Elucidation of the Histone Deacetylase 6 Pharmacophore

BY

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THESIS
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JHK
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Epigenetic Regulation</td>
<td>1</td>
</tr>
<tr>
<td>1.2 HDAC Classification</td>
<td>3</td>
</tr>
<tr>
<td>1.3 HDAC Catalytic Mechanism</td>
<td>7</td>
</tr>
<tr>
<td>1.4 Design of HDAC Inhibitors</td>
<td>9</td>
</tr>
<tr>
<td>1.5 Therapeutic Applications of HDAC Inhibitors</td>
<td>11</td>
</tr>
<tr>
<td>2. DISCOVERY OF HDAC6 SELECTIVE MERCAPTOACETAMIDES</td>
<td>15</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>15</td>
</tr>
<tr>
<td>2.2 Chemistry</td>
<td>16</td>
</tr>
<tr>
<td>2.3 Results and Discussion</td>
<td>29</td>
</tr>
<tr>
<td>2.3.1 HDAC Inhibition</td>
<td>29</td>
</tr>
<tr>
<td>2.3.2 Molecular Modeling</td>
<td>33</td>
</tr>
<tr>
<td>2.3.3 Mercaptoacetamide Oxidation</td>
<td>38</td>
</tr>
<tr>
<td>2.3.4 Neuroprotection and Glutathione Depletion</td>
<td>39</td>
</tr>
<tr>
<td>2.4 Conclusion</td>
<td>48</td>
</tr>
<tr>
<td>2.5 Experimental Section</td>
<td>49</td>
</tr>
<tr>
<td>2.5.1 Chemistry</td>
<td>49</td>
</tr>
<tr>
<td>2.5.2 HDAC Inhibition</td>
<td>71</td>
</tr>
<tr>
<td>2.5.3 Molecular Modeling</td>
<td>72</td>
</tr>
<tr>
<td>2.5.4 Mercaptoacetamide Oxidation</td>
<td>73</td>
</tr>
<tr>
<td>2.5.5 Neuroprotection and Glutathione Depletion</td>
<td>74</td>
</tr>
<tr>
<td>3. OPTIMIZATION OF TRICYLIC HDAC6 SELECTIVE INHIBITORS</td>
<td>76</td>
</tr>
<tr>
<td>3.1 Introduction</td>
<td>76</td>
</tr>
<tr>
<td>3.2 Chemistry</td>
<td>77</td>
</tr>
<tr>
<td>3.3 Results and Discussion</td>
<td>82</td>
</tr>
<tr>
<td>3.3.1 HDAC Inhibition</td>
<td>82</td>
</tr>
<tr>
<td>3.3.2 Regulatory T cell Suppression</td>
<td>89</td>
</tr>
<tr>
<td>3.4 Conclusion</td>
<td>101</td>
</tr>
<tr>
<td>3.5 Experimental Section</td>
<td>101</td>
</tr>
<tr>
<td>3.5.1 Chemistry</td>
<td>101</td>
</tr>
<tr>
<td>3.5.2 HDAC Inhibition</td>
<td>131</td>
</tr>
<tr>
<td>3.5.3 Regulatory T cell Suppression</td>
<td>131</td>
</tr>
<tr>
<td>3.5.3.1 Mitotic Suppression Assays</td>
<td>131</td>
</tr>
<tr>
<td>3.5.3.2 Homeostatic Proliferation Assays</td>
<td>132</td>
</tr>
</tbody>
</table>
# TABLE OF CONTENTS (continued)

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>ARYL AND ALIPHATIC BASED HDAC6 SELECTIVE INHIBITORS</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.</td>
<td>Introduction</td>
<td>134</td>
</tr>
<tr>
<td>4.1</td>
<td>Chemistry</td>
<td>134</td>
</tr>
<tr>
<td>4.2</td>
<td>Results and Discussion</td>
<td>140</td>
</tr>
<tr>
<td>4.3</td>
<td>HDAC Inhibition</td>
<td>140</td>
</tr>
<tr>
<td>4.3.1</td>
<td>Neuroprotection Studies</td>
<td>140</td>
</tr>
<tr>
<td>4.4</td>
<td>Conclusion</td>
<td>146</td>
</tr>
<tr>
<td>4.5</td>
<td>Experimental Section</td>
<td>147</td>
</tr>
<tr>
<td>4.5.1</td>
<td>Chemistry</td>
<td>147</td>
</tr>
<tr>
<td>4.5.2</td>
<td>HDAC Inhibition</td>
<td>166</td>
</tr>
<tr>
<td>4.5.3</td>
<td>Neuroprotection Studies</td>
<td>167</td>
</tr>
<tr>
<td>5.</td>
<td>CONCLUSION AND FUTURE DIRECTIONS</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>CITED LITERATURE</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td>VITA</td>
<td>182</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. HDAC ISOFORM CHARACTERISTICS</td>
<td>6</td>
</tr>
<tr>
<td>II. IN VITRO HDAC ISOFORM INHIBITORY ACTIVITY</td>
<td>30</td>
</tr>
<tr>
<td>III. ELECTROSTATIC ENERGY SCORES OF THE MINIMIZED COMPLEXES FOR (R)-2.14 AND (S)-2.14</td>
<td>34</td>
</tr>
<tr>
<td>IV. IC&lt;sub&gt;50&lt;/sub&gt; AND HDAC6 SELECTIVITY DATA FOR 6-, 7-, 8- AND 9- SUBSTITUTED γ-CARBOLINES</td>
<td>84</td>
</tr>
<tr>
<td>V. IC&lt;sub&gt;50&lt;/sub&gt; AND HDAC6 SELECTIVITY DATA FOR 2-SUBSTITUTED γ-CARBOLINES</td>
<td>86</td>
</tr>
<tr>
<td>VI. IC&lt;sub&gt;50&lt;/sub&gt; AND HDAC6 SELECTIVITY DATA FOR 2-SUBSTITUTED β-CARBOLINES</td>
<td>88</td>
</tr>
<tr>
<td>VII. IC&lt;sub&gt;50&lt;/sub&gt; AND HDAC6 SELECTIVITY DATA FOR ARYL AND ALIPHATIC COMPOUNDS</td>
<td>142</td>
</tr>
<tr>
<td>FIGURE</td>
<td>PAGE</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>1. Interaction between a histone protein and DNA</td>
<td>2</td>
</tr>
<tr>
<td>2. Phylogenetic characterization of the class I, II and IV HDAC isoforms</td>
<td>4</td>
</tr>
<tr>
<td>3. Proposed mechanism of lysine deacetylation</td>
<td>8</td>
</tr>
<tr>
<td>4. Structural characteristics of HDAC inhibitors</td>
<td>10</td>
</tr>
<tr>
<td>5. Structures of known HDAC inhibitors</td>
<td>14</td>
</tr>
<tr>
<td>6. Chromatograms depicting separation of 2.10 enantiomers</td>
<td>20</td>
</tr>
<tr>
<td>7. Geometric isomers of compound 2.28</td>
<td>23</td>
</tr>
<tr>
<td>8. Chromatograms depicting separation of 2.31 enantiomers</td>
<td>26</td>
</tr>
<tr>
<td>9. Putative complexes for (R)-2.14 and (S)-2.14 with HDAC2 and the HDAC6 homology model</td>
<td>36</td>
</tr>
<tr>
<td>10. Rate of oxidation for compounds 2.13, (R)-2.14 and 2.15</td>
<td>39</td>
</tr>
<tr>
<td>11. Neuroprotection plots for selected mercaptoacetamide compounds</td>
<td>41</td>
</tr>
<tr>
<td>12. Prevention of glutathione depletion by selected mercaptoacetamides</td>
<td>47</td>
</tr>
<tr>
<td>13. CFSE-dilution plots for selected tricyclic compounds</td>
<td>90</td>
</tr>
<tr>
<td>14. Standardized suppression curves for selected tricyclic compounds</td>
<td>95</td>
</tr>
<tr>
<td>15. Standardized suppression ratios</td>
<td>98</td>
</tr>
<tr>
<td>16. In vivo assessment of HDAC6i activity using a seven-day homeostatic proliferation model</td>
<td>100</td>
</tr>
<tr>
<td>17. Neuroprotection plots for selected aryl and aliphatic HDACi</td>
<td>145</td>
</tr>
</tbody>
</table>
# LIST OF SCHEMES

<table>
<thead>
<tr>
<th>SCHEME</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Synthesis of amide linked mercaptoacetamides</td>
<td>17</td>
</tr>
<tr>
<td>2. Isolation of 2.14 enantiomers</td>
<td>18</td>
</tr>
<tr>
<td>3. Synthesis of (R)-2.10</td>
<td>19</td>
</tr>
<tr>
<td>4. Synthesis of hydroxamate 2.21</td>
<td>21</td>
</tr>
<tr>
<td>5. Synthesis of alkene linked mercaptoacetamides</td>
<td>22</td>
</tr>
<tr>
<td>6. Isolation of 2.25 geometric isomers</td>
<td>23</td>
</tr>
<tr>
<td>7. Synthesis of ether linker mercaptoacetamides</td>
<td>24</td>
</tr>
<tr>
<td>8. Isolation of 2.32 enantiomers</td>
<td>25</td>
</tr>
<tr>
<td>9. Synthesis of (R)-2.31</td>
<td>25</td>
</tr>
<tr>
<td>10. Synthesis of alkyne- and styryl-linked mercaptoacetamides</td>
<td>28</td>
</tr>
<tr>
<td>11. Preparation of 6-, 7-, 8-, and 9-substituted γ-carbolines</td>
<td>78</td>
</tr>
<tr>
<td>12. Synthesis of 2-substituted γ-carbolines</td>
<td>80</td>
</tr>
<tr>
<td>13. Synthesis of 2-substituted β-carbolines</td>
<td>82</td>
</tr>
<tr>
<td>14. Preparation of bicyclic compounds</td>
<td>136</td>
</tr>
<tr>
<td>15. Preparation of pyrrole and pyrazole compounds</td>
<td>137</td>
</tr>
<tr>
<td>16. Synthesis of 4-pyridyl and dimethylaminophenyl derivatives</td>
<td>138</td>
</tr>
<tr>
<td>17. Preparation of pyrrolidine derivatives</td>
<td>139</td>
</tr>
<tr>
<td>18. Preparation of substituted amine derivatives</td>
<td>140</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>BOC</td>
<td>tert-butyloxycarbonyl</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD4</td>
<td>cluster of differentiation 4</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CMT</td>
<td>Charcot-Marie-Tooth disease</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRO</td>
<td>contract research organization</td>
</tr>
<tr>
<td>CTCL</td>
<td>cutaneous T cell lymphoma</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>cytotoxic T-lymphocyte antigen 4</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>Foxp3</td>
<td>forkhead box P3</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>oxidized glutathione</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyltransferase(s)</td>
</tr>
<tr>
<td>HCA</td>
<td>homocysteic acid</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase(s)</td>
</tr>
<tr>
<td>HDACi</td>
<td>histone deacetylase inhibitor(s)</td>
</tr>
<tr>
<td>HDLP</td>
<td>histone deacetylase like protein</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
</tbody>
</table>
**LIST OF ABBREVIATIONS (continued)**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>HRMS</td>
<td>high resolution mass spectrometry</td>
</tr>
<tr>
<td>Hsp</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>IL-2</td>
<td>interleukin-2</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
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<tr>
<td>LRMS</td>
<td>low resolution mass spectrometry</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloprotease</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>p53</td>
<td>tumor protein 53</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCC</td>
<td>pyridinium chlorochromate</td>
</tr>
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<td>PDB</td>
<td>protein data bank</td>
</tr>
<tr>
<td>PyBOP</td>
<td>benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate</td>
</tr>
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<td>RAG</td>
<td>recombination activating gene</td>
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<td>RCSB</td>
<td>Research Collaboratory for Structural Bioinformatics</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SAHA</td>
<td>suberoylanilide hydroxamic acid</td>
</tr>
<tr>
<td>SIRT</td>
<td>sirtuin</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Teffs</td>
<td>effector T cells</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
</tbody>
</table>
**LIST OF ABBREVIATIONS (continued)**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TIC</td>
<td>total ion chromatogram</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TMS</td>
<td>tetramethylsilane</td>
</tr>
<tr>
<td>Tregs</td>
<td>regulatory T cells</td>
</tr>
<tr>
<td>TSA</td>
<td>trichostatin A</td>
</tr>
<tr>
<td>XIC</td>
<td>extracted ion chromatogram</td>
</tr>
<tr>
<td>ZBG</td>
<td>zinc binding group</td>
</tr>
</tbody>
</table>
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SUMMARY

With the Food and Drug Administration (FDA) approval of suberoylanilide hydroxamic acid (SAHA, 2006) and romidepsin (2009) for the treatment of cutaneous T cell lymphoma, histone deacetylase inhibitors (HDACi) have become the focus of numerous drug discovery efforts both in academia and in the private sector. In fact, there are currently over three hundred ongoing clinical trials involving the use of HDACi for the treatment of a multitude of diseases. Originally identified as epigenetic regulators, histone deacetylases (HDACs) were found to regulate gene expression by controlling the post-translational acetylation status of lysine residues located on histone proteins. In addition to their histone deacetylase function, these proteins were also found to interact with and deacetylate a large number of non-histone substrates. These non-histone substrates include transcription factors, chaperone proteins and redox regulators. While the epigenetic function of these proteins is of paramount importance, especially in regard to cancer therapeutics, it is the non-histone deacetylase function of these proteins that became the focus of our drug discovery efforts.

Inhibition of one isoform in particular, HDAC6, promised to provide significant therapeutic benefits. HDAC6 is a class I/Ib histone deacetylase that is usually localized to the cytosol and thus its primary function is not considered to be the deacetylation of histones. Murine knockout experiments indicated that the loss of this protein was not embryonic lethal and that mice developed normally without any noticeable physiological defects. HDAC6 has two functional deacetylase domains and is known to have a large number of non-histone substrates including α-tubulin and heat shock protein 90 (Hsp90). Aberrant α-tubulin acetylation has been identified in a number of disease states and is especially prominent in neurodegenerative diseases such as Charcot-Marie-Tooth disease (CMT) and Huntington's
SUMMARY (continued)

disease. In addition, recent studies have indicated that the modulation of Hsp90 by HDAC6 could provide significant benefits for the treatment of autoimmune and inflammatory diseases. Thus, the development of selective HDAC6 inhibitors could provide useful therapeutic agents as well as research tools to investigate the specific functions of the HDAC6 isoform.

The work outlined in this dissertation is oriented toward determining the structural features necessary to confer potent and selective HDAC6 inhibition and identifying the potential therapeutic benefits thereof. To do this, we synthesized a number of compounds with various cap groups, linkers and zinc binding group modifications. Compounds identified in this work were found to exhibit subnanomolar potency at HDAC6 and more than 7,000 fold selectivity for HDAC6 compared to HDAC1. Investigation of their pharmaceutical potential revealed several therapeutic benefits in models of neuroprotection, autoimmunity and immunosuppression both in vitro and in vivo. We found that the use of substituted mercaptoacetamide zinc binding groups in place of the traditional hydroxamic acids could provide similar potency at HDAC6. We also found that substitutions to the 2-position of both β- and γ-carboline derivatives were desirable for selective HDAC6 inhibition. In addition, the use of indole or substituted monoaryl based cap groups provided similar HDAC6 potency and selectivity. Inhibition of HDAC6 is able to provide numerous therapeutic benefits without resulting in the same toxicity observed with broad spectrum HDAC inhibition. The work described herein provides a testament to the therapeutic use of selective HDAC6 inhibitors and identifies structural features that are beneficial for the design of such compounds.
CHAPTER 1

INTRODUCTION

1.1 Epigenetic Regulation

Epigenetic modifications such as acetylation, methylation, phosphorylation, ubiquitylation and sumoylation effectively serve to regulate gene expression without altering the genetic sequence itself.\(^1\) In particular, the post-translational acetylation status of lysine residues located on the N-termini of histone proteins is regulated by two competing enzymes, the HDACs and the histone acetyl transferases (HATs). This regulation is accomplished through modulation of electrostatic interactions between the negatively charged phosphate groups of the deoxyribonucleic acid (DNA) backbone and the positively charged ε-amino groups of lysine residues located on histone proteins.\(^3\) At physiological pH, the free amino group on the lysine side chain is protonated and, as a result, is positively charged. The interaction between this positively charged amino group and the negatively charged phosphate group of the DNA backbone causes the DNA to be tightly wound around the histone which prevents ribonucleic acid (RNA) polymerase from accessing and transcribing the DNA. HAT catalyzes the transfer of an acetyl group to the amino group of the lysine side chain resulting in the formation of an acetamide, which is neutral at physiological pH. This disrupts the electrostatic interaction allowing the DNA to uncoil from the histone and undergo transcription (Figure 1).\(^9, 10\) Thus, inhibition of HDAC activity would, theoretically, result in an increase in gene transcription.
Figure 1. Depiction of the interaction between the lysine side chain of a histone protein and the phosphate group of the DNA backbone (left). When acetylated, this electrostatic interaction is disrupted which allows the DNA to uncoil from around the histone (right).(10)

HDAC inhibition, however, also results in the decreased expression of almost the same number of genes as are up regulated. This is believed to result from the hyperacetylation of non-histone substrates as HDAC proteins are also known to act on a variety of transcription factors, chaperone proteins, and redox regulators. (11, 12) As a result, there have been numerous therapeutic applications for HDACi reported in the literature ranging from cancer
to neurodegeneration. Indeed, two HDACi, SAHA (2006) and romidepsin (2009), have been approved by the FDA for the treatment of cutaneous T cell lymphoma (CTCL).\(^{(13, 14)}\) However, one of the major obstacles facing the development of HDACi for clinical uses beyond oncology stems from the toxicity associated with inhibition of the class I isoforms.\(^{(15)}\) While some toxicity may be tolerable and even necessary when designing drugs for oncologic purposes, it may well prevent these drugs from ever reaching clinical trials for the vast majority of other disease states.\(^{(16, 17)}\) Thus, elimination of these side effects is desirable, and one way to accomplish this is to develop inhibitors that are selective for the particular isoform of interest. In our case, HDAC6 has emerged as a promising drug target as recent research has indicated that selective inhibition does not exhibit the cytotoxic profile associated with inhibition of the class I isoforms.\(^{(18)}\)

1.2 **HDAC Classification**

The histone deacetylase family of proteins currently consists of 18 distinct isoforms that comprise four separate classes ranging in size from approximately 33 to more than 130 kDa with HDAC6 being the largest. Of these classes, three are Zn\(^{2+}\) dependent, class I, II and IV, and one is NAD\(^+\) dependent, class III. The class I HDAC proteins include isoforms 1, 2, 3 and 8 which are structurally related to the yeast homolog, RPD3. The class II HDAC proteins are homologous to the yeast deacetylase, HDA1, and are further subdivided into class IIa and class IIb. HDAC class IIa consists of isoforms 4, 5, 7 and 9 while HDAC class IIb consists of isoforms 6 and 10. HDAC11 is the sole member of class IV as it shares structural features with both the class I and class II HDAC proteins; however, its low sequence similarity warrants its separate classification.\(^{(19-21)}\) All of the Zn\(^{2+}\) dependent isoforms are related and their phylogenetic similarities can be observed in Figure 2.\(^{(20)}\) Seven isoforms (SIRT1-7) make up the class III HDACs which are collectively referred to as the sirtuins or
the Sir2 proteins. Despite the significant difference in size and their unique phylogenetic characteristics, the HDAC active site is highly conserved making the design of isoform selective inhibitors difficult.\,(21, 22) However, small structural differences among isoforms do exist and these are what we attempt to exploit when designing compounds. In particular, variations in the residues comprising the entrance to the catalytic channel and the catalytic channel itself have been the focal point of our inhibitor design.\,(23)

Figure 2. Phylogenetic characterization of the class I, II and IV HDAC isoforms.\,(20)
The class I and class II HDACs also differ in a number of other features including subcellular localization, tissue distribution, catalytic activity and non-histone substrates (Table I). (4, 20, 24-26) The class I HDAC isoforms 1 and 2 are localized to the nucleus as these proteins lack a nuclear export signal. (20, 27) HDAC3, however, does contain a nuclear export signal and while this structural feature suggests that HDAC3 can shuttle between the nucleus and cytoplasm, it is almost exclusively found in nuclear extracts upon subcellular fractionation. It has been suggested that proteins associated with HDAC3 are responsible for its nuclear localization and that this observation is likely cell-type specific. (20, 28, 29) In regard to tissue distribution, HDACs 1-3 are considered to be ubiquitously expressed and knockout experiments have indicated that these proteins are necessary for viability. (16) The last member of the class I HDACs, HDAC8, was originally reported to be ubiquitously expressed and restricted to the nuclear compartment as well, however, other reports have indicated that HDAC8 is only expressed during smooth muscle cell differentiation and that, during this process, HDAC8 is primarily localized to the cytosol. (4, 16, 25)
### TABLE I

**HDAC ISOFORM CHARACTERISTICS (4, 16, 20, 30)**

<table>
<thead>
<tr>
<th>Class</th>
<th>Isoform</th>
<th>Subcellular localization</th>
<th>Tissue distribution</th>
<th>Non-histone substrates (partial list)</th>
<th>Associated proteins (partial list)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>HDAC1</td>
<td>nucleus</td>
<td>ubiquitous</td>
<td>androgen receptor, p53, GCMα, YY1, MyoD, E2F-1, Stat3, Smad7, MEF2, NF-κB, SHP</td>
<td>Sin-3, Mi-2/NuRD, CoREST</td>
</tr>
<tr>
<td>I</td>
<td>HDAC2</td>
<td>nucleus</td>
<td>ubiquitous</td>
<td>Glucocorticoid receptor, YY1, Bcl-6, Stat3, Smad7, MEF2, NF-κB</td>
<td>Sin-3, Mi-2/NuRD, CoREST</td>
</tr>
<tr>
<td>I</td>
<td>HDAC3</td>
<td>nucleus</td>
<td>ubiquitous</td>
<td>SHP, MEF2D, GCMα, YY1, GATA-1, GATA-2, RelA, SRY, Stat3, Smad7, NF-κB</td>
<td>N-CoR/SMRT, CDK9, SP1, PP4c</td>
</tr>
<tr>
<td>I</td>
<td>HDAC8</td>
<td>Nucleus/ cytoplasm</td>
<td>ubiquitous/ smooth muscle tissue</td>
<td>---</td>
<td>EST1B</td>
</tr>
<tr>
<td>Ila</td>
<td>HDAC4</td>
<td>Nucleus and cytoplasm</td>
<td>heart, muscle tissue, brain</td>
<td>GCMα, GATA-1, HP-1</td>
<td>ANKRA, RFXANK</td>
</tr>
<tr>
<td>Ila</td>
<td>HDAC5</td>
<td>Nucleus and cytoplasm</td>
<td>Heart, muscle tissue, brain</td>
<td>GCMα, GATA-1, GATA-2, Smad7, HP-1</td>
<td>REA, estrogen receptor, CAMPTA</td>
</tr>
<tr>
<td>Ila</td>
<td>HDAC7</td>
<td>Nucleus and cytoplasm</td>
<td>Heart, pancreas, muscle tissue, placental tissue</td>
<td>PLAG1, PLAG2, MEF2, Foxp3</td>
<td>HIF-1α, Bcl-6, Foxp3, endothelin receptor, α-actinin 1, α-actinin 4, androgen receptor, Tip60</td>
</tr>
<tr>
<td>Ila</td>
<td>HDAC9</td>
<td>Nucleus and cytoplasm</td>
<td>Brain, muscle tissue</td>
<td>Foxp3, Hsp70, MEF2</td>
<td>Foxp3</td>
</tr>
<tr>
<td>IIB</td>
<td>HDAC6</td>
<td>primarily cytoplasmic</td>
<td>Heart, liver, kidney, pancreas, brain</td>
<td>α-tubulin, Hsp90, SHP, Smad7, peroxiredoxin, cortactin</td>
<td>Runx2, HDAC11</td>
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<tr>
<td>IIB</td>
<td>HDAC10</td>
<td>primarily cytoplasmic</td>
<td>liver, kidney, spleen</td>
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<td>---</td>
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<tr>
<td>IV</td>
<td>HDAC11</td>
<td>Nucleus and cytoplasm</td>
<td>Heart, kidney, muscle tissue, brain</td>
<td>---</td>
<td>HDAC6</td>
</tr>
</tbody>
</table>

Note: Deacetylase activity of class Ila HDACs may result from recruitment of other HDAC proteins.
1.3 HDAC Catalytic Mechanism

The mechanism of lysine deacetylation is theoretically similar for the class I, IIb and IV HDAC isoforms. The class IIa HDACs contain a tyrosine to histidine mutation in the active site and are considered to be much less efficient at deacetylating lysine residues.\(^{(21, 31)}\) Based on the known catalytic mechanisms of both metallo- and serine proteases, along with information obtained from the crystal structure of SAHA and trichostatin A (TSA) bound to the yeast homolog, HDLP, the following catalytic mechanism has been proposed (Figure 3).\(^{(32)}\) The acetylated lysine residue enters the catalytic pocket where the carbonyl oxygen atom of the acetamide is coordinated to zinc. Similar to a metalloprotease, the zinc ion polarizes the carbonyl of the acetamide making the carbonyl carbon more electrophilic.\(^{(33)}\) A water molecule is also present in the active site which is oriented for nucleophilic attack on the carbonyl carbon by the zinc ion. This water molecule is also hydrogen bonded to an aspartic acid-histidine charge-relay system which serves to increase the nucleophilicity of the oxygen atom. This interaction is reminiscent of the charge-relay system responsible for polarizing the hydroxyl group of serine residues in the active site of serine proteases.\(^{(33, 34)}\) In the first mechanistic step, the water molecule attacks the carbonyl carbon of the acetamide generating an oxyanion intermediate. The oxyanion formed during this first step is stabilized by hydrogen bonding to the tyrosine residue and coordination to the zinc ion. Similar to metalloproteases, the newly added hydroxyl group is also coordinated to the zinc ion.\(^{(33)}\) The last step of the mechanism involves the cleavage of the carbon-nitrogen bond of the acetamide during which the amino group of the lysine side chain accepts a proton from a second aspartic acid-histidine charge-relay system. The resulting products of the reaction are the deacetylated lysine residue which becomes charged at physiological pH and an acetate anion which is shuttled away from the active site through an adjacent cavity. Modifications to this mechanism have also been proposed.\(^{(35-37)}\)
Figure 3. Proposed mechanism of lysine deacetylation. (32)
1.4 Design of HDAC Inhibitors

The traditional structure of an HDACi consists of a cap group that interacts with the surface of the protein along with a linker that connects the cap group with the zinc chelating moiety (Figure 4).\(^{(19, 38, 39)}\) To inhibit HDAC catalytic activity, functional groups with strong affinity for the active site zinc ion have been incorporated while the rest of the molecule can be designed to impart selectivity for a class or even a particular isoform.\(^{(39-41)}\) Stemming from metalloprotease inhibitors, the first synthesized HDAC inhibitor contained a hydroxamic acid zinc binding group.\(^{(19, 42)}\) The hydroxamic acid functional group is known to be a potent metal chelator and since the development of suberoylanilide hydroxamic acid (SAHA), it has been extensively employed in the design of HDACi. Despite highly conserved residues involved in substrate recognition and catalytic activity across the HDAC isoforms, subtle differences do exist that can be exploited in the design of selective compounds. Thus, there have been extensive cap group, linker and zinc binding group modifications reported in the literature, however, identification of truly isoform selective inhibitors continues to present a significant challenge.\(^{(22, 40, 41, 43-50)}\)
Figure 4. Structural characteristics of HDAC inhibitors.
Structurally, HDAC6 is unique compared to the other HDAC isoforms. In particular, it contains two separate catalytic active sites each responsible for the deacetylation of different substrates. While both sites have been shown to possess histone deacetylase activity, it has been demonstrated that the C-terminal deacetylase domain is the target of both pan-HDAC and selective HDAC6 inhibitors. This domain was also found to be responsible for the deacetylation of α-tubulin, one of its major non-histone substrates.\(^{51}\) In addition to the two catalytic domains, HDAC6 also contains a zinc finger domain responsible for binding ubiquitinated proteins and transporting them to the aggresome for degradation.\(^{52}\) In regard to the tertiary structure, homology modeling has revealed significant structural differences between the region surrounding the entrance to the C-terminal active site of HDAC6 and that of other HDAC proteins. The entrance to the deacetylase domain of HDAC6, upon visual inspection of the model in comparison with crystal structures of HDAC8 and HDLP, is slightly wider and significantly shallower.\(^{39}\) Thus, these structural features have been the focus of selective HDAC6 inhibitor design.

1.5 **Therapeutic Applications of HDAC Inhibitors**

Selective inhibition of HDAC6 has been suggested for the treatment of a variety of disease states including neurodegenerative disorders, autoimmune and inflammatory diseases, hypertension and cancer.\(^{16, 53-63}\) While HDAC6 may possess some histone deacetylase activity, the fact that this protein is primarily cytosolic indicates that the therapeutic benefits related to HDAC6 inhibition are likely related to its non-histone substrates.\(^{4, 51}\) In regard to neuroprotection, HDAC6 inhibition has been linked to the upregulation of brain-derived neurotrophic factor (BDNF) transport, a protein involved in neurogenesis, and the mediation of peroxiredoxins, an important class of redox regulators.\(^{64-67}\) HDAC6 inhibition has also been shown to regulate the acetylation status
of Hsp90, an important chaperone responsible for the proper folding and intracellular transport of numerous client proteins.\(^{(57, 68-70)}\)

Recently, inhibition of HDAC6 was demonstrated to provide therapeutic benefits in a mouse model of CMT.\(^{(56)}\) CMT is an inherited peripheral neuropathy characterized by distal muscle weakness, decreased deep-tendon reflexes and foot deformities.\(^{(71, 72)}\) In this model, transgenic mice were engineered to express mutant Hsp27 and these mice were found to exhibit all the symptoms of CMT. Physiologically, these mice displayed a significant decrease in acetylated α-tubulin levels resulting in severe axonal transport deficits. Aberrant α-tubulin acetylation also disrupted the proper intracellular trafficking of mitochondria, which is likely related to the muscle weakness associated with this disease. Treatment of these mice for 21 days with the selective HDAC6 inhibitor, tubastatin A, completely rescued the CMT phenotype. Immunohistological analysis indicated that tubastatin A treatment significantly increased α-tubulin acetylation and restored proper mitochondrial transport.\(^{(56)}\)

For autoimmune and inflammatory diseases, the specific mechanisms behind the therapeutic effects are less clear, however, HDAC6 inhibition has been shown to enhance the suppressive effects of FOXP3+ regulatory T cells (Tregs) in vitro and in vivo.\(^{(73)}\) Mice with major histocompatibility complex-incompatible cardiac allografts exhibited significantly increased survival times when treated with tubastatin A and low dose rapamycin compared to those treated with rapamycin alone. Interestingly, the treatment regimen for these mice was only once daily for 14 days, however, these mice survived for more than 60 days post treatment. These increased survival times were also observed in mice treated with the Hsp90 inhibitor, 17-AAG, further indicating that the non-histone substrates are responsible for the beneficial effects associated with HDAC6 inhibition.\(^{(57)}\)
Since the discovery of SAHA, there have been numerous HDACi reported in the literature (Figure 5). However, the development of selective HDACi has emerged as a challenging field in drug discovery as only a few selective inhibitors are known and they are of great interest, not only as tools for probing the biological functions of the individual isoforms, but also as potential therapeutic agents with reduced side effect profiles compared to their non-selective counterparts. The remainder of this dissertation will explore the design and synthesis of selective HDAC6 inhibitors in an attempt to identify the HDAC6 pharmacophore. In addition to their HDAC inhibitory profiles, the biological activities of these HDAC6 selective agents are also investigated.
Figure 5. Structures of known HDAC inhibitors.
CHAPTER 2

DISCOVERY OF HDAC6 SELECTIVE MERCAPTOACETAMIDES

2.1 Introduction

HDAC6 selective inhibitors have been suggested to avoid some of the major side effects associated with broad spectrum HDACi. (17, 45, 58) Thus, our particular interest has focused on HDAC6 which is expressed by most neurons and therefore serves an additional role in controlling gene transcription in the brain. (26, 52) In neuronal models of oxidative stress, many hydroxamic acid-based HDACi were found to exhibit some intrinsic toxicity whereas their mercaptoacetamide counterparts were found to be fully neuroprotective suggesting that alternative zinc binding groups (ZBGs) may be preferred depending on the desired therapeutic goal. (18, 43, 74) However, examples of neuroprotective hydroxamic acid HDACi have also been identified. (39)

Many known HDACi such as SAHA and Trichostatin A (TSA) contain a hydroxamic acid zinc binding group (ZBG), however, in addition to being metabolically labile (orally administered SAHA exhibits a half-life of 1.5-2 h in humans), hydroxamates are very potent metal chelating agents which could lead to off-target activity at other zinc containing enzymes such as the matrix metalloproteases, MMP-1, MMP-2 and MMP-3, ultimately resulting in undesirable toxicity. (45, 75-78)

Previously, we reported on the synthesis of a series of mercaptoacetamide-based HDACi that exhibited some HDAC6 selectivity and were found to have better therapeutic profiles in protecting neurons against oxidative stress-induced cell death compared to their hydroxamic acid homologs. (75) The mechanism of HDAC inhibition by mercaptoacetamide-
based HDACi is, theoretically, similar to that of the hydroxamates in that both have the potential to chelate zinc in a monodentate or bidentate fashion depending on the isoform.\(^{(21, 79)}\) In addition to our previously reported analogs, numerous examples of thiol containing HDACi exist in the literature.\(^{(45, 79, 80)}\) However, one potential drawback of these mercaptoacetamide HDACi is their ability to undergo oxidation to the disulfide. While this disulfide bond can be reduced within cells to afford the parent monomers, it can present a problem when profiling the activity of these inhibitors against purified proteins.\(^{(81)}\) Thus, we synthesized a novel series of modestly potent mercaptoacetamide-based HDAC6 selective inhibitors displaying increased resistance to oxidation and evaluated their ability to protect primary cortical neurons from oxidative stress-induced cell death.

### 2.2 Chemistry

The synthetic procedures used for the preparation of our first series of amide-linked mercaptoacetamide isoxazoles are outlined in Scheme 1. Here, a nitrile oxide cycloaddition was employed to generate the tri-substituted isoxazole 2.1 starting from (\(E\))-benzaldehyde chloro-oxime and ethyl-2-butynoate using microwave irradiation. This ester was saponified to yield acid 2.2 and then reacted with a diamine linker in the presence of the coupling agent PyBOP to yield 2.3. The Boc-protecting group was removed to give amine 2.4 and then a second coupling reaction was carried out with different trityl- or pivaloyl-protected mercaptoacetic acids 2.5-2.8. The pivaloyl-protected pro-drug 2.12 was thus made available for study. The trityl group was removed from compounds 2.9-2.11 to afford three mercaptoacetamide ligands with no (2.13), one (2.14) or two (2.15) methyl groups alpha to the thiol of the mercaptoacetamide.
Scheme 1. Synthesis of amide linked mercaptoacetamides.

Reagents and conditions: a) N-Chlorosuccinimide, Al₂O₃, microwave, 40 °C; b) Ethyl-2-butynoate, Et₃N, THF, microwave, 70 °C; c) NaOH, MeOH/H₂O, 60 °C; d) NH₂(CH₂)₃NHBOc, PyBOP, DIPEA, CH₂Cl₂, RT; e) TFA, CH₂Cl₂, RT; f) 2.5-2.8, PyBOP, DIPEA, CH₂Cl₂, RT; g) TFA, Et₃SiH, CH₂Cl₂, 0 °C to RT.
Because it is well known that the absolute stereochemistry of a particular molecule can greatly influence its activity, we also chose to investigate the individual enantiomers of compound 2.14. The enantiomers of compound 2.10 were separated using chiral column chromatography and then subsequently deprotected to afford the single enantiomers, (R)-2.14 and (S)-2.14 (Scheme 2). In order to determine the absolute stereochemistry of the two isolated enantiomers, starting from 2.4 and (S)-2-aminopropanoic acid 2.16, we synthesized the (R)-isomer of compound 2.10, (R)-2.10, and compared the retention time of the pure enantiomer to that of the racemic mixture and determined that the (R)-isomer was the first to elute from the column (Scheme 3 and Figure 6).(82)

Scheme 2. Isolation of 2.14 enantiomers.

Reagents and conditions: a) TFA, Et₃SiH, CH₂Cl₂, 0 °C to RT.
Scheme 3. Synthesis of (R)-2.10.

Reagents and conditions: a) NaNO₂, 5N HCl; b) CsSCOCH₃, DMF; c) 2.18, PyBOP, DIPEA; d) 0.5N NaOH, THF/MeOH; e) Ph₃COH, TFA, CH₂Cl₂.
Figure 6. Chromatograms for a) racemic mixture of 2.10, b) \((R)-2.10\) (obtained using synthetic route described above in Scheme 3) and c) \((S)-2.10\). Wavelength monitoring at 230 nm.
As we were aware of the fact that the hydroxamic acid group may be associated with neuronal toxicity, we synthesized an analog in order to compare it with our mercaptoacetamide-based ligands both in terms of potency and neuroprotective activity (Scheme 4). The hydroxamic acid 2.21 was prepared in 2 steps from the isoxazole acid 2.2 using standard PyBOP coupling protocol and then hydroxylamine to supplant the obtained methyl ester.


Reagents and conditions: a) \( \text{NH}_2(\text{CH}_2)_6\text{CO}_2\text{Me}\cdot\text{HCl}, \text{PyBOP}, \text{DIPEA}, \text{CH}_2\text{Cl}_2, \text{RT} \); b) \( \text{NH}_2\text{OH}\cdot\text{HCl}, \text{KOH}, \text{MeOH}, \text{RT} \).

As shown in Scheme 5, the synthesis of alkene-linked mercaptoacetamides 2.25-2.28 was performed in 5/6 steps starting from the previously obtained acid 2.2. 2.2 was reduced to the alcohol and subsequently oxidized to aldehyde 2.23. A Wittig reaction was then employed using aldehyde 2.23 and a prepared phosphonium salt to produce compound 2.24. Procedures similar to those described in Scheme 1 were applied to
compound 2.24 to generate the alpha-unsubstituted, mono- and dimethylmercaptoacetamides. In order to investigate the difference in activity between the geometric isomers generated during the Wittig reaction, the isomers of compounds 2.25 and 2.28 were isolated using chiral column chromatography and their molecular configuration was assigned based on $^1$H NMR (Scheme 6 and Figure 7).

Scheme 5. Synthesis of alkene linked mercaptoacetamides.

Reagents and conditions: a) Borane-THF, THF, 0 °C to RT; b) MnO$_2$, CH$_2$Cl$_2$, RT; c) BrPh$_3$P(Ch$_2$)$_n$NHBOc, $n$BuLi, THF, 0 °C to RT; d) TFA, CH$_2$Cl$_2$, RT; e) 2.5-2.8, PyBOP, DIPEA, CH$_2$Cl$_2$, RT; f) TFA, Et$_3$SiH, CH$_2$Cl$_2$, 0 °C to RT.
Scheme 6. Isolation of 2.25 geometric isomers.

Reagents and conditions: a) TFA, Et$_3$SiH, CH$_2$Cl$_2$, 0 °C to RT.

Figure 7. Geometric isomers of compound 2.28 isolated via chiral column chromatography.
Ligand 2.32, the compound containing an ether linker, was synthesized from alcohol 2.22 by conversion to the iodo precursor via an Appel-like reaction which was subsequently reacted with tert-butyl 5-hydroxypentylcarbamate to yield intermediate carbamate 2.30. The obtained product was transformed as previously described in Scheme 1 into the desired α-methyl mercaptoacetamide (Scheme 7). Again, in order to investigate the effect of stereochemistry on compound activity, the enantiomers of compound 2.31 were isolated via chiral column chromatography and then deprotected to yield (R)-2.32 and (S)-2.32 (Scheme 8). The absolute stereochemistry was assigned in the same manner as that for compound 2.14 (Scheme 9 and Figure 8).

Scheme 7. Synthesis of ether linked mercaptoacetamides

Reagents and conditions: a) I$_2$, PPh$_3$, imidazole, CH$_2$Cl$_2$, 0 °C to RT; b) HO(CH$_2$)$_5$NHBOc, NaH, DMF, -20 °C to RT; c) TFA, CH$_2$Cl$_2$, RT; d) 2.6, PyBOP, DIPEA, CH$_2$Cl$_2$, RT; e) TFA, Et$_3$SiH, CH$_2$Cl$_2$, 0 °C to RT.
Scheme 8. Isolation of **2.32** enantiomers.

Reagents and conditions: a) TFA, Et$_3$SiH, CH$_2$Cl$_2$, 0 °C to RT.

Scheme 9. Synthesis of **(R)-2.31**.

Reagents and Conditions: a) TFA, CH$_2$Cl$_2$; b) **2.18**, PyBOP, DIPEA; c) 0.5N NaOH, THF/MeOH; d) Ph$_3$COH, TFA, CH$_2$Cl$_2$. 
Figure 8. Chromatograms for a) racemic mixture of 2.31, b) (R)-2.31 (obtained using synthetic route described above in Scheme 9) and c) (S)-2.31. Wavelength monitoring at 254 nm.
Synthesis of the alkyne- and styryl-linked mercaptoacetamides was performed starting from commercially available or readily obtained iodo-isoxazoles 2.34-2.35. The Sonogashira and Heck reactions, followed by conditions similar to those described in Scheme 1, were used with 2.34-2.35 to generate the alkyne- and styryl-linked compounds 2.36-2.40 and 2.42-2.43, respectively (Scheme 10). With the desired compounds in hand, we now studied their activity in both the isolated enzyme assays and in cell based neuroprotection studies.
Scheme 10. Synthesis of alkyne- and styryl-linked mercaptoacetamides.

```
R = H or F
```

```
<table>
<thead>
<tr>
<th>Step</th>
<th>Reaction Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>a, b, c</td>
<td>ClCOCH₃, ZnCl₂, nBuLi, THF, -78 °C</td>
</tr>
<tr>
<td>d, e, f, g</td>
<td>NH₂OMe·HCl, pyridine, Na₂SO₄, MeOH, reflux</td>
</tr>
<tr>
<td>h</td>
<td>ICl, CH₂Cl₂, reflux</td>
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<tr>
<td>i</td>
<td>HCC(CH₂)nNHBoc, PdCl₂(PPh₃)₂, CuI, DIPEA, DMF, microwave, 130 °C</td>
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<tr>
<td>j</td>
<td>TFA, CH₂Cl₂, RT</td>
</tr>
<tr>
<td>k</td>
<td>2.5-2.8, PyBOP, DIPEA, CH₂Cl₂, RT</td>
</tr>
<tr>
<td>l</td>
<td>TFA, Et₃SiH, CH₂Cl₂, 0 °C to RT</td>
</tr>
<tr>
<td>m</td>
<td>tert-butyl 4-vinylbenzylcarbamate, Pd(OAc)₂, nBu₄NCl, Na₂CO₃, DMF, microwave, 130 °C</td>
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```

```
2.34 R=H
2.35 R=F
```

```
2.36 n=3, R=R'=R''=H, R'''=CH₃
2.37 n=3, R=F, R'=R''=H, R'''=CH₃
2.38 n=3, R=R'=H, R''=R'''=CH₃
2.39 n=2, R=R'=H, R''=R'''=CH₃
2.40 n=2, R=R''=R'''=H, R'=Piv
```

```
2.34
2.41
```

```
2.42 R=H
2.43 R=CH₃
```
2.3 **Results and Discussion**

2.3.1 **HDAC Inhibition**

IC\(_{50}\) values were determined for these new mercaptoacetamide ligands at selected HDAC isoforms using a fluorescence-based assay and the results are displayed in Table II. The mercaptoacetamide 2.13 showed submicromolar HDAC6 activity with some selectivity over both the class I (>21 fold) and class IIa (>38 fold) isozymes. The related hydroxamic acid 2.21 exhibited similar activity and selectivity at the class I and class IIa isoforms (>14 and >33 fold respectively). The slight difference in potency observed between 2.13 and 2.21 may have resulted from the ability of 2.21 to form an additional hydrogen bond to the enzyme. It is known that the NH of the hydroxamate ZBG is able to hydrogen bond with a histidine in the HDAC active site whereas the mercaptoacetamide ZBG lacks this functionality.(83) The addition of a methyl group to the mercaptoacetamide ZBG of 2.13, compound 2.14, resulted in a slight reduction in potency (2.13 IC\(_{50}\) = 0.26 μM, 2.14 IC\(_{50}\) = 1.1 μM), however, the activity at all other HDAC isoforms was eliminated. Because 2.14 was a racemic mixture, we isolated the single enantiomers and observed that the (R)-isomer, (R)-2.14, displayed submicromolar activity at HDAC6, similar to the original unsubstituted compound 2.13, whereas the (S)-isomer, (S)-2.14, was completely inactive. Compound (R)-2.14 maintained selectivity over all the other HDAC isoforms except HDAC3 for which the 10-dose IC\(_{50}\) was 15 μM.
<table>
<thead>
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<th>Compound</th>
<th>Class I (IC&lt;sub&gt;50&lt;/sub&gt;, μM)</th>
<th>Class Ila (IC&lt;sub&gt;50&lt;/sub&gt;, μM)</th>
<th>Class Ilib (IC&lt;sub&gt;50&lt;/sub&gt;, μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HDAC1 HDAC2 HDAC3</td>
<td>HDAC4 HDAC5</td>
<td>HDAC6</td>
</tr>
<tr>
<td>2.13</td>
<td>5.7 ± 0.5&lt;sup&gt;e&lt;/sup&gt; 28 14</td>
<td>10 15</td>
<td>0.26 ± 0.03&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;30 &gt;30 &gt;30</td>
<td>&gt;30 &gt;30</td>
<td>1.1 ± 0.01&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>(R)-2.14</td>
<td>&gt;30&lt;sup&gt;e&lt;/sup&gt; &gt;30&lt;sup&gt;e&lt;/sup&gt; 15 ± 1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&gt;30&lt;sup&gt;e&lt;/sup&gt; &gt;30&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.28 ± 0.04&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>(S)-2.14</td>
<td>&gt;30&lt;sup&gt;e&lt;/sup&gt; &gt;30&lt;sup&gt;e&lt;/sup&gt; &gt;30&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&gt;30&lt;sup&gt;e&lt;/sup&gt; &gt;30&lt;sup&gt;e&lt;/sup&gt;</td>
<td>&gt;30&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>2.15</td>
<td>&gt;30 &gt;30 &gt;30</td>
<td>&gt;30 &gt;30</td>
<td>&gt;30</td>
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<tr>
<td>2.21</td>
<td>3.0 ± 0.2&lt;sup&gt;e&lt;/sup&gt; 11 2.6</td>
<td>9.4 6.0</td>
<td>0.18 ± 0.08&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>2.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&gt;30 &gt;30 &gt;30</td>
<td>&gt;30 &gt;30</td>
<td>2.7 ± 0.1&lt;sup&gt;e,f&lt;/sup&gt;</td>
</tr>
<tr>
<td>cis-2.25</td>
<td>&gt;30&lt;sup&gt;e&lt;/sup&gt; --- ---</td>
<td>&gt;30&lt;sup&gt;e&lt;/sup&gt; ---</td>
<td>4.8 ± 1.8&lt;sup&gt;e,f&lt;/sup&gt;</td>
</tr>
<tr>
<td>trans-2.25</td>
<td>28 ± 4&lt;sup&gt;e&lt;/sup&gt; --- ---</td>
<td>&gt;30&lt;sup&gt;e&lt;/sup&gt; ---</td>
<td>3.5 ± 0.5&lt;sup&gt;e,f&lt;/sup&gt;</td>
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<td>9.8</td>
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<tr>
<td>2.27&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>&gt;30 &gt;30</td>
<td>&gt;30</td>
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<tr>
<td>2.28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&gt;30 &gt;30 &gt;30</td>
<td>&gt;30 &gt;30</td>
<td>&gt;30&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>--- ---</td>
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<td>trans-2.28</td>
<td>&gt;30 --- ---</td>
<td>--- ---</td>
<td>&gt;30&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>2.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.6 14 5.5</td>
<td>&gt;30 &gt;30</td>
<td>0.85</td>
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<tr>
<td>(R)-2.32</td>
<td>1.5 ± 0.03&lt;sup&gt;e&lt;/sup&gt; ---</td>
<td>3.4 ± 0.2&lt;sup&gt;e&lt;/sup&gt; ---</td>
<td>0.83 ± 0.27&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>(S)-2.32</td>
<td>&gt;30&lt;sup&gt;e&lt;/sup&gt; --- ---</td>
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</tr>
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<td>2.7 7.5 1.4</td>
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<td>2.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.7 9.4 2.1</td>
<td>&gt;30 13</td>
<td>2.6</td>
</tr>
<tr>
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<td>&gt;30 &gt;30</td>
<td>&gt;30</td>
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<tr>
<td>2.39</td>
<td>&gt;30 &gt;30 &gt;30</td>
<td>&gt;30 &gt;30</td>
<td>&gt;30</td>
</tr>
<tr>
<td>2.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&gt;30 &gt;30 &gt;30</td>
<td>&gt;30 &gt;30</td>
<td>&gt;30</td>
</tr>
<tr>
<td>2.43</td>
<td>&gt;30 &gt;30 &gt;30</td>
<td>&gt;30 &gt;30</td>
<td>&gt;30</td>
</tr>
<tr>
<td>TSA&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.006 ± 0.017 ± 0.008 ±</td>
<td>0.047 ± 0.013 ±</td>
<td>0.0017 ±</td>
</tr>
<tr>
<td></td>
<td>0.003 0.008 0.004</td>
<td>0.061 0.007</td>
<td>0.0005</td>
</tr>
</tbody>
</table>
These results were determined by the CRO Reaction Biology (Malvern, PA). 10-dose IC$_{50}$ values were determined using 3-fold serial dilutions starting at concentrations of 30 μM. Compounds with an IC$_{50}$ > 30 μM were considered to be inactive. A dashed line (---) indicates that the compound was not tested at that isoform.

b Racemic mixture.

c Mixture of geometric isomers.

d IC$_{50}$ values for TSA are reported as the average of five experiments ± SD for HDAC1, 3, 4 and 6 and the average of four experiments ± SD for HDAC2 and 5.

e Evaluated in duplicate.

f $p > 0.05$ (difference not statistically significant), determined using the unpaired $t$-test available in GraphPad Prism 5 (La Jolla, CA).
Additional modifications were made to the linker region and cap group of these mercaptoacetamides. Replacing the amide linker of 2.13 by an alkene, 2.25, resulted in micromolar HDAC6 activity and a good selectivity profile with the IC$_{50}$ at all other tested isoforms being $>$30 μM. In order to investigate the effect of geometric isomerism on compound activity, the isomers of 2.25, cis-2.25 and trans-2.25, were isolated and found to exhibit similar activity at HDAC6 and a less than two-fold difference in activity at HDAC1 suggesting that the geometric configuration of the double bond at this position in the linker has little effect on ligand binding. The pivaloyl prodrug of 2.25, compound 2.28, along with the individual geometric isomers, cis-2.28 and trans-2.28, displayed no inhibition at any of the HDAC isoforms tested. This was expected as these IC$_{50}$ values were measured using purified protein, and as such, no metabolic enzymes were present to release the active form of the drug.(84) The other pivaloyl prodrugs, 2.12 and 2.40, were not screened against the isolated HDAC enzymes for this reason.

When there was an ether linker replacing the amide, as in compound 2.32, HDAC6 activity was still in the submicromolar range and selectivity over the class I (>6 fold) and class IIa (>35 fold) isoforms was similar to that of compound 2.13. Again, we investigated the effect of absolute stereochemistry on compound activity and found that the (R)-isomer of 2.32, (R)-2.32, exhibited HDAC6 activity in the submicromolar range whereas the (S)-isomer, (S)-2.32, was completely inactive. These results, combined with the results obtained for the enantiomers of compound 2.14, led us to conclude that the R-configuration of the monomethyl mercaptoacetamide ZBG is preferred for HDAC6 activity.

Lastly, the compounds containing alkyne linkers and monomethyl mercaptoacetamide ZBGs (2.36-2.37) exhibited micromolar activity with only some selectivity over the HDAC4 isoform. Compounds containing a styryl linker (2.42-2.43) had no
HDAC activity perhaps due to the rigidity of this linker. The addition of two methyl groups to the ZBG, as with 2.38 and 2.39, completely eliminated activity at every tested HDAC isoform and for all cap group and linker modifications. This loss of activity can likely be attributed to steric interactions in the active site. While the HDAC proteins are able to accommodate some steric bulk, the addition of a second methyl group likely renders the compound unable to adopt a conformation that does not clash with active site residues.

2.3.2 Molecular Modeling

With the intent of rationalizing the stereoselectivity observed with the reported mercaptoacetamides, a molecular docking study was carried out considering the two enantiomers of 2.14, (R)-2.14 and (S)-2.14, and three relevant HDAC subtypes. In order to reduce the number of exploited homology models, the following structures were studied: (a) X-ray structure of the human HDAC2 isozyme (PDB Id: 3MAX) as representative of the Class I subtypes; (b) X-ray structure of the human HDAC4 isozyme (PDB Id: 2VQM) as representative of the Class IIa subtypes; (c) homology model of the second catalytic domain of human HDAC6 (Class IIb) as recently generated by us. It is worth mentioning the high sequence homology between HDAC1 and HDAC2 such that the results obtained for HDAC2 can be considered similar to what would be observed with HDAC1. The docking calculations were carried out considering the anionic form of the thiol for the two enantiomers. Also, the two His-Asp dyads which characterize the catalytic core of the HDAC isozymes were considered in their singly protonated state such that only the first histidine residue (His145 for HDAC2, His158 for HDAC4 and His610 for HDAC6-CDII) was protonated while the second histidine residue (His146 for HDAC2, His159 for HDAC4 and His611 for HDAC6-CDII) was considered neutral and in its Nɛ tautomeric form to minimize steric hindrance within the catalytic pocket. This choice was justified by considering the precise planarity
which characterizes the first dyad and increases its basicity whereas the other dyad does not show similar planarity resulting in a less basic histidine residue. This was also in line with other enzymatic studies which evidenced that the first protonation highly disfavored a second protonation of His146 allowing for easy proton transfer between the two dyads. (85)

The electrostatic energy scores, as determined with VEGA using a distance dependent dielectric function, for all minimized complexes between HDAC isozymes and enantiomers \((R)-2.14\) and \((S)-2.14\) were lower for the \((R)\)-isomer compared to the \((S)\)-isomer which confirmed the observed stereoselectivity (Table III).

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Protonated His Residue</th>
<th>((R)-2.14) Energy Score</th>
<th>((S)-2.14) Energy Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC2</td>
<td>His145</td>
<td>-142.37</td>
<td>-108.43</td>
</tr>
<tr>
<td>HDAC4</td>
<td>His158</td>
<td>-129.87</td>
<td>-103.38</td>
</tr>
<tr>
<td>HDAC6</td>
<td>His610</td>
<td>-127.43</td>
<td>-101.42</td>
</tr>
</tbody>
</table>
The observed difference in binding mode for the two enantiomers can be accounted for by considering the interactions available to the amido group of each ligand (Figure 9a). Indeed, in both complexes, the sulfur atom approached the zinc ion which also interacted with protonated His145, however, only the amido group of the (R)-isomer was able to interact with the zinc ion and stabilize hydrogen bonding interactions with the highly conserved Tyr308 residue. Conversely, the amido group of the (S)-isomer was unable to elicit these significant interactions and in addition, clashes with Cys156. This was in line with recent mechanistic studies which found that inhibitors of Class I isozymes are characterized by a tight bidentate chelation with the zinc ion which is reinforced by a H-bonding interaction with the conserved tyrosine residue and is not dependent on the ionization state of the inhibitor.\(^{(86)}\) The differing arrangement of the ZBG moieties also reflected on the interactions stabilized by the cap group such that the (R)-isomer was able to assume a more folded geometry and stabilize two relevant H-bonds with His183 and Tyr209 in addition to a rich set of hydrophobic contacts whereas the (S)-isomer assumed a more extended conformation and does not display these interactions.
Figure 9. a) Putative complexes for (R)-2.14 and (S)-2.14 with the resolved HDAC2 crystal structure (PDB Id: 3MAX) illustrating that only the active (R)-isomer can elicit bidentate chelation which is reinforced by H-bonding interactions between the amido group of the ligand and Tyr308. b) Putative complexes for the two enantiomers, (R)-2.14 and (S)-2.14, with the second catalytic subunit of the HDAC6 subtype (previously published) illustrating that only the active (R)-isomer can elicit bidentate chelation reinforced by H-bonding between the sulfur atom and Tyr782. This difference was also reflected by the position of the cap group since only the active (R)-isomer was able to stabilize H-bonding interactions with Asp567 and Ser568 on the cavity rim.
The role of the cap group moiety was even more preponderant in HDAC4 (complexes not shown), since the ZBG group realized a similar interaction pattern for both enantiomers in that the sulfur atom contacted the zinc ion and the charged His158 residue in both cases. Conversely, the phenyl isoxazole moiety of the \( (R) \)-isomer was able to stabilize hydrophilic interactions between two arginine residues (Arg37 and Arg154) which characterize the cavity rim of HDAC4. This finding was in agreement with the weak monodentate chelation observed with the class IIa isoforms due to the lack of a conserved tyrosine residue which globally renders the obtained complexes less dependent on interactions with the metal ion and more dependent on interactions with the cap group and linker.

As depicted by Figure 9b, the binding modes observed between the two enantiomers, \( (R)-2.14 \) and \( (S)-2.14 \), and HDAC6 were similar to what was observed with HDAC2. The sulfur atom of the ZBG of both enantiomers contacted the zinc ion and protonated His610, however, only the carbonyl of the \( (R) \)-isomer’s ZBG was able to interact with the zinc ion allowing for bidentate chelation. In addition, the sulfur atom of the \( (R) \)-isomer’s ZBG also contacted Tyr782. These putative complexes suggested the importance of bidentate chelation to the zinc ion in regard to activity and also suggested the plausible role of Tyr782 in further stabilizing the thiol group. The different interactions stabilized by the cap group moieties also seemed to possess a relevant role in HDAC6 binding since the phenyl isoxazole group of the \( (R) \)-isomer elicited H-bonding interactions with Asp567 and Ser568, while the cap group of the \( (S) \)-isomer assumed a more lateral arrangement stabilizing only weak hydrophobic contacts.

Altogether, the docking results confirmed the differences in selectivity observed between the considered subtypes which, based on these calculations, resulted from the
different roles of the ZBG and cap group moieties in determining the binding mode. Interestingly, the different binding modes observed between the ZBG of each enantiomer and HDAC2 were essentially due to the different residues surrounding the metal ion; however, they may also be due to the presence of the adjacent foot cavity which characterizes the binding pocket of the class I subtypes and allows for greater diversity amongst the computed poses which was reflected by a larger difference in computed energy scores.

Finally, it was interesting to observe how the different arrangements of the linker, despite its flexibility, had a constraining effect on the interactions stabilized by the cap group (especially in regard to the class II isozymes) which was probably a result of the rigidity induced by the two amido functionalities.

2.3.3 Mercaptoacetamide Oxidation

To determine if the addition of α-methyl groups relative to the thiol in these mercaptoacetamides increased resistance to oxidation, three ligands, 2.13, (R)-2.14, and 2.15, were assayed by LC-MS to determine their qualitative rate of oxidation (Figure 10). We found that compound 2.13, which contained no methyl groups, dimerized at a much faster rate than both the monomethyl, (R)-2.14, and dimethyl, 2.15, compounds. Comparing compound (R)-2.14 and 2.15, we found that the addition of a second methyl group adjacent to the thiol further decreased the rate of oxidation; however, this decrease was not nearly as significant as the addition of the first methyl group.
Figure 10. Qualitative determination of the rate of oxidation for compounds 2.13 (♦), (R)-2.14 (■) and 2.15 (●) as measured by LC-MS.

### 2.3.4 Neuroprotection and Glutathione Depletion

Treating central nervous system (CNS) diseases with HDAC inhibitors, especially HDAC6 inhibitors, is viewed as a rational therapeutic approach and therefore, our ligands were tested for their ability to provide primary cortical neuron protection in an in vitro model of oxidative stress-induced neurodegeneration.\(^{(56, 87)}\) In this model, neurodegeneration is induced by the presence of a 5 mM concentration of the cysteine homolog, homocysteic acid, which depletes the cellular antioxidant glutathione via competitive inhibition of cysteine uptake at the plasma membrane cysteine/glutamate antiporter. Since cysteine is required for the synthesis of glutathione, the inhibition of its uptake results in glutathione depletion. Cellular redox homeostasis therefore becomes disrupted which leads to accumulation of endogenously produced and unopposed oxidants resulting in neuronal degeneration over an approximately 24-hour period of time. Importantly, primary neurons at this early
developmental stage lack ionotropic and metabotropic receptors and are not susceptible to excitotoxicity, rather, cell death is induced by the accumulation of unopposed free radicals and the neurons exhibit a number of apoptotic features.

At concentrations of 10 μM, the hydroxamic acid 2.21 showed little neuroprotective activity but did exhibit some toxicity which was not observed with the mercaptoacetamides. Compound 2.13, which displayed good HDAC6 activity, was moderately neuroprotective at concentrations of 7.5 μM and fully neuroprotective at 10 μM while the prodrug 2.12 was moderately neuroprotective at 5 μM and fully neuroprotective at 7.5 μM concentrations (Figure 11). It has been established that thioesters help to improve cell permeability by acting as prodrugs for the active thiols which are released once they enter the cell.(81, 84, 88, 89) Therefore, it is likely that 2.12 is metabolized to 2.13 inside the cell and that 2.13 is responsible for the observed neuroprotective properties. In addition, the thioester prodrug 2.12 may exhibit enhanced cell permeability compared to the mercaptoacetamide 2.13 which would account for the lower concentrations of 2.12 required to exhibit neuroprotection.(90) The same trend was also observed for the geometric isomers of 2.25, cis-2.25 and trans-2.25, and their respective prodrugs, cis-2.28 and trans-2.28. Alkene-linked mercaptoacetamides cis-2.25 and trans-2.25 displayed moderate neuroprotective activity at concentrations of 20 μM and full neuroprotection at concentrations of 50-100 μM while their respective prodrugs, cis-2.28 and trans-2.28, were neuroprotective at 10 μM concentrations but devoid of any significant HDAC activity (Figure 11).
Figure 11. Neuroprotection plots for selected mercaptoacetamide compounds. Rat primary cortical neurons were incubated with varying concentrations of each compound in the absence (dark gray bars) or presence (light gray bars) of the cysteine homolog, HCA (5 mM).

The monomethyl mercaptoacetamides 2.26, 2.36 and 2.37 displayed neuroprotective activities that correlated well with their HDAC6 inhibitory activity. Compounds 2.36 and 2.37 displayed IC₅₀ values at HDAC6 between 1 and 3 μM and were neuroprotective at 10 μM in vitro. 2.26, however, was between 3- and 12-fold less potent than the other monomethyl mercaptoacetamides in the enzyme assay which was reflected by the need to use concentrations of 20 μM to achieve neuroprotection. Compound 2.15 did not display any neuroprotective activity which was consistent with the results of the HDAC6 enzyme inhibition assay.

For some compounds, however, the HDAC6 activity failed to track closely with the observed neuroprotective activity. The enantiomers of 2.14, compounds (R)-2.14 and (S)-2.14, displayed peculiar neuroprotective profiles such that while compound (R)-2.14 was neuroprotective as expected, compound (S)-2.14 was also neuroprotective even though it
did not display HDAC6 activity. The same trend was observed with \((R)-2.32\) and \((S)-2.32\). Compounds \(2.27, 2.38, 2.39, 2.40, 2.42\), and \(2.43\) were also neuroprotective but had no activity at HDAC6 suggesting that an off-target mechanism may play a role in the neuroprotective properties of these compounds (Figure 11).

Therefore, the ability of selected compounds to prevent glutathione depletion was measured (Figure 12). We found that the \((S)\)-isomer \((S)-2.14\), but not the \((R)\)-isomer \((R)-2.14\), was able to prevent glutathione depletion suggesting this as an additional neuroprotective pathway for these compounds. Moreover, \(2.13\) was also very effective in preventing glutathione depletion which likely contributes to the excellent neuroprotective profile observed with this compound. A similar, albeit less pronounced, effect was observed with \((R)-2.32\) and \((S)-2.32\) in that the \((S)\)-isomer was more effective in preventing glutathione depletion than the \((R)\)-isomer. Many of the other compounds tested displayed a moderate ability to prevent glutathione depletion including \textit{cis}- and \textit{trans}-2.25 as well as their respective prodrugs, \textit{cis}- and \textit{trans}-2.28.
Figure 12. Prevention of glutathione depletion by selected mercaptoacetamides. Rat primary cortical neurons were incubated with 10 μM concentrations of each compound in the absence (dark gray bars) or presence (light gray bars) of the cysteine homolog, HCA (5 mM).

Of note, however, is compound 2.21 which was able to prevent glutathione depletion without being neuroprotective. This can likely be attributed to the intrinsic neuronal toxicity that is often observed with hydroxamic acids such that while this compound is active at HDAC6 and is able to prevent glutathione depletion, the aforementioned zinc binding group precludes any substantial neuroprotective activity. When dealing with epigenetic regulators (and small molecules in general), it is unlikely that only one biological pathway will be affected and thus, the neuroprotective activity of these compounds probably results from a combination of effects. In addition to HDAC6 inhibition and the prevention of glutathione depletion, other mechanisms that may contribute to the observed neuroprotective activity of
these compounds include the inhibition of matrix metalloproteases and other zinc dependent enzymes, interference with HCA itself and/or the intrinsic antioxidant activity of the thiols themselves.\(^{(91)}\) However, we have previously demonstrated that a thiol alone is not sufficient for potent neuroprotection as 2-phenylethanethiol required 1 M concentrations to achieve the same result.\(^{(75)}\) Therefore, further investigation into the potential neuroprotective mechanisms of these compounds will be necessary to fully elucidate all of the biological pathways involved.

### 2.4 Conclusion

Currently, two HDACi are being marketed for the treatment of cutaneous T-cell lymphoma and a host of others are the subject of ongoing clinical trials.\(^{(92)}\) The continued development of novel HDACi as therapeutics is warranted, however, the majority of the reported compounds lack isozyme selectivity, thus leading to various levels of undesirable toxicity. While pan-activity may be acceptable for oncology applications, it is unlikely to be acceptable in other therapeutic areas.\(^{(15)}\) As detailed herein, we have developed a series of selective, stable, low molecular weight HDAC6 inhibitors that exhibit neuroprotection in neuronal models of oxidative stress for potential use in the treatment of neurodegenerative diseases, traumatic brain injury, and other health problems, such as myocardial infarction, in which oxidative stress is an issue. We demonstrated that mercaptoacetamide \(^{2.13}\) (HDAC1 IC\(_{50}\) = 5.7 \(\mu\)M, HDAC6 IC\(_{50}\) = 0.26 \(\mu\)M) exhibited similar potency and selectivity compared to the hydroxamic acid analog \(^{2.21}\) (HDAC1 IC\(_{50}\) = 3.0 \(\mu\)M, HDAC6 IC\(_{50}\) = 0.18 \(\mu\)M) and thus, mercaptoacetamide ZBGs may potentially be used in place of the traditional hydroxamic acids. In addition, replacement of the hydroxamic acid functionality with a mercaptoacetamide alleviated the toxicity observed in cortical neurons suggesting this as a potential method to achieve an improved safety profile. Selective inhibition of HDAC6 also
resulted in dose dependent protection against oxidative stress in cultured cortical neurons, further supporting the idea that HDAC6 inhibition may be a viable target for the treatment of certain neurological diseases. Therefore, HDAC6 selective mercaptoacetamides offer an attractive alternative to the conventional hydroxamic acid-based inhibitors as they allow for a reduction in toxicity as well as the potential for enhanced cell permeability via prodrug formation.

2.5 Experimental Section

2.5.1 Chemistry

$^1$H NMR and $^{13}$C NMR spectra were recorded on a Bruker spectrometer at 300/400 MHz and 75/100 MHz, respectively, with TMS as an internal standard. Standard abbreviations indicating multiplicity were used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, dt = doublet of triplets and br = broad. HRMS experiments were performed on a Q-TOF-2TM instrument (Micromass). TLC was performed with Merck 250-mm 60F$_{254}$ silica gel plates. Column chromatography was performed using Merck silica gel (40-60 mesh). Microwave-assisted reactions were carried out in a sealed tube using a Biotage Initiator. Prep HPLC was carried out with an ACE 5AQ column (250 x 10 mm) on a Shimadzu LC8A with SPD-10avp detector (254 and 280 nm) and flow rate of 3.5 mL/min with a gradient (Method A: gradient from 30% acetonitrile in water to 100% acetonitrile over 30 min; Method B: gradient from 10% acetonitrile in water to 100% acetonitrile over 28 min). Chiral HPLC was carried out with a Chiralpak® AD column (250 x 10 mm) on a Shimadzu LC8A with SPD-10AV detector (230 and 240 nm) and flow rate of 2.4 mL/min with an isocratic mobile phase (80:20 hexane/2-propanal). The purity of all tested compounds was ≥95% as determined by analytical HPLC (Agilent 1100 with a G1314A detector (254 nm) and flow rate of 1.4 mL/min using a Synergi 4μ hydro-RP column.
(150 x 4.6 mm) or a Luna 5μ C₈ column (150 x 4.6 mm) and a gradient from 60% acetonitrile in water to 100% acetonitrile over 25 min).

**Ethyl 5-methyl-3-phenylisoxazole-4-carboxylate (2.1).** (E)-benzaldehyde oxime (2.4 g, 19.8 mmol) was mixed with neutral Al₂O₃ (20g). N-chlorosuccinimide (3.0 g, 22.4 mmol) was added and the reaction mixture was irradiated at 40 °C for 3 x 10 min, mixing between each step. The mixture was extracted with CCl₄ (40 mL) and the solvent was evaporated. The obtained yellow oil was dissolved in anhydrous THF (30 mL). Ethyl 2-butynoate (1.16 mL, 10.0 mmol) and triethylamine (6.0 mL, 43.0 mmol) were added slowly. The reaction mixture was irradiated at 70 °C for 1 h, filtered and washed with EtOAc, and then the filtrate was concentrated in vacuo. Purification of the crude reaction mixture by column chromatography (SiO₂, 0-10% EtOAc/hexanes) afforded the title compound (1.0 g, 43%) as a slightly yellow oil. 

\(^1\)H NMR (400 MHz, CDCl₃): δ 7.62-7.60 (m, 2H), 7.43-7.38 (m, 3H), 4.21 (q, J = 7.2 Hz, 2H), 2.70 (s, 3H), 1.92 (t, J = 7.2 Hz, 3H). \(^13\)C NMR (100 MHz, CDCl₃): δ 175.6, 162.3, 129.6, 129.2, 128.5, 127.9, 108.2, 60.5, 13.7, 13.3.

**5-Methyl-3-phenylisoxazole-4-carboxylic acid (2.2).** To the isoxazole 2.1 (2.68 g, 11.6 mmol) in MeOH/H₂O: 1/1 (80 mL) was added sodium hydroxide (540 mg, 13.3 mmol). The reaction mixture was stirred at 60 °C for 4 h and then the methanol layer was evaporated. The aqueous layer was acidified with 1N HCl to pH 3 and then extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with water (100 mL), brine (50 mL) and dried over anhydrous Na₂SO₄ to afford the title compound (2.31 g, 98%) as a white powder. 

\(^1\)H NMR (400 MHz, CDCl₃): δ 7.65-7.62 (m, 2H), 7.49-7.43 (m, 3H), 2.77 (s, 3H). \(^13\)C NMR (100 MHz, CDCl₃): δ 177.7, 166.8, 162.7, 129.9, 129.4, 128.2, 127.9, 107.5, 13.9.

**tert-Butyl 5-(5-methyl-3-phenylisoxazole-4-carbonylamino)-pentylcarbamate (2.3).** To the isoxazole 2.2 (2.32 g, 11.4 mmol) in CH₂Cl₂ (100 mL) was added tert-butyl 5-
aminopentylcarbamate (2.6 mL, 12.6 mmol), PyBOP (7.12 g, 13.7 mmol), and diisopropylethylamine (6 mL, 34.2 mmol). The reaction mixture was stirred at RT for 20 h and quenched with water. The aqueous layer was extracted with CH₂Cl₂ (3 x 50 mL). The combined organic layers were washed with brine (50 mL) and dried over anhydrous Na₂SO₄. Purification of the crude reaction mixture by column chromatography (SiO₂, 0-20% EtOAc/hexanes) afforded the title compound (4.35 g, 98%) as a white powder. ¹H NMR (400 MHz, CDCl₃):  δ 7.49-7.47 (m, 2H), 7.39-7.36 (m, 3H), 5.82 (br, 1H), 4.71 (br, 1H), 3.13-3.08 (m, 2H), 2.94-2.89 (m, 2H), 2.54 (s, 3H), 1.32 (s, 9H), 1.28-1.22 (m, 4H), 1.08-1.03 (m, 2H). ¹³C NMR (100 MHz, CDCl₃):  δ 172.4, 161.2, 159.6, 155.6, 129.9, 128.5, 128.3, 127.8, 111.1, 78.5, 38.8, 29.1, 28.3, 27.9, 25.9, 25.8, 12.2.

**Methyl-3-phenylisoxazole-4-carboxylic acid (5-aminopentyl)-amide-TFA (2.4).** To the isoxazole 2.3 (4.02 g, 10.4 mmol) in CH₂Cl₂ (60 mL) was added trifluoroacetic acid (TFA) (7.7 mL, 103.7 mmol). The reaction mixture was stirred at RT for 4h and then the solvent was removed in vacuo to afford the title compound (4.09 g, 98%) as a yellow oil. ¹H NMR (400 MHz, CD₂OD):  δ 7.61-7.57 (m, 2H), 7.43-7.39 (m, 3H), 3.27-3.23 (m, 2H), 2.84-2.80 (m, 2H), 2.49 (s, 3H), 1.68-1.58 (m, 2H), 1.54-1.47 (m, 2H), 1.34-1.26 (m, 2H). ¹³C NMR (100 MHz, CD₂OD):  δ 171.6, 164.6, 161.9, 131.2, 129.8, 129.2, 128.1, 113.8, 40.5, 40.3, 29.6, 28.0, 24.7, 12.0.

**2-(Tritylthio)acetic acid (2.5).** To thioglycolic acid (3.5 mL, 50 mmol) in CHCl₃ (50 mL) were added triphenylmethanol (13.0 g, 50 mmol) and TFA (5.0 mL, 65 mmol). The reaction mixture was stirred at RT for 3h. The volatiles were removed in vacuo. The crude product was recrystallized from CH₂Cl₂/hexanes: 1/1 (30 mL) and washed with cold Et₂O to afford the title compound (15.9 g, 95%) as a white powder. ¹H NMR (300 MHz, CDCl₃):  δ 7.43-7.40
(m, 6H), 7.30-7.19 (m, 9H), 3.02 (s, 2H). $^{13}$C NMR (75 MHz, CD$_3$OD): δ 171.7, 144.2, 129.3, 127.6, 126.6, 66.7, 34.3.

2-[(Tritylthio)propanoic acid (2.6). Compound 2.6 (75%) was prepared from 2-mercaptopropanoic acid according to the methodology described for 2.5. $^1$H NMR (400 MHz, CD$_3$OD): δ 7.43-7.41 (m, 6H), 7.28-7.19 (m, 9H), 2.90 (q, J = 7.2 Hz, 1H), 1.01 (d, J = 7.2 Hz, 3H). $^{13}$C NMR (100 MHz, CD$_3$OD): δ 177.1, 145.8, 130.8, 128.9, 127.6, 69.3, 44.0, 19.4.

2-Methyl-2-[(tritylthio)propanoic acid (2.7). Compound 2.7 (80%) was prepared from 2-mercaptoisobutyric acid according to the methodology described for 2.5. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.68-7.66 (m, 6H), 7.38-7.26 (m, 9H), 1.48 (s, 6H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 180.0, 144.2, 129.9, 127.6, 126.7, 68.4, 51.2, 27.1.

2-[(Pivaloylthio)acetic acid (2.8). At 0 ºC, to thioglycolic acid (1.39 mL, 50 mmol) in dioxane (10 mL) were added pivaloyl chloride (2.7 mL, 22 mmol) and triethylamine (6.1 mL, 44 mmol). The reaction mixture was stirred at RT overnight, filtered and acidified with 1N HCl to pH 1. This layer was extracted with CH$_2$Cl$_2$ (3 x 100 mL). The combined organic layers were dried over anhydrous Na$_2$SO$_4$. Purification of the crude reaction mixture by prep HPLC Method B afforded the title compound (0.61 g, 17%) as a colorless oil. $^1$H NMR (300 MHz, CDCl$_3$): δ 9.80 (s, 1H), 3.52 (s, 2H), 1.89 (s, 9H). $^{13}$C NMR (75 MHz, CD$_3$OD): δ 205.1, 174.7, 46.2, 30.8, 27.0.

5-Methyl-3-phenylisoxazole-4-carboxylic acid [5-[(2-tritylsulfanylacetyl)-aminopentyl]-amide (2.9). To the isoxazole 2.4 (3.85 g, 9.6 mmol) in CH$_2$Cl$_2$ (100 mL) was added compound 2.5 (3.85 g, 11.5 mmol), PyBOP (7.0 g, 13.4 mmol), and diisopropylethylamine (5 mL, 29.0 mmol). The reaction mixture was stirred at RT for 20 h and quenched with water. The aqueous layer was extracted with CH$_2$Cl$_2$ (3 x 50 mL). The combined organic layers were washed with brine (50 mL) and dried over anhydrous Na$_2$SO$_4$. Purification of the crude
reaction mixture by column chromatography (SiO$_2$, 0-5% MeOH/CH$_2$Cl$_2$) afforded the title compound (5.32 g, 92%) as a white foam. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.58-7.55 (m, 2H), 7.53-7.49 (m, 3H), 7.42-7.38 (m, 6H), 7.31-7.20 (m, 9H), 5.97 (br, 1H), 5.38 (br, 1H), 3.21-3.17 (m, 2H), 3.09 (s, 2H), 2.89-2.85 (m, 2H), 2.71 (s, 3H), 1.32-1.23 (m, 4H), 1.05-1.01 (m, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 173.3, 167.7, 161.4, 159.9, 143.8, 130.3, 129.3, 128.9, 128.7, 128.1, 128.0, 126.9, 111.2, 67.7, 39.2, 38.9, 35.7, 28.6, 28.5, 23.8, 12.6.

5-Methyl-3-phenylisoxazole-4-carboxylic acid [5-(2-tritylsulfanylpropionyl)-aminopentyl]-amide (2.10). Compound 2.10 (73%) was prepared from compound 2.6 according to the methodology described for 2.9. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.59-7.56 (m, 2H), 7.51-7.47 (m, 3H), 7.44-7.42 (m, 6H), 7.30-7.22 (m, 6H), 7.21-7.19 (m, 3H), 6.01 (br, 1H), 5.75 (br, 1H), 3.21-3.16 (m, 2H), 3.02 (q, $J = 7.6$ Hz, 1H), 2.91-2.86 (m, 1H), 2.67-2.62 (m, 1H), 2.66 (s, 3H), 1.43 (d, $J = 7.6$ Hz, 2H), 1.33-1.28 (m, 2H), 1.24-1.20 (m, 2H), 1.08-1.02 (m, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 173.0, 172.4, 161.5, 159.9, 144.1, 130.3, 129.1, 128.8, 128.6, 128.0, 127.9, 126.8, 111.2, 67.9, 44.3, 39.2, 39.0, 28.5, 28.4, 23.7, 19.9, 12.6.

(R)-5-Methyl-3-phenylisoxazole-4-carboxylic acid [5-(2-tritylsulfanylpropionyl)-aminopentyl]-amide ((R)-2.10). Compound (R)-2.10 was isolated from the racemic mixture of compound 2.10 using chiral prep HPLC as described in the synthetic protocol above. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.59-7.56 (m, 2H), 7.51-7.48 (m, 3H), 7.44-7.42 (m, 6H), 7.31-7.27 (m, 6H), 7.24-7.20 (m, 3H), 5.92 (br, 1H), 5.46 (br, 1H), 3.22-3.17 (m, 2H), 3.02 (q, $J = 7.6$ Hz, 1H), 2.92-2.87 (m, 1H), 2.71 (s, 3H), 2.69-2.61 (m, 1H), 1.43 (d, $J = 7.6$ Hz, 3H), 1.33-1.27 (m, 2H), 1.25-1.20 (m, 2H), 1.07-1.02 (m, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 174.0, 172.4, 161.6, 160.2, 144.5, 130.7, 129.5, 129.3, 129.1, 128.5, 128.3, 127.2, 110.9, 68.3, 44.7, 39.6, 39.3, 28.9, 28.8, 24.1, 20.4, 13.1. $[\alpha]_D^{20} = +39.9$ (c 1, CHCl$_3$).
(S)-5-Methyl-3-phenylisoxazole-4-carboxylic acid [5-(2-tritylsulfanylpropionyl)-aminopentyl]-amide ((S)-2.10). Compound (S)-2.10 was isolated from the racemic mixture of compound 2.10 using chiral prep HPLC as described in the synthetic protocol above. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.58-7.56 (m, 2H), 7.53-7.49 (m, 3H), 7.43-7.41 (m, 6H), 7.30-7.28 (m, 6H), 7.26-7.19 (m, 3H), 5.93 (br, 1H), 5.44 (br, 1H), 3.20-3.18 (m, 2H), 3.02 (q, J = 7.6 Hz, 1H), 2.93-2.86 (m, 1H), 2.70 (s, 3H), 2.68-2.62 (m, 1H), 1.43 (d, J = 7.6 Hz, 3H), 1.32-1.28 (m, 2H), 1.23-1.19 (m, 2H), 1.04-1.02 (m, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 173.5, 172.1, 161.2, 159.6, 143.9, 130.1, 129.0, 128.7, 128.6, 127.9, 127.7, 126.6, 110.7, 67.8, 44.1, 39.1, 38.7, 28.3, 28.2, 23.6, 19.8, 12.5. [$\alpha$]$_D^{20}$ = -36.5 (c 1, CHCl$_3$).

5-Methyl-3-phenylisoxazole-4-carboxylic acid [5-(2-tritylsulfilylisobutyryl)-aminopentyl]-amide (2.11). Compound 2.11 (98%) was prepared from compound 2.7 according to the methodology described for 2.9. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.59-7.56 (m, 2H), 7.47-7.45 (m, 3H), 7.44-7.42 (m, 6H), 7.26-7.22 (m, 6H), 7.20-7.17 (m, 3H), 6.49 (br, 1H), 6.01 (br, 1H), 3.20-3.15 (m, 2H), 3.10-3.05 (m, 2H), 2.62 (s, 3H), 1.35 (s, 6H), 1.33-1.28 (m, 2H), 1.19-1.13 (m, 2H), 1.07-1.02 (m, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 174.1, 172.5, 161.9, 160.2, 144.4, 130.3, 129.1, 129.0, 128.6, 128.0, 127.8, 126.9, 111.7, 67.9, 52.3, 43.3, 39.3, 28.6, 28.5, 28.4, 23.9, 12.6.

5-Methyl-3-phenylisoxazole-4-carboxylic acid [5-(2-pivaloylsulfanylisobutyryl)-aminopentyl]-amide (2.12). Compound 2.12 (14%) was prepared from compound 2.8 according to the methodology described for 2.9. Purification of the obtained crude mixture was done using prep HPLC Method A. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.59-7.57 (m, 2H), 7.54-7.49 (m, 3H), 6.22 (br, 1H), 5.46 (br, 1H), 3.45 (s, 2H), 3.24-3.19 (m, 2H), 3.17-3.12 (m, 2H), 2.71 (s, 3H), 1.42-1.31 (m, 4H), 1.24 (s, 9H), 1.16-1.11 (m, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 207.26, 173.8, 168.7, 161.6, 160.0, 130.5, 129.1, 129.0, 128.3, 111.1, 46.5, 39.3,
5-Methyl-3-phenylisoxazole-4-carboxylic acid [5-(2-mercaptoacetyl)-aminopentyl]-amide (2.13). At 0 °C, to the isoxazole 2.9 (5.31 g, 8.8 mmol) in CH₂Cl₂ (50 mL) were added TFA (6.5 mL, 88.0 mmol) and triethylsilane (2.8 mL, 17.6 mmol). The reaction mixture was stirred at RT for 3 h. DMF (2 mL) was added and volatiles were removed in vacuo. Purification of the crude reaction mixture by prep HPLC Method A afforded the title compound (2.45 g, 77%) as a white powder. ¹H NMR (400 MHz, CDCl₃): δ 7.59-7.54 (m, 2H), 7.52-7.48 (m, 3H), 6.70 (br, 1H), 5.44 (br, 1H), 3.26-3.20 (m, 6H), 2.71 (s, 3H), 1.85 (t, J = 9.0 Hz, 1H), 1.51-1.44 (m, 2H), 1.41-1.34 (m, 2H), 1.22-1.14 (m, 2H). ¹³C NMR (100 MHz, CDCl₃): δ 173.7, 169.1, 161.5, 160.0, 130.5, 129.1, 128.9, 128.2, 111.1, 39.5, 39.0, 28.9, 28.7, 28.2, 23.8, 12.8. ESI-HRMS calc. for [C₁₈H₂₃N₃O₃S+H]⁺: m/z 362.15329, found: m/z 362.1519. HPLC purity: 99.7%.

5-Methyl-3-phenylisoxazole-4-carboxylic acid [5-(2-mercaptopropionyl)-aminopentyl]-amide (2.14). Compound 2.14 (46%) was prepared from compound 2.10 according to the methodology described for 2.13. ¹H NMR (400 MHz, CDCl₃): δ 7.59-7.54 (m, 2H), 7.52-7.47 (m, 3H), 6.54 (br, 1H), 5.50 (br, 1H), 3.40-3.35 (m, 1H), 3.24-3.15 (m, 4H), 2.69 (s, 3H), 1.99 (d, J = 8.4 Hz, 1H), 1.50 (d, J = 7.2 Hz, 3H), 1.47-1.42 (m, 2H), 1.40-1.34 (m, 2H), 1.21-1.15 (m, 2H). ¹³C NMR (100 MHz, CDCl₃): δ 173.7, 172.8, 161.5, 160.0, 130.5, 129.1, 128.9, 128.2, 111.1, 39.4, 39.0, 38.1, 28.8, 28.7, 23.8, 22.2, 12.8. ESI-HRMS calc. for [C₁₉H₂₅N₃O₃S+H]⁺: m/z 376.16894, found: m/z 376.1692. HPLC purity: 98.9%.

(R)-5-Methyl-3-phenylisoxazole-4-carboxylic acid [5-(2-mercaptopropionyl)-aminopentyl]-amide ((R)-2.14). Compound (R)-2.14 (43%) was prepared from compound (R)-2.10 according to the methodology described for 2.13. ¹H NMR (400 MHz, CDCl₃): δ
7.57-7.54 (m, 2H), 7.54-7.50 (m, 3H), 6.56 (br, 1H), 5.53 (br, 1H), 3.40-3.36 (m, 1H), 3.24-3.15 (m, 4H), 2.69 (s, 3H), 1.99 (d, J = 8.4 Hz, 1H), 1.50 (d, J = 7.2 Hz, 3H), 1.48-1.42 (m, 2H), 1.40-1.35 (m, 2H), 1.21-1.15 (m, 2H). 13C NMR (100 MHz, CDCl3): δ 173.3, 172.6, 161.2, 159.7, 130.1, 128.7, 128.5, 127.8, 110.8, 39.1, 38.6, 37.8, 28.5, 28.4, 23.5, 21.9, 12.5. [α]20D = +3.9 (c 0.1, CHCl3). ESI-HRMS calc. for [C19H25N3O3S+H]+: m/z 376.1689, found: m/z 376.1692. HPLC purity: 98.7%.

(S)-5-Methyl-3-phenylisoxazole-4-carboxylic acid [5-(2-mercaptopropionyl)-aminopentyl]-amide ((S)-2.14). Compound (S)-2.14 (45%) was prepared from compound (S)-2.10 according to the methodology described for 2.13. 1H NMR (400 MHz, CDCl3): δ 7.58-7.55 (m, 2H), 7.53-7.48 (m, 3H), 6.51 (br, 1H), 5.48 (br, 1H), 3.41-3.37 (m, 1H), 3.25-3.16 (m, 4H), 2.70 (s, 3H), 1.99 (d, J = 8.4 Hz, 1H), 1.50 (d, J = 7.2 Hz, 3H), 1.46-1.43 (m, 2H), 1.38-1.33 (m, 2H), 1.21-1.15 (m, 2H). 13C NMR (100 MHz, CDCl3): δ 174.0, 173.1, 161.8, 160.2, 130.7, 129.3, 129.2, 128.5, 111.4, 39.7, 39.2, 38.4, 29.1, 29.0, 24.1, 22.5, 13.1. [α]20D = -3.7 (c 0.1, CHCl3). ESI-HRMS calc. for [C19H25N3O3S+H]+: m/z 376.1689, found: m/z 376.1691. HPLC purity: 99.0%.

5-Methyl-3-phenylisoxazole-4-carboxylic acid [5-(2-mercaptoisobutyryl)-aminopentyl]-amide (2.15). Compound 2.15 (57%) was prepared from compound 2.11 according to the methodology described for 2.13. 1H NMR (400 MHz, CDCl3): δ 7.54-7.50 (m, 2H), 7.47-7.43 (m, 3H), 6.96 (br, 1H), 5.63 (br, 1H), 3.21-3.16 (m, 2H), 3.15-3.10 (m, 2H), 2.64 (s, 3H), 2.14 (s, 1H), 1.52 (s, 6H), 1.46-1.38 (m, 2H), 1.36-1.30 (m, 2H), 1.17-1.09 (m, 2H). 13C NMR (100 MHz, CDCl3): δ 175.1, 173.3, 161.5, 159.9, 130.3, 129.0, 128.7, 128.1, 111.1, 47.5, 39.6, 39.0, 30.2, 28.9, 28.6, 23.8, 12.7. ESI-HRMS calc. for [C20H27N3O3S+H]+: m/z 390.1846, found: m/z 390.1861. HPLC purity: 99.0%.

The spectral data for compounds 2.16-2.18 matches that which was previously published.
(R)-S-1-(5-(5-Methyl-3-phenylisoxazole-4-carboxamido)pentylamino)-1-oxopropan-2-yl ethanethioate (2.19). Compound 2.19 (58 mg, 93%) was prepared from amine 2.4 according to the procedure described for 2.9 substituting 2.18 for 2.5. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.56 (m, 2H), 7.48 (m, 3H), 6.25 (br, 1H), 5.56 (br, 1H), 3.97 (q, $J$ = 7.2 Hz, 1H), 3.23-3.08 (m, 4H), 2.68 (s, 3H), 2.31 (s, 3H), 1.43-1.31 (m, 7H), 1.12 (m, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 111.5, 41.1, 39.4, 39.3, 30.4, 29.1, 28.8, 23.9, 16.1, 13.0. ESI-LRMS calc. for [C$_{21}$H$_{27}$N$_3$O$_4$S+H]$^+$: m/z 418.2, found: m/z 418.2.

Methyl 7-(5-methyl-3-phenylisoxazole-4-carbonylamino)-heptanoate (2.20). To the isoxazole 2.2 (0.2 g, 1.0 mmol) in CH$_2$Cl$_2$ (20 mL) was added methyl 5-aminopentanoate (generated from its acid) (0.193 g, 1.0 mmol), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (0.612 g, 1.2 mmol), and diisopropylethylamine (0.5 mL, 3 mmol). The reaction mixture was stirred at RT for 20 h and then quenched with water. The aqueous layer was extracted with CH$_2$Cl$_2$ (3 x 30 mL). The combined organic layers were washed with brine (30 mL) and dried over anhydrous Na$_2$SO$_4$. Purification of the crude reaction mixture by column chromatography (SiO$_2$, 0-5% MeOH/CH$_2$Cl$_2$) afforded the title compound (0.29 g, 86%) as a white solid. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.43-7.41 (m, 2H), 7.36-7.27 (m, 3H), 5.91 (br, 1H), 3.49 (s, 3H), 3.07-3.02 (m, 2H), 2.46 (s, 3H), 2.13 (t, $J$ = 7.2 Hz, 2H), 1.46-1.37 (m, 2H), 1.24-1.18 (m, 2H), 1.13-1.05 (m, 2H), 1.03-2.97 (m, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 173.6, 172.1, 161.2, 159.7, 129.9, 128.2, 127.8, 126.7, 111.3, 51.0, 38.9, 33.4, 28.5, 28.2, 26.0, 24.3, 12.1.

5-Methyl-3-phenylisoxazole-4-carboxylic acid (6-hydroxycarbamoylhexyl)-amide (2.21). To the isoxazole 2.20 (0.1 g, 0.29 mmol) in MeOH (1 mL) was added NH$_2$OH [generated from filtration at 0 ºC of a mixture of NH$_2$OH-HCl (0.4 g, 5.8 mmol) and KOH (325
mg, 5.8 mmol) in MeOH (2mL) heated at 40 °C] and KOH (120 mg, 2.1 mmol). The reaction mixture was stirred at RT for 1.5 h and quenched with water. The aqueous layer was extracted with EtOAc (3 x 10 mL). The combined organic layers were dried over anhydrous Na$_2$SO$_4$. Purification of the crude reaction mixture by prep HPLC Method A afforded the title compound (0.21 g, 21%) as a white solid.

$^1$H NMR (400 MHz, CD$_3$OD): δ 8.20 (br, 1H), 7.66-7.64 (m, 2H), 7.52-7.44 (m, 3H), 3.31-3.27 (m, 2H), 2.54 (s, 3H), 2.08 (t, $J = 7.2$ Hz, 2H), 1.62-1.48 (m, 4H), 1.36-1.30 (m, 4H). $^{13}$C NMR (100 MHz, CD$_3$OD): δ 171.5, 164.6, 161.9, 131.3, 129.8, 129.2, 129.1, 113.9, 40.6, 30.0, 29.7, 27.6, 26.6, 11.9. ESI-HRMS calc. for $[C_{18}H_{23}N_3O_4+H]^+$: m/z 346.17613, found: m/z 346.1768. HPLC purity: 96.2%.

5-Methyl-3-phenylisoxazol-4-ylmethanol (2.22). A borane-THF solution (1.5 ml, 1.0 M) was added dropwise to a solution of acid 2.2 (203 mg, 1 mmol) in anhydrous THF (10 ml) at 0 °C. The mixture was then stirred at room temperature overnight after which the reaction was quenched with H$_2$O (10 mL) and the organic products were extracted with EtOAc (3 x 15 mL). The organic layer was washed with water (10 mL), brine (10 mL) and dried over Na$_2$SO$_4$. After concentration in vacuo, the crude product was purified by column chromatography (SiO$_2$, EtOAc/hexanes = 70:30 - 50:50) to afford the title product 2.22 (170 mg, 90%) as a colorless oil. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.76-7.73 (m, 2H), 7.42-7.39 (m, 3H), 4.47 (s, 2H), 2.37 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 168.5, 162.3, 129.6, 128.8, 128.7, 128.1, 112.9, 53.2, 10.9.

5-Methyl-3-phenylisoxazole-4-carbaldehyde (2.23). The alcohol 2.22 (190 mg, 1 mmol) and activated MnO$_2$ (870 mg, 10 mmol) were dissolved in anhydrous CH$_2$Cl$_2$ (5 mL) under Ar atmosphere. The mixture was stirred at room temperature overnight. After the black powder was filtered off, the filtrate was dried in vacuo and purified by column chromatography (SiO$_2$, EtOAc/hexanes = 75:25) to afford the title product 2.23 (175 mg,
94%) as a white solid. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 9.91 (s, 1H), 7.66-7.65 (m, 2H), 7.49-7.47 (m, 3H), 2.74 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 184.3, 176.6, 162.0, 130.3, 128.8, 114.9, 12.8.

**tert-Butyl 7-(5-methyl-3-phenylisoxazol-4-yl)-hept-6-enylcarbamate (2.24).** At 0 ºC, to the tert-butyl 6-triphenyl-1$^5$-phosphanyhexylcarbamate phosphonium salt (6.26 g, 11.5 mmol) (generated from tert-butyl 6-bromohexylcarbamate and triphenylphosphate in toluene at reflux for 4 days) in THF (100 mL) was added n-butyllithium (10.8 mL, 17.3 mmol). The reaction mixture was stirred at 0 ºC for 30 min then the aldehyde 2.23 (388 mg, 2.07 mmol) in THF (5 mL) was added. The reaction mixture was stirred at RT for 1.5 h and then quenched with water (50 mL). The aqueous layer was extracted with EtOAc (3 x 60 mL). The combined organic layers were washed with brine (50 mL) and dried over anhydrous Na$_2$SO$_4$. Purification of the crude reaction mixture by column chromatography (SiO$_2$, 0-25% EtOAc/hexanes) afforded the title compound Z/E: 8/2 (415 mg, 54%) as a yellow oil. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.73-7.70 (m, 2H), 7.62-7.59 (m, 2H), 7.44-7.38 (m, 6H), 6.06-6.02 (m, 2H), 5.82-5.76 (m, 2H), 4.54 (br, 1H), 4.49 (br, 1H), 3.09-2.99 (m, 4H), 2.45 (s, 3H), 2.31 (s, 3H), 2.17-2.12 (m, 2H), 1.91-1.86 (m, 2H), 1.41 (s, 18H), 1.36-1.27 (m, 8H), 1.23-1.15 (m, 4H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 166.0, 165.4, 161.0, 155.8, 136.7, 134.9, 129.7, 129.3, 128.4, 127.7, 117.6, 117.0, 112.5, 111.1, 78.9, 40.3, 33.2, 29.7, 28.8, 28.5, 28.3, 26.2, 12.0, 11.7.

**N-[7-(5-Methyl-3-phenylisoxazol-4-yl)-hept-6-enyl]-2-mercaptoacetamide (2.25).** Compound 2.25 Z/E: 7/3 (33%) was prepared from compound 2.24 in 3 steps according to the methodology described for 2.4, 2.9 and 2.13. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.75-7.72 (m, 2H), 7.63-7.61 (m, 2H), 7.45-7.41 (m, 6H), 6.97 (br, 1H), 6.60 (br, 1H), 6.09-6.05 (m, 2H), 5.84-5.74 (m, 2H), 3.28-3.15 (m, 4H), 2.47 (s, 3H), 2.33 (s, 3H), 1.94-1.89 (m, 2H),
1.84-1.80 (m, 2H), 1.56-1.20 (m, 12H). $^1$C NMR (100 MHz, CDCl$_3$): $\delta$ 169.1, 166.0, 165.5, 161.0, 136.6, 134.8, 129.6, 129.3, 128.3, 127.6, 117.6, 116.9, 112.5, 111.0, 39.7, 33.1, 29.0, 28.7, 28.4, 28.1, 26.2, 11.7. ESI-HRMS calc. for [C$_{19}$H$_{24}$N$_2$O$_2$S+H]$^+$: m/z 345.16313, found: m/z 345.1638. HPLC purity: 98.1%.

(Z)-2-Mercapto-N-(7-(5-methyl-3-phenylisoxazol-4-yl)hept-6-enyl)acetamide  (cis-2.25). Compound cis-2.25 (65%) was prepared from cis-2.29 according to the procedure described for 2.13 (see Scheme 6 for isolation of cis-2.29). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.73 (m, 2H), 7.43 (m, 3H), 6.79 (s, 1H), 6.08 (d, $J$ = 10.8 Hz, 1H), 5.77 (dt, $J$ = 7.2 Hz, 11.2 Hz, 1H), 3.23-3.15 (m, 4H), 2.33 (s, 3H), 1.91 (q, $J$ = 7.2 Hz, 2H), 1.85 (t, $J$ = 8.8 Hz, 1H), 1.40 (m, 2H), 1.32 (m, 2H), 1.22 (m, 2H). $^1$C NMR (100 MHz, CDCl$_3$): $\delta$ 170.1, 166.4, 161.3, 136.9, 129.8, 129.7, 128.8, 128.0, 117.3, 111.4, 40.1, 29.2, 29.0, 28.7, 28.3, 26.6, 12.0. ESI-HRMS calc. for [C$_{19}$H$_{24}$N$_2$O$_2$S+H]$^+$: m/z 345.1631, found: m/z 345.1628. HPLC purity: 98.5%.

(E)-2-Mercapto-N-(7-(5-methyl-3-phenylisoxazol-4-yl)hept-6-enyl)acetamide  (trans-2.25). Compound trans-2.25 (71%) was prepared from trans-2.29 according to the procedure described for 2.13 (see Scheme 6 for isolation of trans-2.29). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.63 (m, 2H), 7.45 (m, 3H), 6.78 (s, 1H), 6.07 (d, $J$ = 16.0 Hz, 1H), 5.77 (dt, $J$ = 6.8 Hz, 16.0 Hz, 1H), 3.31-3.06 (m, 4H), 2.48 (s, 3H), 2.17 (q, $J$ = 7.2 Hz, 2H), 1.84 (t, $J$ = 9.0 Hz, 1H), 1.57 (m, 2H), 1.48-1.34 (m, 4H). $^1$C NMR (100 MHz, CDCl$_3$): $\delta$ 169.7, 165.8, 161.5, 135.1, 129.8, 129.6, 128.8, 128.7, 118.0, 112.8, 40.2, 33.4, 29.4, 29.1, 28.4, 26.6, 12.3. ESI-HRMS calc. for [C$_{19}$H$_{24}$N$_2$O$_2$S+H]$^+$: m/z 345.1631, found: m/z 345.1630. HPLC purity: 97.5%.

N-[7-(5-Methyl-3-phenylisoxazol-4-yl)-hept-6-enyl]-2-mercaptopropionamide  (2.26). Compound 2.26 Z/E: 6/4 (18%) was prepared from compound 2.24 in 3 steps according to the methodology described for 2.4, 2.10 and 2.14. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.75-7.73
(m, 2H), 7.63-7.62 (m, 2H), 7.46-7.41 (m, 6H), 6.47 (br, 1H), 6.35 (br, 1H), 6.10-6.05 (m, 2H), 5.83-5.79 (m, 2H), 3.43-3.32 (m, 2H), 3.28-3.23 (m, 2H), 3.19-3.13 (m, 2H), 2.48 (s, 3H), 2.34 (s, 3H), 2.19-2.13 (m, 2H), 2.00-1.96 (m, 2H), 1.93-1.91 (m, 2H), 1.55-1.52 (m, 6H), 1.43-1.22 (m, 12H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): δ 172.6, 166.1, 165.6, 161.1, 136.7, 134.8, 129.8, 129.4, 128.6, 127.8, 117.8, 117.2, 112.6, 111.1, 39.7, 38.3, 33.2, 30.3, 29.1, 28.8, 28.5, 26.3, 22.3, 11.9. ESI-HRMS calc. for [C\(_{20}\)H\(_{26}\)N\(_2\)O\(_2\)S\(\)+\(\ +\): m/z 359.1787, found: m/z 359.1785. HPLC purity: 97.4%.

\(N\)-[7-(5-Methyl-3-phenylisoxazol-4-yl)-hept-6-enyl]-2-mercaptoisobutyramide (2.27). Compound 2.27 Z/E: 7/3 (49%) was prepared from compound 2.24 in 3 steps according to the methodology described for 2.4, 2.11 and 2.15. \(^1\)H NMR (400 MHz, CDCl\(_3\)): δ 7.74-7.72 (m, 2H), 7.62-7.60 (m, 2H), 7.44-7.40 (m, 6H), 6.97 (br, 1H), 6.86 (br, 1H), 6.08-6.04 (m, 2H), 5.83-5.77 (m, 2H), 3.24-3.21 (m, 2H), 3.16-3.11 (m, 2H), 2.46 (s, 3H), 2.32 (s, 3H), 2.17-2.13 (m, 2H), 1.92-1.90 (m, 2H), 1.56 (s, 12H), 1.42-1.21 (m, 12H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): δ 175.0, 166.1, 165.5, 161.0, 136.7, 134.8, 129.8, 129.4, 128.6, 127.8, 117.7, 117.1, 112.5, 111.1, 47.7, 39.9, 33.2, 30.1, 29.1, 28.8, 28.5, 26.3, 11.9. ESI-HRMS calc. for [C\(_{21}\)H\(_{28}\)N\(_2\)O\(_2\)S\(\)+\(\ +\): m/z 371.1798, found: m/z 371.1785. HPLC purity: 96.4%.

\(N\)-[7-(5-Methyl-3-phenylisoxazol-4-yl)-hept-6-enyl]-2-pivaloylsulfanylacetamide (2.28). Compound 2.28 Z/E: 7/3 (42%) was prepared from compound 2.24 in 2 steps according to the methodology described for 2.4 and 2.12. Purification of the obtained crude mixture was done using prep HPLC Method B. \(^1\)H NMR (400 MHz, CDCl\(_3\)): δ 7.71-7.68 (m, 2H), 7.59-7.57 (m, 2H), 7.41-7.37 (m, 6H), 6.35 (br, 1H), 6.25 (br, 1H), 6.03-6.00 (m, 2H), 5.79-5.73 (m, 2H), 3.44 (s, 2H), 3.42 (s, 2H), 3.20-3.14 (m, 2H), 3.11-3.06 (m, 2H), 2.43 (s, 3H), 2.28 (s, 3H), 2.14-2.09 (m, 2H), 1.89-1.83 (m, 2H), 1.44-1.14 (m, 30H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): δ 206.8, 168.2, 165.9, 165.4, 160.9, 136.6, 134.8, 129.6, 129.2, 128.4, 127.6, 117.5,
116.9, 112.4, 111.0, 46.3, 39.4, 33.0, 32.3, 29.0, 28.7, 28.4, 27.0, 26.2, 11.7. ESI-HRMS
calc. for \([C_{24}H_{32}N_2O_3S+H]^+\): \(m/z\) 429.22064, found: \(m/z\) 429.2185. HPLC purity: 98.4%.

**(Z)**-S-2-(7-(5-Methyl-3-phenylisoxazol-4-yl)hept-6-enylamino)-2-oxoethyl

2,2-dimethylpropanethioate (cis-2.28). Compound cis-2.28 was isolated from 2.28 using
chiral column chromatography. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 7.71 (m, 2H), 7.41 (m, 3H),
6.16 (s, 1H), 6.06 (d, \(J = 10.8\) Hz, 1H), 5.79 (m, 1H), 3.44 (s, 2H), 3.12 (q, \(J = 6.8\) Hz, 2H),
2.32 (s, 3H), 1.89 (q, \(J = 7.2\) Hz, 2H), 1.37-1.27 (m, 4H), 1.25-1.15 (m, 11H). \(^{13}\)C NMR (100
MHz, CDCl\(_3\)): \(\delta\) 207.3, 168.6, 166.3, 161.3, 129.9, 129.6, 128.7, 128.0, 117.3, 111.3,
46.7, 39.7, 32.7, 29.3, 29.1, 28.8, 27.4, 26.5, 12.0. ESI-HRMS calc. for \([C_{24}H_{32}N_2O_3S+H]^+\):
\(m/z\) 429.2206, found: \(m/z\) 429.2217. HPLC purity: 99.8%.

**(E)**-S-2-(7-(5-Methyl-3-phenylisoxazol-4-yl)hept-6-enylamino)-2-oxoethyl

2,2-dimethylpropanethioate (trans-2.28). Compound trans-2.28 was isolated from 2.28 using
chiral column chromatography. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 7.62 (m, 2H), 7.45 (m, 3H),
6.20 (s, 1H), 6.06 (d, \(J = 16.0\) Hz, 1H), 5.76 (dt, \(J = 7.0\) Hz, 16.0 Hz, 1H), 3.47 (s, 2H), 3.21
(q, \(J = 6.8\) Hz, 2H), 2.48 (s, 3H), 2.15 (q, \(J = 7.2\) Hz, 2H), 1.49 (m, 4H), 1.33 (m, 2H), 1.25
(s, 9H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 207.5, 168.7, 165.8, 161.5, 135.1, 129.9, 129.6,
128.8, 128.7, 118.0, 112.8, 46.8, 39.8, 33.5, 32.7, 29.5, 29.1, 27.5, 26.5, 12.3. ESI-HRMS
calc. for \([C_{24}H_{32}N_2O_3S+H]^+\): \(m/z\) 429.2206, found: \(m/z\) 429.2212. HPLC purity: 99.4%.

**(Z)**-N-(7-(5-methyl-3-phenylisoxazol-4-yl)hept-6-enyl)-2-(tritylthio)acetamide (cis-2.29).

Compound cis-2.29 was prepared from carbamate 2.24 according to the methodology
described for 2.4 and 2.9 followed by isolation via chiral column chromatography. \(^1\)H NMR
(400 MHz, CDCl\(_3\)): \(\delta\) 7.78 (m, 2H), 7.43 (m, 9H), 7.27 (m, 9H), 6.09 (d, \(J = 11.0\) Hz, 1H),
5.82 (m, 1H), 3.12 (s, 2H), 2.90 (q, \(J = 6.7\) Hz, 2H), 2.33 (s, 3H), 1.92 (q, \(J = 6.8\) Hz, 2H),
1.27 (m, 4H), 1.14 (m, 2H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 167.8, 166.1, 161.1, 144.0, 136.8,
(E)-N-(7-(5-Methyl-3-phenylisoxazol-4-yl)hept-6-enyl)-2-(tritylthio)acetamide  \((\text{trans}-2.29)\). Compound \(\text{trans}-2.29\) was prepared from carbamate 2.24 according to the methodology described for 2.4 and 2.9 followed by isolation via chiral column chromatography. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 7.65 (m, 2H), 7.46 (m, 9H), 7.28 (m, 9H), 6.08 (d, \(J = 16.1\) Hz, 1H), 5.78 (dt, \(J = 6.7\) Hz, 16.0 Hz, 1H), 3.14 (s, 2H), 2.97 (q, \(J = 6.6\) Hz, 2H), 2.49 (s, 3H), 2.16 (q, \(J = 6.7\) Hz, 2H), 1.38 (m, 4H), 1.28 (m, 2H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 167.8, 165.6, 161.3, 144.0, 134.9, 129.6, 129.4, 129.3, 128.6, 128.5, 128.2, 127.0, 117.8, 112.6, 67.9, 39.6, 35.9, 33.2, 29.0, 28.9, 26.3, 12.1.

\textit{tert}-Butyl 5-(5-methyl-3-phenylisoxazol-4-ylmethoxy)-pentylcarbamate  \((2.30)\). The alcohol 2.22 (190 mg, 1 mmol), triphenylphosphine (315 mg, 1.2 mmol) and imidazole (88 mg, 1.3 mmol) were dissolved in anhydrous CH\(_2\)Cl\(_2\) (5 mL) under Ar atmosphere. Iodine (305 mg, 1.2 mmol) was added in one portion to the flask through a temporarily opened neck at 0 \(^\circ\)C. The mixture was stirred at RT for 3 h. After evaporating the solvent, the residue was purified by a short column (SiO\(_2\), EtOAc/hexanes = 70:30) to remove baseline impurities and obtain the crude intermediate 4-(iodomethyl)-5-methyl-3-phenylisoxazole.

NaH (60 mg, 1.5 mmol) was added in one portion to a solution of \textit{tert}-butyl 5-hydroxypentylcarbamate (305 mg, 1.5 mmol) in dry DMF (5 ml) at 0 \(^\circ\)C. The mixture was cooled to -20 \(^\circ\)C, then a solution of the crude compound 4-(iodomethyl)-5-methyl-3-phenylisoxazole in anhydrous DMF (5 ml) was added slowly. The mixture was warmed to room temperature and stirred overnight. After quenching with a saturated NH\(_4\)Cl solution (10 mL), the mixture was extracted with EtOAc. The organic layer was washed with water and brine and dried over Na\(_2\)SO\(_4\). The solvent was removed in vacuo and the residue was
purified by column chromatography (SiO$_2$, EtOAc/hexanes = 75:25 - 50:50) to afford the title product 2.30 (112 mg, 30%) as a colorless oil. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.78-7.75 (m, 2H), 7.46-7.43 (m, 3H), 4.31 (s, 2H), 3.45 (t, $J$ = 6.4 Hz, 2H), 3.09-3.08 (m, 2H), 2.48 (s, 3H), 1.63-1.58 (m, 2H), 1.49-1.33 (m, 13H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 168.8, 167.2, 162.7, 129.5, 129.2, 128.7, 128.3, 110.5, 70.0, 61.5, 40.2, 29.8, 29.2, 28.4, 24.0, 11.2.

(R)-N-(5-((5-Methyl-3-phenylisoxazol-4-yl)methoxy)pentyl)-2-(tritylthio)propanamide

((R)-2.31). Compound (R)-2.31 was isolated from a racemic mixture of compound 2.31 using chiral column chromatography. Compound 2.31 was prepared from 2.30 according to the methodology described for 2.4 and 2.10. $^1$H-NMR (400 MHz, CDCl$_3$): $\delta$ 7.79 (m, 2H), 7.47 (m, 9H), 7.25 (m, 9H), 4.33 (s, 2H), 3.45 (t, $J$ = 6.4 Hz, 2H), 2.97 (m, 2H), 2.69 (m, 1H), 2.50 (s, 3H), 1.57 (m, 2H), 1.46 (d, $J$ = 7.5 Hz, 3H), 1.29 (m, 4H). $^{13}$C-NMR (100 MHz, CDCl$_3$): $\delta$ 172.2, 168.9, 162.7, 144.3, 129.6, 129.4, 129.3, 128.7, 128.3, 128.1, 127.0, 110.6, 69.9, 68.1, 61.5, 44.6, 39.6, 29.3, 28.9, 23.6, 20.2, 11.3. $[\alpha]$_{D}^{20} = +36.0 (c 0.2, CHCl$_3$).

(S)-N-(5-((5-Methyl-3-phenylisoxazol-4-yl)methoxy)pentyl)-2-(tritylthio)propanamide

((S)-2.31). Compound (S)-2.31 was isolated from a racemic mixture of compound 2.31 using chiral column chromatography. Compound 2.31 was prepared from 2.30 according to the methodology described for 2.4 and 2.10. $^1$H-NMR (400 MHz, CDCl$_3$): $\delta$ 7.79 (m, 2H), 7.45 (m, 9H), 7.26 (m, 9H), 4.32 (s, 2H), 3.45 (t, $J$ = 6.3 Hz, 2H), 2.97 (m, 2H), 2.72 (m, 1H), 2.49 (s, 3H), 1.58 (m, 2H), 1.45 (d, $J$ = 7.5 Hz, 3H), 1.29 (m, 4H). $^{13}$C-NMR (100 MHz, CDCl$_3$): $\delta$ 172.2, 168.8, 162.7, 144.3, 129.6, 129.4, 129.2, 128.7, 128.3, 128.1, 127.0, 110.6, 69.9, 68.1, 61.5, 44.5, 39.6, 29.3, 28.9, 23.6, 20.2, 11.3. $[\alpha]$_{D}^{20} = -27.0 (c 0.4, CHCl$_3$).

2-Mercapto-N-[5-(5-methyl-3-phenylisoxazol-4-ylmethoxy)-pentyl]-propionamide

(2.32). Compound 2.32 (32%) was prepared from compound 2.30 in 3 steps according to the methodologies described for 2.4, 2.10 and 2.14. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.78-7.76
(R)-2-Mercapto-N-(5-((5-methyl-3-phenylisoxazol-4-yl)methoxy)pentyl)propanamide

((R)-2.32). Compound (R)-2.32 (56%) was prepared from compound (R)-2.31 according to the methodology described for 2.13. 1H-NMR (400 MHz, CDCl3): δ 7.77 (m, 2H), 7.45 (m, 3H), 6.55 (s, 1H), 4.32 (s, 2H), 3.48-3.38 (m, 3H), 3.24 (q, J = 6.8 Hz, 2H), 2.49 (s, 2H), 1.99 (d, J = 8.0 Hz, 1H), 1.63 (m, 2H), 1.51 (m, 5H), 1.40 (m, 2H). 13C-NMR (100 MHz, CDCl3): δ 173.3, 169.1, 163.0, 129.9, 129.4, 129.0, 128.5, 110.8, 70.1, 61.7, 40.0, 38.5, 29.4, 29.3, 23.8, 22.4, 11.5. [α]D20 = +15.0 (c 0.09, CHCl3). ESI-HRMS calc. for [C19H26N2O3S+H]+: m/z 363.17369, found: m/z 363.1740. HPLC purity: 98.7%.

(S)-2-Mercapto-N-(5-((5-methyl-3-phenylisoxazol-4-yl)methoxy)pentyl)propanamide

((S)-2.32). Compound (S)-2.32 (56%) was prepared from compound (S)-2.31 according to the methodology described for 2.13. 1H-NMR (400 MHz, CDCl3): δ 7.78 (m, 2H), 7.45 (m, 3H), 6.51 (s, 1H), 4.32 (s, 2H), 3.48-3.40 (m, 3H), 3.24 (q, J = 6.8 Hz, 2H), 2.49 (s, 2H), 1.99 (d, J = 8.0 Hz, 1H), 1.63 (m, 2H), 1.52 (m, 5H), 1.40 (m, 2H). 13C-NMR (100 MHz, CDCl3): δ 173.2, 169.1, 163.0, 129.9, 129.4, 129.0, 128.5, 110.8, 70.1, 61.7, 40.0, 38.5, 29.5, 29.4, 23.9, 22.5, 11.5. [α]D20 = -13.6 (c 0.08, CHCl3). ESI-HRMS calc. for [C19H26N2O3S+H]+: m/z 363.17369, found: m/z 363.1728. HPLC purity: 96.4%.

(R)-S-1-(5-((5-Methyl-3-phenylisoxazol-4-yl)methoxy)pentylamino)-1-oxopropan-2-yl ethanethioate (2.33). Compound 2.33 (45 mg, 89%) was prepared from carbamate 2.30 according to the procedures described for 2.4 and 2.9 substituting 2.18 for 2.5. 1H NMR (400
MHz, CDCl$_3$): δ 7.75 (m, 2H), 7.45 (m, 3H), 6.22 (s, 1H), 4.30 (s, 2H), 3.99 (q, $J = 7.2$ Hz, 1H), 3.44 (t, $J = 6.4$ Hz, 2H), 3.20 (m, 2H), 2.48 (s, 3H), 2.31 (s, 3H), 1.60 (m, 2H), 1.47 (m, 5H), 1.33 (m, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$): δ 196.9, 171.4, 169.0, 162.9, 129.8, 129.4, 128.9, 128.5, 110.8, 70.1, 61.7, 41.0, 39.7, 30.4, 29.4, 29.3, 23.7, 16.1, 11.5. ESI-LRMS calc. for [C$_{21}$H$_{28}$N$_2$O$_4$S+H]$^+$: m/z 405.1, found: m/z 405.1.

5-(3-Fluorophenyl)-4-iodo-3-methylisoxazole (2.35). At $-78$ °C, to 1-ethynyl-3-fluorobenzene (1.15 mL, 10 mmol) in THF (50 mL) was slowly added n-butyllithium (9.4 mL, 15 mmol). The reaction mixture was stirred at 0 °C for 30 min then cooled to $-78$ °C. ZnCl$_2$ (2g, 15 mmol) in THF (15 mL) and then acetyl chloride (1.42 mL) were added. The reaction mixture was stirred at RT for 1 h and quenched with hexanes (10 mL). The organic layer was washed with brine (15 mL) and dried over anhydrous Na$_2$SO$_4$. Purification of the crude reaction mixture by column chromatography (SiO$_2$, 0-20% EtOAc/hexanes) afforded 4-(3-fluorophenyl)-but-3-yn-2-one (1.6 g, 98%) as a slightly yellow oil. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.38-7.16 (m, 4H), 2.47 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 184.2, 163.4, 160.9, 130.3, 128.8, 121.6, 119.7, 119.4, 118.2, 118.0, 88.4, 88.2, 32.6.

To this ketone in MeOH (30 mL) with Na$_2$SO$_4$ (2.84 g, 20 mmol) were added NH$_2$OH-HCl (1.67 g, 20 mmol) and pyridine (2.83 mL, 35 mmol). The reaction mixture was stirred at reflux for 17 h and then the volatiles were evaporated. The crude mixture was dissolved in EtOAc, washed with H$_2$O, washed with brine and dried over anhydrous Na$_2$SO$_4$ to afford the racemic 4-(3-fluorophenyl)-but-3-yn-2-one O-methyloxime (1.6 g, 85%) as a yellow oil. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.48-7.03 (m, 8H), 3.96 (s, 3H), 3.95 (s, 3H), 2.10 (s, 3H), 2.06 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 163.4, 161.0, 149.8, 130.0, 127.8, 123.7, 118.7, 118.5, 116.6, 116.3, 97.4, 88.6, 62.5, 62.3, 20.9, 20.4.
To this oxime in CH₂Cl₂ (60 mL) was added 1M ICl in CH₂Cl₂ (10.2 mL, 10.2 mmol). The reaction mixture was stirred at RT overnight and saturated Na₂S₂O₃ was added. The aqueous layer was extracted with CH₂Cl₂ (3 x 30 mL). The combined organic layers were dried over anhydrous Na₂SO₄. Purification of the crude reaction mixture by prep HPLC Method A afforded the title compound (526 mg, 41%) as a yellow oil. ¹H NMR (400 MHz, CDCl₃): δ 7.81-7.79 (m, 1H), 7.73-7.71 (m, 1H), 7.45-7.39 (m, 1H), 7.16-7.11 (m, 1H), 2.31 (s, 3H). ¹³C NMR (100 MHz, CDCl₃): δ 165.6, 163.6, 163.0, 161.2, 130.3, 128.8, 122.7, 117.4, 117.2, 114.1, 113.9, 58.6, 12.4.

2-Mercapto-N-[7-(3-methyl-5-phenylisoxazol-4-yl)-hept-6-ynyl]-propionamide (2.36). To the 4-iodo-3-methyl-5-phenylisoxazole 2.34 (1 g, 3.5 mmol) in DMF (16 mL) was added tert-butyl hept-6-ynylcarbamate (1.48 g, 7 mmol) [generated from 6-heptynenitrile by reduction of the nitrile with LiAlH₄ in Et₂O and Boc-protection of the obtained amine], PdCl₂(PPh₃)₂ (245 mg, 0.35 mmol), Cul (133 mg, 0.7 mmol) and diisopropylethylamine (16 mL). The reaction mixture was irradiated at 130 °C for 20 min. EtOAc (30 mL) was added. The organic layer was washed with H₂O (3 x 50 mL) and dried over anhydrous Na₂SO₄. Purification of the crude reaction mixture by column chromatography (SiO₂, 0-100% EtOAc/hexanes) afforded tert-butyl 7-(3-methyl-5-phenylisoxazol-4-yl)-hept-6-ynylcarbamate (0.87 g, 67%) as a yellow oil. ¹H NMR (400 MHz, CDCl₃): δ 8.12-8.09 (m, 2H), 7.49-7.43 (m, 3H), 4.53 (s, 1H), 3.14-3.13 (m, 2H), 2.51 (t, J = 6.4 Hz, 2H), 2.34 (s, 3H), 1.70-1.63 (m, 2H), 1.53-1.49 (m, 4H), 1.43 (s, 9H). ¹³C NMR (100 MHz, CDCl₃): δ 167.6, 162.0, 155.9, 130.2, 128.7, 127.4, 125.9, 99.5, 97.2, 79.1, 70.0, 40.4, 29.6, 28.4, 28.2, 26.0, 19.6, 10.5.

The boc protecting group was removed according to the methodology described for 2.4 to afford 7-(3-methyl-5-phenylisoxazol-4-yl)-hept-6-ynylamine TFA salt. ¹H NMR (400 MHz, CD₃OD): δ 8.09-8.07 (m, 2H), 7.50-7.48 (m, 3H), 2.95-2.89 (m, 2H), 2.57 (t, J = 6.4 Hz, 2H),
2.30 (s, 3H), 1.75-1.68 (m, 4H), 1.62-1.55 (m, 2H). $^{13}$C NMR (100 MHz, CD$_3$OD): δ 168.9, 163.3, 161.0, 131.7, 130.0, 128.5, 126.9, 100.6, 98.6, 70.7, 40.6, 29.2, 28.1, 26.8, 20.0, 10.4.

$N$-[7-(3-Methyl-5-phenylisoxazol-4-yl)-hept-6-ynyl]-2-tritylsulfanylpropionamide (43%) was prepared from the obtained amine according to the methodology described for 2.10. $^1$H NMR (400 MHz, CDCl$_3$): δ 8.12-8.10 (m, 2H), 7.49-7.43 (m, 9H), 7.29-7.26 (m, 6H), 7.22-7.18 (m, 3H), 5.99 (br, 1H), 3.05-2.97 (m, 2H), 2.76-2.71 (m, 1H), 2.50-2.46 (m, 2H), 2.33 (s, 3H), 1.63-1.58 (m, 2H), 1.44 (d, $J$ = 7.2 Hz, 3H), 1.39-1.33 (m, 4H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 172.0, 167.4, 161.8, 144.1, 130.1, 129.2, 128.5, 127.9, 127.3, 126.8, 125.7, 99.3, 97.0, 69.9, 67.9, 44.3, 39.4, 28.4, 28.0, 25.9, 20.0, 19.4, 10.4.

Compound 2.36 (79%) was prepared from the obtained propionamide according to the methodology described for 2.13. $^1$H NMR (400 MHz, CDCl$_3$): δ 8.07-8.05 (m, 2H), 7.45-7.39 (m, 3H), 6.70 (br, 1H), 3.40-3.33 (m, 1H), 3.26-3.21 (m, 2H), 2.47 (t, $J$ = 6.8 Hz, 2H), 2.29 (s, 3H), 1.99 (d, $J$ = 8.4 Hz, 1H), 1.67-1.60 (m, 2H), 1.58-1.47 (m, 7H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 172.9, 167.4, 161.8, 130.2, 128.5, 127.1, 125.7, 99.3, 97.1, 69.8, 39.5, 37.9, 28.8, 28.0, 25.9, 22.1, 19.4, 10.3. ESI-HRMS calc. for [C$_{20}$H$_{24}$N$_2$O$_2$S+H]$^+$: m/z 357.16313, found: m/z 357.1638. HPLC purity: 98.9%.

7-[5-(3-Fluorophenyl)-3-methylisoxazol-4-yl]-hept-6-ynyl-2-mercaptopropionamide (2.37). Compound 2.37 (20%) was prepared from compound 2.35 in 4 steps according to the methodology described for 2.36. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.83-7.79 (m, 2H), 7.42-7.36 (m, 1H), 7.11-7.06 (m, 1H), 6.67 (br, 1H), 3.39-3.34 (m, 1H), 3.27-3.22 (m, 2H), 2.48 (t, $J$ = 6.8 Hz, 2H), 2.29 (s, 3H), 1.99 (d, $J$ = 8.0 Hz, 1H), 1.68-1.61 (m, 2H), 1.58-1.47 (m, 7H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 172.8, 166.0, 163.7, 161.9, 161.3, 130.3, 129.0, 121.4, 117.1, 116.9, 112.7, 112.5, 100.2, 98.0, 69.6, 39.5, 37.9, 28.8, 28.0, 26.0, 22.1, 19.4, 10.3. ESI-
HRMS calc. for \([C_{20}H_{23}FN_2O_2S+H]^+\): \(m/z\) 375.15370, found: \(m/z\) 375.1540. HPLC purity: 99.4%.

2-Mercapto-2-methyl-\(N\)-[7-(3-methyl-5-phenylisoxazol-4-yl)-hept-6-ynyl]-propionamide (2.38). Compound 2.38 (24%) was prepared using compound 2.7 in 4 steps according to the methodology described for 2.36. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 8.12-8.10 (m, 2H), 7.49-7.43 (m, 3H), 6.96 (br, 1H), 3.29-3.24 (m, 2H), 2.52 (t, \(J = 6.8\) Hz, 2H), 2.34 (s, 3H), 2.11 (s, 1H), 1.71-1.66 (m, 2H), 1.62-1.51 (m, 10H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 175.0, 167.6, 162.0, 130.3, 128.7, 127.4, 125.9, 99.5, 97.1, 70.1, 47.8, 39.9, 30.4, 29.0, 28.2, 26.1, 19.6, 10.5. ESI-HRMS calc. for \([C_{21}H_{26}N_2O_2S+H]^+\): \(m/z\) 371.17878, found: \(m/z\) 371.1784. HPLC purity: 96.2%.

2-Mercapto-2-methyl-\(N\)-[6-(3-methyl-5-phenylisoxazol-4-yl)-hex-5-ynyl]-propionamide (2.39). Compound 2.39 (30%) was prepared with tert-butyl hex-5-ynylcarbamate and compound 2.7 in 4 steps according to the methodology described for 2.36. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 8.07-8.05 (m, 2H), 7.45-7.37 (m, 3H), 7.02 (br, 1H), 3.31-3.26 (m, 2H), 2.52 (t, \(J = 6.8\) Hz, 2H), 2.30 (s, 3H), 2.15 (s, 1H), 1.70-1.64 (m, 4H), 1.55 (s, 6H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 175.0, 167.5, 161.8, 130.2, 128.6, 127.2, 125.8, 99.3, 96.7, 70.2, 47.5, 39.3, 30.2, 28.6, 25.8, 19.2, 10.4. ESI-HRMS calc. for \([C_{20}H_{24}N_2O_2S+H]^+\): \(m/z\) 357.16313, found: \(m/z\) 357.1636. HPLC purity: 98.3%.

\(N\)-[6-(3-Methyl-5-phenylisoxazol-4-yl)-hex-5-ynyl]-2-pivaloylsulfanylacetamide (2.40). Compound 2.40 (16%) was prepared with tert-butyl hex-5-ynylcarbamate and compound 2.8 in 3 steps according to the methodology described for 2.36. \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 8.10-8.08 (m, 2H), 7.49-7.44 (m, 3H), 6.42 (br, 1H), 3.51 (s, 2H), 3.33-3.28 (m, 2H), 2.52 (t, \(J = 6.8\) Hz, 2H), 2.33 (s, 3H), 1.70-1.62 (m, 4H), 1.22 (s, 9H). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 207.4, 169.4, 167.7, 162.0, 130.3, 128.7, 127.3, 125.9, 99.4, 96.6, 70.3, 46.5, 39.2, 32.3,
28.5, 27.2, 25.7, 19.3, 10.5. ESI-HRMS calc. for \([C_{23}H_{28}N_2O_3S+H]^+\): \(m/z\) 413.18934, found: \(m/z\) 413.1898. HPLC purity: 99.6%.

**tert-Butyl 4-[trans-2-(3-methyl-5-phenylisoxazol-4-yl)vinyl]-benzylcarbamate (2.41).** To the 4-iodo-3-methyl-5-phenylisoxazole (0.2 g, 0.7 mmol) in DMF (3 mL) was added tert-butyl 4-vinylbenzylcarbamate (327 mg, 1.4 mmol) [generated from Boc-protection of 4-vinylbenzylamine], \(\text{Pd(OAc)}_2\) (16 mg, 0.07 mmol), \(\text{nBu}_4\text{NCl}\) (194 mg, 0.7 mmol) and \(\text{Na}_2\text{CO}_3\) (148 mg, 1.4 mmol). The reaction mixture was irradiated at 130 °C for 1 h. \(\text{EtOAc}\) was added. The organic layer was washed with \(\text{H}_2\text{O}\) (3 \(\times\) 20 mL) and dried over anhydrous \(\text{Na}_2\text{SO}_4\) to afford the title compound (180 mg, 66%) as a brown oil. \(^1\text{H NMR (400 MHz, CDCl}_3\)): \(\delta\ 7.71-7.68\ (m, 2\text{H}), 7.48-7.43\ (m, 3\text{H}), 7.40-7.38\ (m, 2\text{H}), 7.30-7.26\ (m, 2\text{H}), 6.95\) (d, \(J = 16.4\ \text{Hz}, 1\text{H})\), 6.85 (d, \(J = 16.4\ \text{Hz}, 1\text{H})\), 5.28 (s, 1H), 4.31-4.29 (m, 2H), 2.46 (s, 3H), 1.48 (s, 9H). \(^{13}\text{C NMR (100 MHz, CDCl}_3\)): \(\delta\ 165.2, 158.6, 155.8, 138.8, 135.7, 131.3, 129.6, 128.6, 127.8, 127.5, 127.2, 126.2, 116.4, 112.6, 79.1, 44.0, 28.1, 11.8.

**2-Mercapto-N-(4-[trans-2-(3-methyl-5-phenylisoxazol-4-yl)vinyl]-benzyl)-propionamide (2.42).** Compound 2.42 (10%) was prepared from compound 2.41 in 3 steps according to the methodologies described for 2.4, 2.10 and 2.14. \(^1\text{H NMR (400 MHz, CDCl}_3\)): \(\delta\ 7.74-7.72\) (m, 2H), 7.52-7.46 (m, 3H), 7.44-7.42 (m, 2H), 7.30-7.26 (m, 2H), 6.99 (d, \(J = 16.8\ \text{Hz}, 1\text{H})\), 6.89 (d, \(J = 16.8\ \text{Hz}, 1\text{H})\), 6.80 (br, 1H), 4.47-4.45 (m, 2H), 3.51-3.47 (m, 1H), 2.50 (s, 3H), 2.05 (d, \(J = 8.4\ \text{Hz}, 1\text{H})\), 1.60 (d, \(J = 8.4\ \text{Hz}, 3\text{H})\). \(^{13}\text{C NMR (100 MHz, CDCl}_3\)): \(\delta\ 172.7, 165.8, 158.9, 135.7, 136.4, 131.3, 130.0, 128.9, 128.1, 127.5, 126.2, 117.2, 112.8, 43.6, 38.2, 22.2, 12.1.\) ESI-HRMS calc. for \([C_{22}H_{22}N_2O_2S+H]^+\): \(m/z\) 379.14748, found: \(m/z\) 379.1477. HPLC purity: 96.9%.

**2-Mercapto-2-methyl-N-(4-[trans-2-(3-methyl-5-phenylisoxazol-4-yl)vinyl]-benzyl)-propionamide (2.43).** Compound 2.43 (55%) was prepared from compound 2.41 in 3 steps.
according to the methodologies described for 2.4, 2.11 and 2.15. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.72-7.69 (m, 2H), 7.50-7.45 (m, 3H), 7.43-7.41 (m, 2H), 7.35 (br, 1H), 7.28-7.26 (m, 2H), 6.97 (d, $J = 16.4$ Hz, 1H), 6.87 (d, $J = 16.4$ Hz, 1H), 4.45-4.43 (m, 2H), 2.47 (s, 3H), 2.26 (s, 1H), 1.64 (s, 6H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 174.9, 165.4, 158.7, 138.0, 136.0, 131.3, 129.8, 127.8, 127.3, 126.0, 116.8, 112.7, 47.3, 43.5, 30.2, 11.9. ESI-HRMS calc. for [C$_{23}$H$_{24}$N$_2$O$_2$S+H]$^+$: $m/z$ 393.16313, found: $m/z$ 393.1624. HPLC purity: 99.0%.

### 2.5.2 HDAC Inhibition

HDAC inhibition assays were performed by the Reaction Biology Corporation (Malvern, PA) using human, full length recombinant HDAC1, 2, 5 and 6 isolated from a baculovirus expression system in Sf9 cells. A complex of human, full length recombinant HDAC3 co-expressed with human NCOR2 was used in the HDAC3 assays and amino acids 627-1085 of human, recombinant HDAC4 were used for the HDAC4 assays. An acetylated, fluorogenic peptide derived from residues 379-382 of p53 (RHKK$_{Ac}$) was used as the substrate in the assays. The reaction buffer contained 50 mM Tris-HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl$_2$, 1 mg/mL BSA and a final concentration of 1% DMSO. The enzyme was delivered into wells of the reaction plate and compounds were delivered in 100% DMSO into the enzyme mixture by acoustic technology (Echo550; nanoliter range). The plates were spun down and pre-incubated for 5-10 min. The substrate was then delivered to all reaction wells in order to initiate the reaction and the reaction was incubated for 2 h at 30 °C. After incubation, developer and Trichostatin A were added to quench the reaction and generate fluorescence. Then, kinetic measurements were taken for 1.5 h in 15 min intervals to ensure that development was complete. Endpoint readings were taken for analysis after the development reached a plateau. Dose-response curves were generated and the IC$_{50}$ for each compound was extrapolated from the generated plots (10-dose IC$_{50}$...
curves were generated using a 3-fold serial dilution pattern starting with concentrations of 30 μM).

2.5.3 Molecular Modeling

The two enantiomers \((R)-2.14\) and \((S)-2.14\) were simulated considering the thiol function in its anionic form. The conformational behavior of these compounds was investigated using a Monte Carlo procedure (as implemented in the VEGA suite of programs, www.vegazz.net) which generated 1,000 conformers by randomly rotating the rotors. All geometries so obtained were stored and optimized to avoid high-energy rotamers. The 1,000 conformers were clustered according to their similarity in order to discard redundancies; in this analysis, two geometries were considered non-redundant when they differed by more than 60 degrees in at least one torsion angle. For each ligand, the so obtained lowest energy structure was then exploited in the following docking simulations.

As mentioned before, the resolved structures of HDAC2 and HDAC4 were retrieved from the RCSB PDB, while the homology model of the HDAC6 subtype had been recently generated by us.(39) The experimental structures were completed by adding hydrogen atoms; the side-chains of Arg, Lys, Glu, and Asp were ionized to remain compatible with physiological pH values while His residues were considered neutral by default apart from those belonging to cited dyads which are reported in Supplementary Table 1. In particular, in all docking simulations, only the first histidine residue (His145, His158 and His610 for HDAC2, 4 and 6 respectively) was protonated. In all simulated HDAC structures, the second neutral histidine (His146, His159 and His611 for HDAC2, 4 and 6 respectively) was considered in its Nε tautomeric form in order to minimize steric hindrance within the catalytic pocket. The structures so obtained were minimized while keeping the backbone fixed to preserve the experimental folding for HDAC2 and 4 and the predicted folding of HDAC6.
Docking simulations were performed by *GriDock*, a parallel tool based on the AutoDock4.0 engine. In detail, the grid box was set to include all residues within a 15 Å radius around the catalytic metal ion thus comprising the entire catalytic cavity. The resolution of the grid was 60×60×60 points with a grid spacing of 0.450 Å. Each inhibitor was docked into this grid with the Lamarckian algorithm as implemented in AutoDock. For the docking simulations, the flexible bonds of the ligand were automatically recognized by *GriDock* and left free to rotate. The genetic-based algorithm ran 20 simulations per substrate with 2,000,000 energy evaluations and a maximum number of generations set to 27,000. The crossover rate was increased to 0.8, and the number of individuals in each population to 150. All other parameters were left at the AutoDock default settings. The docking results were ranked considering both AutoDock scores and the distance between the zinc ion and sulfur atom. The best complexes were minimized keeping all atoms outside of a 15 Å radius around the bound substrate fixed to favor the mutual adaptability between the ligand and enzyme. The optimized complexes were then used to re-calculate the AutoDock docking scores and VEGA energy scores. This work was done in the laboratory of Dr. Giulio Vistoli at the Università degli Studi di Milano (Milan, Italy).

2.5.4 *Mercaptoacetamide Oxidation*

These experiments were performed on a Shimadzu LCMS – 2010EV Liquid Chromatograph Mass Spectrometer. Electrospray ionization was used in positive mode with a scan range of m/z 300 to 800 with scans occurring every 0.5 seconds. A Halo C8 3.0 x 30 mm column with 2.7 μm particle size was used with a flow rate of 0.2 mL/min. Gradient: 25% MeCN/H2O to 100% MeCN, 8 min; 100% MeCN, 2 min; 100% MeCN to 25% MeCN/H2O, 0.1 min; 25% MeCN/H2O, 49.9 min. LC-MS solvents were purchased from commercial sources and each contained 0.1% formic acid. 100 μg of each compound was dissolved in 1
mL of MeOH and 1 μL aliquots were analyzed every hour for 24 hours. Normalized peaks corresponding to the [M+H]+ and [M+Na]+ ions for both the monomer and dimer were extracted from the total ion chromatogram (TIC) and the area under each respective peak in the extracted ion chromatograms (XIC) was calculated using the automatic integration feature available in Shimadzu’s LCMS Solutions (Columbia, MD) software. The cutoff for peak width was set at 20 seconds and the rest of the parameters were left at default settings. The ratio of total dimer to total monomer was calculated by adding the areas of the [M+H]+ and [M+Na]+ peaks for each respectively and calculating the quotient. The ratio was then plotted against time for each compound.

2.5.5 Neuroprotection and Glutathione Depletion

For the neuroprotection studies, cells were rinsed with warm PBS and then placed in minimum essential medium (Invitrogen) containing 5.5 g/liter glucose, 10% fetal calf serum, 2 mM L-glutamine, and 100 μM cystine. Oxidative stress was induced by the addition of the cysteine homolog, homocysteic acid (HCA; 5 mM), to the media. HCA was diluted from 100-fold concentrated solutions that were adjusted to pH 7.5. In combination with HCA, the novel HDAC inhibitors (10 μM) were added. Viability was assessed after 48 hours by calcein-acetoxymethyl ester (AM)/ethidium homodimer-1 staining (live/dead assay) (Molecular Probes, Eugene, OR) using fluorescence microscopy and the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method.

Total intracellular glutathione (GSH + GSSG) measurements of primary neuron cultures were determined using the GSH-Glo Glutathione Assay kit (Promega) according to the manufacturer’s protocol. Primary cortical neurons were plated at 1 × 10^5 cells per well in a poly-D-lysine coated 96-well plate. After 24 hours of incubation, neurons were exposed to HCA in the presence or absence of drug for 8 hours. Neurons were lysed with DTT
containing lysis buffer to convert any GSSG to GSH, allowing for the determination of total GSH. The lysates were incubated with a reaction buffer containing the luciferin precursor luciferin-NT and the enzyme GST. In the presence of GSH, GST converts luciferin-NT into luciferin. A subsequent reaction buffer containing the enzyme luciferase, which produces light as a by-product of luciferin metabolism, was added to the lysates and the light intensity (which correlates with GSH levels) was measured with a luminometer (LMax II 384; Molecular Devices). The values were normalized to protein concentration as determined by a Bradford protein assay. GSH standards were used to calculate GSH concentrations. This work was done in the laboratory of Dr. Brett Langley at The Burke Medical Research Institute (Ithaca, NY).
CHAPTER 3
OPTIMIZATION OF TRICYCLIC HDAC6 SELECTIVE INHIBITORS

3.1 Introduction

HDACi were recently shown to enhance the suppressive effects of Foxp3+ regulatory T cells (Tregs). (73, 94) The pharmacological enhancement of Treg suppression is a potential therapeutic approach to slow or reverse the pathogenesis of autoimmune disorders and prevent allograft rejection, inflammatory bowel disease and rheumatoid arthritis. (73, 94, 95) It is well known that HDACs are involved in regulating Treg immunosuppression, though the particular isoform or isoforms responsible for this activity remain under investigation. (73, 94) The use of HDACi results in increased acetylation of Foxp3, an important transcription factor responsible for the proper maturation of Tregs and the normal functioning of the immune system. (94) Foxp3 is part of a multi-protein complex that reduces Treg expression of certain cytokines such as interleukin-2 (IL-2), and HDACi use enhances Treg expression of CTLA-4, a protein with an important role in immunosuppression. (73) As a result, in murine models of allograft survival, HDACi in combination with low-dose rapamycin therapy resulted in donor-specific allograft tolerance, in stark contrast to recipients treated with rapamycin alone. (57)

Previously, our lab reported on the synthesis of a first generation, potent and selective HDAC6i, tubastatin A, and showed that tubastatin A enhanced the suppressive functions of murine Foxp3+ Tregs in an HDAC6-specific manner. (39, 57) We now report a second generation of these compounds, a series of substituted β- and γ-carbolines, further optimized for activity, selectivity and physiochemical properties. Usually, HDACi consist of a cap group that interacts with the surface of the protein, a linker that occupies a hydrophobic
channel leading to the active site and a metal chelator that interacts with the zinc ion at the bottom of the catalytic pocket.\(^{(10)}\) Having determined that a benzyl linker was optimal for potent, selective HDAC6 inhibition and keeping with the standard zinc-binding group, a hydroxamic acid, we sought to explore modifications to the cap group of these HDACi. We synthesized compounds with varying substitutions to the carboline cap groups and evaluated their HDAC inhibitory activity using purified recombinant human HDAC proteins isolated from a baculovirus expression system. Selected compounds were then tested for their ability to enhance the function of murine Foxp3+ Tregs in vitro and in vivo.

### 3.2 Chemistry

The three series of tubastatin A derivatives employed in this study were prepared from inexpensive, commercially available starting materials according to the synthetic routes outlined in Schemes 11-13. The first series was synthesized using Fischer indole synthesis to generate 6-, 7-, 8-, and 9- substituted γ-carboline cap groups 3.1-3.8 from the respective phenylhydrazine and 1-methyl-4-piperidone.\(^{(96)}\) Alkylation at the 5-position with 4-bromomethyl-benzoic acid methyl ester returned intermediate esters 3.9-3.16 which were subsequently converted to hydroxamic acids 3.17-3.24 using hydroxylamine hydrochloride and sodium methoxide (Scheme 11).\(^{(39)}\)
Scheme 11. Preparation of 6-, 7-, 8-, and 9-substituted γ-carbolines.

Reagents and conditions: a) H$_2$SO$_4$, 1,4-dioxane, 2 h, 60 °C; b) 4-bromomethyl-benzoic acid methyl ester, KOtBu, DMF, 80 °C, 2 h; c) NH$_2$OH·HCl, NaOMe, MeOH, 0 °C to rt, 16 h.

The second series of compounds consisting of 2-substituted γ-carbolines was prepared from 1-benzylpiperidin-4-one and phenylhydrazine. Fischer indole synthesis was employed to generate the 2-benzyl substituted γ-carboline which was deprotected via catalytic hydrogenation to yield common intermediate 2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole 3.25. (96, 97) Orthogonal alkylation at the 2-position followed by the 5-position with
the appropriate alkyl/benzyl halide and 4-bromomethyl-benzoic acid methyl ester respectively, yielded intermediate esters \(3.26-3.37\). The penultimate esters were then converted to hydroxamic acids \(3.38-3.49\) using hydroxylamine hydrochloride in the presence of sodium methoxide in the same manner as the compounds described in the first series (Scheme 12).\(\text{(39)}\)

Reagents and conditions: a) H$_2$SO$_4$, 1,4-dioxane, 60 °C, 2 h; b) H$_2$, 10% Pd/C, 70% EtOH/H$_2$O, 70 °C, 24 h; c) alkyl/benzyl halide, Et$_3$N, MeCN, 60 °C, 2 h; d) 4-bromomethylbenzoic acid methyl ester, KO'Bu, DMF, 80 °C, 2 h; e) NH$_2$OH·HCl, NaOMe, MeOH, 0 °C to rt, 16 h.
The third series of compounds consisting of 2-substituted β-carbolines was synthesized from commercially available 2,3,4,9-tetrahydro-1H-pyrdo[3,4-b]indole. Boc protection at the 2-position followed by alkylation at the 9-position with 4-bromomethyl-benzoic acid methyl ester yielded common intermediate 3.50. Deprotection and alkylation at the 2-position with the appropriate alkyl/benzyl halide yielded intermediate esters 3.51-3.53 which were subsequently converted to hydroxamic acids 3.54-3.56 again using hydroxylamine hydrochloride and sodium methoxide (Scheme 13). With the desired compounds in hand, we now sought to assay the activity of each at HDAC1 and HDAC6 as well as determine the ability of selected compounds to enhance Treg mediated mitotic suppression.

Reagents and conditions: a) di-tert-butyl dicarbonate, THF, reflux, 8 h; b) 4-bromomethylbenzoic acid methyl ester, KOtBu, DMF, 80 °C, 2 h; c) 10% TFA/DCM, 30 °C, 2h; d) alkyl/benzyl halide, Et3N, MeCN, 60 °C, 2 h; e) NH2OH-HCl, NaOMe, MeOH, 0 °C to rt, 16 h.

3.3 Results and Discussion

3.3.1 HDAC Inhibition

We investigated substitutions to the aromatic portion of the γ-carboline cap group in an effort to increase both potency and selectivity in comparison to our lead, tubastatin A. The results of the HDAC inhibition assays for the compounds in the first series are displayed in Table IV. Introduction of a methyl group to the 6- or 7-position, compounds 3.17 and 3.18 respectively, as well as two methyl groups simultaneously to the 7- and 9-positions, 3.20,
did little to increase the potency at HDAC6, but did increase the potency at HDAC1, resulting in a decrease in selectivity. The introduction of a methyl group to the 8-position, 3.19, increased potency at both isoforms and thus, we continued to look for substitutions at this position that would result in the same increase in potency at HDAC6 but not HDAC1. The introduction of a bulky tert-butyl group at the 8-position, 3.21, reduced potency at HDAC6 while incorporation of a methoxy group at this position, 3.22, had effects similar to that of the methyl group. In general, alkyl substitutions to the 6-, 7- and 9-positions of the γ-carboline cap resulted in an increase in potency at HDAC1 with little effect on potency at HDAC6. Substitutions at the 8-position did increase potency at HDAC6 with the 8-chloro compound 3.23 exhibiting an HDAC6 IC$_{50}$ of about 1 nM and retaining the selectivity of our lead, tubastatin A. However, introduction of an 8-bromo substituent, 3.24, while increasing potency at both HDAC1 and HDAC6, significantly reduced the selectivity for HDAC6.
### TABLE IV

IC$_{50}$ AND HDAC6 SELECTIVITY DATA FOR 6-, 7-, 8- AND 9-SUBSTITUTED γ-CARBOLINES

![Chemical Structure]

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>$R$</th>
<th>HDAC1 IC$_{50}$ (μM)</th>
<th>HDAC6 IC$_{50}$ (nM)</th>
<th>Selectivity HDAC1/HDAC6 (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tubastatin A</td>
<td>--</td>
<td>16.4 ± 2.6</td>
<td>15 ± 1</td>
<td>1093</td>
</tr>
<tr>
<td>3.17</td>
<td>6-Me</td>
<td>22.1 ± 6.2</td>
<td>34.7 ± 5.5</td>
<td>636</td>
</tr>
<tr>
<td>3.18</td>
<td>7-Me</td>
<td>4.05 ± 0.94</td>
<td>11.5 ± 0.0</td>
<td>352</td>
</tr>
<tr>
<td>3.19</td>
<td>8-Me</td>
<td>2.07 ± 0.27</td>
<td>4.90 ± 0.05</td>
<td>422</td>
</tr>
<tr>
<td>3.20</td>
<td>7,9-diMe</td>
<td>10.3 ± 1.5</td>
<td>38.4 ± 0.1</td>
<td>268</td>
</tr>
<tr>
<td>3.21</td>
<td>8-’Bu</td>
<td>14.1 ± 3.5</td>
<td>22.5 ± 4.9</td>
<td>626</td>
</tr>
<tr>
<td>3.22</td>
<td>8-OMe</td>
<td>1.88 ± 0.66</td>
<td>3.06 ± 0.49</td>
<td>614</td>
</tr>
<tr>
<td>3.23</td>
<td>8-Cl</td>
<td>1.02 ± 0.13</td>
<td>1.02 ± 0.13</td>
<td>1000</td>
</tr>
<tr>
<td>3.24</td>
<td>8-Br</td>
<td>0.427 ± 0.133</td>
<td>0.704 ± 0.197</td>
<td>606</td>
</tr>
</tbody>
</table>
In light of these results, it was apparent that substitutions to the 6-, 7-, 8-, and 9-positions of the cap group were not beneficial to increasing the selectivity of these compounds, nor were they increasing the potency at our desired isoform. Therefore, we investigated substitutions to the other side of the cap group, at the 2-position specifically. HDAC IC₅₀s and HDAC6 selectivity data for compounds 3.38-3.49 are given in Table V. The desmethyl derivative of tubastatin A, 3.39, was synthesized to evaluate the necessity of the methyl group at the 2-position. We found that removal of the methyl group resulted in a compound that was twice as potent and selective as tubastatin A. Introduction of an ethyl group instead of a methyl group at this position, 3.40, increased both potency and selectivity from 15 nM to 3 nM and from about 1,000 to nearly 3,500 fold respectively. Introduction of a bulkier iso-propyl group, 3.41, increased the potency at and selectivity for HDAC6 compared to tubastatin A, however, the increase was not as dramatic as with the ethyl group suggesting that smaller or linear substituents might be more desirable. Incorporation of an allylic group at the 2-position, 3.42, dramatically improved the HDAC6 IC₅₀ to less than 1 nM and increased the selectivity for HDAC6 vs. HDAC1 to nearly 6,000 fold.
### TABLE V

IC$_{50}$ AND HDAC6 SELECTIVITY DATA FOR 2-SUBSTITUTED γ-CARBOlines

![Chemical Structure]

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>R</th>
<th>HDAC1 IC$_{50}$ (µM)</th>
<th>HDAC6 IC$_{50}$ (nM)</th>
<th>Selectivity HDAC1/HDAC6 (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tubastatin A</td>
<td>Me</td>
<td>16.4 ± 2.6</td>
<td>15 ± 1</td>
<td>1093</td>
</tr>
<tr>
<td>3.38</td>
<td></td>
<td>8.62 ± 0.06</td>
<td>2.25 ± 0.39</td>
<td>3831</td>
</tr>
<tr>
<td>3.39</td>
<td>H</td>
<td>16.4 ± 0.1</td>
<td>6.56 ± 0.48</td>
<td>2500</td>
</tr>
<tr>
<td>3.40</td>
<td>Et</td>
<td>10.2 ± 0.7</td>
<td>2.99 ± 0.67</td>
<td>3411</td>
</tr>
<tr>
<td>3.41</td>
<td></td>
<td>15.7 ± 3.2</td>
<td>8.96 ± 1.62</td>
<td>1752</td>
</tr>
<tr>
<td>3.42</td>
<td></td>
<td>5.82 ± 0.09</td>
<td>0.972 ± 0.125</td>
<td>5987</td>
</tr>
<tr>
<td>3.43</td>
<td>Benzyl</td>
<td>10.3 ± 1.4</td>
<td>1.44 ± 0.07</td>
<td>7152</td>
</tr>
<tr>
<td>3.44</td>
<td>(\text{OMe})</td>
<td>5.19 ± 0.01</td>
<td>2.96 ± 0.85</td>
<td>1753</td>
</tr>
<tr>
<td>3.45</td>
<td>(\text{OMe})</td>
<td>4.64 ± 0.14</td>
<td>3.05 ± 0.25</td>
<td>1521</td>
</tr>
<tr>
<td>3.46</td>
<td>(\text{N})</td>
<td>2.74 ± 0.31</td>
<td>0.582 ± 0.022</td>
<td>4707</td>
</tr>
<tr>
<td>3.47</td>
<td>(\text{CONH}_2)</td>
<td>1.61 ± 0.45</td>
<td>0.459 ± 0.134</td>
<td>3507</td>
</tr>
<tr>
<td>3.48</td>
<td>(\text{CONH}_2)</td>
<td>3.98 ± 0.33</td>
<td>0.799 ± 0.205</td>
<td>4981</td>
</tr>
<tr>
<td>3.49</td>
<td>(\text{CONH}_2)</td>
<td>7.45 ± 0.07</td>
<td>4.06 ± 0.68</td>
<td>1834</td>
</tr>
</tbody>
</table>
We hypothesized that there could be a benefit to introducing aromatic functionalities at the 2-position for π-stacking purposes. Therefore, we introduced a benzyl substituent, 3.43, and observed a dramatic increase in selectivity to over 7,000 fold versus HDAC1 as well as a moderate increase in potency with an HDAC6 IC\textsubscript{50} of about 1 nM. The introduction of methoxy substituents to the benzyl group, 3.44 and 3.45, resulted in a decrease in potency and selectivity compared to the benzyl group alone. In an attempt to increase aqueous solubility in addition to improving potency and selectivity at HDAC6, we replaced the phenyl substituent of 3.43 with a 4-pyridyl group, 3.46, which resulted in subnanomolar potency at HDAC6 as well as approximately 5,000 fold selectivity over HDAC1.

Once we had determined that the 2-position was optimal for substitution, we decided to investigate the breadth of substituents that would not only maintain the excellent potency/selectivity profile that we had elucidated but enhance the drug-like properties of these molecules. Because amides are known to be very polar and could function to improve aqueous solubility, we decided to incorporate an acetamide group tethered with an ethyl linker to mimic the ethyl substituent of 3.40. This resulted in 3.47 with an IC\textsubscript{50} of 0.5 nM at HDAC6 and about 3,500 fold selectivity over HDAC1. Since this compound exhibited a good activity profile, we wanted to investigate the optimal length of the linker between the acetamide substituent and the nitrogen at the 2-position. We found that a one carbon linker was optimal, 3.48, with an HDAC6 IC\textsubscript{50} of 0.8 nM and a selectivity of nearly 5,000 fold compared to HDAC1. This compound was also found to have a lower cLogP (cLogP\textsubscript{3.48} = 1.38) compared to our lead compound, tubastatin A (cLogP = 2.38), indicating the potential for improved aqueous solubility.

To investigate the position of the nitrogen atom in the tricyclic ring system, a small series of β-carbolines was also synthesized and the results of the HDAC inhibition assays
are displayed in Table VI. The β-carboline regioisomer of tubastatin A, 3.55, was very potent at HDAC6 with an IC$_{50}$ of approximately 1 nM and a selectivity of around 3,500 fold over HDAC1. The desmethyl derivative 3.54 was equipotent at HDAC6 with an IC$_{50}$ of about 2 nM and a selectivity of about 4,000 fold over HDAC1. To determine if increased HDAC6 selectivity could be obtained by incorporating a benzyl substituent at the 2-position as was the case with 3.43, compound 3.56 was synthesized and found to exhibit an improved HDAC6 IC$_{50}$ of less than 1 nM. While 3.56 was not quite as selective as 3.43, it still maintained approximately 5,000 fold selectivity for HDAC6 compared to HDAC1.

TABLE VI

IC$_{50}$ AND HDAC6 SELECTIVITY DATA FOR 2-SUBSTITUTED β-CARBOLINES

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>R</th>
<th>HDAC1 IC$_{50}$ (μM)</th>
<th>HDAC6 IC$_{50}$ (nM)</th>
<th>Selectivity HDAC1/HDAC6 (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.54</td>
<td>H</td>
<td>9.70 ± 0.03</td>
<td>2.49 ± 0.60</td>
<td>3895</td>
</tr>
<tr>
<td>3.55</td>
<td>Me</td>
<td>5.18 ± 0.12</td>
<td>1.40 ± 0.30</td>
<td>3700</td>
</tr>
<tr>
<td>3.56</td>
<td>Benzyl</td>
<td>4.32 ± 0.70</td>
<td>0.872 ± 0.195</td>
<td>4954</td>
</tr>
</tbody>
</table>
3.3.2 Regulatory T Cell Suppression

After having identified a series of potent and selective tubastatin A analogs with improved IC_{50}s against HDAC6 and selectivity versus HDAC1, we sought to evaluate the ability of these compounds to enhance the suppressive function of Tregs in vitro using Tregs and effector T cells (Teffs) isolated from C57BL/6 mice. CFSE-labeled Teffs were incubated in the presence and absence of Tregs, with or without the addition of selected HDAC6 inhibitors at multiple working concentrations. Flow cytometry was used to measure CFSE fluorescence and CFSE-dilution plots were generated with the percentage of cells determined to be undergoing mitotic division displayed in the top left of each plot (Figure 13). As the ratio of Tregs:Teffs increased, the number of cells undergoing mitosis decreased, and this effect was augmented by the addition of HDAC6i to the culture media.
Treg:Teff Ratio  1:1  1:2  1:4  1:8  1:16  0

Control

3.46 (0.25 µM)

3.47 (0.5 µM)

3.47 (1.0 µM)

3.47 (1.5 µM)
Figure 13. CFSE-dilution plots for selected tricyclic compounds.
Raw mitotic division data obtained from the CFSE-dilution plots was standardized with respect to a control for each compound at each tested concentration. The standardized cell division data was then converted to standardized suppression data and plotted against the ratio of Teffs:Tregs (Figure 14).
Figure 14. Standardized suppression curves for selected tricyclic compounds at their highest working concentration. Standardized suppression curves were generated by applying min-max normalization to the raw cell division data which was then converted to percent mitotic suppression (% suppression = 100 - % dividing cells) and plotted against the ratio of Teffs:Tregs.
The area under the standardized suppression curve (AUC) for each compound at each tested concentration, along with the AUC for the respective control, was calculated and the AUC ratio between the compound at a particular concentration and the respective control was determined (Figure 15). All of the compounds tested enhanced Treg suppression of Teff proliferation with tubastatin A and 3.46 exhibiting the most pronounced effect. Both tubastatin A and 3.46 more than doubled the immunosuppressive function of Tregs in vitro, an effect similar to that observed using pan-HDACi. (73)

Figure 15. AUC suppression ratio for each compound at highest working concentration. Relative suppression ratios were determined by comparing the area under the standardized suppression curve for each compound to its respective control using GraphPad Prism 5 (Relative suppression = \( \frac{\text{AUC}_{\text{compound}}}{\text{AUC}_{\text{control}}} \)).
To further validate these results, we undertook seven-day homeostatic proliferation assays to assess the in vivo effects of our HDAC6i (Figure 16). We have previously demonstrated the utility of HDAC6 targeting in these assays.\(^{(57)}\) The principle of the assay is such that conventional T cells adoptively transferred to an immunodeficient (RAG-/-) host undergo rapid proliferation within secondary lymphoid tissues. This homeostatic expansion is decreased by the actions of Foxp3+ Tregs, and HDAC6-/- Tregs are more effective than wild-type Tregs in suppressing the proliferation of the transferred cells.\(^{(57)}\) To ensure that the effects of our HDAC6i were not attributable to inhibition of HDAC6 in proliferating Teffs, we used wild-type CD4+CD25+ Tregs and CFSE-labeled HDAC6-/- CD4+CD25- Teffs. We found that mice treated with HDAC6i had significantly lower numbers of Teffs in their peripheral lymph nodes (\(p = 0.03\)) due to enhancement of the Treg suppressive function. Both 3.24 and 3.46 tended to be more effective than tubastatin A, though the differences were not statistically significant.
Figure 16. In vivo assessment of HDAC6i activity using a seven-day homeostatic proliferation assay with HDAC6−/− CFSE+/low conventional T cells in the presence of HDAC6+ Tregs (2:1 ratio) and daily injections of DMSO, tubastatin A or second-generation HDAC6i (1 mg/kg). Numbers of CD4+ Foxp3− CFSE+/low conventional T cells in peripheral lymph nodes of adoptively transferred RAG−/− mice (3/group) are shown (mean ± SEM, *p < 0.03 vs. DMSO).

Functionalization of the carboline cap group was well tolerated with respect to both inhibition of the recombinant HDAC6 protein and the ability to enhance Treg induced mitotic suppression of Teffs both in vitro and in vivo. Substitution at the 2-position of both the β- and γ-carbolines was found to be optimal with some compounds exhibiting subnanomolar potency at HDAC6 as well as greater than seven thousand fold selectivity versus HDAC1 in the HDAC inhibition assays. The wide range of tolerated functional groups allows for the selection of substituents to enhance the drug-like properties of these compounds. In addition, it is known that placing bulky substituents at the 2-position of the γ-carboline
eliminates undesirable off-target activity sometimes observed with other carbolines such as Dimebolin.\(^{(99)}\)

### 3.4 Conclusion

Pan-HDACi and selective HDAC6i were previously shown to promote the acetylation and function of Foxp3, a key transcription factor responsible for control of Treg-dependent T cell immune responses.\(^{(57, 94)}\) Furthermore, HDAC inhibition enhances the expression of CTLA-4 in Foxp3+ Tregs and directly correlates with their ability to suppress Teff proliferation.\(^{(73)}\) While it is known that broad spectrum HDACi can enhance the suppressive effect of Tregs in vitro, whether one or more particular isoforms are responsible for this action remains to be determined. Here, we have developed a series of second generation HDAC6 selective compounds and demonstrated their ability to enhance Treg suppression of Teff proliferation both in vitro and in vivo. Due to the prevalence of rheumatoid arthritis, which the Center for Disease Control estimates affects 1-2% of the population worldwide, and other autoimmune diseases, as well as the need for new therapies to improve the prognosis after organ transplantation, we believe that further investigation into the immunosuppressive effects of these HDAC6 selective agents is warranted. The development of isoform selective HDAC inhibitors continues to be a widely undertaken endeavor and we believe that their discovery will provide invaluable research tools for identifying the specific functions of each HDAC isoform.

### 3.5 Experimental Section

#### 3.5.1 Chemistry

\(^{1}\)H NMR and \(^{13}\)C NMR spectra were obtained using a Bruker spectrometer with TMS as an internal standard. The following standard abbreviations indicating multiplicity were
used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = double doublet, dt = double triplet and br = broad. HRMS experiments were carried out using a Shimadzu IT-TOF instrument with MeCN and H$_2$O spiked with 0.1% formic acid as the mobile phase. Reaction progress was monitored by TLC using precoated silica gel plates (Merck silica gel 60 F$_{254}$, 250 μm thickness). Automated column chromatography was performed using the CombiFlash Rf apparatus available from Teledyne ISCO and pre-packed 25 g cartridges loaded with Merck silica gel (40-60 mesh) along with the following conditions: Gradient: 100% DCM, 5 min; 0-10% MeOH/DCM, 20 min, 10% MeOH/DCM, 5 min; flow rate = 30 mL/min with wavelength monitoring at 254 and 280 nm. Preparatory HPLC was carried out using a Shimadzu preparative liquid chromatograph with the following specifications: Column: ACE 5 AQ (150 x 21.2 mm) with 5 μm particle size. Gradient: 8-100% MeOH/H$_2$O, 30 min; 100% MeOH, 5 min; 100-8% MeOH/H$_2$O, 4 min; 8% MeOH/H$_2$O, 1 min; flow rate = 17 mL/min with wavelength monitoring at 254 and 280 nm. Both solvents were spiked with 0.05% TFA. Analytical HPLC was carried out using an Agilent 1100 series instrument with the following specifications: Column: Luna 5μ C18(2) 100A (150 x 4.60 mm) with 5 μm particle size. Flow rate = 1.4 mL/min with wavelength monitoring at 254 nm. Gradient: 10-100% MeOH/H$_2$O, 18 min; 100% MeOH, 3 min; 100-10% MeOH/H$_2$O, 3 min; 10% MeOH/H$_2$O, 5 min. Both solvents were spiked with 0.05% TFA. The purity of all tested compounds was ≥ 95% as determined by analytical HPLC.

**General Procedure A.** The appropriate phenylhydrazine and 1-methylpiperidin-4-one (1 molar equivalent) were dissolved in 1,4-dioxane (35 mL). The reaction was placed in an ice bath and concentrated H$_2$SO$_4$ (5 mL) was added dropwise. The reaction was then removed from the ice bath and heated to 60 °C for 2 h. When the reaction was complete as evidenced by TLC, it was cooled to room temperature, then placed on ice and the pH was adjusted to approximately 13 by the addition of saturated NaHCO$_3$ (100 mL) and solid
NaOH. The organic products were extracted with EtOAc (3x30 mL), washed with brine (10 mL), dried with Na₂SO₄, filtered and concentrated in vacuo. The resulting product was purified via automated column chromatography.

**General Procedure B.** The appropriate γ-carboline was dissolved in anhydrous DMF (3 mL) and added to a suspension of potassium tert-butoxide (1 molar equivalent) in anhydrous DMF (2 mL) under argon at room temperature. The reaction mixture was heated to 80 °C for 15 min after which 4-bromomethyl-benzoic acid methyl ester (1 molar equivalent) dissolved in anhydrous DMF (2 mL) was added at 80 °C. The reaction was stirred at 80 °C for 2 h after which the reaction was cooled to room temperature and poured into cold water (15 mL). The organic products were extracted with EtOAc (3x20 mL), washed with water (3x15 mL), brine (10 mL), dried with Na₂SO₄, filtered and concentrated in vacuo. The resulting product was purified via automated column chromatography.

**General Procedure C.** The appropriate ester and hydroxylamine hydrochloride (6 molar equivalents) were dissolved in MeOH (5 mL) and cooled to 0 °C in an ice bath (DMF can be added to improve the solubility of the ester as this is important). A 25% sodium methoxide solution in MeOH (8 molar equivalents) was added upon which a precipitate formed. The reaction was allowed to warm to room temperature and then stirred for 16 h. Upon completion as evidenced by TLC, the reaction was quenched by the addition of a 10% TFA/DCM solution (~5 mL) and then filtered to remove residual NaCl. The filter cake was washed with additional MeOH (5 mL) and the combined filtrate and wash were concentrated in vacuo. The crude product was then dissolved in DMF and purified by preparatory HPLC.

**General Procedure D.** The γ-carboline was placed under argon in a two-neck round-bottom flask fitted with a condenser. Anhydrous MeCN (5 mL) was added followed by the addition of Et₃N (2 molar equivalents) at room temperature. The reaction was heated to
60 °C and then the appropriate alkyl/benzyl halide (1 molar equivalent) was added in anhydrous MeCN (2 mL). The reaction was stirred for 2 h at 60 °C and then poured into a 1:1 mixture of EtOAc/H₂O (20 mL). The organic layer was isolated and the aqueous layer was further extracted with EtOAc (2x15 mL). The combined organic layers were washed with brine (10 mL), dried with Na₂SO₄, filtered and concentrated in vacuo. The desired 2-substituted γ-carboline was isolated by automated column chromatography and then dissolved in anhydrous DMF (3 mL) and added to a suspension of potassium tert-butoxide (1 molar equivalent) in anhydrous DMF (2 mL) under argon at room temperature. The reaction mixture was heated to 80 °C for 15 min after which 4-bromomethyl-benzoic acid methyl ester (1 molar equivalent) dissolved in anhydrous DMF (2 mL) was added at 80 °C. The reaction was stirred at 80 °C for 2 h after which the reaction was cooled to room temperature and poured into cold water (15 mL). The organic products were extracted with EtOAc (3x30 mL) and the combined extracts were washed with water (3x15 mL), brine (10 mL), dried with Na₂SO₄, filtered and concentrated in vacuo. The resulting product was purified via automated column chromatography.

**General Procedure E.** The β-carboline was placed under argon in a two-neck round-bottom flask fitted with a condenser. Anhydrous MeCN (5 mL) was added followed by the addition of Et₃N (2 molar equivalents) at room temperature. The reaction was heated to 60 °C and then the appropriate alkyl/benzyl halide (1 molar equivalent) was added in anhydrous MeCN (2 mL). The reaction was stirred for 2 h at 60 °C and then poured into a 1:1 mixture of EtOAc/H₂O (20 mL). The organic layer was isolated and the aqueous layer was further extracted with EtOAc (2x15 mL). The combined organic layers were washed with brine (10 mL), dried with Na₂SO₄, filtered and concentrated in vacuo. The resulting product was purified via automated column chromatography.
2,6-Dimethyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole (3.1). The title compound was synthesized from o-tolylhydrazine (2.00 g, 16.4 mmol) according to general procedure A (0.40 g, 12%). $^1$H NMR (400 MHz, MeOD): δ 7.18 (d, $J = 7.6$ Hz, 1H), 6.88 (m, 2H), 3.65 (s, 2H), 2.83 (t, $J = 5.5$ Hz, 2H), 2.52 (s, 3H), 2.45 (s, 3H). $^{13}$C NMR (100 MHz, MeOD): δ 135.9, 131.2, 125.3, 121.1, 119.8, 118.6, 114.4, 106.8, 63.8, 53.0, 34.0, 22.9, 15.7. ESI-HRMS: calc. for C$_{13}$H$_{16}$N$_2$: [M+H]$^+$ = m/z 201.1386, found: [M+H]$^+$ = m/z 201.1389.

2,7-Dimethyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole (3.2). The title compound was synthesized from m-tolylhydrazine hydrochloride (2.00 g, 12.6 mmol) according to general procedure A (1.68 g, 67%). $^1$H NMR (400 MHz, CDCl$_3$): δ 8.70 (s, 1H), 7.32 (d, $J = 7.9$ Hz, 1H), 6.91 (d, $J = 7.9$ Hz, 1H), 6.83 (s, 1H), 3.71 (s, 2H), 2.77 (m, 2H), 2.64 (s, 3H), 2.61 (s, 3H). $^{13}$C NMR (100 MHz, MeOD): δ 136.8, 131.3, 130.5, 123.9, 120.6, 116.9, 111.0, 107.7, 52.6, 51.9, 45.8, 23.4, 21.7. ESI-HRMS: calc. for C$_{13}$H$_{16}$N$_2$: [M+H]$^+$ = m/z 201.1386, found: [M+H]$^+$ = m/z 201.1381.

2,8-Dimethyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole (3.3). The title compound was synthesized from p-tolylhydrazine hydrochloride (2.00 g, 12.6 mmol) according to general procedure A (2.18 g, 86%). $^1$H NMR (400 MHz, CDCl$_3$): δ 7.28 (s, 1H), 7.17 (m, 1H), 6.95 (s, 1H), 3.66 (br, 2H), 2.84 (m, 4H), 2.57 (s, 3H), 2.44 (s, 3H). $^{13}$C NMR (100 MHz, MeOD): δ 134.9, 131.5, 127.3, 126.0, 121.8, 116.4, 110.0, 106.0, 52.1, 51.4, 44.3, 22.9, 20.2. ESI-HRMS: calc. for C$_{13}$H$_{16}$N$_2$: [M+H]$^+$ = m/z 201.1386, found: [M+H]$^+$ = m/z 201.1386.

2,7,9-Trimethyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole (3.4). The title compound was synthesized from (3,5-dimethylphenyl)hydrazine hydrochloride (2.00 g, 11.5 mmol) according to general procedure A (1.08 g, 44%). $^1$H NMR (400 MHz, MeOD): δ 6.87 (s, 1H), 6.54 (s, 1H), 3.88 (s, 2H), 2.85 (t, $J = 5.4$ Hz, 2H), 2.78 (t, $J = 5.3$ Hz, 2H), 2.51 (s, 3H), 2.50
(s, 3H), 2.34 (s, 3H). $^{13}$C NMR (100 MHz, MeOD): δ 135.4, 128.5, 128.4, 126.6, 121.4, 119.6, 106.6, 105.2, 51.9, 50.0, 42.8, 21.3, 18.7, 17.1. ESI-HRMS: calc. for C$_{14}$H$_{18}$N$_{2}$: [M+H]$^{+}$ = m/z 215.1543, found: [M+H]$^{+}$ = m/z 215.1534.

8-tert-Butyl-2-methyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole (3.5). The title compound was synthesized from (4-tert-butylphenyl)hydrazine hydrochloride (2.00 g, 10.0 mmol) according to general procedure A (1.53 g, 63%). $^1$H NMR (400 MHz, CDCl$_3$): δ 7.40 (s, 1H), 7.20 (m, 2H), 3.75 (s, 2H), 2.83 (m, 4H), 2.60 (s, 3H), 1.39 (s, 9H). $^{13}$C NMR (100 MHz, MeOD): δ 139.6, 133.2, 129.9, 124.0, 117.0, 110.9, 108.3, 105.0, 50.6, 49.9, 42.8, 32.4, 29.6, 21.4. ESI-HRMS: calc. for C$_{16}$H$_{22}$N$_{2}$: [M+H]$^{+}$ = m/z 243.1856, found: [M+H]$^{+}$ = m/z 243.1855.

8-Methoxy-2-methyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole (3.6). The title compound was synthesized from (4-methoxynphenyl)hydrazine hydrochloride (2.00 g, 11.5 mmol) according to general procedure A (1.71 g, 69%). $^1$H NMR (400 MHz, CDCl$_3$): δ 7.05 (d, J = 8.7 Hz, 1H), 6.77 (s, 1H), 6.66 (dd, J = 2.5 Hz, 8.7 Hz, 1H), 3.75 (s, 3H), 3.55 (s, 2H), 2.72 (m, 4H), 2.46 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 153.8, 132.8, 131.3, 126.4, 111.2, 110.6, 108.4, 100.0, 55.9, 52.5, 51.8, 45.8, 23.9. ESI-HRMS: calc. for C$_{13}$H$_{16}$N$_{2}$O: [M+H]$^{+}$ = m/z 217.1335, found: [M+H]$^{+}$ = m/z 217.1340.

8-Chloro-2-methyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole (3.7). The title compound was synthesized from (4-chlorophenyl)hydrazine hydrochloride (2.00 g, 11.2 mmol) according to general procedure A (1.08 g, 44%). $^1$H NMR (400 MHz, MeOD): δ 7.33 (s, 1H), 7.23 (d, J = 8.6 Hz, 1H), 7.00 (d, J = 8.6 Hz, 1H), 3.67 (s, 2H), 2.92 (m, 4H), 2.61 (s, 3H). $^{13}$C NMR (100 MHz, MeOD): δ 133.4, 131.9, 125.2, 122.5, 118.8, 114.6, 109.9, 104.9, 50.4, 49.5, 42.7, 21.3. ESI-HRMS: calc. for C$_{12}$H$_{13}$ClN$_{2}$: [M+H]$^{+}$ = m/z 221.0840, found: [M+H]$^{+}$ = m/z 221.0838.
8-Bromo-2-methyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole (3.8). The title compound was synthesized from (4-bromophenyl)hydrazine (3.00 g, 16.0 mmol) according to general procedure A (2.82 g, 94%). \(^1\)H NMR (400 MHz, MeOD): \(\delta 7.47 (d, J = 1.6 \text{ Hz}, 1\text{H}), 7.18 (d, J = 8.5 \text{ Hz}, 1\text{H}), 7.12 (dd, J = 1.8 \text{ Hz}, 6.7 \text{ Hz}, 1\text{H}), 3.62 (s, 2\text{H}), 2.90 (t, J = 5.3 \text{ Hz}, 2\text{H}), 2.84 (t, J = 5.2 \text{ Hz}, 2\text{H}), 2.52 (s, 3\text{H}). \(^{13}\)C NMR (100 MHz, MeOD): \(\delta 134.8, 132.9, 127.1, 122.6, 118.9, 111.5, 111.0, 106.0, 51.6, 50.6, 43.9, 22.4\). ESI-HRMS: calced for C\(\text{_{12}}\)H\(\text{_{13}}\)BrN\(\text{_{2}}\): [M+H]\(^{+}\) = m/z 265.0335, found: [M+H]\(^{+}\) = m/z 265.0348.

Methyl 4-((2,6-dimethyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate (3.9). The title compound was synthesized from 2,6-dimethyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole 3.1 (0.40 g, 2.0 mmol) according to general procedure B (0.07 g, 9%). \(^1\)H NMR (400 MHz, CDCl\(\text{_{3}}\)): \(\delta 7.93 (d, J = 8.3 \text{ Hz}, 2\text{H}), 7.03 (m, 2\text{H}), 6.93 (d, J = 8.3 \text{ Hz}, 2\text{H}), 6.86 (d, J = 7.2 \text{ Hz}, 1\text{H}), 5.54 (s, 2\text{H}), 3.89 (s, 3\text{H}), 3.84 (s, 2\text{H}), 2.95 (t, J = 5.9 \text{ Hz}, 2\text{H}), 2.78 (t, J = 5.7 \text{ Hz}, 2\text{H}), 2.64 (s, 3\text{H}), 2.48 (s, 3\text{H}). \(^{13}\)C NMR (100 MHz, CDCl\(\text{_{3}}\)): \(\delta 166.7, 144.7, 135.4, 133.3, 130.2, 129.6, 129.0, 126.5, 125.1, 124.6, 120.5, 119.5, 115.8, 63.7, 52.1, 51.3, 47.9, 45.2, 22.2, 19.5\). ESI-HRMS: calc. for C\(\text{_{22}}\)H\(\text{_{24}}\)N\(\text{_{2}}\)O\(\text{_{2}}\): [M+H]\(^{+}\) = m/z 349.1911, found: [M+H]\(^{+}\) = m/z 349.1898.

Methyl-4-((2,7-dimethyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate (3.10). The title compound was synthesized from 2,7-dimethyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole 3.2 (0.35 g, 1.7 mmol) according to general procedure B (0.36 g, 59%). \(^1\)H NMR (400 MHz, CDCl\(\text{_{3}}\)): \(\delta 7.95 (d, J = 8.3 \text{ Hz}, 2\text{H}), 7.36 (d, J = 7.9 \text{ Hz}, 1\text{H}), 7.07 (d, J = 8.2 \text{ Hz}, 2\text{H}), 6.96 (m, 2\text{H}), 5.27 (s, 2\text{H}), 3.90 (s, 3\text{H}), 3.73 (s, 2\text{H}), 2.83 (t, J = 5.6 \text{ Hz}, 2\text{H}), 2.76 (br, 2\text{H}), 2.58 (s, 3\text{H}), 2.43 (s, 3\text{H}). \(^{13}\)C NMR (100 MHz, CDCl\(\text{_{3}}\)): \(\delta 166.7, 143.2, 134.2, 132.3, 131.0, 129.3, 126.1, 123.7, 121.0, 117.5, 109.2, 108.2, 52.3, 52.1, 51.7, 46.1,
Methyl 4-((2,8-dimethyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate (3.11). The title compound was synthesized from 2,8-dimethyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole 3.3 (0.30 g, 1.5 mmol) according to general procedure B (0.35 g, 56%). 

\[ \text{H NMR (400 MHz, CDCl}_3\text{): } \delta 7.94 (d, J = 8.1 \text{ Hz}, 2H), 7.26 (m, 2H), 7.12 (d, J = 8.1 \text{ Hz}, 1H), 7.08 (d, J = 8.2 \text{ Hz}, 2H), 5.26 (s, 2H), 4.09 (s, 2H), 3.91 (s, 3H), 3.57 (t, J = 5.9 \text{ Hz}, 2H), 3.18 (t, J = 6.3 \text{ Hz}, 2H), 3.01 (s, 3H), 2.42 (s, 3H). \]

\[ \text{C NMR (100 MHz, CDCl}_3\text{): } \delta 166.5, 141.9, 135.6, 130.3, 130.0, 129.8, 129.7, 125.9, 125.0, 124.4, 117.6, 109.4, 101.5, 52.2, 50.7, 50.6, 46.5, 41.6, 21.4, 19.2. \]

ESI-HRMS: calc. for C\textsubscript{22}H\textsubscript{24}N\textsubscript{2}O\textsubscript{2}: [M+H]\textsuperscript{+} = m/z 349.1911, found: [M+H]\textsuperscript{+} = m/z 349.1904.

Methyl 4-((2,7,9-trimethyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate (3.12). The title compound was synthesized from 2,7,9-trimethyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole 3.4 (0.30 g, 1.4 mmol) according to general procedure B (0.40 g, 78%). 

\[ \text{H NMR (400 MHz, CDCl}_3\text{): } \delta 7.93 (d, J = 8.3 \text{ Hz}, 2H), 7.16 (d, J = 8.3 \text{ Hz}, 2H), 6.92 (s, 1H), 6.75 (s, 1H), 5.24 (s, 2H), 4.26 (s, 2H), 3.91 (s, 3H), 3.57 (t, J = 5.8 \text{ Hz}, 2H), 3.19 (t, J = 6.4 \text{ Hz}, 2H), 3.02 (s, 3H), 2.43 (s, 3H), 2.38 (s, 3H). \]

\[ \text{C NMR (100 MHz, DMSO-d}_6\text{): } \delta 166.6, 148.9, 135.7, 131.4, 129.7, 129.6, 128.8, 128.5, 127.5, 124.4, 123.0, 107.4, 107.2, 65.9, 52.4, 50.0, 41.2, 30.5, 21.6, 20.0, 19.9. \]

ESI-HRMS: calc. for C\textsubscript{23}H\textsubscript{26}N\textsubscript{2}O\textsubscript{2}: [M+H]\textsuperscript{+} = m/z 363.2067, found: [M+H]\textsuperscript{+} = m/z 363.2065.

Methyl 4-((8-tert-butyl-2-methyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate (3.13). The title compound was synthesized from 8-tert-butyl-2-methyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole 3.5 (0.50 g, 2.1 mmol) according to general procedure B (0.64 g, 79%). 

\[ \text{H NMR (400 MHz, CDCl}_3\text{): } \delta 7.93 (d, J = 8.2 \text{ Hz}, 2H), 7.45 (s,
1H), 7.20 (m, 1H), 7.09 (m, 3H), 5.38 (s, 2H), 3.92 (s, 3H), 3.78 (s, 2H), 2.85 (m, 2H), 2.78 (m, 2H), 2.61 (br, 3H), 1.39 (s, 9H). $^{13}$C NMR (100 MHz, MeOD): δ 166.7, 144.0, 141.9, 135.1, 132.7, 129.5, 128.9, 126.1, 125.4, 119.1, 113.1, 108.5, 107.5, 52.0, 51.3, 51.2, 45.5, 44.2, 34.0, 31.1, 22.0. ESI-HRMS: calc. for C$_{25}$H$_{30}$N$_2$O$_2$: [M+H]$^+$ = m/z 391.2380, found: [M+H]$^+$ = m/z 391.2397.

Methyl 4-((8-methoxy-2-methyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate (3.14). The title compound was synthesized from 8-methoxy-2-methyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole 3.6 (0.50 g, 2.3 mmol) according to general procedure B (0.40 g, 48%). $^1$H NMR (400 MHz, CDCl$_3$): δ 7.93 (d, $J$ = 8.0 Hz, 2H), 7.04 (m, 3H), 6.93 (d, $J$ = 8.7 Hz, 1H), 6.77 (dd, $J$ = 2.4 Hz, 8.6 Hz, 1H), 5.27 (s, 2H), 3.89 (s, 3H), 3.86 (s, 3H), 3.71 (s, 2H), 2.83 (m, 2H), 2.77 (m, 2H), 2.58 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 166.7, 154.0, 143.2, 134.0, 131.9, 130.1, 129.3, 126.2, 126.1, 110.7, 109.7, 108.5, 100.3, 55.9, 52.5, 52.1, 51.9, 46.3, 45.8, 23.0. ESI-HRMS: calc. for C$_{22}$H$_{24}$N$_2$O$_3$: [M+H]$^+$ = m/z 365.1860, found: [M+H]$^+$ = m/z 365.1859.

Methyl 4-((8-chloro-2-methyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate (3.15). The title compound was synthesized from 8-chloro-2-methyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole 3.7 (0.15 g, 0.7 mmol) according to general procedure B (0.18 g, 72%). $^1$H NMR (400 MHz, CDCl$_3$): δ 7.87 (d, $J$ = 8.1 Hz, 2H), 7.35 (s, 1H), 7.05 (m, 2H), 7.02 (d, $J$ = 8.0, 2H), 5.27 (s, 2H), 3.89 (s, 3H), 3.67 (s, 2H), 2.82 (br, 2H), 2.62 (br, 2H), 2.57 (s, 3H). $^{13}$C NMR (100 MHz, MeOD): δ 166.8, 144.0, 142.0, 135.2, 132.7, 129.5, 128.9, 126.1, 125.3, 119.1, 113.0, 108.4, 107.4, 51.1, 45.5, 44.1, 34.0, 30.9, 22.0. ESI-HRMS: calc. for C$_{21}$H$_{21}$ClN$_2$O$_2$: [M+H]$^+$ = m/z 369.1364, found: [M+H]$^+$ = m/z 369.1373.
Methyl 4-((8-bromo-2-methyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-
yl)methyl)benzoate (3.16). The title compound was synthesized from 8-bromo-2-methyl-
2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole 3.8 (0.50 g, 1.9 mmol) according to general
procedure B (0.37 g, 47%). $^1$H NMR (400 MHz, MeOD): $\delta$ 7.87 (d, $J = 8.4$ Hz, 2H), 7.55 (s, 1H), 7.14 (br, 2H), 7.04 (d, $J = 8.4$ Hz, 2H), 5.33 (s, 2H), 3.84 (s, 3H), 3.74 (s, 2H), 2.93 (t, $J = 5.9$ Hz, 2H), 2.82 (t, $J = 5.5$ Hz, 2H), 2.58 (s, 3H).

13C NMR (100 MHz, MeOD): $\delta$ 166.3, 142.9, 135.2, 134.0, 129.2, 128.7, 126.7, 125.7, 123.3, 119.5, 111.9, 110.3, 106.3, 51.3, 50.8, 50.4, 45.2, 43.5, 21.4. ESI-HRMS: calc. for C$_{21}$H$_{21}$BrN$_2$O$_2$: [M+H]$^+$ = m/z 413.0859, found: [M+H]$^+$ = m/z 413.0859.

4-((2,6-Dimethyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)-N-
hydroxybenzamide-TFA (3.17). The title compound was synthesized from methyl 4-((2,6-
dimethyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.9 (0.07 g, 0.2 mmol)
according to general procedure C and the TFA salt was isolated as a white solid (12 mg, 18%). $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 11.16 (s, 1H), 10.22 (br, 1H), 7.67 (d, $J = 8.1$ Hz, 2H), 7.33 (d, $J = 7.8$ Hz, 1H), 6.93 (m, 4H), 5.62 (m, 2H), 4.67 (br, 1H), 4.35 (br, 1H), 3.78 (br, 1H), 3.60 (br, 1H), 3.01 (br, 5H), 2.44 (s, 3H). 13C NMR (100 MHz, DMSO-$d_6$): $\delta$ 164.2, 143.2, 135.5, 132.3, 132.2, 127.9, 125.8, 125.5, 125.4, 121.2, 120.4, 116.3, 103.1, 51.0, 50.4, 47.8, 42.2, 20.0, 19.4. ESI-HRMS: calc. for C$_{21}$H$_{23}$N$_3$O$_2$: [M+H]$^+$ = m/z 350.1863, found: [M+H]$^+$ = m/z 350.1863.

4-((2,7-Dimethyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)-N-
hydroxybenzamide-TFA (3.18). The title compound was synthesized from methyl 4-((2,7-
dimethyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.10 (0.10 g, 0.3 mmol)
according to general procedure C and the TFA salt was isolated as a white solid (27 mg, 27%). $^1$H NMR (400 MHz, MeOD): $\delta$ 7.68 (d, $J = 8.3$ Hz, 2H), 7.40 (d, $J = 8.1$ Hz, 1H), 7.19
(s, 1H), 7.11 (d, J = 8.1 Hz, 2H), 6.99 (d, J = 8.2 Hz, 1H), 5.44 (m, 2H), 4.74 (d, J = 15.0 Hz, 1H), 4.39 (d, J = 12.4 Hz, 1H), 3.84 (br, 1H), 3.58 (br, 1H), 3.14 (s, 2H), 3.12 (s, 3H), 2.42 (s, 3H). 13C NMR (100 MHz, MeOD): δ 166.2, 141.7, 137.7, 132.4, 131.4, 129.5, 127.2, 126.1, 122.5, 121.6, 117.1, 109.4, 102.0, 51.4, 51.8, 45.5, 41.6, 20.5, 19.7. ESI- HRMS: calc. for C21H23N3O2: [M+H]+ = m/z 350.1863, found: [M+H]+ = m/z 350.1868.

4-((2,8-Dimethyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)-N-hydroxybenzamide·TFA (3.19). The title compound was synthesized from methyl 4-((2,8-dimethyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.11 (0.30 g, 0.7 mmol) according to general procedure C and the TFA salt was isolated as a white solid (39 mg, 15%). 1H NMR (400 MHz, MeOD): δ 7.70 (d, J = 8.2 Hz, 2H), 7.31 (s, 1H), 7.25 (d, J = 8.6 Hz, 1H), 7.03 (d, J = 8.1 Hz, 1H), 7.01 (d, J = 7.8 Hz, 2H), 5.44 (s, 2H), 4.55 (br, 2H), 3.71 (br, 2H), 3.12 (m, 5H), 2.43 (s, 3H). 13C NMR (100 MHz, DMSO-d6): δ 164.2, 141.6, 135.4, 132.3, 131.6, 128.8, 127.6, 126.9, 125.0, 123.8, 117.9, 110.3, 102.2, 51.0, 50.5, 46.0, 42.3, 21.5, 20.0. ESI-HRMS: calc. for C21H23N3O2: [M+H]+ = m/z 350.1863, found: [M+H]+ = m/z 350.1862.

N-Hydroxy-4-((2,7,9-trimethyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzamide·TFA (3.20). The title compound was synthesized from methyl 4-((2,7,9-trimethyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.12 (0.40 g, 1.1 mmol) according to general procedure C and the TFA salt was isolated as a white solid (28 mg, 23%). 1H NMR (400 MHz, MeOD): δ 7.64 (d, J = 8.4 Hz, 2H), 7.18 (d, J = 8.4 Hz, 2H), 7.05 (s, 1H), 6.68 (s, 1H), 5.37 (s, 2H), 4.29 (s, 2H), 3.67 (t, J = 6.0 Hz, 2H), 3.23 (t, J = 6.3 Hz, 2H), 3.12 (s, 3H), 2.41 (s, 3H), 2.34 (s, 3H). 13C NMR (100 MHz,): δ 164.7, 140.2, 136.2, 130.7, 129.7, 127.4, 127.3, 125.6, 124.5, 121.4, 120.5, 105.6, 100.9, 50.9, 49.4, 43.9,
40.1, 18.8, 18.1, 17.0. ESI-HRMS: calc. for C_{22}H_{25}N_{3}O_{2}: [M+H]^+ = m/z 364.2020, found: [M+H]^+ = m/z 364.2016.

4-((8-tert-Butyl-2-methyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)-N-hydroxybenzamide·TFA (3.21). The title compound was synthesized from methyl 4-((8-tert-butyl-2-methyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.13 (0.20 g, 0.5 mmol) according to general procedure C and the TFA salt was isolated as a white solid (55 mg, 27%). ¹H NMR (400 MHz, MeOD): δ 7.66 (d, J = 8.2 Hz, 2H), 7.52 (s, 1H), 7.31 (m, 2H), 7.14 (d, J = 8.0 Hz, 2H), 5.46 (br, 2H), 4.59 (br, 2H), 3.72 (br, 2H), 3.13 (br, 5H), 1.43 (s, 9H). ¹³C NMR (100 MHz, MeOD): δ 142.6, 141.6, 135.2, 132.3, 131.4, 127.7, 126.9, 124.6, 120.4, 114.0, 110.1, 102.7, 51.0, 50.7, 46.1, 42.2, 34.8, 32.2, 20.0. ESI-HRMS: calc. for C_{24}H_{29}N_{3}O_{2}: [M+H]^+ = m/z 392.2333, found: [M+H]^+ = m/z 392.2337.

N-Hydroxy-4-((8-methoxy-2-methyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzamide·TFA (3.22). The title compound was synthesized from methyl 4-((8-methoxy-2-methyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.14 (0.09 g, 0.2 mmol) according to general procedure C and the TFA salt was isolated as a white solid (11 mg, 13%). ¹H NMR (400 MHz, MeOD): δ 7.58 (d, J = 8.0 Hz, 2H), 7.15 (d, J = 7.8 Hz, 1H), 7.01 (d, J = 8.2 Hz, 2H), 6.91 (s, 1H), 6.74 (d, J = 8.6 Hz, 1H), 5.36 (m, 2H), 4.44 (br, 2H), 3.69 (s, 3H), 3.28 (br, 2H), 3.01 (br, 5H). ¹³C NMR (100 MHz, MeOD): δ 168.4, 155.1, 146.3, 130.4, 129.5, 129.1, 128.3, 128.0, 127.7, 111.4, 111.3, 109.2, 100.7, 63.3, 54.7, 50.6, 39.6, 29.1, 19.2. ESI-HRMS: calc. for C_{21}H_{23}N_{3}O_{3}: [M+H]^+ = m/z 366.1812, found: [M+H]^+ = m/z 366.1803.

4-((8-Chloro-2-methyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)-N-hydroxybenzamide·TFA (3.23). The title compound was synthesized from methyl 4-((8-chloro-2-methyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.15 (0.10 g,
0.3 mmol) according to general procedure C and the TFA salt was isolated as a white solid (16 mg, 15%). $^1$H NMR (400 MHz, MeOD): $\delta$ 7.68 (d, $J = 8.0$ Hz, 2H), 7.51 (s, 1H), 7.38 (d, $J = 8.6$ Hz, 1H), 7.12 (d, $J = 7.8$ Hz, 2H), 5.48 (s, 2H), 4.55 (br, 2H), 3.71 (br, 2H), 3.17 (br, 2H), 3.12 (s, 3H). $^{13}$C NMR (100 MHz, MeOD): $\delta$ 166.1, 141.1, 135.7, 132.2, 131.5, 127.3, 126.2, 125.7, 125.6, 122.3, 117.0, 110.9, 102.0, 51.2, 50.7, 45.8, 41.6, 19.7. ESI-HRMS: calc. for C$_{20}$H$_{20}$ClN$_3$O$_2$: [M+H]$^+$ = m/z 370.1317, found: [M+H]$^+$ = m/z 370.1323.

4-((8-Bromo-2-methyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)-N-hydroxybenzamide-TFA (3.24). The title compound was synthesized from methyl 4-((8-bromo-2-methyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.16 (0.25 g, 0.6 mmol) according to general procedure C and the TFA salt was isolated as an off-white solid (75 mg, 30%). $^1$H NMR (400 MHz, DMSO-$_d_6$): $\delta$ 11.18 (s, 1H), 10.23 (s, 1H), 9.03 (s, 1H), 7.75 (s, 1H), 7.68 (d, $J = 7.6$ Hz, 2H), 7.47 (d, $J = 8.8$ Hz, 1H), 7.28 (d, $J = 8.6$ Hz, 1H), 7.10 (d, $J = 7.7$ Hz, 2H), 5.48 (s, 2H), 4.63 (br, 1H), 4.32 (br, 1H), 3.73 (br, 1H), 3.54 (br, 1H), 3.09 (s, 2H), 2.99 (s, 3H). $^{13}$C NMR (100 MHz, DMSO-$_d_6$): $\delta$ 163.8, 140.7, 135.4, 133.1, 132.0, 127.3, 126.5, 126.2, 124.4, 120.4, 112.4, 112.3, 102.2, 50.4, 49.9, 45.8, 41.9, 19.7. ESI-HRMS: calc. for C$_{20}$H$_{20}$BrN$_3$O$_2$: [M+H]$^+$ = m/z 414.0812, found: [M+H]$^+$ = m/z 414.0817.

2,3,4,5-Tetrahydro-1H-pyrido[4,3-b]indole (3.25). 1-Benzyl-4-piperidone (5.0 g, 26.4 mmol) and phenylhydrazine (2.86 g, 26.4 mmol) were dissolved in 1,4-dioxane (35 mL). The reaction was placed in an ice bath and to it was added conc. H$_2$SO$_4$ (5 mL). The reaction was then removed from the ice bath and heated to 60 °C for 2 h. When the reaction was complete as evidenced by TLC, it was cooled to room temperature and the pH was adjusted to approximately 13 by the addition of saturated NaHCO$_3$ (100 mL) and then by solid NaOH. The organic products were extracted with EtOAc (3x30 mL), washed with brine (10 mL),
dried with Na$_2$SO$_4$, filtered and concentrated in vacuo to yield 2-benzyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole (6.13 g, 93%) as a beige solid. Further purification was not required and the compound was used directly in the next step. $^1$H NMR (400 MHz, CDCl$_3$): δ 8.00 (s, 1H), 7.48 (d, $J = 7.0$ Hz, 2H), 7.38 (m, 4H), 7.23 (d, $J = 7.4$ Hz, 1H), 7.13 (m, 2H), 3.87 (s, 2H), 3.79 (s, 2H), 2.91 (t, $J = 5.8$ Hz, 2H), 2.76 (t, $J = 5.5$ Hz, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 138.6, 136.1, 132.2, 129.2, 128.4, 127.2, 126.2, 121.1, 119.2, 117.5, 110.7, 108.7, 62.4, 50.2, 49.8, 23.7. ESI-HRMS: calc. for C$_{18}$H$_{18}$N$_2$: [M+H]$^+$ = m/z 263.1543, found: [M+H]$^+$ = m/z 263.1533.

2-Benzyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole (1.0 g, 3.8 mmol) and 10% Pd/C (150 mg) were suspended in a 70% EtOH/H$_2$O solution (20 mL) and placed under hydrogen at atmospheric pressure. The reaction was heated to 70 °C and stirred for 24 h after which the reaction was filtered through filter paper before cooling to remove Pd/C. The filter cake was washed with 70% EtOH/H$_2$O (3×50 mL) and the combined washes and filtrate were concentrated in vacuo. The crude product was recrystallized from a 70% EtOH/H$_2$O solution and the precipitate was isolated by filtration, washed with cold MeOH and dried in vacuo. The title compound was isolated (0.61 g, 93%) as a light brown solid. $^1$H NMR (400 MHz, DMSO-d$_6$): δ 10.71 (s, 1H), 7.28 (q, $J = 7.7$ Hz, 2H), 6.98 (m, 1H), 6.89 (m, 1H), 3.85 (s, 2H), 3.01 (t, $J = 5.6$ Hz, 2H), 2.66 (t, $J = 5.5$ Hz, 2H). $^{13}$C NMR (100 MHz, DMSO-d$_6$): δ 135.4, 133.4, 125.7, 120.1, 118.2, 117.1, 110.7, 108.4, 43.1, 41.8, 24.2. ESI-HRMS: calc. for C$_{11}$H$_{12}$N$_2$: [M+H]$^+$ = m/z 173.1073, found: [M+H]$^+$ = m/z 173.1065.

**tert-Butyl 5-(4-(methoxycarbonyl)benzyl)-3,4-dihydro-1H-pyrido[4,3-b]indole-2(5H)-carboxylate (3.26).** Di-tert-butyl dicarbonate (0.70 g, 3.2 mmol) was placed in a round-bottom flask fitted with a condenser under argon and to it was added 2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole 3.25 (0.50 g, 2.9 mmol) suspended in THF (20 mL) upon which the
reaction turned from cloudy white to yellow. The reaction was heated at reflux with stirring for 8 h after which the reaction was allowed to cool to room temperature and concentrated in vacuo. The reaction mixture was then poured into a 1:1 mixture of EtOAc/H₂O (30 mL). The organic layer was isolated and the aqueous layer was further extracted with EtOAc (2x10 mL). The organic layers were combined, washed with water (10 mL), brine (10 mL), dried with Na₂SO₄, filtered and concentrated in vacuo. Purification by column chromatography (SiO₂, 0-100% EtOAc/hexane) afforded tert-butyl 3,4-dihydro-1H-pyrido[4,3-b]indole-2(5H)-carboxylate (0.66 g, 83%) as a dark yellow oil. ¹H NMR (400 MHz, MeOD): δ 7.36 (d, J = 7.7 Hz, 1H), 7.60 (d, J = 8.0 Hz, 1H), 7.06 (m, 1H), 6.99 (m, 1H), 4.60 (s, 2H), 3.79 (t, J = 5.6 Hz, 2H), 2.81 (t, J = 5.7 Hz, 2H), 1.51 (s, 9H). ¹³C NMR (100 MHz, MeOD): δ 155.6, 136.4, 131.9, 125.4, 120.6, 118.4, 116.6, 110.4, 105.7, 79.9, 60.1, 40.6, 27.3, 22.9. ESI-HRMS: calc. for C₁₆H₂₀N₂O₂: [M+H]⁺ = m/z 273.1598, found: [M+H]⁺ = m/z 273.1592.

tert-Butyl 3,4-dihydro-1H-pyrido[4,3-b]indole-2(5H)-carboxylate (0.44 g, 1.6 mmol) was dissolved in anhydrous DMF (3 mL) and added to a suspension of potassium tert-butoxide (0.18 g, 1.6 mmol) in anhydrous DMF (2 mL) under argon at room temperature. The reaction mixture was heated to 80 °C for 15 min after which 4-bromomethyl-benzoic acid methyl ester (0.37 g, 1.6 mmol) dissolved in anhydrous DMF (2 mL) was added at 80 °C. The reaction was stirred at 80 °C for 2 h after which the reaction was cooled to room temperature and poured into cold water (15 mL). The organic products were extracted with EtOAc (3x30 mL), washed with water (3x15 mL), brine (10 mL), dried with Na₂SO₄, filtered and concentrated in vacuo. Purification by column chromatography (SiO₂, 0-80% EtOAc/hexane) afforded the title compound (0.50 g, 74%) as a dark orange oil. ¹H NMR (400 MHz, CDCl₃): δ 7.96 (d, J = 8.3 Hz, 2H), 7.53 (d, J = 6.7 Hz, 1H), 7.16 (m, 3H), 7.07 (d, J = 8.1 Hz, 2H), 5.31 (s, 2H), 4.71 (s, 2H), 3.90 (s, 3H), 3.83 (s, 2H), 2.72 (s, 2H), 1.52 (s, 9H). ¹³C NMR (100 MHz, CDCl₃): δ 166.3, 154.7, 142.5, 136.3, 133.3, 129.8, 129.1, 125.7,
125.1, 121.2, 119.2, 117.5, 108.8, 107.3, 79.5, 51.7, 45.8, 41.1, 40.3, 28.1, 22.1. ESI-HRMS: calc. for C_{25}H_{28}N_{2}O_{4}: [M+H]^+ = m/z 421.2122, found: [M+H]^+ = m/z 421.2104.

**Methyl 4-((3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate (3.27).** tert-Butyl 5-(4-(methoxycarbonyl)benzyl)-3,4-dihydro-1H-pyrido[4,3-b]indole-2(5H)-carboxylate (0.24 g, 0.57 mmol) was dissolved in DCM (4 mL) and to it was added TFA (0.5 mL) upon which the reaction turned from light orange to dark orange. The reaction was allowed to stir at room temperature until completion as evidenced by TLC after which volatiles were removed in vacuo. The residue was resuspended in EtOAc (30 mL) and washed with saturated NaHCO$_3$ (2x15 mL), brine (10 mL), dried with Na$_2$SO$_4$, filtered and concentrated in vacuo. Further purification was not required and the title product was obtained (0.16 g, 85%) as a dark orange oil. $^1$H NMR (400 MHz, MeOD): δ 7.88 (d, J = 8.4 Hz, 2H), 7.41 (d, J = 7.6 Hz, 1H), 7.26 (d, J = 8.0 Hz, 1H), 7.07 (m, 4H), 5.34 (s, 2H), 4.15 (s, 2H), 3.84 (s, 3H), 3.28 (t, J = 5.8 Hz, 2H), 2.79 (t, J = 5.8 Hz, 2H). $^{13}$C NMR (100 MHz, MeOD): δ 166.7, 143.7, 136.7, 132.5, 129.5, 128.9, 126.1, 125.5, 121.3, 119.2, 117.3, 109.0, 106.2, 51.2, 45.3, 42.2, 41.1, 21.1. ESI-HRMS: calc. for C$_{20}$H$_{20}$N$_{2}$O$_{2}$: [M+H]^+ = m/z 321.1598, found: [M+H]^+ = m/z 321.1591.

**Methyl 4-((2-ethyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate (3.28).** 2,3,4,5-Tetrahydro-1H-pyrido[4,3-b]indole (0.50 g, 2.9 mmol) and acetaldehyde (0.24 mL, 4.4 mmol) were placed in a round-bottom flask and dissolved in anhydrous MeOH at room temperature. Sodium cyanoborohydride (0.42 g, 6.7 mmol) was added in small portions over a 15 min period and the reaction was then stirred at room temperature for 3 h. The reaction was then quenched by the addition of 2N HCl (20 mL) and stirred for 15 min. The pH was adjusted to 12 with 1N NaOH and the aqueous layer was extracted with EtOAc (3x15 mL). The combined organic extracts were washed with brine (10 mL), dried with
Na₂SO₄, filtered and concentrated in vacuo. The isolated product was then directly subjected to general procedure B to afford the title compound (0.30 g, 29%). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta\) 7.85 (d, \(J = 6.8\) Hz, 2H), 7.38 (m, 2H), 7.13 (d, \(J = 8.2\) Hz, 2H), 7.01 (m, 2H), 5.41 (s, 2H), 3.80 (s, 3H), 3.59 (s, 2H), 2.74 (br, 2H), 2.69 (br, 2H), 2.57 (q, \(J = 7.2\) Hz, 2H), 1.10 (t, \(J = 7.12\) Hz, 3H). \(^1^3\)C NMR (100 MHz, DMSO-\(d_6\)): \(\delta\) 166.0, 144.1, 136.4, 134.2, 129.6, 129.2, 128.9, 126.7, 120.7, 118.9, 117.5, 109.5, 108.1, 63.0, 52.1, 51.3, 48.6, 45.1, 22.6, 12.6. ESI-HRMS: calc. for C\(_{22}\)H\(_{24}\)N\(_2\)O\(_2\): [M+H]\(^+\) = m/z 349.1911, found: [M+H]\(^+\) = m/z 349.1918.

**Methyl 4-((2-isopropyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate (3.29).** The title compound was synthesized from 2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole \(3.25\) (0.50 g, 2.9 mmol) and 2-bromopropane (0.27 mL, 2.9 mmol) according to general procedure D (0.37 g, 35%). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(\delta\) 7.88 (d, \(J = 8.3\) Hz, 2H), 7.39 (m, 2H), 7.14 (d, \(J = 8.3\) Hz, 2H), 7.02 (m, 2H), 5.41 (s, 2H), 3.81 (s, 3H), 3.68 (s, 2H), 2.94 (m, 1H), 2.79 (t, \(J = 5.5\) Hz, 2H), 2.66 (br, 2H), 1.08 (d, \(J = 6.5\) Hz, 6H). \(^1^3\)C NMR (100 MHz, DMSO-\(d_6\)): \(\delta\) 166.4, 144.6, 136.7, 134.7, 130.0, 129.3, 127.1, 126.0, 121.0, 119.2, 117.8, 109.8, 108.9, 53.9, 52.5, 49.0, 45.8, 44.5, 23.6, 18.7. ESI-HRMS: calc. for C\(_{23}\)H\(_{26}\)N\(_2\)O\(_2\): [M+H]\(^+\) = \(m/z\) 363.2067, found: [M+H]\(^+\) = \(m/z\) 363.2072.

**Methyl 4-((2-allyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate (3.30).** The title compound was synthesized from 2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole \(3.25\) (0.50 g, 2.9 mmol) and 3-bromoprop-1-ene (0.25 mL, 2.9 mmol) according to general procedure D (0.24 g, 23%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 7.95 (d, \(J = 8.2\) Hz, 2H), 7.49 (dd, \(J = 3.3\) Hz, 3.0 Hz, 1H), 7.15 (m, 5H), 6.03 (m, 1H), 5.29 (m, 4H), 3.90 (s, 3H), 3.79 (s, 2H), 3.32 (d, \(J = 6.8\) Hz, 2H), 2.90 (t, \(J = 5.7\) Hz, 2H), 2.77 (t, \(J = 5.4\) Hz, 2H). \(^1^3\)C NMR (100 MHz, CDCl\(_3\)): \(\delta\) 166.3, 142.7, 136.4, 135.2, 133.2, 129.7, 129.0, 125.8, 125.6, 120.8, 118.9,
Methyl 4-((2-benzyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate (3.31).

The title compound was synthesized by subjecting the intermediate obtained during the synthesis of compound 3.25, 2-benzyl-2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole (0.50 g, 1.9 mmol), to general procedure B (0.38 g, 48%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.96 (d, $J = 6.7$ Hz, 2H), 7.30 (m, 8H), 7.13 (m, 3H), 5.30 (s, 2H), 3.91 (s, 3H), 3.84 (s, 2H), 3.82 (s, 2H), 2.91 (t, $J = 5.8$ Hz, 2H), 2.75 (t, $J = 5.5$ Hz, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 166.7, 143.1, 138.5, 136.7, 133.6, 130.1, 129.3, 129.1, 128.3, 127.1, 126.2, 126.0, 121.1, 119.3, 117.8, 109.0, 108.8, 62.3, 52.1, 50.0, 49.9, 46.3, 22.8. ESI-HRMS: calc. for C$_{27}$H$_{26}$N$_2$O$_2$: [M+H]$^+$ = m/z 411.2067, found: [M+H]$^+$ = m/z 411.2074.

Methyl 4-((2-(3-methoxybenzyl)-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate (3.32). The title compound was synthesized from 2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole 3.25 (0.50 g, 2.9 mmol) and 1-(bromomethyl)-3-methoxybenzene (0.41 mL, 2.9 mmol) according to general procedure D (0.23 g, 18%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.97 (d, $J = 8.2$ Hz, 2H), 7.44 (m, 3H), 7.18 (m, 5H), 7.06 (d, $J = 7.6$ Hz, 2H), 5.34 (br, 2H), 4.54 (m, 4H), 3.96 (s, 3H), 3.90 (s, 3H), 3.81 (s, 2H), 3.39 (br, 1H), 3.23 (br, 1H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 166.5, 161.1, 141.7, 137.2, 133.2, 132.0, 130.9, 130.6, 130.4, 129.8, 129.5, 125.9, 124.7, 123.0, 120.7, 117.2, 116.6, 109.6, 101.6, 58.3, 55.3, 52.2, 49.1, 48.1, 46.6, 19.1. ESI-HRMS: calc. for C$_{28}$H$_{28}$N$_2$O$_2$: [M+H]$^+$ = m/z 441.2173, found: [M+H]$^+$ = m/z 441.2163.

Methyl 4-((2-(4-methoxybenzyl)-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate (3.33). The title compound was synthesized from 2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole 3.25 (0.50 g, 2.9 mmol) and 1-(chloromethyl)-4-methoxybenzene
(0.39 mL, 2.9 mmol) according to general procedure D (0.64 g, 76%). $^1$H NMR (400 MHz, CDCl$_3$): δ 7.97 (d, J = 8.3 Hz, 2H), 7.46 (dd, J = 2.1 Hz, 4.7 Hz, 1H), 7.36 (d, J = 8.6 Hz, 2H), 7.16 (m, 5H), 6.92 (d, J = 8.6 Hz, 2H), 5.29 (s, 2H), 3.92 (s, 3H), 3.84 (s, 3H), 3.80 (s, 2H), 3.78 (s, 2H), 2.90 (t, J = 5.7 Hz, 2H), 2.75 (t, J = 5.4 Hz, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 166.7, 158.8, 143.1, 136.7, 133.7, 130.6, 130.3, 130.1, 129.3, 126.2, 126.0, 121.1, 119.3, 117.8, 113.7, 109.0, 108.9, 61.7, 55.3, 52.1, 49.9, 49.7, 46.2, 22.8. ESI-HRMS: calc. for C$_{28}$H$_{28}$N$_2$O$_3$: [M+H]$^+$ = m/z 441.2173, found: [M+H]$^+$ = m/z 441.2174.

Methyl 4-((2-(pyridin-4-ylmethyl)-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate (3.34). The title compound was synthesized from 2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole 3.25 (0.50 g, 2.9 mmol) and 4-(bromomethyl)pyridine hydrobromide (0.73 g, 2.9 mmol) according to general procedure D (0.89 g, 74%). $^1$H NMR (400 MHz, CDCl$_3$): δ 8.57 (d, J = 5.1 Hz, 2H), 7.94 (d, J = 8.1 Hz, 2H), 7.43 (d, J = 6.8 Hz, 1H), 7.34 (d, J = 5.2 Hz, 2H), 7.11 (m, 5H), 5.21 (s, 2H), 3.85 (s, 3H), 3.76 (d, J = 9.1 Hz, 4H), 2.84 (t, J = 5.2 Hz, 2H), 2.71 (br, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 166.2, 149.5, 147.7, 142.7, 136.4, 133.1, 129.7, 129.0, 125.8, 125.5, 123.4, 120.9, 119.1, 117.4, 108.8, 108.1, 60.6, 51.7, 49.9, 49.6, 45.8, 22.5. ESI-HRMS: calc. for C$_{26}$H$_{25}$N$_3$O$_2$: [M+H]$^+$ = m/z 412.2020, found: [M+H]$^+$ = m/z 412.2014.

Methyl 4-((2-(3-amino-3-oxopropyl)-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate (3.35). The title compound was synthesized from 2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole 3.25 (0.35 g, 2.3 mmol) and 3-bromopropanamide (0.40 g, 2.3 mmol) according to general procedure D (0.23 g, 29%). $^1$H NMR (400 MHz, CDCl$_3$): δ 7.87 (br, 1H), 7.84 (d, J = 8.2 Hz, 2H), 7.36 (m, 1H), 7.04 (m, 3H), 6.96 (d, J = 8.2 Hz, 2H), 5.76 (br, 1H), 5.17 (s, 2H), 3.78 (s, 3H), 3.71 (s, 2H), 2.81 (m, 4H), 2.65 (br, 2H), 2.43 (t, J = 6.1 Hz, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 175.2, 166.7, 142.9, 136.8, 133.3, 130.1, 129.4, 126.1, 125.7,
Methyl 4-((2-(2-amino-2-oxoethyl)-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate (3.36). The title compound was synthesized from 2,3,4,5-tetrahydro-1H-pyrido[4,3-b]indole 3.25 (0.50 g, 2.9 mmol) and 2-bromoacetamide (0.40 g, 2.9 mmol) according to general procedure D (0.55 g, 50%). $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 7.88 (d, $J$ = 8.1 Hz, 2H), 7.37 (m, 2H), 7.28 (br, 1H), 7.16 (m, 3H), 7.01 (m, 2H), 5.43 (s, 2H), 3.81 (s, 3H), 3.71 (s, 2H), 3.13 (s, 2H), 2.84 (br, 2H), 2.76 (br, 2H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 172.1, 166.0, 144.1, 136.3, 133.9, 129.6, 128.6, 126.8, 125.4, 120.8, 119.0, 117.5, 109.6, 107.6, 60.7, 52.2, 50.3, 49.4, 45.5, 22.3. ESI-HRMS: calc. for C$_{22}$H$_{23}$N$_3$O$_3$: [M+H]$^+$ = m/z 378.1812, found: [M+H]$^+$ = m/z 378.1824.

Methyl 4-((2-(4-amino-4-oxobutyl)-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate (3.37). 2,3,4,5-Tetrahydro-1H-pyrido[4,3-b]indole 3.25 (0.50 g, 2.9 mmol) was placed under argon in a two-neck round-bottom flask fitted with a condenser. Anhydrous MeCN (5 mL) was added followed by the addition of Et$_3$N (2 molar equivalents) at room temperature. The reaction was heated to 60 °C and then methyl 4-bromobutanoate (1 molar equivalent) was added in anhydrous MeCN (2 mL). The reaction was stirred for 2 h at 60 °C and then poured into a 1:1 mixture of EtOAc/H$_2$O (20 mL). The organic layer was isolated and the aqueous layer was further extracted with EtOAc (2x15 mL). The combined organic layers were washed with brine (10 mL), dried with Na$_2$SO$_4$, filtered and concentrated in vacuo. Methyl 4-(3,4-dihydro-1H-pyrido[4,3-b]indol-2(5H)-yl)butanoate was isolated by automated column chromatography (0.66 g, 84%). $^1$H NMR (400 MHz, MeOD): $\delta$ 8.80 (br, 1H), 7.46 (br, 1H), 7.14 (br, 3H), 3.77 (s, 2H), 3.71 (s, 3H), 2.83 (br, 2H), 2.69 (t, $J$ = 7.2 Hz, 2H), 2.59 (br, 2H), 2.46 (t, $J$ = 7.3 Hz, 2H), 2.03 (m, 2H). $^{13}$C NMR (100 MHz, MeOD): $\delta$
Methyl 4-(3,4-dihydro-1H-pyrido[4,3-b]indol-2(5H)-yl)butanoate (0.66 g, 2.4 mmol) was dissolved in MeOH (15 mL) and cooled to 0 °C. A 25% aqueous NH₃ solution (15 mL) was added and the reaction was stirred at 0 °C for 2 h after which the reaction was allowed to come to room temperature and then refluxed for an additional 12 h. After TLC indicated that the reaction was complete, it was concentrated in vacuo and then poured into a 1:1 mixture of EtOAc/H₂O (60 mL). The organic fraction was isolated and the aqueous layer was further extracted with EtOAc (2x15 mL). Combined organic fractions were washed with water (15 mL), brine (10 mL), dried with Na₂SO₄, filtered and concentrated in vacuo. Further purification was not required and the isolated product, 4-(3,4-dihydro-1H-pyrido[4,3-b]indol-2(5H)-yl)butanamide (0.34 g, 54%), was subjected to the next step without further purification. ¹H NMR (400 MHz, DMSO-d₆): δ 10.75 (s, 1H), 7.29 (m, 3H), 6.96 (m, 2H), 6.73 (br, 1H), 3.56 (s, 2H), 2.76 (br, 4H), 2.53 (t, J = 7.06 Hz, 2H), 2.14 (t, J = 7.3 Hz, 2H), 1.79 (m, 2H). ¹³C NMR (100 MHz, DMSO-d₆): δ 174.8, 136.4, 133.3, 126.1, 120.5, 118.6, 117.4, 111.2, 107.6, 57.4, 50.9, 49.7, 33.5, 24.0, 23.4. ESI-HRMS: calc. for C₁₅H₁₉N₃O: [M+H]⁺ = m/z 258.1601, found: [M+H]⁺ = m/z 258.1598.

The title compound was synthesized from 4-(3,4-dihydro-1H-pyrido[4,3-b]indol-2(5H)-yl)butanamide (0.34 g, 1.3 mmol) according to general procedure B (0.21 g, 39%). ¹H NMR (400 MHz, MeOD): δ 7.83 (d, J = 8.2 Hz, 2H), 7.41 (d, J = 6.9 Hz, 1H), 7.17 (d, J = 8.0 Hz, 1H), 7.04 (m, 4H), 5.21 (s, 2H), 3.80 (s, 3H), 3.71 (s, 2H), 2.80 (br, 2H), 2.68 (br, 2H), 2.61 (t, J = 7.1 Hz, 2H), 2.28 (t, J = 7.3 Hz, 2H), 1.96 (m, 2H). ¹³C NMR (100 MHz, MeOD): δ 177.0, 166.7, 143.8, 136.9, 133.3, 129.5, 128.9, 126.1, 125.8, 121.0, 119.1, 117.3, 109.0,
107.5, 56.9, 51.2, 50.2, 49.3, 45.4, 32.9, 22.7, 22.0. ESI-HRMS: calc. for C_{24}H_{27}N_{3}O_{3}: [M+H]^{+} = m/z 406.2125, found: [M+H]^{+} = m/z 406.2133.

**tert-Butyl 5-(4-(hydroxycarbamoyl)benzyl)-3,4-dihydro-1H-pyrido[4,3-b]indole-2(5H)-carboxylate (3.38).** The title compound was synthesized from *tert*-butyl 5-(4-(methoxycarbonyl)benzyl)-3,4-dihydro-1H-pyrido[4,3-b]indole-2(5H)-carboxylate 3.26 (0.11 g, 0.3 mmol) according to general procedure C and isolated as an orange solid (7 mg, 7%).

$^{1}$H NMR (400 MHz, MeOD): δ 7.65 (d, $J = 8.3$ Hz, 2H), 7.46 (d, $J = 7.4$ Hz, 1H), 7.28 (d, $J = 7.9$ Hz, 1H), 7.09 (m, 4H), 5.41 (s, 2H), 4.66 (s, 2H), 3.81 (br, 2H), 2.75 (br, 2H), 1.51 (s, 9H).

$^{13}$C NMR (100 MHz, MeOD): δ 166.4, 155.5, 142.3, 136.9, 133.3, 131.1, 127.1, 126.0, 125.3, 121.1, 119.1, 117.1, 109.0, 106.8, 80.0, 45.3, 41.6, 40.8, 27.3, 22.0. ESI-HRMS: calc. for C_{24}H_{27}N_{3}O_{3}: [M+H]^{+} = m/z 422.2074, found: [M+H]^{+} = m/z 422.2073.

**4-((3,4-Dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)-N-hydroxybenzamide-TFA (3.39).** The title compound was synthesized from methyl 4-((3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.27 (0.38 g, 1.2 mmol) according to general procedure C and the TFA salt was isolated as a yellow solid (152 mg, 38%).

$^{1}$H NMR (400 MHz, MeOD): δ 7.68 (d, $J = 8.2$ Hz, 2H), 7.53 (d, $J = 7.7$ Hz, 1H), 7.38 (d, $J = 8.0$ Hz, 1H), 7.19 (d, $J = 7.8$ Hz, 1H), 7.13 (m, 3H), 5.49 (s, 2H), 4.51 (s, 2H), 3.65 (t, $J = 5.9$ Hz, 2H), 3.09 (t, $J = 5.5$ Hz, 2H).$^{13}$C NMR (100 MHz, MeOD): δ 161.2, 141.6, 137.0, 131.4, 130.8, 127.2, 126.2, 124.9, 122.1, 119.8, 117.3, 109.4, 102.2, 46.9, 41.5, 40.8, 19.2. ESI-HRMS: calc. for C_{19}H_{19}N_{3}O_{2}: [M+H]^{+} = m/z 322.1550, found: [M+H]^{+} = m/z 322.1549.

**4-((2-Ethyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)-N-hydroxybenzamide-TFA (3.40).** The title compound was synthesized from methyl 4-((2-ethyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.28 (0.30 g, 0.8 mmol) according to general procedure C and the TFA salt was isolated as a yellow-orange solid.
N-Hydroxy-4-((2-isopropyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzamide·TFA (3.41). The title compound was synthesized from methyl 4-((2-isopropyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.29 (0.21 g, 0.6 mmol) according to general procedure C and the TFA salt was isolated as an orange solid (35 mg, 17%). $^1$H NMR (400 MHz, MeOD): δ 7.67 (d, $J = 8.3$ Hz, 2H), 7.57 (d, $J = 7.8$ Hz, 1H), 7.39 (d, $J = 8.2$ Hz, 1H), 7.21 (t, $J = 7.1$ Hz, 1H), 7.15 (m, 3H), 5.48 (q, $J = 16.3$ Hz, 2H), 4.59 (dd, $J = 13.8$ Hz, 29.5 Hz, 2H), 3.86 (m, 1H), 3.81 (m, 1H), 3.57 (m, 1H), 3.17 (br, 2H), 1.51 (d, $J = 3.7$ Hz, 6H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 163.8, 141.1, 136.6, 132.0, 131.7, 127.3, 126.7, 124.7, 122.0, 119.7, 118.0, 110.2, 102.6, 57.1, 46.2, 45.7, 44.5, 20.3, 17.1, 16.4. ESI-HRMS: calc. for C$_{22}$H$_{25}$N$_3$O$_2$: [M+H]$^+$ = m/z 364.2020, found: [M+H]$^+$ = m/z 364.2029.

4-((2-Allyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)-N-hydroxybenzamide·TFA (3.42). The title compound was synthesized from methyl 4-((2-allyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.30 (0.21 g, 0.6 mmol) according to general procedure C and the TFA salt was isolated as a yellow solid (49 mg, 25%). $^1$H NMR (400 MHz, MeOD): δ 7.68 (d, $J = 8.1$ Hz, 2H), 7.53 (d, $J = 7.8$ Hz, 1H), 7.39 (d, $J = 8.3$ Hz, 1H), 7.22 (t, $J = 7.1$ Hz, 1H), 7.15 (m, 3H), 6.11 (m, 1H), 5.70 (m, 2H), 5.49
(d, J = 11.3 Hz, 2H), 4.74 (br, 1H), 4.44 (br, 1H), 4.03 (d, J = 6.8 Hz, 2H), 3.91 (br, 1H), 3.56 (br, 1H), 3.16 (s, 2H). $^{13}$C NMR (100 MHz, MeOD): δ 165.8, 141.1, 136.9, 131.1, 130.2, 126.8, 125.9, 125.8, 124.3, 121.9, 119.6, 117.0, 109.2, 101.6, 57.5, 48.9, 48.5, 45.3, 19.3. ESI-HRMS: calc. for $C_{22}H_{23}N_3O_2$: [M+H]$^+$ = m/z 362.1863, found: [M+H]$^+$ = m/z 362.1860.

4-((2-Benzyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)-N-hydroxybenzamide·TFA (3.43). The title compound was synthesized from methyl 4-((2-benzyl-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.31 (0.18 g, 0.4 mmol) according to general procedure C and the TFA salt was isolated as an off-white solid (35 mg, 19%). $^1$H NMR (400 MHz, MeOD): δ 7.66 (d, J = 8.4 Hz, 2H), 7.54 (m, 5H), 7.46 (d, J = 7.7 Hz, 1H), 7.37 (d, J = 8.2 Hz, 1H), 7.19 (m, 1H), 7.12 (m, 3H), 5.47 (s, 2H), 4.57 (m, 4H), 3.75 (br, 2H), 3.14 (s, 2H). $^{13}$C NMR (100 MHz, MeOD): δ 147.5, 137.2, 131.4, 130.8, 130.7, 130.0, 129.4, 129.1, 127.2, 126.2, 124.7, 122.2, 119.9, 117.2, 109.5, 101.9, 59.2, 49.3, 48.9, 45.6, 19.7. ESI-HRMS: calc. for $C_{26}H_{25}N_3O_2$: [M+H]$^+$ = m/z 412.2020, found: [M+H]$^+$ = m/z 412.2034.

N-Hydroxy-4-((2-(3-methoxybenzyl)-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzamide·TFA (3.44). The title compound was synthesized from methyl 4-((2-(3-methoxybenzyl)-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.32 (0.23 g, 0.5 mmol) according to general procedure C and the TFA salt was isolated as a beige solid (112 mg, 48%). $^1$H NMR (400 MHz, DMSO-$d_6$): δ 11.17 (s, 1H), 10.33 (s, 1H), 9.02 (s, 1H), 7.67 (d, J = 8.2 Hz, 2H), 7.47 (m, 3H), 7.15 (m, 5H), 7.07 (m, 2H), 5.46 (dd, J = 17.3 Hz, 25.4, 2H), 4.51 (s, 2H), 4.41 (br, 1H), 3.80 (s, 3H), 3.49 (br, 1H), 3.17 (m, 3H), 3.06 (br, 1H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 163.8, 159.5, 141.1, 136.6, 132.0, 131.7, 131.3, 130.1, 127.3, 126.7, 124.5, 123.3, 122.0, 119.8, 117.8, 116.6, 115.3, 110.2, 101.9, 58.2, 55.3, 48.7,
N-Hydroxy-4-((2-(4-methoxybenzyl)-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzamide-TFA (3.45). The title compound was synthesized from methyl 4-((2-(4-methoxybenzyl)-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.33 (0.23 g, 0.5 mmol) according to general procedure C and the TFA salt was isolated as a beige solid (123 mg, 53%). $^1$H NMR (400 MHz, DMSO-d$_6$): $\delta$ 11.16 (s, 1H), 10.13 (s, 1H), 9.05 (br, 1H), 7.67 (d, $J = 8.2$ Hz, 2H), 7.49 (t, $J = 8.9$ Hz, 4H), 7.11 (m, 6H), 5.46 (dd, $J = 16.8$ Hz, 28.1 Hz, 2H), 4.50 (br, 1H), 4.47 (s, 3H), 4.37 (br, 1H), 3.78 (s, 2H), 3.59 (m, 1H), 3.46 (m, 1H), 3.17 (m, 1H), 3.02 (m, 1H). $^{13}$C NMR (100 MHz, DMSO-d$_6$): $\delta$ 163.8, 160.3, 141.0, 136.6, 132.8, 132.0, 131.7, 127.3, 126.7, 124.5, 122.0, 121.7, 119.8, 117.8, 114.4, 110.2, 101.9, 57.9, 55.3, 48.5, 48.0, 45.7, 19.7. ESI-HRMS: calc. for C$_{27}$H$_{27}$N$_3$O$_3$: [M+H]$^+$ = m/z 442.2125, found: [M+H]$^+$ = m/z 442.2104.

N-Hydroxy-4-((2-(pyridin-4-ylmethyl)-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzamide-TFA (3.46). The title compound was synthesized from methyl 4-((2-(pyridin-4-ylmethyl)-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.34 (0.82 g, 2.0 mmol) according to general procedure C and the TFA salt was isolated as a light yellow solid (327 mg, 40%). $^1$H NMR (400 MHz, MeOD): $\delta$ 8.81 (d, $J = 5.8$ Hz, 2H), 7.85 (d, $J = 6.1$ Hz, 2H), 7.65 (d, $J = 8.2$ Hz, 2H), 7.48 (d, $J = 7.8$ Hz, 1H), 7.37 (d, $J = 8.2$ Hz, 1H), 7.19 (t, $J = 7.1$ Hz, 1H), 7.12 (m, 3H), 5.46 (s, 2H), 4.71 (s, 2H), 4.59 (s, 2H), 3.80 (t, $J = 5.8$ Hz, 2H), 3.17 (t, $J = 5.5$ Hz, 2H). $^{13}$C NMR (100 MHz, MeOD): $\delta$ 166.2, 147.9, 142.3, 141.5, 137.2, 131.4, 130.6, 127.2, 126.7, 126.2, 124.7, 122.2, 119.9, 117.3, 109.5, 101.8, 57.5, 50.1, 49.6, 45.6, 19.7. ESI-HRMS: calc. for C$_{25}$H$_{24}$N$_4$O$_2$: [M+H]$^+$ = m/z 413.1972, found: [M+H]$^+$ = m/z 413.1979.
4-((2-(3-Amino-3-oxopropyl)-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)-N-hydroxybenzamide·TFA (3.47). The title compound was synthesized from methyl 4-((2-(3-amino-3-oxopropyl)-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.35 (0.13 g, 0.35 mmol) according to general procedure C and the TFA salt was isolated as a light yellow solid (111 mg, 43%). $^1$H NMR (400 MHz, MeOD): $\delta$ 7.67 (d, $J = 8.3$ Hz, 2H), 7.54 (d, $J = 7.7$ Hz, 1H), 7.38 (d, $J = 8.1$ Hz, 1H), 7.17 (m, 4H), 5.47 (br, 2H), 4.80 (br, 2H), 4.49 (br, 1H), 3.92 (br, 1H), 3.65 (m, 3H), 3.17 (br, 2H), 2.89 (t, $J = 6.8$ Hz, 2H). $^{13}$C NMR (100 MHz, MeOD): $\delta$ 172.7, 166.2, 141.5, 137.3, 131.4, 130.5, 127.2, 126.2, 124.7, 122.2, 119.9, 117.4, 109.5, 101.8, 51.7, 50.0, 49.3, 45.6, 28.8, 19.5. ESI-HRMS: calc. for C$_{22}$H$_{24}$N$_4$O$_3$: [M+H]$^+$ = m/z 393.1921, found: [M+H]$^+$ = m/z 393.1920.

4-((2-(2-Amino-2-oxoethyl)-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)-N-hydroxybenzamide·TFA (3.48). The title compound was synthesized from methyl 4-((2-(2-amino-2-oxoethyl)-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.36 (0.50 g, 1.3 mmol) according to general procedure C and the TFA salt was isolated as a light yellow solid (75 mg, 15%). $^1$H NMR (400 MHz, MeOD): $\delta$ 7.69 (d, $J = 8.3$ Hz, 2H), 7.52 (d, $J = 7.7$ Hz, 1H), 7.39 (d, $J = 8.2$ Hz, 1H), 7.21 (t, $J = 7.1$ Hz, 1H), 7.15 (m, 3H), 5.49 (s, 2H), 4.66 (br, 2H), 4.19 (s, 2H), 3.79 (br, 2H), 3.18 (br, 2H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 166.6, 163.9, 141.5, 136.9, 132.3, 131.8, 127.7, 126.9, 125.0, 122.3, 120.1, 118.3, 110.5, 102.2, 55.5, 50.3, 49.7, 46.0, 19.8. ESI-HRMS: calc. for C$_{21}$H$_{22}$N$_4$O$_3$: [M+H]$^+$ = m/z 379.1765, found: [M+H]$^+$ = m/z 379.1778.

4-((2-(4-Amino-4-oxobutyl)-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)-N-hydroxybenzamide·TFA (3.49). The title compound was synthesized from methyl 4-((2-(4-amino-4-oxobutyl)-3,4-dihydro-1H-pyrido[4,3-b]indol-5(2H)-yl)methyl)benzoate 3.37 (0.21 g, 0.5 mmol) according to general procedure C and the TFA salt was isolated as a light yellow
solid (42 mg, 20%). $^1$H NMR (400 MHz, MeOD): δ 7.68 (d, $J$ = 8.3 Hz, 2H), 7.54 (d, $J$ = 7.8 Hz, 1H), 7.38 (d, $J$ = 8.1 Hz, 1H), 7.21 (t, $J$ = 7.2 Hz, 1H), 7.14 (m, 3H), 5.48 (s, 2H), 4.82 (br, 1H), 4.44 (br, 1H), 3.94 (br, 1H), 3.59 (br, 1H), 3.42 (t, $J$ = 7.4 Hz, 2H), 3.17 (br, 2H), 2.51 (t, $J$ = 6.6 Hz, 2H), 2.16 (m, 2H). $^{13}$C NMR (100 MHz, MeOD): δ 176.0, 166.2, 141.5, 137.3, 131.4, 130.6, 127.2, 126.2, 124.8, 122.2, 119.9, 117.4, 109.5, 102.1, 55.7, 49.8, 49.3, 45.6, 31.7, 19.8, 19.7. ESI-HRMS: calc. for C$_{23}$H$_{26}$N$_4$O$_3$: [M+H]$^+$ = m/z 407.2078, found: [M+H]$^+$ = m/z 407.2086.

**tert-Butyl 9-(4-(methoxycarbonyl)benzyl)-3,4-dihydro-1H-pyrido[3,4-b]indole-2(9H)-carboxylate (3.50).** Di-tert-butyl dicarbonate (7.60 g, 34.8 mmol) was placed in a round-bottom flask fitted with a condenser under argon and to it was added 2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indole (5.00 g, 29.0 mmol) suspended in THF (100 mL) upon which the reaction turned from cloudy white to yellow. The reaction was then heated to reflux and stirred for 8 h after which the reaction was allowed to cool to room temperature and concentrated in vacuo. The reaction mixture was poured into a 1:1 mixture of EtOAc/H$_2$O (100 mL). The organic layer was isolated and the aqueous layer was further extracted with EtOAc (2x30 mL). The combined organic layers were washed with water (20 mL), brine (20 mL), dried with Na$_2$SO$_4$, filtered and concentrated in vacuo. Purification by column chromatography (SiO$_2$, 0-100% EtOAc/hexane) afforded tert-butyl 3,4-dihydro-1H-pyrido[3,4-b]indole-2(9H)-carboxylate (7.32 g, 93%) as a dark yellow oil.

**tert-Butyl 3,4-dihydro-1H-pyrido[3,4-b]indole-2(9H)-carboxylate (5.00 g, 18.4 mmol) was dissolved in anhydrous DMF (30 mL) and added to a suspension of potassium tert-butoxide (2.06 g, 18.4 mmol) in anhydrous DMF (20 mL) under argon at room temperature. The reaction mixture was heated to 80 °C for 15 min after which 4-bromomethyl-benzoic acid methyl ester (4.21 g, 18.4 mmol) dissolved in anhydrous DMF (20 mL) was added. The
reaction was stirred at 80 °C for 2 h after which the reaction was cooled to room temperature and poured into cold water (75 mL). The organic products were extracted with EtOAc (3x30 mL), washed with water (3x20 mL), brine (15 mL), dried with Na₂SO₄, filtered and concentrated in vacuo. Purification by column chromatography (SiO₂, 0-80% EtOAc/hexane) afforded the title compound (3.78 g, 49%) as a dark orange oil. ¹H NMR (400 MHz, CDCl₃): δ 7.97 (d, J = 7.8 Hz, 2H), 7.56 (d, J = 6.8 Hz, 1H), 7.18 (m, 5H), 5.27 (s, 2H), 4.54 (br, 2H), 3.91 (s, 3H), 3.79 (br, 2H), 2.88 (br, 2H), 1.52 (s, 9H). ¹³C NMR (100 MHz, CDCl₃): δ 166.2, 154.7, 142.2, 136.5, 131.5, 129.8, 129.2, 126.5, 125.9, 121.3, 119.2, 117.9, 108.7, 108.3, 79.8, 51.7, 46.1, 42.1, 40.5, 28.1, 21.1. ESI-HRMS: calc. for C₂₅H₂₈N₂O₄: [M+H]⁺ = m/z 421.2122, found: [M+H]⁺ = m/z 421.2124.

Methyl 4-((3,4-dihydro-1H-pyrido[3,4-b]indol-9(2H)-yl)methyl)benzoate (3.51).

 tert-Butyl 9-(4-(methoxycarbonyl)benzyl)-3,4-dihydro-1H-pyrido[3,4-b]indole-2(9H)-
 carboxylate 3.50 (3.00 g, 7.1 mmol) was dissolved in DCM (24 mL) and to it was added TFA (3 mL) upon which the reaction turned from light orange to dark orange. The reaction was allowed to stir for 2 h at room temperature after which volatiles were removed in vacuo. The residue was resuspended in EtOAc (50 mL) and washed with saturated NaHCO₃ (2x20 mL), brine (15 mL), dried with Na₂SO₄, filtered and concentrated in vacuo. Further purification was not required and the title product was obtained (1.75 g, 76%) as a dark orange oil. ¹H NMR (400 MHz, CDCl₃): δ 7.97 (d, J = 7.8 Hz, 2H), 7.57 (d, J = 7.1 Hz, 1H), 7.24 (m, 1H),
7.19 (m, 2H), 7.10 (d, J = 8.1 Hz, 2H), 5.29 (s, 2H), 4.53 (s, 2H), 3.91 (s, 3H), 3.78 (br, 2H),
2.87 (br, 2H). ¹³C NMR (100 MHz, MeOD): δ 164.8, 141.9, 134.9, 130.5, 127.6, 127.1,
125.3, 124.1, 119.2, 117.0, 115.6, 106.9, 106.0, 49.3, 43.6, 40.9, 39.2, 19.1. ESI-HRMS: calc. for C₂₀H₂₀N₂O₂: [M+H]⁺ = m/z 321.1598, found: [M+H]⁺ = m/z 321.1610.
Methyl 4-((2-methyl-3,4-dihydro-1H-pyrido[3,4-b]indol-9(2H)-yl)methyl)benzoate (3.52).
The title compound was synthesized from methyl 4-((3,4-dihydro-1H-pyrido[3,4-b]indol-9(2H)-yl)methyl)benzoate 3.51 (0.50 g, 1.6 mmol) and iodomethane (0.10 mL, 1.6 mmol) according to general procedure E (0.18 g, 34%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.95 (d, $J$ = 8.2 Hz, 2H), 7.54 (m, 1H), 7.16 (m, 3H), 7.15 (d, $J$ = 8.1 Hz, 2H), 5.25 (s, 2H), 3.89 (s, 3H), 3.53 (s, 2H), 2.90 (m, 2H), 2.80 (m, 2H), 2.51 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 166.7, 142.8, 136.9, 132.4, 129.4, 126.9, 126.1, 121.5, 119.5, 118.3, 109.1, 107.9, 52.7, 52.1, 51.3, 46.3, 45.5, 21.3. ESI-HRMS: calc. for C$_{21}$H$_{22}$N$_2$O$_2$: [M+H]$^+$ = m/z 335.1727, found: [M+H]$^+$ = m/z 335.1724.

Methyl 4-((2-benzyl-3,4-dihydro-1H-pyrido[3,4-b]indol-9(2H)-yl)methyl)benzoate (3.53).
The title compound was synthesized from methyl 4-((3,4-dihydro-1H-pyrido[3,4-b]indol-9(2H)-yl)methyl)benzoate 3.51 (0.50 g, 1.6 mmol) and benzyl bromide (0.19 mL, 1.6 mmol) according to general procedure E (0.36 g, 57%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.96 (d, $J$ = 8.1 Hz, 2H), 7.56 (m, 1H), 7.33 (m, 4H), 7.17 (m, 4H), 7.05 (d, $J$ = 8.0 Hz, 2H), 5.21 (s, 2H), 3.92 (s, 3H), 3.77 (s, 2H), 3.61 (s, 2H), 2.94 (br, 2H), 2.91 (br, 2H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 166.7, 142.9, 138.4, 136.9, 133.2, 130.1, 129.4, 129.0, 128.4, 127.2, 127.1, 126.2, 121.3, 119.4, 118.2, 109.0, 108.5, 61.9, 52.1, 50.6, 49.3, 46.3, 21.3. ESI-HRMS: calc. for C$_{27}$H$_{26}$N$_2$O$_2$: [M+H]$^+$ = m/z 411.2067, found: [M+H]$^+$ = m/z 411.2071.

4-((3,4-Dihydro-1H-pyrido[3,4-b]indol-9(2H)-yl)methyl)-N-hydroxybenzamide·TFA (3.54). The title compound was synthesized from methyl 4-((3,4-dihydro-1H-pyrido[3,4-b]indol-9(2H)-yl)methyl)benzoate 3.51 (0.10 g, 0.5 mmol) according to general procedure C and the TFA salt was isolated as an off-white solid (57 mg, 57%). $^1$H NMR (400 MHz, MeOD): $\delta$ 7.67 (d, $J$ = 8.2 Hz, 2H), 7.58 (d, $J$ = 7.3 Hz, 1H), 7.46 (m, 2H), 7.34 (t, $J$ = 7.4 Hz, 1H), 7.12 (d, $J$ = 8.6 Hz, 2H), 5.47 (s, 2H), 4.39 (s, 2H), 3.62 (t, $J$ = 5.7 Hz, 2H), 3.15 (t, $J$ =
5.1 Hz, 2H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 166.4, 143.8, 137.1, 130.0, 129.2, 128.2, 127.3, 126.2, 122.6, 120.1, 118.7, 110.4, 106.7, 52.6, 46.2, 41.8, 18.6. ESI-HRMS: calc. for C$_{19}$H$_{19}$N$_3$O$_2$: [M+H]$^+ = m/z$ 322.1550, found: [M+H]$^+ = m/z$ 322.1556.

N-Hydroxy-4-((2-methyl-3,4-dihydro-1H-pyrido[3,4-b]indol-9(2H)-yl)methyl)benzamide·TFA (3.55). The title compound was synthesized from methyl 4-((2-methyl-3,4-dihydro-1H-pyrido[3,4-b]indol-9(2H)-yl)methyl)benzoate 3.52 (0.18 g, 0.5 mmol) according to general procedure C and the TFA salt was isolated as an off-white solid (26 mg, 15%). $^1$H NMR (400 MHz, MeOD): $\delta$ 7.70 (d, $J = 6.6$ Hz, 2H), 7.58 (d, $J = 7.9$ Hz, 1H), 7.38 (d, $J = 8.2$ Hz, 1H), 7.22 (t, $J = 7.0$ Hz, 1H), 7.13 (m, 3H), 5.46 (s, 2H), 4.66 (br, 1H), 4.35 (br, 1H), 3.81 (br, 1H), 3.50 (br, 1H), 3.20 (t, $J = 6.5$ Hz, 2H), 3.09 (s, 3H). $^{13}$C NMR (100 MHz, MeOD): $\delta$ 166.3, 141.4, 137.5, 131.2, 127.3, 126.2, 126.1, 125.9, 122.5, 119.8, 118.1, 109.5, 106.0, 52.1, 49.8, 45.8, 41.9, 18.3. ESI-HRMS: calc. for C$_{20}$H$_{21}$N$_3$O$_2$: [M+H]$^+ = m/z$ 336.1707, found: [M+H]$^+ = m/z$ 336.1700.

4-((2-Benzyl-3,4-dihydro-1H-pyrido[3,4-b]indol-9(2H)-yl)methyl)-N-hydroxybenzamide·TFA (3.56). The title compound was synthesized from methyl 4-((2-benzyl-3,4-dihydro-1H-pyrido[3,4-b]indol-9(2H)-yl)methyl)benzoate 3.53 (0.36 g, 0.9 mmol) according to general procedure C and the TFA salt was isolated as an off-white solid (152 mg, 42%). $^1$H NMR (400 MHz, MeOD): $\delta$ 7.60 (d, $J = 8.3$ Hz, 2H), 7.54 (d, $J = 7.8$ Hz, 1H), 7.47 (m, 5H), 7.74 (d, $J = 8.2$ Hz, 1H), 7.21 (dt, $J = 0.9$ Hz, 7.2 Hz, 1H), 7.12 (t, $J = 7.2$ Hz, 1H), 7.03 (d, $J = 8.2$ Hz, 2H), 5.33 (s, 2H), 4.51 (s, 2H), 4.36 (br, 2H), 3.68 (br, 2H), 3.16 (t, $J = 5.3$ Hz, 2H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 164.2, 141.2, 137.3, 132.4, 131.6, 130.2, 130.1, 129.3, 127.7, 127.4, 127.0, 125.9, 122.6, 120.1, 118.9, 110.5, 106.5, 58.4, 49.3, 47.5, 46.3, 18.4. ESI-HRMS: calc. for C$_{26}$H$_{25}$N$_3$O$_2$: [M+H]$^+ = m/z$ 412.2020, found: [M+H]$^+ = m/z$ 412.2034.
3.5.2 **HDAC Inhibition**

HDAC inhibition assays were performed by the Reaction Biology Corporation (Malvern, PA) using human, full length recombinant HDAC1 and 6 isolated from a baculovirus expression system in Sf9 cells. An acetylated, fluorogenic peptide derived from residues 379-382 of p53 (RHKK_{Ac}) was used as the substrate in the assays. The reaction buffer contained 50 mM Tris-HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1 mg/mL BSA and a final concentration of 1% DMSO. The enzyme was delivered into wells of the reaction plate and compounds were delivered in 100% DMSO into the enzyme mixture by acoustic technology (Echo550; nanoliter range). The plates were spun down and pre-incubated for 5-10 min. The substrate was then delivered to all reaction wells in order to initiate the reaction and the reaction was incubated for 2 h at 30 °C. After incubation, developer and Trichostatin A were added to quench the reaction and generate fluorescence. Then, kinetic measurements were taken for 1.5 h in 15 min intervals to ensure that development was complete. Endpoint readings were taken for analysis after the development reached a plateau. Dose-response curves were generated and the IC₅₀ for each compound was extrapolated from the generated plots (10-dose IC₅₀ curves were generated using a 3-fold serial dilution pattern starting with concentrations of 30 μM). All IC₅₀ determinations were done in duplicate and the values expressed in this chapter are the average of both trials ± the standard deviation.

3.5.3 **Regulatory T Cell Suppression**

3.5.3.1 **Mitotic Suppression Assays**

Spleens and lymph nodes from C57BL/6 mice were harvested and single cell suspension of lymphocytes prepared. We used magnetic beads (Miltenyi Biotec, Auburn, CA) for cell separation into effector T-cells (CD4⁺CD25⁻, Teffs), regulatory T-cells
(CD4⁺CD25⁺, Tregs) and antigen presenting cells (Thy1.2⁺, APC). Teffs were CFSE-labeled and then incubated in the presence of irradiated APC and varying concentrations of each compound at different Treg:Teff ratios. CD3ε mAb (1 µg/mL) was added to stimulate cell division and after 3-4 days of incubation, the percent of dividing cells was determined by CFSE dilution. To eliminate false positives due to intrinsic compound toxicity, working concentrations of each compound were defined as those in which the difference between the number of Teffs undergoing mitosis in the presence and absence of drug and with no Tregs present was < 10%. CFSE-dilution plots were generated for each experiment and the percentage of cells undergoing mitosis displayed in the top left of each plot (Figure 13). Raw data regarding the percentage of cell divisions was standardized for each compound and its respective control at each tested concentration by applying min-max normalization in GraphPad Prism 5 (La Jolla, CA). Standardized cell division data was converted to percent mitotic suppression (% mitotic suppression = 100 - % mitotic division) and then plotted against the ratio of Teffs:Tregs (Figure 14). The area under the standardized suppression curves was calculated using GraphPad Prism 5 and the AUC ratios for each compound versus the control were calculated at multiple working concentrations (Figure 15). Compounds exhibiting AUC ratios greater than 1.25 were considered to be significant. This work was done in the laboratory of Dr. Wayne Hancock at the University of Pennsylvania (Philadelphia, PA).

3.5.3.2 Homeostatic Proliferation Assays

We isolated HDAC6⁻/⁻ CD4⁺CD25⁻ Teffs from 3 HDAC6⁻/⁻ mice and WT CD4⁺CD25⁺ Tregs from 19 WT C57BL/6 mice. HDAC6⁻/⁻ cells were labeled with CFSE and injected into 12 RAG1⁻/⁻ mice along with Tregs; each mouse received 4 x 10⁵ Tregs and 8 x 10⁵ Teffs intravenously. Mice were divided into 4 groups (n = 3/group) and treated daily for 7
days with DMSO control, tubastatin A, 3.24 or 3.46 (1 mg/kg, i.p.). On day 8, peripheral lymph nodes were collected and the total number of viable cells calculated separately for each mouse. After cell staining with CD4 and Foxp3 monoclonal antibodies, the absolute number of lymph node CD4+ Foxp3- CFSE+/low cells in each mouse was determined by flow cytometry. Data from each group of mice was expressed as mean ± SEM, and differences between groups were compared using the Mann-Whitney U test. This work was done in the laboratory of Dr. Wayne Hancock at the University of Pennsylvania (Philadelphia, PA).
CHAPTER 4

ARYL AND ALIPHATIC BASED HDAC6 SELECTIVE INHIBITORS

4.1 Introduction

Having recently identified numerous potent and selective tetrahydrocarboline derivatives with low to subnanomolar potency at HDAC6 and in upwards of 7,000 fold selectivity for HDAC6 versus HDAC1, we decided to further investigate the necessary structural features required to confer potent and selective HDAC6 inhibition. In addition to continuing with our pharmacophore development, we also wanted to improve ligand efficiency as much as possible. This would allow us additional chemical space in which to introduce various substitutions in order to fine tune the desired pharmacokinetic and physiochemical properties of these molecules. Due to the diverse set of indications for which HDAC6 selective inhibitors have been suggested, the ability to adjust the solubility, absorption and/or distribution of these compounds would be of significant benefit. For example, HDAC6 inhibition has shown positive effects in models of colitis, an inflammation of the colon. In this instance, polar compounds with limited ability to cross the blood brain barrier would be desired. However, HDAC6 inhibition has also been proven to provide therapeutic benefits in models of CMT. In this case, more lipophilic compounds with a better chance of crossing the blood brain barrier would be ideal. Improving ligand efficiency would allow us to incorporate these changes while still maintaining the drug-like properties of our previously identified HDAC6 selective inhibitors.

4.2 Chemistry

The bicyclic, monocyclic and acyclic derivatives employed in this study were prepared according to the synthetic routes outlined in schemes 14-18. The first series was
synthesized in two steps starting from commercially available or readily prepared indoles and methyl 4-(bromomethyl)benzoate (Scheme 14). Treatment of the respective indole or 9H-purine with sodium hydride followed by the addition of the benzyl halide resulted in nucleophilic substitution at the 1-position which allowed us to obtain intermediate esters 4.1-4.7, 4.9, 4.10 and 4.12. A similar procedure was also used to prepare the acetamide control, 4.13, substituting 4-(bromomethyl)benzamide as the benzyl halide. Subsequent conversion of the appropriate ester to the hydroxamic acid yielded the desired products 4.14-4.23.
Scheme 14. Preparation of bicyclic compounds.

Reagents and conditions: a) methyl 4-(bromomethyl)benzoate, NaH, DMF, 80 °C, 2 h; b) NH₂OH·HCl, NaOMe, MeOH, 0 °C to rt, 16 h or NaOH, NH₂OH (aq), MeOH/THF, 0 °C to rt, 15 min.
Compounds 4.26 and 4.27 were prepared from 1H-pyrrole and 1H-pyrazole respectively (Scheme 15). Following a procedure similar to that used for the previously synthesized esters, either 1H-pyrrole or 1H-pyrazole was treated with potassium tert-butoxide followed by methyl 4-(bromomethyl)benzoate which resulted in intermediate esters 4.24 and 4.25. These esters were then converted to the respective hydroxamic acids 4.26 and 4.27 with hydroxylamine hydrochloride.

Scheme 15. Preparation of pyrrole and pyrazole compounds.

Reagents and conditions: a) methyl 4-(bromomethyl)benzoate, KO\textsuperscript{Bu}, DMF, 80 °C, 2 h; b) NH\textsubscript{2}OH·HCl, NaOMe, MeOH, 0 °C to rt, 16 h.
Compounds employing a six membered ring as the cap group were also investigated (Scheme 16). These compounds were synthesized from their respective bromoic acids and methyl 4-(bromomethyl)benzoate using a Suzuki-Miyaura cross-coupling reaction. The intermediate esters generated were then converted to the hydroxamic acids as previously described.


Reagents and conditions: a) Tetrakis(triphenylphosphine)palladium(0), methyl 4-(bromomethyl)benzoate, Na$_2$CO$_3$, DME or diglyme, H$_2$O, 100 °C, 4 h; b) NH$_2$OH·HCl, NaOMe, MeOH, 0 °C to rt, 16 h.
Lastly, compounds containing pyrrolidine and substituted amine cap groups were also prepared and evaluated (Schemes 17 and 18). These compounds were synthesized in a fashion similar to that of the pyrroles such that the respective amine was treated with potassium tert-butoxide followed by methyl 4-(bromomethyl)benzoate to yield the intermediate esters. Again, hydroxylamine hydrochloride was used to convert the penultimate intermediate to the desired hydroxamic acid.

Scheme 17. Preparation of pyrrolidine derivatives.

Reagents and conditions: a) methyl 4-(bromomethyl)benzoate, KO'Bu, DMF, 80 °C, 2 h; b) NH$_2$OH·HCl, NaOMe, MeOH, 0 °C to rt, 16 h.
Scheme 18. Preparation of substituted amine derivatives.

Reagents and conditions: a) methyl 4-(bromomethyl)benzoate, KO\textsuperscript{t}Bu, DMF, 80 °C, 2 h; b) NH\textsubscript{2}OH·HCl, NaOMe, MeOH, 0 °C to rt, 16 h.

4.3 Results and Discussion

4.3.1 HDAC Inhibition

In order to further investigate the exact amount of steric bulk necessary for selective HDAC6 inhibition, we evaluated the HDAC inhibitory activity of compounds with cap groups of varying size starting with the bicyclic indoles and culminating with simple, substituted amines. The results of the HDAC inhibitory assays are displayed in Table VII. We found that compounds with indole based cap groups having methyl substitutions at the 2- or 3-position exhibited significantly better selectivity for HDAC6 over HDAC1 compared to the unsubstituted indole 4.14. This compound exhibited an HDAC6 IC\textsubscript{50} of approximately 10 nM and was around 600 fold selective for HDAC6. The 2-methylindole derivative 4.15 had a potency of about 2 nM at HDAC6 and a selectivity of approximately 2,100 fold. The 3-methylindole derivative 4.16 exhibited an HDAC6 IC\textsubscript{50} of about 5 nM but was only about 1,300 fold selective. The addition of two methyl groups to the 2- and 3-positions resulted in
the dimethylindole 4.17 which was significantly more selective for HDAC6 (approximately 3,000 fold) than either of the monomethyl compounds and equally as potent with an HDAC6 IC_{50} of approximately 4 nM.

Once we had established that we could maintain activity with indole based cap groups, we decided to investigate the addition of other substituents with varying chemical properties to these HDAC inhibitors. The introduction of a benzyl group to the indole 3-position 4.18 resulted in a decrease in potency at HDAC6 as well as a decrease in selectivity for HDAC6 versus HDAC1. The introduction of basic amines tethered with an ethylene linker to the indole cap group, such as with the N-methylpiperazine derivative 4.21 and the dimethyltryptamine derivative 4.22, resulted in a decrease in selectivity but roughly the same potency at HDAC6 compared with the simple methyl substituted indoles. Introduction of a sulfonamide in this position 4.23, however, resulted in subnanomolar potency at HDAC6 and increased the selectivity to more than 3,600 fold. Replacing the indole cap group with a purine cap group, 4.19 and 4.20, had little effect on potency at HDAC6 but significantly decreased the selectivity. Also, replacing the hydroxamic acid ZBG with an amide, 4.13, completely negated all activity at HDAC1 and HDAC6.
TABLE VII

IC\textsubscript{50} AND HDAC6 SELECTIVITY DATA FOR ARYL AND ALIPHATIC COMPOUNDS

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<th>Compound ID</th>
<th>HDAC1 IC\textsubscript{50} (μM)</th>
<th>HDAC6 IC\textsubscript{50} (nM)</th>
<th>Selectivity HDAC1/HDAC6 (fold)</th>
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</tbody>
</table>
The use of monocyclic cap groups, both aryl and aliphatic, and substituted amine based cap groups resulted in compounds with slightly reduced potency at HDAC6 and significantly reduced selectivity for HDAC6 compared to HDAC1 relative to the indole based derivatives. The pyrrole and pyrazole derivatives, 4.26 and 4.27, had HDAC6 IC\textsubscript{50}s of 30 and 10 nM, respectively; however, selectivity for HDAC6 was reduced to less than 200 fold. Use of a pyridine or meta-dimethylaminophenyl cap group also had similar effects in regard to potency and selectivity. The para-dimethylaminophenyl compound 4.32, however, exhibited low nanomolar potency and selectivity of more than 1,000 fold for HDAC6. Compounds with pyrrolidine cap groups 4.37-4.39 exhibited increased IC\textsubscript{50} values in the 0.1-1.0 μM range at HDAC6 and were less than 100 fold selective for HDAC6 versus HDAC1. It should be noted, however, that the (S)-2-methoxymethyl pyrrolidine derivative 4.38 was significantly more potent at HDAC6 than the corresponding (R) enantiomer. In regard to the compounds containing amine based cap groups, the diethylamino compound 4.43 was significantly less potent and selective than the diisopropylamino derivative 4.44. The diphenylamino compound 4.45, however, was even better with an HDAC IC\textsubscript{50} of 4.5 nM and a selectivity of 1,400 fold for HDAC6 versus HDAC1, similar to that of the 3-methylindole derivative 4.16.

In comparison to tubastatin A (HDAC6 IC\textsubscript{50} = 15 nM, selectivity for HDAC6 vs. HDAC1 = 1,093 fold), some of these compounds exhibited both improved potency at and selectivity for HDAC6. The 2- and 3-methylindole derivatives, 4.15 and 4.16, along with the 2,3-dimethylindole derivative 4.17, were all more potent at and selective for HDAC6 compared to tubastatin A. This is also the case for sulfonamide derivative 4.23. In addition, the p-dimethylaminophenyl derivative 4.32 and the diphenylamino derivative 4.45 displayed improved potency and selectivity for HDAC6 compared to HDAC1. Therefore, these compounds offer alternatives to the tricyclic HDAC6 inhibitors previously reported. These
compounds also provide improved ligand efficiency and the short synthetic route allows for the facile incorporation of a multitude of substituents that could be useful for altering the pharmacokinetic and physiochemical properties of these compounds.

4.3.2 Neuroprotection

In regard to the neuroprotective properties of these compounds, they are not as robust as the mercaptoacetamides.\textsuperscript{(104)} However, three compounds do exhibit promising activity with two of the analogs being very similar in structure (Figure 17). The pyrrole 4.26 exhibits significant neuroprotective activity at concentrations of 10-20 μM. In addition, both of the dimethylaminophenyl compounds were neuroprotective with their neuroprotective activity correlating with the concentration necessary to infer neuroprotection. 4.32 had an HDAC6 IC\textsubscript{50} of approximately 6 nM whereas 4.33 had an HDAC6 IC\textsubscript{50} around 70 nM. This difference in activity is also observed in the neuroprotection plots such that while 4.32 is neuroprotective at 10 μM, 4.33 only begins to display neuroprotective properties at 20 μM. For compound 4.33, even higher concentrations may be required to achieve full neuroprotection as even at 20 μM concentrations, it was not fully neuroprotective. It should also be noted that, with the exception of 4.21, these compounds do not appear to be toxic to cortical neurons. The inability of some of these compounds to display neuroprotective activity may have resulted from permeability issues as compounds such as 4.27 (cLogP = 0.138), 4.19 (cLogP = -0.59) and 4.20 (cLogP = -0.59) are quite polar compared to compounds like 4.26 (cLogP = 1.10), 4.32 (cLogP = 2.49) and 4.33 (cLogP = 2.49). 4.43 is not a potent HDAC6 inhibitor and, in addition, 4.43 and 4.44 are tertiary amines which would be protonated under physiological conditions. Thus, these compounds may also lack neuroprotective activity as a result of permeability issues.
4.4 Conclusion

Herein, we have demonstrated that the desired HDAC6 inhibition profile could be maintained using a variety of different cap groups in combination with a benzyl linker and hydroxamic acid zinc binding group. These results suggest that while moderate HDAC6 potency and selectivity could be obtained using substituted amines and aliphatic rings as cap groups, aryl cap groups are preferred for selective HDAC6 inhibition. We have shown
that using substituted indole and monoaryl cap groups results in compounds with similar, if not better, potency and selectivity compared to tubastatin A. Thus, for potent and selective HDAC6 inhibition, an aryl cap group is preferred in combination with a benzyl linker directly attached to the hydroxamic acid zinc binding group. (39) Tricyclic and bicyclic cap groups provide the most robust activity and selectivity profile at HDAC6, however, potent and selective inhibition can also be achieved using simple, substituted monoaryl cap groups. (39, 101)

In addition, the increased ligand efficiency of these compounds allows chemical space for the inclusion of structural features aimed at improving or modulating both the pharmacokinetic and pharmacodynamic properties for a particular indication. Lastly, we have also demonstrated that these compounds can provide neuroprotection in cortical neuron models of oxidative stress. These results also suggest that permeability across the blood brain barrier may be a determining factor further underlining the importance of developing a diversifiable pharmacophore. Thus, these compounds provide attractive alternatives to the tricyclic HDAC6 inhibitors that we have previously reported and we believe that further investigation regarding their use for additional indications is warranted.

4.5 Experimental Section

4.5.1 Chemistry

$^1$H NMR and $^{13}$C NMR spectra were obtained using a Bruker spectrometer with TMS as an internal standard. The following standard abbreviations indicating multiplicity were used: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, m = multiplet, dd = double doublet, dt = double triplet, and br = broad. LRMS experiments were carried out using an Agilent 1100 series LC/MSD instrument with MeCN and H$_2$O spiked with 0.1% formic acid as the mobile phase. HRMS experiments were carried out using a Shimadzu IT-TOF instrument with MeCN and H$_2$O spiked with 0.1% formic acid as the mobile phase.
Reaction progress was monitored by TLC using precoated silica gel plates (Merck silica gel 60 F254, 250 μm thickness). Automated column chromatography was performed using the CombiFlash Rf apparatus available from Teledyne ISCO and prepacked cartridges (25 or 50 g) loaded with Merck silica gel (40–60 mesh) along with the following conditions: Method 1: 100% hexane, 5 min; 0-50% EtOAc/hexane, 25 min; 50% EtOAc/hexane, 5 min. Method 2: 100% DCM, 5 min; 0–10% MeOH/DCM, 20 min, 10% MeOH/DCM, 5 min. Flow rate = 30-40 mL/min (depending on cartridge size) with wavelength monitoring at 254 and 280 nm. Preparatory HPLC was carried out using a Shimadzu preparative liquid chromatograph with the following specifications: Column: ACE 5 AQ (150 × 21.2 mm) with 5 μm particle size. Method 1: 25–100% MeOH/H₂O, 30 min; 100% MeOH, 5 min; 100–25% MeOH/H₂O, 4 min; 25% MeOH/H₂O, 1 min. Method 2: 8–100% MeOH/H₂O, 30 min; 100% MeOH, 5 min; 100–8% MeOH/H₂O, 4 min; 8% MeOH/H₂O, 1 min. Flow rate = 17 mL/min with wavelength monitoring at 254 and 280 nm. Both solvents were spiked with 0.05% TFA. Where applicable (unless otherwise specified), resin bound bicarbonate was used to neutralize the trifluoroacetic acid salts obtained during preparatory HPLC purification. Analytical HPLC was carried out using an Agilent 1100 series instrument with the following specifications: Column: Luna 5 μ C18(2) 100A (150 × 4.60 mm) with 5 μm particle size. Flow rate = 1.4 mL/min with wavelength monitoring at 254 nm. Gradient: 10–100% MeOH/H₂O, 18 min; 100% MeOH, 3 min; 100–10% MeOH/H₂O, 3 min; 10% MeOH/H₂O, 5 min. Both solvents were spiked with 0.05% TFA.

**General Procedure A:** NaH (1 mol equiv) was dissolved in anhydrous DMF (5 mL/mmol) under argon and cooled to 0 °C. To it was added the appropriate indole (1 mol equiv) dissolved in anhydrous DMF (3 mL/mmol). The reaction was stirred for 15 min at 0 °C followed by the addition of methyl 4-(bromomethyl)benzoate (1 mol equiv) in anhydrous DMF (2 mL/mmol). The reaction was stirred for 2 h at 70 °C and then quenched by the
addition of H$_2$O (30 mL). The organic products were extracted with EtOAc (3 x 30 mL), washed with H$_2$O (2 x 30 mL), brine (15 mL), dried over Na$_2$SO$_4$, filtered and concentrated in vacuo.

**General Procedure B:** The appropriate ester was dissolved/suspended in MeOH (3 mL/mmol) and added to a mixture of NH$_2$OH•HCl (6 mol equiv) in MeOH (3 mL/mmol) which was followed by the addition of NaOMe (8 mol equiv of a 25% solution in MeOH). The mixture was stirred for 2 h at 0 °C followed by stirring for 22 h at RT. When the reaction was complete as evidenced by TLC, the reaction was quenched by the addition of trifluoroacetic acid (5 mol equiv of a 10% solution in DCM), filtered, and the filter cake was washed with additional MeOH (5-15 mL). The combined filtrate and wash were then concentrated in vacuo to yield the crude product which was dissolved in DMF and purified by preparatory HPLC.

**General Procedure C:** Solid NaOH (8 mol equiv) was dissolved in a 50% aq. solution of NH$_2$OH (~50 mol equiv) at 0 °C. Then, a solution of the appropriate ester (1 mol equiv) in THF/MeOH (9:9 mL/mmol) was added dropwise to the aforementioned, vigorously stirred hydroxylamine solution. Upon complete addition, the ice bath was removed and the reaction was allowed to stir 15 min. The reaction was quenched with AcOH (10 mol equiv) and concentrated in vacuo to yield the crude product which was dissolved in DMF and purified by preparatory HPLC.

**General Procedure D:** The appropriate boronic acid (1 mol equiv), methyl 4-(bromomethyl)benzoate (1.2 mol equiv), tetrakis(triphenylphosphine)Pd(0) (0.02 mol equiv), and K$_2$CO$_3$ (2.1 mol equiv) were placed in a dry, sealed tube under Ar atmosphere. Diglyme (4 mL/mmol) and H$_2$O (2 mL/mmol) were added through a rubber septa which was immediately replaced by the screw on cap. The reaction was heated to 100 °C and stirred
for 4 h. Then, the reaction was diluted with H₂O (20 mL) and the organic products were extracted with DCM (3 x 15 mL), washed with brine (15 mL), dried over Na₂SO₄, filtered and concentrated in vacuo.

**Methyl 4-((1H-indol-1-yl)methyl)benzoate (4.1).** The title compound was prepared from 1H-indole (0.500 g, 4.27 mmol) according to General Procedure A and purified using automated column chromatography method 1. The product was isolated as a white solid (0.860 g, 76%). ¹H NMR (400 MHz, CDCl₃): δ 8.05 (d, J = 8.23 Hz, 2H), 7.79 (d, J = 6.9 Hz, 1H), 7.24 (m, 6H), 6.70 (d, J = 3.05 Hz, 1H), 5.35 (s, 2H), 3.97 (s, 3H). ¹³C NMR (100 MHz, CDCl₃): δ 166.7, 142.9, 136.3, 130.1, 129.6, 128.9, 128.4, 126.7, 122.0, 121.2, 119.9, 109.7, 102.2, 52.2, 49.8. ESI-HRMS: calc. for C₁₇H₁₅NO₂: [M+H]⁺ = m/z 266.1176, found: [M+H]⁺ = m/z 266.1182.

**Methyl 4-((2-methyl-1H-indol-1-yl)methyl)benzoate (4.2).** The title compound was prepared from 2-methyl-1H-indole (2.00 g, 15.3 mmol) according to General Procedure A and purified using automated column chromatography method 1. The product was isolated as a white solid (2.87 g, 67%). ¹H NMR (400 MHz, DMSO-d₆): δ 7.89 (d, J = 8.0 Hz, 2H), 7.48 (d, J = 7.6 Hz, 1H), 7.33 (d, J = 7.6 Hz, 1H), 7.09 (d, J = 8.0 Hz, 2H), 7.01 (m, 2H), 6.32 (s, 1H), 5.49 (s, 2H), 3.81 (s, 3H), 2.34 (s, 3H). ¹³C NMR (100 MHz, DMSO-d₆): δ 166.4, 144.5, 137.2, 137.1, 130.0, 128.9, 128.2, 126.8, 120.9, 119.8, 119.7, 109.9, 100.7, 52.5, 45.9, 12.8.

**Methyl 4-((3-methyl-1H-indol-1-yl)methyl)benzoate (4.3).** The title compound was prepared from 3-methyl-1H-indole (0.500 g, 3.81 mmol) according to General Procedure A and purified using automated column chromatography method 1. The product was isolated as a white solid (0.668 g, 63%). ¹H NMR (400 MHz, CDCl₃): δ 8.04 (d, J = 7.9 Hz, 2H), 7.72 (d, J = 7.4 Hz, 1H), 7.24 (m, 5H), 6.94 (s, 1H), 5.31 (s, 2H), 3.97 (s, 3H), 2.46 (s, 3H). ¹³C
NMR (100 MHz, CDCl₃): δ 166.7, 143.1, 136.5, 130.0, 129.4, 129.0, 126.6, 125.7, 1221.8, 119.1, 119.0, 109.3, 71.8, 52.1, 49.5, 9.6. ESI-HRMS: calc. for C₁₉H₁₇NO₂: [M+H]^+ = m/z 280.1332, found: [M+H]^+ = m/z 280.1322.

**Methyl 4-((2,3-dimethyl-1H-indol-1-yl)methyl)benzoate (4.4)**. The title compound was prepared from 2,3-dimethyl-1H-indole (0.500 g, 3.44 mmol) according to General Procedure A and purified using automated column chromatography method 1. The product was isolated as a viscous yellow oil (0.652 g, 65%). ^1H NMR (400 MHz, CDCl₃): δ 7.95 (d, J = 8.3 Hz, 2H), 7.57 (dd, J = 3.1 Hz, 2.2 Hz, 1H), 7.16 (m, 3H), 7.04 (d, J = 8.2 Hz, 2H), 5.36 (s, 2H), 3.91 (s, 3H), 2.32 (s, 3H), 2.29 (s, 3H). ^13C NMR (100 MHz, CDCl₃): δ 166.8, 143.5, 136.3, 132.2, 130.1, 129.2, 128.7, 126.0, 121.0, 119.1, 118.1, 108.6, 107.4, 52.1, 46.4, 10.1, 8.9. ESI-HRMS: calc. for C₁₉H₁₉NO₂: [M+H]^+ = m/z 294.1489, found: [M+H]^+ = m/z 294.1503.

**Methyl 4-((3-benzyl-1H-indol-1-yl)methyl)benzoate (4.5)**. To a solution of methyl 4-((1H-indol-1-yl)methyl)benzoate 1 (0.250 g, 0.94 mmol) and benzaldehyde (96 μL, 0.94 mmol) in DCM (10 mL) at 0 °C was added SiEt₃H (0.45 mL, 2.83 mmol) followed by trifluoroacetic acid (0.14 mL, 1.88 mmol). The reaction was stirred at 0 °C for 1 h. The reaction was then adjusted to pH 10 with 2N NaOH and the organic products were extracted with DCM (3 x 15 mL). The combined organic fractions were washed with brine (15 mL), dried with Na₂SO₄, filtered and concentrated in vacuo. The title compound was isolated using automated column chromatography method 1 (0.262 g, 78%). ^1H NMR (400 MHz, CDCl₃): δ 7.97 (d, J = 8.0 Hz, 2H), 7.56 (d, J = 8.0 Hz, 1H), 7.32-7.27 (m, 4H), 7.23-7.10 (m, 6H), 6.87 (s, 1H), 5.33 (s, 2H), 4.15 (s, 2H), 3.91 (s, 3H). ^13C NMR (100 MHz, CDCl₃): δ 166.8, 143.0, 141.2, 136.8, 130.1, 129.5, 128.7, 128.4, 128.3, 126.6, 126.5, 126.0, 122.1, 119.5, 119.3, 115.4, 109.6, 52.2, 49.7, 31.6.
**Methyl 4-((9H-purin-9-yl)methyl)benzoate (4.6).** The title compound was prepared from 9H-purine (0.500 g, 3.81 mmol) according to General Procedure A (substituting KO\textsubscript{t}Bu for NaH) and purified using automated column chromatography method 2. The pH was adjusted to 10 with 1N NaOH prior to extraction with EtOAc. The product was isolated as a white solid (0.287 g, 64%). \(^1\)H NMR (400 MHz, DMSO-\textsubscript{d}6): δ 9.20 (s, 1H), 8.94 (s, 1H), 8.78 (s, 1H), 7.92 (d, \(J = 8.3\) Hz, 2H), 7.44 (d, \(J = 8.3\) Hz, 2H), 5.62 (s, 2H), 3.82 (s, 3H). \(^{13}\)C NMR (100 MHz, DMSO-\textsubscript{d}6): δ 165.9, 152.3, 151.2, 148.0, 147.2, 141.7, 133.7, 129.7, 129.2, 127.8, 52.2, 46.1. ESI-HRMS: calc. for C\textsubscript{14}H\textsubscript{12}N\textsubscript{4}O\textsubscript{2}: [M+H]\(^+\) = m/z 269.1033, found: [M+H]\(^+\) = m/z 269.1032.

**Methyl 4-((7H-purin-7-yl)methyl)benzoate (4.7).** The title compound was prepared from 9H-purine (0.500 g, 3.81 mmol) according to General Procedure A (substituting KO\textsubscript{t}Bu for NaH) and purified using automated column chromatography method 2. The pH was adjusted to 10 with 1N NaOH prior to extraction with EtOAc. The product was isolated as a viscous yellow oil (0.108 g, 24%). \(^1\)H NMR (400 MHz, DMSO-\textsubscript{d}6): δ 9.16 (s, 1H), 9.02 (s, 1H), 8.97 (s, 1H), 7.94 (d, \(J = 8.2\) Hz, 2H), 7.51 (d, \(J = 8.2\) Hz, 2H), 5.74 (s, 2H), 3.83 (s, 3H). \(^{13}\)C NMR (100 MHz, DMSO-\textsubscript{d}6): δ 165.9, 160.4, 152.0, 150.2, 141.2, 140.6, 129.8, 129.5, 128.0, 125.0, 52.2, 48.4. ESI-HRMS: calc. for C\textsubscript{14}H\textsubscript{12}N\textsubscript{4}O\textsubscript{2}: [M+H]\(^+\) = m/z 269.1033, found: [M+H]\(^+\) = m/z 269.1036.

**3-[2-(4-Methyl-piperazin-1-yl)-ethyl]-1H-indole (4.8).** 2-(1H-Indol-3-yl)ethanol (0.500 g, 3.10 mmol) and Et\textsubscript{3}N (1 mL) were dissolved in DCM (2 mL) and then mesyl chloride (0.24 mL, 3.102 mmol) was added dropwise at RT. The reaction was stirred for 3 h and then volatiles were removed in vacuo. The crude mesylate was taken up in DCM (3 mL) and to it was added 1-methylpiperazine (1.72 mL, 15.5 mmol) and Et\textsubscript{3}N (1 mL). The reaction was heated to 40 °C and stirred overnight. After completion, the reaction mixture was poured into
cold water, the pH was adjusted to 10 with 1N NaOH and the organic products were extracted with DCM (3 x 15 mL). The combined organic extracts were washed with brine (15 mL), dried with Na$_2$SO$_4$, filtered and concentrated in vacuo. Automated column chromatography method 2 was used to isolate the title compound (0.42 g, 56%). $^1$H NMR (400 MHz, CDCl$_3$): δ 7.54 (d, $J = 7.8$ Hz, 1H), 7.34 (d, $J = 8.1$ Hz, 1H), 7.10 (t, $J = 7.1$ Hz, 1H), 7.03 (t, $J = 7.3$ Hz, 1H), 6.98 (s, 1H), 2.87 (t, $J = 7.8$ Hz, 2H), 2.58 (t, $J = 5.9$ Hz, 2H), 2.38 (br, 8H), 2.17 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 136.7, 127.4, 121.8, 121.0, 118.3, 118.0, 112.4, 111.0, 58.9, 54.1, 52.2, 44.6, 22.2. ESI-HRMS: calc. for C$_{15}$H$_{21}$N$_3$: [M+H]$^+$ = m/z 244.1808, found: [M+H]$^+$ = m/z 244.1797.

4-[[3-[[2-(4-Methyl-piperazin-1-yl)-ethyl]-1H-indol-1-ylmethyl]-benzoic acid methyl ester (4.9). The title compound was prepared from 3-[[2-(4-methyl-piperazin-1-yl)-ethyl]-1H-indole 4.8 (0.400 g, 1.64 mmol) according to General Procedure A (substituting KO'Bu for NaH). The pH was adjusted to 10 with 1N NaOH prior to extraction with EtOAc. The product was purified via automated column chromatography method 2 (0.412 g, 64%). $^1$H NMR (400 MHz, CDCl$_3$): δ 7.79 (d, $J = 8.3$ Hz, 2H), 7.56 (d, $J = 7.0$ Hz, 1H), 7.04 (m, 6H), 5.14 (s, 2H), 3.76 (s, 3H), 2.90 (t, $J = 7.6$ Hz, 2H), 2.63 (t, $J = 8.6$ Hz, 2H), 2.46 (br, 8H), 2.23 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 166.2, 143.3, 136.2, 129.1, 128.6, 127.8, 126.1, 125.4, 121.2, 118.4, 118.2, 112.6, 109.1, 58.4, 53.8, 51.9, 50.8, 48.4, 44.3, 21.8. ESI-HRMS: calc. for C$_{24}$H$_{29}$N$_4$O$_2$: [M+H]$^+$ = m/z 392.2333, found: [M+H]$^+$ = m/z 392.2343.

Methyl 4-((3-(2-(dimethylamino)ethyl)-1H-indol-1-yl)methyl)benzoate (4.10). The title compound was prepared from 2-((1H-indol-3-yl)-N,N-dimethylethanamine (0.130 g, 0.69 mmol) according to General Procedure A. The pH was adjusted to 10 with 1N NaOH prior to extraction with EtOAc. The product was purified via automated column chromatography method 2 (0.174 g, 61%). $^1$H NMR (400 MHz, CDCl$_3$): δ 7.97 (d, $J = 8.3$ Hz, 2H), 7.65 (d, $J =$
7.5 Hz, 1H), 7.17 (m, 5H), 6.99 (s, 1H), 5.34 (s, 2H), 3.91 (s, 3H), 3.04 (t, \( J = 7.6 \) Hz, 2H), 2.75 (m, 2H), 2.43 (s, 6H).

**N-(2-(1H-Indol-3-yl)ethyl)methanesulfonamide (4.11).** To a round bottom flask charged with tryptamine (2.0 g, 12.5 mmol) in DCM (20 mL) was added Et\(_3\)N (3.5 mL, 25 mmol) under an atmosphere of Ar. The reaction was cooled to 0 °C, mesyl chloride (1.5 mL, 18.7 mmol) was added and the resulting reaction mixture was stirred at RT for 2 h. The reaction was quenched with water (20 mL) and extracted with DCM (3x 20 mL). The combined organic extracts were washed with brine (30 mL), dried over Na\(_2\)SO\(_4\), filtered and concentrated in vacuo. The desired product was purified via automated column chromatography method 2 (2.1 g, 70%) and isolated as a brown oil. \(^1\)H NMR (400 MHz, CD\(_3\)OD) δ 7.55 (d, \( J = 7.6 \) Hz, 1H), 7.33 (d, \( J = 8.0 \) Hz, 1H), 7.08 (m, 2H), 7.01 (td, \( J = 8.0, 0.8 \) Hz, 1H), 3.35 (t, \( J = 7.2 \) Hz, 2H), 2.99 (t, \( J = 7.2 \) Hz, 2H), 2.76 (s, 3H).

**Methyl 4-((3-(2-(methylsulfonamido)ethyl)-1H-indol-1-yl)methyl)benzoate (4.12).** The title compound was prepared from N-(2-(1H-indol-3-yl)ethyl)methanesulfonamide 4.11 (300 mg, 1.26 mmol) according to General Procedure A (substituting KO\(^t\)Bu for NaH) and purified using automated column chromatography method 2. The product was isolated as a brown waxy solid (0.399 g, 82%). \(^1\)H NMR (400 MHz, CDCl\(_3\)) δ 8.20 (br s, 1 H), 8.01 (d, \( J = 8.0 \) Hz, 2 H), 7.42 (m, 3 H), 7.33 (d, \( J = 8.4 \) Hz, 1 H), 7.17 (t, \( J = 7.6 \) Hz, 1 H), 7.08 (t, \( J = 7.2 \) Hz, 1 H), 6.95 (d, \( J = 1.6 \) Hz, 1 H), 4.45 (s, 2 H), 3.92 (s, 3 H), 3.49 (t, \( J = 7.6 \) Hz, 2 H), 2.96 (t, \( J = 7.6 \) Hz, 2 H), 2.74 (s, 3 H). \(^13\)C NMR (100 MHz, CDCl\(_3\)) δ 166.69, 141.53, 136.17, 136.13, 129.95, 129.80, 126.96, 122.14, 122.13, 119.49, 118.40, 112.09, 111.27, 52.13, 51.23, 48.35, 38.90, 24.82. ESI-LRMS: [M+H]^+ = m/z 386.
**4-((1H-Indol-1-yl)methyl)benzamide (4.13).** Methyl 4-((1H-indol-1-yl)methyl)benzoate 4.1 was dissolved in MeOH (10 mL) and to it was added a 30% solution of ammonium hydroxide (5 mL). The reaction was heated to reflux for 16 h after which it was cooled to RT and H₂O (30 mL) was added. The organic products were extracted with EtOAc (3 x 15 mL), washed with brine (15 mL), dried with Na₂SO₄, filtered and concentrated in vacuo. The crude product was dissolved in DMF and purified by preparatory HPLC method 1. The title compound was isolated as a white solid (37 mg, 13%). ¹H NMR (400 MHz, CD₃OD): δ 7.79 (d, J = 7.9 Hz, 2H), 7.58 (d, J = 7.8 Hz, 1H), 7.29 (m, 2H), 7.19 (d, J = 8.0 Hz, 2H), 7.11 (t, J = 7.2 Hz, 1H), 7.04 (t, J = 7.5 Hz, 1H), 6.53 (d, J = 2.8 Hz, 1H), 5.47 (s, 2H). ¹³C NMR (100 MHz, CD₃OD): δ 170.59, 142.41, 136.24, 132.64, 128.93, 128.14, 127.57, 126.36, 121.13, 120.32, 118.99, 109.29, 101.14, 48.93. ESI-LRMS: [M+H]^+ = m/z 251.1. ESI-HRMS: calc. for C₁₆H₁₄N₂O: [M+H]^+ = m/z 251.1179, found: [M+H]^+ = m/z 251.1179.

**4-((1H-Indol-1-yl)methyl)-N-hydroxybenzamide (4.14).** The title compound was synthesized from methyl 4-((1H-indol-1-yl)methyl)benzoate 4.1 (0.840 g, 3.17 mmol) according to General Procedure B (prep. HPLC method 1) and isolated as a white solid (0.443 g, 53%). ¹H NMR (400 MHz, DMSO-d₆): δ 11.14 (S, 1H), 9.01 (br, 1H), 7.67 (d, J = 8.1 Hz, 2H), 7.56 (d, J = 7.77 Hz, 1H), 7.52 (d, J = 3.1 Hz, 1H), 7.42 (d, J = 8.1 Hz, 1H), 7.23 (d, J = 8.1 Hz, 2H), 7.09 (t, J = 7.2 Hz, 1H), 7.02 (t, J = 7.5 Hz, 1H), 6.50 (d, J = 3.0 Hz, 1H), 5.48 (s, 2H). ¹³C NMR (100 MHz, DMSO-d₆): δ 164.4, 141.9, 136.1, 132.3, 129.6, 128.7, 127.6, 127.3, 121.7, 120.9, 119.6, 110.5, 101.6, 49.2. ESI-HRMS: calc. for C₁₆H₁₄N₂O₂: [M+H]^+ = m/z 267.1128, found: [M+H]^+ = m/z 267.1137.

**N-Hydroxy-4-((2-methyl-1H-indol-1-yl)methyl)benzamide (4.15).** The title compound was synthesized from methyl 4-((2-methyl-1H-indol-1-yl)methyl)benzoate 4.2 (0.259 g, 0.937 mmol) according to General Procedure B (prep. HPLC method 1) and isolated as a light
brown solid (86 mg, 33%). $^1$H NMR (400 MHz, DMSO-$d_6$): δ 11.13 (s, 1H), 8.96 (br, 1H), 7.65 (d, $J = 8.2$ Hz, 2H), 7.46 (d, $J = 7.1$ Hz, 1H), 7.33 (d, $J = 7.5$ Hz, 1H), 7.00 (m, 4H), 6.31 (s, 1H), 5.45 (s, 2H), 2.36 (s, 3H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 166.0, 142.5, 136.6, 136.0, 128.9, 127.9, 127.0, 126.1, 120.6, 119.5, 119.4, 108.6, 100.5, 45.7, 12.3. ESI-MS: calc. for C$_{17}$H$_{16}$N$_2$O$_2$: [M+H]$^+$ = m/z 281.1285, found: [M+H]$^+$ = m/z 281.1283.

$N$-Hydroxy-4-((3-methyl-1H-indol-1-yl)methyl)benzamide (4.16). The title compound was synthesized from methyl 4-((3-methyl-1H-indol-1-yl)methyl)benzoate 4.3 (0.200 g, 0.716 mmol) according to General Procedure B (prep. HPLC method 1) and isolated as a white solid (0.108 g, 54%). $^1$H NMR (400 MHz, DMSO-$d_6$): δ 11.13 (s, 1H), 8.99 (s, 1H), 7.66 (d, $J = 8.4$ Hz, 2H), 7.50 (d, $J = 8.0$ Hz, 1H), 7.37 (d, $J = 8.0$ Hz, 1H), 7.23 (m, 3H), 7.06 (td, $J = 8.0$, 1.2 Hz, 1H), 7.03 (td, $J = 7.6$, 0.8 Hz, 1H), 5.39 (s, 2H), 2.27 (s, 3H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 166.48, 142.50, 136.57, 131.08, 128.99, 126.97, 126.49, 125.78, 121.15, 118.39, 118.35, 110.36, 109.09, 48.62, 8.25. ESI-MS: calc. for C$_{17}$H$_{16}$N$_2$O$_2$: [M+H]$^+$ = m/z 279.1139, found: [M+H]$^+$ = m/z 279.1144.

4-((2,3-Dimethyl-1H-indol-1-yl)methyl)-$N$-hydroxybenzamide (4.17). The title compound was synthesized from methyl 4-((2,3-dimethyl-1H-indol-1-yl)methyl)benzoate 4.4 (0.632 g, 2.15 mmol) according to General Procedure B (prep. HPLC method 1) and isolated as a white solid (0.487 g, 77%). $^1$H NMR (400 MHz, DMSO-$d_6$): δ 11.13 (s, 1H), 9.00 (br, 1H), 7.66 (d, $J = 8.2$ Hz, 2H), 7.45 (d, $J = 6.9$ Hz, 1H), 7.32 (d, $J = 7.3$ Hz, 1H), 7.01 (m, 4H), 5.43 (s, 2H), 2.27 (s, 3H), 2.22 (s, 3H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 164.4, 142.4, 136.4, 133.0, 132.1, 128.6, 127.6, 126.5, 120.9, 119.0, 118.1, 109.6, 106.5, 45.9, 10.3, 9.2. ESI-MS: calc. for C$_{18}$H$_{18}$N$_2$O$_2$: [M+H]$^+$ = m/z 295.1441, found: [M+H]$^+$ = m/z 295.1453.

4-((3-Benzyl-1H-indol-1-yl)methyl)-$N$-hydroxybenzamide (4.18). The title compound was synthesized from methyl 4-((3-benzyl-1H-indol-1-yl)methyl)benzoate 4.5 (0.200 g, 0.563
mmol) according to General Procedure B (prep. HPLC method 1) and isolated as a white solid (80 mg, 40%). $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 11.20 (br, 1H), 7.67 (d, $J = 8.2$ Hz, 2H), 7.45 (d, $J = 7.9$ Hz, 1H), 7.36 (d, $J = 8.2$ Hz, 1H), 7.27 (m, 6H), 7.15 (d, $J = 6.7$ Hz, 1H), 7.07 (t, $J = 7.9$ Hz, 1H), 6.96 (t, $J = 7.8$ Hz, 1H), 7.07 (t, $J = 7.9$ Hz, 1H), 5.40 (s, 2H), 4.05 (s, 2H). $^{13}$C NMR (100 MHz, CD$_3$OD): $\delta$ 164.48, 142.06, 141.91, 136.63, 132.18, 128.83, 128.68, 128.06, 127.57, 127.41, 127.29, 126.17, 121.83, 119.44, 119.19, 114.53, 110.45, 49.01, 31.30. ESI-HRMS: calc. for C$_{23}$H$_{20}$N$_2$O$_2$: [M+H]$^+$ = m/z 357.1598, found: [M+H]$^+$ = m/z 357.1597.

4-((9H-Purin-9-yl)methyl)-N-hydroxybenzamide (4.19). The title compound was synthesized from methyl 4-((9H-purin-9-yl)methyl)benzoate 4.6 (0.287 g, 1.07 mmol) according to General Procedure B (prep. HPLC method 2) and isolated as a white solid (288 mg, 45%). $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 11.18 (br, 1H), 9.20 (s, 1H), 8.95 (s, 1H), 8.78 (s, 1H), 7.71 (d, $J = 8.2$ Hz, 2H), 7.40 (d, $J = 8.2$ Hz, 2H), 5.58 (s, 2H), 3.73 (br, 1H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 164.2, 152.6, 151.5, 148.4, 147.5, 139.8, 134.0, 132.9, 128.0, 127.8, 46.5. ESI-HRMS: calc. for C$_{13}$H$_{11}$N$_5$O$_2$: [M+H]$^+$ = m/z 270.0986, found: [M+H]$^+$ = m/z 270.0992.

4-((7H-Purin-7-yl)methyl)-N-hydroxybenzamide (4.20). The title compound was synthesized from methyl 4-((7H-purin-7-yl)methyl)benzoate 4.7 (0.108 g, 0.40 mmol) according to General Procedure B (prep. HPLC method 2) and isolated as a white solid (50 mg, 46%). $^1$H NMR (400 MHz, DMSO-$d_6$): $\delta$ 11.21 (br, 1H), 9.16 (s, 1H), 9.00 (s, 1H), 8.95 (s, 1H), 7.73 (d, $J = 8.2$ Hz, 2H), 7.47 (d, $J = 8.2$ Hz, 2H), 6.75 (br, 1H), 5.68 (s, 2H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): $\delta$ 164.1, 160.7, 152.5, 150.3, 141.2, 139.3, 133.1, 128.2, 127.9, 125.3, 48.8. ESI-HRMS: calc. for C$_{13}$H$_{11}$N$_5$O$_2$: [M+H]$^+$ = m/z 270.0986, found: [M+H]$^+$ = m/z 270.0984.
**N-Hydroxy-4-{3-[2-(4-methyl-piperazin-1-yl)-ethyl]-indol-1-ylmethyl]-benzamide (4.21).**

The title compound was synthesized from 4-{3-[2-(4-Methyl-piperazin-1-yl)-ethyl]-indol-1-ylmethyl]-benzoic acid methyl ester 4.9 (0.412 g, 1.05 mmol) according to General Procedure B (prep. HPLC method 2) and isolated as a white solid (0.265 g, 64%). $^1$H NMR (400 MHz, DMSO-$d_6$): δ 7.67 (d, $J = 7.8$ Hz, 3H), 7.42 (m, 2H), 7.24 (d, $J = 7.9$ Hz, 2H), 7.12 (t, $J = 7.2$ Hz, 1H), 7.04 (t, $J = 7.4$ Hz, 1H), 5.43 (s, 2H), 3.81 (m, 4H), 3.53 (br, 4H), 3.40 (br, 2H), 2.84 (s, 3H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 163.9, 141.4, 136.1, 131.9, 127.3, 127.2, 127.1, 127.0, 121.8, 119.0, 118.9, 110.3, 55.6, 49.6, 48.7, 48.0, 42.2, 19.4. ESI-HRMS: calc. for C$_{23}$H$_{28}$N$_4$O$_2$: [M+H]$^+$ = m/z 393.2285, found: [M+H]$^+$ = m/z 393.2299.

**4-((3-(2-(Dimethylamino)ethyl)-1H-indol-1-yl)methyl)-N-hydroxybenzamide (4.22).** The title compound was synthesized from methyl 4-((3-(2-(dimethylamino)ethyl)-1H-indol-1-yl)methyl)benzoate 4.10 (0.412 g, 1.05 mmol) according to General Procedure B (prep. HPLC method 2) and isolated as a white solid (18 mg, 11%). $^1$H NMR (400 MHz, CD$_3$OD): δ 7.66 (m, 3H), 7.29 (m, 2H), 7.31 (d, $J = 8.0$ Hz, 2H), 7.13 (m, 2H), 5.41 (s, 2H), 3.48 (t, $J = 7.4$ Hz, 2H), 3.24 (t, $J = 8.3$ Hz, 2H), 2.97 (s, 6H). $^{13}$C NMR (100 MHz, CD$_3$OD): δ 166.33, 141.99, 136.78, 131.29, 127.48, 127.05, 126.74, 126.65, 121.88, 119.23, 118.09, 109.73, 109.73, 108.70, 57.65, 48.90, 42.12, 20.35. ESI-HRMS: calc. for C$_{20}$H$_{23}$N$_3$O$_2$: [M+H]$^+$ = m/z 338.1863, found: [M+H]$^+$ = m/z 338.1865.

**N-Hydroxy-4-((3-(2-(methylsulfonamido)ethyl)-1H-indol-1-yl)methyl)benzamide (4.23).** The title compound was synthesized from methyl 4-((3-(2-(methylsulfonamido)ethyl)-1H-indol-1-yl)methyl)benzoate 4.12 (155 mg, 0.401 mmol) according to General Procedure C (prep. HPLC method 2) and isolated as a beige solid (55 mg, 35%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 11.22 (br s, 1H), 10.82 (s, 1H), 7.87 (d, $J = 8.4$ Hz, 2H), 7.48 (d, $J = 8.4$ Hz,
2H), 7.31 (d, J = 8.0 Hz, 1H), 7.11 (d, J = 2.4 Hz, 1H), 7.05 (td, J = 7.6, 0.8 Hz, 1H), 6.94 (td, J = 7.6, 0.8 Hz, 1H), 4.48 (s, 2H), 3.35 (m, 2H), 2.97 (s, 3H), 2.84 (m, 2H). ^13^C NMR (100 MHz, DMSO-\textit{d}_6) δ 164.00, 140.73, 136.15, 131.99, 128.01, 127.09, 126.84, 122.98, 121.00, 118.32, 118.07, 111.42, 110.70, 50.53, 48.42, 38.02, 24.48.

ESI-LRMS: [M+H]^+ = m/z 388. ESI-HRMS: calc. for C\textsubscript{19}H\textsubscript{21}N\textsubscript{3}O\textsubscript{4}S: [M+H]^+ = m/z 388.1326, found: [M+H]^+ = m/z 388.1319.

**Methyl 4-((1H-pyrrol-1-yl)methyl)benzoate (4.24).** The title compound was prepared from 1H-pyrrole (0.150 g, 2.24 mmol) according to General Procedure A (substituting KO\textsuperscript{t}Bu for NaH) and purified using automated column chromatography method 1. The product was isolated as a clear viscous oil (0.432 g, 90%). ^1H NMR (400 MHz, CDCl\textsubscript{3}): δ 8.01 (d, J = 8.3 Hz, 2H), 7.17 (d, J = 8.3 Hz, 2H), 6.72 (t, J = 2.0 Hz, 2H), 6.24 (t, J = 2.1 Hz, 2H), 5.15 (s, 2H), 3.93 (s, 3H). ^13^C NMR (100 MHz, CDCl\textsubscript{3}): δ 166.7, 143.4, 130.0, 129.5, 126.7, 121.2, 108.9, 53.0, 52.1.

**Methyl 4-((1H-pyrazol-1-yl)methyl)benzoate (4.25).** The title compound was prepared from 1H-pyrazole (0.150 g, 2.20 mmol) according to General Procedure A (substituting KO\textsuperscript{t}Bu for NaH) and purified using automated column chromatography method 1. The product was isolated as a viscous yellow oil (0.387 g, 81%). ^1H NMR (400 MHz, CDCl\textsubscript{3}): δ 7.99 (d, J = 8.2 Hz, 2H), 7.56 (s, 1H), 7.41 (d, J = 2.0 Hz, 1H), 7.20 (d, J = 8.1 Hz, 2H), 6.30 (d, J = 1.9 Hz, 1H), 5.36 (s, 2H), 3.88 (s, 3H). ^13^C NMR (100 MHz, CDCl\textsubscript{3}): δ 166.2, 141.5, 139.5, 129.7, 129.4, 129.1, 126.9, 105.9, 55.0, 51.7. ESI-HRMS: calc. for C\textsubscript{12}H\textsubscript{12}N\textsubscript{2}O\textsubscript{2}: [M+H]^+ = m/z 217.0972, found: [M+H]^+ = m/z 217.0969.

**4-((1H-Pyrrol-1-yl)methyl)-N-hydroxybenzamide (4.26).** The title compound was synthesized from methyl 4-((1H-pyrrol-1-yl)methyl)benzoate 4.24 (0.400 g, 1.86 mmol) according to General Procedure B (prep. HPLC method 1) and isolated as a white solid (0.225 g, 56%). ^1H NMR (400 MHz, CD\textsubscript{3}OD): δ 7.70 (d, J = 8.3 Hz, 2H), 7.20 (d, J = 8.3 Hz,
2H), 6.73 (t, J = 2.0 Hz, 2H), 6.11 (t, J = 2.1 Hz, 2H), 5.17 (s, 2H). $^{13}$C NMR (100 MHz, CD$_3$OD): δ 166.4, 143.0, 131.2, 127.0, 126.6, 120.7, 108.0, 52.0. ESI-HRMS: calc. for C$_{12}$H$_{12}$N$_2$O$_2$: [M+H]$^+$ = m/z 217.0972, found: [M+H]$^+$ = m/z 217.0974.

4-((1H-Pyrazol-1-yl)methyl)-N-hydroxybenzamide (4.27). The title compound was synthesized from methyl 4-((1H-pyrazol-1-yl)methyl)benzoate 4.25 (0.387 g, 1.79 mmol) according to General Procedure B (prep. HPLC method 1) and isolated as a white solid (0.251 g, 65%). $^1$H NMR (400 MHz, CD$_3$OD): δ 7.74 (m, 3H), 7.55 (d, J = 1.5 Hz, 1H), 7.27 (d, J = 8.4 Hz, 2H), 6.37 (t, J = 2.1 Hz, 1H), 5.43 (s, 2H). $^{13}$C NMR (100 MHz, CD$_3$OD): δ 166.3, 140.9, 139.3, 131.7, 130.5, 127.1, 127.0, 105.7, 54.3. ESI-HRMS: calc. for C$_{11}$H$_{11}$N$_3$O$_2$: [M+H]$^+$ = m/z 218.0924, found: [M+H]$^+$ = m/z 218.0917.

Methyl 4-(pyridin-4-ylmethyl)benzoate (4.28). The title compound was prepared from pyridin-4-ylboronic acid (0.123 g, 1.00 mmol) according to General Procedure D and purified using automated column chromatography method 2 (20 mg, 9%). $^1$H NMR (400 MHz, CD$_3$OD): δ 8.59 (d, J = 6.6 Hz, 2H), 7.86 (d, J = 8.3 Hz, 2H), 7.78 (d, J = 6.6 Hz, 2H), 7.28 (d, J = 8.3 Hz, 2H), 4.26 (s, 2H), 3.74 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 166.7, 162.3, 142.4, 141.2, 129.9, 129.3, 129.1, 127.2, 51.3, 40.8. ESI-LRMS: [M+H]$^+$ = m/z 228.

Methyl 4-(4-(dimethylamino)benzyl)benzoate (4.29). The title compound was prepared from 4-(dimethylamino)phenylboronic acid (0.165 g, 1.00 mmol) according to General Procedure D and purified using automated column chromatography method 2. The product was isolated as an orange oil (0.211 g, 78%). $^1$H NMR (400 MHz, CD$_3$OD): δ 7.93 (d, J = 8.4 Hz, 2H), 7.59 (d, J = 8.7 Hz, 2H), 7.45 (d, J = 8.7 Hz, 2H), 7.33 (d, J = 8.4 Hz, 2H), 4.10 (s, 2H), 3.87 (s, 3H), 3.28 (s, 6H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 166.9, 146.0, 142.8, 141.3, 130.6, 129.5, 128.7, 128.1, 120.3, 51.2, 45.6, 40.5. ESI-LRMS: [M+H]$^+$ = m/z 270.
Methyl 4-(3-(dimethylamino)benzyl)benzoate (4.30). The title compound was prepared from 3-(dimethylamino)phenylboronic acid (0.165 g, 1.00 mmol) according to General Procedure D and purified using automated column chromatography method 2. The product was isolated as a clear oil (0.254 g, 94%). \(^1\)H NMR (400 MHz, CDCl\(_3\)): δ 7.99 (d, \(J = 8.1\) Hz, 2H), 7.30 (m, 6H), 4.08 (s, 2H), 3.91 (s, 3H), 3.15 (s, 6H). \(^13\)C NMR (100 MHz, CDCl\(_3\)): δ 166.9, 144.9, 144.5, 143.1, 130.6, 130.0, 128.9, 128.8, 128.6, 119.9, 117.5, 52.1, 45.6, 41.6. ESI-LRMS: [M+H]+ = m/z 270.

N-Hydroxy-4-(pyridin-4-ylmethyl)benzamide (4.31). The title compound was synthesized from methyl 4-(pyridin-4-ylmethyl)benzoate 4.28 (0.020 g, 0.09 mmol) according to General Procedure B (prep. HPLC method 2) and isolated as a white solid (5 mg, 25%). \(^1\)H NMR (400 MHz, CD\(_3\)OD): δ 8.74 (d, \(J = 6.4\) Hz, 2H), 7.93 (d, \(J = 6.3\) Hz, 2H), 7.78 (d, \(J = 8.2\) Hz, 2H), 7.43 (d, \(J = 8.1\) Hz, 2H), 4.41 (s, 2H). \(^13\)C NMR (100 MHz, CD\(_3\)OD): δ 166.1, 162.4, 141.4, 140.8, 131.3, 129.3, 127.5, 127.1, 40.7. ESI-HRMS: calc. for C\(_{13}\)H\(_{12}\)N\(_2\)O\(_2\): [M+H]+ = m/z 229.0972, found: [M+H]+ = m/z 229.0966.

4-(4-(Dimethylamino)benzyl)-N-hydroxybenzamide (4.32). The title compound was synthesized from methyl 4-(4-(dimethylamino)benzyl)benzoate 4.29 (0.211 g, 0.78 mmol) according to General Procedure B (prep. HPLC method 2) and isolated as a white solid (0.140 g, 66%). \(^1\)H NMR (400 MHz, CD\(_3\)OD): δ 7.69 (d, \(J = 8.2\) Hz, 2H), 7.53 (d, \(J = 8.6\) Hz, 2H), 7.42 (d, \(J = 8.56\) Hz, 2H), 7.31 (d, \(J = 8.1\) Hz, 2H), 4.09 (s, 2H), 3.25 (s, 6H). \(^13\)C NMR (100 MHz, CD\(_3\)OD): δ 166.6, 144.5, 142.3, 141.7, 130.5, 130.2, 128.8, 127.1, 119.8, 45.3, 40.4. ESI-HRMS: calc. for C\(_{16}\)H\(_{18}\)N\(_2\)O\(_2\): [M+H]+ = m/z 271.1441, found: [M+H]+ = m/z 271.1448.

4-(3-(Dimethylamino)benzyl)-N-hydroxybenzamide (4.33). The title compound was synthesized from methyl 4-(3-(dimethylamino)benzyl)benzoate 4.30 (0.254 g, 0.94 mmol) according to General Procedure B (prep. HPLC method 2) and isolated as a white solid
(0.117 g, 46%). $^1$H NMR (400 MHz, CDCl$_3$): δ 7.70 (d, $J = 8.2$ Hz, 2H), 7.50 (m, 3H), 7.35 (m, 3H), 4.12 (s, 2H), 3.27 (s, 6H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 166.6, 144.3, 143.7, 143.6, 130.4, 130.2, 129.7, 128.8, 127.1, 120.2, 117.5, 45.4, 40.7. ESI-HRMS: calc. for C$_{16}$H$_{18}$N$_2$O$_2$: [M+H]$^+$ = m/z 271.1450, found: [M+H]$^+$ = m/z 271.1450.

**Methyl 4-(pyrrolidin-1-ylmethyl)benzoate (4.34).** Pyrrolidine (0.12 mL, 1.52 mmol), methyl 4-formylbenzoate (0.250 g, 1.52 mmol), NaBH(OAc)$_3$ (0.52 g, 2.4 mmol) and 5 Å molecular sieves were dissolved in 1,2-dichloroethane (5 mL) under Ar atmosphere and stirred for 24 h at RT. Then, the reaction was diluted with 2N NaOH (30 mL) and extracted with EtOAc (3 x 20 mL). The combined organic fractions were washed with brine (15 mL), dried over Na$_2$SO$_4$, filtered and concentrated in vacuo. The crude product was purified via automated column chromatography (50-100% EtOAc/hexane, 25 g cartridge) to yield the title compound as a clear oil (0.166 g, 50%). $^1$H NMR (400 MHz, CDCl$_3$): δ 8.00 (d, $J = 8.0$ Hz, 2H), 7.43 (d, $J = 8.0$ Hz, 2H), 3.92 (s, 3H), 3.69 (s, 2H), 2.54 (br, 4H), 1.81 (t, $J = 3.2$ Hz, 4H).

**(S)-Methyl 4-((2-(methoxymethyl)pyrrolidin-1-yl)methyl)benzoate (4.35).** The title compound was synthesized from (S)-2-(methoxymethyl)pyrrolidine (0.175 g, 1.52 mmol) according to a procedure similar to that used for compound 4.34. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.99 (d, $J = 8.4$ Hz, 2H), 7.42 (d, $J = 8.0$ Hz, 2H), 4.17 (d, $J = 13.6$ Hz, 1H), 3.92 (s, 3H), 3.45 (m, 2 H), 3.35 (m, 4H), 2.92 (m, 1H), 2.74 (m, 1H), 2.21 (m, 1H), 1.95 (m, 1H), 1.69 (m, 3H).

**(R)-Methyl 4-((2-(methoxymethyl)pyrrolidin-1-yl)methyl)benzoate (4.36).** The title compound was synthesized from (R)-2-(methoxymethyl)pyrrolidine (0.175 g, 1.52 mmol) according to a procedure similar to that used for compound 4.34. $^1$H NMR (400 MHz, CDCl$_3$): δ 7.98 (d, $J = 8.0$ Hz, 2H), 7.41 (d, $J = 8.0$ Hz, 2H), 4.16 (d, $J = 13.6$ Hz, 1H), 3.91
(s, 3H), 3.42 (m, 2H), 3.33 (m, 4H), 2.92 (m, 1H), 2.73 (m, 1H), 2.19 (m, 1H), 1.93 (m, 1H), 1.70 (m, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$): δ 166.7, 144.7, 129.1, 128.4, 128.3, 76.0, 62.8, 58.9, 58.7, 54.2, 51.6, 28.0, 22.4. ESI-LRMS: [M+H]$^+$ = m/z 264.

$\textbf{N-Hydroxy-4-(pyrrolidin-1-ylmethyl)benzamide-TFA (4.37).}$ The title compound was synthesized from methyl 4-(pyrrolidin-1-ylmethyl)benzoate 4.34 (0.120 g, 0.55 mmol) according to General Procedure B (prep. HPLC method 2) and isolated as the trifluoroacetic acid salt (38 mg, 21%). $^1$H NMR (400 MHz, DMSO-$d_6$): δ 11.33 (s, 1H), 10.69 (br, 1H), 9.15 (br, 1H), 7.81 (d, $J = 8.0$ Hz, 2H), 7.58 (d, $J = 8.0$ Hz, 2H), 4.39 (s, 2H), 3.22 (br, 4H), 1.94 (br, 4H). $^{13}$C NMR (100 MHz, CD$_3$OD): δ 165.61, 134.17, 133.62, 130.29, 127.62, 57.21, 53.60, 22.37. ESI-HRMS: calc. for C$_{12}$H$_{16}$N$_2$O$_2$: [M+H]$^+$ = m/z 221.1285, found: [M+H]$^+$ = m/z 221.1286.

$\textbf{(S)-N-Hydroxy-4-((2-(methoxymethyl)pyrrolidin-1-yl)methyl)benzamide-TFA (4.38).}$ The title compound was synthesized from (S)-methyl 4-((2-(methoxymethyl)pyrrolidin-1-yl)methyl)benzoate 4.35 (0.120 g, 0.46 mmol) according to General Procedure B (prep. HPLC method 2) and isolated as the trifluoroacetic acid salt (23 mg, 13%). $^1$H NMR (400 MHz, CD$_3$OD): δ 7.87 (d, $J = 8.0$ Hz, 2H), 7.64 (d, $J = 8.0$ Hz, 2H), 4.67 (d, $J = 12.8$ Hz, 1H), 4.35 (d, $J = 12.8$ Hz, 1H), 3.85 (br, 1 H), 3.60 (d, $J = 4.8$ Hz, 2H), 3.42 (br, 4H), 3.32 (m, 1 H), 2.30 (m, 1H), 2.15 (m, 1H), 1.95 (m, 2H). $^{13}$C NMR (100 MHz, CD$_3$OD): δ 165.55, 133.74, 130.81, 127.55, 69.86, 67.00, 58.08, 57.75, 54.48, 26.03, 21.81. ESI-HRMS: calc. for C$_{14}$H$_{20}$N$_2$O$_3$: [M+H]$^+$ = m/z 265.1547, found: [M+H]$^+$ = m/z 265.1550.

$\textbf{(R)-N-Hydroxy-4-((2-(methoxymethyl)pyrrolidin-1-yl)methyl)benzamide-TFA (4.39).}$ The title compound was synthesized from (R)-methyl 4-((2-(methoxymethyl)pyrrolidin-1-yl)methyl)benzoate 4.36 (0.120 g, 0.46 mmol) according to General Procedure B (prep. HPLC method 2) and isolated as the trifluoroacetic acid salt (21 mg, 12%). $^1$H NMR (400
MHz, DMSO-$d_6$): δ 11.23 (s, 1H), 9.87 (br, 1H), 9.02 (br, 1H), 7.71 (d, $J = 8.4$ Hz, 2H), 7.49 (d, $J = 8.0$ Hz, 2H), 4.45 (d, $J = 12.8$ Hz, 1H), 4.20 (m, 1H), 3.62 (br, 1H), 3.47 (m, 1H), 3.39 (m, 1H), 3.17 (s, 4H), 3.08 (br, 1H), 2.05 (m, 1H), 1.87 (m, 1H), 1.73 (m, 1H), 1.63 (m, 1H).

$^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 163.85, 134.05, 131.35, 127.68, 70.70, 66.37, 58.91, 57.36, 54.37, 26.59, 22.16. ESI-HRMS: calc. for C$_{14}$H$_{20}$N$_2$O$_3$: [M+H]$^+$ = m/z 265.1547, found: [M+H]$^+$ = m/z 265.1551.

Methyl 4-((diethylamino)methyl)benzoate (4.40). The title compound was prepared from diethylamine (0.350 g, 4.79 mmol) according to General Procedure A (substituting KO'Bu for NaH). The pH was adjusted to 10 with 2N NaOH prior to extraction with EtOAc. The product was isolated as a yellow oil and did not require further purification (0.790 g, 75%). $^1$H NMR (400 MHz, CD$_3$OD): δ 8.14 (d, $J = 8.2$ Hz, 2H), 7.66 (d, $J = 8.2$ Hz, 2H), 4.44 (s, 2H), 3.95 (s, 3H), 3.25 (m, 4H), 1.37 (t, $J = 7.3$ Hz, 6H). $^{13}$C NMR (100 MHz, DMSO-$d_6$): δ 166.2, 135.9, 131.8, 130.9, 130.0, 54.8, 52.8, 46.6, 8.8. ESI-HRMS: calc. for C$_{13}$H$_{19}$NO$_2$: [M+H]$^+$ = m/z 222.1489, found: [M+H]$^+$ = m/z 222.1485.

Methyl 4-((diisopropylamino)methyl)benzoate (4.41). The title compound was prepared from diisopropylamine (0.500 g, 4.94 mmol) according to General Procedure A (substituting KO'Bu for NaH). The pH was adjusted to 10 with 2N NaOH prior to extraction with EtOAc. The product was isolated as a yellow oil and did not require further purification (1.10 g, 89%). $^1$H NMR (400 MHz, CD$_3$OD): δ 8.10 (d, $J = 8.4$ Hz, 2H), 7.71 (d, $J = 8.4$ Hz, 2H), 4.51 (s, 2H), 3.93 (s, 3H), 3.85 (m, 2H), 1.46 (dd, $J = 6.1$ Hz, $J = 12.5$ Hz, 12H). $^{13}$C NMR (100 MHz, CD$_3$OD): δ 165.9, 135.9, 130.6, 130.1, 129.4, 54.8, 51.1, 49.4, 17.2, 16.3. ESI-HRMS: calc. for C$_{15}$H$_{23}$NO$_2$: [M+H]$^+$ = m/z 250.1802, found: [M+H]$^+$ = m/z 250.1800.

Methyl 4-((diphenylamino)methyl)benzoate (4.42). NaH (0.220 g, 5.54 mmol) was dissolved in anhydrous DMF (2 mL) under argon and cooled to 0 °C. To it was added
diphenylamine (0.0.750 g, 4.43 mmol) dissolved in anhydrous DMF (2 mL). The reaction was stirred for 15 min at 0 °C followed by the addition of methyl 4-(bromomethyl)benzoate (1.02 g, 4.43 mmol) in anhydrous DMF (2 mL). The reaction was stirred for 2 h at 60 °C and then quenched by the addition of H₂O (20 mL). The organic products were extracted with EtOAc (3 x 30 mL), washed with H₂O (2 x 30 mL), brine (15 mL), dried with Na₂SO₄, filtered and concentrated in vacuo. The title compound was purified using automated column chromatography method 1 (0.746 g, 53%). 

1H NMR (400 MHz, CDCl₃): δ 8.00 (d, J = 8.2 Hz, 2H), 7.45 (d, J = 7.7 Hz, 2H), 7.27 (m, 4H), 7.07 (d, J = 7.9 Hz, 4H), 6.98 (t, J = 7.3 Hz, 2H), 5.06 (s, 2H), 3.92 (s, 3H).

13C NMR (100 MHz, CDCl₃): δ 166.9, 147.8, 144.8, 130.0, 129.4, 128.9, 126.5, 121.7, 120.7, 56.3, 52.0. ESI-LRMS: [M+H]+ = m/z 318.

4-((Diethylamino)methyl)-N-hydroxybenzamide (4.43). The title compound was synthesized from methyl 4-((diethylamino)methyl)benzoate 4.40 (0.518 g, 2.34 mmol) according to General Procedure B (prep. HPLC method 2) and isolated as a viscous, yellow oil (0.448 g, 56%). 

1H NMR (400 MHz, CD₃OD): δ 7.72 (d, J = 8.2 Hz, 2H), 7.42 (d, J = 8.1 Hz, 2H), 3.65 (s, 2H), 2.55 (q, J = 7.2 Hz, 4H), 1.08 (t, J = 5.0 Hz, 6H). 

13C NMR (100 MHz, CD₃OD): δ 166.3, 139.5, 131.9, 129.7, 127.0, 56.1, 46.4, 9.3. ESI-HRMS: calc. for C₁₂H₁₈N₂O₂: [M+H]+ = m/z 223.1441, found: [M+H]+ = m/z 223.1441.

4-((Diisopropylamino)methyl)-N-hydroxybenzamide (4.44). The title compound was synthesized from methyl 4-((diisopropylamino)methyl)benzoate 4.41 (0.750 g, 3.0 mmol) according to General Procedure B (prep. HPLC method 2) and isolated as a viscous, light-yellow oil (0.324 g, 42%). 

1H NMR (400 MHz, CD₃OD): δ 7.84 (d, J = 8.2 Hz, 2H), 7.67 (d, J = 8.2 Hz, 2H), 4.48 (s, 2H), 3.84 (m, 2H), 1.47 (br, 12H). 

13C NMR (100 MHz, CD₃OD): δ 165.6, 134.7, 133.2, 130.5, 127.5, 55.1, 49.7, 17.5, 16.8. ESI-HRMS: calc. for C₁₄H₂₂N₂O₂: [M+H]+ = m/z 251.1754, found: [M+H]+ = m/z 251.1744.
4-((Diphenylamino)methyl)-N-hydroxybenzamide (4.45). The title compound was synthesized from methyl 4-((diphenylamino)methyl)benzoate 4.42 (0.200 g, 0.33 mmol) according to General Procedure B (prep. HPLC method 2) and isolated as a white solid (27 mg, 13%). $^1$H NMR (400 MHz, CD$_3$OD): δ 7.69 (d, $J = 8.0$ Hz, 2H), 7.47 (d, $J = 8.0$ Hz, 2H), 7.23 (t, $J = 7.6$ Hz, 4H), 7.05 (d, $J = 8.0$ Hz, 4H), 6.93 (t, $J = 7.5$ Hz, 2H), 5.07 (s, 2H). $^{13}$C NMR (100 MHz, DMSO-d$_6$): δ 164.5, 147.8, 143.0, 131.8, 129.8, 127.5, 126.9, 121.7, 120.7, 55.5. ESI-HRMS: calc. for C$_{20}$H$_{18}$N$_2$O$_2$: [M+H]$^+$ = m/z 319.1441, found: [M+H]$^+$ = m/z 319.1447.

4.5.2 HDAC Inhibition

HDAC inhibition assays were performed by the Reaction Biology Corporation (Malvern, PA) using human, full length recombinant HDAC1 and 6 isolated from a baculovirus expression system in Sf9 cells. An acetylated, fluorogenic peptide derived from residues 379-382 of p53 (RHKK$_{Ac}$) was used as the substrate in the assays. The reaction buffer contained 50 mM Tris-HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl$_2$, 1 mg/mL BSA and a final concentration of 1% DMSO. The enzyme was delivered into wells of the reaction plate and compounds were delivered in 100% DMSO into the enzyme mixture by acoustic technology (Echo550; nanoliter range). The plates were spun down and pre-incubated for 5-10 min. The substrate was then delivered to all reaction wells in order to initiate the reaction and the reaction was incubated for 2 h at 30 °C. After incubation, developer and Trichostatin A were added to quench the reaction and generate fluorescence. Then, kinetic measurements were taken for 1.5 h in 15 min intervals to ensure that development was complete. Endpoint readings were taken for analysis after the development reached a plateau. Dose-response curves were generated and the IC$_{50}$ for each compound was extrapolated from the generated plots (10-dose IC$_{50}$ curves were
generated using a 3-fold serial dilution pattern starting with concentrations of 30 μM). All IC\textsubscript{50} determinations were done in duplicate and the values expressed in this chapter are the average of both trials ± the standard deviation.

4.5.3 Neuroprotection Studies

For the neuroprotection studies, cells were rinsed with warm PBS and then placed in minimum essential medium (Invitrogen) containing 5.5 g/liter glucose, 10% fetal calf serum, 2 mM L-glutamine, and 100 μM cysteine. Oxidative stress was induced by the addition of the cysteine homolog, homocysteic acid (HCA; 5 mM), to the media. HCA was diluted from 100-fold concentrated solutions that were adjusted to pH 7.5. In combination with HCA, the novel HDAC inhibitors (10 μM) were added. Viability was assessed after 48 hours by calcein-acetoxyethyl ester (AM)/ethidium homodimer-1 staining (live/dead assay) (Molecular Probes, Eugene, OR) using fluorescence microscopy and the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. This work was done in the laboratory of Dr. Brett Langley at The Burke Medical Research Institute (Ithaca, NY).
CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

The area of epigenetics and, more specifically, the development of drugs that modulate the epigenome have been rapidly gaining attention in the scientific literature.\(^1\), \(^2\), \(^105\) The ability to alter gene expression without specifically engineering or tampering with the genetic sequence has resulted in the identification of numerous attractive new drug targets.\(^57\), \(^106\) Inhibitors designed to modulate post-translational acetylation and methylation have been actively pursued in the literature by both academic institutions and pharmaceutical companies alike.\(^39\), \(^51\), \(^107\)-\(^109\) Indeed, the approval of SAHA and romidepsin by the FDA for the treatment of CTCL has legitimized the search for small molecules designed to alter the acetylation status of various protein targets. In addition to these two compounds, there are numerous other HDACi currently undergoing clinical trials for the treatment of various types of cancers, chronic obstructive pulmonary disease, HIV, poly- and thrombocythemia, sickle cell disease, graft-versus-host disease and a few other indications both as a mono- and combination therapy (clinicaltrials.gov). However, of the more than three hundred ongoing clinical trials involving HADC inhibitors, the vast majority of them are designed for cancer and most of the compounds involved in these studies are non-selective or class I selective HDACi.

In order to successfully apply HDACi to areas outside of oncology, it will be necessary to eliminate the toxicity that is often associated with inhibition of the class I isoforms.\(^15\), \(^18\), \(^39\) This would require the development of HDAC isoform or class selective inhibitors.\(^10\), \(^22\) These types of compounds would not only be useful as potential therapeutics, they would also provide invaluable research tools for the elucidation of the specific protein targets, substrates and functions of each HDAC isoform. This information
would help identify the relevant therapeutic target(s) for each disease of interest as well as identify potential downstream targets that could be useful for formulating new combination therapies.

The work outlined in this dissertation was specifically designed to address the aforementioned issues of toxicity and HDAC isoform selectivity. The work described in Chapter 2 resulted in the identification of moderately HDAC6 selective compounds and further validated the use of a mercaptoacetamide zinc binding group in place of the more traditional hydroxamic acid. We found that compounds containing α-methyl substituted mercaptoacetamide zinc binding groups exhibited similar HDAC activity profiles compared to their hydroxamic acid counterparts. In addition, stereochemistry of the methyl group in this position was significant such that the (R)-enantiomer was active whereas the (S)-enantiomer was not. Incorporation of a methyl group in this position also significantly inhibited disulfide formation which, if left unchecked, could confound the HDAC isoform inhibition assays. We also demonstrated the improved toxicity profile of these compounds by comparing the viability of neuronal cells treated with a mercaptoacetamide to that of neuronal cells treated with the respective hydroxamic acid.(104)

In addition to the investigation of zinc binding group modifications for HDAC6 selectivity, we also wanted to improve on the previously disclosed tubastatin A scaffold. At the time of its publication, tubastatin A was the best-in-class HDAC6 selective inhibitor based on its potency, selectivity for HDAC6 and drug-like properties in comparison to other reported HDAC6 selective compounds.(39) This prompted us to attempt to elucidate the exact structural features necessary for potent, selective HDAC6 inhibition. Beginning with modifications to the tricyclic cap, we systematically determined that substitutions to the 2-position of both the β- and γ-tetrahydrocarbolines was optimal. Furthermore, we
demonstrated that a multitude of functional groups could be incorporated in this position, often providing improvements in terms of the activity and selectivity profiles of these compounds. In doing such, we were able to enhance the potency of these inhibitors to subnanomolar levels and improve the selectivity for HDAC6 versus HDAC1 to more than 7,000 fold.\((101)\)

After having significantly improved on the HDAC inhibitory properties of these compounds, we wanted to be certain that their pharmacological attributes remained intact. Therefore, we evaluated the ability of these inhibitors to improve the suppressive effects of regulatory T cells. We demonstrated that these compounds prevented the proliferation of effector T cells in vitro with efficacy similar to that of tubastatin A. We expanded our evaluation to murine models and found that these new derivatives were at least as effective at suppressing effector T cell proliferation in vivo. These results further support the use of selective HDAC6 inhibitors for the treatment of autoimmune disorders and warrant additional investigation.\((101)\)

Lastly, in order to further evaluate the structural features necessary for selective HDAC6 inhibition and, at the same time, improve ligand efficiency, we investigated a number of aryl cap groups as well as aliphatic and acyclic cap groups. In regard to HDAC activity, we found that the indole based derivatives exhibited both improved potency at and selectivity for HDAC6 compared to tubastatin A. In addition, substitutions to the indole cap were well tolerated with alkyl substitutions at the 2- and/or 3-position being optimal. The \(p\)-dimethylaminophenyl derivative was very similar to tubastatin A in regard to both potency and selectivity; however, the ligand efficiency was much improved. Evaluation of these compounds for their neuroprotective properties revealed that membrane permeability, in combination with HDAC6 activity, is a significant factor in these assays. Compounds with a
cLogP < 1 were not neuroprotective, regardless of their HDAC6 activity, while compounds that possessed significant HDAC6 activity and cLogP values > 1 were able to protect neurons from oxidative stress in this model. Unfortunately, not all of the compounds described in Chapter 4 have been evaluated for their neuroprotective properties and none of these new compounds have been tested in the immunosuppressive models described in Chapter 3. These studies are currently underway and should provide us with additional insight as to what physiochemical properties and structural features are optimal for treating different diseases.

We are just beginning to understand how the epigenetic regulatory machinery is able to maintain proper levels of gene expression and how aberrant regulation of these epigenetic processes factors into various diseases. HDACs are major drug targets that have emerged in the field of epigenetics and, if nothing else, the development of novel and selective HDAC inhibitors will provide invaluable research tools to help facilitate our understanding of these epigenetic processes. The identification of non-histone substrates for HDAC proteins has also led to a significant increase in our understanding of regulatory machinery inside the cell. Development of selective HDAC inhibitors will help determine which non-histone substrates are associated with certain diseases and, in addition to potentially serving as therapeutics, may help identify other downstream drug targets for future exploitation. The work described in this dissertation serves to identify certain structural features necessary for selective HDAC6 inhibition as well as identify potential sites of modification for additional fine tuning of these small molecules. These compounds also significantly expand the molecular toolkit available to other researchers for mapping the epigenome. The potential for HDAC inhibitors to be therapeutically relevant to areas outside of oncology exists; however, it can only be realized through the identification and understanding of the isoform specific functions of histone deacetylase proteins.
CITED LITERATURE


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