperscript{41,42} Although the fluorescence intensity of the Zn\textsuperscript{2+}-bound complex is greater than
that of the protonated sensor, the magnitude of the fluorescence change upon metal binding is diminished and background fluorescence from the unmetallated probe is high at physiological pH.\textsuperscript{39} In the second generation ZP sensor reported here, we have addressed these shortcomings with an improved synthetic strategy and a modified Zn\textsuperscript{2+}-binding ligand.

Experimental Section

Materials and Methods. Chlorobenzene and dichloroethane were distilled from CaH\textsubscript{2} under nitrogen. Acetonitrile was distilled from CaH\textsubscript{2} under nitrogen and dried over 3 Å molecular sieves, nitrobenzene and DMF were dried over 3 Å molecular sieves. THF was dried on an aluminum oxide column followed by a column of molecular sieves. CDCl\textsubscript{3} was dried over 3 Å molecular sieves. Di-(2-picoly)amine (DPA) was prepared as previously described.\textsuperscript{43} All other reagents were purchased and used as received. Flash column chromatography was performed with silica gel-60 (230-400 mesh), octadecyl-functionalized silica gel (RP18), or Brockman I activated basic aluminum oxide (150 mesh). Thin layer chromatographic (TLC) analysis was performed with Merck F254 silica gel-60, Merck RP-18 F254S, or Merck F254 aluminum oxide-60 plates and viewed by UV light, or developed with ceric ammonium molybdate, ninhydrin or iodine stain. NMR spectra were recorded on a Varian 500 MHz or a Mercury 300 MHz spectrometer at ambient probe temperature, 283 K, and referenced to the internal \textsuperscript{1}H and \textsuperscript{13}C solvent peaks. Infrared spectra were recorded on a BTS 135 or an Avatar 360 FTIR instrument as KBr pellets or thin films on NaCl plates. Electrospray ionization (ESI) mass spectrometry was
performed in the MIT Department of Chemistry Instrumentation Facility (DCIF) with the use of m-nitrobenzyl alcohol as the matrix. **CAUTION:** Several of the isolated compounds below contains perchlorate ion, which can detonate explosively and without warning. Although we have encountered no incidents with the reported compound, all due precautions should be taken.

**2'-Carboxy-5-chloro-2,4-dihydroxybenzophenone (5).** Phthalic anhydride (3, 9.0 g, 61 mmol) and 4-chlororesorcinol (4, 8.54 g, 59.1 mmol) were combined in C₆H₅NO₂ (150 mL) and chilled to 0°C. Aluminum chloride (AlCl₃, 18.4 g, 138 mmol) was added slowly over 1 h and the slurry was stirred overnight while warming to room temperature. The reaction mixture was heated to 120°C for 6 h, and the reaction mixture was diluted with 0.1 M HCl (700 mL) and hexanes (100 mL) to precipitate a black-brown solid. The solids were collected on a frit and dissolved in a boiling solution of methanol and water (1:1) and decolorizing carbon was added. The solution was filtered through Celite and the product crystallized at -10°C. The solid was recrystallized twice (1:1 CH₃OH/water), washed with ice cold water and dried to give the product as a brown crystalline solid (8.45 g, 48.9 %). TLC Rf = 0.26 (7:3 CHCl₃/MeOH). ¹H NMR (CD₃OD, 500 MHz) δ 6.47 (1 H, s), 6.94 (1 H, s), 7.38 (1 H, dd, J = 1.2, 7.2 Hz), 7.65 (1 H, td, J = 1.5, 7.5 Hz), 7.73 (1 H, td, J = 1.5, 7.2 Hz), 8.11 (1 H, dd, J = 1.5, 7.5 Hz). ¹³C NMR (CD₃OD, 125 MHz) 104.91, 113.32, 115.65, 128.64, 130.70, 131.14, 131.75, 133.78, 134.76, 141.54, 161.80, 164.78, 168.69, 202.41. FTIR (KBr) 3390, 2828, 2663, 2547, 1691, 1615, 1571, 1419, 1293, 1219, 1141, 925, 772. HRMS (ESI): Calcd for MH⁺, 293.0218; Found 293.0212.
2'-Chloro-5'-methylfluorescein Di-\textit{t}-butyldimethylsilyl Ether (7). 2'-Carboxy-5-chloro-2,4-dihydroxybenzophenone (5, 5.00 g, 17.0 mmol) and 2-methylresorcinol (2.12 g, 17.0 mmol) were crushed and melted into a brown liquid at 150 °C. Fused ZnCl₂ (2.33 g, 110 mmol) was added slowly over 35 min, and the temperature was slowly increased to 250 °C over 30 min until the material solidified. The brick red solid was pulverized and boiled in 250 mL of 3 M HCl for 30 min. The red solid was collected on a frit, washed thoroughly with hot water and dried in vacuo. The crude product was filtered through a plug of silica (7:3 CHCl₃:MeOH) to give a red solid after solvent removal. The crude 2'-chloro-5'-methylfluorescein (6, mixture of two components with ~10% 4',5'-dimethylfluorescein) was combined with imidazole (3.40 g, 101 mmol) in DMF (300 mL) and stirred. To the resulting red slurry was added \textit{t}-butyldimethylsilyl chloride (6.92 g, 45.9 mmol). The reaction mixture was stirred for 12 h at room temperature. A portion of the DMF was removed (~250 mL), and the reaction mixture was diluted with saturated brine (~300 mL). The aqueous layer was extracted with EtOAc, and the combined organic extracts were dried over MgSO₄ to give a brown oil after filtration and solvent removal. The crude product was filtered through silica (7:2:1 hexanes/C₆H₅CH₃/EtOAc) and the solvents removed. Flash chromatography (9:1 hexanes/EtOAc) yielded an impure brown solid (3.8 g) containing ~10% of the corresponding 4',5'-dimethylfluorescein disilylether. A yield of 37% was calculated based on the integration of NMR peaks. TLC $R_f = 0.41$ (4:1 hexanes/EtOAc). $^1$H NMR (CDCl₃, 500 MHz) δ 0.21 (6 H, s), 0.31 (6 H, s), 1.02 (9 H, s), 1.05 (9 H, s), 2.34 (3 H, s), 6.46-6.53 (2 H, m), 6.74 (1 H, s), 6.83 (1 H, s), 7.20 (1 H, d, $J = 7.5$ Hz), 7.64 (1 H, t, $J = 8.0$ Hz), 7.70 (1 H, t, $J$
= 7.0 Hz), 8.03 (1 H, d, J = 7.5 Hz). 13C NMR (CDCl3, 125 MHz) δ -4.17, -4.10, -4.03, -3.94, 9.58, 18.47, 18.54, 25.79, 25.85, 25.91, 83.43, 108.69, 111.62, 113.19, 116.70, 121.35, 124.21, 125.32, 125.43, 127.02, 128.95, 130.04, 135.28, 150.64, 151.07, 152.79, 153.37, 155.61, 169.42. FTIR (thin film) 2955, 2930, 2858, 1769, 1606, 1488, 1411, 1281, 1257, 1218, 1183, 1089. HRMS (ESI): Calcd for MH+, 609.2259; Found 609.2254.

2'-Chloro-5'-bromomethylfluorescein Di-t-butyldimethylsilyl Ether (8).

2'-Chloro-5'-methylfluorescein di-t-butyldimethylsilyl ether (7, 3.80 g, 6.24 mmol, contains 10% 4',5'-dimethylfluorescein disilyl ether), 1,3-dibromo-5,5-dimethylhydantoin (2.0 g, 7.0 mmol), and 1,1'-azobis(cyclohexanecarbonitrile) (Vazo 88, 85 mg, 0.347 mmol) were combined in C8H5Cl (150 mL). Acetic acid (100 μL, 1.70 μmol) was added to the stirring solution, and the reaction mixture was stirred at 40°C for 60 h. The crude reaction mixture was washed twice with hot water (100 mL, 80°C), and the solvent was removed. Flash chromatography on silica (7:1 hexanes/EtOAc) yielded the product as a brown oil, (2.83 g, 73.3%). TLC Rf = 0.28 (7:1 hexanes/EtOAc). 1H NMR (CDCl3, 500 MHz) δ 0.28 (6 H, s), 0.30 (6 H, s), 1.04 (9 H, s), 1.05 (9 H, s), 4.83 (1 H, d, J = 9.5 Hz), 4.84 (1 H, d, J = 9.5 Hz), 6.56 (1 H, d, J = 9.0 Hz), 6.63 (1 H, d, J = 9.0 Hz), 6.92 (1 H, s), 7.22 (1 H, d, J = 7.5 Hz), 7.62 (1 H, td, J = 1.0, 7.5 Hz), 7.68 (1 H, td, J = 1.0, 7.5 Hz), 8.01 (1 H, dd, J = 1.0, 7.5 Hz). 13C NMR (CDCl3, 125 MHz) δ -4.30, -4.24, -4.10, -4.01, 18.30, 22.00, 22.33, 24.37, 25.63, 25.76, 33.86, 82.38, 108.68, 111.98, 113.11, 114.57, 118.29, 121.72, 124.03, 125.20, 126.71, 128.68, 128.77, 130.13, 135.30, 150.36, 150.53, 152.12, 153.35, 155.83, 169.87. FTIR (thin film) 3208, 2954, 2931, 2858, 1730, 1608, 1582, 1453, 1429, 1282, 1257, 839. HRMS (ESI): Calcd for MH+, 687.1364; Found 687.1359.
2-[Bis(2-pyridylmethyl)aminomethyl]nitrobenzene (10). DPA (950 mg, 4.77 mmol), K₂CO₃ (6.50 g, 47.0 mmol), 2-nitrobenzylbromide (9, 980 mg, 4.54 mmol), and powdered 3 Å molecular sieves (750 mg) were combined in 20 mL of CH₃CN and stirred for 12 h under Ar. The crude reaction mixture was filtered through Celite to give a brown oil after solvent removal. Flash chromatography on basic alumina (7:3 CH₂Cl₂:EtOAc) yielded the product as an orange oil (997 mg, 65.7%). TLC Rᵣ = 0.26 (7:3 CH₂Cl₂:EtOAc). ¹H NMR (CDCl₃, 500 MHz) δ 3.79 (4 H, s), 4.08 (2 H, s), 7.14 (2 H, td, J = 1.0, 5.0 Hz), 7.34 (1 H, td, J = 1.0, 7.5 Hz), 7.40 (2 H, d, J = 7.5 Hz), 7.49 (1H, dd, J = 1.0, 7.5 Hz), 7.64 (2 H, td, J = 2.0, 7.5 Hz), 7.71 (1 H, dd, J = 0.5, 7.5 Hz), 7.77 (1 H, dd, J = 1.0, 8.0 Hz), 8.51 (2 H, dq, J = 1.0, 5.0 Hz). ¹³C NMR (CDCl₃, 125 MHz) δ 56.06, 60.57, 122.33, 123.48, 124.52, 128.12, 131.57, 132.56, 134.55, 136.68, 149.10, 150.13, 158.77. FTIR (thin film) 3064, 3009, 2925, 2830, 1589, 1526, 1433, 1362, 766, 731. HRMS (ESI): Calcd for MH⁺, 335.1503; Found 335.1493.

2-[Bis(2-pyridylmethyl)aminomethyl]aniline (11). Pd/C (300 mg, 10% activated) and 2-[bis(2-pyridylmethyl)aminomethyl]-nitrobenzene (10, 2.48 g, 7.42 mmol) were combined in 150 mL of MeOH and stirred under a hydrogen atmosphere (1 atm) for 24 h with the addition of 300 mg of Pd/C after 12 h. The reaction mixture was filtered through Celite to give a dark yellow oil after solvent removal. Flash chromatography on basic alumina with a solvent gradient (CHCl₃→9:1 CHCl₃/MeOH) yielded a yellow oil. Additional flash chromatography on basic alumina (99:1 CHCl₃/MeOH) yielded the product as a yellow oil (100 mg, 5%). TLC Rᵣ = 0.41 (97:3 CHCl₃/MeOH). ¹H NMR (CDCl₃, 500 MHz) δ 3.65 (2 H, s), 3.79 (4 H, s), 4.94 (2 H, bs), 6.59-6.64 (2 H, m), 7.02-7.05
(2 H, m), 7.12 (2 H, td, J = 1.0, 5.0 Hz), 7.37 (2 H, d, J = 7.5 Hz), 7.59 (2 H, td, J = 2.0, 7.5 Hz), 8.53 (2H, dt, J = 1.0, 5.0 Hz). $^{13}$C NMR (CDCl$_3$, 125 MHz) $\delta$ 57.90, 60.22, 115.45, 117.23, 122.09, 122.33, 123.49, 128.55, 131.28, 136.43, 147.12, 149.18, 159.29. FTIR (thin film) 3996, 3320, 3209, 3009, 2921, 2805, 1615, 1590, 1494, 1433, 751. HRMS (ESI): Calcd for MH$,^+$, 305.1766; Found 305.1769.

9-(o-Carboxyphenyl)-2-chloro-5-[2-[bis(2-pyridylmethyl)aminomethyl]-N-methylaniline]-6-hydroxy-3-xanthanone (ZP4, 14). 2'-Chloro-5'-bromo-methylfluorescein di-t-butyldimethylsilyl ether (8, 160 mg, 234 $\mu$mol) and pyridine (85 $\mu$L, 1.1 mmol) were combined in 7 mL of CH$_3$CN. AgNO$_3$ (60 mg, 349 $\mu$mol) was added to the stirring solution causing the reaction to change from a yellow solution to a clear solution with the formation of a white precipitate. After 15 min, 2-[bis(2-pyridylmethyl)aminomethyl]-aniline (11) in 20 mL of CH$_3$CN was added to the solution, and the reaction mixture was stirred for 12 h at room temperature. The crude reaction mixture was filtered through Celite and diluted with brine (~300 mL), EtOAc (200 mL) and CH$_2$Cl$_2$ (100 mL). The combined organics were washed with brine (3×100 mL), dried over Na$_2$SO$_4$, and the solvents were removed to give TBS-protected ZP4. The crude product was combined in 15 mL of THF with AcOH (32 $\mu$L, 538 $\mu$mol) and 1.0 M tetrabutylammonium fluoride (TBAF in THF, 485 $\mu$L, 485 $\mu$mol) and stirred for 36 h at room temperature. Upon addition of the TBAF, the solution immediately changed from orange to deep red. The reaction was diluted with 100 mL of H$_2$O, and the aqueous solution was washed with hexanes (2×100 mL) then saturated with NaCl. The product was extracted into EtOAc, and the combined organics were washed with H$_2$O (100 mL) and brine (3×100 mL), dried over Na$_2$SO$_4$ to
give a red solid after filtration and solvent removal. Medium pressure flash chromatography on RP18 silica (3:1 0.1 N HCl:CH$_3$CN) followed by removal of the CH$_3$CN afforded an acidic solution of ZP4. The acidic solution was loaded onto a second RP18 silica column packed with millipure H$_2$O, and the column was washed with millipure water until the eluant from the column reached neutral pH (6-7, pH paper). The product was washed off the column (4:1 CH$_3$CN/H$_2$O), and the solvents were removed to give an orange powder (48 mg, 30%). $^1$H NMR (DMF-d$_7$, 500 MHz) $\delta$ 3.78 (1 H, d, $J = 22$ Hz), 3.88 (1 H, d, $J = 24.5$ Hz), 3.92 (2 H, d, $J = 25.5$ Hz), 4.00 (2 H, d, $J = 24$ Hz), 4.59 (2 H, s), 6.59 (1 H, t, $J = 12$ Hz), 6.68 (1 H, d, $J = 14.5$ Hz), 6.80 (1 H, s), 7.01 (1 H, d, $J = 13.5$ Hz), 7.03 (1 H, d, $J = 14.5$ Hz), 7.16-7.20 (2 H, m), 7.25-7.29 (3 H, m), 7.43-7.51 (3 H, m), 7.69 (2 H, t, $J = 12.5$ Hz), 7.81 (1 H, t, $J = 11.5$ Hz), 7.90 (1 H, t, $J = 10.5$ Hz), 8.07 (1 H, d, $J = 12.5$ Hz), 8.40 (2 H, d, $J = 7.0$ Hz). $^{13}$C NMR (DMF-d$_7$, 125 MHz) $\delta$ 58.57, 60.02, 105.39, 111.18, 111.74, 112.30, 113.76, 113.87, 117.03, 117.61, 123.84, 124.87, 125.40, 125.93, 128.01, 128.74, 129.33, 130.37, 131.45, 132.62, 136.75, 138.95, 148.96, 149.32, 151.91, 152.16, 153.45, 156.91, 160.04, 169.82. FTIR (KBr) 3372, 3062, 1792, 1606, 1450, 1284, 1252, 754. HRMS (ESI): Calcd for MH$^+$, 683.2061; Found 683.2024.

[2-{Bis(2-pyridylmethyl)aminomethyl}-N-methylanilinophenol](BPAMP, 15). 2-{Bis(2-pyridylmethyl)aminomethyl}aniline (11, 2.15 g, 7.06 mmol) was dissolved in 50 mL of EtOAc and salicylateddehyde (0.72 mL, 6.76 mmol) was added dropwise via a syringe. The reaction mixture was stirred for 12 h under Ar, and the solvent was removed. The resulting yellow oil was dissolved in 50 mL of dichloroethane, combined with NaBH(OAc)$_3$ (1.58 g, 7.45 mmol), and stirred vigorously for 12 h at room temperature. The excess
NaBH(OAc)₃ was quenched with saturated NaHCO₃, and the product was extracted into CH₂Cl₂ (3 × 50 mL). The combined organics were washed once with water (50 mL), dried over MgSO₄, filtered, and the solvent was removed to give a dark yellow oil. Flash chromatography on silica (CHCl₃/MeOH/H₂NPr 195:4:1) yielded the product as a yellow solid (1.98 g, 71.2%). TLC R₄ = 0.26 (CHCl₃/MeOH/PrNH₂ 195:4:1). ¹H NMR (CDCl₃, 300 MHz) δ 3.48 (2 H, s), 3.56 (4 H, s), 4.27 (2 H, d, J = 7.0 Hz), 6.45-6.55 (2 H, m), 6.69-6.73 (2 H, m), 6.84-6.93 (4 H, M), 7.00-7.04 (3 H, m), 7.09 (1 H, d, J = 12.5 Hz), 7.28 (2 H, td, J = 1.5, 2.5 Hz), 8.25 (2 H, dt, J = 1.0, 8.0 Hz). ¹³C NMR (CDCl₃, 125 MHz) δ 46.46, 58.74, 60.52, 112.07, 116.91, 117.71, 119.74, 122.15, 123.40, 123.49, 125.38, 128.46, 128.71, 129.16, 130.82, 136.64, 147.69, 148.69, 156.55, 158.40. FTIR (thin film) 3294, 3042, 2818, 2717, 1592, 1455, 1237, 750. HRMS (ESI): Calcd for MH⁺, 411.2179; Found 411.2160.

[Zn(BPAMP)(H₂O)](ClO₄) (16). A portion of BPAMP (15, 25 mg, 61 µmol) was combined with NaOH (0.81 mL, 0.075 M, 61 µmol) and zinc triflate (0.81 mL, 0.075 M, 61 µmol) in 3 mL of CH₃CN. Sodium perchlorate (200 mg, 1.6 mmol) was dissolved in water (1 mL) and added to the CH₃CN solution, then the combined solutions were filtered through Celite. Crystallization at room temperature with concurrent evaporation of some solvent over 2 days yielded yellowish clear blocks suitable for X-ray crystallography. The crystalline material was filtered, powdered and dried under vacuum at 50 °C to yield 14.2 mg of product in 39.4% yield. FTIR (KBr) 3490, 3270, 3062, 2860, 1607, 1478, 1446, 1274, 1099, 761, 622. Anal. Calcd for ZnC₂₅H₂₇Cl₁₄N₄O₆: C, 52.72; H, 4.59; N, 9.46. Found: C, 52.47; H, 4.52; N, 9.28.
[Mn(BPAMP)(H₂O)](ClO₄) (17). BPAMP (15, 40 mg, 97 μmol) was combined with NaOH (1.3 mL, 0.075 M, 97 μmol) and manganese perchlorate (1.3 mL, 0.075 M, 97 μmol) in 3 mL of MeOH. The MeOH solution was layered with water (3 mL) containing sodium perchlorate (300 mg, 2.4 mmol). Crystallization at room temperature with concurrent evaporation of some solvent over 2 days yielded clear yellow blocks suitable for X-ray crystallography. The crystalline material was filtered, powdered and dried under vacuum at 50 ºC to yield 36.2 mg of dried product in 64.1% yield. FTIR (KBr) 3469, 1605, 1477, 1444, 1270, 1109, 1094, 764, 622. Anal. Calcd for MnC₂₆H₁₇Cl₁N₄O₆: C, 53.66; H, 4.68; N, 9.63. Found: C, 50.18; H, 4.30; N, 9.01.

[Cu(BPAMP)](ClO₄) (18). This compound was prepared in a manner identical to that described above for 17 using copper perchlorate (1.3 mL, 0.075 M, 97 μmol) and crystallized as brown needles and green blocks. Only the green blocks were suitable for X-ray crystallography. The crystalline material was filtered, powdered and dried under vacuum at 50 ºC to yield 36.2 mg of dried product in 71.3% yield. FTIR (KBr) 3431, 3239, 3072, 2926, 1607, 1478, 1446, 1276, 1092, 758, 621. Anal. Calcd for CuC₂₆H₂₅Cl₁N₄O₅: C, 54.55; H, 4.40; N, 9.79. Found: C, 53.48; H, 4.24; N, 9.67.

Collection and Reduction of X-ray Data. Crystals were covered with Paratone-N oil and suitable specimens were mounted on the tips of glass fibers at room temperature and transferred to a Bruker (formerly Siemens) APEX X-ray diffraction system with a graphite-monochromatized MoKα radiation (λ = 0.71073 Å) controlled by a Pentium-based PC running the SMART software package. Data were collected at 173 K in a stream of cold N₂ maintained with a
KRYO-FLEX nitrogen cryostat. Procedures for data collection and reduction have been reported previously. The structures were solved by direct methods and refined by full matrix least-squares and difference Fourier techniques with SHELXTL. Empirical absorption corrections were applied with the SADABS program, and structures were checked for higher symmetry by PLATON. Space groups were determined by examining systematic absences and confirmed by the successful solution and refinement of the structures. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were assigned idealized locations and given isotropic thermal parameters 1.2 times the thermal parameter of the carbon atoms to which they were attached. In the structures of 16 and 18, the perchlorate anion is disordered, and a water molecule in both 16 and 17 is partially occupied; all were modeled and refined accordingly. Relevant crystallographic information is summarized in Table 4.1, and the 50% thermal ellipsoid plots are shown in Fig. 4.2. Table 4.2 contains selected bond distances and angles, and the atomic coordinates and equivalent isotropic displacement parameters are provided in Tables 4.3-4.5.

**General Spectroscopic Methods.** Ultrol grade PIPES (piperazine-\(N,N'\)-bis(2-ethanesulfonic acid)) from Calbiochem and KCl (99.997%) was purchased and used as received. All solutions were passed through 0.2-\(\mu\)M cellulose filters before measurements. Except for the fluorescence titration experiment, Zn solutions were prepared by the addition of appropriate amounts of 1.0 M, 100 mM, 10 mM or 1 mM Zn\(^{2+}\) stocks that were checked by atomic absorption spectroscopy for concentration accuracy, or by titration with terpyridine and measurement of the absorption spectra. The titration was performed by treating
a 100 mM solution of 2,2′:6′,2″-terpyridine in buffered solution (50 mM PIPES, 100 mM KCl, pH 7) with aliquots of 10 mM (nominal) ZnCl₂ and determining the equivalence point by monitoring the absorbance of the resulting complex at 321 nm (ε = 35.9 × 10³ M⁻¹ cm⁻¹). The Zn²⁺ stocks were prepared from 99.999% pure ZnCl₂. The purity of the ZP probes was verified by HPLC. ZP was introduced to aqueous solutions by addition of a stock solution in DMSO (0.67 mM). Graphs were manipulated and equations calculated by using Kaleidagraph 3.0. The pH values of solutions were recorded with an Orion glass electrode that was calibrated prior to each use. The experiments for measuring the pH-dependent fluorescence, quantum yield, Kᵦ, metal ion selectivity were performed as previously described.³⁸-⁴⁰,⁴⁹

**UV-Visible Spectroscopy.** Absorption spectra were recorded on a Hewlett Packard 8453A diode array spectrophotometer under the control of a Pentium II-based PC running the Windows NT ChemStation software package, or a Cary 1E scanning spectrophotometer under the control of a Pentium PC running the manufacturer supplied software package. Spectra were routinely acquired at 25 °C, maintained by a circulating water bath in 1-cm path length quartz cuvettes with a volume of 1.0 or 3.5 mL.

**Fluorescence Spectroscopy.** Fluorescence spectra were recorded on a Hitachi F-3010 spectrofluorimeter under the control of a Pentium-based PC running the SpectraCalc software package. Excitation was provided by a 150 W Xe lamp (Ushio Inc.) operating at a current of 5 A. All spectra were normalized for excitation intensity via a rhodamine quantum counter, and emission spectra were normalized by the manufacturer-supplied correction curves. Spectra were
routinely acquired at 25 °C, maintained by a circulating water bath in 1 cm × 1 cm quartz cuvettes using 3 nM slit widths and a 240 nm/min scan speed. Fluorescence emission measurements were also acquired in a 1 cm × 1 cm quartz cell using a Spex Fluorolog-2 instrument with 1-nm bandwidth slits. All spectra were corrected for emission intensity by using the manufacturer-supplied photomultiplier curves.

**Titration of ZP1, ZP4 and BPAMP with Metal Ions.** Solutions (50 mM PIPES, 100 mM KCl, pH 7) containing either 10 μM ZP1, 10 μM ZP4 or 100 μM BPAMP were titrated with 10 mM stock solutions of the divalent metal ion under consideration (CuCl₂, MnCl₂ or ZnCl₂). The absorption spectrum for each trial was measured before and following the addition of each aliquot of stock solution. Subtraction of the initial spectrum from subsequent ones generated absorption difference plots. The absorption changes were plotted as a function of [M²⁺].

**Tissue Preparation and Staining Procedures.** Tissue from normal rats suffering prior seizures was used. Methods of administering pilocarpine by intraperitoneal injection were followed,⁵⁰ and the rats were allowed to survive overnight after drug administration. After seizure or control (no) treatment, rats were killed by an overdose of anesthesia, decapitated, and the brains were removed rapidly and frozen by burying on dry-ice "snow" for 2-3 min. Frozen brains were then mounted on the chuck of a closed-cabinet cryostat (Harris/Jung Reichert), and cut (at -13 degrees C) at anywhere from 6 to 30 μm thickness. Immediately after thawing onto clean glass slides, the sections were allowed to
dry at room temperature for 0.5 to 3 h, then stained and photographed. Through all steps, care was taken to keep sections covered in dust-free containers.

Staining was done by applying 60 μL of staining fluid to sections lying flat in a plastic box. After 1 minute, the excess reagent was washed off by gentle agitation in a 0.8% saline solution. Slides were viewed and photographed while still damp without coverslips, immediately after rinsing. To improve contrast, selected sections were also cleared by immersion in increasing concentrations of glycerol (in water), specifically 30, 60, 90 and 100%. These were then viewed under glycerine immersion (Zeiss 25X, variable aperture) with 1.25 or 2.0-post magnification.

**Microscopy methods.** Images were made on a Zeiss Universal with SPOT II cooled CCD camera, using Olympus Plan-apo 10X (0.87) or Zeiss 25X (glycerine or water immersion, 1.3). Illumination was via 200 W HBO high-pressure mercury illumination, using either a 360 nm or 480 nm band-pass filter, 500 nm dichroic splitter and either a long-pass or a 550 band pass emission filter. Additional images were made on a Nikon Diaphot with Odyssey confocal, illuminating with 488 nm Argon laser and viewing through a 500 nm long-pass filter. In this case, sections were mounted directly on coverslips and viewed (from below) through the coverslip with oil immersion, using Nikon 40 and 100X objectives.

**Results**

**ZP4 Synthesis.** Scheme 4.1 outlines the synthesis of the fluorescein scaffold from commercially available starting materials. Multigram quantities of 2'-carboxy-5-chloro-2,4-dihydroxybenzophenone (5) can be prepared in 49%
yield by reacting one equivalent of phthalic anhydride (3) and 4-chlororesorcinol (4) using AlCl₃ as a catalyst. Subsequent fusion of 5 with 2-methylresorcinol using ZnCl₂ as the catalyst yields a mixture of the desired 2'-chloro-5'-methylfluorescein (6), 2',7'-dichlorofluorescein and 4',5'-dimethylfluorescein. The crude reaction mixture consists of a 4:1 mixture of the desired asymmetric product and the symmetric byproducts. The dichlorofluorescein is removed during workup, leaving a mixture containing ~90% 6. Silylation of the phenols on both fluorescein components with t-butyldimethyl silyl chloride (TBS-Cl) under standard conditions provides 2'-chloro-5'-methylfluorescein di-t-butyldimethylsilyl ether (7) in 37% yield based on NMR integration. Bromination of the mixture of silylated products under mild conditions and removal of the remaining symmetric product by flash chromatography gives 2'-chloro-5'-bromomethylfluorescein di-t-butyldimethylsilyl ether (8) in 73% yield (13% overall). Scheme 4.2 outlines the synthesis of the ligand fragment from the commercially available 2-nitrobenzyl bromide (9) using conventional methods. The overall yield for 11 is only 3% because of the low-yielding reduction of the nitro group.

The combination of fluorescein scaffold 8 and the ligand fragment 11 in the presence of AgNO₃ provides the silyl-protected final product (Scheme 4.3). Upon removal of the silyl groups with fluoride ion, pure ZP4 (14) is obtained after chromatography on reverse phase silica. Analysis of the crude product suggests a >50% conversion to ZP4 that is >80% pure. The most significant loss of product occurs during chromatography. The overall yield of ZP4 is 4% from 4-chlororesorcinol.
**Fluorescence Properties of ZP4.** Fig. 4.3 depicts the pH dependent fluorescence changes measured for ZP4. The plot of the integrated emission intensity reveals three fluorescence-related $pK_a$ values: a slight emission enhancement with a $pK_a$ of 10.0, a larger increase with a $pK_a$ of 7.2, and fluorescence quenching with a $pK_a$ of 4.0.

In buffered solution (50 mM PIPES, 100 mM KCl, pH 7) using EDTA to scavenge adventitious metal ions, ZP4 has a quantum yield of 0.06 and an emission maximum at 521 nm. Upon the addition of 25 $\mu$M Zn$^{2+}$ the quantum yield increases to 0.34 and the emission maximum shifts to 515 nm. The addition of Zn$^{2+}$ is also accompanied by a shift in the excitation maximum from 506 nm ($\varepsilon$ = $61.0 \times 10^3$) to 495 nm ($\varepsilon$ = $66.7 \times 10^3$). The brightness ($\varepsilon \times \Phi$) of the Zn$^{2+}$-bound sensor is ($22.7 \times 10^3$).

The fluorescence response of ZP4 is Zn$^{2+}$- and Cd$^{2+}$-selective. Fig. 4.4 shows the fluorescence response of ZP4 in the presence of various divalent metal ions. Even at high concentrations (2 mM) of Ca$^{2+}$ and Mg$^{2+}$ produce no appreciable change in the fluorescence emission. Fluorescence enhancement of ZP4 by Zn$^{2+}$ occurs in the presence of these two metal alkali earth metal ions. Other first row transition metals including Cu$^{2+}$, Ni$^{2+}$, Co$^{2+}$, Fe$^{2+}$ and Mn$^{2+}$ produce no discernible change in the emission intensity, but only the sample containing Mn$^{2+}$ produces a fluorescence response upon the subsequent addition of Zn$^{2+}$.

The binding affinity of ZP4 was characterized by using the dual-metal single-ligand buffer system described previously.$^{38,40}$ Varying the total Zn$^{2+}$ concentration between 0–1 mM while maintaining constant concentrations of
Ca\(^{2+}\) (2 mM) and EDTA (1 mM) provides buffered free \(\text{Zn}^{2+}\) between 0.17 and 25 nM. The quantum yield of ZP4 increases 5.7-fold upon the addition of \(\text{Zn}^{2+}\); similarly, the integrated emission intensity increases ~5-fold during the binding titration. Fig. 4.5 shows a representative titration of ZP4 and the fitting of the integrated fluorescence emission. The titration was performed four times using different \(\text{Ca}^{2+}/\text{Zn}^{2+}/\text{EDTA}\) buffers. Analysis of the response indicates that the \([\text{Zn(ZP4)}]\) complex has an apparent \(K_d\) of \(0.65 \pm 0.10\) nM.

**Structural Features of ZP4.** Attempts to crystallize the \(\text{Zn}^{2+}\) complex of ZP4 were unsuccessful, so a truncated version of the metal ion binding fragment, BPAMP, was prepared from 11 and salicylaldehyde (Scheme 4.4) in 71% yield. The \(\text{Zn}^{2+}, \text{Mn}^{2+}\) and \(\text{Cu}^{2+}\) complexes of the BPAMP ligand were crystallized from aqueous MeOH or CH\(_3\)CN in the presence of excess NaClO\(_4\). All three complexes are monomers containing a single ligand and metal ion (Fig. 4.2). The Mn and Zn compounds are isomorphous with the pentavalent ligand and a water molecule forming an octahedral complex. The Cu complex lacks the water molecule, adopting trigonal bipyramidal geometry.

Titrations of ZP4 and BPAMP in buffered solution with \(\text{Zn}^{2+}\) show a 1:1 binding by optical spectroscopy. Titration of ZP1 exhibits 2:1 ZP1:Zn\(^{2+}\) binding accompanied by changes similar to those observed for ZP4 in the absorption spectrum. The titration of ZP1 and ZP4 with \(\text{Cu}^{2+}\) and Mn\(^{2+}\) also reveals 2:1 and 1:1 binding respectively.

**Histological Staining Results.** In contrast to 6-methoxy-(8-p-toluenesulfonamido)quinoline (TSQ), which stains all the zinc-containing terminal fields in hippocampal slices (Fig 6b), slices treated with ZP4 only show intense staining of the neurons injured by prior excitotoxic insult (seizures), with
negligible labeling of zinc-containing vesicles (Fig. 4.6a). Control slices show no detectable free zinc except in presynaptic vesicles that are only stained by TSQ.

Discussion

Synthesis. The synthetic approaches to both ZP1 and ZP2 result in a symmetric ligand substitution pattern at the 4' and 5' positions of the xanthenone ring (Fig. 4.1). Computer modeling suggested that binding a single Zn\(^{2+}\) ion between ligands emanating from these two positions would be geometrically and entropically disfavored. One possible strategy for making a sensor with a single Zn\(^{2+}\)-binding site is to incorporate the metal-binding moiety on the bottom (phthalate) ring. Synthesis of bottom ring modified fluorescein derivatives from resorcinol and phthalic anhydride derivatives inevitably requires a difficult separation of structural isomers, however. Fluorescein amine is a commonly employed, commercially available bottom ring derivative; however, it is not an attractive starting material because of its high cost and the relative inertness of the amino group to chemical transformation. In addition to expense and reactivity, bottom ring synthetic strategies often require low yielding, multi-step processes that subject the fluorescein to rigorous reaction conditions and difficult purification after each manipulation. Alternatively, we envisioned adapting the method used to prepare ZP2 to access unsymmetrical fluorescein derivatives with a single zinc-binding moiety on the xanthenone ring.

An unsymmetrical fluorescein-based Ca\(^{2+}\) sensor was the inspiration for our synthetic strategy. In the conventional synthesis of fluoresceins, two equivalents of a resorcinol are allowed to react with phthalic anhydride under
rigorous conditions to give the product. Two molecules of resorcinol condense to form the xanthenone skeleton. To obtain a fluorescein with a single metal-binding arm, a strategy that employs two different resorcinols is required. Dihydroxybenzophenone derivatives like 2'-carboxy-5-chloro-2,4-dihydroxybenzophenone (5) are intermediates in the formation of fluorescein molecules. When prepared independently from 4-chlororesorcinol (4) and phthalic anhydride (3), 5 reacts with 2-methyl resorcinol to give an asymmetric fluorescein (Scheme 4.1). Analysis of the reaction product reveals minor amounts of 2',7'-dichlorofluorescein and 4',5'-dimethylfluorescein. The presence of these symmetric fluoresceins indicates that the formation of the benzophenone is to some extent reversible. A variety of methods and catalysts were screened in an attempt to eliminate the formation of these symmetric products, but the highest conversion to the desired monofunctional fluorescein was 80% under ZnCl₂ fusion conditions.⁵³

Separation of the 2'-chloro-5'-methylfluorescein (6) from the symmetric products proved to be both difficult and unnecessary. Installation of t-butyldimethyl silyl (TBS) ethers on the phenols under standard conditions provides a convenient method to purify and manipulate the desired product. Unprotected fluoresceins are notoriously difficult to handle because of their limited solubility in conventional organic solvents and their propensity to adopt different isomeric forms. Silyl ethers were selected as the protecting groups for their stability to nucleophiles by comparison to esters that were utilized previously.³⁸ The similar polarity of silylated 4',5'-dimethylfluorescein and 2'-chloro-5'-methylfluorescein di-t-butyldimethylsilyl ether (7) prohibits separation,
but following bromination the undesired product could be removed by chromatography. Although the overall yield of 2'-chloro-5'-bromomethyl-fluorescein di-i-butylidemethylsilyl ether (8) is moderately low, multi-gram quantities of this key fluorescein starting material can be obtained in several days.

A pre-assembled fluorescein starting material is an advantage for the convergent synthesis of target molecules. The primary significance of the synthesis outlined in Scheme 4.3 is the ability to modify the Zn$^{2+}$-binding ligand easily and logically to prepare future sensor candidates for screening. In addition to 2-nitrobenzyl bromide (9), a number of commercially available starting materials could serve as useful building blocks for the Zn$^{2+}$-binding fragment. Systematic variation of the ligand building block as well as the metal-binding fragment will permit correlation of structural features with fluorescence properties and the binding affinity of the probes. Another significant improvement in this synthetic method is the ability to access sensor molecules from the bromomethyl fluorescein derivative. Formation of a nitroxy compound in situ by combining 8 with AgNO$_3$ activates the benzylic position toward nucleophilic substitution. Displacement of the bromide facilitated by AgNO$_3$ activation avoids the low yielding oxidation chemistry required to prepare the dialdehyde precursor of ZP2.$^{38}$

**Fluorescence Properties of ZP4.** Under simulated physiological conditions ZP1 and ZP2 exhibit relatively intense fluorescence in the absence of Zn$^{2+}$ ($\Phi \approx 0.3$). This background emission decreases the sensitivity of these probes and interferes with the measurement of Zn$^{2+}$ concentration gradients. The
pKₐ values of the amine responsible for the fluorescence change of ZP1 and ZP2 are 8.4 and 9.4, respectively. When the amines are completely deprotonated at high pH, both probes emit a dim fluorescent signal, and exhibit a dramatic fluorescence enhancement upon the addition of Zn²⁺.³⁸ Since protonation diminishes the magnitude of the fluorescence change, adjusting the pKₐ of the nitrogen responsible for the PET quenching attenuates the fluorescence properties. Aromatic nitrogen atoms typically have lower pKₐ values (pKₐ = 4–6) than their aliphatic counterparts. In addition to having a low affinity for protons, aniline nitrogen atoms seldom form strong bonding interactions with metal ions. In order to avoid proton interference while retaining an appreciable affinity for Zn²⁺, ZP4 integrates an aniline nitrogen into a chelating ligand containing a di(2-picolyl)amine (DPA) moiety. Maintaining the delicate balance between Zn²⁺-binding affinity and proton insensitivity represents the most significant challenge in designing PET-based Zn²⁺ sensors.

In the pH dependent fluorescence changes measured for ZP4, the pKₐ at 10.0 corresponds to the protonation of the tertiary amine. Although the aniline nitrogen would be predicted to dominate PET, the aliphatic amine contributes to the quenching at basic pH. The high pKₐ value of 10.0, compared to the value of ~9 for the previous ZP sensors, indicates that the environment of the aliphatic amine in ZP4 promotes binding of a proton. The pKₐ of 7.2 measured for the aniline nitrogen provides additional evidence that advantageous H-bonding by the chelating ligand facilitates protonation. The pKₐ associated with fluorescence quenching at low pH is consistent with the formation of a nonfluorescent fluorescein isomer that was observed for ZP sensors previously.³⁸ Lowering of
the aniline pKₐ will be addressed in future sensor design. Incorporation of
electron withdrawing groups on the aniline ring, particularly the para position,
should dramatically influence the pKₐ of the aromatic nitrogen. The new
convergent synthesis should facilitate easy access to related compounds.

The lower quantum yield (0.06) of the unmetallated sensor at pH 7, a
value ~5X less than that observed for the previous generation ZP sensors,
demonstrates that the aniline nitrogen more efficiently quenches fluorescence. The
relatively low background emission from the free probe makes ZP₄ a significant
improvement over the first ZP sensors. Correlation of the Zn²⁺-induced
fluorescence enhancement with the pKₐ measurements provides evidence that
the aniline nitrogen also coordinates to the metal ion. Although the brightness of
the Zn²⁺-complexed sensor is sufficient for imaging applications, the quantum
yield of the complex is less than half the value measured for ZP₁ and ZP₂.

Possible explanations for the lower quantum yield of the Zn²⁺-complex of ZP₄
include the existence of a second unidentified quenching mechanism, or
inefficient disruption of PET from the aniline nitrogen by Zn²⁺-binding. Future
investigation of structurally related ZP derivatives may help to understand the
basis for this fluorescence behavior.

**Structural Aspects of ZP₄ and Behavior Toward Transition Metal Ions.**

The sub-nM Zn²⁺-binding affinity of ZP₄ is nearly identical to that of ZP₁ and
ZP₂. In addition to Zn²⁺-binding affinity, the hypsochromatic shift in the
absorption wavelength of ZP₄ upon metal ion binding suggests that the phenolic oxygen coordinates to the Zn²⁺ ion in solution in the same manner as the
previous two ZP sensors. The similarity of the binding strength, the
comparable absorbance and emission properties, and the use of an identical metal-binding ligand indicate that the coordination of Zn$^{2+}$ is probably similar for all three ZP complexes. The Zn$^{2+}$-induced shift in the absorbance spectrum is characteristic of binding by the phenolic oxygen. DPA has a high affinity for Zn$^{2+}$ ($K_d = 70 \text{ nM}$),$^{54}$ and the sub-nM $K_d$ requires a contribution from this ligand fragment. The increase in quantum yield indicated coordination of the aniline nitrogen that is primarily responsible for PET quenching at physiological pH. Scheme 4.5 shows our interpretation of the solution chemistry of ZP4. In the absence of Zn$^{2+}$, the aliphatic amine is protonated, giving the sensor an overall $-1$ charge. Upon binding the chelating moiety wraps around the Zn$^{2+}$ ion, with the final coordination site being occupied by a water molecule.

One of the most interesting aspects of ZP4 is the difference in the fluorescence behavior toward transition metal ions compared to properties of ZP1 and ZP2. This behavior indicates some difference in either the metal ion coordination or the electronic structure of the two generations of ZP metal complexes. Transition metal ions commonly quench the fluorescence of sensors.$^{42}$ Unlike the fluorescence of ZP1 and ZP2, which is quenched significantly by open shell metal ions, the emission of ZP4 is unchanged upon binding Mn$^{2+}$, Fe$^{2+}$, Co$^{2+}$, Ni$^{2+}$ and Cu$^{2+}$. Since subsequent addition of Zn$^{2+}$ can only displace the weakly binding Mn$^{2+}$ ion, it appears that the other transition metal ions bind tightly to the receptor of all three sensors and compete for the binding of Zn$^{2+}$. Examination of the optical spectra from the titration of ZP1 and ZP4 with Zn$^{2+}$, Cu$^{2+}$ and Mn$^{2+}$ supports this conclusion, because the only
apparent difference in the behavior of these two sensors is the stoichiometry of metal ion binding.

The crystal structure of the zinc complex of ZP1 contains two 5-coordinate Zn$^{2+}$ ions each coordinated by the three nitrogen atoms of a DPA ligand, a phenolic oxygen and a water molecule. The zinc coordination is identical to that of zinc$^{55}$ and other transition metal complexes$^{56,57}$ with truncated versions of this N$_3$O ligand. Since attempts to crystallize the Zn$^{2+}$ complex of ZP4 were unsuccessful, BPAMP was used for structural studies. The BPAMP ligand shows similar Zn$^{2+}$-binding behavior to ZP4 in buffered solution. The crystal structures of the Zn$^{2+}$, Mn$^{2+}$ and Cu$^{2+}$ complexes (Fig. 4.2) display coordination consistent with the spectroscopic measurements of ZP4, with all 5 donors from the chelating ligand bound to the metal ions. The only difference between the 3 structures is that the Cu$^{2+}$ complex is trigonal bipyramidal rather than octahedral because it lacks the coordinated water molecule found in the Zn$^{2+}$ and Mn$^{2+}$ structures. From the structural and spectroscopic experiments performed thus far, it remains unclear why the fluorescence response of ZP4 to transition metals is different from that of the first generation of ZP sensors. Investigation of the electronic structure of ZP4 complexes by DFT as well as experiments on the fluorescence properties is planned to investigate this behavior further.

These metal-binding properties have important implications in biological studies where other metal ions may interfere with Zn$^{2+}$ measurements; however, there may be no such pools of available transition metals in healthy cells. The reducing environment within cells precludes the presence of Cu$^{2+}$, which is readily reduced to Cu$^{+}$ and disproportionates to Cu metal and Cu$^{2+}$ in aqueous
solution. Current evidence argues against the presence of free copper in cells and reveals that chaperone proteins transport Cu to its cellular targets.\textsuperscript{58} Free Fe\textsuperscript{2+} readily oxidizes in aqueous solution to insoluble Fe\textsuperscript{3+},\textsuperscript{59} drastically reducing the presence of free metal ion. Such Fe species would serve as a source of oxidative stress to cells,\textsuperscript{60,61} so sequestration of Fe by macromolecules within cells seems extremely probable. Since most "free" transition metal ions seem to have a deleterious effect on living organisms,\textsuperscript{62} numerous unidentified metalloregulatory proteins may exist, even for trace metals like Co\textsuperscript{2+}. Despite the limited probability that other metal ions will interfere with biological assays, the ability to bind Zn\textsuperscript{2+} selectively in the presence of other transition metal ions remains an important goal in sensor design.

**Imaging Damaged Neurons with ZP4.** Since synaptic vesicles remain intact throughout slice preparation,\textsuperscript{63} the inability of ZP4 to stain zinc-containing presynaptic terminals indicates that this probe cannot penetrate cellular membranes. In contrast to ZP4, membrane permeable probes like TSQ and ZP1 stain these vesicles intensely. The impermeability of ZP4 may arise from either its charge at neutral pH, a dipole moment, or an inability to adopt a permeable lactone isomer.\textsuperscript{64} Both the healthy neurons and the cells damaged during seizures by the release of excitotoxic Zn\textsuperscript{2+} are cut open by the microtome blade during tissue slicing, permitting ZP4 to enter the cells. Only the damaged neurons display intense fluorescence that indicates the presence of Zn\textsuperscript{2+} when stained. Since membrane permeable probes like ZP1 and TSQ image both zinc-containing vesicles and damaged neurons, discrimination between the two is
quite difficult. To date, ZP4 provides the most well resolved, detailed images of zinc-damaged neurons using fluorescence microscopy.\textsuperscript{64}

**Conclusions**

We have designed, synthesized and characterized ZP4, a second-generation member of the ZP family of \( \text{Zn}^{2+} \)-selective sensors. ZP4 is prepared by a convergent synthesis where the fluorescein scaffold and the \( \text{Zn}^{2+} \)-binding fragment are prepared independently then linked to yield the final sensor. This new synthetic method, along with a high yielding synthesis of the key fluorescein intermediate, allows the efficient preparation of additional sensor candidates for screening. The key feature of ZP4 is the incorporation of an aniline nitrogen atom into the \( \text{Zn}^{2+} \)-binding ligand. The aniline nitrogen responsible for PET quenching of the unmetallated fluorophore has a lower \( pK_a \) than the aliphatic nitrogen of ZP1 and ZP2, making it is less sensitive to protonation under physiological conditions. Since the background fluorescence from free probe is diminished, ZP4 is more sensitive than previous ZP sensors.

**Acknowledgements**

This work was supported at MIT by grant GM65519 from the National Institute of General Medical Sciences and a grant from the McKnight Foundation for the Neurosciences. The NMR spectrometer at the MIT DCIF was purchased with support from the National Science Foundation under grant no. CHE9808061. We thank C. J. Frederickson for the images of damaged neurons and W. Bu for his assistance with crystallography.
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Table 4.1. Crystallographic Parameters for [Zn(BPAMP)(H$_2$O)](ClO$_4$)$_2$·0.5(H$_2$O) (16·0.5H$_2$O), [Mn(BPAMP)(H$_2$O)](ClO$_4$)$_2$·(CH$_3$OH)·0.5(H$_2$O) (17·CH$_3$OH·0.5(H$_2$O)) and [Cu(BPAMP)(H$_2$O)](ClO$_4$)$_2$ (18).

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$^aR = \sum |F_o| - |F_c|/\sum |F_o|$, $^bwR^2 = \{\sum[w(F_o^2 - F_c^2)^2]/\sum[w(F_o^2)]\}^{1/2}$
Table 4.2. Selected Interatomic Distances (Å) and Angles (deg) for [Zn(BPAMP)(H<sub>2</sub>O)][ClO<sub>4</sub>]<sub>0.5(H<sub>2</sub>O)</sub> <sup>16</sup>(16·0.5H<sub>2</sub>O), [Mn(BPAMP)(H<sub>2</sub>O)][ClO<sub>4</sub>]<sub>·(CH<sub>3</sub>OH)·0.5(H<sub>2</sub>O)</sub> <sup>17</sup>CH<sub>3</sub>OH·(0.5)H<sub>2</sub>O and [Cu(BPAMP)(H<sub>2</sub>O)][ClO<sub>4</sub>]<sup>18</sup>.

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Numbers in parentheses are estimated standard deviations in the last digit(s). Atom labels are provided in Figure
Table 4.3. Atomic coordinates \( (x \times 10^4) \) and equivalent isotropic displacement parameters \( (\AA^2 \times 10^3) \) for \([\text{Zn(BPAMP)}(\text{H}_2\text{O})](\text{ClO}_4)^{-0.5}\text{(H}_2\text{O}) (16 \cdot 0.5\text{H}_2\text{O})\). \( U(\text{eq}) \) is defined as one third of the trace of the orthogonalized \( U_{ij} \) tensor.

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Table 4.4. Atomic coordinates (x $10^4$) and equivalent isotropic displacement parameters (Å² $10^3$) for [Mn(BPAMP)(H₂O)](ClO₄)·(CH₃OH)·0.5(H₂O). U(eq) is defined as one third of the trace of the orthogonalized $U_{ij}$ tensor.

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Table 4.5. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (Å^2x 10^3) for [Cu(BPAMPF)(H_2O)](ClO_4). U(eq) is defined as one third of the trace of the orthogonalized U_ii tensor.

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Scheme 4.1
Scheme 4.2
Scheme 4.3
Scheme 4.4
Scheme 4.5
Figure 4.1. The first members of the ZP family of fluorescein-based Zn$^{2+}$ sensors ZP1 and ZP2. Both sensors incorporate a DPA ligand and have fluorescence properties amenable to biological studies.
Figure 4.2. ORTEP diagrams of [Zn(BPAMP)(H₂O)](ClO₄)·0.5(H₂O) (16·0.5H₂O), [Mn(BPAMP)(H₂O)](ClO₄)·(CH₃OH)·0.5(H₂O) (17·CH₃OH·(0.5)H₂O) and [Cu(BPAMP)(H₂O)](ClO₄) (18) showing 50% thermal elipsoids and selected atom labels. Solvent molecules, hydrogen atoms and perchlorate groups are omitted for clarity.
Figure 4.3. Plot of the normalized integrated emission intensity versus pH for ZP4. The $pK_a$ at 10.0 correspond to the aliphatic nitrogen and the one at 7.2 to the aniline nitrogen. At low pH fluorescein adopts a nonfluorescent isomer corresponding to the decrease in emission intensity. The quantum yield of the sensor at pH 7 is 0.06.
Figure 4.4. Fluorescence response of ZP4 to various metal ions. Bars represent the final integrated fluorescence response ($F_f$) over the initial integrated emission ($F_i$). Initial spectra were acquired in 100 mM KCl, 50 mM PIPES, 10 μM EDTA pH 7.00 at 25 °C. Excitation was provided at 500, and the emission was integrated between 503 and 635 nm. Aliquots of concentrated stock solutions (10 mM) of each metal ion were added to the solution to provide 50 μM total metal ion. Zn$^{2+}$ solution was added to the solution containing the metal ion similarly.
Figure 4.5. Fluorescence emission response of ZP4 to buffered Zn$^{2+}$ solutions. Spectra were acquired in 100 mM KCl, 50 mM PIPES, pH 7.00 at 25 °C. Excitation was provided at 500 nm with 3 nm slit widths. Emission data were corrected for the response of the detector, using the manufacturer supplied curve, and the emission data points at 500 nm, which were perturbed by scatter, has been removed for clarity. The spectra shown are for free zinc buffered at 0, 0.172, 0.424, 0.787, 1.32, 2.11, 3.34, 5.60, 10.2, and 24.1, nM respectively. For the final spectrum (containing 1 mM EDTA and 1 mM Zn$^{2+}$) additional ZnCl$_2$ was added to provide ~25 μM free Zn$^{2+}$. Inset: fluorescence response obtained by integrating the emission spectra between 503 and 635 nm, subtracting the baseline (0 Zn$^{2+}$) spectrum and normalizing to the full scale response (25 μM free Zn$^{2+}$).
Figure 4.6. Hilus of dentate gyrus stained with (a) ZP4 and (b) TSQ. The brain tissue is taken from a rat following seizure activity. The zinc-positive neurons are more distinct when labeled with ZP4 since the neuropil is not stained. The background from Zn\textsuperscript{2+}-containing vesicles that are stained by TSQ makes identification of damaged cells more difficult.
Chapter 5

Identification of Neuronal Cells Suffering Zinc Toxicity by Use of a Novel Fluorescent Sensor*
Introduction

Whereas cells viewed in situ in normal, healthy tissue virtually never display any perikaryal staining by histochemical methods for zinc,\textsuperscript{1,2} injured cells stain intensely for zinc in culture,\textsuperscript{3} acute slice preparations\textsuperscript{4,5} and in tissue harvested in vivo.\textsuperscript{6} Thus, the presence of perikaryal zinc may be taken as an indicator of cell injury.\textsuperscript{7,8}

In the case of neurons, perikaryal zinc staining is routinely observed in scattered locations after any excitotoxic insults, including stroke, ischemia,\textsuperscript{9,10} seizures,\textsuperscript{11} and blunt head trauma.\textsuperscript{4,5} Moreover, because zinc chelation before any of those insults can dramatically reduce the number of apparently injured (acidophilic, eosinophilic) neurons seen subsequently, it is widely presumed that the zinc is a major contributor to cell injury.\textsuperscript{7,12}

Zinc-injured cells were first discovered by the use of the TSQ fluorescent stain,\textsuperscript{13} and TSQ staining has remained the method of choice ever since. The use of silver methods has been tried as an alternative to TSQ, but except for one fairly difficult procedure, the silver techniques have not proved useful for staining zinc-injured cells.\textsuperscript{1,14}

TSQ has several minor and one major liability when used for the purpose of identifying zinc-injured (zinc-filled) neurons in histologic sections. Minor problems are that (i) TSQ material does not keep more than a few minutes after staining; (ii) UVB illumination is required for fluorescence; (iii) the solubility and partition coefficients for TSQ change when it binds zinc.\textsuperscript{6} The major problem is that TSQ easily penetrates all cell membranes. It therefore stains not only the perikaryal zinc in zinc-injured cells, but also the synaptic zinc that is localized
within synaptic vesicles in boutons clustered tightly all around the neuronal somata in tissue.

Therefore, in TSQ treated material, one can see zinc-injured cells that are not surrounded by zinc-rich boutons, but it is difficult if not impossible to visualize zinc-filled somata that are surrounded by equally-bright zinc-filled presynaptic boutons. This property has led to the possibly erroneous notion that zinc-injured cells only appear after the surrounding zinc-filled boutons have released their zinc.

In the present work we introduce a new zinc-responsive fluorescent probe that is easy to use and stains zinc-filled neurons brightly without staining the surrounding zinc-filled vesicles in axonal boutons. The utility of this stain for identifying and counting zinc-injured cells is demonstrated.

**Materials and Methods**

**Synthesis and Handling of ZP1 and ZP4.**\(^{15}\) The synthesis of ZP1 has been described previously.\(^{16}\) The compound is synthesized from commercially available 2',7'-dichlorofluorescein and dipicolyl amine (DPA) by a Mannich reaction. ZP1 is now available from NeuroBioTex. The preparation of ZP4 proceeds from a central asymmetrically derivativized fluorescein in a convergent fashion.\(^{17}\) Both sensors are purified by low-pressure chromatography on RP18 silica and analyzed by analytical HPLC. The chemical structures of the two probes are shown in Fig. 1.

To prevent photobleaching, the probes are routinely stored at \(< -10 \, ^\circ\text{C}\) in the dark. Although no decomposition of solid ZP1 or ZP4 has been observed
under any circumstances, we take additional precautions to guarantee their longevity. ZP1 is soluble in methanol, acetonitrile and halogenated hydrocarbons. ZP4 is only sparingly soluble in methanol and acetonitrile, and insoluble in most other common solvents. Both probes are very soluble in dimethyl formamide and dimethyl sulfoxide. We routinely prepare solutions of probes in DMSO (~1 mM) for spectroscopic studies and store them frozen at -25 °C. The desired portion of a stock solution is thawed and diluted into the buffer of choice prior to use. We have observed no decomposition of these frozen stock solutions over extended periods of time (1 year).

Measurement of ZP1 and ZP4 Spectroscopic Properties. Utrol grade PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid) from Calbiochem and KCl (99.997%) were purchased and used as received for buffer preparation. Measurements of the fluorescence and optical spectra of ZP1 and ZP4 were carried out in buffered aqueous solution (50 mM PIPES, 100 mM KCl, pH 7). Fluorescence spectra were acquired on a Hitachi F-3010 under the control of the SpectraCalc software package running on a Pentium-based PC. Excitation was provided by a 150 W Xe lamp (Ushio Inc.) operating at a current of 5 A. All spectra were normalized for excitation intensity via a rhodamine quantum counter, and emission spectra were normalized by the manufacturer-supplied correction curves. Absorption spectra were recorded on a Cary 1E scanning spectrophotometer under the control of a Pentium PC running the manufacturer supplied software. All spectra were routinely acquired at 25 °C, a temperature maintained by a circulating water bath, in 1-cm path length quartz cuvettes. Additional experimental details on the measurement of quantum yields,
dissociation constants, metal ion selectivity of the fluorescence response, and pH-dependent fluorescence changes are described elsewhere.\textsuperscript{16,17}

Tissue Preparation Procedures Following Head Trauma or Seizure. Tissue was prepared from normal rats and mice, from rats suffering prior seizures, and from rats and mice suffering prior fluid-percussion traumatic brain injury. With seizures, reported methods of administering pilocarpine by intraperitoneal injection were followed exactly,\textsuperscript{11} and the rats were allowed to survive overnight after drug administration. For the blunt head trauma, the fluid percussion methods were used.\textsuperscript{18} Briefly, rats or mice were surgically anesthetized by isoflurane inhalation, maintained in a stereotaxic head holder under deep isoflurane anesthesia, and then prepared for the fluid percussion procedure by placement of a burr-hole in the skull to which was attached a rigid, fluid-filled tube. The tube in turn was attached to a fluid-filled cylinder, in which a piston could be rapidly accelerated by the force of a swinging pendulum.\textsuperscript{18} The resulting fluid percussion to the closed calvarium was a brief (~ 10 ms) pulse of pressure at 2 atmospheres. This calibrated blunt force insult causes survivable brain damage, with zinc-induced neuronal injury.\textsuperscript{4,5} The post trauma survival interval was 6 h.

After seizure, trauma, or no treatment (control), rats were killed by an overdose of anesthesia, decapitated, and the brains were removed rapidly and frozen by burying on dry-ice "snow" for 2-3 min. Frozen brains were then mounted on the chuck of a closed-cabinet cryostat (Harris/Jung Reichert), and cut at -13 °C at anywhere from 6 to 30 µm thickness. Immediately after thawing onto clean glass slides, the sections were allowed to dry at room temperature for
0.5 to 3 h, stained, and photographed. Through all steps, care was taken to keep sections covered in dust-free containers. All use of animals was done in compliance with the spirit and letter of OLAW regulations and was approved by either the UTMB IACUC, NeuroBioTex IACUC or the UCI IACUC.

**Staining Methods.** Staining was done by applying 60 μL of staining fluid to sections lying flat in a plastic box. After 1 min, the excess reagent was washed off by gentle agitation in a 0.8% saline solution. Slides were viewed and photographed while still damp without coverslips, immediately after rinsing. To improve contrast, selected sections were also cleared by immersion in increasing concentrations of glycerine in water, specifically 30, 60, 90 and 100%. The sections were then viewed under glycerine immersion (Zeiss 25X, variable aperture) with 1.25 or 2.0-post magnification.

**Microscopy.** Images were made on a Zeiss Universal with SPOT II cooled CCD camera, using Olympus Plan-apo 10X (0.87) or Zeiss 25X (glycerine or water immersion). Illumination was achieved with a 200 W HBO high-pressure mercury lamp, using either a 360 nm or 480 nm band-pass filter, 500 nm dichroic splitter and either a long-pass or a 550 band pass emission filter. Additional images were made on a Nikon Diaphot with Odyssey confocal microscope, illuminating with 488 nm Ar laser and viewing through a 500 nm long-pass filter. In this case, sections were mounted directly on coverslips and viewed from below through the coverslip with oil immersion, using Nikon 40 and 100X objectives.

**Live Slice Handling and Imaging.** Slice manipulations including equilibration were performed in covered chambers containing 6 mL of buffer, with slices completely submerged, and protected from vigorous bubbling in the
chamber by a semipermeable nylon mesh (Millicell CM inserts; Millipore, Bedford, MA) through which small needle holes were made to facilitate solution exchange. All chamber solutions were pre-bubbled with 95% O₂ / 5% CO₂ for 30 min before slice immersion, to ensure O₂ saturation. Immediately upon vibratome sectioning, coronal slices (400 μm; the most anterior slice is discarded) were transferred to a chamber with 5 ml of cold (4°C) equilibration buffer 139 mM sucrose, 32.5 mM NaCl, 24 mM NaHCO₃, 0.5 mM CaCl₂, 1 mM NaH₂PO₄, 2.5 mM KCl, 10 mM MgSO₄, 12 mM glucose, pH 7.4), bubbled with 95% O₂ and 5% CO₂ for 30 min.

At the end of the 30 min equilibration period, slices were transferred to a room temperature buffer for 5 min and then treated for 30 min with ZP1 (20 μM in saline). For imaging of slices, we used an Olympus Fluoview confocal system equipped with an argon laser (excitation 488 nm; emission >510 nm) and a 4, 10, 20 and 40× epifluorescence objective (Olympus-America, NY, NY). Experiments were carried out in a HEPES-buffered medium (HSS) with composition: 120 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl₂, 20 mM HEPES, 15 mM glucose, 1.8 mM CaCl₂, 10 mM NaOH, pH 7.4. Slices were scanned at 250-270 μm from the slice surface.

Alternatively, live slices were prepared and incubated essentially as described above except that the thickness was varied from 200 to 400 μm, preincubation was done without the Millipore mesh and, during imaging, the slices were maintained at 34-36 °C and illumination was from either a band-pass (480 nm) filtered High-pressure mercury (200W) or the 488 nm line of an Ar laser.
RESULTS

Chemical and Optical Properties of ZP1 and ZP4. Both ZP1 and ZP4 have relatively broad absorption and emission spectra typical of fluorescein derivatives. In the absence of Zn$^{2+}$, ZP1 and ZP4 have their excitation maxima at 513 (ε = 67,100 M$^{-1}$ cm$^{-1}$) and 505 (ε = 60,900 M$^{-1}$ cm$^{-1}$), respectively, under pseudo-physiological conditions (50 mM PIPES, 100 mM KCl, pH 7). ZP1 has an emission maximum at 530 nm with a quantum yield of 0.38. ZP4 emission reaches a maximum at 520 nm with a quantum yield of 0.06.

Upon addition of Zn$^{2+}$ the excitation maxima are blue-shifted to 507 nm (ε = 77,800 M$^{-1}$ cm$^{-1}$) for ZP1 and 495 nm (ε = 66,700 M$^{-1}$ cm$^{-1}$) for ZP4. Emission wavelengths also shift slightly to 525 nm with a quantum yield of 0.87 for ZP1 and 515 nm with a quantum yield of 0.34 for ZP4.$^{16,17}$

Metal Ion Specificity of ZP1 and ZP4. Both ZP1 and ZP4 show a selective fluorescence response to nM concentrations of Zn$^{2+}$ in the presence of mM concentrations of Mg$^{2+}$ and Ca$^{2+}$. The common physiological ions K$^+$, Na$^+$, Mg$^{2+}$ and Ca$^{2+}$ produce no appreciable change in the excitation or emission of either of these two probes. In addition to these metal ions, high concentrations of glutamate (300 μM), a common neurotransmitter often found in Zn-containing vesicles, produce no effect on the fluorescence properties of ZP1 and ZP4. Other first row transition metals including Mn$^{2+}$, Co$^{2+}$, Fe$^{2+}$, Ni$^{2+}$ and Cu$^{2+}$ quench the fluorescence of ZP1, but do not affect the emission of ZP4.$^{17}$ With the exception of Mn$^{2+}$, which binds only weakly to both probes, the transition metal ions bind strongly to the receptors and cannot be displaced by Zn$^{2+}$. Of all the metal ions
screened other than Zn\(^{2+}\), only Cd\(^{2+}\) induces a positive fluorescence response in ZP1 and ZP4.

The binding affinity of these probes for Zn\(^{2+}\) has been measured by using a dual-metal single ligand buffering system.\(^{16}\) The K\(_a\) values are 0.5 nM and 0.6 nM for ZP1 and ZP4 respectively. ZP1 also has a second, lower affinity (μM) binding site; however, coordination of Zn\(^{2+}\) to the second site induces no change in the fluorescence emission at physiological pH.\(^{16}\)

**Histologic Staining Results.** ZP1 treatment produced vivid and discrete staining of all but one of the known zinc-containing terminal fields in live hippocampal slices. Both the stratum radiatum and stratum oriens were clearly defined by fluorescence, and the stratum lacunosum-molecular and cell strata (pyramidal, granulosum) were devoid of staining (Fig. 2). The region that was not apparent was the trilaminar region of the dentate molecular zone, where the inner and outer 1/3 have very light zinc-containing innervation and the middle 1/3, none. Although visible in TSQ and silver staining, this region could not be resolved in the ZP1 stain. The Olympus Fluoview confocal image of the live slice showed individual bright puncta up to 5-10 μm in size in the stratum lucidum, and just visible in the stratum radiatum (Fig 2, left panel). These undoubtedly correspond to individual axonal boutons of the giant "mossy" (stratum lucidum) and normal (radiatum) varieties.

Whereas ZP1 gave results quite similar to TSQ, ZP4 behaved quite differently (Fig. 3). This difference is most clearly seen in tissue in which injured, zinc-filled neurons had been produced by prior excitotoxic injury. The most striking result of the present work is that ZP4 only faintly stains neuropil that is
rich in zinc-containing presynaptic vesicles, i.e., bouton zinc. This effect is best appreciated by comparison of adjacent sections stained with TSQ and ZP1, both of which are lipophilic and do stain the vesicles, and with ZP4, which does not (Figs. 3, 4). In contrast to ZP4, which is membrane impermeable and did not stain zinc-containing boutons, ZP1, which is lipophilic, stained them vividly. In fact, ZP1 staining was essentially the same as that obtained with any other membrane-permeable zinc staining methods, such as TSQ, or the intravital selenium-silver methods of Danscher (Fig. 2).

In the same sections in which ZP4 only barely stained zinc-containing neuropil, it vividly stained neurons injured by prior excitotoxic insult. This result was observed in tissue from rats suffering prior seizures (Fig. 4), from rats suffering prior blunt head trauma (Fig. 3) and in tissue from mice suffering blunt trauma (Figs. 5, 6). As with all staining of "free" zinc in brain tissue, uninjured tissue showed no detectable staining anywhere except in the presynaptic vesicles (Fig 5).

Due to reduced background staining of zinc-containing boutons, the ZP4 reagent revealed some unappreciated aspects of the pattern of zinc distribution in zinc-injured neurons. First, a population of neurons lying in the subgranular (polymorph) stratum of the dentate gyrus proved to be consistently labeled in animals suffering prior excitotoxic injury. These neurons are almost always obscured in tissue prepared with TSQ, because the bouton zinc (vesicular zinc) is typically intensely fluorescent in this subgranular zone (e.g., Fig. 3).

The second set of differences observable with the ZP4 (as opposed to TSQ) concern the fine structure of staining distribution. Thus ZP4 reveals that staining extends undiminished into the dendrites of labeled cells (Figs. 5, 6). Also, ZP4
shows that the staining of the cell nucleus is especially bright in zinc-positive cells (Fig. 6).

Finally, due to the relative lack of vesicular zinc staining in the brains of both control and injured animals, one may accept the presence of zinc-stained somata in the injured, but not the control, animals as a primary difference in soma staining and not, as might be imagined, merely an artifact of reduced vesicular (background) signal in injured animals.

DISCUSSION

Chemical Properties of ZP1 and ZP4 Relevant to Imaging Studies. ZP1 and ZP4 both have similar chemical structures and optical properties, but differing imaging capabilities in biological tissues. In initial staining studies, a bright perinuclear punctate fluorescence, that was enhanced upon treatment with zinc/pyrithione, was observed upon treatment of non-neuronal cells with ZP1.19 ZP1 is not only membrane permeable, but also permeates vesicular membranes, because the staining pattern was consistent with labeling of the Golgi or an Golgi-associated vesicle.16 In contrast, cell staining using similar procedures with ZP4 shows no evidence of intracellular labeling. These observations suggest that, unlike ZP1, ZP4 is impermeable to cell membranes.

To understand these differences in permeability, preliminary experiments partitioning the two probes between aqueous buffer and 1-octanol were carried out using fluorescence and optical spectroscopy. Except at high pH (> 8), ZP1 is lipophilic, and becomes sequestered in the octanol phase. At high pH, the amines are deprotonated to afford a hydrophilic anionic species. In the presence
of Zn\(^{2+}\), ZP1 is hydrophilic and appears to be almost completely localized in the aqueous phase. The behavior of ZP4 is more complex, distributing between the two phases at physiological pH. At low pH, and as the Zn-complex, ZP4 seems to have significant lipophilicity. More quantitative experiments are in progress to define this behavior more fully.

Scheme 1 depicts our interpretation of the behavior of ZP1 as derived from all our studies to date. At physiological pH, both of the aliphatic amines are protonated, and the phenolic oxygen and carboxylate groups are deprotonated to afford a charge-neutral zwitterion. Protonation of the amines is consistent with the relatively high level of background emission, whereby the protons inhibit the photoinduced electron transfer quenching mechanism of the unmetallated fluorophore.\(^{16}\) Although neutral, the zwitterion is unlikely to be membrane-permeable because of the several charged groups in the molecule. Formation of the lactone yields a neutral ZP1 isomer containing no charged groups. In this form, the two phenols can each hydrogen bond to a dipyridylmethylamino (DPA) group, stabilizing the lactone isomer. Three isomers of fluorescein have been identified and characterized, and the lactone isomer is favored in anhydrous aprotic media.\(^{20}\) In cells and tissues it is predicted that the ring-opened zwitterion of ZP1 isomerizes to the lactone form, permitting the sensor to diffuse passively across cell or vesicular membranes. Upon exposure to Zn\(^{2+}\), the first ion binds in the high affinity site that includes the tridentate DPA arm and a phenolic oxygen atom.\(^{16}\) At high concentrations of Zn\(^{2+}\), the second low affinity site becomes occupied. The weaker binding is
attributed to the loss of the coordination of the phenolic oxygen, and repulsion between the incoming Zn\(^{2+}\) and the positively charged [Zn(ZP1)]\(^+\) complex.

Unlike ZP1, ZP4 is predicted to carry an overall negative charge under physiological conditions (Scheme 2), but the preliminary partitioning experiments suggest a more complicated explanation for the inability of ZP4 to cross cell membranes. In contrast to the symmetric DPA units flanking both the oxygen atoms of ZP1, ZP4 lacks a second ligand arm. Without a ligand to participate in a hydrogen bonding interaction with a second phenolic hydrogen, ZP4 may lack the ability to stabilize effectively a membrane-permeable lactone isomer. In addition to hydrogen bonding ability, the asymmetric structure of ZP4 affords a molecule with a greater dipole moment than the symmetric ZP1, which may contribute to its inability to cross membranes. Upon Zn\(^{2+}\)-binding, ZP4 binds a single metal ion to provide a fluorescent, charge neutral zwitterionic complex.

**Basis of the Preferential Staining of Somata.** Presumably, ZP4 fails to stain presynaptic vesicles in the frozen sections simply because it cannot penetrate them. Classical neurochemical procedures demonstrate that synaptic vesicles will remain intact and sequester their contents through tissue chopping, homogenization, and centrifugation, requiring harsh osmotic shock to rupture them.\(^{21}\) Consistent with this interpretation is that the same lack of staining is observed, for example, with the membrane-impermeable zinc fluorescent probe Newport Green (the hydrophilic salt), and with the membrane-impermeant mixture of apo carbonic anhydrase and 4-(aminosulfonyl)-7-(ethanolamino)-2,1,3-benzoxadiazole (ABD-N), which is a ratiometric zinc probe.\(^{22}\) Finally,
because ZP1 (permeable) stains the synaptic vesicles, whereas ZP4 (impermeable) does not, it seems virtually certain that the membrane permeability required for probes to enter vesicles is controlling the staining of the neuropil.

According to this interpretation, zinc-filled neurons would stain simply because they have been sliced open by the microtome blade. An interesting issue is that the thinner the section is cut, the more likely would be the staining of smaller diameter somata. Specifically, whereas even 20 μm sections should cut open most principal pyramidal neurons (20-30 μm diameter), one would want 5 μm sections to be sure of seeing the smallest neuronal somata. Furthermore, there is probably some systematic bias in our material against the successful imaging of the smallest dendrites. At only a few microns in diameter, these would remain largely uncut and be accessible to stain molecules only by diffusion from other sectioned surfaces of the cell. In short, the stained dendritic neuropil observed in our sections may still be an underrepresentation of the true total density of zinc-filled dendrites.

Source and Amount of the Zinc in Somata. The present ZP4 methods give no quantitative data, but other work suggests that zinc-injured cells found after excitotoxic insult may contain in the range of 0.5 to 2.0 μM of "free" zinc. There appears to be at least two sources of this zinc. First, zinc released from nearby zinc-containing presynaptic terminals can penetrate into the postsynaptic neurons through a variety of zinc-permeable channels, including the Ca-AMPA kainate, NMDA, and L-type calcium channels. In normal physiological function, this transcellular translocation of zinc ions is implicated
as a key factor in synaptic plasticity.\textsuperscript{25,26} In addition to transynaptic translocation, zinc can also be liberated from within the postsynaptic neuron by oxidation and/or nitrosylation of thiol ligands in residues (histidine, cysteine) that coordinate zinc.\textsuperscript{27}

How much zinc is contributed by these two sources is not yet resolved, but, it is well-established that the excess zinc in the cytosol is cytotoxic, or cytolethal. This conclusion is based on the oft-repeated observation that the number of apparently injured neurons, eosinophilic or acidophilic neurons, found after excitotoxic injury is greatly reduced by the simple expedient of administering a zinc chelator before the insult.\textsuperscript{28,29}

**Comparison of Zinc Sensors in the ZP Family.** Although ZP4 has advantages for identifying cells undergoing zinc-induced toxicity, ZP1 has proved to be useful for rendering the zinc-filled vesicles visible in living as well as histological sections. Unlike TSQ, which changes solubility and partition coefficient when complexed with zinc,\textsuperscript{6} or its hydrophilic TSQ congener TFLZn, which slowly changes from ionized to nonionized (i.e., lipophilic) form in solution, the ZP probes appear to be stable and little altered in solubility or partition coefficients by zinc binding. This property makes ZP1 a good probe for detecting and visualizing vesicular zinc. In passing, one should note that the traditional "intracellular" probes, such as Newport Green or Mag-Fura 2, cannot be used to render vesicles visible, because these probes are metabolized to ionized forms (i.e., "trapped") in the cytosol before they can penetrate into vesicles. Thus, ZP1 is uniquely useful for staining zinc in vesicles or other similar secretory granules. Because the weakly bound zinc in vesicles is probably in the
low mM concentration range, a ZP1 variant with a lower stability constant, such as, $K_d = 10^4$, would perhaps be even more useful.

**Conclusions**

We have devised a new methodology for imaging Zn$^{2+}$-injured neurons in mice and rats following seizures and head trauma using the new zinc-specific fluorescent sensor ZP4. In contrast to staining with TSQ, the current state-of-the-art technique for such imaging, ZP4 is particularly successful in imaging Zn$^{2+}$-injured neurons because it does not stain intact Zn$^{2+}$-containing synaptic vesicles in addition to the damaged cells. The unique ability ZP4 to image the injured neurons selectively is attributed to its inability to cross cell membranes, preventing staining of the Zn$^{2+}$-containing vesicles. In addition to ZP4 methodologies, we report the use of ZP1 to image Zn$^{2+}$-containing synaptic vesicles. ZP1 and TSQ are both lipophilic and therefore similar in inability to image Zn$^{2+}$-damaged neurons selectively; however, ZP1 is particularly effective in imaging vesicular zinc because its physical properties appear relatively unchanged upon Zn$^{2+}$-binding.

ZP1 and ZP4 are the first in a family of Zn$^{2+}$-specific fluorescent probes being developed for studying the neurochemistry of zinc. In addition to elaborating on the imaging studies describe here with these existing probes, we are developing new sensors with improved optical properties as well as binding affinities suitable for measuring changes in Zn$^{2+}$ concentration associated with neurological events.
Acknowledgement. This work was supported at MIT by grants from the McKnight Foundation for the Neurosciences and GM65519 from the National Institutes of General Medical Sciences.
References

(*) A slightly altered version of this work has been submitted for publication. Frederickson, C. J.; Burdette, S. C.; Frederickson, C. J.; Sensi, S. L.; Weiss, J. H.; Balaji, R. V.; Bedell, E.; Prough D. S.; Lippard, S. J. 2002. The imaging experiments were carried out by the listed collaborators.


(15) ZP1 and ZP4 are abbreviations for the Zn-specific fluorescent sensor Zinpyr-1 and Zinpyr-4 respectively.


Scheme 5.1
Scheme 5.2
**Figure 5.1.** Chemical structures of ZP1, ZP4 and TSQ. ZP is short for Zinpyr, a name given not only for the pyridine groups but for their ability to "peer" into the concentration of Zn$^{2+}$ in biological specimens. TSQ is 6-methoxy-(8-$p$-toluenesulfonamido)quinoline.
Figure 5.2. ZP1 staining of live hippocampal slices. Right panel is a panorama (4X objective; Olympus IX 50) illustrating the staining of all the cytoarchitectonic regions innervated by zinc-containing axons, including the hilus (h), str. Oriens (o), and str. radiatum (r). Left panel shows the border of the str. lucidum and radiatum from a similar slice imaged in confocal microscopy (40X, Nikon). Note the large distinct puncta (giant mossy boutons) in str. lucidum (arrows and inset). Smaller fluorescent puncta are just visible in radiatum.
Figure 5.3. Hilus of dentate gyrus stained with TSQ (top), ZP4 (middle) and ZP1 (bottom). Each hilus is a 20 μm frozen section from a rat suffering traumatic brain injury. Zinc-positive neurons are visible in all 3 sections (arrows), but only ZP4 stains the neurons preferentially, making detection of the cells easier and more reliable.
Figure 5.4. ZPA staining of the dorsal neocortex of a mouse suffering prior traumatic brain injury. Note the absence of stained neurons in a region of relatively less damaged cortex and the numerous stained neurons in a region extensively damaged (note shredding of the tissue on the left).
Figure 5.5. Enlargement of the cortex shown in the left panel of Fig 5.4.

Note that scattered individual neurons are brightly stained, whereas background (other neurons, glia, neurpil) is completely unstained.
Figure 5.6. MIB staining of 30 nM nevirapine-treated cultures. Left panel shows unstimulated control cells at 24 hours post-treatment. Right panel shows similar control untreated cultures at 24 hours post-treatment.
Chapter 6

Further Applications of ZP Sensors:

Two Photon Microscopy* and Surface Attachment Strategy
Introduction

In order to facilitate studies of Zn$^{2+}$ in neurobiology, we have designed and reported on a new family of Zn$^{2+}$-sensitive fluorescent probes. Compounds ZP1 (1), ZP2 (2), and ZP4 (3) are members in a series of fluorescein-based sensors designed to induce a positive fluorescence response upon complexation of Zn$^{2+}$ (Fig. 6.1). The ZP family offers several advantages over traditional Zn$^{2+}$ sensors, but additional improvements in the fluorescence properties of these probes are desirable. Although altering the chemical nature of existing sensors remains an important goal, the development of methodologies for employing these sensors in investigations of biological systems is also a high priority.

In pursuit of the latter goal we have examined the fluorescence properties of ZP sensors by two photon excitation (TPE) techniques. In two-photon microscopy (TPM), two photons of lower energy are used to excite a fluorophore into an excited state. TPM has several unique advantages over one photon microscopy including reduction of photodamage to living tissues, improvement in spatial resolution and sensitivity, and the ability to image thick specimens. In addition to investigating improved instrumental techniques for study, we were interested to investigate whether a Zn$^{2+}$ sensor could be fastened to a membrane or other surface. Such a material could be a powerful investigative tool. We therefore devised a means to attach a ZP derivative to a solid support by a nucleophilic aromatic substitution reaction of fluorine. Accordingly ZPF1 a new member of the ZP family that is structurally related to ZP1 and ZP2, has been
prepared and characterized. Both the synthesis of ZPF1 and TPM work are described in the present chapter.

**Experimental**

**Materials and Methods.** CDCl$_3$ was dried over 3 Å molecular sieves. 3,4,5,6-Tetrafluoro-2',7'-dichlorofluorescein (12) was prepared as previously described. All other reagents were purchased and used as received. NMR spectra were recorded on a Varian 500 MHz or a Mercury 300 MHz spectrometer at ambient probe temperature, 283 K, and referenced to the internal $^1$H and $^{13}$C solvent peaks. Infrared spectra were recorded on an Avatar 360 FTIR instrument as KBr pellets. Electrospray ionization (ESI) mass spectrometry was performed in the MIT Department of Chemistry Instrumentation Facility (DCIF) with the use of m-nitrobenzyl alcohol as the matrix.

9-(3,4,5,6-Tetrafluoro-2-carboxyphenyl)-2,7-dichloro-4,5-bis[bis(2-pyridylmethyl)-aminomethyl]-6-hydroxy-3-xanthanone (ZinpyrF-1, ZPF1, 5). Di(picolyl)amine (DPA, 4.00 g, 20.1 mmol) and paraformaldehyde (602 mg, 20.1 mmol) were combined in 60 mL of CH$_3$CN and refluxed for 530 min. 3,4,5,6-Tetrafluoro-2',7'-dichlorofluorescein (4, 3.00 g, 6.34 mmol) in 90 mL of CH$_3$CN/H$_2$O (1:1) was added to the solution and the reaction mixture was refluxed for 36 h. The CH$_3$CN was removed and the product was extracted into CH$_2$Cl$_2$. The organic extract was washed once with saturated brine, dried over MgSO$_4$, filtered and the solvents were removed to yield a dark red syrup. The product was dissolved in a minimal amount of boiling EtOH and precipitated at -25 °C. The precipitate was collected, washed thoroughly with ice cold EtOH
and ice cold water and dried to afford a salmon pink solid (825 mg, 14.5%). Additional crops of product were collected to provide a total yield of 29.6% (1.68 g). $^1$H NMR (CDCl$_3$, 300 MHz) $\delta$ 4.01 (8 H, s), 4.17 (4 H, s), 6.80 (2 H, s), 7.20 (4 H, t, $J = 5.1$ Hz), 7.35 (4 H, d, $J = 7.5$ Hz), 7.67 (4 H, td, $J = 1.5$, 7.5 Hz), 8.59 (4 H, d, $J = 4.8$ Hz). $^{19}$F NMR (CDCl$_3$, 300 MHz) $\delta$ 26.33 (1 F, t, $J = 22.5$ Hz), 33.99 (1 F, t, $J = 19.2$ Hz), 34.37 (1 F, s), 38.14 (1 F, m). $^{13}$C NMR (CDCl$_3$, 125 MHz) $\delta$ 49.21, 59.20, 107.11, 110.41 (d, $J = 12$ Hz), 112.34, 118.25, 122.71, 123.16, 123.41. 126.56, 134.06 (d, $J = 13$ Hz), 137.38, 140.54, 141.28 (t, $J = 14$ Hz), 142.55 (d, $J = 8.5$ Hz), 142.98 (d, $J = 10$ Hz), 143.34 (t, $J = 14$ Hz), 144.74 (t, $J = 14$ Hz), 145.12 (d, $J = 11$ Hz), 146.74, 146.83, 147.73, 148.27, 148.82, 156.81, 157.62, 162.74. FTIR (KBr, cm$^{-1}$) 3426, 3604, 3013, 2923, 2858, 2613, 1779, 1624, 1591, 1516, 1501, 1473, 1435, 1287, 1216, 1194, 1114, 1023, 764. HRMS (ESI): Calcd for MH$^+$, 895.1826; Found 895.1831.

**General Spectroscopic Methods.** Ultrol grade PIPES (piperazine-$N,N'$-bis(2-ethanesulfonic acid)) from Calbiochem and KCl (99.997%) was purchased and used as received. All solutions were passed through 0.2-$\mu$M cellulose filters before measurements. Except for the fluorescence titration experiment, Zn solutions were prepared by the addition of appropriate amounts of 1.0 M, 100 mM, 10 mM or 1 mM Zn$^{2+}$ stocks that were checked by atomic absorption spectroscopy for concentration accuracy, or by titration with terpyridine and measurement of the absorption spectra. The titration was performed by treating a 100 mM solution of 2,2':6',2"-terpyridine in buffered solution (50 mM PIPES, 100 mM KCl, pH 7) with aliquots of 10 mM (nominal) ZnCl$_2$ and determining the equivalence point by monitoring the absorbance of the resulting complex at 321 nm ($\varepsilon = 35.9 \times 10^3$ M$^{-1}$ cm$^{-1}$). The Zn$^{2+}$ stocks were prepared from 99.999% pure
ZnCl$_2$. The purity of the ZP probes was verified by HPLC. ZP was introduced to aqueous solutions by addition of a stock solution in DMSO (1 mM). Graphs were manipulated and equations calculated by using Kaleidagraph 3.0. The pH values of solutions were recorded with an Orion glass electrode that was calibrated prior to each use. The experiments for measuring the pH-dependent fluorescence, quantum yield, $K_a$, metal ion selectivity were performed as previously described.$^1$

**UV-Visible Spectroscopy.** Absorption spectra were recorded on a Hewlett Packard 8453A diode array spectrophotometer under the control of a Pentium II-based PC running the Windows NT ChemStation software package, or a Cary 1E scanning spectrophotometer under the control of a Pentium PC running the manufacturer supplied software package. Spectra were routinely acquired at 25 °C, maintained by a circulating water bath in 1 cm path length quartz cuvettes with a volume of 1.0 or 3.5 mL.

**Fluorescence Spectroscopy.** Fluorescence spectra were recorded on a Hitachi F-3010 spectrofluorimeter under the control of a Pentium-based PC running the SpectraCalc software package. Excitation was provided by a 150 W Xe lamp (Ushio Inc.) operating at a current of 5 A. All spectra were normalized for excitation intensity via a rhodamine quantum counter, and emission spectra were normalized by the manufacturer-supplied correction curves. Spectra were routinely acquired at 25 °C, maintained by a circulating water bath in 1 cm × 1 cm quartz cuvettes using 3 nM slit widths and a 240 nm/min scan speed. Fluorescence emission measurements were also acquired in a 1 cm × 1 cm quartz cell using a Spex Fluorolog-2 instrument with 1-nm bandwidth slits. All spectra
were corrected for emission intensity by using the manufacturer-supplied photomultiplier curves.

**General Two Photon Microscopy Methods.** Ultrol grade PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid)) from Calbiochem and KCl (99.997%) were purchased and used as received. Zn was added from concentrated stock solutions of 100 mM, 10 mM or 1 mM ZnCl that were checked by atomic absorption spectroscopy for concentration accuracy. The Zn$^{2+}$ stocks were prepared from 99.999% pure ZnCl$_2$. Samples were prepared on glass microscope slides with a 200 µL sample well, and covered with glass coverslips. All measurements were carried out in buffered solution (50 mM PIPES, 100 mM KCl, pH 7). ZP probes were added from concentrated stock solutions in DMSO. Measurements were carried out at 65 µM probe.

**Cell Preparation.** MCF-7 cells were plated onto Lab-Tek chambered coverglasses and grown to 60-80% confluence at 37 ºC in DMEM supplemented with 10% v/v fetal bovine serum and 2 mM L-glutamine. For labeling, the cells were washed once with PBS, then incubated with 5 µM fluorophore in HBSS at 25 ºC for 0.5 h. Prior to imaging, the labeling solution was aspirated and the cells were washed 3 times with PBS.

**Two-photon Microscopy.** The two-photon excitation was provided by a femtosecond titanium-sapphire laser (Tsunami, Spectra-Physics, Mountain View, CA) pumped by a 5 W diode-pumped, solid-state continuous wave (CW) laser (Millennia, Spectra-Physics, Mountain View, CA). The microscope system is comprised of a modified inverted microscope (Axiovert 100TV, Zeiss, Thornwood, NY), a 40× objective lens (Zeiss F Fluar, NA 0.13), and an objective
microscope (model p-721 PIFOC, Physik Instrumente, Germany). X-Y scans are achieved using a scanner unit (Cambridge Technology, Watertown, MA), and photon pulses from the photomultiplier are discriminated against dark noise with an amplifier-discriminator unit (model F-100T, Advanced Research Instrument Corporation, Boulder, CO).

Results and Discussion

Two-Photon Microscopy. Under simulated physiological conditions (50 mM PIPES, 100 mM KCl, pH 7) ZP1, ZP2 and ZP4 undergo efficient TPE between 740-900 nm with an excitation maximum at 780 nm (Fig. 6.2). The ratio of the emission intensity of the bound:unbound sensor reaches a maximum at 820 nm for ZP2 and ZP4 and 860 nm for ZP1 (Fig. 6.3). Changing the ratio of Zn$^{2+}$ to ZP1 in solution modulates the TPE of the sensor. At a ZP1 concentration of 65 μM, the emission intensity increases as the Zn$^{2+}$ concentration increases from 0.1 to 10 μM (Fig. 6.4). Very little change in emission intensity accompanies the concentration change between 0 and 0.1 equiv of Zn$^{2+}$, although, the fluorescence lifetime (τ) increases from 1.61 to 1.77 ns. The lifetime also changes with 1 equiv (2.41) and 10 equiv (2.59 ns) of Zn$^{2+}$ in solution. Simultaneous measurement of emission intensity and fluorescence lifetime offers the possibility to measure accurately [Zn$^{2+}$] gradients in living systems. Fig. 6.5 depicts the images ZP1 in the absence and presence of Zn$^{2+}$ in MCF-7 cells. Panel (a) shows cells treated with ZP1. Panel (b) shows ZP1 treated cells after the addition of Zn$^{2+}$/pyrithione. Panel (c) shows cells after subsequent treatment with TPEN. These observations are similar to those made with one photon confocal
microscopy in COS-7 cells,\textsuperscript{1} and demonstrate the feasibility of using TPM to image Zn\textsuperscript{2+} in biological systems. Accurate measurement of Zn\textsuperscript{2+} concentration requires additional characterization of the TPE properties of ZP1 in solution.

**Synthesis and Characterization of ZPFI.** A strategy to attach a Zn\textsuperscript{2+} sensor to an external surface offers the possibility to construct fluorescent beads for applications in diagnostic medicine or to modify the outer membranes of neurons for investigation into synaptic Zn\textsuperscript{2+} release. Construction of all the ZP sensors involves installation of the metal-binding ligand on the xanthenone (top) ring system. Given the design of these sensors, the phthalate (bottom) ring provides the ideal site for attaching a tether to an external surface. Previous studies demonstrate that the fluorine in the 5-position of 3,4,5,6-tetrafluorofluoresceins undergoes nucleophilic displacement under mild conditions (Scheme 6.1).\textsuperscript{6}

The synthesis of ZPFI (5) parallels the Mannich reaction conditions used to prepare ZP1 (Scheme 6.2),\textsuperscript{1} using the 3,4,5,6-tetrafluoro-2',7'-dichlorofluorescein (4). The preparation of ZPFI is straightforward and requires no elaborate purification. Washing the crude product with cold EtOH and H\textsubscript{2}O provides product that is over 95% pure.

Under simulated physiological conditions (50 \textmu M PIPES, 100 mM KCl) at pH 7 in the presence of EDTA to scavenge adventitious metal ions, ZPFI has a quantum yield of 0.11. The quantum yields increase to 0.55 in the presence of 25 \textmu M Zn\textsuperscript{2+}. The excitation maximum shifts from 533 nm (\(\varepsilon = 98.8 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}\)) to 525 nm (\(\varepsilon = 119.2 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}\)) upon Zn\textsuperscript{2+} complexation. ZPFI undergoes a slight hypsochromic shift in the absorption wavelength indicative of
coordination of the donor group (phenol) to Zn\(^{2+}\) in the same manner as ZP1 and ZP2. ZPF1 also exhibits a Zn\(^{2+}\)-specific fluorescence response to divalent metal ions that is identical to ZP1 and ZP2. Ca\(^{2+}\) and Mg\(^{2+}\) concentrations as high as 5mM produce no change, and other first row transition metal ions including Cu\(^{2+}\), Ni\(^{2+}\), Co\(^{2+}\), Fe\(^{2+}\) and Mn\(^{2+}\) quench the fluorescence (Fig. 6.6). Only the sample containing Mn\(^{2+}\) produces a fluorescence response upon the subsequent addition of Zn\(^{2+}\) demonstrating that the other transition metal ions bind tightly to ZPF1.

The binding affinity of ZPF1 was characterized by using the dual-metal single-ligand buffer system described previously.\(^1,4\) Varying the total Zn\(^{2+}\) concentration between 0–1 mM while maintaining constant concentrations of Ca\(^{2+}\) (2 mM) and EDTA (1 mM) provides buffered free Zn\(^{2+}\) between 0.17 and 25 nM. The integrated emission intensity increases ~6-fold during the binding titration. Fig. 6.7 shows a representative titration of ZPF1 and the fitting of the integrated fluorescence emission. The titration was performed in triplicate using different Ca\(^{2+}\)/Zn\(^{2+}\)/EDTA buffers. Analysis of the response indicates that the [Zn(ZP4)] complex has an apparent \(K_d\) of 0.9 ± 0.10 nM.

The major difference in the properties between ZPF1 and the previous generation of ZP sensors is the effect of protons on the emission. Three pH-dependent fluorescence changes are observed having \(pK_a\) values of 6.7, 3.9 and 1.2 (Fig. 6.8). The value of 1.2 corresponds to the formation of a nonfluorescent fluorescein isomer, a phenomenon observed for ZP1, ZP2 and ZP4 at \(pK_a\) values of 2.8, 3.9, 4.0 respectively.\(^1,2,4\) Unlike ZP1 and ZP2, which only have one \(pK_a\) value associate with fluorescence enhancement, ZPF1 shows a 2-step increase in emission with \(pK_a\) values of 6.8 and 3.9. This incremental increase corresponds
to the stepwise protonation of the two aliphatic amine atoms of the DPA arms. The pKₐ values for the two amine atoms in ZP1 and ZP2 are similar in value and therefore appear as a single pH-induced fluorescence change. More interestingly is that the fluorescence enhancement of ZPF1 occurs at significantly lower pKₐ value than ZP1 and ZP2. The primary shortcoming of the previous ZP sensors is their sensitivity to protons. The decreased susceptibility of ZPF1 to protonation is significant advancement in the properties of ZP sensors. Since the only difference in the structure of ZP1 and ZPF1 is the fluorination of the phthalate ring, it appears that there is a through-space influence on the pKₐ value of the amine atoms.

Conclusions

The use of two-photon microscopy has been explored with the ZP compounds. Initial results indicate TPM to be a viable technique for measuring [Zn²⁺] induced fluorescence changes in solution and living cells. Complete characterization of the two-photon fluorescence properties of ZP1 and its utility in biological imaging are ongoing. Attempts to fasten ZPF1 to a surface have been initiated as well.

Acknowledgement. This work was supported at MIT by the McKnight Foundation for the Neurosciences and grant GM65519 from the National Institutes of General Medical Sciences. We thanks Olga Burenkova for preparing the cells for imaging.
References

(*) Two-Photon Microscopy was carried out in collaboration with Dr. Michael Previte and Professor Peter T. C. So in the M.I.T. Department of Mechanical Engineering. Cells were prepared by Olga Burenkova.


Scheme 6.1
Scheme 6.2
Figure 6.1. Structures of the fluorescein-based fluorescent sensors for Zn$^{2+}$ ZP1 (1), ZP2 (2) and ZP4 (3).
Figure 6.2. Emission intensity of ZP1, ZP2 and ZP4 (65 μM) as a function of two photon excitation at 740, 780, 820, 860 and 900 nm. A ~2-fold enhancement is observed in the presence of 1 equivalent of Zn$^{2+}$ (65 μM).
Figure 6.3. Ratio of the emission intensity of ZP sensors (65 μM) with the corresponding Zn$^{2+}$ complexes ([Zn$^{2+}$] = 650 μM) with two photon excitation at 740, 780, 820, 860 and 900 nm.
Figure 6.4. Fluorescence emission response of ZP1 (65 μM) to 0.1 (6.5 μM), 1 (65 μM), and 10 (650 μM) equivalents of Zn^{2+} with two photon excitation at 740, 780, 820, 860 and 900 nm.
Figure 6.5. (a) Two photon microscopy images of MCT-7 cells labeled with 5 µM ZPI for 15 min at 25 °C. (b) The bright perinuclear punctate staining is enhanced by the addition of 50 µM Zn²⁺ pyrithione and is reversible upon addition of 25 µM TPEN (c).
Figure 6.6. Fluorescence response of ZPF1 to various metal ions. Bars represent the final integrated fluorescence response ($I_f$) over the initial integrated emission ($I_o$). Initial spectra were acquired in 100 mM KCl, 50 mM PIPES, 10 μM EDTA pH 7.00 at 25°C. Excitation was provided at 525, and the emission was integrated between 500 and 650 nm. Aliquots of concentrated stock solutions (10 mM) of each metal ion were added to the solution to provide 50 μM total metal ion. Zn²⁺ solution was added to the solution containing the metal ion similarly.
Figure 6.7. Fluorescence emission response of ZPF1 to buffered Zn\(^{2+}\) solutions. Spectra were acquired in 100 mM KCl, 50 mM PIPES, pH 7.00 at 25 °C. Excitation was provided at 524 nm with 3 nm slit widths. Emission data were corrected for the response of the detector, using the manufacturer supplied curve. The spectra shown are for free zinc buffered at 0, 0.172, 0.424, 0.787, 1.32, 2.11, 3.34, 5.60, 10.2, and 24.1, mM respectively. For the final spectrum (containing 1 mM EDTA and 1 mM Zn\(^{2+}\)) additional ZnCl\(_2\) was added to provide ~25 μM free Zn\(^{2+}\). Inset: fluorescence response obtained by integrating the emission spectra between 500 and 635 nm, subtracting the baseline (0 Zn\(^{2+}\)) spectrum and normalizing to the full scale response (25 μM free Zn\(^{2+}\)).
Figure 6.8. Plot of the normalized integrated emission intensity versus pH for ZPF1. The \( pK_a \) values at 6.8 and 3.9 correspond protonation of the two nitrogen atoms. At low pH fluorescein adopts a nonfluorescent isomer corresponding to the decrease in emission intensity. The quantum yield of the sensor at pH 7 is 0.11.
Chapter 7

The Rhodafuor Family, an Initial Study of Potential Ratiometric Fluorescent Sensors for Zn$^{2+}$
Introduction

Studying the function of free Na\textsuperscript{+}, K\textsuperscript{+}, Ca\textsuperscript{2+}, Mg\textsuperscript{2+} and Zn\textsuperscript{2+} in biological systems can be challenging because these metal ions lack spectroscopic properties. Their investigation can be facilitated by the use of fluorescent probes\textsuperscript{1-3} however. Fluorescence-based sensors consist of a receptor capable of selectively binding the species of interest, linked to a fluorophore that undergoes a change in optical emission upon analyte binding\textsuperscript{4}. Since the development of efficient synthetic techniques is required to access desired sensor molecules, organic chemistry plays a crucial role in the service of biological application.

The most common class of fluorescent sensors for metal ions is based on photoinduced electron transfer (PET) quenching mechanisms. PET sensors consist of a receptor covalently linked to a fluorophore. In the absence of analyte, electrons localized on a donor station of the receptor participate in back ET with the excited state of the fluorophore, quenching emission. Upon complexation of a metal ion, the electronic structure of the receptor changes, interrupting PET and restoring fluorescence\textsuperscript{4}. Such "off-on" fluorescent probes are often employed as biosensors because typically they are easy to prepare, emit an intense fluorescent signal, and have well understood photophysics.

In an effort to study the function of Zn\textsuperscript{2+} in neurobiology, we have designed and reported on a series of Zn\textsuperscript{2+}-specific PET sensors\textsuperscript{5-8}. ZP1, ZP2 and ZP4 are members in a series of fluorescein-based sensors designed to induce a positive fluorescence response upon Zn\textsuperscript{2+} complexation. Although these dyes offer several advantages over traditional Zn\textsuperscript{2+} sensors, they also illustrate the
challenges of using PET-based probes for biological imaging. Metal-binding atoms in the receptor that are responsible for PET quenching can be susceptible to proton-induced fluorescence under physiological conditions. The magnitude of ZP sensor fluorescence response to Zn$^{2+}$ is greater than to protons, but background fluorescence from protonated probe interferes with quantitation of zinc concentration. Ideally, the fluorescence of PET sensors is independent of pH, the probes being non-emissive in the absence of analyte. Without a fluorescence signal from free probe, however, monitoring the distribution of sensors in cells is difficult. As with ZP1 and ZP2, which tend to localize in vesicles, sensors may disperse unevenly in a dynamic environment like biological tissue because of their chemical properties. Imaging and measurements may therefore reflect the distribution of the free probes rather than analyte.

One possibility to circumvent the obstacles associated with intensity probes is to alter the sensing strategy. Integration of a donor group of the receptor into π-system of fluorophore produces sensors that undergo a spectral shift in the excitation and/or emission wavelength upon metal ion binding. The key mechanistic feature of such ratiometric probes is the decoupling of donor electrons from resonance with the fluorophore, an event that changes the electronic structure. Several ratiometric sensors for Ca$^{2+}$ exist, yet only a handful of Zn$^{2+}$ probes have been reported, none of which is based on a fluorescein scaffold. A ratiometric Ca$^{2+}$ sensor utilizing a hybrid fluorescein and rhodamine fluorophore was proposed originally and reported previously. Although this sensor was successfully obtained, the laborious synthesis is unattractive and lacks versatility. In the present work, we describe methodology
for preparing fluorescein/rhodamine hybrid sensors by applying aryl-
azamacrocycle coupling$^{14}$ to make a first generation ratiometric sensor for Zn$^{2+}$.

**Experimental Section**

**Materials and Methods.** Acetonitrile, dichloromethane, dichloroethane
(DCE), and triethylamine were distilled from CaH$_2$ under nitrogen. Chloroform
was passed through a column of basic aluminum oxide, and dried over 3 Å
molecular sieves. Toluene was distilled from Na/benzophenone ketyl. Methanol
was distilled from Mg/I$_2$. CDCl$_3$ was dried over 3 Å molecular sieves. 2’-
Carboxy-5-chloro-2,4-dihydroxybenzophenone (4) was prepared as previously
described.$^6$ All other reagents were purchased and used as received. Flash
column chromatography was performed with silica gel-60 (230-400 mesh) or
Brockman I activated basic aluminum oxide (150 mesh). Thin layer
chromatographic (TLC) analysis was performed with Merck F254 silica gel-60 or
Merck F254 aluminum oxide-60 plates and viewed by UV light or developed
with ceric ammonium molybdate, ninhydrin or iodine stain. NMR spectra were
recorded on a Varian 500 MHz or Mercury 300 MHz spectrometer at ambient
probe temperature, 283 K, and referenced to the internal $^1$H and $^{13}$C solvent
peaks. Infrared spectra were recorded on a BTS 135 or an Avatar 360 FTIR
instrument as KBr pellets or thin films on NaCl plates. Electrospray ionization
(ESI) mass spectrometry was performed in the MIT Department of Chemistry
Instrumentation Facility (DCIF) with the use of m-nitrobenzyl alcohol as the
matrix.
**N,N-Bis(2-pyridylmethyl)-m-anisidine (2).** Picolylchloride hydrochloride (12.0 g, 73.4 mmol) was dissolved in 3 mL of water, and 18 mL of 5 N NaOH was added to give a pink solution. An additional 18 mL of 5 N NaOH was added to the vigorously stirred solution, after m-anisidine (1, 1.8 mL, 16.0 mmol) was combined with the solution of picolylchloride. An aliquot of cetyltrimethylammonium chloride (250 μL, 25 wt. % in water) was added as a phase transfer catalyst (PTC), and the reaction was stirred vigorously under Ar. Additional picolylchloride hydrochloride (5.6 g, 34.3 mmol) was added to the solution after 48 h, and after 144 h, an additional portion of picolylchloride hydrochloride (10.0 g, 61.2 mmol) and 15 mL of 5 N NaOH. After a total reaction time of 11 d, the product was extracted into CH₂Cl₂ and dried over MgSO₄, to give a brown solid after solvent removal. Flash chromatography on basic alumina with a solvent gradient (24:1→4:1 CH₂Cl₂/EtOAc) yielded the product as a yellow solid (1.72 g, 35.2%). TLC Rf = 0.33 (4:1 CH₂Cl₂/EtOAc). ¹H NMR (CDCl₃, 500 MHz) δ 3.69 (3 H, s), 4.85 (4 H, s), 6.27 (1 H, t, J = 2.5 Hz), 6.30 (2 H, tt, J = 2.0, 7.5 Hz), 7.08 (1 H, t, J = 8.0 Hz), 7.17 (2 H, t, J = 5.0 Hz), 7.31 (2 H, d, J = 7.5 Hz), 7.67 (2 H, t, J = 7.5 Hz), 8.61 (2 H, dd, J = 1.0, 5.0 Hz). ¹³C NMR (CDCl₃, 125 MHz) δ 55.23, 57.5, 99.26, 102.29, 105.75, 120.94, 122.20, 130.19, 137.01, 148.88, 158.93, 160.94. FTIR (thin film, cm⁻¹) 3418, 1612, 1591, 1500, 1471, 1434, 1346, 1264, 1201, 1168, 755. HRMS (ESI): Calcd for MH⁺, 306.1606; Found 306.1606.

**N,N-Bis(2-pyridylmethyl)-3-aminophenol (3).** A solution of N,N-bis(2-pyridylmethyl)-m-anisidine (2, 500 mg, 1.64 mmol) in 20 mL of CH₂Cl₂ was frozen with liquid N₂, and 50 mL of 1.0 M BBr₃ (50 mmol) in CH₂Cl₂ was added via a cannula. The solution was allowed to warm slowly to room temperature
and stirred under Ar for 40 h. The reaction mixture was chilled to \(-40^\circ C\) by an isopropanol/dry ice bath, and MeOH was added slowly to quench the excess BBr₃. The quenched reaction mixture was diluted with \(-300\) mL of water and boiled for 45 min. After the aqueous solution was cooled, neutralized to pH \(\sim 6.5\) with saturated NaHCO₃, and saturated with KCl, the product was extracted into CH₂Cl₂ to give a red solid after solvent removal. Flash chromatography on basic alumina (4:1 CH₂Cl₂/EtOAc) yielded the product as a yellow solid (190 mg, 39.8%). TLC Rₜ = 0.27 (17:3 CH₂Cl₂/EtOAc). $^1$H NMR (CDCl₃, 500 MHz) δ 4.81 (4 H, s), 6.12 (1 H, t, J = 2.0 Hz), 6.23 (1 H, dd, J = 2.5, 8.0 Hz), 6.30 (1H, dd, J = 2.0, 8.0 Hz), 7.04 (1 H, t, J = 8.0 Hz), 7.11 (2 H, t, J = 5.0 Hz), 7.29 (2 H, d, J = 8.0 Hz), 7.64 (2 H, td, J = 1.5, 7.5 Hz), 8.34 (2 H, d, J = 4.5 Hz). $^{13}$C NMR (CDCl₃, 125 MHz) δ 57.28, 99.74, 104.26, 105.56, 121.19, 122.45, 130.69, 137.61, 149.06, 149.36, 158.56, 158.88. FTIR (thin film, cm⁻¹) 3413, 1613, 1595, 1502, 1438, 1355, 1191, 756. HRMS (ESI): Calcd for MH⁺, 292.1450; Found 292.1444.

9-(o-Carboxyphenyl)-2-chloro-6-[bis(2-pyridylmethyl)amino]-3-xanthanone (5, Rhodaflor-1, RF1). N,N-Bis(2-pyridylmethyl)-3-aminophenol (3, 300 mg, 1.03 mmol) and 2'-carboxy-5-chloro-2,4-dihydroxybenzophenone (4, 295 mg, 1.01 mmol) were combined in 5 mL of methane sulfonic acid (CH₃SO₃H). The resulting dark red solution was stirred for 48 h at 70°C. The reaction mixture was diluted with 250 mL of water, chilled to 0°C, and slowly neutralized with saturated NaHCO₃. The aqueous mixture was extracted thoroughly with CH₂Cl₂, and the combined organic extracts were dried over MgSO₄ to give a red solid after filtration and solvent removal. Flash chromatography on silica (93:7 CHCl₃/MeOH) yielded the product as a red solid (322 mg, 57.1%). TLC Rₜ = 0.47
(9:1 CHCl₃/MeOH). ¹H NMR (CDCl₃, 500 MHz) δ 4.84 (4 H, s), 6.43 (2 H, d, J = 10.5 Hz), 6.56 (1 H, d, J = 8.5 Hz), 6.73 (2 H, s), 7.14-7.24 (5 H, m), 7.56-7.69 (4 H, m), 8.02 (1 H, d, J = 7.5 Hz), 8.54 (2 H, d, J = 5.0 Hz). ¹³C NMR (CDCl₃, 125 MHz) δ 56.57, 98.94, 103.81, 108.25, 109.70, 111.85, 121.25, 122.88, 124.94, 125.87, 126.11, 127.94, 128.23, 128.78, 128.99, 129.41, 129.66, 129.93, 134.81, 137.98, 149.35, 149.58, 150.53, 153.01, 157.20, 169.35. FTIR (KBr, cm⁻¹) 3056, 2581, 1760, 1632, 1584, 1514, 1479, 1433, 1388, 1345, 1197, 761, 701. HRMS (ESI): Calcd for MH⁺, 548.1377; Found 548.1372.

**N,N-Bis(2-pyridylmethyl)-4-methyl-α-anisidine** (7). 2-Picolylchloride hydrochloride (10.0 g, 61.0 mmol) was dissolved in 3 mL of water, and 18 mL of 5 N NaOH was added to give a pink solution. An additional 18 mL of 5 N NaOH was added to the vigorously stirred solution, after which 4-methyl-α-anisidine (6, 3.70 g, 27.0 mmol) was combined with the solution of picolylchloride. An aliquot of cetyltrimethylammonium chloride (450 μL, 25 wt. % in water) was added as a phase transfer catalyst (PTC), and the reaction was stirred vigorously under Ar. Additional picolylchloride hydrochloride (10.0 g, 61.0 mmol) and 5 mL of 5.0 N NaOH was added to the solution after 72 h. After 96 h, an additional portion of picolylchloride hydrochloride (6.00 g, 36.6 mmol) was added to the solution, and after 216 h a final addition of picolylchloride hydrochloride (7.00 g, 42.7 mmol) and 5 mL of 5 N NaOH was made. After a total reaction time of 11 days, the product was extracted into CH₂Cl₂ and dried over MgSO₄ to afford a dark red oil after solvent removal. Flash chromatography on basic alumina with solvent gradient (9/1 CH₂Cl₂/EtOAc to 4/1 CH₂Cl₂/EtOAc) yielded the product as an orange oil (1.43 g, 16.6%). TLC Rₜ = 0.29 (4:1 CH₂Cl₂/EtOAc). ¹H NMR
(CDCl₃, 500 MHz) δ 2.39 (3 H, s), 3.65 (3 H, s), 4.37 (4 H, s), 6.50 (1 H, dd, J = 2.5, 8.5 Hz), 6.61 (1 H, d, J = 3.0 Hz), 7.07-7.12 (3 H, m), 7.42 (2 H, d, J = 8.0 Hz), 7.58 (2 H, td, J = 2.0, 7.5 Hz), 8.52 (2 H, dt, J = 1.0, 5.5 Hz). ¹³C NMR (CDCl₃, 125 MHz) δ 18.17, 55.41, 56.70, 108.09, 108.84, 122.09, 122.36, 125.05, 131.89, 136.60, 149.26, 150.22, 158.35, 159.05. FTIR (thin film, cm⁻¹) 3392, 2940, 1608, 1589, 1501, 1433, 1163, 1045, 762. HRMS (ESI): Calcd for MH⁺, 320.1763; Found 320.1741.

_N,N-Bis(2-pyridylmethyl)-3-amino-4-methylphenol (8)._ A solution of _N,N-bis(2-pyridylmethyl)-4-methyl-m-anisidine (7, 1.00 g, 3.13 mmol) _in 10 mL of CH₂Cl₂ _was frozen with liquid N₂, _and 90 mL of 1.0 M BBr₃ (90 mmol) _in CH₂Cl₂ _was added via a cannula. _The solution _was allowed to warm slowly to room temperature and stirred under Ar for 40 h. _The reaction mixture was chilled to −40°C via an isopropanol/dry ice bath, _and MeOH _was added slowly to quench the excess BBr₃. _The quenched reaction mixture was diluted with ~350 mL of water and boiled for 45 min. _After the aqueous solution was cooled, neutralized to pH ~6.5 with saturated NaHCO₃, _and saturated with NaCl, _the product was extracted into CH₂Cl₂ _to give a red solid after solvent removal. Flash chromatography on basic alumina (19:1 CHCl₃/MeOH) yielded the product as a yellow solid (449 mg, 47.0%). TLC Rᵣ = 0.26 (19:1 CHCl₃/MeOH). ¹H NMR (CDCl₃, 500 MHz) δ 2.38 (3 H, s), 4.33 (4 H, s), 6.49 (1 H, dd, J = 2.5, 7.5 Hz), 6.60 (1 H, d, J = 2.5), 7.04 (1 H, d, J = 8.0 Hz), 7.10 (2 H, dd, J = 5.0, 7.0 Hz), 7.45 (2 H, d, J = 7.5 Hz), 7.60 (2 H, td, J = 1.5, 7.5 Hz), 8.38 (2 H, dt, J = 1.0, 5.0 Hz). ¹³C NMR (CDCl₃, 125 MHz) δ 18.15, 59.32, 108.94, 111.42, 122.32, 122.43, 123.95, 132.38, 137.15, 148.77, 149.64, 155.87, 158.87. FTIR (thin film) 3422, 1610, 1599, 1531, 1474,

9-(o-Carboxyphenyl)-2-chloro-6-[(2-pyridylmethyl)amino]-3-xanthanone (9). N,N-Bis(2-pyridylmethyl)-3-amino-4-methylphenol (8, 284 mg, 0.93 mmol) and 2'-carboxy-5-chloro-2,4-dihydroxybenzophenone (4, 272 mg, 0.93 mmol) were combined in 5 mL of methane sulfonic acid (CH₃SO₃H). The resulting dark red solution was stirred for 48 h at 70° C. The reaction mixture was diluted with 250 mL of water, chilled to 0° C, and slowly neutralized with saturated NaHCO₃. The aqueous mixture was extracted thoroughly with CH₂Cl₂, and the combined organic extracts were dried over MgSO₄ to give a red solid after filtration and solvent removal. Flash chromatography on silica (9:1 CHCl₃/MeOH) yielded the product as a red solid (149 mg, 34.1%). TLC Rf = 0.15 (9:1 CHCl₃/MeOH). ¹H NMR (CDCl₃, 500 MHz) δ 2.09 (3 H, s), 4.50 (2 H, s), 6.34 (1 H, s) 6.47 (1 H, s), 6.78 (2 H, s), 7.18 (1 H, d, J = 7.5 Hz), 7.27 (2 H, m), 7.33 (1 H, d, J = 7.0 Hz), 7.64-7.72 (3 H, m), 8.10 (1 H, d, J = 8.0 Hz), 8.61 (1 H, d, J = 4.5 Hz). ¹³C NMR (CDCl₃, 125 MHz) δ 48.30, 95.82, 103.59, 105.16, 111.15, 119.44, 120.97, 122.12, 123.89, 124.56, 124.94, 125.27, 126.96, 127.83, 127.91, 128.26, 129.96, 135.05, 135.72, 136.74, 148.91, 149.05, 150.13, 150.82, 158.74, 168.41. FTIR (KBr, cm⁻¹) 3402, 1643, 1595, 1560, 1494, 1434, 1329, 1287, 1016, 759. HRMS (ESI): Calcd for MH⁺, 471.1112; Found 471.1219.

1,4,7-Tris(benzyloxycarbonyl)-1,4,7,10-tetraazacyclo-dodecane (3CBZ-cyclen, 11). Cyclen (10, 4.35 g, 25 mmol) and Et₃N (10.5 mL) were dissolved in 250 mL of CHCl₃. Dibenzyl dicarbonate (20.0 g, 69.9 mmol) was dissolved in 200 mL of CHCl₃ and added via a syringe pump to the stirring solution of cyclen
over 7 h. The reaction mixture was stirred for 24 h at room temperature. The \( \text{CHCl}_3 \) was removed, and flash chromatography on silica (3:17 hexanes/EtOAc) yielded the product as a white solid (10.6 g, 73.0\%). TLC \( R_f = 0.35 \) (EtOAc). \( ^1\text{H} \) NMR (CDCl\(_3\), 500 MHz) \( \delta 0.91 \) (1 H, s), 2.76-2.87 (4 H, m), 3.29-3.77 (12 H, m), 4.88 (1 H, s), 5.05 (2 H, s), 5.14 (1 H, s), 7.18-7.34 (15 H, m). \( ^{13}\text{C} \) NMR (CDCl\(_3\), 125 MHz) \( \delta 45.43, 48.39, 48.69, 49.06, 49.26, 50.50, 50.95, 51.18, 66.63, 66.85, 66.97, 67.10, 127.54, 127.63, 127.74, 127.91, 128.04, 128.31, 128.37, 128.44, 136.58, 136.66, 136.88, 155.85, 156.20, 156.34. FTIR (thin film) 3213, 2940, 2827, 1696, 1609, 1498, 1419, 1365, 1259, 1159, 769, 698. HRMS (ESI): Calcd for MH\(^+\), 575.2870; Found 575.2868.

\( 1,4,7\)-Tris(benzyloxy carbonyl)-10-(3-methoxyphenyl)-1,4,7,10-tetraaza- cyclo-dodecane (3CBZ-Ar-cyclen, 13). 2-Dicyclohexylphosphino-2′-(N,N- dimethyl- amino)biphenyl (450 mg, 1.14 mol), Pd\(_2\)(dba)\(_3\) (200 mg, 218 \( \mu \)mol) and sodium tert-butoxide (2.6 g, 27 mmol) were combined in a Schlenk tube outfitted with a Teflon screwcap and dried in vacuo for 1 h. A 10.6 g (18.4 mmol) portion of dry 11 was transferred into the tube in 25 mL of toluene while purging with Ar. To the combined was added materials 3-bromo anisidine (12, 2 mL, 15.8 mmol) by a syringe. After thoroughly purging with Ar, the tube was sealed and placed in oil bath at 80 °C and stirred for 36 h. The reaction mixture was cooled, diluted with \( \text{CH}_2\text{Cl}_2 \), filtered through Celite, and the solvent was removed. Flash chromatography on silica (1:1 hexanes/EtOAc) yielded the product as a white solid (3.66 g, 34\%). TLC \( R_f = 0.60 \) (EtOAc). \( ^1\text{H} \) NMR (CDCl\(_3\), 500 MHz) \( \delta 3.35 \) (16 H, bs), 3.73 (3 H, s), 5.02 (2 H, s), 5.11 (4 H, s), 6.28 (2 H, s), 6.40 (1 H, d, \( J = 7.5 \) Hz), 7.10 (1 H, t, \( J = 8.0 \) Hz), 7.27-7.31 (15 H, m). \( ^{13}\text{C} \) NMR (CDCl\(_3\), 125 MHz)
δ 50.06, 50.54, 55.21, 67.15, 67.37, 128.08, 128.14, 128.31, 128.41, 128.60, 128.67, 130.27, 136.54, 136.76, 160.75. FTIR (thin film) 3195, 2924, 2833, 1611, 1499, 1461, 1357, 1278, 1220, 1159, 1055, 754. HRMS (ESI): Calcd for MH⁺, 681.3288; Found 681.3307.

1-(3-Methoxyphenyl)-1,4,7,10-tetraazacyclo-dodecane (Ar-cyclen, 14). Pd/C (2.0 g, 10% activated) and 3CBZ-Ar-cyclen (13, 3.66 g, 5.38 mmol) were combined in 125 mL of MeOH and stirred under a hydrogen atmosphere (1 atm) for 24 h. The reaction mixture was filtered through Celite to give a dark yellow oil after solvent removal. Flash chromatography on silica (72:25:3 CHCl₃/MeOH/NH₄OH) yielded the product as a white solid (965 mg, 64.3%). TLC Rₗ = 0.24 (97:3 CHCl₃/MeOH). "H NMR (CDCl₃, 500 MHz) δ 2.52 (3 H, s), 2.63 (4 H, t, J = 5.0 Hz), 2.83 (4 H, t, J = 5.0 Hz), 2.87 (4 H, J = 5.0 Hz), 3.48 (4 H, t, J = 5.0 Hz), 3.76 (3 H, s), 6.31-6.38 (2 H, m), 6.46 (1 H, dd, J = 2.0, 8.0 Hz), 7.10-7.15 (1 H, m). "C NMR (CDCl₃, 125 MHz) δ 47.04, 47.10, 47.19, 52.54, 55.34, 101.99, 103.134, 129.94, 130.11, 150.90, 160.67. FTIR (thin film) 3230, 2924, 2851, 1727, 1617, 1461, 1409, 1277, 1152, 757. HRMS (ESI): Calcd for MH⁺, 279.2179; Found 279.2172.

1-(3-Methoxyphenyl)-7-(2-pyridylimethyl)-1,4,7,10-tetraazacyclo-dodecane (PyAr-cyclen, 15). Ar-cyclen (14, 451 mg, 1.62 mmol) and 2-pyridinecarboxaldehyde (175 µL, 1.83 mmol) were combined in 50 mL DCE and stirred. NaBH(OAc)₃ (515 mg, 2.43 mmol) was added and the reaction mixture was stirred for 24 h at room temperature. Saturated NaHCO₃ (5 mL) was added to quench unreacted borohydride reagent. The aqueous layer was extracted with EtOAc. The combined organic layers were washed with saturated brine, dried
over MgSO₄, filtered, and the solvent was removed. Flash chromatography on silica (22:2:1 CHCl₃/MeOH/PrNH₂) yielded the product as a white solid (599 mg, 63.5%). TLC Rₜ = 0.35 (22:2:1 CHCl₃/MeOH/PrNH₂). ³¹H NMR (CDCl₃, 300 MHz) δ 2.54-2.56 (4 H, m), 2.62-2.65 (4 H, m), 2.74 (4 H, t, J = 5.1 Hz), 3.37 (4 H, t, J = 4.8 Hz), 3.67 (2 H, s), 3.73 (3 H, s), 6.36 (1 H, dd, J = 2.4, 8.1 Hz), 6.50 (1 H, t, J = 2.4 Hz), 6.60 (1 H, dd, J = 2.4, 8.1 Hz), 6.93-6.98 (2 H, m), 7.10-7.24 (2 H, m), 8.24 (1 H, dd, J = 0.6, 3.9 Hz). ¹³C NMR (CDCl₃, 75 MHz) δ 47.01, 47.24, 52.45, 53.14, 55.21, 62.27, 102.27, 103.37, 109.15, 122.00, 122.87, 129.72, 136.65, 148.71, 152.03, 159.75, 160.52. FTIR (thin film) 3394, 2930, 2833, 1608, 1499, 1458, 1361, 1221, 1163, 1048, 886, 755. HRMS (ESI): Calcd for MH⁺, 370.2607; Found 370.2614.

1-(3-Methoxyphenyl)-4,10-(diethyl)-7-(2-pyridylmethyl)-1,4,7,10-tetraazacyclo-dodecane (PyEt₂Ar-cyclen, 16). PyEt₂Ar-cyclen (15, 675 mg, 1.83 mmol) and acetaldehyde (1 mL, mmol) were combined at 0 °C in 20 mL DCE and stirred. The temperature of the reaction mixture was maintained between 0-20 °C to prevent evaporation of acetaldehyde. NaBH(OAc)₃ (1.63 g, 7.69 mmol) was added, and the reaction mixture was stirred for 24 h while warming to room temperature. Saturated NaHCO₃ (5 mL) was added to quench unreacted borohydride reagent. The aqueous layer was extracted with EtOAc. The combined organic layers were washed with saturated brine, dried over MgSO₄, filtered, and the solvent was removed. Flash chromatography on silica with a solvent gradient (22:2:1 CHCl₃/MeOH/PrNH₂) yielded the product as a white solid (412 mg, 53.0%). TLC Rₜ = 0.40 (22:2:1 CHCl₃/MeOH/PrNH₂). ³¹H NMR (CDCl₃, 300 MHz) δ 0.93 (6 H, t, J = 7.2 Hz), 2.42 (4 H, q, J = 7.2 Hz), 2.56-2.63 (8 H, m), 2.78 (4 H, t, J = 5.7 Hz), 3.55 (4 H, t, J = 6.0 Hz), 3.66 (2 H, s), 3.75 (3 H, s),
6.10-6.19 (2 H, m), 6.26 (1 H, d, J = 9.6 Hz), 7.00-7.13 (2 H, m), 7.51-7.61 (2 H, m),
8.46 (1 H, d, J = 4.8 Hz). $^{13}$C NMR (CDCl$_3$, 75 MHz) δ 12.33, 49.37, 50.47, 51.98,
52.41, 52.79, 55.15, 61.48, 98.02, 99.86, 104.80, 121.80, 123.62, 129.71, 136.13, 148.61,
150.15, 160.07, 160.66. FTIR (thin film) 2962, 2925, 2800, 1610, 1500, 1467, 1356,
1160, 753. HRMS (ESI): Calcd for MH$^+$, 426.3233; Found 426.3227.

1-{9'-({Carboxyphenyl})-6'-amino-2'-chboro-3'-xanthanone}-4,10-diethyl-
7-(2-pyridylmethyl)-1,4,7,10-tetraazacyclo-dodecane (17, Rhodafluor-2, RF2).
PyEt$_2$Ar-cyclohexane (16, 100 mg, 235 μmol) and 2'-carboxy-5-chloro-2,4-
dihydroxybenzophenone (4, 275 mg, 942 μmol) were combined in 5 mL of
methane sulfonic acid (CH$_3$SO$_3$H) and sealed in a thick walled glass tube. The
resulting dark red solution was stirred for 48 hrs at 80 °C. The reaction mixture
was diluted with 100 mL of water, chilled to 0 °C, and slowly neutralized with
NaHCO$_3$. The aqueous mixture was extracted thoroughly with CH$_2$Cl$_2$, and the
combined organic extracts were dried over MgSO$_4$ to give a red solid after
filtration and solvent removal. Flash chromatography on silica (17:2:1
CHCl$_3$/MeOH/PrH$_2$N) yielded the product as a red solid (322 mg, 57.1%). TLC
R$_f$ = 0.41 (17:2:1 CHCl$_3$/MeOH/PrH$_2$N). $^1$H NMR (CDCl$_3$, 300 MHz) δ 1.04 (6 H,
t, J = 6.9 Hz), 2.84-3.18 (16 H, m), 3.72 (4 H, s), 3.83 (2 H, s), 6.44 (1 H, s), 6.77-6.79
(2 H, m), 7.18-7.22 (2 H, m), 7.31 (1 H, quad, J = 1.2, 5.1 Hz), 7.38 (1 H, d, J = 7.5
Hz), 7.63 (2 H, quind, J = 6.0, 7.5 Hz), 7.77 (1 H, td, J = 1.8, 7.8), 8.10 (1 H, dd, J =
1.5, 7.2 Hz), 8.52 (1 H, dt, J = 0.9, 4.2 Hz). $^{13}$C NMR (CDCl$_3$, 75 MHz) δ 8.91, 21.04,
50.88, 49.722, 50.49, 52.95, 60.90, 98.90, 105.19, 113.26, 114.25, 115.39, 124.44,
125.89, 130.36, 130.63, 130.90, 130.74, 131.03, 131.45, 132.45, 134.45, 138.88, 139.06,
141.26, 150.31, 154.85, 157.34, 158.82, 159.97, 173.58, 177.64. FTIR (KBr) 3329, 2963,
2926, 1758, 1582, 1494, 1343, 1195, 1005. HRMS (ESI): Calcd for MH+, 668.2998; Found 668.2978. Anal. Calcd for C_{39}H_{44}Cl_{5}N_{5}O_{4} (17•CH_{2}Cl_{2}): C, 62.19; H, 5.89; N, 9.30. Found: C, 62.03; H, 6.14; N, 9.27.

**General Spectroscopic Methods.** Ultrol grade PIPES (piperazine-\(N,N'\)-bis(2-ethanesulfonic acid)) from Calbiochem and KCl (99.997%) was purchased and used as received. All solutions were filtered through 0.2-\(\mu\)M cellulose filters before measurements. Except for the fluorescence titration experiment, Zn solutions were prepared by the addition of appropriate amounts of 1.0 M, 100 mM, 10 mM or 1 mM Zn\(^{2+}\) stocks that were checked by atomic absorption spectroscopy for concentration accuracy, or by titration with terpyridine and measurement of the absorption spectra. The titration was performed by treating a 100 mM solution of 2,2':6',2''-terpyridine in buffered solution (50 mM PIPES, 100 mM KCl, pH 7) with aliquots of 10 mM (nominal) ZnCl\(_2\) and determining the equivalence point by monitoring the absorption of the resulting complex at 321 nm (\(\epsilon = 35.9 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}\)). The Zn\(^{2+}\) stocks were prepared from 99.999% pure ZnCl\(_2\). The purity of the ZP probes was verified by HPLC. ZP was introduced to aqueous solutions by addition of a stock solution in DMSO (1.0 mM). Graphs were manipulated and equations calculated by using Kaleidagraph 3.0. The pH values of solutions were recorded with an Orion glass electrode that was calibrated prior to each use. The experiments for measuring the pH-dependent fluorescence, quantum yield, metal ion selectivity were performed as previously described.\(^5,6,8\)

**UV-Visible Spectroscopy.** Absorption spectra were recorded on a Hewlett Packard 8453A diode array spectrophotometer under the control of a
Pentium II-based PC running the Windows NT ChemStation software package, or a Cary 1E scanning spectrophotometer under the control of a Pentium PC running the manufacturer supplied software package. Spectra were routinely acquired at 25 °C, maintained by a circulating water bath in 1-cm path length quartz cuvettes with a volume of 1.0 or 3.5 mL.

**Fluorescence Spectroscopy.** Fluorescence spectra were recorded on a Hitachi F-3010 spectrofluorimeter under the control of a Pentium-based PC running the SpectraCalc software package. Excitation was provided by a 150 W Xe lamp (Ushio Inc.) operating at a current of 5 A. All spectra were normalized for excitation intensity via a rhodamine quantum counter, and emission spectra were normalized by the manufacturer-supplied correction curves. Spectra were routinely acquired at 25 °C, maintained by a circulating water bath in 1 cm × 1 cm quartz cuvettes using 3 nm slit widths and a 240 nm/min scan speed. Fluorescence emission measurements were also acquired in a 1 cm × 1 cm quartz cell using a Spex Fluorolog-2 instrument with 1-nm bandwidth slits. All spectra were corrected for emission intensity by using the manufacturer-supplied photomultiplier curves.

**Titration of Zn²⁺ Binding by Absorption and Emission Spectroscopy (K₀).** A 3.0 mL solution containing 10 μM RF2 in buffer was prepared and an initial absorbance and fluorescence measurement was made. Zn²⁺ aliquots were titrated into the solution to give final concentrations of 2.8, 5.6, 8.3, 11.1, 13.9, 16.7, 19.4, 22.2, 25.0, 27.8, 30.6, 33.3, 38.9, 44.4, 50.0, 55.6, 66.7, 77.8, 88.9, 100, 111, 139, 167, 222, 333, 444, 556 μM and the absorption and emission spectra were recorded. The absorption spectra were corrected for dilution, and both data sets
were analyzed with SPECFIT, a nonlinear least-squares fitting program.\textsuperscript{15} The measurements were performed in triplicate to ensure accuracy of the derived $K_d$ value.

**Results and Discussion**

**Synthesis of Rhodafluor Derivatives.** Before embarking on the preparation of complex ratiometric Zn\textsuperscript{2+} sensors, the synthetic methods were tested by making a prototype of the RF structure (Scheme 7.1). Dialkylation of $m$-anisidine (1) with picolyl chloride affords $N,N$-bis(2-pyridylmethyl)-$m$-anisidine (2) in fair yield. Subsequent removal of the methyl protecting group with boron tribromide (BBr\textsubscript{3}) provides $N,N$-bis(2-pyridylmethyl)-3-aminophenol (3), one of the required fragments for the synthesis of RF1 (5). A variety of reagents was screened for catalyzing the condensation of 3 with 2'-carboxy-5-chloro-2,4-dihydroxybenzophenone (4) to yield RF1. Unlike the synthesis of the structurally related Ca\textsuperscript{2+} sensor,\textsuperscript{13} reactions catalyzed by ZnCl\textsubscript{2} in THF (200° C, sealed vessel) failed to yield the desired product. Presumably zinc binding to 3 inhibits the reaction. RF1 was isolated successfully from the reaction of 3 and 4 in neat methane sulfonic acid (CH\textsubscript{3}SO\textsubscript{3}H).

The affinity of RF1 for Zn\textsuperscript{2+} is predicted to be weak since it contains only poor metal-binding units. RF1 has an aniline nitrogen atom with a partial positive charge at neutral pH and two pyridine donors. A practical sensor requires additional metal binding ligands to enhance binding affinity. One approach to synthesizing more elaborate targets involves elaboration of the parent RF1 skeleton. The presence of a methyl group adjacent to the di-(2-
picolyl)amine (DPA) provides potential for modification of the chemical structure using the methods devised for the synthesis of ZP2 and ZP4.\textsuperscript{5,6} Following a parallel synthetic scheme to RF1, the \textit{N,N}-bis(2-pyridylmethyl)-3-amino-4-methylphenol (8) analog, which would afford the desired compound, was prepared in gram quantities. Reaction of 8 with 4 in CH\textsubscript{3}SO\textsubscript{3}H failed to produce the desired compound, however (Scheme 7.2). An unexpected decomposition pathway results in the elimination of one of the 2-picolyl groups from the aniline nitrogen. Elaboration of 9 proves to be impractical because aniline nitrogen atoms are not particularly reactive, and installation of protecting groups on the free phenol is not trivial with these hybrid fluorophores. In addition, the aromatic ring system of RF1 seems inert to electrophilic aromatic substitution reactions like the Mannich reaction used to prepare ZP1.\textsuperscript{5}

Manipulation of an assembled RF compound presents several synthetic difficulties. Alternatively, the preparation of 3-aminophenols is straightforward and elaboration of the Zn\textsuperscript{2+}-binding ligand prior to formation of the RF framework is an appealing strategy. Investigations into macrocyclic polyamines indicate that these ligands act as selective, high affinity receptors for Zn\textsuperscript{2+}.\textsuperscript{16} Recent progress in Pd-catalyzed amination chemistry demonstrates that macrocyclic amines can be coupled with aromatic bromides,\textsuperscript{14,17} thereby avoiding elaborate multi-step synthesis required by more conventional techniques.\textsuperscript{18}

The synthesis of a cyclen-based RF target is outlined in Scheme 7.3. Installation of protecting groups on three nitrogen atoms restricts the Pd-coupling to a single site on the cyclen ring. The preparation of the tri-protected
compound from commercially available cyclen (11) was accomplished by using procedures for the synthesis of analogous Boc-protected cyclen.\textsuperscript{19} Coupling of the 3-bromoanisidine (12) with the 3CBZ-cyclen (11) proceeds in reasonable yield, and provides multigram quantities of the desired product. The efficient synthesis of 11 represents a significant advance in RF synthesis. Removal of the CBZ protecting groups occurs under mild conditions in high yield to afford Ar-cyclen (14). One commonly encountered feature of Zn\textsuperscript{2+} cyclen complexes is the presence of an additional pendant chelating ligand.\textsuperscript{20} Reaction of 14 with 2-pyridinecarboxaldehyde under reducing conditions yields the 1,7-substituted PyAr-cyclen (15) with excellent selectivity. None of the 1,4-product is observed, and only small amount of the compound containing a second pyridyl arm is recovered when the quantity of aldehyde is restricted to 1 equivalent. When submitted to identical reductive amination conditions using an excess of acetaldehyde, the remaining two secondary nitrogen atoms of 15 undergo alkylation yielding the PyEt\textsubscript{2}Ar-cyclen (16).

Methyl ether protecting groups are robust and deprotection often requires harsh conditions. Only limited quantities of the corresponding 3-aminophenol could be recovered following removal of the methyl group of 16 with BBr\textsubscript{3}. Test reactions substituting of the methyl ether 2 for the phenol 3 in the synthesis of RF1 indicated that removal of the ether was unnecessary for assembly of the final compound. Avoiding a deprotection step eliminates a low yielding step in the synthesis. Reaction of 16 with 4 in neat CH\textsubscript{3}SO\textsubscript{3}H provides RF2 (17) in high yield. RF2 is prepared in 3.1% overall yield from cyclen in six steps.
Fluorescence Properties of RF2. The absorbance maximum of RF2 under simulated physiological conditions (50 mM PIPES, 100 mM KCl, pH 7) occurs at 514 nm (ε = 82.7 × 10^3 M^{-1} cm^{-1}) with an emission maximum at 539 nm. The quantum yield of the RF2 in the presence of EDTA to scavenge adventitious metal ions is 0.36. In the presence of Zn^{2+}, the absorbance maximum shifts slightly to 510 nm (ε = 50.8 × 10^3 M^{-1} cm^{-1}), the maximum emission remains at 539 nm, and the quantum yield increases to 0.56. The two limiting resonance forms of the RF fluorophore are the iminophenoxy mesomer, which has rhodamine-like fluorescence properties, and the aminoquinone mesomer, which has optical properties characteristic of fluorescein (Fig. 7.1). A change in the contribution from these two limiting resonance forms to the electronic structure of the fluorophore in the complexed and uncomplexed states is required to alter significantly the optical properties of the sensor. The small shift in the absorbance spectrum, and the fluorescein-like wavelengths observed in the presence and absence of Zn^{2+}, suggest that RF2 begins and remains in the aminoquinone form during metal ion binding.

The negligible Zn^{2+}-induced spectral shifts prompted an examination of the pH dependent absorption and emission wavelengths of RF2. Under basic conditions (pH 12, 100 mM KCl), the absorption maximum occurs at 526 nm with an emission maximum at 539 nm. Under acidic conditions (pH 5, 100 mM KCl), the absorption maximum occurs at 507 nm with an emission maximum at 529 nm. The fluorescence intensity of RF2 is dependent on protons. Fitting of the integrated emission intensity reveals pK_a values at 11.5 and 6.4, corresponding to increases in fluorescence, and one value at 2.9 as fluorescence decreases (Fig. 7.2).
Diminished fluorescence under acidic conditions is associated with the formation of a non-fluorescent isomer of RF2 similar to the one adopted by ZP sensors at low pH. The pK\textsubscript{a} values at 11.5 and 6.4 indicate protonation of amine atoms in the cyclen macrocycle. When deprotonated, the free electrons of amine atoms participate in quenching of the fluorophore excited state by a PET mechanism. This proposed quenching mechanism is consistent with the observed increase in the quantum yield upon Zn\textsuperscript{2+}-binding. The pH dependence of RF2 absorption and emission also provides an explanation for the minimal Zn\textsuperscript{2+}-induced wavelength shifts. Protonation of the macrocycle at neutral pH forces RF2 to adopt the aminoquinone form in the both the Zn\textsuperscript{2+}-free and complexed forms, limiting the contribution of the iminophenoxy mesomer to the fluorescence properties (Scheme 7.4).

The fluorescence increase and binding affinity of RF2 for Zn\textsuperscript{2+} were characterized by titration of the sensor with \(\mu\)M concentrations of metal ion and measurement of the absorption and emission spectra (Fig. 7.3). The absorption changes fit a \(K_d\) of 13.5 \(\pm\) 0.5 \(\mu\)M. The affinity of RF2 for Zn\textsuperscript{2+} is lower than for some cyclen complexes, but comparable in value to compounds that contain an aniline nitrogen atom in the macrocycle. With the exception of Cu\textsuperscript{2+} and Cd\textsuperscript{2+}, which bind to RF2 and quench the fluorescence, other divalent transition metals induce only slight quenching in the fluorescence intensity (Fig. 7.4). Binding by these metal ions appears to be weak since subsequent addition of Zn\textsuperscript{2+} to these RF2 solutions enhances fluorescence.
Conclusions

We have prepared a first generation ratiometric sensor for Zn$^{2+}$ exploiting organometallic methodology for coupling aryl bromides with azamacrocycles. This approach provides a straightforward and convenient means to access a compound that otherwise would require a difficult, multi-step route using conventional organic techniques. In addition to synthetic ease, this method permits assembly of the Zn$^{2+}$ binding ligand prior to formation of the fluorophore skeleton, avoiding manipulation of a more sensitive compound.

Since the amine atoms of RF2 are susceptible to protonation, the sensor prefers the aminoquinone mesomer in the presence and absence of Zn$^{2+}$ at physiological pH. The insignificant contribution from the iminophenoxy resonance form in the uncomplexed state prevents RF2 from exhibiting the desired shifts in the excitation and emission wavelength upon Zn$^{2+}$-binding. RF2 does act as a modest PET sensor, however, with an \(~50\%\) enhancement in quantum yield upon Zn$^{2+}$-binding. This property renders RF2 of immediate value for application in situations where our previous tight binding (< 1 nM) sensors are inadequate. Variation of the metal ion receptor should allow attenuation of the properties of future RF sensors to achieve the desired wavelength changes. Application of the methodologies described here will allow these changes to be made in a simple and efficient manner.

Acknowledgement. This work was supported at MIT by the McKnight Foundation for the Neurosciences and grant GM65519 from the National Institutes of General Medical Sciences. We thank X.-X. Zhang for his guidance and helpful suggestions in running the Pd-catalyzed coupling reactions.
References

(*) A slightly altered version of this work has been submitted for publication. Burdette, S. C.; Lippard, S. J. 2002.


Scheme 7.1
Scheme 7.2
Scheme 7.3
Scheme 7.4
Figure 7.1. The two limiting resonance forms of the rhodafluor fluorophore. The aminoquinone mesomer has fluorescence properties characteristic of fluorescein, and the iminophenoxy has rhodamine-like fluorescence properties.
Figure 7.2. Plot of the normalized integrated emission intensity versus pH for RF2. The increase in fluorescence corresponds to protonation of the amine atoms in the cyclen macrocycle, and the decrease corresponds to the formation of a nonfluorescent isomer.
Figure 7.3. The absorption (a) and emission (b) spectra of RF2 after addition of Zn$^{2+}$ to give final concentrations of 5.6, 11.1, 16.7, 22.2, 25.0, 27.8, 33.3, 44.4, 55.6, 66.7, 77.8, 88.9, 100, 111, 139, 167, 222, 333, 444, 556 μM. Emission increases at 539 nm while absorption decreases at 514 nm over the course of the titration.
Figure 7.4. Fluorescence response of RF2 to various metal ions. Bars represent the final integrated fluorescence response \( (F_f) \) over the initial integrated emission \( (F_i) \). Initial spectra were acquired in 100 mM KCl, 50 mM PIPES, 10 \( \mu \)M EDTA pH 7.00 at 25 °C. Excitation was provided at 514 nm, and the emission was integrated between 490 and 625 nm. Aliquots of
Biographical Note

The author was born on August 12, 1975 in Charleston, West Virginia. His secondary schooling included attending the Mountaineer Montessori School for elementary education, Elkview Junior High and Herbert Hoover High School. After graduating from high school, he attended Case Western Reserve University, earning a B.S. in Chemistry in 1997. During his college years, he spent four summers interning at the Union Carbide Co. Technical Center in South Charleston, West Virginia under the supervision of several Ph.D. scientists. Following his graduate work with Professor Stephen J. Lippard, he will pursue postdoctoral research in the labs of Jean M. J. Fréchet at the University of California, Berkeley.
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Bibliography:


Presentations


