The Design and Synthesis of Neuroprotectants Based on an Endogenous Platform

by

Mary I. Purcell

A thesis submitted in conformity with the requirements for the degree of Master of Science

Department of Pharmaceutical Sciences
University of Toronto

© Copyright by Mary Purcell 2015
The Design and Synthesis of Neuroprotectants Based on an Endogenous Platform

Mary Purcell

Master of Science

Department of Pharmaceutical Sciences
University of Toronto

2015

Abstract

There is a pressing need for the development of a neuroprotective therapy that limits neuronal injury in ischemic stroke patients. A diversity of neurotoxic factors, including divalent metal cations, are released during ischemia that result in brain damage. The goal of this research is to develop a method to mitigate the neurotoxic influxes of such cations (focusing on Zn\(^{2+}\)) using histidine as a model chelating molecule, thereby limiting neuronal death during an ischemic stroke. Through the use of computational methods, several small molecules have been identified that bind favourably with Zn\(^{2+}\); nuclear magnetic resonance experiments involving Zn\(^{2+}\) in combination with these small molecules supports these \textit{in silico} predictions. A subset of active molecules shows measurable binding constants through calorimetric methods. Several molecules provide protection from Zn\(^{2+}\) toxicity \textit{in vitro}; Zn\(^{2+}\) imaging experiments confirm the mechanism of protection is metal chelation.
Acknowledgments

I would like to thank my supervisor, Donald Weaver, for his support and assistance with conducting and completing this research. I would also like to acknowledge the members of the Weaver lab for their support and guidance; Dr. Fan Wu has been an essential chemistry mentor and Dr. Luzhe Pan a patient biology instructor. Drs. Braden Sweeting, Seung-Pil Yang, Prachi Vilekar and others provided helpful discussions and insights. As well, I gratefully thank my family and friends for personal support. CIHR is acknowledged for CGS-M funding.
Table of Contents

Acknowledgments ........................................................................................................ iii

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

List of Tables .............................................................................................................. ix

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

List of Schemes .......................................................................................................... xiv

List of Abbreviations .................................................................................................. xv

1 Introduction .............................................................................................................. 1

1.1 Stroke .................................................................................................................... 2

1.1.1 The Ischemic Cascade ..................................................................................... 2

1.1.2 Cations Implemented in Stroke .................................................................... 4

1.2 The Importance of Zn$^{2+}$ ................................................................................. 4

1.2.1 Zn$^{2+}$ Classification .................................................................................... 4

1.2.2 The Role of Labile Zn$^{2+}$ ........................................................................... 4

1.2.3 Zn$^{2+}$ Release and Transport Systems ....................................................... 5

1.2.4 Evidence for Excitotoxicity ......................................................................... 5

1.2.5 Experimental Interventions Against Excitotoxicity ..................................... 7

1.3 Current Treatment for Stroke ........................................................................... 7

1.3.1 Non-Pharmacological Treatments ............................................................... 7

1.3.2 Pharmacological Treatments ....................................................................... 8

1.3.2.1 Thrombolytics ......................................................................................... 8

1.3.2.2 Neuroprotectants .................................................................................... 9

1.3.2.2.1 Difficulties with Neuroprotectant Trials ........................................... 10

1.4 Metal Chelation as a Neuroprotective Strategy .............................................. 11

1.4.1 Histidine as a Potential Neuroprotectant .................................................... 12

1.4.1.1 Histaminergic System Activation in Potential Neuroprotection ............ 12

1.4.1.2 Antioxidant Behaviour of Histidine ....................................................... 13

1.4.1.3 Neuroprotection by Histidine Metal Chelation .................................... 13

1.5 Project Outline .................................................................................................... 14
2 A Computational Study of the Interaction Between Zn$^{2+}$ and Histidine-Based Small Molecules

2.1 Computer Aided Drug Design
2.1.1 Molecular Mechanics
2.1.1.1 MMFF94x Force Field
2.1.2 Energy Minimization
2.1.3 Semi-Empirical Methods
2.2 Computational Methods
2.2.1 Interactions Between Histidine-Based Small Molecules and Divalent Metal Cations
2.2.1.1 In vacuo simulations of the interaction between histidine-based small molecules and divalent metal cations
2.2.1.2 Solvated simulations of the interaction between histidine-based small molecules and divalent metal cations
2.2.2 Interactions Between N/S-Heterocycles and Divalent Metal Cations
2.2.3 Optimization of 2,5-Diketopiperazine and 2,4-Imidazolidinedione Substitution Patterns for Zn$^{2+}$ Chelation
2.2.4 Modeling of Biologically Relevant Zn$^{2+}$ Binding Sites
2.3 Computational Results and Discussion
2.3.1 Results of the Interactions Between Histidine-Based Small Molecules and Divalent Metal Cations
2.3.2 Results of the Interactions Between N/S-Heterocycles and Divalent Metal Cations
2.3.3 Results for the Optimization of 2,5-Diketopiperazine and 2,4-Imidazolidinedione Substitution Patterns for Zn$^{2+}$ Chelation
2.3.4 Results for the Modeling of Biologically Relevant Zn$^{2+}$ Binding Sites

3 Synthesis of Histidine-Based Small Molecules as Potential Neuroprotectants for Ischemic Stroke
3.2.4 Synthetic Routes for the Production of Imidazolidinediones ............................................. 41
3.2.5 Synthetic Routes for the Production of Atypical Amino Acids ................................................. 42

3.3 Synthetic Considerations in the Selection of Target Compounds ................................................. 42

3.3.1 Description of Ester Synthesis ........................................................................................................ 43
3.3.2 Description of Dipeptide Synthesis ................................................................................................. 43
3.3.3 Description of Diketopiperazine Syntheses .................................................................................... 44
3.3.4 Description of Imidazolidinedione Syntheses .............................................................................. 46
3.3.5 Description of Atypical Amino Acid Synthesis ............................................................................. 49

3.4 Experimental Details ...................................................................................................................... 50

3.4.1 General Materials and Methods .................................................................................................... 50
3.4.2 Procedure for the synthesis of L-histidine benzyl ester (3) ............................................................ 51

3.4.3 Synthesis of Dipeptides .................................................................................................................. 51

3.4.3.1 General procedure for the coupling of Boc-protected amino acids with amino acid methyl esters ........................................................................................................................................................................ 51

3.4.3.2 General procedure for the demethylation of Boc-protected dipeptide methyl esters ............ 52

3.4.3.3 General procedure for the removal of Boc from Boc-protected dipeptides .............................. 54

3.4.4 Synthesis of Non-Symmetrical Diketopiperazines ........................................................................ 55

3.4.4.1 General procedure for the removal of Boc from Boc-protected dipeptide methyl esters 55

3.4.4.2 General procedure for intramolecular cyclization of dipeptide methyl esters ...................... 55

3.4.5 Synthesis of Symmetrical Diketopiperazines ................................................................................ 56

3.4.5.1 Procedure for the methylation of L-methionine (56) ................................................................. 56

3.4.5.2 General procedure for the intermolecular cyclization of amino acid methyl esters ............... 57

3.4.6 Synthesis of N,N-Substituted Diketopiperazines ........................................................................... 57

3.4.7 Synthesis of Substituted 2,4-Imidazolidinediones ....................................................................... 59

3.4.7.1 General procedure for the synthesis of 5-substituted 2,4-imidazolidinediones 59

3.4.7.2 General procedure for the synthesis of 3-substituted 2,4-imidazolidinediones .................... 60

3.4.7.3 Procedure for detritylation of 3-((1-trityl-1H-imidazol-4-yl)methyl)imidazolidine-2,4-dione (71) ................................................................................................................................................................................. 61

3.4.7.4 Procedure for the synthesis of 3,5-substituted 2,4-imidazolidinedione 62

3.4.8 Synthesis of Atypical Amino Acids .................................................................................................. 63

3.4.8.1 General procedure for the Wittig-Horner reaction ................................................................. 63

3.4.8.2 General procedure for the hydrogenation of N-Cbz-a-pyridinylmethylene glycine methyl esters ........................................................................................................................................................................ 64

3.4.8.3 General procedure for the demethylation of a-pyridinylmethyl glycine methyl esters . . . 65
4 Qualitative and Quantitative Analysis of Zn\(^{2+}\) Chelation ..............................67
  4.1 Qualitative Assessment of Chelation by NMR Spectroscopy .................................. 68
    4.1.1 NMR Spectroscopy Methods ............................................................................. 68
    4.1.2 NMR Spectroscopy Results and Discussion ....................................................... 69
      4.1.2.1 Results for the addition of Zn\(^{2+}\) to histidine in \(^1\)H NMR experiments .......... 69
      4.1.2.2 Results for the addition of Zn\(^{2+}\) to esters in \(^1\)H NMR experiments .......... 70
      4.1.2.3 Results for the addition of Zn\(^{2+}\) to dipeptides in \(^1\)H NMR experiments .......... 70
      4.1.2.4 Results for the addition of Zn\(^{2+}\) to diketopiperazines in \(^1\)H NMR experiments .......... 72
      4.1.2.5 Results for the addition of Zn\(^{2+}\) to imidazolidinediones in \(^1\)H NMR experiments .......... 73
      4.1.2.6 Results for the addition of Zn\(^{2+}\) to atypical amino acids in \(^1\)H NMR experiments .......... 74
  4.2 Quantitative Assessment of Chelation by Isothermal Titration Calorimetry ................. 75
    4.2.1 An Introduction to Isothermal Titration Calorimetry ......................................... 75
    4.2.2 ITC Methods ..................................................................................................... 76
      4.2.2.1 One-Site Binding Model for ITC Curve Fitting .............................................. 78
      4.2.2.2 Sequential Site Binding Model for ITC Curve Fitting ..................................... 79
    4.2.3 ITC Results and Discussion ................................................................................ 80
      4.2.3.1 Analysis of ITC data for the titration of histidine into Zn\(^{2+}\) and Ca\(^{2+}\) .......... 80
      4.2.3.2 Analysis of ITC data for the titration of dipeptides into Zn\(^{2+}\) ....................... 81
      4.2.3.3 Analysis of ITC data for the titration of diketopiperazines into Zn\(^{2+}\) .......... 83
      4.2.3.4 Analysis of ITC data for the titration of imidazolidinediones into Zn\(^{2+}\) .......... 84
      4.2.3.5 Analysis of ITC data for the titration of atypical amino acids into Zn\(^{2+}\) .......... 85

5 In Vitro Testing of Histidine-Based Small Molecules Against Zn\(^{2+}\)-Induced Toxicity ..................................................................................................................87
  5.1 In Vitro Zn\(^{2+}\) Toxicity Studies ................................................................................. 88
  5.2 In Vitro Methods ..................................................................................................... 88
    5.2.1 Cell Toxicity Assay ............................................................................................ 88
    5.2.2 Zn\(^{2+}\) Imaging .................................................................................................. 89
  5.3 Results and Discussion for In Vitro Data ................................................................... 89
    5.3.1 Results for the use of histidine in a Zn\(^{2+}\)-induced cell toxicity assay ................. 89
    5.3.2 Results for the use of esters in a Zn\(^{2+}\)-induced cell toxicity assay ................. 91
    5.3.3 Results for the use of dipeptides in a Zn\(^{2+}\)-induced cell toxicity assay ........... 92
    5.3.4 Results for the use of diketopiperazines in a Zn\(^{2+}\)-induced cell toxicity assay ....... 93
    5.3.5 Results for the use of imidazolidinediones in a Zn\(^{2+}\)-induced cell toxicity assay .... 94
    5.3.6 Results for the use of atypical amino acids in a Zn\(^{2+}\)-induced cell toxicity assay .... 94
6 Conclusions and Future Directions ..........................................................97
  6.1 Conclusions ......................................................................................... 98
  6.2 Future Directions .................................................................................. 99
References ........................................................................................................102
List of Tables

Table 1.1. Summary of phase III clinical trials involving neuroprotective agents for the treatment of ischemic stroke. ................................................................. 9

Table 1.2. Guidelines for preclinical evaluation of neuroprotective agents (STAIR 1999). ...... 10

Table 2.1. Functional energy terms of a general MM force field. Visual representations of the interactions are included (Leach 1996). ............................................................................. 18

Table 2.2. Values for the change in energy upon complex formation between histidine analogues in Figure 2.4 and Zn\(^{2+}\) using both MM and semi-empirical calculations. .................... 29

Table 2.3. Values for the change in energy upon complex formation between histidine analogues in Figure 2.4 and Cu\(^{2+}\) and Ca\(^{2+}\) using MM. ................................................................. 29

Table 2.4. Values for the change in energy upon complex formation (in vacuo) between N/S-heterocycles and divalent metal cations using both MM (Zn\(^{2+}\), Cu\(^{2+}\), and Ca\(^{2+}\) complexes), and semi-empirical calculations (Zn\(^{2+}\) complexes). ......................................................... 32

Table 2.5. Values for the change in energy upon complex formation (under solvated conditions) between atypical amino acids and divalent metal cations using both MM (Zn\(^{2+}\), Cu\(^{2+}\), and Ca\(^{2+}\) complexes), and semi-empirical calculations (Zn\(^{2+}\) complexes). ......................................................... 33

Table 2.6. Values for the change in energy upon complex formation between substituted 2,5-diketopiperazines and Zn\(^{2+}\). Substitution positions are indicated in the compound structure. ..... 34

Table 2.7. Values for the change in energy upon complex formation between substituted 2,4-imidazolidinediones and Zn\(^{2+}\). Substitution positions are indicated in the compound structure. 35

Table 2.8. Summary of results for modeling of Zn\(^{2+}\) containing proteins. ............................. 36

Table 3.1. Summary product table for synthesized dipeptides. ................................................. 44

Table 3.2. Summary product table for synthesized non-symmetric diketopiperazines. .......... 45

Table 3.3. Summary product table for synthesized 5-substituted 2,4-imidazolidinediones. ...... 47

Table 3.4. Summary product table for synthesized 3-substituted 2,4-imidazolidinediones. ...... 48

Table 3.5. Summary product table for synthesized atypical amino acids. ............................... 50

Table 4.1. Experimental conditions for the qualitative assessment of metal (Zn\(^{2+}\) or Ca\(^{2+}\)) :ligand interactions in D\(_2\)O. NMR spectra of samples were collected on a Bruker 400 MHz spectrometer. ........................................................................................................ 69

Table 4.2. Experimental concentrations for ITC runs performed at 25°C on a MicroCal VP-ITC calorimeter. .......................................................................................................................... 77
Table 4.3. Thermodynamic parameters for histidine 1 binding to Zn$^{2+}$ at pH 7.0 obtained from ITC measurements in 100 mM HEPES buffer at 25°C.......................................................... 81

Table 4.4. Thermodynamic parameters for 4, 5, and 6 binding to Zn$^{2+}$ at pH 7.0 obtained from ITC measurements in 100 mM HEPES buffer at 25°C.......................................................... 83

Table 4.5. Thermodynamic parameters for 75 binding to Zn$^{2+}$ at pH 7.0 obtained from ITC measurements in 100 mM HEPES buffer at 25°C.......................................................... 85

Table 5.1. Treatments of N2a cells; ‘compound’ represents histidine 1 and analogues. All experiments were performed in triplicate and cell viability assessed at 6 and 24-hour time points. ........................................................................................................................................... 89
List of Figures

Figure 2.1. Graphical representation of the Lennard-Jones 12-6 potential................................. 19

Figure 2.2. Schematic representation of the stretch-bend energy between atoms \(i, j, \) and \(k\) ..... 21

Figure 2.3. Schematic representation of the out-of-place bending energy between the plane described by \(ijk\), and atom \(l\) ............................................................... 21

Figure 2.4. Chemical structures of histidine-based small molecules used for \textit{in silico} predictions of divalent metal binding potential in Section 2.2.1......................................................... 24

Figure 2.5. Identification of cation placement sites around histidine \(1\) by bolded letter labels \(A, B, \) and \(C\) ........................................................................................................... 25

Figure 2.6. Chemical structures of N/S-heterocycles used for \textit{in silico} predictions in Section 2.2.2......................................................................................................................... 27

Figure 2.7. Chemical structures of atypical amino acids used for \textit{in silico} predictions in Section 2.2.2......................................................................................................................... 27

Figure 2.8. Typical \(Zn^{2+}\) complex pose with histidine \(1\) as an example ligand under A) evacuated and B) solvated conditions............................................................... 30

Figure 2.9. Common pose assumed by complexes of \(Zn^{2+}\) and substituted diketopiperazines, shown with substitution pattern \(3S\) from Table 2.6 as an example. ......................................................... 34

Figure 2.10. Important residues in A) 2D and B) 3D in the Zif268-DNA complex. Bond distance and energies are shown in A), units are \(\text{Å}\) and kcal/mol, respectively. In A) purple dashed lines represent ionic interactions and green dashed lines represent sidechain interactions. ................................................................. 36

Figure 3.1. General structure of a 2,5-diketopiperazine and 2,4-imidazolidinedione, shown with atom numbering. ................................................................. 39

Figure 4.1. Chemical shifts of imidazole protons from histidine \(1\) where samples 1 to 5 have incremental increases of \(Zn^{2+}\), described in Table 4.1. Protons responsible for shifts shown are red. ........................................................................................................... 70

Figure 4.2. NMR spectra of \(6\) where samples 1 to 5 have incremental increases of \(Zn^{2+}\), described in Table 4.1. ........................................................................................................... 71

Figure 4.3. Chemical shifts of H7 and H10 protons from \(6\) where samples 1 to 5 have incremental increases of \(Zn^{2+}\), described in Table 4.1. Protons responsible for shifts shown are red. ........................................................................................................... 71

Figure 4.4. Chemical shifts of imidazole protons from \(6\) where samples 1 to 5 have incremental increases of \(Zn^{2+}\), described in Table 4.1. Protons responsible for shifts shown are red........... 72
Figure 4.5. Lowest energy dipeptide 4:Zn$^{2+}$ complex showing Zn$^{2+}$ interacting with the C-terminal portion of the molecule. ................................................................. 72

Figure 4.6. Chemical shifts of imidazole protons from where samples 1 to 5 have incremental increases of Zn$^{2+}$, described in Table 4.1. Protons responsible for shifts shown are red .......... 73

Figure 4.7. Chemical shifts of imidazole protons from where samples 1 to 5 have incremental increases of Zn$^{2+}$, described in Table 4.1. Protons responsible for shifts shown are red. ........................................................................................................... 74

Figure 4.8. Chemical shifts of alkyl protons from where samples 1 to 5 have incremental increases of Zn$^{2+}$, described in Table 4.1. Protons responsible for shifts shown are red. ........................................................................................................... 74

Figure 4.9. Chemical shifts of imidazole protons from where samples 1 to 5 have incremental increases of Zn$^{2+}$, described in Table 4.1. Protons responsible for shifts shown are red. ........................................................................................................... 75

Figure 4.10. Schematic representation of a calorimeter used in ITC ......................................................... 76

Figure 4.11. ITC data for the titration of 0.33 mM Zn$^{2+}$ with 10 mM histidine 1 in 100 mM HEPES buffer at pH 7.00. Parameters of best fit for the binding curve can be found in Table 4.3. ........................................................................................................... 80

Figure 4.12. ITC data for the titration of 0.33 mM Zn$^{2+}$ with 10 mM 4 (plot A), 5 (plot B), and 6 (plot C) in 100 mM HEPES buffer at pH 7.00. Parameters of best fit for the binding curves can be found in Table 4.4. ........................................................................................................... 82

Figure 4.13. ITC data for the titration of 0.33 mM Zn$^{2+}$ with 10 mM 75 in 100 mM HEPES buffer at pH 7.0. Parameters of best fit for the binding curve can be found in Table 4.5. ........... 84

Figure 4.14. ITC data for the titration of 0.33 mM Zn$^{2+}$ with 10 mM 42 in 100 mM HEPES buffer at pH 7.0. ........................................................................................................... 85

Figure 5.1. Assessment of cell viability following Zn$^{2+}$ exposure with or without 1 mM treatment solutions as indicated. Cell viability was quantified using fluorescence 24 hours after treatment and normalized against the cell viability of the assay control. Bars represent SE (*** = P < 0.001, unpaired t-test, comparisons with respect to corresponding control group). ............... 90

Figure 5.2. Zn$^{2+}$ imaging of N2a cells with FluoZin-3AM. A) control (no addition); B) 200 µM Zn$^{2+}$; C) 300 µM Zn$^{2+}$; D) 200 µM Zn$^{2+}$ and 1 mM histidine 1; and E) 300 µM Zn$^{2+}$ and 1 mM histidine 1. Magnification at 20x, bar=200 µm ........................................................................................................... 91

Figure 5.3. Assessment of cell viability following Zn$^{2+}$ exposure with or without 1 mM treatment solutions as indicated. Cell viability was quantified using fluorescence 24 hours after treatment and normalized against the cell viability of the assay control. ........................................... 91

Figure 5.4. Assessment of cell viability following Zn$^{2+}$ exposure with or without 1 mM treatment solutions as indicated. Cell viability was quantified using fluorescence 24 hours after treatment and normalized against the cell viability of the assay control. ........................................... 92
**Figure 5.5.** Assessment of cell viability following Zn$^{2+}$ exposure with or without 1 mM treatment solutions as indicated. Cell viability was quantified using fluorescence 24 hours after treatment and normalized against the cell viability of the assay control. Bars represent SE (** = $P \leq 0.01$; * = $P \leq 0.05$, unpaired t-test, comparisons with respect to corresponding control group). ................................................................. 93

**Figure 5.6.** Assessment of cell viability following Zn$^{2+}$ exposure with or without 1 mM treatment solutions as indicated. Cell viability was quantified using fluorescence 24 hours after treatment and normalized against the cell viability of the assay control. Bars represent SE (* = $P \leq 0.05$, unpaired t-test, comparisons with respect to corresponding control group). ......................... 94

**Figure 5.7.** Assessment of cell viability following Zn$^{2+}$ exposure with or without 1 mM treatment solutions as indicated. Cell viability was quantified using fluorescence 24 hours after treatment and normalized against the cell viability of the assay control. ................................................. 95

**Figure 5.8.** Assessment of cell viability following 200 µM Zn$^{2+}$ exposure with or without administration of 42 (2 mM). Cell viability was quantified using fluorescence 24 hours after treatment and normalized against the cell viability of the assay control. Bars represent SE (** *= $P \leq 0.001$ relative to control; ### = $P < 0.001$ relative to 200 µM Zn$^{2+}$, unpaired t-test). ........... 95
List of Schemes

**Scheme 3.1.** General mechanism for Fischer esterification. .......................................................... 40

**Scheme 3.2.** General procedure of the Bucherer-Bergs reaction. ................................................. 41

**Scheme 3.3.** General mechanism for the Gabriel synthesis of α-amino acids. ............................. 42

**Scheme 3.4.** Acid-catalyzed esterification of histidine 1 in BnOH/CHCl₃ to form 3 (41% yield). .......................................................... 43

**Scheme 3.5.** General peptide coupling procedure for the synthesis of dipeptides. ......................... 43

**Scheme 3.6.** General procedure for intramolecular dipeptide cyclization. ................................. 44

**Scheme 3.7.** Synthesis of symmetric diketopiperazine 7 via intermolecular cyclization (yield 22%). ....................................................................................................................... 45

**Scheme 3.8.** Methylation procedure of methionine followed by intermolecular cyclization (overall yield 4.8%). ....................................................................................................................... 45

**Scheme 3.9.** Trityl protection and chlorination of 4(5)-(hydroxymethyl)imidazole to form 59 (overall yield 96%). ....................................................................................................................... 46

**Scheme 3.10.** Procedure for N,N-alkylation of 2,4-diketopiperazine (overall yield 21%). ............ 46

**Scheme 3.11.** Application of the Urech reaction to the synthesis of 5-substituted 2,4-imidazolidinediones. ....................................................................................................................... 47

**Scheme 3.12.** Attempted procedure for the synthesis of 3-substituted 2,4-imidazolidinediones. 47

**Scheme 3.13.** General procedure for the synthesis of 3-substituted 2,4-imidazolidinediones. .... 48

**Scheme 3.14.** Modified procedure for the synthesis of 3-substituted 2,4-imidazolidinediones with imidazole (overall yield 11%). ....................................................................................................................... 48

**Scheme 3.15.** Procedure for the synthesis of 75 (overall yield 8.2%). .......................................... 49

**Scheme 3.16.** General procedure for the synthesis of atypical amino acids. ............................... 49
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3D</td>
<td>three dimensional</td>
</tr>
<tr>
<td>ACN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AMPA</td>
<td>$\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>adenosine triphosphatase</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-bis(2-amino-phenoxo)ethane-$N,N,N',N'$-tetraacetic acid</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>1,2-bis(2-amino-phenoxo)ethane-$N,N,N',N'$-tetraacetic acid tetrakis(acetoxymethyl ester)</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>Boc-</td>
<td>tert-butyloxycarbonyl protecting group</td>
</tr>
<tr>
<td>C-</td>
<td>carbon-</td>
</tr>
<tr>
<td>CaEDTA</td>
<td>calcium ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Cbz-</td>
<td>carboxybenzyl protecting group</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DART</td>
<td>Direct Analysis in Real Time</td>
</tr>
<tr>
<td>D-</td>
<td>D-stereocenter</td>
</tr>
<tr>
<td>DBU</td>
<td>1,8-diazabicyclo[5.4.0]undec-7-ene</td>
</tr>
<tr>
<td>DCC</td>
<td>dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DIPEA</td>
<td>$N,N$-diisopropylethylamine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DP-b99</td>
<td>1,2-bis(2-amino-phenoxo)ethane-$N,N,N',N'$-tetraacetic acid-$N,N'$-di[2-(octyloxy)ethyl ester].-$N,N'$-disodium salt</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>$E_{\text{ele}}$</td>
<td>electrostatic energy</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>$E_{\text{tot}}$</td>
<td>total energy</td>
</tr>
<tr>
<td>$E_{\text{vdw}}$</td>
<td>van der Waals energy</td>
</tr>
<tr>
<td>EXTEND-IA</td>
<td>Extending the Time for Thrombolysis in Emergency Neurological Deficits- Intra-Arterial</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GABA_A</td>
<td>gamma-aminobutyric acid receptor A</td>
</tr>
<tr>
<td>H-bonding</td>
<td>hydrogen bonding</td>
</tr>
<tr>
<td>H_1 receptor</td>
<td>histamine receptor 1</td>
</tr>
<tr>
<td>H_2 receptor</td>
<td>histamine receptor 2</td>
</tr>
<tr>
<td>HBTU</td>
<td>$N,N,N',N'$-tetramethyl-$(1H$-benzotriazol-1-yl)uronium hexafluorophosphate</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
</tr>
<tr>
<td>Hex</td>
<td>hexanes</td>
</tr>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HRMS</td>
<td>high resolution mass spectrometry</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IMS-III</td>
<td>Interventional Management of Stroke III</td>
</tr>
<tr>
<td>IPA</td>
<td>isopropyl alcohol</td>
</tr>
<tr>
<td>ITC</td>
<td>isothermal titration calorimetry</td>
</tr>
<tr>
<td>KINARM</td>
<td>Kinesiological Instrument for Normal and Altered Reaching Movement</td>
</tr>
<tr>
<td>L-</td>
<td>L-stereocenter</td>
</tr>
<tr>
<td>LNAA</td>
<td>large neutral amino acid transporter</td>
</tr>
<tr>
<td>M.p.</td>
<td>melting point</td>
</tr>
<tr>
<td>MCAO</td>
<td>middle cerebral artery occlusion</td>
</tr>
<tr>
<td>MM</td>
<td>molecular mechanics</td>
</tr>
<tr>
<td>MMFF94</td>
<td>Merck molecular force field 94</td>
</tr>
<tr>
<td>MMFF94x</td>
<td>Merck molecular force field 94x</td>
</tr>
<tr>
<td>MOE</td>
<td>Molecular Operating Environment</td>
</tr>
<tr>
<td>MOPAC</td>
<td>Molecular Orbital PACkage</td>
</tr>
<tr>
<td>MR RESCUE</td>
<td>Mechanical Retrieval and Recanalization of Stroke Clots Using Embolectomy</td>
</tr>
<tr>
<td>N-</td>
<td>nitrogen-</td>
</tr>
<tr>
<td>N/S-</td>
<td>nitrogen and sulphur containing</td>
</tr>
<tr>
<td>N2a</td>
<td>Neuro-2a</td>
</tr>
<tr>
<td>NDDO</td>
<td>neglect of diatomic differential overlap</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PM3</td>
<td>Parametric Method 3</td>
</tr>
<tr>
<td>pTsOH</td>
<td>para-toluene sulphonic acid</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>STAIR</td>
<td>The Stroke Treatment and Academic Industry Roundtable</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue plasminogen activator</td>
</tr>
<tr>
<td>TPEN</td>
<td>N,N,N′,N′-tetrakis(2-pyridylmethyl)ethylenediamine</td>
</tr>
<tr>
<td>TrCl</td>
<td>trityl chloride</td>
</tr>
<tr>
<td>TsCl</td>
<td>tosyl chloride</td>
</tr>
<tr>
<td>TSQ</td>
<td>N-(6-methoxy-8-quinolyl)-p-toluenesulfonamide</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>ZIP</td>
<td>zrt/irt-like protein</td>
</tr>
<tr>
<td>ZnT</td>
<td>zinc transporter</td>
</tr>
<tr>
<td>ZnT1</td>
<td>zinc transporter 1</td>
</tr>
<tr>
<td>ZnT3</td>
<td>zinc transporter 3</td>
</tr>
</tbody>
</table>
CHAPTER 1

1 Introduction
1.1 Stroke

A stroke is the acute onset of neurological disability due to altered blood flow. Strokes may be hemorrhagic or ischemic in origin, together representing the third leading cause of death in Canada and 6% of total annual deaths (Heart and Stroke Foundation 2012). Hemorrhagic strokes are caused by the rupture of a weakened blood vessel, while ischemic strokes are caused by the blockage of a blood vessel.

According to a 2000 statistic, stroke costs the Canadian economy $3.6 billion a year in physician services, lost wages, and hospital costs (PHAC 2009). In addition to these societal and personal monetary burdens, the detrimental physical and mental disabilities experienced by stroke survivors strains the health care system. Commonly, patients are left with severe speech, balance, and mental deficiencies. In Canada alone, 50 000 strokes are experienced annually (Heart and Stroke Foundation 2012). These statistics serve to emphasize the necessity of developing agents to aid in the treatment of ischemic stroke.

1.1.1 The Ischemic Cascade

Approximately 90% of strokes are ischemic and result from the blockage of a brain blood vessel by a platelet aggregate. This blockage results in an insufficient supply of blood causing oxygen and glucose deprivation to that area. Following an ischemic event, complex cellular and molecular events are initiated, known as the ischemic cascade. The site of the occlusion is known as the ischemic core. In this region, blood flow is reduced to 15% of normal levels, resulting in ATP present at less than 25% of basal levels. The ischemic penumbra is the tissue area surrounding the ischemic core where blood flow is reduced to below 40% normal and ATP is reduced to ~50-70% of basal levels (Lipton 1999). The ischemic penumbra represents an area of brain tissue that may be able to be rescued through the implementation of post-ischemic interventions. Oxygen and glucose deprivation are known to cause ATP disturbances resulting in synaptic release of glutamate and disruption of Ca\(^{2+}\) homeostasis, decreases in brain pH, initiation of inflammatory processes and, more recently, the synaptic release of Zn\(^{2+}\).

Neurons subjected to ischemic conditions rapidly become ATP deficient. At the ischemic core, the inability of cells to maintain Na\(^{+}\)-K\(^{+}\) ATPase due to energy deficiencies result in Na\(^{+}\) and Ca\(^{2+}\) accumulation and necrotic cell death (Lipton 1999). At the level of the ischemic penumbra,
reductions in blood flow are insufficient to cause neuronal death through direct energy failure. Instead, ATP deficient neurons become depolarized, causing release of synaptic glutamate. Excessive concentrations of extracellular glutamate cause overstimulation of AMPA, kainate, and NMDA-type glutamate receptors, resulting in an influx of Ca\(^{2+}\) and Na\(^+\) through the channels gated by these receptors. Increased levels of intracellular Ca\(^{2+}\) leads to various routes of neuronal death including generation of reactive oxygen species, disruption of mitochondrial processes, and activation of lipases and proteases (Kristian and Siesjo 1998).

Intracellular brain pH is maintained at approximately 7.2 (Casey, Grinstein, and Orlowski 2010). Drops in intracellular pH between 0.5 and 1 unit have been seen in ischemic models, and contribute to ischemia induced cell death (Lipton 1999). Oxygen deprivation results in insufficient ATP levels to maintain cellular function causing anaerobic metabolism of glycogen stores. This causes lactate, pyruvate, and proton accumulation subsequently dropping brain pH (Casey, Grinstein, and Orlowski 2010). This accumulation of acidic species causes an eventual increase in intracellular Ca\(^{2+}\) leading to various detrimental pathways, discussed briefly in the paragraph above.

Inflammation is recognized as a key factor in ischemic cell death. Inflammatory processes are prompted by oxygen deprivation, production of reactive oxygen species, and oxidative stress, for example. Injured neurons release inflammatory signals that activate the immune system. In addition to inflammatory processes, immunosuppression of the brain causes increased infections in stroke patients, influencing stroke morbidity and mortality. The highly complex interplay between ischemia and immunity is still being elucidated (Iadecola and Anrather 2011).

Synaptic release of Zn\(^{2+}\) from glutamatergic neurons has, more recently, been implemented in the pathogenesis of ischemia-induced cell death. Zn\(^{2+}\) is released following depolarization and taken up in post-synaptic neurons through voltage-gated Ca\(^{2+}\) channels, Na\(^+\)/Zn\(^{2+}\) exchange, NMDA receptor-gated channels, and kainate and AMPA-gated channels in a similar fashion as Ca\(^{2+}\) (J.-M. Lee, Zipfel, and Choi 1999). Zn\(^{2+}\) may then induce apoptosis or necrosis, dependent on its concentration (Manev et al. 1997). Initiation of apoptotic pathways involving caspase activation as well as the release of pro-apoptotic proteins occur following Zn\(^{2+}\) exposure (Sensi et al. 2009). As well, Zn\(^{2+}\) causes the generation of reactive oxygen species and disruption of mitochondrial processes which lead to neuronal degeneration (J.-M. Lee, Zipfel, and Choi 1999).
1.1.2 Cations Implemented in Stroke

As mentioned previously, Ca$^{2+}$ and Zn$^{2+}$ are the main divalent cations implemented in the pathophysiology of ischemic stroke. Ca$^{2+}$ antagonists have been widely assessed as potential therapeutics for ischemic stroke. However, Zn$^{2+}$ is a comparatively novel neurotoxic factor in the ischemic cascade and has not been explored as thoroughly. Therefore effort should be put into investigating its possible utility as a target for neuroprotection in ischemic stroke.

1.2 The Importance of Zn$^{2+}$

Zn$^{2+}$ is a borderline transition metal, with characteristics that allow for chelation of both hard and soft ligands. The divalent cation has a full d shell of electrons, which makes it chemically inert as well as limits ligand field effects (Laitaoja, Valjakka, and Jänis 2013). This allows for Zn$^{2+}$ complexes to be of varying coordination numbers as well as allows ligand interactions with the cation to occur with diverse geometries.

1.2.1 Zn$^{2+}$ Classification

Zn$^{2+}$ is one of the most abundant trace metals in the human body. It is essential in numerous physiological systems, including the neural system (Vallee and Falchuk 1993). In the brain, Zn$^{2+}$ exists in two forms: static (or bound) Zn$^{2+}$ and labile (or chelatable) Zn$^{2+}$. Static Zn$^{2+}$ plays both structural and catalytic roles in transcription factors and various metalloproteins, while labile Zn$^{2+}$ is thought to be involved in neurotransmitter function and also act as a neurotoxin (Fukada et al. 2011; Cuajungco and Lees 1997).

1.2.2 The Role of Labile Zn$^{2+}$

Labile, or chelatable, Zn$^{2+}$ is found throughout the forebrain, concentrated in hippocampal mossy fiber boutons of glutamatergic neurons (Vallee and Falchuk 1993; Fukada et al. 2011; Frederickson, Maret, and Cuajungco 2004). These labile Zn$^{2+}$ stores were first recognized by Maske in 1955 with use of a dithizone based stain (Maske 1955). In the brain, extracellular labile Zn$^{2+}$ concentrations are approximately 19 nM (Frederickson et al. 2006). Physiologically, Zn$^{2+}$ is known to inhibit both excitatory and inhibitory receptors. As well, high concentrations of Zn$^{2+}$ in regions of the brain including the amygdala, hippocampus, and neocortex suggest that Zn$^{2+}$ plays a role in synaptic plasticity.
1.2.3 Zn$^{2+}$ Release and Transport Systems

Once released into the synaptic cleft, Zn$^{2+}$ is taken up into post-synaptic neuronal bodies through similar pathways as Ca$^{2+}$ entry, including voltage-gated calcium channels, and NMDA, kainate and AMPA receptor-gated channels. Uptake can also occur through the zrt/irk-like (ZIP) proteins, which are also carriers of other divalent metal cations such as Fe$^{2+}$ and Mn$^{2+}$ (Que, Domaille, and Chang 2008).

It is the zinc transporter family of proteins, ZnTs, that are responsible for the vesicular packaging and regulation of Zn$^{2+}$ homeostasis. ZnT1, a membrane protein, is largely responsible for maintaining intracellular Zn$^{2+}$ concentrations. It is expressed ubiquitously and is up or down regulated in response to dietary Zn$^{2+}$ levels (Que, Domaille, and Chang 2008; McMahon and Cousins 1998). ZnT3 is expressed in the brain and is solely responsible for packaging Zn$^{2+}$ into vesicles in the glutamatergic boutons. This was confirmed by a study involving ZnT3 knockout mice, which showed no chelatable Zn$^{2+}$ in mossy fiber boutons (J.-Y. Lee et al. 2000).

1.2.4 Evidence for Excitotoxicity

Zn$^{2+}$ excitotoxicity is a relatively new phenomenon. As mentioned previously, the first evidence of labile Zn$^{2+}$ in the brain was found by Maske in 1955 (Maske 1955). Klitenick et al. later found, through acid-vapour decomposition of rat hippocampal tissue, that labile Zn$^{2+}$ pools were in the concentration range of 300 µM (Klitenick, Frederickson, and Manton 1983). Furthermore, concentrations of Zn$^{2+}$ in this range (250-300 µM) were shown to be toxic to cultured primary cortical neurons (Yokoyama, Koh, and Choi 1986). It was also established that Zn$^{2+}$ is released from hippocampal slices following excitation (Howell, Welch, and Frederickson 1984; Assaf and Chung 1984). In such experiments, increases of Zn$^{2+}$ in the perfusate of stimulated hippocampal slices was detected. More recent literature makes use of direct Zn$^{2+}$ imaging experiments involving fluorescent probes and has shown that the concentration of Zn$^{2+}$ released from brain slices appears to be more moderate than initially thought, in the 10-30 µM range. However, this range represents an average concentration throughout the brain following excitation, and therefore transient concentrations in the synaptic cleft following ischemia would be presumed to be in the higher micromolar range (Frederickson, Koh, and Bush 2005).
In vivo experimentation in the following decades echoes the in vitro findings described in the previous paragraph. One of the first accounts of Zn\(^{2+}\) release causing neuronal degeneration in vivo was in a seizure model (Frederickson, Hernandez, and McGinty 1989). Seizures induced by kainic acid resulted in reduced TSQ (a Zn\(^{2+}\) specific fluorescent dye) staining in the neuropil and increased TSQ staining in individual neurons. The former represents loss of Zn\(^{2+}\) from presynaptic terminals, whereas the latter represents accumulation of Zn\(^{2+}\) in neuronal bodies. Neurodegeneration was observed in TSQ positive regions. Similar results were replicated in several studies following cerebral ischemia in rats (Tonder et al. 1990; J.-M. Lee et al. 2002). In these studies the uptake of Zn\(^{2+}\) was monitored following middle cerebral artery occlusion (MCAO) in vivo using TSQ. Abnormal amounts of Zn\(^{2+}\) were found in some cortical neurons, all of which showed early signs of degenerative changes 24 hours after insult (J.-M. Lee et al. 2002; Tonder et al. 1990). This phenomenon was also shown using Timm’s stain, a silver sulphide stain that causes precipitation of metal sulphides which subsequently reduce the silver ions present and makes them visible. Following MCAO in rats Timm’s stain was used to show a loss of zinc sulphide precipitates in the mossy fiber system following occlusion initiation, representing Zn\(^{2+}\) translocation from the pre-synaptic boutons (Sørensen et al. 1998).

In addition to Zn\(^{2+}\) translocation from pre-synaptic to post-synaptic neurons causing degeneration, it has been hypothesized that Zn\(^{2+}\) is released intracellularly from metallothioneins (Sensi et al. 2009; Shuttleworth and Weiss 2011). Metallothionein proteins are capable of binding seven Zn\(^{2+}\) in a multitude of cysteine containing pockets. Decreases in pH and oxidative stress can induce such intracellular Zn\(^{2+}\) release. In a study where ZnT3-null mice (i.e. mice with no vesicular Zn\(^{2+}\) stores in synaptic vesicles) were subjected to kainate-induced seizures, Zn\(^{2+}\) accumulation was still detected in degenerating neuronal bodies. This suggests that non-vesicular Zn\(^{2+}\) is also capable of causing neurotoxicity. Intracellular Zn\(^{2+}\) release from metallothioneins was deduced as a mechanism for Zn\(^{2+}\) accumulation. However, intracerebral injection of CaEDTA (a Zn\(^{2+}\) specific chelator) still blocked Zn\(^{2+}\) accumulation, indicating Zn\(^{2+}\) may be extruded into the extracellular space (by ZnT1) and then taken up by into post-synaptic cells, causing cell death (J.-Y. Lee et al. 2000).
1.2.5 Experimental Interventions Against Excitotoxicity

Inhibition of Zn\(^{2+}\)-induced neuronal degeneration has been achieved by employing Zn\(^{2+}\) chelators \textit{in vitro} and \textit{in vivo}. For example, \textit{in vitro} addition of the membrane permeant Zn\(^{2+}\)-chelator, TPEN, to cultures of cortical neurons and glia showed protective effects from Zn\(^{2+}\) exposure. However, TPEN alone was highly toxic at concentrations above 3 µM (Canzoniero et al. 2003). Toxicity of TPEN may be related to the interruption of normal physiological processes by total removal of Zn\(^{2+}\), an essential metal for proper brain functioning. The translocation of labile Zn\(^{2+}\) from pre-synaptic to vulnerable post-synaptic neurons was interrupted \textit{in vivo} by intracerebral injection of CaEDTA. Injection of CaEDTA reduced the number of neurons showing early signs of degeneration in comparison to the same experiments conducted without the addition of CaEDTA (J.-M. Lee et al. 2002). Koh et al. presented similar results as above from an \textit{in vivo} study involving transient forebrain ischemia and subsequent CaEDTA administration (Koh et al. 1996).

To combat the toxicity associated with chelation of physiological levels of divalent metal cations, analogues of BAPTA (a known Ca\(^{2+}\) chelator) with added lipophilic moieties have been recently synthesized and studied. One such analogue, DP-b99, is a moderate-affinity Zn\(^{2+}\)-chelator, developed by D Pharm Ltd., that serves to chelate excessive extracellular Zn\(^{2+}\) in the vicinity of cellular membranes, but not interrupt the physiological processes that require Zn\(^{2+}\) (Barkalifa et al. 2009). In phase II clinical trials, an improved 90-day recovery rate in the DP-b99 treated cohort was seen in comparison to the placebo treated cohort. However, no significant improvement in the 90-day survival probability was found (Diener et al. 2008). A phase III study was recently completed and no efficacy for the treatment of human ischemic stroke with DP-b99 was found (Lees et al. 2013).

1.3 Current Treatment for Stroke

1.3.1 Non-Pharmacological Treatments

Currently, treatment focuses on a preventative approach, encouraging exercise and a balanced diet to mediate risk factors. In some cases, surgery may be performed to remove potential platelet aggregates. However, if the blockage is over 70%, then antiplatelet agents (aspirin or clopidogrel, for example) are used to treat the potential stroke.
1.3.2 Pharmacological Treatments

There are two main categories of pharmacological treatment avenues following ischemic stroke: thrombolysis or neuroprotection.

1.3.2.1 Thrombolytics

Thrombolytic agents, or ‘clot busters’, lyse the occlusion thereby restoring oxygen and glucose to the affected tissues. Tissue plasminogen activator (tPA, also known as alteplase) is one such agent, released in 1996 by the FDA (J.-M. Lee, Zipfel, and Choi 1999). It is a recombinant serine protease that binds to fibrin in the occlusion and catalyzes the conversion of plasminogen to plasmin, which in turn promotes clot breakdown. Alteplase was originally approved for administration up to 3 hours following stroke symptom onset, although this window has been widened to 4.5 hours. Before administration of tPA, all cases must be confirmed to be ischemic, as hemorrhagic symptoms would be exacerbated by the action of a thrombolytic. The narrow therapeutic window and increased risk of intracranial hemorrhage result in less than 5% of stroke patients receiving this treatment (Green and Shuaib 2006). Since the approval of alteplase in 1996, no new entities have entered the market for the treatment of stroke.

Desmoteplase, another thrombolytic agent, has recently completed clinical trials. It differs from alteplase in its high specificity for fibrin, increasing the potential therapeutic window and minimizing neurotoxicity (Green and Shuaib 2006). Phase II trial results were promising, however, the follow-up phase III study showed a lack of efficacy and the primary trial outcome was not reached (Hacke et al. 2005, DIAS-III on clinicaltrials.gov, 2014).

Recently, several clinical trials evaluating endovascular thrombectomy in ischemic stroke patients have resulted in positive outcomes (Berkhemer et al. 2015; Goyal et al. 2015; Campbell et al. 2015). One such trial will be highlighted below. In March 2015, results for the phase III trial Extending the Time for Thrombolysis in Emergency Neurological Deficits- Intra-Arterial (EXTEND-IA) were released. The trial indicated endovascular thrombectomy as an adjunctive therapy to tPA administration. Endovascular thrombectomy is a surgical procedure that restores blood flow to the occluded area by employing a retrievable stent to capture the clot. Campbell et al. showed that patients who received tPA within the recommended 4.5 hours and underwent endovascular thrombectomy had improved reperfusion to the salvageable brain tissue, early
neurologic recovery, and improved functional outcomes, such as increases in independent living, in comparison to the tPA control arm. This trial utilized the advances in brain imaging technology to ensure patients with salvageable brain tissue and occlusion size of less than 70 mL were selected. These results represent a much-needed boost in the field of stroke research. Previous trials comparing tPA plus thrombectomy to tPA alone had produced only neutral results (see trials IMS-III (Broderick et al. 2013) and MR RESCUE (Kidwell et al. 2013), for example). The success of the EXTEND-IA trial showcases the importance of trial design.

1.3.2.2 Neuroprotectants

The goal of neuroprotective therapies is to interrupt one or more of the pathways in the ischemic cascade, consequently minimizing the neuronal tissue damage caused by those pathways. The main area of action for neuroprotection is the region of the ischemic penumbra, which represents a tissue region that is salvageable if interventions are employed. There have been numerous trials involving neuroprotective agents over the past several decades which have been reviewed extensively (Green and Shuaib 2006; Tymianski 2013). Types of interventions include glutamate antagonists, anti-inflammatory agents, ion-channel modulators, free radical scavengers, GABA receptor agonists, and serotonin agonists. Table 1.1 summarizes several agents from each of these categories that have entered phase III trials.

**Table 1.1.** Summary of phase III clinical trials involving neuroprotective agents for the treatment of ischemic stroke.

<table>
<thead>
<tr>
<th>Study Name</th>
<th>Study Title</th>
<th>Agent</th>
<th>Mechanism of Action</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICTUS</td>
<td>International Citicoline Trial on acute Stroke</td>
<td>Citicoline</td>
<td>Free radical scavenger</td>
<td>Negative</td>
</tr>
<tr>
<td>ALIAS</td>
<td>Albumin in Acute Stroke Trial</td>
<td>Albumin</td>
<td>Several; e.g. reverse blood aggregation</td>
<td>Negative</td>
</tr>
<tr>
<td>SAINT-II</td>
<td>Safety and Effectiveness of NXY-059 for the Treatment of Patients Who Have Suffered From a Stroke</td>
<td>NXY-059</td>
<td>Free radical scavenger</td>
<td>Negative</td>
</tr>
<tr>
<td>IMAGES</td>
<td>Intravenous Magnesium Efficacy in Stroke</td>
<td>Magnesium sulphate</td>
<td>NMDA channel blocker, Ca channel blocker</td>
<td>Negative</td>
</tr>
</tbody>
</table>
Table 1.1 references: (Ashfaq et al. 2007; Becker 2002; Dávalos et al. 2012; Franke et al. 1996; Ginsberg et al. 2013; Muir, Lees, Ford, and Davis 2004; Rosenberg et al. 2011; Saver et al. 2015; Wahlgren, MacMahon, De Keyser, Indredavik, and Ryman 1994, Repinotan on clinicaltrials.gov 2009)

### 1.3.2.2.1 Difficulties with Neuroprotectant Trials

Clinical trials involving neuroprotective agents in ischemic stroke have been largely disappointing. Attributes such as selection of appropriate animal models to verify preclinical efficacy, heterogeneity of the patient population, time to intervention administration, and selection of end points should be carefully considered.

These types of considerations are summarized in the Stroke Therapy Academic Industry Roundtable (STAIR) criteria, which was introduced in the late 1990’s (STAIR 1999). Original criteria are found in Table 1.2. The STAIR guidelines underscore the difficulties faced by stroke trial investigators.

**Table 1.2.** Guidelines for preclinical evaluation of neuroprotective agents (STAIR 1999).

<table>
<thead>
<tr>
<th>FAST-MAG</th>
<th>Field Administration of Stroke Therapy – Magnesium Trial</th>
<th>Magnesium sulphate</th>
<th>NMDA channel blocker, Ca channel blocker</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>FIST</td>
<td>Flunarizine in Stroke Treatment</td>
<td>Flunarizine</td>
<td>Ca channel blocker</td>
<td>Negative</td>
</tr>
<tr>
<td>MACSI</td>
<td>Efficacy and Safety Study of DP-b99 in Treating Acute Ischemic Stroke</td>
<td>DP-b99</td>
<td>Metal chelation</td>
<td>Negative</td>
</tr>
<tr>
<td>HALT</td>
<td>Hu23F2G Phase 3 stroke trial</td>
<td>Anti-leukocyte antibody: LeukArrest</td>
<td>Anti-inflammatory</td>
<td>Negative</td>
</tr>
<tr>
<td>INWEST</td>
<td>Intravenous Nimodipine West European Stroke Trial</td>
<td>Nimodipine</td>
<td>Ca channel blocker</td>
<td>Negative</td>
</tr>
<tr>
<td>Repinotan</td>
<td>Repinotan in Patients With Acute Ischemic Stroke</td>
<td>Repinotan</td>
<td>Serotonin (5-HT(1A)) agonist</td>
<td>Negative</td>
</tr>
</tbody>
</table>

**STAIR Recommendations for Preclinical Assessment of Neuroprotectant Drugs**

- Adequate dose-response curve
- Define the time window in a well-characterized model
- Blinded, physiologically controlled reproducible studies
- Histological and functional outcomes assessed acutely and long-term
- Initial rodent studies, then consider gyrencephalic species
- Permanent occlusion then transient in most cases
Timing of intervention administration is an essential aspect of stroke trials; there must be salvageable brain tissue remaining in the ischemic penumbra for agents to act in a neuroprotective manner. Recently, Tymianski outlined the emergence of pre-hospital treatment in stroke trials (Tymianski 2013). The FAST-MAG study mentioned in Table 1.1 was designed not only to assess the neuroprotective potential of intravenous MgSO$_4$, but also to look at the feasibility of pre-hospital clinical trials. This type of trial cannot categorize the stroke as ischemic or hemorrhagic before administration of the intervention, however unlike thrombolytics, neuroprotectants pose little threat of exacerbating hemorrhagic strokes. Trials of this nature would need to factor in that a proportion of the study cohort will not have the indication under investigation. Therefore, trials would need to be powered to achieve significance with approximately 20% of cases enrolled to be hemorrhagic, as was seen in the FAST-MAG trial (Saver et al. 2015).

1.4 Metal Chelation as a Neuroprotective Strategy

Chelation therapies are used for the treatment of diseases involving metal-ion imbalances, such as iron chelation in sickle cell anemia and removal of toxic heavy metals from the blood, and have been proposed as a means to regulate toxic influxes of divalent metal cations following ischemic strokes (Angel et al. 2002). In the past, research has focused on Ca$^{2+}$ chelation. Tymianski et al. showed the first successful use of metal-ion chelation to treat neuronal ischemic injury \textit{in vivo}. Pretreatment with infusion of the membrane permeant Ca$^{2+}$-chelator, BAPTA-AM and several BAPTA analogues attenuated ischemic neuronal damage following focal cerebral ischemia in rats (Tymianski et al. 1993). However, as mentioned previously, Zn$^{2+}$ has emerged as a major neurotoxin in the ischemic cascade, thus development and implementation of Zn$^{2+}$ chelating molecules as possible neuroprotectants for ischemic stroke should be evaluated.

Although clinical studies involving metal-ion chelation for the treatment of ischemic stroke have met little clinical success thus far, the \textit{in vitro} and \textit{in vivo} evidence of neuroprotection is appealing. Exploring a new scaffold, such as histidine, that is endogenous to the body and brain represents another approach to designing chelators that may have clinical utility in treating ischemic stroke.
1.4.1 Histidine as a Potential Neuroprotectant

Histidine in an essential amino acid obtained through diet and is required for maintaining nitrogen balance, contributes to the buffering capacity of the blood, and plays central roles as an acid-base catalyst in various enzymes (Bender 2012). L-Histidine is rapidly absorbed in the intestine through sodium-dependent amino acid transporters, and has a bioavailability of 80% (Wade and Tucker 1998). It is also blood-brain penetrant, taken up through the large-neutral amino acid transporter (LNAA) in the blood-brain barrier (BBB).

In addition to being an essential amino acid for protein synthesis and proper enzyme functioning, histidine exhibits neuroprotective properties. Its neuroprotective actions are thought to occur through activation of the histaminergic system as well as antioxidant actions. In addition to these mechanisms, histidine is known to chelate the divalent metal cation Zn\(^{2+}\) implemented in the ischemic cascade. Therefore, metal chelation represents another possible mechanism for its neuroprotective effects.

1.4.1.1 Histaminergic System Activation in Potential Neuroprotection

Histidine is the primary source of histamine, which is biologically synthesized by L-histidine decarboxylase. Histamine acts as both a neurotransmitter and mediator of immune responses. Activation of the H\(_1\) histamine receptor augments allergic reactions, while activation of the H\(_2\) receptor functions conversely, suppressing immunological responses. H\(_2\) receptor agonists suppress lymphocyte proliferation, cytokine production, and neutrophil accumulation (Adachi 2005).

Adachi et al. have shown in vivo that post-ischemic intraperitoneal administration of histidine substantially reduced infarct volume following focal cerebral ischemia in a dose-dependent manner (Adachi, Liu, and Arai 2005). These positive effects were diminished by co-administration of an H\(_2\) receptor antagonist, but were not affected by co-administration of an H\(_1\) receptor antagonist. Therefore the authors surmise that the positive effects of histidine in this model are linked to activation of the H\(_2\) receptor of the histaminergic system. As discussed briefly in Section 1.1.1, inflammatory processes are acknowledged as pathways that lead to ischemic cell death (Iadecola and Anrather 2011). Therefore, it is possible that the suppression of such inflammatory processes through activation of the H\(_2\) receptor would lead to
neuroprotection in ischemic stroke models.

1.4.1.2 Antioxidant Behaviour of Histidine

In addition to histaminergic system activation, histidine has antioxidant properties. Histidine is known as a hydroxyl radical scavenger (Zs.-Nagy and Floyd 1984). Fe$^{2+}$ can form complexes with ADP and ATP, which subsequently react with hydrogen peroxide to produce damaging hydroxyl radicals. Electron-spin resonance can be used to quantify the hydroxyl radicals produced by these reactions through using a reagent to trap the radicals produced. Histidine is also able to scavenge these radicals, observed by decreased signals in electron-spin resonance experiments (Zs.-Nagy and Floyd 1984). Hydroxyl radicals are the most active biological free radical, responsible for detrimental actions such as lipid peroxidation and DNA damage (Halliwell 1994).

As well, histidine is able to scavenge the reactive oxygen species singlet oxygen ($^1$O$_2$), which is molecular oxygen with an electron promoted to an excited state (Matheson and Lee 1979; J. W. Lee et al. 1999). Histidine rapidly reacts with singlet oxygen, rendering it nonreactive and thereby reducing its toxicity (Matheson and Lee 1979). The production of reactive oxygen species following ischemia is a main cause of cell death; therefore the employment of an antioxidant could modulate the detrimental effects of reactive oxygen species in the ischemic cascade.

However, in an in vivo model of focal ischemia, L-histidine did not prevent formation of malondialdehyde, which is an indicator of lipid peroxidation and oxidative stress (Irisawa et al. 2008). This evidence contradicts the likelihood of histidine exerting its neuroprotective effects solely as an antioxidant.

1.4.1.3 Neuroprotection by Histidine Metal Chelation

Histidine was first co-crystallized with Zn$^{2+}$ in 1963 (Harding and Cole 1963; Kretsinger, Cotton, and Bryan 1963). Since then, an extensive amount of computational and experimental literature on the interactions between histidine and Zn$^{2+}$ has been published ((Trzaskowski, Adamowicz, and Deymier 2008), for example), largely due to the structural importance of their interactions in enzymes and transcription factors. In addition to computational literature supporting the favorable interaction between histidine and Zn$^{2+}$, potentiometric titrations lend
more evidence to this story. In potentiometric titration experiments, the stability of complexes in solution are determined by titration and subsequent monitoring of pH or voltage. L-Histidine and several histidine based analogues including dihistidine form Zn\(^{2+}\) complexes with binding affinities in the range of \(6.3 \times 10^5\) to \(4 \times 10^6\) M\(^{-1}\) (Vogler and Vahrenkamp 2002). For comparison, BAPTA (mentioned in Section 1.2.5 as a neuroprotective agent) interacts with Ca\(^{2+}\) with a binding affinity of \(9.4 \times 10^6\) M\(^{-1}\) (107 nM dissociation constant, Tsien 1980). Therefore, one can compare the potential of histidine and histidine analogues to chelate Zn\(^{2+}\) in a manner similar to BAPTA chelating Ca\(^{2+}\) and hence potentially act as neuroprotectants.

In 2013, through a convoluted experiment involving the extracts from various agricultural products and fish, it was found that D- and L-histidine were able to attenuate Zn\(^{2+}\)-induced toxicity \textit{in vitro} (Kawahara et al. 2013). The crystallographic and computational literature on Zn\(^{2+}\)-histidine interactions, as well as the \textit{in vitro} evidence of its protection from Zn\(^{2+}\) toxicity led us to postulate that histidine may be an endogenous neuroprotectant that exerts its protective effects through metal chelation.

1.5 Project Outline

1.5.1 Hypotheses

Based on the above literature search, it is reasonable to hypothesize that:

1. Chelation of labile Zn\(^{2+}\) as it is released from glutamatergic neurons will attenuate metal-ion neurotoxicity following an ischemic stroke.

2. Histidine may be an endogenous neuroprotectant capable of limiting Zn\(^{2+}\) neurotoxicity and can be used as a platform around which to design new chemical entities to be used as putative therapeutics for the treatment of ischemic stroke.

1.5.2 Research Goals

To evaluate these hypotheses, computational and synthetic chemistry will be employed to design a Zn\(^{2+}\) chelating molecule that is brain penetrant and has drug like properties, to function as a probe. Specific goals of this project are as follows:

- To evaluate the binding interactions of histidine-based small molecules and Zn\(^{2+}\) \textit{in silico}.

- To synthesize 15-20 molecules based on favorable \textit{in silico} results.
➢ To evaluate the ability of synthesized molecules to chelate Zn$^{2+}$ qualitatively using NMR spectroscopy.

➢ To evaluate the ability of synthesized molecules to chelate Zn$^{2+}$ quantitatively using isothermal titration calorimetry.

➢ To evaluate the ability of synthesized molecules to protect against Zn$^{2+}$-induced toxicity *in vitro*.
CHAPTER 2

2  A Computational Study of the Interaction Between Zn$^{2+}$ and Histidine-Based Small Molecules
2.1 Computer Aided Drug Design

Computational chemistry has been widely adopted by medicinal chemists as a tool to intelligently design drug-like molecules. A range of molecular modeling techniques are available to analyze molecules in 3D and predict how a molecule will behave biologically. Lead synthetic targets can be therefore analyzed in silico and designed rationally based on 3D properties and predicted biological function.

Molecular modeling techniques allow for structure- or mechanism-based drug design, by direct or indirect methods. Understanding the correlation between physical properties, chemical structure, and the 3D structure a molecule adopts in space can inform computational chemists about the potential biological performance of novel molecules (Cohen 1996). Several levels of theory are available for use in molecular modeling including molecular mechanics, semi-empirical methods, and ab initio methods. The project described herein utilized calculations based on molecular mechanics and semi-empirical methods; these methods will be briefly introduced.

2.1.1 Molecular Mechanics

Molecular mechanics (MM) uses empirical energy functions derived from classical mechanics to describe atomic bonding and does not explicitly consider electrons. Therefore, MM methods are able to handle large systems of molecules, but are unable to yield results about bond formation or breakage. Functions that describe molecular energy are known as force fields; there are numerous force fields available to the computational chemist, parameterized to cover a broad range of applications and molecular systems.

Force fields contain both bonding and non-bonding terms. Bond lengths and angles are described by bonding terms while van der Waals and electrostatic interactions are described by non-bonding terms. Most modern force fields use an expression of the following potential energy function (Leach 1996; Cohen 1996):

\[
E_{total} = E_1 + E_2 + E_3 + E_4
\]  

[2.1]

where \( E_1, E_2, \) and \( E_3 \) are bonding terms and \( E_4 \) is a non-bonding term. Each energy term in Eq.
[2.1] is described in Table 2.1; visual representations of the interactions described are also included.

**Table 2.1.** Functional energy terms of a general MM force field. Visual representations of the interactions are included (Leach 1996).

<table>
<thead>
<tr>
<th>Energy Equation</th>
<th>Visual Representation</th>
</tr>
</thead>
<tbody>
<tr>
<td>( E_1 = \sum_{\text{bonds}} \frac{k_l}{2} (l - l_0)^2 )</td>
<td>bond stretching</td>
</tr>
<tr>
<td>( E_2 = \sum_{\text{angles}} \frac{k_\theta}{2} (\theta - \theta_0)^2 )</td>
<td>angle bending</td>
</tr>
<tr>
<td>( E_3 = \sum_{\text{torsions}} \frac{k_t}{2} [1 + \cos(n\phi - \delta)] )</td>
<td>bond rotation</td>
</tr>
<tr>
<td>( E_4 = \sum_{\text{non-bonding}} \left( 4\varepsilon \left( \frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left( \frac{\sigma_{ij}}{r_{ij}} \right)^6 \right) + \frac{q_i q_j}{4\pi\varepsilon_0 r_{ij}} )</td>
<td>( \delta^\pm ) van der Waals + electrostatic</td>
</tr>
</tbody>
</table>

*\( k_l, k_\theta, \) and \( k_t \) are constants based on empirical data

The term \( E_1 \) represents the energy associated with the interaction between two bonded molecules as the bond length \( (l) \) deviates from the reference bond length \( (l_0) \) using a harmonic potential function. \( E_2 \) is also modeled using a harmonic function and represents the energy summation of all bond angles in the molecule. In this term, \( \theta \) describes the bond angle and \( \theta_0 \) is the reference bond angle. All reference variables are described by the force field parameters. The energy associated with bond rotation, or torsions, is represented by \( E_3 \), where \( \phi \) is the torsional (or dihedral) angle, \( n \) is the multiplicity or the number of minima in the cosine function as the bond is rotated 360°, and \( \delta \) is the phase factor which describes where the torsional angle passes through its minimum value. Van der Waals forces and electrostatic interactions are described by \( E_4 \). The energy associated with van der Waals forces are commonly represented by the Lennard-Jones 12-6 function, where \( \sigma_{ij} \) is the collision diameter (distance at which the force between the two nuclei is zero), \( r_{ij} \) is the bond length, and \( \varepsilon \) is the well depth of the Lennard-Jones function (Leach 1996). Figure 2.1 shows the Lennard-Jones 12-6 potential function graphically, with
variables labeled.

![Graphical representation of the Lennard-Jones 12-6 potential.](image)

**Figure 2.1.** Graphical representation of the Lennard-Jones 12-6 potential.

In MM calculations, atoms are treated as point charges, therefore the attractive or repulsive electrostatic interactions between atoms can be approximated by Coulomb’s law, where $q_i$ and $q_j$ in $E_4$ (of Eq. [2.1]) are the partial atomic charges on atoms $i$ and $j$, respectively, $r_{ij}$ is the $i$-$j$ bond length, and $\varepsilon_0$ is the dielectric constant *in vacuo*. Dielectric constants are used based on the solvation of the system under study. (Leach 1996)

Force field parameters include constants that describe atomic properties such as hybridization state, atomic number, and radius, for example. These parameters are used to verify computed results against reference values. Therefore, each force field will generate different results, limited by the quality of the data used in the parameterization process. Force fields should be chosen such that the molecules used in the parameterization process are similar to the types of molecules that will be assessed computationally. This ensures that the most appropriate parameter values are used, giving the most accurate results. Using a force field parameterized for a different family of molecules may give unpredictable erroneous results. The specific force field used in this project, MMFF94x, is described below.

### 2.1.1.1 MMFF94x Force Field

MMFF94x is a modified version of MMFF94, which stands for the Merck Molecular Force Field, initially designed in 1994 (Halgren 1996). In the original MMFF94 force field conjugated nitrogen atoms assume a tetrahedral geometry, whereas the MMFF94x force field has an increased energy of inversion for conjugated nitrogen atoms and they therefore assume a planar geometry (Molecular Operating Environment 2010). The potential energy function for this force
field is as follows (Halgren 1996):

\[ E = E_B + E_A + E_{BA} + E_{OOP} + E_T + E_{VDW} + E_Q \]  \[2.2\]

where \( E_B = \) bond stretching energy, \( E_A = \) angle bending energy, \( E_{BA} = \) stretch-bend interactions, \( E_{OOP} = \) out-of-plane bending energy, \( E_T = \) torsional energy, \( E_{VDW} = \) van der Waals interactions, and \( E_Q = \) electrostatic energy. The first four energy terms in Eq. \[2.2\] are bonding terms, while the last two terms are non-bonding terms.

Bond stretching energy is calculated using Eq. \[2.3\], where \( k_{B,ij} \) is the force constant, \( r_{ij} \) is the bond length, \( r_{ij}^0 \) is the reference bond length specified by the force field, and \( cs \) is the cubic-stretch constant (Halgren 1996). Unlike \( E_I \) in Table 2.1, this functional form of the bond stretch energy is quartic. Higher order terms have been added to more accurately model the Morse curve, which represents the energy change associated with increases in atomic bond distance (Leach 1996).

\[ E_{B,ij} = \frac{k_{B,ij}}{2} \left( r_{ij} - r_{ij}^0 \right)^2 \left( 1 + cs \left( r_{ij} - r_{ij}^0 \right) + \frac{7}{12} cs^2 \left( r_{ij} - r_{ij}^0 \right)^2 \right) \]  \[2.3\]

The angle bending energy is expressed as a cubic function (Eq. \[2.4\]), where \( k_{A,ij} \) is the force constant, \( \theta_{ij} \) describes the bond angle, \( \theta_{ij}^0 \) is the reference bond angle, and \( cb \) is the cubic-bend constant (Halgren 1996).

\[ E_{A,ij} = \frac{k_{A,ij}}{2} \left( \theta_{ij} - \theta_{ij}^0 \right)^2 \left( 1 + cb \left( \theta_{ij} - \theta_{ij}^0 \right) \right) \]  \[2.4\]

The stretch-bend energy (\( E_{BA} \)) describes the energy associated with consequent changes in bond length as bond angle is varied. This is represented schematically in Figure 2.2. As the angle \( \theta_{ijk} \) decreases, \( r_{ij} \) and \( r_{jk} \) tend to increase. This is mathematically represented in Eq. \[2.5\], where \( k_{BA,ijk} \) and \( k_{BA,kij} \) are the force constants that relate \( i-j \) and \( k-j \) bond stretches, respectively, with the \( ijk \) angle, \( r \) is bond length, and \( \theta \) is bond angle (Halgren 1996).

\[ E_{BA,ijk} = \left( k_{BA,ijk} \left( r_{ij} - r_{ij}^0 \right) + k_{BA,kij} \left( r_{jk} - r_{jk}^0 \right) \right) \left( \theta_{ijk} - \theta_{ijk}^0 \right) \]  \[2.5\]
An energy term for out-of-plane bending ($E_{OOP}$, Eq. [2.6]) is incorporated into the MMFF94x force field (Halgren 1996). This term maintains planarity in sp$^2$ hybridized carbons by increasing the energy barrier for inversion, for example, and is shown schematically in Figure 2.3. $E_{OOP}$ is described by Eq. [2.6] where $k_{OOP}$ is the force constant and $\chi$ is the angle between the out-of-plane atom and the plane (see Figure 2.3).

\[ E_{OOP,ijk;l} = \frac{k_{OOP}}{2} \chi_{ijk;l}^2 \]  

[2.6]

**Figure 2.3.** Schematic representation of the out-of-place bending energy between the plane described by $ijk$, and atom $l$.

Torsional energy is given the functional form found in Eq. [2.7], which is similar to $E_3$ of Eq. [2.1], with the addition of higher order terms that aid in accurately representing torsional energy in systems with heteroatoms. Force constants are represented by $k_{T1}$, $k_{T2}$, and $k_{T3}$, while $\phi$ describes the dihedral angle (Halgren 1996). The additional terms in Eq. [2.7] account for the interaction between atoms 1 and 4 as the dihedral angle between them changes (Leach 1996).

\[ E_{T,ijkl} = \frac{k_{T1}}{2} (1 + \cos \phi) + \frac{k_{T2}}{2} (1 - \cos 2\phi) + \frac{k_{T3}}{2} (1 + \cos 3\phi) \]  

[2.7]

The energy associated with van der Waals interaction is given the following functional form, introduced by Halgren in 1992 (Halgren 1992):

\[ E_{VDW,ij} = \varepsilon_{ij} \left( \frac{1 + \delta}{\rho_{ij} + \delta} \right)^{n-m} \left( \frac{1 + \gamma}{\rho_{ij}^m + \gamma} - 2 \right) \]  

[2.8]
where $\varepsilon_{ij}$ is the well depth (see Figure 2.1 for example), $\rho_{ij} = R_{ij}/R_{ij}^*$, $\delta$ and $\gamma$ are buffering constants, and $R_{ij}$ is the atomic distance between atoms $i$ and $j$. $R_{ij}^*$ is described by Eq. [2.9] where $A_i$ is a constant and $\alpha_i$ is atomic polarizability (Halgren 1992).

$$R_{ij}^* = A_i\alpha_i^{1/4}$$  \[2.9\]

If $n = 12$, $m = 6$, and $\delta = \gamma = 0$ are substituted into Eq. [2.8], the conventional Lennard-Jones 12-6 potential is recreated. In MMFF94x, specific values assigned are $n = 14$, $m = 7$, $\delta = 0.07$, and $\gamma = 0.12$ such that the function takes the form in Eq. [2.10].

$$E_{VDW,ij} = \varepsilon_{ij} \left( \frac{1.07 R_{ij}^*}{R_{ij} + 0.07 R_{ij}^*} \right)^7 \left( \frac{1.12 R_{ij}^*^7}{R_{ij}^7 + 0.12 R_{ij}^*^7} - 2 \right)$$  \[2.10\]

Halgren developed this ‘buffered 14-7’ energy equation of van der Waals interactions in order to more accurately model the high quality data available for the interaction of rare gases helium, argon, and neon. Constants were fit using such data. This form keeps the potential finite as the interatomic distance approaches zero, whereas the conventional Lennard-Jones 12-6 function goes to infinity at zero atomic separation (Leach 1996). Atomic polarizability also factors into this equation, therefore adding another empirical parameter to bring consensus between calculations and experimental data.

Electrostatic energy is represented by a buffered Coulombic form, shown in Eq. [2.11], where $k$ is a constant, $q_i$ and $q_j$ are the partial atomic charges on atoms $i$ and $j$, respectively, $r_{ij}$ is the $i$-$j$ bond length, $\varepsilon$ is the dielectric constant of the system under study, and $\delta$ is the electrostatic buffering constant, equal to 0.05 Å (Halgren 1996). The distance buffering constant ensures that the infinitely attractive electrostatic forces do not overwhelm the finite repulsive van der Waals forces as oppositely charged nuclei interact.

$$E_{Q,ij} = \frac{kq_iq_j}{\varepsilon(r_{ij} + \delta)}$$  \[2.11\]

### 2.1.2 Energy Minimization

Energy minimization is carried out to find the lowest energy conformation of the molecule under study by using the Born-Oppenheimer approximation, which states that for molecules in the
ground state, energy is solely dependent on nuclear position (Cohen 1996). Therefore, by varying the nuclear coordinates, a potential energy surface that is a function of atomic position can be constructed. The goal of the energy minimization process is to find the global minimum on the potential energy surface, indicating equilibrium geometry has been achieved. One can imagine an analogy in which the surface “valleys” correspond to minima, for which the first derivative of the potential energy function is zero, and the second derivative is positive (i.e. the minima is a concave valley that goes “uphill” in all directions). There are various minimization algorithms that can be used find the geometries that correspond to such minima, which are generally selected based on the number of molecules in the system and the relative energy of the starting conformation (Leach 1996).

2.1.3 Semi-Empirical Methods

Semi-empirical calculations use approximations based on empirical parameters to simplify *ab initio* calculations, decreasing the time and cost of computing. This is a powerful tool for medicinal chemistry applications where the size of the system under study is usually large. Unlike calculations using MM, which do not take into account electrons, semi-empirical methods explicitly consider valence shell electrons. These calculations use the Hartree-Fock approximation, which states that each electron experiences all other electrons as a field of charge, rather than considering single electron-electron interactions (Leach 1996). The motions of valence electrons are described by wavefunctions, which are approximated in space by the linear combination of molecular orbitals. Semi-empirical methods start with an initial guess of the valence orbital occupation, and the potential energy each electron would experience due to the other electrons is calculated and compared against the field that the electrons produce. When the wavefunction of the electron is consistent with the field generated by it and the other electrons, a self-consistent field has been reached and the system is minimized (Cohen 1996).

There are various models available for semi-empirical calculations, most of which are based on the Neglect of Diatomic Differential Overlap (NDDO) method. In this method, all two-electron integrals in the Schrodinger equation involving two-center charge distributions are neglected. In other words, electron-electron repulsions for adjacent atoms are ignored, and rather replaced using parameters based on experimental data or *ab initio* results (Leach 1996). Parametric Method 3 (or PM3) was used in this project due to its suitability for calculations involving
transition metals (Stewart 2004). PM3 is based on the NDDO method, and unlike earlier models, the parameterization process was automated (Stewart 1989). However, the PM3 model is insufficient in predicting amide bond rotational energy, therefore an additional torsional energy term for amide bonds has been appended (Molecular Operating Environment 2010).

2.2 Computational Methods

2.2.1 Interactions Between Histidine-Based Small Molecules and Divalent Metal Cations

*In silico* simulations of the interaction between a series of histidine-based small molecules, shown in Figure 2.4, and divalent metal cations (Zn\(^{2+}\), Cu\(^{2+}\), and Ca\(^{2+}\)) were performed using the Molecular Operating Environment (MOE) software suite (Molecular Operating Environment 2010). Gas phase and solution phase optimizations were conducted. The focus of this modeling is to assess the ability of small molecules to chelate Zn\(^{2+}\). However, Cu\(^{2+}\) and Ca\(^{2+}\) are also divalent metal cations present in the brain, therefore their chelation capacity was also assessed *in silico*. Ideally, a molecule with specificity for Zn\(^{2+}\) over Ca\(^{2+}\) will be found.

![Chemical structures of histidine-based small molecules](image)

**Figure 2.4.** Chemical structures of histidine-based small molecules used for *in silico* predictions of divalent metal binding potential in Section 2.2.1.

2.2.1.1 *In vacuo* simulations of the interaction between histidine-based small molecules and divalent metal cations

Ligands (shown in Figure 2.4) were built *in silico* and a systematic search methodology within the MOE suite was utilized to identify the lowest-energy conformations using the MMFF94x
force field, parameterized for small organic molecules and suitable for use in solvated systems (Halgren 1996). Simulations were performed at pH 7 to represent ischemic brain conditions, where drops in pH of 0.5 to 1 unit is typical (Lipton 1999). The total system ($E_{tot}$), van der Waals ($E_{vdw}$), and electrostatic ($E_{ele}$) energies were recorded. Following this energy minimization process, a cation ($Zn^{2+}$, $Ca^{2+}$, or $Cu^{2+}$) was placed at 3.0 Å distances from the lowest-energy conformers in a variety of positions to fully sample all possible in silico metal:ligand complex interactions. A distance of 3.0 Å was selected as previous studies suggest that it is an optimal distance for positive and negative interactions while maintaining structural integrity (Weaver Lab, personal communication). Placement of cations at further distances may not allow all of the possible interactions to be captured, while placing cations at closer distances may produce unfavorable and unrealistic repulsions between cations and partial positive charges on the ligands. Figure 2.5 shows cation placement sites for histidine 1, represented by bolded letter labels A, B, and C, as an example. The metal:ligand starting poses underwent energy minimization and $E_{tot}$, $E_{vdw}$, and $E_{ele}$ were recorded. The initial and final cation orientations, as described by cation proximity to molecular functionalities, were recorded. The difference in energy between the metal:ligand complex and the lowest-energy ligand conformers was computed to obtain relative binding energies. An interaction was classified as occurring if the distance between the metal and atoms involved was less than or equal to 3.0 Å.

![Figure 2.5](image)

**Figure 2.5.** Identification of cation placement sites around histidine 1 by bolded letter labels A, B, and C.

More robust semi-empirical calculations using the PM3 Hamiltonian were performed for metal:ligand complexes under evacuated conditions using the Molecular Orbital PACkage (MOPAC), a semi-empirical molecular orbital software program, implemented in MOE (Halgren 1996). Ligands were built in silico and energy minimized to produce the lowest-energy conformation using the restricted Hartree-Fock method and the PM3 Hamiltonian. Singlet state energy was recorded. $Zn^{2+}$ was placed at 3.0 Å distances around the ligands and the complexes
were subsequently energy minimized as described above. The difference in singlet state energies of the metal:ligand complex and ligand was computed to obtain relative binding energies.

2.2.1.2 Solvated simulations of the interaction between histidine-based small molecules and divalent metal cations

Metal:ligand complexes were optimized in an aqueous environment to mimic physiologic conditions. Again, pH 7 was used to simulate ischemic brain conditions. Energy minimization was performed using the MMFF94x force field in the MOE program under explicit solvation with periodic boundary conditions. Lowest energy conformers of the ligands from \textit{in vacuo} simulations were solvated and energy minimization was performed as described above. As previously, cations were placed at 3.0 Å distances around the minimized ligand and the system underwent energy minimization. $E_{\text{tot}}$, $E_{\text{vdw}}$, $E_{\text{ele}}$, and initial and final cation orientations were recorded for the ligand and the metal:ligand complexes. Energy differences represent the relative binding energies for the metal:ligand complexes in aqueous conditions.

2.2.2 Interactions Between N/S-Heterocycles and Divalent Metal Cations

Similar MM and semi-empirical calculations as described in Section 2.2.1 were performed to assess the interaction of various 4-, 5-, and 6-membered N/S-heterocycles with divalent metal cations; heterocycles used in calculations are shown in Figure 2.6. These simulations produced relative metal:heterocycle binding energies. In all cases, the metal cation was placed 3.0 Å above the heterocycles in Figure 2.6 as a starting orientation. These simulations were performed in order to assess the capability of atypical amino acids to chelate divalent cations preferentially over histidine 1. Comparison of metal:heterocycle binding interactions to metal:imidazole binding interactions allowed for such predictions.
Several N/S-heterocycles were then selected, based on their potential to yield favorable binding interactions, as substitutes for the imidazole ring in histidine 1, forming atypical amino acids. These atypical amino acids, shown in Figure 2.7, were built in silico using the MOE program. Interactions with Zn$^{2+}$, Cu$^{2+}$, and Ca$^{2+}$ were assessed using MM and semi-empirical calculations as described in Section 2.2.1. Initial cation orientations were chosen based on results that indicate favorable interactions are most probable between the heterocycle and carboxylate functionalities in molecules containing these functional groups. The difference in energy between the metal:ligand complex and the lowest-energy ligand conformers was computed to obtain relative binding energies.
2.2.3 Optimization of 2,5-Diketopiperazine and 2,4-Imidazolidinedione Substitution Patterns for Zn$^{2+}$ Chelation

2,4-Imidazolidinediones (hydantoin) represent a drug-like platform, similar to 2,5-diketopiperazines (cyclic dipeptides), which have potential to be exploited as a platform for the design of a Zn$^{2+}$-chelator. 2,5-Diketopiperazines with 3,6-1H-imidazol-4-ylmethyl substitution (7 and 8) were previously assessed for their ability to chelate Zn$^{2+}$ in silico (see Section 2.2.1). By optimizing the substitution pattern of such diketopiperazines, as well as imidazolidinediones, it may be possible to increase such molecules affinity for Zn$^{2+}$ chelation. Therefore, permutations of mono- and di-1H-imidazol-4-ylmethyl substituted 2,5-diketopiperazines and 2,4-imidazolidinediones were built in silico using the MOE program and their interaction with Zn$^{2+}$ was simulated in vacuo and under aqueous conditions as described in Section 2.2.1.

2.2.4 Modeling of Biologically Relevant Zn$^{2+}$ Binding Sites

As briefly mentioned in Section 1.2, Zn$^{2+}$ is a common cofactor in biological systems, playing crucial catalytic and structural roles. Zn$^{2+}$ metalloproteins are one of the most abundant proteins in nature (Laitaoja, Valjakka, and Jänis 2013). In an attempt to learn more about what makes something a high affinity Zn$^{2+}$ chelator in a biological setting, molecular modeling of various proteins with Zn$^{2+}$ binding sites was performed.

Given that the focus of this research project is to use histidine 1 as an endogenous Zn$^{2+}$ binding ligand to rationally design other Zn$^{2+}$ chelators, proteins with histidine residues at the binding site were selected in order to collect information on pertinent structural and electronic molecular features to direct future drug design and increase chelation ability. A literature review also revealed that most Zn$^{2+}$ metalloproteins also commonly contain cysteine residues at the binding site, therefore a portion of the proteins selected were chosen to include this these residues.

Eight proteins were selected by reviewing the literature and the Zn$^{2+}$ binding site was analyzed in silico. Data files of protein crystal structures used (PDB codes 1ZAA, 2OHX, 1CA2, 1XER, 1R4R, 3MV1, 1TON, and 2ATC) were gathered from the Protein Data Bank (PDB). Tools in the MOE software program were used to record interaction lengths and angles, coordination number, relevant interactions with amino acid residues, and any spatially interesting observations (Molecular Operating Environment 2010).
2.3 Computational Results and Discussion

2.3.1 Results of the Interactions Between Histidine-Based Small Molecules and Divalent Metal Cations

The interaction energies of the histidine-based small molecules (shown in Figure 2.4) and Zn\(^{2+}\) \textit{in vacuo} and under solvated conditions can be found in Table 2.2. Total interaction energies for all histidine-based analogues shown in Figure 2.4 with Cu\(^{2+}\) and Ca\(^{2+}\) cations can be found in Table 2.3.

Table 2.2. Values for the change in energy upon complex formation between histidine analogues in Figure 2.4 and Zn\(^{2+}\) using both MM and semi-empirical calculations.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Δ(E_T)</th>
<th>Δ(E_{vdw})</th>
<th>Δ(E_{ele})</th>
<th>Δ(E_T)</th>
<th>Δ(E_{vdw})</th>
<th>Δ(E_{ele})</th>
<th>Δ(E_T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-109.95</td>
<td>49.18</td>
<td>-158.20</td>
<td>-94.78</td>
<td>34.20</td>
<td>-128.37</td>
<td>-230.76</td>
</tr>
<tr>
<td>3</td>
<td>-41.39</td>
<td>25.06</td>
<td>-69.48</td>
<td>-31.95</td>
<td>31.74</td>
<td>-72.62</td>
<td>-22.67</td>
</tr>
<tr>
<td>4</td>
<td>-131.97</td>
<td>60.62</td>
<td>-194.16</td>
<td>-95.78</td>
<td>24.01</td>
<td>-122.69</td>
<td>-225.61</td>
</tr>
<tr>
<td>5</td>
<td>-100.84</td>
<td>44.41</td>
<td>-148.96</td>
<td>-97.18</td>
<td>33.75</td>
<td>-141.83</td>
<td>-259.49</td>
</tr>
<tr>
<td>6</td>
<td>-100.86</td>
<td>44.36</td>
<td>-148.91</td>
<td>-101.18</td>
<td>34.86</td>
<td>-135.51</td>
<td>-252.62</td>
</tr>
<tr>
<td>7</td>
<td>-62.10</td>
<td>30.62</td>
<td>-111.08</td>
<td>-41.61</td>
<td>11.40</td>
<td>-49.00</td>
<td>-126.74</td>
</tr>
<tr>
<td>8</td>
<td>-86.17</td>
<td>39.73</td>
<td>-137.98</td>
<td>-46.26</td>
<td>10.41</td>
<td>-57.47</td>
<td>-137.92</td>
</tr>
</tbody>
</table>

Table 2.3. Values for the change in energy upon complex formation between histidine analogues in Figure 2.4 and Cu\(^{2+}\) and Ca\(^{2+}\) using MM.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Δ(E_T) (\textit{in vacuo})</th>
<th>Δ(E_T) (solvated)</th>
<th>Δ(E_T) (\textit{in vacuo})</th>
<th>Δ(E_T) (solvated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-113.92</td>
<td>-103.29</td>
<td>-79.25</td>
<td>-66.17</td>
</tr>
<tr>
<td>2</td>
<td>-36.15</td>
<td>-23.41</td>
<td>-15.49</td>
<td>-2.09</td>
</tr>
<tr>
<td>3</td>
<td>-43.67</td>
<td>-24.76</td>
<td>-17.13</td>
<td>-6.18</td>
</tr>
<tr>
<td>4</td>
<td>-136.27</td>
<td>-100.75</td>
<td>-96.30</td>
<td>-77.43</td>
</tr>
<tr>
<td>5</td>
<td>-103.93</td>
<td>-97.27</td>
<td>-75.66</td>
<td>-68.53</td>
</tr>
<tr>
<td>6</td>
<td>-103.95</td>
<td>-102.48</td>
<td>-75.67</td>
<td>-74.70</td>
</tr>
<tr>
<td>7</td>
<td>-64.56</td>
<td>-45.30</td>
<td>-41.78</td>
<td>-34.59</td>
</tr>
<tr>
<td>8</td>
<td>-87.25</td>
<td>-51.17</td>
<td>-60.92</td>
<td>-43.00</td>
</tr>
</tbody>
</table>
The most common interactions formed in the Zn$^{2+}$:ligand complexes were of ionic character. This is represented by the highly negative $\Delta E_{ele}$ values in Table 2.2; $\Delta E_{ele}$ values also showed a similar trend for the complexes involving Cu$^{2+}$ and Ca$^{2+}$. In general, all of the compounds assessed were stabilized much more by electrostatic interactions than van der Waals interactions. When present, the carboxylate functional group reduced the energy of the interaction greatly. Compounds 2 and 3, both esters, show increased total complex energy, which relates to the absence of the cation stabilizing carboxylate group. Additionally, the nitrogen at position 3 in the imidazole ring provided a high degree of stabilization to these complexes. As a general trend, ligands containing both carboxylate and imidazole ring functionalities provided the greatest stabilization when both groups were chelated to the Zn$^{2+}$ cation. Figure 2.8 shows a typical complex pose stabilized by both carboxylate and imidazole ring groups for histidine 1.

![Figure 2.8](image)

**Figure 2.8.** Typical Zn$^{2+}$ complex pose with histidine 1 as an example ligand under A) evacuated and B) solvated conditions.

Binding energies under solvated conditions were lower than *in vacuo* as a general trend. This is due to the hydrogen bonding capability of the main stabilizing functional groups (carboxylate and imidazole ring), which detracts from the groups interaction with the respective cations present in the system. The carboxylate interactions with the cations were more conserved than the interactions with the nitrogen of the imidazole ring under solvated conditions. These simulations were conducted with Cu$^{2+}$ and Ca$^{2+}$ to try to assess if there is a degree of specificity for Zn$^{2+}$ chelation over other divalent metal cations present in the brain. *In vacuo*, and to a lesser extent under solvated conditions, Zn$^{2+}$ and Cu$^{2+}$ showed a similar affinity for chelation, while Ca$^{2+}$ did not provide the same degree of stabilization. Therefore, these small molecules are predicted to interact favorably with Zn$^{2+}$ over Ca$^{2+}$. 
The results for the semi-empirical calculations are found in Table 2.2. These results show similar trends to the relative Zn$^{2+}$ binding energies calculated with MM (also found in Table 2.2). Correlation between these two levels of theory lends well to the hypothesis that histidine-based small molecules will be effective at chelating Zn$^{2+}$.

In vacuo and under solved conditions, with MM and semi-empirical calculations, metal:ligand complexes involving histidine 1 or linear dipeptides (compounds 4, 5, and 6) showed the most favorable interactions. Cation complexes with diketopiperazines (7 and 8) showed an intermediate binding affinity, while esters 2 and 3 showed a low potential for the formation of stabilizing interactions, largely due to the loss of the negatively charged carboxylate group. Tables 2.2 and 2.3 show that Zn$^{2+}$ and Cu$^{2+}$ form complexes preferentially over Ca$^{2+}$, which provides in silico evidence for a degree of chelation specificity.

To compare these results to a known Zn$^{2+}$ chelator, EDTA was assessed in the same manner as the compounds in Figure 2.4. The total interaction energy for the EDTA-Zn$^{2+}$ complex was -306.38 kcal mol$^{-1}$ in vacuo and -290.12 kcal mol$^{-1}$ under solvated conditions. This binding energy is substantially larger than that for any assessed small molecule complexed with Zn$^{2+}$ (see Table 2.2 for values). The main stabilizing group was the carboxylate functionalities, which gave a $\Delta E_{ele}$ value of -406.06 kcal mol$^{-1}$. It is unlikely that any histidine-based compound will result in a competitive binding energy in comparison to EDTA. However, such a strong chelator may interrupt physiological processes involving Zn$^{2+}$, such as was hypothesized for the cause of toxicity with TPEN administration mentioned in Section 1.2.5.

### 2.3.2 Results of the Interactions Between N/S-Heterocycles and Divalent Metal Cations

Results for MM and semi-empirical calculations of the change in energy associated with metal:heterocycle complex formation are summarized in Table 2.4. The heterocycles used for these calculations are shown in Figure 2.6. The most favorable binding interaction for all metals resulted from complex formation with imidazole 13, the functional group of the model ligand histidine 1. Interactions between metals and unsaturated heterocycles were more favorable than interactions between metals and comparable saturated heterocycles. Semi-empirical calculations yielded binding energies that echoed the results from MM calculations, however, several of the heterocycles were distorted in the minimization process due to charge-charge repulsions; these
poses were not considered viable and their values are not included in Table 2.4. Several heterocycles form complexes that are energetically similar to complexes containing imidazole, therefore, substitutions of the imidazole ring in histidine 1 with these N/S-heterocycles was explored.

Table 2.4. Values for the change in energy upon complex formation (in vacuo) between N/S-heterocycles and divalent metal cations using both MM (Zn$^{2+}$, Cu$^{2+}$, and Ca$^{2+}$ complexes), and semi-empirical calculations (Zn$^{2+}$ complexes).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Zn$^{2+}$:Ligand complex $\Delta E_T$ (in vacuo)</th>
<th>Cu$^{2+}$:Ligand complex $\Delta E_T$ (in vacuo)</th>
<th>Ca$^{2+}$:Ligand complex $\Delta E_T$ (in vacuo)</th>
<th>Zn$^{2+}$:Ligand complex $\Delta E_T$ (in vacuo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>-0.55</td>
</tr>
<tr>
<td>11</td>
<td>0.00</td>
<td>2.39</td>
<td>-3.20</td>
<td>55.82</td>
</tr>
<tr>
<td>12</td>
<td>1.48</td>
<td>0.35</td>
<td>11.24</td>
<td>molecule distorted</td>
</tr>
<tr>
<td>13</td>
<td>-46.87</td>
<td>-48.43</td>
<td>-33.91</td>
<td>-154.14</td>
</tr>
<tr>
<td>14</td>
<td>0.00</td>
<td>3.99</td>
<td>0.00</td>
<td>91.18</td>
</tr>
<tr>
<td>15</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>molecule distorted</td>
</tr>
<tr>
<td>16</td>
<td>-43.91</td>
<td>-45.60</td>
<td>-30.40</td>
<td>-134.01</td>
</tr>
<tr>
<td>17</td>
<td>-40.80</td>
<td>-41.98</td>
<td>-30.53</td>
<td>-135.74</td>
</tr>
<tr>
<td>18</td>
<td>-35.13</td>
<td>-36.60</td>
<td>-23.57</td>
<td>-136.68</td>
</tr>
<tr>
<td>19</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>-2.79</td>
</tr>
<tr>
<td>20</td>
<td>-39.26</td>
<td>-40.73</td>
<td>-27.33</td>
<td>-143.69</td>
</tr>
<tr>
<td>21</td>
<td>-3.30</td>
<td>-3.30</td>
<td>-3.30</td>
<td>41.81</td>
</tr>
<tr>
<td>22</td>
<td>-3.70</td>
<td>-5.08</td>
<td>5.53</td>
<td>molecule distorted</td>
</tr>
<tr>
<td>23</td>
<td>-33.71</td>
<td>-35.08</td>
<td>-22.71</td>
<td>-128.64</td>
</tr>
<tr>
<td>24</td>
<td>-29.04</td>
<td>-30.25</td>
<td>-19.42</td>
<td>-131.92</td>
</tr>
<tr>
<td>25</td>
<td>-39.95</td>
<td>-41.09</td>
<td>-30.01</td>
<td>-139.10</td>
</tr>
<tr>
<td>26</td>
<td>-21.91</td>
<td>-22.42</td>
<td>-17.97</td>
<td>-123.17</td>
</tr>
<tr>
<td>27</td>
<td>-32.46</td>
<td>-33.44</td>
<td>-23.90</td>
<td>-122.96</td>
</tr>
<tr>
<td>28</td>
<td>-18.79</td>
<td>-19.71</td>
<td>-11.63</td>
<td>-123.17</td>
</tr>
<tr>
<td>29</td>
<td>-17.14</td>
<td>-17.61</td>
<td>-12.92</td>
<td>molecule distorted</td>
</tr>
<tr>
<td>30</td>
<td>-25.43</td>
<td>-26.25</td>
<td>-18.18</td>
<td>molecule distorted</td>
</tr>
<tr>
<td>31</td>
<td>-9.86</td>
<td>-10.12</td>
<td>-7.46</td>
<td>-135.28</td>
</tr>
<tr>
<td>32</td>
<td>-32.18</td>
<td>-33.23</td>
<td>-23.54</td>
<td>-134.94</td>
</tr>
<tr>
<td>33</td>
<td>-35.11</td>
<td>-36.41</td>
<td>-24.40</td>
<td>-140.31</td>
</tr>
<tr>
<td>34</td>
<td>-27.03</td>
<td>-27.73</td>
<td>-20.71</td>
<td>-123.34</td>
</tr>
<tr>
<td>35</td>
<td>-23.60</td>
<td>-24.48</td>
<td>-16.52</td>
<td>-120.04</td>
</tr>
</tbody>
</table>

Results for MM and semi-empirical calculations of the change in energy associated with metal:atypical amino acid complex formation are shown in Table 2.5 (ligands used in simulations shown in Figure 2.7). Similar to complexes discussed in Section 2.2.1, ionic
interactions were the most common stabilizing effects. The carboxylate group chelated cations most commonly, with heterocycles also playing a minor role in stabilizing complexes. As well, interactions between ligands and Zn\(^{2+}\)/Cu\(^{2+}\) were formed favourably over interactions with Ca\(^{2+}\). As previously, solvating the system decreased the interaction energy due to the potential of functional groups to undergo hydrogen bonding. All atypical amino acids assessed performed relatively similarly in their potential to chelate Zn\(^{2+}\), and considering both levels of theory used for these calculations, such metal complexes showed similar binding energetics to histidine 1. Therefore, substitution of the imidazole ring in histidine 1 with varying heterocycles represents a plausible step forward in rationally designing a divalent metal cation chelator.

**Table 2.5.** Values for the change in energy upon complex formation (under solvated conditions) between atypical amino acids and divalent metal cations using both MM (Zn\(^{2+}\), Cu\(^{2+}\), and Ca\(^{2+}\) complexes), and semi-empirical calculations (Zn\(^{2+}\) complexes).

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Molecular Mechanics /kcal mol(^{-1})</th>
<th>Semi-Empirical /kcal mol(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zn(^{2+}):Ligand complex</td>
<td>Cu(^{2+}):Ligand complex</td>
</tr>
<tr>
<td>36</td>
<td>-89.41</td>
<td>-99.32</td>
</tr>
<tr>
<td>37</td>
<td>-80.31</td>
<td>-92.80</td>
</tr>
<tr>
<td>38</td>
<td>-100.29</td>
<td>-108.95</td>
</tr>
<tr>
<td>39</td>
<td>-90.90</td>
<td>-85.56</td>
</tr>
<tr>
<td>40</td>
<td>-81.54</td>
<td>-93.08</td>
</tr>
<tr>
<td>41</td>
<td>-98.50</td>
<td>-91.02</td>
</tr>
<tr>
<td>43</td>
<td>-105.88</td>
<td>-104.73</td>
</tr>
<tr>
<td>44</td>
<td>-82.64</td>
<td>-84.26</td>
</tr>
<tr>
<td>45</td>
<td>-97.42</td>
<td>-96.06</td>
</tr>
</tbody>
</table>

### 2.3.3 Results for the Optimization of 2,5-Diketopiperazine and 2,4-Imidazolidinedione Substitution Patterns for Zn\(^{2+}\) Chelation

Results for MM and semi-empirical calculations of the change in energy associated with Zn\(^{2+}\):diketopiperazine complex formation are shown in Table 2.6, which also includes the substitution patterns sampled. In general, di-substituted molecules provided greater stabilization of Zn\(^{2+}\) complexes than mono-substituted molecules. The most common complex pose was Zn\(^{2+}\) chelated to both the carbonyl group and heterocycles (see Figure 2.9, for example). There are several molecules with higher affinity for Zn\(^{2+}\) chelation than the previously assessed
diketopiperazines 7 and 8 (or 3S,6S and 3S,6R in Table 2.6). In addition, several molecules (3S,3R; 3R,4) form more favorable binding interactions with Zn$^{2+}$ than histidine 1 by semi-empirical results. These results suggest that it is worth exploring 2,5-diketopiperazines with varying substitution patterns for their ability to chelate Zn$^{2+}$.

**Table 2.6.** Values for the change in energy upon complex formation between substituted 2,5-diketopiperazines and Zn$^{2+}$. Substitution positions are indicated in the compound structure.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Substitution Positions</th>
<th>Molecular Mechanics /kcal mol$^{-1}$</th>
<th>Semi-Empirical /kcal mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\Delta E_T$ (in vacuo)</td>
<td>$\Delta E_T$ (solvated)</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>-39.83</td>
<td>-40.01</td>
</tr>
<tr>
<td>3R</td>
<td></td>
<td>-75.47</td>
<td>-41.71</td>
</tr>
<tr>
<td>3S</td>
<td></td>
<td>-80.12</td>
<td>-70.61</td>
</tr>
<tr>
<td>1,4</td>
<td></td>
<td>-83.42</td>
<td>-2.87</td>
</tr>
<tr>
<td>3R, 4</td>
<td></td>
<td>-81.29</td>
<td>-77.56</td>
</tr>
<tr>
<td>3S, 4</td>
<td></td>
<td>-83.61</td>
<td>-76.44</td>
</tr>
<tr>
<td>3S, 3R</td>
<td></td>
<td>-72.23</td>
<td>-67.03</td>
</tr>
<tr>
<td>3S, 6S</td>
<td></td>
<td>-62.10</td>
<td>-41.61</td>
</tr>
<tr>
<td>3S, 6R</td>
<td></td>
<td>-86.17</td>
<td>-46.26</td>
</tr>
</tbody>
</table>

**Figure 2.9.** Common pose assumed by complexes of Zn$^{2+}$ and substituted diketopiperazines, shown with substitution pattern 3S from Table 2.6 as an example.

The relative binding energies for 2,4-imidazolidinediones calculated with MM and semi-empirical methods are found in Table 2.7. As seen with 2,5-diketopiperazines, di-substituted 2,4-imidazolidinediones also provide greater stabilization of Zn$^{2+}$ complexes in comparison to mono-substituted molecules. Again, several molecules (1,5R; 3,5S; 3,5R) form more favorable binding interactions with Zn$^{2+}$ than histidine 1 by semi-empirical methods. This known drug-
like platform with established synthetic routes represents a potential platform for designing molecules to chelate Zn\(^{2+}\).

**Table 2.7.** Values for the change in energy upon complex formation between substituted 2,4-imidazolidinediones and Zn\(^{2+}\). Substitution positions are indicated in the compound structure.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Substitution Positions</th>
<th>Molecular Mechanics /kcal mol(^{-1})</th>
<th>Semi-Empirical /kcal mol(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(\Delta E_T \text{ (in vacuo)})</td>
<td>(\Delta E_T \text{ (solvated)})</td>
</tr>
<tr>
<td>1, 3</td>
<td></td>
<td>-107.07</td>
<td>-36.25</td>
</tr>
<tr>
<td>3, 5R</td>
<td></td>
<td>-78.97</td>
<td>-75.27</td>
</tr>
<tr>
<td>3, 5S</td>
<td></td>
<td>-107.30</td>
<td>-70.06</td>
</tr>
<tr>
<td>1, 5R</td>
<td></td>
<td>-83.05</td>
<td>-34.67</td>
</tr>
<tr>
<td>1, 5S</td>
<td></td>
<td>-80.21</td>
<td>-71.08</td>
</tr>
<tr>
<td>5R, 5S</td>
<td></td>
<td>-64.36</td>
<td>-49.51</td>
</tr>
<tr>
<td>5R</td>
<td></td>
<td>-74.36</td>
<td>-37.99</td>
</tr>
<tr>
<td>5S</td>
<td></td>
<td>-75.19</td>
<td>-69.30</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>-68.49</td>
<td>-44.43</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>-41.33</td>
<td>-34.40</td>
</tr>
</tbody>
</table>

### 2.3.4 Results for the Modeling of Biologically Relevant Zn\(^{2+}\) Binding Sites

A summary of results for active site modeling of proteins containing Zn\(^{2+}\) is found in Table 2.8 for three of the eight proteins analyzed. The Zn\(^{2+}\)-binding site of one of the zinc fingers in the Zif268-DNA complex is shown in Figure 2.10 for a visual representation of active site modeling (Pavletich and Pabo 1991). For the proteins in Table 2.8, cysteine residues were coordinated with Zn\(^{2+}\) through the terminal sulphur atom and histidine residues through their imidazole ring. Zn\(^{2+}\) coordination numbers ranged from 3 to 5, with a coordination number of 4 found most commonly. The average lengths for Zn\(^{2+}\)-histidine and Zn\(^{2+}\)-cysteine residue interactions were 2.04 Å and 2.21 Å, respectively. Angles of interactions were of approximately tetrahedral geometry. In addition, the average interaction energy was lower for Zn\(^{2+}\)-histidine (-8.21 kcal/mol) than it was for Zn\(^{2+}\)-cysteine (-7.88 kcal/mol).

This modeling shows that both sulphur and nitrogen containing side chains are capable of chelating Zn\(^{2+}\), although histidine residues appear to result in slightly more energetically advantageous interactions than cysteine residues. These results suggest that histidine 1 is an appropriate ligand to design Zn\(^{2+}\) chelators around, and also suggests that incorporating cysteine or other sulphur-containing groups may improve chelation ability of such molecules.
Table 2.8. Summary of results for modeling of Zn$^{2+}$ containing proteins.

<table>
<thead>
<tr>
<th>PDB Code</th>
<th>Protein Name</th>
<th>Coordination Number</th>
<th>Active Site Residues</th>
<th>Zn$^{2+}$ Interaction Length /Å</th>
<th>Energy of Interaction /kcal mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2OHX</td>
<td>Alcohol dehydrogenase (horse liver)</td>
<td>4</td>
<td>Cys(97)</td>
<td>2.41</td>
<td>-2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cys(100)</td>
<td>2.34</td>
<td>-2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cys(103)</td>
<td>2.3</td>
<td>-2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cys(111)</td>
<td>2.39</td>
<td>-2.5</td>
</tr>
<tr>
<td>1ZAA</td>
<td>Zif268-DNA complex</td>
<td>4</td>
<td>Cys(7)</td>
<td>2.23</td>
<td>-8.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cys(12)</td>
<td>2.44</td>
<td>-7.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>His(25)</td>
<td>2.14</td>
<td>-4.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>His(29)</td>
<td>1.88</td>
<td>-6.2</td>
</tr>
<tr>
<td>3MV1</td>
<td>Adenosine deaminase</td>
<td>5</td>
<td>His(15)</td>
<td>2.09</td>
<td>-4.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>His(17)</td>
<td>2.12</td>
<td>-20.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>His(214)</td>
<td>2.14</td>
<td>-19.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Asp(295)</td>
<td>2.46</td>
<td>-12.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H$_2$O</td>
<td>2.13</td>
<td>-2.2</td>
</tr>
</tbody>
</table>

**Figure 2.10.** Important residues in A) 2D and B) 3D in the Zif268-DNA complex. Bond distance and energies are shown in A), units are Å and kcal/mol, respectively. In A) purple dashed lines represent ionic interactions and green dashed lines represent sidechain interactions.
CHAPTER 3

3 Synthesis of Histidine-Based Small Molecules as Potential Neuroprotectants for Ischemic Stroke
3.1 Rationale for the Selection of Synthetic Targets

L-Histidine 1 was chosen as a model endogenous ligand from which to design possible Zn\(^{2+}\) chelators for reasons mentioned in Section 1.4.1, including *in vitro* and *in vivo* evidence for neuroprotection. As well, chemical similarity to histidine 1 may make synthesized compounds also substrates for the LNAA transporter and therefore brain penetrant (Hawkins et al. 2006). Analogues to be synthesized were chosen rationally based on their *in silico* chelation potential calculated in Chapter 2 and synthetic accessibility.

A simple esterification of histidine 1 allowed for elucidation of the carboxylate group significance in Zn\(^{2+}\) chelation. *In silico* results, as well as the literature and general chemical knowledge, suggest that esterifying the carboxylate group will diminish interactions with metal cations by decreasing the ionic interactions between the negatively charged carboxylate and divalent cation.

Modification of histidine 1 while maintaining its functional characteristics is possible by synthesizing the dipeptide, therefore, a series of dihistidine compounds were synthesized. Since metal chelation is dependent on the 3D structure of the chelating ligand, several stereoisomers of dihistidine were synthesized. This may also provide insight into any stereospecific chelation effects. D-Stereocenters were also included to increase the drug-like properties of these molecules, as peptides containing D-stereocenters are less likely to undergo enzymatic amide bond cleavage in comparison to peptides containing naturally occurring L-stereocenters.

Peptidomimetics are molecules that have structural elements common to natural peptides but are designed to avoid some of the issues associated with peptides, such as poor stability to proteases and low bioavailability. Peptide cyclization is a common approach in peptidomimetic drug design. Cyclization of peptides dramatically reduces the molecules flexibility and hopefully will hold it in a position favorable for its intended mechanism of action, chelation of Zn\(^{2+}\), in this case. Additionally, by cyclizing dipeptides the charged termini are tied up, which increases lipophilicity and the likelihood that compounds will cross the BBB, essential for their intended application. Therefore, several diketopiperazines, were synthesized; Figure 3.1 shows the general structure of a diketopiperazine.
Imidazolidinediones (structure shown in Figure 3.1), commonly known as hydantoins, are another class of molecules that have been evaluated as peptidomimetics. As seen with diketopiperazines, imidazolidinediones have a rigid backbone that mimics the atoms in a dipeptide backbone unit. By substituting imidazolidinediones with the side chain of histidine 1, it may be possible to synthesize Zn$^{2+}$-chelating molecules. In addition, imidazolidinediones are a privileged drug platform with known CNS activity (Nogrady and Weaver 2005). For these reasons, substituted imidazolidinedione analogues were synthesized.

The side chain of histidine 1 represents a possible functional group for synthetic substitutions. The stability of several N/S-heterocycle:Zn$^{2+}$ complexes was compared to imidazole:Zn$^{2+}$ stability in silico (see Section 2.3.2). Favorable results indicate that substitutions of the imidazole ring in histidine 1 with various N/S-heterocycles are viable synthetic targets. In addition to the in silico results, use of unnatural amino acids is another peptidomimetic approach. As well, due to their structural similarity to histidine 1, it is possible that they will be able to be transported by the LNAA transporter and therefore be brain penetrant.

3.2 A Review of Traditional Synthetic Routes

3.2.1 Synthetic Routes for the Production of Esters

Acid catalyzed esterifications, known as Fischer esterifications, are widely used to form esters from the corresponding carboxylic acid. This general reaction mechanism is shown in Scheme 3.1. Acidic conditions lead to protonation of the carbonyl, rendering a more reactive electrophile. Attack of a nucleophile, an alcohol in this case, leads to formation of a tetrahedral intermediate followed by tautomerization. The reaction is driven towards the ester product by removal of the evolving water, which by Le Châtelier’s principle will drive the reaction towards product formation.
3.2.2 Synthetic Routes for the Production of Dipeptides

Peptide coupling reactions require activation of the C-terminal carboxylic acid of a peptide or amino acid so that nucleophilic attack by a free amine can occur. Carbodiimides, such as dicyclohexylcarbodiimide (DCC), have been and are still commonly used in this activation step. However, these carbodiimides form very reactive activated carboxylic acids, therefore racemization of the reactants is a common problem. Newer generation coupling reagents, such as HBTU, form less reactive intermediates that reduce the risk of racemization.

Following formation of the amide bond mediated by coupling reagents, dipeptides must be deprotected at both the C- and N-termini, with synthetic conditions dependent on the protecting groups. Common N-protecting groups include Boc and Cbz, while common C-protecting groups include methyl and benzyl esters.

3.2.3 Synthetic Routes for the Production of Diketopiperazines

Diketopiperazines (see Figure 3.1 for structure) can be symmetrical, composed of two identical amino acids, or nonsymmetrical, composed of amino acids with differing side chains and/or differing stereochemistry. In the case of symmetrical diketopiperazines, synthesis is straightforward and can be achieved by heating the free amino acid methyl esters causing intermolecular cyclization and release of methanol.

Nonsymmetrical amino acids can also be formed by heating the free amino acid esters, though this strategy will yield a large proportion of unwanted products that are likely difficult to separate due to similar chemical characteristics, and is not commonly employed. Instead, the dipeptide ester is heated under acidic or basic conditions so that the nucleophilic N-terminal
amine can intramolecularly attack the C-terminus, which is followed by subsequent alcohol elimination (Fischer 2003).

Factors such as the rotational barrier of the amide bond, steric hindrance, and side chain interactions can influence the rate of diketopiperazine formation (Fischer 2003). In order for the dipeptide N-terminal amine to attack the C-terminal and close the ring, the amide bond must be in the cis conformation. The rotational energy barrier going from the trans orientation, which is the energetically favorable conformation, to the cis orientation is high due to the double bond character of the amide bond. This barrier must be overcome to achieve cyclization. As well, steric hindrance from the side chain groups may lead to slower rates of cyclization or inhibit cyclization altogether.

3.2.4 Synthetic Routes for the Production of Imidazolidinediones

Imidazolidinedione (see Figure 3.1 for structure) was discovered by Baeyer in 1861. It was isolated as a side product from the hydrogenation of allantoin, hence the common name hydantoin (Ware 1950). Since then, hydantoin chemistry has been widely applied to various systems for diverse applications. Synthesis of 5-substituted 2,4-imidazolidinediones is possible by reaction of α-amino acids with potassium cyanate followed by cyclization under refluxing acidic conditions. This reaction was first introduced by Urech in 1873 and has been applied to a wide variety of α-amino acids and α-amino acid analogues (Ware 1950). Aqueous conditions are employed in this synthetic scheme, making this synthetic route appealing for pharmaceutical applications.

The Bucherer-Bergs reaction is another possible synthetic route used for forming 2,4-imidazolidinediones, shown in Scheme 3.2. This route utilizes an aldehyde or ketone, potassium cyanide, and ammonium carbonate to form 5- or 5,5-substituted 2,4-imidazolidinediones (Ware 1950). The carbonyl compound reacts with potassium cyanide to form a cyanohydrin intermediate, which further reacts with ammonium carbonate to yield the appropriate 2,4-imidazolidinediones.

![Scheme 3.2. General procedure of the Bucherer-Bergs reaction.](image-url)
3.2.5 Synthetic Routes for the Production of Atypical Amino Acids

There are many routes available to synthesize α-amino acids including the Strecker reaction, a modified version of the Gabriel reaction, as well as the Wittig-Horner reaction.

The first step in the Strecker reaction is the reaction of an aldehyde with ammonia in the presence of hydrogen cyanide to form an imine (Wade 2012). Then, a cyanide ion attacks the imine to form a nitrile intermediate, which can be hydrolyzed to form the α-amino acid.

In a modified version of the Gabriel reaction (shown in Scheme 3.3), diethyl phthalimidomalonate is used as a starting material. The malonate is deprotonated under basic conditions and combined with an alkyl halide. This alkylated intermediate then undergoes hydrolysis and decarboxylation to yield the α-amino acid (Wade 2012).

Scheme 3.3. General mechanism for the Gabriel synthesis of α-amino acids.

A literature search for the preparation of α-amino acids containing a heterocyclic side chain group revealed application of the Wittig-Horner reaction to their synthesis (Masquelin et al. 1994). Commercially available Cbz-protected phosphonoglycine can react with α-substituted aldehydes under mild conditions to form the subsequent alkenes, which can then be hydrogenated and subsequently deprotected at the C-terminus to yield the free α-amino acid. Stereospecific hydrogenation catalysts may be used if enantiomerically pure products are desired.

3.3 Synthetic Considerations in the Selection of Target Compounds

As mentioned previously, the compounds synthesized were chosen as targets based on their interaction energy with Zn\(^{2+}\) \textit{in silico} as well as their synthetic accessibility. Synthetic routes from the traditional and literature-based schemes described in Section 3.2 were selected based on
compound availability, ease of synthesis and purification, and successful synthetic experiences by way of discussion with senior chemists in the Weaver laboratory. The procedures utilized for each class of compounds, or each compound in the case of singular analogues, will be discussed below.

3.3.1 Description of Ester Synthesis

Commercially available 2 was used as received from Bachem. This allowed for the elucidation of the carboxylate group importance in Zn$^{2+}$ chelation. The benzyl ester of histidine 1 was synthesized to assess the potential for π-cation interactions between the benzyl moiety and Zn$^{2+}$. Fischer esterification was employed, shown in Scheme 3.4, using a modified constant pressure-dropping funnel filled with anhydrous MgSO$_4$ to trap the evolving water.

![Scheme 3.4. Acid-catalyzed esterification of histidine 1 in BnOH/CHCl$_3$ to form 3 (41% yield).](image)

3.3.2 Description of Dipeptide Synthesis

Peptide coupling was performed using the second generation coupling reagent HBTU activated with Hünig’s base, N,N-diisopropylethylamine (DIPEA). This general procedure is shown in Scheme 3.5. The synthesis of dipeptides involved coupling of a Boc-protected amino acid with an amino acid methyl ester. Following coupling, deprotection involved removal of the C-terminus methyl ester under basic conditions, followed by removal of Boc at the N-terminus under acidic conditions to yield hydrochloride salts.

![Scheme 3.5. General peptide coupling procedure for the synthesis of dipeptides.](image)
The fully protected glycyl-L-histidine dipeptide 49 was synthesized in order to produce the mono-substituted diketopiperazine. Table 3.1 summarizes the dipeptides synthesized.

**Table 3.1.** Summary product table for synthesized dipeptides.

<table>
<thead>
<tr>
<th>R'</th>
<th>R</th>
<th>x1</th>
<th>x2</th>
<th>Compound</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="R1" alt="Diagram" /></td>
<td><img src="R2" alt="Diagram" /></td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>63%</td>
</tr>
<tr>
<td><img src="R1" alt="Diagram" /></td>
<td><img src="R2" alt="Diagram" /></td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>43%</td>
</tr>
<tr>
<td><img src="R1" alt="Diagram" /></td>
<td><img src="R2" alt="Diagram" /></td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>57%</td>
</tr>
<tr>
<td><img src="R1" alt="Diagram" /></td>
<td><img src="R2" alt="Diagram" /></td>
<td>1</td>
<td>2</td>
<td>49</td>
<td>98%*</td>
</tr>
</tbody>
</table>

*This yield represents the 1st reaction in Scheme 3.5 only.

### 3.3.3 Description of Diketopiperazine Syntheses

Nonsymmetrical diketopiperazines were successfully synthesized through intramolecular cyclization of the appropriate dipeptide esters, shown in Scheme 3.6.

![Scheme 3.6](Diagram)

**Scheme 3.6.** General procedure for intramolecular dipeptide cyclization.

The first step in this synthetic route involved deprotection of the N-terminus of Boc-protected dipeptide methyl esters under acidic conditions, followed by refluxing in MeOH under basic conditions to cause ring closure. Synthetic results for this cyclization reaction are summarized in Table 3.2.
Table 3.2. Summary product table for synthesized non-symmetric diketopiperazines.

<table>
<thead>
<tr>
<th>R’</th>
<th>R</th>
<th>x</th>
<th>Compound</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td></td>
<td>3</td>
<td>8</td>
<td>40%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>55</td>
<td>18%</td>
</tr>
</tbody>
</table>

Symmetric diketopiperazines were synthesized through the more simple intermolecular cyclization procedure, as described in Section 3.2.3. Scheme 3.7 shows synthesis of 7 from 2 eq. of 2.

Scheme 3.7. Synthesis of symmetric diketopiperazine 7 via intermolecular cyclization (yield 22%).

In addition to cyclo(L-histidyl-L-histidyl) 7, cyclo(L-methionyl-L-methionyl) 57 was synthesized to evaluate the chelating potential of a diketopiperazine with sulphur-containing side chains, as Zn$^{2+}$ binding sites of metalloproteins commonly contain residues with sulphur (Laitaoja, Valjakka, and Jänis 2013). L-Methionine was first methylated at the C-terminal to form 56 by refluxing in methanolic HCl, formed by addition of acetyl chloride to MeOH at 0°C (see Scheme 3.8). Then, 56 was cyclized intermolecularly under basic conditions to form 57.

Scheme 3.8. Methylation procedure of methionine followed by intermolecular cyclization (overall yield 4.8%).

In silico simulations predict that di-substituted diketopiperazines are better chelators of Zn$^{2+}$ than mono-substituted diketopiperazines. The 3,6-substitution pattern (see Figure 3.1 for substitution
positions) of 2,5-diketopiperazine are accessed through dipeptide cyclization and intermolecular cyclization of amino acid methyl esters described previously. Substitution at the 1,4-positions showed promising Zn$^{2+}$-chelation potential in silico, therefore this compound was selected to be synthesized and tested experimentally. This compound was synthesized by N,N-alkylation of glycine anhydride using sodium hydride and 59 (synthesized via Scheme 3.9) as the electrophile. Scheme 3.10 shows the reaction procedure utilized for the alkylation.

![Scheme 3.9](image)

**Scheme 3.9.** Trityl protection and chlorination of 4(5)-(hydroxymethyl)imidazole to form 59 (overall yield 96%).

![Scheme 3.10](image)

**Scheme 3.10.** Procedure for N,N-alkylation of 2,4-diketopiperazine (overall yield 21%).

The above alkylation reaction was first attempted using 4-(bromomethyl)-1-Boc-1H-imidazole rather than 59 in step 2. However, upon the addition of this bromide compound to the NaH and glycine anhydride mixture, a color change from pale yellow to dark brown was immediately noted. This evidence, coupled with TLC analysis indicated that the reactive bromide electrophile was decomposing. To combat this issue, the more stable alkyl chloride was used with N-trityl protection, which also allowed for reaction monitoring by UV light. When the reaction shown in Scheme 3.10 was attempted using 59, the reaction proceeded without electrophile decomposition (no color change noted). The trityl group was removed by brief stirring in 40% trifluoroacetic acid (TFA) in DCM (v/v) using triethylsilane as a carbocation trap.

### 3.3.4 Description of Imidazolidinedione Syntheses

Imidazolidinediones substituted at the 5-position were synthesized using the Urech reaction described in Section 3.2.4. Addition of potassium cyantate to the appropriate α-amino acid was
followed by intramolecular cyclization under acidic conditions (Scheme 3.11). Following completion of the cyclization reaction, the product mixture was basified to an appropriate pH to allow for recrystallization of the free base in water. Compounds synthesized by the Urech method are summarized in Table 3.3.

**Scheme 3.11.** Application of the Urech reaction to the synthesis of 5-substituted 2,4-imidazolidinediones.

**Table 3.3.** Summary product table for synthesized 5-substituted 2,4-imidazolidinediones.

<table>
<thead>
<tr>
<th>R</th>
<th>Compound</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>62</td>
<td>97%</td>
</tr>
<tr>
<td>N</td>
<td>63</td>
<td>91%</td>
</tr>
</tbody>
</table>

Various heterocycles, as directed by *in silico* simulations from Section 2.3.2, were used as substrates for substitution of 2,4-imidazolidinedione at the 3-position. The acidity of the proton at the 3-position (α to two carbonyl groups) was exploited as a way to deprotonate selectively over the 1-position (refer to Figure 3.1 for atom numbering). An alkyl halide was then added to the reaction mixture to allow for N-alkylation. Deprotonation was first attempted using the inorganic base K$_2$CO$_3$, however, this resulted in a mixture of approximately 1:1 mono-:di-substituted products (shown in Scheme 3.12). It is likely that the poor solubility of the starting material relative to the mono-substituted product lead to further substitution forming the di-substituted product. The mono- and di-substituted products were inseparable by silica-gel chromatography, therefore this synthetic route was not viable.

**Scheme 3.12.** Attempted procedure for the synthesis of 3-substituted 2,4-imidazolidinediones.
To overcome this issue, an organic base (DBU) was utilized, which resulted in a homogenous reaction mixture and formation of only the mono-substituted product, as shown in Scheme 3.13. Compounds synthesized by this route are summarized in Table 3.4.

Scheme 3.13. General procedure for the synthesis of 3-substituted 2,4-imidazolidinediones.

Table 3.4. Summary product table for synthesized 3-substituted 2,4-imidazolidinediones.

<table>
<thead>
<tr>
<th>R</th>
<th>X</th>
<th>Compound</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Br</td>
<td>66</td>
<td>35%</td>
</tr>
<tr>
<td></td>
<td>Br</td>
<td>67</td>
<td>57%</td>
</tr>
<tr>
<td></td>
<td>Br</td>
<td>68</td>
<td>57%</td>
</tr>
<tr>
<td></td>
<td>Cl</td>
<td>69</td>
<td>47%</td>
</tr>
</tbody>
</table>

A slight modification was required for substitution with imidazole, reaction shown in Scheme 3.14. Due to the potential for an irreversible self-reaction between 4-(chloromethyl)-1H-imidazole, the imidazole ring was protected using trityl chloride prior to use. Detritylation of 70 in 40% TFA in DCM allowed for isolation of the TFA salt 71, shown in Scheme 3.14.

Scheme 3.14. Modified procedure for the synthesis of 3-substituted 2,4-imidazolidinediones with imidazole (overall yield 11%).
Di-substituted 2,4-imidazolidinediones were favored to interact with Zn\(^{2+}\) over mono-substituted 2,4-imidazolidinediones, therefore a 3,5-substituted 2,4-imidazolidinedione was synthesized. Using 63 as the starting material, Scheme 3.15 was used to synthesize 75. Purification with preparatory HPLC originally yielded the TFA salt 74, which was highly hygroscopic. The TFA salt was exchanged with hydrochloride using chloride activated Amberlite resin to yield 75.

![Scheme 3.15](image)

**Scheme 3.15.** Procedure for the synthesis of 75 (overall yield 8.2%)  

### 3.3.5 Description of Atypical Amino Acid Synthesis

Due to the *in silico* stability of several heterocycle:Zn\(^{2+}\) complexes in comparison to the imidazole:Zn\(^{2+}\) complex, substitution of the imidazole ring in histidine 1 with pyridine was investigated. These molecules were accessed via the Wittig-Horner reaction shown in Scheme 3.16; Table 3.5 summarizes the atypical amino acids synthesized by this mechanism.

![Scheme 3.16](image)

**Scheme 3.16.** General procedure for the synthesis of atypical amino acids.
Table 3.5. Summary product table for synthesized atypical amino acids.

<table>
<thead>
<tr>
<th>R</th>
<th>Compound</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>42</td>
<td>29%</td>
</tr>
<tr>
<td>![Image]</td>
<td>43</td>
<td>16%</td>
</tr>
<tr>
<td>![Image]</td>
<td>44</td>
<td>7.4%</td>
</tr>
</tbody>
</table>

3.4 Experimental Details

3.4.1 General Materials and Methods

All chemicals were received from Alfa Aesar, Amresco, Bachem, BDH Chemicals, Chem-Impex International Inc., Combi-Blocks or Sigma-Aldrich and used without further purification. Solvents for synthesis purposes were used at General Purpose Reagent grade. Dry solvents were purchased from EMD Millipore. Analytical TLC was performed using Merck 60 F254 silica gel plates. TLC visualization was by UV light (254 nm) and staining with potassium permanganate or ninhydrin solution. Chromatographic columns were run manually with 40-63 µm silica or using a CombiFlash Rf purification system from Teledyne Isco with RediSep Rf silica columns. Preparatory HPLC was performed with a Gilson preparative HPLC system fitted with a C\textsubscript{18} column.

All compounds were pure by TLC analysis and were characterized by NMR and mass spectrometry. NMR spectra (\textsuperscript{1}H, \textsuperscript{13}C, and \textsuperscript{19}F) were recorded on a Bruker 400 MHz spectrometer. Deuterated solvents for NMR experiments were purchased from Sigma-Aldrich. NMR data was processed using Bruker TOPSPIN software or MestReNova-LITE. HRMS spectra were measured on an AB Sciex QStarXL mass spectrometer equipped with an ESI ion source or a JEOL AccuTOF model JMS-T1000LC mass spectrometer equipped with a DART ion source. Melting points were determined using a Mel-Temp II capillary apparatus and are uncorrected.
3.4.2 Procedure for the synthesis of L-histidine benzyl ester (3)

To a stirred solution of benzyl alcohol (13 mL) and chloroform (15 mL), histidine \( \text{I} \) (2.00 g, 12.8 mmol) and para-toluenesulphonic acid (5.32 g, 30.9 mmol) were added. The reaction flask was topped with a constant pressure drop funnel filled with anhydrous MgSO\(_4\), modified to trap the evolving water, and refluxed at 90°C until the reaction went to completion. After cooling to RT the mixture was triturated with diethyl ether (50 mL) to produce a white solid. The solution was decanted and the precipitate washed with ether (3 x 50 mL) to remove excess benzyl alcohol. The solid was then treated with 50 mL of 50% by weight K\(_2\)CO\(_3\) solution, extracted with EtOAc (3 x 50 mL), dried over anhydrous MgSO\(_4\), and concentrated in vacuo to yield 3 (1.28 g, 41% yield) as a brown solid. M.p. 152-160°C (dec.), lit. 138-146°C (dec.) (Jones and Wood 1986); \( \delta_H \) (400 MHz, CDCl\(_3\)): 2.87 (dd, 1H, J\(_{14.70}\), 7.86, CH\(_2\)), 3.08 (dd, 1H, J\(_{14.68}\), 4.39, CH\(_2\)), 3.81 (dd, 2H, J 7.66, 4.43, CH), 5.13 (s, 2H, CH\(_2\)), 6.73 (s, 1H, CH imidazole), 7.32 (m, 5H, 5 x CH phenyl), 7.49 (s, 1H, CH imidazole).

3.4.3 Synthesis of Dipeptides

3.4.3.1 General procedure for the coupling of Boc-protected amino acids with amino acid methyl esters

A round bottom flask was charged with an amino acid methyl ester (3.01 mmol) under inert atmosphere. Anhydrous DMF (5 mL) and DIPEA (9.6 mmol, 3.2 equiv) were added and the mixture stirred for 10 minutes. Subsequently, a Boc-protected amino acid (3.00 mmol, 1.0 equiv) and HBTU (3.6 mmol, 1.2 equiv) were added to the reaction vessel and stirred for 24 hours at RT. The crude product was extracted with a mixture of 80:20 v/v DCM:IPA (3 x 30 mL), washed with brine (50 mL), concentrated in vacuo, and purified by silica-gel chromatography using gradient elution to afford Boc-protected dipeptide methyl esters.

\( N\)-Boc-L-histidyl-L-histidine methyl ester (46)

White solid (0.82 g, 67% yield); chromatography conditions EtOAc:MeOH:NH\(_3\)OH (90:10:0.1 to 85:15:0.1); \( \delta_H \) (400 MHz, d\(_6\)-DMSO): 1.34 (s, 9H, 3 CH\(_3\)), 2.76 (m, 4H, 2 CH\(_2\)), 3.56 (s, 3H, CH\(_3\)), 4.14 (m, 1H, CH), 4.47 (m, 1H, CH), 6.73 (broad s, 1H, CH...
imidazole), 6.80 (broad s, 1H, CH imidazole), 6.91 (d, 1H, J 8.27, NH amide), 7.51 (s, 2H, 2 CH imidazole), 8.21 (d, 1H, J 7.76, NH amide), 11.81 (broad s, 2H, 2 NH imidazole).

**N-Boc-L-histidyl-D-histidine methyl ester (47)**

White solid (0.63 g, 52% yield); chromatography conditions DCM:MeOH:NH$_3$OH (90:10:0.1 to 85:15:0.1); $\delta$$_H$ (400 MHz, d$_6$-DMSO): 1.34 (s, 9H, 3 CH$_3$), 2.74 (dd, 1H, J 15.04, 8.27, CH$_2$), 2.81 (dd, 1H, J 14.95, 5.05, CH$_2$), 2.90 (m, 2H, CH$_2$), 3.59 (s, 3H, CH$_3$), 4.18 (m, 1H, CH), 4.49 (m, 1H, CH), 6.77 (s, 1H, CH imidazole), 6.85 (m, 2H, CH imidazole and NH amide), 7.67 (s, 1H, CH imidazole), 7.75 (s, 1H, CH imidazole), 8.27 (d, 1H, J 7.62, NH amide).

**N-Boc-D-histidyl-L-histidine methyl ester (48)**

White solid (1.06 g, 66% yield); chromatography conditions DCM:Hex:MeOH:NH$_3$OH (75:25:5:0.1 to 75:25:10:0.1); $\delta$$_H$ (400 MHz, d$_6$-DMSO): 1.34 (s, 9H, 3 CH$_3$), 2.72 (m, 1H, CH$_2$), 2.80 (m, 1H, CH$_2$), 2.89 (m, 2H, CH$_2$), 3.59 (s, 3H, CH$_3$), 4.17 (m, 1H, CH), 4.48 (m, 1H, CH), 6.72 (s, 1H, CH imidazole), 6.81 (s, 1H, CH imidazole), 6.85 (d, 1H, J 8.18, NH amide), 7.60 (s, 1H, CH imidazole), 7.64 (s, 1H, CH imidazole), 8.25 (d, 1H, J 7.55, NH amide).

**N-Boc-glycyl-L-histidine methyl ester (49)**

White solid (1.54 g, 98% yield); chromatography conditions EtOAc:Hex:MeOH:NH$_3$OH (75:15:7:0.1 to 75:15:10:0.1); $\delta$$_H$ (400 MHz, d$_4$-MeOD): 3.04 (dd, 1H, J 14.80, 7.89, CH$_2$), 3.13 (dd, 1H, J 15.03, 5.41, CH$_2$), 3.71 (m, 5H, CH$_2$ and CH$_3$), 4.70 (t, 1H, J 6.66, CH), 6.89 (s, 1H, CH imidazole), 7.61 (s, 1H, CH imidazole).

3.4.3.2 General procedure for the demethylation of Boc-protected dipeptide methyl esters

To a solution of NaOH (8.80 mmol, 5 equiv) in distilled water (10 mL) at 0°C, a Boc-protected dipeptide methyl ester (1.76 mmol) was added and stirred for 1 hour at the same temperature.
The mixture was then acidified to pH 2 with 2 N HCl (~5 mL), concentrated in vacuo, and extracted with IPA (100 mL). Following filtration, the IPA was concentrated in vacuo to give a Boc-protected dipeptide hydrochloride salt.

**N-Boc-L-histidyl-L-histidine dihydrochloride (50)**

![Chemical structure](image1)

White solid (0.77 g, 94%); $\delta_H$ (400 MHz, d$_6$-DMSO): 1.32 (s, 9H, 3 CH$_3$), 2.86 (dd, 1H, J 15.23, 9.30, CH$_2$), 3.03 (m, 2H, CH$_2$), 3.16 (dd, 1H, J 15.53, 4.63, CH$_2$), 4.27 (m, 1H, CH), 4.52 (m, 1H, CH), 7.07 (d, 1H, J 7.97, NH amide), 7.26 (s, 1H, CH imidazole), 7.36 (s, 1H, CH imidazole), 8.46 (d, 1H, J 7.44, NH amide), 8.83 (s, 1H, CH imidazole), 8.85 (s, 1H, CH imidazole), 14.004 (broad s, 4H, 4 NH imidazole).

**N-Boc-L-histidyl-D-histidine dihydrochloride (51)**

![Chemical structure](image2)

White solid (0.73 g, quantitative yield); $\delta_H$ (400 MHz, D$_2$O): 1.22 (s, 9H, 3 CH$_3$), 2.92 (dd, 1H, J 15.43, 8.99, CH$_2$), 3.00 (dd, 1H, J 15.35, 8.87, CH$_2$), 3.12 (dd, 1H, J 15.49, 5.18, CH$_2$), 3.19 (dd, 1H, J 15.78, 5.13, CH$_2$), 4.21 (dd, 1H, J 8.91, 5.28, CH), 4.39 (dd, 1H, J 8.81, 4.92, CH), 7.09 (s, 1H, CH imidazole), 7.18 (s, 1H, CH imidazole), 8.47 (s, 1H, CH imidazole), 8.49 (s, 1H, CH imidazole).

**N-Boc-D-histidyl-L-histidine dihydrochloride (52)**

![Chemical structure](image3)

White solid (0.81 g, 87% yield); $\delta_H$ (400 MHz, d$_6$-DMSO): 1.32 (s, 9H, 3 CH$_3$), 2.94 (m, 4H, 2 CH$_2$), 4.25 (dd, 1H, J 13.35, 8.20, CH), 4.46 (dd, 1H, J 12.46, 7.56, CH$_2$), 6.89 (d, 1H, J 8.32, NH amide), 7.08 (s, 1H, CH imidazole), 7.12 (s, 1H, CH imidazole), 8.36 (d, 1H, J 7.79, NH amide), 8.40 (s, 1H, CH imidazole), 8.50 (s, 1H, CH imidazole), 10.16 (broad s, 4H, 4 NH imidazole).
3.4.3.3 General procedure for the removal of Boc from Boc-protected dipeptides

To a 4 N HCl solution (~4.0 mL, 10 equiv) at 0°C, a Boc-protected dipeptide (1.64 mmol) was added and stirred for 1 hour at the same temperature. The mixture was concentrated under reduced pressure to yield a dipeptide hydrochloride salt.

**L-histidyl-L-histidine trihydrochloride (4)**

Hygroscopic white solid (0.68 g, quantitative yield); **M.p.** 230-232°C; \(\delta_H\) (400 MHz, \(d_6\)-DMSO): 3.21 (m, 4H, 2 CH\(_2\)), 4.34 (broad s, 1H, CH), 4.60 (m, 1H, CH), 7.49 (s, 1H, CH imidazole), 7.52 (s, 1H, CH imidazole), 8.66 (broad s, 3H, NH\(_3\)), 9.06 (s, 2H, 2 CH imidazole), 9.33 (d, 1H, J 7.6, NH amide), 14.60 (broad s, 4H, 4 NH imidazole); \(\delta_C\) (100 MHz, \(D_2\)O): 25.99, 26.10, 51.94, 52.27, 117.16, 118.61, 125.42, 128.31, 133.51, 134.34, 167.81, 173.17; **HRMS** (ESI\(^{+}\)): mass [M+H] calc’d for C\(_{12}\)H\(_{17}\)N\(_6\)O\(_3\) 293.1362 Da, measured 293.1364 Da.

**L-histidyl-D-histidine trihydrochloride (5)**

Hygroscopic white solid (0.54 g, 83% yield); **M.p.** 218-221°C; \(\delta_H\) (400 MHz, \(d_6\)-DMSO): 3.18 (m, 4H, 2 CH\(_2\)), 4.29 (broad s, 1H, CH), 4.54 (m, 1H, CH), 7.40 (s, 1H, CH imidazole), 7.48 (s, 1H, CH imidazole), 8.61 (broad s, 3H, NH\(_3\)), 9.04 (s, 1H, CH imidazole), 9.06 (s, 1H, CH imidazole), 9.35 (d, 1H, J 7.75, NH amide), 14.61 (m, 4H, 4 NH, imidazole); \(\delta_C\) (100 MHz, \(D_2\)O): 25.89, 26.03, 51.86, 52.30, 117.04, 118.31, 125.74, 128.63, 133.61, 134.41, 168.05, 173.04; **HRMS** (ESI\(^{+}\)): mass [M+H] calc’d for C\(_{12}\)H\(_{17}\)N\(_6\)O\(_3\) 293.1362 Da, measured 293.1370 Da.

**D-histidyl-L-histidine trihydrochloride (6)**

Hygroscopic white solid (0.71 g, quantitative yield); **M.p.** 226-228°C; \(\delta_H\) (400 MHz, \(d_6\)-DMSO): 3.18 (m, 4H, 2 CH\(_2\)), 4.28 (broad s, 1H, CH), 4.53 (m, 1H, CH), 7.40 (s, 1H, CH imidazole), 7.47 (s, 1H, CH imidazole), 8.63 (broad s, 3H, NH\(_3\)), 9.04 (s, 1H, CH...
imidazole), 9.06 (s, 1H, CH imidazole), 9.36 (d, 1H, J 7.73, NH amide), 14.67 (broad m, 4H, 4 NH imidazole); δC (100 MHz, D2O): 25.92, 26.03, 51.86, 52.40, 117.01, 118.30, 125.75, 128.68, 133.60, 134.40, 168.03, 173.17; HRMS (ESI+): mass [M+H] calc’d for C12H17N6O3 293.1362 Da, measured 293.1365 Da.

3.4.4 Synthesis of Non-Symmetrical Diketopiperazines

3.4.4.1 General procedure for the removal of Boc from Boc-protected dipeptide methyl esters

To a 4 N HCl solution (5.9 mL, 10 equiv) at 0°C, a Boc-protected dipeptide methyl ester (2.36 mmol) was added and stirred for 1 hour at the same temperature. The mixture was concentrated in vacuo to give a dipeptide methyl ester hydrochloride salt.

**L-histidyl-D-histidine methyl ester trihydrochloride (53)**

![L-histidyl-D-histidine methyl ester trihydrochloride](image)

White solid (0.97 g, quantitative yield); δH (400 MHz, D2O): 3.06 (m, 1H, CH), 3.22 (m, 4H, 2 CH2), 3.65 (s, 3H, CH3), 4.22 (m, 1H, CH), 7.17 (s, 1H, CH imidazole), 7.25 (s, 1H, CH imidazole), 8.53 (d, 1H, J 1.29, CH imidazole), 8.59 (d, 1H, J 1.28, CH imidazole).

**Glycyl-L-histidine methyl ester dihydrochloride (54)**

![Glycyl-L-histidine methyl ester dihydrochloride](image)

White solid (1.40 g, quantitative yield).

3.4.4.2 General procedure for intramolecular cyclization of dipeptide methyl esters

To a solution of TEA (7.18 mmol, 3.5 equiv) in MeOH (10 mL), a dipeptide methyl ester (2.05 mmol) was added and stirred under refluxing conditions for 24 hours. The precipitant was filtered and recrystallized in distilled water (30 mL) to afford a 3,6-substituted 2,5-diketopiperazine.
cyclo(L-Histidyl-D-histidyl) (8)

\[
\text{White solid (0.23 g, 40\% yield); M.p. > 230^\circ C (dec., lit. >280^\circ C (Benedetti et al. 1988)); } \\
\delta_H (400 MHz, d_6-DMSO): 2.83 (m, 2H, CH_2), 2.89 (dd, 2H, J 14.32, 3.82, CH_2), 3.65 (s, 2 H, 2 x CH), \\
6.78 (s, 2H, 2 NH amide), 7.53 (s, 2H, 2 CH imidazole), 7.98 (s, 2H, 2 CH imidazole), 11.83 (s, 2H, 2 NH imidazole); } \\
\text{HRMS (ESI+): mass [M+H] calc’d for C_{12}H_{15}N_6O_2 275.1256 Da, measured 275.1264 Da.}
\]

cyclo(Glycyl-L-histidyl) (55)

\[
\text{White solid (0.16 g, 18\% yield); M.p. 200-205^\circ C (dec., lit. 230-235^\circ C (dec.) (Brunetti et al. 2013)); } \\
\delta_H (400 MHz, d_5-DMSO): \\
2.90 (d, 2H, J 3.79, CH_2), 3.28 (d, 1H, J 17.48, CH_2), 3.54 (dd, 1H, J 17.65, 2.32, CH_2), 3.97 (broad t, 1H, CH), 6.80 (s, 1H, CH imidazole), 7.56 (s, 1H, CH imidazole), 7.93 (s, 1H, NH amide), \\
8.04 (s, 1H, NH amide), 11.90 (broad s, 1H, NH imidazole); } \\
\text{HRMS (ESI+): mass [M+H] calc’d for C_8H_11N_4O_2 195.08820 Da, measured 195.08876 Da.}
\]

3.4.5 Synthesis of Symmetrical Diketopiperazines

3.4.5.1 Procedure for the methylation of L-methionine (56)

To a round bottom flask under inert atmosphere, MeOH (50 mL) was added and cooled to 0\(^\circ\)C. Acetyl chloride (2.13 mL, 30 mmol) was added to the flask dropwise and the solution warmed to RT. The addition of L-methionine (3.73 g, 25 mmol) was followed by refluxing at 75\(^\circ\)C for 24 hours. The solution was then cooled to RT and concentrated until approximately half of the MeOH was remaining, maintaining the product in solution. The crude product mixture was triturated with diethyl ether (100 mL) and 56 (4.80 g, 96\% yield) was collected as a white solid; \delta_H (400 MHz, D_2O): 2.00 (s, 3H, CH_3), 2.10 (m, 1H, CH_2), 2.20 (m, 1H, CH_2), 2.57 (t, 2H, J 7.17, CH_2), 3.74 (s, 3H, CH_3), 4.20 (t, 1H, J 6.35, CH).
3.4.5.2 General procedure for the intermolecular cyclization of amino acid methyl esters

To a solution of NaOH (79.7 mmol, 2 equiv) in MeOH (50 mL), an amino acid methyl ester dihydrochloride (40.0 mmol) was added and the solution was filtered. The filtrate was heated at 80°C for 3 hours in vacuo, affording an off-white solid. Recrystallization in distilled water (10 mL) yielded a 3,6-substituted 2,5-diketopiperazine.

cyclo(L-Histidyl-L-histidyl) (7)

White solid (0.22 g, 22% yield); M.p. > 260°C, lit. 290-295°C (dec.) (Jackson et al. 1988); δH (400 MHz, d6-DMSO): 2.43 (dd, 2H, J 14.77, 7.75, CH2), 2.87 (dd, 2H, J 14.67, 3.71, CH2), 4.00 (m, 2H, 2 CH), 6.79 (s, 2H, 2 NH amide), 7.58 (s, 2H, 2 CH imidazole), 7.95 (s, 2H, 2 CH imidazole); HRMS (ESI⁺): mass [M+H] calc’d for C12H13N6O2 275.1256 Da, measured 275.1260 Da.

cyclo(L-Methionyl-L-methionyl) (57)

White solid (0.28 g, 5% yield); M.p. 229-231°C, lit. 235-236°C (Kobler, Haeussner, and Weckbecker 2011); δH (400 MHz, d6-DMSO): 1.92 (m, 4H, 2 CH2), 2.03 (s, 6H, 2 CH3), 2.52 (m, 4H, 2 CH2), 3.95 (t, 2H, J 5.92, 2 CH), 8.24 (s, 2H, 2 NH); HRMS (DART-TOF⁺): mass [M+H] calc’d for C10H19N2O2S2 263.08879 Da, measured 263.08914 Da.

3.4.6 Synthesis of N,N-Substituted Diketopiperazone

(1-trityl-1H-imidazol-4-yl)methanol (58)

To a stirred solution of 4-(hydroxymethyl)-imidazole (1.01g, 10.30 mmol) in anhydrous DMF (5 mL) and triethylamine (2.1 mL, 15.07 mmol), was added trityl chloride (3.06 g, 10.98 mmol). The mixture was stirred for 18 hours at RT. The precipitant was washed with distilled water and dried under vacuum to yield 58 (3.50 g, quantitative yield) as a white solid; δH (400 MHz, CDCl3): 4.58 (s, 2H, CH2), 6.79 (s, 1H, CH imidazole), 7.15 (m, 6H, 6 x CH benzyl), 7.34 (m, 9H, 9 x CH benzyl), 7.44 (s, 1H, CH imidazole).
4-(chloromethyl)-1-trityl-1H-imidazole (59)

A stirred solution of 58 (2.00 g, 5.86 mmol) in DCM (25 mL) was cooled to 0°C. Thionyl chloride (0.86 mL, 11.79 mmol) was added dropwise and the mixture was warmed to RT over 2 hours. The crude product was extracted with DCM (3 x 20 mL) from saturated Na$_2$CO$_3$ (20 mL), dried over anhydrous MgSO$_4$, and concentrated in vacuo to yield 59 (1.53 g, 96% yield) as a white solid; $\delta_H$ (400 MHz, CDCl$_3$): 4.57 (s, 2H, CH$_2$), 6.87 (s, 1H, CH imidazole), 7.14 (m, 6H, 6 x CH benzyl), 7.36 (m, 9H, 9 x CH benzyl), 7.43 (s, 1H, CH imidazole).

1,4-bis((1-trityl-1H-imidazol-4-yl)methyl)piperazine-2,5-dione (60)

A reaction flask was charged with glycine anhydride (0.22 g, 1.93 mmol) and NaH (0.17 g, 4.25 mmol) as a 60% dispersion in mineral oil, evacuated, and placed under inert atmosphere. Anhydrous DMF (5 mL) was added and the reaction mixture was stirred vigourously for 1 hour. 59 (1.53 g, 4.26 mmol) was dissolved in anhydrous DMF (5 mL) and transferred to the NaH solution dropwise. The reaction mixture was stirred for 3 hours at RT, heated to 60°C and stirred for a further 18 hours. The crude product mixture was poured into distilled water (150 mL) and 60 (1.27 g, 39% yield) was collected by filtration as a pale yellow solid; $\delta_H$ (400 MHz, CDCl$_3$): 4.03 (s, 4H, 2 x CH$_2$), 4.52 (s, 4H, 2 x CH$_2$), 6.81 (d, 2H, J 0.96, 2 x CH imidazole), 7.12 (m, 12H, 12 x CH benzyl), 7.34 (m, 18H, 18 x CH benzyl), 7.37 (d, 2H, J 1.26, 2 x CH imidazole).

1,4-bis((1H-imidazol-4-yl)methyl)piperazine-2,5-dione bis(2,2,2-trifluoroacetate) (61)

A stirred solution of 60 (1.26 g, 1.66 mmol) in DCM (10 mL) was cooled to 0°C. Triethylsilane (0.53 mL, 3.32 mmol) was added dropwise, followed by the dropwise addition of TFA (6.0 mL). The mixture was stirred at 0°C for 10 minutes and warmed to RT over 1 hour. The crude product mixture was concentrated in vacuo, and precipitated with EtOAc (50 mL). The precipitate was subsequently filtered, washed with EtOAc (10 mL), and lyophilized from distilled water (5 mL) and ACN (2 mL) to yield 61 (0.45 g, 54% yield) as a pale yellow solid; M.p. 179-182°C; $\delta_H$ (400 MHz, D$_2$O): 4.03 (s, 4H, 2 CH$_2$), 4.57 (s, 4H, 2
CH<sub>2</sub>, 7.37 (s, 2H, 2 CH imidazole), 8.53 (s, 2H, 2 CH imidazole); δ<sub>C</sub> (100 MHz, D<sub>2</sub>O): 39.39, 49.08, 118.27, 126.92, 134.25, 165.54; HRMS (ESI<sup>+</sup>): mass [M+H] calc’d for C<sub>12</sub>H<sub>15</sub>N<sub>6</sub>O<sub>2</sub> 275.1256 Da, measured 275.1260 Da.

3.4.7 Synthesis of Substituted 2,4-Imidazolidinediones

3.4.7.1 General procedure for the synthesis of 5-substituted 2,4-imidazolidinediones

An amino acid (10.12 mmol) and potassium cyanate (12.08 mmol, 1.2 equiv) were dissolved in distilled water (6 mL) and stirred at 70°C for 1.5 hours. A solution of 6 N HCl (36 mmol, 3.5 equiv) was then added and the reaction was refluxed at 120°C for a further 4 hours. The crude product mixture was allowed to cool to RT and basified to an appropriate pH to allow for recrystallization of the free 5-substituted 2,4-imidazolidinedione in water.

**Hydantoin (62)**

![Hydantoin structure](image)

White solid (0.98 g, 97% yield); δ<sub>H</sub> (400 MHz, d<sub>6</sub>-DMSO): 3.84 (s, 2H, CH<sub>2</sub>), 7.72 (s, 1H, NH), 10.62 (s, 1H, NH).

**5-((1H-imidazol-4-yl)methyl)imidazolidine-2,4-dione (63)**

![5-((1H-imidazol-4-yl)methyl)imidazolidine-2,4-dione structure](image)

White solid (1.62 g, 91% yield); M.p. 215-218°C; δ<sub>H</sub> (400 MHz, d<sub>6</sub>-DMSO): 2.77 (dd, 1H, J 14.84, 6.68, CH<sub>2</sub>), 2.91 (dd, 1H, J 14.91, 4.35, CH<sub>2</sub>), 4.21 (dd, 1H, J 5.98, 5.16, CH), 6.79 (s, 1H, CH imidazole), 7.51 (s, 1H, NH), 7.79 (s, 1H, CH imidazole), 10.51 (s, 1H, NH), 11.86 (broad s, 1H, NH imidazole); δ<sub>C</sub> (100 MHz, D<sub>2</sub>O): 28.02, 58.61, 117.32, 131.33, 135.82, 160.01, 178.62; HRMS (DART-TOF<sup>+</sup>): mass [M+H] calc’d for C<sub>8</sub>H<sub>11</sub>N<sub>4</sub>O<sub>2</sub> 195.08820 Da, measured 195.08876 Da.
3.4.7.2 General procedure for the synthesis of 3-substituted 2,4-imidazolidinediones

A reaction vessel was charged with 62 (4.90 mmol) under inert atmosphere. Anhydrous DMF (10 mL) was added and the reactant was dissolved with stirring. DBU (11 mmol, 2.2 equiv) was immediately added dropwise and the mixture was stirred for 30 minutes. An alkyl halide (5.50 mmol, 1.1 equiv) was then added to the reaction vessel and the mixture was stirred for 18 hours at 60°C. The mixture was cooled to RT and concentrated in vacuo. The crude product mixture was diluted with distilled water (20 mL) and extracted into DCM + 6% IPA (3 x 30 mL). The combined organic phases were dried over anhydrous MgSO₄, concentrated in vacuo, and purified by silica-gel chromatography using gradient elution to yield 3-substituted 2,4-imidazolidinedione.

3-(pyridin-2-ylmethyl)imidazolidine-2,4-dione (66)

White solid (0.33 g, 35% yield); chromatography conditions EtOAc:MeOH (100:0 to 95:5); M.p. 139-141°C; δH (400 MHz, d₆-DMSO): 4.02 (s, 2H, CH₂), 4.64 (s, 2H, CH₂), 7.27 (m, 2H, 2xCH aromatic), 7.75 (dt, 1H, J 7.67, 1.68, CH aromatic), 8.15 (s, 1H, NH), 8.47 (d, 1H, J 4.78, CH aromatic); δC (100 MHz, d₆-DMSO): 42.83, 46.54, 121.46, 122.89, 137.25, 149.46, 155.90, 157.85, 172.49; HRMS (DART-TOF⁺): mass [M+H] calc’d for C₉H₁₀N₃O₂ 192.07730 Da, measured 192.07768 Da.

3-(pyridin-3-ylmethyl)imidazolidine-2,4-dione (67)

White solid (0.54 g, 57% yield); chromatography conditions EtOAc:MeOH (100:0 to 95:5); M.p. 150-151°C; δH (400 MHz, d₆-DMSO): 3.98 (s, 2H, CH₂), 4.56 (s, 2H, CH₂), 7.36 (dd, 1H, J 7.83, 4.82, CH aromatic), 7.67 (dt, 1H, J 7.88, 1.74, CH aromatic), 8.17 (s, 1H, NH), 8.48 (dd, 1H, J 4.74, 1.24, CH aromatic), 8.50 (d, 1H, J 1.67, CH aromatic); δC (100 MHz, d₆-DMSO): 39.15, 46.52, 124.05, 132.86, 135.82, 149.10, 149.34, 157.66, 172.42; HRMS (DART-TOF⁺): mass [M+H] calc’d for C₉H₁₀N₃O₂ 192.07730 Da, measured 192.07765 Da.
3-(pyridin-4-ylmethyl)imidazolidine-2,4-dione (68)

White solid (0.54 g, 57% yield); chromatography conditions DCM:MeOH (100:0 to 95:5); **M.p.** 197-199°C; \(\delta_H\) (400 MHz, d\(_6\)-DMSO): 4.02 (s, 2H, CH\(_2\)), 4.56 (s, 2H, CH\(_2\)), 7.24 (d, 2H, J 5.89, 2 x CH aromatic), 8.22 (s, 1H, NH), 8.51 (d, 2H, J 5.89, 2 x CH aromatic); \(\delta_C\) (100 MHz, d\(_6\)-DMSO): 40.43, 46.58, 122.50, 145.97, 150.17, 157.59, 172.47; **HRMS** (DART-TOF\(^{+}\)): mass [M+H] calc’d for C\(_9\)H\(_{10}\)N\(_3\)O\(_2\) 192.07730 Da, measured 192.07756 Da.

3-(thiazol-4-ylmethyl)imidazolidine-2,4-dione (69)

White solid (0.19 g, 47% yield); chromatography conditions EtOAc:MeOH (100:0 to 95:5); **M.p.** 145-147°C; \(\delta_H\) (400 MHz, d\(_6\)-DMSO): 3.97 (s, 2H, CH\(_2\)), 4.65 (s, 2H, CH\(_2\)), 7.50 (d, 1H, J 1.51, CH thiazole), 8.13 (s, 1H, NH), 9.03 (d, 1H, J 1.92, CH thiazole); \(\delta_C\) (100 MHz, d\(_6\)-DMSO): 38.02, 46.48, 116.14, 152.40, 154.81, 157.56, 172.19; **HRMS** (DART-TOF\(^{+}\)): mass [M+H] calc’d for C\(_7\)H\(_8\)N\(_3\)O\(_2\)S 198.03372 Da, measured 198.03413 Da.

3-((1-trityl-1H-imidazol-4-yl)methyl)imidazolidine-2,4-dione (70)

White solid (0.28 g, 13% yield); chromatography conditions EtOAc:Hex:MeOH (50:45:0 to 50:45:5); \(\delta_H\) (400 MHz, CDCl\(_3\)): 3.97 (s, 2H, CH\(_2\)), 4.62 (s, 2H, CH\(_2\)), 6.52 (br s, 1H, NH amide), 6.86 (s, 1H, CH imidazole), 7.12 (m, 6H, 6 CH aromatic), 7.33 (m, 9H, 9 CH aromatic), 7.37 (d, 1H, J 1.31, CH imidazole).

3.4.7.3 Procedure for detritylation of 3-((1-trityl-1H-imidazol-4-yl)methyl)imidazolidine-2,4-dione (71)

To a stirred solution of 70 (0.49 g, 1.16 mmol) and triethylsilane (0.37 mL, 2.32 mmol) in DCM at 0°C was added TFA (4 mL) dropwise. The mixture was stirred for 15 minutes at the same temperature, for 1 hour at RT, and subsequently concentrated *in vacuo*. The resulting residue was taken up in distilled water (10 mL), filtered, and lyophilized to yield 71 (0.29 g, 84% yield) as a white solid; **M.p.** 138-139°C; \(\delta_H\) (400 MHz, D\(_2\)O): 3.95 (s, 2H, CH\(_2\)), 4.66 (s, 2H, CH\(_2\)), 7.35 (s,
1H, CH imidazole), 8.52 (d, 1H, J 1.21, CH imidazole); \( \delta_C \) (100 MHz, D2O): 31.79, 46.59, 117.92, 127.35, 133.81, 158.55, 174.18; HRMS (DART-TOF\(^+\)): mass [M+H] calc’d for C7H9N4O2 181.07255 Da, measured 181.07228 Da.

3.4.7.4 Procedure for the synthesis of 3,5-substituted 2,4-imidazolidinedione

5-((1-trityl-1H-imidazol-4-yl)methyl)imidazolidine-2,4-dione (72)

To a stirred solution of 63 (1.00 g, 5.55 mmol) in DMF (5 mL) was added TEA (1.16 mL, 8.32 mmol) and TrCl (1.85 g, 6.64 mmol) and stirred for 18 hours. The solution was triturated with distilled water (50 mL) to yield 72 (1.43 g, 61% yield) as a white solid; \( \delta_H \) (400 MHz, d\(_6\)-DMSO): 2.77 (dd, 1H, J 14.76, 5.64, CH\(_2\)), 2.83 (dd, 1H, J 14.83, 4.89, CH\(_2\)), 4.20 (t, 1H, J 5.20, CH), 6.67 (d, 1H, J 0.83, CH imidazole), 7.06 (dd, 6H, J 8.00, 1.53, 6 CH aromatic), 7.21 (d, 1H, J 1.24, CH imidazole), 7.38 (m, 9H, 9 CH aromatic), 7.81 (s, 1H, NH), 10.49 (s, 1H, NH).

3,5-bis((1H-imidazol-4-yl)methyl)imidazolidine-2,4-dione ditrifluoroacetate (74)

A reaction vessel was charged with 72 (1.42 g, 3.36 mmol) under inert atmosphere. Anhydrous DMF (10 mL) was added and the reactant dissolved with stirring. DBU (0.55 mL, 3.70 mmol) was added dropwise and the mixture was stirred for 30 minutes. 59 (1.32 g, 3.70 mmol) was then added to the reaction vessel and the mixture was stirred at RT for 1 hour and then at 60°C for 18 hours. The crude product mixture was cooled to RT, triturated with distilled water (100 mL) and the precipitate collected by filtration. Following this, the precipitated white solid 73 (1.28 g, 1.72 mmol) was dissolved in DCM (10 mL), cooled to 0°C, and triethylsilane (0.54 mL, 3.43 mmol) and TFA (4 mL) were added dropwise, respectively. The mixture was stirred at 0°C for 15 minutes followed by stirring at RT for 1 hour, concentrated, and taken up in distilled water (10 mL). The crude product mixture was purified by reverse phase HPLC using gradient elution (H\(_2\)O:ACN:TFA 100:0:0.01 to 10:90:0.01) to yield 74 (0.30 g, 18% yield) as a white solid; \( \delta_H \) (400 MHz, D2O): 3.15 (d, 2H, J 5.07, CH\(_2\)), 4.48 (t, 1H, J 5.02, CH), 4.58 (t, 1H, CH imidazole), 7.12 (s, 1H, CH imidazole), 7.27 (s, 1H,
CH imidazole), 8.44 (s, 1H, CH imidazole), 8.54 (s, 1H, CH imidazole); δC (100 MHz, D₂O): 25.66, 31.42, 56.16, 117.38, 118.14, 126.84, 127.14, 133.48, 133.88, 157.35, 174.01.

3,5-bis((1H-imidazol-4-yl)methyl)imidazolidine-2,4-dione dihydrochloride (75)

The TFA salt 74 was dissolved in distilled water (10 mL) and Amberlite resin in its chloride-activated form (5g/mmol) was added. The mixture was stirred at RT for 24 hours, the Amberlite was removed by filtration, and the solution lyophilized. Disappearance of the TFA salt was checked by ¹⁹F NMR spectroscopy and the stirring in Amberlite procedure was repeated to yield 75 (0.15 g, 75% yield) as yellow solid; M.p. 45-48˚C; δH (400 MHz, D₂O): 3.15 (d, 2H, J 5.02, CH₂), 4.49 (t, 1H, J 5.04, CH), 4.58 (d, 2H, J 2.20, CH₂), 7.12 (s, 1H, CH imidazole), 7.28 (s, 1H, CH imidazole), 8.45 (d, 1H, J 1.22, CH imidazole), 8.55 (s, 1H, CH imidazole); δC (100 MHz, D₂O): 25.70, 31.47, 56.19, 117.43, 118.14, 126.79, 127.15, 133.50, 133.93, 157.38, 174.05; HRMS (DART-TOF⁺): mass [M+H] calc’d for C₁₁H₁₃N₆O₂ 261.11000 Da, measured 261.10955 Da.

3.4.8 Synthesis of Atypical Amino Acids

3.4.8.1 General procedure for the Wittig-Horner reaction

(±)-Benzyloxy carbonyl-α-phosphonoglycine trimethyl ester (5.01 mmol) was dissolved in anhydrous THF (10 mL) and cooled to 0°C under inert atmosphere. Tetramethylguanidine (5.50 mmol, 1.1 equiv) was added to the reaction vessel dropwise and the mixture was stirred at 0°C for 1 hour. Pyridinecarboxaldehyde (5.47 mmol, 1.1 equiv) was then added dropwise and the reaction was stirred for 2 hours at 0°C and 2 hours at RT. The crude product mixture was extracted with EtOAc (3 x 30 mL) from brine, dried over anhydrous MgSO₄, concentrated in vacuo, and purified by silica-gel chromatography using gradient elution to yield N-Cbz-α-pyridinylmethylene glycine methyl ester.

(E)-methyl 2-(((benzylcarbonyl)amino)-3-(pyridin-2-yl)acrylate (76)

Pale yellow oil (1.35 g, 87% yield); chromatography conditions Hex:EtOAc (100:0 to 85:15); δH (400 MHz, CDCl₃): 3.85 (s, 3H, CH₃), 5.20 (s, 2H, CH₂), 6.29 (s, 1H, CH), 7.17 (ddd, 1H, J 7.54, 4.96,
1.09, CH aromatic), 7.23 (d, 1H, J 7.91, CH aromatic), 7.38 (m, 5H, 5 x CH benzyl), 7.69 (dt, 1H, J 7.75, 1.82, CH aromatic), 8.58 (d, 1H, J 4.42, CH aromatic), 11.31 (s, 1H, NH).

\[(E)\text{-methyl 2-((((benzyloxy)carbonyl)amino)-3-(pyridin-3-yl)acrylate (77)}\]

\[
\begin{align*}
\text{Cbz} & \quad \text{N} \\
& \quad \text{O} \\
\end{align*}
\]

Pale yellow oil (1.49 g, 96% yield); chromatography conditions
Hex:EtOAc (75:25 to 50:50); \( \delta_H \) (400 MHz, CDCl\(_3\)): 3.85 (s, 1H, CH\(_3\)), 5.11 (s, 2H, CH\(_2\)), 6.82 (s, 1H, CH), 7.32 (m, 7H, CH, 5 x CH benzyl, NH and CH aromatic), 7.82 (d, 1H, J 7.48, CH aromatic), 8.52 (dd, 1H, J 4.78, 1.15, CH aromatic), 8.70 (d, 1H, J 1.35, CH aromatic).

\[(E)\text{-methyl 2-((((benzyloxy)carbonyl)amino)-3-(pyridin-4-yl)acrylate (78)}\]

\[
\begin{align*}
\text{Cbz} & \quad \text{N} \\
& \quad \text{O} \\
\end{align*}
\]

Yellow oil (1.46 g, 94% yield); chromatography conditions
Hex:EtOAc (75:25 to 50:50); \( \delta_H \) (400 MHz, CDCl\(_3\)): 3.86 (s, 3H, CH\(_3\)), 5.08 (s, 2H, CH\(_2\)), 6.92 (s, 1H, CH), 7.18 (s, 1H, NH), 7.34 (m, 7H, 5 x CH benzyl and 2 x CH aromatic), 8.54 (dd, 2H, J 4.58, 1.59, 2 x CH aromatic).

3.4.8.2 General procedure for the hydrogenation of \( N\text{-Cbz-}\alpha\text{-pyridinylmethylene glycine methyl esters} \)

To a solution of \( N\text{-Cbz-}\alpha\text{-pyridinylmethylene glycine methyl ester (3.43 mmol) in MeOH (20 mL)} \) was added Pd/C (179 mg, 50 mg/mmol). The mixture was hydrogenated at 60 psi for 24 hours and the catalyst was removed by filtration through a short pad of celite, washing with MeOH (25 mL). The mixture was concentrated under reduced pressure and purified by silica-gel chromatography (Hex:EtOAc:MeOH:NH\(_3\)OH 40:50:10:0.15) to yield an \( \alpha\text{-pyridinylmethyl glycine methyl ester} \).

\[\text{methyl 2-amino-3-(pyridin-2-yl)propanoate (79)}\]

\[
\begin{align*}
\text{N} & \quad \text{NH}_2 \\
& \quad \text{O} \\
\end{align*}
\]

Viscous pale yellow oil (0.35 g, 57% yield); \( \delta_H \) (400 MHz, d\(_4\)-MeOD):
3.08 (dd, 1H, J 13.94, 7.56, CH\(_2\)), 3.21 (dd, 1H, J 13.94, 5.89, CH\(_2\)), 3.67 (s, 3H, CH\(_3\)), 3.91 (dd, 1H, J 7.46, 5.97, CH), 7.28 (ddd, 1H, J 7.53, 5.00, 1.00, CH aromatic), 7.32 (d, 1H, J 7.82, CH aromatic), 7.76 (dt, 1H, J 7.69, 1.80, CH aromatic), 8.48 (d, 1H, J 4.97, CH aromatic).
methyl 2-amino-3-(pyridin-3-yl)propanoate (80)

Viscous pale yellow oil (0.18 g, 23% yield); \( \delta_H \) (400 MHz, d\textsubscript{4}-MeOD):

\[
\begin{align*}
3.02 \text{ (dd, 1H, J 13.91, 7.016, CH\textsubscript{2})}, & \quad 3.10 \text{ (dd, 1H, J 13.95, 6.46, CH\textsubscript{2})}, \\
3.71 \text{ (s, 3H, CH\textsubscript{3})}, & \quad 3.86 \text{ (t, 1H, J 6.70, CH)}, \quad 7.41 \text{ (dd, 1H, J 7.98, 4.98, CH aromatic)}, \quad 7.74 \text{ (dt, 1H, J 7.82, 1.81, CH aromatic)}, \quad 8.42 \text{ (d, 1H, J 1.92, CH aromatic)}, \quad 8.44 \text{ (dd, 1H, J 4.86, 1.51, CH aromatic)}.
\end{align*}
\]

methyl 2-amino-3-(pyridin-4-yl)propanoate (81)

Viscous pale yellow oil (0.15 g, 18% yield); \( \delta_H \) (400 MHz, d\textsubscript{4}-MeOD):

\[
\begin{align*}
2.96 \text{ (dd, 1H, J 13.52, 7.23, CH\textsubscript{2})}, & \quad 3.08 \text{ (dd, 1H, J 13.58, 6.32, CH\textsubscript{2})}, \\
3.70 \text{ (s, 3H, CH\textsubscript{3})}, & \quad 3.80 \text{ (t, 1H, J 6.76, CH)}, \quad 7.32 \text{ (dd, 2H, J 4.73, 1.36, 2 x CH aromatic)}, \quad 8.45 \text{ (dd, 2H, J 4.58, 1.54, 2 x CH aromatic)}.
\end{align*}
\]

3.4.8.3 General procedure for the demethylation of \( \alpha \)-pyridinylmethyl glycine methyl esters

An \( \alpha \)-pyridinylmethyl glycine methyl ester (1.94 mmol) was dissolved in MeOH (2 mL) and 5 mL of an aqueous solution of NaOH (19.3 mmol, 10 equiv) was added. The mixture was stirred for 3 hours at RT, concentrated \textit{in vacuo}, purified by reverse phase HPLC using gradient elution (H\textsubscript{2}O:ACN:TFA 100:0:0.1 to 20:80:0.1) and lyophilized to yield the atypical amino acid TFA salt. The TFA salt was then dissolved in distilled water (10 mL) and Amberlite resin in its chloride-activated form (5 g/mmol) was added. The mixture was stirred at RT for 24 hours, the Amberlite was removed by filtration, and the solution was lyophilized. Disappearance of the TFA salt was checked by \(^{19}\text{F}\) NMR spectroscopy and, if necessary, stirring in Amberlite followed by lyophilization was repeated to yield an \( \alpha \)-pyridinylmethyl glycine hydrochloride salt.

2-amino-3-(pyridin-2-yl)propanoic acid dihydrochloride (42)

Pale yellow solid (0.27 g, 59% yield); \textbf{M.p.} >190°C (dec.); \( \delta_H \) (400 MHz, D\textsubscript{2}O): 3.56 (d, 2H, J 7.16, CH\textsubscript{2}), 4.29 (m, 1H, CH), 7.88 (t, 1H, J 6.73, CH pyridine), 7.94 (d, 1H, J 7.92, CH pyridine), 8.46 (t, 1H, J 7.55, CH pyridine), 8.63 (d, 1H, J 4.70, CH pyridine); \( \delta_C \) (100 MHz, D\textsubscript{2}O): 33.60, 52.11,
HRMS (DART-TOF\(^+\)): mass [M+H] calc’d for C\(_8\)H\(_{11}\)N\(_2\)O\(_2\) 167.08205 Da, measured 167.08237 Da.

**2-amino-3-(pyridin-3-yl)propanoic acid dihydrochloride (43)**

Pale yellow solid (0.17 g, 71% yield); \(\textbf{M.p.} >195^\circ\text{C} \) (dec.); \(\delta_H\) (400 MHz, D\(_2\)O): 3.45 (dd, 1H, J 14.96, 6.57, CH\(_2\)), 3.50 (dd, 1H, J 14.94, 7.41, CH\(_2\)), 4.38 (m, 1H, CH), 8.02 (dd, 1H, J 7.70, 6.29, CH pyridine), 8.55 (d, 1H, J 8.12, CH pyridine), 8.70 (d, 1H, J 5.71, CH pyridine), 8.74 (s, 1H, CH pyridine). \(\delta_C\) (400 MHz, D\(_2\)O): 32.67, 53.26, 127.68, 135.80, 140.54, 141.66, 148.23, 170.67. **HRMS** (DART-TOF\(^+\)): mass [M+H] calc’d for C\(_8\)H\(_{11}\)N\(_2\)O\(_2\) 167.08205 Da, measured 167.08242 Da.

**2-amino-3-(pyridin-4-yl)propanoic acid dihydrochloride (44)**

Pale yellow solid (87.3 mg, 44% yield); \(\textbf{M.p.} >190^\circ\text{C} \) (dec.); \(\delta_H\) (400 MHz, D\(_2\)O): 3.51 (dd, 1H, J 15.32, 7.78, CH\(_2\)), 3.57 (dd, 1H, J 14.69, 8.01, CH\(_2\)), 4.45 (t, 1H, J 7.06, CH), 8.01 (d, 2H, J 5.20, 2 x CH pyridine), 8.71 (d, 2H, J 5.47, 2 x CH pyridine). \(\delta_C\) (100 MHz, D\(_2\)O): 35.87, 52.95, 128.31, 141.21, 157.08, 170.66. **HRMS** (DART-TOF\(^+\)): mass [M+H] calc’d for C\(_8\)H\(_{11}\)N\(_2\)O\(_2\) 167.08205 Da, measured 167.08231 Da.
CHAPTER 4

4 Qualitative and Quantitative Analysis of Zn$^{2+}$ Chelation
4.1 Qualitative Assessment of Chelation by NMR Spectroscopy

NMR spectroscopy is a technique that allows for the characterization of chemical entities by use of an external magnetic field. Briefly, nuclei possessing spin have an associated magnetic moment that in the presence of an external magnetic field (applied to the sample by the spectrometer) will align with or against the applied field. When this external magnetic field is relaxed, the high energy nuclei (nuclei aligned against the magnetic field) transition back to the ground state, creating a fluctuating magnetic field that is translated into current and subsequent chemical shift values for the nuclei by the spectrometer. Unique nuclei will have distinct chemical shifts due to their surrounding electronic environment that causes the external applied field to be experienced in a distinct way. Nuclei may be shielded or deshielded (electron poor or electron rich environments, respectively) by neighbouring functional groups or, for example, metal ions.

Experiments are most commonly performed in solution phase, although solid-state NMR is also available. NMR spectroscopy has been widely applied to the analysis of transition metal complexes involving small molecules and low molecular weight proteins with cations such as Zn$^{2+}$, Ca$^{2+}$, Mg$^{2+}$, and Cd$^{2+}$, for example (Nair et al. 2010; Brown and Antholine 1979). Typically these experiments involve assessing how the chemical shifts of nuclei are changed in response to complex formation. Changes in chemical shift correlate to an alteration of the experienced external magnetic field due to a shift in the molecules conformation or electronic environment. Such shifts suggest that interactions between the ligand and metal cation are occurring.

4.1.1 NMR Spectroscopy Methods

The ability of histidine 1 and synthesized compounds to chelate Zn$^{2+}$ and Ca$^{2+}$ was assessed qualitatively using NMR spectroscopy. Compounds were added to D$_2$O containing 50 mM HEPES buffered to pH 7.0±0.1. Solutions of 30 mM ZnSO$_4$$\cdot$7H$_2$O or CaCl$_2$$\cdot$2H$_2$O in D$_2$O were added incrementally to form final buffered solutions with varying metal:ligand molar ratios, where ligand refers to histidine 1 or a synthesized compound. Experimental molar ratios of metal:ligand can be found in Table 4.1. Only a subset of synthesized compounds was assessed for interactions with Ca$^{2+}$. Spectra were collected on a Bruker 400 MHz spectrometer and
assessed for changes in chemical shift, indicative of a change in ligand conformation resulting from an interaction between the ligand and Zn$^{2+}$ or Ca$^{2+}$.

**Table 4.1.** Experimental conditions for the qualitative assessment of metal (Zn$^{2+}$ or Ca$^{2+}$):ligand interactions in D$_2$O. NMR spectra of samples were collected on a Bruker 400 MHz spectrometer.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Metal:Ligand Molar Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>0.25</td>
</tr>
<tr>
<td>4</td>
<td>0.33</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**4.1.2 NMR Spectroscopy Results and Discussion**

NMR spectra for the experiments described in Section 4.1.1 are shown in Figures 4.1-4.4 and 4.6-4.9. Sample numbers are indicated in these figures, and correspond to the sample number in Table 4.1. Numbered compound structures are included on each figure for reference, with protons shown in the spectra highlighted in red. Formation of Zn(OH)$_2$ occurred in samples containing more than 0.25 molar Zn$^{2+}$ at pH 7.0. These samples were passed through a micro filter prior to the collection of their NMR spectra. The addition of 30 mM CaCl$_2$•2H$_2$O to select compounds (histidine 1, 4, 5, 7, 8) in buffered D$_2$O did not produce detectable changes in the $^1$H NMR spectra recorded (data not shown), therefore experiments involving Ca$^{2+}$ were terminated. This evidence affirms the molecular modeling in Section 2.3.1 that suggests histidine 1 has selectivity for interacting with Zn$^{2+}$ over Ca$^{2+}$.

**4.1.2.1 Results for the addition of Zn$^{2+}$ to histidine in $^1$H NMR experiments**

The addition of 30 mM ZnSO$_4$•7H$_2$O to histidine 1 in D$_2$O at pH 7.0 produced detectable changes in the $^1$H NMR spectrum (see Figure 4.1). Notable change in the chemical shift (at addition of 25 mol% Zn$^{2+}$) of the H2 (7.70 to 7.65 ppm) proton indicates that addition of Zn$^{2+}$ initiated interactions between the cation and the imidazole ring. Variation in H7 (3.84 to 3.86 ppm) shifts are less pronounced (data not shown), although the splitting pattern changes from a doublet of doublets to a triplet, suggesting the interactions between H7 and H6 protons become equivalent. Protons at position 6 are hidden in the HEPES buffer peaks and therefore cannot be compared.
Both inductive and anisotropic effects are likely involved in these detected shifts. Inductive effects are the result of neighbouring electronegative atoms or electron withdrawing groups that pull electron density away from the nucleus and decrease the shielding effects of the valence electrons. Magnetic anisotropy refers to non-uniformity in the magnetic field. Electrons in \( \pi \)-systems, such as imidazole, interact with the applied magnetic field resulting in anisotropy. Regions in which the induced field aligns with the external field results in deshielding, while regions where the induced field opposes the external field results in shielding. The interplay of these effects are complex and not easily resolved. In the case of H7, inductive effects likely results in deshielding and the slight downfield shift (i.e. higher chemical shift value), data not shown. However, H2 is involved in the imidazole \( \pi \)-system, so cation addition may alter the induced anisotropic field such that the proton is more shielded, as seen with the upfield shift (i.e. lower chemical shift value).

**Figure 4.1.** Chemical shifts of imidazole protons from histidine 1 where samples 1 to 5 have incremental increases of Zn\(^{2+}\), described in Table 4.1. Protons responsible for shifts shown are red.

### 4.1.2.2 Results for the addition of Zn\(^{2+}\) to esters in \(^1\)H NMR experiments

NMR experiments were attempted with 2 and 3. However, in buffered D\(_2\)O solution the esters decomposed to form the free acid. This was confirmed by both NMR analysis and TLC using ninhydrin stain. Therefore the analysis of esters as potential Zn\(^{2+}\) chelators was unsuccessful.

### 4.1.2.3 Results for the addition of Zn\(^{2+}\) to dipeptides in \(^1\)H NMR experiments

Recorded \(^1\)H NMR spectra for the addition of Zn\(^{2+}\) into buffered dipeptide solutions were similar between diastereomers, therefore representative results for 6 are presented. The full spectra of
samples 1-5 are shown in Figure 4.2, with the solvent peak found at 4.7 ppm and HEPES proton peaks at 2.7 to 3.1 ppm and 3.75 ppm.

Figure 4.2. NMR spectra of 6 where samples 1 to 5 have incremental increases of Zn$^{2+}$, described in Table 4.1.

The chemical shift region containing alkyl protons is expanded in Figure 4.3 with a numbered structure of 6 for reference. It is apparent that an interaction is occurring that causes changes to the environment of H7 (middle peak at ~4.0 ppm) and H10 (leftmost peak at ~4.3 ppm) when they are compared to the HEPES buffer triplet at 3.75 ppm, which does not shift from its original position in sample 1. The chemical shift of H7 is altered significantly more than that of H10, suggesting this region of the molecule is more involved in the interaction with Zn$^{2+}$. Another interesting feature is the increase in $J^3_{\text{trans}}$ from 0.02 to 0.03 Hz ($J^3_{\text{cis}}$ unchanged) experienced by H10, suggesting the H12 proton trans to H10 may also be changed in orientation upon complexation.

Figure 4.3. Chemical shifts of H7 and H10 protons from 6 where samples 1 to 5 have incremental increases of Zn$^{2+}$, described in Table 4.1. Protons responsible for shifts shown are red.
The aromatic region of dipeptide 6 is shown in Figure 4.4; protons in this region also show deviations in chemical shift as Zn$^{2+}$ content is increased through samples 1-5. *In silico* calculations predict that binding energies are lowest for the dipeptide 4 when Zn$^{2+}$ is interacting with the C-terminus portion of the dipeptide. Figure 4.5 shows this visually. Therefore, the two outside peaks in Figure 4.4 likely correspond to H15 (furthest downfield shift) and H17 (furthest upfield shift) as they have the largest deviations in chemical shift for this spectral region as Zn$^{2+}$ content is increased.

![Chemical shifts of imidazole protons from 6](image1)

**Figure 4.4.** Chemical shifts of imidazole protons from 6 where samples 1 to 5 have incremental increases of Zn$^{2+}$, described in Table 4.1. Protons responsible for shifts shown are red.

![Lowest energy dipeptide 4:Zn$^{2+}$ complex](image2)

**Figure 4.5.** Lowest energy dipeptide 4:Zn$^{2+}$ complex showing Zn$^{2+}$ interacting with the C-terminal portion of the molecule.

4.1.2.4 Results for the addition of Zn$^{2+}$ to diketopiperazines in $^1$H NMR experiments

The $^1$H NMR spectra of diketopiperazine 7 shows more pronounced changes to imidazole proton chemical shifts than 8 upon addition of ZnSO$_4$$\cdot$7H$_2$O, and is shown in Figure 4.6. Unlike seen with histidine 1, the upfield proton (H4) peak is shifted to a greater extent than the downfield proton (H2) peak. This suggests that Zn$^{2+}$ may be interacting primarily with the central ring, as
H4 is closer in proximity to this region than H2. Peak broadening is evident as Zn\(^{2+}\) is added to samples, indicating fast exchange of the molecule between the free and complexed forms.

![Figure 4.6. Chemical shifts of imidazole protons from 7 where samples 1 to 5 have incremental increases of Zn\(^{2+}\), described in Table 4.1. Protons responsible for shifts shown are red.](image)

\(^1\)H NMR spectra of 61 show fluctuations in chemical shift of imidazole protons with increasing Zn\(^{2+}\) content (data not shown), with the furthest downfield proton considerably affected by Zn\(^{2+}\) addition (7.61 to 7.74 ppm). Alkyl protons are not significantly affected by Zn\(^{2+}\) addition. However, in these samples the HEPES buffer peaks at 3.75 ppm also shift by 0.05 ppm, which suggests samples may have not been pH matched correctly. A pH mismatch makes comparing spectra difficult because there would be another variable that may be causing \(^1\)H NMR spectral shifts.

55 and 57 were not run in this assay.

4.1.2.5 Results for the addition of Zn\(^{2+}\) to imidazolidinediones in \(^1\)H NMR experiments

No changes in the \(^1\)H NMR spectra were detected upon increasing Zn\(^{2+}\) content in buffered solutions of 66, 67, 68, and 69.

However, Figure 4.7 shows large changes in the chemical shifts of the imidazole protons of 71. Compound 71 contains an imidazole ring, while 66-68 contain pyridine rings and 69 contains a thiazole ring. These results indicate that as seen in silico (refer to Table 2.4), imidazole is a superior Zn\(^{2+}\) chelator compared to these other heterocycles. Modifying the imidazolidinedione substitution position from 3- to 5- reduces peak shifting, as was seen with addition of Zn\(^{2+}\) into
63 (data not shown). This suggests that substitution at the 3-position may result in improved 
Zn\(^{2+}\) chelation over substitution at the 5-position in the 2,4-imidazolidinedione platform.

**Figure 4.7.** Chemical shifts of imidazole protons from 71 where samples 1 to 5 have 
incremental increases of Zn\(^{2+}\), described in Table 4.1. Protons responsible for shifts shown are red.

75 was not run in this assay.

4.1.2.6 Results for the addition of Zn\(^{2+}\) to atypical amino acids in \(^1\)H NMR 
experiments

The addition of 30 mM ZnSO\(_4\)•7H\(_2\)O to a buffered solution of 42 at pH 7.0 produced significant 
changes in the \(^1\)H NMR spectra recorded; two different chemical shift regions of the collected 
spectra are shown in Figures 4.8 and 4.9. Figure 4.8 highlights the alkyl protons; the 
environment of the H7 protons and the H8 proton is altered significantly as Zn\(^{2+}\) is titrated into 
samples suggesting that Zn\(^{2+}\) is interacting with this region of 42.

**Figure 4.8.** Chemical shifts of alkyl protons from 42 where samples 1 to 5 have incremental 
increases of Zn\(^{2+}\), described in Table 4.1. Protons responsible for shifts shown are red.
Peaks of aromatic pyridine protons are shown in Figure 4.9. The shifts of aromatic protons H3 or H4 and H5 (furthest upfield shifts) become resolved from one another as Zn$^{2+}$ content is increased. The chemical shift of H3 or H4 is relatively unchanged, although the peak of H2 (the furthest downfield shift) becomes significantly broadened with Zn$^{2+}$ addition. If Zn$^{2+}$ is interacting with nitrogen in the pyridine ring, H2 will be closest aromatic proton in proximity and therefore broadening of this peak upon complex association/dissociation is plausible.

**Figure 4.9.** Chemical shifts of imidazole protons from 42 where samples 1 to 5 have incremental increases of Zn$^{2+}$, described in Table 4.1. Protons responsible for shifts shown are red.

NMR experiments with 43 showed similar results as 42, with both chemical shift alterations and peak broadening as Zn$^{2+}$ content was increased. 44 was not run in this assay.

### 4.2 Quantitative Assessment of Chelation by Isothermal Titration Calorimetry

#### 4.2.1 An Introduction to Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) is a solution-phase technique that directly measures the heat produced or absorbed as molecules interact, first commercialized by Wiseman et al. in 1989 for the purpose of measuring thermodynamic properties including binding constants and enthalpy (Wiseman et al. 1989). A schematic of the calorimeter is shown in Figure 4.10.

The instrument contains two cells, a reference cell and a sample cell, enclosed in an adiabatic shield to prevent any interactions between the system and the surroundings. The sample cell holds the analyte and an injection syringe injects small aliquots of the titrant into the sample cell at timed intervals, allowing the system to reach equilibrium after each injection. The instrument functions by maintaining a zero temperature difference between the reference and sample cells.
by increasing or decreasing power applied to the reference cell, dependent on the feedback power collected from the sample cell. An endothermic reaction would result in increases of feedback power and therefore heat applied to the reference cell while an exothermic reaction would lead to decreases in feedback power and subsequent cooling of the reference cell (Wiseman et al. 1989). The heat of reaction is obtained by integration of the power influxes over time using the concentration of the analyte and titrant solutions to convert data to units of heat/mol.

**Figure 4.10.** Schematic representation of a calorimeter used in ITC.

The development of increasingly sensitive instruments has made application of isothermal titration calorimetry to bioinorganic applications possible (Grossoehme, Spuches, and Wilcox 2010). A literature review revealed studies assessing the interaction of transition metals (such as Ni$^{2+}$, Cu$^{2+}$, and Zn$^{2+}$) with small molecules, peptides, as well as various proteins (Zhang, Akilesh, and Wilcox 2000; Garrido, Ràfols, and Bosch 2011; Zidane et al. 2012; Garcia-Valls and Hatton 2003; Talmard, Bouzan, and Faller 2007; Grossoehme, Spuches, and Wilcox 2010; Wilcox 2008). Therefore, this technique was used to reveal thermodynamic properties of Zn$^{2+}$ binding to histidine I and synthesized analogues.

### 4.2.2 ITC Methods

ITC measurements were carried out at 25 ± 0.1°C on a MicroCal VP-ITC calorimeter. Ligand and metal solutions were prepared in the same buffer stock, 100 mM HEPES solution, prepared by dissolving the free acid in MilliQ water and adjusting to pH 7.00 ± 0.05 with NaOH.
Experimental solutions were made by dissolving powders in HEPES buffer, ensuring pH of the resulting solutions were maintained at an equivalent pH to the buffer solution. A solution of ZnSO₄•7H₂O was prepared at a concentration of 0.33 mM and used in the sample cell while ligand solutions were prepared at concentrations of 10 mM and used as titrants. The titration of 10 mM histidine 1 into 0.33 mM CaCl₂•2H₂O was also conducted in order to test for binding specificity to Zn²⁺ over Ca²⁺. Exact concentrations were recorded and used in the data analysis. All samples were degassed by being stirred under vacuum for 5 minutes prior to use.

In each ITC experiment, 25 to 35 injections (7 µL each) of 10 mM ligand solution were made into the sample cell containing 0.33 mM metal solution. Exact concentrations can be found in Table 4.2. Injections were spaced by 180 to 240 s, depending on the experiment, to allow for equilibration between injections. The injection syringe stirring speed was set to 307 rpm.

Control experiments were performed by injecting ligand solution into buffer to account for heats of dilution and subtracted from experimental results.

**Table 4.2.** Experimental concentrations for ITC runs performed at 25°C on a MicroCal VP-ITC calorimeter.

<table>
<thead>
<tr>
<th>[Zn²⁺]mM</th>
<th>Titrant</th>
<th>[Titrant]mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.341</td>
<td>1</td>
<td>9.947</td>
</tr>
<tr>
<td>0.348</td>
<td>4</td>
<td>10.008</td>
</tr>
<tr>
<td>0.341</td>
<td>5</td>
<td>9.834</td>
</tr>
<tr>
<td>0.348</td>
<td>6</td>
<td>10.157</td>
</tr>
<tr>
<td>0.348</td>
<td>7</td>
<td>10.209</td>
</tr>
<tr>
<td>0.348</td>
<td>55</td>
<td>10.711</td>
</tr>
<tr>
<td>0.348</td>
<td>61</td>
<td>9.954</td>
</tr>
<tr>
<td>0.348</td>
<td>63</td>
<td>10.193</td>
</tr>
<tr>
<td>0.341</td>
<td>66</td>
<td>9.990</td>
</tr>
<tr>
<td>0.341</td>
<td>67</td>
<td>9.938</td>
</tr>
<tr>
<td>0.348</td>
<td>68</td>
<td>9.624</td>
</tr>
<tr>
<td>0.348</td>
<td>69</td>
<td>10.344</td>
</tr>
<tr>
<td>0.348</td>
<td>71</td>
<td>10.099</td>
</tr>
<tr>
<td>0.348</td>
<td>75</td>
<td>9.855</td>
</tr>
<tr>
<td>0.348</td>
<td>42</td>
<td>9.703</td>
</tr>
<tr>
<td>0.348</td>
<td>43</td>
<td>10.205</td>
</tr>
<tr>
<td>0.348</td>
<td>44</td>
<td>10.665</td>
</tr>
</tbody>
</table>

Recorded data (differential power versus time) was analyzed using MicroCal Origin 7.0; one-site or sequential site binding models were used for curve fitting. The software integrates the area
under the curve of each injection, which corresponds to the heat released or absorbed with each injection volume. This data is further translated to units of heat/mol using appropriate dilution factors. The integrated data is then fit using fitting procedures implemented in the MicroCal software, which will be described briefly below as per the MicroCal Origin data analysis manual (MicroCal 2004).

### 4.2.2.1 One-Site Binding Model for ITC Curve Fitting

In the following situations $M$ will denote Zn$^{2+}$ in the sample cell and $X$ will denote synthesized compounds in the injection syringe. Situations where there is a single independent binding site can be described by the equilibrium expression in Eq. [4.1]. The binding constant for this expression is described by Eq. [4.2], where $[X] = \text{free ligand concentration}$ and $\Theta = \text{fraction of sites on } M \text{ occupied by ligand } X$.

$$M + X \rightleftharpoons MX \quad [4.1]$$

$$K = \frac{\Theta}{(1 - \Theta)[X]} \quad [4.2]$$

The bulk ligand concentration $X_t$ is equal to the sum of the free and bound ligand concentrations where $M_t$ is the bulk concentration of Zn$^{2+}$ in the sample cell volume $V_o$ and $n$ is the number of site in $M$.

$$X_t = [X] + n\Theta M_t \quad [4.3]$$

Curve fitting endeavors to model the change in heat from each injection, or $\Delta Q(i)$:

$$\Delta Q(i) = Q(i) - Q(i - 1) \quad [4.4]$$

The heat at each injection point can be expressed as

$$Q = n\Theta M_t \Delta H V_o \quad [4.5]$$

where $n$ is the number of sites occupied and $\Delta H$ is the enthalpy of binding. Substituting Eq. [4.3] into Eq. [4.2] and solving the quadratic for $\Theta$ allows for calculation of $\Delta Q(i)$ for a given set of $n$, $K$, and $\Delta H$ values. An initial guess of these terms gives a calculated value for $\Delta Q(i)$ which is then compared against the collected experimental data set. MicroCal Origin 7.0 employs
Marquardt methods to refine the values of $n$, $K$, and $\Delta H$ and iterations are completed until no more improvements to the fit are made (MicroCal 2004).

### 4.2.2.2 Sequential Site Binding Model for ITC Curve Fitting

The sequential site binding model describes systems in which there is more than one binding site, and binding events are influenced by one another. This is described for a case with two sequential binding sites below. Eqs. [4.6] and [4.7] show a system where each ligand $X$ is bound sequentially to $M$.

\[
M + X \rightleftharpoons MX \quad [4.6]
\]

\[
MX + X \rightleftharpoons MX_2 \quad [4.7]
\]

The binding constants are defined relative to the progress of saturation such that:

\[
K_1 = \frac{[MX]}{[M][X]} \quad [4.8]
\]

\[
K_2 = \frac{[MX_2]}{[MX][X]} \quad [4.9]
\]

The fraction of $M$ bound with ligand $X$ can be expressed in terms of the free species $M$, where $F_1$ is the fraction of $M$ having one bound ligand and $F_2$ is the fraction of $M$ having two bound ligands.

\[
F_1 = \frac{K_1 [X]}{1 + K_1 [X]} \quad [4.10]
\]

\[
F_2 = \frac{K_1 K_2 [X]^2}{1 + K_1 [X] + K_1 K_2 [X]^2} \quad [4.11]
\]

The heat for each injection can be calculated using Eq. [4.12], then Eq. [4.4] can be employed to find the optimal parameters for the best fit using the same procedure as described in Section 4.2.2.1 (MicroCal 2004).

\[
Q = M_i V_0 (F_1 \Delta H_1 + F_2 [\Delta H_1 + \Delta H_2]) \quad [4.12]
\]
4.2.3 ITC Results and Discussion

4.2.3.1 Analysis of ITC data for the titration of histidine into Zn$^{2+}$ and Ca$^{2+}$

Figure 4.11 shows the ITC data for titration of histidine 1 into Zn$^{2+}$ in 100 mM HEPES buffer at pH 7.0. Parameters for the fit using one-site binding can be found in Table 4.3. These results indicate that at pH 7.00, histidine 1 binds to Zn$^{2+}$ with a molar ratio of 0.83:1. This value is non-integer, and not expected, although the heterogeneous protonation state of histidine 1 at this pH may play a role in this. The curve reaches equilibrium when the molar ratio is 2:1 (histidine 1:Zn$^{2+}$), therefore for Zn$^{2+}$ to be saturated there must be 2-fold excess ligand in the system.

![ITC data for titration of 0.33 mM Zn$^{2+}$ with 10 mM histidine 1 in 100 mM HEPES buffer at pH 7.00. Parameters of best fit for the binding curve can be found in Table 4.3.](image)

The Gibb’s free energy term, calculated using Eq. [4.13], is negative (see Table 4.3) and indicates that this complexation reaction is spontaneous, or exergonic.

$$\Delta G = \Delta H - T\Delta S$$  \hspace{1cm} [4.13]

The binding enthalpy value reflects the strength and specificity of binding between Zn$^{2+}$ and histidine 1. The positive value of $\Delta H$ (refer to Table 4.3) indicates that this reaction is endothermic and enthalpically opposed. However, the entropy term ($-T\Delta S$) for this titration is highly negative, which shows that the reaction is entropically driven. Changes to entropy can arise from several aspects of complexation, including a loss of entropy associated with decreased translational degrees of freedom upon ligand binding, changes in conformational entropy as the free ligand becomes bound, and solvent effects (Bronowska 2011). Solvent effects are the main
entropic contributor. In this case, Zn\(^{2+}\) must release an appropriate number of water molecules from its solvation shell to allow for ligand binding, and as previously organized water molecules are released into the bulk large observable increases in entropy are detected.

In previous literature, Zhou et al. found that at acidic pH there doesn’t appear to be a direct interaction between histidine 1 and Zn\(^{2+}\), while above pH 7.5, the 2:1 complex dominates (Zhou et al. 2013). Therefore, it seems pH is considerably affecting the complexation reaction in these ITC experiments. This is plausible as the pKa of the histidine 1 side chain is ~6, only 1 unit higher than the experimental pH used. Although the predominant species in solution will have a net zero charge, using the Henderson–Hasselbalch equation, at pH 7.0 approximately 10% of the ligand species in solution would have a +1 formal charge and therefore would be less likely to interact with the positively charged cation. This heterogeneity in the ligand species may explain the non-integer binding observed.

**Table 4.3.** Thermodynamic parameters for histidine 1 binding to Zn\(^{2+}\) at pH 7.0 obtained from ITC measurements in 100 mM HEPES buffer at 25°C.

<table>
<thead>
<tr>
<th>Best Fit Parameters using the One-Site Model</th>
<th>Compound 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>N /mol titrant/mol Zn(^{2+})</td>
<td>0.83 ± 0.03</td>
</tr>
<tr>
<td>K /10(^4) M(^{-1})</td>
<td>4.09 ± 0.84</td>
</tr>
<tr>
<td>∆H /cal mol(^{-1})</td>
<td>811.0 ± 39.27</td>
</tr>
<tr>
<td>∆S /cal mol(^{-1}) deg(^{-1})</td>
<td>23.8</td>
</tr>
<tr>
<td>∆G /kcal mol(^{-1})</td>
<td>-6.29</td>
</tr>
</tbody>
</table>

ITC data for the titration 10 mM histidine 1 into 0.33 mM CaCl\(_2\)•2H\(_2\)O did not show any binding in solution (data not shown). This data confirms the *in silico* prediction of binding specificity for Zn\(^{2+}\) over Ca\(^{2+}\); NMR results also mimicked this ITC result.

**4.2.3.2 Analysis of ITC data for the titration of dipeptides into Zn\(^{2+}\)**

Figure 4.12 shows the ITC data for the titration of dipeptides 4, 5, and 6 into Zn\(^{2+}\) in 100 mM HEPES buffer at pH 7.0. Parameters for the fit using sequential binding with two sites can be found in Table 4.4. All binding steps are enthalpically as well as entropically favoured,
demonstrated by negative values for all $\Delta H$ parameters and positive values for $\Delta S$ parameters. The curves in Figure 4.12 show that the first site becomes saturated at a molar ratio of approximately 1:1 in all cases, after which the second site begins to become occupied. The binding constants for the initial binding site are all an order of magnitude higher than for the second binding site. $\Delta H_1$ for titrations with 5 and 6 were more favorable than for 4, whereas $\Delta H_2$, $\Delta S_1$, and $\Delta S_2$ are similar for all dipeptides. Therefore, the main difference found between diastereomers is that 4 is less enthalpically favoured to occupy the initial Zn$^{2+}$ binding site.

![Figure 4.12](image)

**Figure 4.12.** ITC data for the titration of 0.33 mM Zn$^{2+}$ with 10 mM 4 (plot A), 5 (plot B), and 6 (plot C) in 100 mM HEPES buffer at pH 7.00. Parameters of best fit for the binding curves can be found in Table 4.4.

The first binding site for dipeptides has an affinity with the same order of magnitude as histidine 1. The negative values of enthalpy indicate that the interaction between 4/5/6 and Zn$^{2+}$ are stronger and more specific than the interaction between histidine 1 and Zn$^{2+}$, which had a positive enthalpy. However, the observed Gibbs free energy for histidine 1 binding is lower than for any of the dipeptides, therefore this complexation reaction can be considered to be more spontaneous.
Table 4.4. Thermodynamic parameters for 4, 5, and 6 binding to Zn$^{2+}$ at pH 7.0 obtained from ITC measurements in 100 mM HEPES buffer at 25°C.

<table>
<thead>
<tr>
<th>Best Fit Parameters using the Sequential Site Model</th>
<th>Compound 4</th>
<th>Compound 5</th>
<th>Compound 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_1 /10^4$ M$^{-1}$</td>
<td>1.02 ± 0.07</td>
<td>5.07 ± 1.1</td>
<td>2.39 ± 0.27</td>
</tr>
<tr>
<td>$\Delta H_1 /$cal mol$^{-1}$</td>
<td>-268.2 ± 7.87</td>
<td>-861.7 ± 14.2</td>
<td>-772.9 ± 7.59</td>
</tr>
<tr>
<td>$\Delta S_1 /$cal mol$^{-1}$ deg$^{-1}$</td>
<td>17.4</td>
<td>18.6</td>
<td>17.4</td>
</tr>
<tr>
<td>$\Delta G_1 /$kcal mol$^{-1}$</td>
<td>-5.46</td>
<td>-6.41</td>
<td>-5.96</td>
</tr>
<tr>
<td>$K_2 /10^3$ M$^{-1}$</td>
<td>2.72 ± 0.08</td>
<td>5.05 ± 0.36</td>
<td>3.28 ± 0.11</td>
</tr>
<tr>
<td>$\Delta H_2 /$cal mol$^{-1}$</td>
<td>-2548 ± 15.2</td>
<td>-2145 ± 34.0</td>
<td>-2273 ± 20.8</td>
</tr>
<tr>
<td>$\Delta S_2 /$cal mol$^{-1}$ deg$^{-1}$</td>
<td>7.17</td>
<td>9.75</td>
<td>8.46</td>
</tr>
<tr>
<td>$\Delta G_2 /$kcal mol$^{-1}$</td>
<td>-4.69</td>
<td>-5.05</td>
<td>-4.80</td>
</tr>
</tbody>
</table>

4.2.3.3 Analysis of ITC data for the titration of diketopiperazines into Zn$^{2+}$

The binding curve for 7 had an insignificant signal and therefore it is possible that detected binding was the result of an impurity and not true cation binding. No changes in the binding isotherms for 55 and 61 titrated into Zn$^{2+}$ were detected. Compound 61 showed considerable deviations in chemical shift of imidazole protons upon Zn$^{2+}$ addition in NMR experiments (refer to Section 4.1.2.4); the discrepancy between NMR and ITC results may be due to several factors. NMR experiments are sensitive to conformational changes that may not result in net energy changes or complex formation, whereas ITC would not detect such shifts in ligand conformation. Also, the concentrations run in NMR experiments were higher than those used in ITC experiments, so weaker complexation interactions may not have been detected. ITC experiments could be run at higher concentrations in samples that showed promising results by NMR but did not translate into detectable binding curves in ITC experiments.
4.2.3.4 Analysis of ITC data for the titration of imidazolidinediones into Zn\(^{2+}\)

No changes in the binding isotherms of \(63, 66, 67, 68, 69,\) and \(71\) were found. Compound \(63\) and compounds \(66-69\) were not expected to bind with Zn\(^{2+}\), as NMR spectra were not altered with Zn\(^{2+}\) addition, although \(71\) did show changes in NMR spectra with Zn\(^{2+}\) addition in the aromatic proton region.

The binding isotherm for the titration of \(75\) into Zn\(^{2+}\) in 100 mM HEPES buffer at pH 7.0 is shown in Figure 4.13. The curve is fit by a one-site binding model and the parameters of best fit can be found in Table 4.5. The value of \(N\) suggests that \(75\) is binding to Zn\(^{2+}\) in a ratio of \(1.46:1\). This binding stoichiometry is non-integer, which could be the result of several factors. First, the recorded concentration of either \(75\) or ZnSO\(_4\)
\textbullet 7H\(_2\)O solutions may be incorrect and causing errors in the fit. As well, a non-uniform protonation state at pH 7.0 may also be causing non-integer binding stoichiometry. This could be further explored by measuring the pKa of \(75\) and correlating with ITC data.

Similar to what was observed with histidine 1, this complexation reaction is opposed enthalpically although favoured entropically, and exergonic in nature.

\[\text{Figure 4.13. ITC data for the titration of 0.33 mM Zn}^{2+}\text{ with 10 mM 75 in 100 mM HEPES buffer at pH 7.0. Parameters of best fit for the binding curve can be found in Table 4.5.}\]
**Table 4.5.** Thermodynamic parameters for 75 binding to Zn$^{2+}$ at pH 7.0 obtained from ITC measurements in 100 mM HEPES buffer at 25°C.

<table>
<thead>
<tr>
<th>Best Fit Parameters using the One-Site Model</th>
<th>Compound 75</th>
</tr>
</thead>
<tbody>
<tr>
<td>N /mol titrant/mol Zn$^{2+}$</td>
<td>1.46 ± 0.04</td>
</tr>
<tr>
<td>K /10$^4$ M$^{-1}$</td>
<td>1.69 ± 0.22</td>
</tr>
<tr>
<td>ΔH /cal mol$^{-1}$</td>
<td>201.2 ± 7.30</td>
</tr>
<tr>
<td>ΔS /cal mol$^{-1}$ deg$^{-1}$</td>
<td>20.0</td>
</tr>
<tr>
<td>ΔG /kcal mol$^{-1}$</td>
<td>-5.76</td>
</tr>
</tbody>
</table>

**4.2.3.5 Analysis of ITC data for the titration of atypical amino acids into Zn$^{2+}$**

The binding curve for 42, and to a lesser extent 43 and 44, showed a large amount of drift over time, although no binding event was measurable. The binding isotherm of 42 titrated into Zn$^{2+}$ in 100 mM HEPES at pH 7.0 is shown in Figure 4.14. These results do not correspond to the NMR experiments that showed changes in chemical shift of $^1$H NMR spectra of 42 with Zn$^{2+}$ addition; possible reasons for this discrepancy were discussed in Section 4.2.3.3.

![Figure 4.14](image)

**Figure 4.14.** ITC data for the titration of 0.33 mM Zn$^{2+}$ with 10 mM 42 in 100 mM HEPES buffer at pH 7.0.

It appears that measurable binding interactions with Zn$^{2+}$ occur most frequently with compounds that possess both a carboxylate group as well as an imidazole ring. The ITC results presented do
not fully correlate with NMR experiments, although NMR experiments would detect minor changes that might not be the result of a true complexation interaction but rather due to changes in the electronic environment of ligands upon addition of a cationic species. Further investigation could be done to assess if increasing concentration in ITC experiments changes results. The heterogeneity in the protonation state of the ligands described herein at physiologically relevant pH may make ITC an inappropriate method for assessing chelation in solution. Follow up experiments of determining the pKa of compounds would help elucidate if such heterogeneous states are causing inaccuracies in fits for binding isotherms.
CHAPTER 5

5 *In Vitro* Testing of Histidine-Based Small Molecules Against Zn$^{2+}$-Induced Toxicity
5.1 *In Vitro* Zn\(^{2+}\) Toxicity Studies

As described in Sections 1.2.5 and 1.4.1.3, metal chelation is a possible mechanism for neuroprotection in ischemic stroke. Prevention of Zn\(^{2+}\) toxicity has been shown by administering TPEN *in vitro*, for example (Canzoniero et al. 2003). With synthesized molecules in hand, assessment of their ability to prevent Zn\(^{2+}\)-induced cell death *in vitro* was analyzed. This assay also served as a test for any general toxicity associated with synthesized compounds. Imaging experiments were also conducted.

5.2 *In Vitro* Methods

5.2.1 Cell Toxicity Assay

The ability of histidine 1 and synthesized analogues to protect against Zn\(^{2+}\)-induced toxicity was quantified. All experiments were carried out using the Neuro 2a (N2a, mouse brain neuroblastoma) cell line, obtained from American Type Culture Collection. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) high glucose with L-glutamine and sodium pyruvate supplemented with 10% (v/v) fetal bovine serum and 1% Antibiotic-Antimycotic. Cells were grown at 37°C in a humidified environment maintained at 95% O\(_2\) and 5% CO\(_2\). N2a cells were seeded in 96-well plates at a density of 20,000 cells/well in supplemented DMEM/high glucose. Experiments were performed 24 hours after seeding, at approximately 70% confluence. Experimental treatments involved 6 conditions for each histidine analogue, explained in Table 5.1, as well as appropriate control treatments. ZnSO\(_4\)•7H\(_2\)O and compound solutions were made in DMEM/high glucose. Briefly, 200 µM and 300 µM ZnSO\(_4\)•7H\(_2\)O solutions with and without 1 mM solutions of histidine analogues were added to culture wells. The effect of one-hour incubation with ZnSO\(_4\)•7H\(_2\)O solution before compound addition as well as one-hour incubation with compound solutions prior to Zn\(^{2+}\) addition was also assessed (conditions 3-6). Experiments were performed in triplicate and cell viability was measured using PrestoBlue Viability Reagent at 6 and 24-hour time points. PrestoBlue is a cell permeable resazurin based dye that is reduced to a fluorescent red metabolite by living cells, quantifying cell viability by measuring proliferation of cells. Fluorescence was monitored using a Cytation 3 Cell Imaging Multi-Mode Reader or Synergy H4 Hybrid Microplate Reader.
Table 5.1. Treatments of N2a cells; ‘compound’ represents histidine 1 and analogues. All experiments were performed in triplicate and cell viability assessed at 6 and 24-hour time points.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Experimental Treatment</th>
<th>Intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200 µM ZnSO₄•7H₂O</td>
<td>1 mM compound</td>
</tr>
<tr>
<td>2</td>
<td>300 µM ZnSO₄•7H₂O</td>
<td>1 mM compound</td>
</tr>
<tr>
<td>3</td>
<td>1 hour incubation with 1 mM compound</td>
<td>200 µM ZnSO₄•7H₂O</td>
</tr>
<tr>
<td>4</td>
<td>1 hour incubation with 1 mM compound</td>
<td>300 µM ZnSO₄•7H₂O</td>
</tr>
<tr>
<td>5</td>
<td>1 hour incubation with 200 µM ZnSO₄•7H₂O</td>
<td>1 mM compound</td>
</tr>
<tr>
<td>6</td>
<td>1 hour incubation with 300 µM ZnSO₄•7H₂O</td>
<td>1 mM compound</td>
</tr>
</tbody>
</table>

5.2.2 Zn²⁺ Imaging

Intracellular Zn²⁺ was imaged using the cell-permeant Zn²⁺-sensitive fluorescent dye, FluoZin-3AM (Invitrogen). N2a cells were seeded in 96-well plates at a density of 20,000 cells/well in supplemented DMEM/high glucose medium; experiments were performed at approximately 70% confluency, 24 hours after seeding. Experimental treatments involved conditions 1 and 2 from Table 5.1 and only histidine 1 was used in imaging work. Six hours following experimental treatment, treatment media was removed and 100 µL of DMEM/high glucose medium containing 1 µM FluoZin-3AM and 0.02% Plurionic F-127 (Invitrogen) was added to wells. Pluronic F-127 is a non-ionic detergent that assists in dispersing the non-polar FluoZin-3AM in culture media. Cells were incubated at RT for 45 minutes, followed by washing (4X) with Ca²⁺- and Mg²⁺-free phosphate-buffered saline and incubated for a further 30 minutes at RT. Intracellular Zn²⁺ images were acquired with an EVOS FL Auto Imaging System at 20x magnification using the green-fluorescence-protein channel (excitation = 494 nm, emission = 516 nm).

5.3 Results and Discussion for In Vitro Data

Conditions 3-6 shown in Table 5.1 were only performed for a subset of compounds; no difference was found in the viability of N2a cells pretreated with Zn²⁺ (conditions 5 and 6) or compound (conditions 3 and 4) in comparison to N2a cells not pretreated therefore the rest of the compounds were not assessed under these conditions.

5.3.1 Results for the use of histidine in a Zn²⁺-induced cell toxicity assay

Histidine 1 was shown to fully rescue N2a cells from Zn²⁺-induced toxicity at both 200 µM and 300 µM concentrations (results shown in Figure 5.1). In fact, the viability of the control condition for histidine 1 was significantly increased ($P < 0.001$) compared to the vehicle control.
condition (no Zn\(^{2+}\) or histidine I added). Histidine is an essential amino acid, required for protein synthesis and cell growth, therefore it is not surprising that cells grown in supplemented media show increased proliferation.

**Figure 5.1.** Assessment of cell viability following Zn\(^{2+}\) exposure with or without 1 mM treatment solutions as indicated. Cell viability was quantified using fluorescence 24 hours after treatment and normalized against the cell viability of the assay control. Bars represent SE (*** = \(P < 0.001\), unpaired t-test, comparisons with respect to corresponding control group).

This *in vitro* evidence of protection, coupled with NMR and ITC experiments suggest that the toxic cations are being “tied up” by histidine I and cell death subsequently prevented. Since histidine I and Zn\(^{2+}\) are both cell permeant, it is possible that this mechanism is occurring both intracellularly as well as extracellularly. However, although the evidence presented points at metal chelation as the mechanism of action for protection, it does not rule out histaminergic system activation or antioxidant action. To provide further evidence for the metal chelation mechanism, a Zn\(^{2+}\) imaging agent (FluoZin-3AM, Invitrogen) was used to assess whether Zn\(^{2+}\) localization into the cell was diminished with co-administration of histidine I in comparison to the control group. Results for imaging experiments are shown in Figure 5.2.
It is clear from this imaging experiment that histidine 1 is preventing Zn$^{2+}$ entry into cells at both 200 µM and 300 µM Zn$^{2+}$ concentrations tested. This data lends more evidence to the mechanism of protection being metal chelation.

5.3.2 Results for the use of esters in a Zn$^{2+}$-induced cell toxicity assay

Commercially available 2 and synthesized compound 3 were assessed in this assay; results are shown in Figure 5.3.
It was found that 2 rescued cells fully from Zn\textsuperscript{2+} toxicity. However, in Chapter 4 it was found that in buffered HEPES solution at pH 7, the methyl and benzyl esters are partially cleaved to the free acid. Therefore, it is most likely that the protection by 2 is a result of ester cleavage to histidine 1. One would assume that ester hydrolysis and subsequent protection from Zn\textsuperscript{2+} would also occur with administration of 3, however, this was not found. In fact, the benzyl ester seemed to increase toxicity at 200 µM Zn\textsuperscript{2+} concentrations, although the difference was not significant. This ester was poorly soluble due to its lipophilic benzyl moiety; therefore it is possible that the compound was inadequately dispersed before administration.

5.3.3 Results for the use of dipeptides in a Zn\textsuperscript{2+}-induced cell toxicity assay

Results from synthesized dipeptides 4, 5, and 6 in the cell viability assay are shown in Figure 5.4. All dipeptides showed full rescue of N2a cells from both 200 µM and 300 µM Zn\textsuperscript{2+}. Results from NMR and ITC experiments show these molecules are chelators of Zn\textsuperscript{2+}, with dissociation constants in a similar range of histidine 1. The hypothesis that metal chelation is the predominant mechanism of action for Zn\textsuperscript{2+} protection is strengthened by these findings, but imaging experiments could provide further evidence. All dipeptides showed similar levels of protection at 1 mM concentrations. Further work could be done to find the IC\textsubscript{50} of each dipeptide to elucidate if there is a difference in the potency of diastereomers.

![Figure 5.4](image-url)

**Figure 5.4.** Assessment of cell viability following Zn\textsuperscript{2+} exposure with or without 1 mM treatment solutions as indicated. Cell viability was quantified using fluorescence 24 hours after treatment and normalized against the cell viability of the assay control.

It is possible that cleavage of the amide bond in these dipeptides is occurring, releasing two molecules of histidine 1, which are in reality providing the protection from Zn\textsuperscript{2+} toxicity. NMR
and ITC results showing that 4, 5, and 6 form complexes with Zn\(^{2+}\) favor metal chelation as the protective pathway, although another mechanism activated by histidine 1 is not ruled out.

### 5.3.4 Results for the use of diketopiperazines in a Zn\(^{2+}\)-induced cell toxicity assay

Prepared diketopiperazines were not successful at rescuing cells from Zn\(^{2+}\) toxicity (see Figure 5.5). In fact, 61 significantly increased cell death at both Zn\(^{2+}\) concentrations tested. The control for this compound did not cause cell death, however, so it is in combination with Zn\(^{2+}\) that 61 agonizes cell death. The pathway causing increased cell death is unclear from these experiments, although it may result from increased Zn\(^{2+}\) transport across the cell membrane. It has been found *in vitro* and *in vivo* that administration of histidine 1 significantly increased Zn\(^{2+}\) uptake in rat erythrocytes and across the BBB, respectively, possibly due to the facilitated transport of \((\text{Zn}^{2+}\cdot\text{His})^+\) complex by an amino acid transporter (Buxani-Rice, Ueda, and Bradbury 1994; Yokel 2006; Aiken, Horn, and Saunders 1992). Zn\(^{2+}\) imaging experiments could confirm if a similar process if causing increased uptake of Zn\(^{2+}\) in the presence of 61. However, this phenomenon appears to be dependent on the concentration of histidine 1 present and at higher doses, Zn\(^{2+}\) uptake is inhibited (Wensink et al. 1988). Studies also show that when histidine 1 is added to cells preloaded with Zn\(^{2+}\), Zn\(^{2+}\) efflux is increased (Aiken, Horn, and Saunders 1992). The interplay of such entry and exit routes is complex and it is difficult to decipher which will be dominant.

![Figure 5.5](image-url) **Figure 5.5.** Assessment of cell viability following Zn\(^{2+}\) exposure with or without 1 mM treatment solutions as indicated. Cell viability was quantified using fluorescence 24 hours after treatment and normalized against the cell viability of the assay control. Bars represent SE (\(* = P \leq 0.05\), \(** = P \leq 0.01\), unpaired t-test, comparisons with respect to corresponding control group).
5.3.5 Results for the use of imidazolidinediones in a Zn\(^{2+}\)-induced cell toxicity assay

Results for imidazolidinediones assessed in the cell toxicity assay are shown in Figure 5.6. No synthesized compounds protected cells from Zn\(^{2+}\) toxicity. Similar to diketopiperazine 61, imidazolidinedione 75 caused increased cell death in N2a cells. The mechanism of increased cell death is not clear. The minimal success in NMR experiments, the negative results from ITC experiments, and lack of *in vitro* protection from Zn\(^{2+}\) indicates that 2,4-imidazolidinediones substituted with heterocyclic moieties are not a platform to pursue further as possible Zn\(^{2+}\) chelators.

![Graph showing cell viability](image)

**Figure 5.6.** Assessment of cell viability following Zn\(^{2+}\) exposure with or without 1 mM treatment solutions as indicated. Cell viability was quantified using fluorescence 24 hours after treatment and normalized against the cell viability of the assay control. Bars represent SE (* = \(P \leq 0.05\), unpaired t-test, comparisons with respect to corresponding control group).

5.3.6 Results for the use of atypical amino acids in a Zn\(^{2+}\)-induced cell toxicity assay

Synthesized atypical amino acids were not able to rescue cell from Zn\(^{2+}\) toxicity after administration at 1 mM concentrations, shown in Figure 5.6. However, at 2 mM, compound 42 was able to partially rescue cells from 200 \(\mu\)M Zn\(^{2+}\) (results shown in Figure 5.7).
Figure 5.7. Assessment of cell viability following Zn\(^{2+}\) exposure with or without 1 mM treatment solutions as indicated. Cell viability was quantified using fluorescence 24 hours after treatment and normalized against the cell viability of the assay control.

Figure 5.8. Assessment of cell viability following 200 µM Zn\(^{2+}\) exposure with or without administration of 42 (2 mM). Cell viability was quantified using fluorescence 24 hours after treatment and normalized against the cell viability of the assay control. Bars represent SE (** = P ≤ 0.001 relative to control; ### = P < 0.001 relative to 200 µM Zn\(^{2+}\), unpaired t-test).

The control for 42 resulted in significantly lower cell viability than the control condition, albeit the difference was minimal. NMR experiments showed large deviations in spectra of 42 (refer to Figure 4.8) as Zn\(^{2+}\) is added, although ITC experiments did not show measurable binding curves. It is still possible that there is an interaction with Zn\(^{2+}\) occurring, although measurable binding energies are not found experimentally by ITC. Another technique, such as potentiometric titration, could be employed to confirm if 42 is interacting with Zn\(^{2+}\) or not. It is clear that compounds requiring dosing at 2 mM are not practically effective, but this molecule could be another platform from which to explore the design of Zn\(^{2+}\) chelators. Figure 5.8 suggests that although inferior to imidazole, the pyridine ring may be capable of also chelating Zn\(^{2+}\). It would
be relevant to synthesize other atypical amino acids with varying heterocyclic substitutions, as this platform has potential for inhibiting Zn$^{2+}$ toxicity in vitro.

Thus far, it is possible that the compounds successful at inhibiting cell death in the presence of Zn$^{2+}$ (4, 5, 6) are potentially fragmented into histidine 1, which in reality is providing protection from the cation. Therefore, 42 represents a compound that suggests the hypothesis that histidine 1 may be used as a platform around which to design new chemical entities that chelate labile Zn$^{2+}$ and prevent metal-ion toxicity is indeed plausible.
CHAPTER 6

6 Conclusions and Future Directions
6.1 Conclusions

The goals of this research project were to design and synthesize histidine-based small molecules and to evaluate these molecules for their ability to chelate Zn$^{2+}$ as well as inhibit Zn$^{2+}$ toxicity *in vitro*. A preliminary determination of the therapeutic potential of these compounds for preventing Zn$^{2+}$-induced neuronal cell death following ischemic stroke was performed.

Zn$^{2+}$ was chosen as the target of interest in the ischemic cascade as it is the most recently implemented neurotoxic cation in the pathophysiology of stroke and thus has not been fully explored. Histidine was selected as an endogenous platform around which to design neuroprotectants due to its capability of chelating divalent metal cations, its neuroprotective properties, and its ability to cross the BBB, a feature most common chelators do not possess due to their highly charged state. First, a computational study assessing the *in silico* interactions between divalent metal cations and a variety of small molecules based on the endogenous molecule histidine was performed. Compounds were designed rationally through simple modifications of the parent molecule using peptidomimetic approaches and heterocycle substitutions, for example. Preferential cation binding to the carboxylate and imidazole functionalities was revealed; interactions were primarily ionic in nature.

A subset of molecules was then selected and synthesized using standard organic chemistry synthetic routes, purified by column chromatography and HPLC, and characterized by NMR, mass spectrometry, and melting point determination. A high degree of molecular diversity was incorporated into this study as it is the initial study of this nature.

With molecules in hand, complexation with Zn$^{2+}$ was studied qualitatively using NMR spectroscopy and quantitatively using ITC. NMR spectroscopy results revealed histidine, dipeptides, select diketopiperazines and imidazolinediones, and atypical amino acids interacted with Zn$^{2+}$ as evidenced by detectable changes in chemical shift. These results mimic the *in silico* predictions, with di-substituted diketopiperazines having less pronounced changes in chemical shift than dipeptides and atypical amino acids. Generally, imidazolinediones did not interact with Zn$^{2+}$.

In order to quantify the interaction between synthesized analogues and Zn$^{2+}$, ITC was employed. Measurable binding curves were recorded for histidine 1, 4, 5, 6, and 75. These results correlate
with NMR experiments, although several of the compounds that showed distinct changes in NMR spectra with Zn\(^{2+}\) addition did not produce measurable binding curves. This discrepancy could be due to variables such as the difference in sensitivity between the two techniques and the differences in the experimental concentrations used.

An examination of the inhibition of Zn\(^{2+}\) toxicity \textit{in vitro} revealed that histidine 1, 4, 5, 6, and 42 were successful at blocking Zn\(^{2+}\)-induced cell death. Cumulative experimental results suggest that the observed protective effects \textit{in vitro} are the result of Zn\(^{2+}\) chelation prohibiting intracellular metal accumulation and subsequent cell death. It is possible that the protective effects are also due to histaminergic system activation, as suggested by Adachi et al. (Adachi, Liu, and Arai 2005), or another unknown mechanism, however, Zn\(^{2+}\) imaging experiments provide additional evidence for a mechanism of metal chelation.

Of the classes of compounds synthesized and assessed (i.e. esters, dipeptides, diketopiperazines, imidazolidinediones, and amino acids), dipeptides and amino acids showed the most promise for potential to chelate Zn\(^{2+}\) and prevent it from causing damage \textit{in vitro}.

### 6.2 Future Directions

There are a number of further experiments that can be conducted to expand upon the research described in this thesis. Additional imaging experiments that monitor cellular Zn\(^{2+}\) influx using a fluorescence probe could be conducted for 4, 5, 6, and 42. This would help confirm if the mechanism of action that is providing protection from Zn\(^{2+}\) toxicity is also metal chelation for these molecules. These experiments could also help to elucidate if compounds that increased cell death (61 and 75) is the result of increased transport of Zn\(^{2+}\) across the cell membrane.

Another way in which the \textit{in vitro} work could be expanded upon would be to employ antagonists of the histaminergic receptors, specifically the H\(_2\) receptor, in order to rule out this mechanism of action. Although there is only \textit{in vivo} literature suggesting this mechanism of action, it is still a possible route of protection, as the H\(_2\) receptor has been shown to suppress inflammation and Zn\(^{2+}\) exposure results in inflammatory pathways activation.

The discrepancy between some NMR and ITC results (namely the atypical amino acids) could be clarified by using another technique to monitor the interaction of Zn\(^{2+}\) and synthesized
compounds in solution. A literature review revealed that pH-potentiometric titration has been used in the past to monitor such interactions, and could therefore be explored. Such pH titrations may also be valuable as they would provide information about the pKa and protonation state of compounds at pH 7.0, which could be correlated with the ITC results to potentially clarify non-integer binding stoichiometry.

In order to determine if histidine or histidine-based small molecules are viable clinical candidates, a threshold of the Zn$^{2+}$ binding affinity needed for neuroprotection should be determined. For example, in a more physiologically relevant *in vitro* or *in vivo* model, does a strong chelator such as CaEDTA need to be administered to prevent Zn$^{2+}$-induced neuronal death, or can a slightly less effective chelator, like histidine, also prevent neuronal death? Establishing this would help direct the design of future molecules. It should also be noted that even if histidine or similar molecules do not provide measurable functional outcomes *in vivo*, the act of preventing Zn$^{2+}$ entry into neurons is beneficial, as the detrimental pathways initiated by Zn$^{2+}$ release would be diminished. This inhibition could be utilized in other indications involving Zn$^{2+}$ dyshomeostasis, such as status epilepticus or even osteoarthritis. Ischemic stroke is a highly complex disease state and it is unlikely that targeting one pathway would provide full protection of vulnerable tissues.

As it stands, there are no neuroprotectants available for clinical use. Histidine is a non-toxic, low cost, BBB penetrant molecule that shows *in vitro* and *in vivo* protection from Zn$^{2+}$ toxicity and in focal ischemia, respectively, and should therefore be considered as a potentially beneficial molecule. The project described herein assessed drug-like platforms using histidine as a model ligand. It was revealed that diketopiperazines and imidazolidinediones were not effective at chelating Zn$^{2+}$ or preventing Zn$^{2+}$-induced cell death *in vitro*, thus these platforms should not be further pursued. Moving forward, peptidomimetic approaches and bioisosteric substitutions to make dihistidine molecules more drug-like should be performed. Also, substitutions of the histidine imidazole ring with a more in depth series of N/S-containing heterocycles should be explored for utility as Zn$^{2+}$ chelators, as such a substitution with pyridine produced positive results in the cell toxicity assay. It is important to consider the BBB penetrability, as small polar molecules would be dependent on uptake by a transporter and therefore need to be substrates for such. Computational modeling could potentially be used to inform on this aspect.
From this research it became apparent that of the molecules assessed, histidine was the lead compound as a potential clinical candidate; histidine is widely available in pharmaceutical form at a low cost, has shown to be non-toxic when administered at high doses in loading tests, and is BBB penetrant. Based upon this research, collaborators at Queens University, Drs. Albert Jin and J. Gordon Boyd, are initiating a clinical trial in which L-histidine will be administered to individuals who have had a global ischemic event from a cardiac arrest. Inclusion criteria includes the return of spontaneous circulation after either in-hospital or out of hospital cardiac arrest and being in a comatose (unresponsive to 1-step verbal commands) state. The intervention will be twice daily enteral dosing of histidine for three days versus placebo. Follow-up assessment will occur at 3 and 12 months and include neurological testing and testing with a robotic device – the Kinesiological Instrument for Normal and Altered Reaching Movement (KINARM), which monitors improvements in patients movement and coordination.

The road to the development of a drug candidate is long and complex, and the multifaceted nature of ischemic stroke makes developing neuroprotective therapeutics even more challenging. Ischemic stroke is not a trivial target, and traditional preclinical assays (e.g. receptor binding) are not applicable, making research difficult and expensive. Targeting the chelation of toxic Zn$^{2+}$ is a relatively new approach in stroke research, and further evaluation is warranted. Compounds were produced that interacted with Zn$^{2+}$ and were active in vitro, however, these molecules do not offer improvements over histidine. This project has demonstrated that it is possible to interact with Zn$^{2+}$ and prevent associated toxicity; therefore such histidine-based molecules are worthy of further exploration.
References


