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Identification of HDAC6-Selective Inhibitors of Low Cancer Cell Cytotoxicity

Dr. Irina N. Gaisina[a],[b], **Dr. Werner Tueckmantel**[b], **Dr. Andrey Ugolkov**[c], **Dr. Sida Shen**[a], **Dr. Jessica Hoffen**[c], **Dr. Oleksii Dubrovskyi**[c],[d], **Dr. Andrew Mazar**[c],[d], **Dr. Renee A. Schoon**[e], **Dr. Daniel Billadeau**[e], and **Prof. Dr. Alan P. Kozikowski**[a]

[a]Drug Discovery Program, Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago,IL 60612 (USA)

[b]Psychogenics, Inc., Tarrytown, NY 10591 (USA)

[c]Center for Developmental Therapeutics, Feinberg School of Medicine, Northwestern University, Evanston, IL 60208 (USA)

[d]Department of Pharmacology, Feinberg School of Medicine, Northwestern University, Evanston, IL 60208 (USA)

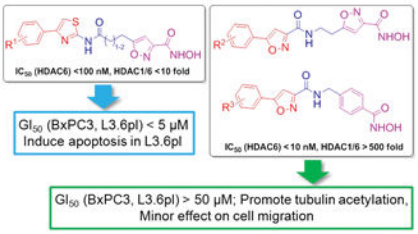
[e]Department of Immunology and Division of Oncology Research, College of Medicine, Mayo Clinic 13–42 Guggenheim, Rochester, MN 55905 (USA)

Abstract

The histone deacetylases (HDACs) occur in 11 different isoforms, and these enzymes regulate the activity of a large number of proteins involved in cancer initiation and progression. The discovery of isoform selective HDAC inhibitors (HDACIs) is desirable, as it is likely that such compounds would avoid some of the undesirable side effects found with the first generation inhibitors. A series of HDACIs previously reported by us were found to display some selectivity for HDAC6 and to induce cell cycle arrest and apoptosis in pancreatic cancer cells. In the present work, we show that structural modification of these isoxazole-based inhibitors leads to high potency and selectivity for HDAC6 over HDAC1-3 and HDAC10, while unexpectedly abolishing their ability to block cell growth. Three inhibitors with lower HDAC6 selectivity inhibit the growth of cell lines BxPC3 and L3.6pl, and they only induce apoptosis in L3.6pl. We conclude that HDAC6 inhibition alone is insufficient for disruption of cell growth, and that some degree of class 1 HDAC inhibition is required. Moreover, the highly selective HDAC6Is reported herein that are weakly cytotoxic may find use in cancer immune system reactivation.

Graphical abstract

Correspondence to: Alan P. Kozikowski.



High or low selectivity: A new class of HDAC inhibitors bearing an isoxazole ring show high potency and selectivity for HDAC6 over HDAC1-3 and HDAC10, while unexpectedly showing little potency in blocking cell growth. These results suggest that HDAC6 inhibition alone is insufficient for disruption of cell growth, and that some degree of class 1 HDAC inhibition is required. The highly selective HDAC6I reported herein that are weakly cytotoxic may find use in cancer immune system reactivation.

Keywords

histone deacetylase inhibitors; hydroxamic acids; HDAC6 selectivity; pancreatic cancer; immune activation

Introduction

Histone acetylation is an important determinant of chromatin organization and gene expression, with the hyperacetylation of histones being associated with “open” euchromatic states of chromatin and hypoacetylation with a “closed” heterochromatic state.[1,2] Although the nuclear histones were identified as the initial target of the HDACs, it was discovered that some HDACs are also located in the cytoplasm where they are involved in the deacetylation of non-histone substrates including PCAF, p300, MyoD, p53, STAT3, p65 (NFκB), HSP90, and α-tubulin.[3] There are four classes of HDACs comprised of 18 different enzymes with a highly conserved catalytic domain. Class I HDACs are homologs of the yeast histone deacetylase RPD3 and are represented by HDAC1-3 and HDAC8; Class II HDACs (HDAC4-7, HDAC9, and HDAC10) share homology with the yeast histone deacetylase HDAC1; Class III HDACs are closely related to the NAD-dependent yeast Sir2 protein and are not affected by inhibitors of other HDACs; and the most recently described HDAC11 is the sole member of the Class IV HDACs.[2,4] HDACs exist in large multimolecular complexes and are recruited to promoter regions of genes in conjunction with other proteins. The acetylation status of histone and non-histone proteins has a wide range of effects on cellular function. In addition to influencing transcriptional activation and the DNA binding affinity of several transcriptional factors, acetylation also affects protein expression, stability, and degradation, and protein-protein interactions within the cell or nucleus.[5] For example, acetylation of α-tubulin results in the stabilization of microtubules, an event not conducive to microtubule reorganization, and acetylation of HSP90 inhibits its ATP-binding and chaperone association with its client proteins, including many proteins that are required for maintenance of cellular transformation.[6] The aberrant activation of HDACs in cancer has been suggested to be involved in the epigenetic silencing of tumor suppressor genes.[1a,7] Yet it is clear that based on their regulatory roles for non-histone targets, the effect of

HDACs in cancer goes far beyond the modulation of histone acetylation and gene expression. Consequently, there have been many efforts to develop HDAC inhibitors (HDACIs) and to investigate their effects on cancer cell growth. Several classes of HDACIs have been found to have potent antitumor activities, with some showing selectivity between cancer cells and normal cells.[8] Most of the known HDACIs are pan-inhibitors, therefore, the identification of compounds showing isoform selectivity is crucial to developing better tools for studying underlying mechanisms in different cancer cell lines. Previously, we have generated several series of HDACIs (Chart 1).[9] Treatment of pancreatic cancer cells with these compounds results in a G1 and/or G2 arrest and induction of apoptosis. In addition, our data indicated that some of these HDACIs cause loss of the DNA damage checkpoint kinase, Chk1, a known HSP90 client protein.[10] Our isozyme analysis indicates that some of these inhibitors, such as compounds **A**–**C**, show low nM potency toward HDAC6 and HDAC3 in vitro. Our objective was to increase the selectivity index in favor of HDAC6 by applying some simple modifications to the scaffold of these lead compounds that would not significantly affect their molecular properties. Such isoform-selective agents should be valuable tools in linking specific HDAC isoforms to tumorigenic activity, especially considering the number of controversial reports on the role of HDAC6-selective inhibitors in cancer.[11]

Results and Discussion

Synthesis of HDAC6-selective inhibitors

With the goal to improve selectivity of the previously synthesized ligands[9a,9c,12] toward HDAC6, we designed new series of compounds with consideration of the unique features of the HDAC6 binding site.[13] The general structure of these inhibitors consists of a hydroxamic acid function as a zinc-binding group (ZBG) attached to an isoxazole or benzene ring incorporated in the linker, and a “cap” group, which in the present work is a heteroaryl group bearing additional functionality. The choice of the terminal motifs was based on HDAC inhibitors previously explored by us.[9b,9c,13] The synthesis of compounds **1–5** is outlined in Scheme 1. Employing a standard procedure,[9c] 4-phenylthiazol-2-ylamine **(19a)** and its *m*- and *p*-nitrophenyl derivatives **(19b**,**c)** were coupled with either 5-hexynoic acid **(20a)** or 4-pentynoic acid **(20b)** to provide corresponding amides **21**. The acetylenic function in **21a,b** was transformed to isoxazole (compounds **23a,b**) by 1,3-dipolar cycloaddition with a nitrile oxide generated *in situ* from ethyl chlorooximinoacetate and a base.[14] The intermediates **21c,d** were reduced to the aniline derivatives **22a,b**, which after treatment with ethyl chloroformate or di-*tert*-butyl dicarbonate to provide corresponding carbamates, were converted to the isoxazoles **23c–e**. Lastly, reaction of **23** with freshly prepared hydroxylamine[15] generated the desired hydroxamic acids **1–5**.

The second series consisting of compounds **6–12** was prepared according to Scheme 2. Coupling of 5-arylisoxazole-3-carboxylic acids **24a–c** with butynylamine provided acetylenes **25a–c**,[9b] and the same procedure as outlined above was followed to generate esters **26a–c**. Deprotection and subsequent acylation of the amino group of **27** with pivaloyl, cyclohexanecarbonyl, and benzoyl chlorides, respectively, provided intermediate esters **28a–c**. Hydroxamate formation yielded the target compounds **6–12**.A small set of compounds

containing a benzene ring in the linker was synthesized as shown in Scheme 3. The precursor acids **24a,b** were coupled with methyl 4–(aminomethyl)benzoate in the presence of PyBOP to provide esters **29a,b**, which were further transformed into hydroxamates **13** and **14**. Alternatively, the *tert*-butoxycarbonyl group was removed, and the resulting amines **30a,b** were acylated with cyclohexanecarbonyl chloride. A two-step procedure was employed to convert intermediate esters **28a,b** into the corresponding hydroxamic acids **15** and **16** (Scheme 3). Carbazole (**32**) was alkylated with propargyl bromide, and the standard reaction sequence was applied to afford compound **17** (Scheme 3). The carbazole-based analog **18** with an elongated linker was made by coupling an intermediate acid formed from the ester **33** with methyl 4-(aminomethyl)benzoate, followed by the aminolysis of the methyl ester with hydroxylamine.

HDAC isoform inhibition

All compounds were tested against both Class I (1, 2, and 3) and Class II (6 and 10) HDACs, and their IC_{50} values are listed in Table 1. We first looked at effects of the linker length on isozyme selectivity. In the phenylthiazole series, compound **2** exhibiting an IC_{50} at HDAC6 of 31 nM, was found to be somewhat more potent than its longer-chain homologs, such as compound **1** (IC_{50} 81.8 nM) and the previously reported compound **A** (IC_{50} 67.7 nM). However, the shorter linker also resulted in increased potency at HDAC3 and HDAC10. In light of previously published results, the introduction of functional groups, such as NH-Boc and NH-COOEt, either in *meta* or *para* position of the benzene ring was hoped to increase the selectivity of these compounds, possibly due to the formation of additional hydrogen bonds with the enzyme. We therefore synthesized ligands **3**–**12**. The introduction of either of the aforementioned groups in *para* position resulted in a twofold improvement in potency (compounds **3** and **4**, IC_{50} at HDAC6 41.2 and 48.9 nM, respectively). However, selectivity against HDAC10 was diminished (HDAC10/6 selectivity ratio 1.0 and 6.6, respectively). On the contrary, the *meta*-(ethoxycarbonyl)amino substituent bestows significant selectivity on the inhibitor **5**. In the bis-isoxazole series, ligands **6** and **7** were equipotent at HDAC6, with the *meta*-NH-Boc substituted analog **7** being more selective against Class 1. Surprisingly, replacement of the Boc group by ethoxycarbonyl was found to be deleterious for activity (**8** *vs* **6**).

Next, we chose to investigate the incorporation of other bulky, electron-withdrawing substituents at an amino group in *meta* position, and synthesized compounds **9**–**12** (Table 1). In ligand **10**, the single-bonded oxygen is deleted, resulting in an amide rather than a urethane function. This replacement caused little reduction of potency or selectivity (IC_{50} at HDAC6 21.2 nM, selectivity index (SI) at least 1900). Compound **9**, bearing a free amino group, was reasonably active at HDAC6, but less selective over all other isoforms. Compound **11** containing a (cyclohexanecarbonyl)amino group showed equal potency to that of the NH-Boc analog **7**. Within this series, the benzoyl-substituted analog **12** was identified as the most potent HDAC6 inhibitor with an acceptable selectivity profile.

Because most of the hydroxamate-based HDACIs are very polar (i. e., have low CLogP values, which could affect cell permeability), we decided to increase their lipophilicity by replacing the heterocycle in the linker with a benzene ring. These structural alterations

resulted in ligands **13** and **14**, which stand out through their picomolar activity at HDAC6 and a selectivity of more than 3 orders of magnitude over HDAC2 and HDAC10, and at least 400-fold selectivity over HDAC1 and HDAC3. These compounds have a higher CLogP value (2.66) compared with their isoxazole analogs **6** and **7** (CLogP = 1.35). Compounds **15** and **16** were as potent as **6** and **7** at HDAC6, but had relatively low SIs. Lastly, the effect of replacement of the amidophenyl group by a more rigid and bulkier substituent, such as carbazole, on the pattern of isozyme selectivity was studied. The isoxazolylhydroxamate **17** was found to be a weak inhibitor at all isozymes tested. On the other hand, the elongated phenylhydroxamate **18** retained the HDAC6 potency of its *meta*-BocNH substituted analog **14**, and showed an improved selectivity over HDAC10.

The selectivity profile of HDACI **6** was more closely evaluated (Table S1 in the supporting information). This compound was found to be highly selective for HDAC6 over all other isoforms.

Growth inhibition of pancreatic cancer cells by new HDACIs

Several HDACIs from different series, demonstrating varying potencies and selectivity profiles in the isozyme assays, were examined for their capacity to decrease pancreatic cancer cell viability (Table 2). Previously, it has been shown that treatment of BxPC3, Panc04.03 and MiaPaCa-2 cells with HDACIs **A–C** led to a significant decrease in pancreatic cancer cell viability.[9b, 9c] Some of our new HDACIs listed in Table 2, such as **1**, **2**, **4**, and **5** showed antitumor activity similar to the reference compound **C** when tested in two pancreatic cancer cell lines, BxPC3 and L3.6pl. HDACIs **1**, **2**, **4**, and **5** demonstrated reasonable growth inhibition in at least one cancer cell line at low-micromolar concentrations, whereas the majority of the more selective HDAC6 inhibitors showed weak antiproliferative effects with GI_{50} values above 50 μM (Table 2). However, exceptions were seen with compound **13** that had GI_{50} values of 8 μM and 2 μM in BxPX3 and L3.6pl, and compound **18** that had GI_{50} values of 2.3 μM and 1.3 μM in BxPX3 and L3.6pl, respectively (Table 2).

Moreover, compounds **1**, **2**, and **4** effectively induced apoptosis as determined by Hoechst staining and PARP cleavage, a marker of apoptosis, in HDACI-treated L3.6pl pancreatic cancer cells 24 hours after the initiation of treatment (Figure 1). On the other hand, none of compounds **1**, **2**, or **4** induced apoptosis in BxPC3 pancreatic cancer cells (data not shown). It is worth noting that the nonselective HDACI, panobinostat, has recently been found to promote apoptosis and reduce tumor growth in the BxPC3 subcutaneous xenograft mouse model.[16] L3.6pl is characterized as a poorly differentiated, fast-growing pancreatic cell line with high tumorigenicity, while BxPC3 is characterized as more differentiated, slow-growing cell line with low tumorigenicity. Our results suggest that selection of HDACIs as effective pancreatic cancer therapies will likely depend on the precise histopathological features of the tumor.

Selective HDAC6 inhibitors do not suppress pancreatic cancer cell migration

Previous studies suggested that levels of HDAC6 but not its deacetylase activity affect both cell migration and cytotoxicity.[17] To gain a better understanding of the utility and

selectivity of HDAC6 inhibitors, PANC1 pancreatic cancer cells were treated with a panel of the new HDAC6-selective inhibitors, and with the non-selective inhibitors **B** and **C** for comparison. We demonstrated that treatment with either group leads to an accumulation of acetylated α-tubulin, a well-known target of HDAC6 (Figure 2A).[18] Pancreatic cancer cell migration was measured using the scratch-wound assay. Importantly, we found that compounds **B** and **C** demonstrated a significant reduction in PANC1 cell migration in this assay, compared to diluent-treated control cells (Figure 2B and Table 3). On the other hand, the HDAC6-selective inhibitors **6**, **9–13**, and **18** had only a minor effect on pancreatic cancer cell migration, in contrast to what has previously been reported for tubacin.[19]

In summary, we have demonstrated that simple structural alterations, such as the incorporation of a rigid aromatic ring into the linker and the introduction of a functional group in the cap residue, can be used to improve selectivity toward the HDAC6 isoform in this series of compounds. We were able to generate compounds possessing low-nanomolar to sub-nanomolar potency and high HDAC6 selectivity. Among those tested, the most selective compounds demonstrated only low antiproliferative activity against pancreatic cancer cell lines as compared to their non-selective analogs. Treatment of pancreatic cancer cells with the newly synthesized HDACIs leads to an accumulation of acetylated α-tubulin, but does not affect cell migration.

Conclusions

In conclusion, while inhibition of Class 1 HDACs has been proven to be essential for reducing the viability of pancreatic cancer cells, the HDAC6 contribution to pancreatic cancer cell viability remains to be investigated. However, the low growth inhibition induced by some of these compounds may make them more valuable as adjuvants in reactivation of the immune system through control of the expression levels of PD-L1.[20] This possibility is currently under study.

Experimental Section

All reactions were conducted under an argon atmosphere and stirred magnetically. Starting materials, reagents, and solvents were purchased from commercial suppliers and used without further purification unless otherwise stated. Microwave-assisted reactions were run in a Biotage Initiator microwave synthesizer. Unless stated otherwise, ^{1}H and ^{13}C NMR spectra were recorded on Bruker DPX-400 or AVANCE-400 spectrometers at 400 MHz and 100 MHz, respectively, with TMS as an internal standard. Standard abbreviations indicating multiplicity were used as follows: s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet, and br = broad. Mass spectra were measured in the ESI mode at an ionization potential of 70 eV with an LC-MS containing a Hewlett-Packard MSD. Analytical TLC was performed on Merck $60F_{254}$ silica gel glass plates, layer thickness 250 μm. Preparative TLC was performed on Analtech silica gel GF plates, layer thickness 1 mm. For flash chromatography, silica gel of 230 400 mesh particle size was used. Analytical HPLC was carried out on an ACE 3AQ C_{18} column (150 × 4.6 mm, particle size 3 μm; flow rate = 2.0 mL/min; from 10% acetonitrile in water to 50% in 10 min and to 100% acetonitrile in 5 min,

both solvents containing 0.05% TFA (Method A), or from 30% acetonitrile in water to 100% acetonitrile in 15 min, both solvents containing 0.05% TFA (Method B)).

General Procedures for Amide Coupling

Method A—To a solution of an aminothiazole (1 eq) and a carboxylic acid (1.1 eq) in dry CH_2Cl_2 (10 mL/mmol) at ambient temperature were added EDCI (1.5 eq) and DMAP (0.1 eq), and the resulting reaction mixture was stirred for 12 h. Upon reaction completion as indicated by TLC, the reaction mixture was diluted with 50 mL of CH_2Cl_2, and then washed with saturated aqueous $NaHCO_3$ (50 mL) and brine (50 mL), dried over anhydrous Na_2SO_4, and concentrated under vacuum. The residue was purified by flash chromatography to afford the product.

Method B—To a mixture of 4-(3- or 4-nitrophenyl)thiazol-2-ylamine (1 eq) and 5-hexynoic acid (1 eq) in anhydrous pyridine (4 mL/mmol) at −15 °C was added phosphorus oxychloride (1 eq) dropwise with vigorous stirring. After 45 min of stirring at −15 °C, the reaction mixture was allowed to warm to ambient temperature, heated for 1 h at 60 °C, and stirred overnight at ambient temperature. The reaction was quenched by addition of ice water, and the mixture was extracted with EtOAc (3 X 50 mL). The organic layer was washed with brine (50 mL), dried over Na_2SO_4, and concentrated under vacuum. The residue was purified by CombiFlash chromatography (EtOAc – hexane; 25% to 40%) to provide the amide.

Method C—To a solution of the carboxylic acid (1 eq) in DMF (0.5 mL/mmol) was added PyBOP (1 eq), and the mixture was stirred for 20 min at ambient temperature. But-3-ynylamine hydrochloride (1.05 eq) and Et_3N (3 eq) were added subsequently, and the reaction mixture was stirred for 1 h. Upon completion of the reaction as ascertained by TLC, Et_3N was evaporated, and the residue was purification by HPLC.

General Procedure for the 1,3-Dipolar Cycloaddition with a Nitrile Oxide (Method D)

To a stirred solution of an acetylene (1 eq) and Et_3N (15 eq) in dry THF (15 mL/mmol) at ambient temperature was added dropwise over 36 h by syringe pump a solution of ethyl chlorooximinoacetate (15 eq) in 20 mL of THF. The white solids formed were separated by filtration and washed with ethyl acetate (200 mL). The combined organic phases were concentrated under vacuum. The residue was purified by column chromatography.

General Procedure for Hydroxamic Acid Formation (Method E)

To a stirred solution of the ester (0.1 g, 1 eq) in CH_2Cl_2 (15 mL/mmol) was added at 0 °C a freshly prepared solution of NH_2OH. After 15 min, the reaction mixture was diluted with 30 mL of CH_2Cl_2, and the product was extracted into H_2O. The water phase was acidified with 1N HCl to pH approx. 4. The precipitate was filtered off and washed with H_2O and hexane to give the hydroxamic acid, which was purified by HPLC.

Preparation of NH_2OH: To a stirring solution of $NH_2OH.HCl$ (1.0 g, 10 wt. eq) in MeOH (10 mL) was added portionwise at 0 °C a solution of KOH (1.0 g) in MeOH (4.0 mL). The

resulting mixture was filtered, and the obtained solution of NH_2OH in MeOH was used in a reaction with an ester.

General Procedure for the Reduction of Nitro Groups (Method F)

A mixture of the nitro compound (1 eq) and NH_4Cl (2 eq) in H_2O-EtOH (3:5, 17 mL/mmol) was heated to reflux, and iron filings (9 eq) and AcOH (1 mL/mmol) were added subsequently. The reaction mixture was refluxed for 2 h, and then allowed to cool to ambient temperature. It was diluted with EtOAc (100 mL), washed with H_2O (40 mL) and brine (40 mL), dried over Na_2SO_4, and evaporated. The residue was purified by CombiFlash chromatography.

N-(4-Phenylthiazol-2-yl)pent-4-ynamide (21b)—General Procedure A was used to couple 2-amino-4-phenylthiazole (**19a**) (1.01 g, 5.67 mmol) and 4-pentynoic acid (**20b**) (0.61 g, 6.24 mmol). The residue was purified by column chromatography (EtOAc – hexane; 30%) to afford **21b** (1.24 g, 85%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.20 (m, 2H), 2.40 (m, 2 H), 7.18 (s, 1H), 7.38–7.47 (m, 3H), 7.85 (m, 2H), 11.24 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.0, 34.4, 69.4, 82.0, 108.0, 126.3, 128.3, 128.9, 134.2, 149.7, 169.2.

5-[2-[*N*-(4-Phenylthiazol-2-yl)carbamoyl]ethyl]isoxazole-3-carboxylic Acid Ethyl Ester (23b)—The title compound was synthesized from **21b** (0.56 g, 2.18 mmol) and ethyl chlorooximinoacetate (4.92 g, 32.7 mmol) according to General Procedure D. The residue was purified by column chromatography (EtOAc – hexane; 25% to 50%). Crystallization from 10% EtOAc – hexane provided the pure ester **23b** (0.41 g, 50%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.44 (t, J = 8.0 Hz, 2H), 2.21 (t, J = 8.0 Hz, 3H), 2.93 (t, J = 8.0 Hz, 2H), 4.45 (q, J = 8.0 Hz, 2H), 6.14 (s, 1H), 7.17 (s, 1H), 7.28–7.43 (m, 3H), 7.49 (d, J = 8 Hz, 2H) (m, 2H), 12.18 (s, 1H); ^{13}C NMR (100 MHz, CDCl3): δ = 14.1, 21.4, 32.3, 62.1, 101.9, 108.3, 126.4, 128.6, 129.0, 134.2, 149.5, 156.2, 159.9, 150.0, 168.9, 172.9.

5-[2-[*N*-(4-Phenylthiazol-2-yl)carbamoyl]ethyl]isoxazole-3-carbohydroxyamic Acid (2)—The title compound was synthesized from the ester **23b** (0.10 g, 0.27 mmol) according to General Procedure E. Yield: 0.05 g, 50%. A sample for biological testing was purified by HPLC. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 2.92 (t, J = 7.2 Hz, 2H), 3.16 (t, J = 7.2 Hz, 3H), 6.58 (s, 1H), 7.31–7.45 (m, 3H), 7.90 (d, J = 7.4 Hz, 2H), 9.35 (s, 1H), 11.49 (s, 1H), 12.38 (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 21.8, 31.1, 32.5, 191.1, 108.4, 126.0, 128.2, 129.1, 134.7, 149.2, 156.6, 157.9, 158.1, 170.1, 173.8; HPLC purity: 97.4%; HRMS-FAB: m/z $[M+H]^+$ calcd for $C_{16}H_{14}N_4O_4S$: 359.0808, found: 359.0820.

5-[2-[*N*-(4-Phenylthiazol-2-yl)carbamoyl]propyl]isoxazole-3-carboxylic Acid Ethyl Ester (23a)—The title compound was synthesized from 2-amino-4-phenylthiazole according to General Procedures A and D in 32% yield over two steps. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.41 (t, J = 8.0 Hz, 2H), 1.96–2.03 (t, J = 8.0 Hz, 3H), 2.93 (m, 2H), 2.21 (t, J = 7.5 Hz, 2H), 2.71 (t, J = 7.5 Hz, 2H), 4.46 (q, J = 8.0 Hz, 2H), 6.30 (s, 1H), 7.34 (s, 1H), 7.36–7.47 (m, 3H), 7.84 (d, J = 8.4 Hz, 2H), 10.62 (s, 1H).

5-[3-[*N*-(4-Phenylthiazol-2-yl)carbamoyl]propyl]isoxazole-3-carbohydroxamic Acid (1)—The title compound was synthesized from the ester **23a** (0.10 g, 0.27 mmol) according to General Procedure E and purified by HPLC (Method A). Yield: (0.053 g, 54%). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 2.02 (m, 2H), 2.54 (t, J = 7.4 Hz, 2H), 2.86 (t, J = 7.4 Hz, 3H), 3.30 (br s, 1H), 6.58 (s, 1H), 7.30–7.45 (m, 3H), 7.88 (d, J = 7.4 Hz, 2H), 9.35 (s, 1H), 12.28 (s, 1H); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 22.8, 25.7, 34.3, 102.3, 108.3, 124.6, 126.0, 128.1, 129.1, 134.7, 149.1, 158.2, 171.2, 171.5; HPLC purity: 98.2%; HRMS-FAB: m/z [M+H]$^+$ calcd for $C_{17}H_{15}N_4O_4S$: 373.0965, found: 373.0978.

***N*-[4-(4-Nitrophenyl)thiazol-2-yl]hex-5-ynamide (21c)—**General Procedure B was used to couple 4-(4-nitrophenyl)thiazol-2-ylamine (**19b**) (0.50 g, 2.26 mmol) and 5-hexynoic acid (**20a**) (0.25 g, 2.26 mmol). The residue was purified by flash chromatography (EtOAc – hexane; 25% to 40%) to provide the acetylene **21c** (0.61 g, 84%).

***N*-[4-(4-Aminophenyl)thiazol-2-yl]hex-5-ynamide (22a)—**The title compound was obtained from the nitro compound **21c** (0.61 g, 1.90 mmol) and NH_4Cl (0.20 g, 3.8 mmol) according to General Procedure F and purified by flash chromatography (EtOAc – hexane; 30% to 50%). Yield: 0.39 g (71%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): δ = 1.75 (m, 2H), 2.20 (m, 2H), 2.56 (m, 2H), 2.82 (s, 1H), 5.23 (br s, 2H), 6.56 (d, J = 9.0 Hz, 2H), 7.16 (s, 1H), 7.53 (d, J = 9.0 Hz, 2H), 12.15 (s, 1H).

5-[3-[*N*-[4-[4-(*tert*-Butoxycarbonylamino)phenyl]thiazol-2-yl]carbamoyl]propyl]isoxazole-3-carboxylic Acid Ethyl Ester (23c)

a. Synthesis of *N*-[4-[2-(hex-5-ynoylamino)thiazol-4-yl)phenyl]carbamic acid *tert*-butyl ester. A mixture of the amine **22a** (0.20 g, 0.70 mmol) and Boc_2O (0.20 g, 0.91 mmol) in 2 mL of toluene was placed in a microwave oven and heated for 20 min at 120 °C. The solvent was evaporated, and the residue was purified by flash chromatography (EtOAc – hexane; 30% to 50%) to provide the intermediate urethane (0.21 g, 79%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.25 (m, 9H), 1.99 (m, 2H), 2.30 (m, 2H), 2.01 (s, 1H), 2.58 (m, 2H), 7.05 (s, 1H), 7.40 (m, 2H), 7.74 (d, J = 9.0 Hz, 2H).

b. The title compound **23c** was synthesized from the above intermediate (0.21 g, 0.54 mmol) and ethyl chlorooximinoacetate (1.23 g, 8.17 mmol) according to General Procedure D and purified by flash chromatography (EtOAc – hexane; 25% to 70%). Yield: 0.12 g (44 %). ^{1}H NMR (400 MHz $CDCl_3$): δ = 1.41 (t, J = 7.1 Hz, 3H), 1.52 (s, 9H), 1.98 (m, 2H), 2.21 (m, 2H), 2.69 (m, 2H), 4.44 (q, J = 7.1 Hz, 2H), 6.37 (s, 1H), 7.04 (s, 1H), 7.33 (m, 2H), 7.64 (m, 2H), 11.50 (br s, 1H).

***N*-[4-[2-[4-[3-(*N*-Hydroxycarbamoyl)isoxazol-5-yl]butyrylamino]thiazol-4-yl]phenyl]carbamic Acid *tert*-Butyl Ester (3)—**The title compound was synthesized from the ester **23c** (0.12 g, 0.24 mmol) according to General Procedure E. Yield: 0.025 g (24.5%). ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.41 (s, 9H), 1.93 (m, 2H), 2.44 (m, 2H), 2.79 (m, 2H), 6.54 (s, 1H), 7.37–7.44 (m 3H), 7.89 (d, J = 5.1 Hz, 2H), 9.37 (s, 1H), 11.40 (br s, 1H), 12.19 (s, 1H); ^{13}C NMR (100 MHz [D$_6$]DMSO): δ = 22.8, 25.6, 28.5, 34.3, 79.6,

101.1, 106.6, 118.5, 126.4, 128.7, 139.5, 149.1, 153.1, 156.6, 157.8, 158.1, 171.1, 174.3; HPLC purity: 97.9%; HRMS-FAB: *m/z* [*M*+H]$^+$ calcd for $C_{22}H_{25}N_5O_6S$: 488.1598, found: 488.1618.

5-[3-[*N*-[4-[4-(Ethoxycarbonylamino)phenyl]thiazol-2-yl]carbamoyl]propyl]isoxazole-3-carboxylic Acid Ethyl Ester (23d)

a. Synthesis of *N*-[4-[2-(hex-5-ynoylamino)thiazol-4-yl]phenyl]carbamic acid ethyl ester. To a mixture of the amine **22a** (0.18 g, 0.64 mmol) and Et_3N (0.17 mL, 1.3 mmol) in THF (5 mL) was added ethyl chloroformate (0.08 g, 0.77 mmol) at ambient temperature. The resulting mixture was stirred for 1 h. Upon completion of the reaction as ascertained by TLC, EtOAc (20 mL) was added. The organic phase was washed with water (20 mL) and brine (20 mL), dried over Na_2SO_4, and evaporated. The residue was purified by flash chromatography (EtOAc – hexane; 25% to 100%) to provide the intermediate urethane (0.23 g, 99%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.23 (t, *J* = 7.1 Hz, 3H), 1.81 (m, 2H), 2.22 (m, 2H), 2.55 (m, 2H), 2.82 (m, 1H), 4.14 (q, *J* = 7.1 Hz, 2H), 7.44 (s, 1H), 7.52 (d, *J* = 8.5 Hz, 2H), 7.77 (d, *J* = 8.5 Hz, 2H), 9.72 (s, 1H), 12.2 (s, 1H); ^{13}C NMR (400 MHz, [D_6]DMSO): δ = 14.9, 17.7, 23.9, 34.1, 60.6, 72.2, 84.2, 106.7, 118.5, 126.5, 129.0, 139.2, 149.0, 153.9, 158.1, 171.3.

b. The title compound **23d** was synthesized from the above intermediate (0.23 g, 0.64 mmol) and ethyl chlorooximinoacetate (0.97 g, 6.43 mmol) according to General Procedure D and purified by flash chromatography (EtOAc – hexane; 30% to 50%). Yield: (0.20 g, 65%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.26 (m, 3H), 2.01 (m, 2H), 2.52 (m, 2H), 2.89 (m, 2H), 4.33 (m, 2H), 6.73 (s, 1H), 7.45 (s, 1H), 7.49 (d, *J* = 8.5 Hz, 2H), 7.77 (d, *J* = 8.5 Hz, 2H), 9.55 (s, 1H), 9.72 (s, 1H), 12.2 (s, 1H), 13.2 (s, 1H).

***N*-[4-[2-[4-[3-(*N*-Hydroxycarbamoyl)isoxazol-5-yl]butyrylamino]thiazol-4-yl]phenyl]carbamic Acid Ethyl Ester (4)—**The title compound was synthesized from the ester **23d** (0.20 g, 0.42 mmol) according to General Procedure E. Yield: (0.075 g, 38%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.25 (t, *J* = 7.1 Hz, 3H), 2.02 (m, 2H), 2.54 (m, 2H), 2.86 (m, 2H), 4.14 (d, *J* = 7.1 Hz, 3H), 6.61 (s, 1H), 7.46 (s, 1H), 7.50 (d, *J* = 7.1 Hz, 2H), 7.78 (d, *J* = 7.1, 2H), 9.72 (s, 1H), 11.48 (s, 1H), 12.27 (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 14.9, 22.8, 25.7, 31.1, 34.2, 60.6, 101.1, 106.8, 118.5, 126.5, 129.0, 139.2, 149.0, 153.9, 157.9, 158.1, 171.1, 174.3; HPLC purity: 98.1%; HRMS-FAB: *m/z* [*M*+H]$^+$ calcd for $C_{20}H_{21}N_5O_6S$: 460.1285, found: 460.1300.

5-[2-[N-[4-[3-(Ethoxycarbonylamino]phenyl)thiazol-2-yl]carbamoyl]ethyl]isoxazole-3-carboxylic Acid Ethyl Ester (23e)

a. Synthesis of N-[3-[2-(pent-4-ynoylamino)thiazol-4-yl]phenyl]carbamic acid ethyl ester. To a solution of the amine **22b** (0.16 g, 0.60 mmol) and Et_3N (0.24 mL, 1.80 mmol) in THF (3 mL) was added dropwise at 0 °C a

solution of ethyl chloroformate (0.07 mL, 0.56 mmol) in THF (1 mL). The reaction mixture was stirred for 20 min, diluted with EtOAc (25 mL), washed with water (20 mL), aqueous $NaHCO_3$ (20 mL), and brine (20 mL), dried over Na_2SO_4, and evaporated. The residue was purified by preparative TLC (EtOAc – hexane; 30%) to provide the intermediate urethane (0.10 g, 48%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.42 (t, J = 7.0 Hz, 3H), 2.84 (t, J = 7.2 Hz, 2H), 2.98 (m, 1H), 4.31 (q, J = 7.0 Hz, 2H), 7.47 (m, 2H), 7.66 (m, 2H), 8.28 (s, 1H), 9.84 (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 18.9, 19.7, 39.1, 52.6, 53.3, 65.3, 76.8, 88.4, 113.2, 120.8, 122.9, 125.0, 134.1, 140.0, 144.7, 144.8, 154.0, 158.7, 162.8, 174.9.

b. The title compound **23e** was synthesized from the above intermediate (0.08 g, 0.23 mmol) and ethyl chlorooximinoacetate (0.35 g, 2.32 mmol) according to General Procedure D and purified by preparative TLC (EtOAc – hexane; 20%). Yield: 0.072 g (65%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.41 (t, J = 8.0 Hz, 3H), 2.65 (t, J = 7.3 Hz, 2H), 3.10 (t, J = 7.3 Hz, 2H), 4.23 (m, 2H), 4.44 (q, J = 8.0 Hz, 2H), 6.33 (s, 1H), 7.12 (m, 2H), 7.29 (m, 2H), 7.43 (m, 1H), 7.87 (br s, 1H), 11.64 (br s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.1, 21.6, 32.6, 62.1, 102.0, 108.5, 121.1, 129.5, 134.8, 138.6, 149.0, 153.9, 156.3, 159.2, 160.0, 169.1, 173.2.

***N*-[3-[2-[3-[3-(*N*-Hydroxycarbamoyl)isoxazol-5-yl]propionylamino]thiazol-4-yl]phenyl]carbamic Acid Ethyl Ester (5)—**The title compound was synthesized from the ester **23e** (0.072 g, 0.163 mmol) according to General Procedure E. Yield: 0.035 g (50%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.30 (t, J = 7.0 Hz, 3H), 2.95 (t, J = 7.3 Hz, 2H), 3.25 (t, J = 7.3 Hz, 2H), 4.19 (q, J = 7.0 Hz, 2H), 6.60 (s, 1H), 7.28–7.32 (m, 3H), 7.55 (d, J = 7.0 Hz, 1H), 8.07 (s, 1H), 9.27 (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 14.9, 21.9, 32.5, 60.6, 102.3, 108.5, 116.1, 118.2, 120.2, 129.4, 135.2, 140.0, 149.3, 153.9, 157.4, 158.0, 161.3, 170.1, 174.6; HPLC purity: 97.6%; HRMS-FAB: m/z [M+H]$^+$ calcd for $C_{19}H_{19}N_5O_6S$: 446.1128, found: 446.1141.

***N*-[4-[3-(*N*-But-3-ynylcarbamoyl)isoxazol-5-yl]phenyl]carbamic Acid *tert*-Butyl Ester (25a)—**General Procedure C was used to couple the acid **24a** (0.60 g, 1.97 mmol)) and but-3-ynylamine hydrochloride (0.22 g, 2.08 mmol). The crude product was purified by HPLC to afford acetylene **25a** (0.126 g, 18%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.45 (s, 9H), 1.98 (t, J = 2.6 Hz, 1H), 2.45 (m, 2H), 3.55 (m, 2H), 6.58 (s, 1H), 6.79 (s, 1H), 7.07 (m, 1H), 7.18 (s, 1H), 7.42 (d, J = 8.6 Hz, 2H), 7.63 (d, J = 8.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 19.3, 28.2, 30.9, 38.0, 70.4, 80.89, 81.2, 98.1, 118.3, 121.3, 126.8, 140.6, 12.2, 158.9, 159.0, 171.4.

5-[2-[[5-[4-(*tert*-Butoxycarbonylamino)phenyl]isoxazole-3-carbonyl]amino]ethyl]isoxazole-3-carboxylic Acid Ethyl Ester (26a)—The title compound was synthesized from the acetylene **25a** (0.126 g, 0.36 mmol) and ethyl chlorooximinoacetate (1.00 g, 6.60 mmol) according to General Procedure D and purified by flash chromatography (EtOAc – hexane; 25% to 70%). Yield: 0.105 g (63%). ^{1}H NMR (400

MHz, $CDCl_3$): δ = 1.34 (t, J = 7.2 Hz, 3H), 1.47 (s, 9H), 3.14 (m, 2H), 3.75 (t, J = 6.8 Hz, 2H), 4.37 (q, J = 7.2 Hz, 2H), 6.51 (s, 1H), 6.80 (s, 1H), 7.46 (d, J = 8.5 Hz, 2H), 7.63 (d, J = 8.5 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 13.9, 26.7, 28.1, 37.1, 62.1, 97.8, 102.5, 118.3, 120.7, 126.7, 141.1, 152.7, 156.4, 158.6, 159.6, 159.9, 171.5, 172.1.

_N_-[4-[3-[_N_-[2-[3-(_N_-Hydroxycarbamoyl)isoxazol-5-yl]ethyl]carbamoyl]isoxazol-5-yl]phenyl]carbamic Acid _tert_-Butyl Ester (6)— The title compound was synthesized from the ester **26a** (0.105 g, 0.223 mmol) according to General Procedure E and purified by HPLC (Method A). Yield: 0.025 g (24.5%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.42 (s, 9H), 3.04 (m, 2H), 3.52 (m, 2H), 6.58 (s, 1H), 7.11 (s, 1H), 7.56 (d, J = 8.4 Hz, 2H), 7.65 (d, J = 8.4 Hz, 2H), 8.92 (m, 1H), 9.28 (br s, 1H), 9.63 (s, 1H), 11.4 (s, 1H); ^{3}C NMR (100 MHz, [D_6]DMSO): δ = 26.4, 28.4, 37.2, 80.0, 98.8, 101.6, 118.5, 120.3, 127.0, 142.3, 153.0, 156.6, 157.8, 159.1, 159.7, 170.9, 172.5; HPLC purity: 97.6%; HRMS-FAB: m/z [M+H]$^+$ calcd for $C_{21}H_{23}N_5O_7S$: 458.1670, found: 458.1669.

_N_-[3-[3-(_N_-But-3-ynylcarbamoyl)isoxazol-5-yl]phenyl]carbamic Acid _tert_-Butyl Ester (25b)—General Procedure C was used to couple the acid **24b** (1.30 g, 4.30 mmol) and but-3-ynylamine hydrochloride (0.50 g, 4.73 mmol). The crude product was purified by HPLC to afford acetylene **25b** (0.96 g, 63%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.54 (s, 9H), 2.07 (t, J = 2.6 Hz, 1H), 2.54 (m, 2H), 3.67 (dd, J = 6.4, 12.8 Hz, 2H), 6.67 (s, 1H), 6.98 (s, 1H), 7.19 (m, 1H), 7.40–7.47 (m, 3H), 7.88 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 19.0, 27.9, 30.5, 37.6, 70.0, 80.5, 80.7, 99.0, 99.4, 115.2, 120.0, 120.1, 127.0, 129.4, 138.8, 152.1, 158.5, 171.0.

5-[2-[[5-[3-(_tert_-Butoxycarbonylamino)phenyl]isoxazole-3-carbonyl]amino]ethyl]isoxazole-3-carboxylic Acid Ethyl Ester (26b)—The title compound was synthesized from the acetylene **25b** (0.96 g, 2.70 mmol) and ethyl chlorooximinoacetate (4.0 g, 27.0 mmol) according to General Procedure D and purified by flash chromatography (EtOAc – hexane; 25% to 40%). Yield: 0.93 g (73%). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.29 (t, J = 7.1 Hz, 3H), 1.55 (s, 9H), 3.21 (t, J = 6.8 Hz, 2H), 3.86 (q, J = 6.8 Hz, 2H), 4.43 (q, J = 7.1 Hz, 2H), 6.55 (s, 1H), 6.69 (s, 1H), 6.99 (s, 1H), 7.13 (m, 1H), 7.43 (m, 3H), 7.89 (s, 1H).

_N_-[3-[3-[_N_-[2-[3-(_N_-Hydroxycarbamoyl)isoxazol-5-yl]ethyl]carbamoyl]]isoxazol-5-yl]phenyl]carbamic Acid _tert_-Butyl Ester (7)— The title compound was synthesized from the ester **26b** (0.10 g, 0.21 mmol) according to General Procedure E and purified by HPLC (Method A). Yield: 0.02 g (20%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.42 (s, 9H), 3.04 (m, 2H), 3.54 (m, 2H), 6.59 (s, 1H), 7.17 (s, 1H), 7.35 (d, J = 7.8 Hz, 2H), 7.45 (m, 2H), 8.01 (s, 1H), 8.99 (t, J = 5.5 Hz, 1H), 9.28 (s, 1H), 9.54 (s, 1H), 11.42 (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 26.4, 28.5, 31.1, 37.2, 79.9, 100.1, 101.6, 114.9, 120.2, 120.8, 127.0, 130.2, 140.8, 153.2, 156.6, 157.8, 158.9, 159.8, 170.9, 172.5; HPLC purity: 97.8%; HRMS-FAB: m/z [M+Na]$^+$ calcd for $C_{21}H_{23}N_5NaO_7$: 480.1490, found: 480.1505.

[4-[3-(But-3-ynylcarbamoyl)-isoxazol-5-yl]phenyl]carbamic Acid Ethyl Ester (25c)—General Procedure C was used to couple the acid **24c** (0.8 g, 2.9 mmol) and but-3-

ynylamine hydrochloride (0.34 g, 3.19 mmol). The crude product was purified by flash chromatography to afford acetylene **25c** (0.94 g, 99%).

5-[2-[[5-[4-(Ethoxycarbonylamino)phenyl]isoxazole-3-carbonyl]amino]ethyl]isoxazole-3-carboxylic Acid Ethyl Ester (26c)—The title compound was synthesized from the acetylene **25c** (0.94 g, 2.87 mmol) and ethyl chlorooximinoacetate (4.35 g, 28.7 mmol) according to General Procedure D and purified by flash chromatography (EtOAc – hexane; 40% to 100%). Yield: 0.36 g (28%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.29 (t, J = 6.5 Hz, 3H), 1.43 (t, J = 7.1 Hz, 3H), 3.21 (t, J = 6.5 Hz, 2H), 3.86 (q, J = 7.1 Hz, 2H), 4.42 (q, J = 7.1 Hz, 2H), 6.56 (s, 1H), 6.78 (s, 1H), 6.90 (d, J = 4.4 Hz, 1H), 7.07 (t, J = 5.6 Hz, 1H), 7.52 (d, J = 8.4 Hz, 1H), 7.65 (d, J = 8.4 Hz, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 14.4, 27.0, 37.2, 61.6, 62.1, 98.1, 102.7, 118.5, 121.5, 126.9, 140.3, 153.1, 156.5, 158.6, 159.3, 159.8, 171.4, 171.8.

***N*-[4-[3-[*N*-[2-[3-(*N*-Hydroxycarbamoyl)isoxazol-5-yl]ethyl]carbamoyl]isoxazol-5-yl]phenyl]carbamic Acid Ethyl Ester (8)—**The title compound was synthesized from the ester **26c** (0.15 g, 0.34 mmol) according to General Procedure E and purified by HPLC (Method A). Yield: 0.065 g (44%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.17 (t, J = 7.1 Hz, 3H), 3.03 (m, 2H), 3.53 (m, 2H), 4.09 (q, J = 6.5 Hz, 2H), 6.58 (s, 1H), 7.12 (s, 1H), 7.54 (d, J = 8.7 Hz, 2H), 7.76 (d, J = 8.7 Hz, 2H), 8.93 (t, J = 5.7 Hz, 1H), 9.27 (s, 1H), 9.90 (s, 1H), 11.4 (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 14.8, 26.4, 37.2, 60.9, 98.9, 101.6, 118.6, 120.6, 127.1, 142.0, 153.8, 156.6, 157.8, 159.1, 159.7, 170.9, 172.5; HPLC purity: 97.6%; HRMS-FAB: m/z $[M+H]^+$ calcd for $C_{19}H_{19}N_5O_7$: 430.1357, found: 430.1374.

5-[2-[[5-(3-Aminophenyl)isoxazole-3-carbonyl]amino]ethyl]isoxazole-3-carboxylic Acid Ethyl Ester (27)—A solution of *tert*-butyl ester **26b** (0.30 g, 0.64 mmol) in a mixture of CH_2Cl_2 and trifluoroacetic acid (3:1, 4 mL) was stirred at ambient temperature for 48 h. The solvents were evaporated, and the residue was purified by flash chromatography (EtOAc – hexane; 50% to 100%) to provide the amine **27** (0.12 mg, 50%).^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.30 (t, J = 7.0 Hz, 3H), 3.13 (m, 2H), 3.61 (m, 2H), 4.34 (q, J = 7.0 Hz, 2H), 6.70 (dd, J = 5.5 Hz, 8.0 Hz, 1H), 6.78 (s, 1H), 7.04–7.19 (m, 4H), 9.02 (t, J = 5.5 Hz, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 14.3, 26.5, 37.1, 62.1, 99.4, 102.8, 110.6, 113.9, 116.8, 127.1, 130.2, 149.5, 156.4, 159.1, 159.6, 159.9, 171.7, 173.7.

5-(3-Aminophenyl)-*N*-[2-[3-(*N*-hydroxycarbamoyl)isoxazol-5-yl]ethyl]isoxazole-3-carboxamide (9)—The title compound was synthesized from the ester **27** (0.06 g, 0.16 mmol) according to General Procedure E and purified by HPLC (Method B). Yield: 0.02 g (34%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 3.04 (m, 2H), 3.53 (m, 2H), 5.36 (br s, 2H), 6.58 (s, 1H), 6.65 (d, J = 8.0 Hz, 1H), 6.98 (m, 2H), 7.06 (s, 1H), 7.10 (m, 2H), 8.95 (t, J = 5.4 Hz), 9.28 (s, 1H), 11.42 (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 26.4, 37.2, 99.4, 101.6, 110.6, 113.9, 116.7, 127.1, 130.2, 149.6, 156.6, 157.8, 159.1, 159.6, 171.7, 172.5; HPLC purity: 97.1%; HRMS-FAB: m/z $[M+H]^+$ calcd for $C_{16}H_{15}N_5O_5$: 358.1146, found: 358.1156.

5-[2-[[5-[3-[(2,2-Dimethylpropionyl)amino]phenyl]isoxazole-3-carbonyl]amino]ethyl]isoxazole-3-carboxylic Acid Ethyl Ester (28a)—To a mixture of the amine 27 (0.06 g, 0.16 mmol) and Et_3N (0.05 mL, 0.36 mmol) in CH_2Cl_2 (1 mL) was added pivaloyl chloride (22 μL, 0.18 mmol) at 0 °C. The resulting mixture was warmed to ambient temperature and stirred for 3 h. The solvent was evaporated, and the residue was purified by flash chromatography (EtOAc – hexane; 40% to 100%) to provide the product (0.054 g, 73%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.42 (t, J = 7.0 Hz, 3H), 3.22 (m, 2H), 3.86 (m, 1H), 4.45 q, J = 7.0 Hz, 2H), 6.56 (s, 1H), 7.45–7.54 (m, 3H), 7.66 (d, J = 8.0 Hz, 1H), 8.04 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.1, 27.0, 27.5, 37.2, 39.7, 62.1, 99.4, 102.7, 117.1, 121.5, 122.0, 127.3, 129.8, 138.8, 158.6, 159.1, 159.8, 171.3, 171.8, 176.8.

5-[2-[[5-[3-[(2,2-Dimethylpropionyl)amino]phenyl]isoxazole-3-carbonyl]amino]ethyl]isoxazole-3-carbohydroxyamic Acid (10)—The title compound was synthesized from the ester **28a** (0.054 g, 0.118 mmol) according to General Procedure E and purified by HPLC (Method A). Yield: 0.022 g (42%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.18 (s, 9H), 3.05 (m, 2H), 3.54 (m, 2H), 6.59 (s, 1H), 7.21 (s, 1H), 7.40 (t, J = 7.8 Hz, 1H), 7.56 (d, J = 7.8 Hz, 1H), 7.76 (d, J = 7.8 Hz, 1H), 8.19 (s, 1H), 9.01 (t, J = 5.0 Hz, 1H), 9.28 (s, 1H), 9.37 (s, 1H), 11.42 (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 26.4, 27.5, 37.2, 100.1, 101.6, 117.2, 121.2, 122.7, 126.8, 130.0, 140.6, 156.6, 157.8, 158.9, 159.8, 170.9, 172.5, 177.2; HPLC purity: 99.9%; HRMS-FAB : m/z [M+H]$^+$ calcd for $C_{21}H_{23}N_5O_6$: 442.1721, found: 442.1738.

5-[2-[[5-[3-[(Cyclohexanecarbonyl)amino]phenyl]isoxazole-3-carbonyl]amino]ethyl]isoxazole-3-carboxylic Acid Ethyl Ester (28b)—To a mixture of the amine 27 (0.06 g, 0.16 mmol) and Et_3N (0.06 mL, 0.48 mmol) in CH_2Cl_2 (1 mL) was added cyclohexanecarbonyl chloride (0.026 mL, 0.19 mmol) at 0 °C. After stirring 3 h at 0 °C, water (2 mL) was added, and the mixture was extracted with CH_2Cl_2 (3 X 5 mL). The combined organic layers were dried over Na_2SO_4 and evaporated. The residue (0.08 g) was used in next step without additional purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.97–1.47 (m, 8H), 1.47–1.84 (m, 5H), 2.35 (m, 1H), 3.14 (m, 2H), 3.62 (m, 2H), 4.38 (m, 2H), 6.80 (s, 1H), 7.26 (s, 1H), 7.47 (t, J = 7.7 Hz, 1H), 7.60 (d, J = 7.7 Hz, 1H), 7.69 (d, J = 7.7 Hz, 1H), 8.25 (s, 1H), 9.07 (m, 1H), 10.05 (s, 1H).

5-[3-[(Cyclohexanecarbonyl)amino]phenyl]-*N*-[2-[3-(*N*-hydroxycarbamoyl)isoxazol-5-yl]ethyl]isoxazole-3-carboxamide (11)—The title compound was synthesized from the ester **26b** (0.08 g, 0.17 mmol) according to General Procedure E and purified by HPLC (Method A). Yield: 0.022 g (25%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.11–1.44 (m, 5H), 1.58 (m, 2H), 1.74 (m, 2H), 2.28 (m, 1H), 3.05 (m, 2H), 3.54 (m, 2H), 6.59 (s, 1H), 7.20 (m, 1H), 7.39 (t, J = 7.6 Hz, 1H), 7.54 (d, J = 7.6 Hz, 1H), 7.60 (d, J = 7.6 Hz, 1H), 8.18 (s, 1H), 9.00 (m, 1H), 9.98 (s, 1H), 11.41 (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 25.6, 25.8, 26.4, 29.5, 37.2, 45.3, 100.2, 101.6, 116.0, 121.0, 126.9, 130.2, 140.7, 156.6, 157.8, 158.9, 159.8, 170.8, 172.5, 175.1; HPLC purity: 98.4%; HRMS-FAB: m/z [M+H]$^+$ calcd for $C_{23}H_{25}N_5O_6$: 468.1877, found: 468.1873.

5-[2-[[5-[3-(Benzoylamino)phenyl]isoxazole-3-carbonyl]amino]ethyl]isoxazole-3-carboxylic Acid Ethyl Ester (28c)—To a mixture of the amine **27** (0.056 g, 0.15 mmol) and Et_3N (0.04 mL, 0.33 mmol) in CH_2Cl_2 (1 mL) was added benzoyl chloride (19 μL, 0.16 mmol) at 0 °C. After stirring for 1 h at 0 °C, water (2 mL) was added, and the mixture was extracted with CH_2Cl_2 (3 X 5 mL). The organic phase was dried over Na_2SO_4 and evaporated, and the residue was purified by preparative TLC (EtOAc – hexane, 50%) to provide the ester **28c** (0.05 g, 69%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.32 (m, 3H), 3.13 (m, 2H), 3.75 (m, 2H), 4.35 (m, 2H), 6.50 (s, 1H), 6.92 (s, 1H), 7.28 (s, 1H), 7.34–7.50 (m, 5H), 7.70 (m, 1H), 7.87 (m, 2H), 7.98 (d, *J* = 5.0 Hz, 1H), 8.03 (s, 1H), 9.14 (s, 1H).

5-[3-(Benzoylamino)phenyl]-*N*-[2-[3-(*N*-hydroxycarbamoyl)isoxazol-5-yl]ethyl]isoxazole-3-carboxamide (12)—The title compound was synthesized from the ester **28c** (0.05 g, 0.10 mmol) according to General Procedure E and purified by HPLC (Method A). Yield: 0.019 g (39%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 3.11 (t, *J* = 6.6 Hz, 2H), 3.63 (dd, *J* = 6.6 Hz, 12.7 Hz, 2H), 6.66 (s, 1H), 7.30 (s, 1H), 7.52–7.64 (m, 5H), 7.67 (d, *J* = 7.9 Hz, 1H), 7.94 (dd, *J* = 1.2 Hz, 6.6 Hz, 1H), 7.98 (m, 2H), 8.39 (m, 1H), 9.08 (t, *J* = 5.8 Hz, 1H), 9.35 (br s, 1H), 10.48 (s, 1H), 11.49 (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 26.1, 36.9, 99.5, 99.9, 101.2, 117.0, 121.4, 122.5, 126.6, 127.7, 128.5, 129.8, 131.8, 134.6, 140.0, 156.3, 157.5, 158.6, 159.5, 165.8, 170.4, 172.2; HPLC purity: 97.3%; HRMS-FAB: *m/z* $[M+H]^+$ calcd for $C_{23}H_{19}N_5O_6$: 462.1408, found: 462.1416.

4-[[[5-[4-[(*tert*-Butoxycarbonyl)amino]phenyl]isoxazole-3-carbonyl]amino]methyl]benzoic Acid Methyl Ester (29a)—To a solution of the acid **24a** (0.50 g, 1.64 mmol) in DMF (3.0 mL) was added PyBOP (0.94 g, 1.80 mmol), and the mixture was stirred for 15 min at ambient temperature. Methyl 4-(aminomethyl)benzoate hydrochloride (0.35 g, 2.16 mmol) and Et_3N (0.65 mL, 4.93 mmol) were added subsequently, and the mixture was heated in a microwave reactor for 20 min at 65 °C. Water (20 mL) was added, and the mixture was extracted with EtOAc (3 X 25 mL). The combined organic phases were washed with brine (40 mL), dried over Na_2SO_4, and evaporated. The residue was purified by flash chromatography (EtOAc – hexane; 40% to 100%) to provide the ester **29a** (0.60 g, 81%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.48 (s, 9H), 4.01 (s, 3H), 4.54 (d, *J* = 6.0 Hz, 2H), 7.14 (s, 1H), 7.45 (d, *J* = 8.1 Hz, 2H), 7.61 (d, *J* = 8.5 Hz, 2H), 7.81 (d, *J* = 8.1 Hz, 2H), 7.95 (d, *J* = 8.6 Hz, 2H), 9.42 (t, *J* = 6.0 Hz, 1H), 9.7 (s, 1H).

***N*-[4-[3-[*N*-[4-(*N*-Hydroxycarbamoyl)benzyl]carbamoyl]isoxazol-5-yl]phenyl]carbamic Acid *tert*-Butyl Ester (13)—**To a solution of the methyl ester **29a** (0.20 g, 0.44 mmol) and hydroxylamine hydrochloride (0.18 g, 2.65 mmol) in a mixture of MeOH and THF (2:1, 15 mL) was added MeONa (0.81 mL, 3.54 mmol, as a 21% solution in MeOH) at ambient temperature. The mixture was stirred overnight, and then 1N hydrochloric acid was added to adjust the pH to approx. 4. The mixture was diluted with EtOAc (50 mL), washed with brine (25 mL), dried over Na_2SO_4, and concentrated under vacuum. The residue was purified by HPLC (Method A) to give the title compound. Yield: 0.02 g (10%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.43 (s, 9H), 4.43 (d, *J* = 5.9 Hz, 2H), 7.15 (s, 1H), 7.31 (d, *J* = 8.0 Hz, 2H), 7.55 (d, *J* = 8.6 Hz, 2H), 7.64 (d, *J* = 8.0 Hz, 2H), 7.75

(d, J = 8.6 Hz, 2H), 9.33 (t, J = 5.9 Hz, 1H), 9.64 (s, 1H), 11.11 (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 28.1, 30.7, 42.1, 79.7, 98.5, 99.5, 118.2, 120.0, 126.6, 127.0, 127.2, 131.5, 142.0, 142.2, 152.6, 158.8, 159.4, 164.1, 170.6; HPLC purity: 97.6%; HRMS-FAB: *m/z* [*M*–H]$^-$ calcd for $C_{23}H_{24}N_4O_6$: 451.1623, found: 451.1639.

4-[[[5-[3-[(*tert*-Butoxycarbonyl)amino]phenyl]isoxazole-3-carbonyl]amino]methyl]benzoic Acid Methyl Ester (29b)—The title compound was synthesized from the acid **24b** (1.00 g, 3.29 mmol), methyl 4-(aminomethyl)benzoate hydrochloride (0.54 g, 3.29 mmol), PyBOP (1.88 g, 3.62 mmol), and *N,N*-diisopropylethylamine (0.96 mL, 7.23 mmol) using the same procedure as for compound **29a**. Yield: 0.77 g (50%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.48 (s, 9H), 4.58 (d, J = 5.2 Hz, 2H), 4.94 (s, 1H), 6.93 (s, 1H), 7.31–7.40 (m, 10H), 7.61 (d, J = 6.3 Hz, 2H), 7.70 (m, 1H), 7.80 (s, 1H), 7.95 (m, 1H).

***N*-[3-[3-[*N*-[4-(*N*-Hydroxycarbamoyl)benzyl]carbamoyl]isoxazol-5-yl]phenyl]carbamic Acid *tert*-Butyl Ester (14)—**The title compound was synthesized from the ester **29b** (0.35 g, 0.78 mmol) using the same procedure as for compound **13**. The crude product was purified by HPLC (Method A) to give the title compound (0.06 g, 17%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 4.43 (d, J = 5.9 Hz, 2H), 7.21 (s, 1H), 7.34 (m, 3H), 7.38 (m, 2H), 7.66 (d, J = 8.1 Hz, 2H), 8.03 (s, 1H), 9.38 (t, J = 6.1 Hz, 1H), 9.55 (s, 1H), 11.12 (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 28.5, 42.5, 79.9, 100.2, 114.9, 120.2, 120.7, 127.0, 127.4, 127.6, 130.2, 131.9, 140.8, 142.5, 153.2, 159.0, 159.9, 164.5, 170.9; HPLC purity: 96.4%; HRMS-FAB: *m/z* [*M*+H]$^+$ calcd for $C_{23}H_{24}N_4O_6$: 453.1774, found: 453.1781.

4-[[[5-[3-[(Cyclohexanecarbonyl)amino]phenyl]isoxazole-3-carbonyl]amino]methyl]benzoic Acid (31b)

a. Synthesis of 4-[[[5-(3-aminophenyl)isoxazole-3-carbonyl]amino]methyl]benzoic acid methyl ester. A solution of *tert*-butyl carbamate **29b** (0.76 g, 1.68 mmol) in 12 mL of a mixture of CH_2Cl_2 and trifluoroacetic acid (1:1) was stirred overnight at ambient temperature. The solvents were evaporated, and the residue was recrystallized from EtOAc-hexane (10%) to provide the intermediate amine (0.50 g) which was used directly in next step.

b. Synthesis of 4[[[5-[3-[(cyclohexanecarbonyl]amino]phenyl]isoxazole-3-carbonyl]amino]methyl]benzoic acid methyl ester **(30b)**. To a solution of the amine (0.50 g, 1.42 mmol) in 20 mL of a mixture of CH_2Cl_2 and THF (1:1) were added Et_3N (0.37 mL, 2.84 mmol) and cyclohexanecarbonyl chloride (0.38 mL, 2.84 mmol) at ambient temperature. After 15 min, water (15 mL) was added, and the mixture was extracted with CH_2Cl_2 (3 X 25 mL). The combined organic phases were washed with brine (40 mL), dried over Na_2SO_4, and evaporated. The residue was purified by flash chromatography (CH_2Cl_2 – MeOH; 5%) to provide the acylation product (0.54 g, 82%), which was used directly in next step.

c. To a stirred solution of intermediate **30b** (0.54 g, 1.17 mmol) in THF (20 mL) was added a freshly prepared solution of LiOH (0.12 g, 4.28 mmol) in H_2O (10 mL) at ambient temperature. After 24 h, NaOH (0.10 g, 2.1 mmol) was added. The mixture was stirred for 30 min, acidified with 1N HCl to pH approx. 2, diluted with EtOAc (25 mL), washed with H_2O (15 mL) and brine (15 mL), dried over Na_2SO_4, and concentrated under vacuum to provide the acid **31b** (0.26 g, 50%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.25-2.33 (m, 10H), 2.33 (t, J = 11.0 Hz, 1H), 4.53 (d, J = 5.7 Hz, 2H), 7.31 (s, 1H), 7.37 (d, J = 8.0 Hz, 2H), 7.46 (t, J = 7.9 Hz, 1H), 7.61 (d, J = 7.9 Hz, 1H), 7.72 (d, J = 7.9 Hz, 1H), 7.90 (d, J = 8.0 Hz, 2H), 8.28 (s, 1H), 9.46 (t, J = 6.0 Hz, 1H), 10.14 (s, 1H).

5-[3-[(Cyclohexanecarbonyl)amino]phenyl]-*N*-[4-(N-hydroxycarbamoyl)benzyl]isoxazole-3-carboxamide (16)—To a solution of the acid **31b** (0.12 g, 0.26 mmol) and *N*-methylmorpholine (0.032 g, 0.32 mmol) in THF (8 mL) was added ethyl chloroformate (0.030 mL, 0.32 mmol) at 0 °C, and the mixture was stirred for 30 min. The precipitate was filtered off, and the filtrate was added to a mixture of hydroxylamine hydrochloride (0.037 g, 0.53 mmol) and Et_3N (0.07 mL, 0.53 mmol) in DMF (2 mL). The reaction mixture was stirred for 30 min at 0 °C. 1N Hydrochloric acid was added to bring the pH to approx. 4, and the mixture was extracted with EtOAc (3 X 25 mL). The combined organic phases were washed with brine (40 mL), dried over Na_2SO_4, and evaporated. The residue was purified by HPLC (Method B) to give the hydroxamic acid. Yield: 0.01 g (8%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.18–1.84 (m, 10H), 2.33 (m, 1H), 4.51 (d, J = 6.0 Hz, 2H), 7.30 (s, 1H), 7.36 (d, J = 8.2 Hz, 2H), 7.47 (t, J = 7.9 Hz, 1H), 7.61 (d, J = 7.9 Hz, 1H), 7.71 (d, J = 7.9 Hz, 1H), 7.73 (d, J = 8.2 Hz, 2H), 8.26 (s, 1H), 9.00 (br s, 1H), 9.46 (t, J = 6.0 Hz, 1H), 10.06 (s, 1H), 11.18 (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 25.6, 25.8, 29.5, 42.5, 45.3, 100.2, 116.0, 121.0, 121.6, 127.0, 127.4, 127.6, 130.2, 131.9, 140.7, 142.5, 159.0, 159.9, 164.5, 170.8, 175.1; HPLC purity: 97.2%; HRMS-FAB: m/z $[M+H]^+$ calcd for $C_{25}H_{26}N_4O_6$: 463.1976, found: 463.1988.

5-[4-[(Cyclohexanecarbonyl)amino]phenyl]-*N*-[4-(*N*-hydroxycarbamoyl)benzyl]isoxazole-3-carboxamide (15)

a. Synthesis of 4-[[[5-[3-[(cyclohexanecarbonyl)amino]phenyl]isoxazole-3-carbonyl]amino]methyl]benzoic acid (**31a**). Intermediate **29a** was subjected to the same three-step sequence as employed in the transformation of intermediate **29b** to **31b**. Compound **31a**: ^{1}H NMR ([D_6]DMSO): δ = 1.16–1.76 (m, 10H), 2.28 (t, J = 11.0 Hz, 1H), 4.47 (d, J = 6.2 Hz, 2H), 7.17 (s, 1H), 7.37 (d, J = 8.2 Hz, 2H), 7.72 (d, J = 8.8 Hz, 2H), 7.80 (d, J = 8.8 Hz, 2H), 7.85 (d, J = 8.2 Hz, 2H), 9.35 (t, J = 6.0 Hz, 1H), 10.03 (s, 1H), 12.79 (br s, 1H).

b. Compound **15** was obtained from the preceding intermediate in the same manner as described for the synthesis of compound **16** from its precursor **31b**. ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 1.11–1.76 (m, 10H), 2.32 (m, 1H), 4.44 (d, J = 6.0 Hz, 2H), 7.16 (s, 1H), 7.33 (d, J = 8.1 Hz, 2H), 7.66

(d, J = 8.1 Hz, 2H), 7.72 (d, J = 8.6 Hz, 2H), 7.80 (d, J = 8.6 Hz, 2H), 8.94 (br s, 1H), 9.35 (t, J = 6.0 Hz, 1H), 10.05 (s, 1H), 11.12 (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 25.6, 25.7, 29.4, 42.5, 45.3, 99.1, 119.6, 121.1, 127.0, 127.4, 127.5, 131.8, 142.1, 142.5, 159.1, 159.8, 164.5, 170.8, 175.2; HPLC purity: 95.3%; HRMS-FAB: m/z [M+H]$^+$ calcd for $C_{25}H_{26}N_4O_6$: 463.1976, found: 463.1988.

5-(Carbazol-9-ylmethyl)isoxazole-3-carboxylic Acid Ethyl Ester (33)

a. Synthesis of 9-prop-2-ynyl-9*H*-carbazole.To a solution of carbazole (1.00 g, 6.00 mmol) in dry DMF (5 mL) was added NaH (0.31 g, 7.8 mmol) as a 60% suspension in mineral oil at 0 °C. The mixture was stirred for 15 min, then propargyl bromide (0.73 mL, 6.6 mmol) was added, and the reaction mixture was allowed to warm to ambient temperature. The reaction mixture was poured into ice-water, and the solution was extracted with EtOAc. The organic phase was washed with water and brine, dried over anhydrous Na_2SO_4, and concentrated under vacuum. The crude product was purified by combiflash chromatography (EtOAc – hexane; 5% to 70%) to give 0.72 g (58%) of the carbazole intermediate. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 2.07 (t, J = 0.4 Hz, 1H), 2.84 (m, 2H), 3.43 (m, 2H), 7.41 (t, J = 8.3 Hz, 2H), 7.50 (t, J = 8.3 Hz, 2H), 8.02 (d, J = 8.3 Hz, 2H), 8.26 (d, J = 8.3 Hz, 2H).

b. To a solution of 9-prop-2-ynyl-9*H*-carbazole (0.72 g, 3.50 mmol) and Et_3N (9.8 mL, 70 mmol) in 20 mL of dry THF under an Ar atmosphere stirred at ambient temperature, was added dropwise over 24 h by syringe pump a solution of ethyl chlorooximinoacetate (5.32 g, 35.0 mmol) in 20 mL of THF. The reaction mixture was filtered and washed with EtOAc, and the filtrate was evaporated. The residue was purified by CombiFlash chromatography (EtOAc – hexane; 5% then 40%) to provide a mixture of the ester **33** and the dimer formed from the reagent (2.35 g).

5-(Carbazol-9-ylmethyl)isoxazole-3-carbohydroxamic Acid (17)—To a stirred solution of the ester **33** (0.33 g, 1.03 mmol) in 5 mL of THF was added at 0 °C a freshly prepared solution of NH_2OH. After 15 min, the reaction mixture was acidified with 1N HCl (pH ~ 4) and diluted with EtOAc. The resulting mixture was washed with H_2O and brine, and the organic phase was dried over Na_2SO_4 and evaporated. The solid residue was purified by HPLC (method A) to give the hydroxamic acid as an off-white solid (0.150 g, 47%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 5.86 (s, 2H), 6.60 (s, 1H), 7.18 (t, J = 7.5 Hz, 2H), 7.40 (t, J = 7.5 Hz, 2H), 7.68 (d, J = 7.5 Hz, 2H), 8.09 (d, J = 7.5 Hz, 2H), 9.27 (s, 1H), 11.36 (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 37.7, 102.2, 109.5, 119.6, 120.4, 122.4, 126.0, 139.7, 155.8, 157.4, 169.6; HRMS-FAB: m/z [M+H]$^+$ calcd for $C_{17}H_{13}N_3O_3$: 308.1029, found: 308.1036.

5-(9*H*-Carbazol-9-ylmethyl)-*N*-[4-(hydroxycarbamoyl)benzyl]isoxazole-3-carboxamide (18)

a. Synthesis of 5-(9*H*-carbazol-9-ylmethyl)isoxazole-3-carboxylic acid. To a stirred solution of the ester **33** (0.83 g, 2.58 mmol) in 5.0 mL of THF was added a freshly prepared solution of LiOH (0.124 g, 5.17 mmol) in 5.0 mL of H_2O at ambient temperature. The mixture was stirred overnight, NaOH (0.103 g, 2.59 mmol) was added, and the reaction mixture was stirred for 30 min. The reaction mixture was acidified with 1N HCl (pH ~ 4), diluted with EtOAc, and washed with H_2O and brine. The organic phase was dried over Na_2SO_4 and evaporated to give the carboxylic acid as an off-white solid (0.63 g, 83%).

b. Synthesis of 4-[[5-(9*H*-carbazol-9-ylmethyl)isoxazole-3-carboxamido]methyl]benzoic acid methyl ester. To a solution of the carboxylic acid (0.33 g, 1.13 mmol) in DMF (3 mL) was added PyBOP (0.64 g, 1.24 mmol), and the mixture was stirred for 15 min at ambient temperature. Methyl 4-(aminomethyl)benzoate hydrochloride (0.24 g, 1.5 mmol) and Et_3N (0.45 mL, 3.4 mmol) were added. The resulting mixture was heated in a microwave oven at 65 °C for 20 min. Water was added, and the mixture was extracted with EtOAc. The combined organic phases were washed with NaCl, dried over Na_2SO_4, and evaporated. The residue was purified by CombiFlash chromatography (EtOAc – hexane; 40% then 100%,) to provide the ester intermediate (1.0 g, 66%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 3.81 (s, 3H), 4.43 (d, J = 6.1 Hz, 2H), 5.94 (s, 2H), 6.65 (s, 1H), 7.24 (t, J = 7.4 Hz, 2H), 7.35 (d, J = 8.3 Hz, 2H), 7.48 (t, J = 7.4 Hz, 2H), 7.75 (d, J = 8.3 Hz, 2H), 7.88 (t, J = 8.3 Hz, 2H), 8.16 (d, J = 7.4 Hz, 2H), 9.31 (t, J = 6.1 Hz, 2H), 11.7 (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 28.2, 43.1, 77.0, 77.9, 99.5, 126.8, 127.1, 128.3, 128.9, 130.7, 136.0, 144.0, 155.8. (c) To a solution of the methyl ester (0.33 g, 0.75 mmol) and hydroxylamine hydrochloride (0.31 g, 4.5 mmol) in MeOH-THF (20 mL, 1:1) was added MeONa (1.3 mL, 6.0 mmol, as a 21% solution in MeOH) at ambient temperature. The mixture was stirred at ambient temperature for 60 h, acidified with 1N HCl (pH ~ 4), and extracted with EtOAc. The combined organic phases were washed with NaCl, dried over Na_2SO_4, and evaporated. The residue was purified by HPLC (method A) to give the hydroxamic acid as a white solid (0.022 g, 6.6%). ^{1}H NMR (400 MHz, [D_6]DMSO): δ = 4.32 (d, J = 5.9 Hz, 2H), 5.88 (s, 2H), 7.18 (m, 4H), 7.42 (t, J = 7.2 Hz, 2H), 7.58 (d, J = 8.2 Hz, 2H), 7.69 (d, J = 8.2 Hz, 2H), 8.10 (d, J = 7.2 Hz, 2H), 8.92 (s, 1H), 9.21 (t, J = 5.9 Hz, 1H), 11.07 (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): δ = 37.8, 42.0, 102.4, 109.5, 119.6, 120.4, 122.5, 126.0, 126.9, 127.1, 131.4, 139.7, 142.0, 158.3, 158.7, 164.1, 170.0; HRMS-FAB: m/z $[M+H]^+$ calcd for $C_{25}H_{20}N_4O_4$: 441.1557, found: 441.1577.

HDAC Inhibition Assays—HDAC inhibition assays were performed by the Reaction Biology Corporation (Malvern, PA) using human, full-length recombinant HDAC1 and 6 isolated from a baculovirus expression system in Sf9 cells. An acetylated, fluorogenic peptide derived from residues 379 382 of p53 (RHKKAc) was used as the substrate in the assays. The reaction buffer contained 50 mM Tris-HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/mL BSA, and a final concentration of 1% DMSO. The enzyme was delivered into wells of the reaction plate, and compounds were delivered in 100% DMSO into the enzyme mixture by Acoustic Technology (Echo550; nanoliter range). The plates were spun down and preincubated for 5 10 min. The substrate was then delivered to all reaction wells to initiate the reaction, and the plates were incubated for 2 h at 30 °C. After incubation, developer and trichostatin A were added to quench the reaction and generate fluorescence. Then, kinetic measurements were taken for 1.5 h in 15 min intervals to ensure that development was complete. End-point readings were taken for analysis after the development reached a plateau. Dose response curves were generated, and the IC_{50} for each compound was extrapolated from the generated plots. (Ten-dose IC_{50} curves were generated using a three-fold serial dilution pattern starting with concentrations of 30 μM.) All IC_{50} determinations were done in duplicate, and the values reported herein are the average of both trials ± the standard deviation.

Measurement of Cell Viability

Pancreatic cancer cell lines BXPC3 and L3.6pl were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The relative number of viable cancer cells was determined 72 hours post-treatment by measuring the optical density using [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] cell proliferation assay kit (Promega, Madison, WI). GI_{50} values for each compound were calculated by non-linear regression model of the standard slope using GraphPad Prism 6.0 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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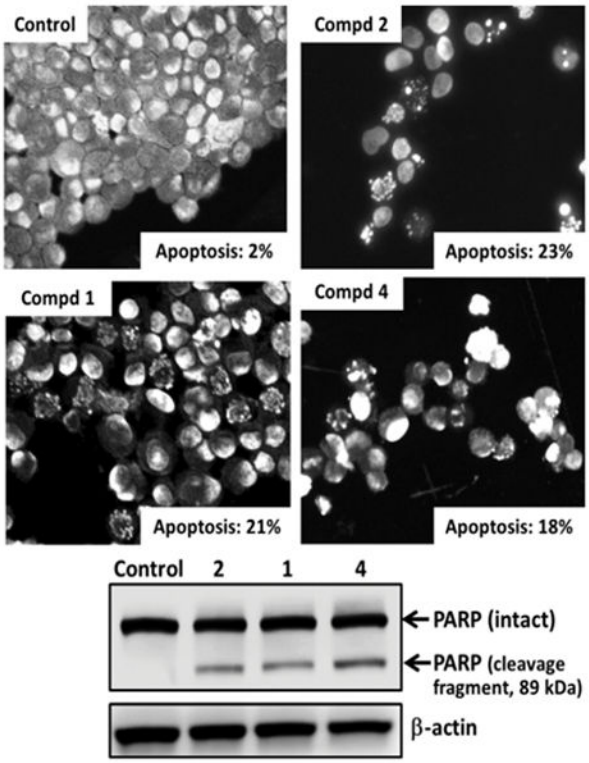


Figure 1.

HDAC inhibitors induce apoptosis in pancreatic cancer cells. L3.6pl pancreatic cancer cells were treated with 10 μM of compounds **1**, **2,** or **4** for 24 h as indicated. Cells were collected and processed for Hoechst staining (upper panel), and cell lysates were prepared for Western immunoblotting (lower panel). 50 μg of the proteins were separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted as indicated.

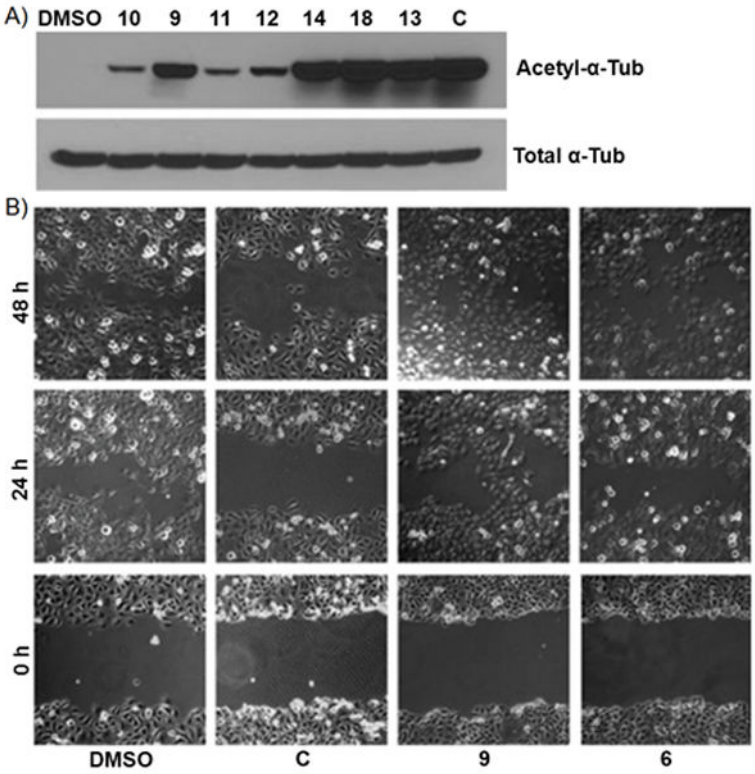
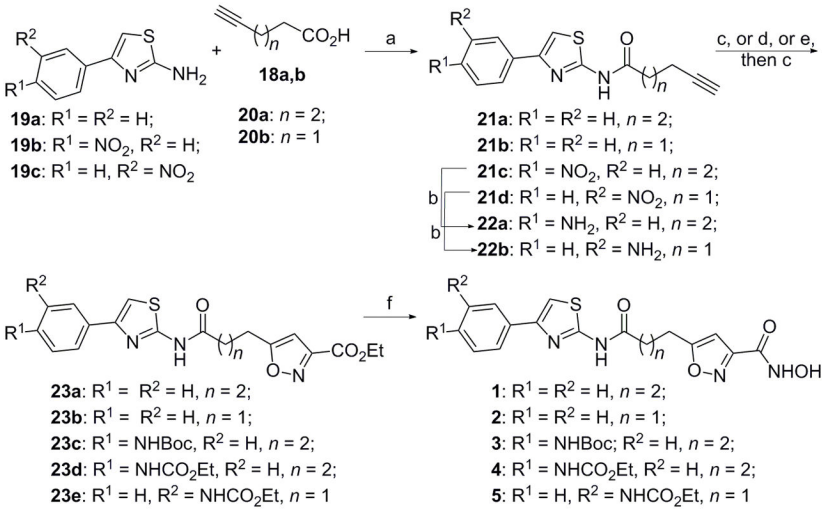


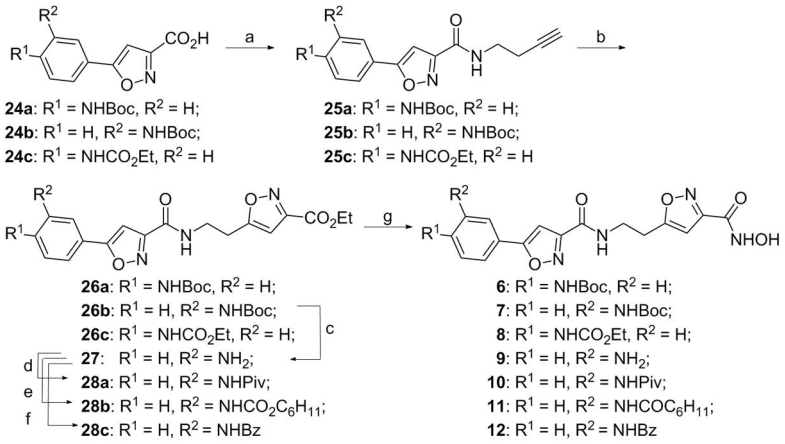
Figure 2.

Evaluation of the ability of novel HDAC6 inhibitors to induce tubulin acetylation and to arrest cancer cell migration. (A) PANC1 cells were treated with DMSO or a 1 μM concentration of HDACIs for 24 h. Cells were harvested and lysed, and equivalent amounts of protein were loaded per lane and immunoblotted as indicated. (B) PANC1 cells were treated with the indicated HDACIs, and a scratch was made through a confluent layer of cells. Images of the cells were taken at 24 and 48 hours post wounding.



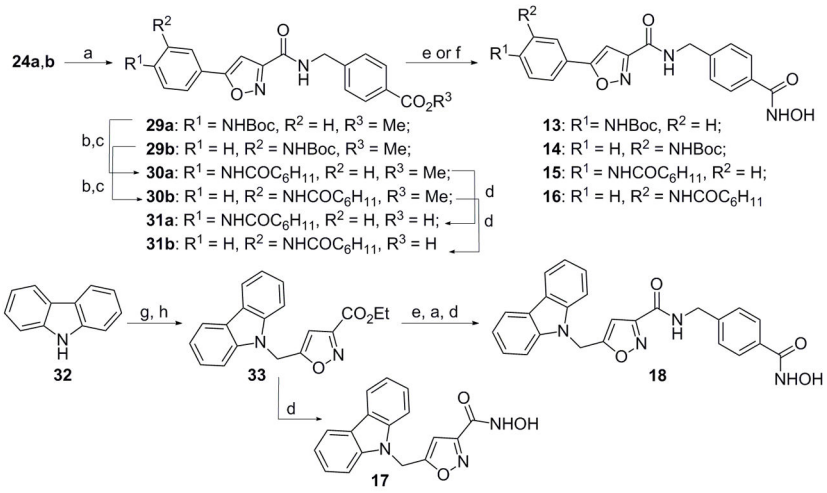
Scheme 1.

Reagents and conditions : (a) (i) EDCI, DMAP, DCM, or (ii) $POCl_3$, pyridine; (b) Fe, NH_4Cl, AcOH, EtOH/H_2O; (c) ethyl chlorooximinoacetate, Et_3N, THF; (d) Boc_2O, toluene, microwave, 120 °C; (e) $ClCO_2Et$, Et_3N, THF; (f) $NH_2OH.HCl$, KOH.



Scheme 2.

Reagents and conditions : (a) But-3-ynylamine hydrochloride, PyBOP, Et_3N, DMF; (b) ethyl chlorooximinoacetate, Et_3N, THF; (c) CF_3COOH, CH_2Cl_2; (d) pivaloyl chloride, Et_3N, CH_2Cl_2; (e) cyclohexanecarbonyl chloride, Et_3N, CH_2Cl_2; (f) benzoyl chloride, Et_3N, CH_2Cl_2; (g) $NH_2OH{\cdot}HCl$, KOH, THF/MeOH.



Scheme 3.

Reagents and conditions: (a) methyl 4-(aminomethyl)benzoate hydrochloride, PyBOP, Et_3N, DMF; (b) CF_3COOH, CH_2Cl_2; (c) cyclohexanecarbonyl chloride, Et_3N, CH_2Cl_2; (d) $NH_2OH{\cdot}HCl$, KOH, THF/MeOH; (e) LiOH/NaOH, THF-H_2O; (f) $ClCO_2Et$, NH_2OH, *N*-methylmorpholine; (g) 3-bromopropyne, NaH, DMF; (h) ethyl chlorooximinoacetate, Et_3N, THF.

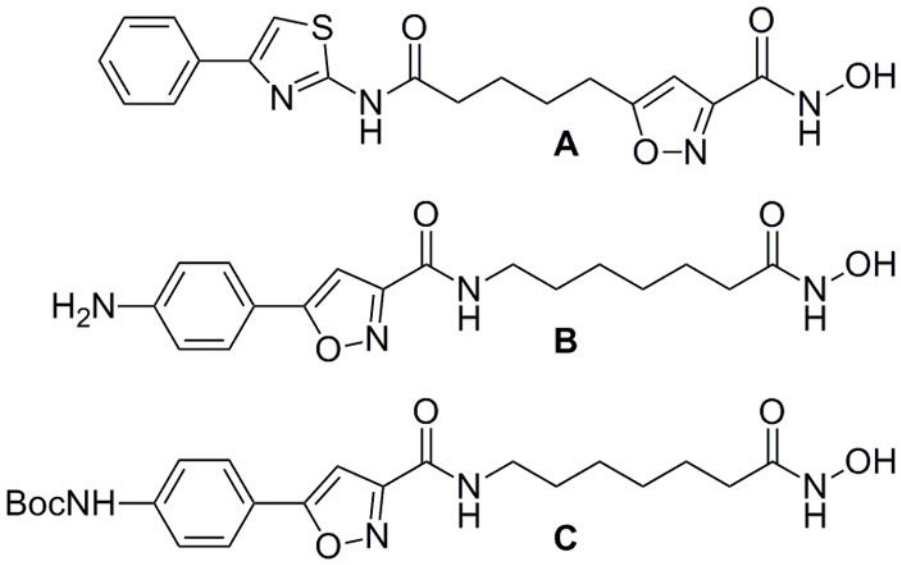


Chart 1.
Selected HDACIs with significant antiproliferative effects on pancreatic tumor cells.

Table 1

In vitro HDAC isozyme inhibition data for new hydroxamate derivatives.

Compd	HDAC Isoform, IC_{50} (nM)[a]								
	Class I						Class II		
	1	SI [b] HDAC1/6	2	SI HDAC2/6	3	SI HDAC3/6	6	10	SI HDAC10/6
A	302	4.5	429	6.3	29.6	0.4	67.7	254	3.8
B	56.3	9.7	146	25	10.2	1.8	5.8	44.9	7.7
C	3.2	0.2	4.8	0.3	22.8	1.7	13.8	90.7	6.6
1	351	4.3	1220	15	934	11	81.8 ± 5.5	854	10
2	307	10	1140	37	320	10	31 ± 3.4	407	13
3	401	10	1140	28	448	11	41.2 ± 4.3	52.9	1.0
4	201	4.1	834	17	354	7.2	48.9 ± 1.5	323	6.6
5	266	80	1100	333	107	32	3.3 ± 0.1	271	82
6	16900	2817	>50000 [c]		22800	3800	6.0 ± 0.3	>50000	
7	>50000		>50000		>50000		7.7 ± 0.8	>50000	
8	3910	39	41300	409	5190	51	101.0 ± 9.7	12500	123
9	6680	398	2360	140	1770	105	16.8 ± 2.5	5290	315
10	>50000		>50000		>50000		21.2 ± 0.9	40100	1910
11	>50000		>50000		>50000		6.7 ± 0.5	>50000	
12	2930	666	10400	2360	7050	1600	4.4 ± 0.1	4630	1050
13	436	1320	1900	5760	135	409	0.33 ± 0.06	3160	9580
14	444	694	1380	2160	789	1230	0.64 ± 0.11	2730	4270
15	350	65	2490	461	215	40	5.4 ± 0.3	204	38
16	212	82	4300	1650	669	257	2.6 ± 0.1	191	73
17	38700	26	>50000		44000	29	1510 ± 65	>50000	
18	495	825	1370	2280	479	798	0.61 ± 0.14	22200	37000
TSA	3.0	3.8	6.4	8.2	7.3	9.3	0.78 ± 0.23	8.9	11.4

[a] Assays were conducted by the Reaction Biology Corp. (Mavern, PA, USA). All the compounds were tested in duplicate in a 10-dose IC_{50} mode with 3-fold serial dilution starting at 30 μM against HDAC6, and tested in singlet in a 10-dose IC_{50} mode with 3-fold serial dilution starting at 30 μM against HDAC1,2,3,10.

[b] Selectivity index.

[c]No inhibition at 50 μM.

Table 2

In vitro growth inhibition (GI) of pancreatic cancer cells by new HDACIs.

Compound	BxPC3 GI_{50} (μM) [a]	SE [b] $LogGI_{50}$ (μM)	L3.6pl GI_{50} (μM) [a]	SE [b] $LogGI_{50}$ (μM)
C	0.6	0.04	0.1	0.11
1	1.5	0.08	1.3	0.17
2	2.3	0.08	0.7	0.08
4	1.7	0.07	1.7	0.12
5	7.0	0.10	2.1	0.12
6	29	0.21	>50	0.17
7	>50	0.22	>50	0.44
8	>50	0.26	47	0.14
9	12	0.06	3.3	0.15
11	>50	0.23	>50	2.48
12	>50	0.49	>50	0.34
13	8.1	0.22	2	0.25
14	7.8	0.09	>50	0.08
18	2.3	0.08	1.3	0.09

[a]Pancreatic cancer cell lines BXPC3 and L3.6pl were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Relative number of viable cancer cells was determined 72 hours post-treatment by measuring the optical density (OD) using [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] cell proliferation assay kit (Promega, Madison, WI). The OD value was determined as a mean of 5 replicates per compound concentration in a 96-well plate. The GI_{50} value for each compound was calculated using a non-linear regression model of the standard slope using GraphPad Prism 6.0 software. b Standard Error

Table 3

Inhibition of cell migration. [a]

Compound	24 h	48 h	Compound	24 h	48 h
DMSO	-	-	DMSO	-	-
B	+++	++	10	-	-
C	+++	++	11	+	-
6	+	-	12	-	-
7	+	+	13	-	-
9	+	-	18	+	-

[a] Level of inhibition (high to low): +++, ++, or +; no inhibition: -.