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Synthesis and histone deacetylase inhibitory activity of cyclic tetrapeptides containing a retrohydroxamate as zinc ligand

Norikazu Nishino,[a,b,*] Daisuke Yoshikawa,[c] Louis A. Watanabe,[c] Tamaki Kato,[a] Binoy Jose,[b] Yasuhiko Komatsu,[b] Yuko Sumida[b] and Minoru Yoshida[b,d]

[a]*Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, Kitakyushu 808-0196, Japan*
[b]*CREST Research Project, Japan Science and Technology Agency, Saitama 332-001, Japan*
[c]*Faculty of Engineering, Kyushu Institute of Technology, Kitakyushu 808-8550, Japan*
[d]*RIKEN, Saitama 351-0198, Japan*

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Abstract—Cyclic tetrapeptide retrohydroxamic acids were prepared as histone deacetylase (HDAC) inhibitors and evaluated the inhibitory activity and found that they have potential as anticancer drugs.
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Histone deacetylases (HDACs) are zinc hydrolases responsible for the deacetylation of *N*-acetyl lysine residues of histone and nonhistone protein substrates.[1] Human HDACs are classified into two distinct classes, the HDACs and sirtuins. The HDACs are divided into two subclasses based on their similarity to yeast histone deacetylases Rpd3 (Class I includes HDAC1, 2, 3, 8, and 11) and Hda1 (Class II includes HDAC4, 6, 7, 9, and 10).[2] All of the HDACs have a highly conserved zinc dependent catalytic domain. There is growing evidence that the acetylation state of proteins and thus HDAC enzyme family plays a crucial role in the modulation of a number of biological processes, including transcription,[3] microtubule structure, and function,[4] and the cell cycle.[5] In the past few years, a number of HDAC inhibitors such as trichostatin A (TSA),[6] SAHA,[7] and CHAPs[8] (Fig. 1) have been reported as useful tools to study the function of chromatin acetylation and deacetylation, and gene expression. Development of more inhibitors for HDAC is necessary not only as probe for biological functions of the different HDACs but also as therapeutics such as inhibition of a specific HDAC that is associated with a particular disease.[9]

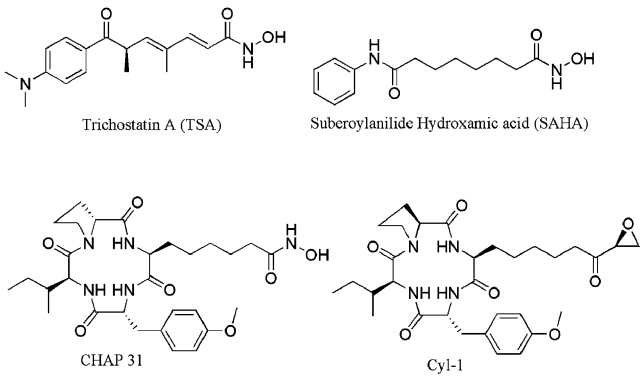

Figure 1. HDAC inhibitors.

The essential feature of an HDAC inhibitor is the zinc binding ligand with a spacer attached to a hydrophobic scaffold. Most of the reported HDAC inhibitors have hydroxamic acid as the zinc binding ligand. We have reported the SAR study of a series of cyclic tetrapeptide hydroxamic acids (CHAPs) and found that CHAPs having the cyclic tetrapeptide part of natural cyclic peptide Cyl-1, as the best of the series with nanomolar activity and therefore we retained the cyclic tetrapeptide scaffold in this study.[8] Recently, a group of researchers from Merck reported the side chain modification of apicidin for the synthesis of more active HDAC inhibitors by replacing the ethyl ketone of apicidin with other zinc binding ligands.[10] Other ligands such as, α-keto

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* Corresponding author. Tel./fax: +81-936956061; e-mail: nishino@life.kyutech.a.jp

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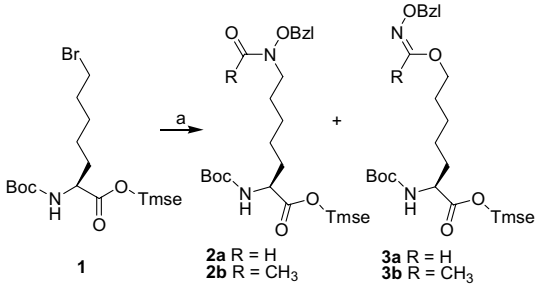
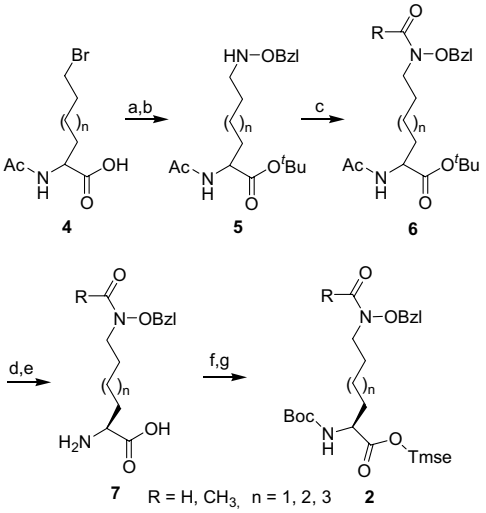
amides[11] and trifluoromethyl ketones[12] were also reported recently. We reported the synthesis and HDAC activity of cyclic tetrapeptide containing sulfhydryl group as zinc binding functionality.[13] For the development of novel HDAC inhibitors with improved biological properties, we proposed to synthesize cyclic tetrapeptides with other suitable zinc binding ligand. Here we report the synthesis and HDAC inhibitory activity of cyclic tetrapeptides with *N*-formyl hydroxylamine (retrohydroxamate) and *N*-acetyl hydroxylamine as zinc binding ligands.

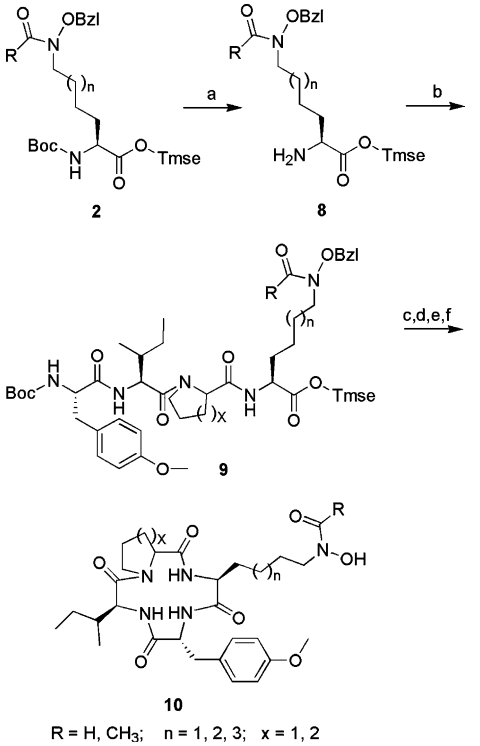
For the synthesis of the cyclic tetrapeptide containing a *N*-formyl hydroxylamine or *N*-acetyl hydroxylamine functional groups at the side chain, we need an amino acid containing retrohydroxamic acid at the ω-position of the side chain with a spacer of four to six methylene units. For this we started with recently reported compound, ω-bromo-α-aminoalkanoic acid.[14] Two routes were selected for the synthesis of retrohydroxamates. First route is, the reaction of protected α-amino-7-bromoheptanoic acid **1** with *N*-formyl-*O*-benzylhydroxylamine.[15] This resulted in the formation of two products as shown in Scheme 1. The required compound **2** was purified by silica gel column chromatography in 25% yield. In the second route, we started with *N*-acetyl-DL-7-bromoheptanoic acid (**4**) to prepare the *t*-butyl ester and reacted it with *O*-benzylhydroxylamine. The product obtained was further treated with acetic anhydride and subsequent trifluoroacetic acid treatment, and enzymatic reaction yielded the desired product **7**. It was then protected with Boc group and 2-(trimethylsilyl)ethanol to yield **2** (Scheme 2). The overall yield obtained by both methods were almost same. After Boc deprotection, the amino acid containing retrohydroxamate **8** was coupled with Boc-D-Tyr(Me)-L-Ile-DL-Pip-OH to yield the diastereomeric linear tetrapeptide **9**. After deprotection of the 2-(trimethylsilyl)ethanol group and the Boc group, the tetrapeptide was cyclized using HATU in DMF under high dilution condition to give the cyclic peptide in 65% yield (Scheme 3). Cyclic tetrapeptides containing D-Pip and L-Pip were separated by silica gel column chromatography and characterized by ^{1}H NMR spectra. The benzyl group was deprotected to yield the proposed cyclic tetrapeptide retrohydroxamic acids **10a**–**l**.[16] Using the same strategy, we synthesized 12 cyclic tetrapeptides containing a *N*-formyl hydroxylamine or *N*-acetyl hydroxylamine as the zinc binding ligand as shown in Table 1.



Scheme 1. Reagents and conditions: (a) KI, K_2CO_3, dry acetone, *N*-formyl-*O*-benzylhydroxylamine or *N*-acetyl-*O*-benzylhydroxylamine, reflux, 36 h (25–35% of **2**).



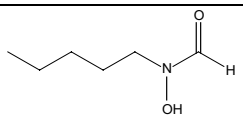
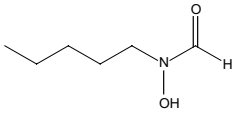
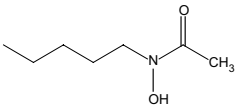
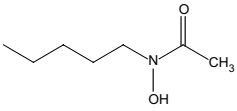
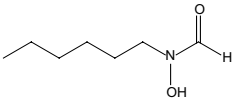
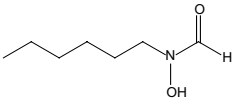
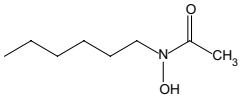
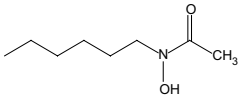
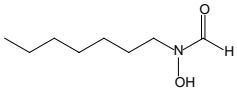
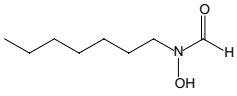
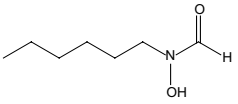
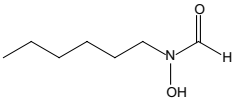
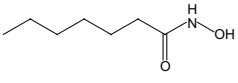
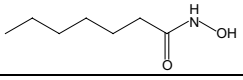
Scheme 2. Reagents and conditions: (a) Boc_2O, DMAP, 100%; (b) *O*-benzylhydroxyl amine, DIEA/MeOH, 40–50%; (c) Ac_2O or HCOOH, 65–70%; (d) TFA, 100%; (e) acylase, 74–80%; (f) Boc_2O, Et_3N, 100%; (g) TmseOH, DCC, DMAP, 90–100%.



Scheme 3. Reagents and conditions: (a) TFA, 100%; (b) Boc-D-Tyr(Me)-L-Ile-X-Xaa-OH, HBTU, HOBt·H_2O, Et_3N, 70–80%; (c) TBAF/THF, 90–100%; (d) TFA, 100%; (e) HATU, DIEA, 50–60%; (f) Pd/C, H_2, 90–100%.

These compounds were tested for the HDAC inhibitory activity using HDAC prepared from mouse melanoma B16/BL6 cells.[17] The IC_{50} values of the compounds are given in Table 1. Compounds **10e** and **10f** having five

Table 1. HDAC inhibitory data for retrohydroxamates and selected CHAPs

Compound	Sequence	Spacer and ligand	Configuration	Chain length	HDAC inhibitory activity IC_{50} (μM)
10a	*cyclo*(-L-Lys(For,OH)-D-Tyr(Me)-L-Ile-L-Pip-)		LDLL	4	1.392
10b	*cyclo*(-L-Lys(For,OH)-D-Tyr(Me)-L-Ile-D-Pip-)		LDLD	4	0.957
10c	*cyclo*(-L-Lys(Ac,OH)-D-Tyr(Me)-L-Ile-L-Pip-)		LDLL	4	88.5
10d	*cyclo*(-L-Lys(Ac,OH)-D-Tyr(Me)-L-Ile-D-Pip-)		LDLD	4	88.4
10e	*cyclo*(-L-Hly(For,OH)-D-Tyr(Me)-L-Ile-L-Pip-)		LDLL	5	0.141
10f	*cyclo*(-L-Hly(For,OH)-D-Tyr(Me)-L-Ile-D-Pip-)		LDLD	5	0.084
10g	*cyclo*(-L-Hly(Ac,OH)-D-Tyr(Me)-L-Ile-L-Pip-)		LDLL	5	58.0
10h	*cyclo*(-L-Hly(Ac,OH)-D-Tyr(Me)-L-Ile-D-Pip-)		LDLD	5	43.8
10i	*cyclo*(-L-Aoc(For,OH)-D-Tyr(Me)-L-Ile-L-Pip-)		LDLL	6	1.350
10j	*cyclo*(-L-Aoc(For,OH)-D-Tyr(Me)-L-Ile-D-Pip-)		LDLD	6	0.893
10k	*cyclo*(-L-Hly(For,OH)-D-Tyr(Me)-L-Ile-L-Pro-)		LDLL	5	NT
10l	*cyclo*(-L-Hly(For,OH)-D-Tyr(Me)-L-Ile-D-Pro-)		LDLD	5	NT
CHAP49[8b]	*cyclo*(-L-Asu(NHOH)-D-Tyr(Me)-L-Ile-L-Pip-)		LDLL	5	0.00396
CHAP50[8b]	*cyclo*(-L-Asu(NHOH)-D-Tyr(Me)-L-Ile-D-Pip-)		LDLD	5	0.00481

Hly is homolysine and Aoc is 2,8-diamino octanoic acid, NT = not tested.

methylene group as spacer and retrohydroxamate as the functional group showed high inhibitory activity. On the other hand, compounds with the same spacer length and same cyclic tetrapeptide scaffold, but containing an acetyl group instead of the formyl group showed very weak activity possibly due to the presence of the bulky methyl group instead of the hydrogen atom. Compounds with four methylene spacers and six methylene

Table 2. HDAC inhibitory data for selected compounds

Compound	HDAC inhibitory activity IC_{50} (μM)				p21 Promoter assay
	HDAC1	HDAC4	HDAC6	HDAC8	EC_{1000} (μM)
TSA	0.019	0.020	0.028	0.04	0.19
CHAP30	0.0044	NT	0.110	NT	NT
CHAP31	0.0004	0.003	0.013	NT	0.022
10f	0.026	0.074	0.782	0.046	NT
10k	0.033	0.089	0.029	0.034	NT
10l	0.067	0.19	0.178	0.059	0.61

NT = not tested.

spacers showed weak activity in comparison with five methylene spacer indicating that the spacer length is optimum with five methylene units as in the case of CHAPs. Compounds with LDLD combination showed better activity than LDLL combination.

The inhibitory activity toward the subclass of HDAC for selected compounds is shown in Table 2.[18] For comparison, the inhibitory activities of TSA and corresponding CHAPs, such as CHAP31 and CHAP 30 are also shown. Retrohydroxamates inhibit HDACs in almost equal extent as TSA. However, the inhibitory activity of retrohydroxamates is lower than that of CHAP31. In an early report on retrohydroxamate inhibitor for thermolysin, the inhibitory activity was also reduced about one tenth of the corresponding hydroxamic acid.[19] The HDAC inhibitory activity of retrohydroxamates is almost similar for all the members of the HDAC family. Proposed mode of interaction of retrohydroxamates with zinc ion at the active site pocket is shown in Figure 2.

Retrohydroxamates are reported as potent, long-lived, and bioavailable matrix metalloproteinase inhibitors.[20] Therefore these cyclic tetrapeptide retrohydroxamates may have improved biological properties than CHAPs even though the inhibitory activity is lower than

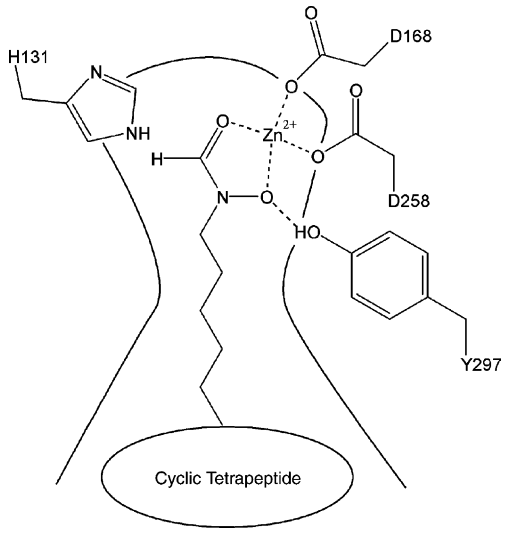


Figure 2. Possible interactions of zinc ion with retrohydroxamate functional group in HDAC. The amino acid numbering correspond to the literature.[22]

CHAPs. During the synthesis of these cyclic tetrapeptides containing retrohydroxamates, a report on straight chain retrohydroxamates was published.[21] Our cyclic tetrapeptide retrohydroxamates are more than 10 times potent than those reported retrohydroxamates indicating the importance of the cyclic tetrapeptide cap group.

We synthesized cyclic tetrapeptides containing a retrohydroxamic acid as suitable replacement of the hydroxamic acid side chain of HDAC inhibitors. Some of these compounds such as **10f** and **10l** showed inhibitory activity in nanomolar concentrations. In conclusion, it has been shown that alternatives to the hydroxamate zinc-binding group can lead to potent inhibitors of HDAC and anticancer agents.

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