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Design and Synthesis of Cyclopeptide Analogues of the Potent Histone Deacetylase Inhibitor FR235222

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*Various structurally modified analogues of FR235222 (**1**), a natural tetrapeptide inhibitor of mammalian histone deacetylases, were prepared in a convergent approach. The design of the compounds was aimed to investigate the effect of structural modifications of the tetrapeptide core involved in enzyme binding in order to overcome some synthetic difficulties connected with the natural product **1**. The modifications introduced could also help identify key structural features involved in the mechanism of action of these compounds. The prepared molecules were subjected to in vitro pharmacological tests, and their potency was tested on cultured cells. Two of the components of the array were found to be more potent than the parent compound **1** and almost as efficient as trichostatin A (TSA). These results demonstrate that it is possible to synthesize highly active cyclic tetrapeptides using commercially available amino acids (with the exception of 2-amino-8-oxodecanoic acid, Ahoda). The nature of the residue in the second position of the cyclic peptide and the stereochemistry of the Ahoda tail are important for the inhibitory activity of this class of cyclic tetrapeptide analogues.*

Introduction

Progress made in biology and medicine over the last two decades has shown that cellular events can be regulated by mechanisms inheritably affecting specific phenotypes without altering the underlying genotypes. These mechanisms include chemical modifications of histones and DNA, which are proposed to convey a part of the so-called epigenetic information.[1,2] Acetylation is one of the best-understood histone modifications, which takes place at the ε-amino group of lysine residues. It is influenced by the antagonistic activities of histone deacetylases (HDACs) and histone acetyltransferases (HATs).[3] By targeting these key histone-modifying enzymes, it has been possible to affect important downstream cellular events.[4] Accordingly, HDAC inhibitors are very promising chemotherapeutic agents capable of blocking angiogenesis[5] and cell proliferation, as well as promoting apoptosis and differentiation.[6,7] Recently, the immunosuppressant fungal metabolite FR235222 (**1**), emerged as a potent natural inhibitor of mammalian HDACs.[8–10] It acts as a reversible zinc chelator and belongs to the class of cyclic tetrapeptide HDAC inhibitors, some of which have already been shown to act as effective anticancer agents.[11] This compound, therefore, represents an attractive basis for new drug designs, and its total synthesis was recently completed by us[12] and others.[13] We also proposed a 3D model for the interaction of this natural ligand with its biological target.[12] The published information on the crystal structure of complexes between known HDAC inhibitors and HDLP,[14] a bacterial homologue of mammalian HDAC enzymes, has proven essential for the establishment of our model and has allowed us to propose a general binding mode of these molecules to the enzyme active site with the following key features involved in HDAC inhibition: a) the α-hydroxyketone function-

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Supporting information for this article is available on the WWW under http://www.chemmedchem.org or from the author: characterization data for compounds **4**–**12**.

ality, which represents the pharmacophore and is responsible for the crucial zinc ion complexation event; b) the carbon chain of the Ahoda residue, which constitutes the linker domain and has the appropriate length for correct insertion into the long, narrow channel of the enzyme active site, and for projecting the zinc chelating element at an optimal distance for metal chelation; and finally, c) the cyclopeptidic core, which plays the role of a surface recognition domain.

Taking into account this information and considering the great interest evoked by the pharmacological properties of FR235222, we undertook the synthesis of a focused array of analogues of the natural compound **1**. Our goal was to produce active compounds that are easier to synthesize and that allow the identification of important structural aspects of the inhibitor–enzyme interactions. In compounds **3**–**7**, (Figure 1) we left the (2*S*,9*R*)-Ahoda fragment, which constitutes both the linker and the zinc ion chelating element, completely unchanged, while the two rare amino acids present in FR235222 were varied in order to simplify the crucial synthetic features of the parent compound. Compounds **8**–**12** (Figure 1) have a different stereochemical arrangement of the Ahoda fragment (2*S*,9*S*) as the functional tail and further chemical diversity at position 2 relative to the parent compound **1**.

Results and Discussion

The array of 11 new FR235222 analogues was synthesized through a convergent strategy of Fmoc-based peptide synthesis (Scheme 1) starting from Fmoc-D-proline (for compounds **2**–**6** and **8**–**12**, Figure 1) and Fmoc-D-4-dimethylproline (for compound **7**)[15] as the first amino acid attached to the resin. The (2*S*,9*R*)- and (2*S*,9*S*)-Ahoda diastereomers were prepared as previously described.[16] To establish a solid-phase peptide chemistry protocol that provides more efficient coupling and cyclization conditions, we started with the construction of compound **2**, in which we used only commercially available amino acids, including the functional tail represented by a homoserine residue.

The 2-chlorotrityl chloride resin was submitted to coupling–deprotection cycles using HOBt and HBTU as activating agents and piperidine (20%) for Fmoc deprotection. To avoid the undesired formation of diketopiperazine at the dipeptide level

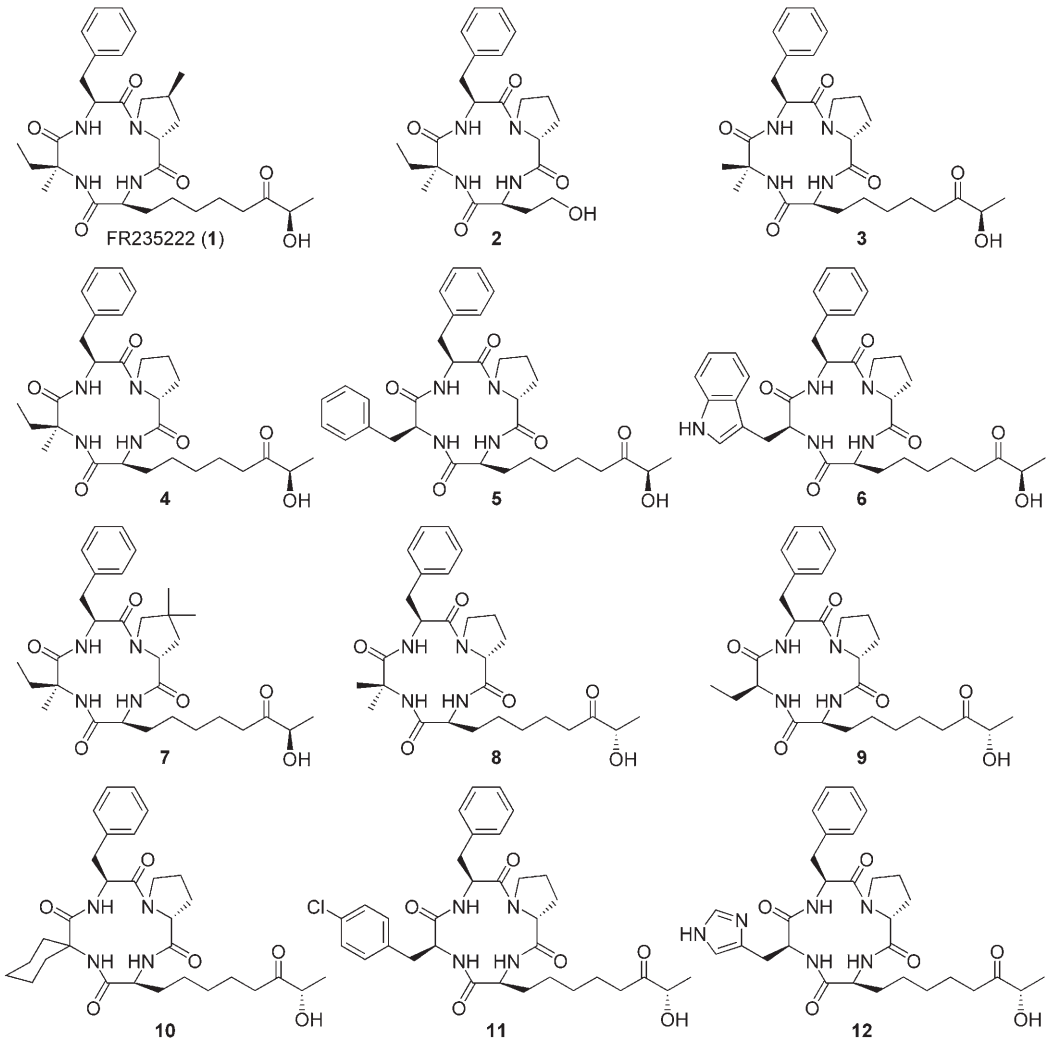
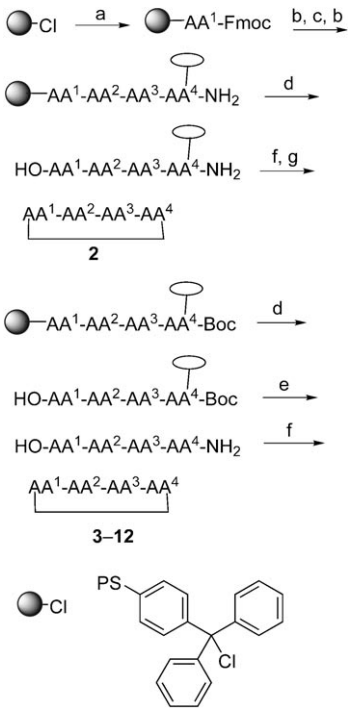


Figure 1. FR235222 (**1**) and analogues **2**–**12**.



Scheme 1. Reagents and conditions: a) DIEA, CH_2Cl_2; b) piperidine (20%) in DMF; c) HOBt-HBTU-NMM, DIEA, DMF; d) AcOH/TFE/CH_2Cl_2; e) TFA/H_2O/TIS (95:4:1); f) HATU, DIEA, DCM/DMF (10–5M); g) TFA/TIS/CH_2Cl_2 (1:5:94).

during Fmoc removal in piperidine, we performed a fast Fmoc deprotection cycle at the level of the second coupling reaction (see Experimental Section below). In the case of the analogues containing an α-disubstituted residue in the third position, we used a larger excess of base and activators (see Experimental Section) to drive the coupling step to completion. The linear tetrapeptides were then cleaved from the resin by treatment with a solution of acetic acid/CH_2Cl_2/TFE followed by removal of the Boc group from Ahoda using TFA/H_2O/TIS. The resulting linear peptides were cyclized in CH_2Cl_2/DMF under highly dilute conditions with HATU as coupling reagent, to give the desired cyclopeptides. The crude products were purified by RP HPLC using H_2O/CH_3CN as the mobile phase. The same procedure was repeated for compounds **3**–**12** and proved to be satisfactory in terms of yields and purity of products obtained. The structures of **2**–**12** were fully elucidated through ESMS and NMR spectroscopic analysis. To evaluate the potency of these modified cyclotetrapeptides as HDAC inhibitors, their activity was compared with that of the parent synthetic compound FR235222 (**1**) and the known small-molecule HDAC inhibitor TSA.[17] The test system employed was the deacetylation of an acetylated peptide substrate, corresponding to the histone H4 tail.

The deacetylation reaction was catalyzed by deacetylases present in a nuclear extract of HeLa cells[18] and was monitored using SELDI-TOF mass spectrometry. Figure 2 shows the dose–response effect observed for the parent compound **1** as a typical example of the results obtained using this assay system. We initially approached compound concentrations that give 50% inhibition by successive dichotomy. For a more precise

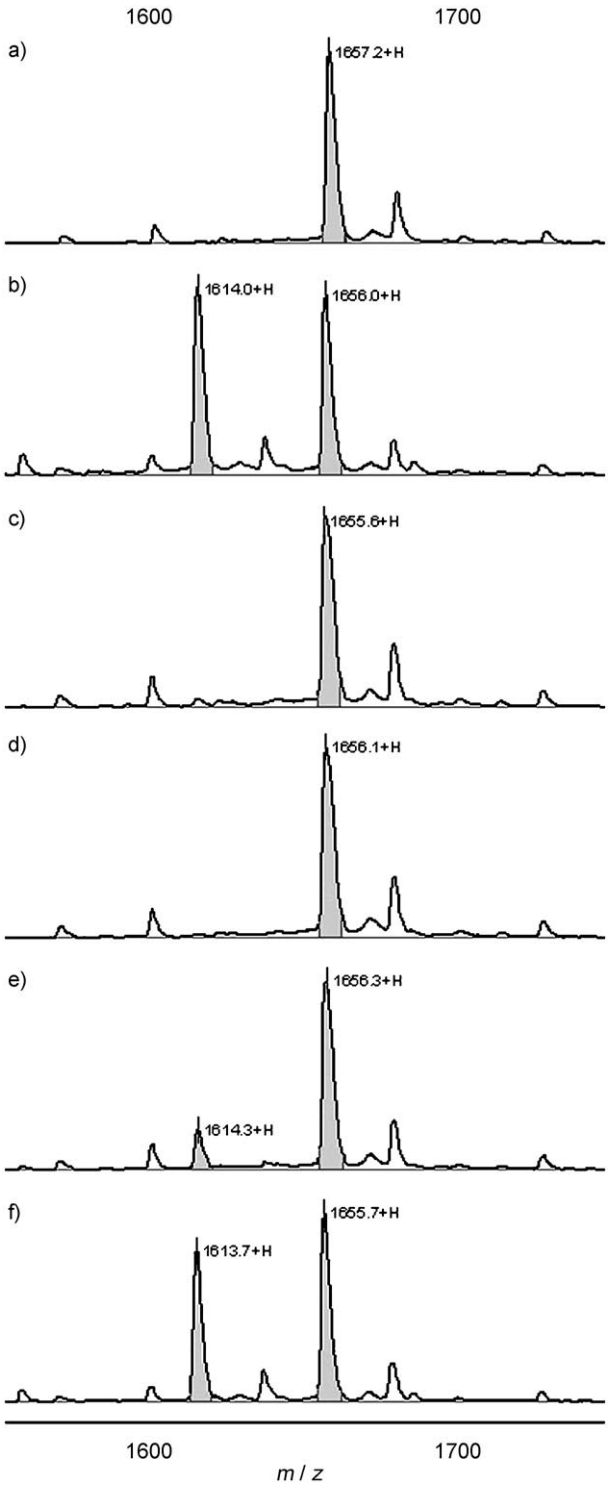


Figure 2. Monitoring the inhibitory activity of compound **1** as a representative assay result. Shown are mass spectra of an acetylated peptide of histone H4 incubated a) without or b) with a HeLa nuclear extract, whereupon ~50% of the peptide is deacetylated by HDACs present in the extract. Inhibition of this deacetylation reaction in the presence of c) TSA and d) compound **1** at 10 μM, e) 100 nM, f) 1 nM. The two major peaks (shaded) correspond to the acetylated (1656 Da) and deacetylated (1614 Da) peptide variants.

determination of the IC_{50} values, we then focused on three concentrations for each compound. The spectra obtained were

used to calculate percent inhibition (see Experimental Section), and Figure 3 shows these values plotted against the concentrations used. Although high reproducibility was observed during the setup of the enzyme-based assay, each experiment was repeated two or three times depending on the variation in observed values. From these data the approximate IC_{50} values were calculated for each compound (Table 1).

Compound **2** has the same tetrapeptide structure of **1**, but lacks the chelating side chain. This compound is inactive under our assay conditions, which confirms the hypothesis that the Ahoda residue acts as a potent functional group for HDAC inhibition, fitting well in the narrow, tubelike active site, indicating that this portion of the molecule tethers the zinc ion. The results obtained from compound **7** indicate that an additional methyl group on the D-MePro residue decreases inhibitory activity (IC_{50} ~ 90 nM) relative to the parent compound (IC_{50} ~ 60 nM). On the other hand, the decrease in steric bulk at this position by removal of the methyl group to give **4** (IC_{50} = 50 nM) does not significantly change the IC_{50} value relative to that of **1** (see Figure 3), indicating that the methyl group on the proline residue does not contribute to inhibitory potency.

Substitution of L-Iva with achiral Aib (in compound **3**) results in a slight increase in inhibitory activity, with an IC_{50} value of 30 nM. Similarly, if L-Iva is substituted by L-Trp, we observe an increase in the inhibitory potency with IC_{50} = 20 nM. The analogues **3** and **6** perform slightly better than the natural product FR235222, with compound **6** being almost as efficient as TSA, which exhibits an IC_{50} value of 15 nM under our assay conditions. Notably, an aryl group in the same position (compound **5**) gives a dramatic decrease in inhibitory activity: **5** is 14-fold less potent than compound **6**. The fact that changes at this position modify the potency of the compounds suggests that the cyclotetrapeptide-based cap group contributes to the high-affinity binding to HDACs, thereby strengthening the inhibitory activity of the zinc chelating element.[19] This high affinity is consistent with the model that selective hydrophobic binding interactions near the exit of the channel establish cooperative effects with the interactions at the rim of the catalytic pocket. The cyclic tetrapeptide with hydrophobic amino acids probably makes extensive contacts at the border of the catalytic site and in the shallow grooves surrounding the

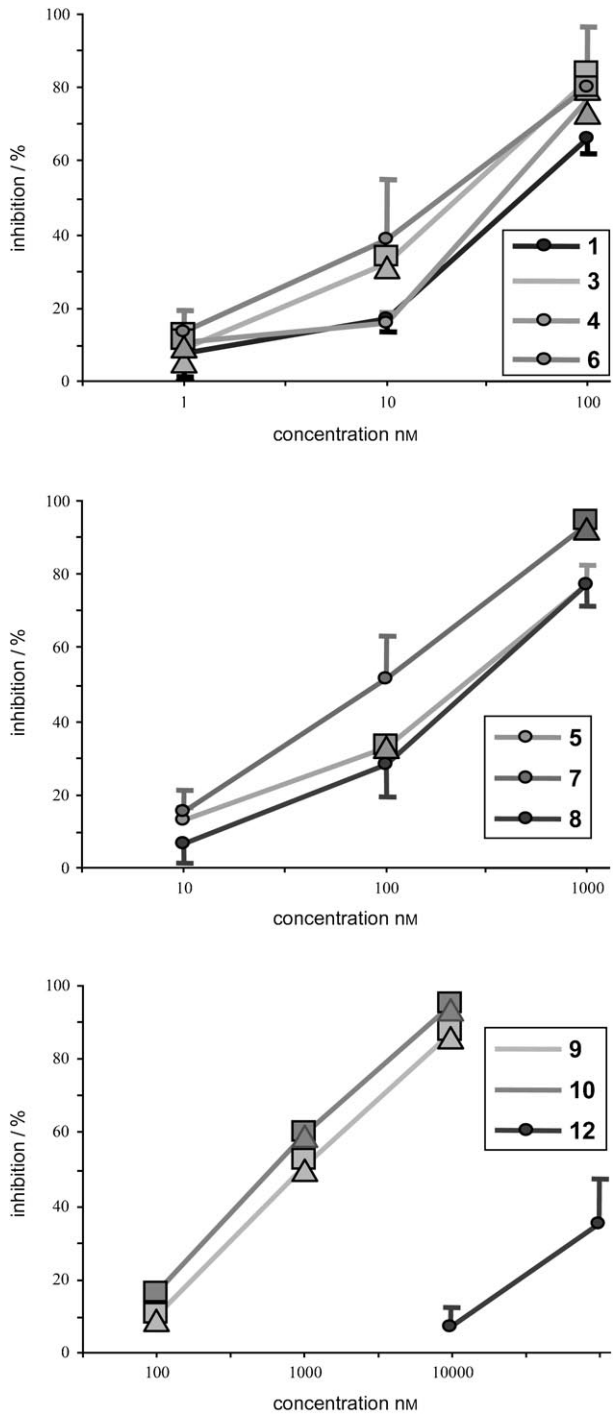


Figure 3. Inhibitory activities of the synthetic compounds at various concentrations measured as described in Figure 2. Values are either obtained from two independent experiments (□,△) or from at least three independent experiments (○). For clarity in distinguishing individual curves, compounds are shown on three different graphs depending on their activity; note the scale differences between the *x* axes.

Table 1. IC_{50} values of FR235222 analogues for the inhibition of histone H4 peptide deacetylation.

Compd	IC_{50} [nM]
TSA	15
sodium butyrate	200000
1	60
2	NA[a]
3	30
4	50
5	280
6	20
7	90
8	330
9	1000
10	670
11	ND[b]
12	~350000

[a] Not active at 100 μM (the highest concentration tested). [b] Not determined.

pocket entrance and may mimic the natural substrate structure, bearing acetylated lysine.

As shown and discussed above, the Ahoda fragment is essential for the inhibitory activity of the cyclic tetrapeptides, and we wanted to investigate whether the stereochemical configuration of this side chain is important for the interactions of the pharmacophore at the catalytic site of the enzyme. Compound **8**, which is identical to compound **3** except for the stereochemistry of the Ahoda fragment, is 10-fold less potent than the epimer **3**, indicating that the stereochemistry of the C9 OH group plays an important role in HDAC inhibition.[20] Also in the *R*-configured series, Aib was replaced with various residues. The corresponding L-Abu analogue **9**, however, is 3-fold less potent than **8**, suggesting that an α,α-disubstitution at the aliphatic amino acid between Phe and Ahoda is preferable. The substitution of the two methyl groups of Aib with a cyclohexyl scaffold, as in **10**, does not improve enzyme inhibition relative to the acyclic amino acids ($IC_{50}=670$ nM). In analogue **11**, Aib was replaced with *p*ClPhe, but we were unable to determine a reliable IC_{50} value for this compound because of its tendency to precipitate, showing low solubility even in DMSO. Finally, the insertion of L-His between Phe and Ahoda (in **12**) drastically decreases the inhibitory activity, showing an IC_{50} in the near millimolar range, almost like that of sodium butyrate. This finding indicates that increasing the polarity at the aryl group does not improve enzyme inhibition, probably because selective hydrophobic contacts at the rim of the catalytic pocket are modified. Although we were not able to improve the inhibitory potency of the stereoisomeric compound by modulating the amino acid residue between Phe and Ahoda, we noted extreme differences in the inhibitory activities of the various compounds. This observation further confirms the importance of the side chain at position 2 in the cyclic tetrapeptides.

The ability of the various compounds to enter the cell and to act on HDACs in live cells was tested by monitoring the acetylation of cellular proteins in rat aortic smooth muscle (A7r5) cells.[21] We used the monoclonal antibody AKL5C1,[22] which is directed against internal, acetylated lysine residues, to monitor acetylated proteins in total cell lysates of A7r5 cells on Western blots. As shown in Figure 4, this antibody recognizes acetylated histones and gives a very faint signal for acetylated tubulin in lysates of untreated cells (Figure 4, lanes 0). As expected, treatment with either sodium butyrate or TSA (Figure 4, lanes B and T, respectively) results in a dramatic increase in acetylated histones, but enhanced tubulin acetylation is observed only after incubation with TSA. This is in agreement with earlier observations showing that TSA acts as a general inhibitor of class I and II HDACs. The Western blot in Figure 4 further shows that the different compounds affect histone acetylation to various degrees, indicating that they are able to enter the cell. Tubulin acetylation, however, is not influenced by these compounds, implying that the tubulin deacetylase HDAC6 is not inhibited.[23–25]

To obtain quantitative data for the effect of these compounds on cultured cells, we scanned the signal for acetylated histone H4 on Western blots of three independent experiments (Figure 5). The potency of the various inhibitors follows the IC_{50} values obtained in vitro except for compounds **5** and **6**, which have a lower potency toward cultured cells in comparison with the corresponding in vitro data. Taking into account the effect of the different compounds on histone and tubulin acetylation, these experiments strongly suggest that our compounds act as class I HDAC inhibitors. In fact, HDAC6 is the only member of class II HDACs shown to have an intrinsic HDAC activity,[26] whereas other class II HDACs seem to use class I members to direct histone deacetylation.[27]

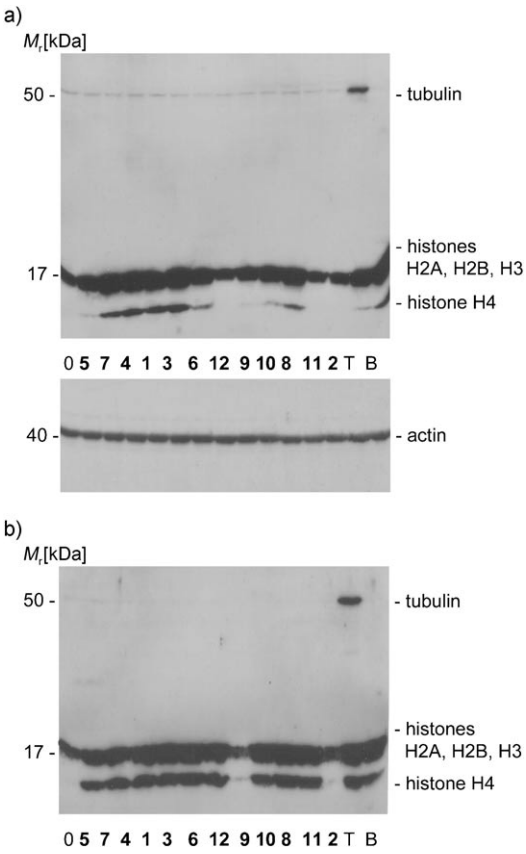


Figure 4. A7r5 cells were cultured for 3 h in the absence of inhibitor (lanes 0) or in the presence of FR235222 (**1**), its various analogues (also as indicated), TSA (T), or sodium butyrate (B). a) Compound concentrations were 1 μM each; TSA concentration was 300 nM. b) Compound concentrations were 10 μM each; TSA concentration was 1 μM. In all cases, sodium butyrate was used at 10 mM. Cells were then lysed directly in Laemmli buffer, and total cell lysates were separated by 12% SDS-PAGE, Western blotted, and acetylated proteins were detected with the AKL5C1 antibody. Part a) includes a Western blot in which a polyclonal antibody against actin was used as loading control; Western blots are representative of three independent experiments.

Finally, a preliminary proliferative test was carried out on A7r5 cells, in which our compounds were added at a concentration of 1 μM and compared with the results obtained with TSA (T, 330 nM) and sodium butyrate (B, 10 μM). As shown in Figure 6, compounds **3**, **4**, and **7** showed behavior similar to that of **1** and to the reference compounds.

In summary, we have shown that by starting from the natural product FR235222 as a model, it is possible to use commercially available amino acids for the synthesis of simplified ana-

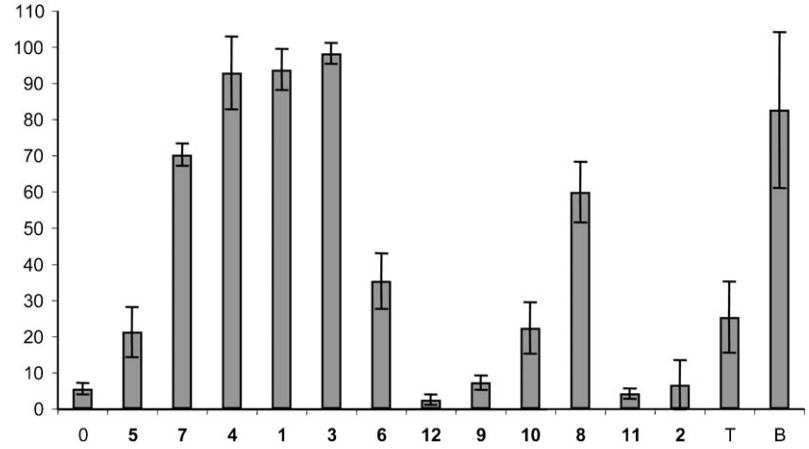


Figure 5. Quantification of the effect of the different compounds (all at 1 μM, except for TSA used at 300nM and sodium butyrate at 10nM) on histone acetylation. The signal for acetylated histone H4 on Western blots, as shown in Figure 4a, was scanned using blots of three independent experiments. Shown are mean values ± SD expressed in arbitrary units.

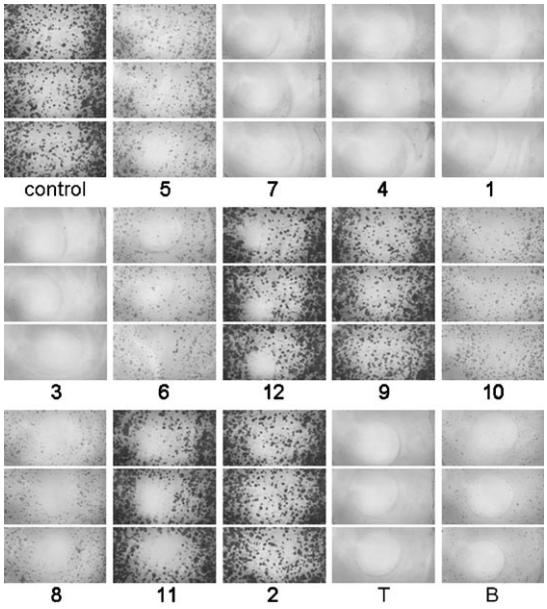


Figure 6. Proliferation of A7r5 cells in the absence of inhibitor (control) or in the presence of compounds as indicated, each at a concentration of 1 μM, or TSA (330 nM), or sodium butyrate (10 mM).

logues that preserve the inhibitory activity of the parent compound. The main structural feature to be maintained is the Ahoda (2-amino-9-hydroxy-8-oxadecanoic acid) side chain, which acts as a potent enzyme-inhibiting group responsible for zinc chelation. Compounds that are composed of an Ahoda fragment in the 2*S*,9*R* configuration are generally more active than compounds bearing a (2*S*,9*S*)-Ahoda side chain. Our results also show that changing the L-Iva residue at position 2 in the cyclic tetrapeptide can modulate the inhibitory potency of the analogues. Compounds that have an α,α-disubstituted amino acid or a bulky aromatic side chain (compounds **3**, **4**, and **6**) next to the chelating element are strong inhibitors, whereas a simple aromatic side chain at that position is less effective (compound **5**). The importance of the Ahoda group and the presence of hydrophobic residues in the cap group, necessary for the optimal interaction with the rim of the active site, support the idea that the side chain that acts as a substrate mimetic, together with the cyclotetrapeptidic structure, play a synergistic role in inhibiting class I HDACs. These results are confirmed by an antiproliferative test on A7r5 cells.

Conclusion

This is one of first studies on the activity of compounds that combine a cyclic tetrapeptide structure as cap group and an Ahoda fragment as zinc chelating element. We have demonstrated that our inhibitors, similarly to other cyclic tetrapeptides lacking the Ahoda side chain,[28] are not active against HDAC6, but inhibit class I HDACs. Compared with other naturally occurring cyclotetrapeptides such as trapoxin,[29] our compounds show a lower in vitro activity (irreversible inhibition of HDAC1 by trapoxin with $IC_{50} = 0.82$ nM).[30] However, trapoxin and its derivatives only show weak activity in vivo, probably due to the chemical instability of the epoxyketone fragment. Our synthetic compounds elude this issue because of the presence of the stable hydroxyketone moiety, as is the case with the CHAP series of synthetic tetrapeptide inhibitors, which are composed of a cyclic tetrapeptide structure and a hydroxamic acid side chain.[28] In comparison with TSA, the CHAP series of synthetic compounds are, however, less active than the inhibitors described herein. Our compounds will therefore be useful for the characterization of cellular events governed by HDAC enzymes and may have great potential for the design and development of more simplified analogues. Further tests in animal models of the most active compounds are in progress.

Experimental Section

General experimental procedures

All NMR spectra (^{1}H, HMBC, HSQC, TOCSY, COSY, ROESY) were recorded on a Bruker Avance DRX600, or on a Bruker Avance 300 MHz, at $T = 298$ K. Compounds **2**–**12** were dissolved in 0.5 mL [D_6]DMSO (99.95%, Carlo Erba, 99.95 atom% D) (^{1}H NMR $\delta =$ 2.50 ppm, ^{13}C NMR $\delta = 39.5$ ppm). NMR data were processed on a Silicon Graphics Indigo 2 workstation using UXNMR software. Chemical shifts are expressed as δ (ppm). Electrospray mass spectrometry (ESMS) was performed on an LCQ DECA TermoQuest mass spectrometer (San José, CA, USA). For estimation of Fmoc amino acids on the resin, absorbance at $\lambda = 301$ nm was recorded on a Shimadzu UV 2101 PC. Analytical and semipreparative reversed-phase HPLC was performed on a Jupiter C_{18} column (250 × 4.60 mm, 5μ, 300 Å, flow rate = 1 mL min^{-1}; 250 × 10.00 mm, 10μ, 300 Å, flow rate = 4 mL min^{-1}, respectively). The binary solvent

system (A/B) was as follows: 0.1% TFA in water (A) and 0.1% TFA in CH_3CN (B). The absorbance was detected at 220–240 nm.

Unless specified, solvents were reagent grade; they were purchased from Aldrich, Fluka, Carlo Erba. CH_2Cl_2 and DMF used for solid-phase reactions were synthesis grade (dried over activated molecular sieves (4 Å)). H_2O and CH_3CN were HPLC grade. 2-Chlorotrityl chloride resin (100–200 mesh), 1% DVB, (ClTrt-Cl, loading level: 1.27 mmol g^{-1} and 1.4 mmol g^{-1}), Fmoc-D-Pro-OH, Fmoc-L-His(Trt)-OH, Fmoc-L-Hse(Trt)-OH, Fmoc-L-Phe-OH, Fmoc-L-Trp(Boc)-OH, HOBt, and HBTU were purchased from Novabiochem. Fmoc-L-Abu-OH, Fmoc-L-Aib-OH, and Fmoc-*p*Cl-(L-Phe)-OH were obtained from Neosystem. (*S*)-(+)-2-amino-2-methylbutanoic monohydrate (Iva) was purchased from Acros Organics. HATU and Fmoc-Cl were purchased from Fluka. Solid-phase peptide syntheses, using the Fmoc-*t*Bu strategy, were carried out on a polypropylene ISOLUTE SPE column on a VAC MASTER system, a manual parallel synthesis device purchased from Stepbio. Fmoc-L-Iva, Boc-Ahoda,[16] Fmoc-D-(4-MePro)-OH, and Fmoc-D-(4-Me_2Pro)-OH were prepared as previously described.[12]

General procedures for the synthesis of compounds 2–12

a) Loading the resin: The PS-ClTrt-Cl (250.0–405.0 mg) resin was placed in a 25-mL polypropylene ISOSOLUTE syringe on a VAC MASTER system, swollen in 3 mL DMF for 1 h, and then washed with 2×3 mL CH_2Cl_2. A solution of Fmoc-AA-OH (1 equiv) and DIEA (4 equiv) in 2.5 mL dry CH_2Cl_2 was added, and the mixture was stirred for 2 h under a stream of N_2. The mixture was then removed, and the resin was washed with 3×CH_2Cl_2/MeOH/DIEA (17:2:1) and sequentially with CH_2Cl_2 (3×3 mL), DMF (2×3 mL), and CH_2Cl_2 (2×3 mL), for 1.5 min each. Resin loading was determined by UV/Vis quantitation of the Fmoc–piperidine adduct. The assay was performed on a duplicate sample: 0.4 mL piperidine and 0.4 mL CH_2Cl_2 were added to two dried samples of Fmoc-AA-resin in two volumetric flasks of 25 mL. The reaction was allowed to proceed for 30 min at room temperature, and then 1.6 mL MeOH were added, and the solutions were diluted to a volume of 25 mL with CH_2Cl_2. A reference solution was prepared in a 25-mL volumetric flask using 0.4 mL piperidine, 1.6 mL MeOH, and CH_2Cl_2 to volume. The solutions were shaken, and the absorbance of the samples versus the reference solution was measured at 301 nm. The substitution level (expressed in mmol AA (g resin)$^{-1}$) was calculated from the equation: mmol $g^{-1} = (A_{301}/7800) \times (25$ mL (g resin)$^{-1})$.[31]

b) Fmoc deprotection: 20% piperidine in DMF (3 mL, 1× 1.5 min), 20% piperidine in DMF (3 mL, 1×10 min or 1×5 min); washings in DMF (2×3 mL), CH_2Cl_2 (2×3 mL), and DMF (2× 3 mL), 1.5 min each.

c) Peptide coupling conditions: The coupling reaction was promoted by a coupling protocol using HOBt/HBTU in DMF: Fmoc-AA (4–5 equiv), HOBt (4–5 equiv), HBTU (4–5 equiv), and NMM (5–6 equiv) or DIEA (8–10 equiv) were stirred under N_2 in 2.5 mL DMF for 2 h. After each coupling, washings were carried out with DMF (3 mL, 3×1.5 min) and CH_2Cl_2 (3 mL, 3×1.5 min). *N*-α-Boc-*O*-TBDMS-Ahoda (2–2.5 equiv) was coupled in the final coupling step using the same procedure as for Fmoc-AAs (HOBt (2–2.5 equiv), HBTU (2–2.5 equiv), and DIEA (4–5 equiv)).

d) Cleavage: The dried peptide resin was treated for 2 h, while stirring, with the following cleavage mixture: AcOH/TFE/CH_2Cl_2 (2:2:6, 10 μL per 1 mg resin). The resin was then filtered off and washed with neat cleavage mixture (3 mL, 3×1.5 min). After addition of hexane (15× volume) to remove acetic acid as an azeotrope, the filtrate was concentrated and lyophilized.

e) N$^{\alpha}$ Boc and side chain deprotection: N-terminus and side-chain deprotections were carried out by treatment with TFA/H_2O/TIS (95:4:1, 10 μL per 1 mg) for 20 min while stirring.

f) Cyclization: The cyclization step was performed in solution at a concentration of 7.8×10^{-5} M with HATU (2.0 equiv) and DIEA (2.5 equiv) in CH_2Cl_2 or DMF. The solution was stirred at 4 °C for 1 h and then allowed to warm to room temperature for 2–3 h. The solvent was removed under reduced pressure.

g) Side chain deprotection (removal of trityl group): Final deprotection was carried out with TFA/TIS/CH_2Cl_2 (1:5:94, 100 μL per 1 mg resin) for 1 h while stirring.

h) N$^{\alpha}$ Boc and side chain deprotection: N-terminus and side-chain deprotections were carried out by treatment with TFA/H_2O/TIS (95:4:1, 10 μL per 1 mg) for 20 min while stirring.

The crude products were purified by semipreparative reversed-phase HPLC (Jupiter C_{18} column: 250×10.00 mm^2, 10μ, 300 Å, flow rate = 4 mL min^{-1}) using the gradient condition reported in Table 2, and were characterized by ESMS and NMR (see Supporting Information).

In vitro activity of cyclopeptide analogues

An acetylated peptide of histone H4 (Ac-S-G-R-G-K-G-G-K-G-L-G-K-G-G-A-K(ac)-MCA (50 pmol), a generous gift from Dr. M. Yoshida) was incubated in a total volume of 10 μL with 0.5 μg HeLa cell nuclear extract (BioVision, Mountain View, CA, USA) in 20 mM Tris, 150 mM NaCl, pH 8 for 1 h at 37 °C in the presence or absence of inhibitors. The reaction was stopped by heating the samples for 5 min at 95 °C. Samples (1 μL each) were spotted onto an H4 protein chip (Ciphergen, Fremont, CA, USA) and allowed to dry. Spots

Table 2. Calculated and observed molecular weights (by MS and HPLC analysis) and HPLC t_R data for compounds **2**–**12**.

Compd	Analogue	Formula	$[M+H]^+$ calcd	$[M+H]^+$ found	t_R [min]
2	Cyclo[D-Pro-Phe-Iva-Hse]	$C_{23}H_{32}N_4O_5$	445.5	445.1	15.35[a]
3	Cyclo[D-Pro-Phe-Aib-(9*R*)-Ahoda]	$C_{28}H_{40}N_4O_6$	529.6	529.1	21.18[b]
4	Cyclo[D-Pro-Phe-Iva-(9*R*)-Ahoda]	$C_{29}H_{42}N_4O_6$	543.6	543.1	17.72[a]
5	Cyclo[D-Pro-Phe-Phe-(9*R*)-Ahoda]	$C_{33}H_{42}N_4O_6$	591.7	591.1	17.85[c]
6	Cyclo[D-Pro-Phe-Trp-(9*R*)-Ahoda]	$C_{35}H_{43}N_5O_6$	630.7	630.2	17.76[c]
7	Cyclo[D-(4-Me_2Pro)-Phe-Iva-(9*R*)-Ahoda]	$C_{31}H_{46}N_4O_6$	571.7	571.3	21.15[a]
8	Cyclo[D-Pro-Phe-Aib-(9*S*)-Ahoda]	$C_{28}H_{40}N_4O_6$	529.6	529.2	21.93[b]
9	Cyclo[D-Pro-Phe-Abu-(9*S*)-Ahoda]	$C_{28}H_{40}N_4O_6$	529.6	529.1	31.22[d]
10	Cyclo[D-Pro-Phe-Acc-(9*S*)-Ahoda]	$C_{31}H_{44}N_4O_6$	569.7	569.2	20.20[a]
11	Cyclo[D-Pro-Phe-*p*ClPhe-(9*S*)-Ahoda]	$C_{33}H_{41}ClN_4O_6$	625.2	625.3	22.87[b]
12	Cyclo[D-Pro-Phe-His-(9*S*)-Ahoda]	$C_{30}H_{40}N_6O_6$	581.6	581.4	18.35[b]

[a] 5–100% of solvent system (A/B) for 30 min. [b] 5–100% of solvent system (A/B) for 45 min. [c] 15–85% of solvent system (A/B) for 40 min. [d] 5–65% of solvent system (A/B) for 80 min.

were then washed twice with 5 μL of 5 mM HEPES. Finally, 2× 0.8 μL 20% CHCA (in 50% CH_3CN and 0.5% TFA) were spotted onto each spot. Peptide masses were acquired by SELDI-TOF (Ciphergen, Fremont, CA, USA) mass spectrometry, and IC_{50} values were calculated using the peak areas in the following way: percent deacetylation was calculated as the percent deacetylated peptide with respect to the sum of deacetylated and acetylated peptides. Inhibitory activities were then calculated as percent inhibition of the deacetylation reaction.

Activity of cyclopeptide analogues on cultured cells

A7r5 smooth muscle cells were cultured in DMEM, 7.5% FCS, penicillin/streptomycin. Cells were trypsinized and resuspended in complete medium, 15% FCS. Into each well of a 24-well plate (already containing 250 μL DMEM without FCS and with various inhibitors) were seeded 250 μL of the cell suspension. Cells were cultured for 3 h, washed once in PBS, and lysed in 100 μL SDS sample buffer. Samples were sonicated for 10 min in a water bath and heated for 5 min at 95 °C. 15 μL (μg) of each sample were then separated by 12% SDS-PAGE and transferred onto PVDF membranes. Acetylated proteins were revealed using the monoclonal antibody AKL5C1, which recognizes acetylated internal lysine residues within proteins.

Proliferation assay

A7r5 cells were trypsinized and plated at a density of 3000 cells $well^{-1}$ in a 12-well plate. After 1 day of culture, compounds **1**–**12** were individually added to a final concentration of 1 μM each, or TSA (final concentration: 330 nM, or sodium butyrate (final concentration: 10 μM). Culture was continued for 5 days, and cells were finally stained using a Coomassie blue solution, rinsed twice in PBS, and dried.

Glossary

Boc, *tert*-butyloxycarbonyl; CHCA, α-cyano-4-hydroxycinnamic acid; ClTrt-Cl, 2-chlorotriphenylmethyl (chlorotrityl) chloride resin; DIEA, *N*,*N*-diisopropylethylamine; DMEM, Dulbecco's modified Eagle's medium; DMF, *N*,*N*-dimethylformamide; FCS, fetal calf serum; Fmoc, 9-fluorenylmethyloxycarbonyl; Fmoc-Acc, *N*-α-Fmoc-1-amino-1-cyclohexane-1-carboxylic acid; Fmoc-D-(4-Me_2Pro)-OH, *N*-α-Fmoc-D-4-dimethylproline; Fmoc-D-Pro-OH, *N*-α-Fmoc-D-proline; Fmoc-L-Abu-OH, *N*-α-Fmoc-L-2-aminobutyric acid; Fmoc-L-Aib-OH, *N*-α-Fmoc-2-aminoisobutyric acid; Fmoc-L-His(Trt)-OH, *N*-α-Fmoc-N^{im}-trityl-L-histidine; Fmoc-L-Hse(Trt)-OH, *N*-α-Fmoc-*O*-trityl-L-homoserine; Fmoc-L-Iva-OH, *N*-α-Fmoc-L-isovaline; Fmoc-L-Phe-OH, *N*-α-Fmoc-L-phenylalanine; Fmoc-L-Trp(Boc)-OH, *N*-α-Fmoc-N^{in}-Boc-L-tryptophan; Fmoc-*p*Cl-(L-Phe)-OH, *N*-α-Fmoc-*para*-chloro-L-phenylalanine; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluoroposphate *N*-oxide; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); HOBt, *N*-hydroxybenzotriazole; NMM, *N*-methylmorpholine; PS, polystyrene, microporous (9*R*)- and (9*S*)-*N*-α-Boc-*O*-TBDMS-Ahoda, *N*-α-Boc-9-*O*-TBDMS-8-oxodecanoic acid; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol, TIS, triisopropylsilane.

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