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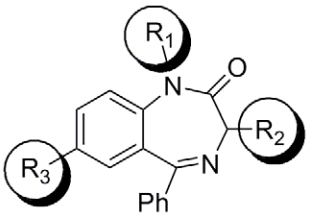
Highlights

► A series of hybrids between SAHA or oxamflatin and 5-phenyl-1,4-benzodiazepines has been synthesized.

► The compounds were evaluated *in vitro* on recombinant hHDAC1 and hHDAC 6 and on HeLa nuclear extracts.

► Antiproliferative activity was tested on different cancer cells types.

► Compound (*S*)-**8** displayed interesting activity against hematological and solid malignancies.



R_1-R_3 = linker-CONHOH, H, CH_3, NH_2

Design, synthesis and preliminary evaluation of a series of histone deacetylase inhibitors carrying a benzodiazepine ring.

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Abstract

A series of new histone deacetylase inhibitors were designed and synthesized based on hybridization between SAHA or oxamflatin and 5-phenyl-1,4-benzodiazepines. The compounds were tested for their enzyme inhibitory activity on HeLa nuclear extracts, and on human recombinant HDAC1 and HDAC6. Antiproliferative activity was tested on different cancer cells types, while proapoptotic activity was primarily tested on NB4 cells. The compounds showed IC_{50} values similar to those of SAHA. Compound (*S*)-**8** displayed interesting activity against hematological and solid malignancies.

Keywords

HDAC inhibitors, 1,4-benzodiazepine, antiproliferative activity, apoptosis, enantioselectivity.

List of abbreviations:

AML, acute myeloid leukemia; BDZ, 5-phenyl-1,4-benzodiazepine; BOP-Cl, bis(2-oxo-3-oxazolidinyl)phosphonic chloride; ER, Eudismic ratio; HDAC, histone deacetylase; SAHA , suberoylanilide hydroxamic acid.

1. Introduction

Histone deacetylases (HDAC) are a family of enzymes playing a crucial role in chromatin remodeling therefore affecting trascriptional processes. These enzymes can be grouped into four classes showing different cellular localization; they act by removing amidic functions on the lysine amino group of histone and non-histone proteins [1].

HDAC inhibition produces anticancer effects in different types of tumors through inhibition of cell growth and induction of cell differentiation and apoptosis; as HDAC are often overexpressed in cells from different tumors, these enzymes have become attractive targets for anticancer therapy [2]. The efficacy of HDAC inhibitors in oncology is highlighted by the number of compounds which entered clinical trials [3]. Only two compounds, namely SAHA (vorinostat) and FK228 (romidepsin) [4,5], have been approved so far for cutaneous T-cell lymphoma, but this class of compounds is undergoing testing for both hematological malignancies and solid tumors, with some adverse effect and not always with success [6]. Therefore, the development of novel HDAC inhibitors endowed with high anticancer properties and low-toxic profile is still a target of basic and clinical research.

The structure of HDAC inhibitors requires a group, often an hydroxamate moiety, able to chelate the zinc ion at the bottom of the active site, a recognition moiety (cap) which binds at the surface of the enzyme, and a suitable spacer which links the two moieties and which can fit the long and narrow active site; this pharmacophore is shown, as examples, in Trichostatin-A, SAHA or oxamflatin (Chart 1). In a previous paper [7] we described the synthesis and preliminary biological evaluation of some HDAC inhibitors carrying a benzodiazepine ring as cap; the design was based on an hybridization between the structure of SAHA, the first inhibitor approved for clinical use, and the 5-phenyl-1,4-benzodiazepine (BDZ) moiety, which has been reported to exert cytostatic, differentiative and apoptotic effects over a wide panel of cancer cell types [8-10]. The most interesting compound of the series were **1** and (*S*)-**2** (Chart 1); the latter was found able to trigger apoptosis in various acute myeloid leukemia (AML) cell lines and blasts from patients with different AML subtypes, as well as in human prostate LNCaP and PC3 cancer cells, showing a low-toxic profile *in vivo* [11,12].

These promising results prompted us to perform structural changes aimed to improve the pharmacological profile of this class of compounds; therefore, the following modifications were introduced. 1) The achiral compound **1**, which proved to be equipotent as (S)-**2** in inhibiting NB4 cell growth, was modified on the spacer: the length was changed into 7 or 5 carbon units (compounds **3** and **4**, general formula A), or it was replaced (compound **5**) by the same spacer found in oxamflatin [13], a compound which has previously inspired our design. 2) The importance of the N^1-methyl group was tested by synthesizing (S)-**6**, the desmethyl analog of (S)-**2**. 3) The effect of changing the position of the linker-chelating group on the benzodiazepine ring was checked by shifting the suberoyl hydroxamate moiety in position 1 (compound **7**, general formula B) or in position 3 (compound **8**, general formula C) on the BDZ ring. Since in the course of this study compound **8** was found endowed with interesting activity on NB4 cells, its enantiomers were separated in order to study enantioselectivity, while the consequences of varying the length of the linker (compounds **9**-**11**) were evaluated on the racemate. 4) With the aim of improving solubility

of **1**, compound **12** (general formula A), carrying a basic amino group in position 3, was prepared as racemate.

2. Results and discussion

2.1. Chemistry

The synthetic pathways are shown in Schemes 1-4. In order to obtain analogs of **1**, the iodo derivative **13** [7] (Scheme 1) was coupled to 6-heptynoic acid under Sonogashira conditions to afford **15**. The same reaction, attempted on 4-pentynoic acid, did not afford the desired acid **16**: a compound was obtained, showing the correct molecular weight but identified, from the NMR spectrum, as the enol-lactone **17** [14]. Compound **16** was therefore obtained by coupling **13** with ethyl 4-pentynoate [15], followed by alkaline hydrolysis of the intermediate ester **18**. Ethyl pent-4-yn-2-enoate [16] was prepared according to Wei as E/Z mixture [17], and coupled with **13**; chromatographic separation of the esters **19**, followed by alkaline hydrolysis afforded acid **20**. Acids **15**, **16** and **20** were then reacted with ethyl chlorocarbonate and hydroxylamine hydrochloride to afford the desired hydroxamate **3**, **4** and **5**. In a similar way, (*S*)-**14** [7] was reacted with hexynoic acid to give (*S*)-**21** which was transformed in the corresponding hydroxamate (*S*)-**6** in the usual way.

The preparation of **7** is reported in Scheme 2. Initially the synthesis of 8-oxo-8-(2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-1-yl)octanoic acid **24a** (R = H, X = O) was attempted, through reaction of 5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-2-one **22** [18] with ethyl 8-chloro-8-oxooctanoate followed by alkaline hydrolysis of the ester intermediate. However, attempts to purify ester **24b** (R = Et, X = O) failed, since the compound proved to be unstable; moreover, the decomposition was much faster when the hydrolysis of the ester group was attempted. Therefore, compound **7** was designed, where the 2-oxo moiety was replaced by CH_2. It was prepared by reacting 5-phenyl-2,3-dihydro-1H-1,4-benzodiazepine **23**, obtained by $LiAlH_4$ reduction of **22** [19], with ethyl hydrogen suberate, followed by alkaline hydrolysis and coupling with hydroxylamine hydrochloride under the usual conditions.

The synthesis of compounds **8**-**11** is reported in Scheme 3. *Rac*-**27** [20] was treated with the suitable dicarboxylic acid as monoesters to give compounds **28**-**30**, which were hydrolyzed to acids **31**-**33**. Reaction with dimethyl-*t*-butylsilylhydroxylamine and removal of the protecting group gave hydroxamates **8**-**10**. Reaction of *rac*-**27** with pimelic acid anhydride gave compound **34**, which was transformed into hydroxamate **11** by reaction with ethyl chlorocarbonate and hydroxylamine.

(*S*)- and (*R*)-**27** were obtained according to Sherrill [21], and transformed into (*S*) and (*R*)-**8** as reported for the racemate. Since the Sherrill's procedure in our hands gave low yields of the enantiopure amines **27** (see experimental section), and given the promising pharmacological results shown by (*S*)-**8**, (*S*)-**27** was later obtained in good yield from *rac*-**27** by fractional crystallization of the (*S*)-camphorsulfonate salt as reported by Reider [22], and converted into (*S*)-**8** as described for the racemate.

The synthesis of the aminoderivative **12** is reported in Scheme 4. The pathway used to prepare **27** was applied to the synthesis of compound **36**, starting from 2-(1H-1,2,3-benzotriazol-1-yl)-2-{[(benzyloxy)carbonyl]amino}acetic acid **35** and 2-amino-5-iodobenzophenone [21,23]. After methylation and exchange of the N-protecting group, the N-Boc derivative **37** was transformed into hydroxamate **38** as reported for analogs **3**-**6**. Treatment with hydrochloric acid gave compound **12**.

2.2. Biological tests

Initially the compounds were tested for their HDAC inhibitory activity on HeLa cell nuclear extracts and on recombinant HDAC1 using a fluorometric assay; the results are reported in Table 1. SAHA was used as the reference compound. Compounds **1** and (*S*)-**2**, previously characterized by us, were also tested in this assay. The data on HDAC1 and on HeLa nuclear extracts show that for compounds with general formula A (Table 1) the activity is only marginally affected by the length of the spacer, as compounds **3** and **4** showed activity in the same range as **1**. On the contrary, the stereogenic center in position 3 on the BDZ nucleus was found important for activity: as a matter of fact, compounds (*S*)-**2** and (*S*)-**6** are 2-5 times more potent than compounds **1**, **3** and **4**, and, as previously reported [7,11], (*S*)-**2** is more potent than (*R*)-**2**. The presence of the methyl group in position 1 did not affect activity, (*S*)-**6** being equipotent with (*S*)-**2** in both HeLa extract and HDAC1. The length of the linker between the triple bond and the hydroxamate moiety is not important for activity, compounds **1**, **3** and **4** showing similar potency; on the contrary, the restriction of flexibility greatly improves activity, since compound **5** showed a potency similar to (S)-**2** and (S)-**6** on HDAC1 and even higher on HeLa nuclear extracts. The introduction of a NH_2 group in position 3 of the BDZ ring, replacing the methyl group of compound **2**, did not improve activity, although *rac*-**12** shows on HDAC1 a potency similar to (S)-**2**.

The linker-hydroxamate group was successfully moved in different position on the BDZ ring: compounds **7** and **8**, carrying this moiety, respectively, in position 1 and 3, show potency in the high nanomolar range. Substitution in position 3 introduces a stereogenic center in the molecule: as it happens for **2**, also for **8** the *S* isomer is the eutomer. For compounds of general formula C the variation of the linker's length affected activity in a different way on HeLa nuclear extracts with respect to HDAC1: while on HDAC1 the most potent compound is the azelate derivative *rac*-**11**, on HeLa nuclear extracts (*S*)-**8** is 2-5 times more potent than its analogs. These small differences in structure-activity relationships can be due to the different experimental conditions or to the presence of additional HDAC isoforms in the HeLa nuclear extracts; however, the two experimental models gave IC_{50} values in the same range.

Since compounds **1** and (*S*)-**2** were shown to increase acetylation of α-tubulin, a substrate for HDAC6, the inhibitory activity of the compounds was tested also by a cell-free assay of this recombinant isoform (Table 1). In general, the synthesized compounds proved to be less active on HDAC6 with respect to HDAC1 with some exceptions, namely (*S*)-**8**, its lower homolog *rac*-**11**, and (*S*)-**6**. Contrary to what happened on HDAC1, the removal of the N^1 methyl group of (*S*)-**2**, to give (*S*)-**6**, produced a 5-fold increase of activity on HDAC6. The variation of chain length also influenced the potency of the compounds with general formula C (Chart 1). On one hand, the removal of one carbon unit from (*S*)-**8** to *rac*-**11** brought a two-fold increase in potency on both HDAC1 and HDAC6; on the other hand, further shortening (*rac*-**10**, 4 methylene units) or the increase (*rac*-**9**, 7 methylene units) of the chain length abolished or, respectively, reduced the activity on HDAC6. On this isoform, *rac*-**11** is the most potent inhibitor of this class of compounds, while *rac*-**10** shows the highest HDAC1/HDAC6 selectivity.

The inhibitory activity of the compounds was also measured on HDAC4, a class IIa HDAC isoform (Table 1). A preliminary test, performed at a fixed concentration (5 μM), showed that the compounds were definitely less potent on this isoform with respect to HDAC1 and HDAC6, the

residual enzymatic activity ranging between 65-100%. This finding suggests for all the compounds a IC_{50} value higher than 5 μM on HDAC4; for this reason, a dose response curve was not performed.

The ability of the compounds to inhibit cancer cell growth was assessed on K562 (human chronic myelogenous leukemia), A549 (human alveolar basal epithelial) and HCT-116 (human colon cancer) cells using an automated assay. IC_{50} values are reported in Table 2. All the compounds were able to reduce cell growth with IC_{50} values in the micromolar range, thus showing that different tumor cell lines were sensitive to the antiproliferative activity of this class of substances. Some compounds showed IC_{50} values in the high nanomolar range, namely (*S*)-**2** and (*S*)-**6** on HCT116 cells, and **5** and (*S*)-**8** on both HCT116 and K562 cell lines, the latter showing a potency similar to the reference compound SAHA. Since our first interest was to find compounds endowed with antileukemic activity, the newly synthesized compounds were tested on human acute promyelocytic leukemia NB4 cell line as the model. The proapoptotic activity of the compounds was assessed by means of the Annexin-V test and it is reported as IC_{50} values (i.e. the drug concentration necessary to induce apoptosis in 50% of the population compared to untreated cultures, Table 2). With the exception of **3** and (*R*)-**8**, all the new compounds were able to induce apoptosis in NB4 cells with IC_{50} values in a low micromolar range, the most active compound being (*S*)-**8**, with an IC_{50} of 0.86 μM.

In previous papers [7,11] we reported that the enantiomers of compound **2** showed different potency in several tests. As a matter of fact, the IC_{50} for antiproliferative activity on NB4 cells was found 12 times lower for (*S*)-**2** than (*R*)-**2**; nevertheless the difference in potency on HDAC1 and HDAC6 of the two enantiomers as determined by a cell-free assay was less than three-fold. The cellular tests reported in this paper (see Table 1) also show an eudismic ratio closer to that previously found in NB4 cells as far as antiproliferative activity is concerned (ER values are 9.5, 8.0 and 9.1 on HCT116, A549 and K562 cell lines, respectively); a similar comparison cannot be done for the proapoptotic effect, since a IC_{50} cannot be derived for (*R*)-**2**. These findings suggest that probably other factors, besides the interaction with HDAC, can affect the cellular activity of (*S*)-**2** and(*R*)-**2**. As far as compound **8** is concerned, enantioselectivity is low on HeLa nuclear extracts, on HDAC1 and also on HCT116, A549 and K562 cell lines, the eudismic ratios being below 3, but it is higher on HDAC6 (ER = 16). The finding that also for compound **8** the eutomer is in all of the performed tests the *S* enantiomer may be just a coincidence, since the two compounds should adopt an opposite orientation when interacting with the enzyme, thus placing the chiral center in different position of the protein domain.

On the basis of these results, (S)-**8** was selected for further study on NB4 cells. As shown in Fig. 1, the drug was capable of inducing histone H3 and H4 acetylation in a dose-dependent manner as assessed by Western blot and immunostaining for the acetylated histones after a 6 hour-treatment with 0.5, 1 and 2 μM concentration (panel A). As it happened for other compounds of this series [7], histone H3 were more sensitive to the drug: densitometric analysis using tubulin as the reference protein (see Materials and Methods) shows that a 0.5 μM concentration of (*S*)-**8** was able to induce 50% acetylation of histone H3 but only 8% of histone H4; at a 1 μM concentration the percentage of acetylated H3 and H4 were 84% and 51% respectively. By extending the time of incubation up to 48 hours a marked decrease in cell viability was observed (IC_{50} 0.46 μM, Fig. 1, panel B) that was accompanied by a significant increase in apoptotic rates in culture. Drug-induced

apoptosis as measured by cytofluorimetric analysis of Annexin/PI stained cells (Fig. 1. Panel C, left) was especially evident in cultures treated with 1 and 2 µM drug where the portion of the population committed to cell death accounted for about 66 and 83%, respectively (see values reported on the upper right quadrants of dot plots). These findings were confirmed by examining the May-Grünwald/Giemsa stained cytosmears (panel C, right) showing that in cultures treated with 1 and 2 µM concentration of the drug there was a massive increase in apoptotic bodies.

In conclusion, a new HDAC inhibitor has been found ((*S*)-**8**) which displays a good potency in cell-free assays and an interesting activity against cells from both hematological (NB4 and K562 cell lines) and solid (A549 and HCT-116 cell lines) tumors. These interesting properties may depend on its ability to inhibit both HDAC1 and HDAC6; as a matter of fact, there are evidences that simultaneous inhibition of both isoforms can be an effective treatment for AML, as well as for other malignancies [24, 25]. The different ability of (*R*) and (*S*)-**8** to inhibit HDAC6 may be a possible explanation for their distinct effects on NB4 cells, nevertheless the activity of this class of compounds on HDAC6 (Table 1) does not completely correlate with the pro-apoptotic properties on NB4 cells (Table 2), highlighting once again that activity in cell-free assay does not necessarily reflect the real potency of these drugs inside the cells [11]. Further studies on this class of compounds, as well as the biological characterization of (*S*)-**8** will be reported in due time.

3. Experimental section

3.1. Chemistry. All melting points were taken on a Büchi apparatus and are uncorrected. NMR spectra were recorded on a Brucker Avance 400 spectrometer (400 MHz for ^{1}H NMR, 100 MHz for ^{13}C). Chromatographic separations were performed on a silica gel column by gravity chromatography (Kieselgel 40, 0.063- 0.200 mm; Merck) or flash chromatography (Kieselgel 40, 0.040-0.063 mm; Merck). Yields are given after purification, unless differently stated. When reactions were performed under anhydrous conditions, the mixtures were maintained under nitrogen. Exact masses of compounds **3-12** were determined with a Thermo Finnigan LTQ Orbitrap mass spectrometer equipped with an electrospray ionization source (ESI). Analysis were carried out in positive ion mode observing protonated molecules $[M+H]^+$ using a proper dwell time aquisition to achieve 60000 units of resolution at Full Width at Half Maximum (FWHM). Elemental composition of compounds were calculated on the basis of their exact masses, accepting only results with an attribution error Delta less than 5 ppm and a not integer RDB (double bond/ring equivalents) value, in order to consider only the protonated species [26]. Compounds were named following IUPAC rules as applied by Reaxys (version 1.0.9619) software.

3.1.1. 7-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-7-yl)hept-6-ynoic acid 15

A mixture of 7-iodo-1-methyl-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-2-one **13** [7] (1.01 g, 2.68 mmol), 6-heptynoic acid (0.51 mL, 4.02 mmol), CuI (0.06 g, 0.32 mmol), $Pd(PPh_3)_4$ (0.12 g, 0.11 mmol) and anhydrous Et_3N (15 mL) were heated at 55 °C for 30 h under nitrogen, then it was partitioned between a saturated solution of NH_4Cl and ethyl acetate. The organic layer was collected and the solvent was removed under vacuum, then the residue was treated with HCl 2N (30 mL) and washed with EtOAc (3 x 30 mL), then additioned with $NaHCO_3$ until pH 7 and extracted with EtOAc. Drying (Na_2SO_4) of the second organic phase and removal of the solvent gave the title compound in 80% yield. Yellow solid, m.p. 51-52 °C. [^{1}H]-NMR ($CDCl_3$) δ: 1.59-1.66 (m, 2H,

CH_2); 1.72-1.80 (m, 2H, CH_2); 2.36-2.44 (m, 4H, $2CH_2$); 3.41 (s, 3H, NCH_3); 3.78 (d, J = 11.4 Hz, 1H, C*H*H); 4.86 (d, J = 11.4 Hz, 1H, CH*H*); 7.29 (d, J = 8.8 Hz, 1H, arom.); 7.33 (d, J = 2.0 Hz, 1H, arom.); 7.42-7.46 (m, 2H, arom.); 7.50-7.53 (m, 1H, arom.); 7.59 (dd, J = 8.8 Hz, 2.0 Hz, 1H, arom.); 7.63-7.67 (m, 2H, arom.) ppm.

3.1.2. 5-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-7-yl)pent-4-ynoic acid 16

Following the same procedure used for **15**, **13** (0.48 g, 1.28 mmol) was treated with 6-pentynoic acid ethyl ester [15] (0.25 g, 1.95 mmol), CuI (0.03 g, 0.15 mmol), $Pd(PPh_3)_4$ (0.06 g, 0.05 mmol) and anhydrous Et_3N (9 mL) at 55 °C for 2 h under nitrogen. Purification with flash chromatography (CH_2Cl_2/MeOH 99:01) gave ethyl 5-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-7-yl)pent-4-ynoate **18** in 88% yield. Yellow waxy solid. [^{1}H]-NMR ($CDCl_3$) δ: 1.23 (t, J = 7.2 Hz, 3H, CH_2CH_3); 2.57 (t, J = 7.0 Hz, 2H, CH_2CH_2); 2.68 (t, J = 7.0 Hz, 2H, CH_2CH_2); 3.40 (s, 3H, NCH_3); 3.77 (d, J = 11.0 Hz, 1H, C*H*H); 4.14 (q, J = 7.2 Hz, 2H, CH_2CH_3); 4.84 (d, J = 11.0 Hz, 1H, CH*H*); 7.28 (d, J = 8.4 Hz, 1H, arom.); 7.32 (d, J = 2.0 Hz, 1H, arom.); 7.41-7.45 (m, 2H, arom.); 7.48-7.52 (m, 1H, arom.); 7.56 (dd, J = 8.8 Hz, 2.0 Hz, 1H, arom.); 7.63-7.65 (m, 2H, arom.) ppm. When the reaction was performed with 6-pentynoic acid, **17** was obtained; this compound was not further purified, nor the stereochemistry on the double bond was investigated. MS (ESI) 347 (M+1). [^{1}H]-NMR ($CDCl_3$) δ: 2.57-2.75 (m, 2H, CH_2CO); 2.97-3.02 (m, 2H, $CH_2C{=}C$); 3.40 (s, 3H, NCH_3); 3.79 (d, J = 11.0 Hz, 1H, C*H*H); 4.82 (d, J = 11.0 Hz, 1H, CH*H*); 6.23 (t, J = 2.2 Hz, 1H, C=CH); 7.12 (d, J = 2.0 Hz, 1H, arom.); 7.33 (d, J = 8.4 Hz, 1H, arom.); 7.38-7.49 (m, 4H, arom.); 7.62-7.66 (m, 2H, arom.) ppm.

A solution of **18** (0.42 g, 1.12 mmol), NaOH (0.07 g, 1.70 mmol) in 2 mL of MeOH, 2 mL of THF and 2 mL of H_2O were stirred at RT for 2 hr, then it was treated with sat. aqueous NH4Cl and extracted with ethyl acetate. Drying (Na_2SO_4) and removal of the solvent gave the title compound in 93% yield. Rose solid, m.p. 50-51 °C. [^{1}H]-NMR ($CDCl_3$) δ: 2.60-2.70 (m, 4H, CH_2CH_2); 3.41 (s, 3H, NCH_3); 3.78 (d, J = 11.6 Hz, 1H, C*H*H); 4.90 (d, J = 11.6 Hz, 1H, CH*H*); 7.31-7.34 (m, 2H, arom.); 7.15-7.49 (m, 2H, arom.); 7.54-7.58 (m, 1H, arom.); 7.63 (dd, J = 8.8 Hz, 2.0 Hz, 1H, arom.); 7.69-7.71 (m, 2H, arom.) ppm.

3.1.3. (2E)-5-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-7-yl)pent-2-en-4-ynoic acid 20

Ethyl pent-4-yn-2-enoate [16] (0.30 g, 2.44 mmol), prepared according to Wei [17] as 4.3:1 E/Z mixture, was reacted with **13** (0.61 g, 1.62 mmol) CuI (0.04 g, 0.19 mmol), $Pd(PPh_3)_4$ (0.07 g, 0.06 mmol) and anhydrous Et_3N (10 mL) at 55 °C for 2 h and 30' under nitrogen, following the same procedure used for **15**. Purification with flash chromatography (cyclohexane/ethyl acetate 7:3) gave ethyl (2E)-5-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-7-yl)pent-2-en-4-ynoate **19** (0.34 g), the 2-Z isomer **19a** (0.06 g) and a mixed fraction (**19**/**19a** 2:1, 0.10 g). **19**: yellow waxy solid. [^{1}H]-NMR ($CDCl_3$) δ: 1.30 (t, J = 7.0 Hz, 3H, CH_2CH_3); 3.43 (s, 3H, NCH_3); 3.79 (d, J = 11.2 Hz, 1H, C*H*H); 4.22 (q, J = 7.0 Hz, 2H, CH_2CH_3); 4.88 (d, J = 11.2 Hz, 1H, CH*H*); 6.28 (d, J = 16.0 Hz, 1H, *CH*=CH); 6.91 (d, J = 16.0 Hz, 1H, CH=*CH*); 7.35 (d, J = 8.8 Hz, 1H, arom.); 7.41-7.47 (m, 3H, arom.); 7.50-7.54 (m, 1H, arom.); 7.64-7.68 (m, 3H, arom.) ppm. **19a**: Yellow waxy solid. [^{1}H]-NMR ($CDCl_3$) δ: 1.24 (t, J = 7.2 Hz, 3H, CH_2CH_3); 3.40 (s, 3H, NCH_3); 3.77 (d, J =

11.0 Hz, 1H, C*H*H); 4.19 (q, J = 7.2 Hz, 2H, *CH*$_2$CH$_3$); 4.84 (d, J = 11.0 Hz, 1H, CH*H*); 6.14 (d, J = 11.6 Hz, 1H, *CH*=CH); 6.29 (d, J = 11.6 Hz, 1H, CH=*CH*); 7.33 (d, J = 8.8 Hz, 1H, arom.); 7.38-7.42 (m, 2H, arom.); 7.45-7.47 (m, 2H, arom.); 7.59-7.61 (m, 2H, arom.); 7.70 (dd, J = 8.4 Hz, 2.0 Hz, 1H, arom.) ppm. **19** was hydrolyzed as reported for the preparation of **16**, obtaining the title compound in 57% overall yields (based on **13**). Yellow solid, m.p. 67-68 °C. [^{1}H]-NMR ($CDCl_3$) δ: 3.43 (s, 3H, NCH_3); 3.79 (d, J = 11.2 Hz, 1H, C*H*H); 4.88 (d, J = 11.2 Hz, 1H, CH*H*); 6.28 (d, J = 16.0 Hz, 1H, *CH*=CH); 6.97 (d, J = 16.0 Hz, 1H, CH=*CH*); 7.36 (d, J = 8.8 Hz, 1H, arom.); 7.42-7.45 (m, 3H, arom.); 7.49-7.53 (m, 1H, arom.); 7.61-7.63 (m, 2H, arom.); 7.67 (dd, J = 8.4 Hz, 2.0 Hz, 1H, arom.) ppm.

3.1.4. *N*-hydroxy-6-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-7-yl)hept-6-ynamide 3

A solution of $NH_2OH{\cdot}HCl$ (0.71 g, 10.18 mmol) in dry MeOH (5 mL) was added to a solution of KOH (0.57 g, 10.18 mmol) in dry MeOH (5 mL) at 0 °C under nitrogen. The resulting mixture was stirred for 15' at 0 °C and filtered in another flask.

Ethyl chloroformate (0.27 mL, 2.88 mmol) and anhydrous Et_3N (0.44 mL, 3.14 mmol) were added to a solution of **15** (0.81 g, 2.00 mmol) in dry THF (5 mL) at 0 °C under nitrogen. The mixture was stirred for 15' at 0 °C and filtered directly in the flask containing the previously prepared solution of NH_2OH in MeOH. The resulting mixture was stirred for 30' at RT under nitrogen, then the solvents were evaporated. The residue was purified by flash chromatography (first eluent: CH_2Cl_2/MeOH 95:5, second eluent: CH_2Cl_2/MeOH 9:1) giving the title compound in 56% yield. Yellow solid, m.p. 80-82 °C. [^{1}H]-NMR (CD_3OD) δ: 1.53-1.60 (m, 2H, CH_2); 1.69-1.76 (m, 2H, CH_2); 2.10 (t, J = 7.2 Hz, 2H, CH_2); 2.40 (t, J = 7.2 Hz, 2H, CH_2); 3.42 (s, 3H, NCH_3); 3.85 (d, J = 11.2 Hz, 1H, C*H*H); 4.62 (d, J = 11.2 Hz, 1H, CH*H*); 7.19 (d, J = 2.0 Hz, 1H, arom.); 7.44-7.48 (m, 2H, arom.); 7.50-7.54 (m, 4H, arom.); 7.63 (dd, J = 8.8 Hz, 2.0 Hz, 1H, arom.) ppm. [^{13}C]-NMR-APT (CD_3OD) δ: 19.60 (CH_2); 25.95 (CH_2); 29.01 (CH_2); 33.19 (CH_2); 35.25 (NCH_3); 56.96 (CH_2); 80.05 (C); 92.17 (C); 121.63 (C arom.); 123.06 (CH arom.); 129.31 (C arom.); 129.66 (CH arom.); 130.88 (CH arom.); 132.51 (CH arom.); 134.62 (CH arom.); 136.35 (CH arom.); 138.89 (C arom.); 144.50 (C arom.); 171.17 (CN); 172.57 (CO); 173.10 (CO) ppm. HRMS-ESI *m*/*z* [*M*+H]$^+$ calcd for $C_{21}H_{20}N_3O_3$: 362.1505, found: 362.1505.

3.1.5. *N*-hydroxy-5-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-7-yl)pent-4-ynamide 4

Following the same procedure used for **3**, **16** (0.36 g, 1.05 mmol) was treated with ethyl chloroformate (0.13 mL, 1.39 mmol), Et_3N (0.21 mL, 1.52 mmol), $NH_2OH{\cdot}HCl$ (0.34 g, 4.93 mmol) and KOH (0.28 g, 4.93 mmol). Purifications with flash chromatography (first eluent: CH_2Cl_2/MeOH/ NH_3 95:05:0.5; second eluent: CH_2Cl_2/MeOH/NH_3 90:10:01) gave the title compound in 36% yield. Pale yellow solid, m.p. 162-163 °C. [^{1}H]-NMR (CD_3OD) δ: 2.32 (t, J = 7.2 Hz, 2H, *CH*$_2$CH$_2$); 2.68 (t, J = 7.2 Hz, 2H, CH$_2$*CH*$_2$); 3.42 (s, 3H, NCH_3); 3.82 (d, J = 11.0 Hz, 1H, C*H*H); 4.62 (d, J = 11.0 Hz, 1H, CH*H*); 7.21 (d, J = 2.0 Hz, 1H, arom.); 7.43-7.46 (m, 2H, arom.); 7.50-7.55 (m, 4H, arom.); 7.63 (dd, J = 8.4 Hz, 2.0 Hz, 1H, arom.) ppm. [^{13}C]-NMR-APT (CD_3OD) δ: 16.47 (CH_2); 32.82 (CH_2); 35.14 (NCH_3); 57.54 (CH_2); 80.60 (C); 90.34 (C); 121.15 (C arom.);

122.90 (CH arom.); 129.54 (CH arom.); 129.91(C arom.); 130.65 (2CH arom.); 131.98 (CH arom.); 134.38 (CH arom.); 135.92 (CH arom.); 139.64 (C arom.); 144.52 (C arom.); 170.81 (CO); 171.55 (CN); 172.44 (CO) ppm. HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{23}H_{24}N_3O_3$: 390.1818, found: 390.1814.

3.1.6. (2E)-*N*-hydroxy-5-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-7-yl)pent-2-en-4-ynamide 5

Following the same procedure used for **3**, **20** (0.28 g, 0.82 mmol) was treated with ethyl chloroformate (0.11 mL, 1.10 mmol), Et_3N (0.17 mL, 1.19 mmol), $NH_2OH{\cdot}HCl$ (0.27 g, 3.87 mmol) and KOH (0.22 g, 3.87 mmol). Purification by flash chromatography (two separations: first with CH_2Cl_2/MeOH 90:10, second with CH_2Cl_2/MeOH/NH_3 90:10:01) gave the title compound in 20% yield. Pale yellow solid, m.p. 125 °C (dec.). [^{1}H]-NMR (CD_3OD) δ: 3.43 (s, 3H, NCH_3); 3.86 (d, J = 11.0 Hz, 1H, C*H*H); 4.65 (d, J = 11.0 Hz, 1H, CH*H*); 6.29 (d, J = 15.6 Hz, 1H, *CH*=CH); 6.82 (d, J = 15.6 Hz, 1H, CH=*CH*); 7.33 (d, J = 1.6 Hz, 1H, arom.); 7.44-7.51 (m, 2H, arom.); 7.51-7.56 (m, 3H, arom.); 7.59 (d, J = 8.8 Hz, 1H, arom.); 7.74 (dd, J = 8.8 Hz, 1.6 Hz, 1H, arom.) ppm. [^{13}C]-NMR-APT (CD_3OD) δ: 35.13 (NCH_3); 57.58 (CH_2); 88.25 (C); 95.04 (C); 119.63 (C arom.); 120.83 (*CH*=CH); 123.23 (CH arom.); 129.58 (2CH arom.); 130.12(C arom.); 130.64 (CH arom.); 131.39 (CH=*CH*); 132.06 (CH arom.); 134.91 (CH arom.); 135.98 (CH arom.); 139.58 (C arom.); 145.55 (C arom.); 164.39 (CO); 171.49 (CN); 172.26 (CO) ppm. HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{21}H_{18}N_3O_3$: 360.1348, found: 360.1345.

3.1.7. 6-[(3S)-3-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-7-yl]hex-5-ynoic acid (*S*)-**21**

Following the same procedure used for **15**, **14** (1.12 g g, 2.99 mmol) was treated with 5-hexynoic acid (0.3 mL, 2.72 mmol), CuI (0.068 g, 0.36 mmol), $Pd(PPh_3)_4$ (0.138 g, 0.12 mmol) and anhydrous Et_3N (15 mL) at 55 °C for 41 h under nitrogen athmosphere. Purification by flash chromatography (CH_2Cl_2/MeOH 9:1) gave the title compound in 61% yield. White solid, m.p. 73-75 °C. [^{1}H]-NMR ($CDCl_3$) δ: 1.71 (d, J = 6.4 Hz, 3H, CH*CH*$_3$); 1.85-1.93 (m, 2H, CH_2); 2.44 (t, J = 7.2 Hz, 2H, CH_2); 2.48 (t, J = 7.2 Hz, 2H, CH_2); 3.73 (q, J = 6.4 Hz, 1H, *CH*CH_3); 7.11 (d, J = 8.4 Hz, 1H, arom.); 7.33 (d, J = 1.6 Hz, 1H, arom.); 7.36-7.45 (m, 3H, arom.); 7.47-7.53 (m, 3H, arom.); 9.82 (s, bs, 1H, NH) ppm.

3.1.8. *N*-hydroxy-6-[(3S)-3-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-7-yl]hex-5-ynamide (*S*)-**6**

Following the same procedure used for **3**, (*S*)-**21** (0.53 g, 1.46 mmol) was treated with ethyl chloroformate (0.19 mL, 1.95 mmol), Et_3N (0.29 mL, 2.12 mmol), $NH_2OH{\cdot}HCl$ (0.48 g, 6.88 mmol) and KOH (0.39 g, 6.88 mmol). Two purifications with flash chromatography (eluents: CH_2Cl_2/MeOH/NH_3 9:1:0.1 and CH_2Cl_2/MeOH 9:1) gave the title compound in 51% yield. White solid, m.p. 198-199 °C. $[\alpha]^{20}_D$(DMSO) +288.3°. [^{1}H]-NMR (CD_3OD) δ: 1.65 (d, J = 6.4 Hz, 3H, CH*CH*$_3$); 1.81-1.88 (m, 2H, CH_2); 2.21 (t, J = 7.2 Hz, 2H, CH_2); 2.41 (t, J = 7.2 Hz, 2H, CH_2); 3.75 (q, J = 6.4 Hz, 1H, *CH*CH_3); 7.19 (d, J = 8.6 Hz, 1H, arom.); 7.22 (d, J = 2.0 Hz, 1H, arom.); 7.42-7.47 (m, 4H, arom.); 7.48-7.54 (m, 1H, arom.); 7.57 (dd, J = 8.6 Hz, 2.0 Hz, 1H, arom.) ppm. [^{13}C]-NMR-APT (CD_3OD) δ: 17.01 (CH_3); 19.45 (CH_2); 25.80 (CH_2); 32.75 (CH_2); 60.01 (CH); 80.76

(C); 90.59 (C); 120.29 (C arom.); 122.33 (CH arom.); 128.78 (C arom.); 129.45 (CH arom.); 130.80 (CH arom.); 131.73 (CH arom.); 135.04 (CH arom.); 135.96 (CH arom.); 139.80 (C arom.); 140.28 (C arom.); 171.14 (CN); 172.22 (CO); 173.15 (CO) ppm. HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{22}H_{22}N_3O_3$: 376.1661, found: 376.1656.

3.1.9. 8-oxo-8-(5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-1-yl)octanoic acid 26

$LiAlH_4$ (0.097 g, 2.55 mmol) was slowly added to a solution of 22 [19] (2.13 g, 9.06 mmol) in dry THF at 0 °C under nitrogen. The mixture is stirred at RT for 1 h, then quenched with ice and partitioned between EtOAc and brine. Drying (Na_2SO_4) of the organic phase, removal of solvent and purification with flash chromatography (eluent: CH_2Cl_2/MeOH 95:5) gave 1.22 g (61% yield) of 5-phenyl-1,3-dihydro-1,4-benzodiazepine **23** together with unreacted **22** (0.72 g). Yellow solid, m.p. 136-137 °C. [^{1}H]-NMR ($CDCl_3$) δ: 3.80-3.82 (m, 2H, CH_2); 3.96-3.98 (m, 2H, CH_2); 4.49 (s, bs, 1H, NH); 6.66-6.70 (m, 1H, arom.); 6.74 (d, J = 8.4 Hz, 1H, arom.); 7.01 (dd, J = 8.0 Hz, 1.2 Hz, 1H, arom.); 7.19-7.23 (m, 1H, arom.); 7.35-7.44 (m, 3H, arom.); 7.53-7.55 (m, 2H, arom.) ppm.

A mixture of **23** (0.6 g, 2.71 mmol), ethyl hydrogensuberate (0.66 g, 3.26 mmol), WSC (0.51 g, 3.26 mmol) and HOBT (0.44 g, 3.26 mmol) in ethanol-free $CHCl_3$ (20 mL) was stirred at room temperature for 3 days, then it was treated with 10% $NaHCO_3$ and the organic layer was separated. Drying (Na_2SO_4) and removal of the solvent gave a residue which was purified by flash chromatography (first eluent: CH_2Cl_2/MeOH/NH_3 99:01:0.1, second eluent CH_2Cl_2/MeOH/NH_3 98:02:0.2, third eluent CH_2Cl_2/MeOH/NH_3 90:10:01) giving unreacted starting material (**23**, 0.44 g) and **25** (0.22 g, 21% yield). Yellow solid, m.p. 85-86 °C. [^{1}H]-NMR ($CDCl_3$) δ: 1.24 (t, J = 7.2 Hz, 3H, $CH_2\mathit{CH_3}$); 1.29-1.35 (m, 4H, $2CH_2$); 1.55-1.65 (m, 4H, $2CH_2$); 2.22-2.28 (m, 4H, $2CH_2$); 3.51-3.54 (m, 2H, NCH_2); 3.65-3.69 (m, 2H, NCH_2); 4.11 (q, J = 7.2 Hz, 2H, $\mathit{CH_2}CH_3$); 6.85 (bs, 1H, arom.); 7.04-7.08 (m, 1H, arom.); 7.46-7.50 (m, 2H, arom.); 7.53-7.60 (m, 3H, arom.); 7.67 (d, J = 7.6 Hz, 2H, arom.) ppm. This compound was hydrolyzed with NaOH (0.032 g, 0.81 mmol) in 1 mL of MeOH, 1 mL of THF and 1 mL of H_2O, the title compound was obtained with 91% yield. Yellow waxy solid. [^{1}H]-NMR ($CDCl_3$) δ: 1.30-1.32 (m, 4H, $2CH_2$); 1.56-1.63 (m, 4H, $2CH_2$); 2.21 (t, J = 7.4 Hz, 2H, CH_2); 2.28 (t, J = 7.2 Hz, 2H, CH_2); 3.47-3.50 (m, 2H, NCH_2); 3.59-3.63 (m, 2H, NCH_2); 6.56 (bs, 1H, arom.); 6.75-6.79 (m, 1H, arom.); 7.12 (d, J = 8.4 Hz, arom.); 7.44-7.48 (m, 3H, arom.); 7.51-7.55 (m, 1H, arom.); 7.61-7.63 (m, 2H, arom.) ppm.

3.1.10. *N*-hydroxy-8-oxo-8-(5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-1-yl)octanamide 7

Following the same procedure used for **3**, **26** (0.44 g, 1.16 mmol) was treated with ethyl chloroformate (0.15 mL, 1.54 mmol), Et_3N (0.23 mL, 1.68 mmol), $NH_2OH{\cdot}HCl$ (0.38 g, 5.45 mmol) and KOH (0.31 g, 5.45 mmol). Purification with flash chromatography (eluent: CH_2Cl_2/MeOH/NH_3 90:10:01) gave the title compound in 75% yield. Yellow solid, m.p. 96-97 °C. [^{1}H]-NMR (CD_3OD) δ: 1.29-1.31 (m, 4H, $2CH_2$); 1.53-1.63 (m, 4H, $2CH_2$); 2.04 (t, J = 7.4 Hz, 2H, CH_2); 2.19 (t, J = 7.4 Hz, 2H, CH_2); 3.46 (s, 4H, t, $2CH_2N$); 6.55 (t, J = 7.6 Hz, 1H, arom.); 6.95 (d, J = 8.8 Hz, 1H, arom.); 7.39-7.43 (m, 2H arom.); 7.46-7.50 (m, 2H, arom.); 7.53-7.56 (m, 3H, arom.) ppm. [^{13}C]-NMR-APT (CD_3OD) δ: 26.51 (CH_2); 26.78 (CH_2); 29.76 (CH_2); 29.82 (CH_2); 33.67 (CH_2); 37.00 (CH_2); 39.64 (CH_2); 42.64 (CH_2); 112.84 (CH arom.); 115.23 (CH arom.);

118.74 (C arom.); 129.19 (CH arom.); 129.93 (CH arom.); 132.02 (CH arom.); 136.30 (CH arom.); 136.43 (CH arom.); 141.83 (C arom.); 152.94 (C arom.); 172.93 (CN); 176.72 (CO) ppm. HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{23}H_{28}N_3O_3.H_2O$: 412.2236, found: 412.2233.

3.1.11. 3-amino-1-methyl-5-phenyl-1H-benzo[e][1,4]diazepin-2(3H)-one (*rac*-27)[20]

To a solution of benzyl 2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-ylcarbamate [21] (1.00 g, 2.60 mmol) in anhydrous DMF (10 mL), kept at 0 °C under N_2, NaH (60% dispersion in mineral oil, 0.10 g, 1 eq) was added in small portions. After 1.5 hr stirring at 0 °C, methyl iodide (0.17 mL, 1.05 eq) was added at once; the mixture was stirred for additional 1.5 h at the same temperature, and then poured into a stirred solution of H_2O (60 mL) containing aqueous sodium hydrogen sulphate (2 mL, 1 N). A solid precipitated, which was filtered, dried under vacuum, and then purified by flash chromatography (CH_2Cl_2/MeOH/NH_3 99:1:0.1 as eluent) giving benzyl 1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e] [1,4]diazepin-3-ylcarbamate in 91% yield. M.p. 78-80 °C. [^{1}H]-NMR ($CDCl_3$): 3.47 (s, 3H, Me); 5.13 (d, J = 12.4 Hz, 1H, C*H*H); 5.17(d, J = 12.4 Hz, 1H, CH*H*); 5.33 (d, J = 8.4 Hz, 1H, CH); 6.72 (d, J = 8.4 Hz, 1H, NH); 7.22-7.28 (m, 1H), 7.28-7.42 (m, 9H), 7.44-7.50 (m, 1H) and 7.58-7.64 (m, 3H) (aromatic protons) ppm. This compound (0.20 g, 0.50 mmol) was dissolved in 5.7 mL of HBr (33% solution in AcOH) under N_2 and the solution was kept stirring at room temperature for 2 hr, then diluted with Et_2O (15 mL) to obtain a suspension. The mixture was filtered and the solid dissolved in H_2O. The aqueous solution was then made alkaline with Na_2CO_3 and extracted with AcOEt. The organic phase was dried (Na_2SO_4), the solvent was removed under vacuum obtaining the title compound in 99% yield. M.p. 62-64 °C. [^{1}H]-NMR (CD_3OD): 3.48 (s, 3H, Me); 4.43 (s, 1H, CH); 7.26-7.32 (m, 2H), 7.39-7.46 (m, 2H), 7.47-7.54 (m, 1H), 7.56-7.61 (m, 3H), 7.65-7.70 (m, 1H) (aromatic protons) ppm.

3.1.12. 8-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-ylamino)-8-oxooctanoic acid *rac*-31

A mixture of *rac*-**27** [20] (0.54 g, 2.02 mmol), ethyl hydrogensuberate (0.43 g, 2.12 mmol), WSC (0.38 g, 2.43 mmol) and HOBT (0.33 g, 2.43 mmol) in ethanol-free $CHCl_3$ (20 mL) was stirred at room temperature for 18 hr, then it was treated with 10% $NaHCO_3$ and the organic layer was separated. Drying (Na_2SO_4) and removal of the solvent gave a residue which was purified by flash chromatography (CH_2Cl_2/MeOH/NH_4OH 99:1:0.1 as eluent). *rac*-**28** was obtained in 66% yield as a pale yellow solid (m.p. 138-140 °C). [^{1}H]-NMR ($CDCl_3$): 1.25 (t, J = 7.2 Hz, 3H, CCH_3); 1.31-1.45 (m, 4H, $2CH_2$); 1.60-1.75 (m, 4H, $2CH_2$); 2.29 (t, J = 7.6 Hz, 2H, CH_2); 2.37 (td, J = 7.6, 2.8 Hz, 2H, CH_2); 3.47 (s, 3H, NCH_3); 4.12 (q, J = 7.2 Hz, 2H, OCH_2); 5.54 (d, J = 8.4 Hz, 1H, CH); 7.23 (t, 1H, J = 7.6 Hz, arom.); 7.31 (d, J = 8.0 Hz, 1H, arom.); 7.33-7.41 (m, 4H, arom.); 7.43-7.49 (m, 1H, arom.); 7.56-7.61 (m, 3H, NH and arom.) ppm. This compound (0.58 g, 1.29 mmol) and NaOH (0.08 g, 1.5 eq) were stirred in a mixture of THF/MeOH/H_2O (2 mL each) for 2 h at room temperature. The mixture was treated with a saturated solution of NH_4Cl and extracted with AcOEt. Drying (Na_2SO_4) and removal of the solvent gave the title compound in 99% yield as a white solid (m.p. 140-141 °C). [^{1}H]-NMR ($CDCl_3$): 1.37-1.44 (m, 4H, $2CH_2$); 1.63-1.75 (m, 4H, $2CH_2$); 2.34 (t, J = 7.2 Hz, 2H, CH_2); 2.39 (td, J = 7.6, 4.4 Hz, 2H, CH_2); 3.48 (s, 3H, NCH_3); 5.57 (d, J = 8.0 Hz, 1H, CH); 7.24 (t, 1H, J = 8.0 Hz, arom.); 7.35 (dd, J = 8.0, 1.2 Hz, 1H, arom.); 7.37-7.42 (m, 3H, arom.); 7.45-7.53 (m, 2H, arom.); 7.57-7.63 (m, 3H, NH and arom.) ppm.

3.1.13. 9-[(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-3-ylamino)-9-oxononanoic acid *rac*-32

Following the same procedure used for *rac*-**28** starting from *rac*-**27** (0.6 g, 2.26 mmol), methyl azelate (0.48 g, 2.38 mmol), WSC (0.42 g, 2.72 mmol) and HOBT (0.37 g, 2.72 mmol) in ethanol-free $CHCl_3$ (20 mL), *rac*-**29** was obtained with 93% yield. Brownish solid, m.p. 116-117 °C. [^{1}H]-NMR ($CDCl_3$) δ: 1.31-1.37 (m, 6H, $3CH_2$); 1.58-1.73 (m, 4H, $2CH_2$); 2.30 (t, J = 7.6 Hz, 2H, CH_2CO); 2.36-2.41 (m, 2H, CH_2CO); 3.47 (s, 3H, NCH_3); 3.66 (s, 3H, OCH_3); 5.56 (d, J = 8.0 Hz, 1H, CH); 7.22-7.26 (m, 1H, arom.); 7.35-7.41 (m, 5H, NH + arom.); 7.46-7.49 (m, 1H, arom.); 7.59-7.61 (m, 3H, arom.) ppm. This compound (0.94 g, 2.09 mmol) was hydrolyzed with NaOH (0.127 g, 3.18 mmol) as reported for *rac*-**31**, obtaining the title compound in 93% yield. White solid, m.p. 158-159 °C. [^{1}H]-NMR ($CDCl_3$) δ: 1.36-1.41 (m, 6H, $3CH_2$); 1.61-1.73 (m, 4H, $2CH_2$); 2.33 (t, J = 7.6 Hz, 2H, CH_2CO); 2.38 (td, J = 7.4 Hz, 2.0 Hz, 2H, CH_2 CO); 3.48 (s, 3H, NCH_3); 5.58 (d, J = 8.6 Hz, 1H, CH); 7.25 (t, J = 7.6 Hz, 1H, arom.); 7.35-7.42 (m, 4H, arom.); 7.46-7.50 (m, 1H, arom.); 7.54 (d, J = 8.6 Hz, 1H, NH); 7.59-7.61 (m, 3H, arom.) ppm.

3.1.14. 6-[(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-3-ylamino)-6-oxohexanoic acid *rac*-33

Following the same procedure used for *rac*-**28**, starting from *rac*-**27** (1.2 g, 4.53 mmol), adipic acid ethyl ester (0.83 g, 4.75 mmol), WSC (0.84 g, 5.43 mmol) and HOBT (0.73 g, 5.43 mmol) in ethanol-free $CHCl_3$ (20 mL), *rac*-**30** was obtained in 98% yield. Light green solid, m.p. 148-149 °C. [^{1}H]-NMR ($CDCl_3$) δ: 1.25 (t, J = 7.2 Hz, 3H, $CH_2\mathit{CH_3}$); 1.69-1.75 (m, 4H, $2CH_2$); 2.30-2.36 (m, 2H, CH_2); 2.38-2.43 (m, 2H, CH_2); 3.47 (s, 3H, NCH_3); 4.12 (q, J = 7.2 Hz, 2H, $\mathit{CH_2}CH_3$); 5.56 (d, J = 8.4 Hz, 1H, CH); 7.23-7.27 (m, 1H, arom.); 7.34-7.43 (m, 5H, NH + 4H arom.); 7.46-7.50 (m, 1H, arom.); 7.59-7.63 (m, 3H, arom.) ppm. This compound (1.88 g, 4.46 mmol) was hydrolyzed with NaOH (0.27 g, 6.79 mmol) in 6 mL of MeOH, 6 mL of THF and 6 mL of H_2O, obtaining the title compound in 92% yield. White solid, m.p. 216-217 °C. [^{1}H]-NMR ($CDCl_3$) δ: 1.73-1.79 (m, 4H, $2CH_2$); 2.38-2.47 (m, 4H, $2CH_2$); 3.49 (s, 3H, NCH_3); 5.57 (d, J = 8.2 Hz, 1H, CH); 7.24-7.27 (m, 1H, arom.); 7.35-7.42 (m, 4H, arom.); 7.46-7.50 (m, 1H, arom.); 7.56 (d, J = 8.2 Hz, 1H, NH); 7.59-7.64 (m, 3H, arom.) ppm.

3.1.15. 7-[(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-3-ylamino)-7-oxoheptanoic acid *rac*-34

2,8-Oxocanedione (0.54 g, 3.77 mmol), prepared from pimelic acid and acetic anhydride, was added to a solution of *rac*-**27** (1 g, 3.77 mmol) in dry THF (20 mL), and the mixture was stirred at RT under nitrogen for 2 h, while the color turned from yellow to green. Water (40 mL) was added, and the mixture was extracted in EtOAc (3 x 50 mL). The organic phase was then extracted with NaOH (2 x 30 mL, 10% aqueous solution) and the aqueous phase additioned with HCl (2N aqueous solution) until pH 2 and extracted with CH_2Cl_2 (3 x 30 mL). Drying (Na_2SO_4), removal of solvent and purification with flash chromatography (eluent: CH_2Cl_2/MeOH 95:5) gave *rac*-**34** in 48% yield. White solid, m.p. 170-171 °C. [^{1}H]-NMR ($CDCl_3$) δ: 1.41-1.48 (m, 2H, CH_2); 1.64-1.77 (m, 4H, $2CH_2$); 2.33-2.41 (m, 4H, $2CH_2$); 3.47 (s, 3H, NCH_3); 5.57 (d, J = 8.4 Hz, 1H, CH); 7.22-7.26 (m,

1H, arom.); 7.33-7.41 (m, 4H, arom.); 7.45-7.49 (m, 1H, arom.); 7.56-7.62 (m, 4H, NH + 3 arom.) ppm.

3.1.16. N^1-hydroxy-N^8-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl)octanediamide (*rac*-8)

To a solution of *rac*-**31** (0.55 g, 1.29 mmol) and Et_3N (0.23 ml, 1.67 mmol) in anhydrous CH_2Cl_2 (5 mL), BOP-Cl (0.56 g, 2.2 mmol), dimethyl-t-butylhydroxylamine (0.29 g, 1.98 mmol) and Et_3N (0.54 ml, 3.87 mmol) were added, and the mixture was stirred at room temperature for 20 h. The solvent was removed under vacuum, the residue was partitioned between H_2O and AcOEt, the organic layer was collected, dried (Na_2SO_4) and the solvent evaporated. The residue was dissolved in MeOH (10 mL) and heated at 50°C for 20 h; then the solvent was removed leaving a residue which was purified by flash chromatography (CH_2Cl_2/MeOH 95:5 as eluent). The title compound was obtained in 27 % yield as a white solid m.p. 84-85 °C. [^{1}H]-NMR (CD_3OD): 1.33-1.48 (m, 4H, $2CH_2$); 1.58-1.73 (m, 4H, $2CH_2$); 2.10 (t, J = 7.2 Hz, 2H, CH_2); 2.41 (t, J = 7.6 Hz, 2H, CH_2); 3.48 (s, 3H, NCH_3); 5.39 (s, 1H, CH); 7.29-7.35 (m, 2H), 7.40-7.45 (m, 2H), 7.47-7.53 (m, 1H), 7.54-7.63 (m, 3H), and 7.66-7.73 (m, 1H) (aromatic protons) ppm. [^{13}C]-NMR-APT (CD_3OD) δ: 24.91 (CH_2); 25.15 (CH_2); 28.05 (CH_2); 32.33 (CH_2); 35.71 (NCH_3); 36.28 (CH_2); 67.31 (CH); 121.69 (CH arom.); 124.33 (CH arom.); 128.47 (CH arom.); 128.92 (C arom.); 130.047 (CH arom.); 130.95 (CH arom.); 131.17 (CH arom.); 132.40 (CH arom.); 137.71 (C arom.); 142.97 (C arom.); 168.10 (CN); 168.96 (CO); 171.28 (CO); 173.79 (CO) ppm. HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{24}H_{28}N_4O_4$: 437.2189, found: 437.2188.

3.1.17. N^1-hydroxy-N^9-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl)nonanediamide (*rac*-9)

Following the same procedure used for *rac*-**8**, *rac*-**32** (0.83 g, 1.91 mmol) was treated with Et_3N (1.14 mL, 8.20 mmol), BOPCl (0.83 g, 3.24 mmol) and dimethyl-t-butylsilylhydroxylamine (0.43 g, 2.92 mmol) in 20 mL of CH_2Cl_2 at RT for 26 h, and after work-up the residue was heated in MeOH (20 mL) at 50 °C for 20 h. Evaporation of the solvent and purification with flash chromatography (eluent: CH_2Cl_2/MeOH 95:5) gave the title compound in 31% yield. Grey solid, m.p. 96-98 °C. [^{1}H]-NMR (CD_3OD) δ: 1.31-1.42 (m, 6H, $3CH_2$); 1.61-1.69 (m, 4H, $2CH_2$); 2.09 (t, J = 7.4 Hz, 2H, CH_2CO); 2.40 (t, J = 7.6 Hz, 2H, CH_2CO); 3.48 (s, 3H, NCH_3); 5.39 (s, 1H, CH); 7.30-7.35 (m, 2H, arom.); 7.41-7.45 (m, 2H, arom.); 7.49-7.53 (m, 1H, arom.); 7.57-7.63 (m, 3H, arom.); 7.69-7.73 (m, 1H, arom.) ppm. [^{13}C]-NMR-APT (CD_3OD) δ: 26.61 (CH_2); 29.91 (CH_2); 29.96 (CH_2); 33.72 (CH_2); 35.69 (NCH_3); 36.77 (CH_2); 69.18 (CH); 123.16 (CH arom.); 125.79 (CH arom.); 129.38 (CH arom.); 130.09 (C arom.); 130.77 (CH arom.); 131.31 (CH arom.); 131.96 (CH arom.); 133.51 (CH arom.); 139.26 (C arom.); 144.34 (C arom.); 169.33 (CN); 169.58 (CO); 172.94 (CO); 176.33 (CO) ppm. HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{25}H_{31}N_4O_4$: 451.2345, found: 451.2346.

3.1.18. N^1-hydroxy-N^6-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl)hexanediamide (*rac*-10)

Following the same procedure used for *rac*-**8**, *rac*-**33** (1.58 g, 4.01 mmol) was treated with Et_3N (2.40 mL, 17.25 mmol), dimethyl-t-butylsilylhydroxylamine (0.90 g, 6.14 mmol) and BOPCl (1.74

g, 6.83 mmol) in 20 mL of CH_2Cl_2 at RT for 22 h, and after work-up the residue was heated in MeOH (30 mL) at 60 °C for 22 h. Evaporation of the solvent and purification with flash chromatography (eluent: CH_2Cl_2/MeOH 95:5) gave the title compound in 18% yield. Light green solid, m.p. 75-76 °C. [^{1}H]-NMR (CD_3OD) δ: 1.68-1.70 (m, 4H, $2CH_2$); 2.12-2.15 (m, 2H, CH_2); 2.40-2.42 (m, 2H, CH_2); 3.47 (s, 3H, NCH_3); 5.39 (s, 1H, CH); 7.29-7.34 (m, 2H, arom.); 7.40-7.44 (m, 2H, arom.); 7.48-7.52 (m, 1H, arom.); 7.57-7.62 (m, 3H, arom.); 7.68-7.72 (m, 1H, arom.) ppm. [^{13}C]-NMR-APT (CD_3OD) δ: 26.10 (CH_2); 26.29 (CH_2); 33.52 (CH_2); 35.68 (NCH_3); 36.34 (CH_2); 69.27 (CH); 123.20 (CH arom.); 125.83 (CH arom.); 129.41 (CH arom.); 130.17 (C arom.); 130.81 (CH arom.); 131.36 (CH arom.); 131.98 (CH arom.); 133.55 (CH arom.); 139.32 (C arom.); 144.40 (C arom.); 169.36 (CN); 169.69 (CO); 172.69 (CO); 175.94 (CO) ppm. HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{22}H_{25}N_4O_4$: 409.1876, found: 409.1871.

3.1.19. N^1-hydroxy-N^7-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl)heptanediamide (*rac*-11)

A solution of $NH_2OH{\cdot}HCl$ (0.16 g, 2.31 mmol) in dry MeOH (5 mL) was added to a solution of KOH (0.13 g, 2.31 mmol) in dry MeOH (5 mL) at 0 °C under nitrogen. The resulting mixture was stirred for 15' at 0 °C and filtered in another flask.

Ethyl chloroformate (0.06 mL, 0.65 mmol) and anhydrous Et_3N (0.1 mL, 0.71 mmol) were added to a solution of *rac*-**34** (0.2 g, 0.49 mmol) in dry THF (5 mL) at 0 °C under nitrogen. The mixture was stirred for 15' at 0 °C and filtered directly in the flask containing the previously prepared solution of NH_2OH in MeOH. The resulting mixture was stirred for 30' at RT under nitrogen, then the solvents were evaporated. The residue was purified by flash chromatography (first eluent: CH_2Cl_2/MeOH 95:5, second eluent: CH_2Cl_2/MeOH 9:1) giving the title compound in 47.2% yield. White solid, m.p. 89-90 °C. [^{1}H]-NMR (CD_3OD) δ: 1.28-1.44 (m, 2H, CH_2); 1.61-1.71 (m, 4H, $2CH_2$); 2.11 (t, J = 7.4 Hz, 2H, CH_2CO); 2.92 (t, J = 7.6 Hz, 2H, CH_2CO); 3.46 (s, 3H, NCH_3); 5.39 (s, 1H, CH); 7.28-7.33 (m, 2H, arom.); 7.40-7.44 (m, 2H, arom.); 7.48-7.51 (m, 1H, arom.); 7.57-7.61 (m, 3H, arom.); 7.67-7.71 (m, 1H, arom.) ppm. [^{13}C]-NMR-APT (CD_3OD) δ: 26.26 (CH_2); 26.40 (CH_2); 29.58 (CH_2); 33.61 (CH_2); 35.67 (NCH_3); 36.61 (CH_2); 69.26 (CH); 123.21 (CH arom.); 125.84 (CH arom.); 129.40 (CH arom.); 130.22 (C arom.); 130.80 (CH arom.); 131.37 (CH arom.); 131.96 (CH arom.); 133.54 (CH arom.); 139.36 (C arom.); 144.42 (C arom.); 169.40 (CN); 169.73 (CO); 172.88 (CO); 176.22 (CO) ppm. HRMS-ESI *m*/*z* $[M+H]^+$ calcd for $C_{23}H_{27}N_4O_4$: 423.2032, found: 423.2028.

3.1.20. Preparation of (*S*)-27 and (*R*)-27.

3.1.20.1. Method A. 1,3-dihydro-5-phenyl-3(*R*,*S*)-{[(*R*)-α-methylbenzyloxycarbonyl]amino]}-1,4-benzodia-zepin-2-one was prepared according to Sherrill [21] as diasteromeric mixture, which was separated by 7 flash chromatographic runs using toluene/ethyl acetate 8:2 as eluent. 1,3-Dihydro-5-phenyl-(3*S*)-{[(*R*)-α-methylbenzyloxycarbonyl]amino]}-1,4-benzodiazepin-2-one (the first eluting isomer) and its 3*R* diastereomer were obtained in 31% and 24% yields, respectively (lit.[21] 41% and 37%, respectively), with ee > 99% (NMR). N^1-Methylation and subsequent removal of the carbamate moiety according to the literature procedure gave (*S*)-**27** and (*R*)-**27** in 75% yields. The NMR spectra of the enantiomers were identical to that of the racemate. (*S*)-**27**: $[\alpha]^{20}_D$ -170.6° (c=1, CH_3OH). (*R*)-**27**: $[\alpha]^{20}_D$ +159.0° (c=1, CH_3OH).

3.1.20.2. Method B. Treatment of *rac*-**27** (0.34 g) with 0.5 eq of (*S*)-camphorsulfonic acid in ethyl acetate afforded, after seeding, the 3(S)-amine-camphorsulfonate salt. A second crystallization from acetonitrile, treatment with NaOH and extraction with CH_2Cl_2 afforded 0.16 g of (*S*)-**27** with enantiomeric excess greater than 99.5 %. The original seeds of the (3*S*)-amine-camphorsulfonate salt were made from the (3S)-amine, obtained through HPLC separation, using the following conditions: Column: LUX Amylose-2 (Phenomenex); particle size: 5μm; length: 250 mm; diameter: 10 mm. Eluent (isocratic conditions): CH_3CN/CH_3OH 95:05 with 0.1% diethylamine. Flow rate: 2ml/min at 40°C for 30 min. The enantiomeric excess was assessed using the following conditions: Column LUX Amylose-2 (Phenomenex); particle size: 3 μm; length: 50 mm; diameter: 4.6 mm. Eluent (isocratic conditions): NH_4OH (10 mM)/CH_3CN 65:35. Flow rate: 1 ml/min at 40°C for 10 min.

3.1.21. (S) and (R) N^1-hydroxy-N^8-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-3-yl)octanediamide [(S)-8] and [(R)-8]

These compounds were prepared starting from (S)-**27** and (R)-**27** as described for the racemate. The NMR spectra of the intermediate and final compounds are identical to those of the racemates. Other physicochemical properties are the following:

(S)-28: waxy solid, 60% yield. $[\alpha]^{20}_D$ -56.0° (c=1, $CHCl_3$).
(R)-28: waxy solid, 68% yield. $[\alpha]^{20}_D$ +55.3° (c=1, $CHCl_3$)
(S)-31: white solid, 82% yield. Mp 48-9°C. $[\alpha]^{20}_D$ -51.8° (c=1, $CHCl_3$)
(R)-31: white solid, 89% yield. Mp 48-9°C. $[\alpha]^{20}_D$ +50.0° (c=1, $CHCl_3$)
(S)-8: white solid, 29% yield. Mp 82-3°C. $[\alpha]^{20}_D$ -53.7° (c=1, CH_3OH)
(R)-8: white solid, 24% yield. Mp 84-5°C. $[\alpha]^{20}_D$ +55.4° (c=1, CH_3OH)

3.1.22. Benzyl N-(7-iodo-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-3-yl)carbamate 36

Anhydrous CH_2Cl_2 (6 mL), oxalyl chloride (1.39 mL, 15.91 mmol) and anhydrous DMF (0.12 mL) were added to a solution of **35** [21] (5.19 g, 15.91 mmol) in anhydrous THF (45 mL) at 0 °C under nitrogen. After 3 h and 30' at 0 °C, a solution of 2-amino-5-iodobenzophenone[23] (4.67 g, 14.46 mmol) and N-methylmorpholine (3.85 mL, 35.00 mmol) in anhydrous THF (15 mL). The mixture was allowed to reach room temperature, then was filtered and the solid washed with THF (15 mL). NH_3 was bubbled in the organic solution for 25', methanol (70 mL) was added and NH_3 was kept bubbling for 20'. The solution was then partitioned between EtOAc (300 mL) and NaOH (1N, aqueous solution), the aqueous phase was washed with EtOAc and the combined organic extracts were washed with brine. Drying (Na_2SO_4) and removal of the solvent gave a residue that was solved in glacial acetic acid (70 mL) and additioned with ammonium acetate (5.19 g, 67.33 mmol). The mixture was stirred at room temperature overnight, evaporated and treated with $NaHCO_3$ (aqueous saturated solution). The resulting slurry was filtered, and the solid residue washed with water and dried under reduced pressure to give the title compound in 37.5 % yield. White solid, m.p. 270-272 °C (dec.). [^{1}H]-NMR (DMSO) δ: 5.06 (d, J = 8.6 Hz, 1H, CHN); 5.08 (s, 2H, CH_2Ph); 7.11 (d, J = 8.4 Hz, 1H, arom.); 7.26-7.39 (m, 5H, arom.); 7.44-7.47 (m, 4H, arom.); 7.50-7.55 (m, 2H, arom.); 7.95 (dd, J = 8.4 Hz, 1.6 Hz, 1H, arom.); 8.50 (d, J = 8.6 Hz, 1H, NHCBZ); 10.95 (s, 1H, ArNH) ppm

3.1.23. *t*-Butyl N-(7-iodo-1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-3-yl)carbamate 37

To a solution of **36** (3.01 g, 5.89 mmol) in dry DMF (20 mL), kept at 0 °C under N_2, NaH (60% dispersion in mineral oil, 0.25 g, 6.18 mmol) was added in small portions. After 1.5 hr stirring at 0 °C, CH_3I (0.38 mL, 6.18 mmol) was added at once; the mixture was stirred for additional 1.5 h at the same temperature, and then poured into a stirred solution of H_2O (60 mL) containing aqueous sodium hydrogen sulphate (2 mL, 1 N). A solid precipitated, which was filtered, dried under vacuum, and then purified by flash chromatography (CH_2Cl_2/MeOH/NH_3 99:1:0.1 as eluent) giving benzyl N-(7-iodo-1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-3-yl)carbamate in 99% yield. Pale yellow solid, m.p. 65-67 °C. [^{1}H]-NMR ($CDCl_3$) δ: 3.43 (s, 3H, NCH_3); 5.13 (d, J = 12.0 Hz, 1H, C*H*HPh); 5.17 (d, J = 12.0 Hz, 1H, CH*H*Ph); 5.30 (d, J = 8.4 Hz, 1H, CHN); 6.66 (d, J = 8.4 Hz, 1H, NHCBZ); 7.12 (d, J = 8.8 Hz, 1H, arom.); 7.27-7.43 (m, 7H, arom.); 7.48-7.53 (m, 1H, arom.); 7.58-7.60 (m, 2H, arom.); 7.66 (d, J = 2.0 Hz, 1H, arom.); 7.88 (dd, J = 8.8 Hz, 2.0 Hz, 1H, arom.) ppm.

This compound was treated with HBr (33% in AcOH, 5 mL) under N_2 and the solution was kept stirring at room temperature for 2 hr, then diluted with Et_2O (15 mL) to obtain a suspension. The mixture was filtered and the solid dissolved in H_2O. The aqueous solution was then made alkaline with Na_2CO_3 and extracted with AcOEt. The organic phase was dried (Na_2SO_4), the solvent was removed under vacuum obtaining 3-amino-7-iodo-1-methyl-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-2-one in 95% yield. Pale yellow solid, m.p. 69-71 °C. [^{1}H]-NMR (CD_3OD) δ: 3.45 (s, 3H, NCH_3); 4.44 (s, 1H, CHN); 7.38 (d, J = 8.8 Hz, 1H, arom.); 7.44-7.48 (m, 2H, arom.); 7.51-7.54 (m, 4H, arom.); 7.98 (dd, J = 8.8 Hz, 2.0 Hz, 1H, arom.) ppm.

This compound was dissolved in anhydrous THF (5 mL) and di-*tert*-butyl dicarbonate (0.17 g, 0.77 mmol) dissolved in anhydrous THF (5 mL) was added dropwise at 0 °C under nitrogen. The solution was left stirring at room temperature overnight, then the solvent was evaporated and the residue partitioned between EtOAc and brine. Drying (Na_2SO_4) of the organic phase and removal of the solvent gave a crude that was purified with flash chromatography (cyclohexane/ethyl acetate 6:4) giving the title compound in 84% yield. White solid, m.p. 186-187 °C. [^{1}H]-NMR ($CDCl_3$) δ: 1.47 (s, 9H, *t*Bu); 3.43 (s, 3H, NCH_3); 5.27 (d, J = 8.6 Hz, 1H, CHN); 6.41 (d, J = 8.6 Hz, 1H, NHBOC); 7.11 (d, J = 8.4 Hz, 1H, arom.); 7.39-7.43 (m, 2H, arom.); 7.47-7.51 (m, 1H, arom.); 7.58-7.60 (m, 2H, arom.); 7.64 (d, J = 2.0 Hz, 1H, arom.); 7.86 (dd, J = 8.4 Hz, 2.0 Hz, 1H, arom.) ppm.

3.1.24. *t*-Butyl N-{7-[5-(hydroxycarbamoyl)pent-1-yn-1-yl]-1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-3-yl}carbamate 38

Following the same procedure used for **15**, **37** (0.24 g, 0.49 mmol) was treated with 5-hexynoic acid (0.10 g, 0.92 mmol), CuI (0.01 g, 0.06 mmol), $Pd(PPh_3)_4$ (0.03 g, 0.02 mmol) and anhydrous Et_3N (5 mL) at 55 °C for 3 h under nitrogen. Purification with flash chromatography (CH_2Cl_2/MeOH 95:0.5) gave 6-(3-(tert-butoxycarbonylamino)-1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-benzo[e][1,4]diaze-pin-7-yl)hex-5-ynoic acid in 99% yield. Yellow solid, m.p. 64-66 °C. [^{1}H]-NMR ($CDCl_3$) δ: 1.46 (s, 9H, *t*Bu); 1.85-1.92 (m, 2H, CH_2); 2.43-2.51 (m, 4H, $2CH_2$); 3.44 (s, 3H, NCH_3); 5.28 (d, J = 8.6 Hz, 1H, CHN); 6.46 (d, J = 8.6 Hz, 1H, NHBOC); 7.28 (d, J = 8.4 Hz, 1H, arom.); 7.34 (d, J = 1.6 Hz, 1H, arom.); 7.38-7.44 (m, 2H, arom.); 7.45-7.49 (m, 1H, arom.); 7.55

(dd, J = 8.8 Hz, 2.0 Hz, 1H, arom.); 7.59-7.61 (m, 2H, arom.) ppm. Following the same procedure reported for **3**, this compound (0.23 g, 0.49 mmol) was treated with ethyl chloroformate (0.06 mL, 0.65 mmol), Et_3N (0.10 mL, 0.71 mmol), $NH_2OH{\cdot}HCl$ (0.16 g, 2.29 mmol) and KOH (0.13 g, 2.29 mmol). Purification by flash chromatography (CH_2Cl_2/MeOH 90:10) gave the title compound in 77% yield. Pale yellow solid, m.p. 80-81 °C. [^{1}H]-NMR ($CDCl_3$) δ: 1.46 (s, 9H, *t*Bu); 1.84-1.87 (m, 2H, CH_2); 2.25 (t, J = 7.6 Hz, 2H, CH_2); 2.38 (t, J = 7.6 Hz, 2H, CH_2); 3.44 (s, 3H, NCH_3); 5.27 (d, J = 8.4 Hz, 1H, CHN); 6.53 (d, J = 8.4 Hz, 1H, NHBOC); 7.28-7.33 (m, 2H, arom.); 7.39-7.42 (m, 2H, arom.); 7.47-7.50 (m, 1H, arom.); 7.56-7.62 (m, 3H, arom.) ppm.

3.1.25. 6-(3-amino-1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H-1,4-benzodiazepin-7-yl)-N-hydroxyhex-5-ynamide 12

A freshly prepared solution of HCl in EtOAc (0.28 N, 7.69 mL) was added to a solution of **38** (0.11 g, 0.22 mmol) in EtOAc (1 mL) under nitrogen. The mixture was stirred at room temperature for 24 h and then filtered. The solid residue was washed with EtOAc and purified with flash chromatography (CH_2Cl_2/MeOH/NH_3 90:10:01) to give the title compound in 58% yield. White solid, m.p. 67-69 °C. [^{1}H]-NMR (CD_3OD) δ: 1.81-1.88 (m, 2H, CH_2); 2.21 (t, J = 7.2 Hz, 2H, CH_2); 2.42 (t, J = 7.2 Hz, 2H, CH_2); 3.46 (s, 3H, NCH_3); 4.43 (s, 1H, CHN); 7.25 (d, J = 1.6 Hz, 1H, arom.); 7.42-7.46 (m, 2H, arom.); 7.49-7.58 (m, 4H, arom.); 7.66 (dd, J = 8.4 Hz, 2.0 Hz, 1H, arom.) ppm. [^{13}C]-NMR-APT (CD_3OD) δ: 19.44 (CH_2); 25.71 (CH_2); 32.71 (CH_2); 35.44 (CH_3); 70.95 (CH); 80.51 (C); 91.37 (C); 121.52 (C arom.); 123.19 (CH arom.); 129.49 (2CH arom.); 130.27 (C arom.); 130.73 (2CH arom.); 131.97 (CH arom.); 133.87 (CH arom.); 136.05 (CH arom.); 139.23 (C arom.); 143.59 (C arom.); 167.85 (CN); 171.32 (CO); 172.13 (CO) ppm. HRMS-ESI *m/z* $[M+H]^+$ calcd for $C_{22}H_{23}N_4O_3$: 391.1770, found: 391.1765. Treatment with 1 eq. of HCl (0.28 N in EtOAc) gave the corresponding hydrochloride

3.2. Biological Assays

3.2.1. Reagents

The compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, MO, USA) to make stock solutions (100 μM) which were stored at room temperature protected from light. Aliquots of this solution were diluted with DMSO according to the need and then added directly to the culture. The amount of DMSO (≤ 0.3%) used as the vehicle did not interfere with activities of compounds

3.2.2. Histone Deacetylase Assay.

3.2.2.1. HeLa nuclear extracts. The *in vitro* activity of HDAC inhibitors was assayed using a BIOMOL Kit AK-500, according to the instructions from the manufacturer (Biomolecular Research Laborator). The assay is automated and performed by a Tecan Freedom EVO® work station. On detail, 15 μl of 30 x diluted nuclear fraction of HeLa cells (9 μg/μl), was diluted to 50 μl with the assay buffer containing the HDAC inhibitor and the substrate (lysine with acetylated amino group on the side chain) at a concentration of 100 μM.

The samples were incubated for 15 min at room temperature (RT) and then exposed to a developer (10 min at RT). In this last step a fluorophore was produced, whose fluorescence was measured

using an excitation wavelength of 355 nm and an emission at 460 nm. All determinations were carried out in triplicate and the IC_{50} was calculated using GraphPad Software

3.2.2.2. Fluorometric human recombinant HDAC1, 4 and 6 Assays. The HDAC Fluorescent Activity Assay for HDAC1, 4 and 6 is based on the Fluor de Lys Substrate and Developer combination (BioMol AK-500) and has been carried out according to supplier's instructions and as previously reported. First, the Fluor de Lys Substrate, which comprises an acetylated lysine side chain, has been incubated with purified recombinant HDAC1 or 6 enzymes in presence or absence of the inhibitors. Briefly, for HDAC1, and HDAC6, 100 ng of recombinant proteins have been used per assay, respectively. Full length HDAC1 and 6 with C-terminal His tag were expressed using baculovirus expression systems. Deacetylation sensitizes the substrates that, in the second step, treatment with the developer produces a fluorophore. Fluorescence has been quantified with a TECAN Infinite M200 station and IC_{50} values has been obtained using GraphPad program. Inhibition of HDAC4 has been measured using a fixed (5μM) concentration of the compounds, finding a residual enzymatic activity not below 65%. For this reason a dose response curve was not performed. On this isoform, the IC50 value for SAHA resulted 0.55 μM [27].

3.2.3. Cell Growth Inhibition Assay

The antiproliferative activity of HDAC inhibitors was evaluated using the CellTiter- Glo®, according the instruction from the manufacture (Promega). The assay is automated and performed by a Tecan Freedom EVO® work station. On detail, K562, A549 and HCT-116 cells (from the ATTC), in exponential growth, were seeded on 96-well plates, 24 hours later the cells were incubated for 72 hours with different concentrations of the inhibitors. After drug treatment, a volume of CellTiter-Glo® Reagent equal to the volume of cell culture medium was added. The content was mixed for 2 min to induce cell lysis. The luminescence was recorded after further 10 min at RT in order to obtain a stabilized luminescent signal. All determinations were carried out in triplicate and the IC_{50} was calculated using GraphPad Software

3.2.4. Experiments with human acute promyelocytic NB4 cells

3.2.4.1. *Culture conditions and determination of IC_{50}.* The all-*trans*-retinoic acid (ATRA)-sensitive human acute promyelcytic cell line NB4 [28] was used as the experimental model. Cells were maintained in RPMI 1640 (Bio-Whittaker Europe, Verviers, Belgium) supplemented with 10% fetal calf serum (FCS, EuroClone, Life Science Division, Milan, Italy) at 37°C in a humidified atmosphere of 5% CO_2. Cells were counted in a Bürker chamber and viability was assessed by the trypan blue exclusion test. Cell number in culture was used to determine the IC_{50} value, i.e. the drug concentration capable of decreasing cell density by 50% as compared to control.

3.2.4.2. *Cell lysate preparation, SDS-PAGE and Western blot*. Harvested cells were resuspended in a lysis buffer [1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 40 mM Tris–HCl pH 8.0, 150 mM NaCl] and sonicated (Microson XL-2000, Misonix, Farmingdale, NY, USA) as described previously. Acetylated isoforms of histone H3 and H4, and α-tubulin (used as the loading control) were determined following 12.5% SDS-PAGE separation of extracts and western blot; then, nitrocellulose membranes were probed using primary rabbit polyclonal antibodies against the acetyl-H3 or -H4 histone (Upstate Biotechnology, Lake Placid, NY, USA) and suitable peroxidase-conjugated secondary antibodies (Santa Cruz Biotech, Santa Cruz, CA, USA) for immunostaining.

The ECL procedure on Hyperfilm ECL (Amersham Biosciences, Buckinghamshire, UK) was used for development. The intensity of each stained band on the gel was then quantified by densitometric analysis with the aid of ImageJ software, using tubulin as the reference protein; values of treated versus untreated samples were from a typical experiment.

3.2.4.3. *Determination of apoptosis.* Apoptotic cells in the populations were measured with a FACScan flow cytometer (Becton-Dickinson) by the Annexin-V-Fluos Apoptosis detection kit (Roche Molecular Biochemicals, Mannheim, Germany). Usually, $0.5x10^6$ cells were used for labeling with Annexin-V-Fluos and propidium iodide (PI) in binding buffer according to the manufacturer's instructions. Moreover, apoptosis was also assessed by examining May-Grünwald/Giemsa stained cytosmears (magnification: x200) with the aid of a microscope (Nikon Eclipse, mod. 50i) equipped with a digital camera DS-5M USB2 (Nikon Instruments, Florence, Italy).

3.2.5. Statistical analysis.

All experiments were independently done at least 3 times and data were statistically analyzed by Student's *t* test.

4. Acknowledgments

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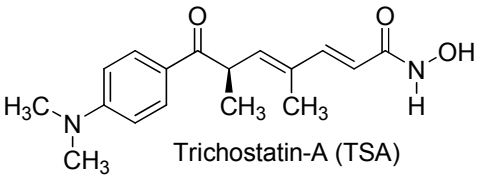
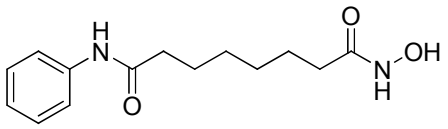
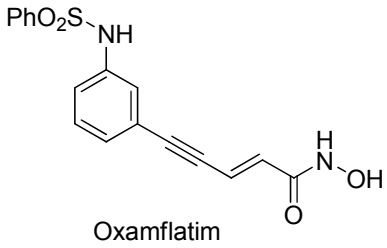
Chart 1



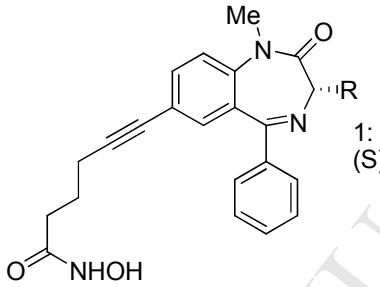
Trichostatin-A (TSA)



SAHA (Vorinostat)



Oxamflatim



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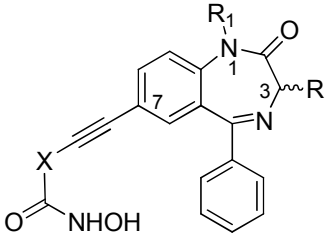
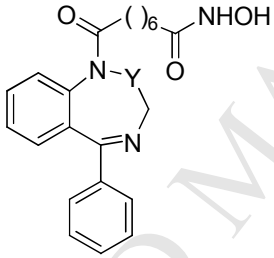
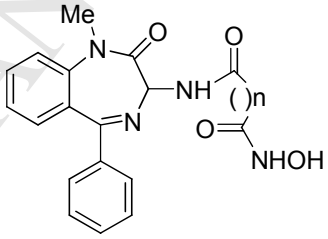


A



B



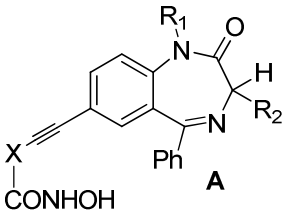
C

3: X = $(CH_2)_2$, R=H, R_1=Me
4: X = $(CH_2)_4$, R=H, R_1=Me
5: X = CH=CH, R=H, R_1=Me
(*S*)-**6**: X = $(CH_2)_3$, R=Me, R_1=H
rac-**12**: X = $(CH_2)_3$, R=NH_2 , R_1=Me

7: Y = CH_2

8: n = 6
9: n = 7
10: n = 4
11: n = 5

Table 1. HDAC inhibition of compounds **1-12** on HeLa nuclear extracts and on recombinant HDAC1 and HDAC6 enzymes.[a]



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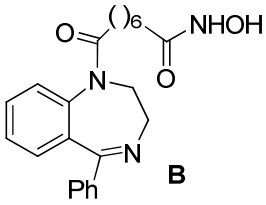

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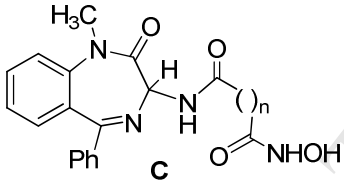
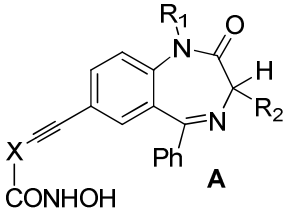


C

N	formula	R_1	X	R_2	n	HeLa [b]		HDAC1 [c]	HDAC6 [c]
						% inhibition at 1µM	IC_{50} (µM)	IC_{50} (µM)	IC_{50} (µM)
1	A	CH_3	$(CH_2)_3$	H	-	39	-	2.15[d] ± 0.041	3.35[d] ± 0.057
(R)-**2**	A	CH_3	$(CH_2)_3$	CH_3	-	21	-	2.18[d] ± 0.043	7.08[d] ± 0.037
(S)-**2**	A	CH_3	$(CH_2)_3$	CH_3	-	63	0.36	0.75[d] ± 0.029	2.70[d] ± 0.038
3	A	CH_3	$(CH_2)_2$	H	-	22	-	2.75 ± 0.046	>100
4	A	CH_3	$(CH_2)_4$	H	-	36	-	1.90 ± 0.039	5.90 ± 0.043
5	A	CH_3	CH=CH	H	-	82	0.16	0.44 ± 0.027	7.72 ± 0.054
(S)-**6**	A	H	$(CH_2)_3$	CH_3	-	65	0.34	0.49 ± 0.042	0.46 ± 0.047
7	B	-	-	-	-	82	0.18	0.25 ± 0.036	1.12 ± 0.038
(R)-**8**	C	-	-	-	6	73	0.31	0.70 ± 0.044	6.50 ± 0.047
(S)-**8**	C	-	-	-	6	88	0.12	0.55 ± 0.047	0.40 ± 0.051
rac-**9**	C	-	-	-	7	57	0.60	0.48 ± 0.024	9.08 ± 0.036
rac-**10**	C	-	-	-	4	70	0.38	0.42 ± 0.046	>100
rac-**11**	C	-	-	-	5	73	0.32	0.27 ± 0.044	0.18 ± 0.036
rac-**12**	A	CH_3	$(CH_2)_3$	NH_2	-	nt	nt	1.09 ± 0.049	>100
SAHA						85	0.13	0.31 ± 0.022	0.038 ± 0.0037

[a] All determinations were carried out in triplicate; values represent the mean. [b] HeLa nuclear extracts; in this automated assay IC_{50} values have been calculated only for compounds whose activity at 1 µM was higher than 50%. SD is < 25%. [c] Human recombinant enzymes. [d] From ref. 11.

Table 2. Antiproliferative activity of compounds **1-11** on cell lines. [a]



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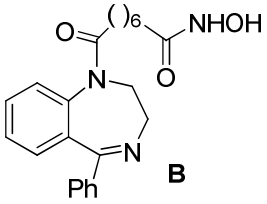

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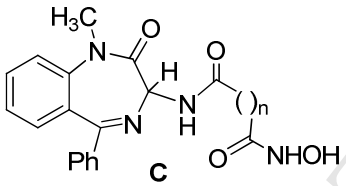
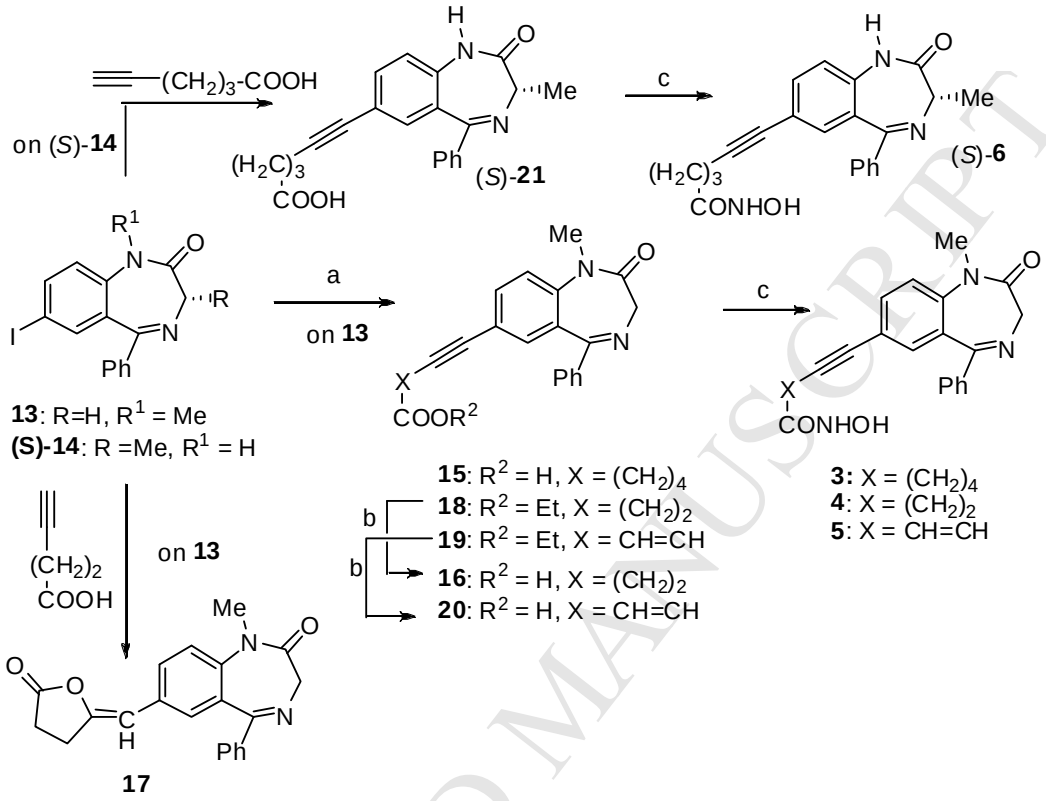


C

N	formula	R_1	X	R_2	n	IC_{50} (μM)			
						HCT 116 [b]	**A549** [b]	**K562** [b]	**NB4** [c]
1	A	CH_3	$(CH_2)_3$	H	-	2.62	5.95	4.97	1.95
(R)-**2**	A	CH_3	$(CH_2)_3$	CH_3	-	7.05	11.83	11.59	> 5[d]
(S)-**2**	A	CH_3	$(CH_2)_3$	CH_3	-	0.74	1.47	1.27	2.06
3	A	CH_3	$(CH_2)_2$	H	-	6.54	10.28	8.22	> 5[d]
4	A	CH_3	$(CH_2)_4$	H	-	1.86	2.69	2.67	4.09
5	A	CH_3	CH=CH	H	-	0.46	1.30	0.78	2.60
(S)-**6**	A	H	$(CH_2)_3$	CH_3	-	0.97	2.82	1.40	3.04
7	B	-	-	-	-	1.44	2.54	1.67	3.99
(R)-**8**	C	-	-	-	6	1.07	2.69	1.72	> 5[d,e]
(S)-**8**	C	-	-	-	6	0.47	1.44	0.72	0.86[e]
rac-**9**	C	-	-	-	7	3.93	7.95	4.59	4.00
rac-**10**	C	-	-	-	4	2.01	5.44	3.28	1.91
rac-**11**	C	-	-	-	5	1.44	4.42	1.84	2.56
SAHA						0.76	1.08	0.64	0.98

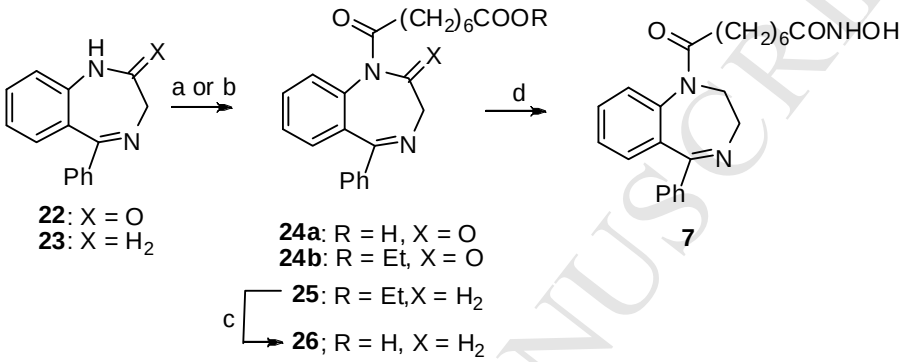
[a] All determinations were carried out in triplicate; values represent the mean. SD are < 25%. [b] Quantification of ATP by luminescence after incubation for 72 hr. [c] Annexin test of apoptosis after incubation for 48 hrs. [d] Highest tested concentration; at this dose, the percentage of apoptotic cells was lower than 50%. [e] The IC_{50} of *rac*-**8** was 1.2 μM.

Scheme 1



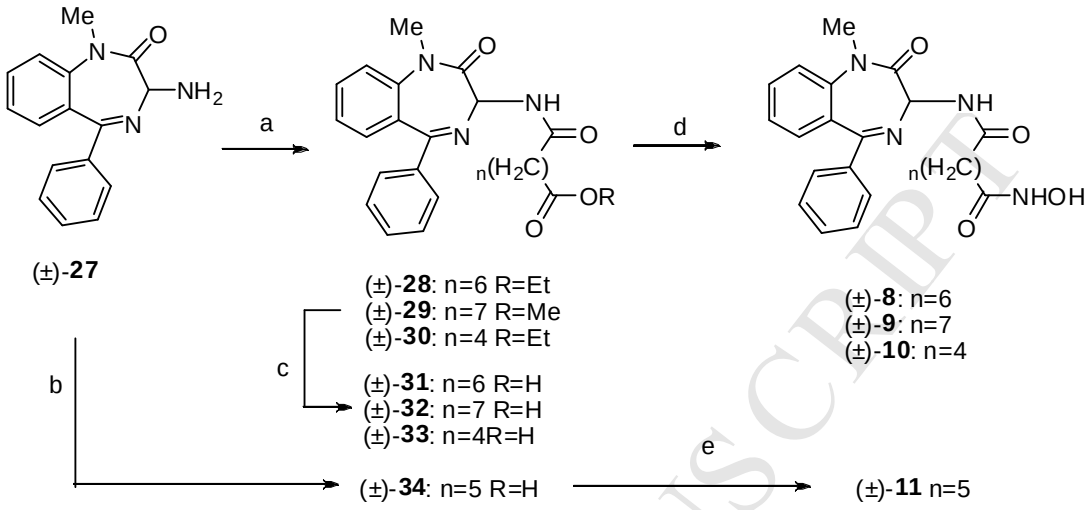
a) HC≡C-X-COOR; b) NaOH; c) ClCOOEt, $NH_2OH.HCl$

Scheme 2



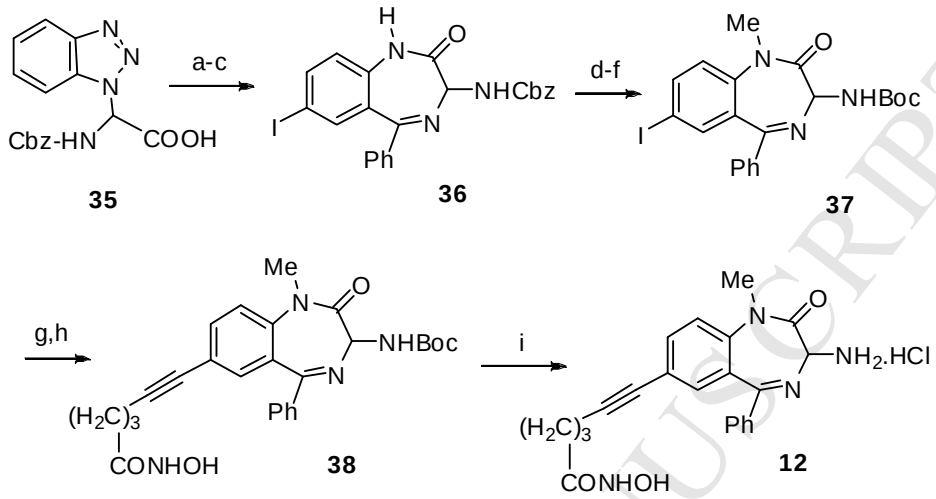
a) $ClCO(CH_2)_6COOEt$ (for **24b**); b) ethyl hydrogensuberate (for **25**); c) NaOH; d) $ClCOOEt$, $NH_2OH.HCl$

Scheme 3

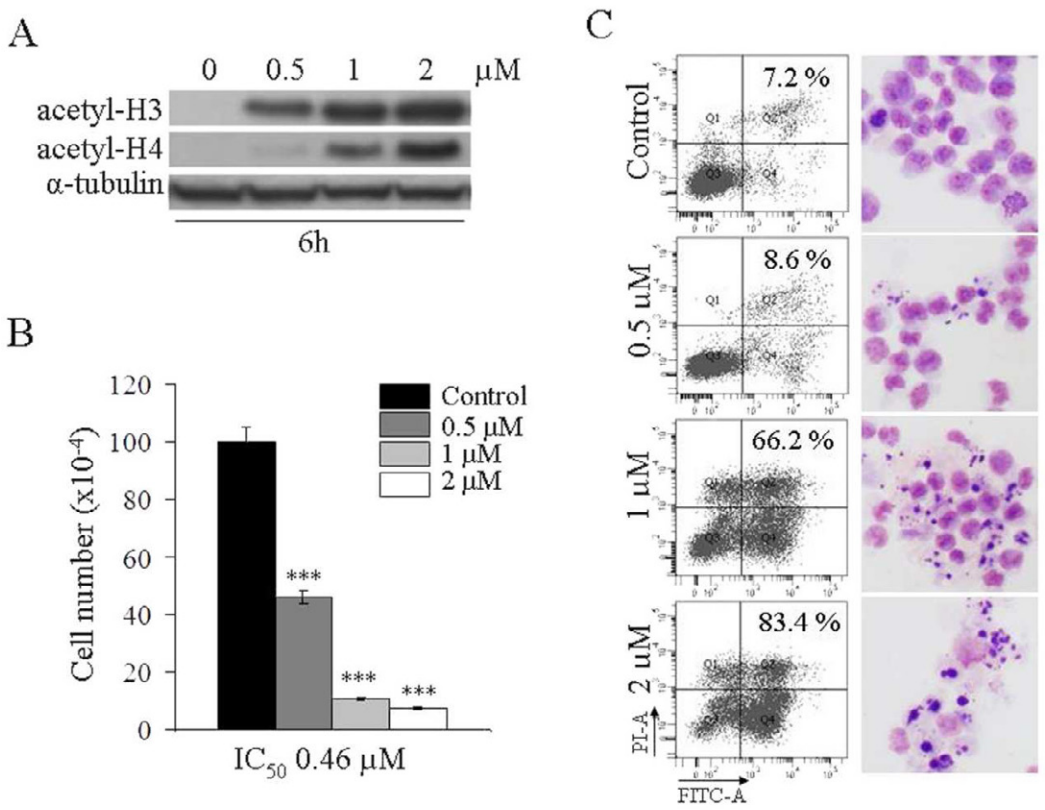


a) $ROCO(CH_2)_nCOOH$ (n = 4, 6, 7; R = Me or Et); b) pimelic anhdyride; c) NaOH; d) Et_3N, BOPCl, NH_2OSiMe_2tBu, MeOH; e) ClCOOEt, $NH_2OH.HCl$. The synthesis of (*S*)-**8** and (*R*)-**8** was performed as reported for the racemate, starting from (*S*) and (*R*)-**27**.

Scheme 4



a) $(COCl)_2$, 2-amino-4-iodobenzophenone; b) NH_3; c) NH_4OAc; d) NaH, MeI; e) HBr, AcOH; f) BOC_2O; g) hexynoic acid; h) $ClCOOEt$, $NH_2OH.HCl$; i) HCl/EtOAc.



Legend to Figure 1 – Biological effects of (*S*)-**8** on NB4 cells.
(**A**) Cells ($1x10^5$/ml) were incubated without/with increasing drug concentrations for 6 hours to determine by Western blot and immunostaining the drug effect on histone H3/H4 acetylation; immunostained signals were visualized on the probed membrane. (**B**) Cells were treated up to 48 hours to monitor drug effects on viable cell number as measured by the trypan blu exclusion test with the aid of a Bürker chamber and then calculate IC_{50} value. (**C**) Number of apoptotic cells in untreated and treated cultures was determined by cyfluorimetric analysis of Annexin-V Fluos/PI stained cells and reported as % of the population on the upper right side of the original dot plots (left panel); consistently, pictures of May-Grünwald/Giemsa stained cytosmears (magnification: x200, right panel) showed a dose-dependent increase in cell shrinking and fragmentation upon treatment. Results were from a typical experiment out of three.