European Journal of Medicinal Chemistry 45 (2010) 4331–4338

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Design and synthesis of novel isoxazole-based HDAC inhibitors

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ARTICLE INFO

Article history:
Received 2 February 2010
Received in revised form
11 June 2010
Accepted 25 June 2010
Available online 30 June 2010

Keywords:
Histone deacetylase
Isoxazole
Zinc binding group
1,3-Dipolar cycloaddition
Enzyme inhibition

ABSTRACT

A series of isoxazole-based histone deacetylase (HDAC) inhibitors structurally related to SAHA were designed and synthesized. The isoxazole moiety was inserted in the vicinity of the Zn^{2+}-binding group in order to check its participation in the coordinating process.

1. Introduction

Altered epigenetic regulations of gene expression play an important role in cancer onset and progression. Epigenetic alterations refer mainly to DNA methylation [1] or to post-translational modifications of histone proteins [2,3]. Histones are nuclear core proteins accountable for the regulation of transcription and cell cycle progression. These activities are dependent on the level of acetylation/deacetylation of specific lysine ε-amino groups of the protein backbones. Histone acetylation/deacetylation is a reversible phenomenon and is tightly regulated by two competing enzymes known as histone acetyltransferases (HATs) [2,3] and histone deacetylases (HDACs) [4,5]. Inhibition of HDACs causes hyperacetylation of histones leading to differentiation, growth arrest and apoptosis of malignant cells. For such a reason such enzymes have recently gained prominence as an emerging target of anticancer agents [6–8]. Several biological investigations have indicated that HDAC enzymes are present in the human genome as an heterogeneous cluster composed by 18 isozymes which have been categorized into four classes (I, II, III and IV) based on their function and on the sequence similarity to their yeast orthologues [9–11]. HDAC enzymes belonging to class I, II and IV are zinc-containing amide hydrolases [9,10], with a conserved catalytic core but differing in size, domain structure and tissue expression pattern, while class III HDACs are sirtuin related proteins and require the cofactor NAD^+ for their deacetylase function [11]. The vast majority of HDAC inhibitors, currently undergoing development as anticancer drugs, target the zinc-dependent isozymes (classes I, II, and IV). Natural and synthetic HDAC inhibitors entered in clinical studies as promising anticancer drug candidates belonging to different structural classes including hydroxamates, cyclic peptides, aliphatic acid and ortho-amino benzamides, e.g. depsipeptide FK228, valproic acid, MS-275, and NVP-LAQ824 (Fig. 1) [12]; the majority of them possess the hydroxamate moiety as the functionality capable of chelating the zinc ion in the active site. In 2006, the US FDA approved suberoylanilide hydroxamic acid (SAHA; Zolinza™, Fig. 1) as first-in-class HDAC inhibitor for once-daily oral treatment of a rare cancer, cutaneous T-cell lymphoma (CTCL) [13,14]. Nevertheless, hydroxamate-based HDAC inhibitors possess some liabilities, e.g. a poor oral absorption and unfavorable pharmacokinetic parameters due to hydrolysis or rapid glucuronidation. Furthermore, the presence in the molecule of such a strong metal chelating group causes the lack of selectivity among the different HDAC isoforms and can also result in inhibition of other metallo-enzymes or sequestration of metal ions giving rise to a number of side effects. Thus, there is a considerable interest in developing non-hydroxamate HDAC inhibitors characterized by a selectivity for individual isoforms or, at least, a small subset of them [5,15].

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doi:10.1016/j.ejmech.2010.06.035

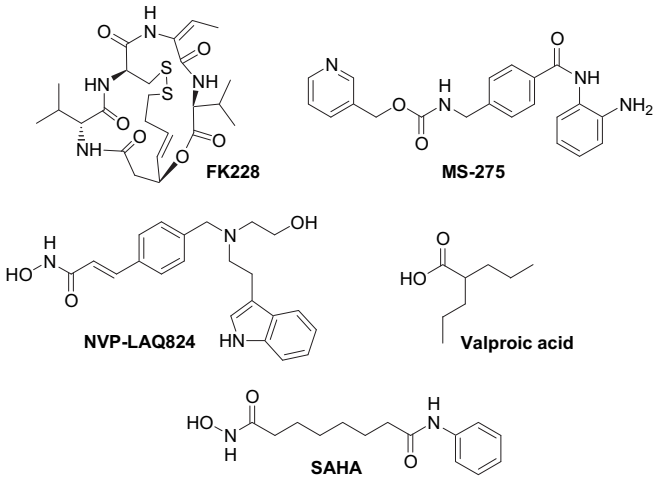


Fig. 1. Structures of HDAC inhibitors.

The X-ray structures of some human and bacterial HDAC enzymes co-crystallized with SAHA and trichostatin A [16–19] revealed the mode of interaction of the inhibitors with the amino acid residues of the enzymes binding pocket, allowing the delineation of the pharmacophore features and the design of a number of inhibitors. Despite the variety of structural motifs, the HDAC inhibitors can be described by a common pharmacophore, which can be segmented into three parts: i) a surface recognition zone (CAP group), which interacts with the amino acid residues on the rim of the active site, ii) a linker domain, usually hydrophobic, which occupies the narrow tube-like channel, and iii) the zinc binding group (ZBG), which chelates the Zn^{2+} ion at the bottom of the active site.

To assess which HDAC isozyme should be targeted for anticancer activity, great efforts are devoted to the discovery of isoform selective HDAC inhibitors. A recent report by Kozikowski et al. [20]. showed that the insertion of substituted aryl-isoxazoles as the CAP group gave HDAC inhibitors potent and selective at HDAC-6 and HDAC-3. Even if the mode of interaction of the isoxazole moiety with the surface residues was not clarified, the same research group, in a subsequent paper, reported the results obtained by shifting this moiety to the linker region, adjacent to the hydroxamate [21]. Some of the novel compounds are provided with an inhibitory activity in the nanomolar range, but with a marginal isozyme selectivity.

The above reported results prompted us to disclose the results of a related investigation carried out by us. The present paper deals with the synthesis of derivatives **1**–**4**, in which the CAP region was kept identical to that of SAHA and an isoxazole or a 4,5-dihydro-isoxazole nucleus was inserted in the linker region adjacent to the ZBG, represented by an hydroxamic acid, to evaluate a possible participation of the heterocyclic nitrogen in the coordination of the zinc ion. In addition, we envisaged that such a nitrogen could mimic the carbonyl oxygen of a classical hydroxamate and we have thus designed derivatives **5**–**13**, in which the length of the spacer is kept to 4 carbon atoms and the 3-substituted isoxazole is intended as the ZBG. In fact, a bidentate interaction with the metal ion could be possible, that involves the heterocyclic nitrogen, mimicking the carbonyl oxygen, and an appropriate substituent appended at the 3 position, i.e. $-NH_2$, $-CH_2OH$, $-CH_2SH$, $-COOH$, $-CH{=}NOH$, $-CONHNH_2$, tetrazolylmethyl-, *o*-phenol, *o*-aniline (Fig. 2).

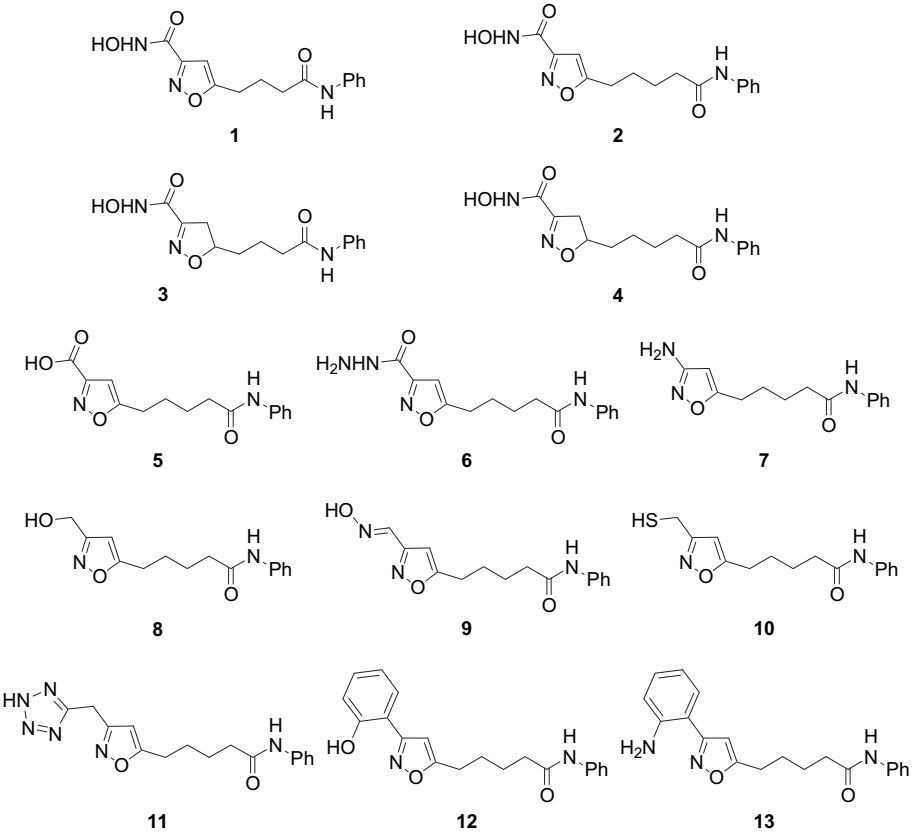
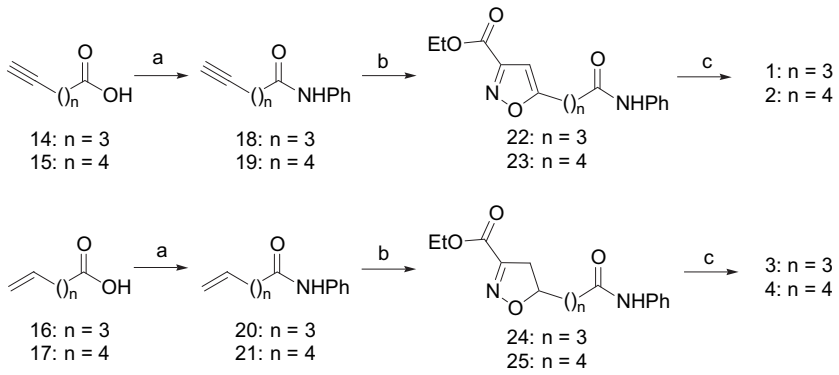


Fig. 2. Compounds synthesized and tested in this study.



Scheme 1. a) DCC, DMAP, aniline, CH_2Cl_2; b) Ethyl 2-chloro-2-(hydroxyimino)acetate, $NaHCO_3$, AcOEt, MW, 100 °C; c) $NH_2OH{\cdot}HCl$, KOH, MeOH.

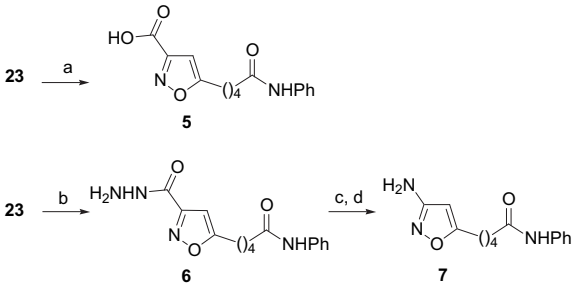
2. Results and discussion

The synthesis of compounds **1**–**13** (Fig. 2) was accomplished by 1,3-dipolar cycloaddition of suitably substituted formonitrile oxides to alkynes **18** and **19**, or alkenes **20** and **21**, to yield the desired isoxazoles or 4,5-dihydro-isoxazoles intermediates, respectively.

The selected dipolarophiles contain i) the cap region, represented by the aromatic ring, ii) the linker, which is an aliphatic chain of 3–4 carbon atoms connected to the cap through an amide bond, and iii) a terminal unsaturation needed to build up the designed isoxazole or isoxazoline ring. Alkynes **18**, **19** and alkenes **20**, **21** were obtained by reacting commercially available carboxylic acids **14**–**17** with aniline under standard reaction conditions (DCC and catalytic DMAP) (Scheme 1).

In order to obtain hydroxamates **1**–**4**, dipolarophiles **18**–**21** were reacted with ethoxycarbonylformonitrile oxide, generated *in situ* from its stable precursor ethyl 2-chloro-2-(hydroxyimino) acetate [22] by adding solid $NaHCO_3$. The pericyclic reaction was carried out under microwave irradiation to speed up the process and afforded the desired cycloadducts **22**–**25**, which were then converted into final products **1**–**4** by treatment with hydroxylamine hydrochloride in a methanolic solution of potassium hydroxide (Scheme 1) [23].

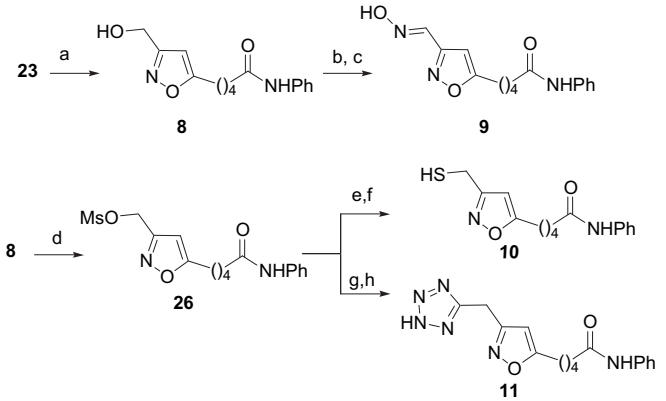
3-Ethoxycarbonyl-isoxazole **23** represented also the key intermediate in the preparation of target derivatives **5**–**11**. In detail, ester derivative **23** was subjected to alkaline hydrolysis to afford the corresponding carboxylic acid **5** (Scheme 2). Alternatively, the same derivative **23** was reacted with hydrazine in ethanol to give hydrazide **6** [24], which, upon treatment with a solution of sodium nitrite in a 1:1 mixture of 10% hydrochloric acid/acetone, followed by heating in a 1:1 mixture of water—acetic acid, was converted into the 3-amino-substituted isoxazole **7** (Scheme 2) [25].



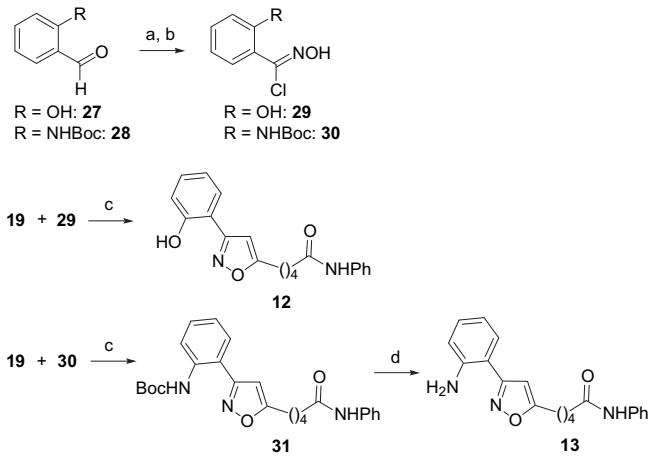
Scheme 2. a) NaOH, EtOH; b) $NH_2NH_2{\cdot}H_2O$, EtOH; c) $NaNO_2$, 10% HCl, acetone, H_2O; d) H_2O/CH_3COOH 1:1, Δ.

An ethanolic solution of ester **23** was also reacted with sodium borohydride supported on alumina to give primary alcohol **8** (Scheme 3). The latter was used as the starting material for the synthesis of target derivatives **9**, **10** and **11** (Scheme 3). Alcohol **8** was mildly oxidized to the corresponding aldehyde, with Dess–Martin's reagent, and immediately condensed with hydroxylamine to afford oxime **9** (Scheme 3). The synthesis of thiol derivative **10** was achieved through the transformation of alcohol **8** into the corresponding mesylate, followed by treatment with potassium thioacetate, to give a *S*-acetyl derivative, easily converted into the desired compound **10** by alkaline hydrolysis (Scheme 3). Finally, mesylate **26** turned out to be a useful intermediate also for the preparation of tetrazolyl-derivative **11**. This transformation was accomplished in two steps: i) the nucleophilic displacement of the mesylate group by the cyanide anion and ii) treatment of the nitrile intermediate with sodium azide and ammonium chloride in DMF at 120 °C (Scheme 3).

For the synthesis of the last two designed derivatives **12** and **13**, it was necessary to prepare suitable 1,3-dipoles to be used in the cycloaddition reaction with alkyne **19**. Chloroximes **29** [26] and **30** were prepared from commercially available salicylaldehyde and 2-*N*-*tert*-butoxycarbonylamino benzaldehyde **28** [27], respectively, following the procedure reported in the literature [26]. Treatment of the freshly prepared chloroximes with excess sodium bicarbonate in the presence of dipole **19**, under microwave irradiation, produced cycloadduct **12** and the *N*-Boc derivative **31**, that, upon treatment with a 30% solution of trifluoroacetic acid in CH_2Cl_2 yielded final derivative **13** (Scheme 4).



Scheme 3. a) $NaBH_4$ 10% on alumina, EtOH; b) Dess–Martin periodinane, CH_2Cl_2; c) $NH_2OH{\cdot}HCl$, TEA, MeOH; d) MsCl, TEA, CH_2Cl_2; e) $KSCOCH_3$, DMF, 90 °C; f) 1 M NaOH, MeOH; g) KCN, DMF; h) NaN_3, NH_4Cl, DMF, 120 °C.



Scheme 4. a) $NH_2OH{\cdot}HCl$, NaOAc, EtOH, H_2O; b) NCS, Py, CH_2Cl_2; 40 °C; c) $NaHCO_3$, AcOEt, MW, 100 °C; d) 30% TFA, CH_2Cl_2.

The new compounds **1**–**13** were submitted to biological evaluation to identify the molecules endowed with HDAC inhibitory activity. The assays were carried out using a commercially available HDAC assay kit, with HeLa cell nuclear extracts as enzyme source and a fluorogenic acetylated histone peptide fragment (Fluor de Lys) as a substrate. As shown in Table 1 the hydroxamates compounds **1**–**4** demonstrated to have a dose dependent moderate biochemical activity (percentage of inhibition in a range between 9 and 23 at 1 μM and between 56 and 76.5% at 10 μM). However, the inhibitory activity was lower than that measured for the reference compound SAHA, especially when measured at low concentrations. Therefore a participation of the heterocyclic nitrogen in the coordination of the zinc ion, in the presence of a hydroxamate moiety, has to be ruled out. We then decided to evaluate whether the insertion of the heterocycle into the SAHA skeleton could play a role in the selectivity profile. To this aim we chose compounds **2** and **4**, having an isoxazole and an isoxazoline moiety, respectively, and tested them for inhibition activity at different HDAC isoforms. The results reported in Table 2 show that both derivatives display 10–70 times lower IC_{50}s at HDAC-6 than at HDAC 1, 2, 3 and 10. This type of selectivity profile parallels that reported for SAHA, therefore a beneficial effect of the heterocycle was not observed.

Concerning the non-hydroxamate derivatives, the only one provided with a moderate inhibitory activity was the thiol compound **10** (22% inhibition at 1 μM and 80% inhibition at 10 μM), whereas all the other derivatives turned out to be inactive. These outcomes demonstrate that none of the 3-substituted isoxazole rings can efficiently vicariate the hydroxamate moiety except for the 3-mercaptomethyl-isoxazole, which probably own its efficacy to the powerful zinc-binding properties typical of the thiol group.

Table 1
In vitro HDAC inhibitory activity.

Compds	Inhibition 0.1 μM (%)	Inhibition 1 μM (%)	Inhibition 10 μM (%)
1	4.5	23.75	78.5
2	1.5	26	78.5
3	3.5	9.75	56
4	4	16.75	77
5	0	0	0.5
6	0	0	8
7	4.5	0	0
8	0	0	0
9	0	0.75	7.5
10	4.5	22	80
11	0	0	5
12	0	0	0
13	0	0	0.5
SAHA	47	85	95

Table 2
In vitro inhibitory activity of compounds **2** and **4** at different HDAC isoforms.

Compds	HDAC-1 IC_{50} (μM)	HDAC-2 IC_{50} (μM)	HDAC-3 IC_{50} (μM)	HDAC-6 IC_{50} (μM)	HDAC-10 IC_{50} (μM)
2	4.95	18.9	26.7	0.43	9.05
4	17.7	36.2	28.1	0.76	23.9
SAHA	0.17	0.39	0.20	0.0093	0.21

3. Conclusion

In conclusion, we have tried to identify new effective isoxazole-based zinc binding groups that could be used instead of the hydroxamate in the design of structurally diverse HDAC inhibitors.

However, though the electronic properties of the 3-substituted isoxazole proposed could have justified an isosterism with the hydroxamate group, the biological results clearly show that, in this case, bioisosterism did not occur, with the exception of the 3-mercaptomethyl-isoxazole. Moreover, the insertion of an isoxazole or isoxazoline ring into the SAHA skeleton did not significantly modify the selectivity profile towards different HDAC isoforms. Structure optimization of compound **10**, including modification of the cap as well as the spacer region, could lead to the development of a new type of non-hydroxamate HDAC inhibitors.

4. Experimental

4.1. Materials and methods

Ethyl 2-chloro-2-(hydroxyimino)acetate [22], *tert*-butyl 2-formylphenylcarbamate **28** [27] and *N*,2-dihydroxybenzimidoyl chloride **29** [26] were prepared following literature procedures.

^1H NMR and ^{13}C NMR spectra were recorded with a Varian Mercury 300 (300 MHz) spectrometer. Chemical shifts (δ) are expressed in ppm and coupling constants (J) in hertz. TLC analyses were performed on commercial silica gel 60 F_{254} aluminium sheets; spots were further evidenced by spraying with a dilute alkaline potassium permanganate solution. Melting points were determined on a model B 540 Büchi apparatus and are uncorrected. Microanalyses (C, H, N) of new compounds agreed with the theoretical value within ±0.4%.

4.2. General procedure for the amide bond formation

A solution of DCC (0.41 g, 2.0 mmol) in CH_2Cl_2 (5.0 ml) was added dropwise to a cooled (0 °C) solution of carboxylic acid **14**–**17** (2.0 mmol), aniline (182 μl, 2.0 mmol) and DMAP (24 mg, 0.2 mmol) in CH_2Cl_2 (5.0 ml). The mixture was allowed to warm at room temperature and stirred for 4 h. The progress of the reaction was monitored by TLC (dichloromethane/methanol 9:1). After disappearance of the starting material, the solid was filtered off and the mixture was washed with 1 N HCl (10 ml), 5% solution of $NaHCO_3$ (10 ml) and brine. The organic layer was dried over anhydrous sodium sulfate and concentrated to give the crude material, which was purified by flash chromatography (petroleum ether/ethyl acetate 8:2) to yield compound **18** [28], **19** [28], **20** [29], and **21** [30] (about 80% yield).

4.3. General procedure for the cycloaddition reaction

Ethyl 2-chloro-2-(hydroxyimino)acetate (0.30 g, 2.0 mmol) and an excess of solid $NaHCO_3$ (0.67 g, 8.0 mmol) were added to a solution of compound **18**–**21** (1.0 mmol) in AcOEt (10 ml). The mixture was irradiated with μW for 90 min at 100 °C. The progress of the reaction was monitored by TLC. Water was added to the reaction mixture and the organic layer was separated and dried over anhydrous sodium sulfate. The solvent was evaporated and the product was purified by crystallization.

4.3.1. Ethyl 5-[4-oxo-4-(phenylamino)butyl]isoxazole-3-carboxylate (**22**)

Crystallized from diisopropyl ether/2-propanol 95/5 as white prisms; mp 81–82 °C; yield 80%; $R_f = 0.26$ (cyclohexane/ethyl acetate 6:4). ^{1}H NMR ($CDCl_3$): 1.40 (t, $J = 7.1$, 3H); 2.16 (m, 2H); 2.43 (t, $J = 7.2$, 2H); 2.87 (t, $J = 7.2$, 2H); 4.41 (q, $J = 7.1$, 2H); 6.45 (s, 1H); 7.10 (t, $J = 7.4$, 1H); 7.24 (bs, 1H); 7.32 (t, $J = 7.4$, 2H); 7.50 (d, $J = 7.4$, 2H). ^{13}C NMR ($CDCl_3$): 14.34, 23.35, 26.15, 36.13, 62.40, 102.17, 120.20, 124.57, 129.19, 138.10, 156.67, 160.30, 170.52, 174.98. Anal. Calcd for $C_{16}H_{18}N_2O_4$ (302.13): C, 63.56; H, 6.00; N, 9.27. Found: C, 63.89; H, 6.28; N, 9.39.

4.3.2. Ethyl 5-[5-oxo-5-(phenylamino)pentyl]isoxazole-3-carboxylate (**23**)

Crystallized from diisopropyl ether/2-propanol 95/5 as white prisms; mp 97–99 °C; yield 86%; $R_f = 0.19$ (cyclohexane/ethyl acetate 7:3). ^{1}H NMR ($CDCl_3$): 1.40 (t, $J = 7.0$, 3H); 1.75–1.85 (m, 4H); 2.35–2.45 (m, 2H); 2.80–2.93 (m, 2H); 4.42 (q, $J = 7.0$, 2H); 6.42 (s, 1H); 7.10 (t, $J = 7.9$, 1H); 7.24 (bs, 1H); 7.31 (t, $J = 7.9$, 2H); 7.52 (d, $J = 7.9$, 2H). ^{13}C NMR ($CDCl_3$): 14.35, 24.95, 26.72, 27.12, 37.09, 62.32, 101.92, 120.08, 124.49, 129.19, 138.12, 156.63, 160.36, 170.92, 175.25. Anal. Calcd for $C_{17}H_{20}N_2O_4$ (316.14): C, 64.54; H, 6.37; N, 8.86. Found: C, 64.26; H, 6.60; N, 9.00.

4.3.3. Ethyl 5-[4-oxo-4-(phenylamino)butyl]-4,5-dihydroisoxazole-3-carboxylate (**24**)

Crystallized from diisopropyl ether/2-propanol 95/5 as white prisms; mp 66–67 °C; yield 98%; $R_f = 0.18$ (petroleum ether/ethyl acetate 7:3). ^{1}H NMR ($CDCl_3$): 1.36 (t, $J = 7.1$, 3H); 1.70–1.95 (m, 4H); 2.35–2.45 (m, 2H); 2.88 (dd, $J = 8.2$, 17.6, 1H); 3.29 (dd, $J = 11.0$, 17.6, 1H); 4.34 (q, $J = 7.1$, 2H); 4.85 (m, 1H); 7.10 (t, $J = 7.4$, 1H); 7.26 (bs, 1H); 7.31 (t, $J = 7.4$, 2H); 7.52 (d, $J = 7.4$, 2H). ^{13}C NMR ($CDCl_3$): 14.39, 21.51, 34.50, 37.06, 38.82, 62.32, 84.18, 120.01, 124.55, 129.26, 138.00, 151.83, 160.93, 170.76. Anal. Calcd for $C_{16}H_{20}N_2O_4$ (304.14): C, 63.14; H, 6.62; N, 9.20. Found: C, 62.89; H, 6.58; N, 9.10.

4.3.4. Ethyl 5-[5-oxo-5-(phenylamino)pentyl]-4,5-dihydroisoxazole-3-carboxylate (**25**)

Crystallized from diisopropyl ether/2-propanol 95/5 as white prisms; mp 62–63 °C; yield 98%; $R_f = 0.22$ (cyclohexane/ethyl acetate 6:4). ^{1}H NMR ($CDCl_3$): 1.27 (t, $J = 7.1$, 3H); 1.58–1.74 (m, 6H); 2.30 (t, $J = 7.4$, 2H); 2.74 (dd, $J = 8.5$, 17.6, 1H); 3.16 (dd, $J = 11.0$, 17.6, 1H); 4.25 (q, $J = 7.1$, 2H); 4.70 (m, 1H); 7.00 (t, $J = 7.7$, 1H); 7.20 (t, $J = 7.7$, 2H); 7.48 (d, $J = 7.7$, 2H); 8.35 (bs, 1H). ^{13}C NMR ($CDCl_3$): 14.33, 25.11, 25.39, 35.03, 37.37, 38.66, 62.28, 84.11, 120.21, 124.36, 129.11, 138.34, 151.77, 160.99, 172.67. Anal. Calcd for $C_{17}H_{22}N_2O_4$ (318.16): C, 64.13; H, 6.97; N, 8.80. Found: C, 64.00; H, 6.84; N, 9.00.

4.4. General procedure for the synthesis of hydroxamic acids

A solution of $NH_2OH \cdot HCl$ (70 mg, 1.0 mmol) in EtOH (0.5 ml) was prepared and refluxed. In a separate flask, a solution of KOH (84 mg, 1.5 mmol) in EtOH (0.5 ml) was refluxed. After cooling at $T < 40$ °C, the solution of KOH was added, in one portion, to the solution of $NH_2OH \cdot HCl$. The mixture was stirred for 5 min and then a solution of the cycloadduct **22**–**25** (0.5 mmol) in EtOH (0.5 ml) was added. The reaction was stirred overnight at room temperature and then was diluted with water (5 ml), made neutral with 2 N HCl and filtered under vacuum. The solid was collected and recrystallized.

4.4.1. N-Hydroxy-5-[4-oxo-4-(phenylamino)butyl]isoxazole-3-carboxamide (**1**)

Crystallized from 2-propanol as white prisms; mp 175–176 °C; yield 54%; $R_f = 0.20$ (dichloromethane/methanol 95:5 + 1% CH_3COOH). ^{1}H NMR (DMSO-d_6): 1.95 (ddd, $J = 7.7$, 7.7, 14.9, 2H); 2.37 (t, $J = 7.7$, 2H); 2.84 (t, $J = 7.7$, 2H); 6.58 (s, 1H), 7.00 (t, $J = 8.5$, 1H); 7.25 (t, $J = 8.5$, 2H); 7.56 (d, $J = 8.5$, 2H), 9.35 (s, 1H), 9.90 (s, 1H), 11.45 (s, 1H). ^{13}C NMR (DMSO-d_6): 23.46, 26.06, 35.87, 101.43, 119.75, 123.63, 129.36, 139.90, 156.99, 158.17, 171.06, 174.81. Anal. Calcd for $C_{14}H_{15}N_3O_4$ (289.11): C, 58.13; H, 5.23; N, 14.53. Found: C, 58.10; H, 5.28; N, 14.60.

4.4.2. N-Hydroxy-5-[5-oxo-5-(phenylamino)pentyl]isoxazole-3-carboxamide (**2**)

Crystallized from 2-propanol as white prisms; mp 175–176 °C; yield 36%; $R_f = 0.20$ (dichloromethane/methanol 95:5 + 1% CH_3COOH). ^{1}H NMR (DMSO-d_6): 1.55–1.75 (m, 4H); 2.32 (m, 2H); 2.84 (m, 2H); 6.54 (s, 1H), 7.00 (t, $J = 8.2$, 1H); 7.26 (t, $J = 8.2$, 2H); 7.56 (d, $J = 8.2$, 2H), 9.35 (s, 1H), 9.87 (s, 1H), 11.45 (s, 1H). ^{13}C NMR (DMSO-d_6): 25.10, 26.28, 27.19, 36.52, 101.25, 119.73, 123.63, 129.35, 139.95, 157.02, 158.14, 171.60, 175.14. Anal. Calcd for $C_{15}H_{17}N_3O_4$ (303.12): C, 59.40; H, 5.65; N, 13.85. Found: C, 59.30; H, 5.58; N, 13.90.

4.4.3. N-Hydroxy-5-[4-oxo-4-(phenylamino)butyl]-4,5-dihydroisoxazole-3-carboxamide (**3**)

Crystallized from 2-propanol as pink prisms; mp 144–146 °C; yield 46%; $R_f = 0.19$ (dichloromethane/methanol 95:5 + 1% CH_3COOH). ^{1}H NMR (DMSO-d_6): 1.50–1.75 (m, 4H); 2.30 (m, 2H); 2.81 (dd, $J = 8.2$, 17.6, 1H); 3.22 (dd, $J = 10.8$, 17.6, 1H); 4.65 (m, 1H); 7.00 (t, $J = 7.5$, 1H); 7.25 (t, $J = 7.5$, 2H); 7.56 (d, $J = 7.5$, 2H), 9.15 (s, 1H), 9.85 (s, 1H), 11.20 (s, 1H). ^{13}C NMR (DMSO-d_6): 21.60, 34.58, 36.57, 82.11, 119.70, 123.64, 129.35, 139.96, 153.41, 157.83, 171.55. Anal. Calcd for $C_{14}H_{17}N_3O_4$ (291.12): C, 57.72; H, 5.88; N, 14.42. Found: C, 57.90; H, 6.00; N, 14.57.

4.4.4. N-Hydroxy-5-[5-oxo-5-(phenylamino)pentyl]-4,5-dihydroisoxazole-3-carboxamide (**4**)

Crystallized from 2-propanol as pink prisms; mp 166–168 °C; yield 40%; $R_f = 0.19$ (dichloromethane/methanol 95:5 + 1% CH_3COOH). ^{1}H NMR (DMSO-d_6): 1.20–1.45 (m, 2H); 1.50–1.65 (m, 4H); 2.30 (t, $J = 7.1$, 2H); 2.79 (dd, $J = 8.2$, 17.6, 1H); 3.23 (dd, $J = 10.7$, 17.6, 1H); 4.65 (m, 1H); 7.00 (t, $J = 7.7$, 1H); 7.25 (t, $J = 7.7$, 2H); 7.56 (d, $J = 7.7$, 2H), 9.15 (s, 1H), 9.85 (s, 1H), 11.20 (s, 1H). ^{13}C NMR (DMSO-d_6): 25.14, 25.55, 34.83, 36.92, 82.25, 119.70, 123.63, 129.35, 140.00, 153.40, 157.86, 171.78. Anal. Calcd for $C_{15}H_{19}N_3O_4$ (305.14): C, 59.01; H, 6.27; N, 13.76. Found: C, 58.90; H, 6.00; N, 13.49.

4.4.5. Synthesis of 5-[4-(phenylcarbamoyl)butyl]isoxazole-3-carboxylic acid (**5**)

Compound **23** (158 mg, 0.5 mmol) was dissolved in EtOH (1.0 ml) and 1 N NaOH (0.5 ml) was added. The mixture was stirred for 1 h at room temperature. The organic solvent was evaporated and the aqueous phase was extracted with Et_2O (1 × 2 ml). The aqueous layer was made acidic with 2 N HCl and newly extracted with AcOEt (4 × 2 ml). The organic extracts were dried over anhydrous sodium sulfate. The solvent was removed under vacuum to give 129 mg (90% yield) of compound **5**.

Compound **5**: crystallized from 2-propanol as white prisms; mp > 148 °C dec; $R_f = 0.18$ (dichloromethane/methanol 95:5 + 1% CH_3COOH). ^{1}H NMR (DMSO-d_6): 1.55–1.78 (m, 4H); 2.28–2.39 (m, 2H); 2.78–2.85 (m, 2H); 6.60 (s, 1H); 7.00 (t, $J = 8.2$, 1H); 7.25 (t,

$J = 8.2$, 2H); 7.56 (d, $J = 8.2$, 2H); 9.30 (s, 1H). ^{13}C NMR (DMSO-d_6): 25.08, 26.32, 27.13, 36.51, 102.45, 119.72, 123.65, 129.33, 139.95, 157.70, 161.75, 171.59, 175.87. Anal. Calcd for $C_{15}H_{16}N_2O_4$ (288.11): C, 62.49; H, 5.59; N, 9.72. Found: C, 62.31; H, 5.41; N, 9.75.

4.4.6. Synthesis of 5-[3-(hydrazinecarbonyl)isoxazol-5-yl]-N-phenylpentanamide (**6**)

To a stirred solution of **23** (474 mg, 1.5 mmol) in EtOH (5.0 ml) at 0 °C was added $NH_2NH_2 \cdot H_2O$ (218 μl, 4.5 mmol). The solution was warmed to room temperature and stirred for 30 min. The solid was filtered off and the solvent was evaporated under reduced pressure to obtain a yellow solid, which was triturated with water and recrystallized from ethanol (263 mg, yield 58%).

Compound **6**: crystallized from ethanol as pale yellow prisms; mp > 148 °C dec; $R_f = 0.35$ (dichloromethane/methanol 95/5). ^{1}H NMR (DMSO-d_6): 1.56–1.74 (m, 4H); 2.32 (t, $J = 7.4$, 2H); 2.81 (t, $J = 7.4$, 2H); 4.45–4.79 (bs, 2H); 6.55 (s, 1H); 7.00 (t, $J = 7.4$, 1H); 7.25 (t, $J = 7.4$, 2H); 7.56 (d, $J = 7.4$, 2H), 9.90 (s, 1H). ^{13}C NMR (DMSO-d_6): 25.11, 26.29, 27.19, 36.52, 101.11, 119.71, 123,65, 129.33, 139.95, 158.62, 158.75, 171.59, 175.07. Anal. Calcd for $C_{15}H_{18}N_4O_3$ (302.14): C, 59.59; H, 6.00; N, 18.53. Found: C, 59.67; H, 6.11; N, 18.78.

4.4.7. Synthesis of 5-(3-aminoisoxazol-5-yl)-N-phenylpentanamide (**7**)

a) The hydrazide **6** (151 mg, 0.50 mmol) was dissolved in a 1:1 mixture of acetone/10% HCl (2.0 ml). The solution was cooled in an ice bath and a solution of $NaNO_2$ (52 mg, 0.75 mmol) in water (0.5 ml) was added dropwise. The mixture was stirred for 30 min. An additional 1.0 ml of water was added and the product was collected by filtration as white plates (137 mg, 88% yield).

b) The acyl azide (137 mg, 0.44 mmol) was suspended in a 1:1 mixture of water/acetic acid (7.0 ml) and the mixture was brought to reflux. After disappearance of the starting material (1 h), the solvents were removed under vacuum and the solid residue was crystallized from ethanol to give compound **7** (88 mg, 77% yield).

Compound **7**: crystallized from ethanol as white prisms; mp > 150 °C dec; $R_f = 0.20$ (cyclohexane/ethyl acetate). ^{1}H NMR (DMSO-d_6): 1.50–1.70 (m, 4H); 2.32 (m, 2H); 2.55 (m, 2H); 5.40 (bs, 2H); 5.56 (s, 1H); 7.00 (t, $J = 7.4$, 1H); 7.26 (t, $J = 7.4$, 2H); 7.56 (d, $J = 7.4$, 2H); 9.85 (s, 1H). ^{13}C NMR (DMSO-d_6): 25.22, 26.54, 27.23, 36.61, 94.34, 119.71, 123.64, 129.33, 139.97, 164.57, 171.66, 175.80. Anal. Calcd for $C_{14}H_{17}N_3O_2$ (259.13): C, 64.85; H, 6.61; N, 16.20. Found: C, 64.98; H, 6.76; N, 16.35.

4.4.8. Synthesis of 5-(3-(hydroxymethyl)isoxazol-5-yl)-N-phenylpentanamide (**8**)

To a stirred solution of compound **23** (316 mg, 1.0 mmol) in ethanol (10 ml) $NaBH_4$ on alumina ($NaBH_4$ 10%, 1.3 mmol, 390 mg) was added. The mixture was stirred at room temperature for 24 h, until disappearance of the starting material. The solid was filtered off and the solvent was evaporated, obtaining compound **8** as a white solid, which was purified by crystallization (260 mg, 95% yield).

Compound **8**: crystallized from 2-propanol white prisms; mp 113–116 °C; $R_f = 0.26$ (dichloromethane/methanol 95:5). ^{1}H NMR (CD_3OD): 1.70–1.82 (m, 4H); 2.38–2.44 (m, 2H); 2.78–2.85 (m, 2H); 4.58 (s, 2H); 6.20 (s, 1H); 7.07 (t, $J = 7.9$, 1H); 7.28 (t, $J = 7.9$, 2H); 7.53 (d, $J = 7.9$, 2H); 9.75 (bs, 1H). ^{13}C NMR (CD_3OD): 25.02, 25.99, 27.01, 36.21, 55.63, 99.91, 120.06, 123.97, 128.59, 138.66, 164.33, 172.91, 173.78. Anal. Calcd for $C_{15}H_{18}N_2O_3$ (274.13): C, 65.68; H, 6.61; N, 10.21. Found: C, 65.40; H, 6.50; N, 10.45.

4.4.9. Synthesis of (E)-5-(3-((hydroxyimino)methyl)isoxazol-5-yl)-N-phenylpentanamide (**9**)

a) To a cooled (0 °C) solution of **8** (192 mg, 0.70 mmol) in dry CH_2Cl_2 (10 ml) was added Dess–Martin periodinane (326 mg, 0.77 mmol). The reaction mixture was warmed to room temperature and stirred for 2 h. The mixture was poured into a solution of 10% $NaHCO_3$ (10 ml) containing 1% $Na_2S_2O_3$, the phases were separated and the organic layer was washed with brine (10 ml) and dried over anhydrous sodium sulfate. The solvent was evaporated to obtain the crude aldehyde that was used in the next step without any additional purification.

b) The aldehyde obtained from the previous step (190 mg, 0.70 mmol) was dissolved in MeOH (2.0 ml). In a separate flask a solution of $NH_2OH \cdot HCl$ (48 mg, 0.70 mmol) and TEA (97 μl, 0.70 mmol) was prepared in MeOH (1.0 ml) and added to the solution of aldehyde. The reaction mixture was stirred for 2 h at room temperature. The solvent was evaporated, the residue was dissolved in AcOEt (5 ml) and washed with 1 N HCl (5 ml). The organic layer was dried over anhydrous sodium sulfate and the solvent was evaporated. The crude material was purified by column chromatography (petroleum ether/ethyl acetate 6:4) to obtain compound **9** (116 mg, 58% yield).

Compound **9**: crystallized from methanol as white prisms; mp 164–167 °C; $R_f = 0.27$ (petroleum ether/ethyl acetate 1:1). ^{1}H NMR (DMSO-d_6): 1.55–1.75 (m, 4H); 2.30 (t, $J = 6.9$, 2H); 2.80 (t, $J = 6.9$, 2H); 6.50 (s, 1H); 7.00 (t, $J = 8.5$, 1H); 7.25 (t, $J = 8.5$, 2H); 7.56 (d, $J = 8.5$, 2H); 8.15 (s, 1H), 9.90 (bs, 1H); 12.00 (s, 1H). ^{13}C NMR (DMSO-d_6): 25.14, 26.23, 27.17, 36.54, 98.83, 119.72, 123.64, 129.33, 139.90, 139.96, 159.04, 171.61, 174.33. Anal. Calcd for $C_{15}H_{17}N_3O_3$ (287.31): C, 62.71; H, 5.96; N, 14.63. Found: C, 62.89; H, 6.18; N, 14.89.

4.4.10. Synthesis of (5-(4-(phenylcarbamoyl)butyl)isoxazol-3-yl) methyl methanesulfonate (**26**)

TEA (208 μl, 1.5 mmol) was added to a stirred solution of compound **8** (274 mg, 1.0 mmol) in CH_2Cl_2 (10 ml). The mixture was cooled at 0 °C and methanesulfonyl chloride (116 μl, 1.5 mmol) was added dropwise. The reaction was stirred at room temperature for 4 h. The organic phase was washed with 1 N HCl (2 × 5 ml), 5% solution of $NaHCO_3$ (2 × 5 ml), brine (5 ml) and dried over anhydrous sodium sulfate. The solvent was evaporated and the product was purified by crystallization with 2-propanol to obtain compound **26** (274 mg, 79% yield).

Compound **26**: crystallized from 2-propanol as white prisms; mp > 78 °C dec; $R_f = 0.32$ (cyclohexane/ethyl acetate 4:6). ^{1}H NMR ($CDCl_3$): 1.70–1.82 (m, 4H); 2.35–2.42 (m, 2H); 2.78–2.85 (m, 2H); 3.03 (s, 3H); 5.25 (s, 2H); 6.18 (s, 1H); 7.05 (t, $J = 7.9$, 1H); 7.32 (t, $J = 7.9$, 2H); 7.38 (bs, 1H); 7.50 (d, $J = 7.9$, 2H). ^{13}C NMR ($CDCl_3$): 24.98, 26.70, 27.08, 37.08, 38.36, 62.30, 100.95, 120.11, 124.53, 129.21, 138.14, 158.27, 171.09, 174.88. Anal. Calcd for $C_{16}H_{20}N_2O_5S$ (352.11): C, 54.53; H, 5.72; N, 7.95. Found: C, 54.78; H, 5.90; N, 7.90.

4.4.11. Synthesis of 5-(3-(mercaptomethyl)isoxazol-5-yl)-N-phenylpentanamide (**10**)

a) Compound **26** (138 mg, 0.39 mmol) was dissolved in DMF (2.0 ml) and CH_3COSK (53 mg, 0.47 mmol) was added. The mixture was stirred overnight at 90 °C. After disappearance of the starting material, water (3 ml) was added and the aqueous phase was extracted with AcOEt (3 × 5 ml). The organic layer was dried over anhydrous sodium sulfate and the solvent was evaporated under reduced pressure. The crude material was purified by column chromatography (cyclohexane/ethyl acetate 7/3) to give the product as a yellow oil (65 mg, 50% yield).

b) The *S*-acetyl intermediate obtained from the previous step (65 mg, 0.19 mmol) was dissolved in MeOH (0.20 ml) and 1 N NaOH was added (0.19 ml). The mixture was stirred 1 h at room temperature. Water (2 ml) was added and the aqueous phase was extracted with Et_2O (1 × 2 ml). The aqueous layer was made acidic with 2 N HCl and newly extracted with AcOEt (4 × 2 ml). The organic extracts were dried over anhydrous sodium sulfate. The solvent was removed under vacuum to give 48 mg (88% yield) of compound **10**.

Compound **10**: crystallized from 2-propanol as white prisms; mp > 135 °C dec; R_f = 0.16 (cyclohexane/ethyl acetate 1:1). ^{1}H NMR ($CDCl_3$): 1.55 (bs, 1H); 1.70–1.85 (m, 4H); 2.30–2.42 (m, 2H); 2.70–2.82 (m, 2H); 3.85 (s, 2H); 6.08 (s, 1H); 7.10 (m, 1H); 7.30 (m, 2H); 7.50 (m, 2H). ^{13}C NMR ($CDCl_3$): 25.12, 25.15, 27.48, 32.16, 34.34, 101.05, 120.51, 124.93, 129.61, 139.04, 158.27, 171.09, 174.88. Anal. Calcd for $C_{15}H_{18}N_2O_2S$ (290.11): C, 62.04; H, 6.25; N, 9.65. Found: C, 62.24; H, 6.35; N, 9.78.

4.4.12. Synthesis of 5-(3-((2H-tetrazol-5-yl)methyl)isoxazol-5-yl)-N-phenylpentanamide (**11**)

a) Compound **26** (138 mg, 0.39 mmol) was dissolved in DMSO (2.0 ml) and KCN was added (38 mg, 0.58 mmol). The solution was stirred at room temperature for 45 min. After disappearance of the starting material, water (3 ml) was added and the aqueous phase was extracted with AcOEt (3 × 5 ml). The organic layer was dried over anhydrous sodium sulfate and the solvent was evaporated under reduced pressure. The crude material was purified by column chromatography (petroleum ether/ethyl acetate 3/7) to give the product as a white solid (73 mg, 66% yield).

b) The intermediate obtained from the previous step (73 mg, 0.26 mmol) was dissolved in DMF (1.0 ml); NaN_3 (270 mg, 4.16 mmol) and NH_4Cl (220 mg, 4.16 mmol) were added and the resulting solution was stirred at 120 °C for 24 h. The mixture was poured into ice cold water (2 ml) and acidified with 1 N HCl. The aqueous layer was extracted with AcOEt (3 × 3 ml); the organic layer was washed with brine, dried over anhydrous sodium sulfate and concentrated to give the crude product which was purified by flash chromatography to yield compound **11** as a white solid (63 mg, 75% yield).

Compound **11**: crystallized from 2-propanol as white prisms; mp > 142 °C dec; R_f = 0.25 (cyclohexane/ethyl acetate 7:3). ^{1}H NMR ($CDCl_3$): 1.75–1.85 (m, 4H); 2.35–2.45 (m, 2H); 2.75–2.85 (m, 2H); 4.38 (s, 2H); 6.06 (s, 1H); 7.02 (t, J = 7.7, 1H); 7.18 (bs, 1H); 7.32 (t, J = 7.7, 2H); 7.51 (d, J = 7.7, 2H). ^{13}C NMR ($CDCl_3$): 24.98, 26.79, 27.15, 37.27, 45.96, 100.46, 119.97, 124.57, 129.27, 137.99, 159.38, 163.80, 170.72, 174.44. Anal. Calcd for $C_{16}H_{18}N_6O_2$ (326.35): C, 58.88; H, 5.56; N, 25.75. Found: C, 59.00; H, 5.66; N, 25.80.

4.4.13. Synthesis of 5-(3-(2-hydroxyphenyl)isoxazol-5-yl)-N-phenylpentanamide (**12**)

Prepared following the general procedure for the cycloaddition reaction, using alkyne **19** (1.0 mmol) and chloroxime **29**.

Compound **12**: crystallized from 2-propanol as pale yellow prisms; mp 124–127 °C; yield 56%; R_f = 0.24 (cyclohexane/ethyl acetate 7:3). ^{1}H NMR ($CDCl_3$): 1.80–1.95 (m, 4H); 2.35–2.45 (m, 2H); 2.83–2.95 (m, 2H); 6.43 (s, 1H); 6.95 (t, J = 7.7, 1H); 7.08 (t, J = 7.7, 2H); 7.14 (bs, 1H); 7.38–7.48 (m, 4H); 7.44–7.55 (m, 2H); 9.55 (s, 1H). ^{13}C NMR ($CDCl_3$): 25.01, 26.58, 27.20, 37.27, 99.03, 113.62, 117.66, 119.91, 119.98, 124.59, 128.12, 129.27, 131.70, 137.95, 156.82, 162.78, 170.66, 172.79. Anal. Calcd for $C_{20}H_{20}N_2O_3$ (336.15): C, 71.41; H, 5.99; N, 8.33. Found: C, 71.56; H, 6.08; N, 8.47.

4.4.14. Synthesis of tert-butyl 2-[chloro(hydroxyimino)methyl] phenylcarbamate (**30**)

a) A solution of NaOAc (328 mg, 4.0 mmol in 2.0 ml of H_2O) was added to a stirred solution of *tert*-butyl 2-formylphenylcarbamate **28** (442 mg, 2.0 mmol), prepared following a literature procedure [27], and $NH_2OH{\cdot}HCl$ (278 mg, 4.0 mmol) in 80% aqueous EtOH (5.0 ml). After refluxing for 3 h, the reaction mixture was diluted with water (2 ml) and then cooled to 0 °C. The solid obtained was filtered off, washed with H_2O, and recrystallized from EtOH to yield the oxime (429 mg, 91% yield).

b) To a solution of the oxime (429 mg, 1.8 mmol), obtained from the previous step, in CH_2Cl_2 (2.0 ml) pyridine (16 μl, 0.2 mmol) was added. The temperature was raised to 40 °C and NCS (266 mg, 2.0 mmol) was added to the resulting solution. The reaction mixture was stirred at this temperature for 4 h and then was diluted with CH_2Cl_2 (10 ml). The organic layer was washed with water (2 × 10 ml) and brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure to afford compound **30**, which was purified by crystallization from EtOH (403 mg, 83% yield).

Compound **30**: crystallized from 2-propanol as pale yellow prisms; R_f = 0.24 (cyclohexane/ethyl acetate 95/5). ^{1}H NMR ($CDCl_3$): 1.55 (s, 9H); 7.02 (t, J = 7.7, 2H); 7.20 (d, J = 7.7, 1H); 7.35 (t, J = 7.7, 1H); 8.38 (bd, J = 8.3, 1H); 9.70 (bs, 1H). ^{13}C NMR ($CDCl_3$): 28.60, 80.69, 117.76, 118.74, 121.96, 131.01, 132.33, 138.73, 145.20, 153.57. Anal. Calcd for $C_{12}H_{15}ClN_2O_3$ (270.08): C, 53.24; H, 5.58; N, 10.35. Found: C, 53.56; H, 5.76; N, 10.59.

4.4.15. Synthesis of tert-butyl 2-(5-(3-(2-phenylacetamido)propyl) isoxazol-3-yl)phenylcarbamate (**31**)

Prepared following the general procedure for the cycloaddition reaction using alkyne **19** (0.7 mmol) and chloroxime **30**.

Compound **31**: crystallized from 2-propanol as pale yellow prisms; mp 145–146 °C; yield 65%; R_f = 0.22 (cyclohexane/ethyl acetate 7/3). ^{1}H NMR ($CDCl_3$): 1.51 (s, 9H); 1.60–1.80 (m, 4H); 2.35–2.45 (m, 2H); 2.65–2.80 (m, 2H); 6.38 (s, 1H); 7.03–7.15 (m, 2H); 7.23 (bs, 1H); 7.28–7.43 (m, 3H); 7.47–7.55 (m, 3H); 8.42 (d, J = 8.5, 1H); 9.68 (bs, 1H). ^{13}C NMR ($CDCl_3$): 25.05, 26.60, 27.22, 28.60, 37.30, 80.58, 100.50, 116.01, 119.78, 119.98, 122.20, 124.56, 129.09, 129.27, 130.86, 137.99, 138.14, 153.45, 162.65, 170.70, 172.80. Anal. Calcd for $C_{25}H_{29}N_3O_4$ (435.22): C, 68.95; H, 6.71; N, 9.65. Found: C, 69.18; H, 6.89; N, 9.78.

4.4.16. Synthesis of 5-(3-(2-aminophenyl)isoxazol-5-yl)-N-phenylpentanamide (**13**)

Compound **31** (196 mg, 0.45 mmol) was treated with a 30% CH_2Cl_2 solution (0.8 ml) of trifluoroacetic acid at 0 °C. The solution was stirred at room temperature for 2 h until disappearance of the starting material. The volatiles were removed under vacuum and the residue was purified by flash chromatography (cyclohexane/ethyl acetate 7/3) to give compound **13** (136 mg, 90% yield).

Compound **13**: crystallized from 2-propanol as white prisms; mp 112–113 °C; R_f = 0.19 (cyclohexane/ethyl acetate 7/3). ^{1}H NMR ($CDCl_3$): 1.80–1.95 (m, 4H); 2.35–2.45 (m, 2H); 2.80–2.93 (m, 2H); 5.42 (bs, 2H); 6.37 (s, 1H); 6.70–6.80 (m, 2H); 7.10 (t, J = 7.4, 1H); 7.15 (bs, 1H); 7.16–7.21 (m, 2H); 7.28–7.36 (t, J = 8.2, 2H); 7.42 (t, J = 7.7, 1H); 7.52 (d, J = 7.7, 1H). ^{13}C NMR ($CDCl_3$): 25.09, 26.58, 27.28, 37.37, 99.85, 112.06, 116.65, 117.23, 119.98, 124.54, 129.26, 129.40, 130.73, 137.99, 146.21, 163.27, 170.80, 171.92. Anal. Calcd for $C_{20}H_{21}N_3O_2$ (335.16): C, 71.62; H, 6.31; N, 12.53. Found: C, 71.78; H, 6.56; N, 12.68.

4.5. Histone deacetylase assay

The *in vitro* activity of HDAC inhibitors was assayed using a BIOMOL Kit AK-500, according to the instructions from the manufacturer (Biomolecular Research Laboratories). The assay is automated and performed by a Tecan Freedom EVO® work station. On detail, 15 μl of 30 × 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.

4.6. Determination of IC_{50} at HDAC 1, 2, 3, 6 and 10

Compounds **2** and **4** were tested by Reaction Biology Corporation, One Great Valley Parkway, Suite 8, Malvern, PA 19355 (www.reactionbiology.com).

Compounds were tested in 10-dose IC_{50} mode in duplicate with 3-fold serial dilution starting at 100 μM. Fluorogenic peptide from p53 residues 379–382 (RHKKAc) was used as general substrate.

Acknowledgment

Financial supports from the Italian Ministry of Education (MIUR – PRIN 2007) and University of Milan are gratefully acknowledged.

References

[1] P.A. Abreu, G. Dellamora-Orriz, L.R. Leao-Ferreira, M. Gouveia, E. Braggio, I. Zalcberg, D.O. Santos, S. Bourguinhon, L.M. Cabral, C.R. Rodrigues, H.C. F. Castro, Exp. Opin. Ther. Targets 12 (2008) 1035.
[2] M. Grunstein, Nature 389 (1997) 349.
[3] W.L. Cheung, S.D. Briggs, C.D. Allis, Curr. Opin. Cell Biol. 12 (2000) 326.
[4] G. Elaut, V. Rogiers, T. Vanhaecke, Curr. Pharm. Des. 13 (2007) 2584.
[5] M. Paris, M. Porcelloni, M. Binaschi, D. Fattori, J. Med. Chem. 51 (2008) 1505.
[6] P.A. Marks, M. Dokmanovic, Expert. Opin. Investig. Drugs. 14 (2005) 1497.
[7] C.B. Yoo, P.A. Jones, Nat. Rev. Drug Discov. 5 (2006) 37.
[8] J.E. Bolden, M.J. Peart, R.W. Johnstone, Nat. Rev. Drug Discov. 5 (2006) 769.
[9] A.J.M. De Ruijter, A.H. van Gennip, H.N. Caron, S. Kemp, A.B.P. van Kuilenburg, Biochem. J. 370 (2003) 737.
[10] I.V. Gregoretti, Y.M. Lee, H.V. Goodson, J. Mol. Biol. 338 (2004) 17.
[11] S. Michan, D. Sinclair, Biochem. J. 404 (2007) 1.
[12] A.V. Bieliauskas, M.K.H. Pflum, Chem. Soc. Rev. 37 (2008) 1402.
[13] see: http://www.fda.gov/cder/foi/nda/2006/021991s000_ZolinzaTOC.htm.
[14] S. Grant, C. Easley, P. Kirkpatrick, Nat. Rev. Drug Discov. 6 (2007) 21.
[15] B. Attenni, J.M. Ontoria, J.C. Cruz, M. Rowley, C. Schultz-Fademrecht, C. Steinkühler, P. Jones, Bioorg. Med. Chem. Lett. 19 (2009) 3081.
[16] M.S. Finnin, J.R. Donigian, A. Cohen, V.M. Richon, R.A. Rifkind, P.A. Marks, R. Breslow, N.P. Pavletich, Nature 401 (1999) 188.
[17] J.R. Somoza, R.J. Skene, B.A. Kats, C. Mol, J.D. Ho, A.J. Jennings, C. Luong, A. Arvai, J.J. Buggy, E. Chi, C. Tang, B.C. Sang, E. Verner, R. Wynands, E.M. Leahy, D.R. Dougan, G. Snell, G. Navre, M.W. Knuth, R.W. Swanson, D.E. McRee, L. W. Tari, Structure 12 (2004) 1325.
[18] A. Vannini, C. Volpari, G. Filocamo, E. Caroli Casavola, M. Brunetti, D. Renzoni, P. Chakravarty, C. Paolini, R. De Francesco, P. Gallinari, C. Steinkuhler, S. Di Marco, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 15064.
[19] T.N. Nielsen, C. Hildmann, A. Dickmanns, A. Schwienhorst, R. Ficner, J. Mol. Biol. 354 (2005) 107.
[20] A.P. Kozikowski, S. Tapadar, D.N. Luchini, K.H. Kim, D.D. Billadeau, J. Med. Chem. 51 (2008) 4370.
[21] S. Tapadar, R. He, D.N. Luchini, D.D. Billadeau, A.P. Kozikowski, Bioorg. Med. Chem. Lett. 19 (2009) 3023.
[22] A.P. Kozikowski, M. Adamcz, J. Org. Chem. 48 (1983) 366.
[23] S. Hanessian, N. Moitessier, C. Gauchet, M. Viau, J. Med. Chem. 44 (2001) 3066.
[24] W.R. Vaughan, J.L. Spencer, J. Org. Chem. 25 (1960) 1160.
[25] R.H. Dodd, C. Ouannĕs, L. Prado de Carvalho, A. Valin, P. Venault, G. Chapouthier, J. Rossier, P. Potier, J. Med. Chem. 28 (1985) 824.
[26] J.W. Bode, Y. Hachisu, T. Matsuura, K. Suzuki, Org. Lett. 5 (2003) 391.
[27] B.M. Nugent, A.L. Williams, E.N. Prabhakaran, J.N. Johnston, Tetrahedron 59 (2003) 8877.
[28] K. Shin, G.B. Dudley, J. Am. Chem. Soc. 128 (2006) 6499.
[29] G. Belanger, M. April, E. Dauphin, S. Roy, J. Org. Chem. 72 (2007) 1104.
[30] E. Hernandez, H. Hoberg, J. Organomet. Chem. 328 (1987) 403.