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Investigation of the effect of different linker chemotypes on the inhibition of Histone Deacetylases (HDACs)

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Abstract

Histone Deacetylases (HDACs) are among the most attractive and interesting targets in anticancer drug discovery. The clinical relevance of HDAC inhibitors (HDACIs) is testified by four FDA-approved drugs for cancer treatment. However, one of the main drawbacks of these drugs resides in the lack of selectivity against the different HDAC isoforms, resulting in severe side effects. Thus, the identification of selective HDACIs represents an exciting challenge for medicinal chemists. HDACIs are composed of a cap group, a linker region, and a metal-binding group interacting with the catalytic zinc ion. While the cap group has been extensively investigated, less information is available about the effect of the linker on isoform selectivity. To this aim, in this work, we explored novel linker chemotypes to direct isoform selectivity. A small library of 25 hydroxamic acids with hitherto unexplored linker chemotypes was prepared. In vitro tests demonstrated that, depending on the linker type, some candidates selectively inhibit HDAC1 over HDAC6 isoform or vice versa. Docking calculations were performed to rationalize the effect of the novel linker chemotypes on biologic activity. Moreover, four compounds were able to increase the levels of acetylation of histone H3 or tubulin. These compounds were also assayed in breast cancer MCF7 cells to test their

antiproliferative effect. Three compounds showed a significant reduction of cancer proliferation, representing valuable starting points for further optimization.

Keywords: HDAC, HDAC inhibitors, linker portion, hydroxamic acids, anticancer drugs.

1. Introduction

Histone deacetylases (HDACs) and histone acetyl-transferases (HATs) play a pivotal role in epigenetic modification of gene expression [1]. In particular, HDACs catalyze the deacetylation of the ε-amino lysine residues on histone tails, causing chromatin condensation and transcriptional repression [2]. Interestingly, the functions of HDACs are not solely restricted to chromatin remodelling. HDACs are also involved in the deacetylation of non-histone proteins such as transcription factors (p53), tubulin, chaperone proteins (Hsp90), and other cytoplasmic proteins (STAT3, BAX, and HMGB1), resulting in the control of cellular proliferation, protein degradation, signal transduction, apoptosis and inflammation [3], [4]. At present, 18 HDACs have been identified and classified into four classes: I (HDACs 1, 2, 3, and 8), II (HDACs 4, 5, 6, 7, 9, and 10) and IV (HDAC11), which are Zn^{2+}-dependent enzymes, whereas class III, better known as sirtuins (SIRT1-7) are NAD^{+}-dependent HDACs [5–7]. The overexpression of HDACs, with the consequent reduction of histone acetylation and transcriptional dysregulation, has been observed in several diseases. Thus, HDAC inhibitors (HDACIs) have gained increasing interest as promising agents for the treatment of cancer, neurodegenerative disorders, inflammation and HIV infections [8–14]. At present, four HDACIs have been approved by the Food and Drug Administration (FDA) to treat some neoplastic diseases (**Figure 1**). Vorinostat (SAHA), a pan-inhibitor against class I, II, and IV, was the first HDACIs approved in 2006 to treat relapsed and refractory cutaneous T-cell lymphoma (CTCL) [15]. Romidepsin was licensed in 2009 for the treatment of CTCL and in 2011 for peripheral T-cell lymphoma (PTCL) [16]. Lastly, Belinostat and Panobinostat were approved in

2014 and 2015, respectively, for relapsed multiple myeloma [17]. Other HDACIs are currently in advanced clinical trials as anticancer agents [18–21].

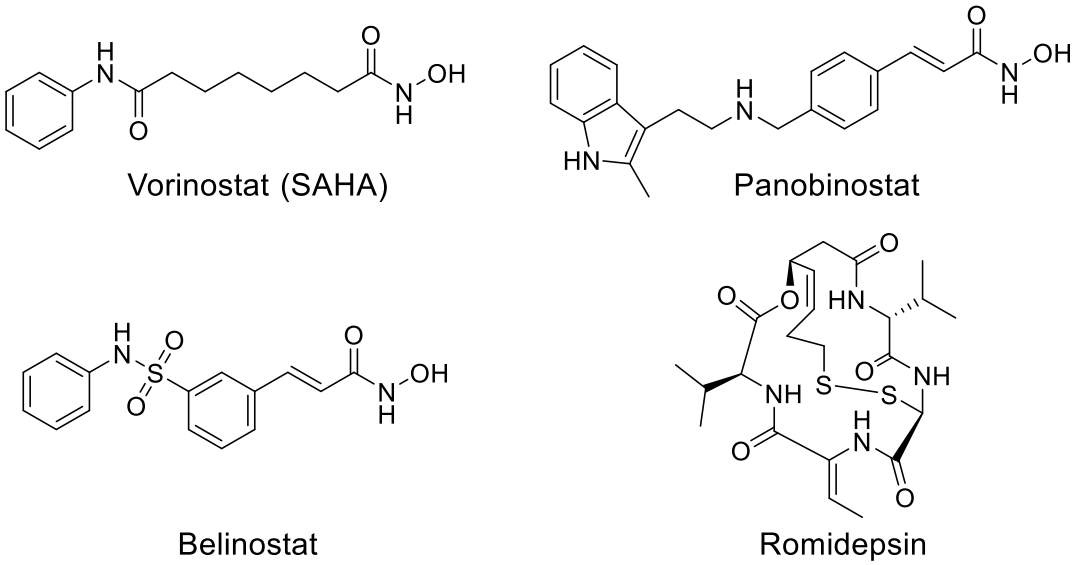


Figure 1. HDACIs approved for clinical use.

Although clinically effective HDACIs have been approved, one of the main drawbacks resides in the lack of selectivity against the different HDAC isoforms. This results in severe side effects, including thrombocytopenia, anorexia, and cardiotoxicity. Thus, the identification of selective HDACIs represents a challenge for the medicinal chemists in order to minimize toxicity and off-target effects [22].

The well-accepted pharmacophoric model for the design of novel HDACIs is composed of: i) a cap group, interacting with the surface of the enzyme; ii) a linker, fitting the tubular access to the zinc atom, and iii) a metal-binding group, coordinating the catalytic zinc ion (zinc-binding group, ZBG) [23,24]. The recent literature shows that isoform selectivity can be achieved in many different ways, focusing mainly on the cap-group and, to a lesser extent, on the ZBG [25,26]. Since the surface groove of HDACs is significantly different between the eleven isoforms, a wide range of cap-groups have been proposed to guide the selectivity of the compounds toward the isoform of interest

[27,28]. Conversely, less information is available about the effect of the linker portion on isoform selectivity. Indeed, modifications of the linker portion are more challenging due to the relatively higher conservation among HDACs substrate-binding tunnel [29]. Therefore only a few chemotypes have been explored (i.e., alkylic chain of SAHA, cinnamate benzylic ring). Senger et al. took the first step in this direction by identifying the thiazole and oxazole rings as novel linker moieties able to direct the selectivity toward the HDAC6 isoform [30]. Thus, with the aim to investigate new linker chemotypes, a small library of twenty-five hydroxamic acids was prepared and tested. Four different and hitherto unexplored classes of linkers were considered: (i) aliphatic heterocyclic linkers such as 1,3-dioxolane and 1,4-dioxa-8-azaspiro[4.5]decane (**1-4**); (ii) aliphatic linear linkers such as emisuccinates (**5, 5a-c**); (iii) aromatic heterocyclic linkers such as benzodioxane, benzodioxin, benzofuran, and benzotriazole (**6-9**); (iv) aromatic/unsaturated linear linkers such as phenyl-oxyacetates/-thioacetates (**10, 10a-b, 11, 11a-b**) and alkynes (**12, 12a**; **Figure 2**).

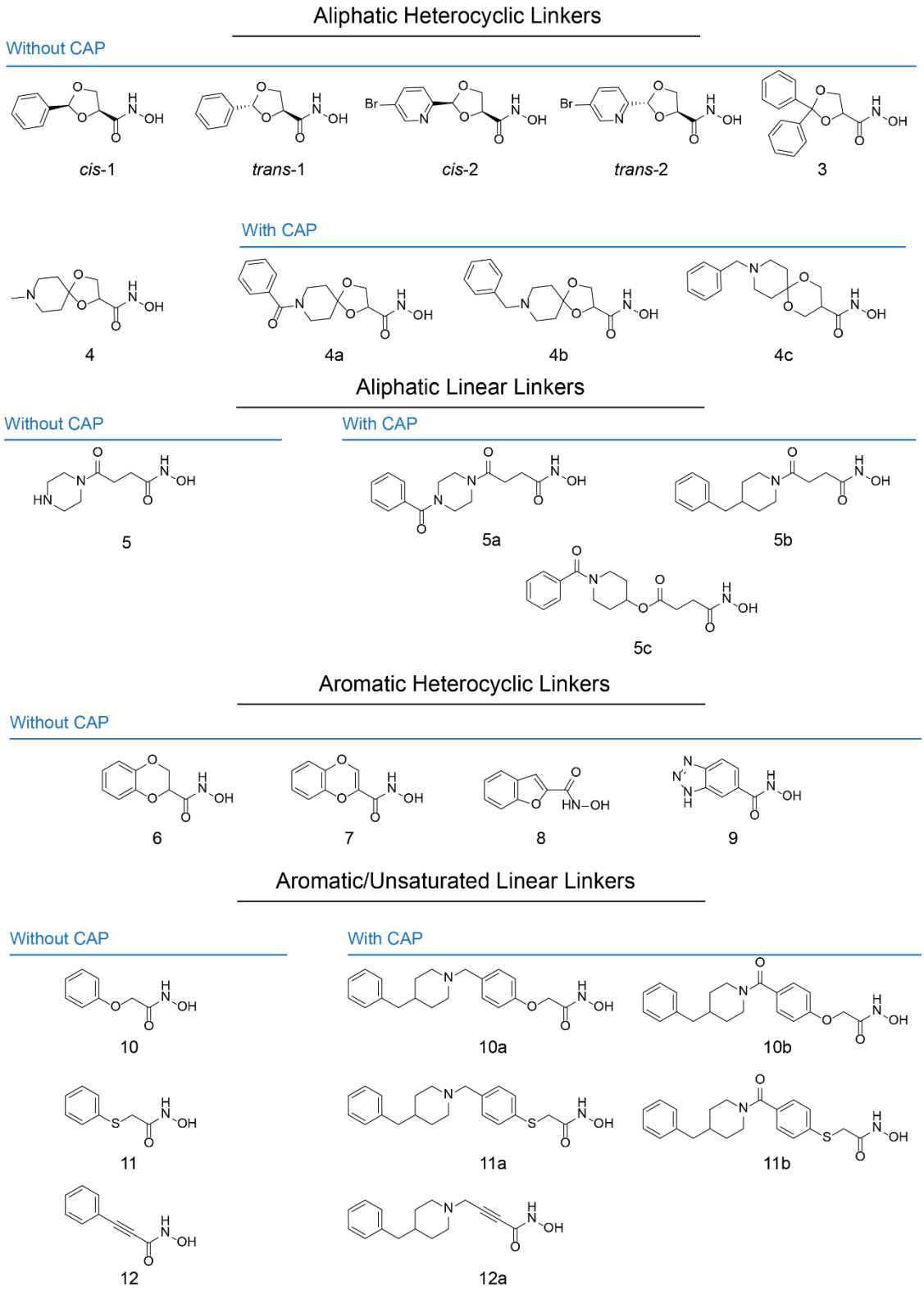


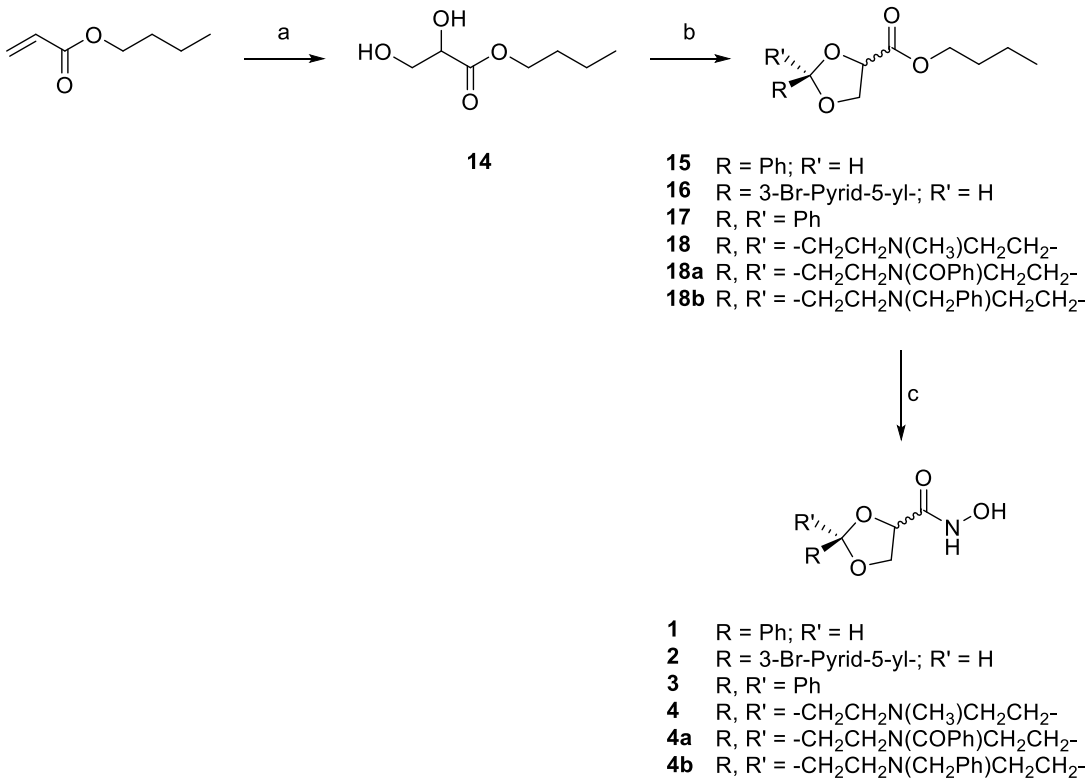
Figure 2. Chemical structures of the newly synthesized HDAC inhibitors.

At first, we focused our work on the search of the minimal structural requirements capable of showing a first sign of activity and selectivity by preparing compounds exclusively formed by the novel linker chemotypes and the hydroxamic acid as ZBG. Then, selected linkers were decorated with the cap groups to validate the novel scaffolds. Twenty-five (**1-5, 5a-c, 6-10, 10a-b, 11, 11a-b, 12, 12a**) hydroxamic acids were synthesized and characterized. All the synthesized compounds were evaluated for their inhibitory activity against purified HDAC1, and HDAC6 enzymes, chosen as representatives of HDACs class I and IIb. The most active compounds were then tested in MCF7 cells to measure histone H3 and α-tubulin acetylation. Moreover, docking calculations were performed to rationalize the observed selectivity profile towards HDAC1 and HDAC6 isoforms.

1. Results and Discussion

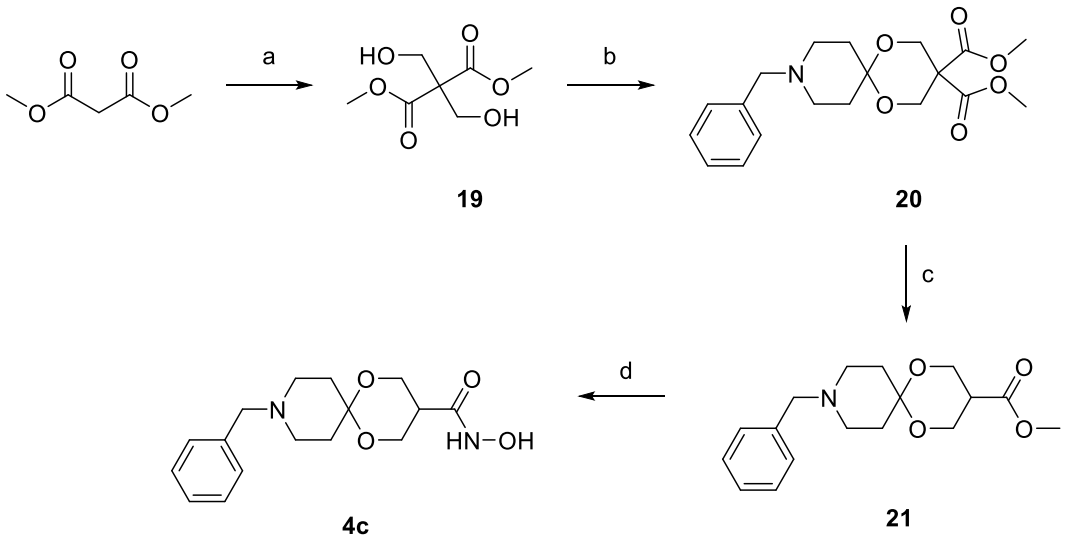
1.1. Chemistry

The synthesis of compounds with an aliphatic heterocyclic linker, such as the 1,3-dioxolanes (**1-3**) and 1,4-dioxa-8-azaspiro[4.5]decane (**4, 4a-b**), was performed as reported in **Scheme 1**. The commercially available *n*-butyl acrylate was oxidized to the corresponding diol (**14**) in neutral conditions using potassium permanganate and TEBAC in dry acetone. The diol (**14)** was then cyclized with the appropriate carbonyl compound (benzaldehyde for ***cis*-1** and ***trans*-1**; 5-bromo-picolinaldehyde for ***cis*-2** and ***trans*-2**; benzophenone for **3**; *N*-methyl-piperidone for **4**; 4-benzoyl-piperidone for **4a** and 4-benzyl-piperidone for **4b**), using the Dean-Stark apparatus to trap the forming water, to provide the corresponding *n*-butyl ester (**15-18** and **18a-b**). The latter was finally converted into the hydroxamic acids **1-4** and **4a-b** by S_NAc with 50% aq. hydroxylamine, in ethanol, at room temperature.



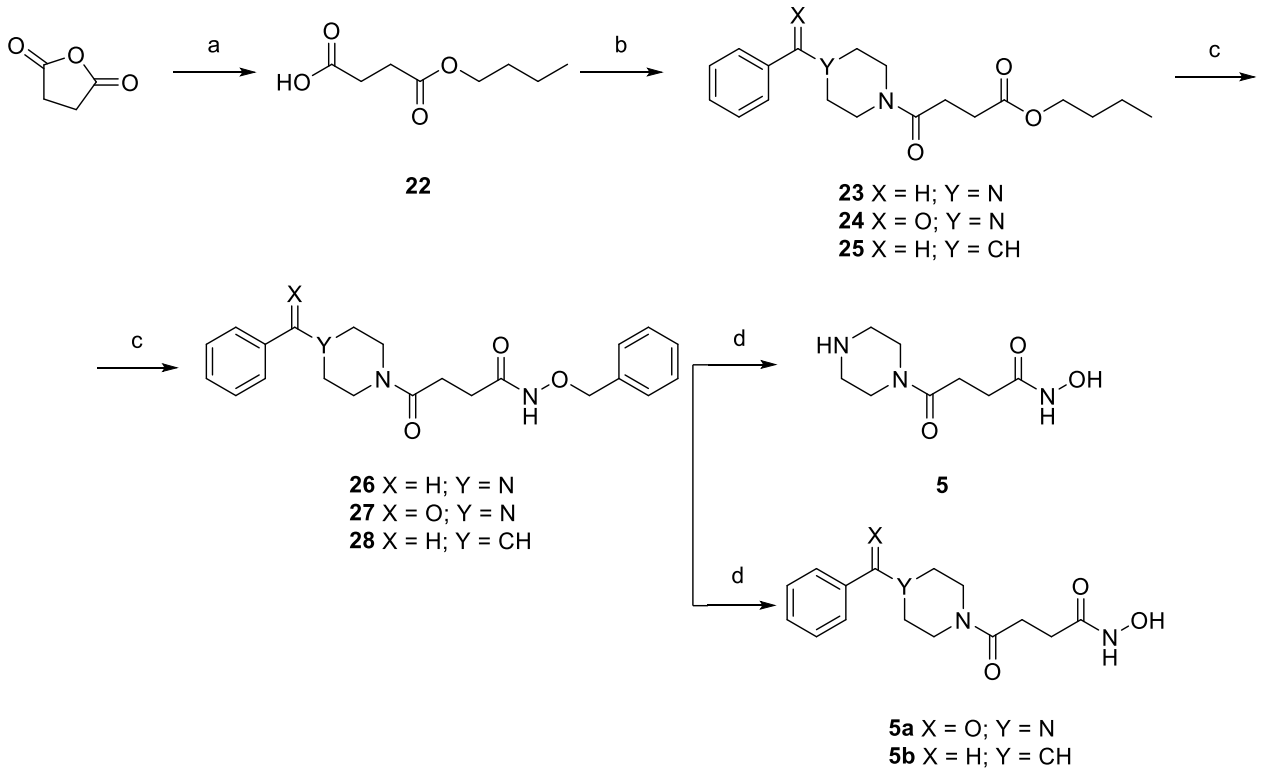
Scheme 1. Reagents and conditions: (a) $KMnO_4$ (1.2 equiv.), TEBAC (1.2 equiv.), Acetone, r.t. (3h) →0 °C (30 min.) → r.t. (1h); (b) carbonyl compound (0.75 equiv.), *p*TSA (cat.), dry Toluene, Dean-Stark trap, N_2, rifl., 6-24 h; (c) 50% aq. NH_2OH (30 equiv.), EtOH, r.t., 24 h.

The 1,3-dioxane derivative **4c** was prepared according to **Scheme 2**. Condensation of dimethyl-malonate with 30% aq. formaldehyde in the presence of $NaHCO_3$, at room temperature, overnight, led to the diol (**19**) in 30% yield. The intermediate **19** was cyclized with 4-benzyl-piperidone to afford the dimethyl ester (**20**), which was further decarboxylated to monomethyl ester (**21**) with NaCl in DMSO at 180°C. Lastly, the ester **21** was converted into hydroxamic acid **4c** under the conditions previously described.



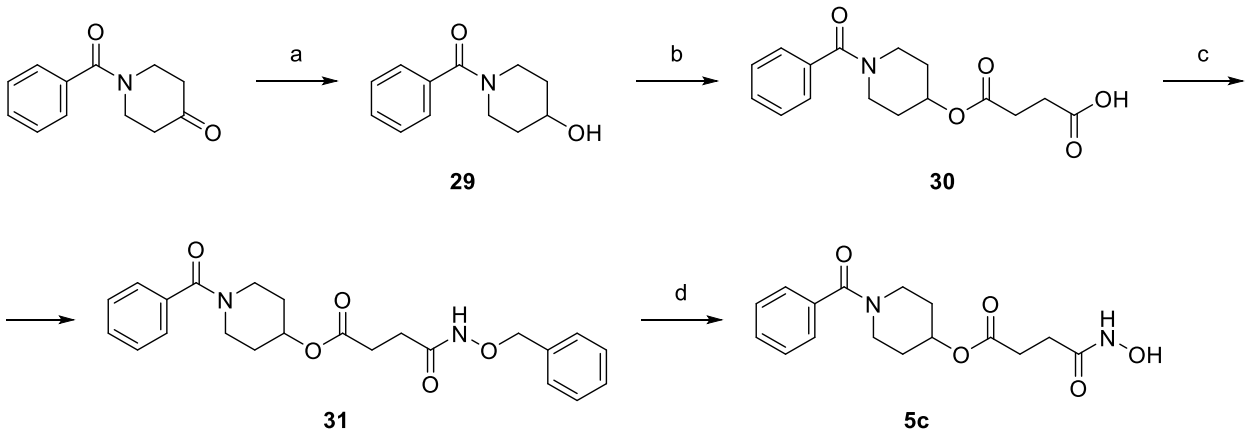
Scheme 2. Reagents and conditions: (a) 30% aq. formaldehyde (3 equiv.), saturated solution of $NaHCO_3$, r.t., overnight, 30% yield; (b) 4-benzyl-piperidone (0.75 equiv.), *p*TSA (cat.), N_2 ,Dean-Stark trap, 24 h, 48% yield; (c) NaCl (1.2 equiv.), DMSO, 180 °C, 4 h, 87% yield; (d) 50% aq. NH_2OH, EtOH, r.t., 24 h, 12% yield.

The synthesis of compounds with the aliphatic linear linker (**5**, **5a-b**) is reported in **Scheme 3**. The aliphatic linker was prepared by reaction of succinic anhydride with butanol, in dry toluene, at 90°C, overnight, to give the methyl hemisuccinate **22**. The free carboxylic group of **22** was reacted with 1-benzylpiperazine (for **23**), N-benzoylpiperazine (for **24**), or 4-benzylpiperidine (for **25**), using EDC and HOBt as coupling agents in DMF, to provide the corresponding amides (**23-25**) [31]. Unexpectedly, the reaction of **23-25** with 50% aq. hydroxylamine did not lead to the corresponding hydroxamate due to the parallel attack of the hydroxylamine to the amide carbonyl group. Nevertheless, using the less reactive O-benzylhydroxylamine in the presence of trimethylaluminum as an activator of the ester carbonyl group, the protected hydroxamic acid **26-28** were successfully obtained in good yield. The deprotection of the O-benzyl hydroxamates **26-28** was performed by hydrogenolysis over palladium/charcoal, using the ThalesNano H-Cube flow reactor, to give **5**, **5a-b** in quantitative yield.



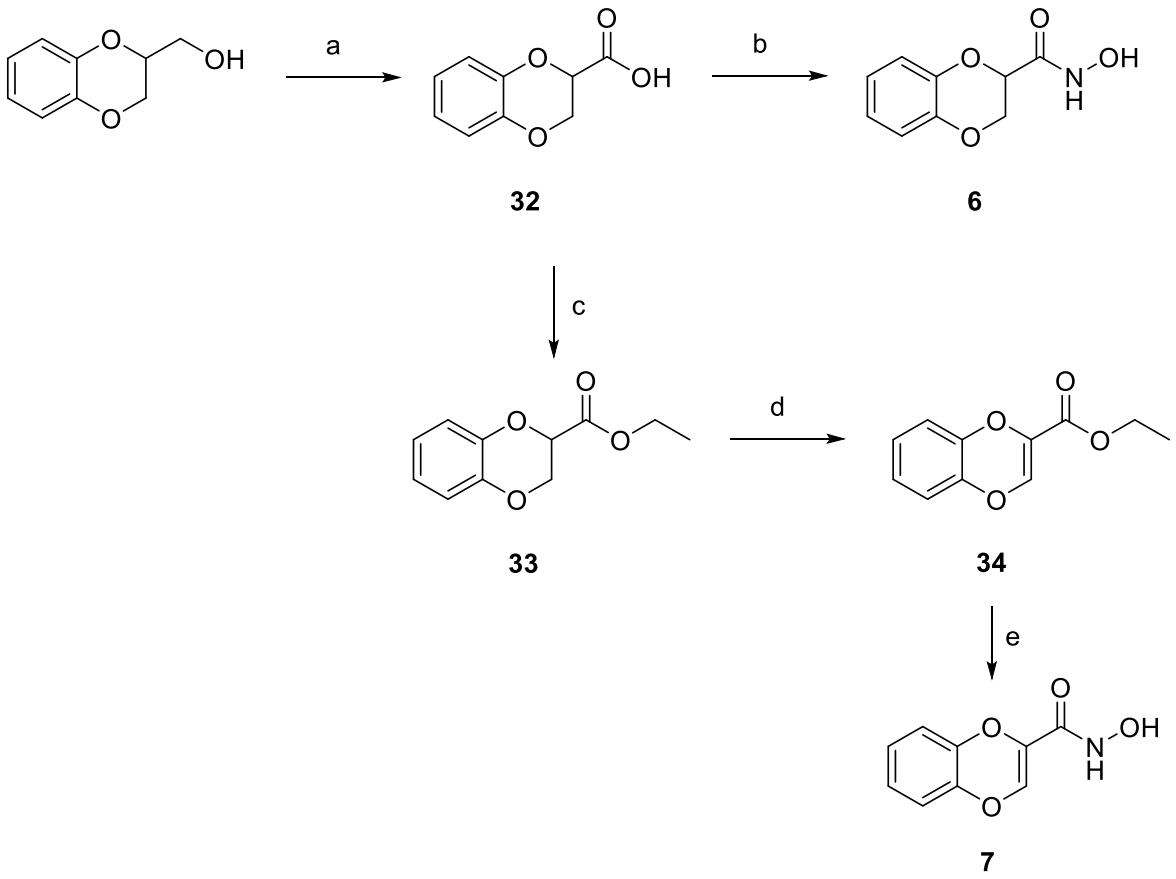
Scheme 3. Reagents and conditions (a) ButOH (1.2 equiv.), dry toluene, 90 °C, overnight, 90% yield. (b) appropriate amine (1 equiv.), EDC·HCl (1 equiv.), HOBt (1 equiv.), DMF, 0 °C to r.t., overnight, 70% yield (for **23**) and 78% yield (for **24**), and 67% yield (for **25**). (c) O-benzylhydroxylamine (4 equiv.), 2M $(CH_3)_3Al$ in diethyl ether (4 equiv.), dry DCM, r.t., N_2, 2h, 72% yield (for **26**), 39% yield (for **27**), and 59% yield (for **28**). (d) H-Cube ThalesNano H_2-Pd/C, MeOH, 60 °C, 20 bar, 1 mL / min, quantitative yield.

For the synthesis of **5c**, 4-benzoyl-piperidone was first reduced to alcohol **29** using sodium borohydride in methanol, at room temperature, overnight (**Scheme 4**). The intermediate **29** was then converted into the corresponding hemisuccinate **30** by reaction with succinic anhydride in DMF, under microwave irradiation. The reaction of **30** with O-protected hydroxylamine, followed by hydrogenolysis, led to the final compound **5c**.



Scheme 4. Reagents and conditions: (a) $NaBH_4$ (1.5 equiv.), methanol, r.t., 12 h, 87% yield; (b) succinic anhydride (2 equiv.), DMF, MW, 150 °C, 150 W, 1.5 h, 57% yield; (c) *i.)* Ethyl-chloroformate (1.2 equiv.), TEA (1.3 equiv.), dry DCM, N_2, 0 °C to r.t., 2 h; *ii.*) O-benzylhydroxylamine (1.2 equiv.), TEA (1.2 equiv.), dry DCM, r.t., nitrogen atmosphere 2 h, 42% yield; (d) H-Cube, 10% Pd/C cartridge, H_2, 20 psi, 60 °C, ethanol, quantitative yield.

The synthesis of compounds with an aromatic heterocyclic linker, such as benzodioxane (**6**) and benzodioxine (**7**), is described in **Scheme 5**. The commercially available (2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methanol was readily oxidized to carboxylic acid **32** with alkaline potassium permanganate, in water, at 60 °C. The acid group was then activated by reaction with ethyl chloroformate to generate *in situ* a mixed anhydride which reacted quickly with hydroxylamine hydrochloride to give the hydroxamic acid **6**. For the synthesis of the benzodioxine **7**, the carboxylic function of **32** was protected first by esterification with thionyl chloride, in ethanol to provide the corresponding ethyl ester **33**.

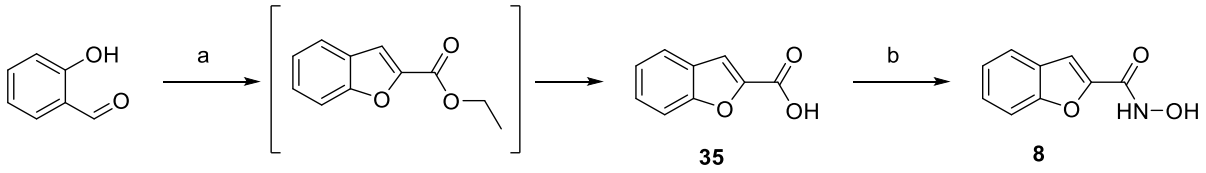


Scheme 5. Reagents and conditions: (a) $KMnO_4$ (1.4 equiv.), KOH (0.5 equiv.), H_2O, r.t. to 60 °C, 4h, 73% yield. (b) *i*. Ethyl chloroformate (1.2 equiv.), DCM dry, N_2, 0 °C, 1 h; *ii*. K^+ $^-$NHOH (2 equiv.), ethanol, 0 °C, 10 min. (c) $SOCl_2$ (1 mL), EtOH, r.t., overnight, 92% yield. (d) *i.* N-Bromosuccinimide (2 equiv.), AIBN (0.30 equiv.), CCl_4, reflux, N_2, h.v., 12h; *ii.* NaI (3 equiv.), acetone, reflux, 2h, 51% yield over two steps. (e) NaOH (8 equiv.), 50% w/w aqueous hydroxylamine (42 equiv.), THF/EtOH 1:1, 0 °C to r.t., 10 minutes, 63% yield.

The oxidation of the benzodioxane **33** to benzodioxine **34** was performed using one pot reaction. **33** was first brominated with N-bromo-succinimide (NBS) and catalytic AIBN as a radical initiator, under UV irradiation, in carbon tetrachloride. Thus the di-halogenated benzodioxane (not isolated) was de-brominated *in situ* with NaI, in refluxing acetone, to afford **34** in 51% yield.

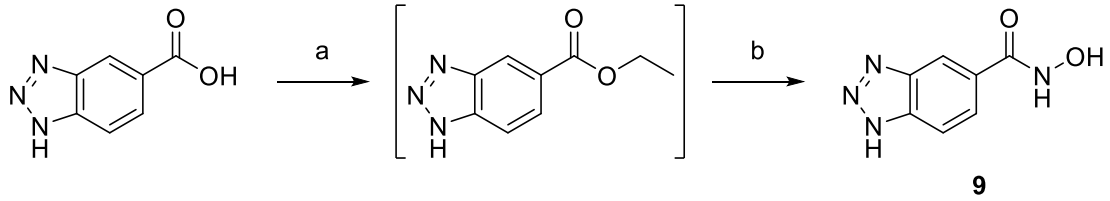
The synthesis of the benzofuran derivative **8** was performed as depicted in **Scheme 6**. Salicylaldehyde was reacted first with ethyl bromoacetate in standard S_N2 conditions followed by spontaneous intramolecular cyclization to give ethyl benzofuran-2-carboxylate, which was directly hydrolyzed to carboxylic acid **35** by refluxing alkaline water. The activation of the carboxylic acid

with ethyl chloroformate, followed by the addition of potassium hydroxamide, afforded the desired hydroxamic acid **8**.



Scheme 6. Reagents and conditions: (a) *i.* K_2CO_3 (2 equiv.) and ethyl 2-bromoacetate (1.1 equiv.), DMF, 150 °C. *ii.* H_2O, reflux, 4 h, 60% yield over two steps. (b) *i.* Ethyl chloroformate (1.2 equiv.), DCM dry, N_2, 0 °C, 1 h; *ii.* $K^+ {}^-NHOH$ (2 equiv.), ethanol, 0 °C, 10 min, 31% yield.

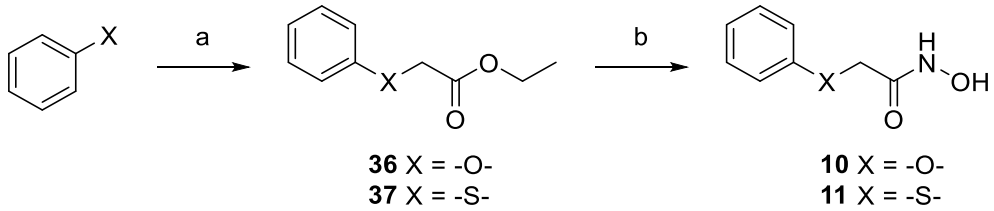
For the synthesis of compound **9**, the commercially available benzotriazole-5-carboxylic acid was first esterified as previously reported. The following addition of a solution of freshly prepared sodium hydroxyamide provided the corresponding hydroxamate **9** (**Scheme 7**).



Scheme 7. Reagents and conditions (a) $SOCl_2$ (1 mL), EtOH, r.t., overnight. (b) NaOH (8 equiv.) in 50% w/w aqueous hydroxylamine (42 equiv.), EtOH/THF 1:1, 0 °C to r.t, 10 minutes, 35% yield.

The synthesis of compounds with an aromatic linear linker (**10** and **11**) was easily obtained, as depicted in **Scheme 8**. Phenol (for **10**) or thiophenol (for **11**) were reacted with ethyl bromoacetate in standard S_N2 conditions, using potassium carbonate as base. The experimental conditions were slightly modified according to the reactivity of the substrate. The less reactive phenol was reacted in DMF at 60 °C to provide the ethyl phenoxyacetate **36** in quantitative yield [32]. On the contrary, the less stable thiophenol was reacted in acetone, at room temperature, to avoid the formation of the

disulphide under alkaline conditions [33]. The ethyl 2-thiophenylacetate **37** was obtained in 54% yield, alongside with the expected disulphide. Lastly, acetates **36** and **37** were converted into the corresponding hydroxamic acids **10-11** using a large excess of 50% w/w aqueous hydroxylamine, in ethanol, at room temperature for 2-24 hours.

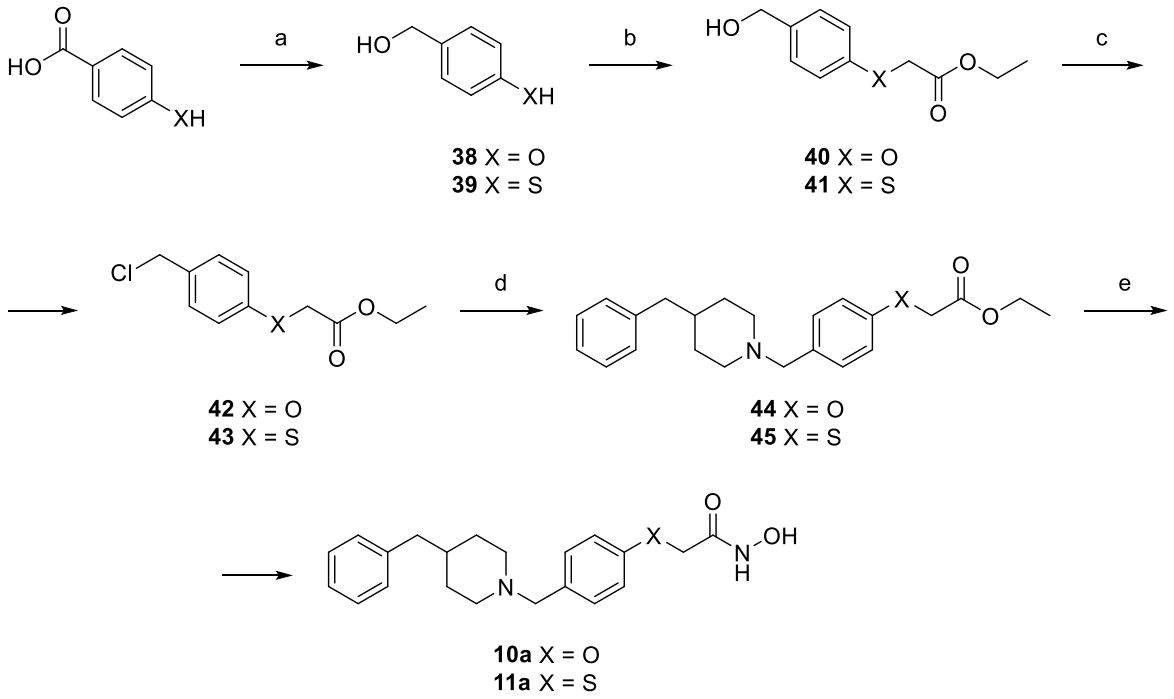


36 X = -O-
37 X = -S-

10 X = -O-
11 X = -S-

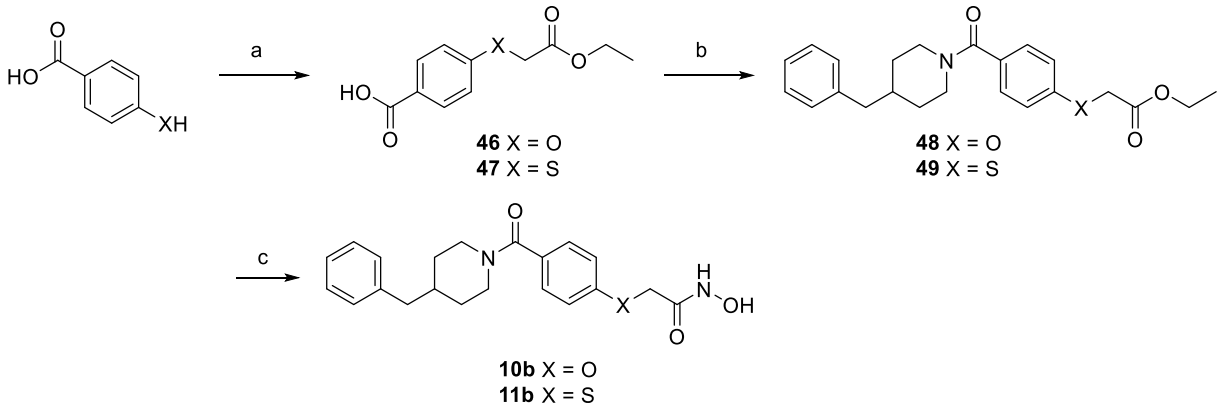
Scheme 8. Reagents and conditions (a) K_2CO_3 (2.5 equiv.), ethyl 2-bromoacetate (1.2 equiv.), DMF, 60 °C, 3h, 94% yield (for **36**). K_2CO_3 (2.5 equiv.), ethyl 2-bromoacetate (1.2 equiv.), Acetone, r.t., overnight, 56% yield (for **37**). (b) 50% w/w aqueous hydroxylamine (30 equiv.), EtOH, r.t., 2 h, 43% yield (for **10**) or 24 h, 87% yield (for **11**).

For the synthesis of compounds **10a** and **11a**, bearing a cap-group, 4-hydroxybenzoic acid or 4-mercaptobenzoic acid was first reduced to the corresponding benzyl alcohol **38** or **39**, which was further reacted with ethyl bromoacetate in the presence of a base to give the ester **40** or **41** (**Scheme 9**) [33]. The benzyl alcohols **40** and **41** were converted into the benzyl chlorides **42** and **43**, and then reacted with benzylpiperidine to provide the intermediates **44** and **45**. These were lastly converted into the hydroxamate **10a** and **11a** as previously described.



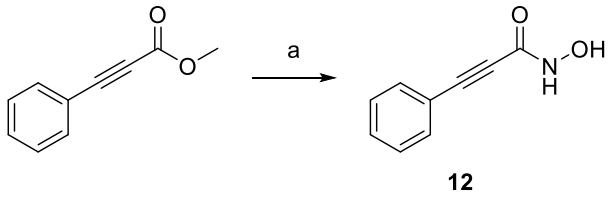
Scheme 9. Reagents and conditions: (a) $LiAlH_4$ (2.2 equiv.), dry THF, 0 °C, nitrogen atmosphere, 6h, 86% yield (for **38**) and 97% yield (for **39**). (b) ethyl 2-bromoacetate (1.2 equiv.), K_2CO_3 (2.5 equiv.), acetone, r.t., 6h, 46% yield (for **40**) and 70% yield (for **41**). (c) $SOCl_2$ (1.2 equiv.), DMF (drop), dry DCM, 0 °C, 2h, quantitative yield. (d) 4-benzylpiperidine (1.1. equiv.), K_2CO_3 (1.5 equiv.), DMSO, r.t. to 60 °C, 6h, 23% yield (for **44**) and 32% yield (for **45**). (e) aqueous hydroxylamine (30 equiv.), EtOH, r.t., overnight, 59% yield (for **10a**) and 37% yield (for **11a**).

Compounds **10b** and **11b** were obtained in three steps (**Scheme 10**). The commercially available 4-hydroxybenzoic or 4-mercaptobenzoic acids was first reacted with ethyl bromoacetate to obtain the intermediate **46** or **47**, respectively. The latter were then coupled with benzylpiperidine, using EDC/HOBt coupling reagents [31], to give the intermediates **48** and **49** which were easily converted into the hydroxamates **10b** and **11b**, using the same reaction conditions described above.



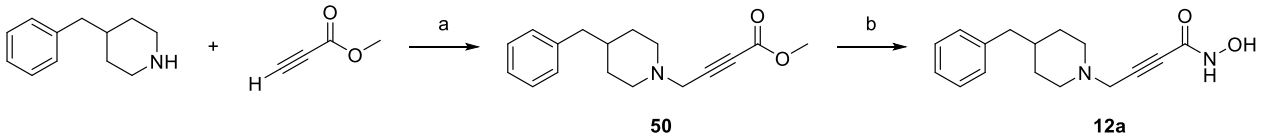
Scheme 10. Reagents and conditions: (a) ethyl bromoacetate (1.2 equiv.), K_2CO_3 (2.5 equiv.), DMF, r.t., 48h, 83% yield (for **46**) and 77% yield (for **47**). (b) 4-benzylpiperidine (1 equiv.), EDC·HCl (1 equiv.), HOBt (1 equiv.), DMF, 0 °C to r.t., overnight, 92% yield (for **48**) and 49% yield (for **49**). (c) aqueous hydroxylamine (30 equiv.), EtOH, r.t., 24h, 65% yield (for **10b**) and 47% yield (for **11b**).

The synthesis of the two compounds with an unsaturated linear linker, without (**12**) and with CAP group (**12a**), was performed as reported in **Scheme 11** and **12**, respectively. **12** was readily obtained from the commercially available methyl 3-phenylpropiolate by reaction with sodium hydroxyamide in 40% yield.



Scheme 11. Reagents and conditions (a) NaOH (8 equiv.), aqueous hydroxylamine (42 equiv.), MeOH/THF 1:1, 0 °C, 5 minutes.

Finally, **12a** was obtained from the ester **50** which was previously prepared by a three-component reaction involving methyl propiolate, benzylpiperidine and paraformaldehyde, using copper(I) iodide as catalyst.



Scheme 12. Reagents and conditions (a) paraformaldehyde (2 equiv.), copper(I) iodide (0.1 equiv.), DMSO, r.t., 30 min., 67% yield (b) 50% w/w aqueous hydroxylamine (3 equiv.), EtOH, r.t., 1h, 63% yield.

1.2. HDACs Inhibition Studies

All the synthesized compounds were evaluated *in vitro* for their inhibitory activity against purified human HDAC1 and HDAC6 enzymes using a fluorescence-based activity assay [34]. Compounds **1-5** were tested as racemic mixtures. The compounds were screened first at 50 μM, and the results were expressed as % of the inhibitory activity. The compounds showing an inhibition >50% were further assessed at lower concentrations (10 and 1 μM). Vorinostat (SAHA), the pan-inhibitor, and tubastatin, an HDAC6 selective inhibitor, were used as reference compounds at 5 μM concentration. The inhibitory activity of all compounds is reported in **Table 1**.

Table 1. Inhibitory activity of the tested compounds against purified HDAC1 and HDAC6 enzymes.

Compound	%inhibition HDAC1[a]			%inhibition HDAC6[a]		
	50 μM	10 μM	1 μM	50 μM	10 μM	1 μM
***Cis*-1**	33±8	-	-	37±9	-	-
***Trans*-1**	25±9	-	-	44±5	-	-
***Cis*-2**	63±3	49±1	-	75±2	55±1	1±2
***Trans*-2**	87±7	56±5	51±2	73±2	63±3	3±2
3	41±9	-	-	31±2	-	-
4	70±4	47±1	-	50±7	-	-
4a	67±8	48±2	-	6±9	-	-
4b	83±6	41±6	-	11±5	-	-
4c	91±4	57±5	47±5	40±2	-	-
5	31±3	-	-	79±8	67±5	62±5
5a	48±3	-	-	74±4	69±2	2±4
5b	74±3	59±1	5±4	83±7	75±7	54±4
5c	50±1	25±6	-	72±9	59±1	33±1
6	78±2	62±3	51±8	20±9	-	-
7	90±5	72±1	60±1	56±2	50±2	17±1
8	77±1	62±2	50±1	87±2	76±9	2±1
9	82±5	64±4	38±1	80±4	68±4	11±2

10	82±5	57±8	49±6	78±4	56±5	3±2
10a	96±8	87±9	67±4	51±6	31±5	-
10b	85±5	85±2	18±14	87±3	29±4	-
11	88±2	73±1	55±1	87±4	65±6	3±1
11a[b]	92±4	92±3	53±8	63±9	52±7	10±1
11b	89±9	79±9	51±4	87±1	59±8	4±1
12	12±5	-	-	77±2	41±6	-
12a	40±6	-	-	92±8	64±4	3±1
SAHA[c]	100			95		
Tubastatin[c]	51			89		

[a] Values were measured in triplicates. Percentages of inhibition are reported as mean ± SD. [b]compound **11a**, IC_{50} = 0.58 μM ± 0.13 against HDAC1 [c] Tested at 5 μM for both HDAC1 and HDAC6.

The tested compounds showed an enzymatic inhibitory activity in the micromolar range, with a percentage of inhibition at 50 μM ranging between 12-88% against HDAC1 and 6-87% against HDAC6. Despite the moderate inhibitory activity, our first goal was to understand if selectivity could be achieved by modulating the linker portion. Indeed these results demonstrate that, depending on the linker type, some candidates show an activity profile shifted toward HDAC1 over HDAC6 (compounds ***trans*-2**, **7**, **10a**, **11a**) or vice versa (**5**, **5b**). Within the series of HDAC1 selective inhibitors, **11a** is the most potent with an IC_{50} value of 0.58 μM (see **Figure SI-1** in the Supporting Information).

Therefore, these novel linker chemotypes, adequately decorated with selective cap groups, could represent valuable starting points for further hit optimization in the search of more potent and isoform-selective HDACs inhibitors. Docking calculations were performed to rationalize the effect of the novel linker on biologic activity and selectivity.

1.3. Molecular modelling

Although HDAC1 and HDAC6 belong to the same protein family, their binding sites present peculiar features, resulting in different ligand selectivity [28]. Superimposition of the 4BKX (HDAC1) [35] and 5EDU (HDAC6) [36] crystal structures (see **Figure SI-2** in the Supporting

Information) shows that the HDAC1 and HDAC6 active sites present similar residues. However, compared to HDAC6, HDAC1 shows a narrower pocket at the cap region entrance but a wider pocket in the zinc and linker regions, thus resulting in different active site shapes. The latter feature is due to a broader separation of Phe150 and Phe205 residues in HDAC1 with respect to the corresponding Phe620 and Phe680 residues of HDAC6. To help rationalize the effect of structurally different linkers on the selectivity of the synthesized compounds, docking calculations were performed into the 4BKX [35] and 5EDU [36] crystal structures, as described in the Methods section. Visual inspection of the predicted docking poses in the two targets shows that the hydroxamic acid moiety of the investigated compounds coordinates the catalytic Zn^{2+} ion, in line with other inhibitors bearing this ZBG [37]. To evaluate the effect of stereochemistry on enzymatic activity, the diastereomeric couple cis/trans-**1** and **2** were prepared and tested. According to the predicted docking poses, the 1,3-dioxolane moiety of *cis*-**2** and *trans*-**2** binds near to the Phe150, His178, Phe205, and Tyr303 (HDAC1), and the corresponding Phe620, His651, Phe680, and Tyr782 residues in HDAC6. Moreover, the pyridine nitrogen of these compounds is located near Phe205 and Leu749 in HDAC1, and the Phe680 and Leu271 residues in HDAC6, with the bromine substituent extending toward the outer enzyme surface. Indeed, *trans*-**2** (**Figure 3**, panels *a* and *b*) fits slightly better than *cis*-**2** within the HDAC1 and HDAC6 binding sites due to the 2R and 4S stereochemistry of the 1,3-dioxolane ring, which results in lower steric hindrance. Interestingly, compounds bearing a bulkier linker portion, namely 1,4-dioxa-8-azaspiro (**4-4b**) and the 1,5-dioxa-9-azaspiro (**4c**), docked better into the HDAC1 active site, mainly because of the wider pocket of HDAC1 in the zinc and linker binding regions described above. Docking calculations and *in vitro* assays also suggested that compounds **5-5c** could not bind efficiently to the hydrophobic tunnel of HDAC1 and reach the catalytic Zn^{2+} ion, due to the steric clashes of the piperazine or piperidine rings with the Phe150 and Phe205 side chains. On the contrary, favourable binding modes, of *e.g.* compound **5**, were found in HDAC6 (**Figure 3**, panel *d*), which has a wider entrance in the cap region. As a result, ligands bearing these linker chemotypes are inactive or significantly less active

against HDAC1 compared to HDAC6 (*e.g.*, **5**, **5a**, **5b** and **5c**). Partially unsaturated aromatic heterocyclic linkers, such as the 1,4-benzodioxane moiety of compound **6**, is well accommodated in the Phe150, His178 and Phe205 tunnel of HDAC1. This compound could not dock into the narrower hydrophobic tunnel of HDAC6, in line with the observed selectivity for HDAC1, due to steric clashes between the 1,4-benzodioxane moiety and the Phe620, His651 and Phe680 side chains of HDAC6 (**Figure 3**, panels *g* and *h*). Molecules with aromatic heterocyclic linkers directly attached to the hydroxamate (*e.g.*, **7**, **8** and **9**) could dock in both HDAC1 and HDAC6 binding sites. This result is in agreement with their enzymatic inhibitor profile. An example of the binding mode is shown in **Figure 3**, panel *e* and *f*, for compound **7**. Compounds based on aromatic/unsaturated linkers, such as the 2-phenoxyethane (*e.g.*, **10-10b**) and 2-(phenylthio)ethane (*e.g.*, **11-11b**) moieties, resulted to be more active on HDAC1. According to the predicted binding modes, the hydroxamic acid of these compounds coordinates the catalytic Zn^{2+} ion in HDAC1, and the phenyl ring establishes favorable π-π stacking interactions with the Phe150 and Phe205 residues. **Figure 3**, panel *c*, shows the binding mode of **10** into HDAC1. Compound **12** with an alkyne linker and a phenyl ring could dock better in HDAC6, due to π- π interactions with the two phenylalanine residues.

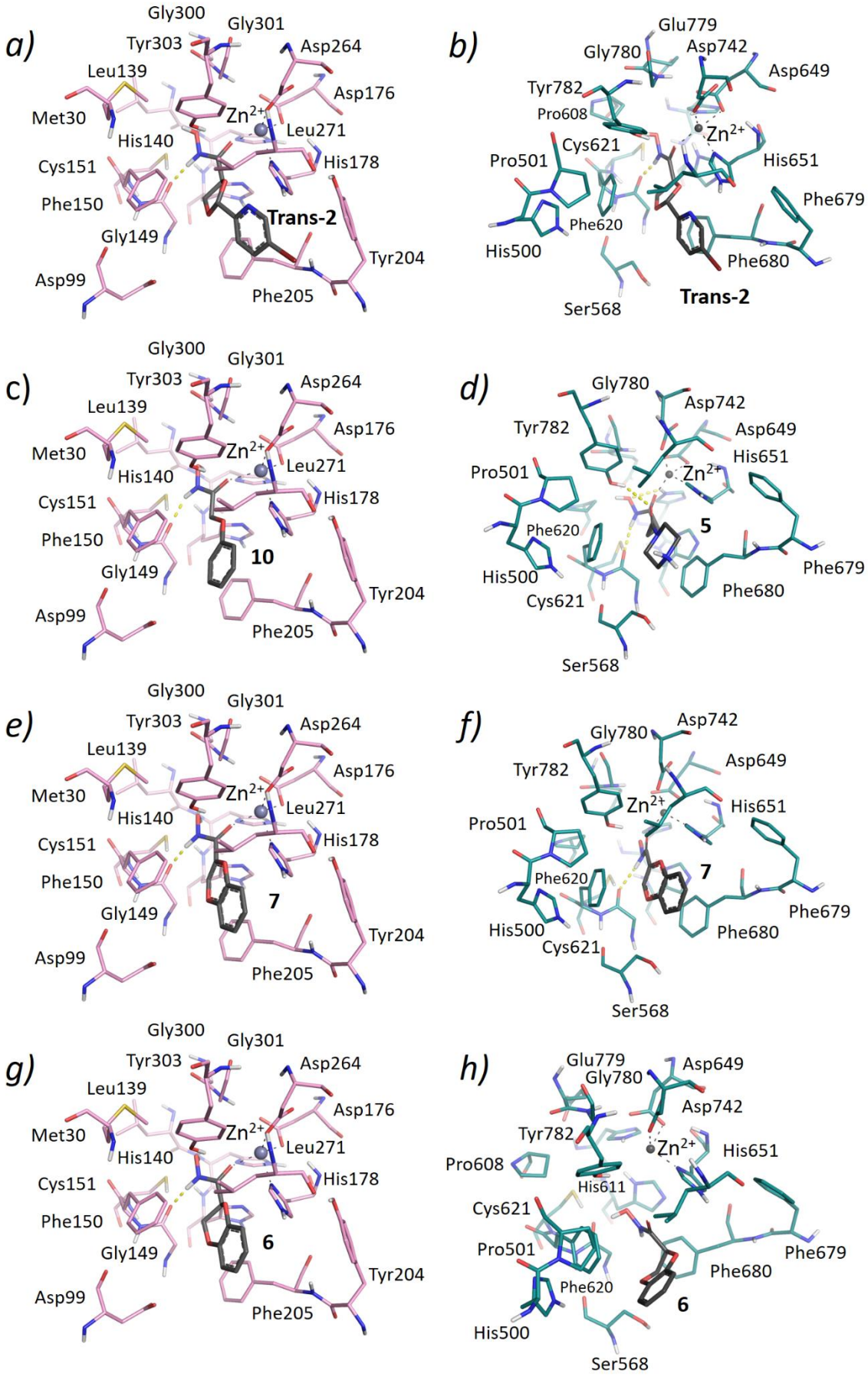

a)
Gly300
Gly301
Tyr303
Asp264
Leu139
Asp176
Met30
Zn2+
Leu271
His140
His178
Cys151
Phe150
Trans-2
Gly149
Tyr204
Asp99
Phe205
b)
Glu779
Gly780
Asp742
Asp649
Tyr782
Pro608
Zn2+
Cys621
Pro501
His651
Phe679
Phe620
His500
Phe680
Ser568
Trans-2
c)
Gly300
Gly301
Tyr303
Asp264
Leu139
Asp176
Met30
Zn2+
Leu271
His140
His178
Cys151
Phe150
10
Gly149
Tyr204
Asp99
Phe205
d)
Gly780
Asp742
Tyr782
Asp649
His651
Pro501
Zn2+
Phe620
5
Phe679
His500
Phe680
Cys621
Ser568
e)
Gly300
Gly301
Tyr303
Asp264
Leu139
Asp176
Met30
Zn2+
Leu271
His140
His178
Cys151
Phe150
7
Gly149
Tyr204
Asp99
Phe205
f)
Gly780
Asp742
Tyr782
Asp649
Zn2+
His651
Pro501
Phe620
7
Phe679
His500
Phe680
Cys621
Ser568
g)
Gly300
Gly301
Tyr303
Asp264
Leu139
Asp176
Met30
Zn2+
Leu271
His140
His178
Cys151
Phe150
6
Gly149
Tyr204
Asp99
Phe205
h)
Glu779
Gly780
Asp649
Asp742
Tyr782
Zn2+
His651
Pro608
His611
Cys621
Pro501
Phe679
Phe680
Phe620
6
His500
Ser568

Figure 3. Binding modes evaluated for representative compounds of the series into 4BKX (HDAC1) and 5EDU (HDAC6). 4BKX and 5EDU are coloured in pink and deep teal, respectively. Predicted docking poses are represented in dark grey sticks. Panels *a*, *c*, *e*, and *g* show the predicted binding mode for *trans*-**2**, **10**, **7,** and **6** into 4BKX, respectively. Panels *b*, *d*, *f*, and *h* show the predicted binding mode for *trans*-**2**, **5**, **7,** and **6** into 5EDU, respectively. The image was created with PyMOL (The PyMOL Molecular Graphics System, Version 1.8, Schrödinger, LLC).

2.4. Cellular studies

Based on *in vitro* inhibition studies, the selected compounds ***cis*-2, *trans*-2, 5, 5b, 7, 8, 10a, 10b, 11a,** and **11b**, showing an activity >50%, were further tested in MCF7 cells to evaluate their effect on the acetylation levels of histone H3, one of the main histones under control of HDAC1, and α-tubulin (HDAC6 target). Compounds that showed a significant increase of the acetylation levels at 50 μM were further investigated at the lower concentrations of 10 μM and 1 μM (**Figure 4**).

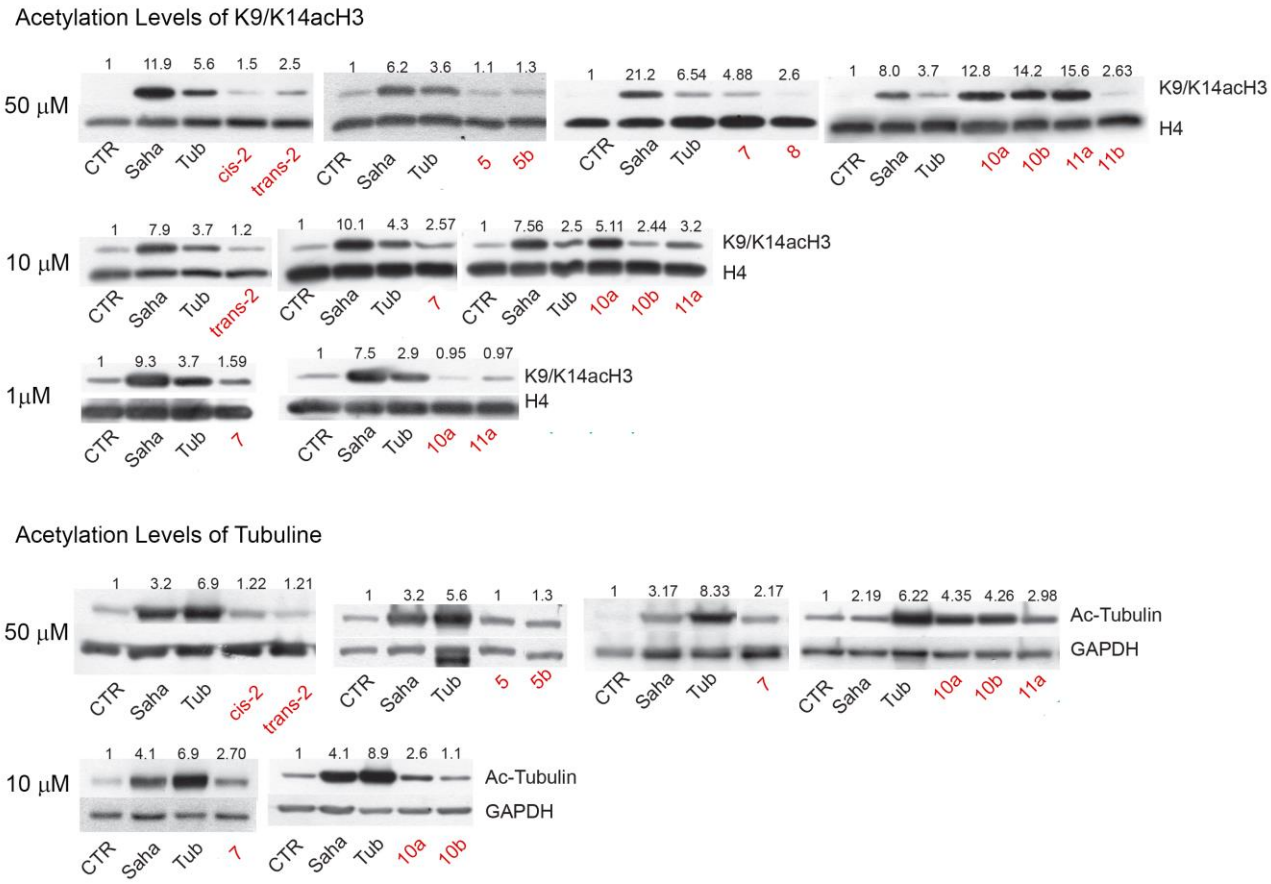


Figure 4. Western blotting analysis of the tested and reference compounds SAHA and Tubastatine on acetylation levels of histone H3 and α-tubulin in MCF7 cells. GAPDH was used as a loading control. Densitometric values were analyzed using ImageJ Software and are reported on the top of bands as the ratio between loading control and acetylated target.

In the series of compounds with an aliphatic heterocyclic linker, ***trans*-2** was the only compound that slightly increased the acetylation of histone H3 at 50 µM (2.5-fold vs control) whilst the effect was not retained at lower concentration (10 µM). As expected, the corresponding ***cis*-2** isomer, less active than the former, showed a reduced level of K14acH3 (1.5-fold). Conversely, regarding the acetylation of α-tubulin, a comparable increase was observed for both ***cis*-** and ***trans*-2** isomers. This is in agreement with their inhibitor activity against HDAC-6 at 50 µM (75% and 73% for ***cis*-2** and ***trans*-2**, respectively).

In the series of compounds with an aliphatic linear linker, **5** and **5b** were chosen for their selectivity profile slightly shifted toward the HDAC-6 isoform (62% and 54% of inhibitor activity at 1 μM, respectively). Unexpectedly, at the cellular level, they did not show an appreciable increment of α-tubulin acetylation at 50 μM concentration. The lack of activity, in the cellular compartment, could be due to the low permeability of these compounds across the plasmatic membrane, probably associated with the high polarity of the piperazinyl moiety. Due to the promising selectivity profile of this series, further SAR studies are needed to better understand the activity/efficacy profile.

In the series of compounds with an aromatic heterocyclic linker, the two constrained analogues of **10**, the benzodioxine **7**, and the benzofuran **8** were able to promote the expression of K14acH3, **7** being more effective than **8** and showing efficacy in a dose-dependent manner (50, 10 and 1 μM). In addition, **7** showed a modest increment of the level of acetylated α-tubulin at 50 μM. These results are in accordance with the enzymatic inhibitor profile of **7** that is a valuable inhibitor of HDAC-1 (60% of inhibition at 1 μM), whereas it is less effective against HDAC-6.

In the series of compounds with an aromatic-linear linker, **10a-b** (phenoxy acetate) and **11a** (phenylthio acetate) are noteworthy for their highest efficacy, with a 12.6-15.6-fold increased level of histone H3 acetylation at 50 μM. This dose-dependent effect persists at the lower concentrations of 1 μM. Although to a lesser extent, **10a** was also able to modulate the expression of acetylated tubulin at 50 μM (4.35-fold) and 10 μM (2.6-fold). Overall, the efficacy of this series of compounds correlates well with isoform selectivity, which is mainly shifted towards HDAC1.

Lastly, **7**, **10a**, and **11a**, the best hit compounds in terms of enzymatic activity and cellular efficacy were selected to assess their antiproliferative activity in human breast cancer cell lines (MCF7). Cell viability was measured at 24, 48, and 72 h. As displayed in **Figure 5**, all compounds showed a significant reduction of cancer proliferation in a time and dose-dependent manner. The antiproliferative activity exerted by these compounds is in agreement with their inhibitory activity that is, in part, associated with the modulation of HDAC1 and HDAC6 isoforms.

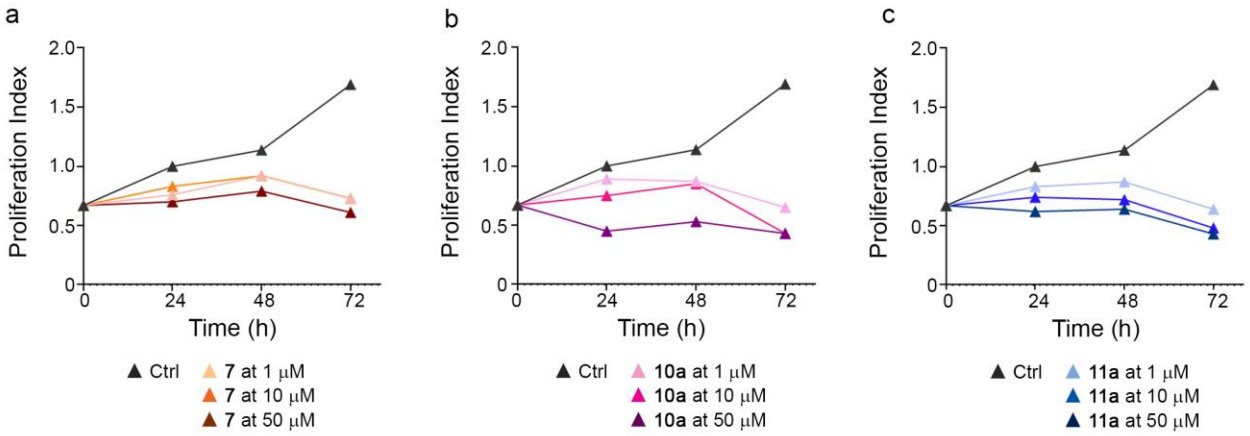


Figure 5. Cell viability assay (MTT) of the test compounds in human breast cancer cell lines (MCF7). Cells were treated with 3 increasing concentrations (1, 10, and 50 μM) of tested compounds and monitored at 24, 48, and 72 hours. Data are expressed as mean ± SEM for three independent experiments, each performed in duplicate.

3. Conclusions

In conclusion, a series of twenty-five novel HDACs inhibitors bearing unexplored linker chemotypes were synthesized and evaluated for their inhibitory activity against the HDAC1 and HDAC6 isoforms. Bulky (aliphatic or aromatic heterocyclic) linkers favor HDAC1 selectivity while aromatic linear (phenoxyacetate/thioacetate) linkers shift the selectivity towards HDAC6. Docking studies were performed to rationalize the effect of the novel linker chemotypes on inhibitory activity and selectivity. So far, the effect of different linker chemotypes has been relatively less explored. Our study shows that, depending on the linker, selectivity can be directed toward HDAC1 or HDAC6 isoform, thus giving the opportunity to medicinal chemists to exit from the conventional chemical space widely explored for HDACs inhibitors. The best compounds were tested in a cell-based study to evaluate the acetylation levels of H3 (HDAC1 target) or α-tubulin (HDAC6 target). Increased levels of acetylation were seen for ***trans*-2**, **7**, **10a**, **11a**. In addition, compounds **7**, **10a,** and **11a** showed a significant reduction of proliferation in MCF7 human breast cancer cell line.

Taken together, these results will set the basis for advancing the design and discovery of more potent and selective HDACs inhibitors to fight cancer.

4. Experimental

4.1. Chemistry

All the reagents, solvents, and other chemicals were used as purchased without further purification unless otherwise specified. Air- or moisture-sensitive reactions were performed under argon atmosphere. Reactions were monitored by thin-layer chromatography on silica gel plates (60F-254, E. Merck) and visualized with UV light, cerium ammonium sulfate, alkaline KMnO4 aqueous solution, or 1% $FeCl_3$ ethanolic solution. Column liquid chromatography (LC) purifications were carried out using Merck silica gel 60 (230-400 mesh, ASTM). Flash chromatography purifications were performed with the ISOLERA-Biotage system. All the hydrogenations were performed with the ThalesNano H-Cube Mini Plus flow reactor. The structures of all isolated compounds were ensured by nuclear magnetic resonance (NMR) and mass spectrometry. ^{1}H and ^{13}C NMR (1D and 2D experiments) spectra were recorded on a DPX-400 Avance (Bruker) spectrometer at 400 MHz or on a DPX-600 Avance (Bruker) spectrometer at 600 MHz. Chemical shifts are expressed in ppm (δ) and calibrated on the residue signal of the solvent: $CDCl_3$ δ 77.04, CD_3OD δ 49.8, DMSO-d_6 δ 39.5. NMR data are reported as follows: chemical shift, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; qnt, quintet; sxt, sextet; m, multiplet; br, broad), coupling constants (Hz) and number of protons/carbons. ^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H-^{13}C heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC) experiments were recorded for the determination of ^{1}H-^{1}H and ^{1}H-^{13}C correlations, respectively. Elemental analysis was performed on a C, H, N, S CE Instruments EA 1110. The following solvent, reactive and chemical moieties were abbreviated: ethyl acetate (AcOEt), dimethylsulfoxide (DMSO), dichloromethane (DCM), cyclohexane (CE), diethyl ether (Et_2O), methanol (MeOH), ethanol

(EtOH), tetrahydrofuran (THF), dimethylformamide (DMF), triethylamine (TEA), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), hydroxybenzotriazole (HOBt), N-bromosuccinimide (NBS), azobisisobutyronitrile (AIBN), piperidine (Pip), piperazine (Pipz), benzyl (Bn), benzoyl (Bz), furan (Fur), diox (Dioxane), dioxi (Dioxin).

4.1.1. General procedure for the synthesis of hydroxamic acids ***1-12***

4.1.1.1. METHOD A: To a solution of aliphatic esters (1equiv.) in ethanol, NH_2OH 50% w/w in H_2O (30 equiv.) was added at room temperature. The reaction was stirred for 1-48 hours and concentrated. The solvent was evaporated under reduced pressure, and the residue was diluted with water. The aqueous solution was neutralized to pH 7 with 1N aqueous HCl and extracted with AcOEt. The organic layer was dried over anhydrous Na_2SO_4 and concentrated. The final compound was purified by chromatography or crystallization.

4.1.1.2. METHOD B: To a solution of carboxylic acid (1 equiv.) in dry DCM, at 0°C and under nitrogen atmosphere, ethyl chloroformate (1.2 equiv.) was added. The mixture was stirred in the same conditions for 1 hour. Thereafter, an ethanolic solution of potassium hydroxamide, prepared by dissolving hydroxylamine hydrochloride (2 equiv.) and potassium hydroxide (2 equiv.) in ethanol, was added dropwise to the first solution at 0 °C. The reaction was stirred for a further 15 minutes and concentrated. The solvent was evaporated under reduced pressure and the residue was diluted with water. The aqueous solution was neutralized to pH 7 with 1N aqueous HCl and extracted with AcOEt. The organic layer was dried over anhydrous Na_2SO_4 and concentrated. The final compound was purified by chromatography or crystallization.

4.1.1.3. METHOD C: NaOH (8 equiv.) was solubilized in 50% w/w aqueous hydroxylamine (42 equiv.) at 0 °C. The appropriate aromatic ester (1 equiv.) solubilized in THF/EtOH 1:1 was added dropwise, and the mixture stirred at room temperature for 10 minutes. The reaction was quenched with glacial acetic acid (8 equiv.) and concentrated under reduced pressure. The residue was diluted with water and neutralized with 1N aqueous HCl. The aqueous solution was extracted in AcOEt,

and the organic phase washed with NH_4Cl, dried over anhydrous Na_2SO_4, and concentrated. The final compound was purified by chromatography or crystallization.

4.1.1.4. METHOD D: Appropriate O-benzylhydroxylamine derivates were solubilized in MeOH, and the hydrogenation was performed with H-Cube Mini Plus ThalesNano with the following conditions: Temperature 60 °C, H_2 20 psi, cartridge Pd/C, solvent MeOH, flow 1mL/min. The solvent was concentrated to give pure hydroxamic acid.

*4.1.1.1.1. Cis-N-hydroxy-2-phenyl-1,3-dioxolane-4-carboxamide (**cis-1**)*

According to Method A. Chromatographed over silica gel: ratio crude/silica gel 1:100 DCM 100%. TLC: DCM:MeOH 95:5 Rf=0.34. Pale yellow liquid (66%yield). **^{1}H NMR** (400MHz, DMSO-d_6) HD48_C δ 4.25 (t,1H, J = 2.0 Hz, O-C$\underline{H}_2$CH); 4.50 (dd,1H, J = 2.0 Hz, OC$\underline{H}_2$CH); 4.71 (dd,1H, J = 1.0 Hz, OC$\underline{H}$CH$_2$); 5.83 (s,1H, OC$\underline{H}$O); 7.50-7.43 (m, 5H, C$\underline{H}_{Ar}$); 8.83 (s,1H, N$\underline{H}$). **^{13}C NMR** (100MHz, DMSO-d_6) δ 69.46 (O-$\underline{C}$H$_2$-CH); 74.14(CH$_2$-$\underline{C}$H-O); 105.46(O-$\underline{C}$H-O); 126.51 ($\underline{C}H_{Ar}$); 1.28.80($\underline{C}H_{Ar}$); 130.09($\underline{C}H_{Ar}$); 135.61($\underline{C}_{Ar}$); 168.15 ($\underline{C}$O). Anal. Calcd. for $C_{10}H_{11}NO_4$: C, 57.41; H, 5.30; N, 6.70; Found C, 57.40; H, 5.45; N, 6.72.

*4.1.1.1.2. Trans-N-hydroxy-2-phenyl-1,3-dioxolane-4-carboxylate (**trans-1**)*

According to Method A. Chromatographed over silica gel: ratio crude/silica gel 1:60 DCM/MeOH 95:5. TLC: DCM:MeOH 95:5 Rf=0.28. Orange liquid (56% yield). **^{1}H NMR** (400MHz, DMSO-d_6) HD49_A δ 3.95 (dd, 1H, *J* = 6.1 Hz, 8.3 Hz, OC$\underline{H}_2$CH); 4.35 (t, 1H, *J* = 8.0 Hz, OC$\underline{H}_2$CH); 4.60 (t, 1H, *J* = 8.0 Hz, OC$\underline{H}$CH$_2$); 5.91 (s, 1H, OC$\underline{H}$O); 7.45 – 7.35 (m, 3H); 7.54 – 7.44 (m, 2H), 9.45-9.96 (brs, 2H, N$\underline{H}$, O$\underline{H}$). **^{13}C NMR** (100MHz, DMSO-d_6) δ 67.97 (O$\underline{C}$H$_2$CH); 73.51 (CH$_2\underline{C}$HO); 103.89 (O$\underline{C}$HO); 126.67 ($\underline{C}H_{Ar}$); 128.17 ($\underline{C}H_{Ar}$); 129.31 ($\underline{C}H_{Ar}$); 137.08 (C_{Ar});166.46 ($\underline{C}$O). Anal. Calcd. for $C_{10}H_{11}NO_4$: C, 57.41; H, 5.30; N, 6.70; Found C, 57.43; H, 5.27; N, 6.68.

*4.1.1.1.3 Cis- 2-(5-bromopyridin-2-yl)-N-hydroxy-1,3-dioxolane-4-carboxamide (**cis-2**)*

According to Method A. Chromatographed over silica gel: ratio crude/silica gel 1:80 DCM/MeOH 95:5. TLC: DCM:MeOH 95:5 Rf=0.30. Yellow solid(49%yield). **^{1}H NMR** (400 MHz, CD_3OD) HD63_A δ 4.28(dd, 1H, *J* = 8.0, 12 Hz, OC$\underline{H}_2$CH); 4.37 (d, 1H, *J* = 8.0 Hz, OC$\underline{H}_2$CH); 4.73 (dd, 1H, *J* = 4.0, 8.0 Hz, OC$\underline{H}$CH$_2$); 5.90 (s, 1H, OC$\underline{H}$O); 7.63 (m, 1H, C$\underline{H}_{Ar}$); 8.13 (m, 1H, C$\underline{H}_{Ar}$); 8.76(d, 1H, *J* = 4.0 Hz, C$\underline{H}_{Ar}$). **^{13}C NMR** (100 MHz, CD_3OD) δ 71.11 (O$\underline{C}$H$_2$CH); 76.43 (O$\underline{C}$HCH$_2$); 105.31 (O$\underline{C}$HO); 122.97 (Br$\underline{C}_{Ar}$); 125.65 ($\underline{C}H_{Ar}$); 141.61 (CH_{Ar}); 151.46 (CH_{Ar}); 155.20 (C_{Ar}); 170.28(C=O). Anal. Calcd. for $C_9H_9BrN_2O_4$: C, 37.39; H, 3.14; N, 9.69; Found C, 37.35; H, 3.10; N, 9.61.

*4.1.1.1.4. Trans- 2-(5-bromopyridin-2-yl)-N-hydroxy-1,3-dioxolane-4-carboxamide (**trans-2**)*

According to Method A. Chromatographed over silica gel: ratio crude/silica gel 1:80 DCM/MeOH 95:5. TLC DCM:MeOH 95:5 Rf=0.27. Yellow liquid (70%yield). **^{1}H NMR** (400 MHz, CD_3OD) HD64_A δ 4.12 (dd, 1H, J = 8.0, 12 Hz, OC$\underline{H}_2$CH); 4.46 (t,1H, J = 8.0Hz, OC$\underline{H}_2$CH); 4.78 (t, 1H, J = 8.0Hz, OC$\underline{H}$CH$_2$); 5.99 (s, 1H, OC$\underline{H}$O); 7.58 (d, 1H, J = 8.0Hz, C$\underline{H}_{Ar}$); 8.09 (t, 1H, J = 8.0Hz, C$\underline{H}_{Ar}$); 8.69(d, 1H, J = 4.0Hz, C$\underline{H}_{Ar}$). **^{13}C NMR** (100 MHz, CD_3OD) δ 69.90 (O$\underline{C}$H$_2$CH); 75.84 (O$\underline{C}$HCH$_2$); 105.17 (O$\underline{C}$HO); 122.59 (Br$\underline{C}_{Ar}$); 123.99 (CH_{Ar}); 141.50 ($\underline{C}H_{Ar}$); 151.18 ($\underline{C}H_{Ar}$); 156.18 ($\underline{C}_{Ar}$); 169.50 ($\underline{C}$=O). Anal. Calcd. for $C_9H_9BrN_2O_4$: C, 37.39; H, 3.14; N, 9.69; Found C, 37.31; H, 3.18; N, 9.65.

*4.1.1.1.5. N-hydroxy-2,2-diphenyl-1,3-dioxolane-4-carboxamide (**3**)*

According to Method A. Chromatographed over silica gel: ratio crude/silica gel 1:60 DCM/MeOH 95:5. TLC: DCM:MeOH 95:5 Rf=0.35. Beige solid (57% yield). **^{1}H NMR** (400MHz, DMSO) HD52_A δ 4.04(dd, 1H, J = 8.0, 6.4 Hz, OC$\underline{H}_2$CH); 4.12(t,1H, J = 6.4 Hz, OC$\underline{H}_2$CH); 4.74(t, 1H, J = 6.4 Hz, OC$\underline{H}$CH$_2$); 7.31-7.51(m, 10H, C$\underline{H}_{Ar}$); 9.14(brs, 1H, N$\underline{H}$); 10.87(brs, 1H, O$\underline{H}$).**^{13}C NMR** (100 MHz, CDCl3) δ 61.74 (O$\underline{C}$H$_2$CH); 73.60 (O$\underline{C}$HCH$_2$); 110.41 (O$\underline{C}$O); 125.73($\underline{C}_{Ar}$);

126.11($\underline{C}_{Ar}$); 127.91($\underline{C}_{Ar}$); 128.24 ($\underline{C}_{Ar}$); 141.49($\underline{C}_{Ar}$); 141.70($\underline{C}_{Ar}$); 165.28($\underline{C}$=O). Anal. Calcd. for $C_{16}H_{15}NO_4$: C, 67.36; H, 5.30; N, 4.91; Found C, 57.30; H, 5.35; N, 4.85.

*4.1.1.1.6. N-hydroxy-8-methyl-1,4-dioxa-8-azaspiro[4.5]decane-2-carboxamide (**4**)*

According to Method A. Chromatographed over silica gel: ratio crude/silica gel 1:20 Acetone/MeOH 6:4. TLC DCM:MeOH 85:5 Rf=0.2. Colorless liquid (20%yield). **^{1}H NMR** (400 MHz, CD_3OD) HD59_A δ 1.78-2.00 (m, 4H, $C\underline{H}_2CHC\underline{H}_2$); 2.34 (s, 3H, $C\underline{H}_3N$); 2.48-2.80 (m, 4H, $C\underline{H}_2NC\underline{H}_2$); 4.09 (dd, 1H, *J* = 8.4, 5.6 Hz $OC\underline{H}_2CH$); 4.27 (dd, 1H, *J* = 8.8, 7.2 Hz, $OC\underline{H}_2CH$); 4.57 (dd, 1H, *J* = 7.6, 5.6 Hz, $OC\underline{H}CH_2$). **^{13}C NMR** (100 MHz, CD_3OD) δ 34.70 ($\underline{C}H_2CCH_2$); 35.50 ($CH_2C\underline{C}H_2$); 45.65 ($\underline{C}H_3N$); 54.12 ($\underline{C}H_2NCH_2$); 54.22 ($CH_2N\underline{C}H_2$); 68.16 ($O\underline{C}H_2CH$); 75.21 (OCH_2CH); 110.02 ($CH_2\underline{C}CH_2$); 170.06 ($\underline{C}$=O). Anal. Calcd. for $C_9H_{16}N_2O_4$: C, 49.99; H, 7.46; N, 12.96; Found C, 50.10; H, 7.38; N, 12.90.

*4.1.1.1.7. 8-benzoyl-N-hydroxy-1,4-dioxa-8-azaspiro[4.5]decane-2-carboxamide (**4a**)*

According to Method A. Chromatographed over silica gel: ratio crude/silica gel 1:100 DCM/MeOH 95:5. TLC DCM:MeOH 95:5 Rf=0.5. Viscous orange liquid (60%yield). **^{1}H NMR** (400 MHz, CDCl3) HD54_A δ 1.60-2.01 (m, 4H, $C\underline{H}_2C\underline{C}H_2$); 3.35–3.80 (m, 3H, $C\underline{H}_2NC\underline{H}_2$); 3.35-4.15(m, 1H, $CH_2NC\underline{H}_2$); 4.17-4.20 (m, 1H, $OC\underline{H}_2CH$); 4.25-4.32 (m, 1H, $OC\underline{H}_2CH$); 4.64 (m, 1H, $OC\underline{H}CH_2$); 7.42 (m, 5H, $C\underline{H}_{Ar}$). **^{13}C NMR** (100 MHz, CDCl3) δ 34.91 (2C, $\underline{C}H_2C\underline{C}H_2$ from HMQC); 44.45 (2C, $\underline{C}H_2N\underline{C}H_2$ from HMQC); 67.27 ($O\underline{C}H_2CH$); 74.05 ($OCH_2\underline{C}H$); 109.86 ($O\underline{C}CH_2$); 126.86 ($\underline{C}H_{Ar}$); 128.59 ($\underline{C}H_{Ar}$); 129.95 ($C\underline{H}_{Ar}$); 135.48 ($\underline{C}_{Ar}$); 167.93 ($N\underline{C}$=0); 173.64 ($NH\underline{C}$=O). Anal. Calcd. for $C_{15}H_{18}N_2O_5$: C, 58.82; H, 5.92 N, 9.15; Found C, 58.75; H, 5.98; N, 9.22.

*4.1.1.1.8. 8-benzyl-N-hydroxy-1,4-dioxa-8-azaspiro[4.5]decane-2-carboxamide (**4b**)*

According to Method A. Chromatographed over silica gel: ratio crude/silica gel 1:80 DCM:MeOH 9:1. TLC DCM:MeOH 95:5; Rf=0.45. White solid (55%yield). **^{1}H NMR** (400MHz, DMSO)

HD57_A δ 1.60–1.85 (m, 4H, C$\underline{H_2}$CC$\underline{H_2}$); 2.25–2.48 (m, 3H, C$\underline{H_2}$NC$\underline{H_2}$); 2.50-2.53 (m, 1H, C$\underline{H_2}$NC$\underline{H_2}$); 3.48 (s, 2H, C$\underline{H_2}$Ar); 3.91 (dd, 1H, *J* = 8.4, 5.6 Hz, OC$\underline{H_2}$CH); 4.13 (dd, 1H, *J* = 8.4, 7.2Hz, OC$\underline{H_2}$CH); 4.40 (dd, 1H, *J* = 5.6, 6.8Hz, (OC$\underline{H}$CH$_2$); 7.24-7.34 (m, 5H, C$\underline{H_{Ar}}$); 8.90 (s, 1H, N$\underline{H}$); 10.61 (s, 1H, O$\underline{H}$). **^{13}C NMR** (100 MHz, DMSO) δ 34.14 (CH$_2$C$\underline{C}$H$_2$); 34.59 ($\underline{C}$H$_2$CCH$_2$); 50.40 ($\underline{C}$H$_2$NCH$_2$); 50.48 (CH$_2$N$\underline{C}$H$_2$); 61.58 (Ph$\underline{C}$H$_2$); 66.31 (O$\underline{C}$H$_2$CH); 73.09 (OCH$_2$$\underline{C}$H); 108.81 (O$\underline{C}CH_2$); 126.82 ($\underline{C}H_{Ar}$); 128.10 ($\underline{C}H_{Ar}$); 128.70 (C$\underline{H_{Ar}}$); 138.47 ($\underline{C}_{Ar}$); 166.54 ($\underline{C}$=O). Anal. Calcd. for $C_{15}H_{20}N_2O_4$: C, 61.63; H, 6.90, N, 9.58; Found C, 61.58; H, 6.95; N, 9.63.

*4.1.1.1.9. 9-benzyl-N-hydroxy-1,5-dioxa-9-azaspiro[5.5]undecane-3-carboxamide (**4c**)*

According to Method A. Yellow liquid (12% yield). **^{1}H NMR** (400 MHz, CDCl3) HD68_C δ 1.65-1.68 (m, 2H, C$\underline{H_2}$ CCH$_2$); 1.98 (brm, 2H, CH$_2$ CC$\underline{H_2}$); 2.44-2.60 (m, 5H, C$\underline{H_2}$NC$\underline{H_2}$, CH$_2$ C$\underline{H}$CH$_2$); 3.48 (s, 2H, PhC$\underline{H_2}$); 3.76-4.01 (m, 4H, OC$\underline{H_2}$CHC$\underline{H_2}$O);7.18-7.24 (m, 5H, C$\underline{H_{Ar}}$). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 35.57 (2C, $\underline{C}$H$_2$C$\underline{C}$H$_2$); 40.70 (OCH$_2$$\underline{C}HCH_2$O); 51.56 (2C, $\underline{C}$H$_2$N$\underline{C}$H$_2$); 59.02 (Ph$\underline{C}$H$_2$); 63.72 (CH$_2$$\underline{C}CH_2$); 98.15 (2C, O$\underline{C}H_2CH\underline{C}H_2$O); 127.64 ($\underline{C}H_{Ar}$); 128.54 ($\underline{C}H_{Ar}$); 128.56 ($\underline{C}H_{Ar}$); 138.22 ($\underline{C}_{Ar}$);167.91 ($\underline{C}$=O). Anal. Calcd. for $C_{16}H_{22}N_2O_4$: C, 62.73; H, 7.24, N, 9.14; Found C, 62.79; H, 7.20; N, 9.21.

*4.1.1.4.1. N-hydroxy-4-oxo-4-(piperazin-1-yl)butanamide (**5**)*

According to Method D. White solid, 80 mg (quantitative yield). **^{1}H NMR** (400 MHz, CD_3OD) δ 3.63 – 3.56 (m, 4H, COC$\underline{H_2}$CH$_2$CO, COCH$_2$C$\underline{H_2}$CO), 2.91 (s, 2H, Pipz), 2.87 – 2.80 (m, 2H, Pipz), 2.68 (t, *J* = 6.9 Hz, 2H, Pipz), 2.54 (t, *J* = 6.7 Hz, 2H, Pipz). **^{13}C NMR** (101 MHz, CD_3OD) δ 177.65 (HONH$\underline{C}$OCH$_2$CH$_2$), 172.65 (N$\underline{C}$OCH$_2$CH$_2$), 46.31 ($\underline{C}$H$_2$-2 Pipz, $\underline{C}$H$_2$-6 Pipz), 45.99 ($\underline{C}$H$_2$-3, $\underline{C}$H$_2$-5 Pipz), 31.16 (NCO$\underline{C}$H$_2$CH$_2$), 28.96 (NCOCH$_2$$\underline{C}H_2$). Anal. Calcd. for $C_8H_{15}N_3O_3$: C, 47.75; H, 7.51, N, 20.88; Found C, 47.82; H, 7.43; N, 20.92.

*4.1.1.4.2 4-(4-benzoylpiperazin-1-yl)-N-hydroxy-4-oxobutanamide (**5a**)*

According to Method D. Colourless liquid, 102 mg (77% yield). **^{1}H NMR** (400 MHz, DMSO) δ 10.37 (s, 1H, CONHOH), 8.65 (s, 1H, CONHOH), 7.45 (dd, *J* = 13.8, 3.2 Hz, 5H, CH_{Ar}-2, CH_{Ar}-3, CH_{Ar}-4, CH_{Ar}-5, C_{HAr}-6), 3.51 (s, 8H, CH_2-2 Pipz, CH_2-3 Pipz, CH_2-5 Pipz, CH_2-6 Pipz), 2.55 (s, 2H, $NCOCH_2CH_2$), 2.21 (t, *J* = 6.9 Hz, 2H, $NCOCH_2CH_2$). **^{13}C NMR** (101 MHz, DMSO) δ 169.88 (CONHOH), 169.18 ($NCOCH_2CH_2$), 168.45 (ArCON), 135.69 (C_{Ar}-1), 129.60 (CH_{Ar}-4), 128.42 (CH_{Ar}-3, CH_{Ar}-5), 126.95 (CH_{Ar}-2, CH_{Ar}-6), 48.56 (CH_2-2 Pipz, CH_2-3 Pipz, CH_2-5 Pipz, CH_2-6 Pipz), 27.63 ($NCOCH_2CH_2$), 27.43 ($NCOCH_2CH_2$). Anal. Calcd. for $C_{15}H_{19}N_3O_4$: C, 59.01; H, 6.27, N, 13.76; Found C, 58.89; H, 6.22; N, 13.81.

*4.1.1.4.3. 4-(4-benzylpiperidin-1-yl)-N-hydroxy-4-oxobutanamide (**5b**)*

According to Method D. Colourless liquid, 180 mg (quantitative yield). **^{1}H NMR** (400 MHz, CD_3OD) δ 7.40 – 7.14 (m, 5H, CH_{Ar}-2, CH_{Ar}-3, CH_{Ar}-4, CH_{Ar}-5, CH_{Ar}-6), 4.47 (d, *J* = 13.1 Hz, 1H, Pip), 3.96 (d, *J* = 13.9 Hz, 1H, Pip), 3.03 (td, *J* = 13.5, 2.6 Hz, 1H, Pip), 2.69 (dd, *J* = 12.1, 7.1 Hz, 2H, $NCOCH_2CH_2$), 2.64 – 2.47 (m, 3H, $ArCH_2$, Pip), 2.38 (t, *J* = 7.1 Hz, 2H, $NCOCH_2CH_2$), 1.91 – 1.76 (m, 1H, CH-4 Pip), 1.70 (t, *J* = 16.0 Hz, 2H, Pip), 1.35 – 1.04 (m, 2H, Pip). **^{13}C NMR** (101 MHz, CD_3OD) δ 172.05 ($HONHCOCH_2CH_2$), 171.92 ($NCOCH_2CH_2$), 141.40 (C_{Ar}-1), 130.16 (CH_{Ar}-3, CH_{Ar}-5), 129.29 (CH_{Ar}-2, CH_{Ar}-6), 127.03 (CH_{Ar}-4), 43.80 (CH_2-2 Pip, CH_2-6 Pip), 43.37 ($ArCH_2$), 39.38 (CH-4 Pip), 33.52 ($NCOCH_2CH_2$), 32.92 ($NCOCH_2CH_2$), 29.14 (CH_2-5 Pip), 28.98 (CH_2-3 Pip). Anal. Calcd. for $C_{16}H_{22}N_2O_3$: C, 66.18; H, 7.64, N, 9.65; Found C, 66.23; H, 7.60; N, 9.61.

*4.1.1.4.4. 1-benzoylpiperidin-4-yl 4-(hydroxyamino)-4-oxobutanoate (**5c**)*

According to Method D. Colorless liquid (quantitative yield). **^{1}H NMR** (400MHz, CD_3OD) HD82 δ 1.60-2.00 (m, 4H, CH_2CHCH_2); 2.41 (t, 2H, J = 8.0Hz, $OCOCH_2$); 2.67 (t, 2H, J = 8.0Hz, $NCOCH_2$); 3.41 (brs, 1H, CH_2NCH_2); 3.60 (brs, 1H, CH_2NCH_2); 3.75 (brs, 1H, CH_2NCH_2); 3.91 (brs, 1H, CH_2NCH_2); 5.07 (q, 1H, J = 4.0Hz, CH_2CHCH_2); 7.37-7.52 (m, 5H, CH_{Ar}). **^{13}C NMR**

(100 MHz, $CDCl_3$) δ 27.4 ($\underline{C}H_2CON$); 30.0 ($\underline{C}H_2CH\underline{C}H_2$); 32.4 ($\underline{C}H_2COO$); 41.7 ($\underline{C}H_2N\underline{C}H_2$); 72.6 ($CH_2\underline{C}HCH_2$); 127.2 ($\underline{C}H_{Ar}$); 128.5 ($\underline{C}H_{Ar}$); 129.7 ($\underline{C}H_{Ar}$); 135.2 ($\underline{C}_{Ar}$); 170.0 (N$\underline{C}$O); 170.6 (O$\underline{C}$NO); 173.1 (O$\underline{C}$O). Anal. Calcd. for $C_{16}H_{20}N_2O_5$: C, 59.99; H, 6.29, N, 8.74; Found C, 60.06; H, 6.25, N, 8.69.

*4.1.1.2.1. N-hydroxy-2,3-dihydrobenzo[b][1,4]dioxine-2-carboxamide (**6**)*

According to Method B. White solid, 85 mg (45% yield). **^{1}H NMR** (400 MHz, DMSO-d_6) δ 9.18 (bs, 2H, N$\underline{H}$O$\underline{H}$), 7.14 – 6.70 (m, 4H, C$\underline{H}_{Ar}$-Dioxi), 4.78 (dd, *J* = 5.5, 2.9 Hz, 1H, C$\underline{H}$), 4.35 – 4.17 (m, 2H, C$\underline{H}_2$). **^{13}C NMR** (101 MHz, DMSO) δ 163.97 ($\underline{C}$ONHOH), 142.71 ($\underline{C}_{Ar}$-1), 122.01 ($\underline{C}_{Ar}$-2), 121.91 ($\underline{C}$H-5 Dioxi), 117.86 ($\underline{C}$H-4 Dioxi), 117.45 ($\underline{C}$H-3 Dioxi), 72.09 ($\underline{C}$H), 65.10 ($\underline{C}H_2$). Anal. Calcd. for $C_9H_9NO_4$: C, 55.39; H, 4.65, N, 7.18; Found C, 55.39; H, 4.65, N, 7.18.

*4.1.1.3.1. N-hydroxybenzo[b][1,4]dioxine-2-carboxamide (**7**)*

According to Method C. White solid, 30 mg (63% yield). **^{1}H NMR** (400 MHz, DMSO) δ 10.94 (s, 1H, CON$\underline{H}$OH), 9.07 (s, 1H, CONHO$\underline{H}$), 7.02 – 6.89 (m, 3H, C$\underline{H}_{Ar}$-4, C$\underline{H}_{Ar}$-5, C$\underline{H}$-3 Dioxi), 6.82 (t, *J* = 7.5 Hz, 2H, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-6). **^{13}C NMR** (101 MHz, DMSO) δ 157.07 ($\underline{C}$ONHOH), 141.44 ($\underline{C}_{Ar}$-1), 140.75 ($\underline{C}_{Ar}$-2), 130.86 ($\underline{C}$-2 Dioxi), 130.63 ($\underline{C}$H-3 Dioxi), 125.27 ($\underline{C}H_{Ar}$-5), 124.89 ($\underline{C}H_{Ar}$-4), 116.50 ($\underline{C}H_{Ar}$-3), 116.41 ($\underline{C}H_{Ar}$-6). Anal. Calcd. for $C_9H_7NO_4$: C, 55.96; H, 3.65, N, 7.25; Found C, 56.04; H, 3.65, N, 7.21.

*4.1.1.2.2. N-hydroxybenzofuran-2-carboxamide (**8**)*

According to Method B. Yellow solid (31% yield). Mp: [129,7 °C – 130,6 °C]. **^{1}H NMR** (400 MHz, DMSO) δ 11.50 (s, 1H, CON$\underline{H}$OH), 9.27 (s, 1H, CONHO$\underline{H}$), 7.76 (d, *J* = 7.5 Hz, 1H, C$\underline{H}$-3 Fur), 7.64 (d, *J* = 7.6 Hz, 2H, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-6), 7.50 – 7.41 (m, 1H, C$\underline{H}_{Ar}$-5), 7.38 – 7.28 (m, 1H, C$\underline{H}_{Ar}$-4).). **^{13}C NMR** (100 MHz, DMSO) δ 155.51 ($\underline{C}$ Benzofur), 154.32 ($\underline{C}$ONHOH), 152.74 ($\underline{C}$-2 Benzofur), 128.43 ($\underline{C}$ Benzofur), 124.74 ($\underline{C}$-6 Benzofur), 123.49 ($\underline{C}$-1 Benzofur), 123.46 ($\underline{C}$-6

Benzofur), 112.40 (C-7 Benzofur), 111.75 (C-3 Benzofur). Anal. Calcd. for $C_9H_7NO_3$: C, 61.02; H, 3.98, N, 7.91; Found C, 61.08; H, 3.94, N, 7.83.

*4.1.1.3.2. N-hydroxy-1H-benzo[d][1,2,3]triazole-5-carboxamide (**9**)*

According to Method C. The product was purified by crystallization with Et_2O to give 100 mg of brown solid (35% yield). **^{1}H NMR** (400 MHz, DMSO) δ 11.38 (s, 1H, CONHOH), 9.14 (s, 1H, CONHOH), 8.33 (s, 1H, CH_{Ar}-6), 7.95 (d, *J* = 8.6 Hz, 1H, CH_{Ar}-3), 7.85 (dd, *J* = 8.7, 1.3 Hz, 1H, CH_{Ar}-5). **^{13}C NMR** (100 MHz, DMSO) δ 165.90 (CONHOH), 141.15 (C Benzotr), 135.07 (C Benzotr), 129.24 (C-6 Benzotr), 129.19 (CCONH Benzotr), 121.24 (C-4 Benzotr), 114.74 (C-7 Benzotr). Anal. Calcd. for $C_7H_6N_4O_2$: C, 47.19; H, 3.39, N, 31.45; Found C, 47.24; H, 3.37, N, 31.46.

*4.1.1.1.10. N-hydroxy-2-phenoxyacetamide (**10**)*

According to Method A. The product was purified by crystallization with Et_2O to give 80 mg of a white solid (43% yield), Melting point Mp: [104,2 °C – 105,6 °C]. **^{1}H NMR** (400 MHz, DMSO) δ 10.82 (s, 1H, CONHOH), 8.96 (s, 1H, CONHOH), 7.30 (t, J = 8.0 Hz, 2H, CH_{Ar}-3, CH_{Ar}-5), 6.95 (d, J = 8.5 Hz, 3H, CH_{Ar}-2, CH_{Ar}-4, CH_{Ar}-6), 4.45 (s, 2H, OCH_2CO). **^{13}C NMR** (101 MHz, DMSO) δ 164.27 (CONHOH), 157.76 (C_{Ar}-1), 129.41 (CH_{Ar}-3, CH_{Ar}-5), 121.09 (CH_{Ar}-4), 114.59 (CH_{Ar}-2, CH_{Ar}-6), 65.74 (OCH_2CO). Anal. Calcd. for $C_8H_9NO_3$: C, 57.48; H, 5.43, N, 8.38; Found C, 57.42; H, 5.51, N, 8.35.

*4.1.1.1.11. 2-(4-(4-benzylpiperidine-1-carbonyl)phenoxy)-N-hydroxyacetamide (**10a**)*

According to Method A. The product was purified by crystallization with Et_2O to give 372 mg of a yellow solid (65% yield). **^{1}H NMR** (400 MHz, Methanol-d_4) δ 7.33 – 7.21 (m, 4H, CH_{Ar}), 7.19 – 7.10 (m, 3H, CH_{Ar}), 6.96 (d, *J* = 8.7 Hz, 2H, CH_{Ar}), 4.54 (s, 2H, OCH_2CO), 3.48 (s, 2H, CH_{2Pip}),

2.89 (dd, *J* = 11.7, 2.7 Hz, 2H, C$\underline{H}_{2Pip}$), 2.53 (d, *J* = 6.9 Hz, 2H, C$\underline{H}_{2Pip}$), 1.99 (td, *J* = 11.9, 2.5 Hz, 2H, C$\underline{H}_{2Pip}$), 1.70 – 1.47 (m, 3H, C$\underline{H}_{2Pip,}$ C$\underline{H}_{Pip}$), 1.31 (td, *J* = 12.3, 11.8, 3.4 Hz, 2H, C$\underline{H}_{2Pip}$). **^{13}C NMR** (101 MHz, DMSO) δ 167.72 (N$\underline{C}$OBz), 164.05 ($\underline{C}$ONHOH), 158.70 ($\underline{C}_{Bz}$-1), 141.66 ($\underline{C}_{Bn}$-1), 132.33 ($\underline{C}_{Bz}$-4), 131.12 ($\underline{C}H_{Bn}$-3, $\underline{C}H_{Bn}$-5), 130.13 ($\underline{C}H_{Bn}$-2, $\underline{C}H_{Bn}$-6, $\underline{C}H_{Bz}$-3, $\underline{C}H_{Bz}$-5), 126.91 ($\underline{C}H_{Bn}$-4), 115.57 ($\underline{C}H_{Bz}$-2, $\underline{C}H_{Bz}$-6), 67.44 (O$\underline{C}H_2$CO), 63.41 ($\underline{C}$H), 54.49 ($\underline{C}H_2$N) 43.94 ($\underline{C}H_2$-2 Pip, $\underline{C}H_2$-6 Pip, Bn$\underline{C}H_2$), 38.94 ($\underline{C}$H-4 Pip), 32.55 ($\underline{C}H_2$-3 Pip, $\underline{C}H_2$-5 Pip). Anal. Calcd. for $C_{21}H_{24}N_2O_4$: C, 68.46; H, 6.57, N, 7.60; Found C, 68.46; H, 6.52, N, 7.64.

*4.1.1.1.12. 2-(4-((4-benzylpiperidin-1-yl)methyl)phenoxy)-N-hydroxyacetamide (**10b**)*

According to Method A. The product was purified by silica gel chromatography (crude:silica gel 1:50; 8 g of silica gel; eluent: DCM/MeOH 9:1) to give 40 mg of white solid (59% yield). **^{1}H NMR** (400 MHz, DMSO) δ 10.85 (s, 1H, CON$\underline{H}$), 8.98 (s, 1H, NHO$\underline{H}$), 7.43-7.24 (m, 4H, C$\underline{H}_{ArO}$-2, C$\underline{H}_{ArO}$-3, C$\underline{H}_{ArO}$-5, C$\underline{H}_{ArO}$-6) 7.19 (dd, *J* = 11.7, 4.6 Hz, 3H, C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-4, C$\underline{H}_{Ar}$-6), 6.98 (d, *J* = 8.7 Hz, 2H, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-5), 4.51 (s, 2H, OC$\underline{H}_2$CONH), 3.65-4.40 bm, 2H, C$\underline{H}_2$Pip), 2.88 (bm, 2H, C$\underline{H}_2$Pip), 2.54 (d, *J* = 6.9 Hz, 2H, ArC$\underline{H}_2$Pip), 1.96 – 1.46 (m, 4H, C$\underline{H}_2$Pip). **^{13}C NMR** (101 MHz, DMSO) δ 167.73 ($\underline{C}$ONHOH), 162.121 ($\underline{C}_{ArO}$-1), 157.91 ($\underline{C}_{Ar}$-1), 140.00 ($\underline{C}_{Ar}$-1), 129.10 ($\underline{C}H_{Ar}$-2, $\underline{C}H_{Ar}$-6), 128.84 ($\underline{C}H_{ArO}$-2, $\underline{C}H_{ArO}$-6), 128.18 ($\underline{C}H_{Ar}$-3, $\underline{C}H_{Ar}$-5), 127.00 ($\underline{C}H_{Ar}$-4), 124.87 ($\underline{C}H_{ArO}$-4), 115.51 ($\underline{C}H_{ArO}$-2, $\underline{C}H_{ArO}$-6) 66.40 (O$\underline{C}$CONHOH), 61.94 (N$\underline{C}H_2$Ar), 51.27 ($\underline{C}H_2$-2 Pip, $\underline{C}H_2$-6 Pip), 41.96 (Ar$\underline{C}H_2$Pip), 36.60 ($\underline{C}$H-4 Pip), 30.16 ($\underline{C}H_2$-3 Pip, $\underline{C}H_2$-5 Pip). Anal. Calcd. for $C_{21}H_{26}N_2O_3$: C, 71.16; H, 7.39, N, 7.90; Found C, 71.15; H, 7.42, N, 7.84.

*4.1.1.1.13. N-hydroxy-2-(phenylthio)acetamide (**11**)*

According to Method A. The product was purified by crystallization with Et_2O to give 203 mg of a white solid (87% yield), Melting point Mp: [99,6 °C – 100,3 °C]. **^{1}H NMR** (400 MHz, DMSO) δ 10.68 (s, 1H, CON$\underline{H}$OH), 8.98 (s, 1H, CONHO$\underline{H}$), 7.35 (ddd, *J* = 23.9, 10.9, 4.7 Hz, 4H, C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-5, C$\underline{H}_{Ar}$-6), 7.21 (dd, *J* = 10.1, 4.3 Hz, 1H, C$\underline{H}_{Ar}$-4), 3.54 (s, 2H, SC$\underline{H}_2$CO). **^{13}C**

NMR (101 MHz, DMSO) δ 164.85 (CONHOH), 135.99 (C_{Ar}-1), 128.93 (CH_{Ar}-2, CH_{Ar}-6), 127.92 (CH_{Ar}-3, CH_{Ar}-5), 125.87 (CH_{Ar}-4), 33.76 (SCH_2CO). Anal. Calcd. for $C_8H_9NO_2S$: C, 52.44; H, 4.95, N, 7.64; S, 17.50; Found C, 52.41; H, 4.86, N, 7.57; S, 14.42.

*4.1.1.1.14. 2-((4-((4-benzylpiperidin-1-yl)methyl)phenyl)thio)-N-hydroxyacetamide (**11a**)*

According to Method A. Waxy solid (75% yield). **^{1}H NMR** (400 MHz, DMSO) δ 10.66 (s, 1H, CONHOH), 8.97 (s, 1H, CONHOH), 7.39 – 7.06 (m, 9H, CH_{Ar}-2, CH_{Ar}-3, CH_{Ar}-4, CH_{Ar}-5, CH_{Ar}-6, CH_{ArS}-2, CH_{ArS}-3, CH_{ArS}-5, CH_{ArS}-6), 3.51 (s, 2H, SCH_2CO), 3.38 (s, 2H, NCH_2ArS), 2.74 (d, *J* = 11.6 Hz, 2H, $ArCH_2$), 2.48 (s, 1H, Pip), 1.84 (t, *J* = 10.7 Hz, 2H, Pip), 1.57 – 1.41 (m, 3H, Pip), 1.29 – 1.05 (m, 3H, Pip). **^{13}C NMR** (101 MHz, DMSO) δ 164.66 (CONHOH), 140.35 (C_{Ar}-1), 137.11 (C_{ArS}-4), 134.99 (C_{ArS}-1), 128.95 (CH_{ArS}-2, CH_{ArS}-6), 198.91(CH_{Ar}-4), 128.06 (CH_{Ar}-3, CH_{Ar}-5), 126.73 (CH_{Ar}-2, CH_{Ar}-6), 125.65 (CH_{ArS}-3, CH_{ArS}-5), 61.94 (NCH_2ArS), 53.10 (CH_2-2 Pip, CH_2-6 Pip), 42.35 ($ArCH_2$), 36.26 (CH-4 Pip), 31.70 (SCH_2CO), 19.75 (CH_2-3 Pip, CH_2-5 Pip). Anal. Calcd. for $C_{21}H_{26}N_2O_2S$: C, 68.08; H, 7.07; N, 7.56; S, 8.65; Found C, 68.05; H, 7.01, N, 7.64; S, 8.56.

*4.1.1.1.15. 2-((4-(4-benzylpiperidine-1-carbonyl)phenyl)thio)-N-hydroxyacetamide (**11b**)*

According to Method A. The product was purified by crystallization with Et_2O to give 200 mg of a waxy solid (47% yield). **^{1}H NMR** (400 MHz, DMSO) δ 10.71 (s, 1H, CONHOH), 9.01 (s, 1H, CONHOH), 7.51 – 7.13 (m, 9H, CH_{Bn}-2, CH_{Bn}-3, CH_{Bn}-4, CH_{Bn}-5, CH_{Bn}-6, CH_{Bz}-2, CH_{Bz}-3, CH_{Bz}-5, CH_{Bz}-6), 4.41 (s, 1H, Pip), 3.59 (s, 3H, SCH_2CONH, Pip), 3.10 – 2.61 (m, 2H, Pip), 2.54 (d, *J* = 7.3 Hz, 2H, $BnCH_2$), 1.90 – 1.72 (m, 1H, CH-4 Pip), 1.60 (s, 2H, Pip), 1.17 (d, *J* = 11.1 Hz, 2H, Pip). **^{13}C NMR** (101 MHz, DMSO) δ 168.35 (NCOBz), 164.68 (SCH_2CONH), 139.98 (C_{Bz}-1), 137.85 (C_{Bn}-1), 133.54 (C_{Bz}-4), 128.97 (CH_{Bn}-3, CH_{Bn}-5), 128.14 (CH_{Bz}-2, CH_{Bz}-6), 127.37 (CH_{Bz}-3, CH_{Bz}-5), 127.06 (CH_{Bn}-2, CH_{Bn}-6), 125.81 (CH_{Bn}-4), 64.87 (CH_2-2 Pip, CH_2-6 Pip), 42.03

(Bn<u>C</u>H_2), 37.45 (<u>C</u>H-4 Pip), 33.20 (S<u>C</u>H_2CONH), 31.56 (<u>C</u>H_2-3 Pip, <u>C</u>H_2-5 Pip). Anal. Calcd. for $C_{21}H_{24}N_2O_3S$: C, 65.60; H, 6.26; N, 7.29; S, 8.34; Found C, 65.51; H, 6.34, N, 7.32; S, 8.26.

*4.1.1.3.3. N-hydroxy-3-phenylpropiolamide (**12**)*

According to Method C. The product was purified by silica gel chromatography (30 g of silica gel; eluent: from CE /AcOEt 1:1 to 100 % of AcOEt) to obtain 136 mg of colourless liquid (32 % yield). **^{1}H NMR** (400 MHz, DMSO) δ 10.17 (s, 1H, CONHO<u>H</u>), 7.67 – 7.36 (m, 5H, C<u>H</u>$_{Ar}$-2, C<u>H</u>$_{Ar}$-3, C<u>H</u>$_{Ar}$-4, C<u>H</u>$_{Ar}$-5, C<u>H</u>$_{Ar}$-6), 4.22 – 2.81 (s, 1H, CON<u>H</u>OH). **^{13}C NMR** (101 MHz, DMSO) δ 150.20 (<u>C</u>ONHOH), 131.86 (<u>C</u>H$_{Ar}$-2, C<u>H</u>$_{Ar}$-6), 128.94 (<u>C</u>H$_{Ar}$-4), 128.87 (<u>C</u>H$_{Ar}$-3, <u>C</u>H$_{Ar}$-5), 124.93 (<u>C</u>$_{Ar}$-1), 92.92 (ArC<u>C</u>CO), 84.02 (Ar<u>C</u>CCO). Anal. Calcd. for $C_9H_7NO_2$: C, 67.08; H, 4.38; N, 8.69; Found C, 66.95; H, 4.29, N, 8.68.

*4.1.1.1.16. 4-(4-benzylpiperidin-1-yl)-N-hydroxybut-2-ynamide (**12a**)*

According to Method A. The product was purified by silica gel chromatography (10 g of silica gel; eluent: from DCM/MeOH 9:1 to 100% MeOH) to give 200 mg of a yellow solid (63% yield). **^{1}H NMR** (400 MHz, CD_3OD) δ 7.23 (m, *J* = 23.1, 19.2, 13.6, 7.8 Hz, 5H, C<u>H</u>$_{Ar}$-2, C<u>H</u>$_{Ar}$-3, C<u>H</u>$_{Ar}$-4, C<u>H</u>$_{Ar}$-5, C<u>H</u>$_{Ar}$-6), 3.39 (s, 2H, NC<u>H</u>$_2$CC), 3.00 (d, *J* = 11.9 Hz, 2H, C<u>H</u>$_2$-3 Pip, C<u>H</u>$_2$-5 Pip), 2.56 (d, *J* = 6.9 Hz, 2H, ArC<u>H</u>$_2$), 2.16 (dd, *J* = 15.8, 6.4 Hz, 2H, C<u>H</u>$_2$-3’ Pip, C<u>H</u>$_2$-5’ Pip), 1.66 (d, *J* = 13.5 Hz, 2H, C<u>H</u>$_2$-2 Pip, C<u>H</u>$_2$-6 Pip), 1.63 – 1.54 (m, 1H, C<u>H</u>-4 Pip), 1.42 – 1.28 (m, 2H, C<u>H</u>$_2$-2’ Pip, C<u>H</u>$_2$-6’ Pip). **^{13}C NMR** (100 MHz, CD_3OD) δ 154.61 (<u>C</u>ONHOH), 140.01 (<u>C</u>H$_{Ar}$-1), 129.10 (<u>C</u>H$_{Ar}$-2, C<u>H</u>$_{Ar}$-6), 128.18 (<u>C</u>H$_{Ar}$-3, C<u>H</u>$_{Ar}$-5), 127.00 (<u>C</u>H$_{Ar}$-4), 81.26 (CH$_2$<u>C</u>≡), 64.93 (C≡<u>C</u>CO), 51.27 (<u>C</u>H$_{Pip}$-2, C<u>H</u>$_{Pip}$-6), 46.88 (C<u>H</u>$_2$N), 41.96 (<u>C</u>H$_2$CH), 36.60 (<u>C</u>H), 30.16 (<u>C</u>H$_{Pip}$-3, C<u>H</u>$_{Pip}$-5). Anal. Calcd. for $C_{16}H_{20}N_2O_2$: C, 70.56; H, 7.40; N, 10.26; Found C, 70.56; H, 7.51, N, 10.33.

*4.1.2. Synthesis of butyl 2,3-dihydroxypropanoate (**14**)*

To a solution of TEBAC (4.3 g, 18.72 mmol, 1.2 equiv.) in dry acetone (20 mL), $KMnO_4$ (2.957 g, 18.72 mmol, 1.2 equiv.) was added, and the reaction mixture stirred at room temperature for 3h. After chilling at 0 °C in an ice bath, a solution of butyl acrylate (2.237 mL, 15.6 mmol, 1 equiv.) in dry acetone (10 mL) was added dropwise. The reaction was stirred in the same conditions for 1 h and quenched with $Na_2S_2O_4$ saturated solution. MnO_2 was filtered on a celite pad and the filtrate concentrated. The residue was solubilized in AcOEt and the organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated to give the titled compound as a colourless liquid. Colorless liquid. 1.650g (64% yield). **^{1}H NMR** (400 MHz, CDCl3) δ 0.95 (t, 3H, *J* = 8.0 Hz, C$\underline{H}_3$); 1.41 (sxt, 2H, *J* = 8.0 Hz, C$\underline{H}_2$CH$_3$); 1.68 (qnt, 2H, *J* = 8.0 Hz, CH$_2$C$\underline{H}_2$CH$_2$); 3.89 (dp, 2H, *J* = 12.0 Hz, C$\underline{H}_2$OH); 4.23-4.28 (m, 3H, C$\underline{H}$OH, -OC$\underline{H}_2$CH$_2$). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 13.78 ($\underline{C}H_3$); 19.23 ($\underline{C}H_2CH_3$); 31.08 ($CH_2\underline{C}H_2CH_2$) 63.83 ($\underline{C}H_2OH$); 64.10 ($O\underline{C}H_2CH_2$); 71.94 ($\underline{C}HOH$);174.64 ($\underline{C}$=O).

*4.1.3. General procedure for the synthesis of dioxolane butyl esters (**15-18, 18a-b**)*

To a solution of the appropriate carbonyl compound (0.75 equiv.) in dry toluene, under argon atmosphere and at room temperature, pTSA (0.1 equiv.), and a solution of butyl 2,3-dihydroxypropanoate (1 equiv.) in dry toluene were added. The Dean-Stark trap was set-up, and the reaction was refluxed overnight. Thereafter, the reaction was cooled at room temperature and diluted with Et_2O. The organic phase was washed with $NaHCO_3$ saturated solution, brine, dried over anhydrous Na_2SO_4, and concentrated. The crude was purified by silica gel chromatography.

4.1.3.1. cis-Butyl 2-phenyl-1,3-dioxolane-4-carboxylate (***cis-15***)

Chromatographed over silica gel: ratio crude/silica gel 1:80, Cyclohexane/AcOEt 9:1; TLC: Cyclohexane: AcOEt 9:1 Rf=0.4. Orange liquid (40%yield) **^{1}H NMR** (400 MHz, $CDCl_3$) δ 0.94 (t, 3H, *J* = 8.0 Hz, C$\underline{H}_3$); 1.38 (sxt, 2H, *J* = 8.0 Hz, C$\underline{H}_2$CH$_3$); 1.66 (qnt, 2H, *J* = 8.0 Hz, C$\underline{H}_2$CH$_2$CH$_3$); 4.18-4.27 (m, 3H, OC$\underline{H}_2$CH, OC$\underline{H}_2$CH$_2$); 4.37(dd, 1H, *J* = 4.0, 8.0 Hz, OC$\underline{H}_2$CH); 4.71 (dd, 1H, *J* =

4.0, 8.0 Hz, OC<u>H</u>CH_2); 5.89 (s, 1H, OC<u>H</u>O); 7.39 (t, 3H, *J* = 4.0 Hz, CH_{Ar}); 7.60 (dd, 2H, *J* = 4.0, 8.0 Hz, CH_{Ar}). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 13.6 (<u>C</u>H_3); 18.7 (<u>C</u>H_2CH_3); 31.0 (<u>C</u>$H_2CH_2CH_3$); 65.0 (O<u>C</u>H_2CH_2); 68.1 (O<u>C</u>H_2CH); 90.1 (O<u>C</u>HCH_2); 108.6 (O<u>C</u>HO); 126.1 (<u>C</u>H_{Ar}); 127.5 (<u>C</u>H_{Ar}); 128.3 (<u>C</u>H_{Ar}); 137.0 (<u>C</u>$_{Ar}$); 170.8 (O<u>C</u>=O).

*4.1.3.2. trans-Butyl 2-phenyl-1,3-dioxolane-4-carboxylate (**trans-15**)*

Chromatographed over silica gel: ratio crude/silica gel 1:80, Cyclohexane/AcOEt 9:1. TLC: Cyclohexane: AcOEt 9:1 Rf=0.5. Orange liquid (40% yield). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 0.96 (t, 3H, *J* = 8.0 Hz, C<u>H</u>$_3$); 1.41 (sxt, 2H, *J* = 4.0 Hz, C<u>H</u>$_2$CH$_3$); 1.68 (qnt, 2H, *J* = 8.0 Hz, C<u>H</u>$_2$CH$_2$CH$_3$); 4.07 (dd,1H, *J* = 8.0, 4.0 Hz, OC<u>H</u>$_2$CH); 4.23(t, 2H, *J* = 8.0 Hz, OC<u>H</u>$_2$CH$_2$); 4.43 (t,1H, *J* = 8.0 Hz, OC<u>H</u>$_2$CH); 4.76 (t, 2H, *J* = 4.0 Hz, OC<u>H</u>CH$_2$); 6.04 (s, 1H, OC<u>H</u>O); 7.39 (m, 3H, C<u>H</u>$_{Ar}$); 7.49 (m, 2H, C<u>H</u>$_{Ar}$). **^{13}C NMR** (100MHz, $CDCl_3$) δ 13.8 (<u>C</u>H_3); 18.9 (<u>C</u>H_2CH_3); 31.1 (<u>C</u>$H_2CH_2CH_3$); 65.2 (O<u>C</u>H_2CH_2); 68.3 (O<u>C</u>H_2CH); 90.3 (O<u>C</u>HCH_2); 108.9 (O<u>C</u>HO); 126.3 (<u>C</u>H_{Ar}); 127.8 (<u>C</u>H_{Ar}); 128.6 (<u>C</u>H_{Ar}); 137.3 (<u>C</u>$_{Ar}$); 170.8 (O<u>C</u>=O).

*4.1.3.3. cis-Butyl 2-(5-bromopyridin-2-yl)-1,3-dioxolane-4-carboxylate (**cis-16**)*

Chromatographed over silica gel: ratio crude/silica gel 1:150 Cyclohexane/AcOEt 9:1. TLC Cyclohexane: AcOEt 9:1 Rf=0.36. Yellow liquid (34%yield). **^{1}H NMR** (400 MHz, CDCl3) δ 0.96 (t, 3H, *J* = 7.2 Hz, CH_2C<u>H</u>$_3$); 1.38 (sxt, 2H, *J* = 7.6 Hz, C<u>H</u>$_2$CH$_3$); 1.65 (qnt, 2H, *J* = 7.2 Hz, CH_2C<u>H</u>$_2$CH$_2$); 4.20 (dt,1H, *J* = 8.0, 4.0 Hz, OC<u>H</u>$_2$CH$_2$); 4.31(t, 2H, *J* = 8.0 Hz, OC<u>H</u>$_2$CH); 4.39 (dd,1H, *J* = 8.0, 4.0 Hz, OC<u>H</u>$_2$CH); 4.76 (dd, 2H, *J* = 7.6, 4.0 Hz, OC<u>H</u>CH_2); 5.95 (s, 1H, OC<u>H</u>O); 7.82 (d, 1H, *J* = 8.4 Hz, C<u>H</u>$_{Ar}$); 7.92 (dd, 1H, *J* = 8.4, 2.0 Hz, C<u>H</u>$_{Ar}$); 8.67(s, 1H, *J* = 2.0 Hz, C<u>H</u>$_{Ar}$). **^{13}C NMR** (100 MHz, CDCl3) δ 13.65 (<u>C</u>H_3); 19.02 (<u>C</u>H_2CH_3); 30.53 (<u>C</u>$H_2CH_2CH_3$); 65.53 (O<u>C</u>H_2CH_2); 69.50 (O<u>C</u>H_2CH); 74.32 (O<u>C</u>H$_2$CH); 104.90 (OCH$_2$<u>C</u>H); 121.67 (C_{Ar}); 123.27 (CH_{Ar}); 140.15 (CH_{Ar}); 149.54 (CH_{Ar}); 154.32 (C_{Ar}); 170.66(C=O).

*4.1.3.4. trans-Butyl 2-(5-bromopyridin-2-yl)-1,3-dioxolane-4-carboxylate (**trans-16**)*

Chromatographed over silica gel: ratio crude/silica gel 1:150 Cyclohexane/AcOEt 9:1. TLC Cyclohexane: AcOEt 9:1 Rf=0.45. Orange liquid (34% yield). **^{1}H NMR** (400 MHz, CDCl3) δ 0.97 (t, 3H, *J* = 7.6 Hz, $CH_2C\underline{H}_3$); 1.42 (sxt, 2H, *J* = 7.6 Hz, $C\underline{H}_2CH_3$); 1.69 (qnt, 2H, *J* = 7.6 Hz, $CH_2C\underline{H}_2CH_2$); 4.16 (dd, 1H, *J* = 8.0, 4.0 Hz, $OC\underline{H}_2CH$); 4.25(t, 2H, *J* = 8.0 Hz, $OC\underline{H}_2CH_2$); 4.47 (t,1H, *J* = 8.0 Hz, $OC\underline{H}_2CH$); 4.84 (dd, 1H, *J* = 7.2, 5.2 Hz, $OC\underline{H}CH_2$); 6.07 (s, 1H, $OC\underline{H}O$); 7.46 (d, 1H, *J* = 8.4 Hz, $C\underline{H}_{Ar}$); 7.90 (dd, 1H, *J* = 8.0, 2.0 Hz, $C\underline{H}_{Ar}$); 8.71(d, 1H, *J* = 2.0 Hz, $C\underline{H}_{Ar}$). **^{13}C NMR** (100 MHz, CDCl3) δ 13.66 ($\underline{C}H_3$); 19.04 ($\underline{C}H_2CH_3$); 30.55 ($\underline{C}H_2CH_2CH_3$); 65.56 ($O\underline{C}H_2CH_2$); 68.55 ($O\underline{C}H_2CH$); 74.49 ($O\underline{C}H_2CH$); 104.13 ($OCH_2\underline{C}H$); 121.49 (C_{Ar}); 122.41 (CH_{Ar}); 139.58 (CH_{Ar}); 150.55 (CH_{Ar}); 154.33 (C_{Ar}); 170.67 (C=O).

*4.1.3.5. Butyl 2,2-diphenyl-1,3-dioxolane-4-carboxylate (**17**)*

Chromatographed over silica gel: ratio crude/silica gel 1:60 Cyclohexane/AcOEt 9:1. TLC: Cyclohexane: AcOEt 9:1 Rf=0.4. Colorless liquid (63%yield). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 0.94 (t, 3H, *J* = 7.6 Hz, $C\underline{H}_3$); 1.36 (sxt, 2H, *J* = 7.6 Hz, $CH_2C\underline{H}_2CH_3$); 1.63 (q, 2H, *J* = 2.4 Hz, $CH_2C\underline{H}_2CH_2$); 4.17 (q, 2H, *J* = 6.0 Hz, $OC\underline{H}_2CH_2$); 4.26 (d, 2H, *J* = 6.4 Hz, $OC\underline{H}_2CH$); 4.71 (t, 1H, *J* = 6.8 Hz, $OC\underline{H}CH_2$); 7.31-7.38 (m, 6H, $C\underline{H}_{Ar}$); 7.55-7.58 (m, 4H, $C\underline{H}_{Ar}$). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 13.66 ($\underline{C}H_3$); 19.01 ($CH_2\underline{C}H_2CH_3$); 30.52 ($CH_2\underline{C}H_2CH_2$); 65.33 ($O\underline{C}H_2CH_2$); 67.84 ($O\underline{C}H_2CH$); 74.41 ($O\underline{C}HCH_2$); 126.33 ($O\underline{C}O$); 128.23($\underline{C}H_{Ar}$); 130.06 ($\underline{C}H_{Ar}$); 132.41($\underline{C}H_{Ar}$); 137.63 ($\underline{C}H_{Ar}$); 170.57(C=O).

*4.1.3.6. Butyl 8-metyl-1,4-dioxa-8-azaspiro[4.5]decane-2-carboxylate (**18**)*

Orange liquid (90% yield). **^{1}H NMR** (400 MHz, CDCl3) δ 0.95 (t, 3H, *J* = 7.2 Hz; $CH_2C\underline{H}_3$); 1.39 (sxt, 2H, *J* = 7.6 Hz; $C\underline{H}_2CH_3$); 1.65 (qnt, 2H, *J* = 7.2 Hz, $CH_2C\underline{H}_2CH_2$); 1.79-1.99 (m, 4H, $C\underline{H}_2CHC\underline{H}_2$); 2.37 (s, 3H, $C\underline{H}_3N$); 2.50-2.70 (m, 4H, $C\underline{H}_2NC\underline{H}_2$); 4.10-4.26 (m, 4H, $OC\underline{H}_2CH_2$, $OC\underline{H}_2CH$); 4.61 (dd,1H, *J* = 7.2, 5.2 Hz, $OC\underline{H}CH_2$). **^{13}C NMR** (100 MHz, CDCl3) δ 13.65

($CH_2\underline{C}H_3$); 19.03 ($\underline{C}H_2CH_3$); 30.55 ($CH_2\underline{C}H_2CH_2$); 34.62($\underline{C}H_2CCH_2$); 34.85 ($CH_2C\underline{C}H_2$); 45.70 ($\underline{C}H_3N$); 53.30 ($\underline{C}H_2NCH_2$); 53.40 ($CH_2N\underline{C}H_2$); 65.29 ($O\underline{C}H_2CH_2$); 67.12 ($O\underline{C}H_2CH$); 73.92($O\underline{C}HCH_2$); 109.57 ($CH_2\underline{C}CH_2$); 171.27 ($\underline{C}$=O).

*4.1.3.7. Butyl 8-benzoyl-1,4-dioxa-8-azaspiro[4.5]decane-2-carboxylate (**18a**)*

Chromatographed over silica gel: ratio crude/silica gel 1:60, Cyclohexane/AcOEt 9:1 TLC: Cyclohexane: AcOEt 9:1. Rf=0.5. Orange liquid (45%yield). **^{1}H NMR** (400 MHz, CDCl3) δ 0.93 (t, 3H, *J* = 7.6 Hz, $C\underline{H}_3$); 1.36 (sxt, 2H, *J* = 7.2 Hz, $CH_2C\underline{H}_2CH_3$);1.61 -1.90 (m, 6H, $CH_2C\underline{H}_2CH_2$, $C\underline{H}_2CC\underline{H}_2$); 3.40 - 3.63 (m, 2H, $C\underline{H}_2NCH_2$); 3.74 - 4.02 (m, 2H, $CH_2NC\underline{H}_2$); 4.10-4.15 (m, 3H, $OC\underline{H}_2CH_2$, $OC\underline{H}_2CHO$); 4.24-4.28 (m, 1H, $OC\underline{H}_2CHO$); 4.62 (t, 1H, *J* = 6.0 Hz, $OC\underline{H}CH_2O$); 7.38-7.40 (m, 5H, $C\underline{H}_{Ar}$). **^{13}C NMR** (100 MHz, CDCl3) δ 13.63 ($\underline{C}H_3$); 19.02 ($\underline{C}H_2CH_3$); 30.54 ($CH_2\underline{C}H_2CH_2$); 33.83 (2C, $\underline{C}H_2C\underline{C}H_2$); 34.74 (2C, $\underline{C}H_2C\underline{C}H_2$); 40.04 ($\underline{C}H_2NCH_2$); 45.59 ($CH_2N\underline{C}H_2$); 65.40 ($O\underline{C}H_2CH_2$); 67.39 ($O\underline{C}H_2CH$); 74.10 ($O\underline{C}HCH_2$); 109.78 ($O\underline{C}O$); 126.82 (2C, $\underline{C}H_{Ar}$); 128.50 (2C, $\underline{C}H_{Ar}$); 129.67 ($\underline{C}H_{Ar}$); 135.94 ($\underline{C}_{Ar}$); 170.41 ($N\underline{C}$=O); 171.04 ($\underline{C}$=O).

*4.1.3.8. Butyl 8-benzyl-1,4-dioxa-8-azaspiro[4.5]decane-2-carboxylate (**18b**)*

Chromatographed over silica gel: ratio crude/silica gel 1:60, Cyclohexane/AcOEt 6:4. TLC: Cyclohexane:AcOEt 6:4 Rf= 0.5. Yellow liquid (15% yield). **^{1}H NMR** (400 MHz, CDCl3) δ 0.95 (t, 3H, *J* = 7.6 Hz, $C\underline{H}_3$); 1.39 (sxt, 2H, *J* = 7.6 Hz, $CH_2C\underline{H}_2CH_3$); 1.65 (qnt, 2H, *J* = 6.4 Hz, $CH_2C\underline{H}_2CH_2$); 1.75 - 2.0 (m, 4H, $C\underline{H}_2CC\underline{H}_2$); 2.45 – 2.70 (m, 4H, $C\underline{H}_2NC\underline{H}_2$); 3.57 (s, 2H, $C\underline{H}_2Ph$); 4.10-4.26(m,4H, $OC\underline{H}_2CH_2$, $OC\underline{H}_2CH$); 4.61 (dd, 1H, *J* = 4.8, 7.2 Hz, $OC\underline{H}CH_2$); 7.33-7.35(m, 5H, CH_{Ar}). **^{13}C NMR** (100 MHz, CDCl3) δ 13.65 ($\underline{C}H_3$); 19.03 ($\underline{C}H_2CH_3$); 30.55 ($CH_2\underline{C}H_2CH_2$); 34.90 ($CH_2C\underline{C}H_2$); 35.02 ($\underline{C}H_2CCH_2$); 51.01 ($\underline{C}H_2NCH_2$); 51.17 ($CH_2N\underline{C}H_2$); 62.56 ($\underline{C}H_2Ph$); 65.25 ($O\underline{C}H_2CH_2$); 67.09 ($O\underline{C}H_2CH$); 73.91 ($O\underline{C}HCH_2$); 110.20 ($O\underline{C}O$); 127.06 ($\underline{C}_{Ar}$); 128.24 (3C, $\underline{C}H_{Ar}$); 129.11 (2C, $\underline{C}H_{Ar}$); 171.34 ($\underline{C}$=O).

*4.1.4. Synthesis of dimethyl 2,2-bis(hydroxymethyl)malonate (**19**)*

To a 35% aqueous solution of formaldehyde (1.7 mL, 22.71 mmol, 3 equiv.), sodium bicarbonate (635 mg, 7.57 mmol, 1 equiv.) was added. The mixture was stirred at 20 °C in a water bath for 10 minutes. Thereafter, dimethylmalonate (1.0 g, 7.57 mmol, 1 equiv.) was added dropwise in the same conditions. The reaction was stirred overnight and quenched with Na_2SO_4 saturated solution. The crude was extracted in Et_2O. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated to afford 440 mg (30% yield) of the target product as a colourless liquid. **^{1}H NMR** (400 MHz, CDCl3) δ 2.402 (brs, 1H, O<u>H</u>); 3.77 (s, 6H, 2C<u>H</u>$_3$); 4.10 (s, 4H, C<u>H</u>$_2$). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 52.92 (<u>C</u>H$_3$); 61.16 (CH$_2$<u>C</u>CH$_2$); 63.91 (CH$_2$); 168.88 (COOCH$_3$).

*4.1.5. Synthesis of dimethyl 9-benzyl-1,5-dioxa-9-azaspiro[5.5]undecane-3,3-dicarboxylate(**20**)*

To a solution of 1-benzylpiperidin-4-one (320 μL, 1.72 mmol, 0.75 equiv.) in dry toluene and THF, under nitrogen atmosphere and at room temperature, pTSA (243 mg, .51 mmol, 1.1eq) and a solution of dimethyl 2,2-bis(hydroxymethyl)malonate (440 mg, 2.28mmol, 1 equiv.) in dry toluene were added in the same conditions. The Dean-Stark trap was set-up, and the reaction was refluxed overnight. Thereafter, the mixture was cooled at room temperature and diluted with AcOEt. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, and concentrated. The crude was pure enough to be used in the next step without further purification.

Yellow liquid. 300mg (48% yield). **^{1}H NMR** (400 MHz, CDCl3) δ 1.90 (br m, 4H, C<u>H</u>$_2$C<u>H</u>$_2$); 2.47 (br m, 4H, C<u>H</u>$_2$NC<u>H</u>$_2$); 3.77 (s, 6H, 2 C<u>H</u>$_3$); 3.79 (s, 2H, C<u>H</u>$_2$Ph); 4.30 (br s, 4H, 2 OC<u>H</u>$_2$); 7.18-7.31 (m, 5H, C<u>H</u>$_{Ar}$). **^{13}C NMR** (100 MHz, CDCl3) δ 31.98 (CH$_2$<u>C</u>H$_2$C<u>C</u>H$_2$); 49.76 (2C, <u>C</u>H$_2$N<u>C</u>H$_2$); 53.13 (2 <u>C</u>H$_3$); 53.80 (OCH$_2$<u>C</u>CH$_2$); 61.84 (2 O<u>C</u>H$_2$); 69.25 (<u>C</u>H$_2$Ph); 93.91 (O<u>C</u>O); 125.30 (<u>C</u>H$_{Ar}$); 128.23 (<u>C</u>H$_{Ar}$); 128.27 (<u>C</u>H$_{Ar}$); 129.14 (<u>C</u>$_{Ar}$); 168.32 (2C, <u>C</u>=O).

*4.1.6. Synthesis of methyl 9-benzyl-1,5-dioxa-9-azaspiro[5.5]undecane-3-carboxylate (**21**)*

To a solution of dimethyl 9-benzyl-1,5-dioxa-9-azaspiro[5.5]undecane-3,3-dicarboxylate (300 mg, 0.83 mmol, l equiv.) in DMSO (3 mL *per* mmol), NaCl (48 mg, 0.83 mmol, 1 equiv.) and water (31 uL, 1.72 mmol, 2 equiv.) was added. The reaction was stirred at 180 °C for 4h, cooled at room temperature, and diluted with AcOEt. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, and concentrated to afford the target product as a brown oil (220 mg, 87% yield), which was used in the next step without further purification.

^{1}H NMR (400 MHz, CDCl3) δ 1.87 (brs, 2H, C$\underline{H_2}$CCH$_2$); 2.00 (brs, 2H, CH$_2$CC$\underline{H_2}$); 2.50 (brs, 4H, C$\underline{H_2}$NC$\underline{H_2}$); 2.85 (m, 1H, CH$_2$C$\underline{H}$CH$_2$); 3.56 (s, 2H, PhC$\underline{H_2}$);4.00-4.05 (m, 4H, OC$\underline{H_2}$CHC$\underline{H_2}$O); 7.29-7.33 (m, 5H, CH$_{Ar}$). **^{13}C NMR** (100 MHz, CDCl3) δ 14.20 (C$\underline{H_2}$C$\underline{C}$H$_2$); 21.04 (OCH$_2$C$\underline{H}$CH$_2$O): 40.12 (2C, C$\underline{H_2}$N$\underline{C}$H$_2$); 49.70 (C$\underline{H_3}$); 51.99 (2C, O$\underline{C}$H$_2$CH$\underline{C}$H$_2$O); 59.82 (Ph$\underline{C}$H$_2$); 60.39 (O$\underline{C}$O); 128.32 (C$\underline{H}_{Ar}$); 128.99 (C$\underline{H}_{Ar}$); 129.26 (C$\underline{H}_{Ar}$); 129.74 (C$_{\underline{Ar}}$); 171.14 (C=O).

*4.1.6. Synthesis of 4-butoxy-4-oxobutanoic acid (**22**)*

Succinic anhydride (2.0 g, 20 mmol, 1 equiv.) and butyl alcohol (2.2 mL, 24 mmol, 1.2 equiv.) were solubilized in dry toluene at room temperature and under argon atmosphere. The reaction was stirred at 90 °C overnight. The solvent was evaporated under reduced pressure. The residue was suspended in water and extracted with AcOEt. The organic layer was dried over anhydrous Na_2SO_4 and concentrated to give a 3.13 g of a yellow oil (90% yield), which was used in the next step without further purification.

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.59 (s, 1H, COO$\underline{H}$), 4.19 – 3.99 (m, 2H, COOC$\underline{H_2}$CH$_2$CH$_2$CH$_3$), 2.72 – 2.47 (m, 4H, HOOCC$\underline{H_2}$CH$_2$, HOOCCH$_2$C$\underline{H_2}$), 1.76 – 1.51 (m, 2H, COOCH$_2$C$\underline{H_2}$CH$_2$CH$_3$), 1.44 – 1.15 (m, 2H, COOCH$_2$CH$_2$C$\underline{H_2}$CH$_3$), 0.89 (dd, *J* = 13.3, 7.3 Hz, 3H, COOCH$_2$CH$_2$CH$_2$C$\underline{H_3}$).

*4.1.7. **General procedure for the synthesis of the emisuccinamides 23-25***

41 (1 equiv.) was solubilized in DMF and subsequentially EDC HCl (1equiv.), HOBt (1 equiv.), and 1-benzylpiperazine (1 equiv.), 4-benzylpiperidine (1 equiv.) or 1-benzoylpiperazine (1 equiv.)

were added at 0 °C. The temperature rose spontaneously at room temperature and the mixture stirred in this condition for 4-18 hours. The reaction was diluted with AcOEt, and the organic phase was washed with a saturated solution of $NaHCO_3$, 1N aqueous HCl, or a saturated solution of NH_4Cl and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated.

*4.1.7.1. Butyl 4-(4-benzylpiperazin-1-yl)-4-oxobutanoate (**23**)*

Prepared according to the general procedure for the synthesis of amides 38 and 39. Yellow liquid, 768 mg (70% yield). Pure enough to be was used without further purification. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.36 – 7.22 (m, 5H, C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-4, C$\underline{H}_{Ar}$-5, C$\underline{H}_{Ar}$-6), 4.19 – 3.99 (m, 2H, COOC$\underline{H}_2$CH$_2$CH$_2$CH$_3$), 3.64 – 3.59 (m, 2H, NCOCH$_2$C$\underline{H}_2$), 3.51 (s, 2H, ArC$\underline{H}_2$), 3.50 – 3.45 (m, 2H, NCOC$\underline{H}_2$CH$_2$), 2.65 – 2.61 (m, 3H, Pipz), 2.46 – 2.39 (m, 5H, Pipz). 1.76 – 1.51 (m, 2H, COOCH$_2$C$\underline{H}_2$CH$_2$CH$_3$), 1.44 – 1.15 (m, 2H, COOCH$_2$CH$_2$C$\underline{H}_2$CH$_3$), 0.89 (dd, *J* = 13.3, 7.3 Hz, 3H, COOCH$_2$CH$_2$CH$_2$C$\underline{H}_3$).

*4.1.7.2. Butyl 4-(4-benzoylpiperazin-1-yl)-4-oxobutanoate (**24**)*

Yellow liquid, 754 mg (78% yield). Pure enough to be was used without further purification. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.46 – 7.40 (m, 5H, C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-4, C$\underline{H}_{Ar}$-5, C$\underline{H}_{Ar}$-6), 4.12 (t, *J* = 6.8 Hz, 2H, COOC$\underline{H}_2$CH$_2$CH$_2$CH$_3$), 3.66 (d, *J* = 47.9 Hz, 8H, C$\underline{H}_2$-2 Pipz, C$\underline{H}_2$-3 Pipz, C$\underline{H}_2$-5 Pipz, C$\underline{H}_2$-6 Pipz), 2.76 – 2.60 (m, 4H; NCOC$\underline{H}_2$CH$_2$COO, NCOCH$_2$C$\underline{H}_2$COO), 1.64 (dd, *J* = 9.7, 5.2 Hz, 2H, COOCH$_2$C$\underline{H}_2$CH$_2$CH$_3$), 1.46 – 1.34 (m, 2H, COOCH$_2$CH$_2$C$\underline{H}_2$CH$_3$), 0.95 (t, *J* = 7.4 Hz, 3H, COOC$\underline{H}_2$CH$_2$CH$_2$C$\underline{H}_3$). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 173.10 (C$\underline{O}$OCH$_2$CH$_2$CH$_2$CH$_3$), 170.08 (Ar$\underline{C}$ON, N$\underline{C}$OCH$_2$CH$_2$), 135.19 (C$\underline{}_{Ar}$-1), 130.10 (C$\underline{H}_{Ar}$-4), 128.66 (C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-5), 127.09 (C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-6), 64.61 (COO$\underline{C}$H$_2$CH$_2$CH$_2$CH$_3$), 45.34 ($\underline{C}$H$_2$-2 Pipz, $\underline{C}$H$_2$-6 Pipz), 41.92 ($\underline{C}$H$_2$-3 Pipz, $\underline{C}$H$_2$-5 Pipz), 30.63 (NCOCH$_2$$\underline{C}H_2$COO), 29.21 (COOCH$_2$$\underline{C}H_2CH_2CH_3$), 27.87 (NCO$\underline{C}H_2CH_2$COO), 19.12 (COOCH$_2CH_2$$\underline{C}H_2CH_3$), 13.71 (COOCH$_2CH_2CH_2$$\underline{C}H_3$).

*4.1.7.3. Butyl 4-(4-benzylpiperidin-1-yl)-4-oxobutanoate (**25**)*

Colourless liquid, 268 mg (70% yield). Pure enough to be was used without further purification. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.30 (dd, *J* = 12.2, 4.5 Hz, 2H; 1a, 3a), 7.22 (t, *J* = 7.3 Hz, 1H; 2a), 7.15 (d, *J* = 7.3 Hz, 2H; 4a, 6a), 4.11 (t, *J* = 6.7 Hz, 2H; 20a, 20b), 2.70 – 2.60 (m, 4H;), 2.56 (d, *J* = 6.9 Hz, 2H; 7a, 7b), 1.84 – 1.57 (m, 2H), 1.47 – 1.34 (m, 1H), 1.18 (qd, *J* = 12.9, 4.4 Hz, 1H), 0.95 (t, *J* = 7.4 Hz, 3H; 23a, 23b, 23c). **^{13}C NMR** (101 MHz, CDCl3) δ 173.36 ($\underline{C}OO$), 169.41 ($_2HC\underline{C}ON$), 139.96 ($\underline{C}_{Ar}$-1), 129.07 ($\underline{C}H_{Ar}$-3,5), 128.30 ($\underline{C}H_{Ar}$-2,6), 126.05 ($\underline{C}H_{Ar}$-4), 64.47 ($COO\underline{C}H_2CH_2CH_2CH_3$), 42.96 ($\underline{C}H_2$-2 Pip, $\underline{C}H_2$-6 Pip), 42.17 ($\underline{C}H_2$-Ar), 38.27 ($\underline{C}$H-4 Pip), 32.45 ($NCO\underline{C}H_2CH_2$), 31.76 ($COOCH_2\underline{C}H_2CH_2CH_3$), 30.65 ($\underline{C}H_2$-3 Pip, $\underline{C}H_2$-5 Pip), 29.43 ($NCOCH_2\underline{C}H_2$), 19.13 ($COOCH_2CH_2\underline{C}H_2CH_3$), 13.73 ($COOCH_2CH_2CH_2\underline{C}H_3$).

*4.1.8. General procedure for the synthesis of the O-benzylhydroxylamine derivates **26-28***

Appropriate ester (1 equiv.) and O-benzylhydroxylamine (4 equiv.) were solubilized in dry DCM under a nitrogen atmosphere at room temperature. After 30 minutes, a solution 1M of trimethylaluminum in toluene (4 equiv.) was added, and the reaction was stirred for one hour in the same condition. The reaction was carefully quenched with $NaHCO_3$ and extracted in DCM. The organic layer was dried over anhydrous Na_2SO_4 and concentrated.

*4.1.8.1. N-(benzyloxy)-4-(4-benzylpiperazin-1-yl)-4-oxobutanamide (**26**)*

The crude was purified by silica gel chromatography (crude:silica gel 1:60; 14 g of silica gel; mobile phase DCM/MeOH 95:5) to give 80 mg of a colourless liquid (70% yield). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.91 (s, 1H, $OCN\underline{H}OCH_2$), 7.66 – 7.32 (m, 10H, CH_{Ar}-2, CH_{Ar}-3, CH_{Ar}-4, CH_{Ar}-5, CH_{Ar}-6, CH_{Bn}-2, CH_{Bn}-3, CH_{Bn}-4, CH_{Bn}-5, CH_{Bn}-6), 4.92 (s, 2H, $OCNHOC\underline{H}_2$), 3.58 (m, 4H, $NCOC\underline{H}_2CH_2$, $NCOCH_2C\underline{H}_2$), 3.51 ($ArC\underline{H}_2$), 2.90 – 2.26 (m, 8H, $C\underline{H}_2$-2 Pipz, $C\underline{H}_2$-3 Pipz, $C\underline{H}_2$-5 Pipz, $C\underline{H}_2$-6 Pipz).

*4.1.8.2. 4-(4-benzoylpiperazin-1-yl)-N-(benzyloxy)-4-oxobutanamide (**27**)*

The crude was purified by silica gel chromatography (70 g of silica gel; mobile phase: DCM/MeOH 98:2) to obtain 170 mg of colourless liquid (19% yield). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.33 (dd, *J* = 6.4, 3.1 Hz, 10H, C$\underline{H}_{Bn}$-2, C$\underline{H}_{Bn}$-3, C$\underline{H}_{Bn}$-4, C$\underline{H}_{Bn}$-5, C$\underline{H}_{Bn}$-6, C$\underline{H}_{Bz}$-2, C$\underline{H}_{Bz}$-3, C$\underline{H}_{Bz}$-4, C$\underline{H}_{Bz}$-5, C$\underline{H}_{Bz}$-6), 4.83 (s, 2H, CONHOC$\underline{H}_2$), 3.62 (ddd, *J* = 60.8, 37.7, 27.3 Hz, 8H, C$\underline{H}_2$-2 Pipz, C$\underline{H}_2$-3 Pipz, C$\underline{H}_2$-5 Pipz, C$\underline{H}_2$-6 Pipz), 2.61 (s, 2H, NCOCH$_2$C$\underline{H}_2$CONH), 2.35 (s, 2H, NCOC$\underline{H}_2$CH$_2$CONH). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.66 (C$\underline{O}$NHO), 170.35 (BzC$\underline{O}$N, N$\underline{C}$OCH$_2$CH$_2$), 135.51 ($\underline{C}_{Bz}$-1), 135.09 ($\underline{C}_{Bn}$-1), 130.15 (C$\underline{H}_{Bz}$-4), 129.16 (C$\underline{H}_{Bn}$-3, C$\underline{H}_{Bn}$-5), 128.68 (C$\underline{H}_{Bn}$-2, C$\underline{H}_{Bn}$-6), 128.55 (C$\underline{H}_{Bz}$-3, C$\underline{H}_{Bz}$-5), 127.10 (C$\underline{H}_{Bz}$-2, C$\underline{H}_{Bz}$-6), 78.15 (CONHOC$\underline{H}_2$), 50.85 (C$\underline{H}_2$-2 Pipz, C$\underline{H}_2$-3 Pipz, C$\underline{H}_2$-5 Pipz, C$\underline{H}_2$-6 Pipz), 28.41 (NCOC$\underline{H}_2$CH$_2$CONH), 25.41 (NCOCH$_2$C$\underline{H}_2$CONH).

*4.1.8.3. N-(benzyloxy)-4-(4-benzylpiperidin-1-yl)-4-oxobutanamide (**28**)*

White solid, 180 mg (59% yield). Pure enough to be was used in the next step without further purification. **^{1}H NMR** (400 MHz, CDCl3) δ 7.38 – 7.01 (m, 10H, C$\underline{H}_{Ar}$-1, C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-4, C$\underline{H}_{Ar}$-5, C$\underline{H}_{Bn}$-1, C$\underline{H}_{Bn}$-2, C$\underline{H}_{Bn}$-3, C$\underline{H}_{Bn}$-4, C$\underline{H}_{Bn}$-5), 4.83 (s, 2H, OCNHOC$\underline{H}_2$), 4.45 (s, 1H; Pip), 3.71 (s, 1H, Pip), 2.85 (t, *J* = 12.4 Hz, 1H, Pip), 2.62 – 2.51 (m, 2H, ArC$\underline{H}_2$CH), 2.50 – 2.20 (m, 4H, NCOC$\underline{H}_2$CH$_2$, NCOCH$_2$C$\underline{H}_2$), 1.77 – 1.65 (m, 1H, C$\underline{H}$-4 Pip), 1.61 (d, *J* = 13.3 Hz, 3H, Pip), 1.06 (s, 2H, Pip). **^{13}C NMR** (101 MHz, CDCl3) δ 169.80 (N$\underline{C}$OCH$_2$, O$\underline{C}$NHOCH$_2$), 151.21 ($\underline{C}_{Ar}$-1), 139.83 ($\underline{C}_{Bn}$-1), 129.14 (C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-5), 129.07 (C$\underline{H}_{Bn}$-3, C$\underline{H}_{Bn}$-5), 128.57 (C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-6), 128.33 (C$\underline{H}_{Bn}$-2, C$\underline{H}_{Bn}$-6), 127.66 (C$\underline{H}_{Bn}$-4), 126.10 (C$\underline{H}_{Ar}$-4), 65.39 (OCNHOC$\underline{H}_2$), 42.88 (C$\underline{H}_2$-2 Pip, C$\underline{H}_2$-6 Pip), 42.48 (ArC$\underline{H}_2$), 38.15 (C$\underline{H}$-4 Pip), 32.33 (NCOC$\underline{H}_2$CH$_2$), 31.86 (C$\underline{H}_2$-3 Pip, C$\underline{H}_2$-5 Pip), 28.71 (NCOCH$_2$C$\underline{H}_2$).

*4.1.9. Synthesis of (4-hydroxypiperidin-1-yl)(phenyl)methanone (**29**)*

To a solution of 1-benzoyl-piperidin-4-one (500mg, 2.46 mmol,1 equiv.) in MeOH (5 mL), $NaBH_4$ (140 mg, 5 mmol, 2 equiv.) was added. The reaction was stirred at room temperature for 12 h. Thereafter, 1N aqueous HCl was added until neutrality. The crude was extracted in AcOEt, washed with brine, dried over anhydrous Na_2SO_4, and concentrated to give the desired product as an analytically pure colorless liquid (445 mg, 87% yield).

LC: Cyclohexane: AcOEt 3:6 Rf= 0.3. **^{1}H NMR** (400 MHz, CDCl3) δ 1.39-1.62(m, 3H, C$\underline{H_2}$CHC$\underline{H_2}$); 1.70-1.95(m, 1H, CH_2CHC$\underline{H_2}$); 3.00-3.40 (m, 2H, C$\underline{H_2}$NCH_2); 3.24(brs, 1H, CH_2NC$\underline{H_2}$); 3.88-3.93(m, 1H, CH_2NC$\underline{H_2}$); 7.30-7.40(m, 5H, CH_{Ar}). **^{13}C NMR** (100 MHz, CDCl3) δ 34.83 (2C, $\underline{C}H_2$CH$\underline{C}H_2$), 44.04 (2C, $\underline{C}H_2$N$\underline{C}H_2$), 67.05 ($\underline{C}$OH), 128.31 ($\underline{C}H_{Ar}$), 128.95 ($\underline{C}H_{Ar}$), 131.87 ($\underline{C}H_{Ar}$), 136.29 ($\underline{C}_{Ar}$), 170.27 ($\underline{C}$=O).

*4.1.10. Synthesis of 4-((1-benzoylpiperidin-4-yl)oxy)-4-oxobutanoic acid (**30**)*

To a solution of (4-hydroxypiperidin-1-yl)(phenyl)methanone (445 mg, 2.17mmol, 1 equiv.) in DMF, succinic anhydride (435 mg, 4.34 mmol, 2 equiv.) was added. The reaction mixture was performed under microwave irradiation (two cycles at 150 °C, 150 W for 20 min, followed by two cycles at 160 °C, 150 W, 20 min). The reaction was quenched with water, alkalized with a saturated aqueous solution of $NaHCO_3$, and extracted in AcOEt. The aqueous phase was acidified with 1N aqueous HCl and retroextraced in AcOEt. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, and concentrated to give a yellow liquid (376 mg, 57% yield).

LC: DCM:MeOH 95:5 Rf=0.4. **^{1}H NMR** (400 MHz, CDCl3) δ 1.50-2.00 (m, 4H, C$\underline{H_2}$CHC$\underline{H_2}$); 2.64-2.68 (m, 4H, OCOC$\underline{H_2}$C$\underline{H_2}$CON); 3.33 (brs,1H, C$\underline{H_2}$NCH_2); 3.60 (brs,2H, C$\underline{H_2}$NC$\underline{H_2}$); 3.97 (brs, 1H, CH_2NC$\underline{H_2}$); 5.00-5.06 (m, 1H, CH_2C$\underline{H}CH_2$); 7.40-7.52 (m, 5H, CH_{Ph}). **^{13}C NMR** (100 MHz, CDCl3) δ 28.73 (OCOC$H_2\underline{C}H_2$COOH); 29.24 (OCO$\underline{C}H_2CH_2$COOH); 69.70 ($CH_2\underline{C}HCH_2$); 126.85 ($\underline{C}H_{Ar}$); 128.54 ($\underline{C}H_{Ar}$); 129.78 ($\underline{C}H_{Ar}$); 135.75 ($\underline{C}H_{Ar}$); 170.62 (N$\underline{C}$O); 171.36 (O$\underline{C}$O); 176.14 ($\underline{C}$OOH).

*4.1.11. Synthesis of 1-benzoylpiperidin-4-yl 4-((benzyloxy)amino)-4-oxobutanoate (**31**)*

To a solution of **49** (210 mg, 0.69 mmol, 1 equiv.) in DCM dry (10 mL) at 0 °C and under nitrogen atmosphere, TEA (114 uL, 0.90 mmol, 1.3 equiv.) and ethyl chloroformiate (77 uL, 0.83 mmol, 1.2 equiv.) were added. The reaction was stirred for 2h at room temperature and, thereafter, O-benzylhydroxylamine (132 mg, 0.83 mmol, 1.2 equiv.) and TEA (110 uL, 0.83 mmol, 1.2 equiv.) were added in the same condition. After 2h, the reaction was diluted with AcOEt, washed with a saturated solution of $NaHCO_3$, a saturated solution of NH_4Cl, and brine. The organic phase was dried over anhydrous Na_2SO_4 and concentrated. The crude was purified by silica gel chromatography (ratio crude:silica gel 1:100, DCM:MeOH 98:2) to give **12** as a colorless liquid (120 mg, 42% yield).

TLC: DCM:MeOH 95:5 Rf=0.3. **^{1}H NMR** (400 MHz, CDCl3) δ 1.60-2.00 (m, 5H, C$\underline{H_2}$CHC$\underline{H_2}$,OCOC$\underline{H_2}$); 2.37 (br s, 1H, OCOC$\underline{H_2}$); 2.69 (br s, 2H, C$\underline{H_2}$CONH); 3.20-4.10 (m, 4H, C$\underline{H_2}$NC$\underline{H_2}$); 4.91 (s, 2H, C$\underline{H_2}$Ph). **^{13}C NMR** (100 MHz, CDCl3) δ 27.7 (C$\underline{H_2}$CON); 30.0 (C$\underline{H_2}$CHC$\underline{H_2}$); 32.4 (C$\underline{H_2}$COO); 41.7 (C$\underline{H_2}$NC$\underline{H_2}$); 72.6 (CH_2C$\underline{H}$CH_2); 78.5 (C$\underline{H_{Ar}}$); 127.1 (C$\underline{H_{Ar}}$); 127.2 (C$\underline{H_{Ar}}$); 127.6 (C$\underline{H_{Ar}}$); 128.5 (C$\underline{H_{Ar}}$); 128.9 (C$\underline{H_{Ar}}$); 129.7 (C$\underline{H_{Ar}}$); 135.2 (C$\underline{_{Ar}}$); 136.5 (C$\underline{_{Ar}}$); 170.0 (NC$\underline{O}$); 170.6 (OC$\underline{N}$O); 173.1 (OC$\underline{O}$).

*4.1.12. Synthesis of 2,3-dihydrobenzo[b][1,4]dioxine-2-carboxylic acid (**32**)*

A solution of $KMnO_4$ (1.300 g, 8.42 mmol, 1.4 equiv.) and KOH (168.8 mg, 3.01 mmol, 0.5 equiv.) in water was added to (2,3-dihydrobenzo[b][1,4]dioxin-2-yl)methanol (1.000 g, 6 mmol, 1 equiv.). The reaction was stirred for one hour at room temperature and then at 60 °C for 2 hours. After 2 hours, a second amount of $KMnO_4$ (450 mg, 0.5 equiv.) was added, and the reaction was stirred for an additional hour. The solution was filtered through a celite pad, diluted with water, acidified with 1N aqueous HCl, and extracted in AcOEt. The organic layer was dried over anhydrous Na_2SO_4 and concentrated to give 800 mg of a yellow solid (73% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.33 – 4.36 (m, 2H, C$\underline{H_2}$-3 Diox), 4.82 (m, 1H, C$\underline{H}$-2 Diox), 6.83 – 6.95 (m, 4H, C$\underline{H_{Ar}}$-3, C$\underline{H_{Ar}}$-4, C$\underline{H_{Ar}}$-5, C$\underline{H_{Ar}}$-6). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 165.0 (CHC$\underline{O}$OH),

143.3 (C_{Ar}-2), 142.7 ($\underline{C}_{Ar}$-1), 120.9 ($\underline{C}H_{Ar}$-4, $\underline{C}H_{Ar}$-5) , 120.2 ($\underline{C}H_{Ar}$-6), 115.0 ($\underline{C}H_{Ar}$-3), 78.2 ($\underline{C}$H-2 Diox), 64.9 ($\underline{C}H_2$-3 Diox).

*4.1.13. Synthesis of Ethyl 2,3-dihydrobenzo[b][1,4]dioxine-2-carboxylate (**33**)*

32 (1 equiv.) was solubilized in EtOH at 0 °C. Thionyl chloride (1 mL) was added dropwise, and the reaction was stirred at room temperature overnight. The solvent was evaporated under reduced pressure. The residue was diluted in AcOEt and washed with a saturated solution of $NaHCO_3$. The organic layer was washed with brine, dried over anhydrous Na_2SO_4 and concentrated, to give 600 mg (64% yield) of a yellow liquid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.06 – 7.00 (m, 2H, C$\underline{H}_{Ar}$-4, C$\underline{H}_{Ar}$-5), 6.95 – 6.85 (m, 2H, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-6), 4.84 (t, *J* = 3.9 Hz, 1H, C$\underline{H}$-2 Diox), 4.41 (d, J = 3.9 Hz, 2H, C$\underline{H}_2$-3 Diox), 4.35 – 4.23 (m, 2H, COOC$\underline{H}_2$CH$_3$), 1.31 (t, *J* = 7.1 Hz, 3H, COOCH$_2$C$\underline{H}_3$). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 167.95 ($\underline{C}$OOCH$_2$CH$_3$), 142.96 ($\underline{C}_{Ar}$-1), 142.34 ($\underline{C}_{Ar}$-2), 122.13 ($\underline{C}H_{Ar}$-5), 121.83 ($\underline{C}H_{Ar}$-4), 117.40 ($\underline{C}H_{Ar}$-6), 117.25 ($\underline{C}H_{Ar}$-3), 72.02 ($\underline{C}$H-2 Diox), 64.97 ($\underline{C}H_2$-3 Diox), 61.96 (COO$\underline{C}$H$_2$CH$_3$), 14.11 (COOCH$_2\underline{C}$H$_3$).

*4.1.14. Synthesis of ethyl benzo[b][1,4]dioxine-2-carboxylate (**34**)*

33 (450 mg, 2.16 mmol, 1 equiv.) was solubilized in dry CCl_4 under nitrogen atmosphere at room temperature. NBS (1.272 g, 7.13 mmol, 3.3 equiv.) and AIBN (105 mg, 0.66 mmol, 0.30 equiv.) were added to the solution in three portions at intervals of one hour, under reflux and hv irradiation. After the last addition of NBS and AIBN the reaction was stirred for additional four hours. The solvent was evaporated, and the residue was diluted with acetone. NaI (973 mg, 6.49 mmol, 3 equiv.) was added to the solution, and the reaction was stirred for 2 hours at reflux. The mixture was cooled, filtered, and concentrated. The residue was suspended in AcOEt and washed with a saturated solution of $Na_2S_2O_3$. The organic layer was dried over anhydrous Na_2SO_4 and

concentrated. The residue was purified over silica gel (30 g of silica gel; eluent phase CE/AcOEt 9:1), obtaining 230 mg of brown solid in 51% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.98 – 6.81 (m, 4H, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-4, C$\underline{H}_{Ar}$-5, C$\underline{H}_{Ar}$-6), 6.72 (d, *J* = 7.6 Hz, 1H, C$\underline{H}$-3 Dioxi), 4.30 (q, *J* = 7.1 Hz, 2H, COOC$\underline{H}_2$CH$_3$), 1.35 (t, *J* = 7.1 Hz, 3H, COOCH$_2$C$\underline{H}_3$). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 161.25 (C$\underline{O}$OCH$_2$CH$_3$), 142.19 (C$\underline{}_{Ar}$-1), 140.66 (C$\underline{}_{Ar}$-2), 135.79 (C$\underline{H}$-3 Dioxi), 129.19 (C$\underline{}$-2 Dioxi), 125.42 (C$\underline{H}_{Ar}$-5), 124.54 (C$\underline{H}_{Ar}$-4), 116.93 (C$\underline{H}_{Ar}$-3), 116.38 (C$\underline{H}_{Ar}$-6), 26.92 (COO$\underline{C}$H$_2$CH$_3$), 14.23 (COOCH$_2\underline{C}$H$_3$).

*4.1.15. Synthesis of benzofuran-2-carboxylic acid (**35**)*

2-hydroxybenzaldehyde (873 µL, 8.20 mmol, 1 equiv.) was solubilized in dry DMF. K_2CO_3 (2.263 g, 16.4 mmol, 2 equiv.) and ethyl 2-bromoacetate (1.000 mL, 9.02 mmol, 1.1 equiv.) was added, and the reaction was stirred at 150 °C for 4 hours. The reaction was quenched with water and refluxed for 1 hour. The mixture was chilled in an ice bath and acidified with 1N aqueous HCl. The yellow precipitated formed was collected by filtration, rinsed with water, and dried to give 800 mg of the desired product as a brown solid (60% yield).

^{1}H NMR (400 MHz, DMSO) δ 7.80 (d, *J* = 7.7 Hz, 1H, C$\underline{H}$-3 Fur), 7.74 – 7.63 (m, 2H, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-6), 7.51 (ddd, *J* = 8.4, 7.3, 1.3 Hz, 1H, C$\underline{H}_{Ar}$-4), 7.41 – 7.29 (m, 1H, C$\underline{H}_{Ar}$-5). **^{13}C NMR** (101 MHz, DMSO) δ 160.05 (C$\underline{}$OOH), 154.95 (C$\underline{}_{Ar}$-1), 146.12 (C$\underline{}$-2 Fur), 127.54 (C$\underline{}_{Ar}$-2), 126.81 (C$\underline{H}_{Ar}$-5), 123.80 (C$\underline{H}_{Ar}$-3), 123.07 (C$\underline{H}_{Ar}$-4), 113.48 (C$\underline{H}_{Ar}$-6), 112.03 (C$\underline{H}$-3 Fur).

*4.1.16. General procedure for the synthesis of ethyl esters **36-37, 40-41, 46-47***

The appropriate phenol or thiophenol (1 equiv.) and K_2CO_3 (2.5 equiv.) were solubilized in 5 mL of DMF (for phenols) or acetone (for thiophenols) and stirred at room temperature for 30 minutes. Ethyl 2-bromoacetate (1.086 mL, 9.83 mmol, 1.2 equiv.) was added, and the reaction was stirred for 2-48 hours at 60 °C. The reaction was quenched with water and extracted with AcOEt. The organic layer was washed with 1N aqueous NaOH, brine, dried over anhydrous Na_2SO_4, and concentrated.

*4.1.16.1. Ethyl 2-phenoxyacetate (**36**)*

Dark yellow oil, 900 mg (94% yield). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.31 (dt, *J* = 7.0, 6.3 Hz, 2H, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-5), 7.02 (t, *J* = 7.4 Hz, 1H, C$\underline{H}_{Ar}$-4), 6.97 – 6.89 (m, 2H, C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-6), 4.64 (s, 2H, ArOC$\underline{H}_2$COO), 4.29 (q, *J* = 7.1 Hz, 2H, COOC$\underline{H}_2$CH$_3$), 1.32 (t, *J* = 7.2 Hz, 3H, COOCH$_2$C$\underline{H}_3$).

*4.1.16.2. Ethyl 2-(phenylthio)acetate (**37**)*

The crude was purified on silica gel (70 g of silica gel; mobile phase CE/AcOEt 95:5) to give 530 mg of colourless liquid (56% yield). **^{1}H NMR** (400 MHz, CDCl3) δ 7.44 (d, *J* = 7.8 Hz, 2H, C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-6), 7.32 (t, *J* = 7.5 Hz, 2H, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-5), 7.26 (dd, *J* = 13.8, 6.8 Hz, 1H, C$\underline{H}_{Ar}$-4), 4.19 (q, *J* = 7.1 Hz, 2H, COOC$\underline{H}_2$CH$_3$), 3.66 (s, 2H, SC$\underline{H}_2$COO), 1.25 (t, *J* = 7.1 Hz, 3H, COOCH$_2$C$\underline{H}_3$). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 169.70 (C$\underline{O}$O), 135.01 (C$\underline{}_{Ar}$-1), 130.04 (C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-6), 129.02 (C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-5), 126.97 (C$\underline{H}_{Ar}$-4), 61.54 (COO$\underline{C}$H$_2$CH$_3$), 36.76 (S$\underline{C}$H$_2$COO), 14.08 (COOCH$_2$$\underline{C}H_3$).

*4.1.16.3. ethyl 2-(4-(hydroxymethyl)phenoxy)acetate (**40**)*

The crude was purified by flash chromatography on ISOLERA Biotage. A gradient was delivered at 1 mL/min using (A) CE and (B) AcOEt. Samples were eluted with 10% B (3 column volume, CV); 10-80% B (20 CV); 80% B (3 CV) to give 745 mg of a yellow oil (43% yield). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.94 – 7.80 (m, 2H, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-5), 7.03 (d, *J* = 8.7 Hz, 2H, C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-6), 4.73 (s, 2H, OC$\underline{H}_2$COO), 4.69 (s, 2H, ArC$\underline{H}_2$OH), 4.30 (q, *J* = 7.1 Hz, 2H, COOC$\underline{H}_2$CH$_3$), 1.32 (t, *J* = 7.1 Hz, 3H, COOCH$_2$C$\underline{H}_3$).

*4.1.16.4. Ethyl 2-((4-(hydroxymethyl)phenyl)thio)acetate (**41**)*

The crude was purified by silica gel chromatography (60 g of silica gel; mobile phase CE/ AcOEt 1:1) to give 500 mg of colourless liquid (70% yield). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.34 (dt, *J* = 15.4, 5.7 Hz, 4H, C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-5, C$\underline{H}_{Ar}$-6), 4.69 (s, 2H, ArC$\underline{H}_2$OH), 4.19 (q, *J* = 7.1 Hz,

2H, COOC$\underline{H}_2$CH$_3$), 3.64 (s, 2H, SC$\underline{H}_2$COO), 1.25 (t, *J* = 7.1 Hz, 3H, COOCH$_2$C$\underline{H}_3$). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 169.66 (S$\underline{C}$H$_2$COO), 139.84 ($\underline{C}_{Ar}$-4), 134.23 ($\underline{C}_{Ar}$-1), 130.30 ($\underline{C}H_{Ar}$-2, $\underline{C}H_{Ar}$-6), 127.62 ($\underline{C}H_{Ar}$-3, $\underline{C}H_{Ar}$-5), 64.80 (Ar$\underline{C}$H$_2$OH), 61.57 (COO$\underline{C}$H$_2$CH$_3$), 36.83 (S$\underline{C}$H$_2$COO), 14.09 (COOCH$_2\underline{C}$H$_3$).

*4.1.16.5. 4-(2-ethoxy-2-oxoethoxy)benzoic acid (**46**)*

The crude was triturated with Et_2O. The precipitate was filtered, rinsed with Et2O and dried to give 384 mg of a white solid (83% yield). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.29 – 7.68 (m, 2H, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-5), 7.02 (d, J = 7.5 Hz, 2H, C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-6), 4.99 (s, 2H, OC$\underline{H}_2$COO), 4.21 (q, J = 8.0 Hz, 2H, COOC$\underline{H}_2$CH$_3$), 1.22 (t, J = 8.1 Hz, 3H, COOCH$_2$C$\underline{H}_3$). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 169.48(O$\underline{C}$H$_2$COO), 168.59 (Ar$\underline{C}$OOH), 159.26 ($\underline{C}_{Ar}$-1), 131.85 ($\underline{C}H_{Ar}$-3, $\underline{C}H_{Ar}$-5), 122.45 ($\underline{C}_{Ar}$-4), 114.64 ($\underline{C}H_{Ar}$-2, $\underline{C}H_{Ar}$-6), 65.15 (COO$\underline{C}$H$_2$CH$_3$), 61.44 (O$\underline{C}$H$_2$COO), 14.26 (COOCH$_2\underline{C}$H$_3$).

*4.1.16.6. 4-((2-ethoxy-2-oxoethyl)thio)benzoic acid (**47**)*

The crude was triturated with Et2O. The precipitate was filtered, rinsed with Et2O and dried to give 538 mg of a yellowish solid (77% yield). **^{1}H NMR** (400 MHz, DMSO) δ 12.91 (s, 1H, ArCOO$\underline{H}$), 7.86 (d, *J* = 8.6 Hz, 2H, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-5), 7.41 (d, *J* = 8.6 Hz, 2H, C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-6), 4.11 (q, *J* = 7.1 Hz, 2H, COOC$\underline{H}_2$CH$_3$), 4.03 (s, 2H, SC$\underline{H}_2$COO), 1.16 (t, *J* = 7.1 Hz, 3H, COOCH$_2$C$\underline{H}_3$). **^{13}C NMR** (101 MHz, DMSO) δ 168.85 (S$\underline{C}$H$_2$COO), 166.84 (Ar$\underline{C}$OOH), 141.85 ($\underline{C}_{Ar}$-1), 129.72 ($\underline{C}H_{Ar}$-3, $\underline{C}H_{Ar}$-5), 127.76 ($\underline{C}_{Ar}$-4), 126.39 ($\underline{C}H_{Ar}$-2, $\underline{C}H_{Ar}$-6), 61.10 (COO$\underline{C}$H$_2$CH$_3$), 33.55 (S$\underline{C}$H$_2$COO), 13.92 (COOCH$_2\underline{C}$H$_3$).

*4.1.17. General procedure for the synthesis of benzyl alcohols **38** and **39***

To a solution of $LiAlH_4$ (2.2 equiv.) in dry THF at 0 °C and under nitrogen atmosphere, 4-hydroxybenzoic acid, or 4-mercaptobenzoic acid (1 equiv.) were added in portion and stirred for 6 hours at room temperature. The reaction was cooled in an ice bath and quenched with 1N aqueous HCl. The reaction was diluted and extracted in AcOEt. HCl was added to solubilized aluminum.

The organic layer was dried over anhydrous Na_2SO_4 and concentrated to give the desired product, pure enough to be used in the next step without further purification.

*4.1.17.1. 4-(hydroxymethyl)phenol (**38**)*

White solid (86% yield). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.15 (dd, *J* = 7.2, 1.5 Hz, 2H, C$\underline{H}_{Ar}$), 6.78 (d, *J* = 7.5 Hz, 2H, C$\underline{H}_{Ar}$), 4.88 – 4.27 (m, 2H, C$\underline{H}_2$).

*4.1.17.2. 4-mercaptophenyl)methanol (**39**)*

Yellow solid (97% yield). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.54 – 6.99 (m, 4H, C$\underline{H}_{Ar}$), 4.53 (s, 2H, C$\underline{H}_2$).

*4.1.18. General procedure for the synthesis of the chlorinated derivatives **42-43***

Appropriate benzyl alcohol **40** or **41** (1 equiv.) were solubilized in 5 mL of $SOCl_2$ at 0 °C. A drop of DMF was added as a catalyst and heated at reflux. After 2 hours, the solvent was evaporated. The residue was triturated with crushed ice and neutralized with a saturated solution of $NaHCO_3$. The aqueous layer was extracted with AcOEt. The organic phase was dried over anhydrous Na_2SO_4 and concentrated to give the desired product that was used in the next step without further purification.

*4.1.18.1. Ethyl 2-(4-(chloromethyl)phenoxy)acetate (**42**)*

White solid, 186 mg (quantitative yield). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.34 (d, *J* = 8.7 Hz, 2H, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-5), 6.95 – 6.87 (m, 2H, C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-6), 4.64 (s, 2H, OC$\underline{H}_2$COO), 4.58 (s, 2H, ClC$\underline{H}_2$Ar), 4.30 (q, *J* = 7.1 Hz, 2H, COOC$\underline{H}_2$CH$_3$), 1.32 (t, *J* = 7.1 Hz, 3H, COOCH$_2$C$\underline{H}_3$). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 168.86 (C$\underline{O}$O), 158.06 (C$\underline{}_{Ar}$-1), 130.99 (C$\underline{}_{Ar}$-4), 130.26 (C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-5), 115.05 (C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-6), 65.62 (OC$\underline{H}_2$COO), 61.59 (COOC$\underline{H}_2$CH$_3$), 46.15 (ClC$\underline{H}_2$Ar), 14.31 (COOCH$_2$C$\underline{H}_3$).

*4.1.18.2. Ethyl 2-((4-(chloromethyl)phenyl)thio)acetate (**43**)*

Yellow liquid, 594 mg (quantitative yield). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.47 – 7.25 (m, 4H, C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-5, C$\underline{H}_{Ar}$-6),), 4.57 (s, 2H, ArC$\underline{H}_2$Cl), 4.19 (q, *J* = 7.1 Hz, 2H, COOC$\underline{H}_2$CH$_3$), 3.66 (s, 2H, SC$\underline{H}_2$COO), 1.25 (t, *J* = 7.2 Hz, 3H, COOCH$_2$C$\underline{H}_3$). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 169.50 (S$\underline{C}$H$_2$COO), 136.11 ($\underline{C}_{Ar}$-4), 135.63 ($\underline{C}_{Ar}$-1), 129.82 ($\underline{C}$H$_{Ar}$-2, $\underline{C}$H$_{Ar}$-6), 129.23 ($\underline{C}$H$_{Ar}$-3, $\underline{C}$H$_{Ar}$-5), 61.64 (COO$\underline{C}$H$_2$CH$_3$), 45.69 (Ar$\underline{C}$H$_2$Cl), 36.41 (S$\underline{C}$H$_2$COO), 14.08 (COOCH$_2$$\underline{C}H_3$).

*4.1.19. Synthesis of ethyl 2-(4-((4-benzylpiperidin-1-yl)methyl)phenoxy)acetate (**44**)*

4-benzylpiperidine (157.1 µL, 0.89 mmol, 1.1 equiv.) and K_2CO_3 (168 mg, 1.22 mmol, 1.5 equiv.) were solubilized in 1mL of DMSO. Thereafter, a solution of ethyl 2-(4-(chloromethyl) phenoxy) acetate (186 mg, 0.81 mmol, 1 equiv.) in DMSO was added dropwise at room temperature and stirred for 2 hours at 60 °C. The reaction was quenched with water and extracted in AcOEt. The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated to give 300 mg of a colourless liquid. The crude was purified on silica gel (1:50 silica ratio; 15 g of silica gel; eluent phase DCM/MeOH 9:1) to give 70 mg of a colourless oil (23% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 – 7.10 (m, 7H, C$\underline{H}_{Ar}$-Bn, C$\underline{H}_{Ar}$-3,5-Ph), 6.88 (d, J = 9.4 Hz, 2H, C$\underline{H}_{Ar}$-2,6-Ph), 4.62 (s, 2H, OC$\underline{H}_2$CO), 4.34 (q, *J* = 7.1 Hz, 2H, COOC$\underline{H}_2$CH$_3$), 3.78 – 3.37 (m, 2H, C$\underline{H}_2$-2,6 Pipz), 3.5-2.75 (m, 2H, C$\underline{H}_2$-2',6' Pipz), 2.56 (s, 2H, PhC$\underline{H}_2$), 2.15-1.85 (m, 2H, C$\underline{H}_2$-3,5 Pipz), 1.70-1.45 (m, 3H, C$\underline{H}_2$-3',4,5' Pipz), 1.31 (t, *J* = 7.1 Hz, 3H, COOCH$_2$C$\underline{H}_3$). **^{13}C NMR** (151 MHz, $CDCl_3$) δ 168.59 ($\underline{C}$OO), 157.91 ($\underline{C}_{Ar}$-1-Ph), 140.00 ($\underline{C}_{Ar}$-1-Bn), 129.10 ($\underline{C}$H$_{Ar}$-2,6 Bn), 128.84 ($\underline{C}$H$_{Ar}$-3,5 Ph), 128.18 ($\underline{C}$H$_{Ar}$-3,5 Bn), 127.00 ($\underline{C}$H$_{Ar}$-4 Bn), 124.87 ($\underline{C}$H$_{Ar}$-4 Ph), 115.51 ($\underline{C}$H$_{Ar}$-2,6 Ph), 65.15 (O$\underline{C}$H$_2$COO), 61.94 (N$\underline{C}$H$_2$Ar), 61.44 (COO$\underline{C}$H$_2$CH$_3$), 51.27 ($\underline{C}$H$_2$-2,5 pip), 41.96 ($\underline{C}$H$_2$-Bn), 36.60 ($\underline{C}$H-4 pip), 30.16 51 ($\underline{C}$H$_{Ar}$-3,5 Ph), 14.26 (COOCH$_2$$\underline{C}H_3$).

*4.1.20. Synthesis of ethyl 2-((4-((4-benzylpiperidin-1-yl)methyl)phenyl)thio)acetate (**45**)*

43 (594 mg, 2.43 mmol, 1 equiv.) was solubilized in DMSO, and K_2CO_3 (503.9 mg, 3.65 mmol, 1.5 equiv.) was added. After 30 minutes at room temperature, 4-benzylpiperidine (470 µL, 2.68 mmol, 1.1 equiv.) was added, and the reaction was stirred for 6 hours at 60 °C. The reaction was cooled at room temperature, diluted in water, and extracted in AcOEt. The organic layer was washed with a saturated solution of NaCl, dried over anhydrous Na_2SO_4, and concentrated to obtain 680mg of a yellow oil that was purified on silica gel (70 g of silica gel; eluent phase CE /AcOEt 1:1) to give 300 mg of the titled compound as a colourless liquid (32% yield).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 – 7.12 (m, 9H, C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-4, C$\underline{H}_{Ar}$-5, C$\underline{H}_{Ar}$-6, C$\underline{H}_{ArS}$-2, C$\underline{H}_{ArS}$-3, C$\underline{H}_{ArS}$-5, C$\underline{H}_{ArS}$-6), 4.18 (q, *J* = 7.1 Hz, 2H, COOC$\underline{H}_2$CH$_3$), 3.63 (s, 2H, SC$\underline{H}_2$COO), 3.46 (s, 2H, NC$\underline{H}_2$ArS), 2.85 (d, *J* = 11.0 Hz, 2H, Pip), 2.55 (d, *J* = 7.0 Hz, 2H, ArC$\underline{H}_2$), 1.91 (t, *J* = 11.1 Hz, 2H, Pip), 1.62 (d, *J* = 10.8 Hz, 2H, Pip), 1.54 (ddd, *J* = 14.9, 7.3, 3.8 Hz, 1H, C$\underline{H}$-4 Pip), 1.40 – 1.29 (m, 2H, Pip), 1.24 (t, *J* = 7.1 Hz, 3H, COOCH$_2$C$\underline{H}_3$). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 169.76 (SCH$_2\underline{C}$OO), 140.72 ($\underline{C}_{Ar}$-1), 137.92 – 137.33 ($\underline{C}_{ArS}$-4), 133.21 ($\underline{C}_{ArS}$-1), 130.14 ($\underline{C}H_{ArS}$-2, $\underline{C}H_{ArS}$-6), 129.86 ($\underline{C}H_{Ar}$-3, $\underline{C}H_{Ar}$-5), 129.11 ($\underline{C}H_{Ar}$-2, $\underline{C}H_{Ar}$-6), 128.14 ($\underline{C}H_{ArS}$-3, $\underline{C}H_{ArS}$-5), 125.76 ($\underline{C}H_{Ar}$-4), 62.80 (N$\underline{C}$H$_2$ArS), 61.51 (COO$\underline{C}$H$_2$CH$_3$), 53.78 ($\underline{C}$H$_2$-2 Pip, $\underline{C}$H$_2$-6 Pip), 43.18 (Ar$\underline{C}$H$_2$), 37.85 ($\underline{C}$H-4 Pip), 37.00 (S$\underline{C}$H$_2$COO), 32.10 ($\underline{C}$H$_2$-3 Pip, $\underline{C}$H$_2$-5 Pip), 14.09 (COOCH$_2\underline{C}$H$_3$).

*4.1.21. General procedure for the synthesis of amides **48-49***

Appropriate carboxylic acid **46** or **47** (1 equiv.) was solubilized in DMF and subsequentially EDC HCl (1equiv.), HOBt (1 equiv.) and 4-benzylpiperidine (1 equiv.) or 1-benzylpiperazine (1 equiv.) were added at 0 °C. The temperature has risen spontaneously at room temperature and the mixture stirred in this condition for 4-18 hours. The reaction was diluted with AcOEt, and the organic phase was washed with a saturated solution of $NaHCO_3$, 1N aqueous HCl, or a saturated solution of NH_4Cl and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated.

*4.1.21.1. Ethyl 2-(4-(4-benzylpiperidine-1-carbonyl)phenoxy)acetate (**48**)*

Orange liquid, 590 mg (91% yield). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.38 (d, *J* = 8.5 Hz, 2H, C$\underline{H}_{Bz}$-3, C$\underline{H}_{Bz}$-5), 7.34 – 7.26 (m, 2H, C$\underline{H}_{Bn}$-3, C$\underline{H}_{Bn}$-5), 7.22 (t, *J* = 7.0 Hz, 1H, C$\underline{H}_{Bn}$-4), 7.16 (d, *J* = 7.5 Hz, 2H, C$\underline{H}_{Bn}$-2, C$\underline{H}_{Bn}$-6), 6.92 (d, *J* = 8.5 Hz, 2H, C$\underline{H}_{Bz}$-2, C$\underline{H}_{Bz}$-6), 4.66 (s, 3H, OC$\underline{H}_2$COO, Pip), 4.30 (q, *J* = 7.1 Hz, 2H, COOC$\underline{H}_2$CH$_3$), 3.84 (s, 1H, Pip), 2.84 (s, 2H, Pip), 2.59 (d, *J* = 6.9 Hz, 2H, BnC$\underline{H}_2$), 1.94 – 1.54 (m, 5H, Pip), 1.32 (t, *J* = 7.1 Hz, 3H, COOCH$_2$C$\underline{H}_3$). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.00 (N$\underline{C}$O), 168.60 (O$\underline{C}$H$_2$COO), 158.77 ($\underline{C}_{Bz}$-1), 139.94 ($\underline{C}_{Bn}$-1), 129.63 ($\underline{C}_{Bz}$-4), 129.07 ($\underline{C}H_{Bn}$-3, $\underline{C}H_{Bn}$-5), 128.93 ($\underline{C}H_{Bn}$-2, $\underline{C}H_{Bn}$-6), 128.31 ($\underline{C}H_{Bz}$-3, $\underline{C}H_{Bz}$-5), 126.07 ($\underline{C}H_{Bn}$-4), 114.41 ($\underline{C}H_{Bz}$-2, $\underline{C}H_{Bz}$-6), 65.39 (O$\underline{C}$H$_2$COO), 61.49 (COO$\underline{C}$H$_2$CH$_3$), 43.02 ($\underline{C}$H$_2$-2 Pip, $\underline{C}$H$_2$-3 Pip, $\underline{C}$H$_2$-5 Pip, $\underline{C}$H$_2$-6 Pip), 38.37 (Bn$\underline{C}$H$_2$), 32.27 ($\underline{C}$H-4 Pip), 14.17 (COOCH$_2\underline{C}$H$_3$).

*4.1.21.2. Ethyl 2-((4-(4-benzylpiperidine-1-carbonyl)phenyl)thio)acetate (**49**)*

The crude was purified by silica gel chromatography (50 g of silica gel; eluent phase CE/ AcOEt 1:1). 440 mg of colourless liquid (49% yield). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.42 (d, J = 8.2 Hz, 2H, C$\underline{H}_{Bz}$-3, C$\underline{H}_{Bz}$-5), 7.38 – 7.32 (m, 2H, C$\underline{H}_{Bz}$-2, C$\underline{H}_{Bz}$-6), 7.30 (d, J = 8.6 Hz, 2H, C$\underline{H}_{Bn}$-3, C$\underline{H}_{Bn}$-5), 7.25 – 7.12 (m, 3H, C$\underline{H}_{Bn}$-2, C$\underline{H}_{Bn}$-4, C$\underline{H}_{Bn}$-6), 4.21 (q, *J* = 7.1 Hz, 2H, COOC$\underline{H}_2$CH$_3$), 3.76 (d, *J* = 9.3 Hz, 1H, Pip), 3.69 (s, 2H, SC$\underline{H}_2$COO), 2.84 (d, *J* = 78.1 Hz, 2H, Pip), 2.59 (d, *J* = 6.4 Hz, 2H, BnC$\underline{H}_2$), 1.91 – 1.72 (m, 2H, Pip), 1.54 (d, *J* = 75.5 Hz, 4H, Pip), 1.26 (t, *J* = 7.2 Hz, 3H, COOCH$_2$C$\underline{H}_3$). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 169.63 (SCH$_2\underline{C}$OO), 169.40 (N$\underline{C}$O), 139.86 ($\underline{C}_{Bz}$-1), 137.29 ($\underline{C}_{Bn}$-1), 134.50 ($\underline{C}_{Bz}$-4), 129.07 ($\underline{C}H_{Bn}$-3, $\underline{C}H_{Bn}$-5), 128.80 ($\underline{C}H_{Bz}$-2, $\underline{C}H_{Bz}$-6), 128.32 ($\underline{C}H_{Bz}$-3, $\underline{C}H_{Bz}$-5), 127.67 ($\underline{C}H_{Bn}$-2, $\underline{C}H_{Bn}$-6), 126.09 ($\underline{C}H_{Bn}$-4), 61.73 (COO$\underline{C}$H$_2$CH$_3$), 42.98 ($\underline{C}$H$_2$-2 Pip, $\underline{C}$H$_2$-6 Pip), 42.46 (Ar$\underline{C}$H$_2$), 38.32 ($\underline{C}$H-4 Pip), 36.04 (S$\underline{C}$H$_2$COO), 26.92 ($\underline{C}$H$_2$-3 Pip, $\underline{C}$H$_2$-5 Pip), 14.19 (COOCH$_2\underline{C}$H$_3$).

*4.1.22. Synthesis of methyl 4-(4-benzylpiperidin-1-yl)but-2-ynoate (**50**)*

4-benzylpiperidine (300 mg, 1.71 mmol, 1 equiv.) and paraformaldehyde (103 mg, 3.43 mmol, 2 equiv.) were solubilized in DMSO and stirred for 10 minutes. Separately, copper (I) iodide (390 mg, 2.05 mmol, 1.2 equiv.) was reacted with methyl propiolate (174 µL, 2.05 mmol, 1.2 equiv.) in DMSO until a clear yellow solution was formed. The solution containing the preformed organic-cuprate was added dropwise to the solution of 4-benzylpiperidine and paraformaldehyde, and the reaction was stirred at room temperature for one hour. The solution was filtered through a celite pad and diluted with AcOEt. The organic phase was washed with Na_2CO_3, brine, dried over anhydrous Na_2SO_4, and concentrated to give 314 mg (67% yield) of the pure titled compound as a brown oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29 (dd, *J* = 10.0, 4.6 Hz, 2H, C$\underline{H}_{Ar}$-3, C$\underline{H}_{Ar}$-5), 7.21 (t, *J* = 7.3 Hz, 1H, C$\underline{H}_{Ar}$-4), 7.16 (d, *J* = 7.1 Hz, 2H, C$\underline{H}_{Ar}$-2, C$\underline{H}_{Ar}$-6), 3.79 (s, 3H, COOC$\underline{H}_3$), 3.47 (s, 2H, NC$\underline{H}_2$CC), 2.85 (d, *J* = 11.4 Hz, 2H, C$\underline{H}_2$-3 Pip, C$\underline{H}_2$-5 Pip), 2.56 (d, *J* = 7.1 Hz, 2H, ArC$\underline{H}_2$), 2.24 (td, *J* = 11.7, 2.3 Hz, 2H, C$\underline{H}_2$-3’ Pip, C$\underline{H}_2$-5’ Pip), 1.70 (d, *J* = 12.8 Hz, 2H, C$\underline{H}_2$-2 Pip, C$\underline{H}_2$-6 Pip), 1.63 – 1.48 (m, 1H, C$\underline{H}$-4 Pip), 1.40 – 1.31 (m, 2H, C$\underline{H}_2$-2’ Pip, C$\underline{H}_2$-6’ Pip).

4.2. Biological session

4.2.1. In vitro inhibitory activity against HDAC1 and HDAC6 enzymes

The inhibitory potency of the new compounds **1a-3** was evaluated *in vitro* against human HDAC1 and HDAC6 enzymes using a fluorescence-based activity assay [24,34].

HDAC1 and HDAC6 recombinant enzymes (10 ng per reaction; BPS Bioscience Catalog #: 50051 and 50006, respectively) were incubated in HDAC buffer (Tris-HCl pH 8.0 50 mM, NaCl 137 mM, KCl 2.7 mM, MgCl2 1 mM) with specific substrates (50 µM) and epidrugs, at a concentration of 1, 10 and 50 µM. As HDAC1 substrate the derivate of fluorescent tertbutyloxycarbonyl (Boc)-(Ac)-Lys-7-amino-4-methylcoumarin (AMC) called MAL, benzyloxycarbonyl analog Z-MAL was used. For HDAC6 instead, the substrate was (S)-[5-Acetylamino-1-(2-oxo-4- trifluoromethyl-2Hchromen-7-ylcarbamoyl)pentyl]carbamic Acid tert-Butyl Ester. Reactions were carried out for 1

hour at 37°C in black microplates (Corning Costar®, cod:266). Deacetylation sensitizes substrate to the Developer step [trypsin 6 mg/mL in trypsin buffer (Tris-HCl pH 8.0 50 mM, NaCl 100 mM)] and it is directly proportional to the production fluorophore. The fluorophore is excited with a 360 nm light, and the emitted light (460 nm) has been quantified with a TECAN Infinite M200 station. SAHA (5 μM) was used as deacetylation control for HDAC1 assay, Tubastatin (5 μM) for HDAC6. For the IC_{50} evaluation of HDAC1 inhibition, **11a** compound was tested in 9-point dose-response assay in duplicate. The IC_{50} value was determined through linear regression of inhibition data using a free online Very Simple IC50 Tool Kit program (ic50.tk).

4.2.2. Cell line

Breast cancer MCF7 cell line (ATCC) were grown in DMEM medium (Euroclone, Milan, Italy) with 10% fetal bovine serum (FBS) (Euroclone), 2 mM L-glutamine (Euroclone), and antibiotics (100 U/ml penicillin, 100 L g/ml streptomycin) (Euroclone). Cells were stimulated with new compounds for 24 hours at 50, 10, and 1 μM.

4.2.3. Immunoblotting analysis

To quantify the acetylation levels, we performed western blot analysis of the total protein and the histone extraction to evaluate ac-tubulin, as a target for HDAC6, and histone H3acK9,K14, as a target for HDAC1, respectively.

4.2.3.1. Total protein extraction

After the induction with the test compounds at the indicated times and concentrations, MCF7 cells were then harvested, washed with PBS (Euroclone), and lysed for 15 min at 4 °C in lysis extraction buffer with protease and phosphatase inhibitors (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 10 mM sodium fluoride, 0.1 mM sodium orthovanadate, 40 mg/mL phenylmethylsulfonyl fluoride

(PMSF), 20 g/mL aprotinin, 20 mg/mL leupeptin, 2 mg/mL antipain, 10 mM p-nitrophenyl phosphate, 10 mg/mL pepstatin A and 20 nM okadaic acid). Cells were vortexed then centrifuged at 13000 rpm for 30 min at 4 °C. Bradford assay (Biorad, Italy) was used to quantify protein concentration. Cell extracts were separated by SDS-PAGE and western blots were carried out for acetyl-tubulin (Sigma) and anti-GAPDH antibody (Santa Cruz Biotechnology) as the loading control. Densitometric analysis of protein expression was performed by using ImageJ image processing package [38].

4.2.3.2. Histone extraction

After the induction with new compounds at the indicated times and concentrations, MCF7 cells were harvested and washed twice with cold 1X PBS and lysed in Triton extraction buffer (TEB; PBS containing 0.5% Triton X 100 (v/v), 2 mM PMSF, 0.02% (w/v) NaN3) for 10 min on ice, with gentle stirring. After centrifugation (2000 rpm at 4 °C for 10 min), the supernatant was removed, and the pellet was washed in half the volume of TEB and centrifuged as before. The pellet was overnight incubated in 0.2 N HCl at 4 °C on a rolling table. The samples were then centrifuged at 2000 rpm for 10 min at 4 °C and histone content was determined with Bradford assay (Bio-Rad, CA, USA). For the detection of histone H3 acetylation, H3K9,14ac (Diagenode) was used. Histone H4 (Abcam) antibodies and Pounceau Red (Sigma) were used to normalize for equal loading. Semi-quantitative analysis was performed using ImageJ software [39].

4.2.4. Antiproliferative assay

Cell viability was determined on MCF7 cell line using Thiazolyl Blue Tetrazolium Bromide [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT; Sigma-Aldrich) assay, following manufacturer's instructions. A total of 2 × 104 cells/well were plated in a 96-well plate and treated, in triplicates, with new compounds at different concentrations for 24, 48, and 72 hours.

Absorbance was read at a wavelength of 570 nm with a TECAN M-200 reader (Tecan, Männedorf, Switzerland) [40].

4.3. Molecular modeling

Crystal structures of human HDAC1 and HDAC6 proteins were downloaded from the Protein Data Bank (PDB) and prepared for the subsequent structure-based calculations by using the *Protein Preparation Wizard* utility of the Schrödinger suite [41–44]. In particular, the 4BKX [35] and 5EDU [36] crystal structures were first downloaded for HDAC1 and HDAC6, respectively. Then, missing amino acid side chains were rebuilt, and potential atom types and bond connectivity issues into the X-Ray complexes were fixed. Moreover, ionization and tautomerization states potentially present at physiological pH were also calculated for the ligand-protein complexes. Afterward, the pretreated structures were minimized according to the OPLS3 force field [45], and the co-crystallized ions and solvent molecules were removed. Water molecules were also removed from the resulting crystal complexes, as none of them have been reported as conserved or involved in the ligand-binding process to HDAC1 and HDAC6. The prepared crystal structures were aligned with *Structure Alignment Tool* of the Schrödinger suite [46], and receptor grids were prepared for the subsequent docking calculations. In particular, the HDAC1 receptor grid was generated around the residues lining the catalytic site of this protein (i.e., Asp99, Leu139, His140, His141, Gly149, Phe150, Asp176, His178, Tyr204, Phe205, Asp264, Leu271, and Tyr303), similarly to as previously reported [47]. For HDAC6 the receptor grid was centred on the co-crystallized ligand, i.e., Trichostatin A (TSN) [36]. The Glide software was used as an engine for the docking calculations, which were performed with default settings of the Standard Precision (SP) protocol [48,49]. The docking protocol was validated for HDAC6 by redocking the crystallographic ligand into its parent receptor, providing a RMSD value lower than 2.0Å. Redocking calculations on the 4BKX crystal structure (HDAC1) were not performed, as it has not been complexed with ligands [35]. Afterward, a set of representative compounds of the investigated series was prepared for the

subsequent structure-based analyses. In particular, the selected ligands were first drawn and then processed with *LigPrep* utility available within the Schrödinger suite [50]. Potential ionization states and tautomers were generated for the compounds. Moreover, additional metal binding states were also calculated for the ligands, as the HDAC proteins present a Zn^{2+} ion into their binding site that is necessary for their catalytic activity [28]. The prepared ligands were finally docked into the models and the resulting ligand-protein complexes visually inspected.

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