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First non-radioactive assay for *in vitro* screening of histone deacetylase inhibitors

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Inhibitors of histone deacetylase (HD) are of great potential as new drugs due to their ability to influence transcriptional regulation and to induce apoptosis or differentiation in cancer cells. So far only radioactive enzyme activity assays or in-vivo assays with subsequent electrophoresis and immunoblotting existed to study the activity of HD and potential inhibitors. To aid in the search of new inhibitors, a non-radioactive screening assay was sought and we have previously succeeded in establishing this for the first time. The assay uses an aminocoumarin derivative of an Ω-acetylated lysine as substrate for the enzyme. Here we report full experimental details, the evaluation of other potential substrates, and comparative analysis of various inhibitors. This advantageous method should have an impact on further developments in the field.

1. Introduction

The chromatin structure has an influence on key cellular processes such as DNA replication, transcription, DNA-repair and differentiation. The chromatin structure and the binding of regulatory proteins to DNA can be modified by reversible acetylation of the ε-amino groups of conserved lysine residues in the N-terminal tails of core histones [1]. This enzymatic modification is established and maintained by histone acetyltransferases and histone deacetylases, enzymes which have been identified as homologues of transcriptional regulators and nucleolar phosphoproteins [2–4]. Recent studies indicate that proteins involved in the regulation of proliferation and differentiation exert their activity by recruitment of histone acetyltransferases or deacetylases [2, 3].

Increasing evidence is accumulating to support the idea that inhibitors of histone deacetylase (HD) may have a great potential in cancer therapy and chemoprevention. It was demonstrated that inhibitors of HD significantly potentiate retinoid induced differentiation of retinoic acid sensitive acute promyelocytic leukemic cell lines (APL) and that oncogenic retinoic acid receptors mediate leukemogenesis through aberrant chromatin acetylation. Moreover, the HD inhibitor trichostatin A **(2)** is able to overcome retinoid resistance in non-sensitive APL cells [5, 6]. Recently, this new approach was successfully applied to a patient with a highly resistant APL using retinoic acid and the unspecific HD inhibitor phenylbutyrate [7]. Complete clinical remission was achieved and the failure of RT-PCR to detect mRNA of the mutated oncogenic retinoid receptors after treatment showed elimination of the residual disease. The treatment was well tolerated, showing that HD can indeed be inhibited *in vivo* without severe side effects. In the course of our studies to synthesize simple inhibitors of HD and the exploration of their potential for cancer therapy and chemoprevention, we have found promising lead substances [8]. Among them were compounds **3** and **4**, constructed from structural elements of the well-characterized inhibitors trapoxin B **(1a)** and trichostatin A **(2)** [9].

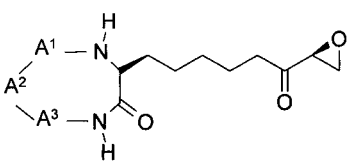
The search for new potent and simple inhibitors of HD is currently hampered by the enzyme substrate used in the activity assay. Either the natural substrate, acetylated histones [10], or synthetic peptides which consist of 8 [11] or 22 [12] amino acids, are used. The latter are composed of sequences from the N-termini of core histones. In either case the substrate is labeled with [^{3}H]acetic acid and the liberation of tritiated acid is measured by extraction and scintillation counting or via a scintillation proximity assay [13]. Thus, radioactivity is always involved, demanding special precautions for handling and waste disposal. The tritiated histones are obtained by a procedure involving treatment of chickens with phenylhydrazine for two weeks and ultimately the death of the animals. For synthesis of the oligopeptides, solid-phase-technology is required and final HPLC purification of the radioactive peptides is necessary. There are non-isotopic assays that allow to estimate inhibition of HD activity but they require isolation of nuclei and subsequently histones, gel electrophoresis and immunoblotting [14]. This procedure is only useful for a limited number of samples and not for the screening of a large number of compounds. Thus, we searched for a simple substrate for HD which would allow an easy and inexpensive non-radioactive assay and we have succeeded in finding the first fluorescent substrate for HD BOC-Lys(Ac)-AMC, also named MAL (*N*-(4-Methyl−7-coumarinyl)-*N*-α-(tert.-butyloxycarbonyl)-*N*-ω-acetyl-lysinamide, **6c**) [15]. This compound can be synthesized in one step from a commercial precursor and is also the first example of a molecule smaller than an octapeptide that is accepted as substrate by HD.

Here we present full experimental details on this compound along with the evaluation of other potential substrates and the comparative analysis of various inhibitors. We have synthesized different fluorescent derivatives of Ω-acetyllysine which is the target structure for HD in the histone proteins and of 6-aminocaproic acid as α-desamino-analogues. The limits of detection for these compounds cover different ranges of concentration and they were investigated for their ability to serve as substrates for HD.

2. Investigations, results and discussion

2.1. Synthesis of potential fluorescent substrates for HD

We have synthesized several substrate analogues labeled with a fluorophore on the C-terminal end. α-BOC-Ω-acetyl-lysine **(5a)** was used as the starting material for the synthesis of the labeled compounds **6a**–**c**. To evaluate whether the substrate structure might be simplified even more, we also used 6-acetylamino-caproic acid **(5b)** that is



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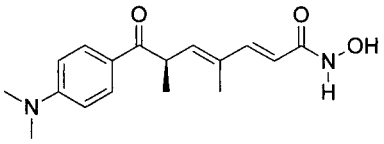
	A^1	A^2	A^3
1a:	L-Phe	L-Phe	D-Pro
1b:	D-Pro	L-Ala	D-Ala



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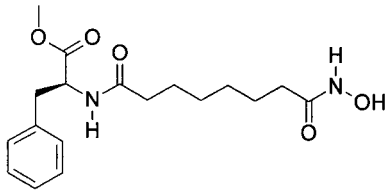

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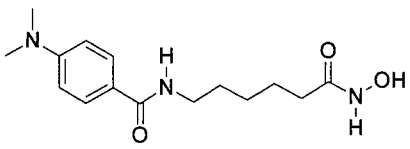

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Inhibitors of HD: **1a:** Trapoxin B, **1b:** HC toxin, **2:** Trichostatin A, **3** and **4:** lead structures for simple inhibitors

lacking the α-amino group of the lysine. Phenylalanine methyl ester, 1-naphthylmethyl amine and 7-amino-4-methyl-coumarin were used as fluorescent labels resulting in the amides **6**. Whereas most of the substrates were easily obtained using EDC or BOP-Cl as activating agents, this was not applicable in the synthesis of **6c**. But we succeeded using a procedure that has been established for the coupling of amines with a lower reactivity such as anilines that involves the use of phosphoryl chloride in pyridine [16] (Scheme 1).

2.2. Fluorometry and HPLC-Chromatography

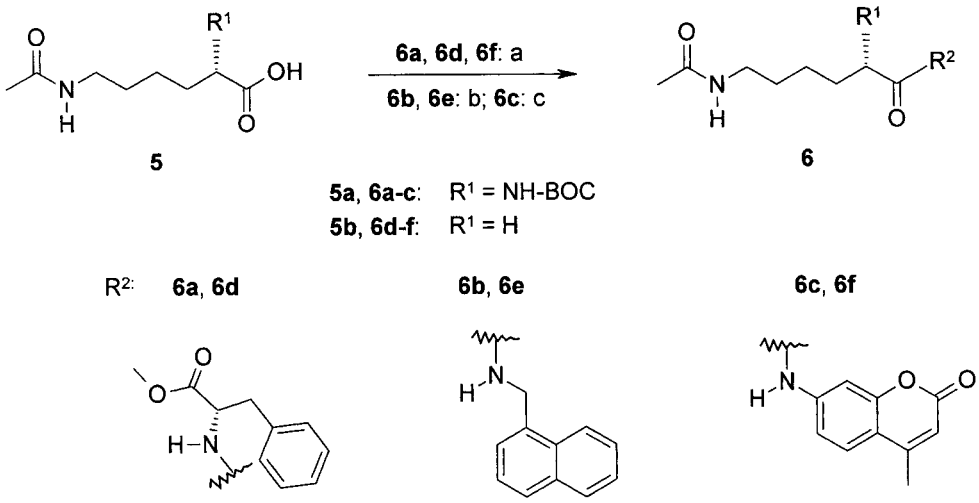
For all compounds fluorescence spectra were recorded to determine the wavelengths for excitation and emission for the HPLC-detection. Then we have established chromatographic systems that allow rapid analysis of the labeled compounds. We have determined the ranges to choose the proper concentration for the enzyme incubation experiments. In all cases the amount of remaining substrate was determined after extraction with an organic solvent from the aqueous phase in order to eliminate deleterious matrix effects on the chromatographic separation (Table 1).

2.3. Reaction of the potential substrates with HD

For the enzyme incubations we used a partially purified rat liver HD, which was obtained by chromatography on Q-sepharose (Fig. 1).

Before evaluation of the potential substrates, we investigated whether they might inhibit the enzyme but this was not observed for any of the compounds when the standard assay involving labeled histones was used [17]. Then the compounds were incubated with the enzyme in concentrations according to the previously determined range of detection. Neither in the case of the phenylalanine derivatives **6a** and **6d** nor with the naphthalin compound **6e** any deacetylation of the potential substrate could be registered after 240 min in the requisite range. There was a second peak of **6a** and **6d** in the chromatograms upon incubation

Scheme 1



a: BOP-Cl, NEM, H_2N-R (**6a, 6d**: as hydrochloride), CH_2Cl_2 (**6a**: 76 %, **6d**: 50 %, **6f**: 74 %); b: EDC, HOBt, NEM, H_2N-R, CH_2Cl_2 (**6b**: 74 %, **6e**: 87 %); c: $POCl_3$, H_2N-R, pyridine (**6c**: 39 %)

Table 1: Chromatographic conditions for fluorescent compounds 6

Compd.	$\lambda_{Exc.}$ (nm)	$\lambda_{Em.}$ (nm)	$t_{Ret.}$ (min)	Range (μg/ml)	Eluent (v/v) H_3CCN/H_2O
6a	260	280	4.62	13.9-223.0	40/60
6b	250	335	3.12	0.1-1.4	50/50
6c	330	395	3.37	5.3-170.6[a]	40/60
6d	260	280	2.39	7.7-42.7	40/60
6e	250	335	3.33	0.3-1.4	40/60
6f	330	395	4.06	ND[b]	30/70

[a] ng/ml
[b] could not be determined properly due to crystallization in the HPLC-system

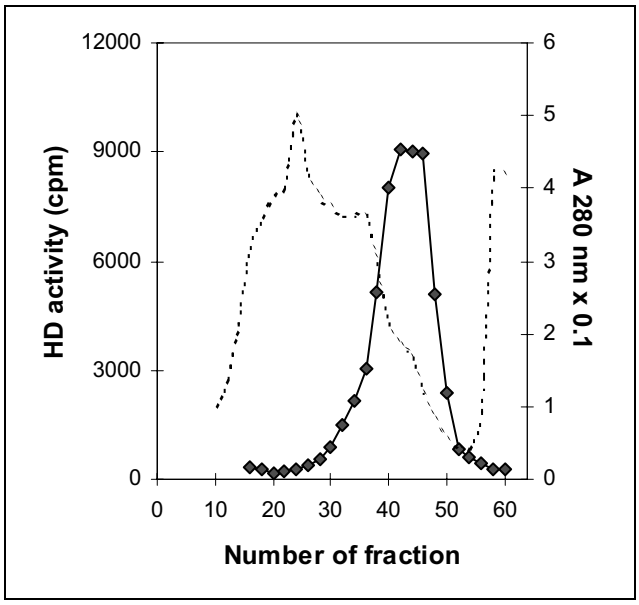


Fig. 1. Purification of rat liver HD. The high speed supernatant of rat liver extract was subjected to chromatography on Q-sepharose using an increasing gradient of NaCl (0–0.5 M) for elution. Fractions of 5 mL were collected and assayed for HD activity. HD activity (–●–) measured in the radioactive assay, expressed as counts per minute (cpm). Protein content (– –) registered by the absorption at 280 nm

with HD but this proved only to be the result of a cleavage of the methyl esters, probably by enzymes with esteratic activity that remained in the partly purified HD preparation. Identity of the newly formed material was proven by HPLC-analysis of the corresponding acids that were synthesized from the esters **6a** and **6d** by LiOH-mediated cleavage and characterized by MS, IR and NMR. The acids were also not deacetylated by incubation with rat liver HD (data not shown). There was some conversion of the naphthalin derivative of the lysine **6b** after 120 and even more after 240 min. As this was limited to a maximum of 56% conversion in the range that can be detected by HPLC after 4 h, we searched for a better substrate for screening purposes. Finally, the coumarin derivative **6c** was found to be converted by the enzyme within 40 min and subjected to further evaluation. The simpler capramide **6e** could not be evaluated due to its extremely low solubility in the aqueous incubation mixture and crystallization problems in the HPLC-system.

By running parallel incubations with **6c** and terminating the reactions in 5 min intervals, we could show a time-dependent decrease of the amount of **6c** after incubation with rat liver HD. A non-enzymatic consumption of **6c** after incubation without the enzyme and workup was not observed and the reaction was completely inhibited in presence of the specific HD inhibitors trichostatin A (100 to 300 nM) **(2)** or the structurally unrelated tetrapeptide inhibitor HC toxin [18] (**1b**, 70 μM) [15]. As a compound with a free amino group is released by the deacetylation reaction, one would expect a new compound in the aqueous layer following workup with acidic buffer. Indeed HPLC of the aqueous phase showed unreacted substrate **6c**, which was not completely extracted, and a second fluorescent material; the same was observed for the whole incubation mixture after terminating the enzymatic reaction with acetonitrile. The new peak increased continuously throughout the incubation period while the substrate peak area decreased over the same time frame [15].

2.4. Proof of deacetylation

We observed a decrease in the concentration of coumarin **6c** in the incubation mixture with HD and formation of a new product but it still had to be shown that deacetylation at the desired position took place. To ensure the identity of the postulated deacetylation product, we undertook its synthesis. α-BOC-Lys(FMOC)-OH **(7)** was coupled with 7-amino-4-methylcoumarin by applying the phosphoryl chloride method used for the synthesis of **6c**. The putative deacetylation product **8** was obtained by piperidine mediated deprotection (Scheme 2).

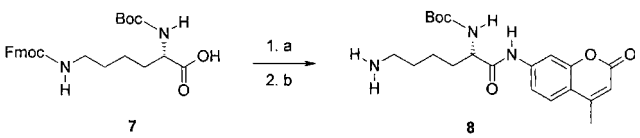
HPLC analysis confirmed the identity of the product of the incubation of **6c** with HD and product **8**. Identity was not only proven by identical retention times but also by HPLC-DAD and HPLC-MS coupling techniques. The new peak showed a MS identical to the synthesized compound **8** and also identical daughter ion fragmentation of m/z = 404 in HPLC-MS-MS coupling (data not shown). The artificial substrate had an even lower K_M-value (0.86 μM/l, see Fig. 2) than the natural one (20 μM/l) [15]. It is also converted by purified maize HD-2 but to a lesser extent compared to the rat enzyme preparation (maize HD-2: 29% conversion; rat liver HD: 68% conversion; both after 60 min, starting concentration 227 ng/ml).

2.5. Fluorescence based inhibition assay

We then investigated if the artificial substrate **6c** could actually be used in determining inhibitory concentrations of various compounds. We have measured inhibitory concentrations for several compounds that show good correlation to assays in which labeled histones are used. We included our lead structures **3** and **4** and the so called hybrid polar compound SAHA (suberoylanilide hydroxamic acid) **10a**, which is an inducer of differentiation [19] and was previously shown to be an inhibitor of HD [20, 21]. Compound **10a** was synthesized according to literature methods [22] and the novel *N*-methyl derivative **10b** was obtained in an analogous fashion (Scheme 3).

For comparison, we determined the IC_{50}-values with radioactively labeled histones and with a highly purified enzyme and labeled histones (Table 2). For these investigations we used maize histone deacetylase HD-2, which was purified by chromatography. As negative controls we

Scheme 2



a: $POCl_3$, pyridine, 7-amino-4-methylcoumarin (65 %); b: piperidine, DMF (38 %)

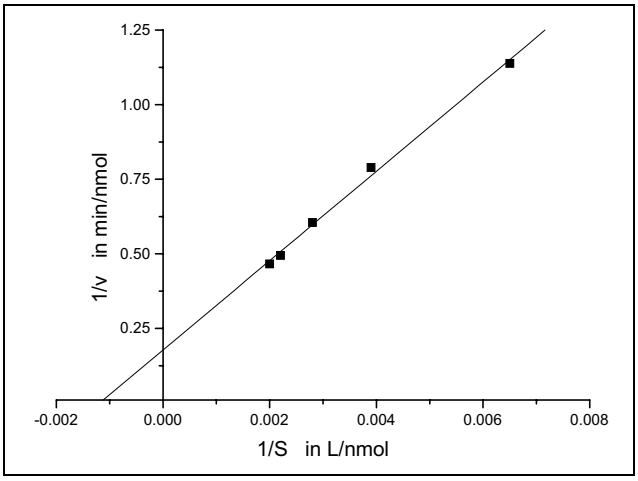


Fig. 2. Lineweaver-Burk-diagram for reaction of **6c** with HD

Table 2: IC_{50}-values (mean ± sd) of various inhibitors of HD activity using fluorescence labeled substrate or prelabeled core histones

Compd.	Rat liver HD and fluorescent substrate	Rat liver HD and labeled histones	Maize HD-2 and labeled histones
2	13.28 ± 1.42 nM	20.96 ± 1.67 nM	3.00 ± 0.09 nM
3	0.99 ± 0.72 μM	13.39 ± 1.02 μM	0.50 ± 0.05 μM
4	0.48 ± 0.02 μM	1.38 ± 0.04 μM	0.10 ± 0.02 μM
10a	0.30 ± 0.004 μM	0.63 ± 0.07 μM	1.00 ± 0.08 μM
10b	7.48 ± 0.47 μM	66.32 ± 15.51 μM	35.0 ± 2.70 μM

included solvent blanks, the NHO-benzyl precursor of **3** and the carboxylic acid corresponding to **4**. These compounds were inactive in all assays at 30–40 μM. This emphasizes the importance of the hydroxamic acid function in those series for the inhibition of HD, which was already known for **2** [23]. This can be explained by the zinc ion in the active site of histone deacetylase that participates in the catalytic cleavage of the amide bond [24].

Although there are some differences in the IC_{50}-values of the various inhibitors, the fluorescent assay gives a good indication for the potency as compared to the standard assay. The largest deviations occur in the system with histones and the rat liver preparation, which might be due to residual proteolytic enzyme activity in the partially purified extract. The *in vitro* assay is mainly a tool for primary screening and the correlation to the biological activity in cancer cells has to be established on a larger set of compounds. We have already shown a good correlation of the *in vitro* enzyme inhibition of such a set of compounds and their differentiating and/or antiproliferative activity in cancer cells [25]. In that study, all compounds that were inactive as enzyme inhibitors are inactive as inducers of differentiation and almost all inhibitors were antiproliferative compounds or inducers of differentiation.

We have shown for the first time that HD is able to accept simple Ω-acetyl-lysinamides **6b** and **6c** as substrates but that substrate specifity is clearly dependent on the amide component. Deacetylation was shown to be enzymatic by the use of specific inhibitors and synthesis of the deacetylation product. Furthermore, simplification to a α-desamino-lysine was not possible in the case of **6b** and could not be investigated due to extreme low solubility in case of the 6-aminocapramide **6f**. The fluorescent substrate **6c** is easily obtained from commercial starting materials. It can be quantified in low micromolar concentrations by HPLC and fluorescence detection and allows for the determination of the inhibitory ability of putative inhibitors of HD with IC_{50}-values from the nanomolar to the micromolar range. This process could be subjected to automation and possibly modified for high-throughput-screening. It may now be possible to replace other testing methods for HD inhibitors that require animal testing or expensive substrates and radioactive labeling and is commercially available (Calbiochem) now. This should simplify and facilitate the search for new inhibitors of HD which have great potential for chemoprevention and treatment of cancer.

3. Experimental

3.1. General

All moisture sensitive reactions were conducted in oven-dried glassware under a dry nitrogen atmosphere. Compound **2** was purchased from Wako (Neuss, Germany), **1b** and lysine derivatives were purchased from Calbiochem (Bad Soden, Germany). Aminomethylcoumarin was purchased from Sigma and all other chemicals were obtained from Aldrich. Compounds **3** and **4** were synthesized according to the literature [8], **10a** according to [22]. Solvents were dried using molecular sieves (3 Å). M.p.'s are uncorrected. Elemental analysis (Foss-Heraeus CHN-O-Rapid) was obtained (CHN, max. ±0.4%) for all compounds unless stated otherwise. IR: KBr pellets, cm^{-1}, Shimadzu 470. ^{1}H-NMR: Varian Gemini 200 (200 MHz). ^{13}C-NMR: Varian Gemini 200 (50.29 MHz). MS: Finnigan MAT 312 (ESI). Flash chromatography was done with silica gel 60, 230–400 mesh (Merck). Fluorescence spectra were recorded on a Shimadzu RF-540. A Shimadzu RF 535 was used as fluorescence detector for HPLC. A LiChrosorb RP-18, 5 μm (150 × 4 mm, Knauer) column was used for the incubation experiments and the inhibition assay. For identification of the incubation product via LC-MS a Multospher 100 RP-18, 5 μm (250 × 4 mm, CS-Chromatography) column and UV-detection (210 nm) were used. A Finnigan LCQ mass spectrometer was used for LC-MS coupling analyses.

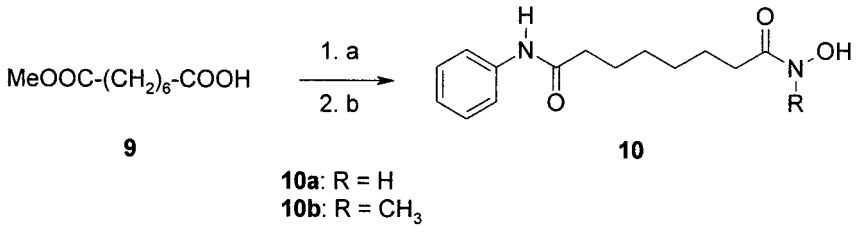
3.2. Chemistry

3.2.1. Amide coupling protocols

Method A: Amide formation using BOP-Cl. To a solution or suspension of the acid in dry CH_2Cl_2 (10 ml/mmol) were added four equivalents of *N*-ethyl morpholine (NEM) under N_2 and the mixture was stirred for 10 min. Then one equivalent of BOP-Cl and one equivalent of the amine or amine hydrochloride were added. After overnight stirring most of the CH_2Cl_2 was removed under reduced pressure and 100 ml ethyl acetate and 50 ml of 3% $NaHCO_3$-solution were added. The organic phase was separated and washed consecutively with H_2O. 2 M HCl and saturated brine (50 ml each). The organic layer was dried over Na_2SO_4 and the solvent was evaporated.

Method B: Amide formation using EDC. To a solution or suspension of the acid in dry CH_2Cl_2 (10 ml/mmol) at 0–5 °C were added 1.2 equiva-

Scheme 3



9 **10**

10a: R = H
10b: R = CH_3

a: $PhNH_2$, BOP-Cl, NEM; b: RHNOH, methanol (**10a**: 25 %, **10b**: 66 %)

lents of each EDC and HOBt. After 10 min at 5 °C 1.1 equivalents of the amine and 2 equivalents of NEM were added. After stirring for another 30 min at 5 °C and 12 h at ambient temperature the resulting precipitate was collected by filtration. The crude product was washed consecutively with 5% citric acid, water, 3% $NaHCO_3$, H_2O and hexane (25 ml each).
Method C: Amide formation using $POCl_3$. The acid was mixed with equimolar amounts of 7-amino-4-methylcoumarin in dry pyridine (6 ml/mmol) at −15 °C. Phosphoryl chloride (0.25 ml/mmol) was then added via a syringe, resulting in a orange-red solution. After stirring for 30 min at −15 °C, the mixture was poured into the 10fold volume of ice/H_2O and extracted three times with 50 ml of ethyl acetate. The combined organic phase was washed consecutively with H_2O, 3% $NaHCO_3$, H_2O and saturated brine (50 ml each). The organic layer was dried over Na_2SO_4 and the solvent was evaporated.

3.2.2. N-(1-(S)-Methoxycarbonyl-2-phenylethyl)-N-α-(tert.-butyloxycarbonyl)-N-ω-acetyl-lysinamide (**6a**)

Synthesized by method A using BOC-Lys-Ac-OH (**5a**; 0.20 g, 0.7 mmol), methyl L-phenylalaninate hydrochloride (0.15 g, 0.7 mmol), BOP-Cl (0.20 g, 0.7 mmol and NEM (0.35 ml, 322 mg, 2.8 mmol). The crude product was recrystallized from CH_2Cl_2. Yield: 260 mg colorless crystals, 76%; m.p. 132 °C; IR 3295, 1729, 1656; ^{1}H NMR (CD_3OD): 1.04–1.76 (m, 15 H), 1.92 (s, 3 H), 2.82–3.24 (m, 4 H), 3.80 (s, 3 H), 3.97 (t, J = 7.28 Hz, 1 H), 4.64 (t, J = 6.91 Hz, 1 H), 7.03–7.44 (m, 5 H); ^{13}C NMR (CD_3OD): 22.58, 24.12, 28.69, 29.93, 33.03, 38.43, 40.19, 52.66, 54.97, 55.91, 80.71, 127.89, 129.50, 130.27, 137.97, 157.66, 173.18, 173.25, 174.96; MS m/z 449 [M^+].
$C_{23}H_{35}N_3O_6$ (449.6)

3.2.3. N-(1-Naphthylmethyl)-N-α-(tert.-butyloxycarbonyl)-N-ω-acetyl-lysinamide (**6b**)

Synthesized by method B using **5a** (0.30 g, 1.04 mmol), EDC (0.24 mg, 1.25 mmol), HOBt (0.17 g, 1.25 mmol), 1-(aminomethyl)-naphthalene (0.17 ml, 0.18 g, 1.14 mmol) and NEM (0.30 ml, 479 mg, 2.08 mmol). The precipitate was recrystallized from ethyl acetate and again from CH_2Cl_2. Yield: 330 mg colorless crystals, 74%; m.p. 133 °C; IR 3295, 1679, 1636; ^{1}H NMR (DMSO-d_6): 1.14–1.48 (m, 13 H), 1.49–1.59 (m, 2 H), 1.76 (s, 3 H), 2.91–2.96 (m, 2 H), 3.90–3.94 (m, 1 H), 4.71–4.74 (m, 2 H), 6.88 (d, J = 8.06 Hz, 1 H), 7.42–7.55 (m, 4 H), 7.76–8.04 (m, 4 H), 8.34 (m, 1 H); ^{13}C NMR (CD_3OD): 22.57, 24.29, 28.67, 29.99, 33.09, 40.18, 42.15, 56.26, 80.66, 124.46, 126.37, 126.84, 127.03, 127.36, 129.30, 129.73, 132.69, 134.67, 135.34, 157.78, 173.12, 174.98; MS: m/z 427.
$C_{24}H_{33}N_3O_4$ (427.54)

3.2.4. N-(4-Methyl-7-coumarinyl)-N-α-(tert.-butyloxycarbonyl)-N-ω-acetyl-lysinamide (**6c**)

Synthesized by method C using **5a** (0.30 g, 1.04 mmol), 7-amino-4-methyl-coumarin (0.17 g, 1.04 mmol) and $POCl_3$ (0.25 ml). The crude product was recrystallized from CH_2Cl_2 and the resulting solid was chromatographed with ethyl acetate/methanol (50 : 1). Yield: 180 mg colorless crystals, 39%; m.p. 175 °C (CH_2Cl_2); IR = 3275, 3205, 1736, 1632; ^{1}H NMR (CD_3OD): 1.06–1.63 (m, 13 H), 1.63–1.82 (m, 2 H), 1.93 (s, 3 H), 2.43 (s, 3 H), 3.15 (t, J = 6.48 Hz, 2 H), 4.10–4.31 (m, 1 H), 6.24 (s, 1 H), 7.50 (dd, J = 8.74 Hz, J = 2.04 Hz, 1 H), 7.70 (d, J = 8.74 Hz, 1 H), 7,80 (d, J = 2.04 Hz, 1 H); ^{13}C NMR (CD_3OD): 18.50, 22.54, 24.31, 28.71, 30.04, 32.99, 40.14, 56.86, 80.75, 108.07, 113.62, 117.20, 126.72, 143.43, 155.24, 155.40, 163.22, 173.23, 174.12; MS: m/z 445 [M^+].
$C_{23}H_{31}N_3O_6$ (445.5)

3.2.5. N-(1-(S)-Methoxycarbonyl-2-phenylethyl)-6-acetylamino-capramide (**6d**)

Synthesized by method B using 6-acetylamino-caproic acid (**5b**, 0.87 g, 5 mmol) methyl L-phenylalaninate hydrochloride (0.87 g, 5 mmol), BOP-Cl (1.27 g, 5 mmol) and NEM (2.53 ml, 2.30 g, 20 mmol). During the extractions the aqueous phases were saturated with NaCl to reduce loss of material. The crude product was crystallized with hexane and recrystallized from CH_2Cl_2. Yield: 840 mg colorless crystals, 50%; m.p. 118 °C; IR = 3275, 3205, 1736, 1632; ^{1}H NMR (CD_3OD): 1.15–1.36 (m, 2 H), 1.39–1.58 (m, 4 H), 1.91 (s, 3 H), 2.15 (m, 2 H), 2.85–3.21 (m, 4 H), 3.68 (s, 3 H), 4.67 (m, 2 H), 7.16–7.31 (m, 5 H); ^{13}C NMR (CD_3OD): 22.56, 26.41, 27.30, 29.96, 36.50, 38.39, 40.43, 52.63, 55.07, 127.85, 129.45, 130.16, 138.26, 173.09, 173.63, 175.89; MS: m/z 334 [M^+].
$C_{18}H_{26}N_2O_4$ (334.4)

3.2.6. N-(1-Naphthylmethyl)-6-acetylamino-capramide (**6e**)

Synthesized by method A using **5b** (0.35 g, 2 mmol), EDC (0.46 g, 2.4 mmol), HOBt (0.32 g, 2.4 mmol), 1-(aminomethyl)-naphthalene (0.33 ml, 0.35 g, 2.2 mmol) and NEM (1.01 ml 0.92 g, 8 mmol). The precipitate was recrystallized from ethyl acetate and again from CH_2Cl_2. Additional material was collected from the mother liquor after evaporation, washing and recrystallization as above. Yield: 600 mg colorless crystals, 87%; m.p. 132 °C; IR = 3235, 1616; ^{1}H NMR (DMSO-d_6): 1.81–2.45 (m, 6 H), 2.58 (s, 3 H), 2.95 (m, 2 H), 3.75 (m, 2 H), 5.50 (d, J = 5.53 Hz, 2 H), 8.06–8.47 (m, 4 H), 8.49–8.96 (m, 4 H), 9.15 (m, 1 H); ^{13}C NMR (DMSO-d_6): 22.52, 25.02, 26.11, 28.86, 35.23, 38.39, 40.09, 123.45, 125.29, 125.45, 125.68, 126.05, 127.43, 128.39, 130.87, 133.25, 134.76, 168.85, 171.91; MS: m/z 312.
$C_{19}H_{24}N_2O_2$ (312.2)

3.2.7. N-(4-Methyl-7-coumarinyl)-6-acetylamino-capramide (**6f**)

Synthesized by method A using **5b** (0.43 g, 2.5 mmol) 7-amino-4-methyl-coumarin (0.44 g, 2.5 mmol), BOP-Cl (0.64 g, 2.5 mmol) and NEM (1.26 ml, 1.15 g, 10 mmol). After stirring for 12 h the reaction mixture was filtered (and not evaporated) and the precipitate was washed consecutively with 5% citric acid, H_2O, 5% $NaHCO_3$, H_2O and hexane and recrystallized from ethyl acetate and again from CH_2Cl_2. Due to its very low solubility, **6f** could not be analyzed by HPLC-analysis and elemental analysis was not obtained. Yield: 830 mg off-white crystals, 74%; m.p. 243 to 254 °C; IR = 3305, 1694; ^{1}H NMR (DMSO-d_6): 1.22–1.56 (m, 6 H), 1.76 (s, 3 H), 2.28–2.46 (m, 5 H); 2.96–3.02 (m, 2 H), 6.22 (s, 1 H), 7.45 (dd, J = 8.10 Hz, J = 1.79 Hz, 1 H), 7.65 (d, J = 8.10 Hz, 1 H), 7.75 (d, J = 1.79 Hz, 1 H), 7.81 (s, 1 H), 10.36 (s, 1 H); ^{13}C NMR (DMSO-d_6): 17.83, 22.60, 24.52, 26.01, 28.84, 36.37, 40.17, 105.38, 111.98, 114.67, 114.99, 125.66, 142.57, 152.94, 153.60, 159.94, 168.83, 171.90; MS: m/z 330 [M^+].
$C_{18}H_{22}N_2O_4$ (330.4)

3.2.8. N-(4-Methyl-7-coumarinyl)-N-α-(tert.-butyloxycarbonyl)-lysinamide (**8**)

3.2.8.1. Synthesis of the Ω-FMOC-compound

Synthesized by method C using BOC-Lys-(FMOC)-OH (**7**, 0.47 g, 1 mmol), 7-amino-4-methylcoumarin (0.18 g, 1 mmol), $POCl_3$ (0.25 ml). After pouring into ice/H_2O the mixture was filtered (and not extracted). The precipitate was washed with 5% $NaHCO_3$ and H_2O. The resulting solid was chromatographed with ethylacetate/hexane (1 : 1) and the product was recrystallized from CH_2Cl_2. Yield: 410 mg colorless crystals, 65%; m.p. 113 °C; IR = 3305, 1694; ^{1}H NMR (CD_3OD): 1.20–1.60 (m, 13 H), 1.61–1.85 (m, 2 H), 2.25 (s, 3 H), 3.15 (m, 2 H), 3.90–4.40 (m, 4 H), 6.10 (s, 1 H), 7.20–7.80 (m, 11 H); ^{13}C NMR (CD_3OD): 18.45, 24.02, 28.57, 30.64, 33.04, 41.16, 56.84, 56.95, 67.62, 80.75, 108.01, 113.58, 117.08, 120.84, 126.05, 126.12, 126.50, 128.09, 128.70, 142.52, 143.24, 145.26, 154.91, 155.04, 155.24, 163.19, 174.03; MS: m/z 625 [M^+].
$C_{36}H_{39}N_3O_7$ (625.8)

3.2.8.2. Deprotection

The FMOC-compound (400 mg) is deprotected with piperidine (2 g) in 10 ml of dry DMF. After 15 min 20 ml diethyl ether were added and the organic layer was extracted three times with 25 ml of 5% citric acid. The aqueous phase is treated with 0.5 M NaOH until white dibenzofulvene precipitated (pH 9). The aqueous phase was filtered and the mother liquor was extracted three times with of ethyl acetate and CH_2Cl_2 (100 ml portions each). The combined organic layers were dried over Na_2SO_4 and evaporated. The resulting oil was crystallized from CH_2Cl_2. Satisfying elemental analysis could not be obtained but the compound was sufficiently pure for identification of the metabolite of **6c** as judged by HPLC. Yield: 100 mg yellow crystals, 38%; m.p. 105 °C; IR = 3290, 1685; ^{1}H NMR (CD_3OD): 1.25–1.60 (m, 13 H), 1.63–1.95 (m, 2 H), 2.40 (s, 3H), 2.65 (t, J = 6.38 Hz, 2 H), 4.05–4.20 (m, 1 H), 6.20 (s, 1 H), 7.50 (dd, J = 8.48 Hz, J = 1.87 Hz, 1 H), 7.70 (d, J = 8.48 Hz, 1 H); 7.80 (d, J = 1.87 Hz, 1 H) ^{13}C NMR (CD_3OD): 18.47, 24.23, 28.73, 32.50, 33.13, 41.94, 56.97, 80.75, 108.12, 113.64, 117.22, 126.68, 143.37, 155.18, 155.40, 163.19, 174.11; MS: m/z 403 [M^+].
$C_{21}H_{30}N_3O_5$ (403.5)

3.2.9. N-Hydroxy-N-methyl-N′-phenyl-suberoylbisamide (**10b**)

Methyl suberoylanilide **(9)** [22] (600 mg, 2.28 mmol) was dissolved in 15 ml of dry methanol and a solution of *N*-methyl hydroxylamine [obtained by treatment of the hydrochloride (5.85 g, 70 mmol) in 50 ml of dry methanol with sodium (2.30 g, 100 mmol)] was added slowly at 0–5 °C. After 1 h at that temperature and another 24 h at room temperature, the reaction mixture was poured into 300 ml of H_2O. The resulting precipitate was filtered off and washed with a small amount of CH_2Cl_2. Yield: 420 mg colorless crystals, 66%; m.p. 112 °C; IR = 3315, 1650, 1590; ^{1}H NMR (DMSO-d_6) δ 1.28 (bs, 4 H), 1.63–1.44 (m, 4 H), 2.49–2.23 (m, 4 H), 3.06 (s, 3 H), 7.03–6.96 (m, 1 H), 7.30–7.22 (m, 2 H), 7.59–7.55 (m, 2 H), 9.81 (bs, 1 H), 9.84 (s, 1 H); ^{13}C NMR (DMSO-d_6): 24.09, 24.50, 28.44, 28.53, 31.42, 35.71 36.32 119.10 122.82 128.50 139.26, 168.5, 171.17; MS: m/z 278 [M^+].
$C_{15}H_{22}N_2O_3$ (278.4)

3.3. Chromatographic data

Fluorescence spectra of the compounds were registered in CH_3CN/H_2O (40/60, v/v). The potential substrates were then subjected to HPLC-chromatography using the eluents as indicated in Table 1 and the chromatographic system as outlined above. Calibration was performed with six different concentrations of the investigated compound in dilutions as in the incubation assays (see below), where the enzyme was substituted by 100 µl of chromatography buffer.

3.4. Enzymes

Maize histone deacetylase HD-2 was purified as previously described [17]. For purification of rat liver HD, fat and ligaments were removed from 30 g of frozen liver and after thawing the liver was cut into small pieces. It was homogenized in 150 ml of buffer solution (50 mM tris-HCl, pH 7.5; 0.25 mM sucrose; 25 mM KCl; 5 mM $MgCl_2$). All further manipulations were done at 4 °C. After filtration and ultracentrifugation (35.000 × g) the supernatant was dialyzed against the chromatography buffer (15 mM tris-HCl, pH 7.9; 0.25 mM EDTA; 10 mM NaCl; 10% (v/v) glycerol; 10 mM 2-mercaptoethanol; thrice for 3 h against 1 l of buffer). A Q-Sepharose column (Big beads, 50 ml, Pharmacia Biosystems) was equilibrated with 500 ml of chromatography buffer (flow rate 1.5 ml/min) and then loaded with 150 ml of the extract. Proteins were eluted with a linear gradient from 10 to 500 mM NaCl in the chromatography buffer (300 ml, 0.3 ml/min). Fractions of 5 ml were collected and assayed for HD activity as previously described [17] and as indicated below. Fractions containing high HD-activity (40 to 50) were pooled and used for the inhibition assays.

3.5. Enyzme incubations and inhibition assays

Stock solutions of potential substrates and the inhibitors in ethanol were made at a concentration of 5 mg/ml ethanol (**2**: methanol, 1 mg/ml) and were further diluted with chromatography buffer. Potential substrates were then incubated with rat liver HD at 3–6 concentrations in the previously determined range under the conditions indicated below and the amount of the remaining potential substrate was registered. It was more than 95% after 40 min, 2 h and 4 h for **6a**, **6d** and **6e**. For **6b** there was a conversion of 6% (40 min), 34% (120 min) and 56% (240 min). Compound **6c** showed a rapid decrease down to approximately 50% after 30 min and below 20% after 120 min (227 ng/ml) [15]. At lower concentrations there is less than 20% of residual **6c** after 30 min. The K_M was determined using five different concentrations of **6c** resp. radioactive labeled histones, terminating the reactions after 30 min and registering the amount of converted substrate resp. liberated [^{3}H]acetic acid.

IC_{50} values are the results of triple determinations. For the radioactive assays 100 µl of rat enzyme preparation (at 37 °C) or 50 µl of maize enzyme (at 30 °C) were incubated (30 min) with 10 µl of total [^{3}H]acetate prelabeled chicken reticulocyte histones (1 mg/ml). Reactions were stopped by addition of 36 µl of 1 M HCl/0.4 M acetate and 800 µl of ethyl acetate. After centrifugation (10.000 g, 5 min) an aliquot of 600 µl of the upper phase was counted for radioactivity in 3 ml liquid scintillation cocktail. For the fluorescence assay an aliquot of 10 µl of a solution of **6c** (14.7 µg/ml H_2O, from a stock solution in ethanol of 5 mg/ml) was incubated with 100 µl of rat enzyme preparation (at 37 °C) for 90 min. Reactions were stopped by addition of 72 µl of 1 M-HCl/0.4 M-sodium acetate and 800 µl of ethyl acetate. After centrifugation (10.000 g, 5 min) an aliquot of 600 µl of the upper phase was taken and the solvent was removed by a stream of nitrogen. The residue was dissolved in 600 µl of the chromatography eluent (CH_3CN/H_2O, 40/60) and 20 µl were injected via autosampler onto the HPLC system (flow rate 0.6 ml/min). The retention time of substrate is 3.40 min.

For the identification of the incubation product of **6c** with rat liver HD, the reaction was stopped by adding 800 µl of CH_3CN and 20 µl of the mixture were analyzed by HPLC without further workup. CH_3CN/H_2O (40/60, 0.01% trifluoroacetic acid; 0.7 ml/min) was used as eluent. The retention time of the product was 7.93 min and that of the substrate was 11.07 min. Synthetic product **8** obtained as indicated above coeluted at 11.07 min. Synthetic and enzymatic product gave identical UV-spectra in HPLC-DAD, identical MS and identical daughter-ion spectra in MS-MS of m/z = 404 by using the HPLC-MS system as described above.

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