Inhibitors of Histone Deacetylase Suppress the Growth of MCF-7 Breast Cancer Cells

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Summary

Inhibitors of histone deacetylase are attracting increasing interest due to their influence on transcription, differentiation, and apoptosis. We have investigated two synthetic inhibitors **3** and **4** of histone deacetylase and the natural product inhibitor trichostatin A for their ability to suppress the growth of MCF-7 breast cancer cells and here present complete and improved synthetic procedures. The compounds show a dose dependent inhibition of growth with activities in the low micromolar and nanomolar range. Trichostatin shows cytocidal effects at 100 nM and still has activity comparable to cisplatin (0.5 μM) at 10 nM. Whereas the synthetic inhibitor **3** has cytocidal activity at 10 μM compound **4** shows a maximum of 40% growth suppression at that concentration.

Introduction

Reversible acetylation of ε-amino groups of highly conserved lysine residues in the N-terminal tails of histones has a modulating impact on chromatin structure. This affects key nuclear processes such as DNA replication, transcription, differentiation, and apoptosis [1]. This modification is established and maintained by histone acetyltransferases and histone deacetylases, enzymes which have been identified as homologs of regulators of transcription and nucleolar phosphoproteins [2–4]. Increasing evidence shows that transcription factors involved in the regulation of proliferation and differentiation exert their function by recruitment of histone acetyltransferases or deacetylases [2,3,5,]. In several cases aberrant chromatin acetylation could be linked to malignant diseases [5,7–9].

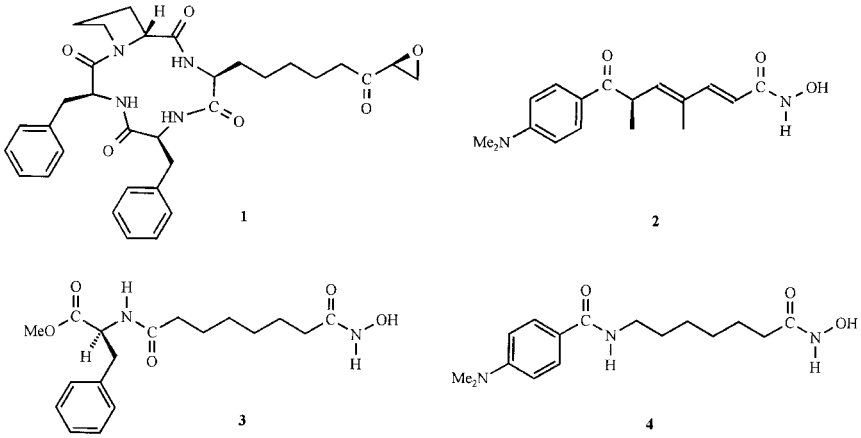
In the course of our studies to synthesize simple inhibitors of histone deacetylase and the exploration of their potential for cancer therapy and chemoprevention we have found promising lead substances [10]. Among them were compounds **3** and **4** constructed from structural elements of the established natural product inhibitors trapoxin B **(1)** and trichostatin A **(2)** (Scheme 1) [11].

Whereas the impact of inhibitors of histone deacetylase on leukaemic cells has been studied at length comparatively little is known about their influence on cell lines derived from solid tumors. Just recently it has been shown that the anticancer drug cisplatin is crosslinking nuclear proteins to the DNA in MCF-7 breast cancer cells and it was suggested that its clinical activity is linked partly to that effect. Among those nuclear proteins were the estrogen receptor and histone deacetylase HDAC-1[12]. So we studied the growth inhibition by reference histone deacetylase inhibitor trichostatin A **(2)** and of our synthetic lead structures **3** and **4** on that human breast cancer cell line.

Results and Discussion

Chemistry

As described previously **3** and **4** are accessible in four synthetic steps using standard peptide coupling methodology.



Scheme 1. Inhibitors of histone deacetylase.

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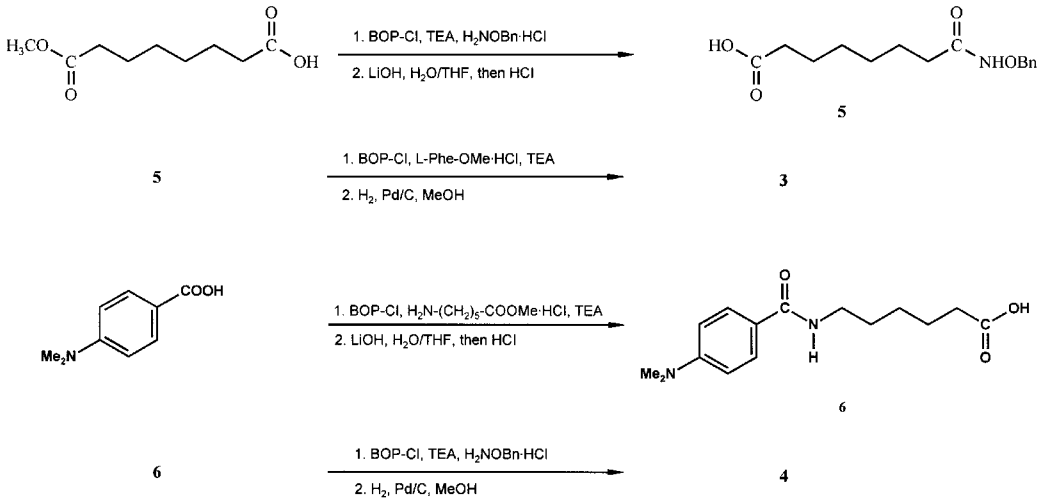

Scheme 2. Synthesis of Inhibitors **3** and **4**.

As the target compounds are very polar we chose a strategy where no chromatography is required after the last synthetic step. Thus a benzyl-protected hydroxylamine is employed that allows for chromatographic purification of the protected hydroxamate. After hydrogenation the compounds can be purified by precipitation from methanolic solutions. The use of BOP-Cl in all coupling steps in the synthesis of **3** led to increased yields compared to the water-soluble carbodiimide EDC. For **4** we had already proven that the use of BOP-Cl is crucial in the first coupling step as mixed anhydride or carbodiimide methods failed due to the inactivating effect of the *para*-dimethylamino group [10] (Scheme 2).

Biological Activities

The impact of the different inhibitors of histone deacetylase on MCF-7 cells was studied in our standard microtiter assay using crystal violet staining to measure total biomass relative to control over a prolonged period of exposure to the antineoplastic agent [13]. Cisplatin was used as a reference compound (Figure 1).

Trichostatin A **(2)** and inhibitor **3** are able to reach cytocidal effects at 100 nM (**2**) and 10 μM (**3**), respectively. In contrast, compound **4** did not exceed 40% suppression of cell growth even at 10 μM. Even at 10 nM, **2** still has an activity comparable to cisplatin at 500 nM. This difference between **2** and our inhibitors reflects the potency in the *in-vitro* enzyme inhibition assay (IC_{50}: **2**: 3 nM, **3**: 500 nM, **4**: 100 nM) [14]. After approximately 60 h there was an increase in cell growth in some of the experiments (**2** at 10 nM, **3** at 10 μM, **4** at 10 μM) which may reflect hydrolysis of the hydroxamates to the inactive carboxylic acids.

Although the dose response curves are rather steep it is encouraging that inhibitors of histone deacetylase can suppress the growth of breast cancer cells and even show cytocidal effects in some cases. It has been shown recently that this enzyme can indeed be inhibited safely in humans. The depsipeptide FR-901228 which was chosen for clinical trials due to its good antitumor activity was discovered to be an inhibitor of histone deacetylase [15]. First results from clinical phase I studies showed encouragingly low toxicity [16]. Moreover, a patient with multi-resistent acute promyelocytic leukaemia was found to respond again to retinoid treatment by combination with the unspecific histone deacetylase inhibitor phenylbutyrate. Six months after treatment RT-PCR did not show any mRNA of the oncogenically transformed retinoid receptors that recruit histone deacetylase to suppress transcription, thus indicating absence of residual disease. Again no severe side effects were observed [17]. Thus this first report of the growth inhibition of MCF-7 breast cancer cells by potent inhibitors of histone deacetylase adds to the mounting evidence that this class of compounds possesses great potential for the treatment of cancer. Additionally it supports the use of *in-vitro* assays of histone deacetylase activity as screening tools in drug discovery [18–20]. Further investigations that involve differences in response depending on estrogen receptor status of the cell and combination with other antineoplastic agents are part of ongoing studies.

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Experimental Part

Melting point: uncorrected.– Elemental analysis: Perkin Elmer.– IR: Shimadzu 470.– ^{1}H-NMR: Varian Gemini 200 (200 MHz).– ^{13}C-NMR: Varian Gemini 200 (50.29 MHz).– MS: Varian MAT 44S, Finnigan MAT 312.– Flash chromatography (FC): silica gel 60, 230–400 mesh (Merck).– Dichloromethane was dried over molecular sieves (3Å).

Chemistry

General Procedure for Amide Formation

To a solution or suspension of the acid in dry CH_2Cl_2 (5 mL/mmol) was added one equivalent of triethylamine (TEA) under nitrogen and the mixture was stirred for 10 min. Then 1.1 equivalents of BOP-Cl, one equivalent of the amine or hydroxylamine hydrochloride, and another three equivalents of

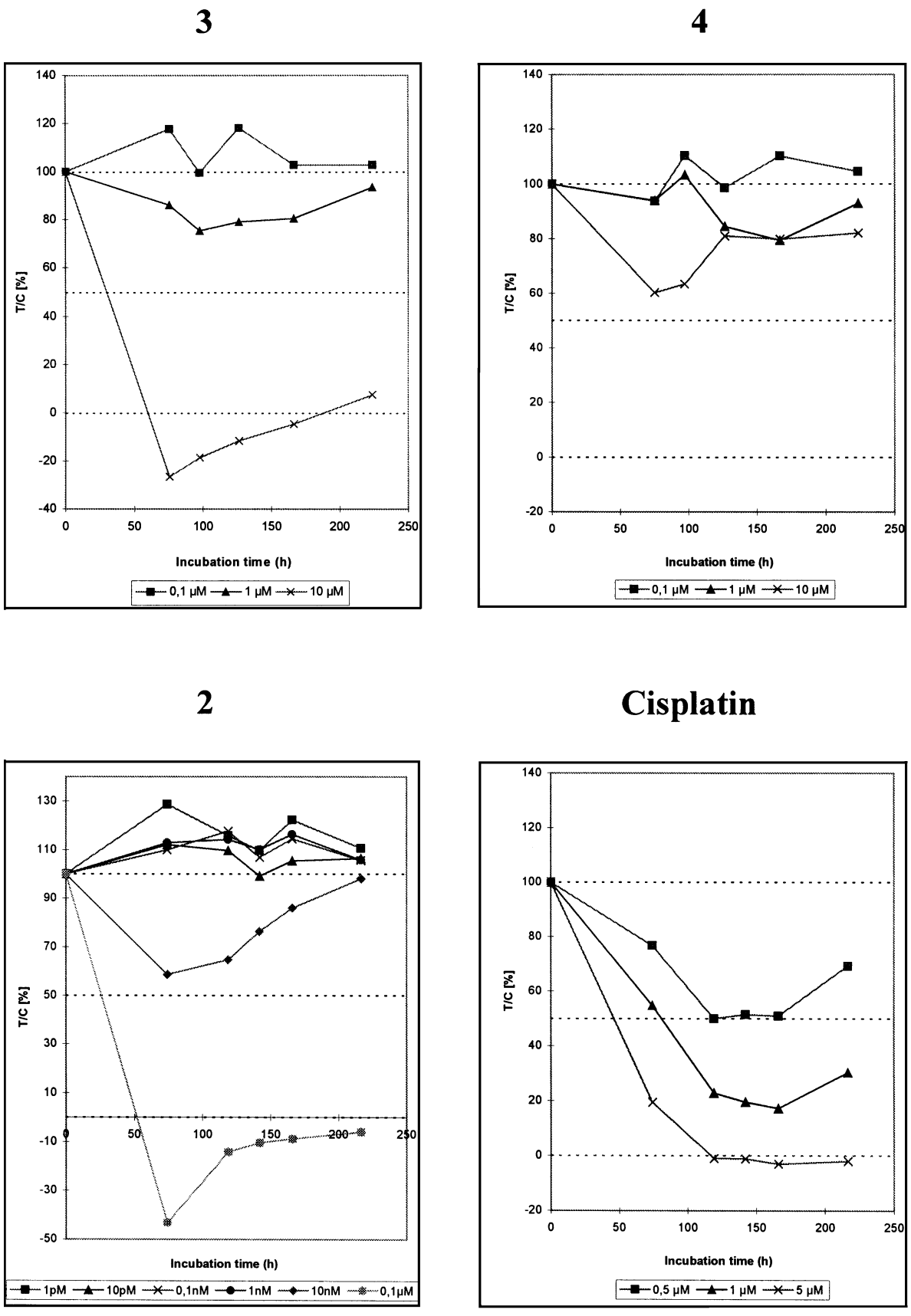

Figure 1. Growth inhibition of MCF-7 breast cancer cells by inhibitors of histone deacetylase.

TEA were added. After stirring overnight 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 50 mL each water and sat. brine. The organic layer was dried over Na_2SO_4 and the solvent was evaporated.

Methyl N-Benzyloxysuberoylamide

General method for amide formation: 1.99 g (10 mmol) monomethyl suberate, 1.39 mL (1.01 g, 10 mmol) TEA, 2.80 g (11 mmol) BOP-Cl, 1.60 g (10 mmol) *O*-benzylhydroxylamine hydrochloride, 4.17 mL (3.03 g, 30 mmol) TEA. The organic phase was also washed with 2 M HCl. The resulting oil was purified by FC using ethyl acetate/hexane (1:1). Yield 5.28 g (95%) colorless oil.– IR (KBr): ν = 3200s, 2940s, 1740s, 1660s.– MS (70 eV); *m/z* (%) = 170 (1), 106 (12), 91 (100).– (^{1}H NMR ($CDCl_3$): δ = 7.47 (bs, 1 H, NH), 7.38–7.34 (m, 5 H, Ar-H), 4.88 (s, 2 H, OCH_2), 3.66 (s, 3 H, OMe), 2.34–2.27 (m, 2 H, CH_2), 1.99–1.88 (m, 2 H, CH_2), 1.67–1.60 (m, 4 H, CH_2), 1.38–1.26 (m, 4 H, CH_2).– ^{13}C NMR ($CDCl_3$): δ = 174.20 (COOMe), 129.18 (Ar-CH), 128. 73 (Ar-1), 128.60 (Ar-CH), 78.26 (OCH_2), 51.44 (OMe), 33.95, 32.99, 32.80, 28.71, 25.00 and 24.71 (CH_2).

N-Benzyloxymonosuberoylamide **(5)**

2.77 g (10 mmol) of methyl *N*-benzyloxysuberoylamide were dissolved in 10 mL of THF and 10 mL (20 mmol) of a 1 M LiOH solution were added. The mixture was stirred for 4 h at room temperature. 10 mL of 0.5 M NaOH and 50 mL of ethyl acetate were added. The aqueous phase was acidified with 2 M HCl (pH 3) and extracted three times with 50 mL portions of ethyl acetate. The organic phase was dried over Na_2SO_4 and evaporated. The resulting solid was sufficiently pure for the next coupling step. Yield 1.78 g (68%) colorless crystals.– Mp 73 °C.– IR (KBr): ν = 3240s, 2940s, 1695s, 1655s.– MS (70 eV); *m/z* (%) = 105 (26), 91 (100), 77 (28).– ^{1}H NMR ($CDCl_3$): δ = 8.29 (bs, 1 H, NH), 7.37 (s, 5 H, Ar-H), 4.89 (bs, 2 H, OCH_2), 2.35–2.27 (m, 3 H, CH_2), 2.03 (bs, 1 H, CH_2), 1.60–1.57 (m, 4 H, CH_2), 1.32–1.27 (m, 4 H, CH_2).– ^{13}C NMR ($CDCl_3$): δ = 178.74 (COOH), 129.23 (Ar-CH), 128. 82 (Ar-1), 128.65 (Ar-CH), 78.39 (OCH_2), 33.94, 32.71, 28.70, 28.62, 24.72 and 24.51 (CH_2).

N-Benzyloxy-N′-[1-(S)-methoxycarbonyl-2-phenylethyl]suberoylbisamide

General method for amide formation; 1.11 g (4 mmol) **5**, 0.5 mL (0.41 g, 4 mmol) TEA, 0.86 g (4 mmol) L-phenylalanine methyl ester hydrochloride, 1.12 g (4.4 mmol) BOP-Cl, 1.68 mL (1.21 g, 12 mmol) TEA. The organic phase was also washed with 2 M HCl. The crude was chromatographed using ethyl acetate/hexane (5:1). Yield 1.25 g (71%) colorless crystals.– Mp 86 °C.– IR (KBr): ν = 3050s, 1730s, 1640s.– MS (70 eV); *m/z* (%) = 440 (3) [M^+], 274 (14), 162 (100).– ^{1}H NMR ($CDCl_3$): δ = 8.79 (bs, 1 H, N*H*OCH_2), 7.37 (s, 5 H, OCH_2*Ph*), 7.33–7.22 (m, 3 H, Phe-H), 7.11–7.06 (m, 2 H, Phe-H), 6.02 (d, ^3J = 7.53 Hz, 1 H, Phe-NH), 4.89–4.81 (m, 3 H, OCH_2 and *CH*COOMe), 3.71 (s, 3 H, OMe), 3.19–3.02 (m, 2 H, CH*CH*$_2$Ph), 2.17–2.09 (m, 2 H, CH_2), 2.02 (bs, 2 H, CH_2), 1.63–1.49 (m, 4 H, CH_2), 1.27–1.24 (m, 4 H, CH_2).– ^{13}C NMR ($CDCl_3$): δ = 172.70 (*C*OOMe), 172.31 (CONH), 135.99 (Phe-1), 135.00 (OCH_2*C*), 129.22, 129.18, 128.70, 128.60 and 127.14 (Ar-CH), 78.33 (OCH_2), 53.06 (OMe), 52.27 (*CH*COOMe), 37.94, 36.18, 32.70, 28.48, 25.20 and 24.82 (CH_2). Anal. ($C_{25}H_{32}N_2O_5$) 440.59: C, H, N.

N-Hydroxy-N′-[1-(S)-methoxycarbonyl-2-phenylethyl]suberoylbisamide **(3)**

1.00 g (2.27 mmol) *N*-benzyloxy-*N′*-[1-(S)-methoxycarbonyl-2-phenylethyl]-suberoylbisamide was dissolved in 15 mL of methanol and 100 mg 10% palladium on charcoal were added. The mixture was treated with hydrogen under atmospheric pressure for 6 h and subsequently filtered. The product was evaporated, redissolved in 5 mL of methanol, and precipitated with diethyl ether. Yield 510 mg (64%) colorless crystals.– Mp 105 °C.– IR (KBr): ν = 3200m, 1730s, 1620s.– MS (70 eV); *m/z* (%) = 350 (9) [M^+], 162 (75), 120 (100).– ^{1}H NMR ($CDCl_3$): δ = 7.34–7.23 (m, 3 H, Ar-H), 7.14–7.10 (m, 2 H, Ar-H), 6.33 (d, ^3J = 7.59 Hz, Phe-NH), 4.89–4.83 (m, 1 H, C*H*COOMe), 3.74 (s, 3 H, OMe), 3.22–3.00 (m, CH*CH*$_2$Ph), 2.20–2.07 (m, 4 H, CH_2), 1.71–1.48 (m, 4 H, CH_2), 1.36–1.21 (m, 4 H, CH_2).– ^{13}C NMR (CD_3OD): δ = 172.19 (CO), 169.06 (CO), 137.28 (Phe-1), 128.95 and 128.11 (Phe-2,3,5,6), 126.41 (Phe-4), 53.31 (OMe), 51.70 (*CH*COOMe), 36.63, 34.89, 32.21, 28.30, 28.11 and 24.97 (CH_2, one resonance is obscured).– Anal. ($C_{18}H_{26}N_2O_5$) 350.46: C, H, N.

Methyl 6-(4-Dimethylaminobenzoylamino)caproate

General method for amide formation: 1.65 g (10 mmol) 4-dimethylamino benzoic acid, 1.39 mL (1.01 g, 10 mmol) TEA, 2.80 g (11 mmol) BOP-Cl, 1.82 g (10 mmol) methyl 6-aminocaproate hydrochloride, 4.17 mL (3.03 g, 30 mmol) TEA. The crude was purified by FC using ethyl acetate/hexane (2:1). Yield 1.70 g (58%) colorless crystals.– Mp 71 °C.– IR (KBr): ν = 3275s, 2935s, 1730s, 1590s.– MS (70 eV); *m/z* (%) = 292 (18) [M^+], 219 (10), 148 (100).– ^{1}H NMR ($CDCl_3$): δ = 7.70–7.65 (m, 2 H, 2′-H, 6′-H), 6.70–6.64 (m, 2 H, 3′-H, 5′-H), 6.12 (bs, 1 H, NH), 3.66 (s, 3 H, OCH_3), 3.48–3.38 m (m, 2 H, CONH*CH*$_2$), 3.01 (s, 6 H, NMe_2), 2.37–2.30 (m, 2 H, *CH*$_2$COOMe), 1.71–1.55 (m, 4 H, NHCH$_2$*CH*$_2$CH$_2$*CH*$_2$), 1.47–1.35 (m, 2 H, NHCH$_2$CH$_2$*CH*$_2$).– ^{13}C NMR ($CDCl_3$): δ = 174.07 (COOMe), 167.49 (CONH), 152.47 (Me_2NC), 128.36 (C-3′, C-5′), 121.76 (*C*CONH), 111.17 (C-2′, C-6′), 51.46 (OCH_3), 40.14 (CONH*CH*$_2$), 39.61 (NMe_2), 33.94 (*CH*$_2$COOMe), 29.53, 26.51 and 24.58 (CH_2).

6-(4-Dimethylaminobenzoylamino)caproic Acid ***(6)***

1.17 g (4 mmol) of methyl 6-(4-dimethylaminobenzoylamino)caproate were dissolved in 10 mL of THF and 4 mL (8 mmol) of a 1 M LiOH solution were added. The mixture was stirred for 4 h at room temperature. 10 mL of 0.5 M NaOH and 50 mL of ethyl acetate were added. The aqueous phase was treated with 2 M HCl to pH 6–7 and some of the acid precipitated. The mother liquor was evaporated to dryness and dried over P_2O_5 in a desiccator overnight. The resulting solid was sufficiently pure for the next coupling step. The spectral data was taken form the precipitated pure acid.– Mp 165 °C.– IR (KBr): ν = 3380s, 2910s, 1700s, 1600s.– MS (70 eV); *m/z* (%) = 278 (36) [M^+], 219 (19), 148 (100).– ^{1}H NMR (CD_3OD): δ = 7.71–7.66 (m, 2 H, 2′-H, 6′-H,), 6.75–6.70 (m, 2 H, 3′-H, 5′-H), 3.37–3.28 (m, 2 H, CONH*CH*$_2$), 3.01 (s, 6 H, NMe_2), 2.34–2.27 (m, 2 H, *CH*$_2$COOH), 1.80–1.55 (m, 4 H, NHCH$_2$*CH*$_2$CH$_2$*CH*$_2$), 1.48–1.35 (m, 2 H, NHCH$_2$CH$_2$*CH*$_2$).– ^{13}C NMR (DMSO-d6): δ = 174.32 (COOH), 165.97 (CONH), 151.94 (Me_2NC), 128.34 (C-3′, C-5′), 121.48 (*C*CONH), 110.71 (C-2′, C-6′), 39.65 (NMe_2), 38.81 (CONH*CH*$_2$), 33.59 (*CH*$_2$COOMe), 29.02, 26.02, 24.23 (CH_2).– Anal. ($C_{15}H_{18}N_2O_3$) 278.32: C, H, N.

N-Benzyloxy-6-(4-dimethylaminobenzoylamino)capramide

General method for amide formation; combined **6** and crude saponification product, 0.56 mL (0.41 g, 4 mmol) TEA, 0.64 g (4 mmol) *O*-benzylhydroxylamine hydrochloride, 1.12 g (4.4 mmol) BOP-Cl, 1.68 mL (1.21 g, 12 mmol), TEA. The crude was chromatographed using ethyl acetate with 1% diethylamine. Yield (from the ester in two steps): 1.06 g (69%) colorless crystals.– Mp 128 °C.– IR (KBr): ν = 3350s, 3150s, 2925s, 1660s, 1605s.– MS (70 eV); *m/z* (%) = 383 (7) [M^+], 212 (49), 148 (100).– ^{1}H NMR ($CDCl_3$): δ = 7.62–7.57 (m, 2 H, 2′-H, 6′-H), 7.30 (s, 5 H, Ph), 6.60–6.55 (m, 2 H, 3′-H, 5′-H), 4.81 (s, 2 H, OCH_2), 3.38–3.29 (m, 2 H, CONH*CH*$_2$), 2.93 (s, 6 H, NMe_2), 2.01 (bs, 2 H, *CH*$_2$CO), 1.68–1.45 (m, 4 H, NHCH$_2$*CH*$_2$CH$_2$*CH*$_2$), 1.36–1.25 (m, 2 H, NHCH$_2$CH$_2$*CH*$_2$).– ^{13}C NMR ($CDCl_3$): δ = 167.61 (*C*ONHCH$_2$), 152.50 (Me_2NC), 129.16 (Ar-CH), 128.67 (OCH_2*C*), 128.58 (Ar-CH), 128.37 (Ar-CH), 121.58 (*C*CONHCH$_2$), 111.18 (C-2′, C-6′), 78.35 (OCH_2), 40.11 (CONH*CH*$_2$), 39.50 (NMe_2), 32.81 (*CH*$_2$CO), 29.40, 26.27 and 24.51 (CH_2).

N-Hydroxy-6-(4-dimethylaminobenzoylamino)capramide **(4)**

383 mg (1 mmol) of *N*-benzyloxy-6-(4-dimethylaminobenzoylamino)-capramide were dissolved in 15 mL of methanol and 40 mg 10% palladium on charcoal were added. The mixture was treated with hydrogen under atmospheric pressure for 6 h and was subsequently filtered. The product was evaporated, redissolved in 5 mL of methanol, and precipitated with diethyl ether. The last step was repeated once. Yield 0.16 g (56%) white powder.– Mp 170 °C.– IR (KBr): ν = 3365s, 3160s, 1650m, 1600s.– MS (70 eV); *m/z* (%) = 293 (5) [M^+], 164 (19), 148 (100).– ^{1}H NMR (DMSO-D6): δ = 10.33 (s, 1 H, CO*NH*OH), 8.67 (s, 1 H, CONH*OH*), 8.07 (t, 1 H, ^3J = 5.45 Hz, CO*NH*CH$_2$), 7.71–7.65 (m, 2 H, 2′-H, 6′-H,), 6.69–6.65 (m, 2 H, 3′-H, 5′-H), 3.23–3.13 (m, 2 H, CONH*CH*$_2$), 2.94 (s, 6 H, NMe_2), 1.97–1.90 (m, 2 H,

CH$_2$CO), 1.53–1.42 (m, 4 H, NHCH$_2$*CH*$_2$CH$_2$*CH*$_2$), 1.30–1.22 (m, 2 H, NHCH$_2$CH$_2$*CH*$_2$).– ^{13}C NMR (DMSO-D6): δ = 169.08 (CONHOH), 166.01 (*CO*NHCH$_2$), 151.96 (Me$_2$N*C*), 128.39 (C-3′, C-5′), 121.42 (*C*CONHCH$_2$), 110.74 (C-2′, C-6′), 39.72 (NMe$_2$), 38.66 (CONH*CH*$_2$), 32.24 (*CH*$_2$CO), 29.11, 26.14 and 24.92 (CH$_2$).– Anal. ($C_{15}H_{23}N_3O_3$) 293.41: C, H, N.

Biological Methods

Cell Culture

The human MCF-7 breast cancer cell line was obtained from the American Type Culture Collection (ATCC, Rockville, Md.; USA). Cell line banking and quality control were performed according to the seed stock concept reviewed by Hay [21]. The MCF-7 cells were maintained as a monolayer culture at 37 °C in a humidified 95% air, 5% CO_2 atmosphere in T-75 flasks using L-glutamine containing Eagle's MEM supplemented with BCS (100mL/L) as growth medium. The cell line was weekly passaged after treatment with trypsin/EDTA.

In Vitro Chemosensitivity Assay

The *in vitro* testing of the inhibitors of histone deacetylase for antitumor activity was carried out on MCF-7 cells according to a previously published microtiter assay [13]. Briefly, 100 µL of a cell suspension at 500 cells/mL culture medium were plated into each well of a 96-well microtiter plate and incubated at growing conditions (see above) for three days. Then each substance was added in three concentrations. After the proper incubation time the medium was removed, the cells were fixed with a glutardialdehyde solution and stored at 4 °C. Cell biomass was determined by a crystal violet staining technique as described earlier [13,22]. The effectiveness (cytostatic effect) of the compounds is expressed as:

$$T/C_{corr}\,[\%] = [(T - C_o) / (C - C_o)] \times 100$$

where T (test) and C (control) are the optical densities at 590 nm of the crystal violet extract of the cells in the wells (i.e. the chromatin-bound crystal violet extracted with ethanol 70%), and C_o is the optical density of the cell extract immediately before treatment. Cytocidal effect:

$$T_{corr}\,[\%] = [(T - C_o) / C_o] \times 100.$$

For the automatic estimation of the optical density of the crystal violet extract in the wells the Microplate Photometer Labsystem Multiscan® Plus was used.

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