Design and Synthesis of Bicyclic Tetrapeptides for Cap Group Optimization toward Histone Deacetylase Inhibitors

A dissertation in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Dedicated to my parents

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Chapter 1

Histone Deacetylase Inhibitors

1. Introduction

Epigenetic changes involve modulations in the expression level of genes, and are recognized as being integral to the pathogenesis of many diseases including cancer, depression, and various neurodegenerative diseases.[1, 2] The regulation of gene expression is essential to the correct functioning of the cell. Cells have many molecular mechanisms for altering gene expression levels; including histone acetylation and methylation, DNA methylation, and the remodeling of chromatin.[3] Many of the modifications to the histones occur at lysine residues near the amino terminus of the histone proteins. These lysine residues are positively charged at physiological pH. The charged lysine residues interact with the oxygen anions of the DNA phosphate backbone, forming strong ionic interactions that keep the DNA wound tightly around the histone. In other words, histone acetylation results in a more transcriptionally active form of DNA, while histone deacetylation causes transcriptional silencing. The balance between acetylation and deacetylation is controlled and maintained by the activity of two families of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). By altering the acetylation status, histone deacetylases determine the accessibility of DNA within chromatin and consequently influence gene expression.[4] Inappropriate recruitment of HDACs provides a molecular mechanism by which genes necessary for proper differentiation or growth arrest can be silenced leading to excessive proliferation. Owing to the link between transcriptional repression and the recruitment of HDACs, inhibitors of this enzymatic activity are expected to repression and to induce re-expression of differentiation-inducing genes.

2. The chromatin

The organization of DNA is accomplished with the help of proteins called histones. Histones are found in octameric form, with DNA wrapped around the exterior of the octamer. This DNA/histone complexes are called nucleosomes, which are packed together to form chromatin (Figure **1**).[5]

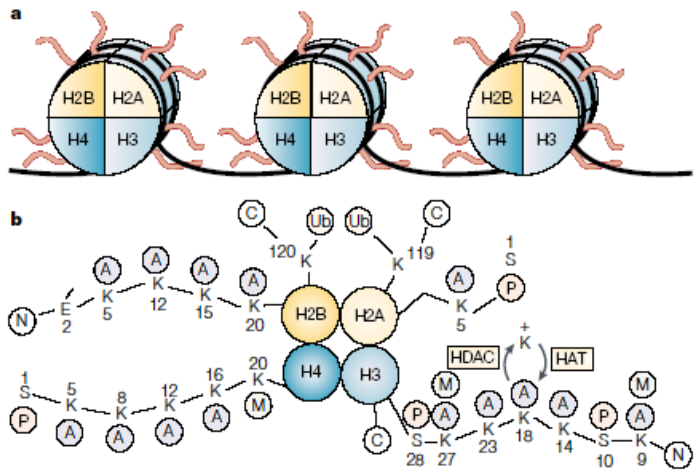


Figure 1. Schematic of the structure of histones in nucleosomes.

The basic unit of chromatin is nucleosome, providing the first order and at least in part, the higher order packaging and about 10,000 fold compactness of the DNA. The core particle of nucleosome consists of highly conserved basic protein, the histone core octamer, around which 146 base pairs of DNA are wrapped. This octamer comprises of eight histones- two each of H2A, H2B, H3 and H4.[6-8] The fifth histone H1, associates with nucleosomes and allows further compaction into chromatin fibres. All four core histones have an amino-terminal tail which is lysine rich and contains about half of the positively charged residues and most of the post-translational modification sites of the core histones. The N-terminal tail of the histones passes through and around the enveloping DNA double helix. It is the electrostatic interaction between the positively charged lysine tail and the phosphate group in helix which bind the DNA tightly around the histone proteins.

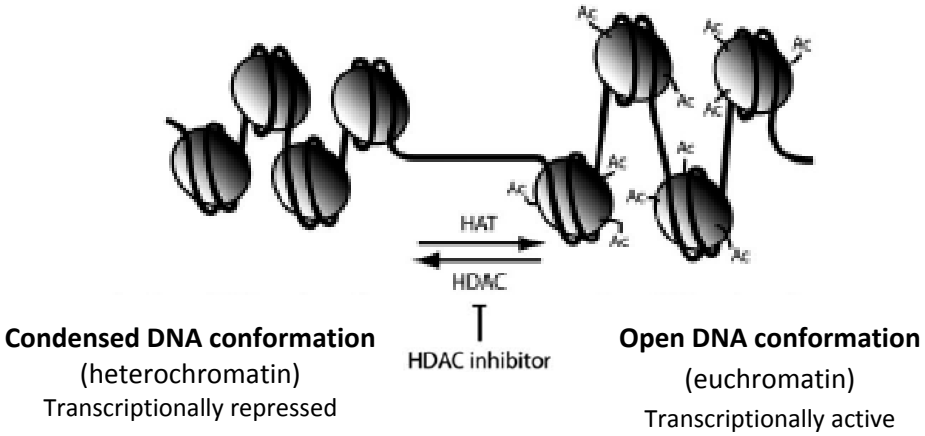


Figure 2. HAT acetylates lysine residues on histone proteins, encouraging the nucleosome to adopt an open conformation that is transcriptionally active. HDAC removes the acetyl groups, promoting a condensed conformation and transcriptional repression.

DNA must be unwound from the histones before it can be accessed by the large complexes responsible for transcription and replication. Nucleosomes exist in either a condensed conformation, where transcription is repressed, or in an open conformation that is transcriptionally active (Figure **2**).[2]

3. Histone tail acetylation and the bromodomain

Post-translational modifications such as acetylation, methylation, phosphorylation and ubiquitination represents as important mechanism for regulating protein function. Among the several post-translational modifications, lysine acetylation or transfer of acetyl group from ε-amino groups of lysine residues clustered near the aminoterminus of nucleosomal histones was discovered four decades ago,[9] and it has become the most studied and appreciated histone modification. Acetylation of specific lysine residues in the amino termini of the core histones plays a fundamental role in transcriptional regulation. All core histone proteins are reversibly and dynamically acetylated at multiple sites in their N-terminal tails (Lysine 9, 14, 18 and 23 in H3; and 5, 8, 12 and 16 in H4). Together with other modifications, histone acetylation forms histone codes to the regulation of chromatin structure and function.[10] Acetylation of lysine residues within the histone tails neutralizes the positive charge of ε-amino group and reduces the interaction between N-terminal tails of histones and the negatively charged DNA, thus correlates with gene activation. Consistent with this notion, transcriptional co-activators and co-repressors have been formed to possess intrinsic HAT and HDAC activities respectively .[11-13]

(i) **(ii)**

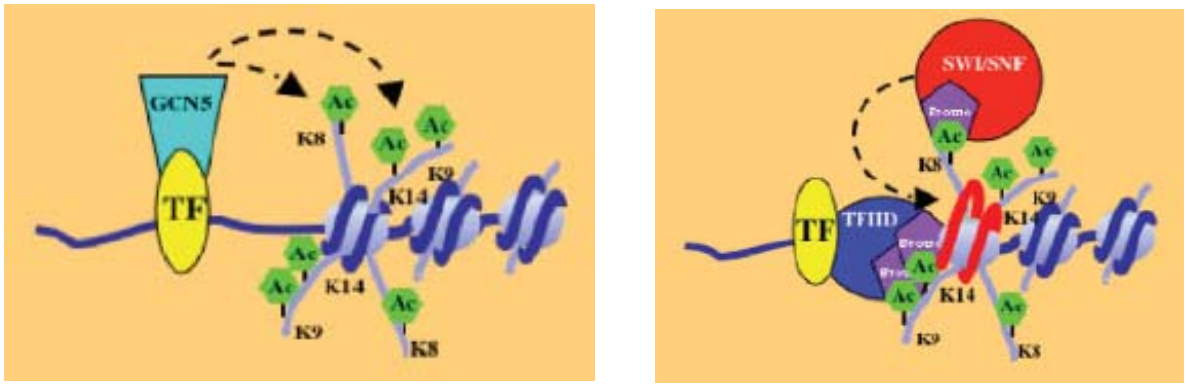


Figure 3. Model for the chromatin modifying enzymes and the bromodomain. **(i)** GCN5 HAT is recruited to the gene promoter by interaction with a transcription factor. **(ii)** The bromodomain-containing transcription complexes SWI/SNF recruited to the promoter by specific interactions between their bromodomain and specifically acetylated histone tails recruit HAT to execute gene specific chromatin acetylation and transcriptional up regulation.

It has been recently reported that, histone tails are specifically recognized and bounded by a protein module termed Bromodomain (Figure 3),[14] which is the docking site for lysine acetylation. Bromodomain play important role in chromatin remodeling by doing the function as acetyl lysine binding domain. This new finding suggests protein-protein interaction mechanism via lysine acetylation, which has broad implications for a wide variety of cellular events, including chromatin remodeling and transcriptional activation. The bromodomain was first identified by sequence alignment as a ~60 residue motif conserved among *Drosophila* Brahma and female-sterile homeotic and four other potential transcription regulators.[15] NMR structural analysis of the bromodomain (Figure 4) reveals four helix bundles with left handed twist.

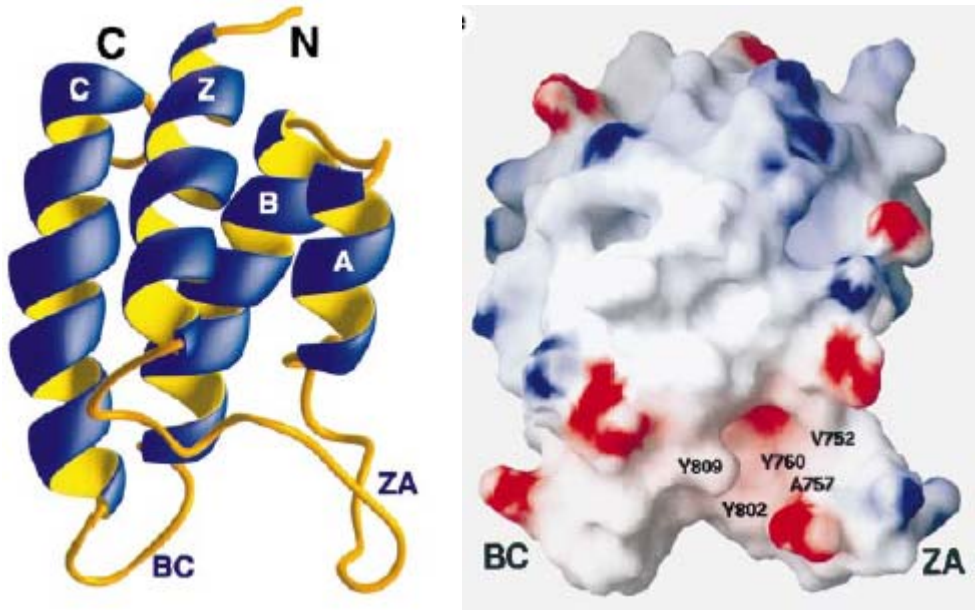


Figure 4. Structure of bromodomain (By NMR studies), reveling four helical model.

Interaction between the bromodomain[16] and acetyl lysine appears to be mediated by conserved residues that line a hydrophobic pocket that is formed by the two long loops. Degree of divergence suggests that, some bromodomain will bind more tightly than others. Such recognition code reveals a mechanism for targeting specific bromodomain containing proteins to particular chromatin targets. Recent studies[17] reveals that association between the amino terminal tails of histones H3 and H4, in terms of physical interactions between the bromodomain from the HAT Gcn5 and GST fusions to the H3 and H4 tails. The association is specific to H3 and H4 histones and not observed with H2A and H2B. Also, particular amino acid changes in the H4 tail abolished the interaction.

4. Role of histone deacetylases (HDACs)

When the gene no longer needs to be transcribed, the histone deacetylase enzymes (HDACs) are recruited to key locations in nucleosomes through their interaction with transcription complexes and remove the acetyl group in the lysines added by HATs.[18-25] The removal of the chemical group restores the positive charge of ε-amino group of lysines and enables histone to interact electrostatically to the negatively charged phosphate backbone of DNA. In this way DNA binds tightly to histone and restricting access of transcription factors to silence genes.

5. Histone deacetylation and cancer

The modification level of histone acetylation and its consequences have received enormous interest in recent years, and increase evidence supports their importance for basic cellular functions such as DNA replication, transcription, differentiation and apoptosis. HDACs and HATs usually act with other proteins in suppressing or activating transcription. Abnormal activities of HATs and HDACs have been linked to the pathogenesis of cancer. Although inactivating mutations of HATs and over expression of HDACs have been described, [19-26] the best link between HDACs and cancer progression comes from models of acute promyelocytic leukemia's, where chromosomal translocations result in chimeric proteins that alter transcriptional events and thereby interfere with normal cell growth, differentiation and apoptosis. Chromosomal translocations between RARa (retinoic receptor a) and PLZF (promyelocytic leukemia zinc finger) or PML (promyelocytic leukemia protein), for example, lead to inappropriate recruitment of co-repressors and HDACs and abolish the ability of RARa to mediate myelocytic differentiation. Another well-documented example is AML1-ETO, where ETO recruits N-CoR and mSin3A together with HDACs, which also interferes with retinoic acid functions and myeloid differentiation. In addition, a gene frequently deregulated in lymphomas is BCL-6, which recruits a complex containing SMRT, mSin3A and HDAC. Together, these examples have in common the aberrant recruitment of HDACs to promoters mediating transcriptional repression, which strengthens the rationale for the development of HDAC inhibitors as cancer therapeutics.[26]

6. The HDAC family

HDAC1 was identified using the HDAC inhibitor trapoxin as an affinity from nuclear extract in 1996.[27] It was found that HDAC1 shares high sequence homology with yeast Rpd3, a

global gene regulator and transcriptional co-repressor with histone deacetylase activity. [28] Subsequently, 18 HDAC family members have been identified in the human genome. HDACs are grouped into class I, class II, class III and class IV based on their sequence homology to their yeast orthologues Rpd3, HdaI and Sir2, respectively.[29, 30] Class I (HDACs 1, 2, 3, and 8) and class II (HDACs 4, 5, 6, 7, 9, and 10) HDACs are Zn^{2+}-dependent amidohydrolases with a conserved catalytic core but differing in size, domain structure, and tissue expression pattern. Class III HDACs are NAD^{+} dependent, unrelated in sequence and mechanism to classes I and II. Class IV consists of HDAC11. Very little information is available about its expression and function.[31] Zn^{2+} dependent enzymes harbor a catalytic pocket with a Zn^{2+} ion at its base that can be inhibited by Zn^{2+} chelating compounds. In contrast, these compounds are not active against sirtuins as class III enzymes have a different mechanism of action requiring NAD^{+} as an essential cofactor.[29] Class II HDAC family members are further subdivided into IIA and IIB (HDAC6 and HDAC10). Class IIA members (HDAC4, 5, 7, 9) are defined by a large, functionally important N-terminal domain regulating nuclear-cytoplasmic shuttling and specific DNA-binding. Class IIB HDAC contains two tandem deacetylase domain and C-terminal zinc finger. HDAC6 has emerged as a major deacetylase functioning as an α-tubulin deacetylase.[32] HDAC10 structurally related to HDAC6, but contains one additional catalytically inactive domain. Its function is largely unknown.[31]

7. HDAC structural study and concept of inhibitor design

Class I HDACs are 350-500 amino acid proteins, while class II enzymes are usually about 1000 amino acid long. Due to the larger size of class II HDACs, they posses many additional structural features, including a lengthy N-terminal sequence. The class I and class II HDACs share a 320 residue region of homology, corresponding to the section of the enzyme that forms the catalytic pocket.[33] The highly conserved catalytic pocket presents challenges to development of selective HDACis. However, some structural differences have been found and have been useful in generating class selective and isoform selective inhibitors.[34]

Histone deacetylase is typically found in the nucleus, at the location of the histone substrates, but can also be found in the cytoplasm. A nuclear import signal (NIS) is required for the histone deacetylase to be transported into the nucleus, and a nuclear export signal (NES) is necessary for transportation out of the nucleus. Regarding the class I enzymes, HDAC1 and 2 are primarily found in the nucleus, as they lack an NES, although HDAC3 can be exported to

the cytoplasm, as they contain an NES. Class II HDACs are generally cytoplasmic proteins, but they may be chaperoned into the nucleus. The 14-3-3 protein forces HDAC enzymes to remain in the cytoplasm. Binding of 14-3-3 to nuclear localization sequences on the class II enzymes interferes with the nuclear import and export signals, and confines the enzymes to the cytoplasm. 14-3-3 binding is regulated by phosphorylation of serine residues on the HDAC.[35, 36]

Class I HDACs tend to be found in all tissue types, while class II enzymes are more tissue specific. This observation has led researchers to speculate that class II enzymes may have an important role in cellular differentiation and development.[37] HDAC6 is the most frequently expressed isoform which may be related to its ability to deactylate a variety of targets, including histones, tubilin, and heat shock protein. The HDAC4 enzyme is quite rare in normal tissues, but is found with much higher incidence in tumor tissues.[29]

HDAC6 is exceptional in that it has two independent catalytic sites. Mutagenesis studies have demonstrated that each site is independent and has a unique set of substrates. Additonally, one site can remain active even if the other is disabled by mutation. A truncated form of HDAC6 that contains no N-terminal catalytic domain retains activity in the C-terminal domain. Mutagenesis and inhibitor studies confirmed that tublin deacetylation takes place only at the C-terminal catalytic site.[38, 39] With the exception of HDAC6, histone deacetylases must form multiprotein complexes with regulatory proteins to be active *in vivo*.

Crystal structures of the HDAC enzymes and their homologues provide essential information for rational drug design of selective HDAC inhibitors. Currently, crystal structures have been reported for HDAC8[40], HDAC7[41], and a variety of histone deacetylase homologues including HDLP[42] and FB188 HDAH.[43]

In 1990s, Yoshida and coworkers found that three different compounds with various functional groups and with different characteristics inhibit HDAC in different extents.[44] One of the inhibitors, trichostatin A (**1**, TSA), is a strong inhibitor of HDAC, on the other hand, butyric acid (**2**) is a weak inhibitor and trapoxin A (**3**) is an irreversible inhibitor. On the basis of the inhibitory activities of the hydroxamic acid **1**, carboxylic acid **2** and epoxy ketone **3** they proposed that HDAC is a metalloprotease enzyme, and suggested the reason for the difference in the activities of these three compounds. Based on this hypothesis, Yoshida et al. synthesized a series of potent HDAC inhibitors with activity in the nanomolar range.

The structural details of the class I/II HDAC inhibitor- enzyme interactions were first elucidated by Finnin et al. in 1999.[42] The crystal structure of histone deacetylase-like protein (HDLP), which was isolated from a hyperthermophilic bacterium, *Aquifex aeolicus*; a homologue of mammalian class I/II HDAC with 35.2% sequence identity over 375 residues, was solved with the HDAC inhibitor (*R*)-trichostatin A (TSA, **1**) (Figure 5) bound to the active site. This structural information provided the first concrete evidence that this enzyme indeed contained an active site zinc-binding pocket, consistent with previous observations[33] that HDAC activity was contingent upon the presence of a metal cofactor and strongly supported by the pronounced activity of certain hydroxamic acid-based inhibitors. The crystal structure reveals that HDLP has a single-domain structure belonging to the open α/β class of folds and the catalytic domain is very closely related to that of HDACs. The enzyme contains two Asp-His charge-relay systems, a structural feature which is conserved across the HDAC family. The prominent architectural feature of the ligand binding site shows the presence of a gently curved tubular pocket approximately 11Å deep (Figure 6) whose walls are lined with hydrophobic and aromatic residues identical to those found in HDAC1.

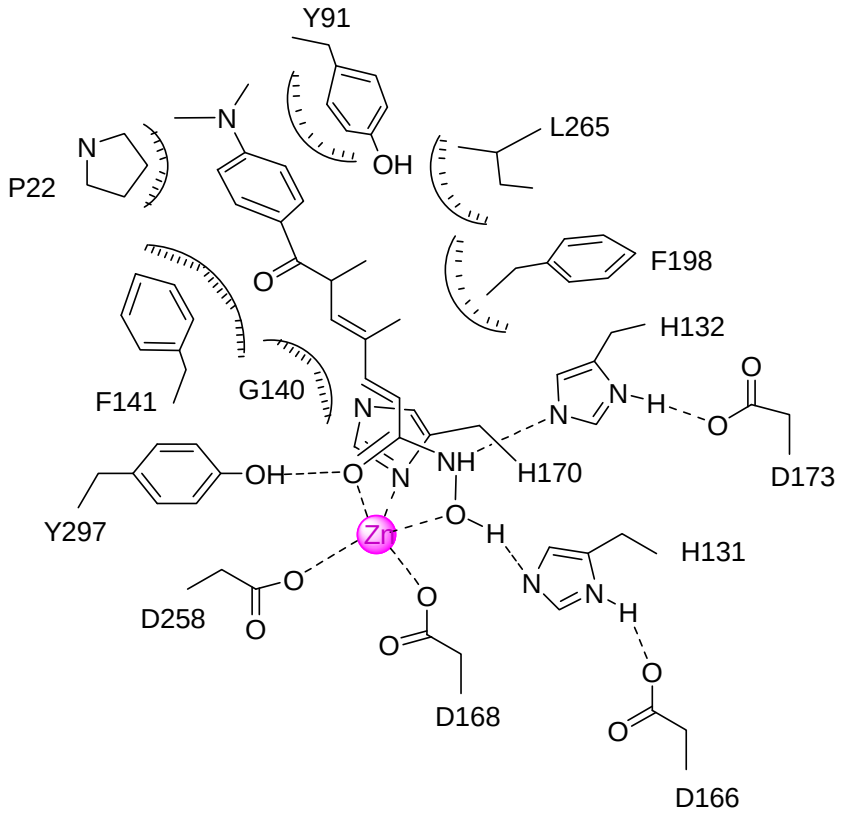


Figure 5: Crystal structure of TSA bound to the HDLP.

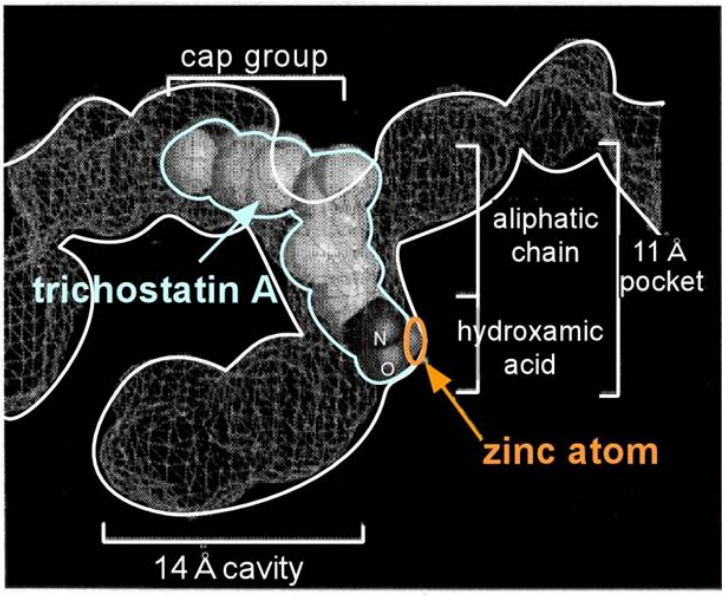
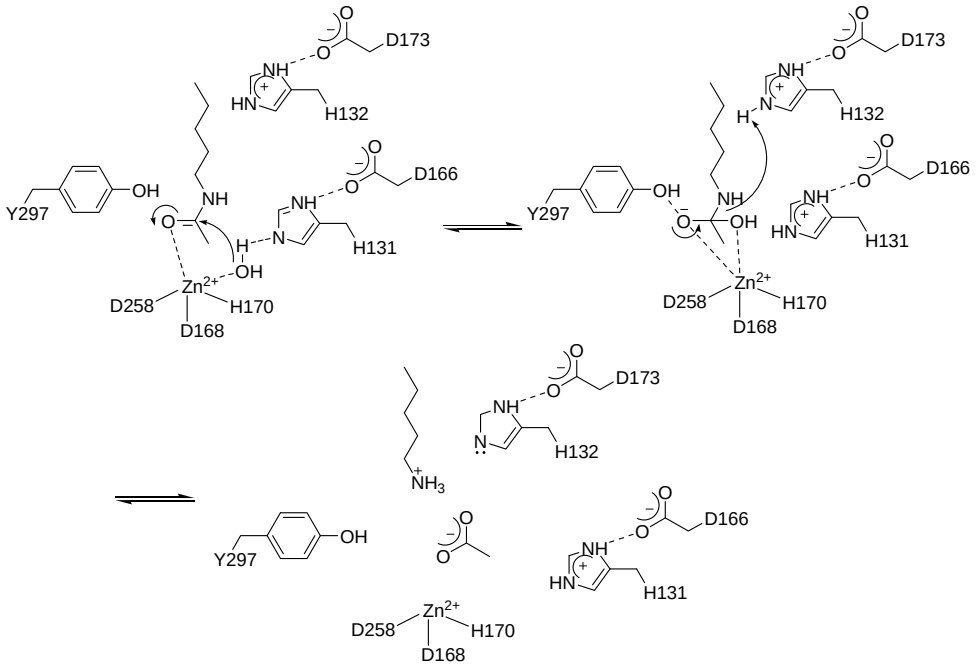


Figure 6. Surface representation of the 11 Å channel and 14 Å internal cavity of HDLP-TSA complex.

The cavity narrows to ~7.5Å near its center, constrained by the parallel disposition of Phe 141 and Phe 198. In the crystal structure of the zinc-reconstituted enzyme, the zinc ion is positioned near the bottom of the pocket. It is coordinated by Asp 168, His 170, Asp 258 and a water molecule. In the crystal structure of HDLP-TSA complex, TSA binds by inserting its long aliphatic chain into the HDAC pocket, making multiple contacts to the tube-like hydrophobic portion of the pocket. The hydroxamic acid group at one end of the aliphatic chain reaches the polar bottom of the pocket where it coordinates the zinc in a bidentate fashion and also contacts active site residues. The aromatic dimethylamino-phenyl group at the other end of the TSA chain makes contacts at the pocket entrance and in an adjacent surface groove, capping the pocket. The length of the aliphatic chain is optimal for spanning the length of the pocket and allowing contacts at the bottom and at the entrance of the pocket.

The structural information mentioned above facilitates elucidation of the precise deacetylation reaction mechanism. The carbonyl and hydroxyl groups of hydroxamic acid along with three tetrahedral-disposed residues (Asp 168, His 170, Asp 258), coordinates the zinc atom and results a penta-coordinated Zn^{2+} and the sole coordinated water molecule is shifted ~25° away from ideal geometry. The catalytic mechanism proposed by Finnin and colleagues is shown in Scheme 1. In HDLP, Y297 and zinc form bonds with the carbonyl oxygen of the acetyl group, causing an increase in the electrophilicity of the carbonyl group. Two charge transfer complexes made up of H132 and D173, and also H131 and D166, hydrogen bond with the free water molecule, increasing the nucleophilicity. Water is further

activated by binding with zinc, which lowers the pKa of water. At this point, the various interactions between the enzyme and the substrate have sufficiently lowered the activation energy of the reaction, and a nucleophilic attack occurs by the water nucleophile upon the carbonyl carbon of acetyl-lysine, generating a tetrahedral intermediate. The tetrahedral intermediate is stabilized by Y297 and Zn^{2+}. Collapse of the tetrahedral intermediate eliminates lysine, and the free acetate picks up a proton from the enzyme. Lysine dissociates from the enzyme, and the enzyme eliminates acetate by shuttling it out of the enzyme through the interior cavity. Hydrogen bonds between an Asp residue on the enzyme surface and two NH groups of the substrate lock the substrate in place for catalysis.



Scheme 1. Mechanism of deacetylation of acetylated lysine.

In addition, separate mutagenesis experiments established that replacement of the two Asp His charge-relay system profoundly disrupted the enzyme's biological activity.[45] More interestingly the crystal structure showed that upon TSA binding the side chain of one tyrosine (Tyr 91), which is at the periphery of the pocket and mostly solvent exposed, changes conformation to make space for the dimethylamino-phenyl group of TSA.

8. Inhibitor structure

The histone deacetylase enzymes are able to be inhibited by small molecules. The inhibitor binds tightly to the active site and likely chelates the zinc cofactor, so that catalysis can not occur. Most HDAC inhibitors, including those in clinical trials, are reversible, although

irreversible inhibitors are known.[46] HDAC enzymes have three primary domains, each with a specific function relating to the structure of the enzyme. The basic functional group of an HDAC inhibitor is the zinc binding group (ZBG), which chelates the zinc ion and engages in multiple hydrogen bonds with the residue at the active site. The zinc binding groups are typically a thiol, hydroxamic acid, carboxylic acid, ketone, or substituted aniline. Recently, HDAC inhibitors with boronic acid ZBG also reported.[47] The zinc binding region is analogous to the acetyl group of the N-acetylated-lysine substrate, and it interacts with many of the same residues of the enzyme. Irreversible inhibitor have an electrophilic group, usually a keto-epoxide, in the ZBG region that may become covalently attached to a nucleophilic residue, such as His, Asp, or Tyr, in the active site.[48, 49] The cyclic tetrapeptide trapoxin was shown experimentally to irreversibly inhibit the HDAC enzymes through reduction of the epoxide ZBG.[49] The linker region is generally a hydrophobic chain, homologous to the hydrophobic portion of the lysine side chain. The linker bridges the zinc binding group and the surface recognition domain, or cap group. The cap group is connected to the linker through a connecting unit, typically an amide bond. The cap group frequently contains one or more aromatic or heteroaromatic ring systems, although many structurally diverse cap groups have been reported. Inhibitors with multiple cap groups are frequently made, and some of these compounds show enhanced potency and selectivity.[50] Because, the residues on the enzyme surface which surround the binding pocket are much less conserved than those in the active site, making the enzyme surface an important source of selectivity.[43] Difference at the enzyme surface between the HDAC isoforms will cause the inhibitor cap group to interact with the enzyme in different ways. The result is that the inhibitor will have a different activity at each isoform. If the difference in activities between isoforms is large enough, the inhibitor is said to be selective.

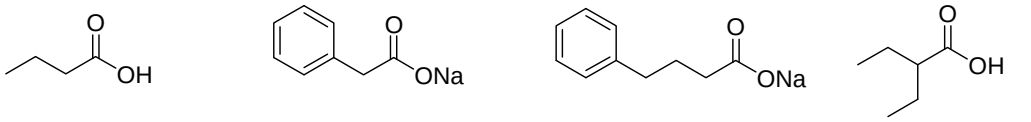
9. The inhibitors of HDACs

From the crystal structural studies of HDLP complexed with TSA or SAHA it is clear that the criteria to be fulfilled by a molecule for an effective inhibitor for HDACs are, a functional group capable of chelating the active site Zn^{2+} ion, (e.g, carboxylic acid, hydroxamic acid, sulfhydryl, etc.) attached to a hydrophobic scaffold, by means of a spacer. The functional group, scaffold and spacer play their specified role in the inhibitory activity. The inhibitory activity is determined by several factors, such as, the length of the spacer and interactions of the spacer with the residues in the channels of catalytic site, the nature of scaffold and its interaction with the residues at the rim region of the catalytic site, and the strength of bonding

between the functional group and the active site. Depending on the zinc binding groups and the construction of hydrophobic surface binding scaffolds, HDAC inhibitors can be roughly categorized in to several structural classes. The following is a brief discussion of different HDAC inhibitors class.

9.1. Carboxylates

Butyric acid (**2**)[51] was the first identified HDAC inhibitor and related compounds such as phenyl butyrate (**5**) one of the first HDAC inhibitors to be tested in proteins, and anticonvulsant valproic acid (**6**)[52] were later found as HDAC inhibitors (Figure 7). However, these carboxylates are far less potent in comparison with other inhibitors. The carboxylic acid, chelating group is less active than the other functional groups such as hydroxamic acid; may be due to weak coordination with Zn^{+2} ions. Despite being much less potent and their pleitropic effects, these are currently among the best studied HDAC inhibitors: valproic acid and phenyl butyrate have already been approved for use in treating epilepsy and some cancers, respectively, where as butanoic acid (or its prodrug forms such as pivaloyloxymethylbutyrate) is undergoing clinical trials.

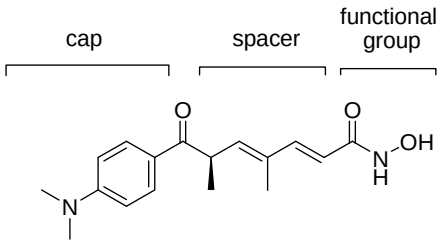


2: Butyric acid **4**: Sodium phenylacetate **5**: Phenylbutyrate **6**: Valproic acid

Figure 7. Carboxylic acids as HDAC inhibitor.

9.2. Hydroxamic acids

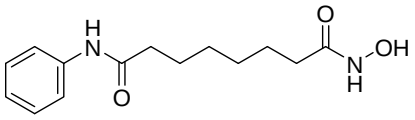
Hydroxamic acid based inhibitors constitute the broadest family of HDAC inhibitors and one of the well-studied ligand for the active site Zn^{2+} of HDAC enzymes and several inhibitors containing a hydroxamic acid functional group are known, including natural products like Trichostatin A (TSA) (**1**). The general structure of these substances consists of a hydrophobic linker that allows the hydroxamic acid moiety to chelate the Zn^{2+} cation at the bottom of HDAC catalytic pocket, while the bulky part of the molecule, which is the surface recognition domain, acts as a cap group. The linker domain can consist of linear either saturated or unsaturated, is generally a hydrophobic group (Figure 8).



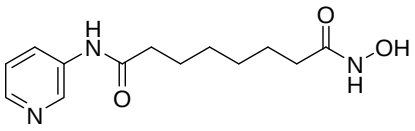
1: Trichostatin

Figure 8. Structural dissection of TSA based on crystal structure of HDLP-TSA complex.

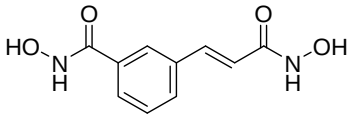
TSA was first isolated in 1976 by Tsuji *et al* from S*treptomyces hygrocopicus* as an fungistatic antibiotic active against *trichophytin,*[53] and the HDAC activity of TSA was found by Yoshida and coworkers in 1990.[54] TSA is a potent inhibitor of histone deacetylase, active at nanomolar concentrations. Whereas the corresponding carboxylate, trichostatic acid, was shown to be ineffective as an HDAC inhibitor, indicating that the hydroxamate is inevitable for the activity.[55] Furthermore, the enantiomer (*S*)-TSA, which was obtained by total synthesis, was demonstrated to be inactive.[55, 56] The interactions of hydroxamic acid functional group at the active site of HDAC are known from the X-ray structure (Figure 5). Based on the structure of TSA and its interaction with HDAC a large number of inhibitors were designed and synthesized.



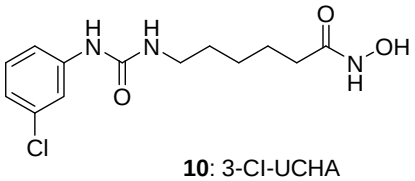
7: SAHA



8: Pyroxamide



9: CBHA



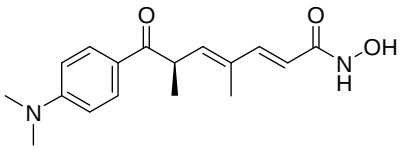
10: 3-Cl-UCHA

Figure 9. Hydroxamic acid HDAC inhibitors having linear linker.

Structurally simple TSA like straight chain hydroxamate, suberoylanilide hydroxamic acid (SAHA, **7**) was reported by Breslow and coworkers, as a cytodifferentiating agent and HDAC inhibitor.[57, 58] After the disclosure of this potent synthetic compound, a large number of synthetic HDAC inhibitors were reported. Examples are, hydroxamates analogous to SAHA, including (Figure 9), pyroxamide (**8**), CBHA (**9**) and 3-Cl-UCHA (**10**).[58, 59] These

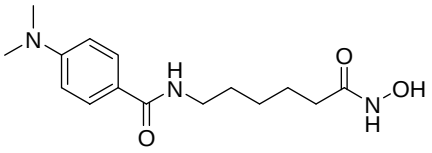
agents have been shown to be effective HDAC inhibitors and antiproliferative agents, both in vitro and in vivo. SAHA (**7**), commercially known as vorinostat (Zolinza; Merck), has recently been approaved by the US FDA for the treatment of cutaneous manifestations in patients with cutaneous T-cell lymphoma.[60] The synthetic SAHA, CBHA and natural TSA, which represent the archetypical hydroxamate HDAC inhibitors, have been instrumental in guiding the design of hydroxamate-derived HDAC inhibitors. Structure activity relationship study of these paradigmatic hydroxamate compounds were performed in a focused way by several research groups.

In order to understand the importance of the methyl-substituted olefinic linker in TSA hybrids, compounds **12** and **13** has been synthesized and evaluated against HDACs.[61] These agents, along with related analogue **11** (Figure 10), demonstrate a highly sensitive and selective SAR features. As for example, the designed addition of a methyl group (**12**) and two double bonds (**13**) caused a 2.3- and 33-fold reduction in activity, respectively, relative to the linear alkyl (**11**) linker.

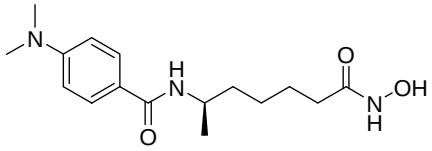


1: Trichostatin

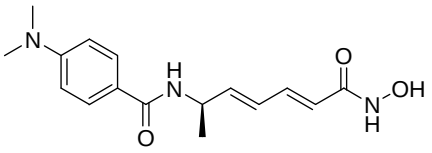
HDAC IC_{50} = 12 nM



11: HDAC IC_{50} = 152 nM



12: HDAC IC_{50} = 359 nM

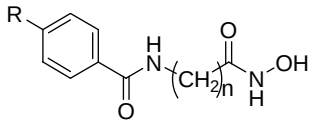


13: HDAC IC_{50} = 12187 nM

Figure 10. TSA analogs.

Jung and coworkers synthesized reverse amide analogues of TSA as HDAC inhibitors.[62] These compounds were tested against both maize histone deacetylase (HD-2) and partially purified rat liver HDAC. Using variation in the substitution at the cap portion

Table 1. SAR of reverse amide analogs of TSA



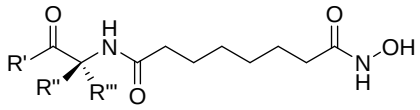
	R	n	HD-2 IC_{50} (nM)	HDAC IC_{50} (nM)
1 TSA			3	26
11	Me_2N	5	100	244
16a	Me_2N	3	>40000	-
16b	Me_2N	4	2000	>10000
16c	Me_2N	6	100	46
16d	Me_2N	7	300	145
16e	MeO	5	140	-
16f	H	5	900	-
16g	Cl	5	150	369
16h	NO_2	5	180	1566
16i	Ph	5	100	265

of the inhibitor and changing the spacer length they studied the inhibitory activity and structure activity relationship (SAR) of simple straight chain hydroxamates (**16**) (Table 1). This study confirmed the earlier observation that compounds with five- and six methylene spacers (**11** and **16c**) are the most active.[56] If the hydroxamic acid is changed to carboxylic acids the activity of these amide analogues diminished drastically.

The same group studied the SAR of several SAHA analogues with a phenylalanine at the cap portion and found that the groups present at the cap region have remarkable effect on the inhibitory activity (**17**).[63] Compounds **17l-o**, with bulky hydrophobic side chains, appear to be among the best inhibitors and differentiating agents (Table 2). Finally, inversion of the phenylalanine chiral center (**17e**,**g** vs **17a** and **17h**) did not affect inhibitor activity against the enzyme or in cells. Woo and coworkers also reported structure-activity studies on similar simple straight chain TSA-SAHA hybrid compounds (**18**, Table 3).[64] These agents were evaluated as inhibitors of recombinant human HDAC1, antiproliferative agents against a panel of human cell lines, and inducers of histone hyperacetylation and p21 expression. This series also demonstrated that a six methylene spacer is optimal (**18a-d**). Heterocyclic

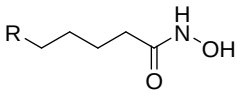
derivatives with the optimized linker (**18e** and **18f**) were also prepared and found to be active. Para-substituted aryl derivatives (**18h-j**) were more active than orthosubstituted derivative **18g**, and it was also shown that increased hydrophobicity in the recognition domain could substantially enhance activity (**18k** and **18l**). The ketone functionality was also replaced with oxime (**18m**,**n**), alcohol (**18s**), alkene (**18o**,**q**), and alkane (**18r**) linkages. All of these compounds were shown to be effective inhibitors of HDAC, with those carrying sp^3 centers (**18r**,**s**) displaying attenuated activity relative to the sp^2-bearing analogues (**18k**,**m**,**o**,**q**). The corresponding carboxylic acids were also tested and found to be inactive with the notable exception of **18t**.

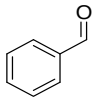
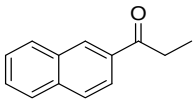
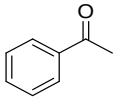
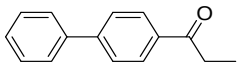
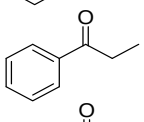
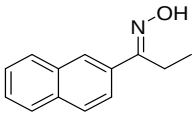
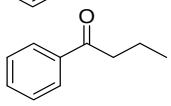
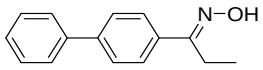
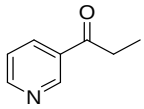
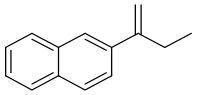
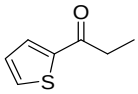
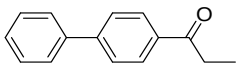
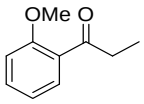
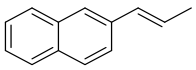
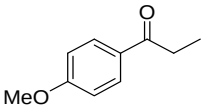
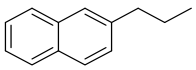
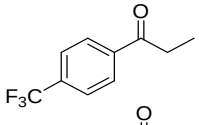
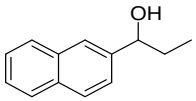
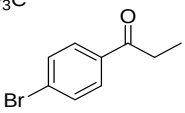
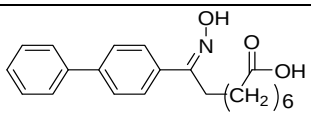
Table 2. SAR of SAHA analogs with phenylalanine cap groups



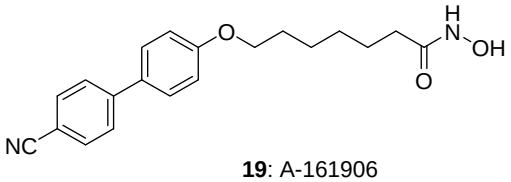
	R´	R´´	R´´´	IC_{50} (nM) Maize HD-2	IC_{50} (nM) Rat liver HDAC
1: TSA				3	12
17a	CH_3O	Bzl	H	500	800
17b	NH_2	Bzl	H	1100	840
17c	PhNH	Bzl	H	80	790
17d	BzlNH	Bzl	H	140	460
17e	$Ph(CH_2)_2NH$	Bzl	H	330	260
17f	$Ph(CH_2)_3NH$	Bzl	H	380	1010
17g	$Ph(CH_2)_2NH$	H	Bzl	340	390
17h	CH_3O	H	Bzl	760	880
17i	OH	H	Bzl	750	-
17j	CH_3O	4-MeOBzl	H	250	-
17k	CH_3O	4-PhBzl	H	210	-
17l	CH_3O	1-napthyl-methyl	H	360	130
17m	CH_3O	2-napthyl-methyl	H	40	510
17n	CH_3O	3-indolyl-methyl	H	120	320
17o	$Ph(CH_2)_2NH$	1-napthyl-methyl	H	35	310

Table 3. Hydroxamic acids with different cap variations



	R	HDAC1 IC_{50}, nM		R	HDAC1 IC_{50}, nM
18a		1500	**18k**		5
18b		500	**18l**		5
18c		65	**18m**		8.5
18d		135	**18n**		4
18e		153	**18o**		8
18f		50	**18p**		2
18g		95	**18q**		6
18h		15	**18r**		25
18i		45	**18s**		35
18j		45			
	TSA	5	**18t**		200

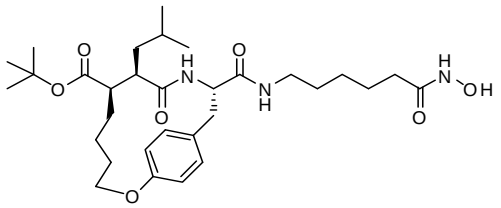
Glaser and co-workers identified the biaryl ether hydroxamate **19** as a mimic of transforming growth factor with proliferation inhibitory properties (Figure 11). This compound inhibits a mixture containing human HDAC1, HDAC2, and HDAC3 (IC_{50} = 9 nM) and to cause histone hyperacetylation and p21 induction.[65]



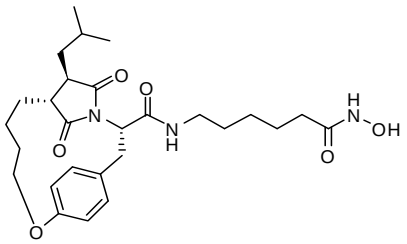
19: A-161906

Figure 11: Biaryl ether hydroxamate.

Curtin and co-workers,[66] reported potent inhibitors with a succinimide surface recognition domain. A macrocyclic compound **20** has good HDAC inhibitory activity while, other non-macrocyclic succinimides such as, **21** were equipotent (Figure 12). To get a complete SAR picture, a series of hydroxamic acids (**22**) were prepared and assayed against human HDAC1 (Table 4)



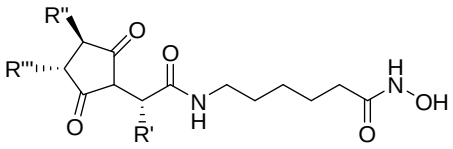
20: HDAC IC_{50} = 2100 nM



21: HDAC IC_{50} = 38 nM

Figure 12: Succinimide as cap group.

Table 4. SAR of hydroxamic acids with succinimide cap groups

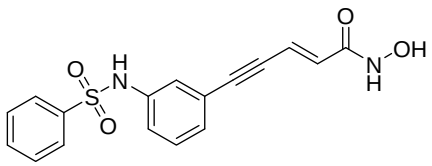


	R´	R´´	R´´´	HDAC IC50 (nM)
7: SAHA				120
22a	Bzl	*i*-Bu	H	99
22b	Bzl	H	H	660
22c	Bzl	*i*-Bu	*n*-Pr	51
22d	Cyclohexyl-CH_2	*i*-Bu	H	640
22e	4-MeO-Bzl	*i*-Bu	*n*-Pr	38
22f	H	*i*-Bu	H	5000

The number, identity, and disposition of macrocycle substituents are vital for activity. The removal of the succinimide substituents (**22b**) or the phenylalanine side chain (**22f**) reduces

activity. Replacement of the succinimide with a lactam or phthalimide also led to reduced activity. The length of the linker domain alkyl group was also found to be critical, with the five-methylene analogue showing maximal activity. This work revealed that the subtle adjustments of the succinimide cap group could lead to large positive changes in the HDAC inhibitory activity.

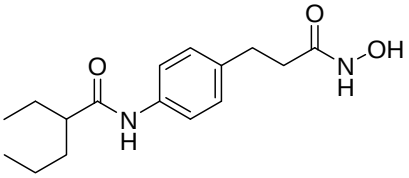
In 1996, Yoshida and coworkers reported another interesting compound, oxamflatin (**24**, Figure 13) containing a phenyl sulfonyl amine moiety as cap group with a spacer and hydroxamic acid functional group as HDAC inhibitor.[67] This compound was structurally different from other HDAC inhibitors. The inhibitory activity against partially purified mouse HDAC (IC_{50} was 15.7 nM), although it was found to be less potent than TSA (**1**) (IC_{50} = 1.44 nM) in the same assay. The disclosure of the potency of the oxamflatin accelerated the research on HDAC inhibitors, and hydroxamic acids containing sulfonamide moiety in the cap group were reported.



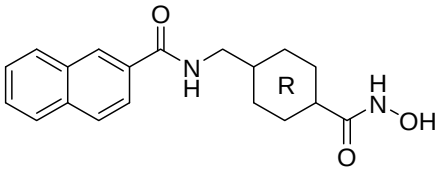
24: Oxamflatin

Figure 13: Phenyl sulfonyl amine moiety as cap group.

Small molecule inhibitors with 1,4-cyclohexelene or 1,4-phenylene linker and bicyclic aryl groups in the cap position were reported by Uesato *et al*.[68] They found that cyclohexeylene containing inhibitors such as, **28** are less potent than phenylene containing inhibitors like **29** (Figure 14). Chen and coworkers reported HDAC inhibitors using a short chain fatty acid such as valproic acid, butyric acid, phenylacetic acid e.t.c.[69] Short chain fatty acids were coupled with Zn^{2+} chelating motifs like hydroxamic acids and *o*-phenylenediamine through aromatic ω-amino acid linkers. These hydroxamic acids (**27**) showed HDAC inhibitory activity in lower micromolar levels. Research group of Mai and Massa reported pyrrole containing HDAC inhibitors with hydroxamic acid as the zinc binding ligand[710-72] (Table 5).



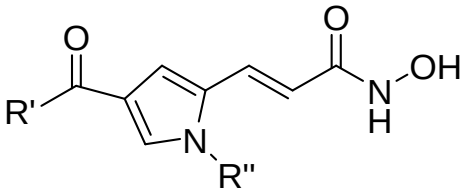
27

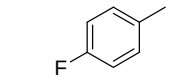
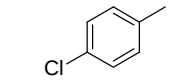
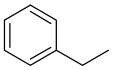
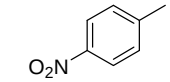
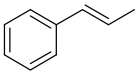
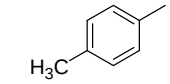
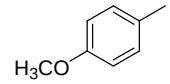
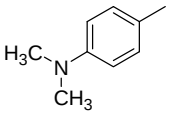


28: R = 1,4-cyclohexelene; HDAC IC_{50} = 2000 nM
29: R = 1,4-phenylene; HDAC IC_{50} = 44 nM

Figure 14: Inhibitors with 1,4-cyclohexelene or 1,4-phenylene linker.

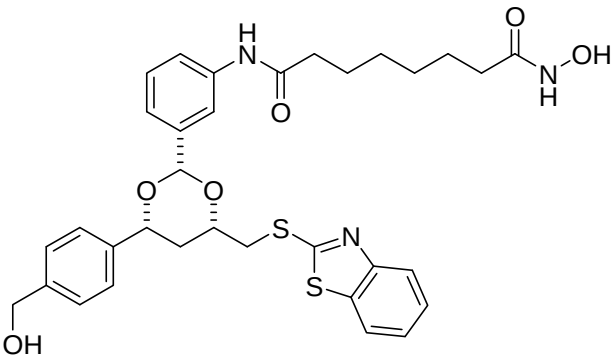
Table 5. Pyrrole-containing HDAC inhibitors



	R'	R"	HDAC IC_{50}, μM		R"	R"	HDAC IC_{50}, μM
30a		CH_3	3.8	**30g**		CH_3	2.4
30b		CH_3	3.8	**30h**		CH_3	0.1
30c		CH_3	2.4	**30i**		CH_3	1
30d		CH_3	3.9	**30j**		H	5
30e		CH_3	1.9	**30k**		*i*-Pr	53
30f		CH_3	2.9	**30l**		Ph	110

These compounds, 3-(4-aroyl- 2-pyrrolyl)-N-hydroxy-2-propenamides, e.g., **30** showed micromolar inhibitory activity toward HDACs. Using extensive docking studies they proposed that the presence of the pyrrole ring in the spacer gave higher flexibility inside the binding pocket of the enzyme. In subsequent work, compound **30h**, in which the benzoyl group on the pyrrole had been replaced by a phenylacetyl, showed a 38-fold increase in enzyme inhibitory activity.[72] The gain in activity was ascribed to the additional flexibility in the surface recognition domain of the inhibitor, which allows overall better contact and an improved binding geometry for the hydroxamate in the active site.

Schreiber and coworkers synthesized a library of 7200 small molecule inhibitors on polystyrene micro beads using one bead one stock solution method.[73] Some of these compounds such as, **31** with a dioxane derived cap group and hydroxamic acid functional group showed micromolar inhibitory activity (Figure 15).



31: HDAC1 IC_{50} = 1.7 μM

Figure 15: Hydroxamic acid inhibitors with dioxane derived cap group.

The research group of C.L.Marson[74] has reported the synthesis of novel series of HDAC inhibitors, based on arylsulfinyl 2,4-hexadienoic hydroxamic acids and their derivatives. Herein they carried out in vitro inhibition and inhibition of CEM (human leukemic cell) viability studies. In the Table 6, some of potent compounds were being listed. Presence of aromatic thioether group units HDAC inhibitors can make the arylpenta-2,4-dienoic acid hydroxamic acids as efficient inhibitors. Sulfides although less similar to TSA than the corresponding sulfones, were generally more potent than the latter, may be due to greater cellular uptake of sulfides. Sulfide **40b** was more metabolically stable than SAHA and for more so than TSA. **32b** was shown to inhibit proliferation of three cell lines by 50% at 0.9-2.7 μM. Saturated chain sulfoxides, **32b-32d** found better inhibitors than saturated chain sulfides **30b** and **30e** in all three assays. The cinnamohydroxamic acid type **19** tolerably good in activity than **27**, in which fused ring is present.

Recently, D.P.Fairlie and et al,[75] have reported the design and synthesis of series of compounds from α-aminosuberic acid, which shown potent activity against cancer cell lines. They emphasized on small hydrophobic groups and chosen several basic groups to mimic dimethyl alanine. Some potent compounds were shown in Table 7. Some of these compounds differing from previously reported cysteine derived series.[76] Compounds **18, 28** and **29** are respectively >30-, 5-, and 3- fold more potent and 7-, 1-, and 2- fold more selective than corresponding cysteine analogues in killing MM96L melanoma cells. Where as **6**, **25**, **26** and

33 are respectively 10-, 10-, 2-, and 3- fold less potent but equally selective. Further optimization at the N-, C- and S- terminus of amino suberic acid or related scaffolds could provide greater potency and selectivity. Also one might consider the replacement of hydroxamate moiety.

Table 6. In Vitro inhibition of HDAC and CEM cell viability

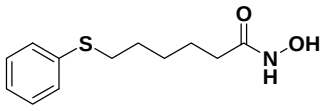
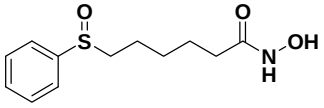
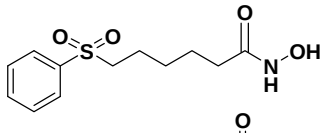
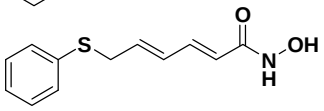
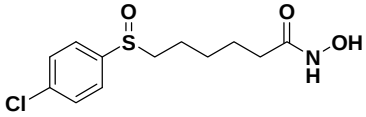
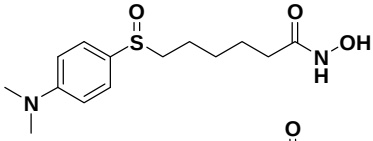
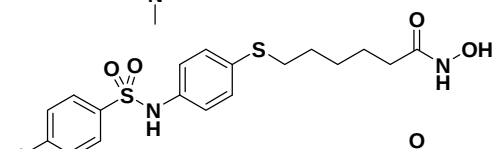
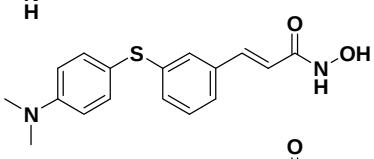
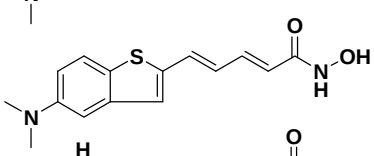
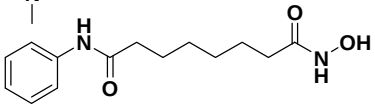
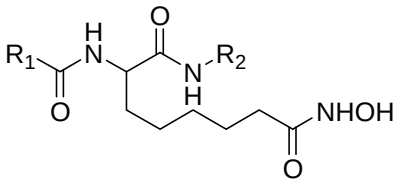
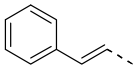
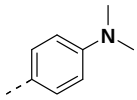
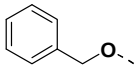
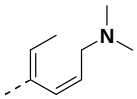
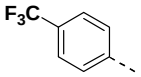
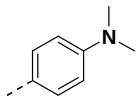
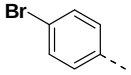
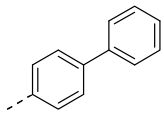
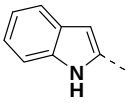
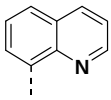
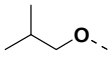
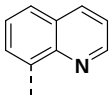
No.	Structure	Purified liver cell IC_{50} (μM)	Purified liver cell IC_{50} (μM)	50% viability CEM cells IC_{50} (μM)
30a		0.12	9.4	>25
32a		0.06	7.7	>25
33		0.04	15.5	>25
4a		0.39	>25	ND
32b		0.15	1.3	2.6
32d		0.18	2.2	3.4
30e		0.11	9.4	>25
19		0.16	8.6	3.4
27		>25	ND	ND
SAHA		0.44	0.33	1.9

Table 7. SAR of antitumor agents

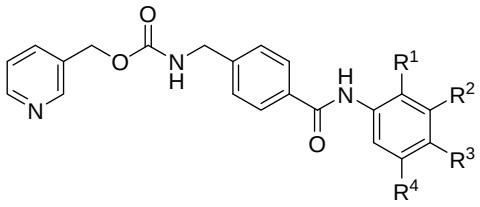


No.	R_1	R_2	IC_{50} (μM) NFF	IC_{50} (μM) MM96L	SI
39			0.24	0.16	1.5
40			0.65	0.61	1.1
41			0.4	0.22	1.8
47			0.19	0.07	2.5
52			0.337	0.021	16
56			0.081	0.023	3.5

9.3. Benzamides

Suzuki and coworkers[77, 78] was the first to report a series of synthetic benzamide-based, non-hydroxamate HDAC inhibitors. Benzamides are the class of compounds which inhibit HDACs by ligating the active site Zn^{2+} ion with a benzamide moiety. These compounds are generally less potent than the corresponding hydroxamate and cyclic tetrapeptide classes. The most potent compound of the series is named as MS-275. It has HDAC inhibitory activity of 5 μM and showed significant anti-tumor activity in vivo.

Table 8. SAR of MS-275 analogs



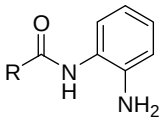
Compd	R^1	R^2	R^3	R^4	IC_{50} (□M)
32: MS-275	NH_2	H	H	H	4.8
32a	H	H	H	H	>100
32b	H	NH_2	H	H	>100
32c	H	H	NH_2	H	>100
32d	NHAc	H	H	H	>100
32e	OH	H	H	H	2.2
32f	NH_2	CH_3	H	H	>100
32g	NH_2	H	CH_3	H	>100
32h	NH_2	H	OCH_3	H	44
32i	NH_2	H	Cl	H	40
32j	NH_2	H	H	CH_3	2.8
32k	NH_2	H	H	OCH_3	4.6
32l	NH_2	H	H	Cl	7.7
32m	NH_2	H	H	F	6.0
1: TAS					0.0046

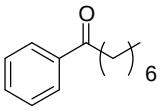
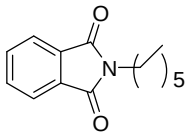
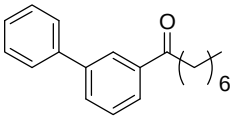
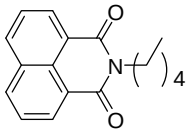
The SAR study of the benzanilide functionality revealed that a 2′-amino (**32**) or 2′-hydroxyl (**32e**) moiety is crucial for inhibitory activity against HDACs, as illustrated in Table 8. Changing the 2′-amino group on the aryl substituent (**32a-c**) like removing, repositioning or capping with acetate (**32d**) makes the compounds inactive. Incorporation of functionality at the R4 site was well tolerated (**32j-m**). Additional evaluation of MS-275 (**32**) demonstrated

that it could inhibit partially purified human HDAC preparations and cause hyperacetylation of nuclear histones in various cell lines.[78, 79] When administered orally to nude mice implanted with tumor lines, seven out of eight lines were strongly inhibited.[78] Importantly, an inactive MS-275 structural analogue was devoid of both in vitro and in vivo activity, further suggesting that the beneficial biological effects are a result of HDAC inhibition. The MS-275 is under clinical trials now.

Researchers of *MethylGene Inc* group reported[80] straight chain benzamides (**34**) as HDAC inhibitors having different cap groups (surface recognition domain). Some of these compounds with hydrophobic cap groups showed HDAC inhibitory activity in low micromolar range as shown in Table 9. However, the *in vivo* anti-tumor activity of selected compounds from this series is lower than that of MS-275. This is probably due to the short half life and poor bioavailability.

Table 9. SAR of benzamides



No.	R	HDAC IC_{50} (μM)	No.	R	HDAC IC_{50} (μM)
34a		9	34c		3
34b		1	34d		1

Although, some of the benzamide inhibitors showed significant HDAC inhibitory activity, there is no clear evidence that benzamide is acting at the zinc active site, and an allosteric binding site cannot be ruled out. Additional studies are required to understand the exact mechanism of action of benzamide-based HDAC inhibitors.

9.4. Electrophilic ketones

There are several electrophilic ketones known to inhibit proteases, including metal-dependent hydrolytic enzymes such as carboxypeptidase-A and metallo-β- lactamase.[81, 82] It has been proved that hydrated form of the ketone acts as a transition-state analogue and coordinates the zinc ion in the active site of carboxypeptidase A (Figure 16).[81] A similar mechanism might also be operative for HDAC inhibition by the electrophilic ketones.

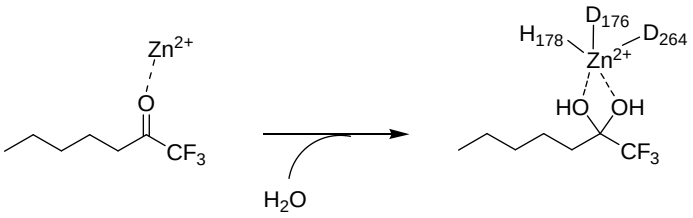
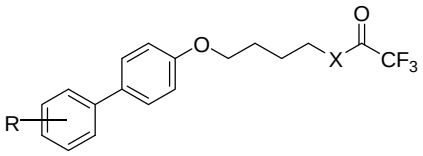


Figure 16: Mechanism of hydration.

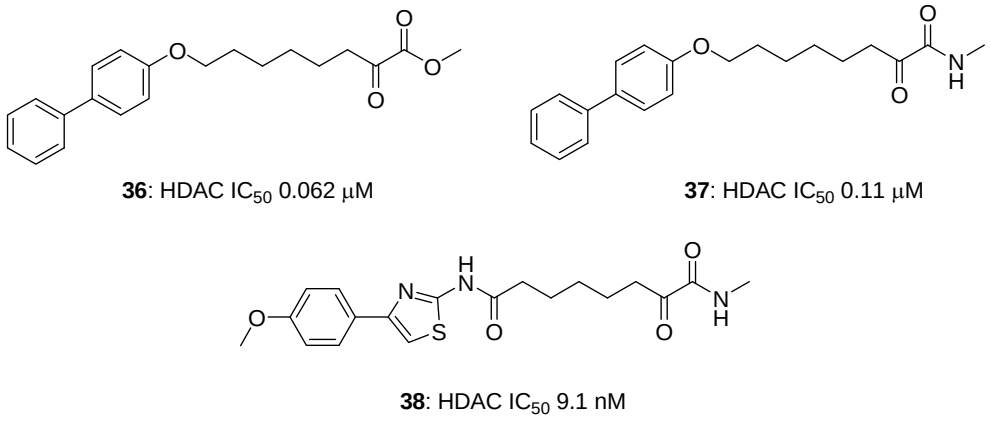
Table 10. Electrophilic ketones with linear cap groups



	R	X	HDAC IC_{50} (μM)
35a	4-Ph	$-CH_2-$	2.6
35b	4-Ph	$-CH_2-CH_2-$	2.9
35c	4-Ph	$-CH_2-CH_2-CH_2-$	72% @ 50
35d	4-Ph	-CH≡CH-	8.6
35e	4-Ph	-CH=CH-	5.0
35f	4-Ph	$-OCH_2-$	11
35g	4-Ph	$-SCH_2-$	4.6
35h	H	$-CH_2-CH_2-$	8.0
35i	3-Ph	$-CH_2-CH_2-$	2.8
35j	4-OPh	$-CH_2-CH_2-$	3.4
35k	Simple ketone analog	$-CH_2-CH_2-$	>50

Frey and coworkers from Abbott Laboratories reported SAHA based straight chain trifluoromethyl ketones as HDAC inhibitors.[83] They synthesized of various linear molecules containing an aromatic ring in the surface recognition domain, connected via ether or amide bonds to an aliphatic chain in the linker domain and carrying a trifluoromethyl ketone in the metal binding domain (Table 10).

α-keto esters and amides also have the hydrating property like trifluoromethyl ketones. Based on this property, Wada and coworkers synthesized α-keto esters and amides as HDAC inhibitors. Several compounds (**36-38**, Figure 17) of the series showed good inhibitory activity and by altering the aryl group, nanomolar α-keto amide inhibitors of HDACs were developed.[84]



36: HDAC IC_{50} 0.062 μM

37: HDAC IC_{50} 0.11 μM

38: HDAC IC_{50} 9.1 nM

Figure 17: α-keto esters and amides.

The electrophilic ketone class represents a relatively new group of HDACs substrate that has not yet been fully explored. More efforts need to develop additional structural variants in order to assess optimal design parameters and tolerances. However, the electrophilic ketone class contains potent inhibitors of HDAC that, despite apparent poor stability, possess antitumor effects in animal models.

9.5. Cyclic peptide based HDAC inhibitors

Cyclic peptide based HDAC inhibitors are particularly interesting because many of them are naturally occurring, they are relatively less toxic with high pharmacokinetic properties, their structural balance between hydrophilicity and hydrophobicity is optimum and structural features are advantageous for their *in vivo* application. The main structural features of the cyclic peptide-based HDAC inhibitors include a macrocycle containing hydrophobic amino acids in the surface recognition domain, an alkyl chain in the linker domain, and a functional

group in the metal binding domain. There is some evidence which support that cyclic peptide based HDAC inhibitors bind to the HDAC enzyme in a manner consistent with TSA (**1**). The aliphatic linker passes down the enzyme's tube-like channel, positioning the binding moiety in proximity to the active site zinc, while the macrocyclic portion binds to the rim of the active site. The macrocycle in naturally occurring cyclic tetrapeptide inhibitors is arranged with a D-amino acid and a cyclic amino acid (Pro or Pip) flanking the amino acid bearing the linker moiety, which generates a constrained 12-membered cyclic structure with extensive internal hydrogen bonding. It is postulated that the D-configuration of the amino acid is necessary for tight association with the rim of the active site, thereby allowing the linker to align and insert into the enzyme's tube-like channel.[85]

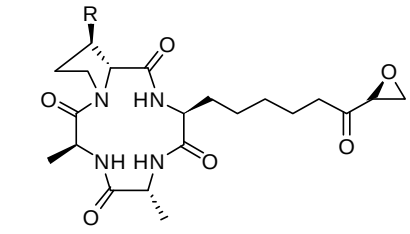
9.5.1. Cyclic tetrapeptide containing epoxyketone moiety

Naturally occurring cyclic tetrapeptide based HDAC inhibitors belong to a class which contain a unique amino acid, (*S*)-2-amino-8-oxo-9,10-epoxydecanoic acid (Aoe). These compounds have two more structural features in common: (i) at least one amino acid with (*R*) configuration and (ii) a proline or pipecolic acid residue. The Aoe side chain is approximately isosteric with an acetylated lysine, suggesting that it functions as a mimic of an acetylated histone protein. Several groups have shown that reduction or hydrolysis of the epoxide results in a complete loss of biological activity, suggesting that Aoe may irreversibly inactivate its receptor through covalent bond formation. (Figure 18).

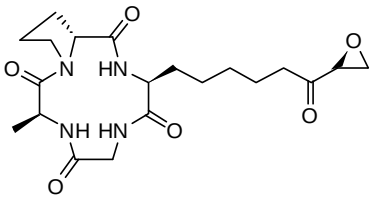
HC-toxin I (**39**, commonly referred to as HC-toxin), produced by *Helminthosporium carbonum*, was one of the first cyclic tetrapeptides in this structural class identified. Its structure, initially characterized by Liesch *et al*.,[86] later was revised by Knoche and co-workers.[87, 88] Further investigations led to the identification of HC-toxin III (**40**),[89] bearing a *trans*-3-hydroxyproline. Also discovered was HC-toxin II (**41**), an analog in which one alanine residue was replaced by a glycine.[90] While HC-toxin III was found to be comparable in potency to **39**, HC-toxin II have low activity (EC_{50} = 7 μg/mL in a maize root inhibition assay) relative to HC-toxin (EC_{50} = 0.2 μg/mL). It is interesting to mention that this 35-fold decrease in efficacy originated from a relatively modest structural change (Ala to Gly).

The cyclic tetrapeptides chlamydocin (**42**) and dihydro-chlamydocin (**43**) were first isolated and characterized by Closse and Huguenin.[91] Chlamydocin, produced by *Diheterospora chlamydosphoria*, exhibited pronounced cytostatic activity *in vitro.* It is found to be a highly potent histone deacetylase (HDAC) inhibitor, inhibiting HDAC activity in vitro with an IC_{50}

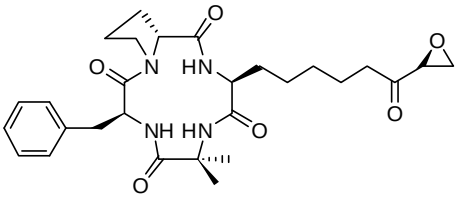
of 1.3 nM. Whereas **43**, with its reduced C8-carbonyl, was inert. Trapoxins A and B, discovered in the fermentation broth of *Helicoma ambiens,*[92] can potentially inhibit cellular transformations (**3** and **47**, respectively). In their structure, they differ solely by the presence of either a cyclic imino acid D-Pip or D-Pro in the tetrapeptide scaffold. The fungal metabolite WF-3161 (**46**) obtained from *Petriella guttulata*, also exhibited interesting antitumor activity *in vitro* and *in vivo*.[93] The prolyl-substituted Cyl-1 (**44**) as well as its pipecolyl-variant Cyl-2 (**45**) produced by *Cylindrocladium scoparium,* were found as potent plant growth regulators.[94-96]



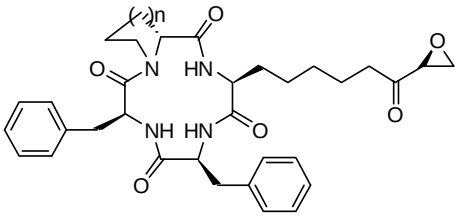
39: HC-Toxin I (R = H)
40: HC-Toxin III (R = OH)



41: HC-Toxin II



42: Chlamydocin (X = O)
43: Dihydrochlamydocin (X = H, OH)



3: Trapoxin A (n = 2)
47: Trapoxin B (n = 1)

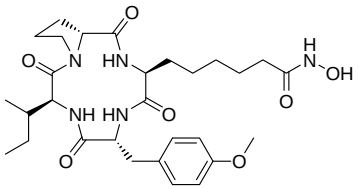
Figure 18. Naturally occurring Aoe containing cyclic tetrapeptides.

Three HDAC inhibitors, HC-toxin, chlamydocin and Cyl-2, were directly compared for phytotoxic efficacy to elucidate the relationship between cyclic tetrapeptide structure and biological activity.[97] The shifts in their respective biological activities correlated to overall cyclic peptide polarity. Consequently, it was postulated that these differences could be ascribed to a given analog's potential ability to penetrate membranes, as increased hydrophobicity enhanced potency. As an example, HC-toxin is 10-fold less potent than chlamydocin against these sensitive maize hybrids[98] This premise was further supported by the dramatically reduced efficacy of the more polar glycine variant of HC-toxin, cyclic tetrapeptide **41** (approximately 3% as active as HC-toxin).

Hydroxamic acids and carboxylic acids are reversible inhibitors of HDAC. On the other hand Trapoxins, Chlamydocin, HC-Toxin, Cyl-1, Cyl-2 and WF-3161 have irreversible HDAC inhibitory activity. On the basis of the irreversible inhibition of trapoxin B with HDAC enzyme, Schreiber and coworkers isolated HDAC1 using affinity chromatography.[99, 100] Due to the irreversible nature of the epoxide based inhibitors; they did not receive much attention in the HDAC inhibitor research.

9.5.2. Cyclic tetrapeptide containing hydroxamic acids

Till date the most potent synthetic HDAC inhibitor reported by Nishino et. al. This inhibitor contains a hydroxamic acid as the zinc binding group attached to a cyclic tetrapeptide scaffold (CHAPs).[85, 101] The biological activity of CHAPs varied by changing the number of the amino acids constituting the ring structure, the pattern of amino acid chiral combinations, and the side chain structure of each amino acids. Comparative study of the activity of cyclic tetrapeptide and cyclic octapeptide revealed the importance of the cyclic tetrapeptide scaffold for high HDAC inhibitory activity. Naturally occurring cyclic tetrapeptide based HDAC inhibitors containing epoxyketone functional group demonstrated the importance of the cyclic tetrapeptide moiety for the nanomolar inhibition of HDAC. Further, there was a 1-25 fold decrease in the activity for cyclic octapeptide confirms the importance of the cyclic tetrapeptide scaffold.



49r:CHAP31;
HDAC IC_{50} = 3.3 nM

Figure 18. Cyclic hydroxamic acid containing peptide (CHAP).

Table 11. SAR of CHAPs

		Structure	HDAC. IC_{50} (nM)
49a	CHAP1	*cyclo*(-L-Asu(NHOH)-L-Phe-L-Phe-D-Pro-)	6.0
49b	CHAP19	*cyclo*(-L-Asu(NHOH)-L-Phe-L-Phe-D-Pro-)2	83.7
49c	CHAP27	*cyclo*(-L-Asu(NHOH)-D-Phe-L-Phe-D-Pro-)	3.4
49d	CHAP38	*cyclo*(-L-Asu(NHOH)-D-Phe-L-Phe-L-Pro-)	5.3

49e	CHAP39	*cyclo*(-L-Asu(NHOH)-L-Phe-D-Phe-L-Pro-)	226
49f	CHAP18	*cyclo*(-L-Api(NHOH)-L-Phe-L-Phe-D-Pro-)	150
49g	CHAP17	*cyclo*(-L-Aza(NHOH)-L-Phe-L-Phe-D-Pro-)	24.7
49h	CHAP131	*cyclo*(-L-Asu(NHOH)-D-Phe-L-Pro-D-Phe-)	91.6
49i	CHAP132	*cyclo*(-L-Asu(NHOH)-D-Pro-L-Phe-D-Phe-)	25.5
49j	CHAP80	*cyclo*(-L-Asu(NHOH)-L-Ala-L-Phe-D-Pro-)	2.0
49k	CHAP88	*cyclo*(-L-Asu(NHOH)-D-Ala-L-Phe-D-Pro-)	3.2
49l	CHAP45	*cyclo*(-L-Asu(NHOH)-D-Phe-L-Ala-D-Pro-)	1.7
49m	CHAP57	*cyclo*(-L-Asu(NHOH)-D-Phe-L-Phe-D-Pip-)	2.9
49n	CHAP56	*cyclo*(-L-Asu(NHOH)-L-Phe-L-Phe-D-Pip-)	4.8
49o	CHAP58	*cyclo*(-L-Asu(NHOH)-D-Phe-L-Phe-L-Pip-)	4.2
49p	CHAP30	*cyclo*(-L-Asu(NHOH)-D-Tyr(Me)-L-Ile-L-Pro-)	3.3
49q	CHAP14	*cyclo*(-L-Asu(NHOH)-D-Tyr(Me)-L-Ile-L-Pro-)2	85
49r	CHAP31	*cyclo*(-L-Asu(NHOH)-D-Tyr(Me)-L-Ile-D-Pro-)	3.3
49s	CHAP42	*cyclo*(-L-Api(NHOH)-D-Tyr(Me)-L-Ile-D-Pro-)	54
49t	CHAP43	*cyclo*(-L-Aaz(NHOH)-D-Tyr(Me)-L-Ile-D-Pro-)	34
49u	CHAP44	*cyclo*(-L-Asu(NHOH)-D-Tyr(Me)-L-Ala-D-Pro-)	3.4
49v	CHAP77	*cyclo*(-L-Asu(NHOH)-D-Tyr-L-Ile-D-Pro-)	3.4
49w	CHAP50	*cyclo*(-L-Asu(NHOH)-D-Tyr(Me)-L-Ile-D-Pip-)	4.0
49x	CHAP49	*cyclo*(-L-Asu(NHOH)-D-Tyr(Me)-L-Ile-L-Pip-)	5.3

Yoshida and coworkers synthesized hydroxamic acid analogues of all these natural products and found that they inhibit HDAC strongly and reversibly. They carried out a SAR study using several combinations of amino acids for the cyclic tetrapeptide scaffold. Details of the SAR studies on CHAPs are given in Table 11.

Table 12. Chlamydocin hydroxamic acid analogues

	Structure	IC_{50} (nM) HDAC1	MHC_{Cx2} (nM)
2	Chlamydocin	0.15	4.6
3	*cyclo*(-L-Asu(NHOH)-Aib-L-Phe-D-Pro-)	5.2	33.2
4	*cyclo*(-L-Asu(NHOH)-Acc5-L-Phe-D-Pro-)	3.02	5.67
5	*cyclo*(-L-Asu(NHOH)-Acc6-L-Phe-D-Pro-)	2.19	5.38
6	*cyclo*(-L-Asu(NHOH)-Acc7-L-Phe-D-Pro-)	2.14	2.76
7	*cyclo*(-L-Asu(NHOH)-Acc8-L-Phe-D-Pro-)	3.99	1.88
8	*cyclo*(-L-Asu(NHOH)-A2in-L-Phe-D-Pro-)	0.98	1.29
9	*cyclo*(-L-Asu(NHOH)-L-Ala-L-Phe-D-Pro-)	1.95	325
10	*cyclo*(-L-Asu(NHOH)-D-Ala-L-Phe-D-Pro-)	3.23	NT
11	*cyclo*(-L-Asu(NHOH)-L-A1in-L-Phe-D-Pro-)	1.12	2.69
12	*cyclo*(-L-Asu(NHOH)-D-A1in-L-Phe-D-Pro-)	1.95	2.29
13	*cyclo*(-L-Asu(NHOH)-L-Pro-L-Phe-D-Pro-)	3303	
14	*cyclo*(-L-Asu(NHOH)-Aib-L-Phe-D-Pip-)	2.98	
15	*cyclo*(-L-Asu(NHOH)-Acc5-L-Phe-D-Pip-)	12.4	37.8
16	*cyclo*(-L-Asu(NHOH)-Acc8-L-Phe-D-Pip-)	2.75	
17	*cyclo*(-L-Asu(NHOH)-A2in-L-Phe-D-Pip-)	1.2	
18	*cyclo*(-L-Asu(NHOH)-A2in-L-Ile-D-Pip-)	2.68	
19	*cyclo*(-D-Asu(NHOH)-L-Pro-D-Phe-Aib-)	8.57	3950
20	*cyclo*(-L-Asu(NHOH)-L-Pro-L-Phe-Aib-)	31.7	57,800
21	*cyclo*(-L-Asu(NHOH)-Aib-L-Phe-L-Pro-)	45.7	717

During the optimization of the CHAP molecules, Nishino *et al.* found that out of many combinations of optically different amino acids, LDLD, LLLD and LDLL combinations have almost similar extent of activity.[85, 101] However, the activity to induce MHC class-1 molecule expression and growth inhibition was high for LDLD configuration, that is, positions 2 and 3 are best occupied with hydrophobic L- and D-amino acids. Further, CHAPs with five methylene units spacer is the best for good inhibitory activity (Table 11). One of these compounds, CHAP31 (Figure 18) was extensively studied *in vivo* and it exhibits antitumor activity in mice bearing B16/BL6 tumor cells. CHAP31 inhibited the growth in four of five

human tumor lines implanted in nude mice. Further, these cyclic tetrapeptide containing HDAC inhibitors are very useful in understanding the factors for the HDAC specificity and selectivity. These cyclic tetrapeptides will help in getting more details about the interaction between the enzyme and the inhibitor, which will lead to the development of selective inhibitors with improved qualities.

Chlamydocin, a natural cyclic tetrapeptide isolated from *Diheterospora chlamydosphoria,* has shown to exhibit potent anticancer activity *in vitro*[102] It belongs to a small family of hydrophobic cyclic tetrapeptide containing the unusual amino acid, 2-amino-8-oxo-9,10-epoxy decanoic acid (Aoe), which is essential for their biological activity. Recently, Nishino *et al.*[103] have synthesized chlamydocin hydroxamic acid analogues by replacing epoxy ketone moiety of Aoe by hydroxamic acid functional group. They also have introduced several aromatic analogues in cyclic framework to study hydrophobic interaction of capping group. The details of HDAC inhibitory data of chlamydocin hydroxamic acids are shown in Table 12.

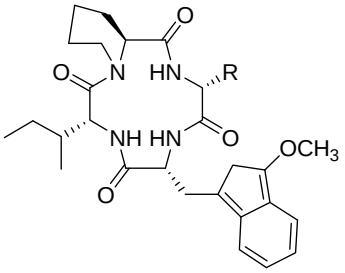
9.5.3. Cyclic tetrapeptide containing ketone functionality

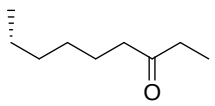
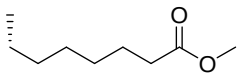
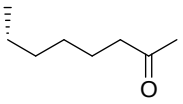
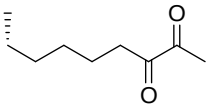
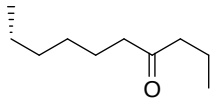
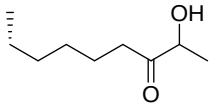
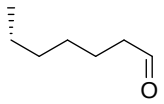
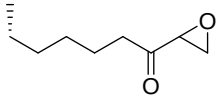
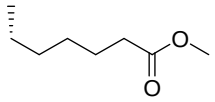
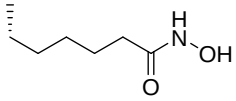
Apicidin (**48a**, Table 13), a cyclic tetrapeptide isolated from *Fusarium Pallodoroseum* which is an endophytic fungus collected from Costa Rica, as a potent and broad spectrum antiprotozoal agent that exerts its biological activity by reversibly inhibiting HDAC activity.[104] A group of scientists from Merck research laboratory extensively studied the HDAC inhibitory activity of apicidin and its synthetic derivatives.[105-111] Apicidin contains an ethyl ketone moiety in its side chain, and so it is different from other natural cyclic peptides like trapoxin or HC-Toxin with epoxyketone side chain. The high potency of HDAC inhibitory activity of apicidin further confirmed the importance of the cyclic tetrapeptide scaffold for the inhibitor design.

The shift of keto group from position C-8 to C-9 decreases potency, confirming that the exact location of the C-8 keto group is critical for proper mimicking of acetylated histone lysine. In addition to that, reduction of the C-8 keto group to epimeric alcohols, formation of olefin or epoxide, or conversion to CH_2 reduce activity. On the other hand, if the ethyl ketone moiety is changed to hydroxamic acid, epoxyketone or methyl ester with a spacer of five methylene groups, the inhibitory activity is further enhanced.[112] Further, they found that the tryptophan moiety of apicidin is a key site and the replacement or modification of this moiety have great influence on the HDAC inhibitory activity which means that the amino acid present in the

cyclic tetrapeptide inhibitor have some interactions with the rim of the HDACs active site pocket.[113] The introduction of a basic group at the tryptophan`s NH increases the HDAC activity and similar enhancement in activity is observed with the introduction of a bulky aryl substituent at the 2-position of tryptophan.

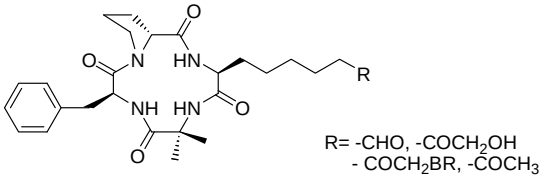
Table 13. SAR of apicidin analogs



	R	HDAC Activity IC_{50} (nM)		R	HDAC Activity IC_{50} (nM)
48a		1	**48f**		7
48b		2	**48g**		30
48c		1	**48h**		0.3
48d		6	**48i**		0.1
48e		0.4	**48j**		0.2

In light of above view that the Apicidin contain an ethyl ketone moiety as zinc ligand, Nishino *et al.* have replaced the epoxy ketone moiety in chlamydocin with several ketones and aldehyde to synthesize potent reversible and selective HDAC inhibitors.[114] They found most of them as potent inhibitors and some compounds have shown remarkable selectivity among HDAC paralogs (Table 14).

Table 14. HDAC inhibitory activity of chlamydocin analogues bearing carbonyl as functional group



	IC_{50} (μM)			EC_{1000} (μM)
	HDAC1	HDAC4	HDAC6	
TSA	0.0190	0.0200	0.0280	0.190
1	>100	>100	>100	>250
2	4.69	4.05	>100	22.7
4	0.0619	0.0595	0.621	0.344
5	1.08	0.290	3.70	1.20
6	>100	>100	>100	NT
7	4.33	1.29	14.1	20.9
10	0.223	0.0604	0.887	0.234

9.5.4. Retrohydroxamic acids

Retrohydroxamic acids (N-formyl hydroxylamine) are reported as potent, long lived orally bioavailable matrix metalloproteinase's (MMPs) inhibitors, which are zinc proteases.[115, 116] Based on the assumption, that retrohydroxamates can also act as good inhibitors of HDACs, Nishino *et al.*[117] designed and synthesized HDAC inhibitors containing retrohydramate (**50**, Figure 19) as the zinc binding ligand with cyclic tetrapeptide cap group and with an optimum spacer length of five methylene units.

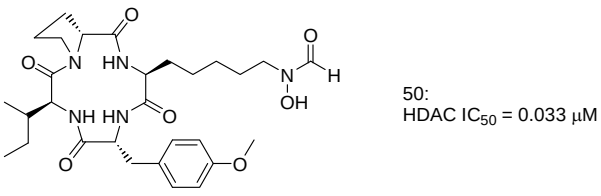


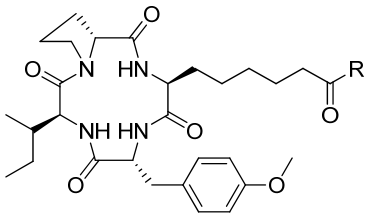
Figure 19. HDAC inhibitors with retrohydroxamic acid group.

Eventhough they have ten times lower inhibitory activity than the corresponding hydroxamic acids, these novel class of inhibitors have potential as anticancer agents. Recently, researchers from Scripps Research Institute reported SAHA based retrohydroxamates (**51** and

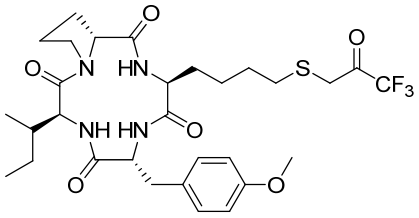
52) as HDAC inhibitors.[118] These compounds are 50 times weaker in activity than the parent compound SAHA. However, retrohydroxamic acids have potential as HDAC inhibitors due to their long half-life and possible oral bioavailability.

9.5.5. Cyclic tetrapeptide containing electrophilic ketones

Jose *et al.*[119] have synthesized cyclic tetrapeptides containing trifluoromethyl ketone and pentafluoroethyl ketone (**53** and **54**) as the zinc binding functionality. Out of these compounds trifluoromethylketone is found to have excellent inhibitory activity (Figure 20). Changing from trifluoro to pentafluoro group did not increase the activity, probably because of the balkiness of the pentafluromethyl group. Incorporation of a sulfur atom to the carbonyl (**55**) increased the activity that may provide different mode of ligation with Zn^{2+} ions, using longer spacer length.



R = CF_3; **53**: HDAC IC_{50} 0.73 μM
R = CF_2CF_3; **54**: HDAC IC_{50} 0.85 μM



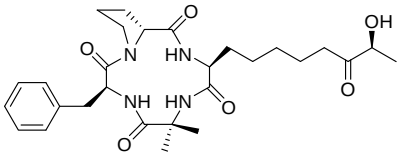
55: HDAC IC_{50} 0.047 μM

Figure 20. Cyclic tetrapeptide containing electrophilic ketone.

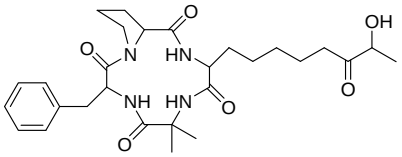
9.5.6. Cyclic tetrapeptide containing hydroxymethyl ketone

So far, several novel fungal metabolites have been characterized which lack the reactive epoxide which is assumed to be essential for significant biological activity. These new compounds include a phytotoxin structurally related to chlamydocin: 9, 10-desepoxy-9(R)-hydroxy-chlamydocin (**56**) produced by *Verticillium coccosporum*[120] as well as the tumorigenic Tan-1746s (**57**) (Figure 21).[121] Both new analogs, as noted, lack the 9,10-epoxide and instead bear a 9-hydroxyl. A published patent has described a desepoxy-trapoxin analog (FR-225497, compound not shown) which had good activity against human tumor cell lines *in vitro* (IC_{50} = 152 and 158 ng/mL against Jurkat and HT-29 cells, respectively).[122] Total synthesis of a potent immunosuppressant FR235222 (**58**), that inhibits mammalian

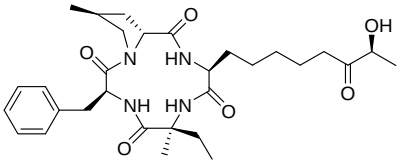
HDACs has been reported.[123] It has a chlamydocin like cyclic tetrapeptide structure, inhibiting the HDACs with IC_{50} = 9.7 ng/mL.



56: 9,10-des epoxy-9-hydroxy-chlamydocin



57: TAN-1746

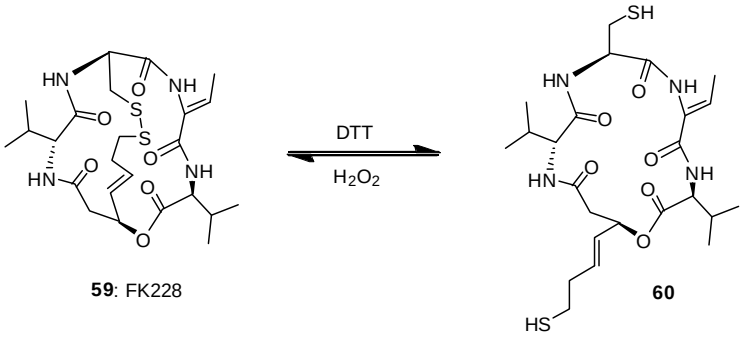


58: FR235222, HDAC IC_{50} 17.4 nM

Figure 21. Cyclic tetrapeptides containing hydroxymethylketone functionality.

9.5.7. Cyclic peptides with thiols

The natural product, FK228 (**59**) (formerly named FR901228 and also known as cyclic depsipeptide) produced by *Chhromobacterium violaceum* showed potent *in vivo* antitumor activity and Ueda *et al.* reported that FK228 have HDAC inhibitory activity.[124-126] There is no visible functional group in FK228 that interacts with the zinc ion in the HDAC binding pocket. It was demonstrated that the zinc-binding site is the sulfhydral functional group and FK228 is converted to its active form by cellular reducing activity (Figure 22).[127]



59: FK228 **60**

Figure 22. Cyclic peptides containing sulfur as zinc ligand.

Table15. Comparative activity study of SCOPs

	IC_{50} (μM)				EC_{1000} (μM)
	HDAC1	HDAC4	HDAC6	HDAC8	
TSA	0.022	0.02	0.028	0.04	0.062
59	0.0358	0.512	>500	NT	0.0031
60	0.0001	NT	0.624	NT	0.0157
62	0.142	0.145	>500	>500	0.0368
62 + DTT	0.0046	0.0021	1.4	1.69	0.0677
63	0.007	0.068	1.61	3.14	0.140
63 + DTT	0.00055	0.0011	2.01	0.494	0.0597

In 2001, the structure of spiruchostatin A and B (**61**, Figure 18) isolated from a *Pseudomonas* extract were discovered with potent HDAC inhibitory activity and the total synthesis of spiruchostatins were reported recently.[128, 129] On the basis of this inhibitory activity of FK228, Nishino and coworkers[130] synthesized many potent inhibitors of HDAC containing a sulfhydral moiety as the functional group for HDAC inhibition. These cyclic tetrapeptides (SCOPs) contain L-2-amino-7-mercaptoheptanoic acid (L-Am7) instead of L-Aoe (2S, 9S)-2-amino-8-oxo-9,10-epoxydecanoic acid of Cyl-1.

The SCOPs were synthesized as stable disulfide dimers (**62**) and hybrids (**63**) with several thiols. These compounds showed potent inhibitory activity as shown in Table 15. To know the interaction of aliphatic cap groups with HDACs, bicyclic peptide disulphide hybrid (Figure 23)[131] was also synthesized without aromatic ring in their macrocyclic framework by ring closing metathesis using Grubbs first generation catalyst. The bicyclic peptide was found to be active in both cell free and cell based condition, and it has some selectivity among HDAC1 and HDAC6.

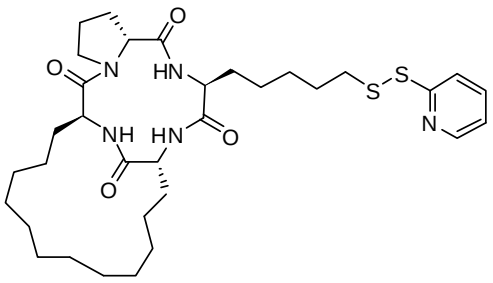


Figure 23. Bicyclic peptide disulphide hybrid.

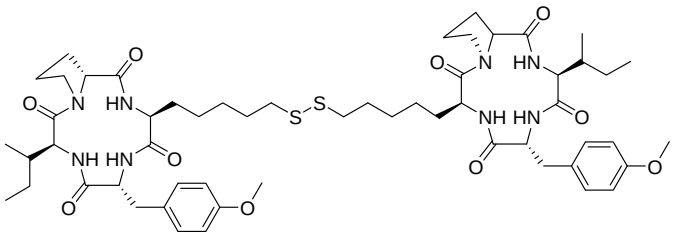
To find out specific inhibitors, Shivashimpi *et al.*[132]have synthesized compounds containing S-S disulphide by shifting benzene ring of L-Phe in chlamydocin framework, and found some potent inhibitors as shown in Table 16.

Table16. Comparative activity study of S-S disulphide cyclic tetrapeptides.

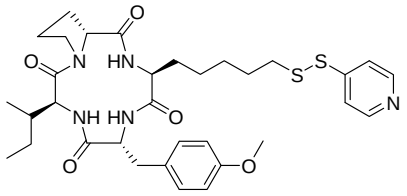
Compound	No.	IC_{50} (nM)			HDAC1/	EC_{1000}
		HDAC1	HDAC4	HDAC6	HDAC4[a]	(nM)
cyclo(-L-Am7(S2Py)-A2in-L-Ala-D-Pro-)	**3**	2.0	1.6	8.8	1.2	140
cyclo(-L-Am7(S2Py)-D-A1in-L-Ala-D-Pro-)	**4**	2.7	2.4	12	1.1	55
cyclo(-L-Am7(S2Py)-L-A1in-L-Ala-D-Pro-)	**5**	36	25	33	1.4	2000
cyclo(-L-Am7(S2Py)-D-2MePhe-L-Ala-D-Pro-)	**6**	170	70	71	2.4	25600
cyclo(-L-Am7(S2Py)-L-2MePhe-L-Ala-D-Pro-)	**7**	3.7	2.2	56	1.6	550
cyclo(-L-Am7(S2Py)-Aib-L-Ala-D-Tic-)	**8**	4.7	4.2	29	1.1	200
cyclo(-L-Am7(S2Py)-Aib-L-Phg-D-Pro-)	**9**	88	56	86	1.5	7400
cyclo(-L-Am7(S2Py)-Aib-L-Ph4-D-Pro-)	**10**	6.2	3.2	36	1.9	320
cyclo(-L-Am7(S2Py)-Aib-L-Ph5-D-Pro-)	**11**	5.3	2.7	43	1.9	110
cyclo(-L-Am7(S2Py)-Aib-L-Ser(Bzl)-D-Pro-)	**12**	3.2	1.6	24	2.0	72
cyclo(-L-Am7(S2Py)-Aib-L-Ser-D-Pro-)	**13**	6.4	4.0	26	1.6	580
cyclo(-L-Am7(S2Py)-Aib-L-Ala-D-Pro-)	**14**	9.4	5.2	43	1.8	750

[a]Seletivity of the enzymes

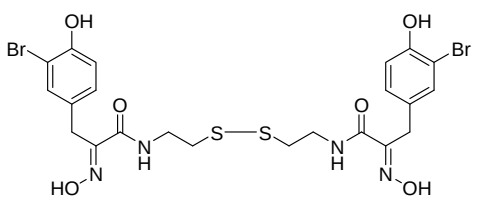
Another group of natural products psammaplins isolated from the sponge *Pseudoceratina purpurea* are potent inhibitors of HDAC.[133] These psammaplins contains a disulphide linkage and therefore the inhibitory activity may be due to the chelation of the zinc ion with sulfhydryl in the active-site pocket. The structure of psammaplin A (**64**) is shown in Figure 23. Studies on the mechanism of action of psammaplin and its synthetic derivatives also supported cellular disulphide cleavage type mechanism of inhibition.[134, 135]



62: SS-Dimer+DTT; HDAC IC_{50} 4.6 nM



63: SS-Hybrid+DTT; HDAC IC_{50} 0.5 nM

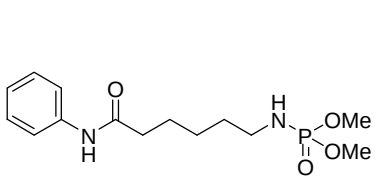


64: psammaplin A

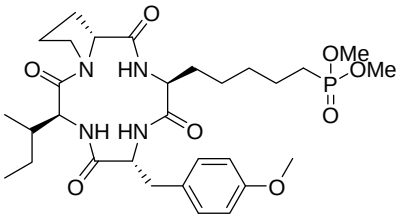
Figure 23. Sulfur containing cyclic tetrapeptides.

9.5.8. HDAC inhibitors with phosphonates

Etzkorn and coworkers synthesized several phosphorus based SAHA analogues **65** and related compounds as HDAC inhibitors.[136] However, these compounds also showed very weak inhibitory activity during HDAC assays. Further, the side chain modification of apicidins ketone moiety to phosphonate by Merck group also showed very low HDAC inhibitory activity.[113] From all these phosphonate compounds studied for HDAC, It is clear that phosphonates are not good inhibitors for the HDAC inhibition, probably due to the highly acidic nature of phosphonate moiety, instead of the basic nature of the HDAC substrate. Jose *et al.*[137] synthesized a phosphonate with a cap group of cyclic tetrapeptide and a spacer of five methylene units. The HDAC inhibitory activity study showed that this phosphonate compound **66** is a very weak inhibitor of HDAC (Figure 24).



65

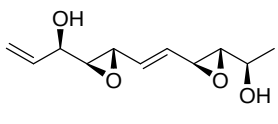


66

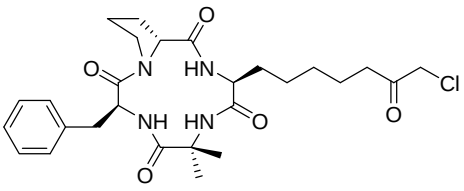
Figure 24. HDAC inhibitors containing phosphonate functional groups.

9.6. Miscellaneous

There are a number of HDAC inhibitors that can not be classified as a structural class. Depudecin, a natural product (**67**, Figure 25) having an epoxide was shown to inhibit HDAC in 1998.[138] Rich *et al.*[139] synthesized a cytostatic chlamydocin derivative having α-chloro ketone functional group as Zn^{2+} ligand. Though the new derivative exhibited somewhat decreased potencies in cell based antiproliferative assay (~3-4 fold) relative to the parent natural product, still it adds a new variation to the zinc ligand library.



67: Depudecin



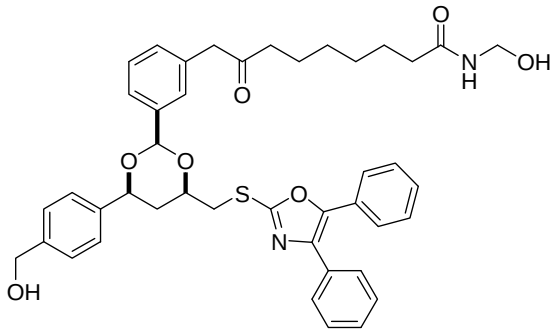
68

Figure 25. Chloro methylketone and epoxy alcohol as zinc ligands.

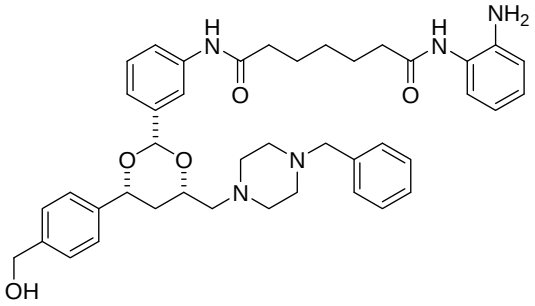
10. Selectivity of HDAC inhibitors

In 2001, Yoshida et al.[101] reported the differential inhibitory activities of a series of CHAP compounds and a number of natural products for class I/II HDACs. This work demonstrated for the first time that the surface recognition domain of the pharmacophore (Figure 6) affects not only inhibitory potency but selectivity as well. For the characterization of individual members in the HDAC family, development of selective inhibitors of individual members of the HDAC family will be a valuable tool. Also specific drugs for different malignant diseases will be the possible from selective inhibitors. Few reports on the selective inhibitors of HDAC families are available so far, and the current research on HDAC inhibitors is mainly directed towards this direction. A class I HDAC specific inhibitors named SCOPs were already reported.[130] Another report on paralog selectivity was recently disclosed from Schreiber's laboratory.[140] They screened mainly two compounds from the library of several 1,3-dioxane based compounds. Out of the two compounds tubacin **69** inhibit HDAC6 and on the other hand, histacin **70** inhibit HDAC1. The main difference between these two compounds is the zinc binding ligand with some difference in the cap group (Figure 26). Tubacin has the hydroxamic acid as the zinc binding ligand and histacin has benzamide as the zinc binding ligand. Also the interactions at the rim of the binding channel are also important for the specific inhibitors. The cap group of histacin and tubacin were based on 1,3-dioxane but with different groups attached to the dioxane base.

Researchers are now concentrating their attention on the synthesis of selective inhibitors of HDAC. However, progress towards the design of selective inhibitors of HDAC is at the preliminary stages. The synthesis of selective inhibitors will help not only in therapeutic discovery but also to elucidate the biological functions of HDACs.



69: Tubacin



70: Histacin

Figure 26. Selective HDAC inhibitors.

11. Conclusions

HDACs appear to be key enzymes in the regulation of gene expression and HDAC inhibitors have potential as chemotherapeutic agents. After the X-ray structure determination of HDLP, the momentum of research on HDAC inhibitors increased and a large number of potent HDAC inhibitors are now available and many of them are in clinical trials. The reported HDAC inhibitors provided information about the active site of HDACs. Cyclic/bicyclic peptides are very much helpful in finding the interactions of each HDAC in the rim region. The design of novel inhibitors based on the reported HDAC inhibitors and on the basis of the information on HDACs will lead to the synthesis of member selective inhibitors. HDAC research is currently focusing on the subtype selective inhibitors, which can be used as bioprobe to find the functions of each member of the HDAC family, and also have potential as drugs for many specific diseases.

12. Purpose of this study

The crystal structure of HDLP and HDLP complexed with TSA reveals that, for a molecule to be an effective inhibitor of HDAC enzymes, a functional group capable of chelating the active site of Zn^{2+} ion need to be attached to a hydrophobic scaffold by means of a spacer. The functional group, scaffold and spacer play their role in the inhibitory activity. The inhibitory activity is determined by several factors, such as type of functional group, length of spacer and its interaction with residues in the channels of catalytic site, nature of scaffold and its interaction with residues at the rim of the enzyme active site, etc. Till date many classes of HDAC inhibitors, starting from non-peptides to cyclic peptides, both synthetic and natural with different types of functional groups have been reported. Some of them were proved to be potent inhibitors of HDAC enzyme, both *in vitro* and *in vivo*. Peculiarity among these inhibitors is that, all of these have aromatic ring in their cap group part and it was been proved that, the aryl group portion may act as cap to bind the surface of HDAC enzyme. The sequence diversity in surface binding region of HDAC enzyme suggests that modification at cap group portion in inhibitor can provide better specificity.

Specificity is a necessary condition for an inhibitor to be useful as a drug with high pharmacokinetic properties and oral bioavailability. Scientists are now concentrating their research on this matter. Very potent HDAC inhibitors including non-peptides and cyclic tetrapeptides, bearing hydroxamic acid or epoxy ketones inhibit with very low selectivity

among HDAC isoforms. Therefore, a lot of effort is needed to develop selective HDAC inhibitors.

As compounds having aromatic ring in the cap group lack in selectivity, cyclic tetrapeptides without aromatic ring were designed and synthesized. Removal of aromatic ring from cyclic tetrapeptide framework resulted in a sharp decrease in activity.[131] As an alternative to aromatic ring, an aliphatic loop was thought to incorporate in cyclic tetrapeptide framework. The loop was assumed to increase hydrophobic interaction between the cap group and the enzyme surface. Moreover, due to less flexibility compared to aromatic side chain, the loop was considered helpful in improving selectivity among the HDAC isoforms.

In the present work, the author intended to develop cyclic tetrapeptide with aliphatic loop (bicyclic tetrapeptide) HDAC inhibitors. A potent cyclic tetrapeptide HDAC inhibitor CHAP31[85] was considered as a lead compound for designing bicyclic tetrapeptide HDAC inhibitors. A series of CHAP31 based bicyclic tetrapeptides have been designed to elucidate the effect of aliphatic loop length on biological activity and conformation of the bicyclic tetrapeptide HDAC inhibitors. In the second chapter, the design, synthesis, interesting biological activity and conformation of CHAP31 based bicyclic tetrapeptides with different aliphatic loop length have been described.

Positioning of aliphatic loop is important for proper interaction in the enzyme rim region. To elucidate effect of aliphatic loop position, three more bicyclic tetrapeptides were designed based on the naturally occurring cyclic tetrapetide HDAC inhibitors Cyl-1, trapxin B and HC-toxin I, using hydroxamic acid instead of epoxyketone as zinc binding group. Due to the difficulty in synthesis of Cyl-1 based bicyclic tetrapeptide, ultimately trapoxin B and HC-toxin I based bicyclic tetrapeptides with ten methylene loop were designed for synthesis. In the third chapter, the design, synthesis of trapoxin B and HC-toxin based bicyclic tetrapeptides have been described. A Comparative study of interesting biological activity and conformation between CHAP31, trapoxin B and HC-toxin based bicyclic tetrapeptides have been described.

One of the bicyclic peptides from CHAP31 based series that shows optimum activity was chosen to investigate the effectiveness of the bicyclic tetrapeptide cap group for other zinc binding groups. In the fourth chapter, the design, synthesis and interesting biological activity of bicyclic tetrapeptide methoxymethyl ketone (Mmk) and boronic acid have been described.

Finally, the findings in the present study are summarized in chapter 5. One the basis of information available for HDAC inhibitors and the findings from the present study, the author expressed his opinion on future prospects of bicyclic tetrapeptides for designing novel drugs.

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Chapter 2

Molecular Design of Bicyclic Tetrapeptide Histone Deacetylase Inhibitors by Changing Aliphatic Loop Length

1. Introduction

Histone deacetylases are enzymes responsible for the regulation of chromatin remodeling and gene transcription by deacetylation of acetyl lysine residues in amino terminal tails of histones.[1-3] As overexpression of HDACs is associated with carcinogenesis of cells, design and synthesis of novel HDAC inhibitors is a promising field on developing anticancer drugs. In fact, there are a variety of HDAC inhibitors in clinical trials and one of them, SAHA (Vorinostat), is already in the market. SAHA have demononsrated the therapeutic benefit in cutaneous T-cell lymphoma and in some malignancies.[4] However, current HDAC inhibitors, including SAHA, broadly inhibit the deacetylase activity of various HDAC enzymes including class I and II, which is associated with reduced potency and toxic side effects. Therefore, it is desirable to release HDAC inhibitors that can selectively inhibit HDAC isoform that is overexpressed in a cancer cell. Up to date, as naturally occurring HDAC inhibitors, trichostatin A (TSA),[5] depsipeptide FK228,[6] and the cyclic tetrapeptide family including trapoxin,[7] chlamydocin,[8] Cyl-1,[9] Cyl-2,[10] HC-toxins,[11] apicidin[12] have been reported. Synthetic TSA-related and SAHA-related inhibitors have been also reported.[13, 14] All these inhibitors exhibit the inhibitory activity for metal-dependent HDAC classes I and II. Recent inhibition model of these metalloenzymes proposed from X-ray crystallography consist of three elements: (1) a metal-binding active site, (2) a surface recognition unit capping the active pocket, (3) a linker unit connecting the metal-binding and surface recognition units.[15] It is plausible that because the active site and linker unit are essential but only bind to the pocket in enzyme, a capping site covering the rim surrounding binding pocket determine the selectivity for HDACs. As the area surrounding the opening to the binding pocket has less homology between HDAC isoforms compared to the active site, the modification of cap group allows to have a significant impact upon isoform selectivity.[16] The surface of the HDAC enzymes that surrounds the binding pocket has multiple grooves, (Figure 1) [17] which could be exploited by inhibitors with appropriate cap groups to improve potency and selectivity of the inhibitors.

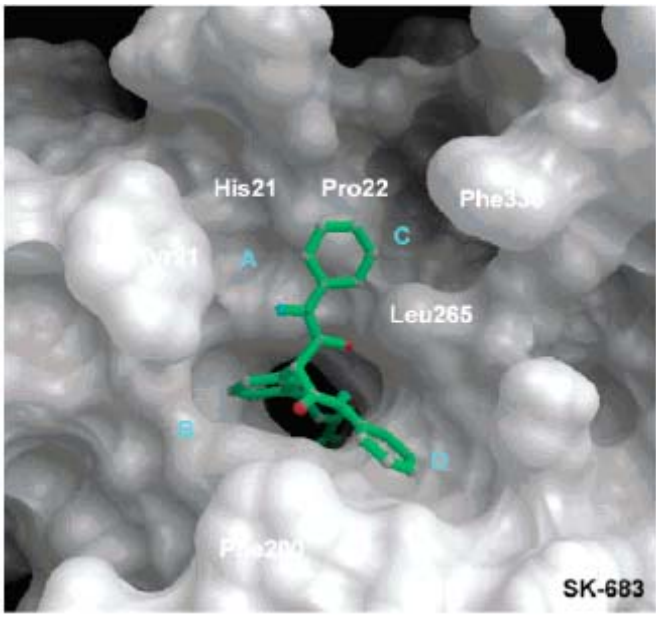


Figure 1. The surface surrounding the active site of HDLP, with an inhibitor in the active site. The grooves are lettered, and important amino acids are labeled.

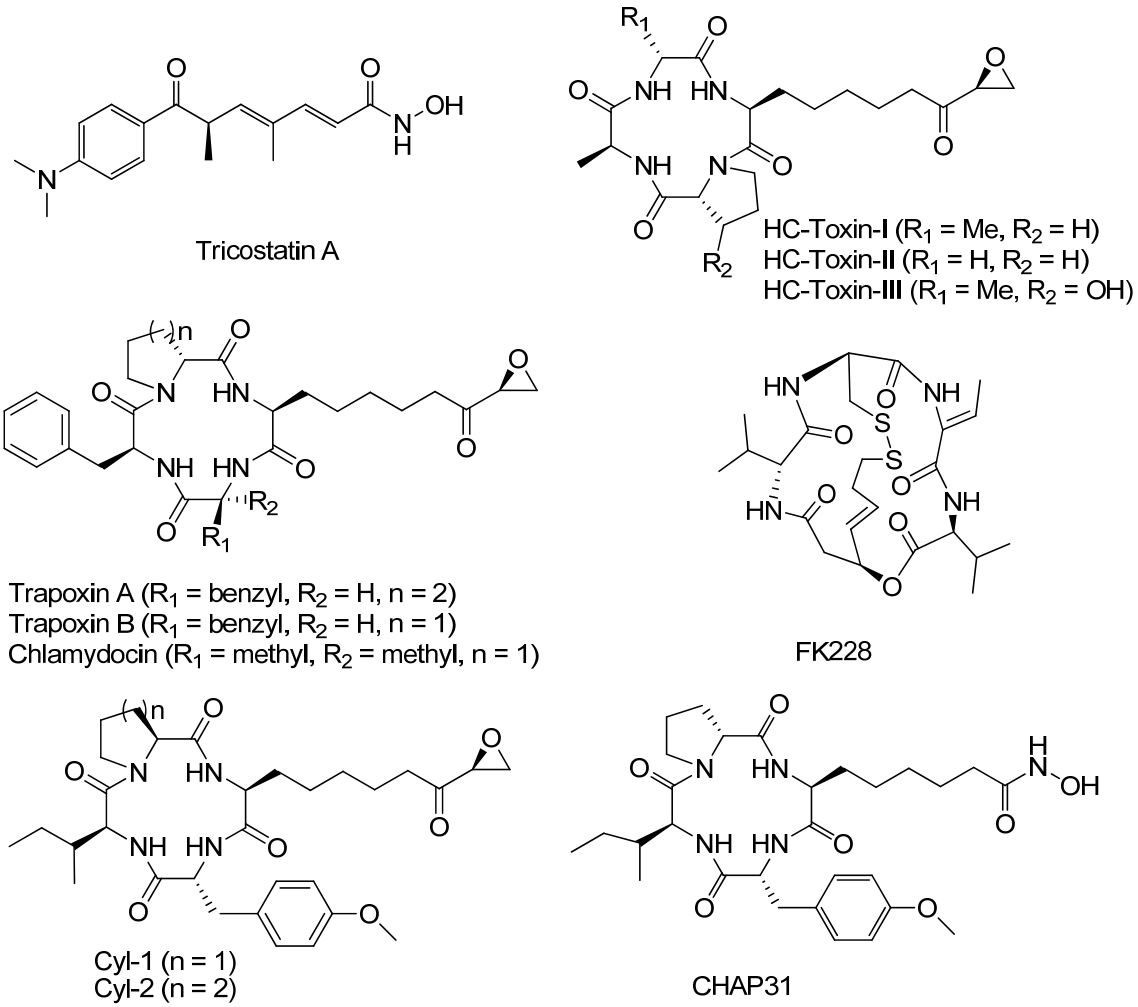


Figure 2. Natural and synthetic HDAC inhibitors.

Extensive approaches using cyclic tetrapeptides CHAPs,[18, 19] SCOPs,[20] and chlamydocin analogs with various binding functional groups[21] have shown that modification of the cap group of HDAC inhibitors led to elevate significantly the selectivity between HDAC isoforms.[22] Nishino and coworkers reported that incorporation of aliphatic loop to the cyclic tetrapeptide framework showed a potent activity and enhanced 2 times selectivity of HDAC4/HDAC1 and 250 times of HDAC4/HDAC6. [23]

In the present study, a series of CHAP31 based bicyclic tetrapeptides have been designed to elucidate the effect of aliphatic loop length on biological activity and conformation of the bicyclic tetrapeptide HDAC inhibitors. One cyclic tetrapeptide with open chains was also design to compare the acitivity and conformation between cyclic and bicyclic tetrapeptides. The author herein describes the design, synthesis, interesting biological activity and conformation of CHAP31 based bicyclic tetrapeptides.

2. Results and discussion

2.1. Design of bicyclic compounds. To introduce aliphatic loop to cyclic tetrapeptides, formation of intramolecular covalent bond between alkyl side-chains of two amino acids will be useful. Olefin metathesis has recently appeared as a versatile and chemoselective means to create carbon-carbon bonds in a range of molecules including natural products, polymers, and biomolecules.[24-28] Modern ruthenium olefin metathesis catalysts tolerate many functional groups common in biomolecules, including amides, alcohols, and carboxylic acids.[29] Several modified amino acids and peptides such as α-helical peptides,[30-37] rigidified amino acids and peptides,[38] antimicrobial peptides[39-41], cyclicpeptide MC4-ligand[42] were synthesized by the aid of intramolecular ring-closing metathesis (RCM) reaction to alter the conformational and metabolic stability. Glucosylated porphyrins bearing a cyclic pseudopentapeptide for photodynamic therapy (PDT) of cancer, and inhibitors of Grb2 SH2 domain signaling, which was associated with breast cancer, have also been synthesized.[43, 44] Recently, synthesis of apicidin analogue has been reported.[45] Nishino and coworkers reported the design and synthesis of a SCOP based bicyclic tetrapeptide.[23] Following these reports, the author design a series of CHAP31 based bicyclic tetrapeptide hydroxamic acids (**1**-**5**) (Figure 3). The sequence and configuration of amino acids in CHAP31 were considered as the basis for designing the bicyclic peptide HDAC inhibitors with different aliphatic loop length. L-2-amino-(n-1)-alkenoate (L-Aen) and D-2-amino-(n-1)-alkenoate (D-Aen) were incorporated in the sequence, and the terminal alkenes were fused by the aid of Grubbs first generation Ru

catalyst (Figure 4). One cyclic tetrapeptide hydroxamic acid (**6**) was also design to compare activity between cyclic and bicyclic tetrapeptides.

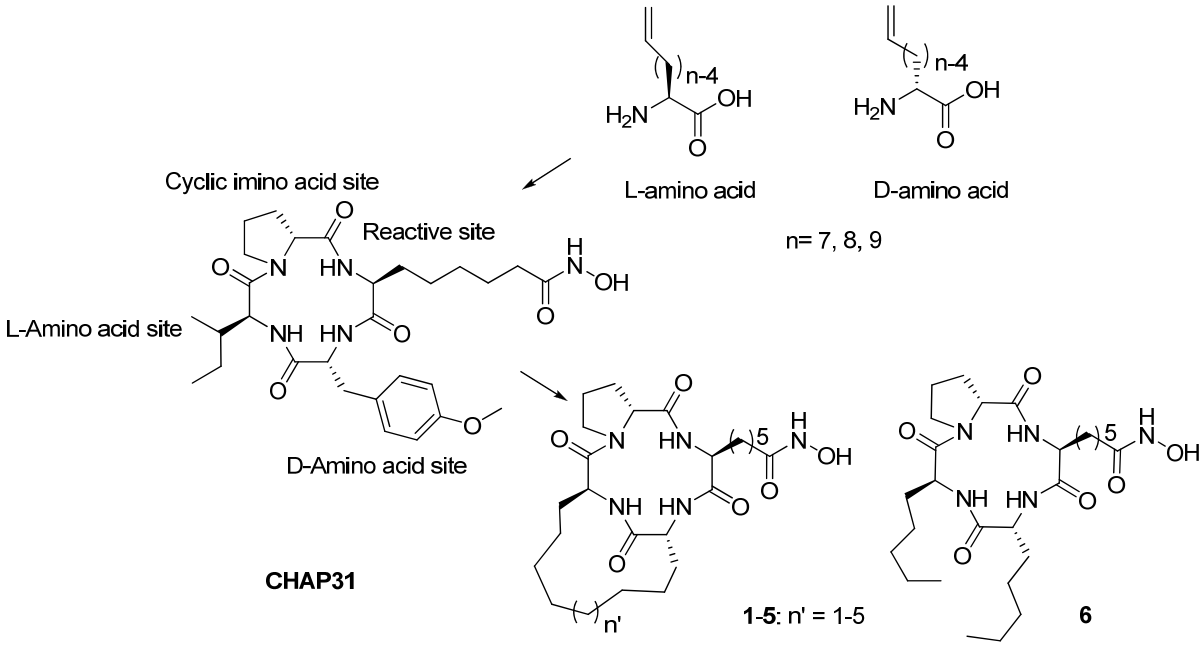


Figure 3. Design of CHAP31 based cyclic/bicyclic tetrapeptide hydroxamic acid HDAC inhibitors.

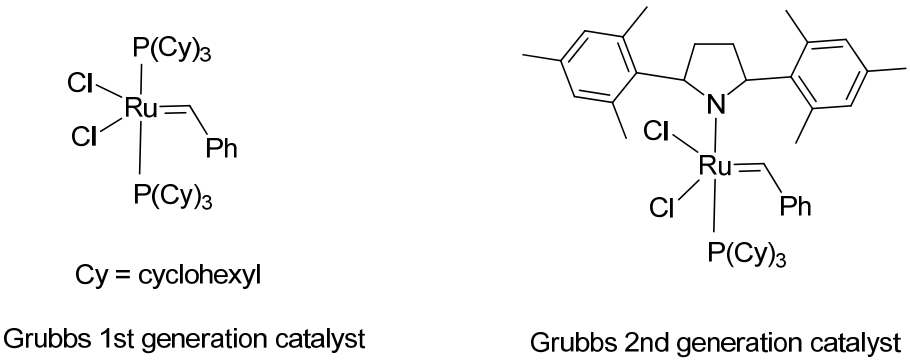
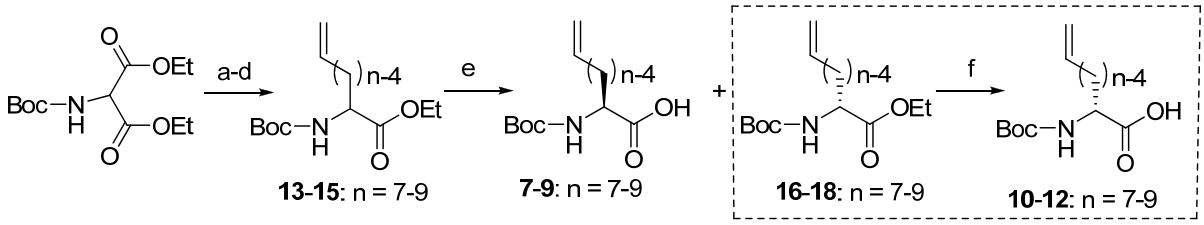


Figure 4. Grubbs Ru catalysts for alkene metathesis.

2.2. Synthesis of building block amino acid derivatives. Synthesis was started from the preparation of the building block amino acids. Boc-L-2-amino-6-heptenoic acid (Boc-L-Ae7-OH) (**7**), Boc-L-2-amino-7-octenoic acid (Boc-L-Ae8-OH) (**8**), Boc-L-2-amino-8-nonenoic acid (Boc-L-Ae9-OH) (**9**), Boc-D-2-amino-6-heptenoic acid (Boc-D-Ae7-OH) (**10**), Boc-D-2-amino-7-octenoic acid (Boc-D-Ae8-OH) (**11**) and Boc-D-2-amino-8-nonenoic acid (Boc-D-Ae9-OH) (**12**) were synthesized by the reported procedure[23] as illustrated in Scheme 1. Compounds **7**-**12** were used for the synthesis of designed cyclic/bicyclic tetrapeptides in Chapter 2 and 3. Bromoalkenes were separately coupled with diethyl Boc-aminomalonate by

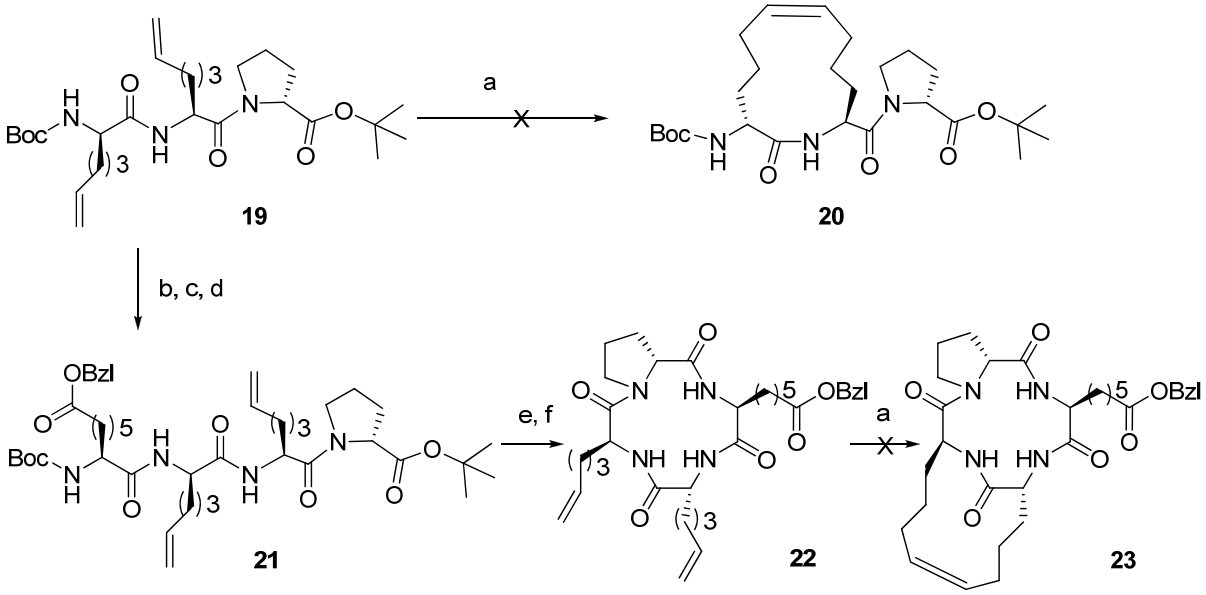
using NaOEt in ethanol. One of the ethyl ester groups of the adduct was hydrolyzed by one equivalent of 1 M NaOH at low temperature (0–5 ^{0}C). The oily monoacid monoester after work-up is then subjected to the decarboxylation by refluxing in toluene. After evaporation of toluene, the resulting oily Boc-DL-Aen-OEt (n = 7-9) was purified by silica gel column chromatography. Finally Boc-DL-Aen-OEt was subjected to the action of subtilisin Carlsberg from *Bacillus licheniformis* (Sigma) in a mixture of DMF and water (1:3, v/v). Boc-L-Aen-OH (**7-9**) was isolated as colorless oil. The recovered Boc-D-Aen-OEt was hydrolyzed by 1 M NaOH to get Boc-D-Aen-OH (**10-12**) with excellent purity in quantitative yield.



Scheme 1. Synthesis of 2-amino-(n-1)-alkenoic acids (Aen). Reagents and conditions: (a) Na, anhyd EtOH, reflux, 30 min; (b) $Br(CH_2)_{n-4}$-$CH{=}CH_2$, reflux, 6 h; (c) NaOH aq., EtOH, 4 h; (d) toluene, reflux, 3 h; (e) subtilisin Carlsberg, DMF/H_2O (1/3), 4 h; (f) 1 M NaOH aq, EtOH, 4h.

2.3. Determination of the lower limit for aliphatic loop length. Determination of the minimum loop length for bicyclic tetrapeptides is illustrated in Scheme 2. In course of synthesis of designed compound **1**, linear tripeptide Boc-D-Ae7-L-Ae7-D-Pro-OtBu (**19)** was synthesized by stepwise peptide elongation from C-terminal. Compound **19** was subjected to ring-closing metathesis (RCM) between D-Ae7 and L-Ae7 using Grubbs first generation Ru catalyst, in dichloromethane (DCM) to obtain linear tripeptide with fused ring (**20**). Instead of obtaining expected compound **20**, it was obtained dimer which was formed by the intermolecular cross metathesis followed by RCM. The dimer was confirmed by LC-MS. By changing the route, cyclic tetrapeptide (**22**) was synthesized. Compound **22** was subjected to (RCM) between D-Ae7 and L-Ae7 to obtain bicyclic tetrapeptide (**23**). Once again RCM yielded dimer, which was confirmed by LC-MS. By increasing the loop length by one methelene group, bicyclic tetrapeptide with nine methelene loop (**2**) was successfully synthesized. By successive increase in the loop length by one methelene group, three more

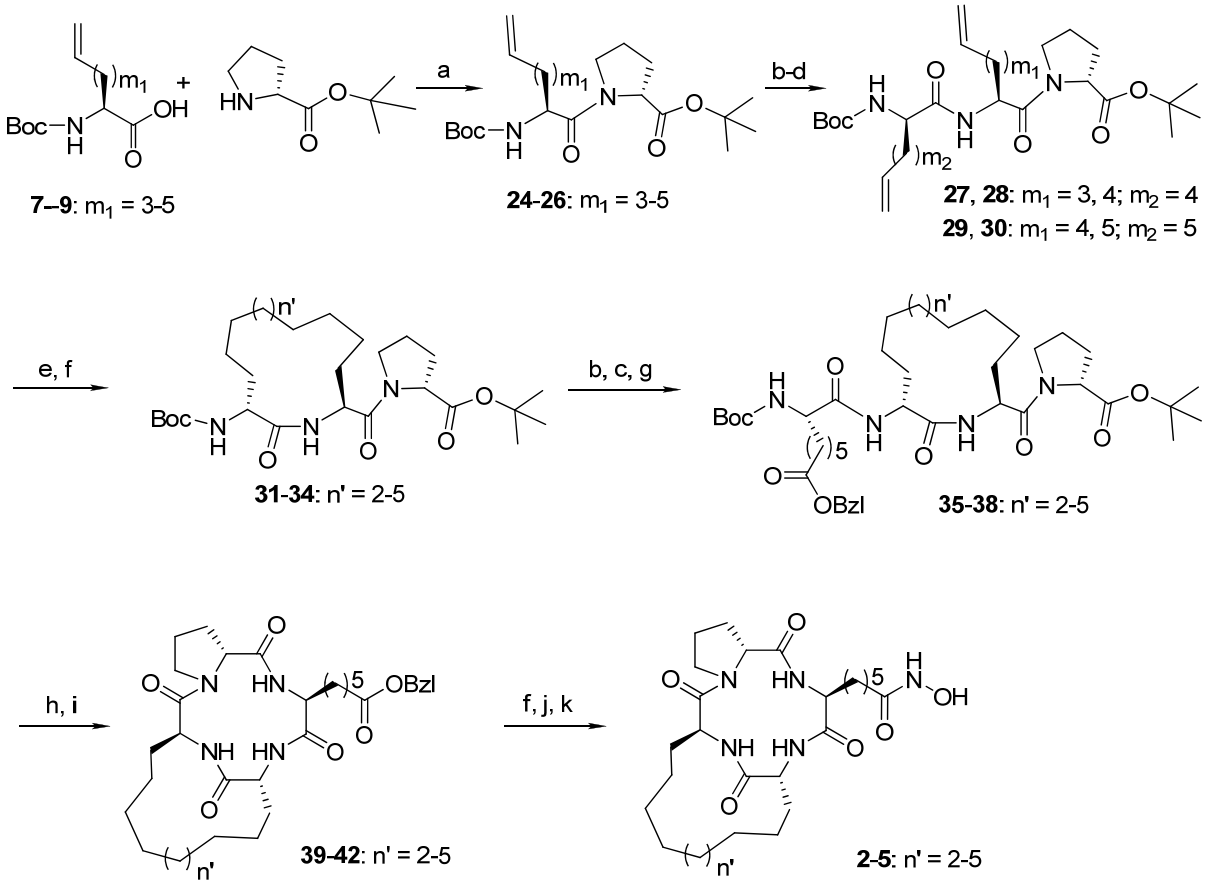
bicyclic tetrapeptides (**3-5**) were synthesized. The synthesis of bicyclic tetrapeptides (**2-5**) is illustrated in Scheme 3.



Scheme 2. Determination of the lower limit for aliphatic loop length of bicyclic tetrapeptides. Reagents and conditions: (a) Grubbs first generation catalyst, DCM, 48 h; (b) 4 M HCl/dioxane, 30 min; (c) saturated Na_2CO_3; (d) Boc-L-Asu(OBzl)-OH, DCC, HOBt, DMF; (e) TFA, 3 h; (f) HATU, DIEA, DMF, 4 h.

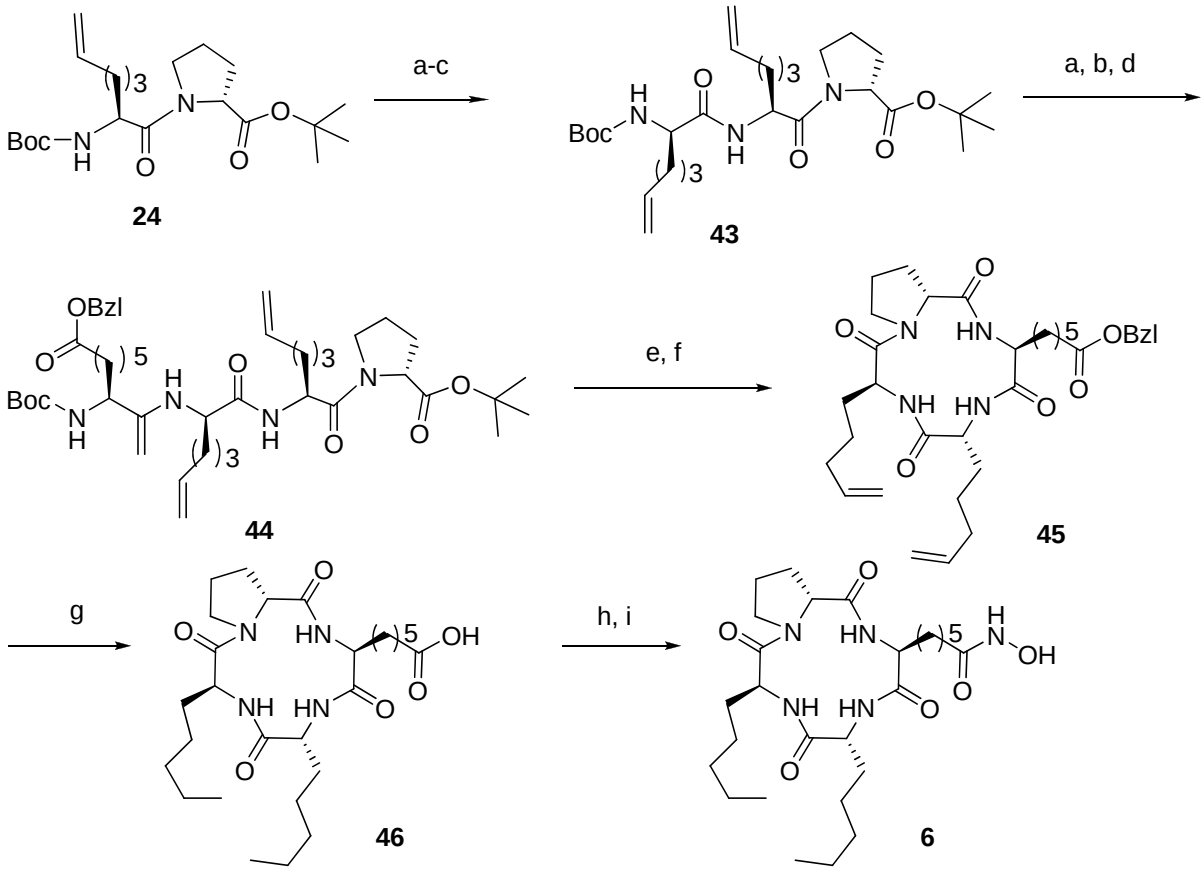
2.4. Synthesis of bicyclic tetrapeptides (2-5) with different aliphatic loop length. CHAP31 based bicyclic tetrapeptides (**2-5**) were synthesized according to Scheme 3 by the conventional solution phase method. H-D-Pro-O^{t}Bu was condensed separately with **7-9** using DCC/HOBt. Boc protection was selectively removed by the reported procedure[46] using 4 M HCl/dioxane, and each amine component was condensed with **11** or **12** by the same DCC/HOBt method to obtain linear tripeptides **27-30**. The linear tripeptides **31-34** with fused side ring were synthesized by ring closing metathesis between D-Ae8/9 and L-Ae7/8/9 using Grubbs first generation Ru catalyst, in dichloromethane (DCM), followed by catalytic hydrogenation. After selective Boc deprotection of **31-34**, Boc protected amino suberic acid benzyl ester [Boc-L-Asu(OBzl)] was incorporated in them to prepare the linear tetrapeptides **35-38**. After removal of both side protections by treating with trifluoroacetic acid (TFA), cyclization reaction was carried out by the aid of *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) in dimethylformamide (DMF) under high dilution conditions with minimum amount of

diisopropylethyl amine (DIEA) (2.5 equiv) to obtain bicyclic tetrapeptides **39-42**. The peptide cyclization yield was 15-67% (Table 1). After Bzl deprotection, by catalytic hydrogenation, the carboxyl group was condensed with hydroxylamine benzyl ester, and finally Bzl protection was removed by catalytic hydrogenation to obtain bicyclic tetrapeptide hydroxamic acids **2-5**.



Scheme 3. Synthesis of CHAP31 based bicyclic tetrapeptide hydroxamic acids (**2-5)**. Reagents and conditions: (a) DCC, HOBt, DMF, 12 h, 75-80%; (b) 4 M HCl/dioxane, 30 min, 70-80%; (c) saturated Na_2CO_3; (d) Boc-D-Ae8-OH, or Boc-D-Ae9-OH, DCC, HOBt, DMF, 12 h, 75-80%; (e) Grubbs first generation catalyst, DCM, 48 h, 50-87%; (f) AcOH, Pd-C, H_2, 12 h, 97-100%; (g) Boc-L-Asu(OBzl)-OH, DCC, HOBt, DMF, 12 h,75-80%; (h) TFA, 3 h, 98-100%; (i) HATU, DIEA, DMF, 4 h, 15-65%; (j) $HCl.H_2NOBzl$, DCC, HOBt, TEA, DMF, 60-80%; (k) Pd-$BaSO_4$, AcOH, H_2, 70-80%.

2.5. Synthesis of cyclic tetrapeptide hydroxamic acid (6) as reference compound. The cyclic tetrapeptide reference compound **6** was synthesized by the same solution phase method as described for compounds **2-5**. In this case ring closing metathesis step was excluded (Scheme 4).



Scheme 4. Synthesis of CHAP31 based cyclic tetrapeptide hydroxamic acid (**6**). Reagents and conditions: (a) 4 M HCl/dioxane, 30 min;(b) saturated Na_2CO_3; (c) Boc-D-Ae7-OH DCC, HOBt, DMF, 12 h; (d) Boc-L-Asu(OBzl)-OH, DCC, HOBt, DMF, 12 h; (e) TFA, 3 h; (f) HATU, DIEA, DMF, 4 h; (g) AcOH, Pd-C, H_2 , 12 h; (h) HCl.H_2NOBzl, DCC, HOBt, TEA, DMF; (i) Pd-$BaSO_4$, AcOH, H_2.

2.6. Effect of loop length on RCM and peptide cyclization. Cyclization yields in the present synthesis are listed in Table 1. For compounds **3**-**5** both RCM and peptide cyclization yields are reasonable, but in case of compound **2** cyclization yield is lower than those of others, particularly on peptide cyclization. Moreover, bicyclic peptide having eight CH_2 loop between D-Ae7 and L-Ae7 was not synthesized. The cyclization of both linear tripeptide Boc-D-Ae7-L-Ae7-D-Pro-O^{t}Bu and *cyclo*(-L-Asu(Bzl)-D-Ae7-L-Ae7-D-Pro-) by RCM gave only the corresponding dimmer that were intermolecularly cyclized. The dimers were confirmed by LC-MS. Since the bicyclic tetrapeptides having nine or more CH_2 groups were successively synthesized, nine CH_2 loop length is the minimum for bicyclic tetrapeptides. Peptide cyclization for compound **2** was of low yield. This may be due to the rigid short loop, which may hinder the peptide bond formation in cyclization step.

Table 1. Effect of loop length on RCM and peptide cyclization.

Compounds	Loop size	RCM yield (%)	Peptide cyclization yield (%)
2	$-(CH_2)_9-$	58	15
3	$-(CH_2)_{10}-$	87	40
4	$-(CH_2)_{11}-$	85	65
5	$-(CH_2)_{12}-$	85	67

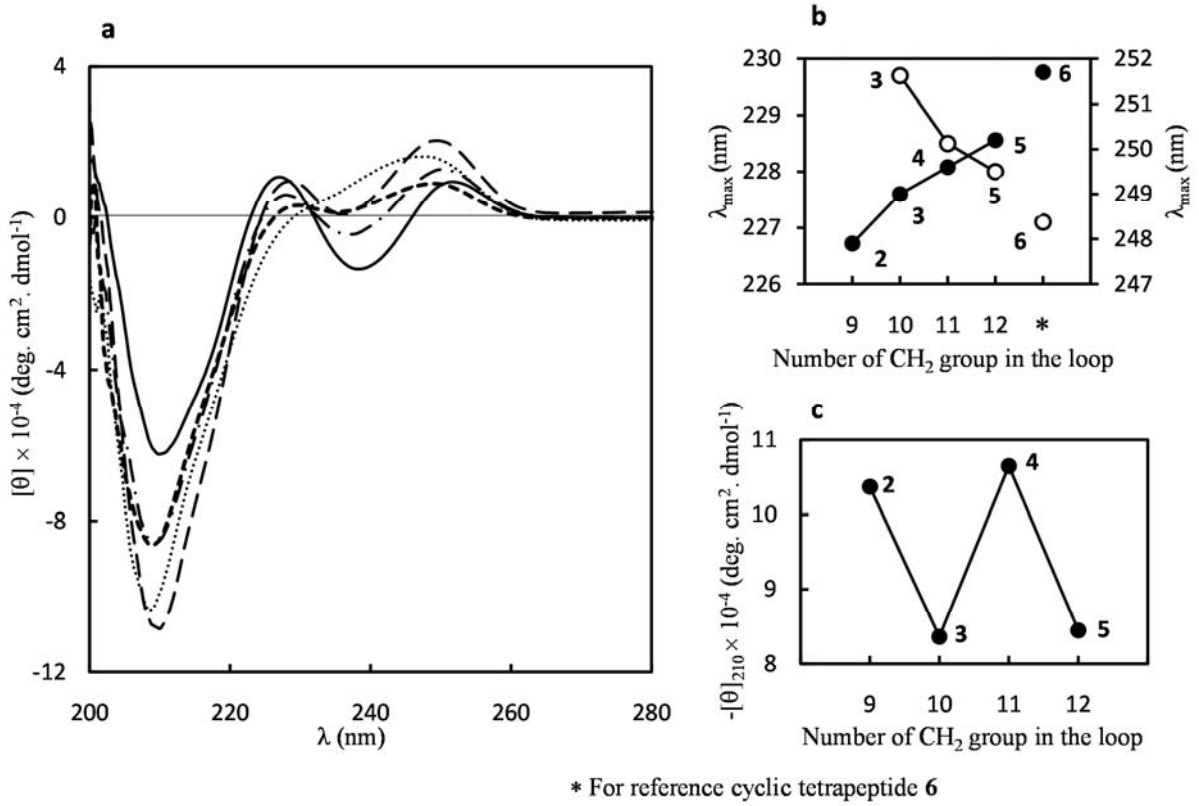


Figure 5. (a) CD spectra of compounds ·····**2**, ----**3**, – – **4**, –·–**5** and —**6** in MeOH (0.1 mM) at 25 ^{0}C; (b) red shift (•) of ellipticity maxima near 228 nm and blue shift (o) of ellipticity maxima near 250 nm with increase in loop length; (c) dependence of ellipticity at the minima near 210 nm on even-odd number of CH_2 group in the loop.

2.7. Conformational studies by CD and NMR. In order to investigate conformational difference, CD measurement of compounds **2-6** was carried out in methanol as solvent. These compounds have one strong negative trough around 210 nm and two weak positive troughs around 228 nm and 250 nm. These similarities on CD spectra suggest that the peptide backbone conformations among these compounds were not so different. However, on details, the ellipticity maxima near 250 nm shift toward longer wavelength (red shift) with increasing loop size and near 228 nm the shift is opposite (blue shift) (Figure 5b). The ellipticity at the

minima near 210 nm depended on even or odd number of CH_2 group in the loop (Figure 5c). These results indicate that there are subtle changes in peptide backbone conformation with aliphatic loop length. The fine change in peptide backbone conformation is also supported by the change in chemical shift of amide protons (Table 2). These results indicate that the aliphatic loop can be used as a regulator to control the cyclic peptide backbone conformation.

Table 2. Chemical shifts of amide protons of compounds **2-6** at 25 ^{0}C in DMSO-d_6.

Residue	δ_H (ppm)				
	2	**3**	**4**	**5**	**6**
L-Asu	8.09	8.35	8.45	8.47	8.43
L-Aen(-)	7.91	7.26	7.27	7.27	7.21
D-Aen(-)	7.18	7.15	7.14	7.17	7.06

2.8. Enzyme inhibition and biological activity. Compounds **2-6** were assayed for HDAC inhibitory activity using HDAC1, HDAC4 and HDAC6 prepared from 293T cells.[47] To examine the inhibitory activity in cell based condition, p21 promoter assay was carried out. The assay methods are schematically represented in Figure 6, and the detail experimental procedures are described in the experimental section. Tricostatin A was used as the reference compound. The results are summarized in Table 3.

All the compounds **2-6** are active in nanomolar range. The activity toward HDAC1 slightly changed with loop length. However, change in activity toward HDAC4 and HDAC6 is not remarkable. All the compounds are specific toward HDAC4 compared with HDAC1 and HDAC6. They are about two times more active toward HDAC4 than HDAC1, and about 50 times more active than HDAC6. HDAC6 is the most frequently expressed isoform which has emerged as a major cytoplasmic deacetylase functions as α-tubulin deacetylase and HSP90 deacetylase thereby regulating cell motility, adhesion and chaperone function. In addition, HDAC6 exerts cellular functions independent from its deacetylase activity.[48] On the other hand, HDAC4 is expressed in tumour tissues, and is rarely found in somatic tissues.[49] Therefore, inhibitors having better selectivity for HDAC4 compare to HDAC6 may be considered more promising as anticancer drug candidates. Compound **3** shows better selectivity (the ratio of IC_{50} value: HDAC6/HDAC4 = 75).

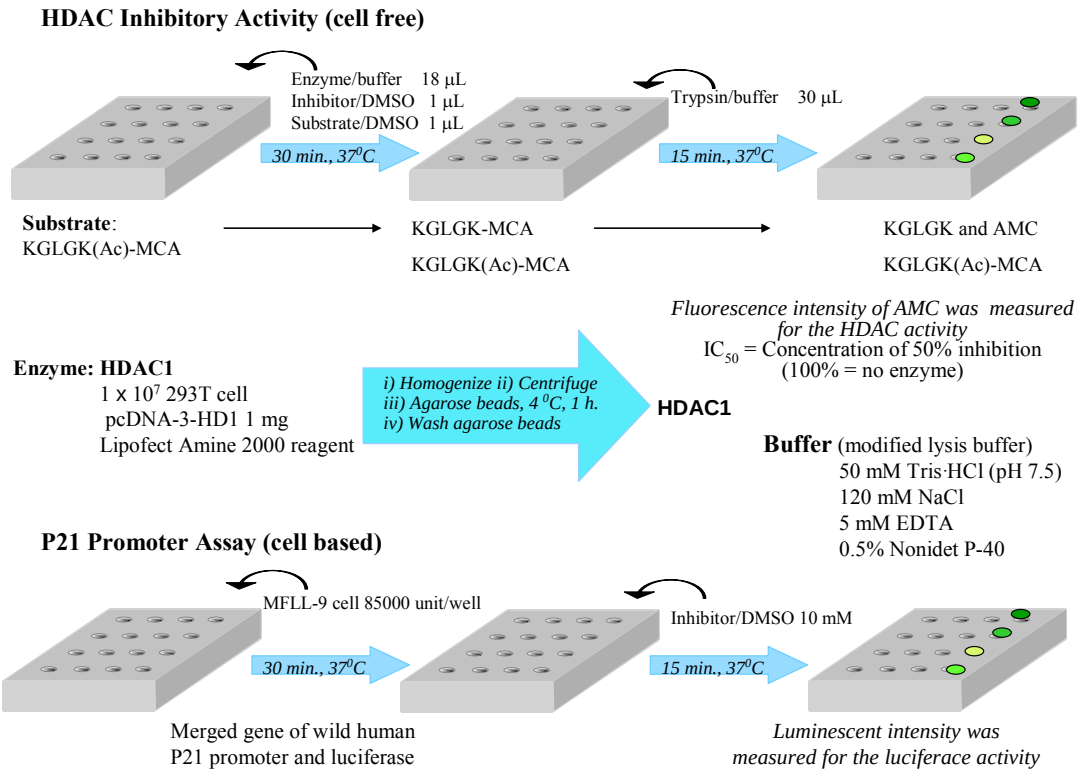


Figure 6. Schematic representation of HDAC inhibitory activity and p21 promoter assay methods.

Table 3. HDAC inhibitory activity and p21 promoter activity data for bicyclic tetrapeptide hydroxamic acids and reference compounds.

Compounds	Loop size	HPLC[a]	IC_{50} (nM)			p21 promoter assay,
		t_R (min)	HDAC1	HDAC4	HDAC6	EC_{1000} (nM)
Tricostatin A	--	--	23	44	65	20
CHAP31[50]	--	--	0.4	3	13	22
2	$-(CH_2)_9-$	4.74	9.1	5.4	330	92
3	$-(CH_2)_{10}-$	6.03	9.1	5.5	410	7.2
4	$-(CH_2)_{11}-$	7.39	11	4.5	280	2.6
5	$-(CH_2)_{12}-$	7.52	13	5.0	240	2.0
6	--	7.51	25	12	340	13

[a]HPLC analysis was performed on a Hitachi instrument equipped with a Chromolith performance RP-18e column (4.6 × 100 mm, Merck). The mobile phases used were A: H_2O with 0.1% TFA, B: CH_3CN with 0.1% TFA using a solvent gradient of A–B over 15 min with a flow rate of 2 mL/min, with detection at 220 nm.

Both CHAP31 and compound **6** are monocyclic. The decrease in *in vitro* biological activity of compound **6** compared to CHAP31 is due to the absence of aromatic side chain. However, in cell based condition compound **6** shows better activity than that of CHAP31. This may be due to the presence of long aliphatic side chains, which facilitate the hydrophobic interactions with cell membrane resulting in better cell permeability. On the other hand, activity of bicyclic compounds **3**, **4** and **5** towards HDAC1 and HDAC4 is comparable to CHAP31. This may be due to the fact that the aliphatic loop facilitates the interaction between cap group and the enzyme surface, and the interaction by the loop is caparable to that of the aromatic ring present in CHAP31. CHAP31 has flexible aromatic side chain of Tyr(Me), which may interact similar extent with the HDAC isoforms resulting in less selectivity. On the other hand, due to the presence of less flexible alkyl loop bicyclic compounds show some selectivity towards HDAC4.

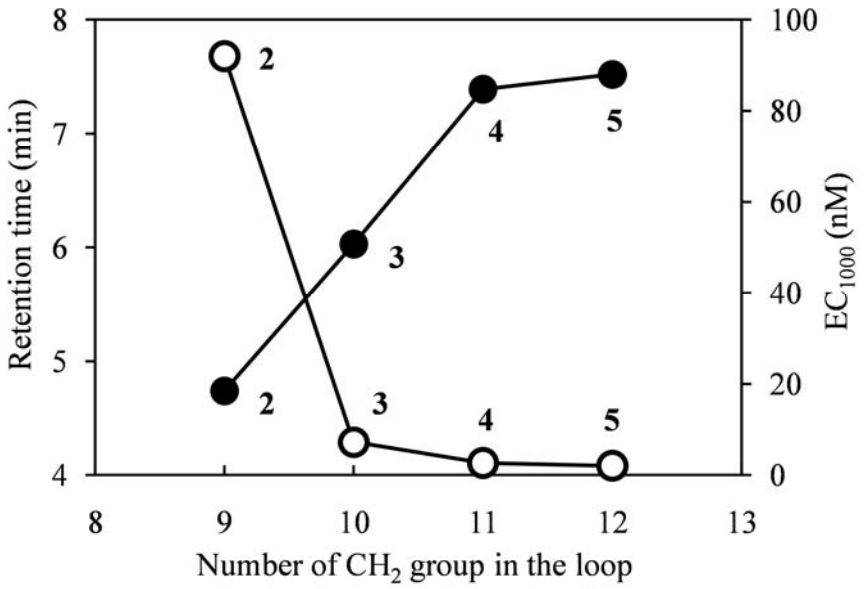


Figure 7. Change in (●) hydrophobicity, and (o) activity in cell based test with loop length of bicyclic tetrapeptides (**2-5**).

An excellent improvement compared to CHAP31 in activity in cell based conditions was observed for most of the compounds. Compounds **3-5** are excellent in p21 promoter-inducing activity. The variation of HPLC retention time, which can be used as a parameter for hydrophobicity, could be correlated with p21 promoter-inducing activity. A linear increase in hydrophobicity was observed when loop length was increased from nine to eleven CH_2 groups. However, increase in hydrophobicity was not remarkable upon further increase in loop length (Figure 7). Similar increasing trend in p21 promoter-inducing activity was

observed. It is assumed that the aliphatic loop helped in penetration through the cell membrane, and resulted in increased activity up to certain loop length. It was found that eleven CH_2 loop length is the optimum for p21 promoter-inducing activity, as no remarkable change in activity was observed for further increase in loop length.

2.9. X-ray crystallographic study. To find out the reason behind the improved activity of the bicyclic tetrapeptides, which do not contain aromatic ring, crystallographic study of compound **3** was performed and the crystal structure was superimposed to that of a potent HDAC inhibitor Ky-2 (contains L-Phe and Aib in its cyclic tetrapeptide framework) (Figure 8b). It was found that both the compounds have similar peptide backbone conformation with four *cis* carbonyl groups. In co-crystal structure of Ky-2 with tHDAC, which has structural similarity with HDAC4, it was found that D84 in the rim region forms three hydrogen bonds with the amide hydrogen (Figure 8a). Due to conformational similarity, D84 also can form similar hydrogen bond with cyclic tetrapeptide framework of compound **3** (Figure 8c). Compound **3** has some extra advantages over Ky-2. The larger hydrophobic loop helps the cap group to bind tightly in the rim region resulting in better inhibitory activity. Moreover, as the loop is less flexible compared to aromatic side chain, the cap group becomes selective to certain HDAC isoforms and results in better selectivity (Table 4).

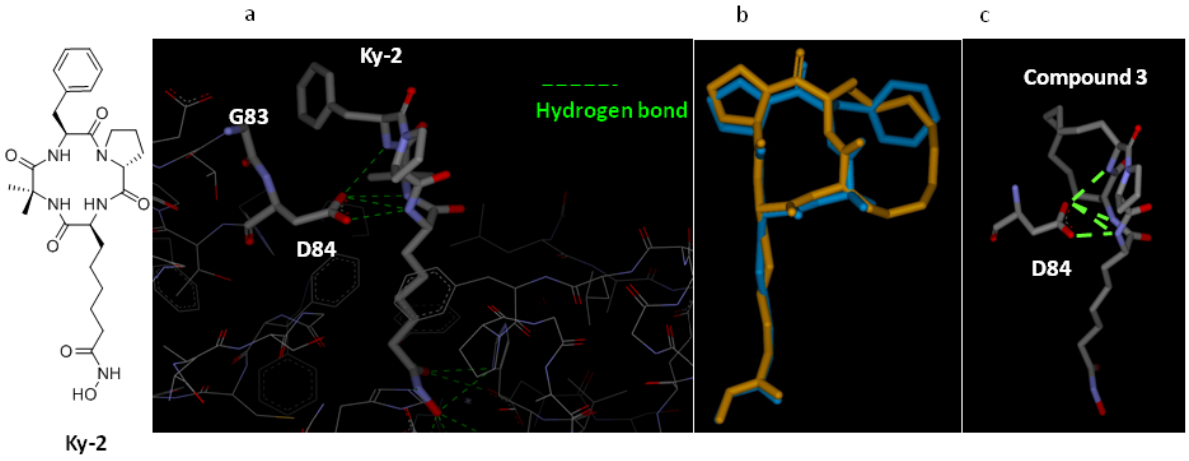


Figure 8. Crystal structures: (a) interaction of Ky-2 with active site of tHDAC; (b) superimposition of Ky-2 and compound **3**; (c) interaction between cyclic tetrapeptide framework of compound **3** with tHDAC D84 through hydrogen bonds.

Table 4. HDAC inhibitory activity and p21 promoter activity data for **Ky-2** and compound **3**.

Compounds	IC_{50} (nM)			HDAC1/	HDAC6/	p21 promoter assay
	HDAC1	HDAC4	HDAC6	HDAC4[a]	HDAC4[a]	EC_{1000} (nM)
Ky-2	18	17	230	1.0	13.5	18
3	9.1	5.5	410	1.7	74.5	7.2

[a]Selectivity toward HDAC4

3. Summary

A series of CHAP31 based bicyclic tetrapeptide hydroxamic acid HDAC inhibitors were designed by changing the aliphatic loop length. First the minimum loop length was determined, and it was found that nine methelene loop length is the minimum for bicyclic tetrapeptides. These inhibitors show potent HDAC inhibitory activity *in vivo* and *in vitro*. They also show some selectivity among the HDAC isoforms. The aliphatic loop length is important, and eleven methelene loop is the optimum for *in vivo* activity. The X-ray crystallographic study reveals that the loop helps in forming such a conformation which is favourable for interaction in the rim region. From the conformational study it was found that the aliphatic loop acts as a regulator to control the cyclic peptide backbone conformation. These results confirm the hypothesis that modification of the cap group of HDAC inhibitors can lead to potent HDAC inhibitors, which may have potential as anticancer agents.

4. Experimental

4.1. General. Unless otherwise noted, all solvents and reagents were reagent grade and used without purification. Flash chromatography was performed using silica gel 60 (230–400 mesh) eluting with solvents as indicated. All compounds were routinely checked by thin layer chromatography (TLC) and/or high performance liquid chromatography (HPLC). TLC was performed on aluminum-backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F_{254}) with spots visualized by UV light or charring. Analytical HPLC was performed on a Hitachi instrument equipped with a chromolith performance RP-18e column (4.6 × 100 mm, Merck). The mobile phases used were A: H_2O with 10% CH_3CN and 0.1% TFA, B: CH_3CN with 0.1% TFA using a solvent gradient of A-B over 15 min with a flow rate of 2 mL/min, with detection at 220 nm. HR FAB-mass spectra were measured on a JEOL JMS-SX 102A instrument. NMR spectra were recorded on a JEOL JNM A500 MHz spectrometer in DMSO-d_6 solutions with reference to TMS. All ^1H shifts are given in parts per million (s = singlet; d

= doublet; t = triplet; m = multiplet). Assignments of proton resonances were confirmed, when possible, by correlated spectroscopy. Ring closing metathesis (RCM) was perform by the aid of benzylidene-bis(tricyclohexylphosphine)dichlororuthenium (Grubbs first generation catalyst). Coupling reactions were performed by standard solution-phase chemistry using dicyclohexyl-carbodiimide (DCC) and 1-hydroxybenzotriazol (HOBt). Peptide cyclization was mediated by *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU).

4.2. Synthesis of Boc-L-2-amino-6-heptenoic acid (Boc-L-Ae7-OH) (7). Metallic sodium (4.0 g, 165 mmol) was dissolved in absolute ethanol (165 mL) and diethyl tert butoxycarbonylaminomalonate (45.42 g, 165 mmol) was added to the solution. The reaction mixture was refluxed for 30 min for the complete removal of the labile hydrogen and then 5-bromo-1-pentene (25 g, 165 mmol) was added and refluxed for 6 h. The reaction mixture was cooled on an ice bath and hydrolysis was carried out by the treatment of 1 M aq NaOH (165 mL, 165 mmol). After selective hydrolysis for 3 h at 0 ^{0}C, ethanol was evaporated and the unreacted 5-bromo-1-pentene was removed by extraction with diethyl ether under basic condition. Then the aqueous solution was extracted with ethyl acetate at pH 3–4, by adding solid citric acid. Ethyl acetate layer was washed with brine and dried over anhydrous $MgSO_4$. Ethyl acetate was evaporated to obtain a colorless oil of monoester monoacid (42.5 g, 82%). HPLC, retention time (t_R) 7.55 min. It was then dissolved in toluene (180 mL) and refluxed for 7 h. The oily ester after evaporation of toluene was then subjected to silicagel column chromatographic purification to get Boc-DL-Ae7-OEt (**13**) as colorless oil (30.8 g, 84%). The resulting oily mass was suspended into a mixture of DMF (114 mL) and water (342 mL) (1:3, v/v) solvent system at 38 ^{0}C using a mechanical stirrer and pH was adjusted at about 7–8 by adding 1 M aq ammonia solution. Then subtilisin Carlsberg from *B. licheniformis* (57 mg, 1 mg enzyme per mmol of substrate) was added and pH was maintained at 7–8 by continuous addition of 1 M aq ammonia solution. The reaction was completed within 4 h. Water and DMF were evaporated and Boc-D-Ae7-OEt (**16**) (15.6 g, 50.5%) was extracted with diethyl ether under basic condition. Then the aqueous solution was extracted with ethyl acetate as described above to get Boc-L-Ae7-OH (**7**) as colorless oil (13.6 g, 49%). HPLC, t_R 7.05 min.

4.3. Synthesis of Boc-L-2-amino-7-octenoic acid (Boc-L-Ae8-OH) (8). Compound **8** was synthesized by using 6-bromo-1-hexene, instead of 5-bromo-1-pentene by the same procedure as described in section **4.2**. HPLC, t_R 8.04 min.

4.4. Synthesis of Boc-L-2-amino-8-nonenoic acid (Boc-L-Ae9-OH) (9). Compound **9** was synthesized by using 7-bromo-1-heptene, instead of 5-bromo-1-pentene by the same procedure as described in section 4.2. HPLC, t_R 8.26 min.

4.5. Synthesis of Boc-D-2-amino-6-heptenoic acid (Boc-D-Ae7-OH) (10). The ester Boc-D-Ae7-OEt (**16**) (15.6 g, 57.5 mmol) was re-resoluted using 5 mg of subtilisin Carlsberg from *B. licheniformis* using the procedure described in Section 4.2. The pure D-ester (14.4 g, 53 mmol) was dissolved in ethanol (60 mL) and hydrolyzed by 1 M NaOH (60 mL, 60 mmol) for 4 h at 0 ^{0}C. After completion of the reaction, ethanol was evaporated and the aqueous solution of Boc-D-Ae7-OH was acidified to pH 3 by solid citric acid. Then the aqueous solution was extracted with ethyl acetate. Finally, ethyl acetate layer was washed with brine, dried over anhydrous MgSO4, and evaporated to obtain Boc-D-Ae7-OH (**10**) as colorless oil (12.75 g, 99%). HPLC, t_R 7.00 min.

4.6. Synthesis of Boc-D-2-amino-7-octenoic acid (Boc-D-Ae8-OH) (11). Compound **11** was synthesized by using Boc-D-Ae8-OEt (**17**) instead of Boc-D-Ae7-OEt (**16**) by the same procedure as described in section 4.5. HPLC, t_R 8.08 min.

4.7. Synthesis of Boc-D-2-amino-8-nonenoic acid (Boc-D-Ae9-OH) (12). Compound **12** was synthesized by using Boc-D-Ae9-OEt (**18**), instead of Boc-D-Ae7-OEt (**16**) by the same procedure as described in section 4.5. HPLC, t_R 8.22 min.

4.8. Synthesis of bicyclic tetrapeptide hydroxamic acid with nine CH_2 loop length (2). To a cooled solution of H-D-Pro-O^{t}Bu (1.71 g, 10 mmol), Boc-L-Ae7-OH (2.67 g, 11 mmol) and HOBt.H_2O (1.53 g, 10 mmol) in dimethylformamide (DMF) (20 mL), DCC (2.47 g, 12 mmol) was added. The mixture was stirred overnight at room temperature. After completion of the reaction, DMF was evaporated and the residue was dissolved in ethyl acetate and successively washed with 10% citric acid, 4% sodium bicarbonate and brine. The ethyl acetate solution was dried over anhydrous $MgSO_4$ and concentrated to remain an oily substance, which was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield Boc-L-Ae7-D-Pro-O^{t}Bu (**24**) (3.30 g, 83%) as oil. The protected dipeptide (1.65 g, 4.1 mmol) was dissolved in 4 M HCl/dioxane (16 mL) and the mixture was kept at room temperature for 40 min. The reaction was monitored by TLC. After completion of the reaction HCl/dioxane was evaporated. The residue was dissolved in EtOAc and washed with saturated Na_2CO_3, dried over anhydrous Na_2CO_3 and EtOAc was evaporated to get H-L-Ae7-D-Pro-O^{t}Bu as heavy oil (0.84 g, 68%). To a cooled solution of H-L-Ae7-D-Pro-O^{t}Bu

(0.84 g, 2.8 mmol), Boc-D-Ae8-OH (0.87 g, 3.4 mmol) and HOBt.H_2O (0.43 g, 2.8 mmol) in DMF (6 mL), DCC (0.70 g, 3.4 mmol) was added and stirred for overnight at room temperature. The product Boc-D-Ae8-L-Ae7-D-Pro-O^{t}Bu (**27**) was obtained in the same manner as described earlier as heavy oil (1.32 g, 88%). HPLC, t_R 7.22 min. To a solution of linear tripeptide Boc-D-Ae8-L-Ae7-D-Pro-O^{t}Bu (**27**) (1.07 g, 2 mmol) in anhydrous and degassed dichloromethane (250 mL), a solution of Grubbs first generation ruthenium catalyst (0.330 g, 0.4 mmol) in anhydrous and degassed dichloromethane (50 mL) was added. The reaction mixture was stirred at room temperature for 48 h. After the completion of reaction, dichloromethane was evaporated and the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield linear tripeptide with fused cycle as foam which on catalytic hydrogenation in presence of Pd-C (0.100 g) in AcOH (10 mL) yield compound **31** (0.590 g, 58%). HPLC, t_R 7.47 min. The protected tripeptide **31** (0.570 g, 1.12 mmol) was dissolved in 4 M HCl/dioxane (10 mL) and the mixture was kept at room temperature for 30 min. The reaction was monitored by TLC. After completion of the reaction HCl/dioxane was evaporated. The residue was dissolved in EtOAc and washed with saturated Na_2CO_3, dried over anhydrous Na_2CO_3 and EtOAc was evaporated to get tripeptide free amine (0.362 g, 79%). To a cooled solution of the free amine (0.362 g, 0.88 mmol), Boc-L-Asu(OBzl)-OH (0.400 g, 1.06 mmol) and HOBt.H_2O (0.136 g, 0.88 mmol) in DMF (2 mL), DCC (0.218 g, 1.06 mmol) was added and stirred for overnight at room temperature. The product linear tetrapeptide (**35**) (0.500 g, 74%) was obtained in the same manner as described earlier as foam. HPLC, t_R 9.21 min. The protected tetrapeptide **35** (0.420 g, 0.54 mmol) was dissolved in TFA (3 mL) at 0 ^{0}C and kept for 3 hours. After evaporation of TFA, the residue was solidified using ether and petroleum ether to yield TFA salt of the linear tetrapeptide (0.380 g, 100%). To DMF solvent (540 mL), the TFA salt (0.380 g, 0.54 mmol), HATU (0.308 g, 0.82 mmol), and DIEA (0.24 mL, 1.36 mmol) were added in separate five portions in every 30 min with stirring, for the cyclization reaction. After completion of the reaction, DMF was evaporated under vacuo, the residue was dissolved in ethyl acetate and washed with citric acid (10%) solution, sodium bicarbonate (4%) solution, and brine, respectively. It was then dried over anhydrous $MgSO_4$ and filtered. After evaporation of ethyl acetate, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield the bicyclic tetrapeptide **39** (0.048 g , 15%). HPLC, t_R 6.12 min. Compound **39** (0.044 g, 0.074 mmol) was dissolved in acetic acid (5 mL) and Pd-C (0.050 g) was added. The mixture was stirred under H_2 for 10 hours. After filtration of Pd-C, acetic acid was evaporated to yield bicyclic tetrapeptide carboxylic acid (0.038 g, 100%). HPLC, t_R

4.36 min. The bicyclic tetrapeptide carboxylic acid (0.038 g, 0.074 mmol) was dissolved in DMF (0.5 mL) at 0 ^{0}C, and O-Benzylhydroxylamine hydrochloride (0.024 g, 0.148 mmol), HOBt.H_2O (0.012 g, 0.074 mmol), triethylamine (0.021 mL, 0.148 mmol) and DCC (0.023 g, 0.111 mmol) were added. The mixture was stirred for 24 h. The product (0.027 g, 60%) was obtained was dissolved in acetic acid (1 mL), and Pd-$BaSO_4$ (0.020 g) was added. The mixture was stirred under H_2 for 24 hours. After filtration of Pd-$BaSO_4$, acetic acid was evaporated and crystallized with ether to yield compound **2** (0.010 g, 45%). ^{1}H NMR (500 MHz, DMSO-d_6): δ_H 1.15-1.40 (m, 18H), 1.47-1.78 (m, 10H), 1.83-1.98 (m, 4H), 3.60 (dd, *J* = 17.5, 8.7 Hz, 1H), 3.85 (ddd, *J* = 9.7, 9.5, 3.5 Hz, 1H), 4.06 (m, 1H), 4.13 (m, 1H), 4.20 (dd, *J* = 17.3, 8 Hz, 1H), 4.61 (d, *J* = 7.9 Hz, 1H), 7.18 (d, *J* = 9.5, 1H), 7.91 (s, 1H), 8.09 (s, 1H), 8.66 (s, 1H), 10.36 (s, 1H); HR FAB-MS $[M+Na]^+$ 544.3141 for $C_{26}H_{43}N_5O_6Na$ (calcd 544.3111).

4.9. Synthesis of bicyclic tetrapeptide hydroxamic acid with ten CH_2 loop length (3). To a cooled solution of H-D-Pro-O^{t}Bu (0.855 g, 5 mmol), Boc-L-Ae8-OH (1.29 g, 5 mmol) and HOBt.H_2O (0.766 g, 5 mmol) DMF (10 mL), DCC (1.24 g, 6 mmol) was added. The mixture was stirred overnight at room temperature. After completion of the reaction, DMF was evaporated and the residue was dissolved in ethyl acetate and successively washed with 10% citric acid, 4% sodium bicarbonate and brine. The ethyl acetate solution was dried over anhydrous $MgSO_4$ and concentrated to remain an oily substance, which was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield Boc-L-Ae8-D-Pro-O^{t}Bu (**25**) (1.60 g, 78%) as oil. The protected dipeptide (1.55 g, 3.8 mmol) was dissolved in 4 M HCl/dioxane (16 mL) and the mixture was kept at room temperature for 30 min. The reaction was monitored by TLC. After completion of the reaction HCl/dioxane was evaporated. The residue was dissolved in EtOAc and washed with saturated Na_2CO_3, dried over anhydrous Na_2CO_3 and EtOAc was evaporated to get H-L-Ae8-D-Pro-O^{t}Bu as heavy oil (0.930 g, 79%). To a cooled solution of H-L-Ae8-D-Pro-O^{t}Bu (0.930 g, 3 mmol), Boc-D-Ae8-OH (0.940 g, 3.3 mmol) and HOBt.H_2O (0.460 g, 3 mmol) in DMF (6 mL), DCC (0.742 g, 3.6 mmol) was added and was stirred for overnight at room temperature. The product Boc-D-Ae8-L-Ae8-D-Pro-O^{t}Bu (**28**) was obtained in the same manner as described earlier as heavy oil (1.40 g, 84%). HPLC, t_R 8.02 min. To a solution of linear tripeptide Boc-D-Ae8-L-Ae8-D-Pro-O^{t}Bu (**28**) (1.10 g, 2 mmol) in anhydrous and degassed dichloromethane (250 mL), a solution of Grubbs first generation ruthenium catalyst (0.330 g, 0.4 mmol) in anhydrous and degassed dichloromethane (50 mL) was added. The reaction mixture was stirred at room

temperature for 48 h. After the completion of reaction, dichloromethane was evaporated and the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield linear tripeptide with fused cycle as foam which on catalytic hydrogenation in presence of Pd-C (0.100 g) in AcOH (10 mL) yield compound **32** (0.920 g, 87%). HPLC, t_R 7.96 min. The protected tripeptide **32** (0.530 g, 1 mmol) was dissolved in 4 M HCl/dioxane (5 mL) and the mixture was kept at room temperature for 30 min. The reaction was monitored by TLC. After completion of the reaction HCl/dioxane was evaporated. The residue was dissolved in EtOAc and washed with saturated Na_2CO_3, dried over anhydrous Na_2CO_3 and EtOAc was evaporated to get tripeptide free amine (0.310 g, 73%). To a cooled solution of the free amine (0.310 g, 0.73 mmol), Boc-L-Asu(OBzl)-OH (0.295 g, 0.78 mmol) and HOBt.H_2O (0.112 g, 0.73 mmol) in DMF (2 mL), DCC (0.161 g, 0.78 mmol) was added and stirred for overnight at room temperature. The product linear tetrapeptide (**36**) (0.450 g, 79%) was obtained in the same manner as described earlier as foam. HPLC, t_R 10.42 min. The protected tetrapeptide **36** (0.445 g, 0.57 mmol) was dissolved in TFA (3 mL) at 0 ^{0}C and kept for 3 hours. After evaporation of TFA, the residue was solidified using ether and petroleum ether to yield TFA salt of the linear tetrapeptide (0.425 g, 100%). To DMF solvent (570 mL), the TFA salt (0.425 g, 0.57 mmol), HATU (0.326 g, 0.86 mmol), and DIEA (0.24 mL, 1.42 mmol) were added in separate five portions in every 30 min with stirring, for the cyclization reaction. After completion of the reaction, DMF was evaporated under vacuum; the residue was dissolved in ethyl acetate and washed with citric acid (10%) solution, sodium bicarbonate (4%) solution, and brine, respectively. It was then dried over anhydrous $MgSO_4$ and filtered. After evaporation of ethyl acetate, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield the bicyclic tetrapeptide **40** (0.143 g , 41%). HPLC, retention time 7.85 min. Compound **40** (0.125 g, 0.2 mmol) was dissolved in acetic acid (5 mL) and Pd-C (0.050 g) was added. The mixture was stirred under H_2 for 10 hours. After filtration of Pd-C, acetic acid was evaporated to yield bicyclic tetrapeptide carboxylic acid (0.104 g, 100%). The bicyclic tetrapeptide carboxylic acid (0.090 g, 0.17 mmol) was dissolved in DMF (0.5 mL) at 0 ^{0}C, and O-Benzylhydroxylamine hydrochloride (0.041 g, 0.26 mmol), HOBt.H_2O (0.026 g, 0.17 mmol), triethylamine (0.037 mL, 0.26 mmol) and DCC (0.054 g, 0.26 mmol) were added. The mixture was stirred for 24 h. The product (0.062 g, 60%) obtained was dissolved in acetic acid (1 mL), and Pd-$BaSO_4$ (0.020 g) was added. The mixture was stirred under H_2 for 24 hours. After filtration of Pd-$BaSO_4$, acetic acid was evaporated and crystallized with ether to yield compound **3** (0.036 g, 67%). ^{1}H NMR (500 MHz, DMSO-d_6, 40 ^{0}C): δ_H 1.14-1.28 (m,

16H), 1.31-1.38 (m, 4H), 1.42-1.54 (m, 6H), 1.64-1.79 (m, 4H), 1.85-2.00 (m, 4H), 3.38-3.45 (m, 2H), 3.57 (dd, *J* = 18, 8.2 Hz, 1H), 3.92 (ddd, *J* = 9.5, 9.5, 3.5 Hz, 1H), 4.21 (m, 1H), 4.44 (m, 1H), 4.58 (d, *J* = 7.9 Hz, 1H), 4.70 (m, 1H), 7.13 (d, *J* = 9.0 Hz, 1H), 7.26 (d, *J* = 10.1 Hz, 1H), 8.29 (d, *J* = 9.1 Hz, 1H), 8.64 (s, 1H), 10.33 (s, 1H); HR FAB-MS $[M+Na]^+$ 558.3263 for $C_{27}H_{45}N_5O_6Na$ (calcd 558.3268).

4.10. Synthesis of bicyclic tetrapeptide hydroxamic acid with eleven CH_2 loop length (4). To a cooled solution of H-D-Pro-O^{t}Bu (1.71 g, 10 mmol), Boc-L-Ae9-OH (2.71 g, 10 mmol) and HOBt·H_2O (1.53 g, 10 mmol) in dimethylformamide (DMF) (20 mL), DCC (2.47 g, 12 mmol) was added. The mixture was stirred overnight at room temperature. After completion of the reaction, DMF was evaporated and the residue was dissolved in ethyl acetate and successively washed with 10% citric acid, 4% sodium bicarbonate and brine. The ethyl acetate solution was dried over anhydrous $MgSO_4$ and concentrated to remain an oily substance, which was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield Boc-L-Ae9-D-Pro-O^{t}Bu (**26**) (3.60 g, 85%) as oil. The protected dipeptide (1.80 g, 4.25 mmol) was dissolved in 4 M HCl/dioxane (20 mL) and the mixture was kept at room temperature for 35 min. The reaction was monitored by TLC. After completion of the reaction HCl/dioxane was evaporated. The residue was dissolved in EtOAc and washed with saturated Na_2CO_3, dried over anhydrous Na_2CO_3 and EtOAc was evaporated to get H-L-Ae9-D-Pro-O^{t}Bu as heavy oil (1.16 g, 84%). To a cooled solution of H-L-Ae9-D-Pro-O^{t}Bu (1.16 g, 3.58 mmol), Boc-D-Ae8-OH (1 g, 3.94 mmol) and HOBt.H_2O (0.55 g, 3.58 mmol) in DMF (8 mL), DCC (0.88 g, 4.30 mmol) was added and stirred for overnight at room temperature. The product Boc-D-Ae8-L-Ae9-D-Pro-O^{t}Bu (**29**) was obtained in the same manner as described earlier as heavy oil (1.57 g, 78%), HPLC, retention time 8.84 min. To a solution of linear tripeptide Boc-D-Ae8-L-Ae9-D-Pro-O^{t}Bu (**29**) (1.13 g, 2 mmol) in anhydrous and degassed dichloromethane (250 mL), a solution of Grubbs first generation ruthenium catalyst (0.330 g, 0.4 mmol) in anhydrous and degassed dichloromethane (50 mL) was added. The reaction mixture was stirred at room temperature for 48 h. After the completion of reaction, dichloromethane was evaporated and the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield linear tripeptide with fused cycle as foam which on catalytic hydrogenation in presence of Pd-C (0.100 g) in AcOH (10 mL) yielded compound **33** (0.825 g, 87%). HPLC, t_R 7.92 min. The protected tripeptide **33** (0.815 g, 1.5 mmol) was dissolved in 4 M HCl/dioxane (15 mL) and the mixture was kept at room temperature for 30 min. The reaction

was monitored by TLC. After completion of the reaction HCl/dioxane was evaporated. The residue was dissolved in EtOAc and washed with saturated Na_2CO_3, dried over anhydrous Na_2CO_3 and EtOAc was evaporated to get tripeptide free amine (0.600 g, 92%). To a cooled solution of the free amine (0.600 g, 1.37 mmol), Boc-L-Asu(OBzl)-OH (0.571 g, 1.51 mmol) and HOBt.H_2O (0.210 g, 1.37 mmol) in DMF (5 mL), DCC (0.339 g, 1.64 mmol) was added and stirred for overnight at room temperature. The product linear tetrapeptide (**37**) (0.890 g, 81%) was obtained in the same manner as described earlier as foam. HPLC, t_R 10.16 min. The protected tetrapeptide **37** (0.825 g, 1.03 mmol) was dissolved in TFA (5 mL) at 0 ^{0}C and kept for 3 hours. After evaporation of TFA, the residue was solidified using ether and petroleum ether to yield TFA salt of the linear tetrapeptide (0.745 g, 98%). To DMF solvent (500 mL), the TFA salt (0.745 g, 1.01 mmol), HATU (0.576 g, 1.52 mmol), and DIEA (0.44 mL, 2.53 mmol) were added in separate five portions in every 30 min with stirring, for the cyclization reaction. After completion of the reaction, DMF was evaporated under vacuo, the residue was dissolved in ethyl acetate and washed with citric acid (10%) solution, sodium bicarbonate (4%) solution, and brine, respectively. It was then dried over anhydrous $MgSO_4$ and filtered. After evaporation of ethyl acetate, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield the bicyclic tetrapeptide **41** (0.425 g , 67%). HPLC, t_R 7.80 min. Compound **41** (0.415 g, 0.66 mmol) was dissolved in acetic acid (5 mL) and Pd-C (0.050 g) was added. The mixture was stirred under H_2 for 10 hours. After filtration of Pd-C, acetic acid was evaporated to yield bicyclic tetrapeptide carboxylic acid (0.355 g, 100%). HPLC, rt 5.61 min. The bicyclic tetrapeptide carboxylic acid (0.350 g, 0.66 mmol) was dissolved in DMF (2 mL) at 0 ^{0}C, and O-Benzylhydroxylamine hydrochloride (0.157 g, 0.98 mmol), HOBt.H_2O (0.101 g, 0.66 mmol), triethylamine (0.137 mL, 0.98 mmol) and DCC (0.202 g, 0.98 mmol) were added. The mixture was stirred for 24 h. The product (0.310 g, 74%) was dissolved in acetic acid (5 mL), and Pd-$BaSO_4$ (0.200 g) was added. The mixture was stirred under H_2 for 24 hours. After filtration of Pd-$BaSO_4$, acetic acid was evaporated and crystallized with ether to yield compound **4** (0.195 g, 75%). ^{1}H NMR (500 MHz, DMSO-d_6 30 ^{0}C): δ_H 1.10-1.99 (m, 32H), 2.50 (m, 4H), 3.57 (dd, *J* = 18.2, 8.4 Hz, 1H), 3.86 (ddd, *J* = 9.5, 9.5, 3.8 Hz, 1H), 4.19 (dd, *J* = 16.7, 7.7 Hz, 1H), 4.41 (m, 1H), 4.53 (d, *J* = 7.5 Hz, 1H), 4.70 (d, *J* = 7.6 Hz, 1H), 7.13 (d, *J* = 9.1 Hz, 1H), 7.23 (d, *J* = 9.8 Hz, 1H), 8.42 (d, *J* = 9.2 Hz, 1H), 8.61 (s, 1H), 10.31 (s, 1H); HR FAB-MS $[M+H]^+$ 550.3603 for $C_{28}H_{48}N_5O_6$ (calcd 550.3605).

4.11. Synthesis of bicyclic tetrapeptide hydroxamic acid with twelve CH_2 loop length (5). Compound **5** was synthesized following the same procedure as described in case of compound **4** using Boc-D-Ae9-OH instead of Boc-D-Ae8-OH. ^{1}H NMR (500 MHz, DMSO-d_6 30 ^{0}C): δ_H 1.17-2.10 (m, 34H), 2.50 (m, 4H), 3.41 (m, 1H), 3.58 (dd, *J* = 17.7, 8.7 Hz, 1H), 3.74 (m, 1H), 3.81 (ddd, *J* = 9.7, 9.5, 3.5 Hz, 1H), 4.06 (m, 1H), 4.19 (dd, *J* = 16.5, 7.5 Hz, 1H), 4.34 (t, *J* = 10.7 Hz, 1H), 4.55 (d, *J* = 8.2 Hz, 1H), 4.70 (d, *J* = 7.3 Hz, 1H), 7.16 (d, *J* = 9.5 Hz, 1H), 7.23 (d, *J* = 10.0 Hz, 1H), 8.44 (d, *J* = 9.2 Hz, 1H), 8.62 (s, 1H), 10.34 (s, 1H); HR FAB-MS $[M+H]^+$ 564.3691 for $C_{29}H_{50}N_5O_6$ (calcd 564.3761).

4.12. Synthesis of cyclic tetrapeptide hydroxamic acid (6). To a cooled solution of H-L-Ae7-D-Pro-O^{t}Bu (0.84 g, 2.8 mmol), Boc-D-Ae7-OH (0.83 g, 3.4 mmol) and HOBt.H_2O (0.43 g, 2.8 mmol) in DMF (6 mL), DCC (0.700 g, 3.4 mmol) was added and stirred for overnight at room temperature. The product Boc-D-Ae7-L-Ae7-D-Pro-O^{t}Bu (**43**) was obtained in the same manner as described earlier as heavy oil (1.3 g, 88%). HPLC, t_R 6.86 min. Boc-D-Ae7-L-Ae7-D-Pro-O^{t}Bu (**43**) (1.3 g, 2.5 mmol) was dissolved in 4 M HCl/dioxane (10 mL) and the mixture was kept at room temperature for 30 min. The reaction was monitored by TLC. After completion of the reaction HCl/ dioxane was evaporated. The residue was dissolved in EtOAc and washed with saturated Na_2CO_3, dried over anhydrous Na_2CO_3 and EtOAc was evaporated to get tripeptide free amine (0.900 g, 85%). To a cooled solution of the free amine (0.900 g, 2.13 mmol), Boc-L-Asu(OBzl)-OH (0.810 g, 2.13 mmol) and HOBt.H_2O (0.326 g, 2.13 mmol) in DMF (5 mL), DCC (0.526 g, 2.55 mmol) was added and stirred for overnight at room temperature. The product Boc-L-Asu(OBzl)-D-Ae7-L-Ae7-D-Pro-O^{t}Bu (**44**) (1.1 g, 66%) was obtained in the same manner as described earlier as foam. HPLC, t_R 9.41 min. Compound **44** (1.05 g, 1.35 mmol) was dissolved in TFA (5 mL) at 0 ^{0}C and kept for 3 hours. After evaporation of TFA, the residue was solidified using ether and petroleum ether to yield TFA salt of the linear tetrapeptide (0.820 g, 84%). To DMF solvent (125 mL), the TFA salt (0.820 g, 1.13 mmol), HATU (0.650 g, 1.71 mmol), and DIEA (0.50 mL, 2.85 mmol) were added in separate five portions in every 30 min with stirring, for the cyclization reaction. After completion of the reaction, DMF was evaporated under vacuo, the residue was dissolved in ethyl acetate and washed with citric acid (10%) solution, sodium bicarbonate (4%) solution, and brine, respectively. It was then dried over anhydrous $MgSO_4$ and filtered. After evaporation of ethyl acetate, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield *cyclo*(-L-Asu(OBzl)-D-Ae7-L-Ae7-D-Pro-) (**45**) (0.450 g , 65%). HPLC, t_R 8.17 min. Compound **45**

(0.330 g, 0.54 mmol) was dissolved in acetic acid (5 mL) and Pd-C (100 mg) was added. The mixture was stirred under H_2 for 20 hours. After filtration of Pd-C, acetic acid was evaporated to yield compound **46** (0.332 g, 100%). HPLC, t_R 5.17 min. Compound **46** (0.332 g, 0.54 mmol) was dissolved in DMF (2 mL) at 0 ^{0}C, and O-Benzylhydroxylamine hydrochloride (0.130 g, 0.81 mmol), HOBt.H_2O (0.083 mg, 0.81 mmol), triethylamine (0.117 mL, 0.81 mmol) and DCC (0.167 g, 0.81 mmol) were added. The mixture was stirred for 24 h. After completion of the reaction, DMF was evaporated under vacuum; the residue was dissolved in ethyl acetate and washed with citric acid (10%) solution, sodium bicarbonate (4%) solution, and brine, respectively. It was then dried over anhydrous $MgSO_4$ and filtered. After evaporation of ethyl acetate, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield cyclic tetrapepetide benzyl ester (0.330 g, 97%) which was dissolved in acetic acid (5 mL), and Pd-$BaSO_4$ (0.200 g) was added. The mixture was stirred under H_2 for 24 hours. After filtration of Pd-$BaSO_4$, acetic acid was evaporated and crystallized with ether to yield compound **6** (0.250 g, 91%). ^{1}H NMR (500 MHz, DMSO-d_6) δ_H 0.81-0.88 (m, 6H),1.18-1.30 (m, 18H), 1.44 (m, 4H), 1.62-1.74 (m, 4H), 1.87-1.97 (m, 4H), 3.52 (dd, *J* = 18.5, 8.5 Hz, 1H), 3.77 (ddd, *J* = 10, 9.5, 4 Hz, 1H), 4.19-4.27 (m, 2H), 4.63 (dd, *J* = 17.3, 8 Hz, 1H), 4.71 (d, *J* = 7.3 Hz, 1H), 7.06 (d, *J* = 10.1 Hz, 1H), 7.21 (d, *J* = 9.5 Hz, 1H), 8.43 (d, *J* = 9.2 Hz, 1H), 8.63 (s, 1H), 10.31 (s, 1H); HR FAB-MS $[M+H]^+$ 538.3543 for $C_{27}H_{48}N_5O_6$ (calcd 538.3605).

4.13. HDACs preparation and enzyme activity assay. In a 100-mm dish, 293T cells (1-2 × 10^6) were grown for 24 h and transiently transfected with 10 g each of the vector pcDNA3-HDAC1 for human HDAC1, pcDNA3-HDAC4 for human HDAC4, or pcDNA3-mHDA2/HDAC6 for mouse HDAC6, using the LipofectAMINE2000 reagent (Invitrogen). After successive cultivation in DMEM for 24 h, the cells were washed with PBS and lysed by sonication in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 5 mM EDTA, and 0.5% NP40. The soluble fraction collected by micro centrifugation was precleared by incubation with protein A/G plus agarose beads (Santa Cruz Biotechnologies, Inc.). After the cleared supernatant had been incubated for 1 h at 4˚C with 4 g of an anti-FLAG M2 antibody (Sigma-Aldrich Inc.) for HDAC1, HDAC4 and HDAC6, the agarose beads were washed three times with lysis buffer and once with histone deacetylase buffer consisting of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 10% glycerol. The bound proteins were released from the immune complex by incubation for 1 h at 4˚C with 40 g of the FLAG peptide (Sigma-Aldrich Inc.) in histone deacetylase buffer (200 mL). The supernatant was collected by

centrifugation. For the enzyme assay, 10 μL of the enzyme fraction was added to 1 μL of fluorescent substrate (2 mM Ac-KGLGK(Ac)-MCA) and 9 μL of histone deacetylase buffer, and the mixture was incubated at 37˚C for 30 min. The reaction was stopped by the addition of 30 μL of tripsin (20 mg/mL) and incubated at 37 ˚C for 15 min. The released amino methyl coumarin (AMC) was measured using a fluorescence plate reader. The 50% inhibitory concentrations (IC_{50}) were determined as the means with SD calculated from at least three independent dose response curves.

4.14. The p21 promoter assay. A luciferase reporter plasmid (pGW-FL) was constructed by cloning the 2.4 kb genomic fragment containing the transcription start site into *Hin*dIII and *Sma*I sites of the pGL3-Basic plasmid (Promega Co., Madison, WI). Mv1Lu (mink lung epitherial cell line) cells were transfected with the pGW-FL and a phagemid expressing neomycin/kanamycin resistance gene (pBK-CMV, Stratagene, La Jolla, CA) with the Lipofectamine reagent (Life Technology, Rockville, MD USA). After the transfected cells had been selected by 400 μg/mL Geneticin (G418, Life Technology), colonies formed were isolated. One of the clones was selected and named MFLL-9. MFLL-9 expressed a low level of luciferase, of which activity was enhanced by TSA in a dose-dependent manner. MFLL-9 cells (1 × 105) cultured in a 96-well multi-well plate for 6 h were incubated for 18 h in the medium containing various concentrations of drugs. The luciferase activity of each cell lysate was measured with a LucLite luciferase Reporter Gene Assay Kit (Packard Instrument Co., Meriden, CT) and recorded with a Luminescencer-JNR luminometer (ATTO, Tokyo, Japan). Data were normalized to the protein concentration in cell lysates. Concentrations at which a drug induces the luciferase activity 10-fold higher than the basal level are presented as the 1000% effective concentration 1000% (EC_{1000}). The human wild-type p21 promoter luciferase fusion plasmid, WWP-Luc, was a kind gift from Dr. B. Vogelstein.

4.15. Circular dichroism measurement. CD spectra were recorded on a JASCO J-820 spectropolarimeter (Tokyo, Japan) using a quartz cell of 1 mm light path length at room temperature. Peptide solutions (0.1 mM) were prepared in methanol and CD spectra were recorded in terms of molar ellipticity, $[\theta]_M$ ($deg \times cm^2 \times dmol^{-1}$).

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Chapter 3

Molecular Design of Bicyclic Tetrapeptide Histone Deacetylase Inhibitors by Changing Aliphatic Loop Position

1. Introduction

Dynamic acetylation and deacetylation of the ε-amino groups of lysines at *N*-terminal tails of core histones are balanced by histone acetyl transferase (HAT) and histone deacetylase (HDAC) enzymes.[1-3] Imbalance in histone acetylation and deacetylation can lead to transcriptional deregulation of genes that are involved in the control of cell cycle progression, differentiation, and/or apoptosis. Aberrant histone deacetylation caused by the disrupted HAT activity or abnormal recruitment of HDACs has been related to carcenogenesis.[4-6] Inhibition of HDAC enzymatic activity is expected to induce re-expression of differentiation-inducing genes. Therefore, design and synthesis of novel HDAC inhibitors is a promising field for research. As described in chapter 1 and 2, several efforts have been made to develop potent and selective HDAC inhibitors. However, isoform selective HDAC inhibitor not yet achieved. The sequence diversity in surface binding region of HDAC enzyme suggests that modification at cap group portion in inhibitor can provide better specificity. Specificity is a necessary condition for an inhibitor to be useful as a drug with high pharmacokinetic properties and oral bioavailability. Scientists are now concentrating their research on this matter. Very potent HDAC inhibitors including non-peptides and cyclic tetrapeptides, bearing hydroxamic acid or epoxy ketones inhibit with very low selectivity among HDAC isoforms. Therefore, a lot of effort is needed to develop selective HDAC inhibitors.

In the present study, a series of bicyclic tetrapeptides have been designed and synthesized to demonstrate the effect of aliphatic loop position on the biological activity of bicyclic tetrapeptide HDAC inhibitors. First, Cyl-1, trapoxin B and HC-toxin I (Figure 1) based bicyclic tetrapeptide hydroxamic acids were designed for synthesis. Trapoxin B and HC-toxin I based bicyclic tetrapeptides with ten methylene loop were successfully synthesized, but the synthesis of Cyl-1 based bicyclic tetrapeptide was failed in the peptide cyclization step. Cyl-1 based cyclic tetrapeptide hydroxamic acid CHAP30[7] was successfully synthesized. It was assumed that due to the steric hindrance imposed by the aliphatic loop, cyclization of tetrapeptide with unfavorable sequence became impossible. By changing the sequence, CHAP31 based bicyclic tetrapeptide with ten methylene loop was successfully synthesized.

Corresponding cyclic tetrapeptides with open chains were also synthesized for comparison in activity and conformation between cyclic and bicyclic tetrapeptide HDAC inhibitors. The author herein describes the account on synthesis of bicyclic peptides using RCM reaction, a brief description of interesting biological results, and conformational change of peptide backbone caused by the aliphatic loop.

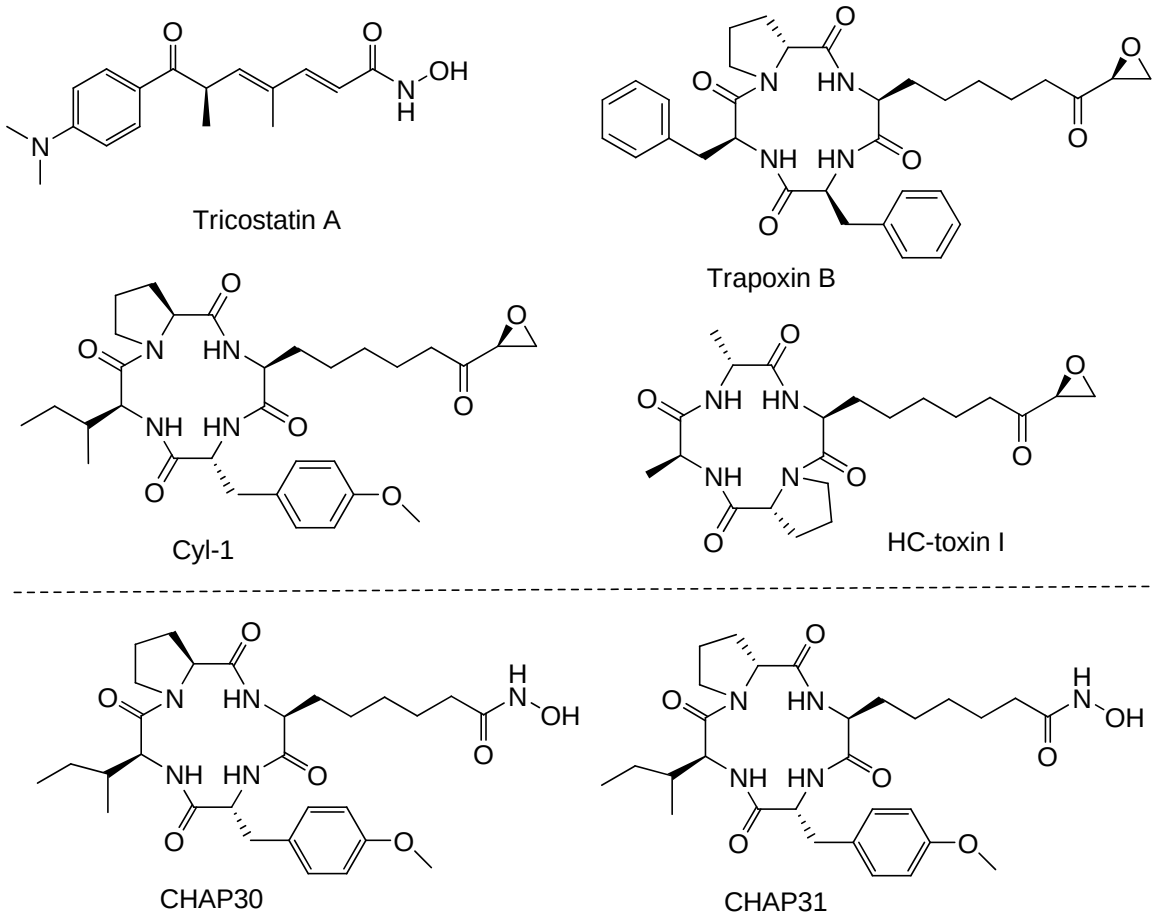


Figure 1. Natural and conjugated cyclic peptide HDAC inhibitors.

2. Results and discussion

2.1. Design of bicyclic compounds. The author design and synthesize CHAP31, trapoxin B and HC-toxin I based bicyclic tetrapeptide hydroxamic acids (**1-3**) (Figur 2) as HDAC inhibitors. The sequence and configuration of amino acids in CHAP31, trapoxin B and HC-toxin I were considered as the basis for designing the three bicyclic peptide HDAC inhibitors. L-2-amino-7-octenoate (L-Ae8) and D/L-2-amino-7-octenoate (D/L-Ae8) were incorporated in the sequence, and the terminal alkenes of two Ae8 were fused by the aid of Grubbs first generation Ru catalyst.

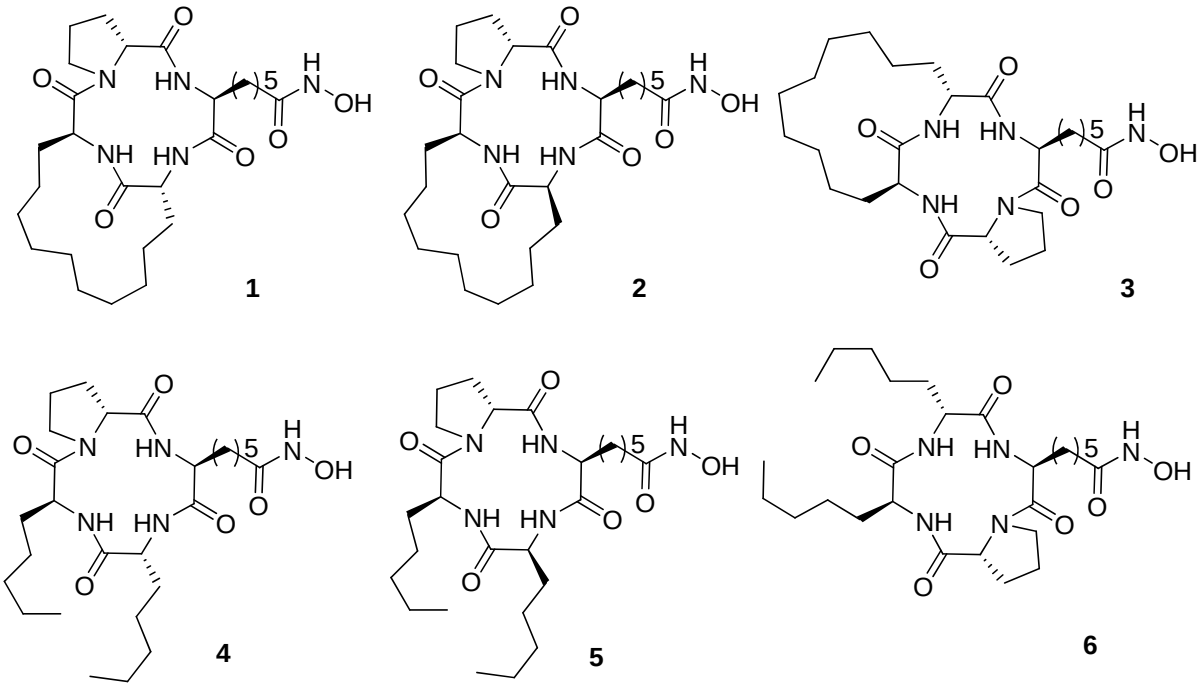
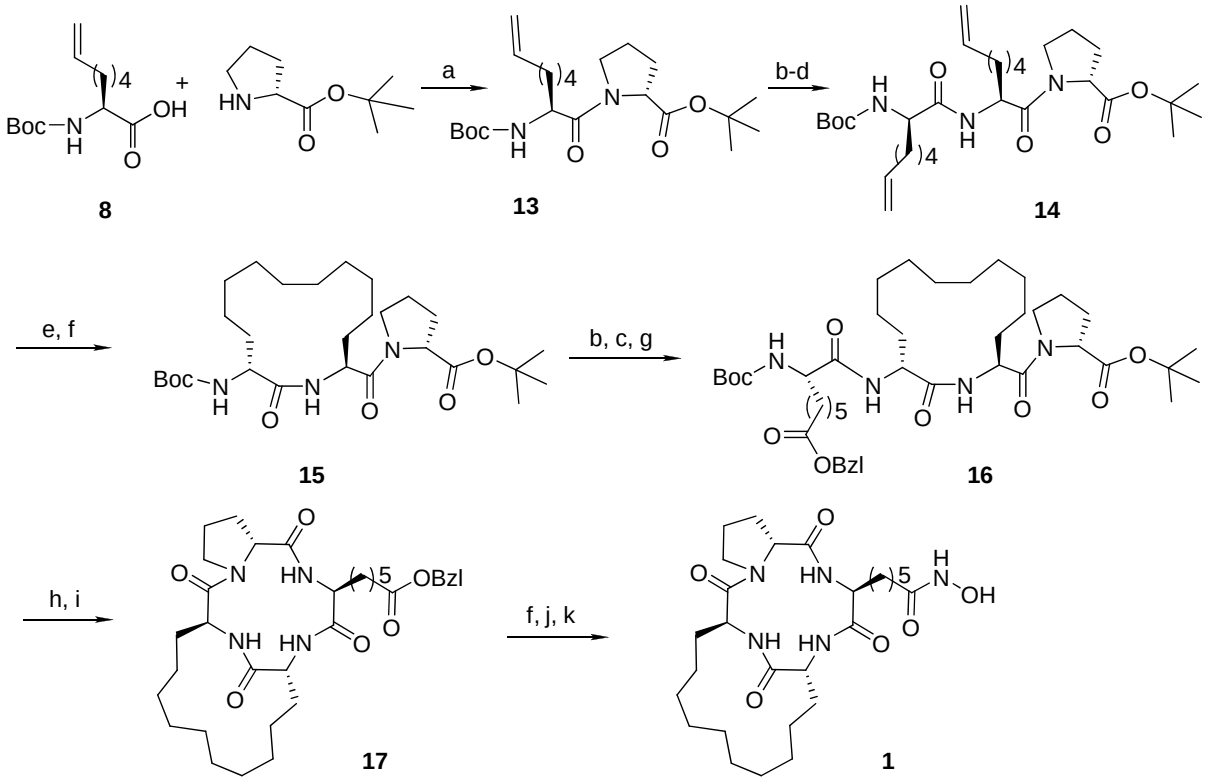


Figure 2. Designed HDAC inhibitors.

2.2. Synthesis of bicyclic tetrapeptides (1-3) and the corresponding open chain cyclic tetrapeptides (4-6). The synthesis of building block amino acid derivatives: Boc-L-2-amino-6-heptenoic acid (Boc-L-Ae7-OH) (**7**), Boc-L-2-amino-7-octenoic acid (Boc-L-Ae8-OH) (**8**), Boc-L-2-amino-8-nonenoic acid (Boc-L-Ae9-OH) (**9**), Boc-D-2-amino-6-heptenoic acid (Boc-D-Ae7-OH) (**10**), Boc-D-2-amino-7-octenoic acid (Boc-D-Ae8-OH) (**11**) and Boc-D-2-amino-8-nonenoic acid (Boc-D-Ae9-OH) (**12**) have been described in chapter 2. CHAP31 based bicyclic tetrapeptide (**1**) was synthesized according to Scheme 1 by the conventional solution phase method. H-D-Pro-O^{t}Bu was condensed with **8** using DCC/HOBt. Boc protection was selectively removed by the reported procedure[8] using 4 M HCl/dioxane, and amine component was condensed with **11** by the same DCC/HOBt method to obtain linear tripeptide **14**. The linear tripeptide **15** with fused side ring was synthesized by ring closing metathesis between D-Ae8 and L-Ae8 using Grubbs first generation Ru catalyst, in dichloromethane (DCM), followed by catalytic hydrogenation. After selective deprotection of Boc, Boc protected amino suberic acid benzyl ester [Boc-L-Asu(OBzl)] was incorporated in them to prepare the linear tetrapeptide **16**. After removal of both side protections by treating with trifluoroacetic acid (TFA), cyclization reaction was carried out by the aid of *N*-

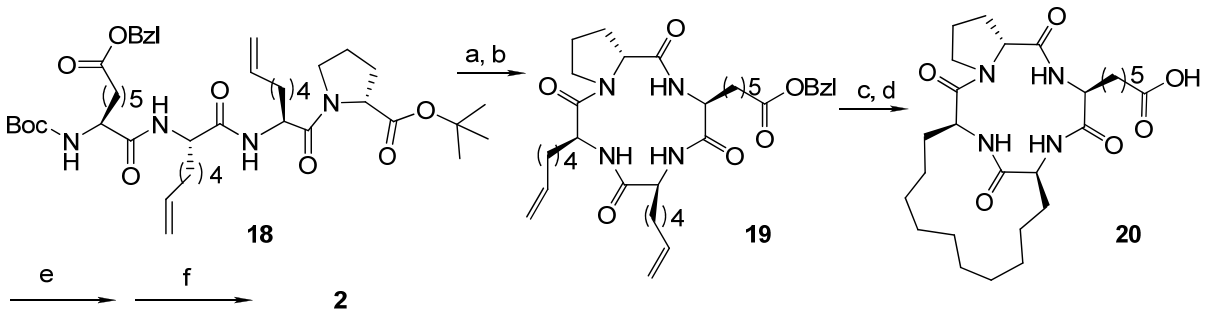
[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU) in dimethylformamide (DMF) under high dilution conditions with minimum amount of diisopropylethyl amine (DIEA) (2.5 equiv) to obtain bicyclic tetrapeptide **17**. After Bzl deprotection, by catalytic hydrogenation, the carboxyl group was condensed with hydroxylamine benzyl ester, and finally Bzl protection was removed by catalytic hydrogenation to obtain bicyclic tetrapeptide hydroxamic acid **1**.



Scheme 1. Synthesis of CHAP31 based bicyclic tetrapeptide (**1**). Reagents and conditions: (a) DCC, HOBt, DMF, 12 h; (b) 4 M HCl/dioxane, 30 min; (c) saturated Na_2CO_3; (d) Boc-D-Ae8-OH (**11**), DCC, HOBt, DMF, 12 h; (e) Grubbs first generation catalyst, DCM, 48 h; (f) AcOH, Pd-C, H_2, 12 h; (g) Boc-L-Asu(OBzl)-OH, DCC, HOBt, DMF, 12 h; (h) TFA, 3 h; (i) HATU, DIEA, DMF, 4 h; (j) $HCl.H_2NOBzl$, DCC, HOBt, TEA, DMF; (k) Pd-$BaSO_4$, AcOH, H_2.

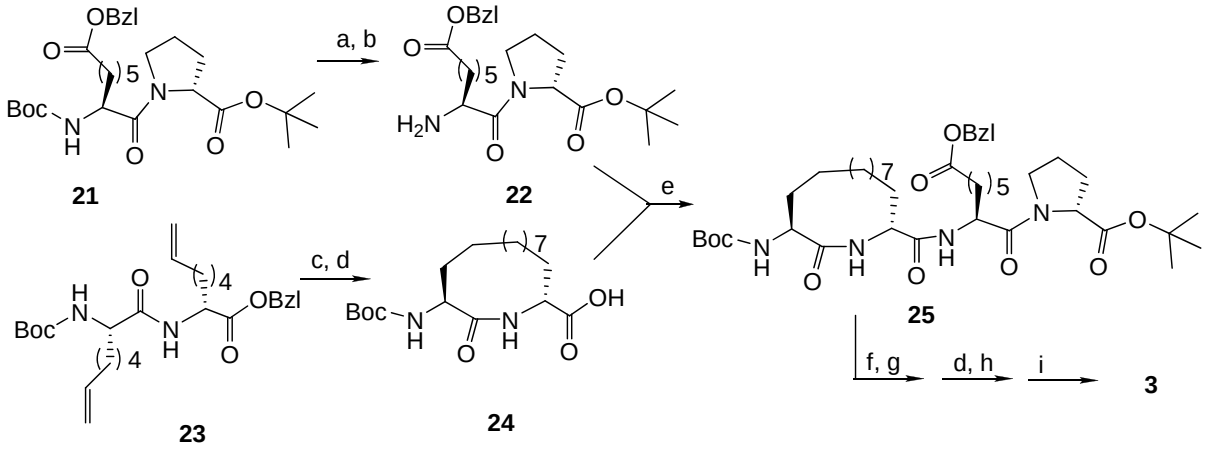
For synthesis of trapoxin B based bicyclic tetrapeptide (**2**), the ring closing metathesis was planned to be carried out after peptide cyclization as shown in Scheme 2. Boc-L-Ae8-OH was used instead of Boc-D-Ae8-OH. Boc-tetrapeptide-O^{t}Bu (**18**) synthesized by stepwise elongation from C-terminal was deprotected by TFA and then cyclized by high dilution

condition in DMF using HATU as described above. Cyclic tetrapaptide **19** obtained was subjected to RCM followed by catalytic hydrogenation to yield a bicyclic tetrapeptide **20**. Successive incorporation of hydroxamic acid benzyl ester and catalytic hydrogenation gave the desired compound **2**.



Scheme 2. Synthesis of trapoxin B based bicyclic tetrapeptide **2**. Reagents and conditions: (a) TFA, 3 h; (b) HATU, DIEA, DMF, 4 h; (c) Grubbs first generation catalyst, DCM, 48 h; (d) AcOH, Pd-C, H_2 , 12 h; (e) HCl.H_2NOBzl, DCC, HOBt, TEA, DMF; (f) Pd-$BaSO_4$, AcOH, H_2.

HC-toxin I based bicyclic tetrapeptide **(3)** was synthesized through fragment condensation of H-L-Asu(OBzl)-D-Pro-O^{t}Bu (**22**) and Boc-fused side chain dipeptide–OH (**24**) by DCC/HOBt, and following cyclization and incorporation of hydroxamic acid gave the desired compound **3** (Scheme 3).



Scheme 3. Synthesis of HC-toxin I based bicyclic tetrapeptide **3**. Reagents and conditions: **(**a) 4 M HCl/dioxane, 30 min; (b) saturated Na_2CO_3; (c) Grubbs first generation catalyst, DCM, 48 h; (d) AcOH, Pd-C, H_2 , 12 h; (e) DCC, HOBt, DMF, 12 h; (f) TFA, 3 h. g) HATU, DIEA, DMF, 4 h; (h) HCl.H_2NOBzl, DCC, HOBt, TEA, DMF; (i) Pd-$BaSO_4$, AcOH, H_2.

Reference cyclic tetrapeptides **4-6** (Figure 2) were synthesized in a similar manner to their corresponding bicyclic tetrapeptides excluding RCM. All the compounds **1-6** were characterized by ^{1}H NMR and high resolution FAB-MS.

2.3. Enzyme inhibition and biological activity. The synthesized bicyclic tetrapeptide and reference monocyclic tetrapeptide hydroxamic acids were assayed for HDAC inhibitory activity using HDAC1, HDAC4, and HDAC6 prepared from 293T cells.[9] To examine the inhibitory activity in cell based condition, p21 promoter assay was carried out. Detailed experimental procedures for the preparation and assays of HDACs and p21 promoter have been described in chapter 2. The results of the HDAC inhibitory activity and the p21 promoter assay of the compounds are shown in Table 1.

Table 1. HDAC inhibitory activity and p21 promoter activity data for bicyclic tetrapeptides and corresponding cyclic tetrapeptide hydroxamic acids.

Compounds	HPLC[a]	IC_{50} (nM)			HDAC1/	HDAC6/	p21 promoter assay
	t_R (min.)	HDAC1	HDAC4	HDAC6	HDAC4[b]	HDAC4[b]	EC_{1000} (nM)
1	6.03	9.1	5.5	410	1.7	74.5	7.2
2	5.80	25	26	230	1	8.8	510
3	7.06	25	31	490	0.8	15.8	58
4	7.51	25	12	340	2.1	28.3	13
5	6.55	37	15	140	2.5	9.3	80
6	7.67	43	43	570	1	13.3	80

[a]HPLC analysis was performed on a Hitachi instrument equipped with a Chromolith performance RP-18e column (4.6 × 100 mm, Merck). The mobile phases used were A: H_2O with 0.1% TFA, B: CH_3CN with 0.1% TFA using a solvent gradient of A–B over 15 min with a flow rate of 2 mL/min, with detection at 220 nm.

[b]Selectivity for HDAC4

All the compounds **1-6** are active in nanomolar range. However, the activity depends upon the sequence of amino acids in the cyclic tetrapeptide framework. During the optimization of the CHAP molecules, Nishino *et al.* found that out of many combinations of optically different amino acids, LDLD, LLLD and LDLL combinations have almost similar extent of activity,[7] which is true for CHAPs that have aromatic side chain in their cyclic framework. However, for bicyclic tetrapeptides and their reference cyclic tetrapeptides, which do not have aromatic ring in their cyclic framework, different results were observed. In HDAC inhibitory activity toward HDAC1 and HDAC4 the order of potency was CHAP31 analogs (LDLD) > HC-toxin analogs (LDLD, reverse to CHAP31) ≈ trapoxin analogs (LLLD). In

p21 promoter-inducing activity the efficacy order was CHAP31 analogs > HC-toxin analogs > trapoxin analogs. As shown in Figure 3, CHAP31 analogs (**1**, **4**) show highest activity and selectivity. Both activity and selectivity improve upon ring closing. Ring closing facilitates the loop to come closer to enzyme surface enhancing interaction between inhibitor cap group and enzyme surface. Also, as closed ring is less flexible than open chain, selectivity toward a certain enzyme also improves upon ring closing. With change in amino acid arrangement in the cyclic framework, aliphatic loop and open chain position get changed, which affect both activity and selectivity. If the loop position is such that the grooves surrounding the entrance to the catalytic pocket to the enzyme are not utilized properly, the inhibitor loose both activity and selectivity. Such case might have happen in case of trapoxin analogs (**2**, **5**) and HC-toxin analogs (**3**, **6**). The HDAC inhibitory activity results are more interesting in case of trapoxin analogs. Due to ring closing, aliphatic chains become more apart from the enzyme surface resulting in less activity and selectivity. The p21 promoter assay results also reflect the similar trend as was observed in case of HDAC inhibitory activity assay. For bicyclic compounds, CHAP31 analog (**1**) is 7 fold more active than HC-toxin analog (**3**), which in turn 6 fold more active than trapoxin analog (**2**).

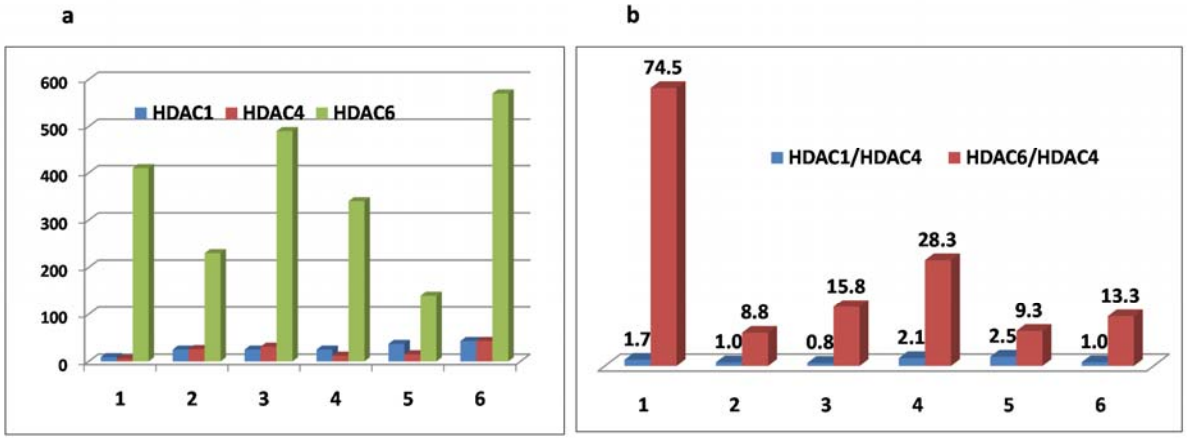


Figure 3. (a**)** HDAC inhibitory activity and **(**b**)** selectivity toward HDAC4.

2.4. Conformational study. Circular dichroism (CD) experiment of the synthesized compounds was carried out in methanol as solvent. The CD spectra are shown in Figure 4. In case of trapoxin analogs (**2, 5**) and HC-toxin analogs (**3**, **6**), the pattern of CD spectra of cyclic tetrapeptide is similar to that of bicyclic tetrpapetide (Figure 4b). This implies that the

cyclic tetrapeptide backbone conformation does not get changed remarkably upon ring closing. However, the situation is different in case of CHAP31 analogs (**1**, **4**). The minima near 240 nm as observed for compound **4** is absent for compound **1** (Figure 4a). This indicates that a remarkable change in peptide backbone conformation has been occurred upon ring closing.

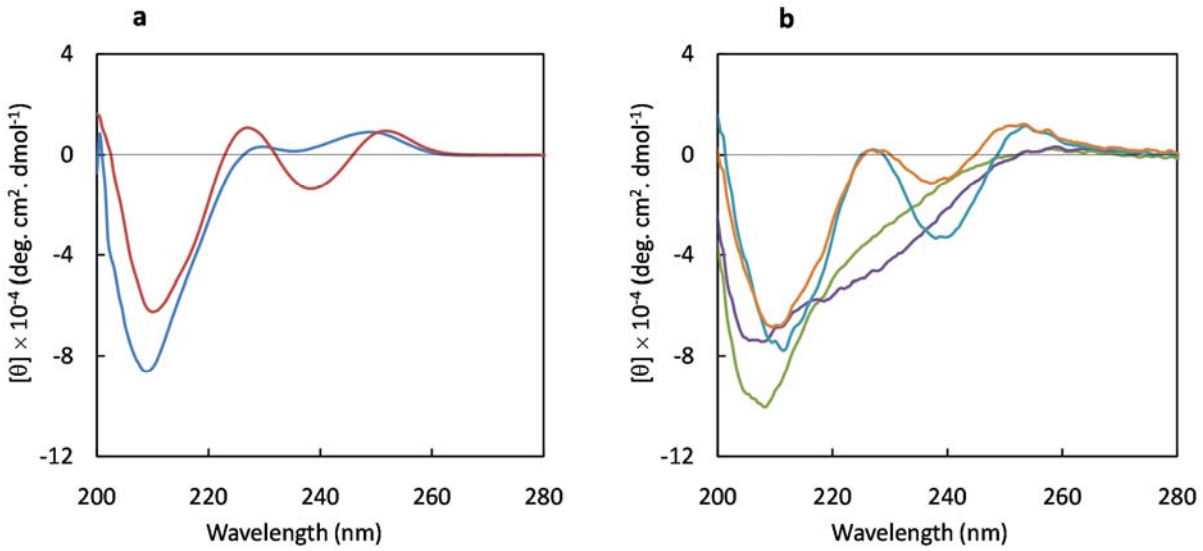


Figure 4. CD spectra of (a) CHAP31 analogs (—**1**, —**4**); (b) trapoxin analogs (—**2**, —**5**) and HC-toxin analogs (—**3**, —**6**).

3. Summary

CHAP31, trapoxin B and HC-toxin I based bicyclic tetrapeptide hydroxamic acid HDAC inhibitors were designed and synthesized successfully. Cyclic tetrapeptides were also synthesized as reference compounds. The cell-free and cell-based HDAC inhibitory activity of the inhibitors was evaluated and it was found that the inhibitors were active against HDAC enzymes in nanomolar range. Both activity and selectivity depend on loop position. In HDAC inhibitory activity against HDAC1 and HDAC4, the order of potency: CHAP31 analogs (LDLD) > HC-toxin I analogs (LDLD, reverse to CHAP31) ≈ trapoxin B analogs (LLLD). In p21 promoter-inducing activity the efficacy order was CHAP31 analogs > HC-toxin I analogs > trapoxin B analogs. For bicyclic compounds, CHAP31 analog is 7-fold more active than HC-toxin I analog, which in turn 6-fold more active than trapoxin analog. CHAP31 analogs show highest activity and selectivity. Both activity and selectivity improve upon ring closing. In case of HC-toxin I analogs, ring closing resulted better activity, but not selectivity.

However, in case of trapoxin analogs, ring closing resulted in less activity. Therefore, aliphatic loop position is important for bicyclic tetrapeptides. Peptide backbone conformation is sensitive to ring closing for CHAP31 analogs. However, for trapoxin analogs and HC-toxin analogs such change is not remarkable. This study may help in better understanding of the interaction of cap group with the ezyme, which may lead to design and synthesize isoform selective inhibitors.

4. Experimental

4.1. General. Unless otherwise noted, all solvents and reagents were reagent grade and used without purification. Flash chromatography was performed using silica gel 60 (230–400 mesh) eluting with solvents as indicated. All compounds were routinely checked by thin layer chromatography (TLC) and/or high performance liquid chromatography (HPLC). TLC was performed on aluminum-backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F_{254}) with spots visualized by UV light or charring. Analytical HPLC was performed on a Hitachi instrument equipped with a chromolith performance RP-18e column (4.6 × 100 mm, Merck). The mobile phases used were A: H_2O with 10% CH_3CN and 0.1% TFA, B: CH_3CN with 0.1% TFA using a solvent gradient of A-B over 15 min with a flow rate of 2 mL/min, with detection at 220 nm. HR FAB-mass spectra were measured on a JEOL JMS-SX 102A instrument. NMR spectra were recorded on a JEOL JNM A500 MHz spectrometer in DMSO-d_6 solutions with reference to TMS. All ^1H shifts are given in parts per million (s = singlet; d = doublet; t = triplet; m = multiplet). Assignments of proton resonances were confirmed, when possible, by correlated spectroscopy. Ring closing metathesis (RCM) was perform by the aid of benzylidene-bis(tricyclohexylphosphine)dichlororuthenium (Grubbs first generation catalyst). Coupling reactions were performed by standard solution-phase chemistry using dicyclohexyl-carbodiimide (DCC) and 1-hydroxybenzotriazol (HOBt). Peptide cyclization was mediated by *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU).

4.2. Synthesis of CHAP31 based bicyclic tetrapeptide hydroxamic acid (1). To a cooled solution of H-D-Pro-O^{t}Bu (0.855 g, 5 mmol), Boc-L-Ae8-OH (**8**) (1.29 g, 5 mmol) and HOBt.H_2O (0.766 g, 5 mmol) in DMF (10 mL), DCC (1.24 g, 6 mmol) was added. The mixture was stirred overnight at room temperature. After completion of the reaction, DMF was evaporated and the residue was dissolved in ethyl acetate and successively washed with

10% citric acid, 4% sodium bicarbonate and brine. The ethyl acetate solution was dried over anhydrous $MgSO_4$ and concentrated to remain an oily substance, which was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield Boc-L-Ae8-D-Pro-O^{t}Bu (**13**) (1.60 g, 78%) as oil. The protected dipeptide (1.55 g, 3.8 mmol) was dissolved in 4 M HCl/dioxane (16 mL) and the mixture was kept at room temperature for 30 min. The reaction was monitored by TLC. After completion of the reaction HCl/dioxane was evaporated. The residue was dissolved in EtOAc and washed with saturated Na_2CO_3, dried over anhydrous Na_2CO_3 and EtOAc was evaporated to get H-L-Ae8-D-Pro-O^{t}Bu as heavy oil (0.930 g, 79%). To a cooled solution of H-L-Ae8-D-Pro-O^{t}Bu (0.930 g, 3 mmol), Boc-D-Ae8-OH (**11**) (0.940 g, 3.3 mmol) and HOBt.H_2O (0.460 g, 3 mmol) in DMF (6 mL), DCC (0.742 g, 3.6 mmol) was added and was stirred for overnight at room temperature. The product Boc-D-Ae8-L-Ae8-D-Pro-O^{t}Bu (**14**) was obtained in the same manner as described earlier as heavy oil (1.40 g, 84%). HPLC, retention time (t_R) 8.02 min. To a solution of linear tripeptide Boc-D-Ae8-L-Ae8-D-Pro-O^{t}Bu (**14**) (1.10 g, 2 mmol) in anhydrous and degassed dichloromethane (250 mL), a solution of Grubbs first generation ruthenium catalyst (0.330 g, 0.4 mmol) in anhydrous and degassed dichloromethane (50 mL) was added. The reaction mixture was stirred at room temperature for 48 h. After the completion of reaction, dichloromethane was evaporated and the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield linear tripeptide with fused cycle as foam which on catalytic hydrogenation in presence of Pd-C (0.100 g) in AcOH (10 mL) yielded compound **15** (0.920 g, 87%). HPLC, t_R 7.96 min. The protected tripeptide **15** (0.530 g, 1 mmol) was dissolved in 4 M HCl/dioxane (5 mL) and the mixture was kept at room temperature for 30 min. The reaction was monitored by TLC. After completion of the reaction HCl/dioxane was evaporated. The residue was dissolved in EtOAc and washed with saturated Na_2CO_3, dried over anhydrous Na_2CO_3 and EtOAc was evaporated to get tripeptide free amine (0.310 g, 73%). To a cooled solution of the free amine (0.310 g, 0.73 mmol), Boc-L-Asu(OBzl)-OH (0.295 g, 0.78 mmol) and HOBt.H_2O (0.112 g, 0.73 mmol) in DMF (2 mL), DCC (0.161 g, 78 mmol) was added and stirred for overnight at room temperature. The product linear tetrapeptide (**16**) (0.450 g, 79%) was obtained in the a similar manner as described earlier as foam. HPLC, t_R 10.42 min. The protected tetrapeptide **16** (0.445 g, 0.57 mmol) was dissolved in TFA (3 mL) at 0 ^{0}C and kept for 3 hours. After evaporation of TFA, the residue was solidified using ether and petroleum ether to yield TFA salt of the linear tetrapeptide (0.425 g, 100%). To DMF solvent (570 mL), the TFA salt (0.425 g, 0.57 mmol), HATU (0.326 g, 0.86 mmol), and DIEA (0.24 mL, 1.42 mmol) were added in separate five

portions in every 30 min with stirring, for the cyclization reaction. After completion of the reaction, DMF was evaporated under vacuum; the residue was dissolved in ethyl acetate and washed with citric acid (10%) solution, sodium bicarbonate (4%) solution, and brine, respectively. It was then dried over anhydrous $MgSO_4$ and filtered. After evaporation of ethyl acetate, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield the bicyclic tetrapeptide **17** (0.143 g , 41%). HPLC, t_R 7.85 min. Compound **17** (0.125 g, 0.2 mmol) was dissolved in acetic acid (5 mL) and Pd-C (0.050 g) was added. The mixture was stirred under H_2 for 10 hours. After filtration of Pd-C, acetic acid was evaporated to yield bicyclic tetrapeptide carboxylic acid (0.104 g, 100%). The bicyclic tetrapeptide carboxylic acid (0.090 g, 0.17 mmol) was dissolved in DMF (0.5 mL) at 0 ^{0}C, and O-Benzylhydroxylamine hydrochloride (0.041 g, 0.26 mmol), HOBt.H_2O (0.026 g, 0.17 mmol), triethylamine (0.037 mL, 0.26 mmol) and DCC (0.054 g, 0.26 mmol) were added. The mixture was stirred for 24 h. The product (0.062 g, 60%) obtained was dissolved in acetic acid (1 mL), and Pd-$BaSO_4$ (0.020 g) was added. The mixture was stirred under H_2 for 24 hours. After filtration of Pd-$BaSO_4$, acetic acid was evaporated and crystallized with ether to yield compound **1** (0.036 g, 67%). ^{1}H NMR (500 MHz, DMSO-d_6, 40 ^{0}C): δ_H 1.14-1.28 (m, 16H), 1.31-1.38 (m, 4H), 1.42-1.54 (m, 6H), 1.64-1.79 (m, 4H), 1.85-2.00 (m, 4H), 3.38-3.45 (m, 2H), 3.57 (dd, *J* = 18, 8.2 Hz, 1H), 3.92 (ddd, *J* = 9.5, 9.5, 3.5 Hz, 1H), 4.21 (m, 1H), 4.44 (m, 1H), 4.58 (d, *J* = 7.9 Hz, 1H), 4.70 (m, 1H), 7.13 (d, *J* = 9.0 Hz, 1H), 7.26 (d, *J* = 10.1 Hz, 1H), 8.29 (d, *J* = 9.1 Hz, 1H), 8.64 (s, 1H), 10.33 (s, 1H); HR FAB-MS $[M+Na]^+$ 558.3263 for $C_{27}H_{45}N_5O_6Na$ (calcd 558.3268).

4.3. Synthesis of trapoxin based bicyclic tetrapeptide hydroxamic acid (2). Boc-L-Asu(OBzl)-L-Ae8-L-Ae8-D-Pro-O^{t}Bu (**18**) was synthesized by stepwise elongation from C-terminal. HPLC, t_R 9.21 min. The protected tetrapeptide **18** (0.920 g, 1.14 mmol) was dissolved in TFA (4 mL) at 0 ^{0}C and kept for 3 hours. After evaporation of TFA, the residue was solidified using ether and petroleum ether to yield TFA salt of the linear tetrapeptide (0.835 g, 97%). To DMF solvent (550 mL), the TFA salt (0.835 g, 1.11 mmol), HATU (0.633 g, 1.66 mmol), and DIEA (0.48 mL, 2.78 mmol) were added in separate five portions in every 30 minutes with stirring for the cyclization reaction. After completion of the reaction, DMF was evaporated under vacuo, the residue was dissolved in ethyl acetate and washed with citric acid (10%) solution, sodium bicarbonate (4%) solution, and brine, respectively. It was then dried over anhydrous $MgSO_4$ and filtered. After evaporation of ethyl acetate, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1)

to yield the cyclic tetrapeptide **19** (0.290 g , 41%). HPLC, t_R 6.12 min. To a solution of **19** (0.270 g, 0.424 mmol) in anhydrous and degassed dichloromethane (55 mL), a solution of Grubbs first generation ruthenium catalyst (0.073 g, 0.09 mmol) in anhydrous and degassed dichloromethane (5 mL) was added. The reaction mixture was stirred at room temperature for 48 h. After completion of the reaction, dichloromethane was evaporated and the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yielded bicyclic tetrapeptide as foam which on catalytic hydrogenation in presence of Pd-C (0.050 g) in AcOH (5 mL) yield bicyclic tetrapeptide carboxylic acid (**20**) (0.090 g, 40%). HPLC, t_R 7.47 min. Compound **20** (0.090 g, 0.17 mmol) was dissolved in DMF (1 mL) at 0 ^{0}C, and O-Benzylhydroxylamine hydrochloride (0.042 g, 0.26 mmol), HOBt.H_2O (0.027 g, 0.17 mmol), triethylamine (0.038 mL, 0.26 mmol) and DCC (0.054 g, 0.26 mmol) were added. The mixture was stirred for 24 h. The product bicyclic tetrapeptide hydroxamic acid benzyl ester (0.073 g, 68%) was obtained was dissolved in acetic acid (5 mL), and Pd-$BaSO_4$ (0.100 g) was added. The mixture was stirred under H_2 for 24 hours. After filtration of Pd-$BaSO_4$, acetic acid was evaporated and crystallized with ether to yield compound **2** (0.042 g, 67%). ^{1}H NMR (500 MHz, DMSO-d_6, 40 ^{0}C): δ_H 1.07-1.13 (m, 2H),1.16-1.39 (m, 16H), 1.43-1.54 (m, 4H), 1.64-1.78 (m, 4H), 1.81 (s, 2H), 1.91-1.98 (m, 3H), 3.29-3.41 (m, 4H), 3.78 (m, 1H), 4.14 (ddd, *J* = 11, 11, 3.6 Hz, 1H), 4.35 (dd, *J* = 11.6, 7.9 Hz, 1H), 5.06 (d, *J* = 7.9 Hz, 1H), 8.33 (d, *J* = 7.6 Hz, 1H), 8.44 (d, *J* = 11 Hz, 1H), 8.90 (s, 1H); HR FAB-MS $[M+Na]^+$ 558.3187 for $C_{27}H_{45}N_5O_6Na$ (calcd 558.3268).

4.4. Synthesis of HC-toxin-I based bicyclic tetrapeptide hydroxamic acid (3).

4.4.1. Synthesis of H-L-Asu(OBzl)-D-Pro-O^{t}Bu (22). To a cooled solution of H-D-Pro-O^{t}Bu (0.555 g, 3.25 mmol), Boc-L-Asu(OBzl)-OH (0.907 g, 3.25 mmol) and HOBt.H_2O (0.497 g, 3.25 mmol) in DMF (7 mL), DCC (0.803 g, 3.9 mmol) was added. The mixture was stirred overnight at room temperature. After completion of the reaction, DMF was evaporated and the residue was dissolved in ethyl acetate and successively washed with 10% citric acid, 4% sodium bicarbonate and brine. The ethyl acetate solution was dried over anhydrous $MgSO_4$ and concentrated to remain an oily substance, which was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield Boc-L-Asu(OBzl)-D-Pro-O^{t}Bu (**21**) (1.40 g, 80%) as oil. The protected dipeptide (**21**) (1.40 g, 2.60 mmol) was dissolved in 4 M HCl/dioxane (13 mL) and the mixture was kept at room temperature for 30 min. The reaction was monitored by TLC. After completion of the reaction HCl/dioxane was evaporated. The residue was dissolved in EtOAc and washed with

saturated Na_2CO_3, dried over anhydrous Na_2CO_3 and EtOAc was evaporated to get H-L-Asu(OBzl)-D-Pro-O^{t}Bu (**22**) as heavy oil (0.840 g, 68%).

4.4.2. Synthesis of linear dipeptide with fused ring (24). To a cooled solution of HCl.H-D-Ae8-OBzl (1.26 g, 4.44 mmol), Boc-L-Ae8-OH (1.14 g, 4.44 mmol), triethylamine (0.639 mL, 4.44 mmol) and HOBt.H_2O (0.679 g, 4.44 mmol) in DMF (10 mL), DCC (1.10 g, 5.33 mmol) was added. The mixture was stirred overnight at room temperature. After completion of the reaction, DMF was evaporated and the residue was dissolved in ethyl acetate and successively washed with 10% citric acid, 4% sodium bicarbonate and brine. The ethyl acetate solution was dried over anhydrous $MgSO_4$ and concentrated to remain an oily substance, which was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield Boc-L-Ae8-D-Ae8-OBzl (**23**) (1.60 g, 80%) as crystalline solid. To a solution of linear dipeptide (0.973 g, 2 mmol) in anhydrous and degassed dichloromethane (250 mL), a solution of Grubbs first generation ruthenium catalyst (0.330 g, 0.4 mmol) in anhydrous and degassed dichloromethane (50 mL) was added. The reaction mixture was stirred at room temperature for 48 h. After the completion of reaction, dichloromethane was evaporated and the residue was purified by silica gel chromatography using chloroform to get fused cyclic dipeptide which on catalytic hydrogenation in presence of Pd-C (0.150 g) in AcOH (7 mL) yielded compound (**24**) (0.465 g, 63%).

4.4.3. Synthesis of bicyclic tetrapeptide hydroxamic acid (3). To a cooled solution of H-L-Asu(OBzl)-D-Pro-O^{t}Bu (**22**) (0.540 g, 1.25 mmol), compound **24** (0.446 g, 1.20 mmol) and HOBt.H_2O (0.184 g, 1.20 mmol) in DMF (3 mL), DCC (0.296 g, 1.44 mmol) was added. The mixture was stirred overnight at room temperature. After completion of the reaction, the product linear tetrapeptide (**25**) (0.670 g, 72%) was obtained in a similar manner as described earlier as foam. HPLC, t_R 9.21 min. In a similar manner as described for compound **1**, compound **3** was obtained from compound **25**. ^{1}H NMR (500 MHz, DMSO-d_6, 40 ^{0}C): δ_H 1.05-1.79 (m, 24H), 1.83-2.08 (m, 10H), 3.37-3.45 (m, 1H), 3.55 (m, 1H), 3.62 (ddd, J = 9.5, 9.5, 3.8 Hz, 1H), 4.34-4.43 (m, 2H), 4.64 (m, 1H), 4.72 (d, J = 7.3 Hz, 1H), 6.85 (d, J = 10.1 Hz, 1H), 7.16 (d, J = 9.8 Hz, 1H), 8.75 (d, J = 8.6, 1H),8.63 (s, 1H), 10.27 (s, 1H); HR FAB-MS $[M+H]^+$ 536.3445 for $C_{27}H_{48}N_5O_6$ (calcd 536.3448).

4.5. Synthesis of CHAP31 based cyclic tetrapeptide (4). Compound **4** was synthesized in a similar manner as described for compound **1** using Boc-L-Ae7-OH (**7**) and Boc-D-Ae7-OH (**10**) instead of Boc-L-Ae8-OH (**8**) and Boc-D-Ae8-OH (**11**), respectively, and by excluding

RCM step. ^{1}H NMR (500 MHz, DMSO-d_6) δ_H 0.81-0.88 (m, 6H),1.18-1.30 (m, 18H), 1.44 (m, 4H), 1.62-1.74 (m, 4H), 1.87-1.97 (m, 4H), 3.52 (dd, *J* = 18.5, 8.5 Hz, 1H), 3.77 (ddd, *J* = 10, 9.5, 4 Hz, 1H), 4.19-4.27 (m, 2H), 4.63 (dd, *J* = 17.3, 8 Hz, 1H), 4.71 (d, *J* = 7.3 Hz, 1H), 7.06 (d, *J* = 10.1 Hz, 1H), 7.21 (d, *J* = 9.5 Hz, 1H), 8.43 (d, *J* = 9.2 Hz, 1H), 8.63 (s, 1H), 10.31 (s, 1H); HR FAB-MS $[M+H]^+$ 538.3543 for $C_{27}H_{48}N_5O_6$ (calcd 538.3605).

4.6. Synthesis of trapoxin based cyclic tetrapeptide (5). Compound **5** was synthesized in a similar manner as described for compound **2** using Boc-L-Ae7-OH (**7**) instead of Boc-L-Ae8-OH (**8**) and by excluding RCM step. ^{1}H NMR (500 MHz, DMSO-d_6) δ_H 0.82-0.88 (m, 6H),1.16-1.33 (m, 16H), 1.43-1.69 (m, 9H), 1.71-1.84 (m, 2H), 1.89-1.97 (m, 2H), 2.01-2.18 (m, 2H), 3.74 (dd, J = 13.2, 7.5 Hz, 1H), 3.88 (m, 1H), 4.08 (m, 1H), 4.29 (m, 1H), 4.38(d, *J* = 8.2 Hz, 1H), 4.60 (d, *J* = 7 Hz, 1H), 7.2 (d, *J* = 4 Hz, 1H), 7.62 (d, *J* = 8.5 Hz, 1H), 8.12 (d, *J* = 8.8 Hz, 1H); FAB-MS $[M+H]^+$ 538.3635 for $C_{27}H_{48}N_5O_6$ (calcd 538.3605).

4.7. Synthesis of HC-toxin based cyclic tetrapeptide (6). Compound **6** was synthesized in a similar manner as described for compound **3** using Boc-D-Ae7-OBzl and Boc-L-Ae7-OH instead of Boc-D-Ae8-OBzl and Boc-L-Ae8-OH, respectively, and by excluding RCM step. ^{1}H NMR (500 MHz, DMSO-d_6, 40 ^{0}C) δ_H 0.84 (m, 6H), 1.19-1.32 (m, 16H), 1.47 (m, 5H), 1.55-1.75 (m, 5H), 1.92 (t, *J* = 7.3, 2H), 2.20 (m, 1H), 2.50 (m, 2H), 3.53 (dd, *J* = 9.5, 8.5 Hz, 1H), 3.74 (ddd, *J* = 9.5, 9.3, 4 Hz, 1H), 4.24 (m, 2H), 4.63 (m, 1H), 4.71 (d, *J* = 7.3 Hz, 1H), 6.99 (d, *J* = 9.8 Hz, 1H), 7.19 (d, *J* = 9.5 Hz, 1H), 8.37 (d, *J* = 8.8, 1H); FAB-MS $[M+H]^+$ 538.3545 for $C_{27}H_{48}N_5O_6$ (calcd 538.3605).

4.8. Circular dichroism measurement. CD spectra were recorded on a JASCO J-820 spectropolarimeter (Tokyo, Japan) using a quartz cell of 1 mm light path length at room temperature. Peptide solutions (0.1 mM) were prepared in methanol and CD spectra were recorded in terms of molar ellipticity, $[\theta]_M$ (deg × cm^2 × dmol^{-1}).

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Chapter 4

Molecular Design of Bicyclic Tetrapeptide Histone Deacetylase Inhibitors by Changing Zinc Binding Groups

1. Introduction

The histone deacetylase enzymes are able to be inhibited by small molecules. The inhibitor binds tightly to the active site and likely chelates the zinc cofactor, so that catalysis cannot occur. HDAC inhibitors have three primary domains: cap group, linker and zinc binding group. Each of the domains has specific role in HDAC inhibitory activity. The basic functional group of an HDAC inhibitor is the zinc binding group (ZBG), which chelates the zinc ion and engages in multiple hydrogen bonds with the residues at the active site. Typical ZBGs include hydroxamic acid, carboxylic acid, mercaptoacetamide, thiol, benzamide.[1] Each class has its own limitations. Hydroxamic acids are very potent ZBGs, and form many hydrogen bonds with zinc and nearby amino acids. However, hydroxamic acid based HDAC inhibitors suffer from poor pharmacokinetic properties, short half-life and low oral bioavailability[2] which limit their use as drug for practical use. Compounds with benzamide ZBGs such as MS-275 have very long half life *in vivo*, but are not as potent as the hydroxamate. On the other hand, the zinc binding group can have a significant effect on the isoform selectivity of an inhibitor. The residues that make up the active site are highly conserved, but there are certain key differences between the active sites of the different isoforms that give certain ZBGs the ability to discriminate between isoforms. Each class of zinc binding group has a stereotypical selectivity profile, whereby most types of compounds with the same ZBG will have similar isoform selectivity.[3] Therefore, it has become increasingly desirable to find HDAC inhibitors with novel zinc binding groups to make the HDAC inhibitors attractive as anticancer drugs.

Several efforts have been made to develop HDAC inhibitors with novel zinc binding groups. Nishino and coworkers[4] synthesized several sulfur-containing cyclic tetrapeptides (SCOPs) based on CHAP31[5] skeleton. The same group designed and synthesized HDAC inhibitors containing retrohydroxamate as the zinc binding ligand with cyclic tetrapeptide cap group.[6] They also reported the synthesis of chlamydocin analog[7] bearing carbonyl group as ligand toward zinc atom in HDAC (Figure 1). Recently, Suzuki and coworkers reported the design and synthesis of non-peptide HDAC inhibitors with boronic acid ZBG.[8]

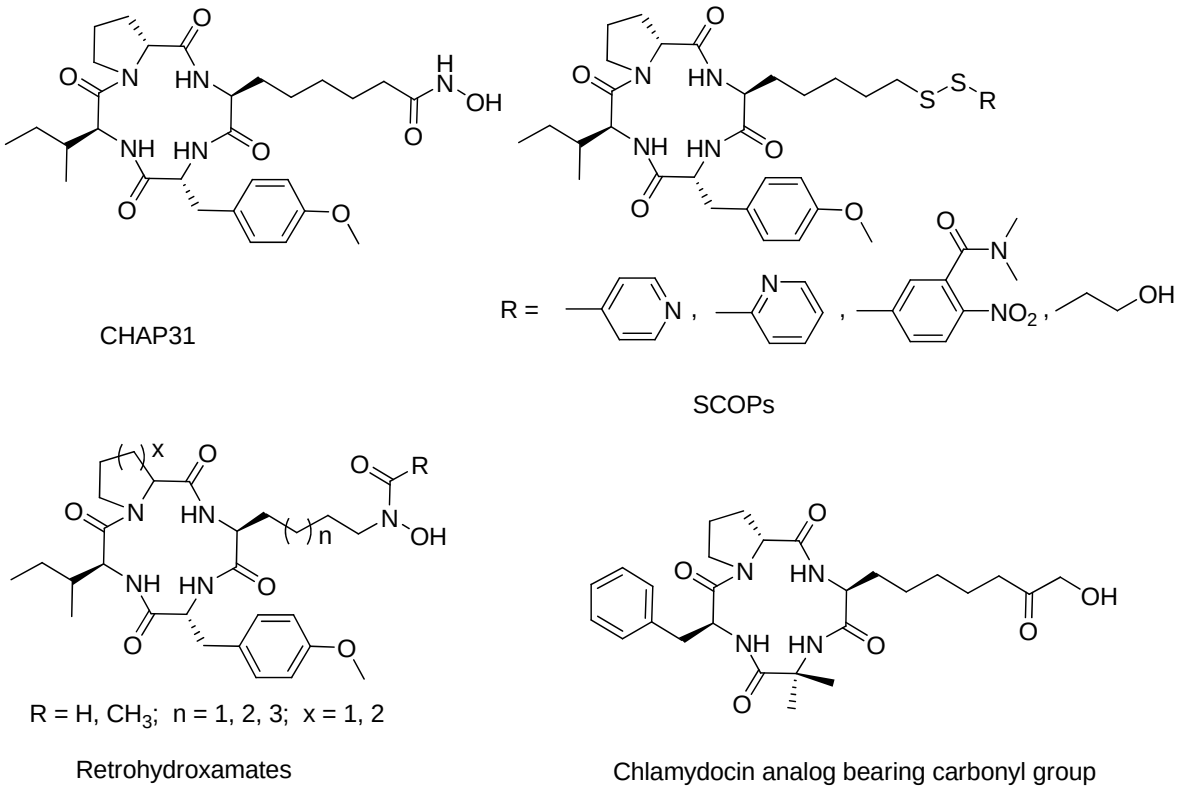


Figure 1. Synthetic cyclic tetrapeptide HDAC inhibitors with different zinc binding groups.

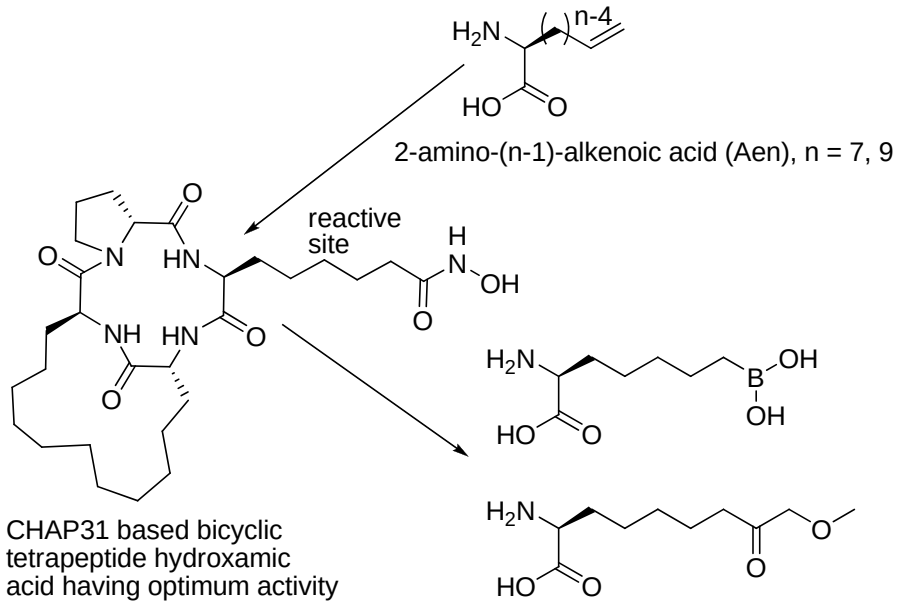


Figure 2. Design of bicyclic tetrapeptide methoxymethyl ketone (Mmk) and boronic acid.

In the present study, the author describes design and synthesis of bicyclic tetrapeptide methoxymethylketone (Mmk) and bicyclic tetrapeptide boronic acid (Figure 2). In chapter 2, the loop length optimization of CHAP31 based bicyclic tetrapeptides have been described.

Bicyclic tetrapeptide hydroxaic acid having optimum activity was considered as the lead compound for designing bicyclic tetrapeptide Mmk and boronic acid. L-Ae9 and L-Ae7 were separately incorporated and the terminal alkene was modified to Mmk and boronic acid, respectively.

2. Results and discussion

2.1. Chemistry

In addition to bicyclic tetrapeptide hydroxamic acid (**1**), bicylcic tetrapeptide methoxymethylketone (Mmk) (**2**) and boronic acid (**3**) have been designed and synthesized to compare the biological activity among the inhibitors bearing different zinc binding groups (Figure 3).

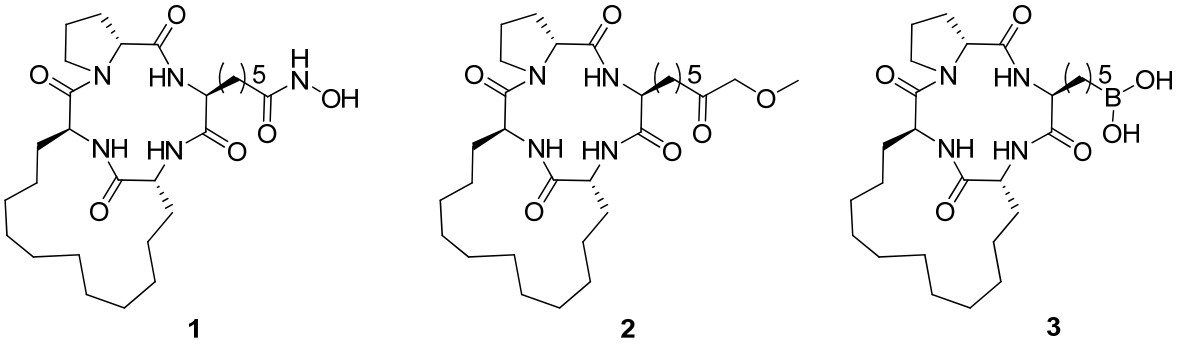
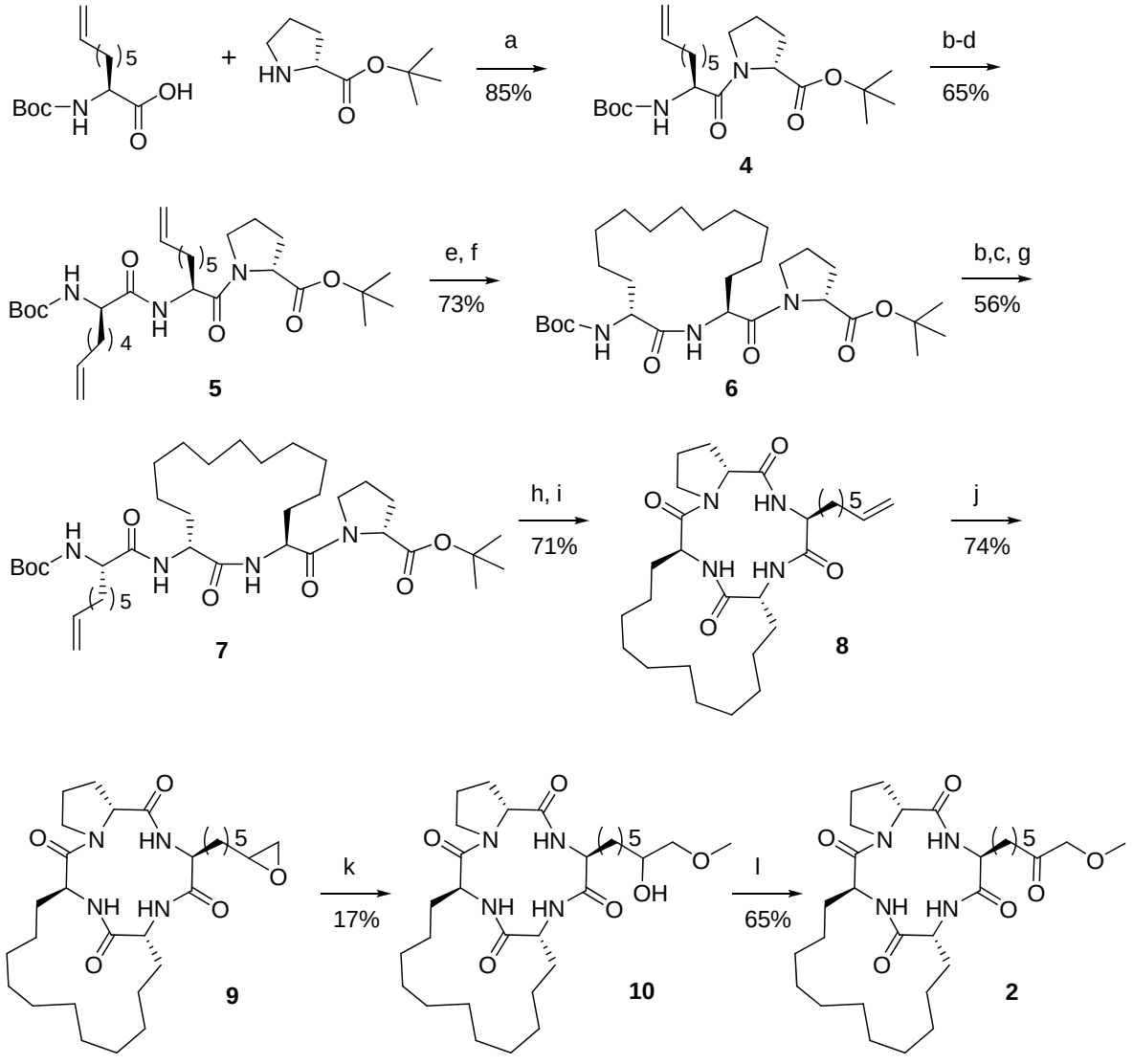


Figure 3. Structure of bicyclic tetrapeptide HDAC inhibitors with different zinc binding groups.

Bicyclic tetrapeptide methoxymethyl ketone (**2**) was synthesized according to Scheme 1 by the conventional solution phase method. After the removal of Z-protection from Z-D-Pro-O^{t}Bu by catalytic hydrogenation, the free amine component was condensed with Boc-L-Ae9-OH using DCC/HOBt to obtain Boc-L-Ae9-D-Pro-O^{t}Bu (**4**). Boc protection was selectively removed by using 4 M HCl/dioxane, and the free amine component was condensed with Boc-D-Ae8-OH by the same DCC/HOBt method to obtain Boc-D-Ae8-L-Ae9-D-Pro-O^{t}Bu (**5**). The linear tripeptide (**6**) with fused side ring were synthesized by ring closing metathesis (RCM) between D-Ae8 and L-Ae9 using Grubbs first generation Ru catalyst, in dichloromethane (DCM), followed by catalytic hydrogenation in presence of Pd-C. After selective deprotection of Boc, Boc-L-Ae9 was incorporated to prepare the linear tetrapeptide (**7**). After

removal of both the side protections by treating with trifluoroacetic acid, cyclization reaction was carried out by the aid of HATU in DMF (0.2 mM) with minimum amount of DIEA (2.5 equiv) to yield bicyclic tetrapeptide (**8**) with terminal alkene in the side chain. The side chain terminal alkene was modified to epoxide by the aid of 3-chloroperbenzoic acid (m-chloroperbenzoic acid, *m*-CPBA) in DCM to obtain compound **9**. Opening of the epoxide ring by the use of NaOMe/MeOH and subsequent oxidation of alcoholic group to keto group using Dess-Martin periodinane (DMP) yielded the bicyclic tetrapeptide methoxymethylketone (**2**).

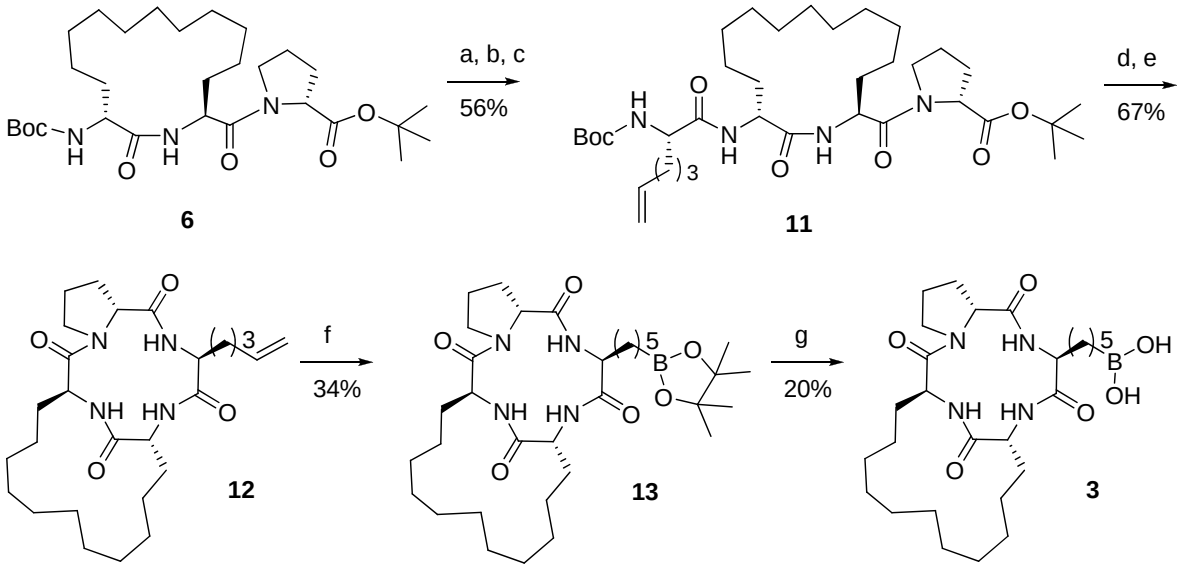


Scheme 1. Synthesis of bicyclic ttetrapeptide methoxymethyl ketone (**2**). Reagents and conditions: (a) DCC, HOBt, DMF, 12 h; (b) 4 M HCl/dioxane, 30 min; (c) saturated Na_2CO_3; (d) Boc-D-Ae8-OH, DCC, HOBt, DMF, 12 h; (e) Grubbs first generation catalyst, DCM, 48 h; (f) AcOH, Pd-C, H_2 , 12 h; (g) Boc-L-Ae9-OH, DCC,

HOBt, DMF, 12 h; (h) TFA, 3 h; (i) HATU, DIEA, DMF, 4 h; (j) m-CPBA, DCM, 12 h; (k) NaOMe/MeOH, 12 h; (l) DMP, DCM, 12 h.

Bicyclic tetrapeptide boronic acid (**3**) was synthesized according to Scheme 2. In this case, the linear tripeptide **6** was used as starting material. After selective deprotection of Boc, Boc-L-Ae7 was incorporated to prepare the linear tetrapeptide. After removal of both the side protections by treating with trifluoroacetic acid, cyclization reaction was carried out by the aid of HATU in DMF (0.2 mM) with minimum amount of DIEA (2.5 equiv) to yield bicyclic tetrapeptide with terminal alkene in the side chain. Pinacole borane was incorporated to the side chain by treating with pinacolborane in presence of $[Ir(cod)Cl]_2$ and bis(diphenylphosphino)methane (dppm) in DCM. Finally, pinacole protection was removed by treating with $NaIO_4$ and NH_4OAc to yield bicyclic tetrapeptide boronic acid (**3**).

The synthesized compounds were characterized by ^1H NMR and high resolution FAB-MS. The purity of the compounds determined by HPLC was above 95%.



Scheme 2. Synthesis of bicyclic tetrapeptide boronic acid (**3**). Reagents and conditions: (a) 4 M HCl/dioxane, 30 min; (b) saturated Na_2CO_3; (c) Boc-L-Ae7-OH, DCC, HOBt, DMF, 12 h; (d) TFA, 3 h; (e) HATU, DIEA, DMF, 4 h; (f) $[Ir(cod)Cl]_2$, Bis(diphenylphosphino)methane (dppm), Pinacolborane, DCM, 48 h; (g) NaIO4, NH_4OAc, Acetone, H_2O, 4 h.

2.2. Enzyme inhibition and biological activity

The synthesized bicyclic tetrapeptide hydroxamic acid and boronic acid were assayed for HDAC inhibitory activity using HDAC1, HDAC4, and HDAC6 prepared from 293T cells.

The detail experimental procedures have been described in chapter 2. The results of the HDAC inhibitory activity and the p21 promoter assay are shown in Table 1.

A remarkable difference in both activity and selectivity were observed among the bicyclic tetrapeptide bearing different zinc binding groups. HDAC inhibitory activity data show that bicyclic tetrapeptide methoxymethyl ketone (**2**) activities are comparable to that of hydroxamic acid (**1**) in both cell free and cell based conditions. Therefore, methoxymethyl ketone seems to possess ability to form necessary hydrogen bonds with zinc and nearby amino acids. Compound **2** also exhibits improved selectivity toward HDAC1 and HDAC4 over HDAC6. Although the residues that make up the active site are highly conserved, there are certain key differences between the active sites of the different isoforms. It seems that Mmk has the ability to discriminate between isoforms by utilizing the small differences between the active sites of the isoforms. The full screening of activity toward the other isoforms can reveal the potentiality of Mmk as ZBG. Bicyclic tetrapeptide boronic acid shows poor activity. In both cell free and cell based conditions it shows activity in micromolar range. Very recently, a series of boronic acid based HDAC inhibitors have been reported, which also showed inhibitory activity in micromolar range.[8] Therefore, boronic acid cannot be considered promising as zinc binding group.

Table 1. HDAC inhibitory activity and p21 promoter activity data for bicyclic tetrapeptides with different zinc binding groups.

Compounds	IC_{50} (nM)			p21 promoter assay, EC_{1000} (nM)
	HDAC1	HDAC4	HDAC6	
TSA	23	44	65	20
1	11	4.5	280	2.6
2	27	26	>100,000	32
3	12,000	9,400	>100,000	10,000

3. Summary

Novel bicyclic tetrapeptide HDAC inhibitors bearing methoxymethyl ketone and boronic acid were designed and synthesized. HDAC inhibitory activity of bicyclic tetrapeptide methoxymethyl ketone is comparable to that of hydroxamic acid in both cell free and cell based conditions. Moreover, bicyclic peptide equipped with methoxymethyl ketone shows

better selectivity among the HDAC isoforms. However, bicyclic tetrapeptide boronic acid fails to show potency against HDAC enzymes. This may be due to the inability of boronic acid to chelate zinc ion properly. This study reveals the potentiality of methoxymethyl ketone as zinc binding group, and at the same time the importance of bicyclic peptide as cap group for HDAC inhibitors.

4. Experimental

4.1. General. Unless otherwise noted, all solvents and reagents were reagent grade and used without purification. Flash chromatography was performed using silica gel 60 (230–400 mesh) eluting with solvents as indicated. All compounds were routinely checked by thin layer chromatography (TLC) and/or high performance liquid chromatography (HPLC). TLC was performed on aluminum-backed silica gel plates (Merck DC-Alufolien Kieselgel 60 F_{254}) with spots visualized by UV light or charring. Analytical HPLC was performed on a Hitachi instrument equipped with a chromolith performance RP-18e column (4.6 × 100 mm, Merck). The mobile phases used were A: H_2O with 10% CH_3CN and 0.1% TFA, B: CH_3CN with 0.1% TFA using a solvent gradient of A–B over 15 min with a flow rate of 2 mL/min, with detection at 220 nm. FAB-mass spectra were measured on a JEOL JMS-SX 102A instrument. NMR spectra were recorded on a JEOL JNM A500 MHz spectrometer. NMR spectra were measured in $CDCl_3$ solutions with reference to TMS. All ^1H shifts are given in parts per million (s = singlet; d = doublet; t = triplet; m = multiplet). Ring closing metathesis (RCM) was performed by the aid of benzylidene-bis(tricyclohexylphosphine)dichlororuthenium (Grubbs first generation catalyst). Coupling reactions were performed by standard solution-phase chemistry using dicyclohexyl-carbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt). Peptide cyclization was mediated by *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU).

4.2. Synthesis of bicyclic tetrapeptide methoxymethylketone (Mmk) (2). To a cooled solution of H-D-Pro-O^{t}Bu (1.71 g, 10 mmol), Boc-L-Ae9-OH (2.71 g, 10 mmol) and HOBt.H_2O (1.53 g, 10 mmol) in DMF (20 mL), DCC (2.47 g, 12 mmol) was added. The mixture was stirred overnight at room temperature. After completion of the reaction, DMF was evaporated and the residue was dissolved in ethyl acetate and successively washed with 10% citric acid, 4% sodium bicarbonate and brine. The ethyl acetate solution was dried over anhydrous $MgSO_4$ and concentrated to remain an oily substance, which was purified by silica

gel chromatography using a mixture of chloroform and methanol (99:1) to yield Boc-L-Ae9-D-Pro-O^{t}Bu (**4**) (3.60 g, 85%) as oil. The protected dipeptide (1.74 g, 4 mmol) was dissolved in 4 M HCl/dioxane (20 mL) and the mixture was kept at room temperature for 35 min. The reaction was monitored by TLC. After completion of the reaction HCl/dioxane was evaporated. The residue was dissolved in EtOAc and washed with saturated Na_2CO_3, dried over anhydrous Na_2CO_3 and EtOAc was evaporated to get H-L-Ae9-D-Pro-O^{t}Bu as heavy oil (1.0 g, 77%). To a cooled solution of H-L-Ae9-D-Pro-O^{t}Bu (1.0 g, 3.1 mmol), Boc-D-Ae8-OH (0.956 g, 3.7 mmol) and HOBt·H_2O (0.474 g, 3.1 mmol) in DMF (8 mL), DCC (0.762 g, 3.7 mmol) was added and stirred overnight at room temperature. The product Boc-D-Ae8-L-Ae9-D-Pro-O^{t}Bu (**5**) was obtained in the same manner as described earlier as heavy oil (1.48 g, 85%), HPLC, retention time (t_R) 8.84 min. To a solution of linear tripeptide Boc-D-Ae8-L-Ae9-D-Pro-O^{t}Bu (1.48 g, 2.64 mmol) in anhydrous and degassed dichloromethane (325 mL), a solution of Grubbs first generation ruthenium catalyst (0.435 g, 0.524 mmol) in anhydrous and degassed dichloromethane (80 mL) was added. The reaction mixture was stirred at room temperature for 48 h. After the completion of reaction, dichloromethane was evaporated and the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield linear tripeptide with fused cycle as foam which on catalytic hydrogenation in presence of Pd-C (0.100 g) in AcOH (10 mL) yield compound **6** (1.02 g, 73%). HPLC t_R 7.92 min. The protected tripeptide (1.02 mg, 1.9 mmol) was dissolved in 4 M HCl/dioxane (8 mL) and the mixture was kept at room temperature for 35 min. The reaction was monitored by TLC. After completion of the reaction HCl/dioxane was evaporated. The residue was dissolved in EtOAc and washed with saturated Na_2CO_3, dried over anhydrous Na_2CO_3 and EtOAc was evaporated to get tripeptide free amine (0.620 g, 75%). To a cooled solution of the free amine (0.310 g, 0.71 mmol), Boc-L-Ae9-OH (0.212 g, 0.78 mmol) and HOBt.H_2O (0.109 g, 0.71 mmol) in DMF (2 mL), DCC (0.176 g, 0.85 mmol) was added and stirred for overnight at room temperature. The product linear tetrapeptide (**7**) (0.370 g, 75%) was obtained in the same manner as described earlier as foam. HPLC t_R 11.43 min. The protected tetrapeptide (0.370 g, 0.53 mmol) was dissolved in TFA (3 mL) at 0 ^{0}C and kept for 3 hours. After evaporation of TFA, the residue was solidified using ether and petroleum ether to yield TFA salt of the linear tetrapeptide (0.335 g, 100%). To DMF solvent (500 mL), the TFA salt (0.335 g, 0.53 mmol), HATU (0.304 g, 0.80 mmol), and DIEA (0.23 mL, 1.33 mmol) were added in separate five portions in every 30 min with stirring, for the cyclization reaction. After completion of the reaction, DMF was evaporated under vacuo, the residue was dissolved in ethyl acetate and washed with citric acid (10%) solution, sodium bicarbonate

(4%) solution, and brine, respectively. It was then dried over anhydrous $MgSO_4$ and filtered. After evaporation of ethyl acetate, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield the bicyclic tetrapeptide **8** (0.195 g, 71%). HPLC t_R 9.14 min. Compound **8** (0.190 g, 0.37 mmol) was dissolved in anhydrous dichloromethane (DCM) (4 mL) in ice bath and 3-chloroperbenzoic acid (*m*-chloroperbenzoic acid, *m*-CPBA) (0.128 g) was added over 30 minutes. The mixture was stirred overnight. After the completion of the reaction, the reaction mixture was diluted to 30 mL by the addition of DCM. The resultant solution was washed with 4% $NaHCO_3$ and brine. The DCM solution was dried over anhydrous $MgSO_4$, concentrated and was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield bicyclic tetrapeptide epoxide (**9**) (0.145 g, 74%) as foam. HPLC t_R 6.50 min. To the epoxide (0.140 g, 0.26 mmol) 0.5 M NaOMe/MeOH (3 mL) was added in ice bath, and was stirred at room temperature for 21 hours. The reaction was quenched by the addition of acetic acid and water, methanol was evaporated. The residue was dissolved in ethyl acetate, and was washed successively with 10% citric acid, 4% $NaHCO_3$ and brine. It was then dried over anhydrous $MgSO_4$ and filtered. After evaporation of ethyl acetate, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield compound **10** (0.025 g, 17%). During the reaction peptide ring opening resulted in low yield. The methoxyalcohol (**10**) (0.025 g, 0.044 mmol) was dissolved in DCM (2 mL) and Dess-Martin periodinane (DMP) (0.025 g, 0.057 mmol) was added. The reaction mixture was stirred overnight at room temperature under argon atmosphere. The reaction was quenched by the addition of saturated $NaHCO_3$ solution (7 mL) containing $Na_2S_2O_3.5H_2O$ (0.056 g, 0.228 mmol), and was stirred vigorously to get two clear layers. The DCM layer was separated and was washed with brine followed by drying over $MgSO_4$. DCM was evaporated and the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield the bicyclic tetrapeptide methoxymethylketone (Mmk) (**2**) (0.016 g, 65%) as crystalline solid. HPLC t_R 7.15 min. ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 1.14-1.17 (m, 2H), 1.20-1.36 (m, 18H), 1.41-1.47 (m, 4H), 1.54-1.63 (m, 4H), 1.78-1.86 (m, 2H), 1.90-2.06 (m, 4H), 2.24-2.33 (m, 1H), 3.42 (t, *J* = 7.4 Hz, 2H), 3.41 (s, 3H), 3.51 (dd, *J* = 17.7, 7.6 Hz, 1H), 3.99 (s, 2H), 4.12 (ddd, *J* = 9.9, 8.8, 4.7 Hz, 1H), 4.24 (dd, *J* = 17.9, 7.5 Hz, 1H), 4.55 (t, *J* = 10.8 Hz, 1H), 4.72 (d, *J* = 7.9 Hz, 1H), 4.86 (t, *J* = 10.1 Hz, 1H), 6.12 (d, *J* = 10.1 Hz, 1H), 6.23 (d, *J* = 10.4 Hz, 1H), 7.07 (d, *J* = 10.1 Hz, 1H); FAB-MS $[M+H]^+$ 563.3812 for $C_{30}H_{51}N_4O_6$ (calcd 563.3809).

4.3. Synthesis of bicyclic tetrapeptide boronic acid (3). The protected tripeptide **6** (1.02 g, 1.90 mmol) was selectively deprotected using the method described above to obtain tripeptide free amine (0.620 g, 75%). The tripeptide free amine (0.305 g, 0.7 mmol) was coupled with Boc-L-Ae7-OH (0.204 g, 0.84 mmol) according to the method described earlier and the fully protected crude linear tetrapeptide was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield linear tetrapeptide **11** (0.350 g, 75%, HPLC, t_R 10.65 min) as foam. Compound **11** (0.340 g, 0.51 mmol) was dissolved in TFA (3 mL) at 0 ºC and kept at room temperature for 3 hours. After evaporation of TFA, the residue was crystalized by using the mixture of ether:pet-ether(1:4) to yield TFA salt of linear tetrapeptide (0.302 g, 98%, HPLC, t_R 6.66 min). To a volume of DMF (500 mL), TFA salt of linear tetrapeptide (0.302 g, 0.5 mmol), HATU (0.285 g, 0.75 mmol) and DIEA (0.218 mL, 1.25 mmol) were added in five aliquots in 30 minutes intervals while the solution was stirred vigorously. After the final addition, the reaction mixture was allowed to stir for an additional hour. Completion of the cyclization reaction was monitored by HPLC, and then DMF was evaporated under reduced pressure. The crude cyclic tetrapeptide was dissolved in ethyl acetate and the solution was washed successively by 10% citric acid, 4% sodium bicarbonate, and brine. Finally, the ethyl acetate solution was dried over anhydrous $MgSO_4$ and filtered. After evaporation of ethyl acetate, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (99:1) to yield bicyclic tetrapeptide **12** (0.166 g, 68%, HPLC, t_R 7.85 min) as foam after drying in vacuo.

To a solution of $[Ir(cod)Cl]_2$ (0.023 g, 0.034 mmol) and bis(diphenylphosphino)methane (dppm) (0.027 g, 0.068 mmol) in anhydrous and degassed DCM (4 mL), compound **12** (0.166 g, 0.34 mmol) and pinacolborane (HBpin) (0.080 mL, 0.51 mmol) were added. The mixture was flashed with Ar for 5 minutes, and was stirred for 48 hours at room temperature. The reaction mixture was concentrated by evaporating DCM and purified by silica gel column chromatography using a mixture of chloroform and methanol (99:1) to yield compound **13** (0.072 g, 34%, HPLC, t_R 9.22 min) as crystalline solid. To a solution of compound **13** (0.072 g, 0.12 mmol) in acetone (2 mL) and H_2O (1 mL), $NaIO_4$ (0.077 g, 0.36 mmol) and NH_4OAc (0.028 g, 0.36 mmol), were added and the suspension was stirred at room temperature for 4 hours. Then acetone was evaporated and the reaction mixture was poured into water and extracted with ethyl acetate. Finally, the ethyl acetate solution was dried over anhydrous $MgSO_4$ and filtered. After evaporation of ethyl acetate, the residue was purified by silica gel chromatography using a mixture of chloroform and methanol (98:2) to yield bicyclic

tetrapeptide boronic acid (**3**) (0.013 g, 20%) as crystalline solid. HPLC, t_R 6.49 min. ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 1.12-1.18 (m, 2H), 1.24-1.33 (m, 18H), 1.37-1.47 (m, 6H), 1.53-1.57 (m, 4H), 1.77-1.86 (m, 2H), 1.90-2.06 (m, 4H), 2.24-2.33 (m, 1H), 3.52 (dd, *J* = 17.7, 7.6 Hz, 1H), 3.63 (t, *J* = 6.4 Hz, 1H), 4.12 (m, 1H), 4.27 (dd, *J* = 17.7, 7.8 Hz, 1H), 4.55 (t, *J* = 9.7 Hz, 1H), 4.72 (d, *J* = 7.6 Hz, 1H), 4.86 (t, *J* = 10.7 Hz, 1H), 6.19 (d, *J* = 10.1 Hz, 1H), 6.27 (d, *J* = 10.4 Hz, 1H), 7.10 (d, *J* = 10.4 Hz, 1H); FAB-MS $[M + Glycerol - 2H_2O - H]^-$ 589 for $C_{30}H_{50}BN_4O_7$ (calcd 589).

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Chapter 5

Bicyclic Tetrapeptides: Potential Tools for Future Drug Design

1. Introduction

As overexpression of histone deacetylases (HDACs) is associated with carcinogenesis of cells, design and synthesis of novel HDAC inhibitors is a promising field on developing anticancer drugs. In fact, there are a variety of HDAC inhibitors in clinical trials and one of them, SAHA (Vorinostat), is already in the market. SAHA have demononsrated the therapeutic benefit in cutaneous T-cell lymphoma and in some malignancies.[1] The approval of SAHA as anticancer drug proves the importance of HDAC inhibitors in cancer treatment. However, current HDAC inhibitors, including SAHA, broadly inhibit the deacetylase activity of various HDAC enzymes including class I and II, which is associated with reduced potency and toxic side effects. Specificity is a necessary condition for an inhibitor to be useful as a drug with high pharmacokinetic properties and oral bioavailability. Scientists are now concentrating their research on this matter. Very potent HDAC inhibitors including non-peptides and cyclic tetrapeptides, bearing hydroxamic acid or epoxy ketones inhibit with very low selectivity among HDAC isoforms. Therefore, a lot of effort is needed to develop selective HDAC inhibitors.

In the present dissertation, the main objective was to design and synthesis of bicyclic tetrapeptide based HDAC inhibitors. The strategy was to modify the cap group of HDAC inhibitor by changing the aliphatic loop in the cyclic tetrapeptide scaffold. As the area surrounding the opening to the binding pocket has less homology between HDAC isoforms compared to the active site, the modification of the cap group allows to have a significant impact upon isoform selectivity.[2] On the other hand, aromatic ring in the cap group is considered important for interaction between cap group and enzyme rim region. As the loop is able to increase hydrophobic interaction in the rim region, it was considered alternative to aromatic ring. Moreover, as the loop is less flexible compared to aromatic side chain, the cap group becomes selective to certain HDAC isoforms and results in better selectivity. Therefore, incorporation of aliphatic loop in the cyclic tetrapeptide framework was expected to increase potency and selectivity of the inhibitors.

In chapter 2, a series of CHAP31[3] based bicyclic tetrapeptide hydroxamic acid HDAC inhibitors were designed by changing the aliphatic loop length. First, the minimum loop

length was determined, and it was found that nine methelene loop length is the minimum for bicyclic tetrapeptides. These inhibitors show potent HDAC inhibitory activity *in vivo* and *in vitro*. They also show some selectivity among the HDAC isoforms. The aliphatic loop length is important, and eleven methelene loop is the optimum for *in vivo* activity. The X-ray crystallographic study reveals that the loop helps in forming such a conformation which is favourable for interaction in the rim region. From the conformational study it was found that the aliphatic loop acts as a regulator to control the cyclic peptide backbone conformation. These results confirm the hypothesis that modification of the cap group of HDAC inhibitors can lead to potent HDAC inhibitors, which may have potential as anticancer agents.

In chapter 3, the author designed bicyclic tetrapeptides based on the naturally occurring cyclic tetrapetide HDAC inhibitors Cyl-1,[4] trapxin B[5] and HC-toxin I,[6] using hydroxamic acid instead of epoxyketone as zinc binding group. Due to the difficulty in synthesis of Cyl-1 based bicyclic tetrapeptide, ultimately trapoxin B and HC-toxin based bicyclic tetrapeptides with ten methylene loop were designed for synthesis. Corresponding cyclic tetrapeptides were also synthesized as reference compounds. The cell-free and cell-based HDAC inhibitory activity of the inhibitors was evaluated and it was found that the inhibitors were active against HDAC enzymes in nanomolar range. Comparative study between CHAP31, trapoxin B and HC-toxin I analogs revealed that both activity and selectivity depend on loop position. In HDAC inhibitory activity against HDAC1 and HDAC4, the order of potency: CHAP31 analogs (LDLD) > HC-toxin I analogs (LDLD, reverse to CHAP31) ≈ trapoxin B analogs (LLLD). In p21 promoter-inducing activity the efficacy order was CHAP31 analogs > HC-toxin I analogs > trapoxin analogs. For bicyclic compounds, CHAP31 analog is 7-fold more active than HC-toxin I analog, which in turn 6-fold more active than trapoxin analog. CHAP31 analogs show highest activity and selectivity. Both activity and selectivity improve upon ring closing. In case of HC-toxin I analogs, ring closing resulted better activity, but not selectivity. However, in case of trapoxin analogs, ring closing resulted in reduced activity. Therefore, aliphatic loop position is important for bicyclic tetrapeptides. Peptide backbone conformation is sensitive to ring closing for CHAP31 analogs. However, for trapoxin analogs and HC-toxin analogs such change is not remarkable. This study may help in better understanding of the interaction of cap group with the ezyme, which may lead to design and synthesize isoform selective inhibitors.

In chapter 4, novel bicyclic tetrapeptide HDAC inhibitors bearing methoxymethyl ketone (Mmk) and boronic acid were design and synthesized to compare the activity of bicyclic

tetrapeptide with different zinc binding groups. Bicyclic tetrapeptide hydroxaic acid having optimum activity was considered as the lead compound for designing bicyclic tetrapeptide Mmk and boronic acid. L-Ae9 and L-Ae7 were separately incorporated and the terminal alkene was modified to Mmk and boronic acid, respectively. HDAC inhibitory activity of bicyclic tetrapeptide methoxymethyl ketone is comparable to that of hydroxamic acid in both cell free and cell based conditions. Moreover, bicyclic peptide equipped with methoxymethyl ketone shows better selectivity among the HDAC isoforms. However, bicyclic tetrapeptide boronic acid fails to show potency against HDAC enzymes. This may be due to the inability of boronic acid to chelate zinc ion properly. This study reveals the potentiality of methoxymethyl ketone as zinc binding group, and at the same time the importance of bicyclic peptide as cap group for HDAC inhibitors.

2. Conclusions

Increasing evidence supports the pleiotropic cellular activities of HDAC isoforms, and that we are only just beginning to understand. Although progress has been made in the understanding of this family of enzymes, the exact molecular role of the various isoforms remains largely unknown. The discovery of novel specific inhibitors will provide invaluable tools for functional studies on the different HDAC isoforms and more importantly, their relevance for treatment of human diseases, particularly cancer. Bicyclic peptide based inhibitors will be helpful in finding the interactions of each HDACs in the rim region. The design of novel inhibitors based on the reported HDAC inhibitors and on the basis of the information on HDACs will lead to the synthesis of isoform selective inhibitors.

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Publications and Presentations at Conferences

Publications from the dissertation

1. Design and Synthesis of Aliphatic Loop Containing Cyclic tetrapeptides as Histone Deacetylase Inhibitors. Nurul M. Islam, Tamaki Kato, Norikazu Nishino, Akihiro Ito, and Minoru Yoshida. To be submitted in *Bulletin of the Chemical Society of Japan.*

2. Bicyclic peptides as potent inhibitors of histone deacetylases: Optimization of alkyl loop length. Nurul M. Islam, Tamaki Kato, Norikazu Nishino, Hyun-Jung Kim, Akihiro Ito, Minoru Yoshida. *Bioorganic & Medicinal Chemistry Letters,* **2010**, *20*, 997-999.

3. Conformational regulation of cyclic tetrapeptide by aliphatic loop: Effect for design of histone deacetylase inhibitors. Nurul M. Islam, Tamaki Kato, Norikazu Nishino. *Peptide Science*, **2009**, in press.

Other Publications

1. Unusual Cleavage of Tripeptides Containing Pipecolic Acid. Islam Md. Nurul, Sonu Ram Shankar, Tamaki Kato and Norikazu Nishino. *Peptides*, **2008**, *Proceedings of the 30th European Peptide Symposium*, 618-619.

2. Pipecolic Acid Disrupts Collagen Triple Helix Structure in Model Peptides. Sonu Ram Shankar, Islam Md. Nurul, Yuji Tanaka, Tamaki Kato and Norikazu Nishino. *Peptides*, **2008**, *Proceedings of the 30th European Peptide Symposium*, 246-247.

Presentations at Conferences

1. Poster presentation: "Conformational regulation of cyclic tetrapeptide by aliphatic loop: Effect for design of histone deacetylase inhibitors." Nurul M. Islam, Tamaki Kato, Norikazu Nishino. *46th Japanese Peptide Symposium,* **2009**, Kitakyushu, Japan.

2. Poster presentation: "Pipecolic Acid Disrupts Collagen Triple Helix Structure in Model Peptides." Sonu Ram Shankar, Islam Md. Nurul, Yuji Tanaka, Tamaki Kato and Norikazu Nishino. *30th European Peptide Symposium,* **2008**, Helsinki, Finland.

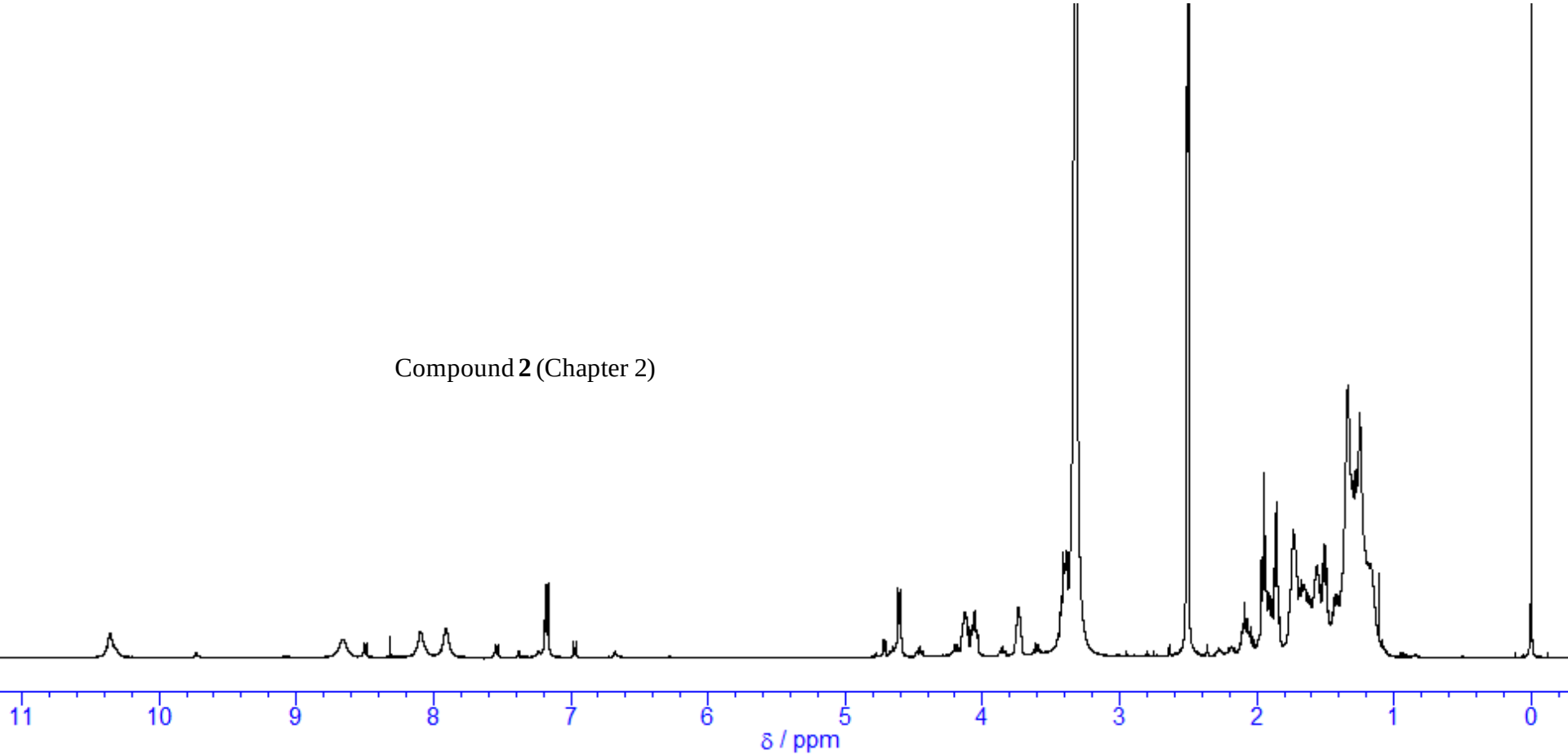
3. Poster presentation: “Unusual Cleavage of Tripeptides Containing Pipecolic Acid.” Islam Md. Nurul, Sonu Ram Shankar, Tamaki Kato and Norikazu Nishino. *30th European Peptide Symposium,* **2008**, Helsinki, Finland.

4. Oral presentation: “Unusual Cleavage of Collagen Model Peptides Containing Pipecolic Acid.” Nurul Md. Islam, Sonu Ram Shankar, Tamaki Kato, and Norikazu Nishino. *88th Japanese Chemical Society Symposium,* **2008**, Tokyo, Japan.

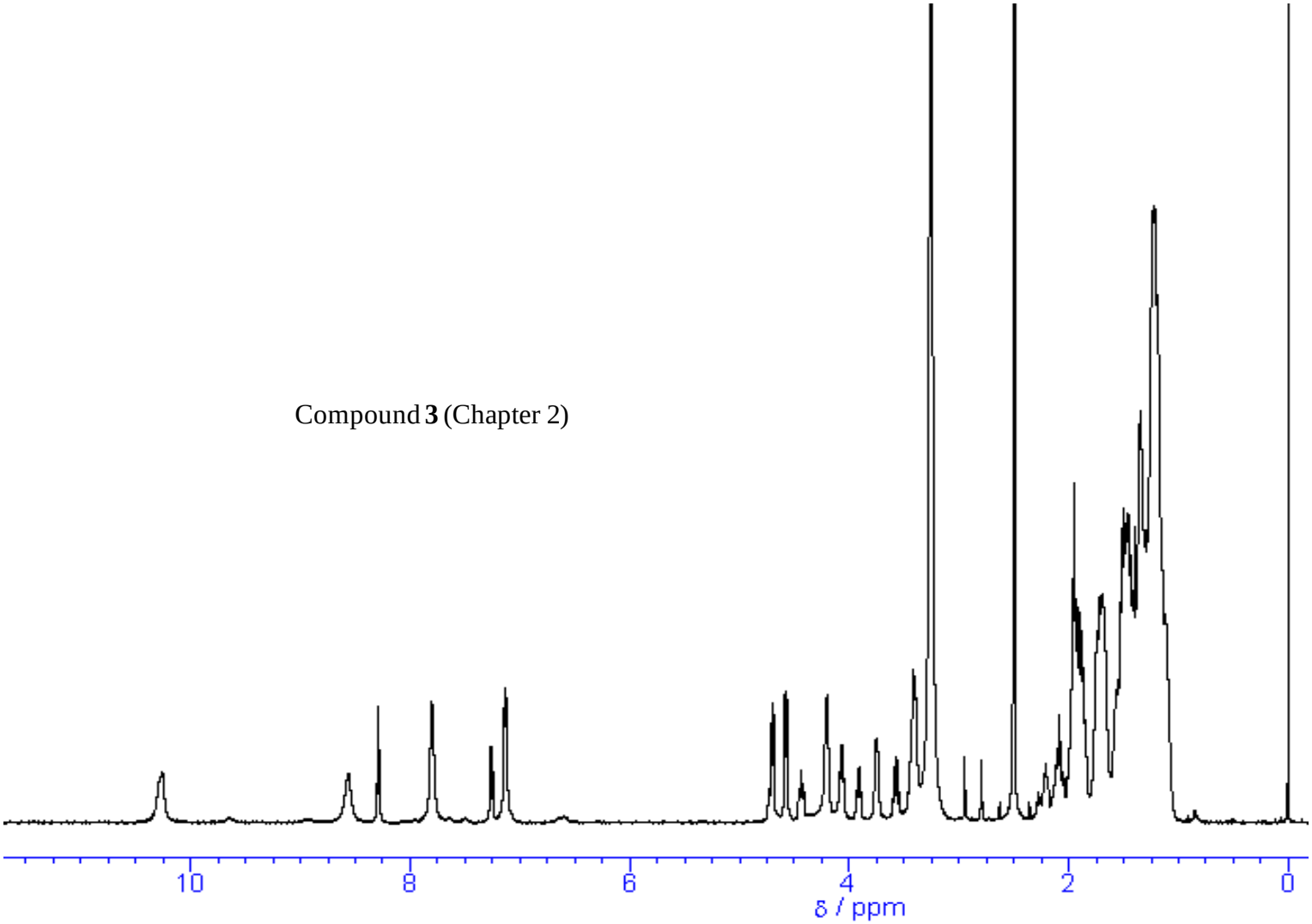
Annex

Proton NMR spectra of cyclic/bicyclic tetrapeptides

Compound **2** (Chapter 2)



Compound **3** (Chapter 2)



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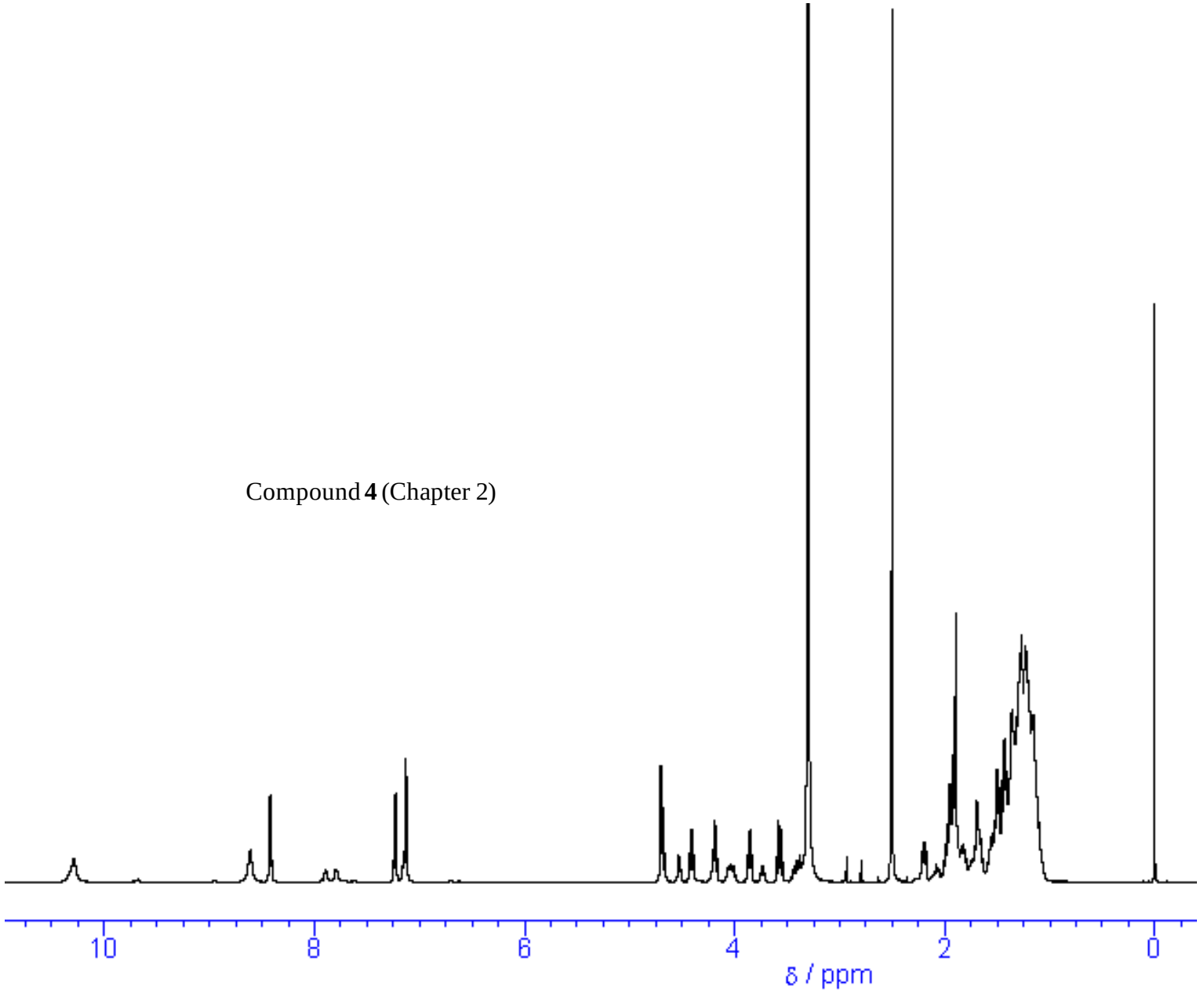

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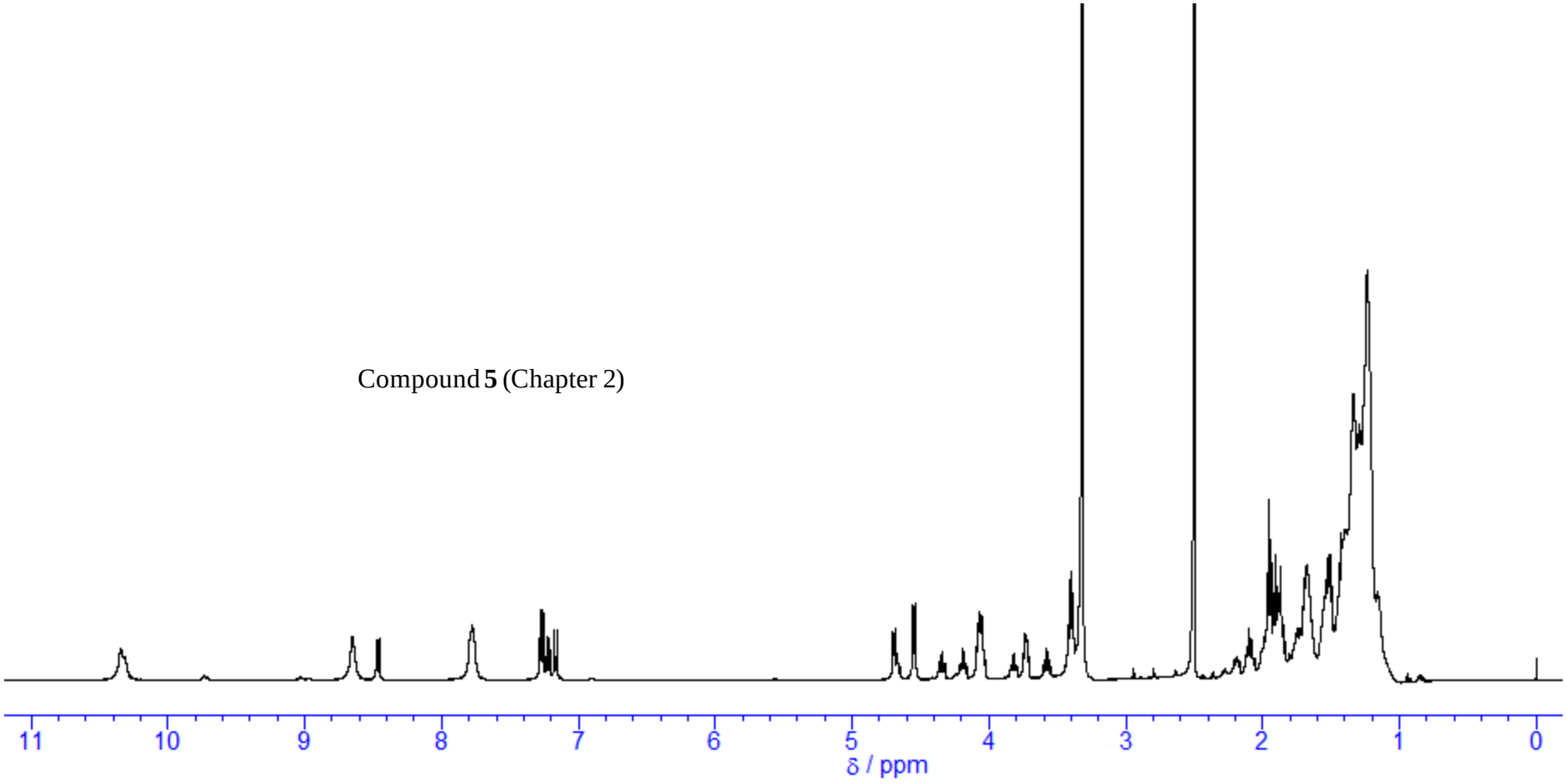

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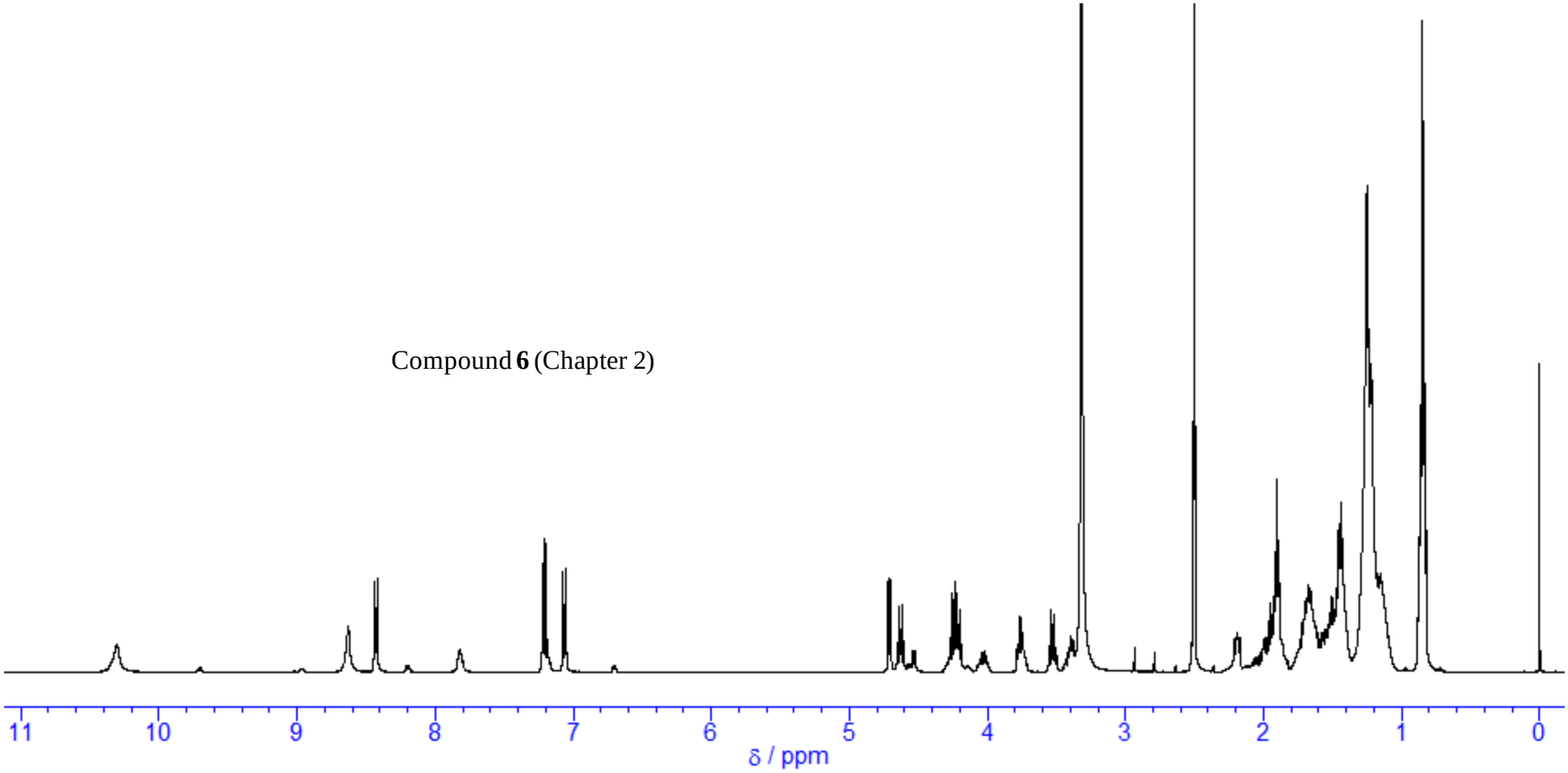
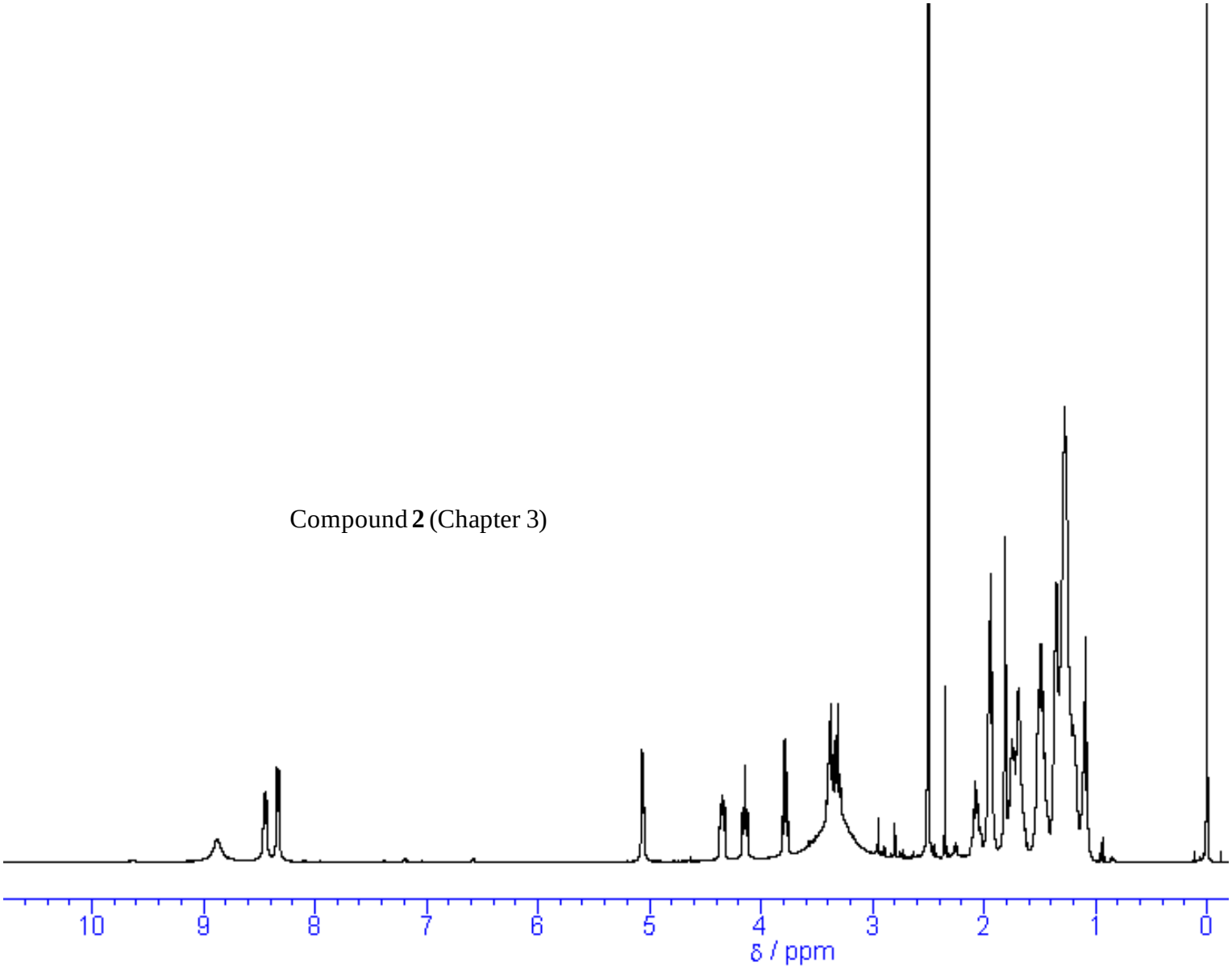


Compound **2** (Chapter 3)



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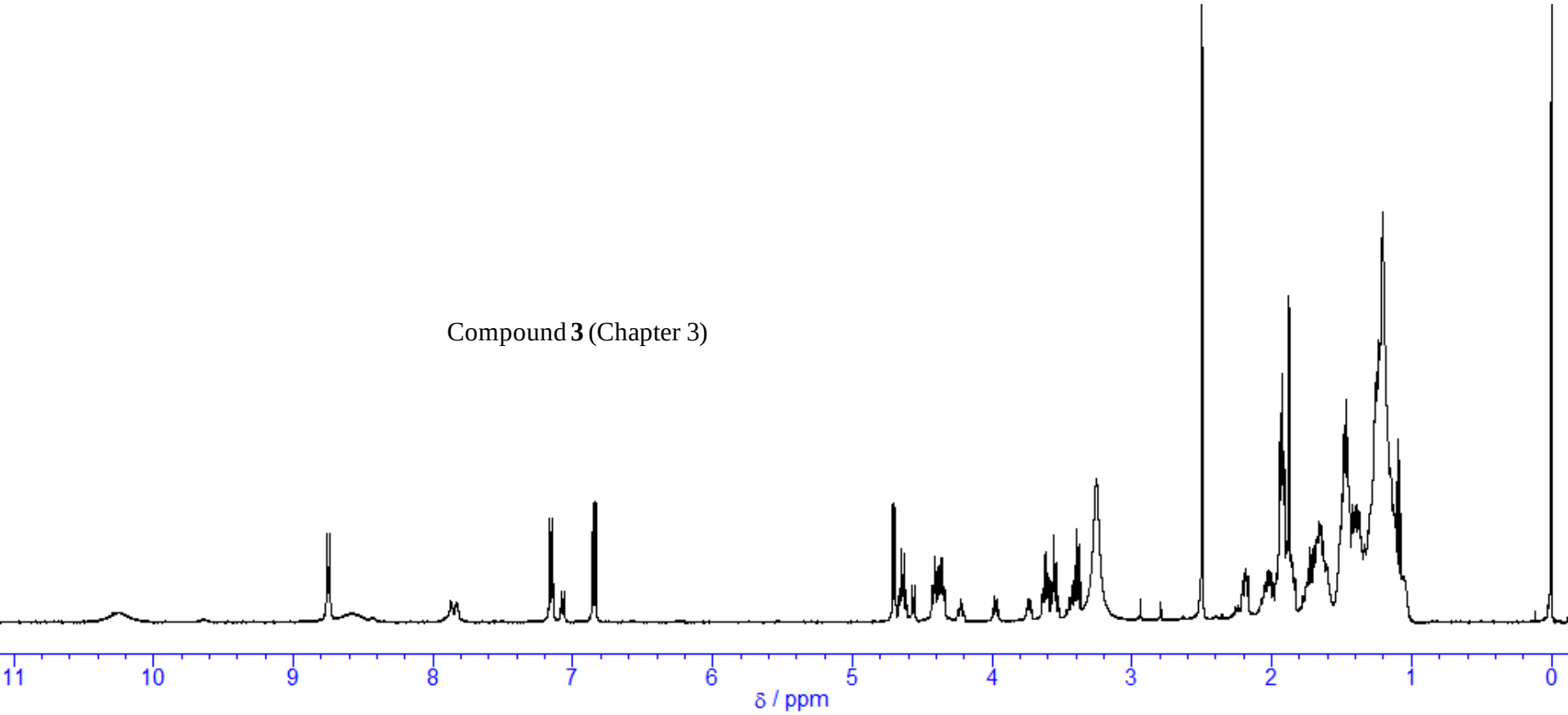

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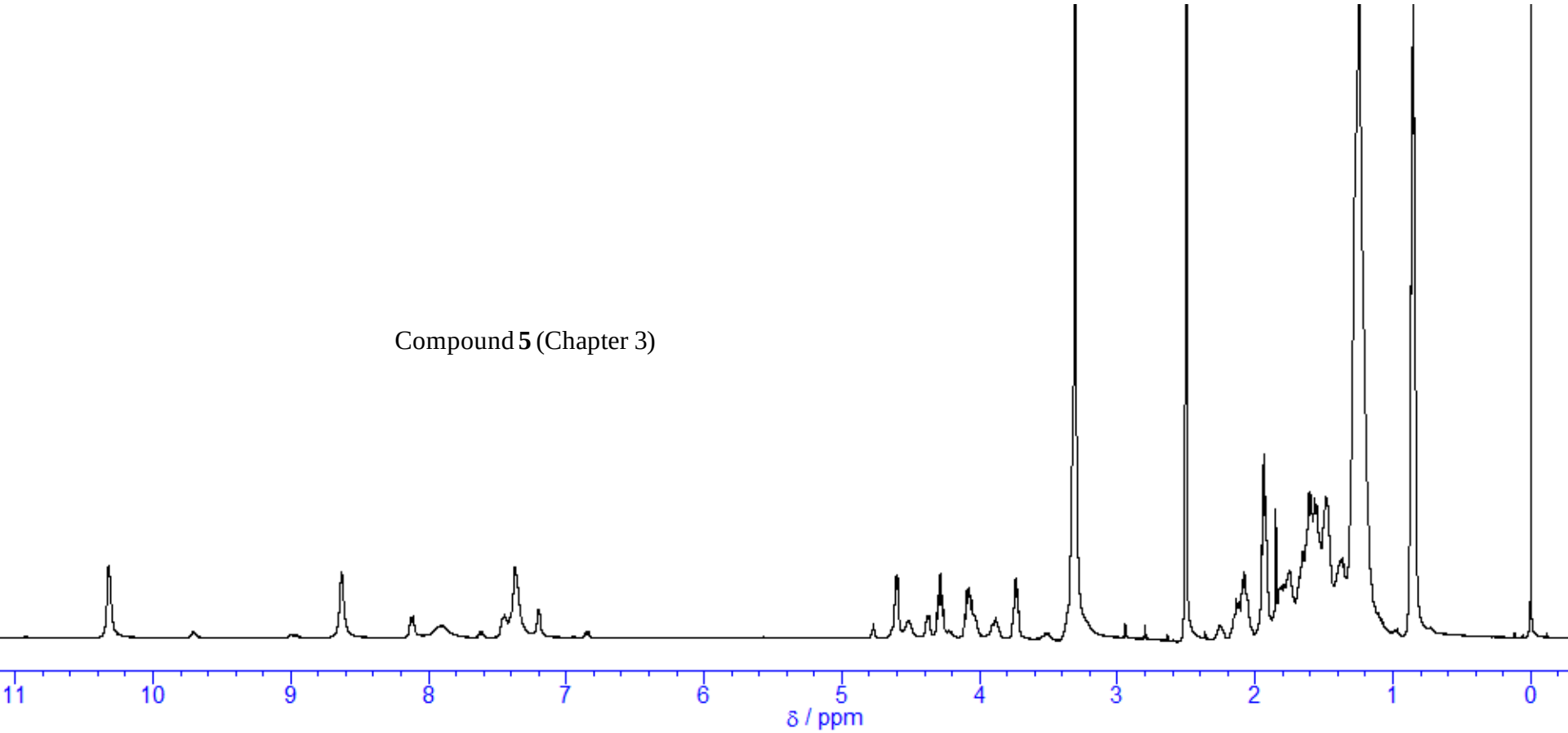

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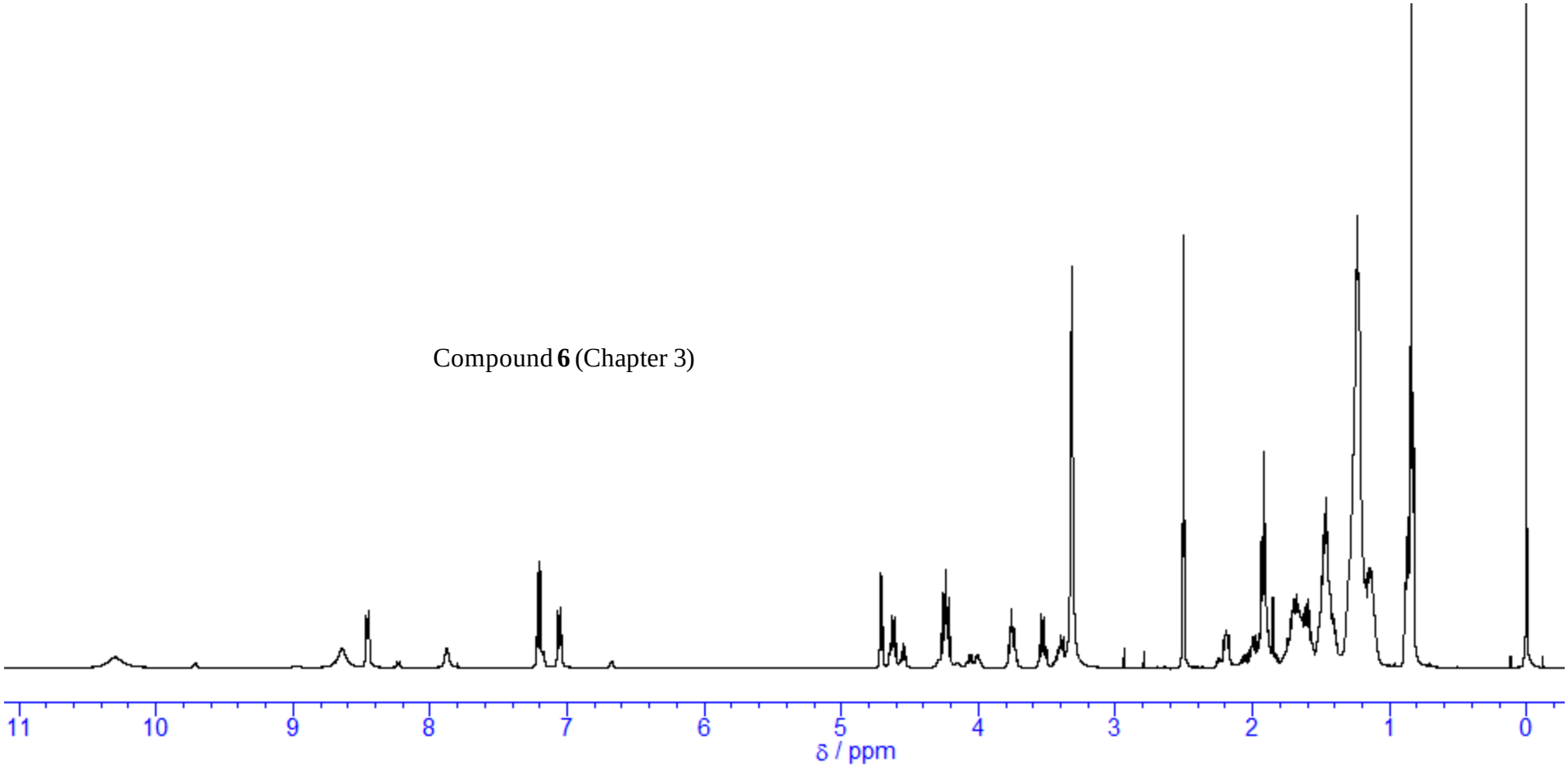

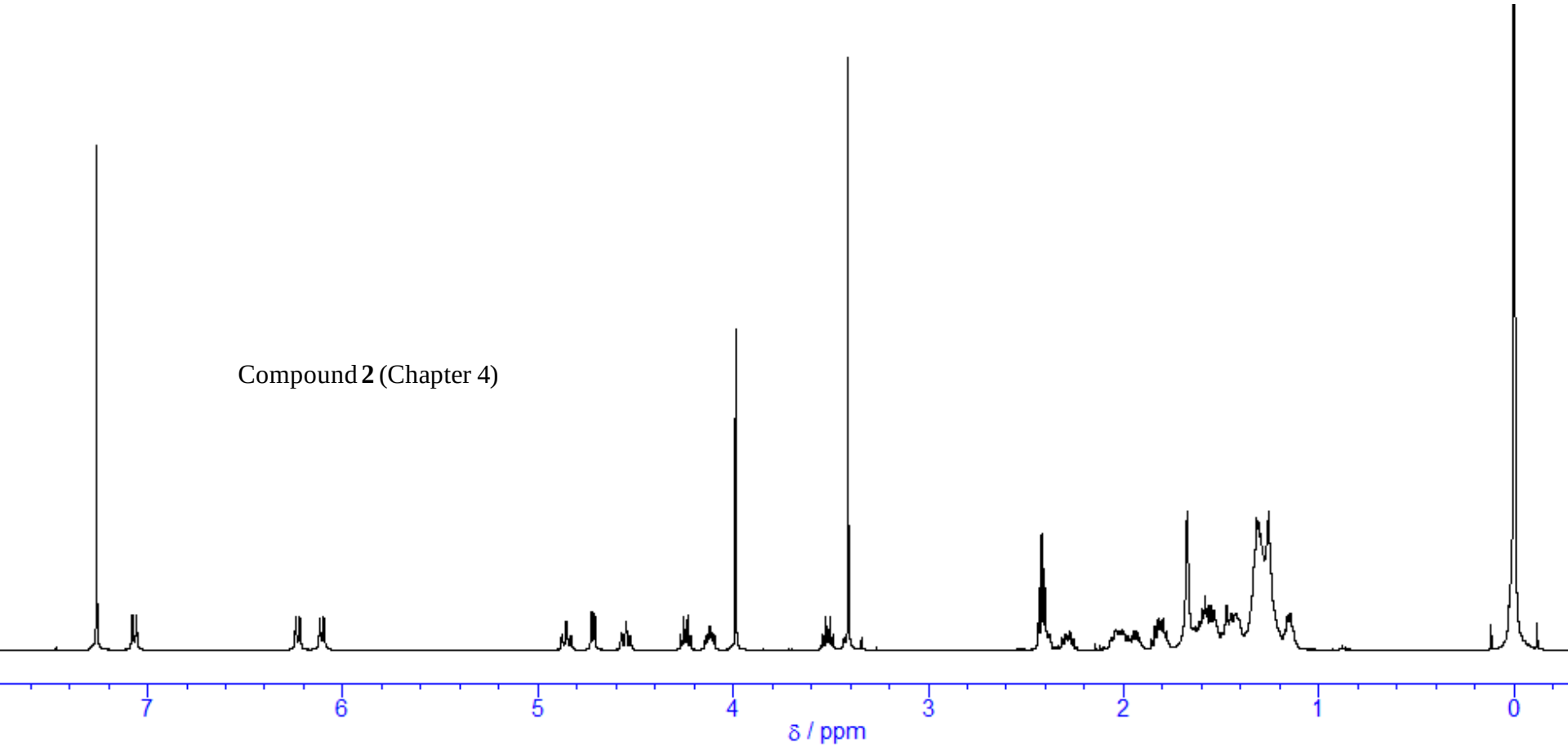

x

Compound **3** (Chapter 4)