Title
Diamidodipyrrins as BODIPY dyes and chelator fragment libraries to identify new scaffolds for metalloprotein inhibitors

Permalink
https://escholarship.org/uc/item/44r540jg

Author
Jacobsen, Jennifer A.

Publication Date
2010

Peer reviewed|Thesis/dissertation
Diamidodipyr reins as BODIPY Dyes and Chelator Fragment Libraries to Identify New Scaffolds for Metalloprotein Inhibitors

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Chemistry

by

Jennifer A. Jacobsen

Committee in Charge:
Professor Seth M. Cohen, Chair
Professor Nathan Gianneschi
Professor Douglas Magde

2010
The Thesis of Jennifer A. Jacobsen is approved and it is acceptable in quality and form for publication on microfilm and electronically:

__________________________________________________________________________

__________________________________________________________________________

__________________________________________________________________________

Chair

University of California, San Diego

2010
# TABLE OF CONTENTS

Signature page.............................................................................................................. iii

Table of Contents........................................................................................................... iv

List of Symbols and Abbreviations.................................................................................. v

List of Figures................................................................................................................ xi

List of Tables.................................................................................................................. xiv

List of Schemes............................................................................................................... xv

List of Equations............................................................................................................ xvi

Acknowledgements......................................................................................................... xvii

Vita and Publications...................................................................................................... xix

Abstract of the Thesis...................................................................................................... xx

Chapter 1: Hydrogen-bond Rigidified BODIPY Dyes

1.1 Introduction................................................................................................................ 1

1.2 Results and Discussion............................................................................................... 10

1.3 Conclusion.................................................................................................................. 19

1.4 Experimental.............................................................................................................. 20

1.5 References................................................................................................................ 33

Chapter 2: Chelator Fragment Libraries for Metalloprotein Inhibitors

2.1 Introduction................................................................................................................ 41

2.2 Results and Discussion............................................................................................... 72

2.3 Conclusion.................................................................................................................. 98

2.4 Experimental.............................................................................................................. 99

2.5 References................................................................................................................ 117
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Abbreviation and Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HPETE</td>
<td>5-Hydroperoxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>5-LO</td>
<td>5-Lipoxygenase</td>
</tr>
<tr>
<td>Å</td>
<td>Ångström; $10^{-10}$ m</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ADA</td>
<td>Adenosine deaminase</td>
</tr>
<tr>
<td>amu</td>
<td>Atomic mass units</td>
</tr>
<tr>
<td>APCI-MS</td>
<td>Atmospheric pressure chemical ionization-mass spectrometry</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATX</td>
<td>Anthrax toxin</td>
</tr>
<tr>
<td>br</td>
<td>Broad peak (NMR)</td>
</tr>
<tr>
<td>BODIPY</td>
<td>4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene</td>
</tr>
<tr>
<td>BoNT</td>
<td>Botulinum neurotoxin</td>
</tr>
<tr>
<td>CA</td>
<td>Carbonic anhydrase</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CFL</td>
<td>Chelator fragment library</td>
</tr>
<tr>
<td>CHF</td>
<td>Congestive heart failure</td>
</tr>
<tr>
<td>CMC</td>
<td>Comprehensive medicinal chemistry database</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CysLT</td>
<td>Cysteinyl leukotriene</td>
</tr>
<tr>
<td>δ</td>
<td>Chemical shift; ppm</td>
</tr>
<tr>
<td>d</td>
<td>Doublet (NMR)</td>
</tr>
<tr>
<td>dd</td>
<td>Doublet of doublets (NMR)</td>
</tr>
<tr>
<td>DADP</td>
<td>Diamidodipyrromethanes</td>
</tr>
<tr>
<td>DDQ</td>
<td>1,4-Dichloro-5,6-dicyanoquinone</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dpa</td>
<td>N-3-(2,4,-dinitrophenyl)-L-α-β-diaminopropionyl</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-Dithiobis-(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DXR</td>
<td>1-Deoxy-d-xylulose-5-phosphate reductoisomerase</td>
</tr>
<tr>
<td>ε</td>
<td>Extinction coefficient</td>
</tr>
<tr>
<td>EF</td>
<td>Edema factor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>ETx</td>
<td>Edema toxin</td>
</tr>
<tr>
<td>FBLD</td>
<td>Fragment based lead design</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>FTase</td>
<td>Farnesyl transferase</td>
</tr>
<tr>
<td>Glx-1</td>
<td>Glyoxylase</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>Δ</td>
<td>Heat</td>
</tr>
<tr>
<td>HBA</td>
<td>Hydrogen bond acceptors</td>
</tr>
<tr>
<td>HBD</td>
<td>Hydrogen bond donors</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HMDO</td>
<td>Hexamethyldisiloxane</td>
</tr>
<tr>
<td>H-PGDS</td>
<td>Human prostaglandin D synthase</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectrometry</td>
</tr>
<tr>
<td>HTS</td>
<td>High throughput screening</td>
</tr>
<tr>
<td>I</td>
<td>Spin quantum number</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Inhibitor concentration leading to 50% enzyme activity</td>
</tr>
<tr>
<td>ID</td>
<td>Identification</td>
</tr>
<tr>
<td>IN</td>
<td>HIV integrase</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>J</td>
<td>Coupling constant (NMR)</td>
</tr>
<tr>
<td>Kₐ</td>
<td>Dissociation equilibrium constant</td>
</tr>
<tr>
<td>Kᵢ</td>
<td>Inhibitor equilibrium constant</td>
</tr>
<tr>
<td>λ</td>
<td>Wavelength; nm</td>
</tr>
<tr>
<td>λₘₐₜ</td>
<td>Wavelength of absorbance; nm</td>
</tr>
<tr>
<td>λₑₐᵐ</td>
<td>Wavelength of emission; nm</td>
</tr>
</tbody>
</table>
LDH     Lactate dehydrogenase
L-DOPA  L-dopamine
LE      Ligand efficiency
LF      Lethal factor
LFi     Lethal factor inhibitor
LT      Leukotriene
LTx     Lethal toxin
M       Monoclinic
m       Multiplet (NMR)
MAPK    Mitogen-activated protein kinase
MAPKK   Mitogen-activated protein kinase kinase
MBL     Metallo-β-lactamase
Mca     (7-Methoxycoumarin-4-yl)-acetyl
MeOH    Methanol
MetAP   Methionine aminopeptidase
MetRS   Methionyl tRNA synthetase
MMP     Matrix metalloprotease
MMPi    Matrix metalloprotease inhibitor
MPO     Myeloperoxidase
mRNA    Messenger ribonucleic acid
MS      Multiple sclerosis
MSS     Musculoskeletal syndrome
MW      Microwave
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NROT</td>
<td>Number of rotatable bonds</td>
</tr>
<tr>
<td>PA</td>
<td>Protective antigen</td>
</tr>
<tr>
<td>PSA</td>
<td>Polar surface area</td>
</tr>
<tr>
<td>ND</td>
<td>Neurodegenerative diseases</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>O/N</td>
<td>Overnight</td>
</tr>
<tr>
<td>π</td>
<td>Pi</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein data bank</td>
</tr>
<tr>
<td>PDF</td>
<td>Peptide deformylase</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>psi</td>
<td>Pound per square inch</td>
</tr>
<tr>
<td>pTSA</td>
<td>p-Toluenesulfonic acid</td>
</tr>
<tr>
<td>φ</td>
<td>Quantum yield</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RECAP</td>
<td>Retrosynthetic combinatorial analysis procedure</td>
</tr>
<tr>
<td>RNApol</td>
<td>Ribonucleic acid polymerase</td>
</tr>
<tr>
<td>RR</td>
<td>Ribonucleotide reductase</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>s</td>
<td>Singlet (NMR)</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>SSAO</td>
<td>Semicarbazide-sensitive amine oxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>STS</td>
<td>Steroid Sulfatase</td>
</tr>
<tr>
<td>( \tau )</td>
<td>Excited state lifetime</td>
</tr>
<tr>
<td>( t )</td>
<td>Triplet (NMR)</td>
</tr>
<tr>
<td>TACE</td>
<td>TNF-( \alpha ) converting enzyme</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TBET</td>
<td>Through bond energy transfer</td>
</tr>
<tr>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitors of metalloproteinases</td>
</tr>
<tr>
<td>TNF-( \alpha )</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Ty</td>
<td>Tyrosinase</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>ZBG</td>
<td>Zinc binding group</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

**Figure 1.1.** Several BODIPY derivatives and their reported photophysical properties................................................................. 2

**Figure 1.2.** Two examples of extended chromophores resulting from structural rigidification............................................................ 4

**Figure 1.3.** Water soluble BODIPY derivatives..................................................... 6

**Figure 1.4.** Various routes towards the synthesis of dipyrrins................................. 8

**Figure 1.5.** Crystal structure of 4c with hydrogen bonds denoted by dashed lines (50% probability ellipsoids). Co-crystallized acetone molecule omitted for clarity.......................................................... 12

**Figure 1.6.** $^{19}$F NMR spectra of 4a in CDCl$_3$. The single resonance appears at ~131ppm and shows the expected $^{11}$B-$^{19}$F coupling........... 13

**Figure 1.7.** Proposed alternative bonding conformation for 4e. The $^{19}$F NMR of 4e does not display the same downfield shift as dyes 4a-4d thus suggesting that 4e does not exist in the same hydrogen-bonding conformation as the other dyes...................... 13

**Figure 1.8.** Normalized excitation (solid line) and emission (dashed line) spectra of 4a in CH$_2$Cl$_2$.................................................................................................................. 14

**Figure 1.9.** Fluorescence decay for 4a: data are black dots, instrument response function is red curve (scaled in amplitude), and fit to the data is the blue curve. The data is well fit by a single exponential decay........... 15

**Figure 1.10.** a) Scheme for the synthesis of 4f. b) Scheme for the synthesis of 4g.......................................................... 18

**Figure 1.11.** $^1$H (a) and $^{13}$C (b) NMR of the product formed upon oxidation and BF$_2$ complexation of 2f. Notably, both spectra display the upfield peaks indicative of two distinct methyl groups and two distinct methylene groups.......................................................... 18

**Figure 2.1.** A schematic representation (left) and a protein crystal structure (right, PDB code: 1QIB) of the active site of MMP-2.................. 46
Figure 2.2. The structures of selected MMP inhibitors are presented here. Inhibitors 1 and 2 bind to the catalytic zinc to inactivate MMPs while 3 is an example of a non-zinc-binding MMPi.

Figure 2.3. Scheme for the activity of mechanism-based MMPi (SB-3CT).

Figure 2.4. A schematic representation (left) and protein crystal structure (right, PDB code: 1YQY) of the active site of LF.

Figure 2.5. Selected LF inhibitors.

Figure 2.6. Leukotriene biosynthetic pathway. Lipoxygenase catalyzes the first two steps of leukotriene synthesis.

Figure 2.7. Select inhibitors of 5-LO.

Figure 2.8. A schematic representation of the active site of 5-LO (left) and a protein crystal (PDB code 2IUJ) structure of soybean lipoxygenase B (right).

Figure 2.9. Melanin biosynthetic pathway.

Figure 2.10. The three oxidation states of the copper center in tyrosinase (left) and a protein crystal structure (PDB code: 2ZMX) of tyrosinase (right).

Figure 2.11. Select inhibitors of tyrosinase. Inhibitors of tyrosinase are based on phenols (arbutin), pyrones (14), pyridinones (17, 19-21), and tropolone (15), among others.

Figure 2.12. Three forms of fragment development.

Figure 2.13. Structures of the components of CFL-1.1.

Figure 2.14. Thermoplot representing the results from the screens of CFL-1.1 against various metalloenzymes.

Figure 2.15: MMP-1 assay results with error bars. CFL-1.1 was screened at 1 mM against MMP-1.

Figure 2.16: MMP-2 assay results with error bars. CFL-1.1 was screened at 1 mM against MMP-2.
Figure 2.17: MMP-3 assay results with error bars. CFL-1.1 was screened at 1 mM against MMP-3

Figure 2.18: MMP-8 assay results with error bars. CFL-1.1 was screened at 1 mM against MMP-8

Figure 2.19: MMP-9 assay results with error bars. CFL-1.1 was screened at 1 mM against MMP-9

Figure 2.20: LF assay results with error bars. CFL-1.1 was screened at 1 mM against LF

Figure 2.21: 5-LO assay results with error bars. CFL-1.1 was screened at 1 mM against 5-LO

Figure 2.22: Tyrosinase assay results with error bars. CFL-1.1 was screened at 1 mM against tyrosinase

Figure 2.23: iNOS assay results with error bars. CFL-1.1 was screened at 1 mM against iNOS

Figure 2.24: 8-Hydroxyquinoline sublibrary

Figure 2.25: Hydroxyquinoline sublibrary screen against MMP-2 at 25 µM

Figure 2.26: Hydroxyquinoline sublibrary screen against 5-LO at 25 µM
LIST OF TABLES

Table 1.1 Photophysical parameters of new BODIPY dyes .................. 16

Table 2.1. Expanded list of metalloenzyme targets and their clinical relevance .............................................................. 43
LIST OF SCHEMES

**Scheme 1.1.** General scheme for the synthesis of the new dyes 4a-e (top).
Structures of dyes 4a-e (bottom)………………………………………….. 10
LIST OF EQUATIONS

Equation 2.1:

\[ LE = -RT \ln[IC_{50}] / (\# \text{ of non-hydrogen atoms}) \]
ACKNOWLEDGEMENTS

I have learned so much over the past four years working in the Cohen lab. To all my labmates past and present, thank you for all you have taught me. Jody, thank you for your example and for teaching me what it takes to be a good scientist. Joe, thank you for all of your help both with software and technological problems and for your advice about presentations and teaching. Matthieu, thank you for taking the time to teach me the details of synthetic chemistry and for being patient with me. Arpita, I am grateful for all the memories from our shared time in this lab.

Seth, thank you for taking a chance on me. Thank you for all the opportunities you have given me. I have learned so much over the past four years and I appreciate all that you have invested in me.

I would also like to thank my family and friends for all the support throughout the years. I could not have gotten this far without you all. Thank you for putting up with me.

Chapter 1, in part, is a reprint of the material as it appears in Dalton Transactions, 2010. Jacobsen, J. A.; Stork, J. R.; Magde, D.; Cohen, S. M. “Hydrogen-bond Rigidified BODIPY Dyes” *Dalton Trans.* **2010**, *39*, 957-962. The thesis author was the primary investigator and author of this paper. Jay R. Stork initiated the BODIPY project as a post-doctoral fellow in the Cohen lab. Doug Magde performed or assisted in many of the photophysical measurements for the BODIPY project.

Chapter 2, in part, is currently being prepared for submission for publication of the material. Jacobsen, J. A.; Fullagar, J. L.; Miller, M. T.; Cohen, S. M. “Chelator fragment libraries: Diversification of the metal-binding scaffold” The thesis author
was the primary investigator and author of this paper. Melissa T. Miller assisted in screening CFL-1.1 and Jessica L. Fullagar assisted in synthesis of the 8-hydroxyquinoline sub-library.
VITA AND PUBLICATIONS

EDUCATION

University of California, San Diego 2010
Masters of Science, Chemistry
Advisor: Professor Seth M. Cohen

University of California, San Diego 2008
Bachelor of Science, Microbiology

HONORS

Chancellor’s Summer Undergraduate Research Scholarship 2006
Chancellor’s Summer Undergraduate Research Scholarship 2007

PUBLICATIONS


ABSTRACT OF THE THESIS

Diamidodipyrrins as BODIPY Dyes and Chelator Fragment Libraries to Identify New Scaffolds for Metalloprotein Inhibitors

by

Jennifer A. Jacobsen

Master of Science in Chemistry

University of California, San Diego, 2010

Professor Seth M. Cohen, Chair

The thesis presented here is split between two distinct projects. The first project explores the synthesis and photophysical evaluation of a new class of BODIPY dyes with handles for functionalization. These dyes may be easily modified for use in biological systems. The BODIPY dyes were synthesized by simple modifications of reported procedures and were evaluated using luminescence and UV-visible spectroscopy. An X-ray diffraction crystal structure and $^{19}$F NMR data indicate that these dyes are rigidified by intramolecular hydrogen bonding.
The second project is directed towards studying the utility of a chelator fragment library in indentifying new metal-binding groups for metalloprotein inhibitors. The chelator fragment library was screened at high concentration in cell-free bioassays against several types of metalloenzymes to yield several new metal-binding scaffolds that result in significant enzyme inhibition. One of these scaffolds was synthetically elaborated at multiple positions to demonstrate the effect of the chelator in a fragment growth strategy.
Chapter 1: Hydrogen-bond Rigidified BODIPY Dyes

1.1 Introduction

4,4-Difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY, Fig. 1.1) derivatives have garnered significant interest over the past twenty years as fluorescent dyes due to their photochemical stability and intense emission profiles. BODIPY dyes typically have sharp absorption bands with emission peaks that can be tuned from the green to the near IR.\(^1\)\(^-\)\(^3\) These dyes generally have high molar absorptivities, high fluorescence quantum yields, and relatively long excited-state lifetimes.\(^4\) These properties make them desirable for a variety of applications, including as synthetic labels for macromolecules in biological studies. The stability of these dyes across a range of pH values and varying solvent polarities makes them particularly suitable for biological experiments.\(^4\)\(^,\)\(^5\) The favorable photophysical properties of this dye class have prompted development of BODIPY frameworks into TBET (Through Bond Energy Transfer) cassettes.\(^4\)\(^,\)\(^6\)\^-\(^8\) TBET is a variation on FRET (Förster Resonance Energy Transfer) in which energy is efficiently transferred from an intense absorber to a bright emitter through a chemical bond. The energy transfer in TBET is more efficient than FRET and results in high-resolution intense emission, which can be applied to advanced biological labeling technology. BODIPY dyes have also been developed as “turn-on” sensors for anions,\(^9\)\^-\(^11\) cations,\(^12\)\^-\(^14\) redox reactions,\(^15\)\^-\(^17\) and a variety of other applications.\(^18\)\^-\(^21\)
Figure 1.1. Several BODIPY derivatives and their reported photophysical properties. Compound 4a is a representative dye from a new series of BODIPYs described here, based on the 3,5-diamidodipyrin template.

In an attempt to modify these dyes for specific applications, numerous derivatives have been prepared.\textsuperscript{1} Interestingly, due to poor chemical stability, the parent compound (Fig. 1.1, A) has only very recently been reported in the literature.\textsuperscript{22} Nonetheless, dyes with a variety of substitution patterns around the core framework have been synthesized and their resultant fluorescence and absorption properties studied and compared.\textsuperscript{1, 4} Among the most significant observations is that the presence of a freely rotating aryl group at the meso-position leads to a decrease in fluorescence quantum yield, which has been attributed to non-irradiative decay pathway from the excited state.\textsuperscript{1, 23} Significantly, this effect can be seen in the comparison of dyes B and C (Fig. 1.1).\textsuperscript{1} The only structural difference between these two dyes is the freely rotating meso-phenyl ring in dye C. While the absorption and emission wavelengths remain unperturbed (an indication that the phenyl ring is not strongly coupled with the π-system), significant fluorescence quenching is observed. By adding the meso-phenyl ring, the quantum yield is reduced from $\phi = 0.81$ in dye B
to $\phi = 0.19$ in dye C. Several groups have demonstrated that the restriction of motion at this position by use of bulky pyrrole substituents$^{24-27}$ or with a bulky *meso*-aryl group$^{26-28}$ will mitigate this loss of fluorescence. As a result, many recently reported BODIPY dyes contain a mesityl group at the *meso*-position.$^{27, 29-32}$

Dyes with highly red-shifted excitation and emission properties are of interest due to the ability of low energy light to penetrate tissue.$^1$ Several strategies have been explored to extend the conjugation of the dipyrrin $\pi$-system in pursuit of red-shifted emission. Early efforts were directed towards the synthesis of dyes with aryl groups at the $\alpha$-positions of the dipyrrin.$^{33, 34}$ The free rotation of these aryl groups often limited the effect of these additions, as conjugation is not enforced with the dipyrrin plane. Larger bathochromic shifts have been observed through the direct expansion of the dipyrrin chromophore using fused aromatic rings$^2, 35-37$ or styryl groups.$^{38-40}$ Two creative examples of chromophore expansion are the direct coordination of peripheral groups to the boron center$^{41}$ (Fig. 1.2a) and the use of metal ion trapping in a BODIPY analogue$^{42}$ (Fig. 1.2b). In Fig. 2a the dye on the left has freely rotating aryl groups at the 3- and 5- position of the dipyrrin core. In the dye on the right, the rotation of the aryl rings is constricted by binding of the oxygen atoms to the boron center, replacing the fluorine atoms. The more rigid dye (right) is red-shifted 85 nm in absorbance and 56 nm in emission relative to the more flexible dye (left). In Fig. 1.2b, the aryl groups at the 1- and 7- position are unrestricted for the dye without metal. In order to chelate mercury the pyridinyl nitrogens must point toward the center cavity. The metal-ligand bond holds the aryl groups in place resulting in a bathochromic shift of 40 nm.
Significantly, there is a correlation between these extremely rigid systems and high quantum yields.

![Diagram of chromophores](image)

**Figure 1.2.** Two examples of extended chromophores resulting from structural rigidification.

Much attention has been paid to the water solubility of BODIPYs in efforts to advance the use of these dyes in biological systems. However, red-shifted emission and water solubility are often competing interests. Specifically, \( \pi \)-systems are commonly extended using hydrophobic unsaturated moieties or aromatic rings.\(^1\) Alternatively, improved water solubility can be achieved through ionization of the molecule, but the accompanying changes in the charge distribution can potentially quench fluorescence.\(^1,43\) Despite these challenges several significant advances toward obtaining water solubility have been accomplished. Two of the most prominent approaches include the aforementioned ionization\(^8,43-48\) and the addition of hydrophilic species such as carboxylic acids\(^45,46\) or polyethylene glycol substituents.\(^49\) Early studies in the solubilization of BODIPY laser dyes involved ionization of the dipyrrin core through electrophilic addition.\(^1\) It was discovered that nitration of the core promotes photoinstability while sulfonation at the same position is tolerated\(^43\) (Fig.
1.3, E). Since this study Burgess and coworkers have used sulfonation to solubilize BODIPYs with applications in biomolecular labeling\textsuperscript{44} and TBET cassettes.\textsuperscript{8} Alternatively, Akkaya and coworkers have successfully probed the strategy of appending repeating ethylene glycol moieties to BODIPY dyes in order to improve their water solubility.\textsuperscript{49} The featured dye from this study (Fig. 1.3, F) has enhanced red emission the presence of Zn\textsuperscript{2+} and is essentially non-emissive in the absence of Zn\textsuperscript{2+} or in the presence of other metal ions. This clearly demonstrates that ethylene glycol groups are compatible components of a functional, highly fluorescent dye. In order to avoid the synthetic challenges associated with including water-soluble functionalities in the dipyrrin framework (need for protecting groups, difficult purification), Niu \textit{et al.} have developed a post-synthetic approach to make bright water-soluble, Zwitterionic BODIPY species\textsuperscript{48} (Fig. 1.3, G). This means that the water-soluble functionalities were added after the boron was coupled to the dipyrrin core. Subsequent studies have revealed a requirement that the ionic functionalities be positioned close to the hydrophobic dipyrrin core in order to achieve water-solubility.\textsuperscript{47}
Dipyrrins are structurally related to porphyrins and as such have been referred to as “porphyrin’s little sister”. Consequently, much of the synthetic chemistry of dipyrrin ligands has been adapted from the porphyrin literature. Dipyrrin synthesis typically involves the condensation of substituted or unsubstituted pyrrole with an electrophilic carbonyl (Fig. 1.4). Symmetric dipyrrins can be synthesized via the condensation of pyrrole rings with an acyl chloride (Fig. 1.4a). This produces a reportedly unstable hydrochloride salt of the dipyrrin. Alternatively, condensation of pyrrole with a cyclic anhydride produces a free carboxylic acid that can be readily
used for molecular labeling (Fig. 1.4b). In another route, pyrrole is condensed with aromatic or aliphatic aldehydes in the presence of acid to produce a dipyrromethane (Fig. 1.4c). This path, however, requires an additional oxidation step with DDQ or p-chloranil to produce the dipyrromethene. Direct condensation between a pyrrole-2-carbaldehyde and a second pyrrole is the most common way to make an asymmetric dipyrrin (Fig. 1.4d). Though this method can be applied to the synthesis of symmetric dyes as well, a higher yielding synthesis has been developed in which the pyrrole-2-carbaldehyde is treated with POCl₃ to generate the dipyrromethene cation. In all these cases, the BF₂ adducts are then formed upon complexation of the dipyrrin with BF₃OE₂ in the presence of triethylamine or other base.
Figure 1.4. Various routes towards the synthesis of dipyrrins. a) Condensation with an acyl chloride leads directly to the dipyrrin. b) Combination of the pyrrole with a cyclic anhydride provides a route to a free carboxylic acid group that can be used for tethering. c) Condensation with an aldehyde produces the dipyrromethane, which can be oxidized to the dipyrrin with DDQ. d) Direct condensation with pyrrole-2-carbaldehyde provides a route to asymmetric dipyrrins.

Several interesting routes have been devised to further modify the BODIPY after BF$_2$ complexation. As discussed earlier, electrophilic addition has provided a means to solubilization of BODIPY dyes in water.$^{43}$ α-Halogenated BODIPYs can be modified through nucleophilic substitution$^{57,58}$ or through organometallic coupling.$^{33,34}$ Another example of post-synthetic organometallic coupling has provided a versatile system for extending conjugation of the dipyrrin π-system.$^{29}$ Wada et al. demonstrated that an extended dipyrrin chromophore with fused rings can be accessed through a retro diels-alder reaction.$^{36}$ Finally, Akkaya and coworkers have applied a
Knoevenagel condensation to α-methylated BODIPY dyes to form styrene derivatives. This chemistry has been exploited by Lee et al. towards the synthesis of a library of BODIPY dyes.

Dipyrrin compounds are widely studied beyond the scope of BODIPY chemistry. Dipyrrins are versatile ligands with the ability to form stable homoleptic complexes with at least 16 different metals, several of which can be formed in various oxidation states. Dipyrrin ligands have been shown to bind Ni(II) in a distorted tetrahedral geometry. This deviation from the preferred square planar geometry of the metal has been attributed to the steric bulk of the dipyrrin ligands. Dipyrrin metal complexes have served as building blocks for supramolecular chemistry.

Dipyrrins have also been developed as porphyrin precursors.

Diamidodipyrromethanes (DADP) have recently been described in the literature as symmetric bipyrrolic ligands with amide substitution at the α-position of the pyrrole rings. Metal complexes of these ligands in their oxidized form (dipyrromethenes) were shown to bind copper and nickel using different atoms to mediate the binding. Inspired by their synthetic accessibility, a series of 3,5-diamidodipyrrin ligands have been developed into BODIPY dyes, represented by 4a (boxed in Fig. 1.1). The BODIPY dyes presented here offer a new scaffold capable of intramolecular hydrogen bonding. The dipyrrin chromophores in these dyes are extended by rigidification of the pendant amido groups. The step-wise synthesis of these dyes allows for the introduction of a variety of substituents.
1.2 Results and Discussion

The general synthesis of 3,5-diamido-BODIPY dyes as presented in Scheme 1.1 begins with the treatment of trichloroacetylpyrrole with neat amine to form an amidopyrrole (1). The precipitated product is condensed with one half equivalent of an aldehyde using acid catalyst to form a dipyrromethane (2). The dipyrromethane is oxidized to the corresponding dipyrrin (3) using 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ)\textsuperscript{66}. The final step is the formation of the boron difluoride adduct (4), which is achieved by reaction of the dipyrrin with BF\textsubscript{3}OEt\textsubscript{2} in the presence of triethylamine. The first three intermediates were generally prepared in high yields though there was significant variation based on the amide substituent. The final step consistently gave poor yields owing to low reactivity and complications in isolation. Notably, the modular synthesis of these dyes enables the introduction of a variety of substituents at both the R and R’ positions, which can be tailored toward solubilization and bioreactivity.

Scheme 1.1. General scheme for the synthesis of the new dyes 4a-e (top). Structures of dyes 4a-e (bottom).
The first evidence obtained for the intramolecular hydrogen bonding exhibited by these dyes was from the X-ray crystal structure of derivative 4c (Fig. 1.5). The acetone solvate of 4c crystallized in the monoclinic space group $P2_1/n$ from a concentrated solution of the complex. This unambiguously confirmed the expected chemical composition and connectivity of this dye with the dipyrrin unit chelating the boron through the two nitrogen atoms. The dipyrrin chromophore is nearly planar with the meso-aryl ring twisted out of plane at a dihedral angle of $\sim51^\circ$ (C4-C5-C26-C31). The N-B bond lengths were similar to other BODIPY complexes at $\sim1.53\,\text{Å}$.\textsuperscript{28,53} Interestingly, the two fluoride ions that are coordinated to the central boron atom are engaged in hydrogen bonding with the amide protons. These hydrogen bonds twist each amide group in opposite directions and bring them slightly out of the plane of the dipyrrin chromophore. The NH\textsuperscript{...}F bond distances are 1.92 Å (hydrogen to fluoride distance, N4-F1) and 2.01 Å (N1-F2). This hydrogen bonding restricts rotation of the $\alpha$-amides and may contribute to the slight red shift of these dyes and provide some protection against intramolecular rotational quenching.
Figure 1.5. Crystal structure of 4c with hydrogen bonds denoted by dashed lines (50% probability ellipsoids). Co-crystallized acetone molecule omitted for clarity.

$^{19}$F NMR resonances of the final dyes were measured to provide evidence of a hydrogen bonding conformation in solution. The characteristic shift of the $^{19}$F NMR resonances of typical BODIPY complexes shows up at approximately -147ppm.\textsuperscript{48, 67, 68} For most of this series (4a, 4b, 4c, 4d), however, the fluorine atoms resonate about 16 ppm downfield of this at approximately -131ppm (Fig. 1.6). This downfield shift is indicative of the fluorine acting as a hydrogen-bond acceptor.\textsuperscript{69} The fluorine atoms display the expected splitting pattern (Fig. 1.6, inset), a quartet resulting from the coupling between the $^{19}$F ($I = 1/2, 100\%$ natural abundance) and the $^{11}$B ($I = 3/2, 80\%$ natural abundance). The final dye, 4e, displays a upfield shift with the fluorine resonance centering around -151 ppm. It may be the case that the fluorine atoms of this dye do not engage in the same hydrogen-bonding interaction as the other dyes. The peripheral oxygen atoms in 4e are also potential hydrogen-bond acceptors and may serve to stabilize an alternative conformation for this dye. One possible alternative bonding conformation is presented in Fig. 1.7.
Figure 1.6. $^{19}$F NMR spectra of 4a in CDCl$_3$. The single resonance appears at ~131 ppm and shows the expected $^{11}$B-$^{19}$F coupling.

![NMR spectrum](image)

Figure 1.7. Proposed alternative bonding conformation for 4e. The $^{19}$F NMR of 4e does not display the same downfield shift as dyes 4a-4d thus suggesting that 4e does not exist in the same hydrogen-bonding conformation as the other dyes.

The photophysical properties of these dyes were evaluated in CH$_2$Cl$_2$ and are summarized in Table 1.1. The fluorescence parameters of 4a will be discussed in detail as a representative example for this series. Compound 4a displays a strong absorption peak at 530 nm with a well-defined shoulder at 370 nm. The excitation and emission spectra of this dye are overlayed in Fig. 1.8. This dye displays two relatively
sharp peaks with the excitation centered at 530 nm and emission at 545 nm. The Stokes shift is therefore approximately 15 nm, which is within the range typical of BODIPY dyes.\textsuperscript{1} The wavelength of emission for this dye gives it a visible yellow-green luminescence upon excitation. Additionally, the emission maximum is \(~25\) nm red-shifted from that of the simple dimethyl analogue (see compound C, Fig. 1.1).\textsuperscript{1} This shift is attributed to the slight overlap of $\pi$-orbitals of the dipyrrin unit and the amide functionality that results from rigidification of the amide by intramolecular hydrogen bonding. The quantum yield of 4a is $\phi = 0.73$. This is nearly 4-times greater than the simple dimethyl analogue (as reported in MeOH). Also, the emission lifetime of 4a is 6.45\(\pm\)0.1 ns. As measured, the lifetime data fits the model of a single exponential decay (Fig. 1.9). The extinction coefficient of 4a is 88,000\(\pm\)3000 M\(^{-1}\)cm\(^{-1}\). These quantitative measurements support the qualitative observation that this is a dye with intense emission.

Figure 1.8. Normalized excitation (solid line) and emission (dashed line) spectra of 4a in CH\(_2\)Cl\(_2\). The quantum yield was determined to be $\phi = 0.73$. 
A series of these dyes were made by varying both the meso-aryl and amido-substituents in order to evaluate their common characteristics. A variety of amide substitutions are well tolerated among these dyes with isopropyl (4a), benzyl (4b), and ethanol (4e) derivatives all displaying long lifetimes and high quantum yields with similar Stokes shifts (Table 1.1). On the other hand, the fluorescence of the tolyl derivative (4c) is severely quenched. 4c has a luminescence lifetime of <10 ps and a quantum yield of $4 \times 10^{-5}$ and is essentially non-fluorescent. Derivative 4d, which has a nitrile substitution on the meso-phenyl ring, exhibits significant fluorescence with a luminescence lifetime of $3.35 \pm 0.1$ ns and a quantum yield of 0.30. Overall, substitutions on the meso-phenyl ring and via the amide moiety are largely tolerated in these dyes. These two sites can be easily modified with functional groups to enable
protein attachment or toward improvement of water solubility. Both of these features will help advance the use of these dyes as biomolecular labels.

Table 1.1 Photophysical parameters of new BODIPY dyes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\lambda_{\text{abs}}$ (nm)</th>
<th>$\varepsilon$ (M$^{-1}$cm$^{-1}$)</th>
<th>$\lambda_{\text{em}}$ (nm)</th>
<th>$\phi$</th>
<th>$\tau$ (ns)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td>530</td>
<td>88000±3000</td>
<td>546</td>
<td>0.73</td>
<td>6.45±0.1</td>
</tr>
<tr>
<td>4b</td>
<td>532</td>
<td>62000±2000</td>
<td>548</td>
<td>0.59</td>
<td>6.7±0.1</td>
</tr>
<tr>
<td>4c</td>
<td>548</td>
<td>39000±2000</td>
<td>563</td>
<td>4×10$^{-5}$</td>
<td>&lt;10ps</td>
</tr>
<tr>
<td>4d</td>
<td>538</td>
<td>61000±4000</td>
<td>557</td>
<td>0.30</td>
<td>3.35±0.1</td>
</tr>
<tr>
<td>4e</td>
<td>530</td>
<td>31000±1000</td>
<td>546</td>
<td>0.41</td>
<td>6.4</td>
</tr>
</tbody>
</table>

$^a$ Average of at least five independent measurements

Due to the high structural similarity of these dyes, we sought to investigate the particularly poor photophysical properties of 4c. We hypothesized that the tolyl groups were quenching the fluorescence via intramolecular rotation. In order to test this hypothesis, we evaluated the photophysics of 4c in a more viscous solvent, dibutylphthalate. 27 Dibutylphthalate was chosen over ethylene glycol or glycerol in order to prevent hydrogen bonding interference. In this solvent the fluorescent lifetime of 4c increased to 10±3 ps and the quantum yield increased 4-fold. Evidence in the literature for a rotational quenching effect can be seen upon comparison of dyes with and without meso-aryl substituents that are otherwise structurally identical (see more complete discussion above). 1 Though this provides some support for this hypothesis, further study is needed. An alternative hypothesis, that the aryl groups are oxidatively quenching the system, should be investigated.
In order to elucidate the effect of the observed intramolecular hydrogen-bonding on the photophysical properties of these dyes, the synthesis of a control dye that lacks hydrogen-bonding capability was pursued. Initially, we attempted to synthesize 4f (see Fig. 1.10), a dye that was structurally identical to 4b except that the amide nitrogens are methylated. Early on, the synthesis of this dye proved to be challenging. The dipyrrromethane intermediate, 2f, was not formed from 1f using the same conditions applied to the other pyrroles. An alternative microwave procedure was developed in which 2f could be synthesized in low yield using InCl$_3$ as the acid catalyst. The $^1$H NMR analysis of intermediates 1f and 2f were plagued by extreme peak broadening that could not be resolved through use of different solvents, a feature that further complicated the synthesis of these dyes. Subsequent oxidation and BF$_2$ complexation steps were attempted via the conditions of the established procedure. These reactions resulted in mixtures of more than 20 compounds. Impurities identified by mass spectrometry and NMR include residual dipyrrromethane, an isomer of the dipyrrromethane (described by Lindsey and coworkers),$^{70, 71}$ a tripyrrane, and an oxidized form of a tripyrrane. Among all of this, a dimly fluorescent product of the correct mass was isolated with extensive column chromatography. Limited to milligram quantities, comprehensive characterization of this product was achieved after multiple syntheses. Fig. 1.11 displays the $^1$H and $^{13}$C NMR of the isolated product. Seen in both spectra are two methyl peaks (each integrating to three protons in the $^1$H NMR) and two methylene peaks (each integrating to two protons in the $^1$H NMR). The NMR evidence indicates that the pyrrole rings are non-equivalent, suggesting that this is a derivative of the “$N$-confused” dipyrrromethane, though the
connectivity is unknown. The correct product could not be identified or isolated from the mixture. The extra steric encumbrance near the site of BF$_2$ complexation in 4f may impede formation of final dye.

![Scheme for the synthesis of 4f and 4g.](image)

**Figure 1.10.** a) Scheme for the synthesis of 4f. b) Scheme for the synthesis of 4g.

![1H NMR and 13C NMR spectra.](image)

**Figure 1.11.** $^1$H (a) and $^{13}$C (b) NMR of the product formed upon oxidation and BF$_2$ complexation of 2f. Notably, both spectra display the upfield peaks indicative of two distinct methyl groups and two distinct methylene groups.
Due to the synthetic difficulties described above, an alternative control dye was pursued. Compound $4g$ is structurally identical to $4b$ except that the amide linkers of $4b$ are replaced with ester linkers in $4g$ (Fig. 1.10). Similar to $4f$, $4g$ lacks the proton of the amide and is therefore incapable of intramolecular hydrogen bonding. In the respect that it is isosteric to $4b$, $4g$ may be a better control for this study. Precursor $1g$ was synthesized from sodium benzylate and trichloroacetylpyrrole using a procedure that was adapted from the literature. A small amount of $2g$ was produced from the condensation of $1g$ in benzaldehyde and $p$-toluenesulfonic acid. This was oxidized and subjected to BF$_2$ complexation conditions. Less than four milligrams of product was isolated from the reaction mixture. The structure of the product was confirmed by mass spectrometry and $^1$H NMR; however, the low yield precluded full characterization. Further silica purification resulted in decomposition of the desired product.

1.3 Conclusion

Five new BODIPY dyes based on a diamidodipyrrin scaffold have been introduced. Their synthesis, characterization, and spectroscopic properties are presented and evaluated. These dyes utilize the unexplored strategy of intramolecular hydrogen bonding to rigidify the extended chromophore and red-shift the emission. The modular synthesis of these scaffolds allows them to be adapted to a wide-range of applications.
Acknowledgements

This chapter, in part, is a reprint of the material as it appears in Dalton Transactions, 2010. Jacobsen, J. A.; Stork, J. R.; Magde, D.; Cohen, S. M. “Hydrogen-bond Rigidified BODIPY Dyes” Dalton Trans. 2010, 39, 957-962. The thesis author was the primary investigator and author of this paper. Jay R. Stork initiated the BODIPY project as a post-doctoral fellow in the Cohen lab. Doug Magde performed or assisted in many of the photophysical measurements for the BODIPY project.

1.4 Experimental

General

Unless otherwise noted, starting materials were obtained from commercial suppliers and used without further purification. Mass spectrometry was performed at the University of California, San Diego Mass Spectrometry Facility in the Department of Chemistry and Biochemistry. A ThermoFinnigan LCQ-DECA mass spectrometer was used for ESI or APCI analysis, and the data were analyzed using the Xcalibur software suite. Elemental analysis was performed at NuMega Resonance Labs, San Diego, California. $^1$H/$^{13}$C NMR spectra were recorded on Varian FT-NMR spectrometers at the Department of Chemistry and Biochemistry, University of California, San Diego. UV-visible absorption spectra were recorded using a Perkin-Elmer Lambda 25 spectrophotometer with the UVWinLab 4.2.0.0230 software package. Absorbance spectra are reported as $\lambda_{\text{max}}$/nm ($\epsilon$/M$^{-1}$ cm$^{-1}$). Microwave reactions were carried out in a CEM Discover S Class microwave reactor.
**BODIPY 4a**

The precursor compounds 1a, 2a, and 3a were prepared as previously published.\(^{66}\) To a suspension of 3a (105 mg, 0.27 mmol) in 10 mL of \(\text{CH}_2\text{Cl}_2\) was added Et\(_3\)N (0.23 mL, 1.6 mmol), followed by BF\(_3\)·OEt\(_2\) (0.27 mL, 2.2 mmol), and the reaction was stirred for 16 h under N\(_2\) (g). The solution was washed with saturated NaHCO\(_3\) (3×10 mL), dried (MgSO\(_4\)), and evaporated. The crude product was purified by flash silica column chromatography with 0-5% MeOH in \(\text{CH}_2\text{Cl}_2\) as eluant. The product was isolated as a dark purple-red solid (14 mg, 0.033 mmol, 12%). \(^1\)H NMR (300 MHz, CDCl\(_3\)): \(\delta\) 7.48-7.63 (m, 7H), 7.12 (d, \(J = 4.40\), 2H), 6.94 (d, \(J = 4.40\), 2H), 4.26-4.37 (m, 2H), 1.31 (d, \(J = 6.60\), 12H). \(^13\)C NMR (CDCl\(_3\)): \(\delta\) 159.4, 152.3, 136.5, 133.5, 132.4, 131.6, 130.9, 128.8, 123.6, 42.5, 22.7. \(^19\)F NMR (282 MHz, CDCl\(_3\)): \(\delta\) 131. UV-vis \(\lambda_{\text{max}} = 530\) nm (\(\epsilon = 88000 \pm 3000\) M\(^{-1}\) cm\(^{-1}\)). \(\lambda_{\text{em}} = 546\) nm (\(\varphi_f = 0.73\)).  \(\tau = 6.45 \pm 0.10\) ns. ESI-MS: \(m/z\) 439.26 [M+H]\(^+\). HRMS calcd for C\(_{23}\)H\(_{26}\)BF\(_2\)N\(_4\)O\(_2\): 438.2148. Found: 438.2151. Anal. Calcd for C\(_{23}\)H\(_{25}\)BF\(_2\)N\(_4\)O\(_2\): C, 63.03; H, 5.75; N, 12.78. Found: C, 63.41; H, 5.95; N, 12.27.

**1,1′-benzylamide-5-phenyl-4,6-dipyrin (3b)**

The precursor compounds 1b and 2b were prepared as previously published.\(^{66}\) Compound 2b (100 mg, 0.20 mmol) was dissolved in 25 mL of THF. DDQ (47 mg, 0.24 mmol) was dissolved in 25 mL of THF and was added dropwise over 15 min to the solution of the dipyrromethane 2b. The reaction was allowed to stir for
2 h. The THF was then removed on a rotary evaporator. The material was not isolated and was used directly for the synthesis of the BODIPY dye (see 4b below). ESI-MS(+): \( m/z \) 487.20 [M+H]^+.

**BODIPY 4b**

The residue from the reaction mixture of the 3b (0.20 mmol) was dissolved in 5 mL of CH\(_2\)Cl\(_2\) to which Et\(_3\)N (0.25 mL, 2.0 mmol) and BF\(_3\)-OEt\(_2\) (0.25 mL, 2 mmol) were added. The reaction was irradiated in a microwave reactor for 2 min at 65 °C, 250 psi, and 300 W. The solvent was evaporated and the residue was purified twice by flash silica column chromatography with 5% MeOH in CH\(_2\)Cl\(_2\) as eluant for the first column and ethyl acetate as eluant for the second column. The product was isolated as a bright red solid (25 mg, 0.047 mmol, 23%). \(^1\)H NMR (500 MHz, CDCl\(_3\)): \( \delta \) 7.88 (m, br, 2H), 7.63 (tt, \( J = 5.5 \) Hz, 1.8 Hz, 1H), 7.58-7.51 (m, 4H), 7.34-7.23 (m, 10H), 7.16 (d, \( J = 4.6 \) Hz, 2H), 6.96 (d, \( J = 4 \) Hz, 2H), 4.61 (d, \( J = 5.8 \) Hz, 4H). \(^{13}\)C NMR (CDCl\(_3\)): \( \delta \) 160.1, 151.6, 151.0, 137.6, 136.8, 133.4, 132.5, 131.8, 130.9, 128.9, 127.9, 127.7, 124.0, 44.4, 29.9. \(^{19}\)F NMR (470 MHz, CDCl\(_3\)): \( \delta \) 131. ESI-MS(+): \( m/z \) 557.28 [M+Na]^+. UV-vis \( \lambda_{\text{max}} \) = 532 nm (\( \varepsilon = 62000 \pm 2000 \) M\(^{-1}\) cm\(^{-1}\)). \( \lambda_{\text{em}} \) = 548 nm (\( \varphi_r = 0.59 \)). \( \tau = 6.70 \pm 0.10 \) ns. HRMS calcd for C\(_{31}\)H\(_{25}\)BF\(_2\)N\(_4\)O\(_4\)Na: 556.1967. Found: 556.1974.

**N-p-tolyl-1H-pyrrole-2-carboxamide (1c)**

To a stirred suspension of p-toluidine (6.1 g, 0.06 mol) in Et\(_3\)N (7.9 mL, 0.06 mol)
was added 2-trichloroacetyl pyrrole (9.9 g, 0.05 mol). The solution was heated to 60 °C for 18 h. The solvent was removed in vacuo and the residue was crystallized from benzene to afford the product as white needles (2.1 g, 0.01 mol, 22%). $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 11.60 (s, br, 1H), 9.64 (s, 1H), 7.60 (d, $J = 8.1$, 2H), 7.12 (d, $J = 7.33$, 2H), 7.04 (s, 1H), 6.94 (s, 1H), 6.15 (s, 1H), 2.26 (s, 3H). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 159.7, 137.4, 132.5, 129.6, 126.8, 123.0, 120.6, 111.7, 109.5, 21.2. ESI-MS: $m/z$ 201.16 [M+H]$^+$. HRMS calcd for C$_{12}$H$_{13}$N$_2$O: 201.1022. Found: 201.1020. Anal. Calcd for C$_{12}$H$_{12}$N$_2$O: C, 71.98; H, 6.04; N, 13.99. Found: C, 72.23; H, 6.30; N, 14.24.

1,1-[$N$-(p-tolyl)carboxamide]-5-(phenyl)dipyrromethane (2c)

Compound 1c (1.0 g, 5.0 mmol) was dissolved in 15 mL of toluene to which benzaldehyde (0.25 mL, 2.5 mmol) and p-TSA (19 mg, 0.10 mmol) were added. The reaction mixture refluxed under N$_2$(g) for 16 hr. The hot mixture was filtered and the precipitate was rinsed with hot toluene followed by benzene. The product was isolated as a light pink powder (0.88 g, 1.8 mmol, 72%). $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 11.57 (s, 2H), 9.59 (s, 2H), 7.58 (d, $J = 8.06$, 4H), 7.18–7.33 (m, 5H), 7.11 (d, $J = 8.1$, 4H), 6.96 (m, sh, 2H), 5.87 (m, sh, 2H), 5.62 (s, 1H), 2.26 (s, 6H). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 159.6, 143.0, 138.3, 137.5, 132.4, 129.6, 128.8, 127.1, 126.2, 120.6, 112.1, 108.8, 43.5, 21.1. ESI-MS: $m/z$ 489.33 [M+H]$^+$. HRMS calcd for C$_{31}$H$_{28}$N$_4$O$_2$Na: 511.2104. Found: 511.2112. Anal. Calcd for C$_{31}$H$_{28}$N$_4$O$_2$: C, 76.21; H, 5.78; N, 11.47. Found: C, 75.85; H, 6.14; N, 11.62.
1,1-[N-(p-tolyl)carboxamide]-5-(phenyl)dipyrrin (3c)

Compound 2c (0.15 g, 0.31 mmol) was dissolved in 7 mL of THF. DDQ (77 mg, 0.34 mmol) was dissolved in 3 mL of THF and was added dropwise over 15 min to the solution of the dipyrromethane 2c. The reaction was allowed to stir for 2 h. The THF was then removed on a rotary evaporator and the crude product was purified by flash silica column chromatography with 0-2% MeOH in CH₂Cl₂ as eluant. Crystallization from benzene produced a red powder (0.10 g, 0.21 mmol, 68%). ¹H NMR (300 MHz, DMSO-d₆): δ 10.40 (s, 2H), 7.73 (d, 4H, J = 8.25), 7.56–7.64 (m, 5H), 7.18 (d, 4H, J=8.25), 7.11 (d, 2H, J=4.40), 6.69 (d, 2H, J=3.85), 2.31 (s, 6H). ¹³C NMR (DMSO-d₆): δ 159.8, 156.4, 150.3, 141.6, 136.8, 133.6, 131.5, 131.0, 129.9, 128.8, 120.6, 119.6, 21.2. ESI-MS: m/z 487.29 [M+H]⁺. HRMS calcd for C₃₁H₂₇N₄O₂: 487.2129. Found: 487.2137.

BODIPY 4c

Compound 4c was prepared according to the same procedure as outlined for 4a starting from 3c (92 mg, 0.19 mmol). The product was isolated as a dark purple-red solid (8.8 mg, 0.016, 8.5%). Diffraction quality crystals of the acetone solvate grew from a saturated solution over two days. ¹H NMR (500 MHz, CDCl₃): δ 9.45 (t, J = 10.5 Hz, 2H), 7.59 (m, 8H), 7.28 (d, J = 4 Hz, 2H), 7.16 (d, J = 8 Hz, 4H), 7.03 (d, J = 4.6 Hz, 2H), 2.37 (s, 6H). ¹³C NMR (CDCl₃): δ 157.7, 152.1, 137.0, 135.4, 133.4, 132.8, 131.9, 131.0, 129.8, 129.0, 124.5, 120.4, 21.3. ¹⁹F NMR (470

1,1-[N-(isopropyl)carboxamide]-5-(4-cyanophenyl)dipyrrromethane (2d)

The precursor compound 1d was prepared as previously published.⁶⁶ Compound 2d was prepared according to the same procedure as outline for 2c starting from N-isopropyl-1H-pyrrole-2-carboxamide (500 mg, 3.29 mmol) and 4-formylbenzonitrile (215 mg, 1.64 mmol). The product was isolated as a light pink powder (0.68 g, 1.63 mmol, 99%). ¹H NMR (400 MHz, DMSO-d₆): δ 11.36 (s, 2H), 7.75 (d, J = 8 Hz, 2H), 7.65 (d, J = 8.4 Hz, 2H), 7.29 (d, J = 8 Hz, 2H), 6.69 (t, J = 2.8 Hz, 2H), 5.78 (t, J = 2.8 Hz, 2H), 5.62 (s, 1H), 4.02 (sxt, J = 6.4 Hz, 2H), 1.11 (dd, J = 6.8 Hz, 2Hz, 12H). ¹³C NMR (DMSO-d₆): δ 165.1, 153.8, 140.4, 137.6, 134.5, 134.3, 133.6, 133.5, 131.7, 130.7, 124.4, 115.4, 114.5, 113.4, 48.1, 28.0. ESI-MS: m/z 418.13 [M+H]⁺. HRMS calcd for C₂₄H₂₈N₅O₂: 418.2238. Found: 418.2245.

BODIPY 4d

A solution of DDQ (0.23 g, 1.0 mmol) in 25 mL of CH₂Cl₂ was added dropwise to a suspension of 2d (0.42 g, 1.0 mmol) in 75 mL of CH₂Cl₂. After stirring for 1.5 h, Et₃N (2.0 mL, 14.0 mmol) and BF₃·OEt₂ (3.0 mL, 24.0 mmol) were added. After
40 h the reaction mixture was washed with 50 mL of H$_2$O, then saturated NaHCO$_3$ (2×30 mL), dried (MgSO$_4$), and concentrated. The crude product was purified by flash silica column chromatography with 0-1% MeOH in CH$_2$Cl$_2$ as eluant and then crystallized from CH$_2$Cl$_2$/hexanes to give a purple-red solid (65 mg, 0.14 mmol, 14%). $^1$H NMR (300 MHz, CDCl$_3$): δ 7.87 (d, $J = 7.98$, 2H), 7.66 (d, $J = 8.25$, 2H), 7.44 (q, br, $J = 8.25$, 2H), 7.13 (d, $J = 4.13$, 2H), 6.84 (d, $J = 4.40$, 2H), 4.25-4.36 (m, 2H), 1.30 (d, $J = 6.60$, 12H). $^{13}$C NMR (CDCl$_3$): δ 159.0, 153.3, 147.1, 137.6, 136.0, 132.6, 131.9, 131.3, 129.6, 124.2, 117.8, 115.3, 42.6, 29.9, 22.6. $^{19}$F NMR (282 MHz, CDCl$_3$): δ 131. UV-vis $\lambda_{\text{max}} = 538$ nm (ε = 61000 ± 4000 M$^{-1}$ cm$^{-1}$). $\lambda_{\text{em}} = 557$ nm (φ$_f$ = 0.30). $\tau = 3.35$ ± 0.10 ns. ESI-MS: m/z 464.13 [M+H]$^+$. HRMS calcd for C$_{24}$H$_{25}$BF$_2$N$_5$O$_2$: 463.2100. Found: 463.2109.

$N$-(2-hydroxyethyl)-1H-pyrrole-2-carboxamide (1e)

Trichloroacetylpyrrole (2.0 g, 9.4 mmol) was dissolved in 10 mL of THF. Ethanolamine (566 µL, 9.4 mmol) was dissolved in 10 mL THF and was added to the pyrrole solution dropwise for 30 min cooled with an ice bath. The reaction was removed from the ice bath after 1.5 h and was allowed to stir overnight at room temperature. The solution was evaporated to an oil and the residue was purified via flash silica column chromatography with 10% MeOH in CH$_2$Cl$_2$ as eluant. The evaporated product was re-dissolved in CH$_2$Cl$_2$ and washed with 1M HCl. A
colorless oil was isolated from the organic layer (1.05 g, 6.8 mmol, 72%). $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 11.40 (s, 1H), 7.95 (t, $J = 5.2$ Hz, 1H), 6.82 (s, 1H), 6.75 (s, 1H), 6.06 (dd, $J = 2.4$ Hz, 6.4 Hz, 1H), 4.72 (t, $J = 5.4$ Hz, 1H), 3.47 (q, $J = 5.6$ Hz, 2H), 3.26 (q, $J = 6.4$ Hz, 2H). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 161.5, 127.0, 121.8, 110.5, 109.1, 60.8, 42.1. ESI-MS(+): $m/z$ 154.82 [M+H]$^+$. HRMS calcd for C$_7$H$_{10}$N$_2$O$_2$: 177.0636. Found: 177.0634.

1,1’-(2-hydroxyethylamide)-5-phenyl-4,6-dipyrrinomethane (2e)

Compound 1e (1.04 g, 6.7 mmol) was dissolved in 30 mL of a 1:1 solution of EtOH and toluene to which benzaldehyde (341 µL, 3.4 mmol) and a catalytic amount of $p$-TSA were added. The reaction mixture was heated to 95 °C and stirred overnight. The solvents were evaporated to an oily residue that was purified via flash silica column chromatography with 10% MeOH in CH$_2$Cl$_2$ as eluant. An orange powder was isolated (423 mg, 1.1 mmol, 31%). $^1$H NMR (300 MHz, CD$_3$OD): $\delta$ 7.27-7.10 (m, 7H), 6.66 (d, $J = 3.9$ Hz, 2H), 5.74 (d, $J = 3.6$ Hz, 2H), 5.47 (s, 1H), 3.59 (q, $J = 5.7$ Hz, 4H), 3.36 (t, $J = 5.4$ Hz, 4H). $^{13}$C NMR (DMSO-$d_6$): $\delta$ 161.4, 143.2, 137.1, 128.7, 127.0, 126.4, 126.2, 110.1, 108.4, 60.8, 43.4, 42.1. ESI-MS(+): $m/z$ 419.15 [M+Na]$^+$. HRMS calcd for C$_{21}$H$_{25}$N$_4$O$_4$: 397.1870. Found: 397.1876.

1,1’-(2-hydroxyethylamide)-5-phenyl-4,6-dipyrin (3e)

Compound 3e was prepared according to the same procedure as outlined for 3c
starting from 2e (200 mg, 0.50 mmol). The material was not isolated and was used directly for the synthesis of the BODIPY dye (see 4a below). APCI-MS(+): $m/z$ 395.17 [M+H]$^+$. 

**BODIPY 4e**

BODIPY dye 4e was prepared according to the same procedure as outlined for 4c starting from crude 3e (0.50 mmol). The dye was isolated as a red solid (20 mg, 0.045 mmol, 9%). $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.91 (t, br, 2H), 7.67-7.55 (m, 4H), 7.11 (d, $J$ = 4.5 Hz, 2H), 6.98 (d, $J$ = 5.1 Hz, 2H), 3.87 (t, $J$ = 4.5 Hz, 4H), 3.66 (q, $J$ = 5.1 Hz, 4H). $^{13}$C NMR (CDCl$_3$): $\delta$ 160.7, 151.5, 133.3, 132.7, 131.8, 130.9, 128.9, 123.4, 61.4, 42.9. $^{19}$F NMR (470 MHz, CDCl$_3$): $\delta$ 151. UV-vis $\lambda_{\text{max}}$ = 530 nm ($\varepsilon$ = 31000 ± 1000 M$^{-1}$ cm$^{-1}$). $\lambda_{\text{em}}$ = 546 nm ($\varphi_t$ = 0.41). $\tau$ = 6.40 ± 0.10 ns. ESI-MS(+): $m/z$ 443.11 [M+H]$^+$. HRMS calcd for C$_{21}$H$_{21}$BF$_2$N$_4$O$_4$Na: 464.1552. Found: 464.1560.

**1,1-Benzyl-1,1’-methylamide-5-phenyl-4,6-dipyrromethane (2f)**

The starting material was prepared according to literature precedent. N-(methyl)-N-(benzyl)-1H-pyrrole-2-carboxamide (1f, 200 mg, 0.93 mmol) was dissolved in 3 mL xylene to which benzaldehyde (50 mL, 0.5 mmol) and a catalytic amount of InCl$_3$ were added. The reaction was carried out in a microwave reactor at 100 °C for 10 min, allowed to cool for two minutes, and reheated to 100 °C for 10 min. The power and pressure for the reaction were 250 psi and 300 W, respectively. The xylene was
evaporated and the crude product was purified by flash silica column chromatography
with 1-2% MeOH in CH₂Cl₂ as eluant. An off white solid was isolated (21 mg, 0.043
mmol, 9%). ¹H NMR (500 MHz, CDCl₃): δ 10.53 (s, br, 2H), 7.46-7.27 (m, 15H),
6.93 (s, 2H), 6.64-6.23 (m, br, 2H), 4.84 (s, br, 4H), 3.24 (s, br, 6H). ESI-MS(+): m/z

1,1-BenzyI-1,1’methylamide-5-phenyl-4,6-dipyrrin (3f)
1,1-BenzyI-1,1’methylamide-5-phenyl-4,6-dipyrromethane (40 mg, 0.077 mmol) was
dissolved in 20 mL of THF. DDQ (21 mg, 0.092 mmol) was dissolved in 20 mL of
THF and was added dropwise over 15 min to the solution of dipyrromethane. The
reaction was allowed to stir for 2 h. The THF was then removed on a rotary
evaporator. The material was used as a crude product for the synthesis of the resultant
dye. APCI-MS(+): m/z 515.18 [M+H]⁺.

BF₂(DADPPh,Me-Bn)- isomer? (4f)
1,1-BenzyI-1,1’methylamide-5-phenyl-4,6-dipyrrin (0.077 mmol) from the prior
reaction was dissolved in 5 mL of CH₂Cl₂. Et₃N (0.25 mL, 1.8 mmol) and BF₃OEt₂
(0.25 mL, 2 mmol) were added. The solution was irradiated in a microwave
synthesizer for 2 min at 65 °C. The reaction mixture was evaporated to dryness. The
residue was run on a silica column with 2% methanol in CH₂Cl₂ as eluant to yield a
bright red solid (25 mg, 0.047 mmol, 23%). ¹H NMR (400 MHz, CDCl₃): 7.62-7.50
(m, 5H), 7.42-7.27 (m, 10H), 7.16 (d, J = 6.4 Hz, 2H), 6.96 (d, J = 3.6 Hz, 1H), 6.90
(d, J = 4.4 Hz, 1H), 6.56 (d, J = 4.0 Hz, 2H), 4.83 (s, 2H), 4.51 (s, 2H), 3.08 (s, 3H),
2.86 (s, 3H). ¹³C NMR (400 MHz, CDCl₃): 164.2, 163.9, 136.3, 135.2, 133.7, 132.4,
132.5, 131.3, 130.7, 129.1, 129.0, 128.9, 128.8, 128.6, 128.0, 127.8, 127.3, 127.2,
118.0, 55.1, 50.5, 32.6, 29.9. ¹⁹F NMR (470 MHz, CDCl₃): δ 144. ESI-MS(+)]: m/z 563.14 [M+H]⁺. UV-vis λmax = 514 nm (ε = 7300 ± 700 M⁻¹ cm⁻¹). λem = 535 nm (Φr = 0.040). τ = 0.50 ± 0.03 ns. HRMS calcd for C₃₃H₂₉BF₂N₄O₂Na: 584.2280. Found: 584.2287.

**Benzyl 1H-pyrrole-2-carboxylate (1g)**

Prepared with an adapted method from literature precedent. Sodium metal (113 mg, 4.9 mmol) was carefully added to freshly distilled benzyl alcohol (2.3 mL, 22.2 mmol). The reaction was allowed to stir overnight. Trichloroacetylpyrrole (4.5 g, 21.2 mmol) was dissolved in 8 mL dry CHCl₃. The sodium benzylate was added to the solution and the reaction was allowed to stir to completion (~3 h). 5 mL 1 M HCl was added and the solution was allowed to stir for 15 min. The solution was evaporated to ~ 5 mL and a grey ppt was filtered off. The product was extracted from the filtrate with CH₂Cl₂. The organic layer was dried with MgSO₄. The resulting residue was purified by flash silica chromatography in 85:15 hexanes to ethyl acetate. 1.8 g (8.9 mmol) of a white powder was obtained. Yield: 42%. ¹H NMR (500 MHz, CDCl₃) δ 9.12 (s, br, 1H), 7.42-7.30 (m, 5H), 6.95 (m, 2H), 6.26 (d, J = 1.8 Hz, 1H), 5.30 (s, 2H). ESI-MS(+)]: m/z 201.93 [M+H]⁺.
1,1’-(Benzyloxy)carbonyl-5-phenyl-4,6-dipyromethane (2g)

Benzyl 1H-pyrrole-2-carboxylate (400 mg, 1.99 mmol), benzaldehyde (100 µL, 0.99 mmol), and p-toluenesulfonic acid (5 mg, catalytic quantity) were heated to 80°C in 30 mL toluene overnight. The reaction mixture was evaporated and the residue was purified by flash silica chromatography using a gradient eluent up to 40% ethyl acetate in hexanes. 61 mg (0.124 mmol) of a white powder was obtained. Yield: 13%. 1H NMR (400 MHz, CDCl₃) δ 9.05 (s, br, 1H), 7.38-7.20 (m, 15H), 6.86 (t, J = 3.2 Hz, 2H), 5.95 (t, J = 3.2 Hz, 2H), 5.46 (s, 1H), 5.24 (s, 4H). 13C NMR (400 MHz, CDCl₃) δ 137.5, 129.3, 128.8, 128.6, 128.4, 128.0, 122.5, 116.3, 110.4, 66.2, 44.5. ESI-MS(+): m/z 490.91 [M+H]⁺. HRMS calcd for C₃₁H₂₆N₂O₄Na: 513.1785. Found: 513.1782.

1,1’-(Benzyloxy)carbonyl-5-phenyl-4,6-dipyrin (3g)

1,1’-(Benzyloxy)carbonyl-5-phenyl-4,6-dipyrrmethane (100 mg, 0.204 mmol) was dissolved in 20 mL of THF. DDQ (56 mg, 0.245 mmol) was dissolved in 20 mL of THF and was added dropwise over 15 min to the solution of dipyrrmethane. The reaction was allowed to stir for 2 h. The THF was then removed on a rotary evaporator. The material was used as a crude product for the synthesis of the resultant dye. ESI-MS(+): m/z 489.08 [M+H]⁺.

BF₂DADP(Ph,BnE) (4g)

1,1’-(Benzyloxy)carbonyl-5-phenyl-4,6-dipyrrin (0.139 mmol) was dissolved in 10
mL of CH₂Cl₂. Et₃N (1 mL, 6.9 mmol) and BF₃OEt₂ (1 mL, 7.9 mmol) were added and the reaction was stirred for 16 h under N₂(g). The solution was washed with satd NaHCO₃ (3×20 mL), dried (MgSO₄), and evaporated. The crude product was purified by flash silica column chromatography with 30% ethyl acetate in hexanes as eluant. 4 mg (0.007 mmol) of a red solid was isolated. Yield: 5%. ¹H NMR (300 MHz, CDCl₃) δ 7.38-7.23 (m, 15H), 7.13 (d, J = 3.6 Hz, 2H), 5.76 (d, J = 3.9 Hz, 2H), 5.21 (s, 4H). ESI-MS(+): m/z 559.10 [M+Na]⁺.

**Crystal data for 4c**

Red needles of the acetone solvate grew at room temperature in a sealed vial (to keep evaporation to a minimum) from a saturated acetone solution over two days. C₃₄H₃₁BF₂N₄O₃, M = 592.44, monoclinic, space group P2₁/n, a = 11.750(3) Å, b = 9.834(3) Å, c = 25.870(7) Å, α = γ = 90°, β = 97.836(3)°, V = 2961.3(14) Å³, T = 100(2) K, Z = 4, 30660 reflections measured, 5421 unique, (Rint = 0.0890), R₁ = 0.0888 (I>2σ(I)), GOF = 1.059. Full details are available in the CIF file (CCDC No. 724399).

**Quantum Yield Measurements**

Emission spectra and luminescence excitation spectra were recorded with a Perkin Elmer LS-55 spectrofluorimeter, supplemented for the weakest emitter by a home-built Raman spectrometer using excitation at 514.5 nm. Fluorescence quantum yields were determined following the protocols set forth by Demas and Crosby.⁷⁴
The reference was rhodamine 6G in methanol with an assumed yield of 0.93±0.02. A major reason for using rhodamine 6G as the reference is that its spectra for both absorption and emission are quite similar to those of the compounds reported here. Solutions were prepared for standard and samples to give absorbances between 0.4 and 0.6 at 500 nm. These were diluted by 10- to 40-fold by gravimetric methods (because of the small amounts of material available and the difficulties involved in pipetting dichloromethane). Plots of emission versus absorption were examined for curvature and the slope determined for the limiting behavior at low concentrations. Spectra were recorded using the correction factors supplied by the manufacturer. In all cases, measurements were carried out at two or more excitation wavelengths as a check, one of which was always 500 nm. Quantum yield values reported in Table 1 have a precision of ~5% for good emitters, and ~50% for the almost nonemissive compound.

1.5 References


Chapter 2: Chelator Fragment Libraries for Metalloprotein Inhibitors

2.1 Introduction

At least a third of all proteins contain metal ions for structural support or catalytic function. Numerous human diseases are associated with misregulated activity of human metalloenzymes or the activity of foreign metalloenzymes from invading pathogens. Inhibitors of metalloenzymes are desired as therapeutics and for applications as pesticides, cosmetics, and scientific tools. Potential therapeutic targets for a variety of diseases include:

- Zinc enzymes: matrix metalloproteinases (MMPs), anthrax lethal factor (LF), histone deacetylases (HDAC)
- Iron enzymes: 5-lipoxygenase (5-LO), nitric oxide synthase (NOS)
- Copper enzymes: tyrosinase (Ty)
- Magnesium enzymes: HIV integrase (IN)
- Other metalloenzymes: see Table 2.1 for an expanded list

The mere presence of a metal ion in these enzymes makes them attractive medicinal targets. Specifically, the metal ion can serve as an anchoring site to which new drugs with metal-binding groups can be directed. Additionally, because metal ions are often directly involved in the catalytic activity of metalloenzymes, compounds that bind at the metal site are likely to inhibit via a competitive mechanism. For these reasons, medicinal chemists have used metal-binding groups in synthetic inhibitors of metalloenzymes for years.
Four metalloprotein targets will be profiled as background for the current study. The function, regulation, structure, associated diseases, and inhibitors of MMP, LF, 5-LO, and tyrosinase will be discussed. This will be followed by an introduction to fragment-based lead design (FBLD) and a discussion of how FBLD might be used to generate metalloenzyme inhibitors.
Table 2.1. Expanded list of metalloenzyme targets and their clinical relevance.

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Enzyme</th>
<th>Abbreviation</th>
<th>Clinical Relevance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Matrix Metalloprotease</td>
<td>MMP</td>
<td>Cancer, OA, MS, COPD, Ischemic injury, others</td>
<td>[14-16]</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Anthrax Lethal Factor</td>
<td>LF</td>
<td>Toxin</td>
<td>[7, 17]</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Botulinum Neurotoxin</td>
<td>BoNT</td>
<td>Toxin</td>
<td>[7, 29]</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Histone Deacetylase</td>
<td>HDAC</td>
<td>Cancer, ND, Inflammatory diseases</td>
<td>[18-20]</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Carbonic Anhydrase</td>
<td>CA</td>
<td>TB, Glaucoma</td>
<td>[30, 31]</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Angiotensin Converting Enzyme</td>
<td>ACE</td>
<td>Hypertension, CHF</td>
<td>[32-34]</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>TNF-alpha Converting Enzyme</td>
<td>TACE</td>
<td>Inflammatory diseases, Cancer</td>
<td>[35-37]</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Farnesyl Transferase</td>
<td>FT or FTase</td>
<td>Cancer, Malaria</td>
<td>[38-40]</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>VanX</td>
<td>VanX</td>
<td>Gram (-) bacterial infection</td>
<td>[6, 41]</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>LpxC</td>
<td>LpxC</td>
<td>Gram (-) bacterial infection</td>
<td>[6, 42, 43]</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Aggrecanase</td>
<td></td>
<td>Arthritis</td>
<td>[44, 45]</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Adenosine Deaminase</td>
<td>ADA</td>
<td>Inflammation, RA, Leukemia, Malaria, others</td>
<td>[46-48]</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Glyoxalase (Note: Bacterial form contains Ni&lt;sup&gt;2+&lt;/sup&gt;)</td>
<td>Glx-1</td>
<td>Cancer</td>
<td>[49-51]</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Hepatitis C Protease</td>
<td>HCV NS2-NS3 protease</td>
<td>Hepatitis C</td>
<td>[52, 53]</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Metallo-beta-Lactamases</td>
<td>MBLs</td>
<td>Bacterial infections</td>
<td>[5, 42, 54]</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>MshB</td>
<td>MshB</td>
<td>TB</td>
<td>[42, 55, 56]</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Thermolysin</td>
<td></td>
<td>Infections</td>
<td>[42, 57]</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Methionyl-tRNA-Synthetase</td>
<td>MetRS</td>
<td>Bacterial infections</td>
<td>[58, 59]</td>
</tr>
<tr>
<td>Heme Fe</td>
<td>Nitric Oxide Synthase</td>
<td>INOS, nNOS, eNOS</td>
<td>Inflammatory diseases, Cancer</td>
<td>[23, 24]</td>
</tr>
<tr>
<td>Heme Fe</td>
<td>Cyclooxygenase</td>
<td>COX</td>
<td>Inflammation</td>
<td>[60-62]</td>
</tr>
<tr>
<td>Heme Fe</td>
<td>Myeloperoxidase</td>
<td>MPO</td>
<td>CVD</td>
<td>[63, 64]</td>
</tr>
<tr>
<td>Fe&lt;sup&gt;2+&lt;/sup&gt;/Fe&lt;sup&gt;3+&lt;/sup&gt;</td>
<td>Lipoxygenase</td>
<td>LO or LOX</td>
<td>Inflammatory diseases, CVD, Cancer</td>
<td>[21, 22]</td>
</tr>
<tr>
<td>Fe&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Peptide Deformylase</td>
<td>PDF</td>
<td>Bacterial infections</td>
<td>[65, 66]</td>
</tr>
<tr>
<td>Fe&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Ribonucleotide Reductase</td>
<td>RR or RNR</td>
<td>Cancer, Viral infections</td>
<td>[67, 68]</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>RNA polymerase</td>
<td>RNAP or RNApol</td>
<td>Infections</td>
<td>[69, 70]</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Hematopoietic Prostaglandin d Synthase</td>
<td>H-PGDS</td>
<td>Inflammatory diseases</td>
<td>[71-73]</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;/Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>HIV Integrase</td>
<td>IN</td>
<td>HIV infection</td>
<td>[27, 28]</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>1-Deoxy-α-Xylulose-5-phosphate Reductoisomerase</td>
<td>DXR</td>
<td>Malaria, Bacterial infections</td>
<td>[6, 74]</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Semicarbazide-Sensitive Amino Oxidase</td>
<td>SSAO</td>
<td>DM, CHF, Inflammatory diseases, AD</td>
<td>[75]</td>
</tr>
<tr>
<td>Cu&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Tyrosinase</td>
<td>Ty</td>
<td>Skin cancer, Cosmetics, Food preservative</td>
<td>[25, 26]</td>
</tr>
<tr>
<td>Ni&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Urease</td>
<td></td>
<td>Bacterial infections</td>
<td>[76]</td>
</tr>
<tr>
<td>Ni&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Glyoxalase (Note: Human form contains Zn&lt;sup&gt;2+&lt;/sup&gt;)</td>
<td>Glx-1</td>
<td>Bacterial infections, Malaria</td>
<td>[49-51]</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Steroid Sulphatase</td>
<td>STS</td>
<td>Endocrine disorders, Estrogen-related cancer</td>
<td>[77, 78]</td>
</tr>
<tr>
<td>Mn&lt;sup&gt;2+&lt;/sup&gt;/Fe&lt;sup&gt;2+&lt;/sup&gt;/Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Methionine Aminopeptidase</td>
<td>MetAP</td>
<td>Bacterial/fungal infections, Cancer</td>
<td>[58, 79]</td>
</tr>
</tbody>
</table>
Zinc Enzymes: MMPs

Matrix metalloproteinases (MMPs) are a family of more than twenty Zn\(^{2+}\)- and Ca\(^{2+}\)-dependant enzymes that are involved in normal tissue growth and repair.\(^{15, 81}\) MMPs are able to degrade and remodel the extracellular matrix and are involved in the innate immune response, angiogenesis, embryonic development, and many signalling pathways.\(^{81}\) MMP activity is tightly regulated by a complex system involving transcriptional regulation, post-translational regulation, and inhibition by endogenous protein inhibitors.\(^{15}\) MMP mRNA transcript levels are normally kept at a basal level; however, expression is upregulated in times of matrix remodelling.\(^{15}\) MMPs are translated and secreted as pro-enzymes and are activated upon cleavage of the pro-domain, thereby exposing the catalytic active site. MMP activity is further regulated by four endogenous protein inhibitors, the tissue inhibitors of MMP (TIMPs).\(^{82, 83}\)

Misregulated MMP activity is problematic and significant research has sought to identify and understand the roles of individual MMPs in various diseases.\(^{84}\) Cancer is a major target of MMPi development due to the role of MMPs in tumorigenesis and tumor metastasis via angiogenesis. MMPs are upregulated in nearly all forms of cancer.\(^{15}\) Potent MMPi have been shown to reduce tumor size and metastasis in animal models thereby validating MMPs as an appropriate target for cancer.\(^{16}\) Aside from cancer, upregulation of MMPs occurs in other pathologies further making MMPs a significant target for therapeutic intervention. MMP-13 may be an important target for osteoarthritis treatment due to its ability to efficiently cleave Type II collagen and significantly impact cartilage degradation.\(^{85}\) MMP-12 is required for TNF\(\alpha\) release that leads to cigarette-smoke-induced inflammation.\(^{86}\) Consequently, MMP-12 may
be a viable therapeutic target for chronic obstructive pulmonary disease (COPD). Elevated levels of MMP-2 and MMP-9 are correlated with the opening of the blood brain barrier during reperfusion following ischemia; thus MMPi may be desirable to mitigate the harm caused by stroke. Misregulated MMP activity is also associated with cardiovascular disease, multiple sclerosis, periodontal disease, and others. Though the potential applications are numerous, MMPi have fared poorly in clinical trials. The failure of broad-spectrum MMPi in clinical trials has been attributed to the dose-limiting musculoskeletal side effects of chronic drug administration, presumably caused by non-specific MMP inhibition. This has established, as the premier goal of MMPi design, the development of inhibitors with significant selectivity between MMP subtypes. To attain biologically significant selectivity a 1000-fold magnitude difference in potency between MMP subtypes is required, which has proved to be a very challenging goal. MMPs have many overlapping substrates and, because of this, they have very similar active sites. MMPs contain a catalytic Zn$^{2+}$ bound by three histidine residues derived from a conserved HExGHxxGxxH motif with the fourth coordination site occupied by a water molecule (Fig. 2.1). Flanking the catalytic zinc ion are “primed” (S1', S2', and S3') and “unprimed” (S1, S2, and S3) substrate binding pockets, which accommodate C-terminus and N-terminus amino acids, respectively, when the natural peptide substrate is bound. The S1' site within MMPs is the most variable pocket in terms of depth and amino acid makeup and, consequently, is the primary site targeted in pursuit of selective MMPi. The S1' pockets in MMP-1 and -7 are significantly occluded and
therefore cannot accommodate inhibitors with bulky substituents. This fact has been exploited in the generation of inhibitors that spare both these MMPs.

![Figure 2.1](image)

**Figure 2.1.** A schematic representation (left) and a protein crystal structure (right, PDB code: 1QIB) of the active site of MMP-2. The catalytic zinc (pink sphere) is coordinated by three histidine residues and a water molecule. A local glutamate residue contributes to water activation.

The vast majority of MMPi in the literature contain a zinc-binding group (ZBG) to chelate the active site Zn$^{2+}$ and a backbone to target one of the substrate binding pockets. Hydroxamic acids were long considered to be the best ZBG due to their ability to bind zinc and form hydrogen bonds with neighboring protein residues. However, this dogma is increasingly challenged as issues arise concerning the metabolic stability of hydroxamic acids and as new ZBGs are introduced and developed into potent inhibitors. Hydrazide, urea, squaric acid, mercaptosulfide, sulfonylamino phosphonate, carbamoyl phosphonate, oxazoline, pyrimidine-2,4,6-trione, hydroxypyrone, and hydroxypyridinone have all been used as ZBGs in MMPi. While many of these inhibitors are semi-selective (inhibit three or
four MMP subtypes potently with selectivity over others), very few have been reported that are selective for a single MMP subtype. One carbamoyl phosphonate MMPi, 1 (see Fig. 2.2), is a simple, but potent inhibitor of MMP-2 ($IC_{50} = 20$ nM, $IC_{50}$ is the concentration that leads to 50% reduction of enzyme activity) with $\geq$1000-fold selectivity over MMP-1, -3, -8, and -9. In a separate example, several of the pyrimidine-2,4,6-triones have been developed into potent and selective inhibitors of MMP-13. The pyrimidine-2,4,6-trione, 2, was found to inhibit bovine cartilage degradation with an $IC_{50}$ of 40 nM (Fig. 2.2). So far the development of selective inhibitors has been limited; however, multiple studies indicate that the ZBG makes an important contribution to the selectivity profile of the overall inhibitor. A better understanding of the role of the ZBG, through systematic studies comparing inhibitors with different ZBGs, is required to effectively use the ZBG as a tool to generate selective inhibitors. While inhibitors with ZBGs continue to dominate the literature, two important counter-strategies for generating selective MMPi have emerged.

![Figure 2.2](image.png)

**Figure 2.2.** The structures of selected MMP inhibitors are presented here. Inhibitors 1 and 2 bind to the catalytic zinc to inactivate MMPs while 3 is an example of a non-zinc-binding MMPi.

In 2007, Johnson *et al.* introduced 3 (Fig. 2.2), one of the most selective MMPi to date. Compound 3 inhibits MMP-13 with an $IC_{50} = 0.67$ nM. The $IC_{50}$ of
compound 3 is $> 30 \mu M$ against MMP -1, -2, -3, -7, and -14, and MMP-8, -9, and -12 are 50% inhibited at $>100 \mu M$. A crystal structure of 3 bound in MMP-13 shows that this inhibitor does not bind the catalytic zinc, with the closest ligand-metal distance being a distant 5.53 Å. Binding of 3 induces Gly227 of MMP-13 to rotate, thereby extending the S1' pocket to accommodate the inhibitor. Gly227 is not conserved across all MMPs and this likely contributes to the selectivity of 3. Consistent with the crystal structure, kinetic observations indicate that 3 acts as a non-competitive inhibitor. Due to its impressive selectivity against MMP-13, compound 3 is in development as an anti-arthritis drug. In a rat model of knee joint damage, 3 was shown to reduce the cartilage legion area of tibial plateaus by 68% with twice daily oral dosing at 30 mg/kg. Furthermore, 3 did not induce the toxic joint side effects indicative of musculoskeletal syndrome (MSS). Inhibitor 3 is among the first reports of a new class of “non-zinc-binding MMPi”.

Another important development in the field of MMPi is the mechanism-based inhibitor, first reported in 2000 by Mobashery and coworkers. The mechanism of inhibition by a compound designated as SB-3CT occurs through a multi-step process beginning with the coordination of the zinc through the sulfur of the thiirane ring (see Fig. 2.3). Upon coordination, the thiirane ring is activated thereby exposing it to nucleophilic attack by an active site glutamic acid residue (Glu$^{404}$). This results in ring-opening and the formation of a covalent-ester bond between inhibitor and protein. The ester bond serves to tether the inhibitor in the active site resulting in slow-binding inhibition of MMP-2, -3, and -9. The selectivity of SB-3CT is derived from the mechanism of MMP inhibition: slow-binding inhibition of MMP-2, -3, and -9 versus
competitive inhibition of other MMPs. Based on this specificity, SB-3CT has been evaluated in pre-clinical models of cancer and stroke. SB-3CT was shown to inhibit liver metastases by 73% and to significantly reduce the tumor colony sizes in a mouse model of T-cell lymphoma when dosed at 50 mg/kg/d. Batamistat, a first generation broad-spectrum MMPi, increased metastasis in the same model. These results indicate that inhibitors with selectivity derived from their mechanism of inhibition may be a viable alternative to traditional, competitive inhibitors.

Figure 2.3. Scheme for the activity of mechanism-based MMPi (SB-3CT). The thirane ring of SB-3CT coordinates the zinc. The active site glutamic acid then attacks the thirane ring to induce ring opening. This results in the formation of a covalent ester bond between inhibitor and protein.

**Zinc Enzymes: LF**

Lethal factor (LF) is a Zn$^{2+}$ metalloendopeptidase that is a potent component of the anthrax toxin (ATX) produced by *Bacillus anthracis*. ATX consists of two toxins: lethal toxin (LTx) and edema toxin (ETx). Both toxins contain protective antigen (PA), which is responsible for cell binding and entry of either edema factor...
(EF) or LF. PA binds to a cell surface receptor on macrophages and is then cleaved by a furin-like surface membrane protease.\textsuperscript{17} Cleavage of the N-terminal domain of PA initiates oligimerization of the remaining peptide to form a membrane-inserting barrel with seven components. Three molecules of either EF or LF can bind to the PA heptamer to gain entry to the cell. Upon binding the complex is endocytosed and trafficked to the endosome. The low pH of the endosome triggers the PA heptamer to insert in the membrane and open a channel for EF and LF to travel to the cytosol. Once inside EF upregulates cAMP to stimulate systemic edema and to disrupt the innate immune response in order to aid the bacteria in host defense evasion. LF cleaves members of the mitogen-activated protein kinase kinase (MAPKK) family to disrupt downstream signaling pathways and cause macrophage apoptosis and ultimately host death.\textsuperscript{17, 102}

In 2001, several letters containing pulverized anthrax spores were sent to public officials in Washington, DC, resulting in several deaths and public panic.\textsuperscript{7} Infection by \textit{B. anthracis}, occurring via inhalational, gastrointestinal, or subcutaneous routes, is difficult to detect and frequently lethal due to the actions of ATX.\textsuperscript{102} \textit{B. anthracis} is generally susceptible to many types of antibiotics including penicillins, tetracyclins, and aminoglycosides, among others.\textsuperscript{7} However, during an anthrax infection it is not sufficient to clear the bacteria; once ATX is released, it may induce death even after the bacteria has been eliminated. Consequently, novel treatments are needed to directly block the activity of ATX. LTx has been identified as the major virulence factor of \textit{B. anthracis} as it plays a major role in bringing about septic shock and death.\textsuperscript{102} Direct administration of LTx is lethal in rats, guinea pigs, mice, and
rabbits. Further, LTx initiates caspase-dependant apoptosis in human endothelial cells to cause vascular damage and, accordingly, a variety of bleeding symptoms. LTx comprised of functioning PA but inactive mutant LF is non-toxic when administered to Swiss mice, indicating that LF activity is essential to the lethality of LTx.

LF has fairly restricted proteolytic specificity as it can cleave the N-terminus of six of the seven forms of MAPKK (excluding MAPKK-5). The catalytic domain of LF, domain IV, contains a HEXXH zinc-binding motif (Fig. 2.4). A mutation to any of these critical residues renders LF impaired or inactive. A catalytic water molecule responsible for peptide hydrolysis is activated by both the catalytic Zn$^{2+}$ (through axial coordination) and by an active site glutamic acid (Glu$^{687}$). The active site residues surrounding the Zn$^{2+}$ are generally hydrophobic.

**Figure 2.4.** A schematic representation (left) and protein crystal structure (right, PDB code: 1YQY) of the active site of LF. The catalytic zinc (pink sphere) is coordinated by two histidine residues, a glutamic acid, and a water molecule. A local glutamate residue contributes to water activation.
Most inhibitors of LF seek to directly block the catalytic activity through zinc chelation. Initial inhibitors of LF used optimized peptide fragments similar to the natural substrate coupled with a hydroxamic acid to chelate the zinc. One extremely potent peptide-based LFi ($K_i = 1$ nM, sequence: AcGYβARRRRRRVLR-NHOH) was found to inhibit macrophage cell death and MAPKK-3 cleavage.\textsuperscript{107} In 2005, Shoop et al. reported a competitive hydroxamate peptide-mimic 4 that inhibited LFi with an IC$_{50} = 54$ nM (see Fig. 2.5).\textsuperscript{108} Compound 4 resulted in 100% survival in a lethal mouse toxemia model in which LTx is administered intravenously. Further, when co-administered with ciprofloxacin, 4 gave 100% protection in a rabbit infected with $B.\ anthracis$. Numerous other LFi have been reported; however, few have submicromolar IC$_{50}$ values and most reports lack cell and animal studies, which are essential indications of efficacy and inhibitor cell penetration. Other zinc-binding inhibitors include hydroxypyrothiones\textsuperscript{109-111} and urea derivatives.\textsuperscript{112} Additionally, non-competitive inhibitors based on hydrazone\textsuperscript{113} and polyphenol\textsuperscript{114} scaffolds have been reported. Comprehensive reviews of LFi are available.\textsuperscript{17,115}
Figure 2.5. Selected LF inhibitors. Compound 4 is a nanomolar inhibitor of LF with promising pre-clinical results. Hydroxypyrothione derivative 5 and urea derivative 6 are two other examples of inhibitors that inactivate LF by chelating the catalytic zinc. Below are hydrazone inhibitor 7 and polyphenol inhibitor 8, two examples of non-competitive inhibitors.

As *B. anthracis* has become a tool of bioterrorism, weaponized versions of the bacteria are of serious concern. Strains of *B. anthracis* have been reported that are genetically engineered to encode genes for antibiotic resistance.\(^7\) In order to effectively address the threat of anthrax, a broad approach including improved diagnostics, vaccines, antibiotics, and small molecules capable of toxin incapacitation is needed.

**Iron Enzymes: 5-LO**

5-Lipoxygenase (5-LO) is an intracellular non-heme iron metalloenzyme that is involved in the inflammatory response. The first enzyme in the leukotriene biosynthetic pathway, 5-LO converts arachidonic acid (AA) to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and subsequently to the unstable
intermediate, leukotriene (LT) A_4 (Fig. 2.6).\textsuperscript{21,117} LTA_4 is further transformed into LTB_4 by LTA_4 hydrolase or into LTC_4 via LTC_4 synthase. LTB_4 serves as a chemoattractant that is responsible for phagocyte adherence to vessel walls.\textsuperscript{21} LTC_4 and its metabolites (LTD_4 and LTE_4, the three collectively referred to as cysteinyll LT or CysLT) function as bronchoconstrictors and also stimulate mucus secretion in the airways.\textsuperscript{21} 5-LO activity has broad physiological implications and necessarily, its regulation is complex.
Figure 2.6. Leukotriene biosynthetic pathway (Adapted from Werz et al.).\textsuperscript{21} Lipoxygenase catalyzes the first two steps of leukotriene synthesis.

Expression of 5-LO is primarily restricted to mature leukocytes; the ability to express 5-LO is acquired during cell maturation\textsuperscript{21} and transcription is blocked in other
cell types by DNA methylation of the 5-LO promoter. Upon synthesis, 5-LO is translocated and becomes associated with the nuclear membrane in a calcium-dependent process. 5-LO activity is further stimulated by ATP and by the phosphorylation of serine residues by a variety of kinases including p38 mitogen activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK). Full activation of 5-LO requires oxidation of the iron from the inactive ferrous (Fe$^{2+}$) form to the catalytic ferric (Fe$^{3+}$) form by lipid hydroperoxides.

5-LO metabolites are associated with several pathologies, including inflammatory diseases, cardiovascular disease, and cancer. 5-LO has long been considered a target for the treatment of asthma due to the roles of CysLT in airway obstruction and hypersensitivity. Treatment with the 5-LO inhibitor, zileuton (Fig. 2.7), significantly reduced symptoms in atopic dermatitis in a pilot clinical trial. Zileuton may also be effective treatment for asthma, allergic rhinitis, COPD, and other inflammatory diseases. LT are upregulated in ischemic tissue and atherosclerotic lesions suggesting a role for 5-LO inhibitors in the treatment of cardiovascular diseases (CVD). Osteoporosis may be an important application of 5-LO inhibitors considering 5-LO metabolites inhibit osteoblastic bone formation in vitro and 5-LO-deficient mice show increased bone thickness. Finally, 5-LO is upregulated in numerous cancer cell types despite its limited expression in healthy non-leukocyte cells, thereby indicating a role in cancer pathology.

Traditionally, inhibitors of 5-LO are one of three types: redox inhibitors, iron chelating inhibitors, and non-redox inhibitors. Both redox inhibitors and chelating inhibitors interact directly with the non-heme iron center of 5-LO, which is
coordinated by two histidine residues and by the carboxylic acid of the C-terminal isoleucine residue (Fig. 2.8).\textsuperscript{126} The iron is critical to the catalytic activity of 5-LO as it converts between ferrous and ferric states to act as an electron shuttle during the reaction.\textsuperscript{127} Redox inhibitors function by competing with the substrate to interfere with the redox chemistry of the reaction.\textsuperscript{22} These inhibitors divert the electron from the natural substrate and also may cycle the radical back to the enzyme to reduce the iron back to its inactive state.\textsuperscript{22} Numerous redox-active 5-LO inhibitors have been developed; however, many inhibitors of this type interfere with other redox reactions in vivo; in particular, several of these inhibitors oxidize the iron in hemoglobin to the ferric form causing significant toxicity (methemoglobinemia).\textsuperscript{22, 128} Other unwanted side effects of some redox inhibitors include genotoxicity and hypersensitivity reactions.\textsuperscript{22} Notably, Abbott labs have produced a potent, orally available redox inhibitor of 5-LO (5, See Fig. 2.7) with in vitro activity that does not cause methemoglobinemia in either rat (per oral, 400 mg/kg/d, 9 d) or dog (per oral, 200 mg/kg/d, 1 month).\textsuperscript{129}
Figure 2.7. Select inhibitors of 5-LO. Compound 9 inhibits 5-LO by interfering with the redox reaction of the enzyme. Zileuton is an example of an inhibitor that chelates the iron to inactivate the enzyme and compounds 10-12 are non-redox inhibitors of 5-LO.

Figure 2.8. A schematic representation of the active site of 5-LO (left) and a protein crystal (PDB code 2IUJ) structure of soybean lipoxygenase B (right). The catalytic ion (orange sphere) is coordinated by two histidine residues and the carboxylic acid of the terminal isoleucine residue. In addition, water, another histidine, and an asparagine residue are all suspected to act as labile ligands.

Alternatively, iron chelating inhibitors bind directly to the metal (in either the ferrous or ferric form) to block catalysis. Among the most common chelating motifs
in the 5-LO inhibitor literature are hydroxamic acids, reverse hydroxamic acids, and N-hydroxyureas.\textsuperscript{130} In fact, the only clinically used 5-LO inhibitor is the N-hydroxyurea inhibitor, Zileuton, which has been approved for the treatment of asthma (Fig. 2.7).\textsuperscript{131} Zileuton is highly specific for 5-LO but must be administered in high doses. Despite significant evidence that inhibition of LT synthesis would yield beneficial effects for a range of pathologies, studies with Zileuton have largely yielded disappointing results. This has spurred speculation that the relative low potency of Zileuton precludes physiologically relevant levels of 5-LO inhibition.\textsuperscript{132} Consequently, this has renewed interest in the development of non-toxic 5-LO inhibitors with improved potency.

In the early 1990's several 5-LO inhibitors were reported that lacked redox activity.\textsuperscript{133, 134} Compounds 10 and 11 (Fig. 2.7) are potent thiazole inhibitors of 5-LO (IC\textsubscript{50} = 400 nM and 60 nM, respectively).\textsuperscript{133, 134} Due to the lack of metal-binding groups and redox active functionalities, the inhibition mechanism of 10 and 11 was examined. Unlike redox inhibitors, 10 and 11 do not stimulate pseudoperoxidase activity in purified human 5-LO.\textsuperscript{135} Further, incubation of 5-LO with these two inhibitors resulted in a decrease in the rate of enzyme inactivation after approximately 3 min, thereby suggesting that these inhibitors may form irreversible enzyme-inhibitor complexes.\textsuperscript{135} Inhibitor 12, a more potent derivative of 7 (IC\textsubscript{50} = 22.0 nM), was reported in 1994.\textsuperscript{136} Compound 12 is able to inhibit LTB\textsubscript{4} production in whole blood with similar potency to zileuton (3.8 and 2 \textmu M, respectively).\textsuperscript{136}

Since 2006, 5-LO inhibitors based on indole,\textsuperscript{132, 137, 138} coumarin,\textsuperscript{139} and pirixinic acid\textsuperscript{140} scaffolds have been reported. Indole and coumarin scaffolds are
known to have antioxidant activities; however, it is not clear that redox interference is the sole mechanism of inhibition by these inhibitors.\textsuperscript{132} Interestingly, there are no obvious metal-binding groups in these inhibitors so the source of their inhibitory activity remains unknown.

Many inhibitors of 5-LO reported over the last decade also inhibit COX.\textsuperscript{141} These inhibitors may have advantages in applications as anti-inflammatory modulators, anti-cancer drugs, and as neuroprotective agents. Dual inhibitors are outside the scope of this introduction. Detailed reviews of 5-LO inhibitors\textsuperscript{22, 142} and 5-LO/COX dual inhibitors\textsuperscript{141} can be found elsewhere.

**Copper Enzymes: Ty**

Tyrosinase is a type III copper metalloenzyme responsible for three catalytic steps in melanogenesis, including the oxidization of L-tyrosine to L-dopamine to initiate the biosynthetic pathway (Fig. 2.9).\textsuperscript{26} Melanogenesis leads to the formation of pheomelanins and eumelanins, which are the primary pigments responsible for skin darkening. Tyrosinase has both monophenolase and diphenolase activity and L-dopamine is oxidized to o-dopaquinone via the diphenolase catalytic cycle. Tyrosinase transcription is regulated by the microphthalmia-associated transcription factor.\textsuperscript{143} Full maturation of tyrosinase requires post-translational N-glycosylation.\textsuperscript{143}
Tyrosinase activity leads to a variety of undesirable effects in both humans and plants. Tyrosinase acts to oxidize phenols following damage to plant-derived foods. This causes browning and results in lost nutritional and economic value. In lettuce, browning intensity correlates with tyrosinase activity. Further, tyrosinase is responsible for wound healing in multiple insect species, and inhibitors of this enzyme may be valuable as pesticides. Consequently, tyrosinase inhibitors are of great interest in the agricultural industry. With regards to human tyrosinase, inhibitors are sought for both cosmetic and medicinal purposes. The tyrosinase inhibitor arbutin reduces tyrosinase activity in a dose-dependant fashion to effectively impede melanin formation and whiten skin color. The presence of tyrosinase mRNA in peripheral blood correlates with a poor prognosis in patients with malignant melanoma. Additionally, tyrosinase modifies \( \alpha \)-synuclein to increase its susceptibility to aggregation and worsen toxicity with respect to Parkinson disease.
Tyrosinase exists in three forms: oxy-tyrosinase, met-tyrosinase, and deoxy-tyrosinase (Fig. 2.10). At rest, tyrosinase is primarily in the met form (85%) though some will remain in the oxy form (15%). In the oxy form the two Cu(II) centers are bound to bridging dioxygen. Monophenols can only bind tyrosinase in the oxy form. The met form has one bridging oxygen between the two Cu(II) centers, which are antiferromagnetically coupled. The met form is only involved with diphenolase oxidation. The deoxytyrosinase consists of two Cu(I) centers that are not bound to oxygen ligands. The deoxy form participates in both mono- and diphenolase activity.

**Figure 2.10.** The three oxidation states of the copper center in tyrosinase (left) and a protein crystal structure (PDB code: 2ZMX) of tyrosinase (right). The active site of tyrosinase consists of two copper ions (red spheres) bound by histidine residues.

Numerous inhibitors of tyrosinase have been reported from natural and synthetic sources. Due to the complexity of the tyrosinase active site many variables affect the mode of inhibition of small molecules. Competitive, non-competitive, uncompetitive, and mixed-type inhibitors have all been reported. Reversible
inhibitors and slow-binders have also been reported. Some inhibitors preferentially bind oxytyrosine while others bind mettyrosinase. Further, small molecules may inhibit the monophenolase activity of tyrosinase more efficiently than the diphenolase activity (or vice versa). Finally, the $K_i$ of certain acidic tyrosinase inhibitors increases by an order of magnitude with increasing pH.$^{152}$

Inhibitors that chelate the copper ion are common. Kaempferol (Fig. 2.11, 13), a flavonol isolated from *Crocus sativus*, is a competitive inhibitor of the diphenolase activity of mushroom tyrosinase ($IC_{50} = 230 \ \mu M$).$^{153}$ Inhibitor 13 chelates the mettyrosinase to block diphenolase activity.$^{153}$ By contrast, kojic acid (14) chelates the oxytyrosinase to block diphenolase activity.$^{26}$ Tropolone (15) also binds to oxytyrosinase to inhibit via a slow-binding mechanism.$^{154}$ Other slow-binding inhibitors include $m$-coumaric acid (16),$^{155}$ L-mimosine (17),$^{156}$ and 4-alkyl-resorcinols (18).$^{157}$ Pyridinones (19-21)$^{158}$ and thiosemicarbazides (22)$^{159,160}$ are other classes of chelator tyrosinase inhibitors.
Figure 2.11. Select inhibitors of tyrosinase. Inhibitors of tyrosinase are based on phenols (arbutin), pyrones (14), pyridinones (17, 19-21), and tropolone (15), among others.

FBLD

Fragment based lead design (FBLD) is an emerging approach of de novo drug design. According to this strategy, low molecular weight compounds (fragments) are screened for low-affinity interactions with drug targets. The hits from these screens are then elaborated into lead compounds. Compared with high-throughput screening (HTS), FBLD uses smaller fragment libraries (10^2-10^3 fragments) and relies on more sensitive methods of screening to accommodate the lower potency of smaller compounds. The use of small compounds allows for a greater sampling of chemical diversity and for more efficient probing of a target active site.\textsuperscript{161, 162}

Fragment collections are typically assembled according to guiding principles set out by Lipinski and others.\textsuperscript{163-165} Lipinski et al. introduced the “rule of 5” in 1997 to inform the selection of compounds for HTS chemical libraries.\textsuperscript{163} Based on analysis of drugs in Phase II clinical trials, recommendations regarding molecular
weight, number of hydrogen-bond donors (HBD) and acceptors (HBA), and lipophilicity were made to describe “drug-like” features that correlate with good oral bioavailability. To these guidelines have been added two more considerations: number of rotatable bonds (NROT) and polar surface area (PSA). These rules have been adapted to FBLD and consequently, the “rule of 3” describes the analogous “drug-like” features desired for a fragment. According to the “rule of 3” fragments should have molecular weight <300 Da, HBA ≤ 3 and HBD ≤ 3, NROT ≤ 3, PSA ≤ 0.6. Additionally, fragments are sought that have good water solubility, lack reactive functional groups, and have some synthetic handle for elaboration.

In general, collections either seek to sample a wide range of diversity or are assembled based on some knowledge of the fragments. In 1996, Bemis et al. set out to study the core shapes of known drugs. An analysis of the 2D scaffolds present in the drugs from the Comprehensive Medicinal Chemistry (CMC) database version 94.1 indicated that 66% were found in only one drug molecule. This suggests that many new pharmaceuticals could be produced from scaffolds that are yet unknown, thus highlighting the importance of investing in collections of molecules with diverse chemical structures. On the other hand, the fact that more than 50% of known drugs (~2560 out of 5120) can be accounted for by 32 scaffolds prompted the authors to propose that it may be advantageous to bias a library to known structures. This general strategy, of enhancing a fragment library with compounds known from other contexts, has appeared in other forms. RECAP is a computational method for breaking down known drugs into fragments for virtual screening. Alternatively, Fesik and coworkers identified “privileged” fragments, known to bind to many
proteins, and used them to augment their own fragment library. Several libraries have been assembled using common motifs from potent inhibitors of kinases and MMPs. A separate strategy seeks to make libraries compatible with NMR screening. In this case fragments are selected that generate distinct NMR peaks.

Screening requires sensitive techniques that can detect weak interactions between fragment and target. Examples of such techniques that have been developed for FBID include NMR, affinity mass-spectrometry, X-ray crystallography, surface plasmon resonance (SPR), high concentration assays, and virtual screening. The information provided by each method varies and so complementary modes of screening are frequently used to verify results. High concentration assays identify fragments that elicit the desired biological response. These are usually fast, quantitative, and have low protein requirements. However, bioassays do not give structural information about the fragments binding mode and location. They also are plagued with problems such as limits to fragment solubility and false positives generated from fragment aggregation. On the other hand, useful structural information can be derived from structure-based techniques such as NMR, X-ray crystallography, and virtual screening. With the exception of virtual screening these structural methods are typically data intensive and require significant amounts of protein. In NMR screens fragment binding is observed in solution and some information regarding the binding mode and affinity is gained. X-ray crystallography yields more detailed information about binding interactions, however, it cannot give affinity information and any given fragment may have a binding mode that is different in solution.
Fragment hits are applied to three main forms of development: link, merge, and growth (Fig. 2.12). First, two fragments that bind at non-overlapping sites can be linked together to generate a more potent compound. Fig. 2.12a presents a schematic representation of the linking strategy applied to MMPi design. It has been reported that maltol binds to the MMP catalytic zinc and the biphenyl group binds in the (S1') substrate-binding pocket. When linked together with an amide bond, a potent inhibitor is formed. Typically the linking strategy requires structural information from the screen to ensure that the binding sites are complementary. However, an alternative strategy is to load the protein with the first fragment and screen for a second fragment while the first site is blocked. The linking of two fragments has also been accomplished using in situ combination. Two fragments that bind at the same site can be merged to form a hybrid compound that utilizes the productive contacts from both the starting fragments with the target protein. Fig. 2.12b presents a plausible, but theoretical example of this principle. In the upper-left, the oxygen of the biphenyl ether forms a hydrogen bond with a protein residue in the substrate-binding pocket. Below, the 4-fluoro-phenylbenzene binds in the same pocket and the fluorine of forms a hydrogen bond with a different part of the protein. The structure bound in the pocket on the right can then be considered a merged version of the two overlapping fragments. Alternatively, multiple fragments that bind at the same site can be compared for improved drug-like properties. Finally, growth of a fragment involves the structural variation of classic medicinal chemistry but starts from a less potent compound. In Figure 2.12c derivatives are made of the first “hit” to expand the fragment. These derivatives are then screened and the best among them is
further modified to grow an even larger inhibitor with improved potency. Evolution of fragments requires an “anchor”, or a starting fragment that does not change its binding mode throughout elaboration. Anchors have been derived from metal binding groups and disulfide tethers. Fragment development must accompany some minimum increase in potency in order to maintain ligand efficiency (LE). LE is a measure used to analyze the potency of an inhibitor as a function of its molecular weight (Equation 2.1).

Equation 2.1: \[ \text{LE} = -\frac{RT \ln[IC_{50}]}{\text{(# of non-hydrogen atoms)}} \]
Figure 2.12. Three forms of fragment development.  a) Two compounds that bind at separate sites may be linked to generate a more potent compound.  b) Two compounds that bind to the same site may be combined to optimize the fragment.  c) One compound may be grown to find more potent derivatives.

Unlike high throughput screening, hits from fragment screening are not true lead compounds.  Instead, fragment screening identifies the productive components of a potential inhibitor so that only constructive scaffolds are included in the final lead structure.  This gives FBLD a distinct advantage for the generation of leads with improved ligand efficiency.  The number of fragments screened is significantly reduced as compared to HTS because smaller fragments more efficiently sample chemical diversity.\textsuperscript{167} The simplicity of fragments and the low-affinity requirements of screening further result in much higher hit rates.\textsuperscript{162, 167, 189} Fragments are less
limited by steric clashes and hydrogen bonding mismatches, which allows them to bind in the most energetically favorable position and to better probe new pockets in the active site of the target.\textsuperscript{167, 189} It is important to note that many fragments are promiscuous binders and because of this, little emphasis should be placed on the selectivity of such small compounds.\textsuperscript{162}

There are several important disadvantages to FBLD versus more traditional lead-generating strategies. First, the hits identified by fragment screening typically have very low potency against the target.\textsuperscript{167} Generation of potent leads from fragments requires either significant rational design or extensive structural optimization. Second, because fragments typically have very low potency, they must be screened at high concentrations, a requirement that can be limited by fragment solubility. Finally, screening techniques that provide structural information about binding, such as NMR and X-ray crystallography, are data-intensive and often require significant amounts of protein.\textsuperscript{167, 189}

**FBLD for Metalloenzymes**

FBLD is particularly well suited for the design of metalloenzyme inhibitors. Chelators are fragments with binding affinities sufficient for the common screening techniques of FBLD ($K_d$ in the micro- to milli-molar range). The mode of binding of chelators is typically known or easily modeled, which makes it unnecessary to obtain structural binding information from the screen. This makes the use of high concentration assays feasible and avoids high protein requirements and data-intensive screening techniques. Further, it is relatively straightforward to bias a library toward a
The use of metal-binding groups as fragments has been reported for the design of inhibitors for MMP-12 and LF.\textsuperscript{111}

Despite the representation of metalloenzymes in FBLD research, most reports use metal-binding groups as anchors for fragment elaboration. Little attention has been paid towards optimizing the metal-binding fragment in its own right. This is significant considering that the metal-binding group can have substantial effects on the properties of inhibitors. Over the past ten years, many new ZBGs have been introduced for MMPi.\textsuperscript{14} Studies have demonstrated the role of the ZBG in inhibitor potency\textsuperscript{183, 193, 194} and MMP selectivity.\textsuperscript{94, 195} Furthermore, novel ZBG scaffolds have been elaborated into potent MMPi.\textsuperscript{92, 94, 96, 183, 196, 197} However, while the use of alternative ZBGs has garnered considerable attention in the MMP community,\textsuperscript{14, 198} the diversity of metal-binding groups in inhibitors of other metalloenzymes is underdeveloped.\textsuperscript{31, 199}

The present study demonstrates the development and evaluation of a chelator fragment library. This collection of compounds introduces new diversity for metal-binding anchors of pharmaceuticals. The incorporation of novel metal-binding groups can be applied to the development of inhibitors for a wide range of metalloenzymes including MMPs, LF, 5-LO, and tyrosinase. A sub-library of derivatives of one chelator, 8-hydroxyquinoline, is presented to illustrate the versatility of new chelators.
2.2 Results and Discussion

Based loosely on conventional principles for fragment libraries, but more specifically tailored to the aims of this project, a chelator fragment library (CFL-1) was assembled. Fragments were chosen that are inexpensive and commercially available or are readily synthetically accessible. Additional considerations include the rigidity of the metal-binding scaffold (cyclic and aromatic compounds were favored), the number of donor atoms (2-4), the variety of donor atoms (oxygen, sulfur, nitrogen), the potential for water solubility, and the similarity to compounds known to be biocompatible. From these basic considerations, 6 metal-binding classes were chosen for representation in the library including picolinic acids (A1-A12), hydroxyquinolines (B1-B12), pyrimidines (C1-C12), hydroxypyrones (D1-D12), hydroxypyridinones (E1-E12), and salicylic acids (F1-F12). Twenty-four miscellaneous compounds were also included (G1-G12 and H1-H12). Several known MMP hits were included as controls and acetohydroxamic acid (G1) was included to represent the common MMPi zinc-binding motif for comparison. Preliminary experiments with CFL-1 indicated that four compounds could not be screened in either fluorimetric or colorimetric MMP assays due to issues with solubility and excessive emission. These four compounds were replaced (by B6, B10, F7, and H2) in order to generate CFL-1.1. The fragments of CFL-1.1 have a low molecular weight; all but 5 compounds are <200 amu (Fig. 2.13).
Figure 2.13. Structures of the components of CFL-1.1. Compounds are arranged by class across each row with A-F representing picolinic acids, hydroxyquinolines, pyrimidines, hydroxypyrones, hydroxypyridinones, and salicylic acids, respectively. Rows G and H contain miscellaneous compounds.
CFL-1.1 was screened against five MMPs (MMP-1, -2, -3, -8, and -9), LF, 5-LO, Ty, and iNOS at a concentration of 1 mM for each fragment. A thermoplot representing the percent inhibition of each of the fragments at this concentration against the screened enzymes is presented in Fig. 2.14. CFL-1.1 has high hit rates against the MMPs, LF, 5-LO, and Tyrosinase (IC₅₀ < 1 mM for ~40% of fragments screened per enzyme) as can be seen in the large regions of the map that are yellow and orange. The LE for a fragment with ten heavy atoms (the average number for this library) and an IC₅₀ of 1 mM is 0.41 kcal/mol. Considering many of these fragments likely have IC₅₀ values much lower than 1 mM this LE is a conservative estimate. Significantly, fragments from the same chelator class evoke similar inhibition for a given enzyme, which is indicative of a conserved binding mode. This information can be used to identify functional groups not involved in binding that can serve as sites for derivitization. Additionally, as expected, many of these chelators are promiscuous binders that inhibit across enzyme classes.
Figure 2.14. Thermoplot representing the results from the screens of CFL-1.1 against various metalloenzymes. Cells are color-coded by a gradient scale from black (representing 0-20% inhibition) to yellow (representing 81-100% inhibition). Grey cells indicate compounds that were not tested and compounds that interfered with the assay resulting in increased enzyme activity compared to the control.

**MMPs**

CFL-1.1 was screened against five MMPs including MMP-1, MMP-2, MMP-3, MMP-8, and MMP-9 at a concentration of 1 mM (Fig. 2.14). Structural differences between these MMPs largely occur outside the primary and secondary coordination spheres of the zinc, specifically in the S1' pocket (vida supra). Because these fragments bind directly to the zinc(II) ion, the variability in peripheral structures
should not differentially affect fragment binding between MMPs. Consistent with this notion, fragments from CFL-1.1 that inhibit one MMP generally inhibit all other family members tested (for example E9-E12, G3-G7). Therefore, this screen can be used to generate new “warheads” to bind zinc for MMPi; however, selectivity between MMP family members will primarily depend on fragment elaboration.

In fact, the major difference among this set of MMPs is the lower pH in the active site of MMP-3 (pH = 6.0). The screen of CFL-1.1 against MMP-3 produced the most distinct results, as ten compounds (A1, A2, A4, A8, A10, A12, B3, B4, B11, and D11) are considerably more potent against MMP-3 than the other MMPs. Compounds A1 and A8 have previously been reported to inhibit MMP-3 with IC50 values of 181 μM and 1.35 mM, respectively. None of the picolinic acid derivatives that inhibit MMP-3 greater than 50% at 1 mM (A1, A2, A4, A10, A12) are substituted at the 6-position. Three quinolines with carboxylic acid groups (B3, B4, and B11) also preferentially inhibit MMP-3.

At 1 mM concentration, the hit rate of CFL-1.1 ranges from 29-43% against the MMPs screened (Fig. 2.15-2.19). The results of the MMP screen are validated by comparison of the percent inhibition by internal control compounds with the IC50 values reported in earlier studies. For the series of hydroxypyrones and hydroxypyridinones, the previously identified hits against MMP-1, -2, and -3 are confirmed (D2, D5, E2, E7, and E9). Consistent with prior reports, compounds D1, D4, E1, E6, and E8 fail to achieve 50% inhibition at 1 mM against these enzymes. The observed trend, that the O,S chelators are more potent than O,O chelators, is conserved with the expanded ligand set (compare O,S chelators E11 and
E12 to O,O chelators E5 and E4, respectively). Compounds G3 and G4 also hit MMP-1, -2, -8 and -9 in addition to MMP-3, which was previously reported.194 Acetohydroxamic acid (G1) was confirmed to have an $IC_{50}$ value greater than 1 mM against all five MMPs.

Several new scaffolds have been identified as good zinc ligands for MMPi. Compound A5, 3-hydroxypicolinic acid, inhibits MMP-1, -2, -3, and -8 at approximately 50% when screened at 1 mM. Nearly all of the 8-hydroxyquinoline derivatives (B2, B7, B8, and B9) strongly inhibit all five MMPs at 1 mM. The 8-hydroxyquinoline-N-oxide (B5) is a potent fragment against the five MMPs. Compound B10, a sulfonamide quinoline chelator, and two pyrimidines (C4 and C5) also inhibit all five MMPs at 1 mM. Tropolone (G11) and its natural product derivative (G12) inhibit all five MMPs greater than 80% at 1 mM.

In contrast, several known metal-binding classes did not significantly inhibit MMPs at 1 mM. Aside from the exceptions noted above, the picolinic acids (A1-A12) are generally poor MMP ligands. Similarly, the majority of pyrimidines screened (C2, C3, and C6-C12) do not appreciably inhibit MMPs at the concentration of the screen. With the exception of F5, which hit MMP-2 and -3, none of the salicylic acid derivatives (F1-F12) inhibit MMPs at 1 mM. Both catechol derivatives (G8-G10, H2, and H4) and acetylacetone derivatives (H8-H12) fail to inhibit MMPs at 1 mM.
**Figure 2.15:** MMP-1 assay results with error bars. CFL-1.1 was screened at 1 mM against MMP-1. The percent inhibition by picolinic acids (A1-A12), quinolines (B1-B12), pyrimidines (C1-C12) and hydroxypyrones (D1-D12) is shown in the top graph. The percent inhibition by hydroxypyridinones (E1-E12), salicylic acids (F1-F12), and miscellaneous compounds (G1-G12 and H1-H12) is shown in the bottom graph.
Figure 2.16: MMP-2 assay results with error bars. CFL-1.1 was screened at 1 mM against MMP-2. The percent inhibition by picolinic acids (A1-A12), quinolines (B1-B12), pyrimidines (C1-C12) and hydroxypyrones (D1-D12) is shown in the top graph. The percent inhibition by hydroxyypyridinones (E1-E12), salicylic acids (F1-F12), and miscellaneous compounds (G1-G12 and H1-H12) is shown in the bottom graph.
Figure 2.17: MMP-3 assay results with error bars. CFL-1.1 was screened at 1 mM against MMP-3. The percent inhibition by picolinic acids (A1-A12), quinolines (B1-B12), pyrimidines (C1-C12) and hydroxypyrones (D1-D12) is shown in the top graph. The percent inhibition by hydroxypyridinones (E1-E12), salicylic acids (F1-F12), and miscellaneous compounds (G1-G12 and H1-H12) is shown in the bottom graph.
Figure 2.18: MMP-8 assay results with error bars. CFL-1.1 was screened at 1 mM against MMP-8. The percent inhibition by picolinic acids (A1-A12), quinolines (B1-B12), pyrimidines (C1-C12) and hydroxypyrones (D1-D12) is shown in the top graph. The percent inhibition by hydroxypyridinones (E1-E12), salicylic acids (F1-F12), and miscellaneous compounds (G1-G12 and H1-H12) is shown in the bottom graph.
Figure 2.19: MMP-9 assay results with error bars. CFL-1.1 was screened at 1 mM against MMP-9. The percent inhibition by picolinic acids (A1-A12), quinolines (B1-B12), pyrimidines (C1-C12) and hydroxypyrones (D1-D12) is shown in the top graph. The percent inhibition by hydroxypyridinones (E1-E12), salicylic acids (F1-F12), and miscellaneous compounds (G1-G12 and H1-H12) is shown in the bottom graph.
CFL-1.1 was screened against LF, another zinc enzyme, at a concentration of 1 mM (Fig. 2.14). The hit rate of CFL-1.1 against LF was 24% (22 out of 93 compounds screened inhibit LF ≥ 50% at 1 mM). Fig. 2.20 shows a graph with error bars of percent inhibition of LF by CFL-1.1. The picolinic acids (A1-A12) are more effective inhibitors of LF when compared to their efficacy against the MMPs. In fact, ten of the twelve fragments screened inhibit LF > 40% at 1 mM. The picolinic acid derivatives (A6-A8) that inhibit LF > 70% at 1 mM are all substituted at the 6-position, which is in contrast to the picolinic acid hits against MMP-3. The 8-hydroxyquinolines (B7-B9, and B12) inhibit LF though not as effectively as they do the MMPs. Both compound B3 and B7 inhibit LF ~50% at 1 mM. Fragment B2 is essentially the “merged” product of B3 with a carboxylic acid at the 2-position and B7 with a hydroxyl group at the 8-position. Interestingly, B2 inhibits LF 89% at 1 mM, far more than any of the other quinolines. This suggests that these two substitutions improve the potency of quinoline synergistically. None of the pyrimidines, including C4, inhibit LF at 1 mM. Similar to MMPs, the hydroxypyrothiones (D2 and D5) completely inhibit LF at 1 mM. In contrast, the inhibition of LF by hydroxypyrones with O,O donor atoms (D1, D3, D4, and D6-D10) is reduced relative to MMPs. The hydroxypyridinones (E1, E3-E6, E8, and E10) are also significantly less potent against LF than MMPs. The most striking difference is the impotency of the hydroxypyridinethiones against LF. While compounds E7, E9, E11, and E12 inhibit the MMPs, 5-LO, and tyrosinase 78% or greater at 1 mM, these compounds only inhibit LF ~50% at this concentration. These results are consistent with the higher
IC$_{50}$ values reported for hydroxypyrones and hydroxypyridinones against LF.\textsuperscript{109} Of the salicylic acids, F$_5$ moderately inhibits LF (56% at 1 mM). This compound also stood out from the rest of the series (F$_1$-F$_{12}$) as the best inhibitor of MMPs. Generally, the miscellaneous compounds that hit MMPs (G$_3$-G$_7$, G$_{11}$, G$_{12}$, H$_1$, and H$_3$) also hit LF. Fragment G$_6$ did not hit LF at all and G$_3$ and G$_4$ are not as potent compared to MMPs. The tropolone fragments (G$_{11}$ and G$_{12}$) inhibited LF ~60% at 1 mM.
Figure 2.20: LF assay results with error bars. CFL-1.1 was screened at 1 mM against LF. The percent inhibition by picolinic acids (A1-A12), quinolines (B1-B12), pyrimidines (C1-C12) and hydroxypyrones (D1-D12) is shown in the top graph. The percent inhibition by hydroxypyridinones (E1-E12), salicylic acids (F1-F12), and miscellaneous compounds (G1-G12 and H1-H12) is shown in the bottom graph.
5-LO

The hits resulting from the screen of CFL-1.1 against 5-LO are different than those identified by the MMP and LF screens (Fig. 2.14). CFL-1.1 has a hit rate of 49% (44 out of 90 compounds screened have IC50 < 1 mM) against 5-LO (Fig. 2.21). All picolinic acids screened (A1-A12) moderately inhibit 5-LO (16-38% inhibition at 1 mM). Similar to the MMPs, the 8-hydroxyquinolines (B2, B7, B8, and B9) and 8-hydroxyquinoline-N-oxide (B5) all hit 5-LO while the other quinoline derivatives do not. Six pyrimidine derivatives (C2, C3, C5, C7, C8, and C10) have IC50 values less than 1 mM. Four of these hits (C3, C5, C7, and C8) have a thiol at the 2-position. Compounds C2 and C10 both have polar substituents at the 4- and 6-positions. The hydroxypyrones (D1-D12) and hydroxypyridinones (E1-E12) all inhibit 5-LO activity >70% at 1 mM. In contrast with the MMP results, the O,O donors and the O,S donors of these two classes inhibit 5-LO relatively evenly (compare D1 and D2, E8, and E9). The difference that is observed in the MMP screens between these two types of compounds may show up in a 5-LO screen performed at a lower concentration. The pyridinone scaffold based on E1 has appeared in several publications of 5-LO inhibitors and 5-LO/COX dual inhibitors. 5-LO is moderately inhibited by all the salicylic acids (F1-F12), but slightly more (31-86%) by those with pendant functional groups capable of hydrogen-bonding (F6, F9-F12). Compound F9 inhibits 5-LO by 86% at 1 mM and is the most potent fragment screened from the salicylate class. Additionally, 5-LO is significantly inhibited by catechol (G8, and H1-H4) and acetylacetone (H8-H12) derivatives, two ligand classes that are traditionally good iron chelators. Nine compounds (B3, D4, D6, D10, E10, F8, G1, G10, and G12)
demonstrate a significant increase in fluorescence in the assay relative to the control with no inhibitor. These ligands potentially activate 5-LO, but more likely interfere with the assay via an unknown mechanism.
Figure 2.21: 5-LO assay results with error bars. CFL-1.1 was screened at 1 mM against 5-LO. The percent inhibition by picolinic acids (A1-A12), quinolines (B1-B12), pyrimidines (C1-C12) and hydroxypyrones (D1-D12) is shown in the top graph. The percent inhibition by hydroxypyridinones (E1-E12), salicylic acids (F1-F12), and miscellaneous compounds (G1-G12 and H1-H12) is shown in the bottom graph. B6, B12, E1, F2, G10, H5, and H6 were not screened due to precipitation.
Tyrosinase

The screen of CFL-1.1 against Ty produced new fragment hits that do not inhibit MMPs and 5-LO (Fig. 2.14). The most commonly referenced inhibitors of Ty include tropolone \((G11, IC_{50} = 0.4 \, \mu M)\), kojic acid \((D7, IC_{50} = 23 \, \mu M)\), and L-mimosine an amino acid derivative of 3,4-hydroxypyridinones \((E3-E5)\).\(^{157, 159}\) Hider et al. have explored the inhibition of maltol \((D4)\) and other hydroxypyridinones \((E3, E4, E6, E8)\) against Ty.\(^{158}\) Compound \(E1\) has also been reported to bind Ty.\(^{204}\) The screening of CFL-1.1 confirmed all of these hits and further expanded the set of hydroxypyrones and hydroxypyridinones that have been evaluated against Ty (Fig. 2.22). Thiopyrones \((D2\) and \(D5)\) and thiopyridinones \((E2, E7, E9, E11\) and \(E12)\) inhibit Ty greater than 80% at 1 mM. As seen with the MMP screens the O,S donors are significantly more potent than their O,O analogs (compare \(D1\) and \(D2, D4\) and \(D5, E8\) and \(E9, E4\) and \(E12)\). Unlike MMPs, many of the hydroxypyrones \((D3, D7, D8, D9, D11,\) and \(D12)\) and hydroxypyridinones \((E1\) and \(E10)\) are also potent against tyrosinase. Generally, 3,4-hydroxypyrones \((D4, D6,\) and \(D10)\) and 3,4-hydroxypyridinones \((E3-E5)\) that are substituted in the 2-position are poor inhibitors of Ty. \(D1\) and \(E1\) are sterically very similar but have significantly different activities. Also, \(D9\) and \(D10\) have remarkably different activities given their structural similarity.

With few exceptions \((A2, B11,\) and \(F6)\), compounds from CFL-1.1 that contain aryl carboxylic acids are potent inhibitors of Ty. All of the picolinic acids \((A1-A12)\) except \(A2\) inhibit tyrosinase by 74% or more at 1 mM. The three quinolines \((B2-B4)\) that hit tyrosinase at 1 mM all contain carboxylic acids. Most of the salicylic acids \((F1, F3-F5,\) and \(F7-F12)\) inhibit tyrosinase by more than 90% at 1 mM. Other
compounds with carboxylic acids (D11, E10, H2, and H4) also hit Ty at 1 mM. Neither of the pyrimidines with aromatic amines (C2 and C9) inhibit Ty at 1 mM. Compound F6 contains an amine group and inhibits tyrosinase at a reduced level (55% inhibition) compared to the other salicylic acids. Other Ty hits include three pyrimidines (C1, C5, and C8), p-toluenesulfonamide (H7), guaiacol (G10), and the acetylacetones (H8-H12).

Given the complexity of Ty and its catalytic cycles it is important to keep in mind that the mechanism of Ty inhibition was not evaluated for these fragments. Although the compounds of CFL-1.1 were chosen because they can chelate metals, some may act as alternative substrates for Ty. Additionally, these compounds may act through competitive, non-competitive, or slow-binding inhibition mechanisms. Similar to the 5-LO assay several compounds (B6-B10, G6-G9, and H3) interfered with the Ty assay resulting in a significant increase in absorbance relative to the negative control.
Figure 2.22: Tyrosinase assay results with error bars. CFL-1.1 was screened against tyrosinase at 1 mM. The percent inhibition by picolinic acids (A1-A12), quinolines (B1-B12), pyrimidines (C1-C12) and hydroxypyrnes (D1-D12) is shown in top graph. The percent inhibition by hydroxypyridinones (E1-E12), salicylic acids (F1-F12), and miscellaneous compounds (G1-G12 and H1-H12) is shown in bottom graph. G10 and H1 were not screened due to precipitation.
iNOS

CFL-1.1 was screened against iNOS at 1 mM concentration for each fragment (Fig. 2.14). iNOS is a heme iron enzyme with only one axial site available for exogenous ligand binding. Because this library was designed with multidentate ligands that have a primary binding mode not optimized for monodentate coordination in conjunction with a heme center, this enzyme generates predictably different outcomes with CFL-1.1. Significantly, there is a much lower hit rate for CFL-1.1 against iNOS (4% of fragments screened or 3 out of 73 have greater than 50% inhibition when screened at 1 mM). As can be seen in Fig. 2.14, the majority of fragments do not appreciably inhibit iNOS. Six fragments (A5, B1, C6, D7, D12, and H12) inhibit iNOS greater than 40% at 1 mM. These compounds span nearly all the metal-binding classes of CFL-1.1 and seem to be specific considering compounds similar to these are ineffective against iNOS (compare C6 with C12, D8 with D7 or D9, D12 with D1, and H12 with H9).

There is much greater variability in the results from screens against iNOS as compared to the other enzymes screened (Fig. 2.23). This error can be accounted for by two major sources. First, the commercial assay is a complex variation of the Griess assay with multiple added steps. Ten components need to be added to each well, which offers many opportunities for human error to bias the assay. Time delay between the additions of various components in the wells is not easily tracked, though such delays may significantly impact the outcome of the assay. Second, this assay is a discontinuous end-point assay, meaning the enzyme activity cannot be monitored in real time. In such assays it is necessary to ensure that the enzyme activity is linear.
over the entire duration of the incubation. Usually, this is achieved by using substrate and enzyme concentrations such that < 20% of the substrate is consumed during the assay. Quality control for a discontinuous assay is much more difficult and in this case the problem is compounded by the high number of assay components required.
Figure 2.23: iNOS assay results with error bars. CFL-1.1 was screened against iNOS at 1 mM. The percent inhibition by picolinic acids (A1-A12), quinolines (B1-B12), pyrimidines (C1-C12) and hydroxypyrones (D1-D12) is shown in top graph. The percent inhibition by hydroxypyridinones (E1-E12), salicylic acids (F1-F12), and miscellaneous compounds (G1-G12 and H1-H12) is shown in bottom graph. B12, C3-C6, C7, C8, D2, D5, E2, E7, E9, E11, E12, F7, G5, G10, H1, H2, H5, and H6 were not screened due to known assay incompatibility or precipitation.
Sub-library

In order to develop fragments into full-fledged inhibitors, handles for synthetic elaboration are needed.\textsuperscript{162, 167, 189} Hydroxamic acids and carboxylic acids each only have one site for derivititation. This limits inhibitor backbones to sites compatible with the metal-binding mode, reduces the binding efficiency of the metal-binding group, or requires the introduction of a highly flexible linker, all of which are undesirable. In order to demonstrate the value of a metal-binding fragment with multiple sites of derivititation, a sub-library based on 8-hydroxyquinoline is was synthesized (Fig. 2.24). 8-Hydroxyquinoline was chosen as an example because it inhibits both MMPs and 5-LO well and the synthetic chemistry of this chelator is reasonably well established. Four different backbone groups were appended to the chelator at four different positions using a sulfonamide linker to generate sixteen novel fragments. The four backbones are all aromatic groups, chosen to bias the sub-library with scaffolds known to be present in potent MMPi. These backbones can also serve as a probe to identify the most productive directions for elaboration. These derivatives generally have twice as many heavy atoms as 8-hydroxyquinoline. In order to maintain an attractive LE (0.3 kcal/mol), fragments of this size should inhibit MMPs >50% at 25 μM.\textsuperscript{188}
Figure 2.24. 8-Hydroxyquinoline sublibrary. Backbones were appended to four different positions (2-, 4-, 5-, and 7-positions) around the quinoline ring. Benzene, fluorobenzene, biphenyl, and thiophene backbones were examined.

The 8-hydroxyquinoline sublibrary was screened against MMP-2 and 5-LO at a concentration of 25 μM. Interestingly, most fragments derived from the 5- and 7-positions of 8-hydroxyquinoline completely inhibit MMP-2 at 25 μM (Fig. 2.25). In contrast, the fragments derived from the 2- and 4- positions of 8-hydroxyquinoline do not significantly inhibit MMP-2 at 25 μM. This demonstrates that the positioning of the backbone around the chelator is having a bigger effect on MMP-2 potency than the variation between backbone structures. Specifically, the biphenyl backbone is made ineffective when positioned off the 2- and 4- positions of 8-hydroxyquinoline. This is significant considering the biphenyl has been used extensively to target the S1’ site of MMPs, but not surprising considering the potency of the B7 fragment against MMP-2.
This data indicates that backbones installed on the 5- and 7-positions are better oriented in the active site upon chelator binding.

Similarly, 5-LO is best inhibited by 5- and 7-substituted 8-hydroxyquinoline fragments (Fig. 2.26). However, these fragments are not as potent against 5-LO at 25 µM as they are against MMP-2. This is expected given the decreased potency of the starting fragment (B7) against this target relative to MMP-2. Generally, the backbone structure does not have as big of an effect on fragment potency as the backbone positioning around the chelator. The one exception may be the fragments with the biphenyl backbone, which all inhibit 5-LO similarly at 25 µM. Compounds 7Bn and 7FB are structurally identical with the exception of one fluorine atom on the phenyl ring of 7FB. The dramatic difference in potency against 5-LO of these two fragments is not seen with MMP-2. One possible explanation for this difference is that there
may be a specific hydrogen-bonding interaction between the fluorine atom of 7FB and the active site of 5-LO.

![Figure 2.26. Hydroxyquinoline sublibrary screen against 5-LO at 25 µM.](image)

### 2.3 Conclusion

The experiments described above illustrate the utility of a chelator fragment library for the identification of metalloprotein inhibitor leads. Numerous new chelators were identified as potent hits of MMPs thereby expanding the arsenal of metal-binding groups for use in MMPi. CFL-1.1 also produced distinct and potent hits for LF, 5-LO, and Ty. Finally, 8-hydroxyquinoline, a potent fragment identified by these screens against MMPs and 5-LO, was elaborated into a sublibrary containing derivatives that extend from the 2-, 4-, 5-, and 7-positions of the 8-hydroxyquinoline scaffold. Derivatives of the 5- and 7- positions preferentially inhibit MMPs and 5-LO, illustrating that the positioning of the backbone around the chelator plays a significant role in inhibitor potency.
Acknowledgements

This chapter, in part, is currently being prepared for submission for publication of the material. Jacobsen, J. A.; Fullagar, J. L.; Miller, M. T.; Cohen, S. M. “Chelator fragment libraries: Diversification of the metal-binding scaffold” The thesis author was the primary investigator and author of this paper. Melissa T. Miller assisted in screening CFL-1.1 and Jessica L. Fullagar assisted in synthesis of the 8-hydroxyquinoline sub-library.

2.4 Experimental

Unless otherwise noted, starting materials were purchased from commercial suppliers (Sigma-Aldrich, Acros Organics) and were used without further purification. Flash silica gel chromatography was performed using Merck silica gel 40-63 µm mesh. ¹H NMR spectra were recorded one of several Varian FT-NMR spectrometers, property of the Department of Chemistry and Biochemistry, University of California San Diego. Mass spectrometry was performed at the Small Molecule Mass Spectrometry Facility directed by Dr. Xu in the Department of Chemistry and Biochemistry, University of California San Diego. Microwave reactions were performed in a 10 mL vial using a CEM discover S-class microwave reactor. Dilute HCl refers to 0.38 M HCl.

Chelator Fragment Library 1.1 (CFL-1.1) – Synthesis and characterization:

CFL-1.1 was assembled from 76 commercially available compounds and 20 synthesized fragments. 13 fragments were prepared according to literature precedent:
Compounds E3, E4, E5, E7, E11, and E12 were prepared according to the general procedure outlined below starting from maltol (D4), thiomaltol (D5), ethylmaltol (D6), and thioethylmaltol (synthesis described below) using methylvamine and ethylamine. The synthesis of B10 is also described below. All compounds were stored in DMSO at 50 mM concentration.

**Thioethylmaltol** (precursor for the preparation of D11).

Ethylmaltol (2.5 g, 17.8 mmol) was dissolved in 100 mL toluene at 100 °C. P₄S₁₀ (1.43 g, 3.2 mmol) and HMDO (4.8 g, 30.4 mmol) were added. The solution was heated to reflux at 100 °C overnight under N₂. The solution was cooled to room temperature and filtered and the filtrate was evaporated to a brown oil. Flash silica chromatography was performed in 1:1 CH₂Cl₂ to hexane to isolate the product (2.28 g, 14.6 mmol). Yield = 82%. ¹H NMR (400 MHz, CDCl₃): δ 1.28 (t, J = 7.2 Hz, 3H), 2.83 (q, J = 7.2 Hz, 2H), 7.32 (d, 5.2 Hz, 1H; ArH), 7.61 (d, J = 4.8 Hz, 1H; ArH), 7.79 (s, 1H; ArOH). APCI-MS(-): m/z 155.00 [M-H]⁻.

**General scheme for the synthesis of E3, E4, E5, E7, E11, and E12**

Starting pyrone (500 mg, 3-4 mmol) was suspended in 5 mL dilute HCl (0.38 M) followed by the addition 2.2 equivalents of the appropriate amine. The solution was irradiated in a microwave synthesizer at 165 °C for 1-10 minutes. The solvent was
evaporated and the crude was purified by flash silica chromatography in 0-6% methanol in CH$_2$Cl$_2$.

**E3 (2-ethyl-3-hydroxy-1-methylpyridin-4(1H)-one)**

**E3** was synthesized according to the above procedure starting from ethylmaltol (D6, 500 mg, 3.57 mmol) and methylamine (244 mg, 7.85 mmol). Yield: 20%. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 7.53 (d, $J = 7.6$ Hz, 1H; ArH), 6.08 (d, $J = 7.6$ Hz, 1H; ArH), 3.67 (s, 3H), 2.70 (q, $J = 7.2$ Hz, 2H), 1.11 (t, $J = 7.2$ Hz, 3H). ESI-MS(+): m/z 154.28 [M+H]$^+$. 

**E4 (2-ethyl-3-hydroxy-1-methylpyridin-4(1H)-one)**

**E4** was synthesized according to the above procedure starting from maltol (D4, 500 mg, 3.97 mmol) and methylamine (393 mg, 8.72 mmol). Yield = 7.1%. $^1$H NMR (400 MHz, CDCl$_3$-$d_1$): $\delta$ 7.23 (d, $J = 6.4$ Hz, 1H), 6.39 (d, $J = 7.6$ Hz, 1H), 4.75-4.25 (br, 1H), 3.95-3.89 (q, $J = 7.6$ Hz, 2H), 2.39 (s, 3H), 1.38 (t, $J = 7.6$ Hz, 3H). ESI-MS(+): m/z 154.2 [M+H]$^+$. 

**E5 (1,2-diethyl-3-hydroxypyridin-4(1H)-one)**

**E5** was synthesized according to the above procedure starting from ethylmaltol (D6, 500 mg, 3.57 mmol) and ethylamine (354 mg, 7.85 mmol). Yield: 16%. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.22 (d, $J = 7.6$ Hz, 1H), 6.40 (d, $J = 6.4$ Hz, 1H), 4.75 (s, br,
1H), 3.93 (q, J = 7.2 Hz, 2H), 2.79 (q, J = 7.2 Hz, 2H), 1.41 (t, J = 7.2 Hz, 3H), 1.23 (t, J = 7.2 Hz, 3H). ESI-MS(+): m/z 168.20 [M+H]⁺.

E7 (3-hydroxy-1,2-dimethylpyridine-4(1H)-thione)

E7 was synthesized according to the above procedure starting from thiomaltol (D₅, 500 mg, 3.53 mmol) and methylamine (241 mg, 7.8 mmol). Yield: 92%. ¹H NMR (400 MHz, CDCl₃-d₁): δ 8.74 (s, 1H), 7.45 (d, J = 6.4 Hz, 1H), 7.11 (d, J = 6.8 Hz, 1H), 3.78 (s, 3H), 2.49 (s, 3H). ESI-MS(+): m/z 156.21 [M+H]⁺.

E11 (1,2-diethyl-3-hydroxypyridine-4(1H)-thione)

E11 was synthesized according to the above procedure starting from thioethylmaltol (400 mg, 2.56 mmol) and ethylamine (254 mg, 5.63 mmol). Yield: 37%. ¹H NMR (400 MHz, CDCl₃): δ 8.75 (s, 1H; ArOH), 7.50 (d, J = 6.4 Hz, 1H; ArH), 7.11 (d, J = 6.8 Hz, 1H; ArH), 4.07 (q, J = 7.2 Hz, 2H), 2.81 (q, J = 7.2 Hz, 2H), 1.49 (t, J = 7.6 Hz, 3H), 1.28 (t, J = 7.2 Hz, 3H). ESI-MS(+): m/z 184.19 [M+H]⁺.

E12 (1-ethyl-3-hydroxy-2-methylpyridine-4(1H)-thione)

E12 was synthesized according to the above procedure starting from thiomaltol (D₅, 500 mg, 3.53 mmol) and ethylamine (350 mg, 7.8 mmol). Yield: 32%. ¹H NMR (400 MHz, CDCl₃-d₁): δ 8.74 (s, 1H), 7.45 (d, J = 7.6 Hz, 1H), 7.13 (d, J = 6.8 Hz, 1H), 4.06 (q, J = 7.2 Hz, 2H), 2.49 (s, 3H) 1.45 (t, J = 7.2 Hz, 3H). ESI-MS(+): m/z 170.16 [M+H]⁺.
B10 N-(quinolin-8-yl)methanesulfonyamide

8-aminoquinoline (200 mg, 1.39 mmol) and methanesulfonyl chloride (162 µL, 2.08 mmol) were dissolved in 3 mL pyridine. The solution was irradiated in a microwave synthesizer for 3 min at 130°C. The reaction mixture was poured over 10 mL H₂O and a dark precipitate (210 mg, 0.945 mmol) was isolated by vacuum filtration. Yield: 68%. ¹H NMR (500 MHz, DMSO-d₆): δ 9.34 (s, br, 1H), 8.90 (dd, J = 4.0, 1.7 Hz, 1H), 8.40 (dd, J = 8.6, 1.7 Hz, 1H), 7.70 (m, 2H), 7.62 (q, J = 4 Hz, 1H), 7.57 (t, J = 8 Hz, 1H), 3.12 (s, 3H). ESI-MS(+): m/z 223.22 [M+H]+.

Hydroxyquinoline sublibrary and precursors:

Scheme for 2-Amino-8-hydroxyquinoline derivatives

2Bn (N-(8-hydroxyquinolin-2-yl)benzenesulfonyamide)

2-Amino-8-hydroxyquinoline (100 mg, 0.624 mmol) and benzenesulfonyl chloride (236 µL, 1.87 mmol) were dissolved in 3 mL of pyridine. The reaction mixture was irradiated in a microwave synthesizer at 130 °C for 6 min. The reaction was quenched with 10 mL water and then extracted with 10 mL CH₂Cl₂. The organic layer was dried with MgSO₄ to yield a white powder. This intermediate was then dissolved in 2-3 mL of MeOH and allowed to heat to reflux. To this solution 3 mL of 2 M NaOH was added drop-wise and the mixture was allowed to reflux for 4-6 hrs under N₂.
atmosphere. The solution was allowed to cool to room temperature and the MeOH was evaporated. 1 M HCl was added drop-wise until precipitate formed. The precipitate was filtered, rinsed with water, and purified by flash silica chromatography in 0-3% methanol in CH$_2$Cl$_2$. A white product (40 mg, 0.133 mmol) was isolated. Yield: 21%. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 12.12 (s, br, 1H), 11.04 (s, br, 1H), 8.18 (d, $J$ = 9.2 Hz, 1H), 7.89 (s, br, 2H), 7.59-7.53 (m, 3H), 7.29-7.22 (m, 2H), 7.12 (d, $J$ = 6.4 Hz, 1H), 7.06 (d, $J$ = 10 Hz, 1H). ESI-MS (+): $m/z$ 301.26 [M+H]$^+$.

2FB (4-fluoro-N-(8-hydroxyquinolin-2-yl)benzenesulfonamide)

2FB was synthesized according to the procedure described for 2Bn starting from 2-amino-8-hydroxyquinoline (100 mg, 0.624 mmol) and 4-fluorobenzene-1-sulfonyl chloride (364 mg, 1.87 mmol). No column necessary. Yield: 37%. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 12.17 (s, br, 1H), 11.05 (s, br, 1H), 8.19 (d, $J$ = 9.2 Hz, 1H), 7.95 (s, br, 2H), 7.38 (t, $J$ = 8.8 Hz, 2H), 7.30-7.23 (m, 2H), 7.13 (d, $J$ = 7.2 Hz, 1H), 7.08 (d, $J$ = 9.6 Hz, 1H). ESI-MS (+): $m/z$ 319.24 [M+H]$^+$.

2BP (N-(8-hydroxyquinolin-2-yl)-[1,1'-biphenyl]-4-sulfonamide)

2BP was synthesized according to the procedure described for 2Bn starting from 2-amino-8-hydroxyquinoline (100 mg, 0.624 mmol) and [1,1'-biphenyl]-4-sulfonyl chloride (473 mg, 1.87 mmol). Yield: 11%. $^1$H NMR (400 MHz, DMSO-$d_6$): δ 12.19 (s, br, 1H), 11.07 (s, br, 1H), 8.19 (d, $J$ = 9.2 Hz, 1H), 7.95 (d, $J$ = 5.6 Hz, 2H), 7.83 (d, $J$ = 8 Hz, 2H), 7.69 (d, $J$ = 7.6 Hz, 2H), 7.48 (t, $J$ = 7.6 Hz, 2H), 7.41 (t, $J$ = 6.8 Hz,
1H), 7.30-7.22 (m, 2H), 7.13 (d, J = 7.6 Hz, 1H), 7.08 (d, J = 10 Hz, 1H). ESI-MS (+): m/z 377.28 [M+H]^+.

2TP (N-(8-hydroxyquinolin-2-yl)thiophene-2-sulfonamide)

2TP was synthesized according to the procedure described for 2Bn starting from 2-amino-8-hydroxyquinoline (100 mg, 0.624 mmol) and thiophene-2-sulfonyl chloride (342 mg, 1.87 mmol). Yield: 15.9%. 1H NMR (400 MHz, CD3OD): δ 8.17 (d, J = 9.2 Hz, 1H), 7.72 (m, 2H), 7.29 (m, 2H), 7.15 (dd, J = 5.6, 2.8 Hz, 1H), 7.10 (m, 2H). ESI-MS (+): m/z 307.30 [M+H]^+.

**Scheme for 4-Amino-8-hydroxyquinoline derivatives**

![Scheme for 4-Amino-8-hydroxyquinoline derivatives](image)

4-amino-8-hydroxyquinoline hydrogen bromide

4-amino-8-hydroxyquinoline hydrogen bromide was prepared according to literature precedent\(^{213}\) starting from 4-amino-8-methoxyquinoline (ChemBridge). Yield: 84%. 1H NMR (400 MHz, D2O): δ 7.77 (d, J = 6.4 Hz, 1H), 7.10 (d, J = 6.4 Hz, 2H), 6.93 (dd, J = 6.8, 2.4 Hz, 1H), 6.40 (d, J = 7.2 Hz, 1H). ESI-MS(+) m/z 161.33 [M+H]^+. 
4Bn (N-(8-hydroxyquinolin-4-yl)benzenesulfonamide)

4-amino-8-hydroxyquinoline hydrogen bromide (50 mg, 0.21 mmol) and benzenesulfonyl chloride (29 µL, 0.23 mmol) were dissolved in 3 mL pyridine. The reaction mixture was irradiated in a microwave synthesizer at 130 °C for 6 minutes. The reaction was quenched with 10 mL water and then extracted with 10 mL CH₂Cl₂. The organic layer was dried with MgSO₄ to yield a white product (16 mg, 0.05 mmol). Yield: 25%. ¹H NMR (400 MHz, CD₃OD): δ 8.05 (d, J = 6 Hz, 1H), 7.98 (d, J = 8.4 Hz, 1H), 7.82 (d, J = 8.4 Hz, 2H), 7.57 (q, J = 6.4 Hz, 2H), 7.42 (t, J = 8 Hz, 2H), 7.35 (t, J = 8 Hz, 1H), 6.53 (d, J = 5.6 Hz, 1H). ESI-MS(+) m/z 301.05 [M+H]⁺.

4FB (4-fluoro-N-(8-hydroxyquinolin-4-yl)benzenesulfonamide)

4FB was synthesized according to the procedure described for 4Bn starting from 4-amino-8-hydroxyquinoline hydrogen bromide (100 mg, 0.41 mmol) and 4-fluorobenzene-1-sulfonyl chloride (126 mg, 0.65 mmol) and requiring 20 minutes of microwave irradiation. A white product (52 mg, 0.16 mmol) was isolated. Yield: 40%. ¹H NMR (400 MHz, CD₃OD) δ 8.49 (s, 1H), 8.04, (t, J = 9.2 Hz, 1H), 7.87-7.84 (m, 2H), 7.59 (d, J = 6.9 Hz, 1H), 7.40 (t, J = 7.4 Hz, 1H), 7.16 (t, J = 6.9 Hz, 2H), 6.58 (d, J = 3.4 Hz, 1H). ESI-MS(+) m/z 319.02 [M+H]⁺.

4BP (N-(8-hydroxyquinolin-4-yl)-[1,1'-biphenyl]-4-sulfonamide)

4BP was synthesized according to the procedure described for 4Bn starting from 4-amino-8-hydroxyquinoline hydrogen bromide (100 mg, 0.41 mmol) and [1,1'-
biphenyl]-4-sulfonyl chloride (157 mg, 0.62 mmol) and requiring 10 minutes of microwave irradiation. A white product (30 mg, 0.08 mmol) was isolated. Yield: 20%. $^1$H NMR (400 MHz, CDCl$_3$): δ 8.30 (s, 1H), 7.00 (d, $J$ = 8.6 Hz, 2H), 7.69 (d, $J$ = 6.9 Hz, 1H), 7.61 (d, $J$ = 8.0 Hz, 2H), 7.55 (t, $J$ = 8.5 Hz, 3H), 7.47 (t, $J$ = 6.3 Hz, 2H), 7.40 (t, $J$ = 7.4 Hz, 1H) 7.32 (t, $J$ = 8.0 Hz, 1H), 6.49 (s, 1H). ESI-MS(+) $m/z$ 377.09 [M+H]$^+$. 

4TP (N-(8-hydroxyquinolin-4-yl)-thiophene-2-sulfonamide)

4TP was synthesized according to the procedure described for 4Bn starting from 4-amino-8-hydroxyquinoline hydrogen bromide (100 mg, 0.41 mmol) and thiophene-2-sulfonyl chloride (112 mg, 0.61 mmol). A white product (54 mg, 0.18 mmol) was isolated. Yield: 44%. $^1$H NMR (400 MHz, CD$_3$OD): δ 8.09 (d, $J$ = 5.2 Hz, 1H), 8.01 (d, $J$ = 8.6 Hz, 1H), 7.79 (d, $J$ = 5.2 Hz, 1H), 7.61 (d, $J$ = 3.4 Hz, 1H), 7.57 (d, $J$ = 8.0 Hz, 1H), 7.38 (t, $J$ = 8.0 Hz, 1H), 7.02 (t, $J$ = 8.6 Hz, 1H), 6.55 (d, $J$ = 5.8 Hz, 1H). ESI-MS(+) $m/z$ 307.03 [M+H]$^+$. 

Scheme for 5-Amino-8-hydroxyquinoline derivatives
**5Bn (N-(8-hydroxyquinolin-5-yl)benzenesulfonamide)**

5-Amino-8-hydroxyquinoline dihydrochloride (100 mg, 0.429 mmol) and benzenesulfonyl chloride (81 µL, 0.644 mmol) were dissolved in 3 mL of pyridine. The reaction was allowed to stir overnight under N₂. In the morning the reaction was quenched with 10 mL water and extracted with 10 mL CH₂Cl₂. The organic layer was dried over MgSO₄ and purified by flash silica chromatography in 0-3% methanol in CH₂Cl₂. The major product was then dissolved in 2-3 ml of MeOH and allowed to heat to reflux. To this solution 3 ml of 2 M NaOH was added drop-wise and the mixture was allowed to reflux for 4-6 hrs under N₂ atmosphere. The mixture was allowed to cool to room temperature and the MeOH was evaporated. 1 M HCl was added drop-wise until precipitate formed. The precipitate was filtered, rinsed with water, and purified by flash silica chromatography in 0-3% methanol in CH₂Cl₂. Yield: 17%. ¹H NMR (400 MHz, DMSO-<d>₆): δ 9.98 (s, 2H), 8.80 (d, J = 4 Hz, 1H), 8.26 (d, J = 8.4 Hz, 1H), 7.59 (d, J = 8 Hz, 3H), 7.51-7.45 (m, 3H), 6.94 (s, 2H). ESI-MS (+): m/z 301.19 [M+H]⁺.

**5FB (4-fluoro-N-(8-hydroxyquinolin-5-yl)benzenesulfonamide)**

5-Amino-8-hydroxyquinoline dihydrochloride (100 mg, 0.429 mmol) and 4-fluorobenzenesulfonyl chloride (125 mg, 0.644 mmol) were dissolved in 3 mL of pyridine. The reaction was allowed to stir overnight under N₂. In the morning the reaction was quenched with 10 mL water and extracted with 10 mL CH₂Cl₂. The organic layer was dried over MgSO₄ and purified by flash silica chromatography in 0-
3% methanol in CH\(_2\)Cl\(_2\). Yield: 21%. \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta\) 10.03 (s, br, 2H), 8.81 (dd, \(J = 4, 1.6\) Hz, 1H), 8.26 (dd, \(J = 8.8, 1.6\) Hz, 1H), 7.63 (dd, \(J = 8.8, 5.2\) Hz, 2H), 7.49 (dd, \(J = 8.4, 4\) Hz, 1H), 7.34 (t, \(J = 8.8\) Hz, 2H), 6.95 (s, 2H). ESI-MS (+): \(m/z\) 319.24 [M+H]\(^+\).

5BP (N-(8-hydroxyquinolin-5-yl)-[1,1'-biphenyl]-4-sulfonamide)

5BP was synthesized according to the procedure described for 5FB starting from 5-amino-8-hydroxyquinoline dihydrochloride (100 mg, 0.429 mmol) and [1,1'-biphenyl]-4-sulfonyl chloride (163 mg, 0.644 mmol). Yield: 37%. \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta\) 10.03 (s, br, 1H), 9.99 (s, br, 1H), 8.79 (dd, \(J = 4, 1.6\) Hz, 1H), 8.31 (dd, \(J = 8.4, 1.2\) Hz, 1H), 7.79 (d, \(J = 8.4\) Hz, 2H), 7.68 (dd, \(J = 13.2, 7.2\) Hz, 4H), 7.50-7.43 (m, 4H), 6.98 (q, \(J = 8\) Hz, 2H). ESI-MS (+): \(m/z\) 377.16 [M+H]\(^+\).

5TP (N-(8-hydroxyquinolin-5-yl)thiophene-2-sulfonamide)

5TP was synthesized according to the procedure described for 5FB starting from 5-amino-8-hydroxyquinoline dihydrochloride (100 mg, 0.429 mmol) and thiophene-2-sulfonyl chloride (118 mg, 0.644 mmol). Yield: 16%. \(^1\)H NMR (400 MHz, CD\(_3\)OD): \(\delta\) 8.77 (dd, \(J = 4, 1.6\) Hz, 1H), 8.33 (dd, \(J = 8.4, 1.6\) Hz, 1H), 7.71 (dd, \(J = 4.8, 1.6\) Hz, 1H), 7.43 (dd, \(J = 8.4, 4\) Hz, 1H), 7.33 (dd, \(J = 4, 1.6\) Hz, 1H), 7.10 (d, \(J = 8.4\) Hz, 1H), 7.03 (dd, \(J = 5.2, 3.6\) Hz, 1H), 6.98 (d, \(J = 8\) Hz, 1H). ESI-MS (+): \(m/z\) 307.21 [M+H]\(^+\).
Scheme for 7-Amino-8-hydroxyquinoline derivatives

5-chloro-8-hydroxy-7-nitroquinoline

5-chloro-8-hydroxy-7-nitroquinoline was prepared according to literature precedent. Yield: 66%. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 9.10 (d, $J$ = 4.4 Hz, 1H), 8.61 (d, $J$ = 9.2 Hz, 1H), 8.21 (s, 1H), 7.95 (dd, $J$ = 8.4, 4.4 Hz, 1H). ESI-MS(-) $m/z$ 223.35 [M-H].

7-amino-8-hydroxyquinoline

7-amino-8-hydroxyquinoline was prepared according to literature precedent. Yield: 95%. $^1$H NMR (300 MHz, DMSO-$d_6$): $\delta$ 10.16 (s, br, 1H), 8.77 (d, $J$ = 7.5 Hz, 1H), 8.71 (d, $J$ = 5.7 Hz, 1H), 8.34 (d, $J$ = 3Hz, 1H), 7.66 (d, $J$ = 8.7 Hz, 1H), 7.48 (t, $J$ = 5.4 Hz, 1H), 7.39 (d, $J$ = 8.7 Hz, 1H). ESI-MS(+) $m/z$ 161.32 [M+H]$^+$. 

7Bn (N-(8-hydroxyquinolin-7-yl)benzenesulfonamide)

7Bn was synthesized according to the procedure described for 4Bn starting from 7-amino-8-hydroxyquinoline (100 mg, 0.625 mmol) and benzenesulfonyl chloride (78 µL, 0.618 mmol). The product was purified flash silica chromatography in 0-3% MeOH in CH$_2$Cl$_2$ followed by recrystallization in EtOH. Yield: 5.6%. $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 9.81 (s, br, 2H), 8.76 (s, 1H), 8.24 (d, $J$ = 8.4 Hz, 1H), 7.74 (d, $J$ =
6.8 Hz, 2H), 7.56-7.51 (m, 1H), 7.47 (d, J = 7.6 Hz, 4H), 7.31 (dd, J = 8.8, 2.8 Hz, 1H). ESI-MS (+): m/z 301.19 [M+H]^+.

7FB (4-fluoro-N-(8-hydroxyquinolin-7-yl)benzenesulfonamide)

7FB was synthesized according to the procedure described for 4Bn starting from 7-amino-8-hydroxyquinoline (100 mg, 0.625 mmol) and 4-fluorobenzene-1-sulfonyl chloride (120 mg, 0.618 mmol). The product was purified by recrystallization in EtOH. Yield: 26%. 1H NMR (300 MHz, DMSO-\(d_6\)): δ 9.84 (s, br, 1H), 8.78 (dd, J = 3.6, 0.9 Hz, 1H), 8.27 (dd, J = 8.4, 1.8 Hz, 1H), 7.77 (dd, J = 9, 5.7 Hz, 2H), 7.51-7.46 (m, 2H), 7.36-7.28 (m, 3H).

7BP (N-(8-hydroxyquinolin-7-yl)-[1,1'-biphenyl]-4-sulfonamide)

7BP was synthesized according to the procedure described for 4Bn starting from 7-amino-8-hydroxyquinoline (100 mg, 0.625 mmol) and [1,1'-biphenyl]-4-sulfonyl chloride (156 mg, 0.618 mmol). The product was purified flash silica chromatography in 0-3% MeOH in CH\(_2\)Cl\(_2\). Yield: 13%. 1H NMR (400 MHz, DMSO-\(d_6\)): δ 9.93 (s, br, 2H), 8.81 (m, 1H), 8.29 (dd, J = 8.4, 2 Hz, 1H), 7.83 (q, J = 8 Hz, 4H), 7.71 (d, J = 6.4 Hz, 2H), 7.57 (dd, J = 8.8, 3.6 Hz, 1H), 7.51-7.47 (m, 3H), 7.44 (dd, J = 8, 3.2 Hz, 1H), 7.38 (dd, J = 8.8, 3.2 Hz, 1H). ESI-MS (+): m/z 377.10 [M+H]^+.
7TP (N-(8-hydroxyquinolin-7-yl)thiophene-2-sulfonamide)

7TP was synthesized according to the procedure described for 4Bn starting from 7-amino-8-hydroxyquinoline (100 mg, 0.625 mmol) and thiophene-2-sulfonyl chloride (113 mg, 0.618 mmol). The product was purified flash silica chromatography in 0-3% MeOH in CH₂Cl₂ followed by recrystallization in EtOH. Yield: 14%. ¹H NMR (400 MHz, DMSO-d₆): δ 9.97 (s, br, 1H), 8.80 (d, J = 4.4 Hz, 1H), 8.28 (d, J = 8 Hz, 1H), 7.83 (d, J = 3.6 Hz, 1H), 7.49 (d, J = 9.2 Hz, 4H), 7.35 (d, J = 8.8 Hz, 1H), 7.06 (t, J = 4.4 Hz, 1H).

Assay Procedures:

Fluorometric Screening against MMP

CFL-1.1 was screened against MMP-1, -2, -3, -8, and -9 at a concentration of 1 mM for each fragment. The assay was carried out in white NUNC 96-well plates as previously described.²⁰⁰ Each well contained a total volume of 90 µL including buffer (50 mM HEPES, 10 mM CaCl₂, 0.05% Brij-35, pH 7.5), human recombinant MMP (BIOMOL International), and the fragment solution (1 mM final concentration). After a 30 min incubation period at 37 °C, the reaction was initiated by the addition of 10 µL fluorogenic MMP substrate (4 µM final concentration, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂·AcOH, BIOMOL International). Kinetic measurements were recorded using a Bio-Tek Flx 800 fluorescence plate reader every minute for 20 min with excitation and emission wavelengths at 320 and 400 nm, respectively. The rate of fluorescence increase was compared for samples against the negative controls.
(wells with diluted DMSO stock instead of inhibitor), which were arbitrarily set as
100% activity. Eighteen compounds from CFL-1.1 were omitted from the screening
due to excessive background fluorescence that interfered with the assay readings. A
separate buffer (50 mM MES, 10 mM CaCl$_2$, 0.05% Brij-35, pH 6.0) was used for all
experiments with MMP-3.

Units of MMP per well:
- MMP-1: 15.3 U
- MMP-2: 1.16 U
- MMP-3: 2U
- MMP-8: 1.84 U
- MMP-9: 0.9 U

**Colorimetric Screening against MMP**

Due to excessive background fluorescence, 18 compounds from CFL-1.1 were
screened in a colorimetric assay against MMP-1, -2, -3, -8, and -9 at a concentration of
1 mM for each fragment. The assay was carried out in clear Costar® 96 well half area
flat bottom assay plates. Each well contained a total volume of 90 µL including buffer
(50 mM HEPES, 10 mM CaCl$_2$, 0.05% Brij-35, 1 mM DTNB, pH 7.5), human
recombinant MMP (BIOMOL International, units of MMP per well as described
above), and the fragment solution (1 mM final concentration). After a 30 min
incubation period at 37 °C, the reaction was initiated by the addition of 10 µL
chromogenic MMP substrate (500 µM final concentration, Ac-Pro-Leu-Gly-[2-mercapto-4-methyl-pentanoyl]-Leu-Gly-OC₂H₅, BIOMOL International). Absorbance was monitored at 405 nm using a Bio-Tek ELx 808 colorimetric plate reader and measurements were recorded every minute for 20 min. The rate of absorbance increase was compared for samples against the negative controls (wells with diluted DMSO stock instead of inhibitor), which were arbitrarily set as 100% activity. A separate buffer (50 mM MES, 10 mM CaCl₂, 0.05% Brij-35, 1 mM DNTB, pH 6.0) was used for all experiments with MMP-3.

**Screening against LF**

CFL-1.1 was screened against LF at a concentration of 1 mM for each fragment. The assay was carried out in white NUNC 96-well plates as previously described.²⁰⁰ Each well contained a volume of 90 µL including buffer (20 mM HEPES, pH 7.4), recombinant LF (10 nM final concentration List Biological Laboratories), and the fragment solution (1 mM final concentration). After a 20 min incubation period at 25 °C, the reaction was initiated by the addition of 10 µL fluorogenic LF substrate (2 µM final concentration, MAPKKide DABCYL/FITC, List Biological Laboratories). Kinetic measurements were recorded using a Bio-Tek Flx 800 fluorescence plate reader every minute for 20 min with excitation and emission wavelengths at 485 and 528 nm, respectively. The rate of fluorescence increase was compared for samples against the negative controls (wells with diluted DMSO stock instead of inhibitor), which were arbitrarily set as 100% activity.
Screening against 5-LO

CFL-1.1 was screened against 5-LO at a concentration of 1 mM for each fragment. The assay was performed according to a literature method. The assay consisted of 80 µL including buffer (50 mM Tris, 2 mM EDTA, 2 mM CaCl₂, pH 7.5), human recombinant 5-LO (0.2 U, Cayman Chemicals), reporter dye H2DCFDA (10 µM, Invitrogen), fragment solution (1 mM), arachidonic acid (AA, 3 µM, Fischer Scientific), and adenosine triphosphate (ATP, 10 µM, Sigma-Aldrich). H2DCFDA and 5-LO were incubated for 5 min prior to the addition of the fragment solution. This was followed by a second incubation for 10 min. The reaction was initiated by the addition of a substrate solution containing AA and ATP. The reaction was monitored at 37 °C using a Bio-Tek Flx 800 fluorescence plate reader. Kinetic measurements were recorded every minute for 20 min with excitation and emission wavelengths at 485 and 528 nm, respectively. The rate of fluorescence increase was compared for samples against the negative controls (wells with diluted DMSO stock instead of inhibitor), which were arbitrarily set as 100% activity. Both incubations were carried out at room temperature.

Screening against tyrosinase

CFL-1.1 was screened against tyrosinase at a concentration of 1 mM for each fragment. The assay was performed according to a literature method. The assay consisted of a total volume of 100 µL including buffer (50 nM phosphate, pH 6.8),
mushroom tyrosinase (30 U, Sigma-Aldrich), fragment solution (1 mM), and L-dopamine (0.5 mM, Sigma-Aldrich). Mushroom tyrosinase and fragment solution were incubated for 10 minutes. A background absorbance reading at 475 nm was recorded using a Bio-Tek ELx 808 colorimetric plate reader. L-dopamine was added to initiate the reaction, which was allowed to proceed for 10 min before a second absorbance reading at 475 nm was taken. After subtracting the background absorbance, the remaining absorbance of the negative controls (wells with diluted DMSO stock instead of inhibitor) was arbitrarily set as 100% activity. The ratio of absorbance between inhibitor and control wells was defined as percent tyrosinase activity. All incubations were carried out at room temperature.

**Screening against iNOS**

CFL-1.1 was screened against iNOS at a concentration of 1 mM per fragment. iNOS assays were performed using a commercially available colorimetric assay kit purchased from Calbiochem. Murine recombinant iNOS protein (0.1 U, Calbiochem), substrate L-arginine (80 μmol, Sigma-Aldrich), and inhibitor fragments (80 μmol) were incubated in assay buffer at a total volume of 60 μL for 5 min. 10 μL of a nitrate reductase solution (prepared as directed by supplier) was added followed by 10 μL of a freshly made stock of 1 mM NADPH to initiate the reaction. NADPH serves as an essential cofactor to iNOS. After 40 min incubation the reaction was stopped by heat inactivation of the iNOS (30 s in boiling water). In order to destroy excess NADPH, 10 μL of a lactate dehydrogenase (LDH) solution and 10 μL of an LDH cofactor solution were added followed by 20 min incubation. A background reading of
absorbance at 540 nm was taken using a Bio-Tek ELx 808 colorimetric plate reader. 50 μL of Griess Reagent 1 (1% sulfanilamide in 5% phosphoric acid) was added to each well immediately followed by 50 μL of Griess Reagent 2 (0.1% N-(1-Naphthyl)ethylenediamine dihydrochloric acid in water). The reagents were allowed to develop for 10 min prior to collecting a second absorbance measurement at 540 nm. After subtracting the background absorbance, the remaining absorbance of the negative controls (wells with diluted DMSO stock instead of inhibitor) was arbitrarily set as 100% activity. The ratio of absorbance between inhibitor and control wells was defined as percent iNOS activity. All incubations were carried out at 37 °C.

**Assays for sublibrary**

The hydroxyquinoline sublibrary was screened against MMP-2 and 5-LO according to the descriptions above with the exception that the final fragment concentration in each well was 25 μM. The sublibrary was screened two times in duplicate for each enzyme.

### 2.5 References


