

THE DESIGN AND SYNTHESIS OF TWO-PHOTON EXCITABLE FLUORESCENT HISTONE DEACETYLASE INHIBITORS

by

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Degree

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ATTENSTATION OF AUTHORSHIP

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

LIST OF ABBREVIATIONS

δ (ppm)	Chemical shift
$^{\circ}\text{C}$	Degrees Celsius
1PE	One-proton excitation
2PE	Two-proton excitation
Ar	Aromatic
CTCL	Cutaneous T-cell lymphoma
d	Doublet
dba	Dibenzylideneacetone
DCM	Dichloromethane
dd	Doublet of doublets
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DMSO-d6	Deuterated dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDCI	N-3-Dimethylaminopropyl-N'-ethyl carbodiimide hydrochloride
EtOAc	Ethyl Acetate
Equiv	Equivalents
FDA	Food and Drug Administration
g	grams
h	hours
HAT	Histone acetyltransferase
HDAC	Histone deacetylase

HOBt	1-Hydroxybenzotriazole hydrate
HRMS	High-resolution mass spectrometry
i-PrOH	Isopropyl alcohol
IC50	Half maximal inhibitory concentration
ICT	Internal charge transfer
<i>J</i>	Coupling constant
<i>m/z</i>	Mass to charge ratio
MeCN	Acetonitrile
MeOH	Methanol
MHz	Megahertz
mmol	Millimoles
NAD	Nicotinamide adenine dinucleotide
NIR	Near infrared
NMR	Nuclear magnetic resonance
pH	Potential of hydrogen
ppm	Parts per million
PTCL	peripheral T-cell lymphoma
RT	Room temperature
SAHA	Suberoylanilide hydroxamic acid
SBD	4-sulfamonyl-7-aminobenzoxadiazole
t-BuOH	<i>tert</i> -butyl alcohol
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography

TSA	Trichostatin A
Xantphos	4,5-Bis(diphenylphosphino)-9,9-dimethylxanthene
ZBG	Zinc binding group

ABSTRACT

This project focuses on the development of HDAC inhibitors that possess innate fluorescent properties. More specifically a potential fluorescent 6-amino-2,3-naphthalimide derived HDAC inhibitor has been synthesised, as a potential tool for cellular imaging studies. The abnormal regulation of HDAC activity has been linked to a number of diseases such as Alzheimer's, Parkinson's, cancer, and many more. HDAC inhibitors are able to restore the regulation of these enzymes to help treat these problems. The synthesis of the target molecule included four main reactions; a nucleophilic acyl substitution to install the imide, followed by an amination at the 6-position of the naphthalimide ring, then hydrolysis of the *tert*-butyl ester, and finally an amide coupling to install the zinc binding group. NMR spectroscopy and mass spectrometry analysis supported that the target molecule has successfully been synthesised. Upon optimisation of the methodology needed to access such compounds, we attempted to synthesise three derivatives of the target compound by changing the amino group at the 6-position. The target compound **43** and the derivative compound **64** were also analysed for their HDAC activity, compound **43** showed incredibly good selectivity for class I HDACs 1 and 3, and compound **64** wasn't as strong but still quite good results in terms of selectivity.

1. INTRODUCTION

Histone Deacetylase (HDAC) Enzymes - What do they do?

Gene expression and chromatin formation are governed by the opposing enzymatic activities of two histone modifying enzymes, histone deacetylase (HDAC) and histone acetyltransferase (HAT) enzymes.¹ HDACs catalyse the removal of acetyl groups on terminal lysine residues² present on histone and non-histone proteins (Figure 1). Deacetylation results in the tightening of the chromatin. This ‘condensed’ structure decreases the occurrence of gene transcription as transcription factors are unable to reach their promoter regions.¹ In contrast, HATs catalyse the acetylation of terminal lysine residues³ (Figure 1). The acetylation of the lysine residue prevents this from interacting with the negatively charged phosphate groups of DNA. As a result, the ‘condensed’ structure unwinds to afford a ‘relaxed’ structure, which then encourages gene transcription because transcription factors are able to bind to DNA.⁴

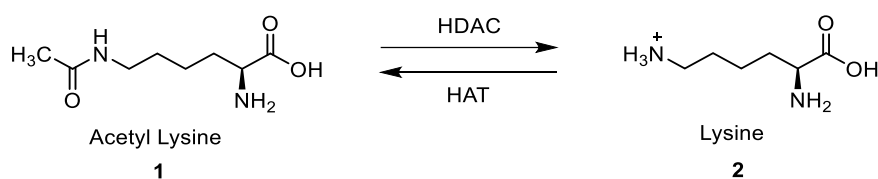


Figure 1. The acetylation and deacetylation of lysine

To date, there are 18 known HDAC enzymes in humans and these can be separated into two groups; zinc-dependant and nicotinamide adenine dinucleotide (NAD)-dependant HDACs.¹ The zinc-dependant HDACs can be further categorised into four classes; class I, class IIa, class IIb, and class IV¹ and are categorised by their similarity to yeast deacetylases.⁵ Class I HDACs are found in the nucleus⁶ and consist of HDAC1, 2, 3, and 8.⁷ Class II HDACs are further sub-divided into class IIa and class IIb. Class IIa includes HDAC4, 5, 7 and 9, while class IIb includes HDAC6 and 10.⁷ In contrast to the class I HDACs, class II HDACs are expressed in both the nucleus and the cytoplasm.⁸ Class IV consists only of HDAC11⁷, and is also found in the nucleus and cytoplasm.⁹

Class III HDACs are NAD-dependant⁷ consisting of sirtuins 1–7. The Class III HDAC isoforms localise in the cytoplasm, mitochondria, and nucleus.¹⁰ More specifically, SIRT1 and SIRT2 are found in the cytoplasm and nucleus, SIRT3 in the mitochondria and nucleus, SIRT4 and SIRT5 only in the mitochondria, SIRT6 exclusively in the nucleus, and SIRT7 in the nucleolus.⁶ As this project focusses on the development of potent HDAC inhibitors that target the zinc-dependent HDACs, class III will not be discussed further.

HDAC Inhibition

As HDACs play a critical role in a number of cellular processes such as regulation of gene transcription, cell growth, proliferation and survival¹¹, the abnormal regulation of HDAC activity has been linked to a number of diseases, including inflammatory and autoimmune diseases, cancer, and interstitial fibrosis.¹² As such, HDAC inhibition has received a significant amount of attention in the scientific community as potential therapeutic treatments for a number of diseases.

HDACs and Disease

Cancer

Tumorigenesis has been related back to abnormal protein acetylation, especially on histones, which HDAC enzymes can be responsible for.¹³ The over expression of HDAC enzymes has also been correlated to many types of cancer.¹⁴ For example, the abnormal regulation of HDAC1 has been reported in breast, hepatocellular, pancreatic, prostate, and lung cancers.¹⁵ Other cancers such as colorectal, gastric, and renal cell have been linked to over expression of HDAC1, 2 and 3.¹⁵ Squamous cell carcinoma has been related to high levels of HDAC6 and advanced stage cancers have been as well.¹⁵ Most HDACs can be linked to at least one type of cancer,¹⁵ so since HDAC inhibitors can help regulate HDAC activity there has been a growing interest in their development as anti-cancer agents.¹³ However, this is still not completely understood but there are ongoing studies and trials working towards finding more out about how we can regulate HDACs to treat cancer.¹⁵

Neurodegenerative disorders

HDAC enzymes are crucial for the regulation of neuronal morphology and brain homeostasis, however, if deregulation occurs it encourages neurodegeneration.¹⁶ In contrast to this, the regulation of DNA damage, metabolic dysfunction, neuronal apoptosis, and inflammatory response by HDACs can have neuroprotective effects as well.¹⁶ In normal conditions HDACs and HATs will be in equilibrium with each other, this homeostasis keeps gene expression regulated which in turn encourages functional neurophysiological components such as learning and memory.¹⁷ But when this balance is disrupted it can lead to neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, neuronal intranuclear inclusion disease as well as others.¹⁸

Inflammation

Inflammation is a very important immunity response that our bodies need, however deregulation of it can be quite harmful.¹⁴ Causing diseases such as asthma, arthritis, chronic obstructive pulmonary disease,¹⁹ inflammatory bowel disease,²⁰ and even cancer.²¹ HDAC inhibitors have been found to have

anti-inflammatory effects for some of these diseases, however, it's hard to know which HDACs are important because these inhibitors are pan inhibitors.¹⁹ This strikes a problem because they have the potential to bind to other enzymes, running the risk of unwanted side effects.¹⁹ Because of this these inhibitors are not likely to become usual treatment for inflammation in a medically practiced setting.¹⁹

HDAC Pharmacophore

The active site of the zinc-dependent HDAC enzymes consists of a narrow tunnel with a zinc ion (Zn^{2+}) at the bottom (Figure 2). As such, potent HDAC inhibitors typically consist of the typical HDAC pharmacophore which is comprised of 3 components:

- The **capping group** which interacts with the amino acid residues that are found at the active site entrance.
- The **linker** is the central part of the molecule, is also hydrophobic and will position itself in the hydrophobic tunnel of the HDAC catalytic domain⁶. This region needs to be a specific length to make sure both the capping group and zinc-binding group will be in their correct positions.⁶
- The **zinc-binding group** is on the other end of the molecule; it is bidentate and will interact with the zinc ion that's buried deep in the active site.⁶

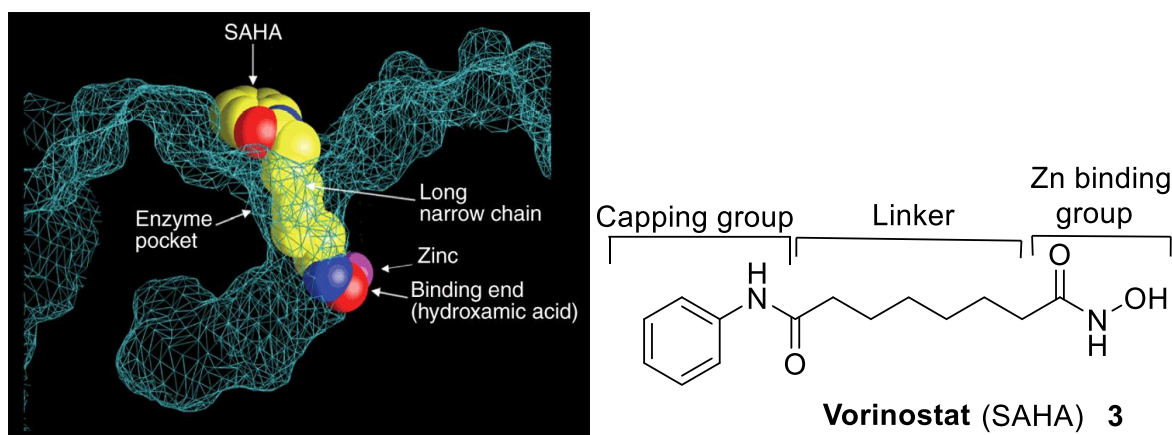


Figure 2. (Left) Active site of Zn dependant HDAC inhibited by SAHA. (Right) The HDAC pharmacophore illustrated using known HDAC inhibitor, SAHA.²²

Over the past 30 years, a large number of diverse and potent HDACs inhibitors consisting of a capping group, linker and zinc binding group, have been reported in the literature. To date, 15 HDAC inhibitors have been used in preclinical and clinical studies, four of which have been Food and Drug

Administration (FDA) approved to be used in the treatment of T-cell lymphoma and myeloma.^{4, 23} These are Vorinostat, Panobinostat, Romidepsin, and Belinostat (Figure 3).⁴

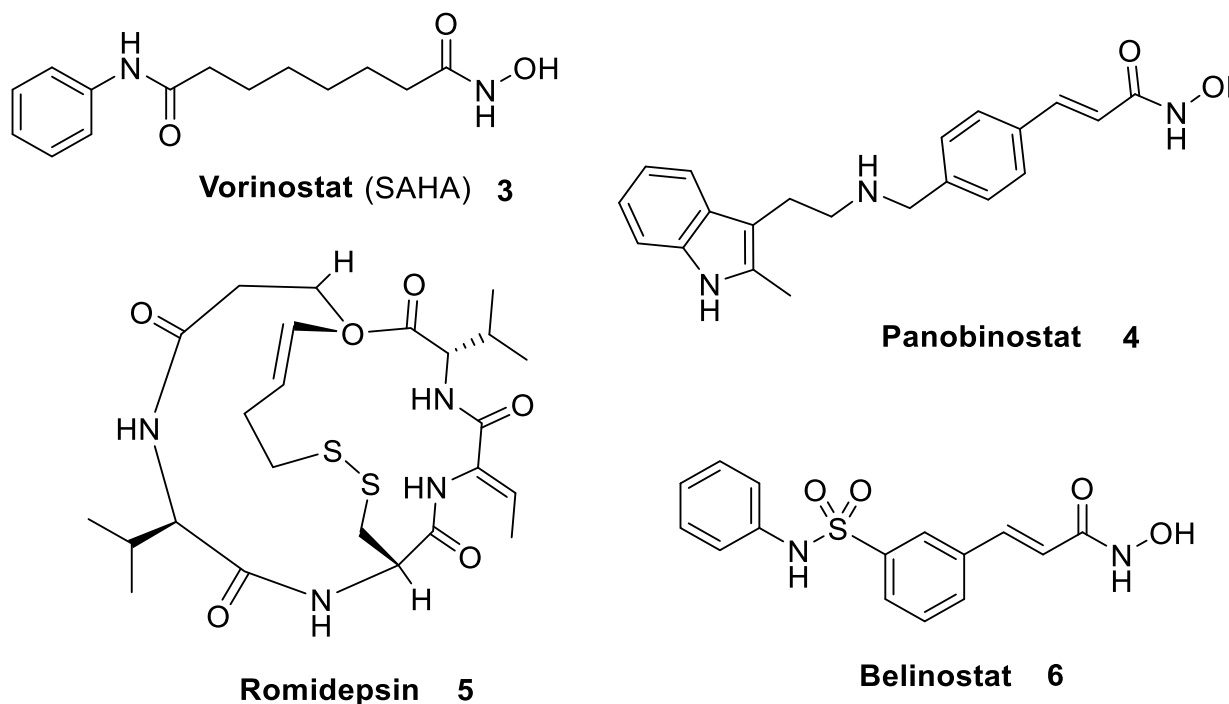


Figure 3. Structure of known HDAC inhibitors Vorinostat, Panobinostat, Romidepsin, and Belinostat.

Zinc Binding Groups

Hydroxamic acids

When it comes to selecting an appropriate zinc binding groups (ZBG), the hydroxamic acid is usually the preferred group of choice as it is most commonly used.^{24, 25} This is likely because the synthesis is easy, it has good solubility, reasonably good *in vitro* stability, and it binds by chelating to the zinc ion in a bidentate fashion.²⁴

SAHA (Figure 2) is a well-known HDAC inhibitor that contains a hydroxamic acid, it is used to treat cutaneous T-cell lymphoma (CTCL) and was actually the first HDAC inhibitor to be approved by the FDA.²⁴ Derivatives of SAHA were made without the hydroxamic acid group and it was found that the inhibitory function was lost which goes to show its importance.²⁵ Another well-known HDAC inhibitor with this zinc binding group is Trichostatin A (TSA) (Figure 4), it is known to show very potent inhibition activity as well as strong antiproliferative properties.²⁵

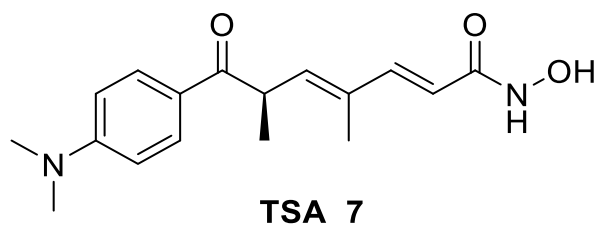


Figure 4: TSA structure

Despite the large number of HDAC inhibitors reported in the literature that consist of a hydroxamic acid ZBG, there are also some disadvantages to using a hydroxamic acid, including poor pharmacokinetics, or the possibility of it binding to other zinc-dependant enzymes and causing unwanted side effects.²⁴ Furthermore, HDAC inhibitors with hydroxamic acid ZBG are often classified as pan inhibitors, which means isoform selective HDAC inhibition is not observed.²⁶ As such, other ZBGs have been considered such as benzamides and aliphatic acids.

Benzamides

In contrast to hydroxamic acids, benzamides are known to have amazing selectivity with class I HDACs.^{24, 25} Good selectivity is related to reduced side effects as the inhibitor is more likely to bind to the desire target than other enzymes.²⁴ However, since benzamides have an amino group that is exposed when it is chelated to the zinc ion it is possible that this could bind with other molecules and cause *in vivo* toxicity²⁴ so there's still potential for unwanted side effects.

No benzamide HDAC inhibitors have been approved by the US FDA yet, possibly due to the fact that in clinical trials it's been observed that these derivatives don't usually exhibit as many therapeutic properties as wanted.²⁴ However, the Chinese FDA have in fact approved Chidamide **8** (Figure 5), the first oral and subtype-selective, benzamide containing HDAC inhibitor, to treat refractory or relapsed peripheral T-cell lymphoma (PTCL).^{24, 25} There are also clinical trials currently ongoing for two other benzamide HDAC inhibitors; MS-275 **9** and MGCD0103 **10** (figure 5), both of which are HDAC1 selective with MGCD0103 also being HDAC2 selective.²⁴ They have been synthesised for the treatment of solid cancers and hematologic malignancies.²⁴

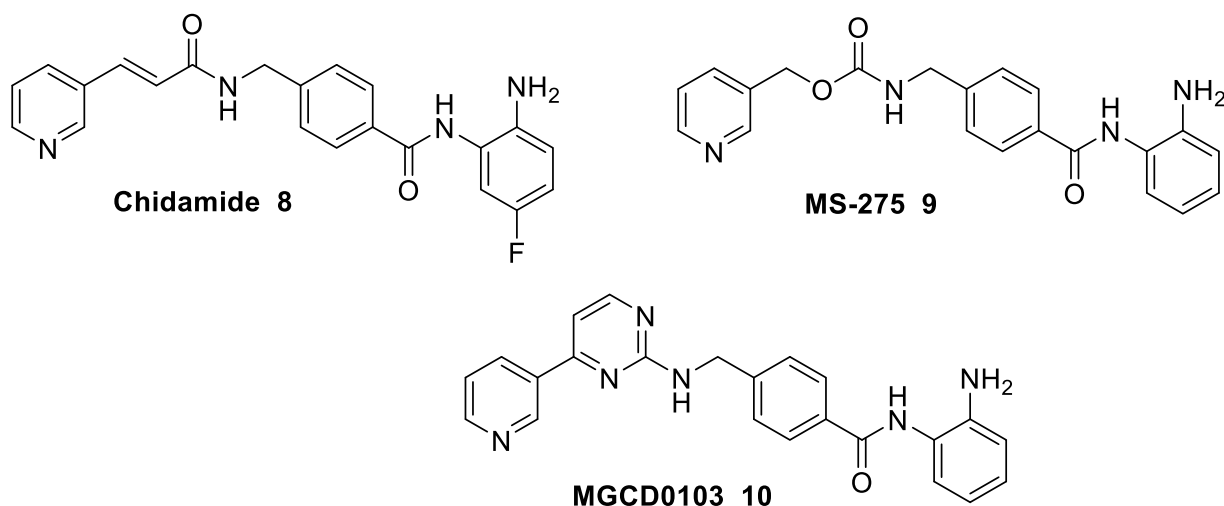


Figure 5: HDAC inhibitors with a benzamide ZBG

HDAC Inhibitors and Fluorescent Imaging

The cellular uptake and subcellular localisation of conventional, non-fluorescent HDAC inhibitors cannot be monitored in real time. This results in a poor understanding of their cellular behaviour and mode of inhibitory action, impeding the development of highly selective and potent HDAC inhibitors. Fluorescent cellular imaging is a powerful tool that allows researchers to study a number of cellular events in real time. In recent years, a number of fluorescent HDAC inhibitors have appeared in the literature, serving as valuable probes to visualise cellular uptake and where in the cell the HDAC inhibitor is located and distributed.²⁷

11 (figure 6) was synthesised in 2011 by Kong et al., it's a fluorescent derivative of SAHA that utilizes a dansyl moiety as the capping group of the HDAC inhibitor.²⁸ This compound targets class II HDACs, it binds strongest to HDAC6 (IC_{50} : 0.13 $\mu\text{mol/L}$) and HDAC4 (IC_{50} : 0.33 $\mu\text{mol/L}$) and has been found to localise in the cytoplasm of treated cells.²⁸ Kong et al. concluded that **11** looked promising as a novel theranostic agent.²⁸

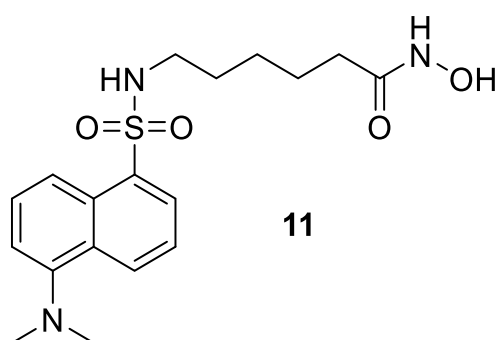
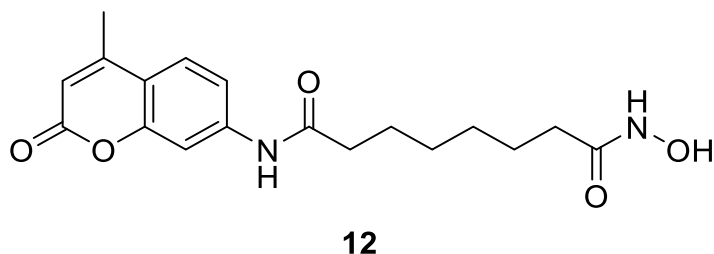
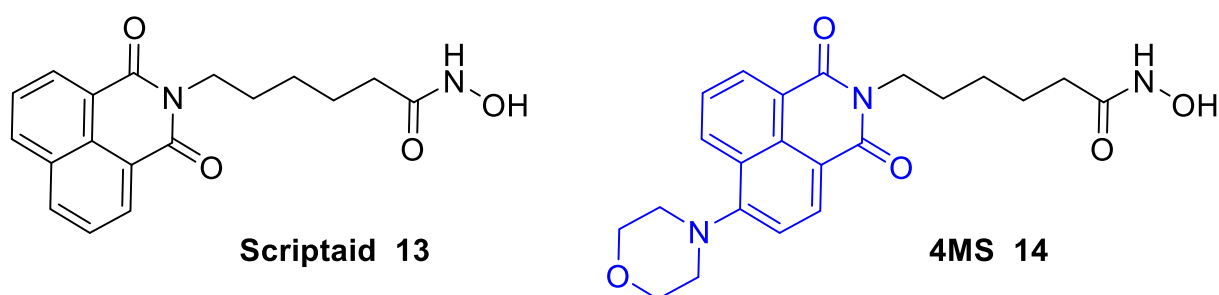


Figure 6: Structure of compound 11

In 2011 Srivastava and co-workers synthesised **12** (figure 7) based on SAHA but with a coumarin derived capping group^{29, 30}. **12** was found to have a strong binding affinity with HDAC8, the K_d value was 160 ± 20 nM.²⁹ It also exhibited an absorption max of 325 nm and an emission band of 400 nm.²⁹

**Figure 7:** Structure of compound 12

In 2015, Pfeffer and co-workers reported the development of 4-morpholinoscRIPTAID (**4MS**, Figure 8). This fluorescent HDAC inhibitor was realised by making a small modification to the capping group of the well-studied, non-fluorescent HDAC inhibitor scriptaid. More specifically, a small amino moiety was introduced at the 4-position of the naphthalimide capping group and gave rise to fluorescent properties that were well suited for cellular imaging studies. Due to the fluorescent nature of **4MS**, it was possible to observe its quick cellular uptake into the cytoplasm, whilst no nuclear penetration was observed.²⁷ Furthermore, cell death was observed after the treatment of the cancer cell line, MDA-MB-231, with **4MS**, demonstrating promising anticancer properties. **4MS** bind the strongest with HDAC6 as it has an IC_{50} value of 12 nM, it also binds reasonably well with HDAC3 and HDAC11 which have IC_{50} values of 0.32 μ M and 029 μ M respectively.²⁷

**Figure 8:** Non-fluorescent HDAC inhibitor, Scriptaid and fluorescent HDAC inhibitor, 4-morpholinoscRIPTAID

In 2019, three novel fluorescent HDAC inhibitors were synthesized and reported in the literature.³¹ This was by incorporating in a fluorescent dansyl group as the capping group and for the zinc binding group either a hydroxamic acid group or benzamide moiety was installed.³¹ **15** (figure 9) binds best with HDAC6 (IC_{50} : 0.132 μ M). **16** (Figure 9) seemed to have the best properties of all three compounds as it was found to have the fastest cellular uptake, and had the strongest anti-proliferative activity.³¹ It had the best affinity of the three compounds, binding strongest with HDAC1 (IC_{50} : 0.034 μ M) and HDAC6 (IC_{50} : 0.046 μ M). **17** had the weakest affinity of the three with its lowest IC_{50} value being 0.668 μ M for HDAC2 and HDAC1 not far behind (IC_{50} : 0.676 μ M).³¹

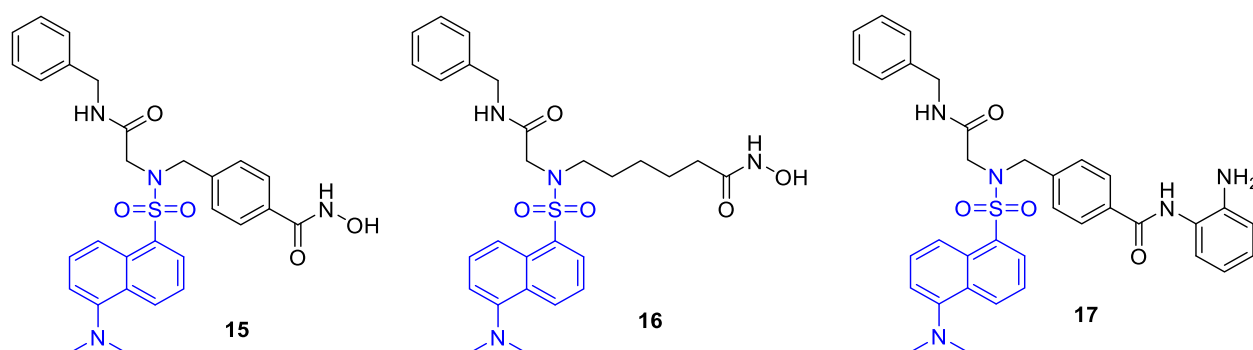


Figure 9: Three fluorescent HDAC inhibitors structures **15**, **16**, and **17**

In 2020, a fluorescent derivative of SAHA **18** was reported, where the capping group of SAHA was replaced with the 4-sulfamoyl-7-aminobenzoxadiazole (SBD) fluorophore (Figure 10). The fluorescent HDAC inhibitor was found to have inhibition activity very similar to vorinostat (SAHA), its IC_{50} data being 30 nM, 304 nM, 37 nM, and 11 nM for HDAC1, 2, 3, and 6 respectively.³² As well as that it was less toxic and more ideal for cellular imaging.³² Co-localization imaging made it possible to observe that the cytoplasm was the primary sub-cellular localisation site for this compound. It was concluded that this compound provided value towards clinical cancer diagnosis.³²

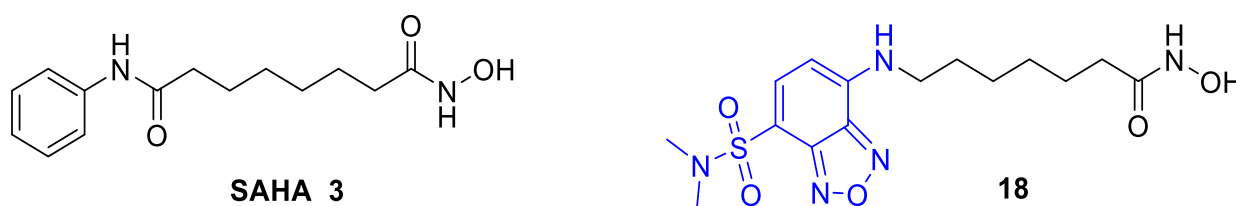


Figure 10. Structure of HDAC inhibitor **1**, derivative of SAHA with (SBD) fluorophore.

19 (figure 11) is another derivative of SAHA that was also developed in 2020³³. This HDAC inhibitor is near infrared (NIR) fluorescent which has been found to have an advantage over visible region fluorescing compounds.³⁴ This is because they have a deeper tissue-permeability and there is a decrease in background signal due to body tissues.³⁴ It was found that **11** has good retention effect in tumors,³³ and when it was measured for inhibitory activity it showed similar results as SAHA against HDAC1, 3, and 6³³.

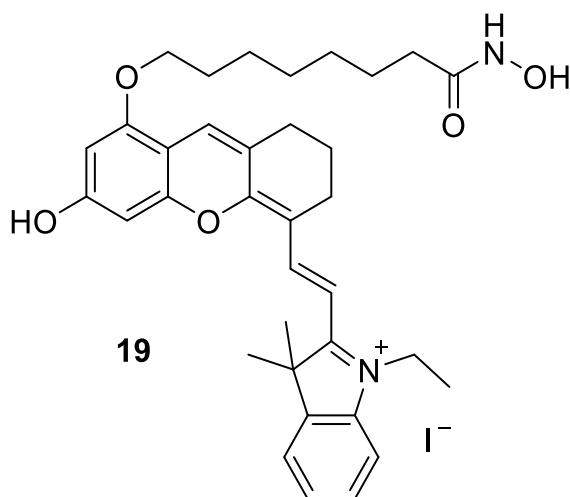


Figure 11: Structure of compound 19

Fluorescent Naphthalimide Derivatives

4-Amino-1,8-naphthalimides

4-Amino-1,8-naphthalimide derivatives are well studied fluorophores, that have been recently used in several processes such as cellular imaging, supramolecular chemistry, and DNA intercalation,³⁵ due to their favourable photophysical properties, including high fluorescence quantum yields, good photostability and large Stokes shifts. 4-Amino-1,8-naphthalimides are fluorescent due to an internal charge transfer (ICT) state in which the lone pair of electrons on the nitrogen donates into the ring and the electrons are accepted by the carbonyl group, as can be seen in Figure 12. This is also known as a push-pull mechanism.

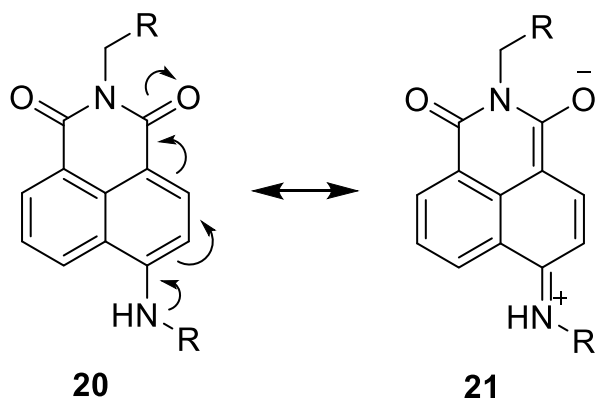


Figure 12. The internal charge transfer state of 4-amino-1,8-naphthalimide derivatives which gives rise to fluorescence.

6-Amino-2,3-naphthalimides

6-Amino-2,3-naphthalimides are structural isomers of the well-studied 4-amino-1,8-naphthalimide fluorophores. Like the 4-aminonaphthalimides, the fluorescent properties of 6-aminonaphthalimides are due to the ‘push-pull’ of electrons between the electron donating amino moiety and the electron withdrawing imide group (Figure 13). In contrast to 4-aminonaphthalimides, there is not a lot of information on 6-amino-2,3-naphthalimides as they have not been studied in great detail. In a recent study by Fujii et al.,³⁶ it was found that 6-aminonaphthalimides exhibit positive solvatochromic properties (*i.e.* emission maximum shifts to longer wavelengths upon increasing the polarity of the solvent system) with large Stokes shifts. The emission of **24** and **25** (Figure 13) range from orange–red in methanol to violet–blue in hexane.³⁶

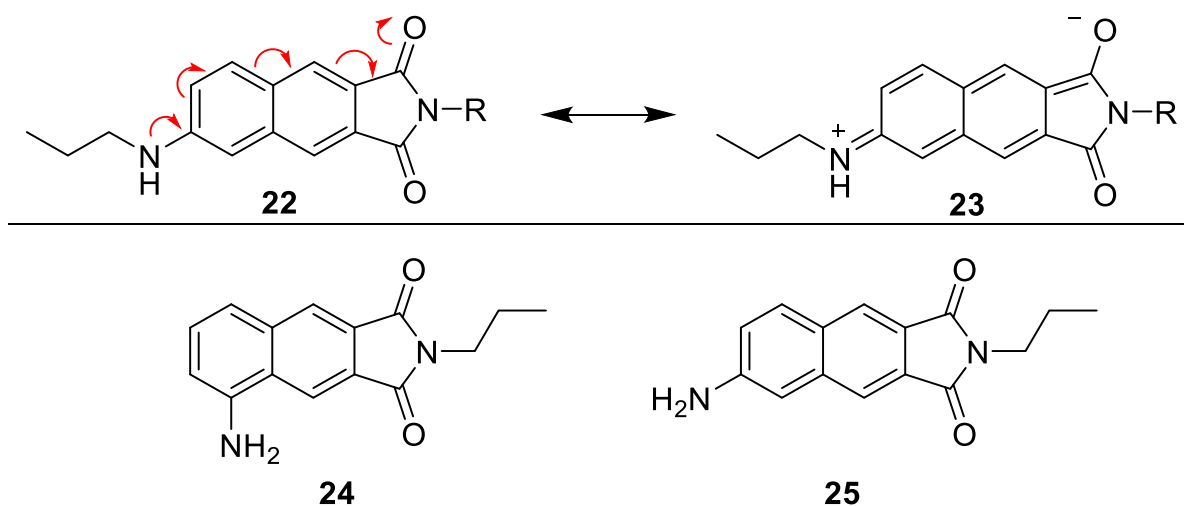


Figure 13: The internal charge transfer state of 6-amino-2,3-naphthalimide derivatives which gives rise to fluorescence.

One- vs Two-Photon Excitation

Two-photon excitation (2PE) is a method of fluorescent imaging that is around 30 years old now.³⁷ The way it works is similar to one-photon excitation (1PE) in which a laser is focused through an objective lens towards the sample except then two excited photons are absorbed, instead of one, they hit the fluorophore at the exact same time exciting the fluorophore and emitting light as a result.²⁴ Essentially two photons end up doing the work of one, each of the two photons being half the energy of the single photon.²⁴ The excitation wavelengths for 1PE and 2PE are 488 nm and 900 nm respectively.³⁸ 2PE uses infrared light while 1PE uses ultraviolet light, the use of infrared light is less toxic to the cell.³⁹ It is also able to image cells deeper in tissue than 1PE can reach.⁴⁰ However, 1PE is known to have less photodamage and photobleaching effects than 2PE so there are pros and cons to each of the methods.³⁹

Project Aims

Previous work in the group has seen the development of highly fluorescent HDAC inhibitors based on the 4-amino-1,8-naphthalimide fluorophore (Figure 14). The highly fluorescent nature of these compounds was well suited for cellular imaging experiments. More recently, 6-amino-2,3-naphthalimide derivatives have demonstrated their utility as two-photon excitable fluorophores. To the best of our knowledge, fluorescent HDAC inhibitors with two-photon excitability properties have yet to be reported. As such, this project focusses on the design, synthesis and characterisation of new HDAC probes suitable for multiphoton imaging techniques. In doing so, the following research objectives have been pursued:

1. How to design and synthesis highly fluorescent 6-amino-2,3-naphthalimide derivatives suitable for multiphoton imaging?
2. What is the relationship between molecular structure and HDAC inhibitory activity?
3. How are the 'reporting' features of the HDAC inhibitors influenced by the different amino functional groups, *i.e.* their structure-photophysical-relationship?

Given the non-critical role of the capping group of the well-studied HDAC inhibitor, scriptaid, this was to be replaced with a 6-amino-2,3-naphthalimide derivative. As the selective inhibition of class I HDACs is of particular interest in the group, the hydroxamic acid was to be replaced with a benzamide moiety, as this zinc binding group has shown preference to binding class I HDACs. As such, this project commenced with the development of 6-aminonaphthalimide scriptaid derivatives, whereby a number of amino moieties are to be installed at the 6-position of the naphthalimide ring (Figure 14).

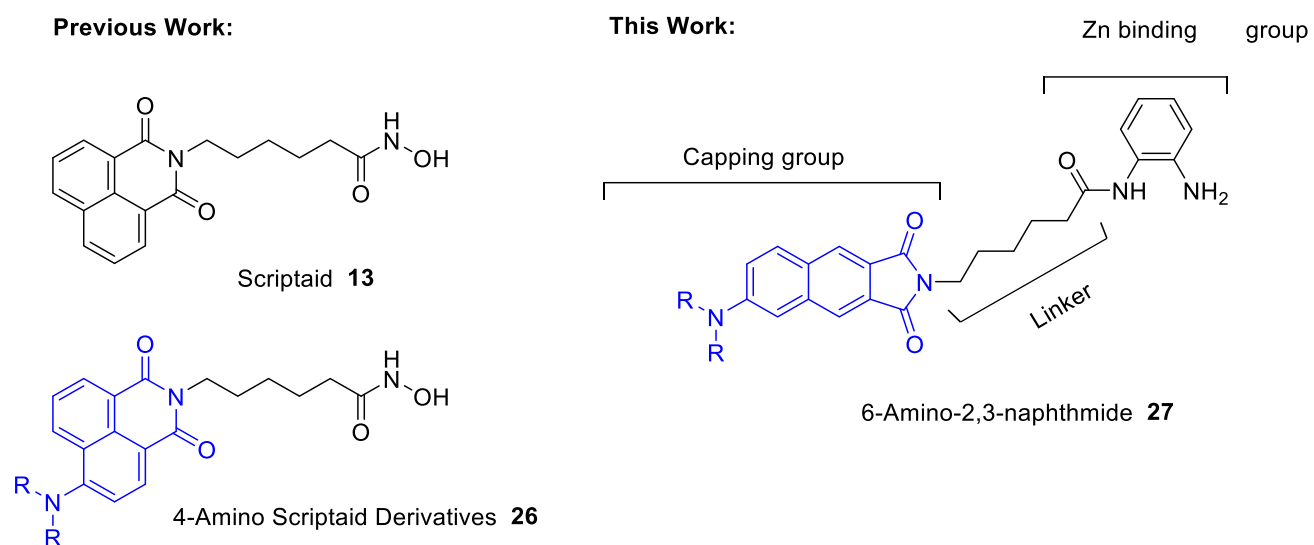


Figure 14. Structure of scriptaid and the 6-amino-2,3-naphthalimide derivatives to be developed in this project.

2. RESULTS AND DISCUSSION

Synthetic Plan.

There are very few examples of 6-amino-2,3-naphthalimides reported in the literature.^{41,42} As such the chemistry needed to access these derivatives remains relatively unexplored. Methods typically employed for the synthesis of these derivatives mainly include the synthesis of the anhydride **33**, with the amino moiety at the 6-position preinstalled before further functionalisation to install the imide (Figure 15).⁴³ The main limitation to this approach is that it's very time consuming as it requires five steps just to synthesise the anhydride. First a protection of the aldehyde, followed by a nucleophilic substitution as well as a deprotection of the aldehyde, then a multi-step process of two addition and one elimination reactions to give the naphthalene derivative, followed by reductive amination, and finally formation of a diacid which is then dehydrated to produce the anhydride.⁴³ Furthermore, this approach does not allow for facile modification of the amino moiety at the 6-position.

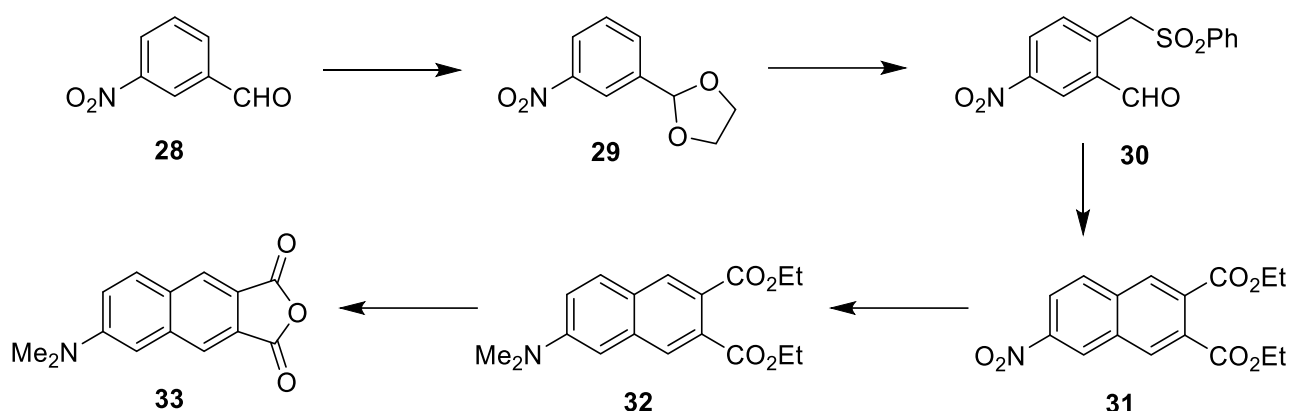


Figure 15: Typical starting pathway for synthesising the anhydride for 6-amino-2,3-naphthalimide derivatives.⁴³

In contrast to this, the chemistry for the preparation of 4-amino-1,8-naphthalimides is well established. The 4-aminonaphthalimides are typically prepared in two steps from the corresponding 4-halo-1,8-naphthalic anhydride starting materials, in which the imide moiety is first installed by nucleophilic acyl substitution followed by amination at the 4-position (Figure 16).^{6,44} As such, it was envisioned that the desired 6-amino-2,3-naphthalimide derivatives to be synthesised in this project could be readily accessed in a similar fashion.

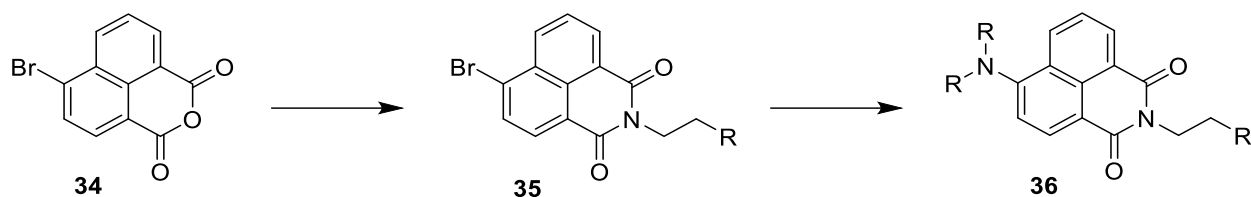


Figure 16: General preparation of 4-aminonaphthalimides

Previous work in the group attempted the synthesis of 6-aminonaphthalimide derived HDAC inhibitors following the synthetic plan outlined in *Pathway 1* in Figure 17. The hydrolysis of the methyl ester **39** to the corresponding carboxylic acid **42**, under basic conditions proved troublesome and it was deemed that the imide ring was susceptible to ring opening to the corresponding amic acid. Due to the poor stability of the naphthalimide moiety under basic conditions, a *tert*-butyl ester was to be installed in place of the methyl ester, as acidic conditions are required for hydrolysis to the corresponding carboxylic acid.

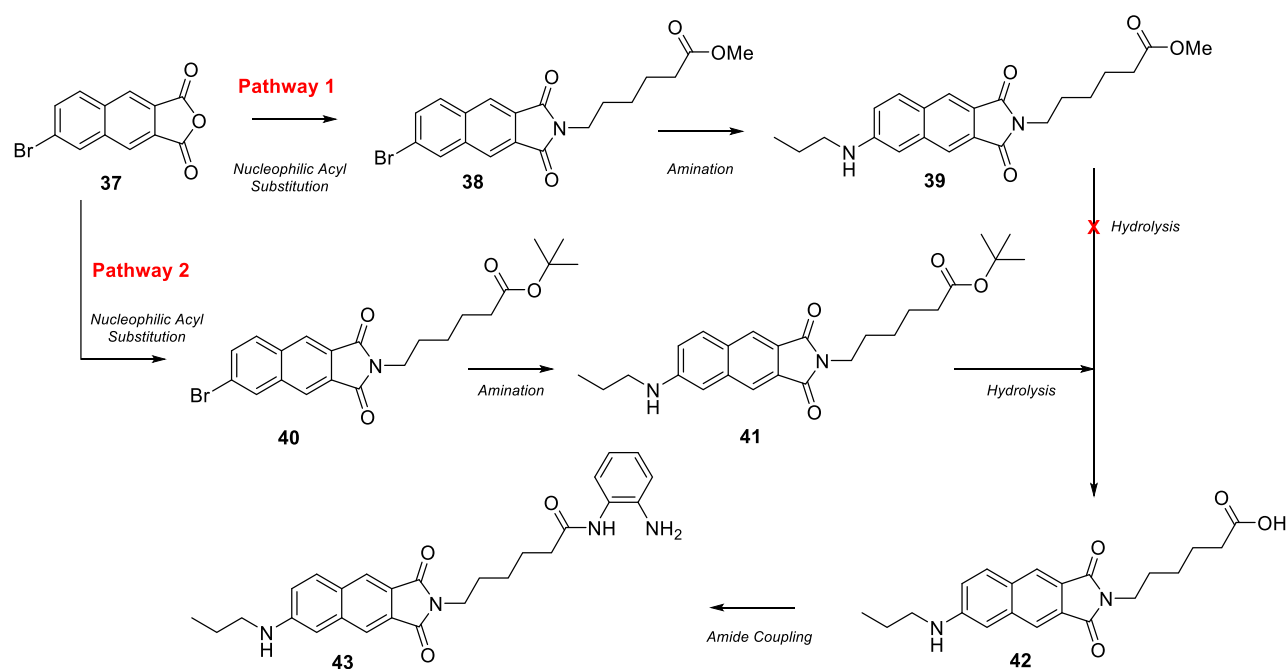


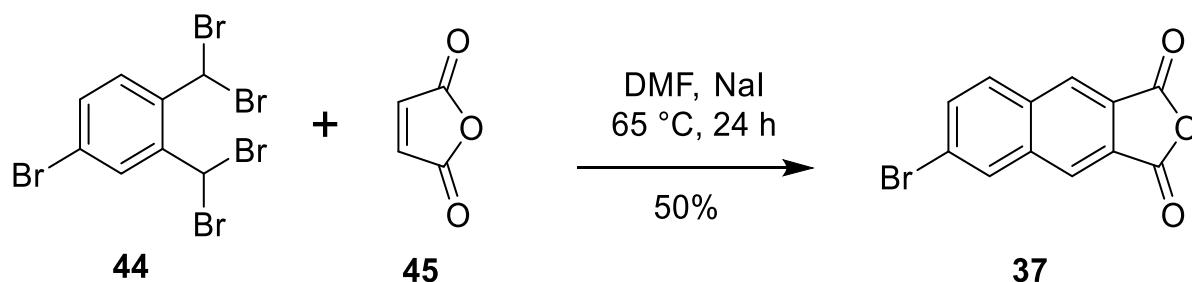
Figure 17: Previous pathway vs current pathway to access the final compound

As such, the proposed synthetic pathway will commence with the synthesis of the naphthalic anhydride **37**, which will then be used to follow the typical synthesis of the 4-aminonaphthalimides of a nucleophilic substitution to form the imide, this time with a *tert*-butyl ester, and an amination to install the amino moiety at the 6-position (*Pathway 2*, Figure 17). The benzamide zinc binding group is then to be installed by hydrolysis of the *tert*-butyl ester followed by an amide coupling with *O*-phenylenediamine.

Nucleophilic Acyl Substitution.

As illustrated in the synthetic plan outlined in Figure 17, the synthesis of the 6-aminonaphthalimide HDAC inhibitors was to commence with nucleophilic acyl substitution to install the imide moiety. As such, the two key starting materials, 6-bromonaphthalic anhydride **37** and *tert*-butyl ester **47** were to be prepared first.

A solution of tetrabromide **44**, malic anhydride **45** and NaI in DMF was left to stir at 65 °C overnight. After cooling the reaction mixture on ice, the reaction mixture was diluted with H₂O, transferred to a beaker and left this to stir for 5 minutes. The resulting precipitate was isolated by vacuum filtration, rinsing with H₂O and MeOH and left to dry overnight. Additional material was further recovered from the filtrate, in which excess solvent was removed and the resulting crude material recrystallized in acetic anhydride. ¹H NMR analysis (figure 18) supports the formation of the desired product as the five key peaks are visible in the aromatic region, each integrating for 1 proton. Two singlets at 8.53 ppm and 8.46 ppm, two doublets at 8.32 ppm and 8.02 ppm, as well as a doublet of doublets at 7.89 ppm.



Scheme 1: Formation of naphthalic anhydride

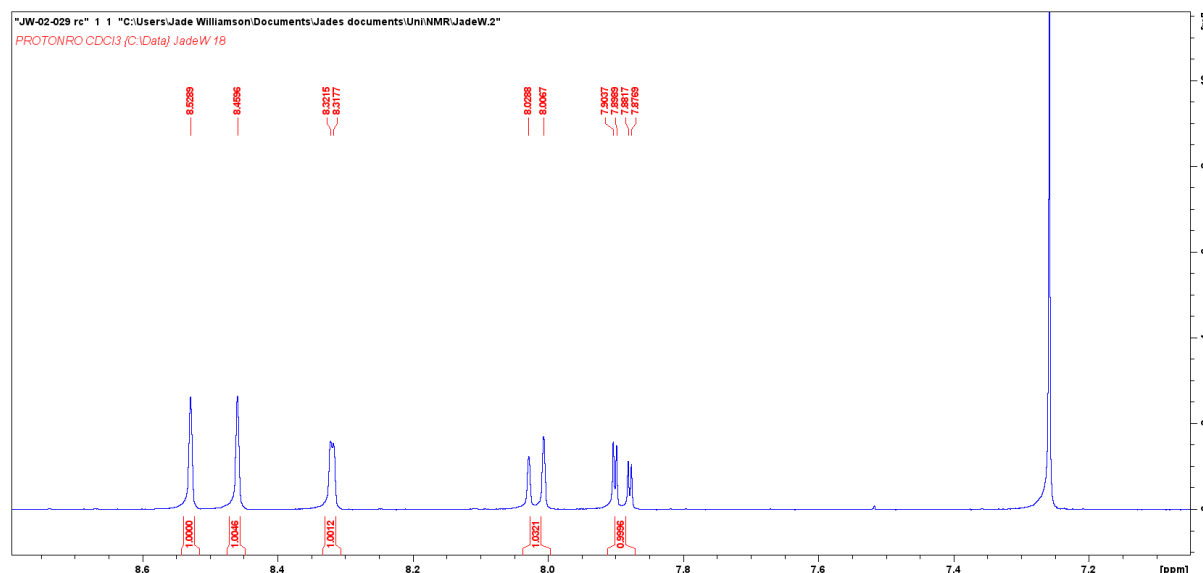
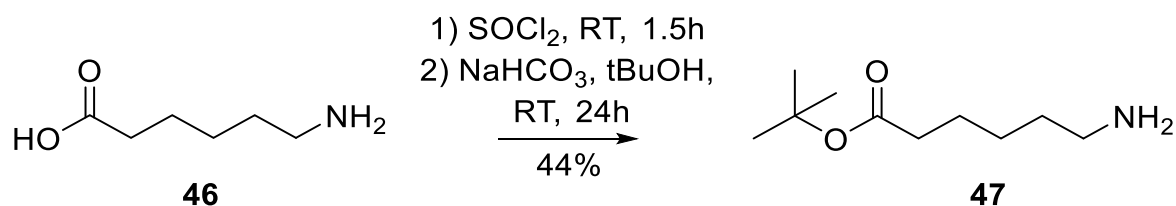


Figure 18: ¹H NMR spectrum of naphthalic anhydride

Next, 6-aminohexanoic acid **46** was to be converted to the corresponding *tert*-butyl ester **47**. Following literature conditions,⁴⁵ a solution of 6-aminohexanoic acid **46** and thionyl chloride was left to stir at

room temperature for 1.5 hours. The thionyl chloride was then carefully removed and a suspension of NaHCO_3 and *t*-BuOH was added. The resulting reaction mixture was then left to stir at room temperature overnight. The solvent was removed, and the reaction was worked up by an aqueous extraction. Analysis of the isolated material by ^1H NMR (figure 19) supported the formation of the desired product, with the presence of the key singlet peak at 1.43 ppm that integrates for 9 protons, this represents the *tert*-butyl group. The other key peak being the broad singlet integrating for 2 protons at 2.65 ppm which is due to the two amine protons. An additional 5 signals corresponding to the aliphatic protons were also observed.



Scheme 2: Formation of *tert*-butyl 6-aminohexanoate

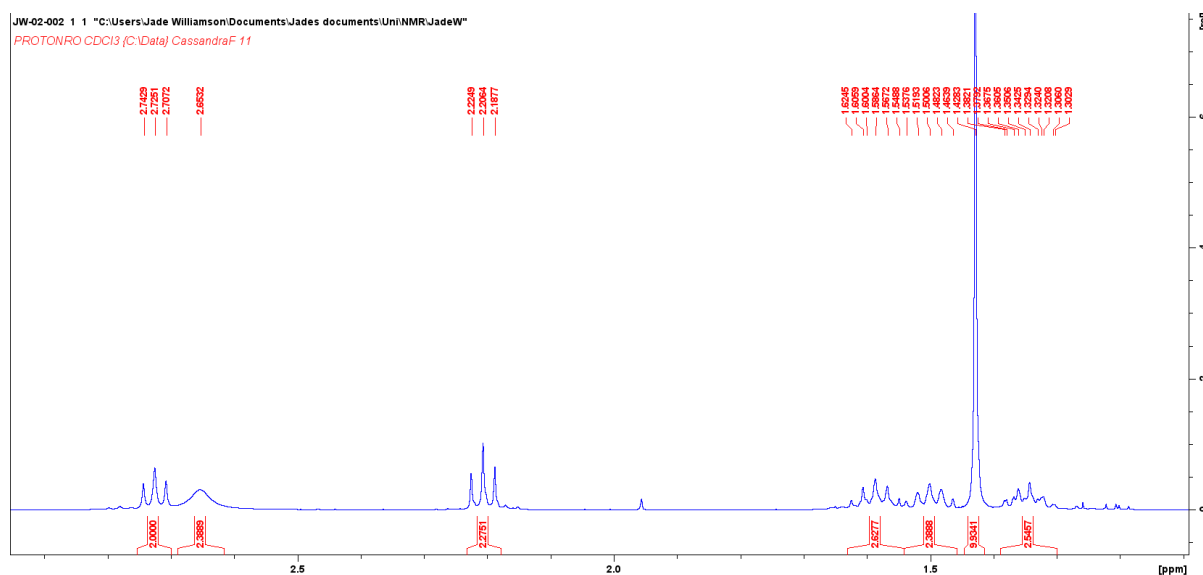


Figure 19: ^1H NMR spectrum of *tert*-butyl 6-aminohexanoate

With the naphthalic anhydride **37** and *tert*-butyl ester **47** in hand, attention turned to the formation of the imide moiety via nucleophilic acyl substitution. In general, the formation of the imide (figure 20) involves nucleophilic attack of the amine to the carbonyl carbon where the electrons go up onto the oxygen then come back down opening the five membered ring. Proton transfer occurs before nucleophilic attack occurs again between the newly installed amine and the other carbonyl carbon in the molecule, pushing the electrons up onto the oxygen. Proton transfer occurs to form water, which is

a good leaving group, the electrons come back down from the oxygen in turn kicking out the water to give the newly installed imide compound.

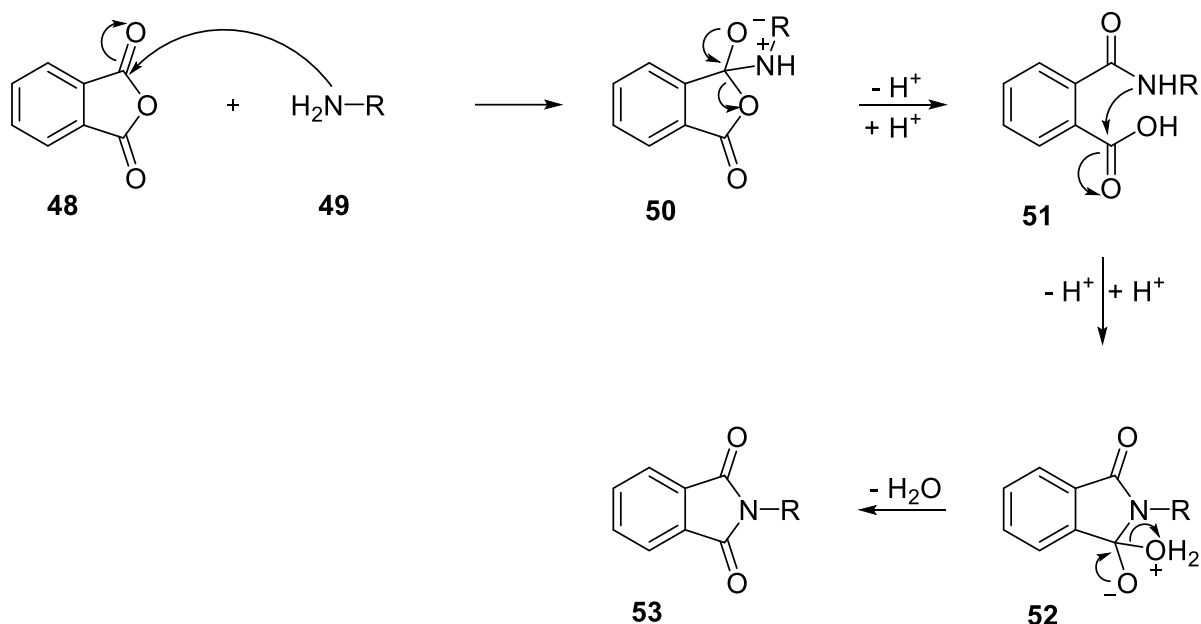
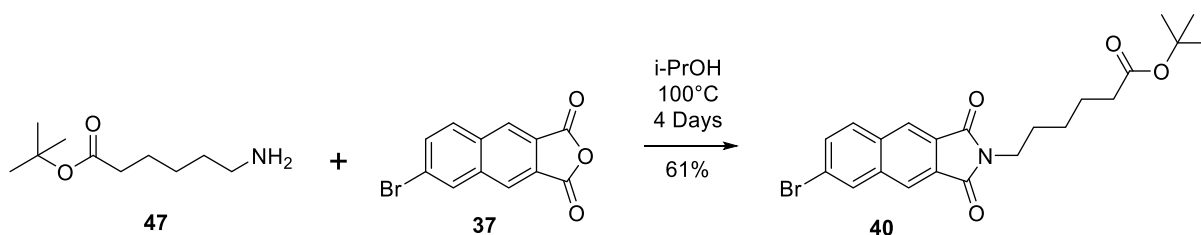


Figure 20: General mechanism for the imide formation vis twin nucleophilic acyl substitution.

A mixture of *tert*-butyl-6-aminohexanoate **47** and naphthalic anhydride **37** was cooled to 0 °C, to which acetic acid was slowly added. After stirring at 90 °C for 2 hours, the reaction mixture was poured over ice and the resulting precipitate was isolated by vacuum filtration. Analysis of the crude material by ^1H NMR spectroscopy showed the desired product is forming but unreacted starting material is still present. Further purification by column chromatography afforded the desired imide **40**, albeit with low yield (22%) and poor purity (Table 1, Entry 1).



Scheme 3: Nucleophilic Acyl Substitution

Table 1: Different conditions trialed to optimize this reaction

Entry	Solvent	Run time	Temperature	Yield
1	Acetic acid	2 hours	90 °C	22%
2	Ethanol, Acetic acid (1 equiv.) & Acetic anhydride (1 equiv.)	48 hours	90 °C	13%
3	Isopropyl alcohol	4 days	100 °C	61%

In efforts to facilitate the reformation of the naphthalimide ring, the reaction was repeated in the presence of both acetic acid and acetic anhydride, using ethanol as the solvent. It was anticipated that the acetic anhydride would result in the acetylation of the carboxylic acid intermediate and in turn, form a better leaving group, thus, facilitating ring closure. After stirring at 90 °C overnight, analysis of the reaction mixture by TLC suggested that ring opening of the imide to the corresponding amic acid still occurred. As such, acetic anhydride was added to the reaction mixture and the temperature was raised to 100 °C and left to stir for an additional night. Unfortunately, no significant improvement in yield was observed (Table 1, Entry 2).

In order to obtain more forcing conditions, pressure vessels can be used in place of traditional round bottom flasks. As such, the reaction was repeated in a pressure vessel in which, *i*-PrOH was also employed as the solvent as literature has shown *i*-PrOH to be a suitable solvent for such transformations.^{46, 47} A solution of naphthalic anhydride **37**, *tert*-butyl-6-aminohexanoate **47** and *i*-PrOH was left to stir at 100 °C for 4 days (Table 1, Entry 3). Aqueous workup and purification by flash column chromatography gave the desired imide **40** in 61% yield. Analysis by ¹H NMR (figure 21) supported that the product had been isolated due the presence of the key *tert*-butyl ester singlet at 1.42 ppm integrating for 9 protons and the five aromatic peaks being present. Also, the other expected aliphatic peaks are all accounted for.

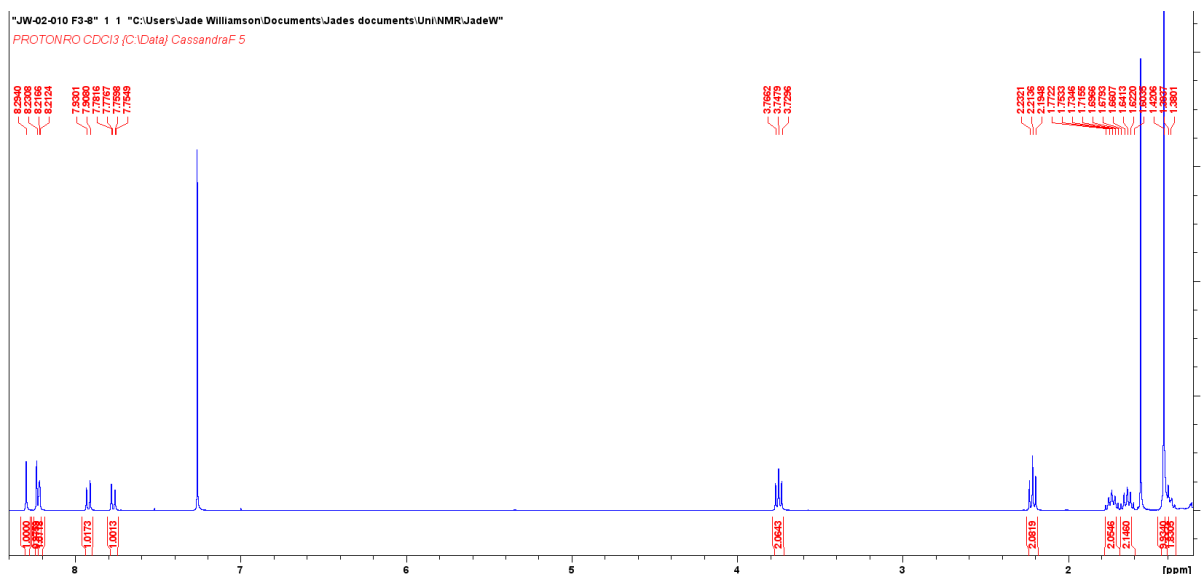


Figure 21: ^1H NMR spectrum of **40**

Buchwald–Hartwig Cross Coupling.

Previous work in the group has successfully introduced various amino moieties at the 4-position of 1,8-naphthalimides employing palladium-mediated cross coupling methodology.²⁷ As such, this approach was investigated for the installation of the amino functionality at the 6-position of the 2,3-naphthalimide ring.

This methodology was established by Stephen L. Buchwald and John F. Hartwig between 1994 and 2000 for the replacement of aryl halides with the C–N bond of an amine by a palladium catalyzed cross coupling.⁴⁸

Below is the catalytic cycle for a Buchwald–Hartwig palladium cross coupling mechanism (Figure 22). To commence, the Pd^0 undergoes **oxidative addition** to the aryl halide, inserting between the aryl carbon-halide bond. In doing so, the oxidation state of the palladium complex increases from Pd^0 to Pd^{II} . Next, the palladium complex undergoes **amine metal coordination**, in which the amine coordinates to the palladium and is subsequently deprotonated by the base, and in turn, replaces the halide. Finally, the palladium complex undergoes **reduction elimination** to release the desired amination product, as well as regenerating the Pd^0 catalyst.

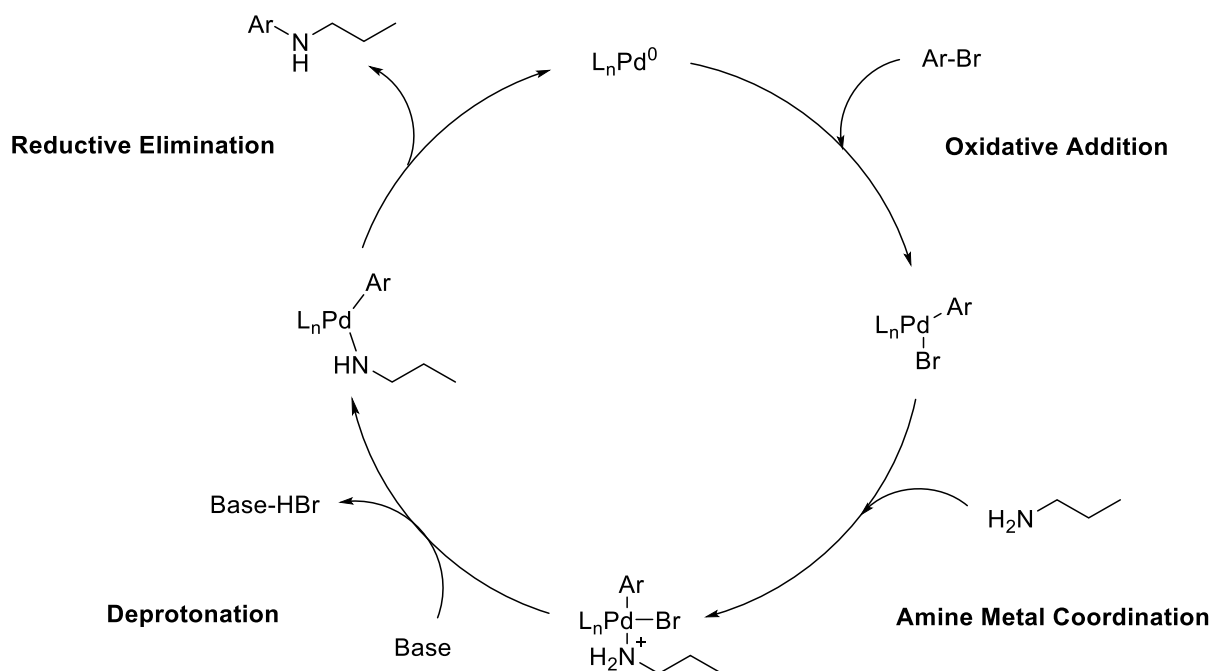
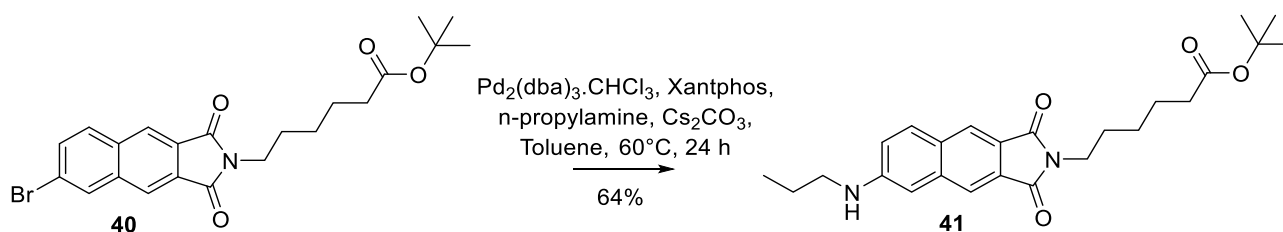


Figure 22: Mechanism of a Palladium catalyzed amination

Employing Pd coupling conditions previously established in the group, a solution of 6-bromonaphthalimide **40**, $\text{Pd}_2(\text{dba})_3 \cdot \text{CHCl}_3$, Xantphos, anhydrous toluene, *n*-propylamine, and Cs_2CO_3 was left to run at 60 °C overnight. The reaction progress was monitored by TLC. Purification by column chromatography gave the desired amino product **41** in a yield of 64%. Analysis by NMR (figure 23) supported the formation of the desired product with the presence of a triplet at 4.19 ppm corresponding to an N-H peak, integrating for 1 proton, which is coupling with the CH_2 to the left of it. This CH_2 group is seen as a quartet at 3.23 ppm and integrates for 2 protons. It is a quartet because its coupling with the N-H and another CH_2 . There is also a triplet at 1.06 ppm integrating for 3 protons that corresponds to the CH_3 at the end of the propyl group. These peaks indicate that the propyl amine moiety has been installed at the 6 position. Another key peak is the singlet at 1.42 ppm that integrates for 3 protons this is characteristic to *tert*-butyl ester group at the end of the molecule.



Scheme 4: Buchwald–Hartwig Amination

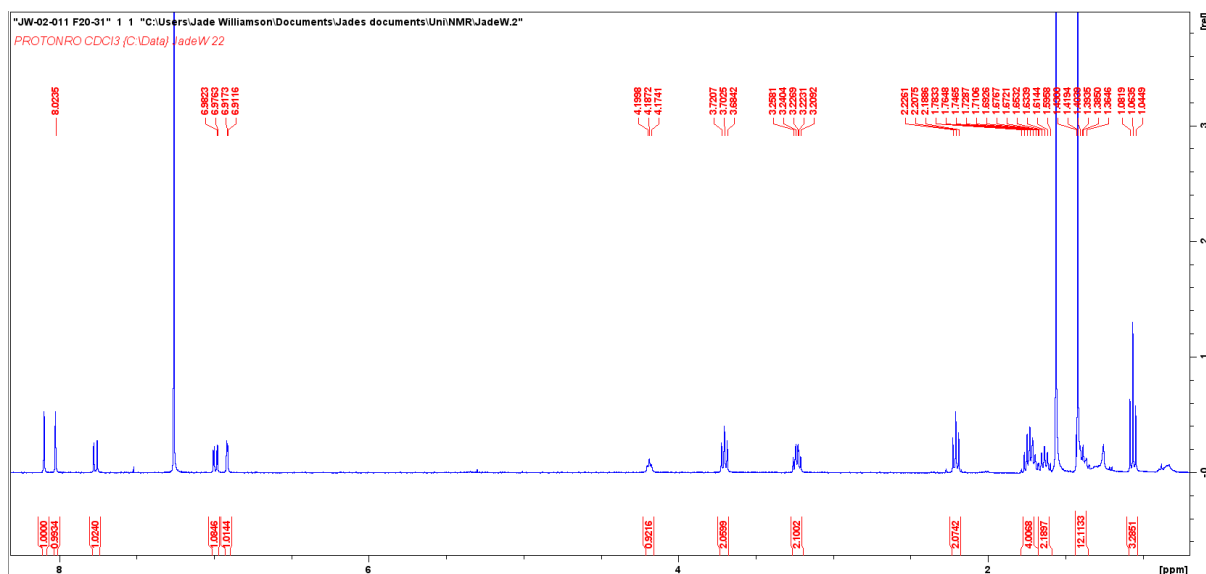
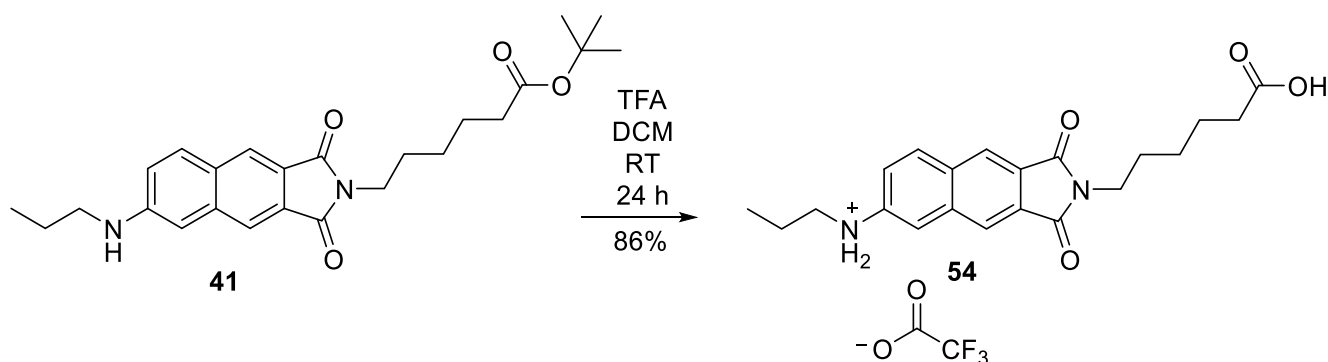


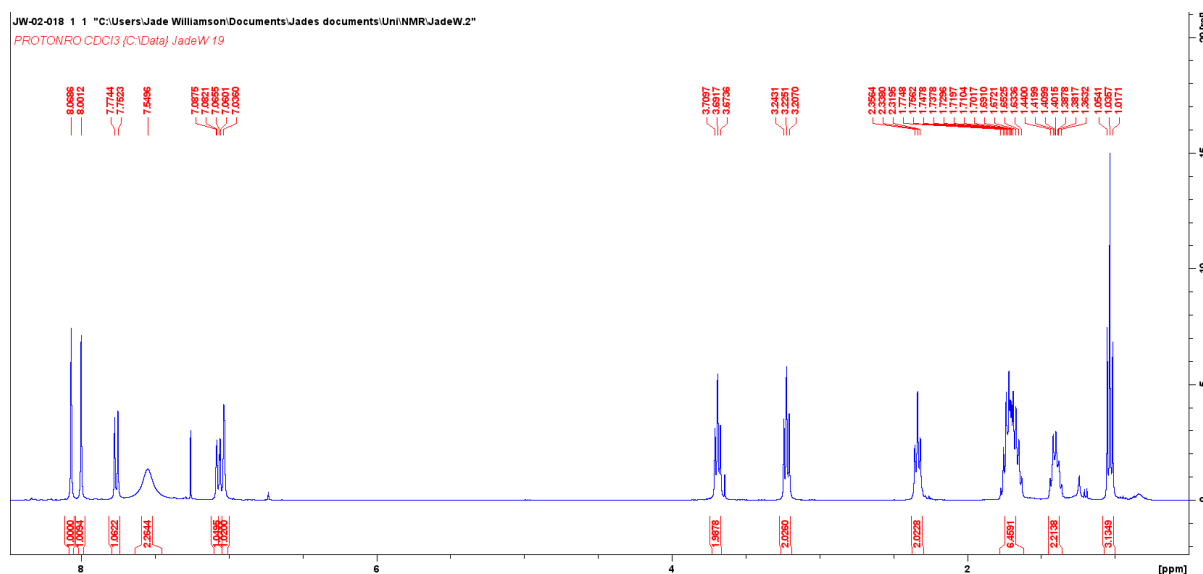
Figure 23: NMR spectrum of **41**

Installation of the Zinc-Binding Moiety

Following the installation of the *n*-propylamino moiety at the 6-position of the naphthalimide ring, attention turned to the synthesis of the zinc-binding moiety – the benzamide.

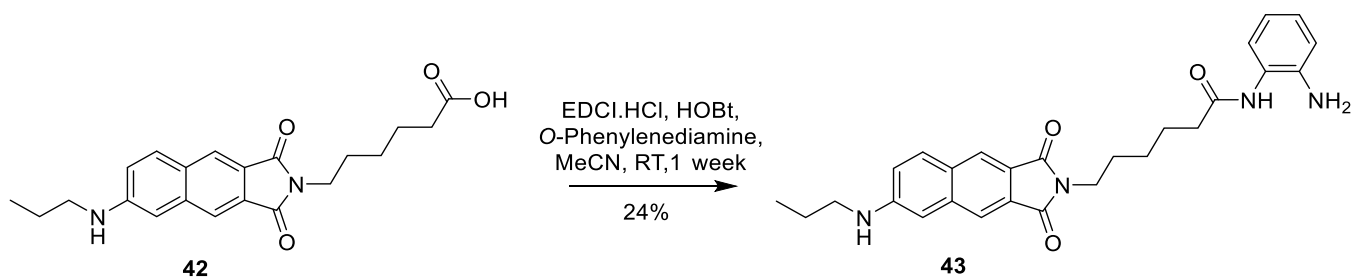
A solution of **41** and trifluoroacetic acid in anhydrous CH_2Cl_2 was left to stir at room temperature overnight. The solvent was removed, and residual TFA was removed via azeotropic distillation with diethyl ether to afford a yellow solid. NMR analysis (figure 24) showed a broad singlet at 7.55 ppm integrating for 2 protons, it is thought that this is due to the amine becoming protonated (scheme 5) as it is likely that the corresponding TFA salt has been isolated. Also, the sharp singlet at 1.42 ppm is no longer there, supporting that the hydrolysis of the *tert*-butyl ester group and the formation of the desired carboxylic acid **54** has occurred.



Scheme 5: Hydrolysis of the *tert*-butyl ester with the TFA salt present**Figure 24:** NMR spectrum for 54

Next, the benzamide zinc binding group was to be installed via amide coupling with the carboxylic acid **42**. In the first attempt, a suspension of **42**, *O*-phenylenediamine, EDCI.HCl, HOBT, and Et₃N in CH₂Cl₂ was left to run at 40 °C overnight. Analysis of the reaction mixture by TLC showed the reaction had gone to completion but a lot of impurities were present. Following aqueous work up, the resulting crude material was purified by flash column chromatography. Despite multiple attempts to obtain a pure sample, NMR analysis showed that only impure material was isolated, making it difficult to decipher whether the desired product had formed.

In another attempt, a suspension of **42**, *O*-phenylenediamine, EDCI.HCl, and HOBT in acetonitrile was left to stir at room temperature for a week. During this time, a yellow precipitate had formed in the reaction mixture. The solvent was then carefully pipetted off to isolate the solid. The solid was then purified twice by flash column chromatography to give the desired HDAC inhibitor **43** in 24% yield. NMR analysis (Figure 25) showed the presence of four new protons in the aromatic region, corresponding to the newly installed benzamide group. There is also a new broad singlet at 3.84 ppm which integrates for 1 proton which likely is due to the N-H proton in the benzamide ring. The usual key peaks are still visible, including the triplet at 4.21 ppm that corresponds to the propyl amine N-H proton, the quartet for the CH₂ next to the N-H which it is coupling with, and the triplet at 1.06 ppm corresponding to the CH₃ in the propyl group.



Scheme 6: Amide coupling

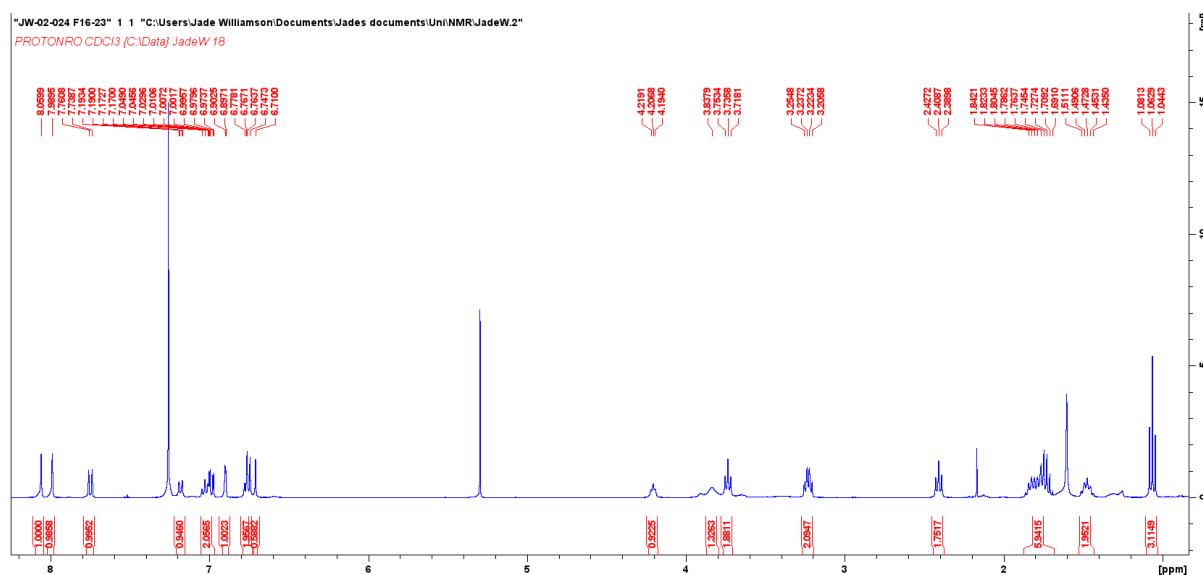
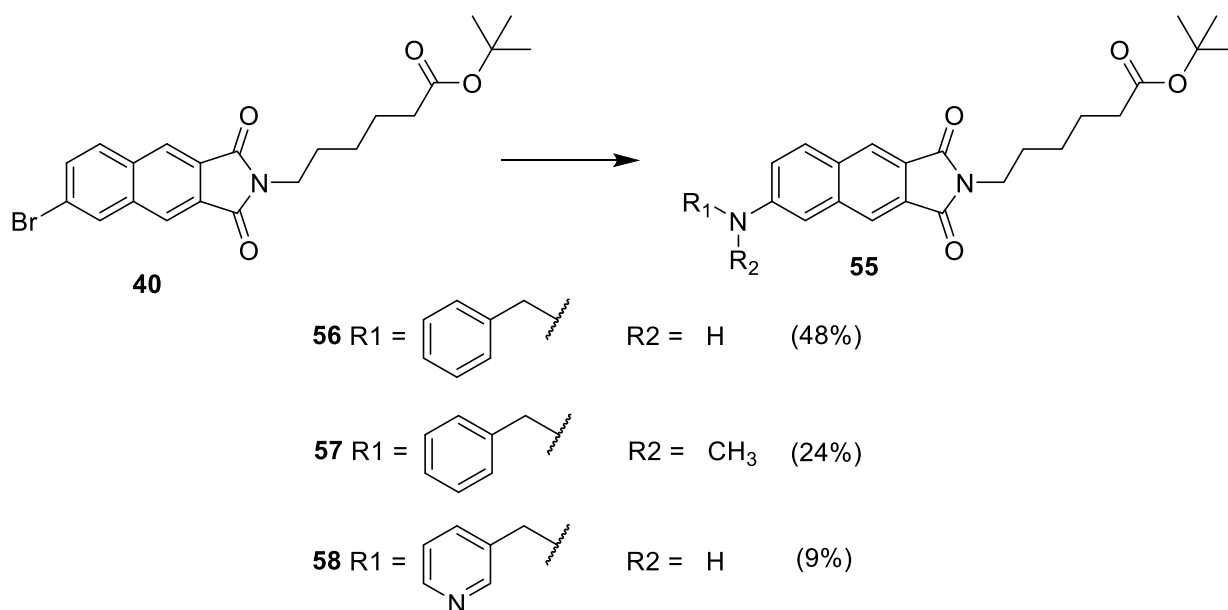


Figure 25: NMR spectrum for 43

With robust methodology in hand to access 6-amino naphthalimide derived HDAC inhibitors, it was therefore of interest to investigate the effects of introducing various amino-substituents at the 6-position of the naphthalimide capping group; not only to modulate photophysical properties but to also potentially influence their ability to bind and inhibit HDAC enzymes.

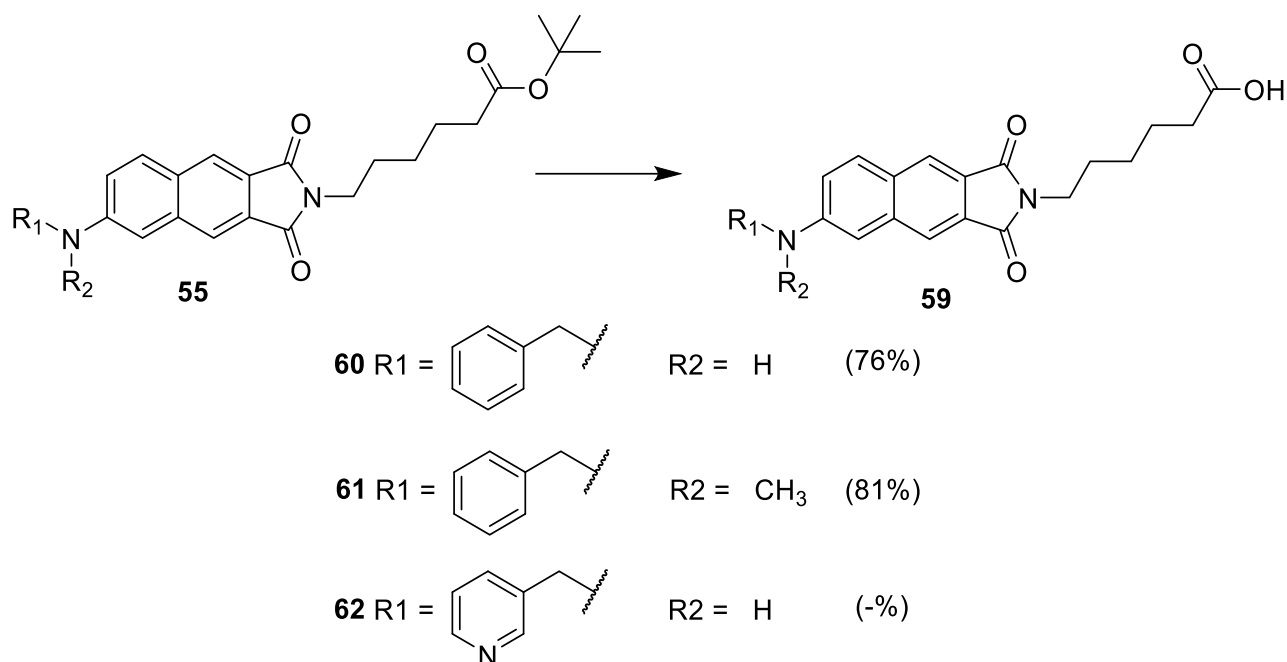
Employing the established for synthesis of **41**, additional derivatives bearing a benzyl-, methyl benzyl-, or picoyl-amine in place of the propylamine were pursued. The success of each reaction was readily determined by NMR spectroscopy and HRMS, with the characteristic shifts being either a singlet or triplet in the aliphatic region integrating for 1 proton. This corresponds to the N-H proton, or in the case that the R-group on the N is the CH₃, there will be a singlet integrating for 3 protons. There should also be 4–5 new protons in the aromatic region and a singlet for a CH₂ in the aliphatic region corresponding to the benzyl group attached to the amine.



Scheme 7: General scheme for benzyl-, methyl benzyl-, and picoyl-amine aminations.

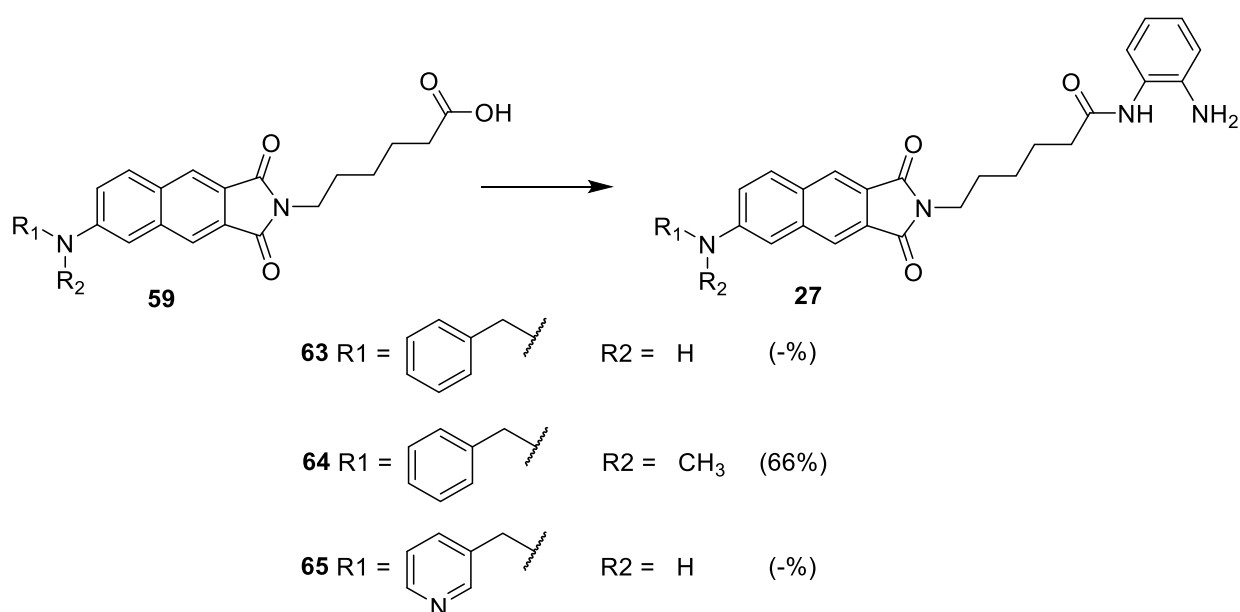
The yields for these derivatives ended up being lower than the amination reaction for the propyl amine **41** (64%), with **57** and **58** being significantly lower 24% and 9% respectively. This is possibly due to each reaction deviating somewhat from the exact original conditions applied for **41**, either by run time or equivalence of reagents added. Optimization for the synthesis of these derivatives needs to be done in future.

Acid-mediated hydrolysis of the *tert*-butyl ester to form the corresponding carboxylic acid was obtained in good yields (76–81%) (scheme 8), using an excess of TFA. The formation of the desired products was supported by NMR spectroscopy, with the disappearance of the singlet corresponding to the *tert*-butyl ester from the ¹H NMR spectrum. However, there were issues trying to purify **62** as the impurity was stuck on the base line and it was found difficult to find a solvent system to remove it.



Scheme 8: General scheme for benzyl-, methyl benzyl-, and picoyl-amine hydrolysis'

Next, a suspension of carboxylic acid, *O*-phenylenediamine, EDCI.HCl, and HOBt in acetonitrile was left to stir at room temperature for 5–7 days. Once the reaction had been worked up it was purified by flash column chromatography 2–5 times in attempts to isolate the product (scheme 9). NMR analysis showed that four new protons were in the aromatic region which would correspond to the installation of the benzamide group. It is also expected for 2 new broad singlets to appear, integrating for 1 and 2 protons, this is will be due to the N-H proton and the NH₂ protons in the benzamide ring.



Scheme 9: General scheme for benzyl-, methyl benzyl-, and picoyl-amine amide couplings

Unfortunately, at this point of time, compounds **63** and **65** were unable to be isolated as clean solid due to difficulties encountered during purification. **63** was purified by flash column chromatography five times and still wouldn't separate from the impurities. **65** was purified twice by flash column chromatography but carried over an impurity from the previous hydrolysis step which proved quite difficult to remove. So, that reaction will need to be optimized to avoid this problem in future. A couple proposed solutions to purifying these compounds include preparative TLC or reverse phase column chromatography. Hopefully these approaches will make it possible to isolate the final products with high purity suitable for further biological studies.

HDAC Profiling

As compound **43** and **64** (figure 26) were isolated in high purity, they were assessed for their inhibitory activity against HDAC isoforms 1, 3 and 8 (class I), HDAC 6 (class IIb), and HDAC 11 (class IV). Inhibitory activity was assessed in a single point assay in which, enzymatic activity has been reported in Table 2 as a percentage of remaining enzyme activity.

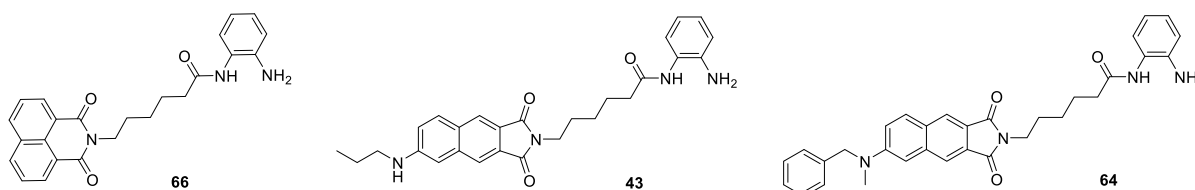


Figure 26: Compounds sent for HDAC activity analysis

Table 2: HDAC Activity (percentage of full enzyme activity) for **43** and **64**

Average Enzyme Isoform Activity (%) at 10 μ M ^a					
Compound	HDAC1	HDAC3	HDAC6	HDAC8	HDAC11
66	12.02	2.25	99.62	97.17	75.93
43	8.41	2.31	99.41	96.18	86.53
64	33.79	45.26	102.33	108.19	81.02

^aAverage of two independent measurements.

For these experiments, **66** was selected as the reference compound, as previous work in the group has shown that this compound exhibits inhibitory activity towards the class I HDACs.⁴⁹

As seen in table 2, it was found that **43** had excellent HDAC activity for HDAC1 (8.41%) and HDAC3 (2.31%), with inhibitor activity towards HDAC1 being slightly better than **66** (12.02%). As anticipated, **43** showed little-to-no inhibitory HDAC activity for the class IIb HDAC, HDAC6 (99.41%), as well as HDAC8 (96.18%, class I), and HDAC11 (86.53%, class IV). This is ideal as it suggests that **43** has class selectivity since it only seems to have activity for HDAC1 and HDAC3 which are part of class I HDACs. It also has isoform selectivity since HDAC8 is a part of class I but the compound has no HDAC activity for it.

64 had similar results in the sense that it only showed significant activity for HDAC1 (33.79%) and HDAC3 (45.26%) but not as well as **43** did. It also showed little to no activity for HDAC6 (102.33%), HDAC8 (108.19%), and HDAC11 (81.02%). Again, just like **43**, **64** showed that it has class selectivity as well as isoform selectivity. This is beneficial because having selectivity means the compound is less likely to bind to other compounds that it's not meant to bind to, reducing the chance of unwanted side effects and complications.

3. FUTURE WORK

Unfortunately, not all the aims of this project were able to be met as there wasn't enough time to get through everything. The aims of this project were:

1. How to design and synthesis highly fluorescent 6-amino-2,3-naphthalimide derivatives suitable for multiphoton imaging?
2. What is the relationship between molecular structure and HDAC inhibitory activity?
3. How are the 'reporting' features of the HDAC inhibitors influenced by the different amino functional groups, *i.e.* their structure-photophysical-relationship?

The design and synthesis step of 6-amino-2,3-naphthalimide derivatives has been mostly achieved as two derivatives have successfully been isolated **43** and **64**. But there are a couple derivatives that need to be isolated in higher purity than they currently are **63** and **65**. To achieve this, the best approach would be to try either prep TLC or reverse phase column chromatography to attempt to remove the impurities. If that is unsuccessful, then we might need to look into changing the synthesis of these compounds so those specific impurities don't form.

At the moment only a very small library of compounds have been synthesized and as stated they're not all clean. So, it would be ideal for a few more derivatives to be made so we have a range of compounds to test and compare the relationship of the structure and the HDAC activity. Also, the IC_{50} values and the fluorescent properties are yet to be measured so once there is a decent sized library of these derivatives it will be important to get this data.

Potentially something else that could be worked on is the optimization of a few of the reactions. The amination reaction never goes to completion, there is always starting material shown on the TLC so being able to figure out conditions that push this to completion would be really beneficial and result in less waste of reagents. This is also the case for the final amide coupling reactions, these don't go to completion either so it would again be ideal to figure out a way to optimize these reactions. Another reaction which needs optimization is the synthesis of **62**, usually the hydrolysis reactions don't require purification but this one showed up with impurities and attempts to removed them were unsuccessful.

4. CONCLUSION

There hasn't been much work done on 6-amino naphthalimide synthesis in the literature, so the methodology for this isn't well established yet. One of the aims of this project was to optimize new methodology for this kind of chemistry and that was, for the most part, successful as multiple novel 6-amino-2,3-naphthalimide derivatives were successfully synthesised. Two of those derivatives (**43** and **64**) being HDAC inhibitors with class I and isoform (HDAC1 and HDAC3) selectivity, it is hoped that these will be highly fluorescent but data analysis for that is yet to be determined. There is some more work that needs to be carried out in order to complete the aims of this project. That being optimization of certain reactions to allow for a larger library of compounds to be synthesized and analysed. Making it possible to be able to investigate the relationship between structure and enzyme activity as well as the structure-photophysical-relationship.

5. EXPERIMENTAL

5.1 General Experimental

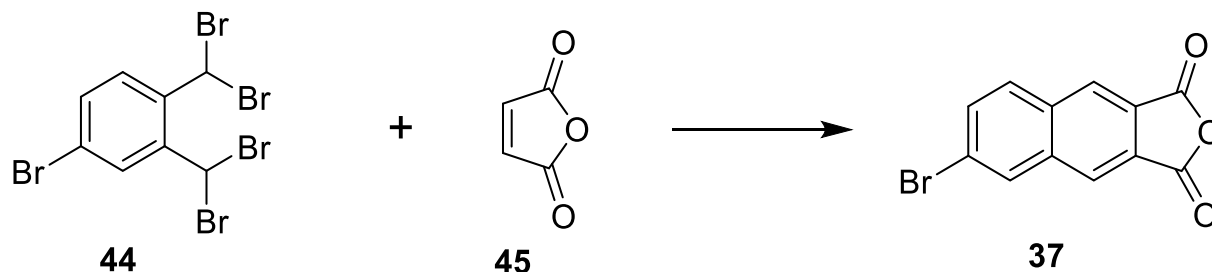
General reagent and solvents for the synthesis of compounds were purchased and used as supplied from commercial sources. Thin-layer chromatography (TLC) was performed using aluminium-backed Merck 60 F₂₅₄ silica plates. Developed plates were initially visualised under 254 nm UV light, followed by an oxidising dip made up of KMnO₄ (1.00 g), K₂CO₃ (1.00 g) and H₂O (100 mL). Column chromatography was performed using silica gel 60 (70–230 mesh).

NMR spectra for ¹H NMR and ¹³C NMR was obtained by using a Bruker Ascend 400 MHz spectrometer. Samples were dissolved (0.5 mL) in either deuterated chloroform (CDCl₃), deuterated dimethyl sulfoxide (DMSO-d₆), or deuterated methanol (CD₃OD). The residual solvent peaks specific to that to the deuterated solvent was used as an internal reference; CDCl₃: 7.26 ppm (¹H NMR), DMSO-d₆: 2.50 ppm (¹H NMR) and 39.52 ppm (¹³C NMR), and CD₃OD: 3.31 ppm (¹H NMR).

High resolution mass spectrometry (HRMS) analysis was conducted and recorded on a Bruker microTOF-Q II mass spectrometer operating at a nominal accelerating voltage of 70 eV. All samples were prepared at a concentration less than 1 mg/mL. HRMS data are reported as: molecular formula, calculated parent ion (*m/z*), observed parent ion (*m/z*).

5.2 Synthetic Details for Compounds 36–65.

6-Bromonaphtho[2,3-*c*]furan-1,3-dione **37**



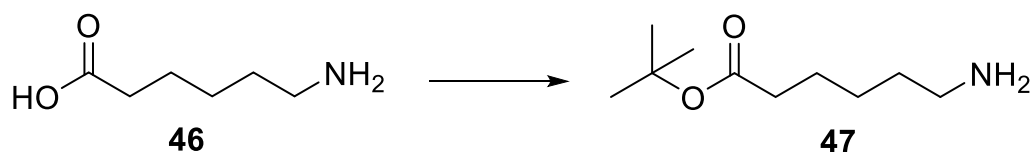
In an round bottom flask, tetrabromide **44** (4.93 g, 9.85 mmol), malic anhydride **45** (965.9 mg, 9.85 mmol) and NaI (8.71 g, 58.1 mmol) were dissolved in DMF (40 mL). This was left to stir at 65 °C overnight. Upon cooling on ice, the reaction mixture was transferred into a beaker to which H₂O was added, the resulting aqueous mixture was left to stir for 5 minutes. The resulting precipitate was

collected by vacuum filtration, washing thoroughly with H₂O and MeOH to afford compound **37** (1.36 g, 50%) as a brown solid.

¹H NMR (400 MHz, CDCl₃): δ 8.53 (s, 1H, Ar), 8.46 (s, 1H, Ar), 8.32 (d, *J* = 1.5 Hz, 1H, Ar), 8.02 (d, *J* = 8.8 Hz, 1H, Ar), 7.89 (dd, *J* = 8.8, 1.9 Hz, 1H, Ar).¹

Unfortunately, no HRMS could be obtained for compound **37**.²

tert*-Butyl-6-aminohexanoate **47*



In a round bottom flask, 6-aminohexanoic acid **46** (2.01 g, 15.15 mmol) and SOCl₂ (11.2 mL, 152.5 mmol) were added. This was left to stir at room temperature for 1.5 hours. Next, excess SOCl₂ was removed. A suspension of NaHCO₃ (28.2 g, 33.55 mmol) in *t*BuOH (50 mL) was added and the resulting reaction mixture was left to stir at room temperature overnight. Excess solvent was removed, and the resulting residue was taken up in EtOAc (50 mL) and NaOH (1 M) and then transferred into a separating funnel. The resulting organic layer was washed with H₂O (50 mL), brine (40 mL × 3), dried over MgSO₄, filtered and excess solvent removed to give the title compound **47** (1.27 g, 44%) as a golden oil.

¹H NMR (400 MHz, CDCl₃): δ 2.73 (t, *J* = 7.1 Hz, 2H), 2.65 (s, 2H), 2.21 (t, *J* = 7.1 Hz, 2H), 1.62–1.55 (m, 2H), 1.54–1.46 (m, 2H), 1.43 (s, 9H), 1.38–1.30 (m, 2H).

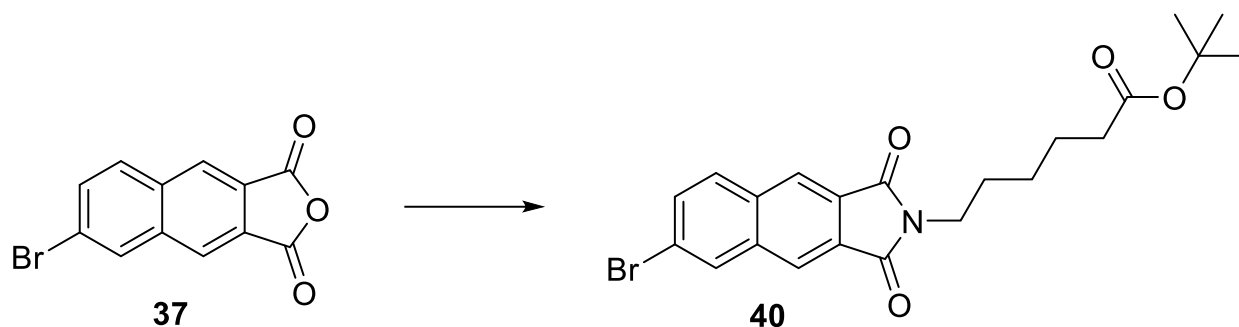
¹³C NMR (101 MHz, CDCl₃): δ 173.0, 79.9, 41.6, 35.4, 32.6, 28.0, 26.2, 24.8.

HRMS (ESI/Q-TOF, *m/z*): calculated for C₁₀H₂₂NO₂ [*M* + *H*]⁺ 188.1645; found 188.1645.

tert*-Butyl 6-(6-bromo-1,3-dioxo-1,3-dihydro-2*H*-benzo[*f*]isoindol-2-yl)hexanoate **40*

¹ ¹³C NMR data was unable to be obtained due NMR machine being down

² Softer ionisation method is needed in order to obtain HRMS data for this sample.



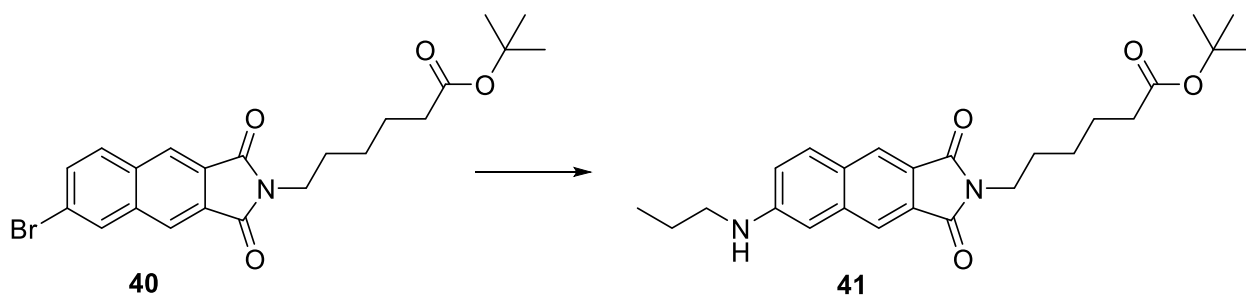
In a pressure vessel, naphthalic anhydride **37** (202.8 mg, 0.732 mmol), *tert*-butyl-6-aminohexanoate **47** (152.2 mg, 0.805 mmol) and isopropyl alcohol (10 mL) were added. This was left to stir at 100 °C for 4 days. Upon cooling to room temperature, the reaction mixture was then extracted with EtOAc (20 mL $\times$ 3). Combined organic layer was washed with brine (40 mL) and dried over MgSO₄, filtered and excess solvent removed. Purification by flash column chromatography (5% MeOH/CHCl₃) gave the title compound **40** (178.9 mg, 61%) as a white solid.

¹H NMR (400 MHz, CDCl₃): δ 8.29 (s, 1H, Ar), 8.23 (s, 1H Ar), 8.21 (d, J = 1.7 Hz, 1H, Ar), 7.92 (d, J = 8.8 Hz, 1H, Ar), 7.77 (dd, J = 8.7, 2.0 Hz, 1H, Ar), 3.75 (t, J = 7.3 Hz, 2H), 2.21 (t, J = 7.4 Hz, 2H), 1.77–1.70 (m, 2H), 1.68–1.60 (m, 2H), 1.42 (s, 9H), 1.41–1.36 (m, 2H).

¹³C NMR (101 MHz, CDCl₃): δ 173.1, 167.84, 167.76, 136.6, 134.0, 132.7, 132.4, 131.7, 129.1, 128.4, 124.6, 123.8, 123.6, 80.2, 38.4, 35.5, 28.4, 28.2, 26.5, 24.8.

HRMS (ESI/Q-TOF, m/z): calculated for C₂₂H₂₄BrNNaO₄ [M + Na]⁺ 468.0781; found 468.0779.

tert*-Butyl 6-(1,3-dioxo-6-(propylamino)-1,3-dihydro-2H-benzo[*f*]isoindol-2-yl)hexanoate **41*



In a round bottom flask, **40** (185.4 mg, 0.415 mmol), Pd₂(dba)₃.CHCl₃ (18.1 mg, 0.0017 mmol), Xantphos (10.3 mg, 0.0017 mmol) and toluene (anhydrous, 15 mL) were added. Next, *n*-propylamine (100 μ L, 1.25 mmol) and Cs₂CO₃ (540 mg, 1.66 mmol) were added. The resulting reaction mixture was left to stir at 60 °C overnight. Upon cooling to room temperature, an appropriate amount of SiO₂ was added and the solvent was removed *in vacuo*. The crude material was loaded onto SiO₂ and purified by

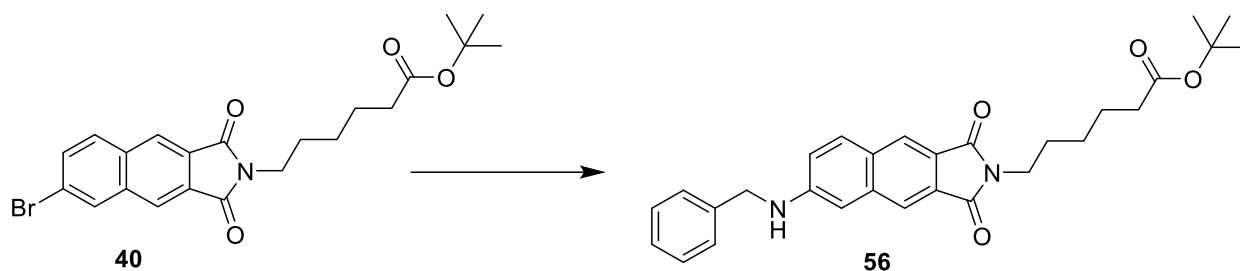
flash column chromatography (2% MeOH in 1:1 EtOAc/Hexane). Further purification by flash column chromatography (20% EtOAc/Hexane) gave the title compound **41** (112.8 mg, 64%) as a yellow solid.

^1H NMR (400 MHz, CDCl_3): δ 8.04 (s, 1H, Ar), 7.97 (s, 1H, Ar), 7.71 (d, J = 8.9 Hz, 1H, Ar), 6.97 (dd, J = 8.8, 2.4 Hz, 1H, Ar), 6.86 (d, J = 2.2 Hz, Ar), 4.32 (s, 1H), 3.67 (t, J = 7.2 Hz, 2H), 3.19 (t, J = 7.1 Hz, 2H), 2.19 (t, J = 7.4 Hz, 2H), 1.75–1.66 (m, 4H), 1.65–1.56 (m, 2H), 1.40 (s, 9H), 1.39–1.32 (m, 2H), 1.03 (t, J = 7.4 Hz, 3H).

^{13}C NMR (101 MHz, CDCl_3): δ 173.1, 168.83, 168.78, 148.9, 138.2, 131.4, 129.0, 128.5, 124.6, 123.4, 122.2, 120.0, 106.3, 80.2, 45.5, 38.0, 35.6, 28.5, 28.2, 26.5, 24.9, 22.6, 11.8.

HRMS (ESI/Q-TOF, m/z): calculated for $\text{C}_{25}\text{H}_{32}\text{N}_2\text{NaO}_4$ [$\text{M} + \text{Na}$] $^+$ 447.2254; found 447.2254.

tert*-Butyl 6-(6-(benzylamino)-1,3-dioxo-1,3-dihydro-2H-benzo[f]isoindol-2-yl)hexanoate **56*

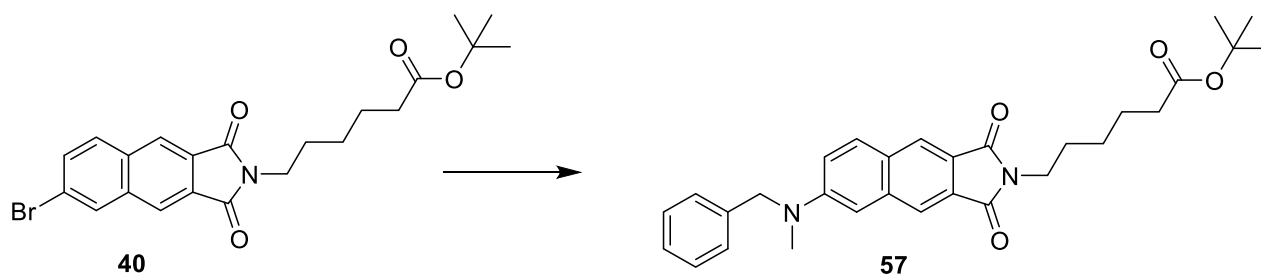


In a round bottom flask, **40** (209.6 mg, 0.470 mmol), $\text{Pd}_2(\text{dba})_3 \cdot \text{CHCl}_3$ (19.5 mg, 0.0188 mmol), Xantphos (10.9 mg, 0.0188 mmol) and toluene (anhydrous, 20 mL) were added. Next, benzylamine (154 μL , 1.41 mmol) and Cs_2CO_3 (612.5 mg, 1.88 mmol) were added. The resulting reaction mixture was left to stir at 60 $^\circ\text{C}$ overnight. Additional $\text{Pd}_2(\text{dba})_3 \cdot \text{CHCl}_3$ (20.2 mg, 0.0195 mmol), Xantphos (11.8 mg, 0.0204 mmol), and, benzylamine (160 μL , 1.46 mmol) was added and the reaction mixture was continued to stir at 60 $^\circ\text{C}$ for a further 3 days. Upon cooling to room temperature, an appropriate amount of SiO_2 was added and the solvent was removed *in vacuo*. The crude material was loaded onto SiO_2 and purified by flash column chromatography (2% MeOH in 1:1 EtOAc/Hexane). Further purification by flash column chromatography (20% EtOAc/Hexane) gave the title compound **56** (106.8 mg, 48%) as a yellow solid.

^1H NMR (400 MHz, CDCl_3): δ 8.03 (s, 1H, Ar), 7.92 (s, 1H, Ar), 7.71 (d, J = 8.8 Hz, 1H, Ar), 7.35–7.28 (m, 4H, Ar), 7.26–7.22 (m, 1H, Ar), 6.97 (dd, J = 8.8, 2.4 Hz, 1H, Ar), 6.88 (d, J = 2.2 Hz, 1H, Ar), 4.55 (t, J = 5.2 Hz, 1H), 4.40 (d, J = 5.2 Hz, 2H), 3.62 (t, J = 7.3 Hz, 2H), 2.15 (t, J = 7.4 Hz, 2H), 1.67–1.60 (m, 2H), 1.58–1.50 (m, 4H), 1.35 (s, 9H), 1.33–1.27 (m, 2H).

^{13}C NMR (101 MHz, CDCl_3): δ 173.2, 168.78, 168.75, 148.5, 138.2, 138.1, 131.5, 129.04, 128.97, 128.8, 127.9, 127.7, 124.6, 123.8, 122.4, 120.1, 107.0, 80.2, 48.1, 38.0, 35.6, 28.5, 28.3, 26.6, 24.9.

***tert*-Butyl 6-(6-(benzyl(methyl)amino)-1,3-dioxo-1,3-dihydro-2*H*-benzo[*f*]isoindol-2-yl)hexanoate**
57



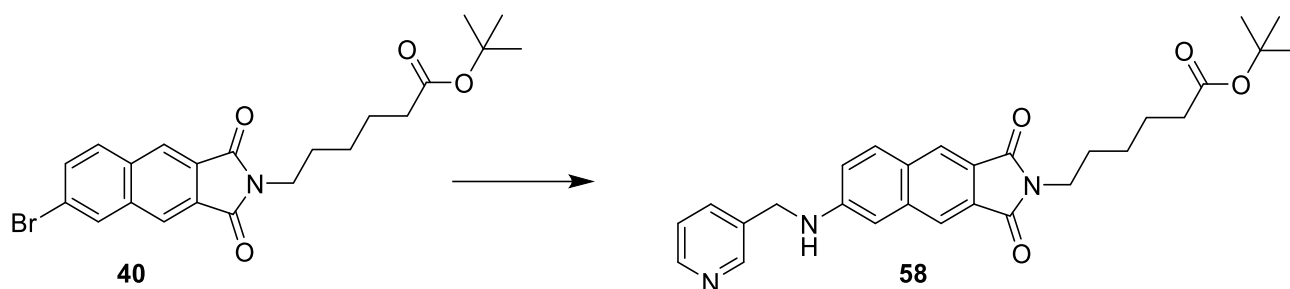
In a round bottom flask, **40** (330.7 mg, 0.741 mmol), $\text{Pd}_2(\text{dba})_3 \cdot \text{CHCl}_3$ (30.6 mg, 0.0296 mmol), Xantphos (17.1 mg, 0.0296 mmol) and toluene (anhydrous, 15 mL) were added. Next, *N*-benzylmethylamine (286 μL , 2.22 mmol) and Cs_2CO_3 (964.4 mg, 2.96 mmol) were added. The resulting reaction mixture was left to stir at 60 $^\circ\text{C}$ for 4 days. Upon cooling to room temperature, an appropriate amount of SiO_2 was added and the solvent was removed *in vacuo*. The crude material was loaded onto SiO_2 and purified by flash column chromatography (2% MeOH in 1:1 EtOAc/Hexane). Further purification by flash column chromatography (20% EtOAc/Hexane) gave the title compound **57** (87.7 mg, 24%) as a yellow solid.

^1H NMR (400 MHz, CDCl_3): δ 8.11 (s, 1H, Ar), 8.03 (s, 1H, Ar), 7.82 (d, J = 9.2 Hz, 1H, Ar), 7.36–7.32 (m, 2H, Ar), 7.29–7.27 (m, 1H, Ar), 7.25–7.22 (m, 3H, Ar), 7.07 (d, J = 2.5 Hz, 1H, Ar), 4.72 (s, 2H), 3.70 (t, J = 7.2 Hz, 2H), 3.22 (s, 3H), 2.21 (t, J = 7.4 Hz, 2H), 1.75–1.68 (m, 2H), 1.67–1.60 (m, 2H), 1.42 (s, 9H), 1.40–1.36 (m, 2H).³

HRMS (ESI/Q-TOF, m/z): calculated for $\text{C}_{30}\text{H}_{34}\text{N}_2\text{NaO}_4$ [$\text{M} + \text{Na}$]⁺ 509.2411; found 509.2401.

***tert*-Butyl 6-(1,3-dioxo-6-((pyridin-3-ylmethyl)amino)-1,3-dihydro-2*H*-benzo[*f*]isoindol-2-yl)hexanoate**
58

³ ^{13}C NMR data was unable to be obtained due NMR machine being down



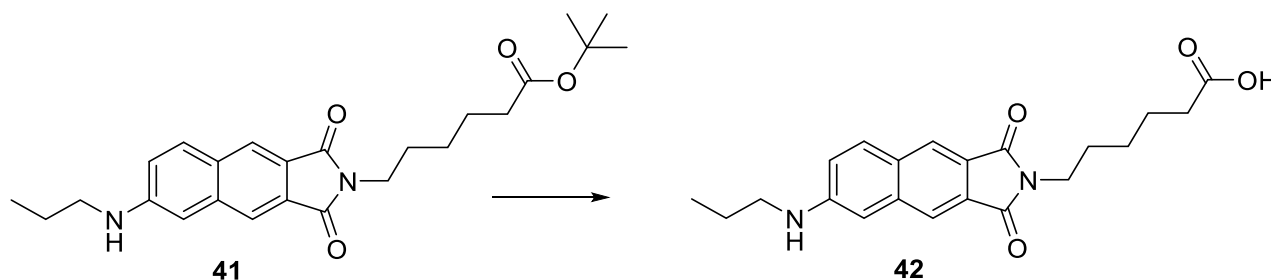
In a round bottom flask, **40** (236 mg, 0.529 mmol), $\text{Pd}_2(\text{dba})_3 \cdot \text{CHCl}_3$ (21.9 mg, 0.0212 mmol), Xantphos (12.3 mg, 0.0212 mmol) and toluene (anhydrous, 30 mL) were added. Next, 3-picoylamine (162 μL , 1.59 mmol) and Cs_2CO_3 (689.4 mg, 2.12 mmol) were added. The resulting reaction mixture was left to stir at 60 °C for 2 days. Additional 3-picoylamine (161.6 μL , 1.587 mmol) was added and the reaction mixture was left to continue stirring at 60 °C for a further 3 days. Upon cooling to room temperature, an appropriate amount of SiO_2 was added and the solvent was removed *in vacuo*. The crude material was loaded onto SiO_2 and purified by flash column chromatography (2% MeOH in 1:1 EtOAc/Hexane). Further purification by flash column chromatography (20% EtOAc/Hexane) gave the title compound **58** (170 mg, 77%) as a yellow solid.

^1H NMR (400 MHz, CDCl_3): δ 8.69 (d, J = 1.7 Hz, 1H, Ar), 8.57 (dd, J = 4.8, 1.6 Hz, 1H, Ar), 8.12 (s, 1H, Ar), 8.00 (s, 1H, Ar), 7.81 (d, J = 8.9 Hz, 1H, Ar), 7.74–7.71 (m, 1H, Ar), 7.32–7.29 (m, 1H, Ar), 7.06 (dd, J = 8.8, 2.4 Hz, 1H, Ar), 6.95 (d, J = 2.3 Hz, 1H, Ar), 4.65 (t, J = 5.5 Hz, 1H), 4.52 (d, J = 5.5 Hz, 2H), 3.70 (t, J = 7.2 Hz, 2H), 2.20, (t, J = 7.4 Hz, 2H), 1.74–1.69 (m, 2H), 1.66–1.57 (m, 2H), 1.42 (s, 9H), 1.36–1.34 (m, 2H).

^{13}C NMR (101 MHz, CDCl_3): δ 173.1, 168.6, 149.29, 149.26, 147.9, 137.9, 135.2, 133.7, 131.6, 129.01, 128.98, 124.5, 124.1, 123.8, 122.4, 119.9, 107.1, 80.1, 77.3, 45.6, 38.0, 35.5, 28.4, 28.2, 26.5, 24.8.

HRMS (ESI/Q-TOF, m/z): calculated for $\text{C}_{28}\text{H}_{31}\text{N}_3\text{NaO}_4$ $[\text{M} + \text{Na}]^+$ 496.2207; found 496.2215.

6-(1,3-Dioxo-6-(propylamino)-1,3-dihydro-2H-benzo[*f*]isoindol-2-yl)hexanoic acid **42**



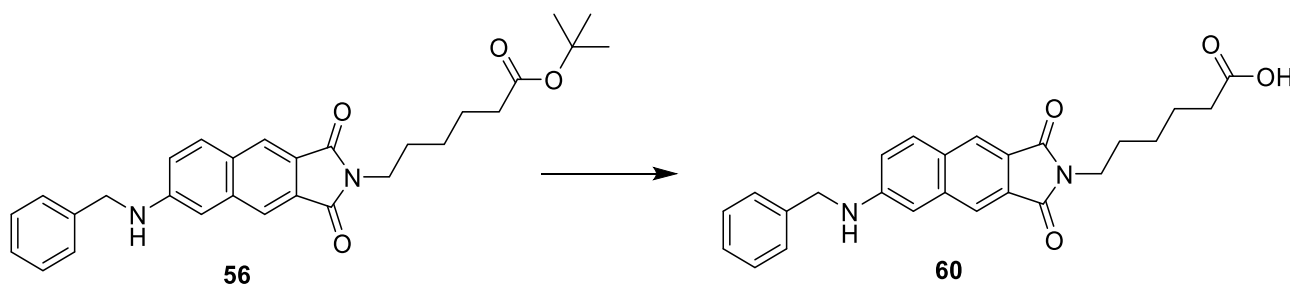
To a solution of **41** (130 mg, 0.306 mmol) in CH₂Cl₂ (20 mL), was added trifluoroacetic acid (2 mL). The resulting reaction mixture was left to stir at room temperature overnight. The solvent was removed and residual TFA was removed by azeotropic distillation with Et₂O (10 mL × 4) to give the title compound **42** (127.7 mg, 86%) as a yellow solid.

¹H NMR (400 MHz, CDCl₃): δ 8.07 (s, 1H, Ar), 8.00 (s, 1H, Ar), 7.76 (d, *J* = 8.8 Hz, 1H, Ar), 7.55 (s, 2H), 7.07 (dd, *J* = 8.7, 2.2 Hz, 1H, Ar), 3.69 (t, *J* = 7.2 Hz, 2H), 3.22 (t, *J* = 7.2 Hz, 2H), 2.34 (t, *J* = 7.4 Hz, 2H), 1.77–1.63 (m, 6H), 1.44–1.36 (m, 2H), 1.03 (t, *J* = 7.4 Hz, 3H).

¹³C NMR (101 MHz, CDCl₃): δ 179.2, 168.60, 168.58, 146.4, 137.6, 131.6, 129.7, 128.9, 124.6, 124.4, 122.7, 120.7, 109.9, 47.3, 37.9, 33.9, 28.3, 26.4, 24.3, 21.9, 11.6.

HRMS (ESI/Q-TOF, *m/z*): calculated for C₂₁H₂₄N₂NaO₄ [*M* + Na]⁺ 391.1628; found 391.1637.

6-(6-(Benzylamino)-1,3-dioxo-1,3-dihydro-2H-benzo[*f*]isoindol-2-yl)hexanoic acid **60**

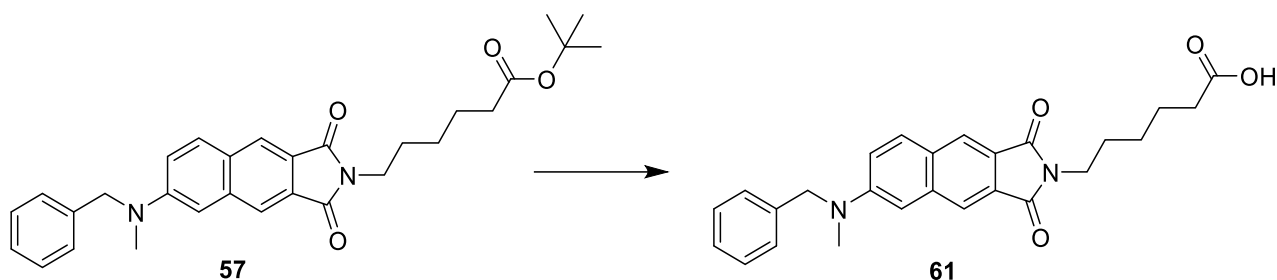


To a solution of **56** (106.8 mg, 0.226 mmol) in CH₂Cl₂ (30 mL), was added trifluoroacetic acid (2 mL). The resulting reaction mixture was left to stir at room temperature for two days. The solvent was removed and residual TFA was removed by azeotropic distillation with Et₂O (10 mL × 4) to give the title compound **60** (91.1 mg, 86%) as a yellow solid.

¹H NMR (400 MHz, CDCl₃): δ 8.11 (s, 1H, Ar), 8.00 (s, 1H, Ar), 7.79 (d, *J* = 8.9 Hz, 1H, Ar), 7.42–7.36 (m, 4H, Ar), 7.33–7.30 (m, 1H, Ar), 7.05 (dd, *J* = 7.7, 2.3 Hz, 1H, Ar), 6.96 (d, *J* = 2.0 Hz, 1H, Ar), 4.48 (s, 2H), 3.71 (t, *J* = 7.3 Hz, 2H), 2.34 (t, *J* = 7.4 Hz, 2H), 1.76–1.65 (m, 4H), 1.45–1.37 (m, 2H).⁴

6-(6-(Benzyl(methyl)amino)-1,3-dioxo-1,3-dihydro-2H-benzo[*f*]isoindol-2-yl)hexanoic acid **61**

⁴ ¹³C NMR data was unable to be obtained due NMR machine being down

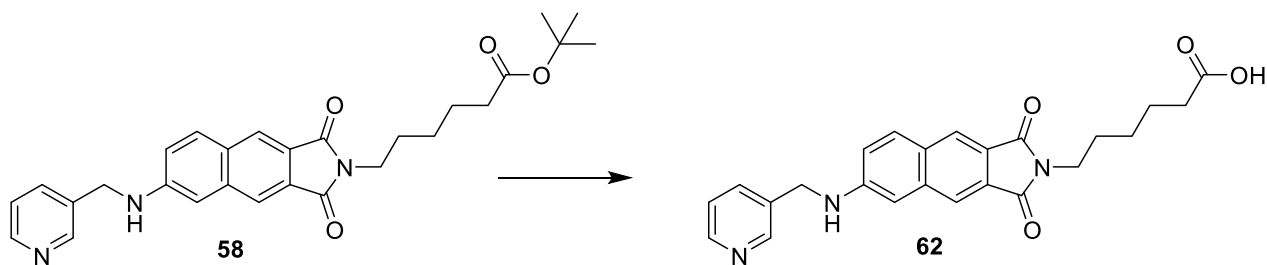


To a solution of **57** (87.7 mg, 0.180 mmol) in CH₂Cl₂ (20 mL), was added trifluoroacetic acid (2 mL). The resulting reaction mixture was left to stir at room temperature overnight. The solvent was removed and residual TFA was removed by azeotropic distillation with Et₂O (10 mL × 4) to give the title compound **61** (79.4 mg, 81%) as a yellow solid.

¹H NMR (400 MHz, CDCl₃): δ 8.11 (s, 1H, Ar), 8.03 (s, 1H, Ar), 7.82 (d, *J* = 9.2 Hz, 1H, Ar), 7.36–7.32 (m, 2H, Ar), 7.29–7.25 (m, 1H, Ar), 7.25–7.22 (m, 3H, Ar), 7.07 (d, *J* = 2.5 Hz, 1H, Ar), 4.72 (s, 2H), 3.71 (t, *J* = 7.2 Hz, 2H), 3.21 (s, 3H), 2.35 (t, *J* = 7.4 Hz, 2H), 1.76–1.65 (m, 4H), 1.45–1.38 (m, 2H).⁵

HRMS (ESI/Q-TOF, *m/z*): calculated for C₂₆H₂₆N₂NaO₄ [*M* + Na]⁺ 453.1785; found 453.1828.⁶

6-(1,3-Dioxo-6-((pyridin-3-ylmethyl)amino)-1,3-dihydro-2H-benzo[*f*]isoindol-2-yl)hexanoic acid
62



To a solution of **58** (151.4 mg, 0.320 mmol) in CH₂Cl₂ (30 mL), was added trifluoroacetic acid (2 mL). The resulting reaction mixture was left to stir at room temperature for 5 days. The solvent was removed and residual TFA was removed by azeotropic distillation with Et₂O (10 mL × 4) to afford the title compound **62** (175 mg) as an orange oil. The product was used in the next step without further purification.

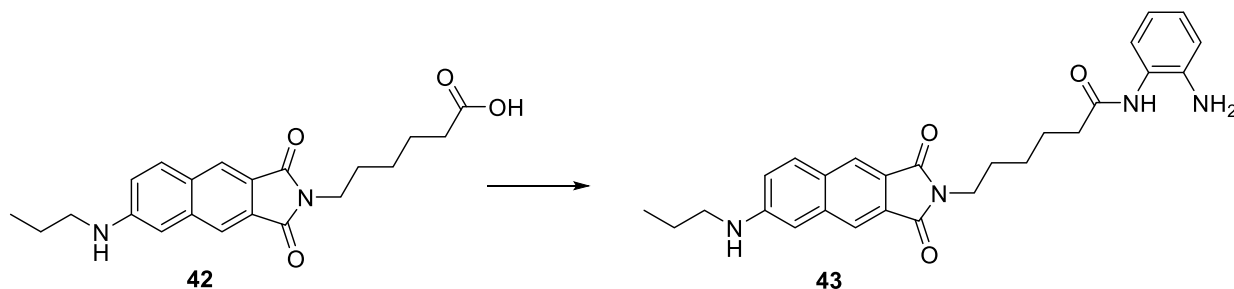
¹H NMR (400 MHz, CDCl₃): δ 8.87 (d, *J* = 0.9 Hz, 1H, Ar), 8.76 (d, *J* = 5.7 Hz, 1H, Ar), 8.69–8.66 (m, 1H, Ar), 8.13 (s, 1H, Ar), 8.07 (dd, *J* = 7.8, 5.8 Hz, 1H, Ar), 7.97 (s, 1H, Ar), 7.90 (d, *J* = 9.0 Hz, 1H,

⁵ ¹³C NMR data was unable to be obtained due to NMR machine being down

⁶ Note that this data has an error of 9.4 ppm. HRMS measurement is to be repeated.

Ar), 7.25 (dd, $J = 8.9, 2.4$ Hz, 1H, Ar), 6.98 (d, $J = 2.3$ Hz, 1H, Ar), 4.79 (s, 2H), 3.67 (t, $J = 7.2$ Hz, 2H), 2.29 (t, $J = 7.4$ Hz, 2H), 1.73–1.61 (m, 6H).⁷

***N*-(2-Aminophenyl)-6-(1,3-dioxo-6-(propylamino)-1,3-dihydro-2*H*-benzo[*f*]isoindol-2-yl)hexanamide 43**



In a round bottom flask, **42** (103 mg, 0.213 mmol) *O*-phenylenediamine (66.7 mg, 0.617 mmol), EDCI.HCl (81.9 mg, 0.427 mmol) and HOBt (15.8 mg, 0.117 mmol) were dissolved in acetonitrile (15 mL). The resulting reaction mixture was left to stir at room temperature for 7 days. Excess solvent was removed and the resulting crude material was purified by flash column chromatography (1:9 MeOH/DCM with 1% NH_4OH) twice. Further purification by flash column chromatography (column loaded with 1:9 MeOH/DCM with 1% NH_4OH ; 2% MeOH/DCM as the eluent) gave compound **43** (23.2 mg, 24%) as a yellow solid.

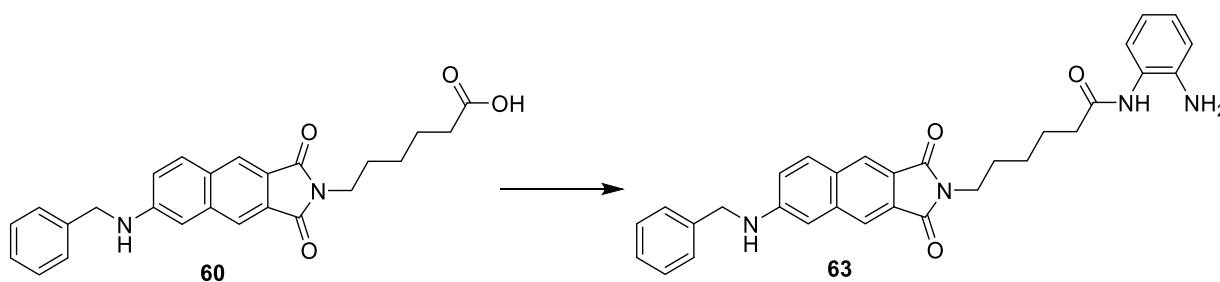
^1H NMR (400 MHz, CDCl_3): δ 8.06 (s, 1H, Ar), 7.99 (s, 1H, Ar), 7.75 (d, $J = 8.8$ Hz, 1H, Ar), 7.18 (dd, $J = 8.2, 1.4$ Hz, 1H, Ar), 7.05–7.01 (m, 1H, Ar), 6.99 (dd, $J = 8.9, 2.4$ Hz, 1H, Ar), 6.90 (dd, $J = 2.2$ Hz, 1H, Ar), 6.78–6.75 (m, 2H, Ar), 4.21 (t, $J = 4.9$ Hz, 1H), 3.84 (s, 1H), 3.74 (t, $J = 7.0$, 2H), 3.25–3.21 (m, 2H), 2.41 (t, $J = 7.4$ Hz, 2H), 1.84–1.69 (m, 6H), 1.51–1.44 (m, 2H), 1.06 (t, $J = 7.4$ Hz, 3H).

^{13}C NMR (101 MHz, CDCl_3): δ 171.6, 169.0, 168.9, 148.9, 140.9, 138.2, 131.4, 128.9, 128.5, 127.2, 125.3, 124.7, 124.6, 123.3, 122.3, 120.1, 119.6, 118.3, 106.3, 45.5, 37.7, 37.0, 28.3, 26.4, 25.3, 22.6, 11.8.

HRMS (ESI/Q-TOF, m/z): calculated for $\text{C}_{27}\text{H}_{30}\text{N}_4\text{NaO}_3$ $[\text{M} + \text{Na}]^+$ 481.2210; found 481.2210.

***N*-(2-Aminophenyl)-6-(6-(benzylamino)-1,3-dioxo-1,3-dihydro-2*H*-benzo[*f*]isoindol-2-yl)hexanamide 63**

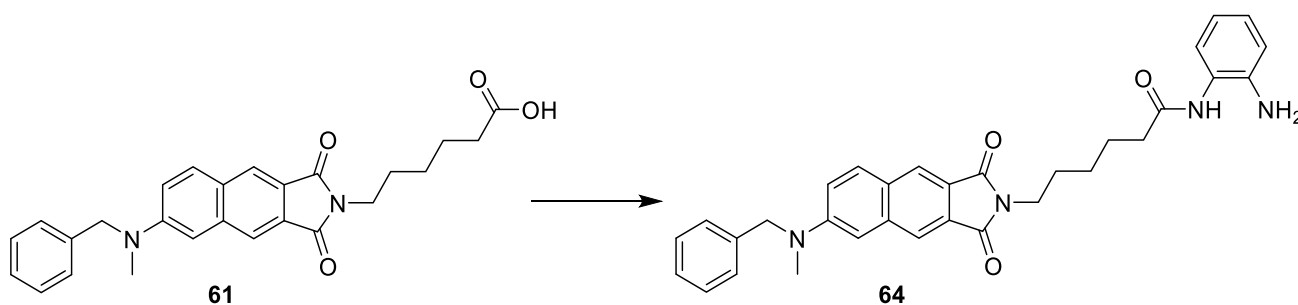
⁷ ^{13}C NMR data was unable to be obtained due the sample not being spectroscopically clean



In a round bottom flask, **60** (91.1 mg, 0.219 mmol), *O*-phenylenediamine (68.7 mg, 0.635 mmol), EDCI.HCl (83.8 mg, 0.437 mmol) and HOBT (16.2 mg, 0.120 mmol) were dissolved in acetonitrile (30 mL). The resulting reaction mixture was left to stir at room temperature for a week. Excess solvent was removed and the resulting residue was dissolved in CH₂Cl₂ (10 mL). To this, was added KH₂PO₄ (20 mL) and the resulting aqueous phase was extracted with CH₂Cl₂ (20 mL × 3). Combined organic layer was washed with brine (40 mL), dried over MgSO₄, filtered and excess solvent removed. Purification by flash column chromatography (1:9 MeOH/DCM with 1% NH₄OH) gave a yellow solid. Further purification by flash column chromatography (column loaded with 1:9 MeOH/DCM with 1% NH₄OH; 2% MeOH/DCM as the eluted solvent) afforded the title compound **63** (53.9 mg) as a yellow solid. Unfortunately, spectroscopically pure sample could not be obtained at this stage and further purification is needed.

¹H NMR (400 MHz, CDCl₃): δ 8.07 (s, 1H, Ar), 7.96 (s, 1H, Ar), 7.77 (d, *J* = 8.8 Hz, 1H, Ar), 7.42-7.36 (m, 4H, Ar), 7.34-7.31 (m, 1H, Ar), 7.17 (d, *J* = 7.1 Hz, 1H, Ar), 7.04 (dd, *J* = 9.6, 2.4 Hz, 1H, Ar), 7.00 (d, *J* = 8.6 Hz, 1H, Ar), 6.94 (d, *J* = 2.0 Hz, 1H, Ar), 6.77-6.73 (m, 2H, Ar), 6.71 (s, 2H, Ar), 4.62 (t, *J* = 5.3 Hz, 1H), 4.48 (d, *J* = 5.4 Hz, 2H), 3.73 (t, *J* = 7.0 Hz, 2H), 2.40 (t, *J* = 7.4 Hz, 2H), 1.85-1.72 (m, 4H), 1.50-1.43 (m, 2H)⁸

N*-(2-Aminophenyl)-6-(6-(benzyl(methyl)amino)-1,3-dioxo-1,3-dihydro-2H-benzo[*f*]isoindol-2-yl)hexanamide **64*



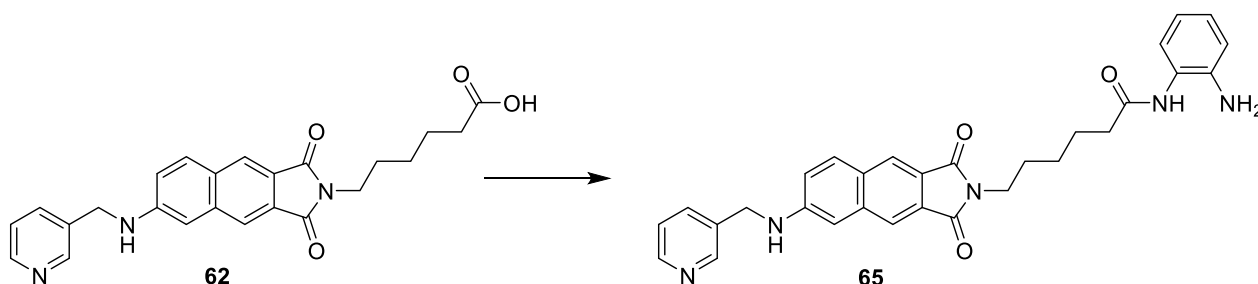
⁸ ¹³C NMR data was unable to be obtained due the sample not being spectroscopically clean

In a round bottom flask, **61** (74.9 mg, 0.137 mmol), *O*-phenylenediamine (42.9 mg, 0.397 mmol), EDCI.HCl (527 mg, 0.275 mmol) and HOBt (10.2 mg, 0.0754 mmol) were dissolved in acetonitrile (15 mL). The resulting reaction mixture was left to stir at room temperature for 5 days. The reaction was filtered through a Buchner funnel. Purification of the precipitate by flash column chromatography (1:9 MeOH/DCM with 1% NH₄OH; 5% MeOH/DCM) gave the title compound **64** (47.4 mg, 66%) as a yellow solid.

¹H NMR (400 MHz, CDCl₃): δ 8.08 (s, 1H, Ar), 7.99 (s, 1H, Ar), 7.81 (d, *J* = 9.2 Hz, 1H, Ar), 7.36–7.32 (m, 2H, Ar), 7.29–7.27 (m, 1H, Ar), 7.25–7.18 (m, 4H, Ar), 7.06 (d, *J* = 2.4 Hz, 1H, Ar), 7.04–7.00 (m, 1H, Ar), 6.78–6.74 (m, 2H, Ar), 4.72 (s, 2H), 3.83 (s, 1H), 3.74 (t, *J* = 7.08 Hz, 2H), 3.22 (s, 3H), 2.42 (t, *J* = 7.4 Hz, 2H), 1.87–1.74 (m, 4H), 1.51–1.44 (m, 2H).⁹

HRMS (ESI/Q-TOF, *m/z*): calculated for C₃₂H₃₃N₄O₃ [M + H]⁺ 521.2547; found 521.2546.

N*-(2-Aminophenyl)-6-(1,3-dioxo-6-((pyridin-3-ylmethyl)amino)-1,3-dihydro-2*H*-benzo[*f*]isoindol-2-yl)hexanamide **65*



In a round bottom flask, **62** (170 mg, 0.320 mmol), *O*-phenylenediamine (100.4 mg, 0.928 mmol), EDCI.HCl (122.7 mg, 0.640 mmol) and HOBt (23.8 mg, 0.176 mmol) were dissolved in acetonitrile (30 mL). The resulting reaction mixture was left to stir at room temperature overnight. Purification by flash column chromatography (1:9 MeOH/DCM with 1% NH₄OH; 5% MeOH/DCM) afforded the title compound **65** (27.6 mg) as a yellow solid. Unfortunately, a spectroscopically pure sample could not be obtained at this stage and further purification is needed.¹⁰

⁹ ¹³C NMR data was unable to be obtained due to NMR machine being down

¹⁰ NMR data was unable to be obtained due the sample not being spectroscopically clean

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