Highly Sensitive Fluorescent Probes for the Detection of Zinc Ion

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HIGHLY SENSITIVE FLUORESCENT PROBES FOR
THE DETECTION OF ZINC ION

BY
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Michael Roper
Committee Member

Approved:

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Joseph B. Schlenoff
Chair, Department of Chemistry

The Office of Graduate Studies has verified and approved the above named committee members.
I would like to dedicate this thesis first and foremost to my parents for bringing me to this world. Thanks for giving me your whole love. Also I would like to dedicate this thesis to my four aunts for being there and encouraging me all the time.

With Love

Sha
ACKNOWLEDGMENTS

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# TABLE OF CONTENTS

LIST OF TABLES ........................................................................................................... vii
LIST OF FIGURES ........................................................................................................ viii
ABBREVIATIONS ......................................................................................................... xi
ABSTRACT .................................................................................................................... xiii

CHAPTER 1 ..................................................................................................................... 1
   INTRODUCTION ........................................................................................................ 1

CHAPTER 2 ..................................................................................................................... 6
   HIGHLY SENSITIVE FLUORESCENT PROBES FOR ZINC ION BASED ON
   TRIAZOYL-CONTAINING TETRADENTATE COORDINATION MOTIFS ............. 6
   2.1 Introduction ....................................................................................................... 6
   2.2 Design and Hypothesis .................................................................................... 8
   2.3 Results and Discussion .................................................................................. 9
      2.3.1 Synthesis of Triazolyl-Containing Tetradeitate Coordination Motifs ...... 9
      2.3.2 X-Ray Crystallography Studies on Zinc Coordination Complexes ........ 12
      2.3.3 \textsuperscript{1}H NMR Studies on Zinc Coordination Complexes .......... 13
      2.3.4 Quantum Yield Studies ........................................................................ 14
      2.3.5 Absorption and Fluorescence Spectrophotometric Studies ................. 15
      2.3.6 Determination of Dissociation Constants ............................................ 22
      2.3.7 Metal Ion Selectivity ............................................................................. 23
   2.4 Experimental Section .................................................................................... 25
      2.4.1 General Information ............................................................................... 25
      2.4.2 Synthesis ............................................................................................... 26
   2.5 Conclusion ..................................................................................................... 29

CHAPTER 3 ................................................................................................................... 30
LIST OF TABLES

Table 1. Quantum yields of 10, 11, 12 and 17 and their respective Zn$^{2+}$ complexes ...... 14
Table 2. Dissociation constants (K_d) of ligands 10, 11, 12 and 17 .............................. 23
LIST OF FIGURES

Figure 1: Schematic representation of a fluorescent metal-ion sensor ..................... 2
Figure 2: Fluorophore structures based on quinoline (1) and fluorescein (2) ............ 3
Figure 3: Di-(2-picolyl)amino group and TPEN ........................................... 3
Figure 4: ZnAF-2 (3) and Zinpyr-1 (4) ............................................................. 4
Figure 5: Benzofuran-derived Zn$^{2+}$ sensor (5) and coumarin-derived Zn$^{2+}$ sensor (6) .... 4
Figure 6: Active site residues of carbonic anhydrase and tris(pyrazolyl)hydroborate .... 7
Figure 7: Concerted Huisgen-type [3+2] cycloaddition (top) and copper(I)-catalyzed
       click reaction (bottom) ............................................................................ 7
Figure 8: Proposed mechanistic steps in the alkyne-azide click reaction ................. 8
Figure 9: Two tetradeutate Zn$^{2+}$ coordination motifs featuring five-membered (A) and
       six-membered (B) coordination rings ..................................................... 9
Figure 10: Synthesized ligands 9-13, 17 and 18 ................................................. 12
Figure 11: ORTEP diagram of [Zn(9)(AN)]$^{2+}$ ..................................................... 12
Figure 12: ORTEP diagram of [Zn(10)(AN)]$^{2+}$ ................................................... 13
Figure 13: $^1$H NMR (acetonitrile-$d_3$, 300 MHz) of (A) 17 and (B) Zn(17)(OTf)$_2$
       from 2.4 to 5.4 ppm ................................................................................ 13
Figure 14: The UV and fluorescence titration of 10 with Zn$^{2+}$ in CH$_3$CN ............... 16
Figure 15: The analysis of fluorescence titration of 10 with Zn$^{2+}$ in CH$_3$CN:
           intensity at 419 nm versus [Zn]/[10] ...................................................... 16
Figure 16: The UV and fluorescence titration of 10 in CH$_3$CN with DIPEA and
           TBAP .................................................................................................... 17
Figure 17: The analysis of fluorescence titration of 10 in CH$_3$CN with DIPEA and
           TBAP: intensity at 418 nm versus [Zn]/[10] ........................................... 18
Figure 18: The fluorescence titration of 10 with Zn$^{2+}$ in aqueous buffer containing HEPES ................................................................. 19

Figure 19: Mechanism of fluorescence signal change of 10 upon binding with Zn$^{2+}$ .......................... 20

Figure 20: The UV and fluorescence titration of 13 in aqueous buffer solution containing HEPES ................................................................. 21

Figure 21: The analysis of the fluorescence titration of 13 in aqueous buffer: intensity at 410 nm versus [Zn]/[13] ................................................................. 21

Figure 22: Metal ion selectivities of compounds 12 and 17 .................................................. 24

Figure 23: The structure of a ditopic cruciform ................................................................. 31

Figure 24: Fluorescent heteroditopic ligands with two coordination sites for Zn$^{2+}$ ............ 32

Figure 25: A fluorescent probe with two Zn$^{2+}$ binding sites ............................................. 32

Figure 26: Fluorescence responses of 22 upon coordinating with one or two Zn$^{2+}$ ............ 33

Figure 27: Mechanism of fluorescence signal change of 22 upon binding with Zn$^{2+}$ ................................................................. 34

Figure 28: Deprotonation of 4-methylesculetin ................................................................. 35

Figure 29: Metal ion-coordination-promoted deprotonation may be the cause of the bathochromic shift of the spectra of 22 ................................................................. 35

Figure 30: Tricarbocyanine-based fluorescent probe for Zn$^{2+}$ ........................................ 40

Figure 31: Compounds 28 and 29 chelate with Zn$^{2+}$ to form six- and five-membered coordination rings, respectively ................................................................. 41

Figure 32: Synthesis of compound 29 ........................................................................ 41

Figure 33: The UV and fluorescence titration of 29 with Zn(OTf)$_2$ in aqueous buffer solution containing EGTA, KNO$_3$, and HEPES ................................................................. 42

Figure 34: The fluorescence titration of 29 with Zn(OTf)$_2$ in aqueous buffer solution containing γ-CD, KNO$_3$, and HEPES ................................................................. 43

Figure 35: The UV and fluorescence titration of 29 with γ-CD in aqueous buffer solution containing KNO$_3$ and HEPES ................................................................. 44
Figure 36: The analysis of fluorescence titration of 28 with γ-CD in aqueous buffer solution containing KNO₃ and HEPES: intensity at 414 nm versus [CD]/[28] ................................................................. 45

Figure 37: The fluorescence titration of 28 with Zn(OTf)₂ in aqueous buffer solution containing γ-CD, KNO₃, and HEPES .................................................... 45

Figure 38: The UV and fluorescence titration of 29 with Zn²⁺ in CH₃CN solution containing DIPEA and TBAP ................................................................. 46

Figure 39: The analysis of fluorescence titration of 28 with Zn²⁺ in CH₃CN solution containing DIPEA and TBAP: intensity ratio at 419 nm and 449 nm versus [Zn²⁺]/[28] ................................................................. 47

Figure 40: ¹H NMR (300 MHz, CDCl₃) of compound 24 ........................................... 51

Figure 41: ¹H NMR (300 MHz, CDCl₃) of compound 26 ........................................... 52

Figure 42: ¹H NMR (300 MHz, CDCl₃) of compound 22 ........................................... 53

Figure 43: ¹H NMR (300 MHz, CDCl₃) of compound 25 ........................................... 54

Figure 44: ¹H NMR (300 MHz, CDCl₃) of compound 29 ........................................... 55
ABBREVIATIONS

t-BuOH \hspace{1cm} \textit{tert}-butanol

\gamma\text{-}CD \hspace{1cm} \text{gamma-cyclodextrin}

CDCl\textsubscript{3} \hspace{1cm} \text{chloroform-}d

CH\textsubscript{3}CN \hspace{1cm} \text{acetonitrile}

Cu/C \hspace{1cm} \text{“copper in charcoal” catalyst}

Cu(NO\textsubscript{3})\textsubscript{2} \hspace{1cm} \text{copper (II) nitrate}

Cu(OAc)\textsubscript{2} \hspace{1cm} \text{copper (II) acetate dihydrate}

DCM \hspace{1cm} \text{dichloromethane}

DIPEA \hspace{1cm} \text{diisopropylethylamine}

DMF \hspace{1cm} \text{dimethyl formamide}

DMSO \hspace{1cm} \text{dimethyl sulfoxide}

EDTA \hspace{1cm} \text{ethylenediamine tetraacetic acid}

EGTA \hspace{1cm} \text{ethyleneglycol tetraacetic acid}

Et\textsubscript{2}O \hspace{1cm} \text{diethyl ether}

EtOAc \hspace{1cm} \text{ethyl acetate}

H\textsubscript{2}SO\textsubscript{4} \hspace{1cm} \text{sulfuric acid}

HEPES \hspace{1cm} \text{4-(2-hydroxyethyl)piperazine-ethanesulfonic acid}

K\textsubscript{2}CO\textsubscript{3} \hspace{1cm} \text{potassium carbonate}
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Description</th>
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<tbody>
<tr>
<td>KNO₃</td>
<td>potassium nitrate</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>sodium sulfate</td>
</tr>
<tr>
<td>NaBH(OAc)₃</td>
<td>sodium triacetoxyborohydride</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>sodium bicarbonate</td>
</tr>
<tr>
<td>NaN₃</td>
<td>sodium azide</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>TBAP</td>
<td>tetrabutylammonium perchlorate</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>Zn(ClO₄)₂</td>
<td>zinc perchlorate</td>
</tr>
<tr>
<td>Zn(OTf)₂</td>
<td>zinc trifluoromethanesulfonate; zinc triflate</td>
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Zinc ions (Zn\(^{2+}\)) perform significant roles in biological processes. Therefore, the detection and imaging of Zn\(^{2+}\) in biological systems are of great interest. In this thesis, several 1,2,3-triazolyl-containing N4-tetradentate ligands and coumarin-containing ligands for Zn\(^{2+}\) are studied. The triazolyl-containing ligand show nanomolar affinity and high selectivity toward Zn\(^{2+}\) under simulated physiological conditions. They are easily derivatizable to afford fluorescent probes which are suitable for sensitive Zn\(^{2+}\) detection. Furthermore, in order to expand the scope of using triazolyl groups to mediate metal coordination and fluorescence modulation upon binding, two new ligands containing the pyrene group as the fluorophore and the triazolyl moiety as the coordination moiety are synthesized and studied. The triazolyl-containing tetradentate ligands upon binding with zinc ion form five- or six-membered coordination rings. In Chapter 3, a heteroditopic ligand containing both dihydroxycoumarin and di-(2-picolyl)amino group was described. This ligand combines the modulation of photoinduced electron transfer (PET) and internal charge transfer (ICT) in one single molecule. Di-(2-picolyl)amino group is expected to be the high-affinity binding site which determines the sensitivity. The dihydroxy-coumarin fragment, which is the low-affinity site, extents the dynamic range of zinc ion detection.

Key Words: fluorescent probes  zinc ion  triazolyl  large dynamic range
CHAPTER 1

INTRODUCTION

Zinc ion (Zn\(^{2+}\)) is the second most abundant transition metal ion in our body.\(^1\),\(^2\) It is involved in many biochemical processes and is essential for effective growth and development of all types of organisms.\(^3\) The influence of zinc derives from its roles in enzymes. There are more than 300 enzymes indispensable of zinc for normal functions, including insulin secretion, the synthesis of nucleic acids and specific proteins, such as hormones and their receptors.\(^4\) Studies show that abnormal concentration levels of zinc ion in human tissue are related to the abnormal states of cells. For instance, the concentration of zinc ion in breast carcinoma is 700% higher than that in normal breast cells.\(^5\) There is also report that zinc is responsible for neurological disorders such as Alzheimer’s disease, Parkinson’s disease, and epilepsy.\(^6\) Therefore, it is evident that zinc plays very important roles in biochemical processes. A detailed understanding of how the chemistry of zinc works is crucial for the understanding of zinc’s physiological and pathological roles. Thus, there is an emergent demand for sensitive real-time detection and imaging of zinc ion in biological systems.

However, the proper means for the accurate measurement of spatiotemporal distributions of zinc in physiological processes have not been developed yet. The reasons are the following: 1) The d\(^{10}\) full valence shell configuration of zinc ion contributes to the lack of spectroscopic signature of this element; 2) although in human body the total concentration of zinc is high, the concentration of free (not chelated to proteins) zinc can be very low; 3) while zinc ion has low abundance in the resting states of most cell types,
its spatiotemporal variations during certain physiological process can be dramatic. The physiological concentration of zinc ranges from 1 nM in cytoplasm to 1 mM in the vesicles of presynaptic neurons.\textsuperscript{7}

The issue on the lack of spectroscopic signature of zinc was successfully resolved by the development of fluorescent probes for the detection and quantification of Zn\textsuperscript{2+} in physiological settings.\textsuperscript{6} A fluorescent probe usually consists of a fluorophore and a zinc ion receptor, which can bind selectively with Zn\textsuperscript{2+}. After binding with zinc ion, a change in the fluorescence intensity and wavelength happens, which in turn can be used to track zinc ion. The mechanism of signal change in fluorescence can be charge transfer, energy transfer, electron transfer, or excimer formation. Among these mechanisms, modulation of photoinduced electron transfer (PET) is very commonly applied.

As shown in Figure 1, an OFF-ON type probe for zinc was designed based on zinc-coordination modulated PET. The fluorescence of the probe molecule in the absence of zinc ion is weak, due to PET from the binding site which is usually electron-rich. After binding with zinc ion, the fluorophore will be turned on, because the coordination makes the zinc ion receptor a less efficient electron donor, thus interrupting PET. In addition, ratiometric type probes which undergo internal charge transfer (ICT) upon excitation, and whose fluorescence intensities change at two different wavelength channels, have also

\textbf{Figure 1.} Schematic representation of a fluorescent metal-ion sensor. ET = electron transfer. (Reprinted from Priya, C.; Sivaramapanicker, S.; Ayyappanpillai, A. Chem. Asian J. 2007, 2, 338 – 348).
been developed.\textsuperscript{8}

For addressing the challenges posed by the extremely low abundance of free zinc ion in biological systems, recent research in the area of zinc ion sensors has contributed significantly to the development of highly sensitive probes. Many probes have the fluorophore structures based on quinoline\textsuperscript{9} or fluorescein,\textsuperscript{10} where the zinc ion receptors are di-2-picolylamine and other linear or cyclic polyamines.\textsuperscript{6}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Fluorophore structures based on quinoline (1) and fluorescein (2).}
\end{figure}

Among them, di-(2-picolyl)amine which is expected to be membrane permeable due to its structural similarity to the membrane-permeable heavy metal chelator N,N,N9,N9-tetra(2-picolyl)ethylenediamine(TPEN), which is the most commonly used zinc-coordinating motif (Figure 3).\textsuperscript{11} Di-(2-picolyl)amine group is a typical electron donor in PET, upon binding with zinc ion, the electron-transfer process from di-(2-picolyl)amine group to the fluorophore is interrupted, and the fluorescence quantum yield is increased.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Di-(2-picolyl)amine group and TPEN.}
\end{figure}
Recently, some new PET-based zinc ion probes such as ZnAF-2$^{12}$ and Zinpyr-1$^{10}$ (Figure 4) were reported to have improved sensitivity, selectivity, and stability compared to those previous ones.

![Figure 4](image1.png)

**Figure 4.** ZnAF-2 (3) and Zinpyr-1 (4).

In addition to the progress in the PET type zinc ion sensors, several ICT probes were also developed for detecting zinc ion ratiometrically. For example, Nagano et al. reported an electron-withdrawing benzofuran derivative (5) (Figure 5) in the design of a ratiometric Zn$^{2+}$ probe.$^{13}$ Brückner et al. reported the coumarin-derived ratiometric sensor (6) (Figure 5) for the detection of zinc ion.$^{14}$

![Figure 5](image2.png)

**Figure 5.** Benzofuran-derived Zn$^{2+}$ sensor (5) and coumarin-derived Zn$^{2+}$ sensor (6).

Although impressive progress has been made in developing zinc ion probes in the past two decades, these reported probes have some disadvantages in terms of synthetic
accessibility, sensitivity, selectivity, optical compatibility with biological samples and in particular, the capacity to cover large concentration ranges of zinc ion.\textsuperscript{2, 6, 13}

Our group initiated a research project to address some of those challenges such as coverage of large concentration ranges of zinc and synthetic accessibility. In this thesis, the progress toward the development of highly sensitive zinc probes are described, which are easily accessible through a Cu(I)-catalyzed triazole ring formation.
2.1 Introduction

In this chapter, the development of triazolyl-containing, highly sensitive fluorescent probes for Zn\(^{2+}\) is described. As mentioned in Chapter 1, the free Zn\(^{2+}\) concentration can be as low as, or even lower than nanomolar. Thus, it is important to develop probe molecules with high affinity to Zn\(^{2+}\) to explore its biological functions.

Parkin, et al.\(^{15}\) reported their research on nitrogen-rich tridentate ligand tris(pyrazolyl)hydroborato as a mimic of the [(His)\(_3\)Zn\(^{II}\)-OH\(_2\)] motif (Figure 6) in the active site of carbonic anhydrase. It was found that the tris(pyrazolyl)hydroborato coordinates with Zn\(^{2+}\) with nanomolar affinity in t-BuOH. There are also other research reports on polydentate zinc-binding ligands with high affinities, such as acyclic N4-tripodal ligand tris(2-pyridylmethyl)amine (TPA).\(^{16}\) However, they are not easily adaptable to synthetic variations as evidenced by the few probe molecules derived from them.\(^{17-19}\)
both easy access to synthetic variations and highly sensitive zinc ion fluorescent probes containing 1,2,3-triazolyl moieties. Huisgen 1,3-dipolar cycloaddition that unites azide and alkyne has been reported as an effective means to form triazole. However, this triazole-forming cycloaddition requires elevated temperature, and results in a mixture of the 1,4 and 1,5 regioisomers, which limit the scope of this reaction. The Sharpless group reported a copper(I)-catalyzed reaction sequence which unites azides and terminal acetylenes to give only 1,4-disubstituted 1,2,3-triazoles, also known as “click reaction” (Figure 7).

According to Sharpless, the Cu(I) catalyst is better prepared in situ by reduction of a Cu(II) salt. The mechanistic proposal for the catalytic cycle is shown in Figure 8. It starts with formation of a Cu(I) acetylide species II via the π complex I, with no reaction...
with internal alkynes. Following azide displacement of one ligand (L), a copper acetylide-azide complex III will be generated. Then nucleophilic attack of acetylide carbon C(4) at N(3) of the azide gives a six-membered copper-containing intermediate IV. Subsequent ring contraction occurs by the association of N(1) lone pair of electrons with the C(5)-Cu π* orbital, which forms intermediate V. Protonation of V followed by dissociation of the product completes the catalytic cycle to regenerate the catalyst. This heterocycle-formation reaction joins small units—azide and a terminal alkyne together in high chemical yield, with 1,4-regiospecificity in a wide range of substrates.22

Figure 8. Proposed mechanistic steps in the alkyne-azide click reaction.22

2.2 Design and Hypothesis

We designed two tetradeinate Zn²⁺ coordination motifs which could chelate with Zn²⁺ to afford a five-membered (Figure 9A) and a six-membered coordination rings (Figure 9B), where N3 and N2 of a 1,2,3-triazolyl participate in binding, respectively.

In addition, 1,2,3-triazolyl groups in these probe molecules function as both chelating ligands and transmitters of a coordination event to the fluorophores. Changing the aryl
group attached to the 1,2,3-triazolyl can fulfill the synthetic variations of the probe molecules.

Figure 9. Two tetradentate Zn\(^{2+}\) coordination motifs featuring five-membered (A) and six-membered (B) coordination rings.

2.3 Results and Discussion

2.3.1 Synthesis of Triazolyl-Containing Tetradeテーテntate Coordination Motifs

The preparations of five-membered ring motifs are outlined in Scheme 1. Aryl azides were prepared by a reported diazotization procedure.\(^{23}\) Seven commercially available aryl amines 7-13 were used to react with NaNO\(_2\) and NaN\(_3\) to form aryl azides. In this diazotization procedure, good yields were obtained for compounds 9-13. The synthesis of 7 and 8 failed. Further studies and syntheses of 7 and 8 will be followed by others in our group. Compound 14 was synthesized from an S\(_{N2}\) reaction between propargyl bromide and di-(2-picolyl)amine. The click reactions between 14 and aryl azides afforded compounds 9-13. Two different types of Cu(I) catalysts were used in the click reactions.
Scheme 1. a) ice bath, HCl, NaNO$_2$, urea, NaN$_3$, 3 h; b) K$_2$CO$_3$, THF, r.t., 16h; c) t-BuOH, sodium ascorbate, Cu(OAc)$_2$ for 10-13.

Compound 9 was synthesized by uniting 14 and benzyl azide with heterogeneous copper-in-charcoal (Cu/C) catalyst, with 75% yield (Scheme 2).

Scheme 2. Heterogeneous copper-in-charcoal-catalyzed click reaction.

In the Lipshutz’s report,$^{24}$ Cu/C heterogeneous catalyst, which can be synthesized according to Scheme 3, can significantly accelerate the reactions between aryl azides and terminal alkynes with yields close to 100%. However, with this Cu/C catalyst, good yield was only obtained for 9. Regarding to other compounds, the yield for 10 was low. The Cu/C catalyst used in preparing 10 may have lost its efficiency after relatively long shelf-time compared with the freshly made materials used in preparing 9.

<table>
<thead>
<tr>
<th>charcoa</th>
<th>Cu(NO$_3$)$_2$</th>
</tr>
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<tbody>
<tr>
<td>1. mix in H$_2$O</td>
<td></td>
</tr>
<tr>
<td>2. ultrasound</td>
<td></td>
</tr>
<tr>
<td>3. filter/wash</td>
<td></td>
</tr>
<tr>
<td>4. dry</td>
<td></td>
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</table>

Scheme 3. Simplified preparation of Cu/C.$^{24}$
Consequently, Cu(OAc)$_2$ in H$_2$O/t-BuOH was used as the precatalyst which was converted into catalytically active Cu(I) *in situ* in the presence of sodium ascorbate. Satisfactory yields for 10-13 were obtained. Cu(I) and Cu(II) species in the reaction mixture can be easily separated from the chelating products by treating with EDTA during work up and subsequent alumina column chromatography.

The preparations of six-membered ring motifs are outlined in Scheme 4. The synthesis of compounds 17 and 18 were accomplished by others in our group. The click reactions between arylacetylene and 1-azidoacetaldehyde dimethylacetal produce compound 15 and 16, which undergo reductive amination with di(2-picolyl)amine to afford probes 17 and 18.

*Scheme 4.* a) NaN$_3$, 18-crown-6, DMF, 75 °C, 2 d; b) t-BuOH, Cu(OAc)$_2$, L-sodium ascorbate, 4-ethynylanisole, r.t., 16 h; c) TFA, reflux, 6 h; d) 1,2-dichloroethane, di-(2-picolyl)amine, r.t. 16 h; e) NaBH(OAc)$_3$, r.t., 6 h.

In summary, the ligands listed in Figure 10 were synthesized.
2.3.2 X-Ray Crystallography Studies on Zinc Coordination Complexes

The formation of the N3-anchored five-membered coordination ring upon binding with Zn$^{2+}$ was confirmed by the X-ray structures of [Zn(L)(AN)](ClO$_4$)$_2$ (Figure 11, L = 9, and Figure 12, L = 10; AN = acetonitrile). The bond lengths of the triazolyl-N3 and pyridyl nitrogen atoms to Zn$^{2+}$ are in the range of 2.02-2.04 Å.

Figure 11. ORTEP diagram of [Zn(9)(AN)]$^{2+}$ (50% probability ellipsoids). Perchlorate counterions are omitted for clarity.
2.3.3 $^1$H NMR Studies on Zinc Coordination Complexes

The formation of six-membered coordination rings was supported by $^1$H NMR binding study of 17.
As a result of the triazolyl coordination to Zn$^{2+}$, restricted rotation of N-CH$_2$-CH$_2$-N single bonds will change the $^1$H NMR spectra as following: the two triplets of H$_a$ assigned to the CH$_2$ adjacent to the tertiary nitrogen and H$_b$ assigned to the CH$_2$ adjacent to N1 of the triazolyl ring (4.50 and 3.01 ppm, Figure 13A), respectively, are transform of into two multiplets (4.50 and 3.35 ppm, Figure 13B). Due to deshielding from the coordinated tertiary nitrogen, the 3.01 ppm triplet of the free ligand of 17 undergoes downfield shift to 3.35 ppm upon coordinating Zn$^{2+}$, while the triplet at 4.50 ppm is not affected. For ligand 18, similar $^1$H NMR response was observed.

2.3.4 Quantum Yield Studies

The fluorescence quantum yields of 10, 11, 12 and 17 were studied in aqueous solutions (0.1 M KNO$_3$, 0.05 M HEPES, pH = 7.2). It can be calculated using Equation 1.

$$\Phi_u = \left(\frac{A_s F_u n^2}{A_u F_s n_0^2}\right) \Phi_s$$

Equation 1

Here, quinine sulfate ($\Phi_f = 0.546$, 0.5 M H$_2$SO$_4$), and 2-aminopyridine ($\Phi_f = 0.60$, 0.5 M H$_2$SO$_4$) were used as standards. In Equation 1, $A_s$ is the absorbance of the standard, $A_u$ is the absorbance of the sample at their respective excitation wavelengths. $F_s$ and $F_u$ are the corresponding integrated fluorescence intensity, and $n$ and $n_0$ are the refractive indexes of the solvents of the sample and standard, respectively. In addition, the absorbance of samples and references was kept below 0.05.

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<tr>
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<th>10</th>
<th>11</th>
<th>12</th>
<th>17</th>
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</thead>
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<td>$\Phi_f$(ligand)</td>
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<td>0.29</td>
<td>0.018</td>
<td>0.0013</td>
</tr>
<tr>
<td>$\Phi_f$(complex)</td>
<td>0.017</td>
<td>0.45</td>
<td>0.14</td>
<td>0.0036</td>
</tr>
</tbody>
</table>

Table 1. Quantum yields of 10, 11, 12 and 17 and their respective Zn$^{2+}$ complexes.

Fluorescence quantum yields were determined in water (HEPES: 50 mM, pH 7.2, KNO$_3$: 100 mM).
Using Equation 1 and the standards stated above, we obtained the quantum yields of 10, 11, 12 and 17 (Table 1), reported values were average values of multiple runs (> 2) at various ligand concentrations and excitation wavelengths.

The fluorescence quantum yields of those ligands were enhanced upon coordinating Zn$^{2+}$. Among those compounds, the enhancements of 10 and 12 (6.5- and 7.8-fold respectively) are larger than that of compound 11 (1.6-fold). The difference in enhancement may relate to the connectivity between the triazolyl and the aromatic group: the aromatic groups in compound 10 and 12 are directly attached to the triazolyl, while in compound 11, the 9-anthryl group is one methylene away from the triazolyl group. For compound 17, both fluorescence enhancement (2.8-fold) and emission band shift upon binding with Zn$^{2+}$ were observed.

2.3.5 Absorption and Fluorescence Spectrophotometric Studies

The absorption and fluorescence spectroscopic studies of 10 were conducted in acetonitrile (CH$_3$CN) solutions firstly. In the absorption titration, an CH$_3$CN solution of 10 (20 µM), Zn(OTf)$_2$ (80 µM) was titrated into an CH$_3$CN solution of 10 (20 µM). Absorbance increased with increasing [Zn$^{2+}$], and the absorbance band underwent subtle bathochromic shift upon binding with Zn$^{2+}$ from 256 nm to 265 nm (Figure 14A).

In the fluorescence titration, an CH$_3$CN solution of 10 (10 µM), Zn(OTf)$_2$ (40 µM) was titrated into a semi-micro quartz fluorometer cuvette (Starna®) containing an CH$_3$CN solution of 10 (10 µM). The samples were excited at 285 nm and emission spectra were collected (Figure 14B). In the absence of Zn$^{2+}$, 10 exhibited weak fluorescence. In the presence of Zn$^{2+}$, the fluorescence was turned on and the intensity increased with increasing [Zn$^{2+}$]. The emission band underwent subtle bathochromic shift upon binding with Zn$^{2+}$.

By plotting $I_{419}$ vs. [Zn]/[10] (Figure 15), the fluorescence intensity of 10 was approaching saturation in the presence of two equivalents of Zn$^{2+}$. 
Figure 14. The UV and fluorescence titration of 10 with Zn$^{2+}$ in CH$_3$CN. (A) The UV titration of 10 (20 µM), in the presence of Zn(OTf)$_2$ (0-40 µM) in CH$_3$CN at 25 °C. Compound 10 was delivered using a stock solution of 10 in DMSO (2.85 mM). Therefore, the sample contained 0.7% DMSO. (B) The fluorescence titration of 10 (10 µM, $\lambda_{ex}$ = 285 nm) in CH$_3$CN in the presence of Zn(OTf)$_2$ (0-20 µM) at 25 °C. Compound 10 was delivered using a stock solution of 10 in DMSO (2.85 mM). Therefore, the sample contained 0.4% DMSO.

Figure 15. The analysis of fluorescence titration of 10 with Zn$^{2+}$ in CH$_3$CN: intensity at 419 nm versus [Zn]/[10].

The UV and fluorescence spectroscopic studies of 10 were also conducted in an CH$_3$CN solution containing DIPEA as the scavenger for H$^+$, and TBAP for controlling ionic strength.

In the UV titration, an CH$_3$CN solution of 10 (20 µM), Zn(OTf)$_2$ (83 µM), DIPEA
(19.2 µM), and TBAP (4.8 mM) was titrated into an CH$_3$CN solution of 10 (20 µM), DIPEA (19.2 µM), and TBAP (4.8 mM). Absorbance increased with increasing [Zn$^{2+}$] (Figure 16A).

In the fluorescence titration, an CH$_3$CN solution of 10 (4.9 µM), Zn(OTf)$_2$ (19.4 µM), DIPEA (5.5 µM), and TBAP (4.8 mM) was titrated into a semi-micro quartz fluorometer cuvette (Starna®) containing an CH$_3$CN solution of 10 (4.9 µM), DIPEA (5.5µM), and TBAP (4.8 mM). The samples were excited at 285 nm and emission spectra were collected (Figure 16B). In the absence of Zn$^{2+}$, 10 exhibited weak fluorescence. After addition of Zn$^{2+}$, the fluorescence was turned on; the intensity increased greatly with increasing [Zn$^{2+}$] until it reached saturation when the concentration of Zn$^{2+}$ was near 2 equiv of the concentration of 10 (Figure 17).

**Figure 16.** The UV and fluorescence titration of 10 in CH$_3$CN with DIPEA and TBAP. (A) The absorption spectra of 10 (20 µM), in CH$_3$CN with DIPEA (19.2 µM), and TBAP (4.8 mM) in the presence of Zn(OTf)$_2$ (0-41µM) at 25 °C. Compound 10 was delivered using a stock solution of 10 in DMSO (2.85 mM). Therefore, the sample contained 0.7% DMSO. (B) The fluorescence spectra of 10 (4.9 µM, $\lambda_{ex}$ = 285 nm), in CH$_3$CN with DIPEA (5.5 µM), and TBAP (4.8 mM) in the presence of Zn(OTf)$_2$ (0-9.7µM) at 25 °C. Compound 10 was delivered using a stock solution of 10 in DMSO (2.85 mM). Therefore, the sample contained 0.2% DMSO.
The fluorescence titration of 10 was further conducted in aqueous buffer solution of HEPES which is suitable for buffering in the physiological pH range of 7.2 - 7.6.

An aqueous solution of 10 (4.9 µM), Zn(OTf)$_2$ (9.7 µM), HEPES (50 mM, pH = 7.2) was titrated into a semi-micro quartz fluorometer cuvette (Starna©) containing an aqueous solution of 10 (4.9 µM), HEPES (50 mM, pH = 7.2) at 25°C. The sample was excited at 285 nm and emission spectra was collected (Figure 18A). In the absence of Zn$^{2+}$, 10 exhibited weak fluorescence. After addition of Zn$^{2+}$, the fluorescence was turned on, the intensity increased with increasing [Zn$^{2+}$]. The fluorescence intensity appeared to saturate when the concentration of Zn$^{2+}$ was over 1 equiv of the concentration of 10. The emission band showed bathochromic shifts upon binding with Zn$^{2+}$ from 350 nm to 400 nm. The emission intensity at wavelength 430 nm were plotted against the concentration ratio of zinc ion and 10 (Figure 18B). The fluorescence intensity increased with increasing [Zn]/[10], and reached a plateau around 1 at the X axis, which suggested the formation of a 1:1 complex between Zn$^{2+}$ and 10.
**Figure 18.** The fluorescence titration of 10 with Zn$^{2+}$ in aqueous buffer containing HEPES. Fluorescence titration of 10 (4.9 µM) with Zn(OTf)$_2$ (0-4.8 µM) in aqueous solution (HEPES: 50 mM, pH = 7.2) at 25 °C. Compound 10 was delivered using a stock solution of 10 in DMSO (2.85 mM). Therefore, the sample contained 0.2% DMSO. (A) Fluorescence titration spectra; (B) Intensity at 430 nm versus [Zn]/[10].

As mentioned in Chapter 1, coordination-modulated photoinduced electron transfer (PET) is a commonly used mechanism of fluorescence signal change of Zn$^{2+}$ probes. And it might be the mechanism for probe 10 too (Figure 19). The probe molecule 10 consists of a fluorophore which is the methoxyphenyl group, a Zn$^{2+}$ chelator which is the triazolyl di-2-picolylamino group. Before binding with Zn$^{2+}$, the fluorophore is only weakly emissive because PET provides a major non-radiative relaxation pathway. Zn$^{2+}$ coordination to the chelator unit especially the tertiary amino group, makes it less efficient electron donor to the attached fluorophore, thus the fluorescence is turned on after binding with Zn$^{2+}$. 
Based on the data analysis of the fluorescence spectroscopic studies in aqueous buffer solution of 10, which showed 1:1 binding ratio between Zn$^{2+}$ and ligand 10, we conducted the UV and fluorescence spectroscopic studies of 13 directly in aqueous buffer solution instead of CH$_3$CN.

In the UV titration, an aqueous solution of 13 (20.4 µM), Zn(OTf)$_2$ (79.6 µM), HEPES (50 mM, pH = 7.2) was titrated into an aqueous solution of 13 (20.4 µM), HEPES (50 mM, pH = 7.2). The absorbance decreased with increasing [Zn$^{2+}$] (Figure 20A). In the fluorescence titration, the aqueous solution of 13 (5.4 µM), Zn(OTf)$_2$ (22.7 µM), HEPES (50 mM, pH = 7.2) was titrated into an aqueous solution of 13 (5.4 µM), HEPES (50 mM, pH = 7.2) at 25 °C. The sample was excited at 365 nm and emission spectra were collected (Figure 20B). The free ligand 13 showed a strong fluorescence in the absence of Zn$^{2+}$, and the fluorescence intensity decreased with increasing [Zn$^{2+}$], which was unexpected. The emission band underwent a bathochromic shifts upon binding with Zn$^{2+}$.
Figure 20. The UV and fluorescence titration of 13 with Zn$^{2+}$ in aqueous buffer solution containing HEPES. (A) The absorption spectra of 13 (20.4 µM) with Zn(OTf)$_2$ (0-39.8 µM) in aqueous solution (HEPES: 50 mM, pH = 7.2) at 25 °C. Compound 13 was delivered using a stock solution of 13 in DMSO (447.8 µM). Therefore, the sample contained 4.5% DMSO. (B) The fluorescence spectra of 13 (5.4 µM, $\lambda_{ex}$ = 365 nm) with Zn(OTf)$_2$ (0-11.7 µM) in aqueous solution (HEPES: 50 mM, pH = 7.2) at 25 °C. Compound 13 was delivered using a stock solution of 13 in DMSO (447.8 µM). Therefore, the sample contained 1.2% DMSO.

The emission intensity at wavelength 410 nm was plotted against the concentration ratio of Zn$^{2+}$ and 13 (Figure 21). The fluorescence intensity decreased with increasing [Zn]/[13], and reached a plateau around 1 at the X axis, which indicated the 1:1 binding between zinc ion and 13.

Figure 21. The analysis of the fluorescence titration of 13 in aqueous buffer solution containing HEPES: intensity at 410 nm versus [Zn]/[13].
2.3.6 Determination of Dissociation Constants

The fluorescence titration data of 10 depicted in Figure 18B shows that Zn\(^{2+}\) binds very tightly to 10 in the HEPES buffer solution. In this titration process, Zn\(^{2+}\) was quantitatively bound to the ligand 10 until 1 equivalent Zn\(^{2+}\) was added. The ratio of the formed complex and the added Zn\(^{2+}\) which is defined as the fractional saturation is always larger than 99%. According to O’Halloran et al.,\(^{25}\) it is not possible to extract reliable K\(_d\) values when the fractional saturation is less than 20% or more than 80%. The measurement of the dissociation constant between Zn\(^{2+}\) and a free ligand can be accomplished by using a second ligand-EGTA, which has well tuned metal affinity. The free Zn\(^{2+}\) concentration is controlled by the EGTA buffer. The Zn\(^{2+}\) binding affinity of EGTA is pH and ionic strength dependent, therefore the titration system must have a fixed pH and ionic strength. In this study, pH = 7.2 was chosen, and the ionic strength was controlled by 0.1M KNO\(_3\).

The dissociation constants (K\(_d\)) of the Zn\(^{2+}\) complexes of 10, 11, 12 and 17 were determined by fitting the fluorescence titration data with a 1:1 association equation.

\[
I = (I_{\text{min}}K_d + I_{\text{max}}[\text{Zn}])/(K_d + [\text{Zn}]) \quad \text{Equation 2}
\]

When the free Zn\(^{2+}\) concentrations are controlled by metal ion buffers (e.g. EGTA in this study), equation 2 was used to fit titration curves using a 1:1 association model. It can be derived as following:

\[
[ZnP]^{2+} \rightleftharpoons Zn^{2+} + P \quad K_d
\]

The total concentration of the probe is P\(_t\). The equilibrated concentrations of Zn\(^{2+}\), P, and [ZnP]\(^{2+}\) following each titration are [Zn], [P], and [ZnP], respectively.

\[
K_d = [\text{Zn}][P]/[\text{ZnP}] = [\text{Zn}](P_t - [\text{ZnP}])/[\text{ZnP}]
\]

\[
[ZnP] = [\text{Zn}]P_t/(K_d + [\text{Zn}])
\]

When the absorbance of all the species is much less than 0.1, the fluorescence intensity is linearly proportional to the concentration of fluorescing species which are free ligand P
and Zn$^{2+}$ complex ZnP in our studies. Define:

$I_{\text{min}} = k_1 P_t$ ($I_{\text{min}}$ is the fluorescence intensity of the free probe, $k_1$ is the proportional constant)

$I_{\text{max}} = k_2 P_t$ ($I_{\text{max}}$ is the fluorescence intensity of the probe/Zn$^{2+}$ complex, $k_2$ is the proportional constant)

$I = k_1(P_t - [\text{ZnP}]) + k_2[\text{ZnP}]$

$I = k_1 P_t - k_1[Zn]P_t/(K_d + [Zn]) + k_2[Zn]P_t/(K_d + [Zn])$

$I = I_{\text{min}} - [\text{Zn}]I_{\text{min}}/(K_d + [Zn]) + I_{\text{max}}[Zn]/(K_d + [Zn])$

$I = (I_{\text{min}}K_d + I_{\text{max}}[Zn])/(K_d + [Zn])$

By fitting the fluorescence titration data with Equation 2, the dissociation constants ($K_d$) of the Zn$^{2+}$ complexes of 10, 11, 12 and 17 were determined (Table 2). The dissociation constants of all those ligands were in the nanomolar range, which matches the basal level of intracellular free Zn$^{2+}$ concentrations.

**Table 2.** Dissociation constants ($K_d$) of ligands 10, 11, 12 and 17

<table>
<thead>
<tr>
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<th>10</th>
<th>11</th>
<th>12</th>
<th>17</th>
</tr>
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<tbody>
<tr>
<td>$K_d$/nM</td>
<td>3.5 ± 0.2</td>
<td>5.7 ± 1.1</td>
<td>12 ± 1.7</td>
<td>7.2 ± 0.4</td>
</tr>
</tbody>
</table>

Dissociation constants ($K_d$) were determined in water (HEPES: 50 mM, pH = 7.2; KNO$_3$: 100 mM, EGTA: 10 mM).

### 2.3.7 Metal Ion Selectivity

Metal ion selectivity of 12 and 17 were studied as the representatives of other probes which form five- and six-membered coordination rings upon binding with zinc ion, respectively. The results are shown in Figure 22. The selectivity was tested using divalent
ions $\text{Ca}^{2+}$, $\text{Mg}^{2+}$, $\text{Cd}^{2+}$, $\text{Fe}^{2+}$, $\text{Mn}^{2+}$, $\text{Pb}^{2+}$, $\text{Cu}^{2+}$, and $\text{Co}^{2+}$. Compound 17 showed better selectivity toward $\text{Zn}^{2+}$ than compound 12. Both compounds 12 and 17 showed little or no fluorescence enhancement in the presence of $\text{Ca}^{2+}$, $\text{Mg}^{2+}$, $\text{Fe}^{2+}$, $\text{Mn}^{2+}$, and $\text{Pb}^{2+}$. $\text{Cu}^{2+}$ and $\text{Co}^{2+}$ were able to coordinate with 12 and 17 and to quench the fluorescence. $\text{Cd}^{2+}$ which is known as a stereoelectronic isostere of $\text{Zn}^{2+}$ coordinated with 12 strongly to result in a large fluorescence enhancement; while it showed significantly weaker coordination with 17. Additionally, monovalent $\text{Na}^{+}$ and $\text{K}^{+}$ were present in the buffer and they did not interfere with the association of the probes with divalent metal ions and the fluorescence modulations.

Figure 22. Metal ion selectivities of compounds 12 and 17. (A) 12 (2.5 $\mu$M, $\lambda_{ex} = 300$ nm) and (B) 17 (2.3 $\mu$M, $\lambda_{ex} = 263$ nm) to divalent metal ions (4.5 $\mu$M) in the absence of $\text{Zn}^{2+}$ (white bars) and in the presence of $\text{Zn}^{2+}$ (4.5 $\mu$M, black bars). All samples were measured in H$_2$O (HEPES: 50 mM, pH = 7.2; KNO$_3$: 100 mM) at 25 °C. Y-axes represent the integrated fluorescence intensity in arbitrary units from (A) 360 nm to 575 nm and (B) 300 nm to 500 nm, respectively.
2.4 Experimental Section

2.4.1 General Information

Reagents and solvents were purchased from various commercial sources and used without further purification unless otherwise stated. Water used in titration experiments were deionized using Barnstead NANO pure Diamond water system. CH$_3$CN (OmniSolv, EMD) and DMSO (ACS Reagent, > 99.9%, Sigma-Aldrich) were directly used in titration experiments without purification. All reactions were carried out under an inert atmosphere of dry argon in oven- or flame-dried glassware. Analytical thin-layer chromatography (TLC) was performed using precoated TLC plates with silica Gel 60 F254 (EMD) or with aluminum oxide 60 F254 neutral. Flash column chromatography was performed using 40-63 µm (230-400 mesh ASTM) silica gel (EMD) as the stationary phase and alumina gel (80-200 mesh EMD). Tetrahydrofuran (THF) was dried by distilling from sodium–benzophenone in a continuous still under an atmosphere of argon. Proton magnetic resonance spectra ($^1$H NMR) were recorded at 300 MHz on Varian Mercury spectrometer. Carbon magnetic resonance spectra ($^{13}$C NMR) were recorded at 75 MHz on a Varian Mercury spectrometer. All chemical shifts were reported in $\delta$ units relative to tetramethylsilane. Chloroform-$d$ was treated with alumina gel prior to use. High resolution mass spectral data were obtained at the Mass Spectrometry Laboratory at FSU: ESI spectra were obtained on a JEOL AccuTof spectrometer; CI spectra were obtained on a JEOL JMS600H spectrometer. Spectrophotometric and fluorometric tritrations were conducted on a Varian Cary 100 Bio UV-Visible Spectrophotometer and a Varian Cary Eclipse Fluorescence Spectrophotometer, respectively. Arylazides were prepared by diazotization of respective arylamines using a published procedure.$^{23}$ The
ionic strengths (0.1) in the titration experiments were assumed to be controlled solely by 100 mM KNO$_3$. The impact of HEPES and EGTA, both of which are relatively weak electrolytes, on ionic strength was not considered. Furthermore, the K$_d$ dependence on ionic strength in the range from 0.1 to 0.2 is negligible under the reported experimental conditions.

**The cuvette and syringe cleaning:**

Using clean, airtight syringes and cuvettes is key to obtaining reproducible data in the following metal ion titration experiments. After usage and removal of the residue solutions, an EDTA (0.1 M) solution is drawn into the syringes and kept for over 10 minutes in order to remove any residue metal ions. The plunger and jacket are then separated and air dried.

Spectrophotometric and fluorometric cuvettes are filled with EDTA (0.1 M) for 10 minutes followed by deionized water and acetone washing. The cuvettes are then air-dried. Base bath (KOH in isopropanol) is used as needed for removing organic residues. Apply special caution when a sample of low fluorescence quantum yield is measured in a cuvette that contained samples of high fluorescence quantum yields in the prior measurements.

**2.4.2 Synthesis**

![Chemical Structure](image)

**N-PROPARGYL-DI-(2-PICOLYL)AMINE (14)** Di-(2-picoly)amine (5.0 mmol, 900 µL) was dissolved in THF (10 mL). K$_2$CO$_3$ (20 mmol, 2.76 g) was added followed by dropwise addition of propargyl bromide (80% in toluene, 5.0 mmol, 557 µL). The reaction mixture was stirred for 16 h before diluted with DCM. The diluted reaction mixture was filtrated through a pad of K$_2$CO$_3$ under vacuum before concentrated. Compound **14** was isolated.
in 96% yield from an alumina column eluted by EtOAc in DCM (0% - 40%). $^1$H NMR (300 MHz, CDCl$_3$): $\delta$/ppm 8.57 (d, $J = 4.2$ Hz, 2H), 7.66 (td, $J = 1.2, 7.8$ Hz, 2H), 7.52 (d, $J = 7.8$ Hz, 2H), 7.17 (td, $J = 5.4, 7.2$ Hz, 2H), 3.93 (s, 4H), 3.43 (d, $J = 2.4$ Hz, 2H), 2.30 (t, $J = 2.4$ Hz, 1H); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$/ppm 159.00, 149.50, 136.72, 123.40, 122.35, 73.87, 59.70, 42.79; HRMS (ESI): calcd. (M+Na$^+$) 260.1164, found 260.1159.

**COMPOUND 9.** Cu/C (25 mg) was suspended in dioxane (1 mL), followed by the addition of triethylamine (70 µL). The reaction mixture was stirred at r. t. Benzyl azide (0.5 mmol, 70 µL) and 14 (0.5 mmol, 129 mg) were added into the reaction mixture subsequently. The reaction mixture was stirred at r. t. for 2 h, then filtered through a pad of celite to remove the catalyst. The filter cake was further washed with EtOAc to ensure complete transfer. The volatiles were removed in vacuo to get the crude product. The crude product was purified on a basic alumina column eluted by EtOAc in hexanes (0% - 30%). The yield was 75%. $^1$H NMR (300 MHz, CDCl$_3$): $\delta$/ppm 8.51 (dd, $J = 1.2, 4.2$ Hz, 2H), 7.63 (td, $J = 1.8, 7.8$ Hz, 2H), 7.55 (s, 1H), 7.52 (d, $J = 4.8$ Hz, 2H), 7.35 (m, 3H), 7.26 (m, 2H), 7.13 (m, 2H), 5.52 (s, 2H), 3.88 (s, 2H), 3.83 (s, 4H); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$/ppm 159.30, 149.13, 144.87, 136.52, 134.95, 129.14, 128.70, 128.01, 123.34, 123.05, 122.08, 59.71, 54.10, 48.77; HRMS (ESI): calcd. (M+H$^+$) 371.1979, found 371.1965.

**COMPLEX [Zn(9)(AN)](ClO$_4$)$_2$.** A solution of 9 in CH$_3$CN (0.1 M, 1.0 mL) was mixed
with a solution of Zn(ClO$_4$)$_2$ in CH$_3$CN (0.1 M, 1.0 mL). Solvent was removed under vacuum. The product was washed with Et$_2$O (5 mL × 3) before dried under vacuum. $^1$H NMR (300 MHz, CD$_3$CN): δ/ppm 8.74 (d, J = 5.4 Hz, 2H), 8.14 (dd, J = 1.2, 7.8 Hz, 2H), 7.98 (s, 1H), 7.68 (t, J = 6.0 Hz, 2H), 7.60 (d, J = 8.4 Hz, 2H), 7.38 (m, 5H), 5.63 (s, 2H), 4.24 (AB system, J$_{AB}$ = 17.1 Hz, 4H), 4.12 (s, 2H).

**COMPOUND 10.** This is a common procedure for the preparation of compounds 10-13. 1-azido-4-methoxybenzene (1.0 mmol, 149 mg) and 14 (1.0 mmol, 237 mg) were dissolved in t-BuOH (2.5 mL). Aqueous sodium ascorbate solution (0.8 M, 2.5 mL) and Cu(OAc)$_2$ solution (0.4 M, 2.5 mL) were added dropwise sequentially. The reaction mixture was stirred at r.t. for overnight before EDTA (0.1 M, 10 mL) was added. The reaction mixture was stirred for another 30 min before pH was raised to 11. The aqueous reaction mixture was extracted using DCM (50 mL × 3). The organic extracts were combined and dried over K$_2$CO$_3$. Compound 10 was purified on a basic alumina column, eluted by EtOAc in DCM (0% - 100%). The yield was 60%. $^1$H NMR (300 MHz, CDCl$_3$): ppm 8.55 (d, J = 4.8 Hz, 2H), 7.97 (s, 1H), 7.71-7.58 (m, 6H), 7.16 (m, 2H), 7.02 (d, J = 9.0 Hz, 2H), 3.96 (s, 2H), 3.90 (s, 4H), 3.88 (s, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$): δ/ppm 159.90, 159.41, 149.32, 145.06, 136.68, 130.85, 123.54, 122.33, 122.26, 121.58, 114.92, 59.91, 55.83, 48.87; HRMS (ESI): calcd. (M+Na$^+$) 409.1752, found 409.1754.
**COMPLEX** [Zn(10)(OTf)](OTf). A solution of 10 in CH$_3$CN (0.1 M, 1.0 mL) was mixed with a solution of Zn(OTf)$_2$ in CH$_3$CN (0.1 M, 1.0 mL). Solvent was removed under vacuum. The product was washed with Et$_2$O (5 mL × 3) before dried under vacuum. $^1$H NMR (300 MHz, CD$_3$CN): δ/ppm 8.78 (d, J = 4.8 Hz, 2H), 8.34 (s, 1H), 8.16 (td, J$_t$ = 7.8 Hz, J$_d$ = 1.8 Hz, 2H), 7.72-7.68 (m, 4H), 7.64 (d, J = 7.8 Hz, 2H), 7.14 (d, J = 9.0 Hz, 2H), 4.33, 4.26 (AB system, J$_{AB}$ = 17.4 Hz, 4H), 4.21 (s, 2H), 3.87 (s, 3H). MS (ESI): calcd. (M+Zn$^{2+}$+OTf) 599.1, 601.1, 603.1, found 599.1, 601.1, 603.1.

**Copper-in-Charcoal-Catalyst.** Activated carbon (10.0 g, 100 mesh, 25% H$_2$O content) was added to a 500 mL round-bottomed flask containing a stir bar. A solution of Cu(NO$_3$)$_2$·3H$_2$O (2.207 g) in deionized water (20 mL) was added to the flask, and another 20 mL deionized water was added to wash down the sides of the flask. The flask was loosely capped and stirred under air for 30 min and then submerged in an ultrasonic bath for 7 h. Toluene was used to wash it. The catalyst was dried by vacuum filtration for 3 h. Finally, 15.2 g wet Cu/C was obtained. The yield was 89%.

### 2.5 Conclusion

1,2,3-triazolyl-containing fluorescent ligands for zinc ion were synthesized and studied. They show nanomolar sensitivity and high selectivity toward zinc ion in the presence of other divalent metal ions. Structural characterizations in both solid and solution supports the hypothesis that N3 and N2 in the 1,2,3-triazolyl coordinate with Zn$^{2+}$ in motifs A and B forming five- and six-membered coordination rings, respectively.
CHAPTER 3

A COUMARIN–CONTAINING, FLUORESCENT HETERODITOPIC LIGAND FOR ZINC ION

3.1 Introduction

The sensitivity of a Zn$^{2+}$ probe is determined by the affinity of the binding site within this probe toward Zn$^{2+}$. A high-affinity binding site may be completely occupied at low Zn$^{2+}$ concentration, which leads to fluorescence signal saturation. Thus, a highly sensitive probe usually results in a narrow effective concentration range, which makes it ineffective in imaging Zn$^{2+}$ over a large concentration range in live cells. This is an important issue for the determination of zinc’s concentration in a biological sample. As mentioned above, there are a lot of cases that the concentrations of Zn$^{2+}$ fluctuate spatially and temporally over large ranges in biological processes. Single fluorescent probes are difficult to cover this large concentration range of physiological Zn$^{2+}$. For example, Zinquin is responsive to subnanomolar of Zn$^{2+}$; however, it is fully saturated at 1µM Zn$^{2+}$. The Zinpyr family usually saturate around 20 nM of Zn$^{2+}$. Some combinations of probes with different affinities have been reported to resolve this problem. However, probe combination methods are laborious and lack of accuracy. Therefore, single probes that can cover the entire physiological range of Zn$^{2+}$ concentration will be superior.

In this chapter, one Zn$^{2+}$ probe with two binding sites - di-2-picolylamino and dihydroxycoumarin will be introduced, which is expected to meet the requirement of
covering large dynamic range of physiological $\text{Zn}^{2+}$.

### 3.2 Design and Hypothesis

The probe demonstrated in this chapter is expected to extend the effective concentration range of zinc ion detection by incorporating two binding sites with different affinities for $\text{Zn}^{2+}$ within one molecule. The high-affinity site which is the dipicolylamino fragment determines the sensitivity of this probe; the low-affinity site, the dihydroxycoumarin fragment extents the dynamic range of $\text{Zn}^{2+}$ detection.

The design of this probe was inspired by earlier reports of ditopic fluorescent molecules. One of the examples is Bunz’s cruciforms\textsuperscript{29} which show fluorescence modulations upon binding with metal ions at different concentrations in dichloromethane.

![Figure 23](image) The structure of a ditopic cruciform.

In the cruciform (Figure 23), the HOMO is located on the distyrylbenzene branch, and the LUMO is located on the bisarylethynyl branch. In the presence of $\text{Zn}^{2+}$, the coordination sites on the HOMO are occupied firstly, which results a large hypsochromic shift of the fluorescence emission spectra. Upon increasing $\text{Zn}^{2+}$ concentration, the coordination sites on the LUMO are also occupied, resulting in a shift of the spectra back...
to longer wavelengths.

Another example is from our group on heteroditopic fluorescent ligands with large dynamic ranges for Zn$^{2+}$ (Figure 24). Designed fluorescent ligands with a general structure of dipicolyl-arylviny1-bipy have a low fluorescence quantum yield in the absence of Zn$^{2+}$. The fluorescence is turned on in the presence of Zn$^{2+}$, and with increasing Zn$^{2+}$ concentration, the spectrum undergoes a bathochromic shift upon binding the second Zn$^{2+}$.

![Figure 24](image-url)  
**Figure 24.** Fluorescent heteroditopic ligands with two coordination sites for Zn$^{2+}$.

In my research, a new fluorescent probe was designed and expected to relate its three coordination states (non-, mono- and di-coordinated) to three fluorescence states as shown in Figure 25.

![Figure 25](image-url)  
**Figure 25.** A fluorescent probe with two Zn$^{2+}$ binding sites (rectangle: high-affinity site; oval: low-affinity site). Three fluorescence states: off, blue, and red represent the coordination states non-, mono-, and di-coordinated respectively.
The first step coordination determines the sensitivity of Zn\(^{2+}\) detection; the second step extends the concentration range of the analysis.

Probe 22 was designed to achieve the coordination and photophysical objectives shown in Figure 25. Incorporated into structure 22 are dipicolylamino group and dihydroxycoumarin as the high- and low-affinity sites, respectively (Figure 26). This probe molecule was designed to have low fluorescence in the absence of Zn\(^{2+}\). In the presence of Zn\(^{2+}\) at low concentration, Zn\(^{2+}\) will bind to the high-affinity dipicolylamino site, which is expected to result in a sensitive fluorescence enhancement. When the Zn\(^{2+}\) concentration is high enough, Zn\(^{2+}\) will bind to the low-affinity site dihydroxycoumarin. Consequently, the fluorescence emission is expected to undergo a bathochromic shift.\(^30\) The short emission wavelength of 22 is defined as the “blue” channel in the presence of Zn\(^{2+}\) at a low concentration; the long emission wavelength of 22 is defined as the “red” channel at a high Zn\(^{2+}\) concentration. The blue channel determines the sensitivity of the probe toward Zn\(^{2+}\), while the red channel determines the effective concentration range of the probe. This “two-channel probe” is expected to possess both high sensitivity and a large effective concentration range in one single molecule.

![Figure 26](image)

**Figure 26.** Fluorescence responses of 22 upon coordinating with one or two Zn\(^{2+}\). Arrows on the top of the structures represent excitation; arrows at the bottom of the structures represent emission at short (blue) and long (red) wavelengths, respectively.
In the absence of Zn$^{2+}$, 22 is designed to exhibit weak fluorescence because PET from the tertiary amino group is expected to quench the excited coumarin fluorophore.$^{30}$ In the presence of Zn$^{2+}$ at low concentration, less than one equivalent, coordination of dipicolylamino with Zn$^{2+}$ stops PET, thereby turning the fluorescence on. The mechanism can be hypothesized as following (Figure 27): in the absence of Zn$^{2+}$, the HOMO of the electron acceptor (the excited fluorophore-coumarin) accepts one electron from the HOMO of the electron donor-dipicolylamino which leads to fluorescence quenching. After Zn$^{2+}$ coordinates with dipicolylamino group, positively charged Zn$^{2+}$ stabilizes the donor’s HOMO, which makes its energy level lower than the HOMO energy level of fluorophore. Consequently, the PET process is terminated and fluorescence is restored.

![Diagram](image.png)

**Figure 27.** Mechanism of fluorescence signal change of 22 upon binding with Zn$^{2+}$ at dipicolylamino. (A) PET from dipicolylamino group quenches the fluorescence of the fluorophore. (B) Upon binding with Zn$^{2+}$, the fluorescence of the fluorophore is turned on.

According to our group’s previous pH profile studies of 4-methylesculetin, the pK$_a$ of the hydroxy group was estimated to be 8.3 (Figure 28).$^{30}$ Therefore, both hydroxyl group are expected to remain protonated in 22 at neutral pH.
4-methylesculetin

Figure 28. Deprotonation of 4-methylesculetin.

Coordination with dihydroxycoumarin to form \([\text{Zn}_2(22)]^{2+}\) is expected to occur at sufficiently high concentration of \(\text{Zn}^{2+}\), higher than one equivalent. Bathochromic shifts of 22 upon interacting with \(\text{Zn}^{2+}\) at dihydroxycoumarin sites are expected.\(^{30}\) The association of dihydroxycoumarin with \(\text{Zn}^{2+}\) promotes the deprotonation of the two hydroxyl group from a neutral molecule to a coordinated dianion at \(\text{pH} = 7\) (Figure 29). At neutral pH, 22 will be fully protonated. \(\text{Zn}^{2+}\)-coordination-promoted deprotonation will elevate electron density, which decreases the energy gap between HOMO and LUMO of the \(\pi-\pi^*\) transition.\(^{30}\)

Figure 29. Metal ion-coordination-promoted deprotonation may be the cause of the bathochromic shift of the spectra of 22.

The synthesis of probe 22 is reported in this thesis. The further photophysical studies of this probe will be conducted by others in our group.
3.3 Experimental Section

3.3.1 General Information

It is the same as that in Chapter 2.

3.3.2 Synthesis

Scheme 5. a) Two drops of H$_2$SO$_4$ (98%), r.t. 16 h; b) DCM, DIPEA, ice bath, 16 h; c) THF, Bu$_4$NI, DIPEA, ice bath; d) CH$_3$OH, stir, HCl, 62°C, reflux.

**COMPOUND 24.** 23$^{31}$ (3 mmol, 0.869 mL) and 1,2,4-benzenetriol (3 mmol, 0.378 g) were charged into a round bottom flask, and stirred to homogeneous mixture. Then two drops of H$_2$SO$_4$ (98%) were added. The reaction mixture was stirred at r.t. over night. The reaction mixture was diluted by EtOAc and dried over Na$_2$SO$_4$. The solvent was carefully removed under vacuum at r.t. to obtain the crude product, which was used directly for the next step. $^1$H NMR (300 MHz, DMSO-d6): $\delta$/ppm 10.38 (s,1H), 9.47 (s,1H), 7.15 (s, 1H), 6.77 (s, 1H), 6.44 (s, 1H), 6.75 (s, 2H).
**COMPOUND 25.** Crude 24 (1.0 mmol, 0.271 g) was dissolved in dry DCM (10 mL), followed by dropwise addition of DIPEA (0.653 mL). The reaction mixture was put in ice bath before methoxy methylchloride (4.0 mmol, 0.304 mL) was added dropwise and stirred at r.t. over night. The reaction mixture was extracted by using DCM (30 mL × 3). The organic extracts were combined and dried over Na₂SO₄. The solvent was removed under vacuum at r.t. Compound 25 was isolated in 16.4% yield over 2 steps from a silica column eluted by EtOAc in DCM (0% - 5%). ¹H NMR (300 MHz, CDCl₃): δ/ppm 7.41 (s, 1H), 7.21 (s, 1H), 6.46 (s, 1H), 5.32 (s, 2H), 5.28 (s, 2H), 4.63 (s, 2H), 3.56 (s, 3H), 3.53 (s, 3H).

**COMPOUND 26.** Catalyst Bu₄NI was suspended in THF (3.5 mL) after addition of 25 (0.34 mmol, 0.123 g). The reaction mixture was put in ice bath before DIPEA (1.376 mmol, 0.25 mL) was added dropwise. Di-(2-picolyl)amine (0.69 mmol, 0.124 mL) was added sequentially. The reaction mixture was stirred at r.t. for overnight. The reaction mixture was extracted by DCM, washed by NaOH (1.0 M), and dried over Na₂SO₄. Compound 26 was purified on a basic aluminacolumn eluted by EtOAc in DCM (5% - 10%). The yield was 22%. ¹H NMR (300 MHz, CDCl₃): δ/ppm 7.36 (d, J = 4.8Hz, 2H), 7.05 (s, 1H), 6.94 (t, J = 7.2Hz, 2H), 6.77 (d, J = 7.8 Hz, 2H), 6.44 (t, J = 5.7 Hz, 2H), 6.26 (s, 1H), 5.77 (s, 1H), 4.55 (s, 2H), 4.49 (s, 2H), 3.15 (s, 2H), 3.09 (s, 4H), 2.74 (s, 3H), 2.69 (s, 3H).
COMPOUND 22. Compound 26 (0.074 mmol, 35.5 mg) was dissolved in CH$_3$OH (4 mL) before 2 drops of HCl (37%) were added. The reaction mixture was refluxed at 62 °C for 30 min. The reaction solvent was removed by vacuum at r.t. 26 was converted into 22 quantitatively. $^1$H NMR (300 MHz, CD$_3$OD): δ/ppm 8.76 (d, J = 6.6Hz, 2H), 8.23 (t, J = 9.6 Hz, 2H), 7.80 (d, J = 8.4Hz, 2H), 7.70 (m, 2H), 7.00 (s, 1H), 6.60 (s, 1H), 6.34 (s, 1H), 4.46 (s, 4H), 4.02 (s, 2H).

3.4 Conclusion

A fluorescent probe for zinc ion that is expected to be effective over a large concentration range was designed. Two different binding sites dipicolylamine and dihydroxycoumarin groups within the single probe molecule are expected to enable the correlation of three coordination states to three fluorescence states, which can be adapted for covering relatively large concentration ranges of Zn$^{2+}$ detection. The combined modulation of PET and ICT was utilized to afford the analytical strategy: in the presence of Zn$^{2+}$ fluorescence is expected to be turned on. When the concentration of Zn$^{2+}$ increases across a relatively broad range, a red shift of the emission band is expected.
CHAPTER 4

PYRENE-BASED TETRADENTATE FLUORESCENT LIGANDS FOR ZINC ION

4.1 Introduction

The final purpose of the research on Zn$^{2+}$ probes is to make zinc visible in living cells. In practical utilization, many factors determine the performance of Zn$^{2+}$ probes *in vivo*, including their sensitivity, selectivity, excitation and emission profiles, aqueous compatibility, etc.\textsuperscript{6} We have carried out research to address some of these challenges that were described in the previous two chapters, such as binding affinity and selectivity. However, the currently available Zn$^{2+}$ probes in our laboratory have several limitations which impede applications in biological samples.\textsuperscript{2,6} Most of them have short excitation wavelengths, usually in UV range, which can be deleterious to living cells. Also, auto fluorescence from the biomolecules can be produced upon UV irradiation, causing inaccurate data collection.

Development of fluorescent probes for Zn$^{2+}$ with long wavelengths is attracting more attention from researchers. For example, Kazuki et al.\textsuperscript{32} reported a new near-infrared region (NIR) fluorescent probe for Zn$^{2+}$ based on tricarbocyanine chromophore (Figure 30), which has an absorbance band centered at 627 nm. Although this probe has good biological compatibility, the dissociation constant $K_d$ for this probe is around 100 nM,
much higher than the probes we reported in Chapter 2.

![Figure 30](image)

**Figure 30.** Tricarbocyanine-based fluorescent probe for Zn\(^{2+}\).

Based on the good results we obtained previously – high sensitivity, probe synthetic variation, and aqueous compatibility, we are now aiming to expand this methodology to other aromatic groups, presumably ones with long excitation and emission wavelengths.

In the past decades, pyrene moiety has attracted a lot of attention from researchers due to their advantageous fluorescent properties.\(^{33,34}\) For example, pyrene has strong UV-Vis absorbance spectra between 310 and 340 nm, and emission spectra between 360 nm and 380 nm.\(^{35}\)

In this chapter, pyrene will be introduced as the first example of expanding this methodology, where triazolyl group is used as both metal chelator and modulator of fluorescence.

### 4.2 Design and Hypothesis

The probes in this chapter incorporate both 1,2,3-triazolyl moiety and pyrene. Similarly to the probes introduced in Chapter 2, structure 28 and 29 were expected to chelate with Zn\(^{2+}\) to afford five-membered and six-membered coordination rings (Figure 31).
Figure 31. Compounds 28 and 29 chelate with Zn$^{2+}$ to form six- and five-membered coordination rings, respectively.

4.3 Results and Discussion

4.3.1 Synthesis

For the synthesis of compound 29, diazotization of 1-aminopyrene afforded 30 which underwent the click reaction with 14 to afford molecule 29. For compound 28, the synthetic work was completed by others in our group. Its photophysical studies will be introduced in this thesis.

Figure 32. Synthesis of compound 29.
4.3.2 Absorption and Fluorescence Spectrophotometric Studies of 29

The UV and fluorescence spectroscopic studies of 29 were conducted in aqueous solutions with and without surfactant gamma-cyclodextrin (γ-CD).

The UV and fluorescence studies of 29 in an EGTA buffer solution was conducted firstly. In the UV titration, a buffered solution of 29 (2.4 µM), Zn(OTf)$_2$ (9.1 mM), EGTA (10 mM), HEPES (50 mM, pH = 7.2), KNO$_3$ (100 mM) was titrated into a buffered solution of 29 (2.4 µM), EGTA (10 mM), HEPES (50 mM, pH = 7.2), KNO$_3$ (100 mM). Absorbance decreased with increasing [Zn$^{2+}$] (Figure 33A).

In the fluorescence titration, a buffered solution of 29 (2.4 µM), Zn(OTf)$_2$ (19.7 mM), EGTA (10 mM), HEPES (50 mM, pH = 7.2), KNO$_3$ (100 mM) was titrated into a semi-micro quartz fluorometer cuvette (Starna®) containing a solution of 29 (2.4 µM), EGTA (10 mM), HEPES (50 mM, pH = 7.2), KNO$_3$ (100 mM). The samples were excited at 341 nm and emission spectra were collected (Figure 33B). In the absence of Zn$^{2+}$, 29 exhibited weak fluorescence, which was enhanced with increasing [Zn$^{2+}$]. A broad excimer peak grew around 430 nm, as a result of Zn$^{2+}$ addition.

![Figure 33](image-url)  
**Figure 33.** The UV and fluorescence titration of 29 with Zn(OTf)$_2$ in aqueous buffer solution containing EGTA, KNO$_3$, and HEPES. (A) The absorption spectra of 29 (2.4 µM) with Zn(OTf)$_2$ (0 - 4.0 mM) in aqueous solution (EGTA: 10 mM, KNO$_3$: 100 mM, HEPES: 50 mM, pH = 7.2) at 25 °C. (B) The fluorescence spectra of 29 (2.4 µM, $\lambda_{ex} = 301$ nm) with Zn(OTf)$_2$ (0 - 9.8 mM) in aqueous solution (EGTA: 10 mM, KNO$_3$: 100 mM, HEPES: 50 mM, pH = 7.2) at 25 °C. Compound 29 was delivered using a stock solution of 29 in DMSO (2.40 mM). Therefore, the sample contained 0.1%
The fluorescence study of 29 was further conducted in the HEPES buffer solution with surfactant \( \gamma \)-CD, which was expected to stop the aggregation of 29. In the fluorescence titration, an aqueous solution of 29 (2.40 \( \mu \)M), Zn(OTf)\(_2\) (9.1 \( \mu \)M), \( \gamma \)-CD (2.8 \( \mu \)M), HEPES (50 mM, pH = 7.2), KNO\(_3\) (100 mM) was titrated into a semi-micro quartz fluorometer cuvette (Starna\textsuperscript{®}) containing an aqueous solution of 29 (2.4 \( \mu \)M), \( \gamma \)-CD (2.8 \( \mu \)M), HEPES (50 mM, pH = 7.2), KNO\(_3\) (100 mM) at 25 °C. The sample was excited at 341 nm and emission spectra were collected (Figure 34A). In the absence of Zn\(^{2+}\), 29 exhibited strong fluorescence, and the excimer peak was not obvious. After addition of Zn\(^{2+}\), the fluorescence intensity of complex 29 decreased. In contrary to the observations in the absence of \( \gamma \)-CD, there was a reduced broad peak around 420 nm. It appeared that \( \gamma \)-CD cannot stop the aggregation fully or the concentration of \( \gamma \)-CD was not sufficient enough to break the excimer. The reason why the fluorescence intensity decreased in the presence of \( \gamma \)-CD is hard to explain now, but further studies will be continued.

The emission intensity at wavelength 402 nm were plotted against the concentration ratio of Zn\(^{2+}\) and 29 (Figure 34B). The fluorescence intensity decreased with the increase of [Zn]/[29] until [Zn]/[29] \( \approx \) 0.75.

**Figure 34.** The fluorescence titration of 29 (2.4 \( \mu \)M) with Zn(OTf)\(_2\) (0 - 4.0 \( \mu \)M) in aqueous solution containing \( \gamma \)-CD, KNO\(_3\), and HEPES (\( \gamma \)-CD: 2.8 \( \mu \)M, KNO\(_3\): 100 mM, HEPES: 50 mM, pH = 7.2) at 25 °C. Compound 29 was delivered using a stock solution of 29 in DMSO (2.40 mM). Therefore, the...
sample contained 0.1% DMSO. (A) Fluorescence titration spectra; (B) Intensity at 402 nm versus [Zn]/[29].

4.3.3 Absorption and Fluorescence Spectrophotometric Studies of 28

In order to study the reaction between 28 (Figure 31) and γ-CD, the fluorescence titration of compound 28 with γ-CD was studied firstly. In the UV titration, a buffered solution of 28 (5.4 μM), γ-CD (12.2 μM), HEPES (50 mM, pH = 7.2), KNO₃ (100 mM) was titrated into a buffered solution of 28 (5.4 μM), HEPES (50 mM, pH = 7.2), KNO₃ (100 mM). Absorbance decreased with increasing γ-CD (Figure 35A).

In the fluorescence titration, a buffered solution of 28 (2.7 μM), γ-CD (6.1 μM), HEPES (50 mM, pH = 7.2), KNO₃ (100 mM) was titrated into a semi-micro quartz fluorometer cuvette (Starna®) containing a solution of 28 (2.7 μM), HEPES (50 mM, pH = 7.2), KNO₃ (100 mM). The samples were excited at 356 nm and emission spectra were collected (Figure 35B). In the absence of γ-CD, 28 exhibited strong fluorescence. As the γ-CD concentration increased, the fluorescent intensity decreased. Excimer band was not observed. Data analysis (Figure 36) showed that at wavelength 414 nm, intensity became stable when the concentration ratio of γ-CD and 28 was close to 1.
stock solution of 28 in DMSO (2.72 mM). Therefore, the sample contained 0.2% DMSO. (B) The fluorescence spectra of 28 (2.7 µM, λex = 356 nm) with γ-CD (0 - 6.1 µM) in aqueous solution (KNO3: 100 mM, HEPES: 50 mM, pH = 7.2) at 25 °C. Compound 28 was delivered using a stock solution of 28 in DMSO (2.72 mM). Therefore, the sample contained 0.1% DMSO.

Figure 36. The analysis of fluorescence titration of 28 with γ-CD in aqueous buffer solution containing KNO3 and HEPES: intensity at 414 nm versus [CD]/[28].

The fluorescence titration of 28 with Zn²⁺ was further conducted in the presence of γ-CD. In the fluorescence titration, an aqueous solution of 28 (2.7 µM), Zn(OTf)₂ (7.6 µM), γ-CD (10.2 µM), HEPES (50 mM, pH = 7.2), KNO3 (100 mM) was titrated into a semi-micro quartz fluorometer cuvette (Starna®) containing an aqueous solution of 28 (2.7 µM), γ-CD (10.2 µM), HEPES (50 mM, pH = 7.2), KNO3 (100 mM) at 25 °C. The sample was excited at 356 nm and emission spectra were collected (Figure 37A).

Figure 37. The fluorescence titration of 28 (2.7 µM) with Zn(OTf)₂ (0 - 3.8 µM) in aqueous solution (γ-CD: 10.2 µM, KNO₃: 100 mM, HEPES: 50 mM, pH = 7.2) at 25 °C. Compound 28 was delivered
using a stock solution of 28 in DMSO (2.72 mM). Therefore, the sample contained 0.1% DMSO. (A) Titration spectra; (B) Intensity at 419 nm versus [Zn]/[28].

In the absence of Zn$^{2+}$, 28 exhibited strong fluorescence, and the excimer peak was not obvious. After addition of Zn$^{2+}$, the fluorescence intensity of complex 28 decreased. Data analysis in Figure 37B showed that at wavelength 419 nm, fluorescence intensity became stable when the concentration ratio of Zn$^{2+}$ and 28 was close to 1. The observation that the fluorescence intensity decreased in the presence of Zn$^{2+}$ is hard to explain now.

The UV and fluorescence spectroscopic studies of compound 28 with Zn$^{2+}$ were also conducted in organic solvent CH$_3$CN with DIPEA which was the scavenger for H$^+$, and TBAP which was serving to control ionic strength.

In the UV titration, an CH$_3$CN solution of 28 (5.4 µM), Zn(OTf)$_2$ (11.1 µM), DIPEA (5.5 µM), and TBAP (5.3 mM) was titrated into an CH$_3$CN solution of 28 (5.4 µM), DIPEA (5.5 µM), and TBAP (5.3 mM). Absorbance decreased with increasing [Zn$^{2+}$] (Figure 38A).

![Figure 38](image_url). The UV and fluorescence titration of 28 with Zn$^{2+}$ in CH$_3$CN solution containing DIPEA and TBAP. (A) The absorption spectra of 28 (5.4 µM) with Zn$^{2+}$ (0 - 5.5 µM) in CH$_3$CN solution (DIPEA: 5.5 mM, TBAP: 5.3 mM) at 25 °C. Compound 28 was delivered using a stock solution of 28 in DMSO (2.72 mM). Therefore, the sample contained 0.1% DMSO. (B) The fluorescence spectra of 28 (2.7 µM, $\lambda_{ex}$ = 360 nm) with Zn$^{2+}$ (0 - 2.8 µM) in CH$_3$CN solution (DIPEA: 2.7 mM, TBAP: 5.3 mM) at 25 °C. Compound 28 was delivered using a stock solution of 28 in DMSO (2.72 mM). Therefore, the sample contained 0.2% DMSO.
In the fluorescence titration, an CH$_3$CN solution of 28 (2.7 µM), Zn(OTf)$_2$ (5.5 µM), DIPEA (2.7 µM), and TBAP (5.3 mM) was titrated into a semi-micro quartz fluorometer cuvette (Starna®) containing an CH$_3$CN solution of 28 (2.7 µM), DIPEA (2.7 µM), and TBAP (5.3 mM). The samples were excited at 360 nm and emission spectra were collected (Figure 38B). Upon binding with Zn$^{2+}$, the fluorescence intensity of 28 decreased, with an isosbestic point at 423 nm.

Data analysis in Figure 39 showed that the fluorescence intensity ratio at wavelength 419 nm and 449 nm decreased when the concentration ratio of Zn$^{2+}$ and 28 increased until [Zn]/[28] approached 1.

![Figure 39](image)

**Figure 39.** The analysis of fluorescence titration of 28 with Zn$^{-}$ in CH$_3$CN solution containing DIPEA and TBAP: intensity ratio at 419 nm and 449 nm versus [Zn$^{2+}$]/[28].

### 4.4 Experimental Section

#### 4.4.1 General Information

It is the same as that in chapter 2.

#### 4.4.2 Synthesis
Scheme 6. a) ice bath, HCl, NaNO₂, urea, NaN₃, 3 h; b) t-BuOH, sodium ascorbate, Cu(OAc)₂.

**COMPOND 30.** HCl (10 mL, 11%) was added into 1-aminopyrene (1.0 mmol, 0.217 g) in an ice bath. Followed by the addition of NaNO₂ (1.5 mmol, 103.5 mg) dissolved in H₂O (1 mL). The reaction mixture was stirred in an ice bath for 30 minutes. Then urea (1.5 mmol, 90 mg) dissolved in H₂O (2 mL) and NaN₃ (4.0 mmol, 260 mg) dissolved in H₂O (2 mL) were added sequentially. The reaction was stirred in an ice bath for 3h. The reaction mixture was neutralized by NaOH (1.0 M). Then the product was extracted by EtOAc before dried over Na₂SO₄. The solvent was removed by vacuum at r.t. The reaction conversion was quantitative.

**COMPOND 29.** 30 (1.0 mmol, 243.3 mg) was mixed with 14 (1.0 mmol, 237.3 mg), followed by the addition of t-BuOH (2.5 mL) and sodium ascorbate (2.0 mmol, 396.2 mg) in H₂O (2.5 mL). Then Cu(OAc)₂ (1.0 mmol, 199.7 mg) dissolved in H₂O (2.5 mL) was added into the mixture. The reaction mixture was stirred in r.t. for 16 h. Cu(I) and oxidized Cu(II) species in the reaction mixture can be easily separated from chelating products by treating with EDTA during workup and subsequent alumina column chromatography. The reaction mixture was added EDTA (10 mL, 0.1 M) to chelate Cu(I).
and Cu(II). The reaction mixture was adjusted by NaOH (1.0 M) to pH > 11. Then the product was extracted by DCM for 3 times. The organic portions were combined and dried over K$_2$CO$_3$. Compound 29 was purified on a basic alumina column. The yield was 24%.

4.5 Conclusion

Compound 28 and 29 were designed and synthesized as new Zn$^{2+}$ probes, which combine pyrene and 1,2,3-triazole moieties together to expand the methodology developed in chapter 2. Initial photophysical studies showed that in aqueous solution, 29 formed excimer, and in the presence of γ-CD the fluorescence emission spectra of both 28 and 29 changed. Further study on these two probes will be conducted by others in our group.
Figure 40. $^1$H NMR (300 MHz, DMSO-d6) of compound 24.
Figure 41. $^1$H NMR (300 MHz, CDCl$_3$) of compound 26.
Figure 42. $^1$H NMR (300 MHz, CD$_3$OD) of compound 22.
Figure 43. $^1$H NMR (300 MHz, CDCl$_3$) of compound 25.
Figure 44. $^1$H NMR (300 MHz, CDCl$_3$) of compound 29.
REFERENCES


7. Cho, K. J.; Trzaska, K. A.; Greco, S. J.; McArdle, J.; Wang, F. S.; Ye, J. H.; Rameshwar, P. Neurons derived from human mesenchymal stem cells show synaptic transmission and can be induced to produce the neurotransmitter substance P by interleukin-1α. Stem Cells 2005, 23, 383-391.


10. Jiang, P. J.; Guo, Z. J. Fluorescent detection of zinc in biological systems: recent


Biophysics, 1975, 72, 3097-3101.


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