Title
A bioinorganic approach to matrix metalloproteinase inhibition

Permalink
https://escholarship.org/uc/item/1pg6q3pm

Author
Puerta, David Thomas

Publication Date
2006

Peer reviewed|Thesis/dissertation
A Bioinorganic Approach to Matrix Metalloproteinase Inhibition

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Chemistry

by

David Thomas Puerta

Committee in charge:

Professor Seth M. Cohen, Chair
Professor William S. Allison
Professor Simpson Joseph
Professor Karsten Meyer
Professor Paul Sung
Professor Francisco Villarreal

2006
The dissertation of David Thomas Puerta is approved, and it is acceptable in quality and form for publication on microfilm.

Chair

University of California, San Diego

2006
To Victoria;

as this dissertation represents the end of another chapter in my life, it fails to compare to all the chapters we have written and have yet to write together.
1.6. Acknowledgements ............................................................................................................ 41
1.7. References .......................................................................................................................... 41

Chapter 2. The Use of Tris(pyrazolyl)borate Model Complexes to Examine the Binding Mode of Known ZBGs ........................................................................................................ 48

2.1. Introduction .......................................................................................................................... 49
2.2. Results .................................................................................................................................. 51
2.2.1. Synthesis of [(Tp^{Ph,Me})ZnOH] .................................................................................. 51
2.2.2. [(Tp^{Ph,Me})Zn(acetohydroxamate)] .............................................................................. 54
2.2.3. Examination of β-Mercaptoketone Based MPIs ................................................................. 57
2.2.4. Examination of β-Mercaptoamide Based MPIs ................................................................. 59
2.2.5. Examination of β-Mercaptoalcohol Based MPIs ............................................................... 62
2.2.6. Binding Modes of Thiol-Based MPIs in Solution ............................................................... 66
2.3. Discussion ............................................................................................................................ 67
2.4. Conclusion ........................................................................................................................... 70
2.5. Experimental Section ........................................................................................................... 70
2.6. Appendix ............................................................................................................................. 77
2.7. Acknowledgements ............................................................................................................. 78
2.8. References ........................................................................................................................... 79

Chapter 3. The Use of Tris(pyrazolyl)borate Model Complexes to Examine Novel ZBGs ................................................................................................................................. 81

3.1. Introduction ........................................................................................................................... 82
3.2. Results .................................................................................................................................. 83
3.2.1. Examination of Hydroxypyridinonate-based ZBGs ......................................................... 83
3.2.2. Examination of N-Methylated Hydroxypyridinonate-based ZBGs ..................... 85
6.5. Experimental Section

6.2.5. Synthesis of Pyrone-Derived MPIs

Chapter 5.2.1. Modeling of the Novel ZBGs in the Active Site of MMP-3

5.2. Results and Discussion

5.1. Introduction

5.2. Modeling of a Futoenone MPI in the Active Site of MMP-3

5.3. Conclusion

5.4. Experimental Section

5.5. Appendix

5.6. Acknowledgements

5.7. References

Chapter 6. The Design, Synthesis, and Inhibitory Profile of Pyrone-Based MPIs and Derivatives

6.1. Introduction

6.2. Results and Discussion

6.2.1. Early Design of Pyrone-Based MPIs

6.2.2. Using the Combined Bioinorganic-Computational Approach to Design Pyrone-Based MPIs AM-1 through AM-6

6.2.3. Synthesis of MPIs AM-1 through AM-6

6.2.4. In Vitro Fluorescence-Based and Cell Invasion Assays of Pyrone-Based MPIs AM-1 through AM-6

6.2.5. Synthesis of Pyrone-Derived MPIs

6.3. Future Pyrone Chemistry

6.4. Conclusion

6.5. Experimental Section

6.6. Acknowledgements

6.7. References
# LIST OF SYMBOLS AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>alpha</td>
</tr>
<tr>
<td>Å</td>
<td>Ångstrøm; $10^{-10}$ m</td>
</tr>
<tr>
<td>Å³</td>
<td>cubic Ångstrøm</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>ADAMT</td>
<td>A disintegrin and metalloproteinase with thrombospondin motif</td>
</tr>
<tr>
<td>atm</td>
<td>atmosphere (1 atm = 101.325 kPa)</td>
</tr>
<tr>
<td>avg</td>
<td>average</td>
</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>br</td>
<td>broad</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>CCDC</td>
<td>Cambridge Crystallographic Data Centre</td>
</tr>
<tr>
<td>CH₂Cl₂</td>
<td>methylene chloride</td>
</tr>
<tr>
<td>CH₃CN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>clcd</td>
<td>calculated</td>
</tr>
<tr>
<td>°</td>
<td>degree</td>
</tr>
<tr>
<td>δ</td>
<td>chemical shift; ppm</td>
</tr>
<tr>
<td>Δ</td>
<td>difference</td>
</tr>
<tr>
<td>d</td>
<td>distance</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
</tr>
<tr>
<td>E</td>
<td>energy</td>
</tr>
<tr>
<td>e⁻</td>
<td>electron</td>
</tr>
<tr>
<td>ε</td>
<td>molar extinction coefficient; M⁻¹ cm⁻¹</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>FKBP</td>
<td>FK506 binding protein</td>
</tr>
<tr>
<td>γ</td>
<td>gamma</td>
</tr>
<tr>
<td>GOF</td>
<td>Goodness Of Fit</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosyl-phosphatidyl inositol</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>IR</td>
<td>infrared⁻¹</td>
</tr>
<tr>
<td>K</td>
<td>Kelvin</td>
</tr>
<tr>
<td>λ</td>
<td>wavelength, lambda</td>
</tr>
<tr>
<td>LADH</td>
<td>Liver alcohol dehydrogenase</td>
</tr>
<tr>
<td>M</td>
<td>molar; mol L⁻¹</td>
</tr>
<tr>
<td>mg</td>
<td>miligram</td>
</tr>
<tr>
<td>µL</td>
<td>microliter</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>mmol</td>
<td>millimol</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MPI</td>
<td>Matrix metalloproteinase inhibitor</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>Membrane type matrix metalloproteinase</td>
</tr>
<tr>
<td>¹⁵N-HSQC</td>
<td>¹⁵N-Heteronuclear single-quantum correlation</td>
</tr>
<tr>
<td>nM</td>
<td>nanomolar</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>ORTEP</td>
<td>Oak Ridge Thermal Ellipsoid Presentation</td>
</tr>
<tr>
<td>Ph</td>
<td>Phenyl</td>
</tr>
<tr>
<td>π</td>
<td>Pi</td>
</tr>
<tr>
<td>ref.</td>
<td>reference</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>σ</td>
<td>sigma</td>
</tr>
<tr>
<td>s</td>
<td>singlet</td>
</tr>
<tr>
<td>SAR by NMR</td>
<td>Structure activity relationship by nuclear magnetic resonance</td>
</tr>
<tr>
<td>t</td>
<td>triplet</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of matrix metalloproteinase</td>
</tr>
<tr>
<td>$\text{Tp}^{\text{Ph,Me}}$</td>
<td>Hydrotris(3,5-phenylmethylpyrazolyl)borate</td>
</tr>
<tr>
<td>$\text{Tp}^*$</td>
<td>Hydrotris(3,5-cumylmethylpyrazolyl)borate</td>
</tr>
<tr>
<td>ZBG</td>
<td>Zinc-binding group</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>$\text{Tp}^-$</td>
<td>hydro-tris(pyrazolyl)borate anion</td>
</tr>
<tr>
<td>$\nu$</td>
<td>wave number, cm$^{-1}$</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1-1. Generalized domain structure for MMPs. The two zinc(II) ions are located in the catalytic domain ................................................................. 5

Figure 1-2. Secondary structure diagram of the catalytic domain of MMP-3. The zinc(II) ions are shown as spheres; the catalytic zinc(II) ion is near the center of the image ................................................................................................ 7

Figure 1-3. Generalized structure for MPIs. The ZBG binds to the catalytic zinc(II) ion. The “P” substituents occupy various subsite pockets in the MMP active site .... 8

Figure 1-4. Molecular surface diagram of the active site of MMP-3 (top). The catalytic zinc(II) ion is shown in black; the enzyme is in gray. Location of 'subsites' are labeled. Schematic figure of the MMP active site before and after inhibition (bottom) ........................................................................................................ 10

Figure 1-5. Chemical diagrams of six hydroxamate-based MPIs: Batimastat (1), Prinomastat (2), WAY-170523 (3), (N-(2-hydroxamatemethylene-4-methylpentoyl)phenylalanyl)methyamine (4), and 3-[4-[3-(cyanomethyl)phenyl]phenoxy]propanohydroxamic acid (5) .............................................. 12

Figure 1-6. Molecular surface diagram of MMP-3 complexed with a hydroxamic acid inhibitor. The catalytic zinc(II) ion is shown in black; the inhibitor and enzyme are in gray (top). The chemical structure and mode of metal binding of the inhibitor are shown at the bottom. Residue Pro221 is shown as a wireframe surface for clarity ......................................................................................................................... 16

Figure 1-7. Definition of τ value for a five-coordinate metal complex. The metal center is designated by the label M and the ligands by labels A-E. The angles α and β are illustrated; for a perfect square pyramid α = β = 180° and for a perfect trigonal bipyramid α = 120°, β = 180° ........................................................................................................ 17

Figure 1-8. Molecular surface diagram of MMP-3 complexed with a carboxylate inhibitor. The catalytic zinc(II) ion is shown in black; the inhibitor and enzyme are in gray (top). The chemical structure and mode of metal binding of the inhibitor are shown at the bottom. Residues Tyr220, Pro221, and Leu222 are shown as a wireframe surface for clarity ................................................................................................. 19

Figure 1-9. Molecular surface diagram of MMP-8 complexed with a thiol inhibitor. The catalytic zinc(II) ion is shown in black; the inhibitor and enzyme are in gray (top). The chemical structure and mode of metal binding of the inhibitor are shown at the bottom. Residue Pro217 is shown as a wireframe surface for clarity .......... 21
Figure 1-10. Top: Molecular surface of the catalytic domain of MMP-8 complexed with a phosphonic inhibitor. The catalytic zinc is shown in black, the inhibitor and enzyme are in gray scale. Bottom: The chemical structure and mode of metal binding of the inhibitor ................................................................. 23

Figure 1-11. Top: Molecular surface of the catalytic domain of MMP-11 complexed with a phosphinic inhibitor. The catalytic zinc is shown in black, the inhibitor and enzyme are in gray scale. Bottom: The chemical structure and mode of metal binding of the inhibitor ................................................................. 24

Figure 1-12. Top: Molecular surface of the catalytic domain of MMP-3 complexed with a thiadiazole inhibitor. The catalytic zinc is shown in black, the inhibitor and enzyme are in gray scale. Bottom: The chemical structure and mode of metal binding of the inhibitor ................................................................. 27

Figure 1-13. Top: Molecular surface of the catalytic domain of MMP-3 complexed with a barbiturate inhibitor. The catalytic zinc is shown in black, the inhibitor and enzyme are in gray scale. Bottom: The chemical structure and mode of metal binding of the inhibitor ................................................................. 28

Figure 1-14. Chemical diagrams of doxycycline (top) and a Futoenone derivative (bottom) ............................................................................................................................................ 30

Figure 1-15. Cis- to trans- isomerization in hydroxamic acid compounds ............... 32

Figure 1-16. Simplified scheme for the Structure Activity Relationship by Nuclear Magnetic Resonance (SAR by NMR) drug discovery method (left). Chemical structure and binding affinity ($K_D$) of several biaryl backbone substituents examined by using the SAR by NMR method (right) ........................................................................ 36

Figure 1-17. Chemical structure and binding affinity ($K_D$) of several ZBGs examined by using the SAR by NMR drug discovery method ................................................................. 37

Figure 1-18. Chemical (left) and crystallographic (right) structure for [(Tp$^*$Zn(acetohydroxamate)] .......................................................................................................................... 40

Figure 2-1. Homoscorpionate ligands tris(pyrazolyl)borate (left) and tris(pyrazolyl)methane (right). ............................................................................................................................................. 49

Figure 2-2. Structural diagrams of asymmetric unit [(Tp$^{Ph,Me}_5$ZnOH] (top) and [(Tp$^{Ph,Me}_5$Zn$_2$(H$_2$O$_2$)] dimer (bottom) with partial atom numbering schemes (ORTEP, 50% probability ellipsoids). The perchlorate counterion, solvent molecules, and selected hydrogen atoms have been omitted for clarity .................................................. 53

Figure 2-3. Structural diagram of [(Tp$^{Ph,Me}_5$Zn(acetohydroxamate)] with partial atom numbering schemes (ORTEP, 50% probability ellipsoids). Hydrogen atoms and solvent have been omitted for clarity ................................................. 55
Figure 2-4. An overlay of all nine atoms (one zinc, two oxygen, two carbon, and four nitrogen atoms) from [(Tp^ph,Me)Zn(acetohydroxamate)] (blue) with that of MMP-13 complexed with the hydroxamate-based MPI (N'- (2-hydroxamatemethylene-4-methyl-pentoyl)phenylalanyl)methylamine (red). The RMS deviation is 0.372 Å ........................................ 55

Figure 2-5. List of representative MPIs (left) and the corresponding small molecules (right) used to model zinc binding. MPI classes from top to bottom: hydroxamates, \( \beta \)-mercaptoamides, \( \beta \)-mercaptoketones, and \( \beta \)-mercaptoalcohols ......................... 58

Figure 2-6. Structural diagram of [(Tp^ph,Me)Zn(3-mercapto-2-butanonate)] with partial atom numbering schemes (ORTEP, 50% probability ellipsoids). Hydrogen atoms and one isomer (partial occupancy disorder, C33B/C34B) have been omitted for clarity ................................................................. 59

Figure 2-7. Structural diagram of [(Tp^ph,Me)Zn(N-methylmercaptoacetamidate)] with partial atom numbering schemes (ORTEP, 50% probability ellipsoids). Hydrogen atoms and solvent have been omitted for clarity ................................................................. 60

Figure 2-8. Structural diagram of [(Tp^ph,Me)Zn(3-mercapto-2-butanonate)] with partial atom numbering schemes (ORTEP, 50% probability ellipsoids). Hydrogen atoms and three isomers (partial occupancy disorder) have been omitted for clarity .................................................................................. 63

Figure 2-9. Structural diagram [(Tp^ph,Me)Zn(3-mercapto-2-propanoate)] with partial atom numbering schemes (ORTEP, 50% probability ellipsoids). Hydrogen atoms and solvent have been omitted for clarity ................................................. 64

Figure 2-10. Structural diagram [(Tp^ph,Me)Zn(2-mercaptoethanoate)] with partial atom numbering schemes (ORTEP, 50% probability ellipsoids). Hydrogen atoms and solvent have been omitted for clarity ................................................. 64

Figure 2-11. Structural diagram of [(Tp^ph,Me)Zn(3-mercapto-2-butanonate)] displaying disorder in carbons 33 and 34 with partial atom numbering schemes (ORTEP, 50% probability ellipsoids). Hydrogen atoms and have been omitted for clarity ................................................................. 77

Figure 2-12. Structural diagram of [(Tp^ph,Me)Zn(3-mercapto-2-butanonate)] displaying disorder from all for isomers with partial atom numbering schemes (ORTEP, 50% probability ellipsoids). Hydrogen atoms have been omitted for clarity ................................................................. 78

Figure 3-1. Synthesis of [(Tp^ph,Me)Zn(ZBG)] model complexes (ZBG = zinc-binding group) ...................................................................................................................... 83
Figure 3-2. Structural diagram of $$[(T_p^{ph,Me})Zn(1-hydroxy-2(1H)-pyridinone)]$$ with partial atom numbering scheme (ORTEP, 50% probability ellipsoids). Hydrogen atoms and solvent have been omitted for clarity ........................................ 84

Figure 3-3. Structural diagram of $$[(T_p^{ph,Me})Zn(3-hydroxy-2(1H)-pyridinone)]$$ with partial atom numbering scheme (ORTEP, 50% probability ellipsoids). Hydrogen atoms, solvent, and partial occupancy disorder have been omitted for clarity ....... 85

Figure 3-4. Structural diagram of $$[(T_p^{ph,Me})Zn(3-hydroxy-1-methyl-2(1H)-pyridinone)]$$ with partial atom numbering scheme (ORTEP, 50% probability ellipsoids). Hydrogen atoms and solvent molecules have been omitted for clarity ...... ................................................................................................................. 87

Figure 3-5. Structural diagram of $$[(T_p^{ph,Me})Zn(3-hydroxy-1,2-dimethyl-4(1H)-pyridinone)]$$ with partial atom numbering scheme (ORTEP, 50% probability ellipsoids). Hydrogen atoms and solvent molecules have been omitted for clarity ......... 87

Figure 3-6. Structural diagram of $$[(T_p^{ph,Me})Zn(1-hydroxy-2(1H)-pyridinethione)]$$ with partial atom numbering scheme (ORTEP, 50% probability ellipsoids). Hydrogen atoms and solvent molecules have been omitted for clarity .................. 90

Figure 3-7. Structural diagram of $$[(T_p^{ph,Me})Zn(3-hydroxy-2-methyl-4-pyrene)]$$ with partial atom numbering scheme (ORTEP, 50% probability ellipsoids). Hydrogen atoms and solvent molecules have been omitted for clarity ...................... 90

Figure 3-8. Infrared spectra for free (solid line) and bound (dashed line) 3-hydroxy-2-methyl-4-pyrene. The spectral region shown is of the carbonyl stretch, which shifts to lower energy upon metal coordination ................................................................. 94

Figure 4-1. Heterocyclic ZBGs proposed for use in MMP inhibitors. AHA was utilized as a benchmark ZBG .................................................................................................................. 107

Figure 4-2. Representative data generated from fluorescent assays of 11 .......... 109

Figure 4-3. Plot of the IC$_{50}$ values of the compounds (AHA, 1-11, excluding 3) against MMP-1 (black), MMP-2 (crossed), and MMP-3 (gray). Inset highlights the most potent compounds (7-11) .................................................................................. 113

Figure 4-4. MTS cell viability assay results. Plot of the relative toxicities of the compounds (AHA, 1-11, excluding 3) in neonatal rat cardiac fibroblasts at concentrations of: 0.001 (black), 0.01 (white), 0.1 (crossed), and 1.0 (gray) mM of the ZBG listed. Colorimetric assay values were normalized to those of untreated cells ........................................................................................................... 117

Figure 4-5. Photomicrograph of CF cells in the absence (A) and presence of two concentrations of compound 9: 0.1 mM (B) and 1.0 mM (C) .................................................. 117
Figure 4-6. Cell invasion assay results. ................................................. 119

Figure 4-7. Representative data generated from fluorescent controls of 11............ 127

Figure 5-1. Heterocyclic ZBGs proposed for use in MMP inhibitors. ZBGs 1-3 and 5-7 were used in the computational modeling studies ............................................. 134

Figure 5-2. Images of the modeling of 1-hydroxy-2(1H)-pyridinone in the active site of MMP-3 ................................................................. 136

Figure 5-3. Images of the modeling of 3-hydroxy-1-methyl-2(1H)-pyridinone in the active site of MMP-3 ................................................................. 138

Figure 5-4. Images of the modeling of 3-hydroxy-1,2-dimethyl-4(1H)-pyridinone in the active site of MMP-3 .................................................... 140

Figure 5-5. Images of the modeling of 3-hydroxy-2-methyl-4-pyrone in the active site of MMP-3 ................................................................. 142

Figure 5-6. Images of the modeling of 1-hydroxy-2(1H)-pyridinethione in the active site of MMP-3 ................................................................. 145

Figure 5-7. Futoenone derivative 12 (left), with ring system 2-methoxybenzenethiol (MBT) in blue and benzo[d][1,3]dioxole in green. Hydroxamate-based MPI, GM6001 is shown on right ......................................................... 147

Figure 5-8. Structural diagram of [(Tp^{Ph,Me})Zn(MBT)] with partial atom numbering schemes (ORTEP, 50% probability ellipsoids). Hydrogen atoms and solvent molecules have been omitted for clarity. Inset displays atoms used for superpositions ........................................................................ 148

Figure 5-9. Three superpositions of [((Tp^{Ph,Me})Zn(MBT))] fragment (Figure 5-8, inset) into the structure of MMP-3 ......................................................... 149

Figure 5-10. Lowest-energy minimized structure of 12 in the active site of stromelysin (MMP-3). Y155 is shown in blue, P156 is shown in red, and the catalytic zinc(II) ion is shown in purple ......................................................... 151

Figure 5-11. Representation of the multiple low energy conformers of 12; red = ~8 kcal/mol. Y155 is shown in blue and P156 is shown in pink .......................... 157

Figure 5-12. Representation of the multiple low energy conformers of 12; orange = ~6 kcal/mol. Y155 is shown in blue and P156 is shown in pink .......................... 158

Figure 5-13. Representation of low energy conformer of 12; yellow = ~5 kcal/mol. Y155 is shown in blue and P156 is shown in pink ........................................ 159
Figure 5-14. Representation of low energy conformer of 12; green = ~4 kcal/mol. Y155 is shown in blue and P156 is shown in pink .................................................. 160

Figure 6-1. Representation of the initial receptor complex used in the LUDI docking experiments with pyrone ZBG (colored by element) in the active site of MMP-3 (gray). In the docking experiments, the backbones would link to the ZBG by overlaying onto the N-C bond ................................. 173

Figure 6-2. LUDI docking image of backbone fragment (green, in S1' subsite) with pyrone ZBG (colored by element) in the active site of MMP-3 (gray) ............... 174

Figure 6-3. LUDI docking image of backbone fragment (green, in S1' subsite) with pyrone ZBG (colored by element) in the active site of MMP-2 (left) and MMP-3 (right)................................................................. 179

Figure 6-4. Neonatal cardiac fibroblast (CF) invasion assay results. Fluorescent measurement (in RFUs) of lysed cells after invasion with: no inhibitor (Control), 250 nM AM-5, and 250 nM AM-6. Increased RFUs indicates increased cell invasion ................................................................. 180
LIST OF SCHEMES

Scheme 2-1. Synthesis of \([\text{Tp}^{\text{Ph,Me}}]_{\text{ZnOH}}\) ................................................................. 52

Scheme 6-1. Synthesis of KA-1 ................................................................................................. 168

Scheme 6-2. Synthesis of CA-2 ................................................................................................. 170

Scheme 6-3. Synthesis of Pyrone-Based MPIs AM-1 through AM-6 ............................ 175

Scheme 6-4. Synthesis of PY-2 ................................................................................................. 182

Scheme 6-5. Synthesis of DH-1 ................................................................................................. 183

Scheme 6-6. Synthesis of AM-2S .............................................................................................. 185

Scheme 6-7. Synthesis of PY-2(N-CH₃) .................................................................................... 186

Scheme 6-8. Proposed synthesis of X-2 ..................................................................................... 189
LIST OF TABLES

Table 1-1. The Classification, Common Names and Substrates of the MMP Family .................................................................3

Table 1-2. List of zinc-binding groups (ZBGs) used in MMP inhibitors...............26

Table 2-1. Crystal data for [(Tp^{Ph,Me})_2Zn_2(H_2O_2)]ClO_4 and [(Tp^{Ph,Me})Zn(acetohydroxamate)] .................................................................56

Table 2-2. Crystal data for [(Tp^{Ph,Me})Zn(3-mercapto-2-butanonate)] and [(Tp^{Ph,Me})Zn(N-methylmercaptoacetamidate)] ........................................61

Table 2-3. Crystal data for [(Tp^{Ph,Me})Zn(2-mercaptoethanoate)], [(Tp^{Ph,Me})Zn(3-mercapto-2-propanoate)], and [(Tp^{Ph,Me})Zn(3-mercapto-2-butanoate)] ........................................65

Table 3-1. Crystal data for [(Tp^{Ph,Me})Zn(1-hydroxy-2(1H)-pyridinone)], [(Tp^{Ph,Me})Zn(3-hydroxy-2(1H)-pyridinone)], and [(Tp^{Ph,Me})Zn(3-hydroxy-1-methyl-2(1H)-pyridinone)] .........................88

Table 3-2. Crystal data for [(Tp^{Ph,Me})Zn(3-hydroxy-1,2-dimethyl-4(1H)-pyridinone)], [(Tp^{Ph,Me})Zn(1-hydroxy-2(1H)-pyridinethione)], and [(Tp^{Ph,Me})Zn(3-hydroxy-2-methyl-4-pyrole)] ........................................91

Table 3-3. Bond lengths for coordinating atoms in complexes of [(Tp^{Ph,Me})Zn(ZBG)]. All bond lengths were obtained from X-ray structure determinations.................................................................92

Table 4-1. IC_{50} values (µM) for ZBGs against MMP-1, MMP-2, MMP-3, and in CF cell culture. Compound 3 was not evaluated due to poor aqueous solubility. Values based on at least three independent experiments.................................................112

Table 5-1. Crystal data and structure refinement for [(Tp^{Ph,Me})Zn(MBT)] ..........155

Table 5-2. List of final minimized energies for conformers of 12........................156

Table 6-1. Structures and IC_{50} Values (µM) for First Generation Pyrone-based MPIs Against MMP-1, MMP-2, and MMP-3.................................................................171

Table 6-2. IC_{50} Values (µM) for MPIs Against MMP-1, MMP-2, and MMP-3. LUDI Scores for MMP-2 (PDB code 1Q1B), and MMP-3 (PDB code 1G4K) are Shown..................................................................................................177

Table 6-3. Structures and IC_{50} Values (µM) of Pyrone-Based MPIs and Derivatives Against MMP-1, MMP-2 and MMP-3. Values are Obtained from Fluorescence-based MMP Assays. ND = not determined.........................................................187
ACKNOWLEDGEMENTS

I would like to first thank my parents. They sacrificed so that I may have a good education. Most importantly, they taught be by example the value of hard work. I thank you for supporting me throughout by academic career.

I would also like to acknowledge my advisor, Prof. Seth Cohen. Seth, you gave me a chance from the moment I walked into your office after faculty talks of orientation and told you ”I don’t remember what your research is but I know I liked it.” Instead of kicking me out of your office, you kindly gave me an in depth talk on all of your research projects. Now, could it be that I was the first student to walk into your office and you had an empty lab to fill in your first year, or was it just the type of person you are? I like to think it is the latter.

I have been truly blessed with wonderful lab mates. Sara, Misha, Tai, and Scot, we began our graduate careers with Seth (as he started his professorship.) As we all had to grow together it gave us all a unique bond and prospective which I wouldn’t replace with a more “established” lab environment.

Jana and Faith, you two both joined me on the MMP project a year after I started in the lab. You two are like the little sisters I never had. Thank goodness, because it would have been hard to grow up with younger sisters who are smarter than me.

This research could not have been accomplished without the help of superb collaborators. I am very grateful for the generosity of Prof. Francisco Villarreal and Prof. J. Andrew McCammon. These two professors, in addition to the wonderful people in their labs are the only reason this highly collaborative research could take
these publications also participated in the research. The permissions to reproduce these papers are granted by Springer, American Chemical Society, Bentham Science Publishers Ltd, John Wiley & Sons Inc, and Elsevier Science.
VITA

Education

2001
Bachelor of Arts in Biochemistry (cum laude), California State University, Dominguez Hills, Carson CA. Advisor: Prof. Leonardo Martinez

2006
Ph.D. in Chemistry, University of California, San Diego, CA. Advisor: Prof. Seth M. Cohen

Research Fellowships, Awards, and Professional Affiliations


2003 Teaching Assistant of the Year, University of California, San Diego


Jan. 2001 – present American Chemistry Society, Member

Presentations


Publications


ABSTRACT OF THE DISSERTATION

A Bioinorganic Approach to Matrix Metalloproteinase Inhibition

by

David Thomas Puerta

Doctor of Philosophy in Chemistry

University of California, San Diego, 2006

Professor Seth M. Cohen, Chair

In an effort to develop potent inhibitors of matrix metalloproteinases (MMPs), a bioinorganic approach was employed.

The synthesis of [(Tp^{Ph,Me})Zn(OH)] provided for a structural analogue of the zinc-(tris-histidine) catalytic site of MMPs. The model complex was used to gain insight into the discrepancy of MMP inhibitor (MPI) potencies between mercapto alcohols and mercapto ketones. This initial experiment validated the use of the inorganic model complex as a structural model of the MMP catalytic site.
Novel ZBGs for incorporation into MPIs were identified. These ZBGs were complexed with [(Tp\(^{\text{Ph,Me}}\)Zn(OH))] to obtain structural information such as binding mode, bond lengths, and coordination geometry. All ZBG examined were found to bind the model complex in a bidentate fashion, indicating promise for incorporation into a full length MPI.

The inhibitory ability of the novel ZBGs was examined in both fluorescent and colorimetric assays using either the catalytic domain of MMPs or native enzyme expressed in a cell culture of neonatal rat ventricular fibroblasts. All novel ZBGs examined were found to be better inhibitors of MMPs in vitro and in cell culture assays than acetohydroxamic acid (the representative ZBG used in the majority of MPIs to date).

In order to design full-length MPIs, a combined computational-bioinorganic method was developed. Using the structural coordinates from the [(Tp\(^{\text{Ph,Me}}\)Zn(ZBG))] complexes, the novel ZBGs were modeled into an X-ray crystal structure of uninhibited MMP-3. This study allowed for the examination of the ZBGs in the active site of an MMP. The novel ZBGs were found to have orientations in the active site of MMP-3 amendable to the attachment of a peptidomimetic backbone, necessary for a full-length MPI.

Finally, the first MPIs based on the heterocyclic ZBGs were developed. The combined computational-bioinorganic method was augmented with the drug discovery program LUDI. Using LUDI enhanced with structural coordinates from [(Tp\(^{\text{Ph,Me}}\)Zn(3-hydroxy-2-methyl-4-pyrone)]}, several MPIs were designed. The potential inhibitors were synthesized and were examined in a fluorescence-based assay of MMP-1, -2, and -
3. The pyrone-based MPIs were found to be more potent than their hydroxamate analogues, demonstrating the efficacy of a bioinorganic approach to the development of metalloprotein inhibitors.
Chapter 1. Introduction
1.1. History of Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a class of hydrolytic enzymes involved in the breakdown of the extracellular matrix and the basement-membrane including components such as aggrecan, collagen, elastin, fibronectin, gelatin, and laminin. Unlike other proteinases, MMPs require a zinc(II) ion cofactor for peptide hydrolysis. MMPs are essential for several processes that are part of normal physiological development including growth, wound healing, and other functions requiring tissue reorganization.[1-4] For example, one of the earliest descriptions of MMP activity in animals was found in the collagenolytic properties of tadpole tissues. The experiment, which consisted of living tail fin tissue placed on a collagen gel, resulted in the degradation of collagen at neutral pH and physiological temperatures.[5] The enzyme collagenase (MMP-1) was found to be responsible for this activity. Since this discovery over 40 years ago, more than twenty mammalian MMPs have been identified and classified.[6-8] Classification of MMPs has been based largely on the different substrate specificities observed during in vitro studies of the individual enzymes.[7] This basis has resulted in the division of MMPs into five groups (Table 1-1): collagenases, gelatinases, stomelysins, membrane-associated, and unclassified.[7, 8] Although some more recent descriptions have categorized MMPs by modular domain structure,[9] the substrate-specificity classification system will be used in this dissertation.
### Table 1-1: The Classification, Common Names and Substrates of the MMP Family.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Common Name</th>
<th>Substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>Collagenase-1; interstitial collagenase</td>
<td>Collagen type 1-3, 7, 8, 10; aggrecan; gelatin; MMP-2, MMP-9</td>
</tr>
<tr>
<td>MMP-2</td>
<td>Gelatinase A; 72kDa gelatinase; MMP-5</td>
<td>Collagen type 1-5, 7, 10, 11, 14; aggrecan; elastin; fibronectin; gelatin; laminin; MMP-9, and MMP-13</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Stromelysin-1; procollagenase; transin-1</td>
<td>Collagen type 2-4, 9-11; aggrecan; elastin; fibronectin; gelatin; laminin; MMP-1, -7, -8, -9, and -13</td>
</tr>
<tr>
<td>MMP-7</td>
<td>Matrilysin-1; PUMP-1</td>
<td>Collagen type 1-3, 5, 7, 8, and 10; aggrecan; elastin; fibronectin; gelatin; laminin; MMP-1, -2, and -9</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Collagenase-2; neutrophil collagenase</td>
<td>Collagen type 4 and 10; aggrecan; elastin; fibronectin; gelatin; laminin</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Gelatinase B; 92kDa gelatinase</td>
<td>Collagen type IV, V, VII, X, XIV; aggrecan; elastin; fibronectin; gelatin</td>
</tr>
<tr>
<td>MMP-10</td>
<td>Stromelysin-2; transin-2</td>
<td>Collagen type 3-5; aggrecan; elastin; fibronectin; gelatin; laminin; MMP-1, and -8</td>
</tr>
<tr>
<td>MMP-11</td>
<td>Stromelysin-3</td>
<td>Aggrecan; fibronectin; laminin; α-1 antitrypsin</td>
</tr>
<tr>
<td>MMP-12</td>
<td>Marcophage metalloelastase</td>
<td>Collagen type 4; elastin; fibronectin; gelatin; laminin; vitronectin</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Collagenase-3</td>
<td>Collagen type 1-4; aggrecan; gelatin; MMP-9</td>
</tr>
<tr>
<td>MMP-14</td>
<td>MT1-MMP (Membrane-Type-1 MMP)</td>
<td>Collagen type 1-3; aggrecan; elastin; fibronectin; gelatin; laminin; MMP-2, and -13</td>
</tr>
<tr>
<td>MMP-15</td>
<td>MT2-MMP</td>
<td>Fibronectin; gelatin; laminin; MMP-2</td>
</tr>
<tr>
<td>MMP-16</td>
<td>MT3-MMP; ovary metalloproteinase</td>
<td>MMP-2</td>
</tr>
<tr>
<td>MMP-17</td>
<td>MT4-MMP</td>
<td>Fibrin; fibrinogen; TNF precursor</td>
</tr>
<tr>
<td>MMP-18</td>
<td>Xenopus MMP</td>
<td>Unknown</td>
</tr>
<tr>
<td>MMP-19</td>
<td>RASI-1; RASI-6</td>
<td>Collagen type 4; aggrecan; COMP; gelatin; laminin; large tenas; nidogen</td>
</tr>
<tr>
<td>MMP-20</td>
<td>Enamelysin</td>
<td>Amelogenin; aggrecan; COMP</td>
</tr>
<tr>
<td>MMP-21</td>
<td>Xenopus MMP</td>
<td>Unknown</td>
</tr>
<tr>
<td>MMP-22</td>
<td>Gallus domesticus MMP</td>
<td>Casein; gelatin</td>
</tr>
<tr>
<td>MMP-23</td>
<td>CA-MMP</td>
<td>Unknown</td>
</tr>
<tr>
<td>MMP-24</td>
<td>MT5-MMP</td>
<td>MMP-2</td>
</tr>
<tr>
<td>MMP-25</td>
<td>MT6-MMP; leukolysin</td>
<td>Gelatin</td>
</tr>
<tr>
<td>MMP-26</td>
<td>Matrilysin-2; endometase</td>
<td>Collagen type 4; α-PI; fibronectin; fibrinogen; gelatin; pro-MMP-9</td>
</tr>
<tr>
<td>MMP-28</td>
<td>Epilysin</td>
<td>Casein</td>
</tr>
</tbody>
</table>
1.1.1. Role of MMPs in Physiology and Disease

The ability of MMPs to degrade components of the extracellular matrix is essential to tasks such as cell growth, cell division, bone growth, wound healing, embryogenesis, and angiogenesis.\textsuperscript{[1-4, 10]} The importance of MMPs in normal physiology is demonstrated by MMP-9 deficient mice, which display impeded bone formation and growth. MMP-9 was found to be vital for primary angiogenesis invading the cartilage matrix in the developmental stages of ossification.\textsuperscript{[11]} In most cases, mice knock out studies have shown that a deletion of individual MMPs have not produced fatal results. This has been attributed to the overlapping substrate selectivity in the MMP family.\textsuperscript{[3]} Nevertheless, several studies have demonstrated that a disruption in the regulation of MMPs is correlated with a number of disease states including arthritis, cardiovascular disease, stroke, atherosclerosis, and tumor metastasis.\textsuperscript{[1, 2, 4, 12, 13]} For example, increased expression of collagenases (MMP-1, MMP-8, MMP-13) and increased collagenolytic activity have been found in the synovial fluid of patients with osteoarthritis and rheumatoid arthritis.\textsuperscript{[14, 15]} Transgenic mice that overproduce MMP-1 have been utilized to determine the role of MMPs in congestive heart failure. After extended MMP-1 expression, these mice displayed a loss of cardiac interstitial collagen and subsequent loss of contractile function.\textsuperscript{[16]} Several other investigations have addressed the role of MMPs in tumor genesis, growth, and metastasis. Experiments with MMP-7 deficient mice demonstrated a 60% reduction in tumor multiplicity and a substantial decrease in tumor size.\textsuperscript{[17]} Other studies have shown that implantation of melanoma and Lewis lung carcinoma cells in
mice lacking MMP-2 results in a decrease in tumor volumes of 39% and 24%, respectively.\textsuperscript{[18]} The role of MMPs in these and several other pathologies has been extensively reviewed.\textsuperscript{[2-4, 19]}

### 1.1.2. Structure of MMPs

MMPs contain the same basic structural features despite the observed variability in substrate specificity (Figure 1-1). MMPs are synthesized as inactive zymogens and excreted prior to activation; this ‘pre-proMMP’ contains a N-terminal signal pre-domain region that directs the protein for secretion, after which, the enzyme exists as the inactive ‘proMMP’.\textsuperscript{[4]} The propeptide domain consists of approximately 80 amino acids containing a conserved cysteine in the sequence -PRCGXPD- that suppresses enzyme activity by coordination of a cysteiny1 sulfur atom (shown in bold) to the active site zinc(II) ion. The enzyme becomes active upon cleavage of the propeptide domain by other MMPs or proteases such as plasmin in what is termed the ‘cysteine switch’ mechanism.\textsuperscript{[3, 4, 6, 7]}

![Figure 1-1. Generalized domain structure for MMPs. The two zinc(II) ions are located in the catalytic domain.](image)

The 170 amino acid catalytic domain (Figure 1-2) is composed of a five-stranded $\beta$-sheet, three $\alpha$-helices, and bridging loop structures.\textsuperscript{[4]} The active site contains two zinc(II) ions; one serves a structural role and the other is the site of
peptide hydrolysis. Buried in the protein, the structural zinc(II) ion is bound by one aspartate and three histidine residues in a tetrahedral geometry. The catalytic zinc is coordinated by three histidine nitrogen atoms (shown in bold) in a conserved sequence -VAAHEXGHXXGXXH-.[20] In the resting state, water molecules occupy the remainder of the coordination sphere and are critical for peptide hydrolysis.[21] Upon binding of a carbonyl oxygen of a peptide to the zinc(II) ion, the amide bond is attacked by a zinc-bound water molecule that is hydrogen bonded to an adjacent glutamate residue. Proton transfer from the water molecule to the glutamate residue and then to the amide nitrogen completes the cleavage of the peptide.[7, 20] All MMPs (except MMP-7 and MMP-23) contain a hemopexin-like domain consisting of ~210 amino acids that is involved in substrate binding. The group of membrane-type MMPs (MT-MMPs) can be divided into two groups according the manner of membrane attachment. The enzymes MT1-MMP, MT2-MMP, MT3-MMP, and MT5-MMP are attached through a transmembrane domain, while MT4-MMP and MT6-MMP are anchored to the membrane through a glycosyl-phosphatidyl inositol (GPI) linkage.[22, 23]
Figure 1-2. Secondary structure diagram of the catalytic domain of MMP-3. The zinc(II) ions are shown as red spheres; the catalytic zinc(II) ion is coordinated by three conserved histidine residues (shown as sticks, colored by atom).
1.2. Inhibitors of Matrix Metalloproteinases

1.2.1. Early Strategies for MMP Inhibition

Due to the variety of diseases that MMPs are correlated with (vide supra), the inhibition of MMPs has grown into an important field for the development of potential chemotherapeutics. Most MMP inhibitors (MPIs) use the same basic design strategy (Figure 1-3); a peptidomimetic backbone is coupled with a metal chelating moiety (often referred to as a zinc-binding group or ZBG).[7,8,24] Following a substrate-based approach, early MPIs were designed to mimic natural substrates of MMPs by using a short peptide derivative attached to a ZBG.[7] Since these early efforts, MPI design has become more structure-based, due to the abundance of NMR and X-ray structural data available for MMPs.[8,25,26] This structure-based approach is dictated by the shape of the active site ‘ subsites’ (vide infra).

![Figure 1-3](image)

**Figure 1-3.** Generalized structure for MPIs. The ZBG binds to the catalytic zinc(II) ion. The “P” substituents occupy various subsite pockets in the MMP active site.

An alternative to small molecule therapeutic strategies for MMP inhibition has been the use of the naturally occurring tissue inhibitors of matrix metalloproteinases (TIMPs).[27] TIMPs are a family of at least four known 20–29 kDa proteins (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) that inhibit MMP activity. This inhibition is due to the reversible formation of a heterodimeric assembly between a TIMP and an MMP.
TIMPs inhibit MMPs by binding of the active site zinc(II) ion (much like synthetic inhibitors) by using residue Cys1; the X-ray crystal structures of TIMP-1 bound to MMP-3 and TIMP-2 bound to MT1-MMP have been determined. The sulfur atom of the cysteine residue is not coordinated, as it is involved in a disulfide bond with Cys70. However, the disulfide bond provides a rigid N-terminus that positions the $\alpha$-amino nitrogen atom and carbonyl oxygen atom in a geometry that facilitates zinc binding. Studies involving mouse models showed TIMP-1 overexpression was able to hinder lymphoma tumor growth, however, this protein-based inhibition strategy has strict limitations due to the inadequate pharmacological stability of TIMPs. Also, TIMPs can inhibit other metalloproteins including ADAMs (A Disintegrin And Metalloproteinase) and ADAMTs (ADAMs with Thrombospondin motifs). Furthermore, studies have found that TIMPs are too large to penetrate cartilage, which may limit their medical usefulness.

1.2.2. Significance of the Active Site ‘Subsites’ in Inhibitor Design

NMR and X-ray crystal structures have yielded valuable information on the zinc(II) ion active site of MMPs. The active site can be described as several substrate interaction sites termed ‘subsites.’ The diversity in substrate selectivity between different MMPs is attributed to the variety of residues found in these subsites. Using Figure 1-4 as a reference, the subsites to the ‘left’ of the zinc (II) ion are classified as ‘unprimed’ (e.g. S1, S2, S3), while the subsites to the ‘right’ of the zinc(II) ion are classified as the ‘primed’ subsites (e.g. S1’, S2’, S3’). The functional
groups of MPIs that are designed to interact with specific subsites (Figure 1-3) are termed ‘P’ or ‘P’ groups,[7] that is, a P1 group is expected to interact with the S1 subsite.

Figure 1-4. Molecular surface diagram of the active site of MMP-3 (top). The catalytic zinc(II) ion is shown in black; the enzyme is in gray. Location of ‘subsites’ are labeled. Schematic figure of the MMP active site before and after inhibition (bottom).
Most efforts in MMP inhibitor design have focused on the primed subsites (termed ‘right-handed’ inhibitors), particularly the S1' pocket. The S1' subsite is generally characterized as a deep, hydrophobic pocket throughout all MMPs, with the exception of MMP-1 and MMP-7.[30, 35] The depth of the S1' pocket varies between MMPs, and the residues in this pocket lack any significant sequence homology. Differences in the S1' pocket have led to the site being titled the “specificity pocket”,[30, 35] and several MPIs have been designed to exploit this feature[36, 37] as in the case of the inhibitor WAY-170523.[38] This inhibitor (Figure 1-5, structure 3), designed by using NMR structural information of the active site, contains a large P1' group that fits tightly into the S1' pocket of MMP-13, but is too bulky for accommodation by the S1' pocket of MMP-1. Consequently, WAY-170523 displays nearly 6000-fold more potency for MMP-13 over MMP-1. However, in some inhibitor-protein complexes with MMP-1 the S1' pocket expands, allowing for an MPI with a large P1' substituent to occupy this cavity.[35]
Figure 1-5. Chemical diagrams of six hydroxamate-based MPIs: Batimastat (1), Prinomastat (2), WAY-170523 (3), (N-(2-hydroxamatemethylene-4-methylpentoyl)phenylalanyl)methylamine (4), and 3-[4-[3-(cyanomethyl)phenyl]phenoxy]propanohydroxamic acid (5).

The solvent exposed S2' pocket is located directly above the opening to the S1' pocket. The S2' cavity is hydrophobic in MMPs with the exception of MMP-1 and MMP-7 where it contains Ser and Thr residues, respectively. The S2' subsite does offer opportunities for selectivity, but has taken a secondary role behind the S1' pocket. A class of inhibitors designed with bulky hydrophobic P2' substituents takes advantage of the differences in the S2' subsites and exhibits selectivity for MMP-2,
MMP-3, MMP-8, MMP-9, and MMP-13 over MMP-1 and MMP-7.\textsuperscript{[39]} Other MPIs have incorporated bulky P2' groups to improve the pharmacokinetic properties of MPIs by shielding amide bonds of the inhibitor from hydrolysis.\textsuperscript{[7, 8, 25, 40]}

The S3' subsite is a solvent exposed region that constitutes the outer rim of the S1' pocket entrance. This subsite has also taken a lesser role in the design of MPIs. Nevertheless, a recent study has shown specificity can be obtained by some P3' substituents. For example, to enhance selectivity of MMP-3 over MMP-2, inhibitors incorporating a-substituted benzylic groups at the P3' position resulted in a 1000-fold decrease in potency against MMP-2, while having little affect on the activity against MMP-3.\textsuperscript{[41, 42]}

The unprimed subsites are more loosely defined and historically have been considered less attractive as targets for selective inhibitor design than the primed subsites. The unprimed region can be described as a more solvent exposed region with less segregation between subsites when compared with the primed subsites. The S2 subsite occupies a region adjacent to the zinc(II) ion, while the S1 and S3 subsites are located further away from the catalytic center. Recent substrate and inhibitor studies have demonstrated the importance of the unprimed subsites. For example, the substrate selectivity between gelatinases (MMP-2 and MMP-9) is partially attributed to the differences in the S2 subsite residues where MMP-2 contains a Glu and MMP-9 contains an Asp in the analogous position. Modeling studies have shown the Glu residue can hydrogen bond with specific substrates while the Asp cannot form these interactions due to the shorter side chain length.\textsuperscript{[43]} Nevertheless, early ‘left-handed’
MPIs showed poor inhibitory activity, which reduced research efforts on the unprimed side of the active site. Although generally less popular, there have been encouraging results demonstrating selectivity can be obtained through left-handed inhibitors.\cite{44} The S1 and S3 subsites have been shown to play a useful role in devising inhibitors for targeting certain MMPs over collagenase (MMP-1).\cite{44} For example, thiadiazole-based inhibitors that target the unprimed subsites demonstrate selective inhibition of MMP-3 ($K_i = 0.018 \ \mu\text{M}$) over MMP-1 (no inhibition).\cite{44}

MPIs have been designed that exploit both the unprimed and primed subsites. A recent example of this type of MPI has yielded potent inhibitors of MMP-13 with selectivity over MMP-1. Substituents designed to interact with the deep S1\textsuperscript{'} pocket of MMP-13 in combination with moieties selected to interact with the S2 subsite produced a greater than 100-fold selectivity for MMP-13 over MMP-1.\cite{45} Clearly the active site subsites have provided a variety of opportunities to design selective inhibitors. However, as described in the following sections, the inhibitor-metal interaction also plays an important role in developing a successful MPI.

1.3. Zinc-Binding Groups of MMP Inhibitors

The primary focus of this section is on the inhibitor-zinc(II) ion interaction, therefore the MPIs discussed will be categorized according to the nature of group used to bind the active site metal ion.\cite{7} Based on this premise, MPIs can be broadly categorized into six groups: hydroxamates, carboxylic acids, thiols, phosphorous-based, other ligands, and natural products. The hydroxamic acid group is
overwhelmingly the most commonly used ZBG in inhibitor design and is generally found to be the most effective. Hydroxamic acids bind the catalytic zinc(II) ion in a bidentate fashion, blocking substrate access to the active site and rendering the metal incapable of peptide hydrolysis. The binding of hydroxamate-based MPIs has been confirmed by X-ray crystallography, which unambiguously displays bidentate coordination with average Zn-O bond lengths of ~2.0 Å (Figure 1-6). To evaluate the coordination geometry of MMPs bound by hydroxamic acid-based MPIs, the parameter \( \tau = (\beta - \alpha)/60 \) was used. \(^{[46]}\) This parameter has been devised for five coordinate metal centers as a ‘degree of trigonality’ between square pyramidal and trigonal bipyramidal structures (Figure 1-7). Perfect trigonal bipyramidal geometry will have a \( \tau \) value of 1.0 \( \alpha = 120^\circ, \beta = 180^\circ \), where perfect square pyramidal structures will have a \( \tau \) value of 0 \( \alpha = \beta = 180^\circ \). Most MMP-inhibitor complexes with hydroxamate-based compounds display a distorted square pyramidal geometry with \( \tau \) values ranging from 0.35 to 0.01. \(^{[8, 25]}\)

The earliest MPIs consisted of a short peptide attached to a ZBG, which has most often been a hydroxamic acid. With the advent of structure-based design, MPIs have been further modified in an effort to obtain greater specificity and potency; however, the majority of these new inhibitors retain the hydroxamic acid ZBG (Figure 1-5).
Figure 1-6. Molecular surface diagram of MMP-3 complexed with a hydroxamic acid inhibitor. The catalytic zinc(II) ion is shown in black; the inhibitor and enzyme are in gray (top). The chemical structure and mode of metal binding of the inhibitor are shown at the bottom. Residue Pro221 is shown as a wireframe surface for clarity.
**Figure 1-7.** Definition of $\tau$ value for a five-coordinate metal complex. The metal center is designated by the label M and the ligands by labels A-E. The angles $\alpha$ and $\beta$ are illustrated; for a perfect square pyramid $\alpha = \beta = 180^\circ$ and for a perfect trigonal bipyramid $\alpha = 120^\circ, \beta = 180^\circ$.

Inhibitors containing carboxylic acid ZBGs have been the most commonly studied group after the hydroxamate-based MPIs.\cite{7} The most apparent reason for the use of carboxylic acids as ZBGs is because these compounds are the synthetic precursors to hydroxamate-based MPIs. If a hydroxamic acid-based MPI has been synthesized and tested against MMPs, often information about the carboxylic acid precursor can also be found. The carboxylate ZBG has been proposed to be a bidentate ligand;\cite{47,48} however, detailed examination suggests that these ZBGs are coordinated in a monodentate fashion to the catalytic zinc(II) ion (Figure 1-8). This conclusion is based upon examination of the Zn-O bond lengths and geometry of the metal centers. Among eight X-ray structures of MMP-inhibitor complexes with carboxylate-based compounds the average Zn-O bond lengths were found to be 1.9 Å ($O_a$, Figure 1-8) and 2.7 Å ($O_b$, Figure 1-8) for the two oxygen atoms, respectively.\cite{48-53} The sum of the covalent radii for oxygen and zinc is only ~2.1 Å, which is
significantly shorter than the reported 2.7 Å ‘bond.’ In addition, a survey of the Cambridge Structural Database (http://www.ccdc.cam.ac.uk/) identified sixteen small molecule structures of zinc coordinated to three nitrogen atoms and a carboxylate; no Zn-O bond distances larger than 2.5 Å are reported, suggesting that longer Zn-O bonds are not true coordinate bonds, or at best, very weak interactions. In the structures that were described as truly five coordinate, the carboxylate oxygen atoms are bound nearly equidistant, contrary to the asymmetric binding found in the MPI complexes.\textsuperscript{54, 55} In addition, examination of the geometry at the metal centers in the MMP-carboxylate MPI structures is most appropriately described as distorted tetrahedral. This supports the contention that the coordination sphere of the zinc(II) ion is essentially unaffected by the more distant oxygen atom, as true bidentate coordination should tend to induce a more standard five-coordinate geometry, such as trigonal bipyramidal or square pyramidal, similar to that found with the hydroxamic acid MPIs discussed above.\textsuperscript{56} Finally, hydrogen bonding is frequently found between the distant carbonyl oxygen atom of the carboxylate ZBG and the protein sidechains,\textsuperscript{49-51, 53} which further reduces the ability of this atom to be strongly coordinated to the zinc(II) ion. As a monodentate ligand, carboxylates should be more weakly bound to the zinc center, due to the loss of a Zn-O bond (relative to hydroxamate-based compounds) and a loss of the chelate effect (vide infra). Consistent with this description of carboxylic acid ZBGs being only monodentate ligands, most carboxylate-based MPIs (with few exceptions) have lower inhibitory activity than their bidentate hydroxamate analogues.\textsuperscript{56}
Figure 1-8. Molecular surface diagram of MMP-3 complexed with a carboxylate inhibitor. The catalytic zinc(II) ion is shown in black; the inhibitor and enzyme are in gray (top). The chemical structure and mode of metal binding of the inhibitor are shown at the bottom. Residues Tyr220, Pro221, and Leu222 are shown as a wireframe surface for clarity.
MPIs that utilize thiol ZBGs have been synthesized and demonstrate, in some cases, inhibition at sub-nanomolar concentrations.\(^{[57-61]}\) Thiol-based MPIs have been of interest due to the apparent thiophilicity of the zinc(II) ion in a number of proteins. Zinc is found coordinated by Cys residues in many proteins including ‘zinc fingers’, liver alcohol dehydrogenase (LADH), and metallothioneins. Greater interest in thiol ZBGs was sparked from the discovery of the ‘cysteine switch’ self-inhibitory mechanism of MMPs (vide supra).\(^{[62]}\) Thiol-containing MPIs use a sulfhydryl group as the lone donating atom or in combination with other donor atoms as in the case of β-mercaptoketones, β-mercaptoalcohols, and β-mercaptoamides. Very few X-ray structures are available for MPI–MMP complexes of thiol-based inhibitors. One of the available structures (Figure 1-9) reveals that the MPI uses the thiol ZBG to bind the zinc(II) ion in a monodentate fashion with a Zn-S distance of 2.2 Å, resulting in a tetrahedral geometry around the metal center.\(^{[63]}\) It has been suggested\(^{[57, 58, 61]}\) that β-mercaptoketones, β-mercaptoalcohols, and β-mercaptoamides bind the MMP zinc(II) ion in a bidentate fashion through the sulfur and oxygen atoms in these ligands. However, no protein structural data for MPIs containing these ZBGs has been reported.
Figure 1-9. Molecular surface diagram of MMP-8 complexed with a thiol inhibitor. The catalytic zinc(II) ion is shown in black; the inhibitor and enzyme are in gray (top). The chemical structure and mode of metal binding of the inhibitor are shown at the bottom. Residue Pro217 is shown as a wireframe surface for clarity.
The discovery that a phosphorous-based compound could inhibit the zinc protease thermolysin prompted efforts to synthesize phosphorous-based MPIs.\textsuperscript{[64]} Despite initial excitement, phosphorous-based MPIs have not shown inhibitory activity comparable to that of hydroxamates. Phosphorous-based inhibitors utilize either phosphonic acid or phosphinic acid groups to bind to the active site zinc(II) ion. Examination of the X-ray crystal structure of a phosphonic acid-based MPI bound to MMP-8 reveals the binding mode of the inhibitor (Figure 1-10).\textsuperscript{[65]} The ZBG is described as binding in a bidentate manner through two of the three oxygen atoms.\textsuperscript{[65]} The Zn-O distances are 1.9 Å and 2.7 Å. Again, based on the long Zn-O bond length monodentate coordination appears to be a more apt description of the binding. Additionally, the coordination geometry of the zinc center in this structure is clearly a distorted tetrahedron and shows little influence from the more distant oxygen atom. The structure of a phosphinic acid MPI has also been obtained (Figure 1-11).\textsuperscript{[66]} The structure of this MPI bound to MMP-11 displays a tetrahedral geometry at the metal center. One phosphinic oxygen atom is bound to the zinc(II) ion at a distance of 2.4 Å. The second phosphinic oxygen is located 2.9 Å from the metal center and is in hydrogen bonding distance to Glu220.
Figure 1-10. Top: Molecular surface of the catalytic domain of MMP-8 complexed with a phosphonic inhibitor. The catalytic zinc is shown in black, the inhibitor and enzyme are in gray scale. Bottom: The chemical structure and mode of metal binding of the inhibitor.
Figure 1-11. Top: Molecular surface of the catalytic domain of MMP-11 complexed with a phosphinic inhibitor. The catalytic zinc is shown in black, the inhibitor and enzyme are in gray scale. Bottom: The chemical structure and mode of metal binding of the inhibitor.
A number of other MPIs have been synthesized that employ ZBGs that do not fall into the any of the previously described categories. The many different functional groups used as ZBGs are listed in Table 1-2 along with the corresponding literature references. Among the many different ZBGs listed, two unconventional MPIs that utilize thiadiazole and 2,4,6-pyrimidine trione (barbituric acid) ZBGs have been structurally characterized bound to their MMP targets. The thiadiazole derived MPIs PNU-142372 and PNU-141803 bind to the active site zinc(II) ion in a monodentate fashion (Figure 1-12) through the sulfur atom of these ZBGs with a Zn-S bond distance of 2.3 - 2.4 Å. The crystal structures of two barbituric acid-based MPIs bound to MMP-3 and MMP-8 reveal their unusual mode of binding (Figure 1-13) where the active site zinc atom is coordinated through the N3 nitrogen atom with a Zn-N bond distance of ~2.1 Å. Of the two reported barbiturate structures, one suggests that the ZBG is bidentate[68] bound through the N3 nitrogen atom and one of the oxygen atoms of the ZBG while the other reference describes the inhibitor as bound in a monodentate fashion. Inspection of the zinc centers in each of these structures indicates that the coordination geometry is best described as distorted tetrahedral. The oxygen atoms of the ZBG are located approximately 3.0 Å away from the zinc(II) ion suggesting they do not coordinate to the metal center. Based on the long Zn-O distance and the tetrahedral coordination geometry these compounds appear to be properly described as monodentate ligands, similar to carboxylate-based ZBGs.
Table 1-2. List of zinc-binding groups (ZBGs) used in MMP inhibitors. The proposed mode of binding, Protein Data Bank (PDB) reference code, and literature citation(s) are also provided.

<table>
<thead>
<tr>
<th>Zinc Binding Group</th>
<th>Structure</th>
<th>Binding Mode</th>
<th>PDB code</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxamic Acid</td>
<td><img src="image1" alt="Hydroxamic Acid Structure" /></td>
<td>Bidentate</td>
<td>1MMQ, 1BIW, 1B3D, 1HFC, 1JAQ, 830C</td>
<td></td>
</tr>
<tr>
<td>Reverse Hydroxamic Acid</td>
<td><img src="image2" alt="Reverse Hydroxamic Acid Structure" /></td>
<td>Bidentate</td>
<td>IGKC, IGKD,</td>
<td></td>
</tr>
<tr>
<td>Carboxylic Acid</td>
<td><img src="image3" alt="Carboxylic Acid Structure" /></td>
<td>Monodentate</td>
<td>1MMP, 1B8Y, 1HFS, 1SLN, 2SRT, 1CGL</td>
<td></td>
</tr>
<tr>
<td>Thiol</td>
<td><img src="image4" alt="Thiol Structure" /></td>
<td>Monodentate</td>
<td>IJAO</td>
<td></td>
</tr>
<tr>
<td>Thio-ketone</td>
<td><img src="image5" alt="Thio-ketone Structure" /></td>
<td>Unknown</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Thio-alcohol</td>
<td><img src="image6" alt="Thio-alcohol Structure" /></td>
<td>Unknown</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Thio-amide</td>
<td><img src="image7" alt="Thio-amide Structure" /></td>
<td>Unknown</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Thiadiazole</td>
<td><img src="image8" alt="Thiadiazole Structure" /></td>
<td>Monodentate</td>
<td>1USN, 2USN, 3USN</td>
<td></td>
</tr>
<tr>
<td>Barbituric Acid</td>
<td><img src="image9" alt="Barbituric Acid Structure" /></td>
<td>Monodentate</td>
<td>IG4K, IJ9</td>
<td>N/A</td>
</tr>
<tr>
<td>Thiobarbituric Acid</td>
<td><img src="image10" alt="Thiobarbituric Acid Structure" /></td>
<td>Unknown</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>Phosphonic Acid</td>
<td><img src="image11" alt="Phosphonic Acid Structure" /></td>
<td>Monodentate</td>
<td>1I73, 1HV5</td>
<td></td>
</tr>
<tr>
<td>Phosphinic Acid</td>
<td><img src="image12" alt="Phosphinic Acid Structure" /></td>
<td>Monodentate</td>
<td>1I73, 1HV5</td>
<td></td>
</tr>
<tr>
<td>Sulfodiimine</td>
<td><img src="image13" alt="Sulfodiimine Structure" /></td>
<td>Monodentate</td>
<td>1MMR</td>
<td></td>
</tr>
<tr>
<td>Thiodiketopiperazine</td>
<td><img src="image14" alt="Thiodiketopiperazine Structure" /></td>
<td>Unknown</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1-12. Top: Molecular surface of the catalytic domain of MMP-3 complexed with a thiadiazole inhibitor. The catalytic zinc is shown in black, the inhibitor and enzyme are in gray scale. Bottom: The chemical structure and mode of metal binding of the inhibitor.
Figure 1-13. Top: Molecular surface of the catalytic domain of MMP-3 complexed with a barbiturate inhibitor. The catalytic zinc is shown in black, the inhibitor and enzyme are in gray scale. Bottom: The chemical structure and mode of metal binding of the inhibitor.
Beyond the many types of synthetic inhibitors that have been explored, various natural products have also been shown to inhibit MMPs. Indeed, the natural products group of inhibitors contains the only clinically approved MPI. The tetracycline antibiotic, Periostat (doxycycline hyclate), is used as an MPI to treat periodontal disease (Figure 1-14, top). Despite modest in vitro inhibition of MMPs, tetracyclines have shown promising anti-tumor potential. The origin of their anti-tumor activity is unclear as tetracyclines can interact with multiple targets in vivo, and may inhibit MMPs directly or by preventing the synthesis or activation of MMPs. No crystal structure of a tetracycline bound to an MMP has been reported; however, studies involving tetracyclines bound to divalent metal ions suggest bidentate coordination through adjacent keto/hydroxyl oxygen atoms. Other natural products found to inhibit MMPs include derivatives of the compound futoenone. Futoenone (Figure 1-14, bottom) is found in the Chinese herbal plant *Piper futokadsura*, which is used to treat inflammatory disease. Futoenone and its derivatives have been studied as MPIs and demonstrate modest inhibitory activity. The mode of binding of these compounds to MMPs is unknown; however, it is proposed that the 2-methoxyphenol moiety in these molecules is responsible for zinc chelation.
**Figure 1-14.** Chemical diagrams of doxycycline (Periostat, top) and a Futoenone derivative (bottom).

### 1.3.1. Limitations of Current Zinc-Binding Groups

Although thousands of MPIs have been synthesized, only Periostat (Figure 1-14) has been approved for therapeutic use and it does not contain a hydroxamate ZBG. One main criticism of the numerous failed MPIs is a lack of specificity and potency resulting in low in vivo activity and unwanted side effects such as musculoskeletal syndrome.\[^{69, 70}\] It has been proposed that inhibition of collagenase (MMP-1) is partially responsible for the development of musculoskeletal pain. However, this pain is observed in patients after treatment with prinomastat (Figure 1-5, compound 2), an MPI that does not inhibit MMP-1.\[^{69}\] This finding suggests that the current method of improving the selectivity of inhibitors for specific MMPs may not completely eliminate the problem of unwanted side effects. In addition, a more alarming study has discovered that the MPI batimastat (Figure 1-5, compound 1) promotes the
metastasis of human breast carcinoma cells in nude mice.\textsuperscript{[71]} It has been suggested that less specific inhibitors may be acceptable when treating cancer if they exhibit potency, but in the remedy of more benign diseases such as arthritis, specific inhibitors with minimal side effects are preferred. Ideally, the development of potent MPIs that are more selective for MMPs over other metalloproteins could reduce the required dosages and minimize the potential side effects.

Attempts to improve the potency and selectivity of MPIs are evidenced by the vast diversity of inhibitor backbones. Efforts placed on developing superior ZBGs are miniscule in comparison. The consensus in the field appears to be that hydroxamic acids are satisfactory metal chelators and that MPI design should focus on the development of more effective backbones. However, an examination of MPI activity with identical backbones, but different ZBGs indicates that both the ZBG and the backbone are essential for obtaining an effective inhibitor, and therefore, adequate efforts should be placed in the development of improved ZBGs. Despite the popularity of hydroxamic acids as a ZBG, there are significant limitations associated with its use in MPIs. Hydroxamic acids are vulnerable to rapid excretion and in vivo hydrolysis; in a study of 5-lipoxygenase inhibitors, hydroxamic acid-based inhibitors were found to rapidly hydrolyze to the corresponding carboxylic acid, which is a significantly weaker ZBG (vide supra).\textsuperscript{[72, 73]} The metal binding selectivity that can be obtained through the ZBGs is a largely overlooked area in MPI development, which is surprising considering that hydroxamic acids have poor selectivity for zinc(II) ions over other divalent first row transition metals. Hydroxamic acids bind tightly to a
variety of metal ions in several oxidation states as evidenced by their widespread use in bacterial siderophores where they act as strong iron(III) chelators.\textsuperscript{[74]} Indeed, the only medically approved chelator for iron overload is the tris(hydroxamate) siderophore desferrioxamine (DFO).\textsuperscript{[75]} In terms of hard/soft acid-base chemistry, the zinc(II) ion is often classified as intermediate and is generally regarded as softer than metals such as iron(III) or manganese(II).\textsuperscript{[76, 77]} In addition to the low selectivity of hydroxamates for various metal ions, the carbon-nitrogen bond in these compounds can undergo a \textit{cis} to \textit{trans} conformational change (Figure 1-15), which reduces its affinity for binding to all metal ions, relative to more rigid ligands.

![Figure 1-15. Cis- to trans- isomerization in hydroxamic acid compounds.](image)

The alternative ZBGs investigated to date, such as carboxylates, thiols, phosphinates, and reverse hydroxamic acids have other limitations. The most significant drawback for these ZBGs is a generally lower affinity for zinc(II) ions than hydroxamic acids. Only thiols are nearly as potent as hydroxamic acids in MPIs, but free thiols suffer from poor pharmokinetic properties. Thiols are known to be unstable in the body due to oxidation and formation of disulfide bonds with exposed Cys residues of various proteins.\textsuperscript{[78]}
1.3.2. Considerations for Improving Zinc-Binding Groups

The discovery of superior ZBGs is expected to benefit the field of MPI development by improving several properties of the inhibitor. First and foremost, an improved ZBG should have a higher affinity for the zinc(II) ion than hydroxamates. As previously discussed, the majority of alternative ZBGs that have been studied have a lower affinity for zinc relative to hydroxamates. Evidence suggests the zinc(II) ion is thiophilic. Therefore, a rational choice for a high affinity ZBG would include sulfur donor atoms; free thiols pose pharmacological problems (vide supra), therefore, less reactive sulfur-containing groups (as found in thiadiazole-based MPIs) should be a suitable alternative to introduce sulfur atoms into the ZBG. A second consideration in designing a novel ZBG should be its mode of binding. According to the chelate effect, the stability of a metal complex can be increased by the binding of two or more donor atoms from the same ligand due to both favorable entropic and enthalpic contributions. Therefore, an effective ZBG should bind in a truly multidentate (bidentate) fashion to obtain enhanced kinetic and thermodynamic stability. The use of ZBGs that have improved in vivo stability will also augment the efficacy of MPIs. Also, by following the example of TIMP-MMP interactions, a rigid ZBG will position the coordinating atoms in a fixed orientation that is predisposed for metal binding, preventing the cis to trans isomerization observed in hydroxamic acids (Figure 1-15).

Through these improvements, novel ZBGs have the potential to increase the in vivo potency, stability, and selectivity of MPIs. To aid in the development of improved ZBGs, a method to evaluate the efficacy of new ZBGs before the synthesis
of an entire MPI should be devised. Such an approach will decrease the time of evaluation, as the preparation of small molecule zinc chelators promises to be easier and more rapid than the synthesis of entire MMP inhibitors.

1.4. Strategies for Identifying Improved Zinc-Binding Groups

Few attempts have been made to systematically improve the ZBG component of MMP inhibitors. One approach that has been applied is known as ‘SAR by NMR’ – Structure Activity Relationship by Nuclear Magnetic Resonance.\textsuperscript{[79]} SAR by NMR was first used to identify ligands for FK506 binding protein (FKBP), and was later developed as a broadly applicable technique to examine and improve biomolecule-drug interactions.\textsuperscript{[79]} In SAR by NMR the small molecule-protein interaction is evaluated by monitoring changes in the $^1\text{H}/^{15}\text{N}$-amide chemical shifts of the protein backbone in a two-dimensional $^{15}\text{N}$-heteronuclear single-quantum correlation ($^{15}\text{N}$-HSQC) experiment. Using $^{15}\text{N}$-HSQC, the affinity of a ligand for a particular binding site can be determined. When a high affinity compound is identified for a given site, additional $^{15}\text{N}$-HSQC spectra can be obtained to evaluate a second ligand that occupies an adjacent binding site. Spectra are taken of the second compound and protein together and also in the presence of the first high-affinity molecule. Once the best combination of compounds is found, a synthetic strategy is devised to link them together (Figure 1-16). Through this process, SAR by NMR can identify optimal chemical moieties for specific sites on a protein that when combined generate high affinity inhibitors.\textsuperscript{[80]}
SAR by NMR was first applied to MMP inhibition to identify new backbones for hydroxamate-based inhibitors of MMP-3. Focusing on hydroxamic acid-based MPIs, acetohydroxamic acid was chosen as the ligand for the first binding site as an appropriate model for the ZBG. Using acetohydroxamic acid, SAR by NMR was then performed to develop a potent inhibitor backbone focusing on the S1' subsite as the main secondary interaction. Biphenyl-derived backbones were selected to interact with the deep, hydrophobic S1' subsite. The study identified preferential trends for the S1' subsite. Groups such as carboxylic acids and aliphatic rings resulted in decreased affinity for the enzyme (Figure 1-16). The addition of cyano groups in the para or meta position increased affinity for MMP-3 ten-fold over a simple biaryl group. The most effective backbone components were synthesized and combined with the hydroxamic acid moiety, resulting in potent inhibitors with nanomolar IC$_{50}$ values.
Figure 1-16. Simplified scheme for the Structure Activity Relationship by Nuclear Magnetic Resonance (SAR by NMR) drug discovery method (left). Chemical structure and binding affinity ($K_D$) of several biaryl backbone substituents examined by using the SAR by NMR method (right).

Although SAR by NMR had successfully identified high-affinity biaryl-substituted MPIs, these hydroxamate-based compounds suffered from poor oral bioavailability attributed to the ZBG. Therefore, SAR by NMR was further utilized in an attempt to identify novel ZBGs with improved pharmokinetics that could exploit the biaryl substituentents identified in the earlier study. In order to find alternate ZBGs, a variety of non-hydroxamate and hydroxamate derivatives were evaluated against MMP-3.\[81] The compounds studied included thiols, trifluoromethyl ketones, salicylates, several substituted aromatic compounds, as well as hydroxamate
derivatives (Figure 1-17). As described above, the affinities of these small molecule ZBGs were assessed based on changes in the chemical shifts of the $^{15}$N HSQC spectrum upon binding of each small molecule. In this manner, SAR by NMR was used to systemically examine these small molecules and to rank the relative affinity of these compounds for the zinc active site. Acetohydroxamic acid, representative of a hydroxamate-based MPI, was used as a benchmark compound to which the other small molecules were compared.

![Chemical structures and binding affinities of ZBGs](image)

**Figure 1-17.** Chemical structure and binding affinity ($K_D$) of several ZBGs examined by using the SAR by NMR drug discovery method.

These experiments successfully identified two ZBGs that bound MMP-3 with at least 100-fold higher affinity than acetohydroxamic acid (Figure 1-17, AHA). One of these
compounds was the thiol-containing compound 2-thenylmercaptan (Figure 1-17, compound 1); the second was a hydroxamate derivative, naphthylhydroxamic acid (Figure 1-17, compound 8). The 2-thenylmercaptan ligand was rejected based on its propensity to react in vivo with proteins other than MMPs;\textsuperscript{[82]} therefore, the naphthylhydroxamic acid compound was pursued as a new ZBG.

Although SAR by NMR is an effective method for determining relative binding, the approach does not provide a detailed determination of the binding conformation for each small molecule. Additional NMR experiments were required to demonstrate direct evidence of zinc binding and the overall orientation of these molecules in the active site. An NOE-based structure of naphthylhydroxamic acid bound to MMP-3 revealed that the naphthyl portion of the ligand interacts with the enzyme backbone, specifically through hydrophobic interactions with residues Phe86, Tyr155, Val163, Tyr168, and His205. The greater affinity of naphthylhydroxamic acid versus acetohydroxamic acid for MMP-3 is attributed to this hydrophobic interaction. This hydrophobic interaction also explained the affinity of phenylhydroxamic acid (Figure 1-17, compound 7) for MMP-3, which was higher than acetohydroxamic acid but lower than the naphthyl derivative. The experiment, therefore, did not unearth a stronger metal-ZBG interaction, but instead has discovered an additional non-covalent (backbone-like) interaction between the bulky naphthyl group and the protein. In addition to increased affinity for MMP-3, the incorporation of the bulky naphthyl group provided steric protection to the hydroxamic acid and reduced ZBG hydrolysis normally observed with hydroxamate-based MPIs.
Ultimately, these results show SAR by NMR can be a useful tool in the discovery of improved ZBGs.

Another approach for evaluating ZBGs is through the use of small molecule model complexes of MMPs. Model complexes have been used to study the structure and reactivity of numerous zinc metalloprotein active sites.\textsuperscript{[83]} In contrast to studying proteins, model complexes are easily synthesized and can be obtained in large quantities. The organic ligands that comprise these complexes are more amenable to modification than proteins. Vahrenkamp and co-workers established the use of model complexes to examine MMP inhibition. These studies utilized tris(pyrazolyl)borate complexes of zinc(II) to provide a model for the tris(histidine) active sites of MMPs.\textsuperscript{[84]} The model complex [(Tp*)Zn(OH)] (Tp* = hydrotris(5,3-methylcumylpyrazolyl)borate) was combined with acetohydroxamic acid to form a complex (Figure 1-18) that is structurally identical to the coordination environment of the MMP active site when hydroxamate-based drugs are bound to the catalytic zinc(II) ion. In an overlay comparison, the corresponding atoms of the model complex (zinc, coordinated pyrazole nitrogen atoms, and acetohydroxamic acid) and a protein X-ray crystal structure of an MMP with a hydroxamate-based MPI bound (active site zinc, coordinating histidine nitrogen atoms, and ZBG of inhibitor) superimpose to within an RMSD of 0.2 Å. In addition, the hydroxamate inhibitor ZINCOV was complexed with [(Tp*)Zn(OH)] to generate a model compound that again accurately reproduced the inhibited MMP active site.\textsuperscript{[84]} Ultimately, these compounds were not further utilized in MPI design as it was suggested that model complexes were not suitable for
developing MPIs due to the significance of backbone interactions within the active site that are not accounted for in the models.

![Chemical and crystallographic structures](image)

**Figure 1-18.** Chemical (left) and crystallographic (right) structure for [(Tp*)Zn(acetohydroxamate)].

### 1.5. Objectives

Our research has focused on the development of non-hydroxamate MPIs. It was decided that the small molecule models described above would be an excellent platform for the examination of the interaction between ZBGs and the catalytic zinc center in MMPs. Therefore, the active site model \([(\text{Tp}^{\text{Ph,Me}})\text{Zn(OH)}]\) (\text{Tp}^{\text{Ph,Me}} = \text{hydrotris}(3,5-\text{phenylmethylpyrazolyl})\text{borate}) was synthesized. The structural examination of novel ZBGs bound to the model complex was performed, allowing for the evaluation of binding modes, bond lengths and potential orientations in the MMP active site. Once novel ZBGs showed promise in the model complex, the compounds were evaluated for potency against MMPs and cellular toxicity in standard biological assays. A computational method was developed to design MPIs using the non-hydroxamate ZBGs. The most promising full-length MPIs generated in silico were
synthesized and evaluated for their inhibitory potency against MMPs. This bioinorganic approach to MMP inhibition has yielded the first pyrone-based MPIs. In fact, the novel MPIs developed are more potent than their hydroxamate analogues.

1.6. Acknowledgements

Text, schemes, and figures of this chapter, in part, are reprints of the materials published in the following paper: Puerta, D.T.; Cohen, S.M. "A Bioinorganic Perspective on Matrix Metalloproteinase Inhibition" Curr. Top. Med. Chem. 2004, 4, 1551-1573. The dissertation author was the primary researcher and author. The permissions to reproduce this paper was granted by Benthan Science Publishers Ltd. Copyright 2004, Benthan Science Publishers Ltd.

1.7. References


Chapter 2. The Use of Tris(pyrazoyl)borate Model Complexes to Examine the Binding Mode of Known ZBGs
2.1. Introduction

As described earlier, the use of tris(pyrazolyl)borate and tris(pyrazolyl)methane complexes and their derivatives to model the active site of an number of metalloenzymes has been well established by the work of Vahrenkamp,[1, 2] Parkin,[3, 4] Trofimenko,[5, 6] and others[7, 8] (Figure 2-1) with Trofimenko coining the term “scorpionate ligands”. These systems have been applied to the modeling of several protein active sites including carbonic anhydrase,[9] liver alcohol dehydrogenase,[2] and various peptidases.[3] The tris(pyrazolyl)borate derivatives have been used by Vahrenkamp and Parkin to model the active site of matrix metalloproteinases, however we sought to use them in a previously unexplored fashion: to elucidate matrix metalloproteinase-drug interactions.[10, 11]

![Figure 2-1. Homoscorpionate ligands tris(pyrazolyl)borate (left) and tris(pyrazolyl)methane (right).](image)

As described previously, MMP inhibitors (MPIs) use a two-fold strategy: the compounds chelate the active site zinc ion with a zinc-binding group (ZBG), while providing non-covalent interactions within the MMP active site through a peptidomimetic backbone.[12, 13] The most commonly used ZBG in MPIs is the hydroxamic acid moiety. Numerous X-ray structures of hydroxamate-based MPIs
bound to MMPs have allowed for detailed information as to the binding mode of the hydroxamate ZBG.\textsuperscript{[13, 14]} Several other ZBGs have been incorporated into MMP inhibitors, including carboxylic acids, phosphates, and thiols.\textsuperscript{[12, 15, 16]} Whereas these ZBGs are not as commonly used, their binding modes with the MMP zinc(II) ion has not been well studied.

To examine both the utility of the tris(pyrazolyl)borate active site model complexes and the binding mode of several MPIs, we have synthesized the zinc complex \([\text{Tp}^{\text{Ph,Me}}\text{ZnOH}]\) \((\text{Tp}^{\text{Ph,Me}} = \text{hydrotris}(3,5\text{-phenylmethylpyrazolyl})\text{borate})\) as a model of the MMP active site. Second, the complex \([\text{Tp}^{\text{Ph,Me}}\text{Zn}(\text{acetohydroxamate})]\) was prepared from \([\text{Tp}^{\text{Ph,Me}}\text{ZnOH}]\) as a structural model for the binding of the numerous hydroxamate-based MPIs to the zinc(II) ion. Finally, again using \([\text{Tp}^{\text{Ph,Me}}\text{ZnOH}]\), we synthesized a series of complexes that are structural models of the binding of thiol-based MPIs to the catalytic zinc(II) ion. The thiol-based compounds examined are known to inhibit MMPs; however, the binding modes of these ZBGs to the catalytic zinc(II) ion are not known.\textsuperscript{[17-19]} With the use of simple model complexes, the binding modes of the MPIs have been elucidated. Additionally, there is a direct correlation between the mode of binding (bidentate or monodentate) and the efficacy of the inhibitor. The data presented here establish that the more potent \(\beta\)-mercaptoketone and \(\beta\)-mercaptoamide MPIs bind the zinc(II) ion in a bidentate fashion, while the less potent \(\beta\)-mercaptoalcohol-based MPIs bind exclusively in a monodentate manner, contrary to previous suggestions (vide infra).\textsuperscript{[18, 19]}
2.2. Results

2.2.1 Synthesis of [(Tp^{Ph,Me})ZnOH]

The foundation of this study is a small molecule model complex of the MMP active site. According to literature procedures, we synthesized [(Tp^{Ph,Me})ZnOH] in three steps from the commercially available starting materials: 1-Benzoylaceton, hydrazine monohydrate, potassium borohydride, and zinc(II)perchlorate (Scheme 2-1).\[^{10,11,20}\] 1-Benzoylaceton was combined with hydrazine monohydrate in toluene to produce 3,5-phenylmethylypyrazole. Three equivalents of the asymmetric pyrazole was then reacted with potassium borohydride in a melt to yield potassium hydrotris(3,5-phenylmethylypyrazolyl)borate (Tp^{Ph,Me}K). Finally, the desired zinc complex was synthesized by reacting one equivalent of Zn(ClO\(_4\))\(_2\)·6H\(_2\)O with (Tp^{Ph,Me}K). Upon crystallization of the [(Tp^{Ph,Me})ZnOH] complex a portion of the material crystallized in a hydrogen-bonded dimer (Table 2-1), in which a lone hydrogen links two [(Tp^{Ph,Me})ZnOH] complexes through their respective hydroxyl groups (Figure 2-2). This unique bonding resembles that found in the native protein, as an activated water molecule bound to the catalytic zinc(II) ion is hydrogen-bonded to an adjacent glutamate residue prior to substrate hydrolysis.\[^{12,21}\] The hydroxide proton refines to an O1-H1 bond distance of 0.733 Å. The bridging hydrogen was found to have an O1-H100 distance of 1.205 Å. This distance is comparable to those reported in other H\(_3\)O\(_2\) species that have been structurally characterized.\[^{20,22-25}\] While the dimer was observed in the solid state, solution \(^1\)HNMR is distinctly that of the monomeric species.
Scheme 2-1. Synthesis of [(Tp\textsuperscript{Ph,Me})ZnOH]. a) H\textsubscript{2}NNH\textsubscript{2}-H\textsubscript{2}O, Toluene, 140 °C 3 h, 91%; b) KBH\textsubscript{4}, neat, 240 °C, 2 h, 75%; c) Zn(ClO\textsubscript{4})\textsubscript{2}-6H\textsubscript{2}O, KOH, CH\textsubscript{2}Cl\textsubscript{2}, MeOH, RT, 16 h, 78%.
Figure 2-2. Structural diagrams of asymmetric unit [(Tp²Ph,Me)²ZnOH] (top) and [(Tp²Ph,Me)²Zn₂(H₂O₂)] dimer (bottom) with partial atom numbering schemes (ORTEP, 50% probability ellipsoids). The perchlorate counterion, solvent molecules, and selected hydrogen atoms have been omitted for clarity.
2.2.2. [(Tp$^{\text{Ph,Me}}$)Zn(acetohydroxamate)]

In order to demonstrate that the ligand, [(Tp$^{\text{Ph,Me}}$)ZnOH] provided an adequate model for the MMP active site, the complex was combined with acetohydroxamic acid in a methanol/methylene chloride mixture to obtain [(Tp$^{\text{Ph,Me}}$)Zn(acetohydroxamate)]. The reaction mixture was evaporated to dryness and dissolved in benzene. Crystals suitable to X-ray diffraction were grown from the benzene solution diffused with pentane. As in similar structures,[10, 26] [(Tp$^{\text{Ph,Me}}$)Zn(acetohydroxamate)] displays a five-coordinate zinc center (Table 2-1) bound by the three pyrazole nitrogen atoms of the Tp$^{\text{Ph,Me}}$ ligand and the two oxygen atoms of the hydroxamate in what is best described as a distorted trigonal bipyramidal environment ($\tau = 0.78$)$^{[27]}$ (Figure 2-3) with the carbonyl oxygen and a pyrazole nitrogen occupying the axial positions. The acetohydroxamate ligand is bound in a bidentate manner with Zn-O distances of 1.98 Å (N-O) and 2.10 Å (C=O), similar to the distances described in early studies.$^{[10]}$ An overlay of the structure of the zinc center in [(Tp$^{\text{Ph,Me}}$)Zn(acetohydroxamate)] indicates, as found in similar complexes,$^{[10]}$ that the coordination geometry is very similar to that found in the structure of the active site zinc(II) ion in MMPs inhibited by hydroxamate MPIs, as determined by the protein-MPI X-ray crystal structure.$^{[26]}$ An overlay of the hydroxamate ZBG, zinc(II) ion, and coordinating nitrogens from [(Tp$^{\text{Ph,Me}}$)Zn(acetohydroxamate)] with that of MMP-13 complexed with the hydroxamate-based MPI ($N'-(2$- hydroxamatemethylene-4-methylpentoyl)phenylalanyl)methylamine,$^{[26]}$ results in a RMS deviation of 0.372 Å (Figure 2-4). The overlay of the two coordination environments around the zinc(II) ions indicates, as anticipated, that [(Tp$^{\text{Ph,Me}}$)Zn(ZBG)] complexes provide a good model for
the binding modes of MPIs. This prompted the use of similar complexes to determine the interaction of inhibitors with MMPs where the mode of binding was not known.\textsuperscript{[17-19]}

Figure 2-3. Structural diagram of \([\text{Tp}^{\text{Ph,Me}}\text{Zn(acetohydroxamate)}]\) with partial atom numbering schemes (ORTEP, 50\% probability ellipsoids). Hydrogen atoms and solvent have been omitted for clarity.

Figure 2-4. An overlay of all nine atoms (one zinc, two oxygen, two carbon, and four nitrogen atoms) from \([\text{Tp}^{\text{Ph,Me}}\text{Zn(acetohydroxamate)}]\) (blue) with that of MMP-13 complexed with the hydroxamate-based MPI (\(\text{N-(2-hydroxamatemethylene-4-methylpentoyl)phenylalanyl)methylamine}\) (red). The RMS deviation is 0.372 Å.
Table 2-1. Crystal data for [(Tp^{Ph,Me})_2Zn_2(H_3O_2)]ClO_4 and [(Tp^{Ph,Me})Zn(acetohydroxamate)].

<table>
<thead>
<tr>
<th></th>
<th>[(Tp^{Ph,Me})_2Zn_2(H_3O_2)]ClO_4</th>
<th>[(Tp^{Ph,Me})Zn(acetohydroxamate)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empirical Formula</td>
<td>ZnC_{42}H_{41.5}N_6O_3BCl_{0.5}</td>
<td>C_{37}H_{46}BN_8O_4Zn</td>
</tr>
<tr>
<td>Crystal System</td>
<td>Monoclinic</td>
<td>Monoclinic</td>
</tr>
<tr>
<td>Space Group</td>
<td>P2_1/c</td>
<td>C2/c</td>
</tr>
<tr>
<td>Unit Cell dimensions</td>
<td>\begin{align*} a &amp;= 13.814(2) \text{ Å} \ \alpha &amp;= 90^\circ \ b &amp;= 13.572(2) \text{ Å} \ \beta &amp;= 107.322(2)^\circ \ c &amp;= 21.506(3) \text{ Å} \ \gamma &amp;= 90^\circ \end{align*} &amp; \begin{align*} a &amp;= 40.321(2) \text{ Å} \ \alpha &amp;= 90^\circ \ b &amp;= 16.979(1) \text{ Å} \ \beta &amp;= 105.043(1)^\circ \ c &amp;= 10.821(1) \text{ Å} \ \gamma &amp;= 90^\circ \end{align*}</td>
<td></td>
</tr>
<tr>
<td>Volume, Z</td>
<td>3848.9(8) \text{ Å}^3, 4</td>
<td>7154.3(6) \text{ Å}^3, 8</td>
</tr>
<tr>
<td>Crystal size</td>
<td>0.50 \times 0.50 \times 0.40 \text{ mm}</td>
<td>0.2 \times 0.1 \times 0.05 \text{ mm}</td>
</tr>
<tr>
<td>Temperature (K)</td>
<td>100(2)</td>
<td>100(1)</td>
</tr>
<tr>
<td>Reflections collected</td>
<td>32125</td>
<td>30093</td>
</tr>
<tr>
<td>Independent reflections</td>
<td>8710 [R(int) = 0.0239]</td>
<td>8088 [R(int) = 0.0235]</td>
</tr>
<tr>
<td>Data/restraints/parameters</td>
<td>8710 / 0 / 514</td>
<td>8088 / 0 / 478</td>
</tr>
<tr>
<td>Goodness-of-fit on F^2</td>
<td>1.044</td>
<td>1.088</td>
</tr>
<tr>
<td>Final R indices l&gt;2σ(l)</td>
<td>\begin{align*} R1 &amp;= 0.0383 \ wR2 &amp;= 0.1000 \end{align*} &amp; \begin{align*} R1 &amp;= 0.0298 \ wR2 &amp;= 0.0807 \end{align*}</td>
<td></td>
</tr>
<tr>
<td>R indices (all data)</td>
<td>\begin{align*} R1 &amp;= 0.0448 \ wR2 &amp;= 0.1042 \end{align*}</td>
<td>\begin{align*} R1 &amp;= 0.0340 \ wR2 &amp;= 0.0870 \end{align*}</td>
</tr>
<tr>
<td>Largest peak/hole difference</td>
<td>1.390 / -0.481 e Å^3</td>
<td>0.418 / -0.231 e Å^3</td>
</tr>
</tbody>
</table>
Since little structural information was known about the mode of binding for thiol-based MPIs namely β-mercaptoketones, \[^{18, 19}\] β-mercaptoamides, \[^{17}\] and β-mercaptoalcohols, \[^{18, 19}\] we sought to synthesize a number of complexes to investigate these compounds. Figure 2-5 shows representative examples of the inhibitors that were studied here and the thiol compounds used to model their zinc binding. All of the model complexes were prepared in an identical fashion as \([\text{Tp}^{\text{Ph,Me}}\text{Zn(acetohydroxamate)}]\) from commercially available thiols, by combining one equivalent of the thiol (Figure 2-5, right) with \([\text{Tp}^{\text{Ph,Me}}\text{Zn(OH)}]\). All of the \([\text{Tp}^{\text{Ph,Me}}\text{Zn(ZBG)}]\) complexes were characterized by elemental analysis, \(^1\text{H}/^{13}\text{C}\) NMR, and X-ray crystallography.

### 2.2.3. Examination of β–Mercaptoketone Based MPIs

The coordination of β-mercaptoketone-based MPIs was evaluated by using 3-mercapto-2-butanone as the ZBG. The structure of \([\text{Tp}^{\text{Ph,Me}}\text{Zn(3-mercapto-2-butanonate)}]\) (Table 2-2) shown in Figure 2-6 reveals that this ZBG binds the zinc atom in a bidentate fashion, through the sulfur and carbonyl oxygen donor atoms. Only one isomer of the 3-mercapto-2-butanonate ligand is shown in Figure 2-6, although the crystal structure possessed a partial occupancy disorder with both the \(R\)- and \(S\)-isomers bound to the zinc(II) ion (Figure 2-11). The coordination environment around the zinc center can be described as distorted trigonal bipyramidal (\(\tau = 0.61\)) with the oxygen donor and one of the pyrazole nitrogens occupying the axial positions of the coordination sphere. The Zn-S bond length is 2.27 Å and the Zn-O bond length is 2.33 Å, demonstrating strong bidentate coordination to the metal center. The
strongly coordinating sulfur atom decreases the Lewis acidity of the zinc center, resulting in a carbonyl Zn-O distance longer (0.23 Å) than observed in the [(Tp\textsuperscript{Ph,Me})Zn(acetohydroxamate)] complex (Figure 2-3). Additionally, the result of the sulfur coordination is a less symmetric bonding geometry as evidenced by the smaller tau value.

**Figure 2-5.** List of representative MPIs (left) and the corresponding small molecules (right) used to model zinc binding. MPI classes from top to bottom: hydroxamates, β-mercaptoketones, β-mercaptoamides, and β-mercaptoalcohols.
Figure 2-6. Structural diagram of [(Tp_{Ph,Me})Zn(3-mercapto-2-butanonate)] with partial atom numbering schemes (ORTEP, 50% probability ellipsoids). Hydrogen atoms and one isomer (partial occupancy disorder, C33B/C34B) have been omitted for clarity.

2.2.4. Examination of β–Mercaptoamide Based MPIs

The β-mercaptoamide-based MPIs were evaluated by using N-methylmercaptoacetamide as the ZBG. The structure of [(Tp_{Ph,Me})Zn(N-methylmercaptoacetamidate)] is shown in Figure 2-7. This complex reveals that the β-mercaptoamide ligand binds the zinc atom in a bidentate fashion as well, utilizing the sulfur and amide oxygen atoms. Earlier work on mercaptoacetyl-derived MPIs had suggested that this ZBG would bind in a bidentate fashion; however, no structural evidence was presented to support this hypothesis.\(^{[17]}\) The coordination environment around the zinc center is more distorted toward square pyramidal than that found in [(Tp_{Ph,Me})Zn(3-mercapto-2-butanonate)] (\(\tau = 0.50\)). The Zn-S distance (2.28 Å) is nearly identical to that found in [(Tp_{Ph,Me})Zn(3-mercapto-2-butanonate)] and the Zn-O
distance (2.26 Å) is slightly shorter, possibly due to the increased donating ability of an amide oxygen versus a keto oxygen atom.

**Figure 2-7.** Structural diagram of [(Tp\textsuperscript{Ph,Me}\textsubscript{Zn(N-methylmercaptoacetamidate)}]) with partial atom numbering schemes (ORTEP, 50% probability ellipsoids). Hydrogen atoms and solvent have been omitted for clarity.
<table>
<thead>
<tr>
<th></th>
<th>[(Tp^Ph,Me)Zn(3-mercapto-2-butanonate)]</th>
<th>[(Tp^Ph,Me)Zn(N-methylmercaptoacetamidate)]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Empirical Formula</strong></td>
<td>C_{34}H_{36}BN_6OSZn</td>
<td>C_{39}H_{40}BN_7OSZn</td>
</tr>
<tr>
<td><strong>Crystal System</strong></td>
<td>Monoclinic</td>
<td>Triclinic</td>
</tr>
<tr>
<td><strong>Space Group</strong></td>
<td>P2/c</td>
<td>P-1</td>
</tr>
<tr>
<td><strong>Unit Cell dimensions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>10.148(1) Å</td>
<td>11.944(2) Å</td>
</tr>
<tr>
<td>α</td>
<td>90°</td>
<td>69.039°</td>
</tr>
<tr>
<td>b</td>
<td>10.942(1) Å</td>
<td>12.241(2) Å</td>
</tr>
<tr>
<td>β</td>
<td>98.793(1)°</td>
<td>85.523°</td>
</tr>
<tr>
<td>c</td>
<td>28.807(1) Å</td>
<td>14.909(3) Å</td>
</tr>
<tr>
<td>γ</td>
<td>90°</td>
<td>62.603°</td>
</tr>
<tr>
<td><strong>Volume, Z</strong></td>
<td>3161.0(3) Å^3, 4</td>
<td>1797.3(5) Å^3, 2</td>
</tr>
<tr>
<td><strong>Crystal size</strong></td>
<td>0.3 × 0.2 × 0.1 mm</td>
<td>0.1 × 0.04 × 0.02 mm</td>
</tr>
<tr>
<td><strong>Temperature (K)</strong></td>
<td>100(1)</td>
<td>100(1)</td>
</tr>
<tr>
<td><strong>Reflections collected</strong></td>
<td>26142</td>
<td>15756</td>
</tr>
<tr>
<td><strong>Independent reflections</strong></td>
<td>7116 (R(int) = 0.0253)</td>
<td>8038 (R(int) = 0.0519)</td>
</tr>
<tr>
<td>**Data/restraints/paramet</td>
<td>7116 / 0 / 422</td>
<td>8038 / 0 / 463</td>
</tr>
<tr>
<td><strong>ers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Goodness-of-fit on F^2</strong></td>
<td>1.068</td>
<td>1.024</td>
</tr>
<tr>
<td><strong>Final R indices I&gt;2σ(I)</strong></td>
<td>R1 = 0.0405</td>
<td>R1 = 0.0604</td>
</tr>
<tr>
<td></td>
<td>wR2 = 0.1063</td>
<td>wR2 = 0.1196</td>
</tr>
<tr>
<td><strong>R indices (all data)</strong></td>
<td>R1 = 0.0458</td>
<td>R1 = 0.0960</td>
</tr>
<tr>
<td></td>
<td>wR2 = 0.1093</td>
<td>wR2 = 0.1328</td>
</tr>
<tr>
<td><strong>Largest peak/hole</strong></td>
<td>0.726 / -0.754 e Å^3</td>
<td>0.981 / -0.519 e Å^3</td>
</tr>
</tbody>
</table>

**Table 2-2.** Crystal data for [(Tp^Ph,Me)Zn(3-mercapto-2-butanonate)] and [(Tp^Ph,Me)Zn(N-methylmercaptoacetamidate)].
### 2.2.5. Examination of β–Mercaptoalcohol Based MPIs

The β-mercaptoalcohol-based MPIs were the final class of compounds examined in this study. The thiol-based inhibitors were evaluated by using 3-mercapto-2-butanol, 3-mercapto-2-propanol, and β-mercaptoethanol as ZBGs. All three complexes, shown in Figure 2-8, Figure 2-9, and Figure 2-10 demonstrate that these ligands bind in a monodentate fashion, exclusively through the sulfur atom (Table 2-3). The binding of three different β-mercaptoalcohols was performed to unambiguously confirm the monodentate coordination mode. The three zinc centers are clearly tetrahedral, with average Zn-N bond distances of 2.07(2) Å and Zn-S distance of 2.22(1) Å. Additionally, the potential coordinating oxygen atoms are positioned away (average Zn-O distance of 5 Å) from the zinc(II) ion.

In contrast to 3-mercapto-2-butanol and N-(methyl)mercaptoacetamide, the β-mercaptoalcohol compounds bind only in a monodentate fashion. The difference in coordination behavior is likely the result of two distinct factors. First, unlike 3-mercapto-2-butanol and N-(methyl)mercaptoacetamide, the β–mercaptoalcohols are not conformationally restricted at the carbon alpha to the oxygen donor. Because 3-mercapto-2-butanol and N-(methyl)mercaptoacetamide have sp² carbonyl carbon atoms beta to the sulfur donor atoms, the ligand has less overall flexibility making it poised for bidentate coordination through the oxygen donors in a manner similar to that observed for the hydroxamate compounds. The second reason why the β–mercaptoalcohols bind in a monodentate fashion is due to the reduced Lewis acidity of the zinc center upon sulfur binding. The deprotonated thiol sulfur atom is a strong
Lewis base that significantly reduces the Lewis acidity of the zinc ion. Coupled with
the conformational freedom of the \( \beta \)-mercaptoalcohols, the zinc center is not
sufficiently electrophilic to deprotonate the alcohol oxygen atom thereby acquiring
another strong ligand. Therefore, the conformationally unrestricted protonated alcohol
donor remains a very weak ligand and does not bind to the zinc center.

\textbf{Figure 2-8.} Structural diagram of \( [(\text{Tp}^{\text{Ph,Me}})\text{Zn}(3\text{-mercapto-2-butanoate})] \) with
partial atom numbering schemes (ORTEP, 50\% probability ellipsoids). Hydrogen atoms and
three isomers (partial occupancy disorder) have been omitted for clarity.
Figure 2-9. Structural diagram [(Tp$^{Ph,Me}$)Zn(3-mercapto-2-propanoate)] with partial atom numbering schemes (ORTEP, 50% probability ellipsoids). Hydrogen atoms and solvent have been omitted for clarity.

Figure 2-10. Structural diagram of [(Tp$^{Ph,Me}$)Zn(2-mercaptoethanoate)] with partial atom numbering schemes (ORTEP, 50% probability ellipsoids). Hydrogen atoms and solvent have been omitted for clarity.
Table 2-3. Crystal data for [(Tp\textsuperscript{Ph,Me}Zn(2-mercaptoethanoate)], [(Tp\textsuperscript{Ph,Me}Zn(3-mercapto-2-propanoate)], and [(Tp\textsuperscript{Ph,Me}Zn(3-mercapto-2-butanoate)].

<table>
<thead>
<tr>
<th></th>
<th>[(Tp\textsuperscript{Ph,Me}Zn(2-mercaptoethanoate)]</th>
<th>[(Tp\textsuperscript{Ph,Me}Zn(3-mercapto-2-propanoate)]</th>
<th>[(Tp\textsuperscript{Ph,Me}Zn(3-mercapto-2-butanoate)]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Empirical Formula</strong></td>
<td>C\textsubscript{38}H\textsubscript{39}BN\textsubscript{6}OSZn</td>
<td>C\textsubscript{41}H\textsubscript{35}BN\textsubscript{6}OSZn</td>
<td>C\textsubscript{34}H\textsubscript{37}BN\textsubscript{6}OSZn</td>
</tr>
<tr>
<td><strong>Crystal System</strong></td>
<td>Triclinic</td>
<td>Triclinic</td>
<td>Monoclinic</td>
</tr>
<tr>
<td><strong>Space Group</strong></td>
<td>P-1</td>
<td>P-1</td>
<td>P\textsubscript{2}/c</td>
</tr>
<tr>
<td><strong>Unit Cell dimensions</strong></td>
<td>(a = 11.349(1) , \text{Å}) (\alpha = 71.114(2)^\circ) (b = 12.020(1) , \text{Å}) (\beta = 88.315(2)^\circ) (c = 14.899(2) , \text{Å}) (\gamma = 62.123(2)^\circ)</td>
<td>(a = 11.580(1) , \text{Å}) (\alpha = 88.252(1)^\circ) (b = 11.674(1) , \text{Å}) (\beta = 82.245(1)^\circ) (c = 15.250(1) , \text{Å}) (\gamma = 60.308(1)^\circ)</td>
<td>(a = 10.0273(6) , \text{Å}) (\alpha = 90^\circ) (b = 11.382(1) , \text{Å}) (\beta = 99.127(1)^\circ) (c = 28.672(2) , \text{Å}) (\gamma = 90^\circ)</td>
</tr>
<tr>
<td><strong>Volume, (Z)</strong></td>
<td>1681.8(3) , \text{Å}^3, 2</td>
<td>1772.8(2) , \text{Å}^3, 2</td>
<td>3230.9(3) , \text{Å}^3, 4</td>
</tr>
<tr>
<td><strong>Crystal size</strong></td>
<td>0.4 \times 0.2 \times 0.2 , \text{mm}</td>
<td>0.6 \times 0.6 \times 0.3 , \text{mm}</td>
<td>0.3 \times 0.2 \times 0.1 , \text{mm}</td>
</tr>
<tr>
<td><strong>Temperature (K)</strong></td>
<td>100(1)</td>
<td>100(1)</td>
<td>100(1)</td>
</tr>
<tr>
<td><strong>Reflections collected</strong></td>
<td>14688</td>
<td>15239</td>
<td>26807</td>
</tr>
<tr>
<td><strong>Independent reflections</strong></td>
<td>7541 ([R(\text{int})=0.0192])</td>
<td>7843 ([R(\text{int})=0.0159])</td>
<td>7299 ([R(\text{int})=0.0297])</td>
</tr>
<tr>
<td><strong>Data/restraints/parameters</strong></td>
<td>7541 / 0 / 437</td>
<td>7843 / 0 / 466</td>
<td>7299 / 0 / 468</td>
</tr>
<tr>
<td><strong>Goodness-of-fit on (F^2)</strong></td>
<td>1.076</td>
<td>1.054</td>
<td>1.058</td>
</tr>
<tr>
<td><strong>Final (R) indices (I&gt;2\sigma(I))</strong></td>
<td>(R1 = 0.0486) (wR2 = 0.1345)</td>
<td>(R1 = 0.0537) (wR2 = 0.1577)</td>
<td>(R1 = 0.0463) (wR2 = 0.1210)</td>
</tr>
<tr>
<td><strong>(R) indices (all data)</strong></td>
<td>(R1 = 0.0589) (wR2 = 0.1392)</td>
<td>(R1 = 0.0556) (wR2 = 0.1594)</td>
<td>(R1 = 0.0558) (wR2 = 0.1270)</td>
</tr>
<tr>
<td><strong>Largest peak/hole difference</strong></td>
<td>1.641 / -0.586 e Å \textsuperscript{3}</td>
<td>2.529 / -0.949 e Å \textsuperscript{3}</td>
<td>1.029 / -0.373 e Å \textsuperscript{3}</td>
</tr>
</tbody>
</table>
2.2.6. Binding Modes of Thiol-Based MPIs in Solution

The above model complexes demonstrate that in the solid-state, some ZBGs bind in a bidentate manner while others are observed to coordinate in a monodentate fashion. In order to confirm binding modes of these model systems in solution, the free and bound ZBGs were studied by using NMR. Comparison of the room temperature $^{13}$CNMR spectrum for 3-mercapto-2-butanone to the corresponding spectrum of [(Tp$_{\text{Ph,Me}}$)Zn(3-mercapto-2-butanoate)], shows downfield shifts in three of the four carbons on the bound ZBG. The change is greatest in the carbons closest to the coordinated S and O atoms. The carbon atom alpha to the sulfur shifts 5.0 ppm (42.5 $\rightarrow$ 47.5) while the binding carbonyl carbon shifts 6.5 ppm (205.4 $\rightarrow$ 211.9). In contrast, only small changes in chemical shift are observed for the monodentate ZBGs. In the comparison of the $^{13}$CNMR of free 3-mercapto-2-propanol and its model complex [(Tp$_{\text{Ph,Me}}$)Zn(3-mercapto-2-propanoate)], the downfield shift of the carbon atom alpha to the sulfur moves only 1.9 ppm (32.8 $\rightarrow$ 34.9) while the carbon alpha to the hydroxyl group shifts 1.0 ppm (68.3 $\rightarrow$ 69.3). The strong downfield shifts of the bidentate ZBGs when compared to the smaller shifts from monodentate ZBGs, suggest that the solid-state structure is maintained in solution at room temperature. The data presented confirm that the binding modes observed in the crystal structures are representative of the interactions in solution, further supporting the utility of these model compounds.
2.3. Discussion

An enormous amount of effort has gone into the design and synthesis of new MPIs,\textsuperscript{[12-15]} however, very little information is available on the mode of binding for many of the non-hydroxamate based MPIs.\textsuperscript{[15, 16]} The traditional means of extracting this data is to obtain structural information through X-ray data of the protein-inhibitor complex.\textsuperscript{[13, 14]} Unfortunately, this requires the isolation and purification of large amounts of high-quality protein, extensive screening of crystallization conditions, and the preparation of heavy-atom derivatives (in some cases). Even with advances in macromolecular structure determination, this venture is a significant investment without the guarantee of the desired results. Without structural information, it is difficult to rationally design MPIs and explain discrepancies in drug activity when comparing ZBGs from different compounds.

Previous work with tris(pyrazolyl)borate model complexes\textsuperscript{[1, 10]} suggested that the inorganic complex [(Tp\textsubscript{Ph,Me}Zn(OH))] would provide an excellent structural model for the MMP active site. We sought to use this model to examine the interaction of these proteases with known MPIs. The structure of [(Tp\textsubscript{Ph,Me}Zn(acetohydroxamate)] established a structural control, as the same binding mode was found for hydroxamate-based drugs as in the model complex. The coordination environment around the zinc(II) ion could be superimposed on the inhibited catalytic site of MMPs (Figure 2-4) with high conformity (RMS deviation of 0.372 Å).\textsuperscript{[26]} This indicated that [(Tp\textsubscript{Ph,Me}Zn(OH))] could be used not only for reproducing known drug-metalloprotein interactions, but perhaps for elucidating unknown interactions. It was apparent that
this structural information could be exceptionally useful for understanding trends in MPI potencies, as well as serving as an aid for future inhibitor design.

Subsequently, a number of thiol-based MPIs were examined. Many thiol-based MPIs demonstrate significant potency against a variety of MMPs, and some are being pursued clinically.\cite{12,15,17-19} Thiol derivatives are a logical progression from the overused hydroxamic acid ZBG, as the strong binding of sulfur groups to zinc is found ubiquitously in biological systems.\cite{28} Despite the investigation of various thiol-derived MPIs, the binding mode of these inhibitors (with the exception of one simple thiol)\cite{29} is unknown.

Complexation of $[(T_{p}^{Ph,Me})Zn(OH)]$ with $\beta$-mercaptoketones, $\beta$-mercaptoamides, and $\beta$-mercaptoalcohols readily revealed the binding mode of these ZBGs. As previously hypothesized,\cite{17-19} the structures of $[(T_{p}^{Ph,Me})Zn(3\text{-mercapto-2\text{-butanionate})}]$ and $[(T_{p}^{Ph,Me})Zn(N\text{-methylmercaptoacetamidate})]$ revealed bidentate zinc coordination for both $\beta$-mercaptoketone- and $\beta$-mercaptoamide-derived ZBGs. The binding of these compounds is much less symmetric than as is found in the acetohydroxamate structures, due to the strong sulfur donors in each ZBG (vide supra). This may contribute to the overall lower efficacy of these compounds when compared with hydroxamic acid derivatives, however a more detailed comparison of MPIs with identical backbones employing the different ZBGs is needed to evaluate this hypothesis.

Interestingly, the complexation of a series of $\beta$-mercaptoalcohols with $[(T_{p}^{Ph,Me})Zn(OH)]$ reliably confirmed monodentate coordination of the ZBGs to the metal center. With three independent compounds and crystal structures, there is little
doubt that $\beta$–mercaptoalcohol-derived drugs bind in a monodentate fashion to the catalytic MMP zinc(II) ion. This result is contrary to the previously suggested bidentate mode of binding espoused by developers of the thiol-based inhibitors.$^{[18, 19]}$

In fact, the model data correlates directly with the inhibitory activities of $\beta$–mercaptoalcohol- and $\beta$–mercaptoketone-derived MPIs. Campbell and coworkers described a series of potent malonyl $\beta$–mercaptoalcohol- and $\beta$–mercaptoketone-based MPIs.$^{[18]}$ Of twelve inhibitors that contained the same backbone substituents and only varied the ZBG between a $\beta$–mercaptoalcohol and a $\beta$–mercaptoketone (for example see Figure 2-5), eleven showed greater potency with the $\beta$–mercaptoketone ZBG (the remaining compound showed equal activity for both derivatives). In more than half of these compounds, the IC$_{50}$ of the $\beta$–mercaptoketone was at least one order of magnitude lower. Although the authors of the papers noted the increased potency of the $\beta$–mercaptoketone compounds, no explanation was provided for the increased efficacy. The data presented here provides a structural explanation for the discrepancies in the MPI potencies. The $\beta$–mercaptoketones are likely to bind the catalytic zinc ion in a bidentate fashion while the $\beta$–mercaptoalcohols bind in only a monodentate fashion, reducing the affinity of the MPIs for the enzyme. In a separate study from the same group,$^{[19]}$ succinyl $\beta$–mercaptoalcohols and a $\beta$–mercaptoketones were compared and the anti- isomer of the alcohols were found to have very poor activity against MMPs, while the syn- isomer of the alcohol and the $\beta$–mercaptoketones were found to be potent compounds. The difference in activity of the two alcohol isomers was speculated to be due to differences in the orientation of
the drug backbone, resulting from different, bidentate binding conformations of the two β-mercaptoalcohol stereoisomers. The drug backbones may reside in the MMP active site differently between the two isomers, but based on the evidence presented here, both isomers will still be coordinated in a monodentate fashion to the zinc(II) ion. Therefore, the differences in potency result solely on non-covalent interactions (Van der Waals, hydrogen-bonding, etc.) where the anti- isomer not only suffers from monodentate binding to the zinc(II) ion, but has no favorable contacts between the backbone and the protein. However, the syn- isomer of the β-mercaptoalcohols overcomes the loss in potency due to monodentate binding with highly optimal interactions of the backbone susituentes and the active site.

2.4. Conclusion

By using a simple structural model of the active site in MMPs, we have rapidly and at high resolution determined the binding modes of the ZBGs from several MPIs. The resulting structural data assists in explaining differences in the inhibitor potency of several MPIs in which the binding modes of these drugs were unknown.

2.5. Experimental Section

General. Unless otherwise noted, starting materials were obtained from commercial suppliers and used without further purification. Elemental analysis was performed at the University of California, Berkeley Analytical Facility. $^1$H/$^13$CNMR spectra were recorded on a Varian FT-NMR spectrometer running at 300 or 400 MHz.
at the Department of Chemistry and Biochemistry, University of California, San Diego. Isolated yields of metal complexes were ~30-75% unless noted otherwise. 

*Caution!* **Perchlorate salts of metal complexes with organic ligands are potentially explosive. Only small amounts of these materials should be prepared and they should be handled with great care.**

**3,5-Phenylmethylpyrazole.** 1-Benzoylacetic acid (12 g, 74 mmol) was dissolved in 100 mL of toluene. Hydrazine monohydrate (3.63 mL, 74 mmol) in 75 mL toluene was added to the 1-benzoylacetone mixture dropwise from a pressure-equalized addition funnel. The hydrazine addition was performed under N$_2$(g) and the mixture was kept cold in an ice bath. After addition was complete, the reaction mixture was fit with a Dean-Stark trap and a reflux condenser in tandem. The reaction mixture was heated to reflux (~140 °C). After 3 h of heating, the orange-yellow solution was cooled to room temperature. This solution was evaporated on a rotory evaporator to obtain a slurry that was filtered and the solid washed with cold hexanes to and dried in a vacuum oven to yield the product as a white solid 10.7 g (91%). $^1$HNMR (CDCl$_3$, 300 MHz, 25 °C): δ 2.27 (s, 3H, CH$_3$), 6.12 (s, 1H, pyrazole-H), 7.28 (m, 1H, phenyl-H), 7.33 (m, 2H, phenyl-H), 7.69 (d, J = 7.2 Hz, 2H, phenyl-H). ESI-MS(+): m/z 159.2 [M+H]$^+$. 

**Potassium Hydrotris(3,5-Phenylmethylpyrazolyl)borate [$^{\text{Tp}}^{\text{Ph,Me}}$K].** 3,5-phenylmethylpyrazole (9.95 g, 63 mmol) and potassium borohydride (1.13 g, 21 mmol) were combine under an N$_2$(g) atmosphere. The solids were heated slowly (2.5 h) to 240 °C affording a yellow liquid. After 2 h at 240 °C, the mixture was cooled to room temperature, whereupon the yellow liquid became a hard solid. The solid was
dissolved in warm acetonitrile and was hot filtered in small amounts. The filtrate was placed in a freezer overnight at 4 °C. White precipitate formed from the filtrate. The mother liquor was decanted off and the solid was dried in a vacuum oven to yield 8.18 g (75%). $^1$H NMR (CDCl$_3$, 300 MHz, 25 °C): δ 1.92 (s, 1H, boro-H), 2.23 (s, 3H, CH$_3$), 6.20 (s, 1H, pyrazole-H), 7.16 (m, 1H, phenyl-H), 7.23 (m, 2H, phenyl-H), 7.48 (d, J = 0.9 Hz, 2H, phenyl-H). ESI-MS(+): m/z 483.3 [M+H]$^+$. 

[(Tp$^{\text{Ph,Me}}$)ZnOH]. [Tp$^{\text{Ph,Me}}$K] (1.57 g, 3.0 mmol) was dissolved in 100 mL CH$_2$Cl$_2$ and added to a stirring solution of Zn(ClO$_4$)$_2$·6H$_2$O (1.11 g, 3.0 mmol) in 20 mL of methanol. After stirring for 2 h at room temperature under a nitrogen atmosphere, KOH (168 mg, 3.0 mmol) was added to the solution. After stirring at room temperature overnight under a nitrogen atmosphere, the solution was filtered through a glass frit and 60 mL of methanol was added to filtrate. The filtrate was evaporated on a rotary evaporator to one third of the original volume (40 mL). The remaining solution was left to stand at room temperature producing the title compound as a white solid. Yield: 78%. $^1$HNMR (CDCl$_3$, 300 MHz, 25 °C): δ 2.52 (s, 9H, pyrazole-CH$_3$), 6.23 (s, 3H, pyrazole-H), 7.28 (m, 3H, phenyl-H), 7.5 (d, J = 6.9, 6H, phenyl-H), 7.65 (d, J = 4.5 Hz, 6H, phenyl-H). $^{13}$CNMR (CDCl$_3$, 100 MHz, 25 °C): δ 13.0, 104.8, 127.1, 127.4, 127.5, 132.7, 144.4, 152.9. Anal. Calcd for C$_{60}$H$_{59}$B$_2$N$_{12}$O$_5$Zn$_2$·ClO$_4$·CH$_2$Cl$_2$: C, 55.63; H, 4.67; N, 12.76. Found C, 55.69; H, 4.51; N, 12.80.

[(Tp$^{\text{Ph,Me}}$)Zn(acetohydroxamate)]. In a 100 mL round-bottom flask, [(Tp$^{\text{Ph,Me}}$)ZnOH] (100 mg, 0.18 mmol) was dissolved in 15 mL of CH$_2$Cl$_2$. To this solution was added 1.0 equiv of acetohydroxamic acid (13 mg, 0.18 mmol) dissolved
in 10 mL of MeOH. The mixture was stirred at room temperature overnight under a nitrogen atmosphere. After stirring, the turbid solution was evaporated to dryness on a rotary evaporator to give a white solid. The solid was dissolved in a minimum amount of benzene (~3 mL) and the material was recrystallized by diffusion with pentane.

$^1$HNMR (CDCl$_3$, 300 MHz, 25 °C): $\delta$ 2.14 (s, 3H, CH$_3$, hydroxamate-CH$_3$), 2.50 (s, 9H, CH$_3$, pyrazole-CH$_3$), 6.16 (s, 3H, pyrazole-H), 7.23 (m, 3H, phenyl-H), 7.34 (m, 6H, phenyl-H), 7.59 (d, $J$ = 8.1 Hz, 6H, phenyl-H). $^{13}$CNMR (CDCl$_3$, 100 MHz, 25 °C): $\delta$ 13.3, 30.9, 38.5, 104.8, 125.8, 127.8, 128.8, 133.0, 145.2, 152.8. Anal. Calcd for C$_{32}$H$_{32}$BN$_7$O$_2$Zn • 0.5 benzene: C, 63.51; H, 5.33; N, 14.81. Found C, 63.71; H, 5.60; N, 14.60.

[(Tp$^{\text{Ph,Me}}$)Zn(3-mercapto-2-butanonate)]. The same procedure was used as in the synthesis of [(Tp$^{\text{Ph,Me}}$)Zn(acetohydroxamate)]. $^1$HNMR (CDCl$_3$, 300 MHz, 25 °C): $\delta$ 0.90 (d, $J$ = 5.7 Hz, 3H, butanonate-CH$_3$), 1.13 (s, 3H, butanonate-CH$_3$), 2.56 (s, 9H, pyrazole-CH$_3$), 2.77 (m, 1H, butanonate-H), 6.16 (s, 3H, pyrazole-H), 7.27 (d, $J$ = 5.4 Hz, 3H, phenyl-H), 7.37 (m, 6H, phenyl-H), 7.69 (d, $J$ = 6.0 Hz, 6H, phenyl-H). $^{13}$CNMR (CDCl$_3$, 100 MHz, 25 °C): $\delta$ 13.4, 23.1, 25.0, 47.5, 105.7, 128.0, 128.6, 128.9, 132.7, 145.0, 153.5, 211.9. Anal. Calcd for C$_{34}$H$_{35}$BN$_6$OSZn: C, 62.64; H, 5.41; N, 12.89. Found C, 62.33; H, 5.62; N, 12.92.

[(Tp$^{\text{Ph,Me}}$)Zn(N-methylmercaptoacetamidate)]. The same procedure was used as in the synthesis of [(Tp$^{\text{Ph,Me}}$)Zn(acetohydroxamate)]. $^1$HNMR (CDCl$_3$, 100 MHz, 25 °C): $\delta$ 1.2 (m, 3H, amidate-CH$_3$), 2.2 (s, 2H, amidate-CH$_2$), 2.6 (s, 9H, pyrazole-CH$_3$), 6.2 (s, 3H, pyrazole-H), 7.3 (m, 3H, phenyl-H), 7.4 (m, 6H, phenyl-H), 7.7 (d, $J$ = 8.0 Hz, 6H, phenyl-H). $^{13}$CNMR (CDCl$_3$, 100 MHz, 25 °C): $\delta$ 12.9, 26.3, 30.9, 105.3,
Anal. Calcd for C₃₃H₃₄BN₇OSZn • 0.5 benzene • H₂O: C, 63.53; H, 5.65; N, 13.09. Found C, 62.58; H, 5.36; N, 13.21.

[(Tp^{Ph,Me})Zn(2-mercaptoethanoate)]. The same procedure was used as in the synthesis of [(Tp^{Ph,Me})Zn(acetohydroxamate)]. ¹H NMR (CDCl₃, 100 MHz, 25 °C): δ 2.1 (m, 2H, ethanoate-CH₂), 2.2 (m, 2H, ethanoate-CH₂), 2.6 (s, 9H, pyrazole-CH₃), 6.2 (s, 3H, pyrazole-H), 7.3 (m, 3H, phenyl-H), 7.4 (m, 6H, phenyl-H), 7.7 (d, J = 7.6 Hz, 6H, phenyl-H). ¹³CNMR (CDCl₃, 100 MHz, 25 °C): δ 13.3, 28.8, 64.7, 105.8, 128.5, 128.6, 128.8, 131.8, 145.8, 154.1. Anal. Calcd for C₃₂H₃₃BN₆OSZn • 0.5 benzene: C, 63.22; H, 5.46; N, 12.64. Found C, 63.34; H, 5.69; N, 12.56.

[(Tp^{Ph,Me})Zn(3-mercapto-2-propanoate)]. The same procedure was used as in the synthesis of [(Tp^{Ph,Me})Zn(acetohydroxamate)]. ¹H NMR (CDCl₃, 400 MHz, 25 °C): δ 0.49 (d, J = 4.5 Hz, 3H, propanoate-CH₃), 1.1 (m, 2H, propanate-CH₂), 1.2 (m, 1H, propanoate-CH), 2.6 (s, 9H, pyrazole-CH₃), 6.2 (s, 3H, pyrazole-H), 7.3 (m, 3H, phenyl-H), 7.4 (m, 6H, phenyl-H), 7.7 (d, J = 8.0 Hz, 6H, phenyl-H). ¹³CNMR (CDCl₃, 100 MHz, 25 °C): δ 13.3, 21.8, 34.9, 69.3, 105.8, 128.6, 128.9, 129.1, 131.7, 145.8, 154.1. Anal. Calcd for C₃₆H₃₈BN₆OSZn • 0.5 benzene: C, 63.68; H, 5.64; N, 12.38. Found C, 63.99; H, 6.01; N, 12.40.

[(Tp^{Ph,Me})Zn(3-mercapto-2-butanolate)]. The same procedure was used as in the synthesis of [(Tp^{Ph,Me})Zn(acetohydroxamate)]. ¹H NMR (CDCl₃, 300 MHz, 25 °C): δ 0.13/0.27 (d, J = 5.1/5.1 Hz, 3H, butanoate-CH₃), 0.50/0.57 (d, J = 5.1/4.2 Hz, 3H, butanoate-CH₃), 2.57 (s, 9H, pyrazole-CH₃), 6.19 (s, 3H, pyrazole-H), 7.35 (m, 3H, phenyl-H), 7.41 (m, 6H, phenyl-H), 7.66 (d, J = 7.2 Hz, 6H, phenyl-H). ¹³CNMR (CDCl₃, 100 MHz, 25 °C): δ 13.4, 18.4/19.3, 21.0/24.1, 42.1/43.7, 72.2/72.8, 105.8,
128.6, 128.7, 128.9, 131.8, 145.7, 154.1. Anal. Calcd for C$_{34}$H$_{37}$BN$_6$OSZn: C, 62.44; H, 5.70; N, 12.85. Found C, 62.79; H, 5.96; N, 12.97.

**X-Ray Crystallographic Analysis.** Data was collected on a Brucker AXS area detector diffractometer. Crystals were mounted on quartz capillaries by using Paratone oil and were cooled in a nitrogen stream (Kryo-flex controlled) on the diffractometer (-173°C). Peak integrations were performed with the Siemens SAINT software package. Absorption corrections were applied using the program SADABS. Space group determinations were performed by the program XPREP. The structures were solved by direct or Patterson methods and refined with the SHELXTL software package. Unless noted otherwise, all hydrogen atoms, except for the boron hydrogen atoms, were fixed at calculated positions with isotropic thermal parameters; all non-hydrogen atoms were refined anisotropically.

[(Tp$^{\text{Ph,Me}}$)ZnOH]. Colorless blocks were grown out of a solution of the complex in benzene diffused with pentane. The structure was found to be a dinuclear complex. The complex co-crystallized with one molecule of a perchlorate counterion. The perchlorate counterion was slightly disordered, and was modeled as a combination of two different orientations of the oxygen atoms. Two benzene solvent molecules were also found in the asymmetric unit.

[(Tp$^{\text{Ph,Me}}$)Zn(acetohydroxamate)]. Colorless blocks were grown out of a solution of the complex in benzene diffused with pentane. The hydrogen atom on the N-H hydrogen atom on the zinc-bound acetohydroxamate was found in the difference map and its positions was not fixed. The complex co-crystallized with one molecule of free
acetohydroxamic acid and one-half of a benzene solvent molecule in the asymmetric unit.

\[ (Tp^{Ph,Me})Zn(3\text{-mercapto-2-butanonate}) \]. Colorless blocks were grown out of a solution of the complex in benzene diffused with pentane. The 3-mercapto-2-butanone used to prepare the complex was a combination of R and S isomers, and the crystals grown are a racemic mixture of the complexes formed with each enantiomer. The two enantiomers did not segregate in the crystal and therefore the structure was disordered with a partial occupancy (55:45) of the carbon atoms a- (C33/C33B) and b- (C34/C34B) to the sulfur atom (Figure 2-11). No hydrogen atoms were calculated or refined for the disordered carbon atoms. No solvent molecules were found in the asymmetric unit.

\[ (Tp^{Ph,Me})Zn(N\text{-methylmercaptoacetamidate}) \]. Colorless blocks were grown out of a solution of the complex in benzene diffused with pentane. The complex co-crystallized with one molecule of benzene in the asymmetric unit.

\[ (Tp^{Ph,Me})Zn(2\text{-mercaptoethanoate}) \]. Colorless blocks were grown out of a solution of the complex in benzene diffused with pentane. The complex co-crystallized with one molecule of benzene in the asymmetric unit.

\[ (Tp^{Ph,Me})Zn(3\text{-mercapto-2-propanoate}) \]. Colorless blocks were grown out of a solution of the complex in benzene diffused with pentane. A severely disordered solvent molecule was found in the asymmetric unit. Despite several attempts, identification of the solvent (benzene or pentane) could not be resolved. However, there is no disorder in the metal complex.
[(Tp^{Ph,Me})Zn(3-mercapto-2-butanoate)]. Large colorless prisms were grown out of a solution of the complex in benzene diffused with pentane. The 3-mercapto-2-butanol used to prepare the complex was a combination of the $R,R$, $S,S$, $R,S$, and $S,R$ isomers, and the crystals grown are a mixture of the complexes formed with each stereoisomer. The two enantiomeric pairs did not segregate during crystal growth nor in the crystal lattice and therefore the structure represents a mixture of all four isomers bound to the zinc center. A disorder (partial occupancy) model of all four isomers was obtained to fit the data (Figure 2-12). The structure clearly shows the mode of binding and indicates that all four isomers bind in a monodentate fashion to the zinc ion. No hydrogen atoms were calculated or refined for the disordered carbon atoms. No solvent molecules were found in the asymmetric unit.

2.6. Appendix

![Structural diagram of [(Tp^{Ph,Me})Zn(3-mercapto-2-butanoate)]](image)

**Figure 2-11.** Structural diagram of [(Tp^{Ph,Me})Zn(3-mercapto-2-butanoate)] displaying disorder in carbons 33 and 34 with partial atom numbering schemes (ORTEP, 50% probability ellipsoids). Hydrogen atoms and have been omitted for clarity.
**Figure 2-12.** Structural diagram of [(Tp^Ph,Me)Zn(3-mercapto-2-butanoate)] displaying disorder from all for isomers with partial atom numbering schemes (ORTEP, 50% probability ellipsoids). Hydrogen atoms have been omitted for clarity.

### 2.7. Acknowledgements

2.8. References


Chapter 3. The Use of Tris(pyrazoyl)borate Model Complexes to Examine Novel ZBGs
3.1. Introduction

In a continuing effort to elucidate drug-metalloprotein interactions using bioinorganic model compounds, the binding of several new ZBGs that show promise for incorporation into second-generation MMP inhibitors is described. The complexes presented herein demonstrate that tris(pyrazolyl)borate complexes of zinc(II) can be used as an initial screen for ZBGs, by providing structural and qualitative binding information, without the need for sophisticated drug synthesis or protein structure determination. Six ligands were selected to demonstrate the wide range of compounds that might serve as effective ZBGs. These ligands share several features in common with the frequently used hydroxamate ZBG, as these compounds are monoanionic ligands that were anticipated to bind the zinc ion in a chelating, bidentate fashion. The compounds studied were also selected because of their potential biocompatibility, based on their known uses in biological systems.[1-5] The data herein demonstrate that the ZBGs, 1-hydroxy-2(1H)-pyridinone, 3-hydroxy-2(1H)-pyridinone, 3-hydroxy-1-methyl-2(1H)-pyridinone, 3-hydroxy-1,2-dimethyl-4(1H)-pyridinone, 1-hydroxy-2(1H)-pyridinethione, and 3-hydroxy-2-methyl-4-pyrone each displace the hydroxide ligand in [(Tp$_{Ph,Me}$)ZnOH] and coordinate the zinc(II) ion in a bidentate fashion. The metal-ligand bond lengths are compared to those found in the corresponding acetohydroxamate complex.[6] The model-based approach described here is expected to provide an unexplored and effective route toward second-generation MPI design.
3.2. Results

Utilizing \([\text{Tp}^{\text{Ph,Me}}\text{ZnOH}]^6\) as a starting point, a number of complexes were synthesized to serve as models of novel ZBGs bound to the active site of MMPs (Figure 3-1). The series of ZBGs presented here can be separated into three groups: hydroxypyridinonate-based, \(N\)-methylated-hydroxypyridinonate-based, and hydroxypyridinonate derivatives. The complexes of \([\text{Tp}^{\text{Ph,Me}}\text{Zn}(\text{ZBG})]\) have all been characterized by X-ray crystallography, \(^1\text{H}/^{13}\text{CNMR, IR, and elemental analysis.}\)

![Figure 3-1. Synthesis of \([\text{Tp}^{\text{Ph,Me}}\text{Zn}(\text{ZBG})]\) model complexes (ZBG = zinc-binding group).](image_url)

3.2.1. Examination of Hydroxypyridinonate-based ZBGs

The X-ray structures of hydroxypyridinonate-based ZBGs bound to the model complex show that these chelators coordinate in a bidentate fashion to the catalytic zinc(II) center (Figure 3-2 and Figure 3-3). The ZBG 1-hydroxy-2(1\(H\))-pyridinone is a cyclic analog of hydroxamic acid, and was therefore anticipated to bind in a chelating manner. In the structure of \([\text{Tp}^{\text{Ph,Me}}\text{Zn}(1\text{-hydroxy-2}(1\text{H})\text{-pyridinone})]\), the zinc center can be described as distorted trigonal bipyramidal (\(\tau = 0.64\))\(^7\) with the 2-hydroxy oxygen atom and one of the pyrazole ring nitrogen atoms occupying the axial positions of the coordination sphere (Figure 3-2). The coordinating oxygen atoms in
this complex have Zn-O bond lengths of 1.97 Å (O1) and 2.09 Å (O2). These bond lengths are similar to the corresponding bond lengths in [(Tp^{Ph,Me})Zn(acetohydroxamate)], suggesting that this ZBG may bind the catalytic zinc ion with an affinity comparable to the hydroxamic acid ZBG.\[6\]

In the structure of [(Tp^{Ph,Me})Zn(3-hydroxy-2(1H)-pyridinone)], the zinc center can also be described as distorted trigonal bipyramidal ($\tau = 0.63$) with one oxygen donor and one of the pyrazole rings occupying the axial positions of the coordination sphere (Figure 3-3). This isomer of 1-hydroxy-2(1H)-pyridinone has Zn-O bond lengths of 1.92 Å (O1) and 2.23 Å (O2), demonstrating strong bidentate coordination to the metal center.

**Figure 3-2.** Structural diagram of [(Tp^{Ph,Me})Zn(1-hydroxy-2(1H)-pyridinone)] with partial atom numbering scheme (ORTEP, 50% probability ellipsoids). Hydrogen atoms and solvent have been omitted for clarity.
3.2.2. Examination of N-Methylated Hydroxypyridinonate-based ZBGs

The second class of ZBGs examined was the N-methylated-hydroxypyridinonate group. These ZBGs show similar binding to the unsubstituted hydroxypyridinonates. In the complex \([(T_{p,Me}^\text{Ph})\text{Zn}(3\text{-hydroxy-1-methyl-2(1H)-pyridinone})]\) (Figure 3-4), the coordination environment around the zinc center can be described as distorted trigonal bipyramidal ($\tau = 0.52$). The coordinating oxygen atoms are bonded to the model complex in a bidentate fashion with a Zn-O1 bond length of 1.94 Å and a Zn-O2 bond length of 2.21 Å (Table 3-3). The ZBG 3-hydroxy-1,2-dimethyl-4(1H)-pyridinone has the shortest averaged phenolic and carbonyl Zn-O
bond lengths of all the novel ligands presented herein. The X-ray structure (Figure 3-5) clearly demonstrates the tight binding of this ZBG with bond lengths of 1.96 Å (Zn-O1) and 2.05 Å (Zn-O2). The coordination environment is best described as distorted tetragonal ($\tau = 0.44$), and is more distorted from trigonal bipyramidal than the other ZBGs described here. The shorter bond lengths and tetragonal coordination geometry can be attributed to the delocalization of the lone pair from the N-methyl group para to the carbonyl in the ZBG. This causes the ring to be more electron rich, subsequently making the carbonyl oxygen a stronger Lewis base resulting in a higher affinity for the zinc(II) ion. In the other ZBGs (except 1-hydroxy-2(1$H$)-pyridinethione) the phenolyic oxygen is the strongest donor to the zinc(II) ion as evidenced by the shorter bond lengths (Table 3-3). However, in the case of 3-hydroxy-1,2-dimethyl-4(1$H$)-pyridinone, the two oxygen donors are more evenly suited donors resulting in a more symmetrical chelation.
**Figure 3-4.** Structural diagram of \([(\text{Tp}^{\text{Ph,Me}})\text{Zn}(3\text{-hydroxy-1-methyl-2(1}H\text{-pyridinone)})]\) with partial atom numbering scheme (ORTEP, 50% probability ellipsoids). Hydrogen atoms and solvent molecules have been omitted for clarity.

**Figure 3-5.** Structural diagram of \([(\text{Tp}^{\text{Ph,Me}})\text{Zn}(3\text{-hydroxy-1,2-dimethyl-4(1}H\text{-pyridinone)})]\) with partial atom numbering scheme (ORTEP, 50% probability ellipsoids). Hydrogen atoms and solvent molecules have been omitted for clarity.
**Table 3-1.** Crystal data for [(Tp\textsuperscript{Ph,Me})Zn(1-hydroxy-2(1H)-pyridinone)], [(Tp\textsuperscript{Ph,Me})Zn(3-hydroxy-2(1H)-pyridinone)], and [(Tp\textsuperscript{Ph,Me})Zn(3-hydroxy-1-methyl-2(1H)-pyridinone)].

<table>
<thead>
<tr>
<th></th>
<th>[(Tp\textsuperscript{Ph,Me})Zn(1-hydroxy-2(1H)-pyridinone)]</th>
<th>[(Tp\textsuperscript{Ph,Me})Zn(3-hydroxy-2(1H)-pyridinone)]</th>
<th>[(Tp\textsuperscript{Ph,Me})Zn(3-hydroxy-1-methyl-2(1H)-pyridinone)]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Empirical Formula</strong></td>
<td>C\textsubscript{35}H\textsubscript{32}BN\textsubscript{7}O\textsubscript{2}Zn</td>
<td>C\textsubscript{41}H\textsubscript{38}B\textsubscript{1}N\textsubscript{7}O\textsubscript{2}Zn</td>
<td>C\textsubscript{42}H\textsubscript{41}BN\textsubscript{7}O\textsubscript{2}Zn</td>
</tr>
<tr>
<td><strong>Crystal System</strong></td>
<td>Monoclinic</td>
<td>Triclinic</td>
<td>Monoclinic</td>
</tr>
<tr>
<td><strong>Space Group</strong></td>
<td>P\textsubscript{2}\textsubscript{1}/c</td>
<td>P\textsuperscript{-1}</td>
<td>P\textsubscript{2}\textsubscript{1}/c</td>
</tr>
<tr>
<td><strong>Unit Cell dimensions</strong></td>
<td>a = 9.7863(15) Å, (\alpha = 90^\circ)</td>
<td>a = 11.0865(10) Å, (\alpha = 111.300(10)^\circ)</td>
<td>a = 19.959(2) Å, (\alpha = 90^\circ)</td>
</tr>
<tr>
<td></td>
<td>b = 23.269(4) Å, (\beta = 98.122(3)^\circ)</td>
<td>b = 12.229(11) Å, (\beta = 96.615(2)^\circ)</td>
<td>b = 16.652(19) Å, (\beta = 92.197(2)^\circ)</td>
</tr>
<tr>
<td></td>
<td>c = 13.607(2) Å, (\gamma = 90^\circ)</td>
<td>c = 15.469(14) Å, (\gamma = 102.364(2)^\circ)</td>
<td>c = 10.988(12) Å, (\gamma = 90^\circ)</td>
</tr>
<tr>
<td><strong>Volume, Z</strong></td>
<td>3067.5(8) Å\textsuperscript{3}</td>
<td>1865.3(3) Å\textsuperscript{3}</td>
<td>3649.4(7) Å\textsuperscript{3}</td>
</tr>
<tr>
<td><strong>Crystal size (mm\textsuperscript{3})</strong></td>
<td>0.20 × 0.07 × 0.07</td>
<td>0.35 × 0.20 × 0.06</td>
<td>0.26 × 0.2 × 0.06</td>
</tr>
<tr>
<td><strong>Reflections collected</strong></td>
<td>26190</td>
<td>10782</td>
<td>31063</td>
</tr>
<tr>
<td><strong>Independent reflections</strong></td>
<td>([R (\text{int}) = 0.0620] 7029)</td>
<td>([R (\text{int}) = 0.0150] 7642)</td>
<td>([R (\text{int}) = 0.0540] 8344)</td>
</tr>
<tr>
<td><strong>Data/restraints/parameters</strong></td>
<td>7029 / 0 / 422</td>
<td>7642 / 0 / 516</td>
<td>8344 / 0 / 486</td>
</tr>
<tr>
<td><strong>Goodness-of-fit on F\textsuperscript{2}</strong></td>
<td>0.882</td>
<td>1.067</td>
<td>0.941</td>
</tr>
<tr>
<td><strong>Final R indices</strong></td>
<td>(R_1 = 0.0435) wR\textsubscript{2} = 0.0797</td>
<td>(R_1 = 0.0589) wR\textsubscript{2} = 0.1714</td>
<td>(R_1 = 0.0374) wR\textsubscript{2} = 0.0866</td>
</tr>
<tr>
<td>(I &gt; 2\sigma(I))</td>
<td>(R_1 = 0.0781) wR\textsubscript{2} = 0.0864</td>
<td>(R_1 = 0.0676) wR\textsubscript{2} = 0.1802</td>
<td>(R_1 = 0.0481) wR\textsubscript{2} = 0.0897</td>
</tr>
<tr>
<td><strong>R indices (all data)</strong></td>
<td>(R_1 = 0.0781) wR\textsubscript{2} = 0.0864</td>
<td>(R_1 = 0.0676) wR\textsubscript{2} = 0.1802</td>
<td>(R_1 = 0.0481) wR\textsubscript{2} = 0.0897</td>
</tr>
<tr>
<td><strong>Largest peak/hole difference</strong></td>
<td>0.836 / -0.567 e Å\textsuperscript{3}</td>
<td>1.464 / -0.458 e Å\textsuperscript{3}</td>
<td>0.767 / -0.484 e Å\textsuperscript{3}</td>
</tr>
</tbody>
</table>
3.2.3. Examination of Hydroxypyridinonate Derived ZBGs

The final group of novel ZBGs explored are classified as hydroxypyridinonate derivatives. This group includes 1-hydroxy-2(1H)-pyridinethione and 3-hydroxy-2-methyl-4-pyrone. The complex \([(Tp^{Ph,Me})\text{Zn}(1\text{-hydroxy-2(1H)-pyridinethione})]\) (Table 3-2) reveals that 1-hydroxy-2(1H)-pyridinethione binds in the expected bidentate fashion (Figure 3-6). The coordination environment around the zinc center can be described as distorted trigonal bipyramidal (\(\tau = 0.54\)). The bond lengths of the coordinating sulfur and oxygen are longer than the coordinating oxygen atoms in the corresponding acetohydroxamic acid complex\[^6\] due to the larger sulfur atom. In addition to the comparably longer Zn-S (2.32 Å) distance, the sulfur binding also affects the binding of the adjacent oxygen atom, increasing the Zn-O bond length to 2.08 Å (Table 3-3).

3-Hydroxy-2-methyl-4-pyrone binds to \([(Tp^{Ph,Me})\text{ZnOH}]\) resulting in a complex (Figure 3-7) with comparable bond lengths as in \([(Tp^{Ph,Me})\text{Zn(acetohydroxamate})]\)^6 The Zn-O1 distance is 1.94 Å and the Zn-O3 distance is 2.18 Å (Table 3-3). The coordination environment is similar to that of the hydroxypyridinonate ZBGs described above. The coordination sphere can be described as distorted trigonal bipyramidal (\(\tau = 0.69\)) with one oxygen and one nitrogen donor occupying the axial positions.
Figure 3-6. Structural diagram of \([(\text{Tp}^{\text{Ph,Me}})\text{Zn}(1\text{-hydroxy-2}(1H)\text{-pyridinethione})]\) with partial atom numbering scheme (ORTEP, 50% probability ellipsoids). Hydrogen atoms and solvent molecules have been omitted for clarity.

Figure 3-7. Structural diagram of \([(\text{Tp}^{\text{Ph,Me}})\text{Zn}(3\text{-hydroxy-2-methyl-4-pyrone})]\) with partial atom numbering scheme (ORTEP, 50% probability ellipsoids). Hydrogen atoms and solvent molecules have been omitted for clarity.
Table 3-2. Crystal data for [(TpPh,Me)Zn(3-hydroxy-1,2-dimethyl-4(1H)-pyridinone)], [(TpPh,Me)Zn(1-hydroxy-2(1H)-pyridinethione)], and [(TpPh,Me)Zn(3-hydroxy-2-methyl-4-pyrole)].

<table>
<thead>
<tr>
<th>Empirical Formula</th>
<th>Crystalline System</th>
<th>Space Group</th>
<th>Unit Cell Dimensions</th>
<th>Volume, Z</th>
<th>Crystal size (mm$^3$)</th>
<th>Temperature (K)</th>
<th>Reflections collected</th>
<th>Independent reflections</th>
<th>Data/restraints/parameters</th>
<th>Goodness-of-fit on $F^2$</th>
<th>Final $R$ indices $I&gt;2\sigma(I)$</th>
<th>$R$ indices (all data)</th>
<th>Largest peak/hole difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>[(Tp$^{\text{Ph,Me}}$)Zn(3-hydroxy-1,2-dimethyl-4(1H)-pyridinone)]</td>
<td>C$<em>{37}$H$</em>{36}$BN$_7$O$_2$Zn</td>
<td>Monoclinic</td>
<td>$a = 12.215(14)$ Å, $b = 11.0738(13)$ Å, $c = 24.646(3)$ Å, $\alpha = 90^\circ$, $\beta = 100.944(2)^\circ$, $\gamma = 90^\circ$</td>
<td>3273.0(7) Å$^3$, 4</td>
<td>0.17 × 0.10 × 0.02</td>
<td>100(1)</td>
<td>27739</td>
<td>7497 / 0 / 422</td>
<td>1.008</td>
<td>0.0410 / 0.0841</td>
<td>0.0416 / 0.0942</td>
<td>0.0617 / 0.0914</td>
<td>0.405 / -0.431 e Å$^{-3}$</td>
</tr>
<tr>
<td>[(Tp$^{\text{Ph,Me}}$)Zn(1-hydroxy-2(1H)-pyridinethione)]</td>
<td>C$<em>{140}$H$</em>{128}$B$<em>4$N$</em>{28}$O$_4$S$_4$Zn$_4$</td>
<td>Monoclinic</td>
<td>$a = 35.847(3)$ Å, $b = 20.813(15)$ Å, $c = 34.388(3)$ Å, $\alpha = 90^\circ$, $\beta = 100.251(10)^\circ$, $\gamma = 90^\circ$</td>
<td>25246(3) Å$^3$, 32</td>
<td>0.34 × 0.13 × 0.05</td>
<td>100(1)</td>
<td>108046</td>
<td>28818 / 0 / 1685</td>
<td>0.990</td>
<td>0.0416 / 0.0942</td>
<td>0.0823 / 0.1104</td>
<td>0.0645 / -0.288 e Å$^{-3}$</td>
<td>0.645 / -0.288 e Å$^{-3}$</td>
</tr>
<tr>
<td>[(Tp$^{\text{Ph,Me}}$)Zn(3-hydroxy-2-methyl-4-pyrole)]</td>
<td>C$<em>{36}$H$</em>{33}$BN$_6$O$_3$Zn</td>
<td>Triclinic</td>
<td>$a = 10.0442(8)$ Å, $b = 20.813(15)$ Å, $c = 17.237(14)$ Å, $\alpha = 93.609(10)^\circ$, $\beta = 101.606(10)^\circ$, $\gamma = 93.206(10)^\circ$</td>
<td>1751.9(2) Å$^3$, 2</td>
<td>0.35 × 0.19 × 0.05</td>
<td>100(1)</td>
<td>7606</td>
<td>7606 / 0 / 459</td>
<td>1.047</td>
<td>0.0192</td>
<td>0.0291 / 0.0792</td>
<td>0.0309</td>
<td>0.418 / -0.397 e Å$^{-3}$</td>
</tr>
</tbody>
</table>
Table 3-3. Bond lengths for coordinating atoms in complexes of [(Tp^{Ph,Me})Zn(ZBG)]. All bond lengths were obtained from X-ray structure determinations.

<table>
<thead>
<tr>
<th>Complex [(Tp^{Ph,Me})Zn(ZBG)]</th>
<th>Zn-O bond length for phenolic oxygen atoms (Å)</th>
<th>Zn-O bond length for carbonyl oxygen atoms (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetohydroxamic acid</td>
<td>1.976(1)</td>
<td>2.102(1)</td>
</tr>
<tr>
<td>1-Hydroxy-2(1H)-pyridinone</td>
<td>1.967(2)</td>
<td>2.093(2)</td>
</tr>
<tr>
<td>3-Hydroxy-2(1H)-pyridinone</td>
<td>1.918(2)</td>
<td>2.232(2)</td>
</tr>
<tr>
<td>3-Hydroxy-1-methyl-2(1H)-pyridinone</td>
<td>1.935(1)</td>
<td>2.209(1)</td>
</tr>
<tr>
<td>3-Hydroxy-1,2-dimethyl-4(1H)-pyridinone</td>
<td>1.965(1)</td>
<td>2.051(1)</td>
</tr>
<tr>
<td>1-Hydroxy-2(1H)-pyridinethione</td>
<td>2.076(2)</td>
<td>2.325(1) (Zn-S)</td>
</tr>
<tr>
<td>3-Hydroxy-2-methyl-4-pyrone</td>
<td>1.937(1)</td>
<td>2.185(1)</td>
</tr>
</tbody>
</table>

3.2.4. Examination of the Binding Mode of the ZBGs in Solution

The solid state structures of these ZBGs clearly demonstrate strong bidentate chelation of the zinc(II) metal centers. NMR were acquired for all of the metal complexes to confirm ZBG coordination in solution. Large changes in the \(^1\)HNMR spectra between the free and bound ZBGs supports the stability of these complexes in solution. In the complex [(Tp^{Ph,Me})Zn(3-hydroxy-1-methyl-2(1H)-pyridinone)], significant shifts are observed in the \(^1\)HNMR spectra. An unambiguous upfield shift is noticed for all protons in the ZBG bound to metal center. These shifts are most likely the result of both the direct interaction of the chelator with the zinc(II) ion, and the inclusion of the ZBG into the aromatic, hydrophobic pocket created by the Tp^{Ph,Me} ligand. For example, the proton para to the carbonyl group shifts from 6.24 ppm in the free ZBG, to 6.01 ppm in the complex. In the complex [(Tp^{Ph,Me})Zn(3-hydroxy-2-
methyl-4-pyrole), the $^1$HNMR also demonstrates from 0.5 to greater than 1.0 ppm changes in the proton resonances of the ZBG. Significant changes are also observed in the spectra of [(Tp$^{\text{Ph,Me}}$)Zn(1-hydroxy-2(1H)-pyridinethione)] relative to the free ZBG where, again, in the $^1$HNMR spectrum, notable shifts in the protons of the ZBG are observed. The $^1$HNMR data suggests that the ligand binding observed in the solid state is preserved in solution at room temperature.

To further confirm the mode of binding, IR spectra were examined of the free ZBGs and were compared to the corresponding zinc complexes. Cast films from CHCl$_3$ solutions onto NaCl plates of the ZBGs and complexes allowed for measurement of the carbonyl stretching frequencies. Appropriate B-H stretches were observed for the [(Tp$^{\text{Ph,Me}}$)Zn(ZBG)] complexes. All six of the ZBGs showed shifts in the C=O stretch to lower energy when complexed as [(Tp$^{\text{Ph,Me}}$)Zn(ZBG)]. In the free ZBG 3-hydroxy-2-methyl-4-pyrene, the carbonyl (1624 cm$^{-1}$) exhibits a 27 cm$^{-1}$ shift to lower wavenumbers in the metal complex (Figure 3-8). Similarly, comparison of the spectra of 1-hydroxy-2(1H)-pyridinone and the complex [(Tp$^{\text{Ph,Me}}$)Zn(1-hydroxy-2(1H)-pyridinone)], demonstrates a carbonyl shift from 1643 cm$^{-1}$ for the free ZBG to 1625 cm$^{-1}$ for [(Tp$^{\text{Ph,Me}}$)Zn(1-hydroxy-2(1H)-pyridinone)]. The data here are consistent with other reports of changes in carbonyl stretching frequencies upon metal coordination.$^{[8-10]}$ All three ZBG classes (as defined above) demonstrate shifts in the carbonyl vibration consistent with the bidentate mode of binding found in the X-ray structures.
Figure 3-8. Infrared spectra for free (solid line) and bound (dashed line) 3-hydroxy-2-methyl-4-pyrone. The spectral region shown is of the carbonyl stretch, which shifts to lower energy upon metal coordination.

3.3. Discussion

It has been shown that tris(pyrazolyl)borate complexes are good structural models for the active site of MMPs.\textsuperscript{[6, 11-14]} Using [(Tp\textsuperscript{Ph,Me})ZnOH] to model of the MMP active site, a number of complexes were synthesized to examine new routes to inhibitor design. All six complexes show that the novel ZBGs presented herein bind in a bidentate fashion. The bond lengths of the ZBGs to the zinc center are comparable to those in [(Tp\textsuperscript{Ph,Me})Zn(acetohydroxamate)].\textsuperscript{[6]} A better understanding of the interactions between the zinc(II) ion and the inhibitor is a recognized component of MPI design.\textsuperscript{[15-20]} Using the successful hydroxamate ZBG as a starting point, six
new chelators were selected that were expected to bind as well as or better than hydroxamates. Hydroxypyridinones (HOPOs) were selected as lead compounds for several reasons. Hydroxypyridinones have a high structural homology to hydroxamic acids, and are known to be strong metal chelators.[21-25] In addition, the cyclic structure of hydroxypyridinones reduces the degrees of freedom in the ligand, preventing the cis- to trans- isomerization that can occur in hydroxamic acids, which ultimately detracts from the thermodynamic affinity of the metal-ligand interaction. The basicity of hydroxypyridinones varies between isomers, which potentially allows for tuning the protonation state of the ligand to accommodate possible hydrogen-bonding interactions in the protein active site.[26, 27] Finally, many hydroxypyridinones and related compounds have been or are used in medical and food industry applications, suggesting a reasonable level of biological tolerance for these chemical moieties.[4, 21, 23]

Several hydroxypyridinone derivatives are also described, including N-methylated hydroxypyridinones, a hydroxypyridinethione, and 3-hydroxy-2-methyl-4-pyrone. These ligands share many of the same features as the hydroxypyridinones, but demonstrate other interesting features as well. The N-methylated hydroxypyridinones, 3-hydroxy-1-methyl-2(1H)-pyridinone and 3-hydroxy-1,2-dimethyl-4(1H)-pyridinone, were examined to show that substitutions on the hydroxypyridinone ring did not effect the binding or present steric problems towards zinc binding. This is important to demonstrate because these ZBGs will need to be appended with a peptidomimetic backbone in order to prepare a fully-functional inhibitor. The hydroxypyridinethione, 1-hydroxy-2(1H)-pyridinethione, was examined to show that these sulfur derivatives
also bind in a bidentate fashion to the zinc center. Sulfur-containing ligands of this sort may be very good ZBGs due to the apparent thiophilicity of zinc(II).\cite{3,28} Indeed, 1-hydroxy-2(1\textit{H})-pyridinethione has been used as an zinc ionophore in several studies to increase the zinc(II) concentrations in cells and to interfere with zinc(II) dependent cellular processes.\cite{29,30} Similarly, other thiol-based MPIs have been studied, and have shown reasonably good activity when compared to hydroxamate-based inhibitors.\cite{16,17,31,32}

Combining the best features of both hydroxamates and thiol inhibitors into a single ZBG such as 1-hydroxy-2(1\textit{H})-pyridinethione should result in promising leads for future inhibitor design. Finally, 3-hydroxy-2-methyl-4-pyrone or “maltol” (an FDA approved food additive) was examined as a ZBG. Maltol also binds in a bidentate fashion with similar bond lengths to that of the other ZBGs. Maltol is an attractive ZBG due in particular to its good biological tolerance as evidenced by use as a food additive\cite{1,2} and potential therapeutic applications as an insulin mimetic when complexed with vanadate.\cite{4,5} These novel ZBGs demonstrated strong bidentate binding similar to that found in the complex \([\text{(Tp}^{\text{Ph,Me}}\text{)}\text{Zn(acetohydroxamate)})\], which has been shown to be an accurate model for the MMP-inhibitor complex (Table 3-3).\cite{6}
3.4. Conclusion

A shift in the design process focused on higher affinity, more selective zinc-binding groups will aid in future drug development breakthroughs. We have successfully synthesized and characterized six [(Tp^{Ph,Me})Zn(ZBG)] complexes, in an effort to demonstrate that new ZBGs are available for incorporation into MPIs. With binding comparable to the standard hydroxamate group, these alternative ZBGs give some fresh directions for the difficult endeavor of efficacious MPI design.

3.5. Experimental Section

General. Unless otherwise noted, starting materials were obtained from commercial suppliers (Aldrich) and used without further purification. [(T^{Ph,Me})ZnOH]^6 and 3-hydroxy-1-methyl-2(1H)-pyridinone^{21} were synthesized as previously described. Elemental analysis was performed at the University of California, Berkeley Analytical Facility. \(^{1}H/^{13}C\)NMR spectra were recorded on a Varian FT-NMR spectrometer running at 300 or 400 MHz at the Department of Chemistry and Biochemistry, University of California, San Diego. Infrared spectra were collected on a Nicolet AVATAR 320 FT-IR instrument at the Department of Chemistry and Biochemistry, University of California, San Diego. Caution! Perchlorate salts of metal complexes with organic ligands are potentially explosive. Only small amounts of these materials should be prepared and they should be handled with great care.
[(Tp\textsuperscript{Ph,Me})Zn(1-hydroxy-2(1\textit{H})-pyridinone)]. In a 100 mL round-bottom flask, [(Tp\textsuperscript{Ph,Me})ZnOH] (145 mg, 0.26 mmol) was added to 10 mL of CH\textsubscript{2}Cl\textsubscript{2}. To this solution was added 1.0 equiv of 1-hydroxy-2(1\textit{H})-pyridinone (29 mg, 0.26 mmol) dissolved in 15 mL of MeOH. The mixture was stirred at room temperature overnight under a nitrogen atmosphere. After stirring, the turbid solution was evaporated to dryness on a rotary evaporator to give a white solid. The solid was dissolved in a minimum amount of benzene (~15 mL), filtered to remove any insoluble material, and the filtrate was recrystallized by diffusion of the solution with pentane. Yield: 94%.

\textsuperscript{1}HNMR (CDCl\textsubscript{3}, 400 MHz, 25 °C): \( \delta \) 2.51 (s, 9H, pyrazole-CH\textsubscript{3}), 5.78 (d, \( J = 8.0 \) Hz, 1H, pyridinone-H), 6.10 (t, \( J = 6.4 \) Hz, 1H, pyridinone-H), 6.17 (s, 3H, pyrazole-H), 6.84 (t, \( J = 7.0 \) Hz, 1H, pyridinone-H), 7.10 (m, 9H, phenyl-H), 7.58 (d, \( J = 6.8 \) Hz, 6H, phenyl-H), 7.67 (d, \( J = 8.0 \) Hz, 1H, pyridinone-H). \textsuperscript{13}CNMR (CDCl\textsubscript{3}, 100 MHz, 25°C): \( \delta \) 13.0, 104.4, 106.8, 113.8, 127.2, 127.6, 127.3, 127.6, 131.4, 132.8, 134.0, 144.7, 152.6, 159.9 (C=O). IR (film from CHCl\textsubscript{3}): \( \nu \) 1370, 1537, 1625, 2546 (B-H) cm\textsuperscript{-1}.

Anal. Calcd for C\textsubscript{35}H\textsubscript{32}BN\textsubscript{7}O\textsubscript{2}Zn: C, 63.80; H, 4.90; N, 14.88. Found C, 63.69; H, 4.91; N, 15.14.

[(Tp\textsuperscript{Ph,Me})Zn(3-hydroxy-2(1\textit{H})-pyridinone)]. The same procedure was used as in the synthesis of [(Tp\textsuperscript{Ph,Me})Zn(1-hydroxy-2(1\textit{H})-pyridinone)]. Yield: 76%.

\textsuperscript{1}HNMR (CDCl\textsubscript{3}, 400 MHz, 25 °C): \( \delta \) 2.51 (s, 9H, CH\textsubscript{3}, pyrazole-CH\textsubscript{3}), 6.00 (m, 1H, pyridinone-H), 6.08 (t, \( J = 7.4 \) Hz, 1H, pyridinone-H), 6.18 (s, 3H, pyrazole-H), 6.39 (d, \( J = 6.8 \) Hz, 1H, pyridinone-H), 7.10 (t, \( J = 6.0 \) Hz, 9H, phenyl-H), 7.61 (m, 6H, phenyl-H). \textsuperscript{13}CNMR (CDCl\textsubscript{3}, 100 MHz, 25 °C): \( \delta \) 13.0, 104.2, 110.2, 113.6, 113.8, 127.1, 127.4, 127.5, 132.4, 144.7, 152.5, 155.8, 162.6 (C=O). IR (film from CHCl\textsubscript{3}):
ν 1305, 1546, 1619, 2547 (B-H) cm⁻¹. Anal. Calcd for C₃₅H₃₂BN₇O₂Zn: C, 63.80; H, 4.90; N, 14.88. Found C, 63.61; H, 5.03; N, 14.91.

[(Tp²Ph,Me)Zn(3-hydroxy-1-methyl-2(1H)-pyridinone)]. The same procedure was used as in the synthesis of [(Tp²Ph,Me)Zn(1-hydroxy-2(1H)-pyridinone)]. Yield: 80%. ¹H NMR (CDCl₃, 400 MHz, 25 °C): δ 2.55 (s, 9H, pyrazole-CH₃), 3.50 (s, 3H, pyridinone-CH₃), 6.01 (t, J = 6.8 Hz, 1H, pyridinone -H), 6.24 (s, 3H, pyrazole-H), 6.68 (t, J = 5.6 Hz, 1H pyridinone-H), 7.22 (m, 9H, phenyl-H), 7.45 (d, J = 5.6 Hz, 1H pyridinone-H), 7.56 (d, J = 6.8 Hz, 6H, phenyl-H). ¹³C NMR (CDCl₃, 100 MHz, 25 °C): δ 13.0, 37.7, 104.0, 105.3, 128.1, 128.2, 128.6, 128.9, 130.6, 135.8, 145.8, 153.9. IR (film from CHCl₃): ν 1301, 1371, 1573, 2544 (B-H) cm⁻¹. Anal. Calcd for C₃₆H₃₄N₇O₂BZn·C₆H₆·H₂O: C, 65.60; H, 5.50; N, 12.75. Found C, 65.65; H, 5.41; N, 12.84.

[(Tp²Ph,Me)Zn(3-hydroxy-1,2-dimethyl-4(1H)-pyridinone)]. The same procedure was used as in the synthesis of [(Tp²Ph,Me)Zn(1-hydroxy-2(1H)-pyridinone)]. Yield: 22%. ¹H NMR (CDCl₃, 400 MHz, 25 °C): δ 2.00 (s, 3H, pyridinone-CH₃), 2.51 (s, 9H, pyrazole-CH₃), 3.57 (s, 3H, pyridinone-CH₃), 5.47 (d, J = 6.4 Hz, 1H, pyridinone-H), 6.16 (s, 3H, pyrazole-H), 6.66 (d, J = 6.0 Hz, 1H, pyridinone-H), 7.04 (m, 9H, phenyl-H), 7.61 (d, J = 5.6 Hz, 6H, phenyl-H). ¹³C NMR (CDCl₃, 100 MHz, 25 °C): δ 12.4, 13.0, 42.4, 104.4, 107.4, 126.8, 127.3, 127.6, 128.2, 132.9, 144.6, 152.5. IR (film from CHCl₃): ν 1367, 1553, 1594, 2547 (B-H) cm⁻¹. Anal. Calcd for C₃₇H₃₆BN₇O₂Zn·H₂O: C, 63.04; H, 5.43; N, 13.91. Found C, 62.89; H, 5.39; N, 13.66.
[(Tp^{Ph,Me})Zn(1-hydroxy-2(1H)-pyridinethione)]. The same procedure was used as in the synthesis of [(Tp^{Ph,Me})Zn(1-hydroxy-2(1H)-pyridinone)]. Yield: 70%.

$^1$HNMR ($d^6$-benzene, 400 MHz, 25 ºC): δ 2.26 (s, 9H, pyrazole-CH$_3$), 5.50 (t, $J = 7.0$ Hz, 1H, pyridinethione-H), 6.01 (s, 3H, pyrazole-H), 6.03 (t, $J = 4.8$ Hz, 1H, pyridinethione-H), 6.67 (d, $J = 8.0$ Hz, 1H, pyridinethione-H), 6.90 (t, $J = 6.2$ Hz, 3H, phenyl-H), 7.01 (t, $J = 6.8$ Hz, 6H, phenyl-H), 7.18 (m, 1H, pyridinethione-H), 7.81 (d, $J = 8.0$ Hz, 6H, phenyl-H).

$^{13}$CNMR (CDCl$_3$, 100 MHz, 25 ºC): δ 13.0, 104.9, 116.0, 125.6, 127.1, 127.4, 128.0, 128.8, 132.9, 135.2, 144.4, 152.9. IR (film from CHCl$_3$): ν 1456, 1546, 1596, 2551 (B-H) cm$^{-1}$. Anal. Calcd for C$_{35}$H$_{32}$N$_7$OSBZn: C, 62.28; H, 4.78; N, 14.53. Found C, 62.17; H, 4.89; N, 14.79.

[(Tp^{Ph,Me})Zn(3-hydroxy-2-methyl-4-pyrone)]. The same procedure was used as in the synthesis of [(Tp^{Ph,Me})Zn(1-hydroxy-2(1H)-pyridinone)]. Yield: 61%.

$^1$HNMR (CDCl$_3$, 400 MHz, 25 ºC): δ 2.23 (s, 3H, pyrone-CH$_3$), 2.51 (s, 9H, pyrazole-CH$_3$), 5.29 (d, $J = 5.2$ Hz, 1H, pyrone-H), 6.17 (s, 3H, pyrazole-H), 7.10 (m, 9H, phenyl-H), 7.18 (d, 1H, , $J = 5.2$ Hz, pyrone-H), 7.59 (d, $J = 4.0$ Hz, 6H, phenyl-H).

$^{13}$CNMR (CDCl$_3$, 100 MHz, 25 ºC): δ 13.0, 14.7, 104.3, 109.2, 127.0, 127.4, 127.5, 128.2, 132.7, 144.7, 150.4, 152.5. IR (film from CHCl$_3$): ν 1282, 1455, 1597, 2544 (B-H) cm$^{-1}$. Anal. Calcd for C$_{36}$H$_{33}$N$_6$O$_3$BZn: C, 64.16; H, 4.94; N, 12.47. Found C, 64.74; H, 5.03; N, 12.23.

**X-Ray Crystallographic Analysis.** Data were collected on a Bruker AXS area detector diffractometer. Crystals were mounted on quartz capillaries by using Paratone oil and were cooled in a nitrogen stream (Kryo-flex controlled) on the diffractometer (-173ºC). Peak integrations were performed with the Siemens SAINT
software package. Absorption corrections were applied using the program SADABS. Space group determinations were performed by the program XPREP. The structures were solved by direct or Patterson methods and refined with the SHELXTL software package. Unless noted otherwise, all hydrogen atoms, except for the boron hydrogen atoms, were fixed at calculated positions with isotropic thermal parameters; all non-hydrogen atoms were refined anisotropically.

\((\text{Tp}^{\text{Ph,Me}})\text{Zn}(1\text{-hydroxy-2(1H)-pyridinone})\). Colorless blocks were grown out of a solution of the complex in benzene diffused with pentane (Table 3-1). The hydrogen atom on the boron was found in the difference map and the position was refined.

\((\text{Tp}^{\text{Ph,Me}})\text{Zn}(3\text{-hydroxy-2(1H)-pyridinone})\). Colorless blocks were grown within a few minutes from a solution of the complex in benzene diluted with pentane (Table 3-1). The hydrogen atom on the boron was found in the difference map and its position was refined. The complex co-crystallized with one molecule of benzene that was refined to half occupancy in the asymmetric unit. The complex also contained a disordered phenyl ring on one of the pyrazole arms that was refined in two orientations (partial occupancy 55:45 split). There was no disorder observed in the coordination environment. No hydrogen atoms were calculated or refined for the disordered phenyl ring.

\((\text{Tp}^{\text{Ph,Me}})\text{Zn}(3\text{-hydroxy-1-methyl-2(1H)-pyridinone})\). Colorless blocks were grown out of a solution of the complex in benzene diffused with pentane (Table 3-1). The hydrogen atom on the boron was found in the difference map and the position was refined. The complex co-crystallized with one disordered molecule of
benzene in the asymmetric unit. No hydrogen atoms were calculated or refined for the disordered benzene solvent molecule.

\[(\text{Tp}^{\text{Ph,Me}}\text{Zn}(3\text{-hydroxy-1,2-dimethyl-4(1H)-pyridinone})]\). Colorless blocks were grown out of a solution of the complex in benzene diffused with pentane (Table 3-2). The hydrogen atom on the boron was found in the difference map and the position was refined.

\[(\text{Tp}^{\text{Ph,Me}}\text{Zn}(1\text{-hydroxy-2(1H)-pyridinethione})]\). Colorless blocks were grown out of a solution of the complex in benzene diffused with pentane (Table 3-2). The hydrogen atoms on the boron atoms were found in the difference map and their positions were refined. The asymmetric unit contains four molecules of the complex.

\[(\text{Tp}^{\text{Ph,Me}}\text{Zn}(3\text{-hydroxy-2-methyl-4-pyrone})]\). Colorless blocks were grown out of a solution of the complex in benzene diffused with pentane (Table 3-2). The hydrogen atom on the boron was found in the difference map and the position was refined. The complex co-crystallized with one half molecule of benzene in the asymmetric unit.

### 3.6. Acknowledgements

Text, schemes, and figures of this chapter, in part, are reprints of the materials published in the following paper: Puerta, D.T. and Cohen, S.M., "Examination of Novel Zinc-Binding Groups for Use in Matrix Metalloproteinase Inhibitors" *Inorg. Chem.* 2003, 42, 3423-3430. The dissertation author was the primary researcher and author. The permission to reproduce this paper was granted by the American Chemical Society. Copyright 2003, American Chemical Society.
3.7. References


Chapter 4. Examination of the Inhibitory Potency and Toxicity of Non-Hydroxamate ZBGs
4.1. Introduction

The inability of hydroxamates to produce clinically viable compounds has been attributed in part to low oral availability, poor in vivo stability, and undesirable side effects associated with these compounds.\cite{1, 2} In an attempt to find alternatives to the hydroxamic acid zinc-binding group (ZBG), eleven compounds were identified as ligands for use in MPIs (Figure 4-1, compounds 1-11).\cite{3-5} The compounds were selected on the basis of several factors, which have been described in chapters 1 and 3. These compounds preserve the positive characteristics of hydroxamates, such as their ability to form monoanionic five-member chelates. Furthermore, these proposed ZBGs benefit from superior properties including: (a) better hydrolytic stability originating from cyclic structures, (b) potentially improved biological tolerance (e.g., compound 5 is a food additive, maltol), and (c) proposed increased affinity for the MMP zinc(II) ion due to ligand rigidity\cite{6} and zinc thiophilicity.\cite{7} In light of these criteria, the ligands selected consisted of hydroxypyridinones (1-3, 6), hydroxypyridinethiones (7-9), pyrones (4, 5), and thiopyrones (10, 11).\cite{4, 5, 8, 9}
Figure 4-1. Heterocyclic ZBGs proposed for use in MMP inhibitors. AHA was utilized as a benchmark ZBG.

The potencies of the proposed ZBGs were tested by in vitro inhibition assays with recombinant MMP-1, MMP-2, and MMP-3 and in a cell culture assay using neonatal rat cardiac fibroblast (CF) cells. In both recombinant and cell culture assays, the new ZBGs were found to be effective inhibitors, typically 10- to 100-fold more potent than acetohydroxamic acid (AHA), the representative chelator for the majority of current MPIs.\cite{10-13} The toxicity of these chelators was examined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) cytotoxicity assays, which demonstrate that most of these compounds are non-toxic at concentrations of almost 100 µM. Finally, thione ZBGs were shown to be effective inhibitors of cell invasion through an extracellular matrix membrane. The data presented herein suggests these heterocyclic ZBGs are potent, non-toxic, and biocompatible compounds that show promise for incorporation into a new family of MMP inhibitors.
4.2. Results and Discussion

4.2.1. In Vitro Fluorescence and Colorimetric Assays of MMPs 1-3

Initially, the compounds in Figure 4-1 were tested for ability to inhibit MMP-3 using a commercially available fluorescence-based assay.\cite{14} In this assay, a self-quenched fluorescent MMP substrate is cleaved by the enzyme, separating the quenching group from the fluorescent group.\cite{14} The result of substrate cleavage is a fluorescent signal that can be measured with a fluorescence plate reader in a 96-well format (Figure 4-2). \textbf{AHA} was used as a representative benchmark ZBG found in most inhibitors.\cite{10-13} All the compounds tested showed greater inhibitory activity than \textbf{AHA}; compounds 1-6 demonstrated inhibitory activities 3- to 16-fold greater than \textbf{AHA}, while the most marked improvement in activity was observed with compounds 7-11 that were 70- to 700-fold more potent (Table 4-1). To confirm the potency of these novel ZBGs, a selected number (1, 4-7) of those used in the initial study were examined in a commercially available colorimetric-based MMP-3 assay.\cite{15} The IC$_{50}$ values (Table 4-1) obtained from this experiment closely parallel those found in the fluorescent assay, with the exception of compound 5, which displayed a three-fold lower potency in the colorimetric assay.
Figure 4-2. Representative data generated from fluorescent assays of 11. A) Raw data from fluorescent assay. Relative fluorescent units (RFU) produced from substrate cleavage in each reaction well: control (0 mM 11, red squares), 11 0.05 mM (blue circles), 11 0.1 mM (green circles), 11 0.2 mM (orange circles), 11 0.3 mM (magenta circles), 11 0.5 mM (yellow circles), and 11 1.0 mM (gray circles) are plotted versus time in minutes. B) IC₅₀ plot of 11. The slopes of kinetic traces from plot A containing inhibitor are compared to the slope of the control (no inhibitor). The [(inhibitor slope/control slope) × 100] (% Control) is plotted versus inhibitor concentration. A linear fit of the data for each experiment (three experiments shown in plot B) gives the IC₅₀ value of the inhibitor where y = 50%.

It is important to note that the modest IC₅₀ values of these compounds are due to the lack of a peptidomimetic backbone; this is best exemplified by AHA that has an IC₅₀ value of ~25 mM alone (against MMP-3), but when attached to the appropriate backbone has generated many nanomolar potency inhibitors.⁹,¹¹ The affinity for the individual components of an MPI (ZBG vs. peptidomimetic backbone) has been revealed by the SAR-by-NMR method, which showed a greater than additive effect by linking the two components together.¹²,¹³

With the promising inhibitory results from the two different assay experiments, we sought to demonstrate that compounds 1-11 inhibit MMPs by zinc(II) chelation
and not through interactions specific to the protein backbone. Therefore, two additional MMPs (MMP-1 and MMP-2) were assayed using the same commercially available fluorescence-based assay.\textsuperscript{[14]} The zinc(II)-tris(histidine) hydrolytic active site is conserved throughout the MMP family, but the surrounding subsites in MMPs are not. MMP-1 (collagenase-1) and MMP-2 (gelatinase A) have distinct active sites and both differ from that of MMP-3 (stromelysin-1).\textsuperscript{[10]} The shallow S1’ pocket in MMP-1 differs distinctly from the large hydrophobic channel found in MMP-2 and MMP-3. Most synthetic inhibitors of MMPs have exploited the S1’ pocket to obtain selectivity over MMP-1.\textsuperscript{[10, 11]} MMP-2 and MMP-3 are more closely related; however, there are disparities in the S3’ subsite of the two proteinases. Inhibitors have been designed that are selective for MMP-2 over MMP-3 by exploiting the differences in the S3’ subsite.\textsuperscript{[16, 17]} In addition to structural differences among these MMPs, the catalytic activities of these enzymes show divergent dependences on pH. MMP-3 has optimal catalytic activity at pH 6, while MMP-1 and MMP-2 are most efficient at pH 7.5.\textsuperscript{[18]}

A subset (1, 2, 5, 7-9, 11) of the compounds from our initial study was tested against MMP-1 and MMP-2. This subset represents all of the ligand motifs examined in the initial study: acetohydroxamic acid (\textbf{AHA}, control), hydroxypyridinones (1, 2), pyrones (5), hydroxypyridinethiones (7-9), and thiopyrones (11) (Figure 4-1). Consistent with inhibitory data on MMP-3, the compounds tested (1, 2, 5, 7-9, 11) were more potent inhibitors of MMP-1 and MMP-2 than AHA (Table 4-1). The O,O donor ligands (1, 2 and 5) were 4- to 10-fold more potent inhibitors of MMP-1 and 3-
to 6-fold more potent inhibitors of MMP-2 than AHA (Figure 4-3). The O,S ligands (7-9, 11) showed notable improvements in inhibition, where they demonstrated a 60- to 280-fold increase in potency over AHA for MMP-1 and a 40- to 260-fold increase for MMP-2. The apparent decrease in efficacy of the compounds against MMP-1 and MMP-2 compared to MMP-3 may be due to the differences in the assay conditions. The assays are run at the optimal pH values for each enzyme; MMP-3 is most active at pH 6, while MMP-1 and MMP-2 are most active at pH 7.5. The change in pH from 6 to 7.5 may have an effect on the protonation state of either the inhibitor or amino acid side chains in the active site of the MMPs. It has been shown that the protonation state of a catalytic glutamate residue in the active site strongly affects the potency of inhibitors. A more acidic inhibitor (e.g. carboxylate) will be less potent at a higher pH, where the glutamate residue is deprotonated, while a basic inhibitor (e.g. hydroxamate) will be less potent when the pH of the system is lower than the pKₐ (~5.6) of the glutamate residue. The two most acidic ZBGs 1 and 7 (pKₐ 5.8 and 4.6, respectively), display the most significant decrease in potency, which is consistent with the aforementioned trends. The other ZBGs examined (including AHA) have pKₐ values that are more basic (~8.1-9.5), and are less affected by the increase in pH. Nevertheless, the overall inhibitory trend for the different ZBGs is consistent for all three MMPs.
Table 4-1. IC$_{50}$ values (mM) for ZBGs against MMP-1, MMP-2, MMP-3, and in CF cell culture. Compound 3 was not evaluated due to poor aqueous solubility. Values based on at least three independent experiments.

<table>
<thead>
<tr>
<th>ZBG</th>
<th>MMP-1$^a$</th>
<th>MMP-2$^a$</th>
<th>MMP-3$^a$</th>
<th>MMP-3$^b$</th>
<th>CF Culture$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHA</td>
<td>41600(±400)</td>
<td>15000(±3000)</td>
<td>25000(±4000)</td>
<td>8700(±400)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5960(±40)</td>
<td>5600(±100)</td>
<td>1600(±100)</td>
<td>1500(±100)</td>
<td>3240(±140)</td>
</tr>
<tr>
<td>2</td>
<td>4200(±300)</td>
<td>2600(±400)</td>
<td>5100(±200)</td>
<td>2430(±130)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>N/D</td>
<td>N/D</td>
<td>7200(±1200)</td>
<td>8300(±900)</td>
<td>850(±40)</td>
</tr>
<tr>
<td>5</td>
<td>4200(±300)</td>
<td>2600(±100)</td>
<td>5700(±100)</td>
<td>16000(±2000)</td>
<td>273(±6)</td>
</tr>
<tr>
<td>6</td>
<td>N/D</td>
<td>N/D</td>
<td>5700(±200)</td>
<td>5000(±1000)</td>
<td>2870(±90)</td>
</tr>
<tr>
<td>7</td>
<td>490(±10)</td>
<td>100(±40)</td>
<td>35(±3)</td>
<td>20(±4)</td>
<td>790(±30)</td>
</tr>
<tr>
<td>8</td>
<td>680(±20)</td>
<td>380(±10)</td>
<td>362(±3)</td>
<td></td>
<td>135(±2)</td>
</tr>
<tr>
<td>9</td>
<td>150(±10)</td>
<td>60(±10)</td>
<td>140(±20)</td>
<td></td>
<td>51(±2)</td>
</tr>
<tr>
<td>10</td>
<td>N/D</td>
<td>N/D</td>
<td>120(±40)</td>
<td></td>
<td>125(±2)</td>
</tr>
<tr>
<td>11</td>
<td>400(±10)</td>
<td>140(±10)</td>
<td>210(±20)</td>
<td></td>
<td>86(±2)</td>
</tr>
</tbody>
</table>

$^a$ values obtained from a fluorescence-based assay; $^b$ values obtained from a colorimetric-based assay; N/D: not determined.

On average, the ZBGs were 2-fold more potent against MMP-2 than MMP-1 (Figure 4-3). Control experiments without inhibitor showed that MMP-1 is less active than MMP-2 (data not shown). Assuming that the ZBGs act as competitive inhibitors, which is consistent with a hypothesis of active site metal binding, then it would be expected that inhibition of the less active MMP-1 will exhibit higher IC$_{50}$ values relative to MMP-2. The fact that AHA exhibits the same difference in potency
between MMP-1 and MMP-2 supports the hypothesis that the differences in IC$_{50}$ values between MMPs are due to differences in enzyme activity and not because of non-specific protein interactions of the ZBGs. All of the compounds presented here are small, simple molecules with little functionality and are unlikely to have any strong, specific interactions with the protein subsites. Overall, compared to AHA, the compounds tested here proved to be superior inhibitors of MMP-1, MMP-2, and MMP-3. The inhibition of three different classes of MMPs by these compounds with similar IC$_{50}$ values strongly suggests that these ligands inhibit the enzyme through enhanced zinc(II) binding and not through non-covalent or other unanticipated protein interactions. This conclusion is further supported by the correlation of IC$_{50}$ values with relative binding affinities of these chelators toward a MMP model complex.[21]

Figure 4-3. Plot of the IC$_{50}$ values of the compounds (AHA, 1-11, excluding 3) against MMP-1 (black), MMP-2 (crossed), and MMP-3 (gray). Inset highlights the most potent compounds (7-11).
4.2.2. MMP Assays in Cell Culture of Neonatal Rat Cardiac Fibroblasts

The promising inhibitory potential of these compounds against recombinant MMPs led us to examine their activities in a cellular assay. CF cultures were selected for evaluating new ZBGs based on two primary reasons. First, CF cells have a well documented capacity to produce and secrete a variety of MMPs into culture media in zymogen form, including MMP-2 and MMP-9.[22] The CF secreted MMPs are amenable to activation by several mechanisms, including protease mediated cleavage by enzymes such as plasminogen.[23] Addition of plasminogen activates MMPs by first itself being cleaved by urokinase plasminogen activator (uPA) present on the cell surface and then cleaving the prodomain of the MMP.[24] A second reason for using CF cultures is an increasing body of experimental work indicating that the use of MPIs may have a therapeutic benefit following myocardial infarction.[25]

The compounds shown in Figure 4-1 were analyzed for the ability to inhibit plasmin-activated MMPs produced by CFs in culture. All of the new ZBGs showed greater potency than AHA, with 9 demonstrating the strongest inhibition (Table 4-1). The order of potency largely paralleled that seen in vitro using purified MMPs. Of notable exception, 5 demonstrated significantly greater potency while 7 showed markedly reduced potency in CF culture. These discrepancies may be due to interactions of these compounds with other components of the cellular media. At the concentrations of ZBG used to inhibit these enzymes these compounds will complex loosely bound metal ions in the culture media and may inhibit metalloenzymes other
than MMPs, which might perturb the observed efficacy in this assay. Therefore, assessing the MMP inhibitory capacity of new compounds in culture provides additional information regarding their efficacy toward a ‘physiological’ mix of MMPs that might remain concealed by in vitro assessment alone.

4.2.3. Toxicity Studies of Non-Hydroxamate ZBGs

Compounds 1-11 have been shown both in vitro and in cell culture to be potent, broad-spectrum inhibitors of MMPs (compound 3 has not been completely evaluated due to poor aqueous solubility). We sought to address the question of cytotoxicity and biocompatibility by using a cell viability assay. In this assay, cellular viability is monitored by the reduction of MTT or MTS by mitochondrial enzymes, producing a characteristic blue color. Several of the O,S donor atom ZBGs (7-11) were found to react with MTT and MTS, producing false positives for cell viability in our initial assay attempts. A control experiment between MTT and compounds 7-11 in the absence of CF cells confirmed this reactivity (data not shown). To eliminate this undesired side reaction, the assay protocol was slightly modified by introducing an additional wash step prior to MTT/MTS addition. After incubating the CF cells with inhibitors for 24 h, the cells were washed with fresh media to remove any excess inhibitors from the wells. Subsequently, the MTT or MTS was added as per the standard protocol to evaluate the viability of the CF cells. This simple rinse step gave reproducible MTT and MTS assay results; the assays along with photomicrographs of the CF cells were used for evaluation of inhibitor cytotoxicity. Figure 4-4 shows the
results of the MTS experiments with CF cells. The new ZBGs showed varying degrees of cytotoxicity, with the sulfur-containing compounds (7-11) generally being more toxic. All of the compounds showed low toxicity at concentrations up to 100 µM (with the exception of 7), and compounds 1-6 were comparable to AHA. The MTT assay gave similar results to those obtained with MTS, although all of the compounds (including AHA) showed lower cell viability, particularly at high concentrations (data not shown). Photomicrographs shown in Figure 4-5 are representative of cell images obtained from control (i.e. untreated) CFs, 0.1 mM, and 1.0 mM doses of compound 9. At the 0.1 mM dose of 9, CFs showed little evidence of disruption in cell morphology and are comparable to those of untreated cells. In contrast, at the 1.0 mM dose of 9, CFs demonstrated disruption of normal cell morphology and evidence of cell death. For all ZBGs, visual evidence for disruption of cell morphology was obtained only at doses $\geq$1.0 mM (data not shown). In summary, none of the compounds examined are acutely toxic, which indicates they are viable moieties for incorporation into full MPIs.
Figure 4-4. MTS cell viability assay results. Plot of the relative toxicities of the compounds (AHA, 1-11, excluding 3) in neonatal rat cardiac fibroblasts at concentrations of: 0.001 (black), 0.01 (white), 0.1 (crossed), and 1.0 (gray) mM of the ZBG listed. Colorimetric assay values were normalized to those of untreated cells.

Figure 4-5. Photomicrograph of CF cells in the absence (A) and presence of two concentrations of compound 9: 0.1 mM (B) and 1.0 mM (C). The image on the right is representative of those observed in cells exposed to high concentrations of several of the ZBGs.
4.2.4. Potency of ZBGs in a Cell Invasion Assay

Several of the ZBGs in Figure 4-1 were tested for their ability to inhibit a biological MMP-dependent process, namely the invasion of fibrosarcoma HT1080 cells through a membrane comprised of ECM proteins. In the cell invasion assay, cells move from an upper to a lower chamber as a function of their ability to degrade a synthetic membrane comprised of ECM proteins. Thus, this cell invasion process is dependent on MMP activity. A standard cell invasion assay kit was used to examine this phenomenon and the results are summarized in Figure 4-6. We chose the highest non-toxic concentrations of ZBGs (as determined in the fibroblast toxicity study) for use in the invasion assay. Most of the O,O donor ligands (AHA, 2, and 5) had little or no effect on invasion at a concentration of 1 mM; however the O,O ligand compound 1 did inhibit invasion by 70% at this same concentration (data not shown). The O,S donor ZBGs (8, 9, and 11) inhibited invasion by 52-62% at a concentration of 100 µM, while compound 7 inhibited invasion by 53% at a concentration of 50 µM. The ability of these compounds to inhibit cell invasion correlates well with their in vitro ability to inhibit MMPs, where the O,S donor compounds are far more potent MMP inhibitors. These experiments demonstrate the ability of these compounds to inhibit an MMP-dependent process in an in vitro model of cell invasion.
Figure 4-6. Cell invasion assay results. Plot of relative fluorescence units (RFU, 516 nm) for several ZBGs; an increase in fluorescence indicates more invasion of fibrosarcoma HT1080 cells through an ECM membrane. The control contains no inhibitor, for all others: AHA (1 mM), 7 (50 μM), 8 (100 μM), 9 (100 μM), and 10 (100 μM).

4.3. Conclusion

The past three decades of MPI development has yielded a vast number of compounds with a variety of backbone substituents that are designed to interact with specific MMP subsites. In comparison, little progress has been made on improving the ZBG of the inhibitor that binds directly to the ubiquitous active site zinc(II) ion. With the limitations of hydroxamic acids as a ZBG clearly established, it is increasingly apparent that improved ligands must be developed for second generation MPIs.\textsuperscript{[11, 13, 26]} As part of an ongoing study of new chelators for use in MPIs, the inhibitory properties of compounds 1-11 have been evaluated against MMPs under a
variety of conditions. Recombinant MMP and cell culture inhibition assays confirm that these compounds are effective inhibitors of MMPs that likely bind to the active site zinc(II) ion. Cytotoxicity assays performed on primary cultures of cardiac fibroblasts show these chelators are essentially non-toxic at micromolar concentrations. The O,S donor ligands can inhibit cellular invasion through a ECM membrane in vitro. In summary, the experiments presented here show that potent, non-toxic, and biocompatable alternatives to the hydroxamic acid chelator are available for use in MPIs.

4.4. Experimental Section

**Preparation of ZBGs.** Compounds 1-11 were purchased from a commercial supplier or prepared as previously described.\(^4,5\) \(^1\)H NMR spectra of the O,S ligands (7-11) were used to show that only monomeric species of each ligand were present in solution. For all compounds, the hydroxyl proton was present in the \(^1\)H NMR spectra and the spectral data on 9 was also consistent with published values for the monomeric O,S ligand.\(^27\) No dimer-associated resonances were present in the NMR spectra and no dimer peaks were found in the mass spectrometry analyses, indicating that the compounds are pure as synthesized.

**Fluorescence-Based Recombinant MMP-3 Assays.** MMP-3 (\textit{E. coli} recombinant human stromelysin catalytic domain, aa 83-255) activity was measured utilizing a 96-well microplate fluorescent assay kit purchased from Biomol Research Laboratories, following the procedure provided with the kit. Experiments were
performed using a Bio-Tek Flx 800 fluorescence plate reader and Nunc white 96-well plates. The inhibitors were dissolved in DMSO and further diluted (500-fold) into the assay buffer (50 mM MES, 10 mM CaCl₂, 0.05% Brij-35, pH 6.0). MMP-3 (~20 nM) was incubated with varying concentrations of inhibitors for 1 h at 37 °C, followed by addition of substrate to initiate the assay. The reactions were agitated by shaking for 1 sec after each fluorescence measurement. Upon cleavage of the fluorescent substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ (0.4 mM concentration in assay, Mca = (7-methoxycoumarin-4-yl)-acetyl, Dpa = N-3-(2,4-dinitrophenyl)-L-α-β-diaminopropionyl) at the Gly-Leu bond, Mca fluorescence (λₑₓ = 335 nm, λₑₘ = 405 nm) was measured at 60-second intervals for 15-18 minutes.

To determine the quenching/competitive absorption properties of the inhibitors and possible effects on the assay data, control experiments were performed. Fluorescence intensity measurements at varying concentrations of fluorescent calibration standard (~80-400 nM of Mca-Pro-Leu-OH) in the presence of the different inhibitors were obtained. Standard assay conditions (as described above) were used, but no enzyme was added to the wells in these fluorescence control experiments. The concentrations of calibration standard (100 nM, 150 nM, 200 nM, and 300 nM) used in these controls were comparable to the concentrations of cleaved substrate produced during the assay. Three different concentrations of each inhibitor, near the IC₅₀ for the respective inhibitor, were measured under these conditions. Fluorescence measurements were taken at 1-minute intervals for 10 minutes and the values were averaged. The resulting RFUs (Relative Fluorescence Units) were plotted
against the concentration of the calibration standard (Figure 4-7, left). The slopes of the three lines corresponding to the three ZBG concentrations were plotted as the percentage of the control slope versus the concentration of ZBG (Figure 4-7, right). These three data points were fitted with a straight line and the linear fit \( y = mx + b \) (\( y = -299.72x + 85.399 \) for compound 11) (Figure 4-7 right) was used to determine the amount of fluorescence quenched from the ZBG. The IC\(_{50}\) values were then corrected based on the fluorescence quenched by each ZBG.

**Colorimetric-Based Recombinant MMP-3 Assays.** MMP-3 (E. coli recombinant human stromelysin catalytic domain, aa 83-255) activity was measured utilizing a 96-well microplate colorimetric assay kit purchased from Biomol Research Laboratories, following the procedure provided with the kit. Experiments were performed using a Bio-Tek µQuant colorimetric plate reader monitoring at 402 nm. The inhibitors were dissolved in DMSO and further diluted (500-fold) into the colorimetric assay buffer (50 mM MES, 10 mM CaCl\(_2\), 0.05% Brij-35, 1 mM DTNB, pH 6.0). MMP-3 (~20 nM) was incubated with varying concentrations of inhibitor at 37 °C for 1 h, followed by addition of substrate to initiate the assay. Cleavage of the thioester bond in the substrate Ac-Pro-Leu-Gly-[2-mercapto-4-methyl-pentanoyl]-Leu-Gly-OC\(_2\)H\(_5\) (1.0 mM concentration in assay) by MMP-3 produces a sulfhydryl group which reacts with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to form 2-nitro-5-thiobenzoic acid (\( \lambda_{max} = 412 \) nm). Absorption readings were taken at 60-second intervals for 60 min at room temperature.
To determine if the chelators reacted with the colorimetric assay components, control experiments were performed. The inhibitors examined were placed in colorimetric assay buffer (without the addition of MMP-3 or the colorimetric substrate), and the absorbance was compared to buffer alone. This was done to determine if the inhibitor showed a reaction with DTNB. Substrate (1.0 mM) was then added to the wells to monitor any additional reaction. Absorption readings were taken at 60-second intervals for 60 min at room temperature. The compounds examined in the assay had no reaction with the assay components.

**Fluorescence-Based Recombinant MMP-1 and MMP-2 Assays.** Activities of *E. coli* recombinant human MMP-1 catalytic domain (amino acids 81-249, 19.9 kDa) and MMP-2 catalytic domain (amino acids 81-423, 40 kDa) were measured utilizing a 96-well microplate fluorescent assay kit purchased from BIOMOL International, following the procedure provided with the kit. Experiments were performed using a Bio-Tek Flx 800 fluorescence plate reader and Nunc white 96-well plates. The compounds (Figure 4-1) were dissolved in DMSO and further diluted (500-fold) in assay buffer (50 mM HEPES, 10 mM CaCl$_2$, 0.05% Brij-35, pH 7.5). MMP-1 and MMP-2 were incubated individually with varying concentrations of different inhibitors for 1 h at 37 °C, followed by addition of substrate to initiate the assay. Reactions were agitated by shaking for 1 sec after each fluorescence measurement. Upon cleavage of the fluorescent substrate, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg$\text{NH}_2$ (0.4 mM in assay; Mca = 7-methoxycoumarin-4-yl)-acetyl; Dpa = N-3-(2,4-dinitrophenyl)-L-a-b-diaminopropionyl) at the Gly-Leu bond, Mca fluorescence ($\lambda_{ex} =$
335 nm, \( \lambda_{em} = 405 \) nm) was measured at 60-second intervals for 30 min. The IC\(_{50}\) values obtained were corrected for competitive absorption as described above.

**Cardiac Fibroblast Preparation.** Neonatal rat cardiac fibroblasts (CF) were prepared as previously described\([28]\). Briefly, CFs were prepared from hearts of 1-2 day old Sprague Dawley rats. Following collagenase digestions (4\( \times \)), non-myocyte cells (mostly fibroblasts) were isolated by Percoll density gradient. The cell suspension was plated onto uncoated tissue culture dishes for 30 min to allow preferential attachment of CFs to the bottom of the dish. The non-adherent cells were removed and fresh media (Dulbecco’s modified Eagle’s media +10% fetal bovine serum) was added. CF were allowed to proliferate to confluence and then trypsinized and frozen at \(-70^\circ\)C. CF stocks were freshly plated for each experiment and used from first or second passages. When reaching 90% confluence, cells were serum deprived for 24 h and then treated according to the experimental design (vide infra).

**Cell Culture Assays.** To activate CF MMPs, cells were treated with plasminogen (60 mg/mL, Sigma) for 16 h. MMP activity was determined by adding 10 mM of substrate (Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg) (BIOMOL International) in assay buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM CaCl\(_2\)) with 8 mg/mL aprotinin to neutralize residual plasmin activity, to the culture media. Fluorescence measurements were collected in a similar manner as described for the purified MMP assays (vide supra). Data were fit to the sigmoidal Hill equation \( y = \frac{[ZBG]}{([ZBG]+k)} \) using Prism 3.0 (GraphPad Software, San Diego, CA). In this equation \( y \) is the rate of substrate hydrolysis as a fraction of maximal substrate
hydrolysis, and \( k \) is the ZBG concentration at which activity is half maximal (IC\(_{50}\) value).

**Cell Viability Assays.** To assess ZBG cell toxicity, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS, Promega) cell viability assays were performed according to the manufacturer instructions. MTT and MTS are substrates that provide a colorimetric signal in response to viable mitochondria. CF cells were incubated with varying concentrations of inhibitors (0.001-10 mM) for 24 h. After incubation, the cell media was removed and the cells were rinsed three times with fresh media to remove the excess ZBGs, which could interfere with the MTT or MTS reaction. The cells were then lysed and combined with MTT or MTS in media for 1-2 h. Following solubilization (required only for MTT), absorbance at 570 nm was measured using a microplate reader (Bio-Tek µ-Quant Instrument, Winooski, VT). To qualitatively assess ZBG cytotoxicity, photomicrographic images of non-treated and inhibitor-treated CFs were collected 24 h after treatment at several does of each ZBG.

**Cell Invasion Assays.** Cell invasion assay kits were obtained from Chemicon International and used according to the manufacturer’s instructions with minor modifications. Fibrosarcoma HT1080 cells were grown in DMEM with 10% FBS and were serum starved for 24 h prior to initiating the assay. The ZBGs were dissolved in DMSO and diluted 500-fold in serum-free DMEM. All wells contained the same concentration of DMSO during the assay. 10% FBS was used as a chemoattractant.
The final solutions, containing digested cells, CyQuant GR Dye, and Lysis Buffer, were transferred to white 96-well Nunc flat-bottom plates. The plates were read using a Bio-Tek Flx 800 fluorescence plate reader using $\lambda_{\text{ex}} = 460$ nm and $\lambda_{\text{em}} = 516$ nm. The ZBGs were monitored for inhibition of invasion at 1 mM (AHA, 1-6), and 100 mM (8-10). 7 was examined at 50 mM due toxicity of this compound at 100 mM. All experiments were repeated in quadruplicate.
4.5. Appendix

**Figure 4-7.** Representative data generated from fluorescent controls of 11. A) Fluorescence quenching/competitive absorption measurement of 11. The relative fluorescent units (RFUs) produced from each reaction well over a 10 minute period is plotted versus concentration of calibration standard (see Experimental above for details). RFUs produced from the calibration standard in each well in the presence of 11: control (0 mM 11, red squares), 0.05 mM 11 (blue circles), 0.1 mM 11 (green circles), and 0.15 mM 11 (orange circles) are plotted versus concentration of calibration standard. B) Fluorescence correction. The slopes of data from plot A containing ZBG (11) are compared to the slope of the control (no inhibitor). The [(ZBG slope/control slope) × 100] (% Control) is plotted versus ZBG concentration. A linear fit of the % Control v. inhibitor concentration is used to adjust the IC$_{50}$ value. The original IC$_{50}$ value determined from the fluorescence assay (see Figure 4-2, IC$_{50}$ value for 11 = 0.11 mM) is adjusted using the linear fit in B (y = -299.72x + 85.399, where the IC$_{50}$ value of 0.11 mM is x, giving y = ~52%). This indicates that only ~52% of the emitted fluorescence is detected during the assay with 11. Taking the original IC$_{50}$ value of 0.11 mM and adjusting it by 52% gives the corrected IC$_{50}$ value of 0.21 mM.
4.6. Acknowledgements


4.7. References


Chapter 5. A Combined Bioinorganic-Computational Method for the Modeling of Non-Hydroxamate Matrix Metalloproteinase Inhibitors
5.1. Introduction

As discussed in Chapter 4, the novel ZBGs examined were far more potent in inhibiting several MMPs than acetohydroxamic acid.\textsuperscript{[1]} However, the ZBGs are only a portion of a full-length MPI, and require a backbone for non-covalent interactions within the MMP active site.\textsuperscript{[2-4]} Traditionally, hydroxamate-based MPIs are developed based on a prior SAR data, which involves the structural determination of the protein-inhibitor complex and the synthesis of numerous MPI analogues.\textsuperscript{[2-6]} Because no complexes of the novel ZBGs (examined in Chapters 3 and 4) bound to MMPs have been obtained, traditional methods would require the co-crystallization of the ZBGs with MMPs.

We have shown that there is a high structural similarity between the data obtained from the [(Tp\textsuperscript{Ph,Me})Zn(acetohydroxamate)] complex and that of X-ray crystal structures of MMPs bound by hydroxamate-based MPIs.\textsuperscript{[7]} Unfortunately, the model complex cannot be used to provide the same structural information for a full-length inhibitor, as the protein-inhibitor backbone interaction cannot be accounted for. Computational chemistry, on the other hand, is an excellent tool for elucidating the non-covalent interactions between inhibitors and protein targets. However, the computational modeling of metalloprotein-drug interactions has proven to be difficult on account of the metal center. Therefore, in collaboration with Prof. Andrew McCammon’s group, we sought to combine the best of these two methods; using the structural coordinates from the [(Tp\textsuperscript{Ph,Me})Zn(ZBG)] complexes to model the ZBG-catalytic zinc interaction and computational methods to design the non-covalent interactions between the inhibitor backbone and the protein. We first used this method
to model the novel ZBGs in the active site of MMP-3 to examine if the chelators would pose any steric problems in the active site and to determine if there were any favorable orientations conducive to building a full-length MPI. We then used the method to model the orientation and binding of a futoenone derivative in the active site of MMP-3. We chose to model a futoenone derived inhibitor for three reasons: it is a known inhibitor of MMP-3, it is a non-hydroxamate based inhibitor, and no structural information regarding the interaction of the compound with MMP-3 was known.[8] This experiment was designed to test the ability of the method to model a full-length non-hydroxamate MPI in the active site of an MMP.

5.2. Results and Discussion

5.2.1. Modeling of the Novel ZBGs in the Active Site of MMP-3

As previously described, we sought to utilize the structural data obtained from the \([(T\text{p}^{\text{Ph,Me}})\text{Zn}(ZBG)]\) complexes to overcome the need for time consuming macromolecular X-ray crystallography and complicated computational modeling associated with traditional drug discovery. We first used the structural coordinates obtained from the model complexes to examine the orientation of the novel ZBGs (Figure 5-1) in the active site of MMP-3.
**Figure 5-1.** Heterocyclic ZBGs proposed for use in MMP inhibitors. ZBGs 1-3 and 5-7 were used in the computational modeling studies.

Using a custom script,\(^9\) several of the ZBGs (1-3, 5-7) in Figure 5-1 were superimposed onto the active site of MMP-3. This was accomplished by superimposing the ZBGs, zinc(II) ions, and coordinating nitrogen atoms from the model complexes into a X-ray crystal structure of human stromelysin-1 (MMP-3) (PDB code: 1CQR, Chain A);\(^{10}\) this structure will be used throughout this chapter. The nitrogen atoms of the pyrazole model complexes do not uniquely correspond to any of the three histidine nitrogen atoms bound to the zinc(II) ion in the protein active site. Therefore, three orientations were scrutinized to reveal which superpositions allowed for the ZBG to reside in the active site without colliding with the protein surface. It should be noted that these evaluations are based only on the X-ray crystallographic data, and the role of protein mobility has not been taken into account.\(^{11, 12}\) These studies generally yielded similar results regardless of the ZBG (Figure 5-2 through Figure 5-6). Upon insertion to the protein crystal structure, the ZBGs either showed severe steric clashes with the protein, no steric clashes but with little or no room for attachment of a backbone (required for MPI design), or a binding
conformation that showed no steric clashes and ample space for both the ZBG and a requisite drug backbone. All ZBGs were found to have at least one favorable position in the active site of MMP-3.

The modeling of 1-hydroxy-2(1H)-pyridinone (1) in the active site of MMP-3 revealed two favorable orientations (Figure 5-2, A and B), and one in which the ZBG was colliding with the protein (Figure 5-2, C, red). The first orientation (Figure 5-2, A) directs the 6-carbon toward the S1' pocket. The attachment of backbone moieties from the 6-position may yield potent MPIs as numerous inhibitors exploit this subsite.[2-4] The chemistry of the pyridinones is well known, in fact the derivative, 1-hydroxy-2(1H)-pyridinone-6-carboxylic acid has been used for other applications such as iron and actinide sequestering agents.[13, 14] A feasible approach to a full-length MPI based on 1 would be to synthesize the 6-carboxylic acid derivative and attach backbone groups through amide coupling chemistry. The second orientation (Figure 5-2, B) directs the 6-carbon toward the S1 subsite. Although this subsite is targeted less frequently by MPIs, there have been inhibitors (see thiadiazoles in Chapter 1) found to interact with a tyrosine residue (Y155) in this subsite.[15] Therefore, these two orientations offer the potential to target both sides of the MMP active site. In the final orientation, there is clashing (Figure 5-2, C, red) between the protein and the ZBG at the 3-carbon. Furthermore, the 6-carbon is pointed away from the protein making this position an unlikely candidate for any backbone interactions.
Figure 5-2. Images of the modeling of 1-hydroxy-2(1H)-pyridinone in the active site of MMP-3. Orientation A demonstrates the favorable positioning of the pyrone ZBG with the 6-carbon pointing towards the S1' pocket. Orientation B displays the positioning of the ZBG with the 6-carbon near the S1 subsite and Y155. Orientation C displays obvious steric obstacles for the ZBG, with the obstructing residues colored in red. The protein is shown in grey (spacefilling), zinc(II) in gold (spacefilling), and the ZBG is shown colored by element (stick models). For clarity, a chemical diagram of 1-hydroxy-2(1H)-pyridinone is shown on the bottom right with labeled carbons.
The ZBG 3-hydroxy-1-methyl-2(1H)-pyridinone (2) was also modeled in the active site of MMP-3. Like 1, this ZBG was found to have two favorable orientations (Figure 5-3, A and B) and one orientation (Figure 5-3, C) in which clashing was evident. Compound 2 can be functionalized on both sides of the pyridinone ring from either the N-methyl group or the 4-carbon.\textsuperscript{[14, 16]} It appears from the modeling that the best route for developing an effective MPI would be to attach backbone groups to the 4-carbon side of the ZBG to target the S1' pocket as seen in Figure 5-3, A or to target the other side of the active site as observed in Figure 5-3, B. Attaching to the N-methyl side would not be as desirable, as this group does not point toward either the S1' pocket or the S1 subsite. In fact, the N-methyl group can best be described as positioned toward the solvent in the two favorable orientations. The pyridinones are known to have poor aqueous solubility, therefore it may be beneficial to attach a more hydrophilic group to the N-methyl side of the ring and a backbone group to the 4-position. Figure 5-3, C displays a severe steric clash between the N-methyl group and the protein. Additionally, the 4-position is directed away from the active site precluding it from any interactions with the enzyme.
Figure 5-3. Images of the modeling of 3-hydroxy-1-methyl-2(1H)-pyridinone in the active site of MMP-3. Orientation A demonstrates a favorable positioning of the ZBG with the 4-carbon pointing towards the S1' pocket. Orientation B displays the positioning of the ZBG with the 4-carbon near the S1 subsite. Orientation C displays obvious steric obstacles for the ZBG, with the obstructing residues colored in red. The protein is shown in grey (spacefilling), zinc(II) in gold (spacefilling), and the ZBG is shown colored by element (stick models). For clarity, a chemical diagram of 3-hydroxy-1-methyl-2(1H)-pyridinone is shown on the bottom right with labeled carbons.
The ZBG 3-hydroxy-1,2-dimethyl-4(1H)-pyridinone (3) has two favorable
orientations (Figure 5-4, A and B), and the third orientation (Figure 5-4, C) displays
clashes with the protein at positions 5 and 6 of the pyridinone ring. The position of
the ZBG in Figure 5-4, A demonstrates that the 2-methyl group is directed toward the
S1' pocket, while the N-methyl substituent is extending into a valine residue (shown in
red) in the S2' pocket. Although this may appear as an undesirable interaction, the
clashing is quite minimal and probably not observed in solution. In fact the N-methyl
group could be participating in hydrophobic interactions. The second orientation
(Figure 5-4, B) directs the 2-methyl group away from the S1' pocket, and towards the
S1 subsite. Additionally, the N-methyl group is positioned towards the S2 pocket. As
with the previous pyridinones, the chemistry to functionalize this ZBG is well
known.\textsuperscript{[16-18]} This ZBG provides additional opportunities for derivatives, as the N-
methyl group can be substituted with any primary amine.\textsuperscript{[19]} Furthermore, if the N-
methyl group appears to be too sterically demanding, the N-H derivative can be
synthesized.\textsuperscript{[20]}
Figure 5-4. Images of the modeling of 3-hydroxy-1,2-dimethyl-4(1H)-pyridinone in the active site of MMP-3. Orientation A demonstrates a favorable positioning of the ZBG with the 2-methyl group pointing towards the S1' pocket, with the minor obstruction colored in red. Orientation B displays the positioning of the ZBG with the 2-methyl group near the S1 subsite and the N-methyl group positioned towards the S2 subsite. Orientation C displays steric obstacles for the ZBG, with the obstructing residues colored in red. The protein is shown in grey (spacefilling), zinc(II) in gold (spacefilling), and the ZBG is shown colored by element (stick models). For clarity, a chemical diagram of 3-hydroxy-1,2-dimethyl-4(1H)-pyridinone is shown on the bottom right with labeled carbons.
The ZBG 3-hydroxy-2-methyl-4-pyrone (maltol) (5) has two favorable orientations (Figure 5-5, A and B) and one orientation where clashes with the protein were observed (Figure 5-5, C) when superimposed in the active site of MMP-3. The three orientations closely resemble those of compound 3. The position of maltol in Figure 5-5, A demonstrates that the 2-methyl group points toward the S1' pocket. The second orientation (Figure 5-5, B) positions the 2-methyl group away from the S1' pocket, however, a backbone may be attached to occupy the S1 subsite as postulated for the previous ZBG examined here. The well-known pyrone chemistry can be used to develop a full-length MPI based on this ZBG. There are known procedures to functionalize the 2-methyl group and the 6-position of the pyrone ring.\textsuperscript{18, 19, 21} This chemistry will be discussed in detail in Chapter 6.
Figure 5-5. Images of the modeling of 3-hydroxy-2-methyl-4-pyrone in the active site of MMP-3. Orientation A demonstrates a favorable positioning of the ZBG with the 2-methyl group pointing towards the S1' pocket. Orientation B displays the positioning of the ZBG with the 2-methyl group near the S1. Orientation C displays steric obstacles for the ZBG, with the obstructing residues colored in red. The protein is shown in grey (spacefilling), zinc(II) in gold (spacefilling), and the ZBG is shown colored by element (stick models). For clarity, a chemical diagram of 3-hydroxy-2-methyl-4-pyrone is shown on the bottom right with labeled carbons.
The ZBG 3-hydroxy-2(1H)-pyridinone (6) was modeled in the active site of MMP-3 and was found to have the same orientations as 3-hydroxy-1-methyl-2(1H)-pyridinone (2). The chemistry for synthesizing as full length MPI based on this ZBG would follow that of 2.[14, 16]

The final ZBG examined in the active site of MMP-3 was 1-hydroxy-2(1H)-pyridinethione (7). This ZBG is different from the previously modeled compounds as it contains a coordinating sulfur atom. After the modeling process, there were found to be two favorable orientations (Figure 5-6, A and B) and one clashes with the protein (Figure 5-6, C). The superpositioning of 7 was quite interesting, as the ZBG is rotated 180° from that of compound 1. The replacement of a carbonyl (in 1) with a thione (in 7) changes the coordination of the ZBG to the zinc. As observed in the complex [(Tp\textsuperscript{Ph,Me})Zn(1-hydroxy-2(1H)-pyridinone)] (Chapter 3), compound 1 binds the zinc in a bidentate fashion with the geometry around the metal center best described as distorted trigonal bipyramidal ($\tau = 0.64$)[22] with the 2-carbonyl oxygen atom and one of the pyrazole ring nitrogen atoms occupying the axial positions of the coordination sphere (Chapter 3). In the case of compound 7, the ZBG also binds in a bidentate fashion to make the complex [(Tp\textsuperscript{Ph,Me})Zn(1-hydroxy-2(1H)-pyridinethione)] (Chapter 3) in a similar, distorted trigonal bipyramidal ($\tau = 0.54$)[22] geometry, but the axial positions are occupied by the N-hydroxy (not the 2-carbonyl) oxygen and one of the pyrazole nitrogen atoms. This change in coordination orientation has been observed in similar complexes where the weaker donor atom occupies the axial position.[23-27] Therefore, the orientation of 7 in the MMP active site is converse to
that of compound 1. It should be noted that this observation could only be made using the structural data from small molecule models as a scaffold for the superpositions of the chelators.

The divergent orientation of 7 requires the attachment of inhibitor backbones to the opposite side of the pyridine ring. The 6-carbon in 1 is positioned toward the S1' pocket (Figure 5-2, A) in the first orientation, while in the case of 7, the 6-carbon is aimed in the opposite direction, and the 3-carbon directed toward the S1' pocket (Figure 5-6, A). The second orientation of 7 positions the 6-carbon toward the solvent and the 3-carbon toward the S1 subsite. While the synthesis of the 6-carboxylic acid derivative of 7 is known,[28] this compound may not be as useful as the 6-carboxylic acid derivative of 1 for preparing full-length MPIs, as the 6-position in 7 does not look to be the most beneficial attachment point for backbone groups.
Figure 5-6. Images of the modeling of 1-hydroxy-2(1H)-pyridinethione in the active site of MMP-3. Orientation A demonstrates a favorable positioning of the ZBG with the 3-carbon pointing towards the S1' pocket. Orientation B displays the positioning of the ZBG with the 2-carbon group near the S1. Orientation C displays steric obstacles for the ZBG, with the obstructing residues colored in red. The protein is shown in grey (spacefilling), zinc(II) in gold (spacefilling), and the ZBG is shown colored by element (stick models). For clarity, a chemical diagram of 1-hydroxy-2(1H)-pyridinethione is shown on the bottom right with labeled carbons.
By superimposing the novel ZBGs into an X-ray crystal structure of MMP-3 \[10\], we were able to examine the orientations of these chelators in the enzyme active site. In all the ZBGs examined, favorable orientations were observed. With the rich, known chemistry of pyridinones, pyrones, and pyridinethiones, there seems to be endless potential for the development of full-length MPIs based on these novel chelators.

5.2.2. Modeling of a Futoenone MPI in the Active Site of MMP-3

The superposition of all ZBGs provided valuable structural information as to the orientations of these chelators in the MMP-3 active site. These results prompted us to model a full length MPI that was not based on a hydroxamic acid ZBG and the mode of binding of the inhibitor had not been previously elucidated. For these reasons, we chose a derivative of futoenone.

Futoenone is a natural product, derivatives of which are known to interfere with MMP activity,\[8\] however, the precise mode of binding for these compounds to MMPs has not been determined. Yeh and coworkers examined a series of futoenone derivatives for potency against MMPs -1, -2, and -3. Overall, the compounds were not potent inhibitors of these enzymes. However, the inhibitors did exhibit selectivity for MMP-3 over the other two enzymes. Compound 12 was examined (Figure 5-7), as it was the most effective inhibitor against MMP-3 reported (IC\(_{50}\) = 600 nM) from in that study.\[8\] Compound 12 was selected because the mode of metal-binding and active site conformation was unknown, it demonstrates significant selectivity for stromelysin
(MMP-3) over other MMPs, and it does not possess a hydroxamate group as the zinc(II) ion chelator. The limited number of heteroatoms in this compound suggested that the 2-methoxybenzenethiol (Figure 5-7, blue) was the most probable ZBG.

**Figure 5-7.** Futoenone derivative 12 (left), with ring system 2-methoxybenzenethiol (MBT) in blue and benzo[d][1,3]dioxole in green. Hydroxamate-based MPI, GM6001 is shown on right.

2-Methoxybenzenethiol (MBT) was reacted with \([\text{Tp}^{\text{Ph,Me}}\text{ZnOH}]\) (\(\text{Tp}^{\text{Ph,Me}} = \) hydrotris(3,5-phenylmethylpyrazolyl)borate) to generate the complex \([\text{Tp}^{\text{Ph,Me}}\text{Zn(MBT)}]\) as a model for the interaction between 12 and the MMP active site zinc(II) ion. The structure of \([\text{Tp}^{\text{Ph,Me}}\text{Zn(MBT)}]\) was determined from single crystal X-ray diffraction (Figure 5-8). The MBT was found to bind to the zinc center in a bidentate fashion in a distorted trigonal bipyramidal geometry (\(\tau = 0.70\))\textsuperscript{[22]} with the methoxy oxygen and one of the pyrazole nitrogens occupying the axial positions with a Zn-S distance of 2.25 Å and a Zn-O distance of 2.37 Å (Table 5-1).
With the metal-ZBG interaction structurally determined, we decided to model the remaining portion of the inhibitor computationally. Starting with the structure of \([\text{Tp}^{\text{Ph,Me}}\text{Zn(MBT)}]\), the modeling of the futoenone derivative bound to stromelysin was performed in four steps. First, the structure of \([\text{Tp}^{\text{Ph,Me}}\text{Zn(MBT)}]\) was used to template the conformation of inhibitor 12 in the active site of stromelysin (MMP-3). A portion of the structure (Figure 5-8, inset) was inserted into the crystal structure of MMP-3 in the same fashion as discussed previously for the other ZBGs. As observed with the modeling of the novel ZBGs, the pyrazole nitrogen donors do not specifically correlate with a particular histidine residue in the protein, so three different orientations were obtained (Figure 5-9). Two of the conformations (Figure 5-9, red...
and orange conformations) were immediately dismissed based on steric conflicts between the MBT fragment and the protein. Furthermore, these two unfavorable orientations precluded the attachment of the inhibitor backbone. The remaining superposition showed no steric clashes with the protein, and was determined to be the most probable binding conformation. As noted in the previous section, these evaluations are based only on the X-ray crystallographic data, and the role of protein mobility has not been accounted for.[11, 12]

**Figure 5-9.** Three superpositions of the [(TpPh,Me)Zn(MBT)] fragment (Figure 5-8, inset) into the structure of MMP-3. The MBT ligand is shown in stick representations and the catalytic zinc(II) ion is shown as a purple sphere. The red and orange orientations clash with the protein in areas shown in the corresponding colors on the protein surface. Only the green orientation is free of steric conflicts.

With the orientation of the MBT fragment resolved, the second step was to generate all possible conformers of 12 by varying each of four dihedral angles (Figure 5-7, bonds in red).[29] Of the conformers produced, 14559 were rejected based on internal steric clashes between non-hydrogen atoms. Of the remaining 6177 conformers, every 60th conformation was chosen to obtain a uniform selection of 100
structures. Each structure was placed into the protein by superimposing the 2-methoxybenzenethiol of the full-length inhibitor (Figure 5-7, blue) from the calculated conformers with that of the protein-imbedded MBT fragment.

In the third stage, AMBER parm99 and gaff force fields were used to model the protein and inhibitor, respectively.\(^{30, 31}\) Hydrogen atoms were added to the protein using the program WHAT IF.\(^{32}\) Charges were derived using a Gaussian optimization (HF/6-31G*) and the AMBER 7 module Antechamber.\(^{33}\) The process was completed by performing minimizations using the SANDER module of AMBER 7.\(^{34}\) The ligand atoms not shared with the MBT fragment were allowed to move while the remaining atoms (including the protein) were kept rigid. Minimizations were run until the RMS deviation of the energy gradient was less than \(1 \times 10^{-4}\) kcal mol\(^{-1}\) Å\(^{-1}\).

The strength of ligand binding was assessed according to the minimized energies (Table 5-2). A single conformation minimized to the lowest energy of 3.9 kcal mol\(^{-1}\), followed by a cluster of less favorable conformations at 8-9 kcal mol\(^{-1}\). It was apparent that the lowest energy structure relaxed in a unique position to the active site cleft (Figure 5-10). The remaining low energy conformations minimized outside the protein subsites, making contacts predominantly with solvent-space (Figure 5-11 through Figure 5-14).
Figure 5-10. Lowest-energy minimized structure of 12 in the active site of stromelysin (MMP-3). Y155 is shown in blue, P156 is shown in red, and the catalytic zinc(II) ion is shown in purple.

The lowest-energy minimized structure of 12 (Figure 5-10) reveals several unusual features about the binding. We have previously discussed (Chapter 1) the majority of MPIs have been designed to occupy the S' subsites (termed “right-handed” inhibitors), in particular the S1' pocket (Figure 5-10). However, the backbone portion of minimized 12 lies in the “left-handed” side of the active site (S subsites), where there is a significant interaction with the solvent exposed S1 and S3 subsites (Figure 5-10).\cite{10,15} In this conformation, π-stacking is observed (π–π contact, 3.7 Å) between the phenyl group of benzo[d][1,3]dioxole (Figure 5-7, green) of 12 and the side chain of Y155. The known efficacy of 12 against MMP-3 correlates well with other ‘left-
handed' inhibitors that show selectivity for MMP-3, in which interactions with the hydrophobic residue Y155 play an important role.\textsuperscript{[15]} For comparison, in collagenase (MMP-1), the corresponding residue is a serine,\textsuperscript{[4]} thereby eliminating any possible $\pi$-interactions. Another hydrophobic interaction occurs between the methyl group from the zinc-bound oxygen atom in 12 and a hydrophobic cleft created by the sidechains of F86 and F210. Specific hydrogen bonding is also present between the backbone carbonyl of P156 and the primary alcohol moiety of 12.

Earlier attempts to model the binding of 12 assumed the compound would occupy the same subsites as the hydroxamate-based MPI, GM6001.\textsuperscript{[8]} While these two inhibitors have very different structures, the authors of the study overlaid the two ZBGs and suggested the benzo[$d$][1,3]dioxole moiety would occupy the S2' subsite as observed with the tyrosine ring system in GM6001. Based on the structural information obtained from our method, it can be concluded that the benzo[$d$][1,3]dioxole group cannot occupy the S2' subsite due to the rigidity of the molecule. This data combined with the selectivity of the inhibitor for MMP-3,\textsuperscript{[8]} points toward the binding conformation elucidated with the combined bioinorganic computational method.

### 5.3. Conclusion

By applying the combined bioinorganic computational method, the ZBGs (1-3, 5-7) (Figure 5-1) were modeled in the active site of MMP-3. The structural information obtained from these superpositions proved useful for determining the orientation of the novel ZBGs in the enzyme active sight without the need for
macromolecular crystallography. All ZBGs were found to have at least one favorable orientation that would allow for the addition of an inhibitor backbone to produce a full-length MPI. Additionally, this method has elucidated the interactions of a non-hydroxamate based MPI with MMP-3 in which no previous structural information was known. Comparing structural data of the ZBG-MMP interactions with the modeling studies would be an excellent way to confirm the method. Based on the results of these two experiments, this method is useful not only for examining known MPIs, but also has potential for the design of new MPIs that utilize non-hydroxamate ZBGs.

5.4. Experimental Section

**Computer Modeling Analysis.** Computer analysis was performed on PC workstations running a Linux (Red Hat) operating system. Superpositions were performed on the structure of human stromelysin-1 (MMP-3) based on coordinates from the Protein Data Bank (entry 1CQR, Chain A). The coordinating pyrazole nitrogen atoms were directly superimposed onto the Ne2 atoms of the coordinating histidine residues in the protein. The superpositions were executed using a custom written script that overlaid the small molecule X-ray coordinates onto the protein structure by using a least squares fitting of the corresponding nitrogen atoms. Three different orientations were constructed for each analysis. The resulting structures were then examined by using Rasmol (v. 2.7.2.1, April 2001) and visually inspected for steric clashes with spacefilling models based on van der Waals radii. Superpositions where the ZBG occupied the same space as the protein were determined to be in steric conflict.
[(Tp$^{\text{Ph,Me}}$)Zn(MBT)]. In a 100mL round-bottom flask, [Tp$^{\text{Ph,Me}}$]ZnOH (200 mg, 0.35 mmol) was dissolved in 20 mL CH$_2$Cl$_2$. To this solution, was added 1.0 equiv of 2-methoxybenzenethiol (0.043 mL, 0.35 mmol) dissolved in MeOH (30 mL). The mixture was stirred at room temperature overnight under a N$_2$(g) atmosphere. After stirring, the turbid solution was evaporated to dryness on a rotary evaporator to give a white solid. The solid was dissolved in benzene (~15 mL) and single crystals suitable for X-ray diffraction were obtained by diffusion of the benzene solution with pentane. Yield: 35%. $^1$HNMR (CDCl$_3$, 400 MHz, 25 ºC): δ 2.11 (s, 3H, CH$_3$, methoxy), 2.56 (s, 9H, CH$_3$,pyrazole-CH$_3$), 5.37 (d, $J$ = 7.2 Hz, 1H, benzenethiol-H), 6.19 (s, 3H, pyrazole-H), 6.35 (t, $J$ = 3.6 Hz, 1H, benzenethiol-H), 6.47 (t, $J$ = 3.8 Hz, 1H, benzenethiol-H), 7.10 (br m, 9H, phenyl-H), 7.20 (d, $J$ = 7.6 Hz, 1H, benzenethiol-H), 7.70 (d, $J$ = 7.2 Hz, 6H, phenyl-H). $^{13}$CNMR (CDCl$_3$, 100 MHz, 25ºC): δ 13.1, 51.9, 104.9, 107.3, 119.6, 121.3, 127.5, 127.7, 127.8, 130.8, 131.4, 132.0, 144.9, 152.9, 153.7. Anal. Calcd for C$_{37}$H$_{35}$BN$_6$OSZn: C, 64.59; H, 5.13; N, 12.22. Found C, 64.55; H, 5.27; N, 12.14.

**X-Ray Crystallographic Analysis.** Data was collected on a Brucker AXS area detector diffractometer. A crystal of MBT was mounted on a quartz capillary by using Paratone oil and was cooled in a nitrogen stream (Kryo-flex controlled) on the diffractometer (-173 ºC). Peak integrations were performed with the Siemens SAINT software package. Absorption corrections were applied using the program SADABS. Space group determinations were performed by the program XPREP. The structure were solved by direct methods and refined with the SHELXTL software package. All hydrogen atoms, except for the boron hydrogen atom, were fixed at calculated
positions with isotropic thermal parameters; all non-hydrogen atoms were refined anisotropically.

5.5. Appendix

Table 5-1. Crystal data and structure refinement for [(Tp\textsuperscript{Ph,Me})Zn(MBT)].

<table>
<thead>
<tr>
<th>Empirical formula</th>
<th>C\textsubscript{37}H\textsubscript{35}N\textsubscript{6}BOSZn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>100(2) K</td>
</tr>
<tr>
<td>Crystal system</td>
<td>Monoclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>(P2_1/c)</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
<td>(a = 11.4729(15) \text{&quot;Å})</td>
</tr>
<tr>
<td></td>
<td>(b = 14.0471(18) \text{&quot;Å})</td>
</tr>
<tr>
<td></td>
<td>(c = 20.877(3) \text{&quot;Å})</td>
</tr>
<tr>
<td></td>
<td>(\beta = 99.315(2)^\circ)</td>
</tr>
<tr>
<td>Volume</td>
<td>3320.3(7) \text{&quot;Å}^3</td>
</tr>
<tr>
<td>(Z)</td>
<td>4</td>
</tr>
<tr>
<td>Density (calculated)</td>
<td>1.376 mg/m\textsuperscript{3}</td>
</tr>
<tr>
<td>Crystal size</td>
<td>0.20 x 0.10 x 0.05 mm\textsuperscript{3}</td>
</tr>
<tr>
<td>Theta range for data collection</td>
<td>1.75 to 27.52(^\circ)</td>
</tr>
<tr>
<td>Reflections collected</td>
<td>27992</td>
</tr>
<tr>
<td>Independent reflections</td>
<td>7591 ([R\text{(int)} = 0.0452])</td>
</tr>
<tr>
<td>Data/restraints/parameters</td>
<td>7591 / 0 / 432</td>
</tr>
<tr>
<td>Goodness-of-fit on (F^2)</td>
<td>0.872</td>
</tr>
<tr>
<td>Final (R) indices ([I&gt;2\sigma(I)])</td>
<td>(R_1 = 0.0347, wR_2 = 0.0673)</td>
</tr>
<tr>
<td>(R) indices (all data)</td>
<td>(R_1 = 0.0611, wR_2 = 0.0721)</td>
</tr>
<tr>
<td>Largest diff. peak and hole</td>
<td>0.597 and -0.573 e \text{&quot;Å}^3 )</td>
</tr>
<tr>
<td>Conformer Number</td>
<td>Energy (kcal/mol)</td>
</tr>
<tr>
<td>------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>00001</td>
<td>1.7807E+01</td>
</tr>
<tr>
<td>00061</td>
<td>8.2241E+00</td>
</tr>
<tr>
<td>00121</td>
<td>8.2241E+00</td>
</tr>
<tr>
<td>00181</td>
<td>1.5651E+02</td>
</tr>
<tr>
<td>00241</td>
<td>1.0196E+01</td>
</tr>
<tr>
<td>00301</td>
<td>5.2295E+00</td>
</tr>
<tr>
<td>00361</td>
<td>8.2240E+00</td>
</tr>
<tr>
<td>00421</td>
<td>5.2295E+00</td>
</tr>
<tr>
<td>00481</td>
<td>5.2294E+00</td>
</tr>
<tr>
<td>00541</td>
<td>8.2240E+00</td>
</tr>
<tr>
<td>00601</td>
<td>6.7583E+00</td>
</tr>
<tr>
<td>00661</td>
<td>6.7583E+00</td>
</tr>
<tr>
<td>00721</td>
<td>1.8593E+01</td>
</tr>
<tr>
<td>00781</td>
<td>8.2240E+00</td>
</tr>
<tr>
<td>00841</td>
<td>8.2240E+00</td>
</tr>
<tr>
<td>00901</td>
<td>8.3788E+00</td>
</tr>
<tr>
<td>00961</td>
<td>8.3788E+00</td>
</tr>
<tr>
<td>01021</td>
<td>6.7643E+02</td>
</tr>
<tr>
<td>01081</td>
<td>1.8053E+01</td>
</tr>
<tr>
<td>01141</td>
<td>2.0607E+01</td>
</tr>
<tr>
<td>01201</td>
<td>6.1661E+00</td>
</tr>
<tr>
<td>01261</td>
<td>2.1345E+01</td>
</tr>
<tr>
<td>01321</td>
<td>6.7583E+00</td>
</tr>
<tr>
<td>01381</td>
<td>6.7583E+00</td>
</tr>
<tr>
<td>01441</td>
<td>6.7583E+00</td>
</tr>
<tr>
<td>01501</td>
<td>6.1661E+00</td>
</tr>
<tr>
<td>01561</td>
<td>1.8335E+01</td>
</tr>
<tr>
<td>01621</td>
<td>3.3801E+02</td>
</tr>
<tr>
<td>01681</td>
<td>1.1845E+01</td>
</tr>
<tr>
<td>01741</td>
<td>1.4154E+03</td>
</tr>
<tr>
<td>01801</td>
<td>8.3786E+00</td>
</tr>
<tr>
<td>01861</td>
<td>8.3788E+00</td>
</tr>
<tr>
<td>01921</td>
<td>8.3788E+00</td>
</tr>
<tr>
<td>01981</td>
<td>8.3788E+00</td>
</tr>
<tr>
<td>02041</td>
<td>1.8053E+01</td>
</tr>
<tr>
<td>02101</td>
<td>8.3788E+00</td>
</tr>
<tr>
<td>02161</td>
<td>1.7936E+01</td>
</tr>
<tr>
<td>02221</td>
<td>6.6405E+02</td>
</tr>
<tr>
<td>02281</td>
<td>2.0584E+01</td>
</tr>
<tr>
<td>02341</td>
<td>6.1661E+00</td>
</tr>
<tr>
<td>02401</td>
<td>1.6470E+01</td>
</tr>
<tr>
<td>02461</td>
<td>8.3788E+00</td>
</tr>
<tr>
<td>02521</td>
<td>8.3788E+00</td>
</tr>
<tr>
<td>02581</td>
<td>8.3788E+00</td>
</tr>
<tr>
<td>02641</td>
<td>8.3788E+00</td>
</tr>
<tr>
<td>02701</td>
<td>6.1661E+00</td>
</tr>
<tr>
<td>02761</td>
<td>1.7888E+01</td>
</tr>
<tr>
<td>02821</td>
<td>2.0607E+01</td>
</tr>
<tr>
<td>02881</td>
<td>6.1661E+00</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 5-11.** Representation of the multiple low energy conformers of 12; red = ~8 kcal/mol. Y155 is shown in blue and P156 is shown in pink.
Figure 5-12. Representation of the multiple low energy conformers of 12; orange = ~6 kcal/mol. Y155 is shown in blue and P156 is shown in pink.
Figure 5-13. Representation of low energy conformer of 12; yellow = ~5 kcal/mol. Y155 is shown in blue and P156 is shown in pink.
Figure 5-14. Representation of low energy conformer of 12; green = ~4 kcal/mol. Y155 is shown in blue and P156 is shown in pink.
5.6. Acknowledgements


5.7. References


Chapter 6. The Design, Synthesis, and Inhibitory Profile of Pyrone-Based MPIs and Derivative
6.1. Introduction

This chapter will discuss the design and examination of the first pyrone-based MPIs. Previous experiments have shown that the ZBGs tested are superior to AHA (Figure 5-1) as inhibitors of MMPs (Chapter 4).\[^1,\,2\] Additionally, a combined bioinorganic-computational approach to design non-hydroxamate MPIs proved fruitful in determining the binding mode of a known non-hydroxamate MPI (Chapter 5).\[^3\] Therefore, we sought to use this new design approach to develop full-length MPIs based on the superior ZBGs, namely the pyrones.

The first step in the inhibitor development was to chemically modify the pyrone ring, in order to attach a backbone moiety. Several pyrone-based MPIs were synthesized. While these first generation MPIs were not potent inhibitors, their facile synthesis demonstrated the versatility of the pyrone ZBGs. Using the combined computational-bioinorganic method for inhibitor design (Chapter 5), second generation pyrone-based MPIs were designed. The initial computational method (Chapter 5) was enhanced with the drug discovery program LUDI (Accelrys), which allows for a more efficient screening of potential inhibitor backbones. The second generation MPIs were then synthesized and evaluated for potency against MMPs -1, -2, and -3 and in a cell invasion assay. Three pyrone-based MPIs were found to be potent against MMP-3. Additionally, efforts have been made to take full advantage of the pyrone versatility and potency by developing derivatives of the pyrone-based MPIs.
A new method has been developed to design novel MPIs through a bioinorganic approach. Starting from an inorganic model complex of the zinc tris(histidine) active site of MMPs (Chapter 2), several novel ZBGs were identified and structurally characterized (Chapter 3). These ZBGs were assayed and found to be more potent inhibitors of MMPs than acetohydroxamic acid (Chapter 4). In an effort to design full-length MPIs based on the novel ZBGs, a new method for the development of metalloprotein inhibitors was created, combining structural information from the small molecule models and computational chemistry (Chapter 5). This final chapter represents the culmination of the bioinorganic approach to metalloprotein inhibitor development with the design and synthesis of potent and chemically versatile pyrone-based MPIs.

6.2. Results and Discussion

6.2.1. Early Design of Pyrone-Based MPIs

Pyrones were selected for incorporation into full-length MPIs due to their synthetic versatility,[4] known biocompatibility,[5] and good aqueous solubility. The computational modeling of maltol (3-hydroxy-2-methyl-4-pyrone) in the active site of MMP-3 indicated the 2-methyl substitutent was favorably oriented toward the hydrophobic S1' (Chapter 5).[6] Several studies show that targeting the S1' pocket of MMPs yields potent and selective MPIs.[7, 8] Additionally, the 6-carbon was positioned towards the “unprimed” subsites.[6] Although few, there are examples of potent inhibitors that have exploited this part of the active site.[9] In order to become familiar
with pyrone chemistry and to address any potential problems regarding the synthesis of full-length MPIs, we decided to first attach simple backbone groups to the pyrone moiety. We also examined this first generation of MPIs to determine if the attachment of drug backbones would improve the efficacy of the inhibitor over the ZBG alone.

The first pyrone-based MPIs, **KA-1** through **KA-4** were synthesized from kojic amine (3) in modest yields. During the development of inhibitors **KA-1** through **KA-4**, a new method for the synthesis of 3 from commercially available kojic acid was developed (Scheme 6-1), as the literature synthesis requires a complicated and low yielding reduction of kojic azide (2).[10] We found that reducing 2 with Lindlar Catalyst in ethanol or methanol gave a clean, high yielding route to 3. The synthesis of inhibitors **KA-1** through **KA-4** from 3 was accomplished using standard amide coupling conditions. Commercially available aryl carboxylic acids were activated with 2-mercapto-1,3-thiazolidine in the presence of DCC and a catalytic amount of DMAP in dry methylene chloride. The coupling of the activated amides with 3 in the presence of triethylamine in a methylene chloride/methanol mixture gave the desired inhibitors in modest yields. The synthesis of **KA-1** is shown in Scheme 6-1. These initial syntheses demonstrated the feasibility of coupling backbones to the pyrone ring. We were able to quickly produce a small group of inhibitors with the same phenyl substituent while varying the linker lengths from 0 (**KA-1**) to 3-carbons (**KA-4**). Three of these inhibitors (**KA-2**, -3, and -4) were examined for potency against MMP-3 under assay conditions described previously for the ZBGs (Chapter 4). The poor inhibitory efficacy of the compounds (Table 6-1) was disappointing, but not unexpected, as these MPIs were not developed using any rational drug design
methods. On a positive note, the compounds did inhibit several MMPs more efficiently than the pyrone chelators alone, which encouraged us to attach higher affinity backbone groups to the ZBGs.$^2$

![Chemical Structures]

**Scheme 6-1.** Synthesis of KA-1. a) SOCl, dry CH$_2$Cl$_2$, RT, 85%; b) NaN$_3$, DMF, 80%; c) Lindlar Catalyst, H$_2$(g) 35-40 psi, EtOH, RT, 90%; d) N(CH$_2$CH$_3$)$_3$, CH$_2$Cl$_2$, MeOH, RT, 51%.

It has been established that large hydrophobic groups, such as the ones used in a study by Hadjuk et. al have a high affinity for the S1' pocket in MMPs.$^7, 11$ Using biaryl backbones, Hadjuk et. al were able to develop several hydroxamate-base MPIs with potencies in the nanomolar range.$^7$ We postulated that by using these same backbone groups in combination with the more potent pyrone ZBGs,$^1, 2$ we could develop effective pyrone-based MPIs. We turned our focus toward attaching these backbones to 5-benzyloxy-pyran-4(1H)-one-2-carboxylic acid (5) for two reasons.$^{12}$ First, we wanted to explore more synthetic options for backbone attachment. Second, we found that using this functionalized pyrone gave more options for inhibitor designs, as there are more biaryl amines commercially available than biaryl carboxylic acids. 5-Benzyloxy-pyran-4(1H)-one-2-carboxylic acid (5) was synthesized from
commercially available kojic acid in two steps. Kojic acid was benzyl protected, followed by oxidation using Jones Reagent to produce 5 in good yields. Compound 5 was activated with N-hydroxysuccinamide (NHS) instead of 2-mercapto-1,3-thiazolidine because the later reaction was known to be poor yielding. The activated ester of 5 was used in situ for the coupling to the desired biaryl amine, followed by removal of the benzyl protecting group to produce MPIs CA-1 and CA-2 in modest yields. The synthesis of CA-2 is shown in Scheme 6-2. CA-2 was examined for potency against MMP-3 under the same assay conditions as described previously. This MPI was found to be a slightly more potent inhibitor of MMP-3 than the KA series (Table 6-1). However, this inhibitor still did not demonstrate potency in the nanomolar range (necessary for worthwhile MPIs). With this second disappointing result, it was determined that it was in the best interest of the project to rationally design pyrone-based inhibitors using the combined computational-bioinorganic method, which had been successful in modeling the interaction of a non-hydroxamate MPI in the active site of MMP-3 (Chapter 5). The modeling of maltol in the active site of MMP-3 (Chapter 5) suggested that the biaryl groups attached to the 6-carbon in compounds CA-1 and CA-2 may be pointing towards the unprimed subsites (not high affinity sites for the biaryl moieties). We hypothesized that by attaching the same biaryl backbones to the 2-position of the pyrone, we could direct these hydrophobic groups toward the S1' pocket.
Scheme 6-2. Synthesis of CA-2. a) Jones Reagent, acetone, RT, 74%; b) NHS, DCC, dry THF, RT; c) 4-Phenylbenzylamine, dry THF, RT, 78% (2 steps); d) 10% Pd/C, H₂(g), 35psi, MeOH, 43%.
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Structure</th>
<th>MMP-1</th>
<th>MMP-2</th>
<th>MMP-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>KA-1</td>
<td><img src="image1" alt="Structure" /></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>KA-2</td>
<td><img src="image2" alt="Structure" /></td>
<td>1100</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>KA-3</td>
<td><img src="image3" alt="Structure" /></td>
<td>1000</td>
<td>1100</td>
<td>900</td>
</tr>
<tr>
<td>KA-4</td>
<td><img src="image4" alt="Structure" /></td>
<td>500</td>
<td>400</td>
<td>400</td>
</tr>
<tr>
<td>CA-1</td>
<td><img src="image5" alt="Structure" /></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CA-2</td>
<td><img src="image6" alt="Structure" /></td>
<td>ND</td>
<td>ND</td>
<td>25% @ 50uM</td>
</tr>
</tbody>
</table>

Table 6-1. Structures and IC$_{50}$ Values (µM) for First Generation Pyrone-based MPIs Against MMP-1, MMP-2, and MMP-3. Values are Obtained from Fluorescence-based MMP Assays. ND = not determined. If the IC$_{50}$ value could not be determined due to poor solubility, the inhibition at the highest soluble concentration is shown.
6.2.2. Using the Combined Bioinorganic-Computational Approach to Design Pyrone-Based MPIs AM-1 through AM-6

To design pyrone-based inhibitors in a more rational manner, we used the drug discovery program LUDI (Accelrys) augmented with parameters from a bioinorganic model complex.[13, 6, 13] LUDI uses a constrained docking approach that identifies optimal fragments to link to the pyrone moiety at a specified point of attachment. Structural coordinates from [(Tp^{Ph,Me})Zn(3-hydroxy-2-methyl-4-pyrone)] were used to superposition the pyrone ZBG into an X-ray crystal structure of MMP-3 (PDB code: 1CQR) (Chapter 5) to generate the initial receptor complex.[6] The orientation that allows the R groups (see Scheme 6-3) access to the S1' pocket[6] was used in all docking studies. Hydrogen atoms were added to the aligned maltol molecule and an amide group was built at the 2-position of the ring using Cerius² 4.8.1. The point of attachment to the ZBG was defined as an N-H bond (Figure 6-1, red arrow) from an amide moiety on the 2-position of the maltol ring (Figure 6-1).
**Figure 6-1.** Representation of the initial receptor complex used in the LUDI docking experiments with pyrone ZBG (colored by element) in the active site of MMP-3 (gray). In the docking experiments, the backbones would link to the ZBG by overlaying onto the N-H bond designated with red arrow. All docking was performed using LUDI link mode, where docked fragments are constrained such that a methyl group on the fragment must be aligned with a link site. The terminal N-H amide bond (Figure 6-1, red arrow) was selected as the link site. Initial docking used the default LUDI link library, which contains 1100 different fragments. Fragments were screened and ranked using a LUDI scoring function. Higher LUDI scores indicate higher predicted affinity, with each 100 points representing a predicted order of magnitude decrease in IC$_{50}$. The results from the initial screen with MMP-3 using the LUDI link library yielded modest scores for several compounds. The two highest scoring fragments (n-pentane and a
propylbenzene) had scores of less than 450. Consequently, we created a custom library primarily based on the work of Hadjuk et al., which resulted in three high, one moderate, and two low scoring fragments. The result of the LUDI docking for one of the high scoring compounds (AM-5, vide infra) is shown in Figure 6-2. The fragment in Figure 6-2 was found to reside in the S1' pocket of MMP-3. The high and low scoring fragments from the custom library were similar in structure (see R-groups Scheme 6-3); therefore, all six compounds were synthesized to test the accuracy of the LUDI docking and scoring function.

![Figure 6-2](image)

*Figure 6-2.* LUDI docking image of backbone fragment (green, in S1' subsite) with pyrone ZBG (colored by element) in the active site of MMP-3 (gray). This fragment combination leads to the compound designated AM-5 (see Scheme 1). The zinc(II) ion is shown as a magenta sphere.

### 6.2.3. Synthesis of MPIs AM-1 through AM-6

The synthesis of AM-1 through AM-6 was performed according to Scheme 6-3. Two synthetic routes were utilized, based on the commercial availability of the
desired amine backbones. 2-Carboxy-3-benzyloxy-6-methyl-pyran-4(1H)-one (6) was prepared by a literature method.\textsuperscript{[14]} Compound 6 was then activated with NHS, followed by coupling to the desired amine, and removal of the benzyl protecting group to yield compounds AM-1, AM-2, AM-3, and AM-4. The synthesis of AM-5 and AM-6 was accomplished similarly, but required the Suzuki coupling of 3-benzyloxy-6-methyl-pyran-4(1H)-one-2-carboxy-N-(4-iodobenzylamide) (7) with 4-cyanophenylboronic acid and 4-biphenylboronic acid, respectively, as an intermediate step.

\textbf{Scheme 6-3.} Synthesis of Pyrone-Based MPIs AM-1 through AM-6. (I) a) NHS, DCC, dry THF; b) ‘amine’, dry THF, 88%; c) 10% Pd/C, H\textsubscript{2} (g), 35psi, MeOH or 1:1 HCl:CH\textsubscript{3}COOH, 60–89%. (II) d) ArB(OH)\textsubscript{2}, 2M K\textsubscript{2}CO\textsubscript{3}, Pd(C\textsubscript{6}H\textsubscript{5}O\textsubscript{2})\textsubscript{2}, PPh\textsubscript{3}, toluene, 135 \degree C, 40–85%; e) 10% Pd/C, H\textsubscript{2} 35psi, MeOH or 1:1 HCl:CH\textsubscript{3}COOH, 60–91\%.
6.2.4. In Vitro Fluorescence-Based and Cell Invasion Assays of Pyrone-Based MPIs AM-1 through AM-6

The inhibitory activity of compounds AM-1 through AM-6 was evaluated under the same assay conditions as described earlier,[15] the IC\textsubscript{50} values are listed in Table 6-2. AM-2, AM-5, and AM-6 were the most potent compounds against MMP-3, with IC\textsubscript{50} values in the nanomolar range. The IC\textsubscript{50} values against MMP-3 correlate well with the scores obtained for each fragment using the program LUDI. Although the LUDI scores do not perfectly parallel the relative inhibitory activity, the approach presented here did clearly distinguish between poor, moderate, and exceptional MPIs for MMP-3. There was not, however, the same correlation between score and potency for the inhibitors against MMP-2 (vide infra).

Interestingly, the pyrone-based MPIs presented here are more potent than the analogous hydroxamate-based inhibitors,[7] which is contrary to the accepted dogma that hydroxamic acids are the best ZBGs.[16] It was also observed that an optimal distance between the ZBG and the backbone was crucial for inhibitory potency (compare AM-1, AM-2, and AM-3). The effect of backbone substituents was also noted, as the addition of a cyano group to the para position of the biphenyl increased the potency of the MPI (compare AM-2 and AM-5). Similar effects of linker length and backbone substituents were also observed with hydroxamate analogues.[17] These results strongly support the concept that ZBGs equal or superior to hydroxamates can be identified and utilized in novel MPI designs.[2, 18]
Table 6-2. IC$_{50}$ Values (μM) for MPIs Against MMP-1, MMP-2, and MMP-3. LUDI Scores for MMP-2 (PDB code 1QIB), and MMP-3 (PDB code 1G4K) are Shown.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>MMP-1</th>
<th>MMP-2</th>
<th>MMP-3</th>
<th>LUDI Score (MMP-2)</th>
<th>LUDI Score (MMP-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM-1</td>
<td>&gt;50</td>
<td>36(5)</td>
<td>&gt;50</td>
<td>NS$^a$</td>
<td>NS$^a$</td>
</tr>
<tr>
<td>AM-2</td>
<td>&gt;50</td>
<td>9.3(0.5)</td>
<td>0.24(0.01)</td>
<td>530</td>
<td>600</td>
</tr>
<tr>
<td>AM-3</td>
<td>&gt;50</td>
<td>27(2)</td>
<td>36(1)</td>
<td>NS$^a$</td>
<td>NS$^a$</td>
</tr>
<tr>
<td>AM-4</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>2.4(0.2)</td>
<td>440</td>
<td>440</td>
</tr>
<tr>
<td>AM-5</td>
<td>&gt;50</td>
<td>0.61(0.01)</td>
<td>0.010(0.002)</td>
<td>570</td>
<td>640</td>
</tr>
<tr>
<td>AM-6</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>0.019(0.002)</td>
<td>690</td>
<td>700</td>
</tr>
</tbody>
</table>

$^a$ NS = No Score, no acceptable conformations were found.

The observed trends in the IC$_{50}$ values of the MPIs described here against MMP-3 suggest that the large aromatic backbone substituents of these compounds occupy the S1' subsite. This hypothesis was further examined by determining the selectivity of these compounds against different MMPs. Traditionally, the incorporation of bulky groups directed toward the S1' pocket results in selectivity over MMP-1, which has a shallow S1' pocket.\[^{11, 19}\] All six MPIs were found to be poor inhibitors of MMP-1 (Table 6-2). The poor activity of these compounds against MMP-1 is wholly consistent with the aryl backbone groups occupying the S1' pocket, which supports the LUDI results (Figure 6-2) and ZBG orientation predicted by the bioinorganic modeling studies.\[^{6}\]

The inhibitors were also tested for potency against MMP-2. Like MMP-3, MMP-2 has a deep S1' pocket and potency against these two enzymes is expected to be comparable, as found with hydroxamate-based MPIs.\[^{11, 19}\] Interestingly, although
AM-2, AM-4, AM-5, and AM-6 showed a range of potencies against MMP-3, all four compounds were substantially less potent against MMP-2. Indeed, AM-5 showed >2500-fold selectivity for MMP-3, which, to the best of our knowledge, is the highest selectivity reported for an MPI for MMP-3 over MMP-2.

The observed selectivity of these compounds for MMP-3 over MMP-2 is in contrast to the selectivity observed for most deep S1' pocket MPIs. Hydroxamate-based MPIs that occupy the S1' pocket are almost exclusively more potent for MMP-2 than MMP-3, with few exceptions.[11, 19-21] MPIs reported to be selective for MMP-3 over MMP-2 generally target the S3' subsite;[20, 21] however, based on the LUDI docking (Figure 6-3), the MPIs presented here have no significant interactions in the S3' subsite, and indeed give similar LUDI scores when docked to MMP-2 or MMP-3 (Table 6-2). Therefore, it is plausible that the observed selectivity originates from the pyrone ZBG. It has been reported that more acidic ZBGs, such as carboxylates (a weaker ZBG than the hydroxamate),[16] are generally more potent for MMP-3 than MMP-2,[20-22] which is attributed to the difference in the optimal pH for the two enzymes. MMP-3 prefers a more acidic environment (pH ~6.0) compared with other MMPs (including MMP-2), which favor a higher pH (~7.5).[23] By analogy, we propose that the selectivity of the MPIs reported here is due to the greater acidity of the pyrone versus hydroxamate chelator (ΔpKₐ ~1).[24] These results suggest that the ZBG, and not only the MPI backbone, can provide selectivity between different MMPs without compromising potency.
Figure 6-3. LUDI docking image of backbone fragment (green, in S1' subsite) with pyrone ZBG (colored by element) in the active site of MMP-2 (top) and MMP-3 (bottom). This fragment combination leads to the compound designated AM-6 (see Scheme 6-3). The zinc(II) ion is shown as a gold sphere. There are no interactions apparent in these docking experiments that explain the observed selectivity for MMP-3 over MMP-2 (similar results are obtained for AM-2 and AM-5).

We next examined the ability of AM-5 and AM-6 to inhibit a biological MMP-dependent process, namely the invasion of fibrosarcoma HT1080 cells through a membrane comprised of ECM proteins. As described in Chapter 4, during the cell invasion assay, cells move from an upper to a lower chamber as a function of their ability to degrade a synthetic membrane comprised of ECM proteins. Thus, this cell
invasion process is dependent on MMP activity. A standard cell invasion assay kit was used to examine this phenomenon and the results are summarized in Figure 6-4. At a concentration of 250 nm, the two inhibitors were found to reduce invasion by 67% (AM-5) and 55% (AM-6).

![Figure 6-4](image)

**Figure 6-4.** Neonatal cardiac fibroblast (CF) invasion assay results. Fluorescent measurement (in RFUs) of lysed cells after invasion with: no inhibitor (Control), 250 nM AM-5, and 250 nM AM-6. Increased RFUs indicates increased cell invasion.

### 6.2.5. Synthesis of Pyrone-Derived MPIs

With the promising results of the AM series of inhibitors, we decided to expand our inhibitor library by synthesizing derivatives that take advantage of the potent and chemically versatile pyrones while changing the properties of the MPIs. We sought to synthesize a variety of pyrone-derived MPIs. In this section, four derivatives will be discussed, they include: more water-soluble derivatives, bifunctional derivatives, thiol-based derivatives, and pyridinone derivatives. Through these derivatives, we can examine the full potential of pyrone-based MPIs.
Using a literature method, compound 10 was synthesized in three steps from maltol (an inexpensive, commercially available starting material). Maltol was first benzyl-protected, then oxidized using SeO$_2$ in bromobenzene to 9. Compound 9 was oxidized to the desired carboxylic acid (10) under the same conditions as for the synthesis of 6. This compound resembles 6, however it is lacking the 6-methyl group. With this hydrophobic group eliminated, the MPIs derived from this functionalized pyrone should be more water-soluble. Additionally, the removal of the 6-methyl group (an electron donor) decreases the p$K_a$ of the inhibitor, potentially making these inhibitors more selective for MMP-3. We initially made the inhibitor PY-2 (Scheme 6-4) from compound 10 as a direct analogue of AM-2 (via the same synthetic route). This inhibitor was found to be more potent (Table 6-3) against MMP-3 than its AM analogue. The 5-fold increase in potency may be attributed to the lower p$K_a$ ($\Delta pK_a$ $\sim$1)$^{[26]}$ of the inhibitor. We have already described the preference of MMP-3 for more acidic environments.$^{[23]}$ One cannot rule out the possibility that the 6-methyl group in AM-2 was too bulky, and its removal led to a more potent derivative. The modeling studies with the AM series of inhibitors however, does not suggest that the 6-methyl group had any unfavorable interactions with the protein. Future directions involve the synthesis of PY analogues of AM-5 and AM-6. These derivatives promise to be quite potent.
One drawback of hydroxamate-based MPIs is that it is difficult to synthesize inhibitors that interact with both the primed and unprimed sites of the MMP active site. In fact, the few examples of bi-functional or “double handed” MPIs contain carboxylate or phosphinate ZBGs (see Chapter 1). Pyrones, as shown previously, can be functionalized on each side of the pyrone ring. We developed the chemistry to asymmetrically attach backbones to each side of the pyrone ring. The synthesis of the first “double-handed” pyrone-based MPI is shown in Scheme 6-5. Kojic azide (2) was formylated and benzyl protected to produce 12. Using “click chemistry”,\textsuperscript{[27]} compound 12 was coupled with phenylacetylene to produce the triazole 13. The free hydroxyl group of 13 was oxidized to 14 using Jones Reagent. Further activation and
amide coupling, followed by acid deprotection yielded the compound DH-1. While this compound was not a potent inhibitor of MMP-3 (Table 6-3), the newly developed chemistry will allow for a new library of “double-handed” pyrone MPIs that can take advantage of both sides of the MMP active site.

Scheme 6-5. Synthesis of DH-1. a) HCHO, NaOH, H₂O, RT, 70%; b) BnBr, NaOH(aq), MeOH, 75 °C, 70%; c) Phenylacetylene, CuSO₄, Cu powder, MeOH, 7 days, RT, 85%; d) Jones Reagent, 5 °C, 10%; e) NHS, DCC, dry THF, RT; f) 4-Phenylbenzylamine, dry THF, RT, 88% (2 steps); g) 1:1 HCl:CH₃COOH, RT, 70%.
The ZBG, 3-hydroxy-2-methyl-4-thiopyrone (thiomaltol)\textsuperscript{[28, 29]} was found to be a \(~30\)-fold more potent inhibitor of MMP-3 than maltol. Therefore, we sought to synthesize an MPI based on this O,S donor. Previous work in our lab allows for the facile thionation of maltol\textsuperscript{[29]} Under similar conditions (doubling the amount of P\textsubscript{4}S\textsubscript{10} and hexamethyldisiloxane, HMDO), AM-2 was thionated. Interestingly, the thionation of AM-2 proceeded much more rapidly than that of maltol. Furthermore, the thionation of AM-2 resulted in only the mono-thionated product AM-2S (Scheme 6-6). What was most remarkable about this compound is that it was found to be a less effective inhibitor than AM-2 (Table 6-3). A likely explanation for this is that the same inversion of ZBG orientation has occurred as in the case of 1-hydroxy-2(1\textit{H})-pyridinone and 1-hydroxy-2(1\textit{H})-pyridinethione (Chapter 5). This would position the biaryl backbone towards the unprimed subsites, resulting in a poor drug-protein interaction. Therefore, in the design of thiol-based MPIs, one needs to be mindful of the differences in the ZBG orientations relative to their O,O analogues. If the inversion in orientation did occur as observed in the previous ZBGs (Chapter 5), positioning the backbone on the other side of the pyrone ring might yield a potent inhibitor. The thio-derivative of CA-2, CA-2S was synthesized similarly to AM-2S, however it decomposed in the presence of air and light and could not be examined for potency.
Finally, we sought to further examine the effect of the pK\textsubscript{a} of the pyrone-based MPIs. We have found that MPIs based on the more acidic pyrone ZBGs (vide supra) are potent inhibitors of MMP-3 than hydroxamate-based MPIs with similar backbone substituents. Conversely, we hypothesize that by increasing the pK\textsubscript{a} of the inhibitor we can increase the potency for other MMPs, which prefer a more basic environment relative of MMP-3.\textsuperscript{23} Pyridinones are more basic chelators than pyrones, and we have shown pyridinones to be potent ZBGs (Chapter 4). Using known nitrogen-insertion chemistry,\textsuperscript{4, 14, 24, 30, 31} we have synthesized the pyridinone analogue of PY-2 (Scheme 6-7). Heating a solution of the protected inhibitor PY-2P and ethanol/water mixture with three equivalents of methylamine, followed by deprotection gave the inhibitor PY-2(N-CH\textsubscript{3}). This compound was found to be a worse inhibitor of MMP-3 than the PY analogue, while having the same potency against MMP-2 (Table 6-3). The poor potency for both enzymes may be due to the N-methyl group clashing with the protein backbone. Therefore a less sterically demanding derivative, PY-2P(NH) (Table 6-3) was synthesized by substituting ammonia for methylamine under the same conditions as described for PY-2P(N-CH\textsubscript{3}). The assay of PY-2P(NH) has not yet been completed.
Scheme 6-7. Synthesis of PY-2(N-CH₃). a) methylamine, EtOH, H₂O, 61%; b) 1:1 HCl:CH₃COOH, RT, 85%.
**Table 6-3.** Structures and IC$_{50}$ Values (µM) of Pyrone-Based MPIs and Derivatives Against MMP-1, MMP-2 and MMP-3. Values are Obtained from Fluorescence-based MMP Assays. ND = not determined.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Structure</th>
<th>MMP-1</th>
<th>MMP-2</th>
<th>MMP-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM-2</td>
<td><img src="image" alt="Structure AM-2" /></td>
<td>&gt;50</td>
<td>9.3(0.5)</td>
<td>0.24(0.01)</td>
</tr>
<tr>
<td>PY-2</td>
<td><img src="image" alt="Structure PY-2" /></td>
<td>ND</td>
<td>6</td>
<td>0.054(4)</td>
</tr>
<tr>
<td>DH-1</td>
<td><img src="image" alt="Structure DH-1" /></td>
<td>ND</td>
<td>ND</td>
<td>&gt;10</td>
</tr>
<tr>
<td>AM-2S</td>
<td><img src="image" alt="Structure AM-2S" /></td>
<td>ND</td>
<td>ND</td>
<td>5.5(0.6)</td>
</tr>
<tr>
<td>PY-2(N-CH$_3$)</td>
<td>![Structure PY-2(N-CH$_3$)]</td>
<td>ND</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>PY-2(NH)</td>
<td>![Structure PY-2(NH)]</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
6.3. Future Pyrone Chemistry

The previous sections in this chapter have shown the versatility of the pyrones. In addition to the vast amount of derivatives one could make of the compounds described, there is also the potential to combine different pyrone modifications, such as the thionation of PY-2(NH) or the N-insertion of a double handed inhibitor. In this final section however, a potential MPI containing a new functional group and the scheme for developing this inhibitor will be discussed.

The proposed scheme (Scheme 6-8) details the synthesis of a derivative of AM-2, where the 6-methyl group is replaced with a 6-hydroxymethyl group. This free hydroxyl group should increase the aqueous solubility of the MPI, as well as provide an additional functional group for the attachment of backbone groups directed toward the unprimed subsites. The synthesis of X-2 begins with the methyl-protection of the 6-hydroxymethyl group of benzyl kojic acid (15). The benzyl-protecting group can be removed selectively with acid as described for previous MPIs. Compound (17) can be formylated and then benzyl-protected again to produce 19. Oxidation of 19 in two steps will lead to the acid 21. Activation with NHS and coupling with 4-phenylbenzylamine, followed by deprotection using BBr₃ should yield the MPI.
Scheme 6-8. Proposed synthesis of X-2. a) BnBr, NaOH(aq), MeOH, 75 °C; b) MeI, NaH, dry DMF, 24 h; c) 1:1 HCl:CH₃COOH, RT, 70%; d) HCHO, NaOH, H₂O, RT, 70%; e) BnBr, NaOH(aq), MeOH, 75 °C; f) SO₃ • pyridine, Et₃N, DMSO, CHCl₃, RT; g) NaClO₂, NH₄SO₃H, H₂O/acetone, RT; h) NHS, DCC, dry THF, RT; i) 4-Phenylbenzylamine, dry THF, RT; j) BBr₃, CH₂Cl₂.
6.4. Conclusion

The use of pyrone ZBGs results in more potent inhibitors than those produced with the widely employed hydroxamate group. Our results indicate that the use of a non-hydroxamate ZBG reveals a novel route to MMP inhibitor selectivity. Additionally, the versatility of pyrone chemistry has enabled the development multiple derivatives including more water-soluble, bi-functional, thiol, and pyridinone-based MPIs. Overall, this thesis provides a novel method for the development of matrix metalloprotein inhibitors.

6.5. Experimental Section

General. Unless otherwise noted, starting materials were obtained from commercial suppliers and used without further purification. Compounds 1,\textsuperscript{14} 5,\textsuperscript{12} and 6\textsuperscript{14} were synthesized as previously described. Elemental analysis was performed at NuMega Resonance Labs, San Diego, California. \textsuperscript{1}H/\textsuperscript{13}C NMR spectra were recorded on a Varian FT-NMR spectrometer running at 300 or 400 MHz at the Department of Chemistry and Biochemistry, University of California, San Diego. Mass spectra were acquired at the Small Molecule Mass Spectrometry Facility located in the Department of Chemistry and Biochemistry, University of California, San Diego. A ThermoFinnigan MAT 900XL mass spectrometer was used to acquire the data for the high resolution mass spectra (HRMS). The HRMS spectra were obtained with fast atom bombardment (FAB) as the ion source with 3-nitrobenzylalcohol as the matrix and polyethylene glycol as a reference.
**Kojic Azide (2).** Chlorokojic acid (1) (9.8 g 0.06 mol) was added to a suspension of sodium azide (3.97 g 0.06 mol) in DMF (40 mL). The solution was stirred overnight at room temperature under a N\(_2\) atmosphere. The solution was then poured into water (100 mL), precipitating the product as a cream colored solid. The solid was filtered and dried in vacuo to obtain 7.8 g (76%). \(^1\)HNMR (CDCl\(_3\), 300 MHz, 25 °C) \(\delta 4.19 \text{ (s, 2H, CH}_2\text{), 6.47 (s, 1H), 7.85 (s, 1H).} \) IR (KBr): \(\nu 1231 \text{ (N}_3\text{), 1659 (C=O), 2120 (N}_3\text{) cm}^{-1}\).

**Kojic Amine (3).** To a solution of (2) (325 mg 1.94 mmol) in EtOH (100 mL) was added Lindlar catalyst (100 mg). The solution was placed under H\(_2\)(g) at 35psi for 1 hour at room temperature. The catalyst was removed by filtration and the filtrate was evaporated to light brown solid. The solid was dissolved in hot water and filtered to remove any insolubles. The filtrate was evaporated to a light tan solid 250 mg (91%). \(^1\)HNMR (d\(^6\)-DMSO, 400 MHz, 25 °C): \(\delta 3.52 \text{ (s, 2H, CH}_2\text{), 6.38 (s, 1H), 7.98 (s, 1H).} \) IR (KBr): \(\nu 1216, 1289, 1575, 1659 (C=O), 3374-2683 \text{ (b, NH}_3\text{, OH) cm}^{-1}\). ESIMS(+): 142.1 [M+H]\(^+\).

**Phenyl(2-thioxothiazolidin-3-yl)methanone.** Benzoic acid (2 g 0.016 mol) and 2-mercapto-1,3-thiazolidine (2.1 g 0.17 mol) were dissolved in dry CH\(_2\)Cl\(_2\) (100 mL). To this solution was added DCC (3.6 g 0.017 mol) and a catalytic amount of DMAP (15 mg). The solution was stirred at room temperature under a N\(_2\)(g) atmosphere overnight. The reaction mixture was filtered to remove DCU and concentrated to a yellow oil. The product was purified by column chromatography on silica gel (elutant: CH\(_2\)Cl\(_2\)). Yield 70%. \(^1\)H NMR (d\(^6\)-DMSO, 400 MHz, 25 °C): \(\delta 3.6 \)
(t, $J = 14.7$ Hz, 2H, thiazole-CH$_2$), 4.5 (t, $J = 14.1$ Hz, 2H, thiazole-CH$_2$), 7.2 (d, $J = 7.6$ Hz, 2H, phenyl-H), 7.3 (t, $J = 15.2$ Hz, 3H, phenyl-H).

**2-Phenyl-1-(2-thioxothiazolidin-3-yl)ethanone.** The same procedure for the synthesis of phenyl(2-thioxothiazolidin-3-yl)methanone was used. Yield 73%. $^1$H NMR ($d^6$-DMSO, 400 MHz, 25 ℃): $\delta$ 2.9 (t, $J = 15.6$ Hz, 2H, CH$_2$), 3.4 (t, $J = 15$ Hz, 2H, CH$_2$), 3.5 (t, $J = 15.3$ Hz, 2H, thiazole-CH$_2$), 7.5 (m, 5H, phenyl-H).

**3-Phenyl-1-(2-thioxothiazolidin-3-yl)propan-1-one.** The same procedure for the synthesis of phenyl(2-thioxothiazolidin-3-yl)methanone was used. Yield 81%. $^1$H NMR ($d^6$-DMSO, 400 MHz, 25 ℃): $\delta$ 2.9 (t, $J = 15.6$ Hz, 2H, CH$_2$), 3.4 (t, $J = 15$ Hz, 2H, CH$_2$), 3.5 (t, $J = 15.3$ Hz, 2H, thiazole-CH$_2$), 4.5 (t, $J = 15.6$ Hz, 2H, thiazole-CH$_2$), 7.2 (m, 5H, phenyl-H).

**4-Phenyl-1-(2-thioxothiazolidin-3-yl)butan-1-one.** The same procedure for the synthesis of phenyl(2-thioxothiazolidin-3-yl)methanone was used. Yield 88%. $^1$H NMR ($d^6$-DMSO, 400 MHz, 25 ℃): $\delta$ 1.9 (quintuplet, $J = 15.2$ Hz 2H, CH$_2$), 2.6 (t, $J = 15.2$ Hz, 2H, CH$_2$), 3.1 (t, $J = 14.4$ Hz, 2H, CH$_2$), 3.7 (t, $J = 15.6$ Hz, 2H, thiazole-CH$_2$), 4.5 (t, $J = 15.6$ Hz, 2H, thiazole-CH$_2$), 7.2 (d, $J = 7.6$ Hz, 2H, phenyl-H), 7.3 (t, $J = 15.2$ Hz, 3H, phenyl-H).

**General Procedure for MPIs KA1-4.** Kojic amine (3) (200 mg 1.4 mmol) was dissolved in methanol (60 mL). To this solution was added triethylamine (197.4 uL 1.4 mmol). The corresponding activated amide (see above) was dissolved in CH$_2$Cl$_2$ and added to the above solution. The solution was stirred overnight at room temperature under a N$_2$(g) atmosphere. The reaction mixture was evaporated to an oil
which was purified by column chromatography on silica gel (elutant: 5-10% CH₂Cl₂/MeOH).

**KA1.** Yield 51%. ¹H NMR (d⁶-DMSO, 400 MHz, 25 °C): δ 4.3 (d, J = 5.4 Hz 2H, CH₂), 6.3 (s, 1H, pyrone-H), 7.5 (m, 3H, phenyl-H), 7.9 (d, J = 6.9 Hz, 2H, phenyl-H), 8.0 (s, 1H, pyrone-H), 9.1 (t, J = 12 Hz, 1H, amide-H).

**KA2.** Yield 30%. ¹H NMR (d⁶-DMSO, 400 MHz, 25 °C): δ 3.5 (s, 2H, CH₂), 4.1 (d, J = 5.4 Hz 2H, CH₂), 6.2 (s, 1H, pyrone-H), 7.3 (m, 5H, phenyl-H), 8.0 (s, 1H, pyrone-H), 8.5 (t, J = 12 Hz, 1H, amide-H). ¹³C NMR (d⁶-DMSO, 100 MHz, 25 °C): δ 42.1, 110.3, 126.4, 128.2, 128.9, 135.8, 139.3, 145.5, 164.9, 170.4, 173.5.

**KA3.** Yield 43%. ¹H NMR (d⁶-DMSO, 400 MHz, 25 °C): δ 2.5 (t, J = 16 Hz, 2H, CH₂), 2.8 (t, J = 15.6 Hz, 2H, CH₂), 4.1 (d, J = 6.0 Hz 2H, CH₂), 6.2 (s, 1H, pyrone-H), 7.2 (m, 5H, phenyl-H), 8.0 (s, 1H, pyrone-H), 8.4 (t, J = 12 Hz, 1H, amide-H), 9.1 (br s, 1H, pyrone-OH).

**KA4.** Yield 70%. ¹H NMR (d⁶-DMSO, 400 MHz, 25 °C): δ 1.8 (quintuplet, J = 15.2 Hz 2H, CH₂), 2.2 (t, J = 16 Hz, 2H, CH₂), 2.6 (t, J = 15.6 Hz, 2H, CH₂), 4.1 (d, J = 6.0 Hz 2H, CH₂), 6.2 (s, 1H, pyrone-H), 7.2 (d, J = 7.6 Hz, 2H, phenyl-H), 7.3 (t, J = 15.2 Hz, 3H, phenyl-H), 8.0 (s, 1H, pyrone-H), 8.4 (t, J = 12 Hz, 1H, amide-H), 9.1 (br s, 1H, pyrone-OH).

**5-Benzylxoy-2-hydroxymethyl-pyran-4(1H)-one (4).** To a solution of kojic acid (27.6 g 0.2 mol) in methanol (200 mL) was added NaOH (8.4 g 0.21 mol) in water (80 mL). The solution was heated to reflux at 90 °C. Benzyl bromide (25.1 mL, 0.21 mol) was added dropwise via syringe over 20 min. The reaction was kept at reflux over night under a N₂(g) atmosphere. The methanol was removed by rotary
evaporation and the product precipitated out as white needles. The solid was washed with cold water and dried to yield 35 g (80%). $^1$H NMR ($d^6$-DMSO, 300 MHz, 25 °C): δ 4.27 (d, $J = 6.0$ Hz, 2H, CH$_2$), 4.92 (s, 2H, benzyl-CH$_2$), 5.70 (t, $J = 6.0$ Hz, 1H, OH), 6.30 (s, 1H, pyrone-H), 7.4 (m, 5H, phenyl-H), 8.26 (s, 1H, pyrone-H).

5-Benzylxy-pyran-4(1H)-one-2-carboxylic acid (5) was synthesized according to a previous procedure. Yield 74%. $^1$H NMR ($d^6$-DMSO, 300 MHz, 25 °C): δ 4.96 (s, 2H, benzyl-CH$_2$), 6.91 (s, 1H, pyrone-H), 7.4 (m, 5H, phenyl-H), 8.35 (s, 1H, pyrone-H).

CA1P. To a solution of (5) (1.0 g 4.3 mmol) in dry THF (60 mL) was added NHS (500 mg 4.3 mmol). The reaction mixture was stirred for 30 minutes at room temperature under a N$_2$(g) atmosphere. DCC (890 mg 4.3 mmol) was added to the stirring solution and the mixture was continued to stir for 3 hours until TLC showed consumption of starting material. The DCU was filtered and a portion (2.1 mmol) of the resulting filtrate was used in situ to synthesize CA1P. To the filtered solution, was added 4-Biphenylamine (355 mg 2.1 mmol). The reaction was stirred at 60 °C for 5 days under a N$_2$(g) atmosphere. The product precipitated as a white solid, which was filtered, washed with 20 mL methanol and dried to yield 420 mg. Yield 78%. $^1$H NMR ($d^6$-DMSO, 400 MHz, 25 °C): δ 5.04 (s, 2H, benzyl-CH$_2$), 7.05 (s, 1H, pyrone-H), 7.40 (m, 8H, phenyl-H), 7.65 (t, 4H, $J = 15.0$ Hz, phenyl-H), 7.85 (d, $J = 7.8$ Hz, 2H, phenyl-H), 8.30 (s, 1H, pyrone-H), 10.75 (br, 1H, amide-H). APCIMS(+): m/z 398.02 [M+H]$^+$.  

CA1. To a suspension of CA1P (210 mg 0.63 mmol) in methanol (100 mL) was added 10% Pd/C (20 mg) and placed under H$_2$(g) at 35 psi overnight at room
temperature. The catalyst was filtered off and the filtrate was evaporated to an off-white solid, which was washed with 20 mL benzene and dried to yield 120 mg of a white solid (43%). $^1$H NMR ($d^6$-DMSO, 300 MHz, 25 °C): $\delta$ 7.06 (s, 1H, pyrone-H), 7.35 (br s, 1H, phenyl-H), 7.43 (t, $J = 15.2$ Hz, 2H, phenyl-H), 7.64 (t, $J = 15.2$ Hz, 4H, phenyl-H), 7.88 (d, $J = 8.8$ Hz, 2H, phenyl-H), 8.21 (s, 1H, pyrone-H), 9.69 (br, 1H, amide-H), 10.74 (s, 1H, OH).

**CA2P.** The same procedure was used as in the synthesis of CA1P to yield a white solid. Yield 73%. $^1$H NMR ($d^6$-DMSO, 400 MHz, 25 °C): $\delta$ 4.46 (d, $J = 5.6$ Hz, 2H, CH$_2$), 5.00 (s, 2H, benzyl-CH$_2$), 6.91 (s, 1H, pyrone-H), 7.44 (m, 10H, phenyl-H), 7.65 (m, 4H, phenyl-H), 8.26 (s, 1H, pyrone-H), 9.55 (t, $J = 12$ Hz, 1H, amide-H). ESI-MS(+): $m/z$ 434.1 [M+Na]$^+$.  

**CA2.** The same procedure was used as in the synthesis of CA1 to yield a white solid. Yield 62%. $^1$H NMR ($d^6$-DMSO, 300 MHz, 25 °C): $\delta$ 4.45 (d, $J = 6.0$ Hz, 2H, CH$_2$), 6.92 (s, 1H, pyrone-H), 7.44 (m, 5H, phenyl-H), 7.63 (m, 4H, phenyl-H), 8.13 (s, 1H, pyrone-H), 9.51 (t, $J = 12$ Hz, 1H, amide-H), 9.59 (s, 1H, OH). ESI-MS(-): $m/z$ 320.02 [M-H].

**3-Benzylxoy-6-methyl-pyran-4(1H)-one-2-carboxy-N-(4-iodobenzylamide)** (7). To a suspension of 6 (2.4 g, 9.4 mmol) in dry THF (100 mL) was added N-hydroxysuccinamide (NHS) (1.1 g, 9.4 mmol) and stirred at room temperature under N$_2$(g) for 30 min. 1,3-Dicyclohexylcarbodiimide (DCC) (1.9 g, 9.4 mmol) was then added and the reaction was stirred at room temperature under N$_2$(g) for 3 h. The N,N-dicyclohexylurea (DCU) was filtered and to the resulting filtrate was added 4-iodobenzylamine (2.2 g, 9.4 mmol) as a solid. The reaction was stirred overnight
under N$_2$(g) at 60 °C. The solvent was removed by evaporation and the residue was taken up in CHCl$_3$. The compound was purified by silica column chromatography (CHCl$_3$) to yield a white solid (3 g, 67%). $^1$HNMR (CDCl$_3$, 300 MHz, 25 °C): δ 2.37 (s, 3H, pyrone-CH$_3$), 4.33 (d, $J$ = 6.0 Hz, 2H), 5.34 (s, 2H, benzyl-CH$_2$), 6.29 (s, 1H, pyrone-H), 6.89 (d, $J$ = 8.4 Hz, 2H), 7.30 (m, 5H), 7.60 (d, $J$ = 8.4 Hz, 2H), 8.07 (br, 1H, amide-H). $^{13}$CNMR (CDCl$_3$, 100 MHz, 25 °C): δ 20.1, 43.2, 75.3, 94.2, 115.3, 128.7, 128.9, 129.0, 129.7, 135.0, 136.7, 137.6, 145.9, 165.6, 175.8. ESI-MS(+): $m/z$ 497.94 [M+Na]$^+$. 

3-Benzylxoy-6-methyl-pyran-4(1H)-one-2-carboxy-N-(4-biphenylamide) (AM-1P). To a suspension of 6 (250 mg, 0.95 mmol) in dry THF (25 mL) was added NHS (110 mg, 0.95 mmol) and stirred at room temperature under N$_2$(g) for 30 min. DCC (200 mg, 0.95 mmol) was then added and the reaction was stirred at room temperature under N$_2$(g) for 3 h. The DCU was removed by filtration, and to the resulting filtrate was added 4-biphenylamine (161 mg, 0.95 mmol) as a solid. The reaction was stirred for six days under N$_2$(g) at room temperature. The solvent was removed by evaporation and the residue was taken up in CHCl$_3$. The compound was purified by silica column chromatography (CHCl$_3$ with 0-1% MeOH) to yield a white solid (150 mg, 38%). $^1$HNMR (CDCl$_3$, 400 MHz, 25 °C): δ 2.38 (s, 3H, pyrone-CH$_3$), 5.50 (s, 2H, benzyl-CH$_2$), 6.31 (s, 1H, pyrone-H), 7.25 (d, $J$ = 8.8 Hz, 2H), 7.34 (m, 1H), 7.41 (m, 4H), 7.47 (m, 4H), 7.56 (m, 3H), 9.78 (br, 1H, amide-H). $^{13}$CNMR (CDCl$_3$, 100 MHz, 25 °C): δ 20.1, 76.3, 115.4, 120.0, 126.7, 127.1, 127.4, 128.7, 129.0, 129.3, 129.4, 135.0, 136.1, 137.6, 140.1, 146.1, 156.4, 165.7, 175.7. ESI-
MS(+): \( m/z \) 434.04 \([\text{M+Na}]^+\). HR-FABMS Calcd for \( \text{C}_{26}\text{H}_{22}\text{NO}_4 \): 412.1543. Found: 412.1547.

**3-Hydroxy-6-methyl-pyran-4(1H)–one-2-carboxy-N-(4-biphenylamide) (AM-1).** To a solution of \( \text{AM-1P} \) (100 mg, 0.24 mmol) in methanol (100 mL) was added 10\% Pd/C (10 mg, 10\% w/w). The reaction was placed under \( \text{H}_2(g) \) at 35 psi for 12 h. The catalyst was removed by filtration, and the resulting filtrate was evaporated to an off-white solid and recrystallized from EtOH (60 mg, 77\%). \(^1\)HNMR (\( d^3 \)-MeOD, 300 MHz, 25 °C): \( \delta \) 2.46 (s, 3H, pyrone-CH\(_3\)), 6.39 (s, 1H, pyrone-H), 7.34 (m, 1H), 7.43 (t, \( J = 7.2 \) Hz, 2H), 7.64 (t, \( J = 8.1 \) Hz, 4H), 7.80 (d, \( J = 8.7 \) Hz, 2H). \(^1^3\)CNMR (CDCl\(_3\), 100 MHz, 25 °C): \( \delta \) 20.5, 112.6, 120.8, 126.8, 127.3, 127.7, 128.7, 135.3, 135.7, 140.0, 147.2, 155.4, 165.2, 175.7. ESI-MS(+): \( m/z \) 322.06 \([\text{M+H}]^+\). Anal. Calcd for \( \text{C}_{19}\text{H}_{15}\text{NO}_4 \): C, 69.76; H, 5.27; N, 4.07. Found C, 69.54; H, 5.48; N, 4.30.

**3-Benzylxoy-6-methyl-pyran-4(1H)–one-2-carboxy-N-(4-phenylbenzylamide) (AM-2P).** To a suspension of \( \text{6 (700 mg, 2.7 mmol) in dry THF (40 mL) was added NHS (310 mg, 2.7 mmol) and stirred at room temperature under N\(_2(g)\) for 30 min. DCC (557 mg, 2.7 mmol) was then added and the reaction was stirred at room temperature under N\(_2(g)\) for 3 h. The DCU was removed by filtration, and the resulting filtrate was added 4-phenylbenzylamine (495 mg, 2.7 mmol) as a solid. The reaction was stirred overnight under N\(_2(g)\) at room temperature. The solvent was removed by evaporation and the residue was taken up in CHCl\(_3\). The compound was purified by silica column chromatography (CHCl\(_3\) with 0-1\% MeOH) to yield a white solid (800 mg, 90\%). \(^1\)HNMR (\( d^6 \)-DMSO, 400 MHz, 25 °C): \( \delta \) 2.29
(s, 3H, pyrone-CH₃), 4.45 (d, J = 6.0 Hz, 2H), 5.15 (s, 2H, benzyl-CH₂), 6.38 (s, 1H, pyrone-H), 7.35 (m, 8H), 7.46 (t, J = 7.4 Hz, 2H), 7.57 (d, J = 8.4 Hz, 2H), 7.62 (d, J = 8.4 Hz, 2H), 9.12 (t, J = 5.6 Hz, 1H, amide-H). \(^{13}\)CNMR (\(d^6\)-DMSO, 100 MHz, 25 °C): \(\delta\) 19.1, 42.2, 73.4, 114.5, 126.4, 126.5, 127.2, 127.9, 128.0, 128.1, 128.3, 128.7, 136.1, 137.4, 138.7, 139.6, 143.4, 149.0, 158.6, 165.1, 175.1. ESI-MS(+) : \(m/z\) 448.07 [M+Na]^+. HR-FABMS Calcd for C\(_{27}\)H\(_{24}\)NO\(_4\): 426.1700. Found: 426.1702.

3-Hydroxy-6-methyl-pyran-4(1H)–one-2-carboxy-N-(4-phenylbenzylamide) (AM-2). To a solution of AM-2P (100 mg, 0.24 mmol) in methanol (100 mL) was added 10% Pd/C (10 mg, 10% w/w). The reaction was placed under H₂(g) at 35 psi for 12 h. The catalyst was removed by filtration, and the resulting filtrate was evaporated to an off-white solid (60 mg, 76%). \(^1\)HNMR (\(d^6\)-DMSO, 400 MHz, 25 °C): \(\delta\) 2.31 (s, 3H, pyrone-CH₃), 4.53 (d, J = 6.4 Hz, 2H), 6.33 (s, 1H, pyrone-H), 7.35 (t, J = 7.4 Hz, 1H), 7.43 (q, J = 10.8 Hz, 4H), 7.63 (d, J = 10.4 Hz, 4H), 9.35 (t, J = 6.2 Hz, 1H, amide-H). \(^{13}\)CNMR (\(d^6\)-DMSO, 100 MHz, 25 °C): \(\delta\) 19.4, 42.0, 112.5, 126.4, 126.5, 127.2, 127.9, 128.7, 136.0, 137.4, 138.8, 139.6, 147.3, 162.5, 164.5, 173.3. APCI-MS(+) : \(m/z\) 335.95 [M+H]^+. Anal. Calcd for C\(_{20}\)H\(_{17}\)NO\(_4\): C, 71.63; H, 5.11; N, 4.18. Found C, 71.27; H, 5.43; N, 4.56.

3-Benzyloxy-6-methyl-pyran-4(1H)–one-2-carboxy-N-[2-(4-biphenyl)ethylamide)] (AM-3P). To a suspension of 6 (297 mg, 1.1 mmol) in dry THF (25 mL) was added NHS (131 mg, 1.1 mmol) and stirred at room temperature under N₂(g) for 30 min. DCC (236 mg, 1.1 mmol) was then added and the reaction was stirred at room temperature under N₂(g) for 3 h. The DCU was removed by filtration, and to the resulting filtrate was added 2-(4-biphenyl)ethylamide) (217 mg,
1.1 mmol) as a solid. The reaction was stirred overnight under \( \text{N}_2(\text{g}) \) at room temperature. The solvent was removed by evaporation and the residue was taken up in CHCl\(_3\). The compound was purified by silica column chromatography (CH\(_2\)Cl\(_2\)) to yield a white solid (450 mg, 90%). \(^1\)HNMR (CDCl\(_3\), 300 MHz, 25 °C): \( \delta \) 2.29 (s, 3H, pyrone-CH\(_3\)), 2.72 (t, \( J = 6.9 \) Hz, 2H), 3.50 (q, \( J = 6.9 \) Hz, 2H), 5.25 (s, 2H, benzyl-CH\(_2\)), 6.23 (s, 1H, pyrone-H), 7.20 (m, 4H), 7.30 (m, 4H), 7.40 (t, \( J = 6.9 \) Hz, 2H), 7.53 (t, \( J = 6.5 \) Hz, 4H), 7.83 (t, \( J = 5.1 \) Hz, 1H, amide-H). \(^{13}\)CNMR (CDCl\(_3\), 100 MHz, 25 °C): \( \delta \) 22.0, 36.7, 43.1, 75.0, 117.2, 128.8, 129.1, 129.2, 130.6, 130.7, 130.9, 131.0, 131.1, 137.2, 139.4, 141.4, 142.5, 147.6, 148.4, 160.8, 167.5, 177.9. ESI-MS(+) \( m/z \) 462.10 [M+Na]\(^+\).

3-Hydroxy-6-methyl-pyran-4(1H)-one-2-carboxy-N-[2-(4-biphenyl)ethylamide)] (AM-3). To a suspension of AM-3P (300 mg, 0.68 mmol) in methanol (50 mL) was added 10% Pd/C (30 mg, 10% w/w). The reaction was placed under H\(_2(\text{g})\) at 35 psi for 12 h. The catalyst was removed by filtration, and the resulting filtrate was evaporated to an off-white solid (205 mg, 86%). \(^1\)HNMR (CDCl\(_3\), 400 MHz, 25 °C): \( \delta \) 2.31 (s, 3H, pyrone-CH\(_3\)), 3.00 (t, \( J = 7.2 \) Hz, 2H), 3.75 (q, \( J = 6.8 \) Hz, 2H), 6.26 (s, 1H, pyrone-H), 6.89 (br, 1H, amide-H), 7.35 (m, 3H), 7.45 (t, \( J = 7.2 \) Hz, 2H), 7.59 (d, \( J = 6.8 \) Hz, 4H). \(^{13}\)CNMR (CDCl\(_3\), 100 MHz, 25 °C): \( \delta \) 20.3, 35.1, 40.8, 113.0, 126.8, 127.2, 127.3, 128.7, 129.0, 133.3, 136.9, 137.1, 137.3, 139.6, 140.4, 182.0. HR-FABMS Calcd for C\(_{21}\)H\(_{20}\)NO\(_4\): 350.1387. Found 350.1379. Anal. Calcd for C\(_{21}\)H\(_{19}\)NO\(_4\)•0.20 H\(_2\)O: C, 71.46; H, 5.54; N, 3.97. Found C, 71.31; H, 5.66; N, 4.34.
3-Benzyloxy-6-methyl-pyran-4(1H)-one-2-carboxy-N-(4-methoxybenzylamide) (AM-4P). To a suspension of 6 (515 mg, 1.98 mmol) in dry THF (40 mL) was added NHS (228 mg, 1.98 mmol) and stirred at room temperature under N₂(g) for 30 min. DCC (409 mg, 1.98 mmol) was then added and the reaction was stirred at room temperature under N₂(g) for 3 h. The DCU was removed by filtration, and to the resulting filtrate was added 4-methoxybenzylamine (271 mg, 260 µL, 1.98 mmol) in portions. The reaction was stirred overnight under N₂(g) at room temperature. The solvent was removed by evaporation and the residue was taken up in CHCl₃. The compound was purified by silica column chromatography (CHCl₃ with 0-3% MeOH) to yield a colorless oil which crystallized upon cooling at 4 °C (630 mg, 84%). ¹HNMR (CDCl₃, 400 MHz, 25 °C): δ 2.23 (s, 3H, pyrone-CH₃), 3.70 (s, 3H, methoxy-CH₃), 4.30 (d, J = 5.6 Hz, 2H), 5.19 (s, 2H, benzyl-CH₂), 6.15 (s, 1H, pyrone-H), 6.75 (d, J = 8.8 Hz, 2H), 7.05 (d, J = 8.4 Hz, 2H, 7.20 (m, 5H), 8.02 (t, J = 5.4 Hz, 1H, amide-H). ¹³CNMR (CDCl₃, 100 MHz, 25 °C): δ 19.5, 42.8, 54.9, 74.5, 113.6, 114.8, 128.2, 128.4, 128.6, 128.8, 128.9, 134.7, 145.1, 146.2, 158.2, 158.5, 165.1, 175.4. ESI-MS(+) m/z 402.05 [M+Na]⁺.

3-Hydroxy-6-methyl-pyran-4(1H)-one-2-carboxy-N-[4-methoxybenzylamide)] (AM-4). AM-4P (120 mg, 0.32 mmol) was dissolved in 7.3 mL of a 1:1 solution of concentrated HCl and glacial acetic acid. The solution was stirred under N₂(g) for 24 h at room temperature. The reaction was co-evaporated with methanol (2×20 mL), and dried under vacuum to yield a crude off-white solid, which was recrystallized from EtOH (55 mg, 60%). ¹HNMR (CDCl₃, 400 MHz, 25 °C): δ 2.31 (s, 3H, pyrone-CH₃), 3.80 (s, 3H, methoxy-CH₃), 4.55 (d, J = 6.0 Hz, 2H),
6.24 (s, 1H, pyrone-H), 6.88 (d, J = 8.4 Hz, 2H), 7.20 (br, 1H, amide-H), 7.28 (d, J = 8.8 Hz, 2H). $^{13}$CNMR (CDCl$_3$, 100 MHz, 25 °C): $\delta$ 20.2, 43.0, 55.3, 113.1, 114.1, 128.7, 129.3, 132.4, 148.2, 159.1, 174.0, 181.0. HR-FABMS Calcd for C$_{15}$H$_{15}$NO$_5$: 290.1023. Found: 290.1022. Anal. Calcd for C$_{15}$H$_{15}$NO$_5$: C, 62.28; H, 5.23; N, 4.84. Found: C, 61.89; H, 5.16; N, 4.95.

3-Benzyloxy-6-methyl-pyran-4(1H)–one-2-carboxy-N-(4,4-cyanophenylbenzylamide) (AM-5P). To a solution of 7 (300 mg, 0.63 mmol) in toluene (20 mL) and 2M aqueous K$_2$CO$_3$ (20 mL) was added 4-cyanophenylboronic acid (139 mg, 0.95 mmol), Pd(C$_2$H$_3$O$_2$)$_2$ (28 mg 0.13 mmol), and PPh$_3$ (34 mg, 0.13 mmol). The reaction was heated to reflux at 135 °C under N$_2$ (g) for 10 days. The reaction was extracted with 3×30 mL toluene and the organic layer was washed with 30 mL water. The organic layer was dried over anhydrous MgSO$_4$ and filtered. The resulting filtrate was evaporated to yield a light yellow solid. The product was purified by silica column chromatography (CH$_2$Cl$_2$ with 0-1% MeOH) to yield an off-white solid (100 mg, 40%). $^1$HNMR (CDCl$_3$, 400 MHz, 25 °C): $\delta$ 2.35 (s, 3H, pyrone-CH$_3$), 4.45 (d, J = 6.0 Hz, 2H), 5.33 (s, 2H, benzyl-CH$_2$), 6.27 (s, 1H, pyrone-H), 7.23 (m, 7H), 7.50 (d, J = 8.0 Hz, 2H), 7.64 (d, J = 8.0 Hz, 2H), 7.71 (d, J = 8.4 Hz, 2H), 8.13 (br, 1H, amide-H). $^{13}$CNMR (CDCl$_3$, 100 MHz, 25 °C): $\delta$ 20.0, 43.3, 75.2, 110.9, 115.3, 118.7, 127.4, 127.5, 128.5, 128.6, 128.9, 129.0, 132.5, 137.7, 138.3, 144.8, 145.9, 158.9, 165.6, 175.8. ESI-MS (+): m/z 473.13 [M+Na]$^+$. 

3-Hydroxy-6-methyl-pyran-4(1H)–one-2-carboxy-N-(4,4-cyanophenylbenzylamide) (AM-5). To a solution of AM-5P (75 mg, 0.17 mmol) in methanol (80 mL) was added 10% Pd/C (8 mg, 10% w/w). The reaction was placed
under H₂(g) at 35 psi for 20 h. The catalyst was removed by filtration, and the resulting filtrate was evaporated to a white solid (40 mg, 62%). ¹H NMR (CDCl₃, 400 MHz, 25 °C): δ 2.36 (s, 3H, pyrone-CH₃), 4.71 (d, J = 6.0 Hz, 2H), 6.29 (s, 1H, pyrone-H), 7.23 (br, 1H, amide-H), 7.47 (d, J = 8.4 Hz, 2H), 7.60 (d, J = 8.0 Hz, 2H), 7.67 (d, J = 8.0 Hz, 2H), 7.74 (d, J = 8.4 Hz, 2H). 7.41 (m, 4H), 7.47 (m, 4H), 7.56 (m, 3H). ¹³C NMR (CDCl₃, 100 MHz, 25 °C): δ 19.6, 42.6, 110.3, 112.6, 118.5, 127.1, 127.3, 128.1, 132.3, 136.0, 137.7, 138.0, 144.8, 147.7, 147.8, 162.8, 165.1. HR-FABMS Calcd for C₂₁H₁₇N₂O₄: 361.1183. Found: 361.1188. Anal. Calcd for C₂₁H₁₆N₂O₄•1.5H₂O: C, 65.11; H, 4.94; N, 7.23. Found: C, 64.95; H, 4.88; N, 6.87.

3-Benzylxy-6-methyl-pyran-4(1H)–one-2-carboxy-N-(4,4-

biphenylbenzylamide) (AM-6P). To a solution of 7 (200 mg, 0.42 mmol) in toluene (20 mL) and 2M aqueous K₂CO₃ (20 mL) was added 4-biphenylboronic acid (83 mg, 0.42 mmol), Pd(C₂H₅O₂)₂ (9.2 mg 0.04 mmol), and PPh₃ (11 mg, 0.04 mmol). The reaction was heated to reflux at 135 °C under N₂(g) for 24 h. The reaction was extracted with 3×30 mL toluene and the organic layer was washed with 30 mL water. The organic layer was dried over anhydrous MgSO₄ and filtered. The resulting filtrate was evaporated to yield a yellow solid. The product was purified by silica column chromatography (CHCl₃ with 0-5% MeOH) to an off-white solid (180 mg, 85%). ¹H NMR (CDCl₃, 400 MHz, 25 °C): δ 2.38 (s, 3H, pyrone-CH₃), 4.48 (d, J = 6.0 Hz, 2H), 5.35 (s, 2H, benzyl-CH₂), 6.29 (s, 1H, pyrone-H), 7.23 (m, 7H), 7.38 (t, J = 8.4 Hz, 1H), 7.46 (t, J = 8.0 Hz, 2H), 7.58 (d, J = 8.0 Hz, 2H), 7.67 (m, 6H), 8.13 (t, J = 5.4 Hz 1H, amide-H). ¹³C NMR (CDCl₃, 100 MHz, 25 °C): δ 20.1, 43.6, 75.2, 115.3,
3-Hydroxy-6-methyl-pyran-4(1H)-one-2-carboxy-N-(4,4-
benzhydrylbenzamide) (AM-6). To AM-6 (160 mg, 0.32 mmol) was added 5 mL of a 1:1 solution of concentrated HCl and glacial acetic acid. The suspension was stirred under N₂(g) for 24 h at room temperature. The reaction was co-evaporated with methanol (2×20 mL), and dried under vacuum to yield a white solid (120 mg, 91%).

\[ \text{1HNMR (CDCl}_3, 400 MHz, 25 ^\circ C): \delta 2.35 (s, 3H, pyrone-CH\textsubscript{3}), 4.70 (d, J = 5.6 Hz, 2H), 6.28 (s, 1H, pyrone-H), 7.14 (br, 1H, amide-H), 7.25 (m, 3H), 7.37 (t, J = 7.4 Hz, 1H), 7.46 (t, J = 8.4 Hz, 3H), 7.67 (m, 6H). \]

\[ \text{13CNMR (CDCl}_3, 100 MHz, 25 ^\circ C): \delta 20.4, 43.3, 113.1, 126.9, 127.2, 127.3, 127.4, 127.5, 128.4, 128.7, 135.3, 135.8, 139.1, 140.2, 140.3, 148.1, 140.4, 162.9, 164.3, 173.9. \]

HR-FABMS Calcd for C\textsubscript{26}H\textsubscript{22}NO\textsubscript{4}: 412.1543. Found: 412.1548. Anal. Calcd for C\textsubscript{26}H\textsubscript{21}NO\textsubscript{4}•0.9H\textsubscript{2}O: C, 73.08; H, 5.26; N, 3.06. Found: C, 73.02; H, 5.37; N, 3.28.

3-Benzyloxy-2-methyl-4-pyrone (8). To a suspension of 3-hydroxy-2-methyl-4-pyrone (maltol) (200 g 1.6 mol) in methanol (500 mL) was added 10 M NaOH (175 mL, 1.75 mol). The solution was heated to reflux at 80 °C and benzyl chloride (274 mL, 2.4 mol) was added in portions. The reaction was kept at reflux over night under a N₂(g) atmosphere. The inorganic salts were filtered off and the filtrate was evaporated to a red-orange residue which was taken up in 600 mL CHCl\textsubscript{3}. The solution was washed with 1 M NaOH (2 × 500 mL) and dried over MgSO\textsubscript{4} and filtered. The filtrate was evaporated to a brown oil (300 g, 86%).

\[ \text{1HNMR (CDCl}_3, \]
2-Formyl-3-benzyloxy-pyran-4(1H)–one (9). To a solution of 8 (20 g, 0.09 mol) in bromobenzene (175 mL) at 160 °C, was added SeO₂ (30.8 g, 0.27 mol). The suspension was stirred vigorously at 145 °C for 48 h. The reaction must be closely monitored by TLC (ether). After the reaction had shown no further signs of conversion to the product (~ 70% product by TLC), the reaction mixture was filtered and the filtrate was evaporated to a red-orange oil. The oil was dissolved in ether:hexanes (80:20) and purified by silica column to collect the crude product as a golden oil (12.6 g). The crude product was determined by ¹H NMR to be a mixture of 66% product and 33% starting material, giving a yield of 8.3 g of product (40%). The crude product was used without any further purification in the next step. ¹H NMR (CDCl₃, 400 MHz, 25 °C): δ 5.51 (s, 2H), 6.50 (d, J = 5.6 Hz, 1H, pyrone-H), 7.36 (m, 5H), 7.76 (d, J = 6.0 Hz, 1H, pyrone-H), 9.87 (s, 1H, formyl-H).

2-Carboxy-3-benzyloxy-pyran-4(1H)–one (10). To a solution of crude 9 (12.6 g total, 8.3 g 9, 0.04 mmol) in acetone (100 mL) and water (100 mL) was added sulfamic acid (5.5 g, 0.056 mol) and sodium chlorite (3.8 g, 0.42 mol). The reaction mixture instantly heated up and turned bright yellow in color. The reaction mixture was stirred in an open vessel for 90 minutes. The product precipitated as a white solid after 1 h of stirring. The acetone was removed by rotary evaporation, precipitating more product as a white solid, which was filtered and was washed with a minimal amount diethyl ether. The solid was dried (9.7 g, 98%). ¹H NMR (d₆-DMSO, 400 MHz, 25 °C): δ 5.10 (s, 2H), 6.54 (d, J = 5.6 Hz, 1H, pyrone-H), 7.35 (q, J = 6.0 Hz,
2-Hydroxymethyl-3-hydroxy-6-azidomethyl-pyran-4(1H)–one (11). To a solution of 2 (10 g, 0.06 mol) in water (75 mL) was added 10 M NaOH (6.6 mL, 0.066 mol) and stirred for 10 min at RT. To this solution was added formaldehyde (37% w/w, 5.4 mL, 0.2 mol) was added dropwise over 20 min. This reaction mixture was stirred overnight at RT under N₂(g). The reaction mixture was acidified to pH 1 with HCl (conc), turning the solution a bright red. The acidified solution was evaporated to 1/2 volume and placed in the freezer overnight to crystallize the product as a yellow solid. After thawing the frozen suspension, the product was filtered off and washed with diethyl ether to yield 6.9 g (59%). ¹HNMR (d⁶-DMSO, 300 MHz, 25 °C): δ 4.41 (d, J = 4.5 Hz, 4H), 6.43 (s, 1H, pyrone-H), 9.19 (br s, 1H, OH). ¹³CNMR (d⁶-DMSO, 100 MHz, 25 °C): δ 50.2, 55.0, 111.5, 141.8, 149.9, 160.9, 173.5. APCIMS(+): m/z 197.94 [M+H]+.

2-Hydroxymethyl-3-benzyloxy-6-azidomethyl-pyran-4(1H)–one (12). To a suspension of 11 (2 g, 10.1 mmol) in methanol (20 mL) was added 10 M NaOH (1.12 mL, 11.2 mmol) and heated to reflux at 75 °C. To this solution was added benzyl bromide (1.33 mL, 11.2 mmol) was added dropwise via syringe over 10 min. This reaction mixture was stirred overnight at 75 °C under N₂(g). The reaction mixture was evaporated to a brown residue. The residue was taken up in CHCl₃ and purified by a silica column (elutant: CHCl₃ with 0-5% MeOH) to yield 1.2 g of a light yellow crystalline solid (41%). ¹HNMR (d⁶-DMSO, 300 MHz, 25 °C): δ 4.27 (d, J = 6.0 Hz,
2H), 4.44 (s, 2H), 5.02 (s, 2H), 5.51 (t, J = 6.0 Hz, 2H, OH), 6.47 (s, 1H, pyrone-H), 7.34 (m, 5H). APCIMS(+) : m/z 287.86 [M+H]⁺.

2-Hydroxymethyl-3-benzyloxy-6-phenyltriazole-pyran-4(1H)-one (13). To a suspension of 12 (1.5 g, 5.2 mmol) and phenylacetylene (573 µL, 5.2 mmol) in methanol (25 mL) was added Cu powder (209 mg, 3.3 mmol) and CuSO₄ (47 mg in 15 mL H₂O). The solution became cloudy after stirring for 1 min at RT under N₂(g). The suspension was monitored by TLC and was stirred for a total of 7 days. Water (20 mL) was added to the reaction mixture, and the product was filtered off to yield 1.7 g of a light yellow solid (85%). ¹HNMR (CDCl₃, 400 MHz, 25 ºC): δ 2.38 (br s, 1H, OH), 4.32 (d, J = 5.6 Hz, 2H), 5.16 (s, 2H), 5.36 (s, 2H), 6.26 (s, 1H, pyrone-H), 7.35 (m, 5H), 7.41 (t, J = 8.0 Hz, 2H, phenyltriazole-H), 7.77 (d, J = 8.0 Hz, 2H, phenyltriazole-H), 7.86 (s, 1H, triazole-H). ¹³CNMR (CDCl₃, 100 MHz, 25 ºC): δ 50.7, 57.2, 73.9, 115.4, 120.1, 125.7, 128.5, 128.6, 128.7, 128.8, 129.1, 129.6, 135.9, 142.6 159.1. ESIMS(+): m/z 412.03 [M+Na]⁺.

2-Carboxy-3-benzyloxy-6-phenyltriazole-pyran-4(1H)-one (14). To a solution of 13 (1.2 g, 3.1 mmol) in acetone (200 mL) was added Jones Reagent (2.6 M, 2.0 mL, 6.2 mmol) dropwise over 20 min on ice. The solution was stirred on ice in an open vessel for 2 h. The ice was allowed to thaw and the reaction was monitored by TLC. The reaction was stirred overnight at RT under N₂(g). The reaction mixture was filtered and the filtrate was evaporated to ~50mL. The solution was poured into water (150 mL) to precipitate the product was a yellow solid which was dried to yield 140 mg (11%). Note: the Jones Reagent in this reaction was added in 0.5 mL portions over 24 h. The addition of 2 mL at the initiation of the reaction may produce the
desired product much quicker. \(^1\)HNMR (CDCl\(_3\), 300 MHz, 25 °C): \(\delta\) 5.49 (s, 2H), 5.52 (s, 2H), 6.37 (s, 1H, pyrone-H), 7.37 (m, 8H), 7.82 (d, \(J = 8.0\) Hz, 2H, phenyltriazole-H), 8.00 (s, 1H, triazole-H). APCIMS(+): \(m/z\) 403.97 [M+H]\(^+\).

**DH-1P.** To a solution of 14 (160 mg, 0.4 mmol) in dry THF (25 mL) was added NHS (46 mg, 0.4 mmol) and stirred at room temperature under N\(_2\)(g) for 30 min. DCC (82 mg, 0.4 mmol) was then added and the reaction was stirred at room temperature under N\(_2\)(g) for 3 h. The DCU was removed by filtration, and to the resulting filtrate was added 4-phenylbenzylamine) (73 mg, 0.4 mmol). The reaction was stirred overnight under N\(_2\)(g) at room temperature. The solvent was removed by evaporation and the brown residue was taken up in CHCl\(_3\). The compound was purified by silica column chromatography (CHCl\(_3\) with 0-1% MeOH) to yield a light yellow solid (160 mg, 70%). \(^1\)HNMR (CDCl\(_3\), 400 MHz, 25 °C): \(\delta\) 4.47 (d, \(J = 5.6\) Hz, 2H), 5.34 (s, 2H), 5.52 (s, 2H), 6.36 (s, 1H, pyrone-H), 7.23 (m, 7H), 7.38 (m, 2H), 7.45 (m, 4H), 7.53 (d, \(J = 8.0\) Hz, 2H), 7.58 (d, \(J = 7.2\) Hz, 2H), 7.87 (d, \(J = 7.2\) Hz, 2H), 8.09 (m, 2H). ESI-MS(+): \(m/z\) 591.08 [M+Na]\(^+\).

**DH-1.** DH-1P (75 mg, 0.13 mmol) was dissolved in 3 mL of a 1:1 solution of concentrated HCl and glacial acetic acid. The solution was stirred under N\(_2\)(g) for 24 h at room temperature. The reaction was co-evaporated with methanol (3 × 30 mL), and dried under vacuum to yield a white solid (40 mg, 65%). \(^1\)HNMR (\(d^6\)-DMSO, 300 MHz, 25 °C): \(\delta\) 4.55 (d, \(J = 6.0\) Hz, 2H), 5.68 (s, 2H), 6.45 (s, 1H, pyrone-H), 7.40 (m, 8H), 7.57 (m, 4H), 7.86 (d, \(J = 9.6\) Hz, 2H), 8.79 (s, 1H), 9.36 (t, \(J = 6.0\) Hz, 1H, amide-H). HR-FABMS Calcd for C\(_{28}\)H\(_{23}\)N\(_4\)O\(_4\): 479.1714. Found: 479.1722.
**AM-2S.** To a solution of **AM-2** (200 mg, 0.6 mmol) in benzene (80 mL) at 65 °C was added **P₄S₁₀** (97 mg, 0.22 mmol, 0.366 eq) and **HMDO** (323 mg, 2 mmol, 3.339 eq). The reaction mixture was heated to reflux at 90 °C for 90 minutes. The reaction mixture was filtered to give a yellow filtrate, which was evaporated to a red-orange residue. The compound was purified by a silica column (elutant: **CH₂Cl₂** with 0-5% **MeOH**) (pre-loaded with a maltol solution in **CH₂Cl₂**/**MeOH** to remove any metal contaminants, and washed with the eluting solvent until no maltol could be detected by TLC) to an orange solid (105 mg, 50%). **¹H NMR (CDCl₃, 400 MHz, 25 °C):** δ 2.43 (s, 3H, pyrone-CH₃), 4.71 (d, J = 5.6 Hz, 2H), 7.32 (s, 1H, pyrone-H), 7.36 (m, 1H), 7.43 (m, 4H), 7.63 (m, 4H), 7.99 (br s, 1H, OH), 9.53 (br t, 1H, amide-H). **APCI-MS(-): m/z 350.09 [M-H].** Anal. Calcd for C₃₀H₁₇NO₃S: C, 68.36; H, 4.88; N, 3.99. Found: C, 67.98; H, 4.89; N, 4.16.

**PY-2P(N-CH₃).** To a solution of **PY-2P** (300 mg, 0.73 mmol) in ethanol/water 50:50 (60 mL) was added methylamine by syringe (192 µL, 2.2 mmol). The solution was heated to reflux at 90 °C for 48 h. The solution was allowed to cool, causing the product to crystallize. The off-white solid was filtered and dried to yield 190 mg 61%. **¹H NMR (d₆-DMSO, 400 MHz, 25 °C):** δ 3.60 (s, 3H, N-CH₃), 4.47 (d, J = 6.0 Hz, 2H), 5.06 (s, 2H, benzyl-CH₂), 6.13 (d, J = 6.0 Hz, 2H, pyridinone -H), 7.00 (d, J = 6.0 Hz, 2H, pyridinone -H), 7.25 (m, 6H), 7.35 (m, 2H), 7.44 (m, 4H), 7.53 (m, 2H), 8.01 (t, J = 5.6 Hz, 1H, amide-H). **APCI-MS(+) m/z 425.02 [M+H].**

**PY-2(N-CH₃).** To **PY-2P(N-CH₃)** (150 mg, 0.35 mmol) was added 8 mL of a 1:1 solution of concentrated **HCl** and glacial acetic acid. The suspension was stirred under **N₂(g)** for 5 days h at room temperature. The reaction was co-evaporated with
methanol (2×20 mL), and dried under vacuum to yield a white solid (100 mg, 85%).

\[^1\]HNMR (DMSO , 400 MHz, 25 °C): δ 3.90 (s, 3H, N-CH₃), 4.55 (d, J = 5.6 Hz, 2H), 7.22 (d, J = 7.2 Hz, 1H, pyridinone-H), 7.37 (t, J = 8.0 Hz,1H), 7.48 (m, 4H), 7.65 (m, 4H), 8.18 (d, J = 7.2 Hz, 1H, pyridinone-H), 9.53 (t, J = 5.6 Hz, 1H, amide-H). HR-FABMS Calcd for C_{26}H_{22}NO₄: 335.1390. Found: 335.1393.

**PY-2P(NH).** PY-2P(NH) was synthesized in a similar procedure to PY-2P(N-CH₃) using NH₃ (2 M in ethanol) to yield a beige solid (50%). \[^1\]HNMR (CDCl₃, 300 MHz, 25 °C): δ 4.46 (d, J = 6.0 Hz, 2H), 5.49 (s, 2H, benzyl-CH₂), 6.55 (d, J = 6.0 Hz, 2H, pyridinone-H), 7.25 (m, 6H), 7.38 (m, 1H), 7.46 (t, J = 5.6 Hz, 4H), 7.53 (d, J = 5.6 Hz, 2H), 7.59 (d, J = 6.0 Hz, 2H, pyridinone-H), 8.70 (br t, amide-H), 9.54 (br d, pyridinone-NH). ESI-MS(+): m/z 433.10 [M+Na]^+.

**PY-2(NH).** To a solution of PY-2P(NH) (150 mg, 0.37 mmol) in methanol (75 mL) was added 10% Pd/C (15 mg). The reaction mixture was placed under H₂(g) at 35psi for 16 h at RT. The catalyst was filtered and the filtrate was evaporated to a red solid (the solid was red due to iron contamination). Note: the strong acid solution failed to deprotect this compound. \[^1\]HNMR (DMSO , 300 MHz, 25 °C): δ 4.59 (d, J = 6.0 Hz, 2H), 7.12 (d, J = 6.0 Hz, 1H, pyridinone-H), 7.34 (t, J = 8.0 Hz, 1H), 7.45 (m, 4H), 7.62 (d, J = 6.0 Hz, 2H), 7.93 (d, J = 6.0 Hz, 1H, pyridinone-H), 9.53 (t, J = 6.0 Hz, 1H, amide-H). ESI-MS(+): m/z 320.88 [M+H]^+.

**Recombinant MMP Assays.** Activities of *E. coli* recombinant human MMP-1 catalytic domain (amino acids 81-249, 19.9 kDa), MMP-2 catalytic domain (amino acids 81-423, 40 kDa), and MMP-3 catalytic domain (amino acids 83-255, 19.5 kDa) were measured utilizing a 96-well microplate fluorescent assay kit purchased from
BIOMOL International, following the procedure provided with the kit. Experiments were performed using a Bio-Tek Flx 800 fluorescence plate reader and Nunc white 96-well plates. The inhibitors (Scheme 6-3) were dissolved in DMSO and further diluted 500-fold in assay buffer: 50 mM HEPES, 10 mM CaCl₂, 0.05% Brij-35, pH 7.5 (MMP-1 and MMP-2) and 50 mM MES, 10 mM CaCl₂, 0.05% Brij-35, pH 6.0 (MMP-3). MMP-1, MMP-2 and MMP-3 were incubated individually with varying concentrations of different inhibitors for 1 h at 37 °C, followed by addition of substrate to initiate the assay. Reactions were agitated by shaking for 1 sec after each fluorescence measurement. Upon cleavage of the fluorescent substrate, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ (0.4 mM in assay; Mca = 7-methoxycoumarin-4-yl)-acetyl; Dpa = N-3-(2,4-dinitrophenyl)-L-α-β-diaminopropionyl) at the Gly-Leu bond, Mca fluorescence (λ<sub>ex</sub> = 340 nm, λ<sub>em</sub> = 400 nm) was measured at 60-second intervals for 20 min. Experiments were repeated at least three times. IC<sub>50</sub> values were calculated as the inhibitor concentration at which the enzyme is at 50% control activity (no inhibitor present).

**Computational Methods.** PDB structures 1G4K (MMP-3) and 1QIB (MMP-2) were used for docking with LUDI version 60a, as part of the InsightII 2000L framework. Calculations were run a dual-processor Xeon 2 GHz Linux workstation. For structures having more than one protein in the asymmetric unit, the “A” chain was selected. Proteins were protonated using the “Hydrogens” command of the Biopolymer module of InsightII. Crystal waters and inhibitors were removed from each structure. The zinc binding group (ZBG) 3-hydroxy-2-methyl-4-pyrene (maltol) was positioned in the active site of each protein based on crystal structure coordinates.
of maltol bound to \([(T{_{\text{Ph,Me}}}^{\text{Ph,Me}})\text{ZnOH}]\) \(\text{ZnOH} = \text{hydrotris}(3,5\text{-phenylmethylpyrazolyl})\text{borate}\) as described in Chapter 5.\(^{[3,6]}\)

Docking studies using LUDI link mode were performed with the following parameters: maximum alignment angle 20°; maximum alignment RMSD 0.6 Å; search radius 11 Å; rotate bonds two at a time; preselect 4.0; minimum separation 3.0; lipophilic density 40; polar density 40; minimum surface 0; link weight 1.0; lipophilic weight 1.0; H-bond weight 1.0; aliphatic aromatic off; reject bifurcated off; no unpaired polar off; electrostatic check off; minimum score 0; maximum fits 8000; maximum hits all; maximum unfilled cavity 0; energy estimate 1 scoring function;\(^{[13]}\) and best fit. These parameters were chosen to maximize the quality and thoroughness of the docking. Despite this, it was found that results were somewhat dependent on the search sphere center, and favorable fragment poses could be missed with some search sphere centers, particularly for the larger fragments. To minimize this problem, multiple dockings were performed using different search sphere centers within the S1' pocket; the results presented represent the union of these results.

Further docking was performed using a custom link library consisting of the substituents illustrated in Scheme 6-3. Due to the limited ability of LUDI to handle rotational flexibility in fragments (only 120° or 180° rotations can be performed), all possible rotamers with 30° increments of rotation were generated for each substituent, and each rotamer was added to the library as a separate fragment. The number of rotamers generated varied from 1 (no rotatable bonds) to 72 conformations, depending the on the number of rotatable bonds in each individual fragment. Portions of the fragment with two-fold rotational symmetry were rotated through 180°; asymmetric
portions were rotated through 360°. Bonds between phenyl groups were treated as non-rotatable, and rotamers with steric clashes or eclipsed conformations were excluded. Docking with this custom library was performed using the same parameters as above, except bond rotation was set to one at a time. It would seem that because the library already included all rotamers, bond rotation could be set to none, but using one at a time seemed to reduce the differences in results caused by using different search sphere centers. Reported LUDI scores (Table 6-2) represent the highest scoring pose for a fragment in the specified protein structure, rounded to the nearest 10.

**Cell Invasion Assay.** A cell invasion assay kit was purchased from Chemicon International (Cat. No. ECM 555) and was performed as per kit instructions with minor modifications. Rat cardiac fibroblasts (CF) were prepared as previously described.[35] Briefly, CF were prepared from hearts of 1-2 day old Sprague Dawley rats. Following collagenase digestions (4×), non-myocyte cells (mostly fibroblasts) were isolated by Percoll density gradient. The cell suspension was plated onto uncoated tissue culture dishes for 30 min to allow preferential attachment of CF to the bottom of the dish. The non-adherent cells were removed and fresh media (Dulbecco’s modified Eagle’s media +10% fetal bovine serum) was added. CF were allowed to proliferate to confluence and then trypsinized and frozen at –70 °C. Stocks were freshly plated for the experiment and used from second passage. When reaching 90% confluence, cells were serum deprived for 24 h and then treated according to the experimental design (vide infra).
The CF were trypsinized once more and suspended in fresh serum-free media. Inhibitors AM-5 and AM-6 were dissolved in DMSO and diluted 10-fold in serum free media. The inhibitor solution (2 µL) was added to 100 µL of the cell suspension. The cell suspension containing inhibitors was added to the upper chamber of the well. Media with 10% fetal bovine serum (as chemoattractant) was added to the lower chamber of the well. The plate was incubated for 20 h in a CO₂ incubator (10% CO₂). The cells that had invaded into the lower chamber were dislodged from the underside of the ECMatrix™ membrane and lysed. The lysed cells were treated with CyQuant GR Dye, and monitored for fluorescence using a Bio-Tek Flx 800 fluorescence plate reader and Nunc white 96-well plates (λ<sub>ex</sub> = 485 nm, λ<sub>em</sub> = 516 nm). Increased fluorescence indicates the presence of invaded cells (Figure 6-4).

6.6. Acknowledgements

Text, schemes, and figures of this chapter, in part, are reprints of the materials published in the following paper: Puerta, D.T.; Mongan, J.; Tran, B.L.; McCammon, J.A.; Cohen, S.M. "Potent, Selective Pyrone-Based Inhibitors of Stromelysin-1" J. Am. Chem. Soc. 2005, 127, 14148-14149. The dissertation author was the primary researcher and author. The permission to reproduce this paper was granted by the American Chemical Society. Copyright 2004, American Chemical Society.
6.7. References


