Design, Synthesis, and Biological Characterization of Largazole Analogues

by

Bumki Kim

Department of Chemistry
Duke University

Date: ______________________
Approved:

___________________________
Jiyong Hong, Supervisor

___________________________
Steven W. Baldwin

___________________________
Dewey G. McCafferty

___________________________
Michael C. Fitzgerald

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
Chemistry in the Graduate School
of Duke University

2016
ABSTRACT

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Abstract

Histone deacetylases (HDACs) have been shown to play key roles in tumorigenesis, and have been validated as effective enzyme target for cancer treatment. Largazole, a marine natural product isolated from the cyanobacterium *Symploca*, is an extremely potent HDAC inhibitor that has been shown to possess high differential cytotoxicity towards cancer cells along with excellent HDAC class-selectivity. However, improvements can be made in the isoform-selectivity and pharmacokinetic properties of largazole.

In attempts to make these improvements and furnish a more efficient biochemical probe as well as a potential therapeutic, several largazole analogues have been designed, synthesized, and tested for their biological activity. Three different types of analogues were prepared. First, different chemical functionalities were introduced at the C2 position to probe the class I-selectivity profile of largazole. Additionally, docking studies led to the design of a potential HDAC8-selective analogue. Secondly, the thiol moiety in largazole was replaced with a wide variety of other zinc-binding group in order to probe the effect of Zn$^{2+}$ affinity on HDAC inhibition. Lastly, three disulfide analogues of largazole were prepared in order to utilize a different prodrug strategy to modulate the pharmacokinetic properties of largazole.

Through these analogues it was shown that C2 position can be modified significantly without a major loss in activity while also eliciting minimal changes in isoform-selectivity. While the Zn$^{2+}$-binding group plays a major role in HDAC inhibition, it was also shown that the thiol can be replaced by other functionalities while still retaining inhibitory activity. Lastly, the use of a disulfide prodrug strategy was shown to affect pharmacokinetic properties resulting in varying functional responses *in vitro* and *in vivo*. 
Largazole is already an impressive HDAC inhibitor that shows incredible promise. However, in order to further develop this natural product into an anti-cancer therapeutic as well as a chemical probe, improvements in the areas of pharmacokinetics as well as isoform-selectivity are required. Through these studies we plan on building upon existing structure–activity relationships to further our understanding of largazole’s mechanism of inhibition so that we may improve these properties and ultimately develop largazole into an efficient HDAC inhibitor that may be used as an anti-cancer therapeutic as well as a chemical probe for the studying of biochemical systems.
Dedication

To my parents for their continuous love and support
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<th>Full Form</th>
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<tbody>
<tr>
<td>aCNI</td>
<td>averaged NonCovalent Interaction</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>α-KG</td>
<td>α-ketoglutarate</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>Amnaa</td>
<td>(Z)-(3R)-amino-(2S)-methyl-5-nonedioic acid, 9-amide</td>
</tr>
<tr>
<td>Amnda</td>
<td>(Z)-(3R)-amino-(2S)-methyl-5-nonediodic acid</td>
</tr>
<tr>
<td>Å</td>
<td>angstrom</td>
</tr>
<tr>
<td>Aoda</td>
<td>(2S)-amino-8-oxodecanoic acid</td>
</tr>
<tr>
<td>Aoe</td>
<td>(2S)-amino-8-oxo-9,10-epoxydecanoic acid</td>
</tr>
<tr>
<td>BER</td>
<td>base-excision repair</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenic protein</td>
</tr>
<tr>
<td>Bn</td>
<td>benzyl</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-Butyloxycarbonyl</td>
</tr>
<tr>
<td>brs</td>
<td>broad singlet</td>
</tr>
<tr>
<td>BRSM</td>
<td>based upon recovered starting material</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>CMT</td>
<td>Charcot-Marie-Tooth disorder</td>
</tr>
<tr>
<td>CNV</td>
<td>corneal neovascularization</td>
</tr>
<tr>
<td>CoA</td>
<td>co-enzyme A</td>
</tr>
<tr>
<td>d</td>
<td>doublet</td>
</tr>
<tr>
<td>DABCO</td>
<td>1,4-diazabicyclo[2.2.2]octane</td>
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DCC  
dicyclohexylcarbodiimide

DDQ  
2,3-dichloro-5,6-dicyano-1,4-benzoquinone

DIAD  
diisopropyl azodicarboxylate

DIC  
N,N-Diisopropylcarbodiimide

DMAP  
4-(N,N-dimethylamino)-pyridine

DMF  
N,N-dimethylformamide

DNA  
deoxyribonucleic acid

DNMT  
DNA methyltransferase

EDCI  
1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride

FDA  
food and drug administration

FKBP  
FK506 binding-partner

Fmoc  
fluorenylmethyloxycarbonyl group

GCV  
ganciclovir

GI\textsubscript{50}  
the concentration required for 50% growth inhibition

GSH  
glutathione

HATU  
O-(7-azabenzotriazo-1-yl)-1,1,3,3-tetramethyluronium

HAT  
histone acetyl transferase

HDAC  
histone deacetylase

HDLP  
histone deacetylase like protein

HOAt  
1-hydroxy-7-azabenzotriazole

HOBt  
Benzotriazol-1-ol

HRMS  
high-resolution mass spectrometry

HSC  
hepatic stellate cells
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>IC50</td>
<td>inhibitory concentration 50%</td>
</tr>
<tr>
<td>IL-2</td>
<td>interleukin 2</td>
</tr>
<tr>
<td>kD</td>
<td>kilodaltons</td>
</tr>
<tr>
<td>m</td>
<td>multiplet</td>
</tr>
<tr>
<td>MBCP</td>
<td>macroporous biphasic calcium phosphates</td>
</tr>
<tr>
<td>MBD</td>
<td>methyl-CpG-binding domain</td>
</tr>
<tr>
<td>MECP</td>
<td>methyl CpG binding protein</td>
</tr>
<tr>
<td>MEL</td>
<td>murine erythroleukemia</td>
</tr>
<tr>
<td>MTM</td>
<td>methylthiomethyl</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NCI</td>
<td>national cancer institute</td>
</tr>
<tr>
<td>NHC</td>
<td>N-heterocyclic carbene</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>OPN</td>
<td>osteopontin</td>
</tr>
<tr>
<td>PPTS</td>
<td>Pyridinium p-toluenesulfonate</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>Runx2</td>
<td>runt-related transcription factor 2</td>
</tr>
<tr>
<td>s</td>
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<tr>
<td>SAHA</td>
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</tr>
<tr>
<td>SAM</td>
<td>S-adenosylmethionine</td>
</tr>
<tr>
<td>SUMO</td>
<td>small ubiquitin-like modifier</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TBAF</td>
<td>tetra-$n$-butylammonium fluoride</td>
</tr>
<tr>
<td>TBAI</td>
<td>tetra-$n$-butylammonium iodide</td>
</tr>
<tr>
<td>TBS/TBDMS</td>
<td>$tert$-butyldimethylsilyl</td>
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<tr>
<td>TCE</td>
<td>trichloroethyl</td>
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<tr>
<td>TDG</td>
<td>thymine-DNA-glycosylase</td>
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<tr>
<td>TEPM</td>
<td>thioglycollate-elicited peritoneal macrophages</td>
</tr>
<tr>
<td>TET</td>
<td>ten-eleven translocation enzyme</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
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<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TIPS</td>
<td>triisopropylsilyl</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
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<tr>
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<td>toll-like receptor</td>
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<td>trimethylsilyl</td>
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<td>TSA</td>
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<td>TSE</td>
<td>trimethylsilylethyl</td>
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<td>Trt</td>
<td>trhphenylmethyl</td>
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<tr>
<td>ZBG</td>
<td>zinc-binding group</td>
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<tr>
<td>$^{13}$C NMR</td>
<td>carbon 13 nuclear magnetic resonance</td>
</tr>
<tr>
<td>$\delta$</td>
<td>chemical shift (ppm)</td>
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</table>
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First and foremost, I would like to thank my advisor, Professor Jiyong Hong, for his intellectual guidance and support as well as his constant drive for excellence. I feel very fortunate to have worked for someone that constantly pushed me to improve. I would also like to thank my committee members, Professor Steven W. Baldwin, Professor Dewey G. McCafferty, and Professor Michael C. Fitzgerald for all the time they have spent giving me invaluable advice regarding my research.

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1. Introduction

1.1 The role of natural products in drug discovery and chemical biology

For thousands of years, nature has provided us with countless remedies for the treatment of a variety of ailments. Today we understand that these remedies owe their efficacy to secondary metabolites contained within these natural sources.\(^1\)\(^-\)\(^3\) These compounds, known as natural products, have been and continue to be an irreplaceable source of drug discovery as well as probes for the investigation of biomolecular function.\(^4\) The importance of natural products in these fields cannot be overexpressed and we must continue to explore this excellent resource of potential drugs and chemical biology probes. The significance of natural products is particularly highlighted in the field of drug discovery, as the majority of therapeutics, including some of the most significant and widely used drugs, has been inspired by natural products collected from a variety of natural sources.\(^1\)\(^-\)\(^3\)

One famous example is the isolation of penicillin from the fungus *Penicillium notatum* by Scottish scientist Sir Alexander Fleming (figure 1).\(^3\) As one of the earliest discoveries of an antibiotic agent, this discovery revolutionized the field of medicine. Despite the fact that many strains of bacteria have developed resistance to penicillin today, it continues to be used in clinical settings.\(^5\)\(^,\)\(^6\) Anti-cancer agent paclitaxel (*Taxol\(^®\)*), is a more recent drug, which was isolated by Wall/Wani and co-workers in 1967 from the bark of *Taxus brevifolia*, more commonly known as the Pacific Yew tree (figure 1).\(^7\) Commercially distributed by Bristol-Myers Squibb, paclitaxel is used for the treatment of various cancers, including ovarian, breast, lung, and pancreatic cancer.\(^3\)\(^,\)\(^8\)
Recently, marine life has presented itself as a novel and interesting source of drug discovery. While the majority of natural products isolated to date are of terrestrial origins, marine sources have been relatively underexplored.\textsuperscript{3,9} Despite the fact that the ocean covers 70% of the earth’s surface, until recently, inaccessibility has prevented extensive examination of marine sources for natural products. Advancement in SCUBA technology, submersibles, and remote operated vehicles (ROVs) have greatly aided in the isolation of marine natural product and the subsequent development of therapeutics.\textsuperscript{3,9} Eribulin mesylate (Halaven\textsuperscript{®}) is one result of these efforts.\textsuperscript{3,9,10} In 1986, Hirata and Umeda isolated halichondrin B from the marine sponge \textit{Halichondria okadai}.\textsuperscript{11} The excellent \textit{in vivo} anti-cancer activity of this natural product eventually led to FDA approval of the structurally simplified compound, eribulin mesylate (Halaven\textsuperscript{®}), in 2010 for the treatment of metastatic breast cancer (figure 2).\textsuperscript{3,9}
The utility of natural products are not only limited to drug discovery. Chemical biology has seen many impactful advances from the use of natural products as probes for the elucidation of cellular pathways and protein functions. One such example is the use of bacterial natural products FK506 and rapamycin to elucidate cell signaling transduction pathways that play key roles in the immune response (figure 3). FK506 was isolated from the bacterium *Streptomyces tsukubaensis* Japanese soil samples in 1987 and showed excellent *in vivo* and *in vitro* immunosuppressive activity. It was shown that FK506 binds to FK506-binding protein (FKBP) which consequently causes a cascade of inhibition of several of biological markers of T-cell activation, including calcineurin, interleukin 2 (IL-2) production, and IL-2 receptor expression.
Rapamycin, a structurally similar natural product isolated from the bacterium *Streptomyces hygroscopicus*, also binds to FKBP.\textsuperscript{14,15} However, instead of inhibiting calcinuerin like the FK506-FKBP complex, it targets the mechanistic target of rapamycin (mTOR) in order to inhibit the T-cell response to IL-2 without affecting IL-2 production.\textsuperscript{16} Studies involving FK506 and rapamycin and their roles in the immune response demonstrated how two structurally similar natural products can elicit two discrete cell signaling pathways. While there are countless natural products that have been used as chemical biology probes for the elucidation of cellular pathways, this example particularly emphasizes the significance of the role of natural products.

![Figure 3: Structure of FK506 and rapamycin.](image)

The impact natural products have had on the pharmaceutical industry has also recently been quantified. In 2012, Newman and Cragg compiled a list of all new approved drugs from 1981 to 2010 and categorized them by sources.\textsuperscript{17} These categories were broken down into biological (B), natural products (N), natural products botanical (NB), derived from a natural products with semisynthetic modifications (ND), totally synthetic drugs (S), made by total synthesis (S*), natural product mimics (NM), and vaccines (V). The data shows that 51\% of all
new approved drugs and 62% of all anti-cancer drugs from this time period are natural products or inspired by natural products.

Figure 4: Approved drugs by source (1981-2010). Data from ref.17
Despite the success natural products have demonstrated as therapeutics, there has been a slow decline in interest in natural products from the pharmaceutical industry. The emergence of combinatorial chemistry and high-throughput screening methods has somewhat diminished the perceived importance and shifted the focus towards these new methods. However, these combinatorial libraries have proven ineffective in some cases due to limited structural diversity. Additionally, advancements in natural product screening technology and supply methods have facilitated many aspects of testing natural products as potential therapeutics. The impact natural products have had on drug discovery and chemical biology is undeniable. It is imperative, however, that we continue to examine natural products in order to drive innovation and progress in these fields. Given the structural diversity, target affinity, and specificity natural products exhibit, there is little doubt that they will continue to be an essential resource of novel therapeutics and chemical biology probes used to elucidate unknown molecular pathways.

1.2 Epigenetics and histone modifications

Epigenetics is the study of gene expression changes not caused by variations in the DNA sequence, but rather by enzyme-mediated chemical modifications. DNA is tightly compacted in the nucleus in a complex known as chromatin, which is comprised of many nucleosomes. Each nucleosome has about 146 base pairs of DNA wrapped around an octamer of four histone core proteins (H2A, H2B, H3, and H4). By chemically modifying either the DNA or the histones, the chromatin architecture can be perturbed, and consequently, gene expression can be altered. These chemical modifications are controlled by three classes enzymes, categorized as ‘writers’, ‘erasers’, and ‘readers’. ‘Writers’ are responsible for the incorporation of epigenetic marks into DNA or histones, while ‘erasers’ remove them. This dynamic equilibrium of incorporating and removing
epigenetic markers from DNA and histones forms an epigenetic code, which is recognized by enzymes called ‘readers’ that contain recognition domains for specific epigenetic marks, and subsequently affect gene expression.\textsuperscript{22} Deregulation of epigenetic mechanisms has been linked to a variety of disorders including cancer, immunodeficiency, and learning disabilities.\textsuperscript{20,22} There are two main categories of epigenetic control: DNA methylation and histone modifications (figure 5).

**Figure 5**: DNA packaging in chromatin and epigenetic mechanisms DNA is wrapped around histone octamers (2 copies of H2A, H2B, H3, and H4) to form a nucleosome. Histones can be modified by ‘writer’ proteins (i.e. histone acetyltransferase, histone methyltransferase) to incorporate an epigenetic mark. These marks can be removed by ‘eraser’ proteins (i.e. histone deacetylase, lysine demethylase) and recognized by ‘reader’ domains (i.e. bromodomain, chromodomain). DNA base pairs can be methylated by similar mechanisms. Figure reprinted with permission from ref.\textsuperscript{22}

DNA methylation is mediated by DNA methyltransferase enzymes (DNMT1, DNMT3a, and DNMT3b) and utilizes S-adenosylmethionine (SAM) as a cofactor in order to the methylate the 5’ position of cytosines.\textsuperscript{24} This process is also reversible either through passive DNA replication, or through the active oxidation by ten-eleven translocation (TET) enzymes followed by thymine-DNA-glycosylase (TDG)-mediated base excision repair (figure 6).\textsuperscript{25} 5-
methylcytosine (5mC) can be iteratively oxidized by cellular oxygen and α-ketoglutarate (α-KG) as a cofactor to 5-hydroxycytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). These intermediates can act as substrates for TDG-mediated base excision repair (BER), which can reinstate an unmethylated cytosine (C). The role of TDG in the process of cytosine excision repair is not fully understood.

Figure 6: Methylation and Demethylation of DNA. Cytosines are methylated at the 5-position by DNMT in the presence of SAM to form 5mC. In order to demethylate 5mC, TET utilizes oxygen and α-KG as a co-factor to iteratively oxidize 5mC to 5hmC, 5fC, and 5caC. These intermediates can then undergo TDG-mediated base excision repair to reinstate natural cytosine. Figure adapted from ref.25
The additional methyl group lies in the major groove of the DNA double helix and interferes with transcription factor binding. Additionally, methylated DNA-binding proteins, such as methyl CpG binding proteins (MECPs) and proteins containing methyl-CpG-binding domains (MBDs), bind to methylated cytosines and block transcription factor access to repress gene transcription. The methylation of cytosines specifically in CpG dinucleotides has been shown to
be able to directly silence tumor-suppressing genes. It has also been shown that during carcinogenesis, DNA methylation is highly deregulated, resulting in hypomethylation of CpG dinucleotides in distal regulatory regions and repetitive elements, as well as hypermethylation of CpG islands. In almost every type of cancer an overall deficiency in methylated cytosine has been reported and a unique CpG methylation profile has been observed.\textsuperscript{27}

There are several histone modifications that occur post-translationally including histone phosphorylation, ubiquitination, biotinylation, SUMOylation, crotonylation, methylation, arginine citrullination, ADP-ribosylation, histone tail clipping, and proline isomerization. While all of these post-translational modifications play important role as epigenetic regulators, some of them are more common than others (figure 7) with histone methylation and acetylation being the most abundant and extensively investigated epigenetic marks.\textsuperscript{26,28,29}

\textbf{Figure 8:} Potential methylation states of lysine and arginine on histone tails. HKMTs methylate lysine residues. Type I PRMTs asymmetrically di-methylate arginine residues, and type II PRMTs symmetrically di-methylate arginine residues. Both types are able to mono-methylate arginine residues. Jumonji demethylases and LSDs remove methyl marks. Methylation states are recognized by several recognition domains including PHD, chromo, WD\textsubscript{40}, Tudor, double/tandem Tudor, MBT, Ankyrin Repeats, zf-CW, and PWWP.\textsuperscript{26}
Histone methylation occurs on lysine and arginine residues on the N-terminal histone tails by lysine methyl transferases (HKMTs) or arginine methyl transferases (PRMTs). HKMTs can mono-, di-, or trimethylate lysine residues. Arginine residues are also subject to mono-, or dimethylation. The latter can occur asymmetrically by type I PRMTs, or symmetrically by type II PRMTs (figure 8). Both families of methyl transferases use SAM as a co-factor in order to incorporate the methyl groups into their substrates. These methyl groups can be removed by α-ketoglutarate-dependent Jumonji demethylases or flavin-dependent lysine-specific histone demethylases (LSD, KDM), which possess extremely high substrate specificity.

Methylation states do not directly affect transcriptional activity, but rather recruit reader enzymes which determine the functional outcome. These reader enzymes are highly specific for different methylation patterns and include conserved recognition domains including PHD, chromo, WD40, Tudor, double/tandem Tudor, MBT, Ankyrin Repeats, zf-CW and PWWP domains.

Histone acetylation has been studied extensively and garnered considerable interest due to their implications in early stages of tumorigenesis and cancer progression. The acetylation state of histones is controlled by histone acetyl transferases (HATs) and histone deacetylases (HDACs). Unlike histone methylation, which recruits specific transcriptional regulators to unique methylation sites, histone acetylation regulates gene transcription in a more direct manner. HATs are composed of three families of enzymes: MYST, GNAT, and p300/CBP. These enzymes transfer acetyl groups to the ε-amino group of lysine residues on histone tails by utilizing acetyl-CoA as a cofactor. The uncharged lysine residue has reduced interactions with the negatively charged DNA backbone, resulting in euchromatin states, and a consequent increases in gene transcription. HDACs remove the acetyl groups, restore the positive charge of the amino group, forming heterochromatin states, which increases interaction with the DNA backbone, and represses gene expression (figure 9). Much like the methylation states of histones, the acetylation
states can also be recognized by recognition domains such as bromo and PHD domains. Improper regulation of histone acetylation states have been observed in a variety of different diseases, especially in various types of cancers. The overexpression of HDAC activity and consequent hypoacetylation of histones have been of particular interest.

Figure 9: Epigenetic mechanism of histone acetylation. HATs utilize acetyl-CoA as a co-factor to introduce acetyl groups to the ε-amino group of lysine residues. The decreased electrostatic interaction between the negatively charged DNA backbone and now neutral lysine residues allows more access for transcriptional machinery, thereby increasing transcription. Removal of acetyl groups by HDACs leave charged amino groups which increase electrostatic interactions with negatively charged DNA backbone, limit access by transcriptional machinery, and repress transcription. Figure adapted from ref. 31

As studies of these enzymes continue, it becomes more apparent that transcriptional control is a convoluted mechanism that involves complexes of several epigenetic regulators through recognition of uniquely marked sites and recruitment of co-enzymes including other
epigenetic regulators.\textsuperscript{32} Transcriptional control is also a very delicately balanced equilibrium and disruption of this balance can lead to physiological issues in the form of various disease states. The implications of these enzymes have made them attractive drug targets for these diseases, with histone deacetylases garnering much interest as a therapeutic target in the last few decades.\textsuperscript{22,27}

1.3 Histone deacetylase

In 1964, Allfrey and co-workers reported that the acetylation state of histones had a direct effect on RNA synthesis in cells. They reported that while unmodified histones effectively inhibited RNA polymerase activity in calf thymus cells and \textit{E. Coli}, acetylation of the histones decreased this effect significantly.\textsuperscript{33} Shortly thereafter, in 1969, Fujimoto and co-workers found that enzymatic action deacetylated histones in calf thymus extracts. Radiolabeled acetyl histones were subjected to calf thymus extract, which resulted in rapid release of \textsuperscript{14}C-acetate, indicating that a deacetylation event occurred.\textsuperscript{34} Additionally, when calf thymus extract was omitted, no \textsuperscript{14}C-acetate was observed. Based on this data, Fujimoto and co-workers hypothesized that there was a specific enzyme responsible for the deacetylation of histones. It wasn’t until 1996 that the existence of this enzyme was validated by Schreiber and co-workers\textsuperscript{35,36} (page 25).

The experiment utilized natural product trapoxin B, which was known to cause cell cycle arrest in the G\textsubscript{1} and G\textsubscript{2} phase. It was hypothesized that trapoxin B achieved cell cycle arrest by irreversibly inhibiting HDACs. This was validated as trichostatin A (TSA), a known HDAC inhibitor, competed with tritium-labeled trapoxin B for protein binding in crude bovine thymus extracts, indicating that the two compounds shared a molecular target. Schreiber and co-workers hypothesized that if trapoxin B caused cell cycle arrest by inhibiting HDAC, the binding of trapoxin B to the enzyme in question should co-purify. A synthetic analogue of trapoxin B called
K-trap was prepared and immobilized on an affinity matrix. By using this affinity matrix, Schreiber and co-workers isolated a novel protein. This protein was named HD1 (now known as HDAC1) and marked the first time a mammalian HDAC was cloned and characterized.\cite{35,36} Since then a total of 18 HDAC isoforms have been discovered.\cite{37-39}

![HDAC isoforms classification](classification.png)

**Figure 10:** Classification of HDACs. Figure reprinted with permission from ref.\cite{39}

The HDAC isoforms have been divided into 4 different classes of HDACs based on their sequence homology to different yeast transcriptional regulators. Along with HDAC1, HDACs 2, 3, and 8 are part of class I HDACs and share sequence homology with RPD3. This class of HDACs is generally smaller than the other isoforms. Additionally, they are almost exclusively found in the nucleus with the exception of HDAC3, which is found in the cytoplasm as well. Class II HDACs include HDACs 4, 5, 6, 7, 9, and 10 and are related to HDA1.\cite{37-39} HDACs 6 and
10 are further categorized as class IIb HDACs, due to their unique secondary catalytic domain, even though only HDAC6 possesses a functional secondary catalytic domain.\textsuperscript{40,41} HDAC11, originally classified as a class I HDAC, is a class IV HDAC due to low sequence homology with the other isoforms (figure 10).\textsuperscript{42} The remaining HDACs are class III HDACs, also known as sirtuins for their sequence homology with the yeast transcriptional regulator Sir2.\textsuperscript{37-39}

\textbf{Figure 11}: Mechanism of deacetylation of HDACs. Zn\textsubscript{2+} is held in place in the active site by a catalytic triad (D258, D168, H170) and two water molecules. Lysine enters the active site and displaces one water molecule and coordinates to the Zn\textsuperscript{2+} through the carbonyl oxygen of the acetyl group. The other water molecule attacks the carbonyl carbon and the resulting alkoxide is stabilized by Y297. Subsequent protonation by H132, cleaves the C-N bond, completing deacetylation. Figure adapted from ref.\textsuperscript{43}

Unlike the 11 “canonical” HDACs, which are zinc-dependent due to the Zn\textsuperscript{2+} in their active sites, sirtuins are NAD\textsuperscript{+}-dependent and are not inhibited by classical HDAC inhibitors. This results in a fundamentally different mechanism of action from classical HDACs. In the
latter, the highly conserved active site is shaped like an 11 Å-long channel made up of hydrophobic and aromatic residues. The Zn$^{2+}$ in the bottom of this channel is held in place by a catalytic triad consisting of a histidine residue and two aspartic acid residues. Acetylated lysines are inserted into this channel, where the acetyl group coordinates to the Zn$^{2+}$ to catalyze the deacetylation process (figure 11).

Despite the high sequence homology that some HDAC isoforms share, possibly suggesting redundant function, knockout studies in mice have shown that each isoform has a unique physiological role. For example, deletion of HDACs 1, 2, and 3 has led to lethality in embryonic development due to proliferation defects. Deletion of HDAC4 leads to inhibition of skeletogenesis in the form of ectopic ossification of endochondral cartilage, preventing rib cage expansion and consequent inability to breathe. While HDAC5 or HDAC9 knockout mice are viable, deletion of both isoforms leads to abnormal cardiovascular growth and maturation. HDAC6 knockout mice have shown dramatic increases in tubulin acetylation levels, and HDAC7 knockout mice suffer from endothelial cell integrity during embryogenesis.

Similarly, individual HDAC isoforms have also shown implications in different disease states. For example, HDAC2 levels are highly elevated in the hippocampal area of and the prefrontal cortex of mice that display Alzheimer’s disease symptoms. Additionally, HDACs 1 and 3 were not elevated in these mice, and upregulation of HDAC2 was not seen in control mice. Also RNA-knockout of HDAC2 showed improvement in synaptic plasticity and reversal of neurodegeneration associated memory impairments. HDAC7 activates Edn1 promoter gene in thioglycollate-elicited peritoneal macrophages (TEPM) which leads to Toll-like receptor (TLR)-induced response prevalent in inflammatory diseases. HDAC6 has recently been implicated in a variety of diseases such as colitis, allograft rejection, charcot-marie-tooth disease (CMT), hypertension, and stroke.
1.4 Natural product HDAC inhibitors

The significant implications of HDACs in several disease states as well as normal physiological functions has led to the discovery and development of several HDAC inhibitors from a myriad of sources. Additionally, the validation of HDAC inhibition as anti-cancer treatment has furthered interest in developing effective HDAC inhibitors. In this regard, natural products have played a vital role and provided a variety of HDAC inhibitors that have shown promising biological activities for potential therapeutics. The structural diversity, target affinity, and specificity have also facilitated the examination of the HDAC molecular mechanism in great detail. The contributions of natural products in HDAC research have been extremely impactful. Structurally, natural product HDAC inhibitors can be broken down into, linear, cyclic tetrapeptide, and cyclic depsipeptide HDAC inhibitors.31,51,52

1.4.1 Linear HDAC inhibitors

Over the years, several different linear natural product HDAC inhibitors have been isolated and characterized. Trichostatin A (TSA), isolated from the bacterium Streptomyces hygroscopicus in 1979, was the first of this class of compounds to be discovered.53,54 4 years after its isolation, Krebs/Fleming and co-workers reported the first racemic synthesis of TSA.55 Aldehyde 1 was subjected to Mukaiyama aldol addition conditions, followed by a Wittig reaction to produce intermediate 4. Subsequent DDQ oxidation and coupling with hydroxylamine fashioned (±)-TSA in 22% overall yield (scheme 1). While several enantioselective syntheses of TSA have since been established, this marked the first synthesis of a natural product HDAC inhibitor.
Originally explored as an antifungal agent, it wasn’t until 1990 that Yoshida and co-workers demonstrated that TSA induces \textit{in vitro} hyperacetylation of histones in a variety of mammalian cancer cell lines.\textsuperscript{53,54} Later, it was also shown that TSA inhibits cell proliferation \textit{in vivo} in rat mammary carcinoma models and induces cell differentiation in Friend leukemia cells.\textsuperscript{56,57} It was also interesting to find that synthetic \textit{ent}-TSA did not show HDAC inhibitory activity. TSA was also co-crystalized in the active site of the HDAC-like protein (HDLP) which is a bacterial HDAC homologue from \textit{A. aeolicus}.\textsuperscript{43} This crystal structure showed that TSA non-covalently binds to the active site of HDAC by inserting the terminal hydroxamic acid group into the active site channel, where it can chelate the Zn\textsuperscript{2+} in a bidentate fashion, thereby blocking the active site from its natural substrate (figure 12). This crystal structure has aided in the design of novel HDAC inhibitors as well as the elucidation of mechanism of action of other HDAC inhibitors. It has also helped define a structural requirement for efficient HDAC inhibitors. As shown in figure 12, HDAC inhibitors consist of a cap group, a linker region, and a Zn\textsuperscript{2+}-binding
group (ZBG) “warhead”. The cap group occupies the rim region of the HDAC active site, the linker region is inserted into the channel composed of hydrophobic and aromatic residues, while the ZBG chelates to the Zn\(^{2+}\) in the bottom of the active site.\(^{43}\) Today, TSA still remains as one of the more potent HDAC inhibitors and benchmark for HDAC inhibitory activity, despite it being a pan-HDAC inhibitor with limited isoform-selectivity.

![Figure 12: Space-filling representation of TSA in A. aeolicus HDAC homologue active site and structural features of HDAC inhibitors. Figure reprinted with permission from ref.\(^{43}\)](image)

Suberanilohydroxamic acid (SAHA, vorinostat, Zolinza\(^{®}\)) is an HDAC inhibitor that is structurally similar to TSA. Even though SAHA is not a natural product, it is worth mentioning because of its excellent potency and wide application as a benchmark in HDAC inhibition research. SAHA was discovered in a small molecule screen for murine erythroleukemia (MEL) cell differentiation inducers.\(^{58,59}\) Much like TSA, SAHA has an aromatic cap group and a hydroxamic acid ZBG that is inserted into the HDAC active site in order to prevent the natural substrate from entering. However, instead of the conjugated diene linker that TSA possesses, SAHA has a completely saturated linker (figure 13).\(^{43}\) SAHA is also a pan-HDAC inhibitor with
limited isoform-selectivity, but demonstrates excellent in vivo efficacy against CWR22 prostate cancer mouse models with limited toxicity.\textsuperscript{60,61}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure13.png}
\caption{Crystal structure of A. aeolicus HDAC homologue with SAHA and structure of SAHA. Figure reprinted with permission from ref.\textsuperscript{43}}
\end{figure}

Psammaplin A is a marine natural product isolated from the sponge \textit{Psammaplysilla} sp. and structurally is a monobromotyrosine derived oxime containing cystamine. It has demonstrated potent cytotoxicity against a variety of human cancers including colon, lung, and ovarian carcinomas while also inhibiting angiogenesis and bacterial proliferation.\textsuperscript{62-64} Additionally, psammaplin A inhibits H3 deacetylation, without affecting tubulin acetylation levels, which indicates that it’s a class I-specific HDAC inhibitor.\textsuperscript{65} It has been hypothesized that psammaplin A undergoes glutathione (GSH) mediated disulfide bond reduction to release two thiols which can act as the ZBG (scheme 2). Psammaplin A is the only linear natural product HDAC inhibitor that contains a thiol in the form of a disulfide bond as its ZBG.
Scheme 2: Structure of psammaplin A and disulfide bond reduction to form active thiol.\textsuperscript{65}

Depudecin is an irreversible HDAC inhibitor that was isolated from the fungus *Alternaria brassicicola* RF-328.\textsuperscript{66} Structurally it consists of six asymmetric centers and a bis-\textit{trans}-epoxide moiety, which is unprecedented in HDAC inhibitors to this day. It is hypothesized that depudecin covalently binds to nucleophilic residues in the HDAC active site through its epoxide moiety.\textsuperscript{67} Depudecin has shown the ability to revert ras/src transformed fibroblast cells to their normal flat phenotype.\textsuperscript{67} Additionally, depudecin is a potent angiogenesis inhibitor \textit{in vitro} and \textit{in vivo}.\textsuperscript{68} The first total synthesis of depudecin was reported by Schreiber and co-workers (scheme 3).\textsuperscript{69}
Scheme 3: Total synthesis of depudecin by Schreiber and co-workers.69
Methylation of a d-threose derivative 6 followed by TBS deprotection and Swern oxidation resulted in aldehyde 8. Subsequent coupling with 2-trimethylsilylthiazole afforded the anti-alcohol, which was subjected to an oxidation-reduction sequence to epimerize the hydroxyl group configuration. After reductive thiazole cleavage, and Cu (II)-assisted hydrolysis to form the aldehyde, intermediate 12 was condensed with ketophosphate 13 and asymmetrically reduced in order to, upon TBS deprotection, afford diol 15. Treatment of 15 with 2,2,2-trichloroethyl chloroformate, followed by treatment with anhydrous methanolic hydrochloric acid led to the formation of bis-cyclic carbonate 17, which underwent bis-MTM protection and basic hydrolysis to result in tetraol 19. Conversion of 19 to a diacetoxy dichloride 20 followed by base-mediated epoxide formation furnished the bis-trans epoxide moiety. Lastly, mercuric chloride-mediated MTM cleavage completed the synthesis of depudecin.69

Lastly, santacruzamate A was isolated from a marine cyanobacterium that morphologically resembles the genus Symploca.70 Structurally it contains a carbamate ZBG that is unique and has not been seen in any other natural product HDAC inhibitors (figure 14). Santacruzamate A showed differential cytotoxicity for HCT-116 colon cancer cells and Hut-78 cutaneous T-cell lymphoma cells over normal human dermal fibroblast cells. This differential cytotoxicity is explained by the class I-selectivity of santacruzamate A.70

![Santacruzamate A](image)

**Figure 14:** Structure of santacruzamate A.
1.4.2 Cyclic tetrapeptide HDAC inhibitors

Cyclic tetrapeptide HDAC inhibitors possess a much larger cap group than linear HDAC inhibitors, giving it more opportunity to interact with the rim of the active site. Structurally they consist of 4 amino acids of which one contains the ZBG. HC toxin, Cyl-1, and Cyl-2 were among the first cyclic tetrapeptide HDAC inhibitors that were isolated (figure 15). HC toxin was isolated from the fungus *Helminthosporium carbonum*, which has been known to cause corn leaf spot disease.\(^{71}\) It consists of D-proline, D-alanine, L-alanine, and (2S)-amino-8-oxo-9,10-epoxydecanoic acid (Aoe), which acts as the ZBG.\(^{72}\) The Aoe group is isosteric with an acetylated lysine residue and thought to bind irreversibly to the HDAC active site, much like depudecin (page 21). While the stereochemistry of the epoxide in the Aoe group has not been determined, it has been shown that removal of the epoxide abolishes the HDAC inhibitory activity of HC toxin.\(^{73}\) Cyl-1 and Cyl-2 were isolated from the fungus *Cylindrocladium scoparium* and are structurally similar to HC toxin, containing the same Aoe group.\(^{55,74,75}\) All three compounds are able to inhibit HDAC activity in a variety of different organisms, with Cyl-2 showing preference for HDAC1 over HDACs 4 and 6.\(^{76,77}\)

![HC toxin, Cyl-1, and Cyl-2](image)

**Figure 15:** Structure of HC toxin, Cyl-1, and Cyl-2.

Another group of structurally related compounds are chlamydocin and trapoxins A and B (figure 16), which also contain Aoe as their ZBG. Chlamydocin was isolated from the fungus...
**Diheterospora chlamydosporia** and showed excellent potency against cell proliferation in various human cancer cell lines, while also inducing histone H3 and H4 acetylation.\(^78,79\) In A2780 ovarian cancer cells, chlamydocin has demonstrated effective HDAC inhibition and induction of p21\(^{cip1/waf1}\)-mediated apoptosis. Additionally, it was observed that chlamydocin inhibits HDAC1 (IC\(_{50}\) = 0.15 nM) much more efficiently than HDAC6 (IC\(_{50}\) = 1,100 nM).\(^{79}\) Trapoxins A and B, isolated from fungal fermentation broth of *Helicoma ambiens* RF-1023, were initially investigated for their antibiotic activity.\(^{80}\) However, the ability to induce flattening in *sis* oncogene-transformed NIH3T3 cells, much like TSA and depudecin (page 17), prompted an investigation of their HDAC inhibitory activity. As expected, trapoxins A and B efficiently inhibited HDACs *in vitro*.\(^{81}\) They also demonstrated class I and class IIa-selectivity.\(^{77}\)

![Chlamydocin and Trapoxins A and B](image)

**Figure 16:** Structure of chlamydocin and trapoxins A and B.

The most significant contribution of these natural products is the use of trapoxin B in the isolation of the first mammalian HDAC.\(^{35,36}\) As previously mentioned, a synthetic analogue of trapoxin B called K-trap was prepared and immobilized on an affinity matrix (figure 17). The design of K-trap was based on the fact that chlamydocin’s lack of a phenylalanine residue, which is present in trapoxins A and B, did not show a significant decrease in potency. Therefore, this site of the molecule was chosen to be modified to be immobilized as an affinity matrix. Incubation of the K-trap affinity matrix with bovine thymus lysates resulted in the isolation of six
major proteins between 45 and 50 kD. Further large scale purification led to the resolution of two bands of 46 kD and 50 kD. The interaction of the K-trap affinity matrix and these proteins also seemed to be specific as co-incubation of trapoxin B or TSA led to competition for these proteins, indicating one common target. Upon sequencing, it was found that the 46 kD protein showed homology with yeast transcriptional regulator RPD3 and was responsible for the deacetylation of histones. This protein was named HD1 (now known as HDAC1) and marked the first time a mammalian HDAC was cloned and characterized.

**Figure 17:** Structure of trapoxin B, Chlamydocin, and K-trap affinity matrix K-trap affinity matrix was used to isolate HDAC1. Incubation in bovine thymus extracts resulted in isolation of 6 proteins between 45–50 kD. Large scale purification led to the isolation of a 46 kD protein that showed sequence homology with yeast transcriptional regulator RPD3 (HD1, HDAC1). Figure reprinted with permission from ref. 36

Apicidins are a large family of fungal natural products that showed potent anti-malarial activity, presumably through the inhibition of malarial Apicomplexan parasite HDACs. 82 Anti-malarial activity had not been observed from HDAC inhibitors before the discovery of the
apicidin family. Apicidin and apicidin A were isolated from cultures of the *Fusarium pallidoroseum* fungus.\textsuperscript{60} Based on this discovery further targeted biological screening of fermentation extracts resulted in the isolation of apicidins B, C, D1, D2, and D3.\textsuperscript{83} Structurally apicidins contain either \(N\)-methoxy-\(L\)-triptophan or \(L\)-triptophan, as well as either \(D\)-proline or \(D\)-pipecolinic acid. The ZBG is (2S)-amino-8-oxodecanoic acid (Aoda), or a derivative thereof (figure 18). Apicidins were the first cyclic tetrapeptide HDAC inhibitors that did not contain Aoe group as their ZBG. All members of the apicidin family have demonstrated potent HDAC inhibition in the malarial Apicomplexan parasite, as well as the *Eimeria tenella* parasite.\textsuperscript{82,83} Additionally apicidin has demonstrated the ability to induce dose-dependent H4 acetylation in HeLa cells as well as the ability to revert HeLa cell morphology to normal cell morphology.\textsuperscript{84} It is also able to inhibit cell proliferation in a variety of human cancer cell lines through induction of cyclin-dependent kinase (CDK) inhibitor p21\textsuperscript{cip1/waf1}. Apicidin has shown impressive for HDACs 2, 3 and 8 over other HDAC isoforms.\textsuperscript{60}

![Figure 18: Structure of apicidin family.](image-url)
Fermentation broths of the fungus *Acremonium* sp. led to the isolation of FR235222.\cite{85-87}

During structure–activity relationship studies of this natural product, AS1387392 (LGP1) was synthesized, but later isolated as a natural product from the same source.\cite{88,89} Both natural products contain a unique (2S)-amino-8-oxo-(9R)-hydroxydecanoic acid (Ahoda) as the ZBG (figure 19). While hydroxyl groups are known to be weak ZBGs, the stereochemistry of the hydroxyl group has shown to play a significant role as analogues with the opposite configurations were not able to inhibit HDAC activity.\cite{88} Both compounds showed potent inhibition of murine lymphocyte proliferation, histone H4 deacetylation, and human HDAC inhibition.\cite{85,88,89}

![Figure 19: Structure of FR235222 and AS1387392.](image)

The last group of cyclic tetrapeptide HDAC inhibitors is the azumamide family. These marine natural products are structurally unique as they are completely composed of D-amino acids and contain one β-amino acid, making the macrocyclic ring one atom larger. The ZBG comes from the β-amino acid, which is either (Z)-(3R)-amino-(2S)-methyl-5-nonenedioic acid, 9-amide (Amnaa) or (Z)-(3R)-amino-(2S)-methyl-5-nonedioic acid (Amnda) (figure 20). Along with the total synthesis of all 5 azumamide compounds, Olsen and co-workers also reported the HDAC inhibitory activity of these compounds against a full panel of recombinant human HDACs (table 1).\cite{90} This showed that the compounds with carboxylic acid ZBG were much more potent than amide ZBG and that azumamides showed a preference towards HDACs 1, 2, 3, 10, and 11 over other HDAC isoforms. Because carboxylic acids bind to Zn$^{2+}$ tighter than amides, the
increase in potency has been attributed to the increase in Zn\(^{2+}\) affinity. This was further validated by Ganesan and co-workers as they changed the ZBG to an even stronger hydroxamic acid, which resulted in a significant increase in potency.\(^9\)

![Structure of azumamide family](image)

**Figure 20:** Structure of azumamide family.

**Table 1:** HDAC inhibition of azumamides A–E (IC\(_{50}\), nM). Data from ref. \(^9\)

<table>
<thead>
<tr>
<th></th>
<th>HDAC1</th>
<th>HDAC2</th>
<th>HDAC3</th>
<th>HDAC8</th>
<th>HDAC6</th>
<th>HDAC10</th>
<th>HDAC11</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Azumamide A</strong> (R(^1) = H, R(^2) = NH(_2), R(^3) = i-Pr)</td>
<td>&gt;5000</td>
<td>&gt;5000</td>
<td>3200</td>
<td>&gt;5000</td>
<td>inactive(^b)</td>
<td>inactive(^b)</td>
<td>&gt;5000</td>
</tr>
<tr>
<td><strong>Azumamide B</strong> (R(^1) = OH, R(^2) = NH(_2), R(^3) = i-Pr)</td>
<td>5000</td>
<td>3000</td>
<td>3000</td>
<td>inactive(^b)</td>
<td>inactive(^b)</td>
<td>inactive(^b)</td>
<td>&gt;5000</td>
</tr>
<tr>
<td><strong>Azumamide C</strong> (R(^1) = OH, R(^2) = OH, R(^3) = i-Pr)</td>
<td>32 ± 1</td>
<td>40 ± 20</td>
<td>14 ± 1</td>
<td>&gt;5000</td>
<td>2000</td>
<td>10 ± 4</td>
<td>35 ± 3</td>
</tr>
<tr>
<td><strong>Azumamide D</strong> (R(^1) = H, R(^2) = NH(_2), R(^3) = CH(_3))</td>
<td>&gt;5000</td>
<td>&gt;5000</td>
<td>3700</td>
<td>inactive(^b)</td>
<td>inactive(^b)</td>
<td>inactive(^b)</td>
<td>&gt;5000</td>
</tr>
<tr>
<td><strong>Azumamide E</strong> (R(^1) = H, R(^2) = OH, R(^3) = i-Pr)</td>
<td>67 ± 7</td>
<td>50 ± 30</td>
<td>25 ± 1</td>
<td>4400</td>
<td>&gt;5000</td>
<td>20 ± 12</td>
<td>60 ± 16</td>
</tr>
</tbody>
</table>

\(^a\)Class IIa HDAC inhibition data was omitted due to the inactivity. \(^b\) <50% inhibition at 50 \(\mu\)M.

### 1.4.3 Cyclic depsipeptide HDAC inhibitors

Cyclic depsipeptide HDAC inhibitors are a relatively new group of natural products that have shown very promising biological activity. They all share some structural similarities, such as the existence of a protected thiol that is able to act as ZBG upon disulfide bond reduction (figure 21).
Figure 21: Structure of cyclic depsipeptide HDAC inhibitors.

The first compound of this kind to be isolated was the bacterial natural product FK228 (FR901228, romidepsin, Istodax®). Isolated from bacterial fermentation broth of *Chromobacterium violaceum* No. 968, structurally it consists of L-valine, D-valine, (Z)-dehydrobutyrine, D-cysteine, and (3S)-hydroxy-7-mercapto-4-heptenoic acid, where the last two groups are connected by an internal disulfide bond.\(^{92-94}\) Initial interest in FK228 stemmed from its extremely selective cytotoxicity for cancer cells over normal cells in mouse and human fibroblast. In addition to being able to revert oncogenic morphological phenotypes, FK228 treatment also led to inhibition of tumor growth \textit{in vivo} in mammary adenocarcinoma (MDF-7) and lung adenocarcinoma (A549) mouse models.\(^{94}\) FK228 was found to be a pro-drug that undergoes disulfide bond reduction in order to release the active free thiol, which is able to inhibit HDACs.\(^{95}\) This hypothesis was further supported by an increase in potency of when the disulfide bond of FK228 was reduced. In addition to being a very potent HDAC inhibitor and also exhibiting strong class I-selectivity, FK228 also demonstrated anti-angiogenic activity through downregulation of
hypoxia inducible factor 1α (HIF-1α) expression and consequent decrease in vascular endothelial growth factor (VEGF) mRNA as well as VEGF protein levels.\textsuperscript{96}

\textbf{Scheme 4:} Total synthesis of FK228 by Simon and co-workers.\textsuperscript{97}

The first total synthesis of FK228 was reported by Simon and co-workers in 1996.\textsuperscript{97} One of the key steps in this synthesis was the asymmetric synthesis of \(\beta\)-hydroxy mercaptoheptenoic acid 24, which was accomplished through a titanium-mediated asymmetric aldol reaction. Tetrapeptide 25 was accessed through standard peptide coupling procedures and subsequently
underwent DABCO-mediated elimination of the threonine hydroxyl group to furnish the (Z)-dehydrobutyrine group. Coupling of intermediate 24, was followed by macrolactonization through Mitsunobu conditions. Iodine-mediated trityl deprotection and disulfide bond formation furnished the final product (scheme 4).

**Scheme 5:** Total synthesis of spiruchostatin A by Ganesan and co-workers.98

The internal disulfide bond connecting D-cysteine and (3S)-hydroxy-7-mercapto-4-heptenoic acid as well as the disulfide bond reduction mechanism are also common features in the
natural products of the spiruchostatin family (figure 21). Spiruchostatins were isolated from the bacterium *Pseudomonas sp.* and showed cancer cell growth inhibition across various human cancer cell lines with GI$_{50}$ values in the nanomolar ranges.$^{99-101}$ Much like FK228, spiruchostatins are also class I-selective HDAC inhibitors that undergo disulfide bond reduction to reveal the active thiol ZBG.$^{100}$

To date, several different total syntheses of different members of the spiruchostatin family have been reported, but the total synthesis of spiruchostatin A reported by Ganesan and co-workers is particularly noteworthy (scheme 5).$^{98}$ In this case, the $\beta$-hydroxy mercaptoheptenoic acid 33 was accessed through Vilarrasa’s titanium-mediated aldol reaction which utilizes the Nagao chiral auxiliary. Synthesis of tripeptide 32 began with Claisen condensation of commercially available Boc-L-valine (29), followed by diastereoselective reduction to form the desired alcohol 31. Coupling with D-cysteine and D-alanine furnished tripeptide 32, which was then coupled to $\beta$-hydroxy mercaptoheptenoic acid 33. Tce deprotection and Yamaguchi esterification formed the macrolactone, which then underwent iodine-mediated trityl deprotection and disulfide bond formation and ultimate desilylation to complete the synthesis.

Burkholdacs/thailandepsins are also class I-selective HDAC inhibitors that are structurally very similar to FK228 and spiruchostatins and the most recent discoveries for cyclic depsipeptide HDAC inhibitors. They contain D-amino acids and (3S)-hydroxy-7-mercapto-4-heptenoic acid unit tied up in an internal disulfide bond (figure 21).$^{102-106}$ Also, much like spiruchostatins, bulkholdacs/thailandepsins show broad spectrum anti-cancer activity against a variety of cancer cell lines with GI$_{50}$ values in the nanomolar ranges for over 90% of the NCI-60 Developmental Therapeutics Program (DTP) Human Tumor Cell Line Screen.$^{104}$ Burkholdacs/thailandepsins showed extremely potent HDAC inhibition against HDAC1 when
compared to HDACs 4 and 6. Additionally, reduction of the disulfide bond resulted in a significant increase in potency, further validating the prodrug hypothesis for these types of HDAC inhibitors.\(^\text{103}\)

### 1.4.4 HDACs in drug discovery

Since their initial discovery by Schreiber and co-workers, HDACs have been examined extensively, mainly due to their implications in various disease states with a particular focus on cancer. Suberanilohydroxamic acid (SAHA, vorinostat, Zolinza\(^\text{®}\)) and FK228 (FR901228, romidepsin, Istodax\(^\text{®}\)) were approved by the FDA for the treatment of cutaneous T-cell lymphoma (CTCL) in 2006 and 2009, respectively.\(^\text{59,107}\) More recently, LBH-589 (panobinostat, Farydak\(^\text{®}\)) and PXD101 (belinostat, Beleodaq\(^\text{®}\)) were approved in 2015 for the treatment of multiple myeloma and peripheral T-cell lymphoma, respectively (figure 22).\(^\text{108,109}\) Along with these FDA approved drugs, there are several natural product and synthetic HDAC inhibitors that are currently in clinical trials that show exciting promise.

![Structure of FDA approved HDAC inhibitors.](image.png)
While many aspects of HDAC research have been elucidated, there is much we still don’t understand. One major challenge in the field is the development of an isoform-selective HDAC inhibitor.\textsuperscript{110} While many inhibitors have been examined that demonstrate class-selectivity, a true single isoform-selective HDAC inhibitor has yet to be discovered. It is clear that in order to develop such a specific inhibitor, we must first fully understand the mechanism of inhibition of HDACs. With many natural product HDAC inhibitors showing promising class-selectivity, they will serve as a starting point for the ultimate goal of developing a single isoform-selective HDAC inhibitor.

\textbf{1.5 Goals of dissertation}

Largazole is the last member of the cyclic depsipeptide natural product HDAC inhibitor family. While largazole is structurally and functionally similar to the other inhibitors in this class of molecules, it also possesses many unique characteristics that have garnered considerable interest. For example, largazole has demonstrated excellent differential cytotoxicity for cancer cells over healthy cells, making it an attractive potential anti-cancer drug from a therapeutic perspective.\textsuperscript{111} Largazole has also shown strong class I-selectivity which could potentially explain the differential cytotoxicity that largazole possesses.\textsuperscript{112,113} This class-selectivity is also desirable in a chemical probe for HDAC inhibition research since it allows for examination of the role of fewer HDAC isoforms. Over the past several years, many different studies have been conducted in order to fully understand the mechanistic basis for largazole’s biological activities. While significant progress has been made, there are still underexplored areas. One such challenge is the structural basis for largazole’s class-selectivity and the potential to leverage this into isoform-selectivity.
This dissertation will focus on the design, synthesis, and biological evaluation of several largazole analogues in efforts towards defining a more specific structure–activity relationship. Chapter 2 will discuss the class I-selectivity of largazole and efforts towards improving single isoform-selectivity. Several previous structure–activity relationships have identified crucial structural features of largazole for the efficient inhibition of HDACs. However, only very limited work has been done on the isoform-selectivity of largazole. This chapter aims to further elucidate structural requirements necessary for isoform-selective HDAC inhibitors. Chapter 3 will focus on the importance of the ZBG and the effect of ZBG modulations on the biological activity of largazole. The ZBG is an important structural feature for the HDAC inhibitory activity of largazole. However, the extent to which this metal binding group drives HDAC inhibition is not fully understood. By modulating the Zn\(^{2+}\) affinity of this group, chapter 3 will further examine the importance of the ZBG for the HDAC inhibitory activity of largazole. Lastly, chapter 4 will address the prodrug characteristics of largazole and the effect of different prodrug strategies on pharmacokinetic properties. From a drug development perspective, a major challenge for largazole is the metabolic stability and bioavailability of the compound. By changing the thioester moiety with disulfide prodrug analogues, which is more stable in cellular metabolism, chapter 4 aims to investigate how different prodrug strategies can influence drug like properties of largazole. By investigating these various facets, the goal of this dissertation is to build a more complete understanding about the HDAC inhibitory activity of largazole in order to aid in the future development of an optimal HDAC inhibitor as well as a potential anti-cancer therapeutic.
2. Synthesis and biological characterization of largazole C2 analogues

2.1 Largazole

2.1.1 Discovery, identification, and initial biological activity of largazole

Largazole, a marine natural product, was isolated by Luesch and co-workers from a cyanobacterium of the *Symploca* genus off the coast of Key Largo, Florida in 2008 (figure 23).\(^{111}\) It was named after the collection site (Key Largo) and a structural feature (thiazole) of the compound. A cytotoxic crude organic extract of *Symploca* sp. was subjected to bioassay-guided fractionation by solvent partition, silica gel chromatography, and reversed-phase HPLC to result in the natural product. Extensive 1D- and 2D-NMR along with high-resolution mass spectrometry led to structure elucidation of largazole. It is a 16-membered depsipeptide macrocycle with a variety of unusual structural features, such as a 4-methylated thiazoline unit linearly fused to a thiazole, a 3-hydroxy-7-mercaptohept-4-enoic acid unit, as well as a thioester moiety (figure 23).

![Figure 23: Structure of largazole.](image-url)

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111 Numbers correspond to the reference sources provided at the end of the document.
Initial biological testing of largazole showed impressive selectivity for cancer cells (table 2).\textsuperscript{111} While potently inhibiting the growth of transformed human mammary epithelial cells (MDA-MB-231) in a dose-dependent manner (GI$_{50}$ = 7.7 nM) and inducing cytotoxicity at a higher concentration (LC$_{50}$ = 117 nM), largazole was significantly less potent for nontransformed murine mammary epithelial cells (NMuMG) (GI$_{50}$ = 122 nM and LC$_{50}$ = 272 nM). A similar level of selectivity was observed in transformed fioroblastic osteosarcoma cells (U2OS) (GI$_{50}$ = 55 nM and LC$_{50}$ = 94 nM) over nontransformed fibroblast cells (NIH3T3) (GI$_{50}$ = 480 nM and LC$_{50}$ > 8 $\mu$M). When compared to other natural products that possess anti-cancer activity, largazole demonstrated the greatest level of selectivity. Additionally, largazole showed potent anti-proliferative activity in colon (HT29) (GI$_{50}$ = 12 nM and LC$_{50}$ = 22 nM) and neuroblastoma (IMR-32) (GI$_{50}$ = 16 nM and LC$_{50}$ = 22 nM) cancer cell lines.

<table>
<thead>
<tr>
<th></th>
<th>Largazole</th>
<th>Paclitaxel</th>
<th>Actinomycin D</th>
<th>Doxorubicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>7.7</td>
<td>7.0</td>
<td>0.5</td>
<td>310</td>
</tr>
<tr>
<td>NMuMG</td>
<td>122</td>
<td>5.9</td>
<td>0.3</td>
<td>63</td>
</tr>
<tr>
<td>U2OS</td>
<td>55</td>
<td>12</td>
<td>0.8</td>
<td>220</td>
</tr>
<tr>
<td>NIH3T3</td>
<td>480</td>
<td>6.4</td>
<td>0.4</td>
<td>47</td>
</tr>
</tbody>
</table>

This potent anti-cancer activity combined with excellent selectivity for cancer cells prompted further biological studies of largazole. However, due to a lack of material from natural sources, synthetic efforts were necessary to supply more of the compound for further studies, such as molecular target identification and mode of action elucidation. These efforts culminated in 13 different total syntheses.\textsuperscript{110,111}
2.1.2 Total syntheses of largazole

Scheme 6: Total synthesis of largazole by Hong/Luesch and co-workers.112

The first total synthesis of largazole was completed by Hong/Luesch and co-workers in 2008 (scheme 6).112 The synthesis began with the condensation of nitrile 36 with (R)-2-methyl cysteine methyl ester (37) to produce thiazole-thiazoline 38. Removal of the Boc group was followed by coupling to β-hydroxy acid 39, which was accessed through an asymmetric aldol reaction with N-acyl thiazolidinethione and acrolein. Yamaguchi esterification with N-Boc-L-valine (41) resulted in the acyclic intermediate 42, which underwent hydrolysis, Boc-deprotection,
and HATU/HOAt mediated macro lactonization to form cyclic depsipeptide 43. Lastly, cross-metathesis with thioester 44, which was prepared by coupling thioacid 45 with 4-bromo-1-butene (46) (scheme 7), in the presence of Grubbs’ 2nd generation catalyst provided largazole as a final product in 19% overall yield over 8 steps.

![Scheme 7: Preparation of thioester 44 for cross-metathesis.](image)

Short before the first total synthesis was published, Cramer and co-workers submitted a manuscript for another total synthesis of largazole, utilizing a different approach approach (scheme 8).\textsuperscript{114} β-hydroxy ester 47, which was prepared by enzymatic resolution, was coupled with Fmoc-L-valine (48) and thiazole-thiazoline unit 50 to produce the acyclic intermediate 51. Unlike the first total synthesis, Cramer and co-workers performed the macrolactamization at the bottom amide bond after simultaneous Boc/tert-butyl ester cleavage to access macrocycle 43.

![Scheme 8: Preparation of macrocycle 43 for the total synthesis of largazole by Cramer and co-workers.](image)
The most problematic step in the synthesis was the cross-metathesis step, which is also the last step. The low conversion and yield of the reaction made it challenging to produce preparative amounts of largazole. To this end, Cramer and co-workers have made significant contributions to improve this challenging step. After systematic screening of catalysts, solvents, and reaction temperatures, they reported that 15 mol% of Grela catalyst in 1,2-dichloroethane at 90 °C resulted in the most optimized conditions (table 3).\textsuperscript{114}

Table 3: Optimization of cross-metathesis reaction, and completion of synthesis of largazole by Cramer and co-workers.\textsuperscript{114}

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Yield (%)</th>
<th>Starting material recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grubbs I</td>
<td>5</td>
<td>68</td>
</tr>
<tr>
<td>Grubbs II</td>
<td>11</td>
<td>68</td>
</tr>
<tr>
<td>Hoveyda-Grubbs II</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>52</td>
<td>13</td>
<td>77</td>
</tr>
<tr>
<td>53</td>
<td>75</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{44} (4 equiv), catalyst (10%, +5% after 2 h), 0.1 M in 1,2-dichloroethane, 90 °C, 3–12 h.
Around the same time, Williams and co-workers also submitted a manuscript describing yet another total synthesis of largazole (scheme 9). Here, again an asymmetric aldol reaction was used to form thiazolidinethione 54, which was converted into the TSE ester (55).

Esterification with Fmoc-L-valine, followed by Fmoc deprotection and PyBOP mediated coupling with thiazole-thiazoline 50 resulted in the acyclic precursor 57. Simultaneous TSE and Boc deprotection followed by HATU/HOBt mediated macrolactamization at the bottom amide bond resulted in the trityl protected thiol 58. This intermediate then underwent trityl deprotection and acylation with octanoyl chloride to complete the synthesis.

Scheme 9: Total synthesis of largazole by Williams and co-workers.113

Since these three total syntheses of largazole were published, ten more total syntheses have been reported in the literature.110,112-124 Main synthetic differences include the macrolactamization site of the 16-membered cyclic depsipeptide core, installation of the thioester
moiety, as well as the asymmetric synthesis of the \( \beta \)-hydroxy acid subunit. Figure 24 summarizes the different approaches that have been used by different groups. Hong,\textsuperscript{112} Doi,\textsuperscript{116} Xie,\textsuperscript{122} de Lera,\textsuperscript{121} and Tillekeratne\textsuperscript{124} performed a macrocyclization at the top amide bond, while Williams,\textsuperscript{113} Cramer,\textsuperscript{114} Phillips,\textsuperscript{117} Ghosh,\textsuperscript{118} Ye,\textsuperscript{115} Forsyth,\textsuperscript{119} Jiang,\textsuperscript{120} and Ganesan\textsuperscript{123} closed the macrocycle at the bottom amide bond. Several groups also attempted to perform a macrolactonization in order to close the macrocycle, but no such attempts were successful. For the incorporation of the thioester moiety, Hong, Cramer, Phillips, Ghosh, and de Lera utilized ruthenium catalysts in a cross-metathesis reaction. Williams, Ye, Doi, Xie, Ganesan, and Tillekeratne on the other hand carried a protected thiol throughout the entire synthesis and performed a late stage acylation in order to install the thioester. Jiang and co-workers uniquely utilized the Julia–Kocienski olefination to incorporate the thioester, which also gave them access to the \( Z \)-alkene as an analogue. Forsyth and co-workers carried the complete octanoyl thioester through the entire synthesis. The most significant differences in the total syntheses come from the asymmetric synthesis of the \( \beta \)-hydroxy acid subunit. The most common route to access the desired enantiomer, used by Hong, Williams, Ye, Doi, Xie, Ganesan, and Tillekeratne was an asymmetric aldol reaction utilizing a chiral thiazolinethione auxiliary. Cramer, Phillips and Ghosh utilized enzymatic resolution techniques in order to separate the desired enantiomer, while de Lera kinetically resolved a racemic mixture of enantiomers to access the necessary building block. Forsyth and co-workers had a unique approach of utilizing an NHC-mediated amidation reaction on an \( \alpha, \beta \)-epoxy aldehyde intermediate. The sheer number of total syntheses that have been reported underlines the attention largazole has garnered from not only chemical biology groups, but also organic synthesis groups. Additionally, with a completed total synthesis and a new source of largazole, further biological characterization of largazole was possible.
2.1.3 Target identification and mode of action of largazole

One interesting structural feature of largazole is the thioester moiety. This functional group should hydrolyze rapidly, in what is suspected to be a protein-assisted process, in normal cellular metabolism to generate the corresponding thiol. The resulting 3-hydroxy-7-mercaptohept-4-enoic acid unit has been seen in other natural products, spiruchostatins and FK228. FK228, in particular, shows a large structural overlap with largazole when the disulfide bond is reduced to form reduced FK228 (redFK228) and the thioester in largazole is hydrolyzed (figure 25). This structural similarity led to the initial hypothesis by Hong/Luesch and co-workers,
that largazole and FK228 shared a common molecular target. Additionally, it was hypothesized that largazole acted as a prodrug for the active species, largazole thiol.\textsuperscript{112}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure25}
\caption{Structural similarities between redFK228 and largazole thiol.}
\end{figure}

When largazole was discovered, FK228 was a known HDAC inhibitor. In order to see if largazole also showed HDAC inhibitory activity, HCT-116 colon carcinoma cells were treated with largazole (figure 26).\textsuperscript{112} The intrinsically high HDAC activity in HCT-116 cells was inhibited by largazole in a dose-dependent manner. More importantly, the HDAC inhibition very closely correlated with largazole’s growth inhibitory activity of the HCT-116 cell line. This strongly indicated that the HDAC inhibition by largazole is responsible for the anti-proliferative activity. Immunoblot analysis of endogenous HDAC substrate, histone H3, confirmed the dose-response relationship of largazole and HDAC inhibition (figure 26).\textsuperscript{112}
Figure 26: HDAC inhibitory activity of largazole. (a) Largazole inhibits cellular HDAC activity in HCT-116 colon cancer cells. HDAC inhibition correlates with growth inhibition. (b) Immunoblot analysis of endogenous histone H3 deacetylation shows dose-dependent HDAC inhibition by largazole. Trichostatin A (TSA) was used as a positive control. Figure reprinted with permission from ref. 112

Williams and co-workers also reported the same conclusions independently, based on the structural similarities of largazole, FK228, and spiruchostatins. 113 The hypotheses were also verified when largazole, largazole thiol, and known HDAC inhibitors SAHA and FK228 were tested for their HDAC1 inhibitory activity. All compounds showed very potent HDAC1 inhibitory activity, strongly suggesting largazole is indeed an HDAC inhibitor like FK228 and spiruchostatins (figure 27). Additionally, the increased potency of largazole thiol over largazole supports the idea of a prodrug strategy.
Figure 27: HDAC1 inhibition by largazole (●), largazole thiol (○), SAHA (■), and FK228 (□). Figure reprinted with permission from ref. 113

Largazole also demonstrated class I-selectivity, like FK228. When tested against recombinant human HDAC1 and HDAC6, largazole was found to be much more potent against the class I HDAC (HDAC1) than the class II HDAC (HDAC6).112 In comparison, trichostatin A (TSA), a pan-HDAC inhibitor, showed a significantly lower level of selectivity. Additionally, the prodrug hypothesis of largazole was further validated as largazole thiol proved to be 10 times more potent than largazole in inhibiting HDAC1 (table 4).

Table 4: HDAC1 and HDAC6 inhibition of largazole, largazole thiol, and TSA (IC50, nM). Data from ref. 112

<table>
<thead>
<tr>
<th></th>
<th>HDAC1 (class I)</th>
<th>HDAC6 (class II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Largazole</td>
<td>25 ± 11</td>
<td>5700 ± 3600</td>
</tr>
<tr>
<td>Largazole thiol</td>
<td>2.5 ± 1.4</td>
<td>380 ± 76</td>
</tr>
<tr>
<td>Trichostatin A</td>
<td>4.9 ± 0.8</td>
<td>18 ± 12</td>
</tr>
</tbody>
</table>

Williams and co-workers further validated these results independently by reporting inhibitory constants of largazole against HDACs 1, 2, 3, and 6 (table 5).113 Once again largazole showed selectivity towards the class I HDACs (HDACs 1, 2, and 3), and was much weaker
against class II HDAC, HDAC6. The same selectivity was observed for FK228, which is known to possess class I-selectivity, while SAHA, a pan-HDAC inhibitor, did not show such bias. Additionally, largazole thiol was again shown to be significantly more potent than natural largazole, further validating the prodrug hypothesis.

**Table 5:** HDAC inhibition of largazole, largazole thiol, FK228, and SAHA ($K_i$, nM). Data from ref.\textsuperscript{113}

<table>
<thead>
<tr>
<th></th>
<th>HDAC1</th>
<th>HDAC2</th>
<th>HDAC3</th>
<th>HDAC6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Largazole</td>
<td>20</td>
<td>21</td>
<td>48</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Largazole thiol</td>
<td>0.07</td>
<td>0.07</td>
<td>0.17</td>
<td>25</td>
</tr>
<tr>
<td>FK228</td>
<td>0.12</td>
<td>0.14</td>
<td>0.28</td>
<td>35</td>
</tr>
<tr>
<td>SAHA</td>
<td>10</td>
<td>10</td>
<td>15</td>
<td>9</td>
</tr>
</tbody>
</table>

Based on the general structure of HDAC inhibitors and the known ability of thiols to chelate to Zn$^{2+}$, a putative mechanism of action of largazole was formulated. This hypothesis was also predicted by a variety of homology models put forth by various groups (figure 28). In the presence of plasma or cellular proteins, largazole presumably undergoes thioester bond hydrolysis to liberate the free thiol. This thiol then inserts into the channel like active site of HDAC in order to chelate the Zn$^{2+}$ in the bottom. The macrocycle interacts with the rim portion of the active site and blocks entry of the natural substrate.\textsuperscript{112,113}
This hypothesis was verified in 2011, when visualization of the interaction between largazole thiol and HDAC8 was achieved by an X-ray co-crystal structure by Christianson and co-workers (figure 29). This 2.14 Å resolution crystal structure marked the first time an HDAC complexed with a macrocyclic inhibitor was reported. Surprisingly, the crystal structure showed very minimal conformational change by the macrocyclic core and significant conformational changes by HDAC8 upon binding. The thiol is inserted into the active site channel where it chelates to the Zn$^{2+}$ in a near tetrahedral coordination geometry with ligand–Zn$^{2+}$–ligand angles between 107.6° and 111.8°. The thiolate moiety exhibits preferred thiolate–metal coordination geometry with a thiolate S–Zn$^{2+}$ separation of 2.3 Å, a C–S–Zn$^{2+}$ angle of 97.5°, and a C–C–S–Zn$^{2+}$ dihedral angle of 92.4°. The crystal structure revealed that this ideal binding geometry by the thiol is responsible for high binding affinity of largazole for HDAC. It was also the first structure that showed the thiolate–Zn$^{2+}$ interaction in an HDAC complex. Access to this structural
information provided a rationale for the results of structure–activity relationship studies from numerous largazole analogues prepared by various groups.

Figure 29: Co-crystal structure of HDAC8-largazole thiol complex. Figure reprinte with permission from ref.\textsuperscript{125}

2.1.4 Previously reported largazole analogues and their structure–activity relationship studies

The attention largazole has received from the field has manifested itself into the synthesis and characterization of numerous analogues. In attempts to improve potency and isoform-selectivity, many different structural changes have been examined, specifically the thioester linker, the L-valine subunit, and the thiazole-thiazoline subunit. The most important structural features of largazole are the length of the thiol linker, the C17 configuration, and the olefin geometry. Lengthening the chain resulted in a significant decrease in potency, while shortening the chain resulted in a complete loss of activity (table 6).\textsuperscript{126} Changing the C17 configuration from (S) to (R) resulted in a very significant loss of activity. This data can be rationalized by the HDAC8-largazole thiol complex.\textsuperscript{110,125} If the chain is shortened, the thiol is not able to bind to the
Zn\(^{2+}\) at the bottom of the active site, thereby abolishing the HDAC inhibitory activity. If the chain is lengthened, while the thiol is still able to reach the Zn\(^{2+}\) and coordinate to it, the analogue deters from optimal binding geometry in the macrocycle region, thereby significantly reducing the activity of the compound. The C17 configuration change presumably affects the macrocycle configuration which leads to the observed loss of activity.

**Table 6**: Cancer cell growth and HDAC inhibition of largazole and largazole analogues. Data from ref.\(^{126}\)

<table>
<thead>
<tr>
<th></th>
<th>HCT-116 GI(_{50}) (nM)</th>
<th>HDAC IC(_{50}) (nM)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 1</td>
<td>&gt; 10000</td>
<td>&gt; 20000</td>
</tr>
<tr>
<td>n = 2</td>
<td>6.8 ± 0.6</td>
<td>32 ± 13</td>
</tr>
<tr>
<td>(largazole)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = 3</td>
<td>620 ± 50</td>
<td>7600 ± 900</td>
</tr>
<tr>
<td>n = 4</td>
<td>2500 ± 600</td>
<td>4100 ± 430</td>
</tr>
<tr>
<td>17R</td>
<td>3900 ± 450</td>
<td>&gt; 20000</td>
</tr>
</tbody>
</table>

\(^a\)HDACs from HeLa nuclear extracts were used.

Changing the olefin geometry from *trans* to *cis* resulted in complete loss of activity. Jiang and co-workers prepared the tested a variety of largazole analogues both with *trans* and *cis* olefins for their growth inhibitory activity against human colorectal carcinoma (HCT-116) and lung cancer (A549) cells.\(^{120}\) As table 7 shows, all *trans* analogues have comparable activity to largazole with sub-micromolar GI\(_{50}\) values. On the other hand, all *cis* analogues completely lack
the ability to inhibit cell proliferation. Molecular docking studies show that the geometry of the cis olefin does not allow the thiol to reach the Zn$^{2+}$ in the bottom of the HDAC active site, explaining the lack of activity.$^{120}$

Table 7: Cancer cell growth inhibition of largazole and largazole analogues. Data from ref.$^{120}$

<table>
<thead>
<tr>
<th>R = iPr (largazole)</th>
<th>Olefin geometry</th>
<th>HCT-116 GI$_{50}$ (μM)</th>
<th>A549 GI$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>trans</td>
<td>0.08</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>cis</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td></td>
</tr>
<tr>
<td>R = iBu trans</td>
<td>0.56</td>
<td>3.28</td>
<td></td>
</tr>
<tr>
<td>cis</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td></td>
</tr>
<tr>
<td>R = Bn trans</td>
<td>0.26</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>cis</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td></td>
</tr>
<tr>
<td>R = p-OH-Bn trans</td>
<td>0.39</td>
<td>1.46</td>
<td></td>
</tr>
<tr>
<td>cis</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td></td>
</tr>
</tbody>
</table>

The study conducted by Jiang and co-workers also revealed an important structural modification, which is the amino acid at C2. The L-valine subunit was replaced with L-leucine, L-phenylalanine, and L-tyrosine.$^{120}$ These analogues all showed only slightly weaker anti-proliferative activity against cancer cell lines than largazole, indicating that the C2 position can be modified with minimal loss of activity. Several other analogues with C2 amino acid replacements, such as alanine, glycine have been characterized and corroborate the same
Both analogues were slightly weaker HDAC inhibitors than their corresponding parent compounds (table 8). In the HDAC8–largazole thiol complex, the valine subunit faces the solvent and does not interfere with the enzyme–inhibitor interaction, which explains the minimal affect structural modifications have had at this position.

**Table 8**: HeLa nuclear extract HDAC inhibition of largazole, largazole thiol, and C2 analogues (IC$_{50}$, nM). Data from ref.\textsuperscript{123,126}

<table>
<thead>
<tr>
<th></th>
<th>Largazole</th>
<th></th>
<th>Largazole thiol</th>
</tr>
</thead>
<tbody>
<tr>
<td>59</td>
<td>72 ± 21</td>
<td>60</td>
<td>0.17 ± 0.05</td>
</tr>
<tr>
<td>60</td>
<td>32 ± 13</td>
<td></td>
<td>0.043 ± 0.026</td>
</tr>
</tbody>
</table>

However, when D-amino acids such as D-naphthylalanine or 2-\textit{epi} largazole were tested, a significant reduction in HDAC inhibitory activity was observed (figure 30).\textsuperscript{127,128} D-naphthylalanine analogue 61 was completely inactive in HCT-116 colon carcinoma cell proliferation assay up to 10 \textmu M and 2-\textit{epi} largazole (62) was 25 times less potent in \textit{in vitro} inhibition of HDACs 1, 2, and 3. Interestingly, despite the observed decrease in potency, 2-\textit{epi} largazole (62) was more potent than largazole against prostate cancer (LNCaP and PC-3) cell proliferation.\textsuperscript{128}
Replacement of the thioester with different metal-binding groups such as \( \alpha \)-aminobenzamide or \( \alpha \)-thioamide led to significant reduction in \textit{in vitro} HDAC inhibitory activity (table 9).\textsuperscript{129} 2-thiomethyl pyridine, 2-thiomethyl thiophene, and 2-thiomethyl phenol have also been incorporated. However, when these analogues were tested for their growth inhibition against HCT-116 cells, they were shown to be much less effective than largazole (figure 31).\textsuperscript{124}

\textbf{Table 9:} HDAC inhibition of largazole thiol, \( \alpha \)-aminobenzamide (63), and \( \alpha \)-thioamide (64) analogues (IC\textsubscript{50}, \( \mu \)M). Data from ref.\textsuperscript{129}

<table>
<thead>
<tr>
<th></th>
<th>HDAC1</th>
<th>HDAC2</th>
<th>HDAC3</th>
<th>HDAC6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Largazole thiol</td>
<td>0.0012</td>
<td>0.0035</td>
<td>0.0034</td>
<td>0.049</td>
</tr>
<tr>
<td>\textbf{63}</td>
<td>0.27</td>
<td>4.1</td>
<td>4.1</td>
<td>\textgreater 30</td>
</tr>
<tr>
<td>\textbf{64}</td>
<td>0.67</td>
<td>1.6</td>
<td>0.96</td>
<td>0.7</td>
</tr>
</tbody>
</table>
Figure 31: Structure and growth inhibition of largazole, 2-thiomethyl pyridine (65), 2-thiomethyl thiophene (66), and 2-thiomethyl phenol (67) analogues against HCT-116 cells. Figure reprinted with permission from ref.¹²⁴

Replacement of the thioester with different disulfide groups resulted in similar activity to largazole (table 10).¹³⁰ Because the disulfide bond can be reduced to liberate the active free thiol like the hydrolysis of the thioester it is not surprising that these compounds would have similar activity. This further underlines the importance of the thiol as a ZBG in largazole’s ability to inhibit HDAC activity.
Table 10: Cancer cell growth inhibition of largazole and largazole disulfide analogues (GI<sub>50</sub>, µM).

Data from ref.\textsuperscript{130}

\[
\begin{array}{|c|c|c|c|c|}
\hline
R = & Molt-4 & U937 & MCF-7 & BGC823 \\
\hline
\text{Largazole} & 0.027 & 0.033 & 2.35 & 0.145 \\
\text{Et} & 0.098 & 0.28 & 2.22 & 0.50 \\
\text{n-Bu} & 0.071 & 0.46 & 2.25 & 0.39 \\
\text{t-Bu} & 0.033 & 0.066 & 4.87 & 0.20 \\
\text{n-Hex} & 0.045 & 0.068 & 2.61 & 0.25 \\
\text{n-Oct} & 0.051 & 0.070 & 2.11 & 0.29 \\
\hline
\end{array}
\]

The thiazole-thiazoline unit is also able to undergo significant structural changes without dramatic loss in activity. The replacement of the methyl group at C7 with a hydrogen atom, ethyl, or benzyl group has resulted in minimal changes in largazole’s biological activity (table 11).\textsuperscript{121}

The HDAC8–largazole thiol complex shows that the methyl group is parallel to the protein surface with no interaction with the protein, thereby limiting the effect it has on the inhibitory activity of largazole.\textsuperscript{110,125}
Table 11: HDAC1 inhibition of largazole and C7 analogues (IC$_{50}$, nM). Data from ref.\textsuperscript{121}

<table>
<thead>
<tr>
<th>R =</th>
<th>HDAC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Me (largazole)</td>
<td>11.4</td>
</tr>
<tr>
<td>H</td>
<td>13.7</td>
</tr>
<tr>
<td>Et</td>
<td>161.3</td>
</tr>
<tr>
<td>Bn</td>
<td>4.5</td>
</tr>
</tbody>
</table>

However, when the thiazoline ring is replaced with a thiazole (68), triazole (69), or tetrazole (70), the potency of largazole decreases significantly.\textsuperscript{129,131,132} This is most likely due the conformational change of the macrocycle caused by these structural modifications. Surprisingly when the thiazoline is completely removed and replaced with a simpler $\alpha$-aminoisobutyric acid (71), potency of largazole was largely maintained (figure 32).\textsuperscript{123}
Williams and co-workers also reported the synthesis of analogues in which the thiazole was replaced with a pyridine (72) or an oxazole (73) (table 12). In these cases, similar activity was observed for the oxazole analogue (73) while slight enhancements of activity were observed for the pyridine analogue (72). It is possible that this position could potentially tolerate further modifications without significant loss of activity. The HDAC8–largazole thiol complex further shows that the thiazole-thiazoline faces the solvent, indicating that it may undergo significant structural changes as long as the conformational integrity of the macrocycle stays intact.
Table 12: Structure and HDAC1 inhibition of largazole thiol and thiazole analogues (IC_{50}, nM). Data from ref.\textsuperscript{129,133}  

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
 & HDAC1 \\
\hline
Largazole thiol & 1.2 \\
72 & 0.32 \\
73 & 4.4 \\
\hline
\end{tabular}
\end{table}

The roles of the amide and ester linkages were also examined. Replacement of the ester oxygen to form an amide linkage (74) resulted in very similar potency, which was not surprising because the conformational change induced by the isostere replacement was expected to be minimal.\textsuperscript{134} Methylation of the amide nitrogens in largazole (75 and 76) resulted in a 100–1000 fold decrease in potency, indicating that potential hydrogen bonding of these amide nitrogens could be vital to the conformational structure of the macrocycle and consequently the biological activity of largazole.\textsuperscript{135} Testing of homolargazole (77), which contains an additional methylene in the macrocycle also proved to be detrimental to HDAC inhibitory potency (figure 33).\textsuperscript{136} Once again this is most likely due to the perturbation of the macrocyclic conformation.
Figure 33: Structure and activity of largazole amide isostere (74), N-methyl amide analogues (75 and 76), and homolargazole analogue (77). Data from ref.136

To date, numerous analogues of largazole have been prepared and characterized for their anti-proliferative activity against cancer cell lines and their HDAC inhibitory activity, and these efforts continue. Figure 34 summarizes the structure–activity relationships discussed in this section.110 While the majority of studies have focused on largazole’s ability as an HDAC inhibitor, its downstream mechanism of action as well as its in vivo efficacy have been explored in great detail and are of particular interest. The details of these studies will be discussed further in the next section.
2.1.5 Downstream mechanism of action and *in vivo* efficacy of largazole

While largazole demonstrated anti-proliferative activity in HCT-116 colon carcinoma cells, studies conducted by Hong/Luesch and co-workers showed that largazole also possessed anti-proliferative activity in all cancer cell lines of the NCI’s cell viability screen, which is a collection of 60 cancerous cell lines. It was not surprising that potent cytotoxicity against colon cancer cell lines was observed, but largazole also demonstrated effective cytotoxicity against cell lines originating from melanoma and renal cancer. In order to rationalize the anti-proliferative activity of largazole, the effect of largazole on the cell cycle of HCT-116 cells was examined by DNA content analysis. It was shown that largazole causes $G_1$ cell cycle arrest at low concentrations (1–3.2 nM) and $G_2/M$ cell cycle arrest at higher concentrations ($\geq$ 10 nM),

Figure 34: Summary of structure–activity relationship of largazole. Figure adapted from ref.110
indicating that different genes are regulated at different concentrations. The higher concentrations of largazole also caused apoptosis as evidenced by the population of cells in the sub-G₁ and sub-G₂ phase. This was also consistent with the caspase 3/7 activity induction, which are downstream mediators of apoptosis (figure 35).

**Figure 35:** Effect of largazole on the cell cycle. (A) Treatment of HCT-116 cells with increasing concentrations of largazole shows G₁ arrest at lower concentrations and a shift to G₂/M arrest for higher concentrations. (B) Treatment of HCT-116 cells with increasing concentrations of largazole shows induction of caspase 3/7 activity. Figure reprinted with permission from ref. 135

Hong/Luesch and co-workers also examined transcriptional targets related to the antiproliferative activity of largazole. Since HDACs play a major role in gene transcriptional control, it was not surprising that transcriptomic profiling showed a large number of genes induced or repressed either directly or indirectly by largazole. Additionally, when the human
genome genechip was used with largazole, SAHA, and FK228 a large overlap of affected genes was observed. Among them was the induction of cyclin dependent kinase (CDK) inhibitors p21, p19, p15, and p57, which can all be linked to drug-induced G1 arrest. Corresponding CDK6 and cyclin D1 were strongly downregulated, which would also explain the anti-proliferative activity of largazole. Additionally, various receptor tyrosine kinases (RTKs), such as HER-2, EGFR, and MET were significantly downregulated. The overexpression of RTKs, which drive proliferation, is a common biomarker. The downregulation of these kinases is most likely intimately tied to largazole’s anti-proliferative activity. At higher concentrations of largazole, elevation of the BCL2L11 gene, which codes for a pro-apoptotic BCL2 protein, was observed. Of particular interest was the strong downregulation of insulin growth factor (IGF) receptor substrate 1 (IRS-1). IGF signaling is known to upregulate anti-apoptotic activity via protein kinase B (AKT) activation, providing a rationale for the apoptotic activity observed at high concentrations of largazole. The transcriptional regulation was also validated by analyzing the protein level of these transcriptional regulators by Western blot analysis (figure 36).

Figure 36: Effect of largazole on protein levels of key transcriptional regulators. Various concentrations of largazole were incubated with HCT-116 cells for 24 hours and protein levels analyzed by Western blot analysis. Figure reprinted with permission from ref. 135.
Largazole also demonstrated effective *in vivo* activity in solid tumors. Immunoblot analysis of HCT-116 xenograft mouse models showed histone H3 acetylation at doses starting at 5 mg/kg, with no toxicity at the highest tested concentration (50 mg/kg). Mice treated at 5 mg/kg over 2 weeks also showed severely inhibited tumor growth, as well as induction of caspase-3 which leads to apoptosis (figure 37). The large therapeutic window shown in these mouse models suggest that largazole may be a safer anti-cancer agent compared to some of its more toxic counterparts.

**Figure 37**: *In vivo* efficacy of largazole in a HCT-116 xenograft mouse model. (A) Tumor-bearing mice (*n* = 9) were injected intraperitoneally with largazole (5 mg/kg i.p.) or DMSO (vehicle) daily. (B) Xenograft tumor sections stained for caspase-3 expression in control mice. (C) Xenograft tumor sections stained for caspase-3 expression in mice injected with largazole. Figure reprinted with permission from ref.135
2.1.6 Other biological activities of largazole

The ability of HDACs to affect gene regulation has led to reports that these enzymes play key roles in osteogenesis and could potentially be targeted for the treatment of bone disease. Largazole in particular has demonstrated in vitro and in vivo osteogenic activity. Upon treatment with largazole, murine pluripotent mesenchymal precursor cells (C2C12), used as an in vitro model of osteogenesis, have shown a dose-dependent expression induction of several osteoblast differentiation biomarker, such as alkaline phosphatase (ALP) and osteopontin (OPN). Co-treatment of largazole with noggin, an osteogenic bone morphogenic protein (BMP) inhibitor, ALP induction was significantly reduced (figure 38).

This decrease of ALP expression induction due to noggin indicated that largazole’s osteogenic activity could result from an increase in BMP expression. Additionally, runt-related transcription factor 2 (Runx2) has been shown to be critical in osteogenesis by controlling bone-specific genes like ALP and OPN. In order to rationalize the osteogenic activity as well as the ALP and OPN induction, the effect of largazole on Runx2 and BMPs was examined. At 50 nM treatment of largazole, Runx2 activity increased 29-fold, while the mRNA level increased 3-fold. At the same concentration, largazole also upregulated the expression of BMP-2, 4, 6, 7, and 9 (figure 39). This suggest that the osteogenic activity of largazole is mediated through the
upregulation of Runx2 and BMP expression and activity. Largazole was also shown to inhibit the formation of multinucleated osteoclasts. This induction of bone formation along with suppression of bone resorption suggests a dual nature of largazole in osteogenesis.

![Graph A: Runx2 activity vs Largazole concentration](image1)

![Graph B: mRNA expression levels](image2)

**Figure 39:** Effect of largazole on the Runx2 activation and mRNA induction of Runx2 and BMPs in C2C12 cells. (A) Runx2 activity was measured by luciferase reporter assay. (B) mRNA expression levels of Runx2 and BMPs when treated with largazole or rhBMP-2 (300 ng/mL) as a reference of osteoblastogenesis inducer. Figure reprinted with permission from ref.137

Perhaps more impressive, however, is the ability of largazole to induce *in vivo* bone formation in mouse and rabbit calvarial bones.137 In the mouse models, largazole soaked collagen sponges were implanted in calvarial bones, which lead to woven bone formation over the periosteum (figure 40). In the rabbit fracture healing model, macroporous biphasic calcium phosphates (MBCPs) in the presence of largazole led to new bone formation.
Figure 40: *In vivo* osteogenic activity of largazole in mouse calvaria. Collagen sponges containing 5 μL of PBS vehicle (A) or largazole (B, 10 μM; C, 50 μM) were placed onto mouse calvarial bones. After a 3-week implantation, the mice were sacrificed, and calvarial bones removed. Arrows indicate region of new woven bone formation. Figure reprinted with permission from ref. 137

Largazole has also shown the ability to inhibit ubiquitin activating enzyme (E1) and consequently the ubiquitination of CDK inhibitor p27. 138 This ability is particularly interesting, because ubiquitination-dependent degradation of p27 is a phenomenon present in approximately 50% of all human cancers. Additionally, the mode of inhibition of E1 is extremely different as structure–activity relationship studies revealed that both the aliphatic tail and the macrocycle are needed to inhibit E1, suggesting a unique mechanism of action. Additionally, largazole displayed remarkable selectivity for human E1 over related enzymes such as SUMO E1.

HDAC inhibitors, including largazole have also demonstrated the ability to sensitize EBV+ tumor cells to cytotoxicity in the presence of the anti-herpes drug ganciclovir (GCV). 139 In alkali-induced corneal neovascularization (CNV) mouse models, largazole displayed anti-angiogenic activity by downregulating the expression of the pro-angiogenic factors but upregulating the expression of the anti-angiogenic factors. 140 It also demonstrated inhibition of migration, proliferation and tube formation by human microvascular endothelial cells (HEMC-1) *in vitro*. Lastly, largazole was also able to induce apoptosis in hepatic stellate cells (HSCs) in liver fibrosis as well as ameliorating liver fibrosis models *in vivo*. 141
2.1.7 Previous analogues for the examination of isoform-selectivity of largazole

Since its discovery, largazole has been an extremely popular target for synthetic and biological groups alike. Through many different studies that have been conducted, it has been established that largazole is one of the most potent and selective HDAC inhibitors with a wide variety of potential therapeutic applications. We have elucidated many aspects of largazole’s mechanism of action and its structure–activity relationships. Access to structural information in the form of the HDAC8–largazole thiol X-ray co-crystal structure has provided further structural rationale for these structure–activity relationships. However, while largazole demonstrates excellent class I-selectivity for HDAC inhibition, isoform-selectivity has not been improved significantly to date.

Some groups have looked at the effect of structural modifications on the HDAC isoform-selectivity of largazole. For example, as mentioned previously (page 58), Jiang and co-workers prepared several largazole analogues that contain a triazole or a tetrazole moiety instead of the methyl thiazoline group. These compounds were tested for their ability to inhibit HDAC1 and HDAC9 in order to assess the isoform-selectivity of the analogues. While largazole intrinsically showed a strong preference towards HDAC1 over HDAC9 (57-fold), that selectivity was increased in the case of tetrazole analogue 79 (346-fold), and decreased in the case of triazole analogue 78 (14-fold) (table 13). While the analogues were not as potent as previous reports of largazole thiol, they demonstrated that certain structural modifications could affect the potency of one isoform more severely than another to modulate the isoform-selectivity.
Table 13: HDAC inhibition and isoform selectivity of largazole and thiazoline analogues (IC$_{50}$, μM). Data from ref. $^{132}$

<table>
<thead>
<tr>
<th></th>
<th>HDAC1</th>
<th>HDAC9</th>
<th>HDAC9/HDAC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Largazole</td>
<td>0.146</td>
<td>8.33</td>
<td>57.1</td>
</tr>
<tr>
<td>78</td>
<td>2.35</td>
<td>32.0</td>
<td>13.6</td>
</tr>
<tr>
<td>79</td>
<td>0.1</td>
<td>34.6</td>
<td>346.0</td>
</tr>
</tbody>
</table>

Williams and co-workers have also prepared and tested various analogues for their isoform-selectivity.$^{133,134}$ While replacement of the ester linkage with an amide linkage or the thiazole replacement with a pyridine (80–83) did not result in any significant selectivity changes, oxazole analogue 84 showed inhibitory activity against HDAC7 while maintaining similar activity against HDAC1 compared to previous reports. Largazole thiol has previously been reported to be inactive against HDAC7. While the HDAC7 activity of analogue 84 is much less potent than its HDAC1 activity (HDAC1 IC$_{50}$: 4.4 nM, HDAC7 IC$_{50}$: 0.96 μM), it is interesting to see that a single atom replacement can elicit inhibitory activity against a previously inactive isoform of HDAC (figure 41).
**Figure 41:** Structures of largazole isostere/pyridine analogues and oxazole analogue. Oxazole analogue 84 shows weak HDAC7 inhibitory activity no present in largazole thiol.\textsuperscript{133,134}

**Table 14:** HDAC inhibition and isoform selectivity of largazole disulfide analogues (IC\textsubscript{50}, \(\mu\text{M}\)). Data from ref.\textsuperscript{130}

<table>
<thead>
<tr>
<th>R</th>
<th>HDAC1</th>
<th>HDAC7</th>
<th>HDAC7/HDAC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Et</td>
<td>0.0246</td>
<td>22.2</td>
<td>902.4</td>
</tr>
<tr>
<td>n-Bu</td>
<td>0.0338</td>
<td>5.57</td>
<td>164.8</td>
</tr>
<tr>
<td>t-Bu</td>
<td>0.131</td>
<td>4.0</td>
<td>30.5</td>
</tr>
<tr>
<td>n-Hex</td>
<td>0.015</td>
<td>7.1</td>
<td>473.3</td>
</tr>
<tr>
<td>n-Oct</td>
<td>0.009</td>
<td>2.92</td>
<td>324.4</td>
</tr>
</tbody>
</table>

The disulfide analogues discussed earlier (page 56) prepared by Jiang and co-workers also showed the ability to modulate HDAC1 selectivity over HDAC7.\textsuperscript{130} By modifying the disulfide moiety on largazole thiol from a tert-butyl group to an ethyl group, the HDAC1 selectivity over HDAC7 increased from 31-fold to 902-fold (table 14). This data was particularly...
interesting due to the fact that all analogues possess the same active compound (largazole thiol) masked by a disulfide group, making it extremely difficult to leverage any structural differences to result in isoform-selectivity.

The studies described above have provided us with valuable information regarding the structure–activity relationship of largazole in the context of isoform–selectivity. However, the ultimate goal of developing a single isoform-selective largazole analogue has not yet been achieved. From a therapeutic standpoint, a single isoform-selective HDAC inhibitor would be extremely desirable as it would minimize off target effects by only inhibiting a single target. In chemical biology, possessing a compound that is capable of inhibiting only one isoform of a family of enzymes would be useful in determining the role that particular isoform has in a certain molecular pathway. The ability to examine the inhibition of just one enzyme instead of a group of enzymes would be an invaluable tool in chemical biology. Here, efforts towards the design, synthesis, and biological characterization of a single isoform-selective HDAC inhibitor will be described.

2.2 Results and Discussion

2.2.1 Synthetic goals

Compared to other structural modifications in largazole analogues, the valine subunit has been relatively underexplored. While valine replacements have been synthesized, many of them have similar chemical functionalities and have not been studied for their isoform-selectivity. Nonetheless, previous structure–activity relationships from valine replacement analogues have shown that this position can withstand significant variations with minimal loss in HDAC
inhibitory activity, as long it is an L-amino acid.\textsuperscript{123,126,127} Therefore, several amino acid analogues with a variety of chemical functionalities were designed in order to investigate the effect of these functionalities on the class I HDAC isoform-selectivity.\textsuperscript{142}

Another approach that was utilized was the computational docking of largazole analogues with HDAC8. While largazole displays class I-selectivity, HDAC8 is distinctly different from HDACs 1, 2, and 3, despite sequence homology. This is reflected by the fact that largazole’s ability to inhibit HDAC8 is much lower than the other 3 HDACs.\textsuperscript{110} Previous docking studies have relied on homology models of HDAC enzymes, however, with access to structural information that the X-ray crystal structure of HDAC8-largazole thiol complex provides,\textsuperscript{125} more accurate docking experiments could be performed. With the help of Dr. Robin Chaudret in the Yang group at Duke University, several computational largazole analogues were docked in the HDAC8 active site and the binding affinity was quantified. This led to one promising lead compound which was synthesized and tested for its HDAC inhibitory activity.

\subsection*{2.2.2 Retrosynthetic analysis}

Initially, four C2 analogues were designed, for which the valine subunit was replaced by several chemically different amino acids. The amino acids that were chosen were phenylalanine (aromatic hydrophobic), tyrosine (aromatic hydrophilic), histidine (basic), and aspartic acid (acidic). Since it was already known that the thioester group in largazole only serves as a protecting group for the free thiol, we decided to synthesize and test the free thiols of these analogues (85–88). The octanoyl thioester versions of these specific analogues also have shown to retain anti-proliferative activity in HCT-116 colon cancer cells.\textsuperscript{135} In order to access these analogues, we amended the original total synthesis that was reported by our group in 2008. Since
the thiol linker is conserved in all analogues, we envisioned a divergent synthesis that took advantage of common advanced intermediate 89, which contains a trityl-protected thiol.

Esterification of the appropriate amino acid with 89 would result in easy access to analogues 85–88. This approach would also allow us to avoid the low yielding late stage cross metathesis step. Intermediate 89 would be easily accessible through coupling of *syn*-aldol product 90 and known thiazole-thiazoline 38 (scheme 10).

Scheme 10: Retrosynthetic analysis of C2 analogues (85–88).
HDAC8-selective analogue 91 was designed by computationally docking various largazole analogues in the active site of HDAC8, utilizing the X-ray co-crystal structure of the HDAC8–largazole thiol complex. The addition of the hydroxyl group showed an increase in binding affinity, calculated by Averaged NonCovalent Interaction (aCNI) analysis, most likely due to the existence of many polar amino acids that can participate in hydrogen bonding in the region of the hydroxyl group. We envisioned that analogue 91 could be built from commercial Fmoc-L-valine (60), known thiazolidinethione 90, and thiazole-thiazoline unit 92. Intermediate 92 is closely related to thiazole-thiazoline 38, but contains a protected hydroxyl group. This can be accessed by the condensation of known (R)-2-methyl cysteine (93) and nitrile 94, which can be prepared from commercially available D-serine 95 (scheme 11).

Scheme 11: Retrosynthetic analysis of HDAC8-selective analogue (91).
2.2.3 Synthesis of 1\textsuperscript{st} generation C2 analogues

Synthesis commenced with the coupling of known thiazole-thiazoline unit 38 with syn-aldol product 90. Yamaguchi esterification with commercially available Fmoc-L-phenylalanine (96) and Fmoc-L-tyrosine(\textit{O}-\textit{tBu}) (97) produced the acyclic precursors 98 and 99. Subsequent base-mediated methyl ester hydrolysis, Fmoc deprotection, and HATU-mediated macrolactamization formed trityl-protected thiols 100 and 101. Finally trityl deprotection, and in the case of 101, simultaneous \textit{t}-butyl ether cleavage, resulted in the completion of the phenylalanine and tyrosine analogues (85 and 86) (scheme 12).

Scheme 12: Synthesis of phenylalanine and tyrosine analogues (85 and 86).
Unfortunately, the same reaction conditions were not suitable for the preparation of the histidine and aspartic acid analogues (87 and 88). Specifically, during the base-mediated hydrolysis of the methyl ester, cleavage of the internal ester bond formed during the Yamaguchi esterification was observed (scheme 13). Addition of lower concentrations of lithium hydroxide at lower temperatures were also not successful in achieving selective methyl ester cleavage and resulted in the formation of the undesired side-product in all cases. In order to overcome this issue the methyl ester was substituted with 9-fluorononylmethyl ester (Fm). Since Fm esters are easily deprotected by milder bases such as diethylamine and piperidine, the internal ester bond should stay intact during deprotection.

Scheme 13: Internal ester bond cleavage during cyclization of histidine and aspartic acid analogues (87 and 88).
Scheme 14: Synthesis of histidine and aspartic acid analogues (87 and 88).

EDCI-mediated coupling of thiazole-thiazoline 50 with 9-fluorenemethanol resulted in Fm protected thiazole-thiazoline 102, which was then Boc-deprotected and coupled to syn-aldol
product 90. Yamaguchi esterification of intermediate 103 with Fmoc-L-histidine(N-Boc) (104) or Fmoc-L-aspartic acid(\(O\text{-tBu}\)) (105) resulted in acyclic precursors 106 and 107. Simultaneous Fm/Fmoc deprotection, followed by HATU-mediated macrolactamization afforded trityl protected thiols 108 and 109. Final deprotection of the trityl groups, as well as the Boc group, completed the synthesis of histidine analogues 87 (scheme 14).

Silica gel purification of aspartic acid analogue 88 was not successful, as no product eluded from the stationary phase, potentially due to the highly polar nature of the compound. Therefore, the trityl deprotection and tert-butyl ester cleavage steps were separated. First, TFA-mediated trityl deprotection in the presence of TIPS was performed and the corresponding thiol was isolated. With this intermediate in hand, TFA was once again utilized to remove the tert-butyl group. Upon completion of the reaction, volatiles were evaporated from the reaction mixture to afford aspartic acid analogue 88 in acceptable purity (based on NMR) for further use (scheme 14). With all four analogues in hand, we next turned our attention to testing the HDAC inhibitory activity of these C2 analogues.

**2.2.4 Biological activity of 1\(^{st}\) generation C2 analogues**

The class I HDAC isoform-selectivity profiles of the four C2 analogues were determined with the help of the Luesch group at the University of Florida. In order to accomplish this, the HDAC inhibitory activity of these analogues was tested against class I HDACs (HDACs 1, 2, 3, and 8). The analogues were incubated at various concentrations with recombinant human HDACs, which was followed by incubation with an HDAC developer, a fluorescent HDAC activity probe, to measure the HDAC activity. The inhibition profile shows that all compounds are extremely weak HDAC8 inhibitors. Additionally, all analogues retained comparable potency in HDACs 1, 2,
and 3 to largazole thiol, except for the aspartic acid analogue (88). This analogue experienced a very significant reduction in potency for all HDAC isoforms (table 15). What is perhaps more interesting is the isoform-selectivity of the other analogues. Phenylalanine, tyrosine, and histidine analogues (85–87) all experienced a decrease in potency for HDAC2. Histidine analogue 87 also experienced a potency decrease in HDAC3 to result in a minor preference for HDAC1 over HDACs 2 and 3 (7- and 5.5-fold, respectively) (table 16).

**Table 15:** Class I HDAC inhibition of 1st generation C2 largazole analogues (IC$_{50}$, nM).

<table>
<thead>
<tr>
<th></th>
<th>HDAC1</th>
<th>HDAC2</th>
<th>HDAC3</th>
<th>HDAC8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Largazole Thiol</td>
<td>0.40</td>
<td>0.90</td>
<td>0.70</td>
<td>102</td>
</tr>
<tr>
<td>Phenylalanine (85)</td>
<td>0.29</td>
<td>1.70</td>
<td>0.68</td>
<td>NI$^a$</td>
</tr>
<tr>
<td>Tyrosine (86)</td>
<td>0.21</td>
<td>1.10</td>
<td>0.38</td>
<td>NI$^a$</td>
</tr>
<tr>
<td>Histidine (87)</td>
<td>0.20</td>
<td>1.40</td>
<td>1.10</td>
<td>NI$^a$</td>
</tr>
<tr>
<td>Aspartic Acid (88)</td>
<td>39</td>
<td>150</td>
<td>100</td>
<td>NI$^a$</td>
</tr>
</tbody>
</table>

$^a$ No inhibition up to 1 μM.

**Table 16:** HDAC1 selectivity of largazole thiol and 1st generation C2 largazole analogues.

<table>
<thead>
<tr>
<th></th>
<th>HDAC2/HDAC1</th>
<th>HDAC3/HDAC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Largazole Thiol</td>
<td>2.3</td>
<td>1.8</td>
</tr>
<tr>
<td>Phenylalanine (85)</td>
<td>5.9</td>
<td>2.3</td>
</tr>
<tr>
<td>Tyrosine (86)</td>
<td>5.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Histidine (87)</td>
<td>7.0</td>
<td>5.5</td>
</tr>
<tr>
<td>Aspartic Acid (88)</td>
<td>3.9</td>
<td>2.6</td>
</tr>
</tbody>
</table>
Although the difference in potency is not significant enough to call the histidine analogue (87) an HDAC1-specific inhibitor, it prompted further examination. Based on the HDAC inhibition profile of these 1st generation C2 analogues, a second set of largazole analogues was designed in order to investigate different potential interactions between HDAC1 and the imidazole ring of the histidine analogue (87). Boc-histidine analogue 110 was designed to investigate any potential hydrogen bonding effects of the imidazole ring, by blocking this interaction with an additional Boc group. Diaminobutyric acid and ornithine analogues (111 and 112) are primary amine analogues, where the nitrogens mimic the position of the two different nitrogens in the imidazole ring of the histidine analogue (87). Additionally, the corresponding Boc protected analogues (113 and 114) were also prepared (figure 42). We envisioned that these 2nd generation C2 analogues would be prepared in a similar fashion to the 1st generation analogues.

![Structures of 2nd generation C2 analogues](image)

**Figure 42:** Structures of 2nd generation C2 analogues (110–114).
2.2.5 Synthesis of 2\textsuperscript{nd} generation C2 analogues

Synthesis of the proposed 2\textsuperscript{nd} generation C2 analogues proceeded in a very similar divergent fashion as the 1\textsuperscript{st} generation analogues. Yamaguchi esterification of Fmoc-L-diaminobutyric acid(N-Boc) (115) or Fmoc-L-ornithine(N-Boc) (116) with advanced intermediate 103, resulted in acyclic precursors 117 and 118. Fm/Fmoc deprotection and HATU-mediated macrolactamization afforded trityl-protected thiols 119 and 120. All analogues 111–114 were accessible from these two intermediates. By performing the TFA-mediated deprotection at 0 °C in the presence of TIPS for a short period of time (15 minutes), selective trityl deprotection in the was achieved in order to access the Boc-diaminobutyric acid and Boc-ornithine analogues (113 and 114). Further deprotection of these analogues in the presence of TFA at room temperature for an extended period of time (1 hour) resulted in Boc deprotection to result in diaminobutyric acid and ornithine analogues 79 and 80. Purification of these last two analogues proved problematic as silica gel purification did not result in any final product, potentially due to the high polarity of the compounds. In order to overcome this issue, HPLC purification was employed to complete the synthesis of these analogues (scheme 14).
Scheme 15: Synthesis of diaminobutyric acid and ornithine analogues (111–114).

We initially designed methyl-histidine analogue 121 instead of the Boc-histidine analogue (110). However, various attempts to produce analogue 121 were unsuccessful. We had envisioned the conversion of advanced intermediate 103 to the desired analogue by a similar route as the other C2 analogues. However, neither Yamaguchi esterification nor EDCI/DMAP mediated coupling of Fmoc-L-(N-Me)-histidine and intermediate 103 were successful. Additionally, esterification utilizing intermediate 122 (diastereomer of advanced intermediate 104) under Mitsunobu inversion conditions were also attempted with no success. Lastly,
methylidation of histidine analogue 124 was attempted under several reaction conditions. These attempts were once again met with no success (scheme 16).

We also concurrently tried to prepare pyridine analogue 126. Similar to methyl-histidine analogue 121, we envisioned that the lack of a basic nitrogen would elucidate the role of hydrogen bonding in the perceived HDAC1-selectivity of the histidine analogue (87). However, once again, we encountered issues with the incorporation of Fmoc-3-(2'-pyridyl)-L-alanine into

Scheme 16: Unsuccessful attempts for the synthesis of methyl-histidine analogue 121.
the compound. As before, Yamaguchi esterification, EDCI/DMAP mediated coupling and Mitsunobu inversion conditions all failed to produce intermediate 127 (scheme 17).

Scheme 17: Unsuccessful attempts for the synthesis of pyridine analogue 126.

Due to the lack of success for the synthesis of methyl-histidine and pyridine analogues (121 and 126), we decided to change the design of the molecule to the Boc-histidine analogue (110). Based on the synthesis of diaminobutyric acid and ornithine analogues (111 and 112), we envisioned that advanced intermediate 108, used for the synthesis of the histidine analogue (87), could be selectively trityl deprotected in the presence of the Boc-group. Additionally, the presence of the Boc-group should still block the hydrogen bonding ability of the imidazole, thereby allowing us to investigate the effect of hydrogen bonding on HDAC inhibition. The approach previously used was successful as low reaction temperature (0 °C) and short reaction times (15 minutes), resulted in selective trityl deprotection in a similar manner as diaminobutyric
acid and ornithine analogues (111 and 112) (scheme 17). With the 2nd generation C2 analogues in hand, their class I HDAC inhibitory activity was once again tested.

![Scheme 18: Synthesis of Boc-histidine analogue 110.](image)

**2.2.6 Biological activity of 2nd generation C2 analogues**

Once again all analogues were tested against class I HDACs (HDACs 1, 2, 3, and 8) with the help of the Luesch group at the University of Florida as previously described (page 78) (table 17). As before all analogues showed very weak HDAC8 inhibitory activity compared to the other three class I HDAC isoforms. The Boc-histidine analogue (110) did not show a significant change in potency compared to the histidine analogue (87), indicating that the hydrogen bonding donating ability of the imidazole nitrogen may not be an essential interaction for HDAC inhibition or isoform-selectivity. Also all ornithine and diaminobutyric acid analogues (111–114), designed to mimic the nitrogens in the imidazole ring, showed significant decreases in potency and minimal signs of isoform-selectivity. However, certain trends can be observed from this HDAC inhibitory data. For all HDAC isoforms, ornithine analogue 112 shows higher potency than diaminobutyric acid analogue 111. The same trend is observed when comparing the corresponding Boc protected analogues (113 and 114). Additionally, the existence of a Boc group
increases potency in all isoforms when compared to the Boc deprotected analogues. This seems to suggest that potency of HDACs 1, 2, and 3 inhibition is correlated to steric bulk of the C2 substituent.

Table 17: Class I HDAC inhibition of 2nd generation C2 largazole analogues (IC50, nM).

<table>
<thead>
<tr>
<th></th>
<th>HDAC1</th>
<th>HDAC2</th>
<th>HDAC3</th>
<th>HDAC8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Largazole Thiol</td>
<td>0.40</td>
<td>0.90</td>
<td>0.70</td>
<td>102</td>
</tr>
<tr>
<td>Boc-histidine (110)</td>
<td>0.62</td>
<td>1.10</td>
<td>1.10</td>
<td>540</td>
</tr>
<tr>
<td>Diaminobutyric acid (111)</td>
<td>5.50</td>
<td>21.00</td>
<td>7.80</td>
<td>NIa</td>
</tr>
<tr>
<td>Ornithine (112)</td>
<td>3.30</td>
<td>6.30</td>
<td>6.00</td>
<td>NIa</td>
</tr>
<tr>
<td>Boc-diaminobutyric acid (113)</td>
<td>2.30</td>
<td>3.10</td>
<td>4.20</td>
<td>NIa</td>
</tr>
<tr>
<td>Boc-ornithine (114)</td>
<td>0.96</td>
<td>1.40</td>
<td>1.80</td>
<td>790</td>
</tr>
</tbody>
</table>

a No inhibition up to 1 μM.

2.2.7. Synthesis of HDAC8-selective analogue

As stated in the retrosynthetic analysis, the synthesis of the HDAC8-selective analogue was to start with a protected D-serine derivative. Therefore, we decided to name this compound 91, the serine analogue. The serine analogue (91) was designed with the help of Dr. Robin Chaudret in the Weitao Yang group at Duke University by docking studies of several structural modifications of largazole in the active site of HDAC8, utilizing the X-ray co-crystal structure of the HDAC8–largazole thiol complex. By calculating the binding affinity by Averaged NonCovalent Interaction (aCNI) analysis it was revealed that the additional hydroxyl group increased the ligand–protein binding affinity significantly. This was most likely due to the
existence of many polar amino acids that can participate in hydrogen bonding in the region of the hydroxyl group, as well as the stabilization of several water molecules in the nearby region.

After screening of a few protecting groups, we decided that TBDPS would be the most appropriate protecting group for the synthesis. Initial synthetic efforts utilizing a PMB ether were not successful due to the inability to selectively cleave the Boc group on thiazole-thiazoline intermediate 128 during coupling with syn-aldol product 90. During this reaction two coupling products were produced (129 band 130) (scheme 19). Both of them did not contain the PMB protecting group and therefore could not be carried further in the synthesis.

Scheme 19: Problem with use of PMB ether for the synthesis of the serine analogue (91).

The use of a TBDPS protecting group proved to be much more applicable for this analogue. Known TBDPS protected Boc-D-serine 131 converted to thioamide 132, which was subjected to Hantzsch thiazole condensation conditions to form thiazole ethyl ester 133. The ethyl ester was used to fashion nitrile 134, which was condensed with known (R)-2-methyl cysteine (135) and Fm protected to access the desired thiazole-thiazoline intermediate 136 (scheme 20).
Scheme 20: Synthesis of thiazole-thiazoline subunit (136) for the serine analogue (91).

With thiazole-thiazoline intermediate 136 in hand, the completion of the synthesis progressed similarly to other largazole analogues described above (page 82). Coupling with syn-adol product 90 was followed by Yamaguchi esterification with commercially available Fmoc-L-valine 138, to result in the acyclic precursor 139. Fm/Fmoc deprotection was followed by HATU-mediated macrolactamization to result in the trityl and TBDPS protected intermediate 141. Desytilation through TBAF and TFA-mediated trityl group deprotection completed the synthesis of the serine analogue (91) (scheme 21).
2.2.8 Biological activity of HDAC8-selective analogue

In order to assess the HDAC8-selectivity of serine analogue 91, it was tested for its HDAC inhibitory activity against HDAC1 and HDAC8 with the help of the Luesch group at the University of Florida as described for the C2 largazole analogues previously (page 78). This activity was then compared to the activity of largazole thiol to see the effect of the hydroxyl
group on the HDAC8-selectivity. Unfortunately, for both HDAC1 and HDAC8, a small decrease in potency was observed in the serine analogue (91) (table 18) compared to largazole thiol. The analogue was not able to inhibit HDAC8 more efficiently than largazole, nor was it less active against HDAC1 in order to provide HDAC8 selectivity.

**Table 18**: HDAC inhibition of largazole thiol and the serine analogue (91) (IC$_{50}$, nM).

<table>
<thead>
<tr>
<th></th>
<th>HDAC1</th>
<th>HDAC8</th>
</tr>
</thead>
<tbody>
<tr>
<td>largazole thiol</td>
<td>0.25</td>
<td>1,551</td>
</tr>
<tr>
<td>serine analogue (91)</td>
<td>0.65</td>
<td>2,166</td>
</tr>
</tbody>
</table>

The serine analogue (91) proved to be an all-around weaker HDAC inhibitor for both isoforms rather than an HDAC8-selective inhibitor. The docking studies used for the design of this molecule predicted increased interactions between the hydroxyl group and a variety of polar amino acids present in the area of the structural modification on the HDAC8 surface. It is clear that this predicted model has not increased the binding affinity of the inhibitor to its molecular target. However, despite the decrease in potency, this data shows us that the region of the additional hydroxyl group of the analogue does not completely destroy HDAC activity. This suggests that this region can be subjected to future structural modifications in efforts to find an HDAC8-selective inhibitor. It is possible that a functional group more polar than an alcohol could be utilized to leverage increased interaction with key amino acids found in this particular region.

**2.3 Conclusion**

In summary, we designed, synthesized, and biologically characterized several largazole analogues in pursuit of developing an isoform selective HDAC inhibitor. The valine substituent in
the C2 position was replaced with four amino acids with distinct chemical functionalities. In order to access these analogues, the original synthetic route for largazole was revisited and modified to a more divergent synthetic approach. The four analogues (85–88) were prepared and tested for the in vitro HDAC inhibitory activity. Based on the class I HDAC inhibition profile, we determined that the histidine analogue (87) showed promise as a starting point for an HDAC1-selective inhibitor. In order to further study possible interactions of the histidine analogue (87) with HDAC1, several 2nd generation analogues were designed and prepared (110–114). The class I HDAC inhibition profile of the 2nd generation analogues provided us with further important structure–activity relationships that may be used for future inhibitor design.

While a single isoform-selective inhibitor was not discovered, several new structure–activity relationships have been elucidated. First, the largazole scaffold has an innate preference for HDACs 1, 2, and 3 over HDAC8. This was concluded based on the fact that all analogues showed much lower potency against minimal of HDAC1-selectivity, which was further examined. It was concluded that the hydrogen bonding of the imidazole ring is not an essential interaction for HDAC inhibition. Most importantly, it was shown that steric bulk at the C2 position plays a vital role in efficient HDAC inhibition. This is supported by the fact that the ornithine analogue (112) is more potent than the diaminobutyric acid analogue (111) in all HDAC isoforms tested. The same trend was observed as the corresponding Boc-protected ornithine analogue (114) is more potent than the Boc-protected diaminobutyric acid analogue (113). Lastly, the Boc-protected analogues are all more potent and any of the deprotected analogues. This hypothesis of optimal steric bulk is also further supported by the significantly weaker HDAC inhibitory activity of the aspartic acid analogue (88) compared to other 1st generation analogues (85–87).

In order to develop an HDAC8-selective inhibitor, serine analogue (91) was designed by computational docking studies using the HDAC8–largazole thiol complex X-ray crystal
structure. This analogue was then synthesized and tested for its *in vitro* HDAC inhibitory activity against HDACs 1 and 8. However, it was unsuccessful in reversing largazole’s innate preference for HDAC1 over HDAC8. The predicted increase in binding affinity between the additional hydroxyl group and polar amino acids on the surface of HDAC8 did not increase the HDAC inhibition of the analogue. However, the serine analogue (91) showed similar potency as largazole thiol with minimal activity loss. Keeping the results of the serine analogue (91) in mind, currently, there are ongoing docking experiments for future design of HDAC8-selective largazole analogues, with the help of the Yang group at Duke University.

Along with the C2 analogues described above we have identified two regions in the largazole scaffold that can be modified without detrimental loss in potency. This could play an important role in the future design of largazole analogues in efforts to develop an isoform-selective HDAC inhibitor. Additionally, the change in the synthetic route from a methyl ester to an Fm ester should be applicable in the future for the synthesis of diverse largazole analogues due to their ability to avoid global ester hydrolysis during macrolactamization.

### 2.4 Experimental section

**General Methods**

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich, Acros, Chem-Impex, or Fischer and were used without further purification. All solvents were ACS grade or better and used without further purification except THF which was freshly distilled each time before use. Thin layer chromatography was performed with glass backed silica gel (60 Å) plates purchased from Whatman and visualized with 254 nm UV light. All chromatographic purifications were conducted via flash chromatography using ultra-pure silica gel (230-400 mesh,
60 Å) purchased from Silicycle as the stationary phase unless otherwise noted. All spectra were recorded in CDCl$_3$ unless otherwise noted, using a 400 MHz (Varian) or 500 MHz (Bruker) NMR spectrometer. All NMR shifts are given in ppm, and all $J$-values are given in Hz. High-resolution mass spectra (HRMS) were obtained by an Agilent 6224 time-of-flight liquid chromatography-electrospray ionization spectrometer. Infrared (IR) absorption spectra were determined with a Thermo–Fisher (Nicolet 6700) spectrometer. Optical rotation values were measured with a Rudolph Research Analytical (A21102. API/1W) polarimeter.

**Preparation of 89**

![Chemical structures and reaction scheme]

To a solution of 38 (950 mg, 2.56 mmol) in CH$_2$Cl$_2$ (16 mL) was added TFA (4.0 mL) at 25 °C. After stirring for 2 h at 25 °C, the reaction mixture was concentrated in vacuo and washed with Et$_2$O. To a solution of the crude mixture in CH$_2$Cl$_2$ (50 mL) were added 90 (1.1 g, 1.96 mmol) in CH$_2$Cl$_2$ (5 mL) and DMAP (1.2 g, 9.79 mmol) at 25 °C. After stirring for 3 h at 25 °C, the reaction was quenched by the addition of H$_2$O. The layers were separated and the aqueous layer was extracted with CH$_2$Cl$_2$. The combined organic layers were dried over anhydrous Na$_2$SO$_4$ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes/CH$_3$OH = 10/10/1) to afford 89 (1.2 g, 91% for 2 steps): $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.91 (s, 1 H), 7.40–7.41 (m, 6 H), 7.26–7.29 (m, 6 H), 7.19–7.22 (m, 3 H), 5.55 (ddd, $J$ = 7.0, 7.0, 15.0 Hz, 1 H), 5.42 (dd, $J$ = 5.5, 15.0 Hz, 1 H), 4.68 (dd, $J$ = 5.5, 5.5 Hz, 1 H), 4.45 (m, 1 H), 3.86 (d, $J$ = 11.0 Hz, 1 H), 3.79 (s, 3 H), 3.26 (d, $J$ = 11.5 Hz, 1 H), 2.44 (dd, $J$ = 4.5, 15.0 Hz, 1 H).
Hz, 1 H), 2.38 (dd, J = 8.0, 15.0 Hz, 1 H), 2.21 (dd, J = 7.0, 7.0 Hz, 2 H), 2.06 (ddd, J = 7.0, 7.0, 7.0 Hz, 2 H), 1.64 (s, 3 H).

**Preparation of 98**

![Reaction Scheme]

To a cooled (0 ºC) solution of 96 (35.4 mg, 0.092 mmol) in THF (5 mL) were added 2,4,6-trichlorobenzoyl chloride (15.3 µL, 0.0980 mmol) and Et$_3$N (14.6 µL, 0.105 mmol). After stirring for 2 h at 0 ºC, 89 (43.9 mg, 0.065 mmol) in THF (3 mL) and DMAP (9.58 mg, 0.078 mmol) were added at 0 ºC. After stirring for 1 h at 25 ºC, the reaction was quenched by the addition of saturated NH$_4$Cl solution and diluted with EtOAc. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na$_2$SO$_4$ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes/CH$_3$OH = 10/10/1) to afford 98 (62.7 mg, 92%): $^1$H NMR (400 MHz, CDCl$_3$) δ 7.85 (s, 1H), 7.73 (d, J = 7.6 Hz, 2H), 7.51 (dd, J = 7.2, 3.2 Hz, 2H), 7.37 (dd, J = 7.6, 7.6 Hz, 8H), 7.23–7.32 (m, 9H), 7.18 (dd, J = 7.2, 7.2 Hz, 5H), 7.04 (d, J = 6.4 Hz, 2H), 6.85 (dd, J = 6.0, 6.0 Hz, 1H), 5.45–5.55 (m, 2H), 5.32 (dd, J = 15.4, 7.2 Hz, 1H), 4.63 (d, J = 5.6 Hz, 2H), 4.43 (dd, J = 13.8, 6.4 Hz, 1H), 4.37 (dd, J = 10.8, 7.2 Hz, 1H), 4.28 (dd, J = 10.6, 6.8 Hz, 1H), 4.14 (dd, J = 6.8, 6.8 Hz, 1H), 3.83 (d, J = 11.2 Hz, 1H), 3.75 (s, 3H), 3.21 (d, J = 11.0 Hz, 1H), 3.02 (dd, J = 14.0, 6.8 Hz, 1H), 2.96 (dd, J = 14.0, 6.8 Hz, 1H), 2.50 (d, J = 6.0 Hz, 2H), 2.16 (dd, J = 7.6, 7.6 Hz, 2H), 2.03 (br s, 2H), 1.61 (s, 3H).
Preparation of 100

To a cooled (0 °C) solution of 98 (45.1 mg, 0.043 mmol) in THF/H\textsubscript{2}O (4:1, 1.5 mL) was added 0.25 N LiOH (191 µL). After stirring for 3 h at 0 °C, the reaction mixture was acidified by 0.25 N KHSO\textsubscript{4} until the pH of the solution reached 3. After dilution with EtOAc, the layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na\textsubscript{2}SO\textsubscript{4} and concentrated \textit{in vacuo}. To a solution of the crude mixture in CH\textsubscript{3}CN (5 mL) was added Et\textsubscript{2}NH (0.5 mL) at 25 °C. After stirring for 2 h at 25 °C, the reaction mixture was concentrated \textit{in vacuo} and washed with toluene. After removal of toluene \textit{in vacuo}, to a solution of the crude mixture in CH\textsubscript{2}Cl\textsubscript{2} (43.4 mL) were added HATU (33 mg, 0.087 mmol) and i-Pr\textsubscript{2}NEt (22.6 µL, 0.130 mmol) at 25 °C. After stirring for 18 h at 25 °C, the reaction mixture was concentrated \textit{in vacuo}. The residue was diluted with H\textsubscript{2}O and EtOAc. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na\textsubscript{2}SO\textsubscript{4} and concentrated \textit{in vacuo}. The residue was purified by three iterations of column chromatography (silica gel, EtOAc/hexanes/CH\textsubscript{3}OH = 10/10/1, EtOAc/hexanes = 1/1, EtOAc 100%) to afford 100 (5.8 mg, 17% for 3 steps): \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) δ 7.63 (s, 1H), 7.16–7.34 (m, 15H), 6.86 (d, J = 6.4 Hz, 2H), 6.77–6.79 (m, 3H), 6.04 (br s, 1H), 5.65–5.72 (m, 2H), 5.39 (dd, J = 15.8, 6.8 Hz, 1H), 4.87 (m, 1H), 4.75 (dd, J = 17.6, 7.2 Hz, 1H), 4.25 (dd, J = 17.4, 4.0 Hz, 1H), 4.11 (d, J = 11.6 Hz, 1H), 3.25 (d, J = 11.2 Hz,
1H), 3.17 (dd, J = 14.0, 3.6 Hz, 1H), 3.03 (dd, J = 14.0, 5.6 Hz, 1H), 2.55–2.68 (m, 2H), 2.14 (dd, J = 7.2, 7.2 Hz, 2H), 2.01 (dd, J = 10.0, 10.0 Hz, 2H), 1.77 (s, 3H).

**Preparation of 85**

![Chemical Structure](image)

To a cooled (0 °C) solution of 100 (10 mg, 0.013 mmol) in CH$_2$Cl$_2$ (3 mL) were added TFA (300 µL) and i-Pr$_3$SiH (5 µL, 0.025 mmol). After stirring for 2 h at 25 °C, the reaction mixture was concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, EtOAc/hexanes/CH$_3$OH = 15/15/1) to afford 85 (4.7 mg, 68%): $^1$H NMR (500 MHz, CDCl$_3$) δ 7.67 (s, 1H), 7.32 (d, J = 7.5 Hz, 1H), 6.89 (d, J = 7.0 Hz, 2H), 6.85 (dd, J = 7.5, 7.5 Hz, 1H), 6.79 (dd, J = 7.5, 7.5 Hz, 2H), 5.93 (dd, J = 4.5, 4.5 Hz, 1H), 5.83 (ddd, J = 15.0, 7.5, 7.5 Hz, 1H), 5.75 (br s, 1H), 5.57 (dd, J = 15.5, 5.5 Hz, 1H), 4.92–4.96 (m, 1H), 4.73 (dd, J = 17.5, 6.5 Hz, 1H), 4.44 (dd, J = 17.5, 4.0 Hz, 1H), 4.14 (d, J = 11.5 Hz, 1H), 3.28 (d, J = 11.5 Hz, 1H), 3.21 (dd, J = 13.5, 3.0 Hz, 1H), 3.07 (dd, J = 13.5, 6.0 Hz, 1H), 2.68 (dd, J = 16.0, 7.0 Hz, 1H), 2.61 (dd, J = 16.0, 3.0, 1H), 2.52 (ddd, J = 7.5, 7.5, 7.5 Hz, 2H), 2.31–2.36 (m, 2H), 1.82 (s, 3H), 1.44 (dd, J = 7.5, 7.5 Hz, 1H).
Preparation of 99

To a cooled (0 °C) solution of 97 (103 mg, 0.225 mmol) in THF (5 mL) were added 2,4,6-trichlorobenzoyl chloride (37.7 µL, 0.241 mmol) and Et₃N (35.8 µL, 0.257 mmol). After stirring for 2 h at 0 °C, 99 (108 mg, 0.161 mmol) in THF (3 mL) and DMAP (23.6 mg, 0.193 mmol) were added at 0 °C. After stirring for 1 h at 25 °C, the reaction was quenched by the addition of saturated NH₄Cl solution and diluted with EtOAc. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes/CH₃OH = 10/10/1) to afford 99 (139 mg, 78%): ¹H NMR (400 MHz, CDCl₃) δ 7.85 (s, 1H), 7.73 (d, J = 7.6 Hz, 2H), 7.51 (d, J = 7.2 Hz, 2H), 7.18–7.42 (m, 19H), 6.94 (d, J = 8.4 Hz, 2H), 6.84 (d, J = 8.4 Hz, 2H), 5.52–5.63 (m, 2H), 5.32 (dd, J = 16.2, 7.6 Hz, 1H), 4.64 (d, J = 6.4 Hz, 2H), 4.41 (dd, J = 14.0, 6.4 Hz, 1H), 4.15–4.43 (m, 4H), 3.82 (d, J = 11.6 Hz, 1H), 3.75 (s, 3H), 3.21 (d, J = 11.6 Hz, 1H), 3.00 (dd, J = 14.0, 6.4 Hz, 1H), 2.89 (dd, J = 14.0, 6.4 Hz, 1H), 2.51 (d, J = 6.0 Hz, 2H), 2.18 (dd, J = 7.2, 7.2 Hz, 2H), 2.05 (m, 2H), 1.60 (s, 3H), 1.29 (s, 9H).
Preparation of 101

To a cooled (0 ºC) solution of 99 (81.5 mg, 0.073 mmol) in THF/H₂O (4:1, 2.5 mL) was added 0.25 N LiOH (322 µL). After stirring for 3 h at 0 ºC, the reaction mixture was acidified by 0.25 N KHSO₄ until the pH of the solution reached 3. After dilution with EtOAc, the layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated in vacuo. To a solution of the crude mixture in CH₃CN (5 mL) was added Et₂NH (0.5 mL) at 25 ºC. After stirring for 2 h at 25 ºC, the reaction mixture was concentrated in vacuo and washed with toluene. After removal of toluene in vacuo, to a solution of the crude mixture in CH₂Cl₂ (73.3 mL) were added HATU (55.9 mg, 0.147 mmol) and i-Pr₂NEt (38.3 µL, 0.220 mmol) at 25 ºC. After stirring for 18 h at 25 ºC, the reaction mixture was concentrated in vacuo. The residue was diluted with H₂O and EtOAc. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was purified by three iterations of column chromatography (silica gel, EtOAc/hexanes/CH₃OH = 10/10/1, EtOAc/hexanes = 1/1, EtOAc 100%) to afford 101 (7.8 mg, 13% for 3 steps): ¹H NMR (400 MHz, CDCl₃) δ 7.66 (s, 1H), 7.15–7.34 (m, 15H), 6.73 (d, J = 8.4 Hz, 2H), 6.33 (d, J = 8.4 Hz, 2H), 5.99 (br s, 1H), 5.62–5.70 (m, 2H), 5.44 (dd, J = 15.4, 6.8 Hz, 1H), 4.86 (m, 1H), 4.71 (dd, J = 17.6, 6.4 Hz, 1H), 4.37 (dd, J = 17.4, 4.4 Hz, 1H), 4.09 (d, J = 11.2 Hz, 1H), 3.24 (d, J = 11.6 Hz, 1H), 3.10 (m, 1H), 2.99 (dd, J = 13.6, 5.2 Hz, 1H), 2.60 (dd, J = 16.0, 6.4 Hz, 1H), 2.53 (dd, J = 17.2, 5.2 Hz, 1H), 2.49 (dd, J = 17.4, 4.4 Hz, 1H), 2.30 (d, J = 11.2 Hz, 1H), 1.97 (dd, J = 17.4, 4.4 Hz, 1H), 1.80 (d, J = 11.2 Hz, 1H), 1.66 (d, J = 11.6 Hz, 1H), 1.48 (d, J = 11.6 Hz, 1H), 1.30 (m, 1H), 1.09 (m, 1H), 0.90 (d, J = 11.2 Hz, 1H), 0.80 (d, J = 11.2 Hz, 1H), 0.70 (m, 1H), 0.50 (m, 1H), 0.40 (m, 1H), 0.30 (m, 1H), 0.20 (m, 1H), 0.10 (m, 1H), 0.00 (m, 1H).
$J = 16.2, 2.8 \text{ Hz, 1H}$, 2.14 (dd, $J = 7.2, 7.2 \text{ Hz, 2H}$), 2.00 (ddd, $J = 6.8, 6.8, 6.8 \text{ Hz, 2H}$), 1.76 (s, 3H), 1.20 (s, 9H).

**Preparation of 86**

![Chemical structure of 101 and 86 with reaction conditions](image)

To a cooled (0 °C) solution of 101 (6 mg, 0.007 mmol) in CH$_2$Cl$_2$ (3 mL) were added TFA (300 μL) and i-Pr$_3$SiH (3 μL, 0.015 mmol). After stirring for 2 h at 25 °C, the reaction mixture was concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, EtOAc/hexanes/CH$_3$OH = 15/15/1) to afford 86 (2.5 mg, 60%): $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 7.98 (s, 1H), 7.29 (d, $J = 8.0 \text{ Hz, 1H}$), 6.64 (d, $J = 8.5 \text{ Hz, 2H}$), 6.27 (d, $J = 8.5 \text{ Hz, 2H}$), 5.85 (ddd, $J = 14.5, 7.0, 7.0 \text{ Hz, 1H}$), 5.75 (dd, $J = 4.5, 4.5 \text{ Hz 1H}$), 6.12 (dd, $J = 15.5, 7.0 \text{ Hz, 1H}$), 5.02 (d, $J = 17.5 \text{ Hz, 1H}$), 4.72–4.76 (m, 1H), 4.34 (d, $J = 17.5 \text{ Hz, 1H}$), 3.93 (d, $J = 11.5 \text{ Hz, 1H}$), 3.35 (d, $J = 11.5 \text{ Hz, 1H}$), 3.08 (dd, $J = 14.0, 4.0 \text{ Hz, 1H}$), 2.94 (dd, $J = 16.0, 10.0 \text{ Hz, 1H}$), 2.90 (dd, $J = 14.0, 5.5 \text{ Hz, 1H}$), 2.65 (dd, $J = 16.5, 2.5 \text{ Hz, 1H}$), 2.54 (dd, $J = 7.5, 7.5 \text{ Hz, 2H}$), 2.35 (ddd, $J = 7.5, 7.5, 7.5 \text{ Hz, 2H}$), 1.76 (s, 3H).
Preparation of 102

To a solution of 50 (468.7 mg, 1.31 mmol) in CH$_2$Cl$_2$ (35 mL) we re added 9-fluorenemethanol (386.6 mg, 1.97 mmol), EDCI (504.2 mg, 2.63 mmol), and DMAP (160.0 mg, 1.31 mmol) at 25 °C. After stirring for 2 h at 25 °C, the reaction mixture was concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/3) to afford 102 (441.7 mg, 63%): [α]$^{25}_D$ = −25.9 (c 1.0, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$) δ 7.98 (s, 1H), 7.74 (dd, $J$ = 7.6, 4.4 Hz, 2H), 7.63 (dd, $J$ = 7.2, 0.4 Hz, 2H), 7.38 (ddd, $J$ = 10.4, 7.2, 7.2 Hz, 2H), 7.24 (ddd, $J$ = 23.6, 7.6, 7.6 Hz, 2H), 5.47 (br s, 1H), 4.66 (d, $J$ = 6.0 Hz, 2H), 4.51(d, $J$ = 7.6 Hz, 2H), 4.27 (dd, $J$ = 6.8, 6.8 Hz, 1H), 3.79 (d, $J$ = 11.2 Hz, 1H), 3.24 (d, $J$ = 11.6 Hz, 1H), 1.67 (s, 3H), 1.41 (s, 9H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 172.9, 169.8, 163.0, 155.7, 148.5, 143.5, 143.4, 141.2, 141.2, 127.8, 127.7, 127.1, 127.0, 125.2, 125.1, 121.6, 120.0, 119.9, 84.5, 80.2, 77.4, 67.3, 46.9, 42.3, 41.6, 28.3, 24.1; IR (neat) 3339, 2976, 1714, 1605, 1514, 1450, 1367, 1277, 1249, 1167, 1120, 759, 740 cm$^{-1}$; HRMS (ESI) $m/z$ 536.1671 [(M+H)$^+$, C$_{28}$H$_{29}$N$_3$O$_4$S$_2$ requires 531.1672].
Preparation of 103

To a solution of **102** (603.9 mg, 1.13 mmol) in CH$_2$Cl$_2$ (32 mL) was added TFA (8.0 mL) at 25 °C. After stirring for 1 h at 25 °C, the reaction mixture was concentrated *in vacuo* and washed with Et$_2$O. To a solution of the crude mixture in CH$_2$Cl$_2$ (40 mL) were added **90** (478.6 mg, 0.852 mmol) in CH$_2$Cl$_2$ (5 mL) and DMAP (690.3 mg, 5.565 mmol) at 25 °C. After stirring for 2 h at 25 °C, the reaction was quenched by the addition of H$_2$O. The layers were separated and the aqueous layer was extracted with CH$_2$Cl$_2$. The combined organic layers were dried over anhydrous Na$_2$SO$_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/1) to afford **103** (660.6 mg, 93% for 2 steps): $^1$H NMR (400 MHz, CDCl$_3$) δ 8.04 (s, 1H), 7.73 (dd, J = 6.8, 6.8 Hz, 2H), 7.43 (d, J = 7.2 Hz, 2H), 7.17–7.40 (m, 19H), 6.84 (dd, J = 5.6, 5.6 Hz, 1H), 5.56 (ddd, J = 15.2, 5.6, 5.6 Hz, 1H), 5.42 (dd, J = 15.6, 6.0 Hz, 1H), 4.75 (dd, J = 6.0, 6.0 Hz, 2H), 4.51 (d, J = 7.2 Hz, 2H), 4.44 (br s, 1H), 4.26 (dd, J = 6.8, 6.8 Hz, 1H), 3.77 (d, J = 11.6 Hz, 1H), 3.24 (d, J = 11.6 Hz, 1H), 2.46 (dd, J = 15.2, 3.6 Hz, 1H), 2.39 (dd, J = 15.2, 8.4 Hz, 1H), 2.19 (dd, J = 8.0, 8.0 Hz, 2H), 2.06 (dd, J = 15.0, 6.8 Hz, 2H), 1.70 (s, 3H).
Preparation of 106

To a cooled (0 ºC) solution of 104 (65.4 mg, 0.137 mmol) in THF (5 mL) were added 2,4,6-trichlorobenzoyl chloride (32.2 µL, 0.206 mmol) and Et₃N (33.6 µL, 0.241 mmol). After stirring for 2 h at 0 ºC, 103 (57.4 mg, 0.069 mmol) in THF (3 mL) and DMAP (16.8 mg, 0.138 mmol) were added at 0 ºC. After stirring for 1 h at 25 ºC, the reaction was quenched by the addition of saturated NH₄Cl solution and diluted with EtOAc. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/1) to afford 106 (105 mg, 96%): ¹H NMR (400 MHz, CDCl₃) δ 8.02 (s, 1H), 7.72 (dd, J = 7.2, 7.2 Hz 4H), 7.60 (d, J = 7.2 Hz, 2H), 7.53 (dd, J = 7.2, 7.2 Hz, 2H), 7.34–7.40 (m, 10H), 7.23–7.29 (m, 11H), 7.13–7.21 (m, 4H), 6.15 (d, J = 7.2 Hz, 1H), 5.62 (ddd, J = 17.6, 7.6, 7.6 Hz, 1H), 5.49–5.52 (m, 2H), 4.69 (d, J = 6.4 Hz, 2H), 4.47 (dd, J = 7.2, 2.4 Hz, 4H), 4.30 (d, J = 7.2 Hz, 1H), 4.23 (dd, J = 7.2, 7.2 Hz, 1H), 4.16 (dd, J = 15.4, 6.8 Hz, 1H), 3.75 (d, J = 11.6 Hz, 1H), 3.19 (d, J = 11.6 Hz, 1H), 2.99 (dd, J = 4.4, 4.4 Hz, 2H), 2.61 (br s, 2H), 2.19 (dd, J = 6.4, 6.4 Hz, 2H), 2.05 (br s, 2H), 1.63 (s, 3H), 1.58 (s, 9H).
Preparation of 108

To a solution of S10a (75 mg, 0.058 mmol) in CH$_3$CN (10 mL) was added Et$_2$NH (5.0 mL) at 25 °C. After stirring for 1 h at 25 °C, the reaction mixture was concentrated in vacuo and washed with toluene. After removal of toluene in vacuo, to a solution of the crude mixture in CH$_2$Cl$_2$ (65.8 mL) were added HATU (45.2 mg, 0.119 mmol) and i-Pr$_2$NEt (30.3 µL, 0.174 mmol) at 25 °C. After stirring for 18 h at 25 °C, the reaction mixture was concentrated in vacuo. The residue was diluted with H$_2$O and EtOAc. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na$_2$SO$_4$ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes/CH$_3$OH = 30/30/1) to afford 108 (14.6 mg, 27% for 2 steps): $^1$H NMR (400 MHz, CDCl$_3$): δ 7.62 (s, 1H), 7.16–7.38 (m, 16H), 6.87 (s, 1H), 6.54 (br s, 1H), 5.66–5.71 (m, 2H), 5.42 (dd, $J = 15.6$, 7.2 Hz, 1H), 4.96 (dd, $J = 17.6$, 4.0 Hz, 1H), 4.80 (ddd, $J = 7.6$, 4.8, 4.8 Hz, 1H), 4.32 (dd, $J = 12.4$, 3.2 Hz, 1H), 4.10 (d, $J = 11.2$ Hz, 1H), 3.24 (d, $J = 11.2$ Hz, 1H), 3.07 (d, $J = 4.0$ Hz, 2H), 2.77 (dd, $J = 16.0$, 2.7 Hz, 1H), 2.68 (dd, $J = 16.4$, 8.0 Hz, 1H), 2.17 (dd, $J = 7.2$, 7.2 Hz, 2H), 2.04 (m, 2H), 1.79 (s, 3H), 1.57 (s, 9H).
**Preparation of 87**

![Chemical Structure](image)

To a cooled (0 °C) solution 108 (8.5 mg, 0.010 mmol) in CH₂Cl₂ (3 mL) were added TFA (300 μL) and i-Pr₃SiH (7 μL, 0.025 mmol). After stirring for 1 h at 25 °C, the reaction mixture was concentrated in vacuo. The residue was purified by column chromatography (silica gel, CHCl₃/CH₃OH = 10/1) to afford 87 (2.5 mg, 48%): ¹H NMR (500 MHz, CD₃OD) δ 7.99 (s, 1H), 7.28 (d, \( J = 7.5 \) Hz, 1H), 7.25 (s, 1H), 6.53 (s, 1H), 5.86 (ddd, \( J = 22.5, 7.0, 7.0 \) Hz, 1H), 5.83 (dd, \( J = 4.5, 4.5 \) Hz, 1H), 5.63 (dd, \( J = 16.0, 7.0 \) Hz, 1H), 5.18 (d, \( J = 17.5 \) Hz, 1H), 4.75–4.77 (m, 1H), 4.38 (d, \( J = 17.5 \) Hz, 1H), 3.86 (d, \( J = 11.5 \) Hz, 1H), 3.19 (dd, \( J = 15.5, 6.0 \) Hz, 1H), 3.10 (dd, \( J = 15.5, 4.0 \) Hz, 1H), 3.05 (dd, \( J = 17.0, 11.0 \) Hz, 1H), 2.77 (dd, \( J = 17.0, 2.5 \) Hz, 1H), 2.56 (dd, \( J = 7.5, 7.5 \) Hz, 2H), 2.37 (ddd, \( J = 6.0, 6.0, 6.0 \) Hz, 2H), 1.75 (s, 3H).

**Preparation of 107**

![Chemical Structure](image)

To a cooled (0 °C) solution of 105 (52.0 mg, 0.126 mmol) in THF (10 mL) were added 2,4,6-trichlorobenzoyl chloride (21 μL, 0.134 mmol) and Et₃N (20 μL, 0.142 mmol). After stirring for 2 h at 0 °C, 103 (75 mg, 0.089 mmol) in THF (4 mL) and DMAP (13 mg, 0.197
mmol) were added at 0 ºC. After stirring for 1 h at 25 ºC, the reaction was quenched by the addition of saturated NH₄Cl solution and diluted with EtOAc. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/1) to afford 107 (82.7 mg, 93%): ³H NMR (500 MHz, CDCl₃) δ 7.91 (s, 1H), 7.74 (dd, J = 7.0, 7.0 Hz, 4H), 7.73 (d, J = 7.5 Hz, 2H), 7.55 (dd, J = 8.0, 8.0 Hz, 2H), 7.35–7.41 (m, 10H), 7.26–7.30 (m, 10H), 7.20 (dd, J = 7.5, 7.5 Hz, 3H), 6.86 (br s, 1H), 5.84 (d, J = 8.5 Hz, 1H), 5.66 (dd, J = 15.0, 7.0, 7.0 Hz, 1H), 5.59 (dd, J = 12.8, 6.5 Hz, 1H), 5.44 (dd, J = 15.3, 6.5 Hz, 1H), 4.71 (d, J = 6.0 Hz, 2H), 4.56 (m, 1H), 4.50 (dd, J = 6.5, 4.0 Hz, 2H), 4.41 (dd, J = 10.0, 7.5 Hz, 1H), 4.35 (dd, J = 10.0, 7.5 Hz, 1H), 4.25 (dd, J = 7.0, 7.0 Hz, 1H), 4.20 (dd, J = 7.0, 7.0 Hz, 1H), 3.78 (d, J = 11.5 Hz, 1H), 3.22 (d, J = 11.5 Hz, 1H), 2.88 (dd, J = 17.0, 5.0 Hz, 1H), 2.75 (dd, J = 17.0, 5.0 Hz, 1H), 2.59 (d, J = 5.5 Hz, 2H), 2.20 (dd, J = 6.5, 6.5 Hz, 2H), 2.05 (m, 2H), 1.65 (s, 3H), 1.40 (s, 9H).

Preparation of 109

To a solution of 107 (100 mg, 0.081 mmol) in CH₃CN (10 mL) was added Et₂NH (2.0 mL) at 25 ºC. After stirring for 1 h at 25 ºC, the reaction mixture was concentrated in vacuo and washed with toluene. After removal of toluene in vacuo, to a solution of the crude mixture in CH₂Cl₂ (100 mL) were added HATU (61.5 mg, 0.162 mmol) and i-Pr₂NEt (42.3 µL, 0.243 mmol)
at 25 °C. After stirring for 18 h at 25 °C, the reaction mixture was concentrated in vacuo. The residue was diluted with H₂O and EtOAc. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes/CH₃OH = 30/30/1) to afford 109 (41 mg, 62% for 2 steps): ¹H NMR (400 MHz, CD₃OD) δ 8.02 (s, 1H), 7.56 (d, J = 6.8, 1H), 7.34 (m, 6H), 7.28 (m, 6H), 7.20 (m, 3H), 5.66–5.73 (m, 2H), 5.47 (dd, J = 15.4, 6.4 Hz, 1H), 5.51 (dd, J = 17.6, 4.4, 4.4 Hz, 1H), 4.56–4.60 (m, 1H), 4.45 (dd, J = 17.2, 3.6 Hz, 1H), 3.87 (d, J = 11.6 Hz, 1H), 3.40 (d, J = 12.0 Hz, 1H), 2.96 (dd, J = 16.4, 10.4 Hz, 1H), 2.75 (dd, J = 13.6, 3.6 Hz, 1H), 2.68 (dd, J = 16.8, 5.2 Hz, 1H), 2.17 (dd, J = 7.2, 7.2 Hz, 2H), 2.04 (m, 2H), 1.77 (s, 3H), 1.20 (s, 9H).

Preparation of 88

To a cooled (0 °C) solution of 109 (10 mg, 0.012 mmol) in CH₂Cl₂ (2 mL) were added TFA (200 μL) and i-Pr₂SiH (5 μL, 0.0149 mmol). After stirring for 15 min at 0 °C, the reaction mixture was concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes/CH₃OH = 10/10/1): ¹H NMR (500 MHz, CDCl₃) δ 7.85 (d, J = 7.5 Hz, 1H), 7.62 (s, 1H), 7.37 (br s, 1H), 5.91 (ddd, J = 15.0, 7.0, 7.0 Hz, 1H), 5.75 (dd, J = 15.0, 7.0 Hz, 1H), 5.68 (dd, J = 4.5, 4.5 Hz, 1H), 5.04 (dd, J = 17.0, 6.5 Hz, 1H), 4.66–4.69 (m, 1H), 4.63 (dd, J = 17.0, 3.5 Hz, 1H), 4.01 (d, J = 11.5 Hz, 1H), 3.27 (d, J = 11.5 Hz, 1H), 2.97 (dd, J = 17.5, 3.0 Hz,
1H), 2.91 (dd, J = 15.0, 2.5 Hz, 1H), 2.80 (dd, J = 17.5, 4.0 Hz, 1H), 2.69 (dd, J = 15.0, 6.0 Hz, 1H), 2.54 (ddd, J = 7.0, 7.0, 7.0 Hz, 2H), 2.38 (ddd, J = 7.0, 7.0, 7.0 Hz, 2H), 1.84 (s, 3H), 1.54 (dd, J = 8.0 Hz, 1H), 1.10 (s, 9H). To a solution of the resulting intermediate in CH₂Cl₂ (2 mL) was added TFA (200 μL). After stirring for 1 h at 25 °C, the reaction mixture was concentrated to afford 88 (1.4 mg, 22% for 2 steps).

**Preparation of 117**

To a cooled (0 ºC) solution of 115 (105.7 mg, 0.240 mmol) in THF (10 mL) were added 2,4,6-trichlorobenzoyl chloride (56.2 µL, 0.360 mmol) and Et₃N (58.5 µL, 0.420 mmol). After stirring for 2 h at 0 °C, 103 (100.3 mg, 0.120 mmol) in THF (4 mL) and DMAP (29.3 mg, 0.240 mmol) were added at 0 °C. After stirring for 1 h at 25 ºC, the reaction was quenched by the addition of saturated NH₄Cl solution and diluted with EtOAc. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/1) to afford 117 (146.4 mg, 91%): 

$^1$H NMR (400 MHz, CDCl₃) δ 7.84 (s, 1H), 7.73 (d, J = 7.2 Hz, 4H), 7.56 (d, J = 7.2 Hz, 4H), 7.18–7.38 (m, 23H), 6.99 (br s, 1H), 5.60 (s, 1H), 5.58–5.68 (m, 2H), 5.42 (dd, J = 14.8, 6.8 Hz, 1H), 4.65 (dd, J = 5.6, 5.6 Hz, 2H), 4.48 (d, J = 6.8 Hz, 2H), 4.16–4.37 (m, 5H), 3.67 (d, J = 11.2 Hz, 1H), 3.18 (d, J = 11.2 Hz, 1H), 2.85 (br s, 2H), 2.59 (dd, J = 14.8, 8.0 Hz, 1H), 2.53 (dd, J = 15.2, 4.8 Hz, 107
2H), 2.19 (dd, \( J = 6.4, 6.4 \text{ Hz}, 2H \)), 2.05 (d, \( J = 6.8 \text{ Hz}, 2H \)), 1.90 (br s, 2H), 1.58 (s, 3H), 1.42 (s, 9H).

**Preparation of 119**

To a solution of 117 (29.4 mg, 0.022 mmol) in CH\(_3\)CN (10 mL) was added Et\(_3\)NH (1.0 mL) at 25 °C. After stirring for 2 h at 25 °C, the reaction mixture was concentrated in vacuo and washed with toluene. After removal of toluene in vacuo, to a solution of the crude mixture in CH\(_2\)Cl\(_2\) (25.0 mL) were added HATU (16.7 mg, 0.044 mmol) and i-Pr\(_2\)NEt (11.5 µL, 0.066 mmol) at 25 °C. After stirring for 36 h at 25 °C, the reaction mixture was concentrated in vacuo. The residue was diluted with H\(_2\)O and EtOAc. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na\(_2\)SO\(_4\) and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes/CH\(_3\)OH = 30/30/1) to afford 119 (3.3 mg, 18% for 2 steps): \(^1\)H NMR (400 MHz, CD\(_3\)OD) \( \delta 8.11 \text{ (s, 1H)}, 7.38 \text{ (d, } J = 7.2 \text{ Hz, 6H)}, 7.28 \text{ (dd, } J = 7.2, 7.2 \text{ Hz, 6H)}, 7.21 \text{ (dd, } J = 8.4, 8.4 \text{ Hz, 3H)}, 6.56 \text{ (br s, 1H)}, 5.73 \text{ (ddd, } J = 15.2, 6.8, 6.8 \text{ Hz, 1H)}, 5.47–5.56 \text{ (m, 2H)}, 5.04 \text{ (d, } J = 17.6 \text{ Hz, 1H)}, 4.52 \text{ (dd, } J = 5.6, 5.6 \text{ Hz, 1H)}, 4.35 \text{ (d, } J = 17.6 \text{ Hz, 1H)}, 3.67 \text{ (d, } J = 11.6 \text{ Hz, 1H)}, 3.37 \text{ (d, } J = 11.6 \text{ Hz, 1H)}, 3.00 \text{ (dd, } J = 16.4, 1.4 \text{ Hz, 1H)}, 2.85 \text{ (m, 2H)}, 2.72 \text{ (dd, } J = 17.2, 2.8 \text{ Hz, 1H)}, 2.21 \text{ (dd, } J = 5.6, 5.6 \text{ Hz, 2H)}, 2.07 \text{ (dd, } J = 6.8, 6.8 \text{ Hz, 2H)}, 1.77 \text{ (s, 3H)}, 1.40 \text{ (s, 9H}).
Preparation of 113

To a cooled (0 °C) solution of 119 (11.2 mg, 0.013 mmol) in CH₂Cl₂ (2 mL) were added TFA (200 µL) and i-Pr₃SiH (5 µL, 0.026 mmol). After stirring for 15 min at 0 °C, the reaction mixture was concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc 100%) to afford 113 (4.3 mg, 54%). ¹H NMR (400 MHz, CD₃OD) δ 8.12 (s, 1H), 7.45 (d, J = 8.0 Hz, 1H), 6.58 (br s, 1H), 5.83 (ddd, J = 14.4, 6.8, 6.8 Hz, 1H), 5.61–5.67 (m, 2H), 5.06 (d, J = 17.6 Hz, 1H), 4.54 (ddd, J = 8.0, 5.2, 5.2 Hz, 1H), 4.41 (d, J = 17.6 Hz, 1H), 3.97 (d, J = 11.6 Hz, 1H), 3.38 (d, J = 11.6 Hz, 1H), 3.07 (dd, J = 10.4, 8.4 Hz, 1H), 2.77 (dd, J = 18.0, 2.4 Hz, 1H), 2.55 (dd, J = 7.2, 7.2 Hz, 2H), 2.36 (ddd, J = 6.8, 6.8 Hz, 2H), 1.89 (m, 2H), 1.79 (s, 3H), 1.41 (s, 9H).

Preparation of 111

To a solution of 119 (4.0 mg, 0.0067 mmol) in CH₂Cl₂ (0.1 mL) was added TFA (10 µL). The reaction mixture was stirred for 1 h at 25 °C until there was no more starting material left (detected by MS). The reaction mixture was purged with N₂ and dried in vacuo to remove TFA and CH₂Cl₂. The crude mixture was purified by HPLC (Syringe-Hydro RP, 4 µm, flow rate 2.0
mL/min) eluting with of CH$_3$OH/H$_2$O (CH$_3$OH was kept at 80% in the first 30 min, then increased to 100% in 25 min) to afford 111 (1.2 mg, 36%, $t_r = 23.0$ min, broad): $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 8.13 (s, 1H), 5.85 (ddd, $J = 15.6, 7.2, 7.2$ Hz, 1H), 5.73 (dd, $J = 6.8, 6.8$ Hz, 1H), 5.62 (dd, $J = 15.2, 7.2$ Hz, 1H), 5.06 (d, $J = 17.6$ Hz, 1H), 4.66 (dd, $J = 6.0, 6.0$ Hz, 1H), 4.63 (br s, 1H), 4.41 (d, $J = 17.6$ Hz, 1H), 3.96 (d, $J = 11.6$ Hz, 1H), 3.42 (d, $J = 11.6$ Hz, 1H), 3.10 (dd, $J = 17.2, 11.2$ Hz, 1H), 2.80 (dd, $J = 19.2, 2.0$ Hz, 1H), 2.76 (m, 2H), 2.56 (dd, $J = 6.8, 6.8$ Hz, 2H), 2.36 (ddd, $J = 6.4, 6.4, 6.4$ Hz, 2H), 1.94 (m, 2H), 1.81 (s, 3H).

**Preparation of 118**

To a cooled (0 °C) solution of 116 (118.6 mg, 0.261 mmol) in THF (10 mL) were added 2,4,6-trichlorobenzoyl chloride (61.1 µL, 0.391 mmol) and Et$_3$N (63.7 µL, 0.457 mmol). After stirring for 2 h at 0 °C, 118 (109 mg, 0.130 mmol) in THF (4 mL) and DMAP (31.9 mg, 0.261 mmol) were added at 0 °C. After stirring for 1 h at 25 °C, the reaction was quenched by the addition of saturated NH$_4$Cl solution and diluted with EtOAc. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na$_2$SO$_4$ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/1) to afford 118 (146.4 mg, 83%): $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.84 (s, 1H), 7.72 (d, $J = 7.6$ Hz, 4H), 7.57 (d, $J = 6.8$ Hz, 4H), 7.17–7.39 (m, 23H), 5.55–5.67 (m, 2H), 5.40 (dd, $J = 15.2, 7.2$ Hz, 1H), 4.66 (s, 2H), 4.47 (d, $J = 6.8$ Hz, 2H), 3.82 (s, 3H), 3.69 (t, $J = 6.4$ Hz, 2H), 2.85 (dd, $J = 17.6, 11.2$ Hz, 1H), 2.76 (m, 2H), 2.54 (dd, $J = 6.8, 6.8$ Hz, 2H), 2.36 (ddd, $J = 6.4, 6.4, 6.4$ Hz, 2H), 1.94 (m, 2H), 1.81 (s, 3H).
4.14–4.33 (m, 5H), 3.67 (d, J = 11.2 Hz, 1H), 3.18 (d, J = 11.2 Hz, 1H), 3.05 (dd, J = 5.6, 5.6 Hz, 2H), 2.55 (br s, 2H), 2.19 (dd, J = 6.4, 6.4 Hz, 2H), 2.04 (d, J = 7.2 Hz, 2H), 1.75 (br s, 4H), 1.58 (s, 3H), 1.42 (s, 9H).

**Preparation of 120**

To a solution of 118 (51.6 mg, 0.038 mmol) in CH$_3$CN (20 mL) was added Et$_2$NH (2.0 mL) at 25 °C. After stirring for 2 h at 25 °C, the reaction mixture was concentrated *in vacuo* and washed with toluene. After removal of toluene *in vacuo*, to a solution of the crude mixture in CH$_2$Cl$_2$ (43.4 mL) were added HATU (29.1 mg, 0.076 mmol) and i-Pr$_2$NEt (20 µL, 0.115 mmol) at 25 °C. After stirring for 36 h at 25 °C, the reaction mixture was concentrated *in vacuo*. The residue was diluted with H$_2$O and EtOAc. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na$_2$SO$_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, EtOAc/hexanes/CH$_3$OH = 30/30/1) to afford 120 (10.7 mg, 33% for 2 steps): H NMR (400 MHz, CD$_3$OD) δ 8.09 (s, 1H), 7.38 (d, J = 8.0 Hz, 6H), 7.28 (dd, J = 7.2, 7.2 Hz, 6H), 7.21 (dd, J = 8.4, 8.4 Hz, 3H), 6.20 (br s, 1H), 5.71 (ddd, J = 15.2, 7.2, 7.2 Hz, 1H), 5.65 (dd, J = 12.4, 12.4 Hz, 1H), 5.47 (dd, J = 15.6, 7.2 Hz, 1H), 5.07 (d, J = 18.0 Hz, 1H), 4.55 (dd, J = 4.8, 4.8 Hz, 1H), 4.36 (d, J = 18.0 Hz, 1H), 3.98 (d, J = 11.6 Hz, 1H), 3.35 (d, J = 11.6 Hz, 1H), 2.99 (d, J = 16.8,
11.2 Hz, 1H), 2.75 (m, 2H), 2.70 (dd, $J = 16.8$, 2.8 Hz, 1H), 2.20 (dd, $J = 6.8$, 6.8 Hz, 2H), 2.06 (dd, $J = 7.6$, 7.6 Hz, 2H), 1.78 (s, 3H), 1.70 (m, 2H), 1.41 (s, 9H).

**Preparation of 114**

![Chemical structure 120](image1)

To a cooled (0 °C) solution of 120 (10.7 mg, 0.013 mmol) in CH$_2$Cl$_2$ (2 mL) were added TFA (200 μL) and i-Pr$_3$SiH (5 μL, 0.015 mmol). After stirring for 15 min at 0 °C, the reaction mixture was concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, EtOAc/hexanes/CH$_3$OH = 10/10/1) to afford 114 (4.8 mg, 63%): $^1$H NMR (400 MHz, CD$_3$OD) δ 8.10 (s, 1H), 7.40 (d, $J = 8.0$ Hz, 1H), 5.83 (ddd, $J = 14.8$, 7.2, 7.2 Hz, 1H), 5.59–5.70 (m, 2H), 5.09 (d, $J = 17.6$ Hz, 1H), 4.57 (dd, $J = 4.8$, 4.8 Hz, 1H), 4.40 (d, $J = 17.6$ Hz, 1H), 3.98 (d, $J = 11.2$ Hz, 1H), 3.36 (d, $J = 11.2$ Hz, 1H), 3.05 (dd, $J = 16.8$, 10.8 Hz, 1H), 2.72–2.78 (m, 3H), 2.55 (dd, $J = 6.4$, 6.4 Hz, 2H), 2.35 (ddd, $J = 6.8$, 6.8, 6.8 Hz, 2H), 1.80 (s, 3H), 1.70 (m, 2H), 1.41 (s, 9H).
Preparation of 112

To a solution of 114 (4.0 mg, 0.007 mmol) in CH$_2$Cl$_2$ (0.1 mL) was added TFA (10 μL). The reaction mixture was stirred for 1 h at 25 °C until there was no more starting material left (detected by MS). The reaction mixture was purged with N$_2$ and dried in vacuo to remove TFA and CH$_2$Cl$_2$. The crude mixture was purified by HPLC (Syringe-Hydro RP, 4 µm, flow rate 2.0 mL/min) eluting with of CH$_3$OH/H$_2$O (CH$_3$OH was kept at 80% in the first 30 min, then increased to 100% in 25 min) to afford 112 (1.5 mg, 40%, $t_R$ = 23.0 min, broad): $^1$H NMR (400 MHz, CD$_3$OD) δ 8.12 (s, 1H), 5.85 (ddd, $J = 15.2, 6.8, 6.8$ Hz, 1H), 5.60–5.73 (m, 2H), 5.06 (d, $J = 17.6$ Hz, 1H), 4.62 (br s, 1H), 4.59 (dd, $J = 5.2, 5.2$ Hz, 1H), 4.42 (d, $J = 17.6$ Hz, 1H), 3.97 (d, $J = 11.6$ Hz, 1H), 3.39 (d, $J = 11.6$ Hz, 1H), 3.10 (dd, $J = 17.2, 10.8$ Hz, 1H), 2.80 (dd, $J = 14.8, 1.2, 1H$), 2.73 (dd, $J = 8.0, 8.0$ Hz, 2H), 2.56 (dd, $J = 6.8, 6.8$ Hz, 2H), 2.36 (ddd, $J = 7.2, 7.2, 7.2$ Hz, 2H), 1.88 (m, 2H), 1.80 (s, 3H), 1.74 (m, 2H)

Preparation of 110

To a cooled (0 °C) solution of 108 (8.4 mg, 0.0096 mmol) in CH$_2$Cl$_2$ (1 mL) were added TFA (50 μL) and $i$-Pr$_3$SiH (4 μL, 0.019 mmol). After stirring for 15 min at 0 °C, the reaction
mixture was concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, EtOAc 100%) to afford **110** (2.7 mg, 44%): $^1$H NMR (400 MHz, CD$_3$OD) δ 8.03 (s, 1H), 7.51 (s, 1H), 7.37 (d, $J$ = 8.0 Hz, 1H), 6.93 (s, 1H), 5.83 (ddd, $J$ = 15.6, 6.4, 6.4 Hz, 1H), 5.71 (dd, $J$ = 8.0, 8.0 Hz, 1H), 5.62 (dd, $J$ = 15.2, 6.8 Hz, 1H), 5.09 (d, $J$ = 17.6, 1H), 4.76 (ddd, $J$ = 8.0, 4.4, 4.4 Hz, 1H), 4.37 (d, $J$ = 17.6 Hz, 1H), 3.89 (d, $J$ = 11.6 Hz, 1H), 3.35 (d, $J$ = 11.6 Hz, 1H), 3.06 (dd, $J$ = 14.8, 5.2 Hz, 1H), 2.99 (dd, $J$ = 16.0, 4.8 Hz, 1H), 2.97 (dd, $J$ = 9.6, 6.0 Hz, 1H), 2.70 (dd, $J$ = 17.2, 2.4 Hz, 1H), 2.55 (dd, $J$ = 6.8, 6.8 Hz, 2H), 2.35 (ddd, $J$ = 7.2, 7.2, 7.2 Hz, 2H), 1.75 (s, 3H), 1.60 (s, 9H).

### Preparation of **132**

To a cooled (−10 °C) solution of **131** (4.04 g, 9.11 mmol) in THF (75 mL) were added ethyl chloroformate (868 μL, 9.11 mmol) and Et$_3$N (1.26 mL, 9.11 mmol). After stirring for 30 min at −10 °C, NH$_4$OH (40 mL) was added at −10 °C. After stirring for 1 h at −10 °C, the reaction mixture was extracted with EtOAc. The combined organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated *in vacuo*. After removal of the solvent, to a solution of the crude mixture in CH$_2$Cl$_2$ (100 mL) was added Lawesson reagent (2.78 g, 5.63 mmol) at 25 °C. After stirring for 16 h at 25 °C, the reaction mixture was concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/7 to 1/2) to afford **132** (3.06 g, 73% for 2 steps): $^1$H NMR (400 MHz, CDCl$_3$) δ 7.62–7.66 (m, 4H), 7.38–7.46 (m, 6H), 5.43 (d, $J$ = 6.8 Hz, 1H), 4.53 (br s, 1H), 4.09 (dd, $J$ = 10.0, 4.8 Hz, 1H), 3.85 (dd, $J$ = 10, 6.4 Hz, 1H), 1.45 (s, 9H), 1.46 (s, 9H).
Preparation of 133

To a solution of 132 (3.06 g, 6.67 mmol) in EtOH (100 mL) were added ethyl bromopyruvate (1.3 mL, 10.0 mmol) and CaCO$_3$ (750.0 mg, 7.50 mmol). After stirring for 9 h at 25 °C, the reaction was quenched by addition of saturated NaHCO$_3$. The mixture was stirred vigorously for 1 h. The layers were separated and the aqueous layer was extracted with CHCl$_3$. The combined organic layers were dried over anhydrous Na$_2$SO$_4$ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/7 to 1/2) to afford 133 (1.41 g, 38%). $^1$H NMR (400 MHz, CDCl$_3$) δ 8.10 (s, 1H), 7.54–7.57 (m, 2H), 7.30–7.46 (m, 8H), 5.62 (br s, 1H), 5.16 (br s, 1H), 4.43 (ddd, $J = 7.2, 7.2, 7.2$ Hz, 2H), 4.19 (br s, 1H), 4.00 (dd, $J = 10.0, 4.8$ Hz, 1H), 1.47 (br s, 9H), 1.41 (dd, $J = 6.8, 6.8$ Hz, 3H), 0.98 (s, 9H).

Preparation of 134

To a solution of 133 (1.58 g, 2.85 mmol) in EtOH (30 mL) in a pressure tube was added NH$_4$OH (10 mL). The mixture was cooled (−78 °C) and NH$_3$ was bubbled through the solution for 5 min, and the tube was sealed and warmed (25 °C). After stirring for 48 h at 25 °C, the mixture was cooled (−78 °C), the tube was unsealed, and the mixture was concentrated in vacuo. The residue was taken up in CH$_2$Cl$_2$ and diluted with H$_2$O. The layers were separated and the aqueous
layer was extracted with CHCl₃. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated in vacuo. After removal of CHCl₃, to a cooled (0 °C) solution of the crude mixture in CH₂Cl₂ (100 mL) were added TFAA (500.0 μL, 3.54 mmol) and Et₃N (1.0 mL, 7.08 mmol). After stirring for 1 h at 0 °C, the reaction was quenched by the addition of H₂O. The layers were separated and the aqueous layer was extracted with CH₂Cl₂. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/7) to afford 134 (1.15 g, 79% for 2 steps): ¹H NMR (400 MHz, CDCl₃) δ 7.92 (s, 1H), 7.54–7.57 (m, 2H), 7.35–7.47 (m, 8H), 5.57 (br s, 1H), 5.09 (br s, 1H), 4.16 (br s, 1H), 4.00 (dd, J = 10.0, 4.0 Hz, 1H), 1.48 (s, 9H), 0.99 (s, 9H).

**Preparation of 136**

![Chemical Structure](image)

To a solution of 134 (632.7 mg, 1.25 mmol) in CH₃OH (50 mL) were added 135 (314.2 mg, 1.83 mmol) in CH₃OH (5 mL) and Et₃N (2.0 mL, 14.3 mmol). The mixture was brought to reflux. After stirring for 12 h at reflux, the mixture was cooled (25 °C) and concentrated in vacuo. The residue was taken up in H₂O and acidified by 1.0 N HCl until the pH of the solution reached 3. After dilution with CH₂Cl₂ the layers were separated and the aqueous layer was extracted with CH₂Cl₂. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated in vacuo. After removal of CH₂Cl₂, to a solution of the crude mixture in CH₂Cl₂ (50 mL) were added 9-fluorenemethanol (222.5 mg, 1.13 mmol), EDCI (538.7 mg, 2.81 mmol), and DMAP.
(172.3 mg, 1.41 mmol) at 25 °C. After stirring for 4 h at 25 °C, the reaction mixture was concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/7) to afford 136 (373.9 mg, 37% for 2 steps): \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\)

8.00 (s, 1H), 7.74 (dd, \(J = 7.2, 7.2\) Hz, 2H), 7.64 (d, \(J = 6.4\) Hz, 2H), 7.58 (d, \(J = 8\) Hz, 2H), 7.48 (br s, 2H), 7.32–7.44 (m, 8H), 7.19–7.29 (m, 4H), 5.59 (br s, 1H), 5.17 (br s, 1H), 4.52 (d, \(J = 7.2\) Hz, 2H), 4.27 (dd, \(J = 7.2, 7.2\) Hz, 1H), 4.19 (br s, 1H), 3.99 (dd, \(J = 10.4, 4.8\) Hz, 1H), 3.79 (d, \(J = 11.2\) Hz, 1H), 3.24 (d, \(J = 11.2\) Hz, 1H), 1.67 (s, 3H), 1.48 (s, 9H), 0.99 (s, 9H).

**Preparation of 137**

To a solution of 136 (68.4 mg, 0.085 mmol) in CH\(_2\)Cl\(_2\) (8 mL) was added TFA (2.0 mL) at 25 °C. After stirring for 2 h at 25 °C, the reaction mixture was concentrated in vacuo and washed with Et\(_2\)O. To a solution of the crude mixture in CH\(_2\)Cl\(_2\) (10 mL) were added 90 (66.7 mg, 0.119 mmol) in CH\(_2\)Cl\(_2\) (2 mL) and DMAP (73.0 mg, 0.594 mmol) at 25 °C. After stirring for 3 h at 25 °C, the reaction was quenched by the addition of H\(_2\)O. The layers were separated and the aqueous layer was extracted with CH\(_2\)Cl\(_2\). The combined organic layers were dried over anhydrous Na\(_2\)SO\(_4\) and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/5 to EtOAc 100%) to afford 137 (52.7 mg, 56% for 2 steps): \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\)

8.00 (s, 1H), 7.77 (dd, \(J = 7.2, 4.8\) Hz, 2H), 7.65 (d, \(J = 7.2\) Hz, 2H), 7.59 (d, \(J = 8.0\) Hz, 2H), 7.50–7.53 (m, 2H), 7.20–7.44 (m, 25H), 5.58 (ddd, \(J = 117\) Hz, 2H).
15.6, 6.0, 6.0 Hz, 1H), 5.41–5.50 (m, 2H), 4.54 (d, J = 7.2 Hz, 2H), 4.46 (m, 1H), 4.28 (dd, J = 6.8, 6.8 Hz, 1H), 4.23 (dd, J = 10.0, 3.6 Hz, 1H), 3.95 (dd, J = 10.0, 4.8 Hz, 1H), 3.80 (d, J = 11.6 Hz, 1H), 3.25 (d, J = 11.2 Hz, 1H), 2.40 (d, J = 5.6 Hz, 2H), 2.22 (dd, J = 7.2, 7.2 Hz, 2H), 2.08 (ddd, J = 6.4, 6.4, 6.4 Hz, 2H), 1.68 (s, 3H).

**Preparation of 139**

To a cooled (0 ºC) solution of 138 (115.7 mg, 0.341 mmol) in THF (5 mL) were added 2,4,6-trichlorobenzoyl chloride (80.0 µL, 0.511 mmol) and Et₃N (80.0 µL, 0.568 mmol). After stirring for 1 h at 0 ºC, 137 (125.4 mg, 0.114 mmol) in THF (2 mL) and DMAP (42.0 mg, 0.341 mmol) were added at 0 ºC. After stirring for 1 h at 25 ºC, the reaction was quenched by the addition of saturated NH₄Cl solution and diluted with EtOAc. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/5 to 1/2) to afford 139 (146.9 mg, 90%): ¹H NMR (400 MHz, CDCl₃) δ 8.01 (s, 1H), 7.70–7.75 (m, 4H), 7.48–7.63 (m, 8H), 7.16–7.40 (m, 29H), 6.75 (d, J = 8.0 Hz, 1H), 5.58–5.70 (m, 2H), 5.32–5.42 (m, 2H), 5.28 (d, J = 9.2 Hz, 1H), 4.10–4.52 (m, 7H), 3.91 (dd, J = 9.6, 5.6 Hz, 1H), 3.76 (d, J = 11.2 Hz, 1H), 3.21 (d, J = 11.2 Hz, 1H), 2.48–2.59 (m, 2H), 2.14–2.15 (m, 2H), 2.00–2.02 (m, 3H), 1.68 (s, 3H), 1.00 (s, 9H), 0.87 (d, J = 6.8 Hz, 3H), 0.77 (d, J = 6.8 Hz, 3H).
Preparation of 140

To a solution of 139 (60.4 mg, 0.042 mmol) in CH$_3$CN (10 mL) was added Et$_3$NH (2.0 mL) at 25 °C. After stirring for 2 h at 25 °C, the reaction mixture was concentrated in vacuo and washed with toluene. After removal of toluene in vacuo, to a solution of the crude mixture in CH$_2$Cl$_2$ (42.4 mL) were added HATU (32.3 mg, 0.085 mmol) and i-Pr$_2$NEt (22.1 µL, 0.127 mmol) at 25 °C. After stirring for 12 h at 25 °C, the reaction mixture was concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/2) to afford 140 (7.3 mg, 17% for 2 steps); $^1$H NMR (400 MHz, CDCl$_3$) δ 7.80 (s, 1H), 7.60 (dd, $J$ = 8.0, 8.0 Hz, 3H), 7.13–7.46 (m, 22H), 6.32 (d, $J$ = 10.0 Hz, 1H), 5.66 (ddd, $J$ = 15.6, 6.8, 6.8 Hz, 1H), 5.58 (dd, $J$ = 8.0 Hz, 1H), 5.48–5.53 (m, 1H), 5.31 (dd, $J$ = 15.6, 7.2 Hz, 1H), 4.59 (dd, $J$ = 9.6, 3.2 Hz, 1H), 4.04 (d, $J$ = 11.2 Hz, 1H), 3.96 (dd, $J$ = 10.0, 5.2 Hz, 1H), 3.85 (dd, $J$ = 9.6, 7.2 Hz, 1H), 3.75 (dd, $J$ = 6.4, 6.4 Hz, 1H), 3.27 (d, $J$ = 11.2 Hz, 1H), 2.74 (dd, $J$ = 16.4, 11.2 Hz, 1H), 2.56 (dd, $J$ = 14.4, 4.0 Hz, 1H), 2.17 (dd, $J$ = 6.8, 6.8 Hz, 2H), 1.98–2.12 (m, 3H), 1.86 (s, 3H), 1.07 (s, 9H), 0.66 (d, $J$ = 6.8 Hz, 3H), 0.453 (d, $J$ = 6.8 Hz, 3H).
Preparation of 141

To a cooled (0 °C) solution of 140 (7.3 mg, 0.007 mmol) in THF (2 mL) was added TBAF (1.0M in THF) (50 μL, 0.050 mmol). After stirring for 1 h at 0 °C, the reaction was quenched by addition of saturated NH₄Cl and diluted with CH₂Cl₂. The layers were separated and the aqueous layer was extracted with CH₂Cl₂. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/2 to EtOAc 100%) to afford 141 (4.9 mg, 88%):

$^1$H NMR (400 MHz, CDCl₃) δ 7.66 (s, 1H), 7.39–7.41 (m, 3H), 7.26–7.31 (m, 6H), 7.19–7.23 (m, 3H), 6.82 (d, $J = 9.6$ Hz, 1H), 5.59 (ddd, $J = 15.2$, 6.8, 6.8 Hz, 1H), 5.54 (dd, $J = 8.4$, 8.4 Hz, 1H), 5.34–5.41 (m, 2H), 4.51 (dd, $J = 9.6$, 3.2 Hz, 1H), 4.17 (d, $J = 9.6$ Hz, 1H), 4.06 (d, $J = 11.2$ Hz, 1H), 3.97 (d, $J = 12.4$ Hz, 1H), 3.23 (d, $J = 11.2$ Hz, 1H), 3.05 (dd, $J = 16.0$, 10.8 Hz, 1H), 2.58 (dd, $J = 16.0$, 1.6 Hz, 1H), 2.20 (dd, $J = 6.8$, 6.8 Hz, 2H), 2.04–2.15 (m, 3H), 1.87 (s, 3H), 0.58 (d, $J = 6.8$ Hz, 3 H), 0.33 (d, $J = 6.8$ Hz, 3 H).
Preparation of 91

To a solution of 141 (4.9 mg, 0.006 mmol) in CH₂Cl₂ (1 mL) were added TFA (500 μL) and TIPS (2.6 μL, 0.012 mmol). After stirring for 30 min at 25 °C, the reaction mixture was concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc 100%) to afford 91 (2.7 mg, 80%): ¹H NMR (400 MHz, CDCl₃) δ 7.74 (s, 1H), 7.06 (d, J = 9.2 Hz, 1H), 6.98 (d, J = 9.6 Hz, 1H), 5.81 (ddd, J = 15.2, 7.2, 7.2 Hz, 1H), 5.64 (dd, J = 8.0, 8.0 Hz, 1H), 5.53 (dd, J = 15.2, 6.8 Hz, 1H), 5.40–5.45 (m, 1H), 4.57 (dd, J = 9.6, 3.2 Hz, 1H), 4.18 (dd, J = 11.6, 2.4 Hz, 1H), 4.06 (d, J = 11.6 Hz, 1H), 4.00–4.03 (m, 1H), 3.25 (d, J = 11.6 Hz, 1H), 3.03 (dd, J = 16.4, 10.8 Hz, 1H), 2.67 (dd, J = 15.6, 2.0 Hz, 1H), 2.38 (dd, J = 7.2, 7.2 Hz, 2H), 2.22 (dd, J = 7.2, 7.2 Hz, 1H), 1.94–2.11 (m, 3H), 1.89 (s, 3H), 0.63 (d, J = 6.8 Hz, 3H), 0.40 (d, J = 6.8 Hz, 3H).

Enzymatic Assays

Enzyme inhibitory assays were carried out by BPS Bioscience. In brief, compounds were incubated with an HDAC enzyme, an appropriate HDAC substrate, bovine serum albumin, and HDAC buffer. Duplicate reactions were carried out at 37 °C for 30 min. The reactions were quenched at the end of the incubation period with the addition of HDAC developer. Reactions were further incubated for 15 min at room temperature prior to fluorescence measurement (ex 360 nm/em 460 nm). The % inhibitory activity was calculated according to the equation (F-Fb)/(Ft-Fb), where F- fluorescent intensity of compound treated wells, F₀- fluorescent intensity of blank
wells, $F_r$ fluorescent intensity of solvent control wells. IC$_{50}$ values were calculated using GraphPad Prism.
3. Synthesis and biological characterization of zinc binding group analogues of largazole

3.1 Types of zinc-binding groups in HDAC inhibitors

One of the most important structural features of largazole is the thiol moiety. Its ability to coordinate to the Zn$^{2+}$ in the active site of HDACs is crucial to the inhibitory activity that largazole displays. Several analogues that have been prepared to date have demonstrated the importance of the zinc-binding group (ZBG). Hong/Luesch and co-workers prepared macrocycle 43 and hydroxyl analogue 142 (figure 43).\textsuperscript{112} The replacement of the thiol with a hydroxyl group, which is unable to coordinate to Zn$^{2+}$, rendered the analogue inactive in growth inhibition as well as cellular HDAC inhibition of HCT-116 cells up to concentrations of 10 μM. Additionally, it was also unable to inhibit HeLa nuclear extract HDACs at 10 μM concentrations. This showed that without a functional ZBG, the HDAC inhibitory activity of largazole was abolished. The same inactivity was observed when macrocycle 43 was tested, further highlighting the importance of a ZBG.

\textbf{Figure 43:} Structure of macrocycle analogue (43) and hydroxyl analogue (142).\textsuperscript{112}

Phillips and co-workers further supported this theory by also preparing macrocycle analogue (43) as well as an ester analogue (143) and a ketone analogue (144) (figure 44).\textsuperscript{117}
While largazole showed potent anti-proliferative activity against human breast cancer cells (MDA-MB231) with an IC$_{50}$ of 71 nM, none of the analogues prepared in the study were active up to 600 nM. Hydrolysis of the ester analogue (143) would result in a hydroxyl group, which cannot coordinate to Zn$^{2+}$, while the ketone analogue (144) is not able to undergo hydrolysis to unmask and appropriate ZBG.

![Figure 44: Structure of ester analogue (143) and ketone analogue (144).](image)

The ester analogue (143) was also tested by Cramer and co-workers for its anti-proliferative activity against human epithelial carcinoma cells (A432), along with an alkyl analogue (145) (figure 45). Again, the lack of a ZBG proved detrimental as neither compound showed any activity up to concentrations of 5 μM.

![Figure 45: Structure of alkyl analogue (145).](image)
Table 19: HDAC inhibition of largazole thiol and ZBG analogues (IC$_{50}$, μM). Data from ref.\textsuperscript{129}

<table>
<thead>
<tr>
<th></th>
<th>HDAC1</th>
<th>HDAC2</th>
<th>HDAC3</th>
<th>HDAC6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Largazole thiol</td>
<td>0.0012</td>
<td>0.0035</td>
<td>0.0034</td>
<td>0.049</td>
</tr>
<tr>
<td>146</td>
<td>&gt;30</td>
<td>&gt;30</td>
<td>&gt;30</td>
<td>&gt;30</td>
</tr>
<tr>
<td>147</td>
<td>0.27</td>
<td>4.1</td>
<td>4.1</td>
<td>&gt;30</td>
</tr>
<tr>
<td>148</td>
<td>0.67</td>
<td>1.6</td>
<td>0.96</td>
<td>0.7</td>
</tr>
</tbody>
</table>

In attempts to replace the thiol moiety with other known ZBGs, Williams and co-workers also prepared largazole analogues with a carboxylic acid (146), α-aminobenzamide (147), and α-thioamide (148) (table 19).\textsuperscript{129} The analogue with the carboxylic acid ZBG was called the “largazole–azumamide hybrid” because the ZBG tail was taken directly from natural product HDAC inhibitors azumamides C and E. The α-aminobenzamide group is a ZBG found in well-known synthetic HDAC inhibitor MS-275. Lastly, the α-thioamide motif was identified as a potentially promising ZBG through computational studies performed by Vanommeslaeghe and co-workers. Table 19 shows the HDAC inhibitory activity of these analogues compared to largazole thiol. The largazole–azumamide hybrid (146) was inactive up to concentrations of 30 μM. This is not surprising as studies performed by Jiang and co-workers have previously shown that the largazole analogues with the olefin in the Z-configuration are unable to coordinate to the Zn$^{2+}$ in the HDAC active site.\textsuperscript{120} α-aminobenzamide and α-thioamide analogues (147 and 148) were active against HDACs 1, 2, 3, and 6, but severely less potent than largazole thiol. These
analogues emphasized the importance of an efficient ZBG group, such as a thiol, for the HDAC inhibitory activity of largazole. However, it also proved that the thiol can be replaced with other ZBGs without total loss of activity. To date, the thiol group remains the most effective ZBG for largazole as an HDAC inhibitor.

Figure 46: Structure and HDAC inhibition of largazole, 2-thiomethyl pyridine \((65)\), 2-thiomethyl thiophene \((66)\), and 2-thiomethyl phenol \((67)\) analogues. 1 \(\mu\)M of each compound was incubated with HDAC1 or HDAC6 for 1 h. Figure reprinted with permission from ref.\textsuperscript{124}

As previously mentioned (page 55), Tillekeratne and co-workers designed and prepared three ZBG analogues of largazole that contained a secondary heteroatom that should help chelate the \(\text{Zn}^{2+}\) in a bidentate 5- or 6-membered cyclic transition state. 2-thiomethyl pyridine analogue \((65)\), 2-thiomethyl thiophene analogue \((66)\), and 2-thiomethyl phenol analogue \((67)\) were prepared and tested for their HDAC1 and HDAC6 inhibitory activity.\textsuperscript{124} All analogues demonstrated target enzyme inhibition at concentrations of 1 \(\mu\)M at similar levels to largazole.
However, their IC$_{50}$ values were much higher than previous reports of largazole thiol IC$_{50}$ values (HDAC1: 0.4 nM, HDAC6: 42 nM).

![Figure 47: Structures of simplified largazole analogues. Figure adapted from ref. 143](image)

Recently, Marshall and co-workers tested a variety of simplified largazole analogues with different ZBGs for their inhibitory activity of HDACs 1, 3, 6, and 8. Several structural modifications were undertaken in order to simplify the synthesis of largazole analogues. The ester linkage was replaced with an amide linkage, converting the depsipeptide largazole into a cyclic tetrapeptide. Secondly, the thiazole-thiazoline unit was replaced with either a pro-D-pro or D-pro-pro unit to stabilize the reverse turns in the peptide backbone. Lastly, the β-hydroxy acid group was replaced with either L- or D-aspartic acid so that several ZBGs could be easily linked through amide bond formation. These four novel largazole scaffolds were easily prepared through
traditional peptide synthesis methods greatly simplifying the synthesis of largazole analogues. At the same time several ZBGs were easily introduced by utilizing the aspartic acid moiety. The ZBGs that were introduced were a thiol, thioether, sulfoxide, sulfone, alcohol, amine, carboxylic acid, ester, phosphate, hydroxamic acid, and a β-aminosulfide (figure 47). Initial conformational analysis showed promising overlap between these simplified largazole analogues and largazole thiol in the HDAC8-largazole thiol crystal structure. However, when the simplified largazole analogues containing a thiol was tested for their HDAC inhibition, all analogues were nearly inactive. Most of them showed >20% inhibition even at 30 μM concentrations, with the most potent analogue showing 50% inhibition at 30 μM. In fact, no simplified largazole analogue came close to the potency largazole thiol demonstrated with IC₅₀ values in the nanomolar ranges. The hydroxamic acid analogues were the only ones with any significant inhibitory activity with IC₅₀ values between 5 and 14 μM, while all other analogues showed >50% inhibition at 30 μM.

The analogues prepared by the Williams, Tillekeratne, and Marshall group are the only ones to date that have probed the effect of ZBGs other than a thiol on the HDAC inhibitory activity of largazole. These efforts have been met with limited success as all analogues tested showed significant decreases in potency. However, there is a variety of ZBGs that exist in both natural product and synthetic HDAC inhibitors. As described in chapter 1, largazole, along with all natural product cyclic depsidpeptide HDAC inhibitors, such as FK228, spiruchostatins, contain thiols that act as ZBGs (page 29). For cyclic tetrapeptide inhibitors, there are a larger variety of ZBGs. While the majority of these inhibitors contain epoxy ketones, like in HC toxin, Cyl-1, Cyl-2, chlamydosins, and trapoxins, many inhibitors of the apicidin family contain an ethyl ketone ZBG, while FR235222 and AS1387392 contain α-hydroxy ketones. Members of the azumamide family are unique as they are the only natural product HDAC inhibitors that contain either carboxylic acids or primary amides to be used as ZBGs (page 28). In linear natural product
HDAC inhibitors, depudecin contains an α-hydroxy epoxide ZBG, while santacruzamate A contains a carbamate.

**ZBGs found in HDAC inhibitors**

\[
\begin{align*}
\text{OH} & \quad \text{R'N\text{\text{\text{\text{\text{n}}}O}} \quad \text{SO}_2 \text{Me} \quad \text{PO}_2 \text{O} \text{Li} \\
\text{R'N\text{\text{\text{\text{\text{n}}}O}} & \quad \text{SH} \quad \text{SH} \quad \text{R'N\text{\text{\text{\text{\text{n}}}O}} \quad \text{NH} \text{CO} \text{OH} \quad \text{R'N\text{\text{\text{\text{\text{n}}}O}} \quad \text{Br} \quad \text{R'\text{\text{\text{\text{\text{n}}}O} \quad \text{CO} \text{O}}}
\end{align*}
\]

**ZBGs found in MMP inhibitors**

\[
\begin{align*}
\text{R'\text{\text{\text{\text{\text{n}}}O}} \quad \text{H} \quad \text{N} \quad \text{N} \quad \text{OH}
\end{align*}
\]

**Other ZBGs**

\[
\begin{align*}
\text{R'\text{\text{\text{\text{\text{n}}}O}} \quad \text{H} \quad \text{N} \quad \text{N} \quad \text{OH}
\end{align*}
\]

**Figure 48:** Structures of various ZBGs.

However, the most common ZBGs that have been seen in HDAC inhibitors are hydroxamic acids. Trichostatin A is one of the first examples of a hydroxamic acid HDAC inhibitor.\(^5^4\) Since its discovery, numerous HDAC inhibitors with hydroxamic acid ZBGs have been developed, including Suberanilohydroxamic acid (SAHA, vorinostat, Zolinza\(^6^9\)), LBH-589 (panobinostat, Farydak\(^5^8\)), and PXD101 (belinostat, Beleodaq\(^5^9\)), which are all FDA approved drugs currently being administered for the treatment of various lymphomas. Hydroxamic acids are known to be extremely efficient zinc binders,\(^1^4^4\) which in part explains the success it has shown in HDAC inhibitors. Ganesan and co-workers have also demonstrated that changing the ZBGs in azumamides to a hydroxamic acid significantly increases the potency of the inhibitor.\(^9^1\) However, there are also several disadvantages to using hydroxamic acids as ZBGs. In general hydroxamic acids have been associated with poor pharmacokinetics as well as severe toxicity.\(^1^4^5\)
It is possible that the high metal affinity of hydroxamic acids lead to binding of other metals and consequent off target effects. Additionally, all HDAC inhibitors that contain hydroxamic acids have shown poor isoform-selectivity, which could also be a result of high zinc affinity.\textsuperscript{146} Lastly, it has been reported that in some cases hydroxamic can be metabolized by dehydroxylation or cleaved by exopeptidases to render the inhibitor inactive.\textsuperscript{144}

In addition to these naturally found ZBGs several synthetic HDAC inhibitors have been reported with novel ZBGs, such as $N$-formyl hydroxyl amines, mercaptoamides, sulfones, and phosphones.\textsuperscript{146} The use of ZBGs in chemical biology, however, is not limited to HDAC inhibitors. Cohen and co-workers accumulated various ZBGs that have been reported in the literature for the use of matrix metalloproteinases (MMPs).\textsuperscript{144} Beyond these ZBGs, several other chemical functionalities have been identified as zinc binding moieties (figure 48).\textsuperscript{147}

While previous structure–activity relationship studies have highlighted the importance of the thiol in largazole’s ability to inhibit HDACs, compared to other structural modifications, ZBGs have not been studied in depth. Specifically, there has been very little exploration into the effect of Zn$^{2+}$ affinity of the ZBGs on the potency as well as the isoform-selectivity of largazole in a systematic manner. Additionally, there are disadvantages associated with using a thiol ZBG with largazole, mainly the metabolic instability of largazole thiol.\textsuperscript{123,135} This leads to a short half-life, which limits its use as an anti-cancer therapeutic. While previous structure–activity relationships have shown that the thiol can be replaced, it has always come with a very large decrease in potency. There are numerous options of ZBGs to choose from and here we describe the efforts towards the synthesis and biological evaluation of several ZBG analogues of largazole. A wide variety of ZBGs were chosen to cover a large spectrum of zinc affinities in order to systematically assess importance of zinc binding in HDAC inhibition. Once the analogues were prepared, they were tested for their HDAC inhibition as well as their anti-proliferative activity for
various cancer cell lines. Understanding the role ZBGs play in HDAC inhibition will be indispensable for the improvement of largazole as an effective therapeutic.

3.2 Results and discussion

3.2.1 Synthetic goals

In order to fully assess the importance of a ZBG in HDAC inhibition, a variety of ZBGs that span a wide range of zinc affinities were chosen to be incorporated into the largazole scaffold. A hydroxamic acid (148) was chosen because of its prevalence in other HDAC inhibitors and because it is generally accepted as one of the highest affinity ZBGs with an association constant (Log K) of 5.4 (figure 49). Another high affinity ZBG that was chosen was a mercaptosulfide (149). While mercaptosulfides have not been used in HDAC inhibition, they have been reported as ZBGs for the inhibition of matrix metalloproteinases (MMPs), which are a family of Zn$^{2+}$-dependent endopeptidases. While an exact association constant has not been reported, due to its prevalence in MMP inhibitors as well as its generally accepted excellent Zn$^{2+}$ affinity, we decided to examine this ZBG further. For medium affinity ZBGs, an amine (150) and a β-ketoamide (151) were chosen. Primary amines have demonstrated log K values of 4.2 (figure 49), but have not yet been used as ZBGs in HDAC inhibitors. 1,3-dicarbonyls, such as β-ketoamides, are known to bind zinc in a bidentate six-membered transition state with a similar Zn$^{2+}$ affinity as amines (Log K = 4.6) (figure 49). A ketone (152) and a carboxylic acid (153) were chosen as low affinity ZBGs. Apicidin A contains ethyl ketone as its ZBG, and while no dissociation constant for Zn$^{2+}$ has been reported, ketones generally have very low affinities for most metals. Carboxylic acids have been seen in natural product cyclic tetrapeptide HDAC inhibitors, azumamides C and E.
They are hypothesized to bind zinc in a bidentate fashion with extremely low affinity (Log K = 1.1) (figure 49). Lastly, a simple alkyl chain (154) that contains no ZBG was also chosen to see the effects of removing the ZBG on HDAC inhibition (figure 49).

**Figure 49:** Structures of ZBG analogues of largazole (148–154). Association constant values are given in Log K and refer to the ZBG of the analogue.\textsuperscript{144,147}
3.2.2 Retrosynthetic analysis

Due to the fact that the macrocyclic portion of all analogues was identical, we envisioned using advanced intermediate 43 with different olefins 155 in a cross metathesis reaction to access all analogues in a quick and divergent manner (scheme 22).

However, issues with the cross metathesis route, which will be discussed in more detail in the next section, forced us to reconsider the synthetic route. In this new synthetic scheme, known thiazole-thiazoline intermediate 102 and Fmoc-L-valine (138) would still be preserved for all analogues. Each ZBG could then be incorporated into the largazole scaffold by preparing the appropriate syn-aldol product 156 and coupling it to the two other fragments. The syn-aldol product can be prepared from the corresponding α,β-unsaturated aldehyde 157 and known N-acyl thiazolidinethione (158) (scheme 23).
3.2.3 Synthesis of zinc binding group analogues

We began the synthesis of the ZBG analogues by preparing known macrocycle intermediate 43 following previously published procedures. Cross metathesis of 43 and 1-pentene (159) in the presence of Grubbs’ 2nd catalyst afforded the alkyl analogue (154), albeit in poor yield (scheme 24). The reaction required high catalyst loading which seemed to decompose both starting material and product, explaining the poor yield of the reaction.

Scheme 23: Revised retrosynthetic analysis of ZBG analogues of largazole.

Scheme 24: Synthesis of the alkyl analogue (154).
Scheme 25: Unsuccessful cross metathesis attempts for the synthesis of the hydroxamic acid analogue (148) and the carboxylic acid analogue (153). Grubbs 2nd generation and Hoveyda-Grubbs 2nd generation were utilized in toluene, dichloromethane, and 1,2-dichloroethane as the reaction solvent. No attempts were successful.

Problems with the cross metathesis reaction further manifested themselves during attempted reactions for the hydroxamic acid analogue (148) and the carboxylic acid analogue (153). We prepared several protected hydroxamic acids and carboxylic acids that could be used as substrates for the cross metathesis reaction. However, none of the substrates resulted in the desired products. At low catalyst loading (<20 mol%) there was no consumption in the starting material, while at higher catalyst loading (>20 mol%) only decomposition of the starting material was observed with no product formation. Previous reports of using macrocycle 43 as a cross metathesis substrate had suggested that the use of Hoveyda-Grubbs 2nd generation catalyst was more efficient. Additionally, Cramer and co-workers have also identified 1,2-dichloroethane as
the most suitable solvent for cross metathesis reactions involving macrocycle 43. However, none of these reaction conditions resulted in any desired product (scheme 25).

In order to overcome the issues encountered during the cross metathesis route, we decided to incorporate the appropriate ZBGs by preparing various syn-aldol products. We began...
with the carboxylic acid analogue (153) because we envisioned being able to access several other designed analogues through the carboxylic acid group. The synthesis began with the cross metathesis of known PMB protected carboxylic acid 164 and crotonaldehyde (165) to result in $\alpha,\beta$-unsaturated aldehyde 166. Asymmetric aldol reaction with $N$-acetyl thiazolidinethione (158) resulted in the syn product (167). This intermediate was then coupled to thiazole–thiazoline intermediate 103 to form intermediate 168, which subsequently underwent Yamaguchi esterification with $N$-Fmoc-$L$-valine (138) to produce the acyclic precursor 169. Fm/Fmoc deprotection and HATU-mediated macrolactamization afforded the PMB-protected intermediate 170, which was deprotected by TFA to complete the synthesis of the carboxylic acid analogue (153) (scheme 26).

Scheme 27: Unsuccessful attempts for the synthesis of the hydroxamic acid analogue (148).

The carboxylic acid analogue (153) was then utilized to install the hydroxamic acid moiety for the hydroxamic acid analogue (148). Initial attempts to couple TBS-protected hydroxylamine to the carboxylic acid of (153) by using EDC as a coupling reagent were
unsuccessful as no reaction occurred. Interestingly, ethyl chloroformate-mediated activation of the carboxylic acid resulted in successful coupling of hydroxylamine and subsequent over-activation of the hydroxyl group (scheme 27).

In order to access the hydroxylamine, we used ethyl chloroformate activation with TBS-protected hydroxylamine to prevent over-activation. While this reaction has not been fully optimized yet, it resulted in the TBS-protected intermediate 171, which was deprotected by TFA to complete the synthesis of the hydroxamic acid analogue (148) (scheme 28).

Scheme 28: Synthesis of the hydroxamic acid analogue (148).

For the preparation of the ketone analogue (152), we also thought functionalization of the carboxylic acid analogue (153) would be the most efficient route. We envisioned transforming the carboxylic acid to either a Weinreb amide or an acid chloride and installing the ethyl group through the use of a Grignard reagent. However, while the preparation of the Weinreb amide and acid chloride were both successful, neither reaction with ethyl magnesium bromide resulted in the
desired product. Addition of more ethyl magnesium bromide simply resulted in the decomposition of starting material (scheme 29).

Scheme 29: Unsuccessful attempts for the synthesis of the ketone analogue (152).

Once again, the route was revised to include the ethyl ketone in the appropriate syn-aldol product. Known ketone 175 underwent cross metathesis with crotonaldehyde (165) in the presence of Grubbs 2nd generation catalyst to afford α,β-unsaturated aldehyde 176. Asymmetric aldol reaction with N-acetyl thiazolidinethione (158) resulted in the syn-product (177), which was then coupled to thiazole–thiazoline 102 to result in intermediate 178. Yamaguchi esterification with N-Fmoc-L-valine (138) provided acyclic precursor 179, which was then subjected to Fm/Fmoc deprotection and subsequent HATU-mediated macrolactamization to complete the
synthesis of the ketone analogue (152) (scheme 30). The macrolactamization reaction for this substrate has not yet been optimized.

Scheme 30: Synthesis of the ketone analogue (152).
For the synthesis of the mercaptosulfide analogue (149), we planned on using largazole thiol (180) as an advanced intermediate in an S_n2 reaction with a bromide containing the second sulfur atom (181) (scheme 31). However, the reaction did not result in any conversion of starting material nor the formation of the desired product (182). Using cesium carbonate instead of potassium carbonate with TBAI as a phase-transfer catalyst also did not result in any desired product. Attempts to complete the reaction by switching the nucleophile and leaving group positions were also unsuccessful. Tosylate 183 was prepared from known hydroxyl analogue 142. Once again potassium carbonate or cesium carbonate with TBAI as a phase-transfer catalyst were used to couple the tosyalte (183) with several mono-protected ditihols (184). However all conditions resulted in isolation of the starting material (scheme 31).

Scheme 31: Unsuccessful attempts for the synthesis of the mercaptosulfide analogue (149).

The mercaptosulfide analogue (149) was finally prepared when a bromide instead of a tosylate was used. Known bromide analogue (186) was coupled with trityl-protected ethane
dithiol (187) using potassium carbonate as a base to form the trityl-protected mercaptosulfide analogue precursor (182). Unoptimized TFA-mediated trityl deprotection completed the synthesis of the mercaptosulfide analogue (149) (scheme 32).

Scheme 32: Synthesis of the mercaptosulfide analogue (149).

The amine analogue (150) was prepared much like the carboxylic acid analogue (153) and the ketone analogue (152). The synthesis began with the cross metathesis of Boc-protected amine 188 and crotonaldehyde (165) using Grubbs 2nd generation catalyst to afford α,β-unsaturated aldehyde 189. Asymmetric aldol reaction with N-acetyl thiazolidinethione (158) provided the syn-aldol product (190), which was then coupled with thiazole-thiazoline 102 to result in intermediate 191. Subsequent Yamaguchi esterification with N-Fmoc-L-valine (138) resulted in the acyclic precursor 192, which underwent Fm/Fmoc deprotection and HATU-mediated macrolactamization to form the Boc-protected intermediate 193. Lastly, TFA-mediated deprotection of the Boc group completed the synthesis of the amine analogue (150) (scheme 33).
Scheme 33: Synthesis of the amine analogue (150).
Scheme 34: Synthesis of the β-ketoamide analogue (151).
The last analogue that was prepared was the β-ketoamide analogue (151). We envisioned installing the dicarbonyl moiety as the last step in the synthesis utilizing the appropriate amine and diketene. In order to pursue this route, we required an intermediate that closely resembled the amine analogue (150), with one less methylene unit. Therefore, a large portion of the synthesis of the β-ketoamide analogue (151) closely followed the synthesis of the amine analogue (150).

Cross metathesis of Boc-protected allylamine 194 and crotonaldehyde (165) using Grubbs 2nd generation catalyst afforded α,β-unsaturated aldehyde 195. Asymmetric aldol reaction with N-acetyl thiazolidinethione (158) provided the syn-aldol product (196), which was then coupled with thiazole-thiazoline 102 to result in intermediate 197. Yamaguchi esterification with N-Fmoc-L-valine (138) resulted in the acyclic precursor 198, which underwent Fm/Fmoc deprotection and HATU-mediated macrolactamization to form the Boc-protected macrocycle 199, which is currently unoptimized. TFA-mediated deprotection of the Boc group produced the free amine 200, which set the stage for the last coupling step with diketene (201) to provide the β-ketoamide analogue (151) (scheme 34).

### 3.2.4 Biological activity of zinc binding group analogues

Will all 7 analogues in hand (148–154), we turned our attention to exploring the biological activity of the ZBG analogues with the help of the Luesch group at the University of Florida. First, largazole and all ZBG analogues were tested against three different human cancer cell lines for their anti-proliferative activity: colon cancer (HCT-116), prostate cancer (PC3), and breast cancer (MDA-MB-231). Several concentrations of largazole and ZBG analogues were incubated with each cancer cell line and the cell viability was measured utilizing an MTT assay. Largazole demonstrated extremely potent growth inhibition against HCT-116 cells with an IC₅₀
value of 1.7 nM. This agreed with previous reports of largazole’s potent cytotoxicity towards colon cancer cells. Largazole also showed relatively potent cytotoxicity in MDA-MB-231 cells (IC\textsubscript{50} = 2.8 nM). Against PC3 cells, largazole was much less potent as 50% of cell proliferation was inhibited at 3.2 \( \mu \text{M} \). The hydroxamic acid analogue (148) also showed strong cytotoxicity in HCT-116 and MDA-MB-231 cells with IC\textsubscript{50} values of 1.0 \( \mu \text{M} \) and 471.1 nM respectively, and significantly weaker potency in PC3 cells (39% inhibition at 10 \( \mu \text{M} \)). The same trend was observed for the mercaptosulfide analogue (149). While it proved to be very cytotoxic against HCT-116 and MDA-MB-231 cells with IC\textsubscript{50} values of 146.3 nM and 472.5 nM respectively, its potency was greatly decreased in PC3 cells (52% inhibition at 10 \( \mu \text{M} \)) (table 20). Unfortunately none of the other analogues showed any activity up to concentrations of 10 \( \mu \text{M} \).

Table 20: Anti-proliferative activity of largazole, hydroxamic acid analogue (148) and mercaptosulfide analogue (149) (IC\textsubscript{50}, nM).

<table>
<thead>
<tr>
<th></th>
<th>HCT-116</th>
<th>MDA-MB-231</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Largazole</td>
<td>1.7</td>
<td>2.8</td>
<td>50%^a</td>
</tr>
<tr>
<td>148</td>
<td>1,000</td>
<td>471.0</td>
<td>39%^a</td>
</tr>
<tr>
<td>149</td>
<td>146.3</td>
<td>472.5</td>
<td>52%^a</td>
</tr>
</tbody>
</table>

^aPercent inhibition at concentration tested (10 \( \mu \text{M} \) for 148 and 149, 3.2 \( \mu \text{M} \) for largazole).

In addition to testing the ZBG analogues for their cytotoxicity against cancer cells, we also tested them for their HDAC inhibitory activity. It was interesting to see that the two highest high affinity ZBG analogues showed cytotoxic activity while the lower affinity analogues did not. The HDAC inhibitory data was therefore crucial in determining whether the inhibition of HDACs by these analogues was responsible for the cytotoxicity in cancer cells. Table 2 summarizes the full HDAC inhibitory profile of largazole thiol and the ZBG analogues. Compounds were
incubated at various concentrations with the appropriate HDAC at 37 °C for 30 minutes.

Subsequent incubation with an HDAC developer, a fluorescent HDAC activity probe, for an additional 15 minutes resulted in collection of fluorescence signals used to calculate IC_{50} values. Due to their inactivity, data for HDACs 4, 7, 9, 11, and sirtuins have been omitted. Once again, a similar trend as seen in the cytotoxicity data was observed.

**Table 21: HDAC inhibition profile of largazole thiol and ZBG analogues (IC_{50}, nM).**

<table>
<thead>
<tr>
<th>HDAC</th>
<th>180</th>
<th>148</th>
<th>149</th>
<th>150</th>
<th>151</th>
<th>152</th>
<th>153</th>
<th>154</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.4</td>
<td>26</td>
<td>40</td>
<td>NI^a</td>
<td>NI^a</td>
<td>30%^b</td>
<td>NI^a</td>
<td>NI^a</td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
<td>69</td>
<td>90</td>
<td>NI^a</td>
<td>NI^a</td>
<td>24%^b</td>
<td>NI^a</td>
<td>NI^a</td>
</tr>
<tr>
<td>3</td>
<td>0.7</td>
<td>29</td>
<td>43</td>
<td>NI^a</td>
<td>NI^a</td>
<td>8,000</td>
<td>NI^a</td>
<td>NI^a</td>
</tr>
<tr>
<td>5</td>
<td>NI^a</td>
<td>21%^b</td>
<td>22%^b</td>
<td>NI^a</td>
<td>NI^a</td>
<td>NI^a</td>
<td>NI^a</td>
<td>NI^a</td>
</tr>
<tr>
<td>6</td>
<td>42</td>
<td>600</td>
<td>2,800</td>
<td>NI^a</td>
<td>22%^b</td>
<td>NI^a</td>
<td>17%^b</td>
<td>NI^a</td>
</tr>
<tr>
<td>8</td>
<td>102</td>
<td>3,500</td>
<td>9,600</td>
<td>NI^a</td>
<td>NI^a</td>
<td>NI^a</td>
<td>20%^b</td>
<td>24%^b</td>
</tr>
<tr>
<td>10</td>
<td>0.5</td>
<td>21</td>
<td>3.6</td>
<td>NI^a</td>
<td>NI^a</td>
<td>49%^b</td>
<td>19%^b</td>
<td>NI^a</td>
</tr>
</tbody>
</table>

^aNo significant inhibition (< 15%) at the highest concentration tested (1 μM for 180, 10 μM for other compounds). ^bPercentage inhibition at the highest concentration tested (1 μM for 180, 10 μM for other compounds).

Largazole thiol (180) was the most potent HDAC inhibitor showing sub nanomolar activity against HDACs 1, 2, 3, and 10, while showing nanomolar activity against HDACs 6, and 8. The hydroxamic acid analogue (148) and the mercaptosulfide analogue (149) also showed extremely potent inhibitory activity against HDACs 1, 2, 3, and 10, much like largazole thiol (180). However, they were significantly less potent than largazole thiol (180), with their HDAC8 IC_{50} values reaching the micromolar ranges. Much like the cytotoxicity data, the other analogues with weaker affinity ZBGs show little to no activity.
Based on the fact that the high affinity ZBG analogues demonstrating activity in both the proliferation assay and the HDAC inhibition assay, it is evident that there is a strong correlation between the two biological activities. This is not surprising as previous reports have also shown that largazole’s cytotoxicity correlates extremely well with its ability to inhibit cellular HDAC activity. It is also not surprising that the higher affinity ZBG analogues show higher potency than the lower affinity analogues. However, this trend doesn’t seem to hold up in all cases as the thiol, which should have a lower zinc affinity than the hydroxamic acid or the mercaptosulfide, demonstrated a significantly higher potency than the other high affinity ZBG analogues.

Additionally, the significant decrease in potency between the high affinity ZBG analogues and the medium and low affinity ZBG analogues gives rise to several new questions. Lastly, while the hydroxamic acid analogue (148) and the mercaptosulfide analogue (149) showed decreased potency in the enzymatic assay, that decrease was much more emphasized in the cellular assay, especially in the HCT-116 cells. Largazole was 629-fold more potent than the hydroxamic acid analogue (148) and 88-fold more potent than the mercaptosulfide analogue (149) (table 20). Since the decrease in potency in the enzymatic assay was much less significant, decrease in HDAC inhibition alone cannot fully explain the decrease in anti-proliferative activity.

When rationalizing the HDAC inhibition data, one possibility is that the ZBG in largazole must have a certain affinity for zinc in order to be able to inhibit HDACs. However, what is more likely is that the linker that connects the macrocycle to the ZBG in largazole must be optimized in order to achieve efficient inhibition. Since the metal–ligand distance for the ZBGs and Zn$^{2+}$ is relatively constant, it is likely that the hydroxamic acid analogue (148), the β-ketoamide analogue (151), the ketone analogue (152), and the carboxylic acid analogue (153) all have linkers that result in sub-optimal Zn$^{2+}$ binding. As previously reported, having a linker that is too long can result in a very significant decrease in potency. It is possible that if the linker length of
these analogues was shortened, HDAC inhibitory activity could be observed. The amine analogue (150), however, also showed no inhibitory activity, despite a linker that is a methylene unit shorter. This can potentially attributed to the the protonation of the amine in assay conditions, which would result in a significant decrease in Zn$^{2+}$ affinity. Another potential cause of this perceived inactivity of analogues is a potential change in the mode of inhibition of the analogue. If the change in ZBG results in slow-binding inhibition, it is possible that the onset time exceeds the incubation time of the assay, thereby giving the illusion of an inactive inhibitor. In order to fully understand the role of the ZBG in largazole’s HDAC inhibitory activity, both the linker length and the time-dependency of the inhibitor need to be examined further.

When evaluating the discrepancy in the anti-proliferative assay and enzymatic assay, it is possible that the increased polarity of the hydroxamic acid and the mercaptosulfide impeded absorption of the analogue into cells. For hydroxamic acids in particular, poor pharmacokinetic properties have been previously reported. While these issues remain to be addressed in the future, this data shows that largazole can be incorporated with a ZBG other than thiol and still remain a very efficient HDAC inhibitor with IC$_{50}$ values in the nanomolar ranges.

### 3.3 Conclusion

In summary, we designed, synthesized, and biologically characterized several largazole analogues that contain a variety of ZBGs with a wide range of zinc affinities, in order to assess the extent to which the ZBG affects largazole’s anti-proliferative activity and HDAC inhibitory activity. A convergent synthetic approach was utilized to prepare all of the analogues and each was tested for its anti-proliferative activity against human colon, prostate, and breast cancer, as well as its full HDAC inhibition profile. In both assays, the hydroxamic acid analogue (148) and
the mercaptosulfide analogue (149) proved to be potent anti-proliferative agents as well as HDAC inhibitors, albeit significantly less potent than largazole and largazole thiol, respectively. These two analogues, which contain high affinity ZBGs, also share an HDAC inhibitory profile with largazole thiol as they show excellent potency (nanomolar IC_{50} values) against HDACs 1, 2, 3, and 10, while demonstrating good potency against HDACs 6 and 8. The medium and low affinity analogues were inactive in both the cytotoxicity and the HDAC inhibition assay showing a strong correlation between zinc affinity of the ZBG, HDAC inhibition, and cytotoxicity.

However, it is most likely that the complete lack of activity for some of the medium affinity analogues is caused by other factors, such as linker length and inhibitor mode of action. In order to fully characterize the role that ZBGs play in HDAC inhibition for largazole, these factors must be examined in depth. Nevertheless, these results demonstrated that the thiol can be replaced by other ZBGs without detrimental loss in HDAC inhibitory activity. One of the biggest drawbacks of using largazole as a therapeutic is its short half-life caused by the instability of the thiol in cellular metabolism. It is therefore essential to assess the role of the ZBG in HDAC inhibition in order to improve largazole’s pharmacokinetic properties if we hope to use largazole as a therapeutic in the future.

For future direction of this project, optimization of the linker length will provide essential information that must be considered when assessing the role of the ZBG. Once the linker lengths of the different ZBGs have been optimized a full Zn^{2+}-affinity–HDAC inhibition relationship can be established. Additionally, the stability of the analogues will need to be tested to see if pharmacokinetic properties of largazole, such as half-life and clearance, can indeed be improved by modulation of the ZBG. This information will prove useful in future desing of HDAC inhibitors as well as usage of largazole as a potential therapeutic.
3.4 Experimental

General Methods

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich, Acros, Chem-Impex, or Fischer and were used without further purification. All solvents were ACS grade or better and used without further purification except THF which was freshly distilled each time before use. Thin layer chromatography was performed with glass backed silica gel (60 Å) plates purchased from Whatman and visualized with 254 nm UV light. All chromatographic purifications were conducted via flash chromatography using ultra-pure silica gel (230-400 mesh, 60 Å) purchased from Silicycle as the stationary phase unless otherwise noted. All spectra were recorded in CDCl₃ unless otherwise noted, using a 400 MHz (Varian) or 500 MHz (Bruker) NMR spectrometer. All NMR shifts are given in ppm, and all J-values are given in Hz. High-resolution mass spectra (HRMS) were obtained by an Agilent 6224 time-of-flight liquid chromatography-electrospray ionization spectrometer. Infrared (IR) absorption spectra were determined with a Thermo–Fisher (Nicolet 6700) spectrometer. Optical rotation values were measured with a Rudolph Research Analytical (A21102. API/1W) polarimeter.

Preparation of 154

\[
\begin{align*}
\text{Grubbs II (40 mol%)} & \quad \text{toluene, reflux, 3 h} \\
43 + 159 & \quad 26 \% \quad 154
\end{align*}
\]
To a solution of 43 (12.6 mg, 0.029 mmol) in toluene (1 mL) in a pressure vial were added 159 (31.6 μL, 0.289 mmol) and Grubbs 2nd generation catalyst (4.9 mg, 0.006 mmol) and the vial was sealed. After stirring for 1 h at 100 °C, more 159 (31.6 μL, 0.289 mmol) and Grubbs 2nd generation catalyst (4.9 mg, 0.006 mmol) were added to the mixture. After stirring for 2 h at 100 °C, the mixture was cooled (25 °C) and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/1) to afford 154 (3.9 mg, 26%): [α]D = +29.4 (c 1.0, EtOAc); 1H NMR (500 MHz, CDCl3) δ 7.75 (s, 1H), 7.13 (d, J = 9.5 Hz, 1H), 6.49 (dd, J = 9.0, 2.5 Hz, 1H), 5.81 (ddd, J = 15.5, 7.0, 7.0 Hz, 1H), 5.64 (dd, J = 8.0, 8.0 Hz, 1H), 5.40 (dd, J = 15.5, 7.0 Hz, 1H), 5.26 (dd, J = 17.0, 9.0 Hz, 1H), 4.58 (dd, J = 9.5, 3.5 Hz, 1H), 4.24 (dd, J = 18.0, 3.5 Hz, 1H), 4.02 (d, J = 11.5 Hz, 1H), 3.25 (d, J = 11.5 Hz, 1H), 2.84 (dd, J = 16.5, 10.5 Hz, 1H), 2.66 (dd, J = 16.0, 2.5 Hz, 1H), 2.07–2.11 (m, 1H), 1.99 (ddd, J = 7.0, 7.0, 7.0 Hz, 2H), 1.84 (s, 3H), 1.36 (ddd, J = 7.0, 7.0, 7.0 Hz, 2H), 0.85 (dd, J = 7.0, 7.0 Hz, 3H), 0.66 (d, J = 7.0 Hz, 1H), 0.48 (d, J = 7.0 Hz, 1H); 13C NMR (125 MHz, CDCl3) δ 173.6, 169.6, 169.0, 168.0, 164.6, 147.5, 135.8, 126.4, 124.3, 84.4, 72.9, 57.7, 43.4, 41.2, 40.6, 34.3, 24.2, 22.0, 18.9, 16.6, 13.6; IR (neat) 3370, 2959, 2930, 1734, 1676, 1636, 1376, 1143, 1027, 972 cm⁻¹; HRMS (ESI) m/z 479.1778 [(M+H)+, C22H30N4O4S2 requires 479.1781].

Preparation of 166

To a solution of 164 (500 mg, 2.27 mmol) in CH2Cl2 (10 mL) was added 165 (2.0 mL, 22.7 mmol) and Grubbs 2nd generation catalyst (38.5 mg, 0.045 mmol). After stirring for 3 h at reflux, the mixture was concentrated in vacuo. The residue was purified by column
chromatography (silica gel, EtOAc/hexanes = 1/4) to afford 166 (608.7 mg, > 99%). Due to instability of the compound 166 was immediately used for subsequent steps.

**Preparation of 167**

![Chemical Reaction Diagram](attachment:image.png)

To a cooled (0 °C) solution of 158 (247.6 mg, 1.22 mmol) in CH₂Cl₂ (15 mL) was added TiCl₄ (1.0 M in CH₂Cl₂, 1.34 mL, 1.34 mmol). After stirring for 5 min at 0 °C, the mixture was cooled (−78 °C) and i-PrNEt (233.0 μL, 1.34 mmol) was added. After stirring for 1 h at −78 °C, 166 (184.6 mg, 0.744 mmol) in CH₂Cl₂ (2 mL) was added dropwise. After stirring for 1 h at −78 °C, the reaction was quenched by the addition of saturated NH₄Cl solution. The layers were separated and the aqueous layer was extracted with CH₂Cl₂. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/2 to 1/1) to afford 167 (232.8 mg, 69%): [α]₂⁵D = −175.2 (c 1.0, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 7.28 (d, J = 8.4 Hz, 2H), 6.88 (d, J = 8.8 Hz, 2H), 5.73 (ddd, J = 14.4, 6.4, 6.4 Hz, 1H), 5.56 (dd, J = 15.6, 6.0 Hz, 1H), 5.14 (dd, J = 6.4, 6.4 Hz, 1H), 5.04 (s, 2H), 4.60 (br s, 1H), 3.80 (s, 3H), 3.58 (dd, J = 14.4, 3.2 Hz, 1H), 3.52 (dd, J = 11.6, 8.0 Hz, 1H), 3.26 (dd, J = 17.6, 8.8 Hz, 1H), 3.02 (dd, J = 11.6, 1.2 Hz, 1H), 2.33–2.44 (m, 5H), 1.06 (d, J = 6.8 Hz, 3H), 0.97 (d, J = 7.2 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 203.0, 172.8, 172.4, 159.6, 131.9, 130.1, 129.9, 128.1, 114.0, 71.4, 68.5, 66.1, 55.3, 45.4, 33.8, 30.9, 30.8, 27.5, 19.1, 17.8; IR (neat) 2961, 1731, 1698, 1514, 1247, 1159, 1036, 823 cm⁻¹; HRMS (ESI) m/z 474.1379 [(M+Na)⁺, C₂₂H₂₉NO₅S₂ requires 474.1368].
Preparation of 168

To a solution of 102 (129.7 mg, 0.242 mmol) in CH$_2$Cl$_2$ (12 mL) was added TFA (3.0 mL) at 25 °C. After stirring for 2 h at 25 °C, the reaction mixture was concentrated in vacuo and washed with Et$_2$O. To a solution of the crude mixture in CH$_2$Cl$_2$ (15 mL) were added 167 (104.0 mg, 0.230 mmol) in CH$_2$Cl$_2$ (2 mL) and DMAP (148.0 mg, 1.21 mmol) at 25 °C. After stirring for 2 h at 25 °C, the reaction was quenched by the addition of H$_2$O. The layers were separated and the aqueous layer was extracted with CH$_2$Cl$_2$. The combined organic layers were dried over anhydrous Na$_2$SO$_4$ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/4 to EtOAc 100%) to afford 168 (137.3 mg, 82% for 2 steps): $[\alpha]^{25}_D = -29.5$ (c 1.0, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.97 (s, 1H), 7.71 (dd, $J = 7.2, 4.8$ Hz, 2H), 7.59 (d, $J = 7.6$ Hz, 2H), 7.32–7.41 (m, 2H), 7.16–7.26 (m, 4H), 6.86 (d, $J = 8.0$ Hz, 2H), 5.65 (ddd, $J = 15.6, 6.4, 6.4$ Hz, 1H), 5.47 (dd, $J = 15.6, 6.0$ Hz, 1H), 5.01 (s, 2H), 4.66–4.76 (m, 2H), 4.50 (d, $J = 6.8$ Hz, 2H), 4.45 (br s, 1H), 4.22 (dd, $J = 6.8, 6.8$ Hz, 1H), 3.76 (s, 3H), 3.73 (d, $J = 11.6$ Hz, 1H), 3.19 (d, $J = 11.6$ Hz, 1H), 2.29–2.44 (m, 6H), 1.61 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 172.8, 172.0, 168.3, 163.3, 159.5, 148.0, 143.4, 143.4, 141.3, 141.2, 132.2, 130.1, 129.8, 128.0, 127.9, 127.8, 127.1, 127.0, 125.1, 125.0, 122.5, 120.1, 120.0, 113.9, 84.3, 77.4, 69.0, 67.3, 66.1, 55.3, 46.7, 42.8, 41.5, 40.8, 33.6, 27.3, 24.0; IR (neat) 3323, 1731, 1655, 1611, 1515, 1450, 1246, 1160, 1114, 1032, 972, 827, 759, 741 cm$^{-1}$; HRMS (ESI) m/z 748.2122 [(M+Na)$^+$, C$_{39}$H$_{39}$N$_3$O$_7$S$_2$ requires 748.2120].
Preparation of 169

To a cooled (0 ºC) solution of 138 (128.3 mg, 0.189 mmol) in THF (10 mL) were added 2,4,6-trichlorobenzoyl chloride (100.0 µL, 0.567 mmol) and Et₃N (100.0 µL, 0.662 mmol). After stirring for 30 min at 0 ºC, 168 (137.3 mg, 0.189 mmol) in THF (2 mL) and DMAP (50.0 mg, 0.378 mmol) were added at 0 ºC. After stirring for 1 h at 25 ºC, the reaction was quenched by the addition of saturated NH₄Cl solution and diluted with EtOAc. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 2/1) to afford 169 (191.7 mg, 97%): [α]²⁵D = −20.8 (c 1.0, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 7.94 (s, 1H), 7.71–7.75 (m, 4H), 7.59 (dd, J = 19.6, 7.6 Hz, 4H), 7.20–7.40 (m, 10H), 7.02 (dd, J = 4.8, 4.8 Hz, 1H), 6.88 (d, J = 8.4 Hz, 2H), 5.82 (ddd, J = 15.2, 6.4, 6.4 Hz, 1H), 5.66 (ddd, J = 6.8, 6.8, 6.8 Hz, 1H), 5.39 (d, J = 8.0 Hz, 1H), 5.03 (s, 2H), 4.75 (d, J = 5.6 Hz, 2H), 4.10–4.48 (m, 6H), 3.78 (s, 3H), 3.76 (d, J = 8.0 Hz, 1H), 3.20 (d, J = 11.6 Hz, 1H), 2.57 (d, J = 6.0 Hz, 2H), 2.34–2.40 (m, 4H), 2.07–2.10 (m, 1H), 1.63 (s, 3H), 0.94 (d, J = 6.4 Hz, 3H), 0.89 (d, J = 6.4 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 173.0, 172.6, 171.3, 169.1, 168.6, 162.9, 159.6, 156.5, 148.4, 143.8, 143.7, 143.6, 143.5, 141.3, 141.2, 133.7, 130.2, 127.9, 127.8, 127.7, 127.4, 127.2, 127.1, 125.3, 125.2, 125.1, 125.0, 122.1, 120.1, 120.0, 114.0, 84.7, 77.4, 72.3, 67.4, 67.1, 61.2, 59.5, 55.3, 47.2, 46.8, 41.6, 41.4, 41.2, 33.4, 30.9,
Preparation of 170

To a solution of 169 (57.1 mg, 0.049 mmol) in CH$_3$CN (10 mL) was added Et$_2$NH (2.0 mL) at 25 °C. After stirring for 2 h at 25 °C, the reaction mixture was concentrated in vacuo and washed with toluene. After removal of toluene in vacuo, to a solution of the crude mixture in CH$_2$Cl$_2$ (60.0 mL) were added HATU (37.0 mg, 0.098 mmol) and i-Pr$_2$NEt (25.0 µL, 0.146 mmol) at 25 °C. After stirring for 24 h at 25 °C, the reaction mixture was concentrated in vacuo.

The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/1 to EtOAc 100%) to afford 170 (18.0 mg, 38% for 2 steps): $^1$H NMR (400 MHz, CDCl$_3$) δ 7.73 (s, 1H), 7.25 (d, $J = 7.2$ Hz, 2H), 7.14 (d, $J = 9.2$ Hz, 2H), 6.54 (dd, $J = 9.2$, 3.2 Hz, 1H), 5.83 (ddd, $J = 15.6$, 6.8, 6.8 Hz, 1H), 5.58–5.63 (m, 1H), 5.46 (dd, $J = 15.6$, 6.8 Hz, 1H), 5.22 (dd, $J = 17.6$, 9.2 Hz, 1H), 4.99 (dd, $J = 18.4$, 12.0 Hz, 2H), 4.55 (dd, $J = 9.6$, 3.6 Hz, 1H), 4.25 (dd, $J = 17.6$, 3.2 Hz, 1H), 4.00 (d, $J = 11.2$ Hz, 1H), 3.77 (s, 3H), 3.24 (d, $J = 11.2$ Hz, 1H), 2.73 (dd, $J = 16.0$, 9.6 Hz, 1H), 2.57 (dd, $J = 16.4$, 3.2 Hz, 1H), 2.33–2.41 (m, 4H), 2.05–2.08 (m, 1H), 1.83 (s, 3H), 0.66 (d, $J = 7.2$ Hz, 3H), 0.49 66 (d, $J = 7.2$ Hz, 3H); IR (neat) 3367, 2964, 1733, 1671, 1514, 1248, 1158, 1032, 845 cm$^{-1}$; HRMS (ESI) $m/z$ 651.1918 [(M+Na)$^+$, C$_{30}$H$_{36}$N$_4$O$_7$S$_2$ requires 651.1917].
Preparation of 153

To a solution of 170 (15.7 mg, 0.025 mmol) in CH$_2$Cl$_2$ (5 mL) was added TFA (500 μL). After stirring for 1 h at 25 °C, the reaction mixture was concentrated in vacuo. The residue was purified by column chromatography (silica gel, CHCl$_3$/CH$_3$OH = 9:1 + 1% TFA) to afford 153 (11.9 mg, 94%): [α]$^\text{D}$ = +43.9 (c 0.48, CH$_3$OH); $^1$H NMR (400 MHz, CD$_3$OD) δ 8.15 (s, 1H), 7.29 (d, $J$ = 9.2 Hz, 1H), 5.88 (ddd, $J$ = 14.0, 6.4, 6.4 Hz, 1H), 5.59–5.62 (m, 2H), 5.12 (d, $J$ = 17.6 Hz, 1H), 4.49 (d, $J$ = 9.6, 3.2 Hz, 1H), 4.35 (d, $J$ = 17.6 Hz, 1H), 3.93 (d, $J$ = 11.6 Hz, 1H), 3.40 (d, $J$ = 11.6 Hz, 1H), 3.01 (dd, $J$ = 16.0, 10.0 Hz, 1H), 2.69 (dd, $J$ = 16.8, 2.0 Hz, 1H), 2.33–2.37 (m, 4H), 2.06–2.10 (m, 1H), 1.82 (s, 3H), 0.70 (d, $J$ = 6.8 Hz, 1H), 0.49 (d, $J$ = 6.8 Hz, 1H); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 176.5, 175.9, 172.2, 170.0, 168.0, 148.1, 134.5, 19.0, 126.8, 85.1, 74.0, 59.0, 43.6, 40.6, 38.9, 35.4, 34.1, 28.1, 24.5, 24.3, 19.7, 17.1; IR (neat) 3368, 2964, 2932, 1732, 1668, 1514, 1257, 1184, 1033, 974 cm$^{-1}$; HRMS (ESI) m/z 509.1523 [(M+H)$^+$], C$_{22}$H$_{28}$N$_4$O$_6$S$_2$ requires 509.1523].
Preparation of 171

To a solution of 153 (30.8 mg, 0.061 mmol) in THF (4 mL) were added ethyl chloroformate (10.0 μL, 0.122 mmol) and Et₃N (16.9 μL, 0.122 mmol). After stirring for 1 h at 25 °C, TBS-hydroxylamine (26.8 mg, 0.182 mmol) in CH₃OH (1 mL) was added to the reaction. After stirring for 1 h at 25 °C, the mixture was concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc 100%) to afford 171 (22.4 mg, 59%): [α]²⁵_D = +47.2 (c 0.38, CH₃OH); ¹H NMR (400 MHz, CD₃OD) δ 8.14 (s, 1H), 7.28 (d, J = 9.6 Hz, 1H), 5.86 (ddd, J = 14.4, 6.8, 6.8 Hz, 1H), 5.58–5.62 (m, 2H), 5.15 (d, J = 17.6 Hz, 1H), 4.48 (dd, J = 9.6, 3.2 Hz, 1H), 4.36(d, J = 17.6 Hz, 1H), 3.92 (d, J = 11.6 Hz, 1H), 3.39 (d, J = 11.6 Hz, 1H), 3.01 (dd, J = 16.4, 10.4 Hz, 1H), 2.68 (dd, J = 16.8, 2.4 Hz, 1H), 2.36 (ddd, J = 6.8, 6.8, 6.8 Hz, 2H), 2.18 (dd, J = 6.8, 6.8 Hz, 2H), 2.06–2.09 (m, 1H), 1.81 (s, 3H), 0.96 (s, 9H), 0.69 (d, J = 6.8 Hz, 3H), 0.49 (d, J = 6.8 Hz, 3H), 0.16 (s, 6H); ¹³C NMR (125 MHz, CD₃OD) δ 175.8, 172.2, 172.1, 170.0, 168.0, 148.1, 134.6, 129.2, 126.8, 85.0, 74.8, 40.5, 35.5, 32.9, 29.1, 26.3, 24.4, 19.7, 17.1, 5.4; IR (neat) 3276, 2959, 2929, 1735, 1659, 1509, 1253, 834 cm⁻¹; HRMS (ESI) m/z 660.2316 [(M+Na)⁺, C₂₈H₄₁N₄O₆S₂Si requires 660.2316].
Preparation of 148

To a solution of 171 (22.4 mg, 0.035 mmol) in CH₂Cl₂ (5 mL) was added TFA (400 μL). After stirring for 1 h at 25 °C, the reaction mixture was concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, CHCl₃/CH₃OH = 9:1) to afford 148 (14.9 mg, 81%): [α]₂⁵^D = +33.9 (c 0.24, CH₃OH); ′H NMR (400 MHz, CD₃OD) δ 8.25 (br s, 1H), 8.14 (s, 1H), 7.29 (d, J = 9.2 Hz, 1H), 5.83 (ddd, J = 14.0, 7.2, 7.2 Hz, 1H), 5.56–5.62 (m, 2H), 5.10 (dd, J = 18.0, 5.6 Hz, 1H), 4.49 (dd, J = 9.2, 3.2 Hz, 1H), 4.38 (d, J = 17.6 Hz, 1H), 3.92 (d, J = 11.6 Hz, 1H), 3.39 (d, J = 11.6 Hz, 1H), 3.00 (dd, J = 16.4, 10.0 Hz, 1H), 2.69 (d, J = 16.4 Hz, 1H), 2.35 (ddd, J = 7.2, 7.2, 7.2 Hz, 2H), 2.17 (dd, J = 7.2, 7.2 Hz, 2H), 2.05–2.09 (m, 1H), 1.81 (s, 3H), 0.70 (d, J = 6.8 Hz, 3H), 0.49 (d, J = 6.8 Hz, 3H); ′C NMR (125 MHz, CD₃OD) δ 175.9, 172.2, 170.0, 168.1, 148.1, 1342, 129.3, 136.8, 85.0, 74.1, 59.0, 43.7, 41.8, 40.6, 35.4, 33.0, 29.1, 24.3, 19.7, 17.1; IR (neat) 3272, 2963, 2931, 1657, 1515, 1257, 1185, 1139, 1035, 974 cm⁻¹; HRMS (ESI) m/z 524.1632 [(M+H)+, C₂₂H₂₉N₅O₆S₂ requires 524.1631].

Preparation of 176

To a solution of 175 (375.6 mg, 1.51 mmol) in CH₂Cl₂ (10 mL) was added 165 (1.3 mL, 15.1 mmol) and Grubbs 2nd generation catalyst (40.0 mg, 0.045 mmol). After stirring for 3 h at
reflux, the mixture was concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/5 to 1/3) to afford 176 (225.6 mg, > 99%). Due to instability of the compound, 176 was immediately used for subsequent steps.

**Preparation of 177**

![Chemical diagram]

To a cooled (0 °C) solution of 158 (333.3 mg, 1.64 mmol) in CH₂Cl₂ (10 mL) was added TiCl₄ (1.0 M in CH₂Cl₂, 1.64 mL, 1.64 mmol). After stirring for 5 min at 0 °C, the mixture was cooled (−78 °C) and i-PrNEt (285.0 μL, 1.64 mmol) was added. After stirring for 1 h at −78 °C, 176 (138.0 mg, 0.984 mmol) in CH₂Cl₂ (2 mL) was added dropwise. After stirring for 1 h at −78 °C, the reaction was quenched by the addition of saturated NH₄Cl solution. The layers were separated and the aqueous layer was extracted with CH₂Cl₂. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/5 to 1/3) to afford 177 (133.1 mg, 53%): [α]²⁵_D = −208.8 (c 1.0, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 5.73 (ddd, J = 15.6, 6.4, 6.4 Hz, 1H), 5.56 (dd, J = 15.6, 6.4 Hz, 1H), 5.15 (dd, J = 6.8, 6.8 Hz, 1H), 4.59–4.63 (m, 1H), 3.61 (dd, J = 17.6, 3.5 Hz, 1H), 3.54 (dd, J = 11.2, 8.0 Hz, 1H), 3.28 (dd, J = 17.6, 8.8 Hz, 1H), 3.03 (dd, J = 11.6, 1.2 Hz, 1H), 2.51 (dd, J = 7.2, 7.2 Hz, 2H), 2.42 (ddd, J = 7.2, 7.2, 7.2 Hz, 2H), 2.92–2.37 (m, 3H), 1.03–1.07 (m, 6 H), 0.98 (d, J = 6.8 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 210.7, 203.1, 172.5, 131.5, 130.6, 71.5, 68.6, 45.4, 41.5, 36.0, 30.9, 30.7, 26.4, 19.1, 17.8, 7.8; IR (neat) 3470, 3100, 2930, 2870, 1730, 1500, 1400, 1300, 1200, 1100, 1000, 900, 800, 700, 600, 500 cm⁻¹.
To a solution of 102 (65.8 mg, 0.123 mmol) in CH₂Cl₂ (4 mL) was added TFA (1.0 mL) at 25 °C. After stirring for 1 h at 25 °C, the reaction mixture was concentrated in vacuo and washed with Et₂O. To a solution of the crude mixture in CH₂Cl₂ (5 mL) were added 177 (37.7 mg, 0.108 mmol) in CH₂Cl₂ (2 mL) and DMAP (66.1 mg, 0.541 mmol) at 25 °C. After stirring for 1 h at 25 °C, the reaction was quenched by the addition of saturated NH₄Cl solution and diluted with EtOAc. The layers were separated and the organic layer was washed with additional saturated NH₄Cl solution. The organic layer was dried over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc 100%) to afford 178 (53.3 mg, 80% for 2 steps): [α]²⁵D = –25.5 (c 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 7.91 (s, 1H), 7.71 (dd, J = 7.0, 5.5 Hz, 2H), 7.58 (d, J = 7.5 Hz, 2H), 7.34 (ddd, J = 11.5, 2.5, 2.5 Hz, 2H), 7.23 (dd, J = 26.0, 7.5, 7.5 Hz, 2H), 5.64 (ddd, J = 15.5, 6.5, 6.5 Hz, 1H), 5.46 (dd, J = 15.5, 6.0 Hz, 1H), 4.71 (d, J = 6.0 Hz, 2H), 4.49 (d, J = 7.0 Hz, 2H), 4.45 (br s, 1H), 4.22 (dd, J = 6.5, 6.5 Hz, 1H), 3.71 (d, J = 11.0 Hz, 1H), 3.18 (d, J = 11.0 Hz, 1H), 2.35–2.48 (m, 5H), 2.23 (ddd, J = 7.0, 7.0 Hz, 2H), 1.58 (s, 3H), 0.99 (dd, J = 7.0, 7.0 Hz, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 210.8, 172.7, 172.1, 168.3, 162.9, 148.0, 143.3, 143.2, 141.2, 141.1, 131.7, 130.3, 127.7, 127.6,
127.0, 126.9, 125.0, 124.9, 122.4, 119.9, 84.4, 77.4, 68.9, 67.2, 46.6, 42.6, 42.3, 41.4, 41.2, 40.6, 35.8, 26.1, 23.9, 11.1, 7.6; IR (neat) 3303, 2977, 2935, 1711, 1670, 1540, 1450, 1169, 1119, 975, 198, 159 cm\(^{-1}\); HRMS (ESI) \text{m/z} 640.1918 [(M+Na\(^{+}\)), C\(_{33}\)H\(_{35}\)N\(_{3}\)O\(_{5}\)S\(_{2}\) requires 640.1917].

Preparation of 179

To a cooled (0 °C) solution of 138 (43.8 mg, 0.129 mmol) in THF (5 mL) were added 2,4,6-trichlorobenzoyl chloride (27.0 µL, 0.173 mmol) and Et\(_{3}\)N (36.0 µL, 0.259 mmol). After stirring for 1 h at 0 °C, 178 (53.3 mg, 0.086 mmol) in THF (1 mL) and DMAP (15.8 mg, 0.129 mmol) were added at 0 °C. After stirring for 30 min at 25 °C, the reaction was quenched by the addition of saturated NH\(_{4}\)Cl solution and diluted with EtOAc. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na\(_2\)SO\(_4\) and concentrated \textit{in vacuo}. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 3/2) to afford 179 (76.0 mg, 94%): \([\alpha]_{D}^{25} = -14.1 \) (c 0.36, CHCl\(_3\)); \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta 7.93 \) (s, 1H), 7.71–7.76 (m, 4H), 7.55–7.61 (m, 4H), 7.17–7.45 (m, 8H), 7.08 (br s, 1H), 5.81 (ddd, \(J = 15.5, 6.5, 6.5 \) Hz, 1H), 5.65 (ddd, \(J = 6.0, 6.0, 6.0 \) Hz, 1H), 5.52 (ddd, \(J = 15.0, 7.0 \) Hz, 1H), 5.34 (d, \(J = 8.5 \) Hz, 1H), 4.74 (d, \(J = 6.0 \) Hz, 2H), 4.04–4.50 (m, 6H), 3.75 (d, \(J = 11.0 \) Hz, 1H), 3.19 (d, \(J = 11.0 \) Hz, 1H), 2.60 (d, \(J = 5.5 \) Hz, 2H), 2.44 (d, \(J = 7.5 \) Hz, 2H), 2.37 (ddd, \(J = 7.5, 7.5, 7.5 \) Hz, 2H), 2.80 (ddd, \(J = 7.0, 7.0, 7.0 \) Hz, 2H).
2H), 2.06–2.10 (m, 1H), 1.62 (s, 3H), 1.02 (dd, J = 7.0, 7.0 Hz, 3H), 0.93 (d, J = 6.5 Hz, 3H), 0.89 (d, J = 6.5 Hz, 3H); 13C NMR (125 MHz, CDCl3) δ 210.3, 172.9, 171.2, 169.1, 168.5, 162.9, 156.4, 148.3, 143.7, 143.6, 143.5, 143.4, 141.2, 134.4, 127.9, 127.8, 127.0, 126.9, 125.1, 125.0, 122.16, 119.9, 84.5, 72.4, 67.3, 67.0, 66.9, 59.5, 47.2, 47.1, 46.7, 41.6, 41.4, 41.0, 35.9, 30.8, 26.2, 24.0, 19.2, 19.0, 17.8, 17.7, 7.7; IR (neat) 3326, 3064, 2965, 1715, 1528, 1477, 1187, 1114, 1032, 977, 759, 740 cm⁻¹; HRMS (ESI) m/z 961.3275 [(M+Na)+], C₅₃H₅₄N₄O₈S₂ requires 961.3273.

Preparation of 152

To a solution of 179 (15.6 mg, 0.017 mmol) in CH₃CN (2 mL) was added Et₂NH (0.5 mL) at 25 °C. After stirring for 2 h at 25 °C, the reaction mixture was concentrated in vacuo and washed with toluene. After removal of toluene in vacuo, to a solution of the crude mixture in CH₂Cl₂ (20.0 mL) were added HATU (12.6 mg, 0.033 mmol) and i-Pr₂NEt (9.0 µL, 0.050 mmol) at 25 °C. After stirring for 24 h at 25 °C, the reaction mixture was concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/1 to EtOAc 100%) to afford 152 (2.3 mg, 27% for 2 steps): [α]D²⁵ = +16.6 (c 0.13, EtOAc); 1H NMR (500 MHz, CDCl₃) δ 7.76 (s, 1H), 7.17 (d, J = 9.5 Hz, 1H), 6.56 (d, J = 6.5 Hz, 1H), 5.88 (ddd, J = 15.5, 6.5, 6.5 Hz, 1H), 5.62–5.68 (m, 1H), 5.49 (dd, J = 6.5, 1.5 Hz, 1H), 5.26 (dd, J = 17.5, 9.0 Hz, 1H), 4.57 (dd, J = 9.5, 4.0 Hz, 1H), 4.28 (dd, J = 17.5, 3.0 Hz, 1H), 4.03 (d, J = 11.5 Hz, 1H), 3.28 (d, J = 11.5 Hz, 1H), 2.83 (dd, J = 16.0, 9.5 Hz, 1H), 2.68 (dd, J = 16.0, 3.0 Hz, 1H), 2.45–
2.57 (m, 2H), 2.25–2.40 (m, 4H), 2.05–2.08 (m, 1H), 1.86 (s, 3H), 0.98 (dd, \( J = 7.5, 7.5 \) Hz, 3H), 0.69 (d, \( J = 7.0 \) Hz, 3H), 0.52 (d, \( J = 7.0 \) Hz, 3H); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) \( \delta \) 210.8, 173.4, 169.4, 168.8, 168.1, 164.8, 147.4, 133.9, 127.1, 124.2, 84.3, 71.8, 57.9, 43.3, 41.1, 40.9, 40.5, 36.0, 34.0, 26.3, 24.2, 18.9, 16.7, 7.7; IR (neat) 3367, 2964, 2933, 1732, 1712, 1668, 1508, 1244. 1114, 1030, 975, 843 cm\(^{-1}\); HRMS (ESI) \( m/z \) 543.1706 [(M+Na\(^+\)), \( C_{24}H_{32}N_4O_5S_2 \) requires 543.1708].

**Preparation of 182**

![Chemical structure](image)

To a solution of 186 (14.6 mg, 0.027 mmol) in DMF (5 mL) was added 187 (41.8 mg, 0.124) and K\(_2\)CO\(_3\) (37.1 mg, 0.269). After stirring for 4 h at 25 \(^\circ\)C, the reaction was quenched by the addition of H\(_2\)O and diluted with EtOAc. The layers were separated and the organic layer was washed with more H\(_2\)O. The organic layer was dried over anhydrous Na\(_2\)SO\(_4\) and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes: 2/1) to afford 182 (15.3 mg, 71%): \([\alpha]^{25}_{D} = +4.9\) (c 0.26, CHCl\(_3\)); \(^1\)H NMR (500 MHz, CDCl\(_3\)) \( \delta \) 7.77 (s, 1H), 7.42–7.44 (m, 6H), 7.29–7.35 (m, 6H), 7.18–7.25 (m, 3H), 6.52 (d, \( J = 5.5 \) Hz, 1H), 5.84 (ddd, \( J = 15.5, 7.0,7.0 \) Hz, 1H), 5.67–5.69 (m, 1H), 5.48 (dd, \( J = 16.0, 7.0 \) Hz, 1H), 5.29 (dd, \( J = 17.5, 9.5 \) Hz, 1H), 5.62 (dd, \( J = 9.5, 3.5 \) Hz, 1H), 4.26 (dd, \( J = 17.5, 3.0 \) Hz, 1H), 4.05 (d, \( J = 11.5 \) Hz, 1H), 3.29 (d, \( J = 11.5 \) Hz, 1H), 2.84 (dd, \( J = 16.5, 10.0 \) Hz, 1H), 2.69 (dd, \( J = 16.0, 3.0 \) Hz, 1H), 2.45 (dd, \( J = 6.5, 6.5 \) Hz, 2H), 2.33–2.36 (m, 4H), 2.19 (ddd, \( J = 7.5, 7.5, 7.5 \) Hz, 2H), 2.10–
2.14 (m, 1H), 1.87 (s, 3H), 0.72 (d, J = 7.0 Hz, 3H), 0.55 (d, J = 7.0 Hz, 3H); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) δ 173.5, 169.3, 168.8, 167.8, 164.5, 147.4, 144.7, 133.0, 129.6, 127.9, 126.7, 124.1, 84.4, 71.9, 67.1, 57.8, 43.3, 41.1, 40.5, 34.1, 32.2, 31.2, 31.0, 24.2, 18.9, 16.7; IR (neat) 3374, 2961, 2928, 1734, 1675, 1594, 1505, 1244, 1182, 1031, 972, 742, 701 cm\(^{-1}\); HRMS (ESI) \(m/z\) 821.2294 [(M+Na)\(^+\), \(C_{42}H_{46}N_4O_4S_4\) requires 821.2293].

**Preparation of 149**

To a cooled (0 °C) solution of \(182\) (9.7 mg, 0.012 mmol) in CH\(_2\)Cl\(_2\) (1 mL) were added TFA (10.0 µL) and \(i\)-Pr\(_3\)SiH (5 µL, 0.025 mmol). After stirring for 30 min at 25 °C, the reaction mixture was concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/1 to EtOAc 100%) to afford \(149\) (3.0 mg, 45%): \([\alpha]^{25}\)D = +8.1 (c 0.14, CHCl\(_3\)); \(^1\)H NMR (500 MHz, CDCl\(_3\)) δ 7.76 (s, 1H), 7.17 (d, J = 10.0 Hz, 1H), 6.47 (d, J = 7.5 Hz, 1H), 5.88 (ddd, J = 15.5, 6.5, 6.5 Hz, 1H), 5.66–5.69 (m, 1H), 5.52 (dd, J = 15.0 7.5 Hz, 1H), 5.28 (dd, J = 17.5, 9.5 Hz, 1H), 4.61 (dd, J = 9.5, 3.5 Hz, 1H), 4.27 (dd, J = 17.5, 2.5 Hz, 1H), 4.03 (d, J = 11.5 Hz, 1H), 3.28 (d, J = 11.5 Hz, 1H), 2.85 (dd, J = 16.0, 10.0 Hz, 1H), 2.68–2.75 (m, 5H), 2.58 (dd, J = 7.0, 7.0 Hz, 2H), 2.33 (ddd, J = 6.5, 6.5, 6.5 Hz, 2H), 2.08–2.12 (m, 1H), 1.89 (s, 3H), 1.73 (dd, J = 8.0, 8.0 Hz, 1H), 0.69 (d, J = 7.0 Hz, 3H), 0.51 (d, J = 7.0 Hz, 3H); \(^{13}\)C NMR (125 MHz, CDCl\(_3\)) δ 173.6, 169.4, 168.9, 168.8, 168.0, 164.7, 147.4, 133.1 133.0, 128.1, 124.2, 84.4, 72.0, 57.8, 53.5, 43.3, 41.1, 40.5, 36.2, 34.2, 32.5, 32.1 31.4, 31.2, 31.1, 24.7 24.2,
18.9, 16.7; IR (neat) 3369, 2962, 2930, 1734, 1672, 1510, 1245, 1180, 1033, 973, 702 cm⁻¹; HRMS (ESI) m/z 579.1199 [(M+Na)⁺, C_{23}H_{32}N_{4}O_{4}S_{4} requires 579.1198].

Preparation of 189

\[
\text{NHBOC} + \text{O} \xrightarrow{\text{Grubbs II, CH}_2\text{Cl}_2 \text{ reflux, 4 h}} > 99\% \xrightarrow{\text{O}} \text{NHBOC}
\]

To a solution of 188 (250.0 μL, 1.36 mmol) in CH₂Cl₂ (10 mL) was added 165 (1.1 mL, 13.6 mmol) and Grubbs 2ⁿᵈ generation catalyst (23.0 mg, 0.027 mmol). After stirring for 3 h at reflux, the mixture was concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/3 to 1/2) to afford 189 (304.4 mg, > 99%). Due to instability of the compound, 189 was immediately used for subsequent steps.

Preparation of 190

\[
\text{NHBOC} + \text{O} \xrightarrow{\text{TiCl}_4, \text{i-Pr}_2\text{NEt, CH}_2\text{Cl}_2, -78 \degree \text{C, 1 h}} 65\% \xrightarrow{\text{BochN}} \text{NHBOC}
\]

To a cooled (0 °C) solution of 158 (260.2 mg, 1.28 mmol) in CH₂Cl₂ (10 mL) was added TiCl₄ (1.0 M in CH₂Cl₂, 1.70 mL, 1.70 mmol). After stirring for 5 min at 0 °C, the mixture was cooled (–78 °C) and i-PrNEt (250.0 μL, 1.44 mmol) was added. After stirring for 1 h at –78 °C, 189 (160.8 mg, 0.807 mmol) in CH₂Cl₂ (2 mL) was added dropwise. After stirring for 1 h at –78 °C, the reaction was quenched by the addition of saturated NH₄Cl solution. The layers were separated and the aqueous layer was extracted with CH₂Cl₂. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was purified by column...
chromatography (silica gel, EtOAc/hexanes = 1/1) to afford 190 (182.3 mg, 65%): [α]$_{D}^{25}$ = -177.9 (c 1.0, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$) δ 5.69 (ddd, $J$ = 15.6, 6.4, 6.4 Hz, 1H), 5.60 (dd, $J$ = 15.6, 4.8 Hz, 1H), 5.15 (dd, $J$ = 7.2, 7.2 Hz, 1H), 4.61–4.67 (m, 1H), 3.63 (dd, $J$ = 17.6, 3.2 Hz, 1H), 3.52 (dd, $J$ = 11.6, 8.0 Hz, 1H), 3.27 (dd, $J$ = 17.6, 8.8 Hz, 1H), 3.17 (br s, 2H), 3.02 (d, $J$ = 11.6 Hz, 1H), 2.35 (m, 1H), 2.22 (ddd, $J$ = 6.4, 6.4, 6.4 Hz, 2H), 1.43 (s, 3H), 1.05 (d, $J$ = 6.8 Hz, 3H), 0.97 (d, $J$ = 6.8 Hz, 3H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 202.9, 172.2, 155.8, 133.1, 128.4, 79.1, 71.3, 68.3, 45.3, 39.7, 32.6, 60.7, 30.6, 28.3, 19.0, 17.7; IR (neat) 3367, 2965, 2931, 1688, 1514, 1364, 1250, 1161, 1040, 969 cm$^{-1}$; HRMS (ESI) m/z 425.1539 [(M+Na)$^+$, C$_{18}$H$_{30}$N$_2$O$_4$S$_2$ requires 425.1541].

**Preparation of 191**

To a solution of 102 (190.2 mg, 0.355 mmol) in CH$_2$Cl$_2$ (8 mL) was added TFA (2.0 mL) at 25 °C. After stirring for 1 h at 25 °C, the reaction mixture was concentrated *in vacuo* and washed with Et$_2$O. To a solution of the crude mixture in CH$_2$Cl$_2$ (10 mL) were added 190 (128.6 mg, 0.319 mmol) in CH$_2$Cl$_2$ (2 mL) and DMAP (216.8 mg, 1.78 mmol) at 25 °C. After stirring for 1 h at 25 °C, the reaction was quenched by the addition of saturated NH$_4$Cl solution and diluted with EtOAc. The layers were separated and the organic layer was washed with additional saturated NH$_4$Cl solution. The organic layer was dried over anhydrous Na$_2$SO$_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/1
to EtOAc 100%) to afford 191 (181.1 mg, 85% for 2 steps): \([\alpha]^2_{D} = -22.6 (c 1.0, \text{CHCl}_3); \]^1H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.95 (s, 1H), 7.72 (dd, \(J = 7.2, 4.0\) Hz, 2H), 7.60 (d, \(J = 7.6\) Hz, 2H), 7.36 (ddd, \(J = 7.6, 7.6, 7.6\) Hz, 2H), 7.17–7.23 (m, 2H), 5.63 (ddd, \(J = 15.2, 6.8, 6.8\) Hz, 1H), 5.53 (dd, \(J = 15.6, 5.2\) Hz, 1H), 4.70–4.79 (m, 3H), 4.49 (d, \(J = 6.8\) Hz, 2H), 4.24 (dd, \(J = 6.8, 6.8\) Hz, 1H), 3.75 (d, \(J = 11.6\) Hz, 1H), 3.21 (d, \(J = 11.6\) Hz, 1H), 3.12 (ddd, \(J = 6.0, 6.0, 6.0\) Hz, 2H), 2.49 (dd, \(J = 15.2, 3.2\) Hz, 1H), 2.41 (dd, \(J = 15.6, 8.4\) Hz, 1H), 2.15 (ddd, \(J = 6.4, 6.4, 6.4\) Hz, 2H), 1.63 (s, 3H), 1.41 (s, 9H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 172.9, 172.0, 168.2, 162.9, 156.0, 148.3, 143.5, 143.4, 141.3, 141.2, 133.4, 128.6, 127.8, 127.8, 127.1, 127.0, 125.2, 125.1, 122.2, 120.0, 119.9, 84.6, 79.2, 77.3, 69.0, 67.4, 46.7, 42.8, 41.6, 40.8, 39.9, 62.7, 28.4, 24.1; IR (neat) 3317, 2976, 2931, 1732, 1688, 1524, 1450, 1272, 1164, 1043, 973, 739 cm\(^{-1}\); HRMS (ESI) \(m/z\) 677.2462 [(M+H)+, C\(_{35}\)H\(_{40}\)N\(_{7}\)O\(_{6}\)S\(_{2}\) requires 677.2460].

**Preparation of 192**

To a cooled (0 °C) solution of 138 (136.1 mg, 0.401 mmol) in THF (10 mL) were added 2,4,6-trichlorobenzoyl chloride (84.0 µL, 0.535 mmol) and Et\(_3\)N (112.0 µL, 0.803 mmol). After stirring for 1 h at 0 °C, 191 (181.1 mg, 0.268 mmol) in THF (2 mL) and DMAP (50.0 mg, 0.401 mmol) were added at 0 °C. After stirring for 30 min at 25 °C, the reaction was quenched by the addition of saturated NH\(_4\)Cl solution and diluted with EtOAc. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na\(_2\)SO\(_4\)
and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/1 to 2/1) to afford **192** (256.2 mg, 98%): $[\alpha]_{D}^{25} = -19.5$ (c 1.0, CHCl$_3$); $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.96 (s, 1H), 7.70–7.73 (m, 4H), 7.58 (dd, $J$ = 22.0, 7.5 Hz, 4H), 7.17–7.38 (m, 8H), 5.75 (ddd, $J$ = 15.0, 7.0, 7.0 Hz, 1H), 5.67 (ddd, $J$ = 6.0, 6.0, 6.0 Hz, 1H), 5.55 (dd, $J$ = 15.5, 7.0 Hz, 1H), 5.48 (d, $J$ = 7.5 Hz, 1H), 4.86 (br s, 1H), 4.74 (d, $J$ = 5.5 Hz, 2H), 4.10–4.49 (m, 6H), 3.73 (d, $J$ = 11.0 Hz, 1H), 3.183 (d, $J$ = 11.0 Hz, 1H), 2.62 (d, $J$ = 5.5 Hz, 2H), 2.17–2.19 (m, 2H), 2.07–2.11 (m, 1H), 1.62 (s, 3H), 1.42 (s, 9H), 0.95 (d, $J$ = 7.0 Hz, 3H), 0.90 (d, $J$ = 7.0 Hz, 3H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 172.7, 171.2, 169.1, 168.5, 163.0, 156.3, 155.8, 148.1, 143.6, 143.5, 143.3, 143.2, 141.1, 132.2, 128.4, 127.7, 127.6, 126.9, 125.0, 124.9, 122.1, 119.8, 84.3, 79.0, 77.4, 72.1, 67.2, 66.9, 59.4, 46.9, 46.6, 41.4, 41.2, 40.9, 39.5, 32.4, 30.6, 28.3, 23.9, 18.9, 17.8; IR (neat)3323, 2970, 1708, 1510, 1477, 1265, 1161, 970, 732 cm$^{-1}$; HRMS (ESI) $m/z$ 1020.3646 [(M+Na)$^+$, C$_{55}$H$_{59}$N$_5$O$_9$S$_2$ requires 1020.3649].

**Preparation of 193**

To a solution of **192** (129.0 mg, 0.132 mmol) in CH$_3$CN (20 mL) was added Et$_2$NH (2.0 mL) at 25 °C. After stirring for 2 h at 25 °C, the reaction mixture was concentrated *in vacuo* and washed with toluene. After removal of toluene *in vacuo*, to a solution of the crude mixture in CH$_2$Cl$_2$ (170.0 mL) were added HATU (100.0 mg, 0.264 mmol) and $i$-Pr$_2$NEt (70.0 µL, 0.396 mmol) at 25 °C. After stirring for 24 h at 25 °C, the reaction mixture was concentrated *in vacuo*. 169
The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/1 to EtOAc/hexanes/CH₂OH = 10/10/1) to afford 193 (15.3 mg, 20% for 2 steps): [α]²⁵⁺D = +14.3 (c 0.13, EtOAc); ¹H NMR (500 MHz, CDCl₃) δ 7.77 (s, 1H), 7.20 (d, J = 9.0 Hz, 1H), 6.58 (br s, 1H), 5.87 (ddd, J = 15.5, 7.5, 7.5 Hz, 1H), 5.67 (br s, 1H), 5.53 (dd, J = 15.5, 6.5 Hz, 1H), 5.24 (dd, J = 17.5, 8.5 Hz, 1H), 4.85 (br s, 1H), 4.58 (dd, J = 9.5, 7.5 Hz, 1H), 4.30 (d, J = 16.5 Hz, 1H), 4.03 (d, J = 11.5 Hz, 1H), 3.28 (d, J = 11.5 Hz, 1H), 3.17 (m, 2H), 2.84 (dd, J = 16.5, 9.5 Hz, 1H), 2.70 (dd, J = 16.5, 3.0 Hz, 1H), 2.23–2.24 (m, 2H), 2.07–2.09 (m, 1H), 1.86 (s, 3H), 1.41 (s, 9H), 0.69 (d, J = 7.0 Hz, 3H), 0.53 (d, J = 7.0 Hz, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 173.5, 169.4, 168.9, 168.0, 164.9, 156.0, 147.5, 132.3, 128.7, 124.4, 124.4, 84.4, 79.2, 72.0, 58.0, 43.3, 41.2, 40.5, 34.1, 32.9, 28.5, 24.3, 18.9, 16.9; IR (neat) 3355, 2968, 2930, 1667, 1510, 1247, 1169, 1030, 972, 844 cm⁻¹; HRMS (ESI) m/z 602.2077 [(M+Na)⁺, C₂₆H₃₇N₅O₆S₂ requires 602.2077].

Preparation of 150

To a solution of 193 (7.5 mg, 0.013 mmol) in CH₂Cl₂ (1 mL) was added TFA (200 µL). After stirring for 1 h at 25 °C, the reaction mixture was concentrated in vacuo. The residue was purified by column chromatography (silica gel, CH₂Cl₂/CH₃OH = 5/1 + 1% NH₄OH) to afford 150 (4.7 mg, 68%): [α]²⁵⁺D = +15.7 (c 0.07, CH₃OH); ¹H NMR (500 MHz, CD₃OD) δ 8.14 (s, 1H), 5.80 (dd, J = 15.5, 6.5, 6.5 Hz, 1H), 5.63–5.72 (m, 2H), 5.06 (d, J = 17.5 Hz, 1H), 4.44 (d, J = 17.5 Hz, 1H), 3.92 (d, J = 11.5 Hz, 1H), 3.40 (d, J = 11.5 Hz, 1H), 2.99 (dd, J = 16.5, 10.0 Hz, 1H), 2.34 (s, 3H), 2.23 (m, 2H), 1.86 (s, 3H), 1.41 (s, 9H), 0.69 (d, J = 7.0 Hz, 3H), 0.53 (d, J = 7.0 Hz, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 173.5, 169.4, 168.9, 168.0, 164.9, 156.0, 147.5, 132.3, 128.7, 124.4, 124.4, 84.4, 79.2, 72.0, 58.0, 43.3, 41.2, 40.5, 34.1, 32.9, 28.5, 24.3, 18.9, 16.9, 1030, 972, 844 cm⁻¹; HRMS (ESI) m/z 602.2077 [(M+Na)⁺, C₂₆H₃₇N₅O₆S₂ requires 602.2077].
1H), 2.84 (dd, J = 6.5, 6.5 Hz, 2H), 2.71 (dd, J = 16.5, 3.0 Hz, 1H), 2.32 (ddd, J = 7.0, 7.0, 7.0 Hz, 2H), 2.05–2.08 (m, 1H), 1.80 (s, 3H), 0.71 (d, J = 7.0 Hz, 1H), 0.53 (d, J = 7.0 Hz, 1H); $^{13}$C NMR (125 MHz, CD$_3$OD) δ 174.4, 170.7, 168.6, 166.7, 146.8, 130.1, 129.9, 125.3, 83.7, 72.6, 57.7, 42.2, 40.4, 39.3, 39.2, 33.9, 32.3, 23.0, 18.2, 15.8; IR (neat) 3369, 2962, 2930, 1673, 1513, 1246, 1201, 1133, 1032, 974 cm$^{-1}$; HRMS (ESI) $m/z$ 502.1533 [(M+Na)$^+$], C$_{21}$H$_{29}$N$_5$O$_4$S$_2$ requires 502.1533.

Preparation of 195

![Chemical Reaction]

To a solution of 194 (203.3 mg, 1.29 mmol) in CH$_2$Cl$_2$ (10 mL) was added 165 (1.0 mL, 12.9 mmol) and Grubbs 2nd generation catalyst (22.0 mg, 0.026 mmol). After stirring for 3 h at reflux, the mixture was concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/4 to 1/2) to afford 195 (223.3 mg, > 99%). Due to instability of the compound, 195 was immediately used for subsequent steps.

Preparation of 196

![Chemical Reaction]

To a cooled (0 °C) solution of 158 (285.4 mg, 1.40 mmol) in CH$_2$Cl$_2$ (20 mL) was added TiCl$_4$ (1.0 M in CH$_2$Cl$_2$, 1.81 mL, 1.81 mmol). After stirring for 5 min at 0 °C, the mixture was cooled (−78 °C) and i-PrNEt (315.0 μL, 1.81 mmol) was added. After stirring for 1 h at −78 °C,
195 (223.3 mg, 1.21 mmol) in CH₂Cl₂ (2 mL) was added dropwise. After stirring for 1 h at –78 °C, the reaction was quenched by the addition of saturated NH₄Cl solution. The layers were separated and the aqueous layer was extracted with CH₂Cl₂. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/1) to afford 196 (264.8 mg, 56%): [α]²⁵_D = −184.1 (c 1.0, CHCl₃); °H NMR (500 MHz, CDCl₃) δ 5.70 (ddd, J = 15.5, 5.0, 5.0 Hz, 1H), 5.62 (dd, J = 15.5, 5.5 Hz, 1H), 5.08 (dd, J = 7.0, 7.0 Hz, 1H), 4.78 (br s, 1H), 4.60 (br s, 1H), 3.68 (br s, 2H), 3.47–3.52 (m, 2H), 3.25 (dd, J = 17.5, 9.0 Hz, 1H), 2.98 (d, J = 11.5 Hz, 1H), 2.26–2.33 (m, 1H), 1.37 (s, 9H), 1.00 (d, J = 6.5 Hz, 1H), 0.91 (d, J = 6.5 Hz, 1H); °C NMR (125 MHz, CDCl₃) δ 202.9, 172.0, 155.7, 132.2, 127.9, 79.2, 77.3, 71.4, 67.8, 45.1, 41.8, 45.1, 41.8, 30.7, 30.6, 28.3, 19.0, 17.7; IR (neat) 3364, 2966, 1695, 1515, 1390, 1306, 1251, 1166, 1041 cm⁻¹; HRMS (ESI) m/z 411.1383 [(M+Na)⁺, C₁₇H₂₈N₂O₄S₂ requires 411.1383].

Preparation of 197

To a solution of 102 (172.9 mg, 0.323 mmol) in CH₂Cl₂ (8 mL) was added TFA (2.0 mL) at 25 °C. After stirring for 1 h at 25 °C, the reaction mixture was concentrated in vacuo and washed with Et₂O. To a solution of the crude mixture in CH₂Cl₂ (10 mL) were added 196 (103.2 mg, 0.266 mmol) in CH₂Cl₂ (2 mL) and DMAP (197.0 mg, 1.62 mmol) at 25 °C. After stirring for 1 h at 25 °C, the reaction was quenched by the addition of saturated NH₄Cl solution and
diluted with EtOAc. The layers were separated and the organic layer was washed with additional saturated NH₄Cl solution. The organic layer was dried over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc 100%) to afford 197 (139.6 mg, 79% for 2 steps): [α]²⁵D = -25.3 (c 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 7.93 (s, 1H), 7.71 (dd, J = 7.0, 6.0 Hz, 2H), 7.59 (d, J = 7.5 Hz, 2H), 7.46 (dd, J = 5.5, 5.5 Hz, 1H), 7.35 (ddd, J = 12.0, 7.5, 7.5 Hz, 2H), 7.21 (ddd, J = 29.0, 5.0, 5.0 Hz, 2H), 5.68 (ddd, J = 15.5, 5.5, 5.5 Hz, 1H), 5.59 (dd, J = 16.0, 6.0 Hz, 1H), 4.83 (dd, J = 5.5, 5.5 Hz, 1H), 4.67–4.77 (m, 2H), 4.47–4.55 (m, 3H), 4.23 (dd, J = 7.0, 7.0 Hz, 1H), 3.73 (d, J = 11.5 Hz, 1H), 3.66 (br s, 2H), 3.20 (d, J = 11.5 Hz, 1H), 2.47 (dd, J = 15.5, 3.5 Hz, 1H), 2.41 (dd, J = 15.0, 8.5 Hz, 1H), 1.61 (s, 3 H), 1.40 (s, 9H); ¹³C NMR (125 MHz, CDCl₃) δ 172.9, 171.9, 168.3, 162.9, 155.8, 148.3, 143.5, 143.4, 141.3, 141.3, 132.5, 128.0, 127.9, 127.8, 127.1, 127.0, 125.2, 125.1, 122.1, 120.0, 84.6, 79.5, 77.3, 68.5, 67.4, 46.7, 42.7, 41.8, 41.6, 40.8, 28.4, 24.1; IR (neat) 3315, 2981, 1732, 1695, 1528, 1478, 1366, 1277, 1248, 1167, 759 cm⁻¹; HRMS (ESI) m/z 663.2306 [(M+H)⁺, C₃₄H₃₈N₄O₆S₂ requires 663.2307].

Preparation of 198

![Reaction Scheme](image)

To a cooled (0 °C) solution of 138 (107.2 mg, 0.316 mmol) in THF (10 mL) were added 2,4,6-trichlorobenzoyl chloride (65.0 µL, 0.421 mmol) and Et₃N (88.0 µL, 0.632 mmol). After stirring for 1 h at 0 °C, 197 (139.6 mg, 0.211 mmol) in THF (2 mL) and DMAP (38.6 mg, 0.316
mmol) were added at 0 °C. After stirring for 30 min at 25 °C, the reaction was quenched by the addition of saturated NH₄Cl solution and diluted with EtOAc. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/1 to 2/1) to afford 198 (206.3 mg, 99%): [α]²⁵ⁿ = −18.2 (c 1.0, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 7.93 (s, 1H), 7.71–7.74 (m, 4H), 7.58 (dd, J = 24.5, 7.5 Hz, 4H), 7.12–7.38 (m, 8H), 5.81 (ddd, J = 15.5, 5.5, 5.5 Hz, 1H), 5.64–5.73 (m, 2H), 5.40 (dd, J = 8.0 Hz, 1H), 4.67–4.75 (m, 3H), 4.10–4.49 (m, 6H), 3.71–3.76 (m, 3H), 3.19 (d, J = 11.5 Hz, 1H), 2.60–2.62 (m, 2 H), 2.07–2.11 (m, 1H), 1.63 (s, 3H), 1.42 (s, 9H), 0.94 (d, J = 7.0 Hz, 3H), 0.90 (d, J = 7.0 Hz, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 173.0, 171.3, 169.0, 168.5, 162.9, 156.5, 155.7, 148., 143.7, 143.6, 143.5, 143.4, 141.3, 131.5, 127.8, 127.7, 127.5, 127.1, 125.2, 125.1, 125.0, 122.0, 120.0, 84.6, 79.5, 77.3, 71.7, 67.4, 67.1, 59.6, 47.1, 46.7, 41.6, 41.3, 41.1, 30.8, 28.4, 24.1, 19.1, 17.9; IR (neat) 3322, 2971, 1715, 1519, 1478, 1244, 1164, 738 cm⁻¹; HRMS (ESI) m/z 984.3670 [(M+H)+, C₅₄H₅₇N₅O₉S₂ requires 984.3673].

**Preparation of 199**

To a solution of 192 (206.3 mg, 0.210 mmol) in CH₃CN (10 mL) was added Et₂NH (2.0 mL) at 25 °C. After stirring for 2 h at 25 °C, the reaction mixture was concentrated in vacuo and washed with toluene. After removal of toluene in vacuo, to a solution of the crude mixture in
CH₂Cl₂ (260.0 mL) were added HATU (159.4 mg, 0.419 mmol) and i-Pr₂NEt (100.0 µL, 0.629 mmol) at 25 °C. After stirring for 24 h at 25 °C, the reaction mixture was concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/1 to EtOAc/hexanes/CH₃OH = 10/10/1) to afford 199 (45.5 mg, 38% for 2 steps): [α]²⁵_D = +17.9 (c 0.38, EtOAc). ¹H NMR (400 MHz, CDCl₃) δ 7.75 (s, 1H), 7.15 (d, J = 9.2 Hz, 1H), 6.55 (dd, J = 8.4, 2.4 Hz, 1H), 5.85 (dd, J = 14.0, 6.4, 6.4 Hz, 1H), 5.60–5.72 (m, 2H), 5.24 (dd, J = 17.6, 9.2 Hz, 1H), 4.71 (br s, 1H), 4.58 (dd, J = 9.2, 3.6 Hz, 1H), 4.26 (dd, J = 17.6, 3.6 Hz, 1H), 4.01 (d, J = 11.2 Hz, 1H), 3.72 (br s, 2H), 3.26 (d, J = 11.2 Hz, 1H), 2.82 (dd, J = 15.6, 9.2 Hz, 1H), 2.72 (d, J = 16.4 Hz, 1H), 2.06–2.11 (m, 1H), 1.84 (s, 3H), 1.42 (s, 9H), 0.68 (d, J = 6.8 Hz, 3H), 0.51 (d, J = 6.8 Hz, 3H), ¹³C NMR (125 MHz, CDCl₃) δ 173.6, 169.3, 169.0, 168.0, 164.7, 155.8, 147.5, 131.2, 127.7, 124.3, 84.5, 79.6, 71.7, 57.8, 43.4, 41.8, 40.5, 34.2, 28.4, 24.3, 18.9, 16.7; IR (neat) 3359, 2972, 2932, 1732, 1666, 1509, 1042, 843 cm⁻¹; HRMS (ESI) m/z 566.2102 [(M+H)⁺], C₂₅H₃₅N₅O₆S₂ requires 566.2101.

Preparation of 200

To a solution of 199 (45.5 mg, 0.080 mmol) in CH₂Cl₂ (5 mL) was added TFA (1.0 mL). After stirring for 1 h at 25 °C, the reaction mixture was concentrated in vacuo to afford 200 (76.7 mg, > 99%), which was used for subsequent steps without further purification: [α]²⁵_D = +29.0 (c 0.47, CH₃OH); ¹H NMR (500 MHz, CD₃OD) δ 8.16 (s, 1H), 7.31 (d, J = 9.5 Hz, 1H), 6.01 (dd, J = 15.5, 6.5 Hz, 1H), 5.87 (ddd, J = 15.5, 6.5, 6.5 Hz, 1H), 5.68–5.72 (m, 1H), 5.09 (d, J = 17.5 Hz, 1H).
1H), 4.52 (dd, \( J = 9.5, 3.5 \) Hz, 1H), 4.42 (d, \( J = 17.5 \) Hz, 1H), 3.93 (d, \( J = 11.5 \) Hz, 1H), 3.57 (d, \( J = 6.0 \) Hz, 2H), 3.41 (d, \( J = 11.5 \) Hz, 1H), 3.02 (dd, \( J = 16.5, 11.0 \) Hz, 1H), 2.77 (dd, \( J = 16.5, 3.0 \) Hz, 1H), 2.08–2.12 (m, 1H), 1.81 (s, 3H), 0.72 (d, \( J = 7.0 \) Hz, 3H), 0.51 (d, \( J = 7.0 \) Hz, 3H); \(^{13}\)C NMR (125 MHz, CD$_3$OD) \( \delta = 175.9, 171.8, 170.0, 168.4, 162.0, 161.6, 148.0, 134.9, 126.9, 126.0, 85.0, 73.1, 58.4, 43.7, 41.8, 41.5, 40.1, 35.4, 24.3, 19.6, 17.1; IR (neat) 2965, 1675, 1201, 1178, 1139, 843 cm$^{-1}$; HRMS (ESI) m/z 466.1577 [(M+H)$^+$, \( C_{20}H_{27}N_{5}O_{4}S_{2} \) requires 466.1679].

**Preparation of 151**

To a solution of 200 (10.1 mg, 0.009 mmol) in ether (500 \( \mu \)L) were added 201 (7.0 \( \mu \)L, 0.087 mmol) and Et$_3$N (15.0 \( \mu \)L, 0.087 mmol). After stirring for 3 h at 25 °C, the reaction mixture was concentrated in vacuo. The residue was purified by column chromatography (silica gel, CHCl$_3$/CH$_3$OH = 5/1) to afford 151 (2.4 mg, 20%): \([\alpha]^{25}_{D} = -1.3 \) (c 0.09, CHCl$_3$); \(^{1}H\) NMR (500 MHz, CDCl$_3$) \( \delta = 7.75 \) (s, 1H), 7.16 (d, \( J = 9.0 \) Hz, 1H), 6.59 (br s, 1H), 5.89 (ddd, \( J = 14.0, 5.5 \) Hz, 1H), 5.70–5.74 (m, 2H), 5.22 (ddd, \( J = 17.5, 9.0 \) Hz, 1H), 4.56 (ddd, \( J = 9.0, 4.0 \) Hz, 1H), 4.30 (d, \( J = 15.5 \) Hz, 1H), 4.00 (d, \( J = 7.0 \) Hz, 1H), 3.35 (s, 2H), 3.27 (d, \( J = 11.0 \) Hz, 1H), 2.81 (dd, \( J = 16.5, 9.0 \) Hz, 1H), 2.70 (d, \( J = 13.5 \) Hz, 1H), 2.22 (s, 3H), 2.05–2.08 (m, 1H), 1.84 (s, 3H), 0.71 (d, \( J = 7.0 \) Hz, 3H), 0.0.55 (d, \( J = 7.0 \) Hz, 3H); \(^{13}\)C NMR (125 MHz, CDCl$_3$) \( \delta = 173.4, 169.1, 168.8, 168.2, 165.7, 164.8, 147.5, 130.0, 128.2, 124.2, 84.4, 71.3, 58.0, 49.3, 43.3, 41.2, 176.
40.6, 33.9, 31.0, 24.3, 18.9, 16.9; IR (neat) 3324, 2965, 2931, 1731, 1663, 1517, 1257, 1181, 1031, 762 cm⁻¹; HRMS (ESI) m/z 550.1788 [(M+H)+, C₂₄H₃₁N₅O₆S₂ requires 550.1788].

**Cell viability assay**

HCT116, MDA-MB231, and PC3 cells were plated in 96-well plates (10,000 cells/well) and 24 h later treated with various concentrations of largazole, largazole ZBG analogues or solvent control. After another 48 h of incubation, cell viability was measured using MTT according to the manufacturer’s instructions (Promega).
4. Synthesis and biological characterization of largazole prodrug analogues

4.1 Metabolic stability of largazole

While potency and selectivity are both important factors for largazole, the therapeutic potential also relies heavily on its pharmacokinetic properties. However, to date, there have only been limited reports about such factors regarding largazole. Hong/Luesch and co-workers reported that largazole is surprisingly stable in aqueous solutions. Largazole showed no significant hydrolysis of the thioester moiety in the presence of different buffer solutions (pH 4.0–8.0) along with no detection of largazole thiol. However, in the presence of mouse serum or mouse microsomes, largazole was hydrolyzed to largazole thiol extremely rapidly (>99% within 5 minutes) (figure 50). Similarly, when largazole was incubated with whole cell lysates from HCT-116 colon cancer cells, rapid thioester ester hydrolysis was observed. This suggests that the thioester hydrolysis is promoted by plasma proteins. This is further corroborated by the fact that inactivated microsomes (incubated at 95 °C for 5 minutes) did not aid in the hydrolysis of largazole. The hydrolyzed largazole thiol then exists mostly in a protein-bound largazole thiol adduct that is relatively stable in mouse serum. This protein binding is expected to the reversible and seems to play a “trans-protection” role of largazole thiol.
Figure 50: Plasma stability of largazole, largazole thiol, and largazole thiol adduct 10 μL of largazole was added to 100 μL of mouse serum and incubated. Rapid conversion of largazole to largazole thiol at early time points can be observed. At later time points, largazole thiol adduct is formed. Figure reprinted with permission from ref. 135.

Once hydrolyzed, largazole thiol seems to be surprisingly stable in cellular conditions. Ganesan and co-workers reported that the half-life of largazole thiol in murine liver homogenate is 51 minutes at 37 °C. 123 This metabolic stability however could be modulated by structural changes made in the macrocyclic core. While analogue 202, which maintained the structural rigidity of the macrocycle, had a very similar half-life to largazole thiol, analogues 203 and 204 saw a significant reduction in metabolic stability when the structural integrity of the macrocycle was compromised (figure 51).
Figure 51: Metabolic stability of analogues reported by Ganesan and co-workers. Compounds were incubated with murine liver homogenate at 37 °C and final compound concentration of 100 μM. Time points were taken over 2 hours and samples were analyzed by UPLC/MS. Data from ref.123

Luesch and co-workers have also reported the rapid clearance of largazole thiol from rats via rapid tissue distribution and/or biotransformation.149 A bolus injection of 10 mg/kg of largazole led to undetectable levels of largazole thiol within 2 hours post injection. These data present a potential issue for largazole as a potential therapeutic, specifically in its rapidly hydrolyzed thioester group. Yoshida and co-workers have reported that the half-lives of FK228 and redFK228 are >12 h and 32 min, respectively, in growth medium and 4.7 h and < 20 min in serum, demonstrating the increased metabolic stability through a much more stable disulfide linkage compared to a thioester group.95 While FK228 contains an internal disulfide bond, which makes it difficult to structurally tune the molecule, largazole thiol has a free thiol that can be modified with a disulfide linkage. Therefore, we envisioned that we could use a disulfide linkage to modify largazole thiol in attempts to improve pharmacokinetic parameters of the parent compound.150
4.2 Results and discussion

4.2.1 Synthetic goals

As stated before, we envisioned utilizing largazole thiol in a disulfide prodrug strategy to modulate the pharmacokinetic parameters of largazole. To that end, we designed three disulfide analogues of largazole to see the effect on these pharmacokinetic parameters. In order to accomplish this goal, we proposed the disulfide homodimer of largazole (205), and two cysteine “hetero-dimers” (206 and 207). Since homodimer 205 would reduce to form 2 molecules of largazole thiol, we expected it to be twice as potent on a molar basis with possibly distinct bioavailability. Hetero-dimer 206 was also prepared by incorporating N-Boc-L-cysteine tert-butyl ester through a disulfide linkage. Similarly heterodimer 207 was prepared as the free carboxylic acid as an improved water solubility analogue. These compounds would be tested for their in vitro and in vivo HDAC inhibition, metabolic stability, and oral bioavailability to examine the effect of the disulfide prodrug strategy.
4.2.2 Retrosynthetic analysis

We envisioned that all three analogues would be easily prepared from advanced largazole intermediate 208 by using standard oxidation conditions for disulfide bond formation as previously demonstrated in the synthesis of other cyclic depsipeptide inhibitors (scheme 35). Trityl protected largazole thiol 208 would be easily prepared by synthetic routes similar to the ones described in previous chapters.
4.2.3 Synthesis of disulfide analogues

In collaboration with Dr. Heekwang Park in our group, we began the preparation of advanced intermediate 208 with Yamaguchi esterification of advanced intermediate 103 and commercially available N-Fmoc-L-valine (138) resulted in intermediate 209. Fmoc/Fm deprotection, followed by HATU-mediated macrolactamization furnished key intermediate 208 (scheme 36).
With intermediate 208 in hand, we proceeded to the synthesis of the disulfide analogues (205–207). Homodimer 205 was prepared by utilizing iodine-mediated concurrent trityl group cleavage and disulfide bond formation. This reaction resulted in the desired analogue 205 in excellent yield (88%) (scheme 37).

The same approach was used to prepare heterodimer disulfide analogues 206 and 207.

When the iodine-mediated detritylation/disulfide bond formation was performed in the presence
of a cysteine derivative, the thiol of the cysteine was able to participate in the disulfide bond formation to produce the corresponding heterodimer in excellent yields (93% and 89% for 206 and 207, respectively) (scheme 38).

Scheme 38: Synthesis of disulfide analogues 207 and 208.

With all three compounds in hand, the effects of these structural modifications were tested for their in vitro and in vivo HDAC inhibition, metabolic stability, and oral bioavailability. The Luesch group at University of Florida was instrumental in the generation of the data discussed below.

4.2.4 Biological characterization of disulfide analogues

First, homodimer 205 and heterodimer 206 were tested for their in vitro HDAC inhibitory activity against the complete panel of canonical HDACs. As shown in table 22, both analogues show very similar HDAC inhibition profiles to largazole thiol with low or sub-nanomolar IC$_{50}$ values against HDACs 1, 2, 3, 10, and 11. All three of these compounds were also slightly more
active when compared to FK228. Based on previous studies of largazole’s class I-selectivity it is not surprising to see a lack of inhibition or significant decrease in potency for other class II HDACs.

Table 22: HDAC inhibition of largazole thiol and disulfide analogues (205–207) (IC$_{50}$ ± SD, nM).

<table>
<thead>
<tr>
<th></th>
<th>Largazole thiol</th>
<th>205$^a$</th>
<th>206$^a$</th>
<th>FK228</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC1</td>
<td>0.4 ± 0.05</td>
<td>0.3 ± 0.01</td>
<td>0.4 ± 0.03</td>
<td>0.8 ± 0.03</td>
</tr>
<tr>
<td>HDAC2</td>
<td>0.9 ± 0.09</td>
<td>0.5 ± 0.09</td>
<td>0.9 ± 0.01</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td>HDAC3</td>
<td>0.7 ± 0.003</td>
<td>0.3 ± 0.02</td>
<td>0.6 ± 0.06</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>HDAC4</td>
<td>NI$^c$</td>
<td>NI$^c$</td>
<td>NI$^c$</td>
<td>647 ± 20</td>
</tr>
<tr>
<td>HDAC5</td>
<td>NI$^c$</td>
<td>NI$^c$</td>
<td>NI$^c$</td>
<td>22%$^b$</td>
</tr>
<tr>
<td>HDAC6</td>
<td>35 ± 10</td>
<td>61 ± 8</td>
<td>135 ± 10</td>
<td>45%$^b$</td>
</tr>
<tr>
<td>HDAC7</td>
<td>NI$^c$</td>
<td>NI$^c$</td>
<td>NI$^c$</td>
<td>34%$^b$</td>
</tr>
<tr>
<td>HDAC8</td>
<td>102 ± 7</td>
<td>40%$^b$</td>
<td>35%$^b$</td>
<td>36%$^b$</td>
</tr>
<tr>
<td>HDAC9</td>
<td>NI$^c$</td>
<td>NI$^c$</td>
<td>NI$^c$</td>
<td>45%$^b$</td>
</tr>
<tr>
<td>HDAC10</td>
<td>0.5 ± 0.02</td>
<td>0.2 ± 0.004</td>
<td>0.5 ± 0.01</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>HDAC11</td>
<td>3 ± 0.3</td>
<td>0.1 ± 0.0002</td>
<td>0.2 ± 0.03</td>
<td>0.3 ± 0.0003</td>
</tr>
</tbody>
</table>

$^a$Treated with DTT to reduce disulfide bond. $^b$Percent inhibition at highest concentration tested (1 μM for all compounds). $^c$No significant inhibition observed (>20% at highest concentration tested).

This similarity in inhibition profile to largazole thiol was expected since both analogues liberate largazole thiol upon disulfide bond reduction. Since the assay was performed with pretreatment of the analogues with reducing agent DTT, it is not surprising to see similarities in the HDAC inhibition profile. However, differences became apparent when all analogues were tested for their anti-proliferative activity against HCT-116 colon cancer cells. Analogues 205 and 206 showed similar activity with only a 2-fold decrease in potency compared to largazole thiol.
Analogue 207 on the other hand demonstrated a 7-fold decrease in potency, suggesting that the free carboxylic acid in this analogue may significantly alter its properties, resulting in the decreased anti-proliferative activity (table 23). A similar phenomenon was observed when the HCT-116 cellular HDAC inhibition of the disulfide analogues was investigated. Immunoblot analysis of histone H3 acetylation levels (Lys9/14) of HCT-116 cells upon incubation with respective compounds showed interesting results. While analogues 205 and 206 showed dose-dependent increases in acetylation for both 8 hour and 24 hour incubation periods, analogue 207 only showed an increase in acetylation levels after 24 hour incubation (figure 53). This suggests that the structural changes made to analogue 207 cause a late onset of HDAC inhibitory activity.

Table 23: Anti-proliferative activity of largazole thiol and disulfide analogues (205–207) against HCT-116 human colorectal adenocarcinoma cells (IC_{50} ± SD, nM).

<table>
<thead>
<tr>
<th>Largazole thiol</th>
<th>205</th>
<th>206</th>
<th>207</th>
<th>FK228</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.9 ± 0.7</td>
<td>12 ± 2</td>
<td>13 ± 1</td>
<td>34 ± 5</td>
<td>3.0 ± 0.2</td>
</tr>
</tbody>
</table>
Figure 53: In vitro histone hyperacetylation level monitoring. HCT-116 cells were incubated with various concentrations of largazole, disulfide analogue, or control for 8 hours (A) or 24 hours (B). Hyperacetylation of histone H3 on L9/14 were quantified by immunoblot analysis.

There are several explanations for the late onset of HDAC inhibition by analogue 207. The decreased cell permeability as well as slower biotransformation of the disulfide prodrug to the active largazole thiol could both play a role in the perceived delay in HDAC inhibition. In order to assess the extent to which the biotransformation rate of the prodrug to the active compound plays a role in HDAC inhibition, the prodrug stability and the largazole thiol formation was studied in the presence of HCT-116 cell lysates (figure 54). It was shown that largazole rapidly liberates largazole thiol while reaching a maximum rate of largazole thiol
formation at 30 minutes. Heterodimer 207, on the other hand, didn’t liberate any appreciable level of largazole thiol until about 4 hours after incubation. Homodimer 205 seemed to show similar levels of largazole thiol formation as largazole and heterodimer 206 was not monitored due to the lability of the tert-butyl group leading to inaccurate quantification (figure 54). It seems likely that the disulfide linkage results in a slowed biotransformation from prodrug to active compound as seen in analogue 207. In homodimer 205, however, this effect is offset by the fact that each reduction liberates 2 molecules of largazole thiol, thereby restoring the largazole thiol levels in the presence of HCT-116 lysates.

Figure 54: Largazole thiol formation from largazole and disulfide analogues. HCT-116 protein lysates were incubated with 10 μL of 25 μg/mL solutions of largazole and disulfide analogues 205 and 207. Largazole and 205 liberate similar levels of largazole thiol, while 207 demonstrates significantly decreased levels of largazole thiol liberation.

Next, the in vivo efficacy of the disulfide analogues was investigated. HCT-116 mouse xenograft models were utilized to assess the consequences of varying doses of analogues 205 and 207 through both intraperitoneal injections as well as oral delivery on histone hyperacetylation levels in tumors. Administration of 10 mg/kg of largazole by i.p. resulted in histone H3 hyperacetylation (Lys9/14) after 4 hours in excised tumors, which rapidly declined over the next
24 hours. Analogue 205 showed slightly higher levels of hyperacetylation, however, these levels were sustained up to 24 hours. The same late onset was observed for analogue 207 as no hyperacetylation was seen until 12 hours post injection. This data mirrored the in vitro histone H3 hyperacetylation data discussed above (figure 55).

**Figure 55:** *In vivo* histone hyperacetylation level monitoring. 10 mg/kg (A) or 50 mg/kg (B) of largazole or analogues 205 and 207 were i.p. injected into HCT-116 mouse models. Tumors were excised after 4, 12, and 24 hours. Hyperacetylation levels were monitored by immunoblot analysis. Heterodimer 207 shows same late onset of HDAC inhibition as in vitro assays.

Increasing the dosage to 50 mg/kg resulted in similar trends (figure 55). Largazole showed maximum hyperacetylation at 4 hours with a rapid decline thereafter. Analogue 205 showed slightly comparable levels of hyperacetylation initially, but much higher levels of hyperacetylation at later time points. The ability to affect hyperacetylation by heterodimer 207
was higher at later time points compared to earlier time points, once again suggesting a later onset
in activity. When mice were orally treated with 50 mg/kg of disulfide analogues 205 and 207, no
functional response was observed. This lack of functional response highly suggests that the
bioavailability of the oral route is significantly lower than the i.p. administration. This may be due
to the increased polar surface area and number of hydrogen bond donors and acceptors present in
the disulfide analogues compared of largazole, which are both factors that have been correlated
with bioavailability.

4.3 Conclusion

We have successfully designed and prepared three largazole disulfide analogues by
utilizing a late state common intermediate and an iodine-mediated disulfide bond formation
reaction that is applicable for all three intermediates. The goal of these analogues was to
investigate the pharmacokinetic changes that can be elicited by utilizing a disulfide prodrug
approach. While the analogues showed similar biological activity to the parent compound in in
vitro experiments, in cell and mouse xenograft models, significant differences were apparent. It
was shown that by changing the prodrug strategy from a thioester to a disulfide, pharmacokinetic
changes could be leveraged. Specifically, analogue 207 showed a late onset of histone H3
(Lys9/14) hyperacetylation in HCT-116 cells as well as in mouse tumors. This effect was most
likely due to a combination of poor cell permeability due to the carboxylic acid group on the
cysteine moiety as well as a slower biotransformation from the prodrug to the active compound.
Additionally, the bioavailability of the analogues was investigated. This particularly highlighted
the benefit of using a disulfide prodrug strategy as homodimer 205 showed a more sustained
ability to inhibit HDAC activity and heterodimer 207 again showed a late onset of activity,
suggesting that the onset of the analogue can be modulated through structural changes. It was also demonstrated that oral treatment of the analogues resulted in very poor bioavailability, suggesting that i.p. administration should be the preferred method of treatment for these analogues. Modulation of onset times and duration of HDAC inhibition could prove useful for personalized treatment regiments for patients as well as use of differential probes for various biochemical experiments.

4.4 Experimental

General Methods

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich, Acros, Chem-Impex, or Fischer and were used without further purification. All solvents were ACS grade or better and used without further purification except THF which was freshly distilled each time before use. Thin layer chromatography was performed with glass backed silica gel (60 Å) plates purchased from Whatman and visualized with 254 nm UV light. All chromatographic purifications were conducted via flash chromatography using ultra-pure silica gel (230-400 mesh, 60 Å) purchased from Silicycle as the stationary phase unless otherwise noted. All spectra were recorded in CDCl$_3$ unless otherwise noted, using a 400 MHz (Varian) or 500 MHz (Bruker) NMR spectrometer. All NMR shifts are given in ppm, and all $J$-values are given in Hz. High-resolution mass spectra (HRMS) were obtained by an Agilent 6224 time-of-flight liquid chromatography-electrospray ionization spectrometer. Infrared (IR) absorption spectra were determined with a Thermo–Fisher (Nicolet 6700) spectrometer. Optical rotation values were measured with a Rudolph Research Analytical (A21102. API/1W) polarimeter.
Preparation of 209

To a cooled (0 °C) solution of 138 (269.8 mg, 0.495 mmol) in THF (10 mL) were added 2,4,6-trichlorobenzoyl chloride (116.1 µL, 0.743 mmol) and Et₃N (120.8 µL, 0.867 mmol). After stirring for 1 h at 0 °C, 209 (192.9 mg, 0.231 mmol) in THF (5 mL) and DMAP (60.5 mg, 0.495 mmol) were added at 0 °C. After stirring for 1 h at 25 °C, the reaction was quenched by the addition of saturated NH₄Cl solution and diluted with EtOAc. The layers were separated and the aqueous layer was extracted with EtOAc. The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The residue was purified by column chromatography (silica gel, EtOAc/hexanes = 1/1) to afford 209 (262 mg, 98%): ¹H NMR (400 MHz, CDCl₃) δ 7.94 (s, 1H), 7.74 (dd, J = 4.0, 4.0 Hz, 4H), 7.59 (dd, J = 20.0, 8.0 Hz, 4H), 7.19–7.40 (m, 23H), 6.88 (dd, J = 4.0, 4.0 Hz, 2H), 5.61–5.72 (m, 2H), 5.43 (dd, J = 16.0, 8.0 Hz, 1H), 5.28 (d, J = 8.0 Hz, 1H), 4.72 (d, J = 8.0 Hz, 2H), 4.9 (d, J = 8.0 Hz, 2H), 4.18–4.40 (m, 4H), 4.09 (dd, J = 8.0, 4.0 Hz, 1H), 3.77 (d, J = 12.0 Hz, 1H), 3.21 (d, J = 12.0 Hz, 1H), 2.59 (d, J = 8.0 Hz, 1H), 2.17–2.19 (m, 2H), 2.06 (dd, J = 12.0, 8.0 Hz, 2H), 1.65 (s, 3H), 0.90 (d, J = 8.0 Hz, 3H), 0.85 (d, J = 8.0 Hz, 3H).
Preparation of 208

To a solution of 209 (43.3 mg, 0.037 mmol) in CH$_3$CN (5 mL) was added Et$_2$NH (200 µL) at 25 ºC. After stirring for 2 h at 25 ºC, the reaction mixture was concentrated in vacuo and washed with toluene. After removal of toluene in vacuo, to a solution of the crude mixture in CH$_2$Cl$_2$ (46.0 mL) were added HATU (34.9 mg, 0.092 mmol) and i-Pr$_2$NEt (24.0 µL, 0.138 mmol) at 25 ºC. After stirring for 24 h at 25 ºC, the reaction mixture was concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc 100%) to afford 208 (15.2 mg, 55% for 2 steps): $^1$H NMR (500 MHz, CDCl$_3$) δ 7.73 (s, 1 H), 7.36–7.38 (m, 6 H), 7.25–7.28 (m, 6 H), 7.18–7.21 (m, 4 H), 6.53 (dd, $J = 9.0$, 3.0 Hz, 1 H), 5.71 (ddd, $J = 15.0$, 7.0, 7.0 Hz, 1 H), 5.62 (m, 1 H), 5.40 (dd, $J = 15.5$, 7.0 Hz, 1 H), 5.19 (ddd, $J = 17.5$, 9.0 Hz, 1 H), 4.56 (dd, $J = 9.0$, 3.5 Hz, 1 H), 4.10 (dd, $J = 17.5$, 3.0 Hz, 1 H), 4.02 (d, $J = 11.0$ Hz, 1 H), 3.26 (d, $J = 11.0$ Hz, 2 H), 2.77 (dd, $J = 16.0$, 9.5 Hz, 1 H), 2.65 (dd, $J = 16.0$, 3.0 Hz, 1 H), 2.20 (m, 2 H), 2.05 (m, 3 H), 1.83 (s, 3 H), 0.68 (d, $J = 6.5$ Hz, 3 H), 0.52 (d, $J = 6.5$ Hz, 3 H).
Preparation of 205

To a solution of 208 (110 mg, 0.149 mmol) in CH$_2$Cl$_2$/CH$_3$OH (9:1, 15 mL) was added I$_2$ (76 mg, 0.298 mmol) at 25 °C. After stirring for 30 min at 25 °C, the reaction mixture was quenched by the addition of saturated Na$_2$S$_2$O$_3$ solution. The layers were separated, and the aqueous layer was extracted with CH$_2$Cl$_2$. The combined organic layers were dried over anhydrous Na$_2$SO$_4$, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes/CH$_3$OH = 5/5/1) to afford 205 (135 mg, 88%): 

[α]$^25_D$ = +23.1 (c 0.1, CHCl$_3$); $^1$H NMR (500 MHz, CDCl$_3$) δ 7.76 (s, 2 H), 7.17 (d, J = 9.5 Hz, 2 H), 6.40 (dd, J = 9.0, 3.0 Hz, 2 H), 5.89 (ddd, J = 16.0, 7.5, 7.0 Hz, 2 H), 5.69 (m, 2 H), 5.54 (dd, J = 15.5, 7.0 Hz, 2 H), 5.24 (dd, J = 17.5, 9.5 Hz, 2 H), 4.61 (dd, J = 9.5, 3.5 Hz, 2 H), 4.21 (dd, J = 17.5, 3.5 Hz, 2 H), 4.02 (d, J = 11.5 Hz, 2 H), 3.27 (d, J = 11.0 Hz, 2 H), 2.88 (dd, J = 16.5, 10.5 Hz, 2 H), 2.72 (m, 2 H), 2.71 (dd, J = 7.0, 7.0 Hz, 4 H), 2.43 (m, 4 H), 2.10 (m, 2 H), 1.86 (s, 6 H), 0.70 (d, J = 7.0 Hz, 6 H), 0.53 (d, J = 7.0 Hz, 6 H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 173.7, 169.41, 169.07, 168.17, 164.7, 147.6, 132.9, 128.5, 124.3, 84.6, 72.1, 57.9, 43.4, 41.2, 40.6, 37.8, 34.3, 31.9, 24.4, 19.0, 16.8; HRMS (ESI) m/z 991.2456 [(M+H)$^+$], C$_{42}$H$_{54}$N$_8$O$_8$S$_6$ requires 991.2462].
Preparation of 206

To a solution of 208 (32 mg, 0.043 mmol) in CH$_2$Cl$_2$/CH$_3$OH (9:1, 4 mL) was added N-Boc-Cys(STrt)-OrBu (223 mg, 0.43 mmol) and I$_2$ (220 mg, 0.87 mmol) at 25 °C. After stirring for 30 min at 25 °C, the reaction mixture was quenched by the addition of saturated Na$_2$S$_2$O$_3$ solution. The layers were separated, and the aqueous layer was extracted with CH$_2$Cl$_2$. The combined organic layers were dried over anhydrous Na$_2$SO$_4$, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes/CH$_3$OH = 10/10/1) to afford 206 (31 mg, 93%): [$\alpha$]$^D_{25} = -9.7$ (c 1.5, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$) δ 7.76 (s, 1 H), 7.16 (d, $J = 9.2$ Hz, 1 H), 6.51 (d, $J = 7.2$ Hz, 1 H), 5.86 (d, $J = 7.2$ Hz, 1 H), 5.66 (m, 1 H), 5.53 (dd, $J = 15.6$, 6.8 Hz, 1 H), 5.30 (m, 1 H), 5.27 (dd, $J = 17.6$, 9.2 Hz, 1 H), 4.58 (dd, $J = 9.2$, 3.2 Hz, 1 H), 4.43 (m, 1 H), 4.26 (dd, $J = 17.6$, 2.8 Hz, 1 H), 4.02 (d, $J = 11.2$ Hz, 1 H), 3.26 (d, $J = 11.6$ Hz, 1 H), 3.15 (dd, $J = 13.6$, 4.8 Hz, 1 H), 3.06 (dd, $J = 13.6$, 4.8 Hz, 1 H), 2.85 (dd, $J = 16.4$, 10.0 Hz, 1 H), 2.72 (dd, $J = 6.4$, 6.4 Hz, 2 H), 2.68 (dd, $J = 13.6$, 2.4 Hz, 1 H), 2.42 (ddd, $J = 7.2$, 7.2, 7.2 Hz, 2 H), 2.08 (m, 1 H), 1.85 (s, 3 H), 1.46 (s, 9 H), 1.43 (s, 9 H), 0.68 (d, $J = 6.8$ Hz, 3 H), 0.50 (d, $J = 6.8$ Hz, 3 H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 173.6, 169.7, 169.3, 168.9, 168.0, 164.6, 115.1, 47.5, 132.5, 128.5, 124.3, 84.4, 82.7, 79.9, 72.0, 57.8, 53.8, 43.3, 41.8, 41.1, 40.4, 37.6, 34.2, 31.7, 28.4, 28.0, 24.3, 18.9, 16.7; HRMS (ESI) $m/z$ 772.2537 [(M+H$^+$), $C_{33}H_{49}$N$_5$O$_8$S$_4$ requires 772.2537].
Preparation of 207

To a solution of 208 (22 mg, 0.030 mmol) in CH$_2$Cl$_2$/CH$_3$OH (9:1, 4 mL) was added N-Boc-Cys(STrt)-OH (138 mg, 0.298 mmol) and I$_2$ (151 mg, 0.596 mmol) at 25 °C. After stirring for 30 min at 25 °C, the reaction mixture was quenched by the addition of saturated Na$_2$S$_2$O$_3$ solution. The layers were separated, and the aqueous layer was extracted with CH$_2$Cl$_2$. The combined organic layers were dried over anhydrous Na$_2$SO$_4$, and concentrated in vacuo. The residue was purified by column chromatography (silica gel, EtOAc/hexanes/CH$_3$OH = 10/10/1) to afford 207 (19 mg, 89%); [α]$^D_{25}$ = −375.1 (c 0.8, CHCl$_3$); $^1$H NMR (400 MHz, CDCl$_3$) δ 7.81 (s, 1 H), 7.65 (dd, J = 8.2, 2.8 Hz, 1 H), 7.06 (d, J = 9.6 Hz, 1 H), 5.78 (dd, J = 16.0, 3.6 Hz, 1 H), 5.60 (m, 2 H), 5.25 (dd, J = 17.6, 9.2 Hz, 1 H), 5.00 (d, J = 7.2 Hz, 1 H), 4.71 (m, 1 H), 4.64 (dd, J = 9.2, 3.2 Hz, 1 H), 4.16 (dd, J = 17.6, 3.6 Hz, 1 H), 4.04 (d, J = 11.2 Hz, 1 H), 3.33 (d, J = 11.6 Hz, 1 H), 3.22 (m, 2 H), 3.14 (dd, J = 14.0, 2.4 Hz, 1 H), 2.84 (dd, J = 16.4, 2.4 Hz, 1 H), 2.57 (m, 3 H), 2.18 (m, 2 H), 1.83 (s, 3 H), 1.41 (s, 9 H), 0.75 (d, J = 7.2 Hz, 3 H), 0.53 (d, J = 7.2 Hz, 3 H); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 175.8, 174.6, 172.2, 170.3, 170.0, 168.2, 157.9, 148.0, 133.5, 130.0, 126.9, 85.0, 80.6, 79.5, 73.9, 59.0, 54.3, 43.7, 41.8, 41.5, 40.6, 38.6, 35.4, 32.7, 28.8, 24.3, 19.7, 17.1; HRMS (ESI) m/z 716.1927 [(M+H)$^+$, C$_{29}$H$_{41}$N$_5$O$_8$S$_4$ requires 716.1911].
**Enzymatic assays**

Enzyme inhibitory assays were carried out by BPS Bioscience. In brief, compounds were incubated with an HDAC enzyme (HDACs 1–11), an appropriate HDAC substrate, bovine serum albumin and HDAC buffer with DTT. Duplicate reactions were carried out at 37 °C for 30 min, except for HDAC11, done at room temperature for 3 h. The reactions were quenched at the end of the incubation period with the addition of HDAC developer. Reactions were further incubated for 15 min at room temperature prior to fluorescence measurement (ex 360 nm/em 460 nm). The % inhibitory activity was calculated according to the equation (F – Fb)/(Ft – Fb), where F- fluorescent intensity of compound treated wells, Fb- fluorescent intensity of blank wells, Ft- fluorescent intensity of solvent control wells. IC50 values were calculated using GraphPad Prism.

**Cell culture**

HCT116 colorectal adenocarcinoma cells were cultured in Dulbecco’s modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone) under a humidified environment with 5% CO₂ at 37 °C.

**Cell viability assay**

HCT116 cells were plated in 96-well plates (10,000 cells/well) and 24 h later treated with various concentrations of largazole, largazole homodimer (205), largazole heterodimer 1 (206) and largazole heterodimer 2 (207) or solvent control. After another 48 h of incubation, cell viability was measured using MTT according to the manufacturer’s instructions (Promega).
**Immunoblot analysis**

HCT116 cells (400,000 cells/well) were seeded in 6-well plates and 24 h later treated with various concentrations of compounds or solvent control. Following incubation for 8 or 24 h, whole-cell protein lysates were prepared using PhosphoSafe lysis buffer (Novagen) and protein concentration measured using the BCA Protein Assay kit (Pierce). Cell lysates containing 20 µg of protein were separated by SDS-PAGE, transferred to PVDF membranes, probed with antibodies and detected with the SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Anti-acetyl-histone H3 (Lys9/18) antibody was obtained from Millipore, anti-β-actin and anti-rabbit antibody were from Cell Signaling.

**Metabolite Analyses General**

HPLC-MS was done on a 3200 QTRAP (Applied Biosystems) equipped with a Shimadzu UFLC System.

**Cellular stability**

Analysis of the cellular stability was performed based on the method of Liu et al.1, with modifications. In brief, to the diluted HCT116 protein lysate solution (0.75 mg/mL) was added 10 µL aliquot of largazole, largazole homodimer (205), or largazole heterodimer (207) (25 µg/mL). The solutions were incubated for varying durations: 0.25, 30, 60, 120, 240, 480, 720, 1440 min. To prevent the auto-oxidation of largazole thiol during workup and accurately compare the stability of the prodrugs, the free largazole thiol was adducted to N-ethyl maleimide (NEM). A 100 µL aliquot of NEM (1.9 mg/mL) in ethyl acetate was added to the incubation solution to quench the reaction and form the largazole thiol-NEM adduct. Additional ethyl acetate and internal standard harmine were added after adduct formation. Following workup, the ethyl acetate
layer was collected and evaporated to dryness under nitrogen. Samples were reconstituted in 50 μL methanol. A volume of 10 μL of the reconstituted solution was injected into the HPLC-MS system.

**HPLC-MS Parameters**

Analysis of largazole, largazole thiol-NEM adduct and heterodimer 2 (207) was done using HPLC-MS [column, Onyx Monolithic C18 (4.6 × 125 mm), Phenomenex; solvent, 0.1% aqueous formic acid (solvent A) − 0.1% formic acid in CH₃OH (solvent B); flow rate, 0.5 mL/min; detection by ESIMS in positive ion mode (MRM scan)]. A step-wise gradient elution was employed starting at 60% B and 40% A, then increased to 100% B at 5 min and maintained at this condition for 5 min. Parameters were optimized prior to analysis using direct syringe infusion. The retention times (tᵣ, min; MRM ion pair) of the analytes and internal standard are as follows: harmine (1.5; 213→170), thiol-NEM adduct (2.5; 622→79), largazole (4.3; 623→497), heterodimer (207) (3.1; 716→497). Compound dependent parameters used were as follows: Largazole: DP 65, EP 7.0, CE 37, CXP 23, CEP 28, thiol-NEM adduct: DP 90, EP 4.0, CE 73, CXP 3.0, CEP 38, Harmine: DP 50, EP 8.0, CE 41, CXP 2.0, CEP 12.0. Source gas parameters used were as follows: CUR 10, CAD Low, IS 4500, TEM 450.0, GS1 50.0, GS2 40.0. Homodimer (205) (3.7; 989→461) was analyzed using the same chromatographic conditions in the negative ion mode, using harmine (1.5; 211→194) as internal standard. Compound-dependent parameters are as follows: homodimer (205): DP -105, EP -11, CE -50, CXP -9.0, CEP -48, Harmine: DP -45, EP -6.0, CE -46, CXP -6.0, CEP 14. Source gas parameters used were as follows: CUR 10, CAD Medium, IS -3000, TEM 450.0, GS1 50.0, GS2 50.0.
**In vivo studies**

Female nude mice (nu/nu), 3 to 5 weeks old, were obtained from Charles River Laboratories Inc. (Wilmington, MA) and used for human tumor xenografts. Tumors were established by subcutaneous injection of $1 \times 10^6$ HCT116 cells on the right rear flank of a nude mouse in a volume of 100 µL of sterile saline. When the tumor reached the expected volume (250 – 300 mm$^3$), mice were treated with largazole, largazole homodimer (205), largazole heterodimer 2 (207) by intraperitoneal injection (in DMSO, 25 µL/mouse) or oral gavage (in 60% polyethylene glycol, 15% glycerol, 15% ethanol, 10% DMSO, 100 µL/mouse). Tumors were harvested at 4, 12, and 24 h after the treatment. All studies were carried out under the protocol approved by the Institutional Animal Care and Use Committee at the University of Florida. Tumor samples were homogenized through sonicatation in PhosphoSafe lysis buffer, centrifuged, and the supernatants were collected and used for immunoblot analysis probing with acetyl histone H3 antibody. Immunoblot analyses of the tumor samples were carried out similar to the procedure stated previously.

**In vivo largazole thiol levels in tumors**

Excised tumors were homogenized and extracted with methanol (1:10 w:v) spiked with internal standard harmine. The homogenates were incubated on ice for 10 min and centrifuged at 16000g (15 min at 4 °C). The supernatant was collected and the pellet was reextracted with methanol. All the supernatant was pooled, dried under nitrogen and partitioned with ethyl acetate-water. The ethyl acetate layer was collected, dried and reconstituted in 50 µL CH$_3$OH. A 10 µL aliquot was injected for HPLC-MS analysis according to the validated method of Yu et al.$^2$
References


105. Liu, J. Y.; Ma, X.; Liu, Y. Q.; Wang, Z.; Kwong, S. Q.; Ren, Q.; Tang, S. B.; Meng, Y.; Xu, Z. S.; Ye, T., Total synthesis and stereochemical revision of Burkholdac A. Synlett 2012, 783–787.


Biography

Bumki Kim was born on December 2nd, 1988 in Wiesbaden, Germany. After moving to Williamsburg, VA in the spring of 2001 and then again to Philadelphia, PA in the spring of 2003, he attended Carnegie Mellon University in Pittsburgh, PA in the fall of 2006. In the spring of 2010, he received his Bachelor of Science degree in Chemistry with a minor in Physics while graduating with university honors. In the fall of 2010, he began his graduate career at Duke University, and received his Doctor of Philosophy in organic chemistry in January of 2016.

Honors & Awards:

Burroughs Wellcome Fellowship 2014

Publications:


Presentations:
