

TOWARDS NOVEL SMALL MOLECULE EPIGENETIC INHIBITORS

A Thesis Submitted by

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DECLARATION OF ORIGINALITY

I, Matthias Baud, hereby confirm that I solely produced the presented thesis under the supervision of Doctor Matthew J. Fuchter at the Department of Chemistry, Imperial College London, and that I did not use any other material than cited or known to the public domain.

London, 14th October 2011

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ABSTRACT

Studies during the last decades have shown the cellular epigenetic machinery to be of major importance in the control of gene expression in living organisms. Misregulation of these mechanisms has been described in a variety of cancers, and small molecule inhibitors of enzymes involved in epigenetic gene regulation have emerged as promising agents for the development of new anticancer therapies.

Naturally isolated products continue to play a significant role in anticancer drug discovery. We are interested in the structural and biological properties of the natural product Psammaplin A. Recent studies highlighted its extreme potency against both Histone Deacetylases (HDACs) and DNA Methyltransferase 1 (DNMT1) enzymes, which both play a central role in the regulation of gene expression and tumour development. In enzyme-based assays, Psammaplin A displayed potent activity (IC_{50} s) against an HDAC extract (4.2 nM) and Dnmt1 (18.6 nM). Subsequent studies have shown Psammaplin A to exhibit significant cytotoxicity against a number of cancer cell lines. *In vivo*, studies showed inhibition of tumour growth in the A549 lung xenograph mouse model while maintaining low toxicity.

The goal of our project is to establish a structure-activity relationship (SAR) of Psammaplin A, in order to understand the structural features responsible for such HDAC and DNMT inhibition and help us in the design of new generations of drug-like molecules for epigenetic anticancer therapies. An important number of Psammaplin A analogues could be obtained in few steps from versatile reagents. We will present our synthetic routes towards libraries of Psammaplin A analogues, results on their biological activity, and recent developments towards novel epigenetic modulators.

Based on our high interest in HDACs, results of a number of other novel and early stage projects aimed at dissecting the chemical biology of HDACs will be presented.

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ABBREVIATIONS

°C	Degree Celsius
Å	Angström
δ	Chemical shift
Ac	Acetyl
AcOEt	Ethyl acetate
AcOH	Acetic acid
Anal.	Analysis
<i>app</i>	Apparent
aq.	Aqueous
Ar	Aryl
Bn	Benzyl
Boc	<i>tert</i> -Butoxycarbonyl
BOP	Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate
br	Broad
brsm	Based on the recovery of starting material
Bu	Butyl
Bz	Benzoyl
C	Concentration
<i>ca.</i>	<i>Circa</i>
Calc.	Calculated
Cat.	Catalytic
CDI	Carbonyldiimidazole
CI	Chemical ionisation
Cy	Cyclohexyl
d	Doublet
<i>d</i>	Deuterated
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC	1,3-Dicyclohexylcarbodiimide
dd	Doublet of doublets
DIAD	Diisopropyl azodicarboxylate

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DIPEA	Diisopropylethylamine
DMF	Dimethylformamide
DMAP	4-(Dimethylamino)pyridine
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
EDCI	1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide
e.g.	Exempli gratia
EI	Electron ionisation
Enz	Enzyme
eq.	Equivalent
ESI	Electron spray ionisation
Et	Ethyl
FDA	Food and Drug Administration
g	Gram
h	Hour
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HDAH	Histone deacetylase-like amidohydrolase
HOBt	1-Hydroxybenzotriazole
HRMS	High resolution mass spectrometry
Hz	Hertz
<i>i</i>	<i>Iso</i>
<i>i.e.</i>	<i>Id est</i>
IR	Infrared
<i>J</i>	Coupling constant
K	Kelvin
Lit.	Literature
m	Multiplet
μ	Micro (10 ⁻⁶)
M	Molar or mega (10 ⁶)
MD	Molecular dynamics
Me	Methyl
min	Minute

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MOM	Methoxymethyl
mp	Melting point
MS	Mass spectrometry or molecular sieves
<i>m/z</i>	Mass to charge ratio
NBS	<i>N</i> -Bromosuccinimide
NHS	<i>N</i> -Hydroxysuccinimide
nm	Nanometer
nM	Nanomolar
NMO	4-Methylmorpholine <i>N</i> -oxide
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser effect
NOHP	<i>N</i> -hydroxyphthalimide
pdb	Protein data bank
PDT	Photodynamic therapy
PE	Petroleum ether
Ph	Phenyl
pm	Part per million
Pr	Propyl
PS	Photosensitiser
PTM	Post translational modification
Py	Pyridine
q	Quartet
R _f	Retention factor
RNA	Ribonucleic acid
rt	Room temperature
s	Singlet
SAH	<i>S</i> -adenosyl homocysteine
SAHA	Suberoylanilide hydroxamic acid
SAM	<i>S</i> -adenosyl methionine
SAR	Structure-activity relationship
t	Triplet
T	Temperature
TBS	<i>tert</i> -Butyldimethylsilyl
TCEP	Tris(2-carboxyethyl)phosphine hydrochloride

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TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
THP	Tetrahydropyran
TLC	Thin layer chromatography
TMS	Trimethylsilyl
Ts	Toluene sulfonyl
TSA	Trichostatin A
TSG	Tumour suppressor gene
UV	Ultra violet
VDW	van der Waals
ZBG	Zinc binding group

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Towards novel small molecule epigenetic inhibitors

The scientist does not study nature because it is useful; he studies it because he delights in it, and he delights in it because it is beautiful. If nature were not beautiful, it would not be worth knowing, and if nature were not worth knowing, life would not be worth living.

Jules Henri Poincaré (1854-1912)

Every honest researcher I know admits he's just a professional amateur. He's doing whatever he's doing for the first time. That makes him an amateur. He has sense enough to know that he's going to have a lot of trouble, so that makes him a professional.

Charles Franklin Kettering (1876-1958)

INTRODUCTION

1. The genetic basis of cancer

Nowadays, cancer represents one of the most important causes of mortality worldwide. Significant progress was made during the 20th century towards a better understanding of its origin and development. A number of important studies, including Peyton Rous' pioneering works^{1,2} (1910) on the Rous Sarcoma Virus (RSV) and studies of cellular tumour inducing genes by Bishop and Varmus in the 1970s,³ led to the discovery of the now called “proto-oncogenes” in the genome of a variety of living organisms. Upon specific modifications at the molecular level, the so-called genes can be converted to “oncogenes”, which are genes able to transform normal cells and induce tumours.

The concept of proto-oncogenes marked a great advance, implying that the genome of normal vertebrate cells, including human, carry genes that have the potential, under certain conditions, to induce cell transformation and thus cancer. These discoveries, and the following studies, brought strong support to the idea of misregulation of the host genome as a major cause of cancer. Since the discovery of proto-oncogenes in 1974 by Varmus and Bishop, a variety of proto-oncogenes have been discovered in the human genome, such as *myc* and *Ras*. Following these discoveries, cellular proto-oncogenes and the mechanisms leading to oncogenic activation were intensively studied. Activation of oncogenes was shown to arise through different pathways, notably through misregulation of the level of expression of proto-oncogenes and/or alteration of the structure of their encoded proteins *via* point mutation. Misregulation of the level of expression of a proto-oncogene could itself be the result of different factors, such as gene amplification or chromosomal translocation.⁴ For example, the widely studied H-Ras was the first oncogene to be discovered to have a point mutation in its reading frame. Within a decade, a large number of tumours were found that carried point mutations in one of the three *Ras* (H-Ras, K-Ras, N-Ras) genes present in mammalian genomes, these point mutations were always observed in specific codons. More than 20% of human tumours, from varying tissues, were found to carry these specific point mutations in their *ras* genes.⁵

While the discovery and study of (proto-)oncogenes provided a powerful tool to explain, at least partially, mechanisms leading to cancer, a number of important discoveries in the 1970s and early 1980s were difficult to reconcile with the known tumour inducing character of oncogenes at the time. Importantly, studies by Harris and co-workers from 1965 to 1969 demonstrated the recessivity of the malignant versus wild type phenotype.⁶⁻⁸ These observations, emerging from cell fusion (Figure 1) between mutant and wild-type cells, suggested the existence of a second class of genes involved in the regulation of cell growth and division, which were apparently acting as negative regulators (in opposition to positive regulation by proto-oncogenes). Further studies led to the identification of such genes,^{9,10} later called “Tumour Suppressor Genes” (TSGs), which marked a new important step forward in the understanding of the mechanisms regulating normal cell proliferation, and the consequences of their alteration on cancer pathogenesis. Their discovery provided a new model to explain tumour development: normal cells carry genes able to constrain or suppress their proliferation. During the development of a tumour, the evolving cancer deactivates these genes in addition to oncogenic activation. The loss or deactivation of these growth-suppressing genes then allows rapid progression of cancer. As long as the cancerous cells lack these genes, the proliferation can continue in a malignant fashion. However, as soon as the wild-type, intact versions of these genes operate again in the cell (such as after introduction by cell fusion experiment), cancer progression is shut down and finally stops.

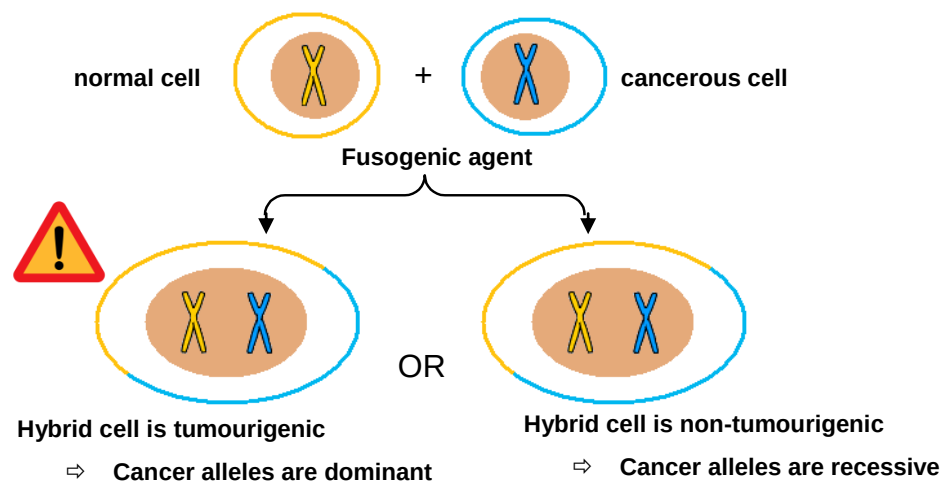


Figure 1 - Illustration of the experimental fusion of cells used to test the dominance or recessiveness of the cancer cell phenotype

An important number of TSGs have been discovered so far, including for example RB1 (cell cycle) and TP53 (DNA repair, cell cycle, apoptosis) and their deactivation seems to be required in order to induce cell transformation and malignant behaviour. TSGs are grouped into two main classes, under the generic name of “gatekeepers” and “caretakers”. “Gatekeepers” are genes that operate on cell multiplication, but also cell differentiation and cell death (e.g. TP53). “Caretakers” are genes that encode proteins responsible for maintaining the integrity of the genome, such as ATM and BRCA1/2 which are involved in DNA repair. Importantly, deactivation of both wild-type alleles of a given TSG is required in order to fully abolish its effect. While genetic mutations were already known as a factor able to alter gene expression, the elimination of both copies of a TSG by successive genetic mutations is statistically unlikely. In particular, alternative mechanisms such as loss of heterozygosity^{11,12} and epigenetic modifications have been shown to be implicated in the silencing of TSGs.¹³ Epigenetic gene regulation will be the subject of the next section of this thesis.

2. EPIGENETICS

2.1. Overview

The human genome is packaged into chromatin, a macromolecular assembly of deoxyribonucleic acid (DNA), histones and non histone proteins (e.g. DNA polymerase, Heterochromatin Protein 1, Polycomb-group proteins). The nucleosomes, forming the repeating unit of chromatin, are composed of approximately 146 base pairs of DNA wrapped around a histone octamer. The latter is composed of an H3/H4 tetramer and two H2A/H2B dimers. In nucleosomes, the *N*-terminal regions of histones, or histone tails, project outward of the assembly. The succession of nucleosomes forms a higher order structure (Figure 2) whose three dimensional characteristics are partly responsible for the expression of genes, making these genes accessible (or not) to the transcriptional machinery.¹⁴⁻¹⁶ In particular, chromatin can be found in an open form (euchromatin), associated with active transcription, or in a condensed form (heterochromatin), associated with transcriptional repression (Figure 3).

While the genetic information is contained in the DNA, regulation of phenomena such as transcription, replication, recombination or DNA repair is controlled by the “epigenome” (from the greek epi: upon/in addition to). “Epigenetic” is a term used to describe heritable and reversible changes in gene expression that do not involve changes in the DNA sequence.

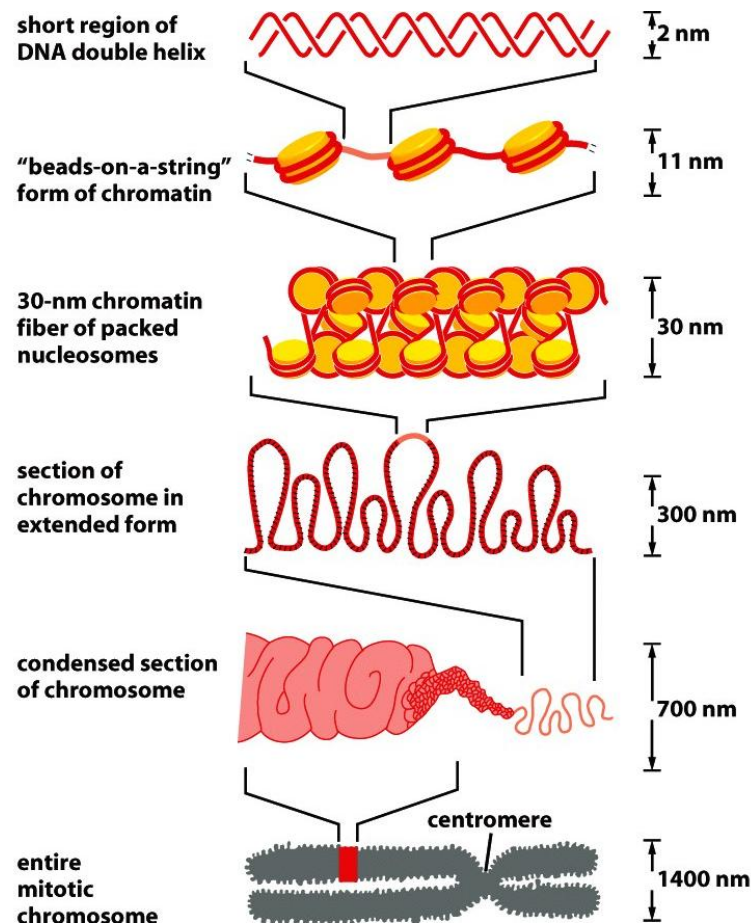


Figure 2 - Model illustrating the different level of organisation in chromatin. Yellow cylinders: histone octamers. Non histone proteins are omitted for clarity. Reproduced and adapted from Bruce Alberts *et al*, *Molecular Biology of The Cell*. 5th ed.; Garland Science: 2008¹⁷

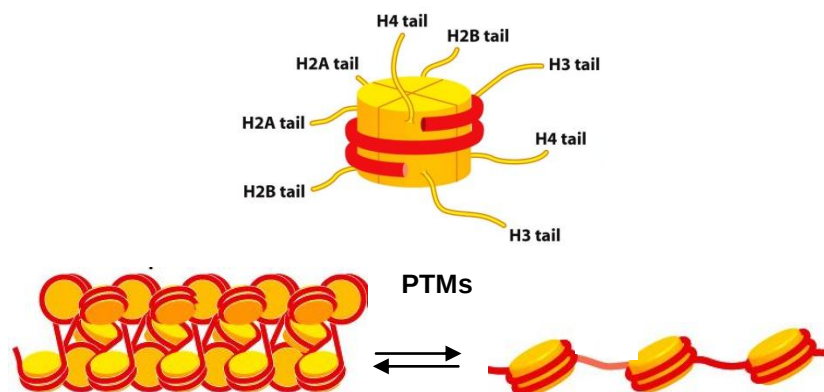


Figure 3 - Top: DNA (red wire) is wrapped around a histone octamer (yellow cylinder). Bottom: Post-translational modifications of histone tails dynamically affects the three dimensional structure of chromatin, and consequently gene expression. In particular, heterochromatin (left) and euchromatin (right) are associated with low and high transcriptional activity respectively. Reproduced and adapted from Bruce Alberts *et al*, *Molecular Biology of The Cell*. 5th ed.; Garland Science: 2008¹⁷

A number of biological processes are regulated by epigenetic gene regulation, such as apoptosis or X-chromosome inactivation.¹⁸ The epigenetic regulation of gene expression notably plays a major role in cell differentiation during embryogenesis. In some ways, epigenetics can be seen as a link between genotype and phenotype (Figure 4).



Figure 4 - The caterpillar and the butterfly are classical examples of how epigenetic modifications affect the phenotype of an organism. While sharing the same genome, major phenotypic characteristics differentiate them. These phenotypic differences are due to differences in their gene expression patterns,¹⁹ controlled by epigenetic modifications

Epigenetic gene regulation can be achieved *via* a myriad of mechanisms, such as covalent modifications of DNA and terminal amino groups of histone tails pointing out of the nucleosomes (Figure 5), and *via* silencing RNA.²⁰⁻²² In particular, covalent modifications of DNA and histones have a direct effect on the higher order chromatin structure, and subsequently on the ability of DNA to interact with the transcriptional machinery. Numerous modifications of chromatin affecting the epigenetic regulation of gene expression are known,²⁰⁻²³ including for example DNA methylation, histone acetylation/deacetylation, methylation/demethylation, phosphorylation, ADP-ribosylation, citrullination, ubiquitination, sumoylation, prolyl isomerisation, biotinylation. The function of the recently discovered DNA hydroxymethylation, originating from the oxidation of methylated DNA, is poorly understood.²⁴⁻²⁷

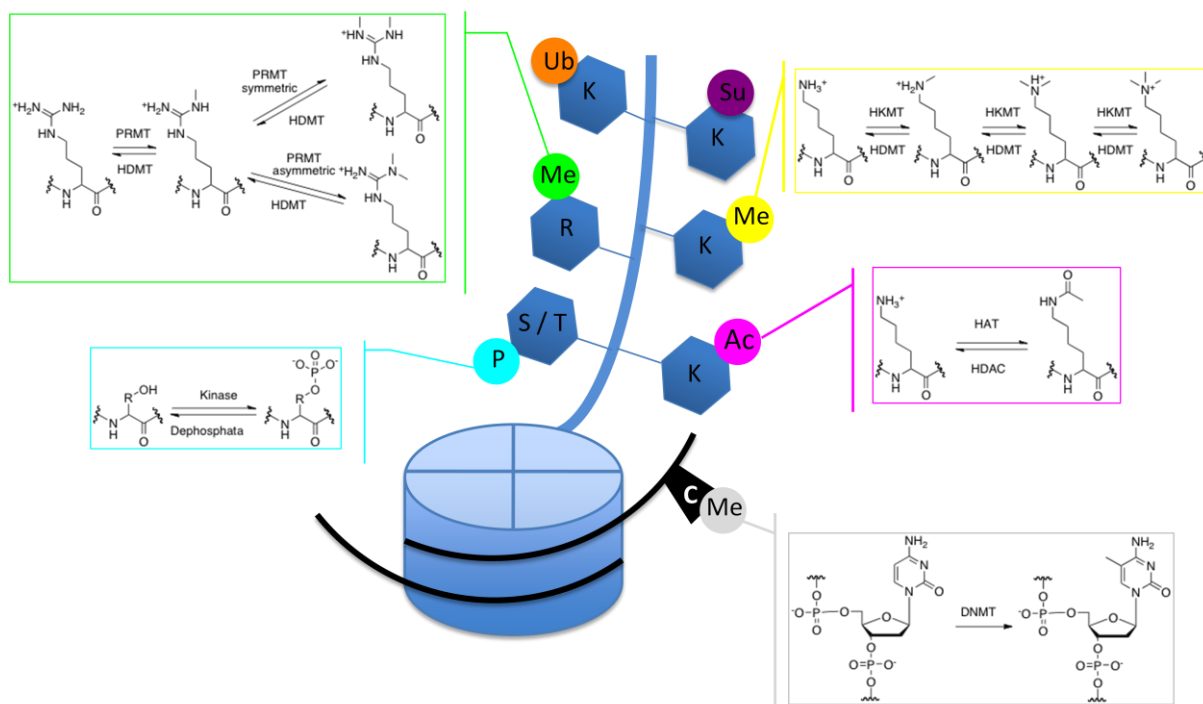


Figure 5 - Examples of dynamic epigenetic modifications of chromatin occurring on DNA (black) and histone tails (blue line). Lysine residues (K) can be acetylated (pink), methylated (yellow), sumoylated (purple) or ubiquitinated (orange); arginine residues (R) can be methylated (green), while serine (S) and threonine (T) residues can be phosphorylated (cyan); DNA can be methylated (grey) on cytosine residues (black). Blue cylinder: histone octamer. Reproduced and adapted from Cherblanc et al.²⁸

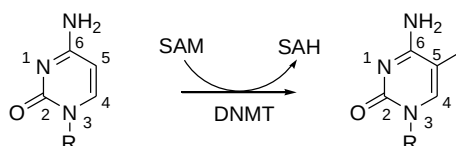
These modifications come within the framework of a large model called the “histone code hypothesis”, specifying the local status of the chromatin with respect to three dimensional characteristics and transcriptional activity. This model suggests that a unique combination of epigenetic states in chromatin can be related to a particular biological outcome.²⁹

Epigenetics represent a relatively new area of research, arousing an increasing interest in the scientific community in term of its importance in the regulation of gene expression, and its omnipresence in the mechanisms leading to the development of tumours. In particular, the phenomena of DNA methylation and histone acetylation/deacetylation have been shown to be at the centre of the epigenetic network, and will be the subject of the next section.

2.2. DNA methylation and histone acetylation/deacetylation

DNA methylation

DNA methylation plays a central role in the epigenetic regulation of gene expression. This phenomenon, occurring on the C5-position of cytosine residues (Scheme 1), is mediated by enzymes called DNA methyltransferases (DNMTs) which use S-adenosyl methionine (SAM or AdoMet) as a cofactor. It generally takes place in particular regions of the DNA called CpG islands, which are rich in cytosine residues and generally located in the proximity of gene promoters.^{15,30} CpG islands are found in more than half of genes in the genome. Hypermethylation of these regions has been found to be associated with transcriptional silencing.³¹⁻³⁴ While the study of the implication of DNA methylation in the regulation of gene expression became a particularly hot topic during the last few decades, first reports mentioning the characteristics, synthesis and natural occurrence of the 5-methylcytosine motif can be traced back to the beginning of the 20th century.³⁵⁻³⁸



Scheme 1 - Methylation of the C5-position of cytosine (left) by DNMTs, using SAM as a cofactor, affords the product C5-methylcytosine (right)

DNA methylation is involved in a myriad of biological processes, and the DNA methylation pattern of CpG dinucleotides in a given organism is tissue specific, and varies from one cell type to another, with largest divergences observed for embryonic stem cells.^{39,40} DNA methylation is notably involved in gene regulation, DNA-protein interactions, cellular differentiation, embryogenesis, genomic imprinting, stabilisation of X-chromosome inactivation, protection against endogenous retroviruses and transposons, development of the immune system, brain function and behaviour.⁴¹ In terms of gene regulation, DNA methylation can induce downregulation of gene expression through different mechanisms. First, it can prevent the binding of transcription factors to target

DNA sequences by decreasing binding affinity, which consequently inhibits transcriptional activity.^{31,32} Second, methylated DNA sequences can also be recognised and bound by methyl-CpG binding proteins (MBPs). Binding of MBPs can directly prevent interaction with transcription factors, but can also promote the recruitment of transcriptional repressor multiprotein complexes, for example involving HDACs.^{42,43}

To date, three active DNMTs -called DNMT1, DNMT3a and DNMT3b- and a related protein lacking the catalytic domain -called DNMT3L- have been identified in mammals. The function of a fifth postulated DNMT, called DNMT2 and lacking DNA methyltransferase activity, is still enigmatic.⁴⁴

DNMT1 was the first mammalian DNA methyltransferase cloned⁴⁵ and characterised. It preferentially methylates hemimethylated DNA over unmethylated DNA^{46,47} and has been implicated in the maintenance of the DNA methylation pattern during cellular division (Figure 6). For this reason, DNMT1 is sometimes called *maintenance* methylase. DNMT1 is present in the nucleus of most somatic cells, however its subnuclear distribution varies dynamically during the cell cycle. Two tissue specific DNMT1s isoforms have been identified. DNMT1o (DNMT1 oocyte) and DNMT1p (DNMT1 pachytene) have been described in oocytes and spermatocytes respectively.⁴⁸ While DNMT1 is a maintenance methylase, its presence has been shown to be required for *de novo* methylation by DNMT3a and DNMT3b.^{49,50}

DNMT1 is at the centre of a complex epigenetic network and interacts with a multitude of proteins. It has notably been shown to interact with histone deacetylase 1 (HDAC1), HDAC2, DNMT3a, DNMT3b, Rb and E2F1 transcription factors, the histone lysine methyltransferases Suv39H1, SET7/9, G9a and EZH2, the silencing effectors HP1 and MeCP2, the chromatin factor UHFR1, PCNA and DMAP1.⁴¹

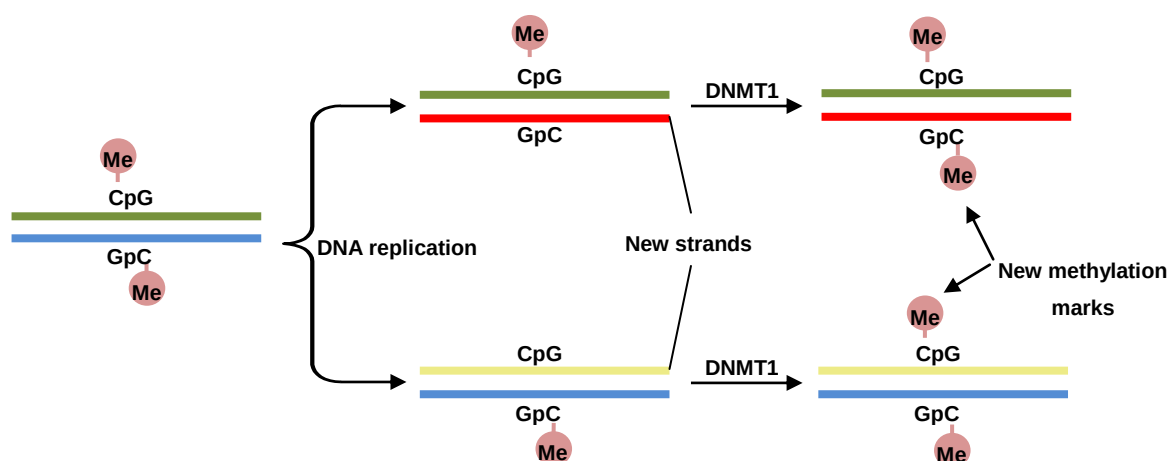


Figure 6 - Maintenance of DNA methylation patterns by DNMT1 during replication

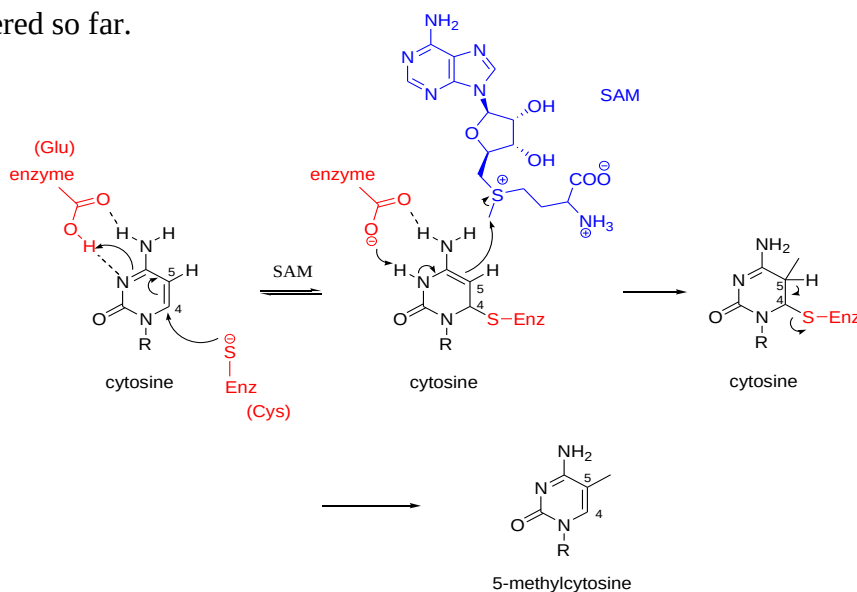
The mammalian DNMT3 family contains three members: DNMT3a, DNMT3b and DNMT3L. DNMT3a and DNMT3b are catalytically active and known as *de novo* methylases, able to methylate previously unmethylated DNA.^{51,52} They play a vital role in the establishment of DNA methylation patterns during embryogenesis.⁵³ Although they are primarily involved in *de novo* DNA methylation, they have been shown to be involved in the maintenance of DNA methylation in heterochromatic regions. The molecular mechanisms underlying the establishment of the *de novo* methylation pattern in mammal is still under intensive investigation. In particular, the dynamic and sequence specific methylation of DNA by DNMTs is poorly understood. A recent review by Jeltsch and co-workers⁴¹ provides a good overview of current models proposed to explain such selectivity. DNMT3L is catalytically inactive but has a regulatory role in germ cells.⁵⁴⁻⁵⁶ Whereas devoid of a catalytic domain, DNMT3L has been found to interact with *de novo* DNMTs.^{55,57-61}

DNMT3a and DNMT3b are present at high level in embryonic tissues and undifferentiated embryonic stem (ES) cells. In contrast, their expression is down-regulated in differentiated cells. DNMT3a is expressed in most tissues. Variants of DNMT3a and DNMT3b have also been reported. DNMT3a2⁶² is cell type specific and generally present in ES cells, germ cells, embryonal carcinoma cells, spleen and thymus. However, it is not expressed in adult tissues. Similarly, the expression level and localisation of DNMT3b isoforms varies during

development, potentially illustrating differences in their function and target sequences.⁶³ The catalytically inactive DNMT3L is expressed in germ cells during gametogenesis and embryogenesis.^{54,55}

DNMT3a and DNMT3b are associated with chromatin containing methylated DNA⁶⁴ and localise in pericentromeric heterochromatin.⁶⁵⁻⁶⁷ The localisation of DNMT3L is dependent on its interaction with DNMT3a and DNMT3b. In the absence of the two *de novo* methylases, DNMT3L is distributed in both the nucleus and the cytoplasm. However, the concentration of DNMT3L in chromatin was shown to increase upon binding to DNMT3a or DNMT3b.⁶⁸

A mechanism for the catalytic mechanism of DNA methylation by DNMTs has been previously proposed (Scheme 2).⁶⁹⁻⁷¹ This model involves the nucleophilic attack of position 6 of the cytosine residue by a cysteine in the active site of the DNMT. This results in a covalent complex which is nucleophilic in position C-5. The reactive intermediate, stabilised by a glutamic acid from the protein, can then accept a methyl group from SAM, resulting in a 5-methyl covalent adduct and S-adenosyl homocysteine (SAH). Abstraction of the proton in the C5-position, followed by elimination of the thiol finally liberates the 5-methylcytosine residue. Importantly, while the reversibility of DNA methylation *in vivo* is known, enzymes exhibiting DNA demethylating activity have not been discovered so far.



Scheme 2 - Proposed catalytic cycle for the methylation of cytosine (black) by DNMTs (red), using SAM (blue) as a cofactor

Histone acetylation/deacetylation

Another highly studied key process in the epigenetic regulation of gene expression is the dynamic phenomenon of acetylation/deacetylation of histones. The dynamic regulation of the acetylation pattern of lysine residues of histone tails is controlled by enzymes called histone acetyl transferases (HATs) and histone deacetylases (HDACs) respectively. In particular, HDACs are able to remove acetyl groups from the lysine residues of these histone tails (Figure 5), and are associated with transcriptional repression *via* chromatin condensation. At the molecular level, the dynamic modification of the histone acetylation pattern by HDACs is thought to control the interactions between positively charged lysine histone tails with the negatively charged DNA. Modulation of these interactions has a direct impact on the three dimensional chromatin structure, and subsequently on transcriptional activity. So far, 18 human genes encoding HDACs have been identified.²⁰ HDACs^{20,21} can be divided into two groups: zinc-dependent HDACs, and NAD⁺ dependent HDACs also called Sirtuins (silent information regulator 2-related proteins) or SIRTs. Zinc dependent HDACs possess a catalytic Zn(II) cation in their active site, whereas SIRTs use the oxidised form of nicotinamide adenine dinucleotide (NAD⁺) as a cofactor in the deacetylation process.

Zinc dependent HDACs can be subdivided into different classes, according to their sequence homology and structural characteristics (Figure 7). Class I HDACs are expressed in the nucleus of most cell lines and include HDAC1, 2, 3 and 8. Class II HDACs are larger than class I enzymes and are tissue specific, shuttling between the cytoplasm and nucleus.^{14,72,73} While called “histone deacetylases”, certain HDACs have been shown to be able to deacetylate non-histone proteins, such as hormone receptors, chaperone proteins, transcription factors and cytoskeletal proteins.^{73,74} While the term “lysine deacetylase” (KDAC) would be more appropriate as a name for these enzymes owing to their lack of specificity for histones, the term HDAC is still used extensively. Class II is further subdivided into class IIa (HDAC4, 5, 7 and 9) and class IIb (HDAC6 and 10) according to their sequence homology, structural characteristics and number of catalytic domains. The recently identified HDAC11 constitutes its own class, IV.

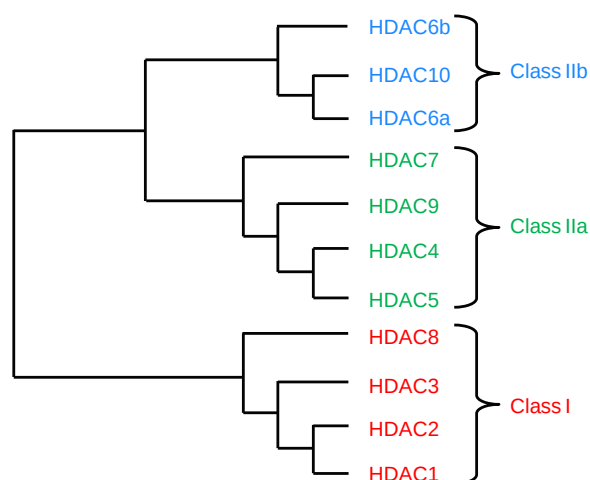


Figure 7 - Zinc dependent HDACs can be subdivided into different classes. HDAC11 constitutes its own class IV

The cleavage of acetyl groups of acetylated lysine histone tails by class I, class II and class IV HDACs proceeds through a zinc(II)-activated water molecule in the active site. Class III HDACs share a common active site of 275 amino acids and use nicotinamide adenine dinucleotide (NAD⁺) dependent as a cofactor. The function of the recently discovered HDAC11 is still under investigation.

In class I, II and IV HDACs, the active site containing the Zn(II) catalytic centre is connected to the surface of the protein via a narrow 11Å long hydrophobic channel (Figure 8).

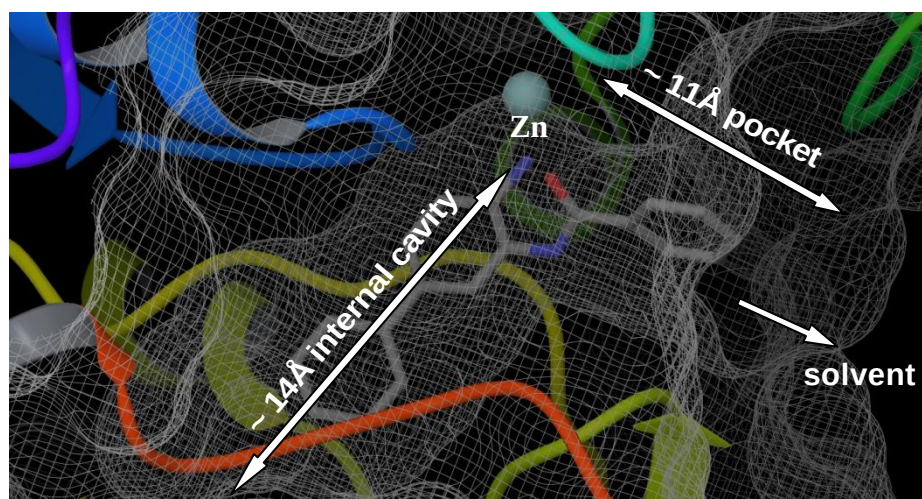
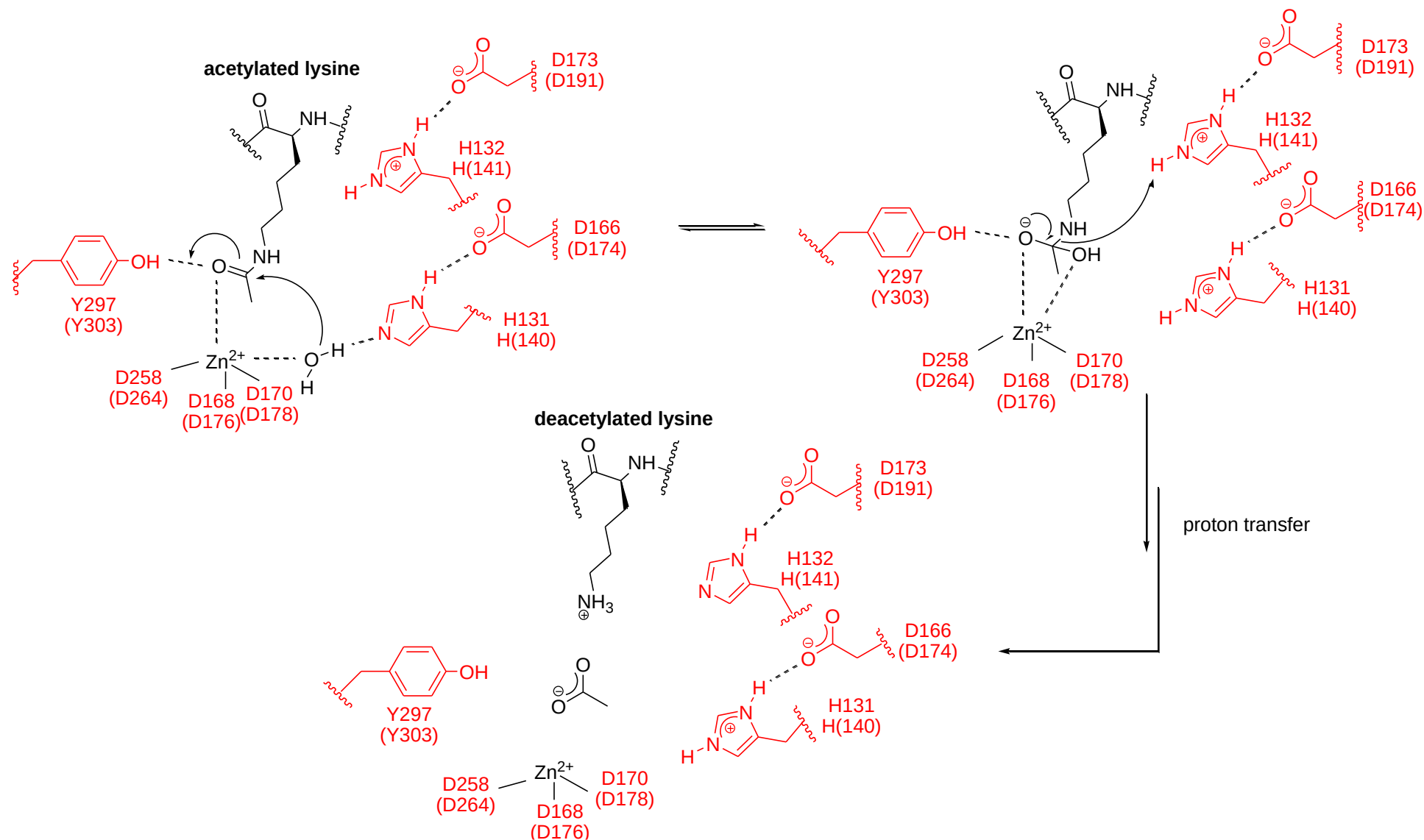


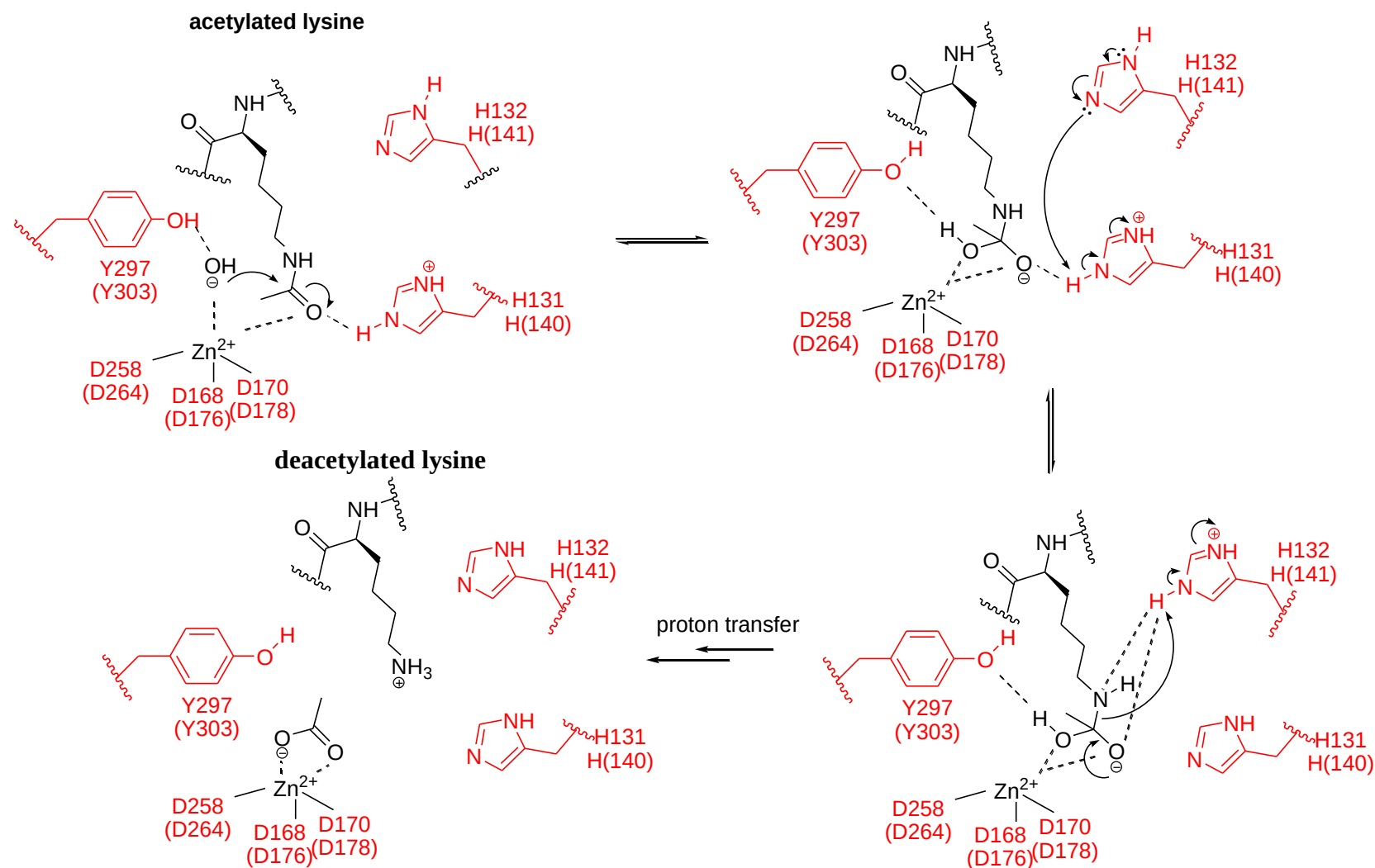
Figure 8 - Crystal structure (pdb 3MAX) of HDAC2 in complex with a benzamide derivative.⁷⁵ The ~ 11Å hydrophobic channel is common to all Zn(II) dependent HDACs and connects the active site to the surface of the protein. A ~ 14Å internal cavity is visible, but it is not conserved among all family members

In the active site, the zinc cation is generally found in a tetrahedral environment in its native state, and is complexed by two aspartates, one histidine and a water molecule. A model for Zn(II) dependent HDACs catalytic activity was proposed⁷⁶ in 1999 (Scheme 3) based on crystallographic studies of a HDAC homologue from the hyperthermophilic bacterium *Aquifex aeolicus* (sharing 35.2% identity with human HDAC1 over 375 residues). This model involves the coordination of the carbonyl oxygen of the acetylated lysine substrate to the zinc cation, positioning the acetate group in ideal position for nucleophilic attack by a zinc-activated water molecule. The tetrahedral oxyanion intermediate is stabilised by both electrostatic interactions with Zn(II), and hydrogen bonding with the neighbouring tyrosine hydroxyl group. Decomposition of the tetrahedral intermediate and proton transfer affords the deacetylated lysine and the acetate moiety. Class IIa HDACs display a highly reduced activity, in part due to the replacement of the catalytic tyrosine by a histidine residue. Indeed, mutagenesis experiments have shown class IIa HDACs to display a highly increased activity when this histidine was mutated by a tyrosine.⁷⁷

However, recent DFT studies by Vanommeslaeghe⁷⁸ and co-workers suggested a slightly different mechanism for the deacetylation process. This model mainly differs in the initial protonation state in the active site, in particular the two histidines forming the charge relay system and the zinc activated water molecule. In this model (Scheme 4) the carbonyl of the substrate undergoes nucleophilic attack of a hydroxide ion bound to Tyr303 (HDLP numbering) and the zinc cation. The negative charge of the resulting tetrahedral oxyanion intermediate is stabilised by the protonated His131 and the zinc cation. Subsequent proton transfer from His131 to neutral His132 occurs through a low potential channel, followed by decomposition of the intermediate and new proton transfer from His132 to the amino group of the deacetylated lysine.



Scheme 3 - Deacetylation mechanism by Zn(II) dependent HDACs of acetylated lysine residues, proposed by Finnin. HDLP active-site residues and their proposed human HDAC1 counterparts (in parenthesis) are labelled



Scheme 4 - Alternative deacetylation mechanism by Zn(II) dependent HDACs of acetylated lysine residues, proposed by Vanommeslaeghe. HDLP active-site residues and their proposed human HDAC1 counterparts (in parenthesis) are labelled

Class III HDACs are homologous of the yeast enzyme Sir2. To date, 7 sirtuins isoforms have been discovered in human (SIRT1-7), with SIRT1⁷⁹ and SIRT2⁸⁰ having received the most attention. They are structurally and mechanistically different from zinc dependent HDACs, and use NAD⁺ as a cofactor in the deacetylation process.^{81,82} SIRTs target a wide range of proteins playing important roles in biological processes such as differentiation, apoptosis, inflammation, energy metabolism, glucose and lipid metabolism, DNA repair and transcriptional silencing.⁸³ Like zinc dependent HDACs, SIRTs are not exclusively restricted to histone deacetylation but are able to deacetylate non histone proteins, such as Ac-CoA, α -tubulin and p-53. Importantly, dysregulation of SIRTs activity has been shown to be involved in cancer and aging related diseases, making the search of novel SIRT inhibitors an intensive area of research. As the results presented in this thesis will be mainly focused on Zn(II) dependent HDACs, SIRTs will not be discussed in depth here.

2.3. Aberrant DNA methylation and histone deacetylation in cancer

It is now recognised that both genetic and epigenetic modifications are critical to every stage of cancer and epigenetic silencing has been shown to play an important role in the misregulation of genes involved in all the hallmarks of cancer.^{84,85} In this context, DNA methylation and histone (de)acetylation are at the center of a complex regulatory network controlling gene expression *via* interrelated epigenetic modifications of chromatin. Aberrant DNA methylation and histone acetylation patterns have been observed in many tumours, often involving aberrant TSGs silencing through hypermethylation of TSG promoters, in addition to hypoacetylation of histones associated with TSG promoters. While hypermethylation of gene promoters is usually associated with transcriptional silencing, global hypomethylation has been shown to lead to chromosomal instability.⁸⁶ An important number of TSG promoters have been found abnormally hypermethylated in various cancer types, leading to their silencing.⁸⁷ Genes involved in DNA repair (e.g. BRCA1, MLH1, MGMT), signal transduction (e.g. RASSF1), cell cycle (e.g. p16^{INK4a}, p15^{INK4b}), metabolism of mutagens (e.g. GSTP1), cell adhesion (e.g. CDH1, CDH13), apoptosis (e.g. DAPK, TMS1, p73), angiogenesis (e.g. THBS1) are examples. As an illustration, the promoter

hypermethylation frequency of a selected set of known or postulated TSGs in various tumour types is shown in Figure 9.⁸⁷ In contrast, promoters of these genes were found unmethylated (or methylated to an insignificant extent) in normal tissues.

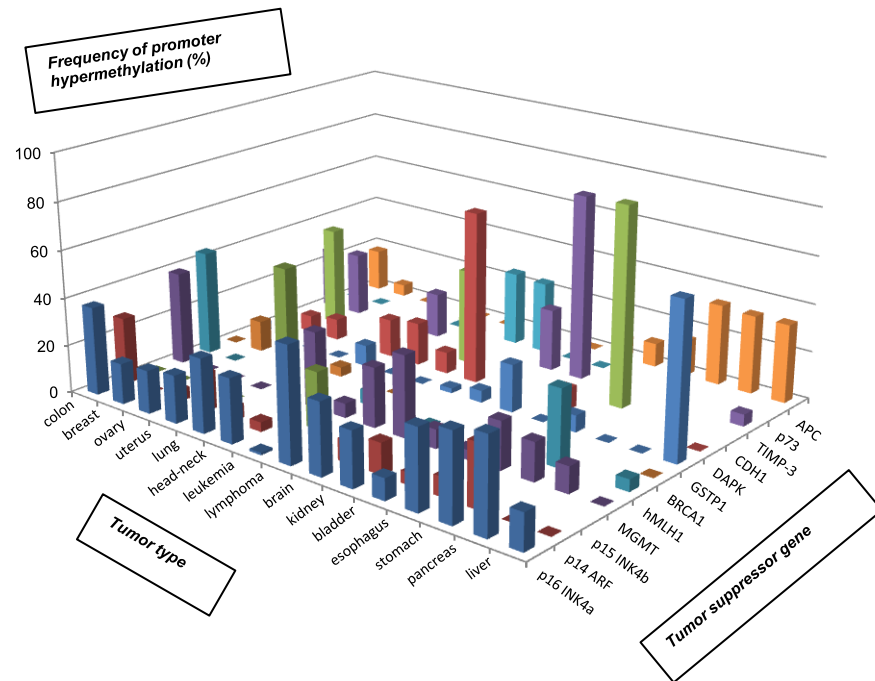


Figure 9 - Methylation of multiple tumour suppressor gene promoters within tumour cells genome, adapted from Esteller *et al.*⁸⁷

The downstream cascade linking promoter hypermethylation to final gene silencing is not completely understood, however it is now generally accepted that DNA methylation and histone modifications are intricately interrelated, acting in concert to determine cell fate.⁸⁸ As previously mentioned, DNA methylation can induce downregulation/silencing of genes by directly or indirectly (MBPs) preventing the binding of transcription factors,^{31,32} but can also promote the recruitment of HDAC containing transcriptional repressor complexes.^{42,43}

As stated earlier, epigenetic modifications of chromatin are reversible (in opposition to genetic mutations), and it is nowadays well accepted that the potential reversibility of the DNA methylation and histone acetylation patterns are viable targets for the treatment of cancer. While a number of approaches for therapy in the past involved the inhibition of proteins acting in an oncogenic way, such as kinases, glycosidases and telomerase, the goal of “epigenetic therapies”⁸⁹

lies in the modulation of the activity of enzymes involved in epigenetic gene regulation. The goal of such a modulation is to restore a regular epigenetic landscape in cancer cells and stop cancer progression. In particular, reversing the aberrant epigenetic patterns in cancer cells is expected to reactivate aberrantly silenced TSGs, and subsequently induce cell growth down regulation. In this context, the development of new inhibitors aimed at correcting the unregulated activity of DNMTs and HDACs has arisen as one of the most promising approaches to cancer therapy.

2.4. HDAC inhibitors

In recent years, there have been significant efforts made towards the discovery of new inhibitors of enzymes involved in the epigenetic regulation of gene expression. In particular, HDACs and DNMTs have been intensely studied for their potential application as potential therapeutic agents but also as potential probes for deeper investigation into epigenetic phenomena at the molecular level. HDAC inhibitors have received a lot of attention, partially due to the success of suberoylanilide hydroxamic acid^{90,91} **1** (SAHA, also known as Vorinostat or Zolinza) and Romidepsin **2**⁹² in clinical trials, and their recent FDA approval (Figure 10). In particular, SAHA was the first HDAC inhibitor approved by the FDA in 2006 (marketed as Zolinza), and is used for the treatment of cutaneous T-cell lymphomas (CTCL).

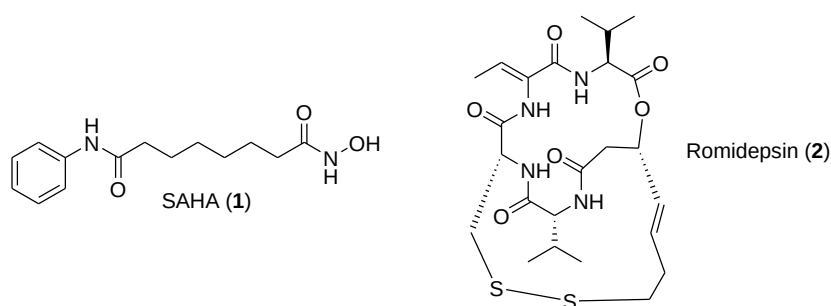


Figure 10 - FDA approved HDAC inhibitors

To date a large number of HDAC inhibitors (HDACis) have been reported, with inhibitors of class I and class II HDACs having received most attention. The majority of these inhibitors possess a zinc binding group (ZBG) coordinating to

the zinc cation in the HDAC active site, a “cap” unit interacting with amino acids at the entrance of the channel leading to the active site, and a linker lying in the connecting hydrophobic channel, positioning the ZBG and the “cap” at an adequate distance (Figure 11).



Figure 11 - Pharmacophore of the majority of HDAC inhibitors, the cap-linker-ZBG model

The majority of class I and class II HDAC inhibitors fall in one of the following categories: short chain fatty acids, hydroxamic acids, cyclic peptides and benzamides. Short-chain fatty acids such as butyrate **3**, phenylbutyrate **4** and valproic acid **5** were the first HDAC inhibitors identified (Figure 12). These compounds are structurally very simple, however exhibit only moderate inhibition, usually in the millimolar range, and lack isoform specificity and bioavailability.

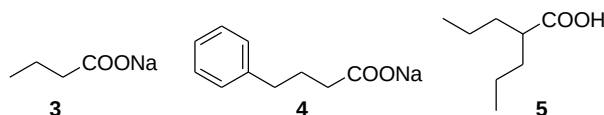


Figure 12 - Examples of short-chain fatty acid HDAC inhibitors

Hydroxamic acids represent the largest and most studied class of HDAC inhibitors. Examples of hydroxamic acid HDACis are shown in Figure 13. Trichostatin A (**6**, TSA), isolated from *Streptomyces Hygroscopicus*, was the first hydroxamic acid inhibitor reported.^{93,94} TSA and SAHA are two of the most studied HDAC inhibitors and display low nanomolar activity *in vitro*. While highly potent, both TSA and SAHA display low isoform selectivity. Crystal structures⁷⁶ of an HDAC1 homologue complexed with TSA^{94,95} **6** and SAHA^{96,97} **1** provided important structural information regarding their binding mode. In these crystal structures, the two inhibitors complex the zinc cation in the active site and engage in a number of H-bonds *via* their hydroxamic acid moiety. The hydroxamic acid is connected to an aromatic capping unit by the intermediate of a hydrophobic linker participating in van der Waals interactions throughout a

hydrophobic tubular channel connecting the active site to the surface of the protein.⁷⁶ The capping group is involved in hydrophobic interactions at the entrance of the tubular pocket. Hydroxamic acids are potent HDACis *in vitro*, and usually exhibit IC₅₀s in the low μ M to low nM range. However, they generally exhibit low isoform selectivity. PCI-34051⁹⁸ **7** and tubacin⁹⁹ **8** are two of the rare examples of potent and isoform selective hydroxamic acid based HDACis *in vitro*, inhibiting HDAC8 and HDAC6 respectively.

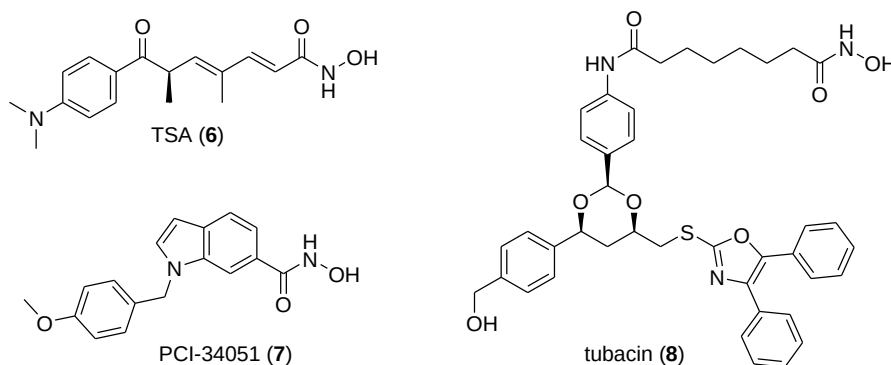


Figure 13 - Examples of hydroxamic acid HDAC inhibitors

A number of cyclic tetrapeptide based natural products have been reported to be highly potent HDACis. Examples include the recently FDA approved Romidepsin (**2**, Figure 10) epoxyketone Trapoxin B^{100,101} (**9**) ethylketone Apicidin¹⁰² **10** and thioester Largazole^{103,104} **11** (Figure 14). Cyclic peptides HDACis efficiently inhibit HDACs at low nanomolar concentration *in vitro*.

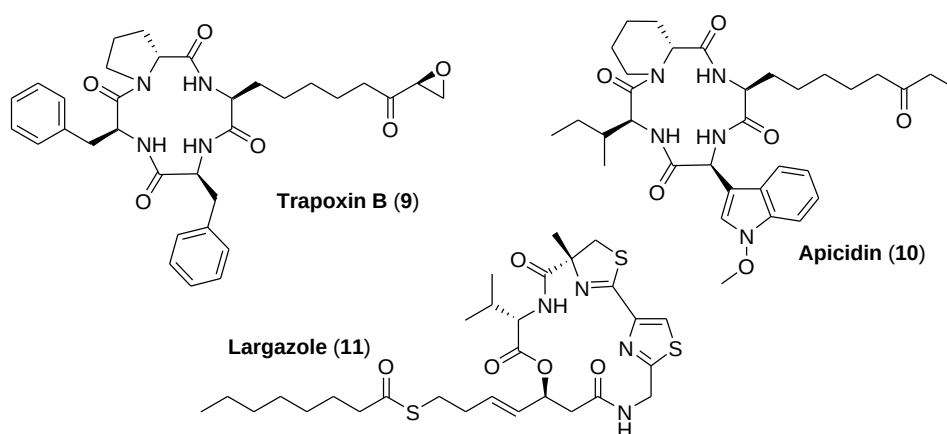


Figure 14 - Examples of naturally occurring cyclic peptide based HDACis

Ortho-aminoanilide derivatives constitute a fourth class of inhibitors. This category includes the class I selective inhibitor MS-275¹⁰⁵⁻¹⁰⁷ (**12**, Figure 15). This class of compounds has received significant attention due to their ability to selectively inhibit class I HDACs while being weakly active against class II.¹⁵ Other examples of HDAC inhibitors, mainly varying in the nature of their ZBG, include electrophilic ketones (e.g. **13**), *N*-formylhydroxylamine derivatives (e.g. **14**) and thiols (e.g. **15**).²⁰ Thiols are a good ZBG, as exemplified by Romidepsin **2** or Largazole **11** which are both low nanomolar HDAC inhibitors and thought to exhibit a thiol as the active species against HDAC activity. Other examples include the thiol analogue of SAHA **15**, which was found to be as potent as SAHA itself.¹⁰⁸ A number of sulfur based inhibitors was synthesized and tested by Suzuki, Miyata and co-workers,¹⁰⁸⁻¹¹² involving notably thiol, thioacetate, alkylsulfone, methylthioether as zinc binding groups.

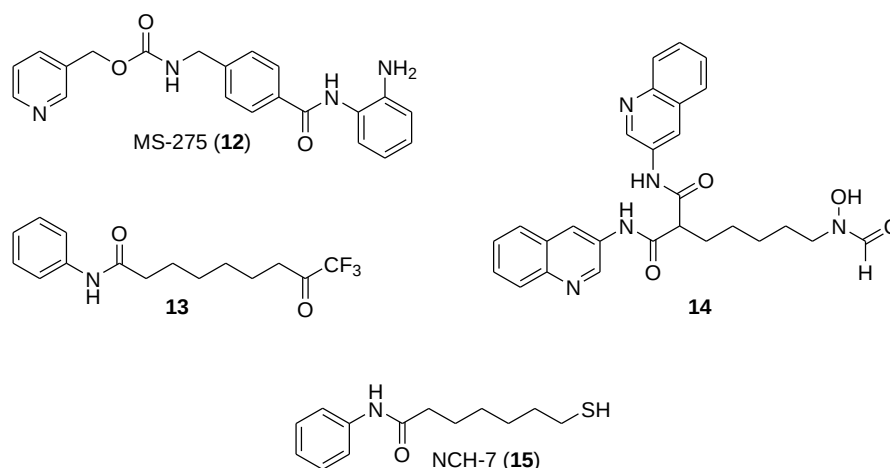


Figure 15 - Examples of non hydroxamate HDAC inhibitors

Despite the success of SAHA and Romidepsin in clinical trials, the majority of HDAC inhibitors suffer from a number of drawbacks. In particular, the correlation between specific epigenetic signalling pathway alteration and their cytotoxicity still needs to be established. This is mainly due to their lack of isoform selectivity, inducing modifications of a variety of downstream HDAC mediated signalling pathways. Despite the discovery of the isoform selective PCI-34051 and Tubacin (Figure 13), novel isoform selective HDACis are still needed. Clearly, not only would such compounds be useful for the development of probes aimed at studying the biological role of individual isoforms, but also for the

development of new anticancer drug candidates able to target specific pathways and therefore potentially display less side effects during therapy. Interestingly, recent preliminary studies have shed light on molecular features exploitable for the development of isoform selective probes. These studies notably demonstrated that structural alteration of the ZBG and capping units of HDACs could lead to class and/or isoform selective compounds.^{99,113,114} In particular the *o*-aminoanilide motif, while being less potent than the hydroxamic acid moiety for zinc binding, was found to selectively target class I HDACs. Other functional groups, such as thiol, have also been reported to exhibit class selectivity.¹¹⁵ Concerning the capping group, a number of studies demonstrated its importance for both potency and selectivity. For example, it was found that alteration of the capping unit of the unselective compound SAHA (**1**) could be exploited for the development of the HDAC6 selective compound Tubacin **8**, demonstrating the importance of the interactions at the surface of the protein for both binding and selectivity.

2.5. DNMT inhibitors

The list of DNMT inhibitors is much smaller compared to the cohort of HDAC inhibitors reported.^{15,20,21} They fall into two classes: nucleoside and non-nucleoside analogues. Among nucleoside analogues are several cytidine mimics such as 5-azacytidine **16**,¹¹⁶ 5-aza-2'-deoxycytidine **17** (also known as decitabine),¹¹⁷ 5-fluoro-2'-deoxycytidine **18**,¹¹⁸ 5,6-dihydro-5-azacytidine **19**¹¹⁹ and zebularine **20**^{120,121} (Figure 16). In particular, **16** and **17** have received FDA approval for the treatment of myelodysplastic syndromes. These analogues act as prodrugs since they require incorporation into DNA to exhibit DNMT inhibition. The inhibition of DNMT activity is mechanism-based. During the methylation process, proton elimination in position 5 is made impossible, inducing trapping of the DNMT in an irreversible covalent adduct (Figure 17). However, it is still unclear whether the effect of these molecules can be attributed to induction of DNA hypomethylation or DNMT depletion.¹²²

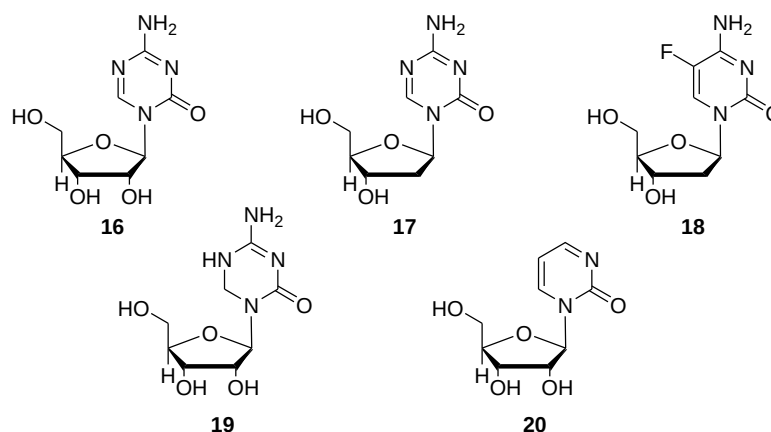


Figure 16 - Cytidine analogues inhibitors of DNA methylation

However, cytidine analogues suffer from a number of drawbacks, such as significant toxicity and poor stability in aqueous media. While zebularine **20** possesses an improved stability in aqueous solution, it still suffers from poor bioavailability.¹²¹ With the limitations encountered with nucleoside DNMT inhibitors, such as poor stability, lack of sequence specificity and incorporation into DNA, efforts have been focused towards the development of non-nucleoside analogues with improved properties.

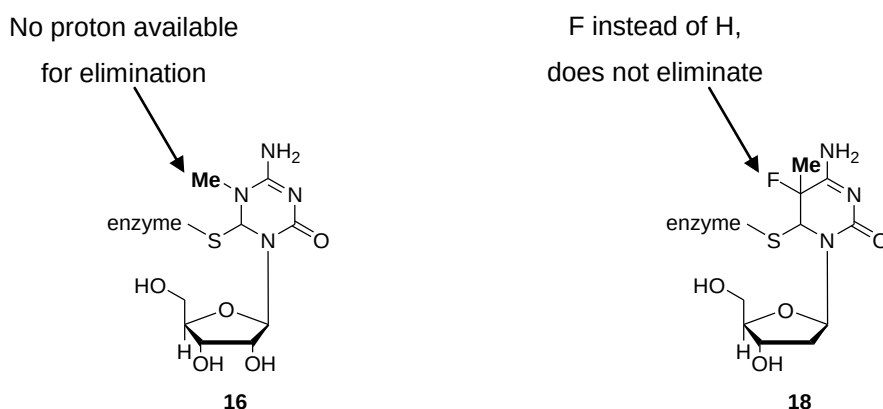


Figure 17 - Irreversible covalent adduct formed during methylation of cytidine analogues by DNMTs (examples of **16** and **18**)

Several non nucleoside inhibitors have been reported. Procaine (**21**), an anesthetic drug, was able to reduce DNA methylation in MCF7 cells. However, it failed to inhibit DNMT *in vitro*. Procainamide¹²³⁻¹²⁵ (**22**) (Figure 18) was also shown to be a demethylating agent with antiproliferative activity, its action is thought to be mediated by its binding to CpG rich sequences in DNA. Lyko and co-workers

discovered the phthaloyl tryptophan derivative RG108¹²⁶ (**23**) *via* virtual screening. RG108 is able to inhibit methyltransferase activity and has been shown to induce DNA hypomethylation *in vitro*. However its ability to induce significant DNA hypomethylation in cells is limited. Hydrazaline (**24**) is a vasodilating drug, and has been reported to inhibit DNA methylation¹²⁷ and to reactivate TSGs in cervical cancer patients.¹²⁸ Several polyphenols were also reported as inhibitors of DNMT activity; among them are EGCG¹²⁹ (**25**), Caffeic acid (**26**) and Chlorogenic acid¹³⁰ (**27**).

Despite their increased stability, non nucleoside analogues have so far shown only reduced potency compared to nucleoside analogues.¹³¹ Intensive research is still ongoing towards potent non nucleoside DNMT inhibitors.

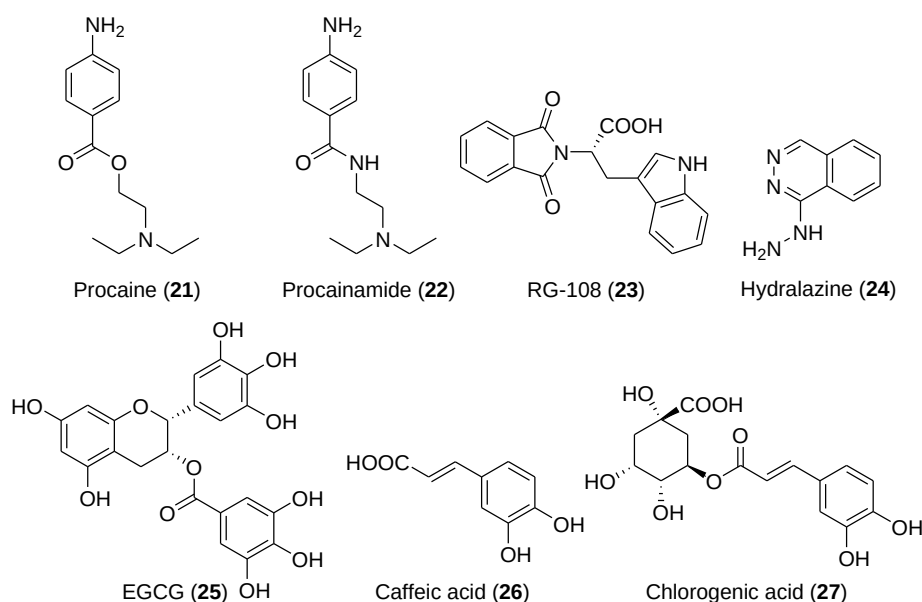


Figure 18 - Examples of non-nucleoside inhibitors of DNA methylation

Nowadays, epigenetic chemotherapies using HDAC and DNMT inhibitors are considered amongst the most promising approach in cancer therapies, notably through the reactivation of TSGs. However in most instances, it has been shown that epigenetically silenced genes cannot be reactivated by HDACis alone, but require the synergistic functions of other agents. In particular, HDACis with DNMT inhibitors (DNMTis) has been recently shown to have synergistic effects against cancer cells when used in combination.¹³²⁻¹³⁴ This synergy between HDACis and DNMTis is considered as a new promising approach for therapy.

RESULTS AND DISCUSSION

1. Design and synthesis of Psammaphin A analogue libraries

1.1. Introduction

Naturally isolated products continue to play a significant role in anticancer drug discovery. Of relevance, a number of HDAC inhibitors isolated from natural sources have been reported,^{15,20,21,108,109,112} including for example Trichostatin A (**6**), sodium butyrate (**3**), Trapoxin B (**9**), and Largazole (**11**) (Figure 19). Naturally occurring DNMT inhibitors have also been reported, such as polyphenols EGCG (**25**)¹²⁹, caffeic acid (**26**) and chlorogenic acid (**27**).¹³⁰

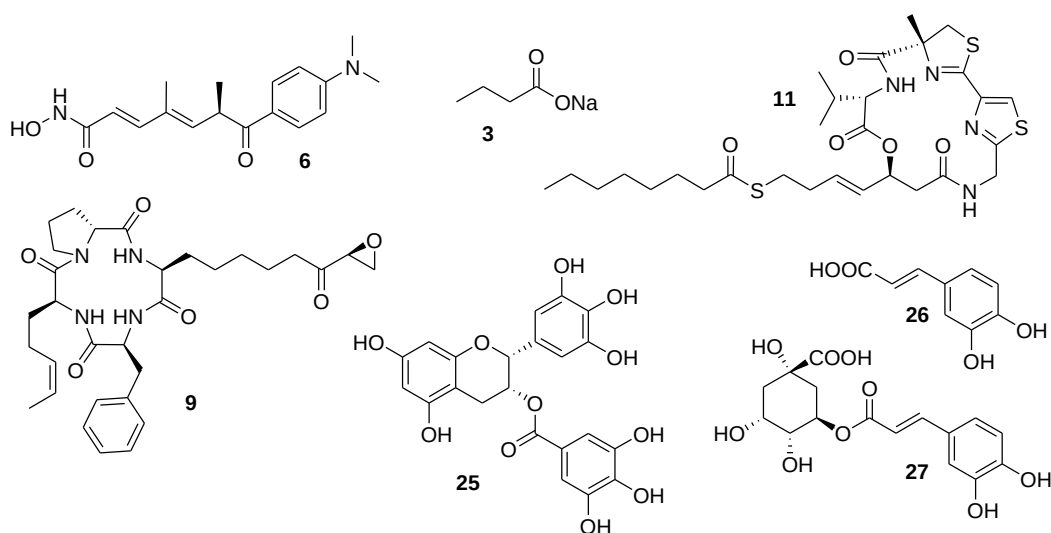


Figure 19 - Examples of naturally occurring HDAC and DNMT inhibitors

In this project, we were particularly interested in the natural product Psammaphin A (**28**, Figure 20), which has been reported to exhibit a range of interesting biological activities.

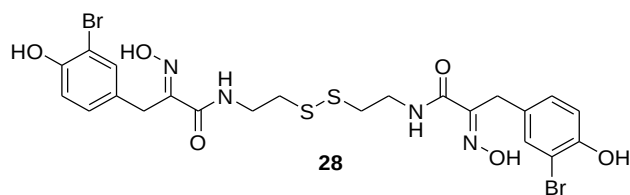


Figure 20 - Structure of the natural product Psammaplin A

Psammaplin A is a member of the psammaplin family of natural products (Figure 21).¹³⁵ All the family members have in common a bromotyrosine scaffold, and differ in the nature of their pendant terminal end group (**Y**, Figure 21, top). Psammaplins belong to the larger bromotyrosine based metabolite family. Numerous bromotyrosine metabolites were isolated from marine sponges,^{135a-g, 135j} notably from marine sponges of the order *Verongida*, known to be a rich source of such metabolites.¹³⁶ Psammaplin A was isolated from several marine sponges including *Pseudoceratina purpurea*.^{135g} It is a symmetrical, dimeric natural product, and was characterised and reported in several publications in 1987,^{135a-c} representing the first example of disulfide and oxime containing metabolite isolated from a marine sponge. The presence of the very unusual α -hydroxyimino amide motif, in addition to the bromophenol functionality, confers Psammaplin A a very unusual structure.

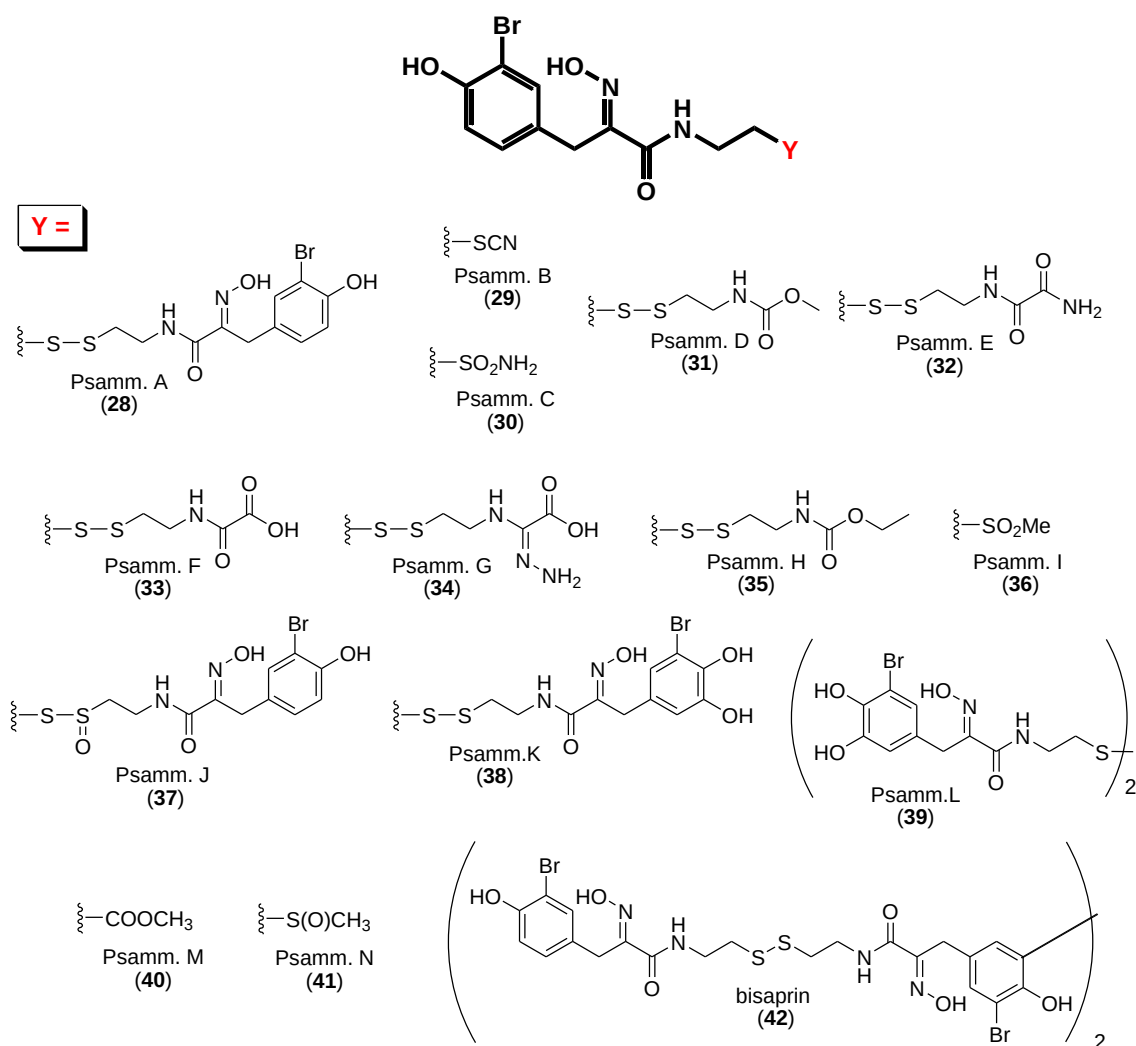


Figure 21 - The psammmaplin family of natural products. A number of other derivatives have also been reported, such as psammmaplin sulfates^{135j} or psammmaplin trisulfides (not shown)

The geometry of the two oximes of Psammmaplin A was assigned as (*E*) by Arabshahi and Schmitz, based on extensive NMR studies.^{135c} Indeed it is known that the ¹H and ¹³C chemical shifts for a methylene group in the α position to an oxime are dependent on the oxime geometry.¹³⁷ In 1987 Arabshahi and Schmitz isolated several new brominated metabolites from an unidentified sponge.^{135c} Interestingly two of these compounds exhibited very similar NMR spectra. One was found to be the natural product now called Psammmaplin A (Figure 20), having an (*E,E*) oxime geometry and the second was found to be the product with an (*E,Z*) geometry, one oxime being isomerized. They later observed that the (*E,Z*) product completely isomerized to the (*E,E*) one after a few weeks, demonstrating

the increased stability of the (*E*) geometry. Notably, it was shown that in the (*Z*) geometry the methylene carbon (α to the oxime) shifted downfield ($\delta = 35.7$ ppm) compared to the (*E*) geometry ($\delta = 27.7$ ppm) (Figure 22). The benzylic protons were found to be upfield ($\delta = 3.68$ ppm) in the (*Z*) geometry compared to the (*E*) geometry ($\delta = 3.87$ ppm) in Psammaplin A, which was in accordance with previous oxime studies made by Karabatsos.¹³⁸

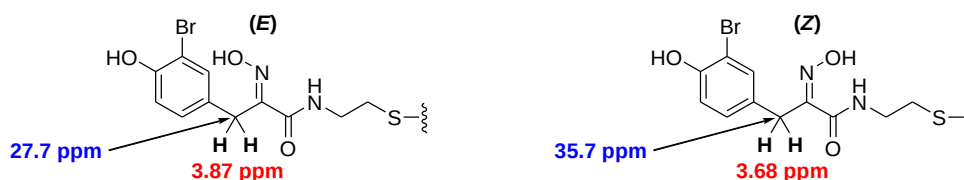


Figure 22

The (*E*) geometry proposed by Arabshahi and Schmitz^{135c} for Psammaplin A was later confirmed by X-ray analysis of the bromotyrosine derived metabolites Bastadin 5 (**43**) and Bastadin 4 (**44**),¹³⁹ and a synthetic Psammaplin A analogue (**45**) (Figure 23).¹⁴⁰

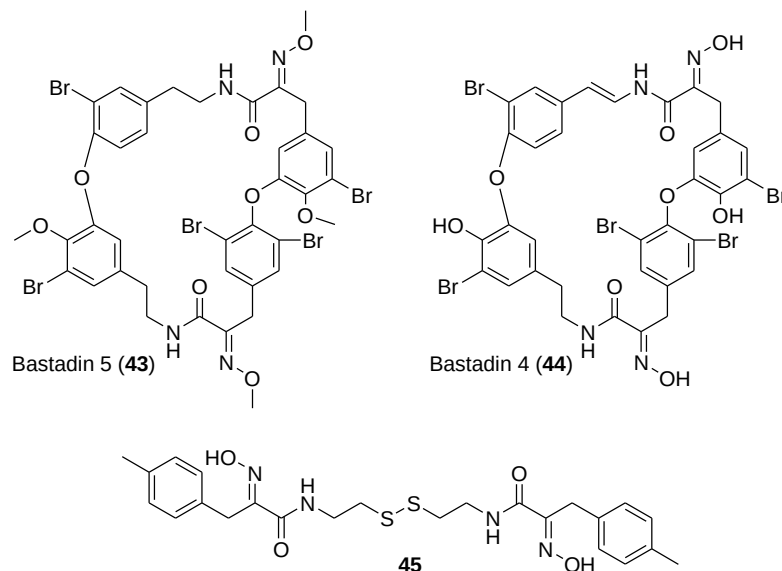


Figure 23

Interestingly, Psammaplin A has been shown to exhibit a wide range of biological activities. While it has been implicated as an inhibitor of numerous targets such as Topoisomerase II,¹⁴¹ DNA gyrase,¹⁴² Leucine aminopeptidase,^{135e} Farnesyl protein transferase,^{135e} Chitinase B,^{135f} Mycothiol-S-conjugate amidase,¹⁴³ Aminopeptidase N¹⁴⁴ and DNA polymerase α -primase,¹⁴⁵ screening of

numerous psammaplins by Crews and co-workers showed Psammaplin A to be an extremely potent inhibitor of both HDAC and DNMT enzymes.^{135g} In particular, Psammaplin A was found to be the most effective inhibitor among all psammaplins tested. In enzymatic assays, Psammaplin A displayed potent activity (IC₅₀) against an HDAC cell extract (4.2 nM) and semi purified DNMT1 (18.6 nM). Subsequently, studies on the antiproliferative properties of Psammaplin A *in vitro* have shown it to have significant cytotoxicity (ED₅₀, µg/mL) against human lung (A549, 0.57), ovarian (SK-OV-3, 0.14), skin (SK-MEL-2, 0.13), CNS (XF498, 0.57), and colon (HCT15, 0.68) cancer cell lines.¹⁴⁶ *In vivo*, it inhibited tumour growth in the A549 lung xenograph mouse model while maintaining low toxicity.^{135g}

Based on our ongoing interest in epigenetics and potential exciting applications in anticancer therapies, we thought Psammaplin A to be a highly valuable starting point for our project. We decided to elucidate the reasons for the observed HDAC and DNMT inhibition by this natural product, by establishing structure-activity relationships (SAR) against both enzymes. We envisaged that such studies would not only allow dissection of the epigenetic activity of psammaplin A, but would inspire new leads in cancer drug discovery. Indeed, non-nucleoside DNMT inhibitors and isoform selective HDAC inhibitors are severely underdeveloped, vindicating the need for new small molecule leads. Furthermore, epigenetic pathways such as histone deacetylation and DNA methylation are intimately interrelated, with the efficacy of HDAC inhibitors potentiated by co-administration of DNMT inhibitors.^{14,132} Clearly, inhibitory molecules with dual activity against several classes of epigenetic enzymes may provide novel approaches to therapy.

As previously mentioned, epigenetically silenced genes cannot be reactivated by HDAC inhibitors alone, and require the synergistic functions of demethylating agents, such as DNMT inhibitors.^{14,132} The discovery of the highly potent inhibitory effect of Psammaplin A against both HDAC and DNMT activity represents a very valuable starting point for our anticancer drug discovery project. Whereas the highly potent HDAC inhibitory properties of several members of the

psammaplins have been reported,^{135g} the molecular mechanisms underlying this inhibitory effect are not completely understood. In 2007, studies performed by Kwon and co-workers¹¹⁵ were consistent with the cap-linker-ZBG model for HDAC inhibitors (Figure 11), as discussed in section 2.4. Their work strongly suggested that Psammaplin A could be a natural prodrug, with the species responsible for HDAC inhibition, a thiol, revealed *via* reduction of the disulfide moiety. Indeed there were precedents, several thiol containing HDAC inhibitors had already been reported previously,^{108,109,112,147,148} such as the thiol analogue of SAHA (**15**, Figure 24), the cyclic peptide based natural products FK228 (Romidepsin, **2**),¹⁴⁸ Spiruchostatin A (**46**) and Spiruchostatin B (**47**). In an enzymatic assay, **15** was found to be as potent as SAHA.¹⁰⁸ In 2002, studies by Furumai and co-workers showed that the HDAC inhibitory potency of Romidepsin (**2**), containing an internal disulfide moiety, was significantly enhanced when in its reduced form.¹⁴⁷ On the contrary, incubation with 1% H₂O₂ led to a drop in potency. Reduction of **2**, followed by methylation of the resulting thiols led to a completely inactive compound against HDACs. Taken together, these observations were in agreement with **2** being a natural prodrug. The potency of **2** was also considerably reduced in glutathione deficient cells, bringing additional support to the prodrug hypothesis.

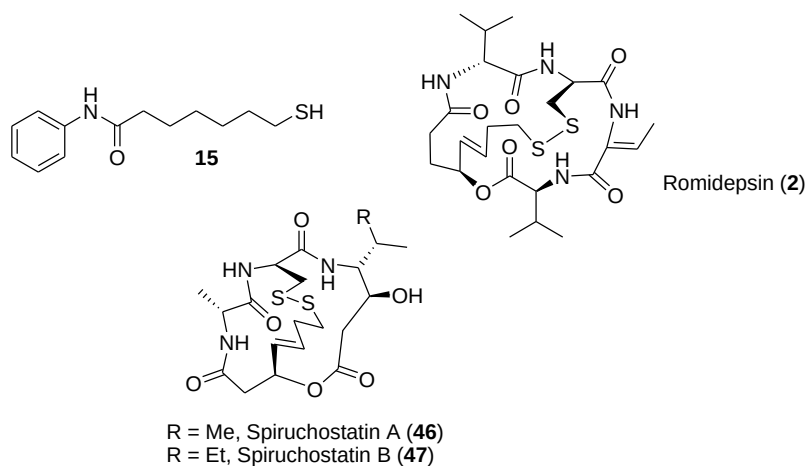
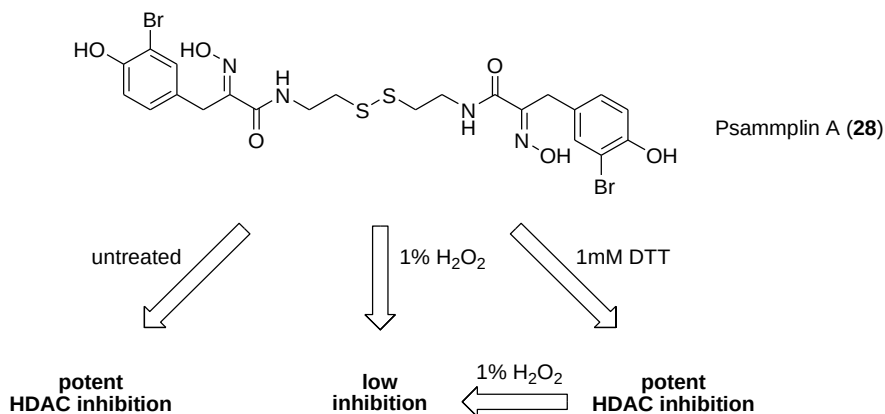


Figure 24

In 2007, Kwon and co-workers showed in a similar manner the HDAC inhibitory potency of Psammaplin A to be dependent on the oxidation state around the sulfur atom (Scheme 5).¹¹⁵ Enzymatic assays performed with Psammaplin A in the presence of 1% H₂O₂ led to very low HDAC inhibition compared to non

treated Psammaplin A. The same experiment performed in the presence of dithiothreitol (DTT) instead of H_2O_2 led to results similar to those obtained with the non pre-treated Psammaplin A. This latter result could be explained by the usual presence of cellular reducing agents in the extracts used for the enzymatic assay, potentially reducing Psammaplin A *in situ*; further addition of H_2O_2 permitted recover of enzymatic activity. During the same study, it was shown that Psammaplin A exhibited greatly decreased HDAC inhibitory potency in glutathione deficient cells, an observation which parallels previous results obtained with other disulfide containing HDAC inhibitors, such as Romidepsin (2). Taken together, all these observations strongly suggested Psammaplin A to be a natural prodrug for HDAC inhibition (Figure 25). Other cell based assays by Kwon and co-workers showed that Psammaplin A, in its reduced form, was a selective class I HDAC inhibitor, efficiently inducing histone hyperacetylation but failing to significantly affect the level of acetylated tubulin. This class selectivity has already been observed with other non hydroxamic acid HDAC inhibitors.^{21,113} Although Psammaplin A induced an upregulation of acetylated histones (substrate for class I HDACs) while leaving the level of acetylated tubulin (substrate for the class II HDAC6) unaffected, subclass isoform specificity was not thoroughly studied.



Scheme 5 - HDAC inhibition by Psammaplin A is affected by the presence of oxidants and reducing agents

Whereas the presence of a thiol as a ZBG seems to be important for HDAC inhibition by Psammaplin A, the importance of the amide, the oxime functionality, and the aromatic “cap” for HDAC inhibition is still unclear.

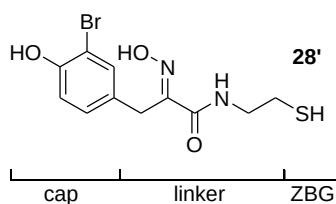
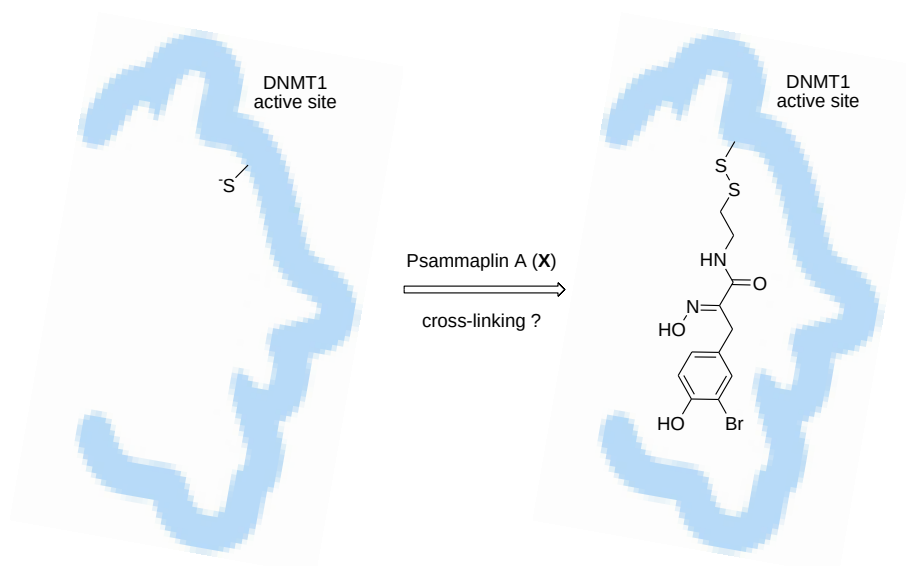


Figure 25 - Proposed cap-linker-ZBG model for Psammaplin A

The mechanism underlying the reported DNMT1 inhibition by Psammaplin A is poorly understood. There is no evidence as to whether the species responsible for DNMT inhibition is a thiol or a disulfide. An initial hypothesis we formulated was that inhibition could proceed *via* crosslinkage between the disulfide bond of Psammaplin A and the thiol residue in the active site of DNMT1 (Scheme 6).^{69,70,149} However this hypothesis is difficult to reconcile with the usual presence of reducing agents (such as DTT) in methyltransferase enzyme based assays, used to stabilize the enzyme and its substrate. Indeed, as previously mentioned Psammaplin A is chemically sensitive to reducing agents, the latter potentially converting it to the corresponding thiol. Alternatively, inhibition *via* an allosteric mechanism cannot be neglected.



Scheme 6 - Hypothesis for the inhibition of DNMT1 by Psammaplin A

1.2. Results and discussion

1.2.1. Library design

Initial work was therefore undertaken with the aim of establishing a structure-activity relationship (SAR) of Psammaplin A against HDAC and DNMT enzymes, in order to shed light on the required structural features of Psammaplin A for HDAC and DNMT inhibition. In order to establish the SAR for Psammaplin A around our targets of interest, we designed and synthesised a library of compounds (Figure 26), in order to probe the nature of the sulfur end group (disulfide, thioether, thiol, etc), the oxime functionality (AB, Figure 26) and to fully explore variation in the substituents of the aromatic “capping” group.

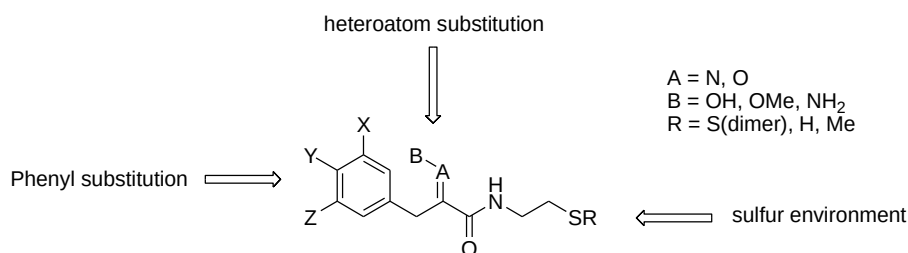


Figure 26: Library design, variation of aromatic substitution, oxime unit and oxidation state of the sulfur

In terms of HDAC inhibition, variation of the disulfide moiety should give a definitive answer to the question of whether the active species is the thiol or the disulfide. In addition to the original disulfide, preparation of the corresponding thiols and methylthioethers was planned. Based on our hypothesis of the thiol as active species against HDACs, we initially expected greatly decreased HDAC inhibitory properties for methylthioether containing compounds.

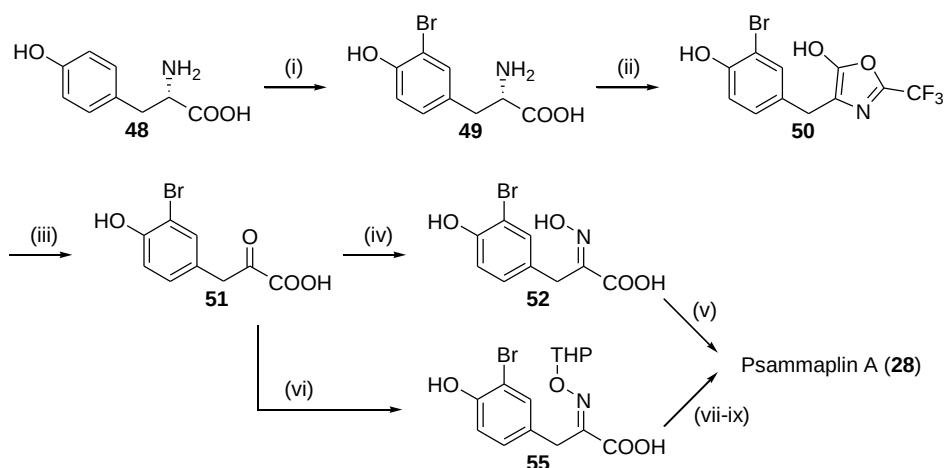
The role of the oxime unit for HDAC inhibition is undocumented. Variation around it should provide useful information about its role in binding.

Finally, variations at the level of the phenol (OH vs OMe) should provide clues about the possible implication of this O-H group in H-bond interactions. Further variation of aromatic substitution should allow the study of the

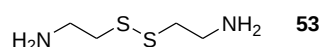
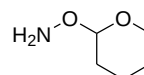
importance of aromatic substitution on potency and selectivity. For example, electron-withdrawing functionalities should enhance arene-arene (π - π) interactions,¹⁵⁰ whereas electron-donating groups, in combination with halogen substituents should enhance a potential sulfur-halogen bond.^{151,152} Likewise, should a sulfur-halogen bond be important, replacement of the bromide moiety in the psammaplin structure with an iodine atom, will increase the halogen atom's polarisability, and subsequently enhance the sulfur-halogen bond.

1.22. Previous syntheses of Psammaplin A and other bromotyrosine metabolites

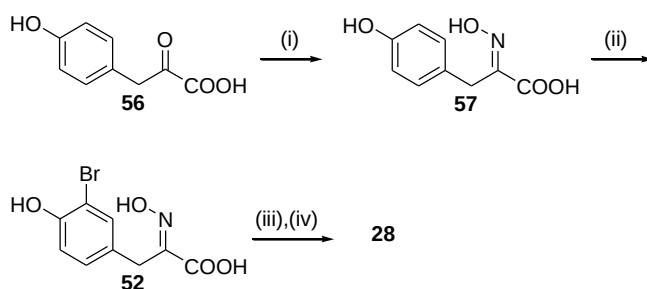
Previously reported synthetic routes towards Psammaplin A, and in general, hydroxyimino bromotyrosine based natural products usually suffer from drawbacks when it comes to the synthesis of libraries of analogues. Several total syntheses of Psammaplin A have been reported previously. The first synthesis was reported by Hoshino in 1992 and involved tyrosine **48** as starting material (scheme 7).¹⁵³ Bromination afforded bromotyrosine **49** in high yield. Refluxing of **49** in trifluoroacetic anhydride afforded trifluorooxazolone **50**, which was then hydrolyzed in aqueous acidic conditions to afford the arylpyruvic acid precursor **51** in good yield. Further condensation with hydroxylamine, followed by direct DCC/*N*-hydroxyphthalimide (NOHP) coupling with cystamine **53** afforded Psammaplin A in 18% overall yield. Nicolaou's approach¹⁵⁴ was similar, except that *O*-(tetrahydro-2*H*-pyran-2-yl)hydroxylamine (*O*-THP protected hydroxylamine, **54**)¹⁵⁵ was employed as a precursor for the final oxime units, generated at the final step by acidic deprotection.

**Reagents and conditions:**

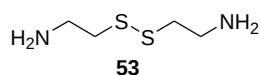
- (i) KBr, KBrO₃, 0.25N HCl, 23 °C, 12h, 81%
 (ii) TFAA, 80-120°C, 1h, 61%
 (iii) 70% aq. TFA, rt, 24h, 97%
 (iv) HONH₂.HCl, AcONa, EtOH, rt, 57%
 (v) **53**, DCC, NOHP, Et₃N, dioxane, rt, 12h, 67%
 (vi) **54**, EtOH, rt, 12h
 (vii) EDC, NHS, dioxane, rt
 (viii) **53** (dihydrochloride salt), Et₃N, dioxane/MeOH, rt
 (ix) HCl, CH₂Cl₂, Et₂O, MeOH, reflux, 2h, 36% from **3**

**53****54****Scheme 7** - Total synthesis of Psammaplin A, Hoshino's and Nicolaou's routes

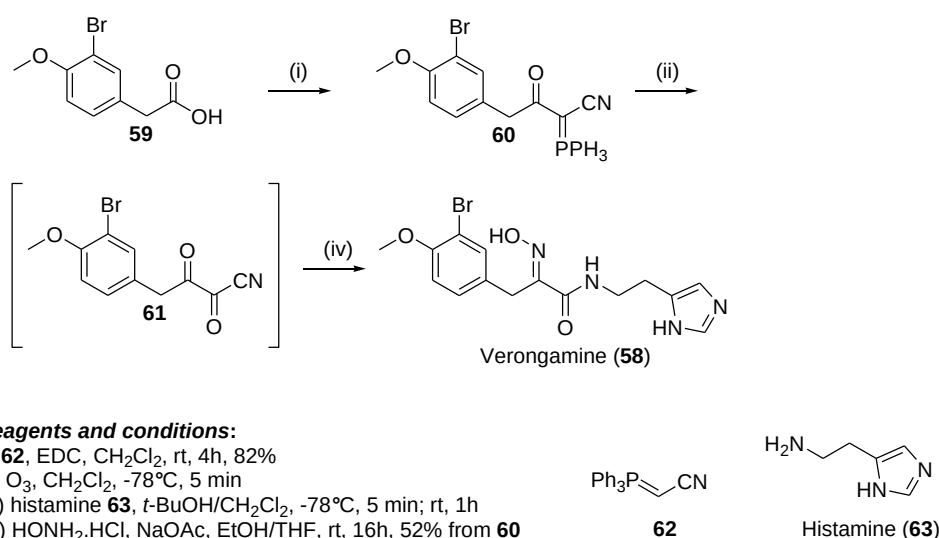
While both Hoshino and Nicolaou used tyrosine as a starting material, Sufrin based her synthesis on the commercially available 4-hydroxyphenylpyruvic acid **56** (Scheme 8).¹⁵⁶ Condensation with hydroxylamine, followed by bromination and final DCC/NOHP mediated coupling with cystamine **53** afforded Psammaplin A in 43% overall yield, representing a substantial yield improvement compared to previous syntheses.

**Reagents and conditions:**

- (i) H₂NOH, pyridine, rt, 3h, 79%
 (ii) KBr, KBrO₃, 0.25N HCl, rt, 40 min, 81%
 (iii) DCC, NHS, DMF, rt, 8h
 (iv) Et₃N, **53**, rt, 14h, 67% from **52**

**53****Scheme 8** - Total synthesis of Psammaplin A, Sufrin's route

As an alternative for the introduction of the arylpyruvate motif, Wasserman and co-workers used a cyano ylide coupling strategy, allowing for the synthesis of the natural product Verongamine (**58**) from a phenylacetic acid derived starting material (scheme 9).¹⁵⁷ The applicability of this methodology for the synthesis of Psammaplin A analogues is relatively limited. Indeed, in addition to the fact that only a limited number of phenylacetic acid derivatives are commercially available, the strong oxidative conditions necessary to generate the phenylpyruvoyl cyanide intermediate **61** limit the scope of aromatic substituents compatible with this chemistry (*i.e.* amines, thioether), not to mention the high toxicity of the formed cyanides.

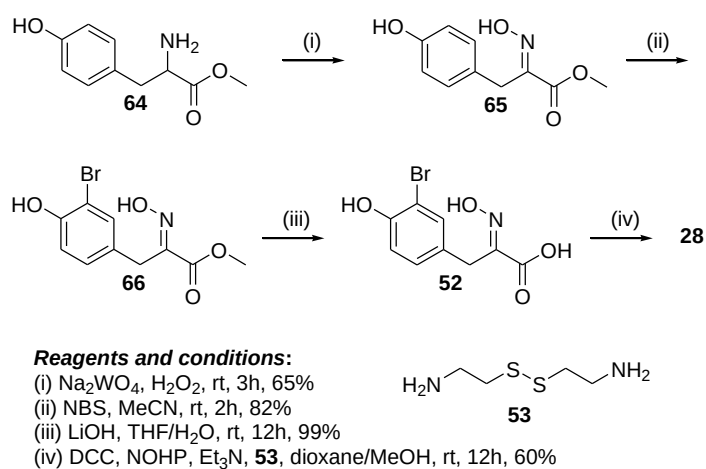


Scheme 9 - Wasserman's total synthesis of Verongamine *via* a cyano ylide coupling

Using a different approach, De Lera synthesised Psammaplin A from tyrosine methyl ester **64**, employing conditions previously reported by Spiling and co-workers for the synthesis of the natural product Verongamine **57** (Scheme 10).¹⁵⁸ Direct oxidation of the amino group¹⁵⁸⁻¹⁶⁰ using Na₂WO₄ afforded oxime **65** in moderate yield. Further bromination afforded bromophenol **66** in good yield. Hydrolysis of ester **66** and DCC/NOHP mediated coupling with cystamine **53** using conditions similar to those employed by Hoshino afforded Psammaplin A in 29% overall yield.

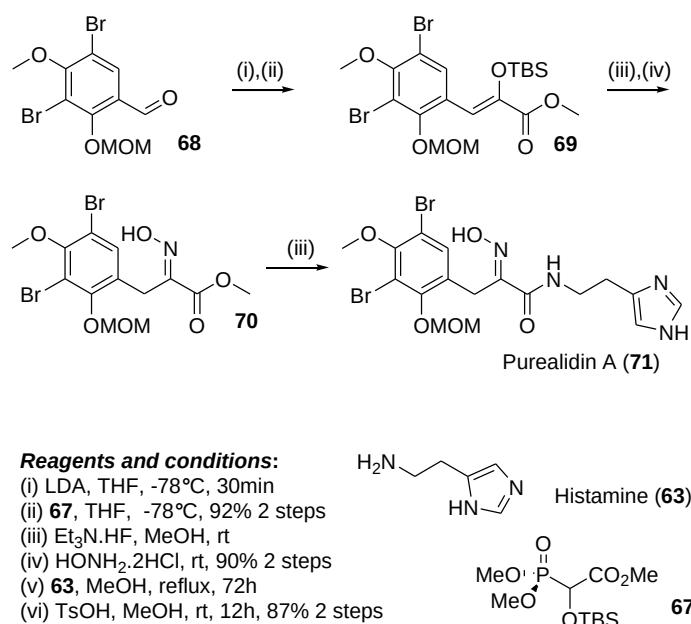
Despite the fact that the previously described synthetic routes afforded several Psammaplin A analogues,^{140,154,156,161a-c} they suffer from a number of

drawbacks. While they allow diversity introduction for the AB system (Figure 26) and the amine nucleophile [and therefore R (Figure 26)], diversity introduction in the aromatic substitution mainly depends on the relatively limited number of commercially available phenylalanine, tyrosine and arylpyruvic acid derivatives. This considerably limits variation of aromatic substitution patterns of Psammaplin A analogues. Early stage aromatic functionalisation of these building blocks is a potential solution to overcome this problem, however it increases the number of steps of the whole sequence. Another general approach was therefore required, allowing cheap and easy access to a wide range of substituted arylpyruvic acid derivatives.



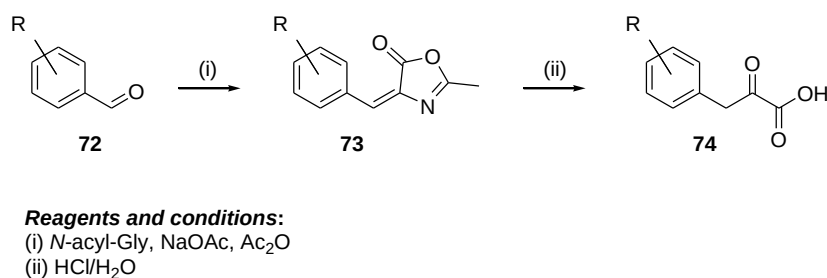
Scheme 10 - Total synthesis of Psammaplin A, de Lera's route

The use of Nakamura's α -OTBS-functionalised dimethylphosphonate **67**¹⁶² by Spiling and co-workers, in a Horner-Wadsworth-Emmons (HWE) reaction with aromatic aldehyde **68** afforded the protected and functionalised methyl arylpyruvate **69** in high yield (scheme 11). The silylenol ether of **69** could be then converted into the corresponding oxime derivative **70** in high yield. Final amidation and MOM deprotection gave access to the natural product Puralidin A (**71**) in good overall yield.¹⁶² The use of an aromatic aldehyde as starting building block represents an important advantage. Indeed a wide range of structurally diverse aromatic aldehydes are available from commercial sources and usually cheap, which is ideal for the construction of a library of analogues. However, conditions used in the initial HWE reaction are incompatible with the presence of deprotonable and/or base sensitive aromatic substituents (*i.e.* hydroxyl) and therefore involves the use of protecting groups.



Scheme 11 - Spilling's total synthesis of Puralidin A using Nakamura's α -OTBS-functionalized dimethylphosphonate¹⁶²

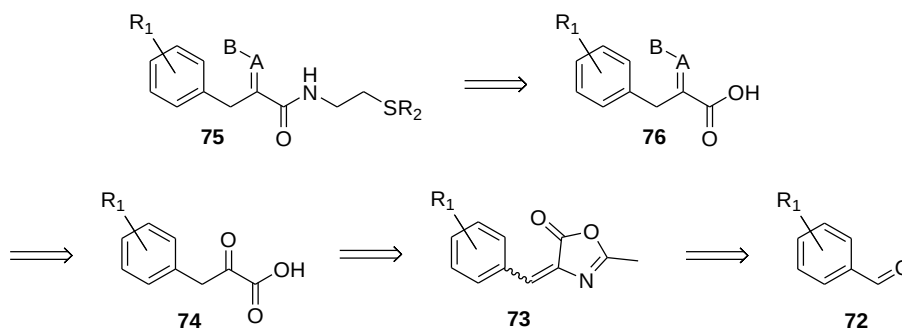
While starting the synthesis of our libraries from aromatic aldehydes would represent a valuable synthetic advantage for diversity introduction on the aromatic ring, milder conditions had to be found in order to convert them to the corresponding arylpyruvic acid derivatives. It was thought that the well known Erlenmeyer-Plöchl azlactone synthesis¹⁶³/hydrolysis sequence¹⁶⁴ was the method of choice to achieve this goal (Scheme 12).



Scheme 12 - Erlenmeyer-Plöchl azlactone synthesis/hydrolysis for the expedient access to a wide range of substituted arylpyruvic acids

The retrosynthetic analysis of target compounds **75** is shown in Scheme 13. Psammaplin A analogues with general structure **75** should be accessible from the corresponding acids **76** using conditions reported previously by others for

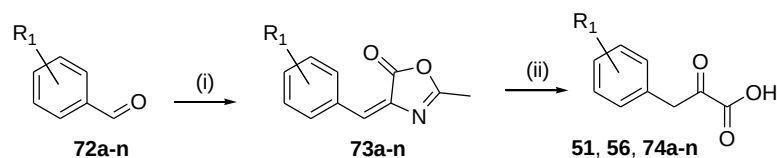
amide bond formation.^{153,154,156} Acids **76** should be obtained easily after condensation of arylpyruvic acids **74** with a hydroxylamine derivative (in the case AB = oxime or *O*-methyloxime). Finally Erlenmeyer azlactone synthesis followed by acidic hydrolysis should provide acids **74** from aldehydes **72**.



Scheme 13 - Retrosynthetic analysis of Psammaplin A analogues

1.23. Synthesis of oxime and *O*-methyloxime containing analogues

Our synthetic route to the various arylpyruvic acids is described in Scheme **14** (+ see Table 1 for structures). Upon exposure to *N*-acetyl glycine and acetic anhydride in the presence of sodium acetate, aromatic aldehydes **72a-n** were converted to the corresponding azlactones **73a-n** in moderate (*i.e.* **73g**) to high (*i.e.* **73h**) yield. Simple filtration, trituration in cold water, and a second filtration afforded the crude azlactones. Importantly, these were pure enough to be used for the next step. Further reflux in 10% aqueous HCl afforded arylpyruvic acids **51**, **56**, **74a-n** in moderate (*i.e.* **74f**) to excellent (*i.e.* **74n**) yield. Once again, simple isolation by filtration afforded arylpyruvic acids **74a-p** as crude products which were used for the next step without further purification. Indeed, attempted purification by normal phase column chromatography proved unsuccessful owing to the high polarity and poor stability of these compounds on silica. Other purification methods were not investigated. Characterisation by NMR showed that these compounds are present exclusively in the enol form in solution (CD_3OD).

**Reagents and conditions:**(i) *N*-Ac-Gly, NaOAc, Ac₂O, reflux, 3h, 37-90%

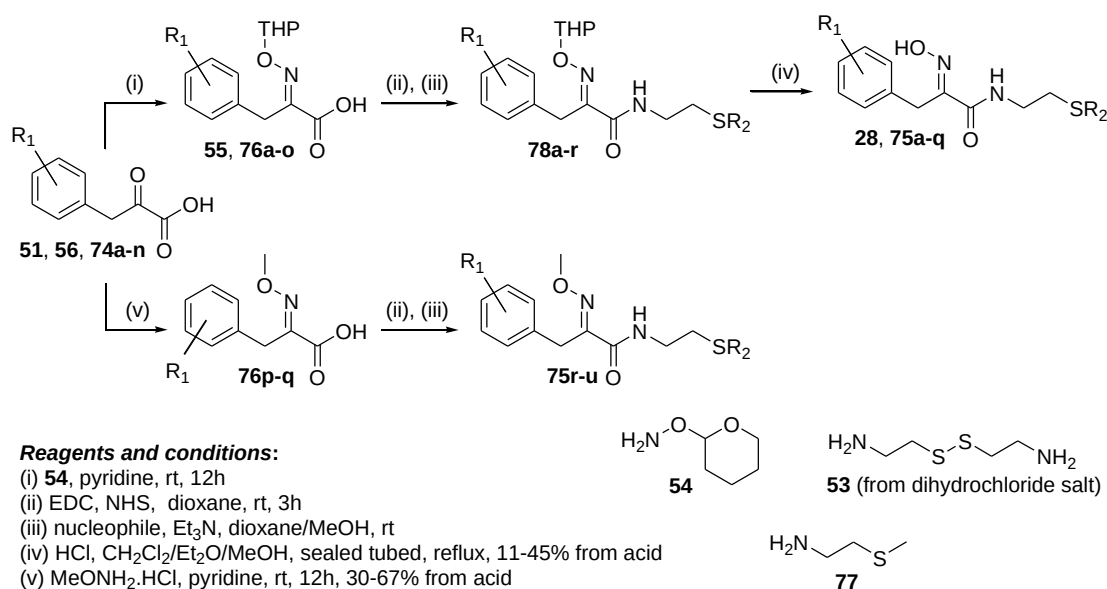
(ii) 10% aq. HCl, reflux, 48-98%

Scheme 14 - Synthesis of substituted arylpyruvic acids

The structurally diverse arylpyruvic acids generated were used as precursors to Psammaplin A analogues following procedures inspired by the previously reported routes (Scheme 15).^{151,152,158b} Condensation of arylpyruvic acids **51**, **56**, **74a-n** with *O*-THP protected hydroxylamine afforded oximes **55**, **76a-o**, which were used as a crude product for the next step after a simple acidic aqueous work-up. EDC mediated amide bond formation with **53** or **77** afforded protected precursors **78a-r**, which were purified *via* a short silica column. Final THP deprotection in anhydrous acidic conditions afforded Psammaplin A (**28**) and analogues **75a-q** in moderate (*i.e.* **75o**) to high (*i.e.* **75c**) yield after purification by flash column chromatography. The synthesis of *O*-methoxime containing analogues was achieved in a similar manner, except methoxylamine was used instead of the *O*-THP protected hydroxylamine. A summary of the structures of azlactones, arylpyruvic acids and analogues **75a-u**, in addition to the yields for the different synthetic steps, can be found in Table 1. Phenylpyruvic acid **74b** and 4-hydroxyphenylpyruvic acid **56** were obtained from commercial sources.

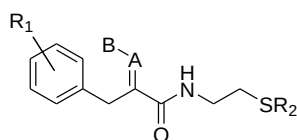
Initially, a comparative study using selected substrates was undertaken (data not shown) in order to determine the best conditions to be used for amide bond formation among those reported by Hoshino, Nicolaou and Godert.^{153,154,156} While the DCC/NOHP mediated amide bond formation used by Hoshino afforded Psammaplin A in only two steps from arylpyruvic **51** (Scheme 7), we found the yield of the coupling between hydroxyimino acids (e.g. **52**) and cystamine **53** to be relatively capricious and substrate-dependent. It therefore decided to switch towards more robust and general coupling conditions. While Godert and Nicolaou both reported efficient coupling procedures using DCC/NHS and EDC/NHS respectively, the latter conditions were found to proceed in a much cleaner

fashion. First, it is well known that dicyclohexylurea, the byproduct of DCC mediated couplings, can be difficult to remove completely by standard purification techniques. The use of the water-soluble EDC represents a considerable advantage, as its corresponding urea is also water-soluble and usually completely removed during standard aqueous work-up. Efficient and general purification methods are particularly important when synthesising large libraries of analogues. Moreover, the low boiling point solvents and the simple intermediate aqueous work-up involved in Nicolaou's synthesis were found to greatly facilitate the purification of the final products. An *O*-THP protected hydroxylamine was preferred as a precursor of the final oxime, as described by Nicolaou,¹⁵⁴ once again for ease of purification of the final product.



Scheme 15 - Synthesis of oxime and *O*-methyloxime containing analogues

In summary, this synthetic route involved clean and high-yielding transformations, allowing the access to oxime and *O*-methyloxime containing compounds after minimum purification by flash column chromatography throughout the whole synthetic sequence.



Entry	aldehyde	Az lactone yield	acid yield	AB	Amine nucleophile	Product, yield from pyruvic acid
72a		73a, 71%	51, 88%	NOH	80	75a, 40%
				NOH	53	28, 35%
				NOMe	80	75r, 43%
				NOMe	53	75s, 30%
72b		73b, 75%	74a, 83%	NOH	80	75b, 38%
				NOH	53	75c, 49%
				NOMe	80	75t, 67%
				NOMe	53	75u, 41%
72c		73c, 54%	74b, R ₁ = H Commercial	NOH	53	75d, 24%
				NOH	53	75e, 20%
				NOH	53	75f, 29%
				NOH	53	75g, 20%
72d		73d, 66%	74c, 72%	NOH	53	75h, 36%
				NOH	53	75i, 30%
				NOH	53	75j, 25%
				NOH	53	75k, 23%
72e		73e, 69%	74d, 71%	NOH	53	75l, 20%
				NOH	53	75m, 20%
				NOH	53	75n, 20%
				NOH	53	75o, 20%
72f		73f, 66%	74e, 94%	NOH	53	75p, 20%
				NOH	53	75q, 20%
				NOH	53	75r, 20%
				NOH	53	75s, 20%
72g		73g, 37%	74f, 48%	NOH	53	75t, 20%
				NOH	53	75u, 20%
				NOH	53	75v, 20%
				NOH	53	75w, 20%
72h		73h, 90%	74g, 92%	NOH	53	75x, 20%
				NOH	53	75y, 20%
				NOH	53	75z, 20%
				NOH	53	75aa, 20%

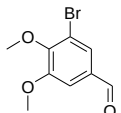
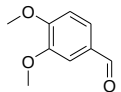
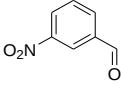
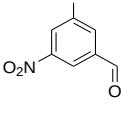
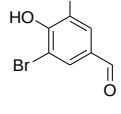
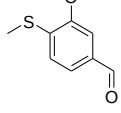
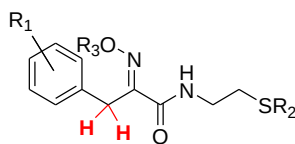
72i		73i, 70%	74i, 93%	NOH	53	75i, 45%
72j		73j, 69%	74j, 62%	NOH	53	75m, 13%
72k		73k, 57%	74k, 72%	NOH	53	75n, 30%
72l		73l, 60%	74l, 95%	NOH	53	75o, 11%
72m		73m, 56%	74m, 82%	NOH	53	75p, 22%
72n		73n, 63%	74n, 98%	NOH	53	75q, 36%

Table 1 - Structure of oxime and O-methyloxime containing analogues, and yields for the different synthetic steps

The oxime geometry of final compounds was assigned as (*E*) in each case, based on the NMR chemical shifts (Table 2) of the benzylic protons and carbon (see section 3.1). Indeed, as previously mentioned NMR studies performed on Psammaplin A by Arabshahi and Schmitz^{135c} have shown that in the (*Z*) geometry the methylene carbon (α to the oxime) shifted downfield ($\delta = 35.7$ ppm) compared to the (*E*) geometry ($\delta = 27.7$ ppm) (Figure 22). In their study, the benzylic protons were found to be upfield ($\delta = 3.68$ ppm) in the (*Z*) geometry compared to the (*E*) geometry $\delta = (3.87$ ppm) in Psammaplin A. Structures can be found in Table 1 above. NMR data reported in Table 2 were in full agreement with an (*E*) geometry in each case.



Entry	Benzylic protons δ (ppm)	Benzylic carbon δ (ppm)
75a	3.79	28.7
28	3.79	28.7
75r	3.81 ^a	28.5 ^a
75s	3.81 ^a	28.5 ^a
75b	3.90 ^a	28.0 ^a
75c	3.87 ^a	28.0 ^a
75t	3.83 ^a	28.5 ^a
75d	3.91	30.0
75e	3.80	29.1
75f	3.88	29.2
75g	3.76 ^b	28.3 ^b
75h	3.81	28.6
75i	3.87	29.0
75j	3.85	29.1
75k	3.83	29.2
75l	3.85	29.5
75m	3.85	29.5
75n	4.03	29.8
75o	4.02	29.8 ^c
75p	3.79	28.5
75q	3.88	29.9

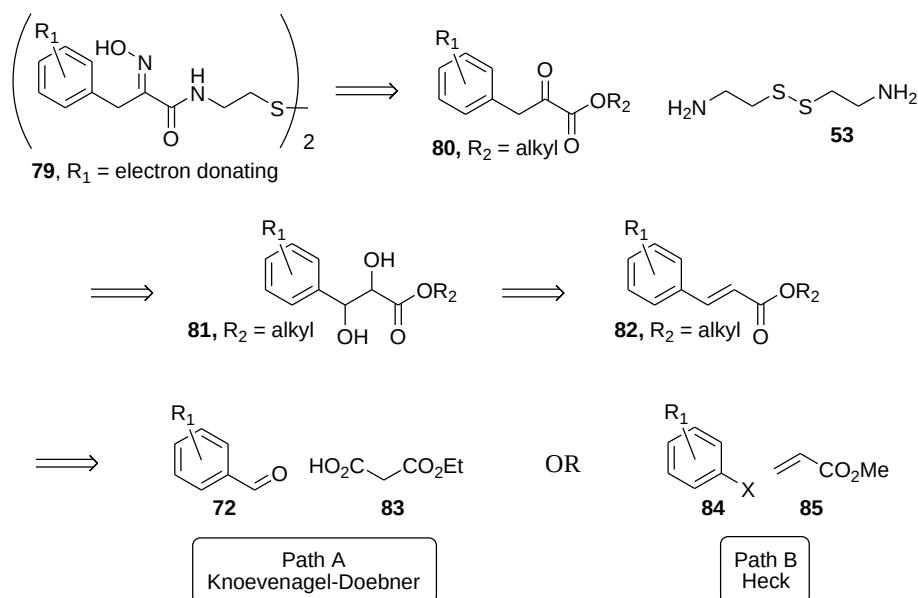
^a CDCl₃; ^b d⁶-DMSO; ^c CD₃OD/d⁶-DMSO

Table 2 - chemical shifts (ppm) of the benzylic protons and carbons for oxime and *O*-methyloxime containing analogues confirmed the (*E*) geometry in each case

While the synthetic route described in Scheme 15 allowed us to access an important number of analogues, we faced several synthetic issues however when starting with electron-rich benzaldehydes, such as *p*-dimethylaminobenzaldehyde. While the Erlenmeyer azlactone synthesis is sufficient in the case of electron-poor aromatic aldehydes, low yields were generally obtained for electron rich substrates. Moreover, adjustment of the pH and isolation of the subsequent arylpyruvic acids was difficult when the aromatic ring contained basic functionalities. To overcome these problems and allow access to electron-rich aromatic Psammaplin A analogues, we developed alternative routes to Psammaplin A and analogues not accessible *via* the previously employed route.

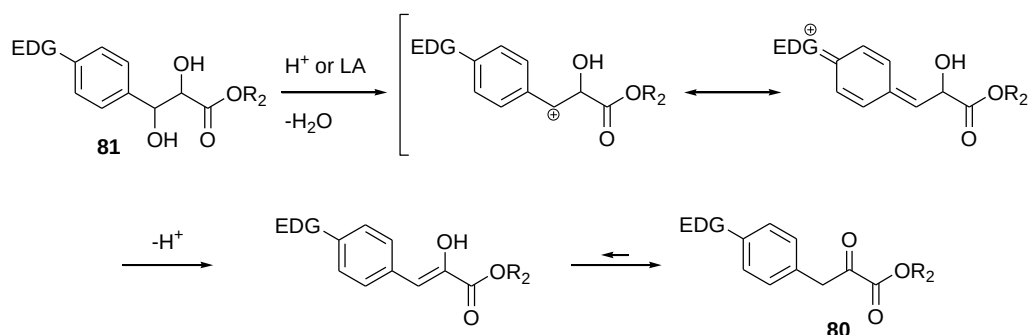
1.24. New synthetic route towards Psammaplin A analogues bearing electron rich aromatics

The retrosynthetic analysis of **79** is given in Scheme 16. The product **79** was considered to be accessible through condensation of ester **80** with hydroxylamine and subsequent double amidation with cystamine **53**. The key step in this synthesis was initially thought to be the introduction of the pyruvate motif. Alternatively, we considered accessing it *via* dihydroxylation of alkene **82** followed by regioselective dehydration of the diol under acidic conditions.



Scheme 16 - Retrosynthetic analysis of Psammaplin A analogues bearing electron rich aromatics (EDG = electron donating group)

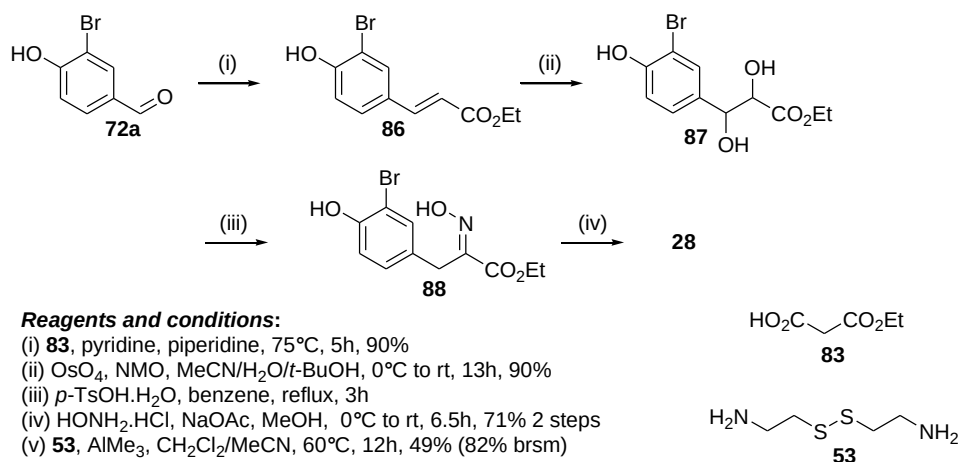
This process is likely to occur *via* an E1 mechanism and should be facilitated by the electron donating ability of the aromatic substituent, stabilising the positively charged benzylic position during dehydration (Scheme 17). Finally, the unsaturated ester should be easily accessible from a Knoevenagel-Doebner condensation between 3-ethoxy-3-oxopropanoic acid **83** and aromatic aldehyde **72**, or a Heck reaction between aryl halide **84** and methyl acrylate **85**. Again, this synthetic route would employ commercially available, diverse and cheap starting materials.



Scheme 17 - Expected regioselective dehydration of diol **81**, facilitated by the presence of electron donating groups on the aromatic ring

In order to test the applicability of this synthetic route for the synthesis of Psammaphin A analogues, we first decided to attempt the synthesis of Psammaphin A itself. Knoevenagel-Doebner condensation between aromatic aldehyde **72a** and 3-ethoxy-3-oxopropanoic acid **83** afforded unsaturated ester **86** in excellent yield (Scheme 18). The latter was converted in high yield to diol **87** using osmium tetroxide in a water/acetonitrile mixture. We were pleased to observe regioselective dehydration of diol **87** with catalytic amounts (0.1 eq.) of *p*-TsOH in refluxing benzene. Further condensation with hydroxylamine afforded α -hydroxyimino ester **88** in 71% yield after 2 steps, as a single isomer (NMR). Compound **88** has been previously reported in the literature as an intermediate for the synthesis of the natural product Verongamine (**58**, Scheme 9) by Spilling *et al.*,¹⁵ and its structure confirmed by X-ray crystallography.¹⁶ Comparison of our data with that reported matched perfectly. This protecting group free sequence employed mild reaction conditions, and represents a considerable advantage compared to the use of Nakamura's α -OTBS-protected dimethylphosphonate in a Horner-Wadsworth-Emmons reaction with aromatic aldehydes.^{14,17,18}

Direct double amidation of **88** with cystamine was preferred to a two step hydrolysis-coupling sequence. Heating ester **88** in the presence of 0.5 equivalents of cystamine (**53**) in MeOH led to no reaction after extended periods of time, despite the successful application of these conditions to the synthesis of the natural product Verongamine (**58**) using histamine (**63**, Scheme 9) as nucleophile.¹⁵ The direct conversion of esters to amides in neutral conditions is known to be relatively substrate dependent, and usually operates at very high temperatures/pressures and reaction times. High temperatures, pressures or the use of strong alkali metal catalysts however are often incompatible with delicate substrates. In our case, the presence of the oxime, the phenol and the disulfide was a concern and therefore milder reagents had to be found. A number of aluminium-based reagents¹⁶⁵ have been reported for this purpose, trimethylaluminium being the most popular.^{165a} Ammonia, primary, and secondary amines readily react with AlMe_3 to form the corresponding aluminium amide, with evolution of methane gas. In the presence of esters, aluminium amides react smoothly to afford amides.



Scheme 18 - Alternative total synthesis of Psammaplin A and analogues bearing electron rich aromatic groups

We were pleased to observe the formation of Psammaplin A when submitting ester **88** and cystamine (**53**) to these coupling conditions. However, the choice of solvent, reaction time and quantity of aluminium reagent were found to be crucial. Indeed, relatively apolar organic solvents, such as CH_2Cl_2 and CHCl_3 , led to no reaction or very low yields respectively. This was principally attributed to low substrate solubility. Solvent polarity was therefore screened and the use of a $\text{CH}_2\text{Cl}_2/\text{MeCN}$ mixture facilitated the formation of Psammaplin A (**28**) in

moderate yield, with partial recovery of the starting material. Since yields for the amidation of esters using AlMe_3 are known to be capricious, partially owing to the moisture sensitivity of the aluminium reagent, more than one equivalent of amine and AlMe_3 is generally used in order to obtain high conversion (usually 1.5 eq.). While the yield of the reaction could be increased with additional equivalents of AlMe_3 , the quantity of cystamine **53** had to remain constant (0.5 eq.) in order to avoid the formation of the monoamidated intermediate as the major product. While an improved yield would be potentially achievable using other coupling reagents, such transformation was not investigated further. For example, the DABAL- Me_3 reagent (Bis(trimethylaluminum)-1,4-diazabicyclo[2.2.2]octane) developed by Woodward^{165f} has been shown to be an improved reagent for the conversion of esters to amides. In particular, it overcomes a number of drawbacks generally encountered with AlMe_3 , such as moisture sensitivity, handling, lifetime and substrate scope.

With this new synthesis of Psammaphin A established, we decided to attempt the synthesis of new analogues, bearing strong electron donating aromatic substituents. Analogues **89** and **90** (Figure 27) were thought to be good candidates.

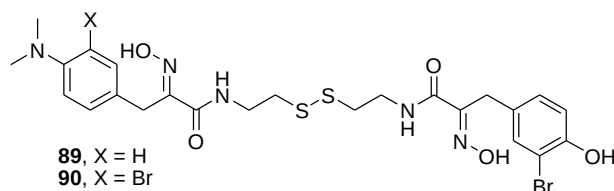
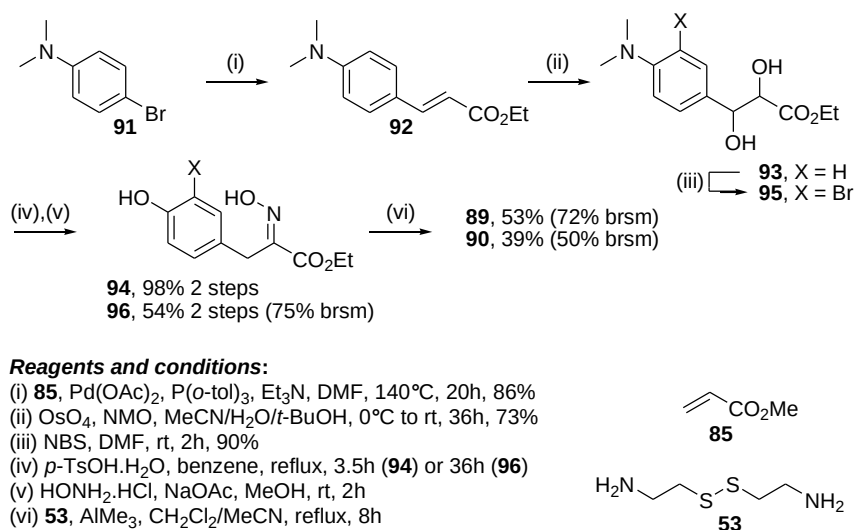


Figure 27

The synthesis is described in Scheme 19. Unsaturated ester **92** was prepared via a Heck reaction between 4-bromo-*N,N*-dimethylaniline **91** and methyl acrylate **85**. Dihydroxylation afforded diol **93** in good yield. We were pleased to observe quantitative formation of ester **94** in 2 steps. Notably, dehydration proceeded smoothly using only 0.05 eq. *p*-TsOH. Again, only one isomer could be observed by NMR for oxime **94**. Amidation as before afforded **89** in moderate yield. Bromination of diol **93** using NBS afforded brominated compound **95** in 90% yield. Under the same conditions, the dehydration/condensation sequence afforded hydroxyimino ester **96** in only 54% yield (75% brsm), even by using 0.2 eq. *p*-

TsOH. The presence of the bromine was believed to have an important influence on the reaction rate. Despite extensive effort, double amidation as before afforded final product **90** in only 39% yield.

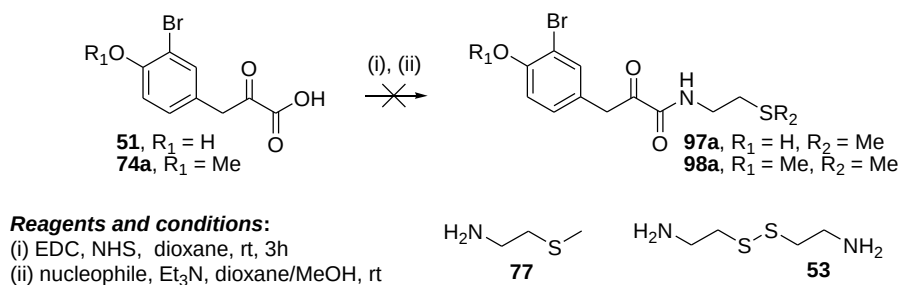


Scheme 19 - Synthesis of analogues **89** and **90** using our newly developed synthetic route

In summary, we have developed two new routes towards new Psammaplin A analogues bearing an oxime or an *O*-methyloxime functionality. The azlactone route (Schemes 14 and 15) was proven particularly efficient for the synthesis of analogues bearing electron poor aromatics, while the diol route (Schemes 18 and 19) was efficient for the synthesis of analogues bearing electron donating aromatic substituents. These routes employed aromatic aldehydes or aryl halides as starting material, which are generally commercially available, diverse and inexpensive.

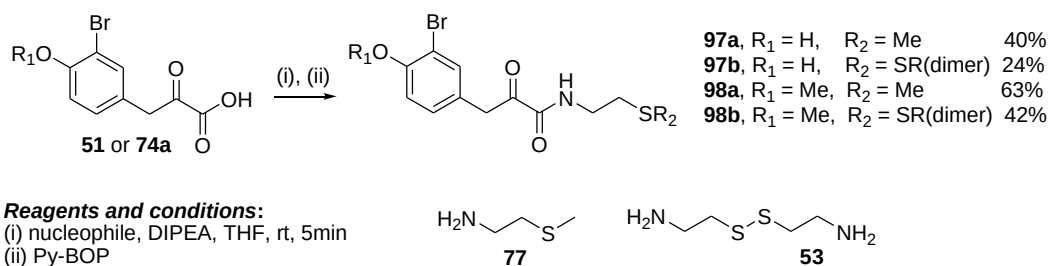
1.25. Synthesis of ketoamide derivatives

Disappointingly, amide coupling between arylpyruvic acids **51** and **74a**, and amine nucleophiles **53** and **77** was unsuccessful (Scheme 20) using EDC and NHS as described earlier (Scheme 15).



Scheme 20

The best results were obtained using Py-BOP as the coupling reagent, affording the title analogues **97a-b** and **98a-b** in low to moderate yields. The yields were particularly poor for the coupling of **51** with diamine **53** (Scheme 21).



Scheme 21 - Synthesis of ketoamide containing analogues using Py-BOP

In parallel, a number of other conditions was employed in order to improve the yield of this coupling. Acid **74a**, bearing a methylated phenol, and monoamine **77** were used as a model system for investigation. A set of representative attempted conditions are reported in Table 3. All conditions involving carbodiimides led to low and non reproducible yields or complete degradation of the starting material. Carbonyl diimidazole (CDI) or POCl_3 also led to complete degradation. Changing the order of addition of reagents did not lead to any improvement.

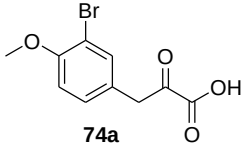
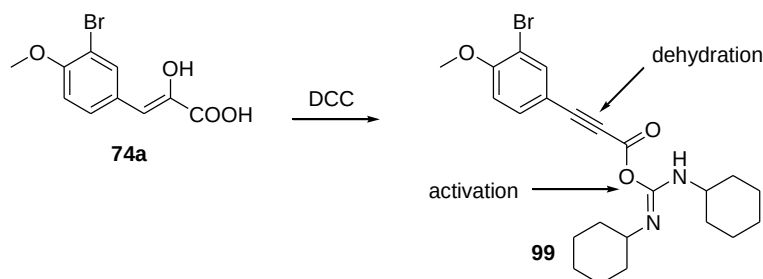
Substrate	Entry	conditions	product
 74a	1	i) EDC, HOBT dioxane, rt, 3h ii) 77 , Et ₃ N, rt	Degradation
	2	i) EDC, NHS dioxane, rt, 0.5h ii) 77 , Et ₃ N, rt	Degradation
	3	i) DCC, NHS, dioxane, rt, 1h ii) 77 , Et ₃ N, rt	Low and non reproducible yield
	4	i) DCC, NHS, DMF, rt, 1h ii) 77 , Et ₃ N, rt	Degradation
	5	DCC, DMAP, 77 , CH ₂ Cl ₂ , 0°C to rt	Low and non reproducible yield
	6	i) CDI, CH ₂ Cl ₂ , rt ii) 77	Degradation
	7	i) POCl ₃ , pyridine, -20°C ii) 77	degradation

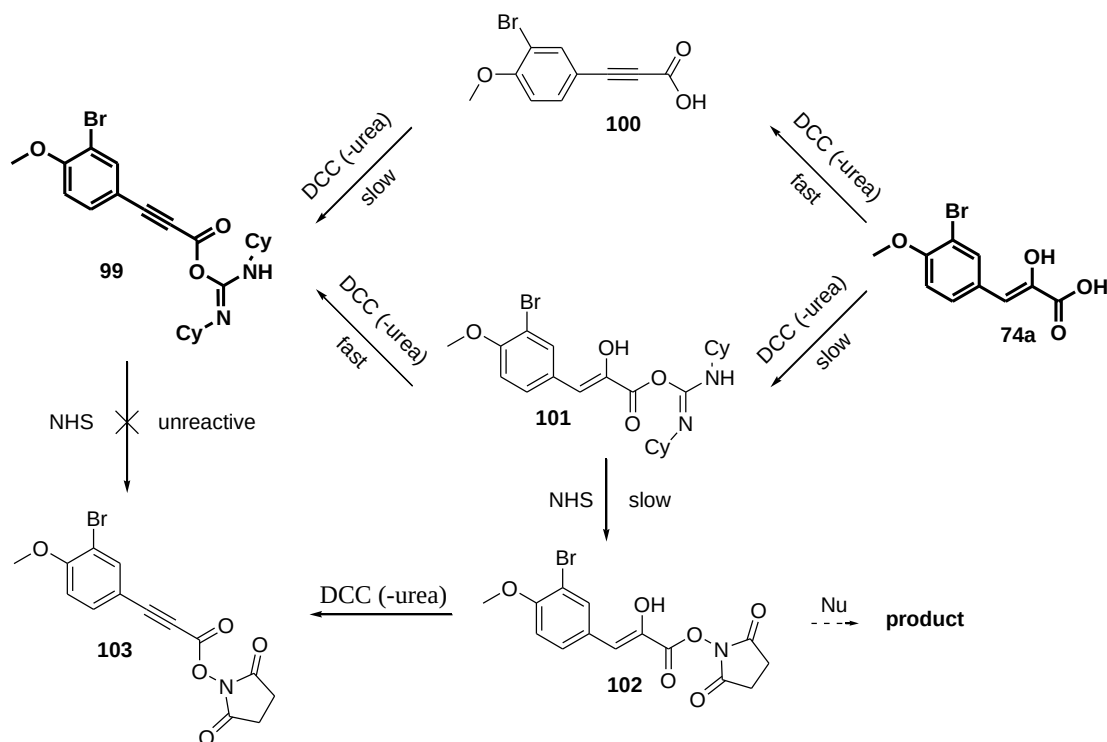
Table 3 - Attempted alternative synthesis of ketoamide containing analogues, conditions attempted for amide bond formation

The problematic nature of the coupling was thought to be attributable to the enol form of acid **74a**, which is the only species visible by NMR (CD₃OD). This can be exemplified by conditions 3 and 4. In these conditions, compound **99** (Scheme 22) was obtained as the major product. Dehydration by DCC, followed by activation of the acid is thought to be responsible for the formation of **99**.



Scheme 22 - Main product of the reaction of acid **74a** with DCC

A mechanism for the formation of **99** is shown in Scheme 23. In this mechanism, dehydration is thought to occur faster than the activation of the carboxylic acid. Varying the temperature (-20°C to rt) did not have any significant influence on the outcome of the reaction.

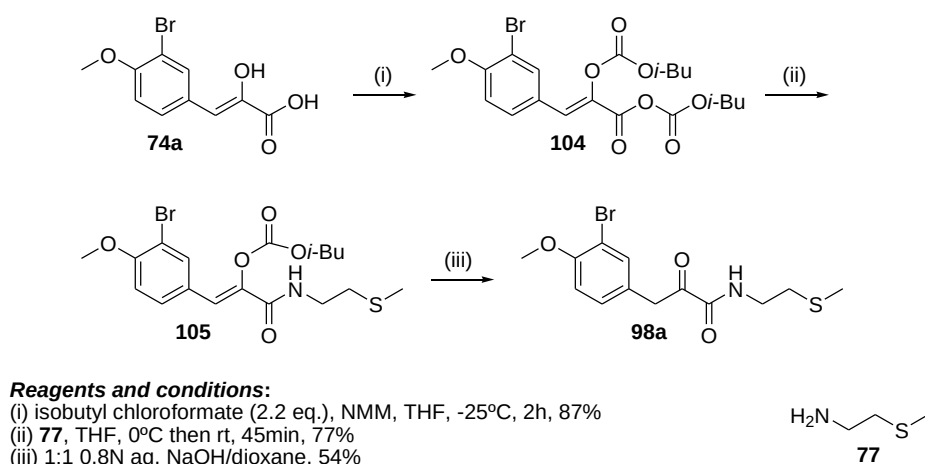


Scheme 23 - Proposed mechanism for the formation of **99**

The low yields obtained for the formation of **97a-b** and **98a-b** lead us to investigate new routes towards these compounds:

The chloroformate route: The increased reactivity of the enol functionality of **74a** led us to consider new synthetic routes, involving protection of the enol. The first way we envisaged involved the use of a chloroformate reagent, allowing protection of the enol as a carbonate and subsequent activation of the carboxylic acid as a mixed anhydride (Scheme 24). The reaction proceeded smoothly upon exposure of **74a** to 2.1 equivalents of isobutyl chloromormate at low temperature, affording mixed anhydride **104** in high yield. Interestingly, **104** was stable enough to be purified by flash column chromatography and characterized by ^1H NMR, ^{13}C NMR and mass spectrometry. Using only 1 equivalent of isobutyl

chloroformate did not allow selective formation of the mixed anhydride. Treatment of **104** with amine **77** afforded amide **105** in good yield. Amide **105** could also be obtained in one step (75%) from acid **74a** without intermediate purification of the mixed anhydride, however necessitating a larger quantity of the expensive nucleophile **77** to drive the reaction to completion. Despite extensive efforts, carbonate deprotection could only be achieved in moderate yield under basic conditions. A representative set of conditions can be found in Table 4.



Scheme 24

Conditions	product
BF ₃ ·OEt ₂ , 4Å MS, CH ₂ Cl ₂ , -10°C to rt ¹⁶⁶	No reaction
1% K ₂ CO ₃ , 25:75 MeOH/H ₂ O, rt	Degradation
LiOH, aq. H ₂ O ₂ , THF, rt	Degradation
8:2 AcOH/H ₂ O, reflux	oxazolidine-2,4-dione 106 , quantitative
1:1 0.8N aq. NaOH/dioxane	54%

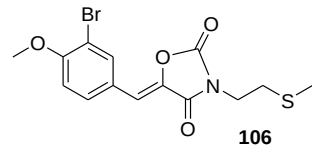
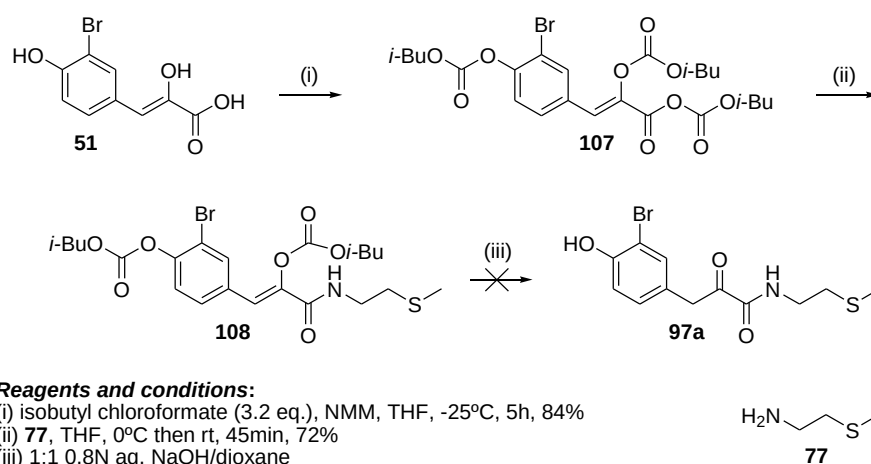


Table 4 - conditions attempted for the carbonate deprotection

This sequence afforded **98a** in 36% overall yield, therefore not representing a particular improvement to the one step Py-BOP route (Scheme 21). The applicability of this route was also found to be relatively limited when

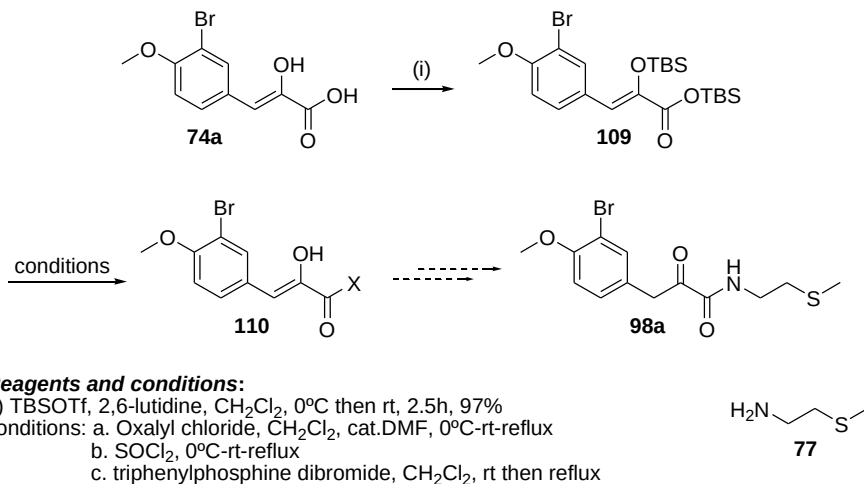
applied to other substrates (Scheme 25). Indeed, attempted double carbonate deprotection of **108** under the same conditions led to a complex mixture. Only traces of the product **97a** could be observed by ^1H NMR and mass spectrometry. The use of milder conditions such as DIPEA in MeOH, aqueous NH_3 in THF, 7M NH_3 in MeOH, did not give rise to any improvement, usually affording complex mixtures or recovery of the starting material. This route was therefore judged unsuitable and not investigated further.



Scheme 25

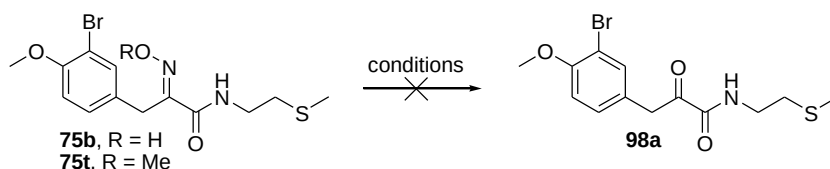
The silyl ester route: It is well known that silyl esters can be converted to the corresponding acyl halides upon exposure to halogenating agents. Double TBS protection of acid **74a** afforded diprotected compound **109** in nearly quantitative yield after purification by column chromatography (Scheme 26). Exposure to oxalyl chloride¹⁶⁷ in CH_2Cl_2 or CHCl_3 only allowed poor conversion (TLC) to the corresponding acyl chloride, even after extended reaction time (up to 1 day) and increased temperature (reflux). The use of thionyl chloride led to a slightly higher conversion, but a significant amount of starting material was still observed. Triphenylphosphine tribromide, known to be an excellent reagent for the conversion of bulky silyl esters to the corresponding acyl bromides,¹⁶⁸ did not lead to significant improvement. In each case, addition of the nucleophile before complete conversion to the acyl halide afforded complex mixtures with no trace (NMR, MS) of the product **98a**. Based on these unconvincing results, this route was not investigated further. The reason for this lack of reactivity is unclear, but

can possibly be attributed to the presence of the second TBS group, locally increasing steric hindrance and therefore slowing conversion. Although less robust, the use of the smaller TMS instead of TBS was not investigated but represents a possible alternative.



Scheme 26

Hydrolysis of oximes: In the final alternative synthetic route explored, it was envisaged that we could obtain ketoamide compounds **97a-b** and **98a-b** by deprotection of their *O*-methyloxime or oxime containing analogues. **75b** and **75t** were chosen as a model system for investigation. Surprisingly, few examples in the literature report the deprotection of the *O*-methyloxime functionality, owing to its high stability.^{169,170} Indeed all reductive conditions employed, inspired by literature examples,^{171,172} led to no reaction (Table 5, entries 1-3). Strong aqueous acids did not lead to a significant improvement (entry 4). After several unsuccessful attempts, following literature procedures involving either reductive¹⁷³ (entry 5) or oxidative conditions with the Dess-Martin periodinane (entry 6),¹⁷⁴ encouraging results were obtained for the hydrolysis of oxime **75b**, using 1.2 M HCl in an acetonitrile-water mixture. These conditions afforded **98a** in 34% yield. Despite intensive effort, this yield could not be improved. Moreover, when the same conditions were applied to Psammaphin A (**28**), mostly degradation and only traces of the doubly deprotected product were observed (HRMS). Once again, this synthetic route did not present any particular advantage compared to the Py-BOP route in term of yields, and was therefore abandoned.

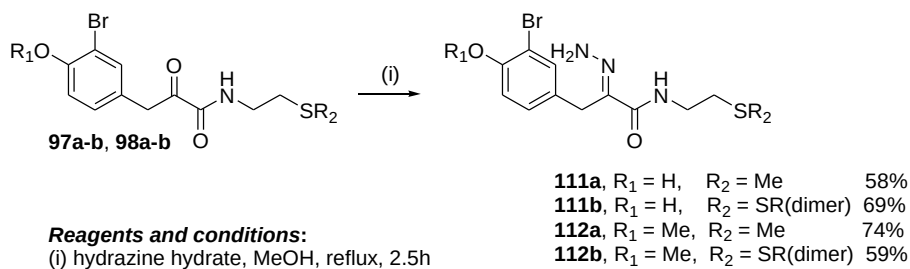


Entry	Substrate	R	conditions	product
1	75t	Me	Zn, AcOH, rt, 1h, 24h, 48h	Starting material
2			Zn, AcOH, 60°C, 24h	Starting material
3			Amberlyst 15, paraformaldehyde, acetone/H ₂ O, rt to 80°C, 12h	Starting material
4			1M HCl, MeCN/H ₂ O, acetaldehyde, 50°C, 12h	Starting material
5	75b	H	TiCl ₄ , NaI, MeCN, rt, 0.25h	Starting material
6			Dess-Martin periodinane, CH ₂ Cl ₂ , rt, 0.75h	degradation
7			1.2M HCl, MeCN/H ₂ O, acetaldehyde, 50°C, 12h	34%

Table 5 - Conditions attempted for the hydrolysis of oximes

1.26. Synthesis of hydrazonoamide derivatives

The synthesis of the title analogues was achieved as described in Scheme 27. Reflux of the keto derivatives **97a-b**, **98a-b** with hydrazine monohydrate in MeOH afforded the corresponding hydrazones in moderate to high yield after purification by flash column chromatography. The formation of only one product could be observed in each case by ¹H NMR of the crude mixtures.



Scheme 27

The geometry of the hydrazone could be assigned as (*E*) based on NOE studies performed with analogue **111b** (Figure 28). Indeed, correlation could be observed between the hydrazone hydrogen (NNH₂) and **H^A** (weak), **H^B** (weak) and **H^C** (benzylic H, strong). However, no correlation could be observed between the hydrazone protons (NNH₂) and **H^D** (NH amide), **H^E** (N-CH₂-) and **H^F** (-CH₂-S).

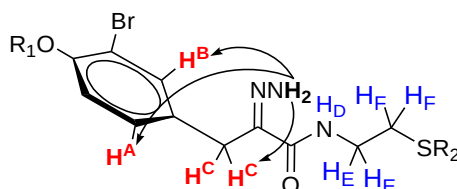


Figure 28 - Correlation between the hydrazone hydrogens and **H^A**, **H^B**, **H^C** (**111b**)

The (*E*) geometry was finally confirmed by Dr. Andrew J. P. White (service of crystallography, Imperial College London) with the X-ray crystal structure of analogue **111a** (Figure 29). Of interest, the geometry adopted by the molecule in the solid state suggests the presence of an intramolecular H-bond between the hydrazone and the amide hydrogen.

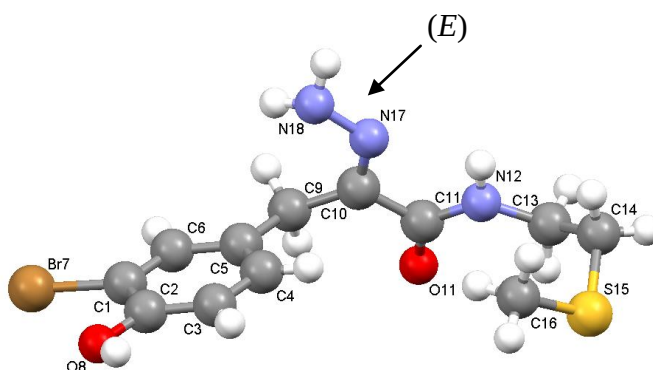
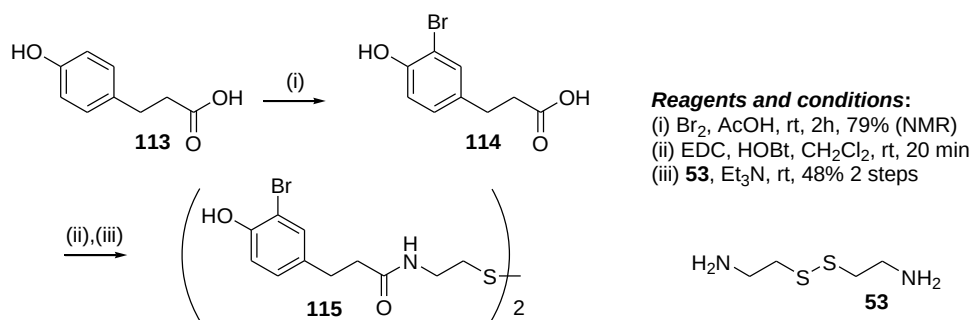


Figure 29 - Crystal structure of **111a**

1.27. Synthesis of the a Psammaplin A analogue lacking the oxime units

In order to further study the importance of the oxime unit of Psammaplin A for potency against HDACs and DNMTs, an analogue of Psammaplin A completely lacking the oxime unit was prepared (Scheme 28) for biological

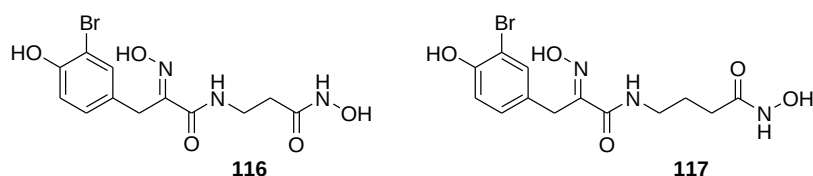
evaluation. Bromination of 3-(4-hydroxyphenyl)propanoic acid **113** afforded **114**, and subsequent coupling with cystamine **53** using EDC and HOBT as coupling reagents afforded simple analogue **115**. The latter has been recently reported by de Lera.^{161a} The bromination step afforded a 1:0.3 mixture of monobrominated compound **114** and its dibrominated analogue. The reported yield was calculated from the ratios observed by ¹H NMR. Flash column chromatography afforded pure samples of **114** to be used for the final coupling step. The relatively low yield for the coupling step was principally due to the presence of the free phenolic functionality, reacting as a competitive nucleophile. Reaction conditions for both bromination and coupling were not optimised, suggesting higher yields are potentially achievable. In particular, coupling *via* a more robust activated ester, such as a succinic ester, could potentially avoid side reaction with the phenol functionality.



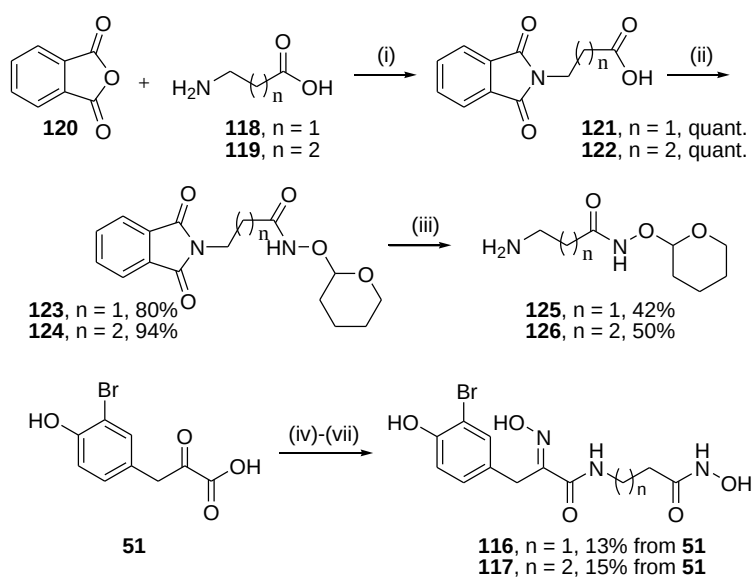
Scheme 28

1.28. Synthesis of hydroxamic acid analogues of Psammaplin A

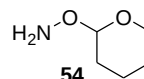
Since in terms of HDAC inhibition it is likely that the sulfur end group of Psammaplin A functions as a zinc binding group, we also decided to prepare a hydroxamic acid analogue of Psammaplin A **116** and its one carbon homologue **117** (Figure 30). Hydroxamic acids are well known to be strong zinc-binding groups for HDACs, and therefore we envisaged **116** and **117** would offer useful additional information regarding the Psammaplin A HDAC pharmacophore.

**Figure 30**

The synthesis of these two analogues is described in Scheme 29. Heating 3-aminopropanoic acid **118** or 4-aminobutanoic acid **119** in the presence of phthalic anhydride **120** under solvent free conditions afforded the phthalimide protected intermediates **121** and **122** in nearly quantitative yield.¹⁷⁵ Further EDC mediated coupling with *O*-THP hydroxylamine **54** afforded intermediates **123** and **124** in high yield. Classical phthalimide deprotection then allowed access to primary amines **125** and **126** in moderate yield after purification by flash column chromatography. Analogues **116** and **117** were obtained in moderate yield after condensation of **51** with *O*-THP hydroxylamine **54**, further amide coupling with **125** or **126** and final THP double deprotection under acidic conditions.

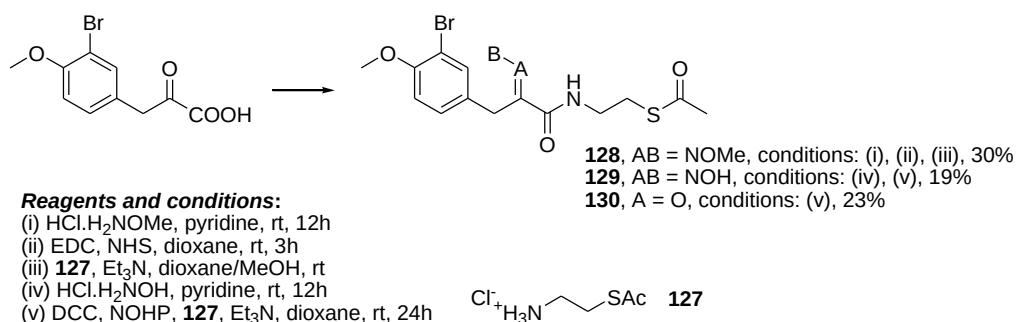
**Reagents and conditions:**

- (i) 170-180°C, neat, 2h
- (ii) **54**, EDC, DMAP, CHCl₃, rt, 12h
- (iii) hydrazine monohydrate, MeOH, reflux, 3h
- (iv) **54**, pyridine, rt, 12h
- (v) EDC, NHS, dioxane, 3h
- (vi) **125** or **126**, Et₃N, dioxane/MeOH, rt, 12h
- (vii) 0.5N HCl, 9:1 dioxane/*i*-PrOH, rt, 20h

**Scheme 29**

1.29. Synthesis of acetate-protected thiol analogues of Psammaplin A

In line with our design for the first library, a number of thioacetate containing analogues were prepared (Scheme 30). We envisaged that these compounds may generate the corresponding thiols *in situ* in cell-based assays following cleavage of the acetyl group *in vivo* by non-selective esterases. Moreover, protection as a thioester is likely to improve cell permeability during cell based assays. Condensation between acid **74a** and *O*-methylhydroxylamine, and subsequent EDC coupling with *S*-2-aminoethyl ethanethioate **127**¹⁷⁶ afforded thioacetate analogue **128**. Condensation of acid **74a** with hydroxylamine, followed by coupling with **127** using DCC and *N*-hydroxyphthalimide¹⁵³ as coupling reagents afforded thioacetate **129** in poor yield. The latter conditions were applied to **98a** and afforded thioacetate analogue **130**, in relatively low yield.



Scheme 30

1.3. Conclusions

In this section are reported the new synthetic routes we developed towards libraries of Psammaplin A analogues. In particular, two new routes were developed, allowing access to Psammaplin A analogues inaccessible *via* currently reported routes, notably bearing electron-rich aromatics. Both routes allow expedient access to the target analogues and employ commercially available, diverse and inexpensive starting materials.

2. Biological evaluation

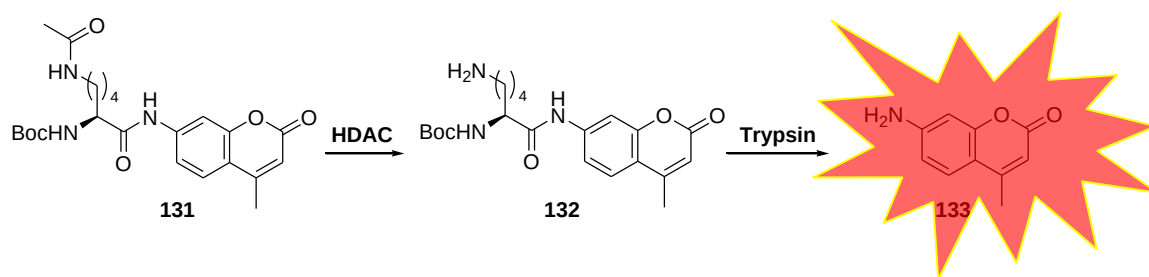
2.1. Introduction

Our libraries of analogues were then assayed against HDACs and DNMT1 in order to establish the SARs of Psammaplin A against these enzymes. In addition, cytotoxicity data for selected analogues were obtained against A549 (human lung carcinoma), MCF7 (human breast carcinoma) and WI38 (human lung fibroblast) cell lines. Finally, cellular studies were performed in order to propose a mode of action for Psammaplin A and analogues in cells. All HDAC assays were performed in collaboration with Pr. Franz-Josef Meyer-Almes' group in Darmstadt (Germany). DNMT1 assays were performed in collaboration with Dr. Fabrice Turlais' group at Cancer Research Technology in London. Finally, cellular studies were performed in collaboration with Pr. Stephen Neidle's group at School of Pharmacy in London.

2.2. Results and discussion

2.2.1. HDAC inhibition

HDAC assays were performed as described by Wegener et al.¹⁷⁷ with minor modifications. Briefly, The fluorogenic HDAC assay consists of two steps: In the first step the Boc-Lys(Ac)-AMC substrate is deacetylated by the corresponding HDAC. The deacetylated substrate, therefore containing a basic amino acid residue serves as substrate for trypsin in the subsequent second step. The second step generates the signal due to the release of fluorescent AMC, whose fluorescence serves as an indirect measure of HDAC enzyme activity (Scheme 31).



Scheme 31 - HDAC assay principle. Fluorescence of the AMC is used as a measurement of HDAC activity

Our synthetic analogues were assayed against recombinant human HDAC1 (rHDAC1, class I), recombinant human HDAC6 (rHDAC6, class II), and purified bacterial histone deacetylase like amidohydrolase (HDAH)^{178,179} which exhibits 35% sequence identity with the second domain of the human HDAC6. The results are shown in Table 6.

As previously mentioned, Psammaplin A has been reported by Crews to be a potent inhibitor of HDAC activity (4.2 nM on a cell extract). Indeed, in our hands, Psammaplin A was found to be a potent HDAC1 inhibitor (**28**, 45 nM), however less potent than previously reported albeit using a recombinant enzyme rather than cell extract.^{135g} Reference compound SAHA (**1**) displayed an IC_{50} of 30 nM, correlating nicely with previously reported values (27 nM).¹⁸⁰ Importantly, Psammaplin A was found 96 fold more potent against HDAC1 (45 nM) compared to the class II HDAC6 (4.3 μ M). There is a pressing need for isoform selective HDAC inhibitors to further validate these important targets, with most of the known inhibitors being non- or class-selective. Previously, psammaplin A was defined as a Class I selective inhibitor by Kwon and co-workers, through its selective effects of histone acetylation (versus tubulin acetylation) in HeLa cells. To the best of our knowledge, our data provides the first isoform selectivity profile of psammaplin A and analogues in cell-free assays. In Table 6, $IC_{50}^{6/1}$ is defined by the ratio $IC_{50}^{HDAC6}/IC_{50}^{HDAC1}$ and was used as an indicator of isoform selectivity. The SAR around our library can be described as follows:

Nature of the sulfur end group. Since previous studies have postulated psammaplin A to be a natural prodrug, whereby the disulfide functionality is reduced in cells to give a free thiol that functions as a zinc binding group,^{115,161a} we felt it was important to assay the corresponding thiol directly. Indeed, direct assay of reduced psammaplin A analogues in cell-free assays have not been previously reported. Thus all monomeric compounds ($R_2 = \text{SH}$) were prepared immediately prior to assay from the corresponding disulfide analogues ($R_2 = \text{SR}$ dimer), using tris(2-carboxyethyl)phosphine hydrochloride (TCEP) as the reducing agent, in a 9:1 DMSO/water solution (see experimental section). Importantly, control experiments have shown TCEP to have no influence in our assay conditions at concentrations up to 25 μM . When the sulfur end group was present as a free thiol ($R_2 = \text{SH}$, **28'**, **75c'**, **75s'**, **75u'**, **97b'**, **98b'**, **111b'**, **112b'**, **115'**), highly potent compounds were observed in each case, efficiently inhibiting HDAC1 in the nanomolar range, while still showing important isoform selectivity. The impressive selectivity of these compounds was found to be particularly accentuated in the case of oxime ($\text{AB} = \text{NOH}$) and hydrazone ($\text{AB} = \text{NNH}_2$) containing compounds **28'/75c'** and **97b'/98b'** respectively, which were found to be highly selective for HDAC1 over HDAC6. The reduced natural product **28'** was found to be the most potent HDAC1 inhibitor of this series (IC_{50} of 0.9 nM), 50 fold more potent than its dimeric parent Psammaplin A (**28**, 45 nM). Overall the disulfide analogues **28**, **75c**, **75s**, **75u**, **97b**, **98b**, **111b**, **112b**, and **115** were found to be less potent than their reduced counterparts in every case. "Protection" of the free thiol as either a thioether or a thioacetate had vastly different effects on potency. Analogues containing a thioether end group ($R_2 = \text{SMe}$, **75a**, **75b**, **75r**, **75t**, **97a**, **98a**, **111a**, **112a**) displayed low to no inhibition of both HDAC1 and HDAC6. In contrast, potencies of acetate protected analogues ($R_2 = \text{SAc}$) **128-130** were approaching those of their corresponding thiols **75u'**, **75c'** and **98b'** respectively. Notably, **129** was found to be highly potent against HDAC1, displaying an IC_{50} of 5 nM. With the exception of **130**, acetyl protected compounds were found 2.8 (**129**) to 8 times (**128**) less potent than the corresponding free thiol analogues against HDAC1. Curiously, acetyl protected compounds **128-130** were found to be moderately active to completely inactive

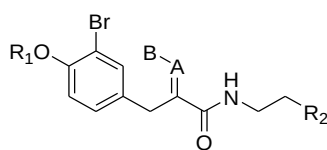
against HDAC6 in each case, highlighting the important isoform selectivity of these compounds. Very recently, de Lera and coworkers reported compound **134**, where the disulfide functionality of Psammaplin A has been replaced by two methylene groups.^{161a} In their assay, **134** did not have any significant influence against HDAC activity. Finally, hydroxamic analogue **116** was found to be highly potent against HDAC1 (2 nM), and HDAC6 (190 nM) with a similar potency to the thiol **28'** derived from Psammaplin A. Hydroxamic acid **117** was found to be less potent than **116**, demonstrating the importance of the alkyl chain length for high potency.

Requirement of the oxime functionality (A-B). Among the different AB systems tested (Table 6), the hydrazone compounds **111b'** (5 nM) and **112b'** (6 nM) were found to retain high potency against HDAC1, with values approaching those of their respective oxime analogues **28'** (0.9 nM) and **75c'** (1.8 nM). Compounds **75s'** (27 nM) and **75u'** (15 nM), bearing an *O*-methyloxime moiety (AB = NOME), were found to be 8-30 fold less active against HDAC1 compared to their respective oxime containing analogues **28'** (0.9 nM) and **75c'** (1.8 nM), suggesting the requirement of a free oxime for potent HDAC1 inhibition. Indeed, ketone analogues (AB = O) **97b'** (0.40 μ M) and **98b'** (1.1 μ M) were found to be 448 and 622 times less potent against HDAC1 respectively than their corresponding oxime containing analogues **28'** (0.9 nM) and **75c'** (1.8 nM). Moreover, **97b'** and **98b'** were found to be in the same range of potency than **115'** (0.42 μ M), for which the A-B functionality had been removed completely.

Interestingly, variation of the AB system had little effect on the potency against HDAC6, despite its impact on potency against HDAC1. This possibly suggests a design criterion for isoform selectivity. Indeed, while analogue **115'** (AB = H₂) and the α -ketoamide containing compounds (AB = O, **97b'** and **98b'**) did not significantly discriminate between HDAC1 and HDAC6, introduction of the *O*-methyloxime functionality induced a 15-75 increase in potency against HDAC1, while maintaining a similar potency against HDAC6. This effect is even more accentuated in the case of oxime containing analogues **28'** and **75c'** (AB = NOH) and hydrazone analogues **111b'** and **112b'** (AB = NNH₂), which were 448-622 and 81-187 fold more potent respectively than **97b'**, **98b'** and **115'** against HDAC1, while being similarly potent against HDAC6.

Phenol methylation status. Compounds bearing a methylated phenol ($R_1 = \text{Me}$, Table 6) led to similar potencies against HDAC1 compared to the corresponding free phenol ($R_1 = \text{H}$) in each case. Methylation of **28'** and **75s'** led to compounds **75c'** and **75u'** which were 9 and 12 times less active respectively against HDAC6, whereas this pattern was found reversed for **97b'** and **111b'** which were found to be 4 and 3 times less potent respectively than their corresponding methylated analogues.

While these data are limited to HDAC1 and HDAC6, several representative compounds were also assayed against HDAH.^{178,179} Surprisingly, all our synthetic analogues were found completely inactive under the assay conditions, even at concentrations of up to 100 μM . The exception to this rule were the hydroxamic analogues **116** and **117** however. For example, compound **116** was found to be significantly potent against HDAH (310 nM).

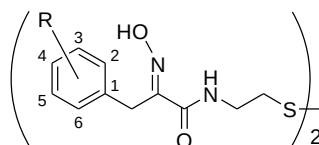


Cpd	R ₁	AB	R ₂ ^a	rHDAC1 IC ₅₀ [μM]	rHDAC6 IC ₅₀ [μM]	IC ₅₀ ^{6/1}
75a	H	NOH	SMe	7.7	> 50	
75b	Me	NOH	SMe	23	> 50	
28	H	NOH	S ₂ R (dimer)	0.045	2.8	62
75c	Me	NOH	S ₂ R (dimer)	0.043	2.0	46
28'	H	NOH	SH	0.0009	0.36	360
75c'	Me	NOH	SH	0.0018	3.2	1772
75r	H	NOMe	SMe	11	> 50	
75t	Me	NOMe	SMe	> 50	> 50	
75s	H	NOMe	S ₂ R (dimer)	0.38	9.5	25
75u	Me	NOMe	S ₂ R (dimer)	0.22	> 50	> 230
75s'	H	NOMe	SH	0.027	0.22	8
75u'	Me	NOMe	SH	0.015	2.7	180
97a	H	O	SMe	7.3	ca 30	
98a	Me	O	SMe	> 50	> 50	
97b	H	O	S ₂ R (dimer)	5	9	1.8
98b	Me	O	S ₂ R (dimer)	9	8	0.9
97b'	H	O	SH	0.40	1.0	2.6
98b'	Me	O	SH	1.1	0.25	0.22
111a	H	NNH ₂	SMe	ca 50	> 50	
112a	Me	NNH ₂	SMe	13	> 50	
111b	H	NNH ₂	S ₂ R (dimer)	6.1	5	0.8
112b	Me	NNH ₂	S ₂ R (dimer)	3	>50	> 16
111b'	H	NNH ₂	SH	0.005	1.0	204
112b'	Me	NNH ₂	SH	0.006	0.32	53
115	H	H ₂	S ₂ R (dimer)	> 50	20	< 0.4
115'	H	H ₂	SH	0.42	1.1	2.6
116	H	NOH	C(O)NHOH	0.002	0.19	95
117	H	NOH	CH ₂ C(O)NHOH	0.31	1.7	5
129	Me	NOH	SAc	0.005	23	4560
128	Me	NOMe	SAc	0.12	>50	> 417
130	Me	O	SAc	0.48	9.5	> 20
SAHA				0.030	0.21	7
TCEP				> 25	> 25	

Table 6 - HDAC inhibition data. IC₅₀s for Psammaplin A and synthetic analogues against HDAC1 and HDAC6. ^aThiols were prepared by *in situ* reduction of the respective disulfides using tris(2-carboxyethyl)phosphine hydrochloride, and used immediately. Details about the procedure used for reduction can be found in the Experimental section

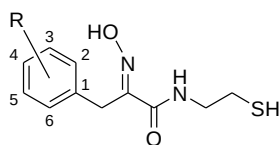
We then further explored the effects of aromatic substitution patterns on HDAC potency and selectivity. Data for disulfides and thiols are given in Table 7 and Table 8 respectively. Once again derivatives containing a free thiol functionality (Table 8) were found to be more potent than their dimeric counterparts. The unsubstituted compound **75d'** was found to be the least active against both HDAC1 and HDAC6. Despite this, compound **75d'** (IC₅₀ of 12 nM) was still more potent than the reference compound SAHA (30 nM, lit. 27 nM).¹⁸⁰ Importantly, several compounds exhibited subnanomolar IC₅₀ values against HDAC1: **75q'** (0.6 nM), **75j'** (0.48 nM) and the extremely potent compound **75p'** (0.2 nM). To the best of our knowledge, compound **75p'** represents the most potent HDAC1 inhibitor not containing a cyclic tetrapeptide core. This compound also exhibited the highest selectivity with an IC₅₀^{6/1} value of 3950. Analogue **75p'** was found to be 60 times more active than unsubstituted analogue **75d'** and demonstrates that aromatic substitution plays a role for potency against HDAC1, however its influence on potency is minor compared to the presence of the free thiol, or the free oxime.

Several important trends emerged from the HDAC6 assays. Compounds **75k'** (0.27 μM), **75h'** (0.24 μM) and **75j'** (0.19 μM) were found to be more potent than the reduced form of Psammaplin A (**28'**, 0.36 μM). The most potent compounds were found to involve a halogen in the *meta* position. This can be clearly seen by comparing the IC₅₀ values (μM) for **75f'** (2.9) vs **75i'** (0.64), **75m'** (2.7) vs **75l'** (0.51) and **75k'** (0.27), **89'** (2.3) vs **90'** (0.70), **75e'** (1.7) vs **28'** (0.36), **75g'** (1.3) vs **75j'** (0.19) and **75n'** (1.2) vs **75o'** (0.74). In each case the presence of a halogen was found to enhance potency against HDAC6. Moreover, iodinated compounds **75k'** (0.27) and **75h'** (0.24) were found to be more potent than their respective brominated analogues **75l'** (0.51) and **75c'** (3.2). Comparison of the IC₅₀ values (μM) for **75c'** (3.2), **90'** (0.70), **75i'** (0.64), **28'** (0.36), and **75j'** (0.19) allowed a preliminary ranking of functional groups in the *para* position: OMe < NMe₂ < F < OH < SMe. Indeed, comparison of IC₅₀s (μM) for the corresponding *para* monosubstituted compounds **75f'** (2.9), **89'** (2.3), **75e'** (1.7) and **75g'** (1.3) confirms this trend, indicating that the hydroxyl and thiomethylether substituents are most favourable in the *para* position for potency against HDAC6.



cpd	R	rHDAC1	rHDAC6	IC ₅₀ ^{6/1}
		IC ₅₀ [μM]	IC ₅₀ [μM]	
28	3-Br-4-OH	0.045	2.8	62
75c	3-Br-4-OMe	0.043	2.0	46
75d	/	6.0	35	6
75e	4-OH	0.18	3.7	21
75f	4-F	0.42	> 50	> 118
75g	4-SMe	0.20	20	99
75h	3-I-4-OMe	0.0050	3.5	704
75i	3-Br-4-F	0.051	15	298
75j	3-Br-4-SMe	0.34	> 50	> 146
75k	3-I-4,5-OMe	0.040	3.9	96
75l	3-Br-4,5-OMe	0.053	10	194
75m	3,4-OMe	3.9	13	3
75n	3-NO ₂	ca 0.50	7.8	16
75o	3-Br-5-NO ₂	0.12	> 50	> 420
75p	3,5-Br-4-OH	nd	5.0	nd
75q	3-OMe-4-SMe	1.7	> 50	> 30
89	4-NMe ₂	3.6	> 50	> 14
90	3-Br-4-NMe ₂	0.18	ca 50	278

Table 7 - HDAC inhibition data, variation of aromatic substitution. IC₅₀s for synthetic Psammaplin A analogues (dimers) against HDAC1 and HDAC6



cpd	R ₁	rHDAC1 IC ₅₀ [μM]	rHDAC6 IC ₅₀ [μM]	IC ₅₀ ^{6/1}
28'	3-Br-4-OH	0.0010	0.36	360
75c'	3-Br-4-OMe	0.0018	3.2	1772
75d'	/	0.012	3.3	271
75e'	4-OH	0.0040	1.7	418
75f'	4-F	0.0070	2.9	420
75g'	4-SMe	0.0020	1.3	630
75h'	3-I-4-OMe	0.0010	0.24	240
75i'	3-Br-4-F	0.0010	0.64	640
75j'	3-Br-4-SMe	0.00048	0.19	388
75k'	3-I-4,5-OMe	0.0010	0.27	270
75l'	3-Br-4,5-OMe	0.0010	0.51	510
75m'	3,4-OMe	0.0030	2.7	900
75n'	3-NO ₂	0.0010	1.2	1210
75o'	3-Br-5-NO ₂	0.0050	0.74	148
75p'	3,5-Br-4-OH	0.00020	0.79	3950
75q'	3-OMe-4-SMe	0.00060	1.4	2372
89'	4-NMe ₂	0.0010	2.3	2262
90'	3-Br-4-NMe ₂	0.0040	0.70	164

Table 8 - HDAC inhibition data, variation of aromatic substitution. IC₅₀s for synthetic Psammaplin A analogues (thiols) against HDAC1 and HDAC6. Thiols were prepared by *in situ* reduction of the respective disulfides using Tris(2-carboxyethyl)phosphine hydrochloride, and used immediately. Details of the procedure used for reduction can be found in the Experimental section

In light of the exciting isoform selectivity of some of our library compounds, we decided to further investigate this effect. The reduced form of Psammaplin A (**28'**) and its hydrazone containing analogue **111b'** were chosen as representative compounds. **28'** and **111b'** were assayed against rHDAC1 and rHDAC8 as Class I representatives, rHDAC6 as representative of class IIb and rHDAC7 as representative of class IIa. The results are shown in Table 9. Both compounds displayed similar selectivity profiles. As previously mentioned, **28'** and **111b'** were found extremely potent against HDAC1. Interestingly, **28'** and

111b' displayed important isoform selectivity within the Class I HDACs. Indeed, they were 1340 and 1663 times less potent against the other representative member of class I, HDAC8. While having previously been shown to be less potent against the Class IIb HDAC6, **28'** and **111b'** were also found to have only weak activity against HDAC7.

cpd ^a	AB	rHDAC1	rHDAC6	rHDAC7	rHDAC8
		IC ₅₀ [μM]	IC ₅₀ [μM]	IC ₅₀ [μM]	IC ₅₀ [μM]
28'	NOH	0.0010	0.36	17	1.3
111b	NNH ₂	0.0050	1.0	45	13

Table 9 - Isoform selectivity profile of **28'** and **111b'**. ^aThiols were prepared by *in situ* reduction of the respective disulfides using Tris(2-carboxyethyl)phosphine hydrochloride, and used immediately. Details about the procedure used for reduction can be found in the Experimental section

Docking and Molecular Dynamics simulations for HDAC inhibition. Since our data were consistent with the postulate that psammaplin A is a natural prodrug, with the free thiol functioning as a zinc binding group, we initiated computational studies to study its interactions with HDAC1. Recently, de Lera and co-workers performed preliminary computational studies on the binding of Psammaplin A to HDACs, using the crystal structure of HDAC8.^{161a} It would seem that in light of our isoform selectivity data (Table 9) that HDAC8 is not a particularly good model to study the binding of Psammaplin A. Recently, the X-Ray structure of HDAC2 in complex with a small ligand was solved⁷⁵ (PDB-entry 3MAX), and since HDAC1 and HDAC2 are 93.7% identical in a 318 amino acid overlap, homology modelling of HDAC1 based on the 3D-structure of HDAC2 should provide a realistic structural model of HDAC1. The homology model of HDAC1 was calculated by Pr. Franz-Josef Meyer-Almes using SWISS-MODEL.¹⁸¹ A set of 120 conformers of deprotonated **28'** was generated using MarvinSketch 5.1.5 (ChemAxon Ltd.). Atomic charges were applied using ANTECHAMBER.¹⁸² The conformers were extensively docked into the active site pocket of HDAC1 using DOCK6.4 with a maximum of 5000 orientations and 10000 simplex iterations per conformer.¹⁸³ The ten conformers with best scores

were refined in a subsequent docking using AMBER score.¹⁸⁴ The best AMBER docking pose was used as a starting point for subsequent molecular dynamics calculations using GROMACS¹⁸⁵ and AMBER99¹⁸⁶ as an implemented forcefield. After *in vacuo* minimization using steepest descent and conjugate gradient algorithms, the complex consisting of HDAC1 and **28'** was solvated in a box containing 15526 TIP4 water molecules and 250 mM NaCl. A position-restrained dynamics simulation was run to equilibrate the water around the protein and to position water molecules and ligand within the active site of the enzyme. Then the temperature of the system was raised stepwise from 100K to 300K. In the last step at 300K a period of 2 ns was simulated.

The trajectory traces were analyzed using VMD 1.8.7 (University of Illinois). The psammaplin A monomer **28'** was found to form three hydrogen brigdes to Y303 (64%), D99 (77%) and the protonated H141 (26%) (see Figure 31). The percentages in brackets denote the fractional period of simulation where the hydrogen bonds are formed. In addition, there appeared to be a structural water molecule with extraordinary restrained position during the simulation. This water molecule brigdes the amide-oxygen of **28'** and H178. The hydrogen bond between the ligand and this water molecule is formed through 96% of the simulation run whereas the hydrogen bond between the ligand and H178 is formed about 50 % of the run period. Moreover a strong interaction between the thiolate and the catalytic Zn^{2+} ion could be observed, with a very sharp radial pair distribution between Zn^{2+} and the sulfur atom of the ligand with a mean distance of (0.20 ± 0.01) nm.

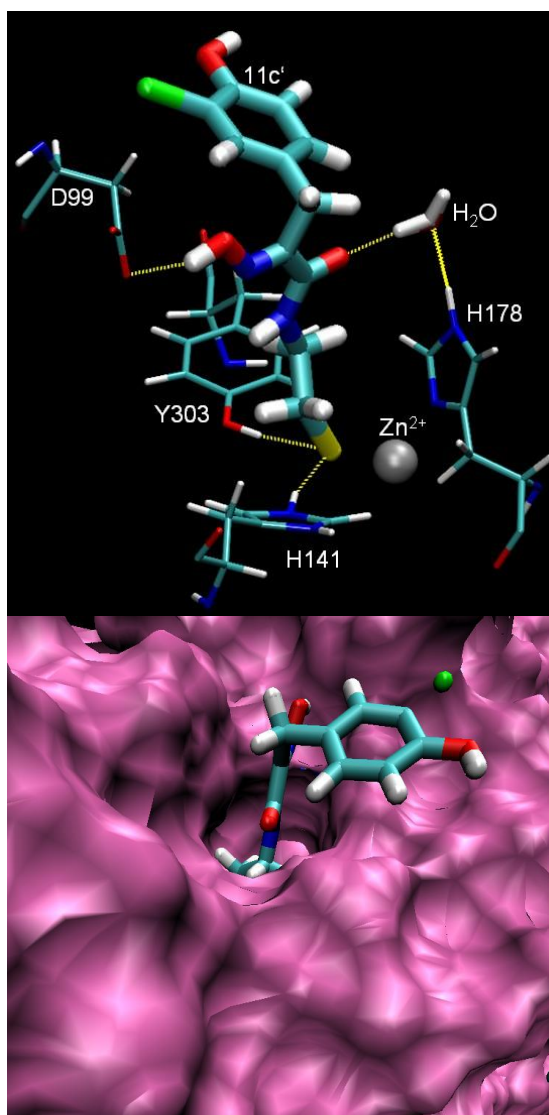


Figure 31 - Snapshot of MD simulation of **28'** in complex with HDAC1 (homology model). **28'** forms hydrogen bonds (dotted yellow lines) with Y303, H141 and D99. A structural water molecule forms bridged hydrogen bonds with inhibitor and H178

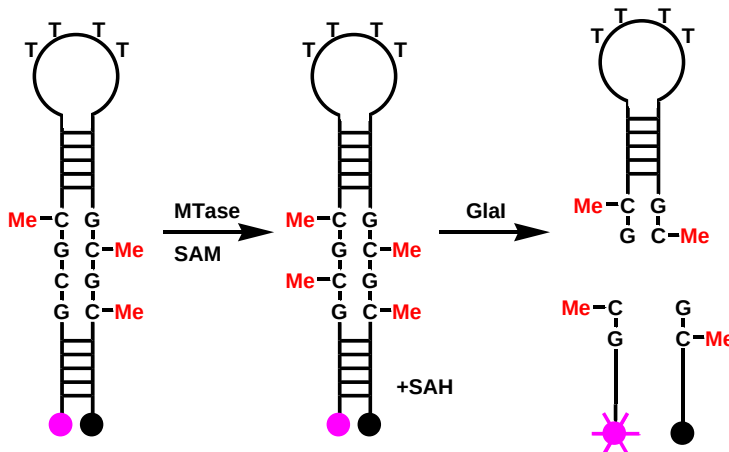
2.22. DNMT inhibition

Since small molecule, non-nucleoside DNMT inhibitors are severely underdeveloped in the literature, we were particularly interested to assess the DNMT activity of our libraries. Despite the initial report by Crews and co-workers that Psammaplin A is highly potent against semi purified DNMT1 (18.6 nM),^{135g} recent studies by de Lera and co-workers observed no inhibition of DNMT1 immunoprecipitated from K562 cells or recombinant DNMT3A.^{161a} We

therefore felt we could further study the potential for DNMT1 inhibition using a high-throughput fluorescence intensity assay. Purified full length *N*-terminal His₆-tagged human DNMT1 was used in the assay, under conditions similar to those described by Roach and co-workers.¹⁸⁷ In this assay, a hemimethylated break light oligonucleotide is used as a substrate for DNMT1. A break light is a single-stranded self complementary oligonucleotide able spontaneously to form a hairpin structure, and bearing a fluorophore and a quencher at the 5' and 3' termini respectively.¹⁸⁸ The formation of the hairpin structure places the fluorophore and the quencher in close proximity, therefore inducing efficient quenching of the fluorescence. Following methylation by DNMT1, the fully methylated oligo is preferentially cleaved¹⁸⁹ over the hemi-methylated substrate by a restriction endonuclease, *Gla* I.¹⁹⁰ Further melting of the short cleaved sequence induces the separation of the fluorophore from the quencher, and therefore an increase in fluorescence which is used as an indicator of enzymatic activity (Scheme 32). Further experimental details can be found in the Experimental section of this thesis.

T : Thymine rich sequence

● Fluorophore
● Quencher



Scheme 32 - “break-light” cytosine-C5 methyltransferase activity assay.
Drawing adapted from Roach et al.¹⁸⁷

Psammaphin A and our synthetic analogues were therefore assayed against DNMT1 using the assay described above. A counterscreen assay against *Gla* I was also performed in order to detect any potential *Gla* I inhibition by our compounds.

Disappointingly, none of our synthetic analogues assessed showed significant inhibition of DNMT1 at 30 μM . The dimeric analogues were also assayed as free thiols, but no significant inhibition could be observed, even at 120 μM . *S*-Adenosyl homocysteine (SAH) was used as a positive control in the assay (Figure 32), and gave an IC_{50} of 0.9 μM (160 replicates, lit. 2-4 μM ^{191,192}).

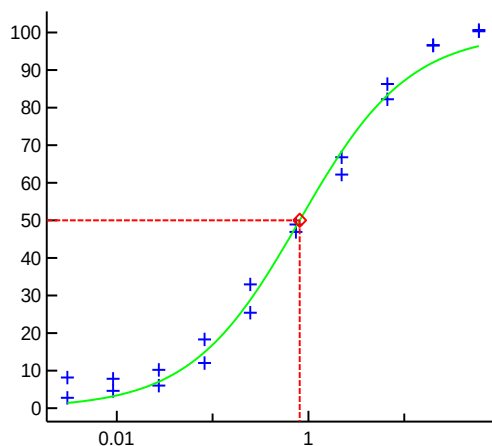


Figure 32 - DNMT1 assay, dose response curve for SAH

One potential concern was the reductive assay conditions required (5mM DTT), which would result in the reduction of any disulfide analogues assessed. Should it be the parent, dimeric structure that was active, it is likely this would not be present under the assay conditions. Analogues **134**^{161a} and **135** (obtained from diamines **136** and **137** respectively, Figure 33), were prepared to replace the native disulfide functionality of Psammaphin A with 2 methylene groups and a thioether respectively (Scheme 33). Such analogues should be chemically stable under the reaction conditions.

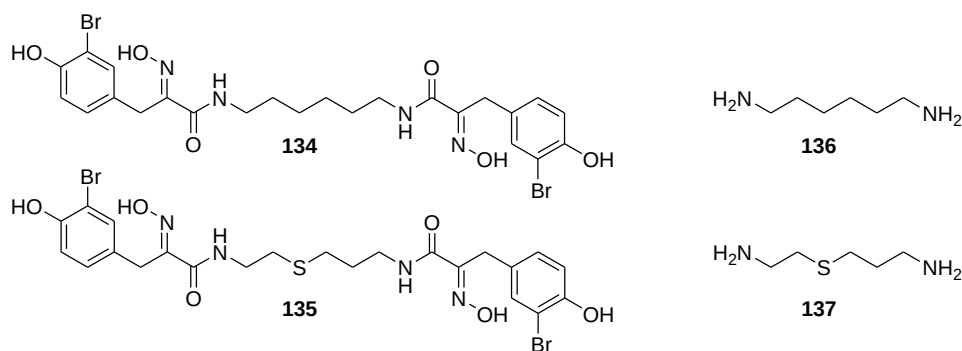


Figure 33

Unfortunately, **134** and **135** were also found inactive against DNMT1 under our assay conditions. As an alternative assay, we decided to study the effects of Psammaplin A (**28**) on the bacterial methyltransferase MSssI. MSssI is a widely used model to study the effects of DNA methylation on mammalian genomes, since this bacterial enzyme is structurally related to mammalian DNA methyltransferases and recognizes the same sequence (CpG).¹⁹³ Indeed, it has been previously used as the basis of an *in vitro* model to study the activities of inhibitors of mammalian methyltransferases.^{130,194} Furthermore, Sufrin and co-workers have previously reported Psammaplin A to inhibit bacterial methyltransferases.^{129,156} Following incubation of the enzyme with its DNA substrate, examination of the degree of DNA digestion with the restriction enzyme BstU1 (CGCG), which cannot cut if the site is cytosine methylated, can conveniently be used to assess the degree of MSssI activity in the presence of inhibitors. As can be seen in Figure 34, Psammaplin A (**28**) was found to potently inhibit MSssI at 50 μ M. The assay was repeated four times, including positive and negative controls, and in each case Psammaplin A was seen to inhibit MSssI activity equivalently down to 5 μ M.

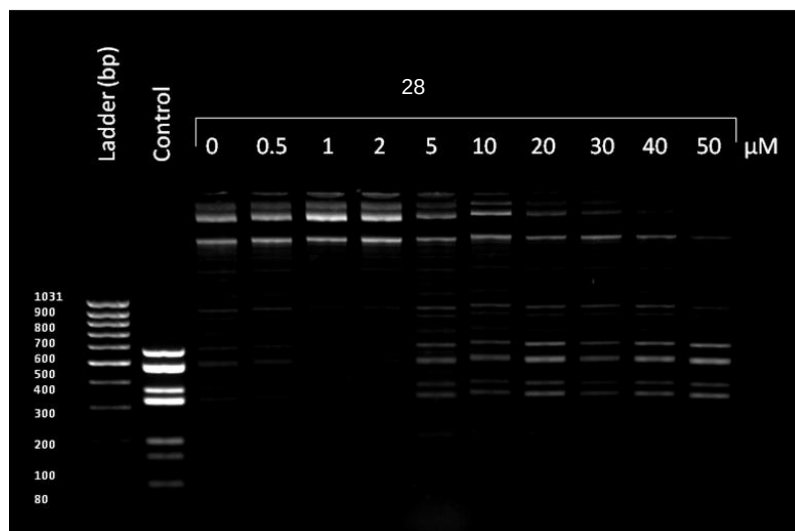
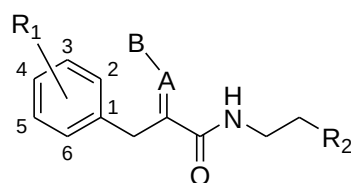


Figure 34 - MSssI assays. Psammaplin A inhibits the bacterial methyltransferase MSssI¹²⁹ *in vitro*

2.23. Cell studies

A large selection of compounds from our library were assessed for cytotoxicity. Our synthetic analogues were assayed against A549 (human lung carcinoma), MCF7 (human breast carcinoma) and WI38 (normal human lung fibroblast) cell lines. The results are given in Tables 10 and 11. Methylthioether containing analogues **75a**, **75b**, **75r**, **75t**, **97a**, **98a**, **111a** and **112a** were found almost inactive against the three cell lines tested. Most of disulfides and thiols exhibited micromolar activities. Compound **75c'** was found particularly active (0.16 μM against A549, 0.61 μM against MCF7). Interestingly, thiol **111b'** was found particularly selective against MCF7 (3.42 μM) with a 10-fold increase in potency compared to the other cell lines.

In order to shed light on the mode of action of Psammaplin A and our synthetic analogues in cells, western blot analysis was performed in MCF7 cells with compounds **28'** and Trichostatin A which was used as a positive control. Untreated cells were used as a reference. Variations in the level of expression of acetylated histone H3 (Ace-H3), histone H3 (H3), Ace-tubulin, tubulin and p21 were studied. The results are shown in Figure 35. **28'** and Trichostatin A were all found to induce a significant upregulation of Ace-H3, what is in accordance with their HDAC inhibitory potency. p21, which is known to be upregulated by HDAC inhibitors, was indeed observed. While important upregulation of p21 by Trichostatin A was observed below 1 μM , a concentration of 5 μM was necessary in order to observe a similar result with **28'**. Compound **28'** was however found to have only marginal influence on the level of Ace-tubulin compared to the upregulation of Ace-H3, whereas significant upregulation of Ace-tubulin was observed in the case of Trichostatin A. This is consistent with the HDAC isoform selectivity observed *in vitro* for **28'** and other Psammaplin A analogues. As control experiments, the total level of histone H3, tubulin and actin (not shown) were found unchanged in each case.



compound	R ₁	AB	R ₂ ^a	A549	MCF7	WI38
				IC ₅₀ [μM]	IC ₅₀ [μM]	IC ₅₀ [μM]
75a	H	NOH	SMe	> 50	> 50	> 50
75b	Me	NOH	SMe	22.8	46.7	> 50
28	H	NOH	S ₂ R (dimer)	7.5	1.27	3.44
75c	Me	NOH	S ₂ R (dimer)	4.09	0.63	2.2
28'	H	NOH	SH	2.53	2.35	3.4
75c'	Me	NOH	SH	0.16	0.61	1.13
75r	H	NOMe	SMe	> 50	> 50	> 50
75t	Me	NOMe	SMe	> 50	49.5	> 50
75s	H	NOMe	S ₂ R (dimer)	49.5	nd	> 50
75u	Me	NOMe	S ₂ R (dimer)	45.9	12.8	nd
75s'	H	NOMe	SH	10.6	19.3	24.2
75u'	Me	NOMe	SH	5.11	12.4	10
97a	H	O	SMe	> 50	18.3	nd
98a	Me	O	SMe	> 50	> 50	> 50
97b	H	O	S ₂ R (dimer)	15.2	13.1	33.1
98b	Me	O	S ₂ R (dimer)	10.3	3.86	4.12
97b'	H	O	SH	43	35.4	42.6
98b'	Me	O	SH	11.4	3.42	14.1
111a	H	NNH ₂	SMe	> 50	> 50	> 50
112a	Me	NNH ₂	SMe	> 50	> 50	> 50
111b	H	NNH ₂	S ₂ R (dimer)	18.8	3.81	nd
112b	Me	NNH ₂	S ₂ R (dimer)	10.1	>50	nd
111b'	H	NNH ₂	SH	33.4	3.42	37.2
112b'	Me	NNH ₂	SH	17.5	10.8	34.0
129	Me	NOH	SAc	8.33	3.23	5.2
128	Me	NOMe	SAc	44.6	12.2	16.6
130	Me	O	SAc	> 50	21.1	> 50

Table 10 - Psammaplin A and synthetic analogues display micromolar cytotoxicity against A549, MCF7 and WI38 cell lines. ^aThiols were prepared by *in situ* reduction of the respective disulfides using Tris(2-carboxyethyl)phosphine hydrochloride, and used immediately. Details about the procedure used for reduction can be found in the Experimental section

compound	R ₁	R ₂ ^a	A549	MCF7	WI38
			IC ₅₀ [μM]	IC ₅₀ [μM]	IC ₅₀ [μM]
11c	3-Br-4-OH	S ₂ R dimer	7.5	1.27	3.44
11c'		SH	2.53	2.35	3.4
11d'	3-Br-4-OMe	S ₂ R dimer	4.09	0.63	2.2
11d'		SH	0.16	0.61	1.13
11i	/	S ₂ R dimer	16.3	11.1	> 50
11i'		SH	15.2	9.38	10.9
11j	4-OH	S ₂ R dimer	19.5	5.55	nd
11j'		SH	19.6	15.5	31.8
11k	4-F	S ₂ R dimer	12.8	2.96	nd
11k'		SH	16.6	11.9	12.8
11m	3-I-4-OMe	S ₂ R dimer	1.08	0.52	nd
11m'		SH	2.6	1.14	1.03
11n	3-Br-4-F	S ₂ R dimer	2.73	1	1.8
11n'		SH	6.24	2.87	7.72
11q	3-Br-3',4-OMe	S ₂ R dimer	2.51	0.72	2.14
11q'		SH	4.4	2.25	4.31
11r	3,4-OMe	S ₂ R dimer	8.11	2.92	8.67
11r'		SH	nd	nd	nd
11t	3-Br-3'-NO ₂	S ₂ R dimer	2.53	0.94	2.23
11t'		SH	2.4	nd	4.84

Table 11 - Psammaplin A and synthetic analogues (variation of aromatic substitution) display micromolar cytotoxicity against A549, MCF7 and WI38 cell lines. ^aThiols were prepared by *in situ* reduction of the respective disulfides using tris(2-carboxyethyl)phosphine hydrochloride, and used immediately. Details about the procedure used for reduction can be found in the Experimental section

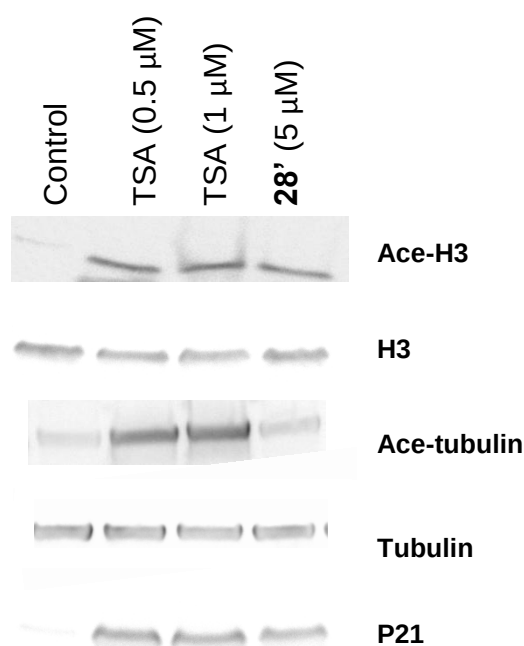


Figure 35 - Western blot analysis of **28'** in MCF7. Untreated cells and Trichostatin A (TSA) were used as negative and positive control respectively

2.24. Discussion

HDAC inhibitory activity and selectivity of Psammaplin A and analogues. It has been previously reported by Kwon and co-workers that in terms of its HDAC activity, psammaplin A functions as a natural prodrug; the active free thiol being revealed following disulfide reduction *in vivo*.¹¹⁵ The evidence presented for this proposal was the fact that pre-treatment of both psammaplin A and HDAC1 with H₂O₂ as an oxidant led to low inhibition of HDAC activity,¹¹⁵ coupled with the decreased potency of Psammaplin A against glutathione deficient cells. Recently, an analogous proposal was presented by de Lera and co-workers based on the fact that a psammaplin A analogue lacking the disulfide bound (**134**) was inactive, as were analogues where the disulfide unit was replaced by methyl ether, methyl thioether and alcohol functionalities.^{161a} Similar conclusions have been drawn for other disulfide bearing cyclic peptidic natural products, such as the HDAC inhibitor Romidepsin (**2**).¹⁴⁷ Since the common pharmacophore of most HDAC inhibitors requires a zinc-binding end group (ZBG), and the fact that thiols are known to be excellent ligands for zinc,¹⁹⁵ we felt the prospect of a free thiol as the active species is highly plausible. Indeed, synthetic HDAC inhibitors utilizing thiol zinc binding groups (protected as thioesters) have been previously reported.¹⁰⁸⁻¹¹² The SAR described for our psammaplin A derived libraries is in line with the hypothesis that psammaplin A is first reduced to the monomeric thiol, and this corresponding monomer is the active HDAC inhibitor. Indeed, by directly assaying the free thiol species, we observed the inhibitory activity of these compounds to be highly potent. In contrast, the corresponding methyl thioethers were not active at all. A parallel observation was previously made by Horinouchi and co-workers during their study of Romidepsin.¹⁴⁷ To validate this hypothesis further, computational studies of the active site of HDAC enzymes such as HDLP (pdb code 1C3P, 1C3R, 1C3S), HDAC2 (pdb code 3MAX), HDAC8 (pdb code 1T64, 3F0R, 1T69), HDAC4 (pdb code 2VQJ, 2VQM), HDAC7 (pdb code 3C10, 3C0Z) or HDAH (pdb code 1ZZ1, 1ZZ3, 1ZZ0, 2VCG) were carried out and revealed the parent psammaplin A to be too “large” in its dimeric form to fit into the active site of the HDACs (Figure 36, example of HDAC2). Our model for compound binding to HDAC1 (*vide supra*, Figure 31) is

also consistent for a thiol being the active species. What is unclear however, is why the parent disulfide analogues retain any potency in *cell free* assays. While this may be related to their ability to generate the corresponding thiols *in situ* under the assay conditions, the mechanism for this is still unclear. It is plausible that traces of reducing agents could be present in commercial kits (rHDAC1, rHDAC6), and could therefore be responsible for their observed potency. To check whether contamination of the dimeric disulfides with their thiol counterparts was giving rise to inhibitory effects, we incubated **28** and **75c** in the presence of hydrogen peroxide prior to the assay (5 mM disulfide, 1% H₂O₂, 90 minutes in the dark, then dilution to 5 μ M and further dilutions for assay). The resultant IC₅₀ values for **28** (45 nM, HDAC1) and **75c** (43 nM, HDAC1) showed a loss in potency. The enzyme activity was found unaffected by the presence of H₂O₂. This result confirms the preliminary results obtained by Kwon and coworkers,¹¹⁵ and strongly suggests the thiol as the active species. Regardless of mechanism, the initial incubation time of the disulfide species under the assay conditions is thought to play a role in the final recorded potency, suggesting that the active free thiol is generated *in situ*.

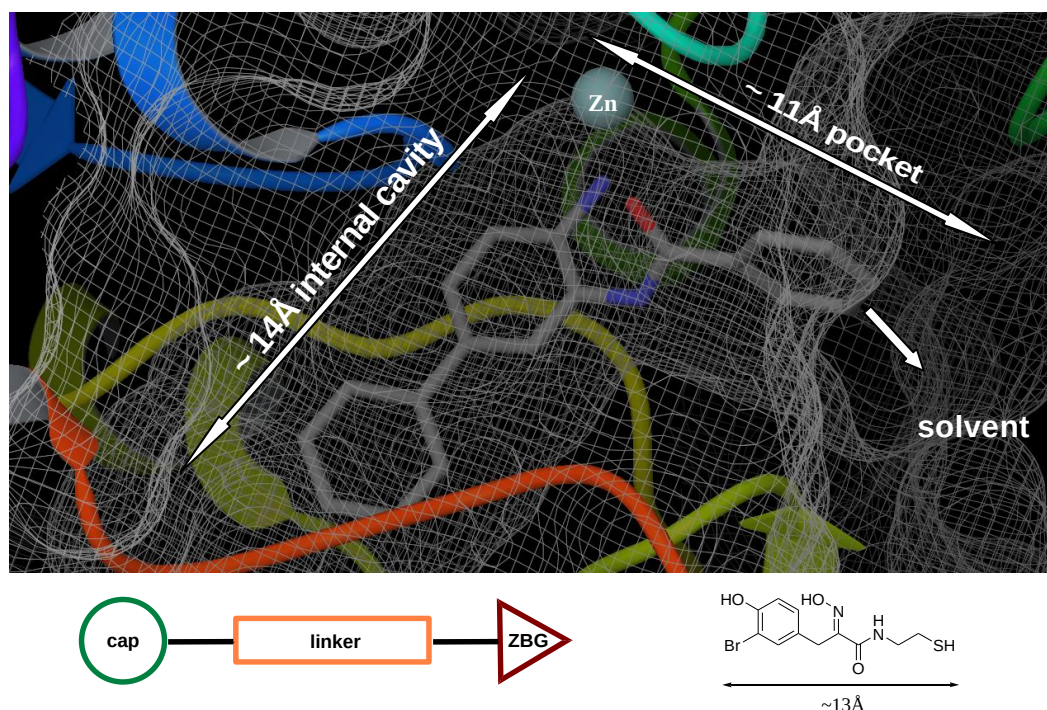


Figure 36 - Top: crystal structure of HDAC2 (pdb 3MAX) in complex with a *N*-(2-aminophenyl)benzamide derivative.⁷⁵ Bottom left: pharmacophore for common HDAC inhibitors. Bottom right: reduced Psammaplin A, proposed active species for HDAC inhibition

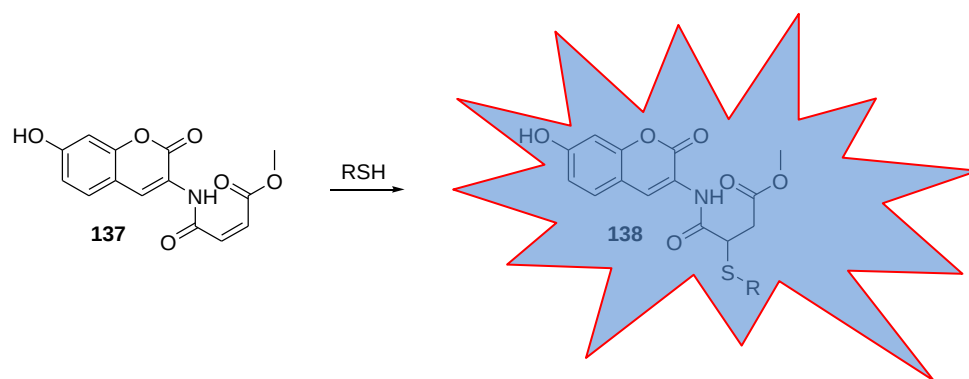
As final confirmation of the monomeric thiol species being the active compound, we found hydroxamic acid analogue **116** to be highly potent against HDAC1, HDAC6 and HDAH. Not only does this illustrate the compatibility of the hydroxamic acid group with the hydroxiimino bromotyrosine based scaffold of Psammaplin A, but also supports the hypothesis that the thiol end group is functioning as a zinc-binding group. One caveat is that in contrast with hydroxamic acids **116** and **117**, all thiols tested were found very weakly active against HDAH (IC₅₀ of **116**: 0.31 μ M; IC₅₀ of **117**: 5.25 μ M). Indeed, in our assay, **28'** and **111b'** displayed IC₅₀s of 30.2 μ M and 32.0 μ M respectively against HDAH. The reason for the lack of activity of thiols against HDAH is still unclear and is under further investigation.

While the use of the disulfide functionality is one strategy for a thiol prodrug, we also envisaged that our acetate-protected thiols **128-130** may generate the corresponding thiol *in situ* after cleavage of the acetyl group *in vivo*. Indeed, during their research of SAHA-based non hydroxamate inhibitors of HDACs, Miyata and co-workers synthesized a number of thioesters analogues which exhibited moderate to high potency in enzymatic and cell based assays.^{109,112} The potency of their compounds was interestingly higher than the potency of their corresponding dimeric analogues, and was thought to reflect a rate of thioester hydrolysis versus the rate of disulfide reduction.

We were surprised therefore to observe HDAC inhibition *in vitro* by acetate protected compounds **128-130**. Previously observed during assays against rat liver extracts (RLE), we hypothesized this to be due to the potential presence of non specific esterases in the extract. However, the same pattern was observed with purified rHDAC1 and purified rHDAC6. Recently, Williams reported the high potency of the natural product largazole, bearing an octanoyl protected thiol, against HDACs.¹⁰³ While the deprotected analogue displayed nanomolar to subnanomolar potency *in vitro* against purified HDACs, it was observed that the native thioester was still highly potent. While reasonably hypothesizing that the octanoyl group was cleaved *in situ* to liberate the active thiol, no rigorous studies were reported to explain these observations.

We began preliminary studies in order to shed some light on the mechanisms leading to acetate deprotection of our compounds *in situ*. While using

purified HDACs, we envisaged our buffered conditions to be potentially responsible for thioester deprotection. We prepared and used synthetic probe **137** (Scheme 34) in order to test this hypothesis.¹⁹⁶ Coumarin based probe **137** is known to react very fast with thiols through a Michael addition reaction. Fluorescence of the coumarin moiety of **137** is efficiently quenched by the intramolecular double bond. However, reaction between a thiol and **137** affords a highly fluorescent conjugate **138**. This system has been found to be particularly efficient to quantify thiol concentration in biological systems. We thought this probe to be a valuable tool to detect any formation of the free thiol from our thioacetates in our buffer. **137** was prepared as previously described.¹⁹⁶



Scheme 34 - Probe **137** reacts very fast with thiols to form the highly fluorescent conjugate **138**

Control experiments were performed using 2-mercaptoethanol as the source of thiol.¹⁹⁶ Fluorescence spectra of **137** (10^{-6} M in buffer FB-188¹⁷⁸) were recorded in the presence of 0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.5 eq. 2-mercaptoethanol **139** (Figure 37, top).

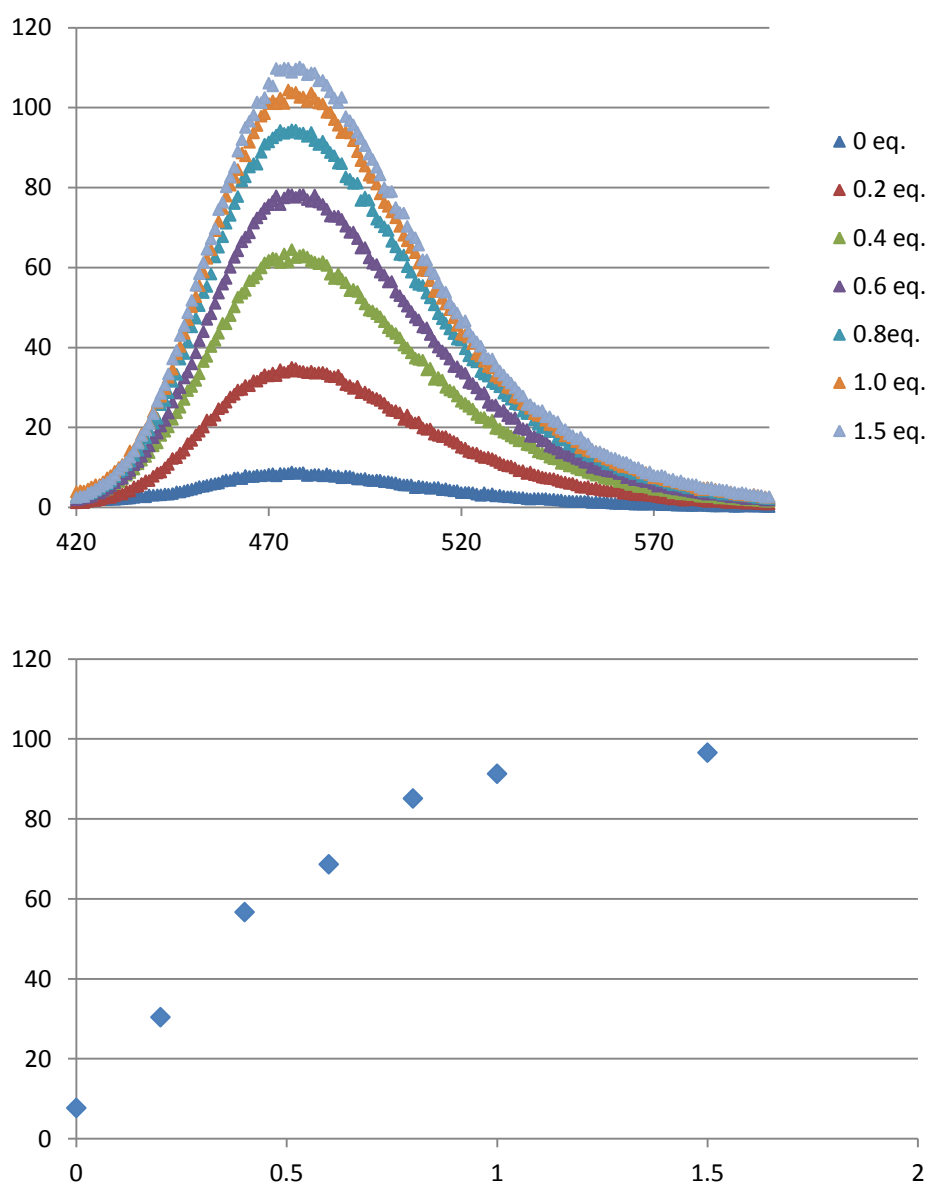


Figure 37 - Top: Fluorescence spectra (a. u.) of **137** (1 μ M) in buffer FB188¹⁷⁸ in the presence of 0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.5 eq. 2-mercaptoethanol. Excitation: 400 nm, emission: 420-600 nm. **137** and different quantities of 2-mercaptoethanol were initially incubated at 1 mM (**137**) for 5h, and further /1000 dilution in buffer was performed before recording the fluorescence spectra.. Bottom: fluorescence intensity (a. u.) at 465 nm as a function of the number of equivalent of 2-mercaptoethanol

As expected, a linear relationship between fluorescence intensity and the concentration of thiol could be observed between 0 and 1.0 eq., confirming the sensitivity and reliability of this system (Figure 37, bottom).

However, no significant variation of the fluorescence intensity could be observed when 2-mercaptoethanol was replaced by thioester **129** in our experiment (Figure 38). While the classical incubation time of thioesters are about 20-30 minutes in HDAC assays, the incubation time for this fluorescence experiment (100 minutes) was chosen to be about 3 times higher in order to ensure the observation of any potential variation, and to exclude the incubation time as a possible systematic error in the final results. Two explanations can be envisaged to explain this last result. 1) The thioester is stable in these conditions and therefore the buffer does not contribute to the *in situ* generation of the free thiol. 2) The quantity of free thiol generated *in situ* is lower than the limit of detection of the experiment, and therefore can't be quantified by this technique. Simplistic calculations suggested that the presence of only 2-3% of active thiol among the thioester (or disulfide) samples should theoretically be enough to result in an IC_{50} in the low nanomolar range.

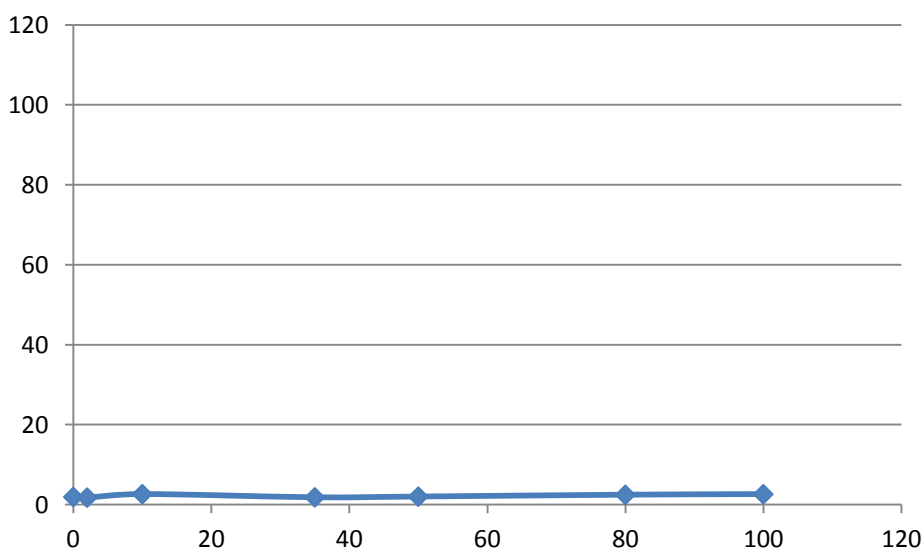


Figure 38 - Time (minutes, X-axis) dependent fluorescence intensity (465 nm, a. u., Y-axis) of **137** (1 μ M) in buffer FB188¹⁷⁸ in the presence of 2.0 eq. thioester **129**. **137** and **129** were incubated at 1 mM (**137**) in buffer FB188¹⁷⁸ (3% DMSO), and further /1000 dilution in buffer was performed before recording the fluorescence spectra.

Another study was later undertaken by our collaborator Pr. Franz Meyer-Almes and his group (Hochschule Darmstadt, University of Applied Sciences, Germany) in order to investigate the potential role of HDAC1 itself in the

hydrolysis of the thioesters. Indeed, HDACs are enzyme able to remove acetyl groups from their substrates and are therefore obvious candidates for investigation. In particular, the initial incubation time of the thioester substrate with HDAC1 was thought to play a role in the final recorded potency. In this experiment (data not shown), inhibition of HDAC1 was measured (single concentration) as a function of the initial incubation time (1-60 minutes) with different thioesters. o variation of enzymatic inhibition could be observed in each case, which seems to exclude HDAC1 as the source of hydrolysis.

In conclusion, the reasons for the observed potency of our thioesters against HDAC1 are still elusive. While inhibition by the thioester itself cannot be excluded, it seems relatively unlikely. Investigations are still in progress in order to shed light on the mechanisms underlying these observations.

One striking aspect of our data is the high selectivity of Psammaplin A and many of our analogues for HDAC1. As described, the oxime functionality in Psammaplin A seems to play an important role, not only for potency against HDAC1, but also HDAC1 selectivity. Indeed, while ketone analogues **97b'** and **98b'** and compound **115'** bearing no functionality in this position displayed similar potencies against both HDAC1 and HDAC6, introduction of the oxime (and to a lower extent, the hydrazone) resulted in an increase in potency against HDAC1, while keeping potency against HDAC6 relatively unaffected. These result thus suggest a possible design criterion for HDAC1 selective inhibitors. At the molecular level, we suspect the selectivity may be due to structural differences between the active sites of class I and class II HDACs. The structural basis for the observed pronounced selectivity of the reduced psammaplin analogues for HDAC1 versus HDAC8,6,7 is still under discussion. We hypothesize that among other structural features hydrogen bonding to Y303 contributes to selectivity because it is substituted by histidine in class IIa HDACs. This tyrosine is assumed to stabilize the oxyanion during the catalytic deacetylation process.¹⁹⁷ Crystal structures of enzyme-inhibitor complexes involving HDAC2 (3MAX), HDAC8 (1T64) and HDAH (1ZZ1) show hydrogen bonds between the hydroxyl group of the catalytic tyrosine and the corresponding inhibitors. In contrast, in the crystal structures of enzyme-inhibitor complexes of HDAC4 (e.g. 2VQJ, 2VQM) and

HDAC7 (3COZ,3C10) the histidine residues corresponding to the catalytic tyrosine are rotated outward from the active site.

L271 (HDAC1) is conserved through class II and class I HDACs unless HDAC8, where leucine is replaced by methionine. This difference might contribute to the observed differences in substrate and inhibitor selectivities within class I between HDACs 1, 2, 3 and HDAC8.

D99 (HDAC1) is conserved through all members of zinc dependent HDACs, but is substituted by an isoleucine in HDAH which might be a possible explanation for the low potencies of the reduced psammaplin analogues against the bacterial HDAC homologue.

In terms of the substitution pattern of the aromatic “capping” unit, we found this feature to have less of an effect on potency and selectivity than the thiol or the oxime functionality. This is agreement with the SAR observed by de Lera and co-workers.^{161a} Our model (see Figure 31) suggests that the aromatic ring is positioned at the entrance of the channel leading to the active site, potentially interacting with one or both of the conserved phenylalanines at the channel entrance. It is therefore clear that a number of our analogues could make interactions with surface residues, resulting in the observed changes in potency and selectivity.

Methyltransferase inhibitory activity of Psammaplin A and analogues

Psammaplin A and other members of the Psammaplin family have been previously reported to be potent inhibitors of the human DNMT1 *in vitro* (IC₅₀ = 18.6 nM for Psammaplin A against semi-purified DNMT1).^{135g} In our DNMT1 assay, Psammaplin A and all synthetic analogues were found inactive at concentrations of up to 30 μ M and 120 μ M (free thiols). These data are in agreement with the recent data reported by de Lera and coworkers, who reported the lack of potency of several Psammaplin A analogues against immunoprecipitated DNMT1 in an alternative methyltransferase assay.^{161a} The origin of the discrepancy between our data and the values reported by Crews and co-workers^{135g} is still unclear, but several differences in protein purity and the assay conditions are apparent. Crews and coworkers used semi-purified DNMT1,

whereas highly purified enzyme was used in our assay. The methylation substrate (and its concentration) was also different in each case, with biotinylated poly(dl-dC) used by Crews and a hairpin DNA mimic oligomer being used in our assay. This difference is likely to be important and could account for the inactivity observed in our assay. It is germane to mention however that the de Lera study also used a poly(dl-dC) substrate.^{161a} Since most DNMT assays involve substantial quantities of reducing agents (usually DTT) in order to avoid degradation of the DNA substrate by external oxidative agents (such as O₂), it is important to highlight the chemical instability of our dimeric compounds under these conditions. Our results on the non reducible analogues **134**^{161a} and **135** (*vide supra*), which were also found to be inactive, suggest however that chemical reduction of psammaplin A analogues may not be responsible for the lack of potency against DNMT1. Taken together, the lack of activity observed in our *in vitro* assays, coupled with the lack of activity observed by de Lera and co-workers, lead us to question the activity of Psammaplin A (and analogues) against DNMT1. While such a supposition is based on data from cell-free assays, it is important to highlight that Sufrin and co-workers have reported a lack of DNA hypomethylation as a result of exposure of HCT116 human colon carcinoma cells to Psammaplin A. Psammaplin A also failed to induce the hypomethylation or reactivation of methylation silenced genes.^{156,198} Since psammaplin A has been reported to have low physiological stability,^{143,144,199} it is plausible the lack of an effect on DNA methylation in cells is due to transport and stability issues. An alternative explanation is that psammaplin A is largely inactive against human DNMTases.

Interestingly, Psammaplin A was found to inhibit the bacterial methyltransferase MSsSI potently at low μ M concentrations (Figure 34). This result is in accordance with preliminary data reported by Sufrin, who also reported activity against bacterial methyltransferases.¹⁵⁶ Importantly, we observed that both Psammaplin A and its reduced form were active against the bacterial methyltransferase, DTT contained in the buffer likely generated substantial amounts of reduced Psammaplin A *in situ* regardless of how the compound was dosed. It is difficult to draw a firm conclusion from the fact that our data show lack of activity against DNMT, but activity against MSsSI, however it is important

to note that our MSssI assay used bacteriophage λ DNA as the substrate at it therefore remains possible that psammaplin A is instead affecting the analysis through interaction with the DNA substrate (for example by intercalation), rather than a direct interaction with the methyltransferase. Further studies are needed to elucidate this possibility.

Cellular studies. Our synthetic analogues showed significant toxicity against A549 (human lung carcinoma), MCF7 (human breast carcinoma) and WI38 (normal human lung fibroblast) cell lines (see Tables 10 and 11). For example several compounds were found to inhibit cell growth at sub-micromolar concentrations, including **75c'** (A549: 0.16 μ M MCF7: 0.61 μ M) and **11q** (MCF7: 0.72 μ M). This data is in agreement with previous studies that have reported Psammaplin A to have significant cytotoxicity (ED_{50} , μ g/mL) against human lung (A549, 0.57), ovarian (SK-OV-3, 0.14), skin (SK-MEL-2, 0.13), CNS (XF498, 0.57), and colon (HCT15, 0.68) cancer cell lines.¹⁴⁶ Furthermore, it has been reported to cause G1 arrest in human endometrial Ishikawa cancer cells²⁰⁰ and U937 leukaemia cells,^{161a} which correlates to the effect of known HDAC inhibitors. We found that methylthioether-containing analogues **75a**, **75b**, **75r**, **75t**, **97a**, **98a**, **111a** and **112a** (Table 10) were largely inactive against all cell lines tested, correlating with our SAR against HDAC inhibition. Additionally, compounds bearing an oxime motive (AB = NOH, Table 10) were found more potent than their respective analogues bearing an *O*-methyloxime (AB = NOME, Table 10), ketone (AB = O, Table 10) or hydrazone (AB = NNH₂, Table 10) unit, once again correlating with SAR data previously obtained in HDAC assays. Taken together this suggests that Psammaplin A mediates its cellular effects through HDAC inhibition.

HDAC1 knockdown in MCF-7 cells has been previously reported to result in cell cycle arrest in G1 and G2.²⁰¹ In light of the fact that our studies indicated psammaplin A to have significant potency and selectivity against HDAC1, and that HDAC inhibitors are being clinically evaluated for breast cancer treatment,²⁰² we decided to investigate further the mode of action of our inhibitors in MCF7 cells, through study of reported HDAC biomarkers. Western blot analysis revealed **28'** to induce an important upregulation of Ace-H3, nicely correlating

with its HDAC inhibitory potency. Interestingly the inability of **28'** to significantly upregulate Ace-tubulin, a substrate for HDAC6, nicely correlates with the low inhibitory potency of this compound against HDAC6 *in vitro*. Finally, **28'** was able to upregulate the level of p21, known to be affected by HDAC inhibitors. This result is again suggestive that HDAC inhibition could account for **28'** (and related analogues) cytotoxicity. Interestingly, preliminary studies have shown that similar results could be observed with hydrazone analogue **111b'** and the hydroxamic acid analogue of Psammaplin A **116** (data not shown). Additional studies are still in progress in order to establish a more accurate picture of the mode of action of **28'** and related synthetic analogues. The results of this study will be reported in future manuscripts

2.3. Conclusions

In this study, we demonstrated that Psammaplin A was a prodrug for HDAC inhibition, requiring reduction of the disulfide unit in order to generate the active species, a thiol. Assaying our libraries of analogues allowed us to draw a robust SAR of Psammaplin A against HDACs, and to propose a binding mode to HDACs. The important isoform selectivity exhibited by our compounds *in vitro* against HDACs allowed us to highlight important structural features potentially exploitable for the development of novel and selective lead compounds. We were however disappointed by the lack of potency of our libraries against DNMT1, which is inconsistent with results previously reported by Crews.^{135g} While differences between our assay conditions and those reported by Crews, notably the nature of the DNMT substrate and enzyme purity, could be invoked in order to explain such differences, this led us seriously to doubt of the DNMT1 inhibitory activity reported by Crews. Finally, cytotoxicity assays and Western blot analysis allowed us to characterise HDAC inhibition by **28'** in MCF7 cells. Interestingly, the ability of **28'** to upregulate histone acetylation while having only a marginal effect on the level of acetylated tubulin was consistent with its HDAC selectivity *in vitro*. Further studies are underway in order to obtain a more accurate picture of the mode of action of **28'** and related synthetic analogues.

3. Further investigation of the role of the oxime unit in Psammaplin A and design of novel lead compounds

3.1. Introduction

As seen earlier in section 3, the synthesis and biological evaluation of the libraries of Psammaplin A analogues allowed us to develop a robust SAR against HDAC inhibition. The work described in section 3 allowed us to dissect the HDAC activity of Psammaplin A and to understand the structural features necessary for potency and selectivity. The impressive selectivity of our compounds for HDAC1 inhibition *in vitro*, mediated by the presence of either the oxime or hydrazone functionality, has not been documented previously. This selectivity represents a highly valuable asset for the design of new generations of novel, potent and selective HDAC inhibitors for anticancer therapies. Indeed, class I HDACs, comprising HDAC1, have been shown to play an important role in the silencing of tumour suppressor genes (TSGs) in cancer and to be amongst the most promising HDAC targets.²⁰³ While Psammaplin A and analogues have been shown to be highly potent and selective HDAC inhibitors *in vitro*, their low stability *in vivo* prevents their potential use in anticancer therapy. Their first drawback lies in the presence of the oxime functionality, potentially source of cytotoxicity *in vivo*. A number of oximes, and the parent compound hydroxylamine, have been shown to exhibit a wide range of cytotoxic effects.²⁰⁴ These effects are mainly of haematological nature,^{204a,204d,e} and are usually related to oxidative stress. Also, Cytochrome P450 mediated metabolic degradation of oximes has been shown to produce reactive species, such as nitriles^{204c} or nitronates.^{204b} The second and probably most important drawback lies in the presence of the pendant terminal thiol. While being necessary for high potency, the low stability of thiols *in vivo* generally prevent them from being incorporated into drug candidates. Although a number of thiols, such as cysteine or glutathione (GSH), play an essential role in various biochemical processes,²⁰⁵ they are in general well known for their ability to produce highly reactive and harmful species *in vivo* during redox reactions in human cells.²⁰⁶ Finally, metalloproteins, such as zinc-dependent proteins,²⁰⁷⁻²¹⁰ represent approximately one third of the

human proteome. The presence of a thiol is therefore likely to induce off-target effects, as thiols are well known to be particularly good ligands for a variety of metals in biological systems.

While a binding mode of Psammaplin A (reduced form) to HDAC1 has been proposed in section 3 based on extensive docking and MD studies, this binding mode is still under discussion in our laboratory. In particular, the role of the oxime and the coordination mode of the zinc cation are still subject to discussion. The results of additional computational studies will be presented in the next sections, with the aim to improve our understanding of the binding mode of Psammaplin A analogues to HDAC1. We hoped these studies to help us in the design of novel drug-like lead structures with the aim of overcoming the *in vivo* instability of Psammaplin A analogues. The design, synthesis and biological evaluation of novel inhibitors will be discussed.

3.2. Results and discussion

3.21. Computational studies

As seen previously in section 3, the oxime present in Psammaplin A and analogues (thiols) is proposed to hydrogen bond with Asp99 (HDAC1 homology model), which is thought to participate to their isoform selectivity. It is therefore surprising that methylated oxime derivatives **75s'** and **75u'**, which are unable to form this hydrogen bond, are still highly potent (IC_{50}) and selective for HDAC1 (**75s'**: 27 nM, **75u'**: 15 nM). This led us to question the importance of the oxime hydrogen for binding, and investigate the possible involvement of the oxime oxygen or nitrogen. Indeed, while the SAR data previously collected did not exclude the implication of the oxime hydrogen in H-bonding interactions with the protein, they strongly suggest that this hypothetical H-bond may not be the causative factor for the observed potency and selectivity observed with oxime containing analogues. For example, the potency (IC_{50}) of *O*-methyloxime containing analogues **75s'** (27 nM) and **75u'** (15 nM) should likely be comparable to the potencies of keto analogues **97b'** (0.40 μ M) and **98b'** (1.1 μ M) and analogue **115'** lacking the oxime unit (0.42 μ M).

We therefore performed additional docking (Schrodinger) and MD (Gromacs) studies, and proposed other plausible binding modes. In particular, there is still the question of whether Psammaplin A and thiol analogues bind to HDACs in a monodentate or bidentate fashion. Indeed, due to its particular electronic properties, Zn(II) has been shown to tolerate different coordination numbers in biological systems.²¹⁰ Its “borderline” character confers Zn(II) the ability to form stable complexes with oxygen and/or nitrogen and/or sulfur containing ligands. In biological systems, Zn(II) is generally tetracoordinated by a combination of His, Asp, Glu, Cys and water. While often found in a tetracoordinated environment, it is not rare to find Zn(II) pentacoordinated, notably in the presence of bidentate ligands. This is, for example, the case for hydroxamic acids (Figure 39).⁷⁶

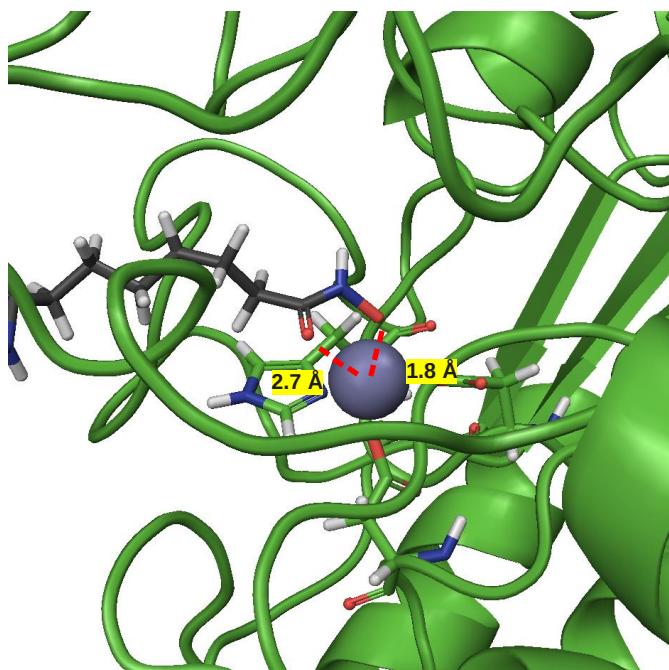


Figure 39 – Crystal structure (1C3S) of SAHA (dark grey, stick representation) bound in the active site of a histone deacetylase like protein (HDLP, green, ribbon representation); Zn(II) (grey sphere) is visible; the bidentate coordination of Zn(II) by SAHA is clearly visible (red dots)

Docking studies performed with analogue **75d'** and the recently published crystal structure of human HDAC2 (pdb code 3MAX)⁷⁵ revealed orientations in which the ligand coordinates to the zinc in a bidentate fashion, with the thiol and the amide oxygen interacting with the metal centre (Figure 40). Although van der Waals radii for the ligand and/or the protein had to be artificially decreased (x 0.7)

during docking in order to allow the ligand to adopt such a conformation, this may be acceptable considering that the conformation of a protein in its crystal structure and its conformation in solution may differ. A bidentate complexation mode was already proposed by Anandan and coworkers²¹¹ for the *N*-(2-mercaptoethyl)amide motif during their study of mercaptoamide based inhibitors, however this result has not been experimentally validated.

In our new docking experiments, His145 (HDAC2 numbering) was protonated and the thiol docked as a thiolate anion. The choice of the protonation state of the two His residues forming the charge relay system was particularly important and had a crucial influence on the outcome of docking and MD experiments. In this model (Figure 41, left), the thiol is deprotonated by His145 and interacts strongly with Zn(II). The amide interacts with Tyr308 (H-bond) and Zn(II) (O-Zn) *via* the oxygen, and with Gly154 (H-bond) *via* the hydrogen. Interestingly, a similar binding mode can be observed in the crystal structure of HDAC2 with a cocrystallized benzamide derivative (pdb code 3MAX, Figure 41 (right)),⁷⁵ in support of our model. A MD simulation using our HDAC1 homology model has shown this bidentate coordination for **75d'** to remain unchanged for at least 2.5 ns.

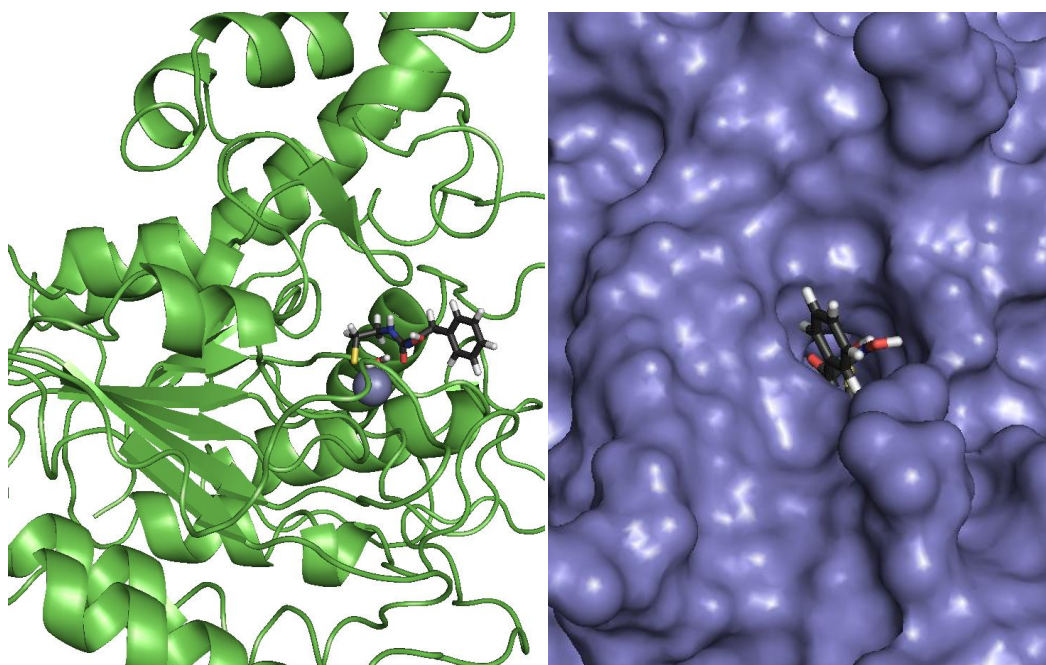


Figure 40 - Left: **75d'** (dark grey, sticks representation) docked in the active site of HDAC2 (green, ribbon representation). Zn(II) (grey sphere) is visible; right: **75d'** (stick representation) docked in the active site of HDAC2 (surface representation)

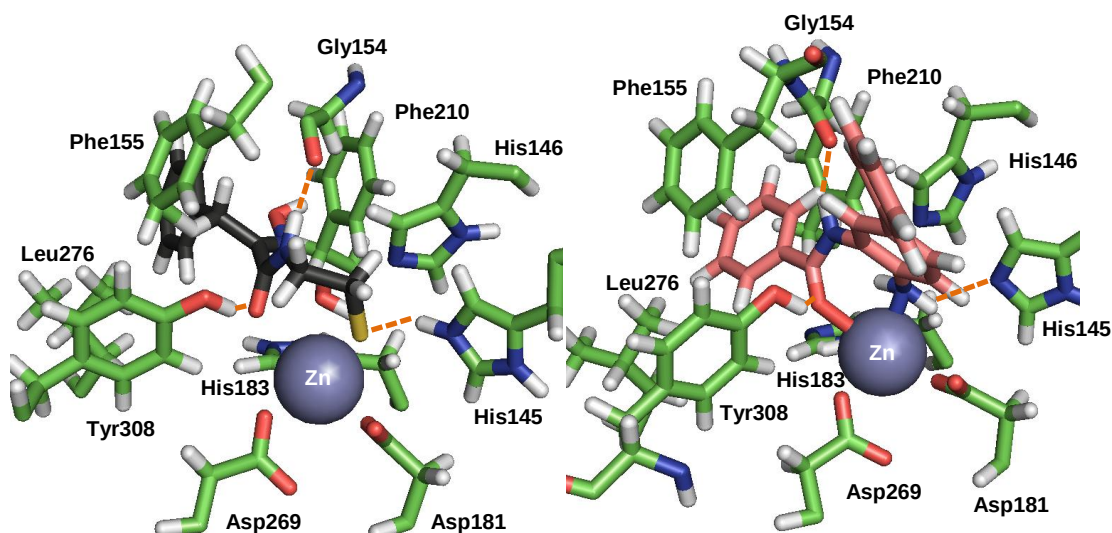


Figure 41 - Left: bidentate binding mode proposed for the *N*-(2-mercaptoethyl)acetamide motif of **75d'** (dark grey: carbon, white: hydrogen, red: oxygen, blue: nitrogen, yellow: sulfur; stick representation). Hydrogen bonds of interest with the protein (green: carbon, white: hydrogen, red: oxygen, blue: nitrogen; stick representation) are highlighted in orange; right: crystal structure (pdb 3MAX) of an *o*-aminobenzamide derivative (pink: carbon, white: hydrogen, red: oxygen, blue: nitrogen; stick representation) in the active site of HDAC2. Hydrogen bonds of interest are highlighted in orange

However, the model shown in Figure 41 (left) fails to reveal the role of the oxime in binding, since no H-bond donor or acceptor can be observed in its direct vicinity. Several hypotheses were proposed in order to explain this observation:

Alternative model 1: It is possible that one or several structural water molecules are involved in oxime binding. The position of these hypothetical water molecules is still unknown and subject to investigation. A plausible candidate for interacting with the ligand *via* a structural water molecule was identified. This candidate is His146 (HDAC2 numbering), which appears to be at approximately 3.6 Å from the oxime nitrogen (Figure 42). However, MD experiments have so far not been able to provide evidence for the presence of such a water molecule. Interaction between the oxime nitrogen and His146 *via* a structural water molecule would allow the oxime to occupy a position that avoids an internal steric clash with the aromatic ring. In the meantime, this would potentially allow the aromatic ring of the ligand to be involved in hydrophobic interactions at the entrance of the active site, possibly *via* π -stacking interactions with the phenyl rings of Phe155 and Phe210. In this conformation, the oxime hydrogen atom could plausibly interact

with Phe210 *via* an O-H- π bond. In this model the *meta* and *para* positions of the aromatic ring are solvent-exposed and therefore potency should not be drastically affected by the introduction of substituents, which correlates to SAR data described in section 3. Then, the slightly reduced potency of *O*-methyloxime analogues could be explained by the steric clash appearing between the methyl group and the rest of the ligand-enzyme complex at the entrance of the active site (Figure 42), notably Phe155 and Phe210. The drop in potency for ketone analogues could be partially explained by the reduced length of the C=O double bond (therefore the position of the oxygen with respect to His146) and the reduced H-bond acceptor character of the carbonyl compared to the nitrogen atom of an oxime or an hydrazone.

The model proposed here alone does give insight to the isoform selectivity profile observed for Psammaplin A analogues, as the two histidine residues involved in the charge relay system are conserved in all members of class I and class IIb. It can be hypothesized that this selectivity, mediated by the oxime or the hydrazone, comes from variation in the orientation and basicity of the two histidine residues among the different isoforms. To date, study of the flexibility and basicity of this system is limited to few examples.⁷⁸ A full study, extended to all isoforms, would be of considerable use for this study.

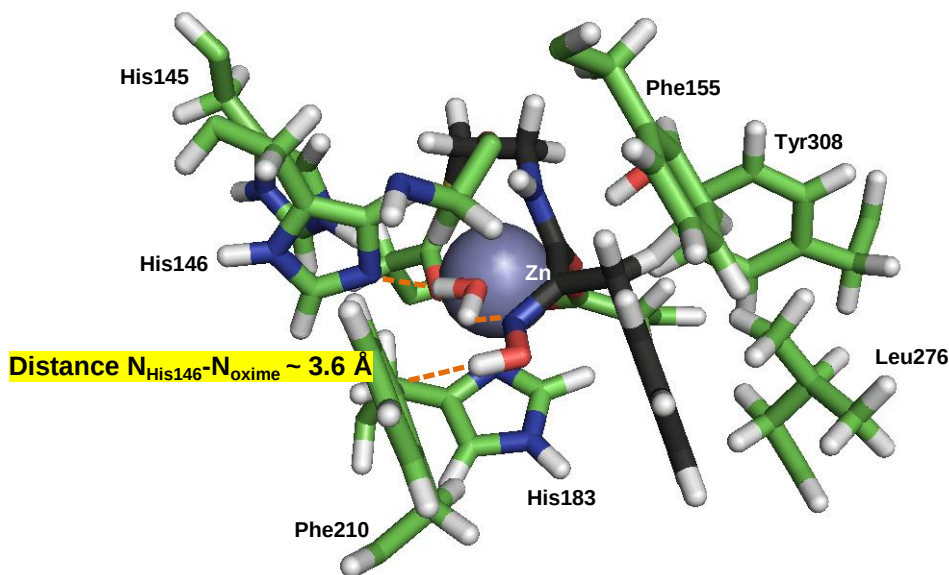


Figure 42 - Ligand **75d'** (dark grey, stick representation) docked in the active site of HDAC2). A structural water molecule bridges the oxime nitrogen and His146.

The water molecule was introduced manually (by the user) after docking, and subsequent steric clashes were reduced manually by rotation around the appropriate bonds. Hydrogen bonds of interest are highlighted in orange

Alternative model 2: MD simulations (Gromacs) were performed on HDAC2 (pdb 3MAX)⁷⁵ in which the native ligand has been removed, and trajectories of the residues in the active site were analysed (Figure 43). While Asp181, Asp269, His183 and a water molecule together form a relatively stable tetracoordinated environment for the catalytic zinc (Figure 43, Figure 44 (top)), the position of His183 was found to be altered during the 0.5 ns simulation. The imidazole ring was notably found to deviate significantly from the starting position during 5-6% of the simulation, in particular it was found to be able to achieve a $\sim 90^\circ$ flip from its original position (Figure 44 (bottom) and Figure 45 (top)). Interestingly, in this conformation His183 is located at an acceptable distance (ca. 2 Å) to interact with the oxime of 75d' (Figure 45, bottom), potentially H-bonding with the sp^2 nitrogen. While this flipped conformation for His183 is not favoured due to the important disturbance of the Zn(II) coordination sphere, addition of the ligand could account for its stabilisation.

While isoform selectivity of our Psammaplin A analogues could potentially be related to the relative flexibility of the zinc coordination sphere among the different HDAC isoforms, this flexibility is poorly documented. Recent *ab initio* QM/MM molecular dynamics studies by Zhang allowed a theoretical assessment of the stability of the zinc coordination sphere in HDAC8,²¹² however no experimental validation has been reported. Moreover, no study has been thoroughly performed on other isoforms, making an accurate comparison impossible at this time.

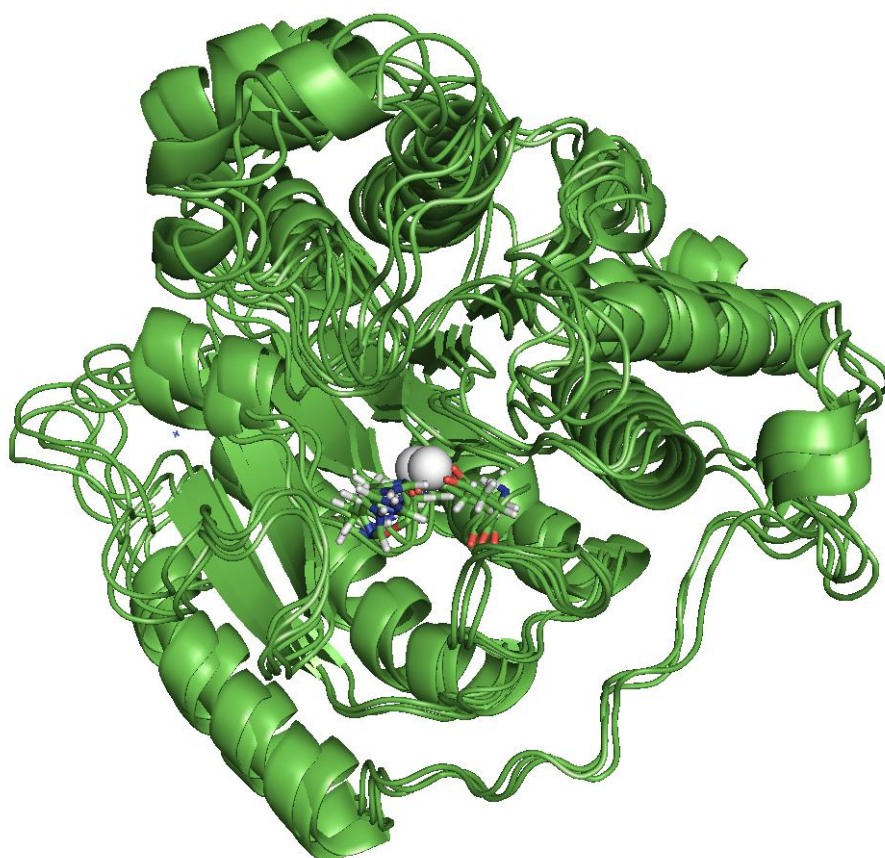


Figure 43 – Superimposed snapshots of MD simulations with HDAC2 (green, ribbon representation) were superimposed. Zn(II) (grey sphere) is visible)

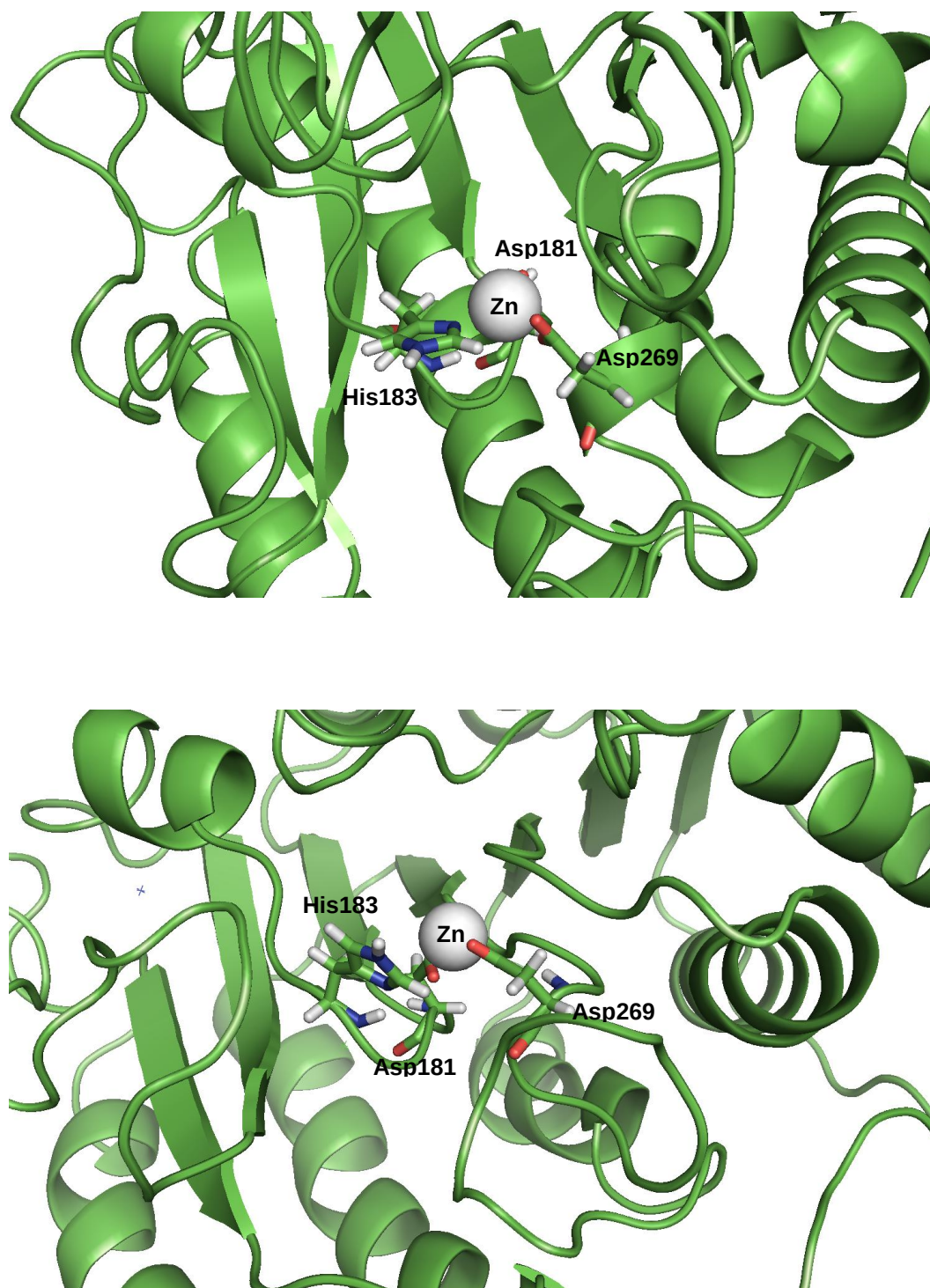


Figure 44 – top: Asp181, Asp 269, His183 (stick representation) and a water molecule (not shown) efficiently coordinate the Zn(II) cation (grey sphere) in the active site of HDAC2 (green, ribbon representation); bottom: His183 was able to flip from its initial/stable position, therefore perturbing the tetracoordinated environment of Zn(II)

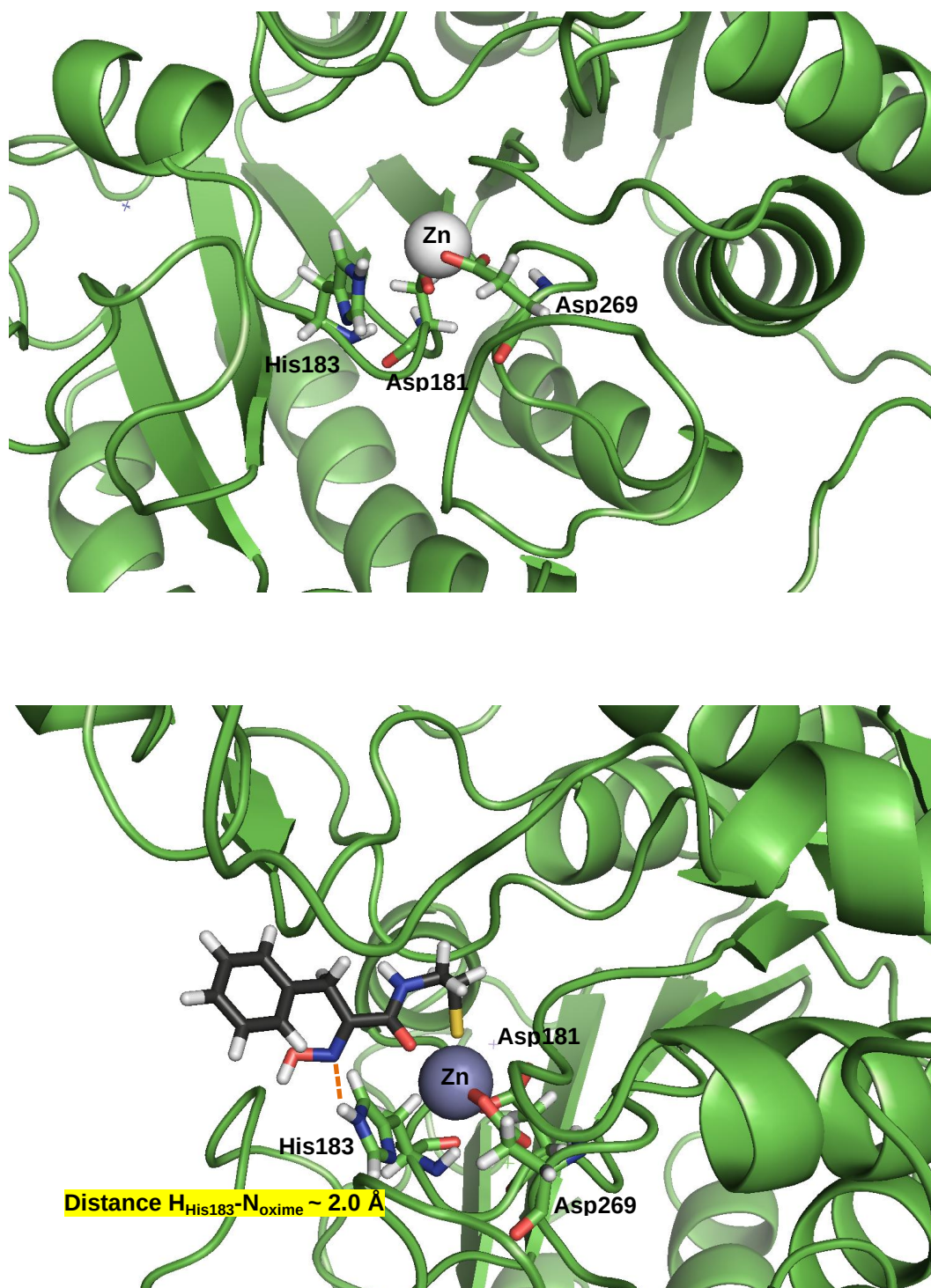


Figure 45 - Top: ~ 90° flip of His183 from its initial/stable position. Bottom: ligand **75d'** (dark grey, stick representation) docked in the active site of HDAC2. The oxime nitrogen of **75d'** forms a hydrogen bond (orange) with flipped His183.

Alternative model 3: An alternative binding mode could involve an isomerised oxime. Indeed, in the (*Z*) geometry the oxime hydrogen is positioned at approximately 2.2 Å from His146, therefore potentially able to form a H-bond (Figure 46). Isomerisation of the oxime of Psammaplin A has already been experimentally observed,^{135c} demonstrating the (*Z*) geometry to be stable under certain conditions. Indeed, in 1987 Arabshahi and Schmitz isolated several new brominated metabolites from an unidentified sponge.^{135c} Interestingly two of these compounds exhibited very similar NMR spectra. One was found to be the natural product now called Psammaplin A, having an (*E,E*) oxime geometry and the second was found to be the product with an (*E,Z*) geometry, one oxime being isomerized. They later observed that the (*E,Z*) product completely isomerized to the (*E,E*) one after a few weeks, demonstrating the increased stability of the (*E*) geometry. We therefore hypothesised that *in situ* isomerisation of the oxime upon binding could induce stabilisation of the HDAC2-ligand complex *via* hydrogen bonding with His146. However, this binding mode is still difficult to reconcile with the observed potency of methylated oxime derivatives.

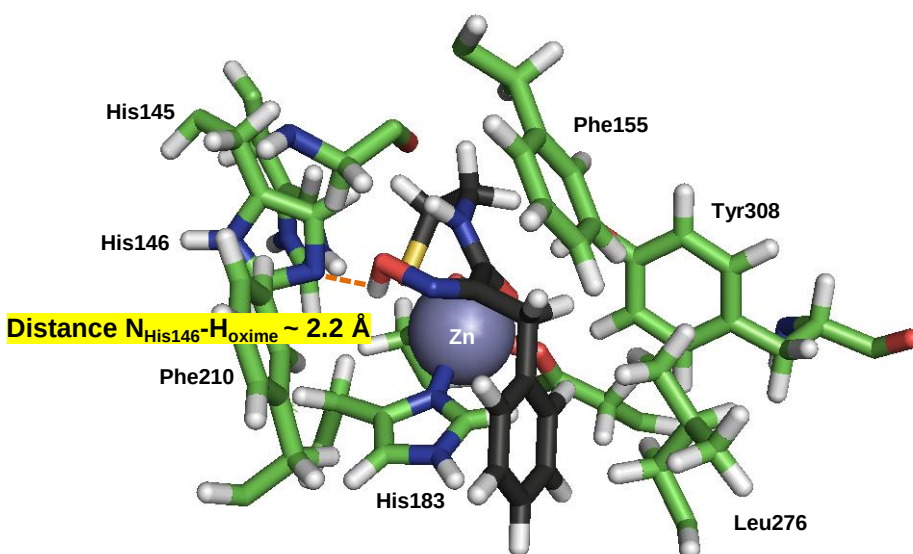


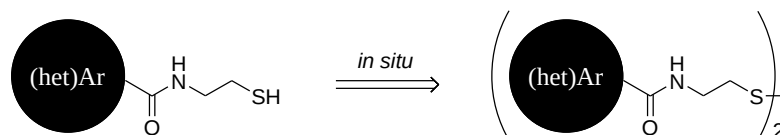
Figure 46 - Isomerized **75d'** (dark grey, stick representation) docked in the active site of HDAC2 (green, stick representation). A hydrogen bond (orange) between the oxime hydrogen and His146 is visible

In summary, while a binding mode for Psammaplin A and analogues to HDAC1 has been proposed in previous sections, difficulties to rely it to the potency observed for methylated oxime derivatives led us to question this model and to hypothesise three alternative binding modes. In those, the zinc(II) cation is

pentacoordinated, by Asp181, Asp269, His183, the thiol and the amide oxygen of the ligand. The aromatic ring is involved in hydrophobic interactions at the entrance of the active site, possibly π -stacking with Phe210 and Phe155. In this model, several possible interaction partners were identified to interact with the oxime:

- His146 *via* a structural water molecule (C=N - - - HOH - - - His, Figure 42)
Phe210, π -H interaction
- flipped His183 (C=N - - - HHis, Figure 45)
- His146 *via* an isomerised oxime (C=N-OH - - - His, Figure 46)

In order to bring additional support to one of these three proposed binding modes, and in particular the type of interactions underlying their impressive isoform selectivity, a library of new and structurally simplified compounds was designed. These new compounds combine an (hetero)aromatic unit linked to the native *N*-(2-mercaptoethyl)acetamide motif present in Psammaplin A (Scheme 35).

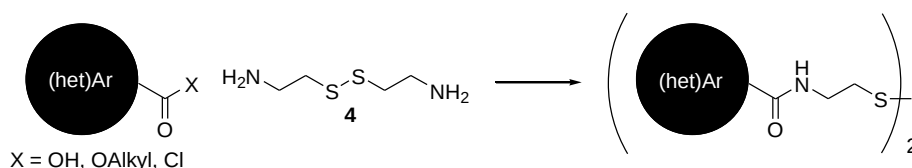


Scheme 35

We felt the use of functionalised aromatics and heteroaromatics to be an obvious choice in order to mimic the oxime or hydrazone of Psammaplin A and analogues. For example, the oxime sp^2 nitrogen can potentially be mimicked by another strong σ -donor atom, such as a pyridine nitrogen. The hydrogen bond donor character of a hydrazone could potentially be reproduced using heterocycles such as pyrrole or pyrazole.

3.22. Synthesis of (hetero)aromatic amides as potential oxime mimics

The synthesis of the different library members was relatively straightforward and was usually achieved through amide bond formation between an aromatic or heteroaromatic carboxylic acid derivative and cystamine **53**. A summary of structures, conditions and yields can be found in Table 12. Yields were generally good, except for compounds **146**, **148**, **152**, **158** and **162**. The low yields obtained for **148**, **152** and **162** were mostly attributed to poor solubility of both the starting materials and products. AlMe₃-mediated amide bond formation between esters and cystamine were capricious, and afforded **146** and **158** in low yields. Carboxylic acids and benzoyl chloride were all obtained from commercial sources. Ethyl 1*H*-pyrrole-3-carboxylate **145** and methyl pyrimidine-2-carboxylate **157** were prepared according literature procedures.^{213,214}



Entry	Starting material	Conditions	product yield	Mimic
139		(i)	140 , 95%	reference
141		(ii), (iii)	142 , 62%	
143		(ii), (iii)	144 , 81%	
145		(iv)	146 , 27% (69% brsm)	

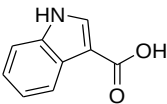
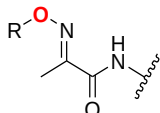
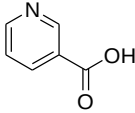
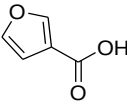
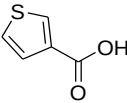
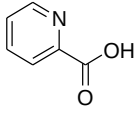
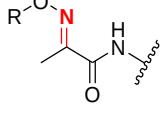
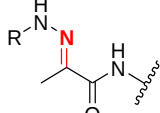
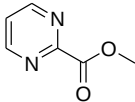
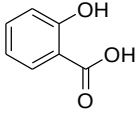
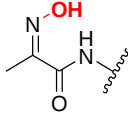
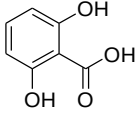
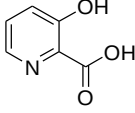
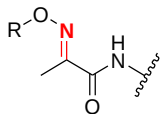
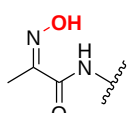
147		(ii), (iii)	148, 9%	
149		(ii), (iii)	150, 64%	
151		(ii), (iii)	152, 20%	
153		(ii), (iii)	154, 78%	
155		(ii), (iii)	156, 80%	 
157		(iv)	158, 31%	
159		(ii), (iii)	160, 59%	
161		(ii), (iii)	162, 16%	
163		(ii), (iii)	164, 53%	 and 

Table 12 - R = H, alkyl

Conditions: (i) PhC(O)Cl, cystamine **53**, Et₃N, CH₂Cl₂, 0°C to rt, 20h; (ii) ArCOOH, EDC, HOBT, CH₂Cl₂, rt, 20 minutes; (iii) cystamine **53**, Et₃N, rt, 14-40h; (iv) ArCOOR, cystamine **53**, AlMe₃, CH₂Cl₂, reflux, 24h

3.23. HDAC inhibition

Dimeric compounds were reduced with TCEP as described previously and the corresponding thiols were assayed against HDAC1 and HDAC6. The results are given in Table 13. Important trends emerged during our search for oxime/hydrazone mimics.

HDAC1: Analogues bearing an H-bond acceptor in a 1,3 relationship with the amide were found to be only moderately potent (IC_{50} s), as exemplified with furan **152'** (12 μ M) which was in the same range of potency as the reference compound **140'** (3.7 μ M), bearing a simple phenyl ring. Varying the polarisability of the acceptor, for example with a thiophene **154'** (6.0 μ M) did not have a significant effect on potency. Introduction of the strong σ -donor pyridine **150'** again did not lead to any improvement. Replacing the H-bond acceptor by a H-bond donor did not have any significant influence. Indeed, pyrrole **146'** (7.2 μ M) and indole **148'** (2.1 μ M) still displayed potencies comparable to reference compound **140'** (3.7 μ M).

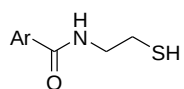
In contrast, analogues bearing a hydrogen bond acceptor, typically a nitrogen, in a 1,2 relationship with the amide were found to be more potent than the corresponding analogues lacking this nitrogen in each case. This is clear when comparing potencies of analogues **152'** (11.8 μ M) vs. **142'** (0.51 μ M), **146'** (7.2 μ M) vs. **144'** (0.48 μ M), **140'** (3.8 μ M) vs. **156'** (0.15 μ M) and **160'** (2.0 μ M) vs. **164'** (0.24 μ M). In particular, **156'** (0.15 μ M) was the best HDAC1 inhibitor and was 25 times more potent than reference compound **140'** (3.8 μ M). 6-Membered ring systems were found to give increased potency compared to 5-membered ones. Indeed, both pyridine **156'** (0.15 μ M) and pyrimidine **158'** (0.22 μ M) were slightly more potent than isoxazole **142'** (0.51 μ M) and pyrazole **144'** (0.48 μ M). In addition to the differences in electronic properties, this could be related to the value of the C-C=N angle and therefore the spatial position of the nitrogen respect to other functional groups on the molecule.

The *o*-hydroxy substituent of **160'**, designed to imitate an isomerized ((*Z*) geometry) was not found to be significantly more potent than reference compound **140'**. However, functionalisation of both *ortho* positions led to a slight increase in

potency, compound **162'** (0.61 μM) being 3 and 6 times more potent than **160'** (2.0 μM) and **140'** (3.7 μM) respectively.

As seen previously, analogues bearing an sp^2 nitrogen adjacent to the amide were found to be very potent and selective against HDAC1. Although the steric and electronic properties of an oxime/hydrazone and the heterocycles analysed are different, we hypothesise these compounds to bind to HDAC1 in a similar way to Psammaplin A. Thus, this study strongly suggests the potency and selectivity of the oxime unit of Psammaplin A to be mostly related to the effect of its nitrogen atom, rather than the H-bond donor character of its hydroxyl functionality. Indeed, every (hetero)aromatic unit employed to imitate the H-bond donor ability of an oxime, in both the (*E*) or (*Z*) geometry, were found not to be significantly more active than the reference compound **140'**. Taken together, these observations disfavour the model previously proposed in section 3, involving a monodentate Zn(II) coordination and a hydrogen bond between the oxime and Asp99 (HDAC1 numbering) at the entrance of the active site. The important difference in potency between **142'**, **144'**, **156'**, **158'**, **164'** and reference compound **140'** is likely to reflect the involvement of the nitrogen in specific interactions with HDAC1.

Interestingly, preliminary results suggest hit compounds, bearing a nitrogen atom in *ortho* position, to be highly selective for HDAC1 compared to HDAC6. This is clearly exemplified by **158'** and **164'**, both displaying $\text{IC}_{50\text{S}} > 35 \mu\text{M}$ against HDAC6, while being submicromolar inhibitors of HDAC1.



Compound	Ar	HDAC1 IC ₅₀ s (μM)	HDAC6 IC ₅₀ s (μM)	Mimic
140'		3.8		Reference
142'		0.51		
144'		0.48		
146'		7.2		
148'		2.1		
150'		5.2		
152'		11.8		
154'		6.0		
156'		0.15		

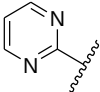
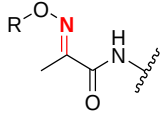
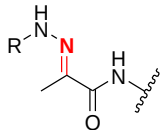
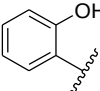
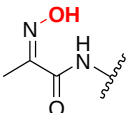
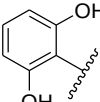
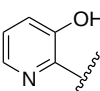
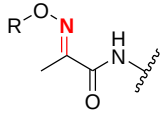
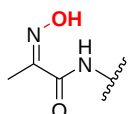
158'		0.22	> 35	
				
160'		2.0		
162'		0.61		
164'		0.24	> 35	 and 

Table 13 - HDAC inhibition data for (hetero)aromatic amide containing analogues

3.24. Further SAR around *N*-(2-mercaptoethyl)picolinamide **156'**

Based on the SAR data around this new series, we decided to consider the potent and structurally simple inhibitor **156'** as a starting point for further investigation. New docking studies were performed using the crystal structure of HDAC2 (pdb 3MAX)⁷⁵ and the best pose is presented in Figure 47 and Figure 48. In this model, the *N*-(2-mercaptoethyl)amide motif coordinates the Zn(II) cation in a bidentate way, as previously proposed for analogue **75d'** (Figure 41). Interestingly, VDW radii did not have to be decreased in order to allow the ligand to adopt this conformation. This can potentially be explained by the difference in size between the pyridine ring and the hydroxyiminophenylalanine motif of **75d'**,

allowing easier access of ligand **156'** to the active site *via* the narrow entrance channel. As expected, no specific interaction between the pyridine nitrogen and the protein could be realised by docking, as the software is unable to predict the existence and position of potential structural water molecules or enzyme dynamics. According to this model, substituents in positions 2, 3 and 4 should be tolerated, and substituents in position 5 would be expected to induce a steric clash with the enzyme and therefore a drop in potency.

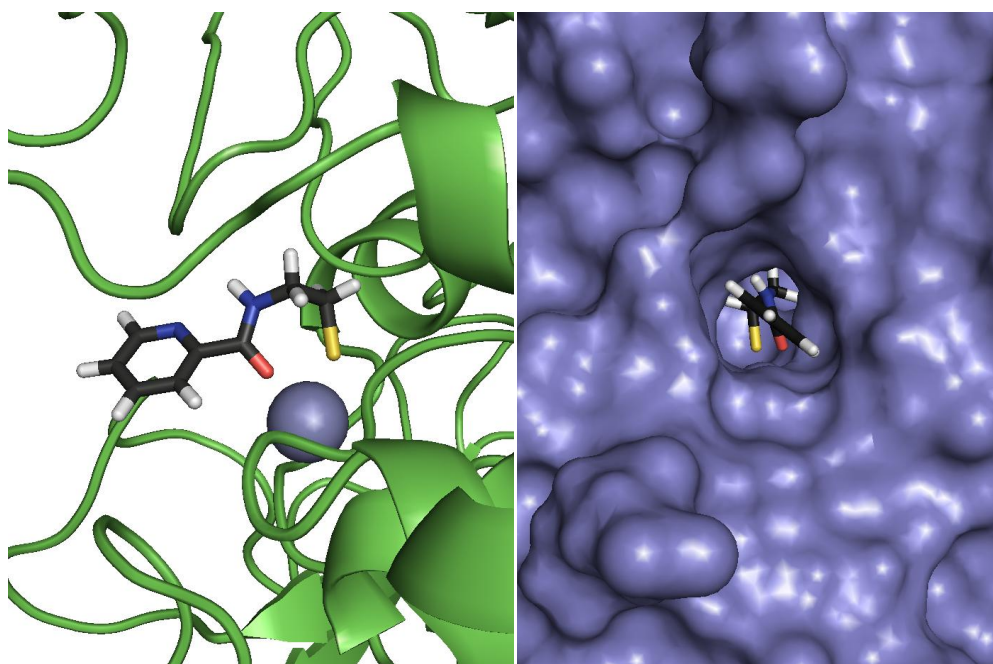


Figure 47 - Left: ligand **156'** (dark grey, stick representation) docked in the active site of HDAC2 (green, ribbon representation). Zn(II) (grey sphere) is visible; right: ligand **156'** (sticks representation) docked in the active site of HDAC2 (surface representation)

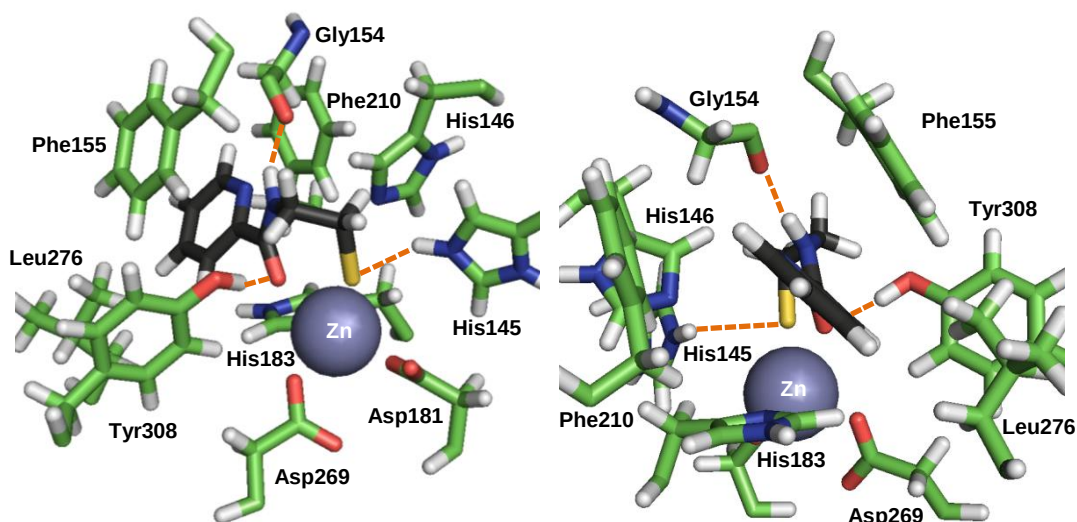


Figure 48 - Left: bidentate binding mode proposed for the *N*-(2-mercaptoethyl)acetamide motif of **156'** (dark grey: carbon, white: hydrogen, red: oxygen, blue: nitrogen, yellow: sulfur; stick representation). Hydrogen bonds of interest with the protein (green: carbon, white: hydrogen, red: oxygen, blue: nitrogen; stick representation) are highlighted in orange; right: backview of ligand **156'** in the active site of HDAC2. Hydrogen bonds of interest are highlighted in orange

Based on this new model, we hypothesized that careful introduction of aromatic substituents may have an influence on the potency against HDAC1 (Figure 49). In addition to steric considerations, the introduction of electron withdrawing groups (EWG) would be expected to induce a decrease of the electronic density of the ring and notably the pyridine nitrogen. Conversely, introduction of electron donating groups (EDG) may counterbalance the deactivating effect of the electron-withdrawing amide and increase the electronic density of the ring, which should potentially strengthen any proposed H-bonding with the pyridine nitrogen.

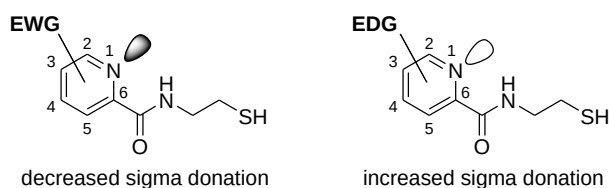
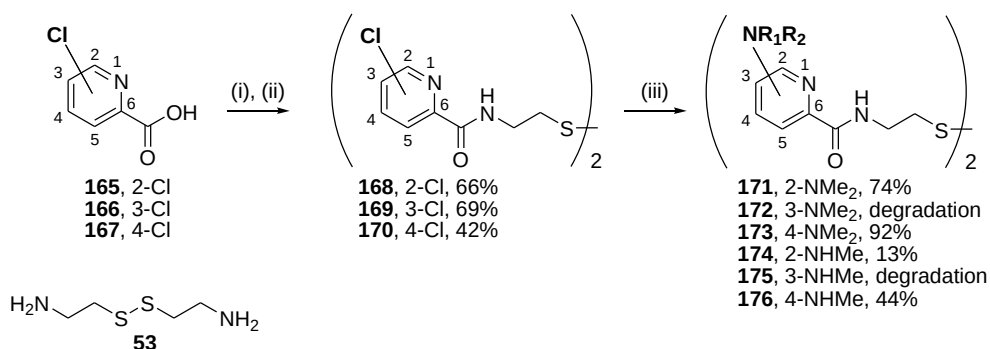


Figure 49

For a preliminary study, a chlorine or amine as substituent was chosen. Indeed, the electronegative chlorine was thought to be a particularly good deactivating group, moreover a number of chloropyridine carboxylic acids are commercially available and inexpensive. On the contrary, the strong +M effect of amine substituents should be particularly efficient for increasing the electronic density of the ring. Simple methylamine and dimethylamine were therefore chosen as model substituents. Variation of the amine substitution pattern should not only inform on the electronic influence on potency, but also the effect of substituents size in relation to its location.

The synthesis of target compounds is described in Scheme 36. EDC-mediated coupling of chloropyridine carboxylic acids **165-167** with cystamine **53** afforded analogues **168-179** in moderate to good yields. Subsequent S_NAr afforded the corresponding amino derivatives **171-176**. In each case, substitution on position 4 was found to be more efficient, due to the increased stabilisation by the pyridine nitrogen. Substitution at positions 2 and 4 with methylamine afforded **174** and **176** in low yields, owing to the lower reactivity of methylamine, but also possibly due to oligomerisation. Substitution with either methylamine or dimethylamine led to degradation when attempted on position 3.



Reagents and conditions:

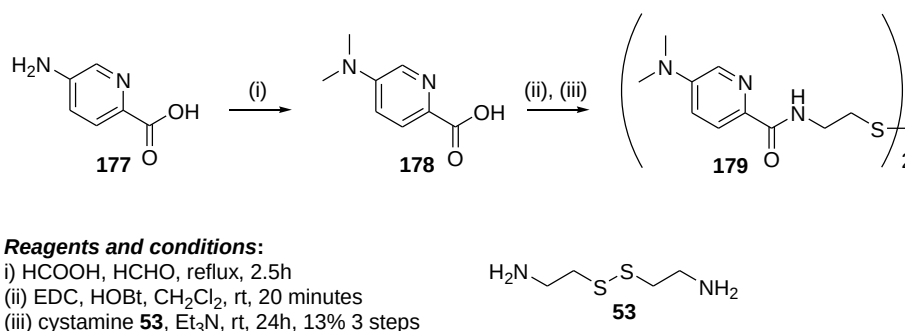
(i) EDC, HOBT, CH₂Cl₂, rt, 20 minutes

(ii) cystamine **53**, Et₃N, rt, 24h

(iii) H₂NMe or HNMe₂ (40% w/w in water), THF, microwave, 160°C

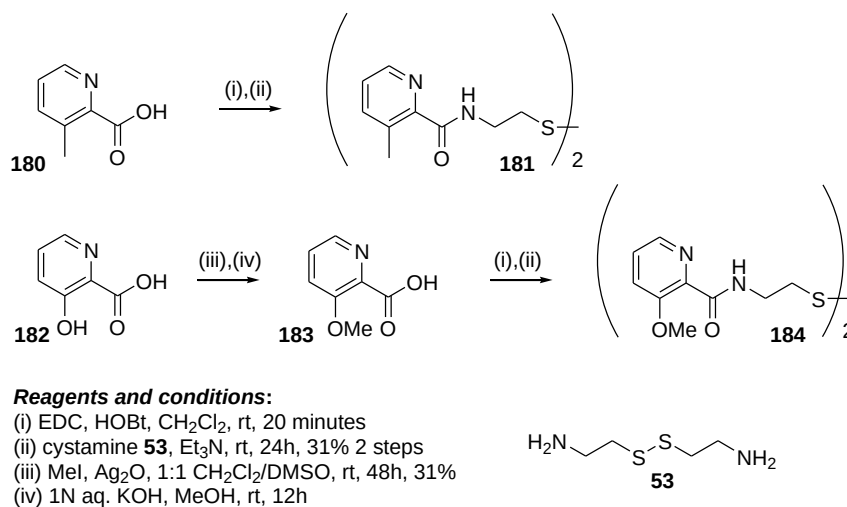
Scheme 36

Since introduction of amine substituents in position 3 was unsuccessful by S_NAr , other conditions were attempted. An Eschweiler-Clarke reaction between 5-aminopicolinic acid **177**, formic acid and formaldehyde as previously described²¹⁵ afforded 5-(dimethylamino)picolinic acid **178**, which was then coupled with cystamine and afforded analogue **179** in 13% yield after 3 steps.



Scheme 37

In order to study the influence of substitution in position 5 (Figure 49), two analogues were synthesised (Scheme 38). EDC mediated coupling between 3-methylpicolinic acid **180** and cystamine **53** afforded 5-methyl substituted analogue **181** in high yield. Double methylation of 3-hydroxypicolinic acid **182** using methyl iodide and silver oxide afforded methyl 3-methoxypicolinate **183** in 31% yield. Hydrolysis under basic conditions, followed by EDC-mediated amide bond formation with cystamine **53** afforded 5-methoxy substituted analogue **184** in 31% yield over 2 steps.



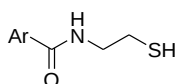
Scheme 38

3.25. HDAC inhibition

Dimeric compounds were reduced with TCEP as described previously and the corresponding thiols were assayed against HDAC1 and HDAC6. The results are given in Table 14.

HDAC1: Chlorinated pyridines **168'**-**170'** were found highly potent (IC_{50}) against HDAC1. In particular, analogue **168'** (0.021 μ M) was found 7 times more active than the reference pyridine compound **156'** (0.15 μ M). In contrast, introduction of amines in positions 2, 3 or 4 did not lead to any improvement in potency, as exemplified by analogues **171'**, **179'**, **173'** and **176'** being similarly potent or slightly less potent (0.17-0.54 μ M) than **156'**. Finally, substitution of position 5 with a methyl or a methoxy group did not have a dramatic influence on potency.

While introduction of the electronegative chlorine led to a 7-fold increase in potency, introduction of the electron-donating amines did not lead to any improvement, suggesting the electronic properties of the pyridine ring to be of importance. Moreover, it seems the position of the chlorine (2, 3 or 4) does not have a dramatic influence, and affords a highly potent compound in each case. At this time, the reasons for these observations are still unclear and subject to further investigation.



Compound	Ar	HDAC1 IC_{50} s (μ M)	HDAC6 IC_{50} s (μ M)
156' (reference)		0.15	
168'		0.021	

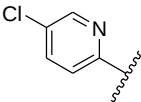
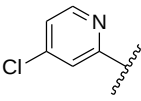
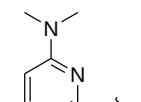
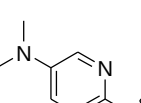
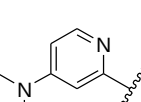
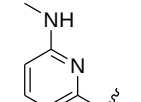
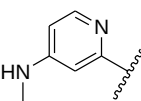
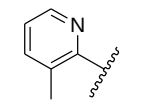
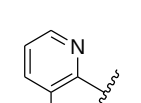
169'		0.087
170'		0.052
171'		0.54
179'		0.42
173'		0.29
174'		
176'		0.17
181'		0.45
184'		0.22

Table 14 - HDAC inhibition data for substituted pyridine containing analogues

In conclusion, our search for oxime mimics led us to the discovery of structurally simple and highly potent pyridine containing compounds against HDAC1 (Figure 50). New computational studies, in addition to biological assessment of these compounds against HDACs led us to propose several alternative binding modes for Psammaplin A and analogues. Interestingly, the

isoform selectivity (HDAC1 and 6) previously observed for Psammaplin A was retained with our new inhibitors, which represent a highly valuable advantage for the further development of isoform selective probes for *in vivo* investigation. The establishment of a more accurate isoform selectivity profile for these inhibitors is in progress. In particular, assessment of selected analogues (**156'** and **168'**) against other HDAC isoforms, notably HDAC7 and HDAC8, will be reported in future manuscripts.

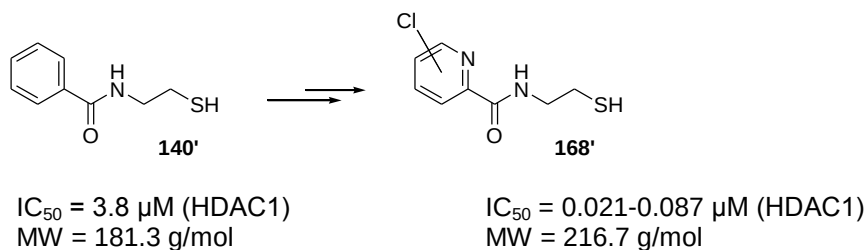


Figure 50 - progress achieved in our search of novel isoform selective inhibitors

3.3. Current/future work and conclusions

Current work (Figure 51, general structure **185'**) is involving further variation of the aromatic substitution pattern and its influence on potency. In particular, other halogens (Cl vs F, Br, I), deactivating groups (e.g. NO₂, CO₂R) and electron donating groups (e.g. NR₂, OR, alkyl) will be probed in order to draw a robust SAR around this pyridine series and improve the potency of our compounds.

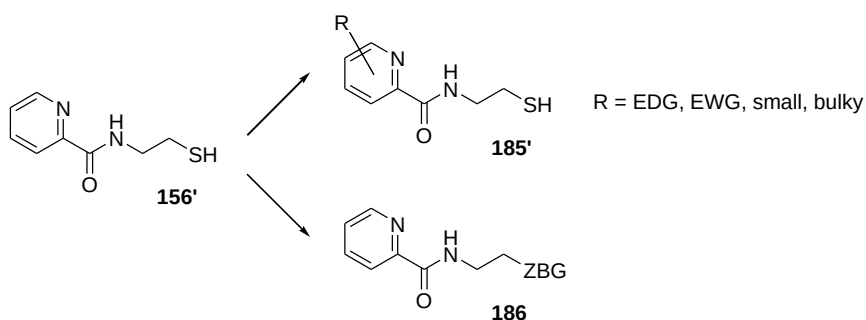


Figure 51 - current work

In parallel, a number of more drug like chemical functionalities will be probed with a view to replacing the thiol moiety of our compounds, while

retaining potency (Figure 51, general structure **186**). For this purpose, the simple pyridine derivative **156'** will be used as a starting point. The presence of the thiol in our pyridine based analogues is very important for potency, as similarly observed previously in section 3 for Psammaplin A derivatives. Indeed, whereas the reduced form of Psammaplin A (**28'**) was highly potent (IC_{50}) against HDAC1 (0.0009 μ M), its methylthioether analogue **75a** was 8500 times less active (7.7 μ M). Interestingly, while the potency of **75a** was low, it proved that low micromolar potency is potentially achievable using a ZBG as weak as a methylthioether. The discovery of potent HDAC inhibitors not primarily relying on their ability to strongly bind to the active site zinc would represent a major advance in the field of HDACis. Indeed, the potency of most HDACis reported in the literature relies on the presence of a strong ZBG (e.g. hydroxamic acid, thiol, electrophilic ketone...), which possibly contributes to their usual toxicity and possible off-target effects.

We envisage the current study has the potential to lead to the discovery of new small molecule HDAC inhibitors whose potency does not involve zinc coordination as the principal factor for binding. In particular, we hope that optimisation of the pyridine substitution pattern in combination with a weak zinc binding group will lead us to the discovery of submicromolar selective HDAC1 inhibitors. This would be unprecedented and would represent a highly valuable tool not only for the development of selective probes for *in vivo* studies, but also for the discovery of potential drug candidates for clinical trials. Results of this study will be reported in further manuscripts.

4. Towards photoswitchable HDAC inhibitors

4.1. Introduction

Cellular processes are mediated by a myriad of complex and intricate biochemical pathways, involving thousands of biomolecules interacting with each other in a temporal and spatial manner. As such, probing the spatiotemporal roles of a biomolecule in its native environment is highly challenging. The use of light as a means to control biomolecules is of particular interest, since irradiation of samples can be controlled with high spatial and temporal precision. Moreover, technological advances during the last decades allowed for routine access to a variety of light sources with accurately adjusted wavelength, power and delivery mode.

In this context, the development of systems which allow the control of biological processes using light has become an increasingly attractive area of research during the last decades.²¹⁶ A number of chemical functionalities have been reported to allow a variety of effects to be produced *in vivo* as a result of light irradiation. Well-known examples of photoresponsive systems include porphyrin-based metal complexes and azobenzene derivatives²¹⁷ (Figure 52). Upon irradiation, porphyrin complexes are able to catalyse oxidoreduction reactions and are notably able to convert triplet oxygen ($^3\text{O}_2$) into cytotoxic singlet oxygen ($^1\text{O}_2$). Irradiation of azobenzene induces a *cis/trans* isomerisation, which can be exploited to modify the conformation of biological systems and therefore influence their behaviour. Another example of light-sensitive systems which have found applications in biology are caged organic molecules, whose effect is revealed after cleavage of a photo-labile group with light.²¹⁸

In particular, a number of studies aimed at controlling biological systems using photoswitchable azobenzene containing small molecules have shown significant success. The photocontrol of biological processes using azobenzene functionalities is usually achieved *via* either covalent introduction of the photoswitch into the biological system (e.g. protein) itself, or *via* the development of azobenzene containing small molecules able to interact with this biological system. Recent examples include notably the development of photochromic ion channel ligands,²¹⁹ photoswitchable oligonucleotides, lipids and carbohydrates.²¹⁷

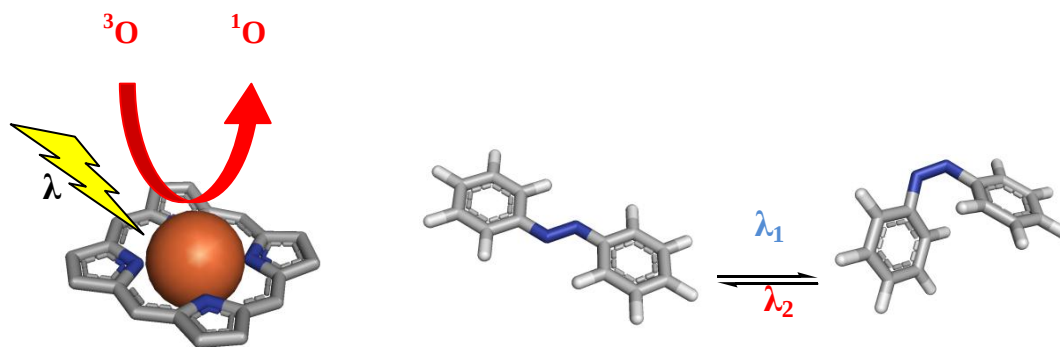


Figure 52 - Examples of photoresponsive systems; Left: photoinduced generation of singlet oxygen using porphyrin derivatives. Grey: carbon; blue: nitrogen; white: hydrogen; orange: metal; Right: *cis/trans* azobenzene photoisomerisation. Grey: carbon; blue: nitrogen; white: hydrogen

While controlling biological systems with light represents a powerful tool for probing the role of biomolecules *in vivo*, important downstream applications are possible in therapy. One of the most famous applications of photoresponsive systems in the clinic is the development of the concept of “photodynamic therapy”.

Photodynamic therapy:

“Photodynamic therapy” (PDT) refers to the use of a light-sensitive drug (photosensitizer, PS) in combination with light in order to destroy a particular subset of target cells (e.g. cancerous cells).²²⁰ PDT can be used as a primary treatment or in conjunction with others such as chemotherapy, radiotherapy or surgery. PDT has been used in the treatment of various invasive and non-invasive tumours, age-related macular degeneration, cardiovascular disease, psoriasis, acne vulgaris and viral warts.²²¹ In particular, an important number of studies have been focusing on the development of porphyrins inspired PS for PDT.^{216,222} Upon irradiation in cells, these compounds are able to generate $^1\text{O}_2$ and other highly reactive and cytotoxic oxygenated species (e.g. H_2O_2).²²³

PDT involves several steps (Figure 53): **i)** administration of the PS into the organism, **ii)** accumulation of the PS into the target cell population, **iii)** irradiation

of the tumour with a regulated dose of light, and at an adequate wavelength, **iv**) activation of the drug upon irradiation and subsequent induction of the cytotoxic effect. Wavelengths between 600 and 1200 nm are particularly efficient for penetrating living tissues. Highly energetic (e.g. UV, X-ray, γ -ray) wavelengths should be avoided in order to prevent damaging of neighbouring tissues.

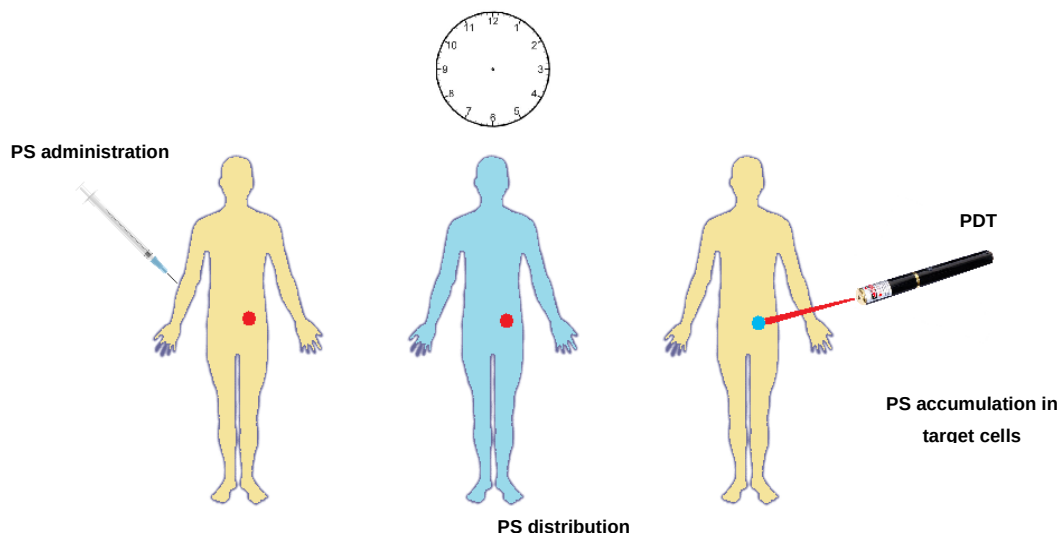


Figure 53 - Principle of PDT

One particular advantage of using PDT lies in the possibility to achieve high regioselectivity for tissue targeting. Indeed, provided the PS is not intrinsically cytotoxic, only the irradiated areas (e.g. tumour) will be affected.

PDT is considered to be a new and promising strategy for cancer treatment. While a number of PSs have already been evaluated in clinical trials, the full potential of the PDT approach is still to be shown, alone or in combination with other therapies. As previously mentioned, the vast majority of currently developed PSs are porphyrin derivatives.²¹⁶ In this study, we were particularly interested in the design and synthesis of novel light-responsive small molecules with the view in the long term to develop new agents for PDT. We were particularly interested in developing new molecules which cytotoxicity upon irradiation in cell does not rely on $^1\text{O}_2$ formation. Indeed, while hundreds of these compounds (e.g. porphyrin based) have been reported, the use of non-porphyrin-based PSs in PDT is severely undocumented. The development of such molecules could therefore open the door to a number of new agents for PDT, with novel modes of action.

4.2. Results and discussion

4.21. Design of photochromic HDAC inhibitors

Based on our high interest in HDACs, we decided to undertake a preliminary investigation in order to study the possibility of controlling HDAC activity *in vitro* with light. In the long term, the major goal of such a study is to develop novel azobenzene containing small molecules which would enable us to control HDAC activity in cells using light, and possibly reprogramme gene expression as a downstream consequence of irradiation. Ultimately, optimisation of such a system would be of interest for the development of the concept of “epigenetic photodynamic chemotherapy” (EPDCT) against cancer. In particular, photocontrolled reactivation of important genes, such as TSGs, could be of particular interest for inducing cell cytotoxicity in cancer cells. While an important number of small molecule PSs are based on the generation of singlet oxygen, the development of epigenetic modulator PSs and applications in PDT have never been reported.

We therefore decided to conduct a proof of concept study *in vitro*, through the derivatisation of simple ligand **187** (Figure 54). **187** has been previously reported to inhibit HDAC activity.²²⁴ It contains a hydroxamic acid, which is thought to bind the Zn(II) cation in the active site of HDACs.

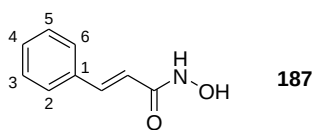


Figure 54

Docking studies using the crystal structures of HDAC2 (pdb 3MAX),⁷⁵ revealed the molecular features of **187** responsible for inhibiting its HDAC targets (Figure 55). The hydroxamic acid moiety complexes the zinc as previously reported.⁷⁶ The two carbon linker positions itself in the hydrophobic channel and the aromatic ring is flanked between the two phenylalanine residues lying at the entrance of the active site.

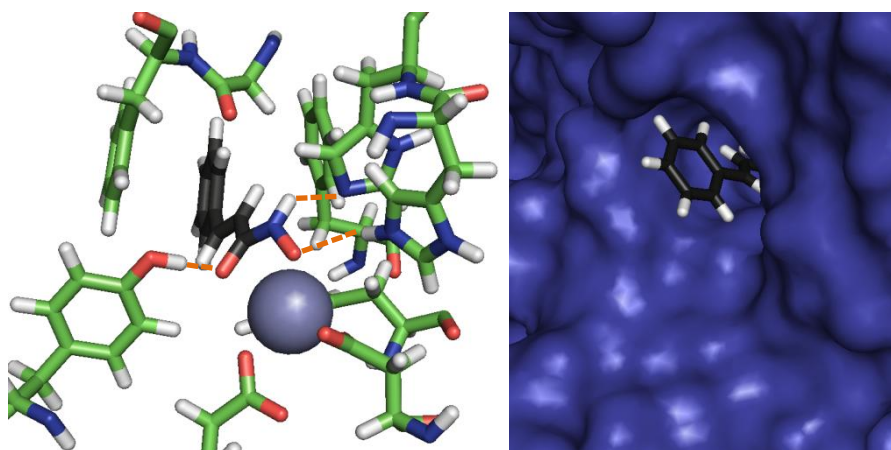


Figure 55 - Left: **187** (dark grey, stick representation) docked in the active site of HDAC2 (green, stick representation, pdb 3MAX). The Zn(II) cation is shown as a grey sphere. Right: ligand **187** docked in HDAC2. The aromatic ring of **187** positions at the entrance of the channel

For the photoswitch we chose an azobenzene moiety. The rationale behind this choice lies in the narrow channel within the HDAC active site. Indeed, the tubular pocket leading to the active site of HDACs is only 3-5Å wide and only ligands exhibiting a cross-section smaller than the diameter of the entrance channel are therefore able to bind. Introduction of an azobenzene at the level of the aromatic ring of **187** would ideally position it at the entrance of the tubular channel. In an ideal case, while the *trans* isomer of the inhibitor would be able to inhibit the HDAC, the *cis* isomer would induce a steric clash with amino acids lining the channel (Figure 56) and decrease the stability of the whole complex. Rigorously, the two different isomers should exhibit different affinities, and we hoped this difference in affinity to be important enough to observe a significant alteration of the enzyme activity/inhibition upon switching.

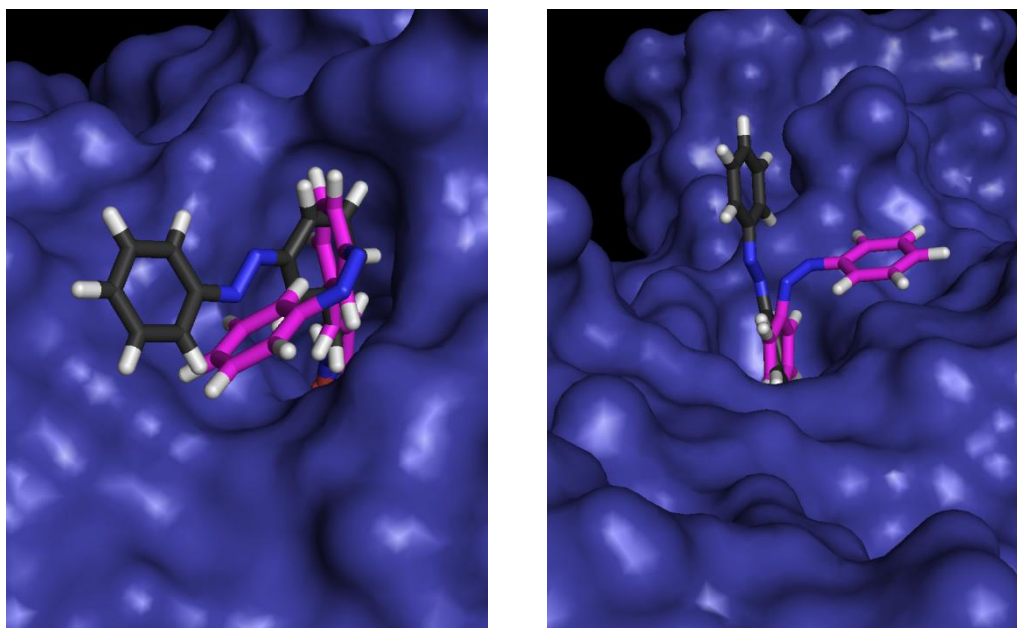


Figure 56 - Left: *trans*-**201** (dark grey, stick representation) and *cis*-**201** (magenta) docked in the active site of HDAC2 (blue, surface representation). Left: *trans*-**202** (dark grey, stick representation) and *cis*-**202** (magenta) docked in the active site of HDAC2 (blue, surface representation)

The docking poses generated with HDAC2 (Figure 56) suggested the functionalisation of positions 1 and 2 to be particularly appropriate for a model study. Functionalisation of position 3 was thought not to be suitable, potentially sterically destabilising the enzyme-ligand complex for both isomers (Figure 56).

4.22. Synthesis of azobenzene-hydroxamic acid conjugates

We therefore undertook the preparation of two derivatives of **187**, derivatised with an azobenzene moiety in positions 1 and 2 respectively. The structures of the two derivatives **188** and **189** are given in Figure 57. A simple, unfunctionalised azobenzene moiety was chosen for this preliminary study, both for proof of concept and ease of synthesis.

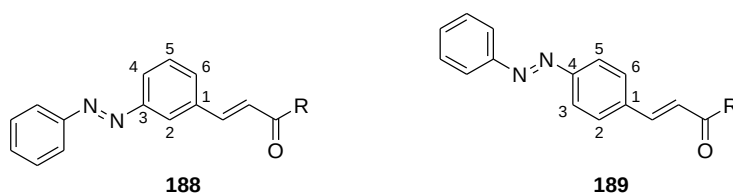
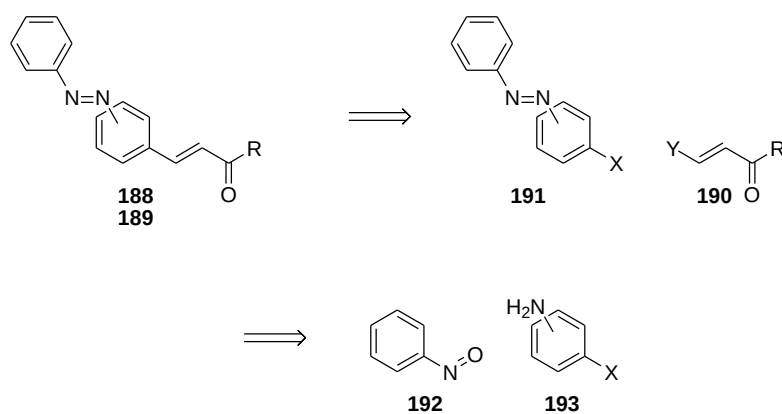


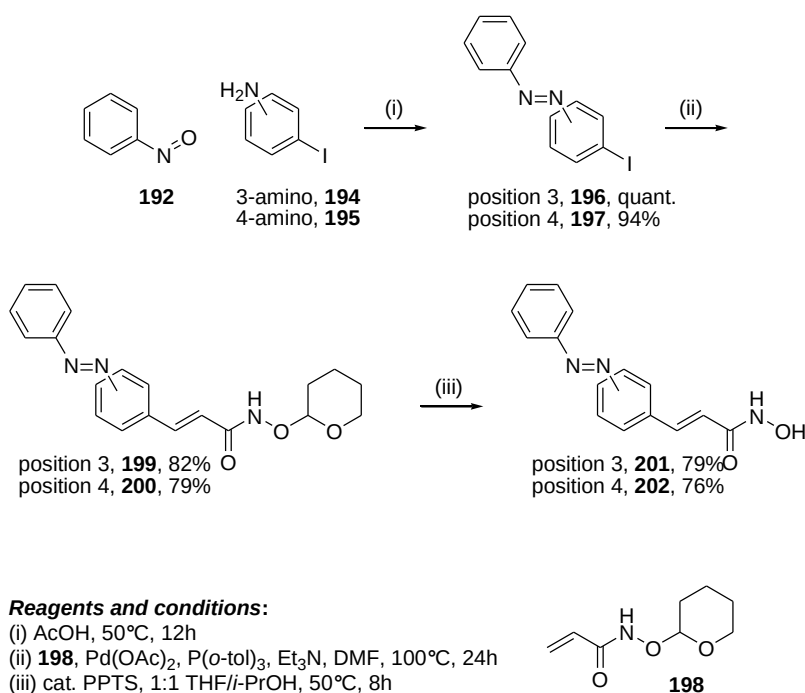
Figure 57

The retrosynthesis of compounds **188** and **189** is shown in Scheme 39. It was thought **188** and **189** could be obtained *via* a cross coupling between an *O*-protected *N*-hydroxyacrylamide derivative **190** and a halogenated azobenzene derivative. Halogenated azobenzenes **191** should finally be accessed *via* the condensation of nitrosobenzene **192** with haloaniline **193** as previously reported.^{219d}



Scheme 39

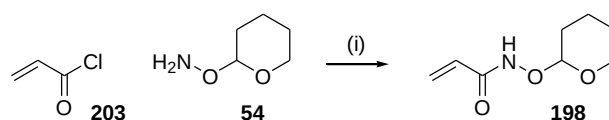
The synthesis of both compounds is shown in Scheme 40. Condensation of nitrosobenzene **192** with 3-iodoaniline **194** and 4-iodoaniline **195** afforded iodinated azobenzene compounds **196** and **197** respectively, in nearly quantitative yield. Coupling of **196** and **197** with *N*-(tetrahydro-2*H*-pyran-2-yl)oxy)acrylamide **198** could be achieved in one step and in high yield *via* a Heck reaction, afforded intermediates **199** and **200**. Heck reactions between aryl halides and *N*-hydroxyacrylamide derivatives have already been reported.²²⁵⁻²²⁷ In particular, a similar Heck coupling between aryl bromides and **198** has already been reported by Bressi and co-workers during their research on new HDAC inhibitors.²²⁷



Scheme 40

Final THP deprotection was achieved by using 0.1 eq. pyridinium *p*-toluenesulfonate (PPTS) at 50°C in a 1:1 THF/*i*-PrOH mixture. These mild conditions afforded 3-diazo and 4-diazo substituted analogues in 79% and 76% yield respectively. This three step sequence allowed for the synthesis of **201** and **202** in 65% and 56% overall yield respectively. Interestingly, this synthetic route was found to be particularly robust and easily scalable. The two first steps could be achieved on a multigram scale (**196**, **197**: ~ 5.6 g; **199**, **200**: ~ 1.7 g). Starting from 500 mg of **199** and **200**, 302 mg of **201** and 288 mg of **202** could be obtained as amorphous orange solids. Attempted recrystallisation, using a number of solvent systems, yielded an amorphous solid in each case.

The synthesis of the THP protected *N*-hydroxyacrylamide fragment **198** was straightforward (Scheme 41). Acylation of *O*-(tetrahydro-2*H*-pyran-2-yl)hydroxylamine **54**¹⁵⁵ with acryloyl chloride **203** afforded **198** in 60% yield.



Reagents and conditions:
(i) Et₃N, CH₂Cl₂, 0°C to rt, 24h, 60%

Scheme 41

4.23. HDAC inhibition

As a starting point for our study, the *in vitro* potency (IC_{50}) of *trans* azobenzene containing analogues **201** and **202** against HDAC1 was determined. The results were obtained in collaboration with Pr. Franz-Josef Meyer-Almes (University of Applied Sciences, Darmstadt, Germany) and are given in Table 15. *Trans* isomers **201** and **202** exhibited submicromolar IC_{50} s against HDAC1.

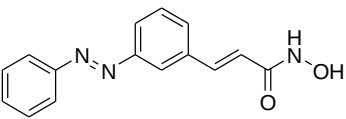
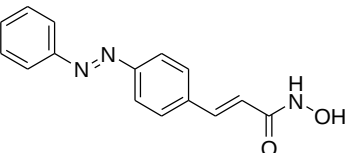
Compound	Structure	HDAC1 IC_{50} s (μ M)
201		0.186
202		0.478

Table 15

4.24. Photophysical study of **201** and **202**

The photophysical properties of **201** and **202** in solution (DMSO) were then studied in collaboration with Dr. Marina Kuimova (Department of Chemistry, Imperial College London). All data were obtained in collaboration with Dr. Marina Kuimova (Imperial College London). The UV-Vis spectra of pure *trans*-**201** and *trans*-**202** are shown in Figure 58. All samples were prepared in the dark prior to irradiation. Absorption maxima around 350 nm could be observed for both compounds (Table 16). $\lambda_{\max}$ and $\epsilon_{\max}$ for **201** and **202** are given in Table 16.

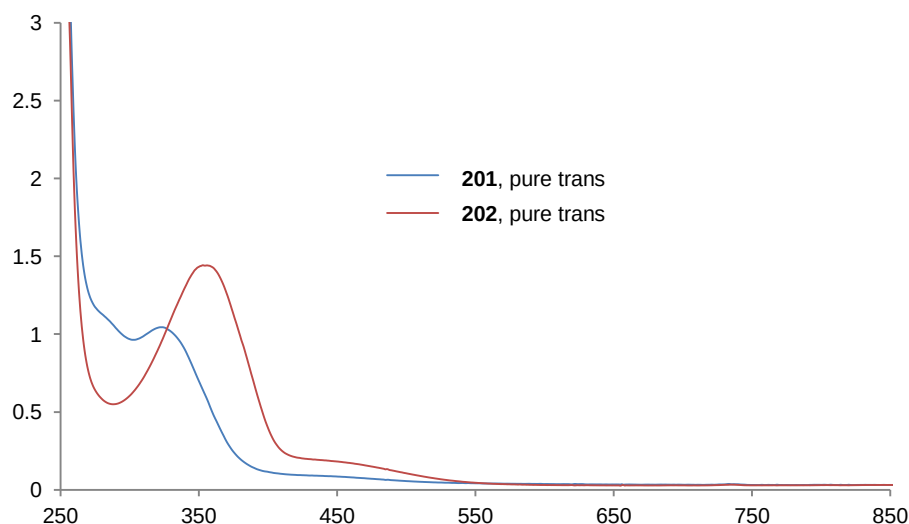


Figure 58 - UV spectra in of **201** and **202** in their pure *trans* state (DMSO, $C = 5 \times 10^{-5}$ M, 293K). X-axis: wavelength (nm), Y-axis: absorbance

	$\lambda_{\max}$ (nm)	$\epsilon_{\max}$ (L.mol ⁻¹ .cm ⁻¹)
201	324	20800
202	353	28800

Table 16

We then studied the photophysical properties of **201** and **202** upon irradiation. As previously mentioned, irradiation of azobenzene derivatives at a given wavelength rarely induces a quantitative *trans*→*cis* photoisomerisation, but generally affords a mixture of both isomers at the photostationary state (PhS). Both compounds were irradiated at 355 nm, close to their maximum absorption, and UV spectra were recorded at regular intervals (Figures 59 and 60). In these conditions, the photostationary state was reached after only a few minutes in both cases. In particular, it was nearly reached after 2 minutes in the case of **202**. A clear isobestic point could be observed at approximately 320 nm in the spectrum of **202**. A significant decrease in the absorption in the 350 nm region could be observed in each case, illustrating the photoconversion of the *trans* compounds. Notably, a new absorption maximum at 302 nm could be observed for **202**.

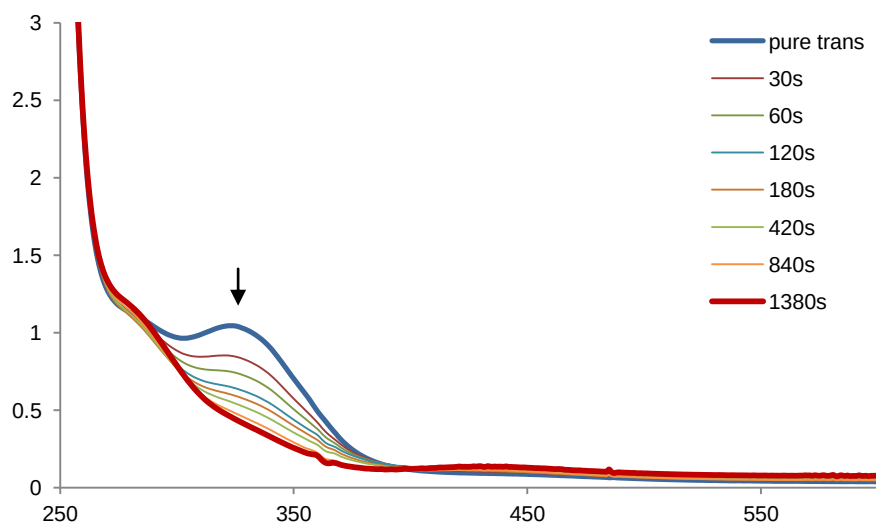


Figure 59 - UV spectrum of **201** as a function of irradiation time at 355 nm (DMSO, $C = 5 \times 10^{-5}$ M, 293K, 20 mW). X-axis: wavelength (nm), Y-axis: absorbance.

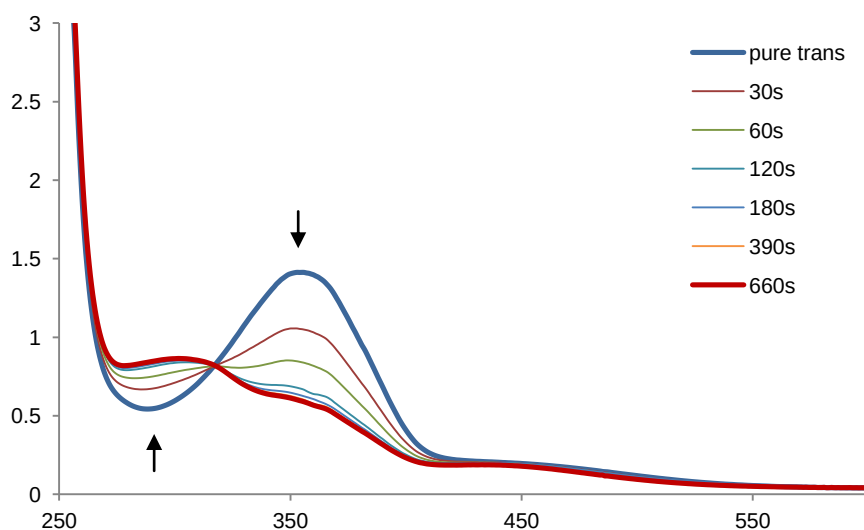


Figure 60 - UV spectrum of **202** as a function of irradiation time at 355 nm (DMSO, $C = 5 \times 10^{-5}$ M, 293K, 20 mW). X-axis: wavelength (nm), Y-axis: absorbance.

The reversibility of this system was then studied. Following irradiation at 355 nm (Figures 59 and 60), it was found that irradiation of the same cuvettes at 430 nm displaced the photostationary state back towards the *trans* isomer (Figures 61 and 62). The new photostationary states were rapidly reached in both cases. It was notably nearly reached in 2 minutes in the case of **202** (Figure 62). While the absorption of **201** and **202** at 430 nm is low and does not change significantly as a function of irradiation (Figures 59 and 60), the photochemical

isomerisation under irradiation at this wavelength is likely to reflect a higher quantum yield for the *cis*→*trans* transition. Interestingly, reversibility of this system could be illustrated by performing two successive isomerisation cycles (*trans*→*cis*→*trans*→*cis*, data not shown) without significant degradation of the system observed by UV.

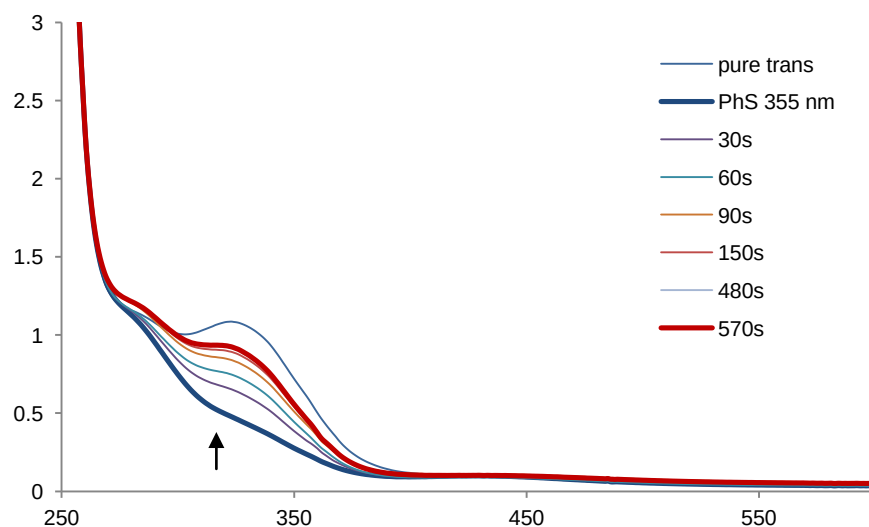


Figure 61 - UV spectrum of **201** as a function of irradiation time at 430 nm, starting from the photostationary state reached under irradiation at 355 nm (PhS 355 nm, t_0), see Figure 59 for comparison. (DMSO, $C = 5 \times 10^{-5}$ M, 293K, 20 mW). X-axis: wavelength (nm), Y-axis: absorbance. The spectrum of pure *trans*-**201** is given as a reference

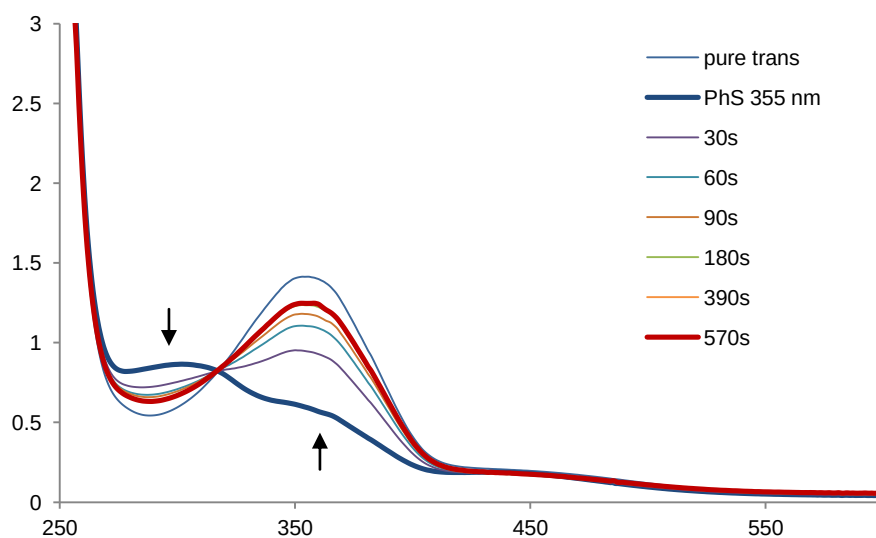


Figure 62 - UV spectrum of **202** as a function of irradiation time at 430 nm, starting from the photostationary state reached under irradiation at 355 nm (PhS 355 nm, t_0), see Figure 60 for comparison. (DMSO, $C = 5 \times 10^{-5}$ M, 293K, 20 mW). X-axis: wavelength (nm), Y-axis: absorbance. The spectrum of pure *trans*-**202** is given as a reference

Following irradiation at 355 nm (Figures 59 and 60), laser promoted *cis*→*trans* isomerisation could also be achieved using a different, higher wavelength. Indeed, while the absorption of **201** and **202** at 532 nm is low and does not change significantly as a function of irradiation (Figures 59 and 60), *cis*→*trans* isomerisation could be observed (Figures 63 and 64).

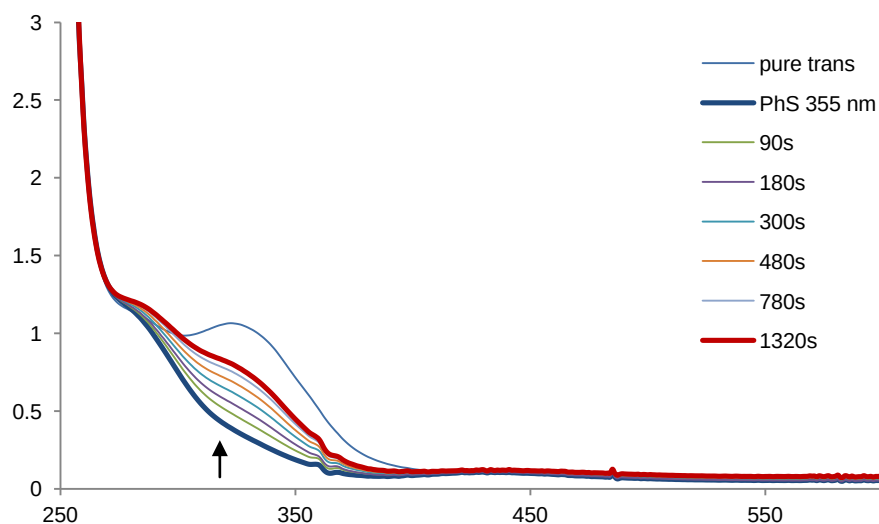


Figure 63 - UV spectrum of **201** as a function of irradiation time at 532 nm, starting from the photostationary state reached under irradiation at 355 nm (PhS 355 nm, t_0), see Figure 59 for comparison. (DMSO, $C = 5 \times 10^{-5}$ M, 293K, 20 mW). X-axis: wavelength (nm), Y-axis: absorbance. The spectrum of pure *trans*-**201** is given as a reference

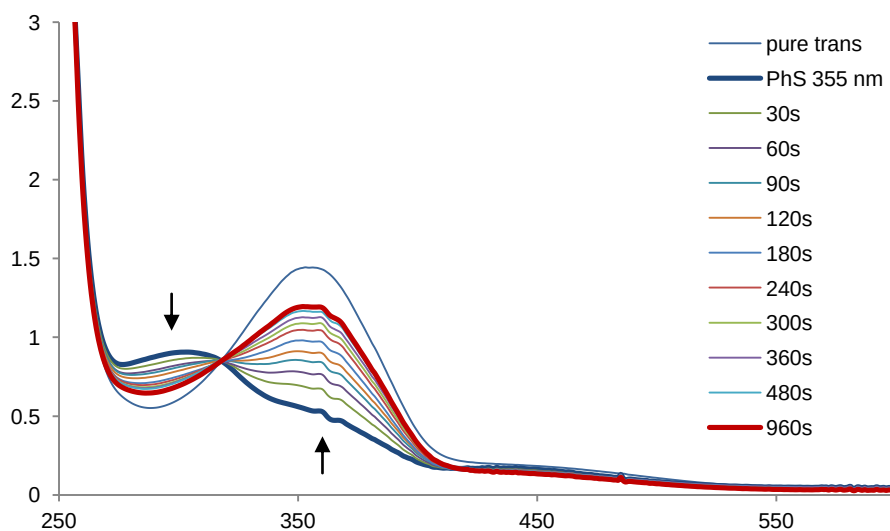


Figure 64 - UV spectrum of **202** as a function of irradiation time at 532 nm, starting from the photostationary state reached under irradiation at 355 nm (PhS 355 nm, t_0), see Figure 60 for comparison. (DMSO, $C = 5 \times 10^{-5}$ M, 293K, 20 mW). X-axis: wavelength (nm), Y-axis: absorbance. The spectrum of pure *trans*-**202** is given as a reference

While irradiation at 532 nm allowed for the photoreversion of both systems after reaching the first photostationary state at 355 nm, it was found less efficient than irradiation at 430 nm (Table 17). Indeed, the absorbance at 324 nm (maximum absorbance of pure *trans* **201**) under 532 nm irradiation was lower (0.80) than under irradiation at 430 nm (0.92), directly reflecting a lower proportion of *trans* **201**. In a similar way, the absorbance at 353 nm (maximum absorbance of pure *trans* **202**) under 532 nm irradiation was lower (1.19) than under irradiation at 430 nm (1.25). Taken together, these data showed the slightly higher efficiency of the 430 nm wavelength for the *cis*→*trans* process in each case.

	A ₃₂₄ (PhS 430 nm)	A ₃₂₄ (PhS 532 nm)	A ₃₅₃ (PhS 430 nm)	A ₃₅₃ (PhS 532 nm)
201	0.92	0.80		
202			1.25	1.19

Table 17

4.25. Current and future work

Determination of *cis/trans* ratios at photostationary states

We are currently performing studies in order to determine the proportions of *cis*-**201** and *cis*-**202** formed at the different photostationary states, and extrapolate their UV-vis spectra using the method developed by Fischer.²²⁸ This method allows the determination of the proportions of two related species at a given photostationary state when only the UV-vis spectrum for the pure starting material and the spectra of the mixture of the two species at two different photostationary states are known. This method has already been successfully applied in the context of diazobenzenes.^{219d} Preliminary data (not shown) suggest a conversion higher than 80% for the *trans* to *cis* photoisomerisation at 355 nm, demonstrating the efficiency of the process. Results of the full study will be reported in future manuscripts.

Half-life of thermal relaxation

While we demonstrated the efficient reversibility of our systems by using the appropriate wavelength for irradiation, their *cis*→*trans* thermal relaxation has not been thoroughly studied. Preliminary NMR experiments suggested the *cis* state to exhibit a relatively long half-life of thermal relaxation. A solution of **202** ($\sim 15 \times 10^{-3}$ M) in d^6 -DMSO was irradiated at 350 nm for 6 hours using a custom set up. The photostationary state could not be reached in these conditions, likely due to the high concentration of the sample and reduced power of the UV source compared to the laser source used for previous studies. However, a new set of signals corresponding to the *cis* isomer was unambiguously observed. Recording NMR spectra at different time intervals after irradiation and integrating the NMR signals of *cis* and *trans* **202** as a function of time (Figure 66) allowed us to obtain a first approximation of the thermal stability of *cis*-**202** in DMSO. The spectrum of pure *trans*-**202** is shown in Figure 65.

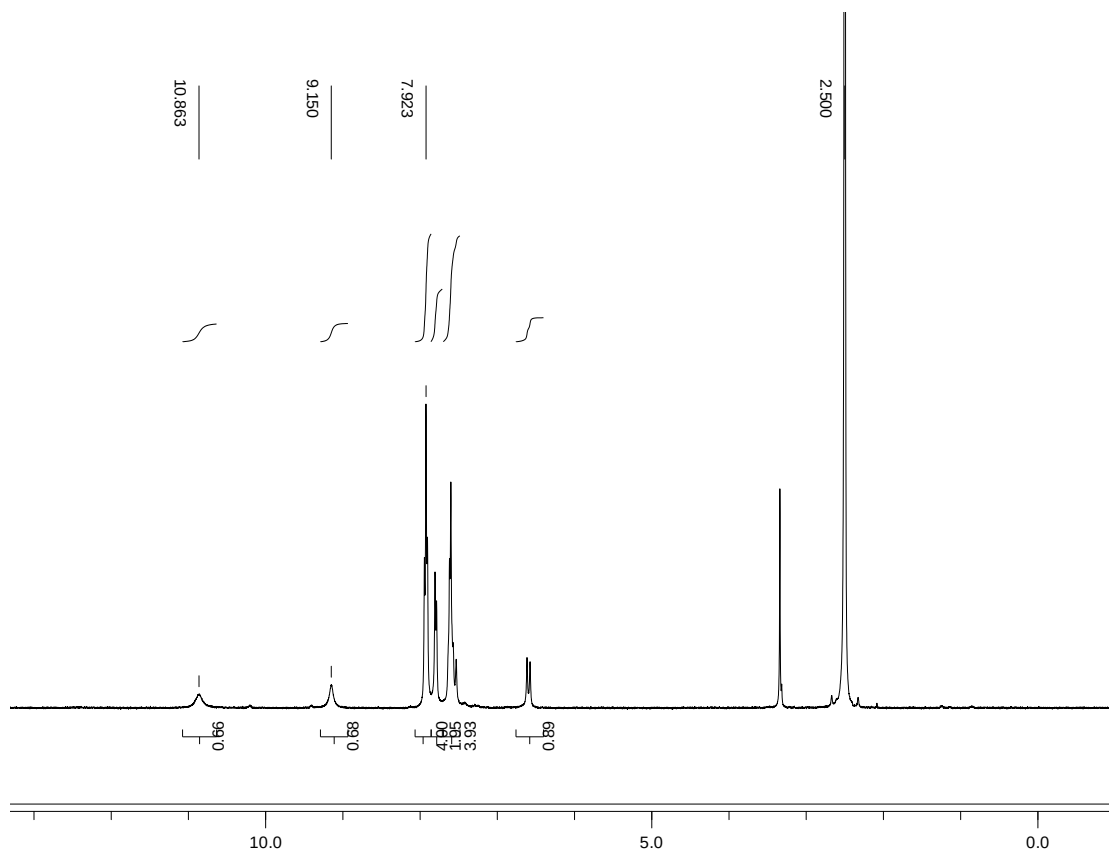


Figure 65 - NMR spectrum of **202** in the pure *trans* state (before irradiation)

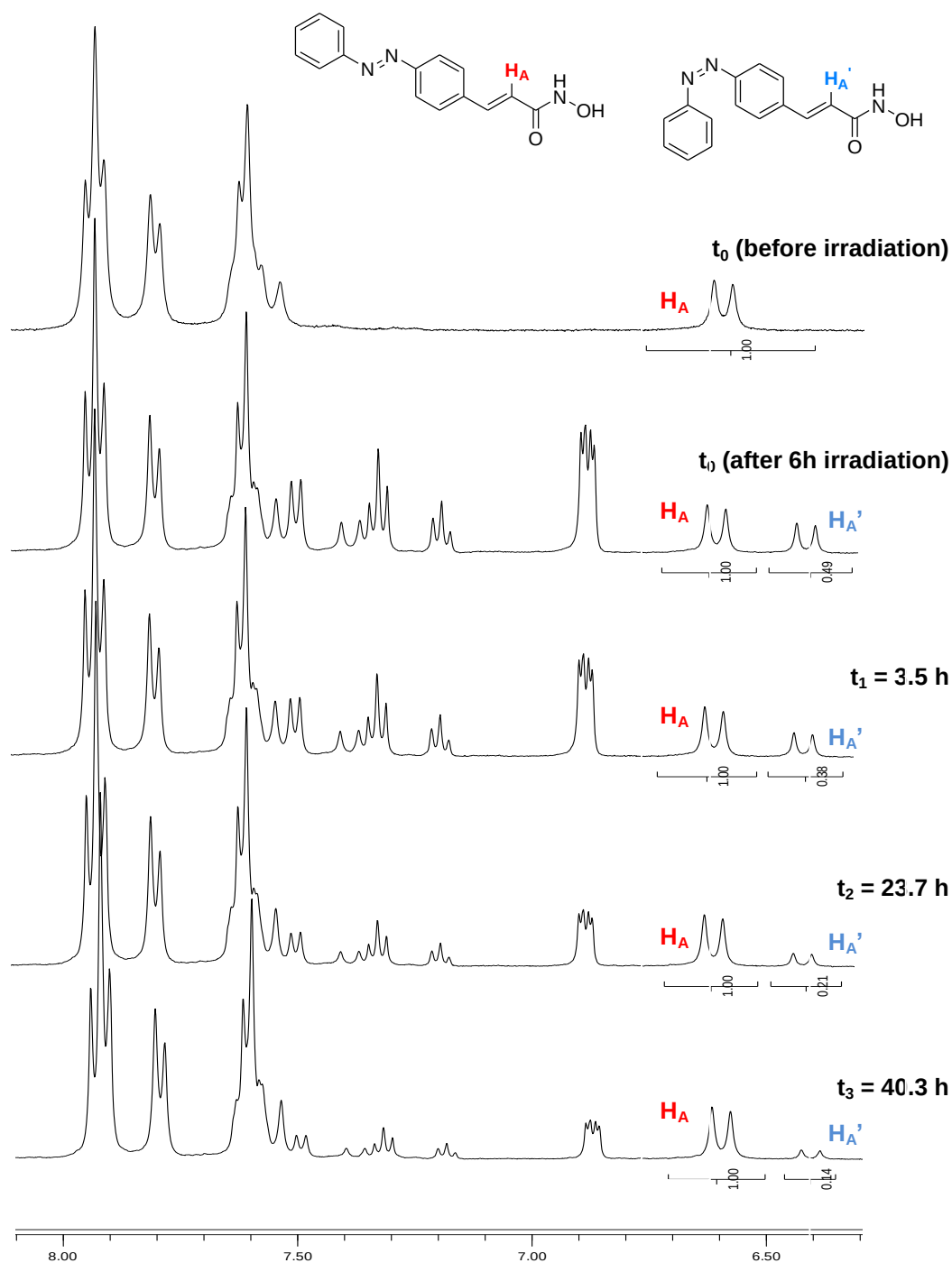


Figure 66 - Time dependent NMR spectrum of a mixture of *trans* and *cis* 202 obtained after 6 hours irradiation. Signals corresponding to H_A and $H_{A'}$ (top) were integrated and normalised to 1 in respect to *trans*-202

Integration of H_A and H_A' signals (Figure 66) gave the proportions (%) of *cis* and *trans* isomers in solution (Table 18). However, it is of note that complete thermal relaxation could not be achieved at rt after more than 40 h.

t (h)	Integration (<i>Trans</i>)	Integration (<i>Cis</i>)	% <i>trans</i>	% <i>cis</i>
0 (before irradiation)	1	0	100	0
0 (after irradiation)	1	0.49	67	33
0.033	1	0.49	67	33
0.066	1	0.49	67	33
3.5	1	0.38	72	28
15.2	1	0.25	80	20
23.7	1	0.21	83	17
34.2	1	0.15	87	13
40.3	1	0.14	88	12

Table 18 - Integration of NMR signals for H_A and H_A' (Figure 66) and determination of the proportion (%) of *cis*-**202** in solution as a function of time (h)

A simple plot of the concentration of *cis*-**202** versus time allowed us to fit the data to a single exponential (Figure 67) with an equation of type:

$$C_{cis}(t) = C_{cis0} \cdot e^{-t/\tau} \quad (1)$$

This gave an approximate $\tau \approx 42$ h.

$$t_{1/2} = \tau \cdot \ln(2) \quad (2)$$

The value determined for τ was then introduced in equation (2), affording a half-life of thermal relaxation for *cis*-**202** $t_{1/2} \approx 29$ hours (Figure 69).

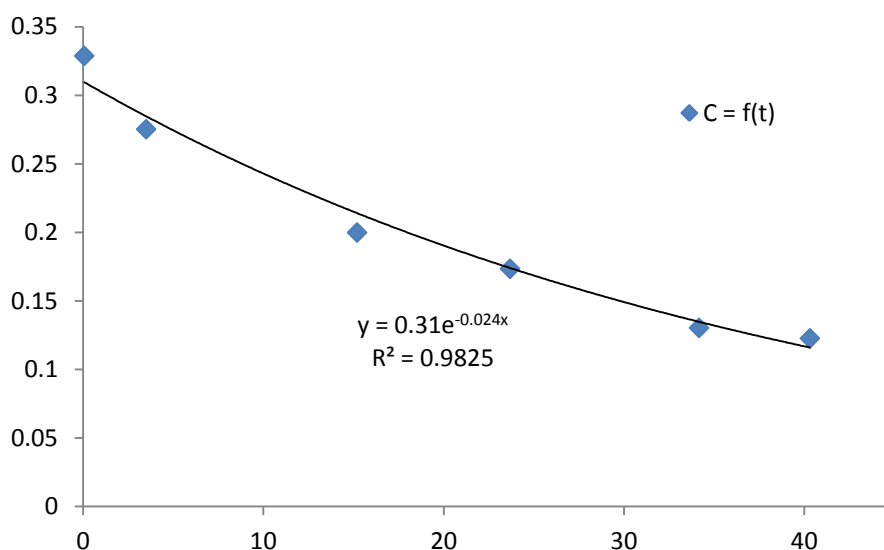
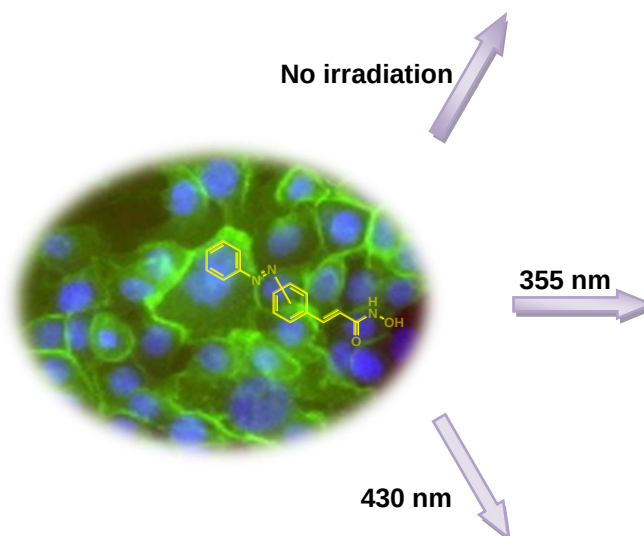


Figure 67 - Thermal relaxation of *cis*-**202** at room temperature (20°C). The relative concentrations of *cis*-**202** in solution, obtained from NMR (Figure 66) were plotted versus time. X-axis: time (h), Y-axis: concentration of *cis*-**202** (a.u.)

While these data allowed for an approximate determination of the half-life of *cis*-**202**, more accurate UV studies are underway in order to confirm this value, in addition to determining the half-life of *cis*-**201**.

***In vitro* and *in vivo* potency of *cis*-**201** and *cis*-**202** against HDAC1**

The first step following completion of the photophysical study of **201** and **202** in solution (DMSO, H₂O, buffer) will consist of the evaluation of the potency of *cis*-**201** and *cis*-**202** against HDAC1 *in vitro*. In a second step, the cytotoxicity of **201** and **202** as a function of irradiation will be studied (Figure 68). We hope to observe a differential potency between *cis* and *trans* isomers *in vitro* that could be exploited to modulate the cytotoxicity of **201** and **202** with light. Results on these preliminary studies will be presented in future reports.

**Figure 68**

4.3.Conclusions

In conclusion, we have developed two new photoswitchable HDAC inhibitors which combine a hydroxamic acid as a ZBG and an azobenzene unit in the cap region. UV-vis studies demonstrated these inhibitors to be light-responsive, efficiently isomerising from *trans* to *cis* (and *vice versa*) under light irradiation. Studies are in progress in order to determine the *cis/trans* ratios at the different photostationary states, in addition to the half times of thermal relaxation of the *cis* isomers. Further studies are in progress in order to evaluate the HDAC inhibitory potency of the *cis* compounds *in vitro*, possibly using the corresponding alkenes as model systems. Finally, the cytotoxicity of **201** and **202** as a function of irradiation will be evaluated in the near future. It is hoped that a significant difference in cytotoxicity will be observed upon irradiation of these compounds at different wavelengths. Such a study would be unprecedented in the field of HDACis. This would potentially open the way towards novel photoswitchable HDACis as candidates for photodynamic chemotherapy.

5. Development of new probes for a Fluorescence Resonance Energy Transfer (FRET) based inhibitor assay for histone deacetylases

5.1. Introduction

Elucidation of the molecular mechanisms leading to HDAC inhibition by HDACis is of crucial importance not only for the development of new probes for the understanding of the role of individual HDAC isoforms but also to improve our knowledge for the rational design of novel, potent and selective inhibitors for anticancer therapy. Progress in this field is mainly dependent on the efficiency of the methods available for inhibitor screening and access to information regarding the molecular mechanisms of binding of these inhibitors to HDACs. In particular, access to thermodynamic parameters for inhibitor binding is complementary of conventional functional assays. Indeed, while commonly used functional assays provide information regarding the structural features responsible for the activity of an inhibitor, they do not provide informations regarding the binding energy (ΔG) and its components (ΔH and ΔS). Access to these parameters is crucial for a deeper understanding of the nature of the interactions involved in the enzyme-ligand complex. In particular, access to thermodynamic data is important for the optimization of the thermodynamic signature of an inhibitor. Indeed, inhibitors should exhibit more enthalpic (ΔH) than entropic (ΔS) characteristics, reflective of specific (such as H-bonds) rather than non-specific (hydrophobic) interactions. In addition to thermodynamic data, a binding assay can give access to crucial data not accessible *via* conventional functional assays, such as binding kinetics and residence time of inhibitors in a binding pocket. Such tools are of high interest for the rational optimisation of lead compounds.

In 2007, Pr. F-J. Meyer-Almes reported a new binding assay for HDACs presenting a number of advantages over previously reported assays. This assay is particularly well suited for the study of the binding and dissociation kinetics of HDACis, in addition to the screening of compound libraries.²²⁹ Initially developed using histone deacetylase like amidohydrolase (HDAH),^{178,179} this nonisotopic

assay is based on FRET between the intrinsically fluorescent tryptophan residues of the enzyme and the fluorescent ligand 2-furylacryloylhydroxamate (FAHA) (204, Figure 69). Binding of FAHA to HDAH causes a FRET signal (Figure 69) leading to a decrease of the tryptophan fluorescence of HDAH ($\lambda_{em} = 350$ nm) and an increase of FAHA fluorescence.

The intensity of the FRET is dependent on the amount of FAHA bound to the enzyme, therefore the ratio of the fluorescence intensities (FIs) FI_{FAHA}/FI_{350} can be directly related to the binding degree (BD) of FAHA to HDAH. As a consequence, it becomes possible to determine the corresponding dissociation constant K_d and its temperature dependency by performing titration experiments under isothermal conditions at different temperatures. This gives access to the value of ΔG at different temperatures (eq. 1).

$$\Delta G(T) = -R \cdot T \cdot \ln(1/K_d(T)) \quad (1)$$

This temperature dependent study of K_d and ΔG allows a Van't Hoff plot of ΔG versus temperature, giving a straight line which intercepts the Y-axis at ΔH and whose slope is $-\Delta S$ (eq. 2)

$$\Delta G(T) = \Delta H - T\Delta S \quad (2)$$

Determination of the thermodynamic parameters of FAHA further allows the determination of these parameters for a non fluorescent ligand, by engaging FAHA and the non fluorescent ligand in competition experiments.

In order to determine the K_d of a non-fluorescent ligand and its temperature dependency, a number of titration experiments must be performed. In such experiments, a complex of FAHA and HDAH is preformed. When the system reaches equilibrium, a titration is performed in which the concentration of the non-fluorescent ligand (e.g. SAHA) is increased. Increasing the concentration of the non fluorescent ligand leads to a decrease of the FRET signal due to the displacement of the bound FAHA. Assuming that the displacement of FAHA

occurs competitively, it is possible to calculate the K_d of the non-fluorescent ligand from the EC_{50} of the titration curve. ΔG can subsequently be obtained from K_d (eq. 1). Performing titration experiments under isotherm conditions at different temperatures further allows a Van't Hoff plot of ΔG versus temperature, giving the parameters ΔH and ΔS (eq. 2).

While FAHA has been very useful tool for a proof of concept during the development of this assay, it suffers from a number of limitations. In the following section, the synthesis of new ligands expected to overcome these limitations, and preliminary results obtained with these ligands when introduced in the FRET assay will be given.

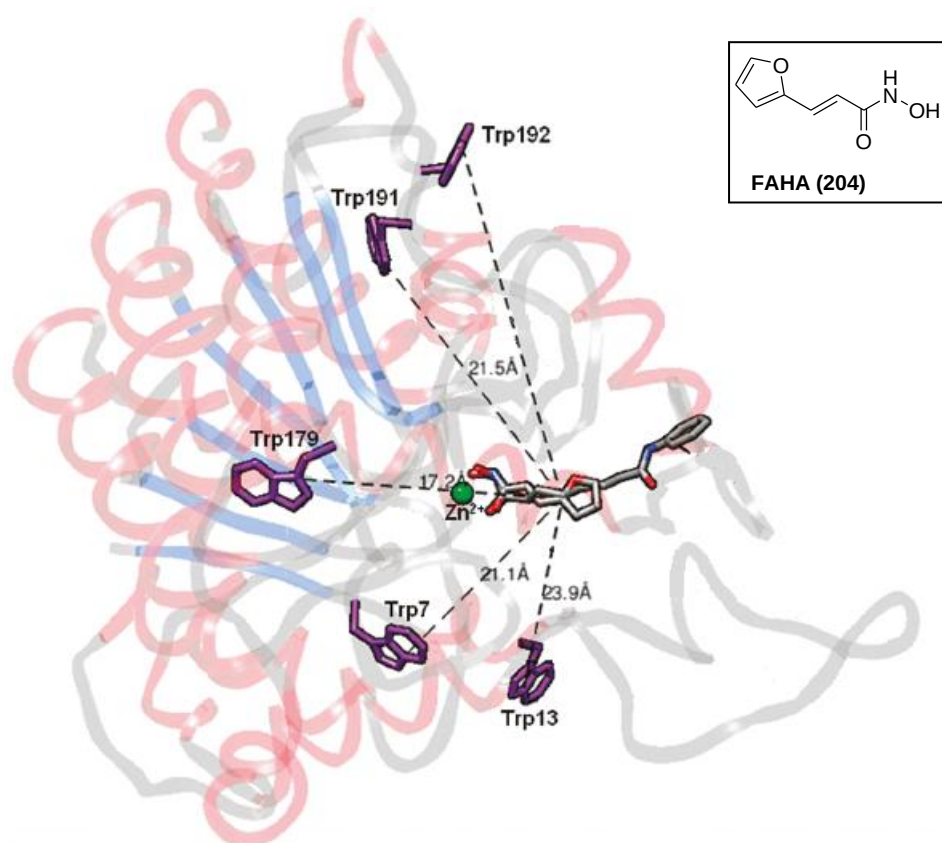


Figure 69 - Overlay of the HDAH-SAHA structure (1ZZ1)¹⁷⁹ and FAHA. FAHA and SAHA are shown as stick models colored by element (grey: carbon; red: oxygen; blue: nitrogen). The five intrinsic tryptophan residues (violet) and active site Zn^{2+} (green) are highlighted. FAHA is superimposed with SAHA in the active site of the HDAH-SAHA complex. Distances (Angstroms) between the chromophores of the tryptophan residues and FAHA are shown. Reproduced and adapted from Sykora and Meyer-Almes.²³⁰ Upon binding, a FRET appears between the HDAH tryptophan and FAHA^{229,230}

5.2. Results and discussion

5.2.1. Design of the fluorescent conjugates

The general structure of the target compounds is shown in Figure 70 (general structure **205**). They combine a dansyl moiety expected to participate in FRET through interactions with tryptophan residues of the enzyme, and a hydroxamic acid moiety able to bind the zinc cation in the active site. In each case, the dansyl moiety and the ZBG are connected by simple, linear amino acids of various length. Dansyl derivatives are well known for their strong absorption maxima between 300 and 350 nm, which coincide with the emission wavelength of HDAC tryptophans, and therefore the dansyl group appeared to be optimal for our study. The use of a dansyl group is particularly advantageous in comparison to FAHA, because it allows the variation of the distance between the fluorescent moiety and the ZBG. Indeed, the distance between the ZBG and the fluorophore and consequently between the fluorophore and tryptophan residues of the enzyme is likely to have a significant influence on the FRET efficiency. Since FAHA has been shown to induce the expected FRET (Figure 69), variation of the distance between the acryloyl part and the furan ring (e.g. with an alkyl chain) is not possible, as it would abolish the fluorescence and therefore the FRET (Figure 70). This potentially limits the applicability of this assay to other HDAC isoforms, as the position of tryptophan residues is likely to differ according to the HDAC isoform considered (Figure 71). Consequently, introduction of alkyl chains of different length between the dansyl group and the ZBG was thought to present a particular advantage for the optimisation of the assay. Firstly, it should improve the FRET efficiency with HDAH and secondly it should broaden the versatility and applicability of this assay to other HDAC isoforms.

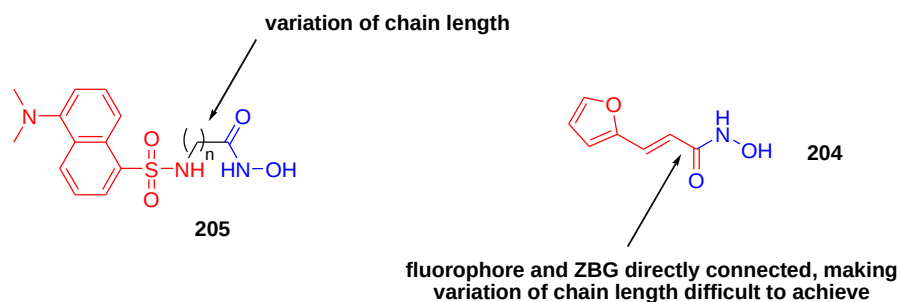


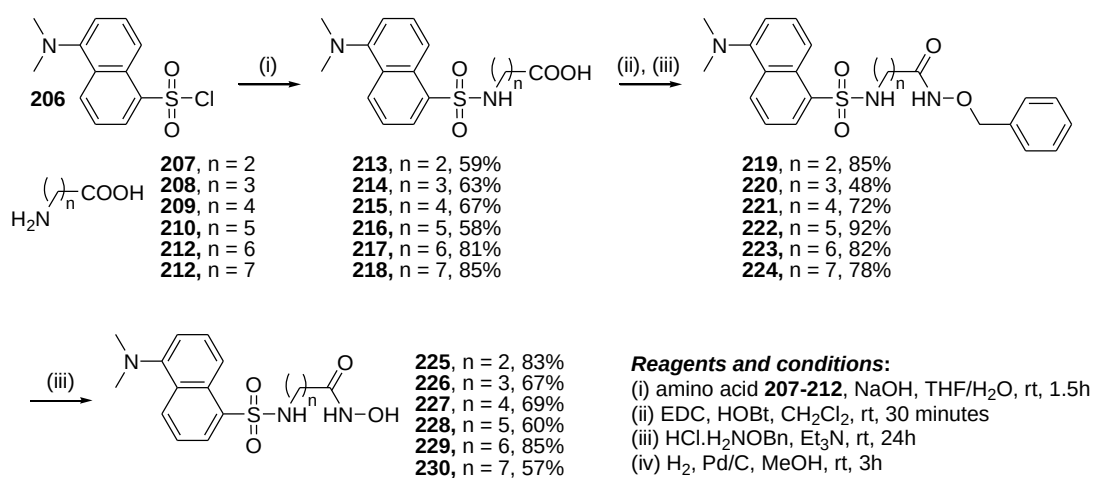
Figure 70 - general structure of target ligands (left). While variation of the chain length between the fluorophore (red) and the ZBG (blue) is easily achievable in the case of the new conjugates (left), it is difficult to achieve in the case of FAHA, as the fluorophore is directly connected to the ZBG



Figure 71 - Crystal structures of HDAH (1ZZ1), HDLP (1C3S), HDAC2 (3MAX) and HDAC8 (1T69) were aligned using Pymol. The relative positions of tryptophan residues in HDAH (yellow), HDLP (pink), HDAC2 (orange) and HDAC8 (red) are highlighted. For clarity, the cocrystalised ligands have been removed and only the structure of HDAH (1ZZ1, green, ribbon representation) is displayed. The Zn(II) cation is displayed as a grey sphere

5.22. Synthesis of target compounds

The synthesis of the dansyl-hydroxamic acid conjugates was straightforward (Scheme 42). Condensation between commercially available dansyl chloride **206** and aminoacids **207-212** afforded sulphonamides **213-218** in 58-85% yields. Further EDC mediated coupling with *O*-benzylhydroxylamine afforded hydroxamic acid precursors **219-224** in 48-92% yields. Final hydrogenolysis of the benzyl protecting group afforded the target conjugates **225-230** in 57-85% yields.



Scheme 42

5.3. Preliminary results and conclusions

Preliminary experiments led by our collaborator F-J. Meyer-Almes and his group (University of Applied Sciences, Darmstadt, Germany) have successfully shown the appearance of a FRET signal between HDAH and our synthetic probes **225-230**. An example is shown in Figure 72. Upon addition of ligand **228**, a new fluorescence signal could be clearly observed at 525 nm, confirming the efficiency of the FRET between HDAH and **228**.

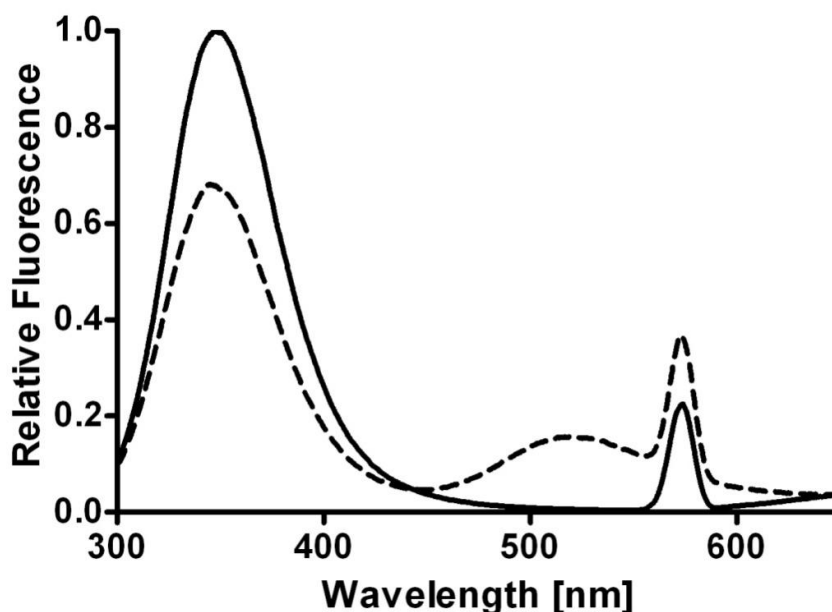


Figure 72 - Example: fluorescence emission spectra of HDAH and a HDAH-**228** complex. The solid line shows the fluorescence emission spectrum of 200 nM HDAH in FB188-buffer¹⁷⁸ at 30° C. The dashed line shows the fluorescence emission spectrum of a mixture of 6.34 μ M **228** and 200 nM HDAH in FB188-buffer¹⁷⁸ pH 8.0 at 30° C. Both samples were excited at 285 nm using the same instrument settings

Optimisation of the assay parameters is underway, and K_{ds} , ΔGs , ΔH and ΔS for each probe are currently being determined against HDAH. Preliminary experiments allowed for the determination of thermodynamic data for common HDACis such as SAHA (data not shown) and will be extended to a number of other inhibitors in the near future. Interestingly, preliminary studies using SAHA (data not shown) revealed the binding of this well known inhibitor to HDAH to be mostly due to entropic factors, rather than enthalpic, highlighting possible reasons for its lack of selectivity among HDAC isoforms. Once the assay is fully established and operational on HDAH, it will be extended to other human HDAC isoforms. In particular, we expect this assay to provide information regarding the thermodynamic signature of Psammaplin A and other synthetic analogues described in previous chapters. Such information would not only enable us to shed light on their binding mode, but also help us in the discovery and design of novel inhibitors displaying high enthalpic contribution to binding as starting points for lead optimisation. All results regarding this study will be reported in future publications.

6. Chemoproteomics of HDAC8

6.1. Introduction

While epigenetics is a very active area of scientific research, there is still much progress to be made in understanding the complex and inter-related signalling pathways mediated by epigenetic enzymes, including HDACs. Specifically, a deeper understanding of the role of individual HDAC isoforms is crucial for establishing an accurate picture of HDAC mediated cellular signalling. To date, a number of isoform-selective HDAC inhibitors have been reported *in vitro*,²³¹ representing important starting points for further in cell investigation. While *in vitro* HDAC assays using recombinant enzymes or protein fragments are a valuable starting point for the evaluation of small molecule inhibitors, they are generally unable to reproduce the native cellular environment of HDAC proteins. The enzymes are therefore unlikely to display accurately a number of their native characteristics, including conformation, post-translational modifications and the presence of regulatory subunits. In particular, it is known that HDACs interact with a number of proteins in cells and usually fulfil their regulatory role as part of multiprotein complexes.²³² A number of HDAC-containing regulatory complexes have been identified. For example, CoREST, NuRD and Sin3 comprise an HDAC1-HDAC2 dimer core, and NCoR is built around an HDAC3 core. The roles of these complexes are multiple and cell-type dependent. For example, CoREST connects histone deacetylation and demethylation, and functions as a neuronal corepressor complex.²³³ NuRD couples histone deacetylation to a chromatin-remodeling ATPase to promote gene silencing,²³⁴ and displays a noticeable DNA-methylation-dependent gene silencing ability.^{32,235} owing to the issues associated with standard HDAC *in vitro* assays, new techniques aimed at studying the proteomics of HDACs to enable the targeting of HDAC complexes directly in cells have been developed.²³⁶⁻²³⁹

Recently, Bantscheff and co-workers developed a new approach combining affinity capture and quantitative mass spectrometry to study the proteomics of zinc-dependent HDACs using small molecule inhibitors.²³⁹

Typically, known non-selective HDAC inhibitors were derivatised and coupled with an activated polymer (Figure 73). Competition experiments were performed between a panel of HDACis and the bead bound unselective probes in the presence of cell extracts. This allowed the potency of HDACis to be quantified against cellular HDAC complexes, and enabled the discovery of previously undocumented cellular HDAC containing complexes, in addition to new non-histone targets. Interestingly, the *in vitro* class and isoform selectivity profile of a number of well known HDACis was found to significantly differ in cells, highlighting the necessity to consider full HDAC complexes in their native environment when attempting to assess the selectivity of HDACis.

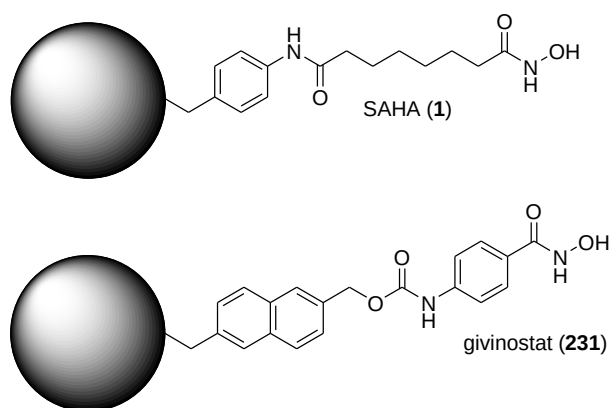
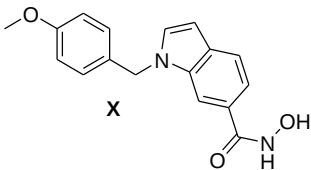


Figure 73

In the present study, we were particularly interested in applying a modified approach to study the proteomics of the fourth class I human deacetylase, HDAC8, in collaboration with F-J. Meyer-Almes and his group. While a number of HDAC-containing repressor complexes containing HDAC1, HDAC2 or HDAC3, HDAC8-containing complexes thus far remain uncharacterised. It was therefore decided to undertake a number of preliminary studies in order to shed light on the still unknown proteomics of HDAC8. The first step of this early stage study is to identify potential HDAC8 interaction partners in cell by affinity capture using a polymer supported HDAC8 selective inhibitor. It was thought the previously reported, HDAC8 selective (*in vitro*) HDACi PCI-34051⁹⁸ (7, Table 19) to be a good starting point for investigation. Indeed, previous studies by Balasubramanian and co-workers have shown this compound would be highly selective against HDAC8 *in vitro* (Table 19). This selectivity for HDAC8 was

therefore thought to be a particular advantage to preferentially bind HDAC8 in cells.



The chemical structure of compound 7 is shown above the table. It consists of a benzimidazole core. At the 2-position of the benzimidazole, there is a 4-methoxybenzyl group. At the 4-position of the benzimidazole, there is a hydroxamic acid group (-C(=O)NHOH). A substituent 'X' is indicated on the benzimidazole ring.

	HDAC1	HDAC2	HDAC3	HDAC6	HDAC8	HDAC10
IC ₅₀ (μM)	4	>50	>50	2.9	0.01	13

Table 19 - HDAC isoform selectivity profile of **7**⁹⁸

6.2. Synthesis of a polymer supported PCI-34051 derivative

We designed compound **232** (Figure 74, right), a new derivative of PCI-34051 **7** bearing a pendant primary amine to be coupled with an activated polymer. The amine was introduced so as to maximise the distance with the hydroxamic acid moiety and therefore minimise potential perturbation of the binding to the protein.

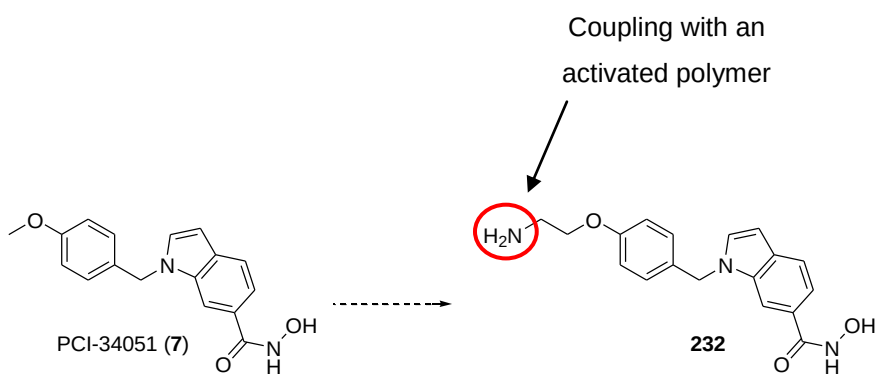
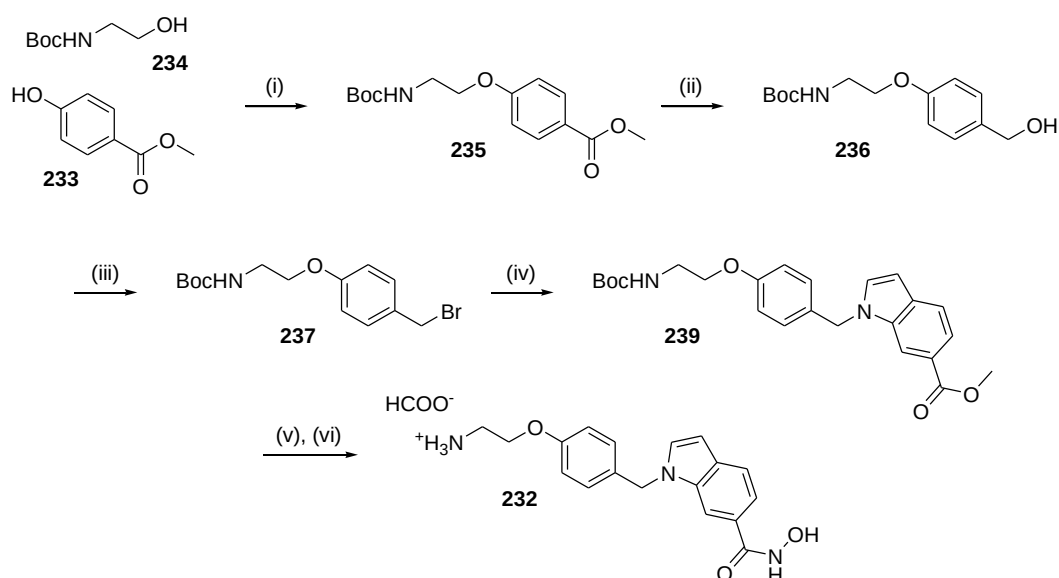


Figure 74 - derivatisation of **7**

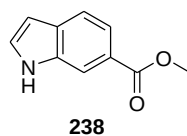
The synthesis of this modified HDAC8-selective inhibitor was straightforward and is described in Scheme 43. The Mitsunobu reaction between

methyl 4-hydroxybenzoate **233** and Boc-protected ethanolamine **234** was performed as previously reported²⁴⁰ and afforded compound **235** in excellent yield. Selective reduction of the ester to the alcohol was achieved in nearly quantitative yield using LiAlH_4 at low temperature and afforded **236**. The subsequent Appel reaction afforded the benzyl bromide derivative **237** in high yield. Slight degradation of **237** on silica was observed by TLCs, purification by flash column chromatography was therefore performed as rapidly as possible using minimal amounts of silica gel. *N*-Alkylation of indole **238** with the benzyl bromide derivative **237** afforded **239** in good yield using NaH in DMF. Subsequent treatment with aqueous hydroxylamine in a THF/MeOH mixture, and a final acidic Boc deprotection afforded **232** in moderate yield after 2 steps. The moderate yield for **232** was mainly due to loss of a substantial amount of material during the final purification by preparative reverse phase LC/MS (low pH, HCOOH). This synthetic route proved to be very robust, and most steps could be performed in multigram quantities.



Reagents and conditions:

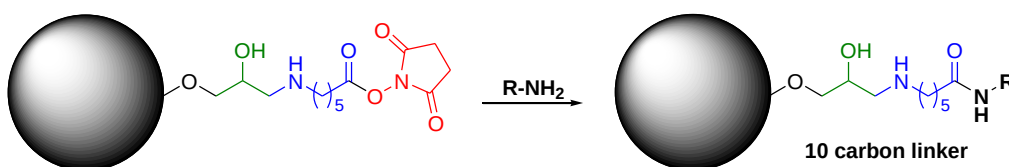
- PPh_3 , DIAD, THF, rt, 24h, 92%
- LiAlH_4 , THF, -20°C , 2h, 95%
- PPh_3 , CBr_4 , CH_2Cl_2 , rt, 2h, 83%
- 238**, NaH, DMF, 0°C , rt, 5.5h, 80%
- H_2NOH , NaOH, THF/MeOH, rt, 8h, 67%
- 4N HCl/dioxane, rt, 5h



Scheme 43 - synthesis of the modified PCI-34051 inhibitor

232 was then coupled with NHS-activated SepharoseTM 4 Fast Flow (GE Healthcare) using a customised procedure (Scheme 44). This NHS-activated polymer is particularly efficient for the immobilisation of ligands bearing primary or secondary amines, and has been shown particularly adapted in previously reported chemoproteomics studies.²³⁹ The resin, supplied in pure isopropanol (16 mL, 256-368 μmol NHS), was filtered and washed with 0.001M HCl (2 x 16 mL, 0-4°C), distilled water (2 x 16 mL, 0-4°C) and anhydrous DMF (3 x 16 mL, 0-4°C). The washed resin was then incubated with **232** (20.0 mg, 53.9 μmol , 0.15-0.21 eq. respect to NHS) and freshly distilled Et₃N (173.0 μL , 3.2 eq. with respect to **232**) dissolved in anhydrous DMF (16 mL, [**232**] $\sim 3 \times 10^{-3}$ M) for 4 hours at rt. The progress of the reaction was followed by UV-Vis (285 nm). After completion of the reaction (80% conversion), the resin was filtered, washed with anhydrous DMF (2 x 16 mL, rt), incubated with aqueous ethanolamine (0.5M ethanolamine, 0.5M NaCl, pH 8.3, 16 mL, rt) for 3 hours and filtered, washed with distilled water (3 x 16 mL, rt) and stored in 20% aqueous EtOH at 0-4°C, in the dark (final volume: 20 mL).

Final loading of **232** $\approx 2.2 \mu\text{mol/mL}$ drained medium



Scheme 44 - coupling of amines with NHS-activated SepharoseTM 4 Fast Flow (GE Healthcare). Grey sphere: spherical, highly cross-linked 4% agarose; mean particle size: 90 μm ; particle size range: 45-165 μm ; NHS density: 16-23 μmol NHS/mL drained medium; green: epichlorohydrin based anchor; blue: 6-aminoheptanoic acid based linker; red: NHS activation; black: ligand bearing an amine (e.g. **232**)

This system, shown in Scheme 44, is expected to selectively immobilise HDAC8 containing complexes on the polymer beads upon exposure to cell extracts (Figure 75). Further custom analytical techniques and comparison with protein databases is hoped to provide crucial information regarding the proteomics of HDAC8 in a number of cell lines. While the selectivity of this compound has been demonstrated *in vitro* only, we expect **232** to exhibit sufficient isoform selectivity in cells in order to preferentially target HDAC8.

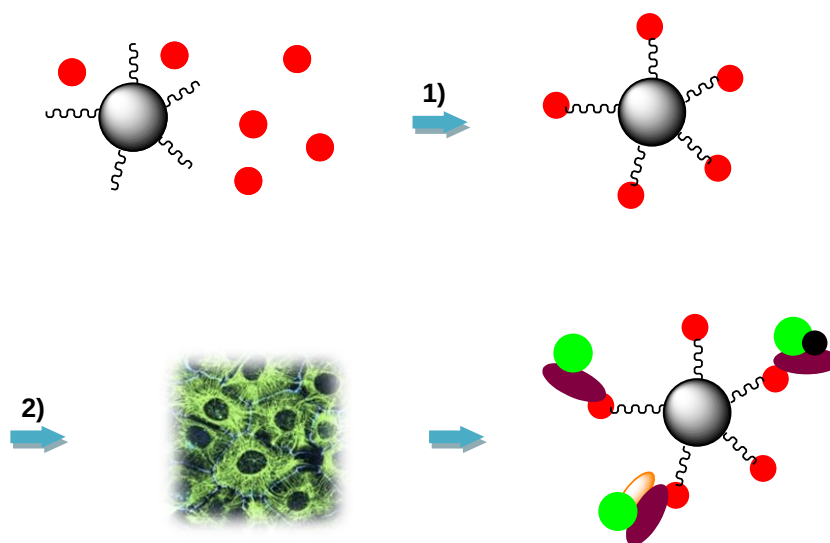


Figure 75 – Study of the proteomics of HDAC8. 1) coupling of HDAC8 selective ligand **232** (red sphere) with NHS-activated Sepharose™ 4 Fast Flow (GE Healthcare); 2) capture of HDAC8 and interaction partners at the surface of the beads upon exposure to cell extracts; purple: HDAC8; green, orange, black: potential HDAC8 interaction partners in cell

Preliminary experiments by Meyer-Almes and his group have shown **232** (unbound) to remain potent against HDAC8 *in vitro*, displaying an IC_{50} of approximately 90 nM. The isolation and characterisation of HDAC8 containing complexes in Hek293 (Human Embryonic Kidney) and K562 (Myelogenous Leukaemia) cell lines, both displaying HDAC8 activity,²⁴¹ is currently attempted and will be reported in future manuscripts.

EXPERIMENTAL

General experimental procedures

All reagents and solvents were supplied from commercial sources, and used as supplied unless otherwise indicated. Reactions requiring anhydrous conditions were conducted in heated glassware (heat gun), under an inert atmosphere (argon), and using anhydrous solvents. CH₂Cl₂ and MeOH were distilled over CaH₂. THF and Et₂O were distilled on Na/benzophenone. Toluene was distilled over Na. All reactions were monitored by analytical thin-layer chromatography (TLC) using indicated solvent systems on E. Merck silica gel 60 F254 plates (0.25mm). TLC plates were visualized using UV light (254nm) and/or by staining in potassium permanganate followed by heating. Solvents were removed by rotary evaporator below 40°C and the compounds further dried using high vacuum pumps. Purification of the different compounds was achieved by column chromatography using Merck Flash Silica Gel 60 (230 – 400 mesh) and reagent/GPR grade solvents.

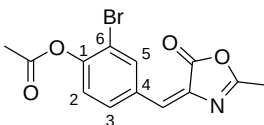
Melting points were obtained on a Reichert-Thermovar melting point apparatus and are uncorrected. Infrared spectra were recorded on a Perkin Elmer Spectrum BX II FT-IR System. Reported absorptions are strong or medium strength unless stated otherwise and given in wavenumbers (cm⁻¹). ¹H and ¹³C NMR were recorded on a Bruker Advance 400 spectrophotometer at 400 MHz and 100 MHz respectively. Chemical shifts (δ H) are quoted in ppm (parts per million) and referenced to residual solvent signals: ¹H δ = 7.26 (CDCl₃), 2.50 (*d*⁶-DMSO), 3.31 (CD₃OD), ¹³C δ = 77.0 (CDCl₃), 39.43 (*d*⁶-DMSO), 49.05 (CD₃OD). Coupling constants (*J*) are given in Hz. Low and high resolution mass spectra (EI, CI, ESI) were recorded by the Imperial College London Department of Chemistry Mass Spectroscopy Service using a Micromass Platform II and Micromass AutoSpec-Q spectrometer. For compounds containing bromine, calculated and experimental masses are reported based on the ⁷⁹Br isotope. Elemental analyses were determined by the University of North London Analytical Service. X-ray crystallographic studies were performed at Imperial College.

1. Design and synthesis of Psammaplin A analogue libraries

Parallel synthesis, general procedure for oxazolone synthesis:

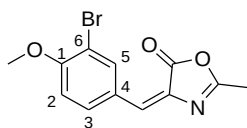
A mixture of the appropriate aromatic aldehyde (1.0 eq.), *N*-acetylglycine (1.3 eq.) and sodium acetate (1.3 eq.) in acetic anhydride (0.45 ml/mmol) was stirred at reflux for 3h, then cooled to room temperature. After complete precipitation the solid was filtered, poured into ice cold water and stirred for 30 minutes. The mixture was then filtered and the remaining solid was dried under vacuum. The crude oxazolone was used for the next step without further purification.

2-Bromo-4-((2-methyl-5-oxooxazol-4(5*H*)-ylidene)methyl)phenyl acetate (73a)

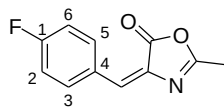


Oxazolone **73a** (yellow solid, 11,6 g, 71%) was prepared from 3-bromo-4-hydroxybenzaldehyde **72a** following the general procedure above. ^1H NMR (400 MHz, CDCl_3) δ 2.38 (s, 3H, $\text{CH}_3\text{C}(\text{O})\text{O}$), 2.42 (s, 3H, $\text{CH}_3\text{C}(\text{N})\text{O}$), 7.03 (s, 1H, 4-CH), 7.20 (d, $J = 8.5$ Hz, 1H, 2-H), 8.00 (dd, $J = 8.5, 2.0$ Hz, 1H, 3-H), 8.43 (d, $J = 2.0$ Hz, 1H, 5-H); ^{13}C NMR (100 MHz, CDCl_3) δ 15.7, 20.8, 116.8, 124.0, 128.3, 132.2, 132.5, 133.5, 136.5, 149.9, 167.0, 167.3, 168.1; HRMS (ESI+) m/z calc. for $\text{C}_{13}\text{H}_{11}\text{BrNO}_4$ $[\text{M}+\text{H}]^+$ 323.9866, found: 323.9860.

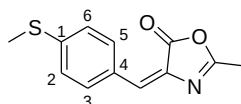
4-(3-Bromo-4-methoxybenzylidene)-2-methyl-4*H*-oxazol-5-one (73b)



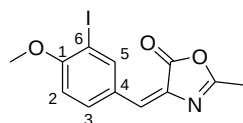
Oxazolone **73b** (yellow solid, 10.3 g, 75%) was prepared from 3-bromo-4-methoxybenzaldehyde **72b** following the general procedure above. ^1H NMR (400 MHz, CDCl_3) δ 2.41 (s, 3H, CH_3O), 3.96 (s, 3H, CH_3C), 6.95 (d, $J = 8.6$ Hz, 1H, 2-H), 7.02 (s, 1H, 4-CH), 7.95 (dd, $J = 8.6, 2.0$ Hz, 1H, 3-H), 8.42 (d, $J = 2.0$ Hz, 1H, 5-H); ^{13}C NMR (100 MHz, CDCl_3) δ 15.7, 56.5, 111.7, 112.3, 127.4, 129.6, 131.5, 133.3, 136.8, 158.0, 165.7, 167.8; HRMS (ESI+) m/z calc. for $\text{C}_{12}\text{H}_{11}\text{BrNO}_3$ $[\text{M}+\text{H}]^+$ 295.9917, found: 295.9919.

4-(4-Fluorobenzylidene)-2-methyloxazol-5(4H)-one (73c)

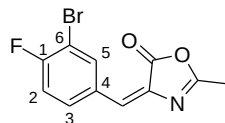
Oxazolone **73c** (yellow solid, 2.30 g, 54%) was prepared from 4-fluorobenzaldehyde **72b** following the general procedure above. ^1H NMR (400 MHz, CDCl_3) δ 2.41 (s, 3H, CH_3), 7.08 - 7.17 (m, 3H, 4-CH + 3-H + 5-H), 8.07 - 8.14 (m, 2H, 2-H + 6-H); ^{13}C NMR (100 MHz, CDCl_3) δ 15.7, 116.1 (d, $J_{\text{CF}} = 22.3$ Hz), 129.5 (d, $J_{\text{CF}} = 2.4$ Hz), 130.0, 132.2, 134.3 (d, $J_{\text{CF}} = 8.7$ Hz), 164.2 (d, $J_{\text{CF}} = 254.5$ Hz), 166.2, 167.7; HRMS (ESI+) m/z calc. for $\text{C}_{11}\text{H}_9\text{FNO}_2$ $[\text{M}+\text{H}]^+$ 206.0612, found: 206.0612.

2-Methyl-4-(4-(methylthio)benzylidene)oxazol-5(4H)-one (73d)

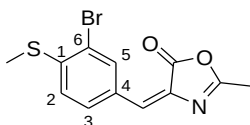
Oxazolone **73d** (orange solid, 3.20 g, 66%) was prepared from 4-methylthiobenzaldehyde **72d** following the general procedure above. ^1H NMR (400 MHz, CDCl_3) δ 2.39 (s, 3H, CH_3C), 2.52 (s, 3H, CH_3S), 7.09 (s, 1H, 4-CH), 7.24 - 7.28 (m, 2H, 2-H + 6-H), 7.96 - 8.02 (m, 2H, 3-H + 5-H); ^{13}C NMR (100 MHz, CDCl_3) δ 14.8, 15.6, 125.5, 129.6, 131.0, 131.6, 132.4, 143.7, 165.4, 167.9; HRMS (ESI+) m/z calc. for $\text{C}_{12}\text{H}_{12}\text{NO}_2\text{S}$ $[\text{M}+\text{H}]^+$ 234.0583, found: 234.0587.

4-(3-Iodo-4-methoxybenzylidene)-2-methyloxazol-5(4H)-one (73e)

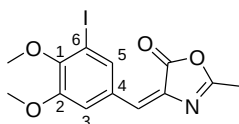
Oxazolone **73e** (orange solid, 3.70 g, 69%) was prepared from 3-iodo-4-methoxybenzaldehyde **72e** following the general procedure above. ^1H NMR (400 MHz, CDCl_3) δ 2.41 (s, 3H, CH_3C), 3.95 (s, 3H, CH_3O), 6.87 (d, $J = 8.6$ Hz, 1H, 2-H), 7.00 (s, 1H, 4-CH), 8.04 (dd, $J = 8.6, 2.1$ Hz, 1H, 3-H), 8.59 (d, $J = 2.1$ Hz, 1H, 5-H); ^{13}C NMR (100 MHz, CDCl_3) δ 15.6, 56.5, 86.4, 110.6, 128.0, 129.4, 131.3, 134.1, 143.1, 160.1, 165.6, 167.7; HRMS (ESI+) m/z calc. for $\text{C}_{12}\text{H}_{11}\text{INO}_3$ $[\text{M}+\text{H}]^+$ 343.9778, found: 343.9791.

4-(3-Bromo-4-fluorobenzylidene)-2-methyloxazol-5(4H)-one (73f)

Oxazolone **73f** (yellow solid, 4.00 g, 66%) was prepared from 3-bromo-4-fluorobenzaldehyde **72f** following the general procedure above. ^1H NMR (400 MHz, CDCl_3) δ 2.42 (s, 3H, CH_3), 7.01 (s, 1H, 4-CH), 7.18 (*app-t*, $J = 8.4$ Hz, 1H, 2-H), 7.98 (m, 1H, 3-H), 8.39 (*app-d*, $J = 5.5$ Hz, 1H, 5-H); ^{13}C NMR (100 MHz, CDCl_3) δ 15.7, 109.8 (d, $J_{\text{CF}} = 22.3$ Hz), 116.9 (d, $J_{\text{CF}} = 22.7$ Hz), 128.2, 130.9 (d, $J_{\text{CF}} = 3.4$ Hz), 133.0 (d, $J_{\text{CF}} = 7.6$ Hz), 133.2 (d, $J_{\text{CF}} = 2.4$ Hz), 137.0, 160.3 (d, $J_{\text{CF}} = 255.0$ Hz), 167.0, 167.3; HRMS (ESI+) m/z calc. for $\text{C}_{11}\text{H}_8\text{BrFNO}_2$ $[\text{M}+\text{H}]^+$ 283.9717, found: 283.9727.

4-(3-Bromo-4-(methylthio)benzylidene)-2-methyloxazol-5(4H)-one (73g)

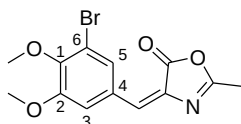
Oxazolone **73g** (yellow solid, 2.47 g, 37%) was prepared from 3-bromo-4-(methylthio)benzaldehyde **72g** following the general procedure above. ^1H NMR (400 MHz, CDCl_3) δ 2.41 (s, 3H, CH_3C), 2.52 (s, 3H, CH_3S), 7.01 (s, 1H, 4-CH), 7.14 (d, $J = 8.4$ Hz, 1H, 2-H), 7.95 (dd, $J = 8.4, 1.8$ Hz, 1H, 3-H), 8.33 (d, $J = 1.8$ Hz, 1H, 5-H); ^{13}C NMR (100 MHz, CDCl_3) δ 15.5, 15.7, 121.3, 124.4, 129.2, 130.5, 131.2, 132.5, 135.2, 144.3, 166.1, 167.5; HRMS (ESI+) m/z calc. for $\text{C}_{12}\text{H}_{11}\text{BrNO}_2\text{S}$ $[\text{M}+\text{H}]^+$ 311.9688, found: 311.9689.

4-(3-Iodo-4,5-dimethoxybenzylidene)-2-methyloxazol-5(4H)-one (73h)

Oxazolone **73h** (orange solid, 2.70 g, 90%) was prepared from 3-iodo-4,5-dimethoxybenzaldehyde **72h** following the general procedure above. ^1H NMR (400 MHz, CDCl_3) δ 2.41 (s, 3H, CH_3C), 3.90 (s, 3H, CH_3O), 3.92 (s, 3H, CH_3O), 6.97 (s, 1H, 4-CH), 7.84 (d, $J = 1.8$ Hz, 1H, 3-H), 7.96 (d, $J = 1.8$ Hz, 1H, 5-H);

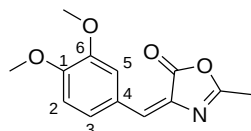
^{13}C NMR (100 MHz, CDCl_3) δ 15.7, 56.0, 60.6, 92.3, 115.7, 129.3, 131.1, 132.4, 135.2, 151.3, 152.4, 166.2, 167.5; HRMS (ESI+) m/z calc. for $\text{C}_{13}\text{H}_{13}\text{INO}_4$ $[\text{M}+\text{H}]^+$ 373.9884, found: 373.9886.

4-(3-Bromo-4,5-dimethoxybenzylidene)-2-methyloxazol-5(4H)-one (73i)



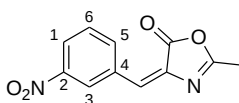
Oxazolone **73i** (orange solid, 3.20 g, 70%) was prepared from 3-bromo-4,5-dimethoxybenzaldehyde **72i** following the general procedure above. ^1H NMR (400 MHz, CDCl_3) δ 2.41 (s, 3H, CH_3C), 3.92 (s, 3H, CH_3O), 3.93 (s, 3H, CH_3O), 6.98 (s, 1H, 4-CH), 7.77 (d, $J = 1.8$ Hz, 1H, 3-H), 7.80 (d, $J = 1.8$ Hz, 1H, 5-H); ^{13}C NMR (100 MHz, CDCl_3) δ 15.7, 56.1, 60.8, 114.7, 117.7, 129.3, 129.4, 130.1, 132.6, 148.8, 153.5, 166.3, 167.5; HRMS (ESI+) m/z calc. for $\text{C}_{13}\text{H}_{13}\text{BrNO}_4$ $[\text{M}+\text{H}]^+$ 326.0022, found: 326.0019.

4-(3,4-Dimethoxybenzylidene)-2-methyloxazol-5(4H)-one (73j)



Oxazolone **73j** (brown solid, 3.60 g, 69%) was prepared from 3,4-dimethoxybenzaldehyde **72j** following the general procedure above. ^1H NMR (400 MHz, CDCl_3) δ 2.38 (s, 3H, CH_3C), 3.94 (s, 3H, CH_3O), 3.95 (s, 3H, CH_3O), 6.90 (d, $J = 8.4$ Hz, 1H, 2-H), 7.08 (s, 1H, 4-CH), 7.50 (dd, $J = 8.4, 1.9$ Hz, 1H, 3-H), 7.93 (d, $J = 1.9$ Hz, 1H, 5-H); ^{13}C NMR (100 MHz, CDCl_3) δ 15.6, 55.9 (2C), 110.8, 113.8, 126.4, 127.3, 130.5, 131.6, 149.1, 151.9, 164.9, 168.1; HRMS (ESI+) m/z calc. for $\text{C}_{13}\text{H}_{14}\text{NO}_4$ $[\text{M}+\text{H}]^+$ 248.0917, found: 248.0916.

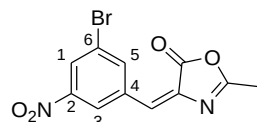
2-Methyl-4-(3-nitrobenzylidene)oxazol-5(4H)-one (73k)



Oxazolone **73k** (yellow solid, 2.12 g, 57%) was prepared from 3-nitrobenzaldehyde **72k** following the general procedure above. ^1H NMR (400

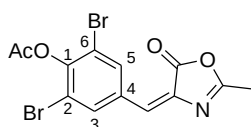
MHz, CDCl₃) δ 2.45 (s, 3H, CH₃C), 7.13 (s, 1H, 4-CH), 7.62 (*app*-t, J = 8.0 Hz, 1H, 6-H), 8.24 (d, J = 7.9 Hz, 1H, 5-H), 8.32 (d, J = 7.7 Hz, 1H, 1-H), 8.99 (s, 1H, 3-H); ¹³C NMR (100 MHz, CDCl₃) δ 15.8, 125.0, 126.3, 127.6, 129.8, 134.6, 134.9, 137.3, 148.6, 166.9, 168.0; HRMS (ESI+) m/z calc. for C₁₁H₉N₂O₄ 233.0557, found: 233.0559.

4-(3-Bromo-5-nitrobenzylidene)-2-methyloxazol-5(4H)-one (73l)



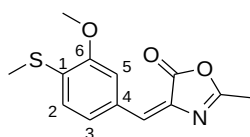
Oxazolone **73l** (brown solid, 1.91 g, 60%) was prepared from 3-bromo-5-nitrobenzaldehyde **72l** following the general procedure above. ¹H NMR (400 MHz, CDCl₃) δ 2.48 (s, 3H, CH₃C), 7.05 (s, 1H, 4-CH), 8.39 (*app*-t, J = 1.9 Hz, 1H, 5-H), 8.53 (*app*-t, J = 1.5 Hz, 1H, 3-H), 8.89 (*app*-t, J = 1.6 Hz, 1H, 1-H); ¹³C NMR (100 MHz, CDCl₃) δ 15.9, 123.2, 124.9, 125.8, 127.8, 135.9, 136.1, 139.7, 149.0, 166.5, 168.9; HRMS (EI+) m/z calc. for C₁₁H₇BrN₂O₄ [M]⁺ 309.9589, found: 309.9595.

2,6-Dibromo-4-((2-methyl-5-oxooxazol-4(5H)-ylidene)methyl)phenyl acetate (73m)



Oxazolone **73m** (yellow solid, 4.04 g, 56%) was prepared from 3,5-dibromo-4-hydroxybenzaldehyde **72m** following the general procedure above. ¹H NMR (400 MHz, CDCl₃) δ 2.42 (s, 3H, CH₃), 2.44 (s, 3H, CH₃), 6.94 (s, 1H, 4-CH), 8.30 (s, 2H, 3-H + 5-H); ¹³C NMR (100 MHz, CDCl₃) δ 15.4, 20.1, 117.5, 125.1, 134.0, 134.6, 134.9, 146.6, 166.6, 167.0, 168.4; HRMS (ESI+) m/z calc. for C₁₃H₁₀Br₂NO₄ [M+H]⁺ 401.8971, found: 401.8990.

4-(3-Methoxy-4-(methylthio)benzylidene)-2-methyloxazol-5(4H)-one (73n)

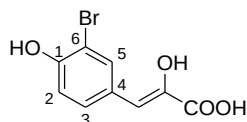


Oxazolone **73n** (orange solid, 4.54 g, 63%) was prepared from 3-methoxy-4-(methylthio)benzaldehyde **72n** following the general procedure above. ^1H NMR (400 MHz, CDCl_3) δ 2.40 (s, 3H, CH_3C), 2.48 (s, 3H, CH_3S), 3.97 (s, 3H, CH_3O), 7.10 (s, 1H, 4-CH), 7.13 (d, $J = 8.1$ Hz, 1H, 2-H), 7.53 (dd, $J = 8.1, 1.5$ Hz, 1H, 3-H), 7.80 (d, $J = 1.5$ Hz, 1H, 5-H); ^{13}C NMR (100 MHz, CDCl_3) δ 13.9, 15.7, 55.9, 111.8, 124.1, 126.2, 130.4, 131.3, 131.5, 133.1, 155.6, 165.2, 167.9; HRMS (ESI+) m/z calc. for $\text{C}_{13}\text{H}_{14}\text{NO}_3\text{S}$ $[\text{M}+\text{H}]^+$ 264.0689, found: 264.0685.

Parallel Synthesis, general procedure for the conversion of azlactones to arylpyruvic acids:

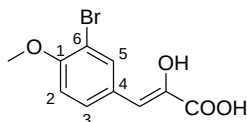
A mixture of the appropriate azlactone in 10% aqueous HCl was heated overnight at reflux. After complete precipitation upon cooling the remaining solid was filtered, washed with a small amount of cold water, and dried under vacuum. The crude product was used for the next step without further purification. Further extraction of the aqueous phase with AcOEt could be achieved in the case of low precipitation of the product.

3-(3-Bromo-4-hydroxyphenyl)-2-hydroxyacrylic acid (51**)**



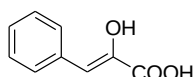
Acid **51** (orange solid, 8.15 g, 88%) was obtained from oxazolone **73a** following the general procedure above. ^1H NMR (400 MHz, CD_3OD) 6.38 (s, 1H, 4-CH), 6.85 (d, $J = 8.5$ Hz, 1H, 2-H), 7.51 (dd, $J = 8.5, 2.1$ Hz, 1H, 3-H), 8.01 (d, $J = 2.1$ Hz, 1H, 5-H); ^{13}C NMR (100 MHz, CD_3OD) 110.5, 110.8, 116.9, 129.6, 131.4, 135.2, 141.1, 154.7, 168.3; HRMS (ESI-) m/z calc. for $\text{C}_9\text{H}_6\text{BrO}_4$ $[\text{M}-\text{H}]^-$ 256.9455, found: 256.9447.

3-(3-Bromo-4-methoxyphenyl)-2-hydroxyacrylic acid (74a**)**



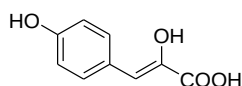
Acid **74a** (yellow solid, 7.71 g, 83%) was obtained from oxazolone **73b** following the general procedure above. ^1H NMR (400 MHz, CD_3OD) 3.89 (s, 3H, CH_3O), 6.40 (s, 1H, 4-CH), 7.01 (d, $J = 8.6$ Hz, 1H, 2-H), 7.65 (dd, $J = 8.6, 2.1$ Hz, 1H, 3-H), 8.09 (d, $J = 2.1$ Hz, 1H, 5-H); ^{13}C NMR (100 MHz, CD_3OD) 56.7, 110.0, 112.4, 112.8, 130.6, 131.4, 135.2, 141.8, 156.5, 168.2; HRMS (ESI-) m/z calc. for $\text{C}_{10}\text{H}_8\text{BrO}_4$ $[\text{M}-\text{H}]^-$ 270.9611, found: 270.9596.

2-Hydroxy-3-phenylacrylic acid (**74b**)



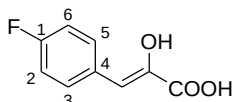
Acid **74b** was purchased from Sigma-Aldrich.

2-Hydroxy-3-(4-hydroxyphenyl)acrylic acid (**56**)



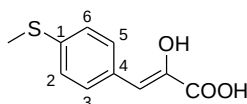
Acid **56** was purchased from Sigma-Aldrich.

3-(4-Fluorophenyl)-2-hydroxyacrylic acid (**74c**)



Acid **74c** (orange solid, 1.48 g, 72%) was obtained from oxazolone **73c** following the general procedure above. ^1H NMR (400 MHz, CD_3OD) 6.47 (s, 1H, 4-CH), 7.02 - 7.09 (m, 2H, 3-H + 5-H), 7.77 - 7.84 (m, 2H, 2-H + 6-H); ^{13}C NMR (100 MHz, CD_3OD) 110.4, 116.0 (d, $^2J_{\text{C-F}} = 22.2$ Hz), 132.6 (d, $^3J_{\text{C-F}} = 8.2$ Hz), 132.9, 142.1, 163.3 (d, $^1J_{\text{C-F}} = 246.3$ Hz), 168.3; HRMS (ESI-) m/z calc. for $\text{C}_9\text{H}_6\text{FO}_3$ $[\text{M}-\text{H}]^-$ 181.0306, found: 181.0296.

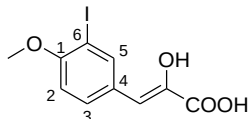
2-Hydroxy-3-(4-(methylthio)phenyl)acrylic acid (**74d**)



Acid **74d** (brown solid, 2.05 g, 71%) was obtained from oxazolone **73d** following the general procedure above. ^1H NMR (400 MHz, CD_3OD) 2.48 (s, 3H, CH_3S), 6.45 (s, 1H, 4-CH), 7.19 - 7.25 (m, 2H, 3-H + 5-H), 7.68 - 7.74 (m, 2H, 2-H + 6-

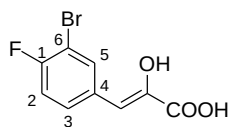
H); ^{13}C NMR (100 MHz, CD_3OD) 15.5, 111.2, 127.1, 131.2, 133.2, 139.4, 142.0, 168.3; HRMS (ESI-) m/z calc. for $\text{C}_{10}\text{H}_9\text{O}_3\text{S}$ $[\text{M}-\text{H}]^-$ 209.0278, found: 209.0269.

2-Hydroxy-3-(3-iodo-4-methoxyphenyl)acrylic acid (74e)



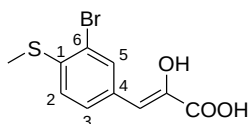
Acid **74e** (orange solid, 3.25 g, 94%) was obtained from oxazolone **73e** following the general procedure above. ^1H NMR (400 MHz, CD_3OD) 3.87 (s, 3H, CH_3O), 6.38 (s, 1H, 4-CH), 6.91 (d, $J = 8.6$ Hz, 1H, 2-H), 7.70 (dd, $J = 8.6, 2.0$ Hz, 1H, 3-H), 8.29 (d, $J = 2.0$ Hz, 1H, 5-H); ^{13}C NMR (100 MHz, $\text{DMSO}-d^6$) 56.3, 86.0, 108.0, 111.2, 129.6, 130.9, 139.3, 140.8, 156.6, 166.2. ; HRMS (ESI-) m/z calc. for $\text{C}_{10}\text{H}_8\text{IO}_4$ $[\text{M}-\text{H}]^-$ 318.9473, found: 318.9472.

3-(3-Bromo-4-fluorophenyl)-2-hydroxyacrylic acid (74f)



Acid **74f** (white solid, 1.76 g, 48%) was obtained from oxazolone **73f** following the general procedure above. ^1H NMR (400 MHz, CD_3OD) 6.42 (s, 1H, 4-CH), 7.16 (app-t, $J = 8.7$ Hz, 1H, 2-H), 7.66 - 7.72 (m, 1H, 3-H), 8.14 (dd, $J = 7.0, 2.1$ Hz, 1H, 5-H); ^{13}C NMR (100 MHz, CD_3OD) 108.7, 109.5 (d, $^2J_{\text{C-F}} = 21.5$ Hz), 117.2 (d, $^2J_{\text{C-F}} = 22.6$ Hz), 131.7 (d, $^3J_{\text{C-F}} = 6.9$ Hz), 134.6, 135.3, 143.3, 159.2 (d, $^1J_{\text{C-F}} = 247$ Hz), 167.8; HRMS (ESI-) m/z calc. for $\text{C}_9\text{H}_5\text{BrFO}_3$ $[\text{M}-\text{H}]^-$ 258.9412, found: 258.9418.

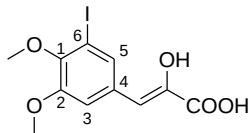
3-(3-Bromo-4-(methylthio)phenyl)-2-hydroxyacrylic acid (74g)



Acid **74g** (brown solid, 1.88g, 92%) was obtained from oxazolone **73g** following the general procedure above. ^1H NMR (400 MHz, CD_3OD) 2.48 (s, 3H, CH_3S), 6.40 (s, 1H, 4-CH), 7.18 (d, $J = 8.4$ Hz, 1H, 2-H), 7.66 (dd, $J = 8.4, 1.8$ Hz, 3-H), 8.06 (d, $J = 1.8$ Hz, 1H, 5-H); ^{13}C NMR (100 MHz, $\text{CD}_3\text{OD}/d^6\text{-DMSO}$) 15.6,

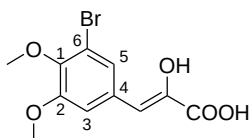
109.5, 121.9, 126.1, 130.4, 134.1, 134.4, 140.2, 143.1, 167.7; HRMS (ESI-) m/z calc. for $C_{10}H_8BrO_3S$ $[M-H]^-$ 286.9383, found: 286.9379.

2-Hydroxy-3-(3-iodo-4,5-dimethoxyphenyl)acrylic acid (74h)



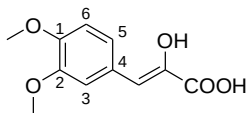
Acid **74h** (brown solid, 2.19 g, 86%) was obtained from oxazolone **73h** following the general procedure above. ^1H NMR (400 MHz, CD_3OD) 3.78 (s, 3H, CH_3O), 3.85 (s, 3H, CH_3O), 6.37 (s, 1H, 4-CH), 7.48 (d, $J = 1.7$ Hz, 1H, 3-H), 7.76 (d, $J = 1.7$ Hz, 1H, 5-H); ^{13}C NMR (100 MHz, CD_3OD) 56.4, 60.8, 92.6, 109.7, 115.3, 132.9, 134.8, 142.6, 149.3, 153.5, 168.0; HRMS (ESI-) m/z calc. for $C_{11}H_{10}IO_5$ $[M-H]^-$ 348.9578, found: 348.9586.

3-(3-Bromo-4,5-dimethoxyphenyl)-2-hydroxyacrylic acid (74i)



Acid **74i** (orange solid, 2.76 g, 93%) was obtained from oxazolone **73i** following the general procedure above. ^1H NMR (400 MHz, CD_3OD) 3.81 (s, 3H, CH_3O), 3.87 (s, 3H, CH_3O), 6.40 (s, 1H, 4-CH), 7.45 (d, $J = 1.8$ Hz, 1H, 3-H), 7.59 (d, $J = 1.8$ Hz, 1H, 5-H); ^{13}C NMR (100 MHz, CD_3OD) 56.5, 61.0, 109.8, 114.3, 118.1, 127.0, 134.1, 142.9, 146.7, 154.7, 168.0; HRMS (ESI-) m/z calc. for $C_{11}H_{10}BrO_5$ $[M-H]^-$ 300.9717, found: 300.9707.

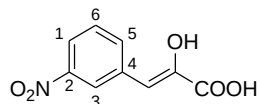
3-(3,4-Dimethoxyphenyl)-2-hydroxyacrylic acid (74j)



Acid **74j** (brown solid, 2.11 g, 62%) was obtained from oxazolone **73j** following the general procedure above. ^1H NMR (400 MHz, CD_3OD) 3.84 (s, 6H, CH_3O), 6.46 (s, 1H, 4-CH), 6.93 (d, $J = 8.4$ Hz, 1H, 6-H), 7.27 (d, $J = 8.4$ Hz, 1H, 5-H), 7.57 (s, 1H, 3-H); ^{13}C NMR (100 MHz, CD_3OD) 56.4 (2C), 112.0, 112.6, 114.4,

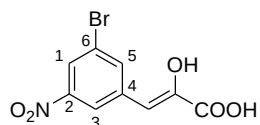
124.5, 129.8, 140.9, 150.1, 150.2, 168.6; HRMS (ESI-) m/z calc. for $C_{11}H_{11}O_5$ $[M-H]^-$ 223.0612, found: 223.0616.

2-Hydroxy-3-(3-nitrophenyl)acrylic acid (**74k**)



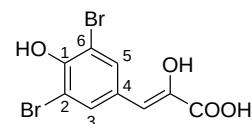
Acid **74k** (brown solid, 1.82 g, 72%) was obtained from oxazolone **73k** following the general procedure above. ^1H NMR (400 MHz, CD_3OD) 6.56 (s, 1H, 4-CH), 7.56 (*app-t.*, $J = 8.0$ Hz, 1H, 6-H), 8.04 (*app-d.*, $J = 7.9$ Hz, 1H, 1-H), 8.08 (ddd, $J = 8.3, 2.4, 0.9$ Hz, 1H, 5-H), 8.77 (*app-t.*, $J = 1.9$ Hz, 1H, 3-H); ^{13}C NMR (100 MHz, CD_3OD) 108.6, 122.6, 124.7, 130.4, 136.4, 138.4, 144.8, 149.8, 167.5; HRMS (ESI-) m/z calc. for $C_9H_6NO_5$ $[M-H]^-$ 208.0251, found: 208.0251.

3-(3-Bromo-5-nitrophenyl)-2-hydroxyacrylic acid (**74l**)

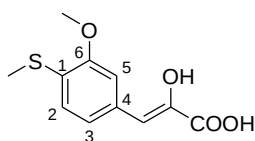


Acid **74l** (grey solid, 1.68 g, 95%) was obtained from oxazolone **73l** following the general procedure above. ^1H NMR (400 MHz, CD_3OD) 6.49 (s, 1H, 4-CH), 8.19 (*app-t.*, $J = 1.9$ Hz, 1H, 5-H), 8.28 (*app-t.*, $J = 1.4$ Hz, 1H, 3-H), 8.63 (*app-t.*, $J = 1.6$ Hz, 1H, 1-H); ^{13}C NMR (100 MHz, CD_3OD) 15.5, 111.2, 127.1, 127.4, 131.2, 132.1, 133.2, 139.4, 142.0, 168.3; HRMS (ESI-) m/z calc. for $C_9H_5BrNO_5$ $[M-H]^-$ 285.9357, found: 285.9353.

3-(3,5-Dibromo-4-hydroxyphenyl)-2-hydroxyacrylic acid (**74m**)



Acid **74m** (brown solid, 2.60g, 82%) was obtained from oxazolone **73m** following the general procedure above. ^1H NMR (400 MHz, CD_3OD) 6.32 (s, 1H, 4-CH), 7.92 (s, 2H, 3-H + 5-H); ^{13}C NMR (100 MHz, CD_3OD) 108.7, 111.9, 131.1, 134.3, 142.5, 151.3, 167.9; HRMS (ESI-) m/z calc. for $C_9H_5Br_2O_4$ $[M-H]^-$ 334.8560, found: 334.8560.

2-Hydroxy-3-(3-methoxy-4-(methylthio)phenyl)acrylic acid (74n)

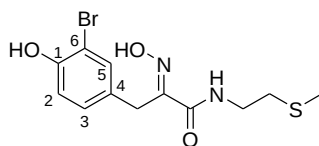
Acid **74n** (off-white solid, 3.75g, 98%) was obtained from oxazolone **73n** following the general procedure above. ^1H NMR (400 MHz, CD_3OD) 2.40 (s, 3H, CH_3S), 3.87 (s, 3H, CH_3O), 6.48 (s, 1H, 4-CH), 7.11 (d, $J = 8.1$ Hz, 1H, 2-H), 7.30 (dd, $J = 8.1, 1.5$ Hz, 1H, 3-H), 7.49 (d, $J = 1.5$ Hz, 1H, 5-H); ^{13}C NMR (100 MHz, CD_3OD) 14.4, 56.2, 111.6, 112.2, 124.2, 126.2, 128.3, 134.2, 141.9, 157.3, 168.3; HRMS (ESI-) m/z calc. for $\text{C}_{11}\text{H}_{11}\text{O}_4\text{S}$ $[\text{M}-\text{H}]^-$ 239.0384, found: 239.0370.

Synthesis of Psammaplin A analogues from arylpyruvic acids, general procedures:

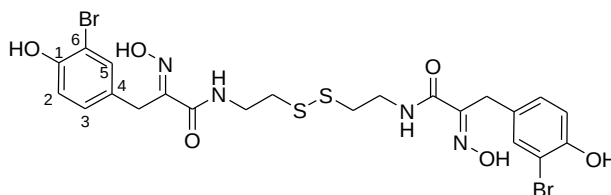
Procedure A, synthesis of oxime derivatives: To a solution of the appropriate crude acid (1 eq.) in freshly distilled pyridine (2 mL/mmol), under an argon atmosphere, was added *O*-(tetrahydro-2*H*-pyran-2-yl)hydroxylamine (1.5 eq.). The resulting mixture was stirred overnight at room temperature. Pyridine was then removed *in vacuo*, the residue was dissolved in 1N HCl (5.5 mL/mmol) and extracted three times with AcOEt (5.5 mL/mmol). The combined organic layers were dried (Na_2SO_4), filtrated and concentrated to afford the crude *O*-THP protected oxime intermediate, used for the next step without further purification. To a solution of the crude *O*-THP protected oxime intermediate (1 eq.) in dry dioxane (2.7 mL/mmol) under an argon atmosphere were added EDCI (1.7 equiv.) and *N*-hydroxysuccinimide (NHS) (1.9 equiv.). The resulting mixture was stirred at room temperature for 3 hours. The solvent was then evaporated *in vacuo*. The residue was dissolved in AcOEt (12 mL/mmol), washed with saturated aqueous NaHCO_3 (2 x 12 mL/mmol), 1N aqueous HCl (2 x 12 mL/mmol) and brine (1 x 12 mL/mmol). The organic phase was then dried (Na_2SO_4) and the solvent evaporated *in vacuo*. The residue was dissolved in dry dioxane (2.7 mL/mmol) under an argon atmosphere and a second solution of the appropriate amine (1 eq. 2-(methylthio)ethanamine or 0.5 eq. cystamine dihydrochloride) and NEt_3 (1.1 eq. for 2-(methylthio)ethanamine or 2.1 eq. for cystamine dihydrochloride) in freshly

distilled MeOH (2.7 mL/mmol) was added. The resulting mixture was then stirred overnight at rt. The solvent was then evaporated *in vacuo* and the product was purified by flash column chromatography (AcOEt/PE₄₀₋₆₀ or AcOEt/CH₂Cl₂ mixtures). The *O*-THP protected precursor was then dissolved in an anhydrous 20 : 1 CH₂Cl₂ : MeOH mixture (6.7 mL / mmol for monomers, 13.4 mL / mmol for disulfides) and 1M HCl in anhydrous Et₂O (6.7 mL / mmol for monomers, 13.4 mL / mmol for disulfides) was added. The resulting mixture was stirred for 3 hours at 60 °C. The solvent was then evaporated *in vacuo* and the product purified by flash column chromatography.

Procedure B, synthesis of *O*-methyloxime derivatives: To a solution of the appropriate crude acid (1 eq.) in freshly distilled pyridine (2 mL/mmol), under an argon atmosphere, was added methoxylamine hydrochloride (1.5 eq.). The resulting mixture was stirred overnight at room temperature. Pyridine was then removed *in vacuo*, the residue was dissolved in 1N HCl (5.5 mL/mmol) and extracted three times with AcOEt (5.5 mL/mmol). The combined organic layers were dried (Na₂SO₄), filtered and concentrated to afford the crude *O*-methylated oxime intermediate, used for the next step without further purification. To a solution of the crude *O*-methylated oxime intermediate (1 eq.) in dry dioxane (2.7 mL/mmol) under an argon atmosphere was added EDCI (1.7 eq.) and NHS (1.9 eq.). The resulting mixture was stirred at room temperature for 3 hours. The solvent was then evaporated *in vacuo*. The residue was dissolved in AcOEt (12 mL/mmol), washed with saturated aqueous NaHCO₃ (2 x 12 mL/mmol), 1N aqueous HCl (2 x 12 mL/mmol) and brine (1 x 12 mL/mmol). The organic phase was then dried (Na₂SO₄) and the solvent evaporated *in vacuo*. The residue was dissolved in dry dioxane (2.7 mL/mmol) under an argon atmosphere and a second solution of the appropriate amine (1 eq. 2-(methylthio)ethanamine or 0.5 eq. cystamine dihydrochloride) and NEt₃ (1.1 eq. for 2-(methylthio)ethanamine or 2.1 eq. for cystamine dihydrochloride) in freshly distilled MeOH (2.7 mL/mmol) is added. The resulting mixture was then stirred overnight at rt. The solvent was then evaporated *in vacuo* and the product was purified by flash column chromatography.

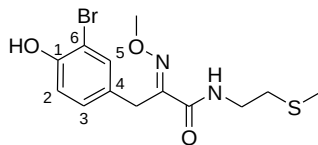
(E)-3-(3-Bromo-4-hydroxyphenyl)-2-(hydroxyimino)-N-(2-(methylthio)ethyl)propanamide (75a)

75a (184 mg, 40% from acid **51**) was prepared according to general procedure A above, and obtained as a white solid after purification by flash column chromatography (MeOH : CH₂Cl₂, 4 : 96) and recrystallization from Et₂O: m.p. 102-104 °C. R_f 0.35 (MeOH : CH₂Cl₂, 5 : 95); IR 3225, 1618, 1548, 1509, 1419, 1013 cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 2.09 (s, 3H, CH₃S), 2.60 (t, *J* = 6.9 Hz, 2H, CH₂S), 3.44 (t, *J* = 6.9 Hz, 2H, CH₂N), 3.79 (s, 2H, 4-CH₂), 6.76 (d, *J* = 8.3 Hz, 1H, 2-H), 7.07 (dd, *J* = 8.3, 2.1 Hz, 1H, 3-H), 7.37 (d, *J* = 2.1 Hz, 1H, 5-H). ¹³C NMR (100 MHz, CD₃OD) δ 15.2, 28.7, 34.2, 39.6, 110.5, 117.0, 130.4, 130.7, 134.5, 153.2, 153.8, 165.8; HRMS (ESI+) *m/z* calc. for C₁₂H₁₆BrN₂O₃S [M+H]⁺ 347.0060, found: 347.0067; Anal. Calc. for C₁₂H₁₅BrN₂O₃S: C, 41.51; H, 4.35; N, 8.07. Found: C, 41.45; H, 4.27; N, 7.96.

(2E,2'E)-N,N'-(2,2'-Disulfanediylobis(ethane-2,1-diyl))bis(3-(3-bromo-4-hydroxyphenyl)-2-(hydroxyimino)propanamide) (natural product PSAMMAPLIN A) (28)

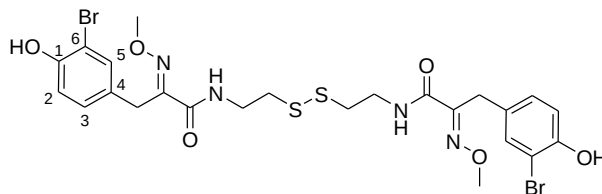
28 (249 mg, 35% from acid **51**) was prepared according to general procedure A above, and obtained as an amorphous light yellow solid after purification by flash column chromatography (gradient AcOEt/PE₄₀₋₆₀, 1 : 1 then 100% AcOEt). The full characterisation of psammaplin A has been reported in the literature;^{153,154,156,135a-c} NMR data obtained matched perfectly those reported. ¹H NMR (400 MHz, CD₃OD) δ 2.81 (t, *J* = 6.7 Hz, 4H, CH₂S), 3.52 (t, *J* = 6.7 Hz, 4H, CH₂N), 3.79 (s, 4H, 4-CH₂), 6.76 (d, *J* = 8.3 Hz, 2H, 2-H), 7.07 (dd, *J* = 8.3, 2.1 Hz, 2H, 3-H), 7.36 (d, *J* = 2.1 Hz, 2H, 5-H); ¹³C NMR (100 MHz, CD₃OD) δ 28.7, 38.6, 39.6, 110.5, 117.1, 130.5, 130.7, 134.5, 153.2, 153.8, 165.9; HRMS (ESI+) *m/z* calc. for C₂₂H₂₅Br₂N₄O₆S₂ [M+H]⁺ 662.9577, found: 662.9590.

(E)-3-(3-Bromo-4-hydroxyphenyl)-2-(methoxyimino)-N-(2-(methylthio)ethyl)propanamide (75r)



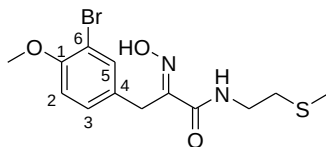
75r (98 mg, 43% from acid **51**) was prepared according to general procedure B above, and obtained as a yellow oil after flash purification by flash column chromatography (AcOEt : PE₄₀₋₆₀°C, 1 : 1). *R*_f 0.55 (AcOEt : PE₄₀₋₆₀°C, 1 : 1); IR 3327, 1657, 1530, 1509, 1493, 1422, 1045 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 2.12 (s, 3H, CH₃S), 2.66 (t, *J* = 6.5 Hz, 2H, CH₂S), 3.52 (m, 2H, CH₂N), 3.81 (s, 2H, 4-CH₂), 4.01 (s, 3H, CH₃O), 5.46 (br. s, 1H, OH), 6.89 (d, *J* = 8.3 Hz, 1H, 2-H), 7.06 (br. t, 1H, NH), 7.15 (dd, *J* = 8.3, 2.0 Hz, 1H, 3-H), 7.40 (d, *J* = 2.0 Hz, 1H, 5-H). ¹³C NMR (100 MHz, CDCl₃) δ 15.2, 28.5, 33.7, 38.0, 63.1, 109.9, 115.9, 129.7, 130.1, 132.5, 150.9, 151.5, 162.5; HRMS (ESI+) *m/z* calc. for C₁₃H₁₈BrN₂O₃S [M+H]⁺ 361.0216, found: 361.0222; Anal. Calc. for C₁₃H₁₇BrN₂O₃S: C, 43.22; H, 4.74; N, 7.75. Found: C, 43.13; H, 4.72; N, 7.65.

(2E,2'E)-N,N'-(2,2'-Disulfaneyldiylbis(ethane-2,1-diyl))bis(3-(3-bromo-4-hydroxyphenyl)-2-(methoxyimino)propanamide) (75s)



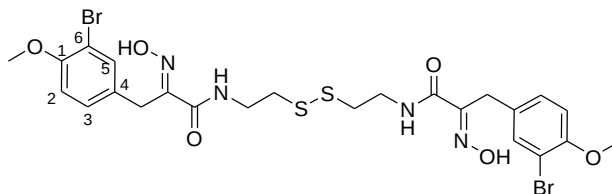
75s (135mg, 30% from acid **51**) was prepared according to general procedure B above, and obtained as an amorphous yellow solid after purification by flash column chromatography (AcOEt : PE₄₀₋₆₀°C : MeOH, 5.8 : 4 : 0.2). *R*_f 0.6 (AcOEt : PE₄₀₋₆₀°C, 7 : 3); IR 3356, 1657, 1528, 1508, 1492, 1422, 1045 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 2.81 (t, *J* = 6.4 Hz, 4H, CH₂S), 3.61 (m, 4H, CH₂N), 3.81 (s, 4H, 4-CH₂), 4.00 (s, 6H, CH₃O), 5.56 (br. s, 2H, OH), 6.88 (d, *J* = 8.3 Hz, 2H, 2-H), 7.06 (br. t, 2H, NH), 7.14 (dd, *J* = 8.3, 2.0 Hz, 2H, 3-H), 7.39 (d, *J* = 2.0 Hz, 2H, 5-H). ¹³C NMR (100 MHz, CDCl₃) δ 28.5, 37.6, 38.3, 63.1, 110.0, 115.9, 129.7, 130.2, 132.5, 150.9, 151.5, 162.6; HRMS (ESI+) *m/z* calc. for C₂₄H₂₉Br₂N₄O₆S₂ [M+H]⁺ 690.9890, found: 690.9893; Anal. Calc. for C₂₄H₂₈Br₂N₄O₆S₂: C, 41.63; H, 4.08; N, 8.09. Found: C, 41.56; H, 3.97; N, 8.15.

(*E*)-3-(3-Bromo-4-methoxyphenyl)-2-(hydroxyimino)-*N*-(2-(methylthio)ethyl)propanamide (75b)

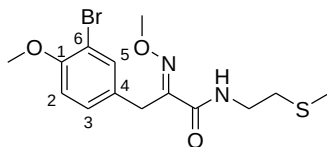


75b (102 mg, 38% from acid **74a**) was prepared according to general procedure A above, and obtained as a yellowish oil after purification by flash column chromatography (AcOEt : PE₄₀₋₆₀, 1 : 1). *R*_f 0.35 (AcOEt : PE₄₀₋₆₀, 1 : 1); IR 1658, 1528, 1495, 1257, 1283, 1055 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 2.10 (s, 3H, CH₃S), 2.65 (t, *J* = 6.4 Hz, 2H, CH₂S), 3.52 (m, 2H, CH₂N), 3.85 (s, 3H, CH₃O), 3.90 (s, 2H, 4-CH₂), 6.79 (d, *J* = 8.5 Hz, 1H, 2-H), 7.05 (br. t., 1H, NH), 7.27 (dd, *J* = 8.5, 2.1 Hz, 1H, 3-H), 7.53 (d, *J* = 2.1 Hz, 1H, 5-H), 7.69 (br. s, 1H, OH). ¹³C NMR (100 MHz, CDCl₃) δ 15.1, 28.0, 33.6, 37.8, 56.2, 111.4, 111.8, 129.5, 129.7, 134.0, 153.2, 154.5, 162.8; HRMS (ESI+) *m/z* calc. for C₁₃H₁₈BrN₂O₃S [M+H]⁺ 361.0216, found: 361.0222; Anal. Calc. for C₁₃H₁₇BrN₂O₃S: C, 43.22; H, 4.74; N, 7.75. Found: C, 43.18; H, 4.63; N, 7.78.

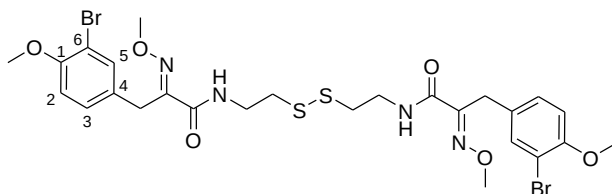
(2*E*,2'*E*)-*N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl)bis(3-(3-bromo-4-methoxyphenyl)-2-(hydroxyimino)propanamide) (75c)



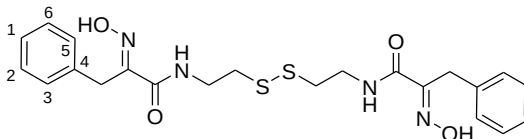
75c (185 mg, 49% from acid **74a**) was prepared according to general procedure A above, and obtained as a yellow amorphous solid after purification by flash column chromatography (AcOEt : PE₄₀₋₆₀, 6 : 4). *R*_f 0.45 (AcOEt : PE₄₀₋₆₀, 7 : 3); IR 1659, 1530, 1495, 1281, 1256, 1055 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 2.77 (t, *J* = 6.2 Hz, 4H, CH₂S), 3.59 (m, 4H, CH₂N), 3.81 (s, 6H, CH₃O), 3.87 (s, 4H, 4-CH₂), 6.74 (d, *J* = 8.5 Hz, 2H, 2-H), 7.15 (br. t, 2H, NH), 7.24 (dd, *J* = 8.4, 2.1 Hz, 2H, 3-H), 7.51 (d, *J* = 2.1 Hz, 2H, 5-H). ¹³C NMR (100 MHz, CDCl₃) δ 28.0, 38.0, 38.3, 56.2, 111.4, 111.8, 129.6, 129.7, 133.9, 152.6, 154.5, 163.5; HRMS (ESI+) *m/z* calc. for C₂₄H₂₉Br₂N₄O₆S₂ [M+H]⁺ 690.9890, found: 690.9882; Anal. Calc. for C₂₄H₂₈Br₂N₄O₆S₂: C, 41.63; H, 4.08; N, 8.09. Found: C, 41.55; H, 4.01; N, 8.15.

(E)-3-(3-Bromo-4-methoxyphenyl)-2-(methoxyimino)-N-(2-(methylthio)ethyl)propanamide (75t)

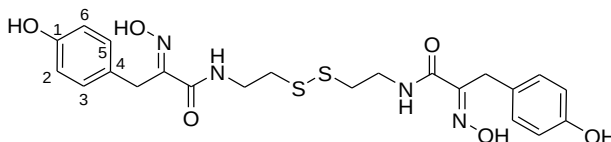
75t (75 mg, 67% from acid **74a**) was prepared according to general procedure B above, and obtained as colourless needles after flash purification by flash column chromatography (AcOEt/PE₄₀₋₆₀°C, 1 : 2), and recrystallization (Bu₂O): m.p. 63-65°C (Bu₂O); R_f 0.65 (AcOEt/PE₄₀₋₆₀°C, 1 : 1); IR 1671, 1519, 1494, 1284, 1255, 1045 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 2.12 (s, 3H, CH₃S), 2.66 (t, *J* = 6.6 Hz, 2H, CH₂S), 3.52 (m, 2H, CH₂N), 3.83 (s, 2H, 4-CH₂), 3.85 (s, 3H, CH₃O-1), 4.02 (s, 3H, CH₃O-N), 6.78 (d, *J* = 8.4 Hz, 1H, 2-H), 7.06 (br. t., 1H, NH), 7.22 (dd, *J* = 8.4, 2.1 Hz, 1H, 3-H), 7.47 (d, *J* = 2.1 Hz, 1H, 5-H); ¹³C NMR (100 MHz, CDCl₃) δ 15.2, 28.5, 33.7, 37.9, 56.2, 63.1, 111.3, 111.7, 129.4, 129.8, 133.9, 151.5, 154.4, 162.4; HRMS (ESI+) *m/z* calc. for C₁₄H₂₀BrN₂O₃S [M+H]⁺ 375.0373, found: 375.0378; Anal. Calc. for C₁₄H₁₉BrN₂O₃S: C, 44.81; H, 5.10; N, 7.46. Found: C, 44.86; H, 5.03; N, 7.41.

(2E,2'E)-N,N'-(2,2'-Disulfanediyldis(ethane-2,1-diyl))bis(3-(3-bromo-4-methoxyphenyl)-2-(methoxyimino)propanamide) (75u)

75u (161mg, 41% from acid **74a**) was prepared according to general procedure B above, and obtained as a white solid after purification by flash column chromatography (AcOEt : PE₄₀₋₆₀°C, 1 : 1) and recrystallisation (AcOEt/hexane): m.p. 81-83 °C (AcOEt/hexane); R_f 0.5 (AcOEt : PE₄₀₋₆₀°C, 1 : 1); **75u** has been reported previously,^{135a-c} ¹H NMR (400 MHz, CDCl₃) δ 2.82 (t, *J* = 6.4 Hz, 4H, CH₂S), 3.62 (m, 4H, CH₂N), 3.82 (s, 4H, 4-CH₂), 3.84 (s, 6H, CH₃O-1), 4.00 (s, 6H, CH₃O-N), 6.78 (d, *J* = 8.4 Hz, 2H, 2-H), 7.06 (br. t., 2H, NH), 7.20 (dd, *J* = 8.4, 2.2 Hz, 2H 3-H), 7.46 (d, *J* = 2.2 Hz, 2H, 5-H); HRMS (ESI+) *m/z* calc. for C₂₆H₃₃Br₂N₄O₆S₂ [M+H]⁺ 719.0203, found: 719.0209.

(2*E*,2'*E*)-*N,N'*-(2,2'-Disulfanediyldis(ethane-2,1-diyl))bis(2-(hydroxyimino)-3-phenylpropanamide) (75d)

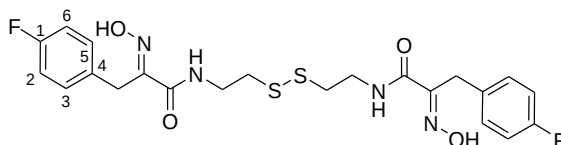
75d (225 mg, 24% from acid 74b) was prepared according to general procedure A above, and obtained as an amorphous white solid after purification by flash column chromatography (gradient acetone : PE₄₀₋₆₀, 3 : 7 to 4 : 6) and precipitation from CH₂Cl₂. *R*_f 0.3 (acetone : PE₄₀₋₆₀, 3 : 7); IR (neat) 1654, 1625, 1522, 1493, 1426, 1000 cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 2.79 (t, *J* = 6.7 Hz, 4H, CH₂S), 3.50 (t, *J* = 6.7 Hz, 4H, CH₂N), 3.91 (s, 4H, 4-CH₂), 7.10-7.16 (m, 2H, 1-H), 7.17-7.30 (m, 8H, 2-H + 3-H + 5-H + 6-H). ¹³C NMR (100 MHz, CD₃OD) δ 30.0, 38.5, 39.6, 127.3, 129.4, 130.1, 138.2, 153.3, 166.0; HRMS (ESI⁺) *m/z* calc. for C₂₂H₂₇N₄O₄S₂ [M+H]⁺ 475.1468, found: 475.1470; Anal. Calc. for C₂₂H₂₆N₄O₄S₂: C, 55.68; H, 5.52; N, 11.81. Found: C, 55.75; H, 5.44; N, 11.89.

(2*E*,2'*E*)-*N,N'*-(2,2'-Disulfanediyldis(ethane-2,1-diyl))bis(2-(hydroxyimino)-3-(4-hydroxyphenyl)propanamide) (75e)

75e (175 mg, 20% from acid 56) was prepared according to general procedure A above, and obtained as an amorphous white solid after purification by flash column chromatography (gradient acetone : PE₄₀₋₆₀, 4 : 6 to 1 : 1). Important degradation was observed during the final deprotection step for reaction times superior to 2h. Only partial conversion to the product (and recovery of the starting material) was achieved during the deprotection step, avoiding degradation and allowing efficient purification of the product. *R*_f 0.3 (acetone : PE₄₀₋₆₀, 1 : 1); IR (neat) 1655, 1613, 1531, 1511, 1437, 1214, 1011, 980 cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 2.79 (t, *J* = 6.8 Hz, 4H, CH₂S), 3.51 (t, *J* = 6.8 Hz, 4H, CH₂N), 3.80 (s, 4H, 4-CH₂), 6.62-6.67 (m, 4H, 2-H + 6-H), 7.05-7.11 (m, 4H, 3-H + 5-H). ¹³C NMR (100 MHz, CD₃OD) δ 29.1, 38.5, 39.6, 116.1, 128.9, 131.2, 153.8, 156.9, 166.2; HRMS (ESI⁺) *m/z* calc. for C₂₂H₂₇N₄O₆S₂ [M+H]⁺ 507.1367, found:

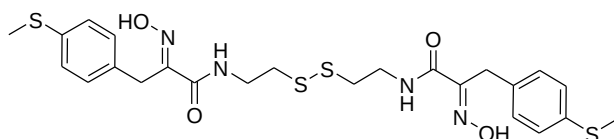
507.1371; Anal. Calc. for $C_{22}H_{26}N_4O_6S_2$: C, 52.16; H, 5.17; N, 11.06. Found: C, 52.10; H, 5.11; N, 10.97.

(2*E*,2'*E*)-*N,N'*-(2,2'-Disulfanediylbis(ethane-2,1-diyl))bis(3-(4-fluorophenyl)-2-(hydroxyimino)propanamide) (75f)



75f (225 mg, 29% from acid **74c**) was prepared according to general procedure A above, and obtained as an amorphous white solid after purification by flash column chromatography (gradient acetone : PE₄₀₋₆₀, 3 : 7 to 4 : 6). R_f 0.25 (acetone : PE₄₀₋₆₀, 3 : 7); IR (neat) 1654, 1625, 1528, 1508, 1427, 1235, 1221, 1023, 1001 cm^{-1} ; ^1H NMR (400 MHz, CD₃OD) δ 2.81 (t, J = 6.6 Hz, 4H, CH₂S), 3.52 (t, J = 6.6 Hz, 4H, CH₂N), 3.88 (s, 4H, 4-CH₂), 6.89-7.00 (m, 4H, 2-H + 6-H), 7.23-7.33 (m, 4H, 3-H + 5-H). ^{13}C NMR (100 MHz, CD₃OD) δ 29.2, 38.6, 39.6, 115.9 (d, J_{CF} = 21.4 Hz), 131.8 (d, J_{CF} = 7.5 Hz), 134.1, 153.1, 162.9 (d, J_{CF} = 243 Hz), 165.9; HRMS (ESI+) m/z calc. for $C_{22}H_{25}F_2N_4O_4S_2$ [M+H]⁺ 511.1280, found: 511.1269; Anal. Calc. for $C_{22}H_{24}F_2N_4O_4S_2$: C, 51.75; H, 4.74; N, 10.97. Found: C, 51.73; H, 4.64; N, 10.92.

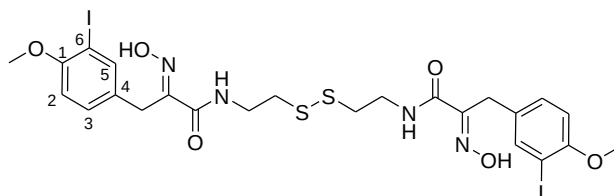
(2*E*,2'*E*)-*N,N'*-(2,2'-Disulfanediylbis(ethane-2,1-diyl))bis(2-(hydroxyimino)-3-(4-(methylthio)phenyl)propanamide) (75g)



75g (114 mg, 20% from acid **74d**) was prepared according to general procedure A above, and obtained as an amorphous light yellow solid after purification by flash column chromatography (acetone : PE₄₀₋₆₀, 3 : 7 then MeOH : CH₂Cl₂ 1 : 9). R_f 0.4 (acetone : PE₄₀₋₆₀, 4 : 6); IR (neat) 1653, 1625, 1523, 1492, 1424, 1000 cm^{-1} ; ^1H NMR (400 MHz, d^6 -DMSO) δ 2.42 (s, 6H, CH₃S), 2.81 (t, J = 6.7 Hz, 4H, CH₂S), 3.41 (m, 4H, CH₂N), 3.76 (s, 4H, 4-CH₂), 7.15 (m, 8H, Ar-H), 8.06 (br. t, J = 5.4, 2H, NH), 11.85 (s, 2H, OH). ^{13}C NMR (100 MHz, d^6 -DMSO) δ 14.9, 28.3, 36.9, 38.1, 126.1, 129.3, 133.5, 135.4, 151.6, 163.2; HRMS (ESI+) m/z calc.

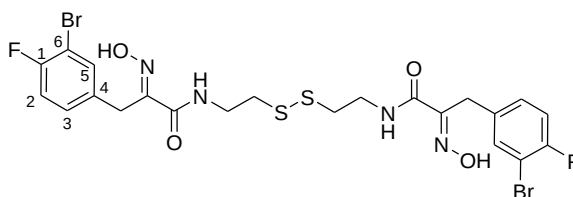
for $C_{24}H_{31}N_4O_4S_4$ $[M+H]^+$ 567.1223, found: 567.1230; Anal. Calc. for $C_{24}H_{30}N_4O_4S_4$: C, 50.86; H, 5.34; N, 9.89. Found: C, 50.91; H, 5.30; N, 9.81.

(2*E*,2'*E*)-*N,N'*-(2,2'-Disulfanediylbis(ethane-2,1-diyl))bis(2-(hydroxyimino)-3-(3-iodo-4-methoxyphenyl)propanamide) (75h)



75h (428 mg, 36% from acid **74e**) was prepared according to general procedure A above, and obtained as an amorphous light yellow solid after purification by flash column chromatography (gradient acetone : PE₄₀₋₆₀, 3 : 7 to 4 : 6). R_f 0.45 (acetone : PE₄₀₋₆₀, 4 : 6); IR (neat) 1657, 1624, 1527, 1487, 1437, 1279, 1251, 1047, 1014, 981 cm^{-1} ; ^1H NMR (400 MHz, CD₃OD) δ 2.80 (t, J = 6.7 Hz, 4H, CH₂S), 3.50 (t, J = 6.7 Hz, 4H, CH₂N), 3.79 (s, 6H, CH₃O), 3.81 (s, 4H, 4-CH₂), 6.79 (d, J = 8.5 Hz, 2H, 2-H), 7.24 (dd, J = 8.5, 2.2 Hz, 2H, 3-H), 7.67 (d, J = 2.2 Hz, 2H, 5-H). ^{13}C NMR (100 MHz, CD₃OD) δ 28.6, 38.7, 39.6, 56.9, 86.2, 112.0, 131.5, 132.3, 141.0, 153.1, 158.3, 165.9; HRMS (ESI+) m/z calc. for $C_{24}H_{29}I_2N_4O_6S_2$ $[M+H]^+$ 786.9612, found: 786.9631; Anal. Calc. for $C_{24}H_{28}I_2N_4O_6S_2$: C, 36.65; H, 3.59; N, 7.12. Found: C, 36.73; H, 3.48; N, 7.07.

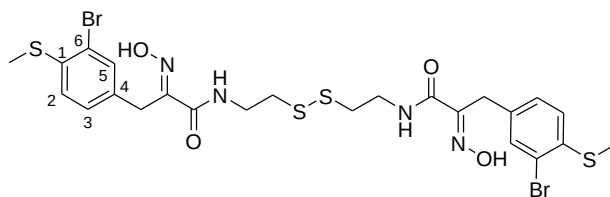
(2*E*,2'*E*)-*N,N'*-(2,2'-Disulfanediylbis(ethane-2,1-diyl))bis(3-(3-bromo-4-fluorophenyl)-2-(hydroxyimino)propanamide) (75i)



75i (201 mg, 30% from acid **74f**) was prepared according to general procedure A above, and obtained as an amorphous white solid after purification by flash column chromatography (gradient acetone : PE₄₀₋₆₀, 3 : 7 to 4 : 6). R_f 0.55 (acetone : PE₄₀₋₆₀, 4 : 6); IR (neat) 1655, 1623, 1528, 1491, 1427, 1404, 1242, 1358, 1013, 984 cm^{-1} ; ^1H NMR (400 MHz, CD₃OD) δ 2.82 (t, J = 6.7 Hz, 4H, CH₂S), 3.53 (t, J = 6.7 Hz, 4H, CH₂N), 3.87 (s, 4H, 4-CH₂), 7.06 (*app*-t, J = 8.5 Hz, 2H, 2-H), 7.27 (ddd, J = 8.5, 4.7, 2.2 Hz, 2H, 3-H), 7.51 (dd, J = 6.7, 2.2 Hz,

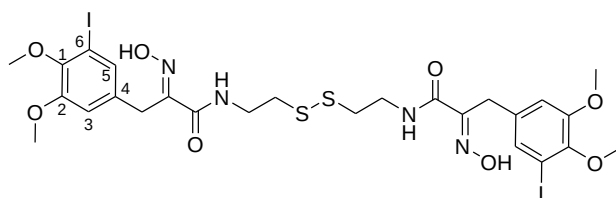
2H, 5-H). ^{13}C NMR (100 MHz, CD_3OD) δ 29.0, 38.6, 39.6, 109.2 (d, $J_{\text{CF}} = 21.1$ Hz), 117.2 (d, $J_{\text{CF}} = 22.5$ Hz), 131.2 (d, $J_{\text{CF}} = 6.9$ Hz), 135.1, 136.1 (d, $J_{\text{CF}} = 3.5$ Hz), 152.5, 159.1 (d, $J_{\text{CF}} = 245$ Hz), 165.7; HRMS (ESI+) m/z calc. for $\text{C}_{22}\text{H}_{23}\text{Br}_2\text{F}_2\text{N}_4\text{O}_4\text{S}_2$ $[\text{M}+\text{H}]^+$ 666.9490, found: 666.9500; Anal. Calc. for $\text{C}_{22}\text{H}_{22}\text{Br}_2\text{F}_2\text{N}_4\text{O}_4\text{S}_2$: C, 39.53; H, 3.32; N, 8.38. Found: C, 39.58; H, 3.30; N, 8.28.

(2*E*,2'*E*)-*N,N'*-(2,2'-Disulfanediyldis(ethane-2,1-diyl))bis(3-(3-bromo-4-(methylthio)phenyl)-2-(hydroxyimino)propanamide) (75j)



75j (270 mg, 25% from acid **74g**) was prepared according to general procedure A above, and obtained as an amorphous light yellow solid after purification by flash column chromatography (gradient acetone : PE_{40-60} , 3 : 7 to 1 : 1). R_f 0.25 (acetone : PE_{40-60} , 3 : 7); IR (neat) 1655, 1623, 1526, 1462, 1432, 1026 cm^{-1} ; ^1H NMR (400 MHz, CD_3OD) δ 2.41 (s, 6H, CH_3S), 2.80 (t, $J = 6.7$ Hz, 4H, CH_2S), 3.51 (t, $J = 6.7$ Hz, 4H, CH_2N), 3.85 (s, 4H, 4- CH_2), 7.09 (d, $J = 8.2$ Hz, 2H, 2-H), 7.25 (dd, $J = 8.2, 1.8$ Hz, 2H, 3-H), 7.45 (d, $J = 1.8$ Hz, 2H, 5-H). ^{13}C NMR (100 MHz, CD_3OD) δ 15.8, 29.1, 38.7, 39.6, 122.4, 126.8, 130.1, 134.2, 136.3, 138.9, 152.7, 165.8; HRMS (ESI+) m/z calc. for $\text{C}_{24}\text{H}_{29}\text{Br}_2\text{N}_4\text{O}_4\text{S}_4$ $[\text{M}+\text{H}]^+$ 722.9433, found: 722.9441; Anal. Calc. for $\text{C}_{24}\text{H}_{28}\text{Br}_2\text{N}_4\text{O}_4\text{S}_4$: C, 39.78; H, 3.90; N, 7.73. Found: C, 39.73; H, 3.85; N, 7.67.

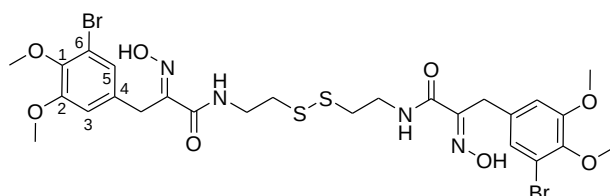
(2*E*,2'*E*)-*N,N'*-(2,2'-Disulfanediyldis(ethane-2,1-diyl))bis(2-(hydroxyimino)-3-(3-iodo-4,5-dimethoxyphenyl)propanamide) (75k)



75k (295 mg, 23% from acid **74h**) was prepared according to general procedure A above, and obtained as an amorphous light yellow solid after purification by flash column chromatography (gradient acetone : PE_{40-60} , 3 : 7 to 4 : 6). R_f 0.15

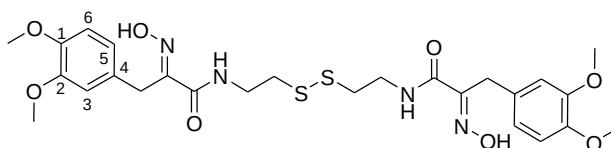
(acetone : PE₄₀₋₆₀, 3 : 7); IR (neat) 1657, 1528, 1480, 1413, 1268, 1137, 1041, 993 cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 2.82 (t, J = 6.7 Hz, 4H, CH₂S), 3.51 (t, J = 6.7 Hz, 4H, CH₂N), 3.71 (s, 6H, CH₃O), 3.80 (s, 6H, CH₃O), 3.83 (s, 4H, 4-CH₂), 6.95 (d, J = 1.7 Hz, 2H, 3-H), 7.23 (d, J = 1.7 Hz, 2H, 5-H). ¹³C NMR (100 MHz, CD₃OD) δ 29.2, 38.7, 39.6, 56.4, 60.8, 92.5, 115.3, 131.9, 136.5, 148.7, 152.8, 153.7, 165.8; HRMS (ESI+) m/z calc. for C₂₆H₃₃I₂N₄O₈S₂ [M+H]⁺ 846.9824, found: 846.9858; Anal. Calc. for C₂₆H₃₂I₂N₄O₈S₂: C, 36.89; H, 3.81; N, 6.62. Found: C, 36.92; H, 3.79; N, 6.57.

(2*E*,2'*E*)-*N,N'*-(2,2'-Disulfanediylbis(ethane-2,1-diyl))bis(3-(3-bromo-4,5-dimethoxyphenyl)-2-(hydroxyimino)propanamide) (75l)



75l (339 mg, 45% from acid **74i**) was prepared according to general procedure A above, and obtained as an amorphous white solid after purification by flash column chromatography (gradient acetone : PE₄₀₋₆₀, 3 : 7 to 4 : 6). R_f 0.35 (acetone : PE₄₀₋₆₀, 4 : 6); IR (neat) 1656, 1625, 1528, 1487, 1413, 1271, 1138, 1044, 998 cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 2.82 (t, J = 6.7 Hz, 4H, CH₂S), 3.52 (t, J = 6.7 Hz, 4H, CH₂N), 3.74 (s, 6H, CH₃O), 3.81 (s, 6H, CH₃O), 3.85 (s, 4H, 4-CH₂), 6.93 (d, J = 1.8 Hz, 2H, 3-H), 7.03 (d, J = 1.8 Hz, 2H, 5-H). ¹³C NMR (100 MHz, CD₃OD) δ 29.5, 38.7, 39.6, 56.6, 60.9, 114.3, 118.0, 126.1, 135.7, 146.1, 152.7, 154.8, 165.8; HRMS (ESI+) m/z calc. for C₂₆H₃₃Br₂N₄O₈S₂ [M+H]⁺ 751.0101, found: 751.0117; Anal. Calc. for C₂₆H₃₂Br₂N₄O₈S₂: C, 41.50; H, 4.29; N, 7.45. Found: C, 41.45; H, 4.21; N, 7.48.

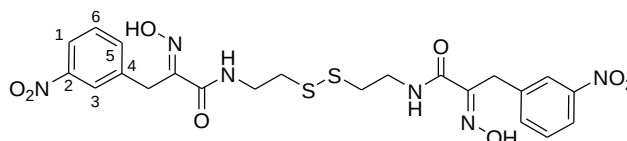
(2*E*,2'*E*)-*N,N'*-(2,2'-Disulfanediylbis(ethane-2,1-diyl))bis(3-(3,4-dimethoxyphenyl)-2-(hydroxyimino)propanamide) (75m)



75m (115 mg, 13% from acid **74j**) was prepared according to general procedure A above, and obtained as an amorphous light yellow solid after purification by flash

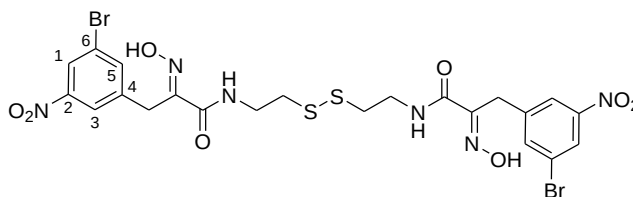
column chromatography (gradient acetone : PE₄₀₋₆₀, 3 : 7 to 4 : 6). Important degradation was observed during the final deprotection step for reaction times superior to 2h. Only partial conversion to the product (and recovery of the starting material) was achieved during the deprotection step, avoiding degradation and allowing efficient purification of the product; R_f 0.25 (acetone : PE₄₀₋₆₀, 4 : 6); IR (neat) 1658, 1628, 1511, 1419, 1259, 1233, 1141, 1022 cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 2.79 (t, *J* = 6.7 Hz, 4H, CH₂S), 3.50 (t, *J* = 6.7 Hz, 4H, CH₂N), 3.76 (s, 6H, CH₃O), 3.77 (s, 6H, CH₃O), 3.85 (s, 4H, 4-CH₂), 6.76-6.84 (m, 4H, 5-H + 6-H), 6.90 (d, *J* = 1.5 Hz, 2H, 3-H). ¹³C NMR (100 MHz, CD₃OD) δ 29.5, 38.7, 39.6, 56.4, 56.6, 113.1, 114.3, 122.7, 131.0, 149.1, 150.3, 153.5, 166.1; HRMS (ESI+) *m/z* calc. for C₂₆H₃₅N₄O₈S₂ [M+H]⁺ 595.1891, found: 595.1897; Anal. Calc. for C₂₆H₃₄N₄O₈S₂: C, 52.51; H, 5.76; N, 9.42. Found: C, 52.54; H, 5.72; N, 9.49.

(2*E*,2'*E*)-*N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl))bis(2-(hydroxyimino)-3-(3-nitrophenyl)propanamide) (75n)



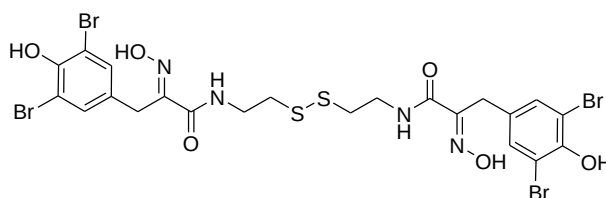
75n (170 mg, 30% from acid **74k**) was prepared according to general procedure A above, and obtained as an amorphous white solid after purification by flash column chromatography (gradient acetone/PE₄₀₋₆₀, 3 : 7 to 4 : 6). R_f 0.35 (AcOEt : PE₄₀₋₆₀, 4 : 6); IR (neat) 1655, 1625, 1520, 1425, 1346, 1208 cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 2.82 (t, *J* = 6.8 Hz, 4H, CH₂S), 3.52 (t, *J* = 6.8 Hz, 4H, CH₂N), 4.03 (s, 4H, 4-CH₂), 7.48 (m, 2H, 6-H), 7.68 (m, 2H, 5-H), 8.04 (m, 2H, 1-H), 8.16 (m, 2H, 3-H); ¹³C NMR (100 MHz, CD₃OD) δ 29.8, 38.6, 39.6, 122.4, 124.9, 130.5, 136.7, 140.6, 149.7, 152.1, 165.6; HRMS (ESI+) *m/z* calc. for C₂₂H₂₅N₆O₈S₂ [M+H]⁺ 565.1170, found: 565.1198; Anal. Calc. for C₂₂H₂₄N₆O₈S₂: C, 46.80; H, 4.28; N, 14.89. Found: C, 46.71; H, 4.23; N, 14.77.

(2*E*,2'*E*)-*N,N'*-(2,2'-Disulfanediyldis(ethane-2,1-diyl))bis(3-(3-bromo-5-nitrophenyl)-2-(hydroxyimino)propanamide) (75o)

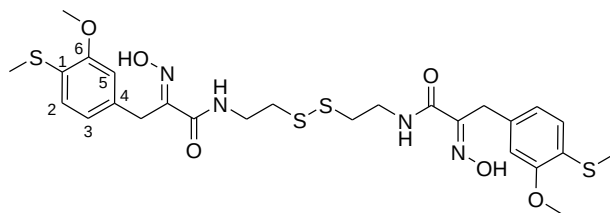


75o (123 mg, 11% from acid **74l**) was prepared according to general procedure A above, and obtained as an amorphous white solid after purification by flash column chromatography (gradient acetone : PE₄₀₋₆₀, 3 : 7 to 4 : 6). *R*_f 0.25 (acetone : PE₄₀₋₆₀, 3 : 7); IR (neat) 1655, 1620, 1526, 1426, 1344, 1026, 988 cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 2.83 (t, *J* = 6.7 Hz, 4H, CH₂S), 3.53 (t, *J* = 6.7 Hz, 4H, CH₂N), 4.02 (s, 4H, 4-CH₂), 7.84-7.87 (m, 2H, 5-H), 8.12-8.15 (m, 2H, 3-H), 8.20 (m, 2H, 1-H). ¹³C NMR (100 MHz, CD₃OD - *d*⁶-DMSO) δ 29.8, 38.7, 39.6, 123.3, 124.1, 125.5, 139.4, 142.8, 150.2, 151.7, 165.0; HRMS (ESI⁺) *m/z* calc. for C₂₂H₂₃Br₂N₆O₈S₂ [M+H]⁺ 720.9380, found: 720.9412; Anal. Calc. for C₂₂H₂₂Br₂N₆O₈S₂: C, 36.58; H, 3.07; N, 11.63. Found: C, 36.64; H, 3.01; N, 11.68.

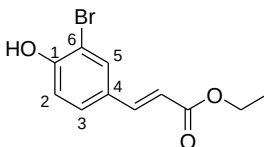
(2*E*,2'*E*)-*N,N'*-(2,2'-Disulfanediyldis(ethane-2,1-diyl))bis(3-(3,5-dibromo-4-hydroxyphenyl)-2-(hydroxyimino)propanamide) (75p)



75p (270 mg, 22% from acid **74n**) was prepared according to general procedure A above, and obtained as an amorphous light yellow solid after purification by flash column chromatography (gradient acetone : PE₄₀₋₆₀, 35 : 65 to 50 : 50). *R*_f 0.3 (acetone : PE₄₀₋₆₀, 4 : 6); IR (neat) 1653, 1625, 1520, 1544, 1474, 1406, 1159 cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 2.82 (t, *J* = 6.7 Hz, 4H, CH₂S), 3.53 (t, *J* = 6.7 Hz, 4H, CH₂N), 3.79 (s, 4H, 4-CH₂), 7.39 (s, 4H, Ar-H). ¹³C NMR (100 MHz, CD₃OD) δ 28.5, 38.6, 39.6, 112.0, 132.3, 134.0, 150.7, 152.6, 165.7; HRMS (ESI⁺) *m/z* calc. for C₂₂H₂₃Br₄N₄O₆S₂ [M+H]⁺ 818.7787, found: 818.7811; Anal. Calc. for C₂₂H₂₂Br₄N₄O₆S₂: C, 32.14; H, 2.70; N, 6.81. Found: C, 32.08; H, 2.70; N, 6.74.

(2*E*,2'*E*)-*N,N'*-(2,2'-Disulfanediyldis(ethane-2,1-diyl))bis(2-(hydroxyimino)-3-(3-methoxy-4-(methylthio)phenyl)propanamide) (75q)

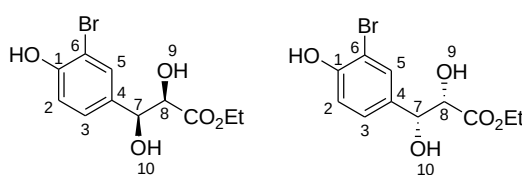
75q (320 mg, 36% from acid **74o**) was prepared according to general procedure A above, and obtained as an amorphous light yellow solid after purification by flash column chromatography (gradient acetone/PE₄₀₋₆₀, 35 : 65 to 50 : 50). *R*_f 0.15 (acetone/PE₄₀₋₆₀, 3 : 7); IR (neat) 1655, 1624, 1525, 1484, 1408, 1248, 1031 cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 2.32 (s, 6H, CH₃S), 2.79 (t, *J* = 6.7 Hz, 4H, CH₂S), 3.49 (t, *J* = 6.7 Hz, 4H, CH₂N), 3.80 (s, 6H CH₃O), 3.88 (s, 4H, 4-CH₂), 6.85 (dd, *J* = 7.9, 1.6 Hz, 2H, 3-H), 6.86 (d, *J* = 1.6 Hz, 2H, 5-H), 7.03 (d, *J* = 7.9 Hz, 2H, 2-H); ¹³C NMR (100 MHz, CD₃OD/*d*⁶-DMSO) δ 14.7, 29.9, 38.6, 39.5, 56.4, 112.6, 123.1, 126.0, 127.0, 136.3, 153.3, 157.5, 165.7; HRMS (ESI+) *m/z* calc. for C₂₆H₃₅N₄O₆S₄ [M+H]⁺ 627.1434, found: 627.1439; Anal. Calc. for C₂₆H₃₄N₄O₆S₄: C, 49.82; H, 5.47; N, 8.94. Found: C, 49.92; H, 5.56; N, 8.93.

(*E*)-Ethyl 3-(3-bromo-4-hydroxyphenyl)acrylate (86)

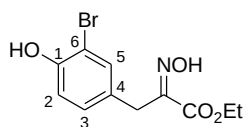
To a solution of 3-bromo-4-hydroxybenzaldehyde **72a** (1 g, 4.98 mmol, 1 eq.) in freshly distilled pyridine (2.5 mL) under an argon atmosphere were added 3-ethoxy-3-oxopropanoic acid **83** (705 μL, 5.97 mmol, 1.2 eq.) and pyrrolidine (41 μL, 0.498 mmol, 0.1 eq.). The resulting mixture was heated to 75°C. After 5 hours, the solvent was evaporated. The residue was dissolved in AcOEt (20 mL), the organic phase was washed with 0.5 N aqueous HCl (4 mL). The aqueous phase was extracted with AcOEt (5 mL). The combined organic layers were dried (MgSO₄) and concentrated. The product (1.21 g, 90%) was obtained after flash column chromatography (AcOEt/PE₄₀₋₆₀ 2 : 8) and recrystallization (CH₂Cl₂/hexane): m.p 103-106°C; *R*_f 0.55 (AcOEt/PE₄₀₋₆₀ 2 : 8). IR (neat) 3323, 1682, 1632, 1599, 1200 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 1.33 (t, *J* = 7.1 Hz,

3H, CH₃), 4.25 (q, J = 7.1 Hz, 2H, CH₂), 5.81 (s, 1H, OH), 6.30 (d, J = 16.0 Hz, 1H, CHC(O)), 7.03 (d, J = 8.4 Hz, 1H, 2-H), 7.40 (dd, J = 8.4, 2.0 Hz, 1H, 3-H), 7.55 (d, J = 16.0 Hz, 1H, 4-CH), 7.65 (d, J = 2.0 Hz, 1H, 5-H); ¹³C NMR (100 MHz, CDCl₃) δ 14.3, 60.5, 110.8, 116.4, 117.3, 128.8, 129.1, 131.7, 142.6, 153.9, 166.9; HRMS (ESI+) m/z calc. for C₁₁H₁₂BrO₃ [M+H]⁺ 270.9964 found: 270.9959; Anal. Calc. for C₁₁H₁₁BrO₃: C, 48.73; H, 4.09. Found: C, 48.85; H, 4.05.

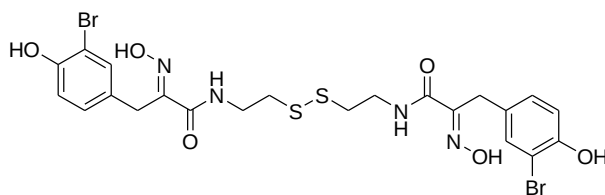
Ethyl 3-(3-bromo-4-hydroxyphenyl)-2,3-dihydroxypropanoate (**87**)



To a mixture of unsaturated ester (**86**) (400 mg, 1.48 mmol, 1 eq.) in a 2 : 1 CH₃CN/H₂O mixture (12 mL) was added 4-methylmorpholine-*N*-oxide (NMO) (225 mg, 1.92 mmol, 1.3 eq.), at 0°C under an argon atmosphere. To this mixture was added a solution of osmium tetroxide (7.5 mg, 0.03 mmol, 0.02 eq.) in *t*-BuOH (1.5 mL). The resulting mixture was stirred at 0°C for 45 minutes, then at rt for 13 hours. Saturated aqueous Na₂SO₃ (1 mL) was added and CH₃CN was evaporated under reduced pressure. The aqueous phase was extracted with AcOEt (4 x 15 mL). The combined organic phases were dried (MgSO₄) and concentrated under reduced pressure. The product (405 mg, 90%) was obtained as a light yellow oil after purification by flash column chromatography (AcOEt/PE₄₀₋₆₀ 6 : 4). R_f 0.4 (AcOEt/PE₄₀₋₆₀ 6 : 4); ¹H NMR (400 MHz, *d*⁶-DMSO) δ 1.11 (t, J = 7.1 Hz, 3H, CH₃), 3.98-4.07 (m, 3H, CH₂CH₃ + 8-H), 4.65-4.72 (m, 1H, 7-H), 5.32 (d, J = 7.2 Hz, 1H, OH), 5.44 (d, J = 5.7 Hz, 1H, OH), 6.86 (d, J = 8.3 Hz, 1H, 2-H), 7.11 (dd, J = 8.3, 1.8 Hz, 1H, 3-H), 7.43 (d, J = 1.8 Hz, 1H, 5-H), 10.07 (s, 1H, 1-OH); ¹³C NMR (100 MHz, *d*⁶-DMSO) δ 13.9, 59.9, 73.2, 75.4, 108.5, 115.5, 126.9, 131.1, 134.1, 152.9, 172.0; HRMS (CI+) m/z calc. for C₁₁H₁₇BrNO₅ [M+NH₄]⁺ 322.0285 found: 322.0291; Anal. Calc. for C₁₁H₁₃BrO₅: C, 43.30; H, 4.29. Found: C, 43.33; H, 4.15.

(E)-Ethyl 3-(3-bromo-4-hydroxyphenyl)-2-(hydroxyimino)propanoate (88)

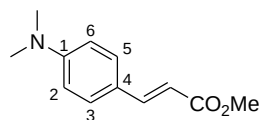
To a solution of diol **87** (215 mg, 0.705 mmol, 1 eq.) in anhydrous benzene (5.6 mL) under an argon atmosphere was added *p*-toluenesulfonic acid monohydrate (13.4 mg, 0.0705 mmol, 0.1 eq.). The resulting mixture was heated at reflux for 3 hours, then cooled to rt. The solvent was evaporated and the residue dissolved in anhydrous MeOH (1.6 mL), under an argon atmosphere. Hydroxylamine hydrochloride (49.0 mg, 0.705 mmol, 1 eq.) and sodium acetate (64 mg, 0.775 mmol, 1.1 eq.) were successively added at 0°C. The resulting mixture was stirred for 6.5 hours. The white precipitate was removed by filtration and washed with MeOH. The filtrate was concentrated and the product (152 mg, 71%) was obtained after purification by flash column chromatography (AcOEt/CH₂Cl₂ 1 : 9). Ester **88** has been previously reported in the literature.^{158,242} ¹H NMR (400 MHz, CDCl₃) δ 1.34 (t, *J* = 7.1 Hz, 3H, CH₃), 3.88 (s, 2H, 4-CH₂), 4.30 (q, *J* = 7.1 Hz, 1H, CH₂CH₃), 5.41 (br. s, 1H, 1-OH), 6.92 (d, *J* = 8.3 Hz, 1H, 2-H), 7.18 (dd, *J* = 8.3, 2.1 Hz, 1H, 3-H), 7.43 (d, *J* = 2.1 Hz, 1H, 5-H); ¹³C NMR (100 MHz, CDCl₃) δ 14.1, 29.3, 62.1, 110.1, 116.0, 129.2, 130.2, 132.5, 151.1, 151.4, 163.1; HRMS (ESI+) *m/z* calc. for C₁₁H₁₃BrNO₄ [M+H]⁺ 302.0022 found: 302.0034.

(2E,2'E)-N,N'-(2,2'-Disulfanediy)bis(ethane-2,1-diyl)bis(3-(3-bromo-4-hydroxyphenyl)-2-(hydroxyimino)propanamide) (28, PSAMMAPLIN A)

To a solution of cystamine **53** (12.6 mg, 0.0828 mmol, 1 eq.) and ester **88** (50 mg, 0.166 mmol, 2 eq.) in an anhydrous 1 : 1 CH₂Cl₂/CH₃CN mixture (2.4 mL), in a sealed tube and under an argon atmosphere, was added trimethylaluminium (2M in hexanes, 166 µL, 0.331 mmol, 4 eq.). Particular care was taken while handling the pyrophoric trimethylaluminium reagent. The resulting mixture was stirred at rt for 30 minutes, then at 60°C for 12h. 0.5M aqueous HCl was added dropwise and the solution was extracted several times with AcOEt over 20 minutes. The

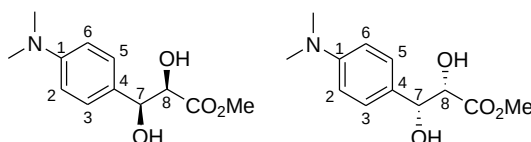
combined organic layers were dried (MgSO₄) and concentrated under reduced pressure. Purification by flash column chromatography (acetone/PE₄₀₋₆₀ 1 : 1) afforded Psammaphin A (27 mg, 49%, 82% brsm).

(E)-Methyl 3-(4-(dimethylamino)phenyl)acrylate (92)



Palladium (II) acetate (280 mg, 1.25 mmol, 0.05 eq.) was added to a mixture of 4-bromo-*N,N*-dimethylbenzenamine **91** (5.0 g, 25.0 mmol, 1 eq.), methylacrylate (22.5 mL, 250 mmol, 10 eq.), triethylamine (10.4 mL, 75 mmol, 3 eq.) and tris(2-methylphenyl)phosphine (760 mg, 2.5 mmol, 0.1 eq.) in anhydrous and degassed DMF (125 mL), at rt, under an inert atmosphere. The resulting mixture was stirred at 140 °C for 20h and monitored by TLC. The mixture was then poured into water (500 mL) and extracted with Et₂O (2 x 400 mL) and CH₂Cl₂ (400 mL). The combined organic phases were washed with brine (300 mL), dried (MgSO₄) and concentrated. The product (4.42 g, 86%) was purified by flash column chromatography (AcOEt/PE₄₀₋₆₀ 15 : 85). Light yellow crystals: m.p. 126-128°C (EtOH/water) (lit.¹⁰ 132-135 °C); R_f 0.5 (AcOEt/PE₄₀₋₆₀, 2 : 8); IR (neat) 1699, 1601, 1524, 1441, 1365, 1328, 1163, 984 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 3.02 (s, 6H, NMe₂), 3.78 (s, 3H, OCH₃), 6.22 (d, *J* = 15.8 Hz, 1H, 4-C_HC_H), 6.64-6.70 (m, 2H, 2-H + 6-H), 7.38-7.46 (m, 2H, 3-H + 5-H), 7.63 (d, *J* = 15.8 Hz, 1H, 4-CH); ¹³C NMR (100 MHz, CDCl₃) δ 40.1, 51.3, 111.8, 112.0, 122.1, 129.7, 145.3, 151.7, 168.3; HRMS (ESI+) *m/z* calc. for C₁₂H₁₆NO₂ [M+H]⁺ 206.1176, found: 206.1179; Anal. Calc. for C₁₂H₁₅NO₂: C, 70.22; H, 7.37; N, 6.82. Found: C, 70.31; H, 7.43; N, 6.93.

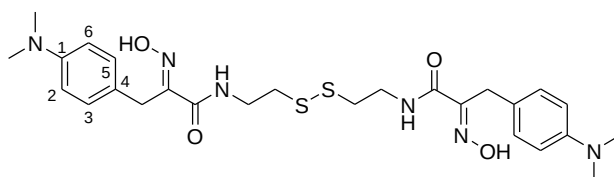
Methyl 3-(4-(dimethylamino)phenyl)-2,3-dihydroxypropanoate (93)



To a mixture of unsaturated ester **23** (3.0 g, 14.6 mmol, 1 eq.) and NMO (3.77 g, 32.1 mmol, 2.2 eq.) in a 2 : 1 CH₃CN/H₂O mixture (130 mL) was added a solution of osmium tetroxide (74 mg, 0.292 mmol, 0.02 eq.) in *t*-BuOH (15 mL). The

resulting mixture was stirred at rt under an inert atmosphere. The reaction was monitored by TLC. After 36h, saturated aqueous Na₂SO₃ was added and the mixture was extracted 3 times with AcOEt. The combined organic layers were washed 2 times with brine, dried (MgSO₄) and concentrated under reduced pressure. Purification by flash column chromatography (AcOEt/CH₂Cl₂ 6 : 4) afforded diol **93** (2.57 g, 73%). Colourless crystals: m.p. 69-71°C (Et₂O); R_f 0.35 (AcOEt/CH₂Cl₂, 6 : 4); IR (neat) 3456, 1743, 1613, 1519, 1437, 1202, 1119, 1061 cm⁻¹; ¹H NMR (400 MHz, *d*⁶-DMSO) δ 2.85 (s, 6H, NMe₂), 3.54 (s, 3H, OCH₃), 4.03 (dd, *J* = 7.3, 4.5 Hz, 1H, 8-H), 4.67 (m, 1H, 7-H), 5.23 (d, *J* = 5.6 Hz, 1H, OH), 5.28 (d, *J* = 7.3 Hz, 1H, OH), 6.62-6.68 (m, 2H, 2-H + 6-H), 7.10-7.17 (m, 2H, 3-H + 5-H); ¹³C NMR (100 MHz, *d*⁶-DMSO) δ 40.2, 51.2, 73.8, 75.8, 111.7, 127.3, 129.3, 149.6, 172.7; HRMS (ESI+) *m/z* calc. for C₁₂H₁₈NO₄ [M+H]⁺ 240.1230, found: 240.1237; Anal. Calc. for C₁₂H₁₇NO₄: C, 60.24; H, 7.16; N, 5.85. Found: C, 60.42; H, 7.03; N, 5.94.

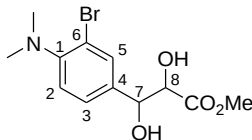
(2*E*,2'*E*)-*N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl))bis(3-(4-(dimethylamino)phenyl)-2-(hydroxyimino)propanamide) (89)



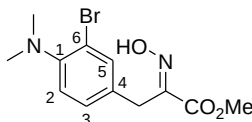
To a solution of diol **93** (1.20 g, 5.01 mmol, 1 eq.) in anhydrous benzene (40 mL) was added *p*-toluenesulfonic acid monohydrate (48 mg, 0.251 mmol, 0.05 eq.). The resulting mixture was stirred at reflux for 3.5h, and then quenched with a saturated aqueous NaHCO₃ solution (20 mL). The two phases were separated, and the aqueous phase extracted with AcOEt (20 mL). The combined organic layers were dried (MgSO₄) and concentrated to afford the crude arylpyruvate ester intermediate (1.11 g, quant.) which was used without further purification. To a solution of the crude arylpyruvic ester intermediate (800 mg, 3.62 mmol, 1.0 eq.) in anhydrous MeOH (8 mL) were added hydroxylamine hydrochloride (277 mg, 3.98 mmol, 1.1 eq.) and sodium acetate (356 mg, 4.34 mmol, 1.2 eq.). The resulting mixture was stirred for 2h under an inert atmosphere and monitored by TLC. The solvent was then evaporated. The residue was dissolved in water and extracted 2 times with AcOEt. The combined organic phases were dried (MgSO₄)

and concentrated, affording methyl 3-(4-(dimethylamino)phenyl)-2-(hydroxyimino)propanoate **94** (835 mg, 98%) which was used without further purification. ^1H NMR (400 MHz, d^6 -DMSO) δ 2.83 (s, 6H), 3.67-3.73 (m, 5H), 6.58-6.66 (m, 2H), 6.97-7.05 (m, 2H), 12.32 (s, 1H); ^{13}C NMR (100 MHz, d^6 -DMSO) δ 28.9, 40.2, 52.0, 112.5, 123.6, 129.1, 149.0, 150.1, 164.2; HRMS (ESI+) m/z calc. for $\text{C}_{12}\text{H}_{17}\text{N}_2\text{O}_3$ $[\text{M}+\text{H}]^+$ 237.1234, found: 237.1239.

A solution of cystamine **53** (161 mg, 1.06 mmol, 1 eq.) and trimethylaluminium (2M in hexane, 1.06 mL, 2.12 mmol, 2 eq.) in anhydrous CH_2Cl_2 (20 mL) was stirred for 0.5h at rt, in a sealed tube and under an inert atmosphere. Particular care was taken while handling the pyrophoric trimethylaluminium reagent. Gas evolution could be observed and the pressure was released several times over 0.5h. Ester **94** (500 mg, 2.12 mmol, 2 eq.) was then added in several portions and the resulting mixture was stirred at reflux for 8h. The formation of a yellow precipitate could be observed. The mixture was cooled to rt and quenched by addition of 0.5M aqueous HCl (pH $\sim$ 3). The biphasic solution was stirred vigorously and the pH was maintained at 3 for 30 minutes. After stirring for 30 minutes the pH was adjusted to 8-9 by addition of aqueous NaHCO_3 . The layers were separated and the aqueous layer was extracted 2 times with AcOEt. The combined organic phases were dried (MgSO_4) and concentrated. The product was purified by flash column chromatography (gradient acetone/ PE_{40-60} , 3 : 7 to 4 : 6). After concentration of the fractions, CH_2Cl_2 was added to the resulting oily residue, allowing precipitation of the product as a white amorphous solid. Compound **89** (315 mg, 53% from **93**, 76% brsm) was collected by filtration and dried under vacuum. White amorphous solid; R_f 0.5 (acetone/ PE_{40-60} , 4 : 6); IR (neat) 3393, 3375, 3265 (br), 1653, 1624, 1518, 1439, 1423, 1342, 1221 cm^{-1} ; ^1H NMR (400 MHz, d^6 -DMSO) δ 2.77-2.84 (m, 16H, $\text{NMe}_2 + \text{CH}_2\text{S}$), 3.40 (m, 4H, CH_2N), 3.68 (s, 4H, 4- CH_2), 6.58-6.63 (m, 4H, 2-H + 6-H), 6.99-7.04 (m, 4H, 3-H + 5-H), 7.99 (t, $J = 5.8$ Hz, 2H, NH), 11.69 (s, 2H, OH); ^{13}C NMR (100 MHz, d^6 -DMSO) δ 27.7, 36.9, 38.0, 40.2, 112.4, 124.1, 129.2, 148.9, 152.3, 163.3; HRMS (ESI+) m/z calc. for $\text{C}_{26}\text{H}_{37}\text{N}_6\text{O}_4\text{S}_2$ $[\text{M}+\text{H}]^+$ 561.2312, found: 561.2321; Anal. Calc. for $\text{C}_{26}\text{H}_{36}\text{N}_6\text{O}_4\text{S}_2$: C, 55.69; H, 6.47; N, 14.99. Found: C, 55.80; H, 6.40; N, 14.95.

Methyl 3-(3-bromo-4-(dimethylamino)phenyl)-2,3-dihydroxypropanoate (95)

N-bromosuccinimide (744 mg, 4.18 mmol, 1 eq.) was added to a solution of diol **93** (1.0 g, 4.18 mmol, 1 eq.) in anhydrous DMF (35 mL) under an inert atmosphere. The resulting mixture was stirred at rt and monitored by TLC. After 2h the mixture was poured into water (100mL) and extracted with AcOEt (2 x 100 mL). The combined organic layers were washed 2 times with brine (100 mL), dried (MgSO₄) and concentrated. Purification by flash column chromatography (AcOEt/CH₂Cl₂ 4 : 6) afforded the brominated product **95** (1.20 g, 90%) as a brown viscous oil. *R*_f 0.5 (AcOEt/CH₂Cl₂, 4 : 6); IR (neat) 3427, 1735, 1602, 1494, 1438, 1402, 1271, 1195, 1113, 1051, 1037, 945 cm⁻¹; ¹H NMR (400 MHz, *d*⁶-DMSO) δ 2.68 (s, 6H, NMe₂), 3.61 (s, 3H, CH₃), 4.14 (dd, *J* = 7.5, 3.5 Hz, 1H, 8-H), 4.81 (dd, *J* = 5.9, 3.5 Hz, 1H, 7-H), 5.31 (d, *J* = 7.5 Hz, 1H, OH), 5.52 (d, *J* = 5.9 Hz, 1H, OH), 7.11 (d, *J* = 8.3 Hz, 1H, 2-H), 7.27 (dd, *J* = 8.3, 1.9 Hz, 1H, 3-H), 7.56 (d, *J* = 1.9 Hz, 1H, 5-H); ¹³C NMR (100 MHz, *d*⁶-DMSO) δ 43.8, 51.4, 72.9, 75.0, 117.8, 120.1, 126.6, 131.7, 138.1, 150.2, 172.5; HRMS (ESI+) *m/z* calc. for C₁₂H₁₇BrNO₄ [M+H]⁺ 318.0335, found: 318.0344; Anal. Calc. for C₁₂H₁₆BrNO₄: C, 45.30; H, 5.07; N, 4.40. Found: C, 45.26; H, 4.97; N, 4.34.

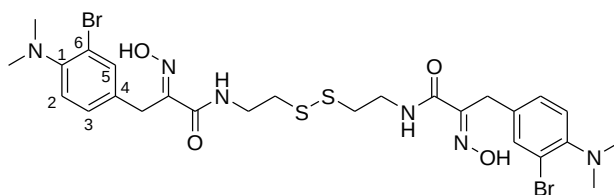
Methyl 3-(3-bromo-4-(dimethylamino)phenyl)-2-(hydroxyimino)propanoate (96)

To a solution of diol **95** (900 mg, 2.83 mmol, 1 eq.) in anhydrous benzene (23 mL) was added *p*-toluenesulfonic acid monohydrate (108 mg, 0.564 mmol, 0.2 eq.). The resulting mixture was stirred at reflux for 36h, and then quenched with a saturated aqueous NaHCO₃ solution (23 mL). The two phases were separated, and the aqueous phase was extracted with AcOEt (1 x 23 mL). The combined organic

layers were dried (MgSO_4) and concentrated to afford 795 mg of a solid 7 : 3 mixture of the arylpyruvic ester intermediate and starting material respectively, which was used without further purification.

To this crude solid (795 mg: 560 mg of dehydrated intermediate, 235 mg of **95**) dissolved in anhydrous MeOH (5.6 mL) were added hydroxylamine hydrochloride (193 mg, 2.78 mmol) and sodium acetate (249 mg, 3.04 mmol, 1.2 eq.). The resulting mixture was stirred for 2h at rt under an inert atmosphere and monitored by TLC. The solvent was then evaporated. The residue was dissolved in water and extracted 2 times with AcOEt. The combined organic phases were dried (MgSO_4) and concentrated. Purification by flash chromatography (AcOEt/PE₄₀₋₆₀ 3 : 7) afforded compound **96** (485 mg, 54% from **95**, 75% brsm) as a yellow viscous oil. R_f 0.45 (AcOEt/PE₄₀₋₆₀, 3 : 7); IR (neat) 3234, 1721, 1492, 1440, 1399, 1299, 1202, 1114, 1002, 945 cm^{-1} ; ^1H NMR (400 MHz, d^6 -DMSO) δ 2.66 (s, 6H, NMe_2), 3.72 (s, 3H, CH_3O), 3.76 (s, 2H, 4- CH_2), 7.09 (d, J = 8.3 Hz, 1H, 2-H), 7.14 (dd, J = 8.3, 1.9 Hz, 1H, 3-H), 7.37 (d, J = 1.9 Hz, 1H, 5-H), 12.53 (s, 1H, OH); ^{13}C NMR (100 MHz, d^6 -DMSO) δ 28.8, 43.7, 52.2, 118.1, 120.8, 128.6, 132.2, 133.3, 149.1, 149.8, 164.0; HRMS (ESI+) m/z calc. for $\text{C}_{12}\text{H}_{16}\text{BrN}_2\text{O}_3$ $[\text{M}+\text{H}]^+$ 315.0339, found: 315.0345; Anal. Calc. for $\text{C}_{12}\text{H}_{15}\text{BrN}_2\text{O}_3$: C, 45.73; H, 4.80; N, 8.89. Found: C, 45.64; H, 4.93; N, 8.79.

(2*E*,2'*E*)-*N,N'*-(2,2'-Disulfanediybis(ethane-2,1-diyl))bis(3-(3-bromo-4-(dimethylamino)phenyl)-2-(hydroxyimino)propanamide) (90)



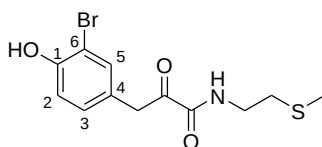
A solution of cystamine **53** (97 mg, 0.634 mmol, 1 eq.) and trimethylaluminium (2M in hexane, 635 μL , 1.27 mmol, 2 eq.) in anhydrous CH_2Cl_2 (12 mL) was stirred for 1h at rt, in a sealed tube and under an inert atmosphere. Particular care was taken while handling the pyrophoric trimethylaluminium reagent. Gas evolution could be observed and the pressure was released several times over 0.5h. Ester **96** (400 mg, 1.27 mmol, 2 eq.) in CH_2Cl_2 (2 mL) was then added dropwise and the resulting mixture was stirred at reflux for 8h. The mixture was cooled to rt and quenched by addition of 0.5M aqueous HCl (pH $\sim$ 3). The

biphasic solution was stirred vigorously and the pH was maintained at 3 for 30 minutes. After stirring for 30 minutes the pH was adjusted to 8-9 by addition of aqueous NaHCO₃. The layers were separated and the aqueous layer was extracted 2 times with AcOEt. The combined organic phases were dried (MgSO₄) and concentrated. The product (175 mg, 34% from **90**, 50% brsm) was purified by flash column chromatography (gradient acetone/PE₄₀₋₆₀, 3 : 7 to 4 : 6) and obtained as a white amorphous solid. R_f 0.5 (acetone/PE₄₀₋₆₀, 4 : 6); IR (neat) 3209 (br), 1655, 1625, 1523, 1490, 1452, 1432, 1204 cm⁻¹; ¹H NMR (400 MHz, *d*⁶-DMSO) δ 2.65 (s, 12H, NMe₂), 2.81 (t, *J* = 6.8 Hz, 4H, CH₂S), 3.41 (m, 4H, CH₂N), 3.73 (s, 4H, 4-CH₂), 7.06 (d, *J* = 8.3 Hz, 2H, 2-H), 7.15 (dd, *J* = 8.3, 1.8 Hz, 2H, 3-H), 7.38 (d, *J* = 1.8 Hz, 2H, 5-H), 8.08 (t, *J* = 5.9 Hz, 2H, NH), 11.89 (s, 2H, OH); ¹³C NMR (100 MHz, *d*⁶-DMSO) δ 27.8, 36.9, 38.1, 43.7, 118.0, 120.7, 128.8, 132.6, 133.4, 149.6, 151.4, 163.1; HRMS (ESI+) *m/z* calc. for C₂₆H₃₅Br₂N₆O₄S₂ [M+H]⁺ 717.0522, found: 717.0531; Anal. Calc. for C₂₆H₃₄Br₂N₆O₄S₂: C, 43.46; H, 4.77; N, 11.70. Found: C, 43.58; H, 4.65; N, 11.59.

General procedure for the synthesis of ketoamide containing analogues:

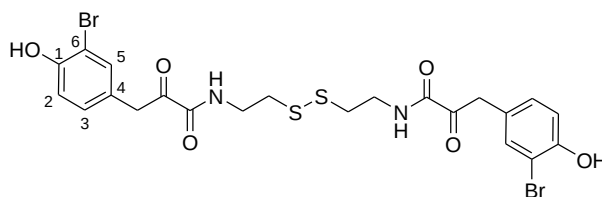
The appropriate crude acid (1 eq.) was added to a solution of the appropriate primary amine (1 eq. for 2-(methylthio)ethanamine, 0.5 eq. for cystamine, obtained from neutralization of cystamine dihydrochloride with sodium hydroxide in water and extraction with chloroform) and diisopropylethylamine (1.1 eq.) at 0°C in freshly distilled THF (13 mL/mmol), followed by py-BOP (1.1 eq.). The resulting mixture was stirred overnight at rt. The day after the solvent was evaporated *in vacuo* and the product was purified by flash column chromatography without any prior work-up.

3-(3-Bromo-4-hydroxyphenyl)-*N*-(2-(methylthio)ethyl)-2-oxopropanamide (97a)



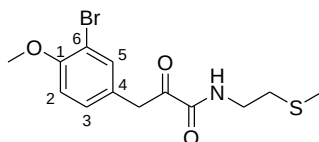
Following the general procedure above, crude acid **51** was coupled with 2-(methylthio)ethanamine **77** to afford product **97a** (370 mg, 40%) as a white solid after purification by flash column chromatography (AcOEt : CH₂Cl₂, 1 : 9) and recrystallization from Et₂O: m.p. 55-59°C; R_f 0.6 (AcOEt : PE₄₀₋₆₀, 1 : 1); IR 3346, 1655, 1543, 1505, 1419 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 2.12 (s, 3H, CH₃S), 2.66 (t, *J* = 6.5 Hz, 2H, CH₂S), 3.52 (m, 2H, CH₂N), 4.13 (s, 2H, 4-CH₂), 5.47 (br. s., 1H, OH), 6.98 (d, *J* = 8.3 Hz, 1H, 2-H), 7.11 (dd, *J* = 8.3, 2.0 Hz, 1H, 3-H), 7.26 (br. t., 1H, NH), 7.37 (d, *J* = 2.0 Hz, 1H, 5-H). ¹³C NMR (100 MHz, CDCl₃) δ 15.1, 33.4, 37.8, 41.8, 110.3, 116.2, 126.0, 130.7, 133.1, 151.5, 159.8, 195.3; HRMS (ESI+) *m/z* calc. for C₁₂H₁₅BrNO₃S [M+H]⁺ 331.9951, found: 331.9945; Anal. Calc. for C₁₂H₁₄BrNO₃S: C, 43.38; H, 4.25; N, 4.22. Found: C, 43.48; H, 4.16; N, 4.13.

***N,N'*-(2,2'-Disulfanediylbis(ethane-2,1-diyl))bis(3-(3-bromo-4-hydroxyphenyl)-2-oxopropanamide) (97b)**



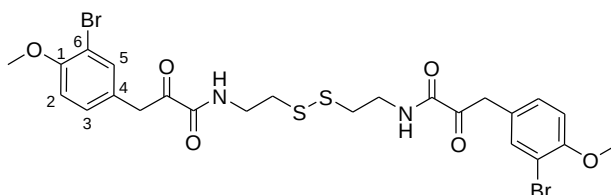
Following the general procedure above, crude acid **51** was coupled with cystamine **53** to afford product **97b** (117 mg, 24%) as an amorphous white solid after purification by flash column chromatography (AcOEt : CH₂Cl₂, 3 : 7). R_f 0.6 (AcOEt : CH₂Cl₂, 4 : 6); IR 3354, 1668, 1495, 1418 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 2.82 (t, *J* = 6.4 Hz, 4H, CH₂S), 3.63 (m, 4H, CH₂N), 4.12 (s, 4H, 4-CH₂), 5.49 (br. s, 2H, OH), 6.97 (d, *J* = 8.3 Hz, 2H, 2-H), 7.10 (dd, *J* = 8.3, 2.0 Hz, 2H, 3-H), 7.30 (br. t., 2H, NH), 7.37 (d, *J* = 2.0 Hz, 2H, 5-H). ¹³C NMR (100 MHz, CDCl₃) δ 37.2, 38.4, 41.8, 110.3, 116.2, 125.9, 130.7, 133.1, 151.6, 160.0, 195.3; HRMS (ESI+) *m/z* calc. for C₂₂H₂₃Br₂N₂O₆S₂ [M+H]⁺ 632.9359, found: 632.9368; Anal. Calc. for C₂₂H₂₂Br₂N₂O₆S₂: C, 41.65; H, 3.50; N, 4.42. Found: C, 41.54; H, 3.58; N, 4.37.

3-(3-Bromo-4-methoxyphenyl)-*N*-(2-(methylthio)ethyl)-2-oxopropanamide (98a)



Following the general procedure above, crude acid **74a** was coupled with 2-(methylthio)ethanamine **77** to afford product **98a** (481 mg, 63%) as a white solid after purification by flash column chromatography (AcOEt : PE₄₀₋₆₀, 4 : 6) and recrystallization from Bu₂O: m.p. 64-66°C; R_f 0.65 (AcOEt : PE₄₀₋₆₀, 1 : 1); IR 1682, 1521, 1498, 1281, 1257, 1055 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 2.12 (s, 3H, CH₃S), 2.66 (t, *J* = 6.5 Hz, 2H, CH₂S), 3.52 (m, 2H, CH₂N), 3.88 (s, 3H, CH₃O), 4.13 (s, 2H, 4-CH₂), 6.86 (d, *J* = 8.4 Hz, 1H, 2-H), 7.17 (dd, *J* = 8.4, 2.1 Hz, 1H, 3-H), 7.26 (br. t., 1H, NH), 7.44 (d, *J* = 2.1 Hz, 1H, 5-H). ¹³C NMR (100 MHz, CDCl₃) δ 15.1, 33.4, 37.8, 41.8, 56.2, 111.7, 112.0, 126.0, 130.0, 134.5, 155.1, 159.8, 195.4; HRMS (ESI⁺) *m/z* calc. for C₁₃H₁₇BrNO₃S [M+H]⁺ 346.0107, found: 346.0111; Anal. Calc. for C₁₃H₁₆BrNO₃S: C, 45.10; H, 4.66; N, 4.05. Found: C, 45.19; H, 4.60; N, 3.92.

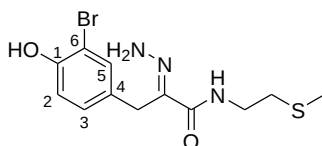
***N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl)bis(3-(3-bromo-4-methoxyphenyl)-2-oxopropanamide) (98b)**



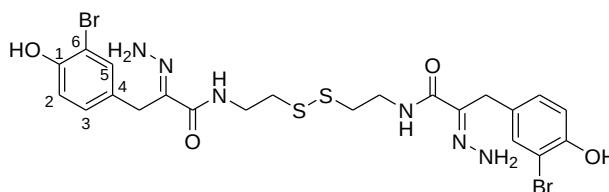
Following procedure A, crude acid **74a** was coupled with cystamine **53** to afford product **98b** (255 mg, 42%) as an amorphous white solid after purification by flash column chromatography (AcOEt : PE₄₀₋₆₀, 6 : 4). R_f 0.4 (AcOEt : PE₄₀₋₆₀, 1 : 1); IR 1660, 1526, 1496, 1256 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 2.83 (t, *J* = 6.4 Hz, 4H, CH₂S), 3.63 (m, 4H, CH₂N), 3.87 (s, 6H, CH₃O), 4.13 (s, 4H, 4-CH₂), 6.85 (d, *J* = 8.5 Hz, 2H, 2-H), 7.16 (dd, *J* = 8.4, 2.2 Hz, 2H, 3-H), 7.31 (br. t., 2H, NH), 7.43 (d, *J* = 2.2 Hz, 2H, 5-H). ¹³C NMR (100 MHz, CDCl₃) δ 37.2, 38.4, 41.8, 56.2, 111.7, 112.0, 125.9, 130.0, 134.5, 155.1, 160.0, 195.3; HRMS (ESI⁺) *m/z* calc. for C₂₄H₂₇Br₂N₂O₆S₂ [M+H]⁺ 660.9672, found: 660.9692; Anal. Calc. for C₂₄H₂₆Br₂N₂O₆S₂: C, 43.52; H, 3.96; N, 4.23. Found: C, 43.62; H, 3.88; N, 4.10.

General procedure for the synthesis of hydrazone containing analogues:

To a solution of the appropriate ketoamide containing compound (1.1 eq.) in freshly distilled MeOH (10 mL/mmol) at rt was added hydrazine monohydrate (compounds **97a** and **98a**: 1.1 eq.; compounds **97b** and **98b**: 2.2 eq.). The resulting mixture was heated to reflux for 2.5 hours then cooled to rt. The solvent was then evaporated *in vacuo* and the product was purified by flash column chromatography.

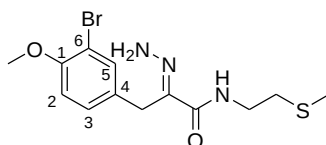
(E)-3-(3-Bromo-4-hydroxyphenyl)-2-hydrazono-N-(2-(methylthio)ethyl)propanamide (111a)

111a (220 mg, 58%) was prepared from **97a** according general procedure above, and obtained as a white solid after purification by flash column chromatography (AcOEt/CH₂Cl₂ 15 : 85) and recrystallization from CH₂Cl₂: m.p. 103-104°C (CH₂Cl₂); R_f 0.25 (AcOEt : CH₂Cl₂, 1 : 9); IR 3414, 3359, 3116, 1633, 1620, 1528, 1509, 1421 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 2.14 (s, 3H, CH₃S), 2.69 (t, *J* = 6.5 Hz, 2H, CH₂S), 3.56 (m, 2H, CH₂N), 3.81 (s, 2H, 4-CH₂), 5.59 (br. s, 1H, OH), 5.68 (br. s, 2H, NH₂), 6.92 (d, *J* = 8.3 Hz, 1H, 2-H), 7.07 (dd, *J* = 8.3, 2.0 Hz, 1H, 3-H), 7.31 (d, *J* = 2.0 Hz, 1H, 5-H), 7.32 (br. t, 1H, NH). ¹³C NMR (100 MHz, CDCl₃) δ 15.2, 27.6, 34.0, 37.9, 110.6, 116.4, 128.8, 129.2, 131.5, 141.6, 151.2, 164.6; HRMS (ESI+) *m/z* calc. for C₁₂H₁₇BrN₃O₂S [M+H]⁺ 346.0219, found: 346.0214; Anal. Calc. for C₁₂H₁₆BrN₃O₂S: C, 41.63; H, 4.66; N, 12.14. Found: C, 41.55; H, 4.60; N, 12.07.

(2E,2'E)-N,N'-(2,2'-Disulfanediylbis(ethane-2,1-diyl))bis(3-(3-bromo-4-hydroxyphenyl)-2-hydrazonopropanamide) (111b)

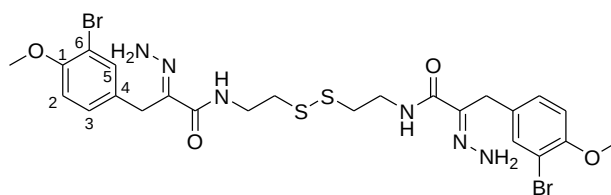
111b (182 mg, 74%) was prepared from **97b** according to general procedure above, and obtained as an amorphous white solid after purification by flash column chromatography (MeOH/CH₂Cl₂ 6 : 94). *R*_f 0.35 (MeOH: CH₂Cl₂, 6 : 94); IR 3359, 3312, 1626, 1530, 1503, 1193 cm⁻¹; ¹H NMR (400 MHz, *d*⁶-DMSO) δ 2.82 (t, *J* = 6.8 Hz, 4H, CH₂S), 3.42 (m, 4H, CH₂N), 3.64 (s, 4H, 4-CH₂), 6.81 (d, *J* = 8.3 Hz, 2H, 2-H), 7.01 (dd, *J* = 8.3, 1.9 Hz, 2H, 3-H), 7.21 (s, 4H, NH₂), 7.31 (d, *J* = 1.9 Hz, 2H, 5-H), 7.79 (t, *J* = 5.9 Hz, 2H, NH), 9.97 (s, 2H, OH). ¹³C NMR (100 MHz, *d*⁶-DMSO) 26.1, 37.3, 38.0, 108.8, 116.0, 128.7, 129.0, 132.4, 136.8, 152.1, 165.0; HRMS (ESI+) *m/z* calc. for C₂₂H₂₇Br₂N₆O₄S₂ [M+H]⁺ 660.9896, found: 660.9910; Anal. Calc. for C₂₂H₂₆Br₂N₆O₄S₂: C, 39.89; H, 3.96; N, 12.69. Found: C, 39.84; H, 3.88; N, 12.61.

(*E*)-3-(3-Bromo-4-methoxyphenyl)-2-hydrazono-*N*-(2-(methylthio)ethyl)propanamide (112a)



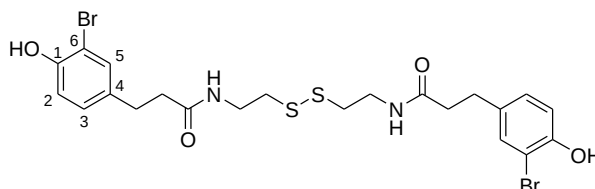
112a (273 mg, 69%) was prepared from **98a** according general procedure above, and obtained as an amorphous white solid after purification by flash column chromatography (AcOEt/CH₂Cl₂ 1 : 9). *R*_f 0.55 (AcOEt : CH₂Cl₂, 1 : 9); IR 3376, 3338, 1637, 1517, 1491, 1251, 1051 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 2.14 (s, 3H, CH₃S), 2.69 (t, *J* = 6.5 Hz, 2H, CH₂S), 3.56 (m, 2H, CH₂N), 3.82 (s, 2H, 4-CH₂), 3.86 (s, 3H, CH₃O), 5.67 (br. s, 2H, NH₂), 6.81 (d, *J* = 8.4 Hz, 1H, 2-H), 7.13 (dd, *J* = 8.4, 2.1 Hz, 1H, 3-H), 7.31 (br. t, 1H, NH), 7.40 (d, *J* = 2.1 Hz, 1H, 5-H). ¹³C NMR (100 MHz, CDCl₃) δ 15.2, 27.6, 34.0, 37.9, 56.3, 112.1, 112.3, 128.3, 128.8, 133.0, 141.7, 154.7, 164.5; HRMS (ESI+) *m/z* calc. for C₁₃H₁₉BrN₃O₂S [M+H]⁺ 360.0376, found: 360.0372; Anal. Calc. for C₁₃H₁₈BrN₃O₂S: C, 43.34; H, 5.04; N, 11.66. Found: C, 43.26; H, 4.93; N, 11.56.

(2*E*,2'*E*)-*N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl))bis(3-(3-bromo-4-methoxyphenyl)-2-hydrazonopropanamide) (112b)



112b (217 mg, 59%) was prepared from **98b** according to general procedure above, and obtained as an amorphous white solid after purification by flash column chromatography (MeOH/CH₂Cl₂ 2 : 98). *R*_f 0.3 (AcOEt : PE₄₀₋₆₀, 3 : 7); IR 3396, 3316, 1637, 1516, 1493, 1253, 1053 cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 2.87 (t, *J* = 6.7 Hz, 4H, CH₂S), 3.56 (t, *J* = 6.7 Hz, 4H, CH₂N), 3.76 (s, 4H, 4-CH₂), 3.81 (s, 6H, CH₃O), 6.90 (d, *J* = 8.4 Hz, 2H, 2-H), 7.16 (dd, *J* = 8.4, 2.0 Hz, 2H, 3-H), 7.39 (d, *J* = 2.0 Hz, 2H, 5-H). ¹³C NMR (100 MHz, CD₃OD) δ 27.7, 39.1, 39.6, 56.8, 112.5, 113.4, 129.8, 131.3, 134.1, 139.2, 156.0, 167.8; HRMS (ESI+) *m/z* calc. for C₂₄H₃₁Br₂N₆O₄S₂ [M+H]⁺ 689.0209, found: 689.0206; Anal. Calc. for C₂₄H₃₀Br₂N₆O₄S₂: C, 41.75; H, 4.38; N, 12.17. Found: C, 41.81; H, 4.30; N, 12.12.

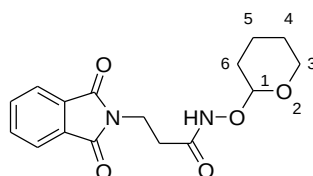
***N,N'*-(2,2'-Disulfanediyldis(ethane-2,1-diyl))bis(3-(3-bromo-4-hydroxyphenyl)propanamide) (115)**



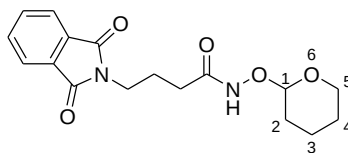
To a solution of cystamine **53** (43.5mg, 0.286 mmol, 1 eq.), triethylamine (239 μL, 1.71 mmol, 6 eq.) and HOBt (92.6 mg, 0.685 mmol, 2.4 eq.) in CH₂Cl₂ (10 mL), under an argon atmosphere, at rt, were added successively EDCI (131 mg, 0.685 mmol, 2.4 eq.) and 3-(3-bromo-4-hydroxyphenyl)propanoic acid **114** (140 mg, 0.571 mmol, 2 eq.). The resulting mixture was stirred for 15h at rt before the solvent was evaporated. The residue was then dissolved in ethyl acetate (10 mL), washed with 1N aqueous HCl (5 mL), saturated aqueous NaHCO₃ (5 mL) and brine (5 mL). The organic phase was dried (MgSO₄) and concentrated. The product (83 mg, 48%) was obtained as a white solid after purification by flash column chromatography (AcOEt). *R*_f 0.2 (AcOEt). IR (neat) 3283, 1635, 1537, 1506, 1495, 1419, 1221 cm⁻¹; ¹H NMR (400 MHz, CD₃OD) δ 2.43 (t, *J* = 7.4 Hz, 4H, CH₂C(O)), 2.70 (t, *J* = 6.7 Hz, 4H, CH₂S), 2.81 (t, *J* = 7.4 Hz, 4H, 4-CH₂),

3.42 (t, $J = 6.7$ Hz, 4H, CH₂N), 6.79 (d, $J = 8.3$ Hz, 2H, 2-H), 6.99 (dd, $J = 8.3$, 2.1 Hz, 2H, 3-H), 7.30 (d, $J = 2.1$ Hz, 2H, 5-H). ¹³C NMR (100 MHz, CD₃OD) δ 31.6, 38.5, 38.9, 39.5, 110.7, 117.2, 129.7, 133.9, 134.7, 153.7, 175.2; HRMS (ESI+) m/z calc. for C₂₂H₂₇Br₂N₂O₄S₂ [M+H]⁺ 604.9774 found: 604.9781; Anal. Calc. for C₂₂H₂₆Br₂N₂O₄S₂: C, 43.58; H, 4.32; N, 4.62. Found: C, 43.66; H, 4.38; N, 4.71.

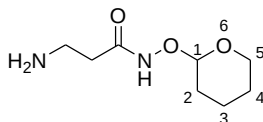
3-(1,3-Dioxoisindolin-2-yl)-N-(tetrahydro-2H-pyran-2-yloxy)propanamide (123)



To a mixture of 3-(1,3-dioxoisindolin-2-yl)propanoic acid **121**¹⁷⁵ (3.5 g, 16.0 mmol, 1 eq.) and O-(tetrahydro-2H-pyran-2-yl)hydroxylamine¹⁵⁵ **54** (1.96 g, 16.8 mmol, 1.05 eq.) in chloroform (135 mL) was added EDCI (3.37 g, 17.6 mmol, 1.1 eq.), followed by DMAP (195 mg, 1.60 mmol, 0.1 eq.). The resulting mixture was stirred overnight at rt. The day after, the reaction mixture was concentrated, water (195 mL) was added and extracted with AcOEt (3 x 135 mL). The combined organic layers were washed with brine (195 mL), dried (Na₂SO₄) and concentrated *in vacuo*. The product (4.06 g, 80%) was obtained as a white solid after purification by flash column chromatography (gradient MeOH/CH₂Cl₂ 2 : 98 to 10:90). R_f 0.45 (AcOEt : CH₂Cl₂, 1 : 1); IR (neat) 1710, 1680, 1653, 1398, 1113 cm⁻¹; ¹H NMR (400 MHz, *d*⁶-DMSO) δ 1.38-1.72 (m, 6H, 4-H₂ + 5-H₂ + 6-H₂), 2.36 (t, $J = 7.0$ Hz, 2H, CH₂C(O)), 3.40 (m, 1H, 3-H_A), 3.78 (t, $J = 7.0$ Hz, 2H, NCH₂), 3.83 (m, 1H, 3-H_B), 4.74 (m, 1H, 1-H), 7.81-7.91 (m, 4H, Ar-H), 11.08 (s, 1H, NH). ¹³C NMR (100 MHz, *d*⁶-DMSO) δ 18.3, 24.5, 27.6, 31.1, 34.0, 61.3, 100.7, 122.9, 131.6, 134.2, 166.2, 167.5; HRMS (ESI+) m/z calc. for C₁₆H₁₈N₂NaO₅ [M+Na]⁺ 341.1108, found: 341.1109; Anal. Calc. for C₁₆H₁₈N₂O₅: C, 60.37; H, 5.70; N, 8.80. Found: C, 60.31; H, 5.62; N, 8.75.

4-(1,3-Dioxoisindolin-2-yl)-N-(tetrahydro-2H-pyran-2-yloxy)butanamide (124)

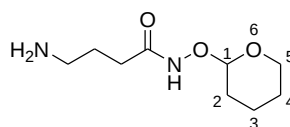
To a mixture of 4-(1,3-dioxoisindolin-2-yl)butanoic acid **122**¹⁷⁵ (4.0 g, 17.2 mmol, 1 eq.) and O-(tetrahydro-2H-pyran-2-yl)hydroxylamine¹⁵⁵ **54** (2.11 g, 18.0 mmol, 1.05 eq.) in chloroform (145 mL) was added EDCI (3.62 g, 18.9 mmol, 1.1 eq.), followed by DMAP (210 mg, 1.72 mmol, 0.1 eq.). The resulting mixture was stirred overnight at rt. The day after, the reaction mixture was concentrated, water (210 mL) was added and extracted with CHCl₃ (3 x 140 mL). The combined organic layers were washed with brine (210 mL), dried (Na₂SO₄) and concentrated *in vacuo*. The product (5.35 g, 94%) was obtained as a white solid after purification by flash column chromatography (MeOH/CH₂Cl₂ 5 : 95). *R*_f 0.45 (AcOEt : CH₂Cl₂, 1 : 1); ¹H NMR (400 MHz, *d*⁶-DMSO) δ 1.40-1.70 (m, 6H, 2-H + 3-H + 4-H), 1.81 (*app*-quintuplet, *J* = 7.2 Hz, 2H, NCH₂CH₂), 2.03 (t, *J* = 7.2 Hz, 2H, CH₂C(O)), 3.47 (m, 1H, 5-H_A), 3.57 (t, *J* = 6.9 Hz, 2H, CH₂N), 3.88 (m, 1H, 5-H_B), 4.75 (m, 1H, 1-H), 7.78-7.92 (m, 4H, ArH), 10.92 (s, 1H, NH). ¹³C NMR (100 MHz, *d*⁶-DMSO) δ 18.2, 23.8, 24.6, 27.7, 29.6, 37.0, 61.2, 100.8, 122.9, 131.6, 134.2, 167.8, 168.2. HRMS (ESI+) *m/z* calc. for C₁₇H₂₀N₂NaO₅ [M+Na]⁺ 355.1264, found: 355.1266. Anal. Calc. for C₁₇H₂₀N₂O₅: C, 61.44; H, 6.07; N, 8.43. Found: C, 61.51; H, 6.02; N, 8.36.

3-Amino-N-(tetrahydro-2H-pyran-2-yloxy)propanamide (125)

To a mixture of 3-(1,3-dioxoisindolin-2-yl)-N-(tetrahydro-2H-pyran-2-yloxy)propanamide **123** (3.23 g, 10.1 mmol, 1 eq.) in freshly distilled methanol (65 mL) was added hydrazine monohydrate (517 μL, 10.7 mmol, 1.05 eq.). The resulting mixture was heated to reflux for 2.5h, then cooled to rt. The white precipitate formed was filtered off and washed with a small amount of methanol. The methanolic phase was concentrated *in vacuo* and the product (805 mg, 42 %) was obtained as a white solid after purification by flash column chromatography

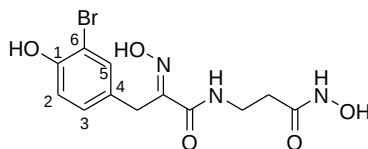
(CHCl₃/MeOH/aq. NH₃ 80 : 18 : 2) without prior workup. R_f 0.2 (CHCl₃/MeOH/aq. NH₃ 80 : 18 : 2); IR (neat) 1659, 1573, 1361, 1112, 1073, 1031 cm⁻¹; ¹H NMR (400 MHz, d⁶-DMSO) δ 1.42-1.74 (m, 6H, 2-H₂ + 3-H₂ + 4-H₂), 2.06 (t, *J* = 6.6 Hz, 2H, CH₂(O)), 2.72 (t, *J* = 6.6 Hz, 2H, NCH₂), 3.44-3.54 (m, 1H, 5-H_A), 3.85-3.96 (m, 1H, 5-H_B), 4.52 (br. s, 2H, NH₂), 4.81 (m, 1H, 1-H). ¹³C NMR (100 MHz, d⁶-DMSO) δ 18.2, 24.6, 27.7, 36.3, 38.1, 61.2, 100.7, 168.3; HRMS (ESI+) *m/z* calc. for C₈H₁₇N₂O₃ [M+H]⁺ 189.1234, found: 189.1232; Anal. Calc. for C₈H₁₆N₂O₃: C, 51.05; H, 8.57; N, 14.88. Found: C, 51.15; H, 8.65; N, 14.94.

4-Amino-*N*-(tetrahydro-2*H*-pyran-2-yloxy)butanamide **126**



To a mixture of 4-(1,3-dioxoisindolin-2-yl)-*N*-(tetrahydro-2*H*-pyran-2-yloxy)butanamide **124** (2.50 g, 7.52 mmol, 1 eq.) in freshly distilled methanol (50 mL) was added hydrazine monohydrate (401 μL, 8.27 mmol, 1.1 eq.). The resulting mixture was stirred overnight at rt. The white precipitate formed was filtered off and washed with a small amount of methanol. The methanolic phase was concentrated *in vacuo* and the product (758 mg, 50 %) was obtained as a white solid after purification by flash column chromatography (CHCl₃/MeOH/aq. NH₃ 80 : 18 : 2) without prior workup. R_f 0.2 (CHCl₃/MeOH/aq. NH₃ 80 : 18 : 2); ¹H NMR (400 MHz, d⁶-DMSO) δ 1.42-1.74 (m, 8H, 2-H₂ + 3-H₂ + 4-H₂ + NCH₂CH₂), 2.00 (t, *J* = 7.3 Hz, 2H, CH₂C(O)), 2.49 (t, *J* = 7.3 Hz, 2H, NCH₂), 3.44-3.54 (m, 1H, 5-H_A), 3.85-3.96 (m, 1H, 5-H_B), 4.56 (br. s, 2H, NH₂), 4.79 (m, 1H, 1-H). ¹³C NMR (100 MHz, d⁶-DMSO) δ 18.2, 24.6, 27.7, 29.0, 29.8, 41.0, 61.2, 100.7, 169.1; HRMS (ESI+) *m/z* calc. for C₉H₁₉N₂O₃ [M+H]⁺ 203.1390, found: 203.1389; Anal. Calc. for C₉H₁₈N₂O₃: C, 53.45; H, 8.97; N, 13.85. Found: C, 53.53; H, 8.90; N, 13.81.

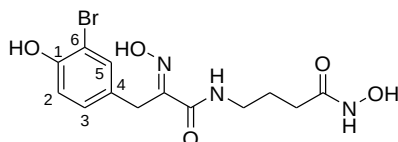
(*E*)-3-(3-Bromo-4-hydroxyphenyl)-*N*-(3-(hydroxyamino)-3-oxopropyl)-2-(hydroxyimino)propanamide (**116**)



To a solution of 3-(3-bromo-4-hydroxyphenyl)-2-hydroxyacrylic acid **51** (1 eq.) in freshly distilled pyridine (2 mL/mmol), under argon, was added *O*-(tetrahydro-2*H*-pyran-2-yl)hydroxylamine **54** (1.5 eq.). The resulting mixture was stirred overnight at room temperature. Pyridine was then removed *in vacuo*, the residue was dissolved in 1N HCl (5.5 mL/mmol) and extracted three times with ethyl acetate (5.5 mL/mmol). The combined organic layers were dried (Na₂SO₄), filtrated and concentrated to afford the crude *O*-THP protected oxime intermediate, used for the next step without further purification. To a solution of the crude *O*-THP protected oxime intermediate (1 eq.) in dry dioxane (2.7 mL/mmol) under argon were added EDCI (1.7 equiv.) and NHS (1.9 equiv.). The resulting mixture was stirred at room temperature for 3 hours. The solvent was then evaporated *in vacuo*. The residue was dissolved in EtOAc (12 mL/mmol), washed with saturated aqueous NaHCO₃ (2 x 12 mL/mmol), 1N aqueous HCl (2 x 12 mL/mmol) and brine (1 x 12 mL/mmol). The organic phase was then dried (Na₂SO₄), filtrated, and the solvent evaporated *in vacuo*. The residue was dissolved in dry dioxane (2.7 mL/mmol) under argon and a second solution of 3-amino-*N*-(tetrahydro-2*H*-pyran-2-yloxy)propanamide **125** (1.0 eq.) and NEt₃ (1.0 eq.) in dry dioxane (2.7 mL/mmol) were added. The resulting mixture was then stirred overnight at rt. The day after the solvent was evaporated and the product was purified by flash column chromatography (1 : 1 AcOEt/PE₄₀₋₆₀). The *O*-THP diprotected precursor was then dissolved in an anhydrous 0.5M solution of HCl in 9:1 dioxane/*i*PrOH (21 mL / mmol). The resulting mixture was stirred at rt and the reaction monitored by TLC. After 20h the solvent was evaporated *in vacuo* and the product (81 mg, 13%) was obtained as a white solid after purification by flash column chromatography (1 : 9 MeOH/CH₂Cl₂). *R*_f 0.15 (MeOH : CH₂Cl₂, 1 : 9); IR (neat) 3202, 1626, 1532, 1492, 1418, 1211, 993 cm⁻¹; ¹H NMR (400 MHz, d⁶-DMSO) δ 2.17 (t, *J* = 7.1 Hz, 2H, CH₂C(O)), 3.32 (*app*-q, *J* = 7.0 Hz, 2H, N-CH₂), 3.67 (s, 2H, 4-CH₂), 6.83 (d, *J* = 8.3 Hz, 1H, 2-H), 7.01 (dd, *J* = 8.3, 2.0 Hz, 1H, 3-H), 7.29 (d, *J* = 2.0 Hz, 1H, 5-H), 7.93 (t, *J* = 5.8 Hz, 1H, NHCH₂), 8.74 (s, 1H, NHOH), 10.03 (s, 1H, 1-OH), 10.44 (s, 1H, NHOH), 11.87 (s, 1H, NOH). ¹³C

NMR (100 MHz, d^6 -DMSO) δ 27.5, 31.9, 35.4, 108.8, 116.1, 128.7, 129.0, 132.7, 151.6, 152.3, 162.8, 167.3; HRMS (ESI+) m/z calc. for $C_{12}H_{15}BrN_3O_5$ $[M+H]^+$ 360.0190, found: 360.0195; Anal. Calc. for $C_{12}H_{15}BrN_3O_5$: C, 40.02; H, 3.92; N, 11.67. Found: C, 40.14; H, 3.83; N, 11.61.

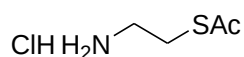
(E)-4-(3-(3-Bromo-4-hydroxyphenyl)-2-(hydroxyimino)propanamido)-N-hydroxybutanamide 117



To a solution of 3-(3-bromo-4-hydroxyphenyl)-2-hydroxyacrylic acid **51** (1 eq.) in freshly distilled pyridine (2 mL/mmol), under argon, was added *O*-(tetrahydro-2*H*-pyran-2-yl)hydroxylamine **54** (1.5 eq.). The resulting mixture was stirred overnight at room temperature. Pyridine was then removed *in vacuo*, the residue was dissolved in 1N HCl (5.5 mL/mmol) and extracted three times with ethyl acetate (5.5 mL/mmol). The combined organic layers were dried (Na_2SO_4), filtrated and concentrated to afford the crude *O*-THP protected oxime intermediate, used for the next step without further purification. To a solution of the crude *O*-THP protected oxime intermediate (1 eq.) in dry dioxane (2.7 mL/mmol) under argon were added EDCI (1.7 equiv.) and NHS (1.9 equiv.). The resulting mixture was stirred at room temperature for 3 hours. The solvent was then evaporated *in vacuo*. The residue was dissolved in EtOAc (12 mL/mmol), washed with saturated aqueous $NaHCO_3$ (2 x 12 mL/mmol), 1N aqueous HCl (2 x 12 mL/mmol) and brine (1 x 12 mL/mmol). The organic phase was then dried (Na_2SO_4), filtrated, and the solvent evaporated *in vacuo*. The residue was dissolved in dry dioxane (2.7 mL/mmol) under argon and a second solution of 4-amino-*N*-(tetrahydro-2*H*-pyran-2-yloxy)butanamide **126** (1.0 eq.) and NEt_3 (1.0 eq.) in dry dioxane (2.7 mL/mmol) were added. The resulting mixture was then stirred overnight at rt. The day after the solvent was evaporated and the product was purified by flash column chromatography (1 : 1 acetone/ PE_{40-60}). The *O*-THP diprotected precursor was then dissolved in an anhydrous 0.5M solution of HCl in 9:1 dioxane/*i*PrOH (21 mL / mmol). The resulting mixture was stirred at rt and the reaction monitored by TLC. After 20h the solvent was evaporated *in vacuo* and the product (170 mg, 15%) was obtained as a white solid after purification by

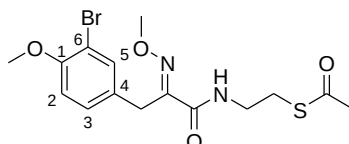
flash column chromatography (1 : 9 MeOH/CH₂Cl₂). R_f 0.15 (MeOH : CH₂Cl₂, 1 : 9); IR (neat) 3184, 1623, 1536, 1493, 1418, 1208, 993 cm⁻¹; ¹H NMR (400 MHz, *d*⁶-DMSO) δ 1.63 (quintet, *J* = 7.3 Hz, 2H, NCH₂CH₂), 1.92 (t, *J* = 7.3 Hz, 2H, CH₂C(O)), 3.11 (m, 2H, NCH₂), 3.68 (s, 2H, 4-CH₂), 6.83 (d, *J* = 8.3 Hz, 1H, 2-H), 7.01 (dd, *J* = 8.3, 2.0 Hz, 1H, 3-H), 7.29 (d, *J* = 2.0 Hz, 1H, 5-H), 8.00 (t, *J* = 6.0 Hz, 1H, NHCH₂), 8.67 (s, 1H, NHOH), 10.03 (s, 1H, 1-OH), 10.34 (s, 1H, NHOH), 11.77 (s, 1H, NOH). ¹³C NMR (100 MHz, *d*⁶-DMSO) δ 25.2, 27.7, 29.8, 38.3, 108.7, 116.0, 128.8, 129.0, 132.7, 151.9, 152.2, 163.1, 168.7; HRMS (ESI+) *m/z* calc. for C₁₃H₁₇BrN₃O₅ [M+H]⁺ 374.0346, found: 374.0359; Anal. Calc. for C₁₃H₁₆BrN₃O₅: C, 41.73; H, 4.31; N, 11.23. Found: C, 41.63; H, 4.23; N, 11.18.

2-Acetylsulfanylethyl ammonium chloride (127)



127 was prepared as previously reported.¹⁷⁶ 2-mercaptoethyl-ammonium chloride (2.0 g, 17.6 mmols, 1 eq.) was stirred under argon in acetyl chloride (10 mL) for a few minutes at room temperature, progressive HCl gas evolution was observed. The mixture was then stirred at 40°C for 2 hours to complete the reaction, then cooled to room temperature. The precipitate was filtered, washed with Et₂O, recrystallized from EtOH, washed with Et₂O and dried *in vacuo* to afford the pure product (1.89 g, 69%) as a white solid: m.p. 142 – 145 °C (EtOH) (lit.¹⁷⁶ 146-147 °C). Anal. Calc. For C₄H₁₀ClNOS: C, 30.87; H, 6.48; N, 9.00. Found: C, 30.76; H, 6.54; N, 8.96.

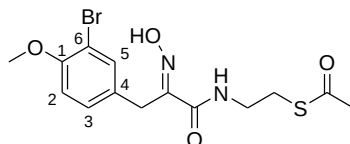
(*E*)-(*S*)-2-(3-(3-Bromo-4-methoxyphenyl)-2-(methoxyimino)propanamido)ethyl ethanethioate (128)



128 (216 mg, 30% from acid **74a**) was prepared according to the general procedure described earlier for the synthesis of *O*-methyloxime containing analogues, and obtained as a yellowish oil after purification by flash column chromatography (AcOEt : CH₂Cl₂, 2 : 98). R_f 0.35 (AcOEt : CH₂Cl₂ 2 : 98); IR

1674, 1520, 1495, 1044 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3) δ 2.33 (s, 3H, CH_3C), 3.04 (t, $J = 6.6$ Hz, 2H, CH_2S), 3.48 (m, 2H, CH_2N), 3.81 (s, 2H, 4- CH_2), 3.84 (s, 3H, CH_3O -1), 4.01 (s, 3H, CH_3O -N), 6.78 (d, $J = 8.4$ Hz, 1H, 2-H), 6.97 (br. t, 1H, NH), 7.21 (dd, $J = 8.4, 2.1$ Hz, 1H, 3-H), 7.46 (d, $J = 2.1$ Hz, 1H, 5-H). ^{13}C NMR (100 MHz, CDCl_3) δ 28.4, 28.6, 30.6, 39.1, 56.2, 63.1, 111.4, 111.8, 129.4, 129.8, 133.9, 151.4, 154.4, 162.6, 195.6; HRMS (ESI+) m/z calc. for $\text{C}_{15}\text{H}_{20}\text{BrN}_2\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 403.0322, found: 403.0336; Anal. Calc. for $\text{C}_{15}\text{H}_{19}\text{BrN}_2\text{O}_4\text{S}$: C, 44.67; H, 4.75; N, 6.95. Found: C, 44.74; H, 4.78; N, 6.89.

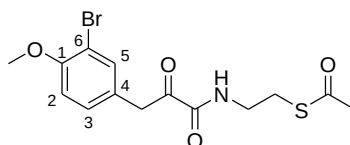
(E)-(S)-2-(3-(3-Bromo-4-methoxyphenyl)-2-(hydroxyimino)propanamido)ethyl ethanethioate (129)



To a solution of crude acid **74a** (1 eq.) in freshly distilled pyridine (2 mL/mmol), under argon, was added hydroxylamine hydrochloride (1.5 eq.). The resulting mixture was stirred overnight at rt. Pyridine was then removed *in vacuo*, the residue was dissolved in 1N HCl (5.5 mL/mmol) and extracted three times with ethyl acetate (5.5 mL/mmol). The combined organic layers were dried (Na_2SO_4), filtered and concentrated to afford the relatively pure oxime intermediate, used for the next step without further purification. To a solution of this crude oxime derivative in dioxane (10 mL/mmol) under argon was added DCC (1 eq.) and *N*-hydroxyphthalimide (1 eq.). After 2 hours of stirring at rt, 2-Acetylsulfanylethyl ammonium chloride **127** (1 eq.) and triethylamine (2.1 eq.) were added and the resulting mixture was stirred overnight at rt. The day after the solvent was evaporated and the product (50 mg, 19 % from acid **74a**) was obtained as a white powder after purification by flash column chromatography ($\text{AcOEt} : \text{CH}_2\text{Cl}_2$ 2 : 8) and trituration in warm AcOEt : m.p. 167-169 $^\circ\text{C}$; R_f 0.6 ($\text{AcOEt} : \text{CH}_2\text{Cl}_2$, 2 : 8); ^1H NMR (400 MHz, CD_3OD) δ 2.29 (s, 3H, CH_3C), 3.01 (t, $J = 6.6$ Hz, 2H, CH_2S), 3.41 (m, 2H, CH_2N), 3.82 (s, 2H, 4- CH_2), 3.83 (s, 3H, CH_3O), 6.91 (d, $J = 8.5$ Hz, 1H, 2-H), 7.22 (dd, $J = 8.5, 2.1$ Hz, 1H, 3-H), 7.44 (d, $J = 2.1$ Hz, 1H, 5-H); ^{13}C NMR (100 MHz, CDCl_3) δ 28.7, 29.4, 30.4, 39.9, 56.7, 112.1, 113.1,

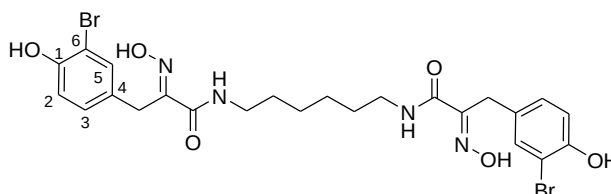
130.5, 131.8, 134.7, 152.9, 155.9, 165.9, 197.1; HRMS (ESI+) m/z calc. for $C_{14}H_{18}BrN_2O_4S$ $[M+H]^+$ 389.0165, found: 389.0164 ($M+H$)⁺; Anal. Calc. for $C_{14}H_{17}BrN_2O_4S$: C, 43.20; H, 4.40; N, 7.20. Found: C, 43.29; H, 4.32; N, 7.32.

(S)-2-(3-(3-Bromo-4-methoxyphenyl)-2-oxopropanamido)ethyl ethanethioate (130)



To a solution of acid **74a** under argon in dioxane (5.5 mL/mmol) was added DCC (1 eq.), *N*-hydroxyphthalimide (1 eq.). After 2 hours of stirring at rt, 2-Acetylsulfanylethyl ammonium chloride **127** (1 eq.) and triethylamine (2.1 eq.) were added and the resulting mixture was stirred overnight at rt. The day after the solvent was evaporated and the product (64 mg, 23 %) was purified by flash column chromatography (AcOEt : CH_2Cl_2 1 : 9) and trituration in Bu_2O : m.p. 93-95°C; R_f 0.65 (AcOEt : CH_2Cl_2 , 1 : 9); IR 1685, 1523, 1498, 1106 cm^{-1} ; ^1H NMR (400 MHz, $CDCl_3$) δ 2.35 (s, 3H, CH_3C), 3.05 (t, J = 6.4 Hz, 2H, CH_2S), 3.50 (m, 2H, CH_2N), 3.88 (s, 3H, CH_3O), 4.13 (s, 2H, $CH_2C(O)$), 6.86 (d, J = 8.5 Hz, 1H, 2-H), 7.16 (dd, J = 8.5, 2.1 Hz, 1H, 3-H), 7.21 (br. t, 1H, NH), 7.42 (d, J = 2.1 Hz, 1H, 5-H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.3, 30.6, 39.3, 41.7, 56.2, 111.7, 112.0, 125.9, 130.0, 134.5, 155.1, 159.9, 195.2, 195.5; HRMS (ESI+) m/z calc. for $C_{14}H_{17}BrNO_4S$ 374.0056 $[M+H]^+$, found: 374.0061; Anal. Calc. for $C_{14}H_{16}BrNO_4S$: C, 44.93; H, 4.31; N, 3.74. Found: C, 45.04; H, 4.27; N, 3.81.

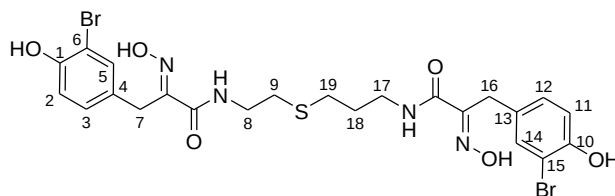
(2E,2'E)-N,N'-(Hexane-1,6-diyl)bis(3-(3-bromo-4-hydroxyphenyl)-2-(hydroxyimino) propanamide) (134)



Following the general procedure described earlier for the synthesis of oxime containing analogues, **134** (44 mg) was obtained in 12% yield from the corresponding arylpyruvic acid **51** and hexane-1,6-diamine, after purification by

flash column chromatography (AcOEt : PE₄₀₋₆₀). Amorphous white solid; **134** has been previously reported in the literature and our data matched reported ones. R_f 0.1 (AcOEt : PE₄₀₋₆₀, 1 : 1); ^1H NMR (400 MHz, CD₃OD) δ 1.22-1.32 (m, 4H, NCH₂CH₂), 1.42-1.52 (m, 4H, NCH₂CH₂), 3.20 (t, J = 7.0 Hz, 4H, NCH₂), 3.79 (s, 4H, 4-CH₂), 6.76 (d, J = 8.3 Hz, 2H, 2-H), 7.06 (dd, J = 8.3, 2.1 Hz, 2H, 3-H), 7.36 (d, J = 2.1 Hz, 2H, 5-H). ^{13}C NMR (100 MHz, CD₃OD) δ 27.5, 28.8, 30.4, 40.2, 110.5, 117.1, 130.4, 130.7, 134.5, 153.6, 153.8, 165.8; HRMS (ESI+) m/z calc. for C₂₄H₂₉Br₂N₄O₆ [M+H]⁺ 627.0448, found: 627.0470.

(*E*)-3-(3-Bromo-4-hydroxyphenyl)-*N*-(3-(2-((*E*)-3-(3-bromo-4-hydroxyphenyl)-2-(hydroxyimino)propanamido)ethylthio)propyl)-2-(hydroxyimino)propanamide (135)



Following the general procedure described earlier for the synthesis of oxime containing analogues, **135** (53 mg) was obtained in 14% yield from the corresponding arylpyruvic acid and 3-(2-aminoethylthio)propan-1-amine **137**,²⁴³ after purification by flash column chromatography (AcOEt : PE₄₀₋₆₀). Amorphous white solid; R_f 0.1 (AcOEt : PE₄₀₋₆₀, 1 : 1); IR (neat) 3248, 1646, 1605, 1539, 1495, 1420; ^1H NMR (400 MHz, CD₃OD) δ 1.74 (quintet, J = 7.2 Hz, 2H, 18-H₂), 2.50 (t, J = 7.2 Hz, 2H, 19-H₂), 2.61 (t, J = 6.7 Hz, 2H, 9-H₂), 3.30 (t, J = 7.2 Hz, 2H, 17-H₂), 3.39 (t, J = 6.7 Hz, 2H, 8-H₂), 3.79 (s, 4H, 7-H₂ + 16-H₂), 6.76 (d, J = 8.3 Hz, 2H, 2-H + 11-H), 7.06 (dd, J = 8.3, 2.1 Hz, 2H, 3-H + 12-H), 7.36 (d, J = 2.1 Hz, 2H, 5-H + 14-H). ^{13}C NMR (100 MHz, CD₃OD) δ 28.7, 28.8, 29.9, 30.4, 32.1, 39.4, 40.0, 110.5 (2C), 117.1 (2C), 130.4 (2C), 130.7 (2C), 134.5 (2C), 153.2, 153.5, 153.8 (2C), 165.8, 166.0; HRMS (ESI+) m/z calc. for C₂₃H₂₇Br₂N₄O₆S [M+H]⁺ 645.0013, found: 645.0045; Anal. Calc. for C₂₃H₂₆Br₂N₄O₆S: C, 42.74; H, 4.05; N, 8.67. Found: C, 42.89; H, 3.98; N, 8.53.

2. Biological evaluation

General procedure A for the *in situ* reduction of disulfides

To the appropriate dimeric compound (neat, 1.0 eq.) was added solid TCEP (1.5 eq.). Dilution with the adequate amount of a 9 : 1 DMSO/distilled water solution and agitation for 20-30 minutes (magnetic stirrer) at rt afforded the required stock solution of thiol. All thiols were used immediately after reduction.

Example: preparation of a 50 mM stock solution of Psammaplin A. To pure Psammaplin A (5.0 mg, 1 eq.) was added solid TCEP (3.2 mg, 1.5 eq.). 301 μ L of a 9 : 1 DMSO/water was added, and the resulting mixture was stirred for 20 minutes at rt. The resulting stock solution was used immediately. Complete and clean reduction was observed by ^1H NMR. HRMS allowed for the observation of $[\text{M}-\text{H}]^-$, $[\text{M}+\text{HCOO}]^-$ and $[2\text{M}-\text{H}]^-$ ions.

General procedure B for the *in situ* reduction of disulfides

An 18.75 mM solution (S1) of TCEP in a 9:1 (v/v) mixture of DMSO/deionized water was prepared. To 40 μ L of S1 were added 10 μ L of a 50 mM solution (S2) of the appropriate dimeric compound. The resulting mixture was incubated for 20 minutes at 25 $^\circ\text{C}$ in the dark, with occasional shaking. The resulting 20 mM stock solution of thiol was used immediately.

HDAC assay

DMSO, Pluronic, TCEP and Trypsin (bovine pancreas) were purchased from Sigma-Aldrich and Boc-Lys(Ac)-AMC from Bachem (Switzerland). The recombinant human histone deacetylases HDAC1 and HDAC6 were obtained from BPS Bioscience (US). All reactions were performed in black half area 96-well microplates (Greiner bio-one, Germany) according to the general procedure described by Wegener et al.¹⁷⁷ with some minor modifications. The reaction buffer contains 50 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, 15 mM Tris/HCl, pH 8, 250 mM NaCl, 0.001% (v / v) Pluronic, and 250 μM EDTA. The buffer components were purchased from Merck (Germany), Roth (Germany) and Sigma-Aldrich.

The fluorogenic HDAC assay consists of two steps: In the first step the Boc-Lys(Ac)-AMC substrate is deacetylated by the corresponding HDAC. The deacetylated substrates, therefore containing a basic amino acid residue serves as substrate for trypsin in the subsequent second step. The second step generates the signal due to the release of fluorescent AMC, whose fluorescence serves as an indirect measure of HDAC enzyme activity. 50 mM stock solutions of test compounds were prepared in DMSO and diluted for testing using reaction buffer.

Thiols were obtained from their corresponding disulfides via general procedure A or B above. Varying the procedure did not show significant influence on the final calculated IC_{50} . Test experiments have shown TCEP to have no influence on enzyme activity at concentrations up to 25 μM .

A serial dilution of test compounds was preincubated with 7.4 nM HDAC1 or 2.8 nM HDAC6, respectively, for 20 min at 21 ± 1 °C in the dark. The enzyme reaction was initiated by the addition of Boc-Lys(Ac)-AMC substrate. The reaction mixture was incubated at 30°C in the dark and stopped after 60 min by the addition of a mixture of 67 μM trypsin and 200 nM SAHA. The fluorescence of AMC serves as an indirect measure of HDAC enzyme activity. The kinetics of AMC release was measured on a fluorescence plate reader PolarStar (BMG) using an excitation wavelength of 340 nm and an emission wavelength of 460 nm. Complete cleavage of deacetylated Boc-Lys-AMC by trypsin was achieved after

about 10-15 min. The fluorescence intensity of the plateau was averaged over at least 5 min and normalized with respect to per cent of enzyme activity. Finally, the normalized fluorescence intensities were plotted versus the concentration of test compounds and fitted to a four-parameter logistic model²⁴⁴ to calculate the IC₅₀ values.

Protein expression and purification of Histone deacetylase-like amidohydrolase (HDAH)

The recombinant HDAH from *Bordetella* strain FB188 (DSM 11172) containing an *N*-terminal His6-affinity tag was prepared as previously reported.¹⁷⁸ Briefly, HDAH was expressed in *E. coli* strain BL21 and the cell lysate was applied to a His-Trap FF (Amersham Biosciences) column loaded with zinc ions. After removing non binding proteins, the pure enzyme was eluted with 500 mM Imidazol. The resulting pure enzyme was dialyzed against assay buffer and directly used to test compounds. The purity of HDAH (> 98%) was determined using polyacrylamide gel electrophoresis and Coomassie Brilliant Blue G-250 to stain protein.

DNMT assay

All reactions were performed in 384 well microtitre plates (Greiner). End point detection reaction was carried out using the restriction endonuclease *GlaI* purchased from SybEnzyme (Novosibirsk, Russia). The break light hemi-methylated oligonucleotide used was obtained from ATDBio Ltd. (Southampton, UK) (5'-FAM-ATCTAGCG5ATCAGTTTTCTGATG5G5TAGAT-dab 3'). The S-(5'-adenosyl)-L-methionine chloride (SAM), methyl donor for the DNMT1 reaction was obtained from Sigma (Gillingham, UK), SAH, S-(5'-Adenosyl)-L-homocysteine and all other reagents were also obtained from Sigma unless otherwise stated. Final fluorescent signal was read using a BMG-LabTech Pherastar (Aylesbury, UK) at 520nm (Em)/485nm (Ex) wavelengths. All single point data were analysed using Excel, dose response curves were analysed and IC₅₀ calculated using XLFit 4.0 (IDBS, Guilford, UK). DNMT1 enzyme was produced in-house (CRT, London, UK) from an *N*-his tagged full length construct

expressed in Sf9 insect cells and protein was harvested after a 50 h infection. The protein was purified and concentrated following nickel affinity and gel filtration chromatography, and glycerol was added to a final concentration of 50%. Approximately 3-5 mg/ml stock was obtained from 500 ml Sf9 cell pellet and this was stored at -20°C. All assay reagents were added to the assay plates using a multidropCombi (ThermoScientific, Basingstoke, UK), all compound dilutions were performed using a BeckmanCoulter Biomek FX (High Wycombe, UK) for the serial dilutions and a PlateMate+ (thermoScientific-Matrix, Basingstoke, UK) for all plate to plate transfers dilutions and compound addition to assay plates.

All assays were carried out as follows, into the wells of a 384 plate was added 1 µl of compound, (at appropriate concentration for testing, normally 30 µM for single point screening), then a 5 µl mix of SAM (10 µM final concentration), and hemi-methylated oligo (125 nM final concentration). The reaction was started by the addition of 5 µl of DNMT1 enzyme, at 80 nM final concentration in DNMT assay buffer (MOPS 100 mM, 125 mM NaCl, 2.5 mM MgCl₂, pH 6.5, 0.05% Triton X-100 and 5 mM DTT). Negative control wells were prepared as above but no SAM was added to mimic a complete inhibition of the DNMT1 reaction. For the positive control wells, no compounds were added. The reaction was incubated for 1 hr at room temperature. The reaction was stopped and signal developed by the addition of a 40 µl mix of a Glu1 0.00156 U/µl and SAH, 125 µM final concentration mix in Glu1 buffer (Tris-HCl 100 mM, NaCl 400 mM, MgCl₂ 3.75 mM, Triton X-100 (0.01%), 0.05%, DTT 5 mM, pH 8). This second enzymatic reaction was incubated for 7 hr at room temperature prior to reading the fluorescence signal at 520 nm.

Thiols were obtained from their respective disulfides according to general procedure A.

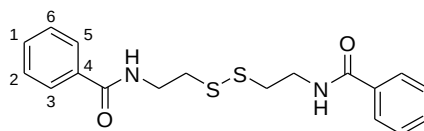
MSsI *in vitro* DNA methylation assay

Enzyme reagents for the MSsI *in vitro* DNA methylation studies were obtained from New England Biolabs. One unit of MSsI enzyme was incubated with 1 µg bacteriophage λ DNA in a 20 µl reaction volume containing 50 mM

NaCl, 10 mM Tris-HCl (pH 7.9 at 25°C), 10 mM MgCl₂, 1mM dithiothreitol (NEBuffer2) and 160 µM S-adenosylmethionine, in the presence and absence inhibitors for two hours at 37°C. Following DNA methylation, the MSssI enzyme was heat inactivated by incubation at 65°C for 20 minutes. The extent of DNA methylation was subsequently assessed by the addition of 30 µl of NEBuffer 2 containing 10 units of BstUI restriction endonuclease and digestion for 1 hour at 60°C. The digested DNA was analysed by gel electrophoresis using a 1.5% agarose gel.

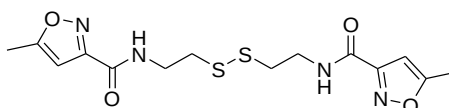
3. Deeper investigation of the role of the oxime unit in Psammaplin A and design of novel lead compounds

N,N'-(2,2'-Disulfanediy)bis(ethane-2,1-diyl)dibenzamide (140)



To an iced cooled solution of benzoyl chloride **139** (0.6 mL, 5.16 mmol, 1 eq.) in freshly distilled CH_2Cl_2 (50 mL), under inert atmosphere, were added triethylamine (1.58 mL, 11.4 mmol, 2 eq.) and cystamine dihydrochloride (581 mg, 2.58 mmol, 0.5 eq.). The resulting mixture was stirred for 2 hours at 0°C , then for 18 hours at rt. The organic phase was washed with 1N aqueous HCl (50 mL). The aqueous phase was extracted one more time with CH_2Cl_2 (50 mL). The combined organic layers were dried (MgSO_4) and the product (885 mg, 95%) was obtained as colourless needles after purification by flash column chromatography ($\text{AcOEt}/\text{CH}_2\text{Cl}_2$ 3 : 7) and recrystallisation ($\text{EtOH}/\text{H}_2\text{O}$): m.p. $122\text{--}124^\circ\text{C}$; R_f 0.4 ($\text{AcOEt}/\text{CH}_2\text{Cl}_2$ 3 : 7). IR (neat) 3268, 1634, 1553, 1325, 1309, 1289; ^1H NMR (400 MHz, CDCl_3) δ 2.98 (t, J = 6.4 Hz, 4H, CH_2S), 3.79 (m, 4H, CH_2N), 7.07 (br. t, 2H, NH), 7.36–7.45 (m, 4H, 2-H + 6-H), 7.46–7.53 (m, 2H, 1-H), 7.79–7.87 (m, 4H, 3-H + 5-H). ^{13}C NMR (100 MHz, CDCl_3) δ 37.9, 39.1, 127.1, 128.5, 131.6, 134.1, 167.8; HRMS (ESI+) m/z calc. for $\text{C}_{18}\text{H}_{21}\text{N}_2\text{O}_2\text{S}_2$ $[\text{M}+\text{H}]^+$ 361.1039 found: 361.1041; Anal. Calc. for $\text{C}_{18}\text{H}_{20}\text{N}_2\text{O}_2\text{S}_2$: C, 59.97; H, 5.59; N, 7.77. Found: C, 59.91; H, 5.48; N, 7.66.

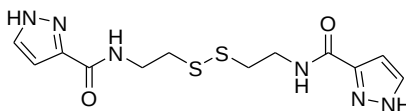
N,N'-(2,2'-Disulfanediy)bis(ethane-2,1-diyl)bis(5-methylisoxazole-3-carboxamide) (142)



To a solution of 5-methylisoxazole-3-carboxylic acid **141** (300 mg, 2.36 mmol, 1.0 eq.) in freshly distilled CH_2Cl_2 (25 mL), under inert atmosphere, were added HOBt (351 mg, 2.60 mmol, 1.1 eq.) and EDCI (498 mg, 2.60 mmol, 1.1 eq.) at rt. After stirring for 10 minutes at rt, cystamine dihydrochloride (266 mg, 1.18 mmol, 0.5 eq.) and freshly distilled NEt_3 (0.823 mL, 5.90 mmol, 2.5 eq.) were added.

The resulting mixture was stirred at rt and followed by TLC. After 24 h, the reaction was stopped. The reaction mixture was diluted with CH₂Cl₂ and the remaining insoluble material was dissolved after addition of small amounts of MeOH. The resulting mixture was washed with water (20 mL). The aqueous phase was back extracted with generous amounts of CH₂Cl₂. The combined organic layers were dried (MgSO₄) and concentrated under reduced pressure. The product (250 mg, 62%) was obtained as a colourless solid after precipitation from MeOH; R_f 0.75 (MeOH/CH₂Cl₂ 6 : 94); IR (neat) 3351, 3143, 1662, 1607, 1549, 1466, 1446, 1281, 1220; ¹H NMR (400 MHz, *d*⁶-DMSO) δ 2.45 (s, 6H, CH₃), 2.90 (t, *J* = 6.8 Hz, 4H, CH₂S), 3.52 (m, 4H, CH₂N), 6.52 (s, 2H, CH), 8.82 (t, *J* = 5.6 Hz, 2H, NH). ¹³C NMR (100 MHz, *d*⁶-DMSO) δ 11.7, 36.6, 38.1, 101.2, 158.7, 158.8, 171.1; HRMS (ESI+) *m/z* calc. for C₁₄H₁₉N₄O₄S₂ [M+H]⁺ 371.0842, found: 371.0852; Anal. Calc. for C₁₄H₁₉N₄O₄S₂: C, 45.39; H, 4.90; N, 15.12. Found: C, 45.29; H, 4.81; N, 14.96.

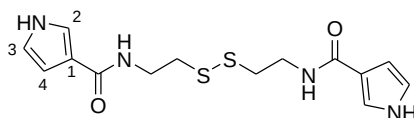
***N,N'*-(2,2'-Disulfanediylbis(ethane-2,1-diyl))bis(1*H*-pyrazole-3-carboxamide) (144)**



To a solution of 1*H*-pyrazole-3-carboxylic acid **143** (400 mg, 3.57 mmol, 1.0 eq.) in freshly distilled CH₂Cl₂ (32 mL), under inert atmosphere, were added HOBT (530 mg, 3.93 mmol, 1.1 eq.) and EDCI (752 mg, 3.93 mmol, 1.1 eq.) at rt. After stirring for 10 minutes at rt, cystamine dihydrochloride (272 mg, 1.78 mmol, 0.5 eq.) and freshly distilled NEt₃ (1.05 mL, 7.49 mmol, 2.1 eq.) were added. The resulting mixture was stirred at rt and followed by TLC. After 36 h, the reaction was stopped. The organic layer was washed with water (32 mL), and the aqueous phase was back extracted with AcOEt (3 x 24 mL). The combined organic layers were dried (MgSO₄) and the product (491 mg, 81%) was obtained as an amorphous white solid after flash column chromatography (MeOH/CH₂Cl₂ 8 : 92). R_f 0.4 (MeOH/CH₂Cl₂ 1 : 9); IR (neat) 3187, 1631, 1547, 1461, 1434, 1351, 1315, 1218, 1189; ¹H NMR (400 MHz, *d*⁶-DMSO) δ 2.90 (t, *J* = 6.9 Hz, 4H, CH₂S), 3.52 (m, 4H, CH₂N), 6.65 (br. s, 2H, CCH), 7.79 (br. s, 2H, NHCH), 8.33 (br. t, 2H, NH amide), 13.24 (br. s, 1H, NH pyrazole). ¹³C NMR (100 MHz, *d*⁶-

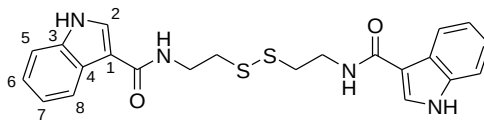
DMSO) δ 37.1, 37.9, 104.9, 129.9, 146.4, 161.6; HRMS (ESI+) m/z calc. for $C_{12}H_{17}N_6O_2S_2$ $[M+H]^+$ 341.0849, found: 341.0856; Anal. Calc. for $C_{12}H_{16}N_6O_2S_2$: C, 42.34; H, 4.74; N, 24.69. Found: C, 42.24; H, 4.65; N, 24.56. 2 tautomeric forms of the pyrazole ring in solution (Scheme 2) were observable by ^1H NMR, what is a known phenomenon for 1*H*-pyrazole-3(5)-(N-*tert*-butyl)carboxamide in solution.²⁴⁵ Recording ^1H NMR spectra at higher temperature did not show significant variation in the spectrum. The signals for the major tautomer are reported.

***N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl))bis(1*H*-pyrrole-3-carboxamide) (146)**



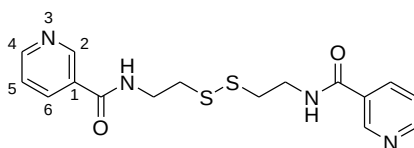
To a solution of cystamine (164 mg, 1.08 mmol, 1.0 eq.) and ethyl 1*H*-pyrrole-3-carboxylate²¹³ **145** (300 mg, 2.16 mmol, 2.0 eq.) in freshly distilled CH_2Cl_2 (7 mL), in a sealed tube and under an inert atmosphere was added trimethylaluminium (2M in hexanes, 2.16 mL, 4.31 mmol, 4.0 eq.) dropwise at 0°C. The reaction mixture was stirred for 5 minutes at 0°C, then at rt for 20 minutes. Particular care was taken while handling the pyrophoric trimethylaluminium reagent. The pressure was released 4-5 times over 20 minutes, then the resulting mixture was refluxed for 24h. After a day, the reaction was cooled to rt, 1N HCl was added, and stirring was continued for 20 minutes. The reaction mixture was then extracted several times at pH = 0, 3, 5 and 7 with generous amounts of AcOEt. The combined organic layers were dried ($MgSO_4$) and concentrated *in vacuo*. The product (100 mg, 27%, 69% brsm) was obtained as an amorphous white solid after purification by flash column chromatography (MeOH/ CH_2Cl_2 12 : 88); R_f 0.3 (MeOH/ CH_2Cl_2 12 : 88); IR (neat) 3236, 1607, 1560, 1518, 1426, 1331, 1204; ^1H NMR (400 MHz, d^6 -DMSO) δ 2.87 (t, J = 6.9 Hz, 4H, CH_2S), 3.46 (m, 4H, CH_2N), 6.45 (m, 2H), 6.73 (m, 2H), 7.30 (m, 2H), 7.97 (t, J = 8.0 Hz, 2H), 11.12 (br. s, 2H). ^{13}C NMR (100 MHz, d^6 -DMSO) δ 37.5, 38.1, 107.1, 118.3, 119.4, 120.3, 164.1; HRMS (ESI+) m/z calc. for $C_{14}H_{19}N_4O_2S_2$ $[M+H]^+$ 339.0944, found: 339.0954; Anal. Calc. for $C_{14}H_{18}N_4O_2S_2$: C, 49.68; H, 5.36; N, 16.55. Found: C, 49.54; H, 5.30; N, 16.46.

***N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl))bis(1*H*-indole-3-carboxamide) (148)**



To a solution of 1*H*-indole-3-carboxylic acid **147** (300 mg, 1.86 mmol, 1.0 eq.) in dry DMF (6 mL), under inert atmosphere, were added HOBt (277 mg, 2.05 mmol, 1.1 eq.) and EDCI (393 mg, 2.05 mmol, 1.1 eq.) at rt. After stirring for 10 minutes at rt, cystamine dihydrochloride (210 mg, 0.93 mmol, 0.5 eq.) and freshly distilled DIPEA (0.972 mL, 5.58 mmol, 3.0 eq.) were added. The resulting mixture was stirred at rt and followed by TLC. After 14 h, the reaction was stopped. The reaction mixture was diluted with AcOEt (50 mL), washed with water (2 x 25 mL) and dried (MgSO₄). The combined organic phases were concentrated and the product (35 mg, 9%) was obtained after precipitation from EtOH. *R*_f 0.4 (AcOEt); IR (neat) 3221, 1546, 1433, 1332, 1208; ¹H NMR (400 MHz, *d*⁶-DMSO) δ 2.95 (t, *J* = 6.5 Hz, 4H, CH₂S), 3.57 (m, 4H, CH₂N), 7.02-7.18 (m, 4H, 6-H + 7-H), 7.42 (d, *J* = 7.8 Hz, 2H, 5-H), 8.01 (d, *J* = 2.5 Hz, 2H, 2-H), 8.08-8.19 (m, 4H, NH amide + 8-H), 11.56 (br. s, 2H, NH indole). ¹³C NMR (100 MHz, *d*⁶-DMSO) δ 37.6, 38.1, 110.3, 111.7, 120.2, 120.9, 121.7, 125.9, 127.7, 136.0, 164.6; HRMS (ESI⁺) *m/z* calc. for C₂₂H₂₃N₄O₂S₂ [M+H]⁺ 439.1257, found: 439.1258; Anal. Calc. for C₂₂H₂₂N₄O₂S₂: C, 60.25; H, 5.06; N, 12.78. Found: C, 60.14; H, 4.95; N, 12.66.

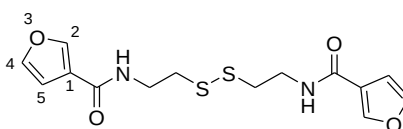
***N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl))dinicotinamide (150)**



To a solution of nicotinic acid **149** (700 mg, 5.69 mmol, 1.0 eq.) in freshly distilled CH₂Cl₂ (60 mL), under inert atmosphere, were added HOBt (845 mg, 6.25 mmol, 1.1 eq.) and EDCI (1.20 g, 6.25 mmol, 1.1 eq.) at rt. After stirring for 10 minutes at rt, cystamine dihydrochloride (640 mg, 2.84 mmol, 0.5 eq.) and freshly distilled NEt₃ (1.66 mL, 11.9 mmol, 2.1 eq.) were added. The resulting mixture was stirred at rt and followed by TLC. After 25 h, the reaction was stopped and the solvent was evaporated. The resulting residue was dissolved in

saturated aqueous NaHCO_3 (60 mL) and extracted with AcOEt (3 x 60 mL). The combined organic layers were dried (MgSO_4) and the product (663 mg, 64%) was obtained as an amorphous white solid after flash column chromatography ($\text{MeOH}/\text{CH}_2\text{Cl}_2$ 1 : 9). R_f 0.45 ($\text{MeOH}/\text{CH}_2\text{Cl}_2$ 1 : 9); IR (neat) 3316, 1641, 1591, 1542, 1301, 1279, 1197, 1279; ^1H NMR (400 MHz, d^6 -DMSO) 2.95 (t, J = 6.8 Hz, 4H, CH_2S), 3.58 (m, 4H, CH_2N), 7.50 (ddd, J = 7.8, 4.8, 0.6 Hz, 2H, 5-H), 8.17 (m, 2H, 6-H), 8.70 (dd, J = 4.8, 1.5 Hz, 2H, 4-H), 8.87 (t, J = 5.4 Hz, 2H, NH), 8.99 (d, J = 2.1 Hz, 2H, 2-H). ^{13}C NMR (100 MHz, d^6 -DMSO) δ 36.9, 38.6, 123.4, 129.6, 134.8, 148.3, 151.8, 164.8; HRMS (ESI+) m/z calc. for $\text{C}_{16}\text{H}_{19}\text{N}_4\text{O}_2\text{S}_2$ $[\text{M}+\text{H}]^+$ 363.0944, found: 363.0960; Anal. Calc. for $\text{C}_{16}\text{H}_{18}\text{N}_4\text{O}_2\text{S}_2$: C, 53.02; H, 5.01; N, 15.46. Found: C, 52.97; H, 4.97; N, 15.34.

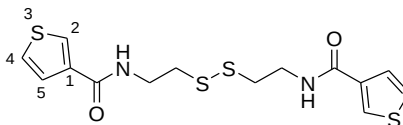
***N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl)difuran-3-carboxamide (152)**



To a solution of furan-3-carboxylic acid **151** (400 mg, 3.57 mmol, 1.0 eq.) in freshly distilled CH_2Cl_2 (38 mL), under inert atmosphere, were added HOBT (530 mg, 3.93 mmol, 1.1 eq.) and EDCI (752 mg, 3.93 mmol, 1.1 eq.) at rt. After stirring for 10 minutes at rt, cystamine dihydrochloride (402 mg, 1.78 mmol, 0.5 eq.) and freshly distilled NEt_3 (1.05 mL, 7.49 mmol, 2.1 eq.) were added. The resulting mixture was stirred at rt and followed by TLC. After 40 h, the reaction was stopped. The organic phase was washed with 1N aqueous HCl (40 mL). The aqueous phase was extracted one more time with CH_2Cl_2 (40 mL). The combined organic layers were washed with saturated aqueous NaHCO_3 (80 mL) and the aqueous layer was back extracted with CH_2Cl_2 (80 mL). The combined organic layers were dried (MgSO_4) and the product (120 mg, 20%) was obtained as an amorphous white solid after flash column chromatography ($\text{MeOH}/\text{CH}_2\text{Cl}_2$ 5 : 95). R_f 0.4 ($\text{MeOH}/\text{CH}_2\text{Cl}_2$ 6 : 94); IR (neat) 3270, 1618, 1568, 1545, 1529; ^1H NMR (400 MHz, d^6 -DMSO) δ 2.88 (t, J = 6.8 Hz, 4H, CH_2S), 3.49 (m, 4H, CH_2N), 6.83 (s, 2H, 5-H), 7.72 (s, 2H, 4-H), 8.16 (s, 2H, 2-H), 8.39 (t, J = 5.2 Hz, 2H, NH). ^{13}C NMR (100 MHz, d^6 -DMSO) δ 37.0, 38.1, 108.8, 122.6, 144.0, 145.1, 161.6; HRMS (ESI+) m/z calc. for $\text{C}_{14}\text{H}_{17}\text{N}_2\text{O}_4\text{S}_2$ $[\text{M}+\text{H}]^+$ 341.0624, found:

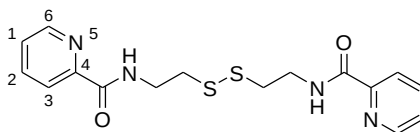
341.0630; Anal. Calc. for $C_{14}H_{16}N_2O_4S_2$: C, 49.40; H, 4.74; N, 8.23. Found: C, 49.04; H, 4.68; N, 8.13.

***N,N'*-(2,2'-Disulfanediyldis(ethane-2,1-diyl))dithiophene-3-carboxamide (154)**



To a solution of thiophene-3-carboxylic acid **153** (300 mg, 2.34 mmol, 1.0 eq.) in freshly distilled CH_2Cl_2 (25 mL), under inert atmosphere, were added HOBt (348 mg, 2.57 mmol, 1.1 eq.) and EDCI (493 mg, 2.57 mmol, 1.1 eq.) at rt. After stirring for 10 minutes at rt, cystamine dihydrochloride (263 mg, 1.17 mmol, 0.5 eq.) and freshly distilled NEt_3 (0.815 mL, 5.85 mmol, 2.1 eq.) were added. The resulting mixture was stirred at rt and followed by TLC. After 24 h, the reaction was stopped. The organic phase was washed with 1N aqueous HCl (25 mL). The aqueous phase was extracted one more time with generous amounts of CH_2Cl_2 and AcOEt. The combined organic layers were washed with saturated aqueous $NaHCO_3$ and the aqueous layer was back extracted with AcOEt. The combined organic layers were dried ($MgSO_4$) and the product (340 mg, 78%) was obtained as an amorphous white solid after flash column chromatography ($MeOH/CH_2Cl_2$ 5 : 95). R_f 0.5 ($MeOH/CH_2Cl_2$ 5 : 95); IR (neat) 3292, 1623, 1552, 1298, 1287 1184; ^1H NMR (400 MHz, d^6 -DMSO) δ 2.91 (t, J = 6.9 Hz, 4H, CH_2S), 3.52 (m, 4H, CH_2N), 7.48 (dd, J = 5.0, 1.1 Hz, 2H, 5-H), 7.58 (dd, J = 5.0, 3.0 Hz, 2H, 4-H), 8.11 (dd, J = 3.0, 1.1 Hz, 2H, 2-H), 8.51 (t, J = 5.5 Hz, 2H, NH). ^{13}C NMR (100 MHz, d^6 -DMSO) δ 37.0, 38.4, 126.6, 126.7, 128.6, 137.5, 162.0; HRMS (ESI+) m/z calc. for $C_{14}H_{17}N_2O_2S_4$ $[M+H]^+$ 373.0167, found: 373.0184; Anal. Calc. for $C_{14}H_{16}N_2O_2S_4$: C, 45.13; H, 4.33; N, 7.52. Found: C, 45.21; H, 4.39; N, 7.54.

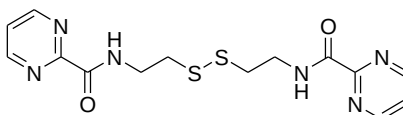
***N,N'*-(2,2'-Disulfanediyldis(ethane-2,1-diyl))dipicolinamide (156)**



To a solution of picolinic acid **155** (700 mg, 5.69 mmol, 1 eq.) in freshly distilled CH_2Cl_2 (60 mL), under inert atmosphere, were added HOBt (845 mg, 6.25 mmol,

1.1 eq.) and EDCI (1.20 g, 6.25 mmol, 1.1 eq.) at rt. After stirring for 10 minutes at rt, cystamine dihydrochloride (640 mg, 2.84 mmol, 0.5 eq.) and triethylamine (1.66 mL, 11.9 mmol, 2.1 eq.) were added. The resulting mixture was stirred at rt and followed by TLC. After 25 h, the reaction was stopped. The solvent was evaporated. Sat. Aq. NaHCO₃ (60 mL) was added and the mixture was extracted with AcOEt (3 x 60 mL). The combined organic layers were dried (MgSO₄) and the product (825 mg, 80%) was obtained as colourless needles after flash column chromatography (AcOEt/CH₂Cl₂ 9 : 1) and recrystallisation (Et₂O): m.p. 74-76°C; R_f 0.6 (AcOEt). IR (neat) 3373, 1660, 1519, 1434, 1289; ¹H NMR (400 MHz, CDCl₃) δ 2.96 (t, *J* = 6.4 Hz, 4H, CH₂S), 3.82 (m, 4H, CH₂N), 7.38-7.47 (m, 2H, 1-H), 7.79-7.89 (m, 2H, 2-H), 8.14-8.23 (m, 2H, 3-H), 8.40 (br. t, 2H, NH), 8.52-8.59 (m, 2H, 6-H). ¹³C NMR (100 MHz, CDCl₃) δ 37.9, 38.1, 122.2, 126.2, 137.3, 148.1, 149.7, 164.5; HRMS (ESI+) *m/z* calc. for C₁₆H₁₉N₄O₂S₂ [M+H]⁺ 363.0944 found: 363.0966; Anal. Calc. for C₁₆H₁₈N₄O₂S₂: C, 53.02; H, 5.01; N, 15.46. Found: C, 53.13; H, 4.94; N, 15.50.

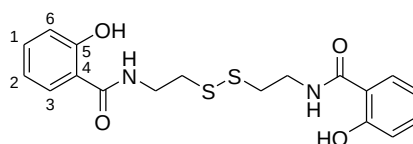
***N,N'*-(2,2'-Disulfanediybis(ethane-2,1-diyl))dipyrimidine-2-carboxamide (158)**



To an ice cold solution of pyrimidine-2-carboxylate **157** (700 mg, 5.07 mmol, 1.0 eq.) and cystamine (386 mg, 2.53 mmol, 0.5 eq.) in CHCl₃ (20 mL), under inert atmosphere, was added dropwise trimethylaluminium (2M in hexanes, 5.07 mL, 10.1 mmol, 2.0 eq.). The resulting mixture was stirred at reflux in a sealed tube. After 24 h, the reaction was stopped. 1N HCl was then added dropwise at 0°C and the resulting mixture was stirred for a few minutes at 0°C then rt for 25 minutes. Particular care was taken while handling the pyrophoric trimethylaluminium reagent. The pH was then adjusted to 9 with saturated aqueous NaHCO₃ and the biphasic mixture extracted with generous amounts of CHCl₃. The combined organic layers were dried (MgSO₄) and the product (288 mg, 31%) was obtained as a yellow solid after flash column chromatography (MeOH/CH₂Cl₂ 7 : 93). R_f 0.45 (MeOH/CH₂Cl₂ 8 : 92); IR (neat) 3272, 1674, 1567, 1524, 1411; ¹H NMR (400 MHz, CDCl₃) δ 2.97 (t, *J* = 6.3 Hz, 4H, CH₂S), 3.86 (m, 4H, CH₂N), 7.43 (d,

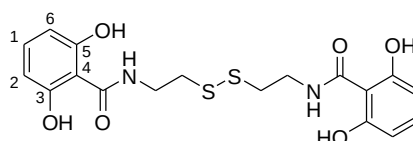
$J = 4.8$ Hz, 2H, N=CHCH), 8.40 (br. t, 2H, NH), 8.87 (d, $J = 4.8$ Hz, 4H, N=CH). ^{13}C NMR (100 MHz, CDCl_3) δ 37.5, 38.4, 122.5, 157.5, 162.3; HRMS (ESI+) m/z calc. for $\text{C}_{14}\text{H}_{17}\text{N}_6\text{O}_2\text{S}_2$ $[\text{M}+\text{H}]^+$ 365.0849, found: 365.0839; Anal. Calc. for $\text{C}_{14}\text{H}_{16}\text{N}_6\text{O}_2\text{S}_2$: C, 46.14; H, 4.43; N, 23.06. Found: C, 46.26; H, 4.44; N, 22.94.

***N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl))bis(2-hydroxybenzamide) (160)**



To a solution of 2-hydroxybenzoic acid **159** (500 mg, 3.62 mmol, 1 eq.) in freshly distilled CH_2Cl_2 (40 mL), under inert atmosphere, were added HOBT (538 mg, 3.98 mmol, 1.1 eq.) and EDCI (763 mg, 3.98 mmol, 1.1 eq.) at rt. After stirring for 10 minutes at rt, cystamine dihydrochloride (408 mg, 1.81 mmol, 0.5 eq.) and triethylamine (1.06 mL, 7.60 mmol, 2.1 eq.) were added. The resulting mixture was stirred at rt and followed by TLC. After 20 h, the reaction was stopped. The organic phase was washed with 1N aqueous HCl (40 mL). The aqueous phase was extracted one more time with CH_2Cl_2 (40 mL). The combined organic layers were dried (MgSO_4) and the product (420 mg, 59%) was obtained as an amorphous white solid after flash column chromatography and recrystallisation ($\text{EtOH}/\text{H}_2\text{O}$). R_f 0.3 ($\text{AcOEt}/\text{CH}_2\text{Cl}_2$ 3:7). IR 3323, 1605, 1545, 1495, 1456, 1370, 1309 cm^{-1} ; ^1H NMR (400 MHz, d^6 -DMSO) δ 2.96 (t, $J = 6.8$ Hz, 4H, CH_2S), 3.61 (m, 4H, CH_2N), 6.85-6.93 (m, 4H, 2-H + 6-H), 7.35-7.44 (m, 2H, 1-H), 7.79-7.87 (m, 2H, 3-H), 8.97 (br. t, 2H, NH), 12.44 (s, 2H, OH). ^{13}C NMR (100 MHz, d^6 -DMSO) δ 36.7, 38.2, 115.1, 117.3, 118.5, 127.7, 133.7, 159.8, 168.9; HRMS (ESI+) m/z calc. for $\text{C}_{18}\text{H}_{21}\text{N}_2\text{O}_4\text{S}_2$ $[\text{M}+\text{H}]^+$ 393.0937 found: 393.0950; Anal. Calc. for $\text{C}_{18}\text{H}_{20}\text{N}_2\text{O}_4\text{S}_2$: C, 55.08; H, 5.14; N, 7.14. Found: C, 55.15; H, 5.05; N, 7.07.

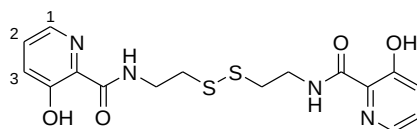
***N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl))bis(2,6-dihydroxybenzamide) (162)**



To a solution of cystamine **53** (345 mg, 2.27 mmol, 1 eq.) in freshly distilled CH_2Cl_2 (40 mL) and dioxane (10 mL), under inert atmosphere, were added HOBT

(736 mg, 5.45 mmol, 2.4 eq.), 2,6-dihydroxybenzoic acid **161** (700 mg, 4.54 mmol, 2 eq.), and EDCI (958 mg, 5.00 mmol, 2.2 eq.) at rt. The resulting mixture was stirred at rt and followed by TLC. After 24 h, the reaction was stopped. The product (155 mg, 16%) was obtained as an amorphous off white solid after flash column chromatography (MeOH/CH₂Cl₂ 5 : 95) and recrystallisation (acetone/hexane). *R*_f 0.3 (MeOH/CH₂Cl₂ 5 : 95). IR (neat) 3270, 1593, 1553, 1452, 1341, 1279, 1237; ¹H NMR (400 MHz, *d*⁶-DMSO) δ 2.97 (t, *J* = 6.4 Hz, 4H, CH₂S), 3.67 (m, 4H, CH₂N), 6.36 (d, *J* = 8.2 Hz, 4H, 2-H + 6-H), 7.16 (d, *J* = 8.2 Hz, 2H, 1-H), 9.14 (br. t, 2H, NH), 12.56 (br. s, 4H, OH). ¹³C NMR (100 MHz, *d*⁶-DMSO) δ 36.7, 37.6, 102.3, 107.1, 133.4, 160.2, 170.0; HRMS (ESI+) *m/z* calc. for C₁₈H₂₁N₂O₆S₂ [M+H]⁺ 425.0836 found: 425.0839; Anal. Calc. for C₁₈H₂₀N₂O₆S₂: C, 50.93; H, 4.75; N, 6.60. Found: C, 50.83; H, 4.70; N, 6.50.

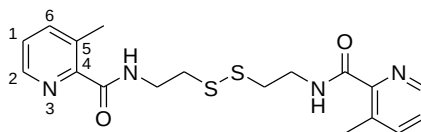
***N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl))bis(3-hydroxypicolinamide) (164)**



To a solution of 3-hydroxypicolinic acid **163** (300 mg, 2.16 mmol, 1.0 eq.) in freshly distilled CH₂Cl₂ (25 mL), under inert atmosphere, were added HOBt (321 mg, 2.37 mmol, 1.1 eq.) and EDCI (455 mg, 2.37 mmol, 1.1 eq.) at rt. After stirring for 10 minutes at rt, cystamine dihydrochloride (164 mg, 1.08 mmol, 0.5 eq.) and freshly distilled NEt₃ (631 μL, 4.53 mmol, 2.1 eq.) were added. The resulting mixture was stirred at rt and followed by TLC. After 48 h, the reaction was stopped. The organic phase was washed with water (25 mL). The aqueous phase was extracted one more time with AcOEt (3 x 25 mL). The combined organic layers were dried (MgSO₄) and the product (225 mg, 53%) was obtained as a white solid after flash column chromatography (MeOH/AcOH/CH₂Cl₂ 4 : 1 : 96). *R*_f 0.8 (MeOH/AcOH/CH₂Cl₂ 4 : 1 : 96); IR (neat) 3381, 3347, 1646, 1575, 1529, 1442, 1440, 1289; ¹H NMR (400 MHz, CDCl₃) δ 2.97 (t, *J* = 6.4 Hz, 4H, CH₂S), 3.82 (m, 4H, CH₂N), 7.31 (dd, *J* = 8.6, 1.5 Hz, 2H, 3-H), 7.35 (dd, *J* = 8.6, 4.1 Hz, 2H, 2-H), 8.07 (dd, *J* = 4.1, 1.5 Hz, 2H, 1-H), 8.42 (br. t, 2H, NH), 12.03 (s, 2H, OH). ¹³C NMR (100 MHz, CDCl₃) δ 37.7, 37.8, 126.2, 128.7, 131.2, 139.5, 157.8, 168.9; HRMS (ESI+) *m/z* calc. for C₁₆H₁₉N₄O₄S₂ [M+H]⁺ 395.0842,

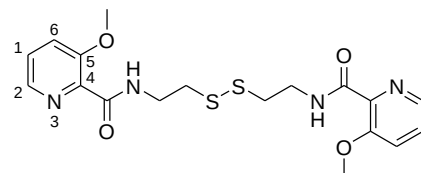
found: 395.0847; Anal. Calc. for $C_{16}H_{18}N_4O_4S_2$: C, 48.72; H, 4.60; N, 14.20. Found: C, 48.85; H, 4.66; N, 14.04.

***N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl))bis(3-methylpicolinamide) (181)**



Compound **181** (334 mg, 67%) was obtained as a white solid, according to the general procedure described above. R_f 0.4 (AcOEt/PE₄₀₋₆₀ 8 : 2); IR (neat) 3291, 1660, 1518, 1440, 1409; ^1H NMR (400 MHz, $CDCl_3$) δ 2.73 (s, 6H, CH_3Ar), 2.96 (t, J = 6.5 Hz, 4H, CH_2S), 3.78 (m, 4H, CH_2N), 7.30 (dd, J = 7.8, 4.5 Hz, 2H, 1-H), 7.58 (dd, J = 7.8, 0.9 Hz, 2H, 6-H), 8.38 (dd, J = 4.5, 0.9 Hz, 2H, 2-H), 8.50 (br. t, 2H, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.5, 38.0, 38.1, 125.7, 135.5, 141.0, 145.3, 146.9, 165.9; HRMS (ESI+) m/z calc. for $C_{18}H_{23}N_4O_2S_2$ $[M+H]^+$ 391.1257, found: 391.1252; Anal. Calc. for $C_{18}H_{22}N_4O_2S_2$: C, 55.36; H, 5.68; N, 14.35. Found: C, 55.30; H, 5.58; N, 14.23

***N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl))bis(3-methoxypicolinamide) (184)**

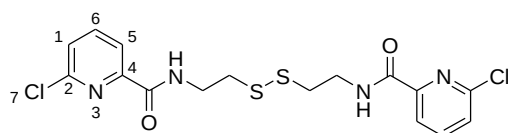


Compound **184** (182 mg, 80%) was obtained as a white solid, according to the general procedure described above. R_f 0.5 (MeOH/ CH_2Cl_2 1 : 9); IR (neat) 3296, 1655, 1509, 1464, 1426, 1279, 1248, 1211; ^1H NMR (400 MHz, $CDCl_3$) δ 2.94 (t, J = 6.4 Hz, 4H, CH_2S), 3.77 (m, 4H, CH_2N), 3.93 (s, 6H, CH_3O), 7.34 (dd, J = 8.5, 1.5 Hz, 2H, 6-H), 7.38 (dd, J = 8.5, 4.1 Hz, 2H, 1-H), 8.12 (br. t, 2H, NH), 8.18 (dd, J = 4.1, 1.5 Hz, 2H, 2-H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.7, 38.0, 56.0, 120.4, 127.0, 138.6, 140.1, 155.8, 164.3; HRMS (ESI+) m/z calc. for $C_{18}H_{23}N_4O_4S_2$ $[M+H]^+$ 423.1155, found: 423.1162; Anal. Calc. for $C_{18}H_{22}N_4O_4S_2$: C, 51.17; H, 5.25; N, 13.26. Found: C, 51.08; H, 5.36; N, 13.16.

General procedure for amide bond formation between cystamine **53** and chloropyridine carboxylic acids **165-167**:

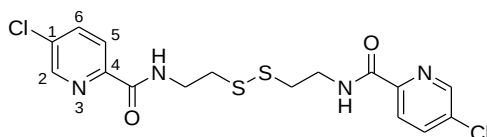
To a solution of the appropriate carboxylic acid (1.0 eq.) in freshly distilled CH_2Cl_2 (11 mL/mmol) at rt was added HOBt (1.1 eq.) and EDCI (1.1 eq.). The resulting mixture was stirred at rt for 10-15 minutes, then cystamine dihydrochloride (0.5 eq.) and freshly distilled triethylamine (2.5 eq.) were added. After 24 hours at rt, the organic phase was washed with sat. aq. NaHCO_3 (11 mL/mmol). The aqueous phase was back extracted two times with CHCl_3 (11 mL/mmol). The combined organic layers were dried (MgSO_4) and concentrated under reduced pressure. The product was purified by flash column chromatography.

***N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl))bis(6-chloropicolinamide) (168)**



Compound **168** (910 mg, 66%) was obtained as a white solid, according to the general procedure described above. R_f 0.45 ($\text{AcOEt}/\text{CH}_2\text{Cl}_2$ 3 : 7); IR (neat) 3324, 1653, 1558, 1535, 1433, 1402, 1308; ^1H NMR (400 MHz, CDCl_3) δ 2.98 (t, J = 6.5 Hz, 4H, CH_2S), 3.82 (m, 4H, CH_2N), 7.46 (dd, J = 7.8, 0.8 Hz, 2H, 1-H), 7.82 (app-t, J = 7.8 Hz, 2H, 6-H), 8.13 (dd, J = 7.8, 0.8 Hz, 2H, 5-H), 8.18 (br. t, 2H, NH). ^{13}C NMR (100 MHz, CDCl_3) δ 37.6, 38.5, 121.0, 127.1, 140.0, 150.1, 150.2, 163.1; HRMS (ESI+) m/z calc. for $\text{C}_{16}\text{H}_{17}\text{Cl}_2\text{N}_4\text{O}_2\text{S}_2$ $[\text{M}+\text{H}]^+$ 431.0164, found: 431.0173; Anal. Calc. for $\text{C}_{16}\text{H}_{16}\text{Cl}_2\text{N}_4\text{O}_2\text{S}_2$: C, 44.55; H, 3.74; N, 12.99. Found: C, 44.55; H, 3.66; N, 12.90.

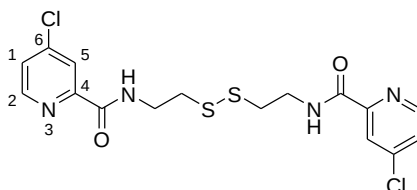
***N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl))bis(5-chloropicolinamide) (169)**



Compound **169** (947 mg, 69%) was obtained as a white solid, according to the general procedure described above. R_f 0.45 ($\text{AcOEt}/\text{CH}_2\text{Cl}_2$ 3 : 7); IR (neat) 3352, 1655, 1525, 1464, 1442, 1302; ^1H NMR (400 MHz, CDCl_3) δ 2.96 (t, J = 6.4 Hz, 4H, CH_2S), 3.81 (m, 4H, CH_2N), 7.81 (dd, J = 8.3, 2.3 Hz, 2H, 6-H), 8.14 (d, J = 8.3 Hz, 2H, 2-H), 8.25 (br. t, 2H, NH), 8.50 (d, J = 2.3 Hz, 2H, 5-H). ^{13}C NMR (100 MHz, CDCl_3) δ 37.8, 38.2, 123.2, 135.1, 137.1, 147.2, 147.8, 163.6; HRMS

(ESI+) m/z calc. for $C_{16}H_{17}Cl_2N_4O_2S_2$ $[M+H]^+$ 431.0164, found: 431.0162; Anal. Calc. for $C_{16}H_{16}Cl_2N_4O_2S_2$: C, 44.55; H, 3.74; N, 12.99. Found: C, 44.63; H, 3.62; N, 12.91.

***N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl))bis(4-chloropicolinamide) (170)**

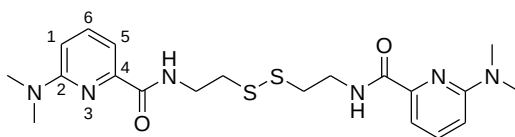


Compound **170** (460mg, 42%) was obtained as a white solid, according to the general procedure described above. R_f 0.65 (AcOEt/ CH_2Cl_2 3 : 7); IR (neat) 3355, 1669, 1558, 1516; ^1H NMR (400 MHz, $CDCl_3$) δ 2.96 (t, J = 6.4 Hz, 4H, CH_2S), 3.82 (m, 4H, CH_2N), 7.43 (dd, J = 5.2, 2.0 Hz, 2H, 1-H), 8.19 (d, J = 2.0 Hz, 2H, 5-H), 8.32 (br. t, 2H, NH), 8.45 (d, J = 5.2 Hz, 2H, 2-H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.7, 38.3, 123.0, 126.4, 146.0, 149.0, 151.1, 163.3; HRMS (ESI+) m/z calc. for $C_{16}H_{17}Cl_2N_4O_2S_2$ $[M+H]^+$ 431.0164, found: 431.0159; Anal. Calc. for $C_{16}H_{16}Cl_2N_4O_2S_2$: C, 44.55; H, 3.74; N, 12.99. Found: C, 44.56; H, 3.65; N, 12.89.

Chlorine displacement, general procedure :

To a solution of the appropriate chlorinated derivative in THF (11 mL/mmol) was added a solution of the appropriate amine (40% w/w in water, 11 mL/mmol). The resulting mixture was heated at 160°C under microwave irradiation and the progress of the reaction was monitored by TLC. The reaction mixture was then concentrated and the product was purified by flash column chromatography.

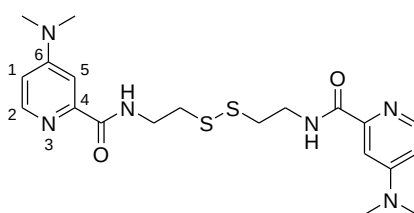
***N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl))bis(6-(dimethylamino)picolinamide) (171)**



Compound **171** (153 mg, 74%) was obtained as a white solid, according to the general procedure described above. R_f 0.2 (MeOH/ CH_2Cl_2 2 : 98); IR (neat) 3338,

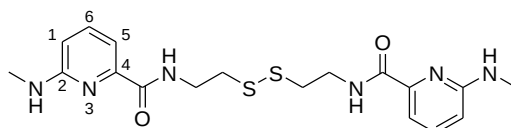
1657, 1520, 1520, 1499, 1428, 1378; ^1H NMR (400 MHz, CDCl_3) δ 2.96 (t, J = 6.4 Hz, 4H, CH_2S), 3.11 (s, 12H, NMe_2), 3.79 (m, 4H, CH_2N), 6.66 (d, J = 8.3 Hz, 2H, 1-H), 7.44 (d, J = 7.0 Hz, 2H, 5-H), 7.57 (dd, J = 8.3, 7.0 Hz, 2H, 6-H), 8.39 (br. t, 2H, NH). ^{13}C NMR (100 MHz, CDCl_3) δ 38.0, 38.1 (2C), 109.1, 110.0, 138.2, 147.4, 157.7, 165.2; HRMS (ESI+) m/z calc. for $\text{C}_{20}\text{H}_{29}\text{N}_6\text{O}_2\text{S}_2$ $[\text{M}+\text{H}]^+$ 449.1788, found: 449.1772; Anal. Calc. for $\text{C}_{20}\text{H}_{28}\text{N}_6\text{O}_2\text{S}_2$: C, 53.55; H, 6.29; N, 18.73. Found: C, 53.46; H, 6.39; N, 18.78.

***N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl))bis(4-(dimethylamino)picolinamide) (173)**



Compound **173** (143 mg, 92%) was obtained as a white solid, according to the general procedure described above. R_f 0.5 (MeOH/ CH_2Cl_2 5 : 95); IR (neat) 3347, 1665, 1603, 1513, 1437 1378 1286, 1222; ^1H NMR (400 MHz, CDCl_3) δ 2.94 (t, J = 6.5 Hz, 4H, CH_2S), 3.05 (s, 12H, NMe_2), 3.78 (m, 4H, NCH_2), 6.54 (dd, J = 5.9, 2.8 Hz, 2H, 1-H), 7.45 (d, J = 2.8 Hz, 2H, 5-H), 8.14 (d, J = 5.9 Hz, 2H, 2-H), 8.50 (br. t, 2H, NH). ^{13}C NMR (100 MHz, CDCl_3) δ 37.9, 38.2, 39.2, 105.2, 108.0, 148.1, 149.7, 155.2, 165.3; HRMS (ESI+) m/z calc. for $\text{C}_{20}\text{H}_{29}\text{N}_6\text{O}_2\text{S}_2$ $[\text{M}+\text{H}]^+$ 449.1788, found: 449.1781; Anal. Calc. for $\text{C}_{20}\text{H}_{28}\text{N}_6\text{O}_2\text{S}_2$: C, 53.55; H, 6.29; N, 18.73. Found: C, 53.46; H, 6.32; N, 18.78.

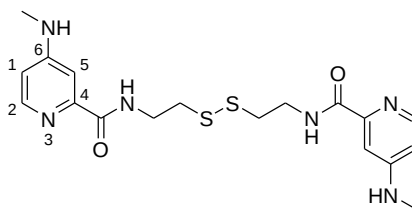
***N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl))bis(6-(methylamino)picolinamide) (174)**



Compound **174** (25 mg, 13%) was obtained as a white solid, according to the general procedure described above. R_f 0.2 (MeOH/ CH_2Cl_2 4 : 96); IR (neat) 3341, 1660, 1601, 1525, 1506, 1466, 1419; ^1H NMR (400 MHz, CDCl_3) δ 2.95 (m, 10H, CH_3N + CH_2S), 3.79 (m, 4H, CH_2N), 4.71 (br. q, 2H, CH_3NH), 6.52 (d, J = 8.3 Hz, 2H, 1-H), 7.46 (d, J = 7.3 Hz, 2H, 5-H), 7.55 (m, 2H, 6-H), 8.36 (br. t, 2H,

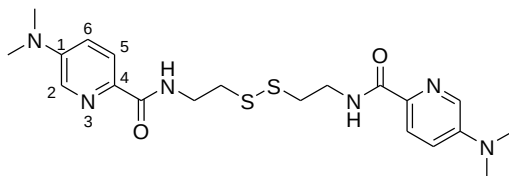
NH amide). ^{13}C NMR (100 MHz, CDCl_3) δ 28.9, 38.0, 38.1, 109.8, 111.3, 138.3, 147.8, 158.1, 165.1; HRMS (ESI+) m/z calc. for $\text{C}_{18}\text{H}_{25}\text{N}_6\text{O}_2\text{S}_2$ $[\text{M}+\text{H}]^+$ 421.1475, found: 421.1464; Anal. Calc. for $\text{C}_{18}\text{H}_{24}\text{N}_6\text{O}_2\text{S}_2$: C, 51.41; H, 5.75; N, 19.98. Found: C, 51.45; H, 5.85; N, 19.88.

***N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl)bis(4-(methylamino)picolinamide) (176)**



Compound **176** (85 mg, 44%) was obtained as a white solid, according to the general procedure described above. R_f 0.2 (MeOH/ CH_2Cl_2 5 : 95); IR (neat) 3350, 1662, 1615, 1537, 1513, 1357; ^1H NMR (400 MHz, d^6 -DMSO) δ 2.73 (d, J = 4.9 Hz, 6H, CH_3), 2.93 (t, J = 6.6 Hz, 4H, CH_2S), 3.56 (m, 4H, CH_2N), 6.57 (dd, J = 5.7, 2.4 Hz, 2H, 1-H), 6.85 (q, J = 4.7 Hz, 2H, CH_3NH), 7.17 (d, J = 2.4 Hz, 2H, 5-H), 8.07 (d, J = 5.7 Hz, 2H, 2-H), 8.75 (t, J = 6.0 Hz, 2H, NH amide). ^{13}C NMR (100 MHz, CDCl_3) δ 28.5, 37.0, 37.9, 105.1, 108.0, 148.2, 149.8, 155.4, 164.5; HRMS (ESI+) m/z calc. for $\text{C}_{18}\text{H}_{25}\text{N}_6\text{O}_2\text{S}_2$ $[\text{M}+\text{H}]^+$ 421.1475, found: 421.1476; Anal. Calc. for $\text{C}_{18}\text{H}_{24}\text{N}_6\text{O}_2\text{S}_2$: C, 51.41; H, 5.75; N, 19.98. Found: C, 51.30; H, 5.89; N, 19.89.

***N,N'*-(2,2'-Disulfanediy)bis(ethane-2,1-diyl)bis(5-(dimethylamino)picolinamide) (179)**

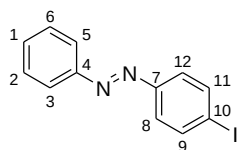


To a solution of acid **178**²¹⁵ (50 mg, 0.30 mmol, 1.0 eq.) in freshly distilled CH_2Cl_2 (3.4 mL) were added HOBt (45 mg, 0.33 mmol, 1.1 eq.) and EDC (63 mg, 0.33 mmol, 1.1 eq.) at rt. The resulting mixture was stirred at rt for 15 minutes. Cystamine dihydrochloride (34 mg, 0.15 mmol, 0.5 eq.) and Et_3N (105 μL , 0.752 mmol, 2.5 eq.) were added, and the resulting mixture was stirred at rt for 24 hours. The solvent was evaporated under reduced pressure, and the product (17 mg,

25%) was purified by flash column chromatography (MeOH/CH₂Cl₂ 3 : 97). R_f 0.2 (MeOH/CH₂Cl₂ 3 : 97); ¹H NMR (400 MHz, CDCl₃) δ 2.95 (t, *J* = 6.4 Hz, 2H, CH₂S), 3.05 (s, 6H, NMe₂), 3.79 (m, 2H, CH₂N), 6.98 (dd, *J* = 8.8, 3.0 Hz, 6-H), 7.96-8.04 (m, 2H, 2-H + 5-H), 8.13 (br. t, 1H, NH). HRMS (ESI+) *m/z* calc. for C₂₀H₂₉N₆O₂S₂ [M+H]⁺ 449.1788, found: 449.1778.

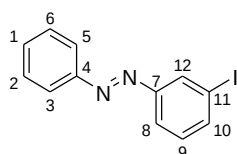
4. Towards photoswitchable HDAC inhibitors

(*E*)-1-(4-Iodophenyl)-2-phenyldiazene (197)



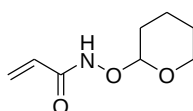
Compound **197** has been previously reported by Trauner and coworkers^{219d} and has been prepared according to the same procedure. Briefly, a solution of nitrosobenzene (2.5 g, 23.3 mmol, 1.0 eq.) and 4-iodobenzenamine (4.24g, 19.4 mmol, 0.83 eq.) in glacial acetic acid (160 mL) was stirred at 40°C for 12h. The resulting mixture was then diluted with water (625 mL) and extracted with CH₂Cl₂ (625 mL). The organic phase was washed with brine (2 x 625 mL), dried (MgSO₄) and concentrated. The product (5.65 g, 94%) was purified by flash column chromatography (AcOEt/PE₄₀₋₆₀ 3 : 7). NMR data matched perfectly with reported ones. ¹H NMR (400 MHz, CDCl₃) δ 7.51 (m, 3H, 1-H + 2-H + 6-H), 7.66 (m, 2H, 8-H + 12-H), 7.87 (m, 2H, 9-H + 11-H), 7.92 (m, 2H, 3-H + 5-H). ¹³C NMR (100 MHz, CDCl₃) δ 97.6, 122.9, 124.5, 129.1, 131.3, 138.3, 151.9, 152.4; HRMS (ESI+) *m/z* calc. for C₁₂H₁₀IN₂ [M+H]⁺ 308.9883, found: 308.9890.

(*E*)-1-(3-Iodophenyl)-2-phenyldiazene (196)



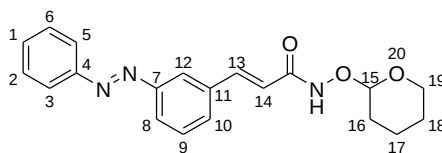
196 was prepared according to the same procedure. ¹H NMR (400 MHz, CDCl₃) δ 7.27 (t, *J* = 7.9 Hz, 1H, 9-H), 7.45-7.57 (m, 3H, 1-H + 2-H + 6-H), 7.79 (m, 1H, 10-H), 7.88-7.97 (m, 3H, 3-H + 5-H + 8-H), 8.26 (t, *J* = 1.7 Hz, 1H, 12-H). ¹³C NMR (100 MHz, CDCl₃) δ 94.6, 123.0, 123.5, 129.1, 130.5, 130.6, 131.5, 139.5, 152.3, 153.3; HRMS (ESI+) *m/z* calc. for C₁₂H₁₀IN₂ [M+H]⁺ 308.9883, found: 308.9877.

N-(Tetrahydro-2*H*-pyran-2-yloxy)acrylamide (198)



To an ice cold solution of O-(tetrahydro-2*H*-pyran-2-yl)hydroxylamine **54**¹⁵⁵ (5.0 g, 42.7 mmol, 1 eq.) and NEt₃ (6.55 mL, 47.0 mmol, 1.1 eq.) in freshly distilled CH₂Cl₂ (375 mL) was added a second solution of acryloyl chloride **203** (3.64 mL, 44.8 mmol, 1.05 eq.) in CH₂Cl₂ (50 mL) over 1 hour. The resulting was then stirred at rt for 24h. The organic phase was then washed with water (250 mL). The organic phase was extracted one more time with CH₂Cl₂ (250 mL). The combines organic layers were dried (MgSO₄) and concentrated. The product (4.41 g, 60%) was purified by flash column chromatography (gradient AcOEt/PE₄₀₋₆₀ 3 : 7 to 7 : 3) and obtained as a white solid. R_f 0.35 (AcOEt : PE₄₀₋₆₀, 7 : 3); ¹H NMR (400 MHz, *d*⁶-DMSO) δ 1.20-1.70 (m, 6H), 3.52 (m, 1H), 3.94 (m, 1H), 4.87 (m, 1H), 5.65 (dd, *J* = 9.0, 3.1 Hz, 1H), 6.04-6.24 (m, 2H), 11.24 (s, 1H). ¹³C NMR (100 MHz, *d*⁶-DMSO) δ 18.2, 24.6, 27.7, 61.3, 100.9, 126.1, 128.4, 161.8; HRMS (ESI+) *m/z* calc. for C₈H₁₃NO₃Na [M+Na]⁺ 194.0788, found: 194.0788; Anal. Calc. for C₈H₁₃NO₃: C, 56.13; H, 7.65; N, 8.18. Found: C, 56.15; H, 7.76; N, 8.08.

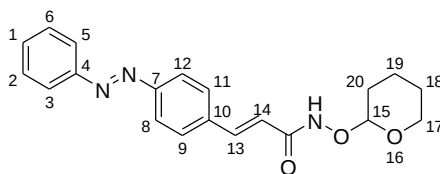
(*E*)-3-(3-((*E*)-Phenyldiazenyl)phenyl)-*N*-(tetrahydro-2*H*-pyran-2-yloxy)acrylamide (199**)**



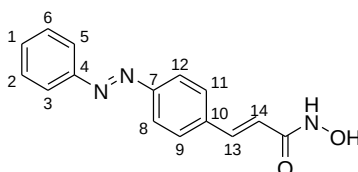
A mixture of compound **196** (1.80 g, 5.84 mmol, 1 eq.), compound **198** (1.50 g, 8.76, 1.5 eq.), NEt₃ (2.44 mL, 17.5 mmol, 3 eq.), tri *o*-tolylphosphine (178 mg, 0.584 mmol, 0.1 eq.), palladium acetate (66 mg, 0.292 mmol, 0.05 eq.) in anhydrous DMF (25 mL) was heated at 100°C for 24h under inert atmosphere. The reaction mixture was then diluted with water (120 mL), extracted with CH₂Cl₂ (2 x 90 mL). The combined organic layers were washed with brine (90 mL), dried (MgSO₄) and concentrated under vacuum. The product (1.69 g, 82%) was obtained as an orange solid after flash column chromatography (AcOEt/PE₄₀₋₆₀ 1 : 1). R_f 0.6 (AcOEt : PE₄₀₋₆₀ 7 : 3); IR (neat) 3177, 1662, 1628, 1206, 1116; ¹H NMR (400 MHz, *d*⁶-DMSO) δ 1.45-1.85 (m, 6H, 16-H₂ + 17-H₂ + 18-H₂), 3.55 (m, 1H, 19-H_A), 3.97 (m, 1H, 19-H_B), 4.94 (m, 1H, 15-H), 6.67 (d, *J* = 15.8 Hz, 1H, 14-H), 7.54-7.70 (m, 5H, 1-H + 2-H + 6-H + 9-H + 13-H), 7.78 (d, *J* = 7.4

Hz, 1H, 10-H), 7.91 (m, 3H, 3-H + 5-H + 8-H), 8.07 (s, 1H, 12-H), 11.30 (s, 1H, NH). ^{13}C NMR (100 MHz, d^6 -DMSO) δ 18.2, 24.6, 27.7, 61.3, 101, 120.1, 120.8, 122.5, 123.5, 129.4, 130.0, 130.5, 131.7, 136.0, 138.4, 151.8, 152.2, 162.1; HRMS (ESI+) m/z calc. for $\text{C}_{20}\text{H}_{22}\text{N}_3\text{O}_3$ $[\text{M}+\text{H}]^+$ 352.1656, found: 352.1660; Anal. Calc. for $\text{C}_{20}\text{H}_{21}\text{N}_3\text{O}_3$: C, 68.36; H, 6.02; N, 11.96. Found: C, 68.49; H, 6.10; N, 11.89.

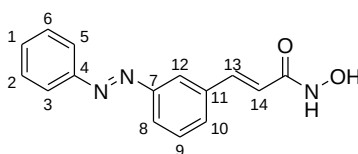
(E)-3-(4-((E)-Phenyldiazenyl)phenyl)-N-(tetrahydro-2H-pyran-2-yloxy)acrylamide (200)



A mixture of compound **200** (1.80 g, 5.84 mmol, 1 eq.), compound **198** (1.50 g, 8.76, 1.5 eq.), NEt_3 (2.44 mL, 17.5 mmol, 3 eq.), tri *o*-tolylphosphine (178 mg, 0.584 mmol, 0.1 eq.), palladium acetate (66 mg, 0.292 mmol, 0.05 eq.) in anhydrous DMF (25 mL) was heated at 100°C for 24h under inert atmosphere. The reaction mixture was then diluted with water (120 mL), extracted with CH_2Cl_2 (2 x 90 mL). The combined organic layers were washed with brine (90 mL), dried (MgSO_4) and concentrated under vacuum. The product (1.63 g, 80%) was obtained as an orange solid after flash column chromatography ($\text{AcOEt}/\text{PE}_{40-60}$ 1 :1). R_f 0.6 (AcOEt : PE_{40-60} , 7 : 3); IR (neat) 3194, 1658, 1622, 1206, 1116; ^1H NMR (400 MHz, d^6 -DMSO) δ 1.45-1.85 (m, 6H, 18- H_2 + 19- H_2 + 20- H_2), 3.55 (m, 1H, 17- H_A), 3.98 (m, 1H, 17- H_B), 4.94 (m, 1H, 15-H), 6.65 (d, J = 15.8 Hz, 1H, 14-H), 7.59 (m, 4H, 1-H + 2-H + 6-H + 13-H), 7.81 (m, 2H, 9-H + 11-H), 7.92 (m, 4H, 3-H + 5-H + 8-H + 12-H), 11.34 (s, 1H, NH). ^{13}C NMR (100 MHz, d^6 -DMSO) δ 18.2, 24.6, 27.7, 61.3, 101.0, 120.5, 122.6, 123.1, 128.7, 129.4, 131.7, 137.5, 138.3, 151.9, 152.1, 162.1; HRMS (ESI+) m/z calc. for $\text{C}_{20}\text{H}_{22}\text{N}_3\text{O}_3$ $[\text{M}+\text{H}]^+$ 352.1656, found: 352.1656; Anal. Calc. for $\text{C}_{20}\text{H}_{21}\text{N}_3\text{O}_3$: C, 68.36; H, 6.02; N, 11.96. Found: C, 68.29; H, 6.00; N, 11.86.

(E)-N-Hydroxy-3-(4-((E)-phenyldiazenyl)phenyl)acrylamide (201)

A solution of compound **199** (500 mg, 1.42 mmol, 1 eq.) and pyridinium paratoluenesulfonate (36 mg, 0.142 mmol, 0.1 eq.) in a 1 : 1 mixture of THF and *i*-PrOH (30 mL) was heated at 50°C for 8h under inert atmosphere. The solvent was then evaporated under vacuum and the product (288 mg, 76%) was obtained as an amorphous dark orange solid after purification by flash column chromatography (MeOH/CH₂Cl₂/AcOH 8 : 92 : 0.5) and attempted recrystallization (AcOH/EtOH, filtration and washings with cold Et₂O). *R*_f 0.3 (MeOH : CH₂Cl₂ 8 : 92); IR (neat) 3244, 1662, 1567, 1353; ¹H NMR (400 MHz, *d*⁶-DMSO) δ 6.59 (d, *J* = 15.7 Hz, 1H, 14-H), 7.48-7.68 (m, 4H, 1-H + 2-H + 6-H + 13-H), 7.80 (m, 2H, 9-H + 11-H), 7.92 (m, 4H, 8-H + 12-H + 3-H + 5-H), 9.15 (br. s, 1H, OH), 10.86 (br. s, 1H, NH). ¹³C NMR (100 MHz, *d*⁶-DMSO) δ 120.9, 122.6, 123.1, 128.6, 129.5 131.7, 137.1, 137.8, 151.9, 152.0, 162.3; HRMS (ESI-) *m/z* calc. for C₁₅H₁₂N₃O₂ [M-H]⁻ 266.0935, found: 266.0919; Anal. Calc. for C₁₅H₁₃N₃O₂: C, 67.40; H, 4.90; N, 15.72. Found: C, 67.52; H, 4.77; N, 15.63.

(E)-N-Hydroxy-3-(3-((E)-phenyldiazenyl)phenyl)acrylamide (202)

A solution of compound **200** (500 mg, 1.42 mmol, 1 eq.) and pyridinium paratoluenesulfonate (36 mg, 0.142 mmol, 0.1 eq.) in a 1 : 1 mixture of THF and *i*-PrOH (30 mL) was heated at 50°C for 8h under inert atmosphere. The solvent was then evaporated under vacuum and the product (302 mg, 79%) was obtained as an amorphous dark orange solid after purification by flash column chromatography (MeOH/CH₂Cl₂/AcOH 8 : 92 : 0.5) and attempted recrystallization (AcOH/EtOH, filtration and washings with cold Et₂O). *R*_f 0.3 (MeOH : CH₂Cl₂ 8 : 92); IR (neat) 3210, 1611, 1535, 1362; ¹H NMR (400 MHz, *d*⁶-DMSO) δ 6.62 (d, *J* = 15.2 Hz,

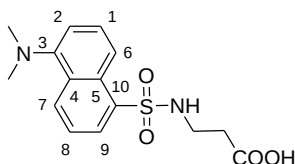
1H, 14-H), 7.50-7.70 (m, 5H, 1-H + 2-H + 6-H + 9-H + 13-H), 7.77 (m, 1H, 10-H), 7.84-7.98 (m, 3H, 3-H + 5-H + 8-H), 8.06 (s, 1H, 12-H), 9.14 (br. s, 1H, OH), 10.83 (br. s, 1H, NH). ¹³C NMR (100 MHz, *d*⁶-DMSO) δ 120.5, 120.9, 122.6, 123.1, 129.5, 130.1, 130.5, 131.7, 136.1, 137.3, 151.8, 152.2, 162.3; HRMS (ESI-) *m/z* calc. for C₁₅H₁₂N₃O₂ [M-H]⁻ 266.0935, found: 266.0921; Anal. Calc. for C₁₅H₁₃N₃O₂: C, 67.40; H, 4.90; N, 15.72. Found: C, 67.30; H, 4.97; N, 15.85.

5. Development of new probes for a Fluorescence Resonance Energy Transfer (FRET) based inhibitor assay for Histone Deacetylases

General procedure for the synthesis of 3-(5-(dimethylamino)naphthalene-1-sulfonamido)alkanoic acids:

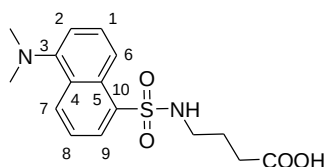
To a solution of the appropriate amino-acid (1.63 mmol, 1.1 eq.) in 1N aqueous sodium hydroxide (2 mL) was added a second solution of dansyl chloride (400 mg, 1.48 mmol, 1 eq.) in THF (13 mL), at rt. The resulting mixture was stirred at rt for 1.5 hour before THF was evaporated under reduced pressure. The resulting aqueous phase was acidified with concentrated aqueous HCl (10%) and extracted several times (AcOEt) from pH 4.5-5 to pH 2.5-3. The combined organic layers were dried (MgSO₄) and concentrated under reduced pressure. The products were obtained as sticky green oil after purification by flash column chromatography (MeOH/CH₂Cl₂).

3-(5-(Dimethylamino)naphthalene-1-sulfonamido)propanoic acid (213)



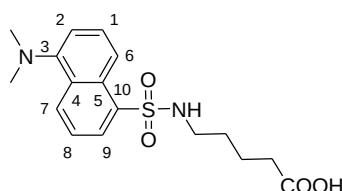
213 was prepared according to the general procedure above. 282 mg, 59%. ¹H NMR (400 MHz, CDCl₃) δ 2.53 (t, *J* = 5.7 Hz, 2H, CH₂COOH), 2.89 (s, 6H, NMe₂), 3.13-3.22 (m, 2H, CH₂N), 5.62 (br. s, 1H, NH), 7.19 (d, *J* = 7.5 Hz, 1H, 2-H), 7.48-7.61 (m, 2H, 1-H + 8-H), 8.21-8.30 (m, 2H, 6-H + 7-H), 8.55 (d, *J* = 8.4 Hz, 1H, 9-H). ¹³C NMR (100 MHz, CDCl₃) δ 34.1, 38.5, 45.4, 115.3, 118.7, 123.2, 128.5, 129.4, 129.5, 129.9, 130.6, 134.6, 151.9, 176.6; HRMS (ESI+) *m/z* calc. for C₁₅H₁₉N₂O₄S [M+H]⁺ 323.1060 found: 323.1054.

4-(5-(Dimethylamino)naphthalene-1-sulfonamido)butanoic acid (214)



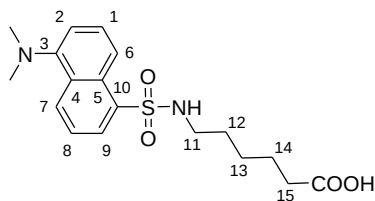
214 was prepared according to the general procedure above. 314 mg, 63%. ^1H NMR (400 MHz, CDCl_3) δ 1.68-1.80 (m, 2H, NCH_2CH_2), 2.32 (t, $J = 7.0$ Hz, 2H, CH_2COOH), 2.89 (s, 6H, NMe_2), 2.92-3.00 (m, 2H, CH_2N), 4.83 (br. s, 1H, NH), 7.19 (d, $J = 7.6$ Hz, 1H, 2-H), 7.47-7.60 (m, 2H, 1-H + 8-H), 8.20-8.30 (m, 2H, 6-H + 7-H), 8.54 (d, $J = 8.5$ Hz, 1H, 9-H). ^{13}C NMR (100 MHz, CDCl_3) δ 24.5, 30.7, 42.4, 45.4, 115.3, 118.7, 123.2, 128.4, 129.5, 129.6, 129.9, 130.5, 134.5, 151.9, 178.2; HRMS (ESI+) m/z calc. for $\text{C}_{16}\text{H}_{21}\text{N}_2\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 337.1217 found: 337.1213.

5-(5-(Dimethylamino)naphthalene-1-sulfonamido)pentanoic acid (215)



215 was prepared according to the general procedure above. 345 mg, 67%. ^1H NMR (400 MHz, CDCl_3) δ 1.40-1.60 (m, 4H, $\text{NCH}_2\text{CH}_2\text{CH}_2$), 2.22 (t, $J = 7.0$ Hz, 2H, CH_2COOH), 2.82-2.95 (m, 8H, $\text{CH}_2\text{N} + \text{NMe}_2$), 4.74 (br. s, 1H, NH), 7.19 (d, $J = 7.3$ Hz, 1H, 2-H), 7.49-7.60 (m, 2H, 1-H + 8-H), 8.24 (d, $J = 7.3$ Hz, 1H, 7-H), 8.27 (d, $J = 8.6$ Hz, 1H, 6-H), 8.54 (d, $J = 8.6$ Hz, 1H, 9-H). ^{13}C NMR (100 MHz, CDCl_3) δ 21.5, 28.9, 33.3, 42.7, 45.4, 115.2, 118.7, 123.1, 128.3, 129.5, 129.6, 129.8, 130.3, 134.7, 151.9, 178.0; HRMS (ESI+) m/z calc. for $\text{C}_{17}\text{H}_{23}\text{N}_2\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 351.1373 found: 351.1374.

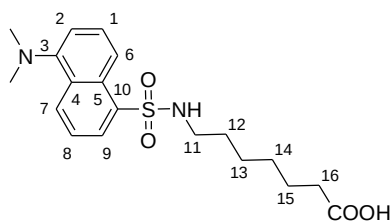
6-(5-(Dimethylamino)naphthalene-1-sulfonamido)hexanoic acid (216)



216 was prepared according to the general procedure above. 314 mg, 58%. ^1H NMR (400 MHz, CDCl_3) δ 1.14-1.27 (m, 2H, 13- H_2), 1.33-1.50 (m, 4H, 12- H_2 +

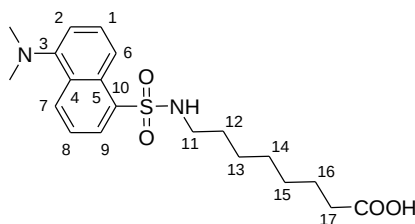
14-H₂), 2.19 (t, $J = 7.3$ Hz, 2H, 15-H₂), 2.83-2.95 (m, 8H, CH₂N + NMe₂), 4.68 (br. s, 1H, NH), 7.19 (d, $J = 7.5$ Hz, 1H, 2-H), 7.48-7.60 (m, 2H, 1H + 8-H), 8.24 (d, $J = 7.3$ Hz, 1H, 7-H), 8.28 (d, $J = 8.7$ Hz, 1H, 6-H), 8.54 (d, $J = 8.6$ Hz, 1H, 9-H). ¹³C NMR (100 MHz, CDCl₃) δ 23.8, 25.7, 29.1, 33.6, 42.9, 45.4, 115.2, 118.7, 123.2, 128.3, 129.6 (2C), 129.8, 130.4, 134.7, 151.9, 179.1; HRMS (ESI+) m/z calc. for C₁₈H₂₅N₂O₄S [M+H]⁺ 365.1530 found: 365.1526.

7-(5-(Dimethylamino)naphthalene-1-sulfonamido)heptanoic acid (217)



217 was prepared according to the general procedure above. 457 mg, 81%. ¹H NMR (400 MHz, CDCl₃) δ 1.10-1.21 (m, 4H, 13-H₂ + 14-H₂), 1.32-1.42 (m, 2H, 15-H₂), 1.42-1.52 (m, 2H, 12-H₂), 2.23 (t, $J = 7.5$ Hz, 2H, CH₂COOH), 2.84-2.93 (m, 8H, 11-H₂), 4.66 (t, $J = 6.1$ Hz, 1H, NH), 7.19 (d, $J = 7.3$ Hz, 1H, 2-H), 7.48-7.60 (m, 2H, 1-H + 8-H), 8.25 (d, $J = 7.3$ Hz, 1H, 7-H), 8.28 (d, $J = 8.7$ Hz, 1H, 6-H), 8.54 (d, $J = 8.5$ Hz, 1H, 9H). ¹³C NMR (100 MHz, CDCl₃) δ 24.3, 25.9, 28.3, 29.2, 33.7, 43.1, 45.4, 115.2, 118.7, 123.2, 128.3, 129.6 (2C), 129.9, 130.4, 134.7, 152.0, 179.1; HRMS (ESI+) m/z calc. for C₁₉H₂₇N₂O₄S [M+H]⁺ 379.1686 found: 379.1684.

8-(5-(Dimethylamino)naphthalene-1-sulfonamido)octanoic acid (218)



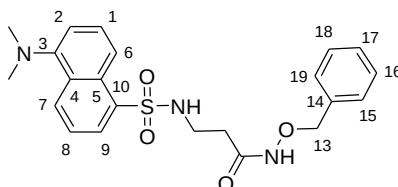
218 was prepared according to the general procedure above. 493 mg, 85%. ¹H NMR (400 MHz, CDCl₃) δ 1.04-1.20 (m, 6H, 13-H₂ + 14-H₂ + 15-H₂), 1.28-1.39 (m, 2H, 16-H₂), 1.45-1.56 (m, 2H, 12-H₂), 2.28 (t, $J = 7.4$ Hz, 2H, 17-H₂), 2.84-2.93 (m, 8H, NMe₂ + 11-H₂), 4.64 (t, $J = 6.0$ Hz, 1H, NH), 7.19 (d, $J = 7.5$ Hz, 1H, 2-H), 7.48-7.60 (m, 2H, 1-H + 8-H), 8.25 (d, $J = 7.3$ Hz, 1H, 7-H), 8.29 (d, $J = 8.7$ Hz, 1H, 6-H), 8.54 (d, $J = 8.5$ Hz, 1H, 9-H). ¹³C NMR (100 MHz, CDCl₃) δ

24.4, 26.1, 28.5, 28.7, 29.3, 33.8, 43.2, 45.4, 115.2, 118.7, 123.2, 128.3, 129.6 (2C), 129.8, 130.4, 134.8, 151.9, 179.3; HRMS (ESI+) m/z calc. for $C_{20}H_{29}N_2O_4S$ $[M+H]^+$ 393.1843 found: 393.1843.

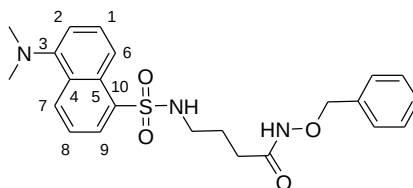
Coupling with benzylhydroxylamine, general procedure:

To a solution of the appropriate acid (1eq.) in freshly distilled CH_2Cl_2 (17 mL/mmol), at rt and under an inert atmosphere (argon) were added successively HOBt (1.1 eq.) and EDCI (1 eq.). After 30 minutes stirring, benzylhydroxylamine hydrochloride (1.1 eq.) and diisopropylethylamine (1.1 eq.) were added, and the reaction was followed by TLC (PE₄₀₋₆₀/acetone). After 24h, the organic phase was washed with water (17 mL/mmol). The aqueous phase was extracted with CH_2Cl_2 (1 x 17 mL/mmol). The combined organic layers were dried (MgSO₄) and concentrated. The coupling product was purified by flash column chromatography (PE₄₀₋₆₀/acetone) and obtained as a light green sticky oil.

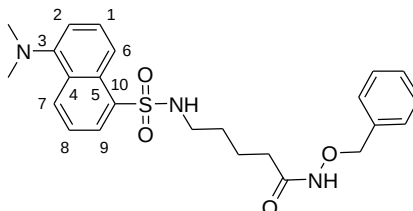
***N*-(Benzyloxy)-3-(5-(dimethylamino)naphthalene-1-sulfonamido)propanamide (219)**



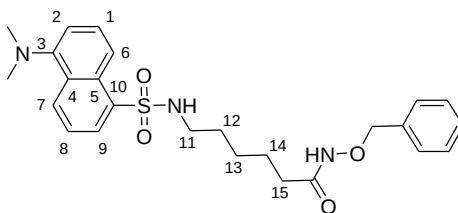
219 was prepared according to the general procedure above. 73mg, 85%. ^1H NMR (400 MHz, d^6 -DMSO) δ 2.12 (t, J = 7.3 Hz, 2H, $CH_2C(O)$), 2.82 (s, 6H, NMe_2), 2.91-3.02 (m, 2H, CH_2N), 4.68 (s, 2H, OCH_2), 7.25 (d, J = 7.4 Hz, 1H, 2-H), 7.28-7.40 (m, 5H, OCH_2ArH), 7.54-7.68 (m, 2H, 1-H + 8-H), 8.01 (t, J = 5.5 Hz, 1H, SNH), 8.11 (d, J = 7.3 Hz, 1H, 7-H), 8.27 (d, J = 8.7 Hz, 1H, 6-H), 8.47 (d, J = 8.5 Hz, 1H, 9-H), 11.01 (s, 1H- $C(O)NH$). ^{13}C NMR (100 MHz, d^6 -DMSO) δ 32.8, 39.0, 45.0, 76.7, 115.1, 119.0, 123.5, 127.8, 128.1, 128.2 (2C), 128.7, 129.0 (2C), 129.4, 135.6, 135.8, 151.3, 166.7. HRMS (ESI+) m/z calc. for $C_{22}H_{26}N_3O_4S$ $[M+H]^+$ 428.1639 found: 428.1642.

***N*-(Benzyloxy)-4-(5-(dimethylamino)naphthalene-1-sulfonamido)butanamide (220)**

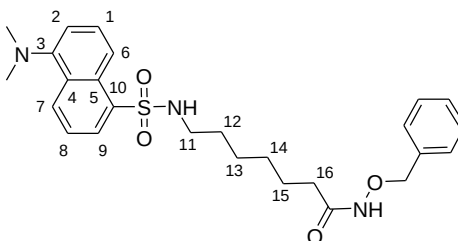
220 was prepared according to the general procedure above. 70 mg, 48%. ^1H NMR (400 MHz, d^6 -DMSO) δ 1.45-1.64 (m, 2H, NCH_2CH_2), 1.91 (t, $J = 7.3$ Hz, 2H, $\text{CH}_2\text{C}(\text{O})$), 2.70-2.78 (m, 2H, CH_2N), 2.82 (s, 6H, NMe_2), 4.70 (s, 2H, CH_2O), 7.26 (d, $J = 7.5$ Hz, 1H, 2-H), 7.28-7.39 (m, 5H, OCH_2ArH), 7.55-7.66 (m, 2H, 1-H + 8-H), 7.91 (t, $J = 5.8$ Hz, 1H, SNH), 8.08 (d, $J = 7.3$ Hz, 1H, 7-H), 8.29 (d, $J = 8.7$ Hz, 1H, 6-H), 8.45 (d, $J = 8.5$ Hz, 1H, 9-H), 10.88 (s, 1H, $\text{C}(\text{O})\text{NH}$). ^{13}C NMR (100 MHz, d^6 -DMSO) δ 25.2, 29.3, 41.9, 45.0, 76.6, 115.0, 119.0, 123.5, 127.7, 128.1 (2C), 128.2, 128.7, 129.0 (2C), 129.3, 135.8, 135.9, 151.3, 168.7. HRMS (ESI+) m/z calc. for $\text{C}_{23}\text{H}_{28}\text{N}_3\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 442.1795 found: 442.1807.

***N*-(Benzyloxy)-5-(5-(dimethylamino)naphthalene-1-sulfonamido)pentanamide (221)**

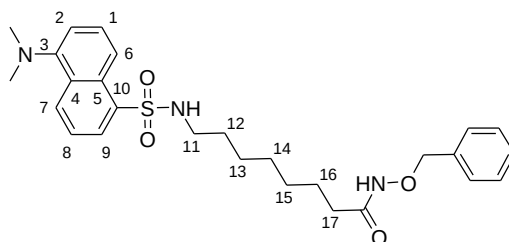
221 was prepared according to the general procedure above. 140 mg, 72%. ^1H NMR (400 MHz, d^6 -DMSO) δ 1.25-1.46 (m, 4H, $\text{NCH}_2\text{CH}_2\text{CH}_2$), 1.83 (t, $J = 7.3$ Hz, 2H, $\text{CH}_2\text{C}(\text{O})$), 2.70-2.79 (m, 2H, CH_2N), 2.82 (s, 6H, NMe_2), 4.73 (s, 2H, CH_2O), 7.25 (d, $J = 7.5$ Hz, 1H, 2-H), 7.29-7.39 (m, 5H, OCH_2ArH), 7.55-7.64 (m, 2H, 1-H + 8-H), 7.87 (t, $J = 5.8$ Hz, 1H, SNH), 8.08 (d, $J = 7.3$ Hz, 1H, 7-H), 8.29 (d, $J = 8.7$ Hz, 1H, 6-H), 8.45 (d, $J = 8.5$ Hz, 1H, 9-H), 10.87 (s, 1H, $\text{C}(\text{O})\text{NH}$). ^{13}C NMR (100 MHz, d^6 -DMSO) δ 21.9, 28.6, 31.5, 42.0, 45.0, 76.6, 115.0, 119.0, 123.5, 127.7, 128.0, 128.1, 128.2, 128.6, 129.0 (2C), 129.2, 135.9, 136.0, 151.2, 168.9. HRMS (ESI+) m/z calc. for $\text{C}_{24}\text{H}_{30}\text{N}_3\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 456.1952 found: 456.1972.

N-(Benzyloxy)-6-(5-(dimethylamino)naphthalene-1-sulfonamido)hexanamide (222)

222 was prepared according to the general procedure above. 178 mg, 92%. ^1H NMR (400 MHz, d^6 -DMSO) δ 1.02-1.12 (m, 2H, 13- H_2), 1.22-1.35 (m, 4H, 12- H_2 + 14- H_2), 1.80 (t, $J = 7.4$ Hz, 2H, 15- H_2), 2.70-2.77 (m, 2H, 11- H_2), 2.82 (s, 6H, NMe_2), 4.74 (s, 2H, CH_2O), 7.25 (d, $J = 7.4$ Hz, 1H, 2-H), 7.28-7.39 (m, 5H, OCH_2ArH), 7.55-7.64 (m, 2H, 1-H + 8-H), 7.85 (t, $J = 5.7$ Hz, 1H, SNH), 8.09 (d, $J = 7.3$ Hz, 1H, 7-H), 8.30 (d, $J = 8.7$ Hz, 1H, 6-H), 8.45 (d, $J = 8.5$ Hz, 1H, 9-H), 10.86 (s, 1H, $\text{C}(\text{O})\text{NH}$). ^{13}C NMR (100 MHz, d^6 -DMSO) δ 24.3, 25.3, 28.7, 31.9, 42.2, 45.0, 76.6, 115.0, 119.1, 123.5, 127.6, 128.1 (3C), 128.6, 129.0 (2C), 129.2, 136.0, 136.1, 151.2, 169.1. HRMS (ESI+) m/z calc. for $\text{C}_{25}\text{H}_{32}\text{N}_3\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 470.2108 found: 470.2118

N-(Benzyloxy)-7-(5-(dimethylamino)naphthalene-1-sulfonamido)heptanamide (223)

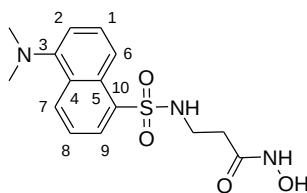
223 was prepared according to the general procedure above. 158 mg, 82%. ^1H NMR (400 MHz, d^6 -DMSO) δ 0.90-1.10 (m, 4H, 13- H_2 + 14- H_2), 1.18-1.35 (m, 4H, 12- H_2 + 15- H_2), 1.82 (t, $J = 7.3$ Hz, 2H, 16- H_2), 2.70-2.79 (m, 2H, 11- H_2), 2.82 (s, 6H, NMe_2), 4.74 (s, 2H, CH_2O), 7.25 (d, $J = 7.6$ Hz, 1H, 2-H), 7.28-7.39 (m, 5H, OCH_2ArH), 7.55-7.64 (m, 2H, 1-H + 8-H), 7.87 (t, $J = 5.6$ Hz, 1H, SNH), 8.09 (d, $J = 7.3$ Hz, 1H, 7-H), 8.30 (d, $J = 8.7$ Hz, 1H, 6-H), 8.45 (d, $J = 8.5$ Hz, 1H, 9-H), 10.88 (s, 1H, $\text{C}(\text{O})\text{NH}$). ^{13}C NMR (100 MHz, d^6 -DMSO) δ 24.6, 25.5, 27.8, 28.8, 32.0, 42.2, 45.0, 76.6, 115.0, 119.1, 123.5, 127.7, 128.1 (2C), 128.2, 128.7, 129.0 (2C), 129.2, 136.0, 136.1, 151.3, 169.2. HRMS (ESI+) m/z calc. for $\text{C}_{26}\text{H}_{34}\text{N}_3\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 484.2265 found: 484.2272.

N-(Benzyloxy)-8-(5-(dimethylamino)naphthalene-1-sulfonamido)octanamide (224)

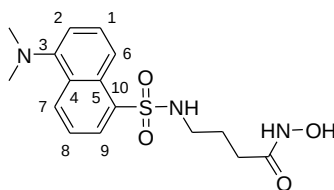
224 was prepared according to the general procedure above. 148 mg, 78%. ^1H NMR (400 MHz, d^6 -DMSO) δ 0.92-1.10 (m, 6H, 13- H_2 + 14- H_2 + 15 H_2), 1.19-1.29 (m, 2H, 16- H_2), 1.29-1.39 (m, 2H, 12- H_2), 1.86 (t, $J = 7.3$ Hz, 2H, 17- H_2), 2.72-2.80 (m, 2H, CH_2N), 2.82 (s, 6H, NMe_2), 4.76 (s, 2H, CH_2O), 7.25 (d, $J = 7.6$ Hz, 1H, 2-H), 7.30-7.39 (m, 5H, OCH_2ArH), 7.55-7.64 (m, 2H, 1-H + 8-H), 7.84 (t, $J = 5.6$ Hz, 1H, SNH), 8.09 (d, $J = 7.3$ Hz, 1H, 7-H), 8.30 (d, $J = 8.7$ Hz, 1H, 6-H), 8.45 (d, $J = 8.5$ Hz, 1H, 9-H), 10.88 (s, 1H, $\text{C}(\text{O})\text{NH}$). ^{13}C NMR (100 MHz, d^6 -DMSO) δ 24.6, 25.6, 28.0, 28.1, 28.9, 32.0, 42.2, 45.0, 76.6, 115.0, 119.1, 123.5, 127.6, 128.1 (2C), 128.2, 128.6, 129.0 (2C), 129.2, 136.0, 136.1, 151.2, 169.2. HRMS (ESI+) m/z calc. for $\text{C}_{27}\text{H}_{36}\text{N}_3\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 498.2421 found: 498.2431.

Benzyl deprotection, general procedure:

To a solution of the appropriate *O*-benzyl protected hydroxamic acid (1 eq.) under an inert atmosphere (argon) were successively added 10% palladium on charcoal (0.1 eq.) and anhydrous methanol (25 mL/mmol) at rt. The solution was saturated with hydrogen by bubbleling for a few minutes while stirring. The system was then closed and kept under H₂. The resulting mixture was stirred at rt and followed by TLC (CH₂Cl₂/MeOH). After 3h, the reaction mixture was filtered through a pad of celite. The latter was washed several times with MeOH. The filtrate was concentrated and the product was purified by flash column chromatography (CH₂Cl₂/MeOH) and obtained as a brown solid.

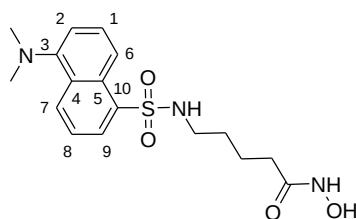
3-(5-(Dimethylamino)naphthalene-1-sulfonamido)-*N*-hydroxypropanamide (225)

225 was prepared according to the general procedure above. 41 mg, 83%. ¹H NMR (400 MHz, *d*⁶-DMSO) δ 2.11 (t, *J* = 7.4 Hz, 2H, CH₂C(O)), 2.83 (s, 6H, NMe₂), 2.90-3.02 (m, 2H, CH₂N), 7.26 (d, *J* = 7.5 Hz, 1H, 2-H), 7.52-7.70 (m, 2H, 1-H + 8-H), 7.96 (t, *J* = 5.8 Hz, 1H, SNH), 8.10 (d, *J* = 7.2 Hz, 1H, 7-H), 8.27 (d, *J* = 8.6 Hz, 1H, 6-H), 8.47 (d, *J* = 8.5 Hz, 1H, 9-H), 8.71 (br. s, 1H, OH), 10.40 (br. s, 1H, C(O)NH). ¹³C NMR (100 MHz, *d*⁶-DMSO) δ 32.8, 39.0, 45.0, 115.1, 119.0, 123.5, 127.8, 128.2, 128.9, 129.0, 129.3, 135.6, 151.3, 166.4. HRMS (ESI+) *m/z* calc. for C₁₅H₂₀N₃O₄S [M+H]⁺ 338.1169 found: 338.1173.

4-(5-(Dimethylamino)naphthalene-1-sulfonamido)-*N*-hydroxybutanamide (226)

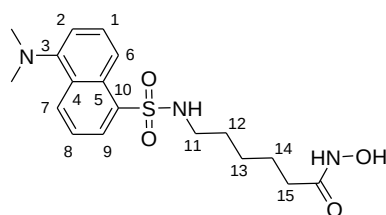
226 was prepared according to the general procedure above. 32 mg, 67%. ^1H NMR (400 MHz, d^6 -DMSO) δ 1.50-1.62 (m, 2H, NCH_2CH_2), 1.89 (t, $J = 7.3$ Hz, 2H, $\text{CH}_2\text{C}(\text{O})$), 2.70-2.80 (m, 2H, CH_2N), 2.83 (s, 6H, NMe_2), 7.26 (d, $J = 7.5$ Hz, 1H, 2-H), 7.54-7.66 (m, 2H, 1-H + 8-H), 7.92 (br. s, 1H, SNH), 8.08 (d, $J = 7.3$ Hz, 1H, 7-H), 8.28 (d, $J = 8.6$ Hz, 1H, 6-H), 8.46 (d, $J = 8.5$ Hz, 1H, 9-H), 8.65 (br. s, 1H, OH), 10.28 (br. s, 1H, $\text{C}(\text{O})\text{NH}$). ^{13}C NMR (100 MHz, CDCl_3) δ 25.0, 29.6, 42.4, 45.4, 115.3, 118.5, 123.2, 128.6, 129.5, 129.6, 129.9, 130.7, 134.3, 152.1, 170.7. HRMS (ESI+) m/z calc. for $\text{C}_{16}\text{H}_{22}\text{N}_3\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 352.1326 found: 352.1333.

5-(5-(Dimethylamino)naphthalene-1-sulfonamido)-N-hydroxypentanamide (227)



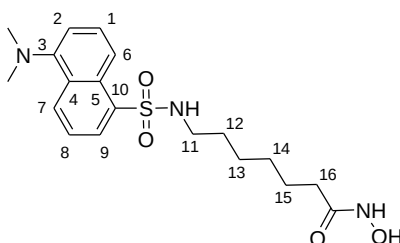
227 was prepared according to the general procedure above. 66 mg, 69%. ^1H NMR (400 MHz, d^6 -DMSO) δ 1.20-1.48 (m, 4H, $\text{NCH}_2\text{CH}_2\text{CH}_2$), 1.83 (t, $J = 7.2$ Hz, 2H, $\text{CH}_2\text{C}(\text{O})$), 2.70-2.80 (m, 2H, CH_2N), 2.83 (s, 6H, NMe_2), 7.25 (d, $J = 7.5$ Hz, 1H, 2-H), 7.52-7.68 (m, 2H, 1-H + 8-H), 7.88 (br. s, 1H, SNH), 8.08 (d, $J = 7.0$ Hz, 1H, 7-H), 8.29 (d, $J = 8.7$ Hz, 1H, 6-H), 8.45 (d, $J = 8.5$ Hz, 1H, 9-H), 8.64 (br. s, 1H, OH), 10.28 (br. s, 1H, $\text{C}(\text{O})\text{NH}$). ^{13}C NMR (100 MHz, CDCl_3) δ 22.0, 28.7, 31.9, 42.6, 45.4, 115.3, 118.8, 123.2, 128.4, 129.5, 129.6, 129.9, 130.4, 134.6, 151.9, 171.2. HRMS (ESI+) m/z calc. for $\text{C}_{17}\text{H}_{24}\text{N}_3\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 366.1482 found: 366.1494.

6-(5-(Dimethylamino)naphthalene-1-sulfonamido)-N-hydroxyhexanamide (228)



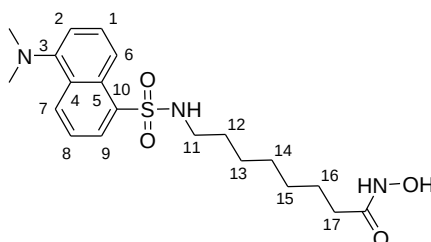
228 was prepared according to the general procedure above. 73 mg, 60%. ^1H NMR (400 MHz, d^6 -DMSO) δ 1-1.18 (m, 2H, 13- H_2), 1.20-1.40 (m, 4H, 12- H_2 + 14- H_2), 1.79 (t, $J = 7.3$ Hz, 2H, 15- H_2), 2.68-2.94 (m, 8H, NMe_2 + 11- H_2), 7.25 (d, $J = 7.5$ Hz, 1H, 2-H), 7.52-7.70 (m, 2H, 1-H + 8-H), 7.86 (t, $J = 5.6$ Hz, 1H, SNH), 8.09 (d, $J = 7.1$ Hz, 1H, 7-H), 8.29 (d, $J = 8.6$ Hz, 1H, 6-H), 8.45 (d, $J = 8.4$ Hz, 1H, 9-H), 8.61 (br. s, 1H, OH), 10.27 (br. s, 1H, C(O)NH). ^{13}C NMR (100 MHz, CDCl_3) δ 24.0, 25.3, 28.7, 32.2, 42.6, 45.5, 115.3, 118.9, 123.3, 128.5, 129.6, 129.7, 129.8, 130.4, 134.7, 151.8, 170.8. HRMS (ESI+) m/z calc. for $\text{C}_{18}\text{H}_{26}\text{N}_3\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 380.1639 found: 380.1644.

7-(5-(Dimethylamino)naphthalene-1-sulfonamido)-N-hydroxyheptanamide (229)



229 was prepared according to the general procedure above. 97 mg, 85%. (400 MHz, d^6 -DMSO) δ 0.92-1.14 (m, 4H, 13- H_2 + 14- H_2), 1.18-1.36 (m, 4H, 12- H_2 + 15- H_2), 1.82 (t, $J = 7.3$ Hz, 2H, 16- H_2), 2.69-2.80 (m, 2H, 11- H_2), 2.83 (m, 6H, NMe_2), 7.25 (d, $J = 7.5$ Hz, 1H, 2-H), 7.53-7.67 (m, 2H, 1-H + 8-H), 7.85 (t, $J = 5.5$ Hz, 1H, SNH), 8.09 (d, $J = 6.7$ Hz, 1H, 7-H), 8.30 (d, $J = 8.6$ Hz, 1H, 6-H), 8.45 (d, $J = 8.5$ Hz, 1H, 9-H), 8.61 (br. s, 1H, OH), 10.26 (br. s, 1H, C(O)NH). ^{13}C NMR (100 MHz, CDCl_3) δ 24.8, 25.5, 28.0, 29.0, 32.4, 43.0, 45.4, 115.2, 118.9, 123.2, 128.4, 129.4, 129.6, 129.8, 130.3, 134.9, 151.8, 171.6. HRMS (ESI+) m/z calc. for $\text{C}_{19}\text{H}_{28}\text{N}_3\text{O}_4\text{S}$ $[\text{M}+\text{H}]^+$ 394.1795 found: 394.1801.

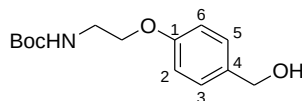
8-(5-(Dimethylamino)naphthalene-1-sulfonamido)-N-hydroxyoctanamide (230)



230 was prepared according to the general procedure above. 60 mg, 57%. ^1H NMR (400 MHz, d^6 -DMSO) 0.92-1.11 (m, 6H, 13-H₂ + 14-H₂ + 15-H₂), 1.17-1.44 (m, 4H, 12-H₂ + 16-H₂), 1.85 (t, J = 7.3 Hz, 2H, 17-H₂), 2.70-2.90 (m, 8H, NMe₂ + 11-H₂), 7.25 (d, J = 7.5 Hz, 1H, 2-H), 7.53-7.68 (m, 2H, 1-H + 8-H), 7.85 (t, J = 5.7 Hz, 1H, SNH), 8.09 (d, J = 7.3 Hz, 1H, 7-H), 8.30 (d, J = 8.6 Hz, 1H, 6-H), 8.45 (d, J = 8.4 Hz, 1H, 9-H), 8.63 (br. s, 1H, OH), 10.28 (br. s, 1H, C(O)NH). δ ^{13}C NMR (100 MHz, CDCl₃) δ 24.8, 25.7, 28.0, 28.4, 29.1, 32.6, 43.0, 45.4, 115.2, 118.9, 123.3, 128.4, 129.6, 129.7, 129.8, 130.3, 134.8, 151.8, 171.1. HRMS (ESI+) m/z calc. for C₂₀H₃₀N₃O₄S [M+H]⁺ 408.1952 found: 408.1961.

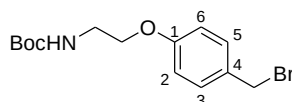
6. Chemoproteomics of HDAC8

Tert-butyl 2-(4-(hydroxymethyl)phenoxy)ethylcarbamate (**236**)



To a cooled (-20°C) solution of methyl 4-(2-(tert-butoxycarbonylamino)ethoxy)benzoate **235**²⁴⁰ (7.0g, 23.7 mmol, 1 eq.) in freshly distilled THF (350 mL) was added dropwise over 10 minutes LiAlH_4 (2M in THF, 13.6 mL, 27.3 mmol, 1.15 eq.). The resulting solution was then stirred at 0°C for 2 hours. The reaction was then cooled to -10°C and were added dropwise water (1.05 mL), 10% NaOH (2.1 mL) and water (3.15 mL). After stirring for another 10 minutes at 0°C , the precipitate was removed by filtration. The filtrate was then concentrated and the product (6.02 g, 95%) was obtained after flash column chromatography (AcOEt/PE₄₀₋₆₀ 1 : 1). R_f 0.5 (AcOEt/PE₄₀₋₆₀ 1 : 1); IR (neat) 3393, 1669 1695, 1518,1251; ^1H NMR (400 MHz, CDCl_3) δ 1.45 (s, 9H, $\text{C}(\text{CH}_3)_3$), 1.87 (br. s, 1H, OH), 3.51 (m, 2H, CH_2N), 4.00 (t, $J = 5.1$ Hz, 2H, CH_2O), 4.61 (s, 2H, ArCH_2), 5.02 (br. s, 1H, NH), 6.87 (m, 2H, 2-H + 6-H), 7.28 (m, 2H, 3-H + 5-H). ^{13}C NMR (100 MHz, CDCl_3) δ 28.4, 40.1, 64.9, 67.2, 79.5, 114.5, 128.6, 133.6, 155.9, 158.1; HRMS (CI⁺) m/z calc. for $\text{C}_{14}\text{H}_{22}\text{NO}_4$ $[\text{M}+\text{H}]^+$ 268.1543, found: 268.1544; Anal. Calc. for $\text{C}_{14}\text{H}_{21}\text{NO}_4$: C, 62.90; H, 7.92; N, 5.24. Found: C, 63.00; H, 7.85; N, 5.15.

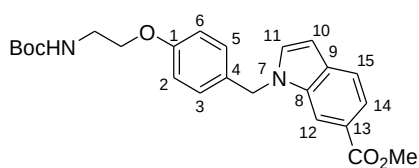
Tert-butyl 2-(4-(bromomethyl)phenoxy)ethylcarbamate (**237**)



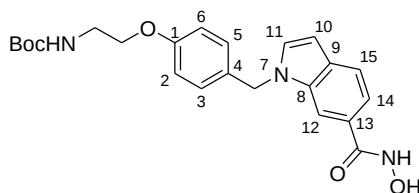
To a solution of alcohol **236** (3.0g, 11.2 mmol, 1 eq.) and CBr_4 (4.84g, 14.6 mmol, 1.3 eq.) in freshly distilled CH_2Cl_2 (85 mL) was added triphenylphosphine (3.83g, 14.6 mmol, 1.3 eq.) by portions at 0°C . The resulting mixture was then stirred at rt and the progress of the reaction was followed by TLC. After complete conversion, the solvent was evaporated under reduced pressure and the product (3.08g, 83%) was obtained after flash column chromatography (AcOEt/PE₄₀₋₆₀ 2 : 8). R_f 0.7 (AcOEt/PE₄₀₋₆₀ 3 : 7); IR (neat) 3364, 1679, 1513; ^1H NMR (400 MHz, CDCl_3) δ 1.45 (s, 9H, $\text{C}(\text{CH}_3)_3$), 3.52 (*app*-q, $J = 5.1$ Hz, 2H, CH_2N), 4.01

(t, $J = 5.1$ Hz, 2H, CH₂O), 4.49 (s, 2H, ArCH₂), 5.00 (br. s, 1H, NH), 6.85 (m, 2H, 2-H + 6-H), 7.31 (m, 2H, 3-H + 5-H). ¹³C NMR (100 MHz, CDCl₃) δ 28.3, 33.7, 40.0, 67.2, 79.5, 114.7, 130.3, 130.4, 155.8, 158.6; HRMS (CI+) m/z calc. for C₁₄H₂₁BrNO₃ [M+H]⁺ 330.0699, found: 330.0707; Anal. Calc. for C₁₄H₂₀BrNO₃: C, 50.92; H, 6.10; N, 4.24. Found: C, 50.83; H, 6.05; N, 4.18.

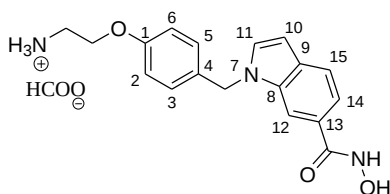
Methyl 1-(4-(2-(tert-butoxycarbonylamino)ethoxy)benzyl)-1H-indole-6-carboxylate (239)



To a solution of commercial methyl 1H-indole-6-carboxylate **238** (1.40g, 7.98 mmol, 1 eq.) in anhydrous DMF (40 mL) was added NaH (60% dispersion in mineral oil, 351 mg, 8.78 mmol, 1.1 eq.) at 0°C. After stirring at 0°C for 20 minutes, bromide **237** (2.90g, 8.78 mmol, 1.1 eq.) was added in one portion. The resulting mixture was stirred at 0°C for 30 minutes, then at rt for 5 hours. The reaction mixture was then cooled to 0°C and water (2 mL) was added dropwise, followed by sat. aq. NH₄Cl (2mL). The reaction was diluted with AcOEt (400 mL), washed with a 1 : 1 water/brine solution (2 x 200 mL), dried (MgSO₄) and concentrated. The product (2.70g, 80%) was obtained after purification by flash column chromatography (AcOEt/PE₄₀₋₆₀ 2 : 8). R_f 0.6 (AcOEt/PE₄₀₋₆₀ 35 : 65); IR (neat) 3373, 1702, 1511, 1274, 1231, 1166; ¹H NMR (400 MHz, CDCl₃) δ 1.44 (s, 9H, C(CH₃)₃), 3.50 (m, 2H, CH₂NBoc), 3.92 (s, 3H, CH₃O), 3.97 (t, $J = 5.1$ Hz, 2H, CH₂O), 4.98 (br. s, 1H, NH), 5.30 (s, 2H, ArCH₂), 6.57 (d, $J = 3.0$ Hz, 1H, 10-H), 6.82 (m, 2H, 2-H + 6-H), 7.06 (m, 2H, 3-H + 5-H), 7.24 (d, $J = 3.0$ Hz, 1H, 11-H), 7.65 (d, $J = 8.3$ Hz, 1H, 15-H), 7.80 (dd, $J = 8.3, 1.4$ Hz, 1H, 14-H), 8.10 (d, $J = 1.4$ Hz, 1H, 12-H). ¹³C NMR (100 MHz, CDCl₃) δ 28.3, 40.0, 49.5, 51.9, 67.1, 79.5, 102.0, 111.9, 114.7, 120.5, 120.6, 123.3, 128.3, 129.3, 131.2, 132.3, 135.6, 155.8, 158.2, 168.1; HRMS (ESI+) m/z calc. for C₂₄H₂₈N₂NaO₅ [M+Na]⁺ 447.1890, found: 447.1874; Anal. Calc. for C₂₄H₂₈N₂O₅: C, 67.91; H, 6.65; N, 6.60. Found: C, 68.03; H, 6.76; N, 6.60.

Tert-butyl 2-(4-((6-(hydroxycarbamoyl)-1H-indol-1-yl)methyl)phenoxy)ethylcarbamate

To a solution of ester **239** (2.2g, 5.18 mmol, 1 eq.) in a 1 : 1 THF/MeOH mixture (25 mL) was added aqueous hydroxylamine (50% w/w, 30 mL) and sodium hydroxide (828 mg, 20.7 mmol, 4 eq.) at rt. After stirring for 8 hours at rt, the reaction mixture was diluted with water (30 mL). The volatiles were removed under reduced pressure. The pH was then adjusted to 6-7 using 1N HCl. The precipitated product that formed was isolated by filtration, washed 3 times with cold acidic (pH 5-6) water, and dried under vacuum. The crude product (2.35g) was used for the next step without further purification. ^1H NMR (400 MHz, d^6 -DMSO) δ 1.35 (s, 9H, $\text{C}(\text{CH}_3)_3$), 3.23 (m, 2H, CH_2NBoc), 3.89 (t, $J = 5.6$ Hz, 2H, CH_2O), 5.36 (s, 2H, ArCH_2), 6.49 (d, $J = 2.9$ Hz, 1H, 10-H), 6.86 (m, 2H, 2-H + 6-H), 6.98 (br. t, $J = 5.1$ Hz, 1H, NH), 7.15 (m, 2H, 3-H + 5-H), 7.44 (d, $J = 8.3$ Hz, 1H, 14-H), 7.53 (d, $J = 8.3$ Hz, 1H, 15-H), 7.59 (d, $J = 2.9$ Hz, 1H, 12-H), 7.92 (s, 1H). ^{13}C NMR (100 MHz, d^6 -DMSO) δ 28.1, 40.0, 48.5, 66.2, 77.7, 100.9, 108.9, 114.4, 117.7, 119.7, 126.6, 128.3, 129.8, 130.1, 130.9, 135.0, 155.6, 157.7, 164.9; HRMS (ESI+) m/z calc. for $\text{C}_{23}\text{H}_{26}\text{N}_3\text{O}_5$ $[\text{M}+\text{H}]^+$ 426.2023, found: 426.2028.

2-(4-((6-(Hydroxycarbamoyl)-1H-indol-1-yl)methyl)phenoxy)ethanaminium formate (232)

Crude boc protected compound **239** (600mg) was stirred at rt in 4N HCl/dioxane for 3 hours. The Solvent was then evaporated under reduced pressure and the product (270 mg, 55% over 2 steps) was purified by preparative reverse phase LC/MS (HCOOH). ^1H NMR (400 MHz, d^6 -DMSO) δ 3.00 (t, $J = 5.4$ Hz, 2H, NCH_2CH_2), 3.99 (t, $J = 5.4$ Hz, 2H, CH_2O), 5.38 (s, 2H, ArCH_2), 6.52 (d, $J = 3.1$

Hz, 1H, 10-H), 6.89 (m, 2H, 2-H + 6-H), 7.18 (m, 2H, 3-H + 5-H), 7.43 (dd, $J = 8.2, 1.3$ Hz, 1H, 14-H), 7.57 (d, $J = 8.2$ Hz, 1H, 15-H), 7.63 (d, $J = 3.1$ Hz, 1H, 11-H), 7.93 (d, $J = 1.3$ Hz, 1H, 12-H), 8.33 (s, 1H, HCOO). ^{13}C NMR (100 MHz, d^6 -DMSO) δ 39, 48.5, 66.2, 101.0, 109.3, 114.6, 117.7, 120.0, 125.5, 128.4, 130.3, 131.4, 134.9, 157.4, 165.0, 165.3; HRMS (ESI+) m/z calc. for $\text{C}_{18}\text{H}_{20}\text{N}_3\text{O}_3$ [free amine +H] $^+$ 326.1499, found: 326.1508.

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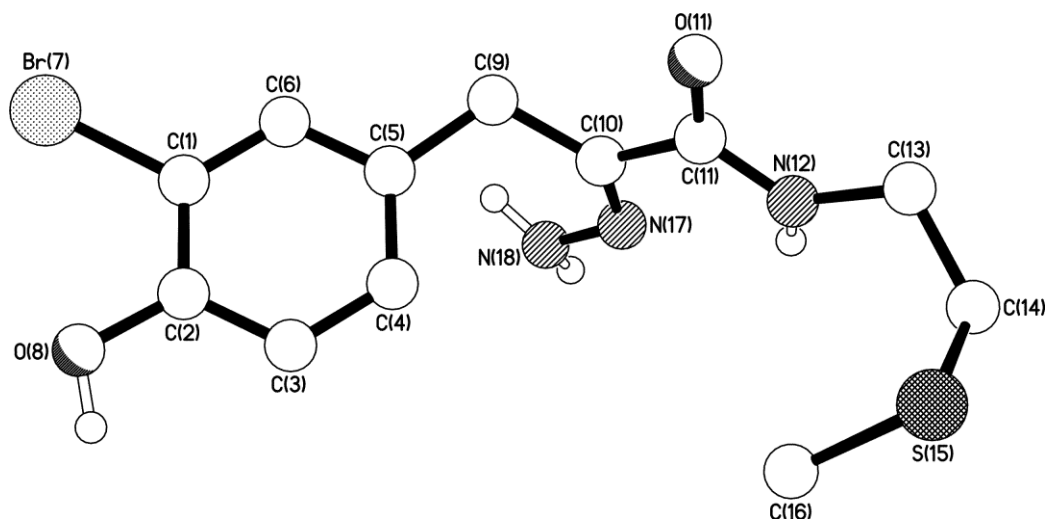
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APPENDICES

Appendix 1: Crystallographic data for compound **111a**.Table 1. Crystal data and structure refinement for **111a**.

Identification code	MF0904
Empirical formula	C ₁₂ H ₁₆ Br N ₃ O ₂ S
Formula weight	346.25
Temperature	173(2) K
Diffractometer, wavelength	OD Xcalibur 3, 0.71073 Å
Crystal system, space group	Monoclinic, P2(1)/c
Unit cell dimensions	a = 10.18411(14) Å α = 90° b = 14.96067(18) Å β = 99.2862(14)° c = 9.13052(13) Å γ = 90°
Volume, Z	1372.91(3) Å ³ , 4
Density (calculated)	1.675 Mg/m ³
Absorption coefficient	3.148 mm ⁻¹
F(000)	704
Crystal colour / morphology	Colourless platy needles
Crystal size	0.48 x 0.27 x 0.04 mm ³

θ range for data collection	3.10 to 32.71°
Index ranges	-14 $\leq h \leq$ 15, -22 $\leq k \leq$ 21, -13 $\leq l \leq$ 13
Reflns collected / unique	15014 / 4603 [R(int) = 0.0222]
Reflns observed [F $>4\sigma$ (F)]	3319
Absorption correction	Analytical
Max. and min. transmission	0.866 and 0.395
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	4603 / 4 / 188
Goodness-of-fit on F ²	0.927
Final R indices [F $>4\sigma$ (F)]	R1 = 0.0240, wR2 = 0.0518
R indices (all data)	R1 = 0.0401, wR2 = 0.0532
Largest diff. peak, hole	0.651, -0.783 eÅ ⁻³
Mean and maximum shift/error	0.000 and 0.001

Table 2. Bond lengths [Å] and angles [°] for **111a**.

C(1)-C(6)	1.3865(18)
C(1)-C(2)	1.3949(18)
C(1)-Br(7)	1.8876(13)
C(2)-O(8)	1.3626(15)
C(2)-C(3)	1.3909(18)
C(3)-C(4)	1.3901(18)
C(4)-C(5)	1.3884(18)
C(5)-C(6)	1.3938(18)
C(5)-C(9)	1.5185(17)
C(9)-C(10)	1.4960(18)
C(10)-N(17)	1.2897(17)
C(10)-C(11)	1.4993(18)
C(11)-O(11)	1.2453(16)

C (11) -N (12)	1.3310 (16)
N (12) -C (13)	1.4574 (17)
C (13) -C (14)	1.5166 (19)
C (14) -S (15)	1.8110 (15)
S (15) -C (16)	1.7977 (16)
N (17) -N (18)	1.3522 (17)

C (6) -C (1) -C (2)	120.91 (12)
C (6) -C (1) -Br (7)	119.49 (10)
C (2) -C (1) -Br (7)	119.59 (10)
O (8) -C (2) -C (3)	123.36 (12)
O (8) -C (2) -C (1)	118.64 (12)
C (3) -C (2) -C (1)	118.00 (12)
C (4) -C (3) -C (2)	120.95 (12)
C (5) -C (4) -C (3)	121.08 (12)
C (4) -C (5) -C (6)	117.99 (12)
C (4) -C (5) -C (9)	124.48 (12)
C (6) -C (5) -C (9)	117.52 (11)
C (1) -C (6) -C (5)	121.06 (12)
C (10) -C (9) -C (5)	116.57 (11)
N (17) -C (10) -C (9)	125.50 (12)
N (17) -C (10) -C (11)	115.04 (12)
C (9) -C (10) -C (11)	119.29 (11)
O (11) -C (11) -N (12)	123.40 (12)
O (11) -C (11) -C (10)	120.60 (12)
N (12) -C (11) -C (10)	115.99 (11)
C (11) -N (12) -C (13)	125.16 (12)
N (12) -C (13) -C (14)	112.18 (11)
C (13) -C (14) -S (15)	115.40 (10)
C (16) -S (15) -C (14)	103.22 (7)
C (10) -N (17) -N (18)	119.25 (12)